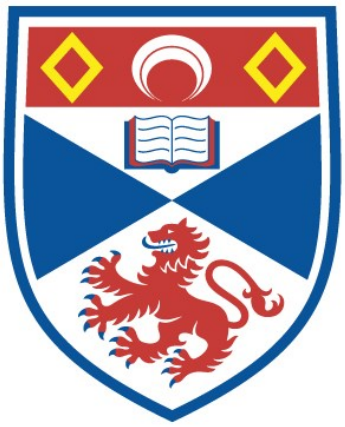
CHEMICAL MODIFICATION OF 5-AMINOLEVULINIC ACID FOR IMPROVED PHOTODYNAMIC THERAPY

Elaine Hilary Brown

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Chemical Modification of 5-Aminolevulinic Acid for Improved Photodynamic Therapy

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Elaine Hilary Brown

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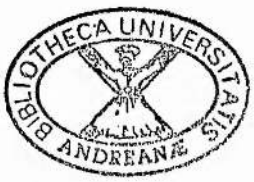

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Abbreviations

ALA	5-aminolevulinic acid
ALA.HCl	5-aminolevulinic acid hydrochloride salt
ALA-OBn	5-aminolevulinic acid benzyl ester
ALA-OEt	5-aminolevulinic acid ethyl ester
ALA-OMe	5-aminolevulinic acid methyl ester
ALA-OHex	5-aminolevulinic acid hexyl ester
ALA-PDT	photodynamic therapy using 5-aminolevulinic acid induced protoporphyrin IX as the photosensitiser
Arklone	1,1,2-trichloro-1,2,2-trifluoroethane, a safer substitute for carbon tetrachloride
Bn	benzyl
b.p.	boiling point
BPD	benzoporphyrin derivative
bs	broad singlet
C^2HCl_3	deuterated chloroform
c-HCl	concentrated hydrochloric acid
CHME	1-cyclohexyl-3,2-morpholinoethyl-p-toluene sulfonate, a peptide coupling agent similar to DCC
CoA	co enzyme A
COSY	correlated spectroscopy, a two dimensional NMR experiment which indicates all spin-spin coupled protons in one spectrum
CP94	1,2-diethyl-3-hydroxypyridin-4-one, an iron chelator
d	doublet
DCC	1,3-dicyclohexyl carbodiimide
DCM	dichloromethane

DHE	dihaematoporphyrin ether
DMF	dimethylformamide
DMSO	dimethylsulfoxide
$^2H_6$-DMSO	deuterated dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetate
EI	elemental ion
Et	ethyl
g	grams
gc	gas chromatography
GMP	guanosine monophosphate
H_2	hydrogen gas
HCl	hydrochloric acid
Hex	hexyl
HIV	human immunodeficiency virus
^2H_2O	deuterated water
Hp	haematoporphyrin
HpD	haematoporphyrin derivative
HSV	herpes simplex virus
HTLV	human T cell leukemic virus
IBC	isobutyl chloroformate
J	coupling constant
KCl	potassium chloride
Lit.	literature value for a melting point
m	multiplet

M	molar
MACE	chlorin consisting of a monoamide with L-aspartic acid
$MgSO_4$	dried magnesium sulfate
mm/Hg	millimetres of mercury
mmol	millimoles
mol	moles
m.p.	melting point
mTHPC	meso-tetrakis(m-hydroxyphenyl)-chlorin, a photosensitiser commercially known as Foscan
N_2	nitrogen gas
NaCl	sodium chloride
n-BuLi	n-Butyl Lithium
$NaHCO_3$	sodium hydrogen carbonate
NaOH	sodium hydroxide
NMM	N-methyl morpholine
NMR	nuclear magnetic resonance spectroscopy
NO	nitric oxide
PII	Photofrin II (porfimer sodium)
PBG	porphobilinogen
PBG synthase	porphobilinogen synthase, the enzyme that coverts two molecules of 5-aminolevulinic acid to porphobilinogen, sometimes known as 5-aminlevulinic acid dehydratase
Pd/C	palladium on charcoal catalyst
PDD	photodynamic detection
PDT	photodynamic therapy
PDV	photodynamic destruction of viruses

P_2O_5	phosphorous pentoxide
PpIX	protoporphyrin IX
PUVA	clinical treatment using a psoralen and UVA light
s	singlet
t	triplet
TB	tuberculosis
t-Boc	tertiary butoxycarbonyl
tlc	thin layer chromatography
THF	tetrahydrofuran
TMEDA	tetramethylethylenediamine
TMS	tetramethylsilane
UV	ultra-violet
Z	carbobenzyloxy

Derivatives of 5-Aminolevulinic Acid

ALA1 / Z-Gly-ALA-OEt
carbobenzyloxyglycinyl-5-aminolevulinic acid ethyl ester

ALA2 / Z-L-Phe-ALA-OEt
carbobenzyloxy-L-phenylalanyl-5-aminolevulinic acid ethyl ester

ALA3 / N-Phthal-ALA-Gluc-OAc
N-phthalimido-5-aminolevulinyl-glucosamine-tetraacetate

ALA4 / N-Phthal-ALA
N-phthalimido-5-aminolevulinic acid

ALA5 / N,N-Dimethyl-ALA
N,N-dimethyl-5-aminolevulinic acid

ALA6 / N-Pent-ALA
N-pentanoyl-5-aminolevulinic acid

ALA7 / N-Hex-ALA
N-hexanoyl-5-aminolevulinic acid

ALA8 / N-Hep-ALA
N-heptanoyl-5-aminolevulinic acid.

ALA9 / N-Ac-ALA
N-acetyl-5-aminolevulinic acid

ALA10 / Z-Gly-ALA-OHex
carbobenzyloxyglycinyl-5-aminolevulinic acid hexyl ester

ALA11 / Z-D-Phe-ALA-OEt
carbobenzyloxy-D-phenylalanyl-5-aminolevulinic acid ethyl ester

ALA12 / N-But-ALA
N-butanoyl-5-aminolevulinic acid.

Abstract

5-Aminolevulinic acid (ALA), one of the body's naturally occurring molecules in the biosynthetic pathway to haem, is being used in Photodynamic Therapy (PDT), a new approach to the treatment of cancer.

It is a highly reactive molecule, which, upon standing in solution, dimerises to give 2,5-di-(β-carboxyethyl) pyrazine. This reaction, and other similar ones, have been studied in some detail as it remains a major problem in the clinic, lowering the dose of active drug being administered and forming a molecule of which the toxicology is not known.

The initial product of dimerisation is a dihydropyrazine, which is immediately oxidised in air to give a pyrazine. The chemistry of dihydropyrazines has also been investigated.

The chemical synthesis of ALA itself has been optimised and derivatives of the molecule have been prepared to try to prevent this dimerisation from occurring and, potentially, increase the tissue selectivity of the drug. Twelve derivatives of ALA have been prepared and biologically tested for their activity in PDT.

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Chapter 5 The Chemistry of Dihydropyrazines

Chapter 6 Synthesis of Derivatives of 5-Aminolevulinic Acid

Chapter 7 Biological Testing of Derivatives of 5-Aminolevulinic Acid

Chapter 8 Experimental

Chapter 1

Introduction

1.1 General Introduction

In the UK over 190 000 new skin tumours are diagnosed every year. This figure is rising, as is the death rate from them.[101] Two thousand four hundred new cases of oral cancer are reported each year in the UK alone and the prognosis is no better than it was forty years ago.[102] Lung cancer is the main cause of cancer deaths in the Western World and, for the majority of patients, conventional treatment fails and tumours continue to progress.[103] Cancer causes more deaths among children under 15 years of age than any other disease, and over half the deaths of people aged less than 65. Most of the diseases that were common killers in the nineteenth and early twentieth centuries, such as leprosy, bubonic plague (black death) and tuberculosis are now curable and have been more or less eradicated. Cancer still remains a major problem.[104] Currently, there are three main approaches to the treatment of cancer. These are radiotherapy, chemotherapy and surgical excision, all of which are harsh. Radiotherapy and chemotherapy are non-selective, killing cells other than tumour cells, and causing many further side effects. Surgical excision is not possible in many cases, but where it is it leads to severe mutilation.[105]

A relatively new and promising approach in the treatment of cancer is photodynamic therapy (PDT) and involves the use of a drug which is activated by light. It is an area where much research is being targeted. Many light sensitive drugs, known as photosensitisers, are being tested for potential use in areas such as dermatology and gynaecology, but the majority of work is aimed at the selective detection and destruction of cancerous tumours.

1.2 What is Cancer?

Any multicellular organism can contract cancer and it is not a recently discovered disease. Ancient Egyptians in 1600 BC were aware of tumours and documented them in hieroglyphics. Autopsies on mummies have shown bone lesions and cancerous growths have been found even in dinosaur bones.

It was Hippocrates who first used the term 'carcinoma' to describe a growth which spread and then destroyed a patient. In the nineteenth century, scientists such as Pasteur were researching the topic but believed that tumours were infectious. By 1914, however, it was known that certain groups of chemicals caused cancer. Today we know that both chemicals, and other infectious agents can cause malignant tumours to develop.

The words 'cancer' and 'tumour' are used commonly to refer to cancer. The medical term is 'neoplasm'. Tumours can be either benign or malignant. Benign tumours do not metastasise, i.e. they do not have secondary growths originating from the primary tumour. They are encapsulated, or contained, and grow very slowly. Malignant tumours are much more dangerous as they do metastasise. They are not contained in one small area but rapidly spread to other parts of the body. Cancer is, therefore, defined as a malignant neoplasm. The term tumour is very general and refers to any abnormal mass of tissue. Neoplasms are tumours but so are any other swellings or lumps, e.g. scars or bruises.[104]

1.3 The History of Photodynamic Therapy

The use of light in medicine originated in Ancient Greece 4000 years ago. Plants containing psoralins (Fig. 1.1) were ingested and, when activated by sunlight, brought about repigmentation of the skin.[106]

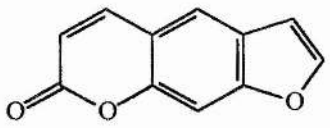

Figure 1.1 - A psoralin (7H-furo[3,2-g][1]benzopyran-7-one)

The Greek physician Herodotus first documented the healing property of light and named it Heliotherapy.[107] This 4000 year old treatment, now known as PUVA (Psoralin and UVA light) is still used today for vitilago, psoriasis, acne, eczema, herpes simplex virus (HSV) and a wide range of other dermatological diseases.[106, 108.]

In the 1890s Finsen treated *lupus vulgaris,* a tubercular skin complaint (TB) with light from a carbon arc lamp. Queen Alexandra helped him to establish the treatment in the UK at the London Hospital in Whitechapel, where they had a light department by the early 1900s. Finsen won the Nobel prize in 1903 and the original light source is now on display in London's Science Museum.[109]

The most promising areas of photomedicine, however, involve the use of a combination of light and a photosensitising drug. It was believed, in the nineteenth

century, that all effective drugs were coloured.[106] Even now, the most effective sensitisers are porphyrins, the word coming from the Greek, *Πορφυροσ* (porphuros), meaning purple. Two years after X-rays were discovered at the turn of the twentieth century, Raab found that acridine dye (Fig. 1.2), in the presence of light, killed Paramecia cells. This was the first published example of a chemical showing a photosensitising effect.[110]

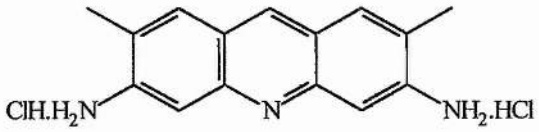

Figure 1.2 - An acridine dye (acridine yellow G)

A few years later, in 1903, the first clinical example of photodynamic therapy involved the treatment of a 70 year old woman with a facial tumour. Von Tappeiner and Jesionek used a 5 % solution of eosin dye (Fig. 1.3).

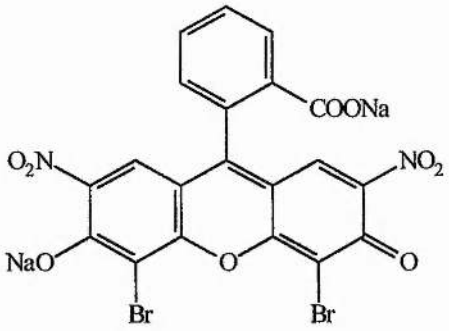

Figure 1.3 - An eosin dye (4'5'dibromo-2'7'-dinitrofluorescein)

This was painted onto the growth at intervals over a two month period. During this time the patient was exposed to both sunlight and light from a lamp. This resulted in regression of the tumour and very little scarring. Although they

showed some positive results, these original photosensitisers, eosin and acridine, are no longer used due to their carcinogenic nature.[111] The term 'photodynamic therapy' was introduced by von Tappeiner. It arises from the German term *photodynamische Wirkung* and describes the damage of living tissue using a drug and light.[109] Von Tappeiner also discovered the necessity for oxygen in a photodynamic reaction.[108]

The first use of porphyrins in PDT was demonstrated by Meyer-Betz in 1913. He injected himself with 200 mg of haematoporphyrin (Hp) (Fig. 1.4) and felt no ill-effects until he spent ten minutes in the sunlight. This caused him to suffer extreme swelling and a painful, disfiguring oedema. He remained sensitive to light for several months. [106, 110, 112] It is, therefore, not surprising that PDT has made little progress until recently.

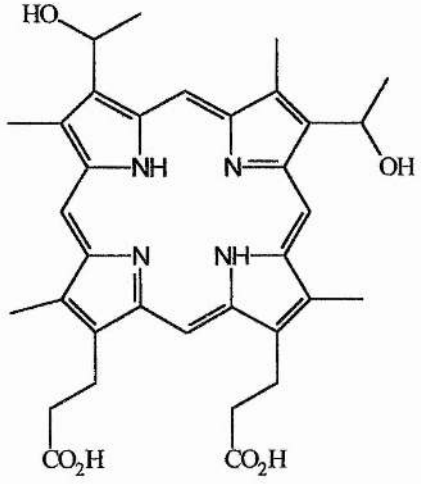

Figure 1.4 - Haematoporphyrin (Hp).

Policard, in 1925, studied the ability of porphyrins to produce a phototoxic reaction.[112] Auler and Banzer in 1942 discovered that Hp accumulated to a

greater extent in tumour cells than healthy ones[106] and this led to the idea of using porphyrins for cancer therapy.[110]

Work in the early 1950s by Schwartz showed that Hp, itself, was not the cause of the long term effects in Meyer-Betz's self-experiment. They were due to oligomeric mixtures in the material which had been extracted from blood. He formed a compound known as haematoporphyrin derivative (HpD)[112] which was shown to have a better tumour localising effect than haematoporphyrin itself.

Single case studies of HpD as a cancer treatment began in the early 1970s,[112] but it was not until 1976 that the first systematic studies on PDT in the clinic were initiated by Dougherty at the Rosewell Park Cancer Institute, Buffalo, USA.[107] He found that further purification of HpD by gel exclusion chromatography removed the monomers leaving an oligomeric substance now known as Photofrin II® (PII).[106] PII has regulatory approval from health boards in the US, Canada, Japan and Europe for use in PDT. Many new second generation photosensitisers are undergoing clinical trials and PDT has become a fourth option for the treatment of cancer. Interest in the area has, therefore, increased very rapidly and now over 10 000 papers have been published.

1.4 Photochemistry - How Does Photodynamic Therapy Work?

Photochemical processes in biology can be divided into two categories. The first concerns positive uses, such as photosynthesis and vision which are advantageous to the organism. The other area, which includes the photodynamic effect, can be thought of as destructive to the organism. PDT, however, can be used to our advantage.[109]

It involves a two step process. The first of these requires the introduction of a light sensitive compound, or a pro-drug into the affected area. This can be done either orally or intravenously by systemic administration. In the case of skin cancer, topical application, in a cream, is a direct form of administration. There must then be an appropriate time interval before the tumour is irradiated with light. This is to allow maximum build-up of the photosensitising agent within the tumour. Light can be delivered directly or via a fibre optic and must be at the correct wavelength to activate the drug (Fig. 1.5).

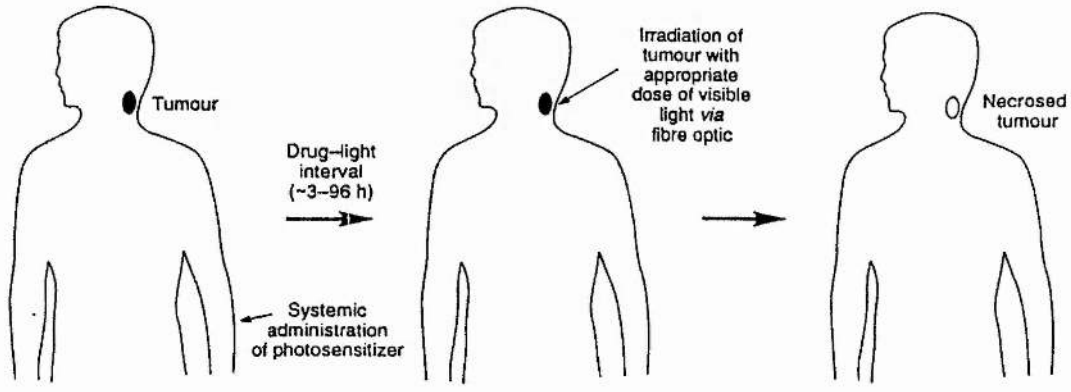

Figure 1.5 - Schematic diagram of PDT.

The light is absorbed by the photosensitiser allowing three competing processes to occur. It can fluoresce, or the activated molecule can be destroyed by a photobleaching process. The third possibility involves a type I or II photochemical reaction and this is the basis for PDT destruction in tumours (Fig. 1.6).[113]

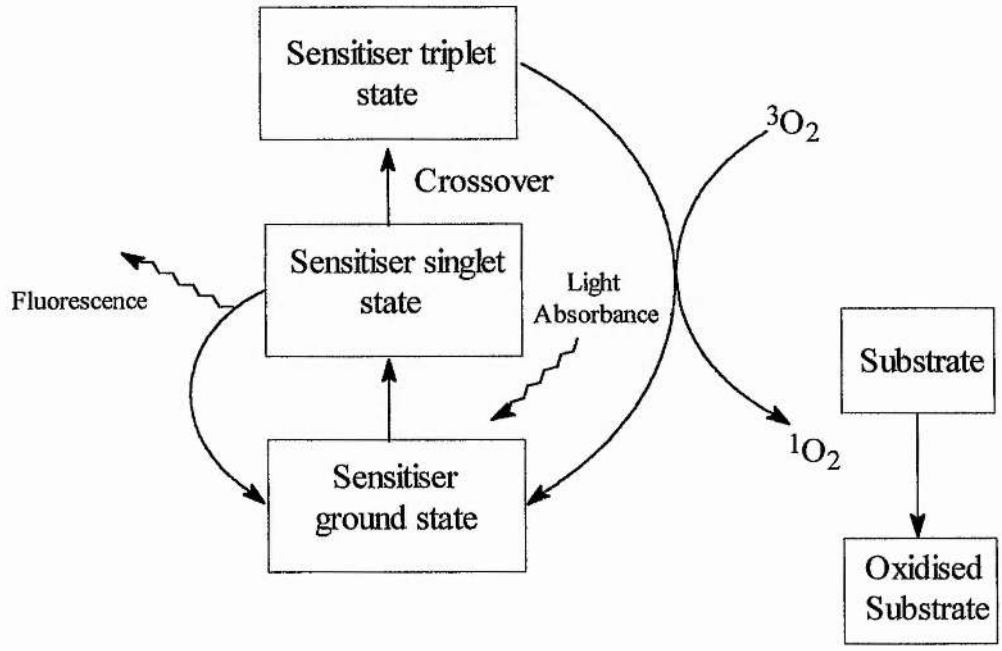

Figure 1.6 - Schematic representation of photochemistry involving a photosensitiser.

1.4.1 Fluorescence

After absorption of a photon of light, photosensitisers are converted to an excited singlet state, which is very short lived. From this state they can decay back to the ground state and, in doing so, emit light. This emission is known as fluorescence[114] and, as it is easily detected, it can be used clinically in photodynamic detection (PDD) of tumours[115] which will be discussed later in Section 1.11.

1.4.2 Photobleaching

Photobleaching effects hinder PDT and are caused by a photoinduced chemical reaction.[115] During light exposure, photosensitisers can be gradually destroyed by singlet oxygen, or other free radicals, yielding products which can no longer contribute to PDT.[114]

1.4.3 Type I and II Photochemical Processes

The excited singlet state of a photosensitiser can undergo an inter-system crossover converting it to a triplet state. This is a spin-forbidden process but, although forbidden processes are less likely to occur, a good photosensitiser will undergo the reaction readily. The sensitiser excited state relaxes by two different mechanisms, either by phosphorescence or by spin exchanging with another molecule which is in its triplet state. As phosphorescence is also a forbidden process, the triplet state can last for microseconds, so is regarded as fairly long-lived and can react with other molecules surrounding it.[112]

A type I photochemical reaction involves the interaction of this triplet state with a biologically active molecule. This occurs by either electron or hydrogen transfer and results in the formation of radicals of the sensitiser. These can react with molecular oxygen producing hydroxyl radicals, hydrogen peroxide and superoxide anions.[114] Although photosynthesis makes use of this,[106] type I reactions play a very minor role in PDT.

The major mechanism for tumour destruction is due to a type II photochemical process (Fig. 1.7).

$D + h\upsilon$	$\longrightarrow$	$D^*(S)$	absorption
$D^*(S)$	$\longrightarrow$	$D^*(T)$	intersystem crossing
$D^*(T) + O_2(T)$	$\longrightarrow$	$D(S) + O_2^*(S)$	energy transfer
$O_2^*(S)$	$\longrightarrow$	$O_2(T)$	deactivation
$O_2^*(S) + X$	$\longrightarrow$	X'	reaction

Figure 1.7 - The type II photochemical process. D is the light absorber (dye) in the ground state, D* (S) is the excited singlet state of D, D* (T) is the excited triplet state of D, O_2 (T) is the ground triplet state of O_2, O_2* (S) is oxygen in its excited singlet state and X is the target of oxidation.

This involves a spin exchange reaction directly between the excited state of the photosensitiser and triplet oxygen, the ground state of molecular oxygen. This results in the generation of the highly reactive singlet oxygen species which undergoes many chemical reactions. In biological molecules these consist mainly of oxidations and cycloadditions which are very disruptive to biological processes (Fig. 1.8).

As proteins and unsaturated lipids, the main constituents of cell membranes, contain many double bonds, they are good targets for singlet oxygen attack. Sulfur containing amino acids are oxidised to sulfoxides. Histidine and tryptophan

react to produce endoperoxides, releasing hydroxyl radicals, which react with DNA.

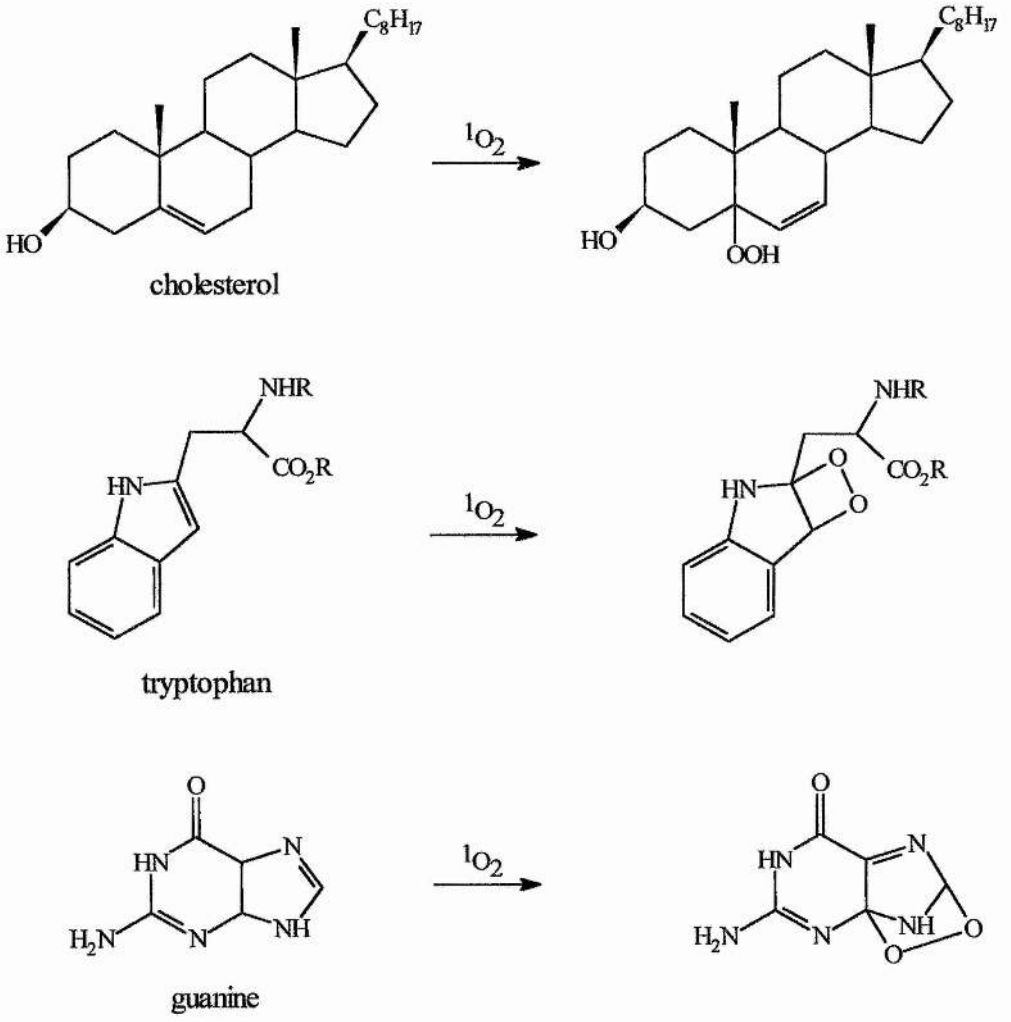

Figure 1.8 - Some chemical reactions involving biological molecules and singlet oxygen

As the photosensitiser transfers its energy to molecular oxygen it is not destroyed. It can return to its ground state to absorb another photon and repeat the cycle,[116] acting as a catalyst (Fig. 1.6).[112]

PDT, therefore, requires only a photosensitising drug, light, and the presence of molecular oxygen to destroy tumours.

1.5 Light Sources and Penetration

The ideal photosensitiser is one which can be activated at wavelengths in the red to near infrared region of the spectrum, as light of longer wavelengths penetrates deeper into the tumour (Fig. 1.9).[112] Even at this wavelength the maximum depth of tumour necrosis in most tissues is only approximately five millimetres.[113]

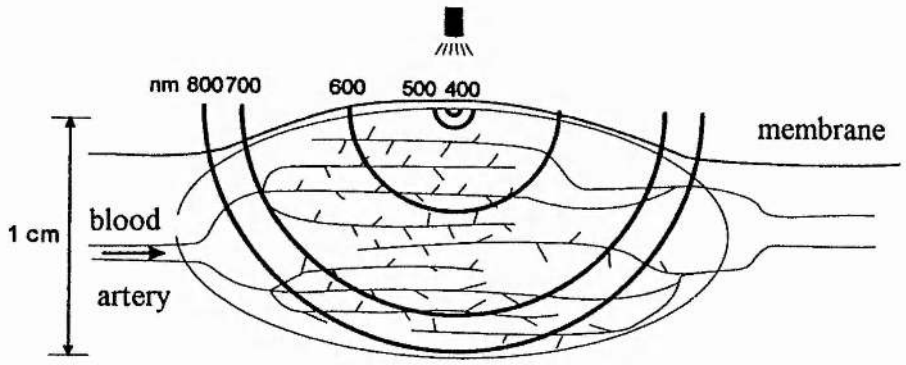

Figure 1.9 - Representation of a tumour section showing light penetration.

The exact wavelength required for photosensitisation varies from drug to drug, e.g. PII and Protoporphyrin IX (PpIX) absorb at 630 nm, phthalocyanines at 675 nm and benzoporphyrin derivative (BPD) at 690 nm.[117]

Originally, sunlight was used to activate the sensitiser but due to its low intensity at 630 nm and problems with control and delivery, it is no longer commonly applied.[118] Slide projectors and gas discharge lamps were also among the original sources used.[111] The two main categories of light delivery systems in PDT today, however, use more sophisticated wavelength filtered lamps and lasers.[118]

Lasers are the most common source of light used in the activation of photosensitisers.[108] Laser beams of a single wavelength can be delivered through single optical fibres easily, allowing light to be delivered to the tumour directly. The most commonly used lasers in PDT are argon ion- and copper vapour-pumped dye systems. These are very expensive, bulky, slow to warm up and require highly trained operators. New technology is producing laser diode arrays which are much more transportable and ready for use in a shorter time but, as yet, they cannot produce light in the 630 nm region.[107]

Extensive work is also underway in the development of non-laser light sources. Arc lamps and fluorescent bulbs are inexpensive but cannot be used with fibre optics. The light must be filtered to give the correct wavelength to maximise absorption of the photosensitiser and tissue penetration.[119]

One non-laser lamp for use in PDT which costs one twentieth of the price of a laser is being developed at the Paterson Institute in Manchester. It has been used to treat two hundred cases of skin cancer successfully using an arc of concentrated light which is cooled down then channelled through a series of optics, taking just forty-five minutes.[120]

As new photosensitisers which absorb at higher wavelengths are discovered, research into new laser and non-laser light sources is vital for PDT.[121]

1.6 Photosensitisers

1.6.1 First Generation Photosensitisers

1.6.1.1 Haematoporphyin (Hp)

Hp (Fig. 1.4) was first isolated from dried blood in 1841 and was one of the first compounds to be tested as a photosensitiser. It was thought that tumours could take up and retain the drug more readily than normal cells.[122]

Experiments in the 1950s, however, showed that it was not haematoporphyrin which caused the long-term effects that were observed, e.g. in Meyer-Betz's self-experiment. It was an oligomeric mixture of porphyrins, resulting from the extraction method used to isolate Hp from blood, that was the photosensitising agent. This mixture is now known as Haematoporphyrin derivative (HpD).[112]

1.6.1.2 Haematoporphyrin Derivative (HpD)

Schwartz prepared the first batch of HpD by treating HP with sulfuric acid in acetic acid. This acetylated the alcohol groups of Hp. He then treated the acetylated mixture with sodium hydroxide to form the oligomer known as HpD (Fig. 1.10).

HpD is a mixture of non-metallic oligomeric porphyrins, each molecule containing up to eight porphyrin building blocks.[107] In 1966, a woman with breast cancer was treated with limited success using HpD and light and this experiment was the first clinical example of the use of PDT in cancer therapy.[112] The selectivity of HpD

into tumours over normal tissue is only about two or three to one in most organs[117] but haematoporphyrin based drugs are not toxic in the absence of light and have been used to treat almost 10 000 patients.[123] The therapy has been shown to be successful. Eleven out of twelve patients suffering from cancer of the larynx who were treated with HpD showed a complete response, i.e. total regression, after only one course of PDT.[124] HpD is still one of the most frequently used photosensitisers.[107]

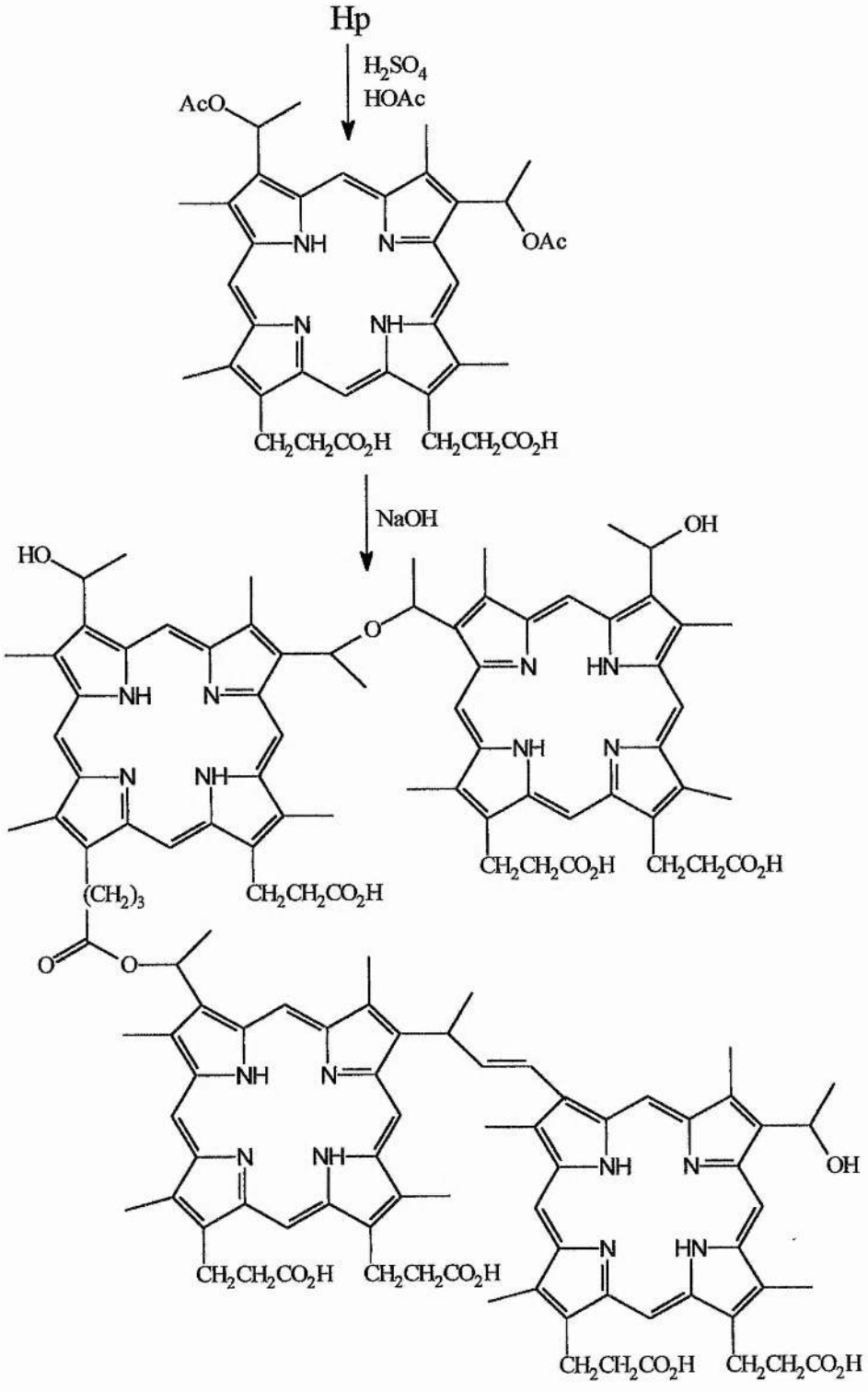

Figure 1.10 - Chemical synthesis of HpD.

1.6.1.3 Photofrin II® (Porfimer Sodium) (PII)

The active compound in HpD was isolated using gel exclusion chromatography at the beginning of the 1980s. It consists of a mixture of haematoporphyrin molecules which are linked together by ether bonds and is known as Photofrin II® or Porfimer Sodium (PII) (Fig. 1.11). It is thought to consist of dihaematoporphyrin ether (DHE) although this has not been confirmed.[119] PII is purer than HpD but still contains a complex mixture of compounds as shown by hplc traces.[112]

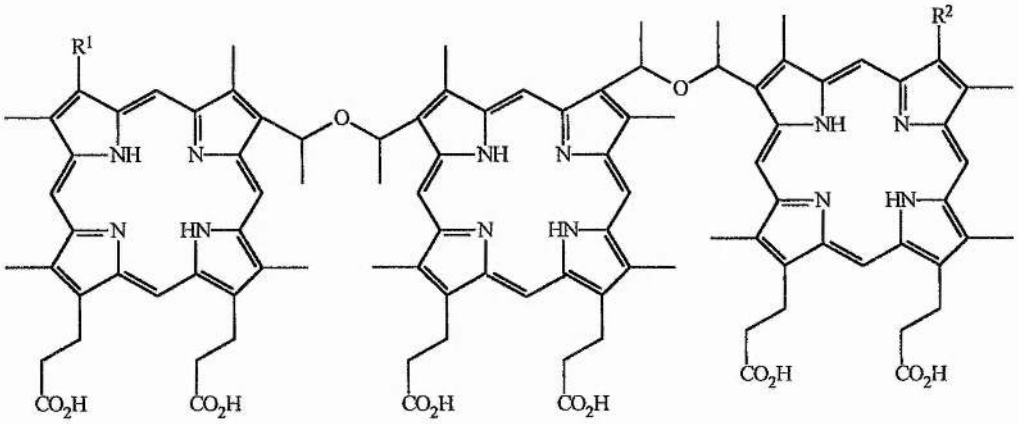

Figure 1.11 - The major component of PII. $R^1 = R^2 = CH(OH)CH_3$ or $CH{=}CH_2$

In 1993, PII gained the first health agency approval for unrestricted use in PDT for the treatment of bladder cancer in Canada.[125] It now has regulatory approval for use in lung cancer in the Netherlands[126] and oesophageal cancers in the US, Japan[127] and France but has not yet been approved for use in the UK.[128]

PII uptake into tumour tissues is comparable with HpD but, of all the currently used drugs, it can kill tumours of a greater depth.[117] PII can be very effective. It was used to treat eleven patients with mouth cancer resulting in a one hundred percent success rate.[129]

1.6.2 Second Generation Photosensitisers

The newer photosensitisers are known as second generation sensitisers and are nearly all synthesised chemically, hence their structures are known. They include the chlorins, phthalocyanines and 5-aminolevulinic acid (ALA) induced protoporphyrin IX (PpIX). These compounds are based mainly upon cyclic tetrapyrrole structures and are designed to absorb light in the red region of the spectrum.[123]

1.6.2.1 Chlorins

Chlorins are reduced, hydrophilic porphyrins which can be derived from chlorophyll A. (Fig. 1.12)[109]

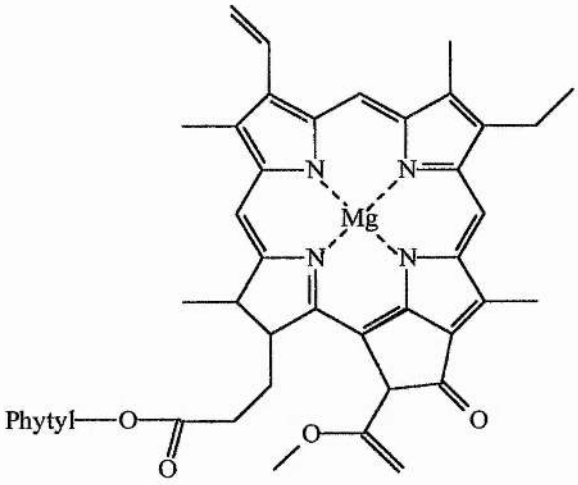

Figure 1.12 - The structure of chlorophyll A.

Phytyl = $CH_3[CH(CH_3)CH_2CH_2CH_2]_3C(CH_3)CHCH_2$

This class of sensitiser includes chlorin e_6 (Fig. 1.13a), MACE (a mono amide with L-Aspartic acid) (Fig. 1.13b) and mTHPC (meso-Tetrakis-m-hydroxyphenyl chlorin), commercially known as Foscan (Fig 1.13c). Benzoporphyrin derivative (BPD) (Fig. 1.13d) is also chlorin,[109] as are the purpurins (Fig 1.13e).[130]

As Chlorins are reduced porphyrins, they tend to oxidise. Exocyclic rings or bulky groups next to the reduced pyrrole ring are usually added to attempt to block this, e.g. the hydroxyphenyl group in Foscan.[116]

Foscan is proving to be a very active photosensitiser which can produce tumour deaths at depths of ten millimetres.[131] Phase I clinical trials are underway.[132, 133]

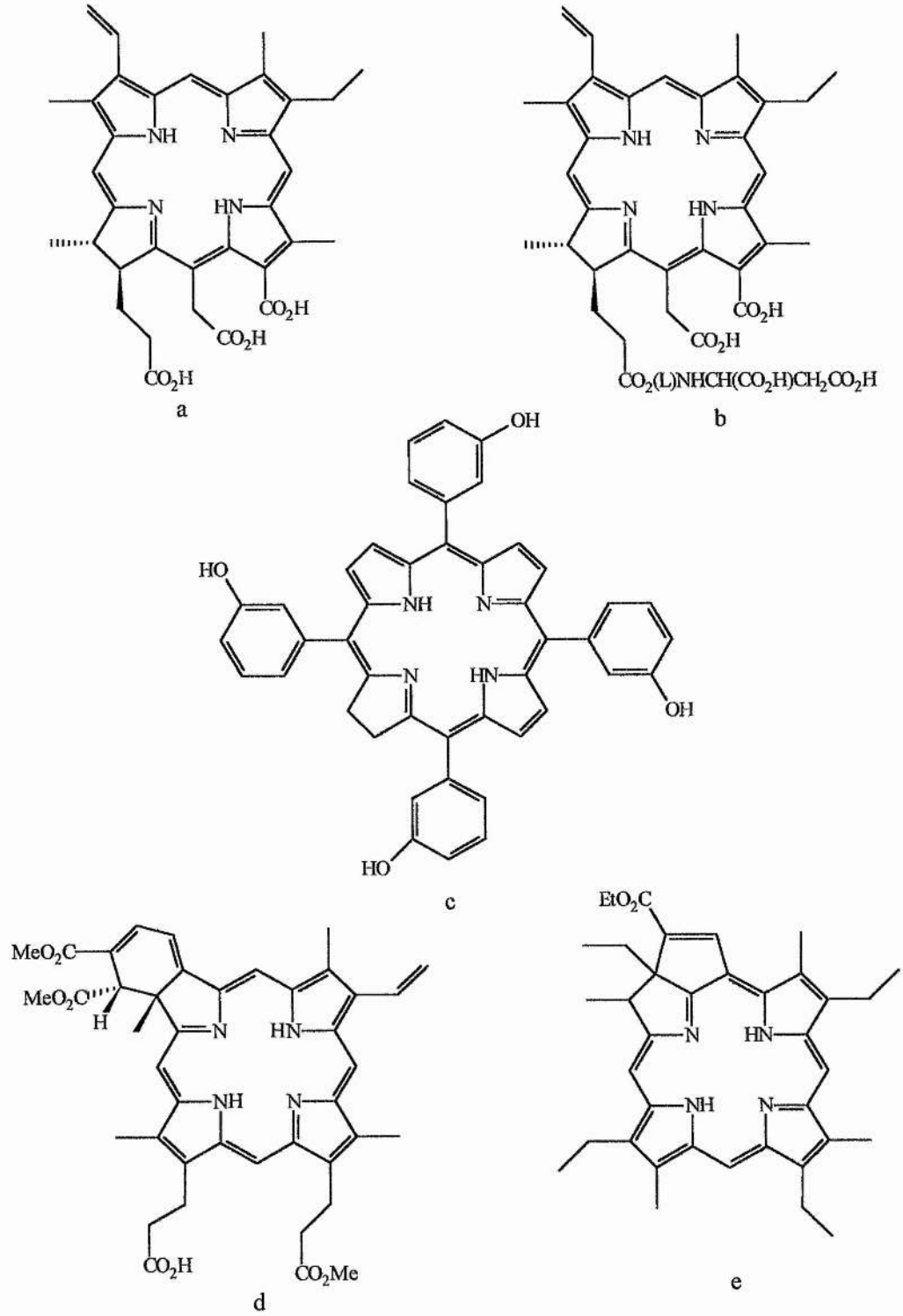

Figure 1.13 - The chlorins. a - chlorin e_6, b - MACE, c - mTHPC, d - BPD and e - a purpurin

1.6.2.2 Phthalocyanines

Phthalocyanines are synthetic porphyrins which can be chelated with metals, especially aluminium and zinc, to enhance their phototoxicity (Fig. 1.14).[130]

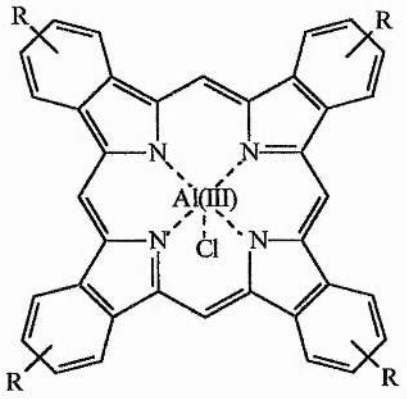

Figure 1.14 - An aluminium sulfonated phthalocyanine. R = H or SO_3H

These compounds have been used commercially, as pigments, since the 1930s but have recently been shown to exhibit photobiological activity, particularly against tumours.[109]

Phthalocyanines that are substituted with sulfonated groups become water soluble. The less sulfonated compounds, however, are more lipophilic and are better at penetrating cell membranes, making them more active derivatives.

Some phthalocyanines have tumour-to-tissue ratios of eight to one in certain cell types and have been shown to be effective photosensitisers.[130]

1.6.2.3 Other Photosensitisers

A variety of non-porphyrin based photosensitisers have been tested for potential use in PDT. These include psoralen derivatives (Fig. 1.15a), cyanines (Fig. 1.15b), phenothiazinium compounds (Fig. 1.15c) and rhodamines (Fig 1.15d). Many of these compounds are commercial dyes and stains.[134]

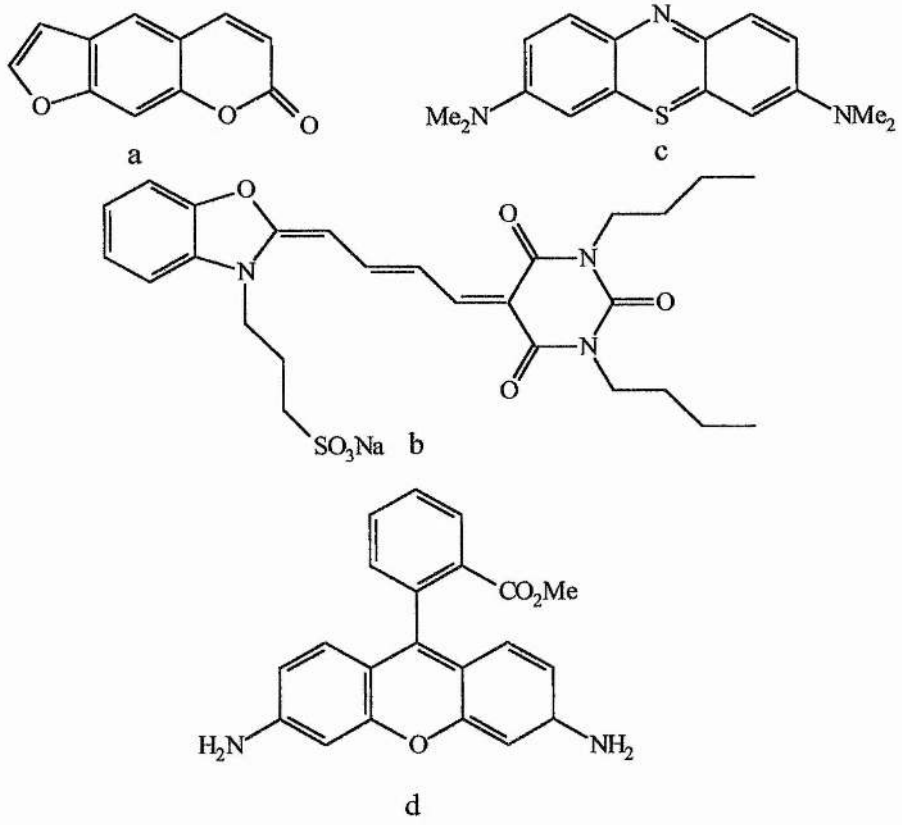

Figure 1.15 - Non-porphyrin based photosensitisers. a - psoralen, b - merocyanine MC540, c - methylene blue, a phenothiazinium and d - rhodamine Rh123

It has even been proposed that, as C_{60} and C_{70} can be converted to their triplet states during UV-irradiation, Buckminster Fullerene derivatives may be able to

cause photodynamic damage. The two fullerenes shown (Fig. 1.16) are cytotoxic to cell lines only after irradiation with light.[135]

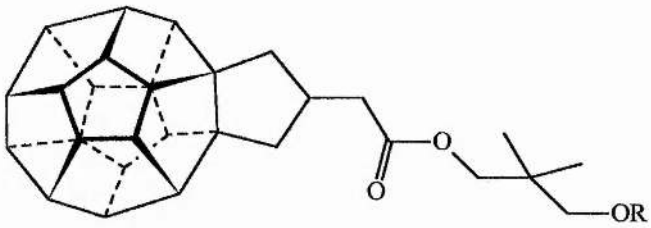

Figure 1.16 - Potential fullerine photosensitisers. R = $CO(CH_2)_2CO_2H$ or $CO(CH_2)_2CO_2H.NEt_3$

Combinations of sensitisers for PDT are being investigated to try to enhance their effects[130] but, as yet, there is no ideal photosensitiser.

1.6.3 'The Ideal Photosensitiser' and Problems Associated With Existing Drugs

There are seven requirements that a good photosensitiser should possess. Ideally

- it should be a pure compound with a reproducible synthesis.
- it should be activated at high wavelengths of light (greater than 600 nm) to enhance tissue penetration.
- in the absence of light the compound should not be toxic
- it must show some specificity for localisation in tumours over normal tissue.
- its triplet excited state must be fairly long-lived to allow time for the generation of singlet oxygen.

- Solubility in the body's tissues, or formulation to enable the drug to be delivered to the active site, is essential.
- It must be removed from the body quickly, because, if the drug is not eliminated shortly after treatment, the patient would remain photosensitive to sunlight.[116]

Unfortunately, there are drawbacks in the use of many of the commercially available photosensitisers. Skin photosensitivity is a major side effect in the use of HpD and PII for PDT. The drugs can be retained by the patient for eight to ten weeks after treatment and, during this time, they have to stay away from all bright lights, particularly sunlight, to avoid formation of a severe sunburn. Chlorins are cleared from the body within three to ten days.[108] Patients treated using Foscan are told to avoid direct sunlight for two weeks after treatment. After this time they must try test exposures on the backs of their hands for five to ten minutes to check for photosensitisation. If none occurs they can gradually increase the time they are exposed to the sun.[133] Benzoporphyrin, phthalocyanines and purpurins require lipid based delivery systems to get to the tumour site.[136] PII and HpD are mixtures of compounds. Some of them are inactive, hence, dosage is inaccurate.[137]

One of the biggest problems to overcome is the synthesis of a compound which is activated by a long enough wavelength. This is normally achieved by expanding the macrocycle to give compounds such as phthalocyanines, or by reducing one, or more, of the pyrrole rings of the porphyrin to give a chlorin.[138] The longest wavelength at which PII can be activated is less than ideal for PDT.[112]

Over the last seven years, a new approach to PDT has been investigated using 5-aminolevulinic acid (ALA). ALA itself is not a photosensitiser but is metabolised within the body to give protoporphyrin IX (PpIX) which is known to be a very effective photosensitising agent.[116] Using ALA as a pro-drug overcomes the delivery problems of PpIX, a highly insoluble molecule and, as PpIX is one of the body's natural compounds (the precursor to haem), it is metabolised quickly leaving the patient light sensitive for just a few hours. ALA induced PpIX is becoming an important area in PDT research.[139]

1.7 5-Aminolevulinic Acid Induced Protoporphyrin IX as a Photosensitiser

ALA itself is not a photosensitiser. It acts as a pro-drug, i.e. a molecule which is metabolised within the body to give the active compound. ALA is an intermediate in the synthesis of tetrapyrollic structures such as vitamin B_{12} and corrins[140] but its use in PDT is because it is a precursor in the haem biosynthetic pathway in mammals, plants and photosynthetic bacteria. It is the immediate precursor to haem, PpIX which is the photosensitising agent.

In the body, ALA is formed from glycine and succinyl CoA using ALA synthase, a pyridoxal phosphate (PLP) activated enzyme. Two molecules of ALA can then combine, in the presence of porphobilinogen synthase (PBG synthase), to give porphobilinogen (PBG). Two molecules of water are eliminated at this stage. PBG deaminase and uroporphyrinogen III cosynthetase cause four molecules of

PBG to react in a head-to-tail fashion and cyclise, yielding uroporphyrinogen III. Decarboxylation with uroporphyrinogen decarboxylase followed by oxidation catalysed by coproporphyrinogen oxidase and protoporphyrinogen oxidase form PpIX (Fig. 1.17), the active molecule in PDT. The formation of haem from PpIX involves insertion of iron into the porphyrin ring in the presence of the enzyme ferrochelatase (Fig. 1.18).[109, 141]

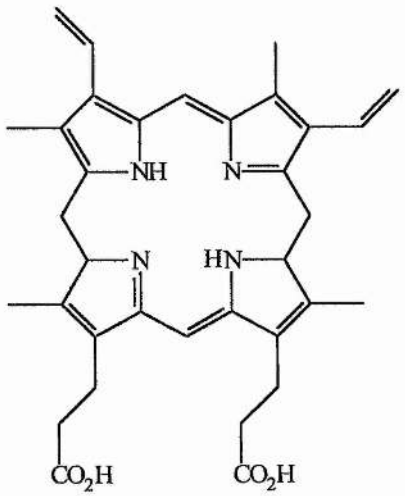

Figure 1.17 - PpIX, the active compound in ALA-PDT.

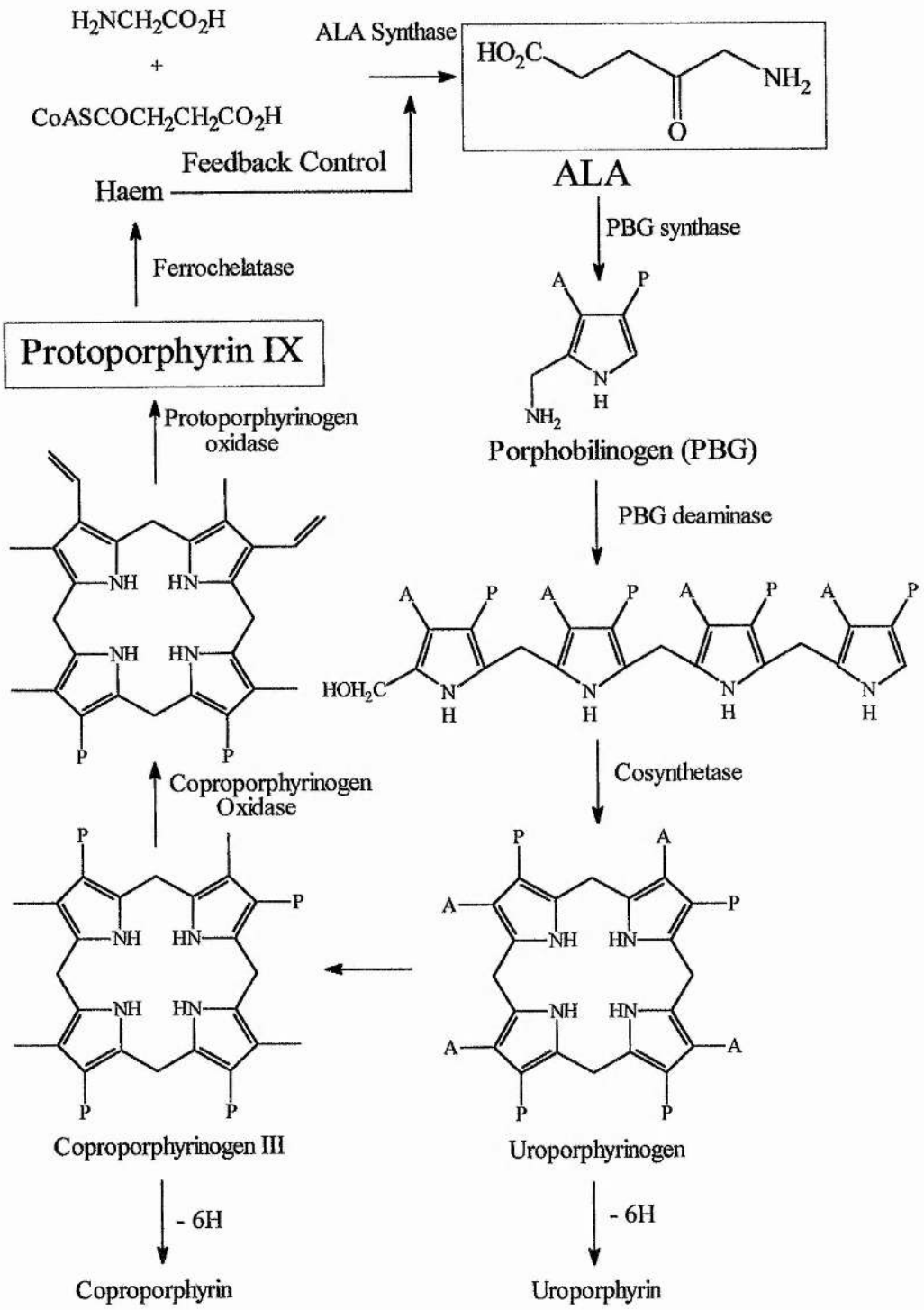

Figure 1.18 - The biosynthetic pathway to haem. A = CH_2CO_2H, P = $CH_2CH_2CO_2H$

The rate of production of PpIX is determined by the rate of synthesis of ALA which, under normal conditions, in healthy tissue, is regulated by a feedback mechanism dependant on the concentration of free haem. By adding extra ALA to the system the feedback control is by-passed and concentrations of PpIX in some cells and tissue build up. This tissue selective mechanism allows the use of ALA induced PpIX in PDT.[139]

PpIX tends to accumulate to a greater extent in abnormal tissue, i.e. preferentially in tumours rather than in healthy cells, which is advantageous. Two possible explanations for this are that cancerous cells grow more rapidly, hence high levels of PpIX are generated faster, or that different enzymes are present in cancerous growths. PpIX may accumulate due to limited capacity of ferrochelatase. PBG deaminase levels are higher in some tissues whereas ferrochelatase is lower.[101] Tumour tissues have a reduced blood flow, hence a lower oxygen content and, as oxygen is a vital reagent in the photodynamic process, this can have an effect in PDT.

1.8 Other Uses of 5-Aminolevulinic Acid

1.8.1 5-Aminolevulinic Acid as a Photodynamic Herbicide and Insecticide

ALA induced PpIX can also be used as a selective herbicide or insecticide. When sprayed with a solution of ALA certain types of plants can be destroyed after exposure to sunlight.[139] Photodynamic herbicides induce accumulation of chlorophyll precursors as well as PpIX in an analogous reaction to the biosynthesis of haem in humans. Light then causes photodamage to the plants by

formation of singlet oxygen.[142] Much of the work on the mechanisms of photodamage by porphyrins has been carried out using plants rather than animals.[143] PpIX is the immediate precursor to protohaem in both plant and animal cells and, as plants and animals share the same tetrapyrrole biosynthetic pathway from ALA to PpIX, the mechanisms for photodamage are the same.[144] These compounds have become known as "photodynamic herbicides" or "laser herbicides" and cause bleaching of the plants leaves, loss of turgidity and, eventually, death of plants.[142]

1.8.2 5-Aminolevulinic Acid as a Plant Growth Regulator

In some plants, e.g. barley and tobacco, ALA does not act as a herbicide. Instead it can be used to inhibit growth. This suppression does not involve photodestruction by porphyrins, but an entirely different biosynthetic pathway. Exogenous ALA acts as a good source of carbon for formation of amino acids, proteins, sugars, lipids and organic acids as well as porphyrin synthesis. It is thought that plant growth is controlled by cytokinins which are derivatives of 6-aminopurine. ALA not involved in porphyrin synthesis is converted initially to dioxovaleric acid, which then utilises the aldehyde group carbon to form a purine ring (Fig. 1.19).

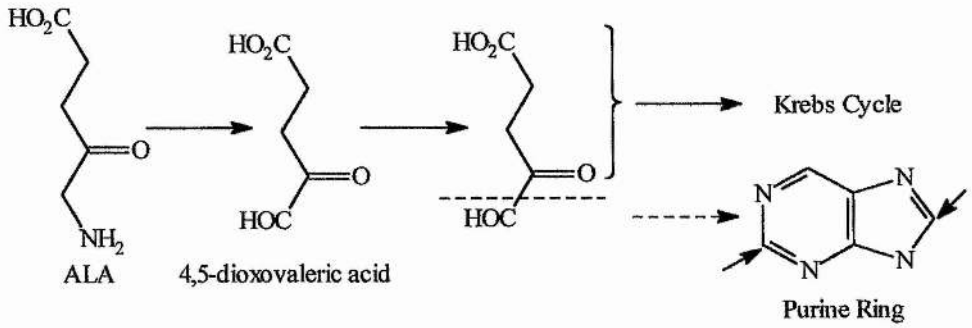

Figure 1.19 - Formation of a purine ring using ALA as a carbon source.

ALA esters and N-acylated derivatives have been patented in Japan as potential plant growth regulators.[145, 146] Interest is also growing in these types of compounds as potential pro-drugs for PDT.[147 - 150]

1.8.3 Early Detection of Lead Poisoning

Lead poisoning has been implicated as a cause of brain damage, behavioural problems, anaemia, mental impairment, kidney failure and coma. Early detection, therefore, is advantageous.[151] Excess lead in the body is known to inhibit several of the enzymes involved in the biosynthetic pathway to haem, particularly ferrochelatase, which is involved in the production of haem from PpIX and PBG synthase, the enzyme that converts two molecules of ALA into PBG.[152] Inhibition of PBG synthase prevents photosensitisation from occurring as the biosynthetic pathway is blocked, therefore, lead poisoning causes ALA levels to build up in the body. High concentrations of ALA measured in blood or urine can give an early indication of lead poisoning[153] which can then be treated easily using chelation therapy.[154]

1.8.4 5-Aminolevulinic Acid and the Porphyrias

The porphyrias are a group of diseases which result from a deficiency in one of the enzymes involved in the biosynthesis of haem.[155] They can lead to accumulation of porphyrin precursors resulting in photosensitisation of the skin.[152] Other symptoms of an acute attack of porphyria include abdominal pain and constipation.[156] More serious cases result in hypertension or psychiatric problems.[155] This was one of the ailments of King George IV.

Diagnosis of the porphyrias depend on detection of increased levels of porphyrin precursors in urine, particularly ALA and PBG.[155] It has been demonstrated, however, that ALA given orally, even in large doses, does not cause neurotoxic effects or any of the other symptoms associated with the acute porphyrias.[157]

1.9 5-Aminolevulinic Acid Based Photodynamic Therapy for the Treatment of Cancer

1.9.1 Lung Cancer

The majority of cancer deaths in the western world are caused by lung cancer. Tumours can block the airways and conventional radiotherapy and chemotherapy are not very successful. ALA-PDT has been used both to detect and treat early stage lung cancers.[103] ALA can be inhaled by the patient, who is then examined using fluorescent light. PpIX fluorescence is much stronger in the tumour than in surrounding tissue. After PDT in one study, seven out of eight tumours showed a complete response and no skin photosensitivity was reported.[101]

1.9.2 Oral Cancer

Two thousand four hundred new cases of oral cancer are detected every year in the UK. A study of eighteen patients with a variety of malignant lesions in the mouth was carried out using ALA-PDT and showed promising results particularly in patients with dysplasia.[102] Another study involving four patients, who were given ALA orally, showed tumour necrosis in three cases. PpIX levels had returned to normal within twenty four hours.

1.9.3 Bladder Cancer

A bladder carcinoma was the first human tumour treated using PDT in 1975.[110] The first health agency approval for PDT using PII was granted in Canada for use in cancer of the bladder. PpIX accumulation in tumours of the human bladder is ten times greater than in normal tissue.[158] In one study ten patients were treated intravenously with ALA. After just one exposure to light four showed a complete remission and two a partial remission. No side effects were observed, hence, multiple treatment sessions were a possibility.[159]

1.9.4 Skin Cancer

Non-melanoma primary skin cancer is the most common disease to affect man.[160] Topically applied ALA-PDT is particularly suited to the treatment of skin cancer as the drug is concentrated in the affected area enhancing the PpIX accumulation within the diseased tissue. The first clinical results of ALA-PDT were published in 1980 and reported a 90% complete response rate in the treatment of eighty basal cell carcinomas. Numerous papers showing similar, or better results have been published since then.[111]

1.9.5 Other Cancers

ALA-PDT is being examined, with some success, as a treatment for several other areas of cancer therapy including cancers of the gastrointestinal tract and rectum[137], the endometrium[161] and the colon.[162]

1.9.6 Enhancing 5-Aminolevulinic Acid Based Photodynamic Therapy

ALA-PDT can be used in conjunction with surgery. The bulk of the tumour is removed by surgery then the residual tissue can be necrosed using PDT.[114]

Topical delivery can be enhanced using DMSO, which assists penetration through the skin.[107]

A new approach to enhancing ALA-PDT is to add iron chelators. This is an attempt to block the final step in haem metabolism. Conversion of the active compound, PpIX, to haem using the enzyme ferrochelatase involves insertion of iron into the porphyrin ring. This should cause a build up of PpIX. Iron chelators include 1,2-diethyl-3-hydroxypyridin-4-one (CP94) (Fig. 1.20a)[163] and desferrioxamine mesylate (Fig. 1.20b).[164] EDTA (Fig. 1.20c), a non-specific metal chelator, is also used.[141]

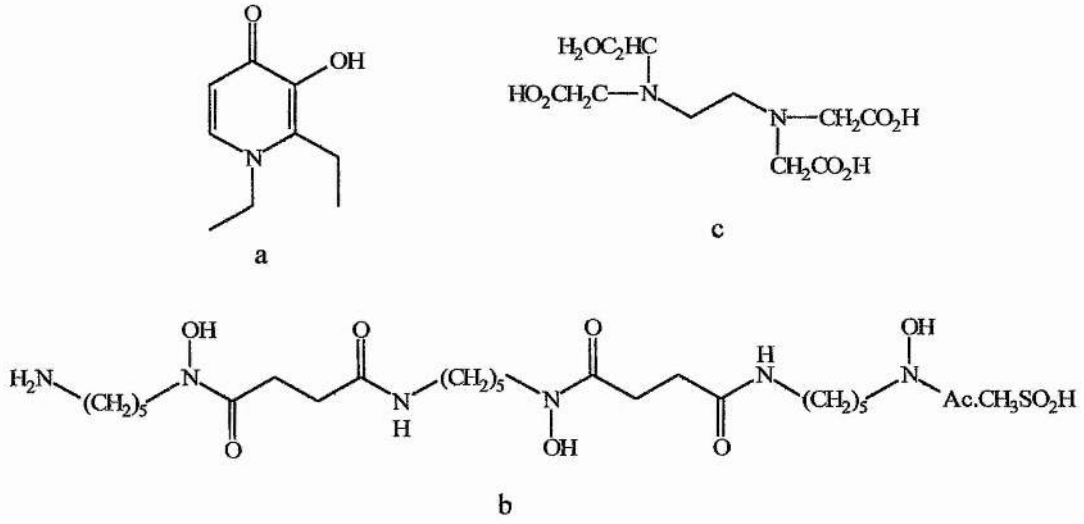

Figure1.20 - Iron chelating agents. a - CP94, b- desferrioxamine mesylate and c - EDTA

1,10 phenanthroline (Fig. 1.21), a tetrapyrrole biosynthesis modulator has also been shown to enhance the PDT effect.[144, 164]

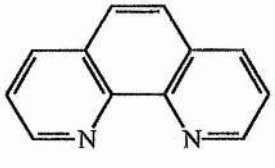

Figure 1.21 - Structure of 1,10-phenanthroline.

PDT using esters of ALA is also proving to be a promising concept. This will be discussed in greater detail later, in Section 6.2.[101]

1.10 5-Aminolevulinic Acid Based Photodynamic Therapy for Other Conditions

PDT is also undergoing trials for many non-cancerous conditions ranging from the supression of superfluous hair growth or very heavy periods to the destruction of bacteria and viruses in transfusion blood.[116]

Psoriasis is a very common ailment caused by a large number of proliferating cells in the epidermis resulting in the formation of plaques on the surface of the skin.[165] It was one of the first diseases treated using PDT with Psoralens and UV light (PUVA). Trials have shown that a single dosage of topical ALA can be successful in treating psoriasis.[166] A clinical improvement of greater than fifty percent can be observed and multiple treatments can be even more successful.[167]

PDT can be used to purge bone marrow in patients with leukamia as porphyrins accumulate in leukemic cells.[168] Atheroschlerotic plaques and rheumatoid arthritis are also well suited to photodynamic damage.[169] As haemoglobin binds to nitric oxide (NO), it blocks the action of NO on cyclic-GMP levels affecting smooth muscle relaxation. Initial results suggest that ALA could be used as a modulator of smooth muscle tone.[170]

Perhaps the most important non-tumour application of PDT, however, is the photodynamic destruction of viruses (PDV). Many viruses, including herpes simplex virus (HSV), some of the hepatitis viruses and retroviruses like human T cell leukemic virus (HTLV) and human immunodeficiency virus (HIV) can be transmitted in blood from a transfusion.[106] Research has shown that these can be destroyed using a suitable photosensitiser and visible light. Singlet oxygen kills the virus, leaving the surrounding essential blood cells intact.[171]

1.11 Photodynamic Detection of Tumours using Fluorescence (PDD)

Early detection of cancer is very important. Many tumours are not discovered until it is too late. An area related to PDT is that of photodynamic detection (PDD). As photosensitisers possess the ability to fluoresce and they can accumulate preferentially in cancerous growths, there is great potential for their use in tumour detection.

In order to avoid photodynamic damage being caused to the cells, lower light levels are used to excite the sensitiser. The resulting fluorescence is, therefore, too low to be detected using just eyesight, so intensified video cameras are used. A disadvantage in the use of some photosensitisers for detection of tumours is long-term skin photosensitisation[115] but, by using ALA induced PpIX, maximum fluorescence levels occur approximately two hours after administration of ALA. After four hours, levels are returned to normal, resulting in skin photosensitisation being short lived. If PDD is to be used for routine clinical diagnosis, the short

incubation time is an additional advantage, as treatment can be on an out-patient basis.[172]

PDD using ALA has been studied mainly for bladder and lung cancer, where a twenty fold difference in PpIX levels between tumour and normal surrounding tissue has been observed.[173] ALA esters may also be suitable for fluorescence detection, as they exhibit even more selectivity between tumour and normal tissue.[101] Work is being carried out in the Physics department, St. Andrews University using ALA induced PpIX fluorescence for tumour detection (Fig 1.22a, b and c).

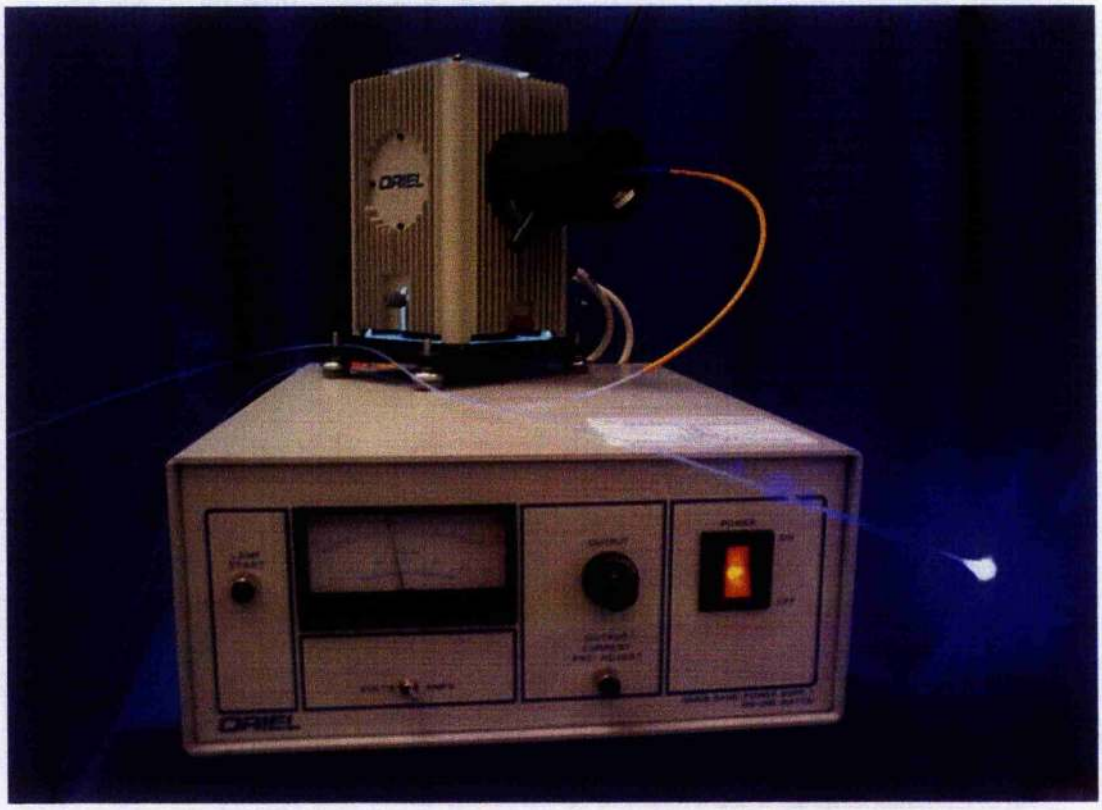

Figure 1.22a - Photograph of apparatus used for PDD in St. Andrews.

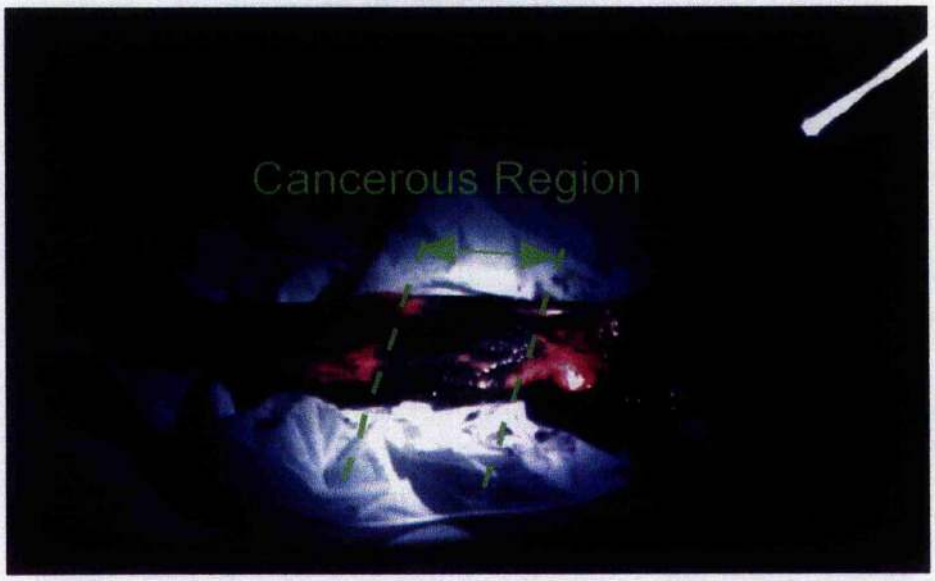

Figure 1.22b - Photograph of ALA induced fluorescence in the human oesophagus

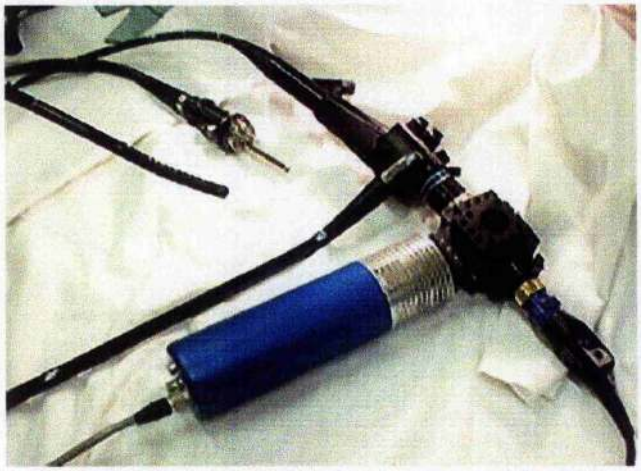

Figure 1.22c - Photograph of endoscope used for PDD.

1.12 Problems With the Use of 5-Aminolevulinic Acid in Photodynamic Therapy and Outline of Thesis

ALA is a fairly expensive compound to purchase. One gram of ALA.HCl costs around £50 from Sigma/ Aldrich, therefore, Chapter 2 deals with the chemical synthesis of the molecule.

In the body, two molecules of ALA dimerise enzymatically using PBG synthase to form PBG in a Knorr type of reaction (Fig. 1.23).

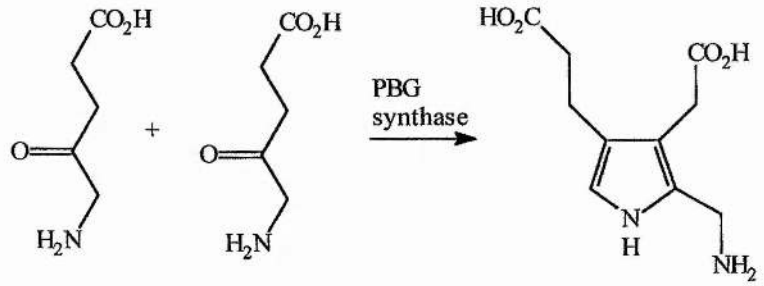

Figure 1.23 - The enzymatic dimerisation of ALA forming PBG

An alternative pyrrole synthesis, known as the Fisher-Fink synthesis, would result in the formation of pseudoporphobilinogen (Fig. 1.24).[174]

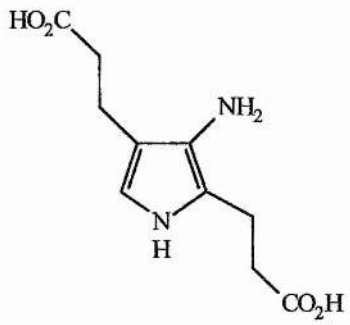

Figure 1.24 - The structure of pseudoporphobilinogen.

Chapter 3 discusses reaction of ALA.HCl with a variety of diketones to investigate whether a Knorr, Fisher-Fink, both reactions or no reaction would occur.

A major problem in PDT using ALA is its stability. ALA is stable as the HCl salt but can not be administered unbuffered, as this causes both pain and hypotension to the patient.[175] Removal of the HCl salt causes a different dimerisation reaction to occur (Fig. 1.25).

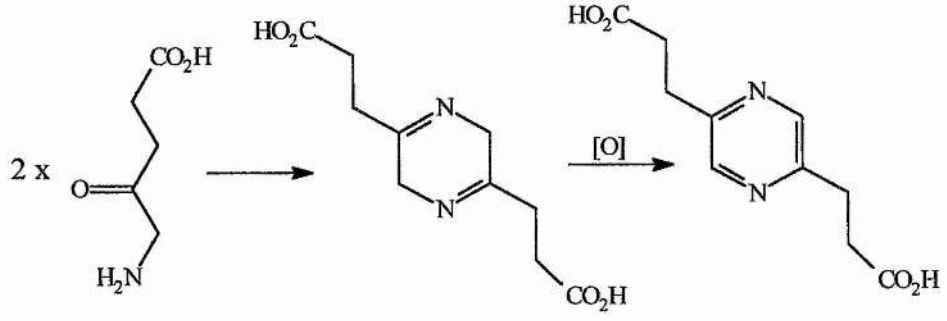

Figure 1.25 - The non-enzymatic dimerisation of ALA.

Two molecules of ALA, in the absence of the enzyme PBG synthase react to form a dihydropyrazine, which is oxidised in air forming a pyrazine. This reaction is irreversible and occurs in solutions prepared in PDT clinics. It is associated with formation of a brown colour (demonstrated in Section 6.5), presumably due to polymerisation reactions. It is not known whether or not this pyrazine is toxic.[176] Its formation also lowers the dosage of the drug being administered to the patient. Chapter 4 investigates this non-enzymatic reaction.

The compound prepared initially in the non-enzymatic reaction is a dihydropyrazine. It is fairly short lived due to aerial oxidation which gives the more stable aromatic compound. Chapter 5 concerns the chemistry of dihydropyrazines, particularly their form in solution.

In order to prevent a brown colouration and pyrazine formation during PDT, derivatives of ALA were prepared with different groups blocking either, or both ends of the molecule. These could have the added advantage of enhancing tissue penetration and selectivity in the body. Chapter 6 deals with their chemical synthesis, and Chapter 7, their biological testing.

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Chapter 2

Synthesis of 5-Aminolevulinic Acid

2.1 Introduction

Many syntheses of ALA.HCl have been published. They fall into three distinct groups. There are routes where the nitrogen atom is introduced into the molecule using cyanide, azide or phthalamide, routes where the nitrogen is already attached to the basic carbon skeleton and routes involving manipulation of functional groups on five or six membered rings followed by ring opening. Several routes to labelled ALA.HCl, both ^{13}C and ^{15}N, have been published but these, in general, involve longer synthetic routes.[201 - 205] The aim of this work was to produce reasonably large quantities of ALA.HCl as quickly, cheaply and safely as possible.

2.1.1 Routes Involving Introduction of a Nitrogen Atom

Syntheses of this type are the most numerous. Several routes involve the introduction of nitrogen into the molecule using cyanide.[206, 207] Others involve sodium azide,[208] but the majority of published syntheses of ALA.HCl involve reacting a brominated or chlorinated compound with potassium phthalimide.[209 -217]

2.1.2 Nitrogen Already Attached to the Basic Carbon Skeleton

Syntheses have been attempted using succinamide,[218] glycine,[219, 220] hippuric acid[221, 222] and diethylacetamidomalonate[223] as starting materials.

2.1.3 Synthesis by Ring Opening

ALA.HCl has also been prepared from N-methoxycarbonylated cyclic amines,[224] 2,5-piperidinedione,[225 - 227] furans[228 - 231] and oxazolinones.[232]

2.2 Experimental Discussion

Initially, the route of Ha, Lee, Ha and Park[208] was followed (Fig. 2.1).

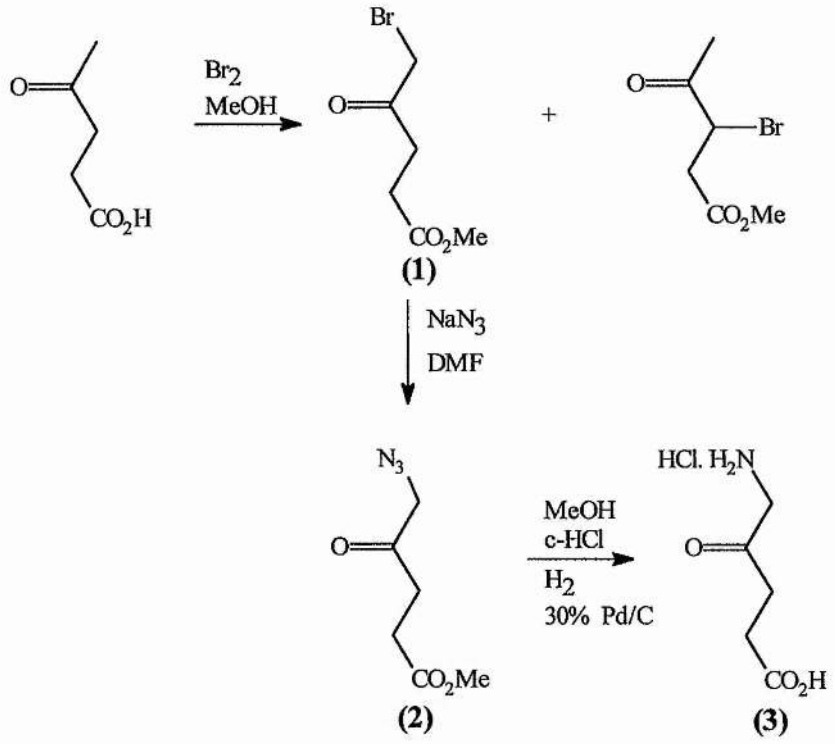

Figure 2.1 - Ha, Lee, Ha and Park synthesis of ALA.HCl.

Levulinic acid was brominated using bromine in methanol (Reaction 8.1) yielding a three to one ratio of methyl 5-bromolevulinate (**1**) to methyl 3-bromolevulinate. Small quantities of the dibrominated compound and free acid were also detected

by gc. The mixture was separated by column chromatography or distillation. The resulting methyl 5-bromolevulinate was then reacted with sodium azide (Reaction 8.2) giving methyl 5-azidolevulinate (**2**), a clear oil which appeared to be reasonably stable. Hydrogenation (Reaction 8.3) yielded ALA.HCl (**3**) as a white solid. Overall yield for this reaction was approximately 25%.

To avoid formation of the potentially dangerous azide, the Gabriel synthesis of Benedikt and Köst[214] was adapted (Fig. 2.2).

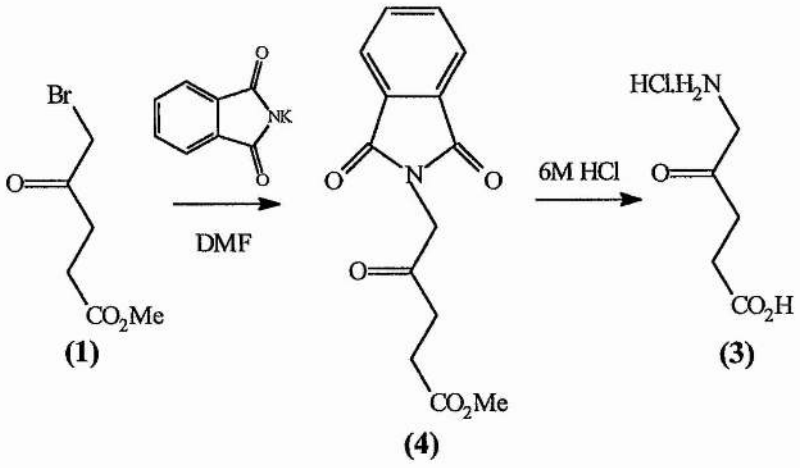

Figure 2.2 - Gabriel synthesis of ALA.HCl.

Methyl 5-bromolevulinate (**1**) was reacted with potassium phthalimide in DMF giving a fairly low yield of methyl 5-phthalimidolevulinate (**4**) (approximately 40-50%) (Reaction 8.4). Heating under reflux conditions in 6M HCl (Reaction 8.5) yielded ALA.HCl (**3**) in an overall yield of only 10%.

Brominated levulinic acid proved to be a severe skin irritant. In order to decrease contact time with the molecule, the brominated mixture was reacted with

potassium phthalimide without purification (Reaction 8.6). Phthalimide reacted with only the bromine on the terminal carbon yielding the desired product (**4**) in an adequate yield (approximately 25%).

To try to increase the overall yield, methyl 5-chlorolevulinate (**7**) was prepared as a pure compound (Fig. 2.3).

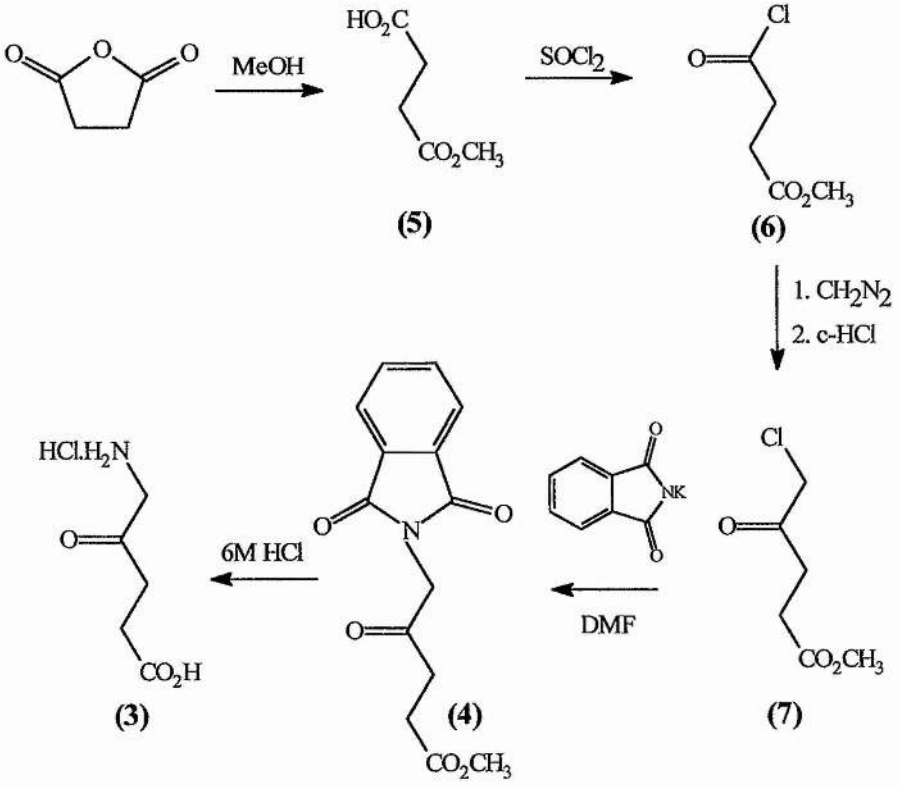

Figure 2.3 - Synthesis of ALA.HCl via methyl 5-chlorolevulinate

Methyl hydrogen succinate (**5**) was prepared from succinic anhydride and methanol (Reaction 8.7) using the method of Cason.[233] Reaction with thionyl chloride yielded methyl 3-chloroformylpropanoate (**6**) (Reaction 8.8). An Arndt-Eislert Synthesis (Fig. 2.4) using diazomethane followed by c-HCl gave methyl 5-

chlorolevulinate (7) via the method of Neuberger and Scott.[215] Overall yield for the three steps was reasonable (approximately 30%).

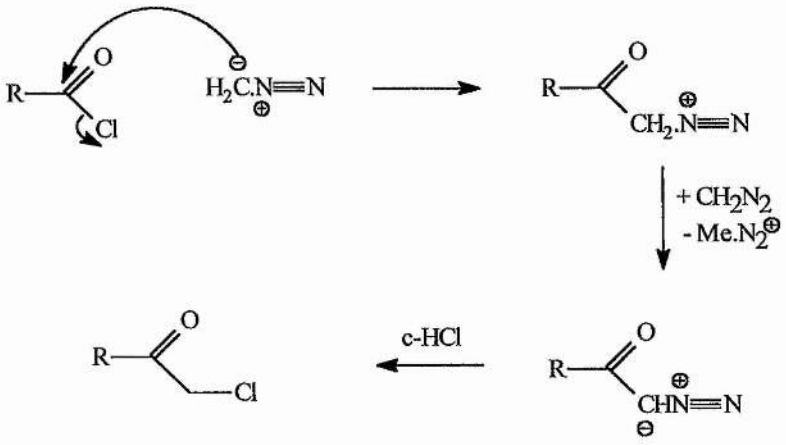

Figure 2.4 - The Arndt-Eislert Synthesis.

Methyl 5-chlorolevulinate was reacted with potassium phthalimide (Reaction 8.10) giving a 45% yield of methyl 5-phthalimidolevulinate (**4**) which was converted to ALA.HCl (**3**) as before (Reaction 8.5).

The low yielding step in all reaction schemes proved to be addition of phthalimide. Soai, Ookawa and Kato[234] found that catalytic amounts of crown ethers, particularly 18-crown-6, increase both reaction rate and yield in the preparation of N-substituted phthalimides from alkyl halides and potassium phthalimide (Fig. 2.5).

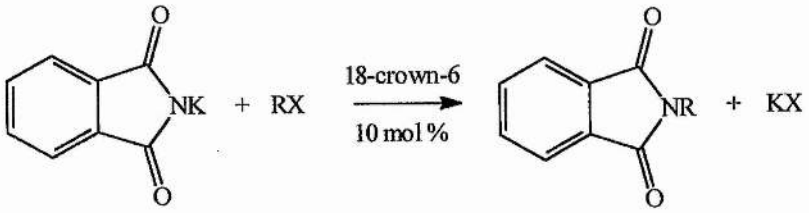

Figure 2.5 - Adapted Gabriel synthesis of N-substituted phthalimides.

18-crown-6 as a phase transfer catalyst in the reaction of methyl 5-chlorolevulinate (7), or methyl 5-bromolevulinate (1) with potassium phthalimide increased the yield from 50 to 75%, hence optimising the lowest yielding step (Reaction 8.11 and 8.12).

The highest overall yielding route for the preparation of ALA.HCl, however, was obtained using an adaptation of the method of Iida, Takao, Ogai and Kajiwara (Fig. 2.6).[230]

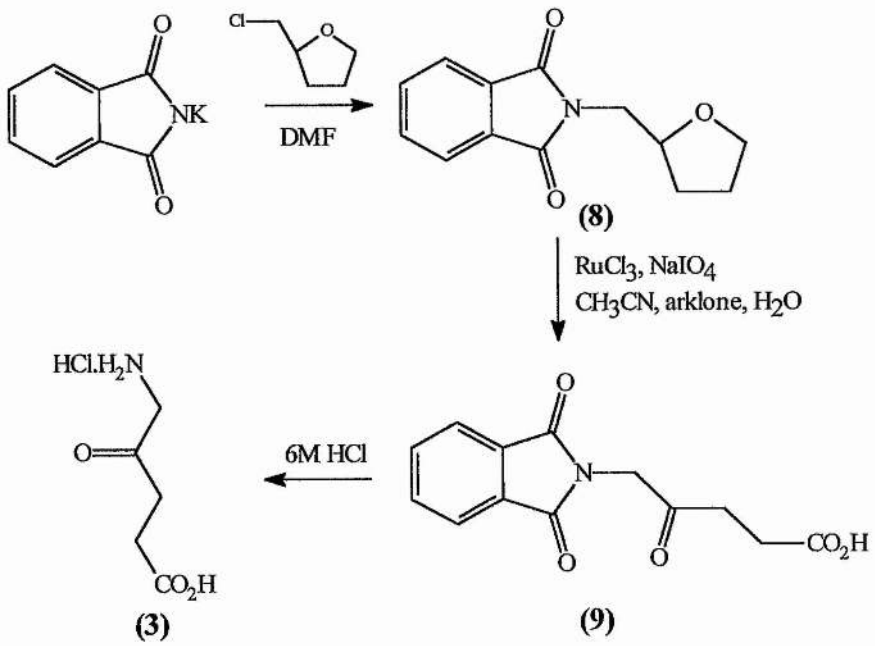

Figure 2.6 - Iida, Takao, Ogai and Kajiwara synthesis of ALA.HCl.

This synthesis was designed for the preparation of ^{15}N-labelled-ALA.HCl but is also a feasible route to ALA.HCl itself. N-Tetrahydrofurfuryl chloride is commercially available and was used in place of N-tetrahydrofurfuryl bromide in the reaction with potassium phthalimide to give N-tetrahydrofurfuryl phthalimide (**8**) (Reaction 8.13) Increasing the reaction time to three hours gave yields comparable to those of Iida *et al.* (approximately 95%). Oxidation using sodium metaperiodate and ruthenium (III) chloride (Reaction 8.14) opened up the furan ring to give phthalimidolevulinic acid (**9**) in very good yield. Heating under reflux conditions in 6M HCl (Reaction 8.15) gave ALA.HCl (**3**) in a yield of greater than 80% over three steps.

2.3 Conclusions

ALA.HCl can be prepared in a matter of days, in good overall yield using an adaptation of a published route shown in Figure 2.6. This route, however, is less cost effective than the longer route shown in Figure 2.3. The first two stages (Reactions 8.7 and 8.8) can be used on a large scale (greater than 100 g) making it, effectively, a three step procedure. Addition of crown ether in the Gabriel synthesis optimises the lowest yielding step. None of the procedures would be suitable for the large scale production of ALA.HCl, due to the high irritant properties of both brominated and chlorinated ketones, the toxicity of ruthenium based compounds, and the explosive nature of diazomethane. As regular laboratory procedures, however, any of the above routes are suitable for the preparation of ALA.HCl.

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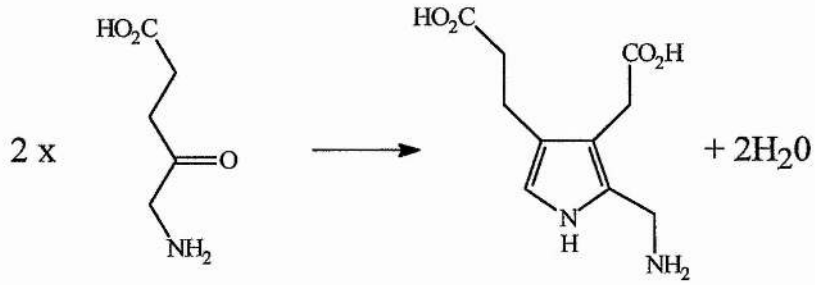
Chapter 3

Reactions of 5-Aminolevulinic Acid with Linear and Cyclic Diketones

3.1 Introduction

When two molecules of ALA combine in the presence of the enzyme porphobilinogen synthase (PBG synthase), porphobilinogen (PBG) is formed in a Knorr type of reaction (Fig 3.1).



porphobilinogen

Figure 3.1 - Formation of PBG from ALA via the Knorr Pyrrole Synthesis.

Knorr first reported the synthesis of 2,4-diethoxy-3,5-dimethylpyrrole in 1886.[301] The condensation of ethyl α-aminoacetoacetate with ethyl acetoacetate produced the molecule now known as Knorr's Pyrrole (Fig. 3.2).

The Knorr Synthesis involves reaction of an α-aminoketone with a carbonyl containing compound which has a reactive methylene group alpha to the carbonyl group.[302] The reaction has been used extensively to prepare building blocks for haem precursors.

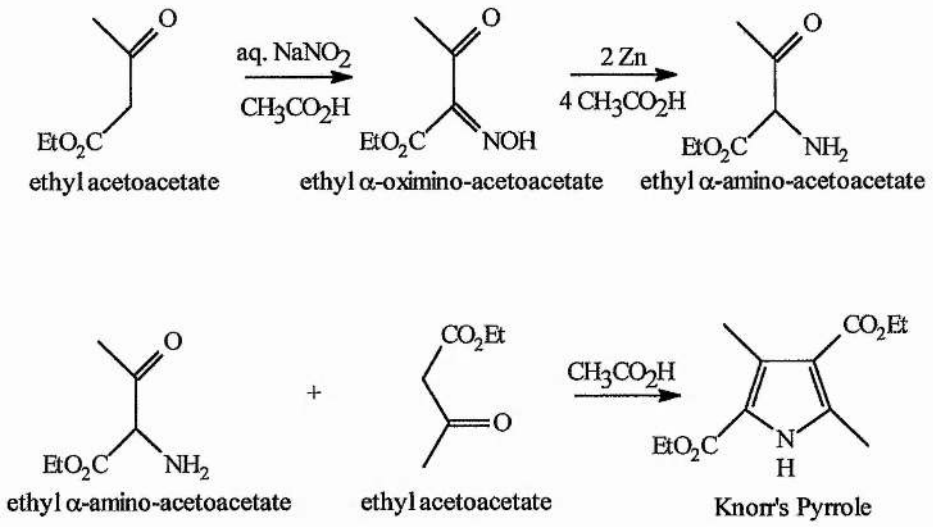


Figure 3.2 - Synthesis of Knorr's Pyrrole.

The two molecules of ALA could potentially cyclise via the 5-methylene group of one of the compounds yielding another product, pseudoporphobilinogen (Fig.3.3). This synthesis is known as the Fischer-Fink Reaction.[303]

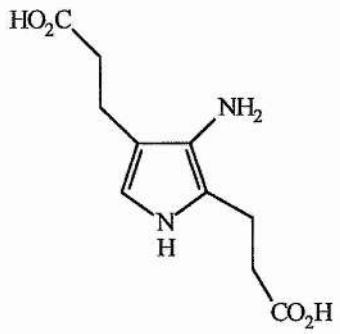


Figure 3.3 - The structure of pseudoporphobilinogen.

Fischer and Fink[303] isolated a minor product in the reaction of ethyl α-oximino-acetoacetate with 2,4-pentanedione (Fig. 3.4).

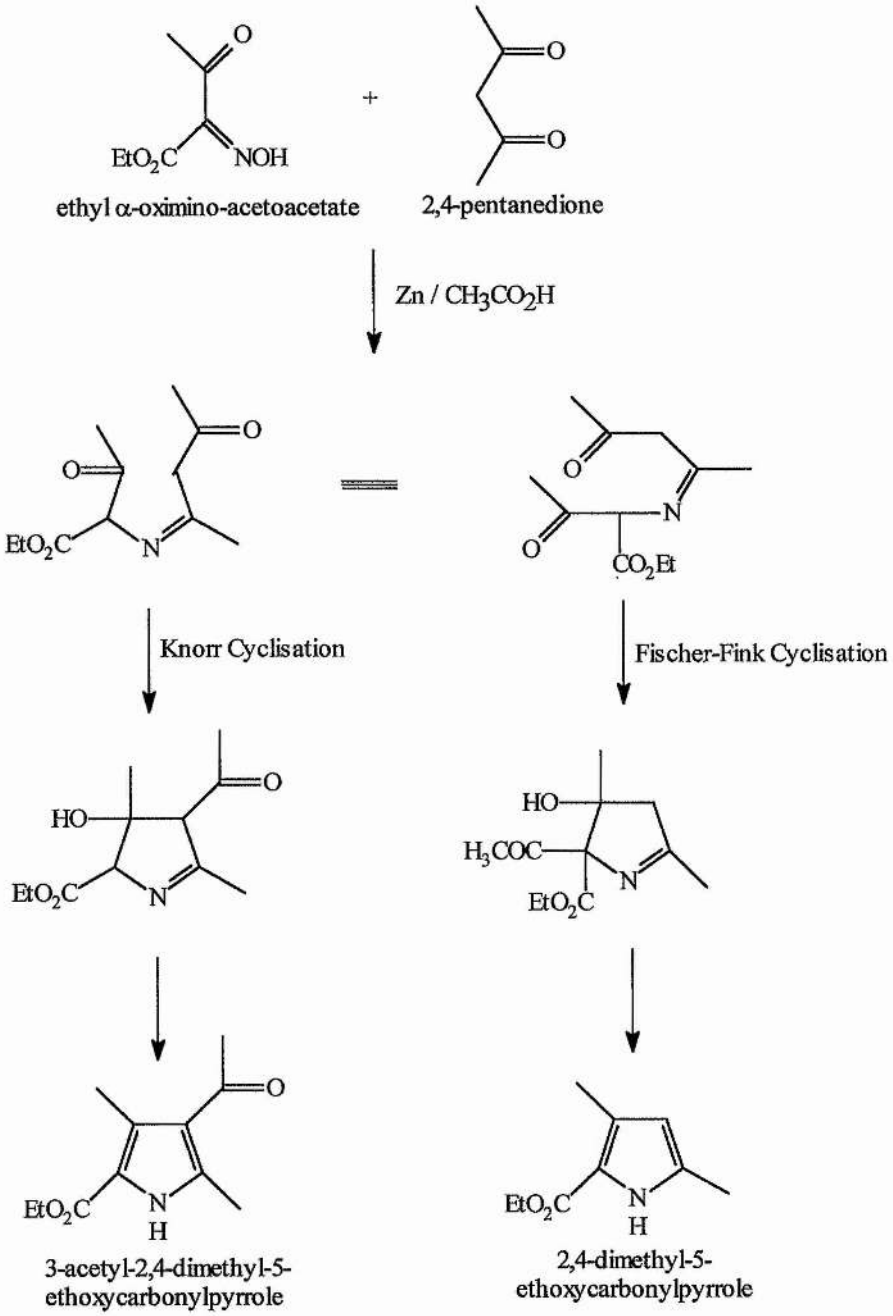


Figure 3.4 - Knorr and Fischer-Fink Pyrrole Syntheses.

The major product found, 3-acetyl-2,4-dimethyl-5-ethoxycarbonylpyrrole, was a result of the Knorr Pyrrole Synthesis. A small quantity of 2,4-dimethyl-5-

ethoxycarbonylpyrrole, however, was due to a different cyclisation mechanism now known as the Fischer-Fink Synthesis.

The general Knorr (Fig. 3.5a) and Fischer-Fink (Fig.3.5b) syntheses are shown below.

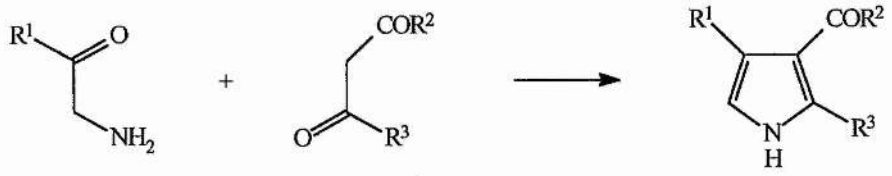


Figure 3.5a - The Knorr Pyrrole Synthesis.

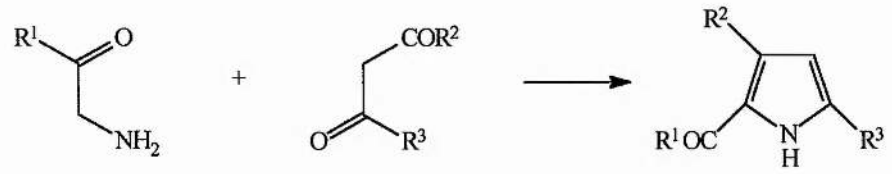


Figure 3.5b - The Fischer-Fink Pyrrole Synthesis.

In order to explore the mechanism for these reactions, a related reaction, that of ALA.HCl and 2,4-pentanedione (acetylacetone), was used. This reaction has also been used as an assay for ALA in biological fluids, particularly for the detection of lead poisoning which inhibits the conversion of ALA into PBG (See Section 1.8.3).

In most cyclisation reactions of this type the Knorr product is the dominant one. The mechanism of cyclisation may depend on the form of ALA in solution. This

has been studied using a number of isotopic probes including ^{13}C NMR spectroscopy of [4-^{13}C] ALA, ^{1}H NMR spectroscopy and ^{1}H/ ^{2}H and ^{16}O/ ^{18}O exchange. The study demonstrated that enols are formed at both C_5 and C_3 but more so at C_5. At pH 6.8, enolisation at C_5 is five times faster than at C_3. In more acidic conditions, relevant to these reactions, the rate is slower but the ratio similar.[304] In aqueous solutions, ALA can exist as a hydrate and in two enolic forms as well as the keto-compound. (See Fig. 4.1).

ALA.HCl has been reacted with a variety of different diketones related to 2,4-pentanedione in order to determine whether the Knorr, Fischer-Fink or both products are produced.[305] As a continuation of that work, ALA.HCl has been reacted with other diketones, particularly cyclic diketones to produce pyrroles and indol-4-ones.

3.2 Experimental Discussion

3.2.1 Reaction of 5-Aminolevulinic Acid with Linear Diketones

Pyrroles are highly insoluble in water, therefore, all pyrrolic products precipitate out of the reaction mixtures and can be analysed. Reaction of ALA.HCl with 2,4-pentanedione (Reaction 8.16) in acetate buffer yielded both 3-acetyl-4-(2-carboxyethyl)-2-methylpyrrole, the Knorr product (**10**) and 2-(3-carboxypropionyl)-3-5-dimethylpyrrole, the Fischer-Fink product (**11**) (Fig. 3.6). The Knorr product was obtained as greater than 90% of the total yield.[305]

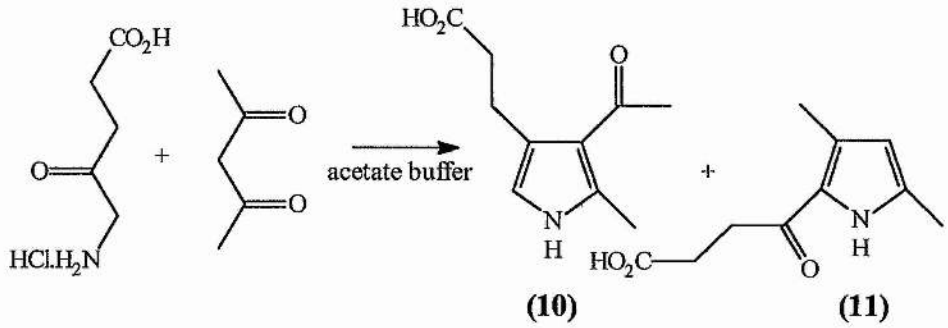


Figure 3.6 - Reaction of ALA.HCl with 2,4-pentanedione.

Reaction of ALA.HCl with 4,6-dioxoheptanoic acid (succinyl acetone, Reaction 8.17), could potentially yield four products, two from the Knorr type reaction (Fig. 3.7a) and two from the Fischer-Fink (Fig. 3.7b). Only the two Knorr products were isolated in approximately a 4:1 ratio of 3-(4-carboxybutionyl)-4-(2-carboxyethyl)-2-methylpyrrole (**12**) to 2-(4-carboxybutionyl)-4-(2-carboxyethyl)-3-methylpyrrole (**13**), presumably due to steric effects caused by interaction between the bulky $CH_2CH_2CO_2H$ groups.

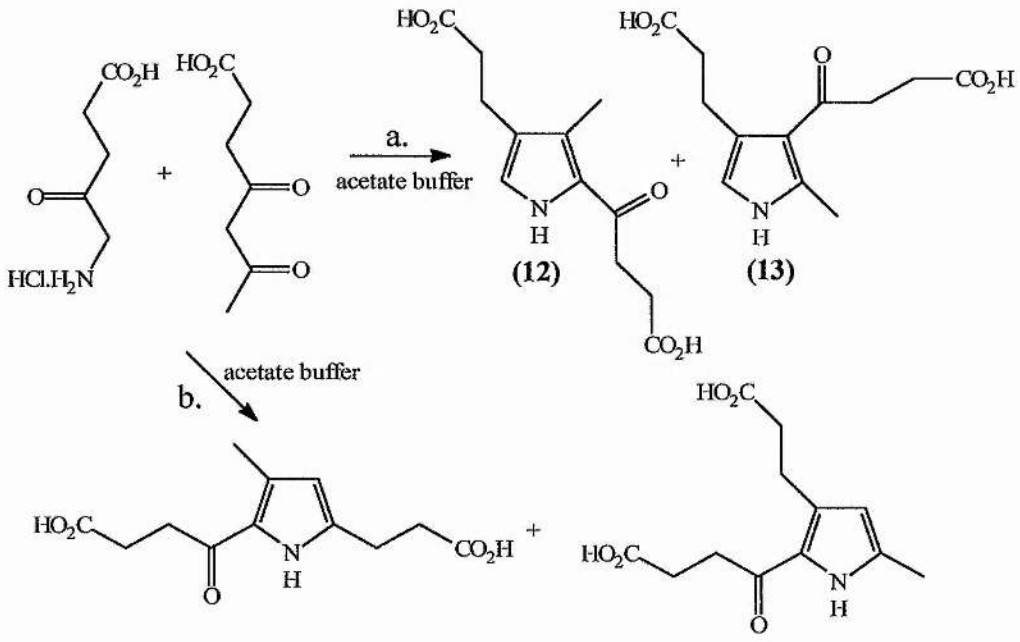


Figure 3.7a and b - Reaction of ALA.HCl with succinyl acetone.

3.2.2 Reaction of 5-Aminolevulinic Acid with Cyclic Diketones

In the reaction of ALA.HCl with cyclic diketones, only the Knorr product is possible and an indol-4-one is formed. Reaction of ALA.HCl with 5-methyl-1,3-cyclohexanedione (Reaction 8.18) under acidic conditions gave, on standing for some days, a small quantity of a white crystalline solid and the analytical data are consistent with the formation of an indol-4-one (**14**) (Fig. 3.8).

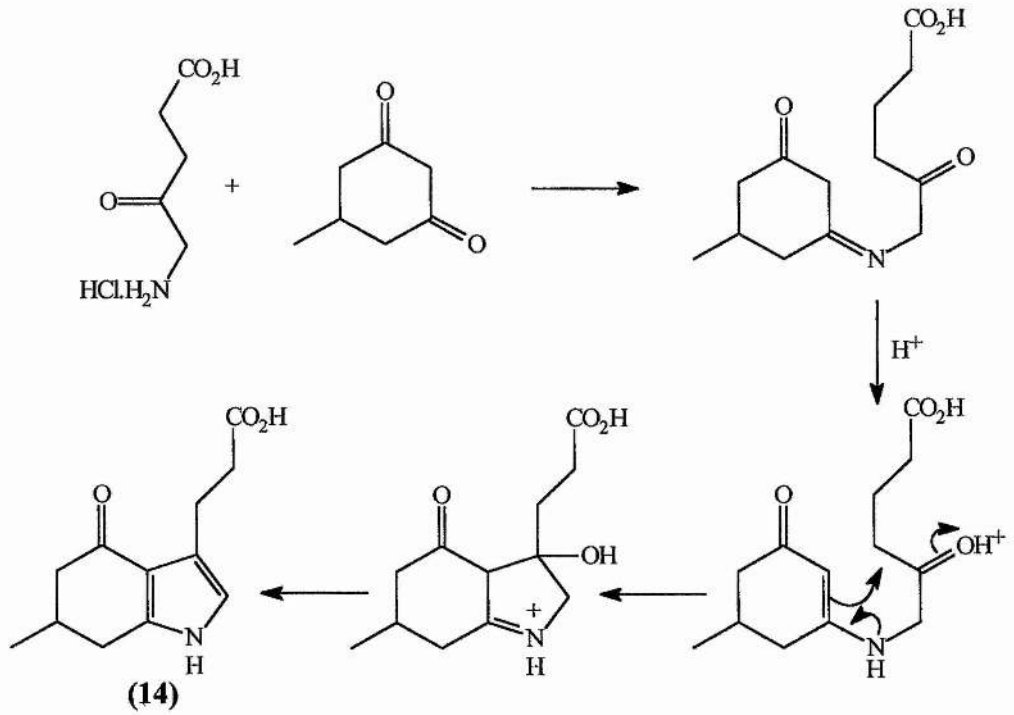
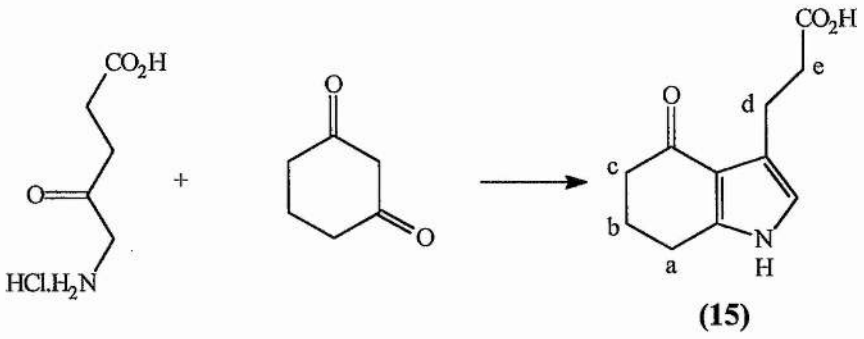


Figure 3.8 - Reaction of ALA.HCl with 5-methyl-1,3-cyclohexanedione.

The mechanism proposed previously[305] for reaction with a non-cyclic diketone and shown in Fig. 3.8 accounts for the formation of this product. The mechanism demands that one hydrogen at the 2 position of the cyclohexane moiety is

sufficiently acidic for tautomerism of the imine to occur. Cyclisation indicates that steric constraints imposed by the cyclic nature of the diketone do not prevent the formation of the transition state of the heterocyclic ring. The presence of the 5-methyl group, however, appears to slow the reaction, for reasons which will be discussed later in Section 3.3.2.

Reaction of ALA.HCl with 1,3-cyclohexanedione (Reaction 8.19) gave some crystalline product immediately after reflux. The spectral data are consistent with the formation of the indol-4-one (**15**) but it was not possible, from simple NMR spectra, to assign chemical shifts for the five methylene groups labelled a-e (Fig. 3.9).

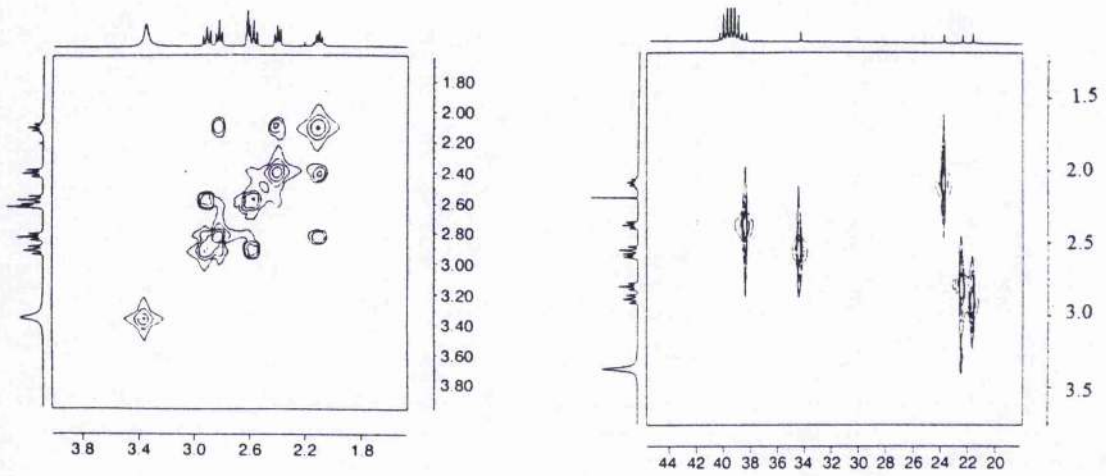


(15)

Figure 3.9 - Reaction of ALA.HCl with 1,3-cyclohexanedione.

Their unambiguous assignments were determined using COSY and ^{1}H-^{13}C correlation two dimensional NMR spectroscopy. In the ^{1}H NMR spectrum all signals, apart from the aromatic proton at δ 6.6 and two signals at δ 11.1 and 11.8, corresponding to NH and OH, are due to methylenes. Four signals are triplets and one, at δ 2.1, is a quintet which must be due to CH_2-b. The signal at

δ 2.4 shows a symmetrical distortion with the quintet suggesting that they are neighbours (i.e. CH_2-a or CH_2-c). In the COSY spectrum (Fig. 3.10) the signal at δ 2.1 has cross peaks with the signals at δ 2.4 and 2.8 and so the last two must be due to CH_2-c and CH_2-a. In the ^1H-^{13}C correlation spectrum (Fig. 3.11) the signal in the ^{13}C NMR spectrum for the methylene with the highest chemical shift, corresponding to CH_2-c because of the proximity to the carbonyl group, is at δ 38.24. This correlates with a signal in the ^1H spectrum at δ 2.4, thus the shifts of CH_2-a and CH_2-c are assigned. The ^1H signals at δ 2.6 and 2.9 have cross-peaks with one another but with no other signals and must, therefore, correspond to CH_2-e and CH_2-d. The one at δ 2.6 correlates with a signal in the ^{13}C spectrum at the high value of δ 34.52 so must be the methylene group next to the carbonyl of the carboxylic acid, i.e. CH_2-e. The signal at δ 2.9, therefore, is due to CH_2-d.



Figures 3.10 and 3.11 - COSY and ^1H-^{13}C correlation two dimensional NMR spectra.

3.3 Conclusions and Mechanism

3.3.1. Reaction of 5-Aminolevulinic Acid with Linear Diketones

With the exception of the acetylacetone reaction, all products obtained were Knorr rather than Fischer-Fink. Previously, when the same experiment was carried out with ethyl acetoacetate only the Knorr product was formed. With 3-methyl-2,4-pentanedione and 3-isopropyl-2,4-pentanedione, however, only the Fischer-Fink product was formed and with 2,4-hexanedione, four products were formed, two from each mechanism.[305]

From an observation that 1,1,1,5,5,5-hexafluoro-2,4-pentanedione, which exists almost entirely in the keto form or its hydrate, does not react with ALA.HCl it can be concluded that the reactive form of the dione is most likely to be an enol. It can be assumed that the intermediate formed as the first product of the reaction is the enaminoketone.

If this enaminoketone can exist in as many forms as ALA then two enolic forms are possible intermediates in the subsequent cyclisation step (Fig. 3.12).

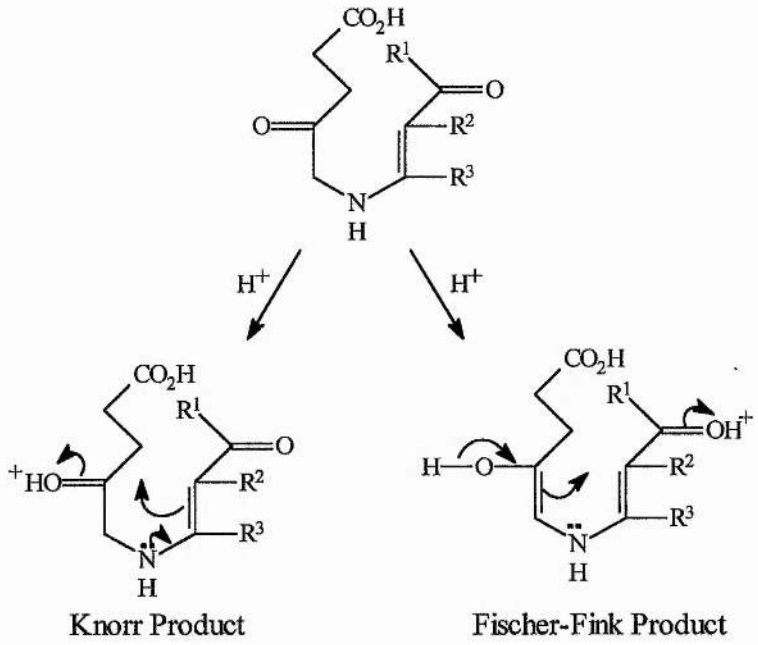


Figure 3.12 - Two possible intermediates formed during the Knorr and Fischer-Fink Pyrrole Syntheses.

Protonation of the 4-keto group of ALA leads readily to cyclisation and elimination of water giving the Knorr product. Enolisation of this keto-group and protonation of the other keto group gives the Fischer-Fink product.

It is known that ALA can enolise at the 5-position, and this should occur in a similar way in the enaminoketone, but there are other factors involved in determining whether the Knorr or Fischer-Fink products are formed.

If you consider the relative ease of protonation of the two keto groups it is not surprising that both products are obtained in some cases. If R^1 is an electron withdrawing group, protonation of the neighbouring keto-group is disfavoured and only the Knorr product is obtained.

If two molecules of ALA.HCl were reacted to form the equivalent enaminoketone this could then, theoretically, be cyclised to give PBG in a non-enzymatic reaction. This, however, does not work as the ketone would be formed only from the C3 enol of ALA and this is disfavoured with respect to the C5 enol. This could possibly explain why two molecules of ALA combine to form a pyrazine (Fig. 1.25) and do not spontaneously cyclise to give PBG.

3.3.2 Reaction of 5-Aminolevulinic Acid with Cyclic Diketones

Reaction of ALA.HCl with cyclic diketones yield indol-4-one products. There is, however, a surprising feature in the NMR spectra of these. The simplicity of the ^{1}H NMR signals, with no geminal coupling for the methylene groups at positions a, b and c indicates that ring inversion of the half-chair form of the six-membered ring must be fast on the NMR timescale. Since the solvent used was $^{2}H_{6}$-DMSO it was not possible to freeze out this inversion by lowering the temperature.

Another interesting observation is that the methyl group at the 5 position substantially lowers the rate of cyclisation. In the transition state for cyclisation, which approximates to a half-chair structure, the methyl group could be in either the pseudo-axial or pseudo-equatorial position. From an examination of models it is clear that there is enough steric interference when in the pseudo-axial position to reduce the rate of reaction. This view was confirmed by our observation that no readily isolable product resulted from the reaction, under the same conditions, of ALA.HCl with 5,5-dimethyl-1.3-cyclohexanedione (dimedone) (Reaction

8.20). This could also be due to the lack of solubility of the compound, and the fact that dimedone is quite acidic. Enols can be formed at both C5 and C3. The protonation step can occur before or after resonance between the carbonyl group and a negatively charged oxygen species. At pH 6.8, enolisation at C5 is five times faster than at C3. In more acidic conditions the ratio is similar but the rate is slower and this may have affected the formation of any products during the reaction (Fig. 3.13).

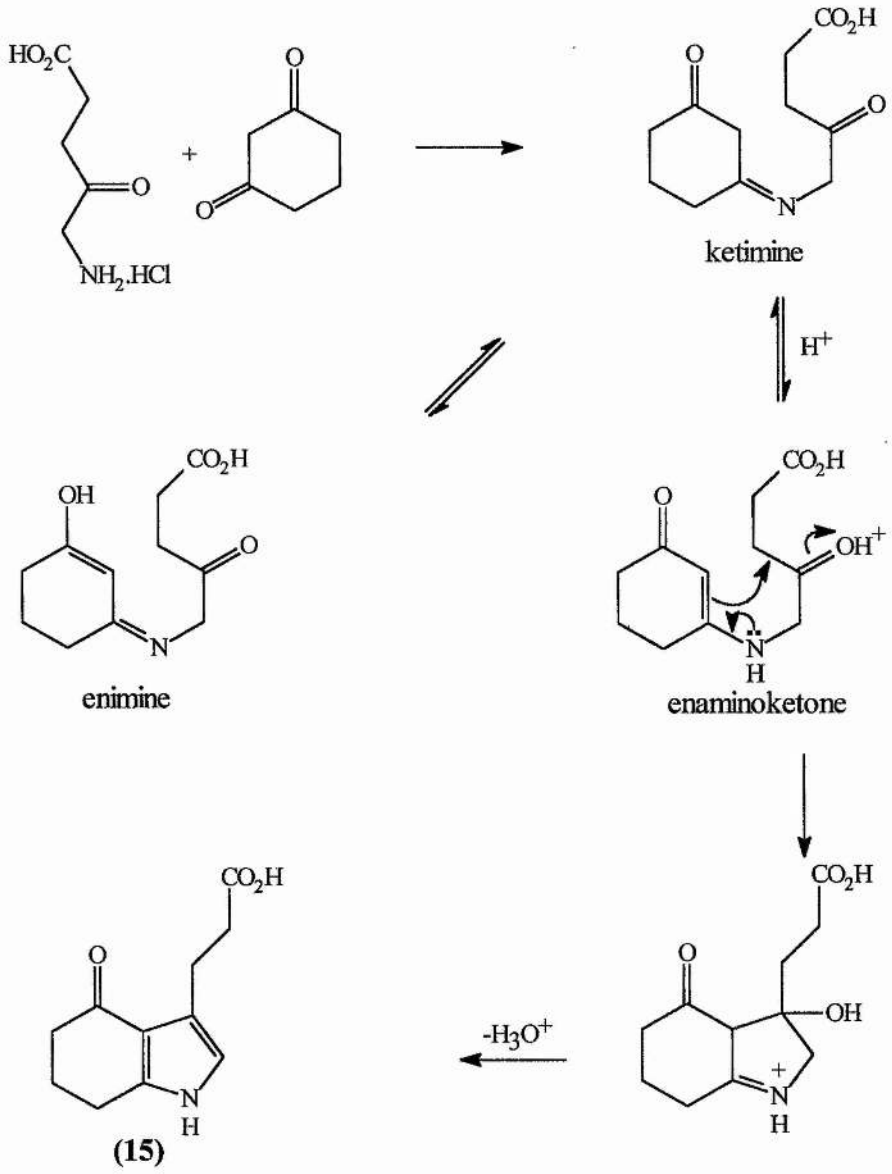


Figure 3.13 - Mechanism for the formation of an indol-4-one.

3.4 References

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Chapter 4

Dimerisation Reactions

4.1 Introduction

ALA, a 4-keto-5-amino acid, is a highly reactive molecule which can exist in a number of forms in aqueous solution (Fig. 4.1).

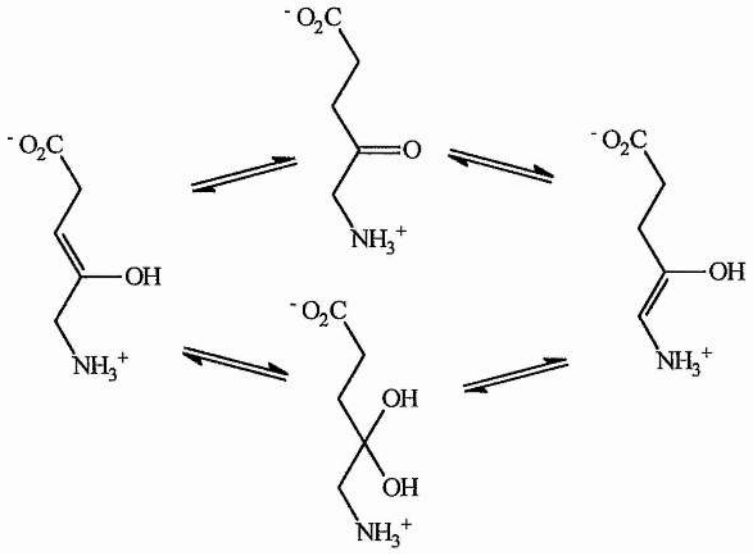


Figure 4.1 - Forms of ALA in solution.

In the body, PBG synthase[401] is the only enzyme involved in the usage of ALA, converting two molecules to PBG (Fig. 4.2).

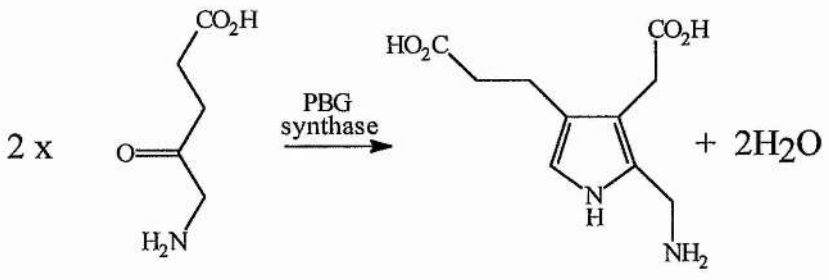


Figure 4.2 - The enzymatic conversion of ALA to PBG.

In the absence of the enzyme, two molecules of ALA react in a different manner forming, firstly, a dihydropyrazine which is oxidised to a pyrazine in air making the reaction irreversible (Fig. 4.3).

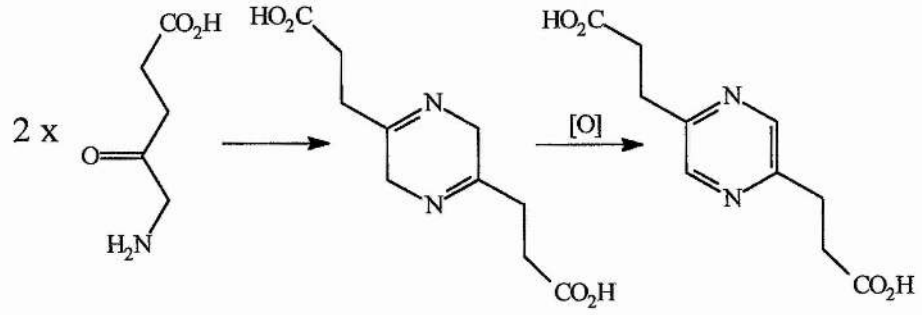


Figure 4.3 - The non-enzymatic dimerisation of ALA.

It has been shown that, under physiological conditions, 2,5-di-(β–carboxyethyl) pyrazine can be formed[402] and that alternative forms of ALA, or its non-enzymatic condensation products, may be involved as neurotoxins in lead poisoning or in some of the diseases known as the porphyrias (See Section 1.8.4).[403] A pyrazine, probably resulting from the non-enzymatic dimerisation of ALA has been detected in the urine of patients with acute intermittent porphyria.[404]

Clinical solutions of ALA for PDT are generally prepared at the beginning of the session. As time wears on, these solutions go a dark brown colour (this is illustrated in Section 6.5) and pyrazine is formed. We believe that two different reactions are taking place, polymerisation of ALA resulting in colour formation and dimerisation giving the pyrazine. These lower the dosage of the drug being given to patients to an unknown amount and the toxicological properties of 2,5-di-(β–carboxyethyl) pyrazine are not known.

We have attempted to prepare a pure sample of the non-enzymatic dimerisation product of ALA for toxicological testing (Section 4.2), and have developed an equation to calculate the percentage of ALA dimerisation in clinically useful solutions (Section 4.3). We have also investigated the kinetics of dimerisation of ALA at various pHs using solution state ^{15}N NMR (Section 4.4). Finally we have investigated the dimerisation reactions of other molecules (Section 4.5).

4.2 Experimental Discussion - The Synthesis of 2,5-Di-(β–Carboxyethyl) Pyrazine

Initially, an attempt was made to prepare 2,5-di-(β-carboxyethyl) pyrazine directly from ALA.HCl (Reaction 8.21) by heating under reflux conditions in acetate buffer, then freeze drying (Fig. 4.4).

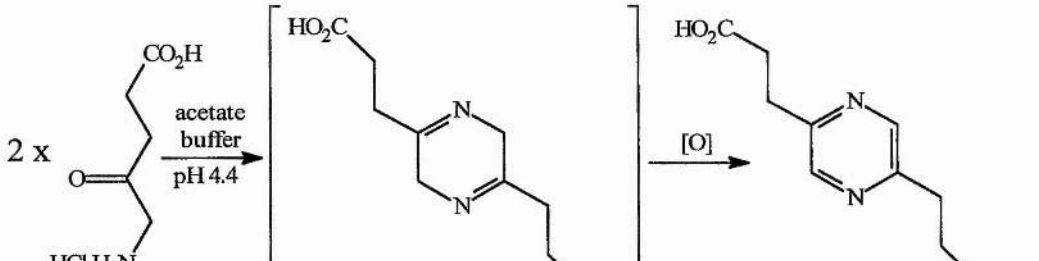


Figure 4.4 - Attempted synthesis of 2,5-di-(β-carboxyethyl) pyrazine.

This gave a brown hygroscopic oil. Attempts to purify the mixture using decolourising charcoal and column chromatography failed and ^{13}C NMR and mass

spectrometry confirmed that the product had polymerised (peaks above the molecular weight of the pyrazine were detected).

An alternative approach was devised, beginning with the pyrazine ring intact (Fig. 4.5).

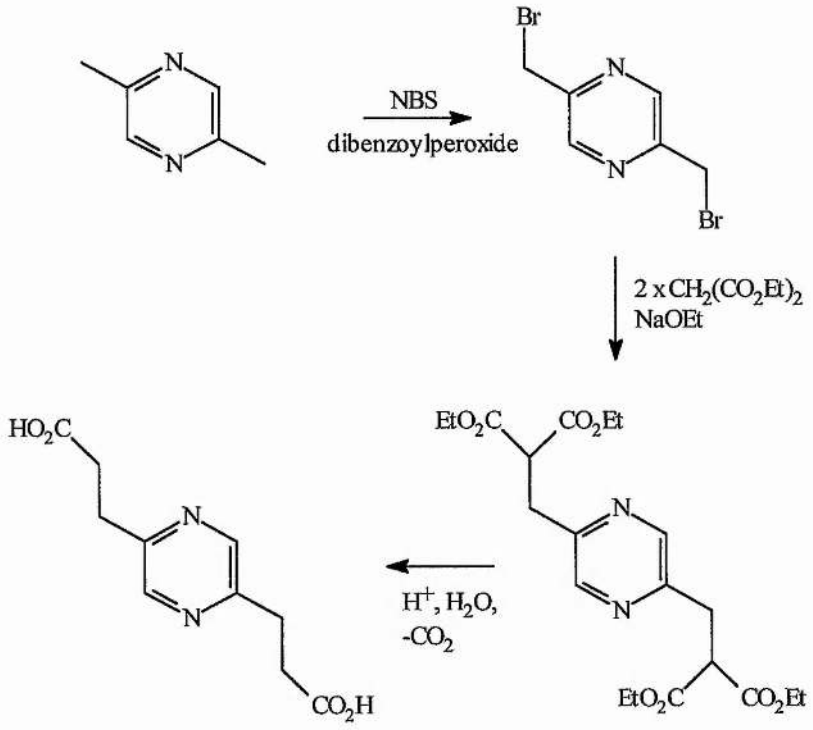


Figure 4.5 - Alternative attempted pyrazine synthesis.

Dibromination of 2,5-dimethylpyrazine (Reaction 8.22) followed by reaction with diethyl malonate and treatment with aqueous acid should yield 2,5-di-(β-carboxyethyl) pyrazine. As the brominated product is known to be highly lachrymatory, extremely irritating to the skin, highly unstable and liable to polymerisation,[405] it was reacted immediately, without purification, with diethyl malonate. Unfortunately, tlc showed there to be at least seven products so this approach was abandoned.

The pyrazine was, therefore, prepared using the route shown in Fig. 4.6.

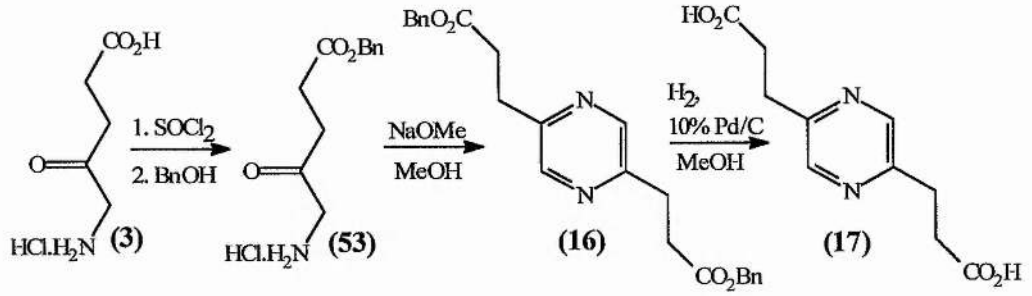


Figure 4.6 - Synthesis of 2,5-di-(β-carboxyethyl) pyrazine.

The HCl salt was removed from the benzyl ester of ALA.HCl using sodium methoxide (Reaction 8.23), causing the benzyl ester of the pyrazine (**16**) to form. Hydrogenation over 10% Pd/C catalyst (Reaction 8.24) yielded the pyrazine (**17**) in good yield. This compound can now be used for toxicological testing.

4.3 Kinetics of the Dimerisation of 5-Aminolevulinic Acid

The convenient use of ALA in a clinic for PDT has not yet been realised. In the concentrated solutions of ALA at the pH required for use, there are two unwanted side reactions, i.e. dimerisation of ALA to give 2,5-di-(β–carboxyethyl)-3,6-dihydropyrazine, which then undergoes immediate oxidation to give 2,5-di-(β-carboxyethyl) pyrazine (**17**) (Fig. 4.3) and a reaction of unknown nature which results in the solution going dark brown. After storage for a few hours a solution of ALA looks rather like Coca-Cola (See Section 6.5). These two reactions, which we think are not necessarily related, make it very difficult to know how the dose of ALA given to patients decreases during the lifetime of the

solution. It should be possible to ascertain the progress of dimerisation by spectrophotometric means as the pyrazine (**17**) has a distinctive spectrum. The gradual deterioration (i.e. the browning) of the solution after a short time, however, prevents this. The absorbance after the completion of the reaction, which could be used to measure the extinction coefficient for the pyrazine, cannot be measured due to this darkening reaction.

For a first order reaction it is possible to deduce the extinction coefficient of the product from spectral changes occurring during early stages of the reaction using the method of Swinbourne[406] and Kedzy.[407] Most second order reactions can be converted into pseudo-first order reactions by appropriate adjustment of the concentration of one of the reactants and the Swinbourne-Kedzy treatment can then be applied. This is not possible for a dimerisation reaction, however, as pseudo-first order conditions can never be realised. We have attempted a treatment of the kinetics of dimerisation reactions which parallels the Swinbourne-Kedzy treatment of a first order reaction and applied it to solutions of ALA to ascertain the extent of dimerisation at timed intervals.

4.3.1 Derivation of the Equation

Consider a general dimerisation reaction:-

$$2A \xrightarrow{k} P \qquad A = ALA;\ P = pyrazine$$

If x is [A] at time t, p is [P] also at time t and x_o is the initial [A] then:-

$$\frac{dp}{dt} = kx^2 = k(x_0 - 2p)^2$$

Separation of the variables gives:-

$$\frac{dp}{(x_0 - 2p)^2} = kdt$$

$$\int_0^p \frac{dp}{(x_0 - 2p)^2} = \int_0^t kdt$$

$$\frac{1}{2(x_0 - 2p)} = kt + C$$

where C is the constant of integration. The reaction is monitored by observing spectral change associated with the formation of P.

If A_t is the absorbance at a fixed wavelength at time t and ε is the extinction coefficient of P, then

$$A_t = \varepsilon p$$

$$\frac{1}{2(x_0 - 2A_t q)} = kt + C$$

where q is the reciprocal of ε. If A_1 is the absorbance at time t_1 and $\Delta t = t_2 - t_1$, then

$$\frac{1}{2(x_0 - 2A_2 q)} - \frac{1}{2(x_0 - 2A_1 q)} = k\Delta t$$

For times t_3 and t_4, where $t_4 - t_3$ is the same Δt, then

$$\frac{1}{2(x_0 - 2A_4q)} - \frac{1}{2(x_0 - 2A_3q)} = k\Delta t$$

$$\frac{1}{2(x_0 - 2A_2q)} - \frac{1}{2(x_0 - 2A_1q)} = \frac{1}{2(x_0 - 2A_4q)} - \frac{1}{2(x_0 - 2A_3q)}$$

$$\frac{(x_0 - 2A_1q) - (x_0 - 2A_2q)}{(x_0 - 2A_2q)(x_0 - 2A_1q)} = \frac{(x_0 - 2A_3q) - (x_0 - 2A_4q)}{(x_0 - 2A_4q)(x_0 - 2A_3q)}$$

$$\frac{A_2q - A_1q}{(x_0 - 2A_2q)(x_0 - 2A_1q)} = \frac{A_4q - A_3q}{(x_0 - 2A_4q)(x_0 - 2A_3q)}$$

This gives a cubic equation which can be solved giving 3 solutions. One solution is $q = 0$, there is a false one and one real one. A range of values of q were obtained by taking a number of combinations of A_1, A_2, A_3 and A_4, keeping Δt constant.

4.3.2 Kinetic Measurements

The spectral changes accompanying the conversion of a 3 mM solution of ALA into 2,5-di-(β-carboxyethyl) pyrazine at 37 °C are shown in Fig. 4.7.

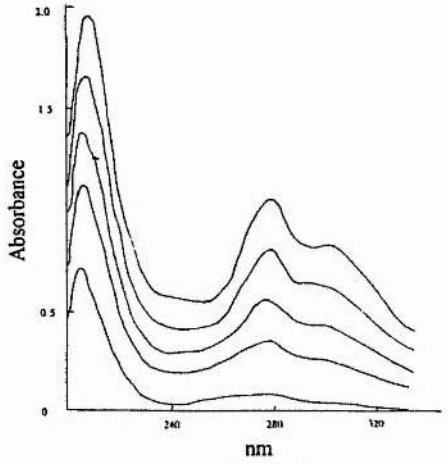


Figure 4.7 - Spectral changes associated with the conversion of ALA into 2,5-di-(β-carboxyethyl) pyrazine.

The absorbance at 280 nm was measured at timed intervals and those values were used to monitor the progress of the reaction in terms of concentration. It is certain that the dimerisation of ALA reaction does not stop at the dihydropyrazine but there is rapid aerial oxidation to the pyrazine (**17**). This was shown to be a product of reaction by Jaffe and Rajagopalan[408] and the dihydropyrazine has only been observed by NMR under the most stringent anaerobic conditions.[409] Various combinations of absorbance values at 280 nm were used to generate the derived cubic equation and this, on solution, gave a range of values for the extinction coefficient of 2,5-di-(β-carboxyethyl) pyrazine of 1361 ± 260 $dm^3mol^{-1}cm^{-1}$.

4.3.3 Conclusions

The derived value of ε was applied to absorbance data for the dimerisation of ALA at both 3.0 mM and 0.3 mM and the results are displayed in Fig. 4.8.

Initial [ALA]/ mM	Time/ mins.	% dimerisation.
3	30	2
3	60	5
3	90	7
3	120	12
0.3	30	3
0.3	60	4
0.3	90	6
0.3	120	8

Figure 4.8 - Table showing dimerisation of a 3.0 or 0.3 mM solution of ALA at a given time.

The significant conclusion is that, in a solution containing concentrations of ALA necessary for PDT, substantial amounts of dimerisation will occur during the lifetime of a clinic and so the dose of ALA being given depends upon the age of the solution. This is unsatisfactory and we have also been working on modifying ALA in ways which will still allow it to be incorporated into the biosynthetic pathway for haem production but which prevent dimerisation and browning in solution during storage (See Chapters 6 and 7).

4.4 Studies on the Dimerisation of ^{15}N Labelled 5-Aminolevulinic Acid by Solution State NMR

^{15}N labelled ALA.HCl was prepared using the method of Iida, Takao, Ogai and Kajiwara.[410] (Scheme 2.6), (Reactions 8.13, 8.14 and 8.15) using ^{15}N labelled potassium phthalimide. This was used to investigate the kinetics of dimerisation of ALA. Kinetic studies using NMR spectroscopy have the distinct advantage that starting materials, intermediates and products can be identified and characterised. The reaction can be followed closely. The spectra of ^{15}N ALA (50 mg) dissolved in phosphate buffer (pH 7, 8 and 11, 2 cm^3) with a few drops of ^2H_2O to provide a lock signal was recorded each hour for 15 hours at room temperature (20 °C). In all three cases no dimerisation was observed. The ^{15}N spectra of ALA with one sharp peak at δ 25.75 was identical 15 hours after preparation of the solution to its initial spectra. The solutions remained colourless. Dimerisation reactions are highly concentration dependent. By multiplying the concentration by two, the amount of dimer will increase four fold. These ALA solutions were 0.15 M which is much less concentrated than a clinically useful solution. The result is, however,

surprising and appears to contradict the results found using the derived equation and UV/visible spectroscopy (Section 4.3). Temperature is an important factor. UV/visible measurements were recorded at 37 °C. An NMR study at higher temperatures would be an interesting exercise. When the samples were heated using a heat gun they became a darker brown colour. ^{15}N NMR spectra showed only one sharp peak, this time at δ 274.88, a value consistent with pyrazine formation.[411] This study indicates that if clinically useful solutions could be kept cold and were less concentrated, they may not dimerise as readily. Further work, however, is required to optimise conditions for the storage of solutions of ALA.

4.5 Investigation of Other Dimerisation Reactions

As part of this study to modify ALA chemically to make it more acceptable for clinical use we thought it would be of value to investigate further the mechanism of a dimerisation reaction.[409, 412]

4.5.1 Kinetics of the Dimerisation of 2'-Amino-2-Hydroxyacetophenone

The UV/visible spectral changes accompanying the conversion of ALA to 2,5-di-(β-carboxyethyl) pyrazine are very small and the reaction is complicated by the formation of a brown colour, thus we are denied one of the most useful tools in the study of reaction mechanism. To counteract this problem we have also investigated the spectral changes accompanying a similar dimerisation reaction involving a compound incorporating a chromophore. Such a reaction is the

conversion of 2'-amino-2-hydroxyacetophenone into 2,5-dihydro-2,5-di-(2'-hydroxyphenyl) pyrazine (Fig. 4.9).

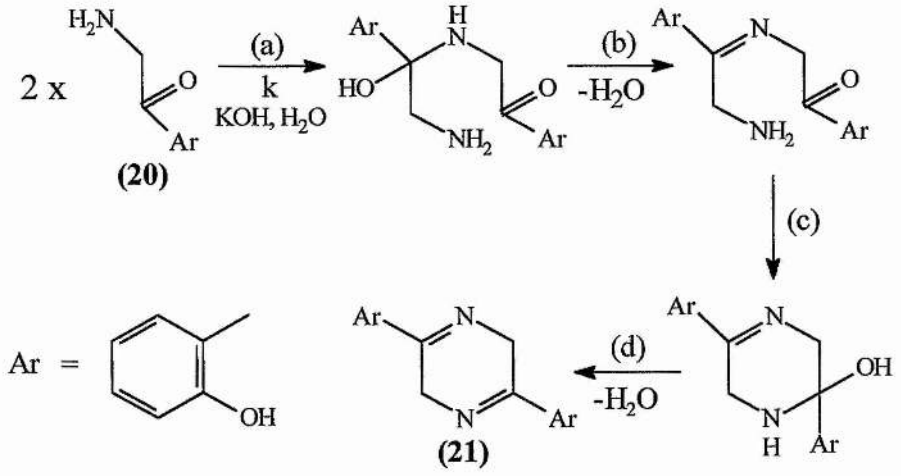
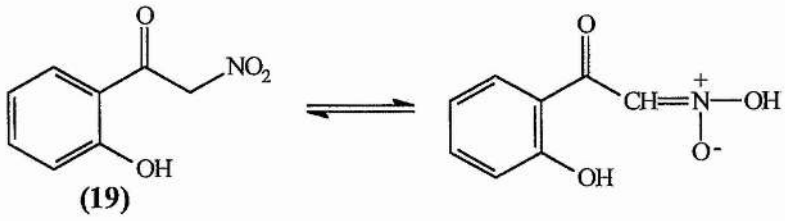


Figure 4.9 - The dimerisation of 2'-amino-2-hydroxyacetophenone.

4.5.1.1 Synthetic Discussion

Initially, the route of Huebner and Link [413] for the preparation of 2'-amino-2-hydroxyacetophenone was followed. 4-Hydroxycoumarin was nitrated using nitric acid in acetic acid in good yield to give 3-nitro-4-hydroxycoumarin (**18**) (Reaction 8.25). Considerable difficulty was found, however, in reproducing the next stage of the reaction. Huebner and Link prepared 2'-amino-2-hydroxyacetophenone hydrochloride by the reaction of 3-nitro-4-hydroxycoumarin (**18**) with hydriodic acid in acetic acid, using hypophosphorous acid to reduce the resulting iodine (Reaction 8.26). After many different attempts, an alternative method was found.[414] 3-Nitro-4-hydroxycoumarin was reacted with aqueous NaOH, then c-HCl, to form 2-hydroxy-2'-nitroacetophenone (**19**) (Reaction 8.27). The NMR spectra of this compound proved to be interesting. Two sets of signals were

visible in the aromatic region and two carbonyl signals were detected in the ^{13}C spectrum. Addition of $^{2}H_6$-DMSO to the pale yellow solid caused the formation of a bright yellow solution. We think that the nitronic acid form of the molecule is being produced in solution in approximately a 1:1 ratio with the expected product. The CH=N+ is not visible in the spectrum (Fig. 4.10).



(19)

Figure 4.10 - Formation of nitronic acid from 2-hydroxy-2'-nitroacetophenone.

The 2-hydroxy-2'-nitro-acetophenone (**19**) was then converted to 2'-amino-2-hydroxyacetophenone (**20**) using tin chloride in HCl (Reaction 8.28). A sample of 2,5-dihydro-2,5-di-(2'-hydroxyphenyl) pyrazine (**21**) was prepared (Reaction 8.29) by dissolving 2'-amino-2-hydroxyacetophenone (**20**) in KOH solution giving the product (**21**) as a highly insoluble, therefore, uncharacterised pink solid (Fig. 4.11).

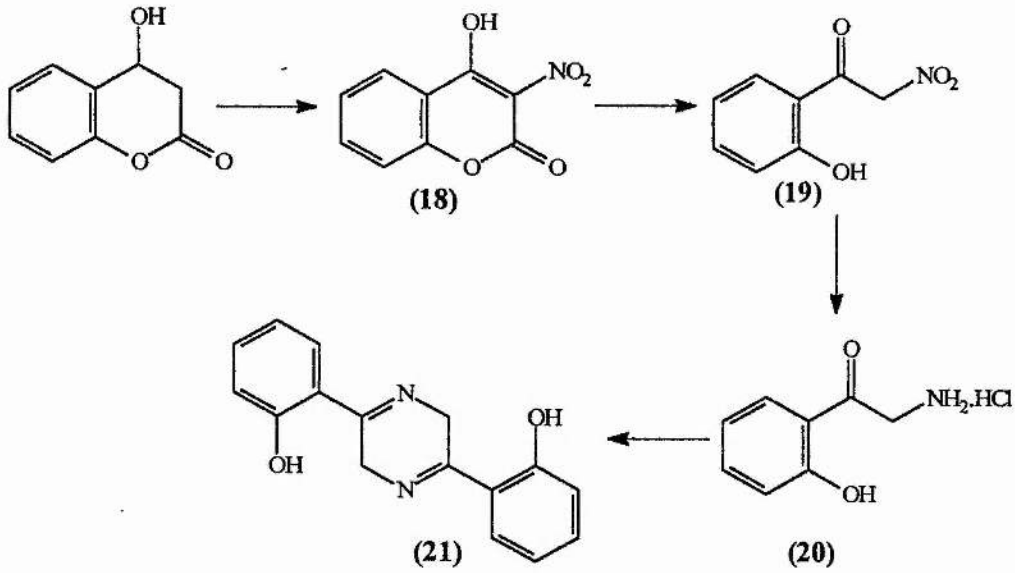


Figure 4.11 - Scheme for the preparation of 2,5-dihydro-2,5-di-(2'-hydroxyphenyl) pyrazine.

4.5.1.2 Kinetic Investigation

The reaction must occur in a number of steps; a possible sequence is shown in Fig. 4.9. More than one of the steps labelled (a-d) could be slow but a single slow step should be indicated by a tight isosbestic point as the spectrum of the reaction mixture changes with time. This was found to be the case (Fig. 4.12).

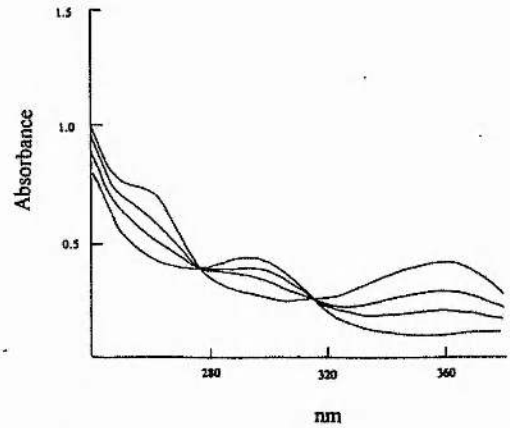


Figure 4.12 - UV/visible spectrum of the dimerisation reaction of 2'-amino-2-hydroxy-acetophenone showing the tight isosbestic point.

The most likely situation, and one that has been observed in other reactions of this type[412] is that (a) is the slow, rate-determining step and this should give rise to simple, second order kinetics. Simplification of the situation to generate pseudo-first order conditions is, again, not possible. As both reactant and product absorb at wavelengths suitable for monitoring the reaction, there is no direct way of converting observed absorbance into concentration of reactant for substitution into an integrated rate equation. However, the extinction coefficients for the starting material and product (ε_1 and ε_2) were readily determined and some trivial algebra indicates how $[SM]_t$ (the concentration of the starting material at time t) may be deduced from A_t (the absorbance at time t)

$$A_t = [SM]_t \varepsilon_1 + [P]_t \varepsilon_2$$

$$= [SM]_t \varepsilon_1 + 0.5 ([SM]_0 - [SM]_t) \varepsilon_2$$

$$= [SM]_t (\varepsilon_1 - 0.5 \varepsilon_2) + 0.5 [SM]_0 \varepsilon_2$$

$$[SM]_t = \frac{2A_t - [SM]_0\varepsilon_2}{2\varepsilon_1 - \varepsilon_2}$$

The term $[SM]_0$ is the initial concentration of the starting material and 2 appears in this equation because two moles of starting material give only one mole of product, P. From the observed values of A_t it was possible to calculate [SM] as a function of time. For a second order reaction with one reactant, a plot of $1/[SM]_t$ against time should be linear with a slope of k. This plot is shown in Fig. 4.13.

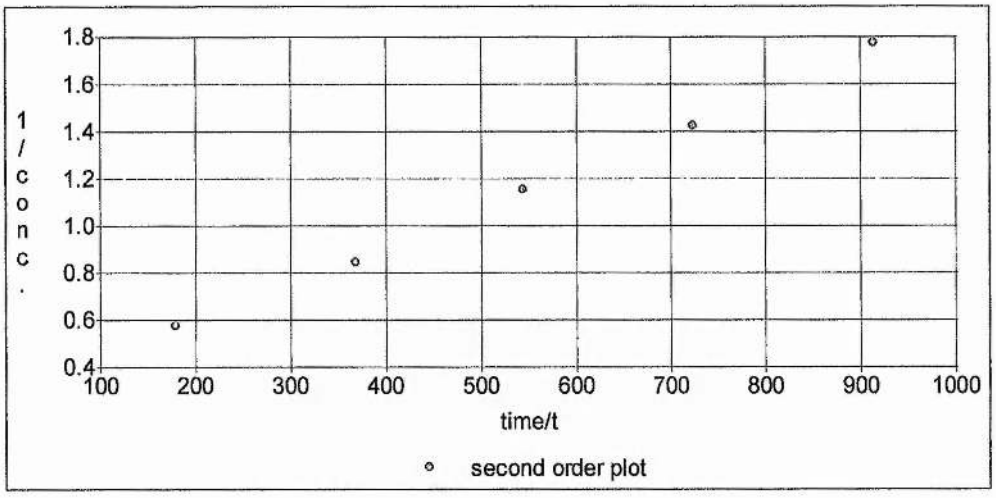


Figure 4.13 - Second order plot of $1/[SM]_t$ against time.

The linearity indicates the second order nature of the reaction and the value of k, taken from the slope, is 0.26 $lmol^{-1}s^{-1}$. Thus the observed kinetics are consistent with the mechanism shown in Fig. 4.9 It is not unreasonable to assume that the mechanism does not involve, for example, the slow enolisation of one molecule of the starting material and rapid reaction with a second molecule.

4.5.2 The Dimerisation of Acetoacetamide

One of the driving forces in the dimerisation of ALA is the nucleophilicity of the amine group so the reaction is prevented by storing the material as the HCl salt. We wondered if compounds containing an amide rather than an amine would show the same readiness to dimerise and selected acetoacetamide for study (Fig. 4.14).

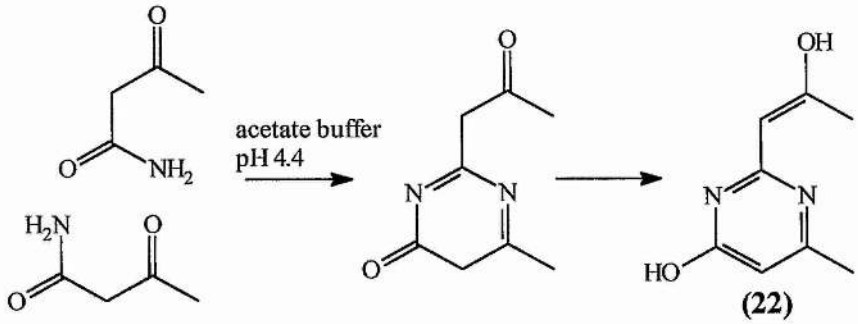


Figure 4.14 - The dimerisation of acetoacetamide.

This material was heated under reflux conditions in acetate buffer and the reaction mixture allowed to stand for some days to give a small quantity of a white crystalline product (Reaction 8.30). All the analytical data points to dimerisation with elimination of water and the NMR spectrum suggests formation of 2-(acetylmethyl)-6-methylpyrimidin-4-one (**22**) with the particular tautomeric form shown in Fig. 4.14. The absence of signals corresponding to methylene groups and the presence of a signal most readily assigned to an olefinic proton are the main pieces of evidence to support this identification. We have been unable to find any references in the chemical literature to a previous observation of this reaction, although the low yield obtained hardly makes it an acceptable synthetic route in the unlikely event of the product ever being required. The mechanism of formation must involve reaction of an amide NH_2 group with both the carbonyl of another amide and an isolated carbonyl and the driving force must be the creation of an aromatic ring. The existence of the exocyclic olefinic double bond is to allow conjugation with theheteroaromatic ring (Fig. 4.15).

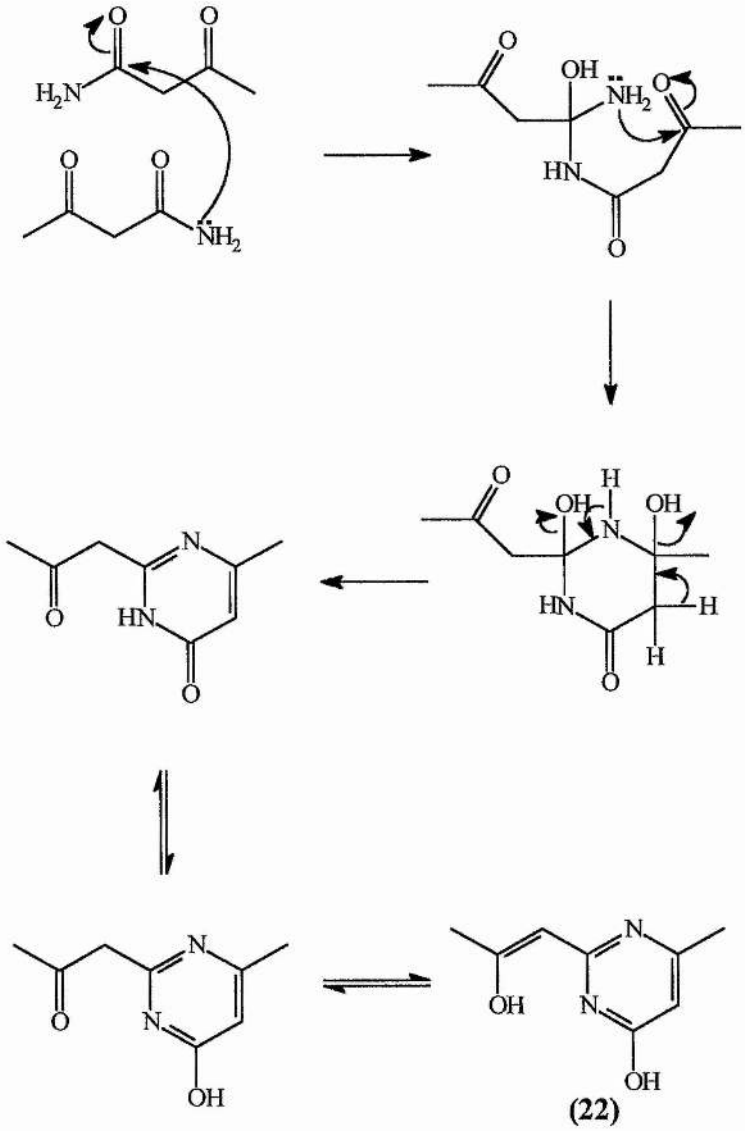


Figure 4.15 - Mechanism for the formation of 2-(acetylmethyl)-6-methylpyrimidin-4-one.

4.6 References

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Chapter 5

The Chemistry of Dihydropyrazines

5.1 Introduction

The initial product formed in the non-enzymatic dimerisation of ALA is a dihydropyrazine (Fig. 5.1). 2,5-Di-(β-carboxyethyl) dihydropyrazine is oxidised immediately in air forming the more stable aromatic pyrazine. The dihydropyrazine has been detected only during anaerobic NMR studies.[501, 502] To try to understand the chemistry of dihydropyrazines we have attempted to prepare a series of more stable derivatives. This chapter deals with the synthesis, use and structure of dihydropyrazines.

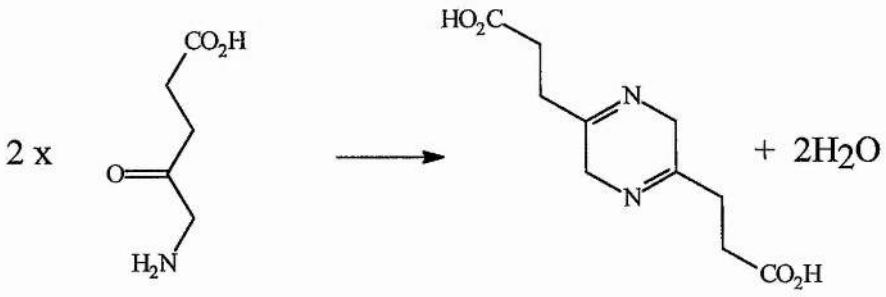


Figure 5.1 - Dimerisation of ALA yielding 2,5-di-(β-carboxyethyl) dihydropyrazine.

5.2 Literature Review of the Synthesis of Dihydropyrazines

All reactions involving preparation of dihydropyrazines must be done under anaerobic conditions to prevent aerial oxidation to the corresponding pyrazine.

5.2.1 Synthesis of 2,5-Dihydropyrazines by the Self-Condensation of α-(Primary Amino) Carbonyl Compounds

The first reported synthesis of a 2,5-dihydropyrazine was in 1902 when aminoacetone, in the presence of potassium, was found to dimerise giving 2,5-dihydro-2,5-dimethyl pyrazine (Fig. 5.2).[503]

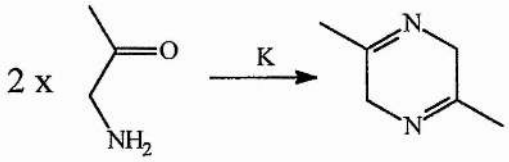


Figure 5.2 - The first reported synthesis of a dihydropyrazine.

In a reaction analogous to the self-condensation of two molecules of ALA, 2,5-dihydropyrazines can be prepared from α-(primary amino) carbonyl compounds. A more recent example is the reaction of alkyl ketones with warm aqueous alkaline potassium ferricyanide to give α-aminoketones. Depending on the reaction conditions used during work-up, either the hydrochloride salt of the α-aminoketone, or a 2,5-dihydropyrazine can be formed (Fig. 5.3).[504]

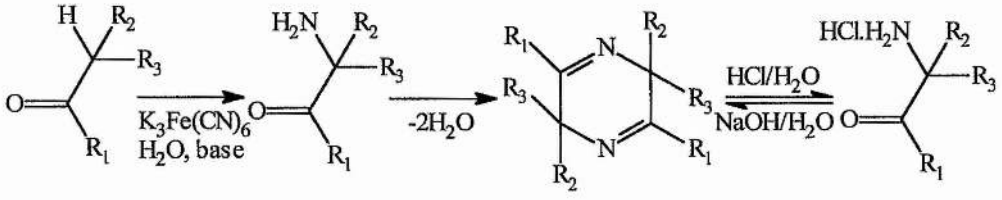


Figure 5.3 - Synthesis of 2,5-dihydropyrazines from alkyl ketones.

Phase transfer catalysis can be used to assist the condensation reaction.[505] Heating the ortho esters of some amino acids also results in dihydropyrazine formation (Fig. 5.4).[506]

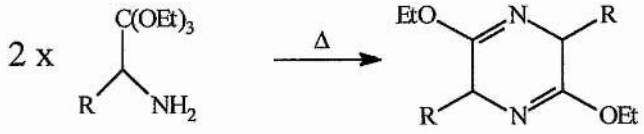


Figure 5.4 - Synthesis of a 2,5-dihydropyrazine from the ortho ester of an amino acid.

5.2.2 Synthesis of 2,5-Dihydropyrazines from 3-Membered Heterocyclic Rings

Azirines dimerise, on standing or heating, to give 2,5-dihydropyrazines (Fig. 5.5).[507]

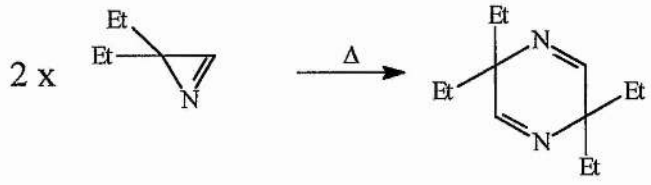


Figure 5.5 - Dimerisation of an azirine to give 2,5-dihydro-3,3,6,6-tetraethylpyrazine.

2-Chlorooxirane can also be converted into a 6 membered heterocycle by forming an α-aminoketone followed by dimerisation (Fig. 5.6).[508]

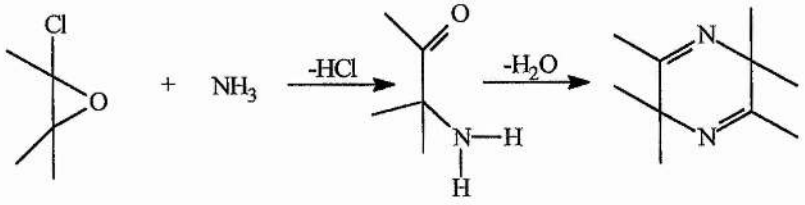


Figure 5.6 - Synthesis of dihydropyrazines from 2-chlorooxiranes.

5.2.3 Preparation of 2,5-Dihydropyrazines via Diketopiperazines

By far the most commonly used route for the preparation of 2,5-dihydropyrazines uses Schöllkopf type chemistry.[509] A diketopiperazine (piperazine-2,5-dione, cyclic dipeptide) can be prepared by coupling a tertiary butoxycarbonyl (t-Boc) protected amino acid to an amino acid protected at the acid functionality as an ester. Formic acid can then be used to generate a dipeptide ester formate which, after reflux in toluene and butanol yields a diketopiperazine (Fig. 5.7).[510]

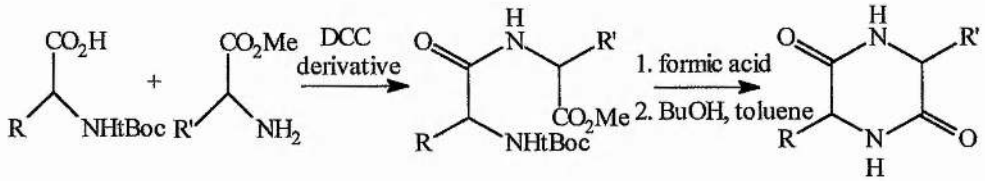


Figure 5.7 - Formation of a diketopiperazine.

Exposure of the dipeptide methyl ester to methanol-ammonia for up to 5 days also yields a diketopiperazine but these conditions can lead to racemisation.[511] Diketopiperazines are converted into 2,5-dihydropyrazines using Meerwein's Salts (triethyloxonium or trimethyloxonium tetrafluoroborate) (Fig. 5.8). [509, 512-518]

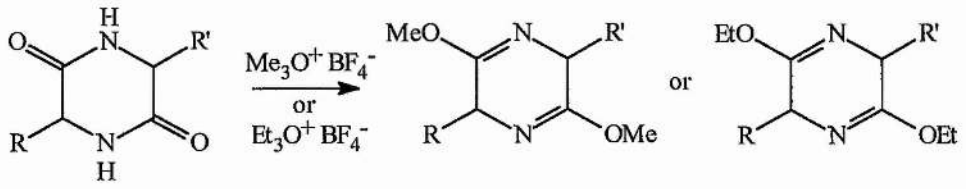


Figure 5.8 - Reaction of a diketopiperazine with Meerweins Salts.

We have used α-aminoketones and Schöllkopf chemistry to generate a series of 2,5-dihydropyrazines. Azirine chemistry was avoided due to the instability and toxic nature of the molecules.

5.3 Applications of Dihydropyrazine Chemistry

Most of the work published concerning dihydropyrazine chemistry is applicable to three main areas of research. These are the browning of foods during the Maillard reaction,[519] studies on antiaromaticity and the synthesis of chiral amino acids.

5.3.1 The Maillard Reaction[519]

The Maillard, or non-enzymatic browning reactions are some of the major pathways causing the flavourings and aromas of cooked foods. The Strecker degradation is one of the most important reaction schemes leading to compounds with an aroma (Fig. 5.9).

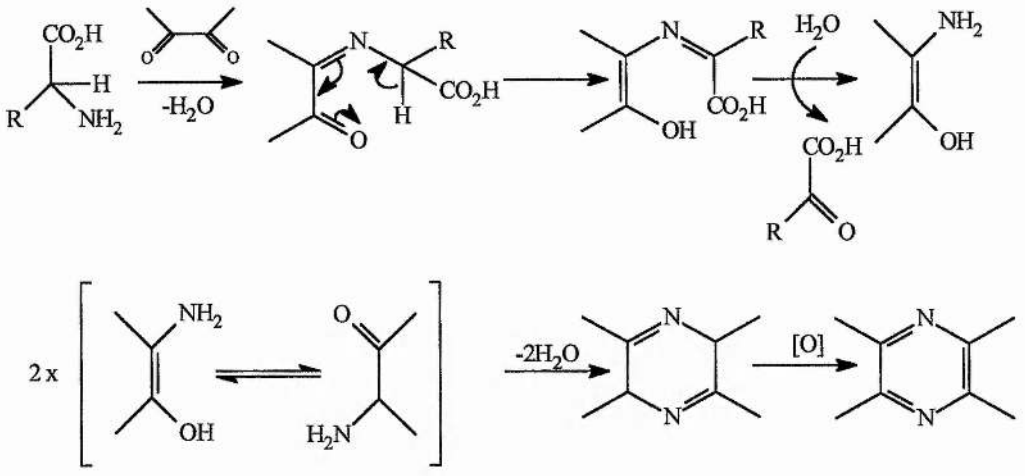


Figure 5.9 - The Strecker Degradation.

It involves a Schiff base formation between an α-dicarbonyl compound and an amino acid. Rearrangement, decarboxylation and hydrolysis yield an α-aminocarbonyl compound, a precursor of dihydropyrazines.[519]

5.3.2 Antiaromaticity

A range of dihydropyrazines have been prepared to assist in the study of antiaromaticity. Cyclic systems containing 4n + 2 π electrons, such as benzene, are defined as aromatic and are very stable. Cyclic conjugated systems with 4n π electrons, on the other hand, are strongly destabilised and are classed as 'antiaromatic'.[520] The dihydropyrazine ring system has been studied as it contains 8 π electrons, resulting in a potential antiaromatic nature.[521]

5.3.3 Chiral Amino Acid Synthesis

Many dihydropyrazines have been prepared as intermediates in the asymmetric syntheses of amino acids. Functional groups can be added to dihydropyrazines via the alkylation of lithiated bis-lactim ethers. Hydrolysis then yields the optically active amino acids (Fig. 5.10).[515]

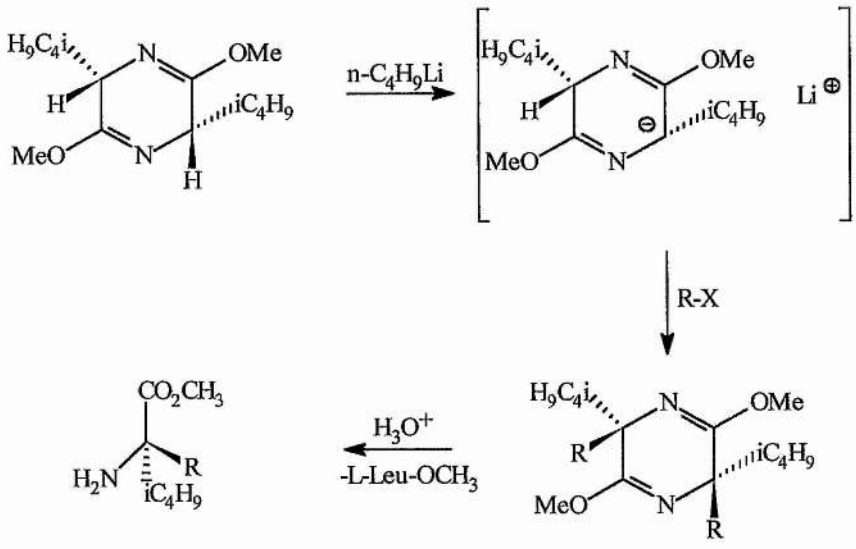


Figure 5.10 - Chiral amino acid synthesis using a dihydropyrazine.

5.4 Experimental Discussion

5.4.1 Dihydropyrazines from the Reaction of an Alkyl Ketone with Potassium Ferricyanide

Initially, the route of Farnum and Carlson[504] (Fig. 5.3) using an alkyl ketone and potassium ferricyanide was used to try to prepare a series of dihydropyrazines. Various ketones were reacted with potassium ferricyanide in water in the presence of ammonia to form the α-(primary amino) carbonyl compound which can lose

two molecules of water to form a dihydropyrazine. The reaction was attempted with 3-methyl-2-butanone (Reaction 8.31), ethyl methyl ketone (Reaction 8.32) and acetone (Reaction 8.33). The results of these reactions are summarised in Fig. 5.11.

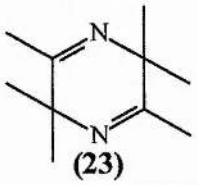
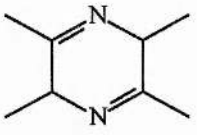
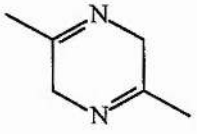
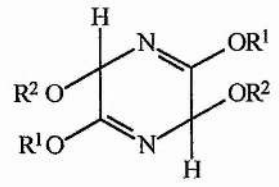
Ketone	Expected Product	Actual Product
3-methyl-2-butanone	 **(23)**	As expected
ethyl methyl ketone		Pyrazine
acetone		None

Figure 5.11 - Summary table of reactions of alkyl ketones with potassium ferricyanide.

Unfortunately, if an exchangeable proton was present, it was possible to isolate only the pyrazine or nothing at all. The yields for the reactions were very low and, as a ten times excess of potassium ferricyanide was required, very wasteful. This approach was abandoned.

5.4.2 Dihydropyrazines from Diketopiperazines

A range of dihydropyrazines was prepared using Schöllkopf chemistry (Fig. 5.7 and 5.8). The amino acids glycine, L-alanine, L-phenylalanine and L-valine were t-Boc protected (Reactions 8.34 (**24**), 8.35 (**25**), 8.36 (**26**), and 8.37 (**27**)) using di-t-butyl pyrocarbonate, then coupled to the methyl ester of the same amino acid (Reactions 8.38 (**28**), 8.39 (**29**), 8.40 (**30**) and 8.41 (**31**)) using a derivative of dicyclohexyl carbodiimide (DCC), 1-cyclohexyl-3,2-morpholinoethyl-p-toluene sulphonate (CHME).[522] Treatment with formic acid removed the t-Boc group causing the molecule to cyclise giving a series of diketopiperazines (Reactions 8.42 (**32**), 8.43 (**33**), 8.44 (**34**) and 8.45 (**35**)).[510] The diketopiperazines were reacted in the presence of Meerwein's Salts, trimethyloxonium tetrafluoroborate[513] (Reactions 8.46 (**36**), 8.47 (**37**), 8.48 (**38**) and 8.49 (**39**)) and triethyloxonium tetrafluoroborate[516] (Reactions 8.50 (**40**) and 8.51 (**41**)) to give a range of dihydropyrazines (Fig. 5.12). The NMR spectra and structures of these compounds are discussed in Section 5.6.



Amino Acid	Compound Number	R^1	R^2
glycine	**(36)**	CH_3	H
L-alanine	**(37)**	CH_3	CH_3
L-phenylalanine	**(38)**	CH_3	CH_2Ph
L-valine	**(39)**	CH_3	$CH(CH_3)_2$
glycine	**(40)**	CH_2CH_3	H
L-alanine	**(41)**	CH_2CH_3	CH_3

Figure 5.12 - Table of dihydropyrazines.

5.5 Literature Review of the Structures of Dihydropyrazines

The simplest, unconjugated known dihydropyrazine which does not incorporate stabilising groups e.g. phenyl or hydroxyphenyl is 2,5-dihydro-2,5-dimethylpyrazine. Three structures for this molecule are possible (Fig. 5.13).[523]

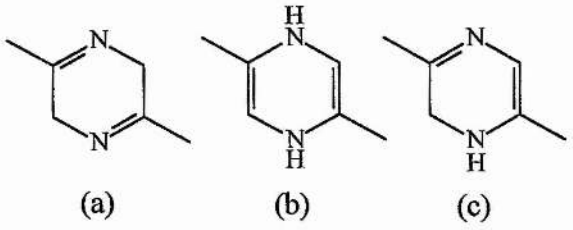


Figure 5.13 - The three possible structures for 2,5-dihydro-2,5-dimethylpyrazine.

The compound was first synthesised by Gabriel and Colman in 1902.[503] Structure (a) is generally assumed to be correct,[524] although structure (b) has also been suggested.[525] Using IR spectroscopy, in 1958, it was suggested that either a mixture of (a) and (b) was present or (c) alone. These conclusions were based upon the presence of a strong band in the IR spectrum at 3260 cm^{-1}, which was interpreted as an NH stretch. This band was later found to be due to the compound being wet.[523] NMR spectroscopy in 1970 concluded that the structure of 2,5-dihydro-2,5-dimethylpyrazine was (a). Small peaks were observed which were assumed to be due to the presence of (b) or (c). As most of the work concerning the structures of dihydropyrazines was done in the 1960s before NMR was a readily available tool, a series of dihydropyrazines were prepared and their NMR and IR spectra used to try to probe their structures in solution (See Section 5.6).

The conformation of the dihydropyrazine ring was investigated by Blake *et al* in 1972.[526] They found that reaction of phenylalanine anhydride with the Meerwein's Salt, triethyloxonium tetrafluoroborate, yielded a cis/trans mixture of dihydropyrazines. They found that extensive isomerisation occurred during the reaction. (Fig. 5.14).

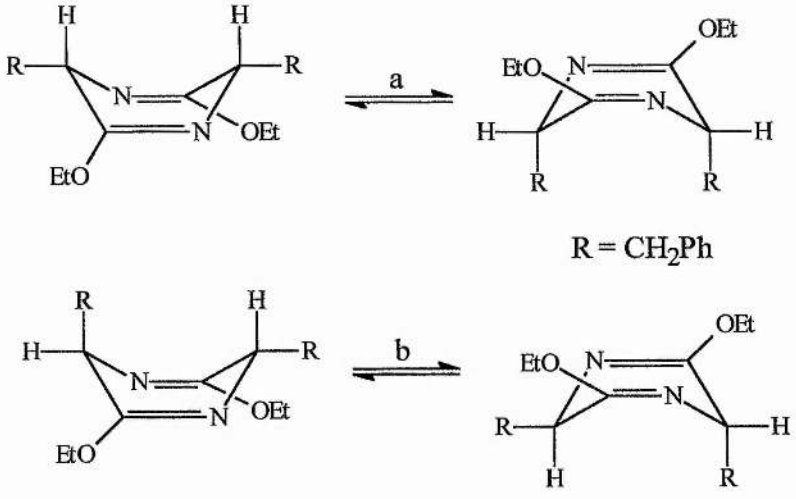


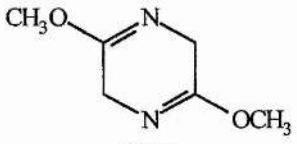
Figure 5.14 - Conformation of a dihydropyrazine which had isomerised during synthesis.

Using variable temperature NMR they concluded that the major isomer is the trans form (Fig. 5.14b). They concluded also that, if there were rapid equilibrium between the two boat conformers, the trans isomer would be equilibrating with an identical conformer (Fig. 5.14b) and temperature change would not affect the ratio. The cis isomer (Fig. 5.14a), however, would equilibrate with a different conformer hence change in temperature would alter the ratio.[526.] We have compared the NMR spectra of compounds containing methyl and ethyl groups where R is either H or CH_3 to try to investigate this further (see Section 5.6).

5.6 NMR Studies on the Structures of Dihydropyrazines

The proton and carbon NMR spectra of 2,5-dihydro-2,5-dimethoxypyrazine (**36**) are very simple. The molecule is symmetrical giving only two singlet peaks in the proton NMR corresponding to OCH_3 and CH_2 and three peaks in the carbon

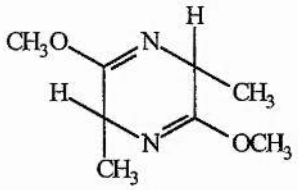
NMR corresponding to OCH_3 CH_2 and $COCH_3$. Regardless of the solvent, solution strength or time only these peaks were visible. On occasion, after some time, additional peaks corresponding to the oxidised pyrazine were detected. No NH stetch was observed in the IR, hence, we conclude that the structure of the molecule is as shown in Fig. 5.15.



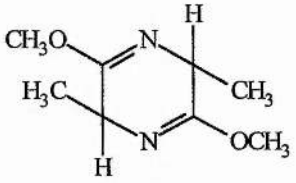
(36)

Figure 5.15 - Structure of 2,5-dihydro-2,5-dimethoxypyrazine.

The proton and carbon NMR spectra of 2,5-dihydro-2,5-dimethoxy-3,6-dimethylpyrazine (**37**) were much more complex. The proton peaks were split and carbon peaks were all doublets indicating cis trans isomerisation. There was still no evidence for any formation of structures similar to those shown in Fig. 5.13b and c. We conclude that the structure of the molecule is as shown in Fig. 5.16.



(37)



(37)

Figure 5.16 - Cis and trans isomers of 2,5-dihydro-2,5-dimethoxy-3,6-dimethylpyrazine.

The compounds prepared from L-phenylalanine, 3,6-dibenzyl-2,5-dihydro-2,5-dimethoxypyrazine (**38**), and from L-valine, 2,5-dihydro-2,5-dimethoxy-3,6-

dipropylpyrazine (**39**), were less susceptible to aerial oxidation than the less hindered compounds but their NMR spectra were even more complicated. There was no evidence for the formation of any compound containing an NH group.

We conclude that dihydropyrazines of this type are in the form shown in Fig. 5.17a and not as in Fig. 5.17b or c.

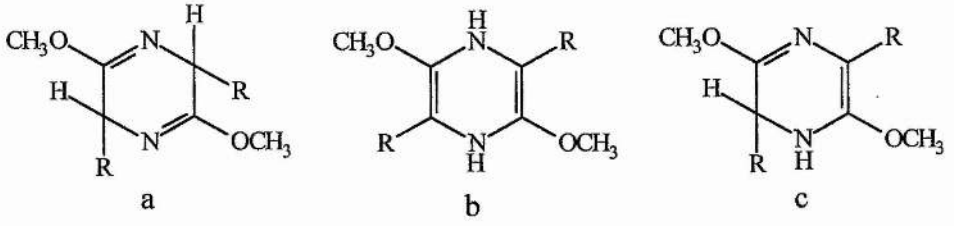


Figure 5.17 - Potential structures of a dihydropyrazine in solution.

The use of triethyloxonium tetrafluoroborate added another degree of complication to the NMR spectra of these compounds. 2,5-diethoxy-2,5-dihydropyrazine (**40**) and 2,5-diethoxy-2,5-dihydro-3,6-dimethylpyrazine (**41**) were prepared. These compounds were both found to be in the same conformation as the molecules synthesised from trimethyloxonium tetrafluoroborate but had even more complex spectra, presumably due to additional couplings between the various groups around the molecule. Their NMR spectra have not been fully assigned as this would be beyond the scope of the study.

5.7 Conclusions

Dihydropyrazines are extremely difficult molecules to work with. In the absence of stabilising groups they are readily oxidised to give the corresponding aromatic pyrazine compound. They must, therefore, be handled under nitrogen at all times. All the dihydropyrazines that we prepared were in the same conformation, although the Meerwein's Salts caused isomerisation, complicating the study. There are two feasible reasons why dihydropyrazines are in this form. Firstly, a C=N bond is, energetically, stronger than a C=C bond making the molecule more stable. The other reason is due to a 'push-pull' mechanism.[527] This concept provides some stability to otherwise unstable systems. An example can be seen in the cyclobutadiene ring system which is stabilised by a push-pull effect between the electron-donating amino groups and the electron-withdrawing ester groups (Fig. 5.18).

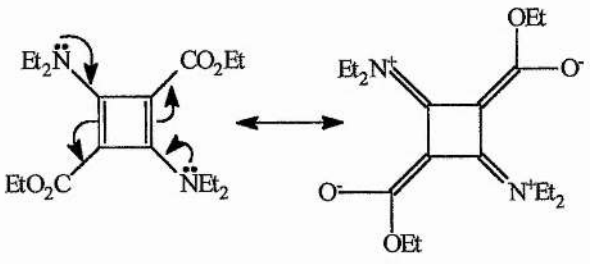


Figure 5.18 - Stabilisation of a cyclobutadiene ring system by a push-pull mechanism.

A similar argument can be used for the dihydropyrazine ring system (Fig. 5.19).

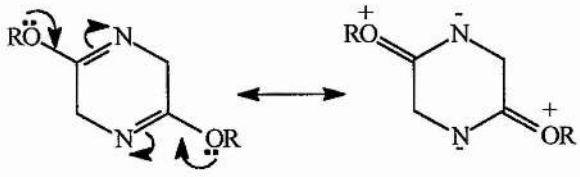


Figure 5.19 - Stabilisation of a dihydropyrazine ring system by a push-pull mechanism.

As 2,5-di-(β-carboxyethyl) dihydropyrazine contains no stabilising groups it is very unlikely that it would be present for any length of time. It would be immediately oxidised to give 2,5-di-(β-carboxyethyl) pyrazine making the reaction irreversible. It would, potentially, be possible for ALA to be in equilibrium with its dihydropyrazine but oxidation to the pyrazine is an irreversible step, causing the percentage of ALA in solution to gradually decrease with time.

5.8 References

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Chapter 6

Synthesis of Derivatives of 5-Aminolevulinic Acid

6.1 Introduction

Although ALA induced PpIX is proving to be one of the more promising techniques in PDT, there are still problems associated with its use. Solutions of ALA go dark brown over time, probably due to a polymerisation reaction (See Chapter 4 and Section 6.5). ALA may also dimerise, in the absence of the enzyme PBG synthase, to give a pyrazine (See Chapter 4). A third problem is the low bioavailability of ALA. Consequently, very high doses are required to produce clinically useful PpIX levels.[601]

ALA exists as a zwitterion at physiological pH (Fig. 4.1), so has very low lipid solubility. This means that it is inefficient at passing through biological barriers, e.g. walls of the stomach or intestine, the stratum corneum (the outer layer of the skin) and cellular membranes.[602] ALA has limited penetration ability through the skin and, as porphyrins are produced in the deepest layers of lesions, there is a need to enhance both ALA absorption and porphyrin production in tumours.[603]

Fortunately, penetration of ALA into normal, healthy skin is poorer than in malignant lesions or in skin which has had chronic sun damage. Also, chemicals such as DMSO can be used to enhance its penetration capacity.[604] However, there is still a need for pro-drugs which have an enhanced lipophilicity. The esters of ALA have proved useful in this respect. By forming an ester, ALA no longer has a negative charge under physiological conditions.[605] It is known that increasing the number of carbon atoms in a chain attached to an existing drug results in an increased lipophilicity of that drug[602] but it is not known how ALA is taken up

into cells. Various non-specific routes such as diffusion, or transport by a specific membrane protein, have been suggested. It is possible, therefore, that the uptake of ALA into cells could be modified by chemically changing groups on the molecule. It is known that transport proteins recognise their substrates by the charged groups and so, by altering these, ALA uptake may be enhanced (or inhibited), or the molecule may be recognised by another transporter. If there is a transport protein which is responsible for regulating levels of ALA, export of the molecule from the cell may be inhibited allowing levels of ALA to increase. This would be advantageous for PDT.[606]

From a chemical perspective, addition of groups to the acid end of ALA such as esters, or amino acids might stop polymerisation and the associated browning from occurring. A better solution, however, would be to attach groups to the amino end stopping dimerisation. The ideal compound would be stable and non-toxic until the appropriate enzymes in the body cleaved the inactive group from ALA leaving the active compound and some other non-toxic group, e.g. an amino acid, sugar or alcohol. Biologically, addition of esters, amino acids, sugars and long chain alkyl groups could enhance the bioavailability of the molecule.

A series of twelve derivatives, with a mixture of functional groups, have been prepared and tested biologically for activity as pro-drugs in PDT.

6.2 Literature Review of 5-Aminolevulinic Acid Pro-Drugs

Only recently has an interest in derivatives of ALA as pro-drugs for PDT developed. The first paper on the subject was published in 1996.[602] Work has centred on alkyl esters of ALA (ALA-methyl ester to ALA-octyl ester),[601 - 604,606] with varied success. In general, shorter chained alcohols (ALA-methyl and ethyl esters) are less effective than ALA itself.[604,606] The longer chained esters, however, particularly the hexyl ester, have been found to be efficient. Prodrugs with long chains are hydrolysed more slowly than ones with a shorter chain but have been shown to penetrate deeper into the cell.[601]

Some success has been found with the tetrahydrofurfuryl and tetrahydropyran esters. N-Acetyl-ALA, however, has been found to be a poor pro-drug for PDT sensitisation.[602] All the alkyl esters and N-alkanoyl protected derivatives of ALA have been patented in Japan for use as potential plant growth regulators,[607, 608] but not, as yet, for use as pro-drugs in PDT.

6.3 Experimental Rationale

Firstly, it was necessary to be able to synthesise alkyl esters of ALA.HCl, for use in both biological testing and as synthetic precursors for NH_2 protected derivatives of ALA.

Initial attempts to prepare ALA-methyl (ALA-OMe) and ALA-ethyl (ALA-OEt) esters followed on from experience gained during the synthesis of ALA.HCl itself

(See Chapter 2). Methyl 5-phthalimidolevulinate was prepared as before (Reactions 8.1 (**1**) and 8.4 (**4**)). Likewise, ethyl 5-phthalimidolevulinate (**43**) was synthesised by substituting ethanol for methanol in the initial bromination reaction (Reactions 8.52 and 8.53). Phthalimide was then removed, leaving the ester intact, using N,N-dimethyl-1,3-propanediamine (Reactions 8.54 and 8.55) giving a very poor yield of both the esters (**44**) and (**46**). The majority of the product in each case was found to be a pyrazine (**45**) and (**47**) (Fig. 6.1).

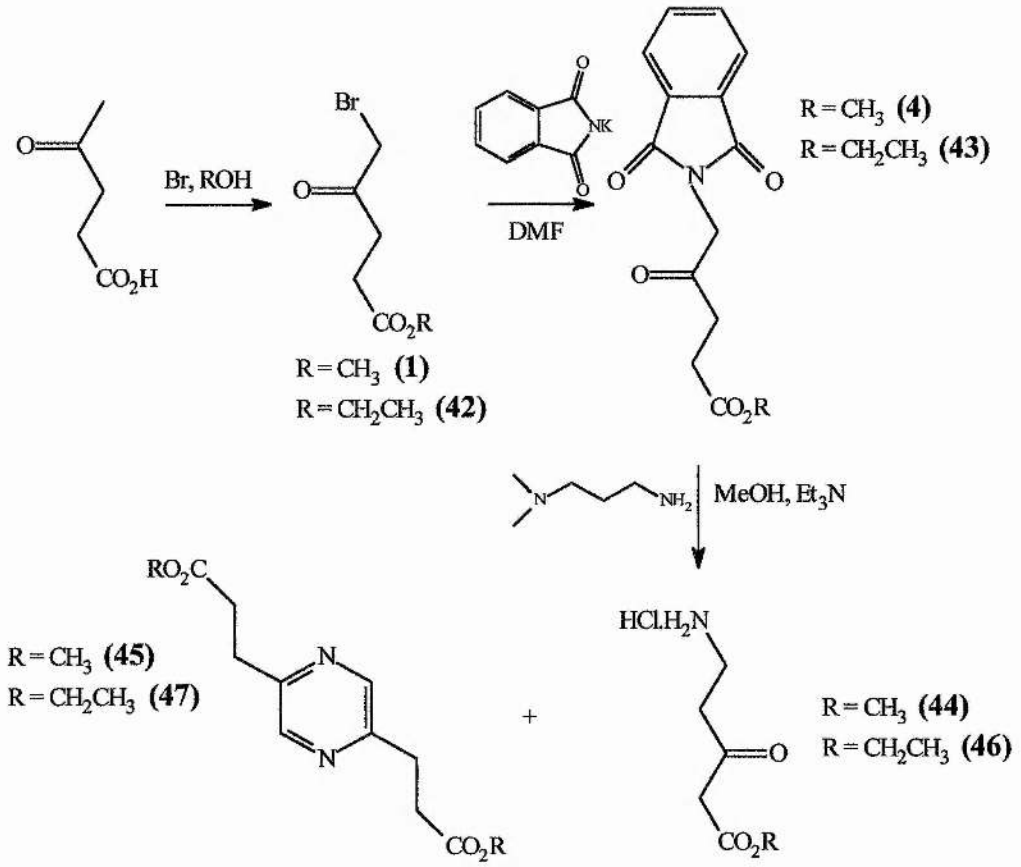


Figure 6.1 - Synthesis of ALA-OMe and ALA-OEt from methyl 5-phthalimidolevulinate.

A route to prepare both ALA-OEt and carbobenzyloxy-ALA (Z-ALA) was attempted.[609] Carbobenzyloxyglycine (Z-Gly) and valine methyl ester (Val-OMe)

were coupled using the mixed anhydride approach (Reaction 8.56, (**48**)). The methyl ester was then removed by saponification (Reaction 8.57 (**49**)). The next stage involved the synthesis of an oxazoline ring. Z-Gly-Val-OH (**49**) was reacted initially with acetic anhydride for 50 minute, then freshly distilled ethyl acrylate and triethylamine for 5 days at 50 °C (Reaction 8.58). Purification of the resulting orange oil was carried out using a polyamide column. Polyamide, a stationary phase, is not acidic, hence, does not break up the oxazoline ring. After passing the same solvent through the column for 1 day, the orange colour remained on the column leaving an almost white solid product (**50**). This was reacted with sodium bicarbonate in aqueous ethanol to produce Z-ALA-OEt (Reaction 8.59, (**51**)). At this stage only a small amount of product was obtained so only the preparation of ALA-OEt was attempted. Hydrogenation, however, yielded 2,5-di-(β-carboxyethyl) pyrazine diethyl ester (Reaction 8.60, (**47**)) rather than the required product (Fig. 6.2).

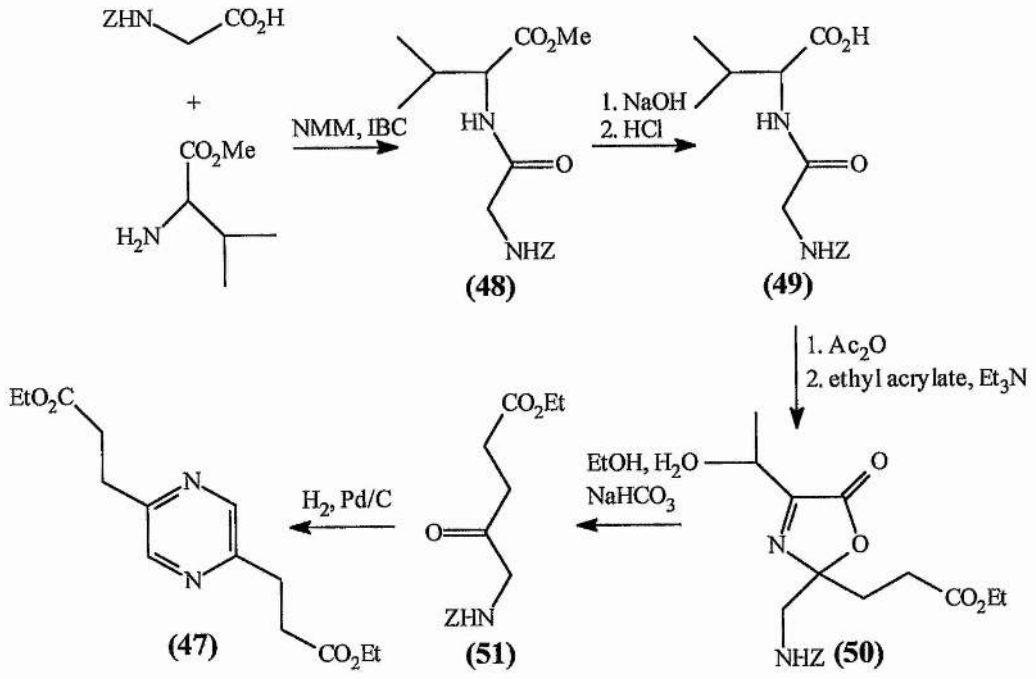


Figure 6.2 - Attempted synthesis of ALA-OEt and Z-ALA.

The use of Z-protecting groups for ALA was abandoned as removal, by hydrogenation, leaves a free NH_2 group which can either cyclise internally, forming piperidine-2,5-dione (**55**) (Fig. 6.3a) or dimerise giving a pyrazine (Fig. 6.3b).

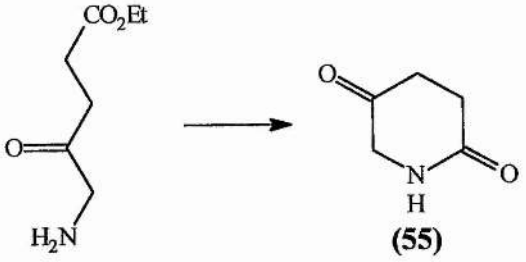


Figure 6.3a - Internal cyclisation of ALA-OEt to form piperidine-2,5-dione.

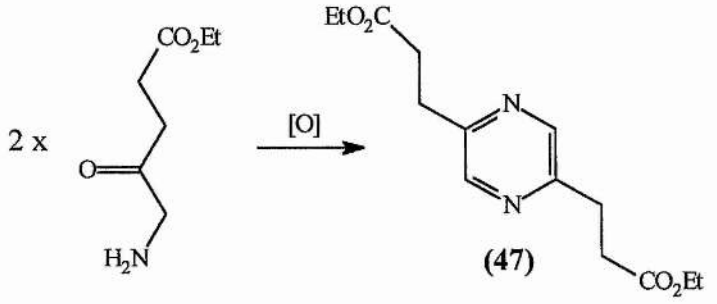


Figure 6.3b - Dimerisation of ALA-OEt to form a 2,5-di-(β-carboxyethyl) pyrazine diethyl ester.

The ester has a much better leaving group than the free acid making ALA esters more reactive and more susceptible to side reactions than ALA itself (See Section 6.5). This approach to the synthesis of protected ALA was, therefore, abandoned.

Instead, ALA esters were prepared directly from ALA.HCl (**3**) using the appropriate alcohol and thionyl chloride.[607] ALA-OEt (Reaction 8.61 (**46**)), ALA0hexyl ester (Reaction 8.62, ALA-OHex, (**52**)) and ALA-benzyl ester (Reaction 8.63, ALA-OBn, (**53**)) were prepared in this way (Fig. 6.4).

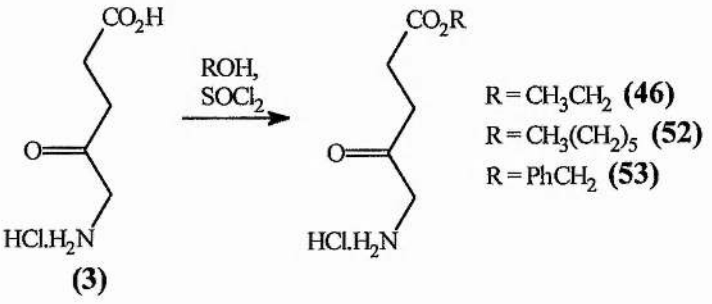


Figure 6.4 - Direct preparation of ALA esters.

ALA esters could then be reacted with various NH_2-protected amino acids and deprotected to form 'dipeptides'. Initial strategy involved reaction of Z-protected

amino acids with ALA-OBn as both protecting groups could be removed together using hydrogenation. Z-Gly was coupled to ALA-OBn using CHME as the coupling reagent (Reaction 8.64, (**54**)) as the mixed anhydride approach resulted in a much lower yield (Reaction 8.65). Deprotection by hydrogenation (Reaction 8.66), however, yielded a diketopiperazine derivative and not the expected fully deprotected product (Fig. 6.5).

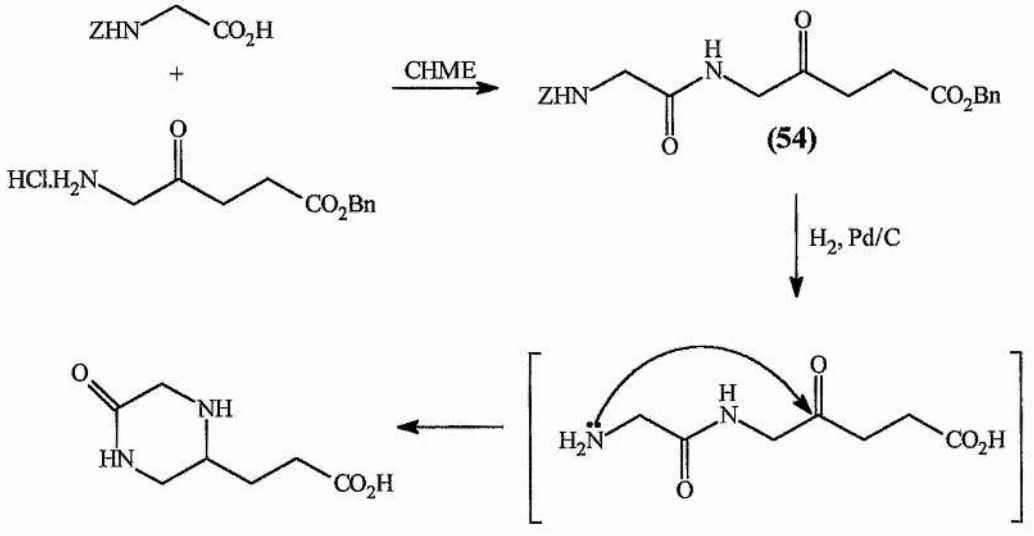


Figure 6.5 - Formation of a diketopiperazine during an ALA derivative deprotection.

Z-Groups can also be removed using HBr/ acetic acid[610] but this would yield an HBr salt which is not ideal for biological testing.

Another method had to be developed where a free NH_2 group was never allowed to form. t-Boc groups can be removed very cleanly using HCl gas, therefore, to prepare deprotected derivatives, this strategy was adopted. t-Boc-Gly was coupled to ALA-OEt using CHME (Reaction 8.67, (**56**)). The product was found to contain a small quantity of piperidine-2,5-dione (Fig. 6.3a (**55**)).The t-Boc

group was then removed using HCl gas to give Gly-ALA-OEt (Reaction 8.68, (**57**)) which was, unfortunately, not prepared early enough to be biologically tested. Preparation of t-Boc-Gly-ALA-OBn followed by hydrogenation to remove the benzyl ester, then treatment with HCl gas to remove the t-Boc group may be a good strategy for the synthesis of dipeptide derivatives (Fig. 6.6).

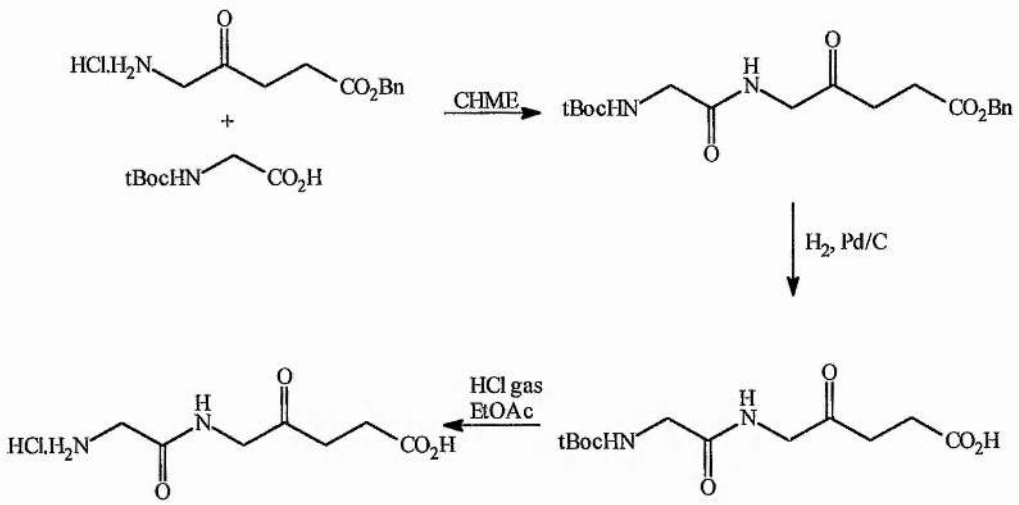


Figure 6.6 - Suggested strategy for the synthesis of deprotected ALA derivatives.

Initially, however, protected derivatives of ALA coupled to amino acids were prepared using Z-protected amino acids with either ALA-OEt or ALA-OHex. Z-Protected amino acids coupled to ALA esters with better yields than t-Boc-protected amino acids and there is some precedent for leaving Z-groups intact on biologically active molecules. Z-Protected amino acids were prepared using standard peptide synthesis techniques (Reactions 8.69 (**58**), 8.70 (**59**) and 8.71 (**60**)).[611] Z-Gly-ALA-OEt (Reaction 8.72, (**61**), ALA1), Z-L-Phe-ALA-OEt (Reaction 8.73, (**62**), ALA2), Z-D-Phe-ALA-OEt (Reaction 8.74, (**63**), ALA11) and Z-Gly-ALA-OHex (Reaction 8.75, (**64**), ALA10) were all prepared using

CHME couplings. Yields for these reactions were fairly low (approximately 45 - 55%) due to the fact the HCl salt could not be removed from ALA.HCl without causing pyrazine formation (Fig. 6.3b). Addition of base to the reaction mixture led to formation of two by-products, piperidine-2,5-dione (**55**) and pyrazine derivative (Fig. 6.3a and 6.3b).

Phthalimidolevulinic acid (Phthal-ALA (**9**)), prepared as an intermediate in the synthesis of ALA.HCl (Reactions 8.13 and 8.14) (Fig. 6.7a) or by removing the methyl ester from methyl 5-phthalimidolevulinate (Reaction 8.76, Fig. 6.7b) was also tested (ALA4).

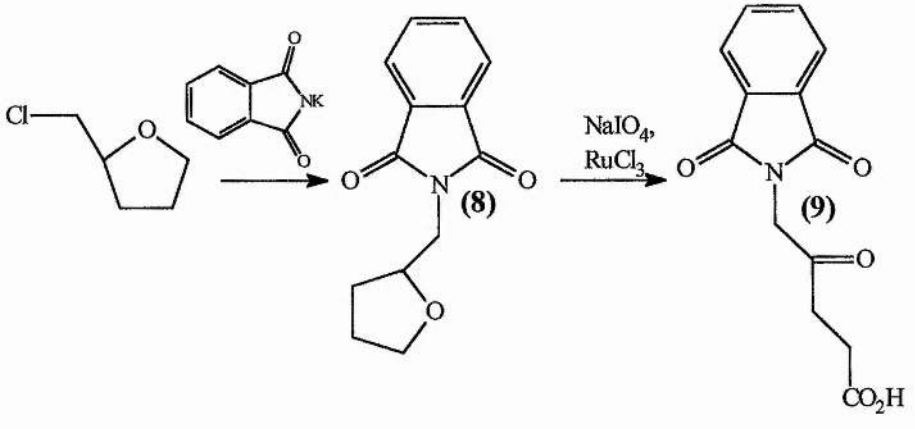


Figure 6.7a - Synthesis of phthalimidolevulinic acid from tetrahydrofurfuryl chloride.

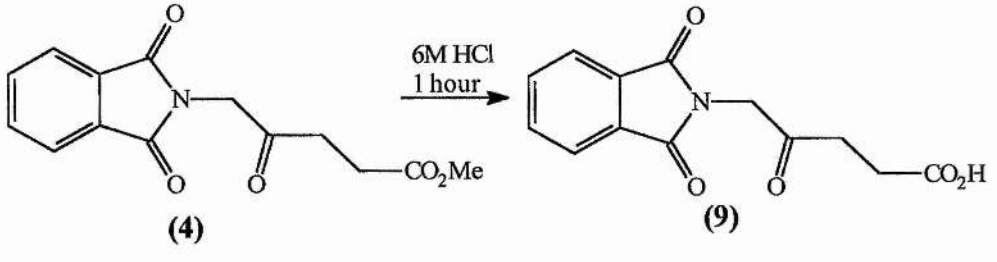
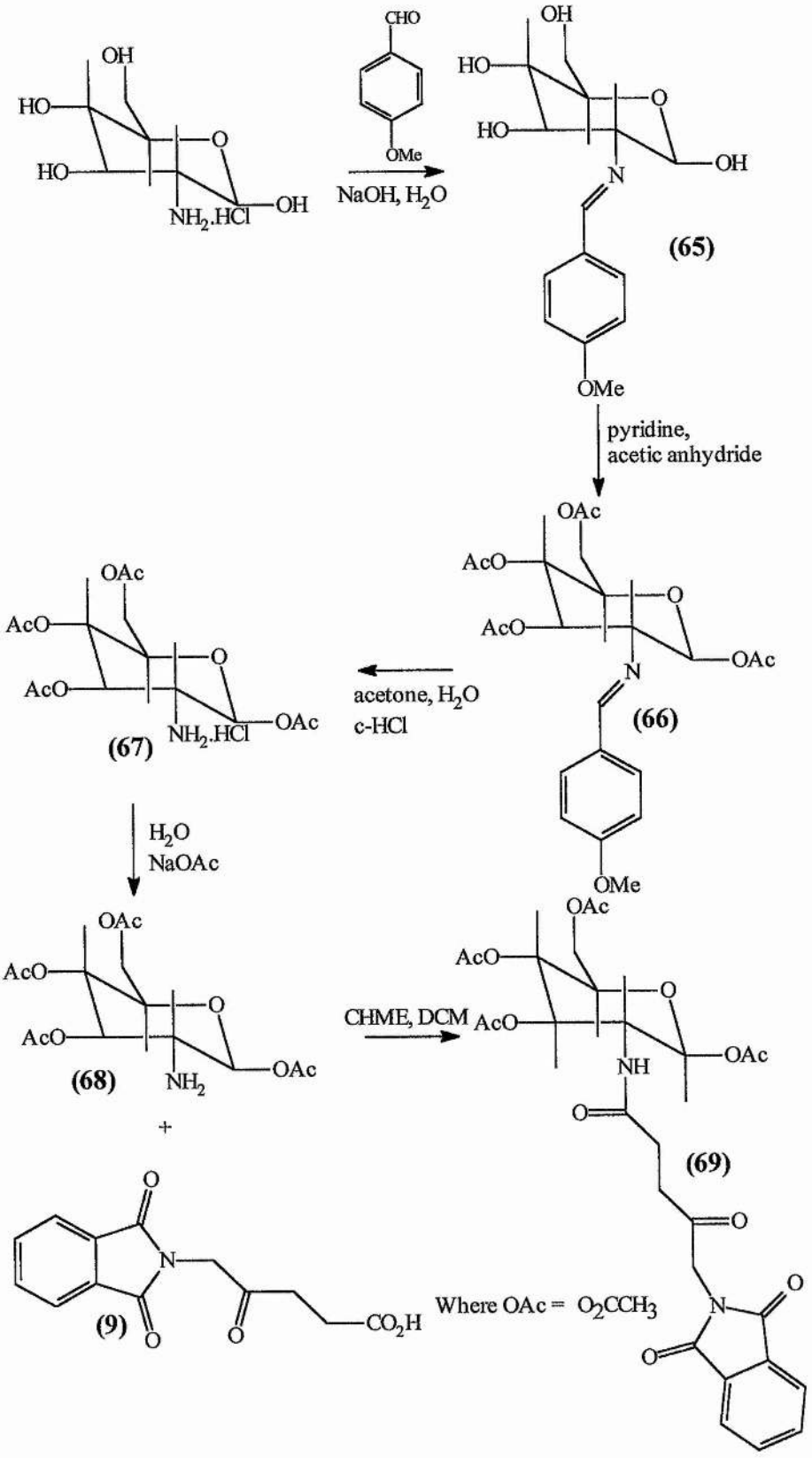


Figure 6.7b - Synthesis of phthalimidolevulinic acid from methyl 5-phthalimidolevulinate.

ALA was attached to a sugar in an attempt to make it more bioavailable. 1,3,4,6-tetra-o-acetyl-2-amino-2-deoxy-β-D-glucopyranose (glucosamine acetate) was prepared in a four step reaction from glucosamine hydrochloride.[612] using the scheme shown in Fig. 6.8 (Reactions 8.77 (**65**), 8.78 (**66**), 8.79 (**67**) and 8.80 (**68**)).



Scheme 6.8 - Scheme for the preparation of Phthal-ALA-Gluc-OAc.

Coupling of Phthal-ALA (**9**) to the protected sugar (**68**) was achieved using CHME (Reaction 8.81 (**69**)) in a fairly high yield (approximately 75%). Various attempts were made to remove the phthalimide group. Reaction with N,N-dimethyl-1,3-propanediamine yielded a mixture of products, including piperidine-2,5-dione (Fig. 6.3a, (**55**), Reaction 8.82), the leaving group this time being a sugar. Reaction with hydrazine, followed by purification using an ion-exchange column yielded a very small quantity of the required product contaminated with a large amount of NaCl (Reaction 8.83). The sugar derivative was, therefore, tested in its protected form (ALA3).

It is possible that piperidine-2,5-dione (**55**), detected as a by-product in many of the reactions, could be a pro-drug of ALA. It is known to open up to give ALA.HCl in c-HCl[613] but, potentially, it may be capable of this reaction under less severe conditions. Attempts were made to prepare the molecule directly from ALA-OEt by removing the HCl salt. Sodium methoxide was chosen as the base, as the resulting sodium chloride could be removed by filtration leaving only methanol, hence, a very clean reaction. Initially a small amount of solvent was used, due to the partial solubility of NaCl in methanol (Reaction 8.84), but, as small quantities of solvent encourage intermolecular reactions the only product isolated was a pyrazine (**47**) (Fig. 6.3b). A large excess of solvent (1000 fold) was used to try to encourage an intramolecular reaction (Reaction 8.85) but this simply lengthened the reaction time and resulted, again, in the formation of pyrazine (**47**) (Fig. 6.3b). A different approach was, therefore, attempted (Fig. 6.9).[614] 2-Hydroxypyridine was oxidised using an Elbs peroxydisulfate reaction (Reaction 8.86) to give 2,5-dihydroxypyridine (**70**) as a black solid. Repeated

recrystallisations from ethanol removed most of the colour and the 2,3-dihydroxypyridine by-product, but, upon contact with air it darkened. Column chromatography produced a white solid but it also darkened upon standing.

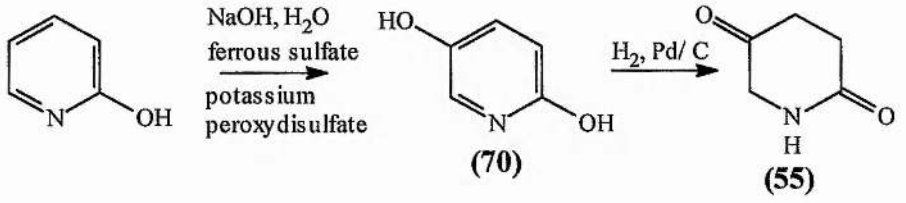


Figure 6.9 - The Elbs Peroxydisulfate oxidation of 2-hydroxypyridine.

A later paper[615] suggested that ferrous sulfate did not improve the reaction (it was thought that a radical process would benefit from its addition). It, in fact, encouraged side reactions and lowered the yield. Repetition of the reaction without ferrous sulfate increased the yield from 20 to 50% (Reaction 8.87 (**70**)). The resulting brown coloured 2,5-dihydroxypyridine was hydrogenated over 10% Pd/C catalyst and purified by column chromatography then recrystallisation[613] (Reaction 8.88) to give a small quantity of piperidine-2,5-dione (**55**) as a slightly brown coloured solid. This was, unfortunately, not prepared in time to be biologically tested.

An attempt was made to couple N-acetyl glycine to ALA-OHex (Reaction 8.89) but this failed, probably due to the poor solubility of N-acetyl glycine in DCM. The product isolated was 2,5-di-(β-carboxyethyl) pyrazine dihexyl ester (**71**).

N-Acetyl-ALA has been tested biologically[602] and found to be a fairly poor ALA pro-drug but, to the best of our knowledge, longer chain alkanoyl derivatives have

not been studied for PDT. A series of N-alkanoyl derivatives were prepared[608] by coupling ALA-OBn to an appropriate acid chloride, then removing the benzyl ester by hydrogenation (Fig. 6.10).

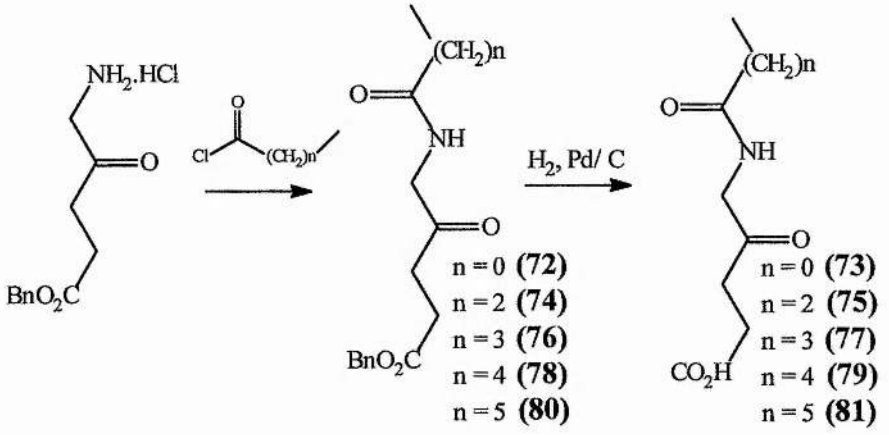
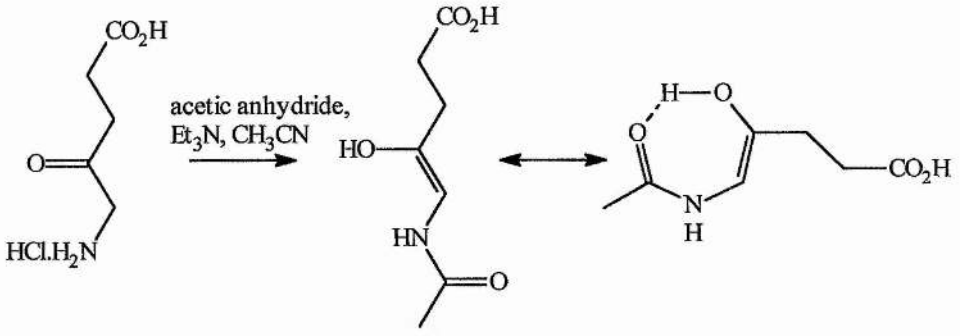


Figure 6.10 - Scheme for the preparation of N-alkanoyl derivatives of ALA.

Derivatives where n = 0 (N-acetyl-ALA, N-Ac-ALA (**73**), Reactions 8.90 and 8.91), n = 2 (N-butanoyl-ALA, N-But-ALA (**75**), Reactions 8.92 and 8.93, ALA12), n = 3 (N-pentanoyl-ALA, N-Pent-ALA (**77**), Reactions 8.94 and 8.95, ALA6), n = 4 (N-hexanoyl-ALA, N-Hex-ALA (**79**), Reactions 8.96 and 8.97, ALA7) and n = 5 (N-heptanoyl-ALA, N-Hept-ALA (**81**), Reactions 8.98 and 8.99, ALA8) were prepared in this way. N-Ac-ALA (**73**) was also prepared directly (Reaction 8.100) from ALA.HCl by reaction with acetic anhydride. Sonication assisted the formation of the product which NMR data suggested was the enol form of the molecule (Fig. 6.11).



(73)

Figure 6.11 - Direct preparation of N-Ac-ALA resulting in enol formation.

Formation of the enol could result in stabilisation by formation of a 7-membered ring. This compound was tested biologically (ALA9).

An attempt was made to couple N-Ac-ALA to the Gly-OMe (Reaction 8.101) but this, like Reaction 8.89, failed due to solubility problems.

Finally N,N-dimethyl-ALA (**82**) was prepared by reacting ALA.HCl with formaldehyde under an atmosphere of hydrogen (Reaction 8.102, ALA5) (Fig. 6.12).

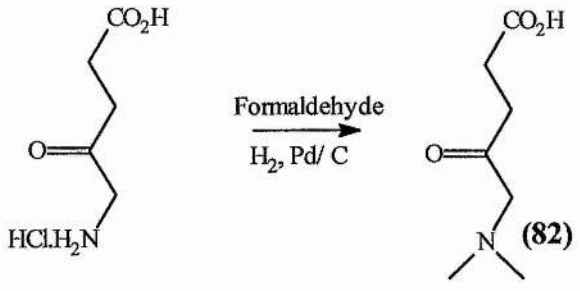
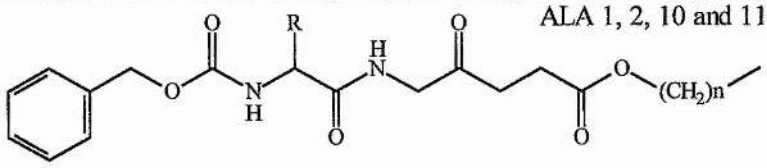
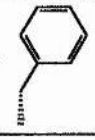
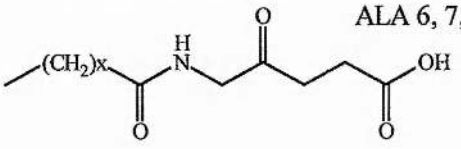


Figure 6.12 - Formation of N,N-dimethyl-ALA.

6.4 Table of 5-Aminolevulinic Acid Derivatives ALA1 - ALA12

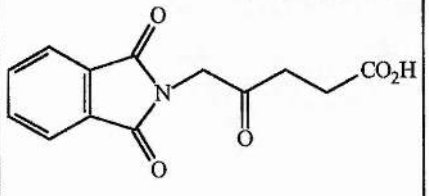


Z-Gly-ALA-OEt ALA1 **(61)**	R = H	n = 1
Z-L-Phe-ALA-OEt ALA2 **(62)**	R = 	n = 1
Z-Gly-ALA-OHex ALA10 **(64)**	R = H	n = 5
Z-D-Phe-ALA-OEt ALA11 **(63)**	R = 	n = 1



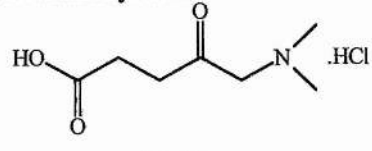
ALA8	**(81)**	x = 5 N-Hep-ALA	ALA6	**(77)**	x = 3 N-Pent-ALA
ALA9	**(73)**	x = 0 N-Ac-ALA	ALA7	**(79)**	x = 4 N-Hex-ALA
ALA12	**(75)**	x = 2 N-But-ALA			

N-Phthal-ALA

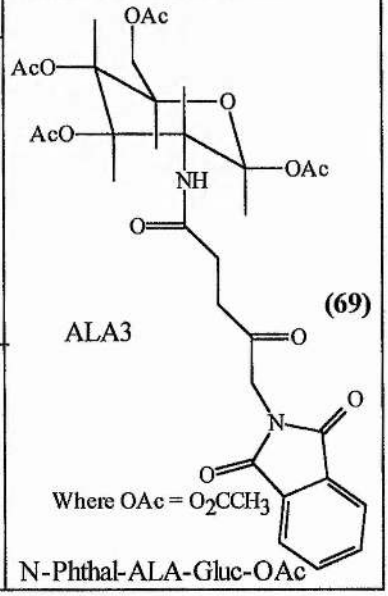


ALA4 **(9)**

N-N-Dimethyl-ALA



ALA5 **(82)**



ALA3 **(69)**

N-Phthal-ALA-Gluc-OAc

Derivatives ALA1 to ALA12 were tested biologically at the National Medical Laser Centre, UCL and the results are discussed in Chapter 7.

6.5 Photographs of the Browning of 5-Aminolevulinic Acid and Derivatives.

One problem encountered in the non-enzymatic dimerisation of ALA to 2,5-di-(β-carboxyethyl) pyrazine is the formation of a brown colour. To demonstrate this, ALA.HCl, ALA-OHex, N-Ac-ALA and Z-L-Phe-ALA-OEt (40 mg) were dissolved in phosphate buffer (1 cm^3, pH 7.4) and DMSO (1 cm^3) and photographed at various time intervals for 24 hours. Fig. 6.13a shows the samples before the solvents were added, Fig. 6.13b was taken 1 hour after sample preparation and Fig. 6.13c shows the solutions 24 hours after their preparation.

The photographs clearly show that the solution of ALA.HCl itself turned yellow but the ALA-OHex solution went brown. This demonstrates that ALA-esters are less stable than ALA itself. The two derivatives of ALA appear to be stable in solution, as they did not change colour with time, hence, if they prove to be effective in the biological testing, they could be efficient pro-drugs for PDT.

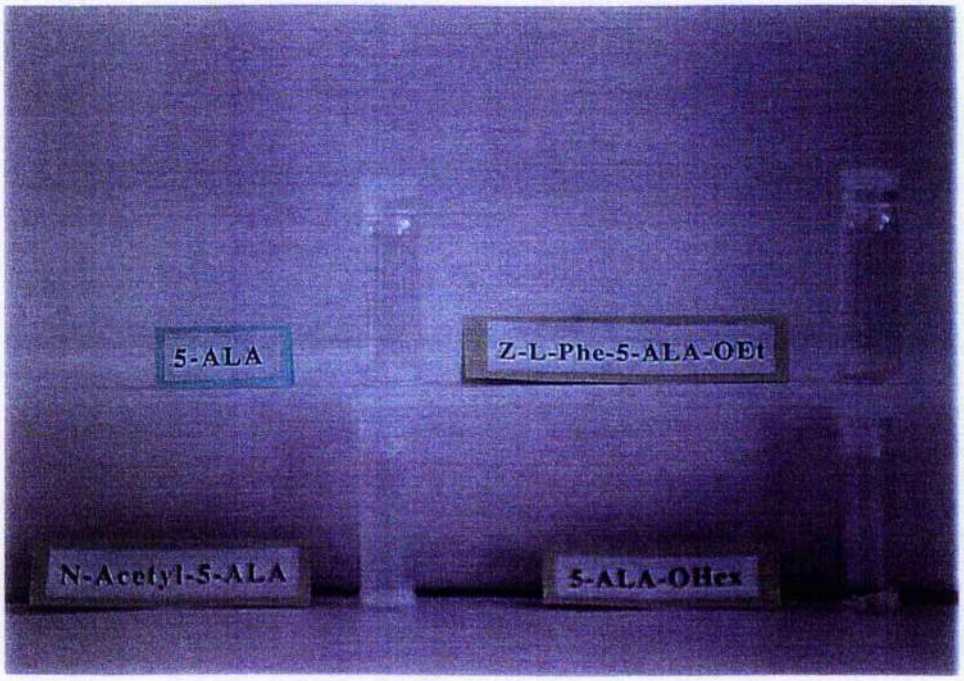


Figure 6.13a - Photograph of ALA.HCl and derivatives before the addition of solvent.

Figure 6.13b - Photograph of ALA.HCl and derivatives 1 hour after addition of solvent.

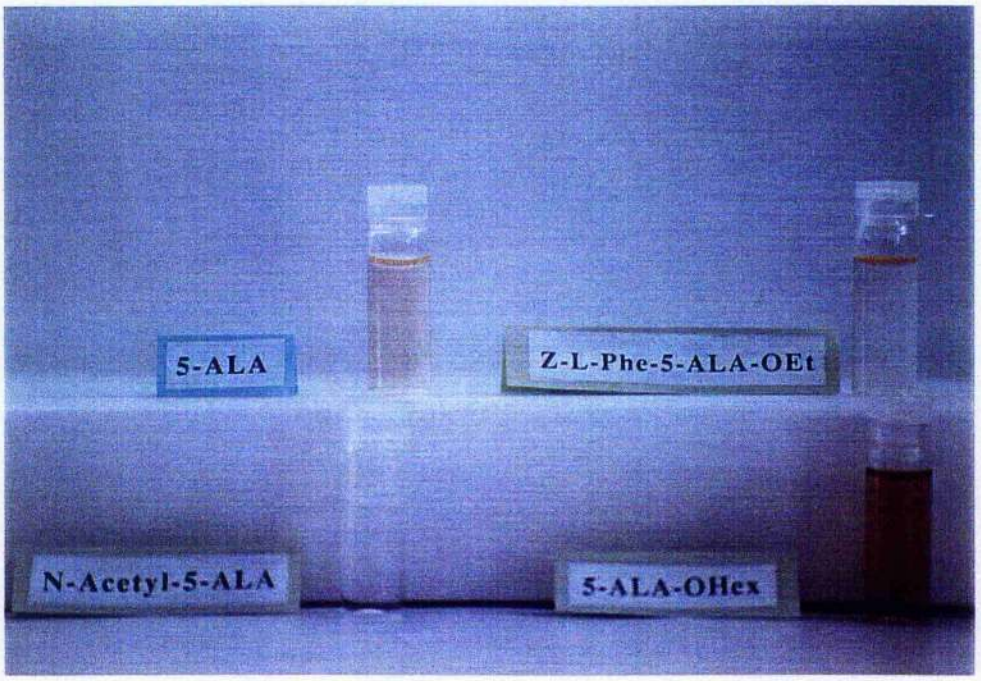


Figure 6.13c - Photograph of ALA.HCl and derivatives 24 hours after addition of solvent.

6.6 References.

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Chapter 7

Biological Testing of Derivatives of 5-Aminolevulinic Acid

7.1 Introduction

ALA derivatives 1-12 and ALA-OHex were tested on samples of normal, healthy human and rat skin (explants) at the National Medical Laser Centre, UCL. Exposure of cells to an external source of ALA results in enhanced porphyrin synthesis and the accumulation of PpIX. The efficiency of the pro-drugs, therefore, was determined by the ability of the skin to produce PpIX, the active compound for PDT, from the derivative. ALA induced PpIX formation has been shown to be greater in tumour cells than normal cells so any effect observed in healthy tissues should be enhanced in tumour cells.

The skin is made up of several layers known as the epidermis, the dermis and the subcutis (Fig. 7.1)

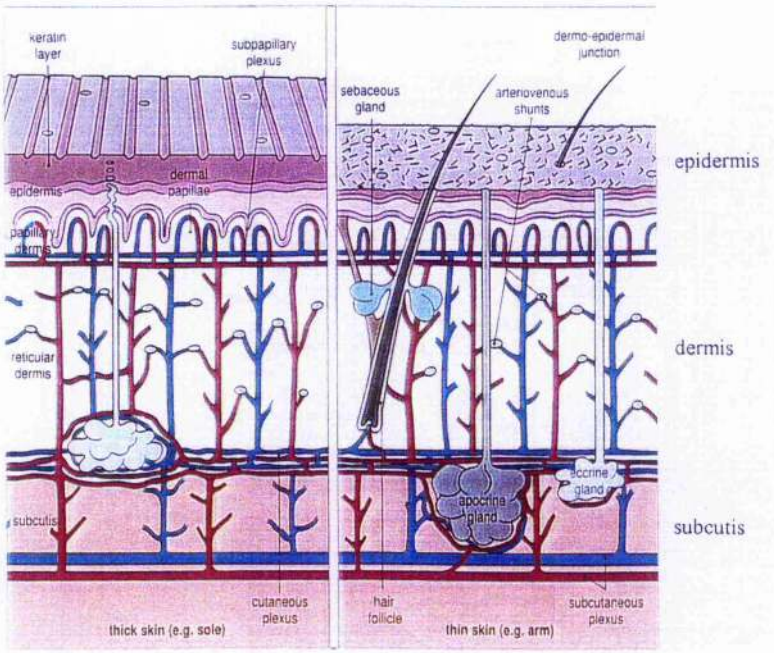

Figure 7.1 - Diagram showing the structure of the skin. Adapted from Ref. 701.

The epidermis is the outer protective layer of the skin and is in contact with the external environment. It consists of stratified epithelium (i.e. layers of tightly packed cells). The outermost layer is composed of very closely packed flat keratin protein cells which form a tough water repellant layer known as the stratum corneum. Growths into the epidermis produce sweat glands and hair follicles. The dermis is the middle, supporting layer and contains blood vessels and nerves. The deepest layer, the subcutis, contains the main network of arteries and veins and is the site of PpIX production in skin.

The four main requirements of the compounds were that they had to

- be taken up by the cells
- diffuse at a reasonable rate through the dermis
- be able to liberate ALA
- be non-toxic to the cells

Normal skin varies in its ability to resist ALA penetration. Thin skin, e.g. the arm, is less of a barrier than thick skin, e.g. soles of the feet, so there is even a variation from site to site on the same patient. Heavily freckled skin, a characteristic of the Celtic race, is penetrated in a very irregular pattern,[702] hence explants containing marks, freckles or bruises are avoided.

7.2 Testing Protocols

7.2.1 Animal and Human Skin Samples

Animal skin samples were obtained from normal female Wistar rats who were sacrificed by CO asphyxia. Their skin was shaved and the stratum corneum removed with a razor. The skin of the rat was removed with scissors and kept on ice until it was used. Fresh samples of human skin were obtained from the plastic surgery department after surgical excisions.

7.2.2 Skin Explant Cultures

Sections of skin (explants) of 70 - 100 mg were floated on plastic meshes in 2.5 cm^3 of serum free Dulbecco's D-MEM / F12 medium without Phenol Red which contained 50 $IUml^{-1}$ penicillin and 50 μg.ml-1 streptomycin. To the serum was added ALA, ALA-OHex or one of the derivatives ALA1-12 dissolved in DMSO. The samples were then incubated at 37 °C in an atmosphere of humidified air with 5% CO_2 in the dark (Fig. 7.2)

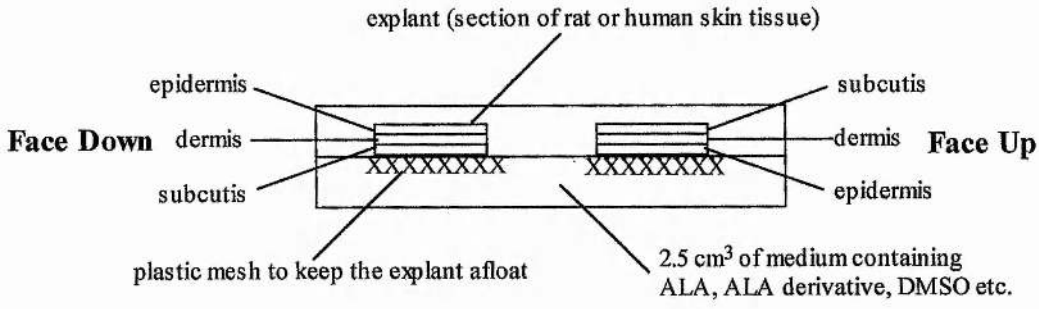

Figure 7.2 - Diagram of skin explant culture

Control experiments were incubated without ALA or a derivative and with DMSO only to ensure the pro-drug itself was causing the effect. Skin samples were placed either face up or face down onto the medium (Fig. 7.2). If the explant was face up the drug had to diffuse through the epidermis and dermis and this mimicks topical application (i.e. application of the drug in a cream or ointment onto the surface of the skin). If the explant was face down the drug was delivered directly to the subcutis and this mimicks intravenous application (injection of the drug).

7.2.3 Measurement of Protoporphyrin IX Content

As PpIX fluoresces, levels can be measured directly and this was done using a plate fluorescence reader (Perkin Elmer) connected to a Perkin Elmer LS 50B fluorescence spectrophotometer using 410 nm excitation and 635 nm emission with slit widths set to 10 nm both on the excitation and emission monochromators. An internal 530 nm highpass filter was used on the emission side and intensity calibrations were performed using a standard of Rhodamine B embedded on a Perspex disc. Measurement of the fluorescence emission spectra from chemically extracted porphyrins showed that fluorescence values were proportional to porphyrin concentration. Chemical extraction was done by homogenizing the tissue samples in a 4:1 solution of ethyl acetate and glacial acetic acid. The homogenates were centrifuged for 30 minutes at 3000 g and the supernatents were extracted with 5% HCl until the aliquots showed no fluorescence. Porphyrins in the media were also extracted with ethyl acetate/ acetic acid and HCl. The direct fluorescence emission spectra of explants exposed

to ALA were characteristic of PpIX with peak emission at 635 and 710 nm (Fig. 7.3).

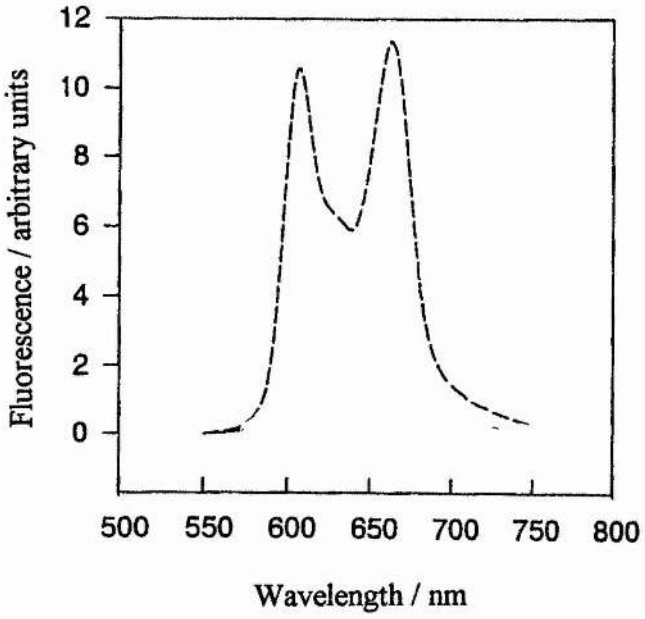

Figure 7.3 - Direct fluorescence emission spectra of PpIX.

Coproporphyrin and uroporphyrin, the precursors to PpIX are water soluble and direct fluorescence spectra of the medium showed peaks which probably correspond to these.[703] The percentage of porphyrins released into the medium was found to be 20% of the total porphyrins synthesised during the 2 to 19 hours of incubation. Medium which was incubated with ALA and no tissue, or tissue only was found to contain no porphyrin peaks.

7.3 Results

7.3.1 ALA and ALA-OHex

In rat tissue exposed to 0.6 mM ALA the PpIX levels increased linearly with time over the 19 hour incubation period. Exposure to the ALA-OHex lead to a maximum of PpIX synthesis after 5 hours of incubation (Fig. 7.4)

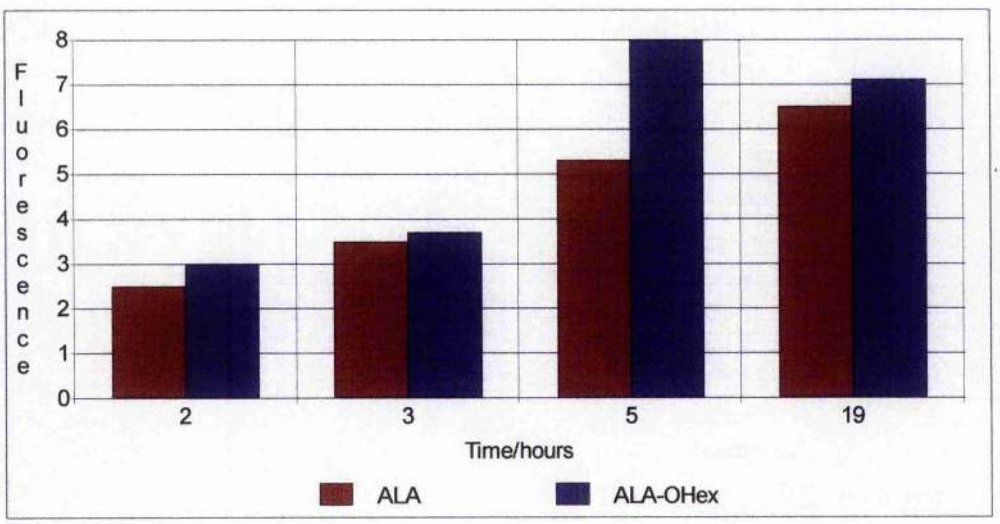

Figure 7.4 - Graph showing PpIX fluorescence after exposure of rat skin explants to 0.6 mM ALA and ALA-OHex for 2, 3, 5 and 19 hours. Direct skin fluorescence was measured at 410 nm excitation and 635 nm emission and autofluorescence of explants incubated without ALA was subtracted. Fluorescence values are in arbitrary units.

As human skin exhibits low fluorescence levels at short incubation times, 19 hours was the time selected for the analysis of PpIX concentration.

In rat skin both ALA and ALA-OHex showed maximum PpIX accumulation between 0.6 and 1.2 mM (Fig. 7.5).

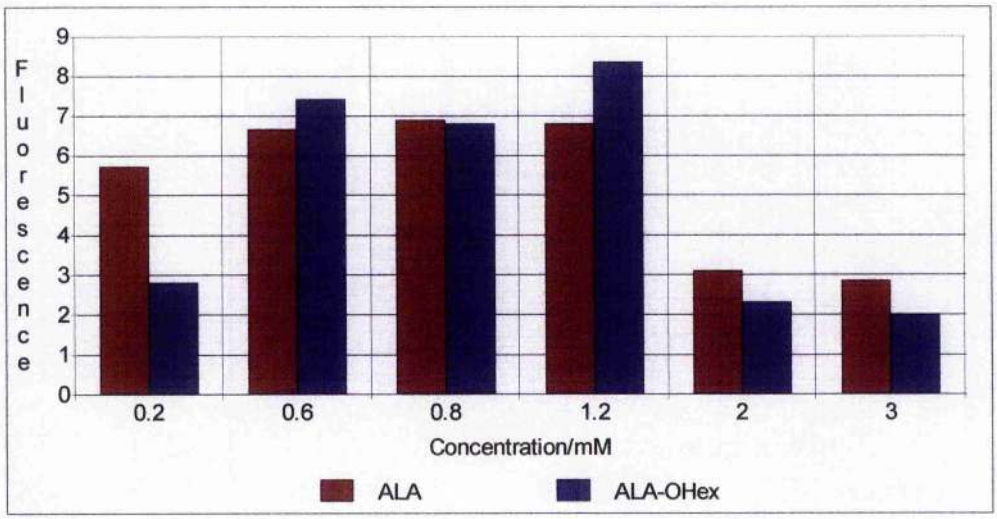

Figure 7.5 - Graph showing PpIX fluorescence after 19 hours exposure of rat skin to different concentrations of ALA and ALA-OHex. Fluorescence values are in arbitrary units.

In human cells, however, ALA-OHex gave significantly lower values than ALA at these concentrations (Fig. 7.6).

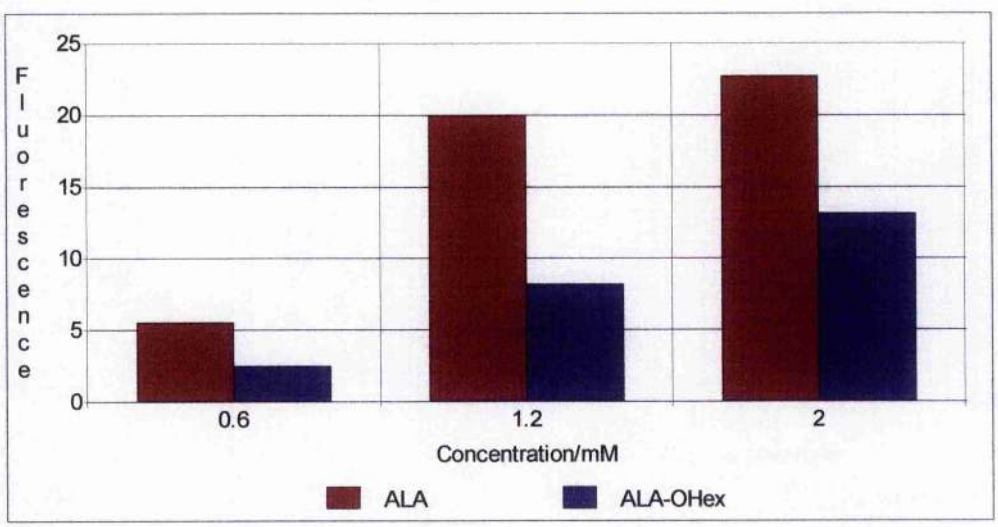

Figure 7.6 - PpIX fluorescence after 19 hours exposure of human skin to different concentrations of ALA and ALA-OHex. Fluorescence values are in arbitrary units.

7.3.2 ALA Derivatives 1-12

For a summary of the chemical structures of the derivatives and the abbreviations used, see Section 6.4.

N-But-ALA, N-Hep-ALA, N-Phthal-ALA-Gluc-OAc, Z-L-Phe-ALA-OEt and N-Phthal-ALA failed totally to induce PpIX fluorescence in human and rat skin. N-But-ALA, N-Hep-ALA and Z-L-Phe-ALA-OEt appeared to have a toxic effect on the cells as lower values of PpIX were recorded than in the control. The results of the other derivatives are illustrated by graphs (Fig. 7.7a, b, and c) and are summarised in the table (Fig. 7.8).

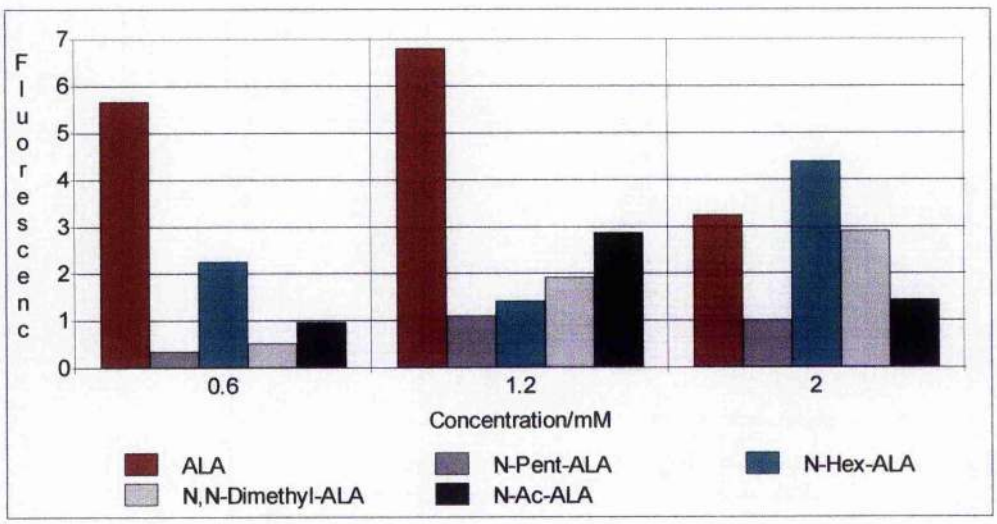

Figure 7.7a - Graph of ALA and derivatives showing PpIX production in rat skin

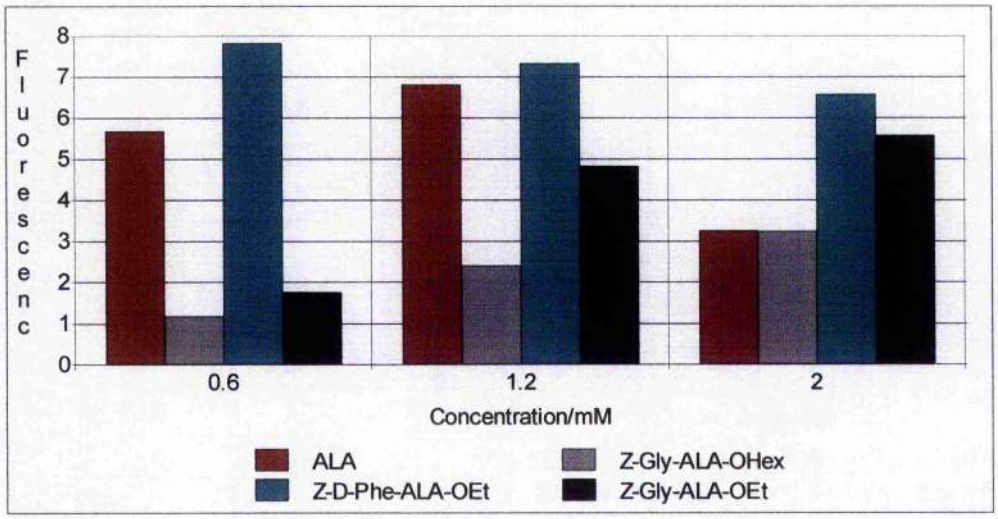

Figure 7.7b - Graph of ALA and derivatives showing PpIX production in rat skin

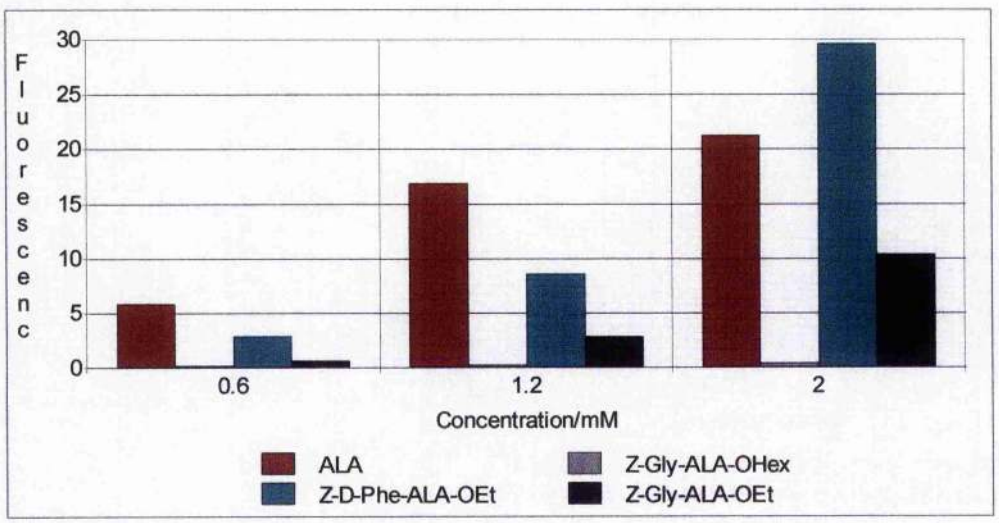

Figure 7.7c - Graph of ALA and derivatives showing PpIX production in human skin.

		Rat	Human
ALA	0.6 mM	5.67 ± 0.43	5.83 ± 0.98
	1.2 mM	6.80 ± 1.31	16.87 ± 3.45
	2.0 mM	3.25 ± 0.48	21.25 ± 5.40
N-Pent-ALA	0.6 mM	0.35 ± 0.52	0.09 ± 0.11
	1.2 mM	1.09 ± 0.09	0.15 ± 0.11
	2.0 mM	1.00 ± 0.60	0.41 ± 0.24
N-Hex-ALA	0.6 mM	2.25 ± 1.31	0.03 ± 0.08
	1.2 mM	1.40 ± 0.90	0.01 ± 0.20
	2.0 mM	4.38 ± 0.01	0.39 ± 0.16
Z-Gly-ALA-OHex	0.6 mM	1.18 ± 0.78	0.22 ± 0.04
	1.2 mM	2.40 ± 0.82	0.24 ± 0.09
	2.0 mM	3.23 ± 1.53	0.41 ± 0.22
Z-D-Phe-ALA-OEt	0.6 mM	7.81 ± 0.95	2.87 ± 0.83
	1.2 mM	7.33 ± 0.95	8.60 ± 1.59
	2 .0 mM	6.57 ± 3.12	29.60 ± 5.26
Z-Gly-ALA-OEt	0.6 mM	1.75 ± 0.86	0.67 ± 0.25
	1.2 mM	4.81 ± 0.76	2.83 ± 0.93
	2.0 mM	5.56 ± 1.25	10.36 ± 2.28
N,N-Dimethyl-ALA	0.6 mM	0.51 ± 0.09	0.08 ± 0.22
	1.2 mM	1.92 ± 0.65	0.06 ± 0.26
	2.0 mM	2.91 ± 0.78	0.24 ± 0.07
N-Acetyl-ALA	0.6 mM	0.96 ± 0.12	0.02 ± 0.09
	1.2 mM	2.87 ± 2.19	0.16 ± 0.09
	2.0 mM	1.43 ± 0.58	0.21 ± 0.39

Fig. 7.8 - Table of results of biological testing of ALA derivatives.

In general, the degree of PpIX induction was less than the corresponding effect of ALA. Z-Gly-ALA-OEt showed an increase in PpIX production in rat skin at 2

mM. Z-D-Phe-ALA-OEt, however, showed promising results. It gave an increase in PpIX production in rat skin and similar levels in human skin to ALA itself at all concentrations.

7.4 Fluorescence Microscopy

Fluorescence microscopy was used to monitor the distribution of PpIX in the skin i.e. where it is produced. Sections of explants of human skin, treated with ALA-OHex at 1.2 mM for 19 hours, were taken and mounted onto microscope slides. The image seen under a microscope (Scale 880 x 550 μ) using ordinary white light was photographed and can be seen in Fig. 7.9. The slide was stained with haematoxylin and eosin. The adjacent section was not stained and was viewed under laser light. The fluorescence of the slide was measured using a fluorescence detector and this image can be seem in Fig. 7.10. The white and blue colours indicate a large quantity of fluorescence, the red colours an intermediate amount and black indicates little or no fluorescence. PpIX is seen to accumulate in areas such as where there are hair follicles and in the epidermis. Production of PpIX from ALA-OHex is much more localised than from ALA itself. The epidermis can be seen on the right hand side of the photographs and has a higher fluorescence intensity than the dermis.

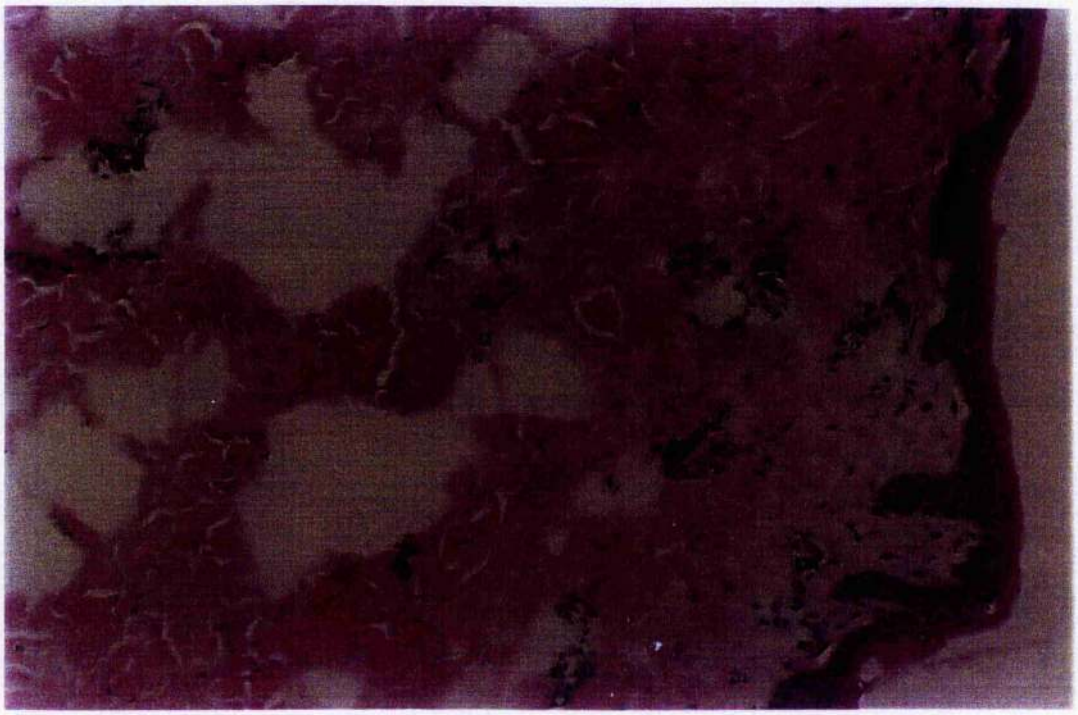

Figure 7.9 - White light microscopy image of a section of human skin stained with haematoxylin and eosin after treatment with 1.2 mM ALA-OHex for 19 hours.

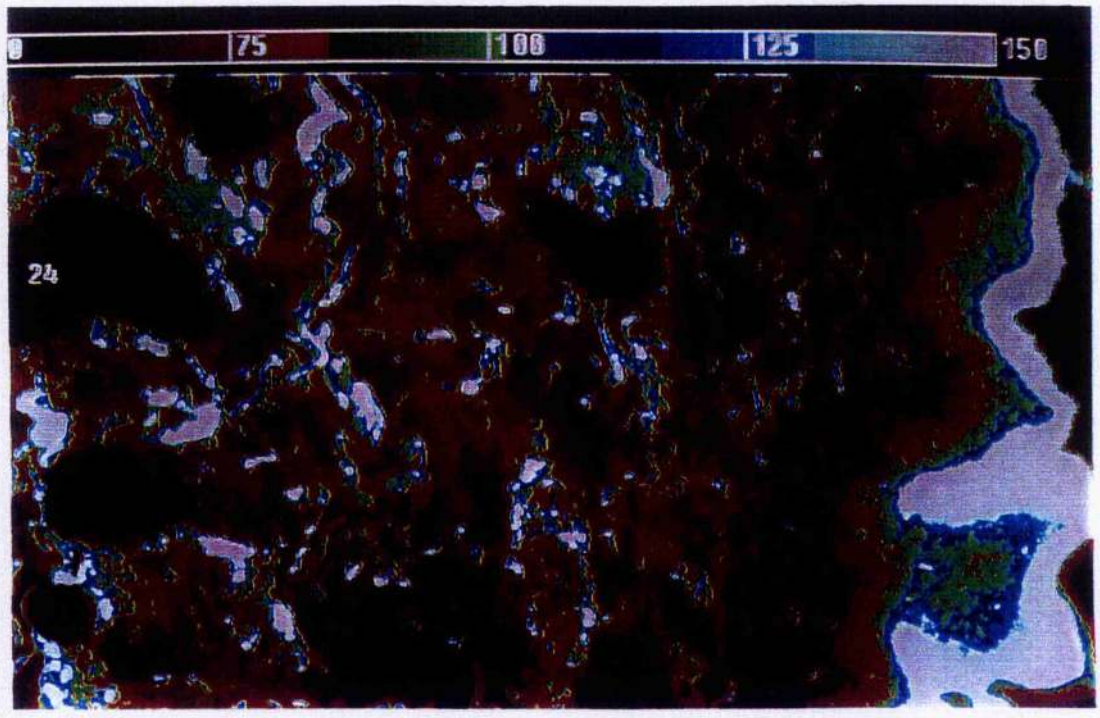

Figure 7.10 - Fluorescence microscopy image of a section, adjacent to that in Fig. 7.9, of human skin treated with 1.2 mM ALA-OHex for 19 hours.

7.5 Conclusions

In this study, the ALA-OHex was found to be very effective in rat skin at producing PpIX, but not as effective in human skin. The hexyl ester has already been found to be the most effective of the esters at producing high levels of PpIX for PDT.[704] It is not known whether the ester is cleaved from ALA before it is incorporated into PpIX. Fluorescence microscopy showed that PpIX formed from the esters of ALA is much more localised than from ALA itself.

Although most of the derivatives were not as effective at producing PpIX in healthy skin samples as ALA itself, most produced some PpIX. This implies that the enzymes required to release ALA from the derivatives must be present in the cells. Presumably proteases or non-specific esterases are required. It would be an interesting exercise to study the enzyme kinetics of the breakdown of some of the derivatives to give ALA itself. The derivatives containing phenylalanine contain a chromophore which would be useful for UV/visible studies. ALA-OBn and N,N-Dimethyl-ALA-OBn would be ideal for the study of ester hydrolysis. It may be possible to monitor the formation of ALA by recording the formation of 2,5-di-(β-carboxyethyl) pyrazine, the non-enzymatic dimer of ALA (See Chapter 4)

All the derivatives of ALA are much more chemically stable than ALA itself (See Section 6.5) so have the advantage that the dosage being given is known. If they are as effective as ALA itself, they may be of use in PDT

N-Acylated derivatives of ALA do not seem to be effective pro-drugs. The shorter alkyl chained derivatives, with the exception of N-Ac-ALA, were cytotoxic. N-Ac-ALA was found to be in its enol form (Fig. 6.11) and produced low levels of PpIX. N-Hex-ALA was more effective than ALA itself at 2 mM in rat skin but no significant results were obtained with these compounds. A potential candidate could be N-phenylacetyl-ALA as this group is known to be cleaved by the enzyme chymotrypsin.

It is not really surprising that the cells were not able to remove the N-phthalimide group from the molecules. An alternative N-protecting group strategy is required. As t-Boc-groups can be put on in non-aqueous conditions and can be removed leaving an HCl salt, this may be a possibility for the preparation of other derivatives. Less heavily protected sugar derivatives may prove to be interesting.

Given the heavy protection of N,N-Dimethyl-ALA, the results are remarkably good. At the highest concentration the PpIX levels in rat skin are comparable to ALA itself.

The most interesting results, however, are from the dipeptide derivatives. It was expected that Z-Gly-ALA-OHex would be more effective than Z-Gly-ALA-OEt. This was not the case. At all three concentrations, the ethyl ester was more effective. Interestingly, Z-L-Phe-ALA-OEt was found to be cytotoxic. It is possible that releasing L-Phe into the cells is disturbing another biochemical pathway as L-Phe is naturally occurring so is bioavailable. Z-D-Phe-ALA-OEt, the other isomer, was the most effective of all the derivatives producing higher

levels of PpIX than ALA itself at all concentrations in rat skin and at 2 mM in human skin. Releasing D-Phe into the cells does not disturb other pathways as it is simply excreted.

There is certainly a great deal of scope for future work. Many of the other naturally occurring amino acids, both L and D, and possibly some of the unnatural ones could be attached to either end of the molecule. Another possibility would be to prepare a dipeptide consisting of two molecules of ALA. This could be more stable than ALA itself and would break down to give only the active compound. The use of solid state chemical techniques could be useful in preparing a large variety of derivatives for further biological testing.

7.6 References

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Chapter 8

Experimental

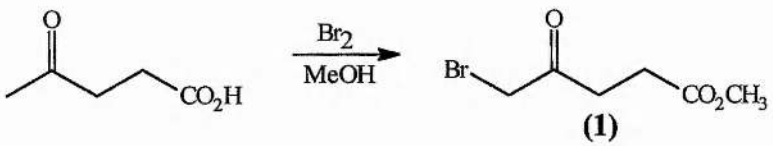
Reagents. All reagents were purchased from Sigma-Aldrich and were reagent grade (unless otherwise stated).

Instrumentation. Melting points were determined with open glass capillaries using a Gallenkamp machine. Elemental analysis were performed on a Carlo-Erba Instrumentazione model 1106 CHN analyser. Mass spectrometry was performed using a Finnigan MAT INCOS 50 mass spectrometer. ^{1}H NMR spectra were obtained either at 200 MHz using a Varian Gemini 200 spectrometer or at 300 MHz using a Varian 300 MHz spectrometer. ^{13}C NMR spectra were recorded at either 50 or 75 MHz using the same instruments. Chemical shifts (δ) for ^{1}H and ^{13}C are recorded in ppm downfield of TMS ($\delta = 0$). ^{15}N NMR spectra were obtained at 30.412 MHz on a Bruker AM 300 spectrometer. All ^{15}N spectra were ^{1}H decoupled and were referenced to external nitromethane (with 20% $C^{2}H_{3}O^{2}H$ to provide the lock signal) set at δ 380.23. UV-Visible spectroscopy used a Phillips PU 8730 scanning spectrophotometer.

Purification of Solvents. Dry DMF[801] was prepared by storing the commercially available solvent over molecular sieves (type 4A) for 48 hours then distilling the resulting liquid under reduced pressure, keeping the temperature below 50 °C. Diethyl ether and THF were dried over sodium wire. Dry ethanol and methanol[802] were prepared using a Grignard reaction. Magnesium turnings (2.5 g) and iodine (0.25 g) were added to a small amount of the ethanol (or methanol). This mixture was warmed gently until hydrogen gas was given off and all the iodine disappeared. Once the magnesium had been converted into magnesium ethoxide (or methoxide), ethanol (or methanol) (500 cm^{3}) was added and the mixture heated under reflux conditions for 30 minutes. The dry solvent was distilled and stored in a flask containing Type 4A molecular sieves. Thionyl chloride[803] was

purified to remove the impurities of sulfur chlorides and sulfuryl chlorides. Technical grade thionyl chloride (250 cm^3) and dipentene (23 cm^3) were distilled until the temperature of the distillation mixture reached 85 °C. The colourless liquid obtained was redistilled and stored in a dark bottle. Hexane used in column chromatography was redistilled prior to use to remove non-volatile impurities. Dry pyridine[802] was prepared by heating pyridine under reflux conditions over potassium hydroxide pellets for 1 hour then distilling the resulting mixture. Dry pyridine has a boiling point of 115.3 °C and this fraction was stored over calcium hydride.

8.1 Synthesis of methyl 5-bromolevulinate.[804]

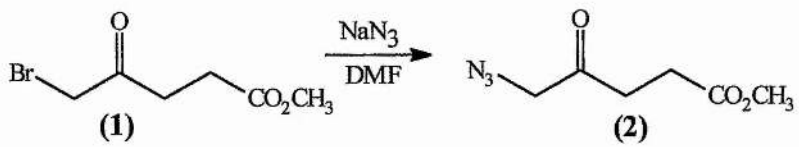


(1)

Bromine (2.6 cm^3, 50 mmol) was added to a solution of levulinic acid (5.1 cm^3, 50 mmol) in methanol (100 cm^3) at room temperature over 15 minutes under an atmosphere of N_2. The reaction mixture was stirred for 1 hour at room temperature then heated under reflux conditions for 3.5 hours. The methanol was removed under reduced pressure and the residue partitioned between diethyl ether and water. The pH of the solution was adjusted to 8 by addition of $NaHCO_3$. The diethyl ether layer was washed with aqueous $NaHCO_3$ and brine and then dried ($MgSO_4$), filtered and concentrated to give a 3 to 1 mixture of methyl 5-bromolevulinate and methyl 3-bromolevulinate. These were separated by distillation (yield of methyl 5-bromolevulinate 2.01 g, 19%), or by silica gel flash column chromatography using 40-60 petroleum ether : DCM 1:5, with 0.5%

acetic acid as the eluant yielding methyl 5-bromolevulinate (**1**) as a pale yellow oil (3.46 g, 33%), b.p. 90 - 95 °C at 0.75 mm/Hg (Lit.[805] 55-64 °C at 0.1 mm/Hg). δ_H (200 MHz, C^2HCl_3) 2.6 (2H, m, CH_2), 2.9 (2H, t, J = 6.7 Hz, CH_2), 3.6 (3H, s, CH_3) and 3.9 (2H, m, $BrCH_2$); δ_C (50 MHz, C^2HCl_3) 27.15 (CH_2COO), 34.82 ($COCH_2CH_2$), 48.21 ($BrCH_2$), 51.35 (CH_3), 174.12 (COO) and 203.69 (CO).

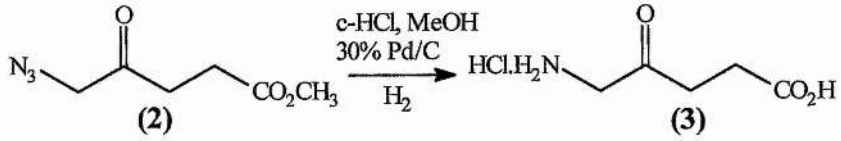
8.2 Synthesis of methyl 5-azidolevulinate.[804]



(1) (2)

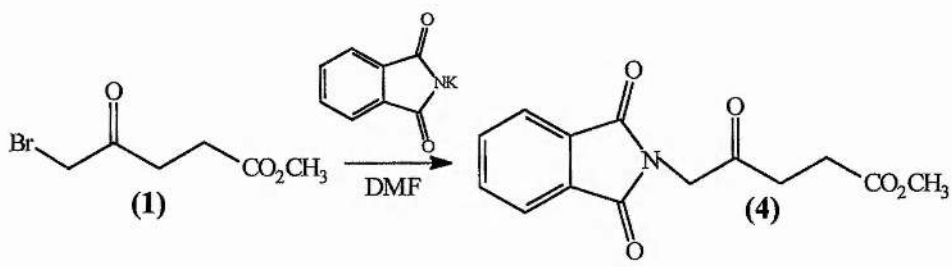
Methyl 5-bromolevulinate (Reaction 8.1, 1.0 g, 4.8 mmol) was added to a solution of sodium azide (0.96 g, 14.4 mmol) in dry DMF (40 cm^3) under an atmosphere of N_2 at -10 °C. The resultant reaction mixture was stirred for 1 hour at this temperature. Diethyl ether was added and the DMF was washed out with brine (3 x 400 cm^3). The organic layer was washed with 5% $NaHCO_3$ solution and water (200 cm^3 of each), dried ($MgSO_4$), filtered and concentrated under reduced pressure giving methyl 5-azidolevulinate (**2**) as a clear oil (0.8 g, 91%), the boiling point was not recorded due to the potentially explosive nature of the compound. δ_H (300 MHz, $^2H_6$-DMSO) 2.5 (2H, t, J = 6.7 Hz, CH_2COO), 2.7 (2H, t, J = 6.7 Hz, $COCH_2CH_2$), 3.5 (3H, s, CH_3) and 4.17 (2H, s, N_3CH_2); δ_C (75 MHz, $^2H_6$-DMSO) 28.12 (CH_2COO), 32.34 ($COCH_2CH_2$), 52.00 (CH_3), 58.31 (N_3CH_2), 174.25 (COO) and 203.42 (CO).

8.3 Synthesis of 5-aminolevulinic acid.[804]



Methyl 5-azidolevulinate (Reaction 8.2, 0.5 g, 2.9 mmol) in methanol (10 cm^3) was stirred with c-HCl (2 cm^3) and 30% Pd/C catalyst (100 mg) under an atmosphere of H_2 at room temperature and atmospheric pressure for 20 hours. The reaction mixture was filtered through Celite and the filtrate concentrated under reduced pressure. Recrystallisation from acetic acid/butanol yielded ALA.HCl (**3**) as white crystals (0.41 g, 84%), m.p. 148 - 150 °C (Lit.[806] 147 °C). m/z (EI^+) 132 (M^+ - HCl salt). Found: C, 35.8; H, 6.0; N, 8.2; Calculated for $C_5H_{10}NO_3Cl$: C, 35.8; H, 6.0; N, 8.4%. δ_H (200 MHz, ^2H_2O) 2.6 (2H, t, J = 6.2 Hz, CH_2COO), 2.8 (2H, t, J = 6.2 Hz, $COCH_2CH_2$) and 4.0 (2H, s, NH_2CH_2); δ_C (50 MHz, ^2H_2O) 28.71 (CH_2COO), 35.81 ($COCH_2CH_2$), 48.00 (NH_2CH_2), 176.15 (COO) and 204.21 (CO).

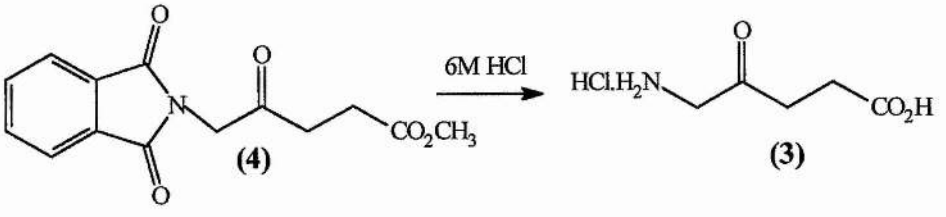
8.4 Synthesis of methyl 5-phthalimidolevulinate.[807]



Potassium phthalimide (1.0 g, 5 mmol) was suspended in dry DMF (5 cm^3). To this was added methyl 5-bromolevulinate (Reaction 8.1, 1.0 g, 5 mmol) and the mixture was stirred for 30 minutes at room temperature and 1 hour at 60 °C. The cooled reaction mixture was filtered to remove KCl and unreacted phthalimide

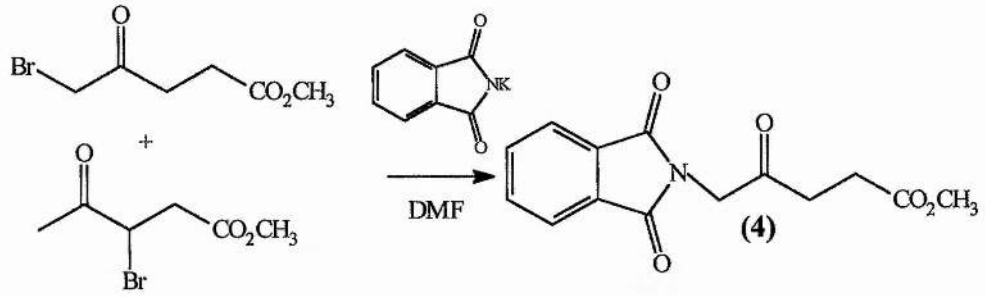
before DCM (20 cm^3) and water (60 cm^3) were added. The aqueous phase was washed with DCM (2 x 10 cm^3). The DCM extracts were washed with 0.2 M NaOH (10 cm^3) and then with water until the washings were colourless (5 x 20 cm^3). Combined DCM extracts were dried ($MgSO_4$), filtered and the solvent removed under reduced pressure. The residue was recrystallised from water yielding methyl 5-phthalimidolevulinate (**4**) as white crystals (0.68 g, 50%), m.p. 96 - 98 °C (Lit.[807] 96- 97 °C). δ_H (200 MHz, C^2HCl_3) 2.7 (2H, t, J = 7.6 Hz, CH_2COO), 2.9 (2H, t, J = 7.6 Hz, $COCH_2CH_2$), 3.7 (3H, s, CH_3), 4.6 (2H, s, NCH_2), 7.8 (2H, m, aromatics) and 7.9 (2H, m, aromatics); δ_C (50 MHz, C^2HCl_3) 28.07 (CH_2COO), 34.98 ($COCH_2CH_2$), 47.02 (NCH_2), 52.45 (CH_3), 123.91, 124.04, 134.55 (3 x aromatics), 168.06 (2 x phthalimide-CO), 173.03 (COO) and 203.10 (ALA-CO).

8.5 Synthesis of 5-aminolevulinic acid.[807]



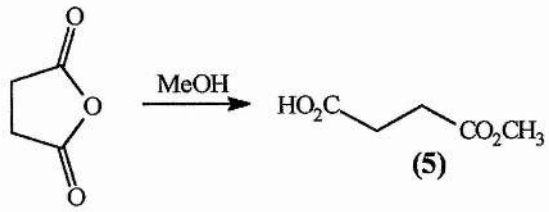
Methyl 5-phthalimidolevulinate (Reaction 8.4, 0.5 g, 1.8 mmol) was heated under reflux conditions in 6 M HCl (6 cm^3) for 8 hours. The solution was cooled, filtered to remove phthalic acid and concentrated under reduced pressure to give ALA.HCl (**3**) as white crystals (0.2 g, 65%). Data as for Reaction 8.3.

8.6 Synthesis of methyl 5-phthalimidolevulinate.



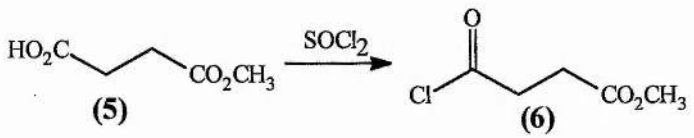
Potassium phthalimide (1 g, 5 mmol) was suspended in dry DMF (5 cm^3). To this was added a mixture of methyl 5-bromolevulinate and methyl 3-bromolevulinate (Reaction 8.1, 1.0 g) and the reaction was stirred for 30 minutes at room temperature and 1 hour at 60 °C. The cooled mixture was filtered to remove KCl and any unreacted phthalimide before DCM (20 cm^3) and water (60 cm^3) were added. The aqueous phase was washed with DCM (2 x 10 cm^3). The DCM extracts were washed with 0.2 M NaOH (10 cm^3) and then with water until the washings were colourless (5 x 20 cm^3). Combined DCM extracts were dried ($MgSO_4$), filtered and the solvent removed under reduced pressure. The residue was recrystallised from water. Unreacted methyl 3-bromolevulinate, an oil, was removed by filtration yielding only methyl 5-phthalimidolevulinate (**4**) as white crystals (0.34 g, 23%, yield calculated from quantity of crude strating material). Data as for Reaction 8.4.

8.7 Synthesis of methyl hydrogen succinate.[808]



A mixture of succinic anhydride (50 g, 0.5 mol) and dry methanol (25 cm^3) was stirred vigorously under reflux conditions for 1 hour. Excess methanol was removed under reduced pressure and the crude product was recrystallised from toluene/cyclohexane to give methyl hydrogen succinate (**5**) as large white crystals (54 g, 82%), m.p. 57 - 59 °C (Lit.[808] 57 - 58 °C). δ_H (200 MHz, ^2H_2O) 2.6 (4H, s, 2 x CH_2) and 3.6 (3H, s, CH_3); δ_C (50 MHz, ^2H_2O) 31.61, 31.81 (2 x CH_2), 55.24 (CH_3), 178.51 and 179.89 (2 x COO).

8.8 Synthesis of methyl 3-chloroformylpropanoate.[808]

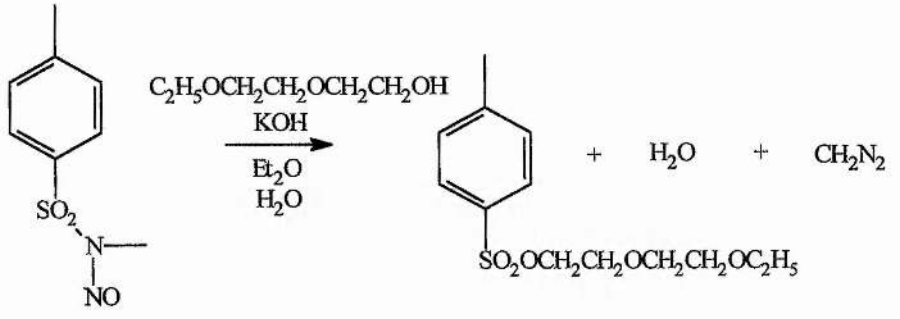


(5) (6)

A mixture of methyl hydrogen succinate (Reaction 8.7, 48.3 g, 0.37 mol) and thionyl chloride (53 cm^3, 0.73 mol) was warmed to 30 °C for 4 hours. Excess thionyl chloride was removed under reduced pressure at the water pump. The crude product was purified by distillation at the oil pump to give methyl 3-chloroformylpropanoate (**6**) as a clear oil (40 g, 73%), b.p. 50 - 60 °C at 2 mm/Hg (Lit.[808] 92- 94 °C at 18 mm/Hg). δ_H (200 MHz, C^2HCl_3) 2.7 (2H, t, J = 6.6 Hz, CH_2COO), 3.2 (2H, t, J = 6.6 Hz, $COCH_2CH_2$) and 3.7 (3H, s, CH_3); δ_C (50 MHz, C^2HCl_3) 29.62 (CH_2COO), 42.21 ($COCH_2CH_2$), 52.74 (CH_3), 171.85 and 173.52 (2 x CO).

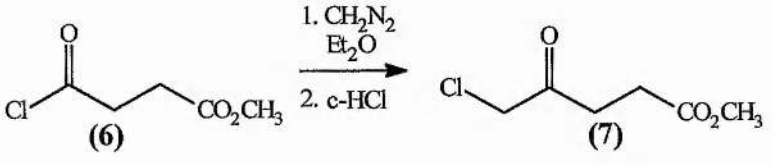
8.9 Synthesis of methyl 5-chlorolevulinate.

8.9.1 Synthesis of diazomethane.[809]



Potassium hydroxide (6.0 g) was dissolved in water (10 cm^3) and carbitol (di(ethyleneglycol) ethyl ether, 35 cm^3) and diethyl ether (10 cm^3) were added. The solution was heated and maintained at 50 °C using a water bath. Diazald (21.4 g), dissolved in diethyl ether (130 cm^3) was added dropwise to the solution over approximately 45 minutes. The bright yellow distillate was collected in a flask immersed in an ice bath and the whole system was fitted with a drying tube. An additional amount of diethyl ether (100 cm^3) was added until the diethyl ether distilling over was colourless. The bright yellow coloured ethereal solution contained approximately 3 g of diazomethane (71 mmol).

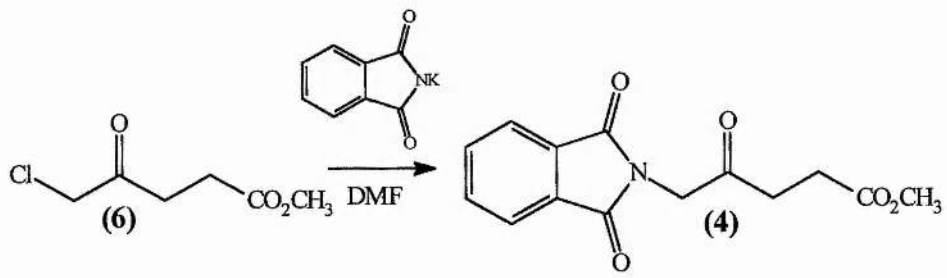
8.9.2 Synthesis of methyl 5-chlorolevulinate.[807]



To a solution of diazomethane (Reaction 8.9.1, 3 g, 71 mmol in 200 cm^3 of diethyl ether) cooled to -5 °C was added, with stirring, a solution of methyl 3-chloroformylpropanoate (4.44 g, 0.029 mol, Reaction 8.8) in dry diethyl ether (14 cm^3), dropwise over 2 hours. The mixture was allowed to warm to room

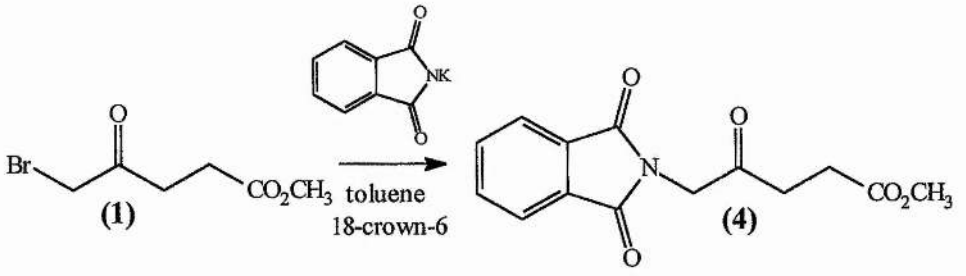
temperature overnight. An equivalent molar amount of c-HCl (2.45 cm^3, 0.029 mol, 37%) was added gradually with stirring to the diazoketone, resulting in a rapid evolution of N_2 after which the solution became a very light yellow colour. The solution was concentrated to 100 cm^3 by leaving the flask unstoppered for 48 hours. It was then washed with water (3 x 30 cm^3) to remove HCl and the combined water washings were extracted with diethyl ether (6 x 20 cm^3) due to the solubility of the ketic ester in water. The combined diethyl ether extracts were dried ($MgSO_4$), filtered and the solvent was removed under reduced pressure giving methyl 5-chlorolevulinate (7) as a clear oil (2.3 g, 47%), b.p. 80 - 85 °C at 1.5 mm/Hg (Lit.[807] 92 - 94 °C at 2 - 3 mm/Hg). δ_H (200 MHz, C^2HCl_3) 2.6 (2H, t, J = 6.2 Hz. CH_2COO), 2.9 (2H, t, J = 6.2 Hz, $COCH_2CH_2$), 3.7 (3H, s, CH_3) and 4.2 (2H, s, $ClCH_2$); δ_C (50 MHz, C^2HCl_3) 28.24 (CH_2COO), 34.80 ($COCH_2CH_2$), 48.83 ($ClCH_2$), 52.30 (CH_3), 173.23 (COO) and 201.74 (CO).

8.10 Synthesis of methyl 5-phthalimidolevulinate.[807]



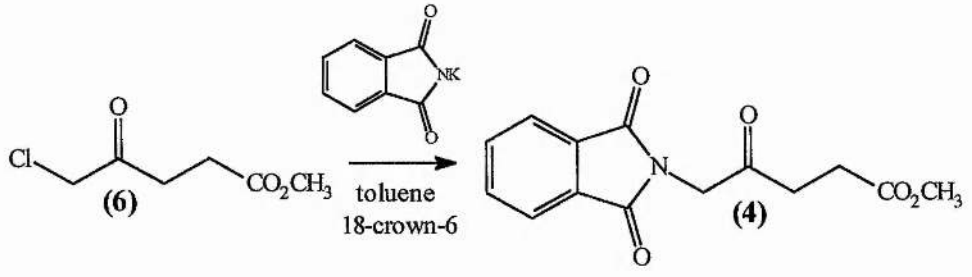
Methyl 5-phthalimidolevulinate (**4**) was prepared using the same method as for methyl 5-bromolevulinate (Reaction 8.4) but using methyl 5-chlorolevulinate (Reaction 8.9.2, 2.3g, 0.014 mol), yielding the product (**4**) as a white crystalline solid (1.74 g, 45%). Data as for Reaction 8.4.

8.11 Synthesis of methyl 5-phthalimidolevulinate.[810]



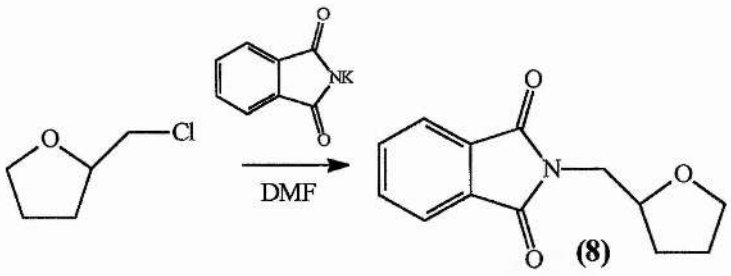
To a mixture of potassium phthalimide (0.22 g, 1.2 mmol) and methyl 5-bromolevulinate (Reaction 8.1, 0.21 g, 1 mmol) in toluene (5 cm^3) was added a catalytic amount of 18-crown-6 (10 mg). The solution was heated to 100 °C for 6 hours under N_2 before water (5 cm^3) was added. The organic layer was separated and the aqueous layer was extracted with DCM (3 x 5 cm^3). The organic fractions were combined, dried ($MgSO_4$), concentrated under reduced pressure and purified by recrystallisation from water, yielding methyl 5-phthalimidolevulinate (**4**) as white crystals (0.21 g, 77%). Data as for Reaction 8.4.

8.12 Synthesis of methyl 5-phthalimidolevulinate.[810]



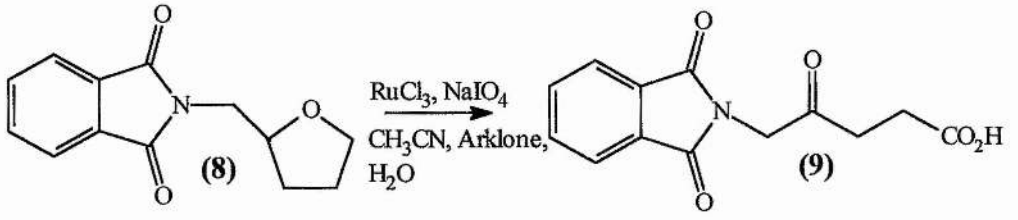
Methyl 5-phthalimidolevulinate was prepared using the same method as for methyl 5-bromolevulinate (Reaction 8.11) but using methyl 5-chlorolevulinate (Reaction 8.9.2, 0.16 g, 1 mmol), yielding the product (**4**) as white crystals (0.2 g, 74%). Data as for Reaction 8.4.

8.13 Synthesis of tetrahydrofurfuryl phthalimide.[811]



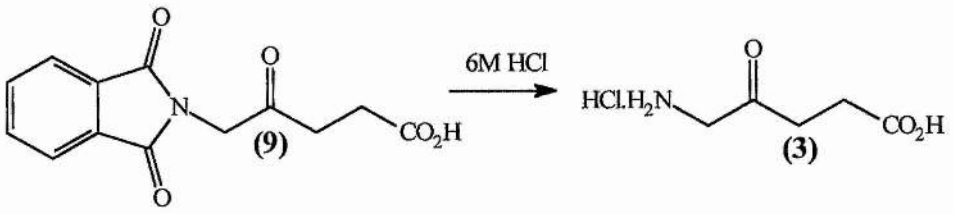
Tetrahydrofurfuryl chloride (2.54 g, 21.1 mmol) was added to a suspension of potassium phthalimide (3 g, 16.15 mmol) in dry DMF (7.5 cm^3) over 12 minutes at 0 °C under N_2. The mixture was heated to reflux for 3 hours then quenched with water (5 cm^3). The reaction was extracted with DCM (3 x 30 cm^3) and the combined extracts were washed with water (2 x 25 cm^3) and brine (25 cm^3). The DCM solution was dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by silica gel flash column chromatography using ethyl acetate : hexane 1:5, and then 1:3, as the eluant yielded tetrahydrofurfuryl phthalimide (**8**) as white crystals (3.01 g, 96%), m.p. 84 - 86 °C (Lit.[811] 82.6 - 86.3 °C). δ_H (200 MHz, C^2HCl_3) 1.7 (1H, m, CH_2CH_2O), 1.8 - 2.1 (3H, m, $CH_2CH_2CH_2O$), 3.7 - 3.8 (2H, m, NCH_2), 3.9 (2H, m, CH_2O), 4.3 (1H, m, NCH_2CH) and 7.7 - 7.9 (4H, m, aromatics); δ_C (50 MHz, C^2HCl_3) 25.78 (CH_2CH_2O), 29.65 ($CH_2CH_2CH_2O$), 42.25 (NCH_2), 68.38 (CH_2O), 76.10 (NCH_2CH), 123.65, 132.56, 134.40 (3 x aromatics) and 168.89 (CO); δ_N (30 MHz, C^2HCl_3) 157.21.

8.14 Synthesis of phthalimidolevulinic acid (ALA4).[811]



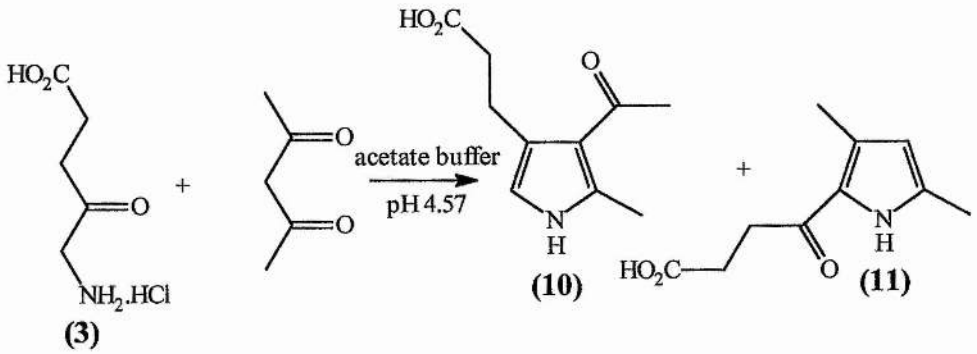
Sodium metaperiodate (6.0 g, 28.2 mmol) and hydrated ruthenium (III) chloride (34 mg) were added to a solution of N-tetrahydrofurfurylphthalimide (Reaction 8.13, 1.63 g, 7 mmol) in 1,1,2-trichloro-1,2,2-trifluoroethane (arklone, 5 cm^3), acetonitrile (25 cm^3) and water (7.5 cm^3) and the mixture was heated at 80 °C for 2 hours, then evaporated under reduced pressure. The residue was taken up in 3M HCl (75 cm^3) and extracted with DCM (6 x 50 cm^3). The combined extracts were dried ($MgSO_4$), filtered, and concentrated under reduced pressure. Purification by silica gel flash column chromatography using ethyl acetate : hexane 1:2 and then ethyl acetate as the eluant yielded phthalimidolevulinic acid (**9**) as a white solid (1.69 g, 92%), m.p. 158 - 160 °C (Lit.[811] 158.9 - 161.7 °C). δ_H (200 MHz, C^2HCl_3 : $^2H_6$-DMSO 9:1) 2.6 (2H, t, J = 6.5 Hz, CH_2COO), 2.8 (2H, t, J = 6.6 Hz, $COCH_2CH_2$), 4.6 (2H, s, NCH_2) and 7.8 - 7.9 (4H, m, aromatics); δ_C (50 MHz, C^2HCl_3 : $^2H_6$-DMSO 9:1) 28.12 (CH_2COO), 34.91 ($COCH_2CH_2$), 46.96 (NCH_2), 123.95, 132.35, 134.41 (3 x aromatics), 168.04 (NCO), 173.10 (CO_2H) and 203.15 (ALA-CO); δ_N (30 MHz, C^2HCl_3 : $^2H_6$-DMSO 9:1) 151.30.

8.15 Synthesis of 5-aminolevulinic acid.[811]



Phthalimidolevulinic acid (Reaction 8.14, 1 g, 3.8 mmol) in 6M HCl (10 cm^3) was heated to reflux for 12 hours then cooled to 0 °C. The resulting phthalic acid was filtered off and the mixture was concentrated under reduced pressure, keeping the temperature below 30 °C to give ALA.HCl (**3**) as a white solid (0.57 g, 89%). Data as for Reaction 8.3. δ_N (30 MHz, ^2H_2O) 25.75.

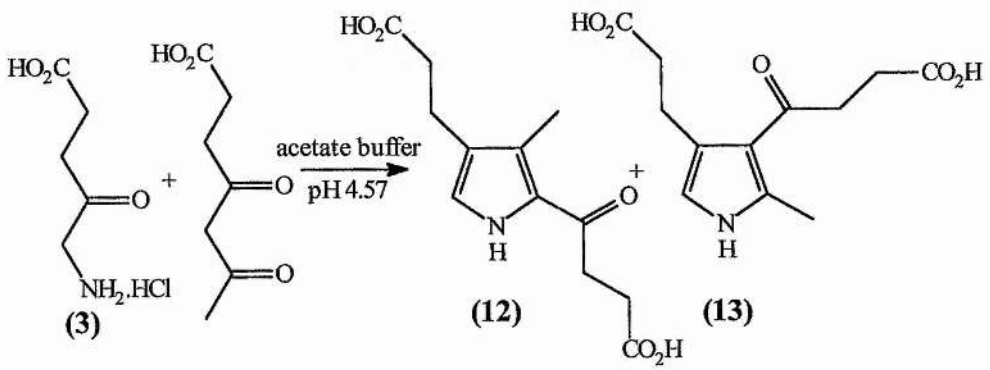
8.16 Synthesis of 3-acetyl-4-(2-carboxyethyl)-2-methylpyrrole and 2-(3-carboxypropionyl)-3,5-dimethylpyrrole.[812]



A solution of ALA.HCl (Reaction 8.15, 0.17 g, 1.0 mmol) and pentane-2,4-dione (0.10 g, 0.10 cm^3, 1.0 mmol) in acetate buffer (4 cm^3, pH 4.57) was heated under reflux conditions for 30 minutes then allowed to cool to room temperature overnight. The resulting brown solid was filtered off, washed thoroughly with cold water and dried to constant weight (0.04 g, 19%), m.p. 192 - 194 °C (Lit.[812]

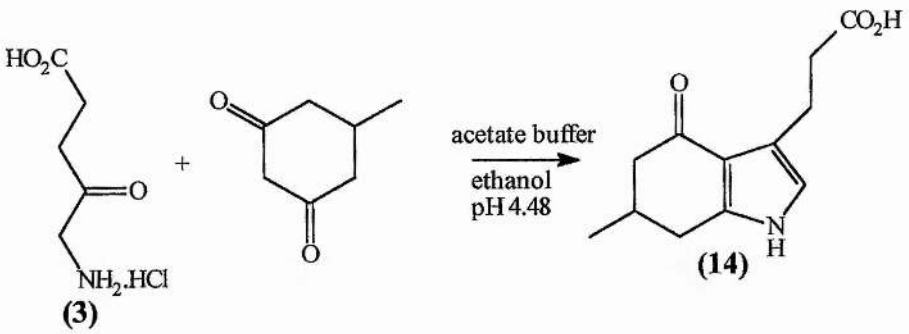
191 °C). m/z (EI^+) 195 (M^+). NMR spectroscopy identifies the two products in approximately a 9:1 ratio: 3 acetyl-4-(2-carboxyethyl)-2-methylpyrrole (**10**); δ_H (300 MHz, $C^2H_3O^2H$) 2.4 (3H, s, $COCH_3$), 2.5 (3H, s, $CHCH_3$), 2.6 (2H, t, J = 7.5 Hz, $CH_2CH_2CO_2H$), 2.9 (2H, t, J = 7.5 Hz, $CH_2CH_2CO_2H$) and 6.4 (1H, s, *CH*NH); δ_C (75 MHz, $C^2H_3O^2H$) 15.41 ($CHCH_3$), 24.22 ($CH_2CH_2CO_2H$), 30.71 ($COCH_3$), 35.92 ($CH_2CH_2CO_2H$), 116.14 (CHNH), 121.26 (C=*C*-CO), 125.87 (C=*C*-CH_2), 138.12 (C=*C*-CH_3), 177.97 (COO) and 197.19 (CO). 2-(3-carboxypropionyl)-3,5-dimethylpyrrole (**11**); δ_H ($C^2H_3O^2H$) 2.2 (3H, s, CH_3C=CCO), 2.3 (3H, s, CH_3CNH), 2.6 (2H, t, J = 6.5 Hz, $CH_2CH_2CO_2H$), 3.0 (2H, t, J = 6.5 Hz, $CH_2CH_2CO_2H$) and 5.8 (1H, s, *CH*CCH_3); δ_c (75 MHz, $C^2H_3O^2H$) 12.85 (CH_3C=CCO), 14.62 (CH_3CNH), 27.14 ($CH_2CH_2CO_2H$), 35.29 ($CH_2CH_2CO_2H$), 113.72 (*CH*CCH_3), 129.14 (*C*CO), 131.96 (CH_3C=CCO), 136.47 (*C*CH_3NH), 177.00 (COO) and 189.12 (C=O).

8.17 Synthesis of 3-(4-carboxybutionyl)-4-(2-carboxyethyl)-2-methylpyrrole and 2-(4-carboxybutionyl)-4-(2-carboxyethyl)-3-methylpyrrole.



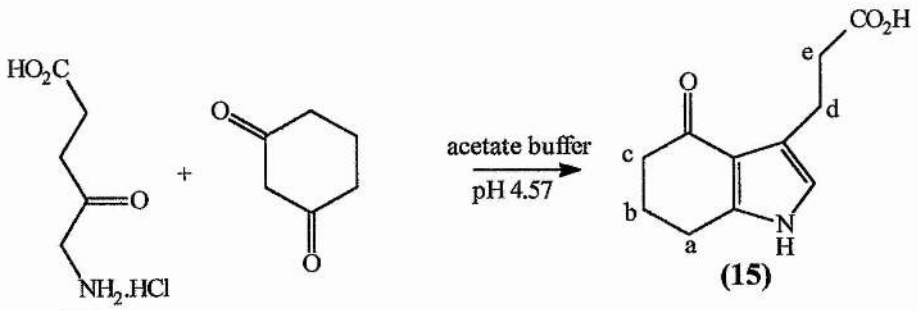
A solution of ALA.HCl (Reaction 8.15, 0.09 g, 5.3 mmol) and 4,6-dioxoheptanoic acid (succinyl acetone) (0.05 g, 5.3 mmol) was heated under reflux conditions in acetate buffer (5 cm^3, pH 4.57) for 30 minutes. The solution was allowed to cool and the resulting white precipitate was filtered off and dried to constant weight (0.06 g, 43%), m.p. 224 - 226 °C. m/z (EI^+) 253 (M^+). Found C, 56.5; H, 6.3; N, 5.5. $C_{12}H_{15}O_5N$ requires C, 56.9; H, 6.0; N, 5.5%. NMR spectroscopy identifies the two products in approximately a 4:1 ratio: 3-(4-carboxybutionyl)-4-(2-carboxyethyl)-2-methylpyrrole (**12**); δ_H (200 MHz, $C^2H_3O^2H$) 2.7 (3H, s, CH_3), 2.8 (3H, m, CH_2), 2.9 (2H, m, CH_2), 3.1 (2H, m, CH_2), 3.2 (2H, m, CH_2) and 6.6 (1H, s, *CH*NH); δ_C (75 MHz, $C^2H_3O^2H$) 12.25 (CH_3), 20.03 ($CH_2CH_2CO_2H$), 25.31 ($CH_2CH_2CO_2H$), 31.75 ($CH_2CH_2CO_2H$), 33.63 ($COCH_2$), 111.71 (CH-aromatic), 116.53 (CCH_2), 120.91 (*C*CO), 131.80 (CNH), 171.35 (COO), 171.52 (COO) and 191.36 (CO). 2-(4-carboxybutionyl)-4-(2-carboxyethyl)-3-methylpyrrole (**13**); δ_H (300 MHz, $C^2H_3O^2H$) 2.6 (3H, s, CH_3), 2.7 (2H, m, CH_2), 2.9 (2H, m, CH_2) 3.2 (2H, m, CH_2), 3.4 (2H, m, CH_2) and 6.7 (1H, s, *CH*NH); δ_C (75 MHz,$C^2H_3O^2H$) 15.25 (CH_3), 21.24 ($CH_2CH_2CO_2H$), 28.04 ($CH_2CH_2CO_2H$), 29.06 ($CH_2CH_2CO_2H$), 34.02 ($COCH_2$), 116.02 (CH-aromatic), 118.05 (CCH_2), 123.04 (*C*CO), 135.21 (CNH), 173.06 (COO), 173.57(COO) and 193.01 (CO).

8.18 Synthesis of 3,2-(carboxyethyl)-6-methyl-4,5,6,7-tetrahydroindol-4-one.



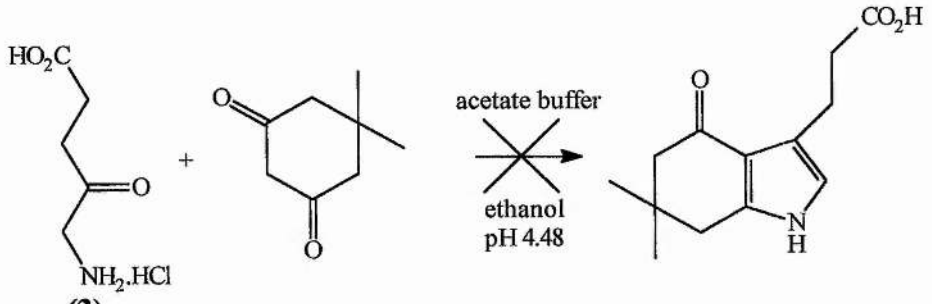
ALA.HCl (Reaction 8.15, 0.17 g, 1 mmol), 5-methyl-1,3-cyclohexanedione (0.13 g, 1 mmol in a saturated solution of ethanol) and acetate buffer (5 cm^3, pH 4.48) were heated under reflux conditions for 30 minutes. The solution was cooled to 0 °C and after a few days the white crystalline product (**14**) that formed was filtered and dried over P_2O_5 to constant weight (0.01 g, 6%), m.p. 198 °C (dec.). m/z (EI^+) 221 (M^+). Found 221.1052. $C_{12}H_{15}NO_3$ requires 221.1052. δ_H (200 MHz, $^2H_6$-DMSO) 1.1 (3H, d, J = 6.1 Hz, CH_3), 2.0 - 2.3 (5H, m, 1 x CH and 2 x CH_2), 2.8 (2H, m, CH_2), 6.5 (1H, s, aromatic) and 11.0 (1H, bs, NH or OH); δ_C (50 MHz, $^2H_6$-DMSO) 20.92 (CH_3), 21.98 ($CH_2CH_2CO_2H$), 28.65 (CH), 30.83, 31.72 (CH_2CCH_3 and CH_2CO), 34.65 (CH_2CO_2H), 116.24 (CH-aromatic), 117.13, 121.14 (CCH_2 and CCO), 143.77 (CNH), 174.62 (COO) and 193.54 (CO).

8.19 Synthesis of 3-(2-carboxyethyl)-4, 5, 6, 7-tetrahydroindol-4-one.



A solution of ALA.HCl (Reaction 8.15, 1 g, 5.97 mmol) and 1,3-cyclohexandione (0.67 g, 5.97 mmol) in acetate buffer (20 cm^3, pH 4.57) was heated under reflux conditions for 30 minutes then allowed to cool to room temperature overnight. The resulting yellow coloured solid was filtered, washed thoroughly with water and recrystallised from ethanol to give 3-(2-carboxyethyl)-4,5,6,7-tetrahydroindol-4-one (**15**) as a white solid (0.14 g, 12%), m.p. 194-196 °C. m/z (EI^+) 207 (M^+). Found C, 63.5; H, 6.5; N, 6.7. $C_{11}H_{13}NO_2$ requires C, 63.8; H, 6.3; N, 6.8%. δ_H (300 MHz, $^2H_6$-DMSO) 2.1 (2H, quin, J = 6.6 Hz, CH_2-b), 2.4 (2H, t, J = 6.6 Hz, CH_2-c), 2.6 (2H, t, J = 7.4 Hz, CH_2-e), 2.8 (2H, t, J = 8.2 Hz, CH_2-a), 2.9 (2H, t, J = 6.6 Hz, CH_2-d), 6.6 (1H, s, aromatic CH), 11.1 (1H, s, NH) and 11.8 (1H, bs, OH); δ_C (75 MHz, $^2H_6$-DMSO) 22.01 (CH_2-a), 23.04 (CH_2-d), 24.06 (CH_2-b), 34.52 (CH_2-e), 38.24 (CH_2-c), 116.01 (aromatic CH), 118.24, 122.43 (*C*CH_2 and *C*CO), 144.06 (CNH), 174.98 (COO) and 195.05 (CO). These interpretations were made using ^1H - ^{13}C and COSY two dimensional NMR spectroscopy (See Section 3.2.2).

8.20 Attempted synthesis of 3-(2-carboxyethyl)-6,6-dimethyl-4,5,6,7-tetrahydroindol-4-one.

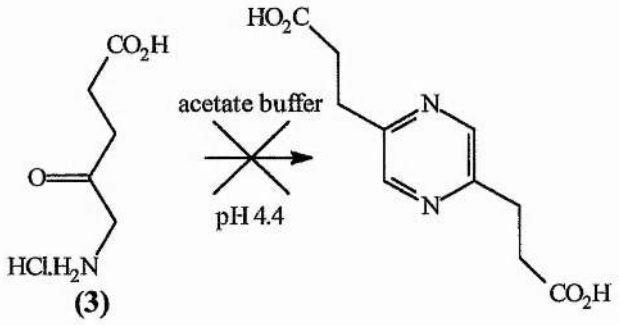


(3)

ALA.HCl (Reaction 8.15, 0.17 g, 1 mmol), 5,5-dimethyl-1,3-cyclohexanedione (dimedone, 0.14 g, 1 mmol in a saturated solution of ethanol) and acetate buffer (5 cm^3, pH 4.48) were heated under reflux conditions for 30 minutes. The solution was allowed to cool to room temperature then refrigerated for several

weeks but no product appeared. The ethanol was removed under reduced pressure and, on cooling, a yellow solid formed which was filtered off and dried to constant weight (0.015 g). From mixed melting point determination, the product was unreacted 5,5-dimethyl-1,3-cyclohexanedione, the starting material.

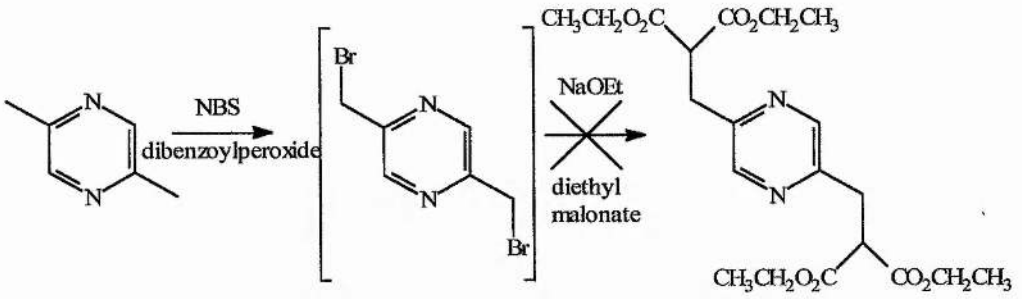
8.21 Attempted synthesis of 2,5-di-(β-carboxyethyl) pyrazine.



(3)

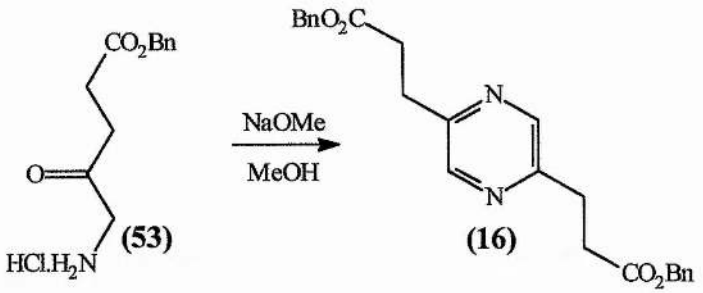
ALA.HCl (Reaction 8.15, 0.5 g, 2.98 mmol) in acetate buffer (10 cm^3, pH 4.4) was heated under reflux conditions for 6 hours causing the solution to go a dark brown colour. The mixture was freeze dried giving brown hygroscopic crystals. Decolourising charcoal failed to remove the colour as did recrystallisation from ethanol or acetone. The product was, therefore, purified by column chromatography using ethyl acetate : DCM 9:1 with 0.5% acetic acid as the eluant. This yielded a brown hygroscopic oil. Mass spectrometry suggested polymerisation had occurred.

8.22 Attempted synthesis of 2,5-di-(β-dicarboxyethyl) pyrazine tetraethyl ester.[813]



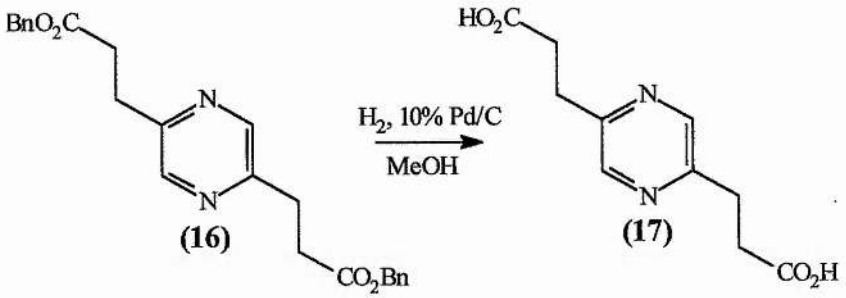
A solution of 2,5-dimethylpyrazine (1.09 cm^3, 1.08 g, 0.01 mol), N-bromosuccinamide (3.56 g, 0.02 mol) and dibenzoyl peroxide (50 mg, 0.20 mmol) in THF (50 cm^3) was heated under reflux conditions for 6 hours then stirred at room temperature for 48 hours. After cooling to 0 °C the mixture was filtered, and the filtrate concentrated under reduced pressure. The resulting residue was reacted immediately to avoid polymerisation. Sodium ethoxide was generated by the addition of sodium metal (3.45 g) to ethanol (100 cm^3). The crude residue was then added (1.98 cm^3) along with diethyl malonate (3.20 g, 0.02 mol, approximately 2 equivalents) and the mixture was heated under reflux comditions for 2 hours. The resulting solution was concentrated under reduced pressure and extracted with diethyl ether (50 cm^3 x 2). The diethyl ether extracts were washed with HCl solution, then brine (50 cm^3 of each), dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a pale orange coloured oil. Tlc showed this to contain at least seven products, therefore the approach was abandoned.

8.23 Synthesis of 2,5-di-(β–carboxyethyl) pyrazine dibenzyl ester.



5-Aminolevulinic acid benzyl ester (Reaction 8.63, 0.5 g, 1.9 mmol) was dissolved in methanol (5 cm^3). Sodium metal (0.044 g, 1.9 mmol) was added causing NaCl to precipitate from the solution. The mixture was stirred at room temperature for 3 hours then filtered to remove the NaCl. The solvent was removed under reduced pressure giving 2,5-di-(β-carboxyethyl) pyrazine dibenzyl ester (**16**) as a slightly yellow coloured oil (0.35 g, 90%). m/z. (EI^+) 404 (M^+). A correct elemental analysis was not obtained as the product was contaminated with a small quantity of NaCl. δ_H (200 MHz, C^2HCl_3) 2.8 (4H, t, J = 9.1 Hz, 2 x CH_2), 3.1 (4H, t, J = 9.1 Hz, 2 x CH_2), 5.1 (4H, s, 2 x CH_2Ph), 7.3 - 7.4 (10H, m, 2 x aromatics) and 8.4 (2H, s, 2 x aromatic CH); δ_C (50 MHz, C^2HCl_3) 29.97 (2 x CH_2CH_2COO), 33.22 (2 x CH_2COO), 66.95 (2 x CH_2Ph), 127.48, 128.10, 128.52, 128.74, 129.03, 136.29 (2 x aromatics), 143.99 (2 x $NCCCH_2$), 153.33 (2 x $NCCCH_2$) and 172.98 (2 x CO).

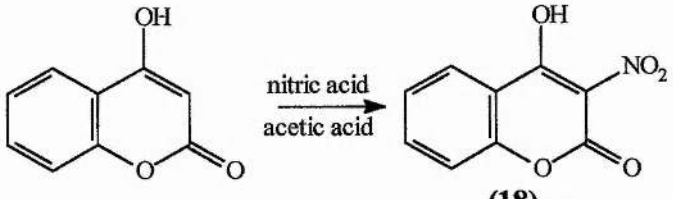
8.24 Synthesis of 2,5-di-(β-carboxyethyl) pyrazine.



(16) (17)

2,5-Di-(β-carboxyethyl) pyrazine dibenzyl ester (Reaction 8.23, 0.3 g, 0.7 mmol) was dissolved in methanol (10 cm^3). 10% Pd/C catalyst (50 mg) was added and the mixture was stirred under an atmosphere of H_2 at room temperature and atmospheric pressure for 24 hours. The resulting mixture was filtered through Celite to remove the catalyst and the solvent was removed under reduced pressure to give 2,5-di-(β-carboxyethyl) pyrazine (**17**) as a light brown coloured oil (0.15 g, 88%). δ_H (200 MHz, $^2H_6$-DMSO) 2.8 (4H, t, J = 9.1 Hz, 2 x CH_2), 3.0 (4H, t, J = 9.1 Hz, 2 x CH_2) and 8.4 (2H, s, 2 x aromatic CH); δ_C (50 MHz, $^2H_6$-DMSO) 29.76 (2 x *CH_2*CH_2COO), 32.83 (2 x *CH_2*COO), 143.81 (2 x NCCCH$_2$), 153.64 (2 x NCCCH$_2$) and 179.29 (2 x CO).

8.25 Synthesis of 3-nitro-4-hydroxycoumarin.[814]

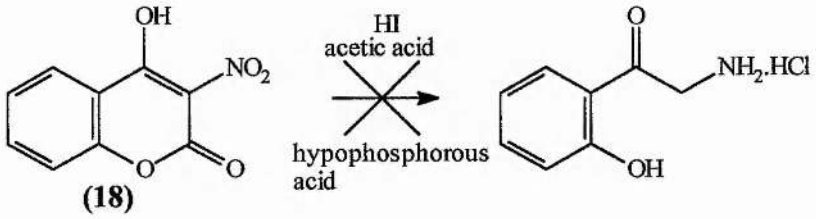


(18)

A suspension of nitric acid (4.6 cm^3, 0.11 mol) in glacial acetic acid (5 cm^3) was added to a suspension of 4-hydroxycoumarin (10.12 g, 0.062 mol) in glacial acetic acid (25 cm^3). The mixture was heated to 70 °C to initiate the reaction then

allowed to cool to room temperature for 2 hours. The product was filtered, washed with ice cold water and dried over P_2O_5 giving 3-nitro-4-hydroxycoumarin (**18**) as a pale orange solid (9.62 g, 75%), m.p. 178 °C (dec.) (Lit.[815] 177 °C (dec.)). δ_H (200 MHz, $^2H_6$-DMSO) 7.3 (2H, t, J = 8.8 Hz, aromatics), 7.6 (1H, t, J = 6.9 Hz, aromatics) and 7.9 (1H, d, J = 8.2 Hz, aromatics); δ_C (50 MHz, $^2H_6$-DMSO)116.54, 120.04, 123.89, 125.76, 128.95, 133.39 (5 x Aromatics and CNO_2), 152.55 (CHCO), 157.00 (CO) and 165.65 (COH).

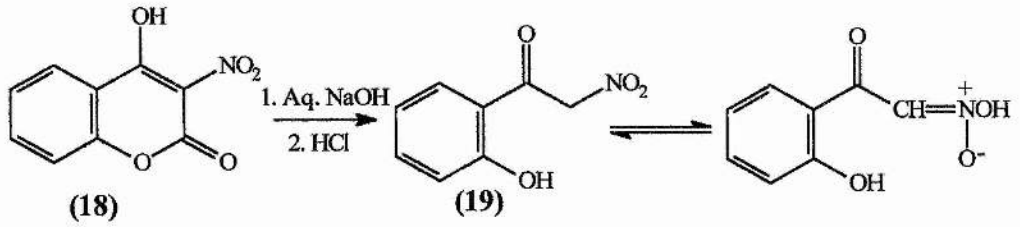
8.26 Attempted synthesis of 2'-amino-2-hydroxyacetophenone hydrochloride.[815]



(18)

3-Nitro-4-hydroxycoumarin (Reaction 8.25, 2 g, 9.7 mmol) was heated under reflux conditions in a mixture of hydriodic acid (5 cm^3, 58%) and glacial acetic acid (10 cm^3) for 15 minutes. Hypophosphorous acid (5 cm^3) was added to reduce the resulting iodine. The mixture was cooled to 0 °C and the crystals that formed were filtered off and recystallised from c-HCl. After numerous different attempts, NMR data shows the resulting solid to be 3-nitro-4-hydroxycoumarin, the starting material.

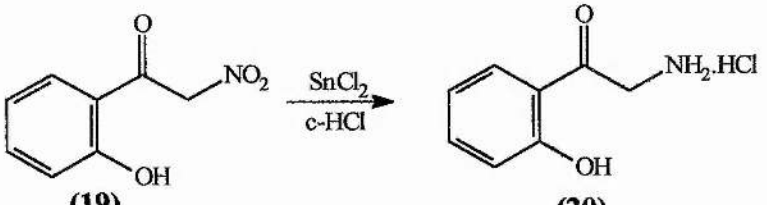
8.27 Synthesis of 2-hydroxy-2'-nitroacetophenone.[814]



(18) (19)

3-Nitro-4-hydroxycoumarin (Reaction 8.25, 4.0 g, 19.3 mmol) was dissolved in aqueous sodium hydroxide solution (5 g in 100 cm^3, 12.5 mM, 5%). The solution was stirred at 50 - 60 °C for 1.5 hours during which time it turned a bright orange colour. The mixture was allowed to cool to room temperature for 2 hours. An excess of c-HCl (15 cm^3) was added causing the immediate precipitation of pale yellow coloured crystals. After standing overnight, the crystals of 2-hydroxy-2'-nitroacetophenone (**19**) were filtered off and washed with water (2.1 g, 60%), m.p. 102 - 104 °C (Lit.[814] 106 °C). m/z (EI^+) 181 (M^+). Found C, 52.9; H, 4.2; N, 7.7; Calculated for $C_8H_7NO_4$: C, 53.0; H, 3.9; N, 7.7%. δ_H (200 MHz, $^2H_6$-DMSO) 6.2 (2H, s, CH_2), 7.0 (2H, m, ar.), 7.6 (1H, m, ar.), 7.8 (1H, m, ar.) and 11.2 (1H, s, OH); δ_C (50 MHz, $^2H_6$-DMSO) 91.05 (CH_2), 122.79, 123.25, 124.88, 125.23 and 135.74, 135.97, 144.37, 142.08 (2 sets of aromatics), 164.97 and 166.83 (2 x C=O). From ^{13}C data, the product seems to isomerise in solution giving a 1:1 ratio of 2-hydroxy-2'-nitro-acetophenone and its nitronic acid.

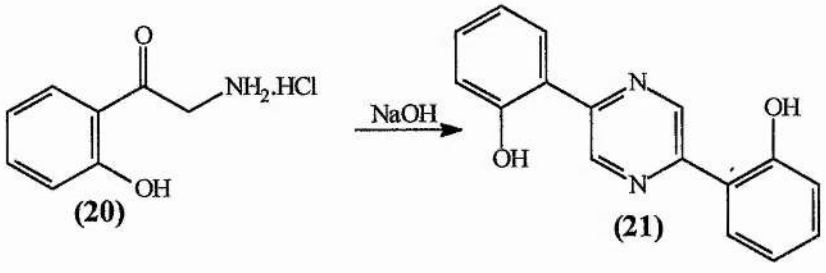
8.28 Synthesis of 2'-amino-2-hydroxyacetophenone hydrochloride.



(19) (20)

2-Hydroxy-2'-nitroacetophenone (Reaction 8.27, 0.88 g, 5.34 mmol), tin (II) chloride dihydrate (4 g, 0.02 mol) and c-HCl (6.66 cm^3) were stirred together causing an exothermic reaction. The temperature rose to 60 °C and after 5 minutes the mixture was heated to 90 °C and left for 30 minutes. Water (6.6 cm^3) was added and the mixture was heated under reflux conditions until a clear yellow solution formed (approximately 30 minutes was required). The solution was cooled to 0 °C and stirred during the dropwise addition of NaOH solution (10M) to pH 5. The resulting light brown precipitate of 2'-amino-2-hydroxyacetophenone hydrochloride (**20**) was filtered off and dried (0.52 g, 70%), m.p. 230 °C (Lit.[815] 229 - 230 °C). m/z (EI^+) 151 (M^+). δ_H (200 MHz, $^2H_6$-DMSO) 4.4 (2H, s, CH_2), 7.0 (1H, t, J = 7.3 Hz, aromatic), 7.2 (1H, d, J = 8.0 Hz, aromatic), 7.6 (1H, t, J = 8.0 Hz, aromatic), 7.8 (1H, d, J = 8.0 Hz, aromatic), 8.3 (2H, bs, NH_2) and 11.3 (1H, bs, OH); δ_C (50 MHz, $^2H_6$-DMSO) 53.76 (CH_2), 123.37, 125.16, 126.18, 135.59, 141.82 (aromatics) and 165.21 (C=O).

8.29 Synthesis of 2,5-dihydro-2,5-di-(2'-hydroxyphenyl) pyrazine.[815]

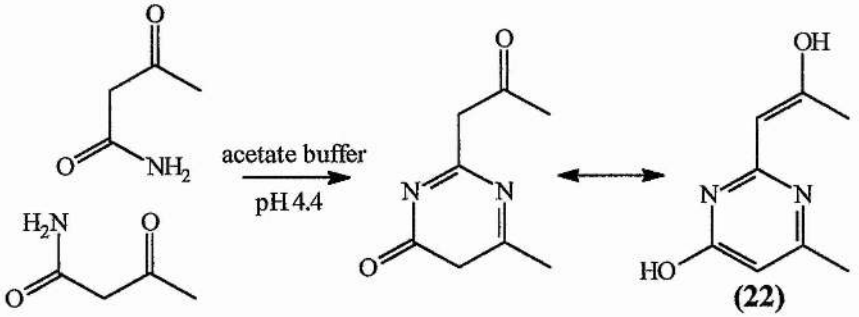


(20) **(21)**

2'-Amino-2-hydroxyacetophenone hydrochloride (Reaction 8.28, 0.32 g, 1.8 mmol) was dissolved in water (10 cm^3). One equivalent of KOH (0.6 M, 3 cm^3, 0.1 g, 1.8 mmol) was added resulting in the formation of a gelatinous pink

precipitate, which was filtered off and recrystallised from dioxane to give 2,5-dihydro-2,5-di-(2'-hydroxyphenyl) pyrazine (**21**) as highly insoluble, therefore uncharacterised, pink crystals (0.10 g, 45%).

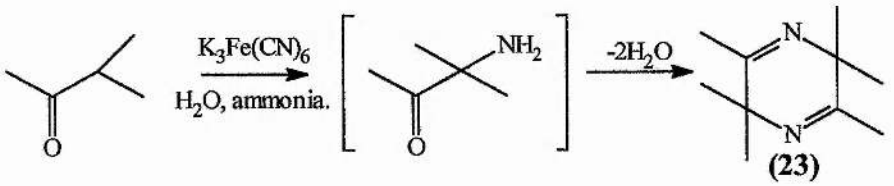
8.30 Synthesis of 2-(acetylmethyl)-6-methylpyrimidin-4-one.



(22)

A solution of acetoacetamide (1 g, 10 mmol) in ethanol (5 cm^3) and acetate buffer (20 cm^3, pH 7.4) was heated under reflux conditions for 30 minutes then allowed to stand at room temperature for 4 days, The resulting white solid was filtered off and recrystallised from ethanol to give 2-(acetylmethyl)-6-methylpyrimidin-4-one (**22**) as white crystals (0.16 g, 10%), m.p. 222 °C (dec.) m/z (EI^+) 166 (M^+). Found C, 57.2; H, 6.1; N, 16.6; Calculated for $C_8H_{10}N_2O_2$: C, 57.8; H, 6.1; N, 16.8%. δ_H (200 MHz, $^2H_6$-DMSO) 2.1 (6H, s, 2 x CH_3), 6.0 (1H, s, CH), 7.5 (1H, s, aromatic), 7.7 (1H, s, OH) and 11.5 (1H, s, OH); δ_C (50 MHz, $^2H_6$-DMSO) 22.59 (*CH_3*CN), 25.15 (*CH_3*COH), 121.52, 121.68 (2 x *CH*COH) 147.32 (N*C*CH_3), 154.82 (NCHN), 167.75 (NCOH) and 174.08 (CH_3*CH*OH).

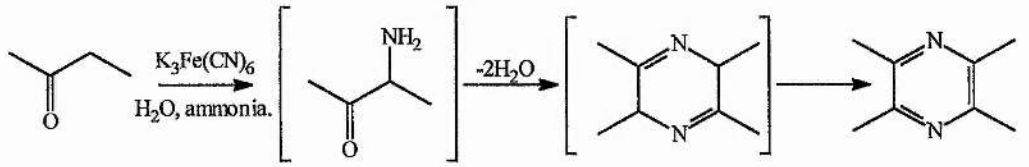
8.31 Synthesis of 2,5-dihydro-hexamethylpyrazine.[816]



(23)

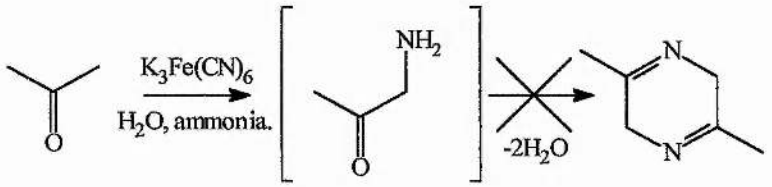
Water (66 cm^3), in a three necked flask fitted with a reflux condenser, a thermometer and a fritted glass nitrogen inlet tube was heated to 80 °C and degassed with a slow stream of nitrogen before potassium ferricyanate (7.82 g, 24 mmol) was added. After 2 minutes of additional degassing the nitrogen bubbler was replaced with a dropping funnel. While a static nitrogen atmosphere was maintained 3-methyl-2-butanone (0.45 g, 5.3 mmol) was added all at once followed by degassed ammonia solution (4 cm^3) dropwise over 30 minutes during which time the temperature remained between 80 and 85 °C. This caused the solution to turn a green colour. The reaction mixture was stirred at 85 °C for 8 hours resulting in formation of a brown coloured solution. It was then cooled and extracted with DCM (5 x 25 cm^3). The combined DCM extracts were washed with brine then 6% HCl solution (50 cm^3 of each). The acid layer was basified using NaOH then re-extracted with DCM (2 x 50 cm^3). DCM layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure to give impure product. Sublimation yielded 2,5-dihydro-hexamethylpyrazine (**23**) as a white solid (0.11 g, 26%), m.p. 88 - 90 °C (Lit.[816] 88 - 89 °C). δ_H (200 MHz, $^2H_6$-DMSO) 1.2 (12H, s, 4 x CH_3) and 1.9 (6H, s, 2 x CH_3); δ_C (50 MHz, $^2H_6$-DMSO) 22.62, 28.41, 55.86 ($C(CH_3)_2$) and 111.14 (CCH_3).

8.32 Attempted synthesis of 2,5-dihydro-2,3,5,6-tetramethylpyrazine.



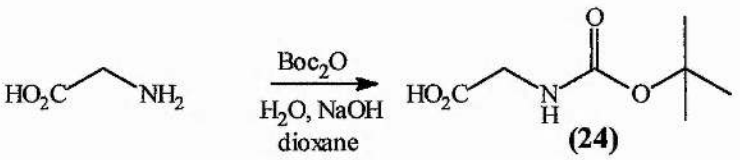
The reaction was carried out using the same method as for 2,5-dihydro-hexamethylpyrazine (Reaction 8.31), but using ethyl methyl ketone (0.38 g, 5.3 mmol). The dihydropyrazine was not isolated. The product of reaction was 2,3,5,6-tetramethylpyrazine (0.08 g, 22%), m.p. 88 - 90 °C (Lit.[817] 85 - 86 °C). δ_H (200 MHz, $^2H_6$-DMSO) 1.4 (12H, s, 4 x CH_3); δ_C (50 MHz, $^2H_6$-DMSO) 19.03 (CH_3) and 153.84 (aromatics).

8.33 Attempted synthesis of 2,5-dihydro-2,5-dimethylpyrazine.



The reaction was carried out using the same method as for 2,5-dihydro-hexamethylpyrazine (Reaction 8.31) but using acetone (0.31 g, 5.3 mmol). No product was isolated.

8.34 Synthesis of t-butoxycarbonylglycine.[818]

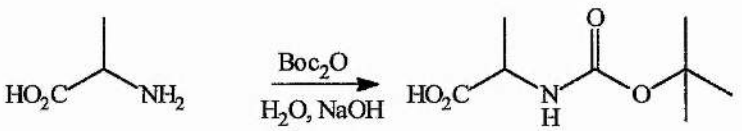


(24)

A solution of glycine (1.5 g, 20 mmol) in a mixture of dioxane (40 cm^3), water (20 cm^3) and NaOH solution (1M, 20 cm^3) was stirred and cooled in an ice water

bath. Di-t-butyl pyrocarbonate (4.8 g, 22 mmol) was added and the mixture was stirred at room temperature for 1 hour then concentrated to 30 cm^3. The solution was cooled to 0 °C and covered with a layer of ethyl acetate (60 cm^3). The mixture was acidified with a dilute solution of $KHSO_4$ to pH 2.5 before the aqueous phase was extracted with ethyl acetate (30 cm^3 x 2). The ethyl acetate extracts were washed with water (60 cm^3 x 2), dried ($MgSO_4$) and concentrated under reduced pressure to give a white solid which was recrystallised from ethyl acetate/hexane to give t-butoxycarbonylglycine (**24**) as white crystals (1.44 g, 41%), m.p. 85 - 87 °C (Lit.[819] 86 - 88 °C). δ_H (200 MHz, C^2HCl_3) 1.4 (9H, s, 3 x Boc-CH_3), 3.9 (2H, m, CH_2), 5.1 (1H, bs, NH or OH) and 6.7 (1H, bs, NH or OH); δ_C (50 MHz, C^2HCl_3) 28.77 (3 x Boc-CH_3), 42.69, 53.89 (CH_2 and $C(CH_3)_3$) 154.06 (COBoc) and 175.49 (COO).

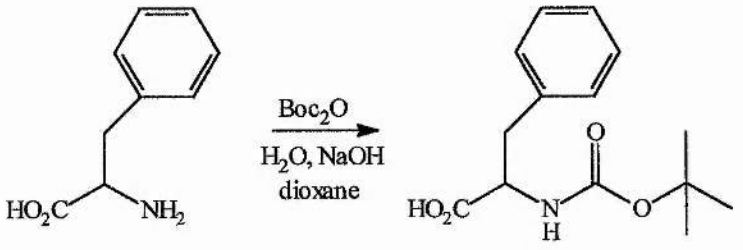
8.35 Synthesis of t-butoxycarbonyl-L-alanine.[818]



(25)

t-Butoxycarbonyl-L-alanine (**25**) was prepared using the same method as for t-butoxycarbonylglycine (Reaction 8.34) but using L-alanine (1.78 g, 20 mmol) yielding the product (**25**) as a white crystalline solid (2.28 g, 60%), m.p. 84 - 86 °C (Lit.[819] 82 - 84 °C). δ_H (200 MHz, C^2HCl_3) 1.3 (12H, s, 3 x Boc-CH_3 and CH_3), 4.2 (1H, m, CH), 5.2 (1H, d, J = 7.7 Hz, NH) and 11.4 (1H, bs, OH); δ_C (50 MHz, C^2HCl_3) 18.84 (CH_3), 28.74 (3 x Boc-CH_3), 48.57, 50.07 (CH and C-Boc), 156.31 (COBoc) and 173.51 (COO).

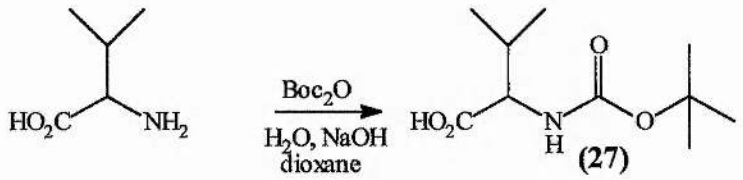
8.36 Synthesis of t-butoxycarbonyl-L-phenylalanine.[818]



(26)

t-Butoxycarbonyl-L-phenylalanine (**26**) was prepared using the same method as for t-butoxycarbonylglycine (Reaction 8.34) but using L-phenylalanine (3.3 g, 20 mmol), yielding the product (**26**) as a white crystalline solid (3.04 g, 53%), m.p. 87 - 89 °C (Lit.[819] 84 - 86 °C). δ_H (200 MHz, C^2HCl_3) 1.3 (9H, s, 3 x Boc-CH_3), 3.2 (2H, m, CH_2), 4.6 (1H, m, CH), 6.6 (1H, d, J = 7.7 Hz, NH), 7.3 (5H, m, aromatics) and 8.8 (1H. bs, OH); δ_C (50 MHz, C^2HCl_3) 28.41 (3 x Boc-CH_3), 38.32 (CH_2), 54.71, 58.79 ($C(CH_3)_3$ and CH), 127.25, 127.52, 129.98, 135.35 (aromatics), 155.85 (COBoc) and 176.46 (COO).

8.37 Synthesis of t-butoxycarbonyl-L-valine.[818]

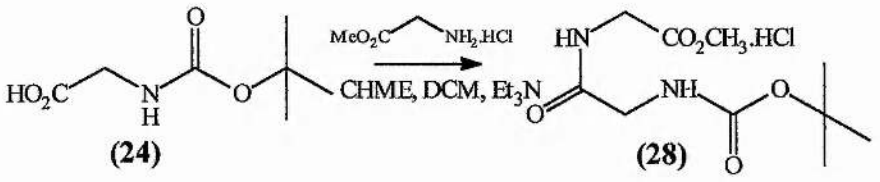


(27)

t-Butoxycarbonyl-L-valine (**27**) was prepared using the same method as for t-butoxycarbonylglycine (Reaction 8.34) but using L-valine (2.34 g, 20 mmol) yielding the product (**27**) as a white crystalline solid (2.27 g, 52%), m.p. 77 - 78 °C (Lit.[820] 77 - 79 °C). δ_H (200 MHz, C^2HCl_3) 1.0 (6H, m, 2 x CH_3), 1.4 (9H, s, 3 x Boc-CH_3), 2.2 (1H, m, *CH*(CH_3)$_2$), 4.3 (1H, m, *CH*NH), 5.1 (1H, d, J = 7.7 Hz, NH) and 6.4 (1H, bs, OH); δ_C (50 MHz, C^2HCl_3) 17.91 (2 x CH_3), 28.76 (3 x

Boc-CH_3), 31.52 ($CH(CH_3)_2$), 58.88, 58.92 ($C(CH_3)_3$ and CHNH), 156.36 (CO) and 177.68 (COO).

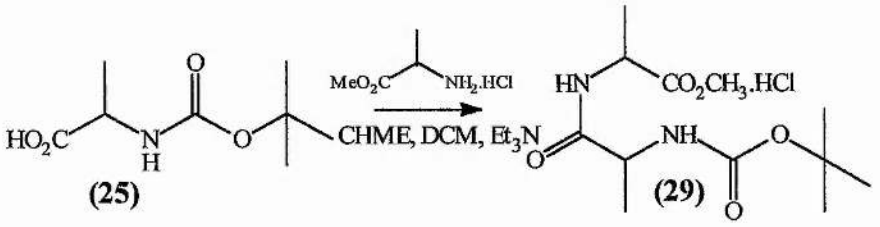
8.38 Synthesis of t-butoxycarbonylglycinyl glycine methyl ester.[818]



(24) (28)

t-Butoxycarbonylglycine (Reaction 8.34, 0.35 g, 2 mmol) was dissolved in DCM (10 cm^3) at 0 °C. Glycine methyl ester hydrochloride (0.25 g, 2 mmol) and triethylamine (0.28 cm^3, 2 mmol) were added. After the addition of CHME (0.85 g, 2 mmol) the solution was stored at 0 °C overnight. The reaction mixture was washed with water, citric acid solution (1M), saturated sodium bicarbonate solution, and water (30 cm^3 of each), dried ($MgSO_4$), filtered and concentrated under reduced pressure to yield t-butoxycarbonylglycinyl glycine methyl ester (**28**) as a white solid (0.49 g, 88%), m.p 116 - 118 °C. δ_H (200 MHz, C^2HCl_3) 1.5 (9H, m, 3 x Boc-CH_3), 3.7 (3H, s, OCH_3), 3.9 (2H, s, CH_2) and 4.1 (2H, s, CH_2); δ_C (50 MHz, C^2HCl_3) 27.25 (3 x Boc-CH_3), 40.05, 43.18 (2 x CH_2), 51.43 (CH_3), 58.88 ($C(CH_3)_3$), 152.15 (CO), 167.29 (COBoc) and 171.98 (COO).

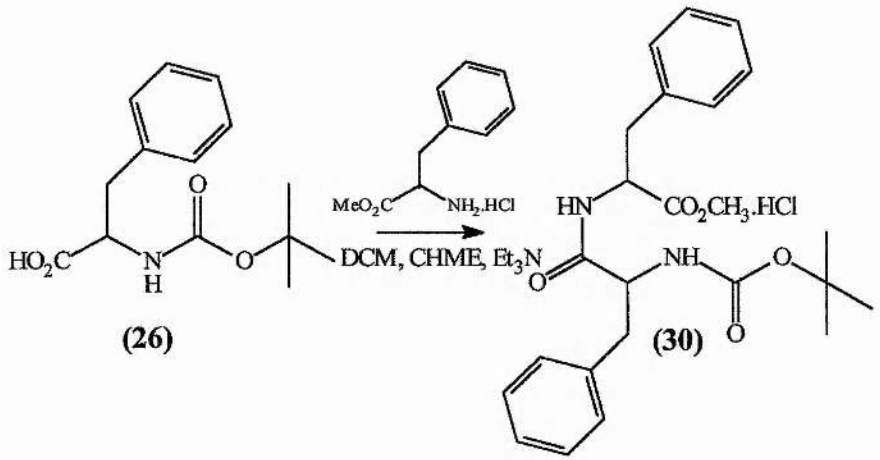
8.39 Synthesis of t-butoxycarbonylalaninyl alanine methyl ester.[818]



(25) (29)

t-Butoxycarbonylalanyl alanine methyl ester (**29**) was prepared using the same method as for t-butoxycarbonylglycinyl glycine methyl ester (Reaction 8.38) but using t-butoxycarbonyl-alanine (Reaction 8.35, 0.38 g, 2 mmol) and alanine methyl ester (0.28 g, 2 mmol), yielding the product (**29**) as a white solid (0.54 g, 87%), m.p 119 - 121 °C. δ_H (200 MHz, C^2HCl_3) 1.5 (15H, m, 3 x Boc-CH_3 and 2 x CH_3), 3.8 (3H, s, OCH_3), 4.2 (1H, m, CH) and 4.6 (1H, m, CH); δ_C (50 MHz, C^2HCl_3) 18.26 (CH_3), 18.76 (CH_3), 28.75 (3 x Boc-CH_3), 46.46 (CH), 51.42 (CH_3), 52.93 (CH), 58.94 ($C(CH_3)_3$), 151.29 (CO-Boc), 169.41 (CONH) and 173.62 (COO).

8.40 Synthesis of t-butoxycarbonylphenylalanyl phenylalanine methyl ester.[818]

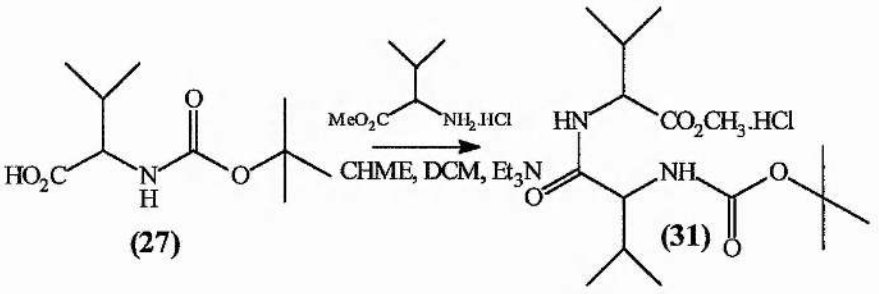


(26) (30)

t-Butoxycarbonylphenylalanyl phenylalanine methyl ester (**30**) was prepared using the same method as for t-butoxycarbonylglycinyl glycine methyl ester (Reaction

8.38) but using t-butoxycarbonyl-phenylalanine (Reaction 8.36, 0.53 g, 2 mmol) and phenylalanine methyl ester (0.43 g, 2 mmol), yielding the product (**30**) as a white solid (0.81 g, 95%), m.p 112 - 114 °C (Lit.[821] 114 - 115 °C). δ_H (200 MHz, C^2HCl_3) 1.3 (9H, s, 3 x Boc-CH_3), 3.3 (4H, m, 2 x CH_2), 3.8 (3H, s, OCH_3), 4.2, 4.5 (2 x 1H, 2 x m, 2 x CH), 6.8 (1H, d, J = 7.6 Hz, NH), 7.1 - 7.4 (10H, m, 2 x aromatics) and 8.4 (1H, d, J = 7.6 Hz, NH); δ_C (50 MHz, C^2HCl_3) 28.40, 28.46, 28.75 (3 x Boc-CH_3), 38.36, 38.77 (2 x CH_2Ph), 52.71 (CO_2CH_3), 53.76 (CCO_2CH_3), 56.17 (*CH*NHBoc), 58.21 ($C(CH_3)_3$), 127 - 129, 136.12, 136.95 (aromatics), 155.84 (*CO*OBoc), 171.39 and 172.54 (2 x CO).

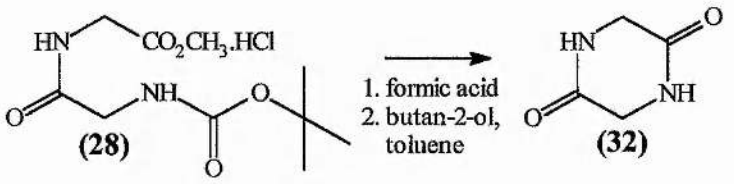
8.41 Synthesis of t-butoxycarbonylvalinyl valine methyl ester.[818]



t-Butoxycarbonylvalinyl valine methyl ester (**31**) was prepared using the same method as for t-butoxycarbonylglycinyl glycine methyl ester (Reaction 8.38) but using t-butoxycarbonyl-valine (Reaction 8.37, 0.43 g, 2 mmol) and valine methyl ester (0.33 g, 2 mmol) yielding the product (**31**) as a white solid (0.59 g, 89%). m.p 115 - 117 °C. δ_H (200 MHz, C^2HCl_3) 1.0 (12H, m, 2 x CH(CH_3)$_2$), 1.4 (9H, s, 3 x Boc-CH_3), 2.0 - 2.2 (2H, m, 2 x *CH*(CH_3)$_2$), 3.8 (3H, s, OCH_3), 3.9 (1H, m, CH) and 4.3 (1H, m, CH); δ_C (50 MHz, C^2HCl_3) 17.24, 19.17 (2 x (CH(CH_3)$_2$), 28.24 (3 x Boc-CH_3), 31.26 (2 x *CH*(CH_3)$_2$), 58.24 ($C(CH_3)_3$),

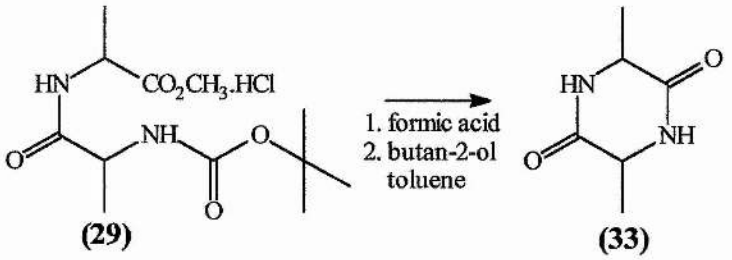
76.14, 77.29 (2 x CHNH under C^2HCl_3), 156.26 (CO-Boc), 162.41 (CONH) and 177.25 (COO).

8.42 Synthesis of glycine anhydride.[821]



t-Butoxycarbonylglycinyl glycine methyl ester (Reaction 8.38, 0.4 g, 1.4 mmol) was dissolved in formic acid (40 cm^3, 98%) and the solution was kept at room temperature for 2 hours. The excess formic acid was removed under reduced pressure, keeping the temperature below 30 °C. The residue containing the crude dipeptide ester formate was then dissolved in butan-2-ol (sec-butyl alcohol, 80 cm^3) and toluene (20 cm^3) and the mixture was heated under reflux conditions for 2.5 hours. The mixture was concentrated under reduced pressure to 20 cm^3 and then cooled to 0 °C. The resulting white crystals of glycine anhydride (**32**) were filtered off and dried over P_2O_5 (0.11 g, 71%), m.p. >250 °C (Lit.[821] 311 - 312 °C). δ_H (200 MHz, $^2H_6$-DMSO) 3.7 (4H, s, 2 x CH_2) and 8.05 (2H, bs, 2 x NH); δ_C (50 MHz, $^2H_6$- DMSO) 36.42 (2 x CH_2) and 171.79 (2 x CO).

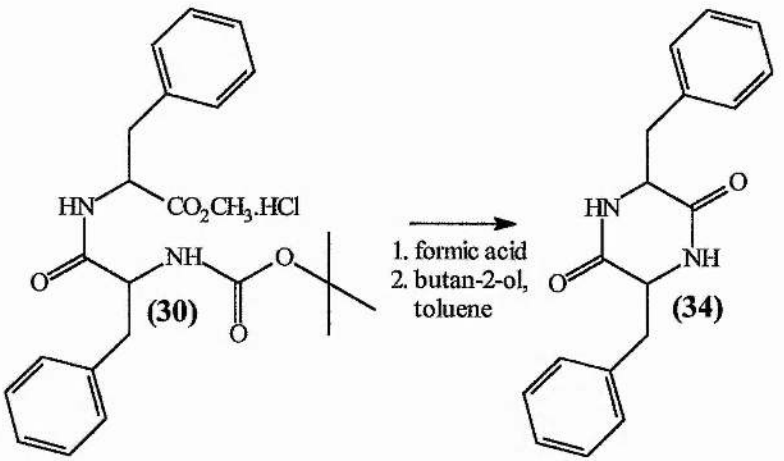
8.43 Synthesis of alanine anhydride.[821]



(29) (33)

Alanine anhydride (**33**) was prepared using the same method as for glycine anhydride (Reaction 8.43) but using t-butoxycarbonylalanyl alanine-methyl ester (Reaction 8.39, 0.44 g, 1.4 mmol), yielding the product (**33**) as a white solid (0.14 g, 70%), m.p. >250 °C (Lit.[823] 291 °C). δ_H (200 MHz, $^2H_6$-DMSO) 1.3 (6H, m, 2 x CH_3), 3.9 (2H, m, CH) and 8.15 (2H, bs, NH); δ_C (50 MHz, $^2H_6$-DMSO) 24.15 (2 x CH_3), 55.55 (2 x CH_3) and 174.78 (2 x CO).

8.44 Synthesis of phenylalanine anhydride.[821]

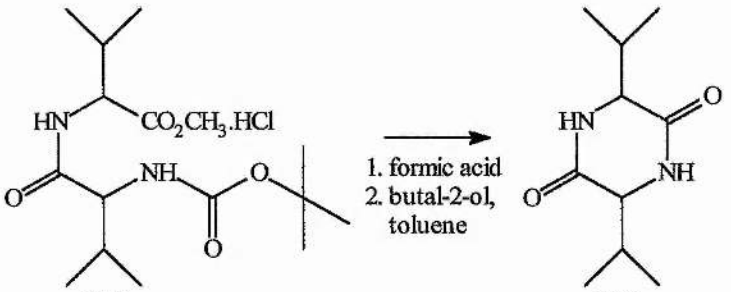


(30) (34)

Phenylalanine anhydride (**34**) was prepared using the same method as for glycine anhydride (Reaction 8.42) but using t-butoxycarbonyl-L-phenylalanyl phenylalanine-methyl ester (Reaction 8.40, 0.6 g, 1.4 mmol), yielding the product (**34**) as a white solid (0.36 g, 86%), m.p. >250 °C (Lit.[824] 311 - 312 °C). δ_H (200

MHz, $^2H_6$-DMSO) 2.3 (4H, m, 2 x CH_2), 4.0 (2H, m, 2 x CH), 7.1 - 7.3 (10H, m, 2 x aromatics) and 8.0 (2H, bs, 2 x NH); δ_C (50 MHz, $^2H_6$-DMSO) 28.76 (2 x CH_2), 60.12 (2 x CH), 132.21, 133.41, 134.62, 134.88, 135.54 (2 x aromatics) and 172.61 (2 x CO).

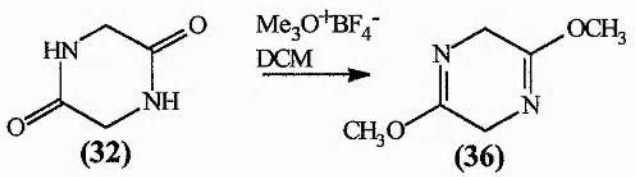
8.45 Synthesis of valine anhydride.[821]



(31) **(35)**

Valine anhydride (**35**) was prepared using the same method as for the glycine anhydride (Reaction 8.42) but using t-butoxycarbonylvalinyl valine-methyl ester (Reaction 8.41, 0.43 g, 1.4 mmol) yielding the product (**35**) as a white solid (0.20 g, 71%), m.p. >250 °C (Lit.[822] 263 °C). δ_H (200 MHz, $^2H_6$-DMSO) 0.9 - 1.0 (12H, m, 2 x CH(CH_3)$_2$), 2.2 (2H. m. 2 x CH(CH$_3$)$_2$), 3.7 (2H, m, 2 x CHNH) and 8.0 (2H, bs, 2 x NH); δ_C (50 MHz, $^2H_6$-DMSO) 17.54, 18.94 (2 x CH(CH_3)$_2$), 51.28 (2 x (CH(CH$_3$)$_2$), 62.45 (2 x CH) and 172.45 (2 x CO).

8.46 Synthesis of 2,5-dihydro-2,5-dimethoxypyrazine.[825]

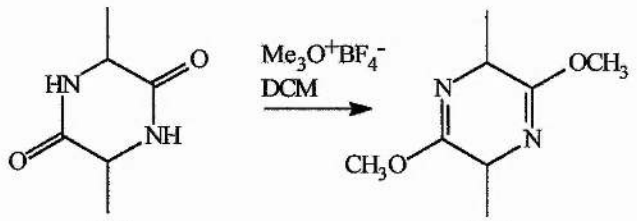


(32) **(36)**

A suspension of glycine anyhydride (Reaction 8.42, 0.11 g, 1 mmol), trimethyloxonium tetrafluoroborate (0.44 g, 3 mmol) and DCM (9 cm^3) was

stirred at room temperature for 1 day then heated under reflux conditions for 4 days. A further quantity of trimethyloxonium tetrafluoroborate (0.074 g, 0.5 mmol) was added after 2 days. The resulting sticky suspension was cooled to 0 °C and NaOH solution (4 cm^3, 2.5 M) was added. The layers were separated and the aqueous layer was extracted with DCM (2 x 10 cm^3). The combined organic layers were re-extracted with water (3 x 10 cm^3), dried ($MgSO_4$), filtered and concentrated under reduced pressure to give 2,5-dihydro-2,5-dimethoxypyrazine (**36**) as a light brown coloured solid (0.05 g, 35%), the melting point was not recorded due to the sensitivity of the product to aerial oxidation. δ_H (200 MHz, C^2HCl_3, $C^2H_3O^2H$ or $^2H_6$-DMSO) 3.6 (6H, s, 2 x OCH_3) and 4.0 (4H, s, 2 x CH_2); δ_C (50 MHz, C^2HCl_3, $C^2H_3O^2H$ or $^2H_6$-DMSO) 46.77 (2 x CH_2), 53.21 (2 x OCH_3) and 159.60 (2 x C-OMe).

8.47 Synthesis of 2,5-dihydro-2,5-dimethoxy-3,6-dimethylpyrazine.[825]

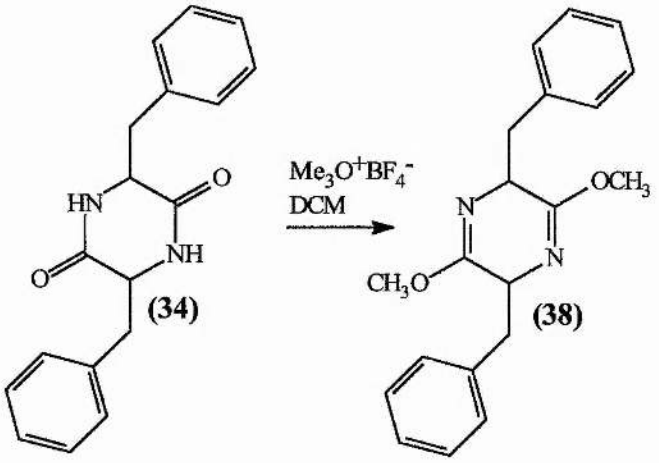


(33) **(37)**

2,5-Dihydro-2,5-dimethoxy-3,6-dimethylpyrazine (**37**) was prepared using the same method as for 2,5-dihydro-2,5-dimethoxypyrazine (Reaction 8.46) but using alanine anhydride (Reaction 8.43, 0.14 g, 1 mmol), yielding the product (**37**) as an off white solid (0.064 g, 38%), the melting point was not recorded due to the sensitivity of the product to aerial oxidation. δ_H (200 MHz, $^2H_6$-DMSO) 1.3 (6H, m, 2 x CH_3), 3.6 (2H, m, 2 x CH) and 4.1 (6H, m, 2 x OCH_3); δ_C (50 MHz, $^2H_6$-

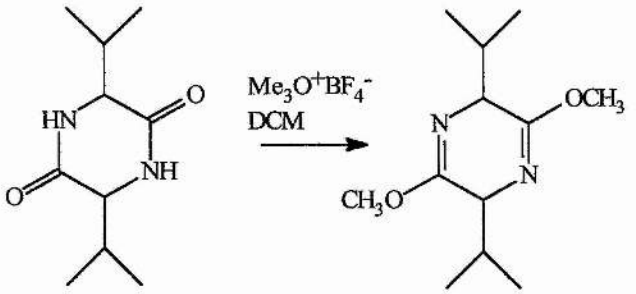
DMSO) 14.62, 14.68 (2 x CH_3), 49.24, 49.31 (2 x OCH_3), 61.02, 61.22 (2 x CH), 160.80 and 161.76 (2 x C-OMe).

8.48 Synthesis of 3,6-dibenzyl-2,5-dihydro-2,5-dimethoxypyrazine.[825]



3,6-Dibenzyl-2,5-dihydro-2,5-dimethoxypyrazine (**38**) was prepared using the same method as for 2,5-dihydro-2,5-dimethoxypyrazine (Reaction 8.46) but using phenylalanine anhydride (Reaction 8.44, 0.29 g, 1 mmol) yielding the product (**38**) as an off white solid (0.085 g, 42%), the melting point was not recorded due to the sensitivity of the product to aerial oxidation. δ_H (200 MHz, $^2H_6$-DMSO) 2.7 (4H, m, 2 x CH_2), 3.4 (6H, m, 2 x OCH_3), 3.6 (2H, m, 2 x CH) and 7.0 - 7.4 (10H, m, aromatics); δ_C (50 MHz, $^2H_6$-DMSO) 46.03, 46.91 (2 x OCH_3), 62.13, 63.06 (2 x CH), 65.08, 65.13 (2 x CH_2), 131.61, 131.70, 131.74, 131.85, 131.89, 132.23, 132.31, 132.48, 133.47, 133.56 (2 x aromatics), 141.63, 143.36 (2 x aromatics), 163.38 and 163.92 (2 x C-OMe).

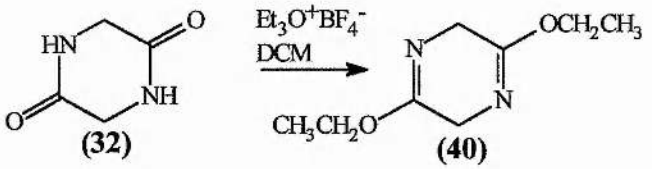
8.49 Synthesis of 2,5-dihydro-2,5-dimethoxy-3,6-dipropylpyrazine.[825]



(35) **(39)**

2,5-Dihydro-2,5-dimethoxy-3,6-dipropylpyrazine (**39**) was prepared using the same method as for 2,5-dihydro-2,5-dimethoxypyrazine (Reaction 8.46) but using valine anhydride (Reaction 8.45, 0.19 g, 1 mmol), yielding the product (**39**) as an off white solid (0.084 g, 37%), the melting point was not recorded due to the sensitivity of the product to aerial oxidation. δ_H (200 MHz, $^2H_6$-DMSO) 1.2 (6H, m, 2 x CH(CH_3)$_2$), 2.0 - 2.2 (2H, m, 2 x CH(CH_3)$_2$), 3.6 (2H, m, 2 x CH) and 4.2 (6H, m, 2 x OCH_3); δ_C (50 MHz, $^2H_6$-DMSO) 21.02, 21.64 (2 x CH(CH_3)$_2$), 31.41, 31.62 (2 x CH(CH_3)$_2$), 163.14 and 163.26 (2 x C-OMe).

8.50 Synthesis of 2,5-diethoxy-2,5-dihydropyrazine.[826]

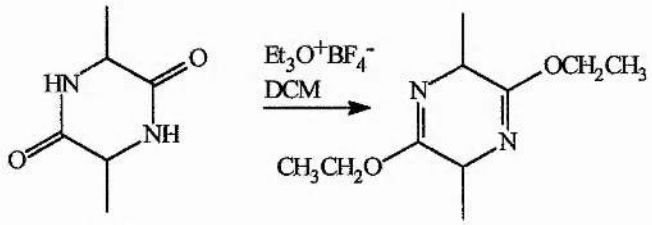


(32) **(40)**

A suspension of glycine anhydride (Reaction 8.42, 0.11 g, 1 mmol) and triethyloxonium tetrafluoroborate (0.62 g, 4.22 mmol, 3.3 cm^3 of a 1M solution in DCM) was vigorously stirred at room temperature under an atmosphere of nitrogen for 72 hours. The mixture was cooled to 0 °C and NaOH solution (2.5M, 5 cm^3 was added. The layers were separated. The aqueous layer was extracted with DCM (2 x 10 cm^3). The combined DCM extracts were washed with water (3

x 10 cm^3) dried ($MgSO_4$), filtered and concentrated under reduced pressure to give 2,5-diethoxy-2,5-dihydropyrazine (**40**) as an off white solid (0.08 g, 47%), the melting point was not recorded due to the sensitivity of the product to aerial oxidation. (200 MHz, $^2H_6$-DMSO) 1.3 (6H, m, 2 x CH_3), 3.5 (4H, m, 2 x CH_2) and 4.1 (4H, m, 2 x CH_2); δ_C (50 MHz, $^2H_6$-DMSO) 14.69, 14.72 (2 x CH_3), 45.12, 16.32, 50.99, 51.52 (2 x CH_2), 162.82 and 163.06 (2 x COEt).

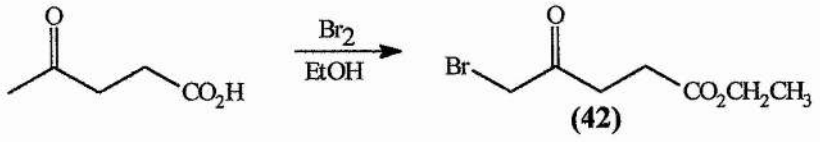
8.51 Synthesis of 2,5-diethoxy-2,5-dihydro-3,6-dimethylpyrazine.[826]



(33) **(41)**

2,5-Diethoxy-2,5-dihydro-3,6-dimethylpyrazine (**41**) was prepared using the same method as for 2,5-diethoxy-2,5-dihydropyrazine (Reaction 8.50) but using alanine anhydride (Reaction 8.43, 0.14 g, 1 mmol) yielding the product (**41**) as an off-white solid (0.10 g, 50%), the melting point was not recorded due to the sensitivity of the product to aerial oxidation. (200 MHz, $^2H_6$-DMSO) 1.3 - 1.5 (12H, m, 4 x CH_3), 3.5 - 3.7 (2H, m, 2 x CH) and 4.0 - 4.1 (4H, m, 2 x CH_2); δ_C (50 MHz, $^2H_6$-DMSO) 14.72, 14.61, 16.22, 16.84 (4 x CH_3), 52.03, 52.57 (2 x CH_2), 61.35, 31.65 (2 x CH), 164.94 and 164.99 (2 x COEt).

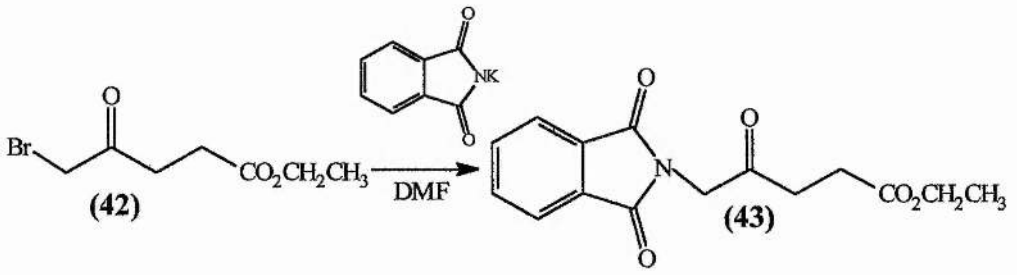
8.52 Synthesis of ethyl 5-bromolevulinate.[807]



(42)

Ethyl 5-bromolevulinate (**42**) was prepared using the same method as for methyl 5-bromolevulinate (Reaction 8.1) substituting ethanol for methanol, giving the product (**42**) as a clear oil (5.25 g, 27%). δ_H (200 MHz, C^2HCl_3) 1.3 (3H, t, J = 7.1 Hz, CH_3), 2.5 (2H, m, CH_2COO), 2.9 (2H, m, $COCH_2CH_2$), 3.9 (2H, m, $BrCH_2$) and 4.0 (2H, m, CH_2CH_3); δ_C (50 MHz, C^2HCl_3) 14.77 (CH_3), 27.15 (CH_2COO), 34.81 ($COCH_2CH_2$), 48.21 ($BrCH_2$), 62.16 (CH_2CH_3), 174.12 (COO) and 203.51 (CO).

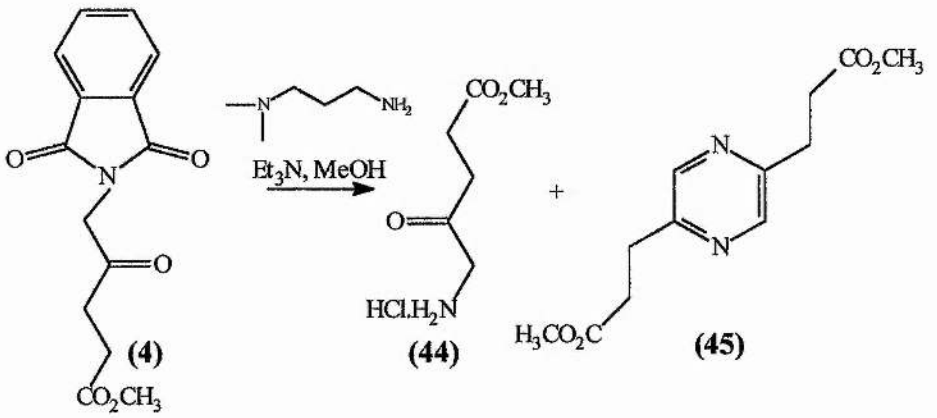
8.53 Synthesis of ethyl 5-phthalimidolevulinate.[807]



Ethyl 5-phthalimidolevulinate (**43**) was prepared using the same method as for methyl 5-phthalimidolevulinate (Reaction 8.4) but using ethyl 5-bromolevulinate (Reaction 8.52, 1.11 g, 5 mmol), yielding the product (**43**) as a white solid (0.81 g, 56%), m.p. 80 - 82 °C (Lit.[807] 79 °C). δ_H (200 MHz, C^2HCl_3) 1.2 (3H, m, CH_3), 2.6 (2H, t, J = 7.5 Hz, CH_2COO), 2.8 (2H, t, J = 7.5 Hz, $COCH_2CH_2$), 4.2 (2H, m, CH_2CH_3), 4.6 (2H, s, NCH_2) and 7.8 to 7.9 (4H, m, aromatics); δ_C (50 MHz, C^2HCl_3) 14.65 (CH_3), 28.33 (CH_2COO), 35.02 ($COCH_2CH_2$), 47.02

(NCH_2), 61.38 (CH_2CH_3), 112.80, 124.08, 132.54, 134.67 (aromatics), 173.21 (COO) and 203.10 (CO).

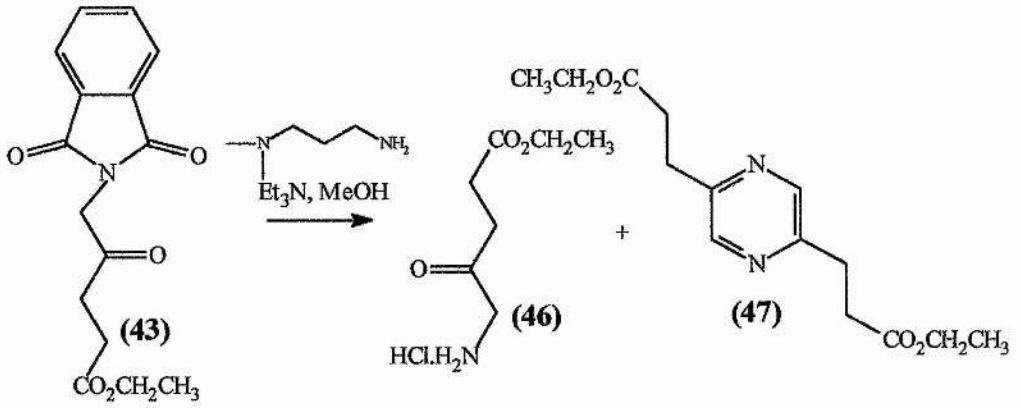
8.54 Synthesis of 5-aminolevulinic acid methyl ester.



Methyl 5-phthalimidolevulinate (Reaction 8.4, 0.47 g, 1.7 mmol) was dissolved in dry methanol (20 cm^3). Triethylamine (0.23 cm^3, 1.7 mmol, 0.17 g) and N,N-dimethyl-1,3-propanediamine (0.64 cm^3, 5.1 mmol, 2.2 equivalents) were added and the mixture was stirred at room temperature overnight causing it to go blue. The methanol was removed under reduced pressure and the mixture was purified by column chromatography, using a 19:1 solution of DCM : methanol with 0.5% HCl as the eluant giving a very small amount of the required product (approximately 10%) and a large amount of 2,5-di-(β–carboxyethyl) pyrazine dimethyl ester (approximately 90%). NMR spectroscopy identifies two products: 5-Aminolevulinic acid methyl ester (**44**); δ_H (200 MHz, $C^2H_3O^2H$) 2.6 (2H, m, CH_2COO), 2.8 (2H, m, $COCH_2CH_2$), 3.6 (3H, s, CH_3) and 4.1 (2H, m, NH_2CH_2); δ_C (50 MHz, $C^2H_3O^2H$) 28.91 (CH_2COO), 35.64 ($COCH_2CH_2$), 49.81 (NH_2CH_2), 52.13 (CH_3), 175.32 (COO) and 203.42 (CO). 2,5-di-(β-carboxyethyl) pyrazine dimethyl ester (**45**); δ_H (200 MHz, $C^2H_3O^2H$) 2.8 (2H, t, J = 5.0 Hz, CH_2), 3.0

(2H, t, J = 5.0 Hz, CH_2), 3.6 (3H, s, CH_3) and 8.4 (1H, s, aromatic); δ_C (50 MHz, $C^2H_3O^2H$) 30.12, 33.24 (CH_2CH_2COO), 52.15 (CH_3), 143.12 (aromatic CH), 154.1 ($NCCH_2$) and 173.02 (CO).

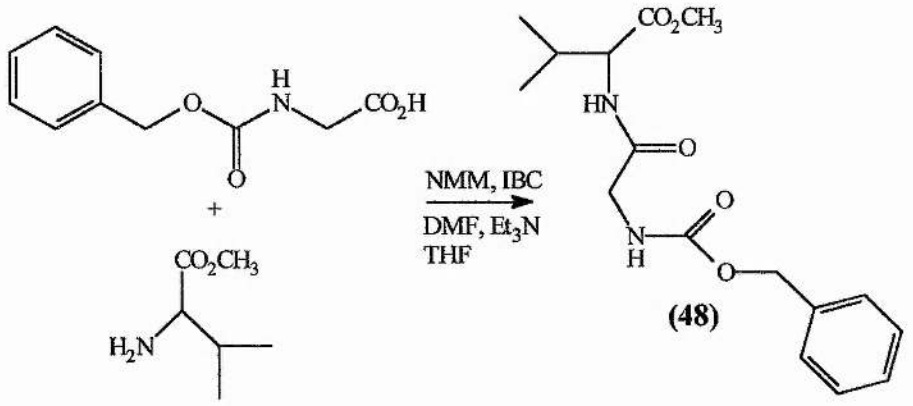
8.55 Synthesis of 5-aminolevulinic acid ethyl ester.



5-Aminolevulinic acid ethyl ester was prepared using the same method as for 5-aminolevulinic acid methyl ester (Reaction 8.54) but using ethyl 5-phthalimidolevulinate (Reaction 8.53, 0.5 g, 1.7 mmol), yielding a small amount of 5-aminolevulinic acid ethyl ester (approximately 10%) and a large amount of 2,5-di-(β-carboxyethyl) pyrazine diethyl ester (approximately 90%). NMR spectroscopy identifies two products: 5-Aminolevulinic acid ethyl ester (**46**); δ_H (200 MHz, $C^2H_3O^2H$) 1.3 (3H, t, J = 7.1 Hz, CH_3), 2.6 (2H, m, CH_2CO), 2.8 (2H, m, $COCH_2CH_2$) and 4.0 - 4.2 (4H, m, NH_2CH_2 and CH_2CH_3); δ_C (50 MHz, $C^2H_3O^2H$) 14.77 (CH_3), 28.88 (CH_2COO), 35.62 ($COCH_2CH_2$), 49.84 (NH_2CH_2), 62.16 (CH_2CH_3), 174.41 (COO) and 203.45 (CO). 2,5-di-β-(carboxyethyl) pyrazine diethyl ester (**47**); δ_H (200 MHz, $C^2H_3O^2H$) 1.2 (3H, m, CH_3), 2.8 (2H, t, J = 5.1 Hz, CH_2), 3.0 (2H, t, J = 5.1 Hz, CH_2), 4.1 (2H, t, J = 5.1 Hz, CH_2) and 8.4 (1H, s, aromatic); δ_C (50 MHz, $C^2H_3O^2H$) 14.61 (CH_3),

30.02, 33.23 ($CH_2CH_2CO_2$), 60.92 (CH_2CH_3), 144.02 (aromatic CH), 153.47 ($NCCH_2$) and 173.08 (CO).

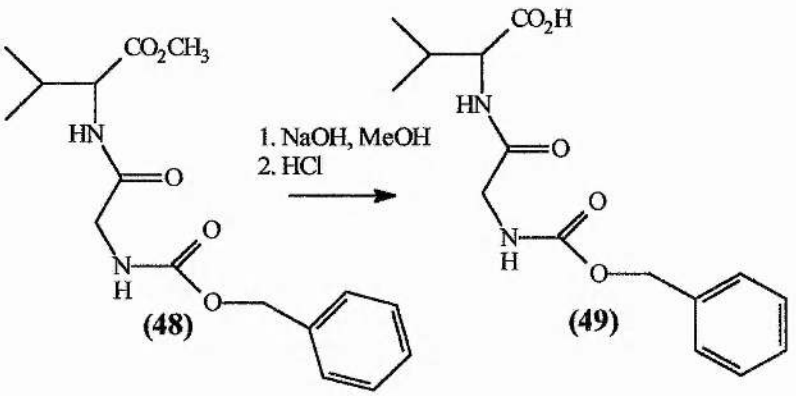
8.56 Synthesis of carbobenzyloxyglycinyl valine methyl ester.[818]



A solution of valine methyl ester (3.0 g, 18 mmol) in dry DMF (30 cm^3) was prepared by gentle warming. This solution was cooled to room temperature and treated wth triethylamine (2.1 cm^3, 15 mmol, 1.5 g). A solution of carbobenzyloxyglycine (3.75 g, 18 mmol) in dry THF (75 cm^3) was cooled in an ethanol/cardice bath and neutralised with N-methyl morpholine (2.04 g, 1.65 cm^3, 15 mmol). Isobutyl chloroformate (1.74 g, 1.98 cm^3, 15 mmol) was added followed by, 3 minutes later, the valine methyl ester solution and the reaction mixture was allowed to warm to room temperature overnight. The hydrochlorides of N-methyl morpholine and triethylamine were removed by filtration and washed with THF. The combined filtrate and washings were concentrated under reduced pressure to approximately 15 cm^3 and diluted with water (75 cm^3). This solution was cooled to 0 °C and filtered. The resulting white solid was washed with water, 0.5 M $KHCO_3$ solution and water (75 cm^3 of each) then dried to give carbobenzyloxyglycinyl valine methyl ester (**48**) as white crystals (2.7 g, 46 %),

m.p. 78 - 80 °C (Lit.[820] 78 °C). δ_H (200 MHz, $^2H_6$-DMSO) 0.9 (6H, m, $CH(CH_3)_2$), 2.0 (1H, m, $CH(CH_3)_2$), 3.4 (2H, s, CH_2NHZ), 3.7 (3H, s, CH_3), 4.2 (1H, t, J = 7.0 Hz, *CH*NH), 5.05 (2H, s, CH_2Ph) and 7.4 - 7.6 (5H, m, aromatics); δ_C (50 MHz, $^2H_6$-DMSO) 18.43, 19.19 ($CH(CH_3)_2$), 30.32 ($CH(CH_3)_2$), 43.35 (CH_2NH), 51.97 (CH_3), 57.58 (CHNH), 65.67 (CH_2Ph), 127.74, 127.94, 128.04, 128.16, 134.21 (aromatics), 169.65 and 172.26 (2 x COO).

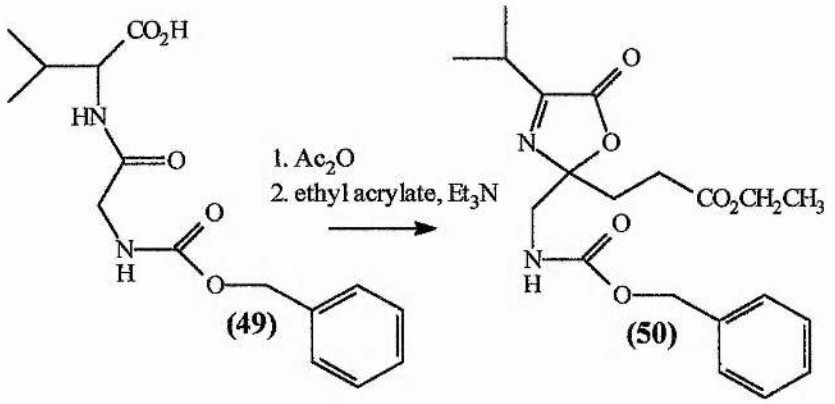
8.57 Synthesis of carbobenzyloxyglycinyl valine.[818]



A solution of carbobenzyoxyglycinyl valine methyl ester (Reaction 8.56, 2.0 g, 6.24 mmol) in methanol (24 cm^3) was surrounded by a water bath at room temperature and 1M NaOH (24 cm^3) added. The mixture was stirred at room temperature for 2 hours. Dilute HCl (24 cm^3, 1M) was added and the methanol was removed under reduced pressure. The aqueous solution was cooled to 0 °C and stirred during acidification to pH 2 (approximately 16 cm^3 of 1M HCl was required). The mixture was stirred at 0 °C for 2 hours and the resulting precipitate was filtered, washed with water and dried over P_2O_5 to give carbobenzyloxyglycinyl valine (**49**) as a white solid (1.52 g, 79%), m.p. 148 - 150 °C (Lit.[827] 146 °C). δ_H (200 MHz, $^2H_6$-DMSO) 0.9 (6H, m, $CH(CH_3)_2$), 2.1 (1H,

m, *CH*(CH_3)$_2$), 3.7 (2H, s, CH_2NHZ), 4.2 (1H, t, J = 7.6 Hz, *CH*NH), 5.05 (2H, s, CH_2Ph) and 7.4 - 7.8 (5H, m, aromatics); δ_C (200 MHz, $^2H_6$-DMSO) 18.16, 19.35 (CH(*CH_3*)$_2$), 30.28 (*CH*(CH_3)$_2$), 41.9 (CH_2NH), 57.30 (CHNH), 65.66 (CH_2Ph), 127.84, 127.97, 128.02, 128.59, 135.02 (aromatics), 169.47 and 173.15 (2 x COO.

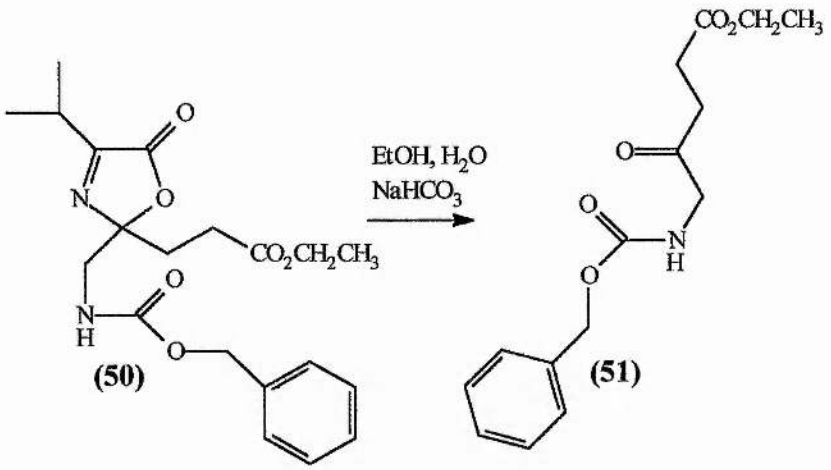
8.58 Synthesis of 2-[(benzyloxycarbonylamino)methyl]-4-isopropyl-5-oxo-3-oxazolin-2-propanoic acid ethyl ester.[828]



Carbobenzyloxyglycinyl valine (Reaction 8.57, 1.5 g, 2 mmol) was warmed to 80 °C for 50 minutes in acetic anhydride (3.75 cm^3). The solvent was then removed under reduced pressure, using toluene (10 cm^3 x 3) as an azeotrope. The resulting orange oil was mixed with freshly distilled ethyl acrylate (3.75 cm^3) and triethylamine (0.7 cm^3) was added dropwise with vigorous stirring at -10 °C. The mixture was allowed to warm to room temperature and was stirred at 50 °C for 5 days. The solvent was removed under reduced pressure and the residue was purified using a polyamide (poly[dimer acid-co-alkyl] polyamine Tm 95 °C d = 0.970) column using 40:60 petroleum ether (150 cm^3) as the eluant. The solvent was passed through the column repeatedly for 12 hours resulting in the orange

colour remaining on the column. The solvent was removed under reduced pressure giving 2-[(benzyloxycarbonylamino)methyl]-4-isopropyl-5-oxo-3-oxazolin-2-propanoic acid ethyl ester (**50**) as an off white coloured solid (1.21 g, 62%), m.p. 60 - 62 °C (Lit.[828] 58 - 61 °C). δ_H (200 MHz, $^2H_6$-DMSO) 1.0 - 1.4 (9H, m, CH_3), 1.7 - 1.9 (2H, m, CH_2), 2.1 - 2.4 (4H, m, 2 x CH_2), 2.6 - 2.8 (1H, m, CH), 3.4 - 3.5 (2H, m, CH_2), 5.0 (2H, s, CH_2Ph) and 7.2 - 7.4 (5H, m, aromatics); δ_C (50 MHz, $^2H_6$-DMSO) 15.90 ($CH_2\mathit{CH_3}$), 19.56 , 19.65 ($CH(\mathit{CH_3})_2$), 32.86 , 34.92 ($\mathit{CH_2CH_2}CO$), 45.01 ($\mathit{CH}(CH_3)_2$), 51.08 ($NH\mathit{CH_2}C$), 65.68, 65.78 (CH_2Ph and CH_2CH_3), 71.11 (NCO), 133.20, 133.32, 133.47, 133.99, 136.96 (aromatics), 141.02 ($\mathit{C}CH(CH_3)_2$), 155.97 ($\mathit{CO}OCH_2$) and 166.14 ($C\mathit{CO}OC$).

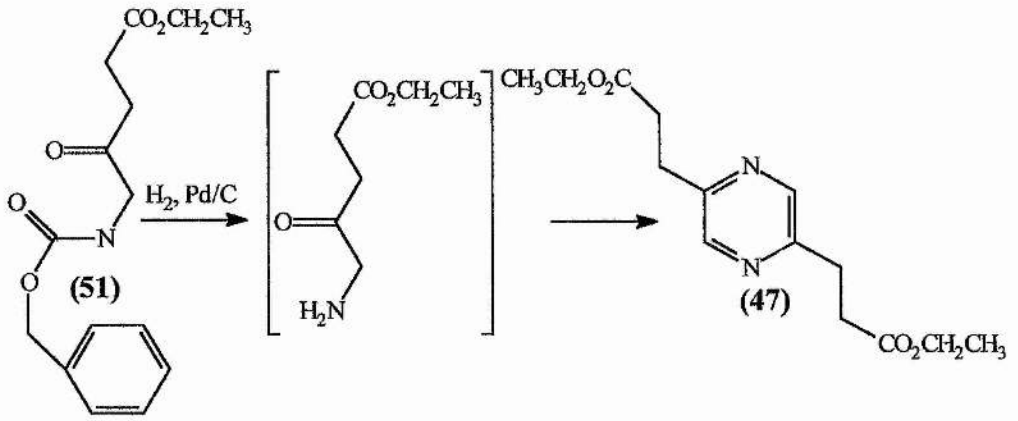
8.59 Synthesis of carbobenzyoxy-5-aminolevulinic acid ethyl ester.[828]



2-[(Benzyloxycarbonylamino)methyl]-4-isopropyl-5-oxo-3-oxazolin-2-propanoic acid ethyl ester (Reaction 8.58, 1 g, 2.56 mmol) was stirred in ethanol (20 cm^3) and saturated sodium bicarbonate solution (20 cm^3) at 60 °C over antibumping granules for 36 hours. The organic solvent was removed under reduced pressure

and the mixture was extracted with DCM (2 x 25 cm^3). The extracts were dried ($MgSO_4$), filtered and concentrated under reduced pressure to give carbobenzyloxy 5-aminolevulinic acid ethyl ester (**51**) as a light brown coloured solid (0.26 g, 35%), m.p. 59 - 61 °C (Lit.[828] 58.5 °C). δ_H (200 MHz, C^2HCl_3) 1.2 (3H, t, J = 7.2 Hz, CH_3), 2.5 - 2.9 (4H, m, $COCH_2CH_2CO$), 4.1 - 4.3 (4H, m, CH_2NH and CH_2CH_3), 5.1 (2H, s, CH_2Ph), 5.4 (1H, s, NH) and 7.4 - 7.6 (5H, m, aromatics) ; δ_C (50 MHz, C^2HCl_3) 14.62 (CH_2CH_3), 29.86(CH_2COO), 32.41 ($COCH_2CH_2$), 43.36 ($COCH_2NH$), 60.01 (CH_2CH_3), 65.52 (CH_2Ph), 127.42, 127.90, 128.92 (aromatics), 156.24 ($COOCH_2$), 172.04 (CO_2Et) and 204.06 ($COCH_2CH_2$).

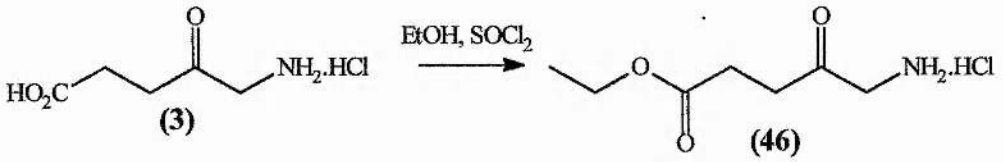
8.60 Attempted synthesis of 5-aminolevulinic acid ethyl ester.



Carbobenzyloxy-5-aminolevulinic acid ethyl ester (Reaction 8.59, 0.2 g, 0.68 mmol) was dissolved in methanol (10 cm^3). 10% Pd/C catalyst (100 mg) was added and the mixture was stirred under an atmosphere of H_2 at room temperature and atmospheric pressure for 6 hours. The catalyst was removed by filtration through Celite and the methanol was removed under reduced pressure leaving a pale yellow coloured oil. NMR data suggested the formation of 2,5-di-

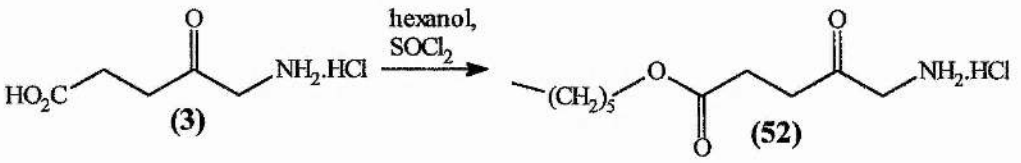
(β-carboxyethyl) pyrazine diethyl ester (**47**) and not the required product. Data as for Reaction 8.55.

8.61 Synthesis of 5-aminolevulinic acid ethyl ester. (ALA-OEt).[829]



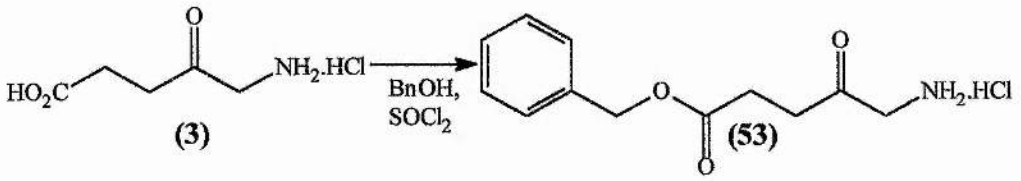
An excess of ethanol (3 cm^3) was cooled to 0 °C. Thionyl chloride (0.5 cm^3, 6.85 mmol) was added dropwise followed by ALA.HCl (Reaction 8.15, 0.5 g, 3.0 mmol). The mixture was heated to 70 °C for approximately 15 minutes until a solution formed and then for a further 10 minutes. After cooling to room temperature, the solvent was removed under reduced pressure. The resulting mixture was dissolved in a small amount of warm ethanol (approximately 5 cm^3). Diethyl ether (10 cm^3) was added and the mixture was cooled to 0 °C overnight. The resulting white crystals of 5-aminolevulinic acid ethyl ester (**46**) were collected on a sinter (0.32 g, 58%), m.p. 105 - 107 °C. δ_H (200 MHz, $C^2H_3O^2H$) 1.3 (3H, t, J = 7.1 Hz, CH_3), 2.6 (2H, m, CH_2CO_2), 2.8 (2H, m, $COCH_2CH_2$) and 4.0 - 4.2 (4H, m, NH_2CH_2 and CH_2CH_3); δ_C (50 MHz, $C^2H_3O^2H$) 14.77 (CH_3), 28.89 (CH_2COO), 35.63 ($COCH_2CH_2$), 49.85 (NH_2CH_2), 62.16 (CH_2CH_3), 174.45 (COO) and 203.4 (CO).

8.62 Synthesis of 5-aminolevulinic acid hexyl ester (ALA-OHex).[829]



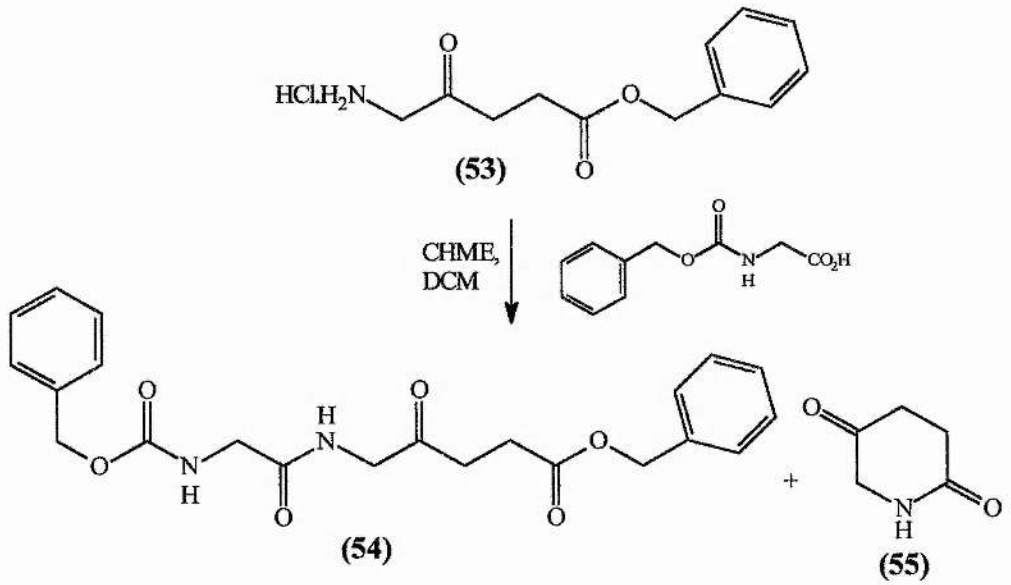
An excess of hexanol (3 cm^3) was cooled to 0 °C. Thionyl chloride (0.5 cm^3, 6.85 mmol) was added dropwise followed by ALA.HCl (Reaction 8.15, 0.5 g, 3.0 mmol). The mixture was heated to 70 °C and stirred for approximately 30 minutes until a solution formed. After a further 10 minutes the mixture was cooled to room temperature. Excess hexanol was removed by silica gel flash column chromatography using acetone (100 cm^3) and then methanol (150 cm^3) as the eluant. Fractions of methanol containing the product were evaporated under reduced pressure. The resulting mixture was dissolved in a small quantity of warm methanol (5 cm^3) and diethyl ether (10 cm^3) was added causing a white precipitate to form. After 12 hours at 0 °C the white crystals of 5-aminolevulinic acid hexyl ester (**52**) were collected on a sinter (0.51 g, 68%), m.p. 100 -102 °C. δ_H (200 MHz, $C^2H_3O^2H$) 1.0 (3H, m, CH_3), 1.4 (6H, m, 3 x CH_2-Hex), 1.6 (2H, m, CH_2-Hex), 2.6 (2H, m, CH_2CO_2), 2.8 (2H, m, $COCH_2CH_2$) and 4.1 (4H, m, NH_2CH_2 and CH_2-Hex); δ_C (50 MHz, $C^2H_3O^2H$) 14.69 (CH_3), 23.92 (CH_2-Hex), 26.97 (CH_2-Hex), 28.29 (CH_2-Hex), 28.83 (CH_2COO), 29.96 (CH_2-Hex), 32.91 (CH_2-Hex), 35.68 ($COCH_2CH_2$), 48.01 (NH_2CH_2), 66.29 (CH_2COO), 174.55 (COO) and 204.16 (CO).

8.63 Synthesis of 5-aminolevulinic acid benzyl ester (ALA-OBn).[829]



5-Aminolevulinic acid benzyl ester (**53**) was prepared using the same method as for 5-aminolevulinic acid hexyl ester (Reaction 8.62) but using an excess of freshly distilled benzyl alcohol (3 cm^3), yielding the product (**53**) as a white solid (0.52 g, 67%), m.p. 111 - 113 °C. δ_H (200 MHz, $C^2H_3O^2H$) 2.7 (2H, m, CH_2COO), 2.9 (2H, m, $COCH_2CH_2$), 4.0 (2H, s, NH_2CH_2), 5.2 (2H, s, CH_2Ph) and 7.4 (5H, m, aromatics); δ_C (50 MHz, $C^2H_3O^2H$) 28.86 (CH_2COO), 35.59 ($COCH_2CH_2$), 48.0 (NH_2CH_2), 67.85 (CH_2Ph), 129.44, 129.84 (aromatics), 174.18 (COO) and 202.16 (CO).

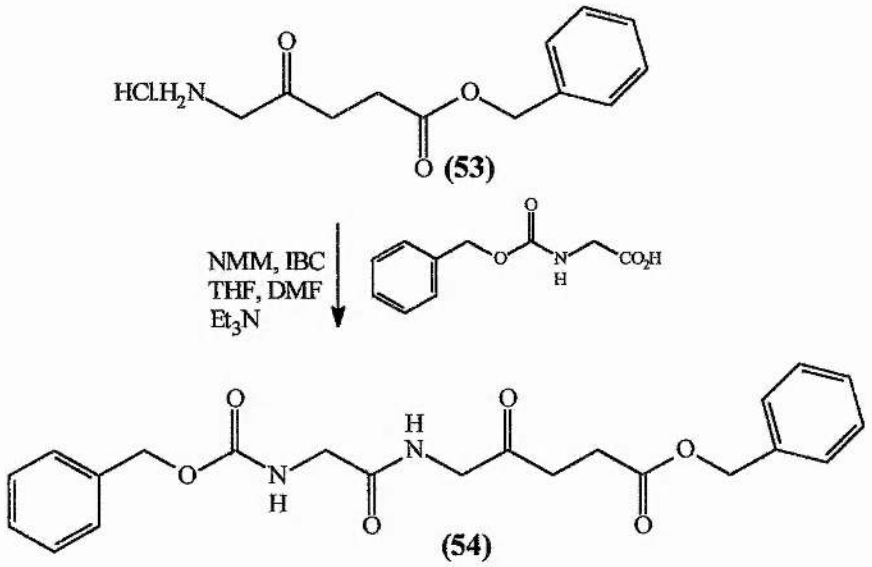
8.64 Synthesis of carbobenzyloxyglycinyl 5-aminolevulinic acid benzyl ester.[818]



5-Aminolevulinic acid benzyl ester (Reaction 8.63, 0.5 g, 1.94 mmol) was dissolved in DCM (10 cm^3) at 0 °C. Carbobenzyoxyglycine (0.41 g, 1.94 mmol)

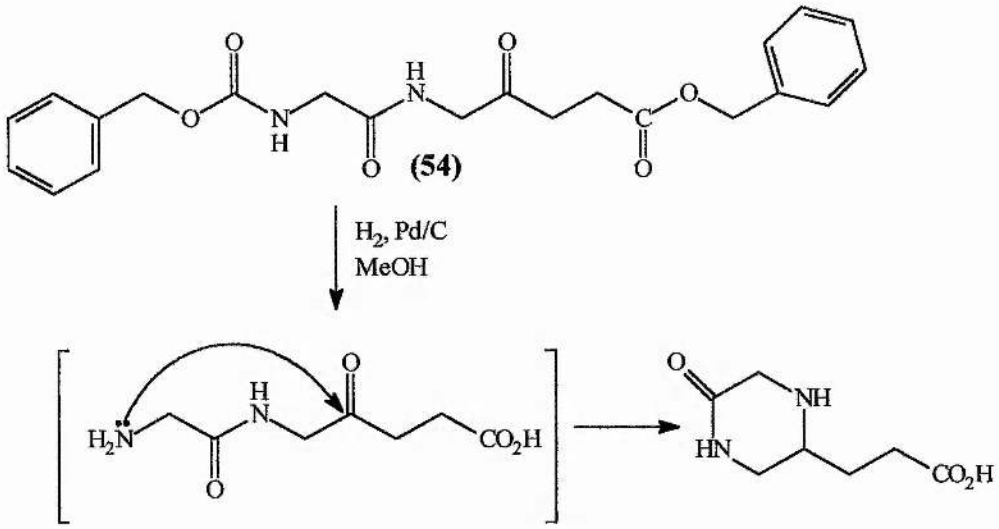
and CHME (0.82 g, 1.94 mmol) were added and the mixture was allowed to warm to room temperature overnight. The resulting reaction mixture was then cooled to 0 °C, filtered to remove any urea produced, washed with water, citric acid (1M), saturated sodium bicarbonate solution and then water (50 cm^3 of each) and dried ($MgSO_4$). Filtration followed by evaporation under reduced pressure yielded carbobenzyloxyglycinyl 5-aminolevulinic acid benzyl ester (**54**) as a white solid (0.52 g, 65%), m.p. 100 - 102 °C. m/z (EI^+) 412 (M^+). A correct elemental analysis could not be obtained due to the product being contaminated with a small amount (<5%) of piperadine-2,5-dione (**55**). NMR spectroscopy identifies two products: carbobenzyloxyglycinyl 5-aminolevulinic acid benzyl ester (**54**); δ_H (200 MHz, C^2HCl_3) 2.5 (2H, m, CH_2COO), 2.8 (2H, m, $COCH_2CH_2$), 3.9 (2H, m, NH_2CH_2), 4.2 (2H, m, $NHCH_2COCH_2$), 5.0, 5.1 (4H, 2 x s, 2 x CH_2Ph), 5.6 (1H, bs, NH), 6.8 (1H, bs, NH) and 7.3 - 7.5 (10H, m, 2 x Ph); δ_C (50 MHz, C^2HCl_3) 28.31 (CH_2COO), 34.96 ($COCH_2CH_2$), 44.79 ($NHCH_2CONH$), 49.52 ($NHCH_2COCH_2CH_2$), 67.22, 67.75 (2 x CH_2Ph), 128.51, 128.74, 128.62, 129.11, 135.10 (aromatics), 156.61 (NHCOO) 169.67 ($NHCH_2CO$), 172.74 (CH_2COOCH_2) and 204.09 ($COCH_2CH_2$). Piperadine-2,5-dione (**55**); δ_H (200 MHz, C^2HCl_3) 2.7 (4H, m, 2 x CH_2), 4.0 (2H, d, J = 3 Hz, CH_2) and 7.1 (1H, m, NH); δ_C (50 MHz, C^2HCl_3) 28.15 (CH_2), 30.21 (CH_2), 51.31 (CH_2), 154.57 and 204.62 (2 x CO).

8.65 Alternative synthesis of carbobenzyloxyglycinyl 5-aminolevulinic acid benzyl ester.[818]



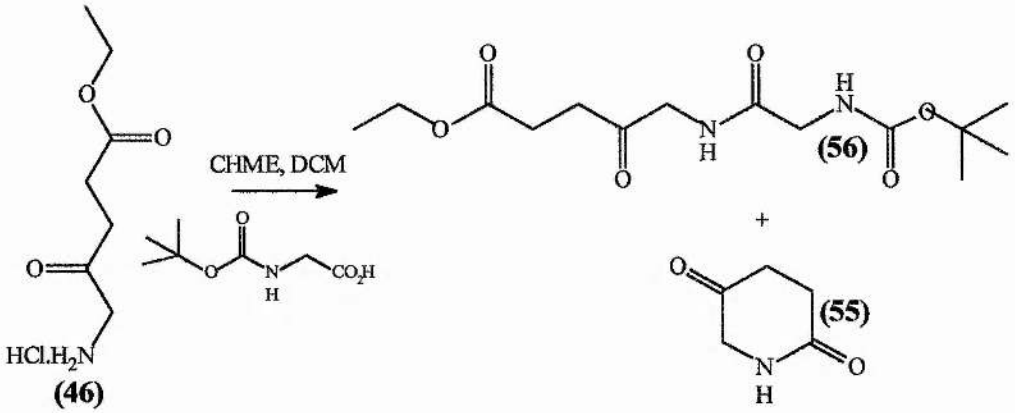
A solution of 5-aminolevulinic acid benzyl ester (Reaction 8.63, 1g, 3.9 mmol) in dry DMF (7.8 cm^3) was treated with triethylamine (0.54 cm^3, 0.39 g, 3.9 mmol). A solution of carbobenzyloxyglycine (0.81 g, 3.9 mmol) in dry THF (20 cm^3) was cooled to -15 °C. Isobutyl chloroformate (0.51 cm^3, 10 mmol) and N-methyl morpholine (0.43 cm^3, 10 mol) were added followed by, 3 minutes later, the 5-aminolevulinic acid benzyl ester solution. The mixture was allowed to warm to room temperature overnight and was then filtered to remove the hydrochloride salts. These were washed with THF and the combined THF fractions were concentrated under reduced pressure to approximately 10 cm^3. Water was added but, after cooling to 0 °C, no crystals precipitated out. The mixture was extracted into DCM (100 cm^3), washed with water, potassium hydrogen carbonate solution (0.5M) then water (100 cm^3 of each), dried ($MgSO_4$), filtered and concentrated under reduced pressure to give carbobenzyloxyglycinyl 5-aminolevulinic acid benzyl ester (**54**) as a brown oil (0.14 g, 17.5%). Data as for Reaction 8.64.

8.66 Attempted synthesis of glycinyl 5-aminolevulinic acid.[818]



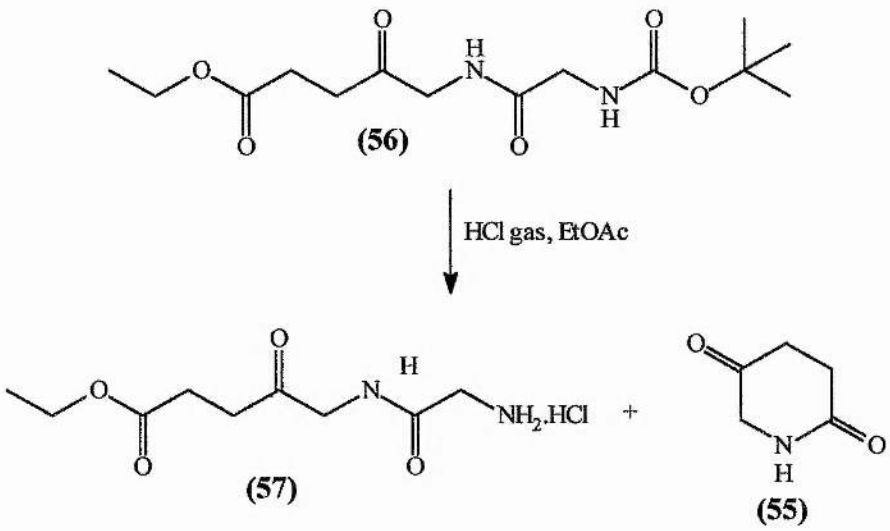
Carbobenzyloxyglycinyl 5-aminolevulinic acid benzyl ester (Reaction 8.64, 0.4 g, 0.97 mmol) was dissolved in methanol (15 cm^3). 10% Pd/C catalyst (100 mg) was added and the mixture was stirred under an atmosphere of H_2 at atmospheric pressure and room temperature for 10 hours. The catalyst was removed by filtration through Celite and the methanol was removed under reduced pressure giving a white solid which, from NMR data, was not the required product.

8.67 Synthesis of t-butoxycarbonylglycinyl 5-aminolevulinic acid ethyl ester.



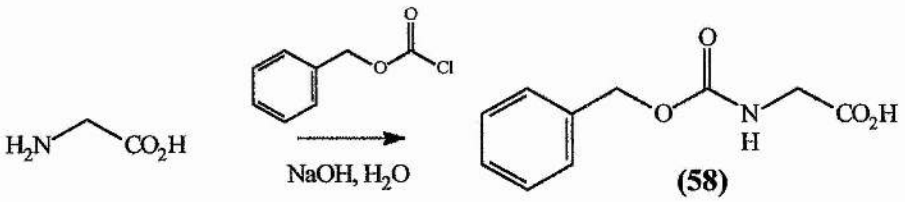
t-Butoxycarbonylglycinyl 5-aminolevulinic acid ethyl ester (**56**) was prepared by a CHME coupling reaction using the same procedure as for carbobenzyloxyglycinyl 5-aminolevulinic acid benzyl ester (Reaction 8.64) but using t-butoxycarbonylglycine (Reaction 8.34, 0.44 g, 2.5 mmol) and 5-aminolevulinic acid ethyl ester (Reaction 8.61, 0.5 g, 2.5 mmol), yielding the product (**56**) as a white solid (0.24 g, 31%), m.p. 92 - 94 °C. m/z (EI^+) 316 (M^+). A correct elemental analysis could not be obtained due to the product being contaminated with a small amount (<5%) of piperadine-2,5-dione (**55**). NMR spectroscopy identifies the two products: t-butoxycarbonylglycinyl 5-aminolevulinic acid ethyl ester (**56**); δ_H (200 MHz, C^2HCl_3) 1.3 (3H, m, CH_3), 1.5 (9H, s, 3 x CH_3-Boc), 2.7 (4H, m, $CH_2CH_2CO_2$), 3.8 (2H, d, $NHCH_2$), 4.1 (2H, m, CH_2), 4.2 (2H, d, J = 4.8 Hz, $NHCH_2$) and 5.1 (1H, s, $CH(CH_3)_3$); δ_C (50 MHz, C^2HCl_3) 14.77 (CH_2CH_3) 28.77 (3 x CH_3-Boc), 34.95 ($COCH_2CH_2$), 35.82 (CH_2CH_3), 44.56 ($NHCH_2CONH$), 157.12 (CO-Boc), 170.25, 172.72,(CONH and COO) and 204.21 (CO-ALA). Piperadine-2,5-dione (**55**); Data as for Reaction 8.64.

8.68 Synthesis of glycinyl 5-aminolevulinic acid ethyl ester.



t-Butoxycarbonylglycinyl 5-aminolevulinic acid ethyl ester (Reaction 8.67, 0.2 g, 0.65 mmol) was dissolved in ethyl acetate (10 cm^3) and cooled to 0 °C. HCl gas was bubbled through the solution for 30 minutes then the mixture was stirred for 1 hour. N_2 gas was bubbled through the solution to remove any excess HCl and the solvent was removed under reduced pressure giving glycinyl 5-aminolevulinic acid ethyl ester (**57**) as a clear oil (0.16 g, 94%). m/z (EI^+) 216 (M^+ - HCl salt). A correct elemental analysis could not be obtained due to the product being contaminated with a small amount (<5%) of piperadine-2,5-dione (**56**). NMR spectroscopy identifies two products: glycinyl 5-aminolevulinic acid ethyl ester (**57**); δ_H (200 MHz, C^2HCl_3) 1.3 (3H, m, CH_3), 2.5 (2H, m, CH_2COO), 2.7 (2H, m, $COCH_2CH_2$), 3.9 (2H, d, $NHCH_2$), 4.1 (2H, m, CH_2) and 4.3 (2H, d, J = 4.8 Hz, $NHCH_2$); δ_C (50 MHz, C^2HCl_3) 14.92 (CH_2CH_3), 34.72, 35.91 ($COCH_2CH_3$ and CH_2CH_3), 45.12 ($NHCH_2CONH$), 170.36, 173.14 (CONH and COO) and 203.96 (CO-ALA). Piperadine-2,5-dione (**55**); Data as for Reaction 8.64.

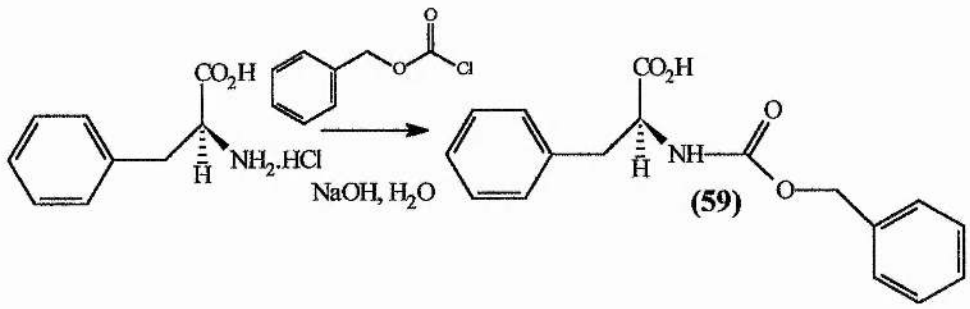
8.69 Synthesis of carbobenzyloxyglycine (Z-Gly).[818]



(58)

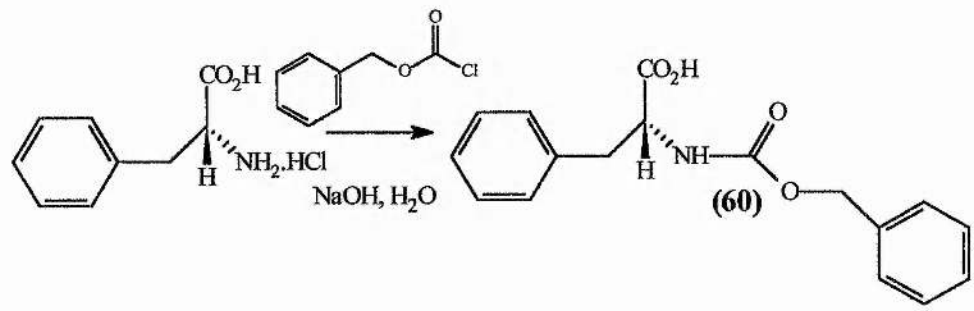
A solution of glycine (11.1 g, 0.1 mol) in 2 M NaOH (50 cm^3) was cooled in an ice/water bath and stirred. Benzyl chloroformate (18.7 g, 15.8 cm^3, 0.11 mol) and 2M NaOH (55 cm^3) were added in ten alternating portions, ensuring the pH remained alkaline throughout, and the temperature was kept between 5 and 10 °C. The mixture was stirred vigorously at room temperature for 30 minutes before the alkaline solution was extracted four times with diethyl ether (4 x 50 cm^3). The diethyl ether extracts were discarded and diethyl ether remaining in the aqueous layer was removed by bubbling a stream of N_2 through the solution. The mixture was acidified using 5 M HCl (22 cm^3) causing a precipitate to form. This product was filtered, washed with water and dried under reduced pressure at room temperature yielding carbobenzyloxyglycine (**58**) as a white solid (12.2 g, 58%), m.p. 122 - 123 °C (Lit.[830] 120 °C). δ_H (300 MHz, $^2H_6$-DMSO) 3.6 (2H, d, J = 8.1 Hz, CH_2), 5.0 (2H, s, CH_2), 7.3 (4H, m, aromatics) and 7.5 (1H, t, J = 7.3 Hz, NH); δ_C (75 MHz, $^2H_6$-DMSO) 37.21 (CH_2), 60.62 (CH_2), 122.92, 123.02, 123.57, 132.26 (aromatics), 151.77 (C=O) and 166.85 (C=0).

8.70 Synthesis of carbobenzyloxy-L-phenylalanine (Z-L-Phe).[818]



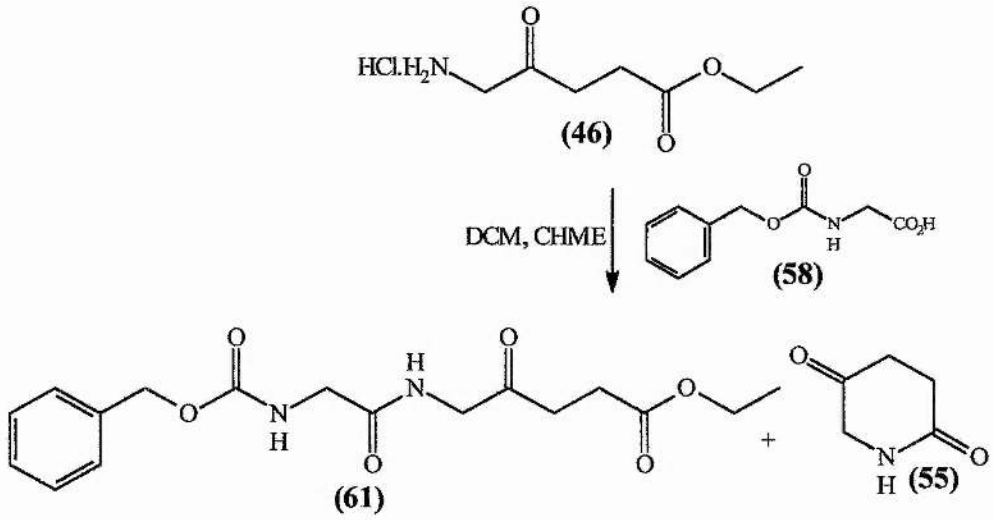
Carbobenzyloxy-L-phenylalanine (**59**) was prepared using the same method as for carbobenzyloxyglycine (Reaction 8.70) using L-phenylalanine (16.5 g, 0.1 mol) yielding the product (**59**) as a white solid (19.4 g, 65%), m.p. 100 - 102 °C (Lit.[830] 103 °C). δ_H (200 MHz,$^2H_6$-DMSO) 3.2 (2H, m, Ph-CH_2), 4.2 (1H, m, CH), 5.0 (2H, s, OCH_2Ph), 7.3 (10H, m, 2 x aromatics) and 7.6 (1H, d, J = 8.7 Hz, NH); δ_C (50 MHz, $^2H_6$-DMSO) 36.82 ($PhCH_2$), 56.14 (CH), 65.55 (CH_2O), 126.58, 127.76, 127.96, 128.39, 128.56, 129.40, 137.32, 138.37 (aromatics), 156.23 (COO) and 173.76 (CO).

8.71 Synthesis of carbobenzyloxy-D-phenylalanine (Z-D-Phe).[818]



Carbobenzyloxy-D-phenylalanine (**60**) was prepared using the same method as for carbobenzyloxyglycine (Reaction 8.70) using D-phenylalanine (16.5 g, 0.1 mol) yielding the product (**60**) as a white solid (16.5 g, 55%), m.p. 98 - 100 °C (Lit.[830] 103 °C). Data as for Reaction 8.70.

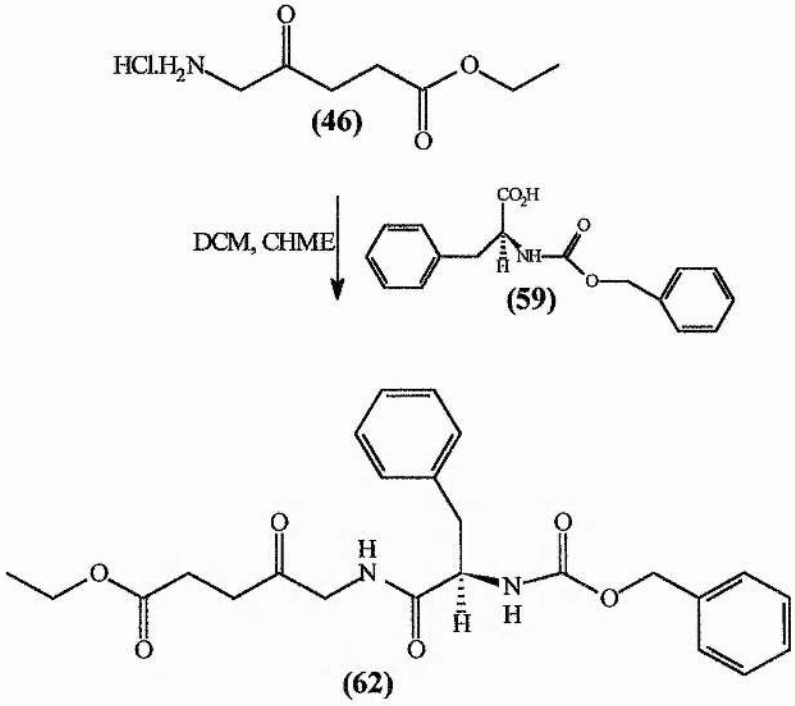
8.72 Synthesis of carbobenzyloxyglycinyl 5-aminolevulinic acid ethyl ester (Z-Gly-ALA-OEt) (ALA1).



Carbobenzyloxyglycinyl 5-aminolevulinic acid ethyl ester (**61**) was prepared by a CHME coupling using the same procedure as for carbobenzyloxyglycinyl 5-aminolevulinic acid benzyl ester (Reaction 8.64) but using 5-aminolevulinic acid ethyl ester (Reaction 8.61, 0.38 g, 1.94 mmol), yielding the product (**61**) as a white solid (0.32 g, 49%), m.p. 88- 90 °C. m/z (EI^+) 350 (M^+). A correct elemental analysis could not be obtained due to the product being contaminated with a small quantity (<5%) of piperadine-2,5-dione (**55**). NMR spectroscopty identifies two products: carbobenzyloxyglycinyl 5-aminolevulinic acid ethyl ester (**61**);δ_H (200 MHz, C^2HCl_3) 1.2 (3H, t, J = 7.7 Hz, CH_3), 2.7 (4H, m, $COCH_2CH_2$), 3.9 (2H, m, CH_2), 4.1 (4H, m, 2 x CH_2), 5.1 (2H, s, CH_2Ph), 5.6 (1H, bs, NH), 6.8 (1H, bs, NH) and 7.4 (5H, m, aromatics.); δ_C (50 MHz, C^2HCl_3) 14.68 (CH_3), 28.34 (CH_2COO), 34.99 ($COCH_2CH_2CO$), 44.83 ($NHCH_2CONH$), 49.41 ($NHCH_2COCH_2$), 61.41 (CH_2CH_3), 67.74 ($PhCH_2$), 128.46, 128.70, 128.89, 129.03 (aromatics), 157.12 (CH_2OCONH), 169.68

(CONH), 172.91 (COO) and 204.15 ($NHCH_2COCH_2$). Piperadine-2,5-dione (**55**); Data as for Reaction 8.64.

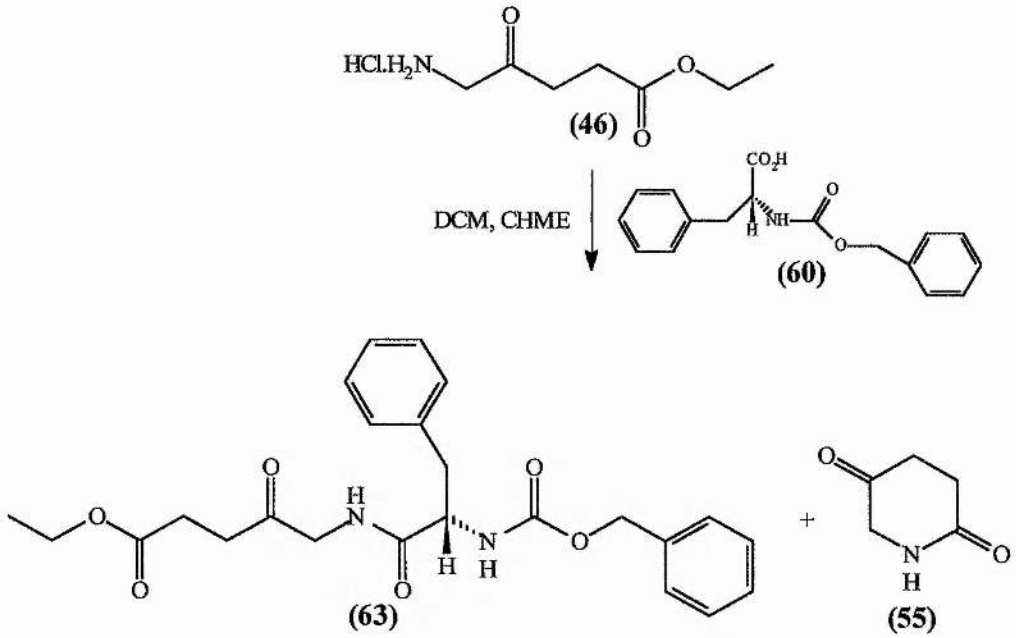
8.73 Synthesis of carbobenzyloxy-L-phenylalanyl 5-aminolevulinic acid ethyl ester (Z-L-Phe-ALA-OEt) (ALA2).



Carbobenzyoxy-L-phenylalanyl 5-aminolevulinic acid ethyl ester (**62**) was prepared by a CHME coupling reaction using the same procedure as for carbobenzyloxyglycinyl 5-aminolevulinic acid benzyl ester (Reaction 8.64) using carbobenzyloxy-L-phenylalanine (Reaction 8.70, 0.58 g, 1.94 mmol) and 5-aminolevulinic acid ethyl ester (Reaction 8.61, 0.38 g, 1.94 mmol), yielding the product (**62**) as a white solid (0.46 g, 54%), m.p. 110 - 111 °C. m/z (EI^+) 440

(M^+). Found: C, 65.1; H, 6.3; N, 6.4; Calculated for $C_{24}H_{28}N_2O_6$: C, 65.4; H, 6.4; N, 6.4%. δ_H (200 MHz, C^2HCl_3) 1.1 (3H, t, J = 7.7 Hz, CH_3), 2.3 (2H, m, $CHCH_2Ph$), 2.5 (4H, m, $COCH_2CH_2CO$), 3.8 (2H, d, $NHCH_2$), 4.0 (2H, m, CH_2), 4.3 (1H, m, CH), 5.0 (2H, s, CH_2Ph), 5.6 (1H, bs, NH), 6.8 (1H, bs, NH) and 7.2 (10H, m, 2 x aromatics); δ_C (50 MHz, C^2HCl_3) 14.31 (CH_3), 28.29 (CH_2COO), 34.99 ($COCH_2CH_2CO$), 38.91 ($CHCH_2Ph$), 49.46 ($NHCH_2COCH_2$), 56.41 (CH), 61.50 (CH_2CH_3), 67.51 ($PhCH_2O$), 127.47, 128.18, 128.44, 128.68, 129.00, 129.11, 129.46, 129.76, 136.63, 136.86 (2 x aromatics), 156.53 (*CO*NHCH), 171.86 ($CHCONHCH_2$), 173.37 (*CO*OEt) and 204.06 ($NHCH_2COCH_2$).

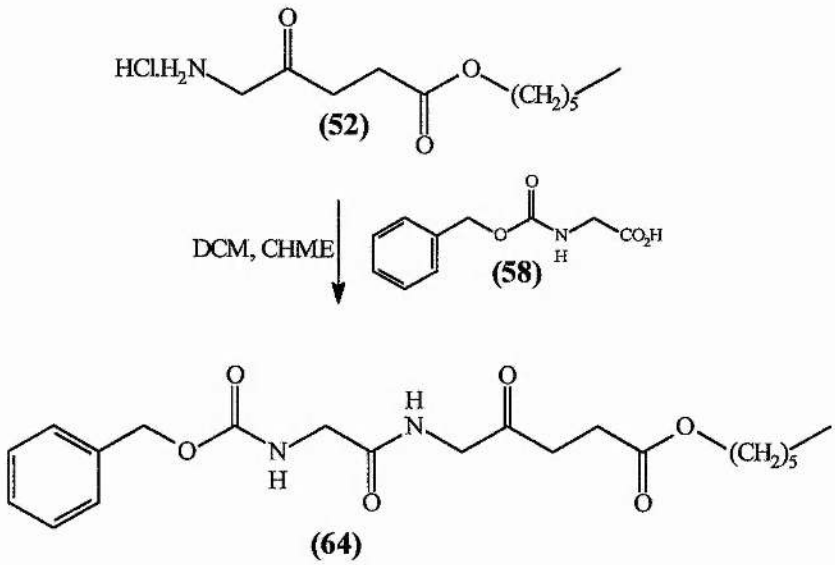
8.74 Synthesis of carbobenzyloxy-D-phenylalanyl 5-aminolevulinic acid ethyl ester (Z-D-Phe-ALA-OEt) (ALA11).



Carbobenzyloxy-D-phenylalanyl 5-aminolevulinic acid ethyl ester (**63**) was prepared by a CHME coupling procedure using the same procedure as for carbobenzyloxyglycinyl 5-aminolevulinic acid benzyl ester (Reaction 8.64) but

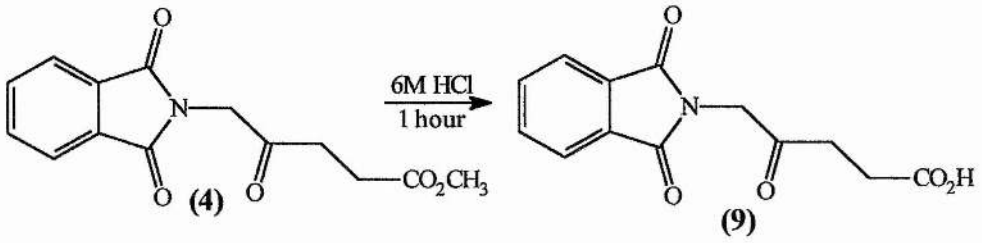
using carbobenzyloxy-D-phenylalanine (Reaction 8.71, 0.58 g, 1.94 mmol) and 5-aminolevulinic acid ethyl ester (Reaction 8.61, 0.38 g, 1.94 mmol), yielding the product (**63**) as a white solid (0.41 g, 48%), m.p. 108 - 110 °C. m/z (EI^+) 440 (M^+). A correct elemental analysis could not be obtained as the product was contaminated with a small quantity (<5%) of piperadine-2.5-dione (**55**). NMR spectroscopy identifies two products: carbobenzyloxy-D-phenylalanyl 5-aminolevulinic acid ethyl ester; δ_H (200 MHz, C^2HCl_3) 1.2 (3H, t, J = 7.7 Hz, CH_3), 2.3 (2H, m, $CHCH_2Ph$), 2.6 (4H, m, $COCH_2CH_2CO$), 3.7 (2H, s, $NHCH_2$), 4.1 (2H, m, CH_2), 4.5 (1H, m, CH), 5.1 (2H, s, OCH_2Ph), 5.4 (1H, bs, NH), 6.6 (1H, bs, NH) and 7.3 (10H, m, 2 x aromatics); δ_C (50 MHz, C^2HCl_3) 14.08 (CH_3), 28.03 (CH_2COO), 34.95 ($COCH_2CH_2$), 38.85 ($CHCH_2Ph$), 49.45 ($NHCH_2COCH_2$), 56.65 (CH), 60.39 (CH_2CH_3), 67.56 ($PhCH_2OCO$), 127.59, 128.54, 128.87, 129.03, 129.48, 129.93, 136.68, 136.72 (2 x aromatics), 155.41 ($OCONHCH$), 171.56 ($CONHCH_2CO$), 173.06 ($COOEt$) and 203.70 ($NHCOCH_2CH_2$). Piperidine-2,5-dione (**55**); Data as for Reaction 8.64.

8.75 Synthesis of carbobenzyloxyglycinyl 5-aminolevulinic acid hexyl ester (Z-Gly-ALA-OHex) (ALA10).



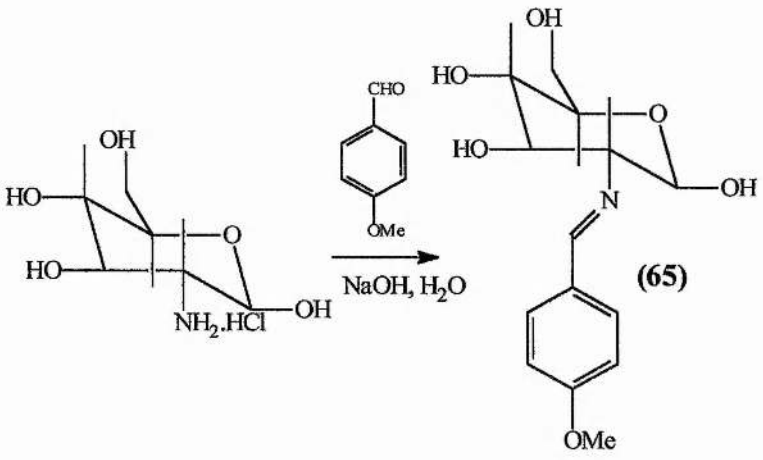
Carbobenzyloxyglycinyl 5-aminolevulinic acid hexyl ester (**64**) was prepared by a CHME coupling reaction using the same procedure as for carbobenzyloxyglycinyl 5-aminolevulinic acid benzyl ester (Reaction 8.64) but using 5-aminolevulinic acid hexyl ester (Reaction 8.62, 0.25 g, 0.9 mmol), yielding the product (**64**) as a white solid (0.20 g, 53%), m.p. 82 - 84 °C. m/z (EI^+) 406 (M^+). Found: C, 62.2; H, 7.4; N, 6.8; Calculated for $C_{21}H_{30}N_2O_6$: C, 62.1; H, 7.4; N, 6.9%. δ_H (200 MHz, C^2HCl_3) 0.9 (3H, t, CH_3), 1.3 (4H, m, 2 x CH_2), 3.9 (2H, d, CH_2), 4.1 (2H, t, CH_2), 4.2 (2H, d, CH_2), 5.2 (2H, s, CH_2Ph), 5.6 (1H, bs, NH), 6.8 (1H, bs, NH) and 7.4 (5H, m, aromatics); δ_C (50 MHz, C^2HCl_3) 14.21 (CH_3), 23.02 (CH_2CH_3), 26.02 (CH_2-Hex), 28.29 (CH_2-Hex), 28.98 (CH_2COO), 31.89 (CH_2-Hex), 35.00 ($COCH_2CH_2$), 44.85 ($NHCH_2CONH$), 49.53 ($NHCH_2COCH_2$), 65.66 ($PhCH_2OCO$), 67.76 ($COOCH_2CH_2$), 128.58, 128.62, 128.76, 128.86, 129.06 (aromatics), 157.14 ($OCONHCH_2$), 169.63 ($NHCH_2CONH$), 173.03 ($COOHex$) and 204.21 ($COCH_2CH_2CO$).

8.76 Synthesis of phthalimidolevulinic acid (ALA4).[831]



Methyl 5-phthalimidolevulinate (Reaction 8.6, 0.5 g, 1.8 mmol) was heated under reflux conditions in 6M HCl (5 cm^3). The solid dissolved slowly over 30 minutes. After 1 hour the mixture was cooled to room temperature, refridgerated overnight and the resulting solid was filtered off, dried and recrystallised from water to give phthalimidolevulinic acid (**9**) as white crystals (0.22 g, 47%). Data as for Reaction 8.14.

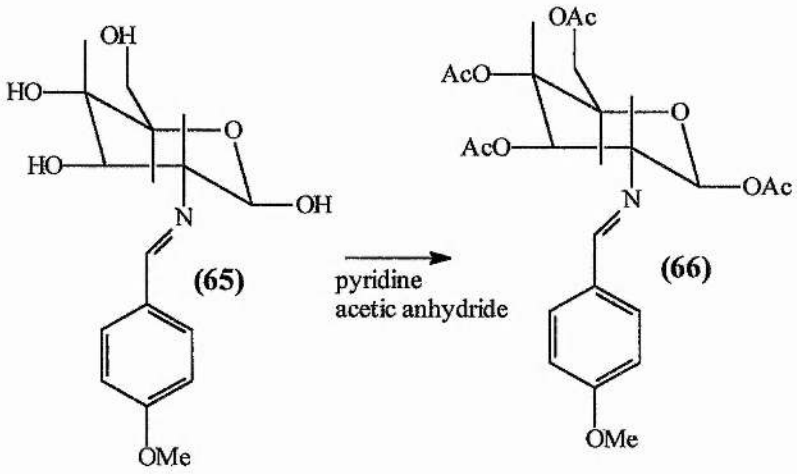
8.77 Synthesis of 2-amino-2-deoxy-N-anisylidene-β-D-glucopyranose.[832]



Glucosamine hydrochloride (10 g, 47 mmol), 1M NaOH solution (47 cm^3, 47 mmol) and p-anisaldehyde (5.7 cm^3, 6.37 g, 47 mmol) were shaken vigorously. After 10 minutes a white precipitate formed and the mixture was cooled to 0 °C for 30 minutes. The solid was filtered off, washed with ice cold water, then ice cold diethyl ether and dried thoroughly yielding 2-amino-2-deoxy-N-anisylidene-

β-D-glucopyranose (**65**) as a white solid (11.5 g, 83%), m.p. 158-161 °C (Lit.[832] 166 °C). δ_H (200 MHz, $^2H_6$-DMSO) 2.8 (1H, t, J = 8 Hz, 2-H), 3.2 (1H, m, 5-H), 3.8 (3H, s, OCH_3), 4.5 (1H, t, J = 8.5 Hz, 4-H), 4.6 (1H, t, J = 8.5 Hz, 3-H), 4.8 (1H, d, J = 8.0 Hz, 6 or 6'-H), 4.9 (1H, d, J = 8.0 Hz, 6 or 6'-H), 6.5 (1H, s, 1-H), 7.0 (2H, d, J = 10.0 Hz, aromatics), 7.6 (2H, d, J = 10.0 Hz, aromatics) and 8.1 (1H, s, N=CH); δ_C (50 MHz, $^2H_6$-DMSO) 55.54 (OCH_3), 61.51 (C2), 70.60, 74.83, 77.10, 78.43 (C3-C6), 95.88 (C1), 114.17, 115.25, 129.90, 130.65 (aromatics) and 161.55 (N=CH).

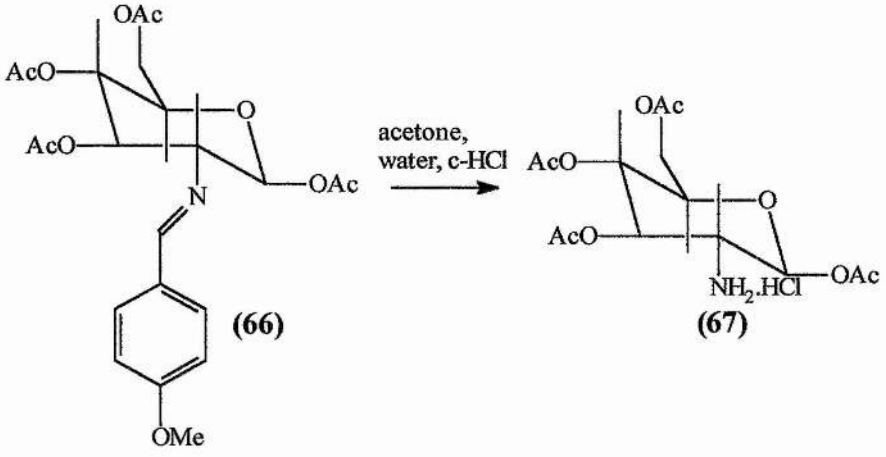
8.78 Synthesis of 1,3,4,6-tetra-O-acetyl-2-amino-2-deoxy-N-anisyl-β-D-glucopyranose.[832]



2-Amino-2-deoxy-N-anisylidene-β-D-glucopyranose (Reaction 8.77, 11.2 g, 37.8 mmol) was dissolved in dry pyridine and cooled to 0 °C. Acetic anhydride (35 cm^3, 37.8 mmol) was added and the mixture was stirred at room temperature overnight then poured onto ice and stirred. The resulting precipitate was filtered, dried and recrystallised from ethanol yielding 1,3,4,6-tetra-O-acetyl-2-amino-2-deoxy-N-anisyl-β-D-glucopyranose (**66**) as white crystals (11.2 g, 64%), m.p. 189

- 190 °C (Lit.[832] 188 °C). δ_H (200 MHz, C^2HCl_3) 1.8 - 2.0 (12H, m, 4 x $COCH_3$), 3.8 (3H, s, OCH_3), 4.0 (1H, t, J = 9.3 Hz, 2-H), 4.2 (1H, m, 5-H), 4.3 (2H, m, 6 and 6'-H), 5.0 (1H, t, J = 9.3 Hz, 3 or 4-H), 5.4 (1H, t, J = 9.3 Hz, 3 or 4-H), 6.0 (1H, d, J = 9.3 Hz, 1-H), 7.0 (2H, m, aromatics), 7.6 (2H, m, aromatics) and 8.2 (1H, s, N=CH); δ_C (50 MHz, C^2HCl_3) 18.95, 21.03, 21.21, 21.33 (4 x $CO\mathit{CH_3}$), 55.92 (OCH_3), 62.30 (C2), 68.48, 73.22, 73.43, 73.72 (C3-C6), 93.63 (C1), 114.56, 115.12, 128.72, 130.78 (aromatics), 164.89 (N=CH), 169.23, 169.65, 170.14 and 170.79 (4 x $\mathit{CO}CH_3$).

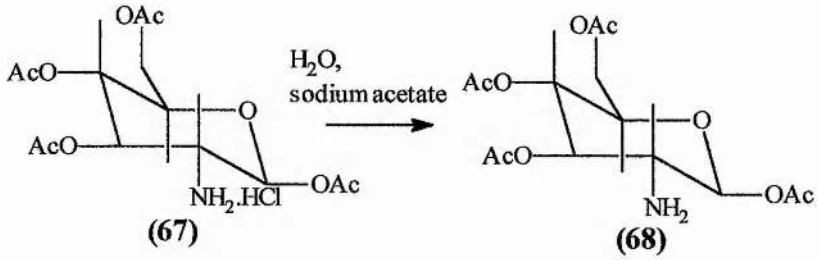
8.79 Synthesis of 1,3,4,6-tetra-O-acetyl-2-amino-2-deoxy-β-D-glucopyranose hydrochloride.[832]



1,3,4,6-Tetra-o-acetyl-2-amino-2-deoxy-N-anisyl-β-D-glucopyranose (Reaction 8.78, 6 g, 0.01 mol) was dissolved in the minimum amount of hot acetone (approximately 20 cm^3). A small quantity of water (1 cm^3) was added and the mixture was cooled to 0 °C. c-HCl (5 cm^3) was added dropwise until a precipitate appeared. The mixture was washed at 0 °C with diethyl ether to remove the anisaldehyde, filtered, and washed with ice cold diethyl ether to give 1,3,4,6-tetra-

O-acetyl-2-amino-2-deoxy-β-D-glucopyranose hydrochloride (**67**) as a white solid (4.4 g, 87%), m.p. 222 - 224 °C (dec.) (Lit.[832] 230 °C (dec.)). δ_H (200 MHz, ^2H_2O) 2.0 - 2.2 (12H, m, 4 x CO*CH*$_3$), 3.8 (1H, t, J = 10.6 Hz, 2-H), 4.3 (3H, m, 5, 6 and 6'-H), 5.2 (1H, t, J = 10.6 Hz, 4-H), 5.4 (1H, t, J = 10.6 Hz, 3-H) and 6.0 (1H, d, J = 9.1 Hz, 1-H); δ_C (50 MHz, ^2H_2O) 19.85, 23.03, 23.08, 23.21 (4 x CO*CH*$_3$), 55.33 (C2), 64.44 (C6), 71.01, 73.81, 75.16 (C3-C5), 93.36 (C1), 174.2, 175.67, 176.01 and 176.56 (4 x *CO*CH$_3$).

8.80 Synthesis of 1,3,4,6-tetra-O-acetyl-2-amino-2-deoxy-β-D-glucopyranose[832]

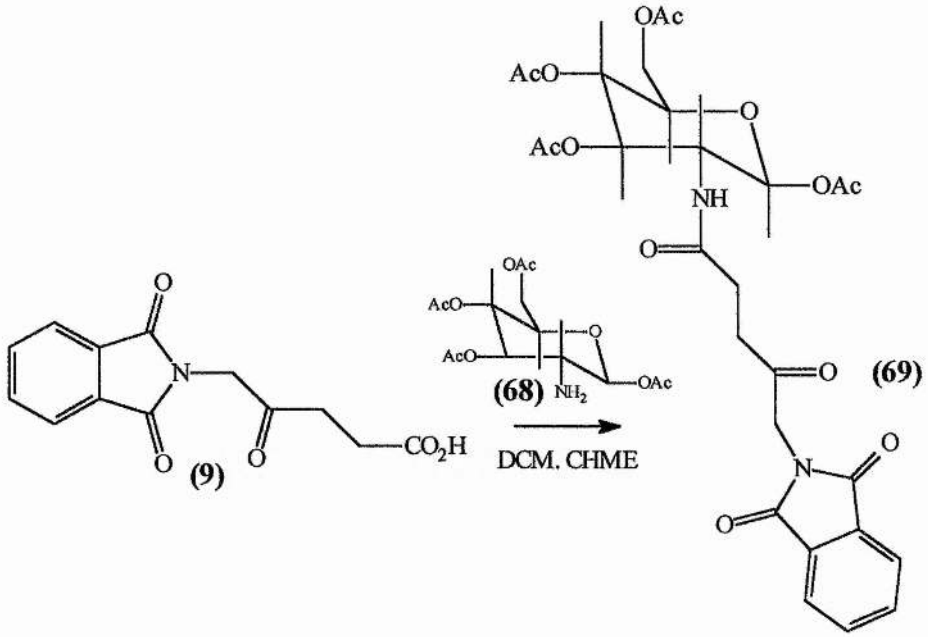


(67) **(68)**

1,3,4,6-Tetra-o-acetyl-2- amino- 2- deoxy -β - D- glucopyranose hydrochloride (Reaction 8.79, 1.18 g, 2.6 mmol,) was dissolved in water (10 cm^3). Sodium acetate (0.85 g, 5.2 mmol) was added resulting in the formation of a white suspension. This was extracted with DCM (30 cm^3 x 3), dried ($MgSO_4$) and concentrated under reduced pressure to give a white solid which was recrystallised from diethyl ether (0.74 g, 68%), m.p. 144 - 146 °C (Lit.[832] 143 °C). δ_H (200 MHz, C^{2}HCl$_3$) 1.9 - 2.2 (12H, m, CO*CH*$_3$), 3.0 (1H, m, 2-H), 4.1 (2H, m, 5 and 6-H), 4.2 (1H, dd, J = 13 Hz and 4 Hz, 6'-H), 5.0 (1H, m, 4-H), 5.4 (1H, m, 3-H) and 5.8 (1H, m, 1-H); δ_C (50 MHz, C^{2}HCl$_3$) 19.61, 19.71, 19.75, 19.93

(4 x CO*CH*$_3$), 54.00 (C2), 60.79 (C6), 67.28, 71.62, 73.96 (C3-C5), 94.07 (C1), 168.25, 168.71, 169.65 and 169.70 (4 x *CO*CH$_3$).

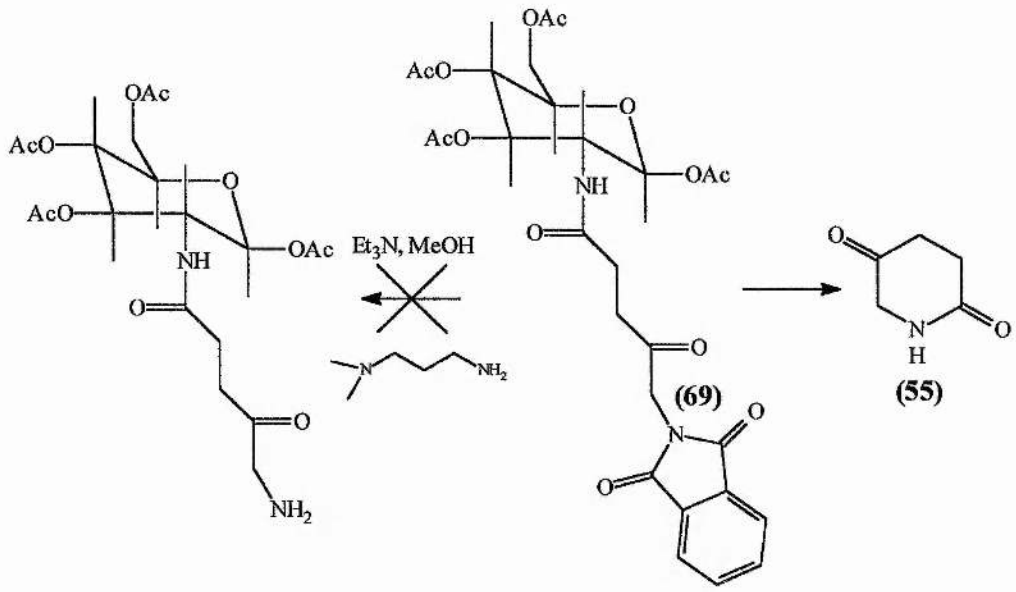
8.81 Synthesis of phthalimido-5-aminolevulinyl glucosamine tetraacetate (Phthal-ALA-Gluc-OAc) (ALA3).



Phthalimidolevulinic acid (Reaction 8.14, 0.25 g, 0.96 mmol) was dissolved in DCM (20 cm^3) and cooled to 0 °C. 1,3,4,5-tetra-O-acetyl-2-amino-2-deoxy-β-D-glucopyranose (Reaction 8.80, 0.40 g, 0.96 mmol) and CHME (0.41 g, 0.96 mmol) were added and the mixture was warmed to room temperature overnight. The reaction was cooled to 0 °C and filtered to remove the resulting urea by-product. The DCM layer was washed with water, 1M HCl solution, saturated sodium bicarbonate solution and water (50 cm^3 of each), dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a white solid (0.48 g, 76%), m.p. 186 - 188 °C (dec.). m/z (EI$^+$) 530 (M^+ - phthalimide). Elemental analysis on sugar derivatives are unreliable as the molecules tend to be very wet. CHN data

for this compound suggested the presence of water in the molecule. δ_H (200 MHz, C^2HCl_3) 2.0 (12H, m, 4 x $COCH_3$), 2.4 (2H, m, CH_2CO_2), 2.9 (2H, m, $COCH_2CH_2$), 3.8 (2H, m, CH_2), 4.1 (1H, m, 2-H), 4.3 (3H, m, 5,6 and 6'-H), 4.5 (2H, s, NCH_2), 5.2 (2H, m, 3 and 4-H), 5.7 (1H, m, NH), 6.2 (1H, m, 1-H) and 7.8 (4H, m, aromatics); δ_C (50 MHz, C^2HCl_3) 19.21, 19.92, 21.07, 21.23 (4 x $COCH_3$), 29.79 (CH_2CONH), 35.14 ($COCH_2CH_2$), 46.81 (NCH_2), 53.26 (C2), 62.21 (C6), 68.49, 72.89, 73.11 (C3-C5), 92.83 (C1), 124.02, 124.36, 133.71, 134.74 (aromatics), 168.05 (N*CO*Phthal), 169.83, 170.10, 171.22, 171.699, 171.99 (*CO*NH and 4 x *CO*CH_3) and 201.02 (CO-ALA).

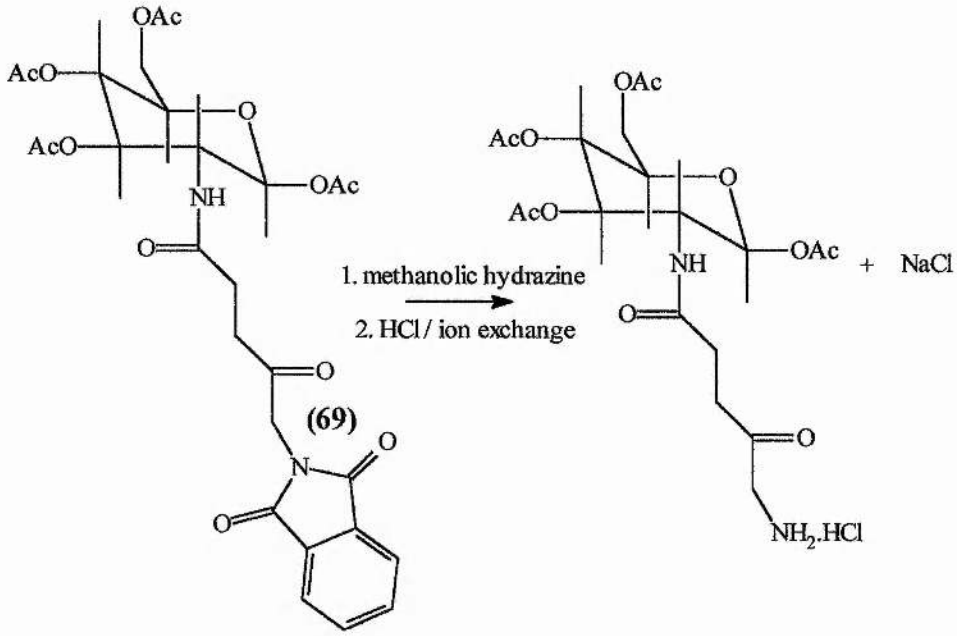
8.82 Attempted synthesis of 5-aminolevulinyl glucosamine acetate.



Phthalimido-5-aminolevulinyl glucosamine tetraacetate (Reaction 8.81, 0.1 g, 0.14 mmol) was suspended in dry methanol (5 cm^3). Triethylamine (0.02 cm^3, 0.14 mmol) and N,N-dimethyl-1,3-propanediamine (0.04 cm^3, 0.33 mmol) were added and the mixture was stirred at room temperature overnight. The mixture was

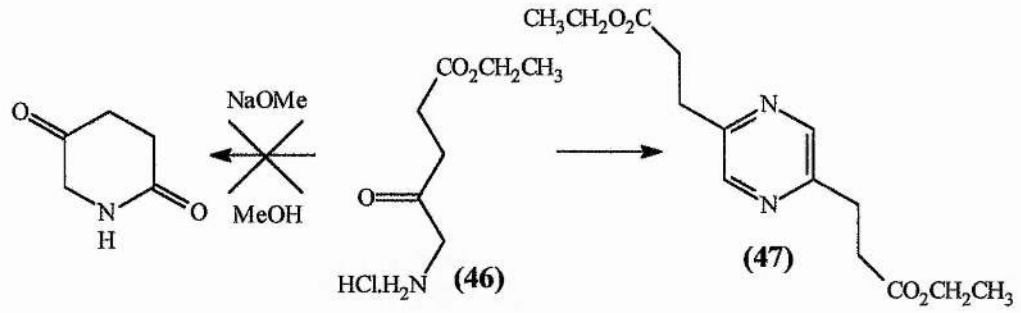
separated by column chromatography yielding piperadine-2,5-dione (**55**) and other products but not the required product. Data as for Reaction 8.67.

8.83 Attempted synthesis of 5-aminolevulinyl glucosamine acetate.



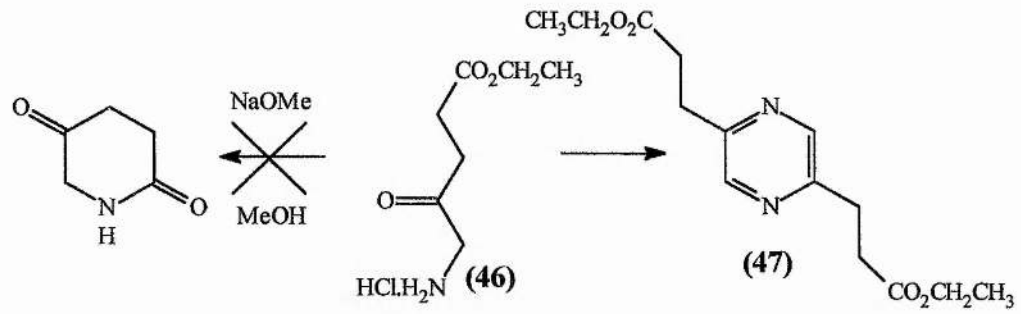
Phthalimido-5-aminolevulinyl glucosamine acetate (Reaction 8.81, 0.25 g, 0.35 mmol) was dissolved in methanolic hydrazine (50 cm^3, 0.2M, 1 g in 100 cm^3 methanol) with gentle warming. The mixture was stirred at room temperature overnight. Excess methanol and hydrazine were removed under reduced pressure leaving a white solid which was purified using ion exchange column chromatography (Amberlyst 100, strongly acidic resin). The column was washed with water, 0.1, 0.5, 1.0 and 6 M HCl solutions and no product was found. Washing with 0.5 M NaOH solution yielded a very small quantity of the required product contaminated with a large quantity of NaCl so this approach was abandoned.

8.84 Attempted synthesis of piperidine-2,5-dione.



5-Aminolevulinic acid ethyl ester (Reaction 8.61, 0.25 g, 1.27 mmol) was dissolved in a small quantity of methanol (5 cm^3). Sodium (0.029 g, 1.27 mmol) was added causing a white precipitate of NaCl to form. The mixture was stirred for 2 hours, filtered and the solvent removed under reduced pressure to give an oily solid. From NMR data the product was 2,5-di-(β-carboxyethyl) pyrazine diethyl ester (**47**) and not the required product. Data as for Reaction 8.55.

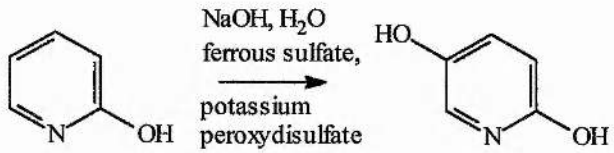
8.85 Attempted synthesis of piperidine-2,5-dione.



5-Aminolevulinic acid ethyl ester (Reaction 8.61, 0.2 g, 1.0 mmol) was dissolved in a large excess of methanol (200 cm^3, 1000 fold excess). Sodium (0.023 g, 1.0 mmol) was added and the mixture was stirred at room temperature for 24 hours. The excess methanol was removed under reduced pressure, DCM (20 cm^3) was added and the mixture was washed with water to remove the NaCl. The organic layer was dried ($MgSO_4$), filtered and concentrated under reduced pressure to

give 2,5-di-(β-carboxyethyl) pyrazine diethyl ester (**47**) and not the required product.

8.86 Synthesis of 2,5-dihydroxypyridine.[833]

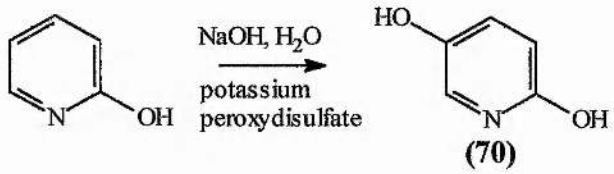


(70)

2-Hydroxypyridine (3.8 g, 0.04 mol) and sodium hydroxide (8 g, 0.2 mol) were dissolved in water (150 cm^3). The solution was cooled to 0 °C and ferrous sulfate (0.2 g) dissolved in water (2 cm^3) was added causing the mixture to go green. Potassium peroxydisulfate (13.5 g, 0.05 mol) was added and the resulting orange mixture was stirred at room temperature for 20 hours. The mixture was filtered, cooled, acidified to pH 1 by the addition of c-H_2SO_4. The acidified mixture was then hydrolysed at 100 °C for 30 minutes under N_2. The red / brown mixture was cooled and brought to pH 6 with 10M NaOH solution under N_2 and the excess water was removed under reduced pressure. The resulting brown solid was dried thoroughly over P_2O_5, then extracted with isopropyl alcohol in a Soxhlet apparatus for 8 hours. Decolourising charcoal failed to remove the brown colour so the excess solvent was removed under reduced pressure to give a brown solid which was recrystallised from ethanol to remove the 2,3-dihydroxypyridine by-product. Column chromatography using DCM as the eluant yielded an off white solid which darkened upon standing in air. The brown crystals of 2,5-dihydroxypyridine (**70**) were, therefore, used (1.2 g, 27%), m.p. 230 °C (dec.) (Lit.[833] 230 °C (dec.)). δ_H (200 MHz, $^2H_6$-DMSO) 6.3 (1H, d, J = 9.5 Hz, CH),

7.0 (1H, d, J = 3.2 Hz, CH) and 7.2 (1H, dd, J = 3.2 and 9.5 Hz, CH) δ_C (50 MHz, $^2H_6$-DMSO) 123.17, 136.99, 138.92, 146.88 and 164.88 (aromatics).

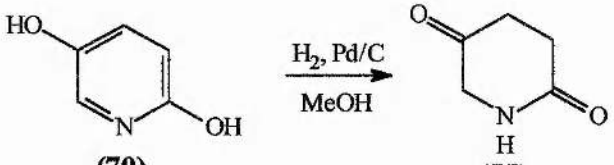
8.87 Synthesis of 2,5-dihydroxypyridine.[833,834]



(70)

2,5-Dihydroxypyridine (**70**) was prepared using the same method as in Reaction 8.86 but omitting the ferrous sulfate[834] yielding the product (**70**) as brown crystals (2.1 g, 47%). Data as for Reaction 8.86.

8.88 Synthesis of piperadine-2,5-dione.[835]

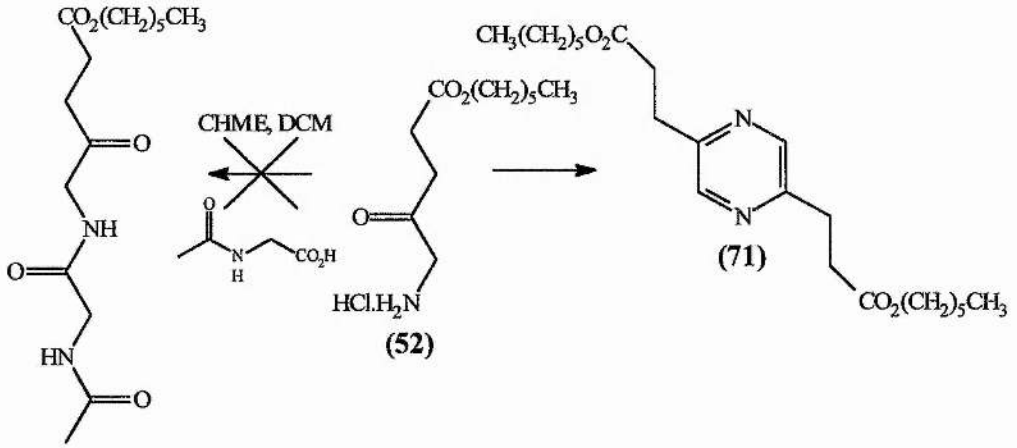


(70) **(55)**

2,5-Dihydroxypyridine (Reaction 8.87, 1.1 g, 10 mmol) was dissolved in methanol (150 cm^3). 10% Pd/C catalyst (200 mg) was added and the mixture was stirred under an atmosphere of H_2 at 30 °C and atmospheric pressure for 5 hours. The catalyst was removed by filtration through Celite and the solvent was removed under reduced pressure. The mixture was purified by silica gel flash column chromatography using DCM:methanol 9:1 as the eluant then recrystallised from acetonitrile to give piperadine-2,5-dione (**55**) as an off white solid (0.12 g, 11%), m.p. 139 - 141 °C (Lit.[835] 139 °C). δ_H (200 MHz, C^2HCl_3) 2.7 (4H, m, 2 x CH_2),

4.0 (2H, d, J = 3.0 Hz, CH_2) and 7.1 (1H, m, NH); δ_C (50 MHz, C^2HCl_3) 28.15 (CH_2), 36.21 (CH_2), 51.31 (CH_2), 174.57 (CO) and 204.62 (CO)

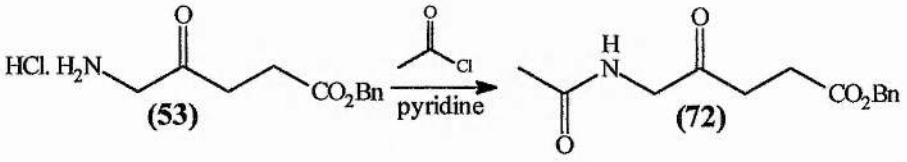
8.89 Attempted synthesis of N-acetylglycinyl 5-aminolevulinic acid hexyl ester.



Acetyl glycine (0.12 g, 0.99 mmol) was suspended in DCM (5 cm^3). N-Methyl morpholine (0.11 cm^3, 0.1 g, 0.99 mmol) was added along with CHME (0.51 g, 1.2 mmol) at 0 °C and the mixture was stirred for 30 minutes to activate the acid. 5-Aminolevulinic acid hexyl ester (Reaction 8.62, 0.25 g, 0.99 mmol) was added along with another equivalent of N-methyl morpholine (0.11 cm^3, 0.1 g, 0.99 mmol) and the mixture was allowed to warm to room temperature overnight. The mixture was cooled, filtered, washed with brine, 1M citric acid solution, saturated sodium bicarbonate solution and water (50 cm^3 of each), dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a yellow oil which, from NMR data was 2,5-di-(β-carboxyethyl) pyrazine dihexyl ester (**71**) and not the required product. δ_H (200 MHz, C^2HCl_3) 0.9 (3H, m, CH_3), 1.2 - 1.4 (6H, m, 3 x CH_2-Hex), 1.6 (2H, m, CH_2-Hex), 2.8 (2H, t, J = 5.1 Hz, CH_2CH_2COO), 3.1 (2H, t, J = 5.1 Hz, $COCH_2CH_2$), 4.0 (2H, m, CH_2-Hex) and 8.3 (1H, s, aromatic); δ_C (50 MHz, C^2HCl_3) 14.52 (CH_3), 23.46 (CH_2CH_3), 28.15 (CH_2-Hex), 29.84 (CH_2-

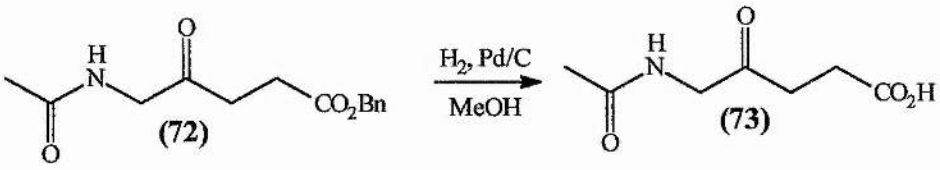
Hex), 31.85 (CH_2CH), 33.26 (CH_2CH_2CH), 65.21 (CO_2CH_2), 144.02 (aromatic CH), 153.41 (NCCH), 173.25 (COO).

8.90 Synthesis of N-acetyl-5-aminolevulic acid benzyl ester. (N-Acetyl-ALA-OBn).[836]



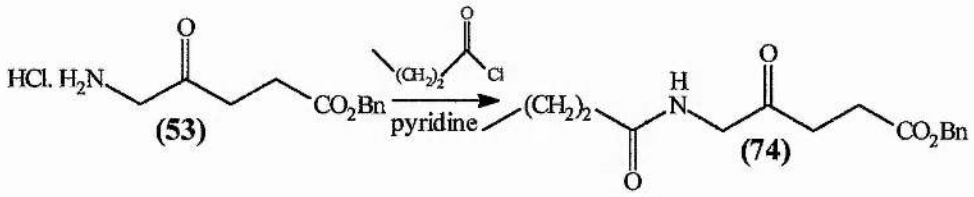
5-Aminolevulinic acid benzyl ester (Reaction 8.63, 0.25 g, 0.97 mmol) was dissolved in pyridine (5 cm^3) at 0 °C. Freshly distilled acetyl chloride (0.11 g, 0.10 cm^3, 1.45 mmol) was added carefully, causing a fairly violent reaction and formation of an orange solution. The reaction was stirred at room temperature overnight. Ethyl acetate (50 cm^3) was added and the mixture was washed with water, 1M HCl solution, saturated sodium bicarbonate solution and water (50 cm^3 of each), dried ($MgSO_4$), filtered and concentrated under reduced pressure to give slightly yellow coloured crystals of N-acetyl-5-aminolevulinic acid benzyl ester (**72**) (0.21 g, 81%), m.p. 76 - 78 °C. δ_H (200 MHz, C^2HCl_3) 2.1 (3H, s, CH_3), 2.8 (4H, m, $COCH_2CH_2CO$), 4.2 (2H, d, J = 5.1 Hz, $NHCH_2$), 5.1 (2H, s, CH_2Ph), 6.3 (1H, bs, NH) and 7.4 (5H, m, Ph); δ_C (50 MHz, C^2HCl_3) 23.42 (CH_3), 28.31 (CH_2COO), 34.94 ($COCH_2CH_2$), 49.75 ($NHCH_2$), 67.25 (CH_2Ph), 128.49, 128.54, 128.76, 129.13 (aromatics), 170.59 (CH_3CO), 172.65 (COO) and 204.78 ($COCH_2CH_2$).

8.91 Synthesis of N-acetyl-5-aminolevulinic acid (N-Acetyl-ALA).[836]



N-Acetyl-5-aminolevulinic acid benzyl ester (Reaction 8.90, 0.2 g, 0.76 mmol) was dissolved in methanol (10 cm^3). 10% Pd/C catalyst (10 mg) was added and the mixture was stirred at room temperature and atmospheric pressure, under an atmosphere of H_2 for 12 hours. The catalyst was removed by filtration through Celite and the solvent was removed under reduced pressure yielding N-acetyl-5-aminolevulinic acid as a clear oil (0.11 g, 85%). δ_H (200 MHz, $C^2H_3O^2H$) 2.0 (3H, s, CH_3), 2.6, 2.8 (2 x 2H, 2 x m, $COCH_2CH_2CO$) and 4.1 (2H, s, $NHCH_2$); δ_C (50 MHz, $C^2H_3O^2H$) 22.58 (CH_3), 29.08 (CH_2COO), 35.72 ($COCH_2CH_2$), 49.02 ($NHCH_2$), 173.91 (CH_3CO), 177.21 (COO) and 204.12 ($COCH_2CH_2$).

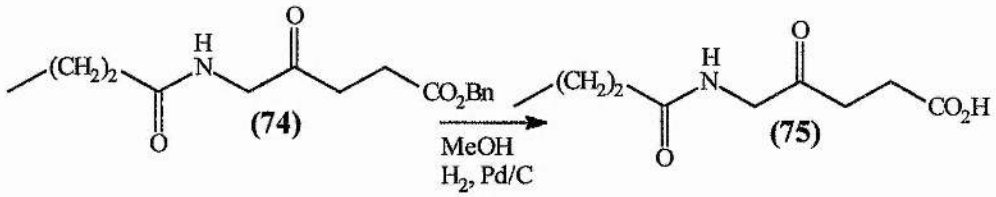
8.92 Synthesis of N-butanoyl-5-aminolevulinic acid benzyl ester (N-But-ALA-OBn).[836]



N-Butanoyl-5-aminolevulinic acid benzyl ester (**74**) was prepared using the same method as for N-acetyl-5-aminolevulinic acid benzyl ester (Reaction 8.90) but using freshly distilled butanoyl chloride (0.15 g, 1.45 mmol), yielding the product (**74**) as a slightly yellow coloured oily solid (0.19 g, 68%), m.p. 62 - 63 °C. δ_H (200 MHz, C^2HCl_3) 1.0 (3H, t, J = 7.5 Hz, CH_3), 1.7 (2H, m, CH_2CH_3), 2.2 (2H,

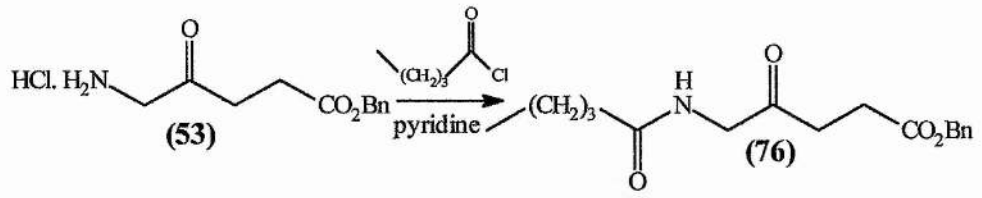
t, J = 7.5 Hz, $CH_2CH_2CH_3$), 2.7 (4H, m, $COCH_2CH_2CO$), 4.2 (2H, d, J = 5 Hz, CH_2NH), 5.2 (2H, s, CH_2Ph), 6.2 (1H, bs, NH) and 7.4 (5H, m, aromatics); δ_C (50 MHz, C^2HCl_3) 14.26 (CH_3), 19.52 (CH_2CH_3), 28.33 (CH_2COO), 35.06 (CH_2CON), 38.74 ($COCH_2$), 49.67 ($NHCH_2CO$), 67.14 (CH_2Ph), 128.38, 128.46, 128.83, 129.05, 136.09 (aromatics), 172.68 (C=O), 173.54 (C=O) and 204.47 ($NHCH_2COCH_2$).

8.93 Synthesis of N-butanoyl-5-aminolevulinic acid (N-But-ALA) (ALA12).[836]



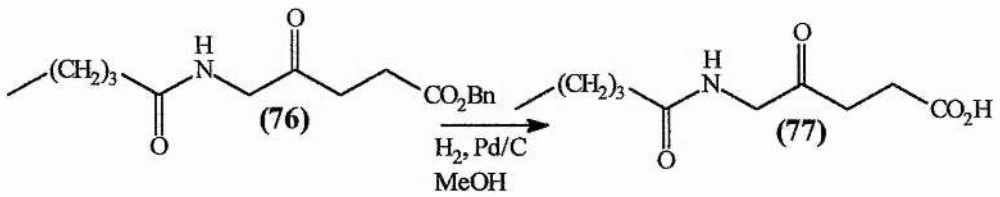
N-Butanoyl-5-aminolevulinic acid (**75**) was prepared using the same method as for N-acetyl-5-aminolevulinic acid (Reaction 8.91) but using N-butanoyl-5-aminolevulinic acid benzyl ester (Reaction 8.92, 0.15 g, 0.76 mmol), yielding the product (**75**) as an off-white solid (0.11 g, 78%), m.p. 114 - 116 °C. δ_H (200 MHz, $C^2H_3O^2H$) 0.9 (3H, m, CH_3), 1.7 (2H, m, CH_2CH_3), 2.2 (2H, m, $CH_2CH_2CH_3$), 2.6 (2H. m. CH_2CO_2H), 2.8 (2H. m. $CH_2CH_2CO_2H$) and 4.1 (2H, s, NH); δ_C (50 MHz, $C^2H_3O^2H$) 17.04 (CH_3), 23.21 (CH_2CH_3), 32.03 (CH_2COO), 38.41 (CH_2CON), 41.69 ($COCH_2$), 49.75 ($HNCH_2CO$), 179.48 (CH_2CONH), 181.35 (CO_2H) and 203.15 ($NHCH_2COCH_2$).

8.94 Synthesis of N-pentanoyl-5-aminolevulinic acid benzyl ester. (N-Pent-ALA-OBn).[836]



N-Pentanoyl-5-aminolevulinic acid benzyl ester (**76**) was prepared using the same method as for N-acetyl-5-aminolevulinic acid benzyl ester (Reaction 8.90) but using freshly distilled pentanoyl (valeryl) chloride (0.17 cm^3, 0.17 g, 1.45 mmol), yielding the product (**76**) as a slightly yellow coloured oily solid (0.26 g, 90%), m.p. 60 - 62 °C. δ_H (200 MHz, C^2HCl_3) 0.9 (3H, t, J = 7.7 Hz, CH_3), 1.2 - 1.5 (2H, m) and 1.5 - 1.8 (2H, m, $CH_2CH_2CH_3$), 2.2 (2H, t, J = 7.7 Hz, $NCOCH_2$), 2.8 (4H, m, $COCH_2CH_2CO$), 4.2 (2H, d, J = 5.1 Hz, $COCH_2N$), 5.1 (2H, s, CH_2Ph), 6.2 (1H, bs, NH) and 7.4 (5H, m, aromatics); δ_C (50 MHz, C^2HCl_3) 14.23 (CH_3), 22.87 (CH_2CH_3), 28.16 ($CH_2CH_2CH_3$), 28.33 (CH_2COOBn), 35.06 (CH_2CONH), 36.61 ($COCH_2CH_2CO$), 49.69 ($NHCH_2COCH_2$), 67.19 (CH_2Ph), 128.73, 128.85, 128.98, 129.08, 129.26 (aromatics), 170.15 (C=O), 171.22 (C=O) and 204.46 ($NHCH_2COCH_2$).

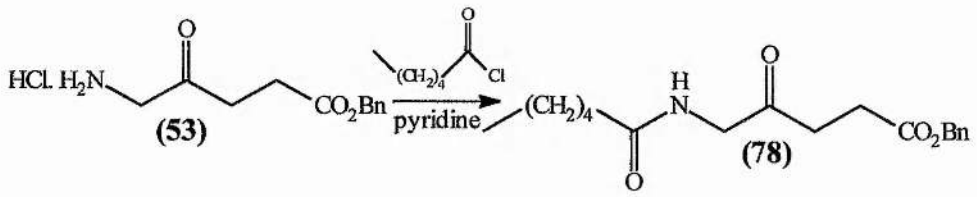
8.95 Synthesis of N-pentanoyl-5-aminolevulinic acid (N-Pent-ALA) (ALA6).[836]



N-Pentanoyl-5-aminolevulinic acid (**77**) was prepared using the same method as for N-acetyl-5-aminolevulinic acid (Reaction 8.91) but using N-pentanoyl-5-

aminolevulinic acid benzyl ester (Reaction 8.94, 0.23 g, 0.76 mmol), yielding the product (77) an off white solid (0.12 g, 86%), m.p. 112-114 °C. δ_H (200 MHz, $C^2H_3O^2H$) 0.9 (3H, t, J = 6.8 Hz, CH_3), 1.4 - 1.6 (4H, m, $CH_2CH_2CH_3$), 2.3 (2H, t, J = 6.8 Hz, $NCOCH_2$), 2.5 - 2.7 (4H, m, $COCH_2CH_2CO$) and 4.1 (2H, s, $COCH_2N$); δ_C (50 MHz, $C^2H_3O^2H$) 14.24 (CH_3), 22.92 (CH_2CH_3), 28.21 ($CH_2CH_2CH_3$), 29.51 (CH_2CO_2H), 35.01 (CH_2CONH), 36.51 ($COCH_2CH_2CO$), 49.69 ($NHCH_2COCH_2$), 170.31 (C=O), 177.54 (CO_2H) and 204.62 ($NHCH_2COCH_2$).

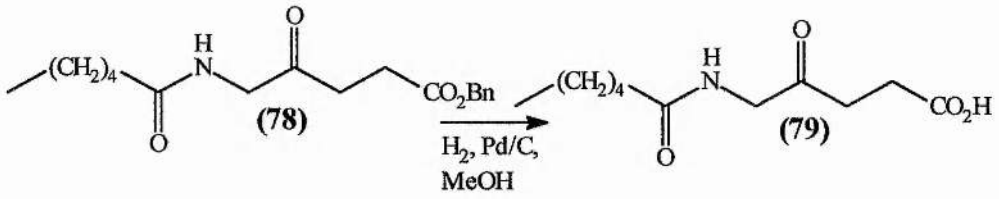
8.96 Synthesis of N-hexanoyl-5-aminolevulinic acid benzyl ester (N-Hex-ALA-OBn).[836]



N-Hexanoyl-5-aminolevulinic acid benzyl ester (**78**) was prepared using the same method as for N-acetyl-5-aminolevulinic acid benzyl ester (Reaction 8.90) but using freshly distilled hexanoyl (caproyl) chloride (0.2 cm^3, 0.19 g, 1.45 mmol), yielding the product (**78**) as a slightly yellow coloured solid (0.29 g, 94%), m.p. 56-58 °C. δ_H (200 MHz, C^2HCl_3) 0.9 (3H, m, CH_3) 1.3 (4H, m) and 1.7 (2H, m, 3 x CH_2-Hex), 2.2 (2H, t, J = 7.1 Hz, $NCOCH_2$), 2.7 (4H, m, $COCH_2CH_2CO$), 4.2 (2H, d, J = 4.8 Hz, $COCH_2N$), 5.1 (2H, s, CH_2Ph), 6.2 (1H, bs, NH) and 7.4 (5H, m, aromatics); δ_C (50 MHz, C^2HCl_3) 14.40 (CH_3), 22.85 (CH_3CH_2), 25.8 (CH_2-Hex), 28.31 (CH_2COOBn), 31.89 (CH_2-Hex), 35.04 (CH_2-Hex), 36.85 ($COCH_2CH_2CO$), 49.68 ($NHCH_2COCH_2$), 67.17 (CH_2Ph), 128.57, 128.70,

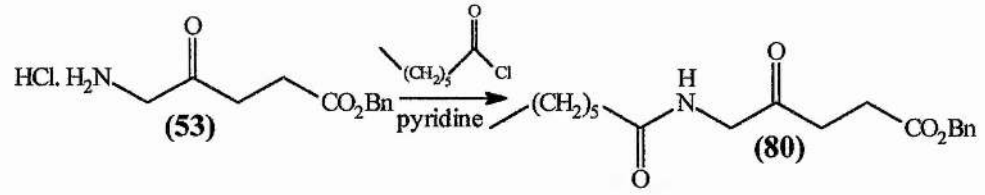
128.78, 128.84, 129.06 (aromatics), 172.67 (*CO*Hex), 173.69 (*CO*OBn) and 204.44 ($NHCH_2\mathit{CO}CH_2$).

8.97 Synthesis of N-hexanoyl-5-aminolevulinic acid (N-Hex-ALA) (ALA7).[836]



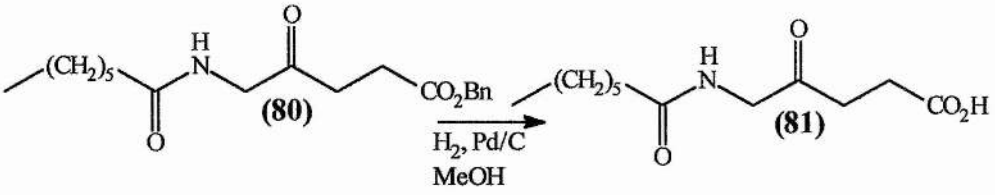
N-Hexanoyl-5-aminolevulinic acid (**79**) was prepared using the same method as for N-acetyl-5-aminolevulinic acid (Reaction 8.91) but using N-hexanoyl-5-aminolevulinic acid benzyl ester (Reaction 8.96, 0.2 g, 0.63 mmol), yielding the product (**79**) as an off white solid (0.12 g, 86%), m.p. 111 - 113 °C. δ_H (200 MHz, $C^2H_3O^2H$) 0.9 (3H, t, J = 6.25 Hz, CH_3), 1.4 (4H, m, 2 x CH_2-Hep), 1.6 (2H, m, CH_2-Hep), 2.2 (2H, t, J = 8.3 Hz, $NCO\mathit{CH_2}$), 2.7 (4H, m, $CO\mathit{CH_2CH_2}CO$) and 4.1 (2H, s, $COCH_2N$); δ_C (50 MHz, $C^2H_3O^2H$) 14.50 (CH_3), 23.68 ($\mathit{CH_2}CH_3$), 26.91 (CH_2-Hex), 28.99 ($COCH_2\mathit{CH_2}CO$), 32.78 (CH_2-Hex), 35.47 (CH_2-Hex), 37.01 ($CO\mathit{CH_2}CH_2CO$), 48.02 ($NH\mathit{CH_2}CO$), 174.37 (NH*CO*Hex), 177.04 (CO_2H) and 206.52 ($NHCH_2\mathit{CO}CH_2$).

8.98 Synthesis of N-heptanoyl-5-aminolevulinic acid benzyl ester (N-Hep-ALA-OBn).[836]



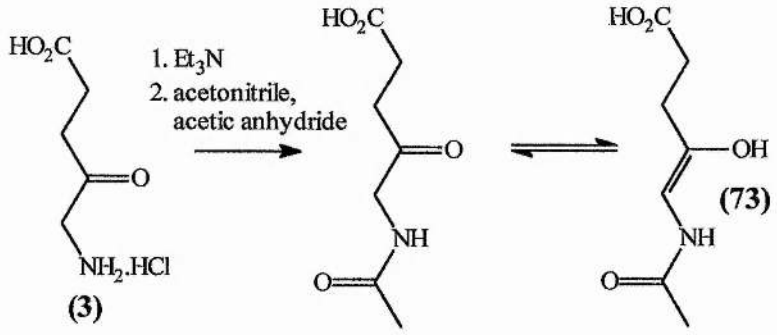
N-Heptanoyl-5-aminolevulinic acid benzyl ester (**80**) was prepared using the same method as for N-acetyl-5-aminolevulinic acid benzyl ester (Reaction 8.90) but using freshly distilled heptanoyl chloride (0.23 cm^3, 0.22 g, 1.45 mmol), yielding the product (**80**) as a slightly yellow coloured solid (0.26 g, 81%), m.p. 55-57 °C. δ_H (200 MHz, C^2HCl_3) 0.9 (3H, m, CH_3), 1.3 (3 x 2H, m, 3 x CH_2-Hep), 1.6 (2H, m, CH_2-Hep), 2.2 (2H, t, J = 7.5 Hz, $NCOCH_2$), 2.8 (4H, m, $COCH_2CH_2CO$), 4.2 (2H, d, J = 5 Hz, $COCH_2N$), 5.1 (2H, s, CH_2Ph), 6.2 (1H, bs, NH) and 7.4 (5H, m, aromatics); δ_C (50 MHz, C^2HCl_3) 14.48 (CH_3), 26.05 (CH_2-Hep), 28.42 ($COCH_2\mathit{CH_2}CO$), 29.42 (CH_2-Hep), 31.99 (CH_2-Hep), 35.06 (CH_2-Hep), 36.90 ($CO\mathit{CH_2}CH_2CO$), 49.69 ($NH\mathit{CH_2}COCH_2$), 67.19 (CH_2Ph), 128.52, 128.73, 128.80, 128.83, 129.04 (aromatics), 172.68 (COHep), 173.74 (COOBn) and 204.46 ($NHCH_2\mathit{CO}CH_2$).

8.99 Synthesis of N-heptanoyl-5-aminolevulinic acid (N-Hept-ALA) (ALA8).[836]



N-Heptanoyl-5-aminolevulinic acid (**81**) was prepared using the same method as for N-acetyl-5-aminolevulinic acid (Reaction 8.91) but using N-heptanoyl-5-aminolevulinic acid benzyl ester (Reaction 8.98, 0.2 g, 0.6 mmol), yielding the product (**81**) as an off white solid (0.15 g, 90%), m.p. 110 - 112 °C. δ_H (200 MHz, $C^2H_3O^2H$) 0.9 (3H, m, CH_3), 1.4 (6H, m, 3 x CH_2-Hep), 1.6 (2H, m, CH_2-Hep), 2.2 (2H, t, J = 7.7 Hz, NCO*CH_2*), 2.7 (4H, m, CO*CH_2CH_2*CO) and 4.0 (2H, s, CO*CH_2*N); δ_C (50 MHz, $C^2H_3O^2H$) 14.69 (CH_3), 23.90 (*CH_2*CH_3), 27.19 (CH_2-Hep), 29.73 (COCH$_2$*CH_2*CO), 30.26 (CH_2-Hep), 33.01 (CH_2-Hep), 35.46 (CH_2-Hep), 37.07 (CO*CH_2*CH_2CO), 48.02 (NH*CH_2*CO), 172.51 (NH*CO*Hep), 177.01 (CO_2H) and 204.01 (NHCH$_2$*CO*CH$_2$).

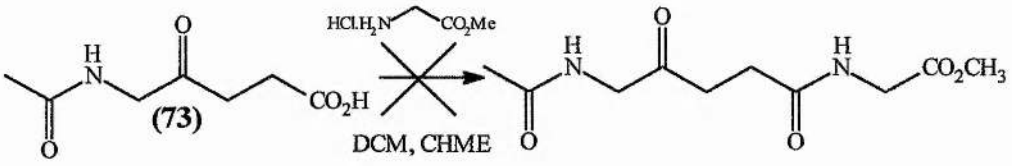
8.100 Synthesis of N-acetyl-5-aminolevulinic acid (N-Ac-ALA) (ALA9).[829]



Triethylamine (1.74 cm^3, 1.26 g, 12.5 mmol) was added to ALA.HCl (Reaction 8.15, 0.42 g, 2.5 mmol). This mixture was sonicated for 15 minutes before acetonitrile (10 cm^3) was added. The solution was stirred and acetic anhydride (0.5 cm^3) was added dropwise. The reaction was stirred at room temperature overnight resulting in formation of a black solution. A small quantity of decolourising charcoal was added and the mixture was refluxed for 30 minutes, filtered through Celite and concentrated under reduced pressure. The mixture was

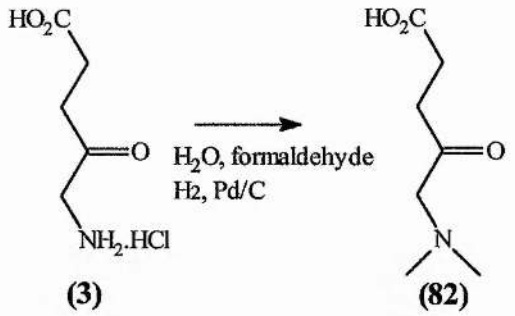
purified by column chromatography using ethyl acetate as the eluant yielding N-acetyl-5-aminolevulinic acid (**73**) as a white solid (0.24 g, 56%), m.p. 150 - 152 °C. δ_H (200 MHz, C^2HCl_3) 2.1 (3H, s, CH_3), 2.8 (2H, t, CH_2CH_2CO), 2.9 (2H, t, CH_2CH_2CO), 6.3 (1H, d, J = 10.1 Hz, NHCH=COH - enol) and 7.1 (1H, bs, NH); δ_C (50 MHz, C^2HCl_3) 23.41 (CH_3), 28.17 and 28.51 ($CH_2CH_2CO_2H$), 101.52 (CH), 135.48 (C-OH), 167.48 (CH_3CO), 174.41 (CO_2H) and 203.12 ($NHCH_2CO$). Peaks corresponding to lit.[829] were observed but there were some unidentified signals.

8.101 Attempted synthesis of N-acetyl 5-aminolevulinyl glycine methyl ester.



An attempt was made to prepare N-acetyl-5-aminolevulinyl glycine methyl ester using the same method as for N-acetyl-glycinyl-5-aminolevulinic acid hexyl ester (Reaction 8.89) but using N-acetyl-5-aminolevulinic acid (Reaction 8.100, 0.1 g, 0.58 mmol) and glycine methyl ester (0.052 g, 0.58 mmol). This yielded only a very small amount (>2%) of impure product so the attempt was abandoned.

8.102 Synthesis of N,N-dimethyl-5-aminolevulinic acid (ALA5).[837]



(3) **(82)**

ALA.HCl (Reaction 8.15, 0.84 g, 0.5 mmol) was dissolved in water (100 cm^3). Formaldehyde (0.6 g, 0.02 mmol) and 10% Pd/C catalyst (0.084 g) were added and the mixture was stirred at room temperature and atmospheric pressure, under an atmosphere of H_2 for 20 hours. The mixture was heated to reflux then filtered through Celite to remove the catalyst. The filtrate was evaporated under reduced pressure and impurities of paraformaldehyde were removed by coevaporation with water. The product was recrystallised from acetone/ether giving N,N dimethyl-5-aminolevulinic acid (**82**) as a slighly yellow coloured solid (0.55 g, 56%), m.p. 201 - 204 °C (Lit.[837] 202 - 204 °C). δ_H (200 MHz, ^2H_2O) 2.7 (2H, t, J = 6 Hz, CH_2COO), 2.8 (2H, t, J = 6 Hz, COCH_2CH_2), 2.9 (6H, s, $N(CH_3)_2$), 4.4 (2H, s, NCH_2) and 7.2 (1H, bs, NH); δ_C (50 MHz, ^2H_2O) 30.54 (CH_2COO), 37.44 ($CH_2CO$$CH_2$), 46.51 ($NCH_2$CO), 67.22 (2 x CH_3), 179.81 (COO) and 206.15 (CO).

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