Synthesis of Labelled Desulfoglucosinolates and Novel Glucosinolate Analogues

A thesis presented for the degree of
Doctor of Philosophy
to the
University of St. Andrews
in December 1999

By

Avril A. B. Robertson

i) I, Avril A. B. Robertson, hereby certify that this thesis, which is approximately 70 000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

date.01.12.99. signature of candidate

ii) I was admitted as a research student in October, 1996 and as a candidate for the degree of Ph.D. in December, 1999; the higher study for which this is a record was carried out in the University of St. Andrews between 1996 and 1999.

date.01.12.99.. signature of candidate.

iii) I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Ph.D. in the University of St. Andrews and that the candidate is qualified to submit the thesis in application for that degree.

date.1.12.99 signature of superviso

In submitting this thesis to the University of St. Andrews I understand that I am giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and abstract will be published, and that a copy of the work may be made and supplied to any *bona fide* library or research worker.

date.01.12.99. signature of candidate.

To my dad,

Thank you for your love and support in all I have done

Acknowledgements

It has been a pleasure to work under the excellent supervision of Dr. Nigel Botting. He has helped in all aspects of my PhD advising on everything from presentations and posters through to my final thesis. He was never too busy to listen and I thank him for teaching me so well.

I would also like to thank my co-workers particularly the current members of the group who have encouraged me through my final year. I wish them well for the future. In addition my flatmates have provided me with a pleasant living environment outwith the department. Thank you David, Ruth, Zoe and Gill.

My collaborators at the Scottish Crop Research Institute provided invaluable LC-MS work and I would like to thank Wynne Griffiths for allowing me to visit the site.

I am grateful to the University of St. Andrews Ettie Steele prize fund for providing the opportunity to study abroad. My work at the ETH was a valuable contribution to my ongoing education. I am indebted to Professor Andrea Vasella for extending me the pleasure and privilege of working in his research group. I also appreciate his generosity in personally funding a third months study. During my stay in Zürich the group made me feel very welcome, taught me many things and left me with pleasant memories.

I would particularly like to thank my fiancé Andrew for his valuable help and support throughout my PhD and I look forward to our future together.

But, above all I would like to thank my dad for providing me with the opportunities to make my life a success. Without his endless strength, encouragement, advice and support through many difficult times, this thesis would not exist. I have therefore dedicated the work to him.

Abbreviations

The following common abbreviations have been used throughout the thesis:

a.m.u.	Atomic mass units
APCI	Atmospheric pressure chemical ionisation
ATP	Adenosine triphosphate
Bn	Benzyl
CI	Chemical ionisation
CID	Collision induced dissociation
Cys	Cysteine
DCM	Dichloromethane
DIECA	Diethyldithiocarbamate
DME	1,2-Dimethoxyethane
DMF	*N,N*-Dimethylformamide
DMF.DMA	*N,N*-Dimethylformamide dimethyl acetal
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC	European Commission
E.C.	Enzyme classification
EDTA	Ethylenediamine tetraacetic acid
EI	Electron impact
ESP	Epithiospecifer protein
FAB	Fast atom bombardment
GC	Gas chromatography
GL	Glucosinolate
G-6-PDH	Glucose-6-phosphate dehydrogenase
GST	Glutathione S-transferase
HK	Hexokinase

HPLC	High performance liquid chromatography
IM	Indolylmethyl
LC-MS	Liquid chromatography-mass spectrometry
MBP	Myrosinase binding protein
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NCS	*N*-Chlorosuccinimide
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
n.m.r.	Nuclear magnetic resonance
o-NPG	*o*-Nitrophenyl-β-D-glucoside
p-NPG	*p*-Nitrophenyl-β-D-glucoside
P450s	Cytochromes P450
PAPS	3-Phosphoadenosine 5'-phosphosulfate
PCC	Pyridinium chlorochromate
PCMB	*p*-Chloromercuribenzoate
PCMS	*p*-Chloromercuribenzene sulfonic acid
PDC	Pyridinium dichromate
PEITC	Phenethyl isothiocyanate
6-PG	6-Phosphoglucose
PNP	*p*-Nitrophenyl
QR	Quinone reductase
TEMPO	2,2,6,6-Tetramethylpiperidine *N*-oxide radical
THF	Tetrahydrofuran
tlc	Thin layer chromatography
TMS	Tetramethylsilane
TSP	Thermospray
UDP(G)	Uridine-5'-diphosphate glucose

CONTENTS

Page

Declaration i

Dedication ii

Acknowledgements iii

Abbreviations iv

Contents vi

ABSTRACT 1

1 INTRODUCTION 2

1.1 Introduction to Glucosinolates 3

1.2 Occurrence and Biological Activity of Glucosinolates 7

1.2.1 Glucosinolate occurrence and variation 7

1.2.2 Compartmentalisation of glucosinolates/myrosinase 10

1.2.3 Glucosinolates and insects 11

1.2.4 Glucosinolates and fungi 16

1.2.5 Glucosinolates and cancer 17

1.2.6 Glucosinolates and goitre 22

1.2.7 Exploitation of a natural reaction 25

1.3 Myrosinase and Glucosinolate Hydrolysis 26

1.3.1 Myrosinase and its mechanism 26

1.3.2 Features of myrosinase 30

1.3.3 Specificity of myrosinase 33

1.3.4 Glucosinolate hydrolysis products 36

1.4 Glucosinolate Analysis..... 41

1.5 Biosynthesis of Glucosinolates..... 44

1.5.1 Amino acid modification..... **44**

1.5.2 Aldoxime and glucosinolate formation..... **45**

1.5.3 Side chain modification..... **48**

1.6 Chemical Synthesis of Glucosinolates..... 49

2 RESULTS AND DISCUSSION..... 57

2.1 Deuterated Desulfoglucosinolates as Internal Standards in LC-MS..... 58

2.1.1 Background and objectives..... **58**

2.1.2 Synthesis of desulfogluconasturtiins..... **60**

2.1.3 Synthesis of desulfoglucobrassicins..... **72**

2.1.3.1 Synthesis of substituted indoles..... **74**

2.1.3.2 Addition of the side chain..... **79**

2.1.3.3 Conversion to desulfoglucobrassicins..... **85**

2.1.4 Synthesis of a deuterated glucopyranose..... **88**

2.1.5 Conclusions..... **99**

2.1.6 APCI-Mass spectrometry of desulfoglucosinolates..... **100**

2.1.7 Internal standards in LC-APCI-MS..... **104**

2.1.8 Gluconasturtiin as an HPLC internal standard..... **108**

2.1.9 Conclusions..... **109**

2.2 Novel Glucosinolate Analogues for Mechanistic Studies with Myrosinase..... 110

2.2.1 Background and objectives..... **110**

2.2.2 Synthesis of novel phenyl glucosinolates..... **111**

2.2.3 Purification of phenyl glucosinolates..... **117**

2.2.4 Isomerism of thiohydroximates..... **121**

2.2.5 Conclusions..... **125**

2.2.6 Studies towards a non-rearrangeable analogue..... **126**

2.2.7 Synthesis of a non-rearrangeable analogue..... **134**

2.2.8 Conclusions..... **138**

2.3 Myrosinase Catalysed Hydrolysis of Glucosinolates..... 139

2.3.1 Incubations of synthetic glucosinolates..... **144**

2.3.1.1 Phenyl glucosinolate homologues..... **144**

2.3.1.2 Novel phenyl analogues..... **148**

2.3.2 Enzyme kinetics and determination of V_{max} and K_M..... **150**

2.3.2.1 K_M and V_{max} for *p*-nitrophenyl glucosinolate... **152**

2.3.2.2 K_M and V_{max} for sinigrin..... **153**

2.3.2.3 Comparisons..... **154**

2.3.3 Studies on a non-rearrangeable glucosinolate analogue..... **155**

2.3.4 Conclusions..... **158**

2.4 Summary..... 159

2.5 Future Work..... 161

3 EXPERIMENTAL 164

3.1 Experimental Details 165
3.2 Gluconasturtiins 168
3.3 Glucobrassicins 180
3.4 Deuterated Glucopyranose 207
3.5 Novel Phenyl Glucosinolates 225
3.6 Non-rearrangeable Glucosinolate Analogues 238
3.7 Studies with Myrosinase 249

4 REFERENCES 270

5 APPENDIX 283

Abstract

A number of novel deuterated desulfoglucosinolates have been prepared. These have been shown to be suitable internal standards for the quantitative LC-MS analysis of glucosinolates. Use of the deuterated desulfoglucosinolates results in a 100-fold improvement in the sensitivity over existing methods. This should allow improved analysis of leaf surface glucosinolates, including determination of the amount present on a single leaf.

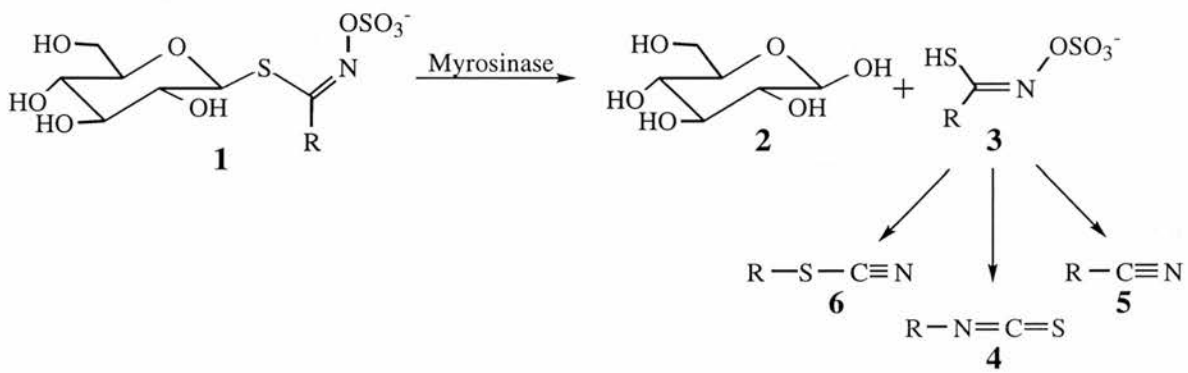
Desulfo-[$^2H_5$]gluconasturtiin was the first compound prepared to establish the methodology. Then two indolyl desulfoglucosinolates were prepared, desulfo-4-[$^2H_3$]methoxyglucobrassicin and desulfo-1-[$^2H_3$]methoxyglucobrassicin, as the corresponding glucosinolates of these derivatives are more potent as oviposition cues when present on the leaf surface. These structures contained deuterium in the aglycone. Finally, β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranosyl phenethyl thiohydroximate was synthesised where the deuterium was incorporated into the glucose fragment. This synthetic methodology should thus be applicable to any glucosinolate of analytical interest.

Some novel glucosinolates and glucosinolate analogues have been prepared in order to probe the chemical mechanism of glucosinolate cleavage by myrosinase. This reaction gives β-D-glucose and an aglycone which undergoes Lossen rearrangement to give sulfate and an isothiocyanate. Three novel substituted phenyl glucosinolates were prepared to probe the involvement of the rearrangement in the overall rate of reaction. Changes in the electronic nature of the side chain were expected to alter the rate of rearrangement (*p*-MeO > H > *p*-NO_2). However the opposite trend was observed, implying that changes in the pKa of the leaving group were more important. An analogue designed to mimic glucosinolate binding to myrosinase but unable to rearrange was designed and synthesised. Surprisingly this was neither a substrate nor an inhibitor for myrosinase.

Introduction

1.1 Introduction to Glucosinolates

Glucosinolates **1** are an important class of sulfur containing glycosides which exist in all members of the *Cruciferae* including the *Brassica* vegetables such as; Brussels sprouts, broccoli and oilseed rape (scheme 1). Damage to these plants, via either food processing or insect attack allows the glucosinolates to come into contact with an enzyme called myrosinase (thioglucoside glucohydrolase, E.C. 3.2.3.1). Myrosinase removes the glycosyl group **2** and initiates a Lossen type rearrangement of the aglycone fragment **3** to yield a variety of biologically active products. The predominant products are isothiocyanates **4** but nitriles **5**, thiocyanates **6**, oxazolidine-2-thiones, hydroxynitriles and epithionitriles can also be formed. These compounds function in plant defence as herbivore toxins, feeding repellents, antifungal and antimicrobial agents.



Scheme 1: *Hydrolysis of glucosinolates by myrosinase*

Glucosinolates represent a structurally diverse class of compounds owing to the variation in the nature of the side chain, **R**. Over 100 glucosinolates occur naturally and are biosynthesised from amino acids. For example phenethyl glucosinolate is derived from phenylalanine. Many more novel glucosinolate analogues have been synthesised mainly to probe the mechanism and active site of the hydrolytic enzyme myrosinase.

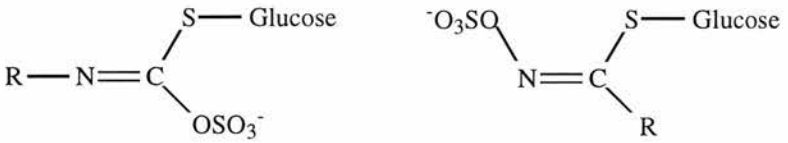
There are two existing systems which are commonly used to name glucosinolates the first semi-systematic approach uses the name of the side chain as a prefix for example, benzyl

glucosinolate. A second method of naming glucosinolates involves using the name of the plant from which the glucosinolate was first isolated for example sinalbin (*p*-hydroxybenzyl glucosinolate) was isolated from *Sinapis alba* seeds.[1] A few of the commonly used trivial names are illustrated in table 1.

Side Chain	**Trivial Name**
2-propenyl	Sinigrin
3-butenyl	Gluconapin
4-pentenyl	Glucobrassicanapin
4-methylthiobutyl	Glucoerucin
4-methylsulfinylbutyl	Glucoraphanin
4-methylsulfinyl-3-butenyl	Glucoraphenin
benzyl	Glucotropaeolin
2-phenethyl	Gluconasturtiin
p-hydroxybenzyl	(Gluco)sinalbin
2-hydroxy-3-butenyl	Progoitrin/epiprogoitrin
3-indolylmethyl	Glucobrassicin
1-methoxy-3-indolylmethyl	Neoglucobrassicin

Table 1: *Trivial nomenclature for glucosinolates occurring in edible plants*

The first glucosinolate to be isolated was sinalbin from *Sinapis alba* (white mustard seed) in 1831 by Robiquet and Boutron.[1] Nine years later Bussy isolated a second glucosinolate sinigrin from *Brassica nigra* (black mustard seed).[2] However, it was not until 1897 that the first glucosinolate structure was proposed by Gadamer.[3] This structure was later revised by Ettlinger and Lundeen in 1956[4] to give the first correct glucosinolate structural formulae (figure 1) as confirmed shortly after by X-ray crystallographic analysis of sinigrin.[56]



Gadamer
1897

Ettlinger and Lundeen
1957

Figure 1: *Ettlinger and Lundeen proposed the first correct glucosinolate structural formulae*

The discovery of new glucosinolates continued with many important contributions by Kjaer and Schultz to give the fascinating class of compounds already described.[7]

Glucosinolates provided a new synthetic challenge which was met by Ettlinger and Lundeen in 1957 with their synthesis of benzyl glucosinolate.[8] However their route was not widely adopted for the synthesis of other glucosinolates due to its low yield and use of carbon disulfide. In 1963 Benn published a more favourable alternative[9] which formed the basis of many future synthetic strategies.

Alongside the glucosinolate investigations came much interest in the enzyme myrosinase responsible for glucosinolate breakdown. In the same year that Bussy isolated sinigrin (1840) Boutron and Fremy reported the action of "myrosin", now called myrosinase,[10] and it was found that myrosinase is actually a group of related isozymes.[11] Ohtsuru and Hata showed that myrosinase catalysed glucosinolate hydrolysis could be greatly accelerated by L-ascorbic acid.[12] In addition Luthy and Matile proposed the "mustard bomb" to describe a possible compartmentalisation of myrosinase, glucosinolates and ascorbate.[13] However the recent publication of the X-ray crystal structure of myrosinase by Burmeister *et al.*[14] provides probably the greatest advancement so far in understanding the enzyme. Thus significant progress has been made in comprehending the glucosinolate-myrosinase system. Further to this is the wealth of biological activity associated with the glucosinolate breakdown products in particular isothiocyanates. Much interest has focused on the interactions of isothiocyanates with biological systems for example as insecticides, oviposition stimulants, biofumigants and also as cancer preventative agents.[15]

This chapter begins by examining glucosinolates. Their occurrence, distribution and compartmentalisation within plants is discussed along with the biological effects associated with their existence. An examination of myrosinase follows detailing its structure, mechanism and specificity. In addition, factors which affect the hydrolytic action of myrosinase on glucosinolates are explored. The modern procedures used to extract and analyse intact glucosinolates are mentioned. Finally, the biosynthesis and chemical synthesis of glucosinolates are discussed in detail.

1.2 Occurrence and Biological Activity of Glucosinolates

1.2.1 Glucosinolate occurrence and variation

Glucosinolates exist as secondary metabolites in families of dicotyledonous angiosperms. In particular they occur in the order Capparales which includes Capparaceae, Brassicacea (Cruciferae), Koeberliniacae, Moringaceae, Resedaceae and Tovariaceae. Other more sporadic occurrences include families such as Caricaceae, Euphorbiaceae, Gyrostemonaceae, Limnanthaceae, Salvadoraceae and Tropaeolaceae.[16] Most scientific interest has centred around crucifers since these plants provide a major contribution to the human diet and also provide feed for animals. Indeed no cruciferous plant has yet been found which does not contain glucosinolates.

It is important to note that although there are many glucosinolates which occur in nature individual plant species do not usually hold a large variety. Horseradish appears to be anomalous possessing a total of 30.[17] In addition, only a few structures will typically predominate in any particular plant organ. Recent studies have shown that glucosinolate types and concentrations could vary within the root system of a single plant. For both Australian oilseed rape and Indian mustard plants, young fine roots (<2 mm diameter) had higher levels of indolyl glucosinolates while the taproot and lateral roots contained more phenethyl and 2-propenyl glucosinolates.[18] Significant changes in glucosinolate types and concentrations are also observed during the growth of a plant. Extensive studies on oilseed rape (*Brassica napus*) showed that during the first stages of growth there was a rapid decrease in the concentrations of glucosinolates found in the seed (2-hydroxybut-3-enyl, but-3-enyl, 4-hydroxyindol-3-ylmethyl and pent-4-enyl glucosinolates); however at the same time other glucosinolates appeared (indol-3-ylmethyl, *N*-methoxyindol-3-ylmethyl and 2-phenethyl glucosinolates).[19] As the plant matured its tissues showed a general decrease in glucosinolate content[19, 20] especially during flowering and seed production.[21] Large variations in glucosinolate levels during the plant's life cycle will lead to

corresponding changes in the protection given by the glucosinolate breakdown products. Therefore at some stages of plant growth insect attraction and infestation could be favoured.[22]

Glucosinolate concentrations do not only change in response to plant growth. Environmental factors such as climate and water, sulfur and nitrogen availability also greatly influence concentrations. These factors are particularly important when considering the economic value of oilseed rape. Glucosinolates contained in rapeseed meal are known to cause antinutritional effects in animals. Therefore from 1992 the European Community only paid subsidies for rapeseed containing less than 20 μmol of glucosinolate per gram of seed.[23]

Studies considering the influence of environmental factors on glucosinolate variation showed that they could be synthesised and degraded under freezing weather conditions,[24] although such temperatures favoured glucosinolate decline. In contrast, drought stimulated oilseed rape to greatly increase its glucosinolate concentrations whether pre- or post-flowering.[25] Disease or insect damage provoked tremendous changes in glucosinolate levels but perhaps the most remarkable observations were provided by Rosa *et al.*[26] who showed large changes in the total and individual glucosinolate content of *Brassica oleracea* within just a single day. Plants therefore have the ability to regulate their glucosinolate levels in response to stress. In 1973 Freeman and Mossadeghi[27] found that stressing a plant by depriving it of water not only increased glucosinolate levels but also increased both inorganic and organic sulfur content. They suggested that under such conditions the plants build up a 'store' of basic metabolites such as sugars and amino acids which can subsequently be converted to secondary metabolites.

Glucosinolates act as a form of storage for nitrogen, carbon and especially sulfur.[19] Consequently biosynthesis of this class of secondary metabolite is influenced by the supply of sulfur and nitrogen. The requirement of oilseed rape for sulfur is often high with as

much as 60-100 kg S Ha^{-1} being used in the growing season.[28] High sulfur concentrations elevate glucosinolate levels[21] while fertilisers with a low sulfur content do not have any marked effect on the amount of glucosinolate contained in oilseed rape.[29] This is largely because sulfur can usually be taken up from the atmosphere. However under sulfur stress *B. juncea* showed a notable decrease in 2-propenyl glucosinolate.[30] Under these conditions glucosinolates are either synthesised in lesser amounts or myrosinase is activated to hydrolyse existing glucosinolates and release sulfurous compounds.[22]

Nitrogen supply also plays an important biosynthetic regulatory role for glucosinolates. Although the effects of nitrogen have not been ratified it appears that an increase will raise the quantities of seed glucosinolate only in the presence of sufficient sulfur.[28] Indeed some studies show a drop in glucosinolate with an increase in nitrogen supply. One suggestion for this phenomenon is that when nitrogen is abundant the plant will synthesise more protein and relatively less carbohydrate. Glucose may therefore become the limiting factor for glucosinolate synthesis at high levels of nitrogen.[29] Alternatively low sulfur concentrations may provide the limiting factor.[31]

These variations in cultivation conditions, climate and agronomic practice can evidently affect the ability of the various plants to produce glucosinolates. It is not known whether these changes serve a defined purpose or appear as a consequence of altered plant metabolism.

1.2.2 Compartmentalisation of glucosinolates/myrosinase

Glucosinolate containing plants release very few volatiles until tissue is damaged in some way so it is reasonable to believe that a degree of compartmentalisation exists within the undamaged plant tissues. As early as 1890 suggestions were made to predict the existence of "myrosin cells"[32] and in 1984 Lüthy and Matile suggested the "mustard bomb" model which illustrated the myrosin cell as shown in figure 2.[13] Myrosinase is shown as membrane bound with the glucosinolates located in the vacuole alongside ascorbate which enhances myrosinase activity. Intact vacuoles treated with exogenous glucosinolates gave hydrolysis products. Furthermore, the association of the thioglucosidase and membrane must be a loose one as nearly all enzymatic activity was lost from membrane preparations after washing.

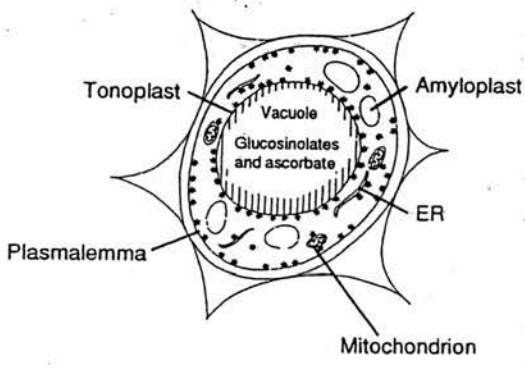


Figure 2: *Myrosin cell as represented by Lüthy and Matile where * represents the localisation of myrosinse*

Despite this, the actual cellular localisation of myrosinase and glucosinolates remains undetermined. In a review by Bones and Rossiter three popular suggestions are given, that myrosinase and glucosinolates are located in different cells, exist in different compartments of the same cell or lie inside the same compartment of the same cell but in an inactive form.[33]

The least likely of the three options is where myrosinase and glucosinolates are localised in different vacuoles of the same cell. In the 14 days after seeds are sown the vacuoles of myrosin cells change considerably via fission and fusion and so regulation of such a system to prevent mixing of myrosinase and glucosinolates would prove highly complex.

Location of myrosinase in special myrosin cells with components such as glucosinolates in separate cells is possible. In this case tissue damage would cause the cells to rupture and their contents to mix.

The final theory involves the coexistence of myrosinase, glucosinolates and ascorbic acid within the vacuole of the myrosin cell. Intact vacuoles would contain a high concentration of ascorbic acid which inhibits myrosinase. On damage to the cell the ascorbic acid is diluted and concentrations fall to an optimum level where it activates myrosinase and promotes glucosinolate hydrolysis.

Unfortunately, the lability of the myrosinase/glucosinolate system has hindered attempts to give a precise account of its organisation and work remains to be done in this area.

1.2.3 Glucosinolates and insects

i) Insecticidal activity of glucosinolates

Glucosinolates act as plant defence agents which are readily activated by myrosinase to give an arsenal of volatiles lethal to many insects. It is rather surprising to find few reports which specifically deal with insecticidal activity and it appears that much work remains to be done to establish good structure-activity relationships in this area.[34] Nonetheless, those reports which are available give a clear indication of the glucosinolate-myrosinase system's toxicity to insects.

Phenethyl glucosinolate is the predominant glucosinolate found in watercress. The hydrolysis product, phenethyl isothiocyanate is toxic to house flies (*Musca domestica L.*) and vinegar flies (*Drosophila melangastor meig*) while confusing flour beetles (*Tribolium confusum duvae*), spider mites (*Tetranchus atlantic*) and pea aphids (*Macrosiphum pisi*).[35] Furthermore, phenethyl isothiocyanate acts as a feeding deterrent to snails (*Phsella spp.*),

caddisflies (*Hesperophylax designatus* and *Limnephilus spp.*) and amphipods (*Gammanus pseudolimnaeus*).[36]

Studies performed on yellow mealworm larvae (*Tenebrio molitor L.*) revealed that glucosinolates and their breakdown products interfere with insect metabolic processes.[37, 38] Primary insect metabolism is essentially the same as it is in animals. However insects are subjected to a wider range of physiological stresses during oviposition, metamorphosis and flight.[39] Due to these increased stresses a particularly efficient glucose transport and transformation ability is required. Trehalose is the main energy storage compound for insects and can be hydrolysed to glucose by the enzyme trehalase.[40] The insecticidal activity of glucosinolates and/or their hydrolytic products is attributed to inhibition of the glycolysis-Krebs cycles which are together responsible for releasing most of the energy contained in glucose. This poisoning of the respiratory metabolism results in a decrease in the total amount of oxygen taken up and carbon dioxide expired by the organism.[37, 38] In addition, any inhibition of glycosidase activity will limit the concentrations of free glucose in the lumen and consequently decrease the amounts of trehalose in the haemolymph.[41] This will result in glucose starvation stimulating the insect to eat more.[42, 43] In this way inactivation of just one essential enzyme system interferes with many other linked digestive activities.

ii) Insect feeding and oviposition stimuli

Pest/plant interactions are complicated because some pests have turned the defence mechanism against the plant. These pests have evolved to use glucosinolates and the volatile breakdown products as cues in identifying a host for feeding or oviposition (egg laying). In addition parasitoids of the specialist insects are also attracted by the volatile compounds.

The first indication of the power of glucosinolates to attract specialist pests was given in a study where *Pieris brassicae* and *Pieris rapae* caterpillars were induced to feed on non-

crucifers and filter paper which were painted with 2-propenyl glucosinolate and *Bunias orientalis* (a crucifer) juice.[44] While little is yet known about the metabolism of glucosinolates by *Brassica*-adapted insects, it has been established that the activity of glucosinolates and breakdown products depends on three main factors: compound concentration, compound structure and type of pest.

Grasshoppers feed preferentially on cultivars with lower isothiocyanate content. The higher the concentration, the less likely the insect is to feed.[45] This trend is common where low concentrations of glucosinolate can have phagostimulant properties and high concentrations deter feeding. While it is usually the overall glucosinolate profile of the plant which governs acceptance for feeding or oviposition, some insects show strong preference for plants containing a particular glucosinolate. For example, alate female aphids (*Brevicoryne brassicae*) are attracted by 2-propenyl isothiocyanate.[46] Similarly, tests using *Phyllotreta cruciferae* and *Phyllotreta striolata* showed 2-propenyl isothiocyanate to be more attractive than ethyl, phenyl and phenethyl isothiocyanate derivatives.[47] Seed weevils (*ceutorhynchus assimilis*) however preferred longer chain alkenyl, benzyl and *p*-hydroxybenzyl isothiocyanates.[39]

These pest/plant interactions have been the subject of many studies particularly with respect to oviposition. Eggs laid in the soil ultimately hatch and the larvae feed on the plant causing large amounts of damage and financial loss to the agriculture industry. There are two main methods for studying the interactions: the first involves use of an artificial leaf and the second is *via* contact chemoreception.[48] The artificial leaf system is constructed of green corrugated card, coated to give a waxy surface and sprayed with a solution of glucosinolate. The oviposition behaviour of the flies can be observed and the number of eggs laid in the soil corresponds to the stimulatory activity of the glucosinolate.

Using this method a pattern of oviposition behaviour has been observed (figure 3). The insect, once it has been attracted to the plant, lands on a leaf exploring it to test the surface

composition and look for specific glucosinolates. It will then either depart or walk down the stem to deposit eggs in the soil.

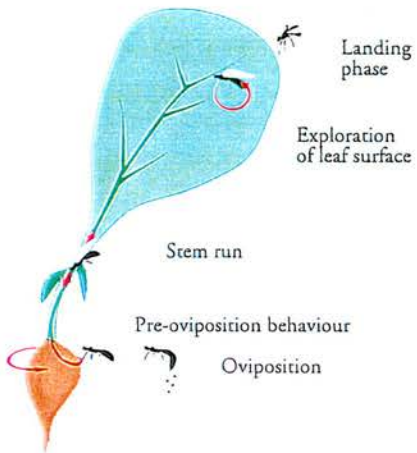


Figure 3: *Behavioural sequence of an adult fly during oviposition*

In studies using artificial leaves there are two processes by which the insect selects its host. The first is an olfactory response to the plant volatiles however this is not enough to induce oviposition. The second part of host selection is known as chemoreception where the insect inspects the plant by contact using special receptor cells. Receptor cells can be found on many major parts of the body including the mouth, wings and ovipositor of flies. Adult females of *Pieris brassicae* and *Delia brassicae* have been observed to use tarsal chemoreceptors in a "drumming" behaviour when ovipositing, allowing contact with compounds lying beneath the waxy leaf surfaces.[49, 50]

Using contact chemoreception an electrical response due to stimulation of a receptor cell, sensitive to glucosinolates, is produced. This response is detected using a tip recording technique where the stimulating solution is contained in a glass capillary and functions as an electrolytic bridge. The capillary acts as a recording electrode linked via an Ag/AgCl junction to the amplifier.[51] In this way the impulses can be measured, amplified and classified according to amplitude (figure 4).

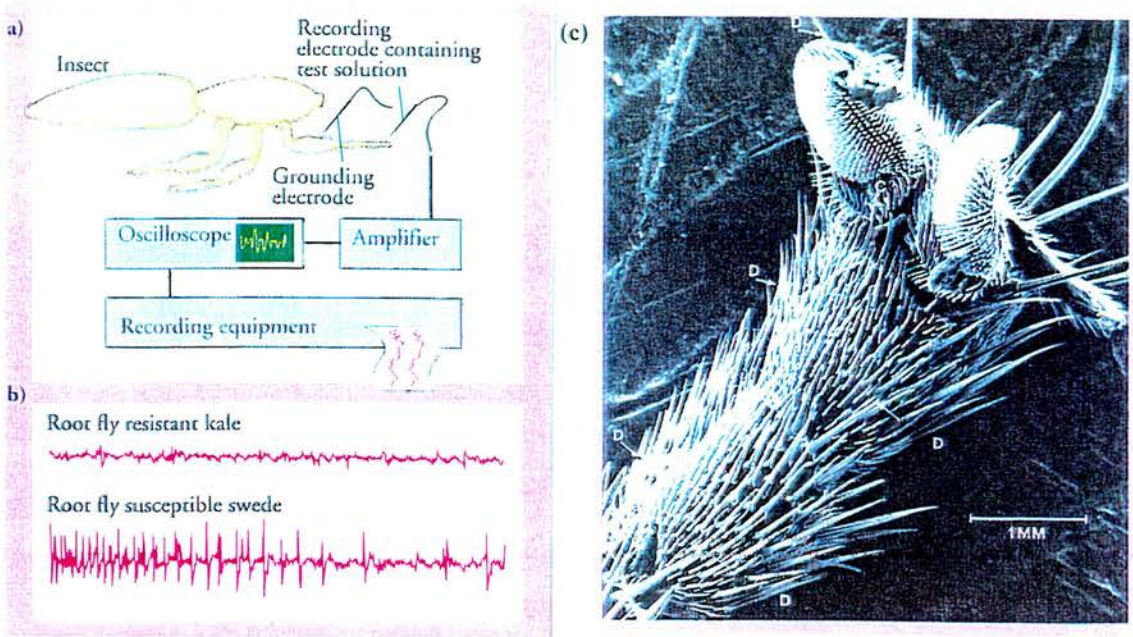


Figure 4: (a) *Diagram of electrophysiological system used to record stimulations of root fly tarsal receptors.* (b) *Example chemosensory responses.* (c) *SEM picture of the female cabbage root fly prothoracic tarsus showing chemoreceptor hairs.*

In general, glucosinolates possessing an indolic side chain are the most potent oviposition kairomones. Leaves of root damaged kale show increased levels of indole glucosinolates and *Delia radicum* prefers to lay its eggs near such plants. Similarly, ovipositing females of *Pieris brassicae* and *Pieris rapae* seek out plants which hold large quantities of tryptophan or its derivatives.[52] Although the reason for this is not clear there are two convincing explanations. Firstly, an amino acid receptor cell which is stimulated by tryptophan, an essential insect nutrient, has been identified in both *Pieris brassicae* and *Pieris rapae* larvae.[53, 52] The amount of tryptophan used in glucobrassicin synthesis is substantial and therefore the presence of this class of compound may act as a marker to the ovipositing adult, indicating the plant's high nutritional potential. The second explanation is that the breakdown products of the glucobrassicins are non-volatile and as such will be less likely to betray the presence of the feeding larvae to generalist predators.[52]

Interestingly it is not only glucosinolates which are capable of stimulating insects such as *Delia radicum* to oviposit. Braven *et al.* showed that other molecules containing an S=O group were also active.[54] Roessingh *et al.* then reported the presence of an unknown non-

glucosinolate oviposition stimulant in *Brassica oleracea*.[55] This unidentified compound is claimed to be even more potent than glucobrassicin for inducing egg laying in *Delia radicum*.

The simultaneous insecticidal and attractant properties of the glucosinolate system are problematic when designing cruciferous crops which are inherently resistant to insect attack. Reducing the quantities of glucosinolate in the crop can give increased attack by non-specialist insects. Increasing the quantities of glucosinolate not only makes the crop unpalatable but can also provide adverse biological effects. Detailed studies on the pest/plant interactions may provide further information on the relative activity of specific glucosinolates and give clues to solving the plant's battle against insects.

1.2.4 Glucosinolates and fungi

The anti-fungal properties of glucosinolates and their enzymatic hydrolysis products have been known for many years. The isothiocyanate products are particularly active against fungi and bacteria. 2-Propenyl isothiocyanate is mutagenic to *Salmonella typhimurium* TA100[56] while phenethyl thiocyanate and 4-methylsulfonylbutyl isothiocyanate inhibit growth of *Staphylococcus aureus* and *Penicillium glaucum*.[57] These properties have prompted studies on the potential use of glucosinolates and their hydrolysis products as natural plant protective agents, biocidal compounds for postharvest fruit storage against fungal pathogens such as *Botrytis cynerea* and as soil biofumigants.[58]

The response of eight pathogenic fungi to the hydrolysis products of 3-methylsulfinylpropyl, benzyl, 2-propenyl and 2-hydroxy-3-butenyl glucosinolates were examined.[59] The study found that the native glucosinolates held no cytotoxic or fungitoxic activity but the isothiocyanates inhibited fungal growth. Isothiocyanates with more lipophilic character were more active and it was postulated that such compounds would pass more easily through cell membranes increasing cellular concentration. Furthermore,

incorporation of sulfur into the side chain enhanced activity. However other studies have shown the fungicidal activity to be more complex. Different fungi tend to show significant variations in their sensitivity to volatiles released from cruciferous tissues. It is therefore likely that each pathogen needs to be tested individually.[60] Nonetheless isothiocyanates may prove to be a promising eco-friendly response to deter soil-borne pathogenic fungi. Some fungal propagules can remain active in soil for considerable periods of time and are difficult to control with fungicides. Studies have suggested that isothiocyanates may be a more effective means of control.[60] It is important to consider that the amount of isothiocyanate released from decomposition of crucifer tissue is relatively small and the biofumigant properties are attributed to the combined effect of low quantities of highly toxic isothiocyanates and large quantities of less toxic volatiles not derived from glucosinolates.[61]

1.2.5 Glucosinolates and cancer

Glucosinolate breakdown products are responsible for the characteristic smell and biting taste of many plants and this has led to their widespread popularity in the human diet, either alone or in condiments such as mustard. Glucosinolate hydrolysis products have therefore been the subject of countless biological studies which all indicate the anticarcinogenic potential of isothiocyanates. This property was first discovered by Sasaki[62] who observed that the incidence of 3'-methyl-4-dimethylaminoazobenzene induced liver tumours was reduced in male wistar rats by prior administration of α-naphthyl isothiocyanate. Additional studies by Wattenberg[16] in the period between 1976 and 1979 illustrated that phenyl, benzyl and phenethyl isothiocyanates (or metabolites therefrom) were effective in mice for suppression of forestomach and lung tumours by the carcinogen 3,4-benzpyrene. Similarly, 7,12-dimethylbenz(a)anthracene induced mammary tumours were suppressed by both benzyl isothiocyanate and thiocyanate. Results such as these prompted recommendations by national committees in the early 1980s for consumption of *Brassica* vegetables to prevent cancer.[63] Since then, an overwhelming number of papers have

illustrated isothiocyanates as potent inhibitors of tumourogenesis. This is particularly evident for colon, lung, stomach and rectal cancer.[64] Other less consistent studies have shown positive effects for prostatic, endometrial and ovarian cancer.[64] Prior to considering inhibition of cancer it is necessary to first consider the three biochemical phases involved in detoxification of cancer inducing compounds.

Phase I - Introduction of new functional groups into lipophilic compounds via oxidation, reduction and hydrolysis. This renders the carcinogen more hydrophilic and therefore more amenable to reaction with nucleophiles and detoxification via phase II enzymes. Such reactions can inhibit activation of the procarcinogen or activate it to a highly reactive carcinogen. The principal phase I enzymes are cytochromes P-450 (P450s).

Phase II - Conjugation reactions between small endogenous molecules and functional groups present (or introduced by phase I metabolism) in the xenobiotic. Alternatively reactive centres can be destroyed for example hydrolysis of epoxides or reduction of quinones. The resulting molecule is then less reactive but is sufficiently polar to be excreted. Examples of phase II enzymes include glutathione S-transferases (GST), UDP-glucuronyl transferases, epoxide hydrolase and NADPH:quinone oxidoreductase (QR).

Phase III - This is the final step whereby the xenobiotic is transported out of the cell

It is the balance between activating and deactivating phase I and II enzymes which determines the degree to which a carcinogen will affect its target organ. Glucosinolate breakdown products are known to alter the enzymatic balance and hence modulate the carcinogen biotransformation. In addition a recently observed effect is the induction of apoptosis which has been observed with allyl isothiocyanate. Here the isothiocyanates cause precancerous gut cells to "self destruct."[65]

Many isothiocyanates are quoted as monofunctional inducers, they induce phase II enzymes selectively. In this respect a great deal of interest has been centred around

sulforaphane an isothiocyanate breakdown product of glucoraphanin which is found in broccoli (figure 5).[66, 63, 67, 68] Sulforaphane is currently the most potent naturally occurring inducer of phase II enzymes known, particularly of QR and GST.[69] Structure activity studies on sulforaphane[66] showed that the oxidised sulfur enhanced potency and 4 or 5 methylene carbons gave optimal activity. Furthermore, loss of the methylsulfinyl group decreased activity but it could be replaced by an acetyl group.[66, 69] It is important to note that sulforaphane was also reported to inhibit P450s from cultured rat hepatocytes in a reversible fashion.[70] In addition it has been shown that 3-methylsulfinylpropyl and *p*-hydroxybenzyl isothiocyanates can inhibit the transcription of P450s.[71] All of these factors probably act in combination to produce the chemoprotective properties.

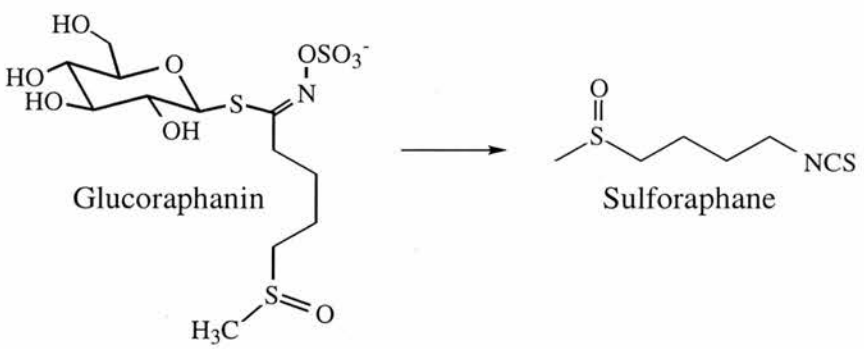
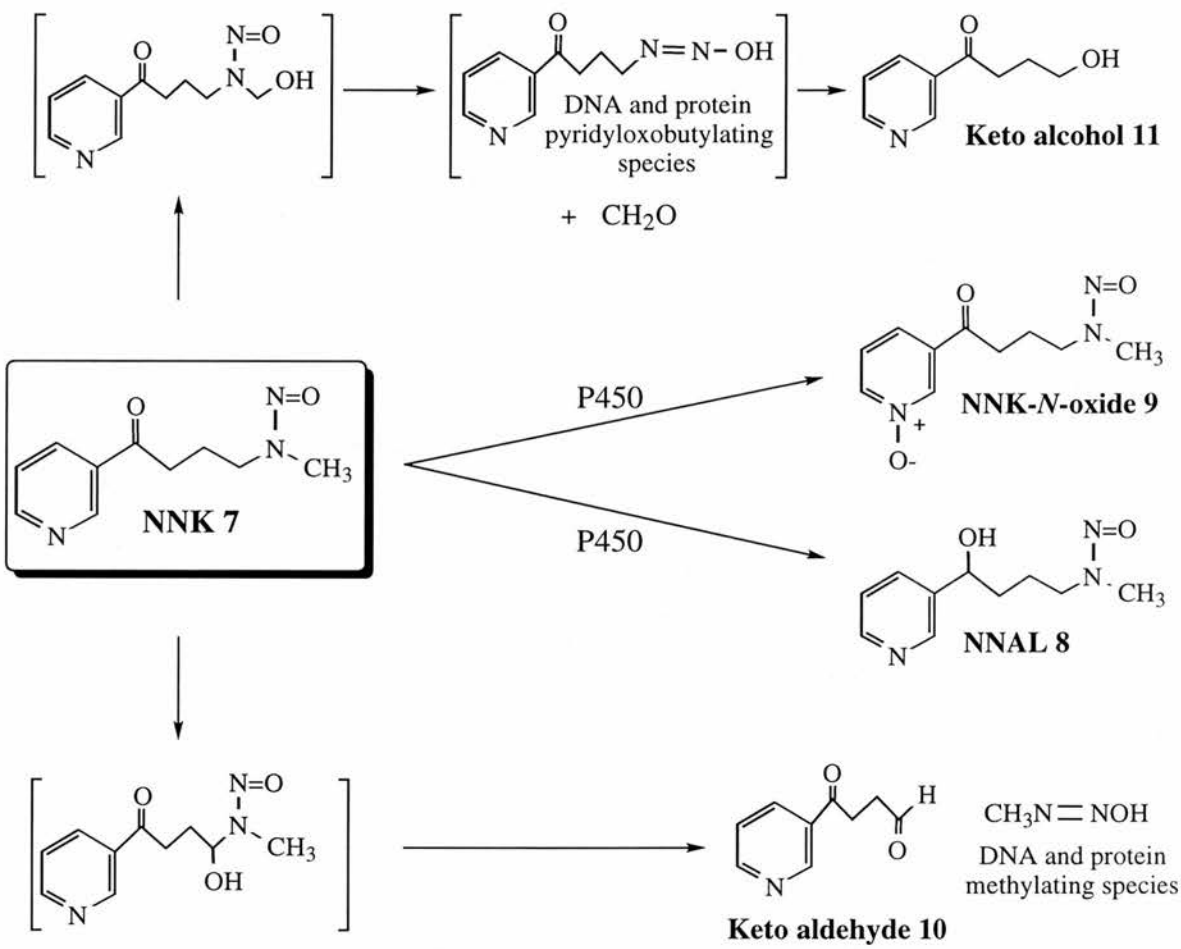


Figure 5: *Hydrolysis of glucoraphanin to yield sulforaphane*

Isothiocyanate inhibition of P450s is particularly significant in suppressing the activation of the carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).[72] NNK is reported as the most potent tobacco-specific carcinogenic nitrosamine ever tested in rodent lung tumourogenesis and the worldwide human exposure to NNK leads to the need to investigate methods of inhibiting its effects.

NNK **7** is activated via three pathways which are illustrated in scheme 2.[73, 74, 75] P450s are involved in catalysing both the α-hydroxylation reaction to give 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) **8** and the *N*-oxidation to NNK-*N*-oxide **9** although participation of flavin-containing monooxygenase (Ziegler's enzyme) has also been

suspected for the *N*-oxidation pathway.[73] NNK *N*-oxidation is thought to be a detoxification process given that the *N*-oxide is not only a weaker lung carcinogen than NNK in mice but it is also more polar and easily excreted. Finally, α-hydroxylation of the methyl or methylene carbon adjacent to the *N*-nitroso group gives either a keto aldehyde **10** and release of a methylating species or a keto alcohol **11** and a pyridyloxobutylating species. These metabolites methylate and pyridyloxobutylate purine bases of DNA causing modification, such as O^6-methylguanine formation, and cancer development.[76]



Scheme 2: *Metabolic pathways of NNK*

A number of studies have focused on the ability of arylalkyl isothiocyanates to inhibit P450s and subsequent metabolic activation of NNK. Phenethyl isothiocyanate (PEITC) has proven to be one of the most effective P450 inhibitors[71] and has been widely studied.

It showed a 70-90% inhibition of NNK α-hydroxylation at a concentration of 50 μM in cultured rat oral tissue.[75] Inhibition of *N*-oxidation was even more striking with 80-90% inhibition at a concentration of 10 μM.[75] Furthermore, PEITC decreased DNA methylation and pyridyloxobutylation by NNK in the lungs of F344 rats by 50%.[77] Increasing the chain length of arylalkyl isothiocyanates shows a corresponding decrease in NNK induced lung tumourogenesis[78, 79, 80, 81, 82] with 5-phenylpentyl and 6-phenylhexyl proving particularly active. Longer alkyl chains may favour binding of the isothiocyanate to the P450 active site. In addition, the isothiocyanate moiety is essential to reduced tumourogenesis.[79] It is important to note that PEITC (and presumably the longer chain derivatives) acts as a blocking agent and as such must be administered before NNK treatment in order to be effective.

Several authors have indicated the ability of indolyl glucosinolate hydrolysis products to reduce the incidence of cancer.[83, 84, 85] Indeed recent research has shown that intact indol-3-ylmethyl and 4-methoxyindol-3-ylmethyl glucosinolates are effective inducers of detoxification enzymes in male Wistar rats,[86] however there has been some conflicting research. It was reported that nitrite treatment of sauerkraut, pickled vegetables, Chinese cabbage and fava beans led to their "direct mutagenic activity."[87] This prompted more work using Chinese cabbage which showed that indole-3-acetonitrile, 4-methoxyindole-3-acetonitrile and 4-methoxyindole-3-aldehyde could act as precursors of *N*-nitroso compounds. However work by Tiedink *et al.* illustrated that the *N*-nitroso compounds were only stable in large amounts of nitrite.[88, 89, 90, 91] Additionally if the food matrix was present glucosinolates and their breakdown products gave an insignificant contribution to nitrosated products and do not therefore appear to pose any risk to human health. Moreover indole-3-methanol and its condensation products have been shown to function as dietary antiestrogens and may therefore protect against breast cancer.[92, 93, 94]

Due to the many naturally occurring isothiocyanates which show very promising chemoprotective properties there exists a real possibility of reducing the incidence of cancer

in humans through dietary methods.[95] Recent research on human consumption of cruciferous vegetables has illustrated a decrease in the metabolic activation of carcinogens in smokers. Furthermore it appears that even cooked vegetables such as watercress, where myrosinase activity has been completely abolished can be of benefit due to the breakdown of glucosinolates by gut microflora.[96] Some reports also suggest that glucosinolate breakdown products contribute in a synergistic way to anticarcinogenic action.[97, 86] However a significant amount of research remains to be done in this field before the full potential of isothiocyanates in cancer prevention can be realised.

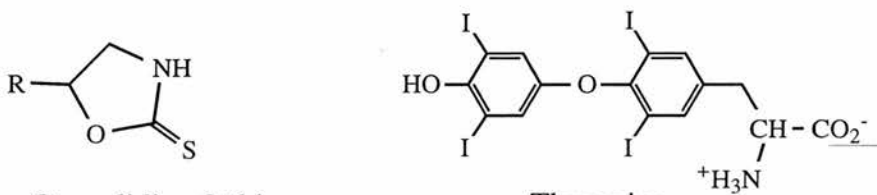
1.2.6 Glucosinolates and goitre

Not all of the biological effects of glucosinolates are beneficial. There are various reports of toxicological effects attributed to glucosinolates and their breakdown products. Hydroxynitriles and glucosinolates found in rapeseed have been implicated in liver haemorrhage development in poultry[98] while in male Fischer rats, the effects were enlargement of adrenal glands, kidneys and liver.[87, 99] In pregnant female Holtzman rats embryotoxic effects of 2-propenyl and 3-methylsulfinylpropyl isothiocyanates and 1-cyano-3,4-epithiobutane have been reported.[100] Furthermore, evidence exists for contact dermatitis development in humans who frequently handle *Brassica* vegetables and leaves.[101]

Possibly the most commercially significant detrimental feature of the glucosinolate products rests with goitrogenetic properties. Oilseed rape contains a good balance of oil, amino-acids and high quality proteins and therefore rape-seed meal provides an excellent source of food for cattle, but the occurrence of glucosinolates in the rape flour limits its nutritive value.[102]

The first report of goitre development as a result of *Brassica* vegetables was published in 1928 when rabbits fed large amounts of cabbage developed the condition.[103] It was also

noted that the symptoms could be alleviated by increasing dietary iodine.[104] In the years since then it has been established that there are two main routes whereby glucosinolates exert their goitrogenic effects.[87] One route is where thiocyanate ion formed *in vivo* competes with iodine for uptake by the thyroid gland. In this case goitre will develop only if there is a deficiency of iodine in the diet.[105] The second goitrogenic pathway involves mainly oxazolidine-2-thiones which are believed to inhibit thyroxine synthesis (figure 6). Increasing dietary iodine in this instance has minimal effect[106] and it is necessary to add thyroxine/thyronine to relieve the symptoms.[107]



Oxazolidine-2-thione Thyroxine

Figure 6: *Oxazolidine-2-thiones inhibit thyroxine synthesis*

On average over 96% of human goitre is caused purely by basic iodine deficiency.[108] A study performed on humans in 1986 involved a diet containing 150 g of Brussels sprouts over a 4 week period.[109] In this study, despite ingestion of significant quantities of glucobrassicin and progoitrin, no effects were observed on human thyroid function when measuring levels of triiodothyronine and thyroxine in the blood. Evidence is therefore lacking for any link between glucosinolate breakdown products and the incidence of human goitre.

As already mentioned the incidence of goitre in cattle as a result of glucosinolate anti-nutritional properties is problematic and a commercial concern. So much so that the EC have recommended maximum levels of glucosinolates in rape-seed and this has led to major programmes to breed safer varieties.[33] It is through such breeding programmes that cultivars containing low levels of erucic acid have been produced and are known as "single-low" or "single-zero" varieties. These cultivars were not ideal since remaining hydroxyalkenyl and aliphatic glucosinolate hydrolysis products reduced the quality of the

rapeseed. As a result plant breeding focused on reducing the levels of glucosinolate to produce cultivars known as "double-low" or "double-zero" low in both erucic acid and aliphatic glucosinolates. However, the levels of indolyl glucosinolates in double-low species remained constant.[16, 110] Chavadej *et al.* succeeded in reducing plant content of this glucosinolate to 3% of its original levels via metabolic engineering.[111] This particular approach redirected tryptophan to give tryptamine rather than indole glucosinolate through incorporating a gene encoding tryptophan decarboxylase into oilseed rape plants (figure 7). This gene provided an artificial metabolic sink with the potential to increase the nutritive value of rapeseed meal.

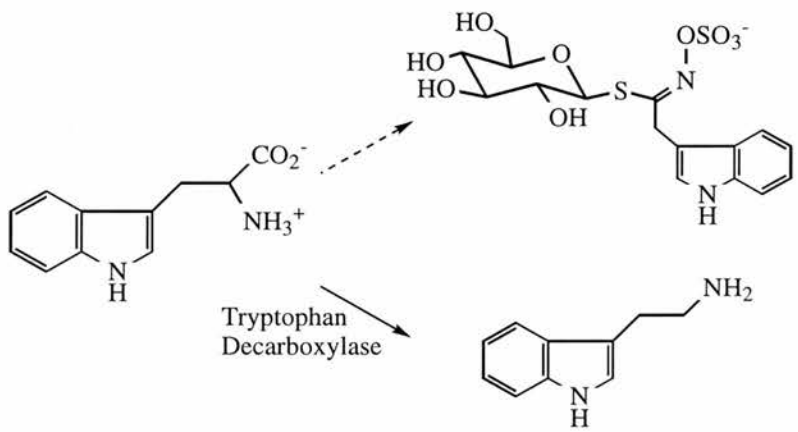


Figure 7: *Redirection of tryptophan in oilseed rape*

A novel chemical method of reducing glucosinolate levels has also been published which involves extraction of glucosinolates using synthetic macromolecular hosts.[112] The host binds quite strongly and selectively to glucose but unfortunately the method uses carbon tetrachloride as a solvent and does not allow recovery of the glucosinolates. Other chemical methods of glucosinolate extraction have shown accompanying reductions in both the vitamin and mineral content of protein isolates and concentrates. The host-guest technique does not seem to suffer these disadvantages and is currently capable of a 66% reduction of glucosinolate.

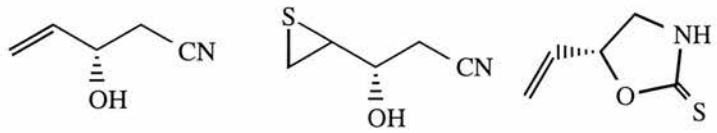
Probably the most promising and recently reported method of glucosinolate extraction involves the use of enzymes to degrade cell walls followed by separation of the aqueous

emulsion.[113] This not only allows recovery of high quality oil and different proteins but also allows the glucosinolates to be isolated and subsequently used to provide a range of useful fine chemicals.

1.2.7 Exploitation of a natural reaction

The ability of myrosinase to produce a variety of hydrolysis products from glucosinolates can be extremely useful as some of these products are not easily synthesised by traditional synthetic procedures. One method of exploiting the enzymatic reaction is to incorporate myrosinase into reverse micelles to produce a bio-reactor.[113, 114, 115, 116] In this low water system myrosinase and glucosinolates are encased in an aqueous environment by surfactant molecules. The surrounding organic solvent provides an ideal medium into which the hydrophobic glucosinolate hydrolysis products can pass. This system would therefore only be useful for glucosinolates containing hydrophobic side chains.

Another promising method of producing fine chemicals is the use of myrosinase immobilised on a support such as nylon 6.6 or gamma-alumina.[117, 118, 119] Using such technology it is possible to produce a variety of products by altering reaction conditions such as pH. Myrosinase is particularly suited to this as it has a broad optimum pH. Furthermore myrosinase would provide an ideal industrial catalyst as it is exceptionally stable when immobilised on a nylon 6.6 membrane and was reported to retain all of its activity after a period of 15 months when it had been used for approximately 1000 assays.[119] Studies using immobilised myrosinase have centred around the hydrolysis of epi-progoitrin which is typically hydrolysed to epi-goitrin but in the presence of Fe^{2+} under acidic conditions will yield 3-hydroxypent-4-enonitrile. Also in the presence of epithiospecifer protein and Fe^{2+} epithionitrile is the product (figure 8).



4-hydroxypent-4-enonitrile epithionitirile epi-goitrin

Figure 8: *Potentially useful breakdown products of epi-progoitrin*

Epi-goitrin itself is useful synthetically as the sulfur atom provides a soft base, while the NH is a hard base. There is also a centre of asymmetry to which is attached a vinyl moiety so chemical modification of this compound becomes a real possibility and work is underway to incorporate the structure into a family of systemic herbicides known as urons.[113, 120]

1.3 Myrosinase and Glucosinolate Hydrolysis

1.3.1 Myrosinase and its mechanism

Myrosinase is the trivial name given to a group of related isoenzymes which catalyse the hydrolysis of glucosinolates[11] and are found in plant tissues, mammalian tissues, bacteria, aphids and fungi.[115] By far the most common source of myrosinase is plant tissues from which various isoenzymes have been separated by several groups using gel electrophoresis. However it is not known whether all the bands observed are in fact real isoenzymes with a distinct amino acid sequence. Furthermore the reason for the presence of different isoenzymes is not yet understood[121] but may relate to different endogenous conditions found in the plant, particular glucosinolates that exist in the tissue or to the conditions found in the organism.[33] The number of isoenzymes identified depends on plant species, tissue type and age. For example, Buchwaldt *et al.*[121] observed the presence of at least 14 isoenzymes in the crude extract of white mustard (*Sinapis alba*) while Lönnerdal and Janson found four in rapeseed (*Brassica napus*).[122] The enzyme's molecular weight typically ranges from 120 to 150 kDa in mustard and rapeseed,[11] while the *Wasabia japonica* enzyme is exceptional with a molecular weight of 580 kDa.[123]

Myrosinase can be viewed as catalysing a reaction which is very similar to the hydrolysis of glycosides by β-glycosidase enzymes. However for myrosinase the substrate is a sulfur rather than an oxygen glycoside and there is a subsequent Lossen rearrangement. Despite the similarity of the two types of enzyme their catalytic machinery appeared to be different as evidenced by the fact that transglycosylation reactions, commonly catalysed by β-glycosidase enzymes, do not occur with myrosinase.[124]

Myrosinase genes were cloned and sequenced and the deduced amino acid sequences strongly resembled several *O*-glycosidases in particular cyanogenic β-glycosidases. However the greatest advancement in the elucidation of the mechanism and structure of myrosinase has been provided by the X-ray crystal structure of *Sinapis alba* myrosinase which was recently solved by Burmeister *et al.*[14]

All myrosinases contain a hydrophobic pocket which lies on the aglycone side of the active site and it is likely to be occupied by the hydrophobic part of the glucosinolate. In agreement with this MacLeod and Rossiter[125] had postulated that glucosinolates with hydrophobic side chains would be hydrolysed at a much faster rate than those possessing more hydrophilic side chains. The sulfate of the glucosinolate is bound by two arginine residues which are not present in β-glycosidases but are conserved amongst myrosinases. This is probably a specific recognition site for myrosinase. A further residue which is likely to be involved in substrate recognition is tryptophan which is often present in β-glycosidases and in myrosinase it is believed to be in Van der Waals contact with the sulfur of the thioglycosidic linkage. Importantly, modelling studies performed using the crystal structure of sinigrin illustrated a significant clash between the enzyme and the aglycone.[14] It was necessary to distort the glucose moiety of sinigrin to a twist boat conformation to allow the glucose moiety, the sulfate and the aglycone to fit into the active site (figure 9).

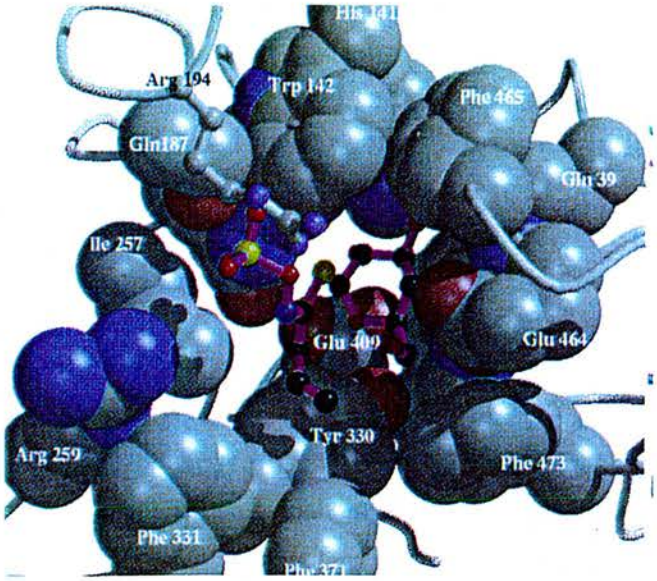
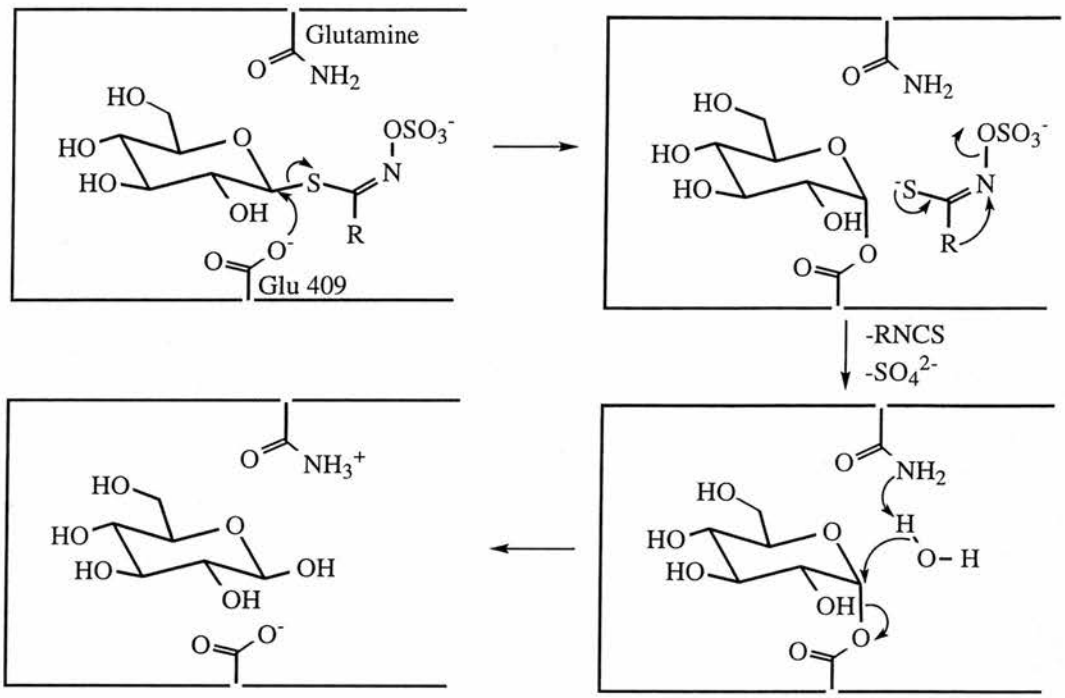


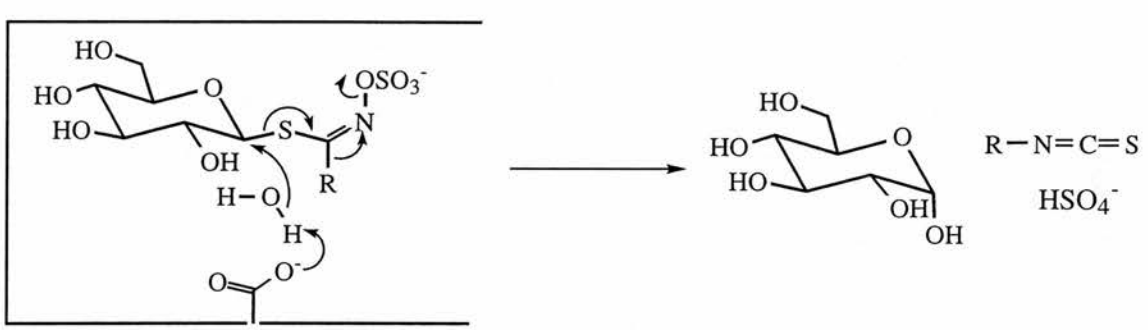
Figure 9: *X-ray crystal structure of myrosinase active site with modelled sinigrin*

The myrosinase catalysed hydrolysis of glucosinolates proceeds in a manner analogous to *O*-glycoside hydrolysis by β-glycosidases. However, unlike the β-glycosidases, myrosinase does not require glutamate as an acid catalyst to protonate the departing aglycone. It appears that the high acidity of the glucosinolate aglycone makes acid catalysis unnecessary. This is supported by the ability of myrosinase to hydrolyse only those *O*-glycosides which have good leaving groups such as *p*-NPG (*p*-nitrophenyl-β-D-glucoside) and *o*-NPG.[124] In myrosinase the catalytic glutamate would give an unfavourable interaction with the sulfate of the substrate and therefore it is replaced by a glutamine (scheme 3).[14] The glutamine is precisely positioned, fixed in place by hydrogen bonds. It is thought that the function of this residue is to bond to a water molecule giving exact delivery for hydrolysis of the glycosyl-enzyme intermediate.



Scheme 3: *mechanism of myrosinase catalysed glucosinolate hydrolysis*

Another important advance in the elucidation of the mechanism of myrosinase was provided by Cottaz *et al.*[126] who used ^1H n.m.r. to study the stereochemistry of the myrosinase catalysed hydrolysis of sinigrin. The work showed that β-glucose was the initial hydrolysis product of the reaction and therefore any α-glucose which existed was due to mutarotation of the β-form. This disproved any speculation that a concerted mechanism could operate (scheme 4).



Scheme 4: *Concerted mechanism of myrosinase catalysed glucosinolate hydrolysis*

Cottaz *et al.*[126] also performed experiments to trap a proposed glycosyl-enzyme intermediate in a similar fashion to the β-glycosidase work of S. Withers and collaborators.[127] 2-Deoxy-2-fluoroglucotropaeolin was thus used as a potential mechanism based inhibitor of *Sinapis alba* myrosinase. It proved to exhibit time dependent inactivation of the enzyme due to formation of a 2-fluoro-2-deoxyglycosyl enzyme intermediate which did not turnover (figure 10). This intermediate existed in the expected α conformation and the pyranose ring was an undistorted $^4C_1$ chair.

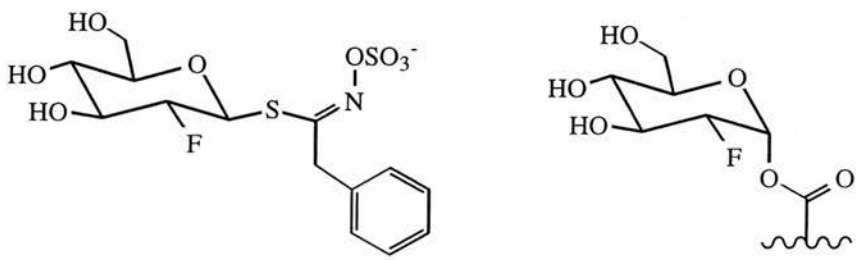


Figure 10: *2-Deoxy-2-fluoroglucotropaeolin reacts to form a 2-fluoro-2-deoxyglycosyl enzyme intermediate*

Spontaneous reactivation of myrosinase was possible by incubating in buffer. Interestingly incubation with 200 mM sodium azide reduced the half life from 46 hrs to 20 hrs. The azide appears to be competing with water to attack the glycosyl-enzyme intermediate. From this evidence it appears that transglycosylation is possible with acceptors that do not require deprotonation by a basic catalyst.

1.3.2 Features of myrosinase

Myrosinase is a remarkably stable enzyme with activity remaining unchanged even after storage for 6 months at pH 6.0.[11] This stability seems to be a consequence of the different environments the enzyme must endure and is achieved through a large number of molecular interactions which exist within its structure. Hydrogen bonds between main-chain atoms and charged residues stabilise the main-chain conformation, along with many salt bridges.[14] Furthermore myrosinase exists as a dimeric species, stabilised by a tetrahedrally

coordinated Zn(II) ion, which serves to reduce the amount of surface exposed.[14] Myrosinase is a glycoprotein with carbohydrate chains distributed over the entire surface, accounting for 9-23% of the total mass. There are commonly five or six glycosylation sites but *Sinapis alba* myrosinase has ten *N*-glycosylation sites which includes a complete plant specific heptasaccharide.[14] The different degrees of glycosylation may be related to the different enzyme environments.

Another recently observed molecular interaction for myrosinase is its association with several polypeptides the role of which is unknown. It is known however that wounding induces the expression of these proteins and they could therefore have some role in response to tissue damage.[128] Furthermore, myrosinase and its binding proteins (MBP97 and MBP70) are co-localised in myrosin grains of myrosin cells in cotyledon tissues of young *Brassica napus* seedlings.[129] A group of proteins called myrosinase-binding protein-related proteins are found in almost all plant parts and have a typical molecular mass of 80-100 kDa. A set of smaller proteins found only in seeds are known as myrosinase-binding proteins (MBP). These have a molecular mass of *ca.* 50 kDa. The different properties, such as solubilities, of these protein sets make it likely that they will have different functions. However much more research is required to establish their importance in the regulation of the myrosinase-glucosinolate system.

The properties of myrosinases can vary quite significantly between plant species and also between cultivars In particular differences are observed in optimum temperature and pH, degree of activation by L-ascorbic acid and relative activity. One of the most exceptional claims of myrosinase stability is provided by Hasapis and MacLeod who refer to a thermal stability of 7 hours 30 minutes at 125 °C for *Lepidium sativum* seed myrosinase under dry heat conditions.[130] A contrasting report by Mcmillan *et al.* states that myrosinase contained within Brussels sprouts does not survive boiling with inactivation occurring after only a few minutes.[109] However, it is generally found that myrosinase has an optimum temperature of 60 °C and above this denaturation occurs. In addition the optimal pH of the

various isozymes from *Sinapis alba* and *Brassica napus* was recorded by Björkman and Lönnerdal and were found to span a wide range from pH 4.5 to 9.0.[11]

Myrosinase is specifically activated by L-ascorbic acid but the degree of activation observed depends once more on the isoenzyme examined. For example, *Sinapis alba* myrosinase is much more active than the enzyme from *Brassica napus* seed but it is not activated as strongly by L-ascorbic acid.[11] Mustard enzyme hydrolysis of sinigrin shows a 25 fold rate enhancement with just 1 mM L-ascorbic acid.[12] Interestingly, high concentrations of L-ascorbic acid are shown to competitively inhibit reaction and analogues of L-ascorbic acid have little or no effect.[124] In 1968 Tsuro and Hata proposed that L-ascorbic acid bound to a specific site on the enzyme and that this binding caused the conformation of the active site to alter in such a way as to improve binding of the glucosinolate aglycone.[131] However at high concentrations L-ascorbic acid can also inhibit myrosinase by competing with the substrate for the active site.

Until 1990 it was accepted that the rate of hydrolysis of *p*-NPG by myrosinase was unaffected by L-ascorbic acid. The reasoning for this was that *p*-NPG bound to the enzyme largely through its sugar moiety and therefore did not occupy the aglycone region of the active site. A change in the aglycone binding site via association with L-ascorbic acid would consequently have no effect on the rate of the hydrolysis. In 1990 Durham and Poultron[132] cast some doubt on this theory when they showed a 2.6 fold activation of *p*-NPG hydrolysis in the presence of 1 mM L-ascorbic acid. This opened up a new theory for myrosinase catalysed glucosinolate hydrolysis where only the glucose and sulfate are involved in binding to the active site. The side chain would therefore lie outwith the binding pocket. This theory would imply that any conformational change provided by L-ascorbic acid lay within this newly defined active site. In light of the myrosinase crystal structure which illustrates that there is a hydrophobic binding pocket which can accommodate the glucosinolate aglycone, Tsuro and Hata's proposal is the more likely, although no L-ascorbic acid binding site has yet been observed in the X-ray structure.

Two further compounds which have been found to enhance myrosinase activity are salicylic acid and methyl jasmonate which are suspected plant defence signal compounds.[133] Chinese cabbage cultures which were treated with 2 μM methyl jasmonate showed 2-3 times greater myrosinase specific activity than a control, while 500 μM methyl jasmonate proved to slightly decrease specific activity. Salicylic acid at 1 mM concentration also decreased the specific activity. This provides the first tentative indication that myrosinase activity could be modulated by a defence signal pathway in *crucifers*.

1.3.3 Specificity of Myrosinase

Myrosinase will hydrolyse all naturally occurring glucosinolates regardless of the wide variety of available side chains. However the rate of hydrolysis varies. Furthermore the sulfate group has been found to be necessary to maintain the enzymatic activity as desulfoglucosinolates were not cleaved by myrosinase.[134] The kinetics of hydrolysis of six different glucosinolates with four different myrosinase isoenzymes from *Sinapis alba* and *Brassica napus* was studied.[11] Each isoenzyme showed the same characteristics with the rate of hydrolysis increasing in the order of methyl, *p*-hydroxybenzyl, 2-hydroxy-3-butenyl, 3-methylsulfonylpropyl, 2-propenyl and benzyl glucosinolate. The one anomaly in this trend was for 2-hydroxy-3-butenyl glucosinolate which was hydrolysed at a faster rate by one of the *Brassica napus* isoenzymes and at a slower rate by a *Sinapis alba* isoenzyme. This investigation illustrated that the isoenzymes had a very similar substrate specificity and had not evolved to hydrolyse any individual class of glucosinolate. In general hydrophobic glucosinolates are hydrolysed at a faster rate than hydrophilic ones while rates of hydrolysis of glucosinolates from a particular class do not vary significantly.

Myrosinase will hydrolyse all naturally occurring glucosinolates but there are few novel analogues which will react. Studies on *Lepidium sativum* myrosinase with a set of 29 natural and synthetic *O*- and *S*-glycosides showed that only four were cleaved. These were sinigrin, benzyl glucosinolate, *o*-NPG and *p*-NPG.[132] The synthetic *o*-NPG and *p*-NPG

were cleaved only because they had an aglycone which was a good leaving group. Amongst the 25 inactive glycosides were PNP-α-D-glucoside and PNP-thio-β-D-glucoside.

The preceding results are typical of many studies which have attempted to find novel sugar derivatives of glucosinolates which will be cleaved by myrosinase. These sugar derivatives included various α-glucosinolates[135] and malto and cellobio disaccharide analogues of indol-3-ylmethyl glucosinolate.[136] It appears that the β-glucose structure is essential to bind the glucosinolate in such a way that it will react. Other derivatives at best show decreased reactivity.

The relative importance of the sugar hydroxyl groups was assessed by Iori *et al.* who synthesised the 2-, 3-, 4- and 6-deoxy derivatives of benzyl glucosinolate and studied their kinetics with *Sinapis alba* seed myrosinase.[137] All of the hydroxyl groups proved to provide important interactions with the enzyme as all deoxy analogues were poorer substrates than native benzyl glucosinolate. This was particularly evident for the 2-deoxy compound which was not hydrolysed by myrosinase. This hydroxyl group is not only important in binding to the active site but is crucial to the enzymatic hydrolysis providing polarisation of the *S*-glucose bond and thereby aiding nucleophilic attack at the anomeric position. Furthermore, this analogue was the first reported compound to show strong competitive inhibition of myrosinase (K_i 1.9 x 10^{-5} M). This feature in itself could have a useful application in the direct isolation of high quality myrosinase from crude plant extracts. Immobilisation of benzyl 2-deoxyglucosinolate on a solid support may give a chromatographic system highly specific for the myrosinase enzyme.

Many thioglucosides have been tested as myrosinase substrates including phenyl and allyl derivatives but none are active (figure 11). Even oxidising the sulfur to the sulfoxide or sulfone, to increase the leaving group ability, has no effect.[138]

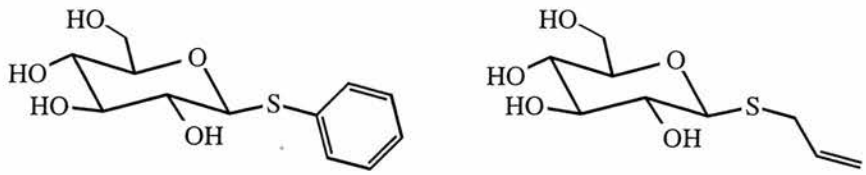


Figure 11: *Example thioglucosides which cannot be hydrolysed by myrosinase*

There are two particularly unusual glucosinolate structures which are substrates for myrosinase and these are shown in figure 12. Phosphoglucotropaeolin **12** was synthesised and found to undergo myrosinase catalysed hydrolytic cleavage although with modified kinetic parameters compared with the sulfate analogue.[139] This is the only published example of a glucosinolate where the *O*-sulfate moiety has been successfully replaced while retaining the activity of myrosinase. Two other novel glucosinolate analogues which have been synthesised are methylselenoglucosinolate and benzylselenoglucosinolate **13** which were hydrolysed to the isoselenocyanates by myrosinase.[140] However, both selenoglucosinolates were hydrolysed relatively slowly in the absence of L-ascorbic acid.

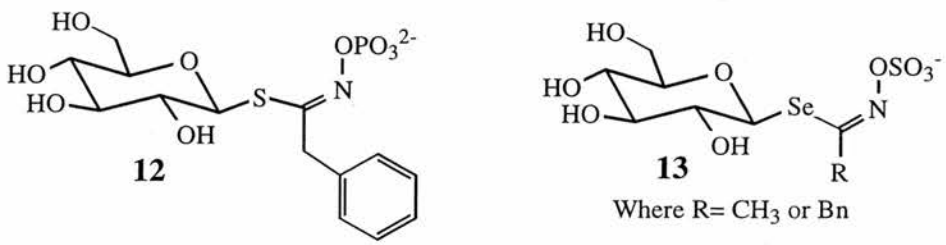


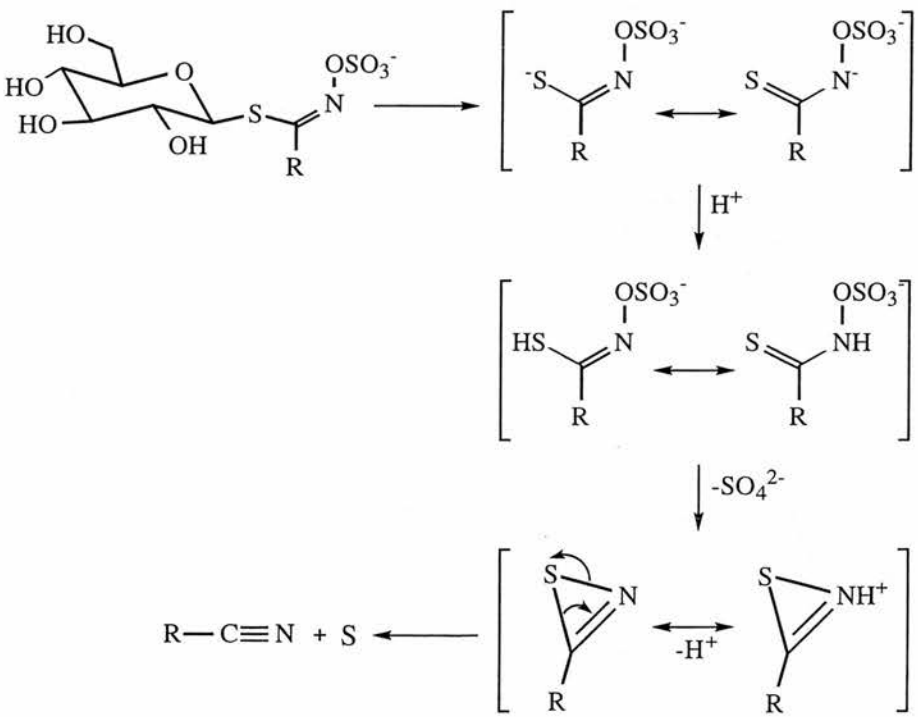
Figure 12: *Novel glucosinolate analogues successfully hydrolysed by myrosinase*

Following from this an independent group have observed that benzylselenocyanate inhibits chemically induced tumour formation in laboratory animals[141] by preventing metabolic activation of carcinogens such as 2-aminoanthracene. The role of selenium in the prevention of cancer susceptibilities remains unclear but like the isothiocyanates it is believed to be due to inhibition of phase I activation or induction of phase II detoxification enzymes. By analogy the isoselenocyanates may also posses this property. It is important to note however that both naturally occurring and inorganic selenium compounds can be extremely toxic.

1.3.4 Glucosinolate hydrolysis products

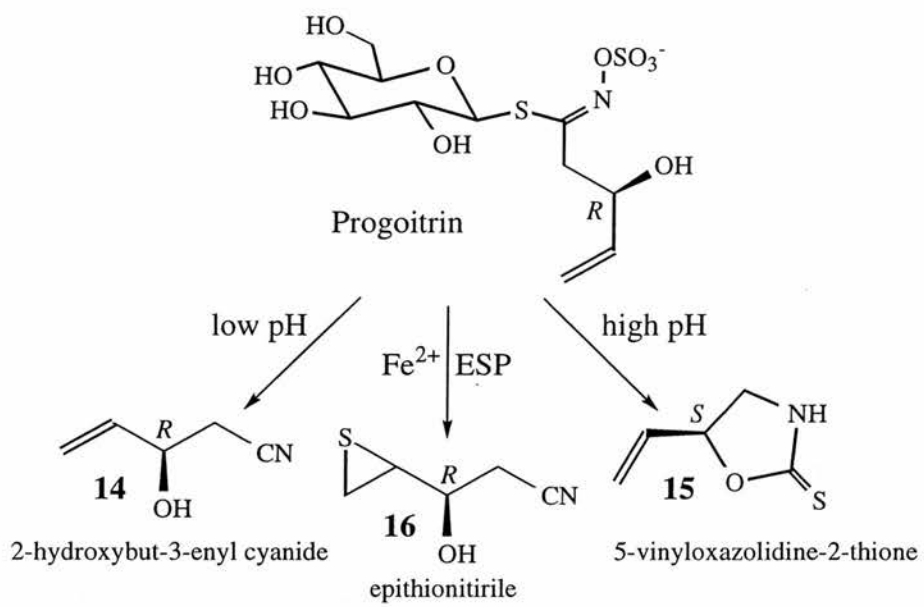
The products of myrosinase catalysed glucosinolate hydrolysis are diverse and the distribution of these products is determined by a number of conditions: the structure of the glucosinolate and specifically its side chain; the pH of the hydrolysis medium and the presence of cofactors.

Glucosinolates can be broadly divided into three subgroups, the main group of which comprises aromatic and aliphatic (both saturated and unsaturated) side chains. Hydrolysis of these glucosinolates by myrosinase at pH 5-7 yields isothiocyanates by Lossen rearrangement while at more acidic pH nitriles predominate at the expense of the isothiocyanates. Isothiocyanates possess more potent biological activity than nitriles and therefore the pH dependence of the enzymatic reaction is an important consideration. The proposed mechanism for nitrile formation is shown in scheme 5.[142] Under these acidic conditions the enzymatically produced aglycone cannot undergo the usual Lossen rearrangement since it is blocked by protonation.



Scheme 5: *Formation of a nitrile from a glucosinolate at low pH*

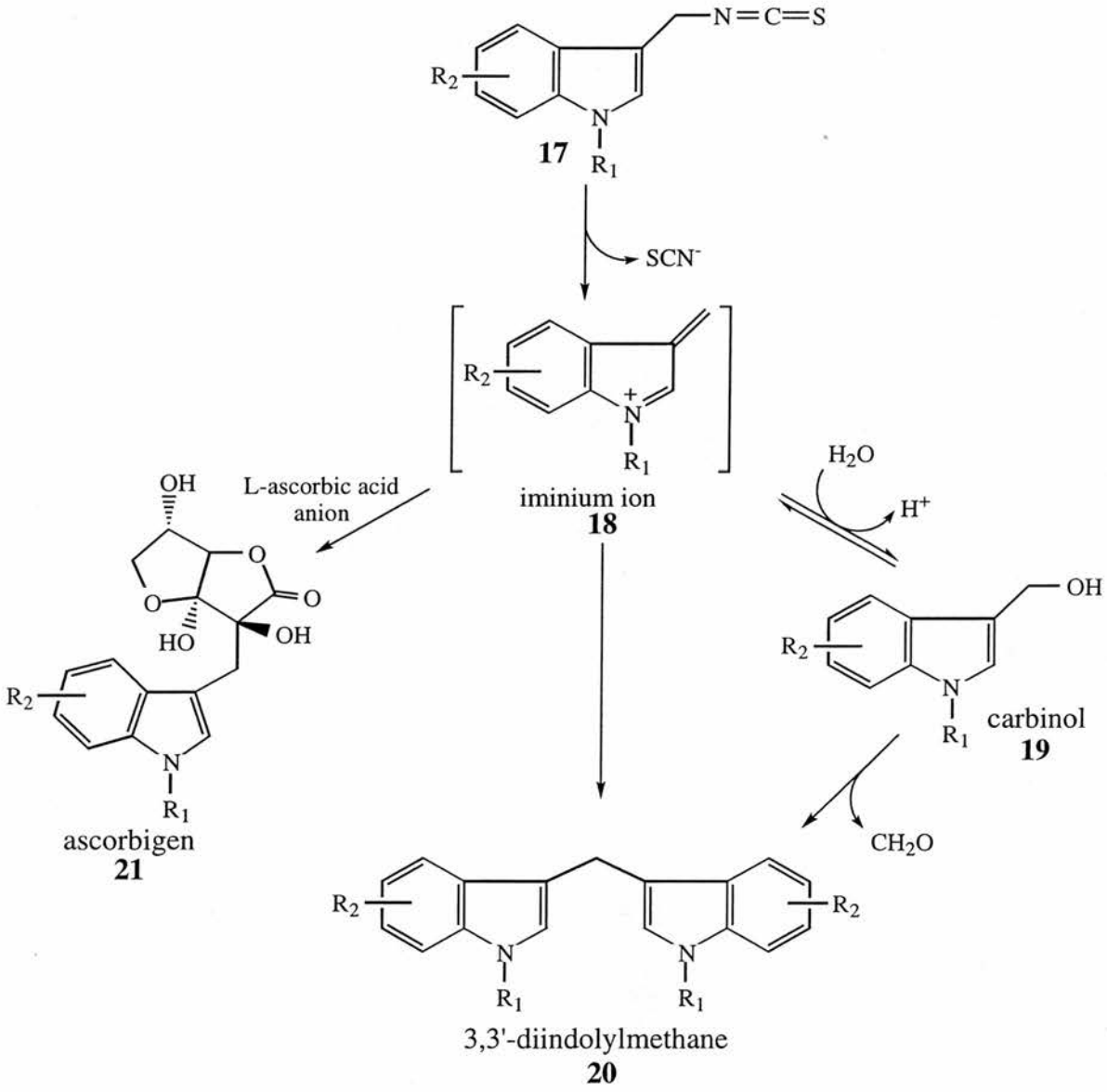
A second much smaller group of glucosinolates which are widespread in major commercial crops have side chains with a β- (or occasionally γ) hydroxyl moiety (scheme 6). As for the aromatic and aliphatic glucosinolates, low pH conditions promote nitrile formation **14** while at pH 5-7 isothiocyanates are believed to form. However in this case the isothiocyanate is rendered unstable by its β-hydroxyl group and undergoes spontaneous cyclisation to give the oxazolidine-2-thione **15** which has anti-thyroid activity. A further reaction product which can result is epithionitrile **16** produced by the presence of a small protein called epithiospecifer protein (ESP) and ferrous ion (scheme 6).[33, 143] ESP is responsible for affecting the transfer of a sulfur atom to the terminal alkene.



Scheme 6: *Products of progoitrin hydrolysis by myrosinase under various conditions*

The third category of glucosinolates are called glucobrassicins and contain indolic or substituted indolic side chains. These are commonly found in oilseed rape and various cabbage and kale species.[144] Enzymatic hydrolysis of glucobrassicins at pH 3-4 yields nitriles with the loss of elemental sulfur and hydrogen sulfide.[144] At higher pH the corresponding isothiocyanates would be expected to form in an analogous manner to other glucosinolates. However efforts to trap, isolate, synthesise or otherwise detect the isothiocyanates have proved largely unsuccessful. As shown in scheme 7 it is believed that the isothiocyanate **17** undergoes a facile hydrolysis with the loss of thiocyanate ion to yield

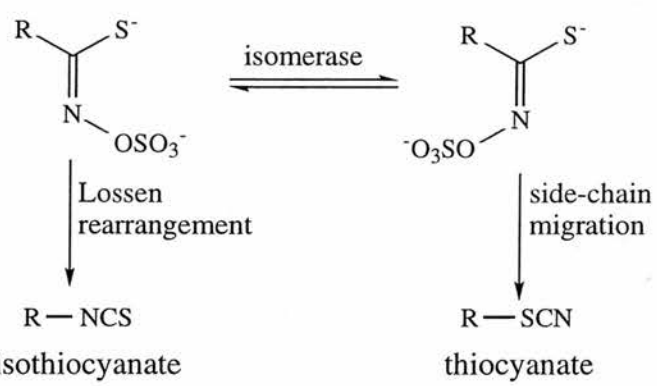
the corresponding carbinol **19** via a stable iminium ion intermediate **18**.[145] The carbinol then self condenses to give 3,3'-diindolylmethane **20** with loss of formaldehyde.[144] In the case of 1-methoxyindol-3-ylmethyl glucosinolate hydrolysis the fleeting existence of an isothiocyanate has been successfully established by low and high resolution mass spectrometry giving credence to the above hypothesis.[146, 147, 148] In this case the isothiocyanate is stabilised by the 1-methoxy substituent on the indole ring which renders the 3-position less reactive.[149]



Scheme 7: *Breakdown products of the glucobrassicins*

In the presence of ascorbic acid glucobrassicins breakdown to give ascorbigens **21**. Their formation is believed to occur via reaction of either the carbonium ion **18** or the isothiocyanate **17** with ascorbic acid. This pathway occurs in competition with the formation of indol-3-ylcarbinols from reaction with water as previously described. At pH 4.0 the yield of ascorbigen is at 80% of the theoretical maximum while an increase in pH to 7.0 gives a greater yield of the 3,3'-diindolylmethane at the expense of ascorbigen.[145]

A final catabolite of myrosinase catalysed glucosinolate hydrolysis is thiocyanate. There are only three glucosinolates which are known to breakdown to give thiocyanates these are allyl, 4-(methylthio)butyl and benzyl glucosinolates. The thiocyanate forming factor has been shown to be independent of pH, inactivated by ascorbate and distinct from thioglucosidase activity.[149] While the exact mechanism of thiocyanate formation remains unknown, it is suggested that the thiocyanate is formed by an isomerase which promotes Z-E isomerisation of the glucosinolate aglycone.[142, 130] Lossen rearrangement is then blocked by the sulfate and the side chain migrates to the sulfur atom (scheme 8).



Scheme 8: *Proposed mechanism for thiocyanate formation*

To facilitate this process it is imperative that the side chain is capable of forming a stabilised carbonium ion after formal loss of thiocyanate. It can be noted that the indole glucosinolates also have the potential to breakdown in this way due to their ability to form a stable cation however this has never been observed.

(i) Effects of Ions and Cofactors

In each of the aforementioned glucosinolate subgroups low pH (*ca.* pH 3) promotes nitrile formation. However nitriles can form under autolysis conditions even at higher pH where isothiocyanates would be the expected products. Uda *et al.* [150, 151] provided the first explanation for this phenomenon by discovering that ferrous ions and perhaps endogenous thiols (L-cysteine, reduced glutathione, cysteamide, thiobenzoic acid and thiophenol) inhibited isothiocyanate formation and accelerated desulfuration of the aglycone. Nitriles are formed at low pH because Lossen rearrangement of the aglycone is blocked by a proton; it is thought that ferrous ions may block the Lossen rearrangement in a similar manner due to affinity for aglycone sulfur atom.[152]

Ferrous ions are also necessary for the formation of epithionitriles by epithiospecifer protein (ESP) and myrosinase. ESP is a small fairly labile protein of 30-40 kDa which was first isolated by Tookey in 1973 and it is important to note that ESP has no enzymatic activity of its own.[153] Kinetic studies conducted by Petroski and Kwolek indicated that ESP non-competitively inhibits myrosinase binding at a site other than the substrate binding site.[154] In addition Brocker and Benn illustrated that hydrolysis of a mixture of 3-butenyl gluco[1-^{35}S]sinolate and 2-propenyl glucosinolate by *Crambe abyssinica* myrosinase gave 4,5-epi[^{35}S]thiopentanonitrile and unlabelled 3,4-epithiobutanonitrile. This work showed that no label had been transferred to the 2-propenyl derivative: thus the sulfur atom was transferred to the terminal alkene by a largely intramolecular mechanism.[155] Not all *brassica* plants contain ESP and it is thought that only those possessing glucosinolates with terminally unsaturated side chains will have ESP.[156]

The presence of cations is known to affect the reactivity of myrosinase and as previously indicated ferrous ions promote nitrile formation. Further studies have examined the effects of ferrous, ferric, cuprous, cupric and manganous ions on the hydrolysis of indol-3-ylmethyl glucosinolate by *Sinapis alba* myrosinase at various pH values.[157] Under low pH conditions ferrous, ferric, cuprous and cupric ions increased the proportion of nitrile

produced at the expense of the other possible hydrolysis products. As the pH was increased from 4 to 7 ferrous, ferric and cuprous ions decreased the ratio of 3-indolylacetonitrile to 3,3'-diindolylmethane and ascorbigen products. Manganous ions however had no effect on the product ratio. In these studies the metal ion concentration was fairly low at 200 μM however other studies have been performed with higher concentrations of metal ions. Hydrolysis of *p*-nitrophenyl β-D-glucoside by light-grown cress (*Lepidium sativum*) myrosinase was strongly inhibited by adding 1 mM lead nitrate, mercury chloride or ferric chloride.[132] Similarly, hydrolysis of epiprogoitrin by *Crambe abyssinica* myrosinase was completely inhibited by cupric ions.[157]

Myrosinase has no real metal requirement as the addition of metal chelators such as EDTA, DIECA, *o*-phenanthroline and 2,2'-dipyridyl displayed no inhibitory properties on the enzyme. Addition of thiol reagents had a drastic effect however with 1 mM PCMS, PCMB or *N*-ethylmaleimide causing a 50-80% decrease in enzyme activity.[132]

1.4 Glucosinolate Analysis

In light of the coexistence of glucosinolates and myrosinase within plant tissues the isolation of glucosinolates for analysis is a complex matter. Any process which damages fresh plant tissue will cause glucosinolate hydrolysis and thus it is necessary to inactivate myrosinase before extraction. The natural sample is firstly frozen in liquid nitrogen or dried (oven or freeze dry) then it is ground and heated to a temperature of 70-110 °C to inhibit myrosinase activity allowing extraction of the glucosinolates.[158, 159] Ion exchange methods are then employed to give preliminary sample purification and from there further purifications differ.

Once the sample has been adequately prepared there is a bewildering array of techniques which are available to assess the glucosinolate content either qualitatively or quantitatively. Furthermore it is often necessary to employ more than one method in order to gain an

accurate analysis. In the past the analysis of glucosinolates depended mainly on the analysis of chemically or enzymatically produced hydrolysis products. Sulfate and glucose are obtained in stoichiometric amounts and so analysis of these products can give a measure of the total glucosinolate content of a sample. Individual glucosinolate content was established through analysis of the aglycone rearrangement products. However at present there is a large amount of interest in the analysis of intact or desulfoglucosinolates and it is this subject which will be discussed hereafter highlighting the importance of the application of GC, HPLC and mass spectrometry in this field.

Analysis of the full glucosinolate skeleton is advantageous since the variable products given from the myrosinase catalysed reaction are avoided. Desulfoglucosinolates are commonly used for analysis and are obtained by removal of the sulfate group from intact glucosinolates using sulfatase (E.C. 3.1.6.1) which is isolated from an edible snail (Helix pomatia). Importantly, the volatility of these desulfoglucosinolates can be increased by conversion to silylated derivatives such as trimethylsilyl.[160] The volatile nature of these compounds has allowed their analysis by GC with the aid of temperature programming.[161] Furthermore glucosinolates which usually yield involatile aglycones such as indolyl and *p*-hydroxybenzyl derivatives can be separated and identified. However the technique is limited as 4-hydroxy-3-indolylmethyl trimethylsilyl desulfoglucosinolate cannot be detected and methylsulfinyl trimethylsilyl desulfoglucosinolates have been observed to give multiple peaks possibly due to decomposition.[161] The advent of HPLC separation partly resolved the aforementioned problems; it has also been noted that both 4-hydroxy-3-indolylmethyl glucosinolate and its desulfo equivalent are rather unstable although HPLC detection is possible.[162]

Potentially all glucosinolates can be identified via GC or HPLC methods but some glucosinolate signals can overlap depending on baseline separation. Sharper separation can be achieved for desulfoglucosinolates if a C_{18} column is employed thus avoiding the use of buffer solutions and ion-pairing reagents. The relative efficiency of HPLC and GC

techniques for the separation of desulfoglucosinolate samples was illustrated by a study which examined the glucosinolate content of rapeseed. HPLC (using desulfoglucosinolates) proved to be more sensitive with 11 glucosinolates detected in the sample while GC (using trimethylsilylated desulfoglucosinolates) could only identify 7.[163]

Coupling together techniques such as GC and HPLC with mass spectrometry gives an increasingly powerful and more popular tool in glucosinolate identification. The potential of this combined technique was illustrated by a study involving separation and identification of desulfoglucosinolates from *Arabidopsis thaliana* by thermospray (TSP) LC-MS.[164] While HPLC gave excellent separation of the glucosinolates, thermospray mass spectrometry allowed their identification. Overall twenty three glucosinolates were identified: sixteen of these had not been previously detected in *Arabidopsis thaliana* and three proved to be novel glucosinolate structures. Co-elution of compounds from the HPLC is detected in the mass spectrum and thus the techniques complement each other well.[165] Although this approach is extremely useful some desulfoglucosinolates do not yield molecular ions and sensitivity of the technique is low.

A milder method of mass spectral detection involves the use of fast atom bombardment (FAB).[166, 167] In this case the intact glucosinolates can be injected directly into the HPLC eliminating the need for desulfation. The negative ion FAB mass spectra gives information on the glucosinolate molecular weights from the $[M\text{-cation}]^-$ anions and the hydrogen sulfate anion (HSO_4^-). In order to obtain further structural information it is necessary to use collision induced dissociation of the characteristic $[M\text{-H}]^-$ anion to give the daughter ion spectra. Typical fragmentation patterns have been established and this aids identification of the glucosinolates. Although a very powerful and useful tool this technique is reminiscent of the LC-TSP-MS method in its relatively low sensitivity.

The most recent advance has been achieved by the use of atmospheric pressure chemical ionisation (APCI) mass spectrometry which provides a particularly intense molecular ion

for desulfoglucosinolates. TSP-MS tends to suffer from inconsistent ionisation conditions and thus the spectrum obtained depends upon aspects such as flow rate, vaporiser temperature and repeller potential. APCI-MS is not hindered by these constraints and since ammonium acetate is not necessary to ensure ionisation the fragmentation pattern is much simpler to interpret than in TSP-MS. The LC-APCI-MS technique was first used by Ishida *et al.* to separate desulfoglucosinolates in rapeseed. They reported that the disadvantage of the technique was its inability to obtain information except from the protonated molecular ions.[168] In this thesis this disadvantage is overcome by the use of collision induced dissociation and more importantly LC-APCI-MS is adapted to allow quantitative determination of very low concentrations of glucosinolate.

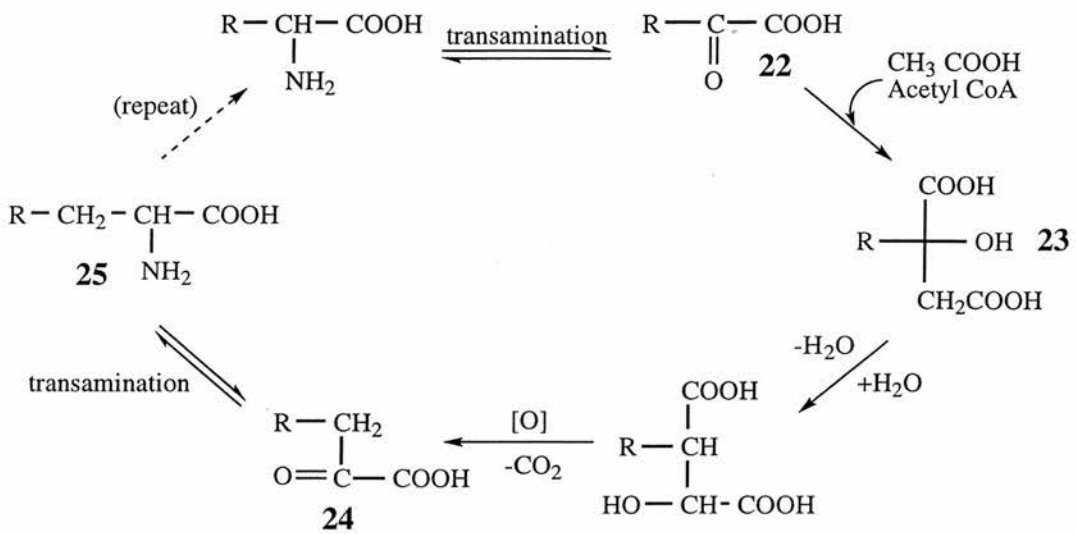
1.5 Biosynthesis of Glucosinolates

Despite the wide variety of natural glucosinolates they are all formed by a common biosynthetic pathway, which starts from amino acids and comprises essentially three stages.[169] Firstly, the amino acid is modified or chain extended. Secondly an aldoxime intermediate is produced which undergoes S-insertion, glycosylation and sulfation. Thirdly, side chain modifications may occur for example oxidation and elimination. These three stages are responsible for the production of glucosinolates and each will be described in turn.

1.5.1 Amino acid modification

Amino acids must undergo homologation to produce the various lengths of glucosinolate side chain typically observed in nature. Glucosinolates derived from methionine for example contain side chains which conform to the formulae $R=CH_3S(CH_2)_n$ where n lies between three and eleven.[170] Similarly, phenethyl glucosinolate is derived from phenylalanine.[171] The biosynthetic sequence illustrated in scheme 9 can be used iteratively

to give the desired degree of homologation before incorporation into the glucosinolate skeleton.[170]



Scheme 9: *Amino acid homologation cycle*

Homologation starts with a transamination catalysed by methionine:glyoxylate aminotransferase to give the α-keto acid **22** which then reacts with acetyl-CoA. The resulting hydroxy acid **23** loses a molecule of water and then is rehydrated, oxidised and decarboxylated to give a homologated α-keto acid **24**. Finally a transamination reaction yields the corresponding amino acid **25**. The transamination catalyst methionine:glyoxylate aminotransferase has been purified and characterised[172] but apart from this very little is known about the regulation of the homologation process and the biochemistry needs to be investigated further.[173]

1.5.2 Aldoxime and glucosinolate formation

Once the amino acid has been chain extended it is converted to the glucosinolate via a series of reactions the first of which involves biosynthesis of an aldoxime figure 13. To date, three different enzymes have been identified which are involved in the aldoxime synthesis. They are flavin containing monooxygenases, cytochromes P450 and peroxidases.

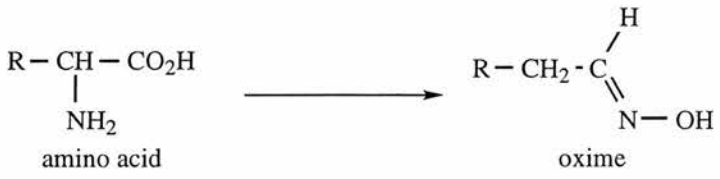


Figure 13: *Oxime biosynthesis*

Using young oilseed rape leaves, flavin containing monooxygenase activity has been established and it is hypothesised that the amino acid-aldoxime conversion could take place via decarboxylation followed by NADPH and oxygen dependent *N*-oxidation.[174] Oilseed rape contains two such monooxygenases, one with substrate specificity for di- and trihomomethionine and a second which has specificity for homophenylalanine (the precursor of phenethyl glucosinolate).[175] The latter monooxygenase has proven to be highly substrate specific and appears to have two domains.[173] The first domain is a hydrophobic one which accepts the side chain of the amino acid while the second binds the α-carboxyl and α-amino groups such that the amino group is orientated towards the flavin ring. The key binding requirements for the substrate are an aromatic ring, an amino acid moiety and a minimum chain length of $(CH_2)_2$; only limited modifications can be made to this before binding is diminished.

It is believed that cytochrome P450 dependent monooxygenases act on tyrosine and phenylalanine derived amino acids and they have been found only in *Tropaeolum majus*, *Sinapis* spp., and *Carica papaya.* They initially catalyse two *N*-hydroxylations before dehydration and decarboxylation occurs to give the oxime.[169] In contrast to this, plasma membrane bound peroxidases operate by a different mechanism where the substrate nitrogen is oxidised by hydrogen peroxide. As for the P450 reaction, the oxidation is followed by dehydration and decarboxylation to yield the aldoxime.[169] Membrane bound peroxidase is the only reported enzyme system to catalyse synthesis of indol-3-yl acetaldoxime the precursor of both indolyl glucosinolates and indolyl acetic acid (a plant growth hormone). These enzymes are commonly found in Chinese cabbage and oilseed rape.[176] It is interesting to note that *S. alba* contains all three types of enzyme, namely a

flavin containing monooxygenase, a cytochrome P450 dependent monooxygenase and a peroxidase.[174] The biosynthesis of oximes is summarised in figure 14.

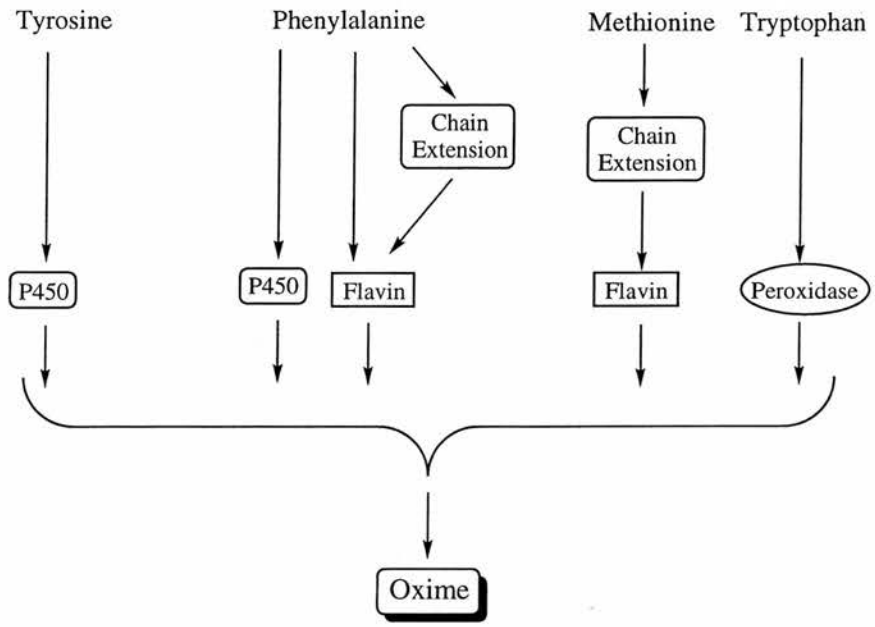
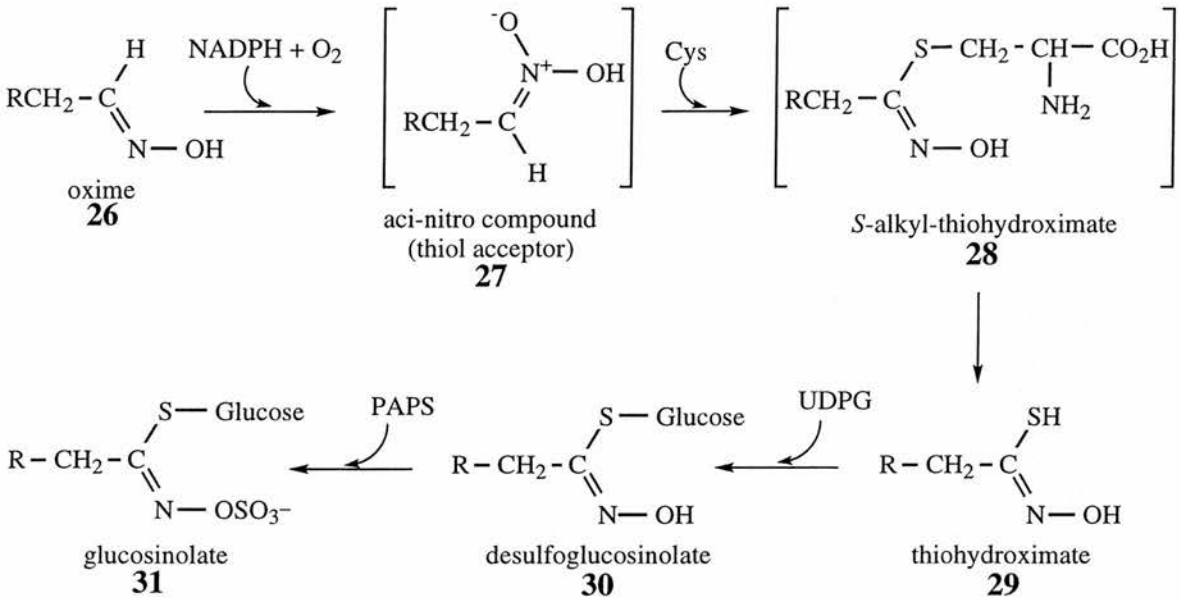


Figure 14: *Oxime biosynthesis*

Following the oxime synthesis are several conjugation reactions which introduce sulfur (scheme 10). Thiohydroximate biosynthesis is not well understood but it is thought that the oxime **26** is converted by oxidation to an aci-nitro compound **27** which is then attacked by a thiol donor.[169] The nature of the sulfur donor remains to be established however cysteine has proven to be a likely candidate and thioglucose has been discounted. The absence of biochemical data regarding the aci-nitro intermediate remains a problem in fully confirming the suspected pathway. It is necessary to determine *in vitro* the conversion of oxime to aci-nitro species then through to the thiohydroximate. Glutathione-*S*-transferase may be responsible for conjugation of cysteine to the aci-nitro compound and the *S*-alkyl-thiohydroximate **28** produced could be cleaved by a *C-S* lyase. However these claims are purely speculative and lack evidence.



Scheme 10: *Biosynthesis of glucosinolates from amino acids*

The later stages of the biochemical pathway, *S*-glycosylation and sulfation, are more fully understood (scheme 10). *S*-Glycosylation of the thiohydroximate **29** is catalysed by a soluble UDPG:thiohydroximate glucosyltransferase (E.C. 2.4.1.-) to give the desulfoglucosinolate **30**. This compound then undergoes sulfation to the glucosinolate **31** by action of a soluble 3-phosphoadenosine 5'-phosphosulfate:desulfoglucosinolate sulfotransferase (E.C. 2.8.2.-). Both enzymes have been partially purified and they exhibit a relaxed specificity with regard to the glucosinolate side chain, unlike the enzymes previously described in the conversion of amino acid to oxime.[177]

1.5.3 Side chain modification

The glucosinolate side chain may or may not undergo a series of final modification steps. For example sulfur containing side chains can undergo oxidation to give the sulfoxide or sulfone. Thus glucoerycolin and glucoraphanin could be produced from glucoerucin (figure 15). Following from this elimination of methane thiol (or an oxidised form of it) can produce alkenyl derivatives.[178]

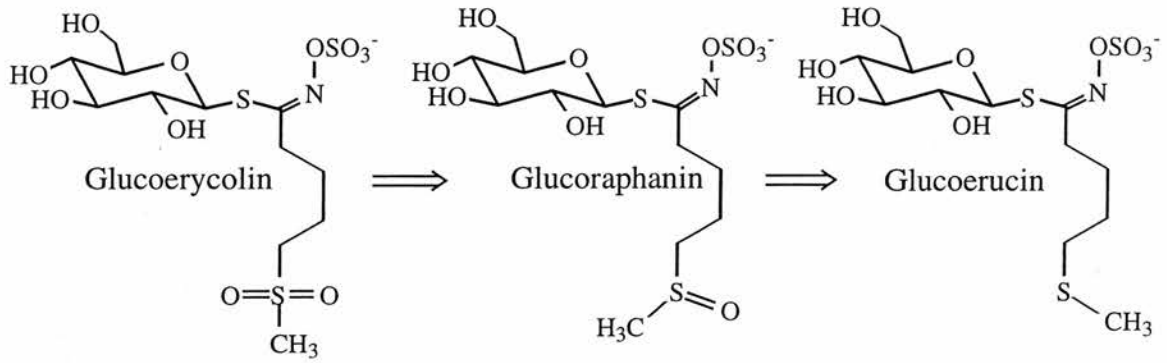
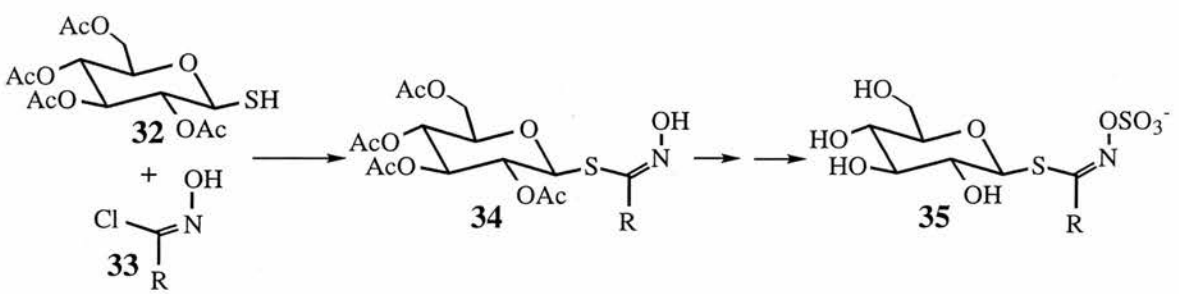


Figure 15: *Biosynthetic oxidation of sulfur containing glucosinolate side chains*

Hydroxylation, methylation, sulfation and stereospecific oxygen insertion are all feasible. These reactions are responsible for the rich variety of glucosinolates which have existed in nature for centuries.

1.6 Chemical Synthesis of Glucosinolates

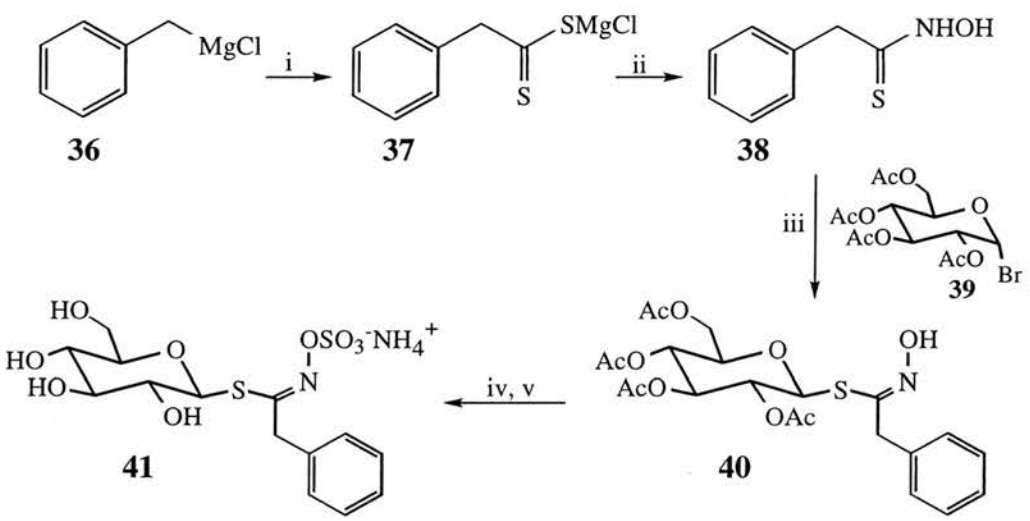
Most glucosinolate synthesis to date follows the same general strategy shown in scheme 11 where a protected thiosugar **32** is coupled under basic conditions to an oximyl chloride **33**. The resulting thiohydroximate **34** is then sulfated using freshly prepared pyridine-sulfur trioxide and the sugar moiety is deprotected using either methanolic ammonia or potassium hydroxide to give the glucosinolate **35**.



Scheme 11: *General strategy for glucosinolate synthesis*

The only exception to the above "nucleophilic sugar" approach lies in the work of Ettlinger and Lundeen in 1957 who published the first glucosinolate synthesis (scheme 12).[8] Their route to benzyl glucosinolate started with a preparation of ethereal magnesium

phenyldithioacetate **37** from benzylmagnesium chloride **36** and carbon disulfide. This was then treated with aqueous hydroxylamine hydrochloride to give phenylacetothiohydroxamic acid **38**. Ettlinger and Lundeen used an "electrophilic sugar" coupling between α-acetobromoglucose **39** and the thiohydroxamic acid in the presence of potassium hydroxide to give a glycosylated thiohydroximate **40**. Sulfation using pyridine-sulfur trioxide and deprotection using methanolic ammonia then afforded tetramethylammonium glucotropaeolin **41**. The electrophilic sugar approach was not widely adopted by other research workers probably because the thiohydroxamic acid is a poor nucleophile and this leads to low yields.

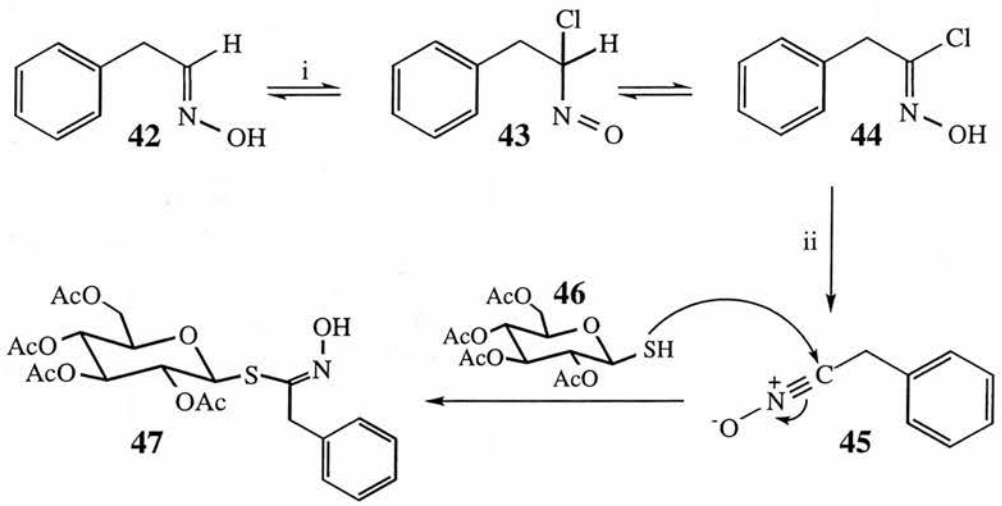


(i) CS_2, Et_2O; **(ii)** $NH_2OH_{(aq)}$, 0 °C, 33%; **(iii)** KOH, CH_3OH, $(CH_3)_2CO$ (1:3), 25 °C, 6 hrs, 47% **(iv)** pyridine-SO_3, pyridine, 25 °C, overnight; **(v)** methanolic ammonia, 94%.

Scheme 12: *'Electrophilic sugar' approach to glucosinolate synthesis*

The more commonly applied nucleophilic sugar strategy was provided by Benn in 1963 in his synthesis of benzyl glucosinolate (scheme 13).[9] He chlorinated phenylacetaldoxime **42** in chloroform using chlorine gas to give phenylmethylhydroxamic chloride **44** via spontaneous rearrangement of a blue chloronitroso compound **43**. The resulting chloride was treated with triethylamine to produce a nitrile oxide **45** which was reacted *in situ* with tetra-*O*-acetyl-thioglucopyranose **46**. The thiohydroximate **47** was sulfated and deprotected as before to give the glucosinolate as its potassium salt. The applicability of

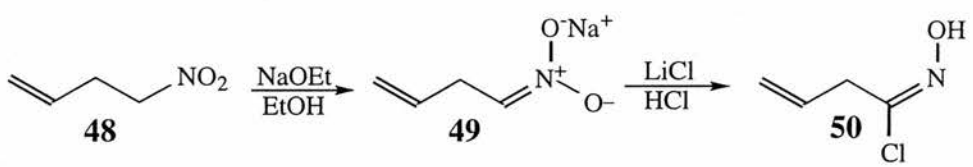
this synthetic route was further illustrated by Benn in the synthesis of *p*-hydroxybenzyl,[179] *p*-methoxybenzyl[179] and phenethyl[180] glucosinolates.



(i) Cl_2, $CHCl_3$, 0 °C, 30 mins; **(ii)** Et_3N, Et_2O, 25 °C, 30 mins, then 0.5 M H_2SO_4, O °C;

Scheme 13: *Benn and Ettlingers 'nucleophilic sugar' approach to glucosinolate synthesis*

The synthesis of sinigrin provided another variant in construction of the glucosinolate skeleton with a new method for synthesis of the oximyl chloride.[181] In this case the hydroximoyl chloride was prepared from a nitro compound using a two step procedure (scheme 14) first described by Kornblum and Brown.[182] Reaction of 4-nitrobut-1-ene **48** with sodium ethoxide gave an intermediate nitronate salt **49** which upon reaction with a lithium chloride-hydrochloric acid mixture yielded but-3-enohydroxamoyl chloride **50**. The usual coupling, sulfation and deprotection gave sinigrin.



Scheme 14: *Nitronate salt route to oximyl chlorides*

So in the period between 1957 and 1965 Benn, Ettlinger and Lundeen had established three fundamental routes to glucosinolates two of which are still used today. From this time

onwards the coupling (triethylamine, aprotic solvent), sulfation (pyridine-sulfur trioxide) and deprotection (methanolic ammonia or potassium hydroxide) reactions have not changed significantly. The greatest variety in glucosinolate synthesis is provided by the different approaches towards construction of the oximyl chlorides. In particular there are a large number of methods which have been developed for the chlorination reaction itself.

During early years the oximes were generally chlorinated at low temperature in chloroform solution using chlorine gas. This method was applied to benzyl,[9] *p*-hydroxybenzyl,[179] *p*-methoxybenzyl,[179] phenethyl[180] and 2-methylbutyl[183] glucosinolates. The phenethyl oximyl chloride proved to be particularly interesting as it existed as a dimer (figure 16) which slowly dissociated to the monomeric oximyl chloride in solution.[184] The authors reported that the compound was unstable above -20 °C and this accounted for low yields in methods which did not employ low temperatures. A second set of reactants involving *N*-chlorosuccinimide and pyridine in chloroform were used successfully by Brochard *et al.* for chlorinating various other natural and novel aromatic oximes.[185]

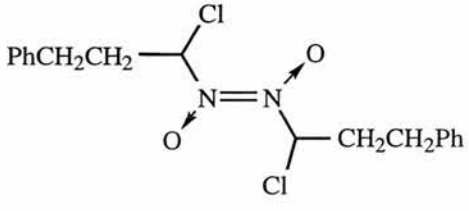


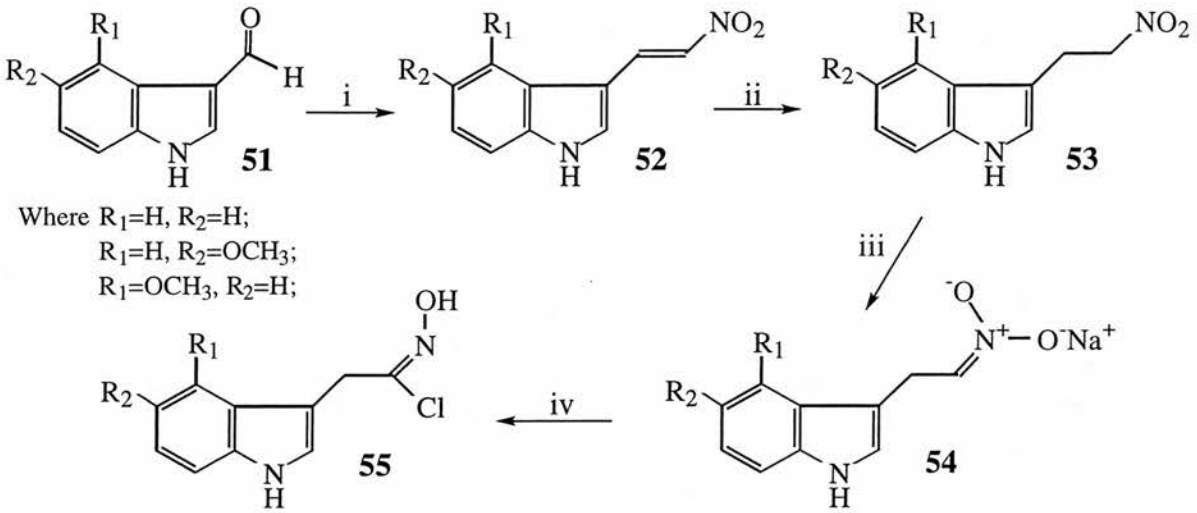
Figure 16: *Dimeric phenethyl oximyl chloride*

However it is interesting to note that literature reports have indicated the use of a much wider range of chlorination conditions for nitronate salts in glucosinolate preparations. During investigations into the synthesis of phenethyl glucosinolate two routes were investigated to produce the oximyl chloride.[184] Direct chlorination of the oxime using chlorine gas as described above afforded 83% of the dimer while chlorination of the nitronate in dry diethyl ether with dry gaseous hydrochloric acid gave a lower yield of 64%. In a similar investigation, Benn and Ettlinger's synthesis of sinigrin[181] was improved by Abramski and Chmielewski.[186] The revised method involved altering the

original chlorination conditions where instead of gaseous hydrochloric acid, a solution of hydrochloric acid in diethyl ether was used. This minor alteration allowed a synthesis of sinigrin which was practical on a ten gram scale and gave good yields.

It is therefore evident that the choice of chlorination conditions can be critical to the success of glucosinolate preparation. Very different yields of oximyl chloride can be obtained from the same starting material if different chlorinations are attempted. In general the chlorinations used for nitronate salts favour sensitive substrates.

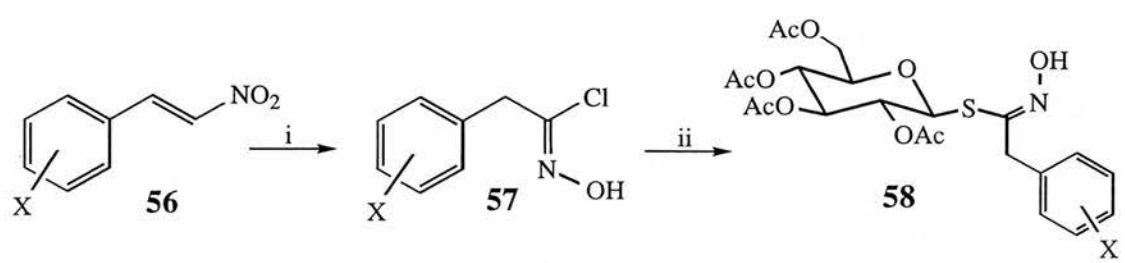
This can be exemplified by the synthesis of indolyl glucosinolates which are renowned to be the most difficult glucosinolates to synthesise. In this case the nitronate salt strategy was successfully applied to the preparation of glucobrassicin and its 4- and 5-methoxy derivatives by Viaud *et al.* (scheme 15).[102, 187, 188] Synthesis started from indole-3-carboxaldehyde **51** which was converted via a Knoevenagel type condensation to 3-(2-nitrovinyl)indole **52**. A chemoselective reduction gave a good yield of the 3-(2-nitroethyl)indole **53** and avoided dimeric side products. Conversion to the sodium nitronate salt **54** was achieved by reaction with sodium methoxide; the nitronate is extremely hygroscopic and thus was not stored but reacted directly in the next step. Chlorination was then undertaken with the use of thionyl chloride in DME at -60 °C to give the oximyl chloride **55**. This compound was unstable and therefore coupled directly to the tetra-*O*-acetyl-thioglucopyranose. This route was adapted to prepare ten sugar variants of glucobrassicin for testing with myrosinase[136, 189] and a [5-^{3}H]-glucobrassicin synthesis was also reported.[190]



(i) CH_3NO_2, $AcONH_4$, 100 °C; **(ii)** $NaBH_4$, SiO_2 230-400 mesh, $CHCl_3$, ^iPrOH, 56%; **(iii)** CH_3ONa, CH_3OH, Et_2O; **(iv)** $SOCl_2$, DME, -78 °C.

Scheme 15: *Synthesis of indolyl oximyl chloride via a nitronate salt*

The continual search for alternative chlorination conditions led to an investigation into the application of Kumaran and Kulkarni's procedure for direct chlorination of arylnitrovinyl compounds.[191] Thus Rollin *et al.* successfully chlorinated various arylnitrovinyl derivatives **56** using titanium tetrachloride and triethylsilane (scheme 16). The resulting arylalkyl hydroximoyl chlorides **57** were subsequently coupled to tetra-*O*-acetyl-thioglucopyranose and the thiohydroximates **58** converted to glucosinolates.[192]



Where X= *p*-OAc, *p*-OCH_3, *o*-OCH_3; **(i)** $TiCl_4$, Et_3SiH, CH_2Cl_2, 25 °C, 1 hr, then aqueous work-up; **(ii)** tetra-*O*-acetyl thioglucopyranose, Et_3N, CH_2Cl_2, Et_2O, 25 °C, 2 hrs.

Scheme 16: *Alternative route to benzyl glucosinolate derivatives*

This method was extended into an exploration of 3-(2-nitrovinyl)indole chlorinations with a view to glucobrassicin synthesis. In this case the indole nitrogen was protected before

chlorination and coupling of the crude product to give the corresponding glycosyl thiohydroximate. The protecting group used proved to significantly affect the yield of the coupled product (table 2). The incorporation of an *N*-methyl protecting group gave an exceptionally good yield considering that the yield of the same transformation in three steps via the nitronate had not previously exceeded 24%.

***N*-Protecting Group**	**Yield of protected glycosyl thiohydroximate**
Acetyl	35%
Methyl	76%
t-Butoxycarbonyl	0%
Phenylsulfonyl	55%

Table 2: *Various yields of indolyl glycosyl thiohydroximates obtained using titanium tetrachloride/triethylsilane chlorinating conditions.*

At this stage it is worth mentioning another group of glucosinolates which have proven tricky to synthesise. These glucosinolates contain an external thio function (figure 17) and account for over one third of the 100 or so naturally occurring glucosinolates. Synthesis has proved difficult since the reactive methylsulfide side chain does not allow chlorination of the oxime with *N*-chlorosuccinimide or chlorine gas. Nevertheless chlorination of the nitronate salt with thionyl chloride in chloroform, as published for the synthesis of 2-hydroxybut-3-enyl glucosinolate, was successful.[193, 194] Additional problems were encountered in the synthesis of the nitroalkylmethylsulfide starting materials from bromochloroalkanes as cyclic sulfonium salts tended to form. A simpler strategy was used in order to synthesise glucoraphanin **60** which contains a sulfoxide moiety in the side chain. In this case glucoerucin (**59** where n=4) was isolated from ripe *Eruca sativa* seeds and then oxidised chemoselectively with hydrogen peroxide at 60 °C.[195]

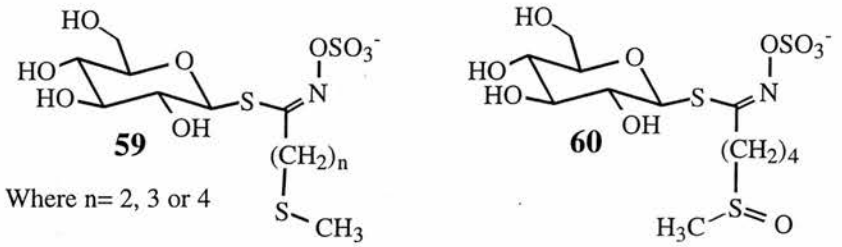
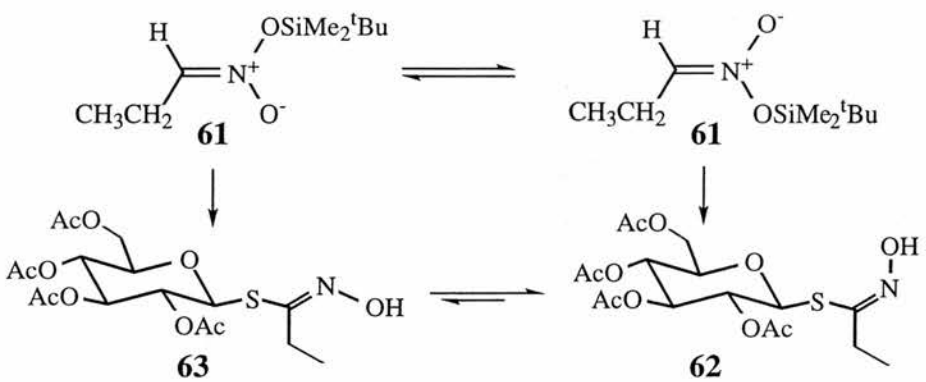


Figure 17: *Glucosinolates containing an external thio function*

Finally there is one new glucosinolate synthesis which does not proceed via an oximyl chloride. This novel glucosinolate forming reaction was reported by Keller *et al.* in the synthesis of ethyl glucosinolate.[196] A modification of the Copenhaver reaction was used where the primary nitroalkane was replaced by a trialkylsilyl nitronate **61** (scheme 17). It was found that rapid oxygen to oxygen migration of the silyl group caused a mixture of E and Z isomers to form upon reaction with tetra-*O*-acetyl thioglucopyranose in 28% and 56% yield respectively. The Z isomer **62** is more stable since the E isomer **63** slowly isomerised to the Z form in solution.



Scheme 17: *Synthesis of a mixture of E and Z glucosinolates*

The rich variety of synthetic routes presented here covers mostly naturally occurring glucosinolates but these methods have also been modified and applied to the synthesis of other novel glucosinolate analogues. These include *C*-glucotropaeolin,[197] α-glucosinolates,[135] selenoglucosinolates,[140] phosphate derivatives,[139] aza analogues,[198] and many more glucosinolates which vary in either their sugar moiety or side chain.[199, 200] Many of these derivatives have been synthesised in order to explore their interaction with myrosinase yet it will be interesting to see if these analogues prove to be useful in other areas of research.

Results and Discussion

2.1 Deuterated Desulfoglucosinolates as Internal Standards in LC-MS

2.1.1 Background and objectives

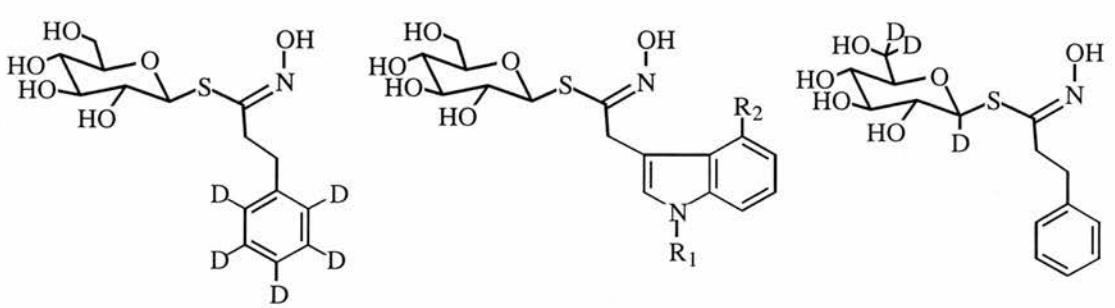
Glucosinolates are known to play a central role in interactions between *Brassica* adapted pests and their target plants. In particular, root flies and other *Brassica* pests use profiles of different plant chemicals on the leaf surface during key stages in host recognition and acceptance for oviposition.[201, 48] In order to study such interactions it is necessary to know which glucosinolates are present in the target plant and in what quantities. This is no simple task as glucosinolate profiles vary from leaf to leaf depending on age and environmental stress.

Currently the best available techniques for glucosinolate analysis are based on high performance liquid chromatographic separation of enzymatically prepared desulfoglucosinolates. However, there are problems with these ultra-violet based methodologies. The glucosinolates which occur in nature are diverse and it is not easy to give an indisputable identification of the peaks given by an ultra-violet trace. Furthermore, there are only a limited number of standards available and results are only semi-quantitative. To perform such HPLC analysis it is necessary to combine 5-10 leaves of similar age to produce an analytical sample of sufficient magnitude. This is clearly undesirable as profiles of individual leaves can vary significantly even within leaves of similar age giving erratic results for bulk samples. Therefore for an accurate account of the leaf surface glucosinolates as perceived by the insect, sampling of individual leaves for chemical analysis and behavioural bioassays is essential.

At the Scottish Crop Research Institute there are excellent LC-MS facilities and soft ionisation techniques can be used to generate molecular ions from desulfoglucosinolates. It is therefore possible to use LC-MS as an improved means of glucosinolate detection with

100-1000 fold increase in sensitivity. This increase in sensitivity can allow quantification and identification of glucosinolates on a single leaf sample. However there are temporal variations in the ionisation efficiency of the instrument. In order to gain quantification it is therefore necessary to have pure internal (or external) glucosinolate standards. These standards must be identical in structure to the glucosinolate being examined and ideally three mass units heavier to give sufficient mass difference from the unlabelled compound. Such labelled materials are not commercially available (indeed only two glucosinolates are commercially available, sinigrin and glucotropaeolin).

Our initial target was a deuterated derivative of desulfogluconasturtiin as this could be synthesised quickly and cheaply. Following on from this two deuterated desulfoglucobrassicin derivatives were synthesised as these glucosinolates are particularly potent oviposition stimuli (figure 18). In each of these target compounds the deuterium is incorporated into the desulfoglucosinolate side chain. This is not an ideal strategy since the side chain of every glucosinolate is different. To allow a more versatile synthesis we incorporated deuterium into the sugar moiety of the glucosinolate since this portion of the glucosinolate does not change (figure 18). This opens up a novel route to synthesis of any deuterated desulfoglucosinolate. It should be noted that for each deuterated target the synthetic route was first developed without incorporation of deuterium.



deuterated desulfogluconasturtiin

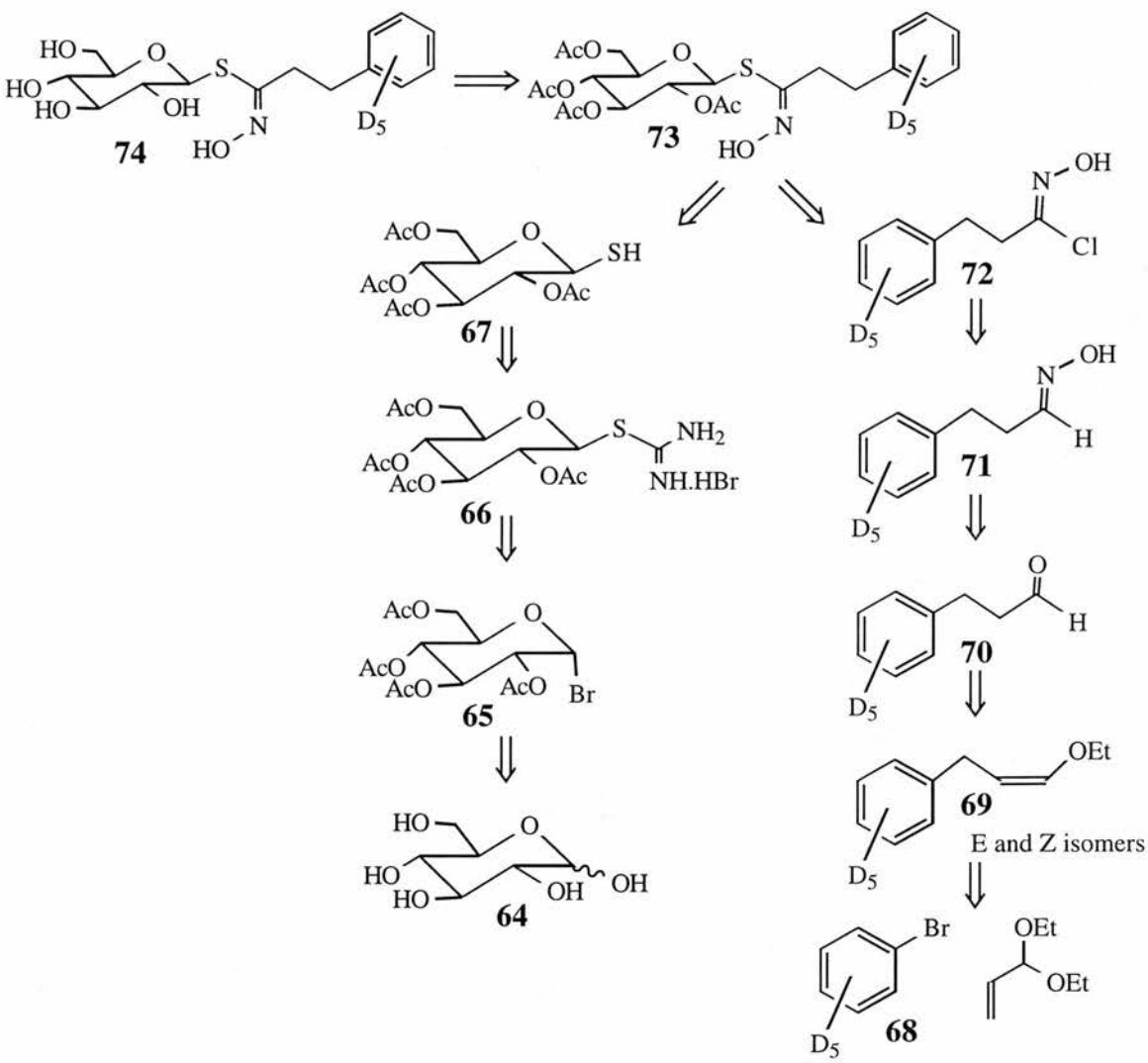
deuterated desulfoglucobrassicins
Where R_1=H, R_2=OCD_3
or R_1=OCD_3, R_2=H

deuterated glucopyranose derivaitve

Figure 18: *Deuterated desulfoglucosinolate target compounds*

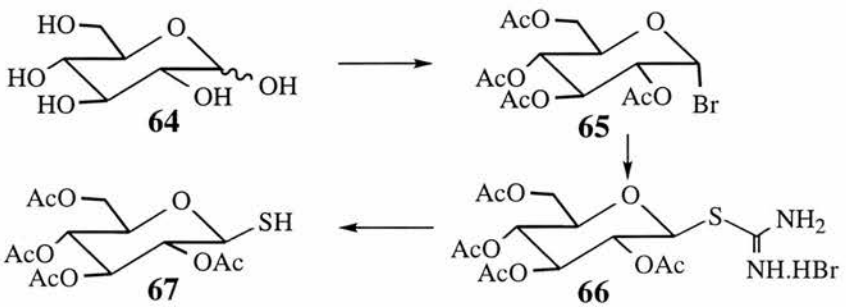
2.1.2 Synthesis of desulfogluconasturtiins

It was proposed to incorporate deuterium into the phenyl ring of desulfogluconasturtiin in the synthesis of deuterated desulfogluconasturtiin **74**. This could be achieved using the readily available, relatively cheap n.m.r. solvent, deuterated bromobenzene **68**. The synthetic route followed is shown in scheme 18 and involves coupling of an oximyl chloride **72** to 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose **67** giving the basic glucosinolate skeleton **73**.



Scheme 18: *Reterosynthetic route to deuterated desulfogluconasturtiin*

2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose **67** can be prepared from D-glucose **64** by a simple three step strategy (scheme 19).



Scheme 19: *Synthetic route to 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranose*

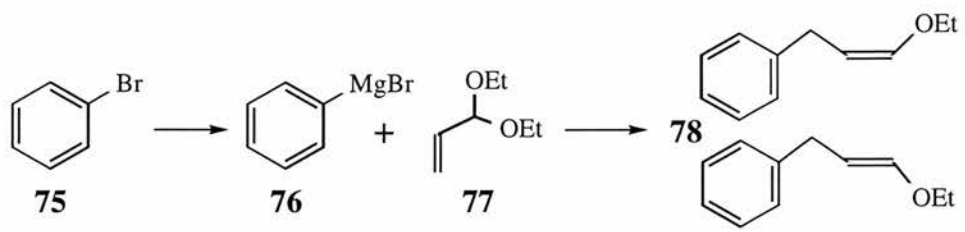
Firstly, D-glucose **64** was reacted with acetic anhydride and 45% hydrobromic acid in acetic acid at 0 °C. This reaction is exothermic and gives 1,2,3,4,6-penta-*O*-acetyl-D-glucopyranose as a mixture of α and β anomers. Upon complete conversion to this product a further portion of 45% hydrobromic acid in acetic acid was added at room temperature. The reaction was stirred overnight under a nitrogen atmosphere to give 2,3,4,6-tetra-*O*-acetyl-1-bromo-α-D-glucopyranose **65**. The crude product was a pale yellow oil which crystallised upon cooling to 0 °C. The resulting solid was recrystallised from a mixture of diethyl ether and 40-60 petroleum ether to give the pure product in 96% yield as a white crystalline solid. This compound is heat labile and must be stored below 0 °C to avoid decomposition. The structure of the compound was established by ^{1}H n.m.r. spectroscopy where four signals were observed at δ 1.9-2.2, corresponding to four acetyl groups. The anomeric proton had shifted from δ 6.3 in 1,2,3,4,6-penta-*O*-acetyl-D-glucopyranose to δ 6.6 in the brominated product. The product given was observed to be exclusively in the α form despite the starting material which existed as a mixture of α and β anomers. This can be attributed to the influence of the anomeric effect.

The second step in the synthesis of acetyl protected thioglucopyranose involved a displacement of the anomeric bromine by thiourea.[202] This reaction is performed in dry acetone and is complete after a period of 15 minutes at reflux. It should be noted that

longer reflux times did not give any increase in yield. The product **66** was obtained in 47% to 51% yield after recrystallisation from acetone. Although the recrystallisation proved to be a slow process it was generally found that improved yields were given in the next step as a result. The ^{13}C n.m.r. spectrum showed a signal at δ 170.3 which corresponded to the *C*=N group in the product. Furthermore the ^{1}H n.m.r. spectrum illustrated a shift of the anomeric proton resonance from δ 6.6 to become part of a multiplet between δ 5.1-5.6. This product proved to be very stable, unlike its precursor and can be stored at room temperature.

Finally, reaction of the 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiouronium bromide **66** with potassium metabisulfite in a water-dichloromethane biphasic reaction gave the required product.[202] The crude product was readily recrystallised from methanol to give 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose **67** in 76-100% yield. This compound can be stored indefinitely if the temperature is kept below 0 °C and proves to be much more stable than the aforementioned bromo compound. The most distinctive feature in the ^{1}H n.m.r. spectrum is the anomeric proton resonance which occurs as a triplet at δ 4.55. The multiplicity indicates a coupling to both the adjacent C*H* at position 2 and the newly formed thiol. In addition the melting point (74-75 °C) is in good agreement with the literature value (75 °C).[202]

Synthesis of the oximyl chlorides for coupling to 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose started from a reaction of bromobenzene **75** with magnesium and a catalytic amount of iodine to successfully produce the expected Grignard reagent **76**. This reacted with acrolein diethylacetal **77** quantitatively to give 1-ethoxy-3-phenylprop-1-ene **78** as a pale-yellow oil containing a mixture of E and Z isomers (scheme 20).[203]

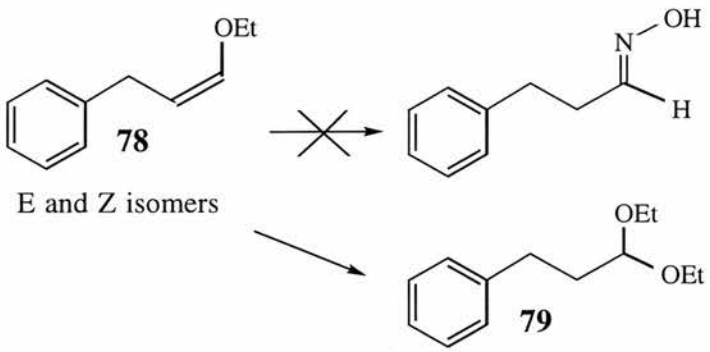


Scheme 20: *Reaction to produce 1-ethoxy-3-phenylprop-1-ene as a mixture of E and Z isomers*

In the ^{1}H n.m.r. spectrum of **78** the ethyl group showed up as two multiplets one at δ 1.40 and one at δ 3.80-4.00 corresponding to the CH_3 and CH_2 respectively. The signals were not well resolved with both E and Z isomers showing similar shifts. However the CH_2 adjacent to the phenyl ring gave two clearly separated double doublets at δ 3.4 and δ 3.7 for the E and Z isomers. The alkenyl protons also gave clear signals. The olefinic proton adjacent to the benzyl group gave a multiplet at δ 4.75 for the Z isomer and a multiplet at δ 5.1 for the E isomer. The olefinic proton next to the ethoxy group however showed as two triplets at δ 6.2 corresponding to the Z isomer and a further two triplets at δ 6.5 for the E isomer. The multiplicity indicates not only a coupling to the other olefinic proton but also an allylic coupling. The E and Z isomers occurred in a ratio of 1:1.4 the assignments being based on the ^{1}H n.m.r. integrals and coupling constants. Correlation spectroscopy aided in correctly assigning the carbon spectrum.

When this experiment was repeated using deuterated bromobenzene and the product purified by distillation at reduced pressure, the spectral data were essentially identical to that detailed above. In this case the aromatic protons were absent from the ^{1}H n.m.r. spectrum as expected. The E and Z isomers occurred in a ratio of 1:1.5 and the mass spectrum showed the expected molecular ion (*m/z* (EI) 167 ($[M]^+$, 87%)).

It was anticipated that 1-ethoxy-3-phenylprop-1-ene **78** would react with hydroxylamine hydrochloride and pyridine in ethanol to yield the corresponding oxime directly. Unfortunately this procedure was unsuccessful and mainly gave the diethylacetal **79** scheme 21.

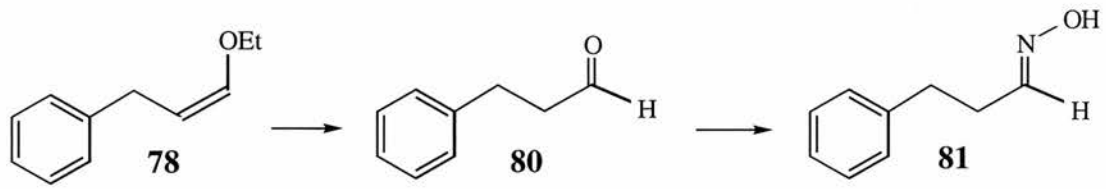


Scheme 21: *Attempted reaction to give the oxime*

The ^{1}H n.m.r. spectrum of the acetal showed the presence of two ethyl groups. The CH_3 occurred at δ 1.25 and integrated to 6 protons while the CH_2 occurred at δ 3.6 and integrated to four protons. The $CH(OEt)_2$ group showed as a triplet integrating to 1 proton at δ 4.5. The two CH_2 groups occurred at δ 2.0 for $CH_2CH(OEt)_2$ and at δ 2.7 for CH_2Ph. The aromatic peaks integrated to 5 protons and were found at δ 7.2. The ^{13}C n.m.r. spectrum provided further evidence that the desired product had not been formed. The ethyl group gave peaks at δ 15.8 and δ 61.5 while the methylene carbons showed at δ 31.6 for $CH_2CH(OEt)_2$ and δ 35.8 for CH_2Ph. The $CH(OEt)_2$ carbon stood out at δ 102.1 while the phenyl resonances occurred further downfield. Both spectra showed that the sample was not entirely pure with some minor contamination from the starting materials but the major product signals were clearly observed.

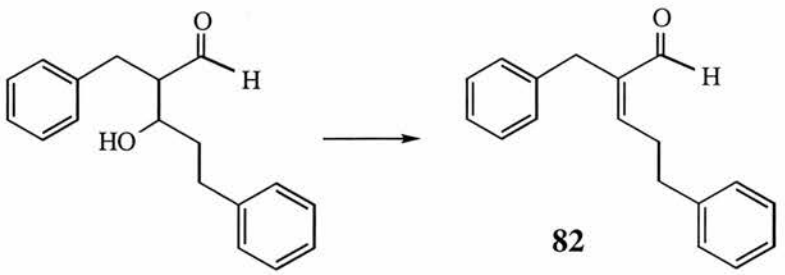
A second attempt involved reaction of 1-ethoxy-3-phenylprop-1-ene with hydroxylamine hydrochloride and sodium acetate trihydrate in a water-tetrahydrofuran solvent. The reaction was warmed in a water bath at 70 °C while stirring vigorously. Extraction of this mixture using diethyl ether and evaporation of the solvent at reduced pressure gave a yellow oil which contained only starting material.

It was thus necessary to convert 1-ethoxy-3-phenylprop-1-ene **78** to the 3-phenylpropionaldehyde **80** before reacting this to give the oxime **81** (scheme 22).



Scheme 22: *Two step reaction to give the oxime*

Several attempts were made at deprotecting 1-ethoxy-3-phenylprop-1-ene. The deprotections involved the use of various concentrations of either hydrochloric or acetic acid in various water-THF mixtures. These reactions were monitored by t.l.c. (silica, ethyl acetate-hexane (1:5)) and compared to an authentic sample of 3-phenylpropionaldehyde **80**. The first reaction involved the reaction of 1-ethoxy-3-phenylprop-1-ene with 5% glacial acetic acid solution. The reaction was initially warmed to 45 °C for a period of 2 hours. No reaction occurred, and so the heat was removed and the solution stirred for 3 days, but still no reaction was observed. Much stronger reaction conditions were then used with reaction in 6.0 M hydrochloric acid. After approximately 1 minute a very faint aldehyde spot appeared but this quickly disappeared to give only one spot on the baseline of the t.l.c.. The reaction mixture was extracted using diethyl ether and the solvent removed at reduced pressure. It was suspected that an aldol product (scheme 23) had formed from reaction of 3-phenylpropionaldehyde **80** with 1-ethoxy-3-phenylprop-1-ene **78** followed by a dehydration reaction to give the enal **82**. The ^{1}H n.m.r. spectrum showed a triplet at δ 5.0 integrating to 1 proton which would correspond to the presence of an alkenyl proton. In addition to this a quartet was observed at δ 2.3 integrating to 2 protons and a multiplet occurred at δ 2.8-3.2 integrating to 4 protons. These signals corresponded to the presence of three methylene groups. In the aromatic region a multiplet was given at δ 7.2-7.8 integrating to 10 protons. Furthermore a singlet was given at δ 9.9 which equates to an aldehyde proton. This reaction occurs very quickly in the presence of 6.0 M hydrochloric acid and so the aldehyde cannot easily be isolated under these conditions.



82

Scheme 23: *Aldol condensation product*

A less concentrated solution of hydrochloric acid was then used in the hope that the aldehyde may be more stable under these conditions and that the aldol reaction would be less facile. Using 0.1 M hydrochloric acid no reaction was observed after 5 hours stirring at room temperature so the acid concentration was increased to 0.5 M. After 2 hours stirring at room temperature some aldehyde had formed however even after stirring for a period of 2 days the reaction remained incomplete. The acid concentration was gradually increased in 0.25 M steps in the hope the reaction would continue to give the aldehyde. After each increase in concentration the reaction was stirred for at least 3 hours. At concentration of 1.75 M hydrochloric acid aldol products started to form in the solution.

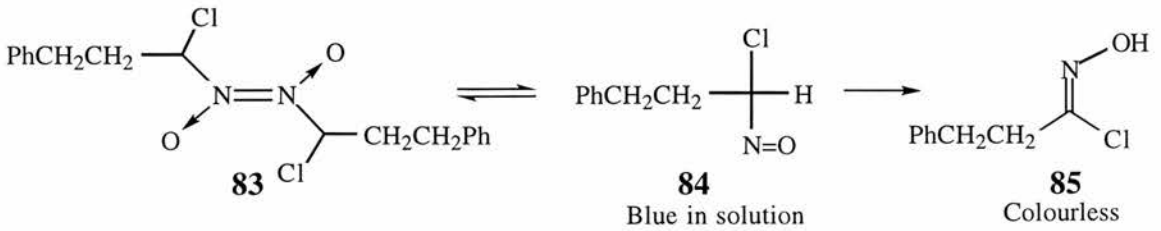
When the reaction was repeated with a 1.6 M concentration of hydrochloric acid the aldol product was formed after only 2 hours reaction time and the starting material had not been consumed. It was considered that perhaps the reaction would progress more readily in the presence of a water miscible organic solvent to aid the solubility of the 1-ethoxy-3-phenylprop-1-ene. A solution of tetrahydrofuran and 1.0 M hydrochloric acid (1:5) was thus investigated but after only 60 minutes the aldol product appeared.

These problems were eventually overcome by reaction in a mixture of acetone-water (4:1) with two drops of concentrated hydrochloric acid.[203] The reaction gave a 73% crude yield of 3-phenylpropionaldehyde **80** as a pale-yellow oil. When repeated with the deuterium labelled material, 87% of the pentadeuterated 3-phenylpropionaldehyde was obtained as a clear oil after distillation at reduced pressure. All spectral data for the 3-

phenylpropionaldehyde product were identical to that of an authentic commercial sample. The spectra of the pentadeuterated 3-phenylpropionaldehyde also illustrated clear formation of the aldehyde with the ^{1}H n.m.r. spectrum showing an aldehyde proton at δ 9.8 and the ^{13}C n.m.r. spectrum giving a carbonyl peak at δ 202.1. The infrared spectrum showed strong carbonyl absorption at 1720 cm^{-1} and the mass spectrum gave the appropriate molecular ion (*m/z* (EI) 139 ($[M]^+$, 91%)). Spectral data implied > 95% incorporation of deuterium.

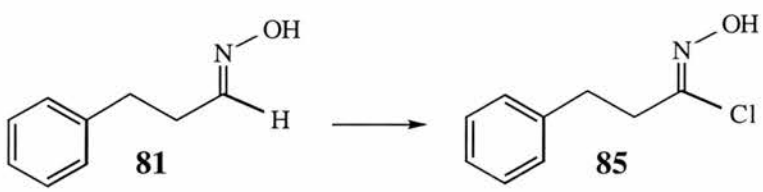
The aldehydes were then reacted with sodium acetate trihydrate and hydroxylamine hydrochloride in a water-ethanol solution to give the corresponding oximes.[204] The non-deuterated oxime was produced in 92% yield and the deuterated oxime formed in 85% yield both were white solids and both existed as E and Z isomers, again spectral data implied > 95% incorporation of deuterium. Microanalysis confirmed the purity of the 3-phenylpropionaldehyde oxime (Found: C, 72.23; H, 7.94; N, 9.35. Calc for $C_9H_{11}NO$: C, 72.46; H, 7.43; N, 9.39%) and the 3-[$^{2}H_5$]phenylpropionaldehyde oxime (Found: C, 70.02; H, 7.25; N, 8.98. Calc for $C_9H_6{}^{2}H_5NO$: C, 70.09; H, 7.19; N, 9.08%).

In chlorinating 3-phenylpropionaldehyde oximes Gil and MacLeod reported that yields could be low.[184] They also noted that the reaction had a strong tendency to give dimeric products (scheme 24). This was clearly observed from their infrared spectrum which showed no band at 3200 cm^{-1} characteristic of the oximyl chloride OH. However a strong band was observed at 1195 cm^{-1} which corresponded to the trans dimer **83**. These compounds are readily formed from the dimerisation of *C*-nitroso compounds **84** (often blue in colour) found in the reaction mixture. The solid dimer was colourless but when dissolved it gave a blue solution which faded with time reverting to a colourless solution of the monomer **85**.



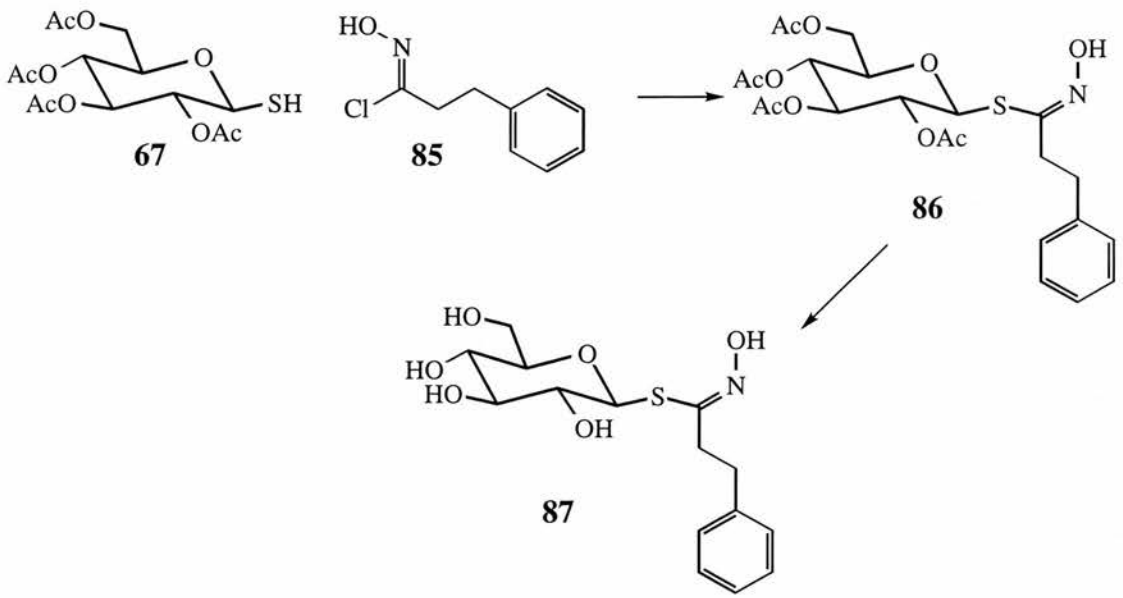
Scheme 24: *Dissociation of dimer in chloroform solution as observed by Gil and MacLeod*

These observations were taken into account when chlorinating the prepared deuterated and non-deuterated 3-phenylpropionaldehyde oximes using *N*-chlorosuccinimide and pyridine in chloroform (scheme 25). In this case the monomeric oximyl chloride **85** was given directly. No blue colour was observed in the solution and the infrared spectrum illustrated the oximyl chloride OH group at 3200 cm^{-1}. The ^1H n.m.r. spectra showed loss of the oxime hydrogen signals at δ 6.8 and δ 7.5 which corresponded to the Z and E isomers respectively. The deuterated and non-deuterated oximyl chlorides were reacted in the next step without purification.



Scheme 25: *Conversion to the oximyl chloride*

The differences observed for the chlorination reactions may be due to the reaction conditions used. Gil and MacLeod chlorinated their oxime using chlorine gas, however in this thesis chlorine gas has been used to chlorinate benzaldehyde oxime (section 2.2.2). Under these conditions a blue solution was observed but these were the only chlorination conditions in this thesis that showed the colouration.



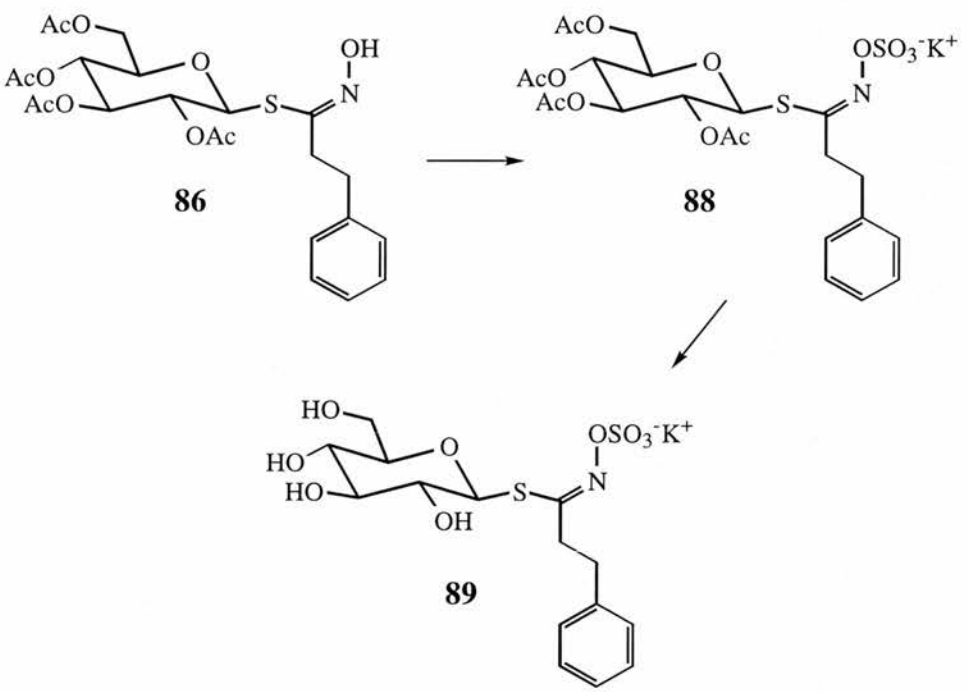
Scheme 26: *Synthesis of the thiohydroximate*

Reaction of the crude oximyl chloride **85** with 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose **67** in tetrahydrofuran with triethylamine successfully produced the corresponding 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl phenethyl thiohydroximate **86** (scheme 26). The product was purified using column chromatography with ethyl acetate-hexane (1:1) to give a white solid in 31% yield. When the reaction was repeated with the deuterated oximyl chloride 37% of the deuterated thiohydroximate was obtained. The ^{1}H n.m.r. spectra of the deuterated and non-deuterated products were identical except, as expected, the phenyl protons are absent in the spectrum of the deuterated product. The acetyl protecting groups appeared in the infrared spectrum at 1750 cm^{-1}. Spectral data implied >95% incorporation of deuterium into the protected deuterated desulfogluconasturtiin.

The final step to give the two desulfogluconasturtiins was to deprotect the glucose moiety using a catalytic amount of potassium methoxide in methanol (scheme 26). The solution was neutralised with Amberlite IR-120 resin and concentrated to give the products as pure white solids. The infrared and both ^{1}H and ^{13}C n.m.r. spectra indicated total loss of the acetyl protecting groups. In addition the microanalysis confirmed successful conversion to

product, desulfogluconasturtiin **87** (Found: C, 51.45; H, 6.15; N, 3.91. Calc for $C_{15}H_{21}NO_6S.0.5H_2O$: C, 51.12; H, 6.29; N, 3.97%) and desulfo-[$^2H_5$]gluconasturtiin (Found: C, 49.55; H, 5.59; N, 3.75. Calc for $C_{15}H_{16}{}^2H_5NO_6S.H_2O$: C, 49.17; H, 5.78; N, 3.82%).

Sulfation and deprotection of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl phenethyl thiohydroximate **86** was carried out (scheme 27) in order that the resulting gluconasturtiin **89** could be tested with myrosinase (section 2.3.1.1) and compared to the novel phenyl analogues synthesised in section 2.2.2. In addition, gluconasturtiin has proven to be of use as an internal standard in studies on glucosinolate metabolism (section 2.1.8).



Scheme 27: *Synthesis of gluconasturtiin*

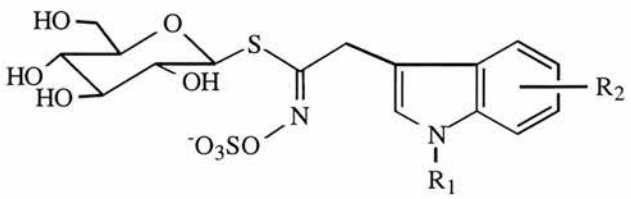
For the sulfation reaction it was necessary to use a freshly prepared solution of pyridine-sulfur trioxide.[138] This complex was formed by reaction of dry pyridine with chlorosulfonic acid in dry dichloromethane at 0 °C. The reaction can be violent and great care was taken upon addition of the chlorosulfonic acid solution to the pyridine solution. Water was rigorously excluded as the chlorosulfonic acid is extremely water sensitive. It is

important to note that the pyridine-sulfur trioxide complex is commercially available however previous studies in our group showed this complex to be ineffective.[138] The complex absorbed water and was thus hydrolysed to give pyridinium hydrogen sulfate which was not active as a sulfating agent precluding formation of the required product.

The thiohydroximate **86** in a solution of dichloromethane was thus added to preformed pyridine-sulfur trioxide complex. The reaction was stirred for a period of 24 hours at room temperature then saturated sodium bicarbonate was added. The mixture released carbon dioxide gas and stirring was continued for a further 30 minutes. The gluconasturtiin **89** was obtained directly from this reaction and thus the deprotection step was unnecessary. The white solid was purified using reverse phase C-18 silica then desalted with G-10 Sephadex using a distilled water eluant. The infrared spectrum illustrated the total loss of the acetyl protecting groups and negative ion electrospray mass spectrometry showed the appropriate molecular ion at 422 corresponding to $[M-K]^-$. The product was pure by microanalysis (Found: C, 39.28; H, 4.74; N, 3.02. Calc for $C_{15}H_{20}NO_9S_2K$: C, 39.04; H, 4.37; N, 3.03%).

2.1.3 Synthesis of desulfoglucobrassicins

Only seven naturally occurring glucobrassicins have been identified, occurring mainly in plants of the *Raphanus* and *Brassica* genera[187, 188] and are illustrated in figure 19.



Glucobrassicin	R_1=H, R_2 = H
Neoglucobrassicin	R_1=OCH_3, R_2 = H
N-Sulfoglucobrassicin	R_1=SO_3^-, R_2 = H
N-Acetylglucobrassicin	R_1=$C(O)CH_3$, R_2 = H
4-Hydroxyglucobrassicin	R_1=H, R_2 = OH
4-Methoxyglucobrassicin	R_1=H, R_2 = OCH_3
5-Methoxyglucobrassicin	R_1=H, R_2 = OCH_3

Figure 19: *There are seven naturally occurring glucobrassicins*

There is some disagreement over the substitution site of the 4/5 methoxy group. However it may be that both exist along with other substituted indole glucosinolates yet to be isolated and identified.[187, 188]

The glucobrassicin family possess potent biological activity for example affecting drug metabolising enzyme systems, altering chemically induced carcinogenesis, goitrogenicity and nitrite trapping.[102] In addition these compounds prove particularly attractive to insects and promote oviposition. Due to this wealth of biological activity a number of synthetic tryptophan-derived glucosinolates exist in the literature. Without exception, all of these glucobrassicins whether naturally occurring, or novel variants, have stemmed from a common synthetic route devised by Viaud and Rollin who first prepared unsubstituted glucobrassicin.[102] Their route was later used to prepare 4- and 5-

methoxyglucobrassicin.[187, 188] However, there is only one existing literature synthesis of a labelled glucobrassicin, figure 20.[190]

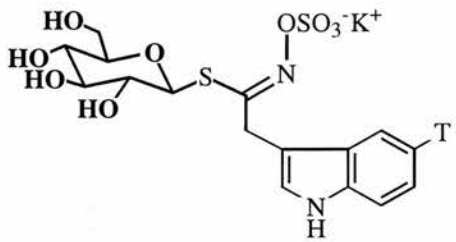
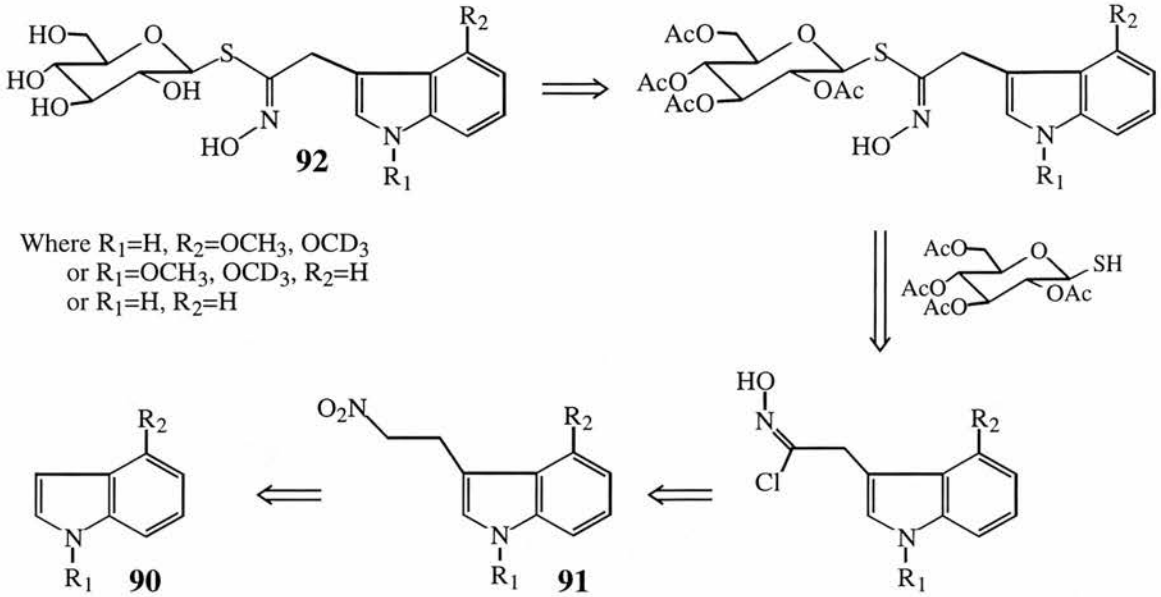


Figure 20: *There is only one labelled glucobrassicin synthesis*

The [5-^{3}H]-indol-3-ylmethylglucosinolate was synthesised from acetylated 5-bromoindol-3-ylmethyl glucosinolate. The radiolabel was incorporated so that the *in vivo* biotransformations of glucobrassicin could be studied. This compound was not useful for our purposes since a difference of at least 3 mass units is required between the natural and labelled compounds. In addition a very high incorporation of the label was required.

In this thesis the synthetic route devised by Viaud and Rollin has been modified to incorporate deuterium into the methoxy functionality of the desulfo-4-methoxyglucobrassicin. In addition, desulfo-*N*-methoxyglucobrassicin (desulfoneoglucobrassicin) has been synthesised for the first time along with its deuterated counterpart. The overall synthetic route used is shown in scheme 28.

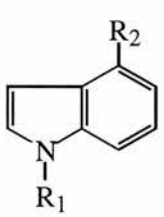


Scheme 28: *Retrosynthesis of desulfoglucobrassicins*

The synthetic route can be divided into three sections and each will be described in turn. Firstly the substituted indoles **90** are synthesised then the side chain is added **91** before the compounds are converted to the desulfoglucobrassicins **92**.

2.1.3.1 Synthesis of substituted indoles

Essentially four indole derivatives were synthesised (figure 21), firstly the 4-methoxyindoles (**93** and **94**) then the *N*-methoxyindoles (**95** and **96**). For all of the compounds synthesised correlation spectroscopy was used to confirm the assignment of the ^{13}C n.m.r. spectra.

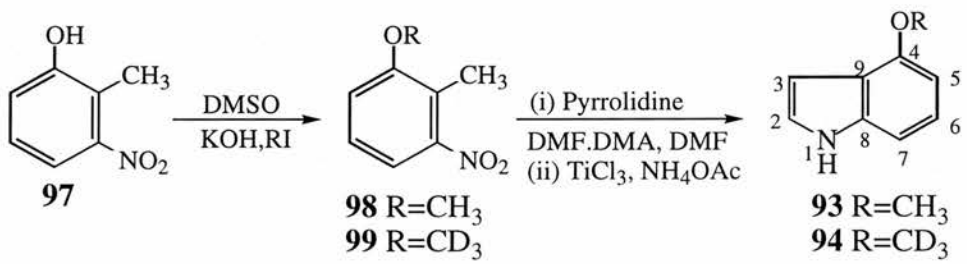


93 R_1=H, R_2=OCH_3
94 R_1=H, R_2=OCD_3
95 R_1=OCH_3, R_2=H
96 R_1=OCD_3, R_2=H

Figure 21: *Substituted indole target compounds*

(i) 4-Methoxyindole synthesis

The 4-methoxyindoles **93** and **94** were prepared via an initial methylation of 2-methyl-3-nitrophenol **97** followed by a cyclisation of 2-methoxy-6-nitrotoluene **98** and **99** (scheme 29).



97

98 R=CH_3
99 R=CD_3

93 R=CH_3
94 R=CD_3

Scheme 29: *Synthetic route to deuterated and nondeuterated 4-methoxyindoles*

The methylation was achieved using **97** and powdered potassium hydroxide in dimethyl sulfoxide to which was added methyl iodide.[205] The reaction produced pale-yellow crystals of **98** in quantitative yield. The infrared spectrum showed complete disappearance of the OH absorption at 3300 cm^{-1} and the ^1H n.m.r. spectrum of the crude product was identical to that of an authentic commercial sample of 2-methyl-3-nitro-methoxybenzene, showing the new methoxy group at δ 3.87.

To cyclise **98** it was first necessary to introduce a one carbon fragment which would add to the aromatic methyl group. This was achieved by treating with *N,N*-dimethylformamide dimethylacetal and pyrrolidine in dry *N,N*-dimethylformamide. The reaction was concentrated under reduced pressure, taken up in acetone then shaken with ammonium acetate buffer and titanium trichloride in a separating funnel. The titanium trichloride reduced the aromatic nitro group to an amine which effected the cyclisation to form the indole ring.[206] A 71% yield of 4-methoxyindole **93** was obtained after column chromatography (silica, 40-60 petroleum ether-diethyl ether (2:1)). From the ^1H n.m.r. spectrum it was seen that the methyl signal had disappeared while the methoxy signal had shifted from δ 3.87 to δ 3.98. All of the other protons occur in the aromatic region of the

spectrum. The microanalysis results (Found: C, 73.37; H, 6.27; N, 9.49. Calc for C_9H_9NO: C, 73.45; H, 6.16; N, 9.52%) and mass spectrum (*m/z* (EI) 147 ($[M]^+$, 100%)) confirmed the identity of the product.

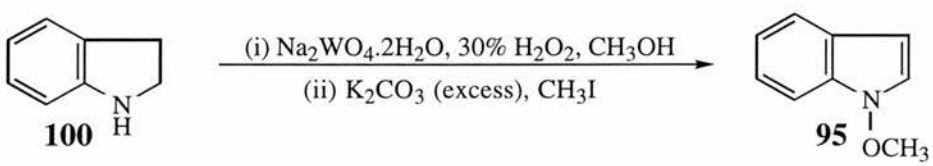
The above reactions were then repeated using [$^2H_3$]methyl iodide to give the 2-methyl-3-nitro-[$^2H_3$]methoxybenzene **99** as a pale-yellow powder. Infrared spectroscopy as before showed loss of the OH absorption and mass spectrometry gave the expected molecular ion peak (*m/z* (EI) 170 ($[M]^+$, 85%)) implying a good incorporation of deuterium. Microanalysis illustrated the purity of the sample (Found: C, 56.35; H, 5.10; N, 8.02. Calc for $C_8H_6{}^2H_3NO_3$: C, 56.46; H, 5.33; N, 8.23%). All spectral data implied >95% incorporation of deuterium, and no signal was observed in the ^1H n.m.r. spectrum at δ 3.87.

Cyclisation of the trideuterated derivative **99** gave the required 4-[$^2H_3$]methoxyindole **94** as white crystals in 57% yield after column chromatography (silica, 40-60 petroleum ether-diethyl ether (2:1)). No methoxy peak showed in the n.m.r. spectrum and correct microanalysis (Found: C, 71.29; H, 6.19; N, 9.26. Calc for $C_9H_6{}^2H_3NO$: C, 71.97; H, 6.04; N, 9.33%) gave conclusive evidence of product formation. In this reaction great care was taken to identify the C^2H_3 group to ensure it had survived the reaction conditions and it did appear that no significant loss of label had occurred.

(ii) *N*-Methoxyindole synthesis

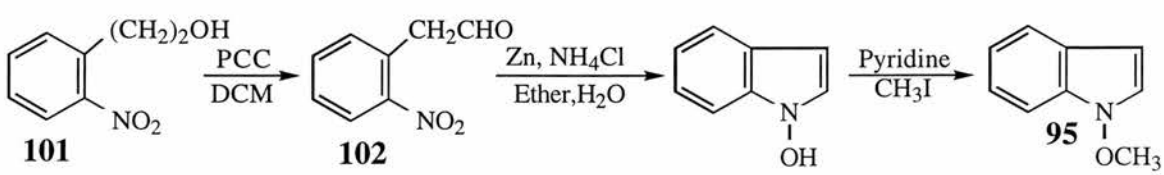
In this section three different routes which were investigated to synthesise *N*-methoxyindole are described.

Route 1: In this route indoline **100** was oxidised by sodium tungstate and 30% H_2O_2 in methanol at 0 °C, followed by methylation *in situ* using K_2CO_3 and methyl iodide (scheme 30).[207, 208] A large mixture of products were given from the reaction mixture and none of the desired product **95** was isolated.



Scheme 30: *First route attempted to synthesise N-methoxyindole*

Route 2: As shown in scheme 31 the oxidation of 2-nitrophenethyl alcohol **101** was effected using PCC in dry dichloromethane. After filtration of the reaction mixture through a column of fluorosil the crude product **102** was obtained in 90% yield.[209] The ^1H n.m.r. spectrum showed formation of the aldehyde with a peak at δ 9.70 and the ^{13}C n.m.r. spectrum showed a similar aldehyde peak at δ 194. This product was not purified as the literature indicated the compound typically decomposed on silica gel and was best used in its crude state.[210]

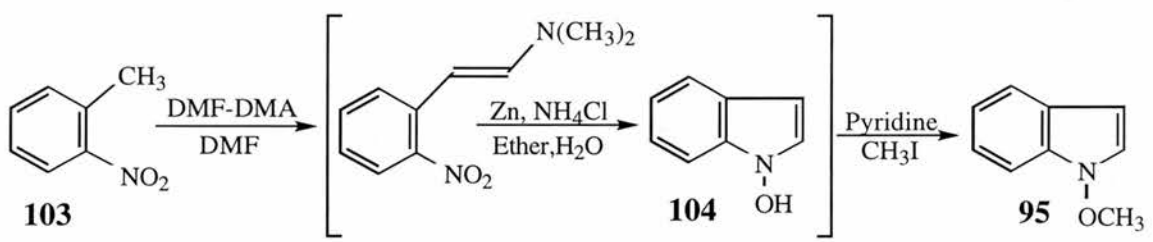


Scheme 31: *Second route attempted to synthesise N-methoxyindole*

Reductive cyclisation of *o*-nitrophenylacetaldehyde **102** was attempted using zinc dust, aqueous ammonium chloride and ether.[211] After stirring for 1 day the organic layer was

added to a mixture of methyl iodide and pyridine. The cyclisation and methylation reactions gave a large mixture of products which proved difficult to separate by column chromatography. *o*-Nitrophenylacetaldehyde is fairly unstable[212] and this may have complicated the reaction. No product was isolated and the method was discontinued in favour of route 3 which proved to be a cleaner more efficient reaction.

Route 3: Using *N,N*-dimethylformamide dimethylacetal an extra carbon unit was added on to 2-nitrotoluene **103** in refluxing *N,N*-dimethylformamide (scheme 32). The product was then reductively cyclised using ammonium chloride and zinc dust in a water-diethyl ether biphasic reaction. The intermediate *N*-hydroxyindole **104** is unstable and was methylated by adding the ethereal layer of the biphasic reaction to methyl iodide in 10% aqueous sodium hydroxide solution with a phase transfer catalyst.[213, 214]



Scheme 32: *Successful synthesis of N-methoxyindole*

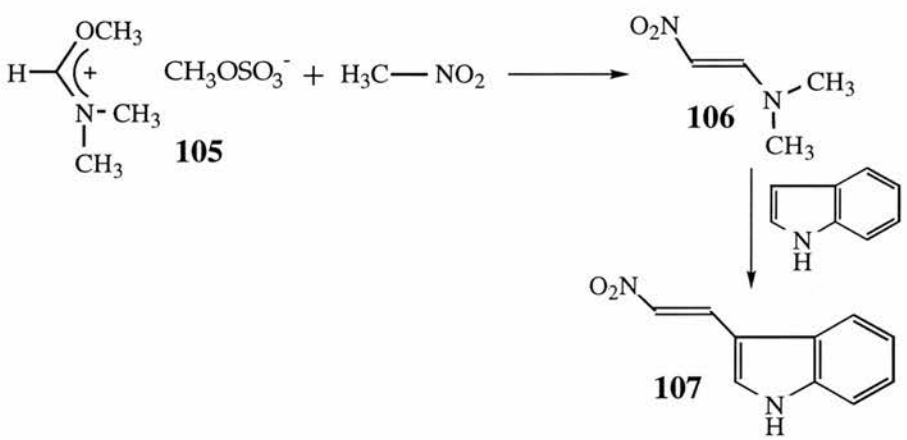
Initial attempts at this reaction gave pure product **95** after column chromatography but in very poor yield, typically of the order 5-7%. Changes were made to the reaction by using longer reaction times, changing the phase transfer catalyst from benzyl triethylammonium chloride to tri(n-octyl)methylammonium chloride and using activated zinc dust. However these changes showed little effect. It was thought that the problem may lie with poor mixing in this heterogeneous reaction mixture. Indeed an increase in yield to 40% was gained by mechanical stirring of the reductive cyclisation. The pure product was a pale-yellow, light sensitive oil which could be stored in the absence of light at room temperature for long periods of time. ^{1}H n.m.r. spectroscopy showed the presence of the *N*-methoxy

functionality at δ 4.15 with the remaining aromatic protons between δ 6.45 and δ 7.8. Mass spectrometry showed the expected molecular ion (*m/z* (EI) 147 ([M]$^+$, 100%)).

The above reaction was repeated using [$^2H_3$]methyl iodide to give *N*-[$^2H_3$]methoxyindole (**96** figure 21) as a golden oil in 44% yield. The ^1H n.m.r. and ^{13}C n.m.r spectra were identical to those of *N*-methoxyindole but the peaks for the methoxy group were absent as expected. Again the mass spectrum gave the correct molecular ion (*m/z*(EI) 150 ([M]$^+$, 100%)).

2.1.3.2 Addition of the side chain

Various methods were investigated for the addition of a nitroethyl side chain onto the 3 position of each indole. The first method involved a reaction of indole with 1-dimethylamino-2-nitroethene **106** to give 3-(2-nitrovinyl)indole **107** (scheme 33).

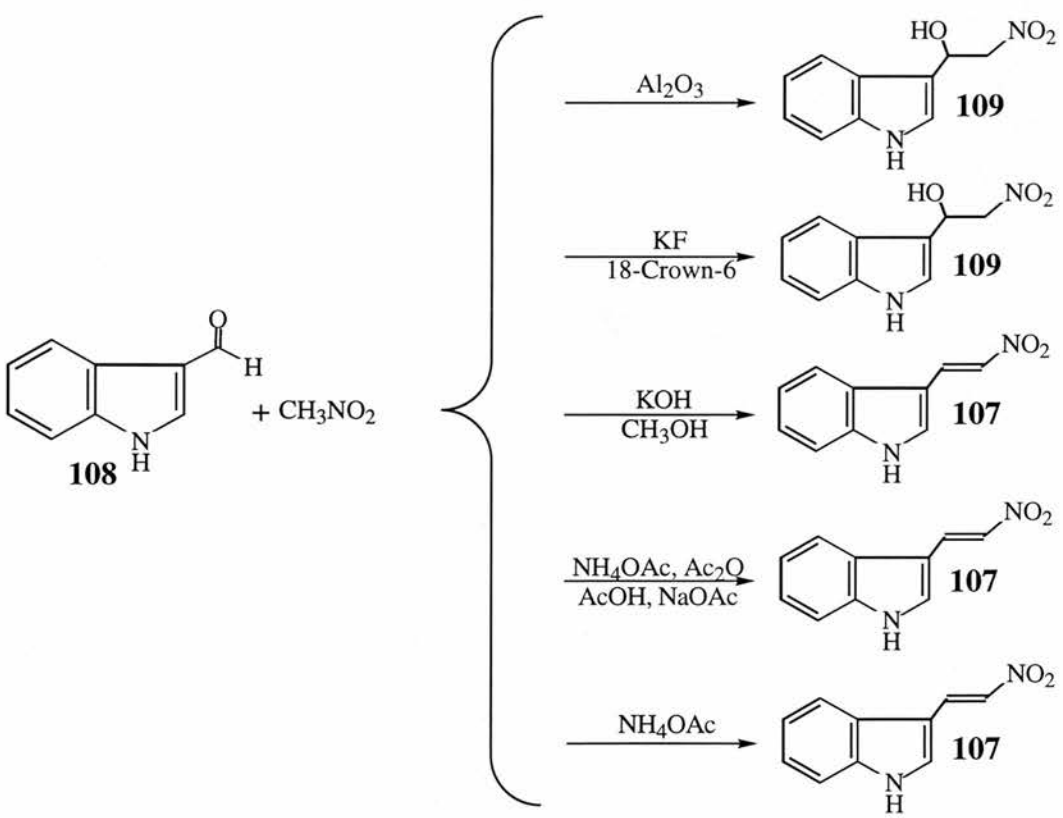


Scheme 33: *Addition of a side chain to indole*

The 1-dimethylamino-2-nitroethene **106** was synthesised by reaction of a *N,N*-dimethyformamide dimethylsulfate complex **105** with nitromethane in sodium ethanoate solution. This gave the product as a stable golden brown crystalline solid. Reaction of this compound with indole in trifluoroacetic acid yielded 3-(2-nitrovinyl)indole **107**.[215] The product was recrystallised from methanol to produce dark brown plate-like crystals of **107**

in 76% yield. The success of this reaction could be determined by ^1H n.m.r. spectroscopy which showed no methyl peaks and a clear shift of the olefin protons. The mass spectrum *m/z*(EI) 188 ($[M]^+$, 100%) and microanalysis (Found: C, 63.45; H, 4.27; N, 14.72. Calc. for $C_{10}H_8N_2O_2$: C, 63.83; H, 4.29; N, 14.89%) gave a more positive identification of the product.

Unfortunately this reaction proved to be rather unreliable and did not always give the desired product. Indeed when the reaction was attempted with 4-methoxyindole a large mixture of products resulted. This prompted an investigation into other methods of side chain addition. Further methods involved Henry-type reaction of indole-3-carboxaldehyde **108** with nitromethane under various conditions (scheme 34).



Scheme 34: *Attempted reactions of 3-formylindole with nitromethane*

The first set of conditions involved reaction of indole-3-carboxaldehyde with nitromethane. In this reaction both reagents are completely adsorbed onto an alumina surface by vigorous stirring. After standing for 24 hours at room temperature the 3-(2-nitro-1-hydroxyethyl)indole **109** should have been produced and could have been washed from the alumina surface using dichloromethane.[216] This product would then have been reacted to give the desired 3-(2-nitroethyl)indole via acetylation and reduction reactions. Unfortunately no product was isolated from the Henry reaction. The carbonyl group of the starting material was observed at δ 10.0 in the ^{1}H n.m.r. spectrum and δ 182.4 in the ^{13}C n.m.r. spectrum.

A second method also involved synthesis of 3-(2-nitro-1-hydroxyethyl)indole **109** followed by acetylation and reduction. Indole-3-carboxaldehyde **108** was stirred with nitromethane in the presence of a catalytic amount of potassium fluoride (0.05 equivalents) in isopropanol.[217] 18-Crown-6 was added to accelerate the reaction but only starting materials could be isolated after a 3 hour reaction period. As above the ^{1}H and ^{13}C n.m.r. spectra illustrated that the carbonyl group of indole-3-carboxaldehyde **108** remained.

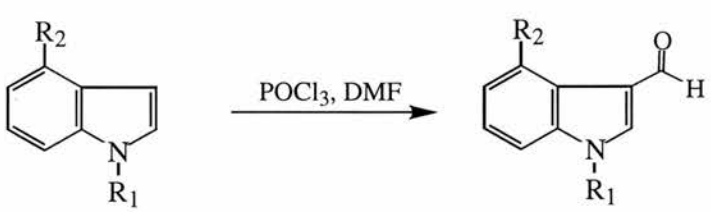
Thirdly indole-3-carboxaldehyde **103**, nitromethane and methanol were cooled to -5 °C upon which 50% potassium hydroxide solution was added.[218] After stirring for a period of 1 hour at 0 °C ice water was added to the reaction mixture then this solution was poured into 3.0 M hydrochloric acid. The precipitate was removed by filtration but the ^{1}H n.m.r. spectrum gave no indication of 3-(2-nitrovinyl)indole **107** formation. Extraction of the aqueous layer using ethyl acetate also gave no product. The reaction was repeated but stirred overnight at room temperature but these reactions gave only starting materials.

Two further methods for the synthesis of 3-(2-nitrovinyl)indole **107** were attempted and both proved to be successful. The first method involved adding a mixture of indole-3-carboxaldehyde **108** and nitromethane to a solution of ammonium acetate and acetic anhydride in acetic acid. The reaction was brought to reflux and sodium acetate added.

During the four hour reflux further acetic anhydride was added periodically.[219] This procedure gave a 43% yield of **107** after column chromatography (silica, ethyl acetate-hexane (1:1)).

The second method proved much simpler. Ammonium acetate, nitromethane and indole-3-carboxaldehyde were brought to reflux for two hours then the reaction was concentrated under reduced pressure.[220] This method gave a 42% yield of product **107** after column chromatography. The ^{1}H n.m.r. spectrum illustrated the loss of the carbonyl group at δ 9.95 while the remaining protons occurred in the region δ 7.45-8.55. The two protons of the vinyl side chain were seen as doublets at δ 8.06 for C*H*NO$_2$ and δ 8.55 for C*H*=CHNO$_2$ in agreement with the literature data.[220] Although these methods were lower yielding than reaction with 1-dimethylamino-2-nitroethene (scheme 33) they were more reliable and were therefore favoured.

Having obtained a reliable method for side chain addition it remained to formylate the 3 position of both the non-deuterated and deuterated 4- and 1-methoxyindoles. A Vilsmeier reaction was employed which involved dropwise addition of the appropriate indole to an ice-cold solution of phosphorous oxychloride in dry *N,N*-dimethylformamide (scheme 35). The reaction was warmed to 45 °C then stirred until complete.[221]



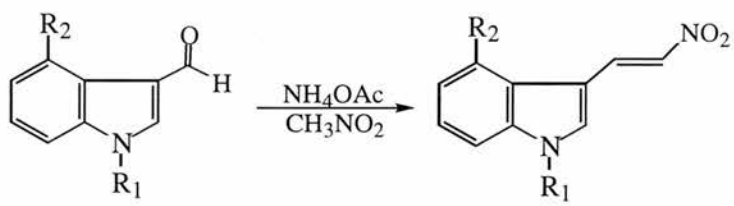
110 R_1=H, R_2=OCH_3; 85%
111 R_1=H, R_2=OCD_3; 73%
112 R_1=OCH_3, R_2=H; 100%
113 R_1=OCD_3, R_2=H; 100%

Scheme 35: *Vilsmeyer formylation of indoles*

Formation of the aldehyde could be clearly seen by ^{1}H and ^{13}C n.m.r. spectroscopy. For both 4-methoxyindoles **110** and **111** the aldehyde peaks occurred at δ 9.75 in the ^{1}H n.m.r. spectrum and at δ 186.6 in the ^{13}C n.m.r. spectrum. For the 1-methoxyindoles **112** and **113** the aldehyde peaks occurred at δ 9.90 in the ^{1}H n.m.r. spectrum and at δ

184.7 in the ^{13}C n.m.r. spectrum. In addition, for all four 3-formyl substituted indoles, the infrared spectra showed strong carbonyl absorptions and mass spectrometry gave the appropriate molecular ions. Spectral data implied >95% incorporation of deuterium for the 4- and 1-[$^{2}H_3$]methoxyindole-3-carboxaldehydes.

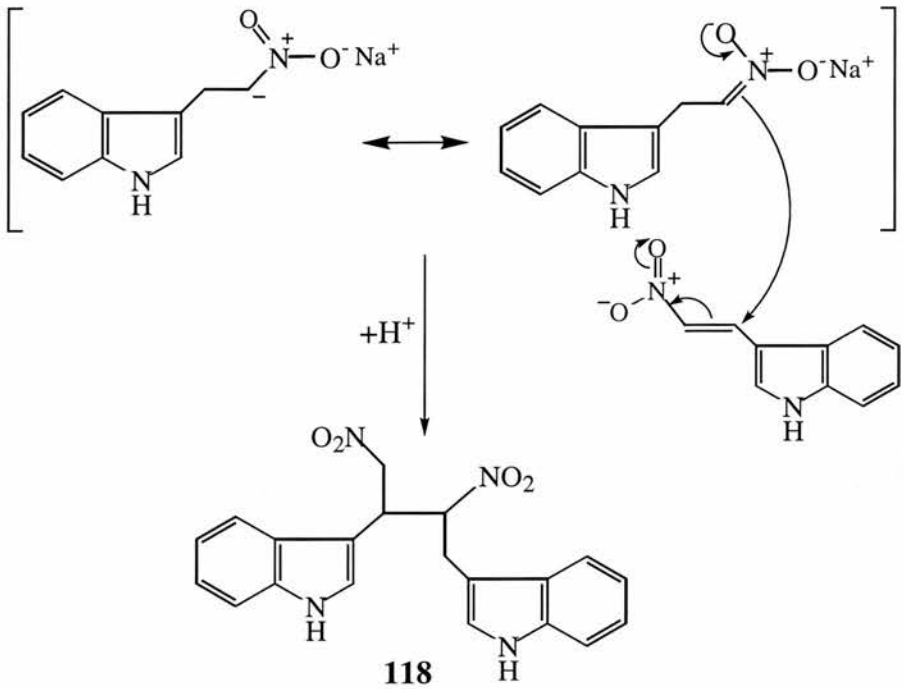
Reaction of the formylated indoles with nitromethane and ammonium acetate in each case provided the corresponding 3-(2-nitrovinyl)indoles (scheme 36) with disappearance of the carbonyl functionality as evidenced by ^{1}H n.m.r., ^{13}C n.m.r. and infrared spectrometry. It was found that the 1-methoxyindoles were less reactive therefore the reaction time was longer. CH Correlation spectroscopy helped to assign the proton and carbon spectra.



114 R_1=H, R_2=OCH_3; 85%
115 R_1=H, R_2=OCD_3; 89%
116 R_1=OCH_3, R_2=H; 75%
117 R_1=OCD_3, R_2=H; 78%

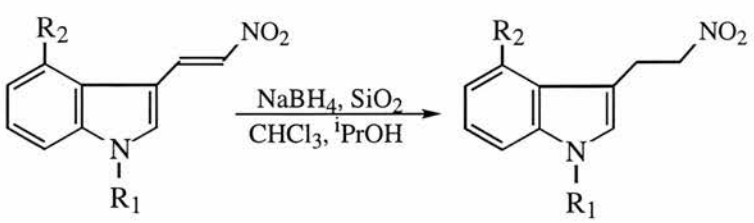
Scheme 36: *Henry reaction to give the nitrovinyl side chains*

With five 3-(2-nitrovinyl)indoles in hand all that remained to do was to reduce their side chains to the nitroethyl equivalents. Two methods were employed to achieve this transformation. The first method involved a simple reduction with sodium borohydride in dry methanol.[222] This method appeared to be largely unselective and gave a complex mixture of products and an alternative method was sought. This problem has been reported in the literature and is attributed to the tendency of the reaction mixture to form dimeric products such as **118**. The formation of **118** is thought to proceed by Michael addition of the resonance stabilised α-carbanion intermediate to the starting nitroalkene (scheme 37). This is favoured if the Michael acceptor and Michael donor exist in the same phase as they would in the methanolic reduction attempted.[223, 224]



Scheme 37: *Dimerisation of 3-(2-nitrovinyl)indole*

An alternative method by Sinhababu and Borchardt involved adding silica gel to a solution of the 3-(2-nitrovinyl)indole in isopropanol-chloroform (3:16). The silica gel suppressed the formation of dimers on addition of the sodium borohydride by acting as an insoluble protic phase on which the negatively charged α-carbanion intermediate could form and accept a proton before having a chance to undergo Michael addition reactions. It was thought that an apolar non protic solvent would provide the best reaction medium but it was found that a small amount of a protic solvent was also required.[223, 224] These conditions were successfully applied to the reduction of the five nitrovinyl indoles already synthesised (scheme 38).



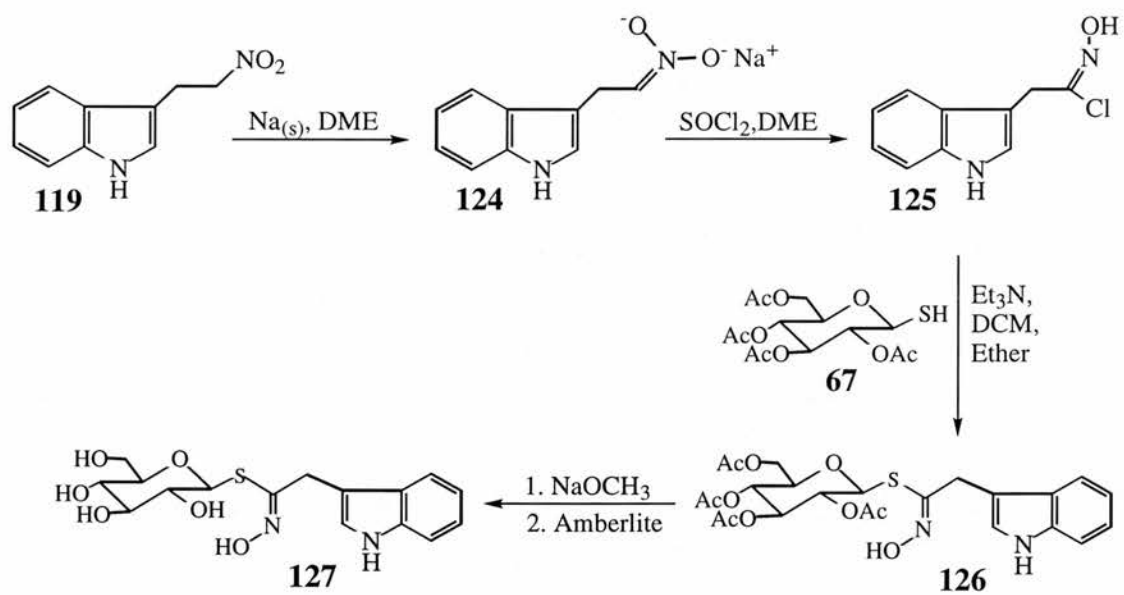
119 R_1=H, R_2=H; 53%
120 R_1=H, R_2=OCH_3; 40%
121 R_1=H, R_2=OCD_3; 37%
122 R_1=OCH_3, R_2=H; 59%
123 R_1=OCD_3, R_2=H; 65%

Scheme 38: *Reduction of 3-(2-nitrovinyl)indoles*

In the ^1H n.m.r. spectra of all the 3-(2-nitroethyl)indoles synthesised two triplets occurred which correspond to the nitroethyl functionality. One triplet was seen around δ 3.5 and corresponded to the CH_2 attached to the indole ring while another occurred higher up at around δ 4.7, and was assigned to CH_2NO_2. In addition the appropriate molecular ions were observed in the mass spectra and microanalysis gave conclusive evidence of product formation.

2.1.3.3 Conversion to desulfoglucobrassicins

Once the 3-(2-nitroethyl)indoles had been synthesised, four steps remained to give the five desulfoglucobrassicin targets according to the route used by Viaud and Rollin (scheme 39).[102]



Scheme 39: *Route used to synthesise desulfo indolyl glucosinolate*

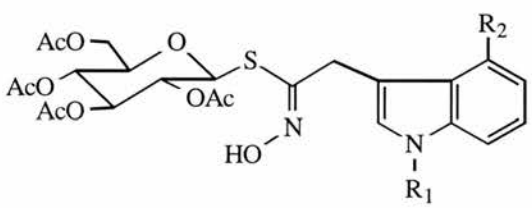
Using 3-(2-nitroethyl)indole **119** starting material, the first three steps proved to be extremely problematic giving dark oils containing inseparable mixtures of products. Titanium tetrachloride[225] and phosphorus pentachloride were tried as alternative chlorinating agents with little success.

Several problems existed, firstly, in producing the sodium nitronate salt **124** moisture must be rigorously excluded. The nitronate is extremely hygroscopic and any water will hydrolyse it back to the starting material. In the second step the oximyl chloride **125** is very unstable and must be reacted on as quickly as possible. Finally the coupling of the oximyl chloride to 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose **67** has a tendency to form the disulfide. Taking these considerations into account the method of Viaud and Rollin was reattempted. The 3-(2-nitroethyl)indole was purified in triplicate via column chromatography to ensure an analytically pure starting material. All solvents were dried immediately prior to use. The thionyl chloride was distilled twice, once from distilled quinoline and once from linseed oil providing a clear colourless liquid. The three problematic steps were carried out under nitrogen using a syringe or cannula tubing to transfer reagents between flasks.

In the reaction of the nitronate **124** to give the oximyl chloride **125** the nitronate in DME and the thionyl chloride solution in DME were cooled to -40 °C in individual flasks then the thionyl chloride solution was added dropwise via cannula tubing to the nitronate. The work-up of the oximyl chloride was performed as quickly as possible due to its lability and was reacted immediately in the next step to give the crude 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl glucobrassicin thiohydroximate **126**. The product was purified by column chromatography using two solvent systems: firstly 40-60 petroleum ether-ethyl acetate (6:4) which removed any unreacted 3-(2-nitroethyl) indole and acetyl protected thioglucose. Secondly column chromatography using dichloromethane-methanol (97:3) removed any disulfide by-product. This afforded pure 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl glucobrassicin thiohydroximate in 39% yield from 3-(2-nitroethyl)indole. The ^{1}H n.m.r. spectral data were identical to those given by Viaud and Rollin[102] and microanalysis (Found: C, 52.37; H, 5.12; N, 4.97. Calc for $C_{24}H_{28}N_2O_{10}S.H_2O$: C, 51.98; H, 5.27; N, 5.05%) gave further confirmation of product.

Final deprotection using a catalytic amount of potassium in methanol proceeded smoothly to give the required desulfoglucobrassicin **127** in 55% yield. The infrared, ^{1}H n.m.r. and ^{13}C n.m.r. spectra all illustrated complete loss of the acetyl groups. Microanalysis (Found: C, 50.78; H, 5.85; N, 6.82. Calc for $C_{16}H_{20}N_2O_6S.0.5H_2O$: C, 50.92; H, 5.61; N, 7.42%) and accurate mass spectrometry (*m/z* (Found: $[M+H]^+$ 369.1130. $C_{16}H_{21}N_2O_6S$ requires 369.1120)) also proved conclusive.

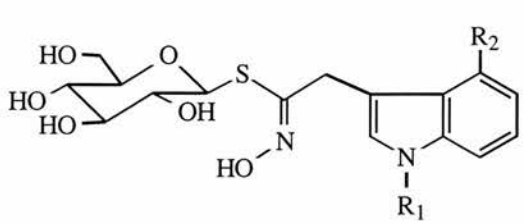
The methoxyglucobrassicin derivatives were synthesised in an identical manner with the acetylated products being produced in 17% yield for the 4-methoxy derivative, 20% yield for the 4-[$^{2}H_3$]methoxy derivative, 34% for 1-methoxy and finally 40% of the 1-[$^{2}H_3$]methoxy derivative (figure 22). It was noted that the 4-methoxy nitronate salts and 4-methoxy oximyl chlorides were less stable than their unsubstituted indole equivalents and correspondingly gave much lower product yields.



128 R_1=H, R_2=OCH_3; 17%
129 R_1=H, R_2=OCD_3; 20%
130 R_1=OCH_3, R_2=H; 34%
131 R_1=OCD_3, R_2=H; 40%

Figure 22: *Acetyl protected desulfomethoxyglucobrassicins*

Deprotection of the acetyl groups as before gave 73% yield for the 4-methoxy derivative, 58% yield for the 4-[$^{2}H_3$]methoxy derivative, 38% for 1-methoxy and finally 51% of the 1-[$^{2}H_3$]methoxy derivative (figure 23). It appears that the larger scale reactions gave lower product yield. The infrared, ^{1}H n.m.r. and ^{13}C n.m.r. spectra all illustrated complete loss of the acetyl groups and the mass spectra showed the appropriate molecular ions.



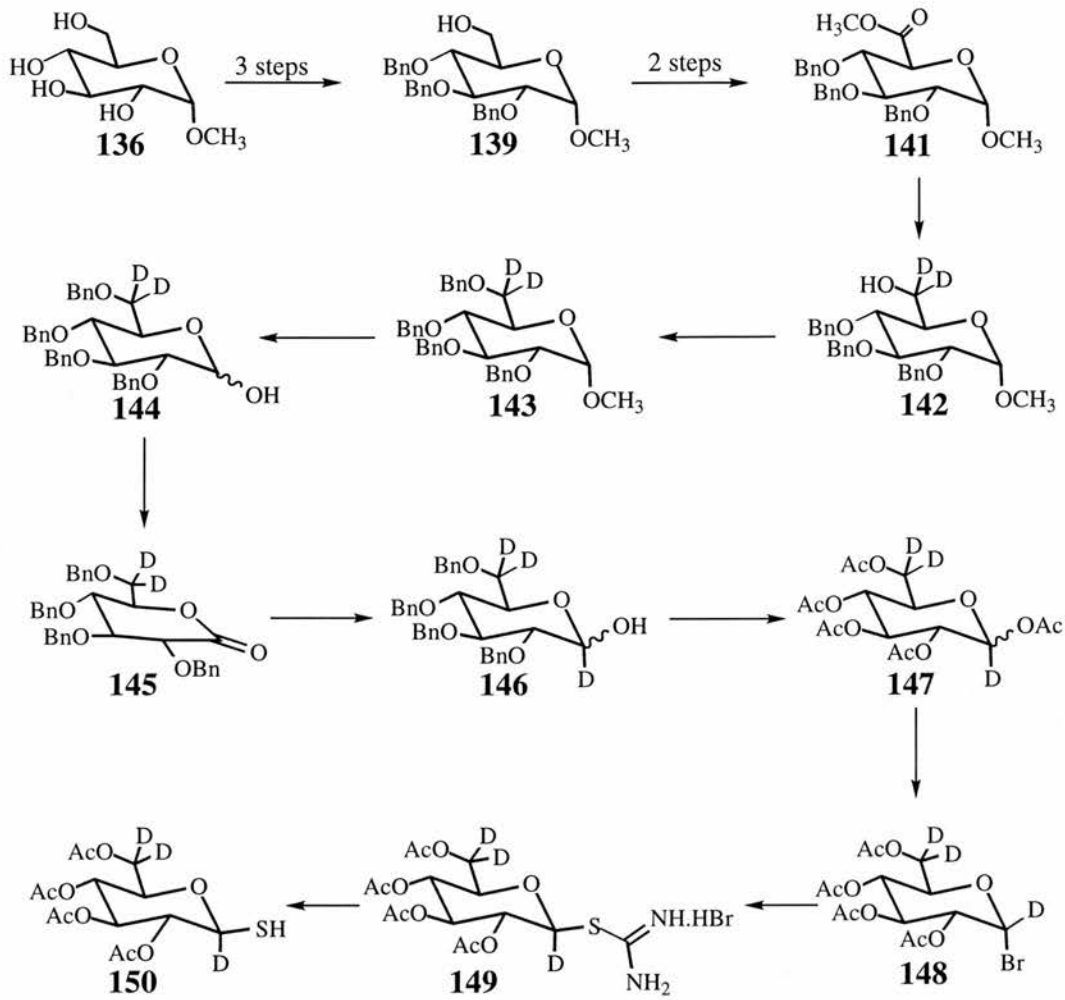
132 R_1=H, R_2=OCH_3; 73%
133 R_1=H, R_2=OCD_3; 58%
134 R_1=OCH_3, R_2=H; 38%
135 R_1=OCD_3, R_2=H; 51%

Figure 23: *Desulfomethoxyglucobrassicins*

This route provided the first ever synthesis of desulfo-1-methoxyglucobrassicin in both labelled and unlabelled form. Deuterated desulfo-4-methoxyglucobrassicin was also synthesised for the first time.

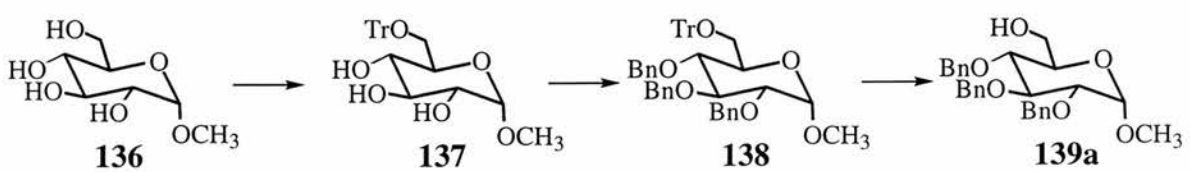
2.1.4 Synthesis of a deuterated glucopyranose

The problem with the aforementioned synthesis (sections 2.1.2 and 2.1.3) is that the deuterium is contained in the side chain of the desulfoglucosinolate. This strategy requires a different synthetic procedure for each compound. In order to give a general route to any deuterated glucosinolate it was decided to develop a route for incorporation of deuterium into the sugar moiety. The most obvious sites for the deuterium were at positions 1 and 6 of glucose. This would give our required 3 mass units difference from the natural compound for use as an internal standard in LC-MS work. The target compound was therefore 2,3,4,6-tetra-*O*-acetyl-thio-β-D-[1-$^{2}H_{1}$, 6-$^{2}H_{2}$]glucopyranose **150** (scheme 40) this compound can be incorporated directly into any established glucosinolate synthesis. Scheme 40 gives an overview of the actual route used while the following discussion illustrates how this route was established. CH-Correlation experiments were used to assign the ^{13}C n.m.r. spectra.



Scheme 40: *Synthesis of 2,3,4,6-tetra-O-acetyl-thio-β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranoside*

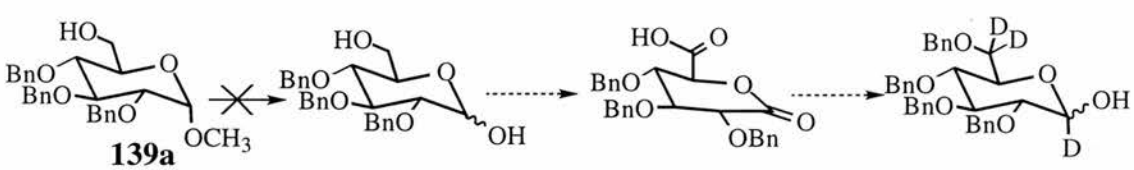
Two different routes were attempted both beginning with methyl-2,3,4-tri-*O*-benzyl-α-D-glucopyranoside **139a** synthesised according to the method of Bernet and Vasella (scheme 41).[226]



Scheme 41: *Bernet and Vasella route to methyl-2,3,4-tri-O-benzyl-α-D-glucopyranoside*

Firstly, a trityl group was introduced at the 6 position of methyl α-D-glucopyranoside **136** using chlorotriphenylmethane and 4-(dimethylamino)pyridine under basic conditions. The product **137** was obtained in quantitative yield after recrystallisation from ethanol. Subsequent benzylation of the 2, 3 and 4 positions with benzyl bromide in *N*,*N*-dimethylformamide using sodium hydride base yielded crude methyl-2,3,4-tri-*O*-benzyl-6-*O*-trityl-α-D-glucopyranose **138**. *p*-Toluenesulfonic acid was then used to remove the trityl group. After purification by column chromatography (silica, ethyl acetate-hexane (1:2)) **139a** was given in 80% yield over the three steps. The structures of these compounds were confirmed from the agreement of their melting points, n.m.r. and mass spectra with those from the literature.[226] In addition microanalysis (Found: C, 71.87; H, 6.93. Calc. for $C_{28}H_{32}O_6$: C, 72.39; H, 6.94%) was obtained for compound **139a**.

It was envisaged that deuterium would be incorporated into the structure via a series of oxidation and reduction reactions (scheme 42). From **139a** the shortest route to the target involved the initial removal of the methyl protecting group at position 1. From here it was hoped that we could simultaneously oxidise positions 1 and 6, however it was acknowledged that the intermediate produced could be very unstable. Subsequent reduction would incorporate deuterium into the sugar.



Scheme 42: *Unsuccessful route to 2,3,4-tri-O-benzyl-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose*

However attempts to remove the methyl group at position 1 were unsuccessful. When **139a** was heated with a mixture of acetic and sulfuric acid the methyl group was unaffected. Mainly starting material was recovered from the reaction but another product occurred further up the t.l.c. plate which had a very similar spectrum to the starting material. From the ^1H n.m.r. spectrum the methyl protecting group was present as were all

three benzyl groups. However, the protons of the glucose ring had shifted considerably. This led to the postulation that perhaps one of the benzyl groups had migrated onto position 6 (figure 24).

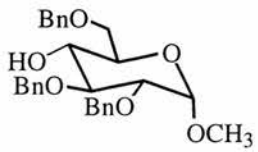
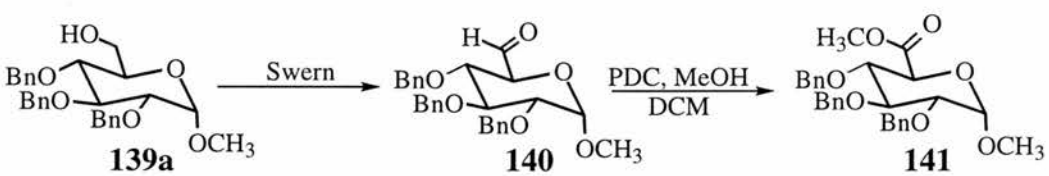


Figure 24: *Side product of demethylation reaction*

A second method involved the use of boron tribromide in dichloromethane however these conditions proved too harsh and resulted in a complex mixture.

It thus seemed more sensible to oxidise the 1 and 6 positions independently of each other. When **139a** was subjected to either a Jones oxidation or a ruthenium trichloride catalysed sodium periodate oxidation,[227] complex mixtures of products resulted. In contrast Pfitzner-Moffat oxidation[228] left large quantities of starting material. A two step oxidation proved much more successful (scheme 43).

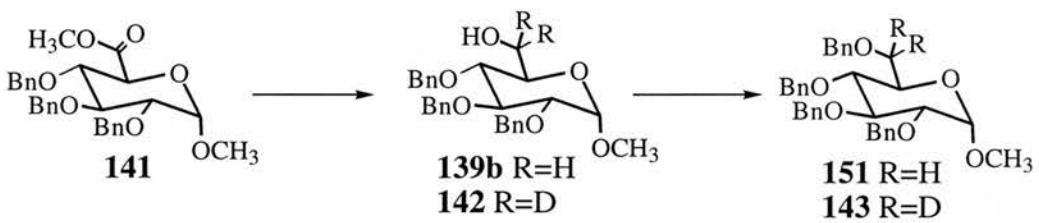


Scheme 43: *Two step oxidation procedure*

Swern oxidation gave methyl 6-aldehydo-2,3,4-tri-*O*-benzyl-α-D-glucopyranoside **140** in 86% yield after column chromatography (silica, ethyl acetate-hexane (1:1)). The infrared spectrum showed loss of the hydroxyl absorption at 3478 cm^{-1} and a new peak showed at 1726 cm^{-1} corresponding to the aldehyde group. The ^{1}H and ^{13}C n.m.r. spectra gave further confirmation with signals at δ 9.7 and δ 198.1 respectively for the aldehyde moiety.

Further oxidation to give the methyl ester **141** was achieved in 68% yield using PDC oxidant in *N,N*-dimethylformamide in the presence of methanol.[229] The infrared spectrum illustrated a shift of the carbonyl absorption to 1748 cm^{-1}. The mass spectrum showed the appropriate molecular ion (*m/z* (EI) 493 ($[M]^+$, 1%)) confirming that oxidation to form the ester had been successful.

To introduce the deuterium into position 6 the ester was reduced and the resulting hydroxyl moiety protected using a benzyl group (scheme 44).



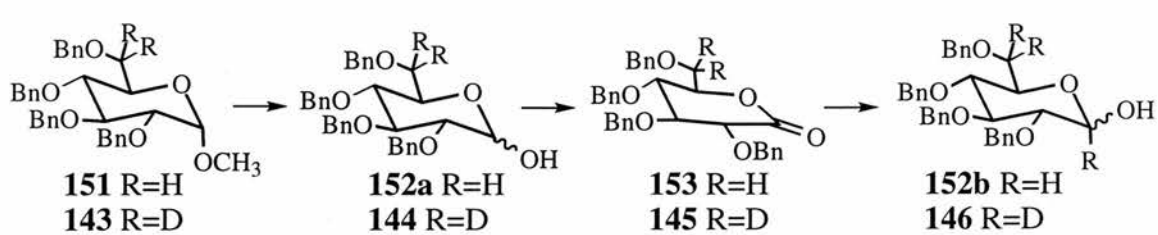
Scheme 44: *Reduction of the ester functionality followed by benzyl protection*

Reduction of the newly formed ester was first tried with lithium aluminium hydride to give **139b** before it was repeated with lithium aluminium deuteride to give **142**. In both cases the reaction proceeded smoothly to give a single product in 100% and 91% yield respectively. The methyl-2,3,4-tri-*O*-benzyl-α-D-glucopyranose data were consistent with the literature values.[226] For both compounds the infrared spectra illustrated the loss of the carbonyl group and showed the hydroxyl peak at 3478 cm^{-1}. Mass spectra showed molecular ions at (*m/z* (CI) 465 ($[MH]^+$, 1%)) and (*m/z* (CI) 467 ($[MH]^+$, 1%)) respectively. Comparing the ^1H n.m.r. spectra of the two products it can be observed that the multiplet at δ 3.52-3.80 which integrated to 5 protons had become less complex for the deuterated product and integrated to just 3 protons. This is consistent with the full incorporation of two deuterium atoms at position 6. Due to the decreased complexity of this signal in the deuterated product assignment became possible. At δ 3.51 there was a double doublet which corresponded to H-2: the multiplicity was due to coupling with H-3 and the anomeric proton. There was a triplet at δ 3.53 which overlapped the double doublet and corresponded to H-4. The third proton observed in the δ 3.45-3.85 region

was H-5 which showed as a doublet. H-3 showed up clearly as a triplet at δ 4.02 which was consistent with the literature data.[226] The remainder of the ^{1}H n.m.r. spectra of the deuterated and non-deuterated products were identical.

Benzylation of the free hydroxyl group was effected as usual with benzyl bromide and sodium hydride base to yield a quantitative amount of methyl-2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranose **151** or 95% of methyl-2,3,4,6-tetra-*O*-benzyl-α-D-[6-$^2H_2$]glucopyranose **143**. The infrared spectra showed loss of the hydroxyl absorption while n.m.r. and mass spectral data showed that the benzylation had been successfully completed with no loss of deuterium in the latter reaction. Again, comparing the ^{1}H n.m.r. spectra of the deuterated and non-deuterated products proved to be useful. The non-deuterated compound gave a multiplet at δ 3.60-3.80 integrating to 5 protons. At the edge of this multiplet (δ 3.60) there appeared to be a double doublet which could correspond to H-2 however this was not conclusive. Considering the same region of the spectrum for the deuterated compound the integral had decreased to correlate with only 3 protons. This was due to the deuterium at position 6 which also decreased the complexity of the δ 3.60-3.80 signal. As suspected earlier H-2 did occur as a double doublet at δ 3.60. At δ 3.70 there was a triplet which corresponded to H-4 while H-5 showed as a doublet at δ 3.77. H-3 showed up as a triplet at δ 4.0. Once more, the remainder of the ^{1}H n.m.r. spectrum proved to be identical to that of the non-deuterated compound.

With two deuterium atoms incorporated at position 6 it remained to introduce deuterium at position 1. The method used is shown in scheme 45.



151 R=H **152a** R=H **153** R=H **152b** R=H
143 R=D **144** R=D **145** R=D **146** R=D

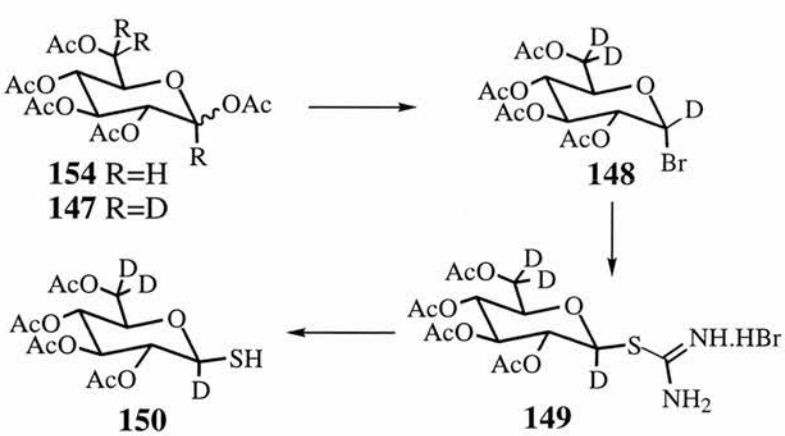
Scheme 45: *incorporation of deuterium at position 1*

The methoxy ether was cleaved by heating **151** or **143** with glacial acetic acid and 1.0 M sulfuric acid under reflux conditions. Again the reaction was first investigated with the unlabelled compound and gave a 54% yield of **152a**. The deuterated compound gave a slightly better yield with 64% of **144**. In both cases a mixture of α and β anomers was obtained. The infrared spectra showed an absorption at 3370 cm^{-1} for the anomeric hydroxyl groups and the melting point of 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose was consistent with the literature value.[230] The ^{1}H and ^{13}C n.m.r. spectra proved to be highly complex due to the presence of two anomers. It was not possible to establish from the ^{1}H n.m.r. spectra what ratio of anomers had been given but it could be seen that the α anomer was the major one. This observation was due to the large doublet at δ 5.20 which corresponded to H-1 of the α anomer with a coupling of 3.6 Hz. The β anomeric proton was lost underneath the resonances from the benzylic protons. The signal occurring at δ 3.9-4.1 corresponded to two protons from the α anomer these were assigned to H-3 and H-5. This assignment was confirmed when considering the spectrum of the deuterated product. H-5α is shown as a doublet at δ 4.04 due to the presence of deuterium at position, H-3α occurs as a triplet at δ 3.98. In the non-deuterated spectrum the multiplet which occurred at δ 3.50-3.80 corresponded to protons 2, 4 and 6 of the α anomer and also protons 3, 4, 5 and 6 of the β anomer. In the deuterated spectrum the integral of this multiplet decreased by an amount equal to 2 protons corresponding to the successful deuteration at position 6. This was further confirmed by the absence of C-6 resonances in the ^{13}C n.m.r. spectrum. These carbon signals would be expected at δ 68.0 (β anomer) and δ 68.5 (α anomer).

Oxidation of **152a** and **144** proceeded smoothly using PCC and powdered molecular sieves in dry dichloromethane.[231] The product was purified using column chromatography (silica, hexane-diethyl ether (7:1)) to give 2,3,4,6-tetra-*O*-benzyl-D-glucono-γ-lactone **153** in 72% yield and 2,3,4,6-tetra-*O*-benzyl-D-[6-$^{2}H_2$]glucono-γ-lactone **145** in 79% yield. The ^{13}C n.m.r. spectrum confirmed the presence of the lactone carbonyl at δ 169.9.

A final reduction was initially investigated with the unlabelled lactone and sodium borohydride. This was then repeated with 2,3,4,6-tetra-*O*-benzyl-D-[6-$^2H_2$]glucono-γ-lactone **145** and sodium borodeuteride.[138] These reactions gave 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose **152b** and 2,3,4,6-tetra-O-benzyl-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose **146** respectively both in quantitative yield. Again the infrared spectra showed the presence of the hydroxyl group at 3370 cm^{-1}. The ^{1}H n.m.r. spectrum showed the total loss of the signal for the α anomeric proton at δ 5.2 while the β proton was again obscured by the signals from the benzylic protons. The ^{13}C n.m.r. spectrum confirmed the deuteration at the anomeric position with no resonances at either δ 91.8 (α anomer) or δ 98.0 (β anomer). Furthermore the resonances for the C-6 carbon atom were absent as expected.

With our three deuterium atoms in place it remained to convert the benzyl protecting groups to acetyl. From this vantage point it was possible to use our already established synthetic route to 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (scheme 46).[202]



Scheme 46: *2,3,4,6-tetra-O-acetyl-thio-β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose synthesis*

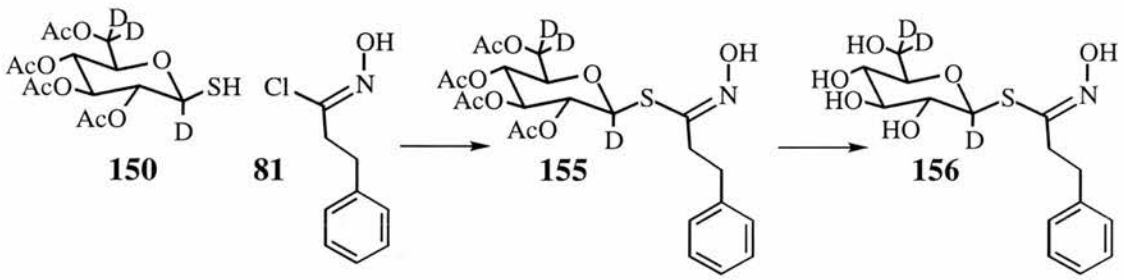
There are many methods available to remove benzyl protecting groups. In our case acetolysis was deemed most suitable as it would remove the benzyl groups to give the desired acetyl protection in a one pot reaction. To change the benzyl protection to acetyl the benzylated sugar was reacted with acetic anhydride and boron trifluoride diethyl etherate as a Lewis acid promoter.[232] 2,3,4,6-Tetra-*O*-benzyl-D-glucopyranose (**152b** scheme 45)

was converted to 1,2,3,4,6-penta-*O*-acetyl-D-glucopyranose **154** in 63% yield after column chromatography (silica, 40-60 petroleum ether-ethyl acetate (3:2)). The α and β anomers were given in a ratio of 5:1. Other fractions from this column contained partially debenzylated sugar products but stirring the reaction for prolonged periods of time did not increase the yield. The melting point corresponded well with the literature value[233] and the infrared spectrum illustrated the presence of the carbonyl groups with an absorption at 1743 cm^{-1}. A molecular ion of *m/z* (CI) 331 ($[M\text{-}OAc]^+$, 100%) was detected but the ^1H and ^{13}C n.m.r. spectra confirmed the presence of 5 acetyl groups. For the reaction using 2,3,4,6-tetra-*O*-benzyl-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose (**146** scheme 45) any partially debenzylated product was recycled to give an increased yield with 82% of **147** obtained. Again the structure was confirmed as above. Comparing the ^1H n.m.r. spectra of the two products it can be observed that the anomeric protons at δ 6.32 (α anomer) and δ 5.7 (β anomer) are not present in the spectrum of the deuterated product. The protons at position 6 which give a signal between δ 4.0 and δ 4.35 also do not show up in the deuterated compound's spectrum. In addition H-5β occurs at δ 3.8 as a doublet while H-5α shows as a doublet at δ 4.10. The H-2, 3 and 4 protons of the β anomer are impossible to assign as they are almost completely hidden by the signals from the predominant α anomer. In the ^{13}C n.m.r. spectrum both signals for the anomeric carbons are lost (δ 89.5, C-1α; δ 92.1, C-1β) along with the C-6 α and β resonance (δ 61.9).

1,2,3,4,6-Penta-*O*-acetyl-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose **147** was converted to 2,3,4,6-tetra-*O*-acetyl-α-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranosyl bromide **148** in 83% yield using 45% w/v hydrogen bromide in acetic acid. The product was unstable and was stored below 0 °C. The mass spectrum showed the appropriate mass peak (*m/z* (CI) 416 ($[MH_2]^+$, 18%)) and the ^1H and ^{13}C n.m.r. spectra illustrated the loss of one acetyl group. Compound **148** was then treated with thiourea in refluxing dry acetone to afford 2,3,4,6-tetra-*O*-acetyl-β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranosylisothiouronium bromide **149** in 98% yield according to the literature method used for the non-deuterated derivative.[202] This compound was hydrolysed in aqueous potassium metabisulfite at 75 °C to form 2,3,4,6-tetra-*O*-acetyl-thio-

β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose **150** in 71% yield.[202] The infrared spectrum showed the presence of both the thiol and carbonyl groups while the microanalysis (Found: C, 45.66; H, 5.45. Calc. for $C_{14}H_{17}{}^2H_3O_9S$: C, 45.77; H, 5.49%) and mass spectrum (*m/z* (CI) 368 ([MH]$^+$, 2%)) provided further evidence of product formation. The ^1H n.m.r. spectrum showed no evidence of signals for the two H-6 protons or for the anomeric proton. The usual multiplet given for H-5 had become a doublet due to coupling to H-4 only. Similarly, H-2 couples with only H-3 and was observed as a doublet at δ 4.98. Overall, spectral data implied >95% incorporation of deuterium in this synthetic route.

To confirm that **150** could be incorporated into the glucosinolate synthesis we chose to include it in the synthesis of desulfogluconasturtiin (scheme 47). Further to this the utility of β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranosyl phenethyl thiohydroximate **156** as an internal standard in LC-MS was assessed.



Scheme 47: *Coupling reaction between 3-phenylpropionaldehyde oximyl chloride and 2,3,4,6-tetra-O-acetyl-thio-β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose*

In the preceding work (section 2.1.2) the coupling reaction between 3-phenylpropionaldehyde oximyl chloride **81** and 2,3,4,6-tetra-*O*-acetyl-thio-β-D-glucopyranose proceeded in very poor yield. This reaction was thus investigated further to see if the yield could be optimised. Initially one equivalent of both 2,3,4,6-tetra-*O*-acetyl-thio-β-D-glucopyranose and oximyl chloride were used and three different solvents were used diethyl ether, dichloromethane and tetrahydrofuran. The reaction was initiated by adding triethylamine and stirring was continued for one hour. The crude yields obtained were poor at 68%, 40% and 32% respectively. The ^1H n.m.r. spectrum of each reaction

product illustrated large amounts of unreacted sugar. Either the reaction time was too short or dimerisation of 2,3,4,6-tetra-*O*-acetyl-thio-β-D-glucopyranose occurred more quickly than anticipated precluding product formation. Examination of each reaction by t.l.c. (silica, 40-60 petroleum ether-ethyl acetate (3:2)) showed, in addition to a small amount of product, both unreacted and dimerised 2,3,4,6-tetra-*O*-acetyl-thio-β-D-glucopyranose. It would appear that although the reaction time is too short, the dimerisation is a fairly facile process and severely restricts the yield of the reaction. Furthermore, the reaction performed in dichloromethane appeared to contain additional impurities and this solvent system was therefore abandoned.

In order to suppress the dimerisation and maximise product yield 2 equivalents of oximyl chloride were used. Under normal circumstances the oximyl chloride is the most difficult to obtain reactant and one does not want to waste it by using excess. However in this case, it is the labelled sugar which is most valuable making this a viable strategy. The purified product of this reaction was given in 80% yield when diethyl ether was used as a solvent and 78% in tetrahydrofuran solvent.

Reaction of 2,3,4,6-tetra-*O*-acetyl-thio-β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose **150** (1 equivalent) and 3-phenylpropionaldehyde oximyl chloride **81** (2.3 equivalents) in diethyl ether solvent gave a quantitative yield of coupled product **155**. Subsequent deprotection using catalytic potassium methoxide in dry methanol afforded the appropriate β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranosyl phenethyl thiohydroximate **156** in 64% yield. This product was extremely hygroscopic. The infrared spectrum showed the loss of acetyl protecting groups and the mass spectrum illustrated the appropriate molecular ion (*m/z* (CI) 347 ([M+H]$^+$, 1%)). Microanalysis illustrated the purity of the product (Found: C, 49.62; H, 6.00; N, 3.58. Calc for $C_{15}H_{18}{}^2H_3NO_6S.0.8H_2O$: C, 49.97; H, 6.32; N, 3.88%).

2.1.5 Conclusions

Four deuterated desulfoglucosinolates have been successfully synthesised for use as internal standards in LC-MS. The indolyl glucosinolate derivatives proved to be extremely challenging to synthesise with many problems encountered both in the construction of the indoles and in the coupling reactions. These problems were overcome but some reactions remain low yielding. The synthesis of each indolyl glucosinolate analogue was achieved in 12 steps from 2-methyl-3-nitrophenol or 2-nitrotoluene and D-glucose starting materials.

The desulfo-[$^2H_5$]gluconasturtiin synthesis was relatively inexpensive due to the deuterated bromobenzene starting material. The synthesis was achieved in 10 steps from [$^2H_5$]bromobenzene and D-glucose and the product obtained was microanalytically pure.

Finally, β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranosyl phenethyl thiohydroximate was synthesised. Incorporation of deuterium into the sugar moiety provides a general strategy for synthesis of any deuterated thiohydroximate. Although most of the reactions are high yielding the synthetic route involves a total of 18 steps. Ideally the deuterated sugar synthesis should be investigated further in order to reduce the number of steps in the sequence. β-D-[1-$^2H_1$, 6-$^2H_2$]Glucopyranosyl phenethyl thiohydroximate was obtained in its microanalytically pure form.

2.1.6 APCI-Mass spectrometry of desulfoglucosinolates

Atmospheric pressure chemical ionisation mass spectrometry was performed at the Scottish Crop Research Institute on all of the desulfoglucosinolates synthesised in this thesis. The phenethyl derivatives proved to be particularly interesting (figure 25).

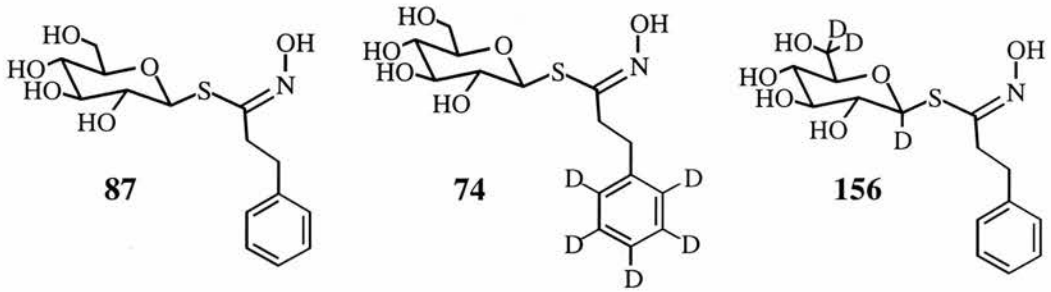


Figure 25: *Three phenethyl desulfoglucosinolates synthesised and investigated by atmospheric pressure chemical ionisation mass spectrometry*

Assignment of the spectral peaks is aided by comparing the deuterated and non-deuterated spectra. Those fragment ions containing the phenyl moiety and therefore originating from the aglycone can be identified by considering the mass spectrum of desulfo-[$^2H_5$]gluconasturtiin **74**. A shift of 5 a.m.u. is observed if the fragment contains the phenyl group.

In the absence of collision induced dissociation (CID) the base peak was consistently $[M+H]^+$. A second characteristic peak found in all spectra was $[M+H-162]^+$ which corresponds to the loss of the carbohydrate portion of the molecule.

When CID (20 V) is used the $[M+H-162]^+$ peak becomes dominant at 100% while the $[M+H]^+$ peak intensity falls from 100% to approximately 4%. This reflects the ease with which the carbohydrate moiety is lost from the desulfoglucosinolate skeleton of all three phenethyl derivatives (table 3).

Desulfo-GL✠	CID* (V)	Ions (Relative Intensity)				
		$[M+H]^+$	$[M+H-162]^+$	$[RCNOH]^+$	$[RCNOH-H_2O]+$	$[R]^+$
D_0	0	344 (100)	182 (18)	-	-	-
	20	344 (5)	182 (100)	148 (24)	130 (23)	105 (55)
	40	-	182 (26)	-	130 (85)	105 (100)
D_3	0	347 (100)	182 (44)	148 (19)	-	-
	20	347 (4)	182 (100)	148 (43)	130	105 (78)
	40	-	182 (21)	148 (5)	130	105 (100)
D_5	0	349 (100)	187 (43)	152 (14)	-	-
	20	349 (4)	187 (100)	152 (32)	-	110 (41)
	40	-	187 (43)	152 (11)	134	110 (100)

✠ Desulfoglucosinolates where D_0=β-D-glucopyranosyl phenethyl thiohydroximate
D_3=β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranosyl phenethyl thiohydroximate
D_5=β-D-glucopyranosyl-[$^2H_5$]phenethyl thiohydroximate

* CID = Collision induced dissociation

Table 3: *Major fragmentation ions (and their relative intensities) produced from phenethyl desulfoglucosinolates with and without collision induced dissociation*

When considering the spectra of desulfo-[$^2H_5$]gluconasturtiin in more detail an interesting fragment ion occurs at *m/z*=152. This signal is present both in the absence of CID and in the presence of CID (20 V and 40V spectra). This fragment ion is possibly due to the loss of HDS and not the expected H_2S from the $[M+H-162]^+$ fragment to give $[RCNOH]^+$. This is an unexpected result as it would involve loss of one of the aromatic deuterium atoms.

An alternative explanation for the *m/z*=152 peak would be the presence of $[RNCO]^+$. It is unclear why this fragment is only observed when the aromatic protons are changed to deuterium atoms. In addition to this apparent anomaly, a minor fragment of $[R-1]^+$ occurs next to the $[R]^+$ fragment in the desulfo-[$^2H_5$]gluconasturtiin spectra. This phenomenon is

not observed for any of the other phenethyl derivatives and further indicates a possible involvement of aromatic deuterons in the fragmentation.

Fragmentation patterns of the indolyl desulfoglucosinolates (figure 26) were similar to the phenethyl derivatives.

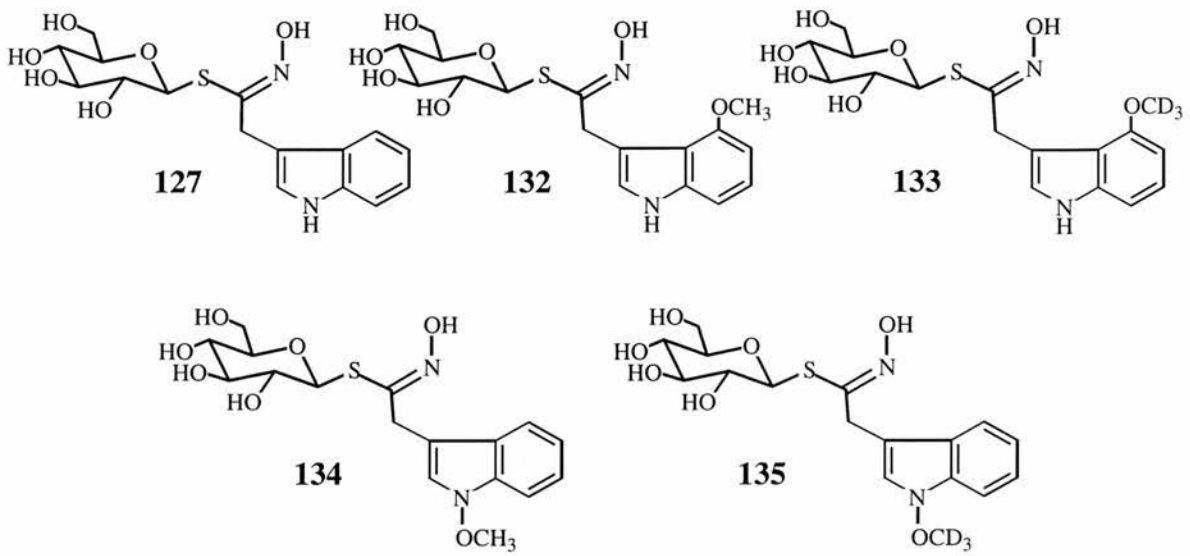


Figure 26 : *Five indolyl desulfoglucosinolates synthesised and investigated by atmospheric pressure chemical ionisation mass spectrometry*

From table 4 it can be seen that in the absence of CID the base peak was again $[M+H]^+$. The $[M+H-162]^+$ peak was also present indicating loss of the carbohydrate moiety. For both the unsubstituted **127** and 4-methoxy **132** spectra the relative intensity of the $[R]^+$ fragment ion was approximately equal to the $[M+H]^+$ base peak. The deuterated-4-methoxy **133** spectrum also showed a large $[R]^+$ signal at a relative intensity of 71%. In contrast to this both of the 1-methoxy derivatives **134** and **135** had a much smaller $[R]^+$ fragment ion with a relative intensity of around 12%.

Desulfo-GL✠	CID* (V)	Ions (Relative Intensity)				
		$[M+H]^+$	$[M+H-162]^+$	$[RCNOH]^+$	$[ROH]^+$	$[R]^+$
3-IM	0	369 (100)	207 (33)	173 (2)	-	130 (97)
	20	369 (3)	207 (58)	173 (2)	147 (1)	130 (100)
4-OCH_3-3IM	0	399 (100)	237 (13)	203 (4)	-	160 (93)
	20	399 (5)	237 (48)	203 (1)	180 (<1)	160 (100)
4-OCD_3-3IM	0	402 (100)	240 (13)	206 (5)	-	163 (71)
	20	402 (13)	240 (50)	206 (2)	180 (<1)	163 (100)
1-OCH_3-3IM	0	399 (100)	237 (25)	203 (7)	-	160 (11)
	20	399 (4)	237 (68)	203 (3)	177 (6)	160 (23)
1-OCD_3-3IM	0	402 (100)	240 (27)	206 (8)	-	163 (12)
	20	402 (6)	240 (79)	206 (28)	180 (<1)	163 (26)

✠ Desulfoglucosinolates where

3-IM	=desulfoglucobrassicin
4-OCH_3-3IM	=desulfo-4-methoxyglucobrassicin
4-OCD_3-3IM	=desulfo-4-[$^2H_3$]methoxyglucobrassicin
1-OCH_3-3IM	=desulfo-1-methoxyglucobrassicin
1-OCD_3-3IM	=desulfo-1-[$^2H_3$]methoxyglucobrassicin

* CID = Collision induced dissociation

Table 4: *Major fragmentation ions (and their relative intensities) produced from indolyl desulfoglucosinolates with and without collision induced dissociation*

As in the case of the phenethyl derivatives, CID (20 V) significantly reduced the relative intensity of the $[M+H]^+$ peak while the $[M+H-162]^+$ fragment peak showed a notable increase in intensity. This again reflects the ease with which the carbohydrate is lost. Unlike the CID spectra of the phenethyl derivatives the $[M+H-162]^+$ peak did not become the base peak. In this instance the unsubstituted, 4-methoxy and deuterated-4-methoxy spectra showed a base peak of $[R]^+$. However, as before, both of the 1-methoxy derivatives proved anomalous. The base peak of the CID 1-methoxy spectrum

corresponded to $[R\text{-}OCH_3]^+$ while the deuterated-1-methoxy spectrum showed a base peak of $[R\text{-}OCD_3]^+$ both occurring at *m/z*=130. In these spectra the $[R]^+$ peak was present but at a much lower intensity (< 20%) than for the other indolyl derivatives investigated.

There are therefore significant differences in the fragmentations of the indole derivatives depending on whether they are substituted at the 1 or 4 position. It appears that the *N*-methoxy substituent is particularly labile. It is possible that this difference in fragmentation could be exploited. The relative intensities of the *m/z*=130 and $[R]^+$ ion could be used to determine whether or not an indolyl desulfoglucosinolate is substituted at the 1 position.

2.1.7 Internal standards in LC-APCI-MS

APCI-MS has previously been reported to generate relatively intense signals for the protonated molecular ions of desulfoglucosinolates.[168] This technique can give very low limits of detection but quantification can prove to be problematic. Temporal variations in the ionisation efficiency of the instrument, principally due to contaminants in the source can produce inconsistent results. With incorporation of deuterated desulfoglucosinolates as internal standards it was hoped that the technique could become quantitative thus providing an increased sensitivity over conventional HPLC methods. Using HPLC it is necessary to combine leaves of similar ages to obtain sufficient sample. This is not ideal as glucosinolate concentrations can vary dramatically within a plant. LC-APCI-MS using an internal standard could accurately determine levels of glucosinolate on a single leaf sample.

It is interesting to note that preliminary LC-MS investigations revealed that the deuterated analogues each eluted earlier than the corresponding desulfoglucosinolate (table 5). This phenomenon is not unknown and has previously been reported for separation of deuterated and non-deuterated palmitic acid by HPLC.[234]

	Retention time (min)	
Desulfoglucosinolate	**non-deuterated**	**deuterated**
Phenethyl	54.5	54.4, 54.2
4-methoxyindol-3-ylmethyl	57.2	57.0
1-methoxyindol-3-ylmethyl	68.5	68.3

Table 5: *Retention times for deuterated and non-deuterated glucosinolates in LC MS*

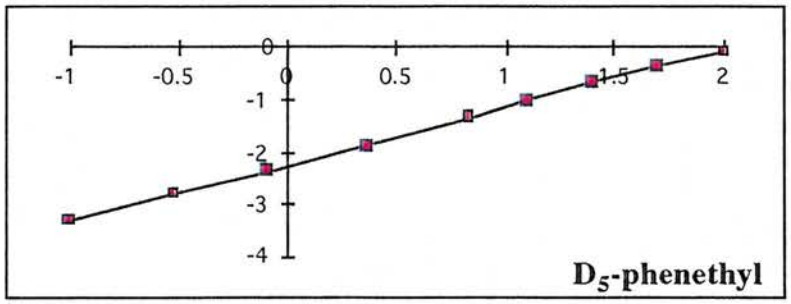
In order to determine the utility of our deuterated desulfoglucosinolates as internal standards for quantification by LC-MS a series of solutions were analysed. A stock solution of each of the non-deuterated glucosinolate analogues was prepared and serially diluted using a stock solution of the appropriate deuterated desulfoglucosinolate. The standard solutions therefore contained between 100 μg and 0.1 μg ml^{-1} of the non-deuterated desulfoglucosinolate while the deuterated desulfoglucosinolate concentration remained constant at 100 μg ml^{-1}.

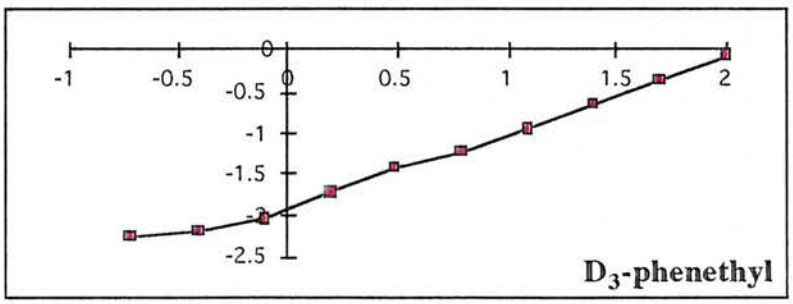
Using LC-APCI-MS the solutions were analysed in either full scan mode or single ion monitoring mode set to detect the $[MH]^+$ ions (phenethyl desulfoglucosinolates: 344, 347 and 349; methoxyindol-3-ylmethyl desulfoglucosinolates:399 and 402). The peak areas given from the analysis were then used to calculate normalised peak areas as follows:

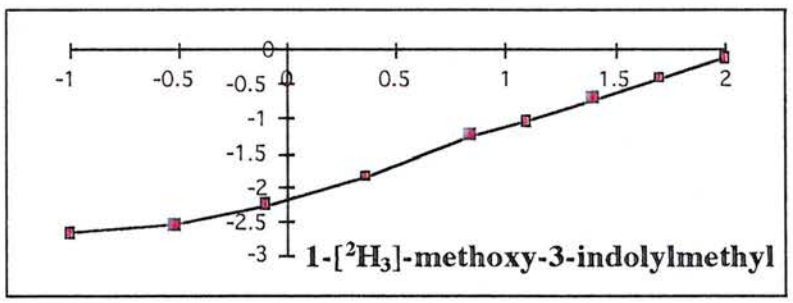
$$NA = \frac{\text{(Area of desulfoglucosinolate peak)}}{\text{(Area of internal standard peak)}}$$

These results were log transformed in order to observe any deviations from linearity at low concentration. In general a linear correlation was given between normalised peak area and concentration of the non-deuterated desulfoglucosinolate (figure 27). However for the indolyl derivatives and the deuterated sugar phenethyl derivative a slight deviation from linearity was observed at very low concentration. Under these circumstances it would be necessary to use a standard calibration curve in order to give accurate results.

Graphs of Log(normalised area) versus Log(Conc.μg/ml) for various deuterated desulfoglucosinolates







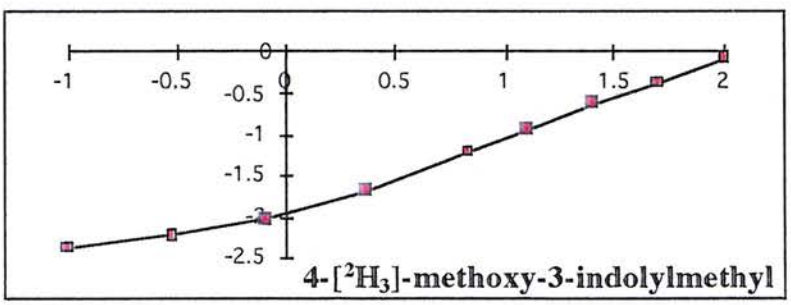


Figure 27: *Graphs illustrating the roughly linear response between concentration of the non-deuterated glucosinolate and the normalised peak areas*

Using these internal standards a large increase in sensitivity has been achieved compared with conventional HPLC methods (table 6). Furthermore if single ion monitoring is employed a 100 fold increase in sensitivity can result for example phenethyl glucosinolate showed an HPLC limit of detection of 68 ng whereas LC-APCI-MS with single ion monitoring gave a limit of 0.5 ng.

	Limit of detection (ng on column)	
Desulfoglucosinolate	**HPLC**	**LC-APCI-MS**
Phenethyl	68	3
3-indol-3-ylmethyl	44	6
1-methoxyindol-3-ylmethyl	40	3

Table 6: *Limits of detection of HPLC and LC-APCI-MS in full scan mode*

Using this technique it is therefore possible to accurately determine the amount of glucosinolate on a single leaf sample for the first time. This also opens up new areas of research including examination of subtle changes in concentrations of leaf surface glucosinolates in response to age, stress and environmental factors.

2.1.8 Gluconasturtiin as an HPLC internal standard

There are countless papers in the literature which detail the activity of isothiocyanates as potent inhibitors of tumorogenesis. However, the majority of these studies have been performed on animals. It is important to establish whether this cancer preventative action on animals and cell lines is consistent in humans.

The bioavailability of dietary isothiocyanates and their factors of variation is under investigation at the Macaulay Land Use Research Institute in Aberdeen. The isothiocyanates which arise in the digestive tract are detected and quantified using urinary end products as biomarkers. However, some glucosinolate survives the digestive system and is excreted intact. Intact glucosinolate contained in faeces is therefore analysed by initially adding an internal standard to the sample then using desulfatase enzymes to give the corresponding thiohydroximates which are detected by HPLC. The addition of internal standard allows quantification of the thiohydroximates in the HPLC analysis.

Using these methods experiments have been conducted where Fischer 344 rats were fed on a diet of Brussels sprouts (high in sinigrin) and dosed with glucotropaeolin. The amount of glucosinolate which passed through the rats without being hydrolysed by either plant or microbial myrosinase was assessed by HPLC. Again the use of an internal standard would allow quantification of results but in this instance neither of the two commercially available glucosinolates (sinigrin and glucotropaeolin) could be used. However the gluconasturtiin synthesised in this thesis (section 2.1.2) was an excellent alternative and was used as the standard.

Using a variety of these experiments the extent of the involvement of plant and microbial myrosinase *in vivo* has been examined. The work has shown that the role of microbial myrosinase is important for glucosinolate degradation particularly when plant myrosinase is inactivated during food processing.[235] To date studies have relied on animal subjects but

this work is currently being extended to include human volunteers. In this instance the differences in the bioavailability of allyl isothiocyanate (from sinigrin) in raw and cooked white cabbage is under investigation.

2.1.9 Conclusions

Comparison of the APCI-MS of deuterated and non-deuterated desulfoglucosinolates proved to be valuable in identifying the peaks given and establishing fragmentation patterns. The desulfo-[$^2H_5$]gluconasturtiin showed an interesting peak at *m/z*=152 and it was postulated that this was due to the loss of HDS from $[M+H-162]^+$ or formation of $[RNCO]^+$. The indole derivatives showed significant differences in fragmentation depending on whether the indole moiety was substituted at the 1 or 4 position. These differences could feasibly be used to determine the site of substitution of indolyl glucosinolates.

LC-APCI-MS using deuterated desulfoglucosinolates as internal standards has provided a technique capable of quantitative determination of glucosinolates from a single leaf sample. This gives approximately 100 fold improvement over current HPLC methods.

Gluconasturtiin has been successfully synthesised and used in metabolism studies as an internal standard for quantitative analysis of glucosinolate in faecal samples by HPLC.

2.2 Novel Glucosinolate Analogues for Mechanistic Studies with Myrosinase

2.2.1 Background and objectives

As mentioned in section (1.1) the breakdown of glucosinolates by myrosinase takes place in two steps. The first step is the hydrolytic cleavage of the glycosidic bond to give glucose and an aglycone. The second step involves a Lossen type rearrangement of the aglycone to give an isothiocyanate and sulfate anion. The degree to which myrosinase is involved in this second step has not yet been established. It is possible that the rearrangement provides the driving force for the overall reaction catalysed by myrosinase.

Lossen rearrangement involves the migration of an alkyl or aryl group with its electron pair to an electron deficient carbon or nitrogen atom (figure 28). In this rearrangement the migrating group does not become free but always remains associated with the substrate. The evidence for this comes from crossover experiments and also from the observation that a chiral migrating group will retain its configuration throughout the rearrangement.

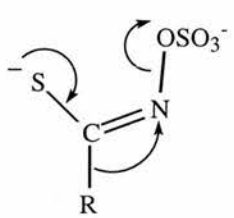


Figure 28: *Lossen rearrangement of the glucosinolate aglycone*

In general the more electron releasing the migrating side chain, the more rapid the rearrangement. Amongst aryl migrating groups it has been found that electron donating groups in either the *para* or *meta* position increases migratory aptitude. In contrast to this, an electron donating group in the *ortho* position decreases migratory aptitude due to steric effects. Electron withdrawing groups on the aryl ring will decrease its ability to migrate regardless of the site of substitution. A reasonable correlation has been found between

migratory aptitude and activation or deactivation of the aryl ring to electrophilic aromatic substitution.

In order to investigate whether the Lossen rearrangement is rate determining in the myrosinase catalysed glucosinolate hydrolysis three novel phenyl glucosinolate derivatives were synthesised (figure 29). These analogues differed only in the electronic nature of the side chain.

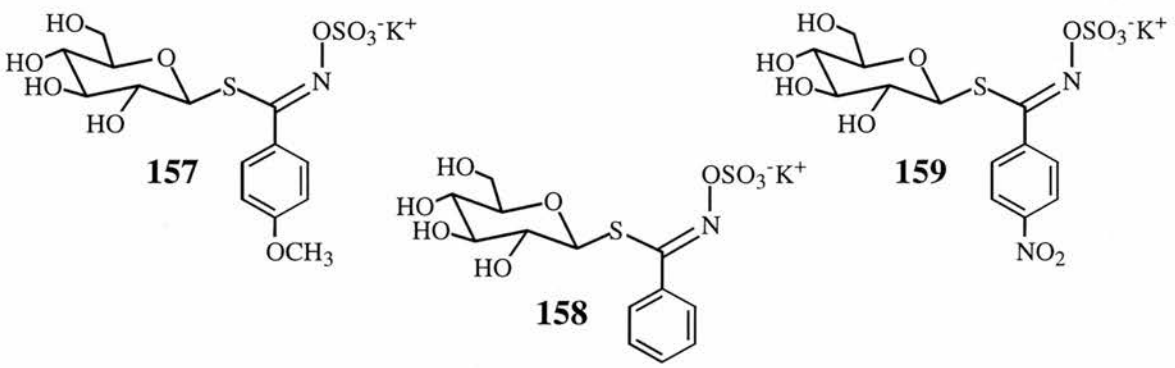


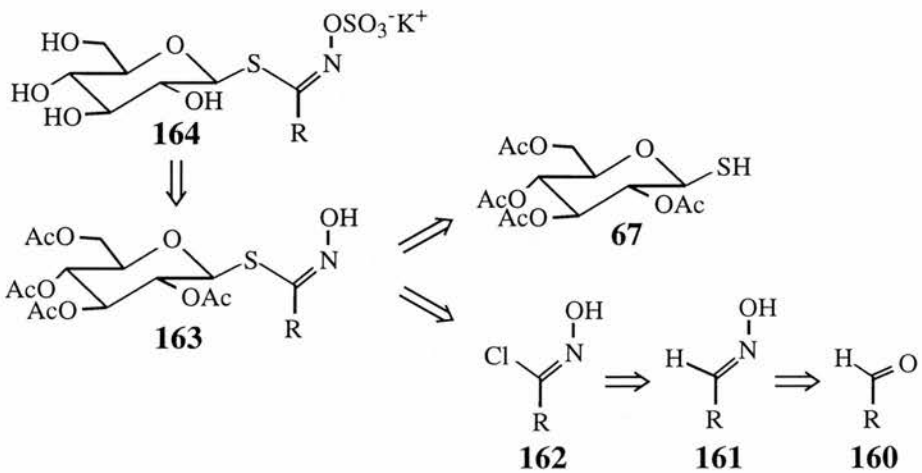
Figure 29: *Three novel phenyl glucosinolate analogues differing in the electronic nature of the side chain*

Considering the Lossen rearrangement, the electron donating methoxy substituent in the *para* position of **157** should accelerate the rearrangement while the *para* nitro substituent of **159** would slow it down. If the rearrangement provides the driving force for the reaction the rate of glucosinolate hydrolysis should be *p*-methoxyphenyl > phenyl > *p*-nitrophenyl.

2.2.2 Synthesis of novel phenyl glucosinolates

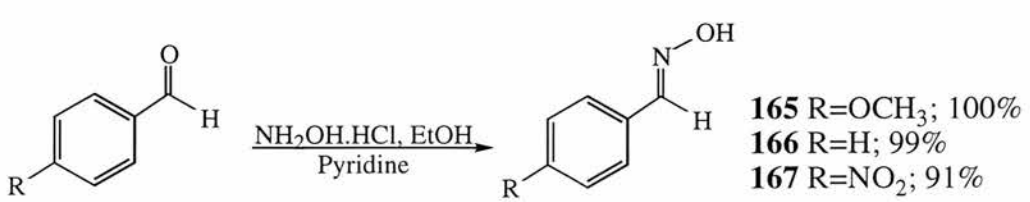
There are a number of possible routes towards the synthesis of glucosinolates. The general route used for the synthesis of novel phenyl glucosinolates is given in scheme 48. The synthesis involved coupling of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose **67** to an oximyl chloride **162** in the presence of triethylamine to give a 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl thiohydroximate **163**. This compound could then be sulfated and deprotected to afford the corresponding glucosinolate **164**. Three different oximyl

chlorides were prepared for coupling to the acetyl protected thioglucopyranose. The oximyl chloride synthesis involved the conversion of the appropriate aldehyde **160** to its oxime **161** and a subsequent chlorination step to give **162**.



Scheme 48: *Retrosynthetic analysis of glucosinolates*

The oximes of *p*-methoxybenzaldehyde, benzaldehyde and *p*-nitrobenzaldehyde were prepared (scheme 49). The procedure involved reaction of the appropriate aldehyde with hydroxylamine hydrochloride and dry pyridine in dry ethanol as solvent.[236] *p*-Methoxybenzaldehyde oxime **165** and benzaldehyde oxime **166** were purified by distillation at reduced pressure while *p*-nitrobenzaldehyde oxime **167** was recrystallised from toluene. The pure products were obtained in 100%, 99% and 91% yield respectively. In addition the ^{13}C n.m.r. spectrum of the *p*-methoxybenzaldehyde oxime showed the presence of E and Z isomers. For all of the oximes the ^{13}C n.m.r. spectra showed a clear peak at approximately δ 145-151 which corresponded to the *C*H=N functionality. To further confirm the correct reaction took place the mass spectra illustrated the appropriate molecular ions.



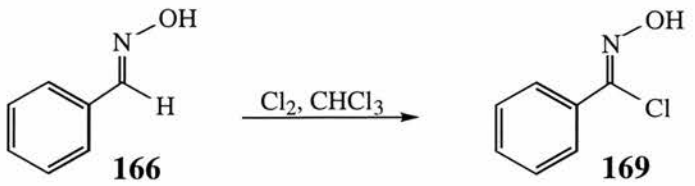
Scheme 49: *Synthesis of oximes*

Chlorination of the three oximes synthesised was effected by three different methods. *p*-Nitrobenzaldehyde oxime was chlorinated via a procedure used by Kjaer and Skrydstrup (scheme 50).[140] In the literature procedure the chlorinated benzyloximes had to be cooled to -78 °C as they proved to be unstable at higher temperatures. However, in our case this proved to be unnecessary as the *p*-nitrobenzaldehyde oxime **167** reacted slowly at 0 °C without any significant decomposition or increase in temperature. Chlorination was therefore achieved in dry *N,N*-dimethylformamide with *N*-chlorosuccinimide at 0 °C. The reaction was quenched using ice/water and extracted into diethyl ether. Concentration at reduced pressure gave the oximyl chloride **168** as a yellow crystalline solid in 92% yield. This derivative proved to be the most stable of the three oximyl chlorides synthesised and could be stored at room temperature. In addition the structure was confirmed by microanalysis (Found: C, 42.39; H, 2.75; N, 13.82. Calc for $C_7H_5N_2O_3Cl$: C, 41.93; H, 2.51; N, 13.97%).

167 **168**

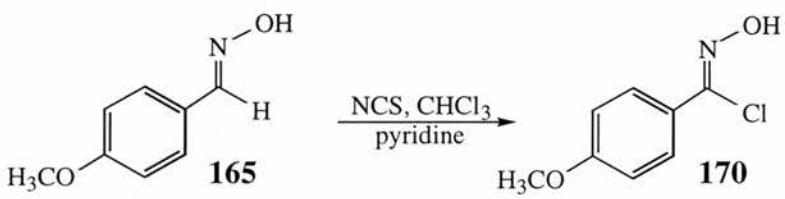
Scheme 50: *Synthesis of p-nitrophenyl oximyl chloride*

The benzaldehyde oxime was chlorinated using a different procedure as the Kjaer and Skrydstrup method was unsuccessful in this case. Benzaldehyde oxime **166** was therefore chlorinated using chlorine gas which was bubbled through a solution of the oxime in chloroform at 0 °C (scheme 51).[184] The reaction turned a brilliant blue colour which persisted until the end point was reached when the colour changed to yellow and gaseous hydrochloric acid was released. The reaction was concentrated at reduced pressure to give a solid which was taken up in hot 60-80 petroleum ether, filtered and the solvent evaporated at reduced pressure. The product of the reaction **169** was a cream coloured solid and was given in 44% yield. Benzaldehyde oximyl chloride was reacted in the next step in its crude form.



Scheme 51: *Synthesis of phenyl oximyl chloride*

Finally, *p*-methoxybenzaldehyde oxime **165** was chlorinated using *N*-chlorosuccinimide and dry pyridine in chloroform at 0 °C (scheme 52).[185] The reaction was quenched using ice/water and extracted into diethyl ether. Concentration at reduced pressure gave the oximyl chloride **170** in 76% yield as a semi-solid which was not purified.

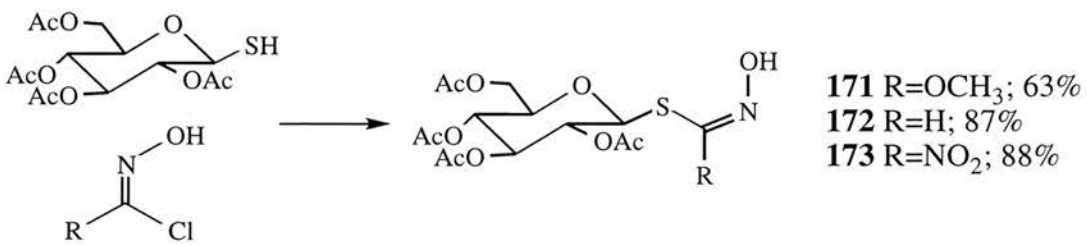


Scheme 52: *Synthesis of p-methoxyphenyl oximyl chloride*

Due to the possibility that these compounds were unstable they were not stored and thus only a limited characterisation was undertaken, however full characterisation was given for the coupled products in the next step. In each case the ^{1}H n.m.r. spectrum of the oximyl chlorides indicated loss of the *CH*NOH signal. Furthermore t.l.c. (silica, 40-60 petroleum ether-ethyl acetate (2:3)) showed that the reactions were complete.

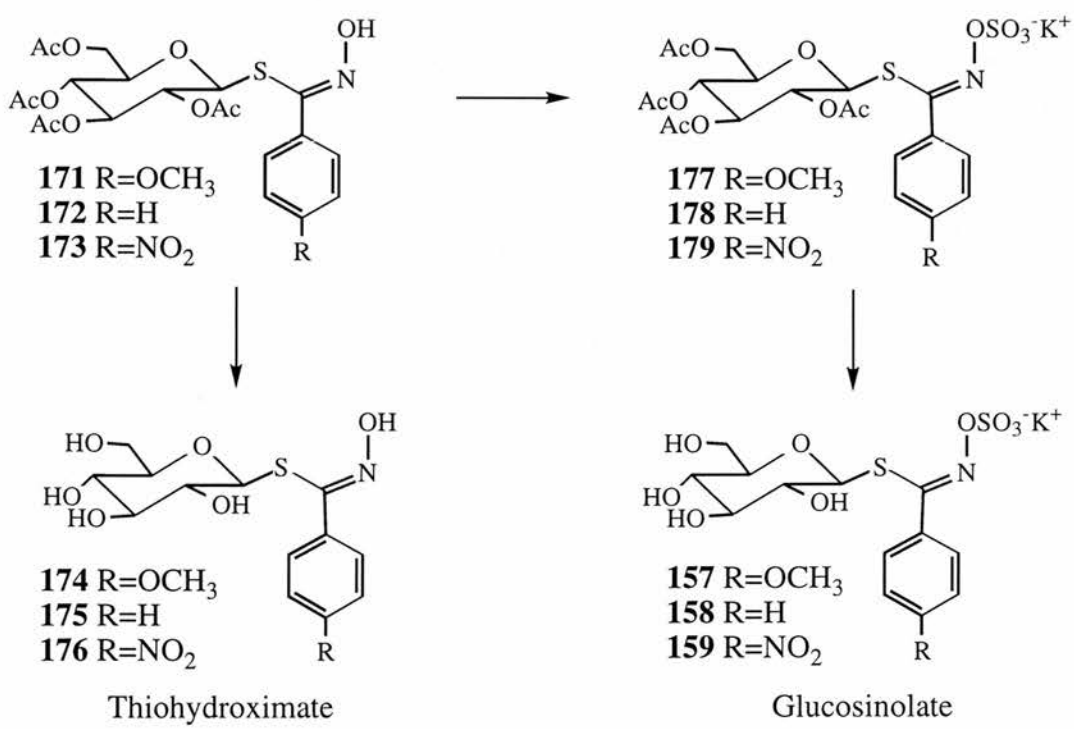
Coupling of the *p*-nitrophenyl, phenyl and *p*-methoxyphenyl oximyl chlorides (scheme 53) to 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose was performed in tetrahydrofuran as solvent and the reaction initiated by the addition of triethylamine.[184] In each case the reaction was stirred overnight at room temperature under a nitrogen atmosphere. The reaction mixture was washed with a 1.0 M solution of sulfuric acid then extracted with diethyl ether and ethyl acetate. The coupled products were purified by dissolving the product in ethanol then adding ice cold water until the product precipitated from the

solution. The 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl thiohydroximates were obtained in 88%, 87% and 63% yield respectively. The structures of the products were confirmed by chemical ionisation mass spectrometry which illustrated the appropriate mass peaks while microanalysis results showed that the products were pure.



Scheme 53: *Coupling reaction to give the glucosinolate skeleton*

This served as a branch point in the synthesis and from here two routes were taken (scheme 54). The first involved a simple deprotection of each acetyl protected thiohydroximate to yield the free thiohydroximate. These compounds were then examined at the Scottish Crop Research Institute by LC-MS (section 2.2.4). The second route involved conversion through to the glucosinolate via sulfation and subsequent deprotection.



Scheme 54: *Synthetic routes to thiohydroximates or glucosinolates*

Deprotection of the tetra-acetyl thiohydroximates was effected using a catalytic amount of potassium methoxide in methanol. The reactions were stirred overnight then Amberlite IR-120 resin was added and stirring continued for a further 30 minutes. The Amberlite was removed by filtration and the resulting solution concentrated at reduced pressure. These reactions proceeded smoothly to give β-D-glucopyranosyl-*p*-nitrophenyl thiohydroximate **176** in 76% yield, β-D-glucopyranosyl phenyl thiohydroximate **175** in 83% yield and β-D-glucopyranosyl-*p*-methoxyphenyl thiohydroximate **174** in 45% yield. Each product was pure by microanalysis and the infrared spectra illustrated total loss of the carbonyl absorption indicating a complete removal of the acetyl protecting groups.

To construct the glucosinolate the protected thiohydroximates had to be sulfated prior to deprotection. The appropriate protected thiohydroximate was therefore added to freshly prepared pyridine-sulfur trioxide complex and the reaction mixture stirred for a period of 24 hours. To quench the reaction aqueous potassium hydrogen carbonate was added which converted the sulfated product to its potassium salt. In this process large volumes of CO_2 gas were released. 2,3,4,6-Tetra-*O*-acetyl-*p*-nitrophenyl glucosinolate **179** was produced as a pale yellow amorphous solid in 93% yield. 2,3,4,6-Tetra-*O*-acetyl-*p*-methoxyphenyl glucosinolate **177** was also given as a pale yellow amorphous solid in 43% yield. Both of these compounds were purified by column chromatography (silica, dichloromethane-methanol (15:1)). The most reliable evidence that product had been formed was provided by negative ion electrospray mass spectrometry which showed molecular ions of 608 and 592 for the *p*-nitro and *p*-methoxy derivatives respectively, corresponding to $[M-K]^-$.

In the case of 2,3,4,6-tetra-*O*-acetyl-phenyl glucosinolate the excess potassium hydrogen carbonate led to immediate deprotection of the glucopyranose moiety to give the phenyl glucosinolate **158** directly in 66% yield. This result was confirmed by the lack of any acetyl signals in either the ^1H or ^{13}C n.m.r. spectra. In addition no carbonyl absorption appeared in the infrared spectrum. Evidence that the sulfation had been successful was

given by the negative ion electrospray mass spectrum which showed a molecular ion of 394 corresponding to $[M-K]^-$.

Finally, deprotection of 2,3,4,6-tetra-*O*-acetyl-*p*-nitrophenyl glucosinolate and its *p*-methoxy equivalent was effected as usual with a catalytic amount of sodium methoxide in methanol. *p*-Nitrophenyl glucosinolate **159** was produced in 50% yield while *p*-methoxyphenyl glucosinolate **157** was produced in 67% yield. Again negative ion electrospray mass spectrometry confirmed product formation with molecular ions at 439 and 424 respectively.

2.2.3 Purification of phenyl glucosinolates

Purifying the synthetic glucosinolates proved to be problematic. Initially, purification was attempted using chromatography on silica gel. Unfortunately the high polarity of the glucosinolates meant that they could not be removed from the column unless large volumes of methanol were used. This method proved totally unsatisfactory as the glucosinolate obtained was contaminated with salts from the silica.

A second purification method attempted was similar to a procedure used by Hanley *et al.*[237] Their approach involved a series of three columns DEAE Sephadex, G-10 Sephadex and finally Amberlite IR-120.

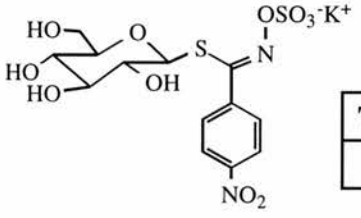
The first column involved the use of anion exchange chromatography with DEAE Sephadex as the stationary phase. The Sephadex was pre-swollen in water for a period of 24 hours before being washed with ammonium bicarbonate. An aqueous solution of glucosinolate was then added to the column and eluted with water. In this process the glucosinolate became attached to the resin due to the attraction of the sulfate on the glucosinolate to the diethylaminoethyl ionogenic groups of the anion exchanger. The impurities were not strongly attracted to the DEAE Sephadex and passed through the

column. Elution of the product using increasing concentrations of ammonium acetate (0.1, 0.5, 1.0 and 2.0 M) typically gave the glucosinolate in the 1.0 or 2.0 M fractions. The glucosinolates used in this study were UV active and were clearly visible on t.l.c. plates. The product was freeze dried then passed onto a desalting column of G-10 sephadex which had been pre-swollen in water overnight. The glucosinolate was added to the column and eluted with water. This gel permeation chromatography column should have excluded the glucosinolate from the pores of the stationary phase matrix while more readily allowing smaller molecules in. In this way the contaminating salts move more slowly through the column allowing the pure glucosinolate to be collected as its ammonium salt. Unfortunately this process did not prove to be as efficient as was hoped. For all three glucosinolates used various quantities of ammonium acetate remained as a contaminant. Therefore use of this method was discontinued.

A third method involved the use of reverse phase chromatography. In this instance compounds are separated on the basis of their attraction to silica with a hydrophobic surface of long chain (C_{18}) alkyl groups. Reverse phase t.l.c. using a water mobile phase had illustrated the presence of two spots. The first spot moved up the plate with the solvent front while the second appeared approximately half way up the plate. In the presence of increasing amounts of methanol or acetonitrile the second spot moved progressively further up the plate. It was thus decided to use a water mobile phase with a C_{18} stationary phase to separate the two spots. This process proved to be very successful and the two samples collected were freeze dried. The ^{1}H n.m.r. spectrum of both samples could show little more than the presence of the glucosinolate skeleton. In order to establish whether or not the glucosinolate was sulfated ^{13}C n.m.r. spectroscopy proved invaluable. If the glucosinolate sample was not sulfated the *C*=N resonance commonly appeared at about δ 150 however if the sulfate moiety was present the resonance shifted by more than 10 ppm to about δ 161. Thus it was established that the sample which was eluted from the column first was the intact glucosinolate while the latter fraction contained the desulfoglucosinolate. Although the glucosinolate appeared to contain no impurities by n.m.r. spectroscopy, the

results of microanalysis proved contradictory. It was suspected therefore that once more salts remained in the samples. In addition, the *p*-methoxyphenyl glucosinolate sample turned cloudy if it was left in aqueous solution for any length of time.

In order to remove the contaminating salts column chromatography using G-10 Sephadex was attempted with mixed success. Phenyl glucosinolate was desalted twice to give a sample which was microanalytically pure. However, *p*-nitrophenyl glucosinolate was desalted three times and it still did not give a microanalytically pure sample. It was suspected that perhaps a sulfate impurity was present which would not show up in the n.m.r. spectra. In order to investigate this *p*-nitrophenyl glucosinolate was analysed for sulfur and oxygen content. The results of this are shown in table 7.



	C	H	N	O	S
Theoretical	32.63	3.16	5.85	36.78	13.40
Found	25.98	3.30	3.67	41.79	14.75

Table 7: *Microanalysis results for p-nitrophenyl glucosinolate*

It can be seen that an extra 1.35% sulfur is present along with an extra 5.01% oxygen. Therefore a sulfate impurity remains in the sample which could not be removed using repeated G-10 Sephadex columns.

In addition, *p*-nitrophenyl glucosinolate was examined using a flame test. If only potassium salts were present a pale lilac flame would be expected however an intense yellow flame was seen. The sample was therefore analysed by atomic absorption spectrometry for both sodium and potassium content.

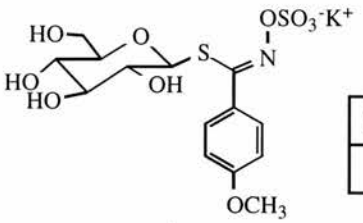
The theoretical amount of potassium in the sample was calculated to be 3.456 mg (0.0884 mmoles) while the actual amount found was 3.29 mg (0.0842 mmoles). Therefore there

was 0.166 mg less potassium than expected in the sample. This deficiency was more than compensated for by the quantity of sodium found which was 1.10 mg (0.0479 mmoles).

It can be concluded that the sample not only contained a sulfate impurity but that it also contained significant quantities of sodium which leads to the glucosinolate occurring as a mixed metal salt. However, previous work in our group had indicated that sulfate is a poor inhibitor of myrosinase and thus this sample was tested in the presence of the impurities found.[138]

Finally, *p*-methoxyphenyl glucosinolate gave poor microanalytical results after purification and freeze drying. As already mentioned, if this glucosinolate was left in aqueous solution a cloudy precipitate formed. This also occurred after desalting the sample and it was suspected that the glucosinolate was decomposing. This was confirmed by ^{1}H n.m.r. where the signals corresponding to the sugar moiety became much more complex and four new doublets appeared. The first two doublets showed up at δ 5.1 and δ 4.5 and were shown to be due to α and β anomeric protons of glucose as their intensity was increased when the solution was spiked with an authentic sample. The origin of the other two doublets at δ 5.3 and δ 5.9 proved to be less obvious. In addition to these signals a mutiplet was observed at δ 6.9. The ^{13}C n.m.r. spectrum was extremely complex and further reinforced the suspected decomposition. Mass spectrometry provided no further clues as to the identity of the decomposition products.

In order to investigate the enzymatic hydrolysis of this sample it was evident that a crude preparation would have to be made and used immediately. Microanalysis of the crude product is shown in table 8.



	C	H	N
Theoretical	36.28	3.91	3.02
Found	33.48	4.05	2.43

Table 8: *Microanalysis results for p-methoxyphenyl glucosinolate*

A flame test showed the presence of sodium and atomic absorption spectroscopy was used to determine the relative amounts of sodium and potassium present in the sample. Again the sample was deficient in potassium with a theoretical amount of 3.815 mg (0.0976 mmoles) while the amount found was 2.65 mg (0.0678 mmoles). Thus there was 1.16 mg less potassium than required. There was a significant quantity of sodium present at 1.81 mg (0.0787 mmoles) which more than accounted for the lack of potassium. Thus the glucosinolate was present as a mixed metal salt.

2.2.4 Isomerism of thiohydroximates

p-Nitrophenyl, phenyl and *p*-methoxyphenyl β-D-glucopyranosyl thiohydroximates were examined by LC-MS at the Scottish Crop Research Institute. An initial investigation displayed one peak at the expected mass for each compound but the *p*-nitrophenyl analogue showed one other very small peak.

After a period of eight days the solutions were re-examined. The phenyl and *p*-methoxyphenyl derivatives showed no change. However, the smaller peak shown in the *p*-nitrophenyl spectrum had increased significantly in size. Both signals were due to molecules of mass 361 corresponding to $[MH]^+$ (figure 30).

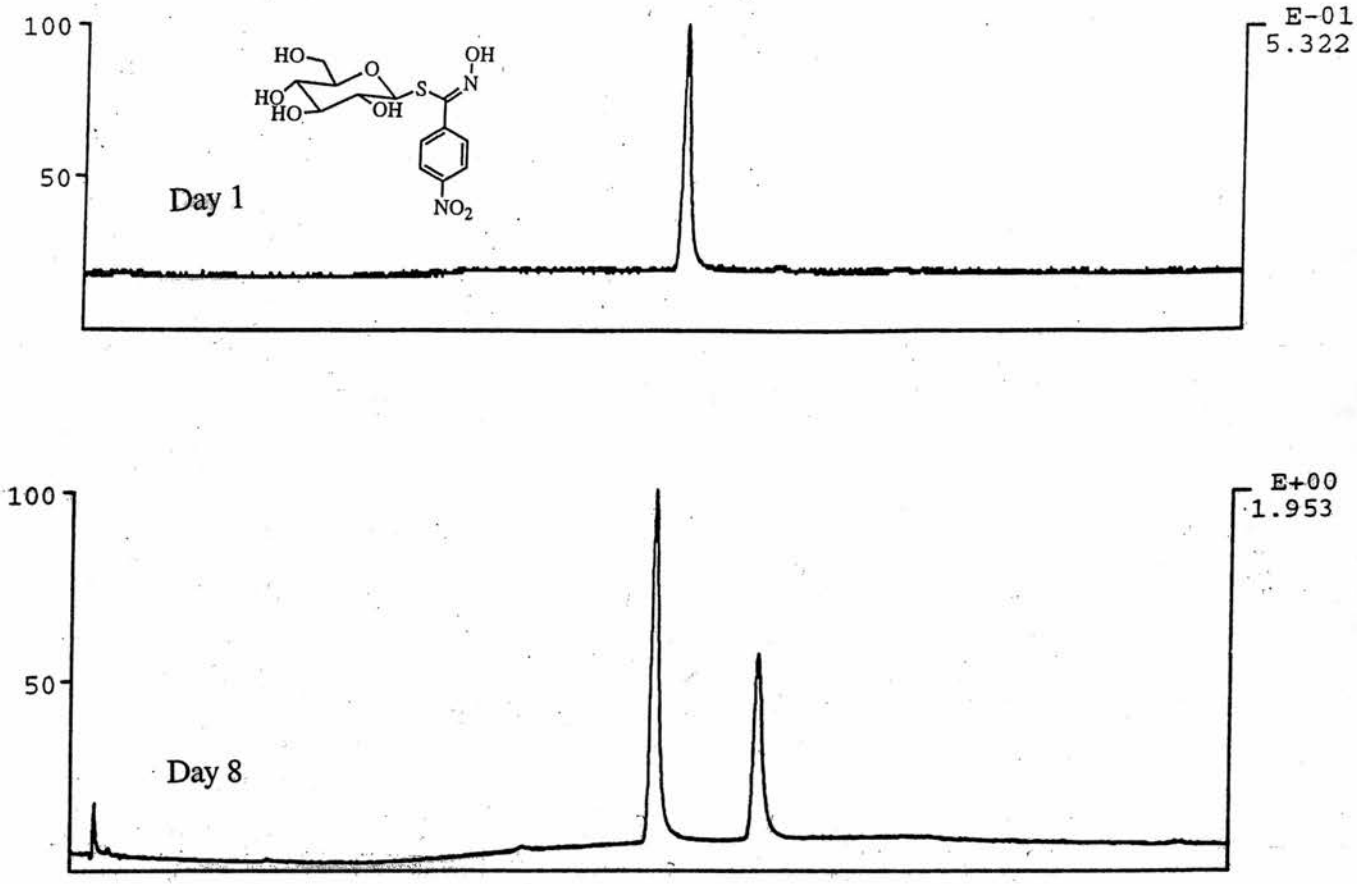


Figure 30: *LC-MS chromatographs illustrating the isomerism of β-D-glucopyranosyl-p-nitrophenyl thiohydroximate*

The three thiohydroximates were further investigated in aqueous solution by ^{1}H n.m.r. spectroscopy. The solutions were stored at room temperature and examined regularly. No change was observed in the spectra of either the phenyl or *p*-methoxyphenyl analogues over a period of four weeks but the *p*-nitrophenyl derivative illustrated a significant change.

On day 1 the anomeric proton resonance of *p*-nitrophenyl glucosinolate was observed at δ 4.20 as a doublet with a coupling of 10 Hz. A minor doublet was also observed at δ 4.35 and had a coupling of 10 Hz. The H-5 proton showed as a multiplet at δ 2.60 and also had a minor signal at δ 2.95. After a period of eight days the minor signals at δ 4.35 for H-1 and δ 2.95 for H-5 had increased in size. The solution contained 23% of the minor compound and 77% of the major one. The minor resonances continued to increase in size over time until the solution contained 38% of the minor component and 62% of the major

one. This ratio was reached after a period of 4 weeks and did not change significantly after this time (figure 31).

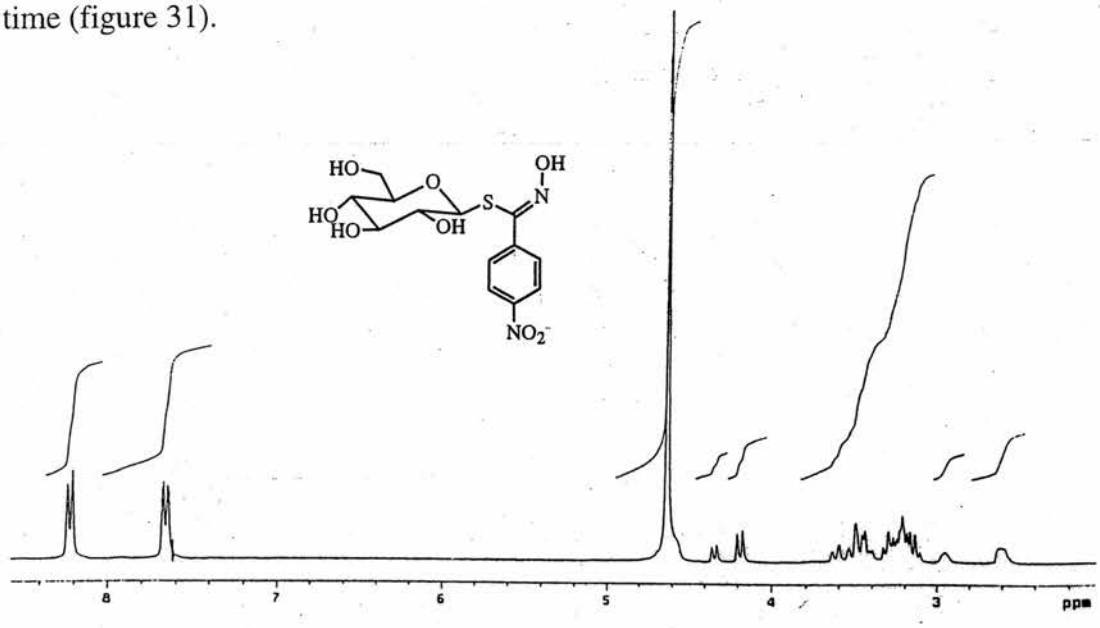
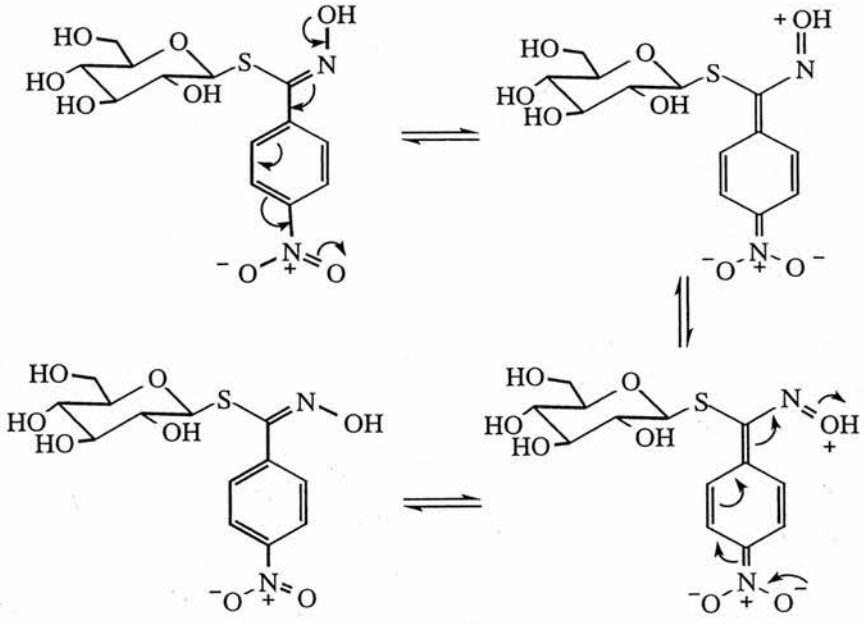


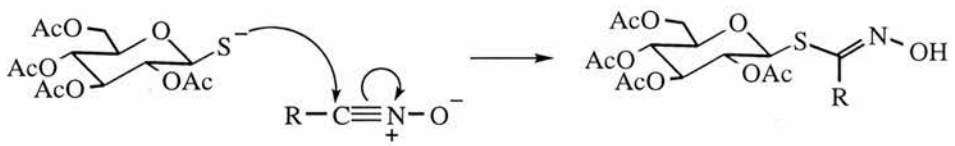
Figure 31: *^1H n.m.r. spectrum illustrating the two isomers of β-D-glucopyranosyl p-nitrophenyl thiohydroximate after a period of 4 weeks*

From this data it was suspected that β-D-glucopyranosyl-*p*-nitrophenyl thiohydroximate could isomerise in aqueous solution. The two forms were most likely to be E and Z isomers (scheme 55).



Scheme 55: *Postulated mechanism for interconversion of E and Z isomers*

The first synthesis of a glucosinolate with E configuration may have been achieved by Benn in 1963[9] although the evidence for this was speculative. He suggested that his synthesis of glucotropaeolin, via attack on a nitrile oxide by a thiol, would be expected to give a thiohydroximate with an E configuration about the carbon-nitrogen double bond (scheme 56).



Scheme 56: *Mechanism for formation of E thiohydroximate*

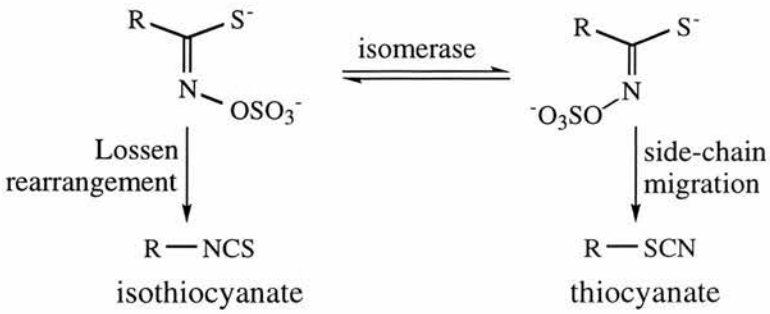
A second report in 1983 by Keller, Yelland and Ben[196] proved to be more conclusive. In this instance a trialkylsilyl nitronate was added to a solution of equimolar amounts of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose and triethylamine in tetrahydrofuran. Two geometric isomers were obtained as homogeneous crystalline solids after purification by column chromatography. It was suggested that rapid oxygen to oxygen migration of the silyl group had been the cause of the isomers occurring in equal amounts (scheme 57).

Scheme 57: *Rapid oxygen to oxygen migration responsible for isomer formation*

The E isomer was stable in its crystalline form if stored in the dark at 0 °C. However it is interesting to note that slow thermal or photochemical isomerism occurred in solution to give the Z isomer. In this instance only the Z isomer was sulfated and deprotected, the E isomer was not investigated further. Therefore there is no record of a glucosinolate occurring in the E configuration.

It would be interesting to synthesise the "unnatural" E-glucosinolate in order to study its decomposition with myrosinase. As mentioned in the introduction (section 1.3.4)

thiocyanates are believed to form as a result of Z-E isomerism of the glucosinolate aglycone catalysed by an isomerase (scheme 58). The E-glucosinolate would therefore be expected to yield thiocyanate products directly due to the conformation of the aglycone.



Scheme 58: *Proposed mechanism for thiocyanate formation*

2.2.5 Conclusions

Three novel phenyl glucosinolate analogues were prepared in order to be used in enzymatic studies. *p*-Nitro and *p*-methoxy analogues were synthesised in eight steps from the corresponding aldehydes and D-glucose. Phenyl glucosinolate was prepared in just seven steps since potassium methoxide deprotection proved to be unnecessary. The glucosinolates were extremely hygroscopic amorphous solids. Purification was extremely challenging nonetheless phenyl glucosinolate was obtained in its microanalytically pure form. However, *p*-nitrophenyl glucosinolate proved to contain a sulfate impurity while also existing as a mixed metal salt. The sulfate impurity should not greatly hamper enzymatic studies using myrosinase. *p*-Methoxyphenyl glucosinolate proved to be the most problematic as it decomposed in aqueous solution. It was concluded that a crude preparation would have to be made and used immediately. Furthermore *p*-methoxyphenyl glucosinolate exists as a mixed metal salt.

p-Nitrophenyl glucosinolate isomerised in aqueous solution as evidenced by LC-MS and ^{1}H n.m.r. spectroscopy. It is likely that the compound exists as E and Z isomeric forms. This is not observed for phenyl or *p*-methoxyphenyl glucosinolates.

2.2.6 Studies towards a non-rearrangeable analogue

As already mentioned in section 2.2.1, the degree to which myrosinase assists the Lossen rearrangement of the aglycone remains to be established. Furthermore it is not known whether the sulfate group is important for binding only or whether it is involved in substrate-assisted catalysis during the rearrangement.[126, 238] Incubations of a glucosinolate analogue which could be cleaved by myrosinase but not rearrange may give an insight into these questions. The ideal substrate for this study would be one where most of the glucosinolate structural features are retained such as in analogue **180** (figure 32). However it was envisaged that this compound would be very unstable[239] causing significant problems (scheme 59).

Scheme 59: *Instability of sulfated compound*

An alternative to this structure, which was envisaged to be simpler to synthesise, was analogue **181** where the sulfate is replaced by a carboxylate and thus the synthesis of this compound was attempted.

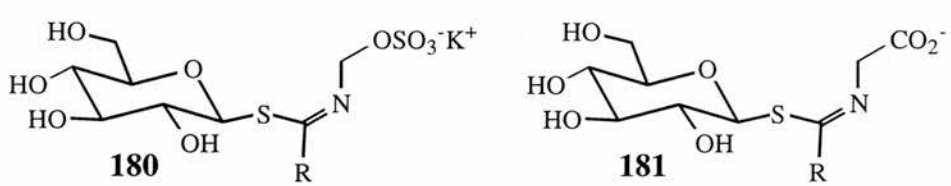
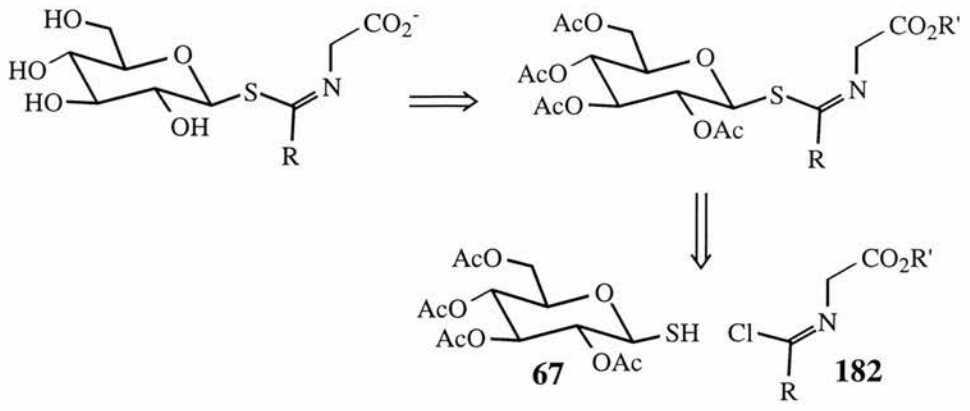


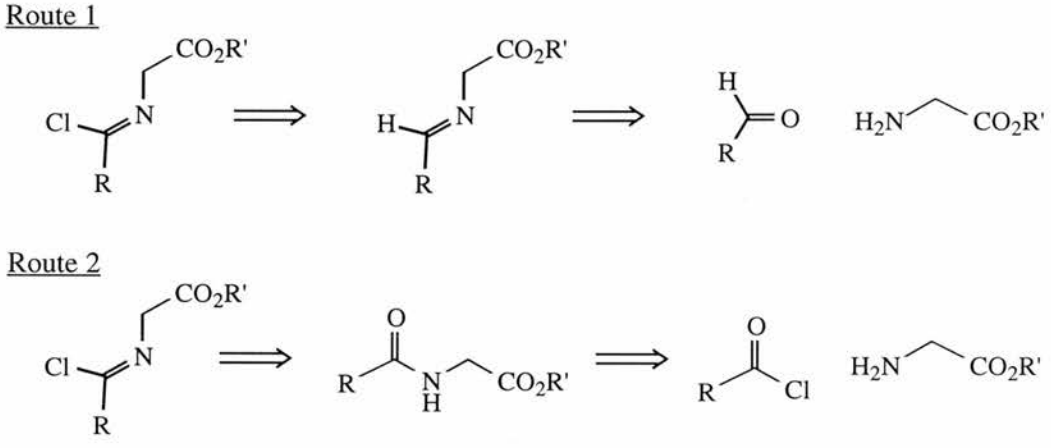
Figure 32: *Two possible non-rearrangeable glucosinolate analogues*

The synthetic route which was investigated is shown in scheme 60 and involves the nucleophilic attack on a chloroimine **182** by a thiosugar **67**.



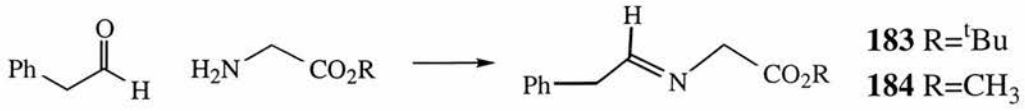
Scheme 60: *Retrosynthesis of a non-rearrangeable glucosinolate analogue*

Two routes were examined for the synthesis of the chloroimine and these are detailed in scheme 61.



Scheme 61: *Two possible routes to synthesise a chloroimine*

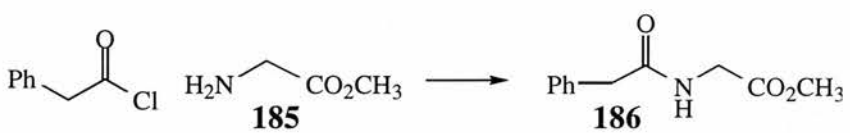
In route 1 the imine synthesis started from the reaction of an aldehyde with a suitably protected glycine derivative (scheme 62). The aldehyde chosen was phenylacetaldehyde as this would introduce a benzyl side chain into the final glucosinolate analogue. The compound produced would then be reminiscent of the naturally occurring benzyl glucosinolate.



Scheme 62: *Attempted synthesis of an imine*

In order to synthesise the imine **183** *t*-Butyl glycinate.AcOH was reacted with phenylacetaldehyde in the presence of dry triethylamine and anhydrous magnesium sulfate. Stirring this reaction at room temperature for a period of 3 days gave no reaction and so the solution was heated at reflux for 2 hours but again no reaction occurred. An identical reaction using methyl glycinate as its hydrochloride salt in order to obtain compound **184** also proved to be unsuccessful and gave only the starting materials. In the ^{13}C n.m.r. spectra no signal was observed around δ 155 which could have corresponded to the *C*=N group. Furthermore the ester, carbonyl and aldehyde signals from the starting materials remained.

In view of this chlorination of an amide via route 2 was investigated to produce the chloroimine. Firstly methyl glycinate hydrochloride **185** was prepared by addition of thionyl chloride to a suspension of glycine in methanol at 0 °C. The solution was heated to reflux for a period of 1 hour then cooled once more to 0 °C. The product crystallised from the solution and was removed by filtration. The crystals were purified by recrystallisation from methanol. The newly formed ester signals were observed in the ^{1}H n.m.r. spectrum at δ 3.0 for the methyl group and in the ^{13}C n.m.r. spectrum at δ 171.5 for the carbonyl.



Scheme 63: *Synthesis of an amide*

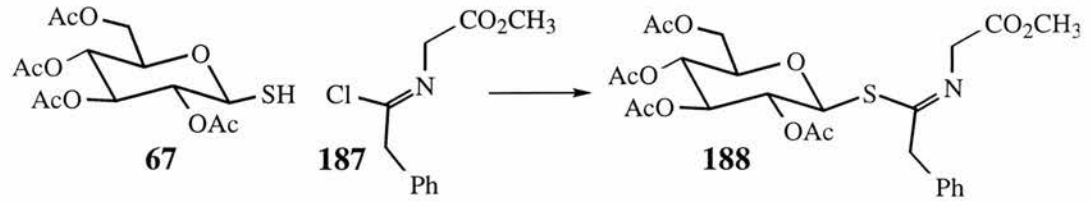
The amide **186** was then synthesised from methyl glycinate hydrochloride by reaction with phenylacetyl chloride in the presence of triethylamine (scheme 63). This reaction was stirred at room temperature for 4 hours and gave a 99% yield after recrystallisation from

60-80 petroleum ether and ethanol. Methyl *N*-(phenylacetyl)glycinate **186** was given as colourless crystals with a melting point of 82-85 °C. In the ^{1}H n.m.r. spectrum the N*H* of the amide could be seen clearly at δ 6.20 as a broad singlet while the C*H*$_2$NH group showed as a doublet at δ 3.95. In addition to this two carbonyl resonances were observed in the ^{13}C n.m.r. spectrum at δ 170.8 and δ 172.2 corresponding to the ester and amide carbonyl groups respectively. Mass spectrometry provided structural confirmation with the correct molecular ion (*m/z* (EI) 207 ([M]$^{+}$, 8%)).

Chlorination of this amide (scheme 64) proved to be challenging. Due to the apparent instability of the resulting chloroimine **187** the compound was not stored but reacted on immediately in the next step. Characterisation of the chloroimine relied mainly on ^{1}H and ^{13}C n.m.r. spectroscopy.

Scheme 64: *Chlorination of an amide*

Chlorination was first investigated by adding oxalyl chloride solution in toluene to a solution of amide in pyridine and tetrahydofuran solvent at 0 °C.[240] The reaction mixture was refrigerated overnight to allow the pyridine hydrochloride to crystallise from the solution. The solid was filtered off and washed with toluene. Concentration of the organic mixture gave a bright orange solid in 64% yield which was reacted on in its crude form. From the ^{1}H n.m.r. spectrum it could be seen that the signal from the N*H* group was no longer present. The singlets which corresponded to PhC*H*$_2$ at δ 3.55 and the ester methyl group at δ 3.65 shifted to δ 3.7 and δ 3.85 respectively. Furthermore the C*H*$_2$NH resonance had disappeared while two new signals occurred at δ 4.05 and δ 4.55 corresponding to the C*H*$_2$N group of the E and Z isomers (ratio 1:1.5).



Scheme 65: *Attempted coupling reaction*

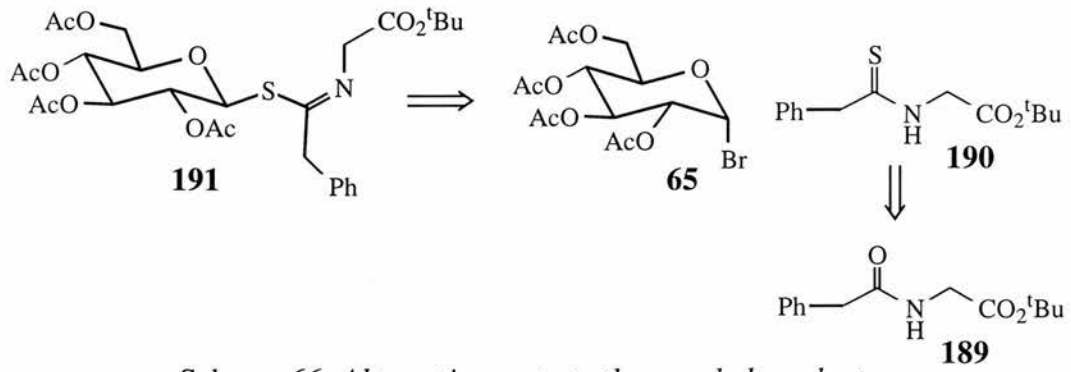
The chloride **187** was reacted in its crude form with 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose **67** in tetrahydrofuran solvent. Triethylamine was added to initiate the reaction and the solution was stirred at room temperature for 2 hours and 30 minutes. A mixture of products were obtained. Separation of the mixture was attempted with some success. A fraction was isolated which although present in very small amount (2% yield) indicated a possible product **188**. The ^{13}C n.m.r. spectrum showed the presence of a *C*=N signal at δ 153.5 and a signal at δ 87.4 which is characteristic of the anomeric proton of a successfully coupled product. Appropriate resonances were also observed for the acetyl, benzyl and sugar carbons but the sample was still very impure and contained an amide starting material contaminant. Because of this the ^{1}H n.m.r. spectrum proved to be very complex and could not be assigned with any certainty. In order to confirm the presence of the desired product a mass spectrum was obtained of the partially purified product. This proved to be inconclusive as it lacked the desired molecular ion at *m/z* (EI) = 554. The mass peak found occurred at *m/z* (EI) = 403 which could correspond to $[M-PhCH_2-CO_2CH_3]^+$. The spectrum did confirm the presence of the appropriate sugar moiety with a characteristic peak at *m/z* (EI) = 331 which corresponded to [M-Aglycone]$^+$. Chemical ionisation mass spectrometry provided no further clues.

Since the coupling reaction had given such a poor yield of product and had proven to be largely unselective the chlorination reaction was reinvestigated in order to determine the best conditions possible to provide the cleanest sequence of reactions. Two further chlorination reactions were studied, the first involved the use of thionyl chloride[241, 242] however the reaction was poor and gave predominantly starting material as evidenced by

the ^{1}H and ^{13}C n.m.r. spectra. The second method was reaction of the amide **186** with phosphorus pentachloride in toluene.[241] Initially, 1.1 equivalents of phosphorus pentachloride were used however, even after 2 hours reaction time starting material remained. The reaction was repeated using 2 equivalents of phosphorus pentachloride but the reaction quickly gave a large mixture of products. However, 1.6 equivalents of phosphorus pentachloride gave a fairly clean looking reaction by t.l.c. (silica, ethyl acetate-hexane (1:1)). If the reaction was left for any longer than 40 minutes decomposition occurred to give a complex mixture. The ^{1}H n.m.r. spectrum gave a singlet at δ 3.75 which integrated to 3 protons and equated to the ester methyl group. A further singlet appeared at δ 4.0 and this was due to the PhCH_2 group. The C$H_2CO_2CH_3$ group showed as two singlets at δ 4.35 and δ 4.5 due to the presence of E and Z isomers. There was little evidence of any residual amide but the product was not entirely clean with additional small peaks occurring in the spectrum. Unfortunately ^{13}C n.m.r. spectroscopy showed the lack of a *C*=N group with no appropriate resonance downfield.

Again an attempted coupling reaction proved to give a complex mixture of products the main compound isolated was 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose **67** indicating that the coupling had been unsuccessful and that the preceding product was unlikely to be the desired chloride.

With the previous approaches proving largely unsuccessful a third route to the product was examined (scheme 66). This involved the attack of 2,3,4,6-tetra-*O*-acetyl-1-bromo-β-D-glucopyranose **65** by a thioamide.



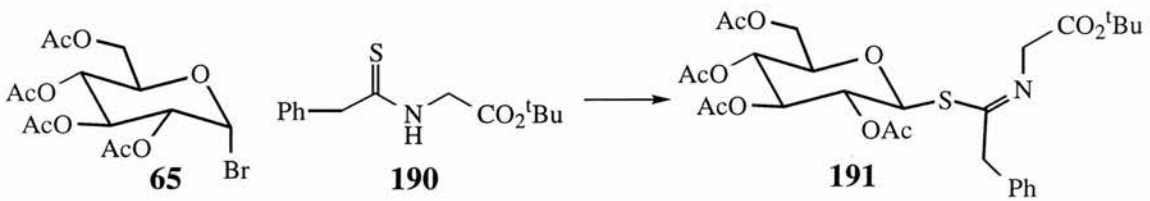
Scheme 66: *Alternative route to the coupled product*

t-Butyl *N*-(phenylacetyl)glycinate **189** was successfully prepared by the reaction of t-butyl glycinate.AcOH with phenylacetyl chloride in dichloromethane with triethylamine base. On completion the reaction was extracted using dichloromethane and the solvent removed by evaporation at reduced pressure. The residue was purified using column chromatography (silica, ethyl acetate-hexane (1:2)) to give the desired amide as a yellow oil in 84% yield. The ^{13}C n.m.r. spectrum confirmed the presence of the t-butyl protecting group with the methyl signals occurring at δ 28.1 and the quaternary carbon at δ 82.4. The carbonyl signal of the ester showed up at δ 169.0 while the amide carbonyl occurred at δ 171.2. In addition the mass spectrum gave the appropriate molecular ion (*m/z* (EI) 249 ([M]$^+$, 5%)).

Thiation of the amide was achieved using Lawesson's reagent in dry tetrahydrofuran solvent.[243] After 2 hours' stirring the reaction was complete. The solvent was removed at reduced pressure and the product was purified using column chromatography (silica, ethyl acetate-hexane (1:2)) to give *t*-butyl *N*-(phenylthioacetyl)glycinate **190** in 81% yield. The ^{13}C n.m.r. spectrum showed a peak at δ 202.5 corresponding to the *C*=S group while the mass spectrum showed the appropriate molecular ion (*m/z* (EI) 265 ([M]$^+$, 41%)). The purity of the product was confirmed by microanalysis (Found: C, 62.94; H, 7.42; N, 5.22. Calc for $C_{14}H_{19}NO_2S$: C, 63.37; H, 7.22; N, 5.28%).

Finally the coupling reaction was carried out according to a literature procedure by Ettlinger and Lundeen (scheme 67).[8] This synthetic strategy has not been widely used for construction of glucosinolates owing to its low yields. Reaction of *t*-butyl *N*-

(phenylthioacetyl)glycinate **190** with 2,3,4,6-tetra-*O*-acetyl-1-bromo-β-D-glucopyranose **65** was performed in a methanol-acetone (1:3) solvent. Powdered potassium hydroxide base was added and the reaction was stirred overnight at room temperature. Under these conditions no product **191** was isolated and only starting material could be recovered.



Scheme 67: *Coupling of the thioamide to 2,3,4,6-tetra-O-acetyl-1-bromo-β-D-glucopyranose*

A second set of conditions involved reaction of the thioamide with 2,3,4,6-tetra-*O*-acetyl-1-bromo-β-D-glucopyranose in acetone using triethylamine base. Since no reaction was observed after stirring overnight at room temperature the reaction was repeated but heated to reflux. Under these conditions *t*-butyl *N*-(phenylthioacetyl)glycinate starting material **190** was recovered quantitatively. 2,3,4,6-Tetra-*O*-acetyl-1-bromo-β-D-glucopyranose **65** was recovered in 53% yield. The remaining 2,3,4,6-tetra-*O*-acetyl-1-bromo-β-D-glucopyranose had reacted to give an alkene containing sugar compound. It was suspected that the 2,3,4,6-tetra-*O*-acetyl-1-bromo-β-D-glucopyranose had undertaken an elimination reaction to give **192** (figure 33).

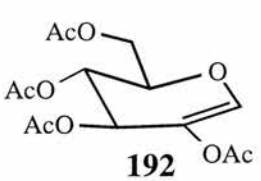


Figure 33: *Suspected elimination product*

The ^{1}H n.m.r. spectrum of the side product contained one alkenyl type proton at δ 6.6 which occurred as a singlet. This corresponded to the proton at position 1. A further two proton signals which were distinctive were a triplet at δ 5.2 and a doublet at δ 5.55. These signals corresponded to protons H-4 and H-3 respectively. Protons H-5, 6a and 6b occurred as a multiplet between δ 4.1-4.5. In addition the four acetyl groups showed at δ 2.00-2.15 and the mass spectrum (CI) showed a molecular ion of 331 ([MH]$^+$, 30%).

2.2.7 Synthesis of a non-rearrangeable analogue

Due to the difficulties encountered in synthesis of **181** a simpler target compound **193** was investigated (figure 34).

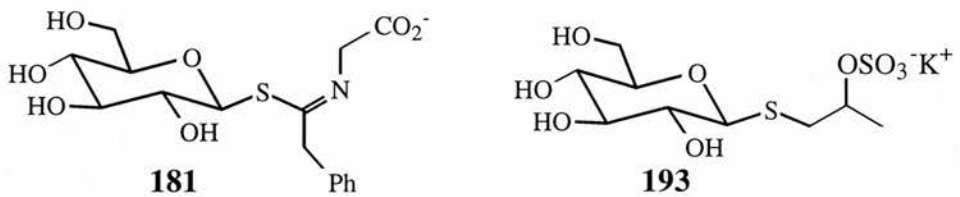
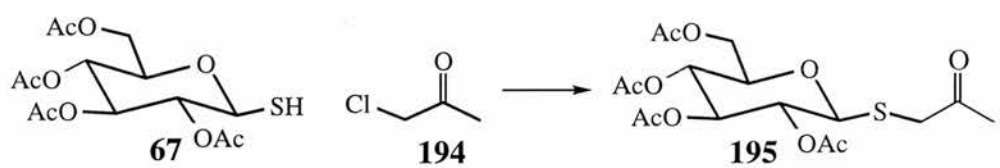


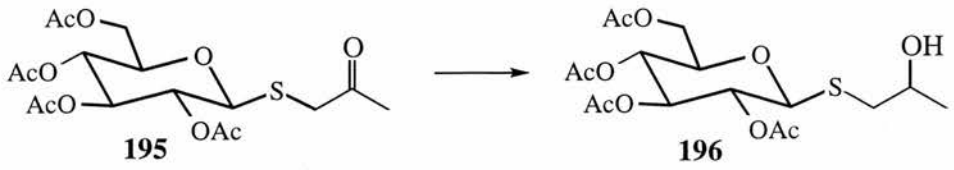
Figure 34: *An alternative non-rearrangeable glucosinolate target*

In analogue **193** the sulfate group of the glucosinolate is retained while also incorporating the appropriate sugar moiety. Both of these features should help the compound to be recognised by myrosinase and thus bind to the active site. The synthesis starts with the nucleophilic attack of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose **67** on chloroacetone **194** in dry diethyl ether as solvent (scheme 68).



Scheme 68: *Coupling of 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranose to choroacetone*

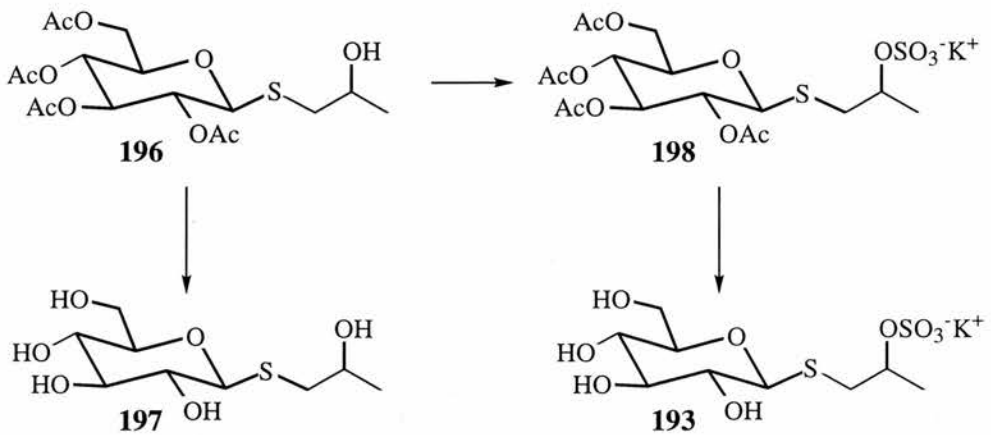
Dry triethylamine was used to initiate the reaction and the solution was stirred for 4 hours. Extraction of the reaction mixture using ethyl acetate and removal of the solvent at reduced pressure gave a colourless oil which was purified using column chromatography (silica, ethyl acetate-hexane (2:1)). 2'-Oxopropyl-2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside **195** was given in 98% yield as a clear oil which crystallised on standing (m.p. 70-72 °C). The mass spectrum illustrated the appropriate molecular ion (*m/z* (CI) 421 ($[M+H]^+$, 5%)) while microanalysis results showed that the product was pure (Found: C, 48.41; H, 5.80. Calc for $C_{17}H_{24}O_{10}S$: C, 48.57; H, 5.75%).



Scheme 69: *Reduction using sodium borohydride*

Reduction was effected using sodium borohydride in water which was added dropwise to a solution of 2'-oxopropyl-2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside **195** in tetrahydrofuran at 0 °C (scheme 69). The reaction was complete after 2 hours when it was quenched with dilute hydrochloric acid and then extracted into ethyl acetate. The solvent was removed at reduced pressure to give a clear syrup which was purified using column chromatography (silica, ethyl acetate-hexane (2:1)). 2'-Hydroxypropyl-2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside **196** was given as a white solid in 74% yield (m.p. 83-85 °C). The infrared spectrum showed the loss of the carbonyl absorption at 1708 cm^{-1} while a broad absorbance due to the newly formed hydroxyl group appeared at 3495 cm^{-1}. The mass spectrum showed the expected molecular ion (*m/z* (CI) 423 ($[M+H]^+$, 5%)) while microanalysis confirmed the purity of the product (Found: C, 48.22; H, 6.28. Calc for $C_{17}H_{26}O_{10}S$: C, 48.33; H, 6.20%). Two isomers were produced in the reaction due to the formation of a new stereogenic centre. From the integrals in the ^1H n.m.r. spectrum it could be seen that the isomers occurred in a ratio of 1:1.1. CH correlation spectroscopy was used to assign the ^{13}C n.m.r. spectrum.

From this compound two routes were taken and are shown in scheme 70. The first involved a simple deprotection to give 2'-hydroxypropyl-1-thio-β-D-glucopyranoside **197** while the second involved sulfation of the hydroxyl moiety followed by deprotection of the sugar to give **193**.



Scheme 70: *Two routes from 2'-hydroxypropyl-2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside*

The deprotection was achieved using a catalytic amount of potassium methoxide in methanol. On completion Amberlite IR 120 resin was added and stirring continued for 15 minutes before the Amberlite was removed by filtration. The reaction mixture was concentrated at reduced pressure and the resulting oil purified by column chromatography (silica, dichloromethane-methanol (9:1)). 2'-Hydroxypropyl-1-thio-β-D-glucopyranoside **197** was given as a clear syrup in 83% yield. The infrared spectrum showed the loss of the carbonyl absorption at 1740 cm^{-1} which indicated that the removal of the acetyl protection had been successful. This was confirmed by ^{13}C n.m.r. spectroscopy which also showed the absence of any carbonyl resonances. In addition microanalysis results indicated that the product was pure (Found: C, 42.02; H, 7.63. Calc for $C_9H_{19}O_6S$: C, 42.34; H, 7.50%). Desulfoglucosinolates are known to typically inhibit myrosinase and thus 2'-hydroxypropyl-1-thio-β-D-glucopyranoside was investigated both as a substrate and as an inhibitor of this enzyme.

Sulfation of 2'-hydroxypropyl-2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside **196** was achieved with a pre-prepared pyridine-sulfur trioxide complex.[138] The product was purified using column chromatography (silica, dichloromethane-methanol (9:1)) to give potassium 1-(2',3',4',6'-tetra-*O*-acetyl-1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate **198** in 62% yield as a colourless syrup. The success of the sulfation could be determined by

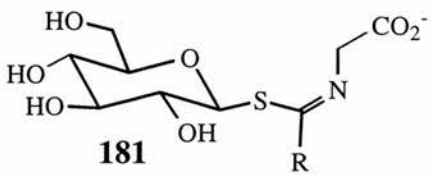
negative ion electrospray mass spectrometry which gave a molecular ion at 501 corresponding to $[M-K]^-$. Microanalysis also provided positive proof that the desired sulfated product had been formed (Found: C, 37.84; H, 4.52. Calc for $C_{17}H_{25}O_{13}S_2K$: C, 37.77; H, 4.66%).

Finally deprotection of potassium 1-(2',3',4',6'-tetra-*O*-acetyl-1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate **198** using a catalytic amount of potassium methoxide in methanol as described previously gave a white amorphous solid. The solid was purified using a series of two columns firstly reverse phase C-18 silica and secondly G-10 Sephadex to remove salts. In both cases distilled water was used as the eluant and the product was freeze dried to give potassium 1-(1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate **193** in 93% yield. The product was pure by microanalysis (Found: C, 28.49; H, 4.54. Calc for $C_9H_{17}O_9S_2K$: C, 29.02; H, 4.60%) and the negative ion electrospray mass spectrum showed the appropriate molecular ion (*m/z* (ES^-) 333 ($[M-K]^-$, 100%)). It is important to note that this product was extremely hygroscopic.

Potassium 1-(1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate **193** was investigated as a substrate for myrosinase. It was suspected that the analogue would cleave to give β-D-glucose but it was not known whether the aglycone would be released from the active site if it could not rearrange.

2.2.3 Conclusions

Synthesis of the initial non-rearrangeable glucosinolate target (figure 35) was largely unsuccessful despite a number of different synthetic approaches. The target was therefore revised.



181

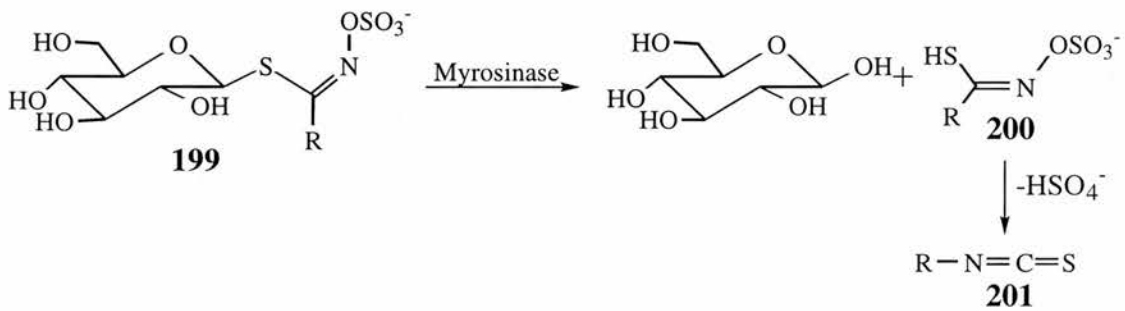
Figure 35: *Non-rearrangeable analogue*

Potassium 1-(1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate was successfully synthesised in four steps from chloroacetone and 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose. The route was generally high yielding but the sulfation step gave only a moderate yield of 62%. Potassium 1-(1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate proved to be extremely hygroscopic but was obtained in its microanalytically pure form.

2'-Hydroxypropyl-1-thio-β-D-glucopyranoside was also synthesised and was pure by microanalysis. This compound should provide an interesting comparison to its sulfated analogue in enzymatic studies using myrosinase.

2.3 Myrosinase Catalysed Hydrolysis of Glucosinolates

Glucosinolates **199** are hydrolysed by myrosinase to give β-D-glucose and an aglycone **200**. The aglycone then rearranges to give isothiocyanate **201** and sulfate anion (scheme 71). As detailed in section 1.3.4 other products can also result depending on the nature of the substrate and conditions of hydrolysis.



Scheme 71: *Hydrolysis of glucosinolates by myrosinase*

There are several methods available to measure the rate of myrosinase catalysed glucosinolate hydrolysis. These methods are centred around three main areas:

(a) measuring the decrease in amount of glucosinolate

(b) measuring glucose release

(c) measuring sulfate release

Decreasing glucosinolate concentrations are determined by monitoring any decline in the ultra-violet absorption of the compound. For this technique it is therefore necessary that the glucosinolate contains a suitable chromophore such as an aromatic group. The standard assay for myrosinase uses this method by measuring the decrease in concentration of sinigrin at 227 nm.[244] The assay is carried out in triplicate using a 0.1 mM sinigrin solution in 33.1 mM potassium phosphate buffer at pH 7.0 in a total volume of 1.0 ml. After equilibration at 37 ± 0.1 °C, 30 μl of myrosinase solution is added and the decrease in absorbance at 227 nm is monitored. The number of units are calculated by dividing the observed initial rate by the theoretical rate (6.7 min^{-1}) calculated for 1 unit of enzyme. One

unit of enzyme is defined as the amount of enzyme required to hydrolyse 1 μmol of sinigrin per minute under standard assay conditions. This assay allows the results of experiments to be corrected to a single unit of enzyme activity thereby allowing comparisons to be drawn between studies.

Measuring glucose release is performed using a hexokinase/glucose-6-phosphate dehydrogenase coupled assay, with the glucose (HK) reagent obtained from Sigma. When this reagent is reconstituted according to the directions it contains approximately the following concentrations of active ingredients:

NAD	1.5 mmol dm^{-3}
ATP	1.0 mmol dm^{-3}
Hexokinase (yeast)	1000 units dm^{-3}
G-6-PDH	1000 units dm^{-3}
magnesium ions	2.1 mmol dm^{-3}
Buffer	pH 7.5± 0.1
Non-reactive stabilisers and fillers	
Sodium azide (as preservative)	0.05%

The enzymatic reactions involved in the assay are as follows:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G-6-P} + \text{ADP}$$
$$\text{G-6-P} + \text{NAD} \xrightarrow{\text{G-6-PDH}} \text{6-PG} + \text{NADH}$$

When the glucosinolate is hydrolysed by myrosinase the glucose produced is phosphorylated at the 6 position by adenosine triphosphate (ATP) in a reaction catalysed by hexokinase (HK). The glucose-6-phosphate produced is oxidised in the presence of nicotinamide adenine dinucleotide (NAD) to give 6-phosphogluconate (6-PG). This reaction is catalysed by glucose-6-phosphate dehydrogenase (G-6-PDH). During this

oxidation an equimolar amount of NAD is reduced to NADH. The consequent increase in absorbance at 340 nm is directly proportional to glucose concentration.

Monitoring sulfate production is a less commonly used technique but can be achieved using turbidimetric methods.[245] In this case inorganic sulfate is determined using a barium polyethylene glycol reagent containing a small amount of preformed barium sulfate. This assay is performed under acidic conditions which avoids complications from phosphate or the preformed barium sulfate. The sample to be assessed is diluted to 3 ml with deionised water and acidified using 1.0 ml of 0.5 M hydrochloric acid. Barium polyethylene glycol (1.0 ml) is then added and the sample mixed thoroughly. To determine the concentration of sulfate present, the absorbance is measured at 600 nm and corrected with respect to a reagent blank. The reading is compared to a standard curve prepared with known amounts of sulfate.

Any one of the above methods would be suitable to investigate the myrosinase catalysed hydrolysis of the glucosinolates synthesised in this thesis. However the non-rearrangeable analogue (section 2.2.7) would not be suitable for UV detection due to the lack of a chromophore. It was therefore decided to use the well established glucose release method to investigate the rate of turnover of the synthetic analogues.

For each assay glucose (HK) reagent (1.0 ml) was equilibrated at 37 ± 0.1 °C in a 1.0 ml volume/1 cm pathlength quartz cuvette in the thermostatted cell holder of the UV spectrophotometer. A 10 µl aliquot of the appropriate reaction mixture was then added and the solution left to stabilise (at least 5 minutes incubation at 37 ± 0.1 °C). The absorbance of the resulting solution was measured at 340 nm. The readings were corrected relative to a blank sample where 10 µl of distilled water was added to 1.0 ml glucose (HK) reagent. The blank reading was taken at 340 nm and 37 ± 0.1 °C. The glucose concentration could then be calculated from the following equation:

$$[\text{Glucose}]/\text{mM} = \left(\frac{(\text{sample absorbance-blank absorbance}) \times 293 \times 0.01}{180.16} \right) \times 1000$$

where 293 is a predetermined factor (mg/dl)
0.01 converts mg/dl to mg/ml
180.16 is the molecular weight of D-glucose

Initially, standard D-glucose solutions were tested to determine the accuracy of the glucose (HK) assay and hence its suitability for our studies.

Standard 5 mM D-glucose solution

Blank absorbance 0.043
Sample absorbance 0.355

$$[\text{Glucose}] = \left(\frac{(0.355-0.043) \times 293 \times 0.01}{180.16} \right) \times 1000$$

[Glucose] = 5.074 mM

Standard 10 mM D-glucose solution

Blank absorbance 0.043
Sample absorbance 0.657

$$[\text{Glucose}] = \left(\frac{(0.657-0.043) \times 293 \times 0.01}{180.16} \right) \times 1000$$

[Glucose] = 9.99 mM

These results indicate that there is an error of less than 2%. This assay is therefore suitable for our purposes.

Reactions to determine the optimum amount of myrosinase

In order to assess how much myrosinase would be necessary to give a reasonable reaction rate such that several measurements could be taken a few test reactions were used. A 10 mM solution of sinigrin in 33.1 mM potassium phosphate buffer at pH 7.0 was thus incubated with myrosinase at 37 ± 0.1 °C. Three different quantities of myrosinase were used 0.041, 0.052 and 0.062 units respectively, accurately measured using the assay described in section 3.7. These reaction mixtures were then sampled in the usual way for D-glucose content at 15 minute intervals until complete reaction had occurred. The times taken to reach completion were 2 hours with 0.041 units, 1 hour 30 minutes with 0.052 units and 1 hour with 0.062 units. For further studies it was decided to use approximately 0.062 units of enzyme activity: this corresponded to a volume of 30 µl from a solution containing 60 mg of myrosinase (lyophilised powder from *Sinapis alba*, 175 units per g of solid, Sigma) in 3 ml of 33.1 mM potassium phosphate buffer at pH 7.0.

Free glucose measurements

For all of the experiments conducted the samples were tested for the presence of free glucose which would cause inaccurate results. In each instance a negligible amount of glucose was found.

In order to check for the presence of enzymes which may degrade D-glucose and again give false results 0.062 units of myrosinase was added to a 10 mM glucose solution. The D-glucose concentration was measured every 30 minutes for 2 hours. The glucose concentration remained constant within the 2% error already established.

2.3.1 Incubations of synthetic glucosinolates with myrosinase

For each glucosinolate examined a 10 mM solution of glucosinolate was prepared using 33.1 mM potassium phosphate buffer at pH 7.0. This solution was prewarmed to 37 ± 0.1 °C and 30 µl of myrosinase solution (20 mg in 1 ml potassium phosphate buffer at pH 7.0) was added to initiate the reaction. This reaction mixture was assayed as before using the glucose (HK) method. Each measurement was performed in triplicate to minimise any error.

2.3.1.1 Phenyl glucosinolate homologues

The hydrolysis of phenyl, benzyl and phenethyl glucosinolates were compared using the glucose (HK) assay method and the results displayed graphically (figure 36). The gradient of the first portion of each graph was used to determine the rate of hydrolysis. In each case the value had to be corrected to 1 unit of enzyme activity to minimise errors incurred by more or less active enzyme preparations.

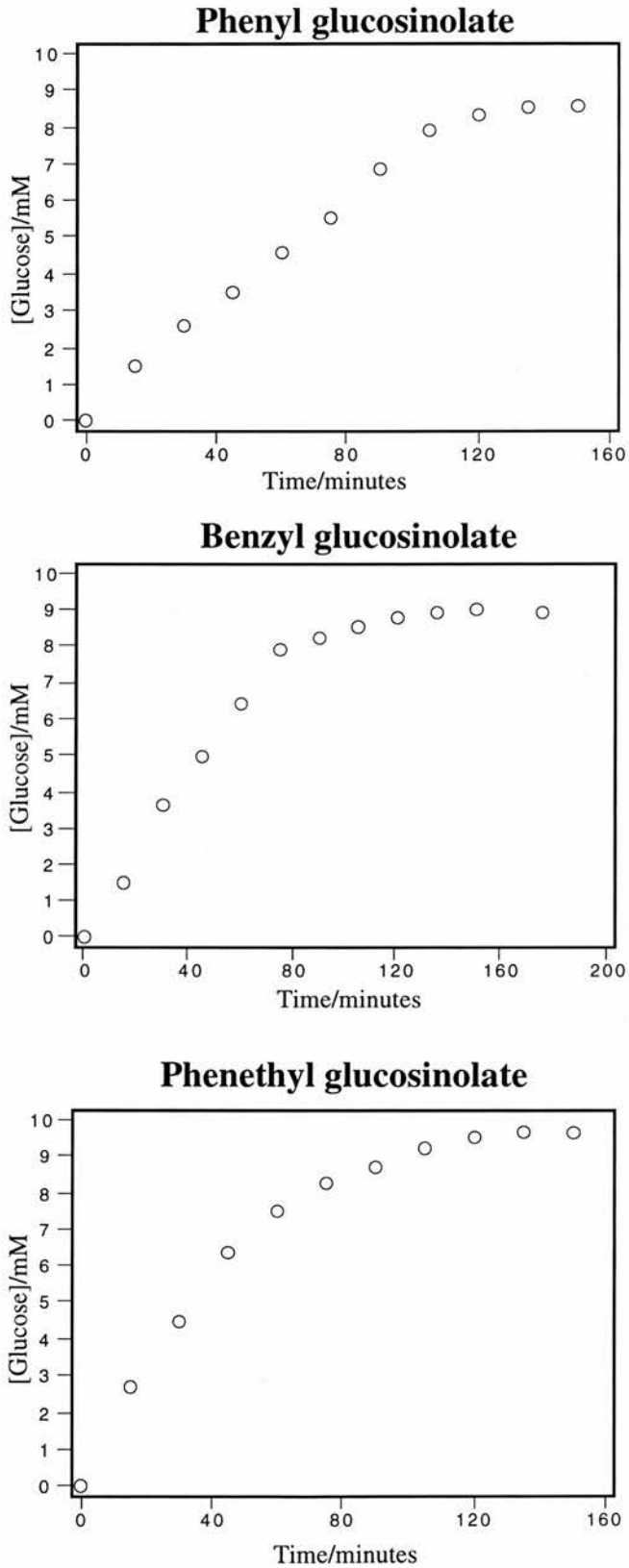


Figure 36: *Graphs illustrating glucose produced from myrosinase catalysed hydrolysis of phenyl glucosinolate homologues*

In each case less than 10 mM of glucose was obtained from the myrosinase catalysed hydrolysis. This was unexpected since all of the glucosinolates used were pure by microanalysis and it was therefore decided to re-examine these reactions.

Considering the initial rates of turnover of each substrate shown in table 9 it can be seen that a trend exists. Phenethyl glucosinolate is cleaved at a faster rate than benzyl glucosinolate and phenyl glucosinolate is slower still. This trend was reflected in the graphs of glucose concentration where phenethyl glucosinolate had yielded 9.64 mM of glucose while benzyl had given 9.0 mM and phenyl 8.5 mM.

Glucosinolate	Rate of turnover[a] (mM min^{-1})
Phenyl	**1.49 ± 0.31**
Benzyl	**1.87 ± 0.23**
Phenethyl	**2.66 ± 0.60**

[a] All values are corrected to one unit of enzyme activity

Table 9: *Turnover rates for phenyl glucosinolate derivatives with myrosinase*

The hydrolysis was thus examined again, leaving the reaction mixture for a longer period of time in order to reach completion. This was more successful but phenyl glucosinolate still failed to give the desired amount of glucose with only 8.9 mmol of glucose given after 220 minutes. Either there is some contaminant present in the sample which was not detected or the glucosinolate picked up water when it was weighed out. In view of the extremely hygroscopic nature of glucosinolates the second explanation is the more likely especially in view of the microanalysis obtained (Found: C, 33.49; H, 3.52; N, 2.62. Calc for $C_{13}H_{16}NO_9S_2K.2H_2O$: C, 33.26; H, 4.29; N, 2.98%).

The trend shown in table 9 for the rate of turnover of the phenyl derivatives is not unexpected. The X-ray crystal structure of myrosinase revealed a binding pocket on the aglycone side of the active site which was lined with hydrophobic residues. It therefore

follows that the longer phenethyl chain may have a greater flexibility and more favourable interaction with the aglycone binding pocket than the phenyl derivative. In agreement with this the rates of hydrolysis of phenyl derivatives decreased in the order phenethyl (2.66 ± 0.60 mM min^{-1}), benzyl (1.87 ± 0.23 mM min^{-1}), phenyl (1.49 ± 0.31 mM min^{-1}).

(i) Effect of adding ascorbic acid to phenyl glucosinolate hydrolysis

L-Ascorbic acid is known to specifically activate myrosinase when it is present in low concentrations whereas at high concentrations inhibition occurs. The binding of L-ascorbic acid occurs at a site other than that used by the substrate. This causes a change in the conformation of the active site and an acceleration of the hydrolysis reaction. At high concentrations, ascorbic acid competes with the substrate for entry into the active site.[124] Interestingly, analogues of ascorbic acid such as glucoascorbate or ascorbyl stearate do not exert this effect.[138]

The extent to which L-ascorbic acid would accelerate the hydrolysis of phenyl glucosinolate was investigated. As in the preceding work a 10 mM solution of glucosinolate in 33.1 mM potassium phosphate buffer at pH 7.0 was used. In addition to this L-ascorbic acid was added such that the reaction mixture contained a 1 mM concentration of this activator. When the solution reached 37 ± 0.1 °C myrosinase was added. The results of this study are illustrated in figure 37. In the presence of L-ascorbic acid (1 mM) the rate of hydrolysis of phenyl glucosinolate was enhanced by 53%.

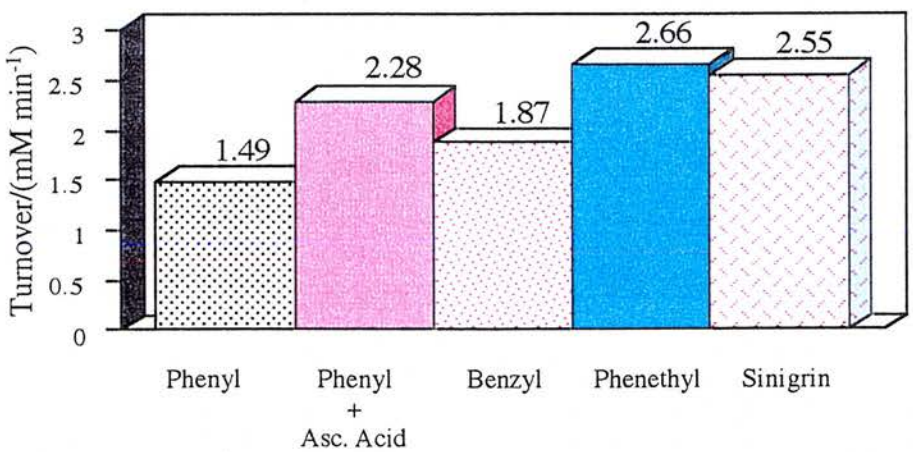


Figure 37: *Graph illustrating rate enhancement by 1.0 mM L-ascorbic acid*

2.3.1.2 Novel phenyl analogues

(i) Incubation of *p*-methoxyphenyl glucosinolate with myrosinase

p-Methoxyphenyl glucosinolate proved to be unstable in aqueous solution. Hydrolysis studies using myrosinase were thus performed on a freshly prepared crude sample. Again the amount of glucose gained was less than expected at 8.0 mmoles. However, in this instance there may well have been impurities in the sample due to the lack of purification.

When *p*-methoxyphenyl glucosinolate was left in either aqueous solution or phosphate buffer in the absence of myrosinase the solution became cloudy. It was thus hypothesised that *p*-methoxyphenyl glucosinolate could breakdown in the absence of myrosinase.

To investigate whether D-glucose was one of the breakdown products, a 10 mM solution of *p*-methoxyphenyl glucosinolate was incubated at 37 ± 0.1 °C for several hours. This did indeed produce glucose although at a much slower rate than in the presence of myrosinase (table 10). In the absence of myrosinase the reaction had given 8.0 mmoles of glucose after 23 hours whereas the same quantity of glucose was given after 2 hours in the presence of myrosinase.

Glucosinolate	Rate of turnover[a] (mM min^{-1})
***p*-methoxyphenyl (myrosinase)**	**1.42 ± 0.32**
***p*-methoxyphenyl (buffer)[b]**	**0.0247 ± 0.0036**
***p*-methoxyphenyl (water)[b]**	**0.0175 ± 0.0028**

[a] All values are corrected to one unit of enzyme activity

[b] Decomposition without myrosinase

Table 10: *Turnover rates for phenyl glucosinolate derivatives with myrosinase*

(ii) Incubation of *p*-nitrophenyl glucosinolate with myrosinase

Microanalytical data indicated that *p*-nitrophenyl glucosinolate contained a sulfate impurity which could not be removed by repeated desalting columns. It was decided to test this analogue in the presence of the sulfate salt. Hydrolysis by myrosinase proceeded smoothly and did in fact give 10 mmoles of glucose after a very short period of time. The turnover rate was 6.44 ± 0.53 mM min^{-1}.

(iii) Comparison of novel phenyl glucosinolate analogues

Comparing the initial rate of turnover of *p*-nitrophenyl, phenyl and *p*-methoxyphenyl glucosinolates (table 11) it could be seen that *p*-nitrophenyl glucosinolate was cleaved at a much faster rate than either the phenyl or *p*-methoxyphenyl derivatives which show a roughly similar rate of reaction. This trend was exactly opposite to that predicted in section 2.2.1. It was therefore suggested that the electron withdrawing nature of the *p*-nitro substituent speeds up the hydrolysis of the glucosinolate by decreasing the pK_a of the thiol and inductively polarising the glycosidic bond. The stability of the aglycone leaving group will also be increased because of the presence of the *p*-nitro group.

Glucosinolate	Rate of turnover[a] (mM min^{-1})
***p*-methoxyphenyl**	**1.42 ± 0.32**
phenyl	**1.49 ± 0.31**
***p*-nitrophenyl**	**6.44 ± 0.53**

[a] All values are corrected to one unit of enzyme activity

Table 11: *Turnover rates for novel phenyl glucosinolate derivatives with myrosinase*

It should be noted that the self decomposition of the *p*-methoxyphenyl glucosinolate probably contributes to the rate of hydrolysis measured in the presence of myrosinase thus giving an enhanced rate of turnover.

2.3.2 Enzyme kinetics and determination of V_{max} and K_M

The two commonly measured parameters for enzyme catalysed reactions are V_{max} and K_M. These parameters are defined as follows:

V_{max} This term describes the maximum velocity of the enzyme. In this instance the enzyme is saturated with substrate and is operating continuously.

K_M This is also known as the binding or Michaelis constant and gives a measure of how well the substrate binds to the enzyme. A small value for K_M indicates that a low concentration of substrate is required to saturate the enzyme; the enzyme has a high affinity for its substrate. Conversely a high value of K_M suggests a weakly bound substrate. K_M is equal to the substrate concentration at which the reaction rate is half of its maximal value.

To determine these parameters it is necessary to monitor the enzyme catalysed reaction with various different substrate concentrations. When the substrate is at low concentration the reaction rate will depend entirely on the affinity of the enzyme for the substrate.

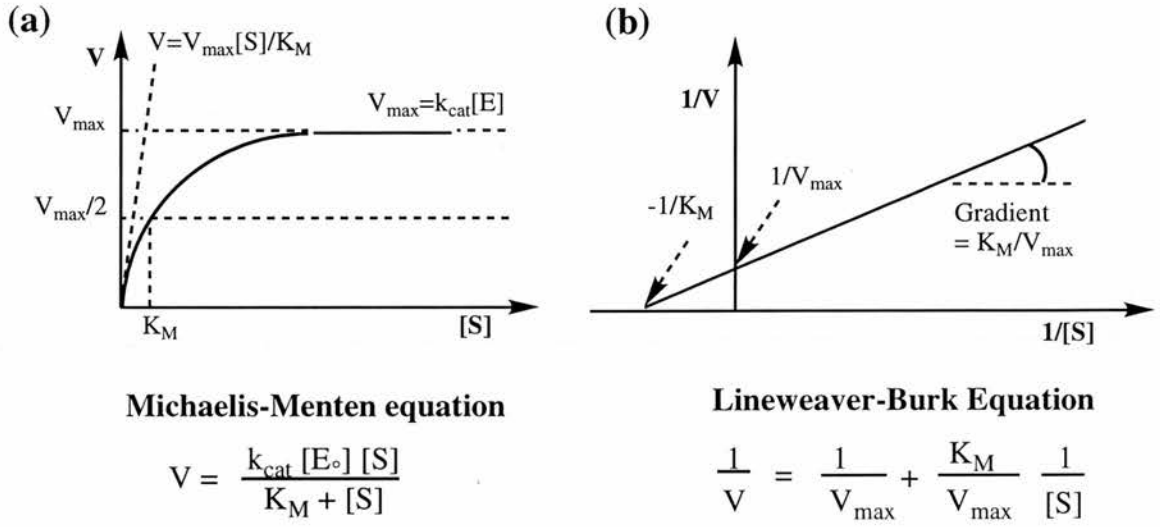
The most simple representation of an enzyme catalysed reaction is as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

It is assumed that the first step is rapid and reversible while the second step is rate determining i.e. $k_2 << k_{-1}$. The corresponding rate equations can be solved to give the Michaelis-Menten equation:

$$V = \frac{k_{cat}\,[E_\circ]\,[S]}{K_M + [S]}$$

This equation was proposed by Lenore Michaelis and Maude-Menten in 1913 and represents the hyperbolic graph shown in figure 38 (a). In practice the reciprocal of the Michaelis-Menten equation is used and gives a straight line plot known as a Lineweaver-Burk plot figure 38 (b).



Michaelis-Menten equation

$$V = \frac{k_{cat}\,[E_\circ]\,[S]}{K_M + [S]}$$

Lineweaver-Burk Equation

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}}\,\frac{1}{[S]}$$

Figure 38: (a) *Saturation kinetics for enzyme catalysed reactions*

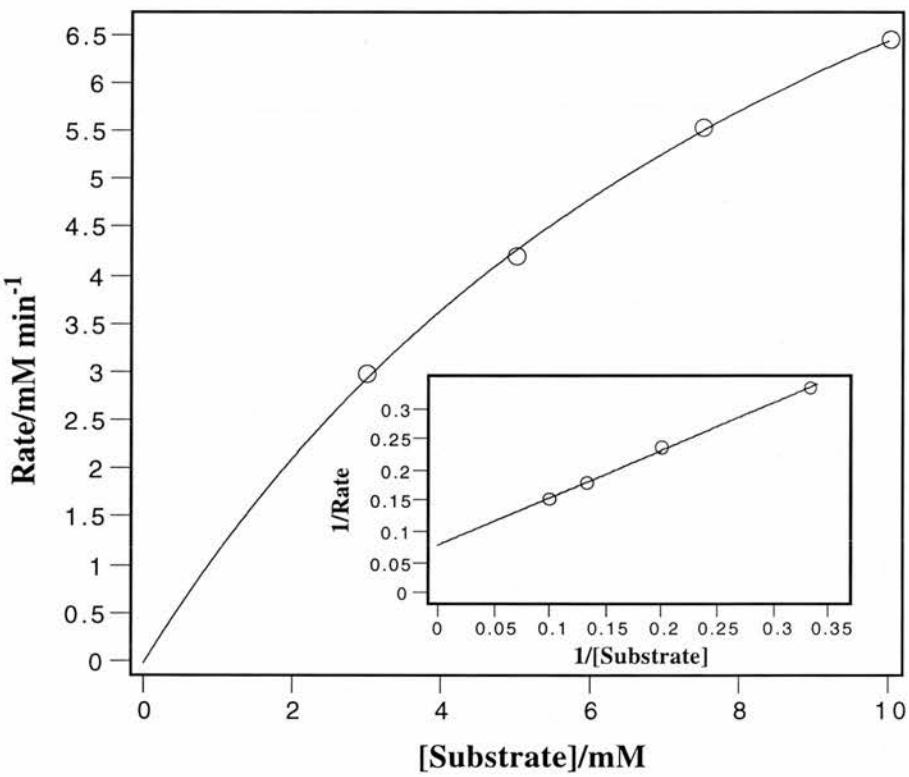
(b) *Lineweaver-Burk plot for enzyme catalysed reactions*

It should be noted that the reciprocal relation in the Lineweaver-Burk plot means that data at low substrate concentrations are emphasised and those at high concentrations are compressed. This makes data less accurate when calculated using these plots. A more accurate method for delivery of K_M and V_{max} is to use non-linear regression to fit the data directly to the Michaelis-Menten equation. This was done using the "Enzfitter" program.

In light of the particularly fast cleavage of the *p*-nitrophenyl glucosinolate analogue the K_M and V_{max} were determined and compared to that for sinigrin.

2.3.2.1 K_M and V_{max} for *p*-nitrophenyl glucosinolate

Using the glucose (HK) assay the turnover of *p*-nitrophenyl glucosinolate was monitored at four different glucosinolate concentrations; 2.0, 5.0, 7.5, 10.0 mM. The rates observed were corrected to 1 unit of enzyme activity to give the graphs shown in figure 39. The K_M and V_{max} values were calculated using the Enzfitter programme. It should be noted that the accuracy of the K_M and V_{max} values are limited by having only four data points.

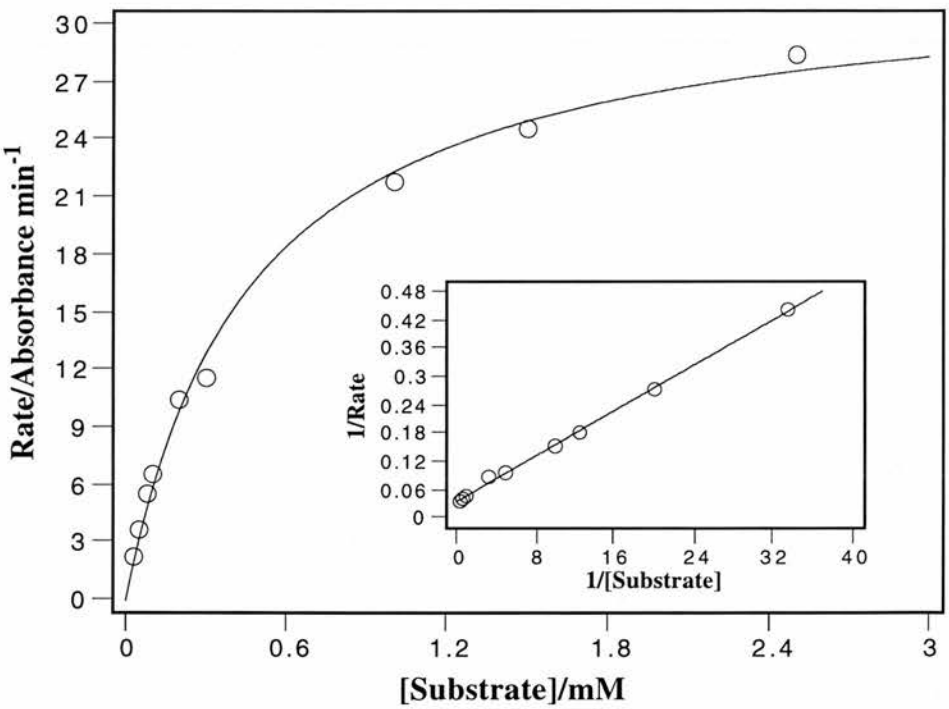


	Value	Standard Error
V_{max}	13.12 mM min^{-1}	0.55 mM min^{-1}
K_M	10.38 mM	0.74 mM

Figure 39: *Determination of V_{max} and K_M for p-nitrophenyl glucosinolate*

2.3.2.2 K_M and V_{max} for sinigrin

The decrease in absorbance due to sinigrin at 227 nm was measured using ultra-violet spectroscopy as described for the sinigrin assay. Several concentrations of sinigrin were assessed (0.03, 0.05, 0.08, 0.1, 0.2, 0.3, 1.0, 1.5, 2.5 mM) and the kinetic values corrected to 1 unit of enzyme activity. The Enzfitter programme was again used to calculate V_{max} and K_M (figure 40). V_{max} has been expressed in mM min^{-1} in order to compare the value of sinigrin to that for *p*-nitrophenyl glucosinolate.



	Value	Standard Error
V_{max}	4.80 mM min^{-1}	0.16 mM min^{-1}
K_M	0.46 mM	0.044 mM

Literature Values[138]

V_{max}	4.83 ± 0.22 mM min^{-1}
K_M	0.42 ± 0.05 mM

Figure 40: *Determination of V_{max} and K_M for p-nitrophenyl glucosinolate*

2.3.2.3 Comparisons

From the V_{max} of the two compounds it can be seen that *p*-nitrophenyl glucosinolate is cleaved by myrosinase at a faster rate than sinigrin. However, it is interesting to note that *p*-nitrophenyl glucosinolate has a much higher K_M than sinigrin. This suggests that *p*-nitrophenyl glucosinolate does not bind as strongly to the active site of myrosinase.

All natural glucosinolates possess a CH_2 "knuckle" in the side chain (figure 41).[185] In the case of the phenyl derivatives synthesised the absence of the knuckle may affect the conformation of the side chain in the active site and impair binding.

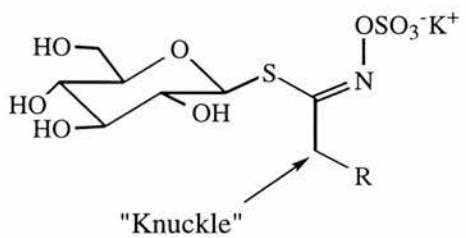
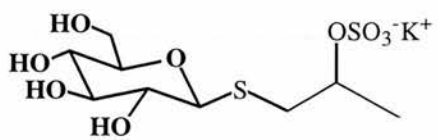


Figure 41: *All naturally occurring glucosinolates possess a CH_2 "knuckle"*

2.3.3 Studies on a non-rearrangeable glucosinolate analogue

Compound **193** contains many of the glucosinolate structural features (figure 42). For example it possesses a glucose moiety with a β-thio link to the aglycone. In addition, the aglycone contains a sulfate group characteristic of glucosinolates. It was thus predicted that **193** would be capable of binding to the active site of myrosinase and that it would be cleaved by the enzyme to give D-glucose. The resulting aglycone would not be capable of undertaking a Lossen rearrangement and may remain in the active site. It should be noted that this compound was tested as a mixture of isomers due to the chirality of the side chain.



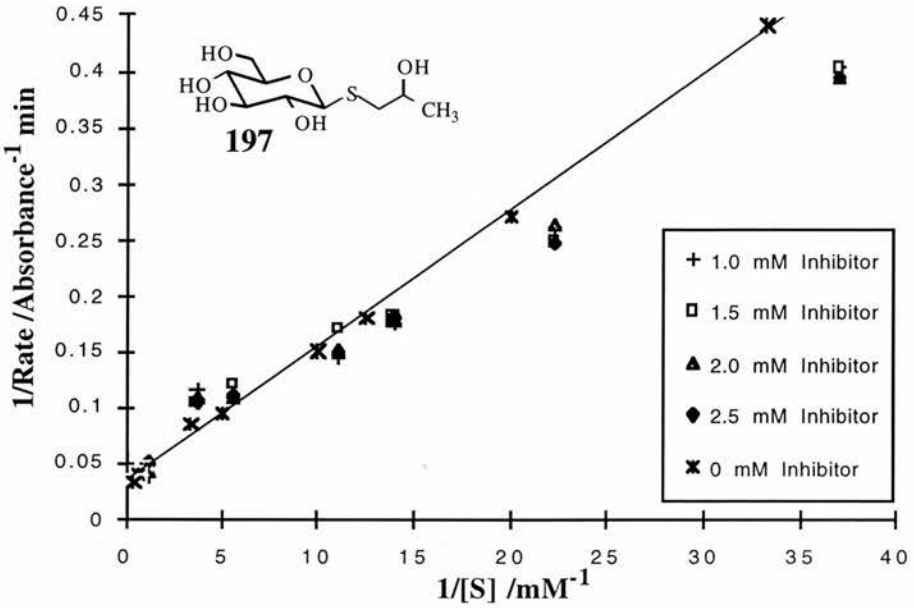
193

Figure 42: *Non-rearrangeable glucosinolate analogue*

A 10 mM solution of **193** in phosphate buffer (33.1 mM at pH 7.0) was incubated at 37 ± 0.1 °C with myrosinase (0.055 units). Using the glucose (HK) assay, samples were taken at 15 minute intervals for 1 hour and 30 minutes. This study showed that no glucose had been liberated. The experiment was repeated using a much greater quantity of myrosinase (0.22 units) however even after incubation overnight no glucose had been produced. A similar experiment was tried using sweet almond β-glucosidase (0.4 units), which also produced no glucose.

In view of the inability of either myrosinase or sweet almond β-glucosidase to cleave **193** its properties as an inhibitor were investigated. The desulfo derivative **197** was also tested in order to provide a comparison and illustrate the importance of the sulfate group in binding to the enzyme. Four inhibitor concentrations (1.0, 1.5, 2.0, 2.5 mmol) were used in the myrosinase catalysed hydrolysis of sinigrin. For compound **193** a 10 mM concentration was also used. The results of these studies are shown in figure 43.

Graph of inhibition of myrosinase catalysed hydrolysis of sinigrin by 2'-hydroxypropyl-1-thio-β-D-glucopyranoside



Graph of inhibition of myrosinase catalysed hydrolysis of sinigrin by potassium 1-(1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate

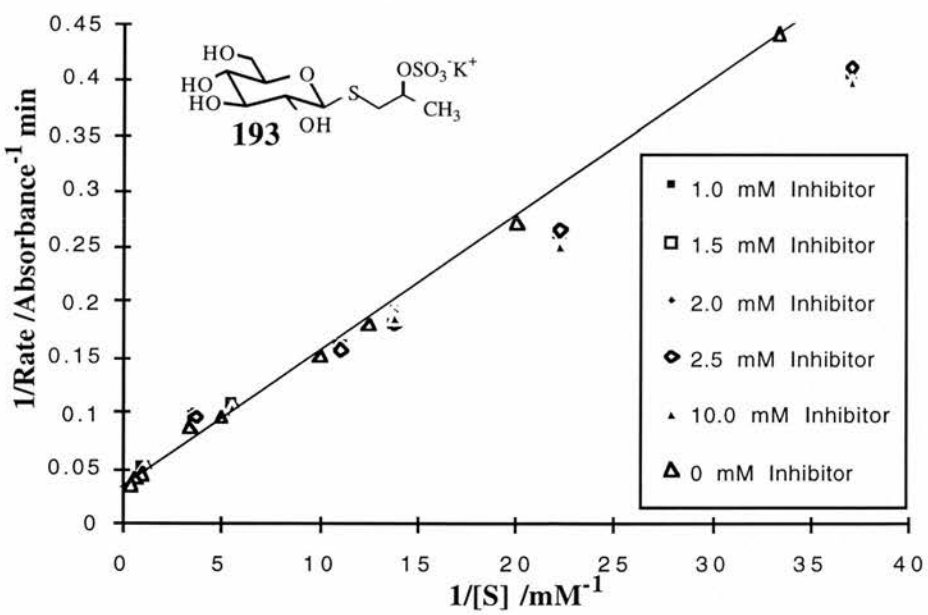


Figure 43: *Graphs illustrating the inability of the synthesised non-rearrangeable analogue to inhibit myrosinase catalysed glucosinolate hydrolysis*

It can be seen from these graphs that neither the sulfated nor unsulfated compounds show any significant effect on the hydrolysis reaction even at 10 mM concentration.

This result was interesting as it implied neither compound bound to the active site. One of the differences between our compound and the glucosinolate skeleton is the lack of the sp^2 hybridised nitrogen in the aglycone. In our compound this has been replaced by an sp^3 hybridised carbon. The site normally associated with the nitrogen lone pair has been replaced by a methyl group and this may affect the binding in some way. Alternatively, the problem may lie with the lack of a suitable side chain to bind to the aglycone recognition site. Nevertheless it is still surprising that the glucose and sulfate moieties were essentially unrecognised by myrosinase.

Regarding the lack of substrate activity, this could be a result of the poorer leaving group ability of the aglycone. The pK_a of the side chain will be much higher than that of the glucosinolate. As myrosinase does not have a residue at the active site to protonate the leaving group this means that a very good leaving group is required for reaction to take place.

It had been proposed[126, 238] that there may be some intramolecular assistance from the sulfate group to aid reaction. The lack of substrate activity in this case would not support this hypothesis.

2.3.4 Conclusions

The phenyl glucosinolate analogues investigated showed the following trend for initial rates of hydrolysis (table 12):

p-Nitrophenyl	6.44 ± 0.53 mM min^{-1}
Phenethyl	2.66 ± 0.60 mM min^{-1}
Phenyl + 1 mM Ascorbic acid	2.28 ± 0.63 mM min^{-1}
Benzyl	1.87 ± 0.23 mM min^{-1}
Phenyl	1.49 ± 0.31 mM min^{-1}
p-Methoxyphenyl	1.42 ± 0.32 mM min^{-1}

Table 12: *Table showing initial rates of hydrolysis for several glucosinolates*

From this it can be seen that the *p*-nitrophenyl glucosinolate is cleaved at a much faster rate than any of the other analogues. The *p*-nitro substituent decreases the pK_a of the thiol group and inductively polarises the glycosidic bond. A comparison of K_M values for *p*-nitrophenyl glucosinolate versus sinigrin illustrated that sinigrin bound more strongly to the enzymes active site (lower K_M). However the V_{max} values indicated that *p*-nitrophenyl glucosinolate was cleaved at a much faster rate than sinigrin.

From table 12 it appears that increasing the length of the alkyl chain gives a corresponding increase in initial rate: phenyl<benzyl<phenethyl. 1 mM Ascorbic acid provided an enhanced rate of turnover for phenyl glucosinolate such that the rate became comparable to the phenethyl derivative.

Finally the sulfo and desulfo non-rearrangeable glucosinolate analogues did not act as substrates for myrosinase. In addition they did not inhibit myrosinase. It remains surprising that potassium 1-(1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate would not bind tightly to the active site given that the glucose and sulfate moieties of the glucosinolates structure are maintained.

2.4 Summary

Four deuterated desulfoglucosinolate were synthesised for use as internal standards in LC-MS. Each of these glucosinolates were obtained in their pure form however the two phenethyl derivatives proved to be especially hygroscopic. Incorporation of deuterium into the sugar moiety of desulfogluconasturtiin provided a general strategy for synthesis of any deuterated desulfoglucosinolate however, the synthesis took 18 steps to complete. APCI-MS was performed on all of the deuterated desulfoglucosinolates at SCRI and compared to their non-deuterated counterparts. In this way many of the mass spectral assignments and fragmentation patterns could be confirmed.

In collaboration with SCRI the deuterated desulfoglucosinolates were successfully tested as internal standards for quantification by LC-APCI-MS. Linear responses were observed between normalised peak area and analyte concentration for each compound but the indolyl derivatives showed slight deviations at very low concentrations. To obtain quantification by LC-APCI-MS a standard calibration curve may be necessary. Using these internal standards combined with single ion monitoring the levels of detection have been improved 100 fold compared to conventional HPLC. It is now possible to quantitatively detect desulfoglucosinolates from a single leaf rather than having to combine leaves of similar ages from different plants to gain sufficient sample.

The hydrolysis of phenyl, benzyl and phenethyl glucosinolates by myrosinase was also examined. It was observed that the increasing length of side chain gave a corresponding increase in initial rate of hydrolysis. Furthermore, 1 mM ascorbic acid enhanced the rate of hydrolysis of phenyl glucosinolate by 53%. The hydrolysis rate was then similar to that observed for phenethyl glucosinolate and also to that of sinigrin.

Three glucosinolates which varied in the electronic nature of the side chain were successfully synthesised. Unfortunately the *p*-nitrophenyl analogue was not obtained in its

microanalytically pure form but the sulfate impurity did not significantly hinder hydrolysis by myrosinase. Furthermore desulfo-*p*-nitrophenyl glucosinolate adopted 2 conformations in aqueous solution as evidenced by LC-MS and ^{1}H n.m.r. spectroscopy. These were postulated to be E and Z isomers. *p*-Methoxyphenyl glucosinolate decomposed in aqueous solution in the absence of myrosinase. This analogue was therefore tested in its crude form. It was also established that both *p*-nitro and *p*-methoxy derivatives existed as mixed metal salts of Na^+ and K^+. Phenyl glucosinolate was obtained in microanalytically pure form.

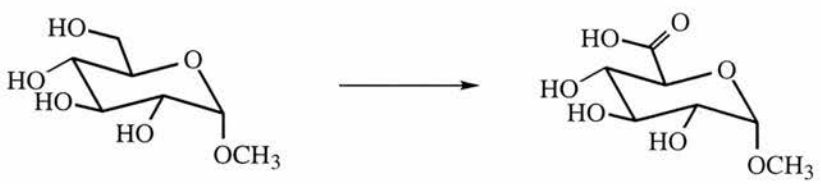
Initial rates of hydrolysis of these three glucosinolates by myrosinase showed the following trend *p*-nitrophenyl>phenyl>*p*-methoxyphenyl. This illustrated that the electron withdrawing nature of the *p*-nitro substituent speeds up the hydrolysis of the glucosinolate by decreasing the pK_a of the thiol and inductively polarising the glycosidic bond.

Several approaches were made towards the synthesis of a glucosinolate analogue which would be capable of hydrolysis by myrosinase but not rearrangement. One analogue was successfully synthesised but interestingly did not act as either a substrate or an inhibitor for myrosinase.

2.5 Future Work

(i) Synthesis of deuterated glucosinolates

It would be interesting to return to the synthesis of 2,3,4,6-tetra-*O*-acetyl-thio-β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose (section 2.1.4). This could be shortened by a direct oxidation of methyl α-D-glucopyranoside to the corresponding β-D-glucuronide (scheme 71). This should be possible via reaction with an oxidising agent such as t-butyl hypochlorite in the presence of TEMPO and sodium hydroxide.[246] However this reaction was reported as low yielding and thus may not provide a significant improvement over the existing route. Improving the synthesis of 2,3,4,6-tetra-*O*-acetyl-thio-β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose would make the route more viable for preparing a wide range of glucosinolates.



Scheme 71: *Direct oxidation of methyl α-D-glucopyranoside*

(ii) Uses of labelled glucosinolates

The LC-APCI-MS technique developed as a result of the compounds synthesised in this thesis allows very small amounts of glucosinolate to be detected accurately (section 2.1.7). This opens up exciting new areas of investigation. Subtle changes in glucosinolate distribution in the various organs of a single plant can be measured. The response of a leaf or plant organ to stress such as insect damage can be monitored. Furthermore, extensive studies during the plant life cycle could provide the basis for understanding glucosinolate synthesis, catabolism, interconversion and flux between plant tissues.

Sulfated versions of the labelled thiohydroximates synthesised are also of a much wider application. The glucosinolate metabolism of insects could be investigated as the metabolic

products of the deuterated glucosinolates would be easily identified. In a similar manner bacterial degradation of leaf surface glucosinolates could be studied using compounds such as [$^2H_5$]gluconasturtiin.

When humans ingest glucosinolate containing plants both plant and bacterial myrosinase are present and involved in glucosinolate breakdown. The resulting isothiocyanates do have a chemopreventative action in animals but studies on humans are scarce. Using studies on rats it has already been established that the role of bacterial myrosinase is important for glucosinolate degradation especially when plant myrosinase is inactivated during food processing (section 2.1.8).[235] However, a significant problem of such studies lies in identification of the released metabolites. Use of labelled glucosinolates *in vitro* and *in vivo* will contribute greatly to clarify the role of gut microflora in glucosinolate breakdown. This may provide further advancement in the understanding of the bioavailability and anticarcinogenic nature of isothiocyanates.

(iii) Mechanistic studies on myrosinase using novel glucosinolate analogues

The successful synthesis of a nonrearrangeable glucosinolate type structure was described. Interestingly this was neither a substrate nor an inhibitor of myrosinase and therefore revealed little about the enzyme. However it would still be of interest to prepare better analogues such as compounds **202** and **203** (figure 44). It would also be of interest to prepare an oxygen version. In this case the aglycone would be a slightly poorer leaving group. Furthermore synthesis of a glucosinolate in the E configuration as described in section 2.2.4 would be informative.

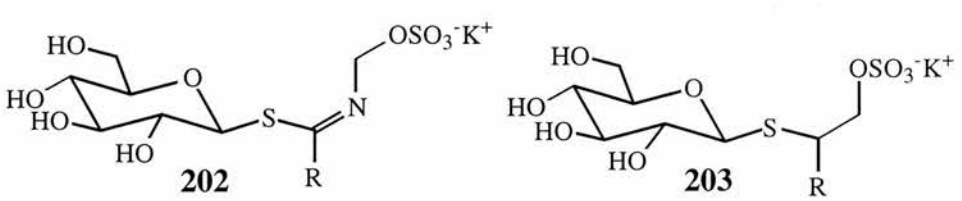


Figure 44: *Alternative non-rearrangeable glucosinolate analogues*

Results appear to show that the rearrangement is indeed spontaneous. However there is still much unknown about the myrosinase mechanism in terms of the identity of the rate limiting step. This leaves scope for much future research including measurement of kinetic isotope effects for myrosinase catalysed reactions.

Experimental

3.1 Experimental Details

Melting points were determined using an electrothermal melting point or Reichert hot-stage microscope apparatus and are uncorrected.

Microanalyses and atomic absorption were carried out by the University of St. Andrews microanalytical laboratory.

Optical rotations were measured at room temperature using an Optical Activity Ltd. AA 1000 polarimeter with 20 cm path-length cells.

Infra-red spectra were recorded on a Perkin-Elmer series 1420 IR spectrophotometer or FT-IR Paragon 1000 spectrophotometer. The samples were prepared as either Nujol mulls or thin films between sodium chloride discs. Absorption maxima are given in wavenumbers relative (cm^{-1}) to a polystyrene standard.

N.m.r. spectra were recorded on a Varian Gemini 200 f.t. spectrophotometer (^1H, 200 MHz; ^{13}C, 50.31 MHz), a Bruker AM 300 f.t. spectrophotometer (^1H, 300 MHz; ^{13}C, 75.4 MHz) or a Varian Unity+ spectrometer (^1H, 500.3 MHz; ^{13}C, 125.8 MHz). Spectra were referenced relative to TMS, chloroform, DMSO, methanol or D_2O. N.m.r. spectra are described in parts per million downfield shift from TMS and are described consecutively as position (δ_H or δ_C), relative integral, multiplicity (s-singlet, d-doublet, t-triplet, dd-doublet of doublets and m-multiplet), coupling constant ($J_{x,y}$ Hz if applicable) and assignment.

Low resolution and high resolution mass spectra were recorded on an A.E.I. MS-902 spectrometer using electron impact (EI) at 70 eV or were obtained on an E.P.S.R.C. service basis based at the University of Swansea using a VG ZAB E. Low and high resolution chemical ionisation (CI) mass spectra were recorded on a VG Autospec using

*iso*butane as the ionising gas. Fast atom bombardment (FAB) was carried out using a 3-nitrobenzyl alcohol matrix. Low resolution spectra were also obtained using a VG Platform electrospray mass spectrometer with VG Masslynx software. Samples were filtered prior to analysis using 4 mm syringe filters (0.45 μm pore size) obtained from Whatman. Major fragments are given as percentages of the base peak intensity.

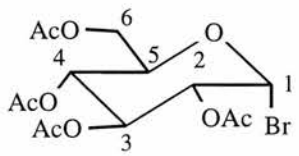
HPLC and LC-APCI-MS were performed at the Scottish Crop Research Institute at Invergowrie. Both analytical and semi-preparative HPLC were undertaken using a Gilson dual pump chromatograph (Anachen Ltd, Luton, UK) with UV detection (230 nm). In analytical mode a Hypersil C18 column (250 x 4.5 mm i.d., 5 μl, Jones Chromatography, Hengoed, Wales, UK) was fitted and an elution profile based on that of Spinks[162] was adopted. The two solvent system consisting of (A) distilled water and (B) 20% v/v acetonitrile (Far UV grade) was set to vary linearly from 1% to 100% over a period of 46 minutes. Over the next 5 minutes the concentration of B was reduced to 1% and then held at this value for a further 9 minutes. Flow rate was maintained at 1.5 ml min^{-1} and the resulting peaks integrated using a Gilson 715 (Anachen Ltd., Luton, UK) integration and control software. For quantification the response factor for each desulfoglucosinolate was determined by the glucose release method of McGregor.[247] All LC-MS was performed on a Finnigan MAT SSQ 710C single quadrupole instrument with an APCI interface (Thermoquest, Hemel Hempstead, UK). The instrument was configured for positive ion chemical ionisation with a coronal discharge of ~5 μA. The samples were loaded via a 5 μl loop onto a reversed phase Ultracarb C18 HPLC column (150 x 2 mm i.d., Phenomenex, Macclesfield, UK) and eluted at 0.25 ml min^{-1} with a water (A) and 20% aqueous acetonitrile (B) gradient, which was set to increase linearly from 1% to 100% over 90 minutes. Full scan spectra were acquired over the range 250 to 410 *m/z* with a scan time of 1.5 seconds. Flow injection analyses were performed with and without collision induced dissociation voltages to induce fragmentation.

UV spectra assays were measured on a Kontron UVICON 932 scanning spectrophotometer fitted with a Grant F15 flow heater and flow cooler.

All solvents were dried according to the methods of Perrin and Armarego or Vogel.[248, 236] Flash chromatography was performed according to the procedure of Still[249] using Sorbisil C60 (40-60 mm mesh) silica gel. Ion-exchange chromatography was carried out using DEAE Sephadex (Sigma). Reverse phase chromatography was performed using C18 reverse phase silica for flash (Baker Bond). Size exclusion chromatography used G10 Sephadex (Sigma, bead size 40-120 μ). Analytical thin layer chromatography was carried out on 0.25 mm precoated silica gel plates (Whatman PE SIL G/UV_{254}) or on precoated reverse-phase silica plates (Merck, RP-18 F_{254s}). Compounds were visualised by UV fluorescence, iodine vapour, aqueous potassium permanganate or 5% sulfuric acid in aqueous ethanol/charring.

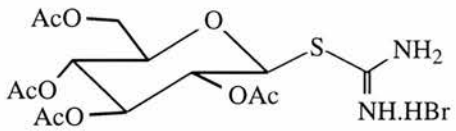
3.2 Gluconasturtiins

2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide (65)



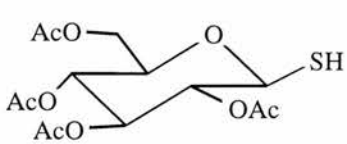
45% w/v Hydrogen bromide in acetic acid (60 ml) was added dropwise to D-glucose (39.0 g, 216 mmol) in acetic anhydride (150 ml) at 0 °C under a nitrogen balloon. After 4 hours further 45% w/v hydrogen bromide in acetic acid (180 ml) was added and the solution stirred at room temperature overnight. The reaction mixture was taken up in dichloromethane (300ml) and poured onto ice/water (500 ml). The organic layer was then added carefully to an ice/saturated sodium hydrogen carbonate solution (600 ml) with stirring. Once the gas evolution became less vigorous the organic phase was added to saturated sodium hydrogen carbonate solution (600 ml). The organic layer was dried ($MgSO_4$) and the solvent evaporated at reduced pressure to give a golden oil which solidified upon cooling to 0 °C. The product was recrystallised from diethyl ether to give 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide as a white crystalline solid (84.9 g, 96%) (the product is heat labile and should be stored below 0 °C); m.p. 86-87 °C (lit.,[250] 88-89 °C); $[\alpha]_D$ +195.9 ° (c 2.42 in $CHCl_3$) (lit.,[251] +197.84 ° (c 2.42 in $CHCl_3$)); ν_{max} (nujol)/cm^{-1} 1730 (CO); δ_H (200 MHz; C^2HCl_3) 1.90-2.20 (12H, 4s, 4 x OC(O)C$\underline{H}_3$), 4.15 (1H, m, H-5), 4.30 (2H, m, H-6a, 6b), 4.85 (1H, dd, $J_{1,2}$ 5 Hz, $J_{2,3}$ 10 Hz, H-2), 5.15 (1H, t, $J_{3,4}$ 10 Hz, $J_{4,5}$ 10 Hz, H-4), 5.55 (1H, t, $J_{2,3}$ 10 Hz, $J_{3,4}$ 10 Hz, H-3), 6.60 (1H, d, $J_{1,2}$ 5 Hz, H-1); δ_C (50.3 MHz; C^2HCl_3) 21.0 & 21.1 (4 x OC(O)$\underline{C}H_3$), 61.4 (C-6), 67.6 (C-4), 70.6 (C-2), 71.0 (C-3), 72.6 (C-5), 86.6 (C-1), 169.9, 170.2, 170.3, 170.9 (4 x O$\underline{C}$(O)CH_3); *m/z* (CI) 428, 430 ($[M + NH_4]^+$, 18%), 331 (8, $[M-Br]^+$) and 213 (29, $[M-Br-2OAc]^+$).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosylisothiouronium bromide (66)



Thiourea (5.4 g, 70.5 mmol) was added to 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (29.0 g, 70.5 mmol) in dry acetone (65 ml) under nitrogen. The solution was heated at reflux for 15 minutes then cooled to 0 °C to give a white precipitate. The precipitate was removed by filtration and recrystallised from acetone to give 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylisothiouronium bromide as a white crystalline solid (17.5 g, 51%); m.p. 200 °C decomposes (lit.[252] 205 °C); $[\alpha]_D$ -17.1° (c 1.0 in MeOH) (lit.,[253] -20.2° (c 1.0 in MeOH)); ν_{max} (nujol)/cm^{-1} 3310-3160 (NH), 1750 (CO) and 1655 (NH); δ_H (200 MHz; ^2H_2O) 2.10 (12H, 4s, 4 x OC(O)C$\underline{H}_3$), 4.19 (1H, m, H-5), 4.34 (2H, m, H-6a, 6b), 5.10-5.60 (4H, m, H-1, 2, 3, 4); δ_C (50.3 MHz; ^2H_2O) 14.9, 18.0, 18.1, 18.2 (4 x OC(O)$\underline{C}H_3$), 59.9 (C-6), 65.7 (C-4), 67.2 (C-2), 71.4 (C-3), 73.9 (C-5), 79.2 (C-1), 170.3, 170.5, 170.7, 171.0, 171.7 (C=N, 4 x O$\underline{C}$(O)CH_3); *m/z* (EI) 331 ([M-CH_4BrN_2S]$^+$, 11%), 169 (35, $[C_8H_9O_4]^+$ and 127 (18, $[C_6H_7O_3]^+$).

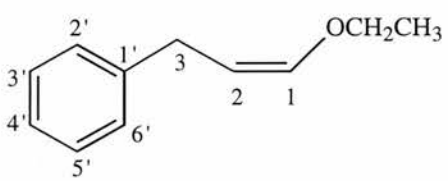
2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (67)



Potassium metabisulfite (1.15 g, 6.0 mmol) was dissolved in water (15 ml) and heated to 75 °C. Dichloromethane (20 ml) was added carefully followed by 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylisothiouronium bromide (3.0 g, 6.0 mmol). The biphasic solution was heated under reflux for 15 minutes then cooled to room temperature. The organic phase was washed with water (3 x 15 ml) then the aqueous layer washed with dichloromethane (20 ml). The combined organic layers were dried ($MgSO_4$) and the solvent evaporated at

reduced pressure to give a white solid. The product was recrystallised from methanol to give 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose as a white crystalline solid (2.2 g, 100%); m.p. 74-75 °C (lit.[202] 75 °C); $[\alpha]_D$ -9.8 ° (c 1.5 in EtOH) (lit.,[254] -8.3 ° (c 1.5 in EtOH)); ν_{max} (nujol)/cm^{-1} 3460 (SH), 1735 (CO); δ_H (200 MHz; C^2HCl_3) 2.00-2.10 (12H, 4s, 4 x OC(O)C$\underline{H}_3$), 3.70 (1H, m, H-5), 4.13 (1H, dd, $J_{5,6a}$ 2.5, $J_{6a,6b}$ 12.3, H-6a), 4.26 (1H, dd, $J_{5,6b}$ 4.8, $J_{6a,6b}$ 12.3, H-6b), 4.55 (1H, t, $J_{1,2}$ 9.4, H-1), 4.98 (1H, t, $J_{1,2}$ 9.4, $J_{2,3}$ 9.4, H-2), 5.09 (1H, t, $J_{3,4}$ 9.4, $J_{4,5}$ 9.4, H-4), 5.18 (1H, t, $J_{2,3}$ 9.4, $J_{3,4}$ 9.4, H-3); δ_C (50.3 MHz; C^2HCl_3) 21.0, 21.2 (4 x OC(O)$\underline{C}H_3$), 62.4 (C-6), 68.5 (C-4), 74.0 (C-2), 76.8 (C-3), 77.5 (C-5), 79.1 (C-1), 169.8, 170.1, 170.6, 171.1 (4 x O$\underline{C}$(O)CH_3); *m/z* (CI) 382 ($[M+NH_4]^+$, 100%), 331 (7, $[M-SH]^+$) and 322 (22, $[M-OAc]^+$).

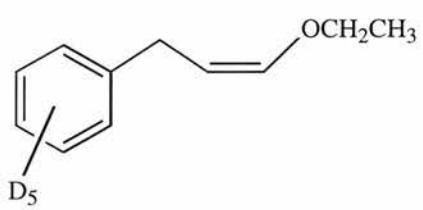
1-Ethoxy-3-phenylprop-1-ene (78)



Bromobenzene (1.0 g, 6.4 mmol) was added to magnesium turnings (1.63 g, 63.25 mmol) and iodine (1 crystal) in dry diethyl ether (10 ml). The reaction was warmed slightly until the iodine colour had disappeared. The remaining dry diethyl ether (20 ml) and bromobenzene (8.0 g, 51.1 mmol) were added dropwise and the reaction stirred for 1 hour. The phenyl magnesium bromide was added dropwise to acrolein diethylacetal (5.0 g, 5.85 ml, 38.4 mmol) and copper (I) bromide (0.275 g, 2.0 mmol) in dry tetrahydrofuran (50 ml) at room temperature. The reaction was exothermic and turned purple in colour on completion. A solution of saturated aqueous ammonium chloride (50 ml) was added before the organic layer was separated, dried ($MgSO_4$) and the solvent evaporated at reduced pressure. A pale yellow oil of 1-ethoxy-3-phenylprop-1-ene was given as a mixture of *E* and *Z* isomers (ratio 1:1.4) in quantitative yield (6.2 g, 100%); δ_H

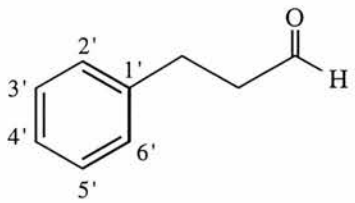
(200 MHz; C^2HCl_3) 1.40 (6H, m, CH_3 *E* and *Z*), 3.40 (2H, dd, $J_{3,2}$ 7, $J_{3,1}$ 2.5, H-3 *E*), 3.70 (2H, dd, $J_{3,2}$ 7, $J_{3,1}$ 2.5, H-3 *Z*), 3.80-4.00 (4H, m, $C\underline{H}_2CH_3$ *E* and *Z*), 4.75 (1H, m, H-2 *Z*), 5.10 (1H, m, H-2 *E*), 6.20 (1H, 2t, $J_{2,1}$ 7, $J_{3,1}$ 2.5, H-1 *Z*), 6.50 (1H, 2t, $J_{2,1}$ 13, $J_{3,1}$ 2.5, H-1 *E*), 7.40 (5H, m, Phenyl); δ_C (50.3 MHz; C^2HCl_3) 15.4 (CH_3, *E*), 16.0 (CH_3, *Z*), 30.9 (C-3, *Z*), 34.7 (C-3, *E*), 65.1 ($\underline{C}H_2CH_3$, *E*), 68.3 ($\underline{C}H_2CH_3$, *Z*), 103.4 (C-2, *Z*), 106.1 (C-2, *E*), 126-129.0 (phenyl *E* and *Z*), 146.0 (C-1, *Z*), 147.8 (C-1, *E*); *m/z* (EI) 162 ($[M]^+$, 36%), 133 (13, $[M\text{-}C_2H_5]^+$), 117 (33, $[M\text{-}C_2H_5O]^+$), 103 (92, $[PhCH_2C]^+$) and 91 (100, $[PhCH_2]^+$).

1-Ethoxy-3-[$^2H_5$]phenylprop-1-ene (69)



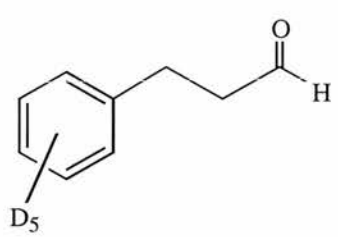
As described for (78) using d_5-bromobenzene. The product was purified by distillation at reduced pressure. A clear oil of 1-ethoxy-3-[$^2H_5$]phenylprop-1-ene was given as a mixture of *E* and *Z* isomers (ratio 1:1.5) (6.4 g, 100%); b.p. 100 °C/0.8 mmHg; δ_H (200 MHz; C^2HCl_3) 1.40 (6H, t, *J* 7, CH_3 *E* and *Z*), 3.40 (2H, dd, $J_{3,2}$ 7, $J_{3,1}$ 2.5, H-3 *E*), 3.70 (2H, dd, $J_{3,2}$ 7, $J_{3,1}$ 2.5, H-3 *Z*), 3.85 (2H, q, *J* 7, $C\underline{H}_2CH_3$ *E*), 4.00 (2H, q, *J* 7, $C\underline{H}_2CH_3$ *Z*), 4.75 (1H, m, H-2 *Z*), 5.10 (1H, m, H-2 *E*), 6.20 (1H, 2t, $J_{2,1}$ 7, $J_{3,1}$ 2.5, H-1 *Z*), 6.50 (1H, 2t, $J_{2,1}$ 13, $J_{3,1}$ 2.5, H-1 *E*); δ_C (50.3 MHz; C^2HCl_3) 15.4 (CH_3, *E*), 16.0 (CH_3, *Z*), 30.9 (C-3, *Z*), 34.7 (C-3, *E*), 65.1 ($\underline{C}H_2CH_3$, *E*), 68.3 ($\underline{C}H_2CH_3$, *Z*), 103.4 (C-2, *Z*), 106.1 (C-2, *E*), 146.0 (C-1, *Z*), 147.8 (C-1, *E*); *m/z* (EI) 167 ($[M]^+$, 87%), 138 (51, $[M\text{-}C_2H_5]^+$), 110 (100, $[M\text{-}C_2H_5OC]^+$), 96 (29, $[C_6{}^2H_5CH_2]^+$) and 83 (20, $[C_6{}^2H_5H]^+$).

3-Phenylpropionaldehyde (80)



Two drops of concentrated hydrochloric acid were added to a mixture of acetone (8 ml), water (2 ml) and 1-ethoxy-3-phenylprop-1-ene (0.25 g, 1.54 mmol). The reaction was heated at reflux for 1 hour then the solvent was evaporated at reduced pressure to give the crude 3-phenylpropionaldehyde as a pale yellow oil (0.15 g, 73%); ν_{max} (thin film)/cm^{-1} 1720 (C=O); δ_H (200 MHz; C^2HCl_3) 2.80 (2H, t, *J* 7, PhC$\underline{H}_2$), 3.00 (2H, t, *J* 7, C$\underline{H}_2$CHO), 7.20-7.40 (5H, m, phenyl), 9.80 (1H, s, C$\underline{H}$O); δ_C (50.3 MHz; C^2HCl_3) 28.6 (Ph$\underline{C}H_2$) 45.8 ($\underline{C}H_2$CHO), 126.9 (C-4'), 128.9 (C-2', 6'), 129.2 (C-3', 5'), 141.0 (C-1') and 202.1 ($\underline{C}$HO); *m/z* (EI) 134 ([M]$^+$, 42%), 104 (35, [M-CH_2O]$^+$) and 91 (100, [$PhCH_2$]$^+$).

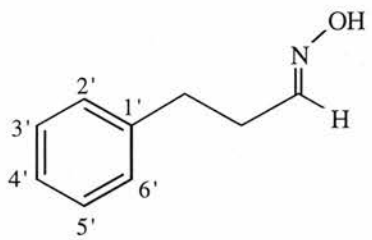
3-[$^2H_5$]Phenylpropionaldehyde (70)



As described for (80) using 1-ethoxy-3-[$^2H_5$]phenylprop-1-ene (5.15 g, 3.08 mmol). The product was purified by distillation at reduced pressure to give 3-[$^2H_5$]-phenylpropionaldehyde as a clear oil (3.74 g, 87%); b.p. 110 °C/0.8 mmHg; ν_{max} (thin film)/cm^{-1} 1720 (C=O); δ_H (200 MHz; C^2HCl_3) 2.80 (2H, t, *J* 7, PhC$\underline{H}_2$), 3.00 (2H, t, *J* 7, C$\underline{H}_2$CHO), 9.80 (1H, s, C$\underline{H}$O); δ_C (50.3 MHz; C^2HCl_3) 28.6 (Ph$\underline{C}H_2$) 45.8

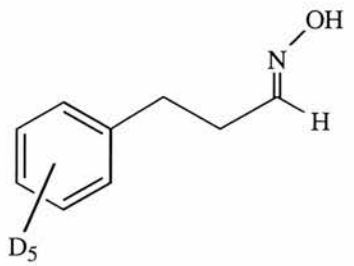
($\underline{C}H_2CHO$), 141.0 (C-1'), 202.1 ($\underline{C}HO$); *m/z* (EI) 139 ($[M]^+$, 91%), 110 (47, $[M-CHO]^+$), 96 (100, $[M-CH_2CHO]^+$) and 83 (45, $[C_6{}^2H_5H]^+$).

3-Phenylpropionaldehyde oxime (81)



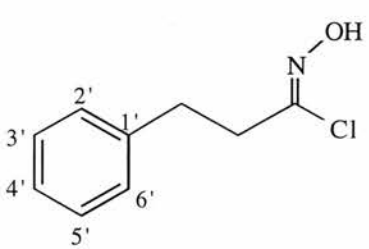
To a solution of sodium acetate trihydrate (5 g), hydroxylamine hydrochloride (3 g) and water (30 ml) was added 3-phenylpropionaldehyde (3.27 ml, 22.36 mmol) and a minimum volume of ethanol to dissolve the aldehyde. The reaction was warmed to 70 °C for 30 minutes with vigorous stirring then cooled to 0 °C to allow the product to crystallise from the solution. The product was removed by filtration, washed with ice-cold water and dried in a desiccator. The 3-phenylpropionaldehyde oxime was given as a mixture of *E* and *Z* isomers (ratio 1:1) and is a white crystalline solid (3.09 g, 93%); m.p. 78-82 °C (lit.,[255] 93-94.5 °C); (Found: C, 72.23; H, 7.94; N, 9.35. Calc for $C_9H_{11}NO$: C, 72.46; H, 7.43; N, 9.39%); ν_{max} (nujol)/cm^{-1} 3200-3110 (OH), 1660 (C=N), 1760 and 700 (monosubstituted phenyl); δ_H (300 MHz; C^2HCl_3) 2.50 (2H, m, $C\underline{H}_2CHN$, *E*), 2.70 (2H, m, $C\underline{H}_2CHN$, *Z*), 2.80-2.90 (4H, m, $PhC\underline{H}_2$, *E* and *Z*), 6.80 (1H, t, *J* 3.5, $C\underline{H}$=N, *Z*), 7.50 (1H, t, *J* 7, $C\underline{H}$=N, *E*), 7.20 (5H, m, Phenyl), 7.70 (1H, s, OH); δ_C (75.45 MHz; C^2HCl_3) 27.2 ($\underline{C}H_2CHN$, *Z*), 31.8 ($\underline{C}H_2CHN$, *E*), 32.3 ($Ph\underline{C}H_2$, *Z*), 33.2 ($Ph\underline{C}H_2$, *E*), 126.0 (C-4'), 129.0 (C-3', 5'), 129.2 (C-2', 6'), 141.2 (C-1', *E*), 141.4 (C-1', *Z*), 151.8 ($\underline{C}H$=N, *E*), 152.1 ($\underline{C}H$=N, *Z*); *m/z* (EI) 149 ($[M]^+$, 10%), 117 (23, $[M-NOH_2]^+$), 104 (51, $[M-CH_2NOH]^+$) and 91 (100, $[PhCH_2]^+$).

3-[$^2H_5$]Phenylpropionaldehyde oxime (71)



As described for (81) using 3-[$^2H_5$]phenylpropionaldehyde (2.0 g, 14.37 mmol). The product was recrystallised from toluene to give a mixture of *E* and *Z* isomers (ratio 1:1) as a white crystalline solid (1.10 g, 49%); m.p. 85-87 °C; (Found: C, 70.02; H, 7.25; N, 8.98. Calc for $C_9H_6{}^2H_5NO$: C, 70.09; H, 7.19; N, 9.08%); ν_{max} (nujol)/cm^{-1} 3200-3110 (OH); δ_H (300 MHz; C^2HCl_3) 2.50 (1H, t, *J* 7, PhC$\underline{H}_2$ *E*), 2.60 (1H, t, *J* 3.5, PhC$\underline{H}_2$ *Z*), 2.70-2.90 (2H, m, C$\underline{H}_2$CHN, *E* and *Z*), 6.70 (1H, s, *J* 3.5, C$\underline{H}$=N *Z*), 7.40 (1H, t, *J* 7, C$\underline{H}$=N *E*); δ_C (75.45 MHz; C^2HCl_3) 27.1 ($\underline{C}H_2$CHN, *Z*), 31.8 ($\underline{C}H_2$CHN, *E*), 32.3 (Ph$\underline{C}H_2$, *Z*), 33.2 (Ph$\underline{C}H_2$, *E*), 141.2 (C-1', *E*), 141.4 (C-1', *Z*), 151.7 ($\underline{C}$H=N, *E*), 151.8 ($\underline{C}$H=N, *Z*); *m/z* (EI) 154 ([M]$^+$, 10%), 121 (13, [M-NH_2OH]$^+$), 109 (37, [M-CH_2NOH]$^+$), 96 (100, [$C_6{}^2H_5CH_2$]$^+$) and 82 (6, [$C_6{}^2H_5$]$^+$).

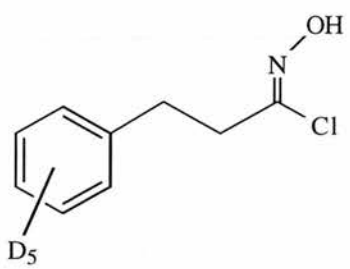
3-Phenylpropionaldehyde oximyl chloride (85)



3-Phenylpropionaldehyde oxime (1.0 g, 6.7 mmol) was dissolved in chloroform (15 ml) and dry pyridine (0.27 ml, 3.35 mmol) then *N*-chlorosuccinimide (0.9 g, 6.7 mmol) was added slowly at 0 °C. After 30 minutes the reaction was poured onto ice water (20 ml) and extracted with diethyl ether (2 x 40 ml). The combined organic layers were washed with

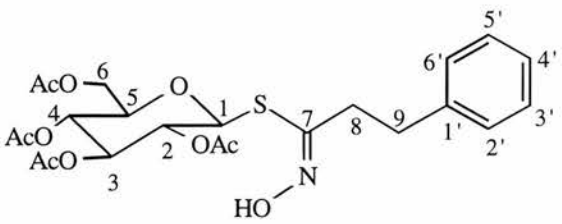
water (2 x 20 ml), dried ($MgSO_4$) and the solvent evaporated at reduced pressure to give a white solid (0.787 g, 64%); m.p. 69-72 °C (lit.,[184] 69-71 °C); ν_{max} (nujol)/cm^{-1} 3200 (OH); δ_H (200 MHz; C^2HCl_3) 2.80 (2H, m, PhC$\underline{H}_2$), 2.90 (2H, m, C$\underline{H}_2$CClN), 7.10-7.30 (5H, m, Phenyl), 8.35 (1H, s, OH); δ_C (50.3 MHz; C^2HCl_3) 33.0 (Ph$\underline{C}H_2$), 38.9 ($\underline{C}H_2$CClN), 126.8 (C-4'), 128.9 (C-3', 5'), 129.0 (C-2', 6'), 144.0 (C-1'), 146.0 (C=N); *m/z* (EI) 184 ($[MH]^+$, 31%), 130 (100, $[M\text{-}ClOH_2]^+$), 91 (30, $[PhCH_2]^+$) and 80 (94, $[CHClNOH_2]^+$).

3-[$^2H_5$]Phenylpropionaldehyde oximyl chloride (72)



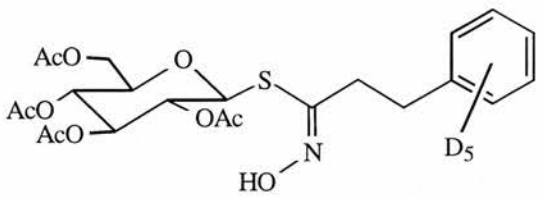
As described for (85) using 3-[$^2H_5$]phenylpropionaldehyde oxime (1.03 g, 6.7 mmol). The product was given as a viscous pale-yellow oil (1.23 g, 97%); ν_{max} (film)/cm^{-1} 3200 (OH); δ_H (200 MHz; C^2HCl_3) 2.80 (2H, m, PhC$\underline{H}_2$), 2.90 (2H, m, C$\underline{H}_2$CClN), 8.35 (1H, s., OH); δ_C (50.3 MHz; C^2HCl_3) 33.0 (Ph$\underline{C}H_2$), 38.9 ($\underline{C}H_2$CClN), 144.0 (C-1'), 146.0 (C=N).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl phenethyl thiohydroximate (86)



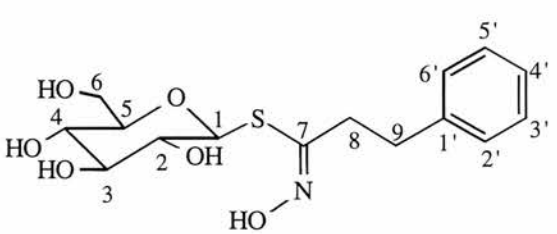
A solution of 3-phenylpropionaldehyde oximyl chloride (0.8 g, 4.35 mmol) was added to a solution of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (0.68 g, 4.35 mmol) in dry tetrahydrofuran (150 ml) under nitrogen with stirring. Dry triethylamine (5.73 ml, 40.95 mmol) was added and the reaction stirred for 18 hours before the solvent was evaporated at reduced pressure. The residue was taken up in diethyl ether (50 ml) and washed with 1 M sulfuric acid (50 ml). The acid layer was extracted with ethyl acetate (20 ml) then the combined organic extracts were dried ($MgSO_4$) and the solvent evaporated at reduced pressure. The resulting solid was purified by column chromatography on silica gel using ethyl acetate-hexane (1:1) as the eluant to give a white amorphous solid (0.66 g, 31%); m.p. >150 °C decomposes (lit.,[184] 198.0 °C); $[\alpha]_D$ +16.4 ° (c 1.0 in $CHCl_3$) (lit.,[184] +11.1 ° (c 0.2 in CCl_4)); (Found: C, 54.30; H, 5.81; N, 2.86. Calc for $C_{23}H_{29}NO_{10}S$: C, 54.00; H, 5.71; N, 2.74%); ν_{max} (nujol)/cm^{-1} 3300 (OH), 1750 (C=O); δ_H (200 MHz; C^2HCl_3) 1.90-2.20 (4 x 3H, 4s, 4 x OC(O)C$\underline{H}_3$), 2.75-2.90 (2H, m, H-8), 2.90-3.00 (2H, m, H-9), 3.70 (1H, m, H-5), 4.10 (2H, m, H-6a, 6b), 4.95-5.15 (3H, m, H-1, 2, 4), 5.25 (1H, t, *J* 10, H-3), 7.30 (5H, m, phenyl); δ_C (50.3 MHz; C^2HCl_3) 21.0-21.3 (4 x OC(O)$\underline{C}H_3$), 33.7 (CH_2), 34.7 (CH_2), 62.8 (C-6), 68.4 (C-4), 70.5 (C-2), 74.1 (C-3), 76.5 (C-5), 80.2 (C-1), 127.1 (C-4'), 128.7 (C-3', 5'), 129.1 (C-2', 6'), 140.9 (C-1'), 152.2 (C=N), 169.8, 169.9, 170.8, 171.2 (4 x O$\underline{C}$(O)CH_3); *m/z* (EI) 511 ($[M]^+$, 3%), 494 (8, $[M-OH]^+$), 331(79, [M-Aglycone]$^+$), 271 (27, [M-Aglycone-AcOH]$^+$), 169 (96, $[C_8H_9O_4]^+$) and 109 (73, $[PhCH_2+OH_2]^+$).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-[$^2H_5$]phenethyl thiohydroximate (73)



As described for (86) using 3-[$^2H_5$]phenylpropionaldehyde oximyl chloride (1.23 g, 6.5 mmol). The product was given as a white amorphous solid (1.24 g, 37%); m.p. >150 °C decomposes; $[\alpha]_D$ +24.2 ° (c 1.0 in $CHCl_3$); (Found: C, 52.62; H, 5.42; N, 2.60. Calc for $C_{23}H_{24}{}^2H_5NO_{10}S.0.5H_2O$: C, 52.56; H, 5.00; N, 2.66%); ν_{max} (nujol)/cm^{-1} 3300 (OH), 1750 (C=O); δ_H (200 MHz; C^2HCl_3) 1.90-2.20 (4 x 3H, 4s, 4 x OC(O)C$\underline{H}_3$), 2.75-2.9 (2H, m, H-8), 2.90-3.00 (2H, m, H-9), 3.70 (1H, m, H-5), 4.10 (2H, m, H-6a, 6b), 4.95-5.15 (3H, m, H-1, 2, 4), 5.25 (1H, t, *J* 10, H-3), 8.20 (1H, s, OH); δ_C (50.3 MHz; C^2HCl_3) 21.0-21.3 (4 x OC(O)$\underline{C}H_3$), 33.7 (CH_2), 34.7 (CH_2), 62.8 (C-6), 68.4 (C-4), 70.5 (C-2), 74.1 (C-5), 76.5 (C-3), 80.2 (C-1), 140.9 (C-1'), 153.2 (C-7), 169.8, 169.9, 170.8, 171.2 (4 x O$\underline{C}$(O)CH_3); *m/z* (CI) 519 ($[M+H_3]^+$, 2.0%), 518 (1.5, $[M+H_2]^+$), 517 (3.0, $[MH]^+$), 331 (94, [M-Aglycone]$^+$), 271 (59, [M-Aglycone-AcOH]$^+$), 169 (73, $[C_8H_9O_4]^+$), 155 (93, [AglyconeH_2-S]$^+$), 137 (100, $[C_6{}^2H_5CH_2CH_2CNH]^+$) and 96 (36, $[C_6{}^2H_5CH_2]^+$).

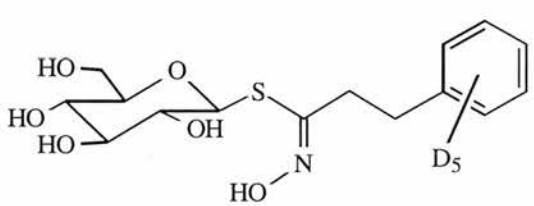
Desulfogluconasturtiin (87)



To 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl phenethyl thiohydroximate (0.5 g, 0.98 mmol) in methanol was added a catalytic amount of potassium metal under nitrogen. The

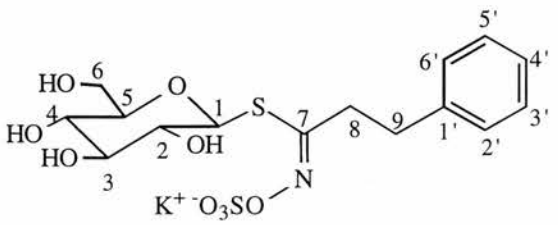
reaction was stirred for 18 hours then Amberlite IR-120 resin was added. Stirring was continued for a further 15 minutes before the Amberlite was removed by filtration and the solvent evaporated at reduced pressure to give a white amorphous solid (0.23 g, 70%); m.p. 50-53.5 °C; $[\alpha]_D$ -49.0 ° (c 1.0 in CH_3OH); (Found: C, 51.45; H, 6.15; N, 3.91. Calc for $C_{15}H_{21}NO_6S.0.5H_2O$: C, 51.12; H, 6.29; N, 3.97%); ν_{max} (nujol)/cm^{-1} 3300 (OH); δ_H (200 MHz; $C^2H_3O^2H$) 2.95-3.00 (4H, m, $C\underline{H}_2C\underline{H}_2$), 3.30-3.50 (4H, m, H-2, 3, 4, 5), 3.65 (1H, dd, $J_{5,6b}$ 5, J_{gem} 12.5, H-6b), 3.85 (1H, dd, $J_{5,6a}$ 2.5, J_{gem} 12.5, H-6a), 4.85 (1H, d, $J_{1,2}$ 10, H-1), 7.25 (5H, s, phenyl); δ_C (50.3 MHz; $C^2H_3O^2H$) 35.1 (CH_2), 35.5 (CH_2), 62.9 (C-6), 71.4 (C-4), 74.7 (C-2), 79.9 (C-3), 82.5 (C-5), 83.6 (C-1), 127.5 (C-4'), 129.8 (C-2', 3', 5', 6'), 142.7 (C-1'), 154.4 (C=N); *m/z* (CI) 344 ($[M+H]^+$, 12.0%), 182 (22, $[AglyconeH_2]^+$), 166 (54, $[glucoseH_2\text{-}O]^+$), 150 (75, $[AglyconeH_2\text{-}S]^+$), 145 (40, $[C_6H_9O_4]^+$) and 132 (100, $[PhCH_2CH_2CNH]^+$).

Desulfo-[$^2H_5$]gluconasturtiin (74)



As described for (87) using 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-[$^2H_5$]phenethyl thiohydroximate (1.116 g, 2.16 mmol). The product was given as a white amorphous solid (0.71 g, 94%); m.p. 89-92 °C; $[\alpha]_D$ -52.0 ° (c 1.0 in CH_3OH); (Found: C, 49.55; H, 5.59; N, 3.75. Calc for $C_{15}H_{16}{}^2H_5NO_6S.H_2O$: C, 49.17; H, 5.78; N, 3.82%); ν_{max} (nujol)/cm^{-1} 3300 (OH); δ_H (200 MHz; $C^2H_3O^2H$) 2.95-3.00 (4H, m, CH_2CH_2), 3.30-3.50 (4H, m, H-2, 3, 4, 5), 3.65 (1H, dd, $J_{5,6b}$ 5, J_{gem} 12.5, H-6b), 3.85 (1H, dd, $J_{5,6a}$ 2.5, J_{gem} 12.5, H-6a), 4.85 (1H, d, $J_{1,2}$ 10, H-1); δ_C (50.3 MHz; $C^2H_3O^2H$) 35.4 (CH_2), 35.8 (CH_2), 62.9 (C-6), 71.4 (C-4), 74.8 (C-2), 79.8 (C-3), 82.4 (C-5), 84.1 (C-1), 142.7 (C-1'), 152.8 (C=N); *m/z* (FAB) 371 ($[MNa]^+$, 21%) and 349 (3, $[M+H]^+$).

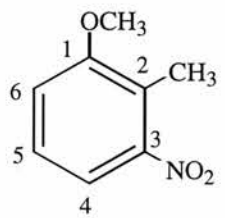
Gluconasturtiin (89)



Dry pyridine (50 ml, 618.5 mmol) and dry dichloromethane (50 ml) were cooled to 0 °C under nitrogen and chlorosulfonic acid (2.3 ml, 33.5 mmol) in dry dichloromethane (50 ml) was added very carefully over a period of 30 minutes. A solution of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl phenethyl thiohydroximate (1.07 g, 2.07 mmol) in dry dichloromethane (25 ml) was added and the reaction stirred for 24 hours at room temperature. Saturated potassium hydrogen carbonate (50 ml) was added and the biphasic mixture stirred for a further 30 minutes. The reaction mixture was evaporated at reduced pressure to give gluconasturtiin as a white solid which was purified using reverse phase C-18 silica then desalted using G-10 Sephadex. In both cases distilled water was used as the eluant. The product was freeze dried to give the pure gluconasturtiin as an extremely hygroscopic white solid (0.24 g, 18%); $[\alpha]_D$ -16.8 (c 0.5 in H_2O) (lit.,[184] -20.7 ° (c 1.0 in H_2O)); (Found: C, 39.28; H, 4.74; N, 3.02. Calc for $C_{15}H_{20}NO_9S_2K$: C, 39.04; H, 4.37; N, 3.03%); ν_{max} (nujol)/cm^{-1} 3600-3000(OH); δ_H (300 MHz; ^2H_2O) 2.80-3.00 (4H, m, CH_2CH_2), 3.20-3.40 (4H, m, H-2, 3, 4, 5), 3.50 (1H, dd, $J_{5,6b}$ 5, J_{gem} 12.5, H-6b), 3.70 (1H, dd, $J_{5,6a}$ 2.5, J_{gem} 12.5, H-6a), 4.85 (1H, d, $J_{1,2}$ 10, H-1), 7.2 (5H, m, C_6H_5); δ_C (75.45 MHz; ^2H_2O) 32.5, 33.8 ($2CH_2$), 60.6 (C-6), 69.1 (C-4), 71.9 (C-2), 77.1 (C-3), 80.1 (C-5), 81.7 (C-1), 126.7 (C-4'), 128.8 (C-3', 5'), 128.9 (C-2', 6'), 140.7 (C-1'), 163.5 (C=N); *m/z* (ES^-) 422.07 ($[M-K]^-$, 100%).

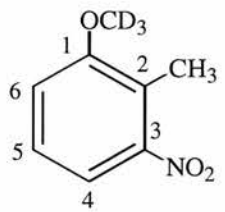
3.3 Glucobrassicins

2-Methyl-3-nitromethoxybenzene (98)



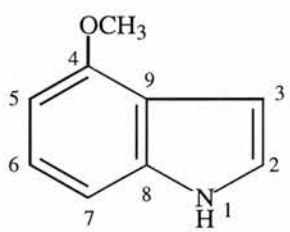
Powdered potassium hydroxide (0.79 g, 14 mmol) was added to dimethyl sulfoxide (7 ml, 98.7 mmol) and stirred for 5 minutes. 2-Methyl-3-nitrophenol (0.54 g, 3.5 mmol) and methyl iodide (0.436 ml, 7.0 mmol) were added in quick succession and the reaction stirred for 15 minutes. On completion the solution was poured into water (70 ml) and extracted with dichloromethane (3 x 70 ml). The combined organic layers were washed with water (5 x 35 ml), dried ($MgSO_4$) and the solvent evaporated at reduced pressure. The pale yellow oil crystallised on standing (0.54 g, 92%); m.p. 51-52 °C (lit.,[256] 52-53 °C); δ_H (200 MHz; C^2HCl_3) 2.34 (3H, s, C<u>H</u>$_3$), 3.87 (3H, s, OC<u>H</u>$_3$), 7.03 (1H, d, *J* 6, H-6), 7.25 (1H, t, *J* 6, H-5), 7.38 (1H, d, *J* 6, H-4); δ_C (50.3 MHz; C^2HCl_3) 12.0 (CH_3), 56.7 (OCH_3), 114.2 (C-4), 116.2 (C-6), 122.4 (C-2), 127.2 (C-5), 151.8 (C-3), 158.9 (C-1); *m/z* (EI) 167 ([M]$^+$, 75%), 150 (80, [M-OH]$^+$), 135 (8, [M-CH_3OH]$^+$), 105 (20, [M-CH_4NO_2]$^+$) and 92 (100, [C_6H_3OH]$^+$).

2-Methyl-3-nitro-[$^2H_3$]methoxybenzene (99)



As described for (98) using [$^2H_3$]methyl iodide. A light yellow powder of 2-methyl-3-nitro-[$^2H_3$]methoxybenzene was obtained (4.42 g, 87%); m.p. 51-53 °C; (Found: C, 56.35; H, 5.10; N, 8.02. Calc for $C_8H_6{}^2H_3NO_3$: C, 56.46; H, 5.33; N, 8.23%); δ_H (200 MHz; C^2HCl_3) 2.35 (3H, s, C<u>H</u>$_3$), 7.00 (1H, d, *J* 6, H-6), 7.25 (1H, t, *J* 6, H-5), 7.40 (1H, d, *J* 6, H-4); δ_C (50.3 MHz; C^2HCl_3) 12.0 (C<u>H</u>$_3$), 114.2 (C-4), 116.2 (C-6), 122.4 (C-2), 127.2 (C-5), 151.8 (C-3), 158.9 (C-1); *m/z* (EI) 170 ([M]$^+$, 85%), 153 (83, [M-OH]$^+$), 135 (17, [M-OC^2H_3H]$^+$), 105 (20, [M-$C^2H_3HNO_2$]$^+$) and 93 (100, [C_6H_4OH]$^+$).

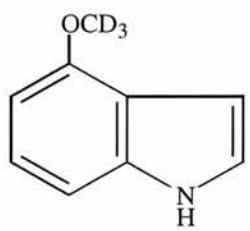
4-Methoxyindole (93)



A stirred solution of 2-methyl-3-nitromethoxybenzene (1.0 g, 6.0 mmol) in *N*,*N*-dimethylformamide (10 ml, 129.4 mmol) was treated with *N*,*N*-dimethylformamide dimethyl acetal (0.88 ml, 6.64 mmol) and pyrrolidine (0.58 ml, 7.08 mmol) then heated under nitrogen at 125 °C for 3 hours. The solvent was evaporated at reduced pressure and the residue taken up in the minimum volume of acetone. This was then added to a mixture of titanium trichloride (30% wt soln in 2 N HCl, 18.2 ml) and ammonium acetate buffer (53 ml, 4 M). The solution was shaken for 10 minutes then extracted with diethyl ether (3 x 75 ml), dried ($MgSO_4$) and the solvent evaporated at reduced pressure. The resulting

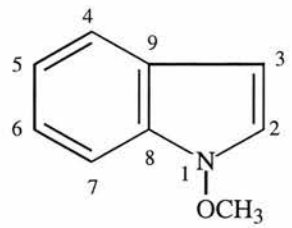
dark oil was purified by flash chromatography on silica gel with 40-60 petroleum ether-diethyl ether (2:1) as the eluant. A white crystalline solid was obtained (0.63 g, 71%); m.p. 68-69 °C (lit.,[206] 68 °C); (Found: C, 73.37; H, 6.27; N, 9.49. Calc for C_9H_9NO: C, 73.45; H, 6.16; N, 9.52%); ν_{max} (nujol)/cm^{-1} 3300 (NH); δ_H (300 MHz; C^2HCl_3) 3.98 (3H, s, $OC\underline{H}_3$), 6.60 (1H, d, *J* 8.1, H-5), 6.72 (1H, m, H-3), 7.04 (1H, d, *J* 8.1, H-7), 7.10 (1H, t, *J* 4.0, H-2), 7.18 (1H, t, *J* 8.1, H-6), 8.15 (1H, s, N<u>H</u>); δ_C (75.45 MHz; C^2HCl_3) 55.3 ($O\underline{C}H_3$), 99.5 (C-5), 99.7 (C-3), 104.5 (C-7), 118.5 (C-9), 122.7 (C-2, 6), 137.2 (C-8), 153.4 (C-4); *m/z* (EI) 147 ($[M]^+$, 100%), 132 (88, $[M-CH_3]^+$), 116 (13, $[M-OCH_3]^+$) and 104 (53, $[C_7H_6N]^+$).

4-[$^2H_3$]Methoxyindole (94)



As described for (93) but using 2-methyl-3-nitro-[$^2H_3$]methoxybenzene. 4-[$^2H_3$]Methoxyindole was obtained as white crystals (1.53 g, 57%); m.p. 68-69.5 °C; (Found: C, 71.29; H, 6.19; N, 9.26. Calc for $C_9H_6{}^2H_3NO$: C, 71.97; H, 6.04; N, 9.33%); *m/z* (Found: $[M+H]^+$ 150.0879. $C_9H_6{}^2H_3NO$ requires 150.0872); ν_{max} (nujol)/cm^{-1} 3300 (NH); δ_H (200 MHz; C^2HCl_3) 6.55 (1H, d, *J* 8.1, H-5), 6.68 (1H, m, H-3), 7.04 (1H, d, *J* 8.1, H-7), 7.10 (1H, t, *J* 4.0, H-2), 7.18 (1H, t, *J* 8.1, H-6), 8.15 (1H, s, N<u>H</u>); δ_C (50.3 MHz; C^2HCl_3) 100.0 (C-5), 100.3 (C-3), 105.0 (C-7), 113.0 (C-9), 123.2 (C-2, 6), 137.7 (C-8), 153.9 (C-4); *m/z* (EI) 150 ($[M]^+$, 100%), 132 (93, $[M-C^2H_3]^+$), 117 (18, $[M-OC^2H_2H]^+$) and 104 (85, $[C_7H_6N]^+$).

1-Methoxyindole (95)



Method 1: 2-Nitrotoluene (3.0 g, 21.9 mmol) was brought to reflux with *N*,*N*-dimethylformamide dimethyl acetal (5.52 g, 6.15 ml, 46.3 mmol) in dry *N*,*N*-dimethylformamide (25 ml) for 18 hours under nitrogen while stirring vigorously. The solvent was evaporated at reduced pressure and the residue was dissolved in diethyl ether (150 ml) then added to a solution of ammonium chloride (4.3 g, 80.4 mmol) in distilled water (30 ml) with zinc dust (27 g, 413 mmol). The reaction was mechanically stirred under nitrogen for 8 hours before it was filtered through silica gel to remove the zinc, washing through with diethyl ether (50 ml). The filtrate was washed with saturated sodium hydrogen carbonate solution (2 x 75 ml) then treated with methyl iodide (16.4 g, 7.2 ml, 116 mmol), 10% aqueous sodium hydroxide solution (150 ml) and tri(n-octyl)methyl ammonium chloride (1.0 g, 2.47 mmol). The reaction was stirred for 20 hours at room temperature, washed with brine (2 x 75 ml), dried ($MgSO_4$) then the solvent was evaporated at reduced pressure to give a dark-red viscous oil. Purification by column chromatography on silica gel using dichloromethane-hexane (3:7) as the eluant gave 1-methoxyindole as a pale-yellow oil (1.30 g, 40%); ν_{max} (thin film)/cm^{-1} 2850 (OCH_3), 1710 ($NOCH_3$); δ_H (200 MHz; C^2HCl_3) 4.15 (3H, s, CH_3), 6.45 (1H, d, $J_{2,3}$ 2.6, H-3), 7.25 (1H, t, *J* 7.7, H-5), 7.34 (1H, d, *J* 2.6, H-2), 7.35 (1H, t, *J* 7.7, H-6), 7.55 (1H, d, *J* 7.7, H-7), 7.70 (1H, d, *J* 7.7, H-4); δ_C (50.3 MHz; C^2HCl_3) 66.0 (CH_3), 98.5 (C-3), 109.0 (C-7), 121.0 (C-4), 122.0 (C-5), 123.0 (C-2), 123.5 (C-9), 123.6 (C-6), 133.0 (C-8); *m/z* (EI) 147 ($[M]^+$, 100%), 132 (61, $[M-CH_3]^+$) and 116 (80, $[M-OCH_3]^+$).

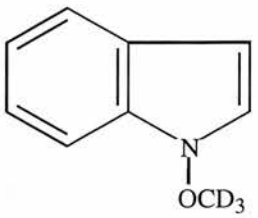
Method 2: Sodium tungstate (0.55 g, 1.68 mmol), 30% hydrogen peroxide (9.5 ml, 84.0 mmol) and methanol (5ml) were cooled to 0 °C. Indoline (0.95 ml, 8.4 mmol) was added

and the solution stirred until complete (30 minutes). Potassium carbonate (1.38 g, 10.0 mmol) was added followed by methyl iodide (0.52 ml, 8.4 mmol) and the reaction stirred overnight. The solution was extracted using diethyl ether (2 x 20 ml), dried ($MgSO_4$) and concentrated at reduced pressure to give a dark oil which contained a large mixture of products. None of the desired product could be isolated.

Method 3: Pyridinium chlorochromate (1.15 g, 5.0 mmol) was added to a solution of 2-nitrophenethyl alcohol (0.5 g, 3.0 mmol) in dry dichloromethane (15 ml). The reaction mixture was stirred for 4 hours at room temperature then passed through a column of fluorosil using dichloromethane eluant. Crude *o*-nitrophenylacetaldehyde was obtained as a pale yellow oil (0.45 g, 91%) which was stored below 0 °C; δ_H (200 MHz; C^2HCl_3) 4.10 (2H, s, CH_2), 7.20-8.00 (4H, m, 4 x C<u>H</u> aromatic), 9.70 (1H, s, CHO); δ_C (50.3 MHz; C^2HCl_3) 49.0 (CH_2), 124.0-136.0 (4 x <u>C</u>H aromatic, <u>C</u>CH_2), 149.8 (<u>C</u>NO_2), 194.0 (CHO).

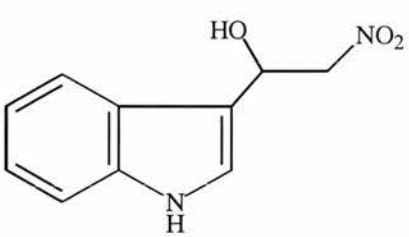
o-Nitrophenylacetaldehyde (0.5g, 3.0 mmol) solution in diethyl ether (17 ml) was added to a solution of activated zinc dust (1.35 g, 20.7 mmol) and ammonium chloride (0.24 g, 4.1 mmol) in water (3 ml). The reaction mixture was stirred at room temperature for 7 hours. The zinc was removed by filtration and the ethereal layer was added to a solution of methyl iodide (0.2 ml, 3.1 mmol) and pyridine (3.5 ml). The solution was stirred overnight then examined by t.l.c. (silica, dichloromethane) at this stage the reaction mixture contained several products. The solvent was evaporated at reduced pressure then the pyridine was coevaporated with toluene to give the crude reaction products. Purification using column chromatography on silica gel with ethyl acetate-dichloromethane (1:1) as the eluant did not yield any of the desired product.

1-[$^2H_3$]Methoxyindole (96)



As described for (95, method 1) using [$^2H_3$]methyl iodide. 1-[$^2H_3$]Methoxyindole was given as a pale-yellow oil (2.40 g, 44%); ν_{max} (thin film)/cm^{-1} 2850 (OC^2H_3), 1710 (N-OC^2H_3); δ_H (200 MHz; C^2HCl_3) 6.60 (1H, d, $J_{2,3}$ 3.0, H-3), 7.35 (1H, t, *J* 7.7, H-5), 7.45 (1H, d, *J* 2.6, H-2), 7.50 (1H, t, *J* 7.7, H-6), 7.70 (1H, d, *J* 7.7, H-7), 7.90 (1H, d, *J* 7.7, H-4); δ_C (50.3 MHz; C^2HCl_3) 98.7 (C-3), 108.9 (C-7), 120.7 (C-4), 122.0 (C-5), 123.1 (C-2), 123.6 (C-9), 123.8 (C-6), 132.6 (C-8); *m/z* (EI) 150 ([M]$^+$, 100%), 132 (68, [M-C^2H_3]$^+$) and 116 (81, [M-OC^2H_3]$^+$).

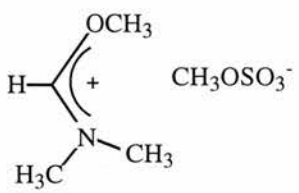
3-(2-Nitro-1-hydroxyethyl)indole (109)



Method 1: Indole-3-carboxaldehyde was added to a solution of nitromethane at 0 °C. The solution was stirred for 5 minutes then chromatographic alumina was added and the reaction stirred vigorously for 1 hour at 0 °C. The solution was warmed to room temperature and after 24 hours the alumina was washed with dichloromethane. The solvent was evaporated at reduced pressure to give a viscous oil. The ^1H n.m.r. spectrum indicated that no reaction had occurred.

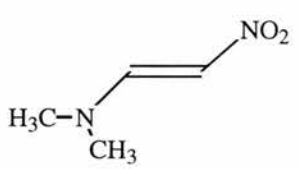
Method 2: Potassium fluoride dihydrate (0.019 g, 0.2 mmol) and nitromethane (0.43 ml, 8.0 mmol) were added to indole-3-carboxaldehyde (0.6 g, 4.0 mmol) in isopropanol (4 ml). 18-Crown-6 was added then the reaction was stirred for 3 hours at room temperature then the solvent was removed at reduced pressure. The ^1H n.m.r. spectrum showed only starting materials.

N, *N*-Dimethylformamide dimethyl sulfate complex (105)



Dimethyl sulfate (12.6 g, 9.45 ml, 100 mmol) was stirred in dry *N*,*N*-dimethylformamide (7.74 ml) at 60-80 °C under nitrogen for 2 hours. The complex was reacted *in situ* to give 1-dimethylamino-2-nitroethylene as follows.

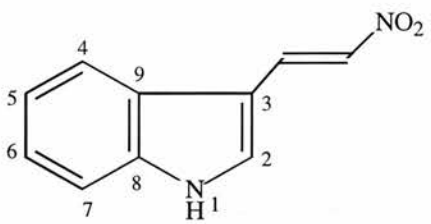
1-Dimethylamino-2-nitroethylene (106)



Dry ethanol (100 ml) was treated with sodium (2.3 g, 100 mmol) under nitrogen. *N*, *N*-Dimethylformamide dimethyl sulfate complex (13) (20 g, 100 mmol) and nitromethane (6.1 g, 98 mmol) were added and the solution heated to boiling for 1-2 minutes. The reaction mixture was cooled to 20 °C then the solvent evaporated at reduced pressure. The residue was taken up in dichloromethane (50 ml) and washed with water (2 x 25 ml). The organic phase was dried ($MgSO_4$) and the solvent evaporated at reduced pressure. The resulting solid was recrystallised from isopropanol to give 1-dimethylamino-2-nitroethylene as golden brown crystals (6.94 g, 61%); m.p. 103.5-104 °C (lit.,[257] 103-105 °C); (Found: C, 41.58; H, 6.95; N, 24.22. Calc for $C_4H_8N_2O_2$: C, 41.38; H, 6.94; N, 24.13%);

ν_{max}(nujol)/cm^{-1} 1630 (C=C), 1500 (NO_2), 1300 (NO_2); δ_H (200 MHz; C^2HCl_3) 2.85 (3H, s, $NC\underline{H}_3$), 3.20 (3H, s, NCH_3), 6.60 (1H, d, *J* 10.3, $=C\underline{H}NO_2$), 8.10 (1H, d, *J* 10.30, $(Me_2N)C\underline{H}=$); δ_C (50.3 MHz; C^2HCl_3) 38.7 ($\underline{C}H_3$), 46.2 ($\underline{C}H_3$), 112.9 ($=\underline{C}HNO_2$), 151.8 ($(Me_2N)\underline{C}H=$); *m/z* (EI) 116 ([M]$^+$, 77%), 99 (7, [M-OH]$^+$), 69 (27, $[C_4H_7N]^+$), 54 (71, $[C_3H_4N]^+$), 44 (18, $[N(CH_3)_2]^+$) and 42 (100, $[C_2H_4N]^+$).

3-(2-Nitrovinyl)indole (107)



Method 1: 1-Dimethylamino-2-nitroethylene (1.75 g, 15 mmol) was dissolved in trifluoroacetic acid (7.5 ml) and the solution cooled to 0 °C. Indole (1.75 g, 15 mmol) was added and the reaction stirred for 80 minutes, during which time it became dark brown in colour. The solution was allowed to reach room temperature then quenched with ice/water (150 ml) giving a yellow semi-solid precipitate. This was extracted with ethyl acetate (175 ml then 2 x 50 ml) washed with saturated sodium hydrogen carbonate solution (75 ml) and brine (50 ml) before being dried ($MgSO_4$) and the solvent evaporated at reduced pressure. The residue was recrystallised from methanol to give 3-(2-nitrovinyl)indole as dark brown plate-like crystals (2.13 g, 76%); m.p. 169 °C (lit.[220] 169-172 °C); (Found: C, 63.45; H, 4.27; N, 14.72. Calc for $C_{10}H_8N_2O_2$: C, 63.83; H, 4.29; N, 14.89%); ν_{max}(nujol)/cm^{-1} 3300 (NH), 1550 (NO_2), 1300 (NO_2), 940 (C=C); δ_H (500 MHz; $C^2H_3O^2H$) 7.45 (2H, m, H-5, 6), 7.66 (1H, m, H-7), 7.99 (2H, m, H-2, 4), 8.06 (1H, d, *J* 13, $C\underline{H}NO_2$), 8.55 (1H, d, *J* 13, $C\underline{H}=CHNO_2$); δ_C (125.8 MHz; $C^2H_3O^2H$) 105.6 (C-3), 113.6 (C-7), 121.3 (C-4, 2), 121.8 (C-9), 123.2 (C-5), 124.7 (C-6), 135.0 (C-8), 135.6 ($\underline{C}H=CHNO_2$), 136.0 ($\underline{C}HNO_2$); *m/z* (EI) 188 ([M]$^+$, 100%), 171 (12, [M-OH]$^+$), 155 (5, [M-HO_2]$^+$), 141 (85, [M-NO_2H]$^+$), 130 (27, [M-CNO_2]$^+$), 115 (80, [M-$C_2H_3NO_2$]$^+$), 89 (29, $[C_7H_5]^+$) and 77 (14, $[C_6H_5]^+$).

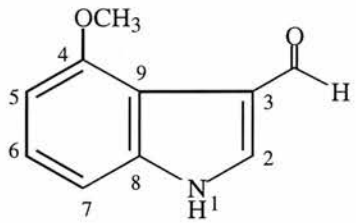
Method 2: A solution of ammonium acetate (0.84 g, 10.9 mmol), acetic anhydride (0.23 ml) and acetic acid (0.67 ml) was stirred for 20 minutes. A solution of indole-3-carboxaldehyde (1.0 g, 6.8 mmol) and nitromethane (4.3 g, 3.83 ml, 71.0 mmol) in acetic acid (4.6 ml) was added and the solution heated to reflux. Sodium acetate trihydrate (0.9 g, 6.6 mmol) was added to the solution then further acetic anhydride (0.8 ml) added dropwise over a 4 hour period at reflux. The solution was cooled to room temperature while adding water (1.7 ml) dropwise. Cooling further to 0 °C gave no precipitate so the reaction mixture was concentrated at reduced pressure and purified by column chromatography on silica using ethyl acetate-hexane (1:1) as the eluant. 3-(2-nitrovinyl)indole (0.56 g, 43%) was obtained as a dark coloured solid; spectral data as for method 1.

Method 3: Dried ammonium acetate (1.5 g, 19.0 mmol) was added to a solution of indole-3-carboxaldehyde (5 g, 34 mmol) in freshly distilled nitromethane (17.5 g, 15 ml, 277 mmol). The solution was stirred vigorously while heating at reflux for a period of 2 hours 30 minutes. The reaction mixture was concentrated at reduced pressure and the residue purified by column chromatography on silica using ethyl acetate-hexane (1:1) as the eluant. 3-(2-Nitrovinyl)indole (2.7 g, 42%) was obtained as a dark coloured solid; spectral data as for method 1.

Method 4: A solution of indole-3-carboxaldehyde (1.0 g, 6.8 mmol) and nitromethane (1.5 g, 1.33 ml, 24.6 mmol) in methanol (25 ml) was cooled to -5 °C. 50% Aqueous potassium hydroxide (7.5 ml) was added dropwise while ensuring that the reaction temperature remained below 0 °C. After stirring for 1 hour at 0 °C ice water (25 ml) was added to the solution and it was then poured into 3.0 M hydrochloric acid (120 ml) at 0 °C. At this point a precipitate formed which was removed by filtration but the ^{1}H n.m.r. spectrum indicated that this precipitate did not contain product. The remaining solution was extracted using ethyl acetate (2 x 100 ml), dried ($MgSO_4$) and concentrated at reduced pressure. Again no product was contained in the residue by ^{1}H n.m.r. spectroscopy. A second attempt where

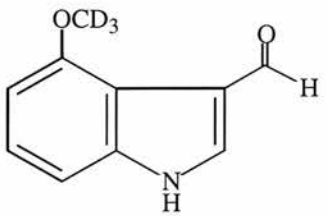
the reaction mixture was stirred overnight at room temperature also proved to be unsuccessful and gave only starting materials.

4-Methoxyindole-3-carboxaldehyde (110)



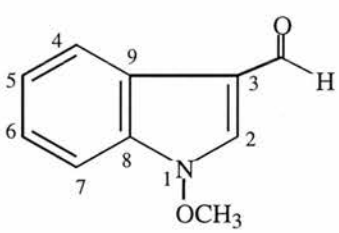
Phosphorous oxychloride (0.48 ml, 5.0 mmol) was added dropwise to dry *N*,*N*-dimethylformamide (3 ml) at 0 °C. A solution of 4-methoxyindole (0.5 g, 3.4 mmol) in dry *N*,*N*-dimethylformamide (2 ml) was then added over a period of 30 mins at 0 °C. The mixture was warmed to 45 °C and stirred for 2 hours. The reaction was poured onto ice water (8 ml) and extracted twice with diethyl ether (2 x 10 ml), these extracts were discarded. The aqueous layer was treated with 2 M sodium hydroxide until the solution was basic then once more extracted with diethyl ether (3 x 20 ml). The organic extracts were washed with brine, dried ($MgSO_4$) and the solvent evaporated at reduced pressure to give the crude product as a pale-yellow solid (0.51 g, 85%); m.p. 163-164 °C (lit.,[258] 162-163 °C); ν_{max} (nujol)/cm^{-1} 3200 (NH), 1600 (CHO); δ_H (200 MHz; C^2HCl_3) 4.00 (3H, s, CH_3), 6.70 (1H, d, *J* 7.5, H-5), 7.10 (1H, d, *J* 7.5, H-7), 7.20 (1H, t, *J* 7.5, H-6), 7.95 (1H, d, *J* 4, H-2), 9.20 (1H, s, NH), 9.75 (1H, s, CHO); δ_C (50.3 MHz; C^2HCl_3) 55.9 (CH_3), 102.9 (C-5), 105.8 (C-7), 115.8 (C-9), 118.5 (C-3), 123.7 (C-6), 129.8 (C-2), 138.1 (C-8), 154.1 (C-4), 186.6 (CHO); *m/z* (EI) 175 ($[M]^+$, 100%), 160 (46, $[M-CH_3]^+$), 144 (40, $[M-OCH_3]^+$) and 146 (38, $[M-CHO]^+$).

4-[$^2H_3$]Methoxyindole-3-carboxaldehyde (111)



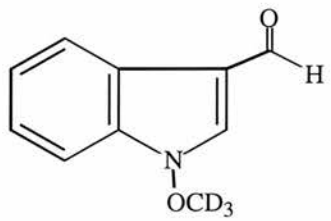
As described for (110) using 4-[$^2H_3$]methoxyindole (1.43 g, 9.52 mmol). The crude product was given as a pale-yellow crystalline solid (1.24 g, 73%); m.p. 158.5-161.0 °C; ν_{max} (nujol)/cm^{-1} 3200 (NH), 1600 (CHO); δ_H (200 MHz; $C^2H_3O^2H$) 6.70 (1H, d, *J* 7.5, H-5), 7.00-7.30 (3H, m, H-2, 6, 7), 8.00 (1H, s, CHO); δ_C (50.3 MHz; $(C^2H_3)_2SO$) 102.4 (C-5), 106.0 (C-7), 115.8 (C-9), 118.4 (C-3), 123.8 (C-6), 129.8 (C-2), 138.2 (C-8), 154.1 (C-4), 186.7 (CHO); *m/z* (EI) 178 ([M]$^+$, 100%), 160 (31, [M-C^2H_3]$^+$) and 149 (15, [M-CHO]$^+$).

1-Methoxyindole-3-carboxaldehyde (112)



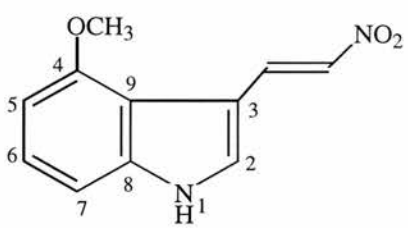
As described for (110) using 1-methoxyindole (1.3 g, 8.8 mmol). The crude product was given as a dark-yellow oil (1.54 g, 100%); ν_{max} (thin film)/cm^{-1} 1600 (CHO); δ_H (300 MHz; C^2HCl_3) 4.10 (3H, s, CH_3), 7.30 (1H, t, *J* 7.5, H-5), 7.35 (1H, t, *J* 7.5, H-6), 7.45 (1H, d, *J* 7.5, H-7), 7.85 (1H, s, H-2), 8.30 (1H, d, *J* 7.5, H-4), 9.90 (1H, s, CHO); δ_C (75.45 MHz; C^2HCl_3) 67.2 (CH_3), 109.2 (C-7), 114.3 (C-3), 121.9 (C-9), 122.4 (C-4), 123.8 (C-5), 125.0 (C-6), 132.9 (C-2), 133.1 (C-8), 184.7 (CHO); *m/z* (EI) 175 ([M]$^+$, 100%), 143 (10, [M-$HOCH_3$]$^+$), 132 (55, [M-CH_3-CO]$^+$) and 116 (74, [M-OCH_3-CO]$^+$).

1-[$^2H_3$]Methoxyindole-3-carboxaldehyde (113)



As described for (110) using 1-[$^2H_3$]methoxyindole (2.0 g, 13.3 mmol). The crude product was given as a dark-yellow oil (2.35 g, 100%); ν_{max} (thin film)/cm^{-1} 1600 (CHO); δ_H (200 MHz; C^2HCl_3) 7.10 (1H, m, H-5), 7.25 (1H, m, H-6), 7.70 (1H, d, *J* 7.5, H-7), 7.75 (1H, s, H-2), 8.10 (1H, d, *J* 7.5, H-4), 9.70 (1H, s, CHO); δ_C (50.3 MHz; C^2HCl_3) 109.2 (C-7), 114.3 (C-3), 121.9 (C-9), 122.4 (C-4), 123.8 (C-5), 125.0 (C-6), 132.9 (C-2), 133.1 (C-8), 184.7 (CHO); *m/z* (EI) 178 ([M]$^+$, 100%), 143 (10, [M-C^2H_3OH]$^+$), 132 (58, [M-C^2H_3-CO]$^+$) and 116 (64, [M-OC^2H_3-CO]$^+$).

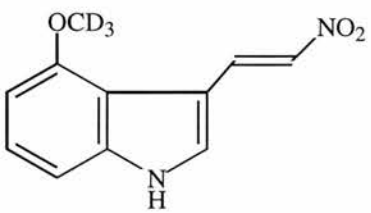
3-(2-Nitrovinyl)-4-methoxyindole (114)



Ammonium acetate (0.13 g, 1.60 mmol) was added to 4-methoxyindole-3-carboxaldehyde (0.5 g, 2.85 mmol) in nitromethane (10 ml) and stirred vigorously while heating at reflux for 2 hours. The reaction was followed by t.l.c. (silica, 1:1, ethyl acetate-hexane) and the product showed as a bright-yellow spot. The solvent was evaporated at reduced pressure and the remaining bright-red solid purified by column chromatography on silica gel with ethyl acetate-hexane (1:1) as the eluant. The product was given as a bright-yellow solid (0.53 g, 85%); m.p. 188 °C decomposes (lit.,[259] 189-190 °C decomposes); ν_{max}

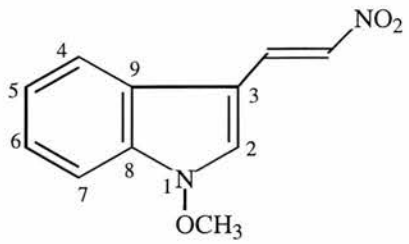
(nujol)/cm^{-1} 3200 (NH), 1300 (NO_2), 950 (C=C); δ_H (200 MHz; $C^2H_3O^2H$) 4.00 (3H, s, CH_3), 6.70 (1H, d, *J* 7.5, H-5), 7.05 (1H, d, *J* 7.5, H-7), 7.20 (1H, t, *J* 7.5, H-6), 7.85 (1H, s, H-2), 8.05 (1H, d, *J* 12.5, $CHNO_2$) 8.60 (1H, d, *J* 12.5, C<u>H</u>=CH); δ_C (50.3 MHz; $(C^2H_3)_2SO$) 55.6 (CH_3), 102.7 (C-5), 106.3 (C-7), 108.2 (C-3), 115.4 (C-9), 124.6 (C-6), 132.5 (C-2), 135.8 (C<u>H</u>=CH and C<u>H</u>NO_2), 139.0 (C-8), 153.9 (C-4); *m/z* (EI) 218 ($[M]^+$, 100%), 187 (7, $[M-OCH_3]^+$), 171 (73, $[M-NO_2H]^+$) and 157 (34, $[M-CH_3NO_2]^+$).

3-(2-Nitrovinyl)-4-[$^2H_3$]methoxyindole (115)



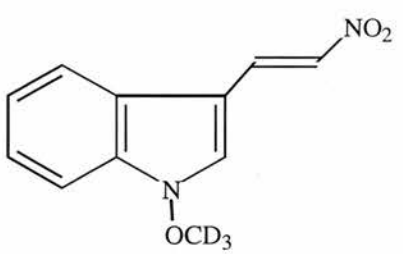
As described for (114) using 4-[$^2H_3$]methoxyindole-3-carboxaldehyde (1.0 g, 5.6 mmol). The purified 3-(2-nitrovinyl)-4-[$^2H_3$]methoxyindole was given as a bright-yellow solid (1.10 g, 89%); m.p. >105 °C decomposes; ν_{max} (nujol)/cm^{-1} 3200 (NH), 1300 (NO_2), 950 (C=C); δ_H (200 MHz; $C^2H_3O^2H$) 6.70 (1H, d, *J* 7.5, H-5), 7.05 (1H, d, *J* 7.5, H-7), 7.20 (1H, t, *J* 7.5, H-6), 7.85 (1H, s, H-2), 8.05 (1H, d, *J* 12.5, $CHNO_2$), 8.60 (1H, d, *J* 12.5, C<u>H</u>=CH); δ_C (50.3 MHz; $(C^2H_3)_2SO$) 102.7 (C-5), 106.3 (C-7), 108.2 (C-3), 115.4 (C-9), 124.6 (C-6), 132.5 (C-2), 135.8 (<u>C</u>H=CH and $CHNO_2$), 139.0 (C-8), 153.9 (C-4); *m/z* (EI) 221 ($[M]^+$, 100%), 174 (56, $[M-NO_2H]^+$) and 157 (35, $[M-C^2H_3NO_2]^+$).

3-(2-Nitrovinyl)-1-methoxyindole (116)



As described for (114) using 1-methoxyindole-3-carboxaldehyde (1.55 g, 8.84 mmol). The purified 3-(2-nitrovinyl)-1-methoxyindole was given as a bright-yellow solid (1.45 g, 75%); m.p. 98-104 °C (lit.,[215] 99.5-100.0 °C); ν_{max} (nujol)/cm^{-1} 1300 (NO_2), 950 (C=C); δ_H (200 MHz; C^2HCl_3) 4.20 (3H, s, CH_3), 7.32 (1H, t, *J* 7.5, H-5), 7.40 (1H, t, *J* 7.5, H-6), 7.52 (1H, d, *J* 7.5, H-7), 7.73 (1H, s, H-2), 7.73 (1H, d, *J* 14.0, $CHNO_2$), 7.74 (1H, d, *J* 7.5, H-4), 8.20 (1H, d, *J* 14.0, C<u>H</u>=CH); δ_C (50.3 MHz; C^2HCl_3) 66.8 (CH_3), 104.4 (C-3), 109.4 (C-7), 120.6 (C-4), 122.4 (C-9), 123.5 (C-5), 125.0 (C-6), 129.6 (C-2), 133.2 (<u>C</u>H=CH), 133.4 ($CHNO_2$), 133.5 (C-8); *m/z* (EI) 218 ([M]$^+$, 75%), 175 (100, [M-CHNO]$^+$), 140 (77, [M-$HOCH_3$-NO_2]$^+$), 132 (62, [M-CH_3-$CCHNO_2$]$^+$) and 114 (50, [C_8H_4N]$^+$).

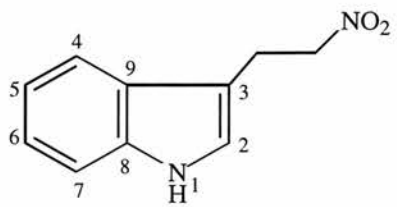
3-(2-Nitrovinyl)-1-[$^2H_3$]methoxyindole (117)



As described for (114) using 1-[$^2H_3$]methoxyindole-3-carboxaldehyde (2.16 g, 12.1 mmol). The purified product was given as a bright-yellow solid (2.08 g, 78%); m.p. 81-85 °C decomposes; ν_{max} (nujol)/cm^{-1} 1300 (NO_2), 950 (C=C); δ_H (200 MHz; C^2HCl_3) 7.32 (1H, t, *J* 7.5, H-5), 7.40 (1H, t, *J* 7.5, H-6), 7.52 (1H, d, *J* 7.5, H-7), 7.73 (1H, s, H-2), 7.73 (1H, d, *J* 14.0, $CHNO_2$), 7.74 (1H, d, *J* 7.5, H-4), 8.20 (1H, d, *J* 14.0,

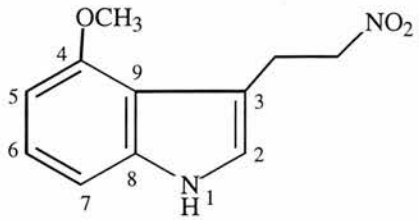
C<u>H</u>=CH); δ_C (50.3 MHz; C^2HCl_3) 104.4 (C-3), 109.4 (C-7), 120.6 (C-4), 122.4 (C-9), 123.5 (C-5), 125.0 (C-6), 129.6 (C-2), 133.2 (C<u>H</u>=CH), 133.4 ($CHNO_2$), 133.5 (C-8); *m/z* (EI) 221 ($[M]^+$, 80%), 178 (100, $[M\text{-}CHNO]^+$), 140 (83, $[M\text{-}HOC^2H_3\text{-}NO_2]^+$), 132 (64, $[M\text{-}C^2H_3\text{-}CCHNO_2]^+$) and 114 (53, $[C_8H_4N]^+$).

3-(2-Nitroethyl)indole (119)



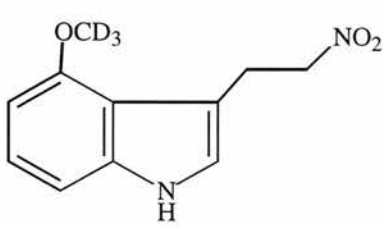
Chloroform (200 ml), isopropanol (38 ml), silica gel (25.2 g) and 3-(2-nitrovinyl)indole (2.7 g, 16.79 mmol) were stirred together for 15 minutes. Sodium borohydride (2.60 g, 68.7 mmol) was then added in portions over 1 hour and the reaction followed by t.l.c. (silica, 1:1, ethyl acetate-hexane). Once the reaction was complete, the excess sodium borohydride was destroyed using 2 M hydrochloric acid then the silica was removed by filtration and washed with dichloromethane. The filtrate was washed with brine (100 ml), dried (Na_2SO_4) and the solvent evaporated at reduced pressure to give a dark oil which was purified three times by column chromatography on silica gel using ethyl acetate-hexane (1:1) as the eluant to give the product as a pale-yellow crystalline solid (1.7 g, 53%); m.p. 66-68 °C (lit.,[260] 67-68 °C); (Found: C, 63.42; H, 5.23; N, 14.72. Calc for $C_{10}H_{10}N_2O_2$: C, 63.15; H, 5.30; N, 14.73%); ν_{max} (nujol)/cm^{-1} 3350 (NH), 1520 (NO_2), 1340 (NO_2); δ_H (300 MHz; C^2HCl_3) 3.50 (2H, t, *J* 7.5, CH_2), 4.70 (2H, t, *J* 7.5, CH_2NO_2), 7.00 (1H, s, H-2), 7.20 (1H, t, *J* 7.5, H-5), 7.25 (1H, t, *J* 7.5, H-6), 7.40 (1H, d, $J_{7,6}$ 7.5, H-7), 7.62 (1H, d, $J_{4,5}$ 7.5, H-4), 8.15 (1H, s, H-1); δ_C (75.45 MHz; C^2HCl_3) 24.1 (CH_2), 74.4 (CH_2NO_2), 110.3 (C-3), 112.2 (C-7), 118.7 (C-4), 120.3 (C-5), 123.0 (C-6), 123.4 (C-2), 127.2 (C-9) 136.8 (C-8); *m/z* (EI) 190 ($[M]^+$, 100%), 143 (52, $[M\text{-}NO_2H]^+$), 130 (15, $[M\text{-}NO_2CH_2]^+$) and 117 (24, $[M\text{-}C_2H_3NO_2]^+$).

3-(2-Nitroethyl)-4-methoxyindole (120)



As described for (119) using 3-(2-nitrovinyl)-4-methoxyindole (0.7 g, 3.2 mmol). The purified product was given as a light-brown coloured solid (0.28 g, 40%); m.p. 90-92.5 °C (lit.,[261] 93-94 °C); (Found: C, 60.18; H, 5.30; N, 12.72. Calc for $C_{11}H_{12}N_2O_3$: C, 59.99; H, 5.49; N, 12.72%); ν_{max} (nujol)/cm^{-1} 3350 (NH); δ_H (300 MHz; C^2HCl_3) 3.55 (2H, t, *J* 7.5, CH_2), 3.95 (3H, s, CH_3), 4.75 (2H, t, *J* 7.5, CH_2NO_2), 6.50 (1H, d, *J* 10, H-5), 6.90 (1H, d, *J* 2.5, H-2), 6.95 (1H, d, *J* 10, H-7), 7.10 (1H, t, *J* 7.5, H-6), 8.00 (1H, s, H-1); δ_C (75.45 MHz; C^2HCl_3) 26.1 (CH_2), 55.6 (CH_3), 77.6 (CH_2NO_2), 100.1 (C-5), 105.3 (C-7), 110.7 (C-3), 117.2 (C-9), 122.4 (C-2), 123.7 (C-6) 138.6 (C-8) 154.7 (C-4); *m/z* (EI) 220 ([M]$^+$, 100%), 173 (50, [M-NO_2H]$^+$), 159 (30, [M-CH_3-NO_2]$^+$) and 130 (25, [M-OCH_3-$CHNO_2$]$^+$).

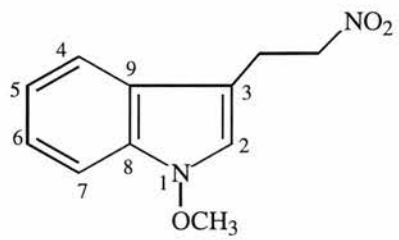
3-(2-Nitroethyl)-4-[$^2H_3$]methoxyindole (121)



As described for (119) using 3-(2-nitroethyl)-4-[$^2H_3$]methoxyindole (2.4 g, 10.8 mmol). The purified product was given as a light-brown coloured solid (0.9 g, 37%); m.p. 85-89 °C; (Found: C, 59.18; H, 5.12; N, 12.52. Calc for $C_{11}H_9{}^2H_3N_2O_3$: C, 59.18; H, 5.42; N, 12.55%); ν_{max} (nujol)/cm^{-1} 3350 (NH); δ_H (200 MHz; C^2HCl_3) 3.60 (2H, t, *J* 7.5, CH_2), 4.80 (2H, t, *J* 7.5, CH_2NO_2), 6.60 (1H, d, *J* 10, H-5), 6.85 (1H, d, *J* 2.5, H-2),

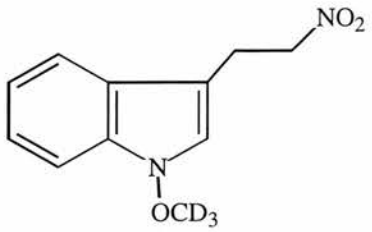
7.00 (1H, d, *J* 10, H-7), 7.20 (1H, t, *J* 7.5, H-6), 8.15 (1H, s, H-1); δ_C (50.3 MHz; C^2HCl_3) 26.2 (CH_2), 77.7 (CH_2NO_2), 100.1 (C-5), 105.4 (C-7), 110.6 (C-3), 117.2 (C-9), 122.6 (C-2), 123.6 (C-6) 138.6 (C-8), 154.8 (C-4); *m/z* (EI) 223 ([M]$^+$, 97%), 176 (100, [M-NO_2H]$^+$), 159 (57, [M-C^2H_3-NO_2]$^+$) and 130 (43, [M-OC^2H_3-$CHNO_2$]$^+$).

3-(2-Nitroethyl)-1-methoxyindole (122)



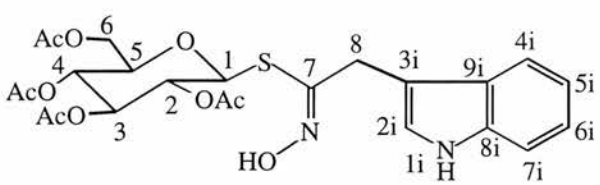
As described for (119) using 3-(2-nitrovinyl)-1-methoxyindole (1.9 g, 8.7 mmol). The product was purified three times using column chromatography on silica gel using ethyl acetate-hexane (1:2) as the eluant to give a pale-yellow oil (1.12 g, 59%); (Found: C, 59.74; H, 5.60; N, 12.99. Calc for $C_{11}H_{12}N_2O_3$: C, 59.99; H, 5.49; N, 12.72%); δ_H (300 MHz; C^2HCl_3) 3.50 (2H, t, *J* 7.5, CH_2), 4.10 (3H, s, CH_3), 4.70 (2H, t, *J* 7.5, CH_2NO_2), 7.20 (1H, s, H-2), 7.25 (1H, t, *J* 7.5, H-5), 7.4 (1H, t, *J* 7.5, H-6), 7.55 (1H, d, *J* 7.5, H-7), 7.65 (1H, d, *J* 7.5, H-4); δ_C (75.45 MHz; C^2HCl_3) 23.9 (CH_2), 66.4 (CH_3), 76.2 (CH_2NO_2), 106.4 (C-3), 109.2 (C-7), 119.1 (C-4), 120.7 (C-5), 122.2 (C-2), 123.5 (C-6), 123.8 (C-9) 132.9 (C-8); *m/z* (EI) 220 ([M]$^+$, 100%), 173 (79, [M-NO_2H]$^+$), 160 (32, [M-CH_2NO_2]$^+$), 142 (79, [M-$HOCH_3$-NO_2]$^+$), 130 (44, [M-OCH_3-CH_2NO_2]$^+$) and 115 (100, [M-OCH_3-$C_2H_4NO_2$]$^+$).

3-(2-Nitroethyl)-1-[$^2H_3$]methoxyindole (123)



As described for (119) using 3-(2-nitrovinyl)-1-[$^2H_3$]methoxyindole (1.9 g, 8.6 mmol). The product was purified three times using column chromatography on silica gel using ethyl acetate-hexane (1:2) as the eluant. The product was given as a pale-yellow oil (1.24 g, 65%); (Found: C, 59.28; H, 4.84; N, 12.10. Calc for $C_{11}H_9H_3N_2O_3$: C, 59.18; H, 5.42; N, 12.55%); δ_H (200 MHz; C^2HCl_3) 3.50 (2H, t, *J* 7.5, CH_2), 4.65 (2H, t, *J* 7.5, CH_2NO_2), 7.20 (1H, s, H-2), 7.25 (1H, t, *J* 7.5, H-5), 7.35 (1H, t, *J* 7.5, H-6), 7.50 (1H, d, *J* 7.5, H-7), 7.65 (1H, d, *J* 7.5, H-4); δ_C (50.3 MHz; C^2HCl_3) 33.9 (CH_2), 76.1 (CH_2NO_2), 106.3 (C-3), 109.2 (C-7), 119.0 (C-4), 120.6 (C-5), 122.2 (C-2), 123.5 (C-6), 123.7 (C-9) 132.9 (C-8); *m/z* (EI) 223 ([M]$^+$, 100%), 176 (73, [M-NO_2H]$^+$), 163 (24, [M-CH_2NO_2]$^+$), 142 (60, [M-HOC^2H_3-NO_2]$^+$), 130 (32, [M-OC^2H_3-CH_2NO_2]$^+$) and 115 (70, [M-OC^2H_3-$C_2H_4NO_2$]$^+$).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl glucobrassicin thiohydroximate (126)



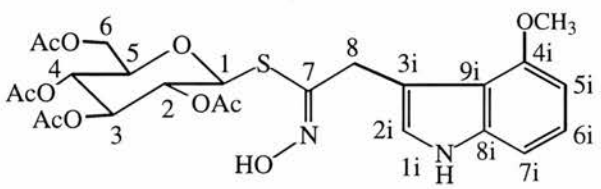
Sodium metal (0.18 g, 7.8 mmol) was added to dry methanol (15 ml) under nitrogen and treated with 3-(2-nitroethyl)indole (1.5 g, 7.9 mmol) in dry diethyl ether (10 ml). After 15 minutes the solvent was evaporated at reduced pressure giving the nitronate as a white solid which was dried under a high vacuum for 15 minutes.

The nitronate was cooled to -40 °C under nitrogen then dry 1,2-dimethoxyethane (37 ml) was added at -40 °C. A solution of purified thionyl chloride (1.86 ml) in dry 1,2-dimethoxyethane (12.5 ml) at -40 °C was added to the nitronate dropwise to give a burgundy coloured solution. After 30 minutes at -40 °C, water (60 ml) was added and the solution extracted with dichloromethane (2 x 100 ml). The organic extracts were dried ($MgSO_4$) and evaporated at reduced pressure to give the hydroximoyl chloride which was left under a high vacuum for 15 minutes and reacted directly in the next step.

The hydroximoyl chloride in dry ether (39 ml) and dry dichloromethane (19.5 ml) was treated with a solution of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (2.51 g, 6.9 mmol) and dry triethylamine (2.5 ml, 17.52 mmol) in dry dichloromethane (19.5 ml). The reaction was stirred for 1 hour giving an orange solution. The reaction mixture was acidified with 0.5 M sulfuric acid (100 ml) then extracted using dichloromethane (2 x 100 ml). The organic extracts were dried ($MgSO_4$) and evaporated at reduced pressure to give a dark, sticky oil. The oil was purified twice by column chromatography on silica gel. Firstly, with 40-60 petroleum ether-ethyl acetate (6:4) as the eluant, secondly, using dichloromethane-methanol (97:3) to give the product as a cream coloured solid (1.46 g, 39%); m.p. 83-86 °C; $[\alpha]_D$ +7.14 ° (c 0.7 in $CHCl_3$) (lit.,[102] +4.0 ° (c 0.70 in $CHCl_3$)); (Found: C, 52.37; H, 5.12; N, 4.97. Calc for $C_{24}H_{28}N_2O_{10}S.H_2O$: C, 51.98; H, 5.27; N, 5.05%); *m/z* (Found: $[M+H]^+$ 537.1550. $C_{24}H_{29}N_2O_{10}S$ requires 537.1543); ν_{max} (nujol)/cm^{-1} 3350 (NH, OH), 1750 ($C(O)CH_3$); δ_H (500 MHz; C^2HCl_3) 1.90-2.10 (4 x 3H, 4s, $OC(O)C\underline{H}_3$), 3.20 (1H, m, H-5), 3.90 (1H, dd, J_{gem} 12.5, $J_{6a,5}$ 2.2, H-6a), 4.00-4.10 (3H, m, H-6b, 8', 8), 4.90-5.00 (4H, m, H-1, 2, 3, 4), 7.10 (1H, s, H-2i), 7.15 (1H, t, *J* 7.5, H-5i), 7.24 (1H, t, *J* 7.5, H-6i), 7.50 (1H, d, $J_{7i,6i}$ 7.5, H-7i), 7.61 (1H, d, $J_{4i,5i}$ 7.5, H-4i), 8.35 (1H, s, NH); δ_C (125.8 MHz; C^2HCl_3) 18.5, 18.6, 21.0, 21.2 (4 x $OC(O)\underline{C}H_3$), 29.0 (C-8), 58.6 (C-6), 68.3 (C-4), 70.5 (C-2), 74.2 (C-3), 75.9 (C-5), 79.9 (C-1), 110.3 (C-3i), 112.2 (C-7i), 118.6 (C-4i), 120.1 (C-5i), 122.8 (C-6i), 123.5 (C-2i), 127.0 (C-9i), 136.8 (C-8i), 151.9 (C-7), 170.0, 170.1, 170.9, 171.5 (4 x

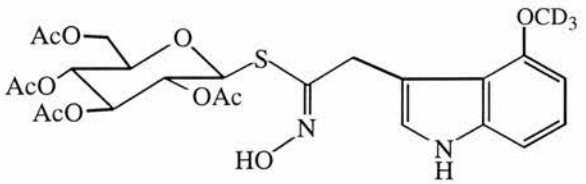
O<u>C</u>(O)CH_3); *m/z* (CI) 538 ($[MH_2]^+$, 1.0%), 537 (3.0, $[MH]^+$), 536 (4.0, $[M]^+$), 332 (59, [MH-Aglycone]$^+$), 271 (64, [M-AglyconeH-AcOH]$^+$) and 169 (100, [M-C_6H_6OSH-4HOAc]$^+$).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl 4-methoxyglucobrassicin thiohydroximate (128)



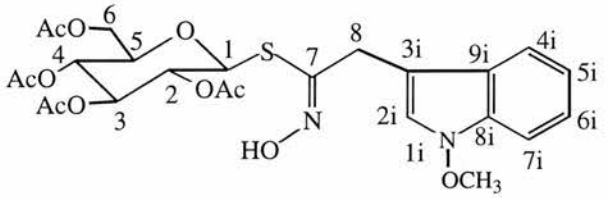
As described for (126) using 3-(2-nitroethyl)-4-methoxyindole (0.69 g, 3.1 mmol). The product was given as a cream coloured solid (0.29 g, 17%); m.p. 154-156 °C; $[\alpha]_D$ -4.0 ° (c 1.1 in $CHCl_3$) (lit.,[187, 188] -3.0 ° (c 1.1 in $CHCl_3$)); *m/z* (Found: $[M+H]^+$ 567.1640. $C_{25}H_{31}N_2O_{11}S$ requires 567.1649); ν_{max} (nujol)/cm^{-1} 3350 (NH), 1750 (C(O)CH_3); δ_H (500 MHz; C^2HCl_3) 1.80-2.10 (4 x 3H, 4s, 4 x OC(O)C<u>H</u>$_3$), 2.90 (1H, m, H-5), 3.65 (1H, d, J_{gem} 12.5, H-6a), 3.80 (3H, s, OC<u>H</u>$_3$), 3.90 (1H, d, J_{gem} 12.5, H-6b), 4.25 (2H, s, H-8, 8'), 4.90-5.00 (3H, m, H-2, 3, 4), 5.10 (1H, d, $J_{1,2}$ 9.0, H-1), 6.45 (1H, d, $J_{5i,6i}$ 8.5, H-5i), 6.85 (1H, s, H-2i), 6.90 (1H, d, *J* 8.5, H-7i), 7.04 (1H, t, *J* 8.5, H-6i), 8.70 (1H, s, NH), 9.90 (1H, s, OH); δ_C (125.8 MHz; C^2HCl_3) 21.0, 21.1, 21.5 (4 x OC(O)<u>C</u>H_3), 30.6 (C-8), 54.5 (OCH_3), 61.0 (C-6), 67.9 (C-4), 70.4 (C-2), 74.4 (C-3), 75.7 (C-5), 80.2 (C-1), 100.2 (C-5i), 105.7 (C-7i), 110.6 (C-3i), 116.7 (C-9i), 122.6 (C-2i), 123.4 (C-6i), 138.3 (C-8i), 153.4 (C-7), 154.7 (C-4i), 169.9, 170.0, 170.8, 171.4 (4 x O<u>C</u>(O)CH_3); *m/z* (CI) 567 ($[MH]^+$, 6%), 566 (2, $[M]^+$), 331 (100, [M-Aglycone]$^+$), 271 (38, [M-Aglycone-AcOH]$^+$), 187 (62, [Aglycone-S-OH]$^+$), 169 (50, [Aglycone-SH_2-$HOCH_3$]$^+$) and 160 (81, [Aglycone-$SCNOH_2$]$^+$).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl 4-[$^2H_3$]methoxyglucobrassicin thiohydroximate (129)



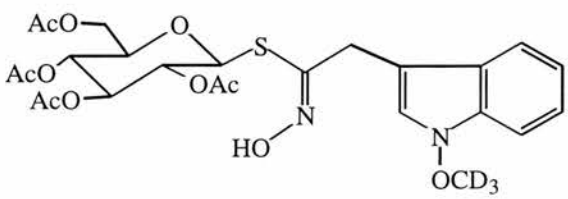
As described for (126) using 3-(2-nitroethyl)-4-[$^2H_3$]methoxyindole (1.0 g, 4.5 mmol). The product was given as a cream coloured solid (0.5 g, 20%); m.p. 90-96 °C; $[\alpha]_D$ -2.6 ° (c 1.1 in $CHCl_3$); (Found: C, 51.66; H, 5.12; N, 4.79. Calc for $C_{25}H_{27}{}^2H_3N_2O_{11}S.0.5H_2O$: C, 51.90; H, 5.40; N, 4.84%); *m/z* (Found: $[M+H]^+$ 570.1848. $C_{25}H_{28}{}^2H_3N_2O_{11}S$ requires 570.1837); ν_{max} (nujol)/cm^{-1} 3350 (NH, OH), 1750 ($C(O)CH_3$); δ_H (200 MHz; C^2HCl_3) 1.80-2.10 (4 x 3H, 4s, 4 x $OC(O)C\underline{H}_3$), 3.00 (1H, m, H-5), 3.70 (1H, d, J_{gem} 12.5, H-6a), 4.00 (1H, d, J_{gem} 12.5, H-6b), 4.25 (2H, s, H-8, 8'), 4.95-5.00 (3H, m, H-2, 3, 4), 5.10 (1H, d, $J_{1,2}$ 10, H-1), 6.50 (1H, d, $J_{5i,6i}$ 8.0, H-5i), 6.90 (1H, s, H-2i), 6.90 (1H, d, *J* 8.0, H-7i), 7.04 (1H, t, *J* 8.0, H-6i), 8.60 (1H, s, NH), 10.00 (1H, s, OH); δ_C (50.3 MHz; C^2HCl_3) 21.0, 21.1, 21.5 (4 x $OC(O)\underline{C}H_3$), 30.6 (C-8), 61.0 (C-6), 67.9 (C-4), 70.4 (C-2), 74.4 (C-3), 75.7 (C-5), 80.2 (C-1), 100.2 (C-5i), 105.7 (C-7i), 110.6 (C-3i), 116.7 (C-9i), 122.6 (C-2i), 123.4 (C-6i), 138.3 (C-8i), 153.4 (C-7), 154.7 (C-4i), 169.9, 170.0, 170.8, 171.4 (4 x $O\underline{C}(O)CH_3$); *m/z* (CI) 570 ($[MH]^+$, 3%), 569 (2, $[M]^+$), 331 (87, [M-Aglycone]$^+$), 271 (24, [M-Aglycone-AcOH]$^+$), 190 (68, [Aglycone-S-OH]$^+$), 169 (41, [Aglycone-SH_2-HOC^2H_3]$^+$) and 163 (100, [Aglycone-$SCNOH_2$]$^+$).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl 1-methoxyglucobrassicin thiohydroximate (130)



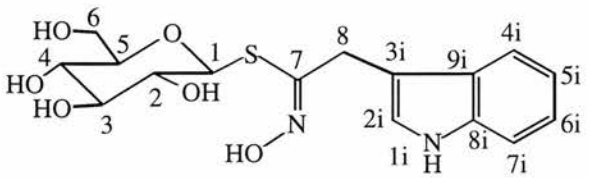
As described for (126) using 3-(2-nitroethyl)-1-methoxyindole (0.8 g, 3.6 mmol). The product was given as a cream coloured solid (0.69 g, 34%); m.p. 67-71 °C; $[\alpha]_D$ +10.6 ° (c 0.5 in $CHCl_3$); (Found: C, 52.47; H, 5.24; N, 4.66. Calc for $C_{25}H_{30}N_2O_{11}S.0.5H_2O$: C, 52.17; H, 5.43; N, 4.87%); *m/z* (Found: $[M+H]^+$ 567.1664. $C_{25}H_{31}N_2O_{11}S$ requires 567.1649); ν_{max} (nujol)/cm^{-1} 1750 ($C(O)CH_3$); δ_H (500 MHz; C^2HCl_3) 1.90-2.10 (4 x 3H, 4s, $OC(O)C\underline{H}_3$), 3.30 (1H, m, H-5), 4.00 (3H, m, H-6a, 8, 8'), 4.05 (3H, s, $OC\underline{H}_3$), 4.10 (1H, m, H-6b), 5.00 (4H, m, H-1, 2, 3, 4), 7.15 (1H, t, *J* 8.5, H-5i), 7.15 (1H, s, H-2i), 7.25 (1H, t, *J* 8.5, H-6i), 7.45 (1H, d, *J* 8.5, H-7i), 7.55 (1H, d, *J* 8.5. H-4i), 9.40 (1H, s, OH); δ_C (125.8 MHz; C^2HCl_3) 21.0, 21.2 (4 x $OC(O)CH_3$), 29.5 (C-8), 62.6 (C-6), 67.0 (OCH_3), 68.3 (C-4), 70.6 (C-2), 74.2 (C-3), 76.1 (C-5), 80.1 (C-1), 106.5 (C-3i), 109.2 (C-7i), 119.0 (C-4i), 120.6 (C-5i), 122.0 (C-9i), 122.2 (C-2i), 123.5 (C-6i), 132.9 (C-8i), 151.4 (C-7), 169.7, 169.9, 170.7, 171.2 (4 x $O\underline{C}(O)CH_3$); *m/z* (CI) 567 ($[MH]^+$, 3%), 566 (1.5, $[M]^+$), 331 (100, [M-Aglycone]$^+$), 271 (30, [M-Aglycone-AcOH]$^+$), 187 (17, [Aglycone-S-OH]$^+$), 169 (40, [Aglycone-SH_2-$HOCH_3$]$^+$) and 160 (45, [Aglycone-$SCNOH_2$]$^+$).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl 1-[$^2H_3$]methoxyglucobrassicin thiohydroximate (131)



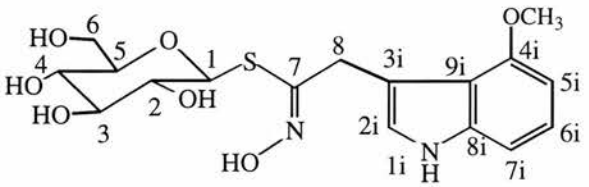
As described for (126) using 3-(2-nitroethyl)-1-[$^2H_3$]-methoxyindole (0.9 g, 4.0 mmol). The product was given as a cream coloured solid (0.82 g, 40%); m.p. 63-67 °C; $[\alpha]_D$ +9.0 ° (c 0.5 in $CHCl_3$); (Found: C, 52.85; H, 4.80; N, 4.63. Calc for $C_{25}H_{27}{}^2H_3N_2O_{11}S$: C, 52.72; H, 5.31; N, 4.92%); *m/z* (Found: $[M+H]^+$ 570.1824. $C_{25}H_{28}{}^2H_3N_2O_{11}S$ requires 570.1837); ν_{max} (nujol)/cm^{-1} 1750 ($C(O)CH_3$); δ_H (200 MHz; C^2HCl_3) 1.90-2.10 (4 x 3H, 4s, 4 x $OC(O)C\underline{H}_3$), 3.30 (1H, m, H-5), 4.00 (3H, m, H-6a, 8, 8'), 4.10 (1H, m, H-6b), 4.95 (4H, m, H-1, 2, 3, 4), 7.15 (1H, t, *J* 7.5, H-5i), 7.15 (1H, s, H-2i), 7.20 (1H, t, *J* 7.5, H-6i), 7.45 (1H, d, *J* 7.5, H-7i), 7.55 (1H, d, *J* 7.5. H-4i), 9.65 (1H, s, OH); δ_C (50.3 MHz; C^2HCl_3) 21.0, 21.2 (4 x $OC(O)CH_3$), 29.5 (C-8), 62.6 (C-6), 68.3 (C-4), 70.6 (C-2), 74.2 (C-3), 76.1 (C-5), 80.1 (C-1), 106.5 (C-3i), 109.2 (C-7i), 119.0 (C-4i), 120.6 (C-5i), 122.0 (C-9i), 122.2 (C-2i), 123.5 (C-6i), 132.9 (C-8i), 151.4 (C-7), 169.7, 169.9, 170.7, 171.2 (4 x $OC(O)CH_3$); *m/z* (CI) 570 ($[MH]^+$, 2%), 569 (1, $[M]^+$), 331 (100, [M-Aglycone]$^+$), 271 (30, [M-Aglycone-AcOH]$^+$), 190 (33, [Aglycone-S-OH]$^+$), 169 (52, [Aglycone-SH_2-HOC^2H_3]$^+$) and 163 (92, [Aglycone-$SCNOH_2$]$^+$).

Desulfoglucobrassicin (127)



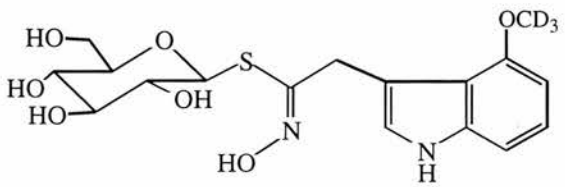
To a stirred solution of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl glucobrassicin thiohydroximate (0.4 g, 0.745 mmol) in anhydrous methanol (5 ml) was added a catalytic amount of potassium metal. After stirring for 18 hours at room temperature under nitrogen, Amberlite IR-120 resin was added. The solution was stirred for a further 15 minutes then the Amberlite was removed by filtration and the solvent evaporated at reduced pressure. The residue was purified by column chromatography on silica gel using dichloromethane-methanol (85:15) to give the product as a cream coloured amorphous solid (0.15 g, 55%); m.p. 105-109 °C; $[\alpha]_D$ +2.7 ° (c 0.7 in CH_3OH) (lit.,[102] +2.0 ° (c 0.70 in CH_3OH)); (Found: C, 50.78; H, 5.85; N, 6.82. Calc for $C_{16}H_{20}N_2O_6S.0.5H_2O$: C, 50.92; H, 5.61; N, 7.42%); *m/z* (Found: $[M+H]^+$ 369.1130. $C_{16}H_{21}N_2O_6S$ requires 369.1120); ν_{max} (nujol)/cm^{-1} 3300-3000 (OH, NH); δ_H (300 MHz; $C^2H_3O^2H$) 3.00-3.40 (4H, m, H-2,3,4,5), 3.60 (1H, dd, $J_{5,6b'}$ 5, $J_{6,6'}$ 12.5, H-6b), 3.85 (1H, dd, $J_{5,6a}$ 2.5, J_{gem} 12.5, H-6a), 3.95 (1H, d, J_{gem} 17.5, H-8'), 4.30 (1H, d, J_{gem} 17.5, H-8), 4.75 (1H, d, $J_{1,2}$ 10, H-1), 7.00 (1H, t, *J* 7.7, H-5i), 7.10 (1H, t, *J* 7.7, H-6i), 7.18 (1H, s, H-2i), 7.35 (1H, d, $J_{7i,6i}$ 7.7, H-7i), 7.70 (1H, d, $J_{4i,5i}$ 7.7, H-4i); δ_C (75.45 MHz; $C^2H_3O^2H$) 30.3 (C-8), 62.7 (C-6), 71.1 (C-4), 74.4 (C-2), 79.3 (C-3), 82.0 (C-5), 82.8 (C-1), 111.4 (C-3i), 112.3 (C-7i), 119.5 (C-4i), 119.9 (C-5i), 122.6 (C-6i), 123.9 (C-2i), 128.2 (C-9i), 138.1 (C-8i), 154.4 (C-7); *m/z* (CI) 369 ($[MH]^+$, 4%), 175 (65, $[AglyconeH_2\text{-}S]^+$), 157 (39, $[C_6H_5O_5]^+$) and 130 (100, $[indole+CH_2]^+$).

Desulfo-4-methoxyglucobrassicin (132)



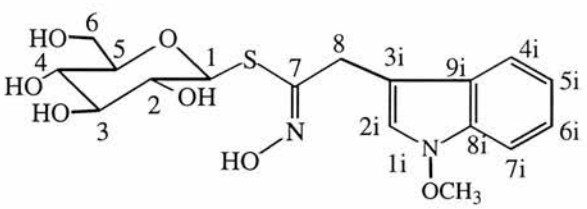
As described for (127) using 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl 4-methoxyglucobrassicin thiohydroximate (0.1 g, 0.18 mmol). The product was given as a cream coloured amorphous solid (0.05 g, 73%); m.p. 117-119 °C; $[\alpha]_D$ +19.4 ° (c 0.4 in CH_3OH) (lit.,[187, 188] -25.0 ° (c 0.4 in CH_3OH)); *m/z* (Found: $[M]^+$ 398.1148. $C_{17}H_{22}N_2O_7S$ requires 398.1147); ν_{max} (nujol)/cm^{-1} 3300-3000 (OH); δ_H (500 MHz; $C^2H_3O^2H$) 3.15 (1H, m, H-5), 3.25 (1H, t, *J* 8.3, H-3) 3.70 (1H, t, *J* 8.3, H-2), 3.55 (1H, t, *J* 8.3, H-4), 3.80 (1H, dd, $J_{5,6b}$ 5, J_{gem} 12.5, H-6b), 3.90 (1H, dd, $J_{5,6a}$ 2.5, J_{gem} 12.5, H-6a), 4.10 (3H, s, CH_3), 4.20 (1H, d, J_{gem} 17.5, H-8'), 4.60 (1H, d, J_{gem} 17.5, H-8), 4.90 (1H, d, $J_{1,2}$ 8.3, H-1), 6.65 (1H, d, $J_{5i,6i}$ 8.6, H-5i), 7.10 (1H, s, H-2i), 7.13 (1H, d, $J_{7i,6i}$ 8.6, H-7i), 7.18 (1H, t, *J* 8.6, H-6i); δ_C (125.8 MHz; $C^2H_3O^2H$) 31.7 (C-8), 55.8 (CH_3), 62.5 (C-6), 70.8 (C-4), 74.5 (C-2), 79.8 (C-3), 81.8 (C-5), 83.7 (C-1), 100.4 (C-5i), 106.3 (C-7i), 112.2 (C-3i), 118.0 (C-9i), 122.7 (C-2i), 123.7 (C-6i), 140.0 (C-8i), 155.6 (C-7), 156.2 (C-4i); *m/z* (APCI) 399 ($[MH]^+$, 100%), 237 (13, $[AglyconeH]^+$) and 160 (93, $[CH_3OindoleCH_2]^+$).

Desulfo-4-[$^2H_3$]methoxyglucobrassicin (133)



As described for (127) using 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl 4-[$^2H_3$]-methoxyglucobrassicin thiohydroximate (0.15 g, 0.26 mmol). The product was given as a cream coloured amorphous solid (0.06 g, 58%); m.p. 94-100 °C; $[\alpha]_D$ -21.6 ° (c 0.4 in CH_3OH); (Found: C, 49.01; H, 5.81; N, 6.63. Calc for $C_{17}H_{19}{}^2H_3N_2O_7S.H_2O$: C, 48.68; H, 5.77; N, 6.68%); ν_{max} (nujol)/cm^{-1} 3300-3000 (OH); δ_H (200 MHz; $C^2H_3O^2H$) 3.00 (1H, m, H-5), 3.05 (1H, t, *J* 10, H-3) 3.20 (1H, t, *J* 10, H-2), 3.35 (1H, t, *J* 10, H-4), 3.65 (1H, dd, $J_{5,6b}$ 5, J_{gem} 12.5, H-6b), 3.70 (1H, dd, $J_{5,6a}$ 2.5, J_{gem} 12.5, H-6a), 4.10 (1H, d, J_{gem} 17.5, H-8'), 4.50 (1H, d, J_{gem} 17.5, H-8), 4.75 (1H, d, $J_{1,2}$ 10, H-1), 6.50 (1H, d, $J_{5i,6i}$ 7.5, H-5i), 6.90 (1H, s, H-2i), 6.95-7.05 (2H, m, H-6i, 7i); δ_C (50.3 MHz; $C^2H_3O^2H$) 31.7 (C-8), 62.5 (C-6), 70.8 (C-4), 74.5 (C-2), 79.8 (C-3), 81.8 (C-5), 83.7 (C-1), 100.4 (C-5i), 106.3 (C-7i), 112.2 (C-3i), 118.0 (C-9i), 122.7 (C-2i), 123.7 (C-6i), 140.0 (C-8i), 155.6 (C-7), 156.2 (C-4i); *m/z* (APCI) 402 ([MH]$^+$, 100%), 240 (13, [AglyconeH]$^+$) and 163 (71, [C^2H_3OindoleCH$_2$]$^+$).

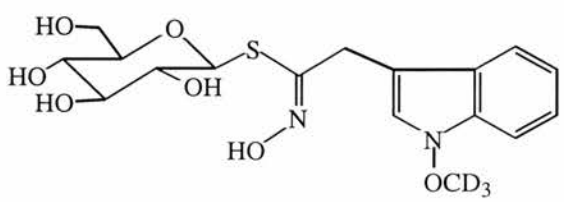
Desulfo-1-methoxyglucobrassicin (134)



As described for (127) using 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl 1-methoxyglucobrassicin thiohydroximate (0.5 g, 0.88 mmol). The product was given as a cream coloured amorphous solid (0.132 g, 38%); m.p. 93-95 °C; $[\alpha]_D$ -11.2 ° (c 0.5 in

CH_3OH); (Found: C, 49.95; H, 5.33; N, 6.19. Calc for $C_{17}H_{22}N_2O_7S.0.5H_2O$: C, 50.11; H, 5.69; N, 6.87%); ν_{max} (nujol)/cm^{-1} 3300-3000 (OH); δ_H (300 MHz; $C^2H_3O^2H$) 3.10-3.45 (4H, m, H-2, 3, 4, 5), 3.65 (1H, dd, $J_{5,6b}$ 5, J_{gem} 12.5, H-6b), 3.75 (1H, dd, $J_{5,6a}$ 2.5, J_{gem} 12.5, H-6a), 3.90 (1H, d, J_{gem} 17.5, H-8'), 4.00 (3H, s, OCH_3), 4.20 (1H, d, J_{gem} 17.5, H-8), 4.70 (1H, d, $J_{1,2}$ 10, H-1), 7.10 (1H, t, *J* 7.5, H-5i), 7.20 (1H, t, *J* 7.5, H-6i), 7.30 (1H, s, H-2i), 7.40 (1H, d, *J* 7.5, H-7i), 7.70 (1H, d, *J* 7.5, H-4i); δ_C (75.45 MHz; $C^2H_3O^2H$) 30.1 (C-8), 62.7 (C-6), 66.3 (OCH_3), 71.2 (C-4), 74.4 (C-2), 79.4 (C-3), 82.2 (C-5), 82.9 (C-1), 108.7 (C-3i), 109.3 (C-7i), 120.3 (C-4i), 120.9 (C-5i), 123.0 (C-9i), 123.7 (C-2i), 124.8 (C-6i), 134.1 (C-8i), 153.7 (C-7); *m/z* (APCI) 399 ($[MH]^+$, 100%), 237 (25, [AglyconeH]$^+$) and 160 (11, $[CH_3OindoleCH_2]^+$).

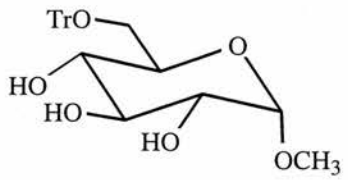
Desulfo-1-[$^2H_3$]methoxyglucobrassicin (135)



As described for (127) using 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl 4-[$^2H_3$]methoxyglucobrassicin thiohydroximate (0.5 g, 0.88 mmol). The product was given as a cream coloured amorphous solid (0.18 g, 51%); m.p. 69-71 °C; $[\alpha]_D$ -10.2 ° (c 0.5 in CH_3OH); (Found: C, 49.50; H, 5.77; N, 6.54. Calc for $C_{17}H_{19}{}^2H_3N_2O_7S.0.5H_2O$: C, 49.75; H, 5.65; N, 6.82%); ν_{max} (nujol)/cm^{-1} 3300-3000 (OH); δ_H (200 MHz; $C^2H_3O^2H$) 3.10-3.45 (4H, m, H-2, 3, 4, 5), 3.65 (1H, dd, $J_{5,6b}$ 5, J_{gem} 12.5, H-6b), 3.75 (1H, dd, $J_{5,6a}$ 2.5, J_{gem} 12.5, H-6a), 4.00 (1H, d, J_{gem} 17.5, H-8'), 4.25 (1H, d, J_{gem} 17.5, H-8), 4.75 (1H, d, $J_{1,2}$ 10, H-1), 7.10 (1H, t, *J* 7.5, H-5i), 7.20 (1H, t, *J* 7.5, H-6i), 7.30 (1H, s, H-2i), 7.40 (1H, d, *J* 7.5, H-7i), 7.70 (1H, d, *J* 7.5, H-4i); δ_C (50.3 MHz; $C^2H_3O^2H$) 30.1 (C-8), 62.7 (C-6), 71.2 (C-4), 74.4 (C-2), 79.4 (C-3), 82.2 (C-5), 82.9 (C-1), 108.7 (C-3i), 109.3 (C-7i), 120.3 (C-4i), 120.9 (C-5i), 123.0 (C-9i), 123.7 (C-2i), 124.8 (C-6i), 134.1 (C-8i), 153.7 (C-7); *m/z* (APCI) 402 ($[MH]^+$, 100%), 240 (27, [AglyconeH]$^+$) and 163 (12, $[C^2H_3OindoleCH_2]^+$).

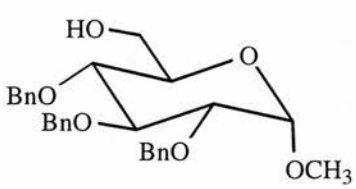
3.4 Deuterated glucopyranose

Methyl-6-*O*-trityl-α-D-glucopyranoside (137)



Methyl α-D-glucopyranoside (10.0 g, 52 mmol) in dry pyridine (100 ml) was treated with chlorotriphenylmethane (18.0 g, 65 mmol) and 4-(dimethylamino)pyridine (1.03 g, 8.4 mmol). The reaction was stirred under nitrogen for 16 hours and the solvent evaporated at reduced pressure. The residue was then recrystallised from ethanol to give methyl-6-*O*-trityl-α-D-glucopyranoside (22.62 g, 100%); m.p. 149-150 °C (lit.,[226] 148-149 °C); $[\alpha]_D$ +102.4 ° (c 0.5 in CH_3OH); ν_{max} (nujol)/cm^{-1} 3400-3000 (OH); δ_H (200 MHz; C^2HCl_3) 3.20-3.90 (6H, m, H-2, 3, 4, 5, 6a, 6b), 3.41 (3H, s, $OC\underline{H}_3$), 4.65 (1H, d, $J_{1,2}$ 3.9, H-1), 7.10-7.60 (15H, m, 3 x Ph); δ_C (50.3 MHz; CH_3OH) 55.6 ($O\underline{C}H_3$), 65.1 (C-6), 72.7 (C-4), 73.7 (C-2), 75.6 (C-3), 87.8 (C-5), 101.3 (C-1), 128.3 (quaternary C, 3 x Ph), 129.0, 130.2 (15CH, 3 x Ph), 145.8 ($\underline{C}Ph_3$); *m/z* (CI) 437 ($[MH]^+$, 1%), 243 (100, $[CPh_3]^+$), 163 (54, $[M\text{-}CPh_3\text{-}OCH_3]^+$) and 145 (25, $[M\text{-}CPh_3\text{-}OCH_3\text{-}HO]^+$).

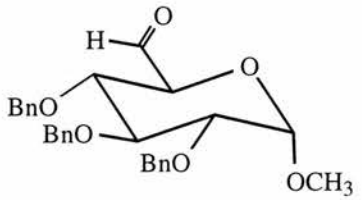
Methyl-2,3,4-tri-*O*-benzyl-α-D-glucopyranoside (139a)



Sodium hydride (60% dispersion in mineral oil) (9.0 g, 351 mmol) was washed with dry diethyl ether (2 x 100 ml) then treated with benzyl bromide (4.75 g, 3.3 ml, 27.8 mmol). Methyl-6-*O*-trityl-α-D-glucopyranoside (12.0 g, 27.6 mmol) in dry *N*,*N*-

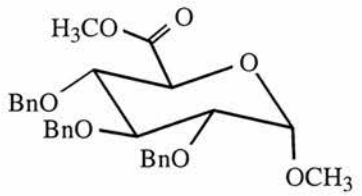
dimethylformamide (130 ml) was added to the reaction and after 4 hours at room temperature the reaction was quenched by dropwise addition of methanol (60 ml). The solution was concentrated then the residue was taken up in diethyl ether (200 ml) before washing with water (150 ml). The aqueous phase was extracted with diethyl ether (2 x 100 ml) then the combined organic extracts were washed with brine (100 ml), dried ($MgSO_4$) and the solvent evaporated at reduced pressure to give methyl-2,3,4-tri-*O*-benzyl-α-D-glucopyranose as a viscous oil. The crude methyl-2,3,4-tri-*O*-benzyl-6-*O*-trityl-α-D-glucopyranoside was taken up in a solution of methanol (120 ml) and dichloromethane (60 ml) then *p*-toluene sulfonic acid was added until pH<4. The reaction was stirred for 12 hours then neutralised with triethylamine and the solvent evaporated at reduced pressure. The residue was taken up in diethyl ether (300 ml) and washed with water (200 ml). The aqueous phase was extracted with diethyl ether (2 x 100 ml) then the combined organic extracts were washed with brine (100 ml), dried ($MgSO_4$) and the solvent evaporated at reduced pressure. The resulting yellow oil was purified by column chromatography on silica gel with ethyl acetate-hexane (1:2) as the eluant to give methyl-2,3,4-*O*-benzyl-α-D-glucopyranoside as a white crystalline solid (10.27 g, 80%); m.p. 66.5-67.0 °C (lit.,[226] 66.5-67.0 °C); $[\alpha]_D$ +26.6 ° (c 1.0 in $CHCl_3$) (lit.,[226] +25.4 ° (c 1.0 in $CHCl_3$); (Found: C, 71.87; H, 6.93. Calc. for $C_{28}H_{32}O_6$: C, 72.39; H, 6.94%); ν_{max} (nujol)/cm^{-1} 3478 (OH); δ_H (200 MHz; C^2HCl_3) 3.37 (3H, s, $OC\underline{H}_3$), 3.52-3.80 (5H, m, H-2, 4, 5, 6a, 6b), 4.00 (1H, t, $J_{2,3}$=$J_{3,4}$ 10, H-3), 4.58 (1H, d, *J* 4, H-1), 4.64 (1H, d, *J* 11, $PhC\underline{H}_2$), 4.67 (1H, d, *J* 11, $PhC\underline{H}_2$), 4.81 (1H, d, *J* 11, $PhC\underline{H}_2$), 4.85 (1H, d, *J* 11, $PhC\underline{H}_2$), 4.90 (1H, d, *J* 11, $PhC\underline{H}_2$), 5.00 (1H, d, *J* 11, $PhC\underline{H}_2$), 7.20-7.40 (15H, m, 3 x $\underline{Ph}CH_2$); δ_C (50.3 MHz; C^2HCl_3) 55.2 ($O\underline{C}H_3$), 61.9 (C-6), 70.7 (C-5), 73.5 ($Ph\underline{C}H_2$), 75.1 ($Ph\underline{C}H_2$), 75.8 ($Ph\underline{C}H_2$), 77.5 (C-4), 80.1 (C-2), 82.0 (C-3), 98.3 (C-1), 127.7-128.6 (15$\underline{C}$H, 3 x $\underline{Ph}CH_2$), 137.9, 138.0, 138.6 (3 x quaternary C, 3 x Ph); *m/z* (CI) 465 ($[MH]^+$, 1%), 464 (5, $[M]^+$), 463 (13, $[M-H]^+$), 433 (43, $[M-CH_3O]^+$), 373 (38, $[M-PhCH_2]^+$), 341 (39, $[M-PhCH_2-CH_3OH]^+$), 271 (42, $[Glucose+PhCH_2]^+$), 181 (81, $[GlucoseH]^+$) and 91 (100, $[PhCH_2]^+$).

Methyl 6-aldehydo-2,3,4-tri-*O*-benzyl-α-D-glucopyranoside (140)



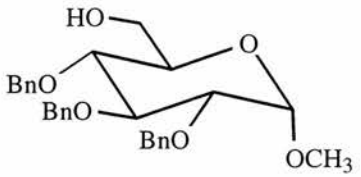
To dichloromethane (120 ml) and oxalyl chloride (2.0 M solution in dichloromethane, 26.7 ml, 53.4 mmol) at -75 °C was added dimethyl sulfoxide (7.55 ml) followed by a solution of methyl-2,3,4-tri-*O*-benzyl-α-D-glucopyranose (23.2 g, 50 mmol) in dichloromethane (145 ml). The reaction was stirred for 1 hour and then triethylamine (32 ml, 233 mmol) was added. The reaction was allowed to warm to room temperature and left to stir for a further 2 hours. The reaction mixture was washed with water (250 ml) and the aqueous layer was extracted with dichloromethane (2 x 150 ml). The organic extracts were washed with brine (150 ml), dried ($MgSO_4$) and the solvent evaporated at reduced pressure to give a viscous oil which was purified by column chromatography on silica gel using ethyl acetate-hexane (1:1) as the eluant to give methyl 6-aldehydo-2,3,4-tri-*O*-benzyl-α-D-glucopyranoside as a clear oil (20 g, 86%); $[\alpha]_D$ +4.9 ° (c 0.38 in $CHCl_3$) (lit.,[262] +13.6 (c 2.6 in $CHCl_3$)); ν_{max} (nujol)/cm^{-1} 1726 (CHO); δ_H (200 MHz; C^2HCl_3) 3.40 (3H, s, $OC\underline{H}_3$), 3.53 (1H, dd, $J_{1,2}$ 3, $J_{2,3}$ 10, H-2), 3.60 (1H, t, $J_{3,4}$=$J_{4,5}$ 10, H-4), 4.12 (1H, t, $J_{2,3}$=$J_{3,4}$ 10, H-3), 4.22 (1H, d, $J_{4,5}$ 10, H-5), 4.64 (1H, d, $J_{1,2}$ 3, H-1), 4.65-5.50 (6H, m, 3 x $PhC\underline{H}_2$), 7.20-7.45 (15H, m, 3 x $\underline{Ph}CH_2$), 9.70 (1H, s, $C\underline{H}O$); δ_C (50.3 MHz; C^2HCl_3) 56.4 ($O\underline{C}H_3$), 74.2 ($Ph\underline{C}H_2$), 74.7 ($Ph\underline{C}H_2$), 75.7 ($Ph\underline{C}H_2$), 76.5 (C-5), 78.3 (C-4), 79.7 (C-3), 82.3 (C-2), 98.9 (C-1), 128.3-129.1 (15$\underline{C}$H, 3 x $\underline{Ph}CH_2$), 137.9, 138.3, 138.8 (3 x quaternary C, 3 x Ph), 198.1 ($\underline{C}HO$); *m/z* (CI) 463 ($[MH]^+$, 3%), 462 (6, $[M]^+$), 461 (21, $[M-H]^+$), 371 (61, $[M-PhCH_2]^+$), 323 (52, $[M-PhCH_2-OCH_3-OH]^+$), 271 (30, $[glucose+PhCH_2]^+$), 233 (40, $[M-2PhCH-OCH_3-OH_2]^+$), 181 (95, $[glucoseH]^+$) and 91 (100, $[PhCH_2]^+$).

Methyl (methyl 2,3,4-tri-*O*-benzyl-α-D-glucopyranosid)uronate (141)



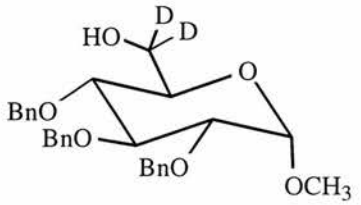
Methyl 6-aldehydo-2,3,4-tri-*O*-benzyl-α-D-glucopyranoside (20 g, 35.3 mmol) was dissolved in dry *N*,*N*-dimethylformamide (430 ml) and dry methanol (8.7 ml, 212 mmol) then the reaction flask was covered to exclude light and cooled to 0 °C. Pyridinium dichromate (80 g, 212 mmol) was added in one portion and the reaction was stirred for 30 minutes before warming to room temperature and stirring overnight. The reaction mixture was added to a column of silica gel with a top layer of ethyl acetate. The column was eluted with ethyl acetate until no further sugar was obtained. The solvent was evaporated at reduced pressure and the product purified using a second column of silica gel with ethyl acetate-hexane (1:6) as the eluant. Methyl (methyl 2,3,4-tri-*O*-benzyl-α-D-glucopyranosid)uronate was given as a clear viscous oil (14.53 g, 68%); $[\alpha]_D$ +26.6 ° (c 1.0 in $CHCl_3$) (lit.,[263] +17.6 (c 0.967 in $CHCl_3$)); (Found: C, 71.07; H, 6.72. Calc. for $C_{29}H_{32}O_7$: C, 70.72; H, 6.55%); ν_{max} (nujol)/cm^{-1} 1748 (C=O); δ_H (200 MHz; C^2HCl_3) 3.40 (3H, s, OC<u>H</u>$_3$), 3.60 (1H, dd, $J_{1,2}$ 3, $J_{2,3}$ 10, H-2), 3.70 (3H, s, C(O)C<u>H</u>$_3$), 3.75 (1H, t, $J_{3,4}$=$J_{4,5}$ 10, H-4), 4.00 (1H, t, $J_{2,3}$=$J_{3,4}$ 10, H-3), 4.20 (1H, d, $J_{4,5}$ 10, H-5), 4.58 (1H, d, J 11, PhC<u>H</u>$_2$), 4.60 (1H, d, $J_{1,2}$ 3, H-1), 4.82-5.00 (5H, m, PhC<u>H</u>$_2$), 7.20-7.40 (15H, m, 3 x <u>Ph</u>CH$_2$); δ_C (50.3 MHz; C^2HCl_3) 53.1 (C(O)O<u>C</u>H$_3$), 56.2 (O<u>C</u>H$_3$), 70.6 (Ph<u>C</u>H$_2$), 74.2 (Ph<u>C</u>H$_2$), 75.7 (Ph<u>C</u>H$_2$), 76.5 (C-5), 79.7 (C-4), 80.0 (C-2), 81.9 (C-3), 99.2 (C-1), 128.3-129.1 (15<u>C</u>H, 3 x <u>Ph</u>CH$_2$), 138.3, 138.4, 139.0 (3 x quaternary C, 3 x Ph), 168.4 (C=O); *m/z* (EI) 493 ($[M]^+$, 1%), 401 (75, $[M\text{-}PhCH_2H]^+$), 369 (32, $[M\text{-}PhCH_2\text{-}CH_3\text{-}OH_2]^+$), 253 (25, $[M\text{-}2PhCH_2\text{-}CO_2CH_2]^+$), 181 (18, [glucoseH]$^+$) and 91 (100, $[PhCH_2]^+$).

Methyl-2,3,4-tri-*O*-benzyl-α-D-glucopyranoside (139b)



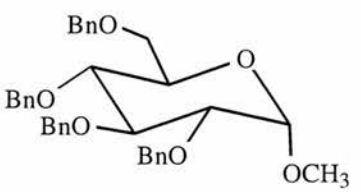
Methyl (methyl 2,3,4-tri-*O*-benzyl-α-D-glucopyranosid)uronate (0.05 g, 0.1 mmol) was dissolved in dry diethyl ether (1 ml) at 0 °C and treated with lithium aluminium hydride (1.0 M solution in diethyl ether) (0.05 ml, 0.05 mmol). The reaction was stirred at room temperature overnight, quenched using 1.0 M sulfuric acid and extracted using ethyl acetate (2 x 10 ml). The organic extracts were washed with brine (5 ml), dried and the solvent evaporated at reduced pressure to yield methyl-2,3,4-*O*-benzyl-α-D-glucopyranoside as a white crystalline solid (0.046 g, 100%); m.p. 66.5-67.0 °C (lit.,[226] 66.5-67.0 °C); $[\alpha]_D$ +26.6 ° (c 1.0 in $CHCl_3$) (lit.,[226] +25.4 (c 1.0 in $CHCl_3$)); (Found: C, 71.87; H, 6.93. Calc. for $C_{28}H_{32}O_6$: C, 72.39; H, 6.94%); ν_{max} (nujol)/cm^{-1} 3478 (OH); δ_H (200 MHz; C^2HCl_3) 3.37 (3H, s, $OC\underline{H}_3$), 3.52-3.80 (5H, m, H-2, 4, 5, 6a, 6b), 4.00 (1H, t, $J_{2,3}=J_{3,4}$ 10, H-3), 4.58 (1H, d, *J* 4, H-1), 4.64 (1H, d, *J* 11, $PhC\underline{H}_2$), 4.67 (1H, d, *J* 11, $PhC\underline{H}_2$), 4.81 (1H, d, *J* 11, $PhC\underline{H}_2$), 4.85 (1H, d, *J* 11, $PhC\underline{H}_2$), 4.90 (1H, d, *J* 11, $PhC\underline{H}_2$), 5.00 (1H, d, *J* 11, $PhC\underline{H}_2$), 7.20-7.40 (15H, m, 3 x $\underline{Ph}CH_2$); δ_C (50.3 MHz; C^2HCl_3) 55.2 ($O\underline{C}H_3$), 61.9 (C-6), 70.7 (C-5), 73.5 ($Ph\underline{C}H_2$), 75.1 ($Ph\underline{C}H_2$), 75.8 ($Ph\underline{C}H_2$), 77.5 (C-4), 80.1 (C-2), 82.0 (C-3), 98.3 (C-1), 127.7-128.6 (15$\underline{C}$H, 3 x $\underline{Ph}CH_2$), 137.9, 138.0, 138.6 (3 x quaternary C, 3 x Ph); *m/z* (CI) 465 ($[MH]^+$, 1%), 464 (5, $[M]^+$), 463 (13, $[M-H]^+$), 433 (43, $[M-CH_3O]^+$), 373 (38, $[M-PhCH_2]^+$), 341 (39, $[M-PhCH_2-CH_3OH]^+$), 271 (42, $[glucose+PhCH_2]^+$), 181 (81, $[glucoseH]^+$) and 91 (100, $[PhCH_2]^+$).

Methyl 2,3,4-tri-*O*-benzyl-α-D-[6-$^2H_2$]glucopyranoside (142)



As described for (139b) using lithium aluminium deuteride. The product was purified by column chromatography on silica gel using ethyl acetate-hexane (1:2) as the eluant to give methyl 2,3,4-tri-*O*-benzyl-α-D-[6-$^2H_2$]glucopyranoside as a white crystalline solid (6.87 g, 91%); m.p. 50-51 °C; $[\alpha]_D$ +21.7 ° (c 1.0 in $CHCl_3$); (Found: C, 71.58; H, 6.71. Calc. for $C_{28}H_{30}{}^2H_2O_6$: C, 72.08; H, 6.91%); ν_{max} (nujol)/cm^{-1} 3479 (OH); δ_H (200 MHz; C^2HCl_3) 3.37 (3H, s, OC<u>H</u>$_3$), 3.51 (1H, dd, $J_{1,2}$ 3, $J_{2,3}$ 10, H-2), 3.53 (1H, t, $J_{3,4}$=$J_{4,5}$ 10, H-4), 3.63 (1H, d, $J_{4,5}$ 10, H-5), 4.02 (1H, t, $J_{2,3}$=$J_{3,4}$ 10, H-3), 4.57 (1H, d, J 3, H-1), 4.60-5.10 (6H, m, 3 x PhC<u>H</u>$_2$), 7.20-7.40 (15H, m, 3 x <u>Ph</u>CH$_2$); δ_C (50.3 MHz; C^2HCl_3) 55.7 (O<u>C</u>H$_3$), 71.0 (C-5), 74.0 (Ph<u>C</u>H$_2$), 75.6 (Ph<u>C</u>H$_2$), 76.3 (Ph<u>C</u>H$_2$), 77.8 (C-4), 80.4 (C-2), 82.5 (C-3), 98.7 (C-1), 128.2-129.0 (15<u>C</u>H, 3 x <u>Ph</u>CH$_2$), 138.5, 138.6, 139.2 (3 x quaternary C, 3 x Ph); *m/z* (CI) 467 ([MH]$^+$, 1%), 465 (4, [M-H]$^+$), 435 (37, [M-C$^2H_2$OH]$^+$), 375 (29, [M-PhC$^2H_2$]$^+$), 343 (29, [M-PhCH$_2$-CH$_3$OH]$^+$), 271 (60, [glucose+PhCH$_2$]$^+$), 181 (94, [glucoseH]$^+$) and 91 (100, [PhCH$_2$]$^+$).

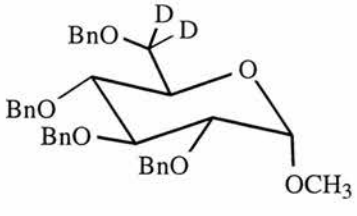
Methyl 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranoside (151)



Sodium hydride (60% dispersion in mineral oil) (0.12 g, 4.6 mmol) was washed with dry diethyl ether (2 x 5 ml) then treated with benzyl bromide (0.37 g, 0.26 ml, 2.1 mmol). Methyl-2,3,4-*O*-benzyl-α-D-glucopyranoside (0.5 g, 1.08 mmol) in dry *N*,*N*-

dimethylformamide (5 ml) was added to the reaction and after 2 hours at room temperature the reaction was quenched by dropwise addition of methanol (10 ml). The solvent was evaporated at reduced pressure then the residue was taken up in diethyl ether (20 ml) and washed with water (15 ml). The aqueous phase was extracted with diethyl ether (2 x 10 ml) then the combined organic extracts were washed with brine (15 ml), dried ($MgSO_4$) and the solvent evaporated at reduced pressure to give the crude product. The resulting oil was purified by column chromatography using ethyl acetate-hexane (1:2) as the eluant to give methyl 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranoside as a clear viscous oil (0.6 g, 100%); $[\alpha]_D$ +37.9 ° (c 1.0 in $CHCl_3$) (lit.,[264] +23.8 ° (c 1.0 in $CHCl_3$) for α form); δ_H (200 MHz; C^2HCl_3) 3.40 (3H, s, $OC\underline{H}_3$), 3.60 (1H, dd, $J_{1,2}$ 3, $J_{2,3}$ 10, H-2), 3.65-3.80 (5H, m, H-4, 5, 6a, 6b), 4.00 (1H, t, $J_{2,3}$=$J_{3,4}$ 10, H-3), 4.40-5.10 (9H, m, 4 x $PhC\underline{H}_2$, H-1), 7.10-7.40 (20H, m, 4 x $\underline{Ph}CH_2$); δ_C (50.3 MHz; C^2HCl_3) 55.7 ($O\underline{C}H_3$), 68.8 (C-6), 70.5 (C-5), 73.9 ($Ph\underline{C}H_2$), 74.0 ($Ph\underline{C}H_2$), 75.6 ($Ph\underline{C}H_2$), 76.3 ($Ph\underline{C}H_2$), 78.1 (C-4), 80.2 (C-2), 82.6 (C-3), 98.7 (C-1), 128.2-129.0 (20$\underline{C}$H, 4 x $\underline{Ph}CH_2$), 138.4, 138.6, 138.7, 139.2 (4 x quaternary C, 4 x Ph); *m/z* (CI) 555 ($[MH]^+$, 1%), 554 (2, $[M]^+$), 553 (5, $[M-H]^+$), 271 (60, $[glucose+PhCH_2]^+$), 217 (33, $[M-3PhCH_2O-CH_4]^+$), 181 (94, $[glucoseH]^+$) and 91 (100, $[PhCH_2]^+$).

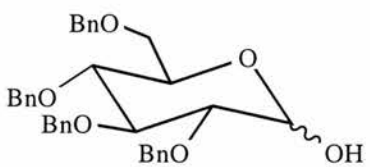
Methyl 2,3,4,6-tetra-*O*-benzyl-α-D-[6-$^2H_2$]glucopyranoside (143)



As described for (151) using methyl 2,3,4-*O*-benzyl-α-D-[6-$^2H_2$]glucopyranoside (6.3 g, 13.5 mmol). Methyl 2,3,4,6-tetra-*O*-benzyl-α-D-[6-$^2H_2$]glucopyranoside was given as a clear viscous oil (7.14 g, 95%); $[\alpha]_D$ +37.4 ° (c 1.0 in $CHCl_3$); (Found: C, 75.53; H, 6.86. Calc. for $C_{35}H_{36}{}^2H_2O_6$: C, 75.51; H, 6.88%); δ_H (200 MHz; C^2HCl_3) 3.40 (3H, s, $OC\underline{H}_3$), 3.60 (1H, dd, $J_{1,2}$ 3, $J_{2,3}$ 10, H-2), 3.70 (1H, t, $J_{2,3}$=$J_{3,4}$ 10, H-4), 3.77 (1H, d,

$J_{4,5}$ 10, H-5), 4.00 (1H, t, $J_{3,4}$=$J_{4,5}$ 10, H-3), 4.40-5.10 (9H, m, 4 x PhC<u>H</u>$_2$, H-1), 7.10-7.40 (20H, m, 4 x <u>Ph</u>CH$_2$); δ_C (50.3 MHz; C^2HCl_3) 55.7 (O<u>C</u>H$_3$), 70.4 (C-5), 73.9 (Ph<u>C</u>H$_2$), 74.0 (Ph<u>C</u>H$_2$), 75.6 (Ph<u>C</u>H$_2$), 76.3 (Ph<u>C</u>H$_2$), 78.1 (C-4), 80.2 (C-2), 82.6 (C-3), 98.7 (C-1), 128.2-129.0 (20<u>C</u>H, 4 x <u>Ph</u>CH$_2$), 138.4, 138.6, 138.7, 139.2 (4 x quaternary C, 4 x Ph); *m/z* (CI) 557 ([MH]$^+$, 1%), 555 (5, [M-H]$^+$), 271 (60, [glucose+PhCH$_2$]$^+$), 219 (33, [M-3PhCH$_2$O-CH$_4$]$^+$), 181 (94, [glucoseH]$^+$) and 91 (100, [PhCH$_2$]$^+$).

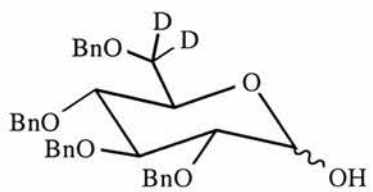
2,3,4,6-Tetra-*O*-benzyl-D-glucopyranose (152a)



Glacial acetic acid (4 ml) and 1.0 M sulfuric acid (1 ml) were heated at 105 °C and a solution of methyl 2,3,4,6-tetra-*O*-benzyl-D-glucopyranoside (0.5 g, 0.9 mmol) in glacial acetic acid was added. The reaction was kept at 105 °C for 1 hour then cooled on ice. The crystalline product was removed by filtration, washed with ice water and dried at reduced pressure. The white crystals were then washed with hexane and dried in a desiccator to give 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose (0.262 g, 54%); m.p. 144-145.5 °C (lit.,[265] 151-152 °C for α form); $[\alpha]_D$ +12.4 ° (c 1.0 in $CHCl_3$) (lit.,[265] +21.7 ° (c 2.2 in $CHCl_3$) for α form); (Found: C, 74.88; H, 6.54. Calc. for $C_{34}H_{36}O_6$: C, 75.53; H, 6.71%); ν_{max} (nujol)/cm^{-1} 3370 (OH); δ_H (200 MHz; C^2HCl_3) 3.45 (1H, t, *J* 10, H-2β), 3.50-3.80 (9H, m, H-2α, 3β , 4 α and β, 5β, 6a α and β, 6b α and β), 3.90-4.10 (2H, m, H-3α, 5α), 4.40-5.00 (17H, m, 4 x PhC<u>H</u>$_2$ α and β, H-1β), 5.20 (1H, d, $J_{1,2}$ 3.6, H-1α), 7.10-7.40 (40H, m, 4 x <u>Ph</u>CH$_2$ α and β); δ_C (50.3 MHz; C^2HCl_3) 68.0 (C-6β), 68.5 (C-6α), 70.4 (C-5α), 73.8, 74.0 (3 x Ph<u>C</u>H$_2$), 75.0 (3 x Ph<u>C</u>H$_2$, 5β), 76.3 (2 x Ph<u>C</u>H$_2$), 78.2, 78.3 (C-4α and β), 80.4 (C-2α), 82.2 (C-3α), 83.5 (C-2β), 85.0 (C-3β), 91.8 (C-1α), 98.0 (C-1β), 128.1-130.0 (20<u>C</u>H, 4 x <u>Ph</u>CH$_2$), 138.4, 138.6, 138.7, 139.2 (4 x

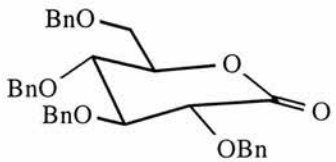
quaternary C, 4 x Ph); *m/z* (CI) 541 ($[MH]^+$, 1%), 523 (21, $[M\text{-}OH]^+$), 415 (61, $[M\text{-}PhCH_2O\text{-}2OH]^+$), 271 (60, $[glucose+PhCH_2]^+$), 235 (30, $[M\text{-}2PhCH_2O\text{-}PhCH_2]^+$), 217 (45, $[MH\text{-}3PhCH_2OH]^+$), 181 (100, $[glucoseH]^+$), 107 (92, $[PhCH_2O]^+$) and 91 (63, $[PhCH_2]^+$).

2,3,4,6-Tetra-*O*-benzyl-D-[6-$^2H_2$]glucopyranose (144)



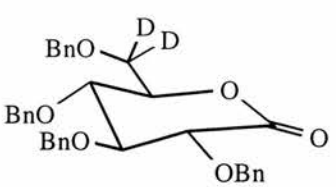
As described for (152a) using methyl 2,3,4,6-tetra-*O*-benzyl-α-D-[6-$^2H_2$]glucopyranoside (6.87 g, 12.3 mmol). 2,3,4,6-tetra-*O*-benzyl-α-D-[6-$^2H_2$]glucopyranose was given as a white crystalline solid (4.28 g, 64%); m.p. 132-133 °C; $[\alpha]_D$ +18.4 ° (c 1.0 in $CHCl_3$); (Found: C, 74.60; H, 6.75. Calc. for $C_{34}H_{34}{}^2H_2O_6$: C, 75.25; H, 6.69%); ν_{max} (nujol)/cm^{-1} 3370 (OH); δ_H (200 MHz; C^2HCl_3) 2.70 (1H, s, OH), 3.45 (1H, m, H-2β), 3.55 (1H, d, $J_{4,5}$ 10, H-5β), 3.60-3.70 (4H, m, H-2α, 3β, 4 α and β), 3.98 (1H, t, $J_{2,3}$=$J_{3,4}$ 10, H-3α), 4.05 (1H, d, $J_{4,5}$ 10, H-5α), 4.40-5.00 (17H, m, 4 x $PhCH_2$ α and β, H-1β), 5.20 (1H, d, $J_{1,2}$ 3.6, H-1α), 7.10-7.40 (40H, m, 4 x $\underline{Ph}CH_2$ α and β); δ_C (50.3 MHz; C^2HCl_3) 70.4 (C-5α), 73.8, 74.0 (3 x $Ph\underline{C}H_2$), 75.0 (3 x $Ph\underline{C}H_2$, 5β), 76.3 (2 x $Ph\underline{C}H_2$), 78.2 (C-4α, 4β), 80.4 (C-2α), 82.2 (C-3α), 83.5 (C-2β), 85.0 (C-3β), 91.8 (C-1α), 98.0 (C-1β), 128.1-130.0 (20$\underline{C}$H, 4 x $\underline{Ph}CH_2$), 138.4, 138.6, 138.7, 139.2 (4 x quaternary C, 4 x Ph); *m/z* (FAB) 566 ($[MH+Na]^+$, 13%), 565 (34, $[M+Na]^+$), 543 (2, $[MH]^+$), 542 (5, $[M]^+$), 541 (12, $[M\text{-}H]^+$), 417 (15, $[M\text{-}PhCH_2O\text{-}OCH_3]^+$) and 181 (100, $[glucoseH]^+$).

2,3,4,6-Tetra-*O*-benzyl-D-glucono-δ-lactone (153)



Pyridinium chlorochromate (2.77 g, 12.8 mmol) was added in one portion to 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranose (1.5 g, 2.77 mmol) and powdered molecular sieves 4 Å (2.77 g) in dry dichloromethane (35 ml). The reaction was stirred at room temperature for 1 hour then hexane (100 ml) and diethyl ether (200 ml) were added. The solution was filtered through silica gel and the solvent evaporated at reduced pressure to give 2,3,4,6-tetra-*O*-benzyl-D-glucono-δ-lactone as a clear viscous oil (1.07 g, 72%); $[\alpha]_D$ +71.1 ° (c 4.5 in $CHCl_3$) (lit.,[266] 79.9 ° (c 4.54 in $CHCl_3$)); ν_{max} (thin film)/cm^{-1} 1765 (C=O); δ_H (200 MHz; C^2HCl_3) 3.70 (1H, dd, $J_{5,6b}$ 4.2, J_{gem} 11, H-6b), 3.80 (1H, dd, $J_{5,6a}$ 2.5, J_{gem} 11, H-6a), 3.95 (1H, t, *J* 7, H-4), 4.00 (1H, t, *J* 7, H-3), 4.15 (1H, d, *J* 7, H-2), 4.50 (1H, m, H-5), 4.45-5.10 (8H, m, 4 x PhC$\underline{H}_2$), 7.10-7.50 (20H, m, 4 x $\underline{Ph}CH_2$); δ_C (50.3 MHz; C^2HCl_3) 66.0 (C-6), 74.0 (Ph$\underline{C}H_2$), 74.3 (2 x Ph$\underline{C}H_2$), 74.5 (Ph$\underline{C}H_2$), 76.5 (C-5), 77.9, 78.5 (C-2, 4), 81.4 (C-3), 125.5-126.3 (20$\underline{C}$H, 4 x $\underline{Ph}CH_2$), 134.7, 135.3, 135.4 (4 x quaternary C, 4 x Ph), 169.9 (C=O).

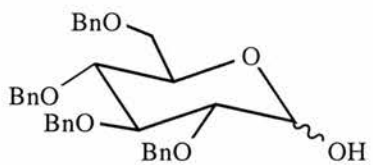
2,3,4,6-Tetra-*O*-benzyl-D-[6-$^2H_2$]glucono-δ-lactone (145)



As described for (153) using 2,3,4,6-tetra-*O*-benzyl-α-D-[6-$^2H_2$]glucopyranose (3.62 g, 6.7 mmol). The product was purified using column chromatography on silica using hexane-diethyl ether (7:1) as the eluant. 2,3,4,6-Tetra-*O*-benzyl-D-[6-$^2H_2$]glucono-δ-lactone was obtained as a clear oil (2.83 g, 79 %); $[\alpha]_D$ +80.2 ° (c 2.0 in $CHCl_3$); ν_{max}

(thin film)/cm^{-1} 1765 (C=O); δ_H (200 MHz; C^2HCl_3) 3.95 (1H, t, *J* 7, H-4), 4.00 (1H, t, *J* 7, H-3), 4.15 (1H, d, *J* 7, H-2), 4.50 (1H, m, H-5), 4.45-5.10 (8H, m, 4 x PhC$\underline{H}_2$), 7.10-7.50 (20H, m, 4 x $\underline{Ph}CH_2$); δ_C (50.3 MHz; C^2HCl_3) 74.0 (Ph$\underline{C}H_2$), 74.3 (2 x Ph$\underline{C}H_2$), 74.5 (Ph$\underline{C}H_2$), 76.5 (C-5), 77.9, 78.5 (C-2, 4), 81.4 (C-3), 125.5-126.3 (20$\underline{C}$H, 4 x $\underline{Ph}CH_2$), 134.7, 135.3, 135.4 (4 x quaternary C, 4 x Ph), 169.9 (C=O); m/z (CI) 541 ($[M+H]^+$, 40%), 540 (5, $[M]^+$), 271 (64, $[MH_4\text{-}3PhCH_2]^+$), 181 (64, $[MH_5\text{-}4PhCH_2]^+$), 107 (65, $[PhCH_2O]^+$) and 91 (100, $[PhCH_2]^+$).

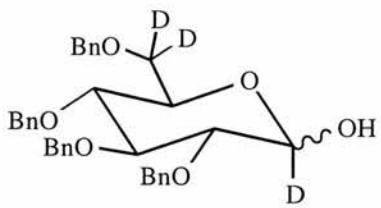
2,3,4,6-Tetra-*O*-benzyl-D-glucopyranose (152b)



2,3,4,6-Tetra-*O*-benzyl-D-glucono-δ-lactone (0.5 g, 0.9 mmol) was dissolved in tetrahydrofuran (3.0 ml) and cooled to 0 °C under nitrogen. Sodium borohydride (0.02 g, 0.53 mmol) was dissolved in water and added to the lactone solution dropwise. The reaction was stirred for 24 hours, quenched with 1 M sulfuric acid then extracted with ethyl acetate (2 x 50 ml) and washed with brine (20 ml). The organic extracts were dried ($MgSO_4$) and the solvent evaporated at reduced pressure to give 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose as a white solid (0.48 g, 99%); m.p. 144-145.5 °C (lit.,[230] 151-152 °C for α form); $[\alpha]_D$ +12.4 ° (c 1.0 in $CHCl_3$) (lit.,[230] 21.7 ° (c 2.2 in $CHCl_3$) for α form); (Found: C, 74.88; H, 6.54. Calc. for $C_{34}H_{36}O_6$: C, 75.53; H, 6.71%); ν_{max} (nujol)/cm^{-1} 3370 (OH); δ_H (200 MHz; C^2HCl_3) 3.45 (1H, t, *J* 10, H-2β), 3.50-3.80 (9H, m, H-2α, 3β, 4 α and β, 5β, 6a α and β, 6b α and β), 3.90-4.10 (2H, m, H-3α, 5α), 4.40-5.00 (17H, m, 4 x PhC$\underline{H}_2$ α and β, H-1β), 5.20 (1H, d, $J_{1,2}$ 3, H-1α), 7.10-7.40 (40H, m, 4 x $\underline{Ph}CH_2$ α and β); δ_C (50.3 MHz; C^2HCl_3) 68.0 (C-6β), 68.5 (C-6α), 70.4 (C-5α), 73.8, 74.0 (3 x Ph$\underline{C}H_2$), 75.0 (3 x Ph$\underline{C}H_2$, 5β), 76.3 (2 x Ph$\underline{C}H_2$), 78.2, 78.3 (C-4α, 4β), 80.4 (C-2α), 82.2 (C-3α), 83.5 (C-2β), 85.0 (C-3β), 91.8 (C-1α), 98.0 (C-1β),

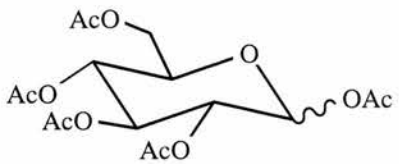
128.1-130.0 (20CH, 4 x $PhCH_2$), 138.4, 138.6, 138.7, 139.2 (4 x quaternary C, 4 x Ph); *m/z* (CI) 541 ($[MH]^+$, 1%), 523 (21, $[M-OH]^+$), 415 (61, $[M-PhCH_2O-2OH]^+$), 271 (60, $[glucose+PhCH_2]^+$), 217 (45, $[MH-3PhCH_2OH]^+$), 181 (100, $[glucoseH]^+$), 107 (92, $[PhCH_2O]^+$) and 91 (63, $[PhCH_2]^+$).

2,3,4,6-Tetra-*O*-benzyl-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose (146)



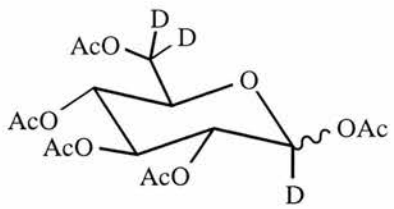
As described for (152b) using 2,3,4,6-tetra-*O*-benzyl-D-[6-$^2H_2$]glucono-δ-lactone (2.7 g, 3.9 mmol) and sodium borodeuteride (0.084 g, 2.3 mmol) in deuterium oxide (0.5 ml). 2,3,4,6-Tetra-*O*-benzyl-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranoside was given as a white crystalline solid (2.7 g, 100%); m.p. 120.5-122 °C; $[\alpha]_D$ +19.2 ° (c 1.0 in $CHCl_3$); ν_{max} (nujol)/cm^{-1} 3370 (OH); δ_H (200 MHz; C^2HCl_3) 3.1 (1H, s, OH), 3.40-3.70 (6H, m, H-2α, 3β, 4 α and β, 5β), 3.98 (1H, t, $J_{2,3}=J_{3,4}$ 10, H-3α), 4.05 (1H, d, $J_{4,5}$ 10, H-5α), 4.40-5.00 (16H, m, 4 x PhCH_2 α and β), 7.10-7.40 (40H, m, 4 x $PhCH_2$ α and β); δ_C (50.3 MHz; C^2HCl_3) 70.4 (C-5α), 73.8, 74.0 (3 x PhCH_2), 75.0 (3 x PhCH_2, 5β), 76.3 (2 x PhCH_2), 78.2 (C-4α, 4β), 80.4 (C-2α), 82.2 (C-3α), 83.5 (C-2β), 85.0 (C-3β), 128.1-130.0 (20CH, 4 x $PhCH_2$), 138.4, 138.6, 138.7, 139.2 (4 x quaternary C, 4 x Ph); *m/z* (FAB) 567 ($[MH+Na]^+$, 4%), 566 (12, $[M+Na]^+$), 544 (2, $[MH]^+$), 543 (5, $[M]^+$), 542 (5, $[M-H]^+$), 418 (14, $[M-PhCH_2O-OCH_3]^+$) and 181 (100, $[glucoseH]^+$).

1,2,3,4,6-Penta-*O*-acetyl-D-glucopyranose (154)



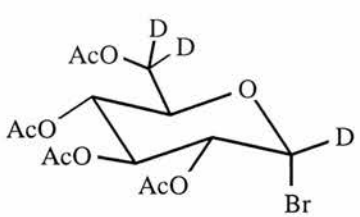
2,3,4,6-Tetra-*O*-benzyl-D-glucopyranose (0.15 g, 0.277 mmol) in acetic anhydride (25 ml) was treated with boron trifluoride diethyl etherate (0.28 ml, 2.22 mmol) at 0 °C and the reaction stirred overnight. Sodium hydrogen carbonate was used to neutralise the reaction mixture which was then extracted using ethyl acetate (2 x 25 ml) and washed with water (4 x 25 ml). The organic extracts were dried ($MgSO_4$) and the solvent evaporated at reduced pressure. The residue was purified by column chromatography on silica gel using 40-60 petroleum ether-ethyl acetate (3:2) as the eluant to give 1,2,3,4,6-penta-*O*-acetyl-D-glucopyranose as a white crystalline solid (0.068 g, 63%); m.p. 115-116 °C (lit.,[233] 112-114 °C α form; 132-135 °C β form); $[\alpha]_D$ +101.4 ° (c 1.0 in $CHCl_3$) (lit.,[233] +102.0 (α), +4.0 (β) (c 1.0 in $CHCl_3$)); ν_{max} (nujol)/cm^{-1} 1743 (CO); δ_H (200 MHz; C^2HCl_3) 2.00-2.20 (30H, s, 5 x OC(O)C$\underline{H}_3$ α and β), 3.80 (1H, m, H-5β), 4.00-4.35 (5H, m, H-5α, 6a α and β, 6b α and β), 5.00-5.20 (4H, m, H-2 α and β, 4 α and β), 5.46 (1H, t, $J_{2,3}$=$J_{3,4}$ 10, H-3 α and β), 5.70 (1H, d, $J_{1,2}$ 10, H-1β), 6.32 (1H, d, $J_{1,2}$ 3.6, H-1α); δ_C (50.3 MHz; C^2HCl_3) 20.4-21.0 (5 x OC(O)$\underline{C}H_3$ α and β), 61.9 (C-6 α and β), 68.1 (C-4β), 68.3 (C-4α), 69.6 (C-2α), 70.3 (C-3α, 5α), 70.6 (C-2β), 73.1, 73.2 (C-3β, 5β) 89.5 (C-1α), 92.1 (C-1β), 169.0, 169.7, 169.9, 170.5, 170.9 (5 x O$\underline{C}$(O)CH_3 α and β); *m/z* (CI) 331 ([M-OAc]$^+$, 100%), 271 (23, [M-2OAc-H]$^+$) and 169 (30, [M-3OAc-AcH]$^+$).

1,2,3,4,6-Penta-*O*-acetyl-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose (147)



As described for (154) using 2,3,4,6-tetra-*O*-benzyl-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose (1.5 g, 2.76 mmol). 1,2,3,4,6-penta-*O*-acetyl-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose was given as a white crystalline solid (0.89 g, 82%); m.p. 111-112 °C; $[\alpha]_D$ +98.1 ° (c 0.6 in $CHCl_3$); (Found: C, 48.34; H, 5.46. Calc. for $C_{16}H_{19}{}^2H_3O_{11}$: C, 48.85; H, 5.64%); ν_{max} (nujol)/cm^{-1} 1735 (CO); δ_H (200 MHz; C^2HCl_3) 2.00-2.20 (30H, m, 5 x OC(O)C$\underline{H}_3$ α and β), 3.80 (1H, d, $J_{4,5}$ 10, H-5β), 4.10 (1H, d, $J_{4,5}$ 10, H-5α), 5.10 (1H, d, $J_{2,3}$ 10, H-2α), 5.14 (1H, t, $J_{3,4}$=$J_{4,5}$ 10, H-4α), 5.46 (1H, t, $J_{2,3}$=$J_{3,4}$ 10, H-3α), 5.00-5.50 (3H, m, H-2β, 3β, 4β); δ_C (50.3 MHz; C^2HCl_3) 20.9-21.4 (5 x OC(O)$\underline{C}H_3$ α and β), 68.1 (C-4β), 68.3 (C-4α), 69.5 (C-2α), 70.1, 70.3 (C-3α, 5α), 70.6 (C-2β), 73.0, 73.2 (C-3β, 5β), 169.3, 169.9, 170.2, 170.7, 171.0 (5 x O$\underline{C}$(O)CH_3 α and β); *m/z* (CI) 395 ($[MH_2]^+$, 1%), 334 (100, $[M\text{-}OAc]^+$), 274 (28, $[M\text{-}2OAc\text{-}H]^+$), 214 (6, $[M\text{-}3OAc\text{-}2H]^+$) and 172 (35, $[M\text{-}3OAc\text{-}AcH]^+$).

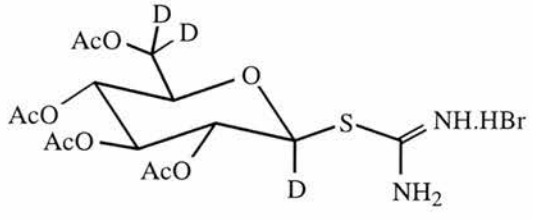
2,3,4,6-Tetra-*O*-acetyl-α-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranosyl bromide (148)



As described for (65) using 1,2,3,4,6-penta-*O*-acetyl-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose (0.7 g, 1.78 mmol). 2,3,4,6-Tetra-*O*-acetyl-α-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranosyl bromide was given as a white crystalline solid (0.614 g, 83%) the product is heat labile and should be stored below 0 °C; m.p. 84-87 °C; $[\alpha]_D$ +186 ° (c 2.42 in $CHCl_3$); ν_{max} (nujol)/cm^{-1} 1730

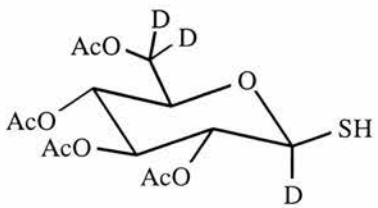
(CO); δ_H (200 MHz; C^2HCl_3) 2.00 (3H, s, OC(O)C$\underline{H}_3$), 2.05 (3H, s, OC(O)C$\underline{H}_3$), 2.10 (6H, 2s, 2 x OC(O)C$\underline{H}_3$), 4.20 (1H, d, $J_{4,5}$ 10, H-5), 4.80 (1H, d, $J_{2,3}$ 10, H-2), 5.15 (1H, t, $J_{3,4}$=$J_{4,5}$ 10, H-4), 5.55 (1H, t, $J_{2,3}$=$J_{3,4}$ 10, H-3); δ_C (50.3 MHz; C^2HCl_3) 21.0 - 21.1 (4 x OC(O)$\underline{C}H_3$), 64.6 (C-4), 70.6 (C-2), 71.0 (C-3), 72.4 (C-5), 169.9, 170.2, 170.3, 171.0 (4 x O$\underline{C}$(O)CH_3); *m/z* (CI) 416 ($[MH_2]^+$, 18%), 415 (5, $[MH]^+$), 414 (17, $[M]^+$), 356 (19, $[MH\text{-}OAc]^+$), 355 (5, $[M\text{-}OAc]^+$), 354 (18, $[M\text{-}HOAc]^+$), 334 (30, $[M\text{-}Br]^+$), 292 (44, $[M\text{-}HAc\text{-}Br]^+$), 216 (7, $[M\text{-}2Ac\text{-}Br]^+$) and 172 (58, $[M\text{-}4OAc\text{-}^2H_3]^+$).

2,3,4,6-Tetra-*O*-acetyl-α-D-[1-$^2H_1$, 6-$^2H_2$]-glucopyranosylisothiouronium bromide (149)



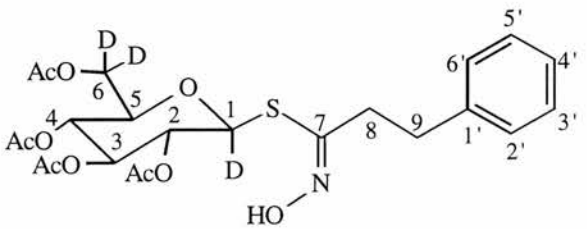
As described for (66) using 2,3,4,6-tetra-*O*-acetyl-α-D-[1-^2H, 6-$^2H_2$]glucopyranosyl bromide (0.6 g, 1.45 mmol). 2,3,4,6-Tetra-*O*-acetyl-α-D-[1-$^2H_1$,6-$^2H_2$]glucopyranosylisothiouronium bromide was given as a white crystalline solid (0.7 g, 98%); m.p. 182.5-183.5 °C; $[\alpha]_D$ -15.6 ° (c 1.0 in CH_3OH); (Found: C, 36.29; H, 4.87; N, 6.42. Calc. for $C_{15}H_{20}{}^2H_3BrN_2O_9S$: C, 36.74; H, 4.73; N, 5.71%); ν_{max} (nujol)/cm^{-1} 3360-3310 (NH), 1730 (CO); δ_H (200 MHz; ^2H_2O) 2.10 (3H, s, COC$\underline{H}_3$), 2.15 (3H, s, COC$\underline{H}_3$), 2.20 (3H, s, COC$\underline{H}_3$), 2.22 (3H, s, COC$\underline{H}_3$), 4.20 (1H, d, $J_{4,5}$ 10, H-5), 5.15 (1H, t, $J_{3,4}$=$J_{4,5}$ 10, H-4), 5.25 (1H, d, $J_{2,3}$ 10, H-2), 5.40 (1H, t, $J_{2,3}$=$J_{3,4}$ 10, H-3); δ_C (50.3 MHz; ^2H_2O) 23.3-23.5 (4 x OC(O)$\underline{C}H_3$), 68.6 (C-4), 70.1 (C-2), 74.1 (C-3), 76.7 (C-5), 170.5 (C=N), 173.2, 173.3, 173.6, 174.4 (4 x $\underline{C}OCH_3$); *m/z* (CI) 492 ($[MH_2]^+$, 8%), 491 (2, $[MH]^+$), 490 (7, $[M]^+$), 444 (32, $[M\text{-}NH_2CH_2NH_2]^+$), 410 (17, $[M\text{-}NH_2CHNH_2S^2HH]^+$), 334 (67, $[M\text{-Aglycone}]^+$), 292 (26, $[M\text{-Aglycone-}C_2H_2O]^+$), 248 (30, $[M\text{-Aglycone-2Ac}]^+$), 216 (24, $[M\text{-Aglycone-2OAc}]^+$) and 172 (25, $[M\text{-Aglycone-2OAc-AcH}]^+$).

2,3,4,6-Tetra-*O*-acetyl-thio-β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose (150)



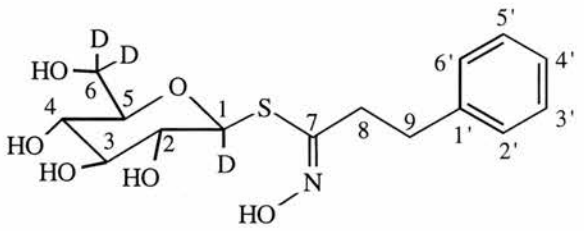
As described for (67) using 2,3,4,6-tetra-*O*-acetyl-α-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranosylisothiouronium bromide (0.24 g, 0.49 mmol). After purification using column chromatography on silica gel with 40-60 petroleum ether-ethyl acetate (1:1) 2,3,4,6-tetra-*O*-acetyl-thio-β-D-[1-^2H, 6-$^2H_2$]glucopyranose was given as a white crystalline solid (0.127 g, 71%); m.p. 117-119 °C; $[\alpha]_D$ -8.7 ° (c 1.0 in CH_3OH); (Found: C, 45.66; H, 5.45. Calc. for $C_{14}H_{17}{}^2H_3O_9S$: C, 45.77; H, 5.49%); ν_{max} (nujol)/cm^{-1} 3460 (SH), 1735 (CO); δ_H (200 MHz; C^2HCl_3) 2.00 (3H, s, OC(O)C$\underline{H}_3$), 2.02 (3H, s, OC(O)C$\underline{H}_3$), 2.10 (3H, s, OC(O)C$\underline{H}_3$), 2.12 (3H, s, OC(O)C$\underline{H}_3$), 3.70 (1H, d, $J_{4,5}$ 10, H-5), 4.98 (1H, d, $J_{2,3}$ 10, H-2), 5.09 (1H, t, $J_{3,4}$=$J_{4,5}$ 10, H-4), 5.18 (1H, t, $J_{2,3}$=$J_{3,4}$ 10, H-3); δ_C (50.3 MHz; C^2HCl_3) 21.1 - 21.3 (4 x OC(O)$\underline{C}H_3$), 68.5 (C-4), 73.9 (C-2), 74.0 (C-3), 76.6 (C-5), 169.9, 170.2, 170.6, 171.2 (4 x O$\underline{C}$(O)CH_3); *m/z* (CI) 368 ([MH]$^+$, 2%), 334 (100, [M-SH]$^+$), 274 (29, [M-OAcS]$^+$) and 172 (62, [M-2Ac-OAc-H_2O-S]$^+$).

2,3,4,6-Tetra-*O*-acetyl-β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranosyl phenethyl thiohydroximate (155)



Hydrocinnamaldehyde oximyl chloride (0.06 g, 0.32 mmol) was suspended in dry diethyl ether (3.0 ml) and a solution of 2,3,4,6-tetra-*O*-acetyl-thio-β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranose (0.052 g, 0.14 mmol) in dry diethyl ether (1.5 ml) was added. The reaction was treated with dry triethylamine (0.3 ml) and stirred overnight. 1 M sulfuric acid (5 ml) was added to the reaction and the solution extracted using ethyl acetate (3 x 15 ml). The organic extracts were dried ($MgSO_4$) and the solvent evaporated at reduced pressure to give a white solid. The product was purified by column chromatography on silica gel using 40-60 petroleum ether-ethyl acetate (3:2) as the eluant. 2,3,4,6-Tetra-*O*-acetyl-β-D-[1-$^2H_1$, 6-$^2H_2$]glucopyranosyl phenethyl thiohydroximate was given as a white solid (0.075 g, 100%); m.p. 152-154 °C; $[\alpha]_D$ +19.6 ° (c 1.0 in $CHCl_3$); (Found: C, 52.66; H, 6.18; N, 2.74. Calc for $C_{23}H_{26}{}^2H_3NO_{10}S.0.5H_2O$: C, 52.76; H, 5.78; N, 2.68%); ν_{max} (nujol)/cm^{-1} 3300 (OH), 1750 (C=O); δ_H (300 MHz; C^2HCl_3) 1.90-2.10 (4 x 3H, 4s, 4 x OC(O)C<u>H</u>$_3$), 2.70-3.00 (4H, m, H-8, 9), 3.65 (1H, d, $J_{4,5}$ 10, H-5), 4.96-5.00 (2H, 2t, *J* 10, H-2, 4), 5.20 (1H, t, *J* 10, H-3), 7.10-7.30 (5H, m, phenyl); δ_C (75.45 MHz; C^2HCl_3) 20.5-20.6 (4 x OC(O)<u>C</u>H$_3$), 33.2 (CH_2), 34.3 (CH_2), 68.1 (C-4), 70.1 (C-2), 73.7 (C-3), 76.0 (C-5), 126.7 (C-4'), 128.4 (C-3', 5'), 128.8 (C-2', 6'), 140.6 (C-1'), 152.0 (C-7), 169.4, 169.5, 170.4, 170.8 (4 x O<u>C</u>(O)CH$_3$); *m/z* (EI) 515 ([M+H]$^+$, 5%), 334 (47, [M-Aglycone]$^+$), 243 (61, [M-Aglycone-OAc-2CH$_3$-2H]$^+$), 172 (30, [$C_8H_6{}^2H_3O_4$]$^+$), 150 (64, [AglyconeH$_2$]$^+$), 132 (100, [Aglycone-O]$^+$), 105 (15, [Ph(CH$_2$)$_2$]$^+$) and 91 (27, [PhCH$_2$]$^+$).

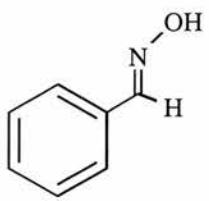
β-D-[1-$^2H_1$, 6-$^2H_2$]Glucopyranosyl phenethyl thiohydroximate (156)



To 2,3,4,6-tetra-*O*-acetyl-β-D-[1-^2H, 6-$^2H_2$]glucopyranosyl gluconasturtiin thiohydroximate (0.5 g, 0.98 mmol) in methanol was added a catalytic amount of potassium metal under nitrogen. The reaction was stirred for 18 hours then Amberlite IR-120 resin was added. Stirring was continued for a further 15 minutes before the Amberlite was removed by filtration and the solvent removed by evaporation at reduced pressure to give an extremely hygroscopic white amorphous solid (0.057 g, 64%); m.p. 78-80 °C; $[\alpha]_D$ -50.3 ° (c 1.0 in CH_3OH); (Found: C, 49.62; H, 6.00; N, 3.58. Calc for $C_{15}H_{18}{}^2H_3NO_6S.0.8H_2O$: C, 49.97; H, 6.32; N, 3.88%); ν_{max} (nujol)/cm^{-1} 3300 (OH); δ_H (300 MHz; $C^2H_3O^2H$) 3.00-3.20 (4H, m, CH_2CH_2), 3.60-3.80 (4H, m, H-2, 3, 4, 5), 7.25 (5H, s, phenyl); δ_C (75.45 MHz; $C^2H_3O^2H$) 34.8 ($\underline{C}H_2$), 35.3 ($\underline{C}H_2$), 71.3 (C-4), 74.5 (C-2), 79.8 (C-5), 82.2 (C-3), 127.4 (C-4'), 129.6, 129.7 (C-2', 3', 5', 6'), 142.7 (C-1'), 154.0 ($\underline{C}$=N); *m/z* (CI) 347 ([MH]$^+$, 1%), 150 (11, [AglyconeH$_2$]$^+$), 131 (46, [Aglycone-OH]$^+$) and 91 (100, [PhCH$_2$]$^+$).

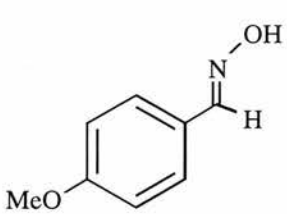
3.5 Novel Phenyl Glucosinolates

Benzaldehyde oxime (166)



Hydroxylamine hydrochloride (4.5 g, 64.8 mmol), benzaldehyde (2.93 g, 2.8 ml, 27.6 mmol), and dry pyridine (6 ml) were added to dry ethanol (60 ml) and the solution heated at reflux for 30 minutes. On completion the reaction was poured into water (300 ml) and extracted with diethyl ether (5 x 90 ml). The organic phase was washed with saturated sodium hydrogen carbonate (2 x 75 ml) then dried ($MgSO_4$) and the solvent evaporated at reduced pressure. The resulting oil was purified by distillation at reduced pressure to give a clear oil (3.31 g, 99%), b.p. 120 °C/0.8 mm Hg; ν_{max} (thin film)/cm^{-1} 3300-3000 (OH); δ_H (200 MHz; C^2HCl_3) 7.40 (3H, m, H-3, 4, 5), 7.65 (2H, m, H-2, 6), 8.20 (1H, s, C<u>H</u>=N), 9.75 (1H, s, N-O<u>H</u>); δ_C (50.3 MHz; C^2HCl_3) 127.6 (C-3, 5), 129.3 (C-2, 6), 130.6 (C-4), 132.7 (C-1), 150.8 (<u>C</u>H=N); *m/z* (EI) 121 ($[M]^+$, 100%), 103 (24, $[M-H_2O]^+$) and 78 (72, $[C_6H_6]^+$).

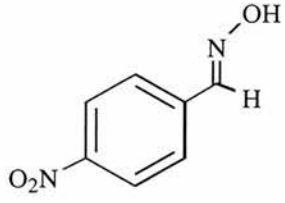
p-Methoxybenzaldehyde oxime (165)



As described for (166) using *p*-methoxybenzaldehyde (3.36 ml, 27.6 mmol). The resulting oil was purified by distillation at reduced pressure 155 °C/0.8 mm Hg to give a white solid (4.17 g, 100%); m.p. 49-57 °C (lit.,[267] 65, 132 °C); (Found: C, 63.54; H, 6.38; N, 9.19.

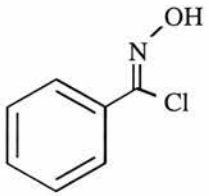
Calc for $C_8H_9NO_2$: C, 63.56; H, 6.00; N, 9.27%); ν_{max} (nujol)/cm^{-1} 3300-3000 (OH); δ_H (200 MHz; C^2HCl_3) 3.85 (3H, s, C<u>H</u>$_3$), 6.90 (2H, d, *J* 9, H-3, 5), 7.55 (2H, d, *J* 9, H-2, 6), 8.13 (1H, s, C<u>H</u>=N), 8.80 (1H, s, N-O<u>H</u>); δ_C (50.3 MHz; C^2HCl_3) 55.1 (O<u>C</u>H$_3$ *E* and *Z* isomers), 113.7, 114.2 (C-3, 5 *E* and *Z* isomers), 123.8, 125.0 (C-1 *E* and *Z* isomers), 128.4, 132.8 (C-2, 6 *E* and *Z* isomers), 145.9, 149.6 (<u>C</u>H=N *E* and *Z* isomers), 160.7 (C-4 *E* and *Z* isomers); *m/z* (EI) 151 ([M]$^+$, 100%), 134 (23, [M-OH]$^+$), 108 (53, [$C_6H_5OCH_3$]$^+$) and 77 (40, [C_6H_5]$^+$)

_p_-Nitrobenzaldehyde oxime (167)



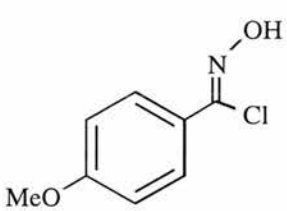
As described for (166) using *p*-nitrobenzaldehyde (8.34 g, 55.2 mmol). The resulting solid was recrystallised from toluene to give *p*-nitrobenzaldehyde oxime as a yellow crystalline solid (8.32 g, 91%); m.p. 129-133.5 °C (lit.,[267] 133 °C); ν_{max} (nujol)/cm^{-1} 3300-3000 (OH), 1380-1300 (conjugated NO_2), 860 (para substituted phenyl); δ_H (200 MHz; C^2HCl_3) 7.80 (2H, d, *J* 9, H-2, 6), 8.15 (1H, s, NO<u>H</u>), 8.20 (1H, s, C<u>H</u>=N), 8.25 (2H, d, *J* 9, H-3, 5); δ_C (50.3 MHz; C^2HCl_3) 123.9 (C-3, 5), 129.2 (C-2, 6), 138.8 (C-1), 148.8 (C-4), 151.2 (<u>C</u>H=N); *m/z* (EI) 166 ([M]$^+$, 100%), 136 (24, [M-NO]$^+$), 120 (13, [M-NO_2]$^+$) and 103 (31, [M-NO_2-OH]$^+$).

Benzaldehyde oximyl chloride (169)



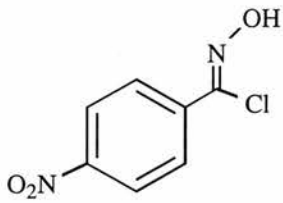
Benzaldehyde oxime (3.0 g, 24.7 mmol) was stirred in chloroform (70 ml) at 0 °C while chlorine gas was bubbled through. The solution changed colour from deep blue to yellow on completion and gaseous hydrochloric acid was released. The reaction mixture was concentrated at reduced pressure to give a solid which was taken up in hot 60-80 petroleum ether, filtered and the solvent evaporated at reduced pressure. The resulting oil crystallised on standing (1.71 g, 44%) and was stored below 0 °C; δ_H (200 MHz; C^2HCl_3) 7.40 (3H, m, H-3, 4, 5), 7.80 (2H, m, H-2, 6), 9.30 (1H, s, N-O<u>H</u>).

***p*-Methoxybenzaldehyde oximyl chloride (170)**



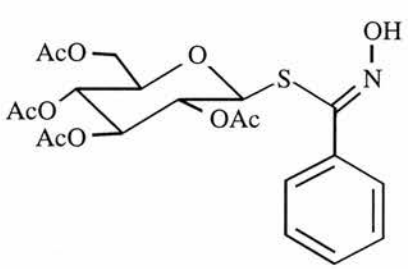
p-Methoxybenzaldehyde oxime (2.0 g, 13.2 mmol) was dissolved in a flask containing chloroform (26 ml) and dry pyridine (0.53 ml). *N*-Chlorosuccinimide (1.76 g, 13.2 mmol) was added slowly at 0 °C then the reaction stirred for 2 hrs at room temperature. The solution was poured onto ice/water and extracted with diethyl ether (2 x 150 ml). The organic phase was washed with water (2 x 100 ml) then dried ($MgSO_4$) and the solvent evaporated at reduced pressure to give *p*-methoxybenzaldehyde oximyl chloride as a yellow semi solid (1.85 g, 76%); δ_H (200 MHz; C^2HCl_3) 3.85 (3H, s, C<u>H</u>$_3$), 6.90 (2H, d, *J* 9, H-3, 5), 7.75 (2H, d, *J* 9, H-2, 6), 9.45 (1H, s, N-O<u>H</u>).

_p_-Nitrobenzaldehyde oximyl chloride (168)



_p_-Nitrobenzaldehyde oxime (1.5 g, 9.0 mmol) was dissolved in dry _N,N_-dimethylformamide (20 ml) and _N_-chlorosuccinimide (1.19 g, 9.0 mmol) added slowly at 0 °C. The solution was stirred for 1 hour at room temperature and then quenched with ice/water (200 ml). The reaction was extracted with diethyl ether (2 x 100 ml) and washed with water (2 x 75 ml). The organic layer was then dried ($MgSO_4$) and the solvent evaporated at reduced pressure to give yellow crystals (1.65 g, 92%); m.p. 108-112 °C (lit.,[268] 116 °C); (Found: C, 42.39; H, 2.75; N, 13.82. Calc for $C_7H_5N_2O_3Cl$: C, 41.93; H, 2.51; N, 13.97%); δ_H (200 MHz; C^2HCl_3) 8.05 (2H, d, _J_ 9, H-2, 6), 8.26 (2H, d, _J_ 9, H-3, 5), 8.91 (1H, s, O<u>H</u>); δ_C (50.3 MHz; C^2HCl_3) 123.9 (C-3, 5), 124.2 (C-2, 6), 138.8 (C-1), 149.8 (C-4), 164.1 (<u>C</u>Cl=NOH); _m/z_ (EI) 200 ($[M]^+$, 60%), 164 (100, $[M-HCl]^+$), 134 (28, $[M-NOHCl]^+$) and 88 (88, $[M-NOHCl-NO_2]^+$).

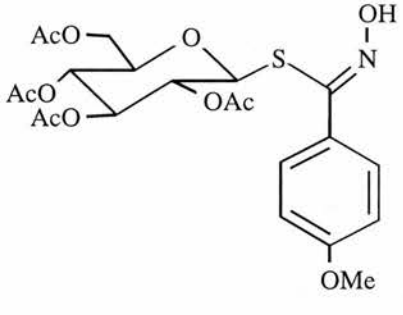
2,3,4,6-Tetra-_O_-acetyl-β-D-glucopyranosyl phenyl thiohydroximate (172)



Benzaldehyde oximyl chloride (0.64 g, 4.1 mmol) in dry tetrahydrofuran (60 ml) was added to 2,3,4,6-tetra-_O_-acetyl-1-thio-β-D-glucopyranose (1.5 g, 4.1 mmol) in dry tetrahydrofuran (100 ml) under nitrogen with stirring. Dry triethylamine (5.4 ml, 38.6 mmol) was added and the reaction stirred at room temperature under nitrogen for 1.5 hours.

The solution was evaporated at reduced pressure and the residue taken up in ethyl acetate (100 ml). The organic phase was washed with 1 M sulfuric acid (100 ml) then dried ($MgSO_4$) and the solvent evaporated at reduced pressure to give 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl phenyl thiohydroximate as a white solid which was recrystallised from ethanol (1.72 g, 87%); m.p. 120-121 °C (lit.,[185] 118 °C); $[\alpha]_D$ +21.2 ° (c 1.0 in $CHCl_3$) (lit.,[185] +19.0 ° (c 1.0 in $CHCl_3$)); (Found: C, 52.48; H, 5.48; N, 2.66. Calc for $C_{21}H_{25}NO_{10}S$: C, 52.18; H, 5.21; N, 2.90%); ν_{max} (nujol)/cm^{-1} 3290 (OH), 1730 (C=O), 1690 (C=N), 1575, 760, 710 (unsubstituted phenyl); δ_H (200 MHz; C^2HCl_3) 1.89-2.20 (12H, 4s, 4 x OC(O)C<u>H</u>3), 3.05 (1H, m, H-5), 3.96 (1H, dd, $J_{5,6a}$ 2.5, $J_{6a,6b}$ 12.5, H-6a), 4.14 (1H, dd, $J_{5,6b}$ 5, $J_{6a,6b}$ 12.5, H-6b), 4.38 (1H, d, $J_{1,2}$ 10, H-1), 5.00 (3H, m, H-2, 3, 4), 7.36 (3H, m, H-3', 4', 5'), 7.45 (2H, m, H-2', 6'), 8.80 (1H, s, N-O<u>H</u>); δ_C (50.3 MHz; C^2HCl_3) 21.0, 21.1, 21.2, 21.3 (4 x OC(O)<u>C</u>H3), 62.3 (C-6), 68.3 (C-4), 70.2 (C-2), 74.2 (C-3), 76.1 (C-5), 81.8 (C-1), 129.0 (C-3', 5'), 129.5 (C-2', 6'), 130.6 (C-4'), 132.7 (C-1'), 153.1 (<u>C</u>=NOH), 169.8, 170.8, 171.2 (4 x O<u>C</u>(O)CH3); *m/z* (CI) 484 ($[M+H]^+$, 7%), 331 (100, [M-Aglycone]$^+$), 271 (21, [M-Aglycone-OAc]$^+$), 213 (10, [Glucose+SH]$^+$), 169 (21, $[C_8H_9O_4]^+$) and 120 (12, $[C_6H_5CNOH]^+$).

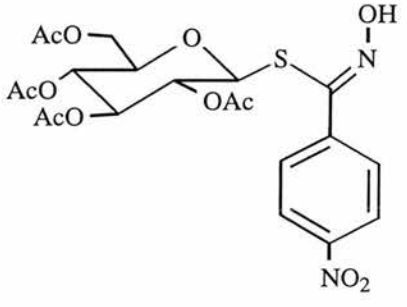
2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-*p*-methoxyphenyl thiohydroximate (171)



As described for (172) using *p*-methoxybenzaldehyde oximyl chloride (0.91 g, 4.9 mmol). 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-*p*-methoxyphenyl thiohydroximate was given as a white crystalline solid (1.58 g, 63%); m.p. 146-150 °C (lit.,[185] 150 °C); $[\alpha]_D$ +18.5 °

(c 1.0 in $CHCl_3$)(lit.,[185] +13.0 ° (c 1.0 in $CHCl_3$)); (Found: C, 51.78; H, 5.41; N, 2.64. Calc for $C_{22}H_{27}NO_{11}S$: C, 51.46; H, 5.30; N, 2.73%); ν_{max} (nujol)/cm^{-1} 3300 (OH), 1700 (C=O), 1580 (C=N); δ_H (200 MHz; C^2HCl_3) 1.97-2.20 (12H, 4s, 4 x OC(O)C<u>H</u>3), 3.15 (1H, m, H-5), 3.87 (3H, s, OC<u>H</u>3), 4.04 (1H, dd, $J_{5,6a}$ 2.5 Hz, $J_{6a,6b}$ 12.5, H-6a), 4.14 (1H, dd, $J_{5,6b}$ 5, $J_{6a,6b}$ 12.5, H-6b), 4.47 (1H, d, $J_{1,2}$ 10, H-1), 5.05 (3H, m, H-2, 3, 4), 6.94 (2H, d, *J* 10, H-3', 5'), 7.46 (2H, d, *J* 10, H-2', 6'); δ_C (50.3 MHz; C^2HCl_3) 21.0 (4 x OC(O)<u>C</u>H3), 55.6 (OCH_3), 62.4 (C-6), 68.4 (C-4), 70.3 (C-2), 74.2 (C-3), 76.1 (C-5), 82.0 (C-1), 114.4 (C-3', 5'), 125.0 (C-1'), 130.9 (C-2', 6'), 153.1 (<u>C</u>=NOH), 161.4 (C-4'), 169.8, 170.8, 171.2 (4 x O<u>C</u>(O)CH3); *m/z* (CI) 514 ($[M+H]^+$, 10%), 331 (100, $[M\text{-Aglycone}]^+$) and 271 (27, $[M\text{-Aglycone-AcOH}]^+$).

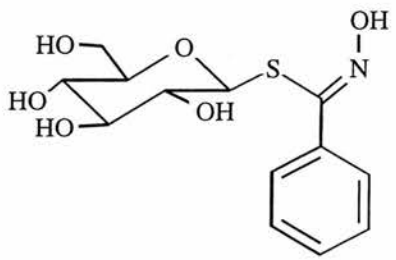
2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-*p*-nitrophenyl thiohydroximate (173)



As described for (172) using *p*-nitrobenzaldehyde oximyl chloride (0.83 g, 4.1 mmol). This gave white crystals of product (1.90 g, 88%); m.p. 145-149 °C; $[\alpha]_D$ +11.0 ° (c 1.0 in $CHCl_3$) (lit.,[185] +8.0 ° (c 1.0 in $CHCl_3$)); (Found: C, 47.86; H, 4.77; N, 5.29. Calc for $C_{21}H_{24}N_2O_{12}S$: C, 47.73; H, 4.58; N, 5.30%); ν_{max} (nujol)/cm^{-1} 3180 (OH), 1720-1680 (C=O), 1370 (conjugated NO_2), 860 (para substituted phenyl); δ_H (200 MHz; C^2HCl_3) 1.98-2.10 (12H, 4s, 4 x OC(O)C<u>H</u>3), 3.25 (1H, m, H-5), 4.00 (1H, dd, $J_{5,6a}$ 2.5, $J_{6a,6b}$ 12.5, H-6a), 4.14 (1H, dd, $J_{5,6b}$ 5, $J_{6a,6b}$ 12.5, H-6b), 4.65 (1H, m, H-1), 5.05 (3H, m, H-2, 3, 4), 7.80 (2H, d, *J* 9.6, H-2', 6'), 8.30 (2H, d, *J* 9.6, H-3', 5'), 8.85 (1H, s, N-O<u>H</u>); δ_C (50.3 MHz; C^2HCl_3) 21.1, 21.2, 21.3 (4 x OC(O)<u>C</u>H3), 62.4 (C-6), 68.2 (C-4),

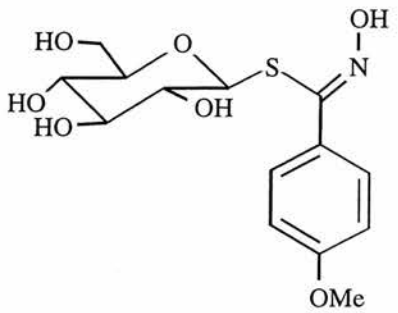
70.6 (C-2), 73.9 (C-3), 76.3 (C-5), 81.8 (C-1), 124.1 (C-3', 5'), 130.4 (C-2', 6'), 139.7 (C-1'), 149.1, 149.9 (C=NOH, C-4), 169.9, 170.7, 171.2 (4 x OC(O)CH_3); *m/z* (CI) 529 ($[M+H]^+$, 4%), 363 (5, $[M-O_2NC_6H_4CHNOH]^+$) and 331 (100, [M-Aglycone]$^+$).

β-D-Glucopyranosyl-phenylthiohydroximate (175)



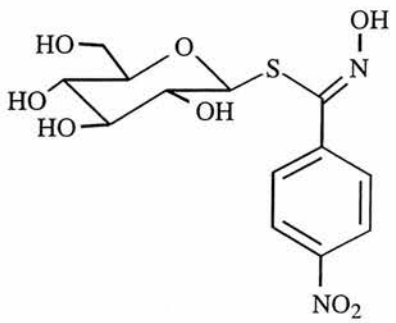
2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl phenyl thiohydroximate (1.5 g, 3.1 mmol) was dissolved in dry methanol and a catalytic amount of potassium added. The reaction was stirred under nitrogen at room temperature for 20 hours. Amberlite IR-120 resin was then added and stirring continued for 30 minutes. The Amberlite was removed by filtration and the solvent evaporated at reduced pressure. The resulting oil was triturated with ethanol to give β-D-glucopyranosyl phenyl thiohydroximate as a white solid (0.82 g, 83%); m.p. 82-95 °C; $[\alpha]_D$ -4.0 ° (c 0.5 in CH_3OH) (lit.,[185] -2.0 ° (c 1.0 in CH_3OH)); (Found: C, 48.54; H, 5.74; N, 3.93. Calc for $C_{13}H_{17}NO_6S.0.5H_2O$: C, 48.14; H, 5.59; N, 4.32%); ν_{max} (nujol)/cm^{-1} 3600-3100 (OH) 760 and 720 (monosubstituted phenyl); δ_H (200 MHz; ^2H_2O) 2.60 (1H, m, H-5), 3.10-3.70 (5H, m, H-2, 3, 4, 6a, 6b), 4.15 (1H, d, *J* 10, H-1), 7.40 (5H, s, H-2', 3', 4', 5', 6'); δ_C (50.3 MHz; ^2H_2O) 62.9 (C-6); 71.6 (C-4), 74.7 (C-2), 79.8 (C-3), 82.5 (C-5), 85.8 (C-1), 131.3 (3', 5'), 131.8 (C-2', 6'), 132.7 (C-4'), 134.9 (C-1'), 153.2 (C=N-OH); *m/z* (CI) 316 ($[M+H]^+$, 27%), 163 (29, $[M-C_6H_6-5OH]^+$) and 138 (100, $[M-C_6H_7OSCH]^+$).

β-D-Glucopyranosyl-*p*-methoxyphenyl thiohydroximate (174)



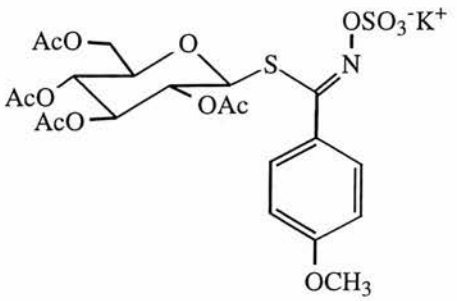
As described for (175) using 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-*p*-methoxyphenyl thiohydroximate (0.3 g, 0.58 mmol) to give β-D-glucopyranosyl-*p*-methoxyphenyl thiohydroximate as a white solid (0.09 g, 45%); m.p. > 260 °C; $[\alpha]_D$ -8.0 ° (c 0.5 in CH_3OH) (lit.,[185] -7.0 ° (c 1.0 in CH_3OH)); (Found: C, 47.39; H, 5.48; N, 3.76. Calc for $C_{14}H_{19}NO_7S.0.5H_2O$: C, 47.45; H, 5.69; N, 3.95%); ν_{max} (nujol)/cm^{-1} 3500-3000 (OH), 1760-1730 (C=N-, conjugated); δ_H (200 MHz; ^2H_2O) 2.60 (1H, m, H-5), 3.10-3.60 (5H, m, H-2, 3, 4, 6a, 6b), 3.75 (3H, s, $OC\underline{H}_3$), 4.10 (1H, d, $J_{1,2}$ 10, H-1), 6.90 (2H, d, *J* 10, H-3', 5'), 7.35 (2H, d, *J* 10, H-2', 6'); δ_C (50.3 MHz; ^2H_2O) 58.2 ($O\underline{C}H_3$) 63.1 (C-6); 71.9 (C-4), 75. (C-2), 80.1 (C-3), 82.6 (C-5), 85.9 (C-1), 116.5 (3', 5'), 129.5 (C-1'), 133.7 (C-2', 6'), 153.5 (C=N-OH), 162.1 (C-4'); *m/z* (CI) 346 ($[M+H]^+$, 1.0%), 345 (4.0, $[M]^+$), 166 (100, $[AglyconeH]^+$) and 134 (83, $[AglyconeH-S]^+$).

β-D-Glucopyranosyl-*p*-nitrophenyl thiohydroximate (176)



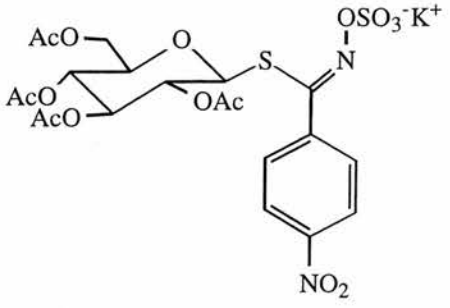
As described for (175) using 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-*p*-nitrophenyl thiohydroximate (0.80 g, 1.51 mmol) to give β-D-glucopyranosyl-*p*-nitrophenyl thiohydroximate as a white solid (0.413 g, 76%); m.p. 184-187 °C; $[\alpha]_D$ +2.8 ° (c 0.5 in CH_3OH) (lit.,[185] +5.0 ° (c 1.0 in CH_3OH)); (Found: C, 43.56; H, 4.68; N, 7.73. Calc for $C_{13}H_{16}N_2O_8S$: C, 43.33; H, 4.48; N, 7.77%); ν_{max} (nujol)/cm^{-1} 3300 (OH), 1360-1330 (NO_2, conjugated); δ_H (200 MHz; ^2H_2O) 2.60 (1H, m, H-5), 3.00-3.70 (5H, m, H-2, 3, 4, 6a, 6b), 4.20 (1H, d, $J_{1,2}$ 10, H-1), 7.65 (2H, d, *J* 10, H-2', 6'), 8.25 (2H, d, *J* 10, H-3', 5'); δ_C (50.3 MHz; ^2H_2O) 62.9 (C-6); 71.5 (C-4), 74.7 (C-2), 79.7 (C-3), 82.7 (C-5), 85.5 (C-1), 126.6 (3', 5'), 133.0 (C-2', 6'), 141.0 (C-1'), 151.2 (C-4'), 156.1 (C=N-OH); *m/z* (CI) 361([M+H]$^+$, 19%), 183 (56, [AglyconeH_3]$^+$), 163 (57, [glucose-OH]$^+$) and 145 (69, [glucose$H$-2H_2O]$^+$).

2,3,4,6-Tetra-*O*-acetyl-*p*-methoxyphenyl glucosinolate (177)



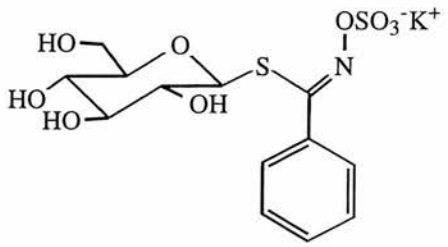
Dry pyridine (36.1 ml, 445 mmol) and dry dichloromethane (75 ml) were cooled to 0 °C under nitrogen and chlorosulfonic acid (1.6 ml, 23.4 mmol) in dry dichloromethane (75 ml) was added very carefully over a period of 30 minutes. A solution of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-*p*-methoxyphenyl thiohydroximate (1.20 g, 2.34 mmol) in dry dichloromethane (50 ml) was added and the reaction stirred for 24 hours at room temperature. Potassium hydrogen carbonate (6.94 g, 70 mmol) in water (80 ml) was added and the biphasic mixture stirred for a further 30 minutes. The reaction mixture was concentrated and coevaporated with toluene at reduced pressure to give the crude product. Purification by column chromatography on silica gel using dichloromethane-methanol (15:1) as the eluant gave 2,3,4,6-tetra-*O*-acetyl-*p*-methoxyphenyl glucosinolate as a pale yellow amorphous solid (0.56 g, 43%); m.p. 120-124 °C; $[\alpha]_D$ +38.0 ° (c 0.5 in CH_3OH); (Found: C, 41.19; H, 3.84; N, 1.96. Calc for $C_{22}H_{26}NO_{14}S_2K.0.5H_2O$: C, 41.24; H, 4.25; N, 2.19%); ν_{max} (nujol)/cm^{-1} 1700 (C=O); δ_H (200 MHz; $C^2H_3O^2H$) 1.80-2.20 (12H, 4s, 4 x OC(O)C$\underline{H}_3$), 3.50 (1H, m, H-5), 3.80 (3H, s, OC$\underline{H}_3$), 3.95 (1H, dd, $J_{5,6a}$ 2.5, $J_{6a,6b}$ 12.5, H-6a), 4.10 (1H, dd, $J_{5,6b}$ 5, $J_{6a,6b}$ 12.5, H-6b), 4.79 (1H, d, *J* 10, H-1), 4.89 (1H, t, *J* 10, H-2), 4.91 (1H, t, *J* 10, H-4), 5.10 (1H, t, *J* 10, H-3), 7.00 (2H, d, *J* 10, H-3', 5'), 7.50 (2H, d, *J* 10, H-2', 6'); δ_C (50.3 MHz; $C^2H_3O^2H$) 19.0, 19.1, 19.3 (4 x OC(O)$\underline{C}H_3$), 54.6 (OCH_3), 61.6 (C-6), 67.9 (C-4), 69.9 (C-2), 73.7 (C-3), 75.6 (C-5), 81.6 (C-1), 113.7 (3', 5'), 123.9 (C-1'), 130.7 (2', 6'), 157.8 (C=N), 161.9 (C-4'), 169.8, 169.9, 170.2, 171.0 (4 x O$\underline{C}$(O)CH_3); *m/z* (ES^-) 592 ([M-K]$^-$, 100%).

2,3,4,6-Tetra-*O*-acetyl-*p*-nitrophenyl glucosinolate (179)



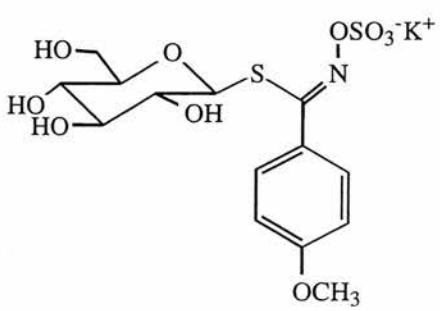
As described for (177) using 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-*p*-nitrophenyl thiohydroximate (0.50 g, 0.95 mmol). 2,3,4,6-Tetra-*O*-acetyl-*p*-nitrophenyl glucosinolate was given as a pale yellow amorphous solid (0.57 g, 93%); m.p. 128-132 °C (decomposes); $[\alpha]_D$ -1.13 ° (c 0.16 in CH_3OH); (Found: C, 37.94; H, 3.83; N, 3.87. Calc for $C_{21}H_{23}N_2O_{15}S_2K.H_2O$: C, 37.95; H, 3.79; N, 4.21%); ν_{max} (nujol)/cm^{-1} 1700 (C=O); δ_H (200 MHz; $C^2H_3O^2H$) 1.90-2.20 (12H, 4s, 4 x OC(O)C$\underline{H}_3$), 3.45 (1H, m, H-5), 3.90 (1H, dd, $J_{5,6a}$ 2.5, $J_{6a,6b}$ 12.5, H-6a), 4.05 (1H, dd, $J_{5,6b}$ 5, $J_{6a,6b}$ 12.5, H-6b), 4.80 (1H, d, *J* 10, H-1), 4.90 (2H, m, H-2, 4), 5.30 (1H, t, *J* 10, H-3), 7.75 (2H, d, *J* 10, H-2', 6'), 8.35 (2H, d, *J* 10, H-3', 5'); δ_C (50.3 MHz; $C^2H_3O^2H$) 19.1, 19.2, 19.4 (4 x OC(O)$\underline{C}H_3$), 61.6 (C-6), 67.9 (C-4), 70.0 (C-2), 73.4 (C-3), 75.4 (C-5), 81.0 (C-1), 123.4 (C-3', 5'), 130.4 (C-2', 6'), 138.2 (C-1'), 149.2 (C-4'), 154.0 (C=N), 169.9, 170.0, 170.2, 171.0 (4 x O$\underline{C}$(O)CH_3); *m/z* (ES^-) 607.8 ($[M-K]^-$, 100%).

Phenyl glucosinolate (158)



As described for (177) using 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl phenyl thiohydroximate (0.46 g, 0.95 mmol). Phenyl glucosinolate was given as a white amorphous solid (0.27 g, 66%); m.p. 110-115 °C; $[\alpha]_D$ -19.8 ° (c 0.5 in H_2O); (Found: C, 33.49; H, 3.52; N, 2.62. Calc for $C_{13}H_{16}NO_9S_2K.2H_2O$: C, 33.26; H, 4.29; N, 2.98%); ν_{max} (nujol)/cm^{-1} 3600-3000 (OH), 770 and 730 (monosubstituted phenyl); δ_H (300 MHz; ^2H_2O) 2.60 (1H, m, H-5), 3.16 (1H, t, *J* 10, H-2), 3.28 (1H, t, $J_{3,4}=J_{4,5}$ 10, H-4), 3.36 (1H, t, $J_{2,3}=J_{3,4}$ 10, H-3), 3.50 (1H, dd, $J_{5,6b}$ 5, $J_{6a,6b}$ 12.5, H-6b), 3.60 (1H, dd, $J_{5,6a}$ 2.5, $J_{6a,6b}$ 12.5, H-6a), 4.19 (1H, d, $J_{1,2}$ 10, H-1), 7.50 (3H, m, H-2', 3', 4', 5', 6'); δ_C (75.45 MHz; ^2H_2O) 60.1 (C-6), 68.8 (C-4), 71.6 (C-2), 77.1 (C-3), 79.9 (C-5), 83.5 (C-1), 128.9 (3', 5'), 129.1 (C-2', 6'), 130.4 (C-1'), 131.2 (C-4'), 164.1 (C=N); *m/z* (ES^-) 393.9 ($[M-K]^-$, 100%).

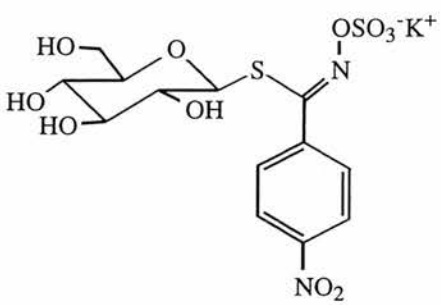
***p*-Methoxyphenyl glucosinolate (157)**



Potassium metal (0.039 g, 0.1 mmol) was added to dry methanol (1 ml) under nitrogen to give 1 N methanolic potassium methoxide. The potassium methoxide was then added

dropwise to 2,3,4,6-tetra-*O*-acetyl-*p*-methoxyphenyl glucosinolate (0.3 g, 0.48 mmol) in dry methanol (10.0 ml) until pH 8-9 was reached. The reaction was stirred for 24 hours then the solvent evaporated at reduced pressure to give *p*-methoxyphenyl glucosinolate as a white amorphous solid (0.148 g, 67%); m.p. 68-71 °C; (Found: C, 33.48; H, 4.05; N, 2.43. Calc for $C_{14}H_{18}NO_{10}S_2K$: C, 36.28; H, 3.91; N, 3.02%); ν_{max} (nujol)/cm^{-1} 3600-3000 (OH), 1150 (C-O stretching), 830 (para disubstituted phenyl); δ_H (300 MHz; ^2H_2O) 2.60 (1H, m, H-5), 3.10-3.40 (3H, m, H-2, 3, 4), 3.50 (1H, dd, $J_{5,6b}$ 5, $J_{6a,6b}$ 12.5, 6b), 3.60 (1H, dd, $J_{5,6a}$ 2.5, $J_{6a,6b}$ 12.5, 6a), 3.80 (3H, s, OCH_3), 4.20 (1H, d, $J_{1,2}$ 10, H-1), 7.00 (2H, d, *J* 10, H-3', 5'), 7.50 (2H, d, *J* 10, H-2', 6'); δ_C (50.3 MHz; ^2H_2O) 54.6 (OCH_3), 60.1 (C-6), 68.8 (C-4), 71.7 (C-2), 77.1 (C-3), 80.0 (C-5), 83.7 (C-1), 114.4 (3', 5'), 123.0 (C-1'), 130.9 (2', 6'), 157.8 (C-4'), 161.9 (C=N); *m/z* (ES$^-$) 424.1 ([M-K]$^-$, 100%).

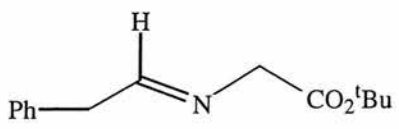
_p_-Nitrophenyl glucosinolate (159)



As described for (157) using 2,3,4,6-tetra-*O*-acetyl-*p*-nitrophenyl glucosinolate (0.3 g, 0.46 mmol). *p*-Nitrophenyl glucosinolate was given as a pale yellow amorphous solid (0.11 g, 50%); m.p. 69-70.5 °C; $[\alpha]_D$ -43.3 ° (c 0.5 in H_2O); (Found: C, 25.98; H, 3.30; N, 3.67; O, 41.79; S, 14.75. Calc for $C_{13}H_{15}N_2O_{11}S_2K$: C, 32.63; H, 3.16; N, 5.85; O, 36.78; S, 13.40%); ν_{max} (nujol)/cm^{-1} 3600-3000 (OH), 860 (para disubstituted phenyl); δ_H (200 MHz; ^2H_2O) 2.60 (1H, m, H-5), 3.10-3.40 (3H, m, H-2, 3, 4), 3.50 (1H, dd, $J_{5,6b}$ 5, $J_{6a,6b}$ 12.5, 6b), 3.55 (1H, dd, $J_{5,6a}$ 2.5, $J_{6a,6b}$ 12.5, 6a), 4.65 (1H, d, $J_{1,2}$ 10, H-1), 7.75 (2H, d, *J* 10, H-2', 6'), 8.30 (2H, d, *J* 10, H-3', 5'); δ_C (50.3 MHz; ^2H_2O) 60.1 (C-6), 68.8 (C-4), 71.8 (C-2), 76.9 (C-3), 80.0 (C-5), 83.2 (C-1), 124.1 (3', 5'), 130.5 (2', 6'), 136.9 (C-1'), 149.1 (C-4'), 161.6 (C=N); *m/z* (ES$^-$) 439 ([M-K]$^-$, 100%).

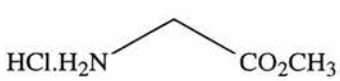
3.6 Non-rearrangeable Glucosinolate Analogues

t-Butyl (2-phenylethylidineamino)acetate (183)



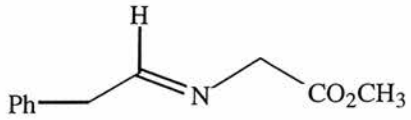
To a solution of t-butyl glycinate.AcOH (1.0 g, 5.0 mmol) in dichloromethane (20 ml) was added magnesium sulfate (0.42 g, 3.5 mmol) followed by triethylamine (1.4 ml, 10.0 mmol) and phenylacetaldehyde (0.6 g, 5.0 mmol). The reaction was monitored by t.l.c. (silica, ethyl acetate-methanol (9:1)) while stirring at room temperature for a period of three days. Both t.l.c. and the ^{1}H n.m.r. spectrum of the crude product showed that no reaction had occurred.

Methyl glycinate hydrochloride (185)



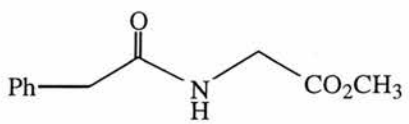
Thionyl chloride (8.8 ml, 121.0 mmol) was added to a solution of glycine (8.26 g, 110.0 mmol) in methanol (120 ml) at 0 °C. The solution was heated to reflux for 1 hour then the solution was cooled to give a white crystalline solid which was removed by filtration. The remaining solvent was removed at reduced pressure to give a white solid. Both solids were recrystallised from methanol to give methyl glycinate hydrochloride (12.5 g, 91%); m.p. 141 °C; (lit.,[269] 142 °C); ν_{max} (nujol)/cm^{-1} 1740 (NH_2), 1580 (C=O, ester); δ_H (200 MHz; $^{2}H_2O$) 3.80 (3H, s, CH_3), 3.90 (2H, s, CH_2); δ_C (50.3 MHz; $^{2}H_2O$) 56.2 (CH_3), 56.0 (CH_2), 171.5 (C=O).

Methyl (2-phenylethylidineamino)acetate (184)



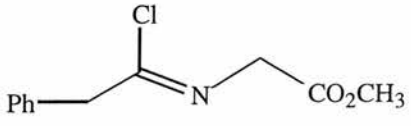
To a solution of methyl glycinate.HCl (0.63 g, 5.0 mmol) in dichloromethane (20 ml) was added magnesium sulfate (0.42 g, 3.5 mmol) followed by triethylamine (1.4 ml, 10.0 mmol) and phenylacetaldehyde (0.6 g, 5 mmol). The reaction was monitored by t.l.c. (silica, ethyl acetate-hexane (9:1)) while stirring at room temperature for a period of three days. Both t.l.c. and the ^{1}H n.m.r. spectrum showed that no reaction had occurred.

Methyl *N*-(phenylacetyl)glycinate (186)



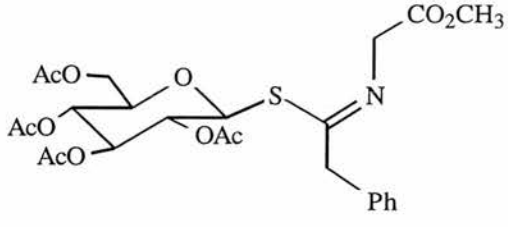
Triethylamine (4.9 ml, 35 mmol) was added to methyl glycinate.HCl (2.0 g, 16 mmol) in dichloromethane (40 ml) at 0 °C. Phenylacetyl chloride (2.1 ml, 16.0 mmol) was added dropwise then the solution was allowed to warm to room temperature and stirred for 4 hours. The reaction mixture was partitioned between water (100 ml) and dichloromethane (100 ml) then the organic layer washed with brine (50 ml), dried ($MgSO_4$) and the solvent removed at reduced pressure. The resulting solid was recrystallised from 60-80 petroleum ether and ethanol to give colourless crystals of methyl *N*-(phenylacetyl)glycinate (3.3 g, 99%); m.p. 89-90 °C (lit.,[270] 89-90 °C); ν_{max} (nujol)/cm^{-1} 1758 (C=O, ester), 750 (monosubstituted phenyl); δ_H (200 MHz; C^2HCl_3) 3.60 (2H, s, PhC$\underline{H}_2$), 3.70 (3H, s, CH_3), 3.95 (2H, d, *J* 5.0, NHC$\underline{H}_2$), 6.20 (1H, s, NH), 7.10-7.40 (5H, m, $C_6\underline{H}_5$); δ_C (50.3 MHz; C^2HCl_3) 41.8 (Ph$\underline{C}H_2$), 43.5 (CH_3), 52.7 (NH$\underline{C}H_2$), 127.8 (C-4'), 129.3 (C-3', 5'), 129.9 (C-2', 6'), 135.1 (C-1'), 170.8 ($\underline{C}O_2CH_3$), 172.2 ($\underline{C}$(O)NH); *m/z* (EI) 207 ([M]$^+$, 8%), 136 (24, [$PhCH_2C(O)NH_3$]$^+$), 118 (6, [PhCHCO]$^+$) and 91 (100, [$PhCH_2$]$^+$).

Methyl (1-chloro-2-phenylethylidineamino)acetate (187)



Oxalyl chloride (0.65 ml, 7.5 mmol) solution in toluene (1.2 ml) was added dropwise to a mixture of methyl *N*-(phenylacetyl)glycinate (1.04 g, 5.0 mmol) and pyridine (0.5 ml, 5.8 mmol) in tetrahydrofuran (7.5 ml) at 0 °C. The solution was refrigerated overnight then the pyridine hydrochloride precipitate was removed by filtration and washed with toluene. The solvent was removed at reduced pressure to give a bright orange solid (0.72 g, 64%); δ_H (200 MHz; C^2HCl_3) 3.70 (2H, s, PhC<u>H</u>$_2$), 3.85 (3H, s, CH_3), 4.05, 4.55 (2H, 2s, C<u>H</u>$_2$N, E and Z isomers), 7.10-7.50 (5H, m, C_6H_5).

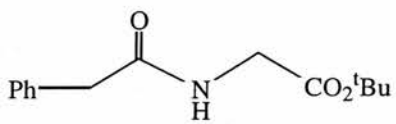
Methyl (1-(2',3',4',6'-tetra-*O*-acetyl-β-D-glucos-1'-ylthio)-2-phenylethylidineamino))acetate (188)



Methyl (1-chloro-2-phenylethylidineamino)acetate (0.72 g, 3.2 mmol) in tetrahydrofuran (40 ml) was added to a solution of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (1.17 g, 3.2 mmol) in tetrahydrofuran (30 ml). Triethylamine (4.22 ml, 30 mmol) was added in one portion and the reaction stirred at room temperature for 2 hours 30 minutes. The solvent was removed at reduced pressure and the residue taken up in diethyl ether (100 ml) then washed with 1.0 M sulfuric acid (100 ml). The organic extract was dried ($MgSO_4$) and the solvent removed at reduced pressure. The residue contained many compounds and was purified using column chromatography on silica with ethyl acetate-dichloromethane (1:1) eluant to give a possible product (0.04 g, 2%); δ_C (50.3 MHz; C^2HCl_3) 20.7, 20.9,

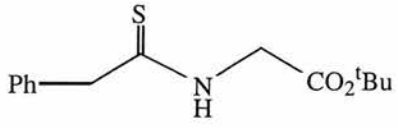
21.1, 21.2 (4 x OC(O)$\underline{C}H_3$), 41.7 (Ph$\underline{C}H_2$), 43.4 (CH_3), 53.0 (N$\underline{C}H_2$), 62.1 (C-6'), 68.3 (C-4'), 70.1 (C-2'), 74.3 (C-5'), 76.5 (C-3'), 87.4 (C-1'), 126.0-131.0 ($C_6\underline{H}_5$, many signals), 153.5 (C=N), 166.0-172.0 (C=O, many signals); *m/z* (EI) 403 ([M-$PhCH_2$-CO_2CH_3]$^+$, 13), 331 (84, [M-Aglycone]$^+$), 169 (100, [$C_8H_9O_4$]$^+$), 109 (58) and 91 (42, [$PhCH_2$]$^+$).

_t_-Butyl _N_-(phenylacetyl)glycinate (189)



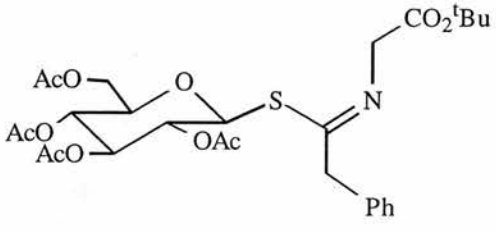
Triethylamine (4.0 ml, 29.0 mmol) was added to t-butyl glycinate.AcOH (2.5 g, 13.1 mmol) in dichloromethane (30 ml) then the solution was cooled to 0 °C. Phenylacetyl chloride (1.74 ml, 13.1 mmol) was added dropwise then the solution was allowed to warm to room temperature. After 4 hours stirring the mixture was extracted with dichloromethane (2 x 20 ml) and the organic extracts dried ($MgSO_4$). The solvent was evaporated at reduced pressure to give *t*-butyl *N*-(phenylacetyl)glycinate (2.75 g, 84%) as a clear oil; ν_{max} (thin film)/cm^{-1} 3299, 1544 (C(O)NH), 1743 (C=O, ester); δ_H (300 MHz; C^2HCl_3) 1.40 (9H, s, C(C$\underline{H}_3$)$_3$), 3.60 (2H, s, PhC$\underline{H}_2$), 3.88 (2H, d, *J* 5.5, C$\underline{H}_2$NH), 5.95 (1H, s, NH) 7.20-7.40 (5H, m, $C_6\underline{H}_5$); δ_C (75.45 MHz; C^2HCl_3) 28.1 (C($\underline{C}H_3$)$_3$), 42.3 (Ph$\underline{C}H_2$), 43.6 ($\underline{C}H_2$NH), 82.4 ($\underline{C}(CH_3)_3$), 127.6 (C-4'), 129.3 (C-3', 5'), 129.7 (C-2', 6'), 134.9 (C-1'), 169.0 ($\underline{C}O_2CH_3$), 171.2 ($\underline{C}$(O)NH); *m/z* (EI) 249 ([M]$^+$, 5%), 193 (12, [M-$CH_2C(CH_3)_2$]$^+$), 176 (19, [M-$OC(CH_3)_3$]$^+$), 149 (52, [M-CO_2-$(CH_3)_2CCH_2$]$^+$), 91 (67, [$PhCH_2$]$^+$) and 57 (100, [$C(CH_3)_3$]$^+$).

t-Butyl *N*-(phenylthioacetyl)glycinate (190)



t-Butyl *N*-(phenylacetyl)glycinate (0.25 g, 1.0 mmol) was dissolved in tetrahydrofuran (1.5 ml) and Lawessons reagent (0.20 g, 0.5 mmol) was added in one portion. The solvent was removed at reduced pressure and the resulting oil purified using column chromatography on silica gel with ethyl acetate-hexane (1:2) as the eluant. *t*-Butyl *N*-(phenylthioacetyl)glycinate was obtained as a pale yellow oil (0.22 g, 81%); (Found: C, 62.94; H, 7.42; N, 5.22. Calc for $C_{14}H_{19}NO_2S$: C, 63.37; H, 7.22; N, 5.28%); ν_{max} (thin film)/cm^{-1} 1743 (C=O, ester), 1100 (C(S)NH); δ_H (300 MHz; C^2HCl_3) 1.40 (9H, s, C(C$\underline{H}_3$)$_3$), 4.15 (2H, s, PhC$\underline{H}_2$), 4.23 (2H, d, *J* 4.5, C$\underline{H}_2$NH), 7.20-7.40 (5H, m, C$_6\underline{H}_5$), 7.60 (1H, s, NH); δ_C (75.45 MHz; C^2HCl_3) 28.0 (C($\underline{C}$H$_3$)$_3$), 48.3 (Ph$\underline{C}$H$_2$), 52.9 ($\underline{C}$H$_2$NH), 83.2 ($\underline{C}$(CH$_3$)$_3$), 128.1 (C-4'), 129.5 (C-3', 5'), 129.7 (C-2', 6'), 134.9 (C-1'), 167.8 ($\underline{C}$O$_2$CH$_3$), 202.5 ($\underline{C}$=S); *m/z* (EI) 265 ([M]$^+$, 41%), 209 (49, [M-(CH$_3$)$_2$CCH$_2$]$^+$), 192 (30, [M-OC(CH$_3$)$_3$]$^+$), 134 (100, [PhCHCS]$^+$), 91 (79, [PhCH$_2$]$^+$) and 57 (69, [C(CH$_3$)$_3$]$^+$).

t-Butyl (1-(2',3',4',6'-tetra-*O*-acetyl-β-D-glucos-1'-ylthio)-2-phenylethylidineamino))acetate (191)

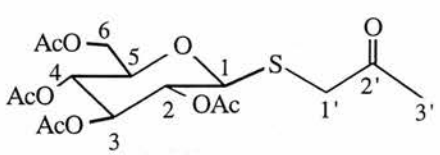


Method 1: 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide (0.30 g, 0.75 mmol) and *t*-butyl *N*-(phenylthioacetyl)glycinate (0.21 g, 0.79 mmol) were dissolved using a mixture of methanol (1 ml) and acetone (3 ml). Powdered potassium hydroxide (0.042 g) was added

in one portion and the solution was stirred overnight. The solvent was removed at reduced pressure and the residue taken up in ethyl acetate (20 ml) then washed with distilled water (15 ml). The organic extract was dried ($MgSO_4$) and the solvent removed at reduced pressure. Purification using column chromatography on silica gel with ethyl acetate-hexane (1:2) as the eluant gave only starting materials.

Method 2: 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide (0.23 g, 0.57 mmol) and *t*-butyl *N*-(phenylthioacetyl)glycinate (0.15 g, 0.57 mmol) were dissolved in acetone (4 ml) and triethylamine (0.2 ml, 1.4 mmol) added to initiate the reaction. After 4 hours at reflux the solution was concentrated at reduced pressure and then extracted using diethyl ether (2 x 20 ml). The ethereal extracts were dried ($MgSO_4$) and the solvent removed at reduced pressure. The residue was purified using column chromatography on silica gel with ethyl acetate-hexane (1:2); starting materials were recovered along with 3,4,6-tri-*O*-acetyl-2-acetoxy-D-glucal (0.069 g); δ_H (200 MHz; C^2HCl_3) 2.00-2.15 (12H, m, 4 x OC(O)C$\underline{H}_3$), 4.10-4.50 (3H, m, H-5, 6a, 6b), 5.20 (1H, t, *J* 5, H-4), 5.55 (1H, d, *J* 5, H-3), 6.60 (1H, s, H-1); δ_C (50.3 MHz; C^2HCl_3) 20.0-20.2 (4 x OC(O)$\underline{C}H_3$), 61.5 (C-6), 67.8 (C-5), 70.2 (C-4), 71.0 (C-3), 72.5 (C-2), 86.9 (C-1), 170.0, 170.2, 170.4, 171.1 (4 x O$\underline{C}$(O)CH_3); *m/z* (EI) 331 ($[MH]^+$, 30%), 288 (20, $[MH-Ac]^+$), 271 (67, $[M-OAc]^+$) and 169 (100, $[C_8H_9O_4]^+$).

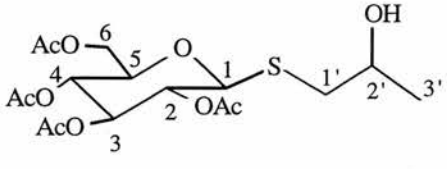
2'-Oxopropyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (195)



2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (1.0 g, 2.74 mmol) was added to chloroacetone (0.22 ml, 2.74 mmol) in dry diethyl ether (90 ml). Dry triethylamine (4.0 ml, 28.6 mmol) was added and a white precipitate formed. After stirring for 4 hours at room temperature the reaction was extracted using ethyl acetate (2 x 50 ml) then the organic

extracts were dried ($MgSO_4$) and the solvent evaporated at reduced pressure to give a clear oil. The oil was purified by column chromatography on silica gel using ethyl acetate-hexane (2:1) as the eluant to give 2'-oxopropyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside as a clear oil which crystallised on standing (1.13 g, 98%); m.p. 70-72 °C; $[\alpha]_D$ +69.7 ° (c 1.0 in $CHCl_3$); (Found: C, 48.41; H, 5.80. Calc for $C_{17}H_{24}O_{10}S$: C, 48.57; H, 5.75%); ν_{max} (nujol)/cm^{-1} 1745 (C=O, ester), 1708 (C=O, ketone); δ_H (300 MHz; C^2HCl_3) 1.90-2.10 (12H, 4s, 4 x OC(O)C<u>H</u>$_3$), 2.29 (3H, s, H-3'), 3.30 (1H, d, J_{gem} 15, H-1a'), 3.50 (1H, d, J_{gem} 15, H-1b'), 3.65 (1H, m, H-5), 4.12 (1H, dd, $J_{5,6a}$ 2.5, J_{gem} 11, H-6a), 4.18 (1H, dd, $J_{5,6b}$ 5, J_{gem} 11, H-6b), 4.50 (1H, d, $J_{1,2}$ 10, H-1), 5.03 (1H, t, $J_{3,4}$ 10, $J_{4,5}$ 10, H-4), 5.05 (1H, t, $J_{1,2}$ 10, $J_{2,3}$ 10, H-2), 5.20 (1H, t, $J_{2,3}$ 10, $J_{3,4}$ 10, H-3); δ_C (75.45 MHz; C^2HCl_3) 17.2, 17.3, 17.4 (4 x OC(O)<u>C</u>H$_3$), 24.5 (C-3'), 37.1 (C-1'), 58.5 (C-6), 64.9 (C-4), 66.3 (C-2), 70.5 (C-3), 72.8 (C-5), 79.5 (C-1), 166.2, 166.3, 167.0, 167.6 (4 x O<u>C</u>(O)CH$_3$), 200.6 (C=O); *m/z* (CI) 421 ($[M+H]^+$, 5%), 331 (100, $[M\text{-Aglycone}]^+$), 271 (27, $[M\text{-Aglycone-AcOH}]^+$), 245 (16, $[MH_2\text{-}3OAc]^+$) and 169 (29, $[C_8H_9O_4]^+$).

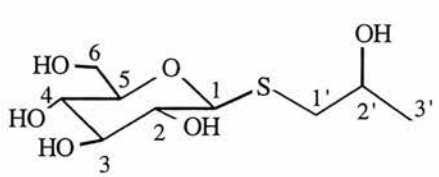
2'-Hydroxypropyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (196)



2'-Oxopropyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (3.41 g, 4.75 mmol) was dissolved in tetrahydrofuran (35 ml) and sodium borohydride (0.16 g, 2.4 mmol) in water (1.2 ml) was added dropwise at 0 °C. The solution was stirred for 2 hours and then quenched using 1 M hydrochloric acid. The reaction was extracted using ethyl acetate (2 x 50 ml) and washed with brine (2 x 25 ml). The organic extracts were dried ($MgSO_4$) and the solvent evaporated at reduced pressure to give a syrup which was purified by column chromatography on silica using ethyl acetate-hexane (2:1) as the eluant. 2'-Hydroxypropyl

2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside was given as a white solid (2.52 g, 74%); m.p. 83-85 °C; $[\alpha]_D$ -24.9 ° (c 1.0 in $CHCl_3$); (Found: C, 48.22; H, 6.28. Calc for $C_{17}H_{26}O_{10}S$: C, 48.33; H, 6.20%); ν_{max} (nujol)/cm^{-1} 3495 (OH), 1740 (C=O); δ_H (300 MHz; C^2HCl_3) 1.80, 1.19 (6H, 2d, *J* 6, 2 x H-3' isomers 1 and 2), 1.96, 1.98, 2.01, 2.04 (24H, 4s, 8 x OC(O)C$\underline{H}_3$ isomers 1 and 2), 2.49 (1H, dd, $J_{1b',2'}$ 9, J_{gem} 15, H-1b' isomer 1), 2.69 (1H, dd, $J_{1b',2'}$ 7, J_{gem} 15, H-1b' isomer 2), 2.73 (1H, dd, $J_{1a',2'}$ 4, J_{gem} 15, H-1a' isomer 1), 2.78 (2H, s, 2 x OH isomers 1 and 2), 2.90 (1H, dd, $J_{1a',2'}$ 3, J_{gem} 15, H-1a' isomer 2), 3.72 (2H, m, H-5 isomers 1 and 2), 3.86 (2H, m, H-2' isomers 1 and 2), 4.01 (4H, m, H-6a, 6b isomers 1 and 2), 4.50 (2H, d, $J_{1,2}$ 10, H-1 isomers 1 and 2), 5.00 (4H, m, H-2, 4 isomers 1 and 2), 5.18 (2H, t, $J_{2,3}$ 10, $J_{3,4}$ 10, H-3 isomers 1 and 2); δ_C (75.45 MHz; C^2HCl_3) 20.5, 20.6, 20.7 (8 x OC(O)$\underline{C}H_3$ isomers 1 and 2), 22.0 (C-3' isomers 1 and 2), 40.4, 40.9 (C-1' isomers 1 and 2), 62.1 (C-6 isomers 1 and 2), 66.9, 67.4 (C-8 isomers 1 and 2), 68.2, 68.3 (C-4 isomers 1 and 2), 69.8, 69.9 (C-2 isomers 1 and 2), 73.8 (C-3 isomers 1 and 2), 77.0 (C-5 isomers 1 and 2), 84.2, 84.6 (C-1 isomers 1 and 2), 169.6, 170.3, 170.8 (8 x O$\underline{C}$(O)CH_3 isomers 1 and 2); *m/z* (CI) 423 ($[M+H]^+$, 5%), 405 (7, $[M-HO]^+$), 363 (19, $[M-(CH_3)_2COH]^+$), 331 (100, [M-Aglycone]$^+$), 271 (40, [M-Aglycone-AcOH]$^+$), 243 (16, [MH-3AcOH]$^+$) and 169 (55, $[C_8H_9O_4]^+$).

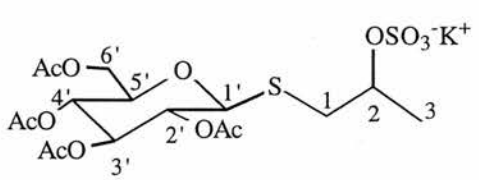
2'-Hydroxypropyl 1-thio-β-D-glucopyranoside (197)



To 2'-hydroxypropyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (0.51 g, 1.2 mmol) in methanol was added a catalytic amount of potassium metal under nitrogen. The reaction was stirred for 18 hours then Amberlite IR-120 resin was added. Stirring was continued for a further 15 minutes before the Amberlite was removed by filtration and the

solvent removed by evaporation at reduced pressure. The resulting oil was purified by column chromatography on silica gel using dichloromethane-methanol (9:1) as the eluant to give 2'-hydroxypropyl 1-thio-β-D-glucopyranoside as a clear syrup (0.25 g, 83%); $[\alpha]_D$ -91.2 ° (c 1.0 in CH_3OH); (Found: C, 42.02; H, 7.63. Calc for $C_9H_{19}O_6S$: C, 42.34; H, 7.50%); ν_{max} (nujol)/cm^{-1} 3300 (OH); δ_H (300 MHz; $C^2H_3O^2H$) 1.22 (6H, 2d, *J* 6, 2 x H-3' isomers 1 and 2), 2.66 (1H, dd, $J_{1b',2'}$ 7, J_{gem} 14, H-1b' isomer 1), 2.71 (1H, dd, $J_{1a',2'}$ 4.5, J_{gem} 14, H-1a' isomer 2), 2.80 (1H, dd, $J_{1b',2'}$ 7, J_{gem} 14, H-1b' isomer 2), 2.90 (1H, dd, $J_{1a',2'}$ 4.5, J_{gem} 14, H-1a' isomer 1), 3.18-3.40 (10H, m, H-2, 3, 4, 5, 2' isomers 1 and 2), 3.65 (2H, m, H-6b isomers 1 and 2), 4.01 (2H, m, H-6a isomers 1 and 2), 4.38, 4.40 (2H, 2d, $J_{1,2}$ 10, H-1 isomers 1 and 2), 5.00 (4H, m, H-2, 4 isomers 1 and 2), 5.18 (2H, t, $J_{2,3}$ 10, $J_{3,4}$ 10, H-3 isomers 1 and 2); δ_C (75.45 MHz; $C^2H_3O^2H$) 21.1 (C-3' isomers 1 and 2), 38.2, 38.5 (C-1' isomers 1 and 2), 61.5 (C-6 isomers 1 and 2), 67.1, 67.2 (C-2' isomers 1 and 2), 70.1 (C-4 isomers 1 and 2), 73.0 (C-2 isomers 1 and 2), 78.2 (C-3 isomers 1 and 2), 80.7 (C-5 isomers 1 and 2), 86.1, 86.2 (C-1 isomers 1 and 2); *m/z* (CI) 256 ($[M+H]^+$, 3%), 255 (28, $[M]^+$), 237 (53, $[M-H_2O]^+$), 219 (30, $[M-2H_2O]^+$), 201 (12, $[M-3H_2O]^+$), 163 (100, [M-AglyconeH]$^+$) and 145 (75, [M-AglyconeH-H_2O]$^+$).

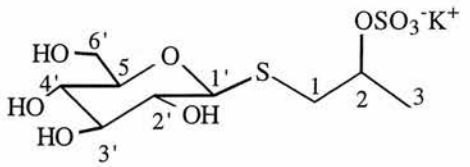
Potassium 1-(2',3',4',6'-tetra-*O*-acetyl-1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate (198)



Dry pyridine (55 ml, 680 mmol) and dry dichloromethane (55 ml) were cooled to 0 °C under nitrogen and chlorosulfonic acid (2.5 ml, 36.4 mmol) in dry dichloromethane (55 ml) was added very carefully over a period of 30 minutes. A solution of 2'-hydroxypropyl-2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (1.5 g, 3.55 mmol) in

dry dichloromethane (25 ml) was added and the reaction stirred for 24 hours at room temperature. Saturated potassium hydrogen carbonate (50 ml) was added and the biphasic mixture stirred for a further 30 minutes. The reaction mixture was evaporated at reduced pressure to give a syrup which was purified by column chromatography on silica gel using dichloromethane-methanol (9:1) as the eluant. Potassium 1-(2',3',4',6'-tetra-*O*-acetyl-1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate was given as a colourless syrup (1.18 g, 62%); $[\alpha]_D$ -56.5 ° (c 1.0 in CH_3OH); (Found: C, 37.84; H, 4.52. Calc for $C_{17}H_{25}O_{13}S_2K$: C, 37.77; H, 4.66%); ν_{max} (nujol)/cm^{-1} 1740 (C=O); δ_H (300 MHz; $C^2H_3O^2H$) 1.72 (6H, 2d, *J* 6, 2 x H-3 isomers 1 and 2), 2.30, 2.32, 2.34, 2.37, 2.41 (24H, 5s, 8 x OC(O)C$\underline{H}_3$ isomers 1 and 2), 3.03 (1H, dd, $J_{1b,2}$ 7.5, J_{gem} 14, H-1b isomer 1), 2.71 (1H, dd, $J_{1b,2}$ 7.5, J_{gem} 14, H-1b isomer 2), 2.73 (1H, dd, $J_{1a,2}$ 4, J_{gem} 14, H-1a isomer 1), 2.90 (1H, dd, $J_{1a,2}$ 4, J_{gem} 14, H-1a isomer 2), 4.25 (2H, m, H-5' isomers 1 and 2), 4.50 (2H, 2dd, $J_{5',6a'}$ 2.5, J_{gem} 14, H-6a' isomers 1 and 2), 4.60 (2H, 2dd, $J_{5',6b'}$ 4, J_{gem} 14, H-6b' isomers 1 and 2), 4.90, 5.05 (2H, 2m, H-2 isomers 1 and 2), 5.18 (2H, d, $J_{1',2'}$ 10, H-1'), 5.24, 5.25 (2H, 2t, $J_{1',2'}$ 10, $J_{2',3'}$ 10, H-2' isomers 1 and 2), 5.36, 5.38 (2H, 2t, $J_{3',4'}$ 10, $J_{4',5'}$ 10, H-4' isomers 1 and 2), 5.61, 5.63 (2H, 2t, $J_{2',3'}$ 10, $J_{3',4'}$ 10, H-3' isomers 1 and 2); δ_C (75.45 MHz; $C^2H_3O^2H$) 15.0, 15.3 (C-3 isomers 1 and 2), 15.9, 16.0, 16.1 (8 x OC(O)$\underline{C}H_3$ isomers 1 and 2), 32.1, 33.2 (C-1 isomers 1 and 2), 58.6, 58.7 (C-6' isomers 1 and 2), 65.2, 65.3 (C-2 isomers 1 and 2), 67.0, 67.4 (C-4' isomers 1 and 2), 70.8 (C-2' isomers 1 and 2), 71.7, 71.9 (C-3' isomers 1 and 2), 72.1, 72.2 (C-5' isomers 1 and 2), 79.8, 81.1 (C-1' isomers 1 and 2), 166.9, 167.1, 168.2 (8 x O$\underline{C}$(O)CH_3 isomers 1 and 2); *m/z* (ES^-) 501 ($[M-K]^-$, 100%).

Potassium 1-(1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate (193)



To potassium 1-(2',3',4',6'-tetra-*O*-acetyl-1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate (0.5 g, 0.92 mmol) in methanol (5 ml) was added a catalytic amount of potassium metal under nitrogen. The reaction was stirred for 18 hours before the solvent was removed by evaporation at reduced pressure to give a white amorphous solid which was purified using reverse phase C-18 silica then desalted using G-10 Sephadex, in both cases distilled water was used as the eluant. The product was freeze dried to give the pure potassium 1-(1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate as an extremely hygroscopic colourless solid (0.321 g, 93%), $[\alpha]_D$ -78.4 ° (c 0.5 in H_2O); (Found: C, 28.49; H, 4.54. Calc for $C_9H_{17}O_9S_2K$: C, 29.02; H, 4.60%); ν_{max} (nujol)/cm^{-1} 3300 (OH); δ_H (200 MHz; ^2H_2O) 1.40 (6H, d, *J* 6, 2 x H-3 isomers 1 and 2), 3.00 (4H, m, H-1 isomers 1 and 2), 3.25-3.60 (8H, m, H-2', 4', 5', 2 isomers 1 and 2), 3.70 (2H, 2dd, $J_{5',6b'}$ 4, J_{gem} 14, H-6b' isomers 1 and 2), 3.90 (2H, 2dd, $J_{5',6b'}$ 4, J_{gem} 14, H-6b' isomers 1 and 2), 4.90 (2H, 2dd, $J_{5',6a'}$ 2.5, J_{gem} 14, H-6a' isomers 1 and 2), 4.60 (2H, t, $J_{2',3'}$ 10, $J_{3',4'}$ 10, H-3' isomers 1 and 2), 4.70 (2H, 2d, $J_{1',2'}$ 10, H-1' isomers 1 and 2); δ_C (50.3 MHz; ^2H_2O) 21.9, 22.1 (C-3 isomers 1 and 2), 38.1, 38.5 (C-1 isomers 1 and 2), 63.7, 63.8 (C-6' isomers 1 and 2), 72.3 (C-2 isomers 1 and 2), 75.2, 75.4 (C-4' isomers 1 and 2), 79.5 (C-2' isomers 1 and 2), 79.9, 80.0 (C-3' isomers 1 and 2), 82.7 (C-5' isomers 1 and 2), 88.2, 88.7 (C-1' isomers 1 and 2); *m/z* (ES^-) 333 ($[M-K]^-$, 100%).

3.7 Studies with Myrosinase

Myrosinase Assay

Myrosinase was constituted by dissolving 60 mg of crude myrosinase (lyophilised powder from *Sinapis alba*, 175 units per g solid, Sigma) in 3 ml of 33.1 mM potassium phosphate buffer solution at pH 7.0. When the enzyme solution was not in use it was stored at -18 °C. Myrosinase activity was assayed according to the method of Palmieri and co-workers.[244] Assays were carried out using 33.1 mM potassium phosphate buffer at pH 7.0 containing 0.1 mM sinigrin in a total volume of 1.0 ml. The solution was placed in a 1.0 ml volume quartz cuvette with a 1 cm pathlength then warmed to 37 ± 0.1 °C in the thermostatted cell holder of the UV spectrophotometer. The reaction was initiated by the addition of myrosinase solution (30 µl) and the decrease in absorbance at 227 nm, due to sinigrin, was measured. The number of units of enzyme activity in the solution was determined by dividing the experimental initial rate by the theoretical rate (6.7 min^{-1}) calculated for 1 unit of enzyme. One unit of enzyme activity was defined as the amount of myrosinase required to catalyse the hydrolysis of 1 µmol of sinigrin per minute under standard assay conditions.

Glucose (HK) Assay

Glucose (HK) reagent was obtained from Sigma and was reconstituted according to the directions with 20 ml of deionised water. The absorbance of this solution was measured at 340 nm to check that the reagent was still active. In each case the absorbance was less than 0.35 and so the reagent activity was acceptable.

Each assay solution (1.0 ml) was equilibrated at 37 ± 0.1 °C in a 1.0 ml volume/1.0 cm pathlength quartz cuvette in the thermostatted cell holder of the UV spectrophotometer. A 10 µl aliquot of the reaction mixture was then added. After a recommended stabilisation period of approximately 5 minutes incubation at 37 ± 0.1 °C the absorbance at 340 nm was

noted. A blank sample of 10 µl distilled water in 1.0 ml glucose (HK) solution was measured at 340 nm and 37 ± 0.1 °C for each sample. The glucose concentration was then calculated from the following equation:

$$[\text{Glucose}]/\text{mM} = \left(\frac{(\text{sample absorbance} - \text{blank absorbance}) \times 293 \times 0.01}{180.16} \right) \times 1000$$

where 293 is a predetermined factor (mg/dl)
0.01 converts mg/dl to mg/ml
180.16 is the molecular weight of D-glucose

From a graphical plot of [Glucose] liberated in mM versus time in minutes the initial rate of turnover can be calculated. This is done by measuring the inital gradient of the graph and correcting for 1 unit of enzyme activity.

Determination of the Accuracy of Glucose (HK) Reagent

Standard 5 mM D-glucose solution

Blank absorbance 0.043
Sample absorbance 0.355

$$[\text{Glucose}] = \left(\frac{(0.355 - 0.043) \times 293 \times 0.01}{180.16} \right) \times 1000$$

[Glucose] = 5.074 mM

Standard 10 mM D-glucose solution

Blank absorbance 0.043
Sample absorbance 0.657

$$[\text{Glucose}] = \left(\frac{(0.657 - 0.043) \times 293 \times 0.01}{180.16} \right) \times 1000$$

[Glucose] = 9.99 mM

These results indicate that there is an error of less than 2%.

Incubations with Varying Amounts of Myrosinase

A 10 mM solution of sinigrin in potassium phosphate buffer (33.1 mM, pH 7.0) was incubated with myrosinase at 37 ± 0.1 °C. The concentration of glucose was measured in triplicate at 15 minute intervals using the glucose (HK) assay method.

20 µl of myrosinase

Time/minutes	Absorbance[a]	[Glucose]/mM
15	0.092	1.50
30	0.176	2.86
45	0.245	3.98
60	0.336	5.46
75	0.396	6.45
90	0.462	7.52
105	0.527	8.57
120	0.589	9.57
135	0.589	9.57

[a] Average of three measurements

25 µl of myrosinase

Time/minutes	Absorbance[a]	[Glucose]/mM
15	0.093	1.52
30	0.205	3.33
45	0.285	4.64
60	0.400	6.50
75	0.513	8.34
90	0.590	9.58
105	0.589	9.57

[a] Average of three measurements

30 μl of myrosinase

Time/minutes	Absorbance[a]	[Glucose]/mM
15	0.157	2.55
30	0.307	5.00
45	0.424	6.90
60	0.580	9.44
75	0.582	9.47
90	0.582	9.47

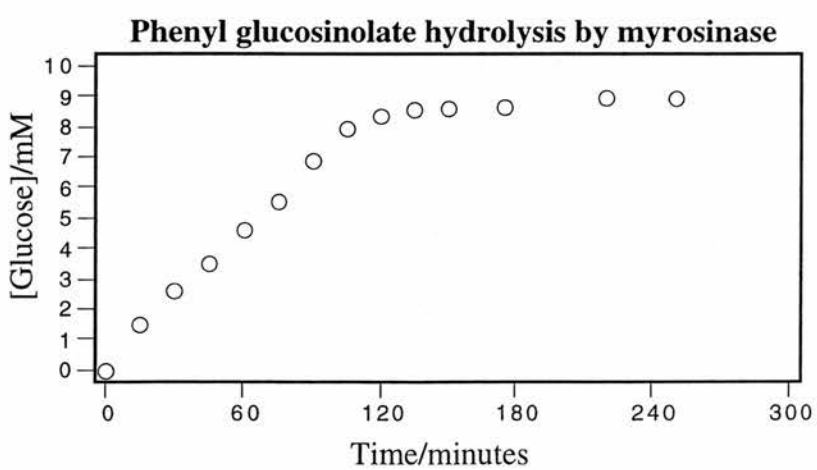
[a] Average of three measurements

Incubation of Phenyl glucosinolate with Myrosinase

A 10 mM solution of phenyl glucosinolate in potassium phosphate buffer (33.1 mM, pH 7.0) was incubated with myrosinase solution (30 μl) at 37 ± 0.1 °C. The concentration of glucose was measured at 15 minute intervals using the glucose (HK) assay method.

Time/minutes	Absorbance[a]	[Glucose]/mM
15	0.092	1.49
30	0.160	2.6
45	0.215	3.5
60	0.282	4.58
75	0.339	5.52
90	0.421	6.85
105	0.485	7.89
120	0.510	8.3
135	0.523	8.51
150	0.526	8.55
175	0.528	8.59
220	0.547	8.9
250	0.546	8.88

[a] Average of three measurements



Rate of turnover* = 1.49 ± 0.31 mM min^{-1}

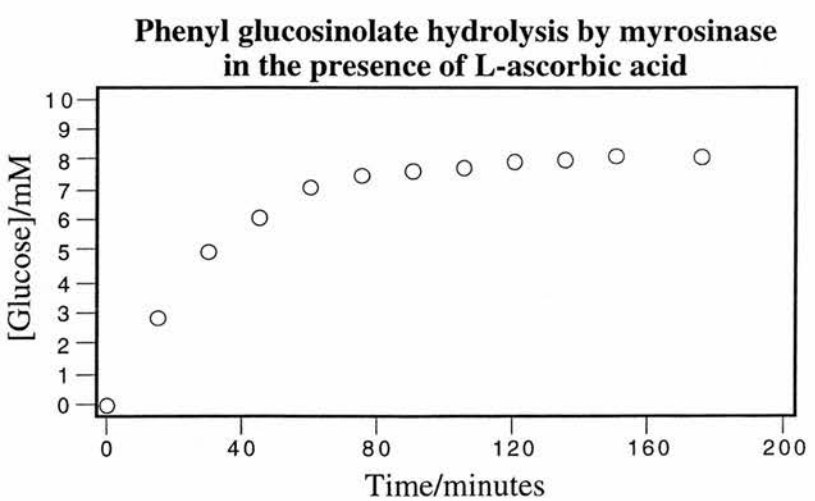
* Corrected for one unit of enzyme activity

Incubation of Phenyl glucosinolate with Myrosinase in the Presence of L-Ascorbic Acid

A solution containing 9.0 mM phenyl glucosinolate and 1.0 mM L-ascorbic acid in potassium phosphate buffer (33.1 mM, pH 7.0) was incubated with myrosinase solution (30 μl) at 37 ± 0.1 °C. The concentration of glucose was measured at 15 minute intervals using the glucose (HK) assay method.

Time/minutes	Absorbance[a]	[Glucose]/mM
15	0.174	2.83
30	0.305	4.98
45	0.374	6.1
60	0.434	7.08
75	0.459	7.47
90	0.468	7.61
105	0.475	7.73
120	0.487	7.92
135	0.491	7.99
150	0.498	8.1
175	0.496	8.07

[a] Average of three measurements



Rate of turnover* **= 2.28 ± 0.63 mM min^{-1}**

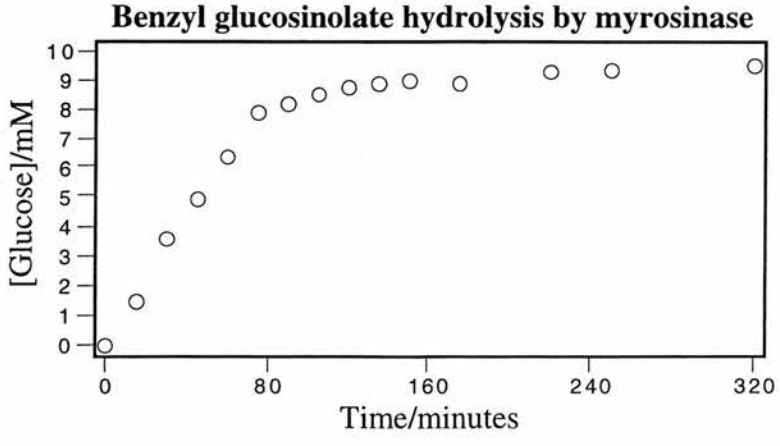
* Corrected for one unit of enzyme activity

Incubation of Benzyl glucosinolate with Myrosinase

As described for phenyl glucosinolate using a 10 mM solution of benzyl glucosinolate.

Time/minutes	Absorbance[a]	[Glucose]/mM
15	0.091	1.48
30	0.222	3.61
45	0.305	4.96
60	0.394	6.4
75	0.485	7.89
90	0.504	8.2
105	0.524	8.52
120	0.539	8.76
135	0.547	8.9
150	0.553	9.0
175	0.547	8.9
220	0.572	9.3
250	0.575	9.35
320	0.584	9.5
420	0.587	9.55

[a] Average of three measurements



Rate of turnover* **= 1.87 ± 0.23 mM min^{-1}**

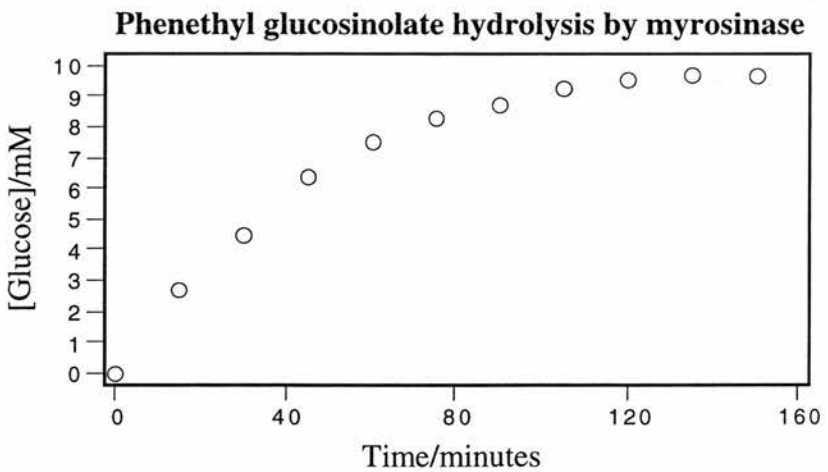
* Corrected for one unit of enzyme activity

Incubation of Phenethyl glucosinolate with Myrosinase

As described for phenyl glucosinolate using a 10 mM solution of phenethyl glucosinolate.

Time/minutes	Absorbance[a]	[Glucose]/mM
15	0.165	2.69
30	0.275	4.47
45	0.390	6.35
60	0.460	7.48
75	0.507	8.25
90	0.534	8.68
105	0.566	9.21
120	0.584	9.5
135	0.593	9.65
150	0.592	9.63
220	0.593	9.64

[a] Average of three measurements



Rate of turnover* = 2.66 ± 0.60 mM min^{-1}

* Corrected for one unit of enzyme activity

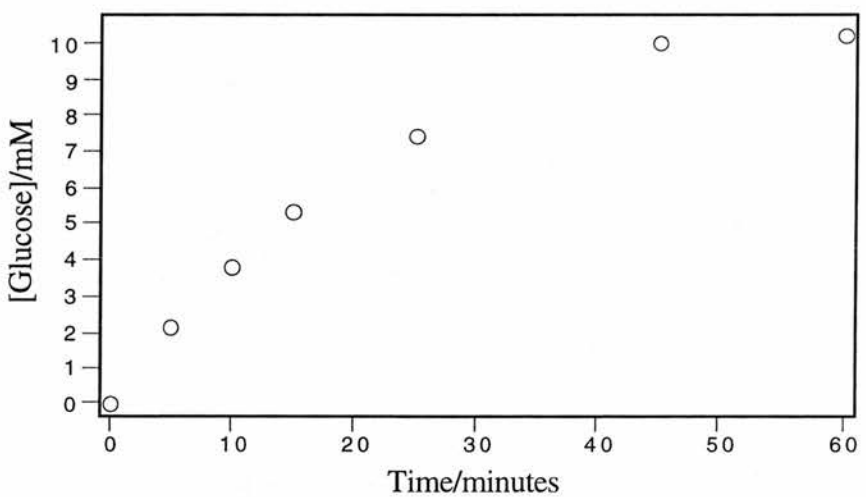
Incubation of *p*-Nitrophenyl glucosinolate with Myrosinase

As described for phenyl glucosinolate using a 10 mM solution of *p*-nitrophenyl glucosinolate.

Time/minutes	Absorbance[a]	[Glucose]/mM
5	0.131	2.13
10	0.233	3.79
15	0.327	5.32
25	0.456	7.41
45	0.615	10.00
60	0.616	10.02

[a] Average of three measurements

***p*-Nitrophenyl glucosinolate hydrolysis by myrosinase**



Rate of turnover* = 6.44 ± 0.53 mM min^{-1}

* Corrected for one unit of enzyme activity

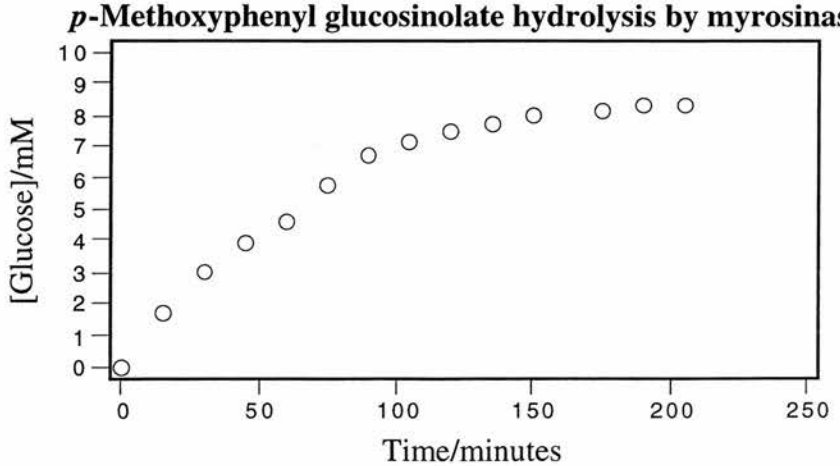
Incubation of *p*-Methoxyphenyl glucosinolate with Myrosinase

As described for phenyl glucosinolate using a 10 mM solution of *p*-methoxyphenyl glucosinolate.

Time/minutes	Absorbance	[Glucose]/mM
15	0.105	1.7
30	0.184	3.0
45	0.240	3.91
60	0.283	4.61
75	0.354	5.76
90	0.411	6.69
105	0.438	7.12
120	0.459	7.46
135	0.473	7.70
150	0.489	7.95
175	0.499	8.11
190	0.510	8.3
205	0.511	8.31

[a] Average of three measurements

***p*-Methoxyphenyl glucosinolate hydrolysis by myrosinase**



Rate of turnover* = 1.42 ± 0.32 mM min^{-1}

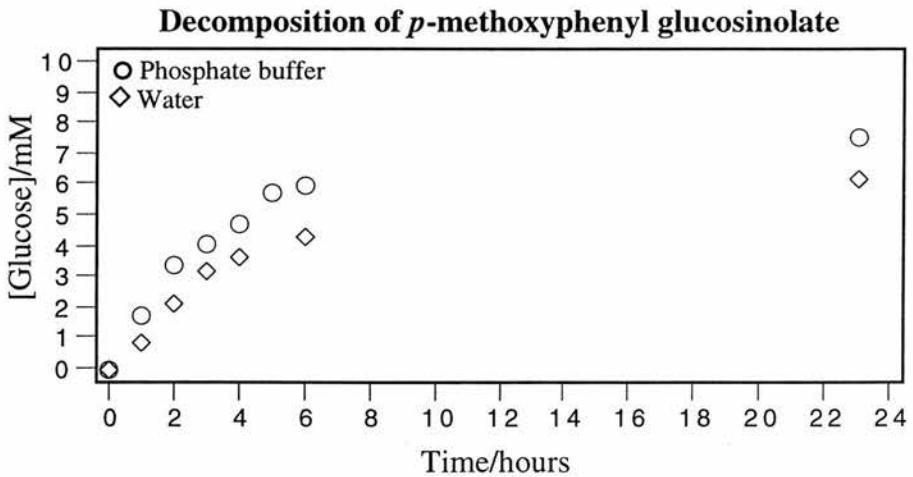
* Corrected for one unit of enzyme activity

Incubation of *p*-Methoxyphenyl glucosinolate without Myrosinase

A 10 mM solution of *p*-methoxyphenyl glucosinolate in either potassium phosphate buffer (33.1 mM, pH 7.0) or distilled water was incubated at 37 ± 0.1 °C. The concentration of glucose was measured at hourly intervals using the glucose (HK) assay method.

Time/hours	Absorbance[a] (Phosphate buffer)	[Glucose] /mM	Absorbance[a] (Water)	[Glucose] /mM
1	0.108	1.75	0.054	0.88
2	0.208	3.39	0.132	2.14
3	0.250	4.06	0.196	3.18
4	0.290	4.71	0.223	3.63
5	0.351	5.71		
6	0.366	5.96	0.265	4.31
23	0.462	7.52	0.379	6.17

[a] Average of three measurements



Rate of decomposition in phosphate buffer* **= 0.0247 ± 0.0036 mM min^{-1}**

Rate of decomposition in water*= 0.0175 ± 0.0028 mM min^{-1}

* Corrected for one unit of enzyme activity

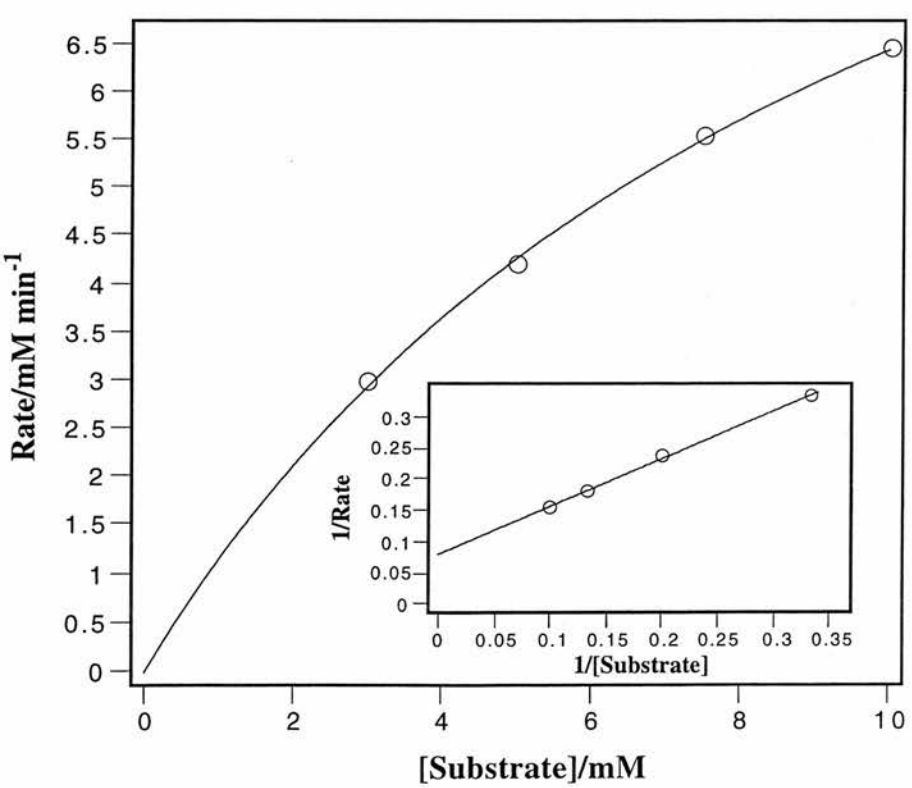
Determination of Kinetic Parameters for the Myrosinase Catalysed Hydrolysis of *p*-Nitrophenyl glucosinolate

Using the glucose (HK) assay the turnover of *p*-nitrophenyl glucosinolate with myrosinase was monitored at four different glucosinolate concentrations 2.0, 5.0, 7.5, 10.0 mM. The rates observed were corrected to 1 unit of enzyme activity. Non-linear regressional analysis was performed on the Michaelis-Menten curve using "ENZFITTER" to give values for V_{max} and K_M.

[*p*-Nitrophenyl glucosinolate]/mM	Rate[a,b]/mM min^{-1}
3.0	2.99 ± 1.26
5.0	4.20 ± 0.20
7.5	5.53 ± 0.81
10.0	6.44 ± 0.53

[a] Average of three measurements

[b] Corrected for one unit of enzyme activity



V_{max} = 13.12 ± 0.55 mM min^{-1}

K_M = 10.38 ± 0.74 mM

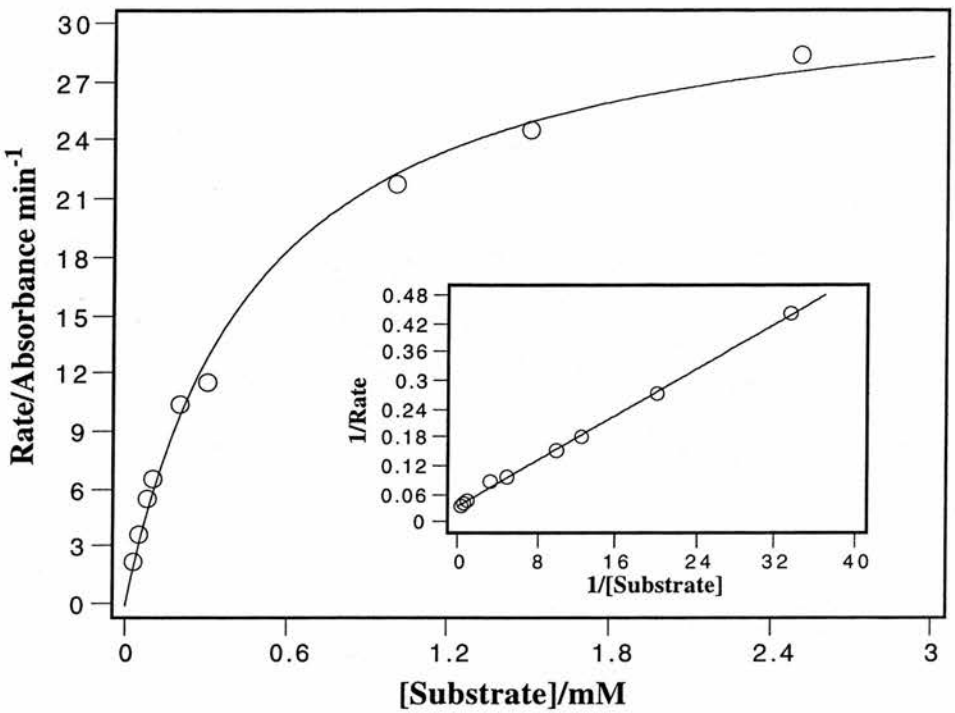
Determination of Kinetic Parameters for the Myrosinase Catalysed Hydrolysis of Sinigrin

Sinigrin incubations were carried out in potassium phosphate buffer (33.1 mM, pH 7.0) and 9 different concentrations of sinigrin were used. The sinigrin solutions were incubated at 37 ± 0.1 °C using the thermostatted cell holder of the UV spectrophotometer. The reaction was initiated by adding of myrosinase solution (30 µl). Quartz cuvettes were used as follows: 0.03-0.30 mM, 1.0 ml volume/1.0 cm pathlength; 1.0-2.5 mM, 0.4 ml volume/0.1 cm pathlength. Initial rate measurements were performed in triplicate for each sinigrin concentration by monitoring the decrease in absorbance at 227 nm (ε= 6784 mol^{-1} dm^3 cm^{-1}) due to sinigrin. The reactions were linear over the measured portion of reaction (approximately 5%). All measurements were corrected to 1 unit of enzyme activity prior to non-linear regressional analysis using "ENZFITTER" to give values for V_{max} and K_M.

[Sinigrin]/mM	Rate[a,b]/Absorbance min^{-1}
0.03	2.27
0.05	3.68
0.08	5.54
0.1	6.59
0.2	10.42
0.3	11.59
1.0	21.78
1.5	24.52
2.5	28.34

[a] Average of three measurements

[b] Corrected for one unit of enzyme activity



V_{max} = 4.80 ± 0.16 mM min^{-1}

K_M = 0.46 ± 0.044 mM

Literature Values[138]

V_{max} = 4.83 ± 0.22 mM min^{-1}

K_M = 0.42 ±0.05 mM

Inhibition Studies

Inhibition studies were performed using sinigrin as the substrate while adding various concentrations of inhibitor to the solutions. Two inhibitors were tested 2'-hydroxypropyl-1-thio-β-D-glucopyranoside and potassium 1-(1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate. Rates of reaction were measured using the procedure described earlier for the kinetic parameter determination with sinigrin.

Inhibition of the Myrosinase Catalysed hydrolysis of Sinigrin using 2'-Hydroxypropyl-1-thio-β-D-glucopyranoside

Four different concentrations of inhibitor were used 1.0, 1.5, 2.0, 2.5 mM. The data for these inhibition studies are given in the following tables.

1.0 mM 2'-hydroxypropyl-1-thio-β-D-glucopyranoside

[Sinigrin]/mM	Rate[a,b]/Absorbance min^{-1}
0.027	2.48
0.045	3.84
0.072	5.67
0.09	6.85
0.18	9.06
0.27	8.63
0.90	25.15

[a] Average of three measurements

[b] Corrected for one unit of enzyme activity

1.5 mM 2'-hydroxypropyl-1-thio-β-D-glucopyranoside

[Sinigrin]/mM	Rate[a,b]/Absorbance min^{-1}
0.027	2.48
0.045	4.01
0.072	5.52
0.09	5.84
0.18	8.21
0.27	9.57
0.90	22.88

[a] Average of three measurements

[b] Corrected for one unit of enzyme activity

2.0 mM 2'-hydroxypropyl-1-thio-β-D-glucopyranoside

[Sinigrin]/mM	Rate[a,b]/Absorbance min^{-1}
0.027	2.54
0.045	3.78
0.072	5.59
0.09	6.58
0.18	9.07
0.27	9.14
0.90	23.21

[a] Average of three measurements

[b] Corrected for one unit of enzyme activity

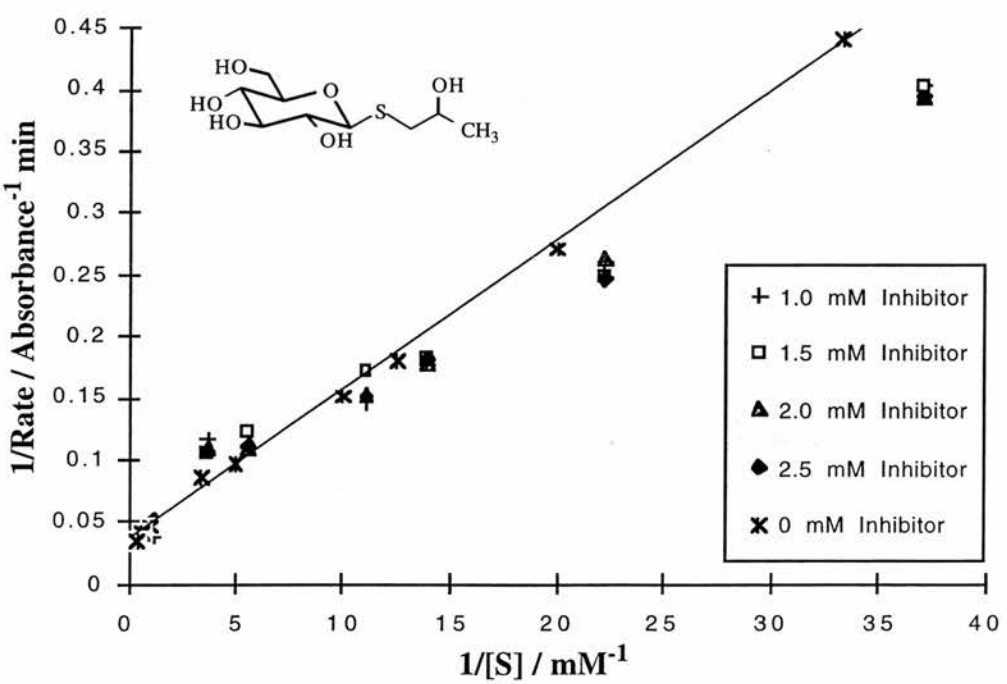
2.5 mM 2'-hydroxypropyl-1-thio-β-D-glucopyranoside

[Sinigrin]/mM	Rate[a,b]/Absorbance min^{-1}
0.027	2.54
0.045	4.04
0.072	5.52
0.09	6.59
0.18	8.95
0.27	9.45
0.90	19.68

[a] Average of three measurements

[b] Corrected for one unit of enzyme activity

Graph of inhibition of myrosinase catalysed hydrolysis of sinigrin by 2'-hydroxypropyl-1-thio-β-D-glucopyranoside



Inhibition of the Myrosinase Catalysed hydrolysis of Sinigrin using Potassium 1-(1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate

Five different concentrations of inhibitor were used 1.0, 1.5, 2.0, 2.5, 10.0 mM. The data for these inhibition studies are given in the following tables.

1.0 mM potassium 1-(1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate

[Sinigrin]/mM	Rate[a,b]/Absorbance min^{-1}
0.027	2.50
0.045	3.80
0.072	5.19
0.09	6.21
0.18	9.91
0.27	10.00
0.90	21.42

[a] Average of three measurements

[b] Corrected for one unit of enzyme activity

1.5 mM potassium 1-(1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate

[Sinigrin]/mM	Rate[a,b]/Absorbance min^{-1}
0.027	2.47
0.045	3.84
0.072	5.54
0.09	6.26
0.18	9.31
0.27	10.29
0.90	19.72

[a] Average of three measurements

[b] Corrected for one unit of enzyme activity

2.0 mM potassium 1-(1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate

[Sinigrin]/mM	Rate[a,b]/Absorbance min^{-1}
0.027	2.46
0.045	3.83
0.072	5.24
0.09	6.19
0.18	9.68
0.27	10.16
0.90	22.43

[a] Average of three measurements

[b] Corrected for one unit of enzyme activity

2.5 mM potassium 1-(1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate

[Sinigrin]/mM	Rate[a,b]/Absorbance min^{-1}
0.027	2.43
0.045	3.77
0.072	5.56
0.09	6.39
0.18	9.42
0.27	10.43
0.90	19.48

[a] Average of three measurements

[b] Corrected for one unit of enzyme activity

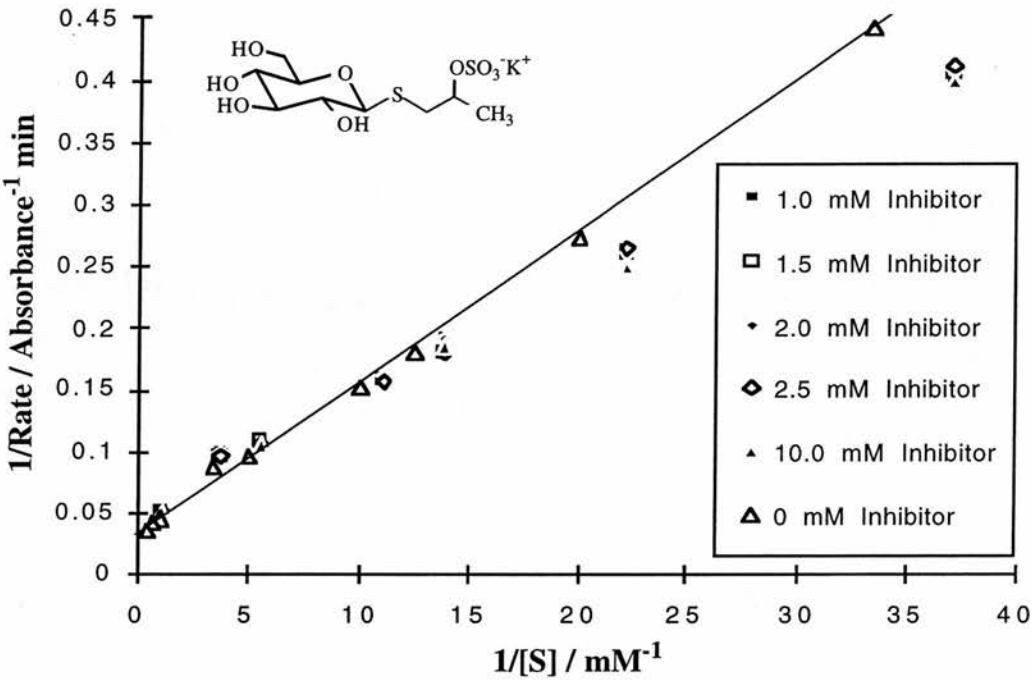
10.0 mM potassium 1-(1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate

[Sinigrin]/mM	Rate[a,b]/Absorbance min^{-1}
0.027	2.52
0.045	3.99
0.072	5.38
0.09	
0.18	9.46
0.27	
0.90	19.71

[a] Average of three measurements

[b] Corrected for one unit of enzyme activity

Graph of inhibition of myrosinase catalysed hydrolysis of sinigrin by potassium 1-(1'-thio-β-D-glucopyranosyl)-propyl-2-sulfate



References

4 References

(1) P. J. Robiquet and F. Boutron, *J. Pharm. Chim.*, 1831, **17**, 279.

(2) A. Bussy, *Annalen*, 1840, **34**, 223.

(3) J. Gadamer, *Ber. Dtsch. Chem. Ges.*, 1897, **30**, 2322.

(4) M. G. Ettlinger and A. J. Lundeen, *J. Am. Chem. Soc.*, 1957, **79**, 1764.

(5) J. Waser and W. H. Watson, *Nature (London)*, 1963, **198**, 1297.

(6) R. E. Marsh and J. Waser, *Acta. Crystallogr. Sect. B*, 1970, **26**, 1030.

(7) A. Kjaer and P. O. Larsen, 'Non-protein amino acids, cyanogenic glycosides and glucosinolates in biosynthesis,' Vol. 5, ed. T. A. Geissman, Specialist periodical reports, The Chemical Society, London, 1976, 179.

(8) M. G. Ettlinger and A. J. Lundeen, *J. Am. Chem. Soc.*, 1957, **79**, 1764.

(9) M. H. Benn, *Can. J. Chem.*, 1963, **41**, 2836.

(10) F. Boutron and E. Fremy, *Annalen*, 1840, **34**, 230.

(11) R. Björkman and B. Lönnerdal, *Biochim. Biophys. Acta*, 1973, **327**, 121.

(12) M. Ohtsuru and T. Hata, *Biochem. Biophys. Acta*, 1979, **567**, 384.

(13) B. Lüthy and P. Matile, *Biochem. Physiol. Pflanz.*, 1984, **179**, 5.

(14) W. P. Burmeister, S. Cottaz, H. Driguez, R. Iori, S. Palmieri and B. Henrissat, *Structure*, 1997, **5**, 663.

(15) S. Palmieri, *Agro. Food Industry Hi-Tech.*, 1999, **10**, 28.

(16) G. R. Fenwick, R. K. Heaney and W. J. Mullin, *CRC Crit. Rev. Food Sci. Nutr.*, 1983, **18**, 123.

(17) K. Grob and P. Matile, *Phytochemistry*, 1980, **19**, 1789.

(18) J. A. Kirkegaard and M. Sarwar, *Aust. J. Agric. Res.*, 1999, **50**, 315.

(19) N. Clossais-Besnard and F. Lahrer, *J. Sci. Food Agric.*, 1991, **56**, 25.

(20) G. F. J. Milford, J. K. Fieldsend, A. J. R. Porter, C. J. Rawlinson, E. J. Evans and P. E. Bilsborrow, *Asp. Appl. Biol.*, 1989, **23**, 83.

(21) E. J. Booth, K. C. Walker and D. W. Griffiths, *J. Sci. Food Agric.*, 1991, **56**, 479.

(22) E. A. S. Rosa, R. K. Heaney, G. R. Fenwick and C. A. M. Portas, 'Horticultural Reviews,' Vol. 19, ed. J. Janick, John Wiley and Sons Inc., New York, 1997, 99.

(23) E. Schnug and J. Ceynowa, *J. Agron. Crop Sci.*, 1990, **165**, 319.

(24) V. I. Shattuck, Y. Kakuda, B. J. Shelp and N. Kakuda, *Hort. Sci.*, 1990, **25**, 1127.

(25) C. F. J. Milford, *J. Sci. Food Agric.*, 1991, **57**, 454.

(26) E. A. S. Rosa, R. K. Heaney, F. C. Rego and G. R. Fenwick, *J. Sci. Food Agric.*, 1994, **66**, 457.

(27) G. C. Freeman and N. Mossadeghi, *J. Hort. Sci.*, 1973, **48**, 365.

(28) F. Zhao, E. J. Evans, P. E. Bilsborrow and J. K. Syers, *J. Sci. Food Agric.*, 1993, **63**, 29.

(29) E. Josefsson, *J. Sci. Food Agric.*, 1970, **21**, 98.

(30) R. P. Bodnaryk and P. Paliswamy, *J. Chem. Ecol.*, 1990, **16**, 2735.

(31) R. C. McDonald, T. R. Manley, T. N. Barry, B. A. Forss and A. C. Sinclair, *J. Agric. Sci. Camb.*, 1981, **97**, 13.

(32) L. Guignard, *J. Bot.*, 1890, **4**, 385.

(33) A. M. Bones and J. T. Rossiter, *Physiologica Plantarum*, 1996, **97**, 194.

(34) C. J. Peterson, T. Tsao and J. R. Coats, *Pestic. Sci.*, 1998, **54**, 35.

(35) E. P. Lichtenstein, F. M. Strong and D. G. Morgan, *Agric. Food Chem.*, 1962, **10**, 30.

(36) R. M. Newman, Z. Hanscom and W. C. Kerfoot, *Oecologia*, 1992, **92**, 1.

(37) P. Pracros, R. Moreau and L. Gourdoux, *Anim. Biol.*, 1992, **1**, 77.

(38) P. Pracros, C. Couranjou and R. Moreau, *Comp. Biochem. Physiol.*, 1992, **103A**, 391.

(39) F. S. Chew, 'ACS symposium series, Biologically active natural products potential use in agriculture,' ed. H. G. Cutler, American Chemical Society, Washington DC, 1988, 170.

(40) K. C. Spencer, 'ACS symposium series, Biologically active natural products potential use in agriculture,' ed. H. G. Cutler, American Chemical Society, Washington DC, 1988, 404.

(41) J. E. Treherne, *J. Exp. Biol.*, 1958, **35**, 611.

(42) A. Gelperin, *J. Insect Physiol.*, 1966, **12**, 331.

(43) E. Bernays, 'Comprehensive Insect Physiology, Biochemistry and Pharmacology,' Vol. 4, ed. G. A. Kerkut, L. I. Gilbert, Pergamon press, New York, 1985, 1.

(44) E. Verschaffelt, *Proc. Acad. Sci. Amsterdam*, 1910, **13**, 536.

(45) S. H. Palowski, P. W. Reigert and J. Krzymanski, *Nature*, 1968, **220**, 174.

(46) J. Petterson, *Symp. Biol. Hung.*, 1976, **16**, 203.

(47) K. A. Pivnick, R. J. Lamb and D. Reed, *J. Chem. Ecol.*, 1992, **18**, 863.

(48) P. Roessingh, E. Städler, G. R. Fenwick, J. A. Lewis, J. K. Nielsen, J. Hurter and T. Ramp, *Entomol. Exp. Appl.*, 1992, **65**, 267.

(49) F. S. Chew and R. K. Robbins, *Symp. R. Ent. Soc. Lond.*, 1984, **11**, 65.

(50) W. A. L. David and B. O. C. Gardiner, *Bull. Entomol. Res.*, 1962, **53**, 91.

(51) E. Städler, 'Chemical Ecology of Insects, Contact Chemoreception,' eds. W. J. Bell, R. T. Cardé, Chapman and Hall, London, 1984, 3.

(52) J. J. A. van Loon, A. Blaakmeer, F. C. Griepink, T. A. van Beek, L. M. Schoonhoven and A. de Groot, *Chemoecology*, 1992, **3**, 39.

(53) V. M. Koritsas, J. A. Lewis and G. R. Fenwick, *Ann. Appl. Biol.*, 1991, **118**, 209.

(54) J. Braven, N. P. Chilcott and C. Hawkes, *J. Chem. Ecol.*, 1996, **22**, 1567.

(55) P. Roessingh, E. Städler, R. Baur, J. Hurter and T. Ramp, *Physiol. Entomol.*, 1997, **22**, 140.

(56) T. Neudecker and D. Henschler, *Mutation Res.*, 1985, **156**, 33.

(57) A. I. Virtanen, *Angew. Chem.*, 1962, **74**, 374.

(58) M. Mari, R. Iori, O. Leoni and A. Marchi, *Ann. Appl. Biol.*, 1993, **123**, 155.

(59) L. M. Manici, L. Lazzeri and S. Palmieri, *J. Agric. Food Chem.*, 1997, **45**, 2768.

(60) U. Smolinska and M. Horbowicz, *J. Phytopathology*, 1999, **147**, 119.

(61) G. D. Bending and S. D. Lincoln, *Soil Biol. and Biochem.*, 1999, **31**, 695.

(62) S. Sasaki, *J. Nara. Ned. Assoc.*, 1963, **14**, 101.

(63) M. Nestle, *Proc. Natl. Acad. Sci. USA*, 1997, **94**, 11149.

(64) D. T. H. Verhoeven, R. A. Goldbohm, G. van Poppel, H. Verhagen and P. A. van der Brandt, *Cancer Epidemiology, Biomarkers and Prevention*, 1996, **5**, 733.

(65) C. Vines, *New Scientist*, 1996, **152**, 46.

(66) Y. Zhang, P. Talalay, C.-G. Cho and G. H. Posner, *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 2399.

(67) J. H. Fahey, Y. Zhang and P. Talalay, *Proc. Natl. Acad. Sci. USA*, 1997, **94**, 10367.

(68) K. Faulkner, R. Mithen and G. Williamson, *Carcinogenesis*, 1998, **19 (4)**, 605.

(69) P. Talalay, J. W. Fahey, W. D. Holtzclaw, T. Prestera and Y. Zhang, *Toxicol. Lett.*, 1995, **82/83**, 173.

(70) K. Mahéo, F. Morel, S. Langouët, H. Kramer, E. L. Ferrec, B. Ketterer and A. Guillouzo, *Cancer Res.*, 1977, **57**, 3649.

(71) G. Williamson, K. Faulkner and G. W. Plumb, *Eur. J. Cancer Prevention*, 1998, **7**, 17.

(72) P. Talalay and Y. Zhang, *Biochem. Soc. Trans.*, 1996, **24**, 806.

(73) Z. Guo, T. J. Smith, H. Ishizaki and C. S. Young, *Carcinogenesis*, 1991, **12**, 2277.

(74) S. M. Hamilton and R. W. Teel, *Anticancer Res.*, 1994, **14**, 1089.

(75) S. E. Murphy, R. Heiblum, P. G. King, D. Bowman, W. J. Davis and G. D. Stoner, *Carcinogenesis*, 1991, **12**, 957.

(76) S. E. Murphy, A. Palomino, S. S. Hecht and D. Hoffmann, *Cancer Res.*, 1990, **50**, 5446.

(77) M. A. Morse, C.-X. Wang, G. D. Stoner, S. Mandal, P. B. Conran, S. G. Amin, S. S. Hecht and F.-L. Chung, *Cancer Res.*, 1989, **49**, 549.

(78) C. C. Conaway, D. Jiao and F.-L. Chung, *Carcinogenesis*, 1996, **17**, 2423.

(79) D. Jiao, T. J. Smith, S. Kim, C. S. Yang, D. Desai, S. Amin and F.-L. Chung, *Carcinogenesis*, 1996, **17**, 755.

(80) F.-L. Chung, M. M. Morse, K. I. Eklind and Y. Xu, *ACS Symposium Series*, 1994, **533**, 232.

(81) S. S. Hecht, M. A. Morse, K. I. Eklind and F.-L. Chung, *Experimental Lung Res.*, 1991, **17**, 501.

(82) M. A. Morse, K. I. Eklind, S. S. Hecht, K. G. Jordan, C.-I. Choi, D. H. Desai, S. G. Amin and F.-L. Chung, *Cancer Res.*, 1991, **51**, 1846.

(83) H. G. Shertzer, *Food Chem. Toxicol.*, 1983, **21**, 31.

(84) H. G. Shertzer, *Chemico.-Biol. Interact.*, 1984, **48**, 81.

(85) H. M. Wortelboer, C. A. de Kruif, A. A. J. van Iersel, H. E. Falke, J. Noordhoek and B. J. Blaauboer, *Biochem. Pharmacol.*, 1992, **43**, 1439.

(86) C. Bonnesen, P. U. Stephensen, O. Andersen, H. Sørensen and O. Vang, *Nutrition and Cancer*, 1999, **33 (2)**, 178.

(87) W. M. F. Jongen, *Proc. Nutr. Soc.*, 1996, **55**, 433.

(88) H. G. M. Tiedink, J. A. R. Davies, L. W. van Broekhoven, H. J. van der Kamp and W. M. F. Jongen, *Food Chem. Toxicol.*, 1988, **26**, 947.

(89) H. G. M. Tiedink, J. A. R. Davies, N. A. Visser, W. M. F. Jongen and L. W. van Broekhoven, *Food Chem. Toxicol.*, 1989, **27**, 723.

(90) H. G. M. Tiedink, A. M. Hissink, S. M. Lodema, L. W. van Broekhoven and W. M. F. Jongen, *Mutation Res.*, 1990, **232**, 199.

(91) H. G. M. Tiedink, C. E. Malingre, L. W. van Broekhoven, W. M. F. Jongen, W. F. M. Lewis and G. R. Fenwick, *J. Agric. Food Chem.*, 1991, **39**, 922.

(92) P. H. Jellinck, P. G. Forkert, D. S. Riddick, A. B. Okey, J. J. Michnovicz and H. L. Bradlow, *Biochem. Pharmacol.*, 1993, **45**, 1129.

(93) H. Liu, W. Wormke, S. H. Safe and L. F. Bjeldanes, *J. Natl. Cancer Inst.*, 1994, **86**, 1758.

(94) J. J. Michnovicz and H. L. Bradlow, *Nutr. Cancer*, 1991, **16**, 59.

(95) T. A. Shapiro, J. W. Fahey, K. L. Wade, K. K. Stephenson and P. Talalay, *Cancer Epidemiology, Biomarkers and Prevention*, 1998, **7**, 1091.

(96) S. M. Getahun and F.-L. Chung, *Cancer Epidemiology, Biomarkers and Prevention*, 1999, **8**, 447.

(97) R. Staack, S. Kingston, M. A. Wallig and E. H. Jeffery, *Toxicol. and Appl. Pharmacol.*, 1998, **149**, 17.

(98) G. R. Fenwick and R. F. Curtis, *Anim. Feed Sci. Technol.*, 1980, **5**, 255.

(99) L. Nugon-Baudon, S. Rabot, O. Szylit and P. Raibaud, *Xenobiotica*, 1990, **20**, 223.

(100) K. Nishie and M. E. Daxenbichler, *Food Cosmet. Toxicol.*, 1980, **18**, 159.

(101) J. C. Mitchell and W. P. Jordan, *Br. J. Dermatol.*, 1974, **91**, 183.

(102) M. C. Viaud and P. Rollin, *Tetrahedron Lett.*, 1990, **31**, 1417.

(103) A. M. Chesney, T. A. Clawson and B. Webster, *Bull. Johns Hopkins Hosp.*, 1928, **43**, 291.

(104) B. Webster and A. M. Chesney, *Bull. Johns Hopkins Hosp.*, 1928, **43**, 291.

(105) E. B. Astwood, *J. Pharmacol. Exp. Ther.*, 1943, **78**, 79.

(106) C. E. Hercus and H. B. Purves, *J. Hyg.*, 1936, **36**, 182.

(107) E. B. Astwood, M. A. Greer and M. G. Ettlinger, *J. Biol. Chem.*, 1949, **181**, 121.

(108) K. A. Steinmetz and J. D. Potter, *Cancer Causes and Control*, 1991, **2**, 427.

(109) M. Mcmillan, E. A. Spinks and G. R. Fenwick, *Hum. Toxicol.*, 1986, **5**, 15.

(110) D. I. McGregor, *Can. J. Plant Sci.*, 1978, **58**, 795.

(111) S. Chavadej, N. Brisson, J. N. McNeil and V. D. Luca, *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 2166.

(112) H. Singh, P. Mehta and A. P. Vig, *J. Agric. Food Chem.*, 1997, **45**, 4522.

(113) S. Palmieri, P. Rollin, H. Sørensen and S. Sørensen, *Agro. Food Industry Hi-Tech.*, 1998, 24.

(114) A. B. Hanley, C. A. Kwiatkowska and G. R. Fenwick, *J. Sci. Food Agric.*, 1990, **51**, 417.

(115) A. Hochkoeppler and S. Palmieri, *Biotechnol. Prog.*, 1992, **8**, 91.

(116) R. Iori, B. Joseph, P. Rollin and S. Palmieri, *GCIRC Bull.*, 1994, **10**, 142.

(117) O. Leoni, F. Felluga and S. Palmieri, *Tetrahedron Lett.*, 1993, **49**, 7967.

(118) O. Leoni, C. Marot, P. Rollin and S. Palmieri, *Tetrahedron Asymm.*, 1994, **5**, 1157.

(119) O. Leoni, R. Iori and S. Palmieri, *J. Agric. Food Chem.*, 1991, **39**, 2322.

(120) P. Daubos, V. Grumel, R. Iori, O. Leoni, S. Palmieri and P. Rollin, *Industrial Crops and Products*, 1998, **7**, 187.

(121) L. Buchwaldt, L. M. Larsen, A. Plöger and H. Sørensen, *J. Chromatogr.*, 1986, **363**, 71.

(122) B. Lönnerdal and J. Janson, *Biochim. Biophys. Acta*, 1973, **315**, 421.

(123) M. Ohtsuru and H. Kawatani, *Agric. Biol. Chem.*, 1979, **43**, 2249.

(124) M. G. Botti, M. G. Taylor and N. P. Botting, *J. Biol. Chem.*, 1995, **270**, 20530.

(125) A. J. MacLeod and J. T. Rossiter, *Phytochemistry*, 1986, **25**, 1047.

(126) S. Cottaz, B. Henrissat and H. Driguez, *Biochemistry*, 1996, **35**, 15256.

(127) I. P. Street, J. B. Kempton and S. G. Withers, *Biochemistry*, 1992, **31**, 9970.

(128) A. Falk, J. Taipalensu, B. Ek, M. Lenman and L. Rask, *Planta*, 1995, **195**, 387.

(129) N. Geshi, E. Andreasson, J. Meijer, L. Rask and A. Brandt, *Plant Physiol. Biochem.*, 1998, **36 (8)**, 583.

(130) X. Hasapis and A. J. MacLeod, *Phytochemistry*, 1982, **21**, 1009.

(131) I. Tsuruo and T. Hata, *Agric. Biol. Chem.*, 1968, **32**, 1420.

(132) P. L. Durham and J. E. Poultron, *Z. Naturforsch*, 1990, **45c**, 173.

(133) D. Cheaves, A. Powell and L. C. Hodges, *Abs. of pap. ACS*, 1997, **213**, 242-CHED.

(134) M. G. Ettlinger, G. P. Dates Jr., B. W. Harrison, T. J. Mabry and C. P. Thompson, *Proc. Natl. Acad. Sci.*, 1961, **47**, 1875.

(135) M. Blanc-Muesser, H. Driguez, B. Joseph, M. C. Viaud and P. Rollin, *Tetrahedron Lett.*, 1990, **31 (27)**, 3867.

(136) C. Gardrat, A. Quinsac, B. Joseph and P. Rollin, *Heterocycles*, 1993, **35**, 1015.

(137) R. Iori, P. Rollin, H. Streicher, J. Thiem and S. Palmieri, *FEBS Lett.*, 1996, **385**, 87.

(138) M. G. Taylor, Ph. D. Thesis, University of St. Andrews, 1997.

(139) S. Lazar and P. Rollin, *Tetrahedron Lett.*, 1994, **35**, 2173.

(140) A. Kjaer and T. Skrydstrup, *Acta. Chem. Scand.*, 1987, **B41**, 29.

(141) T. Shimada, K. El-Bayoumy, P. Upadhyaya, T. R. Sutter, F. P. Guengerich and H. Yamazaki, *Cancer Res.*, 1997, **57**, 4757.

(142) M. Benn, *Pure Appl. Chem.*, 1977, **49**, 197.

(143) E. A. S. Rosa, R. K. Heaney, G. R. Fenwick and C. A. M. Portas, 'Horticultural reviews,' ed. J. Janick, John Wiley and Sons, Inc. New York, 1997, 116.

(144) R. McDanell, A. E. M. McLean, A. B. Hanley, R. K. Heaney and G. R. Fenwick, *Food Chem. Toxicol.*, 1988, **26**, 59.

(145) N. Agerbirk, C. E. Olsen and H. Sørensen, *J. Agric. Food Chem.*, 1998, **46**, 1563.

(146) A. B. Hanley and K. R. Parsley, *Phytochemistry*, 1990, **29**, 769.

(147) A. B. Hanley, K. R. Parsley, J. A. Lewis and G. R. Fenwick, *J. Chem. Soc., Perkin Trans. 1*, 1990, 2273.

(148) K. Monde, M. Takasugi and T. Ohnishi, *J. Am. Chem. Soc.*, 1994, **116**, 6650.

(149) P. M. Dewick, *Natural Prod. Reports*, 1984, 545.

(150) Y. Uda, T. Kurata and N. Arakawa, *Agric. Biol. Chem.*, 1986, **50**, 2735.

(151) Y. Uda, T. Kurata and N. Arakawa, *Agric. Biol. Chem.*, 1986, **50**, 2741.

(152) F. S. Chew, 'ACS symposium series, Biologically active natural products potential use in agriculture,' ed. H. G. Cutler, American Chemical Society, Washington, DC, 1988, 160.

(153) H. L. Tookey, *Can. J. Biochem.*, 1973, **51**, 1654.

(154) R. J. Petroski and W. F. Kwolek, *Phytochemistry*, 1985, **24**, 213.

(155) E. R. Brocker and M. H. Benn, *Phytochemistry*, 1983, **22**, 770.

(156) A. J. MacLeod and J. T. Rossiter, *Phytochemistry*, 1985, **24**, 1895.

(157) L. M. Searle, K. Chamberlain and D. N. Butcher, *J. Sci. Food Agric.*, 1984, **35**, 745.

(158) R. K. Heaney and G. R. Fenwick, 'Methods in plant biochemistry, Methods for glucosinolate analysis,' Vol. 6, ed. P. G. Waterman, Academic, London, 1993, 531.

(159) D. I. McGregor, W. J. Mullin and G. R. Fenwick, *J. Assoc. Off. Anal. Chem.*, 1983, **66**, 825.

(160) D. W. Griffiths, A. N. E. Birch and J. R. Hillman, *J. Hort. Sci. Biotech.*, 1998, **73 (1)**, 1.

(161) R. K. Heaney and G. R. Fenwick, *J. Sci. Food Agric.*, 1980, **31**, 593.

(162) E. A. Spinks, K. Sones and G. R. Fenwick, *Fette Seifen Anstrichmittel*, 1984, **6**, 228.

(163) K. Hrncirík, J. Velísek and J. Davídek, *Z. Lebensm Unters Forsch A.*, 1998, **206**, 103.

(164) L. R. Hogge, D. W. Reed, E. W. Underhill and G. W. Haughn, *J. Chromatogr. Sci.*, 1988, **26**, 551.

(165) F. A. Mellon, J. R. Chapman and J. A. E. Pratt, *J. Chromatogr.*, 1987, **394**, 209.

(166) P. S. Kokkonen, J. van de Greef, W. M. A. Niessen, U. R. Tjaden, G. J. ten Hove and G. van de Werken, *Biol. Mass Spectrom.*, 1991, **20**, 259.

(167) G. Bojesen and E. Larsen, *Biol. Mass Spectrom.*, 1991, **20**, 286.

(168) M. Ishida, I. Chiba, Y. Okuyama, Y. Takahata and N. Kaizuma, *Japan Agric. Res. Quarterly*, 1997, **131**, 73.

(169) B. A. Halkier and L. Du, *Trends in Plant Science*, 1997, **2**, 425.

(170) A. Kjaer, 'The Biology and Chemistry of the Cruciferae,' Ed. Vol. J. G. Vaughan, A. J. MacLeod, B. M. G. Jones, Academic Press, London, 1976, 210.

(171) G. W. Dawson, A. J. Hick, R. N. Bennett, A. Donald, J. A. Pickett and R. M. Wallsgrove, *J. Biol. Chem.*, 1993, **268**, 27154.

(172) C. C. Chapple, J. R. Glover and B. E. Ellis, *Plant Physiol.*, 1990, **94**, 1887.

(173) M. F. Oldfield, R. N. Bennett, G. Kiddle, R. M. Wallsgrove and N. P. Botting, *Plant Physiol. Biochem.*, 1999, **37**, 99.

(174) R. N. Bennett, G. Kiddle and R. M. Wallsgrove, *Plant Physiol.*, 1997, **114**, 1283.

(175) R. N. Bennett, A. J. Hick, G. W. Dawson and R. M. Wallsgrove, *Plant Physiol.*, 1995, **109**, 299.

(176) R. Bennett, J. Ludwig-Muller, G. Kiddle, W. Hilgenberg and R. Wallsgrove, *Planta*, 1995, **196**, 239.

(177) J. C. Jain, J. W. D. GrootWassink, D. W. Reed and E. W. Underhill, *J. Plant Physiol.*, 1990, **136**, 356.

(178) R. N. Bennett, G. Kiddle, A. J. Hick, G. W. Dawson and R. M. Wallsgrove, *Plant, Cell and Environment*, 1996, **19**, 801.

(179) M. H. Benn, *Can. J. Chem.*, 1965, **43**, 1.

(180) M. H. Benn, *J. Chem. Soc.*, 1964, **4**, 4072.

(181) M. H. Benn and M. G. Ettlinger, *J. Chem. Soc., Chem. Commun.*, 1965, 445.

(182) N. Kornblum and R. A. Brown, *J. Am. Chem. Soc.*, 1965, **87**, 1742.

(183) M. H. Benn and L. Yelland, *Can. J. Chem.*, 1967, **45**, 1595.

(184) V. Gil and A. J. MacLeod, *Tetrahedron*, 1980, **36**, 779.

(185) L. Brochard, B. Joseph, M.-C. Viaud and P. Rollin, *Synth. Commun.*, 1994, **24**, 1403.

(186) W. Abramski and M. Chmielewski, *J. Carbohydr. Chem.*, 1996, **15**, 109.

(187) M. C. Viaud, P. Rollin, L. Latxague and C. Gradrat, *J. Chem. Res. (S)*, 1992, 207.

(188) M. C. Viaud, P. Rollin, L. Latxague and C. Gardrat, *J. Chem. Res. (M)*, 1992, 1669.

(189) H. Streicher, L. Latxague, T. Wiemann, P. Rollin and J. Thiem, *Carbohydr. Res.*, 1995, **278**, 257.

(190) S. Chevolleau, B. Joseph, P. Rollin and J. Tulliez, *J. Labelled Cmpd. and Radiopharm.*, 1993, **33**, 671.

(191) G. Kumaran and G. H. Kulkarni, *Tetrahedron Lett.*, 1994, **35**, 9099.

(192) S. Cassel, B. Casenave, G. Déléris, L. Latxague and P. Rollin, *Tetrahedron*, 1998, **54**, 8515.

(193) M. Mavartzotis, V. Dourtoglou, C. Lorin and P. Rollin, *Tetrahedron Lett.*, 1996, **37**, 5699.

(194) A. J. MacLeod and J. T. Rossiter, *J. Chem. Soc., Perkin Trans. 1*, 1983, 717.

(195) R. Iori, R. Bernardi, D. Gueyard, P. Rollin and S. Palmieri, *Bioorg. & Med. Chem. Lett.*, 1999, **9**, 1047.

(196) H. T. Keller, L. J. Yelland and M. H. Benn, *Can. J. Chem.*, 1984, **62**, 437.

(197) V. Aucagne, D. Gueyrard, A. Tatibouët, S. Cottaz, H. Driguez, M. Lafosse and P. Rollin, *Tetrahedron Lett.*, 1999, **40**, 7319.

(198) B. Joseph and P. Rollin, *J. Carbohydr. Chem.*, 1993, **12**, 1127.

(199) S. Cottaz, P. Rollin and H. Driguez, *Carbohydr. Res.*, 1997, **298**, 127.

(200) C. Elfakir, M. Lafosse, M. C. Viaud and M. Dreux, *Journal of High Res. Chromatogr.*, 1992, **15**, 392.

(201) D. W. Griffiths, A. N. E. Birch and W. H. MacFarlane-Smith, *J. Food Agric.*, 1994, **65**, 171.It5.

(202) J. Stanek, M. Sindlerova and M. Cerny, *Coll. Czech. Chem. Commun.*, 1965, **30**, 297.

(203) J. F. Normant, A. Commercon, M. Borgain and J. Villeras, *Tetrahedron Lett.*, 1975, **44**, 3833.

(204) W. Kemp, 'Qualitative Organic Analysis,' McGraw Hill, London, 1979, 58.

(205) R. A. W. Johnstone and M. E. Rose, *Tetrahedron*, 1979, **35**, 2169.

(206) J. G. Buchanan, J. Stoddart and R. H. Wightman, *J. Chem. Soc., Perkin Trans. 1*, 1994, 1417.

(207) M. Somei and T. Kawasaki, *Heterocycles*, 1989, **29**, 1251.

(208) T. Kawasaki, A. Kodama and T. Nishida, *Heterocycles*, 1991, **32**, 221.

(209) P. Molina, M. Alajarín, Sánchez-Andrada, J. S. Carrió, M. Martínez-Rippol, J. E. Anderson, M. L. Jimeno and J. Elguero, *J. Org. Chem.*, 1996, **61**, 4289.

(210) D. M. Gleave and S. J. Brickner, *J. Org. Chem.*, 1996, **61**, 6470.

(211) M. Somei and T. Shoda, *Heterocycles*, 1981, **16**, 1523.

(212) R. M. Acheson, G. H. Aldridge, M. C. K. Choi, J. O. Nwankwo, M. A. Ruscoe and J. D. Wallis, *J. Chem. Res.*, 1984, 101.

(213) R. M. Acheson, P. G. Hunt, D. M. Littlewood, B. A. Murrer and H. E. Rosenberg, *J. Chem. Soc., Perkin Trans. 1*, 1978, 1117.

(214) R. M. Acheson, B. H. Benn, J. Jacyno and J. D. Wallis, *J. Chem. Soc., Perkin Trans. 2*, 1983, 497.

(215) M. Somei, H. Sato, M. Komura and C. Kaneko, *Heterocycles*, 1985, **23**, 1101.

(216) G. Rosini, R. Ballini and P. Sorrenti, *Synthesis*, 1983, 1014.

(217) R. H. Wollenberg and S. J. Miller, *Tetrahedron Lett.*, 1978, **35**, 3219.

(218) J. Harley-Mason and E. H. Pavi, *J. Chem. Soc.*, 1963, 2565.

(219) J. Szmuzkovicz, W. C. Antony and R. V. Heinzelman, *J. Org. Chem.*, 1960, **25**, 857.

(220) L. Canoira, J. G. Rodriguez, J. B. Subirats, J. A. Escario, I. Jimenez and A. R. Martinez-Fernandez, *Eur. J. Med. Chem.*, 1989, **24**, 39.

(221) W. L. Chan, D. D. Ho, C. P. Lau, K. H. Wat, Y. C. Kong, K. F. Cheng, T. T. Wong and T. Y. Chan, *Eur. J. Med. Chem.*, 1991, **26**, 387.

(222) L. I. Kruze and E. L. Hibbert, *Heterocycles*, 1983, **20**, 1373.

(223) A. K. Sinhababu and R. T. Borchardt, *Tetrahedron Lett.*, 1983, **24**, 227.

(224) M. Somei, H. Sato, N. Komura and C. Kaneko, *Heterocycles*, 1983, **20**, 1373.

(225) G. Kumaran, *Tetrahedron Lett.*, 1996, **37**, 6407.

(226) B. Bernet and A. Vasella, *Helv. Chim. Acta*, 1979, **62**, 1979.

(227) H. J. Carlsen, T. Katsuki, V. S. Martin and K. B. Sharpless, *J. Org. Chem.*, 1981, **46**, 3936.

(228) K. E. Pfitzner and J. G. Moffatt, *J. Am. Chem. Soc.*, 1965, **87**, 5661.

(229) P. J. Garegg, L. Olsson and S. Oscarson, *J. Org. Chem.*, 1995, **60**, 2200.

(230) 'Dictionary of Organic Compounds,' Ed. 5, Vol. 5, ed. J. Buckingham, Chapman and Hall, New York, 1982, 5127.

(231) S. Jiang and G. Singh, *Tetrahedron*, 1998, **54**, 4697.

(232) J. Xu, A. Egger, B. Bernet and A. Vasella, *Helv. Chim. Acta*, 1996, **79**, 2004.

(233) 'Dictionary of Organic Compounds,' Ed. 5, Vol. 3, ed. J. Buckingham, Chapman and Hall, New York, 1982, 4504.

(234) C. E. Colson and J. M. Lowenstein, *Methods in Enzymology*, 1981, **72**, 53.

(235) G. Rouzaud, A. J. Duncan, S. Rabot, B. Ratcliffe, J. Durao, S. Garrido and S. Young, *"Food and Cancer prevention 3", conference, Norwich, September 5-8*, 1999, in press.

(236) 'Vogel's Textbook of Practical Organic Chemistry,' Ed. 5, eds. B. S. Furniss, A. J. Hannaford, P. W. G. Smith and A. R. Tatchell, Longman Scientific and Technical, Harlow, 1989.

(237) A. B. Hanley, R. K. Heaney and G. R. Fenwick, *J. Sci. Food Agric.*, 1983, **34**, 869.

(238) Q. Wang and S. G. Withers, *J. Am. Chem. Soc.*, 1995, **117**, 10137.

(239) G. R. Moe, L. M. Sayre and P. S. Portoghese, *Tetrahedron Lett.*, 1981, **22**, 537.

(240) D. C. Smith, S. W. Lee and P. L. Fuchs, *J. Org. Chem.*, 1994, **59**, 348.

(241) M. Casey, C. J. Moody and C. W. Rees, *J. Chem. Soc., Perkin Trans. 1*, 1987, 1389.

(242) J. E. Mills, R. M. Cossgrove, R. D. Shah, C. A. Maryanhoff and V. Paragmian, *J. Org. Chem.*, 1984, **49**, 546.

(243) O. E. Jensen, S.-O. Lawesson, R. Bardi, A. M. Piazzesi and C. Toniolo, *Tetrahedron*, 1985, **41** 5595.

(244) S. Palmieri, O. Leoni and R. Iori, *Anal. Biochem.*, 1982, **123**, 320.

(245) B. Sörbo, *Methods in Enzymology*, 1987, **143**, 3.

(246) F. Melvin, A. McNeill, P. J. F. Henerson and R. B. Herbert, *Tetrahedron Lett.*, 1999, **40**, 1201.

(247) I. McGregor, *Eucarpia Cruciferae Newsletter*, 1985, **10**, 132.

(248) D. D. Perrin, W. L. Armarego and D. R. Perrin, 'Purification of Laboratory Chemicals,' Ed. 3, Pergamon Press, Oxford, 1980.

(249) W. C. Still, M. Khan and A. Mitra, *J. Org. Chem.*, 1978, **43**, 2923.

(250) S. Karjala and K. P. Link, *J. Am. Chem. Soc.*, 1940, **62**, 917.

(251) D. H. Brauns, *J. Am. Chem. Soc.*, 1925, **47**, 1280.

(252) W. Bonner and J. Kahn, *J. Am. Chem. Soc.*, 1951, **73**, 2241.

(253) N. G. Morozova, L. V. Volkova, E. G. Gutsalenko and R. P. Evstigneeva, *Zh. Org. Khim.*, 1976, **12**, 960.

(254) N. K. Richtmeyer, C. J. Carr and C. S. Hudson, *J. Am. Chem. Soc.*, 1943, **65**, 1477.

(255) 'Dictionary of Organic Compounds,' Ed. 5, Vol. 5, ed. J. Buckingham, Chapman and Hall, New York, 1982, 4661.

(256) 'Dictionary of Organic Compounds,' Ed. 5, Vol. 4, ed. J. Buckingham, Chapman and Hall, New York, 1982, 3951.

(257) G. Büchi and C.-P. Mak, *J. Org. Chem.*, 1977, **42**, 1784.

(258) M. Somei, F. Yamada, M. Kunimoto and C. Kaneko, *Heterocycles*, 1984, **22**, 797.

(259) D. B. Repke and W. J. Ferguson, *J. Heterocycl. Chem.*, 1982, **19**, 845.

(260) W. E. Noland and P. J. Hartman, *J. Am. Chem. Soc.*, 1954, **76**, 3227.

(261) D. B. Repke, D. B. Grotjahn and A. T. Shulgin, *J. Med. Chem.*, 1985, **28**, 892.

(262) H. Hashimoto, K. Asano, F. Fuji and J. Yoshimura, *Carbohydr. Res.*, 1982, **104**, 87.

(263) R. R. Schmidt and E. Rücker, *Tetrahedron Lett.*, 1980, **21**, 1421.

(264) 'Dictionary of Organic Compounds,' Ed. 5, Vol. 5, ed. J. Buckingham, Chapman and Hall, New York, 1982, 5127.

(265) 'Dictionary of Organic Compounds,' Ed. 5, Vol. 5, ed. J. Buckingham, Chapman and Hall, New York, 1982, 5127.

(266) H. Kuzuhara and H. G. Fletcher-Jr., *J. Org. Chem.*, 1967, **32**, 2531.

(267) W. Kemp, 'Qualitative Organic Analysis,' McGraw Hill, London, 1979, 90.

(268) *Chem. Abstr.*, 1964, **60**, 14500h.

(269) S. D. Sharma and P. K. Gupta, *Tetrahedron Lett.*, 1979, **14**, 1265.

(270) A. Williams, E. C. Lucas, A. R. Rimmer and H. C. Hawkins, *J. Chem. Soc., Perkin Trans. 2*, 1972, 627.