GLUCOSINOLATES AND ISOTHIOCYANATES : CHEMISTRY AND BIOLOGICAL ACTIVITY

Nicola E. Davidson

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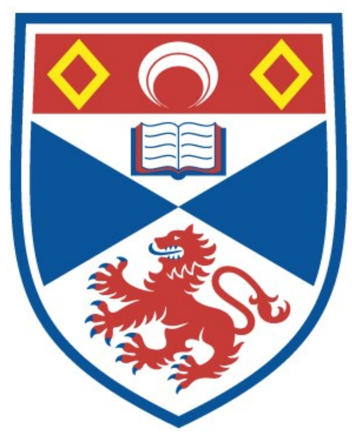

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GLUCOSINOLATES AND ISOTHIOCYANATES: CHEMISTRY AND BIOLOGICAL ACTIVITY

A thesis presented for the degree of

Doctor of Philosophy

to the University of St. Andrews

on the 12th October 1998

by

Nicola E. Davidson

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Dedicated to mum and dad for all your love and support.

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Last but not least, I'd like to thank my parents for putting up with me!

GLOSSARY OF ABBREVIATIONS

BaP	Benzo[a]pyrene
BIC	Benzyl isocyanate
BITC	Benzyl isothiocyanate
bp	Boiling point
BuIC	Butyl isocyanate
BuITC	Butyl isothiocyanate
m-CBA	*m*-Chlorobenzoic acid
CBZ	Benzyloxycarbonyl
CI	Chemical ionisation mass spectrometry
COSY	Correlated spectroscopy
m-CPBA	*m*-Chloroperbenzoic acid
DEAE	Diethylaminoethyl
DIBAL-H	Diisobutyl aluminium hydride
DMBA	7,12-dimethylbenz[a]anthracene
DMD	Dimethyl dioxirane
DMN	Dimethyl nitrosamine
DMSO	Dimethyl sulfoxide
E.C.	Enzyme commission
EDTA	Ethylenediaminetetraaceticacid
EI	Electron impact mass spectrometry
ES^-	Negative ion electrospray mass spectrometry
FT-IR	Fourier transform-infra-red spectrophotometry
GC(/MS)	Gas chromatography(/mass spectrometry)
GLC	Gas-liquid chromatography
GST	Glutathione-*S*-transferase
HK	Hexokinase
HPLC	High performance liquid chromatography
IACR	Institute of Arable Crops Research
IR	Infra-red spectrophotometry
LC-MS	Liquid chromatography-mass spectrometry
MAM	Methylazoxy-methanol
MDMAAB	3'-methyl-4-dimethylaminoazobenzene
mp	Melting point
MPIC	Methoxyphenyl isocyanate
MPITC	Methoxyphenyl isothiocyanate
NDA	*N*-Nitrosodiethylamine

NDMA	*N*-Nitrosodimethylamine
NFA	*N*-2-fluorenyl acetamide
NIC	Naphthyl isocyanate
NITC	Naphthyl isothiocyanate
NMAA	*N*-Nitrosomethylamylamine
NMBA	*N*-Nitrosomethylbenzylamine
NMR	Nuclear magnetic resonance spectroscopy
NNAL	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	*N'*-Nitrosonornicotine
NOE	Nuclear Overhauser Effect
ONPG	*Ortho*-nitrophenyl-β-glucoside
PAPS	3'-Phosphoadenosine 5'-phosphosulfate
PBITC	Phenylbutyl isothiocyanate
PCC	Pyridinium chlorochromate
PEIC	Phenethyl isocyanate
PEITC	Phenethyl isothiocyanate
PHITC	Phenylhexyl isothiocyanate
PIC	Phenyl isocyanate
PITC	Phenyl isothiocyanate
PNPG	*Para*-nitrophenyl-β-glucoside
PPITC	Phenylpropyl isothiocyanate
ppm	Parts per million
PTFA	Peroxytrifluoroacetic acid
RMM	Relative molar mass
SCRI	Scottish Crop Research Institute
THF	Tetrahydrofuran
TLC	Thin layer chromatography
UDPG	Uridine diphosphate glucose
UV	Ultra violet spectrophotometry

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ABSTRACT

The ability of glucosinolates to act as host recognition cues and oviposition stimulants for root flies has been previously established. To further investigate the interactions between pest and glucosinolate a number of simple and complex glucosinolates were synthesised and tested by contact chemoreception. A crude structure-activity relationship was identified whereby the stimulatory activity of the glucosinolate increased as the alkyl side chain was increased from propyl to pentyl, heptyl and nonyl. Comparison of the novel synthetic glucosinolate, naphthylmethyl glucosinolate, with glucobrassicin, a naturally occurring indole derivative, showed the former to have little or no activity whereas the latter is the most active natural stimulant. The synthetic glucosinolates were also demonstrated to act as substrates for the enzyme myrosinase, being hydrolysed to β-D-glucose and the corresponding isothiocyanate. In addition, (7-methoxycarbonylheptyl) glucosinolate, prepared as a precursor to (7-carboxyheptyl) glucosinolate, was found to be a substrate. High resolution NMR studies of the latter compound showed this acidic glucosinolate and indeed alkyl glucosinolates to adopt an unexpected conformation in aqueous solution. Furthermore, a number of alkyl thiohydroximates were synthesised and used as HPLC and LC-MS standards to aid glucosinolate identification.

Isothiocyanates have been identified as chemopreventative agents which inhibit carcinogen activation mediated by cytochrome P450 enzymes. The postulated oxidation of isothiocyanates to isocyanates by these enzymes, was studied using a number of chemical model systems. Oxidation of isothiocyanates was efficiently achieved using dimethyl dioxirane (DMD). Although, the resulting isocyanates could not be isolated, their production was confirmed by GC/MS and FT-IR analysis of reaction solutions. A number of ureas were also prepared by trapping the isocyanates *in situ*. These compounds were demonstrated to arise from the isocyanate and not oxidation of the corresponding thiourea. In addition, peracids were found to produce isocyanates, although less efficiently.

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION TO GLUCOSINOLATES

1.1.1 Background

Glucosinolates are a group of organosulfur secondary metabolites found in a wide variety of plant families.[1] They have been known to exist since ancient times, when plants bearing these compounds were cultivated and used for medicinal purposes.[2] All glucosinolates have the same general structure **1** consisting of a β-thioglucose unit, side chain **R** and sulfated oxime moiety.

1

Most glucosinolates are hygroscopic and display poor crystallinity. In addition, these compounds are highly acidic and can be handled only as salts.[3] Consequently, the isolation of glucosinolates has proved difficult with most being characterised from their breakdown products. Despite this problem, over 100 different glucosinolates have been identified, each varying in the structure of the side chain. A few examples which exemplify the diversity of glucosinolates are given in table 1.

Apart from sinigrin and sinalbin which were the first two glucosinolates to be identified and given classical names,[4,5] most glucosinolates were named after the plant species from which they were isolated i.e. gluconasturtiin was isolated from *Nasturtium officinale*.[6] However, as more glucosinolates were identified the name of the side chain was used as a prefix to the word "glucosinolate" thus gluconasturtiin became phenethyl glucosinolate.

Classification	Side chain	Original Name
Alkyl	CH_3	Glucocapparin
	$CH_3CH(CH_3)$	Glucoputranjivin
Alkenyl	$-CH_2CH=CH_2$	Sinigrin
Sulfurous	$CH_3S(CH_2)_3$	Glucoibervirin
	$CH_3SO(CH_2)_3$	Glucoiberin
	$CH_3SO_2(CH_2)_3$	Glucocheirolin
Aromatic	CH_2	Glucotropaeolin
	CH_2CH_2	Gluconasturtiin
	H_3CO CH_2	Glucoaubrietin
Carbonyl-containing	$CH_3OOC(CH_2)_3$	Glucoerypestrin
Hydroxylated	CH_3 H_2C CH_3 OH	Glucoringiin
Heteroaromatic	CH_2 N H	Glucobrassicin

Table 1 Examples of different glucosinolate side chains.

Glucosinolates are found to exist in plants along with the enzyme myrosinase (E.C. 3.2.3.1) which is responsible for catalysing the hydrolysis of these compounds. When plants are damaged by insect attack or mechanical manipulation, β-D-glucose **2** and an aglycone fragment **3** result. The latter compound is unstable and undergoes a Lossen-type rearrangement to afford primarily the isothiocyanate **4**, although thiocyanates **5** or nitriles **6** can also be formed depending on the reaction conditions (Scheme 1).[7]

HO, HO, HO, HO, O, S, N, $OSO_3^-K^+$, R

1

MYROSINASE

HO, HO, HO, HO, O, OH + HS, N, $OSO_3^-K^+$, R

2 **3**

RNCS **4**

RSCN **5**

RCN **6**

Scheme 1 Hydrolysis of glucosinolates by myrosinase.

These noxious isothiocyanates have not only proved invaluable for glucosinolate identification, but are thought to have a beneficial role in defending the plant from attack by pests. Moreover, these compounds have many toxic and beneficial effects.

In this chapter the occurrence, biosynthesis and synthesis of glucosinolates will be discussed in addition to the beneficial effects of these compounds. Furthermore, the breakdown of glucosinolates by myrosinase will be examined in more detail leading to a discussion of the chemistry and biochemistry of the predominant catabolite, the isothiocyanate.

1.1.2 Occurrence and distribution of glucosinolates

The occurrence of glucosinolates in plants is limited to the order Capparales and certain families of dicotyledonous angiosperms. They have been found to predominate in the Capparaceae, Cruciferae, Moringaceae, Resedaceae and Tavariaceae families and exist sporadically in the Caricaceae, Euphorbiaceae, Gyrostemonaceae, Limnanthaceae, Salvadoraceae and Tropaelaceae. In particular, the glucosinolates found in the Cruciferae have been extensively studied as all plants belonging to this family contain these compounds. The *Brassica* vegetables such as cabbage, Brussels sprouts, broccoli, cauliflower and turnip, which are consumed in large quantities by humans from both eastern and western cultures, are also members of the Cruciferae. These vegetables contain substantial quantities of glucosinolates (table 2).[8]

Vegetable	**Total glucosinolate in unprocessed vegetables (mg/Kg fw)**
Red cabbage	669 (UK), 410-1090 (USA)
White cabbage	510 (Canada), 330-840 (USA)
Savoy cabbage	470-1240 (USA)
Brussels sprouts	1455-3939 (UK), 2562 (Neth.), 1342 (Canada), 600-3900 (UK)
Cauliflower	138-2083 (UK), 530-1140 (UK), 122 (Canada)
Broccoli	611 (UK), 276 (Canada)
Kale	894 (UK)

Table 2 Total glucosinolate concentrations for Brassica vegetables from different countries.

The total glucosinolate concentration, as shown in table 2, is usually measured using a colorimetric assay which detects the quantity of glucose released upon myrosinase hydrolysis. It should be noted that the value differs according to geographical area and this can be attributed to variations in cultivation conditions, climate and agronomic practice. It should also be borne in mind that the total concentration alters depending on the plant part examined, although all plant organs contain glucosinolates.

In addition to differences in the total glucosinolate concentration, the quantity and variety of individual glucosinolates also fluctuates with plant origin. This is demonstrated by examination of Japanese and Kenyan radishes.[9] Although sharing many of the same glucosinolates, only the Japanese variety contains pentyl and benzyl glucosinolates and only the Kenyan variety contains hexenyl and 2-phenethyl glucosinolates.

Analysis of glucosinolate composition was previously performed by gas chromatography (GC) of the isothiocyanates released upon glucosinolate catabolism. However, the intact glucosinolates can now be examined using reverse-phase high performance liquid chromatography (HPLC) or gas-liquid chromatography (GLC). Moreover the sulfate group can be removed enzymatically using sulfatase (E.C. 3.1.6.1) allowing efficient analysis of the corresponding desulfo-glucosinolate. Comparison of GLC and HPLC methods have shown the latter to be most efficient allowing separation and identification of 11 glucosinolates contained in the reference material of rapeseed, with the former separating only 7 glucosinolates.[10]

Fluctuations in the level of total and individual glucosinolates have also been shown to arise at various stages in the plant's life cycle.[11] Accumulation of glucosinolates occurs during vegetative growth and seed maturation, followed by degradation during flowering, germination and the early growth stages of seedlings. This behaviour points to glucosinolates having a role as storage compounds, releasing nitrogen, carbon and sulfur to sustain plant growth when necessary.

In the past decade, a great deal of research has focused on reducing the levels of glucosinolates in certain plants such as oilseed rape.[12] This crop produces large quantities of oil which is valuable in nutrition and as an industrial feedstock. Moreover the seed resulting from oil extraction can be used as animal feed. However, palatability and anti-nutritional problems resulted from feeding livestock with the seed. These were attributed to the wide range of catabolites produced from a variety of glucosinolates in the rape, all of which could exert different effects.

Initially plant breeding strategies allowed levels of erucic acid to be reduced giving rise to "single low" varieties. This has been further improved to give "double low" varieties which contain significantly lower quantities of aliphatic and indolyl glucosinolates. The final aim is to eliminate glucosinolates like progoitrin **7**, as this compound gives rise to 2-hydroxy-3-butenyl isothiocyanate **8**. Subsequent cyclisation affords goitrin **9** which causes goitre in animals (scheme 2).[13]

HO HO HO HO O S $OSO_3^-K^+$ N OH 7 Myrosinase N=C=S OH 8 H N S O 9

Scheme 2 Catabolism of progoitrin to afford goitrin.

1.1.3 Glucosinolate biosynthesis

Glucosinolates are biosynthesised from amino acids and are generally split into three groups depending upon the amino acid from which they are derived: methionine **10** gives rise to aliphatic and alkenyl glucosinolates; phenylalanine **11** affords aromatic compounds; tryptophan **12** is used to produce indolyl glucosinolates.

$CH_3SCH_2CH_2$ COOH H NH_2

10

HOOC H NH_2 C H_2

11

COOH H_2N H N H

12

The pathway from amino acid to final glucosinolate can be split into 3 parts. These are chain elongation of amino acids, assembly of the glucone and finally, modification of the side chain.[14]

Elongation of amino acids is essential for producing the wide range of glucosinolates previously discussed. This process is thought to proceed *via* transamination of the amino acid to afford the corresponding α-keto acid **13**. This compound then condenses with a molecule of acetyl-CoA and undergoes various transformations such as loss of water, rehydration, oxidation and decarboxylation before a further transamination to restore the amino group, and produce the homologated amino acid. Thus methionine **10** can be converted to homomethionine **14**, dihomomethionine **15** and so on (scheme 3).

$CH_3SCH_2CH_2$–CH(NH$_2$)COOH **10** --Trans-amination--> $CH_3SCH_2CH_2$–C(=O)COOH **13** ---> $CH_3SCH_2(CH_2)_2$–CH(NH$_2$)COOH **14** ---> $CH_3SCH_2(CH_2)_3$–CH(NH$_2$)COOH **15**

Scheme 3 Chain extension of methionine.

The enzyme which catalyses the transamination reactions, methionine:glyoxylate aminotransferase, has been purified and characterised.[15] Interestingly, non-glucosinolate producing plants contain low levels of this enzyme or isozymes which are significantly different to those present in glucosinolate producing species.

Next, the amino acid is converted to the glucosinolate **16**, as shown in scheme 4.

R–CH(NH$_2$)-COOH (Amino acid) ⟶ RCH_2–C(H)=N–OH **17** ⟶ RCH_2–C(SH)=N–OH **18** ⟶ RCH_2–C(S-Glu)=N–OH **19** ⟶ RCH_2–C(S-Glu)=N–O–SO_3^- **16**

Scheme 4 Assembly of the glucosinolate skeleton.

The formation of an aldoxime **17** from the amino acid is well established although the enzymology behind this process is still under investigation. It appears that three different types of enzymes are used depending upon the substrate. Microsomal cytochrome P450

enzymes from oilseed rape have been implicated in the conversion of methionine and phenylalanine to the corresponding oximes.[16] Similarly, an enzyme has been isolated from *Tropaeolum majus* L. which was sensitive to cytochrome P450 inhibitors and catalysed the conversion of phenylalanine to phenylacetaldoxime.[17] Conversely, the formation of oximes from homophenylalanine and dihomomethionine has been found to depend upon oxygen, NADPH and a flavin-containing mono-oxygenase.[18] Furthermore, the conversion of tryptophan to indole acetaldoxime is catalysed by a membrane bound peroxidase.[14]

The conversion of the oxime **17** to the thiohydroximate **18** is poorly understood but is postulated to proceed *via* oxidation of the oxime to the nitro-compound which then reacts with the appropriate sulfur donor to yield the thiohydroximate. The identity of the sulfur donor has not yet been confirmed but is thought to be cysteine.

The last two steps in the pathway have been well documented. The formation of the desulfo-glucosinolate **19** is catalysed by UDPG-thiohydroximate glucosyl transferase (E. C. 2.4.1-) with the final step being catalysed by a desulfoglucosinolate sulfotransferase (E. C. 2.8.2-). This latter enzyme was partially purified from Cress (*Lepidium sativum*) and in the presence of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) catalysed the sulfation of desulfobenzyl glucosinolate, desulfo-*p*-hydroxybenzyl glucosinolate and desulfoallyl glucosinolate.[19] The enzyme showed an absolute requirement for the desulfoglucosinolate structure with the reaction rate varying according to the length of the side chain. However, it should be noted that both of these enzymes show wide substrate specificity allowing the synthesis of a wide variety of glucosinolates.

In the final phase of biosynthesis, the side chain of the glucosinolate undergoes secondary modifications. These can be hydroxylations, methylations and oxidations and allow the formation of compounds such as progoitrin (2-hydroxy-3-butenyl glucosinolate) from 3-butenyl glucosinolate.

1.1.4 Synthesis of glucosinolates

Until the 1950's, all the studies performed on glucosinolates were carried out using compounds extracted from plant material. The first glucosinolate was prepared in 1957 by Ettlinger and Lundeen who synthesised benzyl glucosinolate (glucotropaeolin) **20**.[20] They began by reacting a Grignard reagent **21** with carbon disulfide to afford the dithiophenylacetate **22** which was then reacted with hydroxylamine hydrochloride to yield the corresponding thiohydroxamic acid **23**. Coupling with α-acetobromoglucose **24** allowed formation of the glycosylated thiohydroximate **25**. Sulfation using a pyridine-sulfur trioxide complex and deprotection using methanolic ammonia afforded the glucosinolate **20** which was isolated as its tetramethylammonium salt (scheme 5).

Scheme 5 *Reagents and conditions*: i, CS_2, Et_2O; ii, NH_2OH(aq), 0 °C, 33%; iii, KOH, methanol-acetone (1:3), room temp., 6 h, 47%; iv, pyridine-SO_3, pyridine, room temp., overnight; v, methanolic ammonia, 94%.

This strategy can be regarded as an electrophilic sugar approach where substitution occurs at the anomeric position of the sugar moiety initiated by nucleophilic attack of the thiohydroxamic acid. Although this method works well for the synthesis of benzyl glucosinolate, it does not appear to have been used for the synthesis of other glucosinolates. This is most likely a consequence of the inherent stability of thiohydroxamic acids which makes them poor nucleophiles.

An alternative approach to the synthesis of benzyl glucosinolate was published by Benn in 1963.[21] Beginning with phenylacetaldoxime **26** (scheme 6), the corresponding oximyl chloride **27** was prepared by reaction with chlorine gas. This latter compound was reacted with tetra-acetyl-thioglucopyranose **28** in the presence of triethylamine to afford the acetylated thiohydroximate **25**. Sulfation and deprotection were carried out, as in the previous synthesis, to afford the glucosinolate **20** as its potassium salt.

Scheme 6 *Reagents and conditions*: i, Cl_2, $CHCl_3$, 0 °C, 30 minutes; ii, Et_3N, Et_2O, 30 minutes, room temp., then 0.5 M H_2SO_4, 0 °C; iii, Pyridine-SO_3, pyridine, room temp., overnight then $KHCO_3$ (aq); v, methanolic ammonia, 0 °C, overnight.

The key step in this synthesis is the coupling of the oximyl chloride **27** and tetra-acetyl-thioglucopyranose **28**. This is reported to proceed *via* the nitrile oxide **29** which is formed *in situ* from reaction of the oximyl chloride with base (scheme 7).

Scheme 7 Formation of tetra-acetyl benzyl thiohydroximate *via* the nitrile oxide.

This nucleophilic sugar approach has been adopted for the synthesis of a number of naturally occurring glucosinolates. The synthesis of methyl, ethyl, *iso*-propyl and phenethyl glucosinolates has been reported along with preparation of *p*-hydroxybenzyl and *p*-methoxybenzyl glucosinolates.[22] In addition, the syntheses of sinigrin,[23,24] 2-methylpropyl glucosinolate,[25] progoitrin,[26] and glucobrassicin,[27] have been reported. Some of these syntheses, instead of beginning from the oxime, proceed from the corresponding nitro compound. The most interesting example of this route is the synthesis of glucobrassicin (scheme 8). The nitronate salt **30** is prepared from 3-formyl-indole **31** which is extended by reaction with nitromethane to yield the intermediate 3-(2'-nitrovinyl)-indole **32**. Chemoselective reduction using sodium borohydride affords the nitro

compound **33** which is reacted with sodium methoxide to afford the nitronate salt **30**. Chlorination then occurs to afford the oximyl chloride **34** as before.

Scheme 8 *Reagents and conditions*: i, $MeNO_2$, $AcONH_4$, 100 °C; ii, $NaBH_4$, SiO_2, 230-400 mesh, $CHCl_3$, iPrOH, 56%; iii, MeONa, MeOH, Et_2O; iv, $SOCl_2$, DME, -78 °C.

Also, glucoviorylin **35**, glucoibervirin **36** and glucoerucin **37**, which contain an external thio-function, have been prepared *via* the nitronate salt route beginning from bromochloroalkanes.[28]

35 n=2
36 n=3
37 n=4

Despite striking similarities between the synthesis of different glucosinolates, the chlorination method to produce the oximyl chloride has differed significantly. The procedure used depends on whether the precursor is a nitronate salt or an oxime. Reagents used commonly for this transformation are summarised in table 3.

Chlorination of nitronate salt	Chlorination of oxime
Dry HCl(g) (Phenethyl glucosinolate)[29]	Dry Cl_2 in dry ether (Phenethyl)[29]
Thionyl chloride in dry $CHCl_3$ (Progoitrin)[26]	*N*-chlorosuccinimide in $CHCl_3$ (various)[30]
Thionyl chloride in DME (Glucobrassicin)[27]	-
LiCl-HCl (Sinigrin)[23]	-
HCl in dry diethyl ether (Sinigrin)[24]	-

Table 3 Reagents used in the preparation of oximyl chlorides.

The chlorination of nitronate salts and oximes was compared for the preparation of phenethyl glucosinolate.[29] Chlorination of the oxime was most efficient producing the oximyl chloride in 83% yield compared with the analogous reaction of the nitronate which only afforded the product in 64% yield. Moreover, there are less steps in the oxime synthesis making this generally the most efficient route. However, the method used depends on the nature of the side chain with the nitronate salt reagents favouring chlorination of sensitive substrates.

In addition to the chlorination methods above, a further alternative exists. Kularni *et al* recently reported the chlorination of arylnitrovinyl compounds to arylalkyl hydroximoyl chlorides using titanium tetrachloride and triethylsilane.[31] This method has been employed for the synthesis of a number of arylalkyl and indole glucosinolates.[32] Chlorination was found to be very effective for the former compounds with mixed results obtained for the indole derivatives depending on the nature of the *N*-protecting group. The chlorination of *p*-acetoxy-(2-nitro)styrene **38** to afford the corresponding oximyl chloride **39** and tetra-acetyl-*p*-acetoxybenzyl thiohydroximate **40** in 85% overall yield, is given in scheme 9.

Scheme 9 structure labels: AcO, NO2, i,ii, 38, OH, N, Cl, AcO, 39, iii, AcO, O, AcO, AcO, S, OH, N, AcO, 40, OAc

Scheme 9 *Reagents and conditions*: i, $TiCl_4$, Et_3SiH, CH_2Cl_2, rt, 1 h; ii, aqueous work-up; iii, thioglucopyranose **28**, CH_2Cl_2/Et_2O, Et_3N, rt, 2 h, 85%.

In addition to the syntheses already discussed, one other route warrants a mention. An alternative strategy was devised and used for the synthesis of ethyl glucosinolate.[33] The oximyl chloride was substituted with *tert*-butyldimethylsilyl nitronate **41** and this was coupled with tetra-acetylthioglucopyranose **28** as given previously to yield a mixture of *cis* **42** and *trans* **43** thiohydroximates (28% : 56% respectively), as shown in scheme 10. This is contrary to the previous methods which proceed *via* the nitrile oxide and exclusively yield the *trans* product. However, over time the *cis* product isomerised to the *trans* conformer indicating this isomer to be the most stable. This is the only reported synthesis which yields a mixture of geometric isomers.

Scheme 10 *Reagents and conditions*: i, Et_3N, THF, rt, 12 h.

A wide variety of unnatural glucosinolates have been reported from 1987 onwards. Selenoglucosinolates **44**, where the sulfur atom has been substituted by selenium, have been prepared by Kjaer and Skrydstrup.[34] The synthetic route was analogous to that for natural glucosinolates except tetra-acetyl-selenoglucopyranose was used for coupling to the oximyl chloride. These compounds were found to be substrates for myrosinase, producing the corresponding isoselenocyanate upon hydrolysis.

44

R = $PhCH_2$, Me

Rollin and co-workers, in 1990, demonstrated that it was possible to synthesise α-glucosinolates **45** by preparing S-trityl protected α-tetra-acetyl-thioglucopyranose.[35] This compound was transformed into the deprotected α-thiosugar, in 50% yield, by reaction with phenyl mercury acetate followed by hydrogen sulfide. Subsequent reactions afforded phenyl, benzyl, 2-phenethyl, styryl and indol-3-ylmethyl glucosinolate analogues. This

research group has also been responsible for preparing a range of aza-derivatives **46** and a phosphate bio-isostere of benzyl glucosinolate **47**.[36,37]

45

46

R = CH_3, CH_2CH_3, $-CH_2CH{=}CH_2$
phenyl, benzyl, CH=CHPh,
methylindolyl

47

In addition, Rollin has reported a number of aromatic desulfoglucosinolates in which the aromatic group is directly attached to the quartenary carbon of the aglycone.[30] These compounds ranging from phenyl, *p*-fluorophenyl and mesityl to naphthyl, anthracenyl and indole were prepared as possible internal standards for the HPLC analysis of glucosinolates. Furthermore, the synthesis of a number of deoxy-glucosinolates has also been completed.[38] Removal of a hydroxyl group has allowed preparation of 2, 3, 4 and 6-deoxy derivatives of benzyl glucosinolate, as illustrated by the 2-deoxy derivative **48** below. Similarly the 2 and 6-deoxy analogues of glucobrassicin have been prepared.

48

The natural and synthetic glucosinolates have been prepared for a variety of reasons including the synthetic challenge, to further study the biosynthesis and degradation of glucosinolates and as HPLC internal standards to quantify and identify natural glucosinolates from plant extracts. With the exception of two publications, one detailing the use of these compounds as HPLC standards,[39] and the other the use of synthetic glucosinolates as competitive inhibitors of myrosinase,[40] the biological applications and usefulness of these compounds remains largely unexplored.

1.1.5 Biological effects of glucosinolates

Many of the effects related to glucosinolates are actually attributed to their breakdown products and these will be discussed in detail later. However, glucosinolates do have some biological properties such as the ability to act as chemopreventative agents. In particular, glucobrassicin has been shown to inhibit pulmonary adenomas induced by benzo[a] pyrene in mice, with glucotropaeolin and glucosinalbin having similar effects.[41] Furthermore, glucobrassicin and glucotropaeolin are reported to have inhibited the growth of 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumours in rats. In the case of glucobrassicin, studies have shown that feeding this glucosinolate to rats induces mixed function oxidases in the small intestine.[42] These enzymes are commonly employed in the removal of xenobiotic substances.

In addition, glucosinolates have a role as natural insecticides by acting as storage compounds for isothiocyanates which are released when the plant is under attack. The isothiocyanates are noxious compounds with anti-microbial and anti-fungal properties which deter pests and pathogens from further feeding on the plant. However, it has also been found that the relationship between the plant and pest is complicated and glucosinolates can act as oviposition stimuli.[43] The outcome of this egg-laying behaviour, by root flies, is destruction of the plant which has grim financial implications for the agricultural industry.

As a precursor to producing crops which are resistant to root flies, the mechanism behind oviposition has been examined. Roessingh *et al* have examined the role of glucosinolates in oviposition using the cabbage root fly (*D. radicum* (L.)).[43] They used two different types of testing to examine the effect of a range of glucosinolates on oviposition. The first method is electrophysiological recordings or contact chemoreception where the electrical response to a stimulant, generated in a receptor cell, is measured and recorded. The receptor cells are located at specific positions on chemoreceptor hairs on the tarsi of the insect. A scanning electron microscope picture showing the chemoreceptor hairs of this fly is given in figure 1 and a diagrammatic representation of the electrophysiological recording process is given in figure 2.

Figure 1 SEM picture of the female cabbage root fly tarsus showing chemo-receptor hairs.

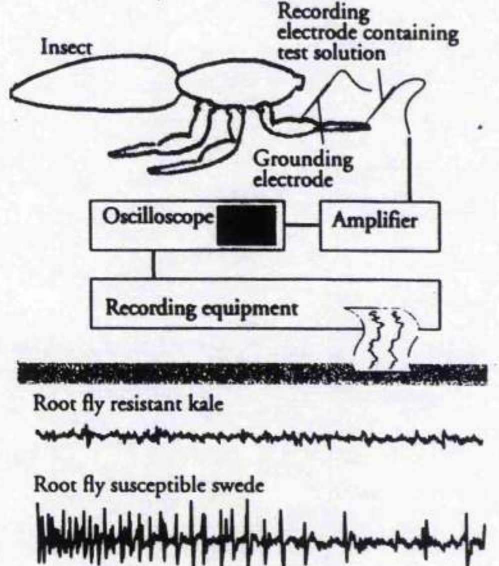


Figure 2 Schematic diagram of electrophysiological recording with recorded chemosensory responses.

The second testing method involves spraying the compound to be analysed onto artificial surrogate leaves and allowing the flies to oviposit. The number of eggs laid correlates with the stimulatory activity of the compound.

Using both of these methods, a range of naturally occurring glucosinolates have been tested to determine their potency as oviposition stimuli.

HO, HO, HO, HO, O, S, N, $OSO_3^-K^+$, R

R = $H_2C{=}CHCH_2$ (sinigrin) $H_2C{=}CHCH_2CH_2$ (gluconapin)

C_6H_5–CH_2 (glucotropacolin) C_6H_5–CH_2CH_2 (gluconasturtiin)

$H_2C{=}CH$–$CH(OH)$–CH_2 (progoitrin) $H_2C{=}CHCH_2CH_2CH_2$ (glucobrassicanapin)

H_3C–$S(=O)$–$(CH_2)_3$ (glucoiberin)

N–OCH_3 indol-3-ylmethyl (neoglucobrassicin) N–H indol-3-ylmethyl (glucobrassicin)

The chemoreceptors of *D. radicum* were particularly sensitive to glucobrassicin, gluconasturtiin and glucobrassicanapin with large differences being observed among individual glucosinolates e.g sinigrin was less active by two orders of magnitude when compared with gluconasturtiin. Similar results were also obtained in the behavioural tests which, unlike the electrophysiological testing, suggested a crude correlation between the overall length of the glucosinolate side chain and the insect response (figure 3).

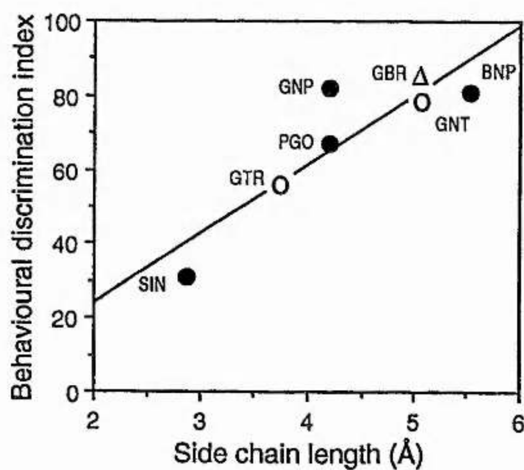


Figure 3 Correlation between side chain length and behavioural discrimination (Δ indole, ● aliphatic, O aromatic glucosinolate.

The authors indicated this correlation required further investigation using other glucosinolates before a structure-activity relationship could be conclusively established. Indeed, this study is complicated by the presence of a wide variety of functional groups. In addition, the ability of other unknown compounds to induce oviposition was recognised.

Many additional studies into the ovipositional behaviour of various species have been recently conducted. Analogous to the research detailed previously, the ovipositional behaviour of the turnip root fly (*Delia floralis*) has been examined using similar methods.[44] Again a good correlation between the behavioural and electrophysiological studies was obtained. The same naturally occurring glucosinolates were tested although sinalbin (*p*-hydroxybenzyl) and glucoerucin (4-methyl thio-butyl) were also included. Once again, the most active compounds were glucobrassicanapin, glucobrassicin and this time gluconapin. The results also indicated *D. floris* to be less responsive to the stimuli than *D. radicum*. Interestingly, it was observed during the behavioural studies that flies either remain on the leaf or depart depending on the glucosinolate present. In fact an oviposition behavioural pattern has been elucidated where the fly explores the leaf surface before displaying stem-run behaviour and finally oviposition (figure 4).

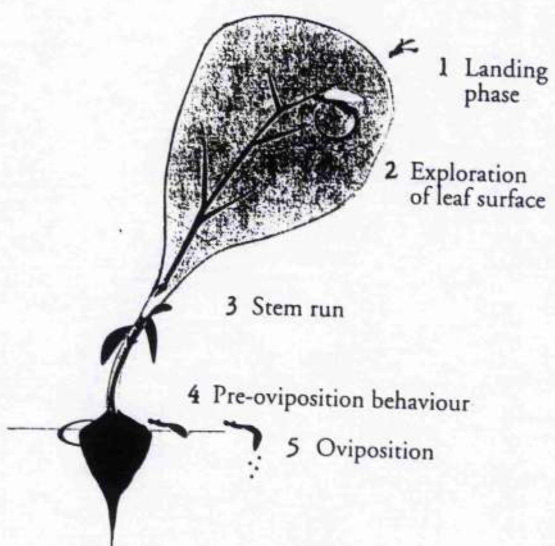


Figure 4 Illustration of the oviposition behavioural pattern of root flies.

In addition to root flies, butterflies such as *Pieris rapae* and *P. napi oleracea* show oviposition stimulated by glucosinolates. Huang and co-workers found that these species were sensitive to the (2*R*) and (2*S*) epimers of glucobarbarin (1-hydroxy-phenethyl glucosinolate), although to different extents, and also to glucobrassicin.[45] *P. rapae* was found to be stimulated by glucobrassicin and gluconasturtiin in a different study, which also determined that *D. floralis*, *D. radicum* and *P. rapae* were more sensitive to the aromatic glucosinolates than the alkyl and thioalkyl compounds.[46]

The waxy leaf surface has also been proven to be necessary for optimum ovipositional activity in studies using the diamondback moth, *Plutella xylostella*.[47] The activity of sinigrin was significantly increased when covered with paraffin or an *n*-alkane mixture although the wax alone was found to be inactive.

A structure-activity study was conducted by Braven *et al* who analysed the ovipositional activity of a few glucosinolate derivatives and a number of other compounds by a bioassay method.[48] They found that changes in the glucone portion of glucosinolates, e.g. by acetylation, led to reduced activity as did removal of the sulfate group which led to a loss of activity. Compounds possessing a sulfonic acid group showed activity as did sulfones indicating that the S=O group was important. However, this study was not comparable to that performed by Roessingh and co-workers as no rational alteration of the glucosinolate structure was performed.

Although much has been learned about the effect of glucosinolates on the ovipositional behaviour of root flies and other species, the research in this area has been restricted by a lack of available glucosinolates. Thus a range of synthetic and naturally occurring glucosinolates were prepared, as detailed in chapters 2 and 3 hereafter, with the aim of establishing a structure-activity relationship where rational side chain modifications were made.

1.2 HYDROLYSIS OF GLUCOSINOLATES BY MYROSINASE

1.2.1 Properties of myrosinase

Glucosinolates are found in plants along with a β-thioglucosidase called myrosinase (E. C. 3.2.3.1) which catalyses the hydrolytic cleavage of the β-glycoside linkage of these compounds. The name suggests the presence of only one enzyme although a family of isoenzymes actually exist.[49] Three forms of the enzyme have been isolated from white mustard seed (*Sinapis alba*) with four isoenzymes being identified in rapeseed (*Brassica napus*). These are glycoproteins having a molecular weight ranging from 125 to 150 kDa, and a total carbohydrate content of 9-23% of the total mass.[12] One exception to this is the myrosinase from *Wasabia japonica* which has a much higher molecular mass of 580 kDa.[50] The enzyme from *S. alba* has two identical subunits, each having a molecular weight of 71.7 kDa,[51] and is encoded by a multigene family which consists of at least 2 subgroups.[52] The distribution of myrosinase is analogous to glucosinolates which is not surprising given that they co-exist. All plant organs contain some myrosinase activity although different patterns of isozymes result depending upon the species, organ and age of the plant.

Björkmann and Lönnerdal examined the properties of isoenzymes from *S. alba* and *Brassica napus* and found them to have a broad pH range, from pH 4.5-9.0, a temperature maximum of 60 °C and a high stability i.e. the activity of the enzyme remained unchanged after storage for 6 months at pH 6.0.[49] Moreover, Palmieri *et al* have reported that myrosinase can be immobilised with negligible loss of activity.[53] They created a myrosinase biosensor to measure the glucosinolate content of crude plant extracts and observed no loss of myrosinase activity after 15 months and approximately 1000 assays.

The activity of myrosinase can be measured using three different methods: determination of glucose release; titrimetric determination of the HSO_4^- released upon hydrolysis; spectrophotometric determination of the consumed substrate.[54,55] The most commonly used

method is the latter due to its speed and simplicity. This is also the most reproducible method although it has been reported to under-estimate the enzyme activity.

The substrate specificity of myrosinase has been examined by several authors. Myrosinase isolated from light-grown cress (*Lepidium sativum*) was incubated with 29 different *O*- and *S*-glycosides which varied not only in the side chain but also in the sugar moiety.[56] Only four compounds were found to be substrates for the enzyme, two of which were benzyl glucosinolate and sinigrin with the other two being the synthetic glycosides *o*-nitrophenyl-β-glucoside **49** (ONPG) and *p*-nitrophenyl-β-glucoside **50** (PNPG). These results showed myrosinase to have a high degree of glucone specificity with only glucosides being accepted at the active site.

49 **50**

The substrate specificity also differs according to the glucosinolate side chain as shown by examination of various naturally occurring compounds.[49] The rate of hydrolysis was found to decrease in the order benzyl, 2-propenyl, 3-methylsulfonylpropyl, 2-hydroxy-3-butenyl, *p*-hydroxybenzyl and methyl glucosinolate. Four different isoenzymes from two different origins showed this pattern although the rate of hydrolysis of 2-hydroxy-3-butenyl glucosinolate increased in one case and decreased in another. Thus it can be concluded that the substrate specificity does not alter significantly between isoenzymes meaning that a particular set of myrosinases are not required for hydrolysis of a defined set of glucosinolates. Generally the rate of hydrolysis differs between different groups of glucosinolates, with aliphatic derivatives being broken down at a higher rate than their indole analogues, but remains virtually unchanged for the members of the same group. Other investigations have shown that the sulfate group is required for activity as desulfoglucosinolates do not act as substrates.[57] In addition, removal of the hydroxyl group

at positions 2, 3, 4 or 6 on the sugar ring leads to a reduction in the rate of hydrolysis implying that these hydroxyls are required for binding the substrate to the enzyme.[40]

Studies on the cress enzyme have also shown that metal ions do not increase the activity of myrosinase but can cause moderate to strong inhibition. The addition of metal chelators such as EDTA also showed no effect implying that the enzyme has no metal requirement. However, thiol reagents such as *N*-ethylmaleimide caused inhibition of enzyme activity.

The most potent activator of myrosinase to be discovered is L-ascorbic acid. Interestingly, this compound activates hydrolysis at low concentration while causing inhibition at high concentrations. In 1968, Tsuro and Hata proposed a mechanism to account for this effect.[58] Two sites are thought to exist on the surface of the enzyme: one for the substrate and one, named the effector site, for L-ascorbic acid. The substrate binding site has two domains for binding the sugar group and aglycon respectively. In the absence of ascorbate, the aglycon region of the glucosinolate does not fit perfectly to the enzyme. However, binding of ascorbate to the effector site causes a conformational change in the structure of myrosinase allowing the perfect glucosinolate fit to be achieved and hence an improved rate of turnover.

Conversely the rate of turnover of PNPG is not affected by ascorbic acid. This can be explained by the fact that PNPG binds to myrosinase through its sugar group with the aglycone having no binding properties. Thus no effect is observed upon addition of ascorbic acid as this molecule only affects the conformation of the aglycone binding region.

Upon addition, of high concentrations of ascorbic acid, the hydrolysis of both sinigrin and PNPG is inhibited indicating that ascorbic acid can also bind to the active site and act as a competitive inhibitor.

Further work on the activating effect of ascorbic acid established that analogues of this molecule had little effect on the activity of myrosinase.[59] Evidence for the proposed

conformational change was provided by spectrophotometry which showed that approximately 1.5 amino acid residues appeared on the surface of the enzyme while 2.3 tryptophan residues were buried in the molecule upon addition of 1 mM ascorbic acid. In addition, the optimum temperature for the hydrolysis of sinigrin decreased from 55 °C to 35 °C, in the presence of ascorbate, while the temperature for PNPG hydrolysis remained unchanged.

1.2.2 Organisation of the glucosinolate/myrosinase system

The research performed in this area has been extensively reviewed by Bones and Rossiter who concluded that three alternatives exist for the location of glucosinolates and myrosinase.[12] The first is that the two components may exist in the same cell but in an inactive form. Myrosinase has been found in the vacuole of myrosin cells which could also house glucosinolates and ascorbic acid. If the latter compound was present in high concentration, myrosinase would be inactive. Cellular disruption upon plant damage would result in dilution of the ascorbic acid, and myrosinase would be activated to catalyse glucosinolate hydrolysis.

The second alternative is that glucosinolates and myrosinase could be located in the vacuoles of different cells. Cellular disruption by insect attack could allow the two components to come into contact. Conversely, a system could operate to transfer one of the components to the site of the other.

The third alternative locates the substrate and enzyme in different vacuoles of the same cell. This is thought to be unlikely given that the vacuoles of myrosin cells undergo changes after sowing which involve the fission and fusion of these compartments. This implies that a highly regulated system would have to operate to keep the glucosinolates and myrosinase apart at this time.

Although it has been established that glucosinolates and ascorbic acid are located in vacuoles and that myrosinase is located in myrosin cells, the exact cellular arrangement of glucosinolates and myrosinase remains unknown.

1.2.3 Chemical mechanism of myrosinase

The chemical mechanism behind the hydrolysis of glucosinolates has been investigated by several researchers and has been proposed to proceed in a manner analogous to *O*-glycoside hydrolysis by β-glucosidases.[40] The greatest progress toward elucidating the mechanism of myrosinase was made by determining the crystal structure of the myrosinase from *S. alba*.[60] This enzyme was found to possess a hydrophobic pocket to accommodate the glucosinolate sidechain and two arginine residues positioned to interact with the sulfate group. Analysis of the glycosyl-enzyme intermediate showed the sugar ring was bound *via* an α-glycosidic linkage to glutamate 409, as shown in scheme 11, and was attached to the active site of myrosinase by six hydrogen bonds. However, unlike the hydrolysis of *O*-glycosides by β-glucosidases, which require an acid catalyst to protonate the leaving aglycone, the acidic residue was absent in myrosinase being replaced by a glutamine residue. Acid catalysis appears to be unnecessary as the high activity of the glucosinolate aglycone makes it an excellent leaving group. This also explains why myrosinase fails to catalyse hydrolysis of *O*-glycosides, which have poor leaving groups that require protonation, whereas the hydrolysis of ONPG and PNPG occurs readily as the nitrophenyl group is a good leaving group. Instead the glutamine residue is thought to have a role in positioning the water molecule for hydrolysis.

Glutamine
Arginines
Glu 409
HSO$_4^-$ + RNCS

Scheme 11 Mechanism of myrosinase catalysed hydrolysis of glucosinolates as evidenced by x-ray crystallography.

This mechanism is supported by Iori and co-workers who have shown that all the sugar hydroxyl groups are required for optimum glucosinolate turnover with various reduced rates of hydrolysis coinciding with removal of one of these from different positions on the sugar ring.[40] Removal of the hydroxyl group at C-2 stopped hydrolysis and the resulting deoxy-glucosinolate actually acted as an inhibitor. It has been postulated that this hydroxyl group is the most important as it causes polarisation of the C-S bond making nucleophilic attack at the anomeric position more favourable. Furthermore, NMR studies of the hydrolysis of sinigrin by *S. alba* showed that the reaction proceeded with retention of configuration at the anomeric carbon,[61] as shown in scheme 11.

Thus despite similarities between myrosinase and β-glucosidases, this research has shown that these enzymes are not identical. This conclusion is also supported by the work of Botti and co-workers who showed that transglycosylation reactions, which are typical of β-glucosidases, could not be catalysed by myrosinase.[62] This is also likely to be due to the inability of myrosinase to protonate the hydrolytic leaving group which means that alternative glycosyl acceptors are not deprotonated and are thus unable to trap out the glycosyl intermediate.

1.2.4 Products of hydrolysis

The hydrolysis of glucosinolates by myrosinase to produce a range of volatile products is shown in scheme 12. The products shown, the isothiocyanate **4**, nitrile **6** and thiocyanate **5**, are the normal products although other compounds can result.

Scheme 12 Normal hydrolysis of glucosinolates to produce isothiocyanates, nitriles and thiocyanates.

As stated earlier, the most common product is the isothiocyanate which results from Lossen-type rearrangement of the corresponding aglycone at neutral pH (scheme 13). The reaction is thought to occur after release of the aglycone from the active site and not be enzyme catalysed.[57] This rearrangement is favourable as the sidechain and sulfate group exist in the *anti* stereochemical configuration as confirmed by x-ray crystallography.[63]

$OSO_3^-K^+$ ... R-N=C=S + SO_4^{2-}

Scheme 13 Lossen-type rearrangement of the aglycone to afford the isothiocyanate.

As the pH of the reaction solution is decreased, production of the isothiocyanate is inhibited probably due to blockage of the Lossen-type rearrangement by protonation. Instead, the formation of the corresponding nitrile is favoured below pH 4.0. The mechanism postulated to account for the production of this species and sulfur is given in scheme 14.[64] Interestingly the formation of nitriles instead of isothiocyanates is also promoted by ferrous ions.[65]

R–C≡N + [S]

Scheme 14 Formation of nitriles under acidic conditions.

The formation of thiocyanates occurs much less, being limited to allyl (sinigrin), benzyl and 4-(methylthio)butyl glucosinolates.[64] Although the exact mechanism is unknown, the

presence of an isomerase which catalyses conversion of the *anti* aglycone to the *syn* isomer has been postulated. The *syn* isomer would not have the correct configuration to undergo the Lossen-type rearrangement, with migration of the side chain occurring instead to afford the thiocyanate **5** (scheme 15).

Scheme 15 Proposed formation of thiocyanates.

Other products are formed upon hydrolysis of β-hydroxy glucosinolates such as progoitrin (2-hydroxy-3-butenyl glucosinolate) **7** (scheme 16). As with the other glucosinolates, the formation of nitriles, and in this case hydroxynitriles **51**, occurs predominately at pH 4-7 and in the presence of ferrous ions. At pH 9 and above, the formation of oxazolidine-2-thiones from the corresponding isothiocyanate predominates. This gives rise to goitrin **9** as shown earlier in scheme 2. In addition, epithionitriles **52** can result in the presence of ferrous ions and a small protein called epithiospecifier protein. This protein interacts with myrosinase to promote sulfur transfer to the terminal alkenyl group.

Scheme 16 Production of hydroxynitriles, oxazolidine-2-thiones and epithionitriles from hydrolysis of β-hydroxy glucosinolates.

Also of interest are the products resulting from hydrolysis of indole glucosinolates. Again, at pH 3-4 the appropriate nitrile is generated while under more basic conditions, the isothiocyanate is virtually undetectable,[66] although the existence of 1-methoxyindol-3-ylmethyl isothiocyanate has been proven.[67, 68] The isothiocyanate is then postulated to decompose to afford the thiocyanate anion. This reaction is favourable due to the stability of the intermediate cation **53** which is readily hydrolysed to indole-3-carbinol **54** in the case of glucobrassicin (scheme 17). This compound then self-condenses to yield 3,3'-diindolylmethane **55** with loss of formaldehyde.

NCS
N
H
OH_2
N+
H
53
OH
N
H
54
N
H
N
H
55

Scheme 17 Hydrolysis products of glucobrassicin.

1.3 INTRODUCTION TO ISOTHIOCYANATES

1.3.1 Background

Isothiocyanates are a group of sulfur and nitrogen containing compounds commonly referred to as "mustard oils" due to their isolation from this condiment. These linear molecules, of the general formula R-NCS, exist mainly as liquids possessing a pungent odour and biting taste, although many crystalline examples are known.[69] They are structural isomers of the thiocyanates, R-SCN, and can be formed from this latter group of compounds by rearrangement. Moreover, they share similar chemistries with their oxygen containing analogues the isocyanates, R-NCO. Isothiocyanates have found application in elucidating the structure and functions of proteins and have been found to inhibit enzymes which require thiol groups for activity. In recent years there has been renewed interest in the chemistry of isothiocyanates as these compounds have been found to have a range of pronounced biological effects.

1.3.2 Chemical properties of isothiocyanates

The isothiocyanate functional group can exist as three different polarised structures (scheme 18), which indicate the electrophilicity of the central carbon atom.

$$R-\bar{N}-\overset{+}{C}=S \longleftrightarrow R-N=C=S \longleftrightarrow R-N=\overset{+}{C}-\bar{S}$$

Scheme 18 Isothiocyanate resonance structures.

It is this feature which accounts for the reactivity of isothiocyanates with aryl isothiocyanates being significantly more reactive that alkyl isothiocyanates due to the electron withdrawing influence of the aromatic ring. As a result of this electrophilicity,

isothiocyanates are highly reactive towards nucleophiles and undergo the following reactions:[1,69]

1. Reaction with alcohols to give monothiourethanes **56**.
2. Reaction with thiols to give *S*-esters of *N*-monosubstituted dithiocarbamic acids **57**.
3. Reaction with ammonia, amines, hydroxylamine, hydrazine and nitrogen-containing heterocycles to form thioureas **58**, **59**, **60**, **61** and **62** respectively.

R–NH–C(=S)–O–R' **56** R–NH–C(=S)–S–R' **57** R–NH–C(=S)–NH_2 **58** R–NH–C(=S)–NH–R' **59**

R–NH–C(=S)–NH–OH **60** R–NH–C(=S)–NH–NH–C(=S)–NH–R' **61** R–NH–C(=S)–N(pyrrole) **62**

4. Addition of carboxylic and thiocarboxylic acids to yield amides **63** (scheme 19).
5. Reaction with compounds containing labile hydrogen (scheme 20).
6. Addition of hydrogen cyanide to form cyanothioamides.
7. Numerous cycloaddition reactions.

R–C(=O)OH —(S=C=N–R')→ [R–C(=O)–O–C(=S)–NH–R'] —(-COS)→ R–C(=O)–NH–R' **63**

Scheme 19 Formation of amides from reaction of carboxylic acids with isothiocyanates.

$$R\text{-}N{=}C{=}S \;+\; CH_3COCH_2COCH_3 \longrightarrow R{-}N(H){-}C({=}S){-}CH(COCH_3)_2$$

Scheme 20 Reaction of an isothiocyanate with acetyl acetone.

In addition, isothiocyanates can combine with simple aromatic compounds, phenols and aryl ethers to form aryl-substituted thioamides when Friedel-Crafts conditions are applied. Isothiocyanates can also be oxidised, using mercuric oxide[70] and palladium chloride[71] and can be reduced by hydride reducing agents (scheme 21).

$$C_6H_5{-}N{=}C{=}S \xrightarrow{LiAlH_4} C_6H_5{-}NH{-}CH_3$$

Scheme 21 Reduction of Phenyl isothiocyanate.

In addition to the reactions of isothiocyanates with amines listed above, these compounds can also react with biological molecules and are used to determine the sequence of polypeptide chains by Edman degradation. The *N*-terminal amino acid is reacted with phenyl isothiocyanate **64**, the Edman reagent, to afford the corresponding phenylthiocarbamoyl peptide **65** (scheme 22). Subsequent treatment with acid affords the phenyl thiohydantoin derivative **66** and the polypeptide chain containing one less amino acid. The derivatised *N*-terminal amino acid can then be identified by chromatography or comparison with the original polypeptide. The degradation can be performed many times to determine the sequence of short peptides. Similarly, fluorescein isothiocyanate is used to label antibodies so that these can be detected inside cells.

Scheme 22 Edman degradation of polypeptides.

It should be noted that the reactivity of isothiocyanates is considerably less than isocyanates and carbodiimides which have similar heterocumulene structures. Isocyanates undergo most of the reactions reported previously for isothiocyanates but with greater ease. For example, isocyanates hydrolyse readily to yield the corresponding amine and carbon dioxide. This leads to the formation of *N,N'*-disubstituted ureas as the amine reacts with another molecule of isocyanate. However, isothiocyanates are relatively stable to water, often being isolated by steam distillation, and only hydrolyse in the presence of acidic or basic catalysts or at high temperature and pressure. Moreover, isocyanates undergo many reactions not reported for isothiocyanates. Isocyanates have a tendency to form dimers and trimers upon heating or in the presence of a catalyst. This is not a common feature of isothiocyanates. They have also been shown to react with hydrogen peroxide derivatives to form peroxycarbamates, another reaction that is not mimicked by isothiocyanates. Furthermore, the reactivity of isocyanates, has been commercially exploited in the formation

of polyurethanes which result from reaction of diisocyanates and diols. The demand for isothiocyanates has been considerably less.

1.3.3 Biological properties of isothiocyanates

Isothiocyanates exhibit a number of toxic and pharmacological properties. Sporadic reports of their disadvantageous features include their goitrogenic,[72] and cytotoxic[73,74] behaviour in addition to inducing chromosome abnormalities and neoplasia.[72] Beneficial effects begin with their anti-fungal, anti-protozoal and anti-bacterial activity which is being investigated further with a view to using isothiocyanates as preservatives in packaged foods.[75] Isothiocyanates have also been shown to inhibit bronchial obstruction and may prove to be promising anti-asthmatic agents.[76] However, it is the capacity of isothiocyanates to prevent carcinogenesis that is of most interest, and which will be reviewed hereafter.

The ability of aromatic isothiocyanates to inhibit chemically induced carcinogenesis was first demonstrated using α-naphthyl isothiocyanate (NITC).[77,78] This compound reduced the formation of liver tumours induced by 3′-methyl-4-dimethylaminoazobenzene (MDMAAB), ethionine and *N*-2-fluorenyl acetamide (NFA) in the liver of male Wistar rats. This finding effectively set the stage for similar investigations into the anti-carcinogenic activities of other aromatic isothiocyanates.

Aryl alkyl isothiocyanates, such as benzyl isothiocyanate (BITC) **67** and phenethyl isothiocyanate (PEITC), **68** have been extensively investigated.

$C_6H_5-CH_2-N=C=S$ **67**

$C_6H_5-CH_2CH_2-N=C=S$ **68**

As mentioned earlier, these compounds are naturally occurring, being derived from the glucosinolates glucotropaeolin and gluconasturtiin respectively. Both compounds have been shown to inhibit carcinogenesis induced by a variety of nitrosamines and polyaromatic hydrocarbons (table 4).

Carcinogen	Test animal	Area of tumour inhibition	Reference
Benzyl Isothiocyanate			
DMBA	Rats ICR/Ha mice	Mammary glands Forestomach, lungs	72, 77, 80, 81
NDA	A/J mice	Forestomach	79
BaP	A/J mice ICR/Ha mice	Lung, forestomach Forestomach	79, 81, 82, 83, 84
DMN	A/J mice	Forestomach	72
NNK	Hamster	Liver microsomes	85, 86, 87
NMBA	F344 rats	Oesophagus	88
MAM-acetate	ACI/N rats	Intestine	89
Phenethyl Isothiocyanate			
DMBA	Mice Rats	Forestomach, lung Mammary glands	72, 79, 81
NBMA	F344 rats	Oesophagus	90
NNN	Rats	Cultured oral tissue	91
NDMA	Rats	Liver microsomes	92
BaP	A/J mice	Forestomach	83, 93
NMAA	Wister rats	Oesophagus, liver	94
NMBA	F344 rats	Oesophagus	88

Table 4 Studies of carcinogen inhibition by BITC and PEITC.

One of the most rigorously investigated carcinogens is 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK) **69**.[95] This nitrosamine is found in tobacco and tobacco smoke, and is

the most potent carcinogen tested on laboratory animals thus far.[93] NNK was found to induce tumours in the lung, nasal cavity, liver and pancreas of F344 rats; nasal cavity and lung tumours in hamsters; and lung tumours in mice.[96] In 1987, 53 million cigarette smokers were being exposed daily to NNK, hence the urgency for an efficient anti-cancer agent.[97]

NNK is one of a number of carcinogens requiring metabolic activation. Once activated, these carcinogens are normally highly electrophilic and reactive towards DNA causing modification and hence cancer development. NNK is metabolically activated by three key processes.[87,91] These are: reduction of the carbonyl group to form 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL); *N*-Oxidation of the pyridine group to form 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone (NNK-*N*-oxide); α-Hydroxylation of the methyl or methylene carbon adjacent to the *N*-nitroso group to produce 4-oxo-1-(3-pyridyl)-1-butanone (keto aldehyde) and a methylating species, or 4-hydroxy-1-(3-pyridyl)-1-butanol (keto alcohol) and a pyridyloxobutylating species (scheme 23).

Scheme 23 Metabolic pathways of NNK.

The methylating and pyridyloxobutylating species, formed upon α–hydroxylation of NNK, are alkyl diazohydroxides which interact with DNA to form adducts.[96] Therefore, inhibition of α-hydroxylation could potentially decrease the carcinogenicity of NNK.

A number of aryl alkyl isothiocyanates have been reported to inhibit NNK induced carcinogenesis. Initially, Chung *et al* [95] showed inhibition of NNK tumorigenesis in rats using PEITC. The study demonstrated that rats treated with PEITC prior to NNK exposure showed approximately 50% less lung tumours compared with those treated with NNK alone. This was extended to an investigation of BITC, PEITC and phenyl isothiocyanate (PITC) on the inhibition of lung tumours induced by NNK in A/J mice.[98] The results showed BITC and PITC had little effect on tumorigenesis but once again PEITC had a profound effect, not only in reducing tumours but in reducing *O*^{6}-methylguanine formation and lung microsomal metabolism of NNK. Further work using synthetic aryl alkyl isothiocyanates with longer alkyl chains, such as phenylpropyl isothiocyanate (PPITC), phenylbutyl isothiocyanate (PBITC) and phenylhexyl isothiocyanate (PHITC), showed that a structure-activity relationship exists for the inhibitory activity of these isothiocyanates.[85,99,100,101] As the length of the alkyl chain of the aryl alkyl isothiocyanate increases so the inhibitory potential of the isothiocyanate increases. This was later attributed to the longer chain analogues possessing higher lipophilicity and lower reactivity than their shorter chain counterparts. Additional tests comparing the inhibition of NNK by dodecyl isothiocyanate, dodecyl alcohol and dodecane proved the isothiocyanate functional group was essential for inhibitory activity.[102]

In addition to tests on laboratory animals exposed to NNK, a range of isothiocyanates have been shown to inhibit the cell growth of human erythroleukemic K562 cells.[103] These results have implications for human health with isothiocyanates being released upon consumption of vegetables, which contain large quantities of glucosinolates (see table 2). Indeed epidemiological studies have shown correlations between consumption of cruciferous vegetables and reduced risk of cancer. Jiao and co-workers recently analysed

the isothiocyanate content of 102 cooked vegetables which are consumed by the Singapore Chinese who show reduced rates of cancer.[104] The vegetables were treated with myrosinase to mimic the breakdown of glucosinolates upon chewing. Watercress was found to contain the highest quantity of isothiocyanates, having a mean value of 81.3 μmol/100g wet weight, and bok choi the lowest, having a mean value of 4.9 μmol/100g wet weight. These values signify the maximum quantity of isothiocyanates to which humans would be exposed after consumption of cooked vegetables assuming full liberation.

It should be noted that it is difficult to assess the intake of isothiocyanates by humans or other animals. The amount of isothiocyanate released depends upon the amount of processing before cooking, the temperature and duration of cooking which can inactivate myrosinase, and the relative uptake of isothiocyanates in the body. Studies on the uptake of BITC in humans have indicated that approximately 50% of the administered amount is excreted as its *N*-acetyl-cysteine conjugate,[105] with PEITC showing a similar reaction.[106] Interestingly, this latter conjugate showed potent inhibition of growth of human leukaemia 60 cells *in vitro* suggesting that these compounds may be anti-cancer agents in their own right.[107]

Furthermore the research conducted also highlights the mode of action of isothiocyanates. Inhibition of carcinogenesis only occurs when the isothiocyanate is administered prior to carcinogen exposure. Morse *et al* fed laboratory animals using a 4-dose protocol and showed that most or even all inhibition was due to a final dose of isothiocyanate two hours prior to treatment with the carcinogen.[108] This indicated that isothiocyanates do not reverse the effects of tumour-inducing agents but act in a chemopreventative capacity. Thus these are extremely promising compounds for reducing the incidence of cancer in humans through dietary consumption. Indeed fortification of the diet with isothiocyanates may have an even greater effect but a great deal more research will be needed to evaluate the toxicity of these compounds and the mechanism by which they achieve chemoprevention.

1.3.4 Mechanisms of chemoprevention

There have been countless reports on the mechanism of chemoprevention by isothiocyanates with the majority of these acknowledging that this area requires further investigation. Currently the chemopreventative effect of isothiocyanates is thought to arise either by isothiocyanates inhibiting the metabolic activation of carcinogenic species or by activating phase II detoxification enzymes which accelerate the disposal of carcinogens.

Most of the research into the inhibition of metabolic activation of carcinogens has concentrated on the NNK model where activation is mediated by cytochrome P450 enzymes located in the liver. The role of these enzymes is to oxidatively introduce polar groups to otherwise hydrophobic xenobiotics to aid their removal from the body. However, these enzymes have been shown to activate carcinogens such as NNK by this oxidative process. Both α-hydroxylation and *N*-oxidation of NNK have been shown to be inhibited by isothiocyanates in tests on cultured rat oral tissue and hamster liver microsomes.[85,86,91] This suggests that isothiocyanates are, in some way, inhibiting the cytochrome P450 isozymes responsible for these processes. Indeed it has been demonstrated that PEITC inhibits a number of cytochrome P450 isozymes including IIE1,IIA1 and IIA2, IIB1, IA1, IA2 and IIIA isozymes.[92,109,110,111] A further study testing the inhibition by a range of isothiocyanates found that isozymes IA1, IA2 and IIB1 were inhibited by longer chain aryl alkyl isothiocyanates with the most potent having a C_6 alkyl chain.[112] Inhibitory potential declined as the chain length was increased to C_8-C_{10} but increased by addition of another phenyl group to phenethyl isothiocyanate to afford the 1,2- and 2,2-diphenyl derivatives.

The second theory, whereby isothiocyanates accelerate the disposal of carcinogens also carries some weight. Studies have shown that BITC acts to increase levels of the phase II detoxification enzyme, glutathione-*S*-transferase (GST).[100,113] In addition, a number of bifunctional isothiocyanates have been shown to be potent inducers of phase II enzymes.[114] These are structural analogues of sulforaphane **70**, an inducer of phase II enzymes isolated

from broccoli. Studies have shown that the highest degree of enzyme induction is achieved when the isothiocyanate group is separated from a methylsulfonyl or acetyl group by three or four carbon atoms. However, other studies indicate levels of GST and quinone oxidoreductase, in the liver and lung microsomes of mice, were unaffected by dietary PEITC.[113] This suggests that carcinogen inhibition by PEITC is not governed by phase II detoxification enzymes and that inhibition of carcinogen activation is the most likely explanation of the isothiocyanate effects. In practice both mechanisms are likely to operate to different extents depending on the compound.

H_3C–S(=O)–$CH_2CH_2CH_2CH_2$–NCS

70

Although the interactions with cytochrome P450 isozymes and GST have been postulated and confirmed, the exact molecular mechanisms involved have not been elucidated. Given that P450s act by substrate oxidation it seems likely that isothiocyanate oxidation could occur. This may result in production of the corresponding isocyanate which, being more reactive than its precursor, could react with nucleophiles at the enzyme active site causing inhibition (scheme 24). This would be an example of "suicide inhibition" with suicide inactivators defined as "compounds activated by the enzyme to intermediates which covalently bind to the enzyme itself thereby irreversibly inhibiting it."[115]

$$\text{R-N=C=S} \xrightarrow{\text{Cyt. P450}} \text{R-N=C=O} \xrightarrow[\sim\text{OH}]{\sim\text{NH}_2,\ \sim\text{SH}} \textbf{Inactivated P450}$$

Scheme 24 Proposed suicide inhibition by isothiocyanates.

The chemical conversion of isothiocyanates to isocyanates was previously addressed in section 1.3.2 where mercuric oxide and palladium chloride were used to prepare isocyanates

under harsh chemical conditions. Moreover, this conversion is also biologically plausible with oxidative desulfurization of compounds containing C=S and P=S having been previously observed.[92,116] For example carbon disulfide (CS_2) is converted to carbonyl sulfide (S=C=O) by cytochrome P450 enzymes.[117] It has been suggested that oxene transfer occurs between the enzyme and carbon disulfide to afford the sulfine and/or an oxathiiranium-type intermediate which rearranges and expels the sulfur atom to afford carbonyl sulfide. A similar mechanism involving isothiocyanates can be postulated involving the sulfine **71** and oxathiiranium intermediate **72** (scheme 25).

R–N=C=S —[O]→ [R–N=C=S^+–O^-] **71**

↓

R–N=C=O + [S] ← [R–N=C(–O–)S] **72**

Scheme 25 Putative mechanism for the oxidation of isothiocyanates by cytochrome P450 enzymes.

The fate of the reactive sulfur species released upon metabolism of thiono-sulfur compounds by cytochrome P450 enzymes has been investigated using ^{35}S labelled compounds.[115] Invariably this species, thought to be atomic sulfur, was found bound to microsomal proteins. Complexation allowed only 75% of sulfur to be removed suggesting that it may exist in more than one chemical environment. The sulfur released upon oxidation is thought to form hydrogen disulfide linkages, which are broken upon complexation, or is thought to undergo carbon-hydrogen insertion reactions to from mercaptans which are chemically more resistant. It has also been suggested that the sulfur atom inactivates the P450 enzymes rather than the activated oxidised species. Inhibition is

hypothesized to be achieved by removal of heme iron by complexation with sulfur or changes in protein structure brought about by sulfur binding to cysteine residues.

Further evidence supporting the biological oxidation of isothiocyanates to isocyanates was provided recently by the work of Lee.[118,119,120] The oxidative metabolism of 2-NITC **73** by rat liver microsomes yielded *N,N'*-di-2-naphthylurea or *N*-2-naphthyl-*N'*-2-fluorenylurea **74** when 2-aminofluorene **75** was added. This suggests that 2-naphthyl isocyanate (2-NIC) **76** was formed from 2-NITC **73** which subsequently reacted with its hydrolysis product to form the di-naphthylurea and with 2-aminofluorene to form the mixed urea **74** (scheme 26).

N=C=S [O] N=C=O 73 76 H_2N 75 O N H N H 74

Scheme 26 Proposed route of formation of *N*-2-naphthyl-*N'*-2-fluorenylurea from NITC.

However, the amounts of these compounds were very small (nanomolar quantities), with a yield of only 0.57% achieved for the di-naphthylurea and 3.16% for the mixed urea. In addition, large quantities of the starting isothiocyanate remained. Further work indicated 1-NIC, benzyl isocyanate (BIC), phenyl, propyl, ethyl and methyl isocyanates could be produced from their corresponding isothiocyanates as evidenced by trapping as mixed ureas. The author conceded that trapping the isocyanate with 2-aminofluorene **75** was a useful but imperfect system as there are many complicating factors. Moreover, conversion

of the mixed thiourea **77** or the symmetrical thiourea to the mixed urea **74** or symmetrical urea could not be ruled out (scheme 27).

Scheme 27 Formation of the mixed thiourea and possible oxidation to the mixed urea.

Thus the chemical oxidation of isothiocyanates was examined as detailed hereafter in chapter 5 as a model for the previously reported biological systems. The initial aim of the work was to show complete conversion of the isothiocyanate to the isocyanate using a chemical reagent to mimic the action of cytochrome P450 oxidation. The free isocyanate would be isolated or detected *in situ* to prove that this species was indeed formed upon oxidation and the mechanism behind formation would be examined. In addition, it was hoped to extend the investigations to more biological systems.

1.4 RESEARCH OBJECTIVES

These were two-fold namely:

1. Synthesis and testing of a number of synthetic and naturally occurring glucosinolates to gain more information on the relationship between insect response and glucosinolate structure. These novel glucosinolates could be tested for a variety of other purposes while providing essential information on the mechanisms behind insect oviposition.

2. Examination of the mechanism behind the chemopreventative effect of isothiocyanates using chemical model systems.

CHAPTER 2

SYNTHESIS OF ALKYL GLUCOSINOLATES AND THIOHYDROXIMATES

2.1 SYNTHETIC TARGETS

In previous studies on the ovipositional behaviour of *D. radicum*, a crude correlation between the side chain length of naturally occurring glucosinolates and the insect response was observed.[43] The main evidence for this correlation came from responses generated from alkenyl glucosinolates of the structure R = CH_2=CH-$(CH_2)_n$ (n =1,2,3). In an attempt to investigate this matter further and simplify the system, a range of alkyl glucosinolates with varying lengths of side chain were envisaged as synthetic targets **78**-**82**. These compounds would allow a large variation in the length of the alkyl chain and would probe the necessity of the alkene functionality for stimulatory activity. Moreover, this range of compounds would allow a mixture of naturally occurring and synthetic analogues to be tested as propyl **78**, butyl **79** and pentyl **80** glucosinolates have been detected in crude plant extracts.[121,9,122] The longer chain derivatives, heptyl **81** and nonyl glucosinolate **82**, have not been reported previously, either synthetically produced or isolated from natural sources.

HO, HO, HO, HO, O, S, N, $OSO_3^-K^+$, R

R = $CH_3(CH_2)_2$ **78**
$CH_3(CH_2)_3$ **79**
$CH_3(CH_2)_4$ **80**
$CH_3(CH_2)_6$ **81**
$CH_3(CH_2)_8$ **82**

The synthesis of more complex derivatives was also envisaged, the first of these being naphthylmethyl glucosinolate **83**. This compound could be compared with glucobrassicin **84** allowing the effect of the NH group in this compound to be assessed.

83 **84**

Moreover, the synthesis of a glucosinolate possessing an alkanoic acid functionality would provide an interesting comparison with the simple alkyl glucosinolates. More importantly, this compound would allow the interaction between the waxy leaf surface and the glucosinolate to be investigated. The enhanced stimulatory potency of sinigrin treated with paraffin could be due to optimal binding of the glucosinolate with the wax.[47] This surface may allow the glucosinolate to be orientated specifically for insect recognition due to the hydrophobic side chain residing in this layer thus exposing the hydrophilic sugar residue and sulfate moiety. This interaction could be increased by lengthening the alkyl side chain and then disrupted by addition of a hydrophilic group to the end of the side chain.

2.2 GENERAL SYNTHESIS OF GLUCOSINOLATES AND THIOHYDROXIMATES

The general synthetic route employed for the synthesis of the glucosinolates and thiohydroximates discussed hereafter, is given in scheme 28. This route was chosen as it has been used extensively for the synthesis of a number of naturally occurring and novel glucosinolates, as detailed in section 1.1.4.

Scheme 28 *Reagents and conditions*: i, Et_3N, THF; ii, NaOMe, Amberlite IR-120; iii, $ClSO_3H$, pyridine, DCM; iv, $KHCO_3$(aq); v, KOMe.

The preformed tetra-acetyl thioglucopyranose **28** is coupled with the appropriate oximyl chloride **85** in the presence of triethylamine to yield the tetra-acetyl-glucopyranosyl thiohydroximate **86**. This compound can then be deprotected by reaction with sodium methoxide to afford the thiohydroximate **19**, or sulfated using pyridine and chlorosulfonic acid. The latter affords the corresponding tetra-acetyl glucosinolate **87** which is deprotected using potassium methoxide to produce the desired glucosinolate **1**. The starting materials, tetra-acetyl thioglucopyranose **28** and the oximyl chloride **85**, can be prepared from β-D-glucose and the corresponding aldehyde respectively. These transformations will be discussed in more detail in the appropriate section.

2.2.1 Preparation of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (thioglucopyranose)

The preparation of thioglucopyranose **28** is common to the synthesis of all the glucosinolates discussed hereafter. This compound was simply prepared in three steps from β-D-glucose (scheme 29).

Scheme 29 *Reagents and Conditions :* i, Ac_2O, 45% (w/v) HBr in AcOH, DCM; ii, $(NH_2)_2CS$, acetone, reflux; iii, $K_2S_2O_5$, H_2O, CH_2Cl_2, reflux.

Firstly, β–D-glucose **88** was reacted with acetic anhydride and 1.5 equivalents of hydrobromic acid in acetic acid to allow formation of penta-acetyl glucopyranose. After approximately one hour a further 4.5 equivalents of hydrobromic acid in acetic acid were added to yield 2,3,4,6-tetra-*O*-acetyl-1–bromo-α-D-glucopyranose (acetobromoglucose) **24** in 71-81% yield. The product was obtained as a gold-coloured oil which crystallised after overnight storage at -20 °C. Recrystallisation was easily achieved by dissolving the crystals in cold diethyl ether and adding petroleum ether (bp 40-60 °C). The presence of the desired compound was confirmed by proton NMR analysis which showed a multiplet integrating to 12 protons, due to the acetyl groups, at 2.04 ppm. Moreover, the anomeric proton had moved from 6.28 ppm in penta-acetyl glucopyranose to 6.59 ppm in acetobromoglucose indicating a change in the environment at this position. However, the

acetobromoglucose was not stored but reacted immediately with an equivalent amount of thiourea in dry acetone to afford 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiouronium bromide **89** following the procedure of MacLeod *et al.*[29] The mixture was heated under reflux for 15-20 minutes until the appearance of the product as a white solid. The product was cooled in ice for approximately 20 minutes before being filtered and washed with acetone to yield the desired isothiouronium bromide in 45-67% yield.

Recrystallisation of the isothiouronium bromide **89** from acetone was found to be extremely time consuming and required large volumes of acetone. Thus in most cases this compound was used without further purification as little sign of impurities could be observed by ^{1}H and ^{13}C NMR.

Conversion of the isothiouronium bromide **89** to thioglucopyranose **28** was carried out in a two-phase reaction system.[123] This involved dissolution of potassium metabisulfite in water at 75 °C before addition of dichloromethane as the lower phase and finally, addition of the bromide. This gave rise to thioglucopyranose **28** in yields ranging from 67% to 85%. Once again, storage of this compound at -20 °C induced crystallisation producing a white solid from a colourless oil. The solid was recrystallised from methanol and the pure compound stored at -20 °C until required for further reaction. This compound could be easily distinguished from the isothiouronium bromide **89** due to altered chemical shifts in both the proton and carbon NMR spectra. In particular, the chemical shift for the anomeric proton changed from δ_H 5.26 to 4.54 and appeared as a triplet due to coupling with the hydrogen of the thiol and also the hydrogen at the 2-position. In the carbon spectrum the signal due to (C-1) moved from 83.87 ppm in the isothiouronium bromide to 79.21 ppm in thioglucopyranose.

2.3 SYNTHESIS OF ALKYL OXIMYL CHLORIDES

The oximyl chlorides required for coupling to thioglucopyranose were prepared in two steps. The corresponding aldehyde **90** was reacted with hydroxylamine hydrochloride to yield the oxime **17** which was then chlorinated, using *N*-chlorosuccinimide, to afford the oximyl chloride **85** (scheme 30).

R–CH=O (**90**) —i→ R–CH=N–OH (**17**) —ii→ R–C(Cl)=N–OH (**85**)

Scheme 30 *Reagents and conditions:* i, $NH_2OH.HCl$, EtOH, pyridine, reflux; ii, *N*-chlorosuccinimide, $CHCl_3$, pyridine.

The required oximes were prepared from butyraldehyde, valeraldehyde, hexanal, octanal and decyl aldehyde using an adaptation of a procedure by Vogel.[124] In each case equivalent amounts of aldehyde and hydroxylamine hydrochloride were dissolved in pyridine and ethanol and the resulting solution was heated under reflux for 1-3 hours. After cooling and concentration under reduced pressure, water was added to the oily residue, and the mixture was cooled in ice to bring about crystallisation. This occurred readily for hexaldoxime **91**, octaldoxime **92** and decaldoxime **93**. However, butaldoxime **94** did not crystallise due to its low melting point. Thus this product was extracted using ethyl acetate, dried and concentrated under reduced pressure to afford the product as a colourless oil in 44% yield. This was used without further purification to prepare the chloro-oxime. Pentaldoxime **95** also failed to yield a solid upon addition of water but the crystalline oxime was recovered, in a yield of 61%, after overnight storage at approximately 4 °C. The oximes **91**, **92** and **93** were recrystallised from ethanol to afford the pure compounds in yields of 76%, 65% and 84% respectively. ^{1}H and ^{13}C NMR spectroscopy of these oximes indicated that they were present as a mixture of geometric isomers. In the case of hexaldoxime **91** a 1:1 mixture of

the two isomers was obtained. The ^{1}H NMR spectrum of this compound showed two triplets integrating for 0.5 H at δ_H 6.73 and 7.42. These were assigned as C*H*NOH *syn* and *anti* respectively after referring to spectral tables.[125] Similarly, two quartets were observed for CH_2CH, integrating to a value of 1 H, at δ_H 2.19 and 2.38. By comparing the ratio of *anti:syn* for the C*H*NOH signals of other oximes, which had different ratios of the two isomers, it was possible to assign these quartets as *anti* and *syn* respective to the values above.

$$CH_3(CH_2)_4-\underset{H}{C}=\underset{OH}{N}$$

91 *Syn*

$$CH_3(CH_2)_4-\underset{H}{C}=\overset{OH}{N}$$

91 *Anti*

In the ^{13}C spectra of these oximes, extra signals due to the methylene protons were observed in every case indicating that a mixture of isomers was present. Moreover, two signals at δ_C 152.84 and 153.42 for *C*HNOH could be assigned to the *anti* and *syn* isomers respectively. The order of these signals was reversed when compared with the proton spectrum which showed the *anti* isomer at highest frequency.

The oximes were then reacted with *N*-chlorosuccinimide in chloroform and pyridine at room temperature, for 2-3 hours, to form the corresponding oximyl chlorides **85**. Analysis by TLC (petroleum ether (bp 40-60 °C):ethyl acetate (4:1)) indicated the oxime had been consumed, and the reaction mixture was then washed with saturated sodium chloride solution and extracted with diethyl ether. Concentration under reduced pressure afforded chlorobutaldoxime **96**, chloropentaldoxime **97**, chlorohexaldoxime **98**, chloro-octaldoxime **99** and chlorodecaldoxime **100** as oils. The yields of these compounds were 100%, 83%, 88%, 88% and 76% respectively. Without exception, these compounds were not purified any further due to fears about their instability. Thus only a limited characterisation was performed for each compound. However, ^{1}H and ^{13}C NMR spectroscopy indicated in each case that the desired product had formed. The proton NMR spectra showed that the C*H*NOH resonance has been lost upon chlorination. In addition, the quartets due to the

adjacent methylene groups were replaced by a single multiplet at a higher ppm value. For example in the case of hexaldoxime **91**, chlorination saw the two triplets in the proton spectrum of hexaldoxime, due to C*H*NOH, both disappear as the hydrogen was replaced by a chlorine. Similarly, the two quartets previously found at δ_H 2.19 and 2.38 were replaced by a 2H multiplet at δ_H 2.50. In the ^{13}C NMR spectrum of chlorohexaldoxime **98** the C-1 resonance was found at 143.39 ppm compared with two signals at 152.84 and 153.42 ppm for the parent oxime. In these spectra only one set of peaks was observed in each case implying the presence of only one isomer

2.4 PREPARATION OF PROTECTED THIOHYDROXIMATES

In the preparation of protected propyl, pentyl, heptyl and nonyl thiohydroximates, equivalent quantities of the oximyl chloride **85** and thioglucopyranose **28** were dissolved in dry THF before dry triethylamine was added. The resulting solutions were then stirred at room temperature under a nitrogen atmosphere for 22-24 hours. The mixtures were subsequently washed with dilute acid and extracted with diethyl ether and ethyl acetate. This afforded the acetylated thiohydroximates **101**, **102**, **103** and **104** respectively (scheme 31).

AcO, AcO, AcO, AcO, O, SH **28** — R–C(Cl)=N-OH **85**, THF, Et_3N → AcO, AcO, AcO, AcO, O, S, N, OH, R

101 R = $CH_3(CH_2)_2$
102 R = $CH_3(CH_2)_4$
103 R = $CH_3(CH_2)_6$
104 R = $CH_3(CH_2)_8$

Scheme 31 Preparation of protected alkyl thiohydroximates.

Difficulties were encountered with the purification of these crude protected thiohydroximates due to the presence of unreacted thioglucopyranose. This contaminant

generally failed to allow the desired product to be recovered by trituration or recrystallisation even though the crude product was usually a solid. Instead, the thiohydroximates **101**, **102**, and **104** were obtained in microanalytical purity after flash chromatography eluting with a mixture of hexane and ethyl acetate (3:2). Conversely, the heptyl derivative **103** was recovered by recrystallisation from the above solvent system. The yields of these pure compounds were 36%, 47% and 69% for propyl, pentyl and heptyl thiohydroximates respectively.

It should be noted that only small amounts of **103** and **104** were purified so that these compounds could be fully characterised. Analysis by TLC indicated that only a small quantity of thioglucopyranose was present which could be removed at the next stage in the synthesis. This was indicated by microanalysis of crude **102** which gave percentage values of 51.11, 6.93 and 2.32% for C, H and N respectively compared with calculated values of 52.26, 6.98 and 2.77%. Therefore the protected heptyl and nonyl thiohydroximates were used without purification for the next stage.

The presence of excess thioglucopyranose, after coupling, was likely a consequence of over-estimating the purity of the oximyl chloride. This meant that less than one equivalent of this latter compound had actually been used. However, the stability of the thioglucopyranose, allowing it to be removed intact after column chromatography, was unexpected.

As an alternative to purification of **104** by removal of unreacted starting material, a small amount of the impure thiohydroximate was reacted with excess chlorodecaldoxime in the coupling reaction described previously. The product of this reaction was triturated in petroleum ether (bp 40-60 °C) at -78 °C to afford a yellow solid which was recrystallised from ethanol:water. Analysis of the recrystallised product by TLC showed only one spot indicating that the remaining thioglucopyranose had been successfully converted to the thiohydroximate.

The synthesis of tetra-acetyl-butyl thiohydroximate **105** used a 40% excess of oximyl chloride to avoid the removal of unreacted thioglucopyranose. Analysis by TLC showed no evidence for the latter compound upon completion of the reaction which afforded the protected butyl thiohydroximate in 49% yield after recrystallisation from ethyl acetate/hexane.

2.5 SYNTHESIS OF ALKYL THIOHYDROXIMATES

Small quantities of the acetylated thiohydroximates **102-105** were deprotected prior to sulfation to produce alkyl thiohydroximates which can be used to identify naturally occurring glucosinolates. Crude plant extracts containing glucosinolates can be analysed by liquid chromatography coupled with mass spectrometry (LC-MS) or HPLC and the individual glucosinolates identified.[39] To improve the analysis, the glucosinolates are desulfated, using the enzyme desulfatase, to produce the corresponding thiohydroximate. By having synthetically prepared authentic alkyl thiohydroximates it should be possible to positively identify the glucosinolates present by comparison of the retention times of these compounds with the crude plant extracts.

The protected alkyl thiohydroximates were dissolved in dry methanol and a catalytic quantity of either sodium or potassium methoxide was added until the pH reached a value of 9 or 10. The resulting solutions were stirred, under a nitrogen atmosphere either overnight or for 18-20 hours before Amberlite IR-120 was added. Removal of the Amberlite and concentration under reduced pressure afforded crude butyl **106**, pentyl **107**, heptyl **108** and nonyl thiohydroximates **109** (scheme 32).

Scheme 32 Synthesis of thiohydroximates.

Initially nonyl thiohydroximate **109** was purified using a C-18 (900 mg) 'Sep-pak.' Using this method, compounds with long hydrophobic side chains are retained on the stationary phase of the cartridge while the impurities are washed through i.e. the crude nonyl thiohydroximate was applied as an aqueous solution allowing the compound to adhere to the reverse-phase silica. Addition of methanol then allowed removal of the compound which was concentrated under reduced pressure to afford the microanalytically pure product, as a white solid, in 35% yield. Analysis of this compound by HPLC at SCRI showed only one signal, due to the product, at 23.22 minutes. This demonstrated that the Sep-pak had been effective and that the thiohydroximate could be used for further analyses. However, Sep-pak purification of nonyl thiohydroximate was found to be irreproducible, possibly due to problems with sample overloading. In addition, the purification of heptyl glucosinolate was attempted using the Sep-pak leading to the production of a colourless oil which was also impure.

However, butyl thiohydroximate **106** was prepared and readily purified using silica chromatography, eluting with ethyl acetate. This afforded the pure compound with a yield of 78%. Thus the purification of heptyl **108** and nonyl thiohydroximates **109** was attempted using this method. The first attempt failed to afford nonyl thiohydroximate **109** in a significant quantity but when repeated, this compound was recovered with microanalytical purity in 40% yield. Heptyl thiohydroximate **108** was also recovered as a

white foam with a yield of 47% and pentyl thiohydroximate **107** was obtained similarly, in 93% yield.

Analysis of butyl, pentyl, heptyl and nonyl thiohydroximates by HPLC at the Institute of Arable Crop Research (IACR) allowed the standard retention times of these compounds to be obtained (table 5).

Thiohydroximate	**Retention time (minutes)**	
	Analysis 1	Analysis 2
Butyl	16.495	16.405
Pentyl	20.287	20.277
Heptyl	27.833	27.827
Nonyl	34.272	34.240

Table 5 HPLC retention times of alkyl thiohydroximates measured at the IARC (Waters 600 LC system with photodiode array detection (200-300 nm) and a 4.6 x 250 mm C_{18} column using a water-acetonitrile mobile phase).

These were then compared with a number of plant extracts thought to contain alkyl glucosinolates. One of these extracts was from the radish (*Raphanus sativus* L.) which has been reported to contain pentyl and hexyl glucosinolates, as determined by GC/MS analysis.[9] The extracts of two different plant parts from a 38 day old plant, grown in compost, were examined to determine the glucosinolates present. An HPLC chromatogram showing the glucosinolates contained in leaf 8 and the leaf primordia is given in figure 5. This spectrum shows many extremely small peaks which are difficult to positively assign to glucosinolates without authentic compounds. By comparing the retention times of the alkyl thiohydroximates in table 5 and the retention times of the plant extract components, it was possible to conclude that these thiohydroximates were not contained in the radish at a level detectable by HPLC. However, HPLC analysis of other plant extracts in the future may

show alkyl thiohydroximates which could be identified by reference to the authentic compounds given previously.

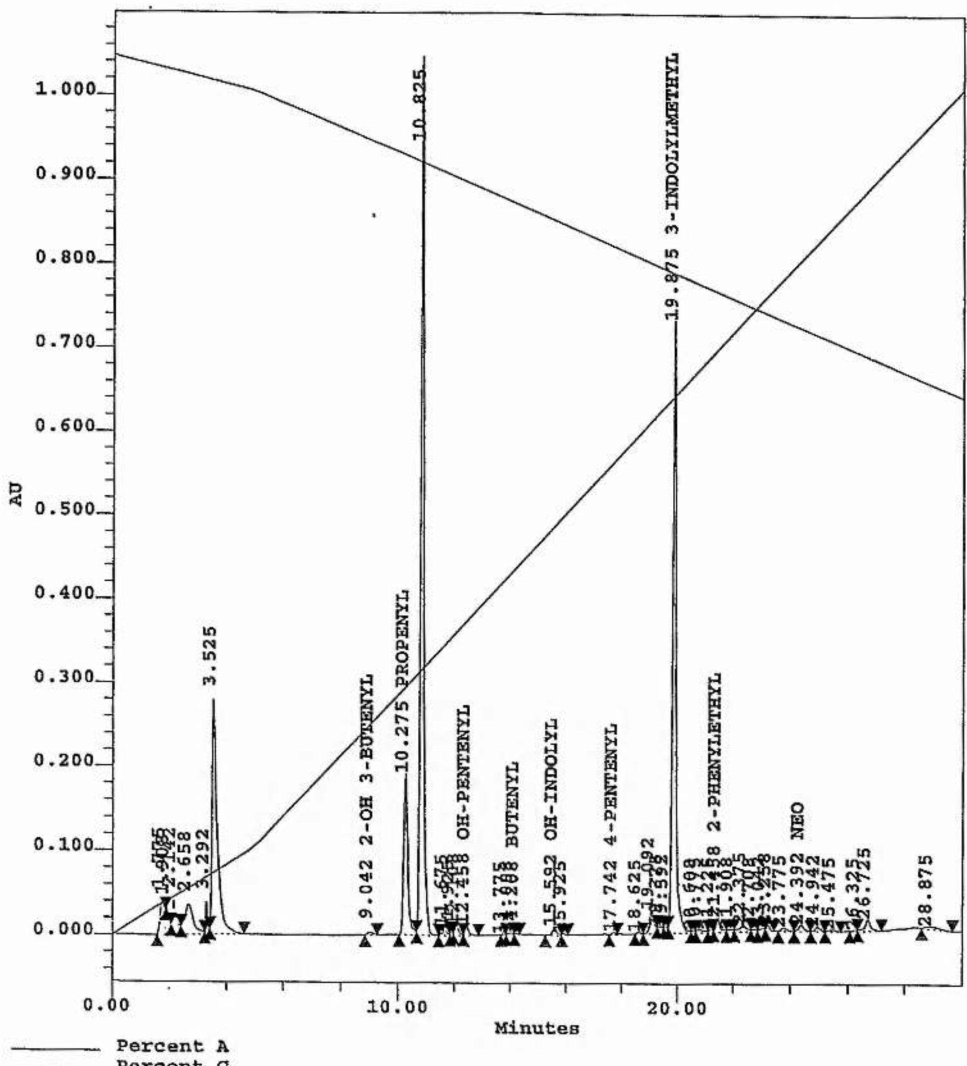


Figure 5 HPLC chromatogram showing glucosinolate composition of the radish (leaf 8 and primordia).

Butyl thiohydroximate was also sent to the Scottish Crop Research Institute (SCRI) and was analysed by LC-MS. In addition, this compound was used to spike a crude extract taken from the surface of the turnip leaf (cultivar *massif*) to determine if one of the components was butyl glucosinolate. This analysis showed the two species to be similar but not identical (figure 6).

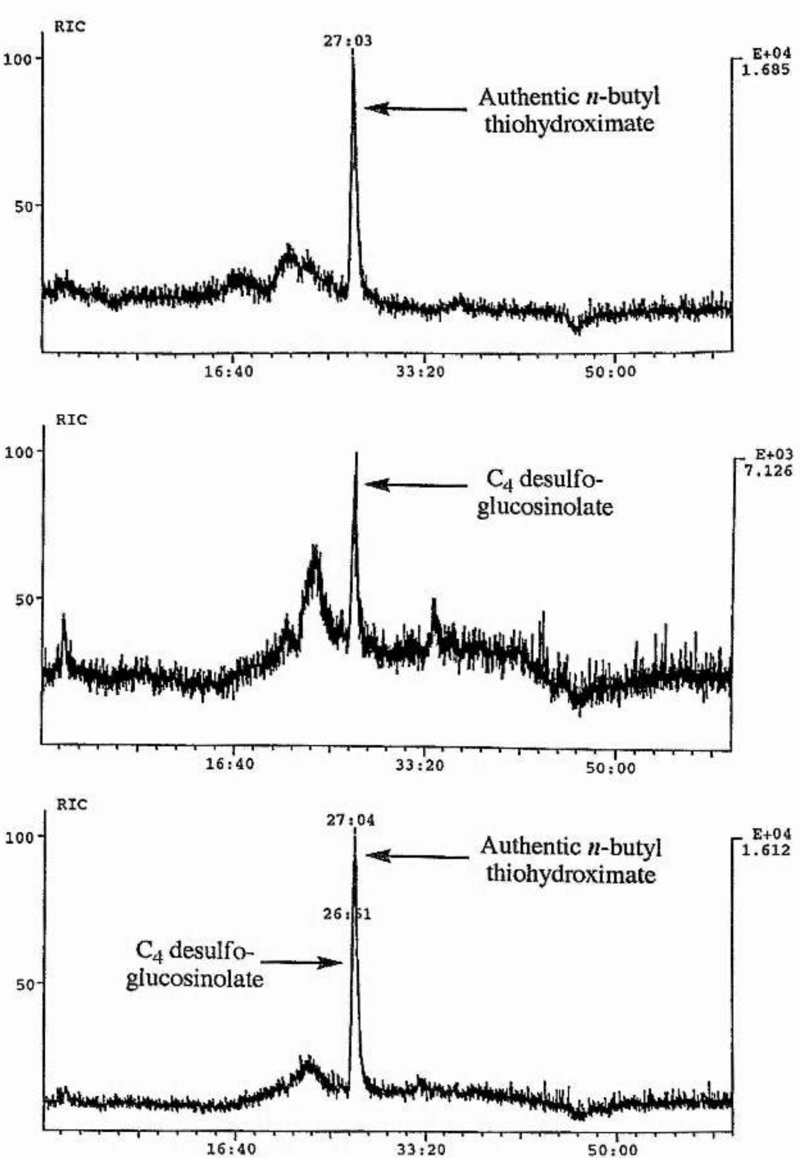


Figure 6 LC-MS chromatogram of the naturally occurring desulfo-glucosinolate spiked with butyl thiohydroximate.

Given that the signals for the desulfo-glucosinolate and butyl thiohydroximate did not overlap exactly it could be postulated that the naturally occurring compound was not the *n*-butyl analogue. However, as the molecular ion was known to be 296, the naturally occurring compound must be an isomer of the *n*-butyl thiohydroximate. To elucidate the structure of this glucosinolate, the other possible isomers, 1-methylpropyl **110** and 2-

methylpropyl thiohydroximate **111**, were synthesised. Indeed, 1-methylpropyl and 2-methylpropyl glucosinolates have been reported to be naturally occurring, thus it was very likely one of these would correlate with the unknown butyl glucosinolate in the plant extract.[2]

110 **111**

The aldehydes, 2-methylbutyraldehyde and 3-methylbutyraldehyde, were treated with hydroxylamine hydrochloride, as before, to afford the corresponding 2- and 3-methylbutyraldoximes, **112** and **113**, in 82% and 72% yield respectively. Subsequent chlorination with *N*-chlorosuccinimide proceeded with surprising results. During previous chlorinations, the reaction solution was almost colourless, perhaps with a faint trace of yellow. Interestingly, in the case of 2-methylbutyraldoxime **112**, the reaction solution turned bright blue after addition of *N*-chlorosuccinimide and upon warming to room temperature. Moreover, a similar phenomenon was witnessed for 3-methylbutyraldoxime **113** except this time a green colour was observed. These colours faded upon concentration under reduced pressure to yield the oximyl chlorides, **114** and **115**, as yellow-coloured oils in 64% and 67% yield respectively for the 2- and 3-methyl derivatives.

Coupling of 1-chloro-2-methylbutyraldoxime **114** with thioglucopyranose afforded the protected thiohydroximate **116** in 76% yield. Analysis of the compound by ^{1}H and ^{13}C NMR, showed a mixture of diastereoisomers as evidenced by doubling of all the signals. For example, two triplets were observed for each methyl group in the side chain and so on. Conversely, the coupling of 1-chloro-3-methylbutyraldoxime **115** with thioglucopyranose did not proceed with such ease, with a yield of only 3% being obtained for the thiohydroximate **117**. Initially a yellow-coloured foam was recovered which was purified

by flash chromatography to afford a sticky white solid. This required recrystallisation to produce the pure compound. The reason for the low yield is unknown although this compound failed to be recovered from three previous attempts at the coupling reaction. It is also unclear whether the coupling reaction failed or the product decomposed upon silica chromatography, which did afford some thioglucopyranose. However, given that no other protected thiohydroximate has decomposed on silica, the decomposition of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-2-methylpropyl thiohydroximate **117** is unlikely. This could suggest that having a branched side chain imparts some steric hindrance which may adversely affect coupling of the oximyl chloride with thioglucopyranose.

Deacetylation of the protected thiohydroximates was carried out as before, using potassium methoxide, and the crude products were purified by column chromatography. This afforded β-D-glucopyranosyl-1-methylpropyl thiohydroximate **110** as a cream-coloured foam, in 82% yield, and β-D-glucopyranosyl-2-methylpropyl thiohydroximate **111** as an off-white powder, in 51% yield.

These hygroscopic compounds were analysed by HPLC at the SCRI in addition to the previous *n*-butyl analogue. It was found that the *n*-butyl and 1-methylpropyl thiohydroximates co-eluted after 21.11 minutes and could not be separated by alteration of the analysis conditions. Conversely, 2-methylpropyl thiohydroximate showed a significantly different retention time of 19.22 minutes (figure 7). To determine the identity of the unknown C_4 glucosinolate present in the turnip leaf extract, which gave a retention time of 20.30 minutes (figure 8), this extract was spiked with a mixture of *n*-butyl and 1-methylpropyl thiohydroximates (1:1). This produced an increase in the size of the peak attributed to the C_4 glucosinolate indicating that one or both of these compounds was present in the turnip leaf extract (figure 9).

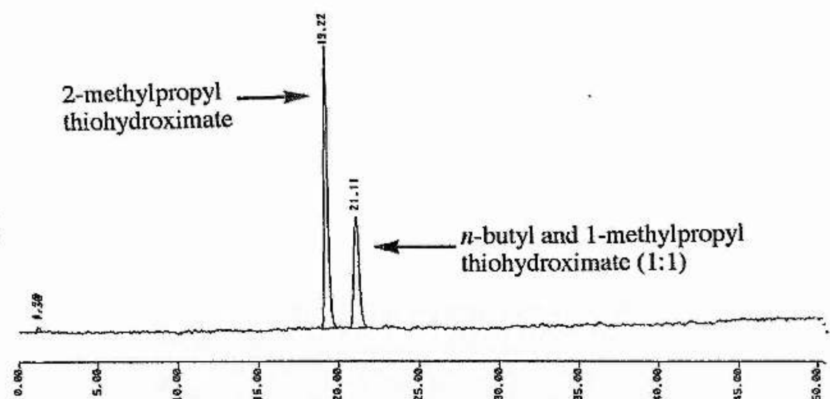


Figure 7 HPLC chromatogram showing the retention times of *n*-butyl, 1-methylpropyl and 2-methylpropyl thiohydroximates (Gilson dual pump HPLC system with UV detector (230 nm), a 4.6 x 250 mm C_{18} column, and a water-acetonitrile mobile phase).

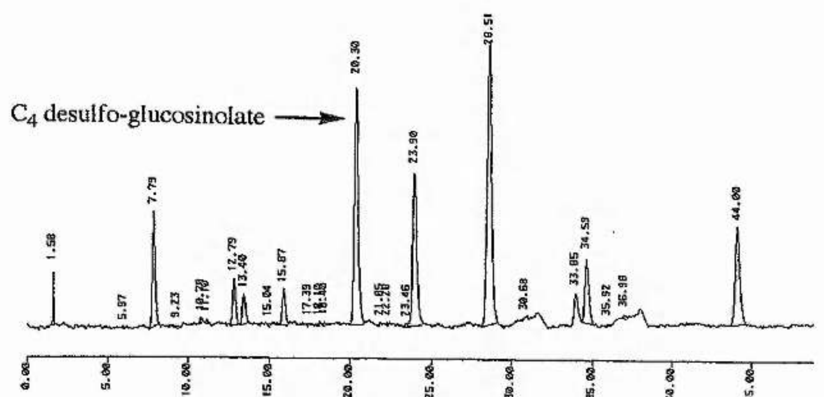


Figure 8 HPLC chromatogram of the turnip leaf extract (cultivar *massif*).

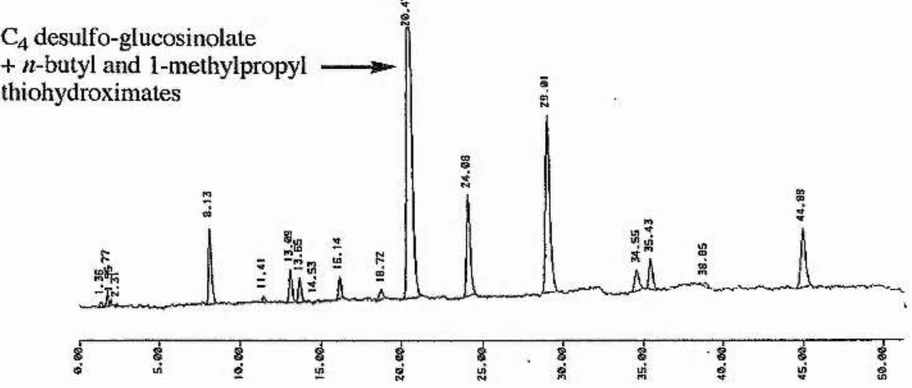


Figure 9 HPLC chromatogram of the turnip leaf extract spiked with *n*-butyl and 1-methylpropyl thiohydroximate.

Unfortunately, no further information could be gathered using HPLC as the authentic compounds were inseparable. Similary LC-MS could not be used as both compounds have the same molecular weight and would likely co-elute as in HPLC. However, while working on a parallel project using GC/MS analysis, Matthew Ball identified a C_4 isothiocyanate arising from myrosinase catalysed hydrolysis of turnip root powder from the same turnip (cultivar *massif*). Analysis of an authentic sample of *n*-butyl isothiocyanate by GC/MS showed the two species to be dissimilar and it was concluded that the C_4 isothiocyanate present had a branched side chain. This presence of 1-methylpropyl isothiocyanate was confirmed by the mass spectrum of the C_4 isothiocyanate (figure 10) which showed a signal at *m/z* 86 corresponding with ($[M-CH_2CH_3]^+$). This fragmentation would only be possible from the 1-methylpropyl isothiocyanate and not the 2-methylpropyl derivative. It could thus be implied that as the 1-methylpropyl isothiocyanate was the only C_4 isothiocyanate released from the root, then the C_4 glucosinolate in the leaf is likely to be the 1-methylpropyl derivative.

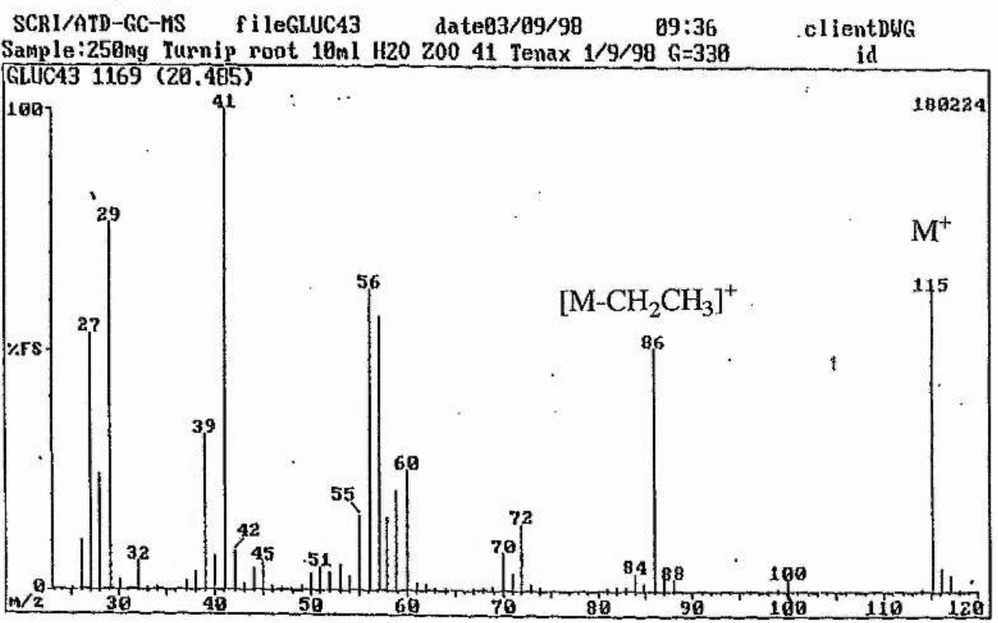


Figure 10 Mass spectrum of the C_4 isothiocyanate identified as 1-methylpropyl isothiocyanate.

2.6 PREPARATION OF PROTECTED GLUCOSINOLATES

The next stage in the preparation of glucosinolates was sulfation of the protected thiohydroximates as shown in scheme 33.

101 R = $CH_3(CH_2)_2$
102 R = $CH_3(CH_2)_4$
103 R = $CH_3(CH_2)_6$
104 R = $CH_3(CH_2)_8$
105 R = $CH_3(CH_2)_3$

118 R = $CH_3(CH_2)_2$
119 R = $CH_3(CH_2)_4$
120 R = $CH_3(CH_2)_6$
121 R = $CH_3(CH_2)_8$
122 R = $CH_3(CH_2)_3$

Scheme 33 Sulfation of acetylated thiohydroximates.

The pyridine-sulfur trioxide complex, used to produce the protected glucosinolates, was generated *in situ* from reaction of pyridine with chlorosulfonic acid at 0 °C. The preformed complex is commercially available but tends to be very hygroscopic and is hydrolysed to give pyridinium hydrogen sulfate. This salt is unreactive as a sulfating agent hence the need to prepare the complex prior to use. However, large excesses of potassium hydrogen carbonate were required to decompose the complex. In the synthesis of 2,3,4,6-tetra-*O*-acetyl-heptyl glucosinolate **120**, a significant quantity of the pyridine-sulfur trioxide complex remained even after the addition of 6.5 molar equivalents of potassium hydrogen carbonate. Increasing the molar equivalency to 19.5 equivalents led to destruction of this complex allowing the product to be purified by flash chromatography, using a mixture of petroleum ether: ethyl acetate (1:1) with up to 10% (v/v) methanol. This afforded micro-analytically pure protected heptyl glucosinolate **120** in 43% yield. The compound was characterised against the initial thiohydroximate **103** and it was found that the chemicals shifts were slightly higher in every case when compared with the starting material. In particular, the 2 H multiplet for $CH_2C{=}N$ of the thiohydroximate, found at δ_H 2.47, became

a triplet at δ_H 2.66 in the sulfated compound. In addition, the signal for *C*=N changed from δ_C 151.66 in the thiohydroximate to δ_C 159.94 after sulfation. The formation of the protected glucosinolate was also confirmed by negative ion electrospray mass spectrometry (ES^-) giving a molecular ion of 584 corresponding with $[(M-K)^-]$. This technique proved invaluable for confirming the synthesis of protected and free glucosinolates.

In the synthesis of the butyl analogue **122**, the sulfation was performed as before with 30 molar equivalents of potassium hydrogen carbonate being added to decompose the pyridine-sulfur trioxide complex. However, this time the two-phase system was concentrated under reduced pressure as soon as CO_2 evolution ceased. This allowed removal of the dichloromethane/pyridine organic layer and precipitation of the protected butyl glucosinolate **122** as a white solid in 73% yield. This was subsequently recrystallised from methanol to afford the pure product. Using this procedure, tetra-*O*-acetyl-pentyl glucosinolate **119** was prepared with a yield of 72%. Upon removal of the organic layer under reduced pressure, diethyl ether was added resulting in the appearance of a white solid. This was identified as the protected pentyl glucosinolate by electrospray mass spectrometry and NMR spectroscopy. Moreover, the protected propyl glucosinolate **118** was prepared similarly in 59% yield.

Difficulties were experienced with the sulfation of the protected nonyl thiohydroximate **104** to afford the corresponding protected glucosinolate **121**. Initial attempts produced a mixture of products by TLC with NMR analysis indicating that unreacted starting material was present. Unfortunately this could not be separated from the product by column chromatography and the sulfation reaction was repeated. When the two-phase system was concentrated directly, a precipitate was recovered which was found to contain a small quantity of the desired product. This enabled a limited characterisation of tetra-*O*-acetyl nonyl glucosinolate **121** to be performed but was insufficient to afford the desired quantity of glucosinolate upon deprotection. Hence, the sulfation reaction was attempted for a third time. Again, a precipitate was recovered upon concentration under reduced pressure which

failed to recrystallise from methanol in an analogous manner to the butyl analogue **122**. Analysis of the solid by negative ion electrospray mass spectrometry showed a molecular ion at 444 which corresponded with nonyl glucosinolate **82**. Thus deprotection had occurred in addition to sulfation in this step. This indicates that base-catalysed hydrolysis brought about by excess hydrogen carbonate had occurred.

2.7 SYNTHESIS OF ALKYL GLUCOSINOLATES

The acetylated propyl **118**, butyl **122**, pentyl **119** and heptyl glucosinolates **120** were all deprotected by the same procedure. Each compound was dissolved in dry methanol and a catalytic amount of potassium methoxide was added. The resulting solutions were stirred under dry conditions overnight, or for a period of 18-20 hours. Concentration under reduced pressure afforded the propyl **78**, butyl **79**, pentyl **80** and heptyl **81** glucosinolates, as hygroscopic foams, all having crude yields of 100%. These compounds were identified as being glucosinolates by NMR and negative ion electrospray mass spectrometry, the latter showing the mass of the compound corresponding to $[(M\text{-}K)^-]$. Proton NMR showed that the acetyl protecting groups had been successfully removed due to the disappearance of the signals due to CH_3COO at 1.98-2.07 ppm. Moreover, the sequence of the carbohydrate signals in the proton spectrum changed upon deacetylation i.e. the signal at highest frequency in the glucosinolate was the anomeric proton instead of the signal due to H-3 being at highest frequency in the protected compound. In addition, the acetate groups disappeared from the ^{13}C spectrum and the signal due to *C*=N moved to a higher chemical shift e.g. from 159.94 ppm to 162.66 ppm in the case of heptyl glucosinolate.

2.8 PURIFICATION OF GLUCOSINOLATES

All of the glucosinolates synthesised were isolated as cream/brown-coloured foams and it was anticipated these would require purification. However, this was found to be extremely challenging.

Initially, as with the alkyl thiohydroximates, it was thought that purification by reverse-phase chromatography in the form of a "Sep-pak" would afford the microanalytically pure product. This theory was reinforced by TLC analysis of propyl and pentyl glucosinolates using C_{18} reverse phase TLC plates which effectively mimic the Sep-pak environment. When water was used as the solvent, two spots were visualised by UV irradiation. The first appeared below the solvent front and was thought to be due to impurities or perhaps any desulfo-glucosinolate. The second was found on the baseline and this moved up the plate when methanol was used as a solvent. This suggested that it should be possible to add the glucosinolate to the cartridge in water, meaning it would adhere but the impurities would not, and then remove the glucosinolate using methanol.

This type of purification was attempted for small quantities (10-60 mg) of propyl, pentyl and heptyl glucosinolates using either "extract clean high load" C_{18} cartridges (2000 mg) from Alltech Associates or "Waters sep-pak plus" C_{18} cartridges. In every case only minute quantities of glucosinolates were recovered upon methanol elution with most of the glucosinolate passing straight through the cartridge with the impurities and the water. It can therefore be concluded that the glucosinolates, although having a hydrophobic alkyl chain, are more hydrophilic than hydrophobic due to the presence of four hydroxyl groups and an ionic sulfate group. This means they have a greater affinity with water rather than with the C_{18} solid support and therefore did not adhere to the cartridge as initially postulated.

The next option was to purify the compounds using chromatography on silica gel. This was not the first option as, being very polar, the glucosinolates could only be removed from the column using methanol in combination with another solvent. This would lead to

dissolution of silica gel from the column which would contaminate the glucosinolate. However, as this contamination would be small, it would not affect the compound testing and this method would allow removal of more undesirable impurities. Therefore silica chromatography was attempted for butyl glucosinolate eluting with methanol and ethyl acetate (1:2.5). This afforded the compound in 63% yield as a white foam. Given the success achieved with this compound, the remaining quantity of pentyl glucosinolate was treated similarly eluting with methanol and ethyl acetate (1:4). Unfortunately no glucosinolate was recovered and the pentyl derivative had to be remade from the aldehyde.

Hanley and co-workers,[126] while reporting an improved isolation of glucobrassicin and some additional glucosinolates, gave a procedure for purifying glucosinolates by ion-exchange chromatography. To test their procedure, the purification of a standard sample of sinigrin (R = propenyl) was attempted.

The first step in the procedure was to apply the glucosinolate to a column of DEAE Sephadex which had been pre-swollen in water and washed with ammonium bicarbonate. This column should allow the ionic glucosinolate to be retained and any non-ionic, desulfated material to pass through or be removed separately on subsequent elutions. Sinigrin was added as an aqueous solution and the column washed first with water, then 0.1, 0.5, 1 and 2 M solutions of ammonium acetate. These elutions were all freeze-dried separately and analysed by NMR spectroscopy and TLC. This showed the presence of sinigrin in the 1 M and 2 M elutions.

The aqueous sinigrin was then passed through a column containing G-10 Sephadex which should allow separation of the glucosinolate and ammonium acetate due to the principles of size-exclusion chromatography. The product of this column was again freeze-dried and analysed by proton NMR. This suggested that only half of the ammonium acetate had been removed and therefore the above procedure was repeated. Analysis by NMR indicated that approximately half of the previous quantity of ammonium acetate had been removed

meaning that several G-10 columns may have to be used in series for the purification of alkyl glucosinolates.

Finally sinigrin was passed through a cation exchange column to restore the potassium counter-ion. This consisted of Amberlite IR-120, washed previously with potassium hydroxide and then with copious amounts of distilled water until the pH was neutral. Sinigrin was added as an aqueous solution and washed off with distilled water. The eluants were freeze-dried and analysed as before. It was then concluded that sinigrin had indeed survived the purification procedure and that this method could be used to purify alkyl glucosinolates.

Nonyl glucosinolate (200 mg) was eluted from DEAE Sephadex using 0.1 M and 0.5 M ammonium acetate, freeze dried, redissolved in water and passed sequentially through two G-10 Sephadex columns. Analysis by NMR, after freeze drying, showed that only half of the ammonium acetate had been removed and that the size-exclusion chromatography was not working effectively. As an alternative, the nonyl glucosinolate sample was dissolved in a mixture of *iso*-propanol, ammonia and water (26:6:5) and passed through cellulose which had been primed with the same solvent. The *iso*-propanol was removed under reduced pressure prior to freeze-drying. Analysis by NMR indicated this procedure had removed a significant quantity of the ammonium acetate with only a small amount remaining. This procedure was repeated leading to a further reduction in the quantity of ammonium acetate but not complete removal of this salt.

Next, the sample was passed through the cationic Amberlite column, freeze-dried and analysed by NMR and microanalysis. Only a quarter of the glucosinolate had been recovered with NMR indicating trace amounts of ammonium acetate to be present and microanalysis failing to show good agreement with the theoretical composition. To determine if this was due to the nature of the compound or the purity of the compound, a standard sample of sinigrin was analysed. This was found to be microanalytically pure indicating the purity of the nonyl glucosinolate was the problem.

It was suggested that conversion of the glucosinolate from the potassium salt to the tetra-methylammonium salt would enhance the crystallinity of the glucosinolate meaning it could be recrystallised from aqueous propan-1-ol. This was attempted for sinigrin which was passed through a column containing Amberlite IR-120 treated with tetra-methylammonium hydroxide. The salt appeared to form readily but failed to recrystallise from the suggested solvent. Hence this procedure was not attempted for the purification of nonyl glucosinolate.

The purification procedure was repeated for the same quantity of nonyl glucosinolate except the G-10 purification was preceded by two cellulose columns. Analysis by NMR showed that a large quantity of ammonium acetate remained. This suggested that the method of purification was not terribly reproducible, given that only a minute amount of acetate remained after the last purification, and also that the glucosinolate was being contaminated by the entire procedure rather than being cleaned. Therefore ion-exchange chromatography was abandoned.

The other glucosinolates, propyl, pentyl and heptyl, were then compared by TLC with their thiohydroximate analogues, and in each case there was no evidence for the presence of any desulfo-glucosinolate. It was therefore determined that these compounds would be used in their present state for testing as no suitable purification method had been found. Indeed, heptyl glucosinolate was found to be microanalytically pure. Impurities in propyl and pentyl glucosinolates were attributed to the salt potassium methoxide as no other compounds could be observed by NMR analysis or mass spectrometry. Thus these compounds were tested in this form using microanalysis to provide an estimate of their purity. In addition, the remaining nonyl glucosinolate was analysed by TLC and was found to contain several impurities. Thus silica chromatography was employed eluting with ethyl acetate then ethyl acetate and methanol (4:1). In the first instance no nonyl glucosinolate was recovered but two further attempts allowed the desired compound to be obtained in microanalytical purity.

2.9 CONCLUSIONS

The alkyl glucosinolates, propyl, butyl, pentyl, heptyl and nonyl glucosinolates, were prepared in eight steps beginning from the corresponding aldehydes and β-D-glucose. These syntheses proceeded similarly with slight variations being observed between the different compounds, especially for nonyl glucosinolate which did not require potassium methoxide deprotection. The glucosinolates were invariably obtained as hygroscopic foams which proved difficult to purify without further contamination by excess salts. Nevertheless butyl, heptyl and nonyl glucosinolates were obtained in microanalytical purity with propyl and pentyl containing small quantities of potassium methoxide. The glucosinolate synthesis also facilitated the preparation of butyl, pentyl, heptyl and nonyl thiohydroximates for use as HPLC standards. Unfortunately, initial attempts to identify these glucosinolates in radish extracts (*R. sativus*) at IACR failed due to low concentrations of these compounds in the extract. Butyl thiohydroximate was also analysed against turnip leaf surface extracts (cultivar *massif*) by LC-MS at the SCRI. This compound did not match exactly with a suspected butyl derivative and 1-methylpropyl and 2-methylpropyl thiohydroximates were thus prepared for further analysis by HPLC. The 2-methylpropyl derivative was shown to be dissimilar to the unknown C_4 glucosinolate with both the 1-methylpropyl thiohydroximate and the *n*-butyl analogue co-eluting with the unknown. Parallel experiments on the identification of volatile compounds from myrosinase catalysed hydrolysis of turnip root powder showed the presence of 1-methylpropyl isothiocyanate as the only C_4 compound. It was thus concluded that the glucosinolate was most likely to be the 1-methylpropyl derivative. This work highlights the difficulties associated with glucosinolate identification from crude plant extracts and the necessity to use a number of analytical techniques. However, the thiohydroximates prepared may prove to be useful standards for future analyses.

CHAPTER 3

SYNTHESIS OF COMPLEX GLUCOSINOLATES

3.1 SYNTHESIS OF NAPHTHYLMETHYL GLUCOSINOLATE

It was envisaged that the target molecule **83** could be synthesised in a similar manner to the alkyl glucosinolates discussed previously in chapter 2. This would involve coupling of preformed oximyl chloride and thioglucopyranose moieties followed by sulfation and deprotection to afford the glucosinolate. Thus the synthesis would begin similarly by oximation of the corresponding aldehyde, 1-naphthylacetaldehyde **123**.

HO, HO, HO, HO, O, S, N, $OSO_3^-K^+$ — **83** ⟹ H, O — **123**

However, 1-naphthylacetaldehyde is not commercially available and had to be synthesised thus adding extra steps to the glucosinolate synthesis.

Initially, it was thought that reduction of 1-naphthylacetonitrile **124** using Raney nickel would afford the desired aldehyde (scheme 34).[127] The nitrile was added to aluminium/nickel alloy suspended in 75% aqueous formic acid and the resulting solution was heated under reflux for either 30 minutes or 2 hours. Unfortunately analysis of the crude product by proton NMR showed no evidence that the desired aldehyde had formed.

NC — **124** --- i ---> H, O — **123**

Scheme 34 *Reagents and conditions:* i, Al/Ni alloy, 75% aq. HCO_2H, reflux.

Following the failure to obtain 1-naphthylacetaldehyde by reduction, the oxidation of 1-naphthaleneethanol **125** was attempted as an alternative route to the aldehyde (scheme 35).

HO
H
O
Oxidation
125
123

Scheme 35 Oxidation of 1-naphthaleneethanol.

This oxidation had been carried out previously using reagents such as chromic acid in benzene, toluene and acetic acid, lead dioxide/sulfuric acid and also lead tetraacetate.[128] Unfortunately these methods generally failed to afford 1-naphthylacetaldehyde **123** in significant quantities with the product of the reaction being the over oxidised product, 1-naphthaldehyde **126**.

H
O
126

However, oxidation using Swern conditions had not been reported and it was hoped that these milder conditions would yield the desired acetaldehyde exclusively. However, a mixture of the starting material and the desired product, 1-naphthylacetaldehyde **123**, were formed along with other side products.

Oxidation of 1-naphthaleneethanol was next attempted using pyridinium chlorochromate (PCC) to determine if any improvement could be found over the Swern oxidation and previous chromic acid oxidations. The reaction was carried out three times with each attempt varying either in the time period allowed for reaction or the molar equivalencies of starting

material and PCC. In every case three compounds could be observed by NMR analysis. These were the starting alcohol **125**, the desired acetaldehyde **123** and also 1-naphthaldehyde **126** but in varying quantities (table 6). The aldehydes were easily identified from the proton NMR spectrum by the signal for the aldehydic proton (C*H*O). 1-Naphthaldehyde **126** gave rise to a singlet at 10.41 ppm whereas the acetaldehyde **123** gave rise to a triplet at 9.79 ppm, due to coupling with the adjacent methylene group. The alcohol was identified by comparison with an authentic spectrum which displayed two triplets at 3.36 and 3.98 ppm due to CH_2CH_2OH.

Reaction No.	Molar equivalency of PCC	Stirring Time	Percentage aldehyde 126	Percentage acetaldehyde 123	Percentage alcohol 125
1	1.5	2 hours	9.8	63.7	26.5
2	1.5	overnight	19.8	69.4	10.7
3	2.0	overnight	28.4	65.3	6.3

Table 6 PCC oxidations of 1-naphthaleneethanol.

It can be concluded from the data that as the time and the amount of PCC increased the amount of starting alcohol decreased. At the same time the amount of the aldehyde **126** increased. Given that the aldehyde **126** and acetaldehyde **123** only differ by a methylene group it is likely that these would be difficult to separate and purify. Conversely it would be easier to separate the acetaldehyde and alcohol meaning the conditions for reaction 1 would be more conducive to recovery of the acetaldehyde. The results also indicated that PCC is a more efficient reagent for the conversion of 1-naphthaleneethanol to the corresponding acetaldehyde than chromic acid, as the acetaldehyde **123** was produced preferentially to the aldehyde **126**.

Before proceeding with this method, one other route to 1-naphthylacetaldehyde was attempted involving reduction of the corresponding ester. 1-Naphthylacetic acid **127** was converted to its ethyl ester **128** in quantitative yield by heating under reflux with dry ethanol and thionyl chloride (scheme 36). The ethyl ester **128** was then reduced smoothly to 1-naphthylacetaldehyde **123**, in 94% yield, using di*iso*-butyl aluminium hydride (DIBAL-H).

Scheme 36 *Reagents and conditions*: i, Dry EtOH, $SOCl_2$, reflux, 3.5 h, 100%; ii, DIBAL-H (1.5 M), toluene, -78 °C, 2 h; iii, MeOH, -78 °C to 20 °C, potassium sodium tartrate, 94%.

Although this route required two steps to produce 1-naphthylacetaldehyde **123**, both steps were efficient and the aldehyde did not require separation from other undesirable side products. Thus this was the route chosen to prepare 1-naphthylacetaldehyde for the synthesis of naphthylmethyl glucosinolate (scheme 37).

The aldehyde **123** was used without purification for the preparation of 1-naphthylacetaldoxime **129**. This was effected, as before, by refluxing with hydroxylamine hydrochloride in a mixture of ethanol and pyridine. Addition of water to the crude product afforded the oxime as a yellow solid which was recrystallised from ethanol to afford the product as a white solid in 52% yield. Once again a mixture of *cis* and *trans* geometric isomers was observed by proton NMR analysis.

Scheme 37 scheme diagram (compounds 123, 129, 130, 28, 131, 83)

Scheme 37 *Reagents and conditions*: i, $NH_2OH.HCl$, EtOH, pyridine, reflux; ii, *N*-chlorosuccinimide, $CHCl_3$, pyridine; iii, THF, Et_3N; iv, $ClSO_3H$, pyridine, DCM; $KHCO_3$(aq); v, K, MeOH.

Reaction of the oxime **129** with *N*-chlorosuccinimide failed to allow complete conversion to the oximyl chloride **130**. The proton NMR spectrum, in addition to showing a singlet at 4.24 ppm due to the methylene group of the oximyl chloride, showed a doublet at 4.06 ppm which was reminiscent of the oxime. Integration indicated that approximately a third of the oxime remained compared with the oximyl chloride. This could be attributed to steric interactions between the naphthyl group and the chlorine, the latter being much larger than its hydrogen predecessor. However, this mixture of oxime and oximyl chloride was not separated, due to fears about the stability of the latter compound, and was coupled as before to 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose **28** in the presence of dry THF and triethylamine. The desired thiohydroximate **131** was recovered as a white solid in 87%

yield after recrystallisation from ethanol. Given the magnitude of this yield it is possible the oxime reacted in addition to the oximyl chloride under the basic conditions of the reaction.

As a precaution, sulfation of the thiohydroximate to afford the protected glucosinolate was carried out in two batches In the first attempt, removal of the organic layer from the biphasic system led to the formation of a brown solid, which was shown by TLC to contain a small quantity of unsulfated starting material. This was easily removed by column chromatography where the unsulfated material was washed off the column using an equal mixture of ethyl acetate and petroleum ether, followed by elution of the protected glucosinolate using a mixture of ethyl acetate and methanol (4:1). This afforded 2,3,4,6-tetra-*O*-acetyl-1-naphthylmethyl glucosinolate **132** as a white solid in 18% yield. This reaction was significantly less efficient than analogous reactions carried out for alkyl thiohydroximates which typically showed yields in the range 43-86%. Again this may be due to the size of the glucosinolate side chain. Given the problems with sulfation of the protected nonyl thiohydroximate and the low yield for the heptyl derivative (43%) it is perhaps not unlikely that an even larger chain resulted in a lower yield.

When attempting this reaction for the second time, a beige-coloured solid was obtained upon removal of the organic layer under reduced pressure. Analysis by negative ion electrospray mass spectrometry indicated the target molecule **83** was present meaning that deacetylation had occurred upon sulfation. Purification by flash chromatography afforded naphthylmethyl glucosinolate **83** in 33% yield. Although the compound showed only one spot by TLC analysis, it was not found to be microanalytically pure thus a further batch was prepared by deprotection of the acetylated glucosinolate **132**. This compound was treated with potassium methoxide in the same manner as the alkyl glucosinolates and purified by flash chromatography to afford the microanalytically pure product **83** in 53% yield. Both batches of naphthymethyl glucosinolate were tested to determine their stimulatory activity with the difference in purity providing a useful comparison.

3.2 STUDIES TOWARDS THE SYNTHESIS OF ACIDIC GLUCOSINOLATES

Several strategies can be envisaged for the preparation of glucosinolates bearing a carboxylic acid functionality as shown below:

In these syntheses the challenge is the production of the corresponding, acid containing, oximyl chloride moiety. The possible routes which could be employed for preparation of this fragment are discussed hereafter.

The first transformation considered was conversion of the corresponding primary nitro compound directly to the oxime using a procedure reported by Johnson and Degering (scheme 38).[129] As they reported for 1-nitropropane, the starting material is added to glacial acetic acid and heated under reflux in the presence of zinc dust to afford the oxime.

$$R{-}CH_2NO_2 \xrightarrow{\text{i}} R{-}\underset{\displaystyle H}{C}{=}N{-}OH$$

Scheme 38 *Reagents and conditions*: i, Zn, glacial acetic acid, reflux, 6 h.

The desired nitro-acid should be easily prepared from the analogous bromo-acid by first protecting the acid functionality and then treating with sodium nitrite (scheme 39) as described by Kornblum *et al.*[130] The bromo-acids are available in a variety of chain lengths

and as such should provide an easy and cost effective starting point for glucosinolate synthesis.

$$R—Br \xrightarrow{i} R—NO_2$$

Scheme 39 *Reagents and conditions*: i, $NaNO_2$, DMF, urea, -20 °C.

However, in practice, the partial reduction of nitro compounds to oximes was found to have little synthetic value. The reduction of 12-nitrododecanoic acid **133** was attempted (scheme 40) yielding a white solid mixed with zinc dust. It was hoped that the product would dissolve in an organic solvent allowing removal of the leftover zinc by filtration. Unfortunately, dissolution proved impossible both in organic solvents and water and, as a result, synthesis of the oxime **134** could not be confirmed.

O_2N ... OH (**133**) $\xrightarrow{i}$ HO–N ... OH (**134**)

Scheme 40 *Reagents and conditions*: i, Zn, glacial acetic acid, reflux, 5 h.

It could be argued that the insolubility of the above compound, assuming it was the oxime, was not unexpected given that the structure is highly polarised yet hydrophobic. This problem could conceivably have been overcome by protection of the acidic functionality as stated earlier. However, it may have been difficult to find a protecting group which was suitable for the above reduction and also the ensuing steps leading to glucosinolate synthesis meaning that a series of steps would be required as illustrated below:

Acid $\xrightarrow{\text{Protection}}$ Protected Acid $\xrightarrow{\text{Reduction}}$ Protected Oxime $\xrightarrow[\text{Reprotection}]{\text{Deprotection then}}$

This was not a practical solution as deprotection of the acidic oxime would result in formation of the same type of insoluble compound obtained earlier and reprotection using another protecting group would be virtually impossible. Bearing these factors in mind direct conversion of the nitro compound to the oxime was deemed unsuitable for the synthesis of acidic glucosinolates.

The next strategy considered also involved the utilisation of nitro compounds. As detailed in section 1.1.4 these are common starting materials for glucosinolate synthesis being converted to oximyl chlorides *via* the nitronate salt (scheme 41).

$$R-CH_2NO_2 \xrightarrow{i} R-\underset{H}{C}=\overset{+}{N}(O^-)(ONa) \xrightarrow{ii} R-\underset{Cl}{C}=N-OH$$

Scheme 41 *Reagents and conditions*: i, Na, ethanol; ii, Diethyl ether, -78 °C then HCl(g).

This route would also be preceded by protection of the acid functionality and preparation of the nitro-acid from the corresponding bromo derivative.

Before determining the merits of this synthetic route another pathway was considered. The oxidation of aliphatic halides to aldehydes has been previously reported and should allow conversion of the analogous bromo-acid to the acidic aldehyde which could then be converted to the oxime as before. The procedure was first reported by Kornblum *et al* who initially converted the alkyl halide to the tosylate which was then treated with a mixture of hot DMSO and sodium bicarbonate.[131] This furnished the aldehyde in good yield. Their protocol was adapted by Bauer and Macomber,[132] who increased the efficiency of the reaction by using iodide to displace the bromide of the starting bromoester (scheme 42). This led to the synthesis of diketones from α-bromo ketones in yields typically ranging from 90-97%.

Scheme 42 Mechanism of oxidation of an α-bromo ketone to a diketone using an iodide-modified Kornblum oxidation procedure.

Essentially the iodide catalyses the reaction by being a better nucleophile than DMSO in the first instance and by being a better leaving group than bromide in the second. By using this approach, the oximyl chloride could be prepared in five steps from the starting bromo-acid. This is analogous to the previous route using the nitro-compound and subsequent nitronate salt. It was considered that the bromide to aldehyde conversion would be more efficient than the bromide to nitro transformation, the latter only yielding 50-60% of the aliphatic nitro derivative due to the ambident nucleophilic nature of the nitrite ion. In addition, preparation of the oximyl chloride *via* the aldehyde would allow the use of previously tried and tested methodology, as given in chapter 2, and for naphthylmethyl glucosinolate.

To test the efficiency of the oxidation reaction on protected bromoacids, the oxidation of ethyl 5-bromovalerate **135** was attempted as a model reaction (scheme 43) using the procedure of Bauer and Macomber.

Scheme 43 *Reagents and Conditions*: i, DMSO, KI, Na_2CO_3, 160 °C, 1h.

Potassium iodide and sodium carbonate were suspended in DMSO and heated to 160 °C before ethyl 5-bromovalerate **135** was added and the resulting mixture stirred at this temperature for 1 hour. The reaction mixture was then rapidly cooled in ice and washed with ice-cold brine. The product was extracted using diethyl ether and the organic layer washed with water, brine, saturated sodium hydrogen carbonate solution and finally more brine. Concentration under reduced pressure afforded the product as golden-coloured oil. NMR analysis showed that some oxidation had occurred but the reaction had not gone to completion. The desired aldehyde **136** was identified by proton signals at 9.75 and 2.51 ppm for the aldehydic proton and adjacent methylene respectively. Integration implied that the bromide accounted for 59% of the material with the remaining 41% being due to the aldehyde, giving at most a poor overall yield of 22% aldehyde by comparison with quantity recovered. In an attempt to improve the yield, the reaction was carried out as before but for 3 hours instead of 1 hour. Proton NMR analysis suggested that the amount of aldehyde had not increased but rather the amount of side-products had increased. This indicated that perhaps the reaction conditions were too harsh and thus the next attempt was carried out at room temperature with the reaction solution being stirred overnight. This time no aldehyde was observed by NMR analysis although some of the bromide had been converted to the iodide. This was signified by a triplet at 3.18 ppm, in the proton spectrum, which is characteristic of CH_2I.

Finally the reaction was attempted at 80 °C for 3 hours. Once again the relative proportions of starting material and product were calculated from the proton NMR spectrum which showed 64% aldehyde and 36% bromide. Overall, from the quantity of material recovered the yield of aldehyde was found to be 68%. This verified that conversion of the bromide to

the aldehyde proceeded well for protected bromoacids, under the right conditions, and this reaction should provide a good starting point for the preparation of acidic glucosinolates.

The next factor considered was the synthetic target. The compound chosen was (7-carboxyheptyl) glucosinolate **137** (scheme 44) which would provide a comparison with the previously prepared heptyl glucosinolate **81**. Moreover, this chain length should minimise any unfavourable intra-molecular interactions which may arise from the incorporation of the acid.

Scheme 44 Retrosynthesis of (7-carboxyheptyl) glucosinolate.

The first step towards the target **137** was protection of 8-bromooctanoic acid **138**. For simplicity, the ethyl ester was chosen as a protecting group. Moreover, this protecting group is stable to manipulations involving mild acid and base meaning it should not be affected during glucosinolate synthesis.

The bromoacid **138** was dissolved in dry ethanol before thionyl chloride was added at 0 °C. The resulting solution was then heated under reflux for 2.5 hours before concentration under reduced pressure to afford a gold-coloured oil. The product was obtained as a

colourless oil in 58% yield, after purification by column chromatography, eluting with a mixture of petroleum ether (bp 40-60 °C) and ethyl acetate (9:1). However, analysis by NMR indicated that in addition to ethyl 8-bromooctanoate **139**, ethyl 8-chlorooctanoate **140**, was also present in a ratio of 31% chloride to 69% bromide. These ratios were determined by comparison of the peak integrals for CH_2Br and CH_2Cl which showed proton resonances at 3.40 and 3.53 ppm respectively. Similar signals were also observed in the ^{13}C spectrum at 45.56 ppm for the chloride and 27.15 ppm for the bromide.

The formation of the ester as mixture of halides was not considered to be a problem as it was postulated that each halide would be displaced by iodide, prior to oxidation, and thus both would be converted to the desired ethyl 8-oxooctanoate **141** (scheme 45).

Br 139 O O
Cl 140 O O
H 141 O O O

Scheme 45 Predicted conversion of haloesters to ethyl 8-oxooctanoic acid.

Oxidation of the haloesters was performed using the conditions derived for the oxidation of ethyl 5-bromovalerate i.e. pre-heating of DMSO, potassium iodide and sodium carbonate to 80 °C then stirring the reaction solution for 3 hours at that temperature. The product was extracted as before and analysed crude by proton NMR. Three main compounds were observed, the starting bromide **139**, chloride **140** and the desired aldehyde product **141**. Only a small amount of the bromide remained indicating that it had been oxidised to the aldehyde. This is contrary to the chloride which was present in a much larger quantity casting doubts about its susceptibility to oxidation. Furthermore, a number of signals were

present in the region 7.2-8.1 ppm which were reminiscent of the alkene **142**, produced by elimination of HX from the haloester. However, this was very much a minor product.

O
O
142

Alteration of the reaction conditions to stirring at 80-85 °C for 4 hours produced little change and the reaction was therefore extended to 10 hours. This allowed complete conversion of the bromoester to the corresponding aldehyde but once again a significant quantity of chloroester remained. This signified that the chloroester was not as reactive towards displacement with iodine and subsequent oxidation by DMSO. Given that almost a third of the ester synthesised was chlorinated and did not react with iodide, low yields of aldehyde were likely to result from exclusive oxidation of the bromoester. Indeed purification of the aldehyde using column chromatography afforded the product in only 38% yield. Thus beginning the synthesis with a mixture of haloesters was deemed unacceptable.

Nevertheless, this problem was overcome with relative ease by preparing the ethyl ester using thionyl bromide instead of thionyl chloride. The reactants were heated under reflux for 2 hours before cooling and purification by column chromatography, eluting with petroleum ether (bp 40-60 °C) and ethyl acetate (9:1). This furnished the desired ethyl 8-bromooctanoate in 81% yield.

Oxidation for 10 hours as before was conducted in two batches. In the first attempt, the product was obtained as a gold-coloured oil which was shown by proton NMR to contain both the aldehyde **141** and bromide **139** in a ratio of 11:1. Despite the impurity, the aldehyde was reacted with hydroxylamine hydrochloride, in the presence of ethanol and pyridine, to produce the oxime **143** (scheme 46).

Scheme 46 *Reagents and Conditions*: i, $NH_2OH.HCl$, ethanol, pyridine, reflux, 2.5 h.

It was hoped that the oxime would crystallise upon concentration under reduced pressure and addition of water, as was experienced with the alkyl analogues. This would eliminate the need to purify the oxoester by chromatography. In practice, the crude oxime was found to exist as an oil, which crystallised upon cooling, only to melt again when warmed to room temperature. Thus the product was extracted using diethyl ether and purified by flash chromatography, eluting with petroleum ether and ethyl acetate as before, to produce ethyl 8-hydroxyiminooctanoate **143**, as a colourless oil, in 47% yield.

To try and improve the yield of oxime, the product of Kornblum oxidation was purified by flash chromatography to afford ethyl 8-oxooctanoate **141** in 61% yield. This was then reacted with hydroxylamine hydrochloride, as given above. This time the oxime **143** crystallised upon addition of water and the product was recovered, in 85% yield, after filtration. This compound was then used without purification to produce the oximyl chloride **144** (scheme 47).

Scheme 47 *Reagents and Conditions*: i, *N*-chlorosuccinimide, $CHCl_3$, pyridine, room temp., 3.5 h.

Once more, treatment with *N*-chlorosuccinimide allowed efficient chlorination affording ethyl 8-chloro-8-hydroxyiminooctanoate **144** in 86% yield.

The next two steps were coupling of the oximyl chloride **144** with thioglucopyranose **28** and sulfation of the protected thiohydroximate **145** (scheme 48). The coupling reaction was performed in the presence of triethylamine and THF and proceeded as before to afford 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-(7-ethoxycarbonylheptyl) thiohydroximate **145** initially as a gold-coloured oil. Dissolution in ethyl acetate, addition of a small volume of hexane and overnight refrigeration afforded the micro-analytically pure thiohydroximate, as a white solid, in 74% yield. This compound was sulfated using the pyridine-sulfur trioxide complex, generated *in situ* from pyridine and chlorosulfonic acid to afford 2,3,4,6-tetra-*O*-acetyl-(7-ethoxycarbonylheptyl) glucosinolate **146**.

Scheme 48 *Reagents and conditions*: i, THF, Et_3N, 24 h, room temp.; ii, Pyridine, $ClSO_3H$, DCM, 24 h, room temp.; iii, $KHCO_3(aq)$.

Prior to the reaction, it was considered that deprotection of the acetyl groups could occur in an analogous fashion to nonyl and naphthylmethyl glucosinolates. Furthermore, the ethyl

ester could be removed by aqueous base hydrolysis mediated by potassium hydrogen carbonate. However, neither of these scenarios were found to occur. After addition of potassium hydrogen carbonate the resulting two-phase system was concentrated under reduced pressure to allow removal of the organic layer. Unlike the previous alkyl analogues the sulfated product failed to appear as a solid hence extraction with ethyl acetate was necessary. This furnished a cream-coloured foam which was shown, by TLC, to contain some of the starting thiohydroximate. Purification by flash chromatography afforded 2,3,4,6-tetra-*O*-acetyl-(7-ethoxycarbonylheptyl) glucosinolate **146** as an off-white solid in 72% yield. Negative ion electrospray mass spectrometry confirmed that sulfation had occurred and the protected glucosinolate had been produced.

The penultimate step in the glucosinolate synthesis was deprotection of the acetyl groups to yield the free sugar (scheme 49).

AcO AcO AcO AcO O S N $OSO_3^-K^+$ O O **146**

i

HO HO HO HO O S N $OSO_3^-K^+$ O OCH_3 **147**

Scheme 49 *Reagents and Conditions*: i, Methanol, cat. KOMe, 24 h, room temp.

This deprotection was carried out as before by dissolving the protected glucosinolate **146** in dry methanol and adding a catalytic quantity of potassium methoxide until the pH reached 8-

9. The reaction solution was stirred at room temperature for 24 hours before being concentrated under reduced pressure to afford a cream-coloured foam. Analysis by ^{1}H and ^{13}C NMR in deuterated methanol showed no acetyl signals, usually observed at 1.99-2.07 ppm, and no signals for the ethyl ester at 4.0-4.3 and 1.25 ppm for OCH_2 and OCH_2CH_3 respectively. However, it could not be ascertained if the methyl ester had formed because of a broad peak at 3.2-3.5 ppm caused by methanol. Thus the compound was reanalysed using deuterated water which showed a singlet integrating to three protons at 3.61 ppm in the proton spectrum and a small signal at 55.05 ppm in the carbon spectrum. Additional evidence was provided by negative ion electrospray mass spectrometry which showed that the ethyl ester had undergone transesterification and had been completely converted to the methyl ester **147** (as shown in scheme 49).

The final step in the synthesis of the acidic glucosinolate was deprotection of the methyl ester to furnish the free acid. This was uncharted territory given that this reaction had not been necessary in the previous syntheses. However, it was foreseen that this protecting group could be easily removed under basic conditions. For example, Corey *et al* reported deprotection of a methyl ester using lithium hydroxide in a mixture of methanol and water (3:1) at 5 °C and for 15 hours.[133]

Thus, the methyl ester **147** was dissolved in methanol before a 1 M solution of potassium hydroxide was added in a ratio of 1.5 molar equivalents of base to 1 molar equivalent of methyl ester. The reaction mixture was then stirred overnight at room temperature before being concentrated under reduced pressure to afford a cream-coloured oil. Analysis of the product by ^{1}H NMR using a 200 MHz spectrometer produced a spectrum with extremely broad peaks making it very difficult to determine if the methyl ester had been completely converted to the acid. However, negative ion electrospray mass spectrometry allowed the starting material to be clearly observed at mass 460 along with the desired product. This showed masses of 445 and 222 due to the singly and doubly ionised species respectively as represented by **148** and **149**.

148

149

The presence of starting material indicated that the conditions were not strong enough for complete deprotection. Thus the procedure was repeated on the same sample meaning that 3 molar equivalents of KOH were added in total using a reaction time of over 24 hours. This time electrospray mass spectrometry showed no sign of starting material although an additional peak was observed which was attributed as an artefact. However, microanalysis of this reaction, and several later attempts, indicated that the product was extremely impure. This was not completely unexpected given that the reaction mixture was merely concentrated under reduced pressure to afford the product. This meant that large amounts of potassium salts were likely to be present. In addition, the product could not be purified by column chromatography on silica gel as it was only miscible with water. However, it was found that impurities could be removed by washing the product with methanol. This acted to dissolve some of the impurities leaving the product behind as a solid. Further to this, impurities could be removed by adding water to dissolve the product and spinning this solution using a centrifuge. The product-containing aqueous layer could then be decanted. Despite this, microanalysis showed deviations from the ideal and the quest for an efficient purification method continued.

Given that silica gel could not be used for chromatography, reverse phase C_{18} silica was seen as a possible alternative. Using this method the product could be added in water and if

it adhered to the solid phase, could be washed off by solvents of reduced polarity. This purification method was attempted for the alkyl glucosinolates (chapter 2) and was found to be unsuccessful. However, in this case, it was anticipated that the glucosinolate would pass straight through the column but would hopefully leave some of the impurities behind. Hence the product was added in water and the column washed with further water and then methanol. The aqueous fractions were combined, freeze-dried and analysed by ^{1}H NMR at 200 MHz. The spectrum obtained was reminiscent of the desired product although the multiplicity of the alkyl signals suggested that another species was present. In an attempt to gain further information about the composition of the sample, NMR analysis was carried out at 500 MHz. This afforded a distinct improvement in the resolution of the spectra obtained. Furthermore, three different species could be identified in the sample.

The major product was the desired acidic glucosinolate **137** which existed along with two other minor products. The first minor product, called unknown 1, was postulated to be the isocyanate **150** with the second minor product, unknown 2, simply being designated as a free sugar of some description.

HO 6 4 5 O HO HO 3HO 2 1 S $OSO_3^-K^+$ N O a,b e,f i,j c,d g,h k,l OH

137

HO O HO O SH O HO k,l g,h c,d N=C=O i,j e,f a,b

150

The proton characterisations of all three compounds are given below (table 7). The acidic glucosinolate **137** and unknown 2 were characterised from the proton spectrum making use of a 2-dimensional proton-proton chemical shift correlation spectrum (COSY). Conversely the characterisation of unknown 1 **150** required the use of more complex NMR experiments discussed hereafter.

Proton	Desired product 137 (major)	Unknown 1 (150)	Unknown 2
H-1	5.00	4.67	4.59
H-2	3.44	3.82	3.59
H-3	3.56	5.11	3.41-3.63
H-4	3.45	3.62	3.41-3.63
H-5	3.54	3.59	3.49
H-6^{a}	3.71	3.76	3.73
H-6^{b}	3.88	3.92	3.91
a,b	2.69	2.46	
c,d	1.71	1.65	
e,f	1.40	1.43	
g,h	1.33	1.36	
i,j	1.55	1.64	
k,l	2.17	2.53	

Table 7 Proton NMR resonance assignments.

The relative amounts of each species were quantified by comparison of the integrals for H-1 of the three components. However, at the normal analysis temperature of 30 °C the anomeric proton of unknown 1 was partially eclipsed by a water signal. By raising the

probe temperature to 55 °C the water signal moved upfield and the anomeric signal was observed unobstructed. Thus the ratio, acidic glucosinolate **137**: unknown 1 **150**: unknown 2, was calculated to be 2.5:1.0:1.1.

When determining the structure of unknown 1 **150**, the first feature considered was the different pattern of signals for the sugar portion of the molecule when compared with the acidic glucosinolate **137**. For unknown 1, the signal at highest frequency was not from the anomeric proton but was found to be from H-3 existing as a triplet at 5.14 ppm. The signal from H-1 was observed upfield at 4.70 ppm. This pattern of signals was observed previously for the acetylated alkyl glucosinolates suggesting that deacetylation may not have gone to completion. However, the other evidence did not fit with this theory i.e. negative ion electrospray mass spectrometry showed no evidence of a partially acetylated glucosinolate and indeed there was little evidence for an acetylated species elsewhere in the ^{1}H and ^{13}C spectra. A signal could be visualised at 1.92 ppm in the proton spectrum but the peak integral did not compare favourably with that for H-3 and was thus assigned as residual acetate remaining from the first deprotection step.

Further information about the structure of unknown 1 was obtained by performing proton-carbon correlation experiments. In particular the long range C-H correlation was extremely useful. The signal for H-3 at 5.11 ppm was found to correlate through two or three bonds with a signal at 175.91 ppm in the carbon spectrum. The chemical shift of this peak was significantly different from COOH, which was found at 186.68 ppm and was more reminiscent of the esters prepared previously which gave rise to signals at 175.89 ppm and 174.37 ppm for the methyl and ethyl esters respectively. Furthermore this signal correlated with the protons (k,l), as shown for **150**, indicating that C-3 was linked to the side chain *via* an ester linkage. Examination of the proton resonances for both the acidic glucosinolate **137** and unknown 1 **150** provided further evidence that *O*-acylation of the hydroxyl attached to C-3 had occurred. Haverkamp *et al* reported α-effects (on the protons attached to the carbon atom which bears the *O*-acyl group) of 1-1.5 ppm downfield shift from TMS,

upon *O*-acylation, and β-effects (the effects on protons attached to adjacent carbon atoms) of approximately 0.2 ppm downfield shift.[134] Looking at the values in table 7, it is clear that the H-3 resonance has moved from 3.56 ppm in the acidic glucosinolate **137** to 5.11 ppm in unknown 1 **150**. This change of 1.55 ppm is consistent with the α-effect. Also the proton resonances of H-2 and H-4 have changed by 0.38 and 0.17 ppm respectively as reported for the β-effect. Thus it was reasonable to conclude that an ester linkage had been formed at C-3.

In addition, the resonances (a,b) from **150** correlated with a signal at 125.00 ppm. This meant that the alkyl chain terminated with a carbon atom showing this resonance frequency. In the major product **137**, protons (a,b) are adjacent to a carbon atom found at 167.63 ppm which was assigned as *C*=N. It is this carbon atom in unknown 1 **150** which was showing a much lower frequency indicating the environment had changed considerably. Moreover, in the major product **137** the *C*=N showed a strong correlation with H-1 whereas in unknown 1 **150** the corresponding signal was absent. This implied that the carbon atom at 125 ppm was not attached to the sugar moiety as found in a normal glucosinolate.

Thus the identity of the terminal carbon atom required elucidation. Several functional groups show signals at 120-130 ppm: nitriles are observed at 119-120 ppm; isocyanates such as octyl isocyanate show a signal at 122 ppm; isothiocyanates such as butyl isothiocyanate show a signal at 129 ppm; alkenes are observed around 121 ppm. Considering this data and all the evidence collected, the structure of unknown 1 was proposed to be the isocyanate **150** shown below:

150

This product could be formed by cyclisation of the glucosinolate **137** initiated by nucleophilic attack of the C-3 hydroxyl at the acid carbonyl group. This would allow formation of the intermediate **151** which, being strained, could undergo hydrolysis at the quaternary centre to afford the second intermediate **152**. Finally a Lossen rearrangement could occur to afford the isocyanate **150** (scheme 50).

Scheme 50 Mechanism of formation of the isocyanate **150**.

Interestingly, hydrolysis of the first intermediate **151** does not occur at the anomeric position in the same manner as hydrolysis of glucosinolates by myrosinase. This was indicated by the fact that only the β-anomer of **150** was observed. If hydrolysis were to take place at the anomeric position a mixture of α and β-anomers would result.

Further evidence for the presence of a species similar to **150** was provided by negative ion electrospray mass spectrometry. Previously a signal was observed of mass 348 which was thought to be an artefact. However, this signal could be due to the isocyanate which has a mass of 348 corresponding with $[M-H]^-$. Moreover, IR analysis of the mixture showed a small absorbance at 2252 cm^{-1} which is also consistent with an isocyanate.

However, there is one problem with the structure postulated and that is the susceptibility of isocyanates to hydrolyse. Given the basic conditions employed during deprotection it could be rationalised that the proposed species **150** would be short lived. However, it may also exist in its ring-closed form **153** and may rapidly switch between the two structures. This could provide stability against hydrolysis and would also explain the frequency of the quaternary carbon which, at 125 ppm, is half way between an isocyanate and an isothiocyanate.

HO, O, HO, O, SH, HO, N=C=O

150

HO, O, HO, O, S, OH, HO, O, N

153

Further to the NMR studies already conducted, the sample was analysed to determine if nuclear Overhauser effects (NOEs) could be observed for the acidic glucosinolate **137** thus providing information on the geometry of this molecule. Indeed strong NOE cross peaks could be observed from H-1 to the protons (a,b) and (c,d) and also from H-5 to the protons (a,b), (c,d), (e,f) and (g,h). This indicates that the alkyl chain is folding across the B-face of the sugar portion of the molecule as below for **137**. This provides further support for the mechanism postulated in scheme 50.

Furthermore the above structure was used to construct a computer model using the AMBER molecular mechanics force field,[135] implementing the parameterisations for carbohydrates.[136] The molecule was constrained using the NOE data previously obtained and the resulting structure was further refined by semi-empirical AM1 energy calculations. This allowed the 3-dimensional structure of (7-carboxyheptyl) glucosinolate to be pictorially represented as given overleaf (figure 11). The structures shown are low energy forms indicating that the folding of the aliphatic chain under the sugar ring is energetically favourable. This conformation allows the alkyl protons to come in close proximity to H-1 and H-5 and also orientates the acidic functional group towards H-3. This again lends credence to the hypothesis that the alkyl chain adds on to H-3 and also the proposal that the ring intermediate **151** undergoes hydrolysis and rearrangement to form an isocyanate type structure.

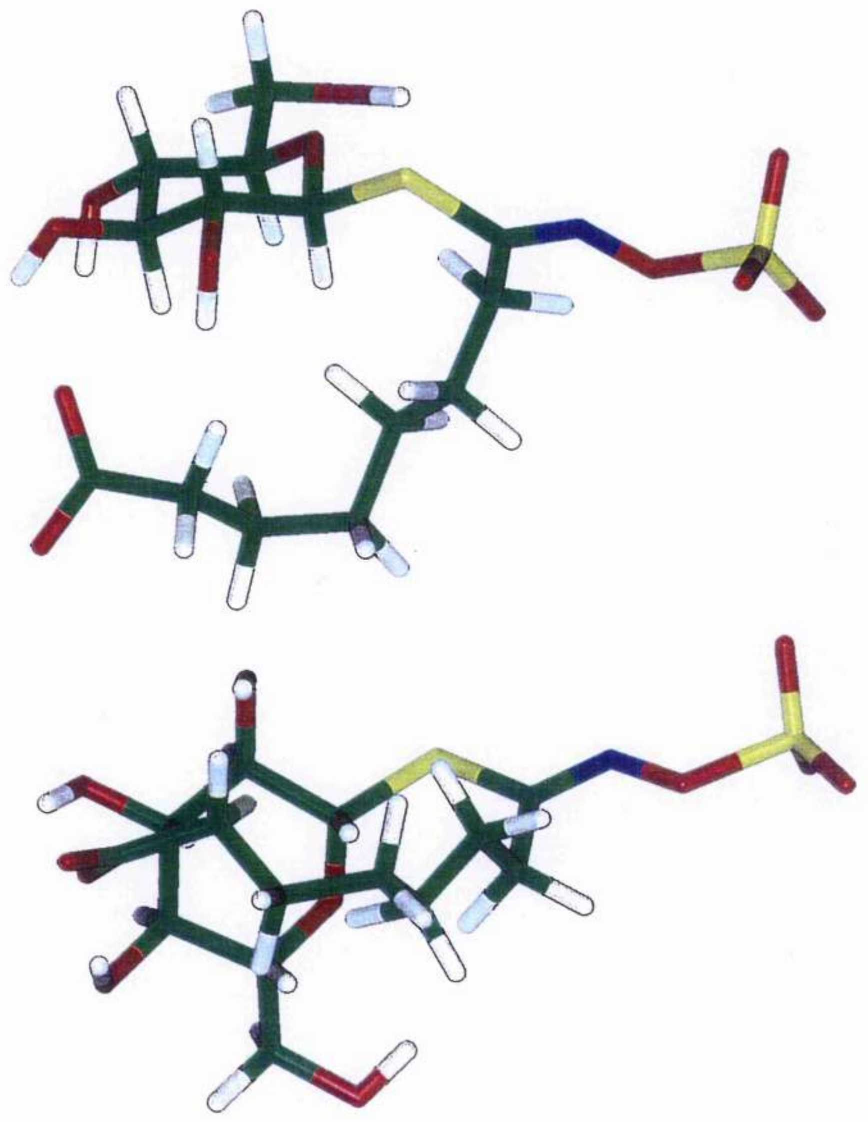


Figure 11 Molecular model of (7-carboxyheptyl) glucosinolate shown in two different orientations.

To determine if this geometry was characteristic of aliphatic glucosinolates, heptyl glucosinolate **81** was analysed as an analogue of (7-carboxyheptyl) glucosinolate **137**. This compound also displayed NOE cross peaks from H-1 to (a,b) and (c,d) and from H-5 to (a,b). However, there were not as many cross peaks as for (7-carboxyheptyl) glucosinolate indicating that the proximity of the alkyl chain to the sugar was not so pronounced for heptyl glucosinolate. A comparison of the NOESY spectra for both glucosinolates is given in appendix A.

Purification of (7-carboxyheptyl) glucosinolate from the other sugar species was further attempted by preparative cellulose TLC. Unfortunately this failed to afford the pure glucosinolate. As an alternative, the methyl ester deprotection was attempted using Pig Liver Esterase.[137] This method was chosen as it uses mildly basic conditions meaning there would not be so much scope for base-catalysed side reactions. The reaction was incubated at 37 °C for almost 48 hours before being heated under reflux for 30 minutes to denature the enzyme. Filtration and lyophilisation afforded a golden-coloured foam which was washed with methanol as before to remove any remaining starting material. Unfortunately, a significant proportion of the methyl ester remained (almost 80% of the starting quantity). Moreover, NMR analysis of the product showed evidence for the occurrence of side reactions. Consequently the preparation of (7-carboxyheptyl) glucosinolate was abandoned.

In addition to (7-carboxyheptyl) glucosinolate **137** the synthesis of a shorter chain analogue (3-carboxypropyl) glucosinolate **154** was also attempted. These compounds may bind in the waxy leaf surface to different extents due to having different chain lengths. Thus by comparing the data from these compounds with the data from the alkyl analogues, it would be possible to assess the effect of the acidic functionality.

Synthesis of the carboxypropyl derivative **154** began as before from the bromoacid **155** (scheme 51).

HO, HO, HO, HO, O, S, $OSO_3^-K^+$, N, O, OH

154

Br, O, OH

155

Scheme 51 Retrosynthesis of (3-carboxypropyl) glucosinolate **154**.

The bromoacid **155** was protected as its ethyl ester by heating under reflux with thionyl bromide and dry ethanol for 2.5 hours. Concentration under reduced pressure afforded a brown oil which was shown by proton NMR to contain the desired ethyl 4-bromobutyrate **156** along with some unreacted starting material. In addition, a second set of ethyl signals were observed which could be caused by displacement of the bromide by ethoxide to yield an ether. The pure product was obtained after purification by flash chromatography as described earlier. Interestingly, the ester could not be visualised by TLC and the product-containing fractions were identified by the compound collecting as an oil around the top of the test tubes and also by its distinctive smell. Unfortunately, a low yield of 36% was realised for ethyl 4-bromobutyrate. Thus the reaction was repeated and an improved yield of 59% was achieved.

The ester **156** was then subjected to Kornblum oxidation in the same manner as for ethyl 8-bromooctanoate (scheme 52).

Br, O, O, **156** —i→ H, O, O, O, **157**

Scheme 52 *Reagents and conditions*: i, DMSO, KI, Na_2CO_3, 80 °C, 10 h.

After extraction and concentration under reduced pressure, the product was obtained as a brown oil. Analysis by TLC showed the presence of six different compounds one of which was shown to be the oxoester **157** by NMR analysis. This was indicated by a proton signal at 9.82 ppm due to C*H*O. Comparison of the integral for this aldehydic signal with the integral for OCH_2CH_3 allowed the relative proportion of aldehyde to be calculated as 15%. Furthermore the yield of aldehyde **157** could be estimated from the weight of material recovered and was found to be 7%. This estimated yield would likely reduce as a consequence of purification.

The reaction was repeated under the same conditions except the reaction time was reduced from 10 hours to 3 hours to determine if less side reactions would occur. However, analysis of the crude product by TLC again showed the presence of six different species. NMR analysis suggested an increase in the amount of aldehyde and an estimated yield was again calculated and found to be 18%.

It was therefore deduced that the route used for the preparation of ethyl 8-chloro-8-hydroxyiminooctanoate **144** was not applicable for preparation of the propyl derivative. The desired oxoester was formed but in insufficient quantities for the ensuing six steps of glucosinolate synthesis.

Before abandoning the synthesis of (3-carboxypropyl) glucosinolate **154**, one other method of aldehyde synthesis was attempted. It was postulated that a diester could be partially reduced using DIBAL-H to give the desired oxoester. Starting from the readily available diethyl succinate **158**, this partial reduction was attempted.

Scheme 53 chemical structures: 158 → 157 (i,ii)

Scheme 53 *Reagents and conditions*: i, DIBAL-H, toluene, -78 °C; ii, MeOH, -78 °C to 20 °C, potassium sodium tartrate.

Initially one molar equivalent of both diethyl succinate **158** and DIBAL-H were added so as to allow reduction of only one of the esters of the starting material. The diester was dissolved in dry toluene and cooled to -78 °C before DIBAL-H was added. The resulting solution was stirred at that temperature for 2 hours before dry methanol was added and the solution slowly allowed to warm to room temperature. An aqueous solution of potassium sodium tartrate was then added and the two-phase system stirred overnight. Separation of the organic layer preceded extraction of the aqueous layer with diethyl ether. Finally, the organics were combined, dried and concentrated under reduced pressure. NMR analysis of the crude product suggested little or no reaction had occurred as only starting material could be observed. The reaction was repeated using 1.1, 1.25 and 2.0 molar equivalents of DIBAL-H to the one molar equivalent of diethyl succinate. In addition, the length of stirring time at -78 °C was increased and on one attempt the reaction was allowed to warm to room temperature immediately after addition of the reducing agent. However, in all these reactions there was little sign of aldehyde formation. It was not practical to increase the amount of DIBAL-H any further as a huge mixture of products could result. Therefore the synthesis of (3-carboxypropyl) glucosinolate was abandoned at this point.

3.3 CONCLUSIONS

The previously unreported synthesis of 1-naphthylmethyl glucosinolate was carried out in 10 steps beginning from β-D-glucose and 1-naphthylacetic acid which was converted to 1-naphthylacetaldehyde *via* the corresponding ethyl ester. Two batches of the glucosinolate were prepared of differing purity for testing.

The synthesis of (7-methoxycarbonylheptyl) glucosinolate was also completed beginning from 8-bromooctanoic acid. This compound was initially prepared as a precursor to the acidic glucosinolate (7-carboxyheptyl) glucosinolate. However this compound was not obtained in a pure form due to difficulties with the final methyl ester deprotection step. High resolution NMR studies showed that one of the by-products resulting from this deprotection was caused by the side chain of (7-carboxyheptyl) glucosinolate folding under the B-face of the sugar molecule causing acylation of the hydroxyl group at C-3. Further evidence suggested that the S-C bond of the aglycone had subsequently hydrolysed to afford an isocyanate type structure. NMR studies of heptyl glucosinolate showed a similar conformation although the folding was less pronounced as indicated by NOE enhancement. In addition, preparation of a shorter chain analogue could not be completed due to low yields being obtained in the initial stages of synthesis.

CHAPTER 4

BIOLOGICAL ACTIVITY OF GLUCOSINOLATES

4.1 INTRODUCTION

In this chapter the properties of the glucosinolates prepared in chapters 2 and 3 are discussed. Two areas were examined. Firstly electrophysiological testing of glucosinolates was carried out to assess the detection of these compounds by root flies and their possible activity as oviposition stimuli. In particular the effect of the glucosinolate side chain structure on the insect response was examined to probe the existence of a structure-activity relationship. The second area was the activity of the synthetically prepared glucosinolates as substrates for myrosinase.

4.2 ELECTROPHYSIOLOGICAL TESTING OF NATURAL AND SYNTHETIC GLUCOSINOLATES

To determine the effect of the glucosinolate structure on the response of root flies, the following compounds underwent electrophysiological testing at Wädenswil, Switzerland. This type of analysis allows determination of the fly's response to the glucosinolate by measuring the electrical current generated by chemoreceptor neurons located within hairs attached to the insect's legs.

Compounds **78-83** were prepared as discussed previously in chapters 2 and 3 with compound **20** and **159-163** being prepared by Professor P. Rollin and co-workers at Institut de Chimie Organique et Analytique, Université d' Orléans, France. By comparing both sets of glucosinolates it was possible, not only to compare a set of synthetic and naturally occurring glucosinolates, but to correlate the results described hereafter with previous test results described in section 1.1.5.

HO, HO, HO, HO, O, S, $OSO_3^-K^+$, N, R

R =

(C3) **78** H_3CS **160**

(C4) **79** H_3CSO **161**

(C5) **80** OH **162**

(C7) **81**

(C9) **82** **163**

20

83

HO **159**

All compounds, dissolved in 10 mM KCl, were tested on the D4 sensilla of *D. radicum* females at the same concentration. The nerve impulses generated were counted in the first second of stimulation to give recordings as illustrated in figure 12.

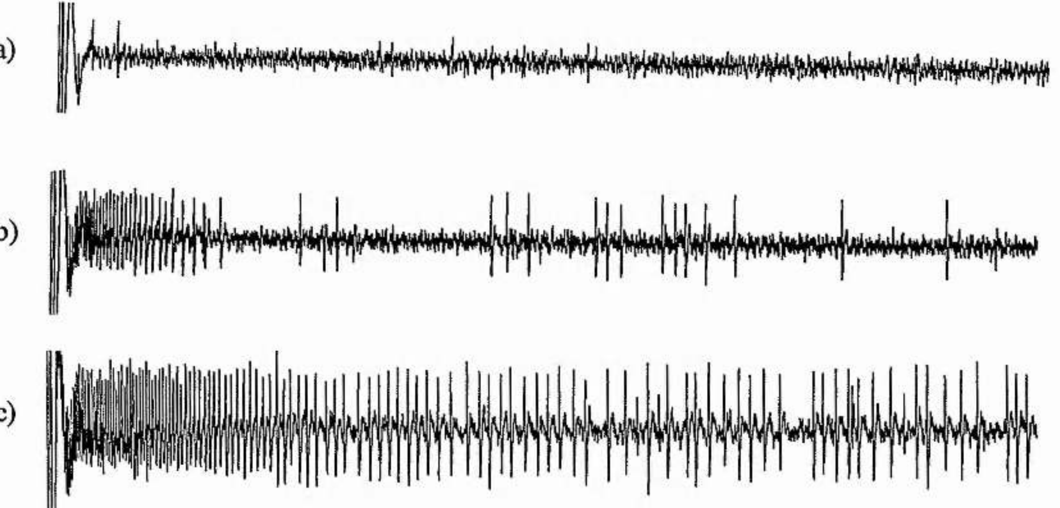


Figure 12 Electrophysiological recordings of a) KCl; b) glucosinalbin **159**; c) nonyl glucosinolate **82**.

These recordings were then converted to numerical results which are given in table 8.

Stimulus	Count	Mean	Standard deviation	Standard error
KCl	9	2.889	4.457	1.486
JMB-15 **81**	9	53.111	13.252	4.417
NED 074R2 **82**	9	61.000	23.864	7.955
NED 083 **81**	9	58.333	21.459	7.153
NED 086R **80**	9	55.333	22.000	7.333
NED 093 **79**	9	33.556	18.642	6.214
NED 094 **78**	9	37.889	24.932	8.311
NED 102R **83**	9	8.889	12.364	4.121
NED 110 **83**	9	2.556	4.902	1.634
Gluco-erucine **160**	8	37.875	20.497	7.247
Gluco-raphenine **161**	8	47.250	23.057	8.152
Gluco-epiprogoitrine **162**	8	28.375	23.513	8.313
Gluco-sinalbine **159**	8	28.500	11.699	4.136
Gluco-tropaeoline **20**	8	45.000	18.800	6.647
Gluco-napine **163**	8	37.000	17.639	6.236

Table 8 Mean electrophysiological data.

The graphical representation of the above data is given in figure 13.

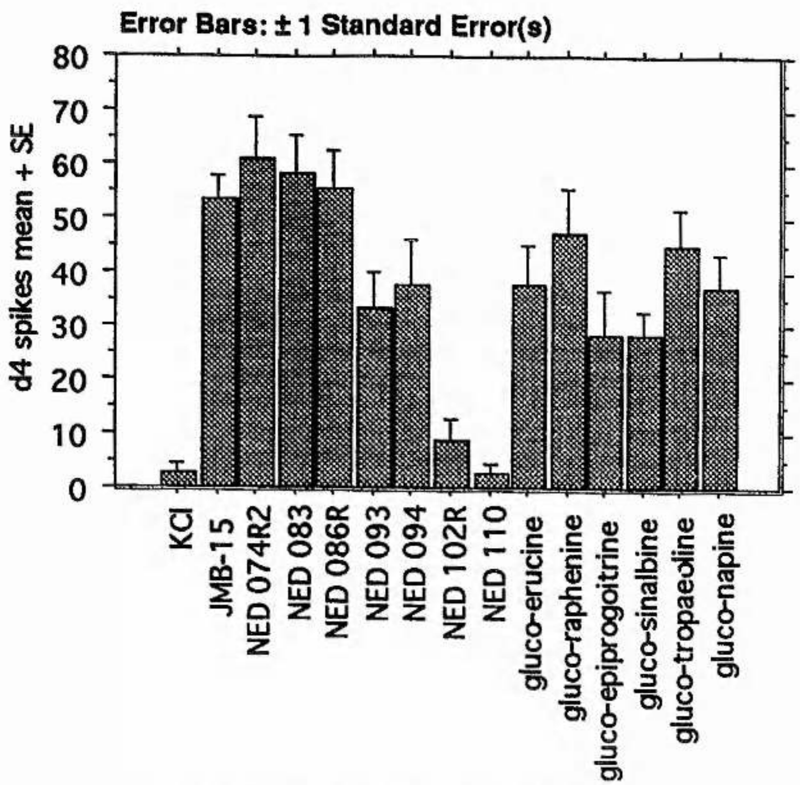


Figure 13 Graph showing the insect chemoreceptor response to the glucosinolates tested. JMB-15, NED 083 = **81**; NED 074R2 = **82**; NED 086R = **80**; NED 093 = **79**; NED 094 = **78**; NED 102R, NED 110 = **83**; gluco-erucine = **160**; gluco-raphenine = **161**; gluco-epigoitrin = **162**; gluco-sinalbine = **159**; gluco-tropaeoline = **20**; gluco-napine = **163**.

To determine if the overall variation in response between the glucosinolates and the background was due to random errors, the data was examined by a single-factor analysis of variance calculation (ANOVA). This indicated that at the 99.9999% confidence limit, the overall variation was significant meaning the differences could not be attributed to random error and must be due to the compounds analysed having a stimulatory effect ($F = 9.473$, $P < 0.0001$). However, in figure 13 a large difference in response between individual glucosinolates and between the glucosinolates and KCl can be observed. The single-factor ANOVA calculation does not determine whether these values are significantly different and the variation between compounds was thus analysed using a Duncan new multiple range test. At the 99.95% confidence limit most of the glucosinolates were found to give

significantly different values from KCl and from NED 102R and NED 110. The complete table of values for this test is given in appendix B.

Examination of figure 13 implied that a correlation existed between the length of the glucosinolate side chain and the stimulatory activity. There appears to be an increase in activity as the length of the side chain increases from propyl to pentyl, heptyl and nonyl, meaning that the structure-activity study previously postulated to exist, may be a reality. This increase is shown graphically in figure 14.

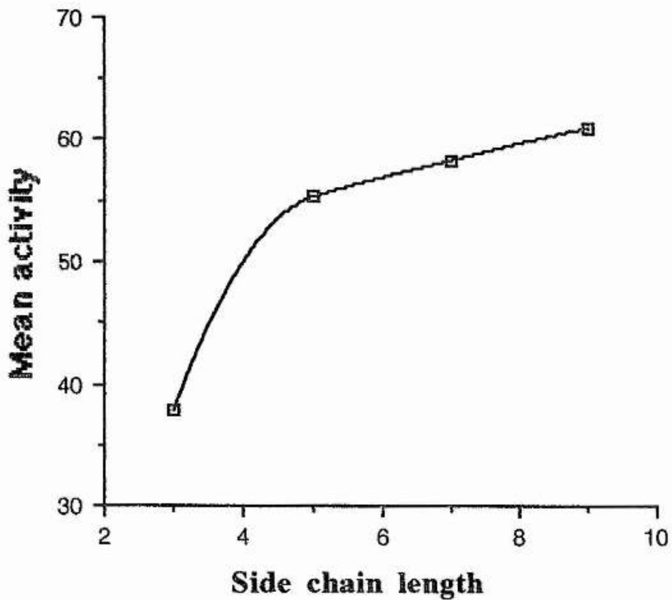


Figure 14 Graph showing the increase in insect response with increasing side chain length.

However, it should be noted that the purities of these compounds differ as shown in table 9. The purity of propyl and pentyl glucosinolates is less than heptyl and nonyl glucosinolates. The impurity in these samples is due to potassium methoxide which was left over from deprotection of the sugar acetyl groups. Given that potassium chloride induced only a small electrophysiological response (shown in figure 13) the potassium methoxide is likely to give a similarly small response. However, the presence of this salt means that there is less active glucosinolate and thus the response values for propyl and pentyl glucosinolates are probably

an underestimate of their true activity. This reduction in activity with reduced purity is shown clearly by comparing the stimulatory activity of JMB-15 and NED 083 which are both heptyl glucosinolate. The former gives a stimulatory value of 53.111 ± 4.417 with the latter a value of 58.333 ± 7.153. Thus, taking account of purity differences could effectively equalise the responses making the alkyl glucosinolates all very similar.

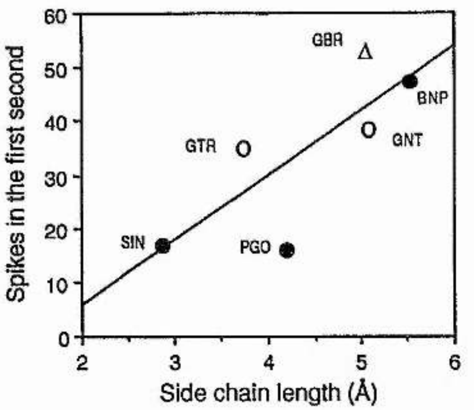
Compound	**Estimated purity (percentage of glucosinolate in sample)**
NED 094 (propyl)	84
NED 093 (butyl)	100
NED 086R (pentyl)	93
NED 083 (heptyl)	100
JMB-15 (heptyl)	76
NED 074R2 (nonyl)	100
NED 102R (naphthylmethyl)	79
NED110 (naphthylmethyl)	85

Table 9 Estimated purity of glucosinolates tested.

Interestingly, butyl glucosinolate appeared to be less stimulatory than propyl glucosinolate. This is unusual given that this compound is microanalytically pure whereas propyl glucosinolate is not. Consequently, the butyl analogue would be expected to be much more active. Unfortunately, the reason for this unusual pattern is unknown.

In previous tests it was shown that glucobrassicin **84** was the most active compound with glucotropaeolin **20** also being relatively potent, as shown in figure 15.[43] However, comparison of naphthylmethyl glucosinolate **83** with glucotropaeolin **20**, in this study, showed that the difference between these compounds was statistically significant with the former being much less active than the benzyl analogue. This implies that naphthylmethyl

glucosinolate and glucobrassicin are dissimilar allowing the conclusion to be drawn that the nitrogen atom in glucobrassicin is important for stimulatory activity. Indeed naphthylmethyl glucosinolate appeared to have no activity as a chemical cue with the sample of highest purity (NED 110) yielding a stimulatory value of 2.556 ± 1.634 which is comparable with KCl (2.889 ± 1.486). Once more this result may be an underestimate of the activity of this compound due to its purity. However, the results indicate that this compound would have little activity even at 100% purity.



HO, HO, HO, HO, O, S, $OSO_3^-K^+$, N, N, H

84

Figure 15 Graph showing activity of glucosinolates towards *D. radicum*, previously measured by contact chemoreception. (GBR = glucobrassicin **84**, GTR = glucotropaeolin **20**).

Comparison of the alkyl glucosinolates with glucotropaeolin **20** suggests that pentyl, heptyl and nonyl glucosinolates are more stimulatory than this naturally-occurring compound. Referring to figure 15, the activity of these alkyl glucosinolates is analogous to, or may even supersede, the activity of glucobrassicin. Thus it can also be concluded that the alkenyl functional group, found in the compounds used in the last study (see section 1.1.5), is not necessary for stimulatory activity and may act to reduce this activity.

4.3 SYNTHETIC GLUCOSINOLATES AS SUBSTRATES FOR MYROSINASE

4.3.1 Background

When glucosinolates **1** are hydrolysed by myrosinase, β-D-Glucose **2** and an isothiocyanate **4** are produced, the latter from rearrangement of the corresponding aglycone **3** (scheme 54).

Scheme 54 Hydrolysis of glucosinolates by myrosinase.

A number of methods are available for measuring the rate of myrosinase catalysed glucosinolate hydrolysis. The decrease in the amount of glucosinolate can only be measured for compounds which display an ultraviolet (UV) absorbance. For example sinigrin **164** is an unsaturated glucosinolate and shows a λ_{max} at 227 nm. Thus the decrease in the amount of sinigrin, caused by myrosinase catalysed hydrolysis, can be monitored at 227 nm. Measured over a given period, the rate of turnover can thus be quantified.

Alternatively, the measurement of glucose and isothiocyanate release can be performed for every glucosinolate.

The glucose release can be measured using a hexokinase (HK)/glucose-6-phosphate dehydrogenase coupled glucose assay which is commercially available. Initially the glucosinolate is incubated with myrosinase which leads to the production of glucose. An aliquot of this reaction solution is added to the glucose (HK) reagent. The following reactions then take place (scheme 55): The glucose is phosphorylated by adenosine triphosphate (ATP) in a reaction catalysed by the enzyme hexokinase (HK). This produces glucose-6-phosphate (G-6-P) which is oxidised in the presence of glucose-6-phosphate dehydrogenase (G-6-PDH) to produce 6-phosphogluconate (6-PG). During this oxidation, an equimolar quantity of nicotinamide adenine dinucleotide (NAD) is reduced to NADH causing an increase in absorbance at 340 nm which is directly proportional to the glucose concentration.

Glucose + ATP —HK→ G-6-P + ADP

G-6-P —G-6-PDH (NAD → NADH)→ 6-PG

Scheme 55 Processes involved in glucose quantification.

The release of isothiocyanates cannot be quantified with such ease as there is no commercially available test-kit. Instead, the isothiocyanate would have to be sampled from the reaction mixture, analysed by GC or GC/MS and quantified using an internal standard.

Given that the compounds prepared previously in chapter 2 are alkyl glucosinolates and possess no chromophores to make them UV active, the direct measurement of glucosinolate turnover by UV spectrophotometry would be impossible. Thus determination of glucose using the glucose (HK) reagent was deemed to be the simplest method to ascertain if these glucosinolates were substrates for myrosinase and to quantify their rate of turnover. The complex glucosinolates, synthesised in chapter 3, could also analysed similarly although it should be possible to examine naphthylmethyl glucosinolate by UV spectrophotometry.

4.3.2 Measurement of glucosinolate turnover by glucose analysis

4.3.2.1 Preliminary investigations

Before testing the synthetic glucosinolates, the accuracy of the glucose (HK) reagent was examined and the optimum analysis conditions deduced.

The accuracy was determined by adding a small volume of 5 and 10 mM D-glucose solution to the (HK) reagent and measuring the absorbance at 340 nm. The readings were compared with that of a blank which was prepared by adding the same quantity of distilled water to the glucose (HK) reagent. The blank and sample absorbance values were used to calculate the amount of glucose as follows:

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

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 allowing conversion to moles

Glucose concentrations were calculated as 5.12 mM and 10.29 mM for the 5 and 10 mM samples respectively. Both analyses produced an error of less that 3% and as a result the glucose (HK) reagent was deemed to be of sufficient accuracy for this study.

Next, the optimum conditions for analysis were ascertained by examining the turnover of a 10 mM solution of sinigrin using varying quantities of myrosinase.

Thus, hydrolysis of sinigrin (10 mM, 1 ml) was carried out using myrosinase solution (prepared as specified in section 7.6.1.1) An aliquot of each reaction solution was added to the glucose (HK) reagent at 15, 45, 120, 130, 300 minutes and the concentration of glucose calculated as before. When 0.09 and 0.18 units of myrosinase were used the reaction was too rapid and complete glucose release was obtained after 15 and 45 minutes respectively. However, using 0.045 units of myrosinase a suitable time course was obtained (table 10).

Time/minutes	[Glucose]/mM[a] for 0.045 units (25 μl) myrosinase
15	1.8
35	5.6
60	10.4
90	10.6

[a] Each concentration was determined from an average of three measured values.

Table 10 Glucose release as a function of time for *ca.* 0.045 units myrosinase.

This time a steady increase in the amount of glucose and thus glucosinolate hydrolysis with time was observed. The reaction appeared to have reached completion after 60 minutes

meaning that the analysis could be completed within a relatively short time period. In addition, a number of analyses could be performed before reaction completion making the study more accurate.

4.3.2.2 Alkyl glucosinolates as substrates for myrosinase

The turnover of the alkyl glucosinolates (propyl, butyl, pentyl, heptyl and nonyl) was measured as follows: A 10 mM solution of each glucosinolate was prepared using potassium phosphate buffer and an aliquot of this (1 ml) was incubated at 37 °C prior to addition of myrosinase (25 μl, *ca.* 0.045 units). The glucose release was measured after 15, 30, 45, 60, 75, 90 and 120 minutes by adding an aliquot of reaction solution to the glucose (HK) reagent, which had been prewarmed at 37 °C. The absorbance at 340 nm was recorded approximately 5 minutes after the addition of reaction solution.

All of the alkyl glucosinolates were found to act as substrates for myrosinase producing glucose. As an example, the time course for heptyl glucosinolate is given in figure 16.

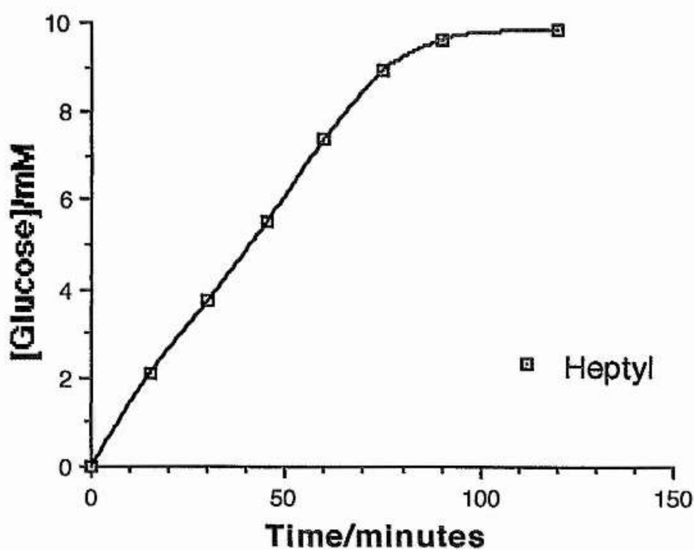


Figure 16 Typical turnover of alkyl glucosinolates by myrosinase, as illustrated by heptyl glucosinolate.

The data was found to be reproducible, as shown for butyl glucosinolate (figure 17) although some rogue data points were obtained. These were eliminated by multiple analyses.

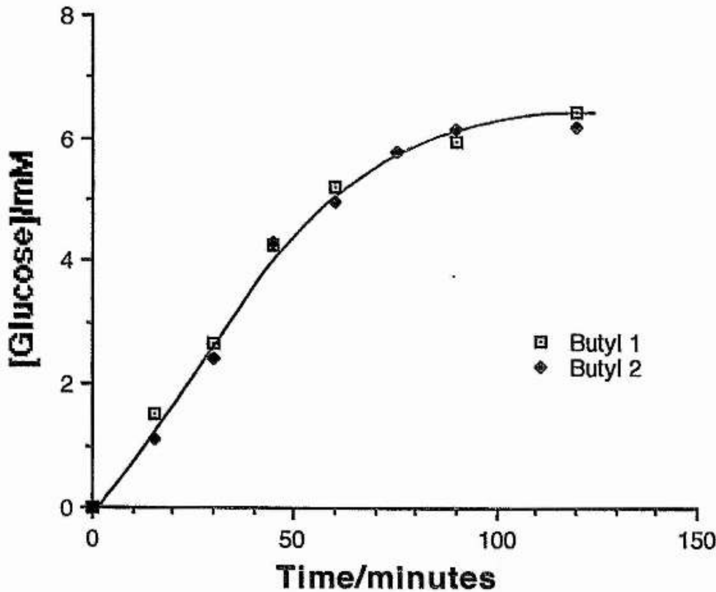


Figure 17 Graph showing reproducibility of data for butyl glucosinolate.

Interestingly, butyl glucosinolate did not yield the expected 10 mmoles of glucose and instead only liberated 6-6.5 mmoles. This is contrary to the other alkyl glucosinolates which generally produced a quantitative yield of glucose. It was hypothesised that the turnover of this glucosinolate may be occurring at a slower rate therefore a further sample was analysed after 210 minutes from the addition of myrosinase. This showed a negligible increase in glucose concentration. A further quantity of myrosinase was added and measurements were taken as before but again only a negligible increase was observed.

The sample of butyl glucosinolate used in these reactions was pure by microanalysis and LC-MS analysis so it is unclear as to why a low yield of glucose was obtained. However, in the electrophysiological studies this compound also seemed to give an anomalously low result. It may be that although this compound appeared to be pure by the same criteria as all the other glucosinolates it in fact contained some type of impurity which meant the actual quantity of glucosinolate was being underestimated.

Before each glucosinolate analysis, the activity of myrosinase was determined. This was carried out in triplicate by adding a specific quantity of myrosinase to a solution of 0.1 mM sinigrin. The decrease in absorbance at 227 nm was measured and the gradient of the first portion of the line was calculated. This allowed the rate of sinigrin metabolism and thus the quantity of active enzyme to be measured. Accordingly, this assay allowed the results to be corrected to one unit of enzyme activity thereby eliminating errors caused by an increase or decrease in enzyme activity between experiments. Thus a comparison of the rate of turnover of alkyl glucosinolates could be made (table 11). The calculation was also performed for sinigrin to allow comparison of this unsaturated glucosinolate with the synthetic saturated analogues.

Glucosinolate (10 mM)	Rate of turnover (mmol/minute)[a]
Sinigrin	3.02
Propyl	2.37
Butyl	2.68
Pentyl	2.43
Heptyl	2.12
Nonyl	2.83 ± 0.39[b]

[a] Corrected to one unit of enzyme activity.

[b] Value taken from the average of three measurements.

Table 11 Turnover rates of alkyl glucosinolates compared with sinigrin.

These results show similar rates of turnover for alkyl glucosinolates by myrosinase irrespective of the length of the alkyl chain i.e. there is little evidence of a trend showing increased turnover with increasing alkyl chain length. In addition, the results show that the synthetic glucosinolates are metabolised at approximately the same rate as the natural substrate, sinigrin. This fits with previous findings that similar glucosinolates are hydrolysed at similar rates.[49]

An addition, to the experiments already conducted, it was also necessary to show that the glucosinolates did not degrade in the absence of myrosinase to afford glucose. Thus butyl glucosinolate was incubated alone at 37 °C and measurements were made as before using glucose (HK) reagent. The results were compared with those obtained previously in the presence of myrosinase (table 12).

Time/m	[Glucose]/mM[a] produced in the presence of myrosinase	[Glucose]/mM[a] produced in the presence of myrosinase	[Glucose]/mM[a] produced in the absence of myrosinase
30	2.66	2.40	0.26
60	5.23	4.97	0.30

[a] Each concentration was determined from an average of three measured values.

Table 12 Comparison of glucose release for butyl glucosinolate in the presence and absence of myrosinase.

The amounts of glucose measured were thus very small and can be accounted for by experimental error. Therefore it can be concluded that glucosinolates do not degrade to afford glucose in the absence of myrosinase.

4.3.2.3 Complex glucosinolates as substrates for myrosinase

The metabolism of naphthylmethyl glucosinolate and (7-methoxycarbonylheptyl) glucosinolate by myrosinase was examined in the same manner as for the alkyl glucosinolates. Both compounds were found to act as substrates for myrosinase as shown in figures 18 and 19.

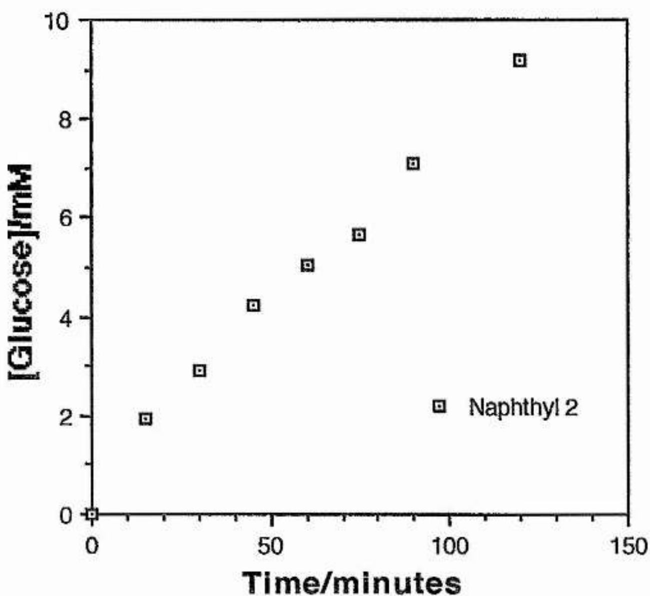


Figure 18 Turnover of naphthylmethyl glucosinolate by myrosinase.

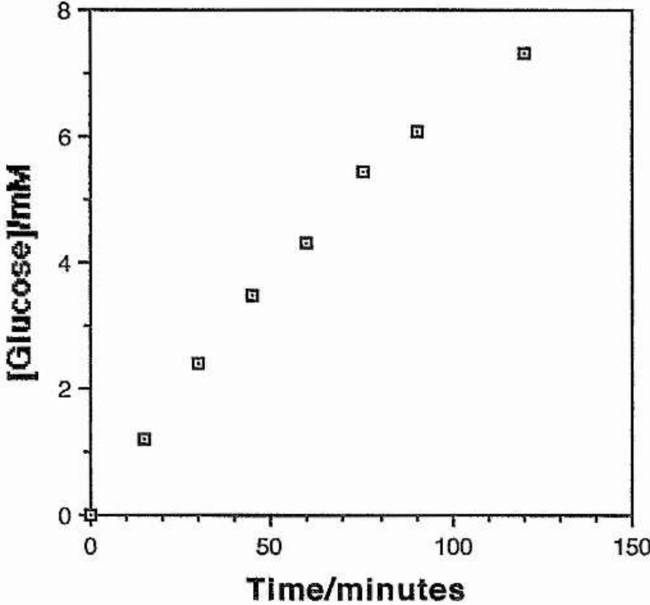


Figure 19 Turnover of (7-methoxycarbonylheptyl) glucosinolate by myrosinase.

The rate of turnover of these compounds was also calculated as before and compared with sinigrin (table 13).

Glucosinolate (10 mM)	Rate of turnover (mmol/minute)[a]
Sinigrin	3.02
Naphthylmethyl	2.17 ± 0.33[b]
(7-methoxycarbonylheptyl)	2.05

[a] Corrected to one unit of enzyme activity.

[b] Value taken from the average of two measurements.

Table 13 Turnover rates of complex glucosinolates compared with sinigrin.

These glucosinolates show slightly slower turnover rates than sinigrin and most of the alkyl glucosinolates. The result for naphthylmethyl glucosinolate is comparable to the correlation given in section 1.2.1 which stated that aliphatic glucosinolates are hydrolysed at a higher rate than their indolyl analogues.[49]

An attempt was also made to measure the turnover of naphthylmethyl glucosinolate directly by UV spectrophotometry. Analysis of this compound in phosphate buffer showed two absorption maxima at approximately 220 and 280 nm. Upon addition of myrosinase these maxima decreased and increased respectively (figure 20).

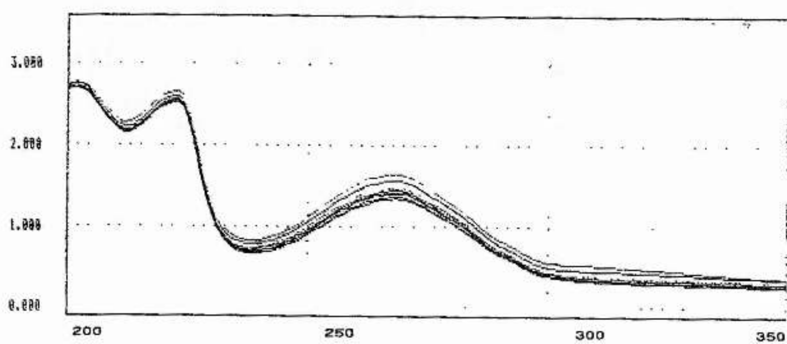


Figure 20 UV trace showing reaction of naphthylmethyl glucosinolate with myrosinase.

Thus by monitoring the decrease in absorbance at 227 nm it was possible to examine the metabolism of naphthylmethyl glucosinolate by myrosinase.

In the previous studies the substrate concentration was high meaning the enzyme was saturated with substrate. Under these conditions, the maximum rate is observed and this is limited only by the catalytic rate of myrosinase. However, if the quantity of substrate is limited then the majority of the enzyme is free. Thus the rate of reaction depends on the binding efficiency between substrate and enzyme at that concentration. The term used to denote this efficiency is K_m, the Michaelis constant. In practice, K_m is defined as the concentration of substrate at which half-maximal rate is observed.[138] This value provides an inverse measurement of how tightly the substrate is bound by the enzyme i.e. high K_m, weakly bound substrate.

Hence the turnover of naphthylmethyl glucosinolate by myrosinase was monitored at a range of glucosinolate concentrations (0.025, 0.0125 and 6.25×10^{-3} mM) in an attempt to determine the binding efficiency (K_m) of this glucosinolate and compare this with sinigrin. The rates obtained were corrected to 1 unit of enzyme and converted to their reciprocals, allowing a Lineweaver-Burk plot to be drawn (figure 21).

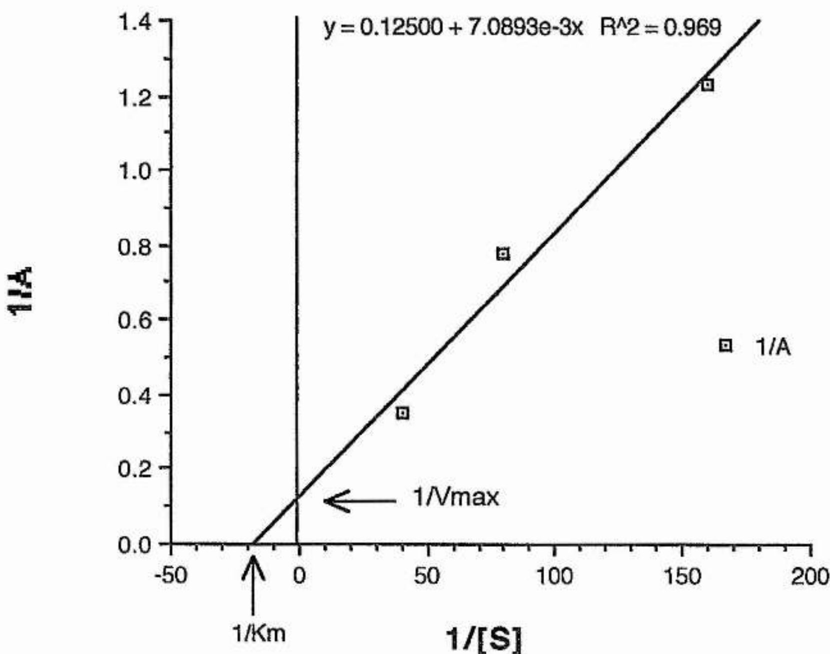


Figure 21 Lineweaver-Burk plot for naphthylmethyl glucosinolate.

From the graph, (figure 21) K_m was found to be 0.06 mM. An error value of ± 0.03 mM was calculated by linear regression analysis using "ENZFITTER." By comparison, sinigrin has a K_m of 0.42 ± 0.05 mM under identical conditions.[123] This result implies that naphthylmethyl glucosinolate binds more tightly at the active site of myrosinase than sinigrin.

In addition, the graph should allow a value for the maximum rate, V_{max}, to be estimated. However, this requires the availability of an accurate value for the extinction coefficient difference between the starting material and product. Given the small amount of glucosinolate prepared, it was not practical to carry out extensive studies to determine this value. Thus the maximum rate was not accurately quantified. Nevertheless, the rate given previously for the turnover of naphthylmethyl glucosinolate, as measured by release of

glucose, should also equate to V_{max} as this was calculated at high substrate concentration where the enzyme operates at maximum rate.

It should be noted that the accuracy of K_m for naphthylmethyl glucosinolate is limited by the presence of only three points on the graph. Analysis of additional concentrations was attempted but this required the use of 1 mm pathlength cells instead of the normal 10 mm cells. These introduced a problem of incomplete mixing of enzyme and substrate resulting in unusual effects.

4.3.3 Measurement of volatiles released upon hydrolysis of alkyl glucosinolates

The volatiles released following hydrolysis of glucosinolates can be sampled and analysed by GC/MS. This was performed as described hereafter for the alkyl glucosinolates.

The volatiles were extracted from the headspace of a reaction vessel containing the alkyl glucosinolate and myrosinase. These were absorbed into a porous polymer (Tenax) which was subsequently thermally desorbed and analysed by GC/MS at the SCRI.

Before analysis of the synthetic glucosinolates it was necessary to define the optimum quantity of substrate and enzyme and also the reaction duration. The equipment at SCRI detects micromolar quantities of volatiles and it was important to use the correct quantity of glucosinolate so that the volatiles could be detected but would not saturate the GC column. Thus preliminary experiments were carried out at 37 °C using sinigrin as shown in table 14.

Experiment no.	[Sinigrin]/ mM	Volume of myrosinase (μl)[a]	Quantity of isothiocyanate produced (μg)[b]	Reaction time/h
1	1×10^{-3}	1000	100	1
2	2×10^{-5}	80	2	4
3	1×10^{-4}	200	10	18

[a] Standard myrosinase solution (see section 7.5.1.1)

[b] Maximum quantity of isothiocyanate formed assuming 100% glucosinolate conversion.

Table 14 Experiments performed to determine optimum conditions for glucosinolate analysis.

GC/MS analysis showed the desired 1-propenyl isothiocyanante, with a retention time of 19.00 minutes, in all three reactions. The reaction conditions employed for experiment 2 were deemed to be the most suitable for analysis of the volatiles from synthetic glucosinolates, as a significant quantity of isothiocyanate had been produced in a relatively short time period.

Thus the alkyl glucosinolates were reacted with myrosinase as given in experiment 2 (table 14) at 37 °C. The headspace of the reaction vessel was sampled allowing absorption of the volatiles into the porous polymer which was analysed by thermal desorption GC/MS. All the glucosinolates were found to release the corresponding isothiocyanates which were identified by comparison with a reference library. The spectra obtained for these compounds were similar to those shown for propyl and pentyl glucosinolates (figures 22 and 23).

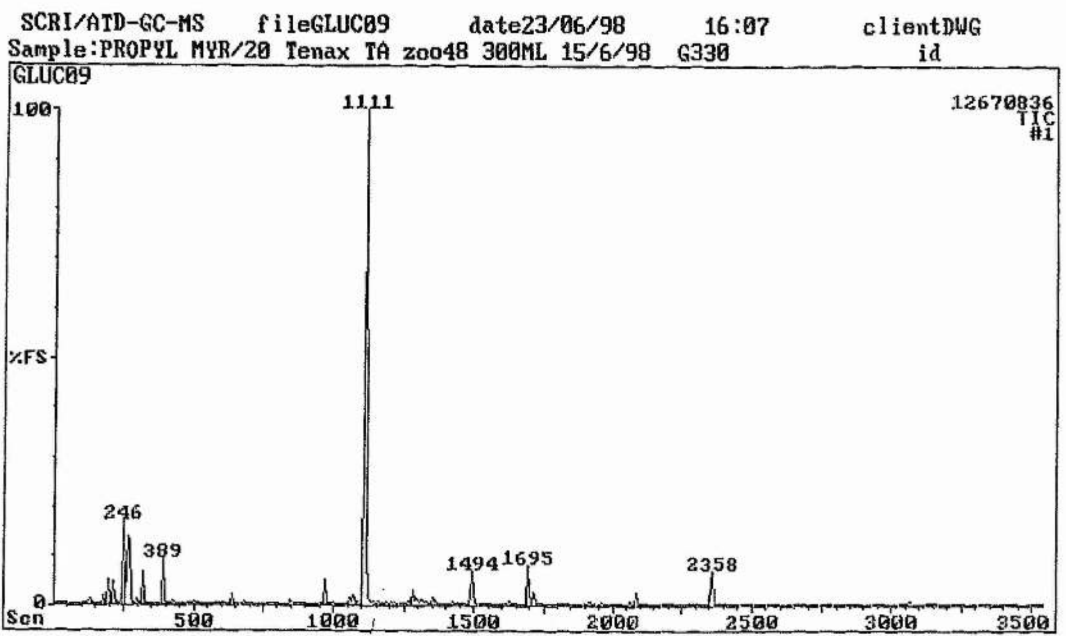


Figure 22 GC/MS chromatogram of the volatiles released upon hydrolysis of propyl glucosinolate.

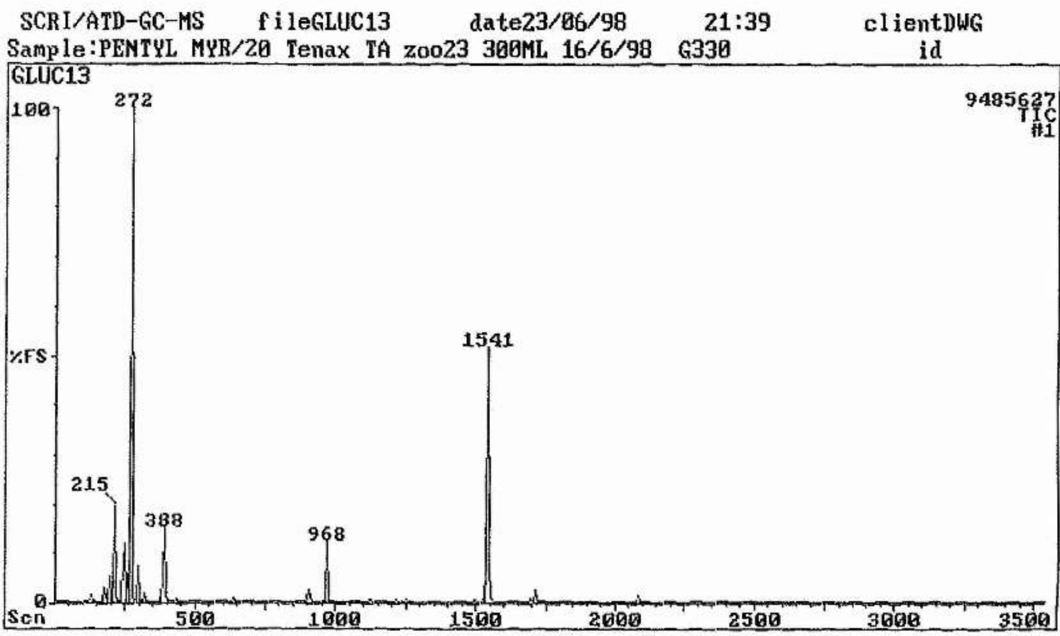


Figure 23 GC/MS chromatogram of the volatiles released upon hydrolysis of pentyl glucosinolate.

In addition, heptyl **165** and nonyl isothiocyanates **166** were identified by comparison with authentic compounds. These were prepared by reaction of the corresponding amine with thiophosgene **167** (scheme 56).

$$R{-}NH_2 + \underset{\mathbf{167}}{Cl_2C{=}S} \xrightarrow{i} R{-}NCS$$

165 $R = (CH_2)_6CH_3$ (25%)
166 $R = (CH_2)_8CH_3$ (54%)

Scheme 56 *Reagents and conditions*: i, Dichloromethane, water, 12 h, room temp.

In addition to the isothiocyanates observed, the corresponding nitriles were observed for butyl and heptyl isothiocyanates (table 15).

Glucosinolate	Retention time of RCN/m	Retention time of RNCS/m	Peak area of RCN	Peak area of RNCS	Ratio of RNCS : RCN
Butyl	15.68	23.14	467351	16183041	3.5:1
Heptyl	27.05	33.18	258348	13891327	54:1

Table 15 Comparison of isothiocyanates and nitriles from butyl and heptyl glucosinolates.

The release of nitriles from these synthetic alkyl glucosinolates was unexpected as the reactions were performed at a buffered pH of 7.0. These conditions are not favourable for nitrile formation which usually occurs around pH 4.0. This is reinforced by the analysis of propyl, pentyl and nonyl glucosinolates which did not afford the corresponding nitrile but yielded the isothiocyanate exclusively. Moreover, the quantity of heptyl cyanide is extremely small. However, the ratio of nitrile to isothiocyanate is significant for butyl glucosinolate although the analysis conditions for this reaction were identical to the others.

The reasons for this are unknown. The nitrile is unlikely to result from thermal decomposition of the glucosinolate as this has been reported to occur at elevated temperatures only, i.e. 125 °C, and would not occur at 37 °C.[139] The only other alternative is thermal decomposition of the isothiocyanate to afford the nitrile. Loss of SH from alkyl isothiocyanates of chain length C_6 and above during mass spectral analysis has been reported.[140]

Interestingly, Benn reported the hydrolysis of propyl glucosinolate by *Thlaspi arvense* L. while examining the mechanism behind thiocyanate formation.[64] Instead of obtaining the predicted thiocyanate he obtained a mixture of propyl isothiocyanate and butyronitrile (3:4) at pH 6.7. This was not mirrored by the myrosinase catalysed hydrolysis of propyl glucosinolate, as discussed earlier, as only the isothiocyanate was obtained.

These results could indicate that although the mechanisms of glucosinolate breakdown are well documented, alternative pathways and exceptions to the rules exist.

The release of isothiocyanates or otherwise from the complex glucosinolates were not examined as these products are unlikely to be volatile.

4.4 CONCLUSIONS

4.4.1 Electrophysiology

Electrophysiological recordings using *D. radicum* female flies were obtained for the alkyl and naphthylmethyl glucosinolates in addition to synthetic samples of a number of naturally occurring glucosinolates. This represents the first electrophysiological data obtained for both synthetic and unnatural glucosinolates. A crude correlation was observed whereby the stimulatory activity of the glucosinolate increased with the length of the side chain. This was postulated previously with a more heterogeneous group of glucosinolates, but had not

been confirmed. Interestingly the insect showed little or no response to naphthylmethyl glucosinolate which was prepared as an analogue of glucobrassicin, the most potent oviposition stimulant. This indicates that the nitrogen of the indole ring in glucobrassicin is important for activity. Furthermore, comparison of the alkyl, naphthylmethyl and naturally occurring glucosinolates showed the alkyl derivatives to be more potent than the aryl alkyl derivatives and alkenyl analogues. Analysis of (7-methoxycarbonylheptyl) glucosinolate has yet to be carried out along with further behavioural studies using the other synthetic glucosinolates. These investigations will allow the glucosinolate activity in the presence of the waxy leaf surface to be determined. Thus the crude electrophysiological correlation between the glucosinolate side chain length and the ovipositional activity will be examined further.

4.4.2 Reaction of synthetic glucosinolates with myrosinase

The glucosinolates were shown to be substrates for the enzyme myrosinase as measured by glucose release upon hydrolysis. The alkyl glucosinolates displayed similar rates of hydrolysis to the natural substrate sinigrin. The complex glucosinolates, naphthylmethyl and (7-methoxycarbonylheptyl) glucosinolates, displayed marginally lower rates of hydrolysis when compared with the alkyl analogues and sinigrin. The catabolism of naphthylmethyl glucosinolate was also followed by UV spectrophotometry allowing an approximate value of 0.06 ± 0.03 mM to be calculated for K_m. This value suggests naphthylmethyl glucosinolate binds more tightly to the active site of myrosinase than sinigrin ($K_m = 0.42 \pm 0.05$).

The release of volatiles upon hydrolysis of alkyl glucosinolates was examined by sampling the head-space of these reactions. The compounds were absorbed into a porous polymer which was subsequently analysed by thermal desorption GC/MS, at the SCRI, showing the corresponding isothiocyanate to be released in every case. The corresponding nitrile was also observed for both butyl and heptyl glucosinolates.

CHAPTER 5

CHEMICAL MODEL STUDIES ON THE OXIDATION OF ISOTHIOCYANATES

5.1 ESTABLISHING A CHEMICAL MODEL SYSTEM

When using a chemical reagent to model a biological process several factors need to be considered carefully. In this case the putative oxidation of isothiocyanates by cytochrome P450 enzymes was to be studied. This meant the choice of an oxidising agent was limited to those which would be relatively specific, efficient, and capable of transferring an oxygen atom to the substrate under mild conditions, thereby mimicking the enzyme. Therefore the reagents used previously to oxidise isothiocyanates, namely mercuric oxide[70] and palladium chloride,[71] were not suitable for this investigation as these produced isocyanates using extremely harsh conditions.

The oxidising nature of cytochrome P450 enzymes has been studied previously using model systems. Peracids, such as peroxytrifluoroacetic acid (PTFA) **168**, have been used in mechanistic models of the cytochrome P450 hydroxylation of aromatic compounds and have proved to be good substitutes.[141] Similarly, PTFA has been used to investigate the microsomal metabolism of the insecticide parathion **169**,[142] which is known to inhibit mono-oxygenase enzymes in the liver and lung.[115] Oxidation of parathion **169** (scheme 57) by PTFA leads to the formation of its oxygen-containing analogue, paraoxon **170**.

$(EtO)_2-P(=S)-O-C_6H_4-NO_2$ **169**

$\xrightarrow{CF_3-C(=O)-O-OH\ \ \mathbf{168}}$

$(EtO)_2-P(=O)-O-C_6H_4-NO_2$ **170** + [S]

Scheme 57 PTFA oxidation of parathion to paraoxon.

This conversion is proposed to proceed *via* transfer of a single oxygen atom to allow formation of a cyclic phosphorus-oxygen-sulfur intermediate **171** which can rearrange to yield paraoxon **170** (scheme 58).

Scheme 58 Mechanism postulated for the formation of paraoxon from parathion.

It is therefore conceivable that isothiocyanates could be converted to isocyanates by PTFA in a similar manner (scheme 59) *via* an oxathiiranium-type intermediate **172**.

Scheme 59 Proposed mechanism for the oxidation of isothiocyanates by PTFA.

Moreover, peracids have been used to oxidise a wide range of sulfur-containing organic compounds,[143] and as such provided an ideal starting point to investigate the oxidation of isothiocyanates.

5.2 DETECTION OF ISOCYANATE PRODUCTS

The next thing to consider was how to monitor the oxidation reactions in terms of detecting and distinguishing between isothiocyanates and isocyanates. The principle techniques available are thin layer chromatography (TLC), infra-red spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry. Analysis of BITC and BIC by TLC, using a number of ethyl acetate:petroleum ether (bp 40-60 °C) solvent systems, showed both compounds to have the same R_f values and to be indistinguishable from each other. This indicated that TLC would be of little use to monitor the oxidation of isothiocyanates to isocyanates. However, analysis of BITC and BIC standards by IR suggested that this technique may be useful in evaluating the reaction. Although the spectra obtained are reasonably similar, the -NCS stretching vibration of BITC is found at 2100 cm^{-1} whereas the -NCO stretching vibration of BIC occurs at 2240 cm^{-1}. Literature values are given as 2050-2150 cm^{-1} for the -NCS stretch of aromatic isothiocyanates and 2275-2230 cm^{-1} for -NCO stretches.[125] This subtle difference should allow differentiation of isocyanates and isothiocyanates.

Similarly, reference spectra of BITC and BIC show that these two compounds may be distinguished by NMR.[144] The ^{1}H NMR spectrum of BITC, recorded in $CDCl_3$, shows a multiplet due to the presence of aromatic protons at δ_H 7.20-7.40 and a singlet corresponding with Ph-CH_2- at δ_H 4.70. The ^{1}H spectrum of BIC shows the position of the aromatic protons to be identical with BITC but the signal due to the methylene protons has moved upfield to δ_H 4.45. ^{13}C NMR also shows differences with the benzylic carbon for BITC occurring at δ_C 48.64 compared with δ_C 46.45 for BIC. The central carbon of

the isothiocyanate and isocyanate functional groups also differs from δ_C 132.40 in BITC to δ_C 123.24 in BIC.

Undoubtedly, mass spectrometry is the simplest technique, of those discussed thus far, for identifying the products of isothiocyanate oxidation due to the compounds of interest having different masses from their isothiocyanate precursors. It should therefore be easy to determine if reaction has occurred. Moreover, the technique of GC/MS should allow efficient separation of reaction mixtures and identification of the species present.

5.3 OXIDATION OF ISOTHIOCYANATES USING *meta*-CHLOROPERBENZOIC ACID (*m*-CPBA)

m-CPBA was the first peracid used as it is readily available and is a moderately powerful oxidising agent which has been used extensively to oxidise sulfur compounds.[143] A number of reactions using *m*-CPBA were performed before oxidation of BITC to BIC was realised. These reactions concentrated on varying the experimental procedure i.e. the solvent, the relative quantities of isothiocyanate and *m*-CPBA, the reaction temperature, and the methods of detecting the isocyanate product. Eventually, the oxidation of BITC was achieved by reaction with 5 molar equivalents of *m*-CPBA. The reaction solution was heated under reflux for 18 hours before the products were extracted using diethyl ether. Analysis by TLC indicated a mixture of products had formed which were purified by column chromatography. The resulting fractions were analysed by NMR, with fraction 5 showing the presence of a compound which could be BIC, identified by a signal at δ_H 4.40. In an attempt to clarify this, the sample was analysed by GC/MS which showed a mixture of products one of which was BIC **173**. This compound showed a retention time of 5.41 minutes and a relative molar mass (RMM) of 133. The fragmentation pattern fitted with the isocyanate and the compound library indicated the product to be BIC. Furthermore, an

authentic sample of BIC was analysed by GC/MS and this showed the same retention time and mass spectrum to the BIC identified from fraction 5.

In addition to BIC **173**, two other species were detected having molecular ions of mass 179 and 245. The latter compound appeared to be the amide **174**. This could arise from nucleophilic attack of *m*-chlorobenzoic acid (*m*-CBA) **175**, upon the isocyanate **173** (scheme 60). This acid is the by-product of all *m*-CPBA oxidations and is usually present as a contaminant in commercial material. Therefore this side reaction could significantly reduce the yield of the isocyanate when *m*-CPBA has not been separated from *m*-CBA. The other product, having a molecular mass of 179, was not identified but may result from the original reaction mixture, work-up, or purification of the reactive isocyanate. From the GC chromatogram, the compound of RMM 179 was observed to be the major product of fraction 5 and **174**, the minor product.

Scheme 60 Formation of **174** from reaction of BIC with *m*-CBA.

This experiment also demonstrated that of all the detection methods discussed previously, GC/MS was the most efficient for detecting isocyanates. NMR spectroscopy, although

giving an indication that the desired product may be present, failed to identify BIC conclusively. This was not due to the instrumentation itself but to the level of purity required for analysis. Mass spectrometry and IR would also have failed in this case as the sample contained three components.

Since it had been established that BITC could be oxidised to BIC by *m*-CPBA, it was then necessary to determine how long complete reaction would take. Therefore, the previous experiment was repeated with aliquots being removed after 1, 5, 10, 26 and 53 hours to determine if a pattern could be deduced. Each aliquot was worked-up and analysed by GC/MS. It was observed that the ratio of BIC/BITC did not alter significantly between aliquots suggesting that the reaction occurred very quickly and the amount of isocyanate and isothiocyanate did not alter thereafter. It was apparent that all the BITC had not been consumed suggesting that perhaps there was not a high enough equivalency of oxidising agent to drive the reaction towards BIC formation. In addition, other products were observed in the GC trace, which could result from the reaction or the conditions of work-up.

To further examine the temperature dependence of the oxidation reaction, two experiments were set up, one performed at 0 °C and the other heated under reflux in acetonitrile. GC/MS analysis showed the presence of BIC in both cases. Integration of the peak areas suggested an increase in product when the reaction mixture was heated under reflux compared with the reaction carried out at 0 °C.

Before proceeding to the next section of work an interesting question was considered: would isocyanate formation be more efficient using a more reactive isothiocyanate? In an attempt to answer this question MPITC **176** was used as a substrate for *m*-CPBA oxidation. This aromatic isothiocyanate is more reactive than BITC due to the electron withdrawing effect of the benzene ring. However, the *p*-methoxy group ensures that it is not too reactive. The main product formed was an insoluble cream-coloured precipitate

identified by mass spectrometry as a mixture of the thiourea derivative of the starting material **177** and the urea derivative of the starting material **178**.

CH_3O–C_6H_4–NCS

176

CH_3O–C_6H_4–NH–C(=S)–NH–C_6H_4–OCH_3

177

CH_3O–C_6H_4–NH–C(=O)–NH–C_6H_4–OCH_3

178

The formation of these ureas is likely to occur in two stages. First hydrolysis of the isocyanate occurs to yield a carbamic acid which, being unstable, undergoes decarboxylation to yield *p*-methoxy aniline. The *p*-methoxy aniline then reacts with the isothiocyanate or isocyanate to form the thiourea or urea respectively. The aromatic isocyanate is very susceptible to hydrolysis and hence dry solvent should have been used. As a result, the reaction was repeated under nitrogen using dry acetonitrile. This time only a small amount of the urea/thiourea precipitate was observed. The main product of the reaction was analysed by GC/MS which showed a large peak corresponding with the starting material and a number of small peaks, one of which corresponded to 4-methoxyphenyl isocyanate **179**. Thus the reaction did not appear to be any more efficient than the oxidation of BITC.

The same reaction was also attempted with 4-nitrophenyl isothiocyanate to investigate the effect of an electron withdrawing substituent. However, no isocyanate was detected.

In retrospect *m*-CPBA proved to be effective in oxidising isothiocyanates to isocyanates and is a good reagent as it is cheap and commercially available. However, the presence of undesirable side-products diminishes the usefulness of this reagent as a model system for P450 enzymes. These could be reduced by separating *m*-CPBA and *m*-CBA prior to use by

washing with a phosphate buffer at pH 7.5 and drying under reduced pressure.[145] However, this would likely prove to be a fruitless procedure as *m*-CBA is produced in the oxidation reaction and could therefore still react with the isocyanate product. The problem of hydrolysis could be remedied by using dry solvent and an inert atmosphere but this again is likely to have little effect as an aqueous work-up is employed upon reaction completion. This is necessary to remove any excess *m*-CPBA and *m*-CBA prior to GC/MS analysis, as these acids may have a detrimental effect on the GC capillary column.

5.4 OXIDATION OF ISOTHIOCYANATES BY PTFA

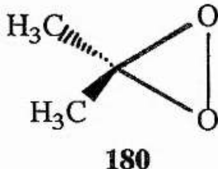
In an attempt to increase the yield of isocyanate obtained, PTFA **168**, being a stronger oxidising agent than *m*-CPBA, was used to investigate BITC oxidation. BITC was added to PTFA, generated *in situ* from trifluoroacetic anhydride and hydrogen peroxide, in dichloromethane at 0 °C. The product was analysed by GC/MS, after work-up, which showed the presence of three components. The major species, appearing in 83% relative abundance, had a molecular ion of mass 151 neatly corresponding with addition of water to BIC (RMM 133) to form the carbamic acid. However, this species should decompose, under the high temperature analysis conditions of the GC/MS and should not be observed. The other two species detected by GC/MS were BIC and a compound of RMM 167 which was not identified. These were found in 10% and 7% relative abundance respectively. It should be noted that there was no trace of BITC indicating the reactant had been completely consumed.

The oxidation of BITC by PTFA appeared to compare favourably to the oxidation using *m*-CPBA regarding the amount of BIC produced. The reaction proceeded fairly rapidly, under mild conditions, without optimisation. Unfortunately, one of the major problems with this oxidising agent is the difficulty of maintaining dry conditions. Generation of PTFA leads to the formation of water which could be removed using molecular sieves. However, the

reaction requires an aqueous work-up to remove unreacted acid and this is likely to lead to hydrolysis of the product. In addition, the oxidising agent must be generated making the procedure more time consuming.

5.5 OXIDATION OF BITC USING DIMETHYL DIOXIRANE (DMD)

The next oxidising agent examined was DMD **180**.[146] This is a relatively new oxidising agent which has been shown to transfer an oxygen atom to a range of substrates, including many sulfur-containing compounds.[147,148] However, it has not been used to oxidise isothiocyanates. An advantageous feature of this reagent is that it is non-nucleophilic and neither it, nor its reduction product acetone, will react with isothiocyanates or isocyanates. It exists as an azeotrope with acetone, being prepared from a mixture of oxone®, water, acetone and sodium hydrogen carbonate at low temperature. It can be conveniently dried prior to use with molecular sieves so that the risk of isocyanate hydrolysis is diminished.



180

Oxidation of BITC was attempted with 5 molar equivalents of freshly prepared DMD at 0 °C. The reaction was performed under nitrogen using dry acetone to minimise hydrolysis of the isocyanate product. Analysis of the reaction mixture after 2 hours, by GC/MS, showed BIC **173** to be the predominant species. Integration of the peak areas, on the chromatogram, implied that this compound accounted for 81% of the material present. One of the other components was thought to be an acetone derivative, having a molecular ion of mass 88, and this accounted for approximately 9% of the total material. The other

component was identified as N,N'-dibenzyl urea **181** and this compound accounted for the remaining 10% of the total material.

181

The formation of the above urea is similar to the formation of the urea and thiourea derivatives observed in the reaction with 4-methoxyphenyl isothiocyanate. This indicates a small amount of water was present in the reaction mixture.

Authentic samples of BIC and BITC were also analysed and the chromatograms obtained were compared with the chromatogram of BIC produced in the oxidation reaction (figure 24). This comparison showed that the isothiocyanate had been completely consumed in the oxidation reaction and that BIC had been produced. It also showed that BITC and BIC exhibited significantly different retention times and could not be mistaken for each other.

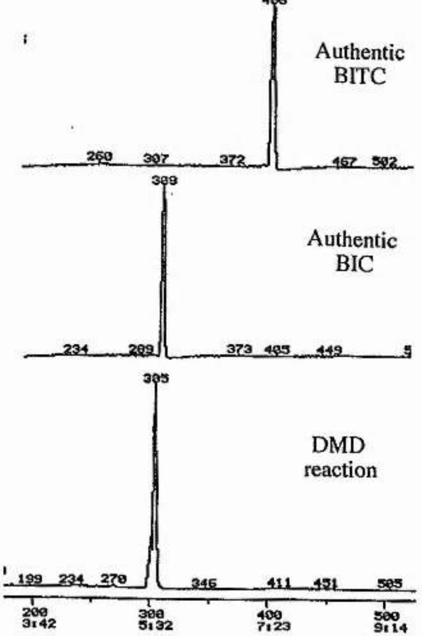


Figure 24 Comparison of chromatograms for authentic BITC, authentic BIC and the oxidation reaction.

This experiment showed that DMD oxidation was very efficient, producing large amounts of BIC in a short period of time, under mild conditions and with very little side-products being formed. The problem of hydrolysis was minimised by drying the oxidising agent prior to use. In addition, the reaction solution could be directly analysed by GC/MS as it contained nothing which would cause damage to the GC column. The only drawback is the generation of DMD which can be laborious. The DMD is produced in low yield as a dilute solution with acetone. Thus in order to carry out oxidations on a large scale a considerable amount of time is needed to prepare the amount of oxidising agent required.

5.6 DIRECT COMPARISON OF OXIDISING AGENTS.

Until now *m*-CPBA, PTFA and DMD have been compared for their availability, ease of use and efficiency in oxidising BITC to BIC. However, this comparison failed to take into account that each oxidising agent used a different set of reaction conditions, different time scales were employed for each oxidation and different quantities of starting materials were used. These factors made it difficult to compare the efficiency of the oxidising agents directly and a controlled experiment had to be performed for this purpose. In planning such an experiment, several factors had to be considered:

1. **Detection of the product.**

   Bearing in mind the reactive nature of BIC, the different work-up procedures required for each oxidising agent and the necessity to control every variable of the experimental procedure, the simplest method was to identify BIC *in situ*.

2. **Instrument of detection.**

The instrument of choice, previously, was GC/MS. However, as accurate quantitative analysis was desired, a GC with a flame ionisation detector (FID) was more attractive than a GC coupled to a mass spectrometer. Furthermore, access to the GC was more exclusive than the GC/MS and it was thought this may help in obtaining reproducible results. Unfortunately, analysis of several different concentrations of BIC failed to show any difference in peak height or area. A number of different detection sensitivities were attempted and different concentrations of BITC were also attempted but to no avail. GC/MS analysis was therefore employed which showed a marked difference in peak area for each concentration of BIC. This technique was used for the remainder of this investigation.

3. **Method of quantitation.**

To perform accurate quantitative analysis it was necessary to calibrate the instrument using a range of standards of known concentration. The standards employed for this experiment were 4.45×10^{-3} M, 8.91×10^{-3} M, 13.40 M x 10^{-3}, 17.80×10^{-3} and 20.00×10^{-3} M BIC, in acetone, where 17.8×10^{-3} M was the maximum concentration of BIC that could be formed in the oxidation reactions. These standards also contained 3.20×10^{-2} M dibenzyl ether which acts as an internal standard to compensate for experimental errors. Analysis of the standards in triplicate, and integration of the peak areas, allowed a calibration graph to be plotted as the ratio of BIC to internal standard against concentration of BIC.

As calibration of the GC/MS had proved successful the comparison study was commenced, whereby BITC was oxidised by *m*-CPBA, PTFA and DMD, with the following factors being kept constant:

i) The quantity of BITC used.

ii) The molar equivalency of each oxidising agent.

iii) The concentration of BITC.

iv) The temperature of each reaction.

v) The reaction time.

vi) The analysis of each reaction mixture by GC/MS.

While the oxidation reactions were taking place, the GC/MS was recalibrated, using the standards detailed above. Due to time constraints, each standard was only analysed once. Recalibration was performed as a precaution to ensure no variation in analysis conditions had occurred since the previous calibration. The graph obtained is shown in figure 25.

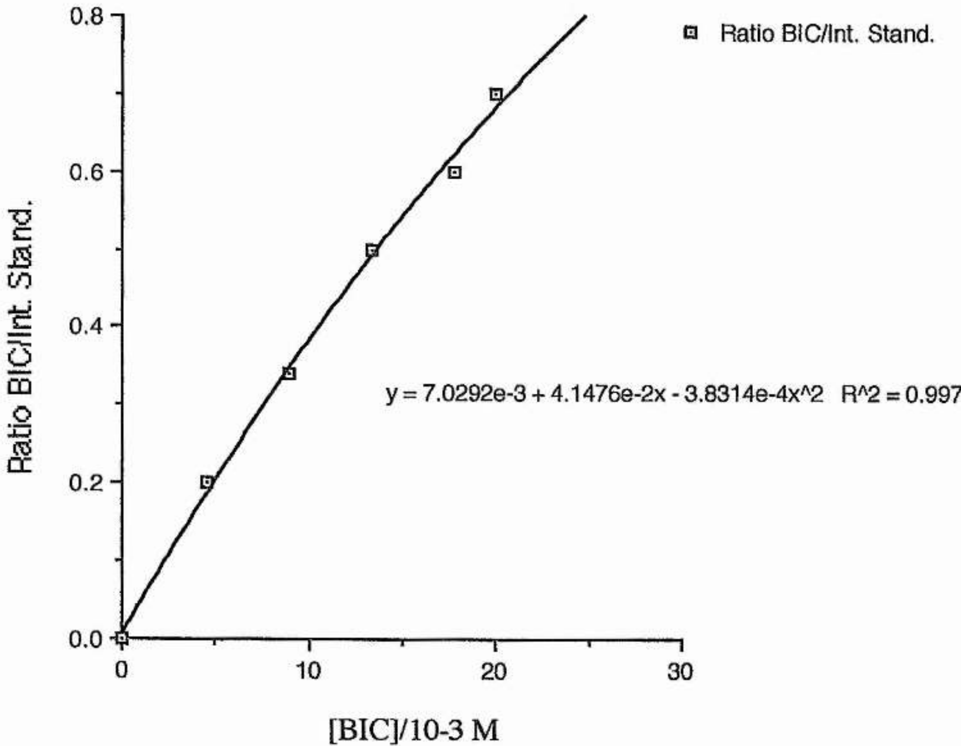


Figure 25 BIC calibration graph.

Analysis of the reaction mixtures by GC/MS and comparison with the calibration graph allowed the concentration and yield of BIC formed in each reaction to be calculated (Table 16). The yields are accurate to ± 10 % based on the triplicate calibration.

Oxidising agent	Percentage yield BIC
DMD	58%
m-CPBA	25%
PTFA	5%

Table 16 Percentage yields for oxidation of BITC.

These results clearly show that DMD is the most efficient oxidising agent, giving the highest yield of BIC under the given set of conditions. Moreover, by comparing the GC traces for all three experiments the oxidation using DMD appeared to be the cleanest giving no side products. The chromatogram for the *m*-CPBA oxidation showed additional peaks which were tentatively assigned to side-products. These may be responsible for the lower yield of BIC. Similarly the PTFA oxidation showed the presence of side-products. However, the major problem with this oxidising agent was the speed of the reaction. This was indicated by the large proportion of unreacted BITC remaining in contrast to the other two oxidation experiments.

It should be noted that the percentage yields of BIC may have been greater if the experiment had been performed over a longer time scale. However, this was impractical as it would have meant analysing the reaction mixtures on a different day from the calibration standards or analysing one or more of the reaction mixtures on a different day from the others. This

would have introduced another potential source of error to the study which is obviously disadvantageous.

5.7 FURTHER WORK ON THE DMD MODEL SYSTEM

5.7.1 Oxidation of a range of isothiocyanates

Thus far DMD had been used to oxidise only BITC to the corresponding isocyanate. In order to probe the generality of the DMD reaction, the oxidations of phenyl isothiocyanate (PITC), phenethyl isothiocyanate (PEITC), methoxyphenyl isothiocyanate (MPITC) and butyl isothiocyanate (BuITC) were investigated. These led to the formation of phenyl isocyanate (PIC) **182**, phenethyl isocyanate (PEIC) **183**, methoxyphenyl isocyanate (MPIC) **184**, and butyl isocyanate (BuIC) **185** respectively. These were all detected by GC/MS analysis and all showed chromatograms similar to that for the oxidation of PITC to PIC (figure 26) with minimal side products and no trace of starting material. This indicated that DMD could be used as a model system to study the oxidation of isothiocyanates as it had been shown to oxidise a range of these compounds.

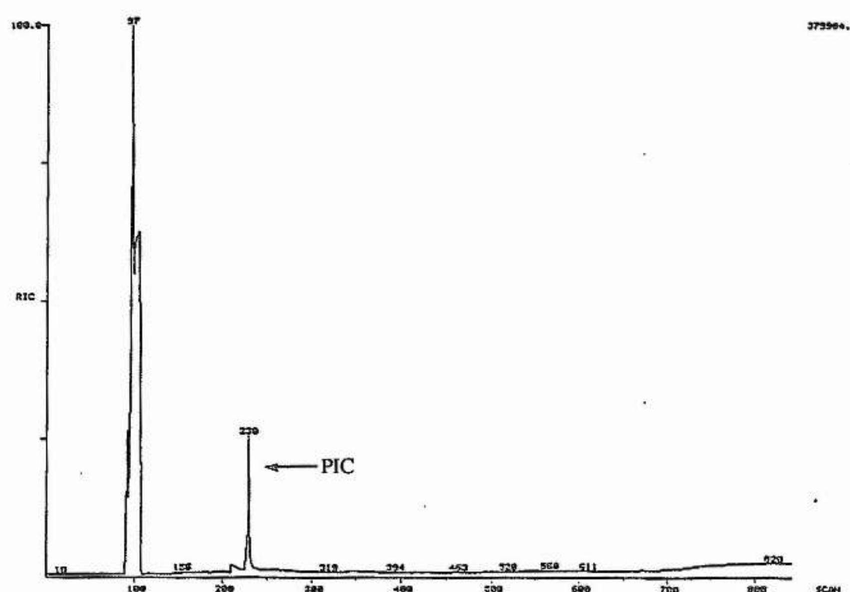


Figure 26 GC/MS chromatogram showing PIC **182** obtained from DMD oxidation of PITC.

5.7.2 Attempted isolation of isocyanates

In the previous experiments the evidence for the formation of isocyanates from isothiocyanates came from GC/MS analysis of DMD, PTFA and *m*-CPBA reaction mixtures. Under these circumstances it could be argued that isocyanates were an artefact of the GC analysis conditions rather than a product of isothiocyanate oxidation. To prove conclusively that isocyanates were being formed from oxidation of isothiocyanates, isolation of the former was attempted.

The first attempt at isolation used BITC which was oxidised by DMD and the progress of the reaction monitored by GC/MS. Once the BITC peak had disappeared from the GC trace, the solvent and excess DMD were removed under reduced pressure. Analysis of the remaining residue by ^1H NMR showed a singlet integrating to two protons at δ_H 4.20 and a multiplet corresponding to the aromatic protons at δ_H 7.20-7.45. Although these signals were more characteristic of BIC than BITC, the singlet at δ_H 4.20 was rather low for the desired product. In addition a vast number of other peaks were observed. In fact GC/MS analysis showed six major components were present, one of which was BIC.

In an attempt to achieve a better result, the experiment was repeated and the product analysed by NMR as before (see Tables 17 and 18 for the NMR data).

Analysis	δ value (ppm)	Assignment
^1H	8.93	COO*H*/CSO*H*
	7.22-7.34	Ar*H*
	4.30	N*H*
	4.45	$PhCH_2NCO$

Table 17 ^1H NMR data for second attempted isolation of BIC.

Analysis	δ value (ppm)	Assignment
^{13}C	207.77	*C*SOH
	139.17	Ar*C*-1
	128.56, 129.00, 129.74, 130.08	Ar*C*
	44.87, 44.99	$PhCH_2NCO$

Table 18 ^{13}C NMR data for second attempted isolation of BIC.

This time the characteristic signal due to the methylene protons of BIC was observed in the correct position at δ_H 4.45. However, both ^1H and ^{13}C spectra indicated the presence of an acidic species of a structure similar to **186** and **187**.

186 **187**

These compounds would result from hydrolysis of BITC and BIC respectively. Infra-red analysis showed evidence for the above structure-type with acid, amine and carbonyl functional groups observed. There was no peak observed at 2240 cm^{-1} corresponding to the N=C=O group of BIC indicating that this species was not present. Analysis of the NMR solution by GC/MS failed to show the presence of an acidic species. This is not unexpected as this type of compound would be unlikely to survive the GC analysis conditions. The resulting amine was not observed either but BITC was found to be present. BIC was also observed, perhaps resulting from decomposition of a urea or a carbamic acid. In addition, the analysis showed a number of other compounds to be present, most of which could not be identified.

The main difficulty with trying to identify isocyanates from the residue remaining after solvent removal is the presence of other species which may, or may not, be reacting with

the isocyanate. To overcome this problem, isocyanates could be separated from the residue by distillation. However, this is not as straight-forward as it seems, the major problem being the scale of the reaction. In order to completely oxidise 500 mg of isocyanate, approximately 500 ml of DMD/acetone is required due to the low yielding DMD preparation, and the requirement to use 5 equivalents for 100% conversion. This presents two problems namely the necessity to remove 500 ml of acetone once the reaction is complete and the small quantity of BIC left for distillation. To overcome this latter difficulty, micro-distillation apparatus could be employed along with a high boiling solvent from which the isocyanate could be distilled without the distillation flask going dry. Careful consideration identified diphenyl ether, which has a boiling point of 258 °C at atmospheric pressure, as an appropriate solvent. A trial distillation was carried out, whereby diphenyl ether, a small amount of acetone and PIC were mixed together and separated by distillation, to determine if the procedure was viable. This proved successful and the oxidation reaction was commenced.

As little success had been realised with BITC, PITC was selected for this experiment. As before, the oxidation reaction was performed under nitrogen using dry DMD and was found to be complete by GC/MS analysis of the reaction mixture. The excess acetone and DMD were removed under reduced pressure to leave a small volume of product, in acetone, to which diphenyl ether was added. This saw the formation of two layers which had not been observed in the trial distillation. This may suggest that the PIC containing layer was more aqueous than organic. Distillation was commenced under nitrogen and with vigorous stirring, to remove the remaining acetone, with reduced pressure being applied to ensure completion. The remaining solution was then allowed to cool to room temperature which saw the appearance of considerable amount of yellow solid. Further distillation under a greater reduced pressure resulted in the entire contents of the reaction flask distilling over, including the diphenyl ether, to leave the yellow solid behind. IR analysis failed to identify any PIC in the distillate therefore the yellow solid was analysed by TLC which suggested the presence of diphenyl ether and perhaps PIC. In an attempt to recover the latter

compound flash chromatography was performed which yielded two major fractions found to be diphenyl ether and *N,N'*-diphenylurea **188**. The formation of the urea **188** indicates that PIC was produced from oxidation of PITC. Hydrolysis then occurred to yield the amine which reacted with another molecule of PIC to form the urea in 94% yield.

O
N N
H H
188

It could also be argued that *N,N'*-diphenylurea came from the thiourea which was oxidised to the urea. This is unlikely as the DMD was removed before distillation and the solid, found to be the urea, was observed after distillation. Hence if oxidation of the thiourea was to occur a solid, due to the thiourea, would have appeared before distillation. To disprove the thiourea oxidation, *N,N'*-diphenylurea and *N,N'*-diphenylthiourea were analysed by GC/MS. The chromatogram for the latter compound showed peaks corresponding to the thiourea itself and also PITC and aniline. No trace of the thiourea or PITC was observed in the trace for *N,N'*-diphenylurea or in the original trace acquired before distillation. This indicates that *N,N'*-diphenylurea was formed from the isocyanate.

However, the problem of recovering pure isocyanate had not been overcome. It was thought recovery of isocyanate may be facilitated by trying to recover a less reactive isocyanate which would be less susceptible to hydrolysis. Hence BuITC was oxidised by DMD to BuIC, the acetone removed under reduced pressure and the remaining residue distilled using a Kugelrohr apparatus under reduced pressure. NMR analysis of the fractions recovered showed minor butyl peaks which were masked by a very large peak, reminiscent of water. The experiment was repeated on the assumption that the DMD had not been properly dried in the previous attempt, with the distillation being performed at atmospheric pressure. ^{1}H NMR suggested the presence of an NH group in addition to a butyl group and the ^{13}C spectrum showed a peak at 166.34 ppm which was characteristic of

an acid. IR analysis also suggested the presence of an acid or water with a broad peak being observed from 2750-3500 cm^{-1}.

Unfortunately, the reason for the difficulty in isolating the isocyanates has not been determined. The evidence strongly suggests that they become converted to carbamic acids as a result of hydrolysis by atmospheric moisture, or water present in the DMD/acetone solution. Indeed it has been reported that DMD solutions are inherently difficult to dry.[149] This hydrolysis must become more prevalent as the solution is concentrated, or upon heating, as there is no sign of hydrolysis in the initial oxidation chromatograms.

The GC traces of the residue, obtained upon solvent removal, indicate there are many species present. Some of these may have been formed from the isocyanate perhaps by reaction with a sulfur or sulfur-containing species which result from the oxidation reaction. This could also be a concentration dependent reaction.

The failure to isolate BIC may be due to the formation of a trimeric species. In their palladium mediated oxidation system, Paraskewas *et al* [71] failed to isolate BIC due to formation of the trimer **189**. However, other isocyanates, such as methyl, ethyl and phenyl isocyanate, were successfully obtained.

Regardless of the many processes occurring, one major drawback, which is likely to have a detrimental effect on the isolation of BIC, is the scale of the reaction i.e. 500 mg maximum. This is difficult to overcome due to the low concentration of DMD in acetone.

O N N O N O

189

5.7.3 FT-IR studies on oxidation of isothiocyanates

Since the attempts to isolate isocyanates from the DMD reaction mixture had met with little success, it was determined that it would be beneficial to detect isocyanates by another technique in addition to GC/MS. It was considered that FT-IR may be the most promising method remaining for detection. As highlighted earlier, the IR group frequencies of isothiocyanates and isocyanates differ by at least 125 cm^{-1} allowing differentiation between the two different classes of compounds. Therefore analysis of standard isothiocyanates and isocyanates followed by analysis of a DMD reaction solution should allow the species present to be identified. However, it was anticipated that difficulties may arise, once more, due to the scale of the reaction. Ideally all the isothiocyanate should be converted to isocyanate which requires 5 equivalents of DMD in acetone. To determine if the isothiocyanate and isocyanate could be visualised at this low concentration, standards of BITC and BIC of a similar concentration to the reaction solution were prepared. These were analysed using solution cells after a background correction had been performed against acetone. This allowed the isothiocyanate and isocyanate peaks to be observed, at 2093 cm^{-1} and 2268 cm^{-1} respectively, despite the low concentration. As this had proved successful, another two samples were prepared where BIC and BITC were dissolved in separate solutions of DMD/acetone in the same quantities as above. This time a background correction was performed against DMD/acetone before the samples were analysed. Unexpectedly, the spectra for BIC and BITC (shown in figures 27 and 28) were not identical.

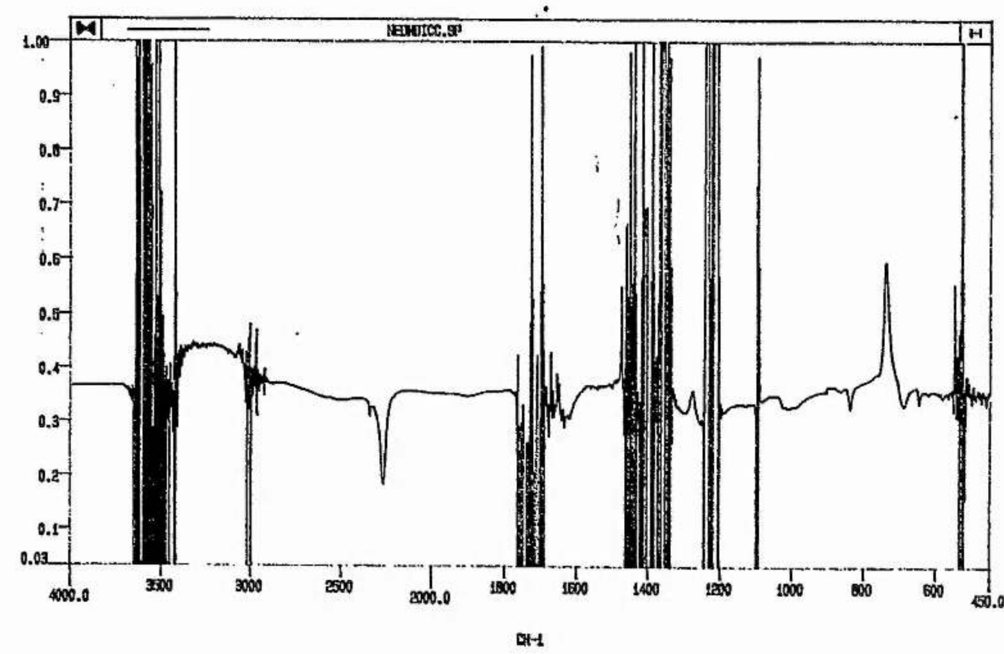


Figure 27 FT-IR spectrum of BIC corrected against DMD/acetone.

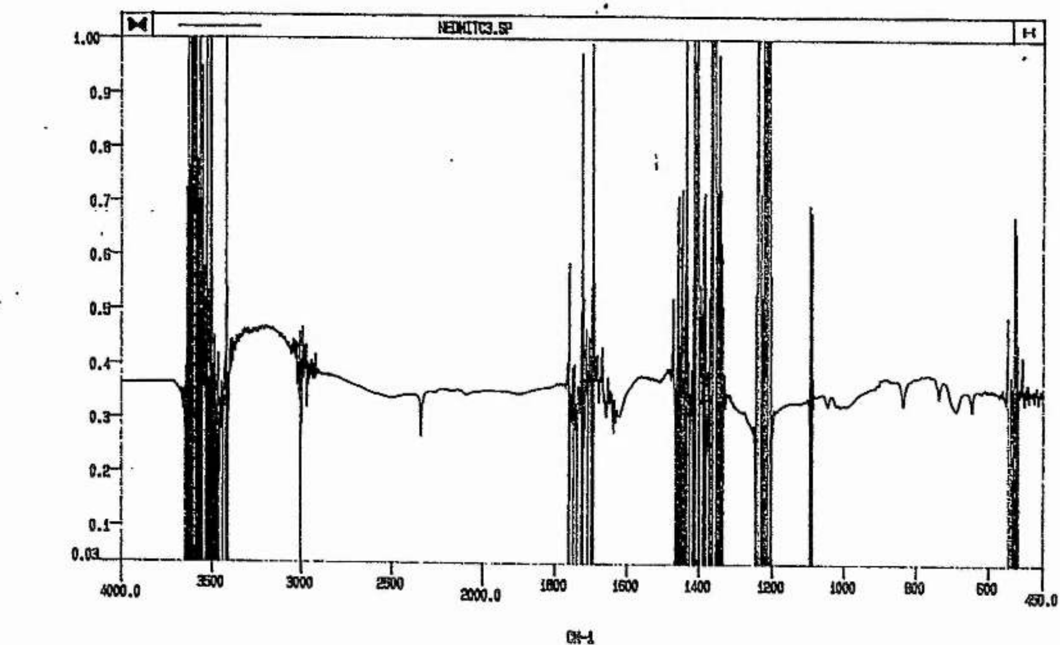


Figure 28 FT-IR spectrum of the reaction of BITC with DMD corrected against DMD/acetone.

Instead of showing a large peak at 2266 cm^{-1} due to BIC, the BITC reaction mixture showed a large peak at 2341 cm^{-1} and a small peak at 2092 cm^{-1}, corresponding to unreacted isothiocyanate. The identity of the peak at 2341 cm^{-1} had still to be verified but reference to standard tables suggested it may be carbon dioxide. This is possible as the newly formed BIC could be hydrolysed to yield benzylamine and carbon dioxide under atmospheric conditions. Indeed the BIC sample in DMD/acetone showed a small peak at 2341 cm^{-1} but the original BITC standard in acetone did not, indicating that only the isocyanate was changing. Expansion of figure 28 showed that four peaks were actually present as illustrated in figure 29. These occurred at 2341, 2276, 2169 and 2092 cm^{-1}. The latter two peaks correspond to BITC, but the peak at 2276 cm^{-1} was thought to be BIC.

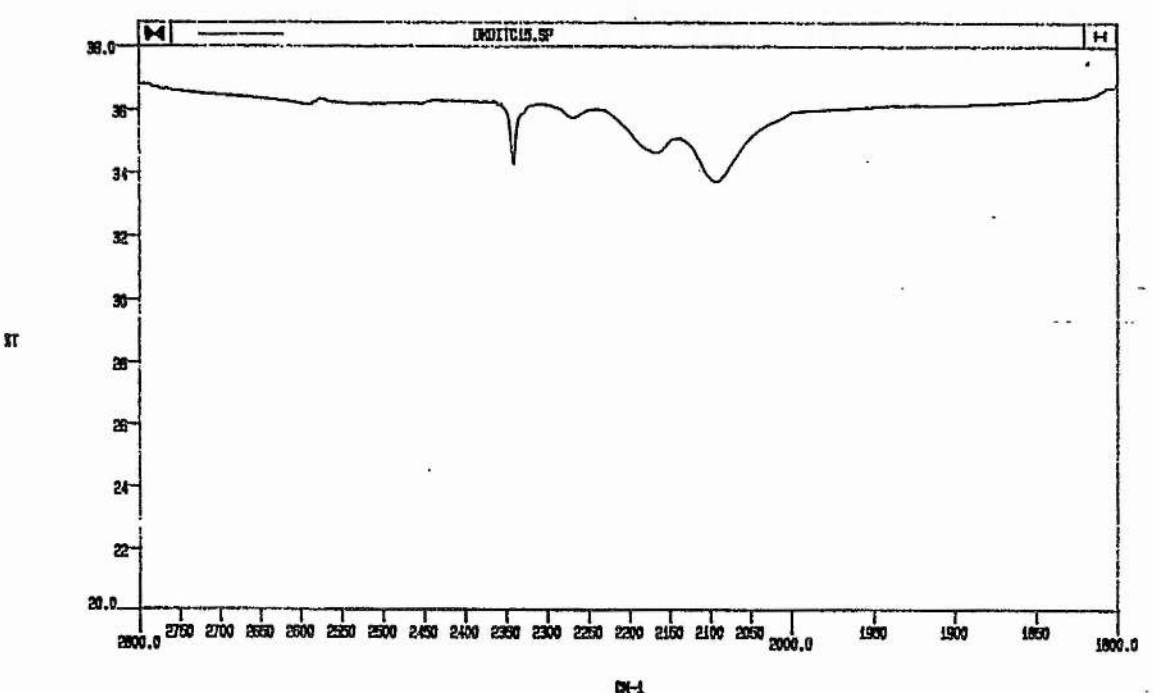


Figure 29 Expanded BITC spectrum.

To verify if this peak was indeed BIC, the BITC reaction mixture was spiked with authentic BIC and re-analysed. The spectrum obtained (figure 30) showed that BIC was indeed present in the BITC reaction mixture as the size of this peak increased upon addition of authentic BIC.

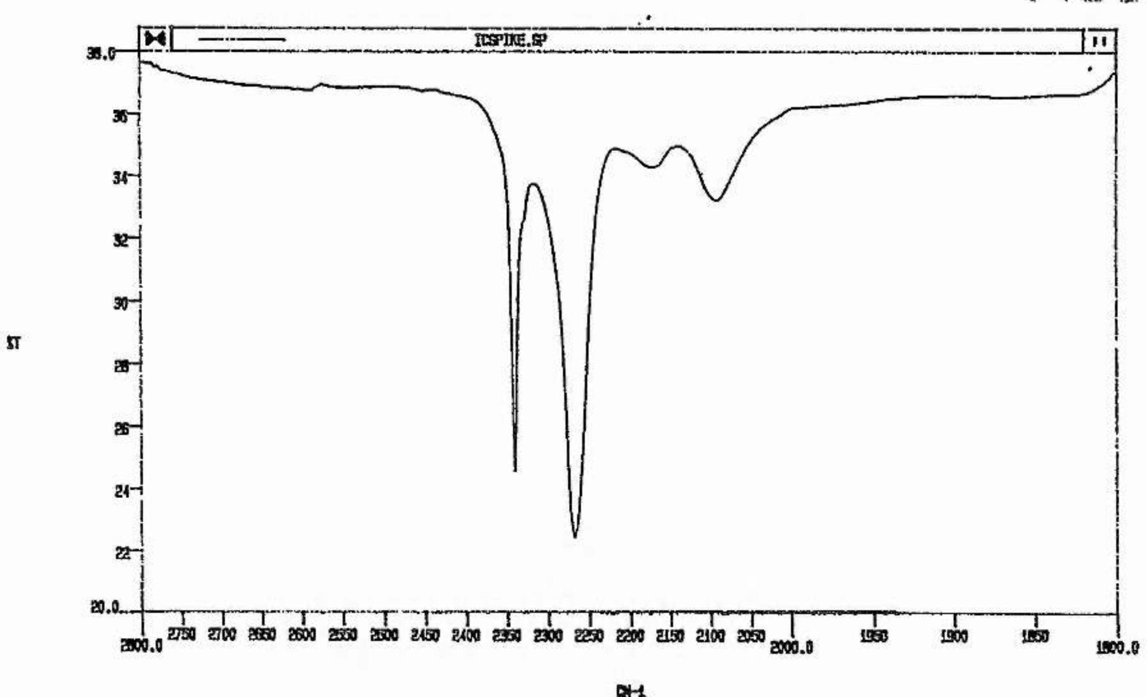


Figure 30 BITC reaction mixture spiked with BIC.

It should also be noted that the area of the peak at 2341 cm^{-1} in figure 30 has increased relative to the size of the isothiocyanate peak when compared with figure 29. This could be due to an increase in hydrolysis as the concentration of BIC increases.

To further investigate the nature of the peak at 2341 cm^{-1}, the decomposition of BIC was measured by FT-IR over the course of an hour. BIC was added to DMD and the resulting solution was analysed, after a background correction against DMD/acetone, at time 0 and after 20, 40 and 60 minutes. The spectra obtained were overlayed and are shown in figure 31.

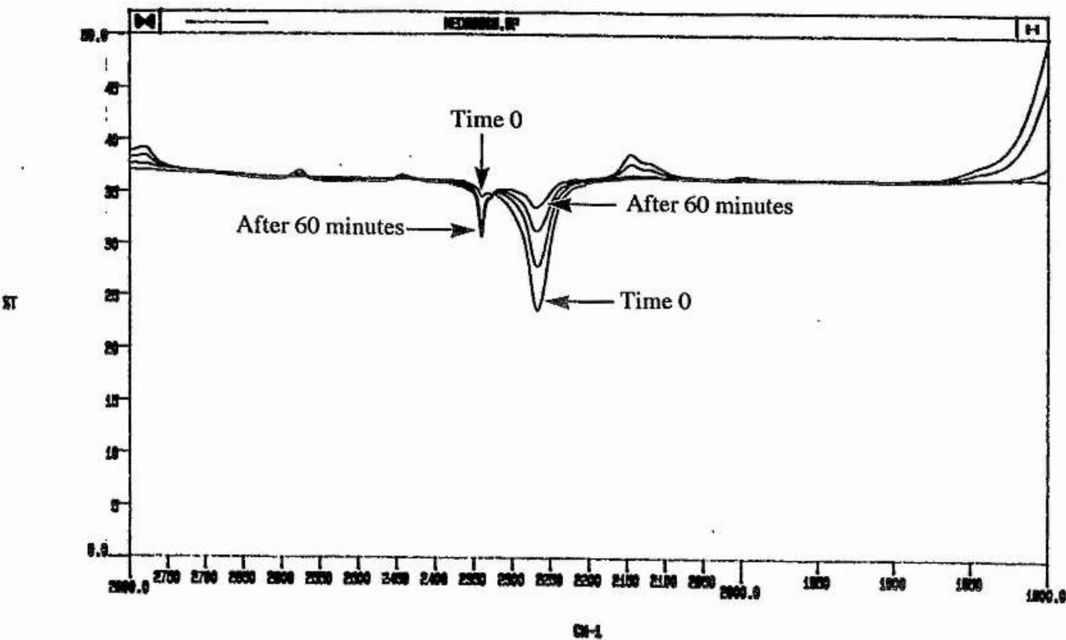


Figure 31 Spectra showing decomposition of BIC with time.

It is apparent that the amount of BIC reduced with time corresponding with an increase in the size of the peak at 2341 cm^{-1}. This indicates that BIC was degrading to form another species and this explains the large peak at 2341 cm^{-1} and the small peak for BIC at 2276 cm^{-1} (figure 29).

The question of whether the peak at 2341 cm^{-1} was due to carbon dioxide, was answered by performing a control experiment. Two aliquots of DMD/acetone were removed from the bulk solution and one was saturated with carbon dioxide while the other remained unchanged. FT-IR analysis of the carbon dioxide-saturated DMD solution, after a background correction against the unaltered DMD solution, showed a peak at 2340 cm^{-1} (figure 32). This clearly identified the peak at 2341 cm^{-1} as carbon dioxide indicating that hydrolysis of the isocyanate was occurring. Unfortunately, it was not possible to view the amine produced from hydrolysis due to interference in the spectrum.

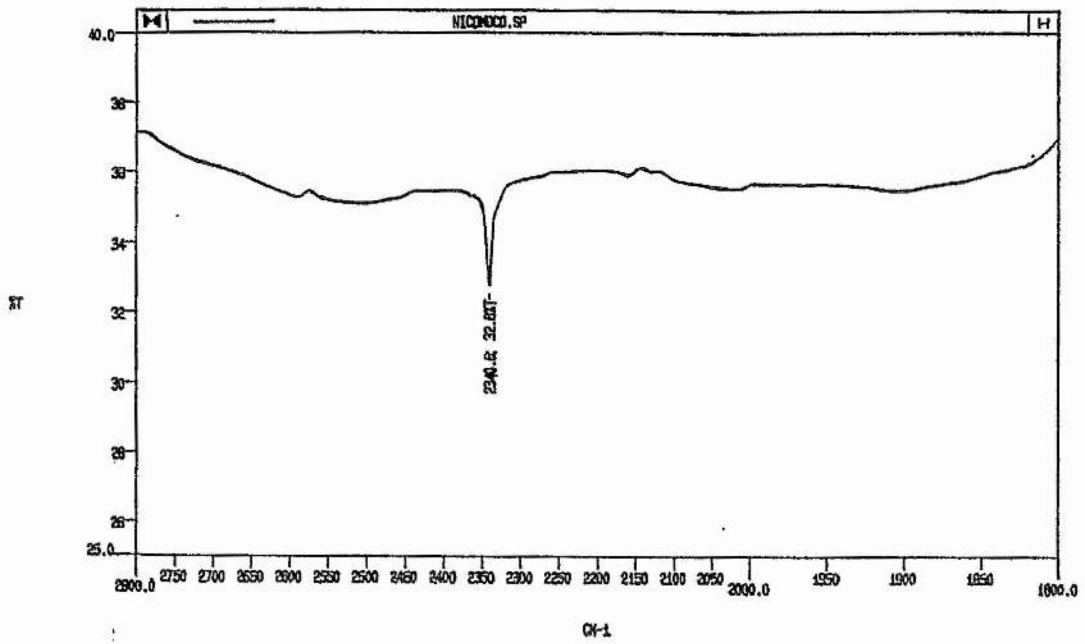


Figure 32 Spectrum showing CO_2-saturated DMD solution corrected against DMD.

5.7.4 Trapping of isocyanates as urea derivatives

Although it had been demonstrated by GC/MS and FT-IR that isocyanates result from oxidation of isothiocyanates, an alternative method of proving the existence of the former was desirable. One way this could be achieved was to trap out the isocyanate with a suitable nucleophile and isolate it as a derivative. As detailed in section 1.3.2, isocyanates react readily with a wide range of nucleophiles including alcohols and amines. Thus carbamates **190** and ureas **191-194** could both be prepared (schemes 61 and 62).

$$R{-}N{=}C{=}S \xrightarrow{\text{i}} \left[R{-}N{=}C{=}O\right] \xrightarrow{\text{ii}} R{-}NH{-}C({=}O){-}OR'$$

190 R = $PhCH_2CH_2$
R' = CH_3

Scheme 61 *Reagents and conditions*: i, DMD/acetone, room temp.; ii, MeOH.

$$R{-}N{=}C{=}S \xrightarrow{\text{i}} [R{-}N{=}C{=}O] \xrightarrow{\text{ii}} R{-}NH{-}C(=O){-}NH{-}R'$$

191 R = Ph
192 R = $PhCH_2$
193 R = $Ph(CH_2)_2$
194 R = $CH_3(CH_2)_3$
R'= $(CH_3)_2CH$

Scheme 62 *Reagents and conditions*: i, DMD/acetone, room temp.; ii, *iso*-propylamine.

Initially, PEITC was oxidised to PEIC and the isocyanate trapped out as methyl phenethylcarbamate **190**, in a yield of 11%, by adding excess dry methanol to the oxidation reaction mixture. Recovery of this compound suggested that the oxidation reaction was not very efficient and this was also reinforced by the recovery of some starting isothiocyanate.

Instead of forming carbamates, trapping the isocyanate as a urea was investigated as an alternative. Using *iso*-propylamine, *N*-*iso*-propyl-*N* '-phenylurea **191** was prepared with a yield of 51%. Although the amount of this compound recovered indicates a greater stability, the urea was compared with methyl phenethylcarbamate using GC/MS. Both compounds were found to decompose back to the isocyanate at elevated temperature although this was less prevalent for the urea. This is contrary to the literature which suggests that ureas decompose at lower temperatures than carbamates giving high yields of isocyanates.[150] As a result of their higher stability and the yield of the urea, *iso*-propylamine was used to trap BITC, PEITC and BuITC as their ureas, **192**, **193** and **194**, in yields of 48%, 44% and 71% respectively.

Although the yields of these ureas were reasonably good, an attempt was made to optimise the reaction conditions to obtain higher yields. Previously, the oxidation reactions were left to stir for 4 to 48 hours prior to addition of amine. This was to ensure the oxidation reaction had gone to completion and to allow the DMD to degrade before addition of amine.

However, analysis of the oxidation reaction by GC/MS after 15 minutes showed the reaction had gone to completion and occurs very rapidly. Therefore, leaving the reaction for extended periods of time may serve only to reduce the amount of isocyanate available to react with the amine as hydrolysis and side-reactions have more time to occur. Consequently, the preparation of *N*-phenyl **191**, benzyl **192**, and phenethyl **193** -*N'*-*iso*-propylureas was repeated with *iso*-propylamine being added no more than 1.5 hours after addition of the isothiocyanate to DMD. This resulted in improved yields (table 19).

Product	Yield (%)
191	89
CH_2 **192**	84
CH_2CH_2 **193**	67
$CH_3CH_2CH_2CH_2$ **194**	71 (Unoptimised)

Table 19 Isocyanate derivatives isolated with improved yields.

To confirm the identity of these ureas, authentic compounds were prepared for the ureas **191**, **192** and **194**, by treating the appropriate isocyanate with *iso*-propylamine in toluene (scheme 63).

$$R{-}N{=}C{=}O \quad + \quad H_2N{-}iPr \xrightarrow{i} R{-}NH{-}C(=O){-}NH{-}iPr$$

195 R = Ph
196 R = $PhCH_2$
197 R = $CH_3(CH_2)_3$

Scheme 63 *Reagents and conditions*: i, toluene, 0 °C.

The yields of these authentics were 88%, 77% and 76% for *N*-phenyl **195**, benzyl **196** and butyl **197** -*N*'-*iso*-propylureas respectively. These were characterised and compared with the ureas **191**, **192** and **194**, prepared from isothiocyanates, and the data for both sets of compounds was found to be identical.

5.7.5 Attempted oxidation of *N*-benzyl-*N*'-*iso*-propylthiourea

It could be argued that the ureas prepared in section 5.7.4 actually result from the isothiocyanates i.e. the isothiocyanate could react with *iso*-propylamine to form the thiourea **198**, which could then be oxidised to the urea by DMD (scheme 64).

$$R{-}N{=}C{=}S \longrightarrow R{-}NH{-}C(=S){-}NH{-}R' \dashrightarrow R{-}NH{-}C(=O){-}NH{-}R'$$

198

Scheme 64 Possible route to the formation of ureas from isothiocyanates.

Therefore to determine if thioureas could be converted to the corresponding ureas by DMD, oxidation of *N*-benzyl-*N*'-*iso*-propylthiourea **199** was attempted. The thiourea **199** was added to DMD (5 equivalents) in acetone and the resulting solution stirred at room temperature for 1 hour prior to GC/MS analysis. This showed a little thiourea but no urea or indeed isothiocyanate or isocyanate, which would be observed due to the high

temperature instability of the former compounds. However, one other species was observed, having a molecular ion of mass 224, which could be tentatively assigned to a compound resulting from oxidation of the sulfur atom of the thiourea. The structure could be similar to the thiourea-*S*-monoxide **200** which has a molecular ion of mass 225. The formation of this compound has been previously reported to arise from hydrogen peroxide oxidation of the corresponding thiourea.[151] Further oxidation can also occur using the same reagent to produce thiourea-*S*-dioxides **201** or using peracetic acid to produce the thiourea-*S*-trioxides **202**.[152,153]

200 **201** **202**

R = $PhCH_2$
R'= $CH(CH_3)_2$

Removal of the solvent approximately 4 hours after the start of the experiment yielded a cream-coloured solid which was highly insoluble in most organic solvents. ^1H NMR of the solid, dissolved in DMSO, suggested that some thiourea remained, as a doublet was observed at *ca.* δ_H 4.58. This is characteristic of the starting compound which gives a doublet at δ_H 4.60 for the benzylic protons. However, there was no doublet at δ_H 4.27-4.32 indicating that *N*-benzyl-*N'*-*iso*-propylurea had not been formed upon reaction of the corresponding thiourea **199** with DMD. However, a number of signals were observed from δ_H 8.85-9.70 which may be reminiscent of an acidic species. Analysis of this NMR sample by GC/MS again showed a species similar to the above compound **200**, along with the starting thiourea **199** and a small amount of compounds of molecular mass 191 and 193 which could be due to the urea **192** (RMM 192), ($[M-H]^+$, 2% and MH^+, 2%).

It can be concluded that if any thiourea was oxidised to the corresponding urea the amount of this compound is negligible. Given that no urea could be visualised by NMR after 4 hours it is highly unlikely that the ureas formed previously, in section 5.7.4, would have resulted from the thioureas. These previous urea-forming reactions were stirred for less than 4 hours after addition of *iso*-propylamine meaning there would insufficient time for conversion of any thiourea to urea.

If any urea was present from oxidation of *N*-benzyl-*N'*-*iso*-propylthiourea it should have been possible to observe the corresponding isocyanate upon GC/MS analysis. This compound was missing, again pointing to the fact that no urea had formed. Instead the evidence dictates that the thiourea underwent sulfur oxidation to yield a species similar to the thiourea-*S*-monoxide **200**. Given that DMD is as powerful an oxidising agent as hydrogen peroxide, this transformation is highly likely. Indeed the sulfonic acid analogue **202** may have formed but could not be visualised by GC/MS. The existence of this latter compound could also explain the insolubility of the product in organic solvents.

5.7.6 Competition between BITC and BIC for reaction with *iso*-propylamine.

This experiment was conducted to determine whether BITC or BIC would react preferentially with *iso*-propylamine under similar conditions to the DMD oxidation experiment (scheme 65).

R—N=C=S
R—N=C=O
i
R—NH—C(=S)—NH—R'
R—NH—C(=O)—NH—R'

Scheme 65 *Reagents and conditions*: i, dry acetone (0 °C), *iso*-propylamine, then stirring at room temp., 3h.

Equivalent quantities of BITC and BIC were added to cold dry acetone before an equimolar quantity of *iso*-propylamine was added. The resulting solution was stirred at room temperature, under nitrogen, for 3 hours and the resulting mixture separated by flash chromatography to afford *N*-benzyl-*N*'-*iso*-propylurea in a yield of 66% with no recovery of the corresponding thiourea. This confirms that the isocyanate reacts preferentially with the amine. In combination with the results from section 5.7.5, this experiment clearly demonstrates that the ureas formed previously, in section 5.7.4, result from the isocyanate and not the isothiocyanate. This is illustrated by scheme 66.

R—N=C=S —DMD, FAST→ R—N=C=O

SLOW* (R—N=C=S → thiourea) FAST (R—N=C=O → urea)

R—NH—C(=S)—NH—R' —DMD, SLOW or NO REACTION→ R—NH—C(=O)—NH—R'

* Slow when compared with the reaction of the isocyanate with *iso*-propylamine.

Scheme 66 Diagram showing relative reaction rates.

As soon as DMD comes into contact with the isothiocyanate, the compound is oxidised rapidly to the corresponding isocyanate. Assuming any isothiocyanate remains (although this is unlikely) the formation of thiourea by reaction with *iso*-propylamine is negligible as the amine will react preferentially with the more electrophilic isocyanate as demonstrated previously. The conversion of any thiourea to urea has proven to be unfavourable meaning the urea present is more likely to have come from the isocyanate. This is even more probable given the conditions employed for trapping the isocyanates as ureas i.e. the amine was added not more than 1.5 hours after addition of DMD meaning that there was unlikely

to be any isothiocyanate remaining after this time which would be capable of reacting with *iso*-propylamine. Moreover, these reactions were analysed by GC/MS before the addition of the amine with no isothiocyanate being observed indicating it had been completely converted to the isocyanate. GC/MS analysis after addition of amine also failed to show any thiourea or isothiocyanate.

Furthermore the previous experiment also lends credence to the proposed oxidation mechanism catalysed by cytochrome P450 enzymes. The reaction of isocyanates with nucleophiles occurs more readily than the analogous reaction with isothiocyanates. Consequently, isocyanate production at the active site of the enzyme is more likely to lead to inhibition of the enzyme than reaction of the isothiocyanate as the inhibitor.

5.7.7 Oxidation of BITC by DMD in the presence of *iso*-propylamine: Attempted *in situ* trapping.

In addition to the previous trapping experiments, the oxidation of BITC in the presence of *iso*-propylamine was also attempted to determine if the isocyanate could be trapped out *in situ* as it was formed. This could mimic a nucleophilic amino acid side chain present at the active site of cytochrome P450 enzymes. By performing this experiment it should be possible to show that reaction of isocyanate with the active site base is likely to occur much faster than reaction of isothiocyanate. This would be indicated by a higher yield of *N*-benzyl-*N*'-*iso*-propylurea **192** compared to *N*-benzyl-*N*'-*iso*-propylthiourea **199**. This would help to show the necessity for conversion of the isothiocyanate to the isocyanate for efficient enzyme inhibition.

Iso-propylamine was added to DMD/acetone before BITC was added. The reaction was carried out in this order as it was anticipated that no reaction would occur until BITC was added. However, addition of *iso*-propylamine was accompanied by a colour change from

pale yellow to bright blue suggesting that a reaction of some kind had occurred. Subsequent GC/MS analysis of the reaction solution approximately 30 minutes after addition of BITC showed peaks due to BITC and BIC and a small peak thought to correspond with *N*-benzyl-*N*'-*iso*-propylurea. The appearance of BITC after 30 minutes was unexpected as the oxidation reaction usually occurs very rapidly. To increase the conversion of BITC to BIC, further DMD was added and the reaction mixture was stirred for another 30 minutes prior to GC/MS analysis. This time a large peak was found for BIC with no BITC evident. However, there was no obvious peak due to the urea and TLC analysis suggested that it had not formed. However, addition of further *iso*-propylamine resulted in the formation of *N*-benzyl-*N*'-*iso*-propylurea **192** in 89% yield.

A control experiment whereby BITC was reacted with *iso*-propylamine in acetone, without DMD, was carried out for approximately the same time scale. This yielded *N*-benzyl-*N*'-*iso*-propylthiourea **199** in 64% yield.

Thus the above experiments showed that formation of the urea occured more readily than formation of the thiourea. However, the relevancy of this study was somewhat diminished by the need to add excess DMD and *iso*-propylamine in order to get urea formation. Also the time scales of the two reactions may have differed slightly due to the need to work-up each reaction separately and by having no means of stopping further reaction occurring.

To overcome the latter problem and provide a better comparison of urea and thiourea formation, it was necessary to develop a method of quenching each reaction after a given time period. It was anticipated that adding a sufficient quantity of 2 M HCl may be effective as the remaining *iso*-propylamine would become protonated and thus non-nucleophilic. This would prevent further reaction occurring with the isocyanate or isothiocyanate. To test this method and also gain an estimate of the speed of reaction between *iso*-propylamine and BITC, the following reaction was carried out: BITC was added to chilled acetone and *iso*-propylamine was then added. The resulting solution was stirred at room temperature for 15

minutes before 2 M HCl was added and the mixture stirred for a further 30 minutes prior to extraction with diethyl ether. The organic layer was washed with water, dried and concentrated under reduced pressure before purification by flash chromatography. This afforded BITC (56%) and *N*-benzyl-*N'*-*iso*-propylthiourea **199** (13%).

The above experiment demonstrated that HCl could be used to quench the reaction between isothiocyanates and *iso*-propylamine. More importantly, it demonstrated that only a small amount of thiourea was formed in 15 minutes, which is the maximum time it would take for isothiocyanate to be oxidised to isocyanate by DMD. This provides more evidence that the ureas formed previously must result from the isocyanate and not the thiourea.

However, the problems experienced with the initial oxidation of BITC in the presence of *iso*-propylamine, and reaction of BIC with the amine, had not been explained. The reaction was repeated again with 2 M HCl being added after 15 minutes. Extraction, as before, and flash chromatography yielded unreacted BITC which accounted for 59% of the BITC initially added. No other compounds were successfully isolated. This indicated that the oxidation reaction had not occurred as expected. This reaction was repeated twice, once with BITC and *iso*-propylamine being pre-mixed before the addition of DMD. This reaction also turned blue in colour, behaving similarly to previous attempts.

The failure of DMD to oxidise BITC in the presence of *iso*-propylamine could be explained by the oxidation of *iso*-propylamine by DMD. This is not improbable as there was a 2-3 fold excess of *iso*-propylamine, compared with BITC, and oxidation of amines to nitro compounds, by DMD, has been previously reported.[154] This reaction would therefore remove DMD, leaving an insufficient quantity for the oxidation of BITC. Moreover, this theory is supported by the failure of BIC to be converted readily to the urea. This would result from a lack of amine present in the reaction solution. This was indeed observed and then rectified by addition of more *iso*-propylamine which lead to urea formation.

No concrete evidence of *iso*-propylamine oxidation was obtained but it seems a plausible reason for the failure of the reaction. It should also be borne in mind that such oxidation of the amine would not occur in the biological system due to the specificity of the enzyme.

5.7.8 Oxidation of BITC in the presence of CBZ-lysine methyl ester

This work was initiated as an extension of section 5.7.7 with the intention of trapping the isocyanate with a biologically relevant nucleophile which could potentially be present at the enzyme active site.

Due to problems with the solubility of CBZ-lysine, the acid group was protected as its methyl ester by refluxing with dry methanol and thionyl chloride to yield the hydrochloride salt. This was subsequently converted to the free amine of CBZ-lysine methyl ester **203** by stirring with methanol and propylene oxide. Next, the authentic urea **204** was prepared, for comparison purposes, by mixing BIC **173** and CBZ-lysine methyl ester **203** in dry acetone. The resulting solution was stirred under nitrogen, at room temperature, for 22 hours. However, TLC analysis suggested that no reaction had occurred indicating that perhaps conversion of the hydrochloride salt to free amine had been unsuccessful. Triethylamine was then added to effect deprotonation which resulted in a mixture of products. Purification by flash chromatography yielded the desired urea **204** in 65% yield.

A further batch of CBZ-lysine methyl ester **203** was then prepared, and reacted with the propylene oxide for 14.5 hours to ensure formation of the free amine. To test that this process had been successful, a small amount of protected lysine was reacted with BIC, in acetone, and the reaction monitored by TLC against the authentic urea. Reaction of the lysine to form the urea occurred within 2 hours indicating the free amine was present. The remaining protected lysine was dissolved in DMD/acetone before BITC was added. The

resulting solution was stirred at room temperature, under nitrogen, for approximately 21 hours. However, analysis by TLC indicated that no reaction had occurred. Given the problems experienced in the previous section, it is conceivable that the protected lysine was oxidised to the nitro compound by DMD. This would explain why no urea was formed when it was demonstrated that the free amine was present.

5.7.9 The fate of sulfur

During the discussions so far, no mention has been made as to the fate of the sulfur atom released from the isothiocyanate upon oxidation. This is because the fate of this atom is unknown. Most of the GC chromatograms showed a small peak with a retention time of 4.02 minutes. The compound responsible for this signal had a mass of 94 and the database suggested that it was dimethyl disulfide **205** or bis-sulfonyl methane (dimethyl sulfone) **206**. Unfortunately no species of this type or any sulfur containing species has ever been recovered. However, if the latter species was recovered it would explain the need for the large excess of DMD required to completely convert the isothiocyanate to the isocyanate i.e. not only is DMD being used to replace sulfur in the isothiocyanate but it is also being used to oxidise the sulfur released.

$H_3C—S—S—CH_3$

205

$H_3C—S(=O)_2—CH_3$

206

These types of species are plausible given that a nucleophilic sulfur would be released which could react with the excess acetone in the reaction mixture. Indeed it has been reported that the sulfur species produced upon oxidative desulfurisation of thiocarbonyl compounds by cytochrome P450 enzymes must be reasonably reactive as it reacts with thiol groups of cysteine to produce hydrodisulfide derivatives.[117]

Analysis by TLC has also indicated that elemental sulfur is not released upon DMD oxidation. The initial reaction was analysed against authentic sulfur and no complimentary spots were observed.

Ptashne and Neal also failed to identify the sulfur species resulting from oxidation of parathion to paraoxon by PTFA and point out that in the enzymatic reaction the sulfur is found covalently bonded to macromolecules.[142, 155]

5.8 OZONOLYSIS OF ISOTHIOCYANATES

It has been indicated in the literature that thiocarbonyl compounds can be oxidised to carbonyl compounds by ozonolysis.[156, 157, 158] By analogy, isothiocyanates could be converted to isocyanates by the same method which could prove to be a comparable model system to the DMD oxidation of isothiocyanates, studied previously. To investigate this hypothesis, the oxidation of BITC using ozone was carried out. BITC was dissolved in dichloromethane and was treated with ozone for 15 minutes initially, at -78 °C. GC/MS analysis of the reaction solution after this period showed that little reaction had occurred, with only a small amount of BIC being observed, and ozonolysis was continued for a further 30 minutes. Again, GC/MS analysis showed that large amounts of starting material remained hence ozonolysis of BITC was attempted at 0-5 °C for 1.25 hours. GC/MS analysis and integration of the peak areas indicated that 77% of BITC, 3% of BIC and 9.5% of benzaldehyde were contained in the reaction mixture.* The presence of benzaldehyde was surprising and not easily explained but if it came from BIC rather than BITC it might explain the very small amount of isocyanate observed. Therefore ozonolysis of BIC was carried out at approximately 0 °C for 1 hour. Integration of the GC/MS peaks showed that

* All percentages are relative to the total integrated area.

67.5% of BIC was present along with 3% of benzaldehyde. Thus it is reasonable to suggest that benzaldehyde results from BIC rather than BITC although its formation from the latter compound cannot be ruled out.

To determine if isocyanate formation would proceed more readily using a more reactive isothiocyanate, the ozonolysis of PITC was attempted at approximately 0 °C for 1.25 hours. GC/MS analysis of the reaction mixture showed that 90% of PITC was present and only 3.25% of PIC had formed.

It can therefore be concluded that oxidation of isothiocyanates to isocyanates by ozonolysis does not proceed readily with only small amounts of isocyanate formed. Thus ozonolysis is not a comparable oxidation system to DMD.

5.9 CONCLUSIONS

The oxidation of isothiocyanates was carried out using *m*-CPBA, PTFA and DMD with the corresponding isocyanate being detected and identified by GC/MS analysis. Quantitative GC/MS analysis showed DMD to be the most efficient reagent capable of oxidising a range of isothiocyanates. Unfortunately, the isolation of the resulting isocyanates proved impossible. This was attributed to the high reactivity of isocyanates meaning they were susceptible to hydrolysis by trace amounts of water in the DMD solution. Instead, the presence of the isocyanate was confirmed by FT-IR analysis of DMD reaction solutions. Not only could the isocyanate be clearly observed, but the hydrolysis of these compounds could also be monitored over time. A decrease in the size of the isocyanate signal was found in addition to an increased signal for carbon dioxide, one of the hydrolysis products. Moreover, the isocyanates resulting from DMD oxidation of isothiocyanates were trapped as their *N*-*iso*-propyl ureas by addition of *iso*-propylamine after oxidation. This allowed the

preparation of *N-iso*-propyl-*N'*-phenyl, benzyl, phenethyl and butyl ureas in 89%, 84%, 67% and 71% yields respectively.

The mechanism behind formation of the mixed ureas was investigated by attempting to oxidise *N*-benzyl-*N'-iso*-propylthiourea. GC/MS analysis failed to show the corresponding urea but suggested that oxidation of the sulfur atom of the thiourea was occurring instead. This proved that the ureas prepared above resulted from the isocyanate and not the corresponding thiourea. Moreover, it was also demonstrated that isocyanates react with amines more rapidly that the corresponding isothiocyanates.

The trapping of isocyanates by an *in situ* nucleophile afforded the mixed urea only after addition of excess amine and DMD. This was postulated to be due to oxidation of the amine to the corresponding nitro compound leaving insufficient DMD to cause isothiocyanate oxidation and insufficient amine to allow urea formation. Further tests using CBZ-lysine methyl ester as a biological nucleophile also failed for the same reason.

Additional studies showed that ozonolysis did not oxidise isothiocyanates to isocyanates and this reagent was not analogous to the peracids, DMD or cytochrome P450 enzymes.

In retrospect, these studies have conclusively demonstrated the oxidation of isothiocyanates to isocyanates which has not been previously reported using mild conditions. Thus it is extremely likely that isothiocyanates are oxidised to isocyanates by cytochrome P450 enzymes and that isocyanates are involved in the suicide inactivation of these enzymes. These studies have allowed a new reaction to be reported with mechanistic implications for the anti-carcinogenic activity of isothiocyanates.

CHAPTER 6

SUMMARY AND FUTURE WORK

6.1 SUMMARY

A range of synthetic and naturally occurring glucosinolates was prepared with the aim of determining their ovipositional activity. The alkyl glucosinolates, propyl, butyl, pentyl, heptyl and nonyl glucosinolates, were prepared in addition to 1-naphthylmethyl glucosinolate and (7-methoxycarbonylheptyl) glucosinolate. These compounds, with the exception of (7-methoxycarbonylheptyl) glucosinolate, were tested by contact chemoreception and were found to be active stimuli. A crude structure-activity relationship was found to exist whereby the insect response increased with an increase in the overall length of the glucosinolate side chain for the alkyl analogues. Naphthylmethyl glucosinolate, prepared as an analogue of glucobrassicin, was found to have little activity. This suggests that the NH group in glucobrassicin is important for stimulatory activity. Comparison with naturally occurring glucosinolates showed the alkyl glucosinolates to be more active indicating that the alkenyl moiety, present in previously tested compounds, is not essential for activity. These compounds are the first synthetic glucosinolates to be tested as oviposition stimuli by contact chemoreception and the results have warranted further behavioural tests which will be conducted in the near future.

Preparation of (7-methoxycarbonylheptyl) glucosinolate was initially performed as a precursor to (7-carboxyheptyl) glucosinolate. Difficulties with the final methyl ester deprotection of the former compound meant the free glucosinolate could not be obtained in pure form. Nevertheless high resolution NMR studies of the impure (7-carboxyheptyl) glucosinolate showed this compound adopted an unexpected conformation in aqueous solution and this was used to explain the formation of unexpected side products upon deprotection. NMR studies of heptyl glucosinolate showed a similar conformation as indicated by NOE enhancement. The conformation of glucosinolates has not been previously reported and may provide an avenue for future research and understanding about the reactivity of these compounds.

Furthermore, the glucosinolates were shown to be substrates for the enzyme myrosinase as measured by glucose release upon hydrolysis. In addition, the release of volatiles upon hydrolysis of alkyl glucosinolates was examined by sampling the head-space of these reactions. The corresponding isothiocyanate was released in every case with nitriles being observed for both butyl and heptyl glucosinolates.

Preparation of alkyl glucosinolates facilitated the synthesis of butyl, pentyl, heptyl and nonyl thiohydroximates for use as HPLC standards at the IACR, and HPLC and LC-MS standards at the SCRI. 1-Methylpropyl and 2-methylpropyl thiohydroximates were also prepared for this purpose. These compounds allowed the identification of a naturally occurring glucosinolate and will aid future studies.

The chemical oxidation of isothiocyanates to isocyanates was studied as a model system for the cytochrome P450 catalysed oxidation of isothiocyanates. This oxidation is proposed to account for the anti-carcinogenic activity of these compounds. The chemical oxidation of isothiocyanates was efficiently achieved using DMD. Although the resulting isocyanates could not be isolated, their existence *in situ* was proven by GC/MS and FT-IR analysis of DMD reaction solutions. In addition, the isocyanates were trapped as their ureas by addition of *iso*-propylamine to the reaction mixture. Thus *N*-*iso*-propyl-*N*'-phenyl, benzyl, phenethyl and butyl ureas were prepared in 89%, 84%, 67% and 71% yields respectively. These ureas were shown to arise from the isocyanate and not from oxidation of the corresponding thiourea. Moreover, peracids were also found to produce isocyanates, although less efficiently, with ozone having no oxidising effect on isothiocyanates. These studies have demonstrated the smooth oxidation of isothiocyanates to isocyanates by mild chemical reagents. Thus it is likely that this transformation is catalysed by cytochrome P450 enzymes and that isocyanates are responsible for the anti-carcinogenic effect of isothiocyanates.

6.2 FUTURE WORK

6.2.1 Glucosinolates

The electrophysiological studies on the synthetic alkyl glucosinolates have shown that these compounds are all active stimuli. However, there is only a small increase in activity as the side chain length increases. The previously observed correlation resulted from behavioural studies using wax coated artificial leaves.[43] An important piece of future work is therefore the examination of the synthetic glucosinolates under similar conditions. Studies are planned at SCRI and at Wädenswil, Switzerland, to examine the same set of compounds as oviposition stimuli for both *D. floralis* and *D. radicum*, to compare with previous studies. These results may then shed more light on the possible interaction of the glucosinolate side chain and the waxy leaf surface and its importance in relation to oviposition activity.

Future synthetic work in this area is mainly concerned with expanding the number of compounds tested as oviposition stimuli to create a much larger picture of the effect of glucosinolate structure on insect response. These studies could allow the identification of glucosinolates which are significantly more stimulatory than the naturally occurring compounds or which cause inhibition of oviposition. These could be useful in future crop protection strategies, using oviposition stimuli to attract insects away from crops to suitable traps. Alternatively, they could be sprayed on 'catch crops.' An interesting possibility is that further studies result in the discovery of oviposition deterents which could be of great value.

The first aim is to complete the synthesis of (7-carboxyheptyl) glucosinolate which is required to investigate the role of the waxy leaf surface in oviposition. This compound will be re-made using an alternative protecting group for the carboxylic acid which can be removed more easily. It is anticipated that the *t*-butyl ester protecting group may be suitable as this can be removed under mildly acidic conditions. This may prevent *O*-acylation of the

C-3 hydroxyl which occurred readily under basic conditions. In addition, the preparation of a shorter chain analogue is also desirable to further probe the effect of the wax as this compound may show different results.

The synthesis of a number of other novel glucosinolates is desirable. Potential targets include aryl alkyl glucosinolates which vary in the length of the alkyl chain. Some examples of these are shown below:

Altering the length of the alkyl chain on either side of the aromatic ring will allow a number of different compounds to be synthesised which could then be compared with the previously tested alkyl analogues. In addition, incorporation of an oxygen atom may provide interesting results and would allow comparison with the naturally occurring sulfur containing glucosinolates. Indeed simple alkyl derivatives containing an oxygen atom may also be of interest.

In addition, the conformation of the above compounds in an aqueous environment could be studied in more detail by high resolution NMR and compared with the previously examined heptyl glucosinolate and (7-carboxyheptyl) glucosinolate.

6.2.2 Isothiocyanates

The scope for future work on the isothiocyanate-isocyanate model system is more limited and would likely only provide further evidence for the isocyanate which has already been conclusively demonstrated. Initially it was hoped to study the oxidation reaction *in situ* using liver microsomes as a source of cytochrome P450 enzymes. This would require the synthesis of ^{15}N labelled isothiocyanates which could be used to follow any reaction with the enzyme by ^{15}N NMR. However, given the reactive nature of isocyanates, it is not unlikely that these compounds would be rapidly hydrolysed under the aqueous conditions and thus be difficult to detect. It would also be interesting to attempt to determine whether these compounds do indeed react to form a covalent linkage to the enzyme active site. This may be possible using radio-labelled isothiocyanates and purified cytochrome P450 enzymes. The fate of the sulfur, in both the model systems discussed previously, and biological systems, is also still unclear and needs to be further examined.

CHAPTER 7

EXPERIMENTAL

7.1 GENERAL

Melting points were recorded using a Gallenkamp melting point apparatus and are uncorrected.

Elemental analyses were performed in the departmental microanalytical laboratory.

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

IR spectra were recorded for liquid films unless otherwise stated on a Perkin-Elmer 1420 instrument. Absorption maxima are given in wavenumbers (cm^{-1}) relative to a polystyrene standard. FT-IR spectra were recorded on a Perkin-Elmer 1710 infrared fourier transform spectrometer using solution cells.

NMR spectra were routinely recorded either on a Varian Gemini 200 spectrometer (^{1}H 200 MHz; ^{13}C 50.31 MHz) or a Varian Gemini 2000 spectrometer (^{1}H 300 MHz; ^{13}C 75.45 MHz). Spectra were referenced relative to tetramethylsilane (TMS) or chloroform, acetone, DMSO, methanol, or D_2O. NMR spectra are described in parts per million downfield shift from TMS and are reported consecutively as position (δ_H or δ_C), relative integral, multiplicity (s=singlet, br s=broad singlet, d=doublet, br d=broad doublet, dd=doublet of doublets, t=triplet, br t=broad triplet, q=quartet, m=multiplet or otherwise), coupling constant ($J_{x,y}$ if applicable; reported in Hz) and assignment. High resolution NMR spectra were recorded using a Varian Unity+ spectrometer (^{1}H 500.3 MHz; ^{13}C 125.8 MHz) operating with a nominal probe temperature of 30 °C. One-dimensional ^{1}H spectra were acquired with 0.2 Hz/point digital resolution and a recycle time of 12 s/transient to allow accurate quantification of peak integrals. NOESY spectra were acquired with 500 ms mixing time, 8 scans per FID and a digital resolution of 1.2 Hz/point in f_2 and 5.0 Hz/point in f_1. High resolution spectra were referenced using dimethyl silapentane sulfonate as an internal standard.

Molecular mechanics computations were performed using the "Discover" package (Biosym, San Diego) with the AMBER force-field parameters. Additional parameters for the sulfate group were taken from Kogelberg and Rutherford.[159] Crude estimates of the parameters for the aglycone linkage (S-C=N) were generated by comparison with model compounds. Partial charges were calculated using AM1 semi-empirical energy calculations, and all simulations were performed *in vacuo* with a fixed dielectric constant appropriate for polar solvent (80.0).

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. 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. GC/MS spectra were recorded using a Hewlett-Packard 5890A gas chromatograph coupled to a Finnigan MAT Incos 50 mass spectrometer. Samples were analysed using an SGE BP1 column (25 m length; 0.2 mm diameter) with a dimethyl siloxane stationary phase (0.1 μm thickness). The following temperature settings were used (°C): ion source, 150; transfer body and nozzle, 299; separator, 20; injector and column, 300. An injection volume of 2.5 μl per sample was used with the following temperature programme: inital temp., 30 °C; final temp., 300 °C; heating rate, 20 °C/minute for 13.5 minutes; fixed temp., 300 °C for 2 minutes; total analysis time, 15.5 minutes.

LC-MS and HPLC chromatograms were recorded at the SCRI, Invergowrie and HPLC chromatograms were recorded at the IACR, Rothamstead. Machine details and conditions of analysis are given in section 7.3.8.

Thermal desorption GC/MS was carried out at the SCRI using a Perkin Elmer ATD 50 Automated Thermal Desorption System linked to a Hewlett Packard 5890 Gas chromatograph with a Finnigan/Masslab (VG) Trio-100 5890 Quadrapole mass spectometer.

Electrophysiological testing was carried out by Erich Staedler and Ruurd De Jong at Eidg. Forschungsanstalt, Schloss, CH-8820 Wädenswil, Switzerland using a method detailed in section 7.5.

Flash chromatography was performed according to the procedure of Still[160] using Kieselgel 60. Analytical thin layer chromatography was carried out on 0.25 mm precoated silica gel plates (Whatman SIL G/UV$_{254}$) or using reverse-phase silica plates (RP-18 F$_{254s}$) from Merck. Compounds were visualised by UV fluorescence, iodine vapour, aqueous potassium permanganate or 5% (v/v) concentrated sulfuric acid in methanol. Ion-exchange chromatography was carried out using DEAE Sephadex from Sigma or analytical grade Amberlite IR-120 (14-52 mesh) from BDH. Size-exclusion chromatography was carried out using G-10 Sephadex (bead size 40-120 μ) from Sigma. Sep-paks were used as stated hereafter.

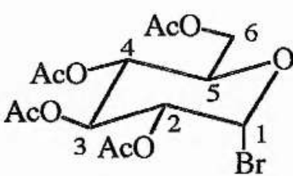
Ozonlysis was carried out using a Fischer Ozon Ozon-generator 500.

UV spectra were recorded using a UVICON 932 spectrophotometer fitted with a Grant F15 flow heater and flow cooler.

Solvents were dried and purified according to the methods of Perrin and Armarego.[161]

7.2 SYNTHESIS OF CARBOHYDRATE PRECURSORS FOR GLUCOSINOLATE SYNTHESIS

2,3,4,6-Tetra-*O*-acetyl-1–bromo-α-D-glucopyranose (α-D-Aceto-bromo-glucose) 24

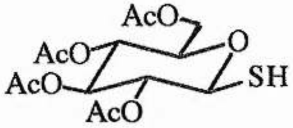


D-glucose (20 g, 111 mmol) was dissolved in acetic anhydride (77 ml) before hydrobromic acid (45% w/v) in acetic acid (13.83 g, 171 mmol) was added dropwise, with stirring, at room temperature under a nitrogen atmosphere. After 1 hour, hydrobromic acid (45% w/v) in acetic acid (41.41 g, 511.76 mmol) was added dropwise and the resulting solution stirrred as before for 18 hours. Dichloromethane (300 ml) was added and the reaction mixture was poured onto ice/water. After vigorous stirring, the organic layer was separated and poured onto ice/ saturated sodium hydrogen carbonate before being washed with more saturated sodium hydrogen carbonate solution (200 ml). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to afford a dark gold oil which crystallised upon storage at -20 °C. Recrystallisation from diethyl ether: petroleum ether (bp 40-60 °C) afforded the title compound as a white solid (37 g, 81%), mp 87 °C (lit.,[124] 88 °C); $[\alpha]_D$ +195.9 ° (c 2.42 in $CHCl_3$) (lit.,[162] +197.84 ° (c 2.42 in $CHCl_3$)); ν_{max}(nujol)/cm^{-1} 1730 (CO); δ_H(200 MHz, $CDCl_3$) 2.04 (12 H, 4 x s, COOC*H*$_3$), 4.09 (1 H, m, *H*-5), 4.29 (2 H, m, *H*-6^a,6^b), 4.81 (1 H, dd, $J_{1,2}$ 4, $J_{2,3}$ 10, *H*-2), 5.14 (1 H, t, $J_{3,4} = J_{4,5}$ 10, *H*-4), 5.53 (1 H, t, $J_{2,3} = J_{3,4}$ 10, *H*-3), 6.59 (1 H, d, $J_{1,2}$ 4, *H*-1); δ_C(50.31 MHz, $CDCl_3$) 21.06, 21.16 (*C*H$_3$), 61.37 (*C*-6), 67.55 (*C*-4), 70.57 (*C*-2), 71.01 (*C*-3), 72.56 (*C*-5), 87.04 (*C*-1), 169.96, 170.28, 170.35, 171.00 (*C*O); *m*/*z*(CI) 428, 430 ([M+NH_4]$^+$, 18%), 331 (8, [M-Br]$^+$) and 213 (29, [M-Br-2 OAc]$^+$).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl isothiouronium bromide 89

To a solution of α-D-acetobromoglucose (37.04 g, 90.1 mmol) in dry acetone (100 ml), thiourea (6.86 g, 90.1 mmol) was added and the resulting solution was heated under reflux, with stirring, under a nitrogen atmosphere for 15 minutes. The product precipitated as a white solid which was removed by filtration and dried under reduced pressure (29.32 g, 67%), mp 176 °C (decomp.) (lit.,[163] 205 °C); $[\alpha]_D$ -20.2 ° (c 1.0 in MeOH) (lit.,[164] -17.3 ° (c 1.0 in MeOH)); ν_{max}(nujol)/cm^{-1} 3310-3160 (NH), 1750 (CO), 1655 (NH); δ_H(200 MHz, D_2O) 1.96, 1.99, 2.01, 2.03 (12 H, 4 x s, CH_3COO), 4.16 (1 H, m, *H*-5), 4.29 (2 H, m, *H*-6^a,6^b), 5.26 (4 H, m, *H*-1,2,3,4); δ_C(50.31 MHz, D_2O) 22.97 (CH_3), 64.66 (*C*-6), 70.35 (*C*-4), 71.88 (*C*-2), 76.07 (*C*-3), 78.61 (*C*-5), 83.87 (*C*-1), 170.26 (*C*=N), 175.15, 175.32, 175.64, 176.32 (*C*O); *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 28



Potassium metabisulfite (3.42 g, 222 mmol) was added to water (75 ml) and the resulting solution was heated, with stirring, to 75 °C. Dichloromethane (100 ml) was added before 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiouronium bromide (7.5 g, 487 mmol), and the two-phase system was heated under reflux for 20 minutes. After cooling, the organic layer was separated, washed with water (3 x 100 ml), dried ($MgSO_4$), and concentrated

under reduced pressure to yield a colourless oil which crystallised upon storage at -20 °C. Recrystallisation from methanol afforded the title compound as a white solid (4.80 g, 85%), mp 75 °C (lit.,[165] 75 °C); $[\alpha]_D$ -9.8 ° (c 1.5 in EtOH) (lit.,[166] -8.3 ° (c 1.5 in EtOH)); ν_{max}(nujol)/cm^{-1} 1735 (CO); δ_H(200 MHz, $CDCl_3$) 2.00, 2.02, 2.08, 2.09 (12 H, 4 x s, CH_3COO), 3.73 (1 H, m, *H*-5), 4.07-4.23 (2 H, m, *H*-6^a,6^b), 4.54 (1 H, t, $J_{1,2}$ 8, *H*-1), 5.08 (3 H, m, *H*-2,3,4); δ_C(50.31 MHz, $CDCl_3$) 21.19, 21.23 (CH_3COO), 62.45 (*C*-6), 68.49 (*C*-4), 73.93 (*C*-2), 73.99 (*C*-3), 76.77 (*C*-5), 79.21 (*C*-1), 169.90, 170.18, 170.66, 171.22 (*C*O); *m/z*(CI) 382 ($[M+NH_4]^+$, 100%), 331 (7, $[M-SH]^+$) and 322 (22, $[M-OAC]^+$).

7.3 SYNTHESIS OF ALKYL GLUCOSINOLATES AND THIOHYDROXIMATES

7.3.1 Preparation of oximes

Butaldoxime 94

$$CH_3(CH_2)_2-\underset{\displaystyle H}{\overset{|}{C}}=N-OH$$

Hydroxylamine hydrochloride (177.7 g, 255.2 mmol) was added to butyraldehyde (10 g, 138.7 mmol) dissolved in a mixture of ethanol (200 ml) and pyridine (20 ml). The resulting solution was heated under reflux, with stirring, for 2.5 hours. After cooling, the solvent was removed under reduced pressure and washed with water (100 ml) before extraction with ethyl acetate (200 ml). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to afford the product as a mixture of two isomers (63% *anti:* 38% *syn*), (5.3 g, 44%), bp 155 °C (lit.,[167] 152 °C); δ_H(200 MHz, $CDCl_3$) 0.94 (3 H, m, CH_3), 1.50 (2 H, hextet, *J* 7, CH_2CH_3), 2.16 (2 H, q, *J* 7, $CH_2CH_2CH_3$ (*anti*)), 2.35 (2 H, q, *J* 6,

$CH_2CH_2CH_3$ (*syn*)), 6.71 (1 H, t, *J* 6, C*H*=N (*syn*)), 7.39 (1 H, m, C*H*=N (*anti*)), 9.70 (1 H, br s, NO*H*); δ_C(50.31 MHz, $CDCl_3$) 14.08, 14.37 (CH_3), 19.92, 20.45, 27.42, 31.90 (CH_2), 152.42 (*C*H=N (*anti*)), 153.04 (*C*H=N (*syn*)).

Pentaldoxime 95

$$CH_3(CH_2)_3-\underset{\displaystyle H}{\underset{|}{C}}=N-OH$$

Valeraldehyde (15 g, 174.2 mmol) and hydroxylamine hydrochloride (22.3 g, 320.5 mmol) were added to a mixture of ethanol (250 ml) and pyridine (25 ml) and the resulting solution heated under reflux as given previously. The product was extracted, as before, to afford an oil which crystallised to yield a white solid, after addition of water and overnight storage at 4 °C. The product was recovered as a mixture of isomers (12% *anti*: 88% *syn*), (10.76 g, 61%), mp 49 °C (lit.,[168] 52 °C); δ_H(200 MHz, $CDCl_3$) 0.92 (3 H, t, *J* 7, CH_3), 1.41 (4 H, m, $(CH_2)_2CH_3$), 2.34 (2 H, q, *J* 7, $CH_2(CH_2)_2CH_3$ (*anti*)), 2.37 (2 H, q, *J* 7, $CH_2(CH_2)_2CH_3$ (*syn*)), 6.72 (1 H, t, *J* 7, C*H*=N (*syn*)), 7.41 (1 H, t, *J* 7, C*H*=N (*anti*)), 9.73 (1 H, br s, NO*H*); δ_C(50.31 MHz, $CDCl_3$) 14.21 (CH_3), 22.63, 22.94, 25.20, 28.81, 29.13, 29.64 (CH_2), 152.74 (*C*H=N (*anti*)), 153.30 (*C*H=N (*syn*)).

Hexaldoxime 91

$$CH_3(CH_2)_4-\underset{\displaystyle H}{\underset{|}{C}}=N-OH$$

Hydroxylamine hydrochloride (10 g, 144 mmol) was added to hexanal (10 g, 99.8 mmol) dissolved in a mixture of ethanol (100 ml) and pyridine (10 ml). The resulting solution was heated under reflux for 2 hours after which water (100 ml) was added resulting in the

formation of a white solid (8.73 g, 76%). Recrystallisation from ethanol afforded the desired product as a mixture of isomers (1:1), (2.88 g, 25%), mp 39-41 °C (lit.,[169] 51 °C); δ_H(200 MHz, $CDCl_3$) 0.89 (3 H, t, *J* 6, C*H*$_3$), 1.30-1.35 (4 H, m, CH_3(C*H*$_2$)$_2$), 1.42-1.52 (2 H, m, C*H*$_2$$CH_2$CH), 2.19 (2 H, q, *J* 6, C*H*$_2$CHN (*anti*)), 2.38 (2 H, q, *J* 6, C*H*$_2$CHN (*syn*)), 6.73 (1 H, t, *J* 6, C*H*=N (*syn*)), 7.42 (1 H, t, *J* 6, C*H*=N (*anti*)), 8.75 (1 H, br s, NO*H*); δ_C(50.31 MHz, $CDCl_3$) 14.44 (CH_3), 22.85, 25.46, 26.19, 26.72, 29.94, 31.72, 32.02 (CH_2), 152.84 (CH=N (*anti*)), 153.42 (CH=N (*syn*)).

Octaldoxime 92

$$CH_3(CH_2)_6—\underset{\displaystyle H}{\underset{|}{C}}=N—OH$$

The same procedure for hexaldoxime was followed except octanal (10 g, 78 mmol) was used instead of hexanal. This afforded the title compound (10.95 g, 98%), as a mixture of isomers (56% *anti*: 44% *syn*), which was recrystallised from ethanol (7.25 g, 65%) mp 42-44 °C (lit.,[170] 60 °C); δ_H(200 MHz, $CDCl_3$) 0.87 (3 H, t, *J* 7, C*H*$_3$), 1.26 (8 H, m, CH_3(C*H*$_2$)$_4$), 1.41-1.51 (2 H, m, C*H*$_2$$CH_2$CH), 2.18 (2 H, q, *J* 7, C*H*$_2$CH (*anti*)), 2.36 (2 H, q, *J* 7, C*H*$_2$CH (*syn*)), 6.71 (1 H, t, *J* 7, C*H*=N(*syn*)), 7.41 (1 H, t, *J* 7, C*H*=N(*anti*)), 8.81 (1H, br s, NO*H*); δ_C(50.31 MHz, $CDCl_3$) 14.54 (CH_3), 23.09, 25.50, 26.51, 27.05, 29.46, 29.51, 29.82, 29.95, 32.18 (CH_2), 152.78 (CH=N (*anti*)), 153.36 (CH=N (*syn*)).

Decaldoxime 93

$CH_3(CH_2)_8—C(H)=N—OH$

Hydroxylamine hydrochloride (8.18 g, 117.7 mmol) was added to decanal (10 g, 64 mmol) and treated as before to afford a white solid which was recrystallised from ethanol to yield the desired product as a mixture of isomers (1:1), (9.23 g, 84%), mp 49-52 °C (lit.,[171] 69 °C); δ_H(200 MHz, $CDCl_3$) 0.87 (3 H, t, *J* 7, CH_3), 1.27 (12 H, br s, $(CH_2)_6$), 1.46 (2 H, m, CH_2CH_2CH), 2.19 (2 H, q, *J* 7, CH_2CH (*anti*)), 2.36 (2 H, q, *J* 7, CH_2CH (*syn*)), 6.72 (1 H, t, *J* 7, C*H*=N (*syn*)), 7.42 (1 H, t, *J* 7, C*H*=N (*anti*)), 8.65 (1 H, br s, NO*H*); δ_C(50.31 MHz, $CDCl_3$) 14.64 (CH_3), 23.19, 25.51, 26.55, 27.07, 29.60, 29.79, 29.84, 29.89, 29.98, 32.38 (CH_2), 152.82 (*C*H=N (*anti*)), 153.43 (*C*H=N (*syn*)).

(*R*, *S*)-2-Methylbutyraldoxime 112

Hydroxylamine hydrochloride (7.42 g, 106.8 mmol) was added to 2-methylbutyraldehyde (5 g, 58.1 mmol), dissolved in a mixture of ethanol (85 ml) and pyridine (8.5 ml), and the resulting solution heated under reflux as before. Concentration under reduced pressure and addition of water failed to afford the product as a solid therefore the solution was extracted with diethyl ether (100 ml). The organic layer was separated, dried ($MgSO_4$) and concentrated under reduced pressure to afford a colourless oil identified as the title compound existing as a mixture of isomers (68% *anti* : 32% syn), (4.74 g, 82%); δ_H(200 MHz, $CDCl_3$) 0.90 (3 H, t, *J* 8, CH_2CH_3), 1.05 (3 H, 2 x d, *J* 8, $CHCH_3$), 1.40 (2 H, m, CH_2CH_3), 2.30 (1 H, septet, *J* 8, $CHCH_3$ (*anti*)), 3.05 (1 H, septet, *J* 8, $CHCH_3$ (*syn*)), 6.50 (1 H, d, *J* 8, C*H*=N (*syn*)), 7.29 (1 H, d, *J* 8, C*H*=N (*anti*)); δ_C(50.31 MHz, $CDCl_3$)

11.89, 12.06 (CH_2CH_3), 17.49, 18.00 ($CHCH_3$), 22.15, 28.00 (CH_2CH_3), 31.58, 36.43 ($CHCH_3$), 156.77 (*C*H=N (*anti*)), 157.62 (*C*H=N (*syn*)).

3-Methylbutyraldoxime 113

H–CH=N–OH

Hydroxylamine hydrochloride (16.26 g, 213.6 mmol) was added to 3-methylbutyraldehyde (10 g, 116.1 mol) dissolved in a mixture of ethanol (100 ml) and pyridine (10 ml) and heated under reflux as before. After cooling, the solvent was removed under reduced pressure to yield a yellow oil to which water (100 ml) was added, and the resulting mixture was extracted with ethyl acetate (2 x 100 ml). The organic layers were combined, dried ($MgSO_4$) and concentrated under reduced pressure to give the title compound as a mixture of isomers (55% *syn* : 45% *anti*), (8.48 g, 72%); δ_H(200 MHz, $CDCl_3$) 0.94 (6 H, m, $(CH_3)_2$), 1.81 (1 H, m, $(CH_3)_2CH$), 2.08 (2 H, t, *J* 6, CH_2CH (*anti*)), 2.28 (2 H, t, *J* 6, CH_2CH (*syn*)), 6.73 (1 H, t, *J* 6, C*H*=N (*syn*)), 7.41 (1 H, *J* 6, C*H*=N (*anti*)), 8.96 (1 H, br s, NO*H*); δ_C(50.31 MHz, $CDCl_3$) 26.62, 27.16 ($(CH_3)_2$), 34.22 ($CH(CH_3)_2$), 38.65 (CH_2), 151.98, 152.10, 152.36, 152.49 (*C*H=N (*syn* and *anti*)).

7.3.2 Preparation of oximyl chlorides

1-Chlorobutaldoxime 96

$$CH_3(CH_2)_2-C(Cl)=N-OH$$

Butaldoxime (6 g, 68.9 mmol) was dissolved in a mixture of chloroform (100 ml) and pyridine (2.72 g, 35.5 mmol) before *N*-chlorosuccinimide (9.2 g, 69 mmol) was added slowly at 0 °C. The resulting solution was stirred at room temperature for 3 hours then poured onto ice/water. The organic layer was separated then washed with water (100 ml) and saturated sodium hydrogen carbonate solution (100 ml). The aqueous layer was extracted with diethyl ether (200 ml) and the combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure to afford a yellow oil which was not purified (6.37 g, 76%); δ_H(200 MHz, $CDCl_3$) 0.89 (3 H, m, CH_3), 1.64 (2 H, m, $CH_2CH_2C=N$), 2.43 (2 H, t, *J* 7, $CH_2C=N$), 9.44 (1 H, br s, NO*H*); δ_C(50.31 MHz, $CDCl_3$) 13.51 ($CH_2CH_2CH_3$), 20.17 ($CH_2CH_2CH_3$), 38.83 ($CH_2CH_2CH_3$), 141.36 (*C*=N); *m/z*(CI) 122, 124 (MH^+, 20, 100%) and 86 (24, $[M-Cl]^+$).

1-Chloropentaldoxime 97

$$CH_3(CH_2)_3-C(Cl)=N-OH$$

Pentaldoxime (10 g, 98.9 mmol) was dissolved in a mixture of chloroform (300 ml) and pyridine (3.9 g, 49.4 mmol) before *N*-chlorosuccinimide (13.2 g, 98.9 mmol) was added as before and the resulting solution stirred for 3.5 hours at room temperature. The product was extracted as given previously to afford a gold-coloured oil which was used without purification (11.09 g, 83%); δ_H(200 MHz, $CDCl_3$) 0.82 (3 H, m, CH_3), 1.32 (2 H, m,

CH_3CH_2), 1.57 (2 H, m, CH_2CH_2C=N), 2.45 (2 H, t, *J* 7, CH_2C=N), 9.61 (1 H, br s, NO*H*); δ_C(50.31 MHz, $CDCl_3$) 14.11 ($CH_2CH_2CH_3$), 21.12, 28.83, 36.71 (CH_2), 141.41 (*C*=N); *m/z*(CI) 136, 138 (MH^+, 31, 100%), 100 (34, $[M-Cl]^+$).

1-Chlorohexaldoxime 98

$$CH_3(CH_2)_4-C(Cl)=N-OH$$

Hexaldoxime (2.5 g, 21.7 mmol) was dissolved in a mixture of chloroform (65 ml) and pyridine (859 mg, 10.86 mmol) before *N*-chlorosuccinimide (2.9 g, 21.7 mmol) was added slowly, with stirring at 0 °C. The resulting solution was treated as above to yield the title compound which was used without purification (3.25 g, 100%); δ_H(200 MHz, $CDCl_3$) 0.90 (3 H, m, CH_3), 1.33 (4 H, m, $CH_3(CH_2)_2$), 1.65 (2 H, m, CH_2CH_2C=N), 2.50 (2 H, m, CH_2C=N), 8.52 (1 H, br s, NO*H*); δ_C(50.31 MHz, $CDCl_3$) 14.40 (CH_3), 22.74, 26.40, 31.14, 37.04 (CH_2), 142.39 (*C*=N); *m/z*(CI) 150, 152 (MH^+, 30, 91%), 114 (47, $[M-Cl]^+$).

1-Chlorooctaldoxime 99

$$CH_3(CH_2)_6-C(Cl)=N-OH$$

Octaldoxime (5 g, 34.9 mmol) was dissolved in a mixture of chloroform (100 ml) and pyridine (1.38 g, 17.46 mmol) before *N*-chlorosuccinimide (4.66 g, 34.9 mmol) was added as above and the resulting solution treated as before. This afforded the desired product as a gold oil which was not purified (5.49 g, 88%); δ_H(200 MHz, $CDCl_3$) 0.88 (3 H, t, *J* 6, CH_3), 1.27 (8 H, br s, $(CH_2)_4$), 1.63 (2 H, m, CH_2CH_2C=N), 2.49 (2 H, t, *J* 7,

CH_2C=N), 8.89 (1 H, br s, NO*H*); δ_C(50.31 MHz, $CDCl_3$) 14.58 (CH_3), 23.10, 26.75, 28.98, 29.37, 32.15, 37.08 (CH_2), 142.50 (*C*=N); *m/z*(CI) 178, 180 (MH^+, 32, 100%), 142 (62, $[M-Cl]^+$) and 126 (7, $[M-OCl]^+$).

1-Chlorodecaldoxime 100

$CH_3(CH_2)_8$—C(Cl)=N—OH

Decaldoxime (6 g, 35.03 mmol) was treated with *N*-chlorosuccinimide (4.68 g, 35.03 mmol) in pyridine (1.39 g, 17.52 mmol) and chloroform (105 ml) as before. This afforded the title compound which was used without purification (6.37 g, 88%); δ_H(200 MHz, $CDCl_3$) 0.87 (3 H, t, *J* 7, CH_3), 1.25 (12 H, br s, $(CH_2)_6$), 1.63 (2 H, m, CH_2CH_2C=N), 2.49 (2 H, t, *J* 7, CH_2C=N), 8.86 (1 H, br s, NO*H*); δ_C(50.31 MHz, $CDCl_3$) 14.60 (CH_3), 23.16, 26.73, 29.00, 29.69, 29.75, 29.91, 32.35, 37.08 (CH_2), 142.55 (*C*=N); *m/z*(CI) 206, 208 (MH^+, 30, 87%), 170 (100, $[M-Cl]^+$).

(*R*,*S*)-1-Chloro-2-methylbutyraldoxime 114

Cl—C(=N—OH)—CH(CH_3)CH_2CH_3

(*R*,*S*)-2-Methylbutyraldoxime (2 g, 19.8 mmol) was dissolved in chloroform (64 ml) and pyridine (0.78 g, 9.9 mmol) before *N*-chlorosuccinimide (2.64 g, 19.77 mmol) was added as before producing a blue-coloured solution. This solution was allowed to stir at room temperature for 3.5 hours before being extracted as given previously. This afforded the product as an orange-coloured oil which was used without purification (1.71 g, 64%); δ_H(200 MHz, $CDCl_3$) 0.85 (3 H, t, *J* 8, $CHCH_3$), 0.96-1.70 (5 H, m, CH_2CH_3), 2.56 (1

H, m, C*H*CH_3); δ_C(50.31 MHz, $CDCl_3$) 11.90 (CH_2*C*H_3), 18.36 (CH*C*H_3), 27.40 (*C*H_2CH_3), 47.72 (*C*HCH_3), 144.36 (*C*=N).

1-Chloro-3-methylbutyraldoxime 115

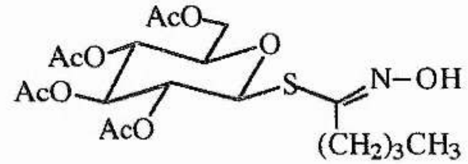
3-Methylbutyraldoxime (8.4 g, 83 mmol) was dissolved in chloroform (250 ml) and pyridine (3.35 ml, 41.5 mmol) before *N*-chlorosuccinimide (11.1 g, 83 mmol) was added at 0 °C with stirring. The resulting solution turned green while warming to room temperature. This was treated as before to afford the product as a dark yellow oil (7.55 g, 67%); δ_H(200 MHz, $CDCl_3$) 0.96 (6 H, m, (C*H*$_3$)$_2$), 2.09 (1 H, m, C*H*(CH_3)$_2$), 2.37, 2.49 (2 H, 2 x d, *J* 8, C*H*$_2$); δ_C(50.31 MHz, $CDCl_3$) 26.68, 27.29 (*C*H_3)$_2$), 31.62 (*C*H), 34.79 (*C*H_2), 157.93(*C*=N).

7.3.3 Synthesis of tetra-*O*-acetyl-alkyl thiohydroximates

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-propyl thiohydroximate 101

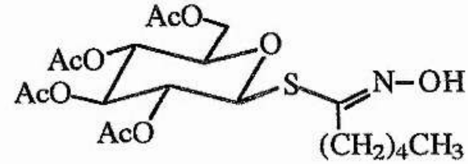
2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (8.99 g, 24.68 mmol) was added to a solution of 1-chlorobutaldoxime (3 g, 24.68 mmol) dissolved in dry THF (250 ml). Dry triethylamine (23.4 g, 231.5 mmol) was then added and the resulting solution stirred at room temperature, under a nitrogen atmosphere, for 21 hours before diethyl ether (200 ml) was added and the mixture washed with 1 M H_2SO_4 (200 ml). After removal of approximately 65% of the organic layer, the remaining organics were recombined with the aqueous layer and extracted with ethyl acetate (200 ml). The organic layers were then combined, dried ($MgSO_4$) and concentrated under reduced pressure to afford a gold-coloured oil. Purification by flash chromatography, on silica gel, eluting with hexane: ethyl acetate (3:2) containing 0.3 % methanol, yielded the title compound (4.0 g, 36%), mp 144 °C; (Found: C, 48.47; H, 6.19; N, 3.03. $C_{18}H_{27}NO_{10}S$ requires C, 48.10; H, 6.05; N, 3.12%); (Found (HRMS): MH^+, 450.1434. $C_{18}H_{28}NO_{10}S$ requires *MH* 450.4799); $[\alpha]_D$ -30.7 ° (c 0.14 in $CHCl_3$); ν_{max}(nujol)/cm^{-1} 3310 (OH), 1700, 1740 (CO), 1600 (C=N); δ_H(200 MHz, CD_3OD) 0.90 (3 H, t, *J* 8, CH_3), 1.67 (2 H, sextet, *J* 8, CH_2CH_3), 2.00, 2.02, 2.03, 2.06 (12 H, 4 x s, CH_3COO), 2.53 (2 H, t, *J* 8, $CH_2CH_2CH_3$), 4.00 (1 H, m, *H*-5), 4.13 (1 H, dd, $J_{5,6^a}$ 3, $J_{6^a,6^b}$ 14, *H*-6^a), 4.24 (1 H, dd, $J_{5,6^b}$ 5, $J_{6^a,6^b}$ 12, *H*-6^b), 4.89 (1 H, br s, O*H*), 4.99, 5.05 (2 H, 2 x t, *J* 9, 10, *H*-2,4), 5.28 (1 H, d, $J_{1,2}$ 10, *H*-1), 5.39 (1 H, t, $J_{2,3}$=$J_{3,4}$ 10, *H*-3); δ_C(50.31 MHz, $CDCl_3$) 14.21 (CH_3), 20.91 (CH_2), 21.06 (CH_3COO), 21.12, 21.18, 34.89 (CH_2), 62.77 (*C*-6), 68.59 (*C*-4), 70.56 (*C*-2), 74.26 (*C*-3), 76.45 (*C*-5), 80.35 (*C*-1), 152.67 (*C*=N), 169.71, 169.91, 170.79, 171.14 (*C*O); *m*/*z*(CI) 450 (MH^+, 7%), 331 (100, $C_6H_7O(OAc)_4^+$).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-butyl thiohydroximate 105



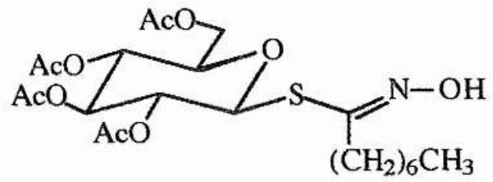
2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (15 g, 41.2 mmol) in dry THF (220 ml) was added to 1-chloropentaldoxime (7.81 g, 57.6 mmol) in dry THF (200 ml). Dry triethylamine (39.1 g, 386.1 mmol) was then added and the resulting solution stirred at room temperature, under a nitrogen atmosphere, for 24 hours. The product was extracted as before to afford a yellow solid which was recrystallised from hexane:ethyl acetate to yield a white solid (9.32 g, 49%), mp 138 °C; (Found: C, 49.23; H, 6.32; N, 3.06. $C_{19}H_{29}NO_{10}S$ requires C, 49.24; H, 6.31; N, 3.02%); (Found (HRMS): MH^+ 464.1590. $C_{19}H_{30}NO_{10}S$ requires *MH* 464.5067); $[\alpha]_D$ -40.0 ° (c 0.14 in $CHCl_3$); ν_{max}(nujol)/cm^{-1} 3310 (OH), 1700, 1740 (CO), 1600 (C=N); δ_H(200 MHz, CD_3OD) 0.96 (3 H, t, *J* 7, CH_3), 1.41 (2 H, sextet, *J* 8, CH_2CH_3), 1.65 (2 H, quintet, *J* 8, $CH_2CH_2CH_3$), 1.99, 2.04, 2.06 (12 H, 3 x s, CH_3COO), 2.55 (2 H, t, *J* 7, $CH_2(CH_2)_2CH_3$), 3.73 (1 H, m, *H*-5), 4.13 (1 H, dd, $J_{5,6^a}$ 2, $J_{6^a,6^b}$ 12, *H*-6^a), 4.25 (1 H, dd, $J_{5,6^b}$ 7, $J_{6^a,6^b}$ 12, *H*-6^b), 4.85 (1 H, br s, O*H*), 4.99, 5.06 (2 H, 2 x t, *J* 10, 10, *H*-2,4), 5.28 (1 H, d, $J_{1,2}$ 10, *H*-1), 5.39 (1 H, t, $J_{2,3}$=$J_{3,4}$ 9, *H*-3); δ_C(50.31 MHz, $CDCl_3$) 14.26 (CH_3), 21.04, 21.11 (CH_3COO), 22.76, 29.64, 32.67 (CH_2), 62.72 (*C*-6), 68.56 (*C*-4), 70.57 (*C*-2), 74.26 (*C*-3), 76.41 (*C*-5), 80.35 (*C*-1), 152.64 (*C*=N), 169.87, 169.89, 170.76, 171.11 (*C*O); *m/z*(CI) 464 (MH^+, 9%), 331 (100, $C_6H_7O(OAc)_4^+$) and 271 (19, $C_6H_7O(OAc)_3^+$).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-pentyl thiohydroximate 102



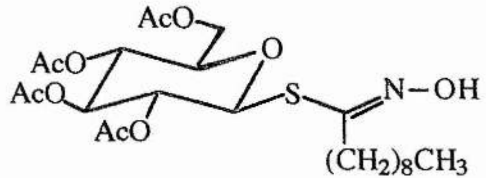
2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (4.38 g, 12.03 mmol) and 1-chloro-hexaldoxime (1.8 g, 12.03 mmol) were dissolved in dry THF (200 ml) before dry triethylamine (11.42 g, 112.84 mmol) was added. The resulting solution was stirred at room temperature, under nitrogen, for 48 hours before diethyl ether (150 ml) was added and the solution was washed with 1 M H_2SO_4 (150 ml). The organic layer was extracted in a similar manner to the previous compound to afford a yellow oil which was purified by flash chromatography, on silica gel, eluting with hexane: ethyl acetate (3:2) containing 0.3% methanol. This afforded the product as a white solid (2.7 g, 47%); mp 131-132 °C; (Found: C, 50.58; H, 6.38; N, 2.79. $C_{20}H_{31}NO_{10}S$ requires C, 50.31; H, 6.54; N, 2.93%); $[\alpha]_D$ -53.6 ° (c 0.14 in $CHCl_3$); ν_{max}(nujol)/cm^{-1} 3310 (OH), 1700, 1740 (CO), 1600 (C=N); δ_H(200 MHz, CD_3OD) 0.94 (3 H, t, *J* 7, CH_3), 1.37 (4 H, m, $(CH_2)_2CH_3$), 1.67 (2 H, quintet, *J* 8, $CH_2(CH_2)_2CH_3$), 1.99, 2.04, 2.04, 2.07 (12 H, 4 x s, CH_3COO), 2.55 (2 H, t, *J* 7, $CH_2(CH_2)_3CH_3$), 3.99 (1 H, m, *H*-5), 4.13 (1 H, dd, $J_{5,6^a}$ 2, $J_{6^a,6^b}$ 11, *H*-6^a), 4.25 (1 H, dd, $J_{5,6^b}$ 5, $J_{6^a,6^b}$ 11, *H*-6^b), 4.91 (1 H, br s, O*H*), 5.03, 5.06 (2 H, 2 x t, *J* 10, 10, *H*-2,4), 5.27 (1 H, d, $J_{1,2}$ 10, *H*-1), 5.39 (1 H, t, $J_{2,3}$=$J_{3,4}$ 9, *H*-3); δ_C(50.31 MHz, $CDCl_3$) 14.48 (CH_3), 21.06 (CH_3COO), 21.17, 22.85, 27.27, 31.87 (CH_2), 62.70 (*C*-6), 68.55 (*C*-4), 70.57 (*C*-2), 74.26 (*C*-3), 76.44 (*C*-5), 80.37 (*C*-1), 152.78 (*C*=N), 169.66, 169.85, 170.76, 171.09 (*C*O); *m/z*(CI) 478 (MH^+, 3%), 331 (100, $C_6H_7O(OAc)_4^+$) and 271 (43, $C_6H_7O(OAc)_3^+$).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-heptyl thiohydroximate 103



2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (3 g, 8.23 mmol) was dissolved in dry THF (100 ml) before 1-chorooctaldoxime (1.46 g, 8.23 mmol), dissolved in dry THF (100 ml), was added. To this, dry triethylamine (7.81 g, 77.2 mmol) was added and the resulting solution was stirred, under a nitrogen atmosphere, at room temperature for 22 hours. The product was extracted as before and recrystallised from ethyl acetate:petroleum ether (bp 40-60 °C) to afford the title compound as a white solid (2.86 g, 69%), mp 127 °C; (Found: C, 52.39; H, 7.12; N, 2.68. $C_{22}H_{35}NO_{10}S$ requires C, 52.26; H, 6.98; N, 2.77%); (Found (HRMS): MH^+ 506.2060. $C_{22}H_{36}NO_{10}S$ requires *MH* 506.6871); $[\alpha]_D$ -17.1 ° (c 0.14 in $CHCl_3$); ν_{max}(nujol)/cm^{-1} 3300 (OH), 1730 (CO), 1600 (C=N); δ_H(200 MHz, CD_3OD) 0.93 (3 H, t, *J* 7, CH_3), 1.35 (8 H, br s, $(CH_2)_4CH_3$), 1.66 (2 H, t, *J* 6, $CH_2(CH_2)_4CH_3$), 2.00, 2.04, 2.05, 2.07 (12 H, 4 x s, CH_3COO), 2.55 (2 H, t, *J* 6, $CH_2(CH_2)_5CH_3$), 3.99 (1 H, m, *H*-5), 4.13 (1 H, dd, $J_{5,6^a}$ 2, $J_{6^a,6^b}$ 12, *H*-6^a), 4.25 (1 H, dd, $J_{5,6^b}$ 7, $J_{6^a,6^b}$ 12, *H*-6^b), 4.92 (1 H, br s, O*H*), 5.05 (2 H, m, *H*-2,4), 5.28 (1 H, d, $J_{1,2}$ 10, *H*-1), 5.40 (1 H, t, $J_{2,3}$=$J_{3,4}$ 9, *H*-3); δ_C(50.31 MHz, CD_3COCD_3) 14.73 (CH_3), 20.92 (CH_3COO), 20.97, 21.03, 28.36, 29.85, 30.63, 32.96 (CH_2), 63.51 (*C*-6), 69.61 (*C*-4), 71.35 (*C*-2), 74.61 (*C*-3), 76.58 (*C*-5), 80.34 (*C*-1), 151.66 (*C*=N), 170.08, 170.39, 170.67, 171.00 (*C*O); *m/z*(CI) 506 (MH^+, 7%), 331 (100, $C_6H_7O(OAc)_4^+$) and 271 (40, $C_6H_7O(OAc)_3^+$).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-nonyl thiohydroximate 104

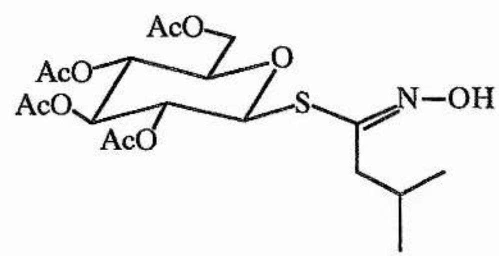


2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (5 g, 13.72 mmol) and 1-chloro-decaldoxime (2.82 g, 13.72 mmol) were dissolved in dry THF (250 ml) before dry triethylamine (13.02 g, 128.69 mmol) was added. The resulting solution was stirred as before for 24 hours then extracted with diethyl ether and ethyl acetate as described previously. This afforded a gold oil which was triturated with petroleum ether (bp 40-60 °C) at -20 °C to yield the product as a pink solid (5.75 g, 79%), mp 109-110 °C; (Found: C, 54.13; H, 7.49; N, 2.52. $C_{24}H_{39}NO_{10}S$ requires C, 54.02; H, 7.37; N, 2.62%); (Found (HRMS): MH^+ 534.2373. $C_{24}H_{40}NO_{10}S$ requires *MH* 534.6407); $[\alpha]_D$ +12.9 ° (c 0.14 in $CHCl_3$); ν_{max}(nujol)/cm^{-1} 3280 (OH), 1730 (CO), 1580 (C=N); δ_H(200 MHz, CD_3OD) 0.92 (3 H, t, *J* 7, CH_3), 1.32 (12 H, br s, $(CH_2)_6CH_3$), 1.66 (2 H, m, $CH_2(CH_2)_6CH_3$), 2.00, 2.04, 2.05, 2.07 (12 H, 4 x s, CH_3COO), 2.55 (2 H, t, *J* 7, $CH_2(CH_2)_7CH_3$), 3.99 (1 H, m, *H*-5), 4.13 (1 H, dd, $J_{5,6^a}$ 2, $J_{6^a,6^b}$ 12, *H*-6^a), 4.25 (1 H, dd, $J_{5,6^b}$ 5, $J_{6^a,6^b}$ 12, *H*-6^b), 4.92 (1 H, br s, O*H*), 5.04, 5.06 (2 H, 2 x t, *J* 8, 4, *H*-2,4), 5.28 (1 H, d, $J_{1,2}$ 10, *H*-1), 5.40 (1 H, t, $J_{2,3}$=$J_{3,4}$ 9, *H*-3); δ_C(50.31 MHz, $CDCl_3$) 14.61 (CH_3), 21.07 (CH_3COO), 21.19, 23.15, 27.59, 29.75, 29.83, 29.99, 32.33, 33.01 (CH_2), 62.68 (*C*-6), 68.52 (*C*-4), 70.55 (*C*-2), 74.26 (*C*-3), 76.45 (*C*-5), 80.36 (*C*-1), 152.95 (*C*=N), 169.67, 169.87, 170.78, 171.10 (*C*O); *m/z*(CI) 534 (MH^+, 8%), 331 (100, $C_6H_7O(OAc)_4^+$) and 271 (25, $C_6H_7O(OAc)_3^+$).

(*R*,*S*)-2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-1-methylpropyl thiohydroximate 116

(*R*,*S*)-1-Chloro-2-methylbutyraldoxime (1.71 g, 12.6 mmol) and 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (3.29 g, 9.0 mmol) were dissolved in dry THF (94 ml) before dry triethylamine (8.56 g, 84.6 mmol) was added with stirring under a nitrogen atmosphere. The resulting solution was treated as before to afford a yellow foam. Purification by flash chromatography, on silica gel, eluting with hexane and ethyl acetate (3:2) afforded the title compound as a mixture of diastereoisomers (3.18 g, 76%), mp 34 °C; (Found: C, 49.12; H, 6.33; N, 2.77. $C_{19}H_{29}NO_{10}S$ requires C, 49.24; H, 6.31; N, 3.02%); $[\alpha]_D$ -20.4 ° (c 0.14 in $CHCl_3$); ν_{max}/cm^{-1} 3330 (OH), 2840, 2920 (alkyl), 1720 (CO), 1600 (C=N); δ_H(200 MHz, CD_3OD) 0.94, 0.95 (3 H, 2 x t, *J* 7, CH_2CH_3), 1.18, 1.20 (3 H, 2 x d, *J* 7, CH_3CH), 1.43-1.84 (2 H, m, CH_3CH_2), 1.99, 1.99, 2.03, 2.05, 2.06 (12 H, 5 x s, CH_3COO), 2.62 (1 H, sextet, *J* 4, C*H*C=N), 4.00 (1 H, m, *H*-5), 4.12 (1 H, d, $J_{6^a,6^b}$ 12, *H*-6^a), 4.23 (1 H, dd, $J_{5,6^b}$ 5, $J_{6^a,6^b}$ 12, *H*-6^b), 4.84 (1 H, br s, NO*H*), 5.01 (2 H, m, *H*-2,4), 5.36 (1 H, t, $J_{2,3}$=$J_{3,4}$ 9, *H*-3), 5.37 (1 H, d, $J_{1,2}$ 10, *H*-1); δ_H(50.31 MHz, CD_3OD) 12.25, 12.52 (CH_2CH_3), 19.77, 20.23 (CH_3CH), 20.88, 20.97 (CH_3COO), 29.38, 29.77 (CH_2CH_3), 40.77, 40.90 (*C*H), 63.75 (*C*-6), 69.94, 70.00 (*C*-4), 72.24 (*C*-2), 75.52 (*C*-3), 76.93, 77.04 (*C*-5), 81.29, 81.39 (*C*-1), 154.66, 154.78 (*C*=N), 171.32, 171.53, 171.87, 172.53 (CH_3COO); *m*/*z*(CI) 464 (MH^+, 6%), 332 (68, $C_6H_8(OAc)_4^+$), 271 (68, $C_6H_7O(OAc)_3^+$), 169 (100, $C_8H_9O_4^+$).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-2-methylpropyl thiohydroximate 117



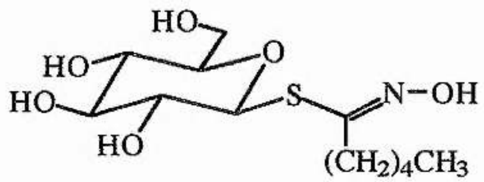
1-Chloro-3-methylbutyraldoxime (2.80 g, 20.7 mmol) and 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (5.37 g, 14.8 mmol) were dissolved in dry THF (150 ml) before dry triethylamine (15.0 g, 138.8 mmol) was added with stirring under a nitrogen atmosphere. The resulting solution was treated as before to afford a yellow foam. Purification by flash chromatography, on silica gel, eluting with hexane and ethyl acetate (3:2) afforded the title compound as a sticky white solid which was recrystallised from ethyl acetate and petroleum ether (216 mg, 3%), mp 147-148 °C; (Found: C, 49.24; H, 6.31; N, 2.86. $C_{19}H_{29}NO_{10}S$ requires C, 49.24; H, 6.31; N, 3.02%); $[\alpha]_D$ +6.67 ° (c 0.09 in $CHCl_3$); ν_{max}(nujol)/cm^{-1} 3330 (OH), 1720 (CO), 1600 (C=N); δ_H(200 MHz, CD_3OD) 0.98, 0.99 (6 H, 2 x d, *J* 6, $(CH_3)_2$), 2.04 (13 H, m, CH_3COO, $CH(CH_3)_2$), 2.40 (2 H, d, *J* 7, $CH_2CH(CH_3)_2$), 3.98 (1 H, m, *H*-5), 4.13 (1 H, dd, $J_{5,6^a}$ 2, $J_{6^a,6^b}$ 12, *H*-6^a), 4.24 (1 H, dd, $J_{5,6^b}$ 5, $J_{6^a,6^b}$ 12, *H*-6^b), 4.98, 5.05 (2 H, 2 x t, *J* 9, 10, *H*-2,4), 5.26 (1 H, d, $J_{1,2}$ 10, *H*-1), 5.39 (1 H, t, *J* 9, *H*-3); δ_C(50.31 MHz, CD_3OD) 20.88 (CH_3COO), 21.04, 22.84 (CH_3), 23.16 (CH_2), 28.15 (*C*H), 63.80 (*C*-6), 69.94 (*C*-4), 72.05 (*C*-2), 75.41 (*C*-3), 77.07 (*C*-5), 81.12 (*C*-1), 151.73 (*C*=N), 171.26, 171.53, 171.85, 172.51 (CH_3COO); *m/z*(CI) 464 (MH^+, 2%), 332 (100, $C_6H_8O(OAc)_4^+$), 271 (40, $C_6H_7O(OAc)_3^+$).

7.3.4 Synthesis of alkyl thiohydroximates

β-D-Glucopyranosyl-butyl thiohydroximate 106

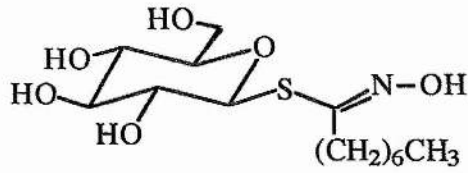
2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-butyl thiohydroximate (1 g, 2.2 mmol) was dissolved in dry methanol (10 ml) before a catalytic amount of potassium methoxide was added until the solution measured pH 9.0. This solution was stirred overnight at room temperature, under a nitrogen atmosphere, before Amberlite IR-120 (acidified using concentrated sulfuric acid and washed with water before use) was added. The reaction was stirred for a further 30 minutes before removal of the Amberlite by filtration, and concentration under reduced pressure to afford a cream-coloured foam. This was purified by chromatography, on silica gel, eluting with ethyl acetate. This yielded the product as a white solid (500 mg, 78%), mp 150 °C; (Found: C, 45.05; H, 7.14; N, 4.64. $C_{11}H_{21}NO_6S$ requires C, 44.73; H, 7.17; N, 4.74%); $[\alpha]_D$ -27.5 ° (c 0.2 in H_2O); ν_{max}(nujol)/cm^{-1} 3000-3450 (OH), 1600 (C=N); δ_H(200 MHz, CD_3OD) 0.96 (3 H, t, *J* 7, C*H*$_3$), 1.41 (2 H, sextet, *J* 8, C*H*$_2$CH$_3$), 1.66 (2 H, quintet, *J* 8, C*H*$_2$CH$_2$CH$_3$), 2.58 (2 H, t, *J* 8, C*H*$_2$(CH$_2$)$_2$CH$_3$), 3.31 (4 H, m, *H*-2,3,4,5), 3.63 (1 H, dd, $J_{5,6^a}$ 5, $J_{6^a,6^b}$ 12, *H*-6^a), 3.85 (1 H, d, $J_{6^a,6^b}$ 12, *H*-6^b), 4.72 (1 H, d, $J_{1,2}$ 9, *H*-1); δ_C(75.42 MHz, CD_3OD) 14.22 (*C*H$_3$), 23.45, 30.91, 33.51 (*C*H$_2$), 62.79 (*C*-6), 71.31 (*C*-4), 74.36 (*C*-2), 79.74 (*C*-3), 82.48 (*C*-5), 83.93 (*C*-1), 162.65 (*C*=N); *m/z*(CI) 296 (MH$^+$, 45%), 163 (41, $C_6H_{11}O_5^+$).

β-D-Glucopyranosyl-pentyl thiohydroximate 107



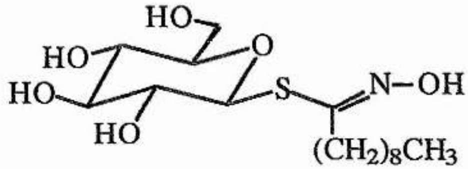
2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-pentyl thiohydroximate (200 mg, 0.4 mmol) was dissolved in dry methanol (10 ml) before a catalytic amount of sodium metal was added, with stirring and under a nitrogen atmosphere. The resulting solution was stirred overnight before Amberlite IR-120 was added and the mixture stirred for a further 20 minutes. Removal of the Amberlite by filtration then concentration under reduced pressure followed by chromatography, as before, afforded the product as a white foam (121 mg, 93%), mp 42-44 °C; (Found: C, 46.60; H, 7.23; N, 4.20. $C_{12}H_{23}NO_6S$ requires C, 46.59; H, 7.49; N, 4.53%); $[\alpha]_D$ -24.3 ° (c 0.2 in H_2O); ν_{max}(nujol)/cm^{-1} 3000-3450 (OH), 1600 (C=N); δ_H(200 MHz, CD_3OD) 0.93 (3 H, t, *J* 7, CH_3), 1.37 (4 H, m, $(CH_2)_2CH_3$), 1.68 (2 H, quintet, *J* 7, $CH_2(CH_2)_2CH_3$), 2.57 (2 H, t, *J* 7, $CH_2(CH_2)_3CH_3$), 3.32 (4 H, m, *H*-2,3,4,5), 3.64 (1 H, dd, $J_{5,6^a}$ 5, $J_{6^a,6^b}$ 12, *H*-6^a), 3.85 (1 H, d, $J_{6^a,6^b}$ 12, *H*-6^b), 4.79 (1 H, d, $J_{1,2}$ 9, *H*-1); δ_C(50.31 MHz, CD_3OD) 14.72 (CH_3), 23.77, 28.88, 32.86, 33.50 (CH_2), 62.96 (*C*-6), 71.48 (*C*-4), 74.66 (*C*-2), 79.96 (*C*-3), 82.50 (*C*-5), 83.66 (*C*-1), 155.18 (*C*=N); *m/z*(CI) 310 (MH^+, 48%), 276 (7, $[MH-CH_3(CH_2)_4]^+$) and 163 (37, $C_6H_{11}O_5^+$).

β-D-Glucopyranosyl-heptyl thiohydroximate 108



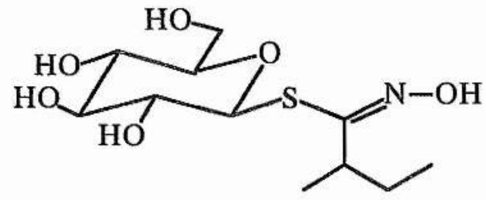
2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-heptyl thiohydroximate (200 mg, 0.4 mmol) was dissolved in dry methanol (10 ml) before a catalytic amount of sodium metal was added, with stirring and under a nitrogen atmosphere. The resulting solution was stirred overnight before Amberlite IR-120 was added and the mixture stirred for a further 30 minutes. Removal of the Amberlite and concentration under reduced pressure followed by chromatography as before afforded the product as a white foam (64 mg, 47%), mp 47-48 °C; (Found: C, 49.54; H, 8.13; N, 3.99. $C_{14}H_{27}NO_6S$ requires C, 49.83; H, 8.06; N, 4.15%); (Found (HRMS): MH^+ 338.1637. $C_{14}H_{27}NO_6S$ requires M*H* 338.4383); $[\alpha]_D$ -13.0 ° (c 0.1 in H_2O); ν_{max}(nujol)/cm^{-1} 3000-3450 (OH), 1600 (C=N); δ_H(200 MHz, CD_3OD) 0.91 (3 H, t, *J* 7, CH_3), 1.33 (8 H, br s, $(CH_2)_4CH_3$), 1.66 (2 H, quintet, *J* 7, $CH_2(CH_2)_4CH_3$), 2.57 (2 H, t, *J* 7, $CH_2(CH_2)_5CH_3$), 3.34 (4 H, m, *H*-2,3,4,5), 3.64 (1 H, dd, $J_{5,6^a}$ 5, $J_{6^a,6^b}$ 12, *H*-6^a), 3.84 (1 H, d, $J_{6^a,6^b}$ 12, *H*-6^b), 4.79 (1 H, d, $J_{1,2}$ 10, *H*-1); δ_C(50.31 MHz, CD_3OD) 14.78 (CH_3), 24.01, 29.18, 30.48, 30.59, 32.29, 33.54 (CH_2), 62.94 (*C*-6), 71.42 (*C*-4), 74.64 (*C*-2), 79.95 (*C*-3), 82.47 (*C*-5), 83.66 (*C*-1), 155.21 (*C*=N); *m*/*z*(CI) 338 (MH^+, 30%), 160 (83, $C_6H_7O_6^+$) and 126 (100, $C_6H_5O_4^+$).

β-D-Glucopyranosyl-nonyl thiohydroximate 109



2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-nonyl thiohydroximate (400 mg, 0.75 mmol) was dissolved in dry methanol (10 ml) before a catalytic amount of sodium metal was added and the resulting solution stirred, under a nitrogen atmosphere, at room temperature, for 20 hours. The reaction was quenched over 30 minutes by addition of Amberlite IR-120, which was removed prior to concentration under reduced pressure to afford a colourless oil (274 mg, 91%). This was purified using a C_{18} sep-pak, eluting with methanol, to afford a white solid (97 mg, 35%), mp 58-60 °C; (Found: C, 52.40; H, 8.87; N, 3.80. $C_{16}H_{31}NO_6S$ requires C, 52.58; H, 8.55; N, 3.83%); $[\alpha]_D$ -19.5 ° (c 0.1 in MeOH); ν_{max}(nujol)/cm^{-1} 3000-3450 (OH), 1600 (C=N); δ_H(200 MHz, CD_3OD) 0.91 (3 H, t, *J* 7, CH_3), 1.31 (12 H, br s, $(CH_2)_6CH_3$), 1.67 (2 H, br m, $CH_2(CH_2)_6CH_3$), 2.57 (2 H, t, *J* 7, $CH_2(CH_2)_7CH_3$), 3.28 (4 H, m, *H*-2,3,4,5), 3.65 (1 H, dd, $J_{5,6^a}$ 5, $J_{6^a,6^b}$ 12, *H*-6^a), 3.85 (1 H, dd, $J_{5,6^b}$ 1, $J_{6^a,6^b}$ 12, *H*-6^b), 4.79 (1 H, d, $J_{1,2}$ 9, *H*-1); δ_C(200 MHz, CD_3OD) 14.76 (CH_3), 24.03, 29.20, 30.64, 30.75, 30.80, 31.00, 33.37, 33.54 (CH_2), 62.94 (*C*-6), 71.44 (*C*-4), 74.66 (*C*-2), 79.96 (*C*-3), 82.50 (*C*-5), 83.67 (*C*-1), 155.35 (*C*=N); *m/z*(CI) 367 (MH^+, 3%), 172 (88, $CH_3(CH_2)_8CNHOH_2^+$) and 154 (100, $CH_3(CH_2)_8CNH^+$).

(*R*,*S*)-β-D-Glucopyranosyl-1-methylpropyl thiohydroximate 110



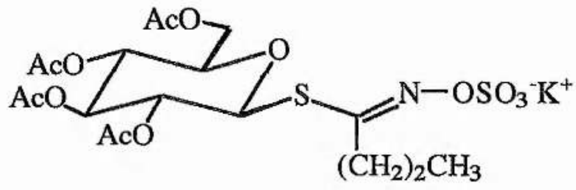
(*R*,*S*)-2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-1-methylpropyl thiohydroximate (1 g, 2.16 mmol) was dissolved in dry methanol (10 ml) before a catalytic quantity of potassium methoxide was added until the pH reached 8-9. The resulting solution was stirred at room temperature, under a nitrogen atmosphere, for 22 hours before being concentrated under reduced pressure to afford a cream-coloured foam. This was identified as the title compound existing as a mixture of diastereoisomers (523 mg, 82%); (Found: C, 44.56; H, 7.49; N, 4.52. $C_{11}H_{21}NO_6S$ requires C, 44.73; H, 7.17; N, 4.74%); $[\alpha]_D$ -29.5 ° (c 0.2 in H_2O); ν_{max}(nujol)/cm^{-1} 3300 (OH); δ_H(200 MHz, CD_3OD) 0.94, 0.95 (3 H, 2 x t, *J* 7, CH_2CH_3), 1.21 (3 H, 2 x d, *J* 7, $CHCH_3$), 1.49, 1.82 (2 H, 2 x m, CH_2CH_3), 2.65 (1 H, m, C*H*), 3.32 (4 H, m, *H*-2,3,4,5), 3.74 (2 H, m, *H*-6^a,6^b), 4.79, 4.81 (1 H, 2 x d, $J_{1,2}$ 10, *H*-1), 4.97 (5 H, br s, O*H*); δ_C(50.31 MHz, CD_3OD) 12.32, 12.75 (CH_2CH_3), 19.29, 20.61 ($CHCH_3$), 29.33, 30.22 (CH_2CH_3), 40.24, 40.46 (*C*H), 62.90 (*C*-6), 71.37, 71.44 (*C*-4), 74.86 (*C*-2), 79.94 (*C*-3), 82.20, 82.34 (*C*-5), 84.13, 84.24 (*C*-1), 157.11 (*C*=N); *m/z*(CI) 296 (MH^+, 7%), 202 (41), 186 (49), 134 (55), 118 (100, $C_5H_{12}NO_2^+$), 100 (74, $C_5H_{10}NO^+$).

β-D-Glucopyranosyl-2-methylpropyl thiohydroximate 111

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-2-methylpropyl thiohydroximate (180 mg, 0.39 mmol) was dissolved in dry methanol (10 ml) and treated as before. After 24 hours Amberlite IR-120 was added and the mixture stirred for 30 minutes. Removal of the Amberlite and concentration under reduced pressure afforded a cream-coloured foam which was purified by flash chromatography, on silica gel, eluting with ethyl acetate. This yielded the title compound as an off-white powder (59 mg, 51%); (Found: C, 45.35; H, 7.30; N, 4.38. $C_{11}H_{21}NO_6S$ requires C, 44.73; H, 7.17; N, 4.74%); $[\alpha]_D$ -6.5 ° (c 0.2 in H_2O); ν_{max}(neat)/cm^{-1} 3318 (OH), 2960 (alkyl); δ_H(200 MHz, CD_3OD) 0.96, 1.00 (6 H, 2 x d, *J* 10, 10, $CH(CH_3)_2$), 2.11 (1 H, m, $CH(CH_3)_2$), 2.34 (1 H, dd, $J_{7^a,8}$ 8, $J_{7^a,7^b}$ 14, *H*-7^a), 2.54 (1 H, dd, $J_{7^b,8}$ 6, $J_{7^a,7^b}$ 14, *H*-7^b), 3.33 (4 H, m, *H*-2,3,4,5), 3.64 (1 H, dd, $J_{5,6^a}$ 5, $J_{6^a,6^b}$ 12, *H*-6^a), 3.85 (1 H, dd, $J_{5,6^b}$ 2, $J_{6^a,6^b}$ 12, *H*-6^b), 4.78 (1 H, dd, $J_{1,2}$ 9, *H*-1); δ_C(50.31 MHz, CD_3OD) 22.60, 23.42 ($(CH_3)_2$), 28.44 (*C*H), 42.37 (CH_2), 62.92 (*C*-6), 71.41 (*C*-4), 74.63 (*C*-2), 79.90 (*C*-3), 82.65 (*C*-5), 83.85 (*C*-1), 156.87 (*C*=N); *m/z*(CI) 295 (M^+, 11%), 279 (38, $[M-O]^+$), 237 (36, $[M-C_4H_{10}]^+$), 218 (46), 163 (35, $C_6H_{11}O_5^+$), 71 (100).

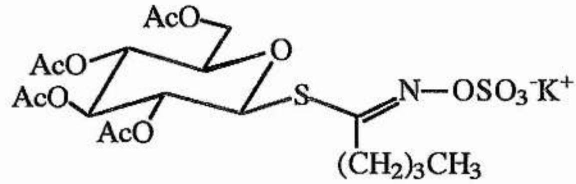
7.3.5 Synthesis of tetra-*O*-acetyl-alkyl glucosinolates

2,3,4,6-Tetra-*O*-acetyl-propyl glucosinolate 118



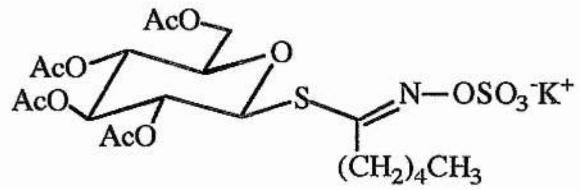
Chlorosulfonic acid (7.87 g, 67.5 mmol) was added to dry dichloromethane (103 ml) and the resulting solution added slowly, with stirring, to a mixture of dry pyridine (103 ml, 1.27 mol) and dry dichloromethane (103 ml). 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-propyl thiohydroximate (3 g, 6.67 mmol) was then added as a solution in dry dichloromethane (50 ml) and the reaction mixture stirred at room temperature for 24 hours. After this period, potassium hydrogen carbonate (20.0 g, 200 mmol) was added in water (100 ml). Once CO_2 evolution had ceased, the two-phase system was concentrated under reduced pressure to allow removal of the organics, yielding a white solid which was removed from the remaining aqueous layer by filtration under reduced pressure. Recrystallisation from methanol afforded the title compound (2.25 g, 59%), mp 146-147 °C (decomp.); (Found: C, 37.79; H, 4.31; N, 2.41. $C_{18}H_{26}NO_{13}S_2K.0.5\ H_2O$ requires C, 37.49; H, 4.72; N, 2.43%); $[\alpha]_D$ -42.9 ° (c 0.14 in H_2O); ν_{max}(nujol)/cm^{-1} 1750 (CO), 1580 (C=N), 1250 (COOC); δ_H(300 MHz, CD_3OD) 1.02 (3 H, t, *J* 8, CH_3), 1.75 (2 H, m, CH_2CH_3), 1.99, 2.02, 2.02, 2.05 (12 H, 4 x s, CH_3COO), 2.63 (2 H, t, *J* 7, $CH_2CH_2CH_3$), 4.02 (1 H, m, *H*-5), 4.15 (1 H, d, $J_{6^a,6^b}$ 11, *H*-6ᵃ), 4.20 (1 H, dd, $J_{5,6^b}$ 5, $J_{6^a,6^b}$ 11, *H*-6ᵇ), 5.01 (2 H, 2 x t, *J* 10, *H*-2,4), 5.35 (1 H, d, $J_{1,2}$ 10, *H*-1), 5.36 (1 H, t, $J_{2,3}$=$J_{3,4}$ 8, *H*-3); δ_C(75.42 MHz, CD_3OD) 13.80 (CH_3), 20.45, 20.57 (CH_3COO), 21.71 ($CH_2CH_2CH_3$), 35.58 ($CH_2CH_2CH_3$), 63.47 (*C*-6), 69.69 (*C*-4), 71.63 (*C*-2), 75.17 (*C*-3), 76.90 (*C*-5), 81.08 (*C*-1), 159.52 (*C*=N), 171.22, 171.47, 171.75, 174.46 (*C*O); *m/z*(ES^-) 529 ($[M-K]^-$, 100%).

2,3,4,6-Tetra-*O*-acetyl-butyl glucosinolate 122



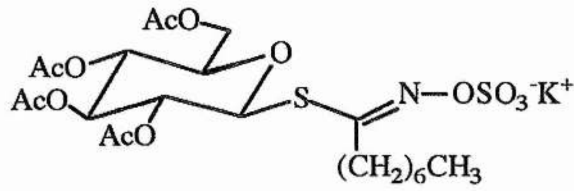
Chlorosulfonic acid (11.45 g, 98.3 mmol) was added to dry dichloromethane (150 ml) and this solution was added dropwise to a mixture of dry dichloromethane (150 ml) and pyridine (145.9 g, 1.84 mol), at 0 °C, with stirring, and under a nitrogen atmosphere. 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-butyl thiohydroximate (4.5 g, 9.7 mmol), dissolved in dry dichloromethane (100 ml), was added and the solution was stirred as before. Potassium hydrogen carbonate (29.16 g, 291.3 mmol) was then added in water (100 ml). The two-phase system was concentrated under reduced pressure to allow removal of the organic layer. This yielded a white solid which was removed by filtration. Recrystallisation from methanol afforded the product as a white solid (4.1 g, 73%), mp 148-150 °C (decomp.); (Found: C, 39.54; H, 4.85; N, 2.36. $C_{19}H_{28}NO_{13}S_2K$ requires C, 39.23; H, 4.85; N, 2.41%); $[\alpha]_D$ +16.4 ° (c 0.14 in H_2O); ν_{max}(nujol)/cm^{-1} 1750 (CO), 1580 (C=N), 1250 (COOC); δ_H(300 MHz, $(CD_3)_2SO$) 1.04 (3 H, t, *J* 7, CH_3), 1.50 (2 H, sextet, *J* 7, CH_2CH$_3$), 1.71 (2 H, m, CH_2CH$_2$CH$_3$), 2.07, 2.12, 2.13, 2.14 (12 H, 4 x s, CH_3COO), 2.66 (2 H, m, CH_2(CH$_2$)$_2$CH$_3$), 4.22 (3 H, m, *H*-5,6^a,6^b), 5.00, 5.05 (2 H, 2 x t, *J* 10, 10, *H*-2,4), 5.58 (1 H, t, $J_{2,3}$=$J_{3,4}$ 9, *H*-3), 5.62 (1 H, d, $J_{1,2}$ 10, *H*-1); δ_C(75.42 MHz, CD_3COCD_3) 13.10 (*C*H$_3$), 19.71, 19.82, 19.89 (*C*H$_3$COO), 21.20, 28.37, 30.61 (*C*H$_2$), 61.65 (*C*-6), 67.65 (*C*-4), 69.12 (*C*-2), 70.28 (*C*-3), 73.86 (*C*-5), 77.69 (*C*-1), 153.74 (*C*=N), 168.85, 169.05, 169.28, 169.66 (*C*O); *m*/*z*(ES$^-$) 542 ([M-K]$^-$, 100%).

2,3,4,6-Tetra-*O*-acetyl-pentyl glucosinolate 119



Chlorosulfonic acid (6.17 g, 53.0 mmol) was added to dry dichloromethane (80 ml) and this solution was added dropwise to a mixture of dry dichloromethane (80 ml) and pyridine (78.75 g, 995.2 mmol), at 0 °C, with stirring, and under a nitrogen atmosphere. 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-butyl thiohydroximate (2.5 g, 5.24 mmol), dissolved in dry dichloromethane (60 ml), was added and the reaction mixture was stirred as before. Potassium hydrogen carbonate (15.74 g, 157.2 mmol) was then added in water (100 ml) and the two-phase system was partially concentrated under reduced pressure to afford a white solid which was removed by filtration (2.67 g, 86%), mp 158 °C (decomp.); (Found: C, 39.61; H, 5.10; N, 2.32. $C_{20}H_{30}NO_{13}S_2K.0.5H_20$ requires C, 39.73; H, 5.17; N, 2.32%); $[\alpha]_D$ -15.7 ° (c 0.14 in H_2O); ν_{max}(nujol)/cm^{-1} 1750 (CO), 1580 (C=N), 1250 (COOC); δ_H(300 MHz, CD_3OD) 0.93 (3 H, br s, CH_3), 1.39 (4 H, m, $(CH_2)_2CH_3$), 1.73 (2 H, m, $CH_2(CH_2)_2CH_3$), 1.97, 2.02, 2.05, (12 H, 3 x s, CH_3COO), 2.64 (2 H, m, $CH_2(CH_2)_3CH_3$), 4.01 (1 H, br s, *H*-5), 4.18 (2 H, m, *H*-6^{a,} 6^b), 5.00, 5.05 (2 H, 2 x t, *J* 10, *H*-2,4), 5.36 (2 H, m, *H*-1,3); δ_C(75.42 MHz, CD_3OD) 14.20 (CH_3), 20.46, 20.57 (CH_3COO), 23.37, 28.10, 32.29, 33.78(CH_2), 63.43 (*C*-6), 69.67 (*C*-4), 71.67 (*C*-2), 75.19 (*C*-3), 76.90 (*C*-5), 81.15 (*C*-1), 159.57 (*C*=N), 171.22, 171.47, 171.78, 172.44 (*C*O); *m*/*z*(ES$^-$) 556 ([M-K]$^-$, 100%).

2,3,4,6-Tetra-*O*-acetyl-heptyl glucosinolate 120



A solution of chlorosulfonic acid (3.03 g, 26.0 mmol) in dry dichloromethane (30 ml) was added to a stirred mixture of dry pyridine (38.6 g, 488.3 mmol) and dry dichloromethane (30 ml) maintained at 0 °C and under a nitrogen atmosphere. 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-heptyl thiohydroximate (1.3 g, 2.6 mmol) dissolved in dry dichloromethane (20 ml) was then added and the resulting mixture stirred as given previously. Potassium hydrogen carbonate (1.67 g, 16.7 mmol) was added in water (100 ml) and this mixture allowed to stir for 30 minutes before partial concentration under reduced pressure followed by co-evaporation with toluene. Further aqueous potassium hydrogen carbonate (5.0 g, 50.1 mmol) was added and the two-phase system partially concentrated under reduced pressure to afford a brown solid. Purification by chromatography, on silica gel, eluting with petroleum ether (bp 40-60 °C): ethyl acetate (1:1) with 10% (v/v) methanol, allowed recovery of the title compound as a white solid (697 mg, 43%), mp 107-109 °C; (Found: C, 42.08; H, 5.52; N, 1.98. $C_{22}H_{34}NO_{13}S_2K$ requires C, 42.36; H, 5.49; N, 2.25%); $[\alpha]_D$ -16.4 ° (c 0.14 in MeOH); ν_{max}(nujol)/cm^{-1} 1750 (CO), 1580 (C=N), 1250 (COOC); δ_H(200 MHz, CD_3OD) 0.91 (3 H, t, *J* 6, CH_3), 1.34 (8 H, br s, $(CH_2)_4$), 1.85 (2 H, br m, $CH_2(CH_2)_4CH_3$), 1.99, 2.04, 2.07 (12 H, 3 x s, CH_3COO), 2.66 (2 H, t, *J* 8, $CH_2(CH_2)_5CH_3$), 4.03 (1 H, m, *H*-5), 4.13 (2 H, dd, $J_{5,6^a}$ 2, $J_{6^a,6^b}$ 12, *H*-6^a), 4.24 (2 H, dd, $J_{5,6^b}$ 4, $J_{6^a,6^b}$ 12, *H*-6^b), 5.03 (2 H, 2 x t, *J* 10, 9, *H*-2,4), 5.36 (1 H, d, $J_{1,2}$ 10, *H*-1), 5.40 (1 H, t, $J_{2,3}$=$J_{3,4}$ 9, *H*-3); δ_C(50.31 MHz, CD_3OD) 14.78 (CH_3), 20.88, 21.02 (CH_3COO), 24.02, 28.78, 30.49, 30.54, 33.25, 33.98 (CH_2), 63.68 (*C*-6), 69.80 (*C*-4), 71.72 (*C*-2), 75.31 (*C*-3), 77.03 (*C*-5), 81.18 (*C*-1), 159.94 (*C*=N), 171.25, 171.55, 171.82, 172.51 (*C*O); *m/z*(ES$^-$) 584 ([M-K]$^-$, 100%).

2,3,4,6-Tetra-*O*-acetyl-nonyl glucosinolate 121

Chlorosulfonic acid (2.21 g, 18.9 mmol) dissolved in dry dichloromethane (30 ml) was added to a stirred mixture of dry dichloromethane (30 ml) and dry pyridine (28.1 g, 355.3 mmol), at 0 °C and under a nitrogen atmosphere. 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-nonyl thiohydroximate (1 g, 1.87 mmol) dissolved in dry dichloromethane (14 ml) was added as before. After 24 hours, potassium hydrogen carbonate (5.62 g, 56.1 mmol) was added in water (100 ml) and the dichloromethane removed under reduced pressure. Addition of diethyl ether allowed the formation of a white precipitate which could not be recovered by filtration. Methanol was then added and the solution was concentrated under reduced pressure to afford a white solid. Recrystallisation failed to purify the desired compound (401 mg, 35%); *m/z* (ES^-) 612 ($[M-K]^-$, 100%).

7.3.6 Synthesis of alkyl glucosinolates

Propyl glucosinolate 78

A solution of potassium methoxide was prepared by adding a catalytic amount of potassium metal to dry methanol (1 ml). A small quantity of this solution was added to 2,3,4,6-tetra-*O*-acetyl-propyl glucosinolate (1 g, 1.8 mmol), dissolved in dry methanol (15 ml), until the

resulting solution measured pH 9 approximately. This solution was then stirred at room temperature and under a nitrogen atmosphere, for 24 hours, before concentration under reduced pressure to afford a cream-coloured foam (703 mg, 100%); (Found: C, 26.53; H, 4.12; N, 3.00. $C_{10}H_{18}NO_9S_2K$ requires C, 30.07; H, 4.54; N, 3.51%); $[\alpha]_D$ -16.5 ° (c 0.2 in H_2O); ν_{max}(nujol)/cm^{-1} 3400 (OH); δ_H(200 MHz, CD_3OD) 1.02 (3 H, t, *J* 7, CH_3), 1.77 (2 H, sextet, *J* 8, CH_2CH_3), 2.68 (2 H, t, *J* 7, $CH_2CH_2CH_3$), 3.33 (4 H, m, *H*-2,3,4,5), 3.65 (1 H, dd, $J_{5,6^a}$ 5, $J_{6^a,6^b}$ 12, *H*-6^a), 3.85 (1 H, dd, $J_{5,6^b}$ 1, $J_{6^a,6^b}$ 12, *H*-6^b), 4.83 (1 H, d, $J_{1,2}$ 9, *H*-1); δ_C(50.31 MHz, CD_3OD) 14.32 (CH_3), 22.32 (CH_2CH_3), 35.83 ($CH_2CH_2CH_3$), 62.91 (*C*-6), 71.44 (*C*-4), 74.45 (*C*-2), 79.83 (*C*-3), 82.59 (*C*-5), 84.01 (*C*-1), 162.41 (*C*=N); *m/z*(ES$^-$) 360 ([M-K]$^-$, 100%).

Butyl glucosinolate 79

HO, HO, HO, HO, O, S, N–$OSO_3^-K^+$, $(CH_2)_3CH_3$

2,3,4,6-Tetra-*O*-acetyl-butyl glucosinolate (2 g, 3.4 mmol) was dissolved in dry methanol (20 ml) before a catalytic amount of potassium methoxide solution was added and the resulting solution stirred as before for 18 hours. A further quantity of potassium methoxide solution was then added and the reaction stirred for a further 6 hours. Concentration under reduced pressure afforded a gold-coloured foam which was purified by chromatography, on silica gel, eluting with methanol: ethyl acetate (1:2.5). This allowed recovery of the title compound as a white foam (892 mg, 63%); (Found: C, 31.70; H, 5.11; N, 3.07. $C_{11}H_{20}NO_9S_2K$ requires C, 31.95; H, 4.88; N, 3.39%); $[\alpha]_D$ -18.5 ° (c 0.2 in H_2O); ν_{max}(nujol)/cm^{-1} 3400 (OH); δ_H(200 MHz, CD_3OD) 0.97 (3 H, t, *J* 7, CH_3), 1.42 (2 H, m, CH_2CH_3), 1.69 (2 H, m, $CH_2CH_2CH_3$), 2.69 (2 H, t, *J* 8, $CH_2(CH_2)_2CH_3$), 3.31 (4 H, m, *H*-2,3,4,5), 3.65 (1 H, d, $J_{6^a,6^b}$ 11, *H*-6^a), 3.85 (1 H, d, $J_{6^a,6^b}$ 11, *H*-6^b), 4.83

(1 H, d, $J_{1,2}$ 10, *H*-1); δ_C(75.42 MHz, CD_3OD) 14.22 (CH_3), 23.45, 30.91, 33.51 (CH_2), 62.79 (*C*-6), 71.31 (*C*-4), 74.36 (*C*-2), 79.74 (*C*-3), 82.48 (*C*-5), 83.93 (*C*-1), 162.65 (*C*=N); *m/z*(ES^-) 374 ($[M-K]^-$, 100%).

Pentyl glucosinolate 80

2,3,4,6-Tetra-*O*-acetyl-pentyl glucosinolate (2.5 g, 4.2 mmol) was dissolved in dry methanol (20 ml) before a catalytic amount of potassium methoxide solution was added and the resulting solution stirred as before for several days. Concentration under reduced pressure afforded the title compound as a cream-coloured foam (1.77 mg, 99%); (Found: C, 31.94; H, 5.01; N, 3.05. $C_{12}H_{22}NO_9S_2K$ requires C, 33.71; H, 5.19; N, 3.28%); $[\alpha]_D$ -19.8 ° (c 0.2 in H_2O); ν_{max}(nujol)/cm^{-1} 3400 (OH); δ_H(200 MHz, CD_3OD) 0.94 (3 H, t, *J* 7, CH_3), 1.41 (4 H, m, $(CH_2)_2CH_3$), 1.75 (2 H, quintet, *J* 7, $CH_2(CH_2)_2CH_3$), 2.69 (2 H, t, *J* 8, $CH_2(CH_2)_3CH_3$), 3.34 (4 H, m, *H*-2,3,4,5), 3.66 (1 H, dd, $J_{5,6^a}$ 5, $J_{6^a,6^b}$ 12, *H*-6^a), 3.86 (1 H, d, $J_{6^a,6^b}$ 12, *H*-6^b), 4.84 (1 H, d, $J_{1,2}$ 9, *H*-1); δ_C(50.31 MHz, CD_3OD) 14.71 (CH_3), 23.74, 28.79, 32.86, 33.99 (CH_2), 62.86 (*C*-6), 71.40 (*C*-4), 74.47 (*C*-2), 75.81 (*C*-3), 82.56 (*C*-5), 84.05 (*C*-1), 162.99 (*C*=N); *m/z*(ES^-) 388 ($[M-K]^-$, 100%).

Heptyl glucosinolate 81

A solution of potassium methoxide was prepared by adding a catalytic amount of potassium (up to 100 mg) to dry methanol (1 ml) at room temperature and under a nitrogen atmosphere. A small volume of this solution was added to 2,3,4,6-tetra-*O*-acetyl-heptyl glucosinolate (460 mg, 0.74 mmol), dissolved in dry methanol (10 ml), until the pH reached 8-9. The resulting solution was stirred overnight under a nitrogen atmosphere before being concentrated under reduced pressure to afford a gold coloured solid (337, 100%); (Found: C, 37.12; H, 5.89; N, 2.84. $C_{14}H_{26}NO_9S_2K$ requires C, 36.91; H, 5.75; N, 3.07%); $[\alpha]_D$ -14.0 ° (c 0.2 in H_2O); ν_{max}(nujol)/cm^{-1} 3400 (OH); δ_H(200 MHz, CD_3OD) 0.91 (3 H, t, *J* 7, CH_3), 1.33 (8 H, br m, $(CH_2)_4$), 1.71 (2 H, br m, $CH_2(CH_2)_4CH_3$), 2.69 (2 H, t, *J* 7, $CH_2(CH_2)_5CH_3$), 3.34 (4 H, m, *H*-2,3,4,5), 3.65 (1 H, dd, $J_{5,6^a}$ 4, $J_{6^a,6^b}$ 10, *H*-6^a), 3.85 (1 H, d, $J_{6^a,6^b}$ 10, *H*-6^b), 4.83 (1 H, d, $J_{1,2}$ 10, *H*-1); δ_C(50.31 MHz, CD_3OD) 14.77 (CH_3), 24.02, 29.08, 30.47, 30.65, 33.25, 33.99 (CH_2), 62.90 (*C*-6), 71.39 (*C*-4), 74.44 (*C*-2), 79.83 (*C*-3), 82.59 (*C*-5), 84.01 (*C*-1), 162.66 (*C*=N); *m/z*(ES$^-$) 416 ([M-K]$^-$, 100%).

Nonyl glucosinolate 82

Chlorosulfonic acid (3.31 g, 28.5 mmol) was added to dry dichloromethane (50 ml) and this solution was added to a stirred solution of dry pyridine (42.23 g, 533.9 mmol) and dry dichloromethane (50 ml) at 0 °C and under a nitrogen atmosphere. 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-nonyl thiohydroximate (1.5 g, 2.81 mmol) was added in dry dichloromethane (30 ml) and the resulting solution stirred at room temperature for 24 hours. Potassium hydrogen carbonate (8.44 g, 84.3 mmol) was added in water (100 ml) and the organics removed under reduced pressure to afford a brown solid. Purification by flash chromatography, on silica gel, eluting with ethyl acetate then ethyl acetate and methanol (4:1) afforded the title compound (277 mg, 20%); (Found: C, 39.41; H, 6.33; N, 2.64. $C_{16}H_{30}NS_2O_9K$ requires C, 39.74; H, 6.25; N, 2.90%); $[\alpha]_D$ -15.75 ° (c 0.2 in H_2O); ν_{max}(nujol)/cm^{-1} 3400 (OH); δ_H(200 MHz, CD_3OD) 0.91 (3 H, t, *J* 7, CH_3), 1.32 (12 H, br s, $(CH_2)_6$), 1.75 (2 H, quintet, *J* 7, $CH_2(CH_2)_6CH_3$), 2.69 (2 H, t, *J* 8, $CH_2(CH_2)_7CH_3$), 3.31 (4 H, m, *H*-2,3,4,5), 3.64 (1 H, dd, $J_{5,6^a}$ 5, $J_{6^a,6^b}$ 12, *H*-6^a), 3.85 (1 H, d, $J_{6^a,6^b}$ 12, *H*-6^b), 4.82 (1 H, d, $J_{1,2}$ 9, *H*-1); δ_C(75.42 MHz, CD_3OD) 13.41 (CH_3), 21.56, 26.66, 28.21, 28.46, 30.78, 31.20, 38.15 (CH_2), 60.27 (*C*-6), 69.21 (*C*-4), 72.32 (*C*-2), 77.70 (*C*-3), 80.84 (*C*-5), 81.59 (*C*-1), 155.89 (*C*=N); *m*/*z*(ES$^-$) 445 ($[M-K]^-$, 100%).

7.3.7 Purification of alkyl glucosinolates

7.3.7.1 Reverse phase (C_{18}) Sep-pak purification

The cartridges used were either C_{18} (900 mg) "maxi-clean" or C_{18} (2000 mg) "extract-clean" Sep-paks obtained from Alltech Associates, or Waters C_{18} "Sep-pak Plus." These were all used following the general procedure: The cartridge was washed with water (10 ml), methanol (10 ml) and water (10 ml); The glucosinolate (*ca.* 150-200 mg) was dissolved in distilled water (100 ml) and an aliquot (20 ml) passed through the cartridge; The Sep-pak was then washed with water (20-40 ml) and methanol (20-40 ml); This procedure was repeated for every aliquot of glucosinolate solution (20 ml).

7.3.7.2 Ion-exchange chromatography

Anion exchange

DEAE Sephadex, pre-swollen in water, was added to a non-sintered column (6-6.5 cm deep, 2.5 cm diameter) and was washed with 2 M ammonium bicarbonate. The glucosinolate (200 mg) was added as a solution with distilled water and the column washed with distilled water (200 ml), 0.1, 0.5, 1 and 2 M ammonium acetate solution (200 ml each). The glucosinolate was recovered by freeze-drying.

Size exclusion

G-10 Sephadex was contained in a burette (10 ml) and the glucosinolate added as a solution with water.

Cellulose chromatography

The column was prepared by suspending cellulose (1 ml) in *iso*-propanol, ammonia and water (26:6:5) and adding to a pipette (5 ml). The freeze-dried glucosinolate was dissolved

in the above solvent and then passed through the column. The *iso*-propanol was removed under reduced pressure prior to freeze-drying the glucosinolate.

Cation exchange

Amberlite IR-120 resin (4 ml) was added to a pipette (5 ml) and washed with 1 M potassium hydroxide solution (30 ml) then distilled water until the pH was neutral (measured using pH indicator paper). The glucosinolate was dissolved in water, added to the column and washed through with distilled water. The glucosinolate was obtained as a solid by freeze-drying.

Conversion of glucosinolate to tetra-methylammonium salt

Amberlite IR-120, in the same quantity as above, was washed with 25% aqueous tetramethylammonium hydroxide (30 ml) and then with water until the pH was neutral. The glucosinolate was added and recovered as before.

7.3.8 HPLC and LC-MS analysis of alkyl thiohydroximates

HPLC chromatograms were recorded at the IACR, Rothamstead, using a Waters 600 LC system equiped with a 996 photodiode array detector. Data was collected between 200 and 300 nm. The column used was a 4.6 x 250 mm C_{18} column (Waters, Symmetry) with a 5 μm particle size. The mobile phase was made up of water-acetonitrile as given in table 20.

Time (minutes)	Flow rate (ml/min)	% Water	% Acetonitrile	Gradient
0	1.5	100	0	-
5	1.5	96	4	Linear
30	1.5	60	40	Linear
35	1.5	60	40	-
35.1	1.5	100	0	Step
45	Next sample	-	-	-

Table 20 Mobile phase during HPLC method (IACR).

HPLC chromatograms were also recorded at the SCRI using a Gilson dual pump HPLC system with a Gilson UV detector set at a fixed wavelength of 230 nm. The instrument used a Rheodyne injector fitted with a 20 μl loop. The column used was a 25 cm x 4.6 mm reverse phase C_{18} column (Hypersil ODS 5μ). The mobile phase had a flow rate of 1.5 ml/min and a composition as detailed in table 21.

Time (minutes)	**% Solvent A (distilled water)**	**% Solvent B (acetonitrile (far UV grade)) and distilled water (1:4)).**
0	99	1
1	99	1
46	0	100
51	99	1
60	99	1

Table 21 HPLC mobile phase composition (SCRI).

LC-MS spectra were recorded using a Finnigan SSQ7 10 C liquid chromatography-mass spectrometer in the atmospheric pressure chemical ionisation mode. A flow rate of 0.3 ml/minute was used with a column and mobile phase as given above.

Volatile products arising from myrosinase catalysed hydrolysis of turnip root powder (cultivar *massif*) were sampled as detailed in section 7.6.3. The powder (250 mg) was added to distilled water (10 ml) which had been prewarmed to 37 °C and the resulting mixture incubated at this temperature for 4 hours before the head space (125 ml) was sampled allowing absorption of the volatiles into a porous polymer (Tenax-TA). The polymer contents was analysed by thermal desorption GC/MS at the SCRI as given in section 7.6.3.

7.4 SYNTHESIS OF COMPLEX GLUCOSINOLATES

7.4.1 Compounds prepared for the synthesis of naphthylmethyl glucosinolate

Attempted raney nickel reduction of 1-naphthylacetonitrile

1-Naphthylacetonitrile (5 g, 167.2 mmol) was added to aluminium/nickel alloy (5 g) suspended in 75% (v/v) aqueous formic acid (100 ml) and the resulting mixture heated under reflux, with stirring, for 2-3 hours. After cooling, the mixture was filtered through celite and washed with ethanol before concentration under reduced pressure to half the original volume. Water (200 ml) was then added and this solution was extracted with dichloromethane (100 ml) and chloroform (100 ml). The organic layer was washed with water (200 ml) and saturated sodium hydrogen carbonate solution (200 ml) before being dried (Na_2SO_4) and concentrated under reduced pressure to afford a yellow/brown oil. Analysis failed to show the presence of 1-naphthylacetaldehyde.

Attempted Swern oxidation of 1-naphthaleneethanol

Oxalyl chloride (406 mg, 3.2 mmol) was added to dry dichloromethane (10 ml) before dry dimethyl sulfoxide (500 mg, 6.4 mmol) was added in dry dichloromethane (2.5 ml), with stirring, under a nitrogen atmosphere and at a temperature of -60 to -50 °C. 1-Napthaleneethanol (500 mg, 2.9 mmol), dissolved in dry dichloromethane (2.5 ml) was added over 5 minutes and the resulting solution stirred at -60 °C for 15 minutes before dry triethylamine (1.47 g, 14.5 mmol) was added. The solution was maintained at -60 °C for a further 5 minutes then allowed to warm to room temperature. Water (60 ml) was added, the organic layer removed and the aqueous layer extracted with dichloromethane (100 ml). The organic layers were combined, washed with saturated brine (100 ml), dried ($MgSO_4$) and concentrated under reduced pressure to afford a gold oil. This was found to contain a mixture of products.

Attempted PCC oxidation of 1-naphthaleneethanol (Sample procedure)

1-Naphthaleneethanol (500 mg, 2.9 mmol) was added to pyridinium chlorochromate (938 mg, 4.4 mmol) dissolved in dry dichloromethane (20 ml). The resulting solution was stirred at room temperature, under a nitrogen atmosphere for approximately 2 hours. Diethyl ether (40 ml) was added and the organic layer decanted from a brown residue which was washed further with diethyl ether (3 x 30 ml). The organics were passed through a florisil column which was further eluted with diethyl ether (200 ml). Concentration under reduced pressure afforded a pale yellow oil found to contain both the starting material and 1-naphthylacetaldehyde (1:2).

1-Naphthylacetic acid ethyl ester 128

1-Naphthylacetic acid (20 g, 107.4 mmol) was dissolved in dry ethanol (500 ml) before thionyl chloride (14.06 g, 118.2 mmol) was added. The resulting solution was heated under reflux for 3.5 hours before concentration under reduced pressure to afford the title compound as a yellow oil (22.95 g, 100%); ν_{max}/cm^{-1} 3050 (aromatic) 2900-3000 (alkyl), 1730 (CO), 1520, 1600 (aromatic), 1150-1275 (COOC); δ_H(200 MHz, $CDCl_3$) 1.27 (3 H, t, *J* 7, OCH_2CH_3), 4.11 (2 H, s, $CH_2COCH_2CH_3$), 4.20 (2 H, q, *J* 7, OCH_2CH_3), 7.54 (4 H, m, *H*-3,5,6,7), 7.88 (2H, m, *H*-4,8), 8.06 (1 H, d, *J* 8, *H*-2); δ_C(50.31 MHz, $CDCl_3$) 14.73, 14.79 (OCH_2CH_3), 39.85 ($CH_2COCH_2CH_3$), 61.54 (OCH_2CH_3), 124.41, 126.01, 126.09, 126.34, 126.89, 128.61, 129.29 (Ar*C*), 131.25 (Ar*C*-1), 132.67 (Ar*C*-9), 134.36 (Ar*C*-10), 172.19 (*C*O).

1-Naphthylacetaldehyde 123

1-Naphthylacetic acid ethyl ester (10 g, 46.7 mmol) was dissolved in dry toluene (200 ml) and the solution cooled to -78 °C before DIBAL-H (1.5 M in toluene; 12.74 g, 89.6 mmol) was added. The resulting solution was stirred at -78 °C for 2 hours under a nitrogen atmosphere before dry methanol (100 ml) was added and the solution allowed to warm slowly to room temperature. A solution of potassium sodium tartrate (60 g in 100 ml H_2O) was added and the biphasic system stirred overnight. The organic layer was removed and the aqueous layer washed with diethyl ether (2 x 150 ml). The organics were combined, dried ($MgSO_4$) and concentrated under reduced pressure to yield a yellow oil (7.43 g, 94%); δ_H(200 MHz, $CDCl_3$) 4.11 (2 H, d, *J* 2, CH_2CHO), 7.46 (4 H, m, Ar*H*), 7.90 (3 H, m, Ar*H*), 9.79 (1 H, t, *J* 2, CH*O*); δ_C(50.31 MHz, $CDCl_3$) 48.89 (CH_2CHO), 124.10, 126.15, 126.22, 126.63, 127.26, 128.89, 129.03, 129.47 (Ar*C*), 132.80 (Ar*C*-9), 134.43 (Ar*C*-10), 200.28 (*C*HO).

1-Naphthylacetaldoxime 129

1-Naphthylacetaldehyde (6.65 g, 39.1 mmol) was dissolved in ethanol (70 ml) and pyridine (7 ml) before hydroxylamine hydrochloride (5 g, 71.9 mmol) was added. The resulting

solution was heated under reflux, with stirring, for 2.5 hours before being concentrated under reduced pressure. Water was then added resulting in the appearance of a yellow solid which was recrystallised from ethanol to afford the title compound (3.78 g, 52%) as a mixture of isomers (40% *anti*: 60% *syn*), mp 127-128 °C (lit.,[128] 123-124 °C); δ_H(200 MHz, $CDCl_3$) 4.02 (2 H, d, *J* 5, CH_2CH (*anti*)), 4.20 (2 H, d, *J* 5, CH_2CH (*syn*)), 6.84 (1 H, t, *J* 5, C*H*=N), 7.38-8.08 (7 H, m, Ar*H*), 8.80 (1 H, br s, NO*H*); δ_C(50.31 MHz, $CDCl_3$) 30.19, 33.95 (CH_2), 124.02, 124.11, 124.22, 126.06, 126.11, 126.44, 126.63, 126.72, 126.94, 127.08, 127.29, 127.36, 127.43, 127.61, 128.29, 128.41, 129.34, 129.52. 133.31, 134.38 (Ar*C* (*syn, anti*)), 151.15 (*C*H=N (*anti*)), 151.49 (*C*H=N (*syn*); *m/z*(CI) 186 (MH^+, 100%), 141 (52, $[M-CH_2NOH]^+$).

Chloro-1-naphthylacetaldoxime 130

N—OH

Cl

1-Naphthylacetaldoxime (6.5 g, 35.1 mmol) was added to a stirred solution of chloroform (50 ml) and pyridine (1.39 g, 17.6 mmol) before *N*-chlorosuccinimide (4.69 g, 35.1 mmol) was added slowly. The resulting solution was stirred at room temperature for 4 hours then poured into ice/water. The organic layer was separated and washed with water (50 ml) and saturated sodium chloride solution (50 ml) while the aqueous layer was extracted with diethyl ether (75 ml). The organics were combined, dried ($MgSO_4$) and concentrated under reduced pressure. This afforded a mixture of the oxime and oximyl chloride (1:2) (5.53 g, 48% oximyl chloride); δ_H(200 MHz, $CDCl_3$) 4.24 (2 H, s, CH_2C=N), 7.32-7.61 (4 H, m, *H*-3,5,6,7), 7.69-8.09 (3 H, m, *H*-2,4,8), 8.86 (1 H, br s, NO*H*); δ_C(50.31 MHz, $CDCl_3$) 40.20 (CH_2C=N), 122.87-133.83 (Ar*C*), 140.07 (*C*=N).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-1-naphthylmethyl thiohydroximate 131

2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (4.32 g, 11.9 mmol) and chloro-1-naphthylacetaldoxime (3.65 g, 16.6 mmol) were dissolved in dry THF (250 ml) before dry triethylamine (11.26 g, 111.3 mmol) was added. The resulting solution was stirred overnight, under a nitrogen atmosphere and at room temperature. Diethyl ether (200 ml) was added and the mixture washed with 1 M H_2SO_4 (200 ml). After removal of approximately 65% of the organic layer, the remaining organics were recombined with the aqueous layer and extracted with ethyl acetate (200 ml). The organic layers were then combined, dried ($MgSO_4$) and concentrated under reduced pressure. Recrystallisation from ethanol afforded the title compound as a white solid (5.62 g, 87%), mp 208-210 °C; (Found: C, 56.10; H, 5.16; N, 2.48. $C_{26}H_{29}NO_{10}S.0.5\ H_2O$ requires C, 56.11; H, 5.43; N, 2.53%); (Found (HRMS): MH^+ 548.1590. $C_{26}H_{30}NO_{10}S$ requires *MH* 548.5880); $[\alpha]_D$ +4.64 ° (c 0.14 in MeOH); ν_{max}(nujol)/cm^{-1} 3300 (OH), 1720, 1750 (CO), 1620 (C=N), 1225, 1250 (COOC); δ_H(200 MHz, $CDCl_3$) 1.85, 1.90, 1.91 (12 H, 3 x s, CH_3COO), 3.05 (1 H, m, *H*-5), 3.79 (1 H, d, $J_{6^a,6^b}$ 12, *H*-6^a), 3.94 (1 H, dd, $J_{5,6^b}$ 5, $J_{6^a,6^b}$ 12, *H*-6^b), 4.26 (2 H, 2 x d, *J* 18, CH_2), 4.64 (1 H, d, $J_{1,2}$ 9, *H*-1), 4.84 (3 H, m, *H*-2,3,4), 7.40 (4 H, m, *H*-3',5',6',7'), 7.80 (3 H, m, *H*-2',4',8'); δ_C(50.31 MHz, $CDCl_3$) 20.78, 20.89 (CH_3COO), 35.57 (CH_2), 62.36 (*C*-6), 68.13 (*C*-4), 70.34 (*C*-2), 74.06 (*C*-3), 75.92 (*C*-5), 80.05 (*C*-1), 123.12, 125.90, 126.02, 126.37, 126.89, 128.33, 129.47, 131.67, 132.33, 134.18 (Ar*C*), 150.17 (*C*=N), 169.88, 169.98, 170.76, 171.34 (CH_3COO); *m/z*(CI) 548 (MH^+, 8%), 331 (100, $C_6H_7O(OAc)_4^+$) and 271 (33, $C_6H_7O(OAc)_3^+$).

2,3,4,6-Tetra-*O*-acetyl-1-naphthylmethyl glucosinolate 132

Chlorosulfonic acid (7.54 g, 64.7 mmol), dissolved in dry dichloromethane (100 ml), was added to a stirred solution of dry pyridine (96.0 g, 1.21 mol) and dry dichloromethane (100 ml) at 0 °C and under a nitrogen atmosphere. 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-1-naphthylmethyl thiohydroximate (3.50 g, 6.4 mmol) was added as a solution with dry dichloromethane (80 ml), and the resulting solution stirred at room temperature and under a nitrogen atmosphere for 23 hours. Potassium hydrogen carbonate (19.19 g, 191.7 mmol) was added in water (100 ml) and once CO_2 evolution had ceased, the organics were removed under reduced pressure to afford a brown solid. Purification by flash chromatography on silica gel, eluting with petroleum ether (bp 40-60 °C): ethyl acetate (1:1) then ethyl acetate:methanol (9:1) afforded the protected glucosinolate as a brown solid, mp 110-112 °C (decomp.); (Found: C, 47.12; H, 4.54; N, 2.07. $C_{26}H_{28}NO_{13}S_2K$ requires C, 46.91; H, 4.24; N, 2.10%); $[\alpha]_D$ -27.5 ° (c 0.14 in H_2O); ν_{max}(nujol)/cm^{-1} 3000 (aromatic), 1750 (CO), 1225 (COOC); δ_H(200 MHz, CD_3OD) 1.95 (12 H, m, CH_3COO), 3.30 (1 H, m, *H*-5), 3.75 (1 H, d, $J_{6^a,6^b}$ 12, *H*-6^a), 4.01 (1 H, dd, $J_{5,6^a}$ 5, $J_{6^a,6^b}$ 12, *H*-6^b), 4.53 (2 H, s, CH_2), 4.89 (4 H, m, *H*-1,2,3,4), 7.56 (4 H, m, *H*-3',5',6',7'), 7.86 (1H, d, *J* 8, *H*-4'), 7.96 (1 H, d, *J* 8, *H*-8'), 8.19 (1 H, d, *J* 8, *H*-2'); δ_C(50.31 MHz, CD_3OD) 20.76, 20.91 (CH_3COO), 36.51 (CH_2), 63.27 (*C*-6), 69.31 (*C*-4), 71.30 (*C*-2), 75.13 (*C*-3), 77.12 (*C*-5), 81.36 (*C*-1), 124.49-135.66 (Ar*C*), 158.87 (*C*=N), 171.07, 171.32, 171.64, 172.42 (CH_3*C*OO); *m/z*(ES$^-$) 626 ([M-K]$^-$, 100%).

1-Naphthylmethyl glucosinolate 83

Method 1

Chlorosulfonic acid (4.26 g, 36.5 mmol) was added to dry dichloromethane (30 ml) and this solution was added to a stirred solution of dry dichloromethane (30 ml) and dry pyridine (27.5 g, 347.7 mmol) at 0 °C and under a nitrogen atmosphere. 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-1-naphthylmethyl thiohydroximate (1 g, 1.8 mmol) was then added as a solution with dry dichloromethane (20 ml) and the resulting solution was stirred at room temperature for 24 hours. Potassium hydrogen carbonate (5.5 g, 54.9 mmol) was added in water (100 ml) and the resulting biphasic system was concentrated under reduced pressure. Further potassium hydrogen carbonate was added (5.5 g, 54.9 mmol) as before and the solution reconcentrated under reduced pressure to afford an off-white coloured solid. Methanol was added and the solid reconcentrated under reduced pressure before purification by flash chromatography, on silica gel, eluting with ethyl acetate then ethyl acetate:methanol (4:1). This produced the title compound as an impure white solid (301 mg, 33%); (Found: C, 36.20; H, 3.78; N, 2.21. $C_{18}H_{20}NO_9S_2K$ requires C, 43.45; H, 4.05; N, 2.81%); $[\alpha]_D$ -3.8 ° (c 0.2 in H_2O); ν_{max}(nujol)/cm^{-1} 3390 (OH); δ_H(200 MHz, CD_3OD) 2.82 (1 H, m, *H*-5), 2.93 (1 H, t, $J_{2,3}$=$J_{3,4}$ 9, *H*-3), 3.17 (2 H, t, *J* 9, *H*-2,4), 3.56 (1 H, dd, $J_{5,6^a}$ 6, $J_{6^a,6^b}$ 12, *H*-6^a), 3.74 (1 H, dd, $J_{5,6^b}$ 2, $J_{6^a,6^b}$ 12, *H*-6^b), 4.37 (2 H, m, CH_2), 4.82 (1 H, d, $J_{1,2}$ 6, *H*-1), 7.41-7.65 (4 H, m, *H*-3',5',6',7'), 7.81 (1H, d, *J* 8, *H*-4'), 7.91 (1 H, d, *J* 8, *H*-8'), 8.22 (1 H, d, *J* 8, *H*-2'); δ_C(50.31 MHz, CD_3OD) 36.35 (CH_2), 62.67, 62.76 (*C*-6), 71.10 (*C*-4), 74.26 (*C*-2), 79.40 (*C*-3), 82.47 (*C*-5), 83.78, 83.81 (*C*-1), 124.65, 126.85, 127.10, 127.40, 128.03, 129.12, 130.20, 132.98, 133.54, 135.59 (Ar*C*), 162.03 (*C*=N); *m/z*(ES^-) 458 ([M-K]$^-$, 100%).

Method 2

2,3,4,6-Tetra-*O*-acetyl-1-naphthylmethyl glucosinolate (500 mg, 0.75 mmol) was dissolved in dry methanol (10 ml) before a catalytic amount of potassium metal was added until the pH reached 8-9. The resulting solution was stirred for 24 hours at room temperature and under a nitrogen atmosphere before being concentrated under reduced pressure to afford a brown foam. Purification by flash chromatography, on silica gel, eluting initially with ethyl acetate then ethyl acetate and methanol (4:1) afforded the product as a gold-coloured foam (198 mg, 53%); (Found: C, 42.48; H, 4.73; N, 2.40. $C_{18}H_{20}NO_9S_2K.0.5\ H_2O$ requires C, 42.68; H, 4.18; N, 2.76%).

7.4.2 Compounds synthesised in the preparation of acidic glucosinolates

Attempted reduction of 12-nitrododecanoic acid

Following a procedure by Johnson and Degering,[129] 12-nitrododecanoic acid (400 mg, 1.6 mmol) and zinc dust (341 mg, 5.2 mmol) were suspended in glacial acetic acid (2.5 ml, 43.7 mmol) and the resulting mixture was heated under reflux for 5 hours. A white precipitate was recovered by filtration and was found to be intractable.

Ethyl 5-oxovalerate 136

A solution of potassium iodide (794 mg, 4.78 mmol), sodium carbonate (507 mg, 4.78 mmol) and DMSO (30 ml) was heated to 80 °C before ethyl 5-bromovalerate (1 g, 4.78 mmol) was added. The reaction mixture was stirred at this temperature for 3 hours before being rapidly cooled and poured onto ice-cold brine (30 ml). The product was extracted with diethyl ether (2 x 20 ml) and the organic layer washed with water (10 ml), brine (10 ml), saturated sodium bicarbonate solution (10 ml) and brine (10 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure to afford a gold-coloured oil (736 mg). This was analysed without purification and found to contain the title compound; δ_H(200 MHz, $CDCl_3$) 2.49 (2 H, 2 x t, *J* 7, CH_2CO), 9.72 (1 H, t, *J* 1, C*H*O).

Ethyl 8-bromooctanoate 139

8-Bromooctanoic acid (5.78 g, 25.9 mmol) was dissolved in dry ethanol (125 ml) before thionyl bromide (5.92 g, 28.5 mmol) was added and the resulting solution heated under reflux with stirring for 2 hours under a nitrogen atmosphere. Concentration under reduced pressure afforded a brown oil which was purified by flash chromatography, on silica gel, eluting initially with petroleum ether (bp 40-60 °C) then petroleum ether and ethyl acetate (9:1). This yielded the title compound as a pale yellow oil (5.26 g, 81%); ν_{max}/cm^{-1} 2858, 2935 (alkyl), 1736 (CO), 1373-1465 (alkyl), 1183 (COOC); δ_H(200 MHz, $CDCl_3$) 1.25 (3 H, t, *J* 7, OCH_2CH_3), 1.39 (6 H, m, $(CH_2)_3$), 1.62 (2 H, quintet, *J* 7, CH_2CH_2COO),

1.85 (2 H, quintet, *J* 7, $BrCH_2CH_2$), 2.29 (2 H, t, *J* 7, CH_2COO), 3.40 (2 H, t, *J* 7, $BrCH_2$), 4.12 (2 H, q, *J* 7, OCH_2CH_3); δ_C(50.31 MHz, $CDCl_3$) 14.72 (CH_3), 25.31, 28.45, 28.89, 29.39, 33.18, 34.41, 34.76 (CH_2), 60.70 (OCH_2CH_3), 174.26 ($COOCH_2CH_3$); *m/z*(CI) 252, 254 (MH^+, 6%), 251, 253 (55, 56, M^+), 205, 207 (5, $[M-C_2H_5OH]^+$).

Ethyl 8-oxooctanoate 141

Following the procedure of Bauer and Macomber,[132] potassium iodide (3.33 g, 20.1 mmol) and sodium carbonate (2.11 g, 20.1 mmol) were suspended in DMSO (150 ml) at 80-85 °C before ethyl 8-bromoooctanoate (5 g, 20.1 mmol) was added with stirring. This mixture was stirred at the above temperature for 11 hours before being rapidly cooled and poured onto ice-cold brine. The product was extracted with diethyl ether (2 x 300 ml) and the organic layer washed with water (150 ml), brine (150 ml), saturated sodium bicarbonate (150 ml) and more brine (150 ml) before being dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was purified by flash chromatography, on silica gel, eluting with petroleum ether (bp 40-60 °C) and ethyl acetate (9:1). This afforded the title compound as a colourless oil (2.27 g, 61%); ν_{max}/cm^{-1} 2722, 2860, 2937 (alkyl), 1735 (CO), 1349-1466 (alkyl), 1182 (COOC); δ_H(200 MHz, $CDCl_3$) 1.15-1.30 (7 H, m, $(CH_2)_2$, CH_3), 1.46 (4 H, m, $(CH_2)_2$), 2.24 (2 H, t, *J* 7, CH_2COO), 2.38, 2.39 (2 H, 2 x t, *J* 7, CH_2CO), 4.07 (2 H, q, *J* 7, $COOCH_2CH_3$), 9.71 (1 H, t, *J* 2, CHO); δ_C(50.31MHz, $CDCl_3$) 14.68 (CH_3), 22.28, 25.14, 29.20, 29.25 (CH_2), 34.64 (CH_2COO), 44.21 (CH_2CHO), 60.65 (OCH_2CH_3), 174.13 ($COOCH_2CH_3$), 203.12 (CHO); *m/z*(CI) 187 (MH^+, 86%), 171 (5, $[M-CH_3]^+$), 141 (100, $[M-OCH_2CH_3]^+$).

Ethyl 8-hydroxyiminooctanoate 143

Ethyl 8-oxooctanoate (2.26 g, 12.11 mmol) was dissolved in ethanol (45 ml) and pyridine (4.5 ml) before hydroxylamine hydrochloride (1.55 g, 22.3 mmol) was added and the resulting solution heated under reflux for 2.5 hours. Concentration under reduced pressure and addition of water afforded a white solid which was removed by filtration under reduced pressure. This was identified as the oxime existing as a mixture of *syn* and *anti* isomers (1:1), (2.06 g, 85%), mp 28 °C; (Found: C, 59.62; H, 9.59; N, 6.87. $C_{10}H_{19}NO_3$ requires C, 59.68; H, 9.51; N, 6.96%); ν_{max}/cm^{-1} 3050-2200 (OH), 2820, 2900 (alkyl), 1700 (CO); δ_H(200 MHz, $CDCl_3$) 1.24 (3 H, t, *J* 7, OCH_2CH_3), 1.34 (4 H, m, $(CH_2)_2$), 1.48 (2 H, m, CH_2), 1.61 (2 H, m, CH_2), 2.18 (2 H, q, *J* 7, CH_2CH), 2.28 (2 H, t, *J* 8, CH_2COO), 4.11 (2 H, q, *J* 8, OCH_2CH_3), 6.72 (1 H, t, *J* 5, C*H*=N (*syn*)), 7.40 (1 H, t, *J* 6, C*H*=N (*anti*)), 7.92 (1 H, br s, NO*H*); δ_C(50.31 MHz, $CDCl_3$) 14.73 (CH_3), 25.25, 25.40, 26.29, 26.80, 29.17, 29.25, 29.47, 29.87, 34.75 (CH_2), 60.76 (OCH_2CH_3), 152.45 (*C*H=N (*anti*)), 152.51 (*C*H=N (*syn*)), 174.39 (*C*O); *m/z*(CI) 202 (MH^+, 100%), 184 (11, $[M-OH]^+$), 138 (18, $[M-C_2H_7O_2]^+$).

Ethyl 8-chloro-8-hydroxyiminooctanoate 144

The oxime **143** (2 g, 10.1 mmol) was dissolved in chloroform (32 ml) and pyridine (397 mg, 5 mmol) before *N*-chlorosuccinimide (1.34 g, 10.1 mmol) was added slowly at 0 °C.

The resulting solution was stirred at room temperature for 3.5 hours before being poured onto ice/water. The organic layer was separated and washed with water (30 ml) then brine (30 ml) and the aqueous layer was extracted with diethyl ether (2 x 30 ml). The organic layers were combined, dried ($MgSO_4$) and concentrated under reduced pressure to afford a pale yellow oil (2.05 g, 86%); δ_H(200 MHz, $CDCl_3$) 1.19-1.47 (7 H, m, $(CH_2)_2$, CH_3), 1.61 (4 H, m, $(CH_2)_2$), 2.27 (2 H, t, *J* 7, CH_2C=N), 2.46 (2 H, t, *J* 7, CH_2COO), 4.10 (2 H, q, *J* 7, OCH_2CH_3); δ_C(50.31 MHz, $CDCl_3$) 14.70 (CH_3), 25.20, 26.51, 28.57, 29.11, 34.72, 36.92 (CH_2), 60.85 ($COOCH_2$), 144.34 (*C*=N), 174.55 (*C*O).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-(7-ethoxycarbonylheptyl) thiohydroximate 145

2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (2.24 g, 6.14 mmol) and the oximyl chloride **144** (2.03 g, 8.59 mmol) were dissolved in dry THF (65 ml) before dry triethylamine (5.83 g, 57.59 mmol) was added. The resulting solution was stirred at room temperature for 24 hours under a nitrogen atmosphere before diethyl ether (65 ml) was added and the solution washed with 1 M H_2SO_4 (65 ml). After removal of approximately 60% of the organic layer, the remaining organics and aqueous layer were extracted with ethyl acetate (65 ml). The organics were combined, dried ($MgSO_4$) and concentrated under reduced pressure to afford a brown solid. Recrystallisation from a mixture of hexane and ethyl acetate afforded the title compound as a white solid (2.57 g, 74%), mp 90 °C; (Found: C, 51.19; H, 6.72; N, 2.49. $C_{24}H_{37}NO_{12}S$ requires C, 51.15; H, 6.62; N, 2.49%); $[\alpha]_D$ -12.1 ° (c 0.14 in $CHCl_3$); ν_{max}(nujol)/cm^{-1} 3240 (OH), 1685, 1715 (CO), 1225 (COOC); δ_H(200 MHz, CD_3OD) 1.25 (3 H, t, *J* 7, OCH_2CH_3), 1.40 (4 H, m, $(CH_2)_2$), 1.65 (4 H,

m, $(CH_2)_2$), 1.98, 1.99, 2.03, 2.06 (12 H, 4 x s, CH_3COO), 2.33 (2 H, t, *J* 7, CH_2COO), 2.55 (2 H, t, *J* 7, $CH_2C{=}N$), 4.00 (1 H, m, *H*-5), 4.07-4.22 (4 H, m, OCH_2CH_3 and *H*-$6^a,6^b$), 4.83 (1 H, br s, NO*H*), 4.98, 5.05 (2 H, 2 x t, *J* 10, 10, *H*-2,4), 5.28 (1 H, d, $J_{1,2}$ 10, *H*-1), 5.39 (1 H, t, $J_{2,3=3,4}$ 10, *H*-3); δ_C(50.31 MHz, CD_3OD) 13.41 (OCH_2CH_3), 19.39, 19.45, 19.54, 24.69, 27.11, 28.50, 28.64, 31.86, 33.81 (CH_2), 60.25 (OCH_2CH_3), 62.27 (*C*-6), 68.49 (*C*-4), 70.54 (*C*-2), 73.95 (*C*-3), 75.48 (*C*-5), 79.50 (*C*-1), 151.06 (*C*=N), 169.78, 170.03, 170.37, 171.00 (CH_3COO), 174.37 ($COOCH_2CH_3$); *m/z*(CI) 564 (MH^+, 2%), 331 (100, $C_6H_7O(OAc)_4^+$), 271 (33, $C_6H_7O(OAc)_3^+$).

2,3,4,6-Tetra-*O*-acetyl-(7-ethoxycarbonylheptyl) glucosinolate 146

Chlorosulfonic acid (9.1 g, 78.1 mmol) was added to dry dichloromethane (65 ml) and the resulting solution was added slowly to an ice-cold solution of dry dichloromethane (65 ml) and dry pyridine (58.6 g, 741 mmol) maintained under a nitrogen atmosphere. The thiohydroximate **145** (2.2 g, 3.9 mmol) was added as a solution in dry dichloromethane (45 ml) at room temperature and the resulting solution was stirred under a nitrogen atmosphere for 24 hours. Potassium hydrogen carbonate (11.7 g, 117 mmol) was added in water (100 ml) and the resulting two-phase system was concentrated under reduced pressure to allow removal of the organics. This yielded a substantial quantity of salts which were removed by filtration before the aqueous layer was extracted with ethyl acetate. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to afford a cream-coloured foam. This was purified by flash chromatography, on silica gel, eluting with petroleum ether (bp 40-60 °C) and ethyl acetate (1:1) then ethyl acetate and methanol (4:1). This afforded the title compound as a cream-coloured solid (1.91 g, 72%), mp 109-

111 °C (decomp.); (Found: C, 42.53; H, 5.41; N, 2.29. $C_{24}H_{36}NO_{15}S_2K$ requires C, 42.28; H, 5.32; N, 2.05%); $[\alpha]_D$ -11.4 ° (c 0.14 in H_2O); ν_{max}(nujol)/cm^{-1} 1700 (CO), 1560 (C=N), 1200 (COOC); δ_H(200 MHz, CD_3OD) 1.25 (3 H, t, *J* 7, OCH_2CH_3), 1.41 (4 H, m, $(CH_2)_2$), 1.67 (4 H, m, $(CH_2)_2$), 1.99, 2.02, 2.04, 2.07 (12 H, 4 x s, CH_3COO), 2.34 (2 H, t, *J* 7, CH_2COO), 2.67 (2 H, t, *J* 7, CH_2C=N), 3.88-4.30 (5 H, m, OCH_2CH_3, *H*-5,6^a,6^b), 4.99, 5.06 (2 H, 2 x t, *J* 10, 10, *H*-2,4), 5.37 (1 H, d, $J_{1,2}$ 10, *H*-1), 5.41 (1 H, t, $J_{2,3=3,4}$ 10, *H*-3); δ_C(50.31 MHz, CD_3OD) 14.89 (OCH_2CH_3), 20.87, 21.03 (CH_3COO), 26.13, 28.45, 29.97, 30.09, 33.83, 35.30 (CH_2), 61.72 (OCH_2CH_3), 63.69 (*C*-6), 69.86 (*C*-4), 71.75 (*C*-2), 75.33 (*C*-3), 77.03 (*C*-5), 81.19 (*C*-1), 159.77 (*C*=N), 171.23, 171.52, 171.80, 172.50 (CH_3COO), 175.89 ($COOCH_2CH_3$); *m/z* (ES$^-$) 642 ([M-K]$^-$, 100%).

(7-Methoxycarbonylheptyl) glucosinolate 147

HO, HO, HO, HO, O, S, N—$OSO_3^-K^+$, O, OCH_3

2,3,4,6-Tetra-*O*-acetyl-(7-ethoxycarbonylheptyl) glucosinolate (750 mg, 681.8 mmol) was dissolved in dry methanol (10 ml) before a catalytic quantity of potassium methoxide was added with stirring until the pH reached 8-9. The resulting solution was stirred at room temperature, under a nitrogen atmosphere, for 24 hours. Concentration under reduced pressure afforded a cream-coloured foam (540 mg, 98 %); (Found: C, 35.26; H, 5.15; N, 2.65. $C_{15}H_{26}NO_{11}S_2K.0.5\ H_20$ requires C, 35.42; H, 5.35; N, 2.75%); $[\alpha]_D$ -12 ° (c 0.2 in H_2O); ν_{max}/cm^{-1} 3400 (OH), 2850, 2930 (alkyl), 1720 (CO), 1250 (COOC); δ_H(200 MHz, D_2O) 1.30 (4 H, m, $(CH_2)_2$), 1.57 (4 H, m, $(CH_2)_2$), 2.32 (2 H, t, *J* 7, CH_2COO), 2.63 (2 H, t, *J* 7, CH_2C=N), 3.42 (4 H, m, *H*-2,3,4,5), 3.61 (3 H, s, OCH_3), 3.63 (1 H, m, *H*-6^a), 3.81 (1 H, dd, $J_{5,6^b}$ 2, $J_{6^a,6^b}$ 11, *H*-6^b), 4.93 (1 H, d, $J_{1,2}$ 9, *H*-1); δ_C(50.31

MHz, D_2O) 27.10, 29.63, 30.67, 30.77, 34.99, 36.57 (CH_2), 55.05 (OCH_3), 63.52 (*C*-6), 72.01 (*C*-4), 74.87 (*C*-2), 79.99 (*C*-3), 83.02 (*C*-5), 84.73 (*C*-1), 167.53 (*C*=N), 180.60 ($COOCH_3$); *m/z* (ES^-) 460 ($[M-K]^-$, 100%).

(7-Carboxyheptyl) glucosinolate 137

The methyl ester **147** (100 mg, 0.20 mmol) was dissolved in methanol (600 μl) before 1 M potassium hydroxide (300 μl, 0.30 mmol) was added and the resulting solution was stirred overnight at room temperature. Analysis by TLC (ethyl acetate and methanol (4:1)) showed the reaction to be incomplete and more potassium hydroxide (100 μl, 0.10 mmol) was added. The resulting solution was stirred as before for several hours prior to concentration under reduced pressure. Methanol was then added and the remaining solid was removed by filtration before purification, on C_{18} reverse-phase silica, eluting with water then methanol. NMR analysis of the aqueous fractions showed (7-Carboxyheptyl) glucosinolate as a mixture with two side-products; δ_H(500 MHz, D_2O) 1.33 (*H*-4'), 1.40 (*H*-3'), 1.55 (*H*-5'), 1.71 (*H*-2'), 2.17 (*H*-6'), 2.69 (*H*-1'), 3.44 (*H*-2), 3.45 (*H*-4), 3.54 (*H*-5), 3.56 (*H*-3), 3.71 (*H*-6^a), 3.88 (*H*-6^b), 5.00 (*H*-1); δ_C(125.8 MHz, D_2O) 28.43, 29.59, 30.67, 31.00 (*C*-2',3',4',5'), 34.80 (*C*-1'), 40.23 (*C*-6'), 63.26 (*C*-6), 71.81 (*C*-4), 74.63 (*C*-2), 79.74 (*C*-3), 82.80 (*C*-5), 84.50 (*C*-1), 167.63 (*C*=N), 186.68 (*C*OOH); *m/z*(ES^-) 222 ($[M-2K]^{2-}$, 100%).

Attempted preparation of (7-Carboxyheptyl) glucosinolate using Pig Liver Esterase

The methyl ester **147** (100 mg, 0.20 mmol) was dissolved in 0.1 M potassium phosphate buffer, pH 8.0, (5.66 ml) and the resulting solution was warmed to 37 °C before addition of pig liver esterase (57 µl, 78.66 units). This reaction solution was incubated at 37 °C for 4 hours after which TLC analysis showed the reaction to be incomplete. Thus the reaction mixture was incubated overnight which failed to allow complete substrate turnover. More pig liver esterase was added (20 µl, 27.60 units) and the mixture incubated for a further 24 hours. Analysis by NMR showed the presence of (7-carboxyheptyl) glucosinolate along with side products and unreacted starting material.

Ethyl 4-bromobutanoate 156

Thionyl bromide (6.95 g, 33.5 mmol) was added to a solution of 4-bromobutryric acid (5.08 g, 30.4 mmol) in dry ethanol (125 ml) and the resulting solution was heated under reflux, with stirring, for 2.5 hours. After cooling, the solvent was removed under reduced pressure to afford a brown oil which was purified by flash chromatography, on silica gel, eluting with petroleum ether (bp 40-60 °C) and ethyl acetate (9:1). This yielded the ester as a pale yellow oil (3.49 g, 59%); ν_{max}/cm^{-1} 2990 (alkyl), 1720 (CO), 1200 (COOC); δ_H(200 MHz, $CDCl_3$) 1.25 (3 H, t, *J* 7, CH_2CH_3), 2.16 (2 H, quintet, *J* 7, $BrCH_2CH_2$), 2.49 (2 H, t, *J* 7, CH_2COO), 3.46 (2 H, t, *J* 7, $BrCH_2$), 4.13 (2 H, q, *J* 7, OCH_2CH_3); δ_C(50.31 MHz, $CDCl_3$) 14.68 (CH_3), 28.24 ($BrCH_2CH_2$), 32.97 (CH_2COO), 33.24 ($BrCH_2$), 61.04 (OCH_2CH_3), 173.02 (*C*O); *m/z*(CI) 195, 197 (MH^+, 59, 59%), 167, 169 (69, 70, $[M-CH_2CH_2]^+$), 115 (45, $[M-HBr]^+$), 87 (100, $[M-Br(CH_2)_2]^+$).

Attempted preparation of ethyl 4-oxobutanoate 157 *via* Kornblum oxidation

Potassium iodide (1.7 g, 10.3 mmol) and sodium carbonate (1.08 g, 10.3 mmol) were suspended in DMSO (77 ml) and heated to 80-85 °C before ethyl 4-bromobutanoate (2 g, 10.3 mmol) was added. This mixture was stirred at the above temperature for 3 hours then cooled rapidly and poured onto ice-cold brine. The product was extracted with diethyl ether (2 x 150 ml) and the organics then washed with water (150 ml), brine (150 ml), saturated sodium bicarbonate solution (150 ml) and more brine (150 ml). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to afford a brown oil which was shown by TLC analysis (using petroleum ether (bp 40-60 °C) and ethyl acetate (9:1)) to contain at least 6 products one of which was identified as the aldehyde (233 mg, 18%); δ_H(200 MHz, $CDCl_3$) 9.82 (1 H, s, C*H*O).

Attempted reduction of diethyl succinate (sample procedure)

Diethyl succinate (1 g, 5.7 mmol) was dissolved in dry toluene (20 ml) and the resulting solution cooled to -78 °C before DIBAL-H (1.5 M in toluene; 816 mg, 5.7 mmol) was added. The resulting solution was stirred at this temperature and under a nitrogen atmosphere for 2 hours before dry methanol (12.5 ml) was added and the solution slowly allowed to warm to room temperature. A solution of potassium sodium tartrate (16g) in water (75 ml) was added and the two-phase system stirred overnight before removal of the organic layer. The aqueous layer was washed with diethyl ether (2 x 100 ml) and the organics were combined, dried ($MgSO_4$) and concentrated under reduced pressure. NMR analysis failed to show the desired aldehyde.

7.5 ELECTROPHYSIOLOGICAL TESTING OF GLUCOSINOLATES

This was performed using a method analogous to that reported by Roessingh *et al*,[43] described hereafter.

Female insects were decapitated and placed in a depression cut into a plexiglass block. The front legs were taped to the surface of the block to expose the sensilla present on the ventral surface of the tarsi. To prevent dessication, a humidified air stream (0.5 m/sec at room temperature, 19-25 °C) was directed over the insect. An indifferent electrode (tip diameter 1 μm), filled with 'insect haemolymph saline' was inserted into this tibia. This preparation allowed stable recordings for many hours using standard tip recording technique. A non-blocking amplifier with a 10^{13} Ω input impedence and a maximal input bias current of 150 fa was used. Recordings were made from D sensilla present on the ventro-lateral surface of each tarsomere. To distinguish the different sensilla an index was used that specifies the tarsal segment (i.e. D_4 in a D sensillum on the 4th tarsal segment, counted from proximal to distal). The nerve impulses generated in the D_4 sensilla were used for this study.

All glucosinolates (1 μg/ml) were dissolved in 10 mM KCl evoking only a few spikes per second by itself (3.7 ± 1.4). The interval between stimulations of the same sensillum was at least 2 minutes. Stimulus pipettes were freshly filled immediately before each series of recordings, and the glucosinolates were normally applied with the lower concentrations first to minimise a reduction of sensitivity due to partial adaptation.

Since the first 50 ms were distorted by the contact artefact, only the total spike from 50-1050 ms were used in the analysis. All counting was carried out from digitised recordings using a data aquisition and analysis programme developed in Wädenswil and running on a Macintosh II computer.

The results obtained are given in section 4.2. Further statistical analysis is contained in appendix B.

7.6 REACTIONS OF SYNTHETIC GLUCOSINOLATES WITH MYROSINASE

7.6.1 Glucose Release

7.6.1.1 Myrosinase Assay

Myrosinase (EC 3.2.3.1) from *Sinapis alba* was obtained from Sigma as a freeze dried powder with an activity of approximately 175 units/g of solid. Sinigrin monohydrate from horseradish was also obtained from Sigma.

The enzyme solution was periodically constituted by dissolving 20 mg of myrosinase in 33.1 mM potassium phosphate buffer at pH 7.0 in a total volume of 1 ml. This solution was stored at -18 °C, when not in use. The enzyme activity was assayed in triplicate according to the method of Palmieri *et al*,[54] using 33.1 mM potassium phosphate buffer at pH 7.0 containing 0.1 mM sinigrin in a total volume of 1 ml. The sinigrin solution was equilibrated at 37 ± 0.1 °C in a quartz cuvette of 1 ml volume/1 cm pathlength in the thermostatted cell holder of the UV spectrophotometer. Reaction was initiated by addition of 30 μl of enzyme solution and the decrease in absorbance at 227 nm, due to sinigrin, was monitored. The number of units in the enzyme solution was calculated by dividing the experimental initial rate by the theoretical rate (6.58 min^{-1}) calculated for one unit of enzyme. One unit of enzyme activity was defined as the amount of enzyme required to catalyse the hydrolysis of 1 μmol of sinigrin per minute under standard conditions.

7.6.1.2 Assessment of Glucose assay

The formation of glucose following incubation of glucosinolates with myrosinase was measured using a hexokinase/glucose-6-phosphate dehydrogenase coupled assay with Glucose [HK] 20 reagent obtained from Sigma. This reagent was periodically constituted by the addition of distilled water (20 ml). Glucose assays were conducted at 37 ± 0.1 °C in a quartz cuvette of 1 ml volume/1 cm pathlength in the thermostatted cell holder of the UV spectrophotometer. The glucose reagent (1 ml) was equilibrated at 37 ± 0.1 °C before addition of the glucose solution (10 μl). The mixture was then allowed to equilibrate for at least 5 minutes before the absorbance was measured at 340 nm. A blank was prepared by adding distilled water (10 μl) to the glucose reagent (1 ml) with all samples analysed against a distilled water reference. The glucose concentration was determined using the following equation:

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

The assay was initially standardised using authentic solutions of glucose (5 mM or 10 mM D-glucose in 33.1 mM potassium phosphate buffer at pH 7.0) which were tested in triplicate.

<u>For standard 5 mM D-glucose solution</u>

Blank absorbance: 0.076 A

Mean sample absorbance : 0.391 A (from 0.388, 0.387 and 0.398 A)

[Glucose] = {(0.391-0.076) x 293 x 0.01 / 180.16} x 1000

[Glucose] = 5.12 ± 0.12 mM.

For standard 10 mM D-glucose solution

Blank absorbance: 0.073 A

Mean sample absorbance: 0.706 A (from 0.718, 0.705 and 0.694 A)

[Glucose] = {(0.706-0.073) x 293 x 0.01 / 180.16} x 1000

[Glucose] = 10.29 ± 0.2 mM.

7.6.1.3 Determination of assay conditions using Sinigrin

The glucose release from incubation of sinigrin with myrosinase was measured as above using Glucose [HK] 20 reagent. Assay solutions were prepared by adding 25, 50 or 100 µl aliquots of myrosinase solution (20 mg in 1 ml buffer) to 1 ml of 33.1 mM potassium phosphate buffer at pH 7.0 containing 10 mM sinigrin, equilibrated at 37 ± 0.1 °C in a plastic cuvette.

Measurement of glucose release was conducted in duplicate after 15, 30, 45, 60 and 90 minutes from the addition of myrosinase. An aliquot (10 µl) of assay solution was added to the prewarmed glucose [HK] reagent (1 ml) and the mixture was then allowed to equilibrate at 37 ± 0.1 °C for at least 5 minutes before the absorbance was measured at 340 nm. A blank was prepared by adding distilled water (10 µl) to the glucose reagent (1 ml) with all samples analysed against a distilled water reference.

The results obtained (table 22) indicated a steady increase in glucose concentration when 25 µl of myrosinase solution (*ca.* 0.045 units) was added to 1 ml 10 mM sinigrin solution.

Time (minute)	Average [Glucose]/mM for 25 μl myrosinase solution	Average [Glucose]/mM for 50 μl myrosinase solution
15	1.81	4.79
30	5.57	10.28
60	10.36	10.80
90	10.60	8.56

Table 22 Turnover of sinigrin with varying quantities of myrosinase.

7.6.1.4 Glucosinolate analysis

Reactions of the synthetic glucosinolates were carried out in 33.1 mM potassium phosphate buffer at pH 7.0 containing 10 mM glucosinolate. Each glucosinolate solution (1 ml or 800 μl) was incubated with myrosinase (*ca.* 0.045 units) at 37 °C. The glucose release was determined in triplicate, as described previously, using the glucose [HK] reagent after 15, 30, 45, 60, 75, 90, and 120 minutes. The absorbance values were corrected with respect to the blank for each determination. Each glucosinolate was found to be a substrate for myrosinase showing an increase in absorbance due to glucose release with time.

7.6.2 Turnover of naphthylmethyl glucosinolate measured by UV spectrophotometry

A solution of naphthylmethyl glucosinolate (0.1 mM) was prepared in 33.1 mM potassium phosphate buffer, as given previously, and was analysed by UV spectrophotometry from 200-350 nm. This showed two absorption maxima at 225 and 280 nm. The decrease in

absorbance at 225 nm, due to the turnover of naphthylmethyl glucosinolate, was then measured at five minute intervals for a total of 10 measurements, after the addition of myrosinase solution (20 μl). Three further solutions of naphthylmethyl glucosinolate, 0.025, 0.0125 and 6.25 x 10^{-3} mM, were prepared using 33.1 mM potassium phosphate buffer. The turnover of naphthylmethyl glucosinolate, at these concentrations, was assayed in triplicate using the same method as the myrosinase activity assay (section 7.5.1.1). The glucosinolate solution was incubated at 37 °C in a quartz cuvette of 1 ml volume/1 cm pathlength. Reaction was initiated by addition of 30 μl of enzyme solution and the decrease in absorbance at 227 nm due to naphthylmethyl glucosinolate was monitored. The rate of turnover was calculated and corrected to one unit of enzyme activity. Mean values of 2.86, 1.28, and 0.81 mmol min^{-1} were obtained for 0.025, 0.0125 and 6.25 x 10^{-3} mM solutions of naphthylmethyl glucosinolate respectively. The reciprocal rates were then used to construct a Lineweaver-Burk plot. This allowed a K_m value of 0.06 mM to be calculated for naphthylmethyl glucosinolate. An error value of ± 0.03 mM was calculated by linear regression analysis using the "ENZFITTER" programme.[172]

7.6.3 Isothiocyanate release from alkyl glucosinolates

7.6.3.1 Materials and apparatus

The isothiocyanates were collected from the headspace of a reaction vessel (figure 33). The charcoal filter was prepared by adding activated charcoal and molecular sieves (4 Å pore size) to a glass bulb which was sealed at each end by glass wool. The porous polymer, Tenax-TA, was contained in a stainless steel tube. The tubes were pre-conditioned at SCRI by passage of a stream of helium at 240 °C for 24 hours. During the reaction, an end cap was placed over the end of the tube instead of the syringe and a water membrane was placed over the other end to minimise absorption of water. Each joint was sealed with PTFE tape to minimise loss of volatiles and Teflon tubing was used where required to minimise

leaching of plasticisers and subsequent absorption onto the polymer. All glassware, tubing and the nitrogen filter were heated at 160 °C for 24 hours to remove any volatiles.

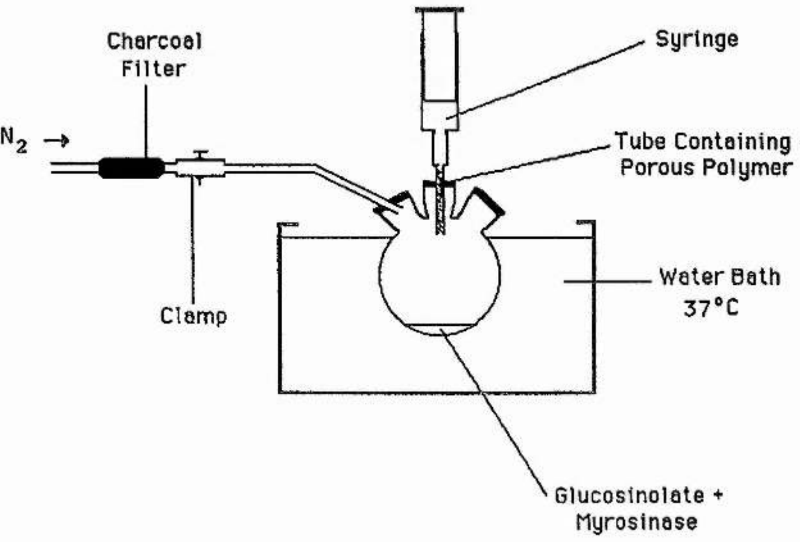


Figure 33 Schematic diagram of the apparatus used for sampling isothiocyanates.

7.6.3.2 Method of sampling isothiocyanates

The reaction vessel was maintained at 37 °C using a water bath. All glucosinolate and myrosinase solutions were prepared as given previously. The glucosinolate (2 x 10^{-5} mmol) was added to the reaction vessel and the system was purged with nitrogen for approximately 10 minutes. The myrosinase solution (80 μl) was then added and the vessel was sealed and incubated for 4 hours. Upon completion of the reaction, the end cap on the tube was replaced by the syringe and the clamp released to allow a flow of nitrogen gas. The volatiles (300 ml) were drawn through the tube by the syringe. The volatiles contained in the tube were analysed at SCRI using thermal desorption GC/MS as given in section 7.1. The volatiles released from nonyl and heptyl glucosinolates were compared with authentic nonyl and heptyl isothiocyanates and found to be identical. The other alkyl glucosinolates were found to release the corresponding isothiocyanate. The corresponding nitrile was also released upon hydrolysis of butyl and heptyl glucosinolates.

7.6.3.3 Preparation of authentic isothiocyanates

Heptyl isothiocyanate 165

$$CH_3(CH_2)_6N{=}C{=}S$$

Heptylamine (2 g, 17.4 mmol) was dissolved in dichloromethane (40 ml) and this solution was added dropwise to an emulsion of thiophosgene (25 ml, 21.7 mmol) and water (60 ml). The resulting biphasic system was stirred at room temperature overnight. The organic layer was then separated, washed with water (2 x 100 ml), dried ($MgSO_4$), and concentrated under reduced pressure to afford a brown oil. This was purified by distillation using a Kugelrohr apparatus to afford the title compound as a colourless oil (690 mg, 25%), bp 125 °C (8 mmHg) (lit.,[173] 241 °C (760 mmHg)); δ_H(200 MHz, $CDCl_3$) 0.83 (3 H, t, *J* 6, CH_3), 1.29 (8 H, m, $(CH_2)_4CH_3$), 1.69 (2 H, quintet, *J* 6, CH_2CH_2NCS), 3.51 (2 H, t, *J* 7, CH_2NCS); δ_C(50.31 MHz, $CDCl_3$) 14.52 (CH_3), 23.02, 27.01, 28.97, 30.45, 32.07 (CH_2), 45.55 (CH_2NCS), 112.81 (NCS).

Nonyl isothiocyanate 166

$$CH_3(CH_2)_8N{=}C{=}S$$

Nonylamine (2 g, 14.0 mmol) was dissolved in dichloromethane (40 ml) and this solution was added to an emulsion of thiophosgene (2.5 ml, 21.7 mmol) and water (60 ml). The resulting biphasic system was treated as before to afford the crude product as a brown oil. Purification by Kugelrohr distillation yielded the title compound as a pale brown oil (1.41 g, 54%), bp 150 °C (15 mmHg); δ_H(200 MHz, $CDCl_3$) 0.90 (3 H, t, *J* 6, CH_3), 1.25 (12 H, br s, $(CH_2)_6CH_3$), 1.70 (2 H, quintet, *J* 8, CH_2CH_2NCS), 3.50 (2 H, t, *J* 8, CH_2NCS);

δ_C(50.31 MHz, $CDCl_3$) 14.59 (CH_3), 23.31, 27.04, 29.29, 29.66, 29.84, 30.44, 32.30 (CH_2), 45.54 (CH_2NCS), 112.80 (NCS).

7.7 STUDIES ON THE OXIDATION OF ISOTHIOCYANATES

7.7.1 Reaction of isothiocyanates with *m*-CPBA

Reaction of BITC with *m*-CPBA

m-CPBA (1.156 g, 6.7 mmol) was dissolved in acetonitrile (40 ml) before BITC (200 mg, 1.3 mmol) was added. The resulting mixture was heated under reflux for 18 hours then allowed to cool to room temperature, before the acetonitrile was removed under reduced pressure. The remaining residue was taken up in diethyl ether (30 ml) and washed with saturated sodium bicarbonate solution (30 ml). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure affording a clear oil which was purified by flash chromatography, on silica gel, eluting with ethyl acetate:petroleum ether (bp 40-60°C) (1:1.5). The fractions were concentrated under reduced pressure and one fraction was analysed by GC/MS. This showed a mixture of products one of which was BIC; *m/z*(EI) 133 (M^+, 65%), 105 (72, $[M-CO]^+$), 92 (97, $PhCH_2^+$), 78 (78, $C_6H_6^+$).

Quantification of BIC formation 173

C_6H_5–CH_2—N=C=O

m-CPBA (6.04 g, 40.2 mmol) was dissolved in acetonitrile (150 ml) before BITC (1.2 g, 7.2 mmol) was added. This mixture was heated under reflux for 53 hours with aliquots removed after 1, 5, 10, 26 and 53 hours. Acetonitrile was removed from each

aliquot, under reduced pressure, before diethyl ether (40 ml) was added. This was washed with saturated sodium bicarbonate solution (60 ml), dried ($MgSO_4$) and concentrated under reduced pressure. The residue was dissolved in dichloromethane (1 ml) for GC/MS analysis; The five aliquots gave relative percentages of BIC as 8.29, 9.57, 4.89, -, and 2.09 respectively.

Preparation of BIC from reaction of BITC with excess *m*-CPBA at 0°C and heating under reflux.

Two experiments were set up as follows:

(i) *m*-CPBA (1.45 g, 8.4 mmol) was added to acetonitrile (20 ml) before BITC (250 mg, 1.7 mmol) was added. The mixture was stirred at 0 °C for 3.5 hours and maintained at 4 °C overnight, before GC/MS analysis. BIC was observed (*m*/*z*(EI) as given previously) with a relative peak area of 162744.

(ii) The procedure was carried out in an identical manner to (i) except the mixture was heated under reflux and left at room temperature overnight before GC/MS analysis. BIC was observed (*m*/*z*(EI) as given previously) with a relative peak area of 199560.

Preparation of urea 178 and thiourea 177 derivatives

m-CPBA (2.09 g, 12.1 mmol) was dissolved in acetonitrile (40 ml) before 4-methoxyphenyl isothiocyanate (1 g, 6.1 mmol) was added. This mixture was heated under reflux for 21 hours before the acetonitrile was removed under reduced pressure to yield a green coloured solid. This was dissolved in diethyl ether (90 ml) and washed with saturated sodium bicarbonate solution (100 ml). A cream coloured precipitate was evident and was filtered off (0.3 g). Spectral data for the thiourea **177**, ν_{max}(nujol)/cm^{-1} 3200 (NH), 1240 (CO); δ_H(200 MHz, d^6-DMSO) 3.39 (3 H, s, OCH_3), 6.93 (2 H, d, $J_{2,3}$=$J_{5,6}$ 10, CH-3,5), 7.35 (2 H, d, $J_{2,3}$=$J_{5,6}$ 10, CH-2,6), 9.90 (1 H, br s, NH); δ_C(50.31 MHz, d^6-DMSO) 55.80 (OCH_3), 113.60 (ArC-3,5), 125.90 (ArC-2,6), 132.80 (ArC-1), 156.80 (ArC-4), 180.20 (CS); m/z(CI) 289 (MH^+, 100%), 255 (18, $[M-HS]^+$), 165 (15, $CH_3OPhNCS^+$), 123 (51, $CH_3OPhNH_2^+$) and 108 (25, CH_3OPh^+). In addition a small amount of urea **178** was observed in the mass spectrum (m/z(CI) 273, (MH^+, 15%)).

Preparation of 4-methoxyphenyl isocyanate 179

To a solution of m-CPBA (2.09 g, 12.1 mmol), dissolved in dry acetonitrile (40 ml), 4-methoxyphenyl isothiocyanate (1 g, 6.1 mmol) was added under a nitrogen atmosphere.

The mixture was heated under reflux for 7 hours before cooling to room temperature to afford a cream-coloured precipitate. This was filtered off prior to solvent removal under reduced presssure. Diethyl ether (60 ml) was added and the resulting solution was washed with saturated sodium bicarbonate solution (2 x 100 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to yield a red/brown solid. GC/MS analysis showed a small amount of the desired product; *m/z*(EI) 149 (M^+).

7.7.2 Preparation of PTFA 168 and reaction with BITC.

$CF_3-C(=O)-O-OH$

Hydrogen peroxide (6 ml, 0.18 mol) was added to dichloromethane (14 ml) at 0 °C. To this mixture, trifluoroacetic anhydride (18 g, 90 mmol) was added slowly over 30 minutes, with stirring, before BITC (0.90 g, 6 mmol) was added. The resulting solution was stirred at 0 °C for 3 hours then maintained at 4 °C for 20 hours. The reaction mixture was washed with distilled water (2 x 20 ml), then dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a yellow/brown oil; δ_H(200 MHz, $CDCl_3$) 4.35 (2 H, s, $PhCH_2$), 7.20-7.40 (5 H, m, C_6H_5); *m/z*(EI) given as before for BIC.

7.7.3 Oxidations using DMD

Preparation of DMD 180

This was performed using the procedure described by Messeguer.[174] A 1000 ml 3-necked reaction flask was cooled to 0-10 °C before water (148 ml), acetone (96 ml) and sodium bicarbonate (18 g) were added. This flask was connected, by means of a side-arm, to a cold finger condenser cooled to -78 °C. This was connected to a 250 ml 3-necked collection flask, attached by rubber tubing to a 100 ml 3-necked collection flask with both flasks being cooled to -78 °C. Oxone (60 g, 98 mmol) was added to the reaction flask in 5 portions over 20 minutes with a moderate vacuum applied thereafter to allow distillation of the DMD/acetone solution. This was dried over molecular sieves and stored at -20 °C.

The concentration of DMD in acetone was determined by phenyl methyl sulfide assay, as described by Adam *et al.*[175] Phenyl methyl sulfide (64.7 μl) was added to d^6-acetone (1 ml) and an aliquot (0.4 ml) added to DMD/acetone (1 ml). The resulting solution was allowed to stand for 5 minutes prior to ^{1}H NMR analysis. Integration of the sulfoxide phenyl protons (δ_H 7.6-7.9) and comparison with the integrated sulfide protons (δ_H 7.1-7.3) allowed the concentration of DMD in acetone to be determined.

Preparation of BIC by reaction of BITC with DMD

A solution of DMD in acetone (13 ml, 0.08 M), was added to dry acetone (10 ml), under a nitrogen atmosphere, before BITC (62.5 mg, 0.8 mmol) was added. This mixture was stirred at 0 °C for 2 hours before analysis by GC/MS; *m/z*(EI) as given previously for BIC; *N,N'*-dibenzylurea, 241 (MH^+, 2%), 149 (3, $[M\text{-}PhN]^+$), 133 (2, $PhCH_2NCO^+$), 106 (28, $PhCH_2NH^+$), 91 (21, $PhCH_2^+$) and 79 (10, $C_6H_6H^+$).

7.7.4 Comparison of Oxidising Agents

GC/MS Analysis Conditions

The column specification and operating conditions are as specified in section 7.1. However the following parameters differed:

Temperature Programme — All analyses were performed using the following GC temperature programme:

Initial temperature	30 °C	Final temperature	251 °C
Heating rate	20.1 °C/minute	Total analysis time	11.0 minutes

Injection Volume — A fixed injection volume of 2 μl was used for each sample.

Calibration of the GC/MS

The GC/MS was calibrated using the standards given in table **23**.

Concentration of BIC (mM)	Volume BIC (μl)	Volume Acetone (ml)	Volume Dibenzyl ether (μl)
4.48	2.75	5	29.7
8.91	5.49	5	29.7
13.40	8.24	5	29.7
17.80	10.98	5	29.7
20.00	12.34	5	29.7

Table 23 Composition of BIC standards.

Note: These standards were prepared using dry acetone and were stored under nitrogen.

Analysis of Reaction Solutions

The reaction solutions were all analysed 8 hours after the the experiment had begun. An aliquot (5 ml) was removed from each reaction flask, dibenzyl ether (29.7 μl) added, and 2 μl of the resulting solution injected to the GC/MS.

Oxidation reactions

Preparation of BIC by DMD oxidation of BITC

$C_6H_5-CH_2-N=C=O$

BITC (200 mg, 1.3 mmol) was added to dry DMD (545.9 mg, 7.4 mmol), prepared as given previously, in acetone (75 ml) at 0 °C. The resulting solution was maintained at this temperature for 8 hours, with stirring, under nitrogen, before being analysed by GC/MS. This showed the presence of BIC (104 mg, 58%); *m/z*(EI) as before.

Preparation of BIC by *m*-CPBA oxidation of BITC

m-CPBA (1.272 g, 7.4 mmol) was dissolved in dry acetonitrile (75 ml) before BITC (200 mg, 1.3 mmol) was added. This mixture was stirred at 0 °C, under nitrogen, for 8 hours. GC/MS analysis showed the presence of BIC (45 mg, 25%); *m/z*(EI) as before.

Formation of BIC by PTFA oxidation of BITC

Hydrogen peroxide (515 mg, 15.1 mmol) was added to dichloromethane (1.20 ml) and the resulting solution stirred at 0 °C while trifluoroacetic anhydride (1.03 ml, 7.4 mmol) was added over 30 minutes. To this solution, BITC (200 mg, 1.3 mmol) and more dichloromethane (74 ml) were added, with stirring, at 0 °C. This mixture was maintained under the same conditions for 8 hours prior to GC/MS analysis. This indicated BIC had formed (8 mg, 5%); *m/z*(EI) as before.

7.7.5 Further work on the DMD model system

7.7.5.1 Oxidation of additional isothiocyanates

Preparation of PIC 182

PITC (100 mg, 0.7 mmol) was added to dry DMD (329 mg, 4.4 mmol) in acetone (61 ml) and the resulting solution was stirred for 18 hours, under nitrogen, at room temperature. GC/MS analysis showed the desired product had formed; *m/z*(EI) 119 (M^+, 100%), 91 (100, $[M-CO]^+$), 77 (5, Ph^+).

Preparation of PEIC 183, BuIC 185, and MPIC 184

The above compounds were synthesised in an analogous manner to PIC except five equivalents of DMD were used, instead of six equivalents as above; *m/z*(EI) PEIC **183**, 147 (M^+, 10%), 119 (2, $[M-CO]^+$), 91 (100, $PhCH_2^+$), 77 (5, Ph^+); BuIC **185**, 99 (M^+, 7%), 71 (10,$[M-CO]^+$), 56 (40, $(CH_2)_4^+$), 43 (40, $CH_3(CH_2)_2^+$); MPIC **184**, 149 (M^+, 100%), 134 (83, $[M-CH_3]^+$), 121 (12, $[M-CO]^+$), 106 (85, CH_3OPh^+) and 78 (62, $C_6H_6^+$).

7.7.5.2 Attempted isolation of isocyanates

Attempted Isolation of BIC

First attempt

BITC (125 mg, 0.84 mmol) was added to dry acetone (10 ml) before dry DMD (311 mg, 4.2 mmol), dissolved in acetone (44.3 ml), was added. The resulting solution was stirred at 0 °C, under nitrogen, for 36 hours while warming to room temperature. GC/MS analysis indicated the product had formed, and the solution was concentrated under reduced pressure. Analysis by ^{1}H NMR indicated a mixture of products and this sample was analysed by GC/MS which showed 8 different compounds to be present. These included benzylamine (*m*/*z*(EI) 107 (M^+, 4%), 105 (41, $[M-H_2]^+$) and 77 (55, Ph^+)) and BIC (*m*/*z*(GC/MS) as given previously).

Second Attempt

BITC (500 mg, 3.4 mmol) was oxidised similarly by DMD (1.36 g, 18.4 mmol) in acetone (243 ml). Concentration of the reaction mixture under reduced pressure afforded a red-coloured residue; ν_{max}/cm^{-1} 3500-2800 (COOH), 3300 (NH), 1700 (CO); δ_H(200 MHz, d^6-acetone) 4.30 (br s, N*H*), 4.45 (2 H, s, PhCH_2), 7.22-7.34 (4 H, m, Ar*H*), 8.92 (2 H, s, COO*H*); δ_C(50.31 MHz, d^6-acetone) 44.87 (PhCH$_2$), 44.99 (Ph*C*H$_2$), 128.56-130.08 (Ar*C*), 139.17 (Ar*C* quartenary), 207.77 (*C*OOH). GC/MS analysis indicated a mixture of 7 compounds.

Attempted Isolation of PIC

PITC (994 mg, 7.4 mmol) was added to DMD (3.02 g, 40.7 mmol) in acetone (558 ml) and the resulting solution was stirred at room temperature, under nitrogen, for 16 hours. GC/MS analysis showed the presence of PIC, thus the solution was concentrated to approximately 10 ml and diphenyl ether (9 ml) was added. The resulting emulsion was

heated, under nitrogen, with vigorous stirring to allow the distillation of acetone. The distillation flask was then allowed to cool to room temperature which saw the appearance of a yellow solid. Reduced pressure was applied before and during further heating, which allowed distillation to occur, leaving a residue of yellow solid. This was purified by flash chromatography, on silica gel, eluting with ethyl acetate:petroleum ether (bp 40-60°C) (1:1) to yield a white solid, identified as *N,N'*-diphenyl urea **188** (738 mg, 94%), mp 241-242 °C (lit.,[176] 239-240 °C); ν_{max}(nujol)/cm^{-1} 3250 (NH), 1625 (CO); δ_H(200 MHz, d^6-DMSO) 6.95-7.50 (5 H, m, Ar*H*), 8.69 (1 H, br s, N*H*); δ_C(50.31 MHz, d^6-DMSO) 118.44 (Ar*C*-2,6), 122.05 (Ar*C*-4). 129.03 (Ar*C*-3,5), 139.99 (Ar*C*-1), 152.79 (*C*O).

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Attempted Isolation of BuIC

First Attempt

BuITC (400 mg, 3.5 mmol) was added to DMD (1.29 g, 17.4 mmol) in acetone (440 ml) and the resulting solution stirred at room temperature, under nitrogen, for 60 minutes. GC/MS analysis showed the presence of BuIC and the solution was concentrated under reduced pressure and distilled using a Kugelrohr apparatus under reduced pressure. This afforded a clear liquid; δ_H(200 MHz, d^6-acetone) 0.85 (t, CH_3(CH_2)$_3$), 1.15-1.55 (m, CH_3(CH_2)$_2$$CH_2$), 3.15 (q, CH_3(CH_2)$_2$CH_2), 4.50 (br s); δ_C(50.31 MHz, d^6-acetone) 14.34 (*C*H_3(CH_2)$_3$), 20.83 (CH_3*C*H_2(CH_2)$_2$) 23.37, 23.79, 32.07 ($CH_3CH_2CH_2$), 40.32 (CH_3(CH_2)$_2$*C*H_2), 42.38, 63.13.

Second Attempt

The same procedure was followed as above except the reaction solution was concentrated prior to GC/MS analysis. This was followed by Kugelrohr distillation at atmospheric pressure which yielded several fractions, none of which contained BuIC and a brown residue; δ_H(200 MHz, $CDCl_3$) 0.90 (3 H, t, *J* 7, $CH_3(CH_2)_3$), 1.20-1.85 (4 H, m, $CH_3(CH_2)_2CH_2$), 2.20 (s), 2.95 (m), 3.15-3.30 (1 H, q), 4.00 (br s), 7.20 (br s), 7.65 (t, N*H*); δ_C(50.31 MHz, $CDCl_3$) 13.98, 14.12, 20.06, 20.42, 29.74, 31.41, 31.48, 40.47, 40.69, 166.34.

7.7.5.3 FT-IR analysis of DMD reaction mixtures

Standards of BITC and BIC were prepared by adding BITC (4.44 μl) and BIC (4.20 μl) to acetone (1.82 ml). These were analysed by FT-IR using solution cells after a background correction against acetone; ν_{max}(soln.)/cm^{-1} BITC 2093; BIC 2268.

Solutions of BITC and BIC in DMD were prepared by adding the same amounts of BITC and BIC, as above, to DMD (1.82 ml). These were analysed by FT-IR after a background correction against acetone; ν_{max}(soln.)/cm^{-1} BITC 2341, 2276, 2169, 2092; BIC 2341, 2266. The BITC sample was spiked with BIC (15 μl) which showed an enhanced peak at 2276 cm^{-1}.

The decomposition of BIC in the FT-IR was monitored using a sample of BIC (10 μl) in DMD (1 ml). An aliquot of this sample was analysed at time 0 and after 20, 40 and 60 minutes after a background correction against DMD; ν_{max}(soln.)/cm^{-1} 2341, 2272.

A carbon dioxide saturated DMD solution was prepared by bubbling CO_2(g), generated from addition of dilute HCl to $CaCO_3$ and dried by passage through conc. H_2SO_4, through

a stirred solution of DMD. The CO_2-saturated DMD solution was analysed by FT-IR after a background correction against untreated DMD; ν_{max}(soln.)/cm^{-1} 2340.

7.7.5.4 Derivatisation of isothiocyanates as carbamates

Preparation of methyl phenethylcarbamate 190

PEITC (163.5 mg, 1.0 mmol) was added to dry DMD (409 mg, 5.5 mmol) in acetone (69 ml) and was stirred under nitrogen, at room temperature, for 1 hour. GC/MS analysis indicated that the isocyanate had formed and methanol (176.86 mg, 5.5 mmol) was added. This mixture was stirred under the same conditions, for 18 hours, before GC/MS analysis showed the reaction to be incomplete. Addition of more methanol (176.86 mg, 5.5 mmol) was followed by heating the mixture under reflux for 4 hours. Concentration under reduced pressure and purification by flash chromatography, on silica gel, eluting with ethyl acetate:petroleum ether (bp 40-60°C) (1:1.5), afforded the product as a colourless oil (18 mg, 11%); ν_{max}/cm^{-1} 3320 (NH), 2980 (CH_2), 1700 (CO), 1260 (COOC), 700, 760 (C_6H_5); δ_H(200 MHz, $CDCl_3$) 2.82 (2 H, t, *J* 7, PhC*H*$_2$), 3.45 (2 H, q, *J* 7, PhCH$_2$C*H*$_2$), 3.67 (3 H, s, OC*H*$_3$), 4.70 (1 H, br s, N*H*), 7.18-7.37 (5 H, m, Ar*H*); δ_C(50.31 MHz, $CDCl_3$) 35.20 (Ph*C*H$_2$), 41.24 (*C*H$_2$NH), 51.09 (O*C*H$_3$), 125.69 (Ar*C*-4), 127.79 (Ar*C*-2,6), 127.94 (Ar*C*-3,5), 207.85 (*C*O); *m/z*(EI) 179 (M^+, 29%), 104 (41, $PhCH_2CH^+$), 88 (100, $CH_2NHCOOCH_3^+$) and 77 (4, Ph^+).

7.7.5.5 Preparation of authentic ureas from isocyanates

Authentic *N*-*iso*-propyl-*N*'-phenylurea 195

Iso-propylamine (7.44 g, 125.9 mmol) was added to dry toluene (50 ml) before PIC (3 g, 25.2 mmol) was added at 0 °C. This mixture was stirred overnight, under nitrogen, while warming to room temperature. Filtration under reduced pressure afforded the product as a white solid (3.94 g, 88%), mp 159 °C (lit.,[177] 156 °C); ν_{max}(nujol)/cm^{-1} 3350 (NH), 1650 (CO), 700, 760 (C_6H_5); δ_H(200 MHz, $CDCl_3$) 1.14 (6 H, d, *J* 6, CH(CH_3)$_2$), 3.85-4.05 (1 H, m, C*H*(CH_3)$_2$), 5.20 (1 H, d, *J* 8, N*H*), 7.01-7.31 (6 H, m, Ar*H*, N*H*); δ_C(50.31 MHz, $CDCl_3$) 23.67 (CH(CH_3)$_2$), 42.36 (*C*H(CH_3)$_2$), 120.39 (Ar*C*-2,6), 123.25 (Ar*C*-4), 129.49 (Ar*C*-3,5), 139.75 (Ar*C*-1), 156.55 (*C*O); *m/z*(EI) 178 (M^+, 16%), 135 (1, $PhNHCONH^+$), 119 (2, $PhNCO^+$), 93 (100, $PhNH_2^+$) and 77 (3, Ph^+).

Authentic *N*-*iso*-propyl-*N*'-benzylurea 196

BITC (2 g, 15 mmol) was added to *iso*-propylamine (4.43 g, 75 mmol) in dry toluene (50 ml) at 0 °C. The resulting solution was stirred for 18 hours under nitrogen before being filtered under reduced pressure to yield the product as a white solid (2.22 g, 77%), mp 127 °C; (Found: C, 69.10; H, 8.60; N, 14.85. $C_{11}H_{16}N_2O$ requires C, 68.72; H, 8.39; N, 14.57%); ν_{max}(nujol)/cm^{-1} 3320 (NH), 1625 (CO), 700, 760 (C_6H_5); δ_H(200 MHz,

$CDCl_3$) 1.00 (3 H, d, *J* 6, CH(C*H*$_3$)$_2$), 1.11 (3 H, d, *J* 6, CH(C*H*$_3$)$_2$), 3.75-3.95 (1 H, m, C*H*(CH_3)$_2$), 4.32 (2 H, d, *J* 6, PhC*H*$_2$), 4.43 (1 H, d, *J* 6, N*H*CH(CH_3)$_2$), 4.87 (1 H, br s, $PhCH_2$N*H*), 7.23-7.32 (5 H, m, Ar*H*); δ_C(50.31 MHz, $CDCl_3$) 23.85 (CH(*C*H$_3$)$_2$), 42.39 (*C*H(CH_3)$_2$), 44.53 (Ph*C*H$_2$), 127.47 (Ar*C*-4), 127.75 (Ar*C*-2,6), 128.94 (Ar*C*-3,5), 140.15 (Ar*C*-1), 158.75 (*C*O); *m/z*(EI) 192 (M^+, 100%), 149 (5, $PhCH_2NHCONH^+$), 106 (74, $PhCH_2NH^+$), 91 (48, $PhCH_2^+$) and 77 (9, Ph^+).

Authentic *N-iso*-propyl-*N*'-butylurea 197

O

$CH_3(CH_2)_3$—N N—

H H

Iso-propylamine (2.98 g, 50 mmol) was added to BuIC (1.00 g, 10 mmol), dissolved in dry toluene (50 ml), at 0 °C. This was stirred under nitrogen for 2 hours while warming to room temperature. The solution was then cooled to -78 °C which saw the appearance of a cream-coloured precipitate. Filtration under reduced pressure afforded the product (1.24 g, 78%), mp 76 °C (lit.,[178] 75-77 °C); ν_{max}(nujol)/cm^{-1} 3300 (NH), 1625 (CO); δ_H(200 MHz, $CDCl_3$) 0.88 (3 H, t, *J* 7, C*H*$_3$(CH_2)$_3$), 1.09 (6 H, d, *J* 7, CH(C*H*$_3$)$_2$), 1.18-1.43 (4 H, m, CH_3(C*H*$_2$)$_2$), 3.10 (2 H, q, *J* 6, $CH_3(CH_2)_2$C*H*$_2$), 3.81-3.84 (1 H, m, C*H*(CH_3)$_2$), 5.16 (1 H, br s, N*H*), 5.35 (1 H, br s, N*H*); δ_C(50.31 MHz, $CDCl_3$) 14.31 (*C*H$_3$(CH_2)$_3$), 20.57 (CH_3*C*H$_2$(CH_2)$_2$), 23.96 (*C*H(CH_3)$_2$), 32.97 (CH_3CH_2*C*H$_2$), 40.44 ($CH_3(CH_2)_2$*C*H$_2$), 42.21 (*C*H(CH_3)$_2$), 158.97 (*C*O); *m/z*(EI) 158 (M^+, 63%), 143 (17, $[M-CH_3]^+$), 129 (6, $[M-C_2H_5]^+$), 116 (12, $[M-C_3H_6]^+$), 101 (8, $(CH_3)_2CHNHCOCH_3^+$), 87 (16, $(CH_3)_2CHNHCOH^+$), 58 (37, $(CH_3)_2CHNH^+$) and 44 (100, $(CH_3)_2CH_2^+$).

7.7.5.6 Trapping of isothiocyanates as ureas

Preparation of *N*-*iso*-propyl-*N*'-phenylurea 191

PITC (150 mg, 1.1 mmol) was added to dry DMD (440 mg, 6.0 mmol) in acetone (180 ml) and stirred under nitrogen at room temperature for 15 minutes. GC/MS analysis indicated the reaction was complete and *iso*-propylamine (1.12 g, 18.9 mmol) was added at 0 °C. This mixture was stirred, under nitrogen for 1.5 hours prior to filtration and concentration under reduced pressure. The filtrate was purified by flash chromatography, on silica gel, eluting with ethyl acetate:petroleum ether (bp 40-60°C) (1:2.33). This afforded the product as a white solid (199 mg, 89%), mp 160 °C (lit.,[177] 156 °C); ν_{max}(nujol)/cm^{-1} 3350 (NH), 1650 (CO), 1450-1600 (CH, CH_3), 700, 750 (C_6H_5); δ_H(200 MHz, $CDCl_3$) 1.23 (6 H, d, *J* 7, CH(C*H*$_3$)$_2$), 3.97 (1 H, m, C*H*(CH_3)$_2$), 5.53 (1 H, d, *J* 7, N*H*CH), 6.97-7.32 (5 H, m, Ar*H*), 7.54 (1 H, br s, ArN*H*); δ_C(50.31 MHz, $CDCl_3$) 23.68 (CH(*C*H$_3$)$_2$), 42.47 (*C*H(CH_3)$_2$), 120.59 (Ar*C*-2,6), 123.42 (Ar*C*-4), 129.54 (Ar*C*-3,5), 139.63 (Ar*C*-1), 156.38 (*C*O); *m/z*(EI) 178 (M^+, 13%), 135 (1, $PhNHCONH^+$), 119 (2, $PhNCO^+$), 93 (100, $PhNH_2^+$) and 77 (4, Ph^+).

Preparation of *N-iso*-propyl-*N*'-benzylurea 192

BITC (250 mg, 1.7 mmol) was added to dry DMD (747 mg, 10.1 mmol) in acetone (360 ml) and stirred, under nitrogen, at room temperature for 15 minutes. GC/MS analysis indicated the reaction was complete and *iso*-propylamine (1.78 g, 30.2 mmol) was added at 0 °C. The resulting solution was stirred for 30 minutes, under nitrogen, before filtration and concentration under reduced pressure. Flash chromatography of the filtrate, on silica gel, eluting with petroleum ether (bp 40-60°C): ethyl acetate (1:1), yielded the product as a white solid (272 mg, 84%) mp 121-124 °C; ν_{max}(nujol)/cm^{-1} 3340 (NH), 1590 (CO), 730, 790 (C_6H_5); δ_H(200 MHz, $CDCl_3$) 1.05 (3 H, d, *J* 5, CH(C*H*$_3$)$_2$), 1.09 (3 H, d, *J* 5, CH(C*H*$_3$)$_2$), 3.80 (1 H, m, C*H*(CH$_3$)$_2$), 4.27 (2 H, d, *J* 5, PhC*H*$_2$), 4.91 (1 H, d, *J* 3, N*H*CH(CH$_3$)$_2$), 5.38 (1 H, br s, PhCH$_2$N*H*), 7.24-7.30 (5 H, m, Ar*H*); δ_C(50.31 MHz, $CDCl_3$) 23.89 (CH(*C*H$_3$)$_2$), 42.53 (*C*H(CH$_3$)$_2$), 44.70 (Ph*C*H$_2$), 127.58 (Ar*C*-4), 127.84 (Ar*C*-2,6), 129.00 (Ar*C*-3,5), 140.03 (Ar*C*-1), 158.50 (*C*O); *m/z*(EI) 192 (M$^+$, 100%), 149 (6, PhCH$_2$NHCONH$^+$), 106 (67, PhCH$_2$NH$^+$), 91 (54, PhCH$_2$$^+$) and 77 (10, Ph$^+$).

Preparation of *N-iso*-propyl-*N*'phenethylurea 193

PEITC (300 mg, 18.4 mmol) was added to dry DMD (681 mg, 9.2 mmol) in acetone (126 ml) and stirred for 60 minutes, under nitrogen and at room temperature. GC/MS analysis showed the isocyanate had formed and *iso*-propylamine (1.63 g, 27.6 mmol) was added at

0 °C. The resulting solution was stirred under nitrogen for 3 hours before filtration and concentration under reduced pressure. Purification of the filtrate by flash chromatography, on silica gel, eluting with petroleum ether (bp 40-60°C):ethyl acetate (1:4) afforded a white solid, identified as the product (252 mg, 67%), mp 94-96 °C; (Found: C, 69.82; H, 9.13; N, 13.66. $C_{12}H_{18}N_2O$ requires C, 69.87; H, 8.79; N, 13.58%); ν_{max}(nujol)/cm^{-1} 3340 (NH), 1625 (CO), 700 (C_6H_5); δ_H(200 MHz, $CDCl_3$) 1.10 (6 H, d, *J* 6, CH(CH_3)$_2$), 2.80 (2 H, t, *J* 7, PhC*H*$_2$), 3.42 (2 H, q, *J* 7, PhCH$_2$C*H*$_2$), 3.81 (1 H, m, C*H*(CH_3)$_2$), 4.20 (1 H, br s, N*H*), 4.32 (1 H, br s, N*H*), 7.12-7.31 (5 H, m, Ar*H*); δ_C(50.31 MHz, $CDCl_3$) 23.92 (CH(*C*H$_3$)$_2$), 29.73 (PhCH$_2$), 37.14 (*C*H$_2$NH), 42.29 (*C*H(CH_3)$_2$), 126.73 (Ar*C*-4), 128.96 (Ar*C*-2,6), 129.28 (Ar*C*-3,5), 139.87 (Ar*C*-1), 158.66 (*C*O).

Preparation of *N-iso*-propyl-*N*'-butylurea 194

BuITC (100 mg, 0.9 mmol) was added to dry DMD (322 mg, 4.3 mmol) in acetone (145 ml) and was stirred, under nitrogen, at room temperature, for 4 hours before *iso*-propylamine (514 mg, 8.7 mmol) was added at 0 °C. The resulting solution was stirred under nitrogen for 12 hours prior to filtration and concentration under reduced pressure. Flash chromatography, on silica gel, eluting with petroleum ether(bp 40-60°C):ethyl acetate (1:1) afforded the product (35 mg, 71%), mp 69 °C (lit.,[178] 75-77 °C); ν_{max}(nujol)/cm^{-1} 3330 (NH), 1580 (CO); δ_H(200 MHz, $CDCl_3$) 0.89 (3 H, t, *J* 7, C*H*$_3$(CH$_2$)$_3$), 1.11 (6 H, d, *J* 7, CH(C*H*$_3$)$_2$), 1.24-1.48 (4 H, m, CH$_3$(C*H*$_2$)$_2$), 3.12 (2 H, q, *J* 6, CH$_3$(CH$_2$)$_2$C*H*$_2$), 3.70-3.84 (1 H, m, C*H*(CH$_3$)$_2$), 4.75 (1 H, br s, N*H*), 4.90 (1 H, br s, *N*H); δ_C(50.31 MHz, $CDCl_3$) 14.28 (*C*H$_3$(CH$_2$)$_3$), 20.53 (CH$_3$*C*H$_2$(CH$_2$)$_2$), 23.95 (CH(*C*H$_3$)$_2$), 32.86 (CH$_3$CH$_2$*C*H$_2$), 40.58 (CH$_3$(CH$_2$)$_2$*C*H$_2$), 42.44 (*C*H(CH$_3$)$_2$), 158.62 (*C*O); *m*/*z*(EI) 158 (M^+, 39%), 143 (9, $[M-CH_3]^+$), 116 (6, $[M-C_3H_6]^+$), 101 (5,

$(CH_3)_2CHNHCOCH_3^+$), 87 (9, $(CH_3)_2CHNHCOH^+$), 58 (33, $(CH_3)_2CHNH^+$) and 44 (100, $(CH_3)_2CH_2^+$).

7.7.5.7 Further investigation of the mechanism of oxidation

Synthesis of *N*-benzyl-*N*'-*iso*-propylthiourea 199

Benzyl isothiocyanate (1 g, 6.7 mmol) was added to *iso*-propylamine (1.98 g, 33.51 mmol) dissolved in dichloromethane (25 ml) and the resulting solution was heated under reflux for 2 hours. Concentration under reduced pressure afforded a yellow solid which was recrystallised from ethanol to yield the product as a white solid (739 mg, 53%), mp 124 °C (lit.,[179] 126 °C); ν_{max}(nujol)/cm^{-1} 3250, 3280 (NH), 3000-3100 (aromatic), 1550 (CN), 1210 (C=S); δ_H(200 MHz, $CDCl_3$) 1.15 (6 H, d, *J* 7, $CH(CH_3)_2$), 4.16 (1 H, m, $CH(CH_3)_2$), 4.60 (2 H, d, *J* 4, $PhCH_2NH$), 5.85 (1 H, br t, *J* 4, $PhCH_2NH$), 6.27 (1 H, br s, $NHCH(CH_3)_2$), 7.27-7.37 (5 H, m, Ar*H*); δ_C(75.42 MHz, $CDCl_3$) 22.53 ($CH(CH_3)_2$), 46.24 ($CH(CH_3)_2$), 48.40 ($PhCH_2NH$), 127.74 (Ar*C*-4), 128.04 (Ar*C*-2,6), 129.05 (Ar*C*-3,5), 137.00 (Ar*C*-1), 180.86 (*C*S); *m/z*(EI) 208 (M^+, 100%), 175 (4, $[M-SH]^+$), 150 (2, $PhCH_2NHCS^+$), 117 (5, $PhCH_2NC^+$), 106 (41, $PhCH_2NH^+$), 91 (89, $PhCH_2^+$), 77 (10, Ph^+).

Attempted oxidation of *N*-benzyl-*N*'-*iso*-propylthiourea

N-Benzyl-*N*'-*iso*-propylthiourea (500 mg, 2.4 mmol) was added to DMD (890 mg, 12 mmol) in acetone (250 ml) and the resulting solution was stirred at room temperature for 1

hour. GC/MS analysis did not show the presence of *N*-benzyl-*N'*-*iso*-propylurea therefore the reaction was left to stir for another 3 hours. Concentration under reduced pressure afforded a cream-coloured solid which was found to be intractable.

Competitive reaction of benzyl isothiocyanate and benzyl isocyanate

Benzyl isothiocyanate (1g, 6.7 mmol) and benzyl isocyanate (892 mg, 6.7 mmol) were added to cold dry acetone (-20 °C; 20 ml) before *iso*-propylamine (396 mg, 6.7 mmol) was added. The resulting solution was stirred under nitrogen for 3 hours before concentration under reduced pressure. Purification by flash chromatography, on silica gel, eluting with petroleum ether (bp 40-60 °C):ethyl acetate (7:3) afforded *N*-benzyl-*N'*-*iso*-propylurea (850 mg, 66%), data as given previously.

7.7.5.8 Oxidation of isothiocyanates in the presence of a nucleophile

Attempted oxidation of benzyl isothiocyanate in the presence of *iso*-propylamine

Method 1

Iso-propylamine (396 mg, 6.7 mmol) was added to DMD (1.24 g, 16.8 mmol) in dry acetone (279 ml) before benzyl isothiocyanate (500 mg, 3.4 mmol) was added and the resulting solution was stirred under nitrogen for 30 minutes. GC/MS analysis showed BITC, and more DMD (0.31 g, 4.2 mmol) was added. The solution was stirred for 30 minutes before being concentrated under reduced pressure. GC/MS analysis showed the reaction was incomplete and further *iso*-propylamine (792 mg, 13.4 mmol) was added and the resulting solution stirred for 1 hour. More *iso*-propylamine was then added (792 mg, 13.4 mmol) and stirred as before. Concentration under reduced pressure afforded a crude product which was purified by flash chromatography, on silica gel, eluting with petroleum

ether (bp 40-60 °C):ethyl acetate (4:1) with increasing ethyl acetate concentration. This afforded the desired product, *N*-benzyl-*N*'-*iso*-propylurea (575 mg, 89%), spectral data see section 7.6.6.

A control experiment was set-up as before except no DMD was added. Purification by flash chromatography as before yielded benzyl isothiocyanate (102 mg, 20%) and *N*-benzyl-*N*'-*iso*-propylthiourea (448 mg, 64%), mp 124 °C (lit.,[179] 126 °C); ν_{max}(nujol)/cm^{-1} 3250, 3280 (NH), 3000-3100 (aromatic), 1550 (CN), 1210 (C=S); δ_H(200 MHz, $CDCl_3$) 1.15 (6 H, d, *J* 7, $CH(CH_3)_2$), 4.16 (1 H, m, $CH(CH_3)_2$), 4.60 (2 H, d, *J* 5, $PhCH_2NH$), 5.85 (1 H, br t, *J* 3, $PhCH_2NH$), 6.27 (1 H, br s, $NHCH(CH_3)_2$), 7.27-7.37 (5 H, m, Ar*H*); δ_C(75.42 MHz, $CDCl_3$) 22.53 ($CH(CH_3)_2$), 46.24 ($CH(CH_3)_2$), 48.40 ($PhCH_2NH$), 127.74 (Ar*C*-4), 128.04 (Ar*C*-2,6), 129.05 (Ar*C*-3,5), 137.00 (Ar*C*-1), 180.86 (*C*S).

Method 2

Iso-propylamine (475 mg, 8.0 mmol) was added to DMD (1.49 g, 20.1 mmol) in dry acetone (265 ml) before benzyl isothiocyanate (600 mg, 4.0 mmol) was added. The resulting solution was stirred at room temperature under nitrogen for 15 minutes before 2 M HCl (50 ml) was added. Stirring continued for a further 30 minutes before diethyl ether (250 ml) was added. The resulting solution was washed with water (50 ml) before being dried ($MgSO_4$) and concentrated under reduced pressure. Purification by flash chromatography, on silica gel, eluting with petroleum ether (bp 40-60 °C):ethyl acetate (7:3) afforded benzyl isothiocyanate (353 mg, 59%).

Method 3

DMD (1.99 g, 26.8 mmol) dissolved in dry acetone (338 ml) was added to *iso*-propylamine (951 mg, 16.1 mmol) before benzyl isothiocyanate (800 mg, 5.4 mmol) was added. The resulting solution was stirred as before for 15 minutes before GC/MS analysis. This showed benzyl isothiocyanate hence DMD (208 mg, 2.8 mmol) in dry acetone (40 ml) was

added and the resulting solution stirred as before. GC/MS analysis after 1.5, 2.5 and 4.5 hours indicated that no reaction had occurred.

Method 4

Iso-propylamine (713 mg, 12.1 mmol) was added to dry acetone (50 ml) before benzyl isothiocyanate (600 mg, 4.0 mmol) was added. DMD (1.49 g, 20.1 mmol) in acetone (402 ml) was added and the resulting solution was stirred at room temperature, under nitrogen for 15 minutes. GC/MS analysis showed the presence of benzyl isothiocyanate and DMD (370 mg, 5 mmol) in acetone (100 ml) was added and stirring continued for 15 minutes. GC/MS analysis showed benzyl isothiocyanate and DMD/acetone (200 ml) was added. GC/MS after 1 hour indicated that benzyl isothiocyanate was still present but the reaction was quenched with 2 M HCl (75 ml). Extraction as before and TLC analysis failed to show the desired *N*-benzyl-*N*'-*iso*-propylurea.

Preparation of *N*-α-CBZ-L-lysine methyl ester 203

$$\begin{array}{rl} & \text{COOMe} \\ & | \\ H_2N(CH_2)_4\text{—} & \text{CH} \\ & | \\ & \text{NHCOOCH}_2\text{Ph} \end{array}$$

N-α-CBZ-L-Lysine (1 g, 3.75 mmol) was added to dry methanol (20 ml) followed by redistilled thionyl chloride (468 mg, 3.9 mmol). This mixture was heated under reflux for 2.5 hours before concentration under reduced pressure to afford a cream solid. To this, dry methanol (15 ml) was added followed by propylene oxide (1.04 g, 17.85 mmol), and the resulting solution was stirred under a nitrogen atmosphere at room temperature for 1 hour. Concentration under reduced pressure afforded the desired product as a white foam which was used without further purification (1.05 g, 100%); ν_{max}(nujol)/cm^{-1} 3350 (NH), 3040 (aromatic), 2900 (CH_2, CH_3), 1600, 1675 (CO); δ_H(200 MHz, CD_3OD) 1.54 (2 H, m,

CH_2CH), 1.78 (4 H, m, $CH_2(CH_2)_2CH_2$), 2.92 (2 H, t, *J* 7, NHCH_2). 3.72 (3 H, s, COOCH_3), 4.21 (1 H, q, *J* 4, α-C*H*), 5.10 (2 H, s, PhCH_2CO), 7.35 (5 H, s, Ar*H*); δ_C(50.31 MHz, CD_3OD) 24.19 ($NH_2(CH_2)_2CH_2$), 28.31 ($NH_2CH_2CH_2$), 32.33 (CH_2CH), 40.84 (NH_2CH_2), 53.13 (COOCH_3), 55.50 (*C*H), 68.00 (COOCH_2Ph), 129.13 (Ar*C*-4), 129.38 (Ar*C*-2,6), 129.82 (Ar*C*-3,5), 138.47 (Ar*C*-1), 159.00 (NH*C*OO), 174.76 (*C*$OOCH_3$); *m/z*(EI) 294 (M^+, 28%), 206 (8), 174 (8, [M-$PhCH_2COH]^+$), 162 (13, $PhCH_2COONHC^+$), 142 (36, $NH(CH_2)_4CHNCOO^+$), 108 (8, $PHCH_2OH^+$) and 91 (100, $PHCH_2^+$).

Preparation of *N*-α-CBZ-*N*-ε-benzyl carbamoyl-L-lysine methyl ester 204

$$C_6H_5-CH_2-NH-C(=O)-NH-(CH_2)_4-CH(COOCH_3)(NHCOOCH_2Ph)$$

N-α-CBZ-L-Lysine methyl ester (900 mg, 3.1 mmol) was dissolved in dry acetone (25 ml) before benzyl isocyanate (370 mg, 2.8 mmol) was added and the resulting solution was stirred, under a nitrogen atmosphere, at room temperature for 22 hours. Analysis by TLC indicated no reaction had occurred and triethylamine (310 mg, 3.1 mmol) was added and the solution stirred for approximately 30 minutes. Concentration under reduced pressure and purification by flash chromatography, on silica gel, eluting with petroleum ether (bp 40-60 °C):ethyl acetate (1:1) afforded the desired product (771 mg, 65%), mp 114-115 °C; (Found: C, 64.79; H, 6.87; N, 9.92. $C_{23}H_{29}N_3O_5$ requires C, 64.62; H, 6.84; N, 9.83%); $[\alpha]_D$ -14.3 ° (c 0.14 in $CHCl_3$); ν_{max}(nujol)/cm^{-1} 3250, 3350 (NH), 3000 (aromatic), 1600, 1675, 1750 (CO), 1550 (CN); δ_H(200 MHz, $CDCl_3$) 1.33 (4 H, m, $NHCH_2(CH_2)_2$), 1.70 (2 H, m, CH_2CH), 3.08 (2 H, q, *J* 6, NHCH_2), 3.70 (3 H, s, COOCH_3), 4.27 (1 H, m, α-C*H*), 4.27 (2 H, d, *J* 6, PHCH_2NH), 5.00 (1 H, br t, *J* 5, CON*H*$(CH_2)_4$), 5.04 (2 H, s, PHCH_2COO), 5.24 (1 H, br t, *J* 5, $PHCH_2$N*H*), 5.86 (1 H,

br d, *J* 8, N*H*COOCH$_2$Ph), 7.19-7.31 (10 H, m, Ar*H*); δ_C(50.31 MHz, CDCl$_3$) 22.81 (NH(CH$_2$)$_2$*C*H$_2$), 30.04 (NHCH$_2$*C*H$_2$), 32.39 (*C*H$_2$CH), 41.00 (NH*C*H$_2$), 44.76 (Ph*C*H$_2$NH), 52.88 (COO*C*H$_3$), 54.23 (*C*H), 67.48 (COO*C*H$_2$Ph), 127.63, 127.84, 128.54, 128.68, 128.79, 129.02 (Ar*C*), 136.65, 139.99 (Ar*C*CH$_2$), 156.73 (NH*C*ONH), 159.14 (NH*C*OO), 173.48 (*C*OOCH$_3$); *m*/*z*(EI) 427 (M^+, 10%), 294 (6, [M-PhCH$_2$NCO]$^+$), 217 (8, NH$_2$(CH$_2$)$_4$CH(COOCH$_3$)NHCOOCH$_2^+$), 164 (6, PhCH$_2$NHCONHCH$_3^+$), 148 (16, PHCH$_2$NHCON$^+$), 133 (3, PhCH$_2$NCO$^+$), 106 (53, PhCH$_2$NH$^+$) and 91 (100, PhCH$_2^+$).

Attempted oxidation of benzyl isothiocyanate in the presence of CBZ-lysine methyl ester

N-α-CBZ-L-Lysine methyl ester (1.25 g, 4.26 mmol) was added to DMD (1.43 g, 19.35 mmol) dissolved in dry acetone (250 ml) before benzyl isothiocyanate (577 mg, 3.87 mmol) was added. The resulting solution was stirred under a nitrogen atmosphere, at room temperature, for 21 hours. Analysis by TLC indicated that no reaction had occurred.

7.7.6 Ozonolysis of isothiocyanates and isocyanates

Attempted ozonolysis of benzyl isothiocyanate

Benzyl isothiocyanate (1 g, 6.7 mmol) was added to dry dichloromethane (65 ml) and the resulting solution was treated with ozone initially for 15 minutes at -78 °C with stirring. Ozonolysis was then continued as before for 30 minutes then at 0 to 5 °C for 75 minutes. The reaction solution was analysed by GC/MS which showed benzaldehyde (9.5%); *m*/*z*(EI) 106 (M^+, 68%), 105 (80, [M-H]$^+$), 77 (100, Ph$^+$); BIC (2.75%) *m*/*z*(EI) as before; and BITC (76.77%).

A benzaldehyde standard was prepared by adding benzaldehyde (10 μl) to dichloromethane (1 ml) and this was analysed as before; *m*/*z*(EI) 106 (M^+, 45%), 105 (76, $[M-H]^+$) and 77 (100, Ph^+).

Attempted ozonolysis of phenyl isothiocyanate

Phenyl isothiocyanate (1 g, 7.4 mmol) was added to dry dichloromethane (70 ml) and the resulting solution was treated with ozone while stirring at -5 °C to 5 °C for 75 minutes. GC/MS analysis after this period showed phenyl isothiocyanate (90% of total); *m*/*z*(EI) 135 (M^+, 90%), 133 (3, $[M-S]^+$), 77 (100, Ph^+). In addition a small amount of phenyl isocyanate was present (3% of total); *m*/*z*(EI) 119 (M^+, 100%), 91 (77, Ph^+).

Attempted ozonolysis of benzyl isocyanate

Benzyl isocyanate (892 mg, 6.7 mmol) was added to dry dichloromethane (70 ml) and the resulting solution was treated with ozone at 0 °C for 1 hour with stirring. GC/MS analysis showed benzyl isocyanate (68% of total) and benzaldehyde (3% of total); *m*/*z*(EI) as given previously.

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APPENDIX

APPENDIX A NOESY SPECTRA

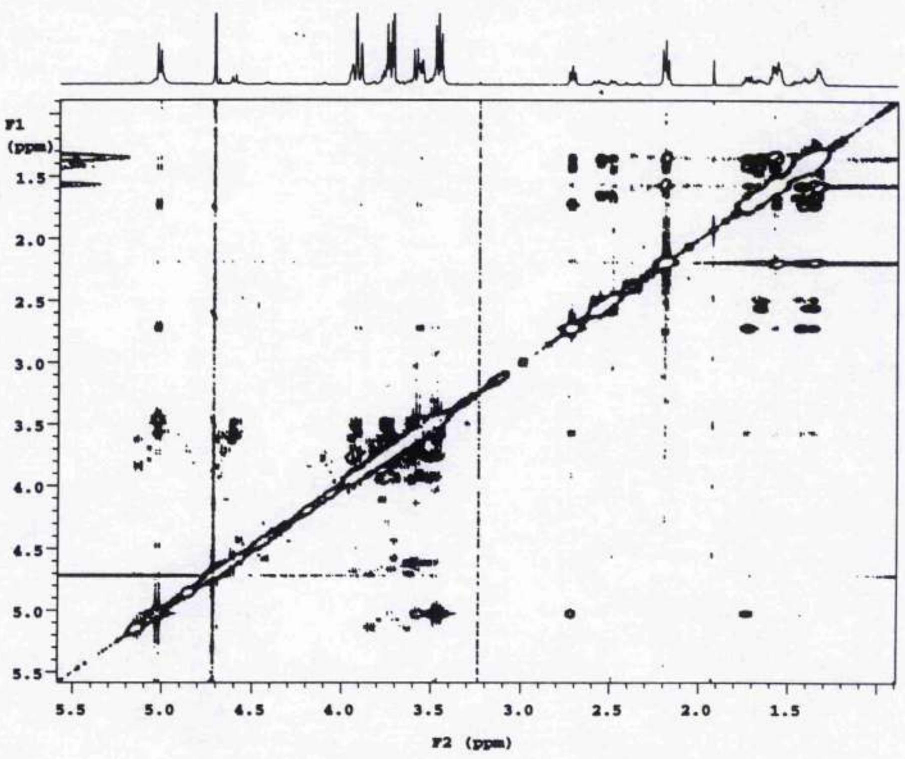

Figure 34 NOESY spectrum of (7-methoxycarbonylheptyl) glucosinolate.

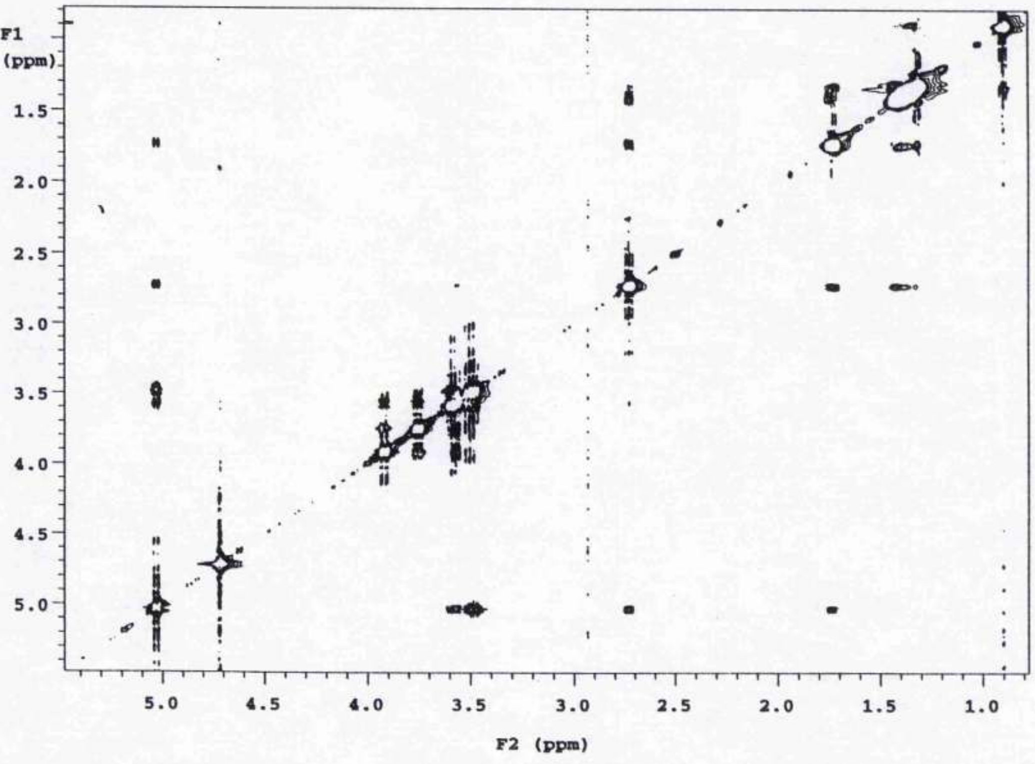

Figure 35 NOESY spectrum of heptyl glucosinolate.

APPENDIX B

STATISTICAL EVALUATION OF ELECTROPHYSIOLOGICAL DATA.

Effect: stimulus
Significance Level: 5 %

	Mean Diff.	Crit. Diff	P-Value	
KCl, JMB-15	-50.222	31.363	<.0001	S
KCl, NED 074R2	-58.111	31.363	<.0001	S
KCl, NED 083	-55.444	31.363	<.0001	S
KCl, NED 086R	-52.444	31.363	<.0001	S
KCl, NED 093	-30.667	31.363	.0006	
KCl, NED 094	-35.000	31.363	.0001	S
KCl, NED 102R	-6.000	31.363	.4927	
KCl, NED 110	.333	31.363	.9698	
KCl, gluco-erucine	-34.986	32.328	.0002	S
KCl, gluco-raphenine	-44.361	32.328	<.0001	S
KCl, gluco-epiprogoitrine	-25.486	32.328	.0054	
KCl, gluco-sinalbine	-25.611	32.328	.0052	
KCl, gluco-tropaeoline	-42.111	32.328	<.0001	S
KCl, gluco-napine	-34.111	32.328	.0002	S
JMB-15, NED 074R2	-7.889	31.363	.3674	
JMB-15, NED 083	-5.222	31.363	.5503	
JMB-15, NED 086R	-2.222	31.363	.7993	
JMB-15, NED 093	19.556	31.363	.0268	
JMB-15, NED 094	15.222	31.363	.0835	
JMB-15, NED 102R	44.222	31.363	<.0001	S
JMB-15, NED 110	50.556	31.363	<.0001	S
JMB-15, gluco-erucine	15.236	32.328	.0927	
JMB-15, gluco-raphenine	5.861	32.328	.5155	
JMB-15, gluco-epiprogoitri ...	24.736	32.328	.0060	
JMB-15, gluco-sinalbine	24.611	32.328	.0072	
JMB-15, gluco-tropaeoline	8.111	32.328	.3686	
JMB-15, gluco-napine	16.111	32.328	.0756	
NED 074R2, NED 083	2.667	31.363	.7602	
NED 074R2, NED 086R	5.667	31.363	.5170	
NED 074R2, NED 093	27.444	31.363	.0021	
NED 074R2, NED 094	23.111	31.363	.0092	
NED 074R2, NED 102R	52.111	31.363	<.0001	S
NED 074R2, NED 110	58.444	31.363	<.0001	S
NED 074R2, gluco-erucine	23.125	32.328	.0114	
NED 074R2, gluco-raphenine	13.750	32.328	.1287	
NED 074R2, gluco-epiprogoi ...	32.625	32.328	.0004	S
NED 074R2, gluco-sinalbine	32.500	32.328	.0004	S
NED 074R2, gluco-tropaeoli ...	16.000	32.328	.0776	
NED 074R2, gluco-napine	24.000	32.328	.0067	
NED 083, NED 086R	3.000	31.363	.7314	
NED 083, NED 093	24.778	31.363	.0053	
NED 083, NED 094	20.444	31.363	.0207	
NED 083, NED 102R	49.444	31.363	<.0001	S
NED 083, NED 110	55.778	31.363	<.0001	S
NED 083, gluco-erucine	20.458	32.328	.0247	
NED 083, gluco-raphenine	11.083	32.328	.2200	
NED 083, gluco-epiprogoitri ...	29.958	32.328	.0012	
NED 083, gluco-sinalbine	29.833	32.328	.0012	
NED 083, gluco-tropaeoline	13.333	32.328	.1406	
NED 083, gluco-napine	21.333	32.328	.0193	
NED 086R, NED 093	21.778	31.363	.0139	
NED 086R, NED 094	17.444	31.363	.0478	
NED 086R, NED 102R	46.444	31.363	<.0001	S
NED 086R, NED 110	52.778	31.363	<.0001	S
NED 086R, gluco-erucine	17.458	32.328	.0545	
NED 086R, gluco-raphenine	8.083	32.328	.3703	
NED 086R, gluco-epiprogoit ...	26.958	32.328	.0033	
NED 086R, gluco-sinalbine	26.833	32.328	.0035	
NED 086R, gluco-tropaeoline	10.333	32.328	.2526	
NED 086R, gluco-napine	18.333	32.328	.0436	
NED 093, NED 094	-4.333	31.363	.6201	
NED 093, NED 102R	24.667	31.363	.0055	
NED 093, NED 110	31.000	31.363	.0005	
NED 093, gluco-erucine	-4.319	32.328	.6317	
NED 093, gluco-raphenine	-13.694	32.328	.1303	
NED 093, gluco-epiprogoitri ...	5.181	32.328	.5654	
NED 093, gluco-sinalbine	5.056	32.328	.5748	
NED 093, gluco-tropaeoline	-11.444	32.328	.2054	
NED 093, gluco-napine	-3.444	32.328	.7022	
NED 094, NED 102R	29.000	31.363	.0012	
NED 094, NED 110	35.333	31.363	<.0001	S
NED 094, gluco-erucine	.014	32.328	.9988	
NED 094, gluco-raphenine	-9.361	32.328	.2997	
NED 094, gluco-epiprogoitri ...	9.514	32.328	.2919	
NED 094, gluco-sinalbine	9.389	32.328	.2983	
NED 094, gluco-tropaeoline	-7.111	32.328	.4304	
NED 094, gluco-napine	.889	32.328	.9214	
NED 102R, NED 110	6.333	31.363	.4690	
NED 102R, gluco-erucine	-28.986	32.328	.0016	
NED 102R, gluco-raphenine	-38.361	32.328	<.0001	S
NED 102R, gluco-epiprogoit ...	-19.486	32.328	.0322	
NED 102R, gluco-sinalbine	-19.611	32.328	.0311	
NED 102R, gluco-tropaeoline	-36.111	32.328	.0001	S
NED 102R, gluco-napine	-28.111	32.328	.0022	
NED 110, gluco-erucine	-35.319	32.328	.0001	S
NED 110, gluco-raphenine	-44.694	32.328	<.0001	S
NED 110, gluco-epiprogoitri ...	-25.819	32.328	.0048	
NED 110, gluco-sinalbine	-25.944	32.328	.0047	
NED 110, gluco-tropaeoline	-42.444	32.328	<.0001	S
NED 110, gluco-napine	-34.444	32.328	.0002	S
gluco-erucine, gluco-raphen ...	-9.375	33.266	.3128	
gluco-erucine, gluco-epipro ...	9.500	33.266	.3064	
gluco-erucine, gluco-sinalbi ...	9.375	33.266	.3128	
gluco-erucine, gluco-tropae ...	-7.125	33.266	.4426	
gluco-erucine, gluco-napine	.875	33.266	.9248	
gluco-raphenine, gluco-epip ...	18.875	33.266	.0435	
gluco-raphenine, gluco-sina ...	18.750	33.266	.0449	
gluco-raphenine, gluco-trop ...	2.250	33.266	.8082	
gluco-raphenine, gluco-napine	10.250	33.266	.2700	
gluco-epiprogoitrine, gluco- ...	-.125	33.266	.9892	
gluco-epiprogoitrine, gluco- ...	-16.625	33.266	.0748	
gluco-epiprogoitrine, gluco- ...	-8.625	33.266	.3529	
gluco-sinalbine, gluco-tropa ...	-16.500	33.266	.0770	
gluco-sinalbine, gluco-napine	-8.500	33.266	.3599	
gluco-tropaeoline, gluco-na ...	8.000	33.266	.3887	

Comparisons in this table are not significant unless the

corresponding p-value is less than ,0005.

APPENDIX C RESEARCH PUBLICATIONS

Oxidation of Isothiocyanates to Isocyanates using Dimethyldioxirane; Relevance to Biological Activity of Isothiocyanates†

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Oxidation of Isothiocyanates to Isocyanates using Dimethyldioxirane; Relevance to Biological Activity of Isothiocyanates†

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The reaction of organic isothiocyanates with dimethyldioxirane in acetone produces isocyanates in good yields, trapped out as the ureas by reaction with isopropylamine.

Organic isothiocyanates are widely distributed in plants, including cruciferous vegetables[1] such as Brussels sprouts, cauliflower and broccoli. They in fact exist as glucosinolates[2] **1** and are only released by the action of the enzyme myrosinase, when the plant is damaged or cooked. The occurrence of isothiocyanates in the diet means that there is considerable interest in their biological activities. Some, including benzyl isothiocyanate **2** and phenethyl isothiocyanate, two of the most commonly occurring compounds, have anti-carcinogenic properties. Thus administration of benzyl isothiocyanate prior to treatment with the carcinogen *N*-nitrosodiethylamine inhibited tumour formation in A/J mice,[3] while phenethyl isothiocyanate inhibits induction of lung tumourigenesis by NNK [4-(methylnitrosamino)-1-(3-pyridyl)butan-1-one] in the forestomach and lung of mice.[4]

Myrosinase → $PhCH_2N{=}C{=}S$

Scheme 1

Two potential mechanisms for this anti-carcinogenic action have been proposed. Firstly, the isothiocyanates have been shown to induce phase 2 detoxification enzymes.[5] Increased levels of these enzymes result in more rapid removal of the carcinogenic species. Secondly, the isothiocyanates have also been found to inhibit metabolic activation of the carcinogen.[6] For example, NNK requires α-hydroxylation by cytochrome *P*-450 enzymes in order to produce the active carcinogen. Phenethyl isothiocyanate specifically inactivates the *P*-450 enzymes responsible for this hydroxylation thus preventing activation. The relative importance of these two effects is still open to debate.

The chemical mechanism of the inhibition of the cytochrome *P*-450 enzymes by isothiocyanates is not understood. Isothiocyanates are reactive compounds, readily attacked by nucleophiles. Indeed, they are often used to label proteins by reaction with free amino groups, *e.g.* using fluorescein isothiocyanate. However, cytochrome *P*-450 enzymes catalyse oxidation reactions and so it is likely that when the isothiocyanates initially interact with the enzyme they undergo an oxidation reaction, producing a more reactive species which is then responsible for the inactivation. This may also explain the specificity observed, in that there is some activation of the isothiocyanate required in order to produce the inhibitor giving an example of suicide (mechanism-based) inhibition.

The insecticides parathion **3** and malathion,[7] containing a P=S group, undergo oxidative desulfuration by mammalian liver microsomes to give a P=O group and elemental sulfur (Scheme 2). Model studies using trifluoroperacetic acid resulted in the same transformation. Furthermore, analogous conversions of thiocarbonyl groups to carbonyl groups have also been observed.[8] If a similar pathway operates for the oxidation of isothiocyanates, a much more reactive isocyanate would be produced at the enzyme active site. This could then acylate an appropriate amino acid side chain causing inactivation of the cytochrome *P*-450. There is some evidence for the conversion of 2-naphthyl isothiocyanate to 2-naphthyl isocyanate by rat liver microsomes, but only in very low yields (<0.6% conversion).[9,10] The chemical oxidation of isothiocyanates has thus been examined as a model for these biological systems.

Liver Microsomes or CF_3CO_3H ; + [S]

Scheme 2

Prior to this work there were only two previous reports of the conversion of isothiocyanates to isocyanates. In 1890 Kuhn and Lieber reported that heating an isothiocyanate with mercuric oxide at 170 °C gave *ca.* 20% of the corresponding isocyanate.[11] More recently,[12] the conversion was achieved using palladium(II) chloride in refluxing 1,4-dioxane. Good to excellent yields of isocyanates were obtained using a range of alkyl and aryl derivatives. The other product in this case was thionyl chloride. However to date no non-metal-catalysed oxidative conversion of isothiocyanates to isocyanates has been reported.

The electrophilic nature of isothiocyanates means that the oxidising agent must be carefully chosen to reduce the possibility of competing nucleophilic attack on the central carbon of the heterocumulene system. Dimethyldioxirane (DMD) thus proved to be the most appropriate reagent, as it is a very reactive non-nucleophilic oxidising agent with acetone as its only by-product.[13] When benzyl isothiocyanate was reacted with a solution of DMD[14] in dry acetone at room temperature analysis by GCMS showed complete consumption of the isothiocyanate after 15 min and only one major product. The product had an identical retention time and mass spectrum to authentic benzyl isocyanate. The identity of the compound was confirmed by addition of isopropylamine to the reaction solution, which resulted in a decrease in the peak due to the isocyanate and the appearance of a new peak, shown to be due to the 1-isopropyl-3-benzylurea by its mass spectrum. The reaction presumably proceeds *via* an oxathiirane type intermediate **4** (Scheme 3), formed *via* either initial transfer of oxygen to sulfur and cyclisation, or direct insertion of oxygen into the carbon–sulfur double bond. Similar mechanisms are proposed for the oxidation of other thiocarbonyl compounds.

A range of isothiocyanates were then reacted with DMD and the isocyanates isolated as their urea derivatives **5** following trapping with isopropylamine. The optimised yields of the 1-isopropylureas were good to excellent for a range of isothiocyanates (Table 1), including both alkyl and aryl deriva-

*To receive any correspondence.

†This is a **Short Paper** as defined in the Instructions for Authors, Section 5.0 [see *J. Chem. Research (S)*, 1997, Issue 1]; there is therefore no corresponding material in *J. Chem. Research (M)*.

Scheme 3

Table 1 Optimised yields of 1-isopropylureas obtained from the oxidation of organic isothiocyanates with DMD and trapping with isopropylamine. Reaction carried out in acetone at room temperature, with a 5-fold excess of DMD, using 1 mmol of isothiocyanate (see General Procedure)

Isothiocyanate (R—N=C=S)	Isolated yield of urea[a] 5 (%)
Benzyl	84
Phenethyl	67
Phenyl	89
Butyl	71

[a]All the products gave satisfactory spectral data.

tives. The best yields were obtained using a five-fold excess of DMD. It could be argued that the amine and isothiocyanate initially react to form the thiourea which can then be oxidised to the urea by DMD. However, examination by both GCMS and TLC, against the authentic thiourea, gave no evidence for its formation during the reaction. It was also observed that reaction of the isothiocyanates with isopropylamine was much slower than the observed oxidation of the isothiocyanate to the isocyanate. It thus seemed most likely that the reaction followed the pathway as given in Scheme 3.

However, attempts were made to isolate the isocyanates directly from the reaction solution. Unfortunately, these were hampered by both the small scale of the reactions, due to the low concentrations of DMD available, and by competing hydrolysis, illustrated by the recovery of 1,3-diphenylurea in 94% yield from the attempted isolation of phenyl isocyanate. The latter arises from the difficulty observed in obtaining sufficiently dry solutions of DMD in acetone. The presence of the free isocyanate in the reaction solution was finally demonstrated by means of FTIR studies. In acetone solution benzyl isothiocyanate gave peaks at 2169 and 2092 cm^{-1}. The addition of DMD gave rise to new peaks at 2276 and 2341 cm^{-1}. The former peak was identical to that produced by authentic benzyl isocyanate. The peak at 2341 cm^{-1} was found to be due to dissolved carbon dioxide, presumably arising from hydrolysis of the isocyanate. Indeed the hydrolysis of authentic isocyanate could be monitored in both acetone and the DMD solution. The fate of the sulfur produced in the reaction has not been elucidated. However in some of the GCMS analyses there is evidence for a species similar to dimethyl disulfide or dimethyl sulfone. The observation of such oxidised sulfur species accounts for the requirement for excess DMD in order to obtain good yields.

A number of other oxidising agents were also examined. *m*-Chloroperbenzoic acid (MCPBA) and trifluoroperacetic acid will carry out the transformation, although in both cases competing reactions lower the yields. With the MCPBA reactions, competing nucleophilic attack by *m*-chlorobenzoic acid, a contaminant and by-product, produces the amide following rearrangement and loss of CO_2. With trifluoroperacetic acid the aqueous conditions employed for reaction cause hydrolysis of the isocyanate. It was found that using equimolar amounts of both these oxidising agents the yields of benzyl isocyanate from the corresponding isothiocyanate were 25 and 5% respectively, compared to a yield of 58% with equimolar DMD. The ozonolysis of benzyl isothiocyanate was also briefly examined, in dichloromethane at 0–5 °C. In this case most of the isothiocyanate remained unchanged, with only small amounts (*ca.* 3%) of the isocyanate and some benzaldehyde (*ca.* 10%). It was not clear whether the latter came from direct reaction of the isothiocyanate or from the isocyanate.

In summary these studies have shown that organic isothiocyanates can be efficiently converted to isocyanates *via* oxidation with DMD. These observations lend credence to the proposal that a similar reaction may be catalysed by cytochrome *P*-450 enzymes, during their reaction with, and inactivation by, isothiocyanates. The non-nucleophilic nature of DMD makes it a good reagent for this transformation, although it has also been observed to much lesser extents with other oxidising agents.

Experimental

Dimethyldioxirane (DMD) was synthesised according to the method of Mello *et al.*[14] as a solution in acetone. The concentration was determined by NMR analysis of the oxidation of methyl phenyl sulfide in $CDCl_3$.[14] The GCMS analyses were carried out using a Hewlett-Packard 5890A gas chromatograph, with SGE BP1 column and a linear temperature gradient from 30 to 300 °C, attached to a Finnigan MAT Incos mass spectrometer. ^1H and ^{13}C NMR spectra were obtained using a Varian 2000 FT spectrometer (^1H, 300 MHz; ^{13}C, 75.42 MHz) and a Varian Gemini FT spectrometer (^1H, 200 MHz; ^{13}C, 50.31 MHz). FTIR spectra were recorded on a Perkin Elmer 1710 spectrophotometer.

General Procedure.—In a typical reaction phenyl isothiocyanate (150 mg, 1.11 mmol) was added to a solution of DMD (440 mg, 6.0 mmol) in dry acetone (180 ml) and stirred under nitrogen at room temperature for 15 min. Analysis by GCMS indicated the reaction was complete and isopropylamine (1.12 g, 18.9 mmol) was added at 0 °C. This mixture was then stirred under nitrogen for 1.5 h prior to filtration and concentration under reduced pressure. The crude product was purified by column chromatography on silica, eluting with ethyl acetate–light petroleum (bp 40–60 °C) (30:70). This afforded the product as a white solid (200 mg, 89%), mp 160 °C (lit.,[15] 156 °C); ν_{max} (nujol)/cm^{-1} 3350 (NH), 1650 (CO), 700, 750 (Ph); δ_H (200 MHz; $CDCl_3$) 1.23 [6 H, d, *J* 7 Hz, $CH(CH_3)_2$], 3.97 [1 H, m, $CH(CH_3)_2$], 5.53 (1 H, d, *J* 7 Hz, NHCH), 6.97–7.32 (5 H, m, ArH), 7.54 (1 H, br s, ArNH); δ_C (50.31 MHz; $CDCl_3$) 23.68 [$CH(CH_3)_2$], 42.47 [$CH(CH_3)_2$], 120.59, 123.42, 129.54 (aromatics), 139.63 (quat. aromatic), 156.38 (CO); *m/z* (EI) 178 (M^+, 13%), 119 (2, $PhNCO^+$), 93 (100, $PhNH_2^+$), 77 (4, Ph^+).

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