# Studies on plant secondary metabolites influencing pest behaviour.

## Thesis

UNRESTRICTED

# STUDIES ON PLANT SECONDARY METABOLITES INFLUENCING PEST BEHAVIOUR

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This thesis has been submitted to the Department of Chemistry of the Open University in partial fulfilment of the requirements of the Doctor of Philosophy degree.

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Many things have helped to keep me sane and I would like to thank the Rothamsted men's tennis team, and the especially the cricket team. During the writing of this thesis I have been kept entertained on many Saturday afternoons by Gloucester RFC, and particularly the inhabitants of the Shed.

Finally, and most importantly my thanks are due to my family, and especially my wife Caroline who has endured a lot to see this manuscript completed.

## ABSTRACT

Synthesis of both [$^{14}$C-1] labelled and unlabelled non-protein amino acids has allowed investigation of the early steps of glucosinolate biosynthetic pathway in *Brassica napus*. Use of the [$^{14}$C-1]amino acids has allowed limited characterisation of the enzymes involved in the initial oxidative decarboxylation reaction. The intermediates between the amino acid and the aldoxime have been studied by synthesis of a range of potential intermediates. Synthesis of [$^{2}$H-2]homophenylalanine has allowed preliminary investigations into the intermediates by examination of the retention or loss of the label in the aldoxime product. The use of $^{19}$F NMR as a technique to examine the intermediates as they are formed *in situ* has been investigated with synthesis of a range of fluorine substituted amino acids and aldoximes.

Synthesis of a range of unlabelled and [$^{14}$C-1]methionine homologues has allowed investigation of the enzymes involved in aliphatic glucosinolate biosynthesis. The synthesis of a range of dihomomethionine analogues, and subsequent competition assays have outlined the substrate specificity of the aliphatic enzyme, and allowed the development of a crude active site model.

The biosynthesis of thiohydroximates from aldoximes has been investigated, by examining the C-S bond cleavage which takes place during thiohydroximate formation. Investigation of these enzymes required the synthesis of the proposed intermediate, a cysteine thiohydroximate conjugate. This was accomplished using nitrile oxide methodology.

Use of regiospecific deuteration has allowed the synthesis of deuterated homophenylalanines, which will allow further investigation of the pathway. [$^{13}$C-2]Dihomomethionine has been synthesised to investigate the possibility of its incorporation into 2-propenylglucosinolate.

Glucosinolate catabolites have been synthesised in order to study their biological activity at electrophysiological, behavioural and field levels.

## ABBREVIATIONS

| | |
|---|---|
| Å | angstrom |
| Ac | acetyl |
| ATP | adenosine triphosphate |
| cDNA | complementary deoxyribonucleic acid |
| CHAPS | 3-[(3-cholamidopropy-l-dimethylammonio]-1-propane sulfonate |
| d. | density |
| DHEt | dihomoethionine |
| DHMet | dihomomethionine |
| DIBAL | diisobutylaluminium hydride |
| DMF | dimethylformamide |
| DMSO | dimethylsulfoxide |
| DSS | 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt |
| Et | ethyl |
| FAD | flavin adenine dinucleotide |
| FID | flame ionisation detector |
| FMN | flavin mononucleotide |
| FMO | flavin-linked monooxygenase |
| GC | gas chromatography |
| GC-MS | gas chromatography-mass spectrometry |
| ha. | hectares |
| HMet | homomethionine |
| HPhe | homophenylalanine |
| HPLC | high pressure liquid chromatography |
| IACR | Institute of Arable Crops Research |

| | |
|---|---|
| IR | infra red |
| Me | methyl |
| Met | methionine |
| mRNA | messenger ribonucleic acid |
| MS | mass spectrometry |
| ms | molecular sieve |
| NADH | nicotinamide adenine dinucleotide, reduced form |
| NADPH | nicotinamide adenine dinucleotide phosphate, reduced form |
| NCS | *N*-chlorosuccinimide |
| NMR | nuclear magnetic resonance |
| nOe | nuclear Overhauser effect |
| PCC | pyridinium chlorochromate |
| pyr | pyridine |
| sat. | saturated |
| TetHMet | tetrahomomethionine |
| THEt | trihomoethionine |
| THMet | trihomomethionine |
| THF | tetrahydrofuran |
| tlc | thin layer chromatography |
| TMS | tetramethylsilane |
| UHP | urea hydrogen peroxide complex |
| UV | ultra violet |

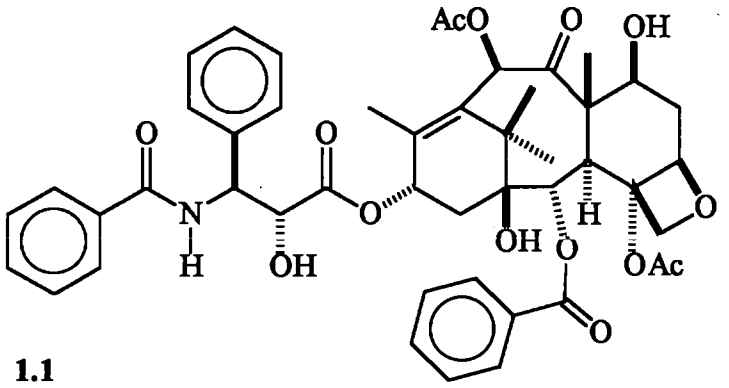
# STUDIES ON PLANT SECONDARY METABOLITES INFLUENCING PEST BEHAVIOUR

# 1 INTRODUCTION

# 1.1 *Plant Secondary Metabolites*

Plant secondary metabolites are a group of organic compounds loosely defined as those not involved in the primary metabolic processes of plants, i.e. those processes essential for survival, including photosynthesis, respiration, growth and reproduction. There have been a number of attempts to define secondary metabolism based on role, distribution within the cell or whole organism, and on chemotaxonomic grounds, but due to the wide variations in functionality and an almost limitless range of chemical structure these definitions have proved inadequate. Any definitions are further complicated by some metabolites being involved in aspects of primary and secondary metabolism.

Many plant secondary metabolites can be categorised according to biosynthesis, such as the phenylpropanoids, which include tannins, lignin and the flavonoids, from the phenylalanine-ammonia lyase pathway. Others, such as glucosinolates, are derived from amino acids, with modifications such as chain extension of the amino acid before entering the main part of the glucosinolate biosynthetic pathway. The role of secondary metabolites within the plant is almost as diverse as their chemical structures, with secondary metabolites being involved in a variety of plant processes, from signalling attraction to defence[1]. Many secondary metabolites have no recognisable role and as such are simply a source of genetic diversity, which may be of use to the plant in future generations[2]. Their use to mankind is also highly varied, with compounds such as taxol (**1.1**) being used as a lead structure for the development of anticancer agents[3], polygodial (**1.2**)[4] and azadirachtin (**1.3**)[5] as insect antifeedants, and pyrethrin 1 (**1.4**)[6] as a natural insecticide, and as a lead for the development of a whole class of synthetic insecticides, the pyrethroids.

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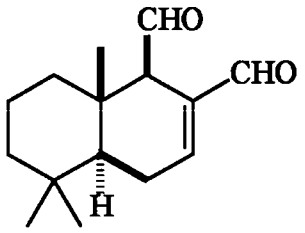


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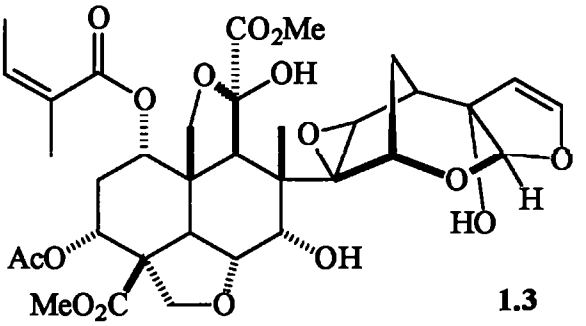


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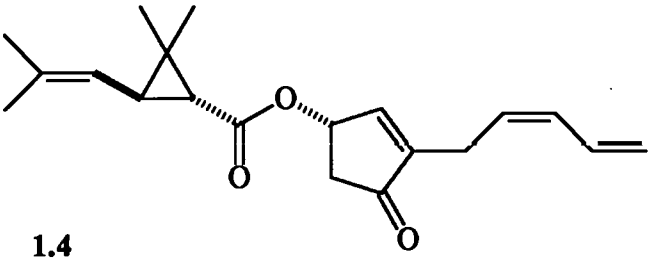


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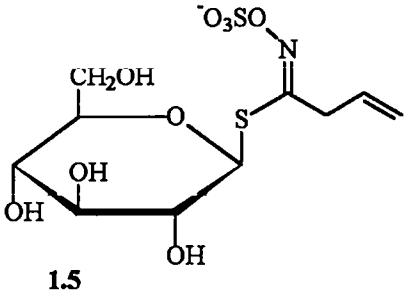
Plant secondary metabolites also give foods their distinctive flavours and aromas, for example sinigrin (**1.5**) (and its catabolite allyl isothiocyanate) in mustard, and vanillin (**1.6**) in vanilla.

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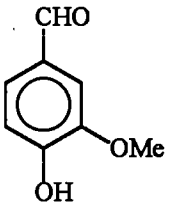


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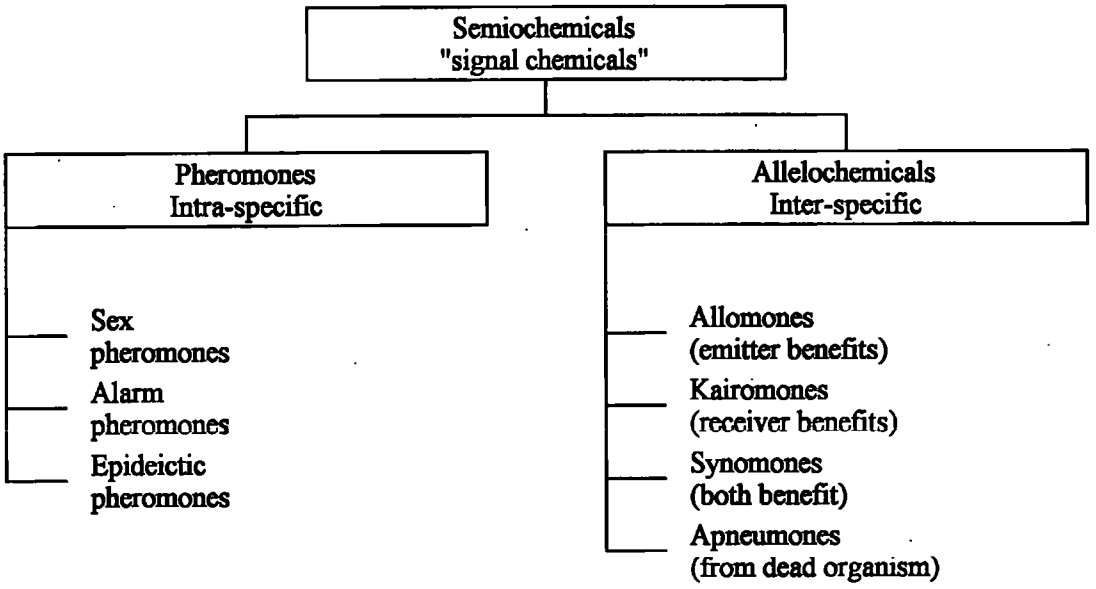
Plant secondary metabolites, and secondary metabolism in general, are well studied fields and are amply covered by a number of excellent reviews and books[1,7-11]. However, despite this large body of knowledge, the field is so wide and varied that some secondary metabolites have not been studied in any detail and nothing is known of their role or biosynthesis, while others have well documented roles, their biosynthesis determined and the associated genes encoding for the enzymes involved sequenced.

## 1.2 *Semiochemicals*

Semiochemicals, or behaviour controlling chemicals, are chemical signals, response to which can cause a change in the behaviour of an organism. Semiochemicals have been particularly well studied for insects although some have been studied in other arthropods and in some species of fish and mammals. They can be divided into two main classes according to the way in which they act[12].



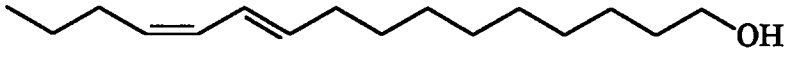
Those acting on organisms of the same species (intra-specifically) are known as pheromones and those acting on different species (inter-specifically) are known as allelochemicals. Within these two classes there are further subdivisions according to the action mediated by the particular behaviour controlling chemical involved.

## 1.2.1 *Pheromones*

Pheromones are produced by an organism in order to mediate the behaviour of other members of the same species. A wide range of behaviour can be influenced, such as aggregation, oviposition, and mate finding, and as such pheromones can act as attractants or repellents. They are further subdivided into three groups according to their role.

Sex pheromones influence behaviour associated with mate finding and courtship behaviour. Numerous insect sex pheromones have been identified, with the first being bombykol (**1.7**) which was isolated as the sex pheromone of the female silkworm, *Bombyx mori* (L.) in 1959[13]. Pheromones often comprise several, or many components. For example, the aphid sex pheromone, first isolated for the vetch aphid, *Megoura viciae,* consists of a mixture of two monoterpenoids, a nepetalactone (**1.8**) and a nepetalactol (**1.9**)[14]. Sex pheromones for different aphid species have been shown to consist of one, or both of these two components, but present in different ratios[15,16]. Stereo- and regiochemistry is very important in determining activity of pheromones, with changes in stereo- and regiochemistry resulting in pheromones which mediate behaviour in different species. For example, the sex pheromone of the damson hop aphid, *Phorodon humuli*, comprises the nepetalactol diasteriomers (**1.10**) as shown[17], and is thereby distinct from

most other aphid species with which it is associated temporally and geographically. The natural stereochemistry is important for activity in most insect pheromones, with the other enantiomers of natural compounds having varying effects. For example, (+)-*exo*-brevicomin (**1.11**), a component of the western pine beetle sex pheromone is active, while (-)-*exo*-brevicomin (**1.12**) is inactive. In other cases the "unnatural" enantiomers can be as active as the natural compound, or inhibitory to the activity, or enhance activity[18].

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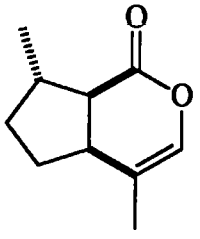


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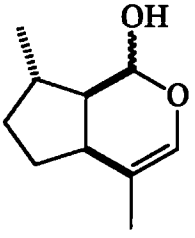


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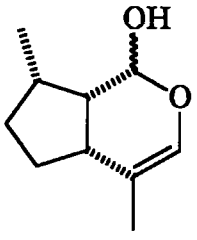


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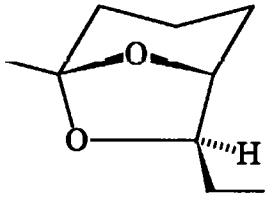


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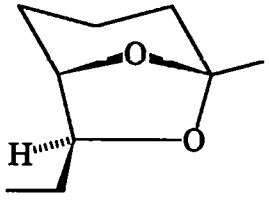
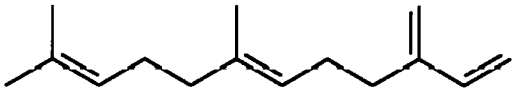



**1.12**

Lepidopteran sex pheromones are a particularly well studied area and usually consist of simple metabolites of long chain fatty acids such as alcohols, aldehydes and esters, with a precise blend of components. Changes in the position, and geometric isomerism, of double bonds in lepidopteran sex pheromones can alter species specificity[19]. These subtle changes in the chemistry of sex pheromones are understood in evolutionary terms, with closely related, but different species needing to be able to differentiate between the sex pheromones of closely related species. This species differentiation can be achieved

by either changes in pheromone composition including changing individual components, or ratios of those components, or by mating at different times of the year.

Alarm pheromones mediate behaviour by warning members of the same species of danger in the immediate area, and these may mediate behaviour in a wider species range than sex pheromones. The sesquiterpene hydrocarbon (*E*)-β-farnesene (**1.13**) is the alarm pheromone for a wide range of aphid species[20]. This structure is typical for an alarm pheromone which will usually have relatively high volatility, to assist the speed of transmission of the signal, and high chemical instability to give the limited persistence required of an alarm pheromone at the site of release. (*E*)-β-Farnesene is readily oxidised in air to inactive products. Other insects such as ants produce alarm pheromones. These can be as simple as straight chain alkanes, or involve stereochemical considerations and functionality, such as (*S*)-4-methylheptan-3-one (**1.14**), the alarm pheromone of the leaf cutting ant, *Atta texana*.



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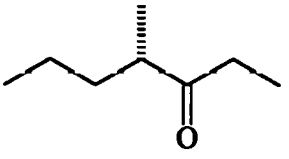
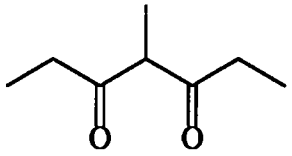



**1.14**

Epideictic pheromones influence the spacing between particular organisms and as such can have either an attractant or repellent effect. Aggregation pheromones such as the polyketide 4-methyl-3,5-heptanedione (**1.15**), the aggregation pheromone of the pea and bean weevil, *Sitona lineatus*, attract adults from both sexes[21]. Oviposition pheromones, such as the mosquito oviposition pheromone (**1.16**), attract females and encourage them to lay eggs[22]. Oviposition deterring pheromones act as repellents and deter egg laying in order to increase the likelihood of success for the larvae by limiting the number of eggs laid in any particular place. Trail pheromones influence behaviour in social insects such as ants, termites and bees, enabling other members of the same species repeatedly to find

a food source. These are often not as species specific as other pheromones, for example the pyrrolecarboxylic ester **1.17**, is used by a number of ants in the tribe Attini as a trail pheromone.



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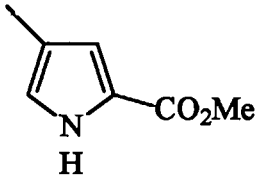

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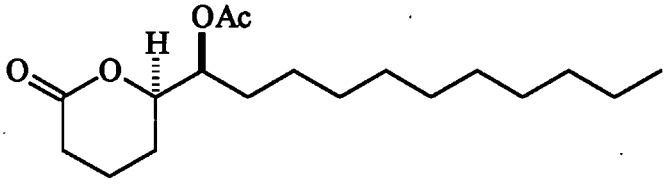


**1.16**

## 1.2.2 *Allelochemicals*

Allelochemicals are chemicals released by one organism which modify the behaviour of organisms from a different species. These are classified according to the source of the signal and whether the signal benefits the emitter or the receiver.

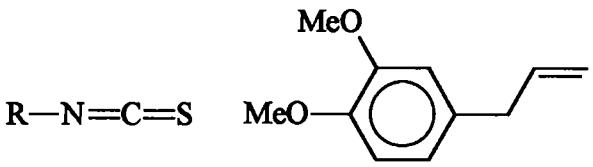
Allomones are signals which benefit the emitter and disadvantage the receiver, usually by deterring one organism from attacking or feeding on another.

Kairomones benefit the receiver and disadvantage the emitter. For example a kairomone will be the chemical signal by which an insect is able to identify a plant suitable for feeding or oviposition. The same chemical can act as both a kairomone and an allomone. Isothiocyanates (**1.18**) which are emitted by members of the Brassicaceae, such as oilseed rape, *Brassica napus*, are repellent to non-specialist pests such as some species of aphids, and are hence allomones, but are attractants to adapted specialist feeders such

as the cabbage seed weevil, *Ceutorhynchus assimilis*, and are hence also kairomones[23-25].

Synomones are of benefit to both the emitter and the receiver, and so include chemical signals such as those which attract pollinators to a flower. The oriental fruit fly, *Dacus dorsalis*, is highly attracted to eugenol methyl ether (**1.19**), and so is attracted to blossoms of plants such as the golden shower tree, *Cassia fistulosa* (Leguminosae), which produce the compound[26].

Apneumones are emitted by a non-living, i.e. dead or decaying, source. As such they can only be of benefit to the receiver.

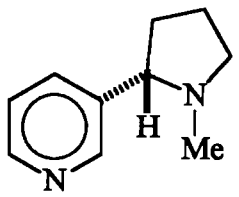



**1.18** **1.19**

## 1.3 *Conventional Strategies for Pest Control*

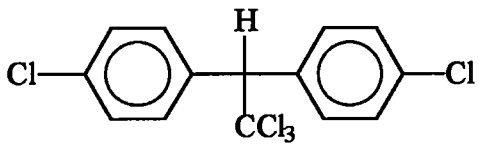
Traditional methods of pest control on crop plants rely on the application of chemicals which are toxic to the target organism, leading to a reduction in pest numbers and damage. The first insecticides developed were inorganic compounds such as the arsenic containing Paris Green (**1.20**), and hydrogen cyanide[27]. These compounds have high mammalian toxicity and were replaced by a series of plant extracts containing toxic organic components, such as tobacco extracts containing nicotine (**1.21**)[27] and pyrethrum daisy, *Tanacetum=Chrysanthanum cinerariaefolium*, extracts containing pyrethrins such as pyrethrin 1 (**1.4**)[6].

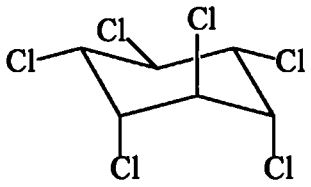
$CH_3CO_2Cu.3Cu(AsO_2)_2$




**1.20** **1.21**

The first compounds designed specifically as insecticides were the organochlorine insecticides, of which the most well known example is DDT (**1.22**)[27]. This has high insect, but low mammalian toxicity. However, use was abandoned because of its persistence in the environment and the development of resistant pest biotypes[28]. The high persistence of organochlorine insecticides has led to their accumulation in the food chain, with higher predators such as fish-eating birds at greatest risk[28]. Other organochlorine insecticides such as γ-hexachlorocyclohexane (γ-HCH, lindane) (**1.23**) are still in use today, although they will probably be removed from the market in the near future.

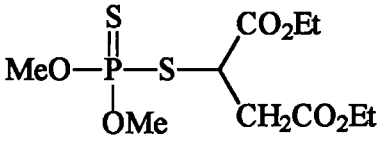



**1.22** **1.23**

Less persistent insecticides were developed to overcome the problems associated with accumulation. The two main classes developed in response to this problem are the organophosphorus (OP) insecticides, developed from nerve gas, such as malathion (**1.24**)[29], and the carbamate insecticides, such as pirimicarb (**1.25**) which contains the carbamate structure[29]. The carbamates were originally developed from the plant-derived compound

physostigmine (**1.26**), which also contains the carbamate functionality, following the observation that physostigmine has the same site of action as the OP's. These classes are still in wide use, but in common with all classes of insecticides there are problems associated with the development of resistant pest biotypes.



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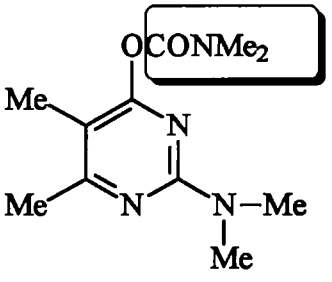

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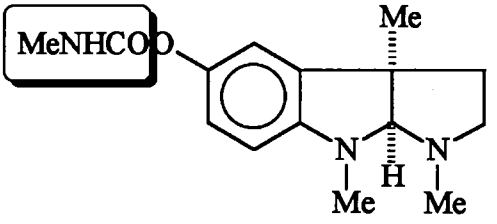
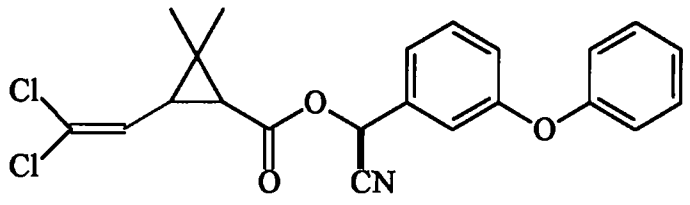
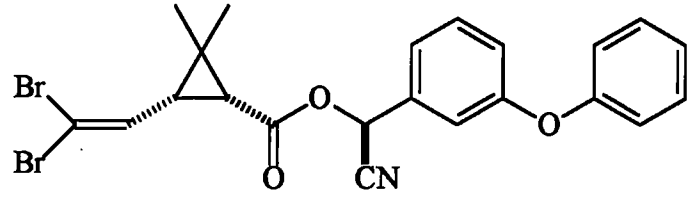


**1.26**

The most recent major advance in conventional pest control is the development of the pyrethroid insecticides from pyrethrin 1 (**1.4**). The first synthetic pyrethroids showed increased photostability from the parent compound, which allowed development of compounds with increased activity, such as cypermethrin (**1.27**) and deltamethrin (**1.28**)[30]. These compounds show extremely high activity with very low mammalian toxicity, resulting in very low application rates. One interesting point about deltamethrin is that the most active isomer contains the opposite stereochemistry from the lead compound pyrethrin 1 (**1.4**). However, despite the success of these compounds, there is the universal problem of resistance as well as problems with high toxicity to beneficial insects and fish[31].




**1.27**




**1.28**

Conventional methods of pest control are now coming under attack from two directions. Environmentalists and pressure groups are demanding a reduction in the use of pesticides with a switch to alternative methods of pest control. New pesticides commonly show resistance problems within a couple of years of launch. As resistance develops, pesticides become increasingly ineffective and cross resistance can also develop to different pesticides with the same mode of action, resulting in severe pest control problems. These factors have encouraged the work on the development of alternative pest control strategies, such as those utilising semiochemicals.

## 1.4 *Alternative Strategies for Pest Control*

Semiochemicals can be used in a number of ways when designing alternative pest control strategies, which can be conveniently split into two categories - direct and indirect. Direct strategies use behaviour controlling chemicals to control pest populations, while

indirect strategies involve the monitoring of populations with the control agent either being a conventional insecticide or a biological agent.

Indirect strategies have so far been developed for a wide range of pests, principally using monitoring traps to gather information which can be used for a number of applications (see Table 1.1)[32]. The chemicals used in monitoring traps will be attractive to the insect pest of interest so, for example, will be a sex pheromone, an aggregation pheromone, or a plant derived kairomone.

**Table 1.1** Uses of monitoring traps

| Information | Application |
|---|---|
| Detection | Early-warning<br>Survey<br>Quarantine |
| "Threshold" | Timing of treatments<br>Timing of sampling<br>Risk assessment |
| Density estimation | Population trends<br>Dispersion<br>Risk assessment<br>Effects of control measures |

Information from monitoring traps can then be used to decide on such questions as the optimum time to spray with conventional insecticides, the need to spray, the effectiveness of control measures, and to survey the population of the particular insect of interest. However, even this information is of little use if it is not interpreted properly. Trap numbers have to be correlated with population numbers and density, climatic conditions must be known, and the range of activity of a particular trap type must be known. Despite these limitations, monitoring traps are of great use to the farmer and have been introduced and widely used for a large number of pest species[33].

Direct strategies have so far been limited in their success due to a number of factors, identified by Silverstein (see Table 1.2)[34].

**Table 1.2** Reasons for failure in direct control strategies

1. Inadequate knowledge of insect behaviour
2. Inadequate knowledge of chemical communication systems
3. High population density
4. Inadequate resources
5. Inadequate pheromone formulations
6. Improper distribution of traps or release sources
7. Invasion from outside the test area
8. Poor timing

The two main direct strategies in current commercial use are mass-trapping and mating disruption. As its name implies, mass-trapping involves attraction of the pest to a target and then killing the pest. The attraction can be caused by a number of lures, from chemical ones such as pheromones and kairomones, to visual ones such as colour and light. The killing mechanisms can be direct, such as trapping on a sticky or water trap, treatment with an insecticide or an electric grid: or indirect with control from a fungal pathogen, insect sterilization or lack of a suitable host plant leading to premature death. One problem associated with the use of sex pheromones as attractants in these systems is that they will usually attract the male of a species and although this may give short term control, enough males will often be left in the general population to mate with the

remaining females so resulting in no long term control. This is also true of other mass trapping strategies which still allow mating to take place. However in specific situations, such as the tsetse fly in southern Africa, the behaviour and biology of the species are such that direct control using semiochemicals is a practical proposition[35].

Mating disruption involves preventing mating from taking place, leading to a long term reduction in overall pest numbers. Typically this will be by preventing mate location by confusing the insect. This can be done using a number of strategies. Synthetic pheromones can disrupt the natural trail following of an insect. The natural pheromone plume can be camouflaged with an additional component, release of only one pheromone component can change the natural pheromone composition, and synthetic compounds can be used as pheromone mimics[36].

## 1.5 *Oilseed Rape*

Oilseed rape, *Brassica napus*, a member of the family Cruciferae(=Brassicaceae) was in 1994 the third largest by area, and the fourth largest by value, arable crop in the UK. It now occupies 496,000 ha (including 92,000 ha on set-aside land), which compares with an average of 262,000 ha in 1983-5, and very limited planting before 1979[37]. This rapid increase in oilseed rape production was started by the introduction of an EU policy to encourage oilseed production within the community, involving the payment of subsidies for oilseeds. The two main products from oilseed rape are derived from the seeds. These are a high quality vegetable oil ( ~ 40% w/w) which is obtained by crushing the seed, and a seed meal which has a high (40%) protein content.

There are five *Brassica* species that are cultivated worldwide as oilseeds. However,

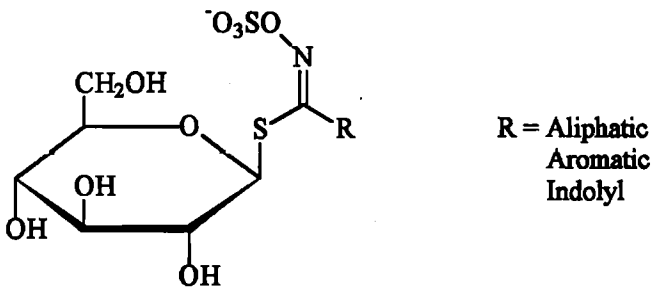
in Europe the crop grown is almost exclusively *Brassica napus*, swede rape. This is a cross derived from turnip rape, *Brassica rapa*, and *Brassica oleracea*. It is thought that *B. napus* originated in southern Europe, and from there spread to other parts of the world[38].

The oil in traditional varieties of *B. napus* contains a high quantity of long chain mono-unsaturated fatty acids, mainly erucic acid (C22:1) and eicosenoic acid (C20:1). However, in the 1940s doubts were raised about the nutritional properties of these long chain fatty acids and when oilseed production started to expand in Canada in the 1950's, efforts were started to reduce the quantity of these long chain fatty acids. Conventional plant breeding techniques, mainly by selection, enabled the production of the first low erucic acid rapeseed in 1968[38], and from 1973 onwards various governments and other governmental organisations have banned the production of high erucic acid varieties for food use. However, high erucic acid varieties are now being re-introduced with the erucic acid produced used as a feedstock for the fine chemicals industry[38,39].

The meal obtained from the seeds after extraction is recognised as high quality due to the high protein content and amino acid balance. However, the meal also contains anti-nutritional components, the glucosinolates, which are known to give Brassicas their distinctive flavour and taste, but have also been shown to be toxic[40]. This has led to the development of oilseed rape with a low glucosinolate concentration in the seed. As with the low erucic acid varieties it has now become compulsory to grow low glucosinolate varieties to obtain subsidies in the EU, resulting in the so-called "double-low" varieties, which have low erucic acid and low seed glucosinolate content.

# 1.6 *Glucosinolates*

Glucosinolates are a group of sulphur and nitrogen containing plant secondary metabolites found mainly, but not exclusively in the Brassicaceae. Over 80 different glucosinolates have been found to date, based on the general structure **(1.29)**[41-43]. Variations occur in the aglycone R-, based on three distinct groups; aliphatic, aromatic and indolyl, according to the amino acid from which the glucosinolate is derived.

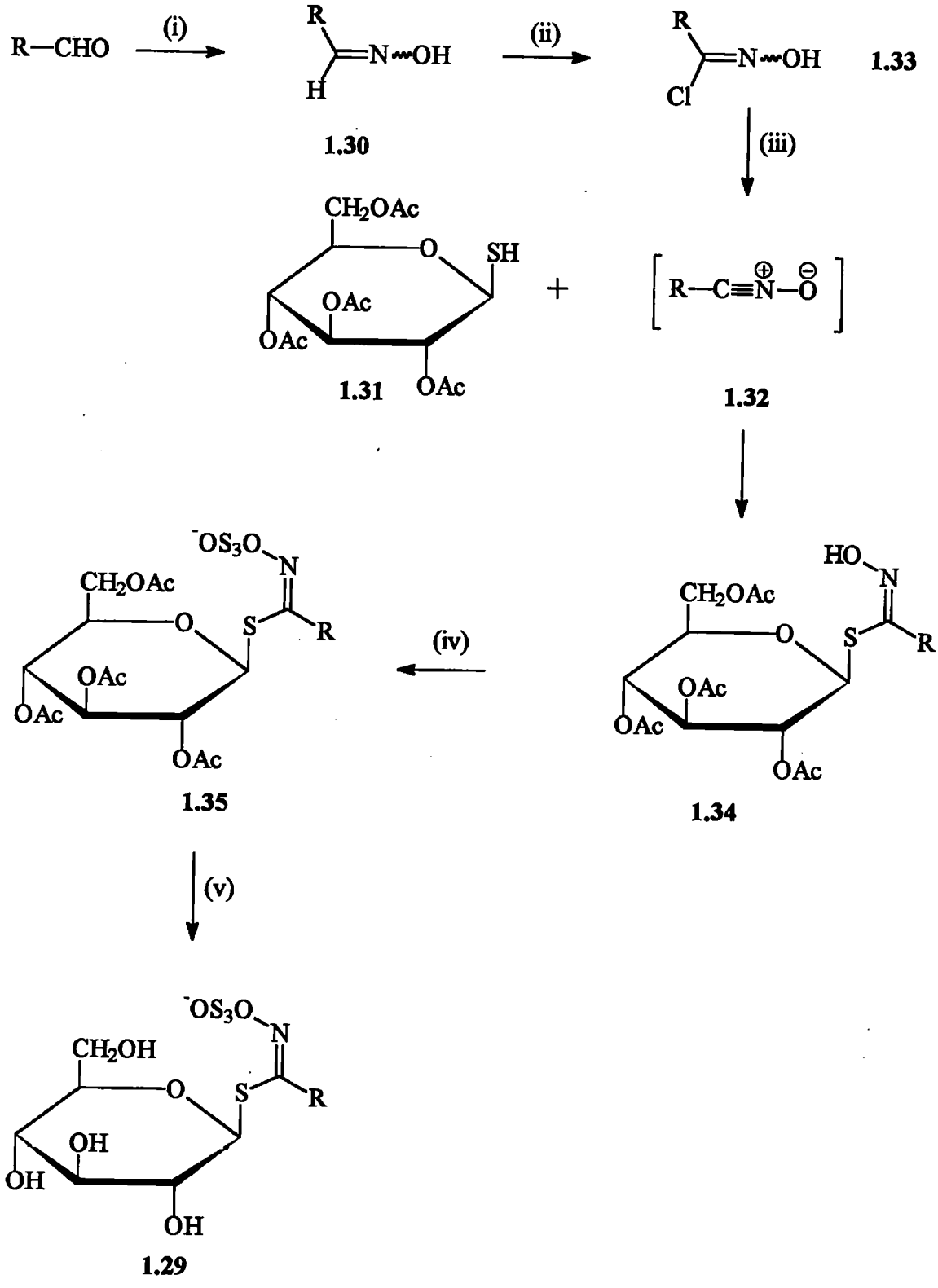



**1.29**

The structure was first proposed based on chemical studies on allylglucosinolate and *p*-hydroxybenzylglucosinolate in 1956[44]. The stereochemistry around the C=N double bond was originally proposed as the *Z* configuration, but this was not proven until 1970 when the X-ray structure for allylglucosinolate was published[45]. To date no further X-ray studies on other glucosinolates have been carried out, but they are all also believed to be the *Z* configuration, based on analogies with allylglucosinolate. The nomenclature of the different glucosinolates is a complex subject because of the use of trivial names given to the early glucosinolates which were isolated. These trivial names refer to both the glucosinolate anion plus the counter-ion, so for instance, sinigrin is potassium allylglucosinolate, while progoitrin is sodium (2*R*)-hydroxy-3-butenylglucosinolate, and in other cases the counter-ion is not specified. This leads to ambiguity when using trivial names, so the use of semi-systematic names derived from aglycone will be used throughout this thesis.

Glucosinolates are non volatile hydrophilic compounds, readily soluble in water, but not in organic solvents. They are stable at neutral pH, but may be hydrolysed by either strong acid or base[43]. Enzymic hydrolysis occurs in the plant when the glucosinolate comes into contact with the enzyme myrosinase (a thioglucosidase EC. 3.2.2.1) to cleave the C-S bond and give an unstable intermediate[46,47]. This will be discussed in detail in section 1.6.1.

Glucosinolates were first chemically synthesised in 1957[48]. The route has since been slightly modified, with the general route now used outlined in Scheme 1.1[49,50]. The key step is the coupling of the aldoxime **(1.30)** with tetraacetylthioglucose **(1.31)** which is accomplished via the highly unstable nitrile oxide intermediate **(1.32)**. This is formed *in situ* from chlorination of the aldoxime to form the hydroximoyl chloride **(1.33)**, and then dehydrochlorination with base to form the nitrile oxide, which undergoes 1,3-addition with tetraacetylthioglucose to give the tetraacetyldesulfoglucosinolate **(1.34)**. Treatment with pyridine-sulfur trioxide complex gives the tetraacetylglucosinolate **(1.35)**, which is converted to the glucosinolate **(1.29)** with methanolic ammonia. Synthesis of the indolyl glucosinolates proved more complicated and the first synthesis was not reported until 1990[51].



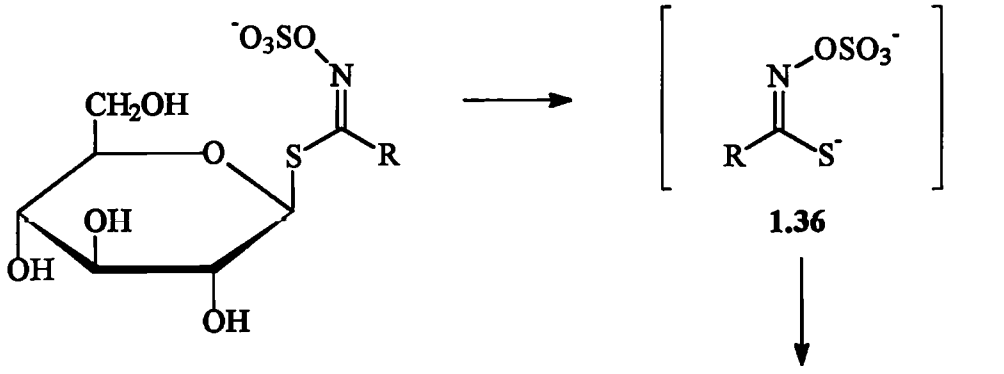

(i) $NH_2OH.HCl$, (ii) $Cl_2$ , (iii) $Et_3N$, (iv), $py.SO_3$ , (v) MeOH, $NH_3$ .

**Scheme 1.1** Synthesis of glucosinolates

Analysis of glucosinolates is a very important area of study, because of the importance of glucosinolate concentrations in seed, so much effort has been expended in order to devise reliable methods for analysis[52]. Analysis can take two forms: determination of total glucosinolate concentration, or determination of the concentration of individual glucosinolates. The total glucosinolate concentration is usually determined by extraction and then enzymic degradation of the glucosinolate, and analysis of one of the degradation products, usually glucose[52]. Individual glucosinolates are analysed as the desulfoglucosinolates by HPLC, following extraction and purification[53,54].

## 1.6.1 *Glucosinolate Catabolism*

As has been already mentioned, glucosinolates (**1.29**) can be hydrolysed by a naturally occurring plant enzyme, myrosinase. This hydrolysis gives glucose and an unstable intermediate (**1.36**) which can undergo a number of different rearrangements to give different products according to the physiological conditions in the plant.

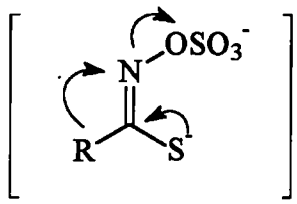



**1.29** **1.36**

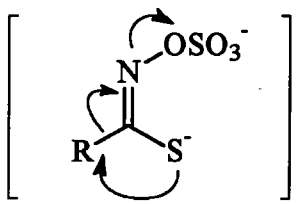
R—SCN R—NCS R—CN

**1.37** **1.18** **1.38**

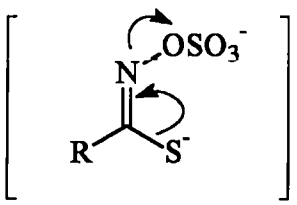
The main products under normal plant physiological conditions are the isothiocyanates (**1.18**) although other products such as thiocyanates (**1.37**) and nitriles (**1.38**) can also be formed. The isothiocyanates come from a Lossen rearrangement (1,2 shift) of the intermediate, while the other products come from different rearrangements as shown[47].




R—N=C=S **1.18**

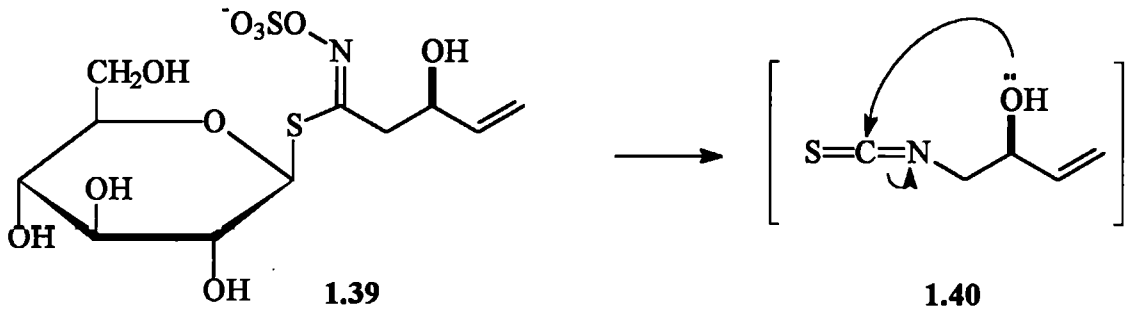



R—S—C≡N **1.37**

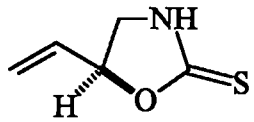



R—C≡N **1.38**

If the isothiocyanate formed contains a ß-hydroxy group it cyclises to give a oxazolidine-2-thione[43]. In the case of progoitrin (**1.39**) the unstable isothiocyanate (**1.40**) undergoes the cyclisation shown to give the product (*S*)-5-vinyloxazolidine-2-thione (**1.41**) (goitrin). This is a highly goitrogenous compound and plant breeders are attempting to lower its concentration in rapeseed meal because of its antinutritional properties[40,55]. When the aglycone is derived from tryptophan the isothiocyanate formed is unstable and decomposes to give indole-3-methanol[56,57].



1.39 1.40

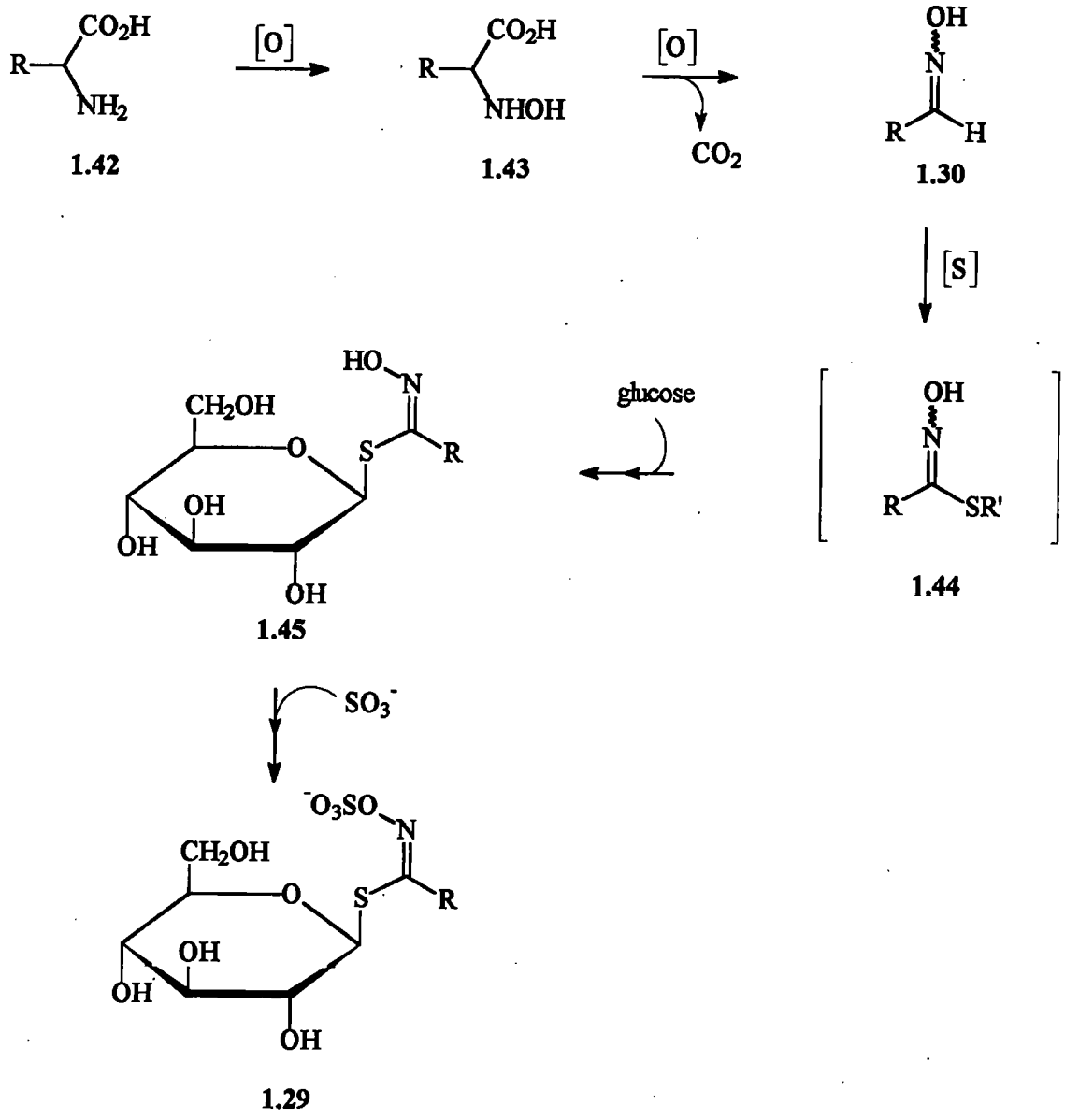


1.41

The toxicity of glucosinolates and their related breakdown products is a well studied area with investigations into occurrence, transfer through from cattle feed to milk, as well as the inherent toxicity of the compounds themselves. Some studies have examined possible anti-cancer properties for some of the breakdown products, based on the observed anti-cancer properties of a number of cruciferous vegetables[58,59].

## 1.6.2 *Glucosinolate Biosynthesis*

Glucosinolates are derived from both protein and, principally, non-protein amino acids as outlined in Scheme 1.2. The amino acid (**1.42**) is oxidised to the *N*-hydroxyamino acid (**1.43**), which then undergoes oxidative decarboxylation to give the aldoxime (**1.30**). This is then transformed to the glucosinolate (**1.29**) via a thiohydroximate intermediate (**1.44**) and the corresponding desulfoglucosinolate (**1.45**).




**Scheme 1.2** Biosynthesis of Glucosinolates

Amino acids were identified as the biogenetic precursors of the glucosinolates by studies using $^{14}C$ labelled amino acids in whole plant feeding experiments[60-62]. [$^{14}C$-1]Amino acids showed no incorporation into the glucosinolate, whereas amino acids labelled in other positions gave incorporation specific to the position of the label in the amino acid. For example [$^{14}C$-2]phenylalanine gave specific incorporation of the label in the thiohydroximate carbon of the glucosinolate. The nitrogen atom in the thiohydroximate group of the glucosinolate is also derived from the parent amino acid which was

demonstrated using double labelled [$^{14}C$,$^{15}N$]L-phenylalanine which was incorporated into glucotropaeolin [63] and double labelled [$^{14}C$,$^{15}N$]dihomomethionine which was incorporated into progoitrin[64].

The first intermediates to be identified on the pathway are the aldoximes (**1.30**). These were shown to be incorporated into glucosinolates, and a number of studies have examined different aldoximes and their subsequent incorporation into glucosinolates[65,66]. There is a change in the $^{14}C/^{15}N$ ratio in double labelled experiments, when racemic [$^{14}C$,$^{15}N$]amino acids are fed to whole plants, but this result is attributed to deamination of the unnatural D-amino acid and utilisation of the corresponding $\alpha$-keto acid in the pathway[64]. This change in ratio does not appear to occur for L-amino acids[63].

There must be at least one intermediate between the amino acids and the aldoximes. The obvious candidates are the *N*-hydroxyamino acids (**1.43**) and experiments with $^{14}C$-labelled *N*-hydroxyphenylalanine appear to confirm this hypothesis[67]. However, the experiments carried out also show evidence for chemical decomposition of the *N*-hydroxyamino acid to the aldoxime, but with increased rates of conversion when the enzyme system is present. The limitations of these results mean that the intermediacy of the *N*-hydroxyamino acids in glucosinolate biosynthesis has not been unequivocally proven. There is further evidence to support the hypothesis based on the related biosynthetic pathway for the cyanogenic glucosides[68]. In this pathway there is a conversion of an amino acid to an aldoxime, which has been shown to occur via the *N*-hydroxyamino acid. This apparent similarity has resulted in a number of papers linking the two pathways in a biosynthetic and evolutionary sense[69-72]. It has been suggested that the two pathways were originally the same, but have diverged at some point in the evolutionary past. This would mean that the enzymes responsible for the conversion of amino acids to aldoximes would be similar in both pathways. If this were the case these results would help in any studies on the glucosinolate biosynthetic pathway. However, care must be taken when making

analogies between apparently similar pathways, without evidence that the enzymes involved are also similar.

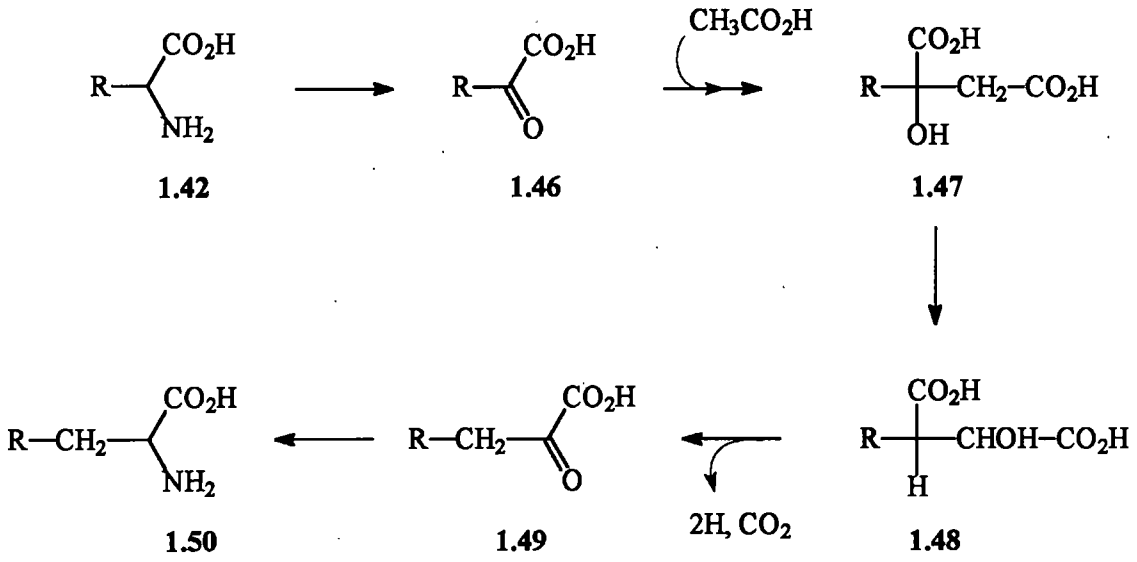
The conversion from aldoximes to glucosinolates has been shown to occur via thiohydroximates (**1.44**) and then desulfoglucosinolates (**1.45**). Labelled studies have shown incorporation of these two intermediates into the glucosinolates[73]. The enzyme responsible for the conversion of the thiohydroximate (**1.44**) and UDP-glucose into the desulfoglucosinolate has been identified for the benzylglucosinolate (**1.29**, R = $PhCH_2$-) system in *Tropaeolum majus*[74,75]. Other enzymes in different *Brassica* species have also been identified[76-78], and appear to show little specificity for the side chain of the thiohydroximate (**1.44**), suggesting a general enzyme for all glucosinolates[76].

The source of the sulfur in the thiohydroximate (**1.44**) is not clear, although it has been shown that it does not come from thioglucose, and there are strong suggestions that cysteine is the most likely sulfur donor[79]. No intermediates have yet been identified for the conversion from the aldoxime to the thiohydroximate, although some studies have suggested the involvement of nitro-compounds[80]. However, incorporation rates for these nitro compounds were very low, and it seems likely that this is only a minor part of the pathway.

The enzyme responsible for the conversion the desulfoglucosinolate (**1.45**) to the glucosinolate (**1.29**) has also been identified. This enzyme is a sulfotransferase which utilises the desulfoglucosinolate and 3'-phosphoadenosine-5'-phosphosulfate (PAPS), and produces the glucosinolate and adenosine-3',5'-diphosphate. Again this enzyme appears to show very little specificity for the side chain of the desulfoglucosinolate[81,82].

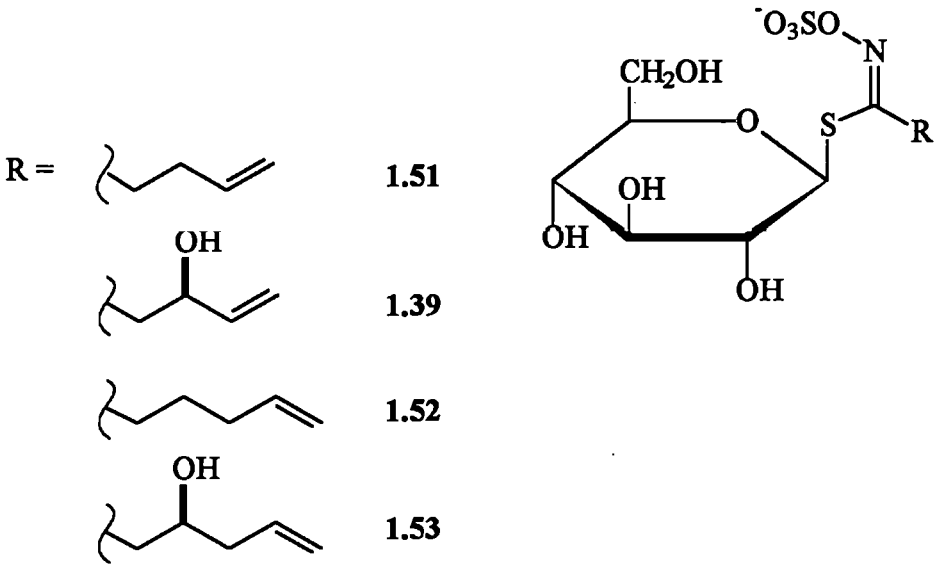
Glucosinolates (**1.29**) have been shown to be derived from amino acids (**1.42**). However a large number of the 80 or so glucosinolates which have been identified in plants are not directly from the 20 protein amino acids. There are two possible places on the pathway for alteration to form the wide range of glucosinolates identified. The first is

at the amino acid stage, and the second is at the glucosinolate stage. As already mentioned a large number of the glucosinolates are derived from non-protein amino acids. These are usually chain extended versions of protein amino acids, and some studies have looked at this chain extension process[83,84]. The pathway is considered to be that shown below, with the additional carbon atom coming from acetate, with loss of $CO_2$.




A transaminase acting on the amino acid (**1.42**) gives the $\alpha$-keto acid (**1.46**), which condenses with acetate to give **1.47**. Isomerisation gives the $\alpha$-hydroxy acid (**1.48**), which undergoes decarboxylation to give the chain extended $\alpha$-keto acid (**1.49**). A further transamination reaction then gives the chain extended amino acid (**1.50**).

Chain extended amino acids can also enter this pathway, allowing the formation of a whole homologous series of amino acids and hence glucosinolates. For example in *B. napus* the 3-butenyl (**1.51**) and 4-pentenylglucosinolates (**1.52**) are found, which are derived from chain extended methionine homologues.

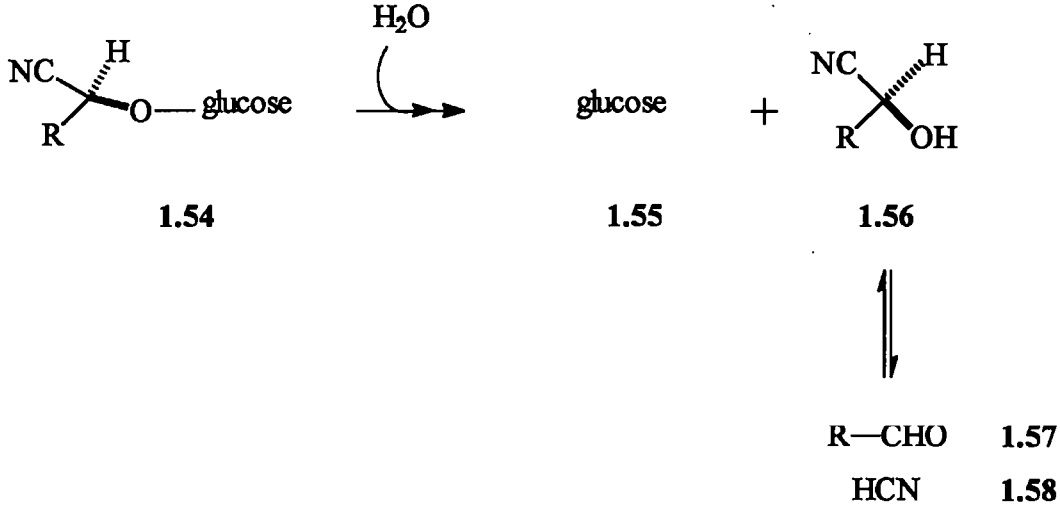



Other possible sites for alteration is also illustrated by these two glucosinolates, which are derived from methionine homologues, but contain no sulfur in the side chain of the final glucosinolate found in *B. napus*. These glucosinolates can be hydroxylated on the side chain to give (*R*)-2-hydroxy-3-butenylglucosinolate (**1.39**) and (*R*)-2-hydroxy-4-pentenylglucosinolate (**1.53**). This modification occurs late in the biosynthetic pathway, which was shown by examining the incorporation of labelled 3-butenylglucosinolate[85,86]. Other modifications known to occur on the alkyl side chain in different plants include oxidation, particularly at the sulfur atom of the side chain, hydroxylation, and elimination of the terminal methanethiol group to leave a double bond.

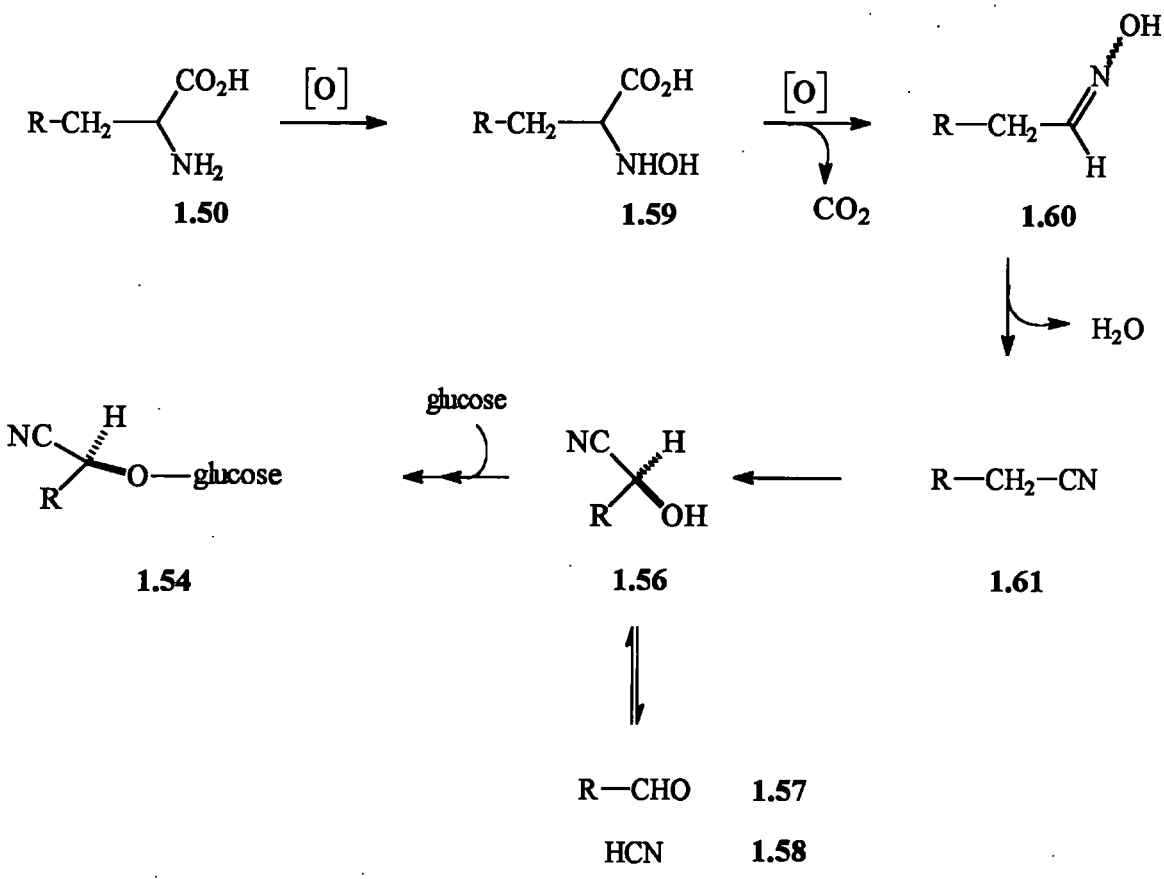
## 1.7 *Cyanogenic Glycosides*

Cyanogenic glycosides are a group of plant secondary metabolites, which like glucosinolates act in defence of the plant by releasing biocidal compounds during tissue

damage. They are of the general structure (**1.54**) and act by release of hydrogen cyanide following plant damage[68]. The structure consists of a sugar moiety, which is normally glucose, linked to a hydroxynitrile aglycone. On damage the sugar linkage is hydrolysed by a ß-glucosidase enzyme, to give glucose (**1.55**) and a hydroxynitrile (**1.56**). The hydroxynitrile then dissociates to give an aldehyde (**1.57**) and hydrogen cyanide (**1.58**). This dissociation process can happen either chemically or enzymically, catalysed by a hydroxynitrile lyase (oxynitrilase)[68].



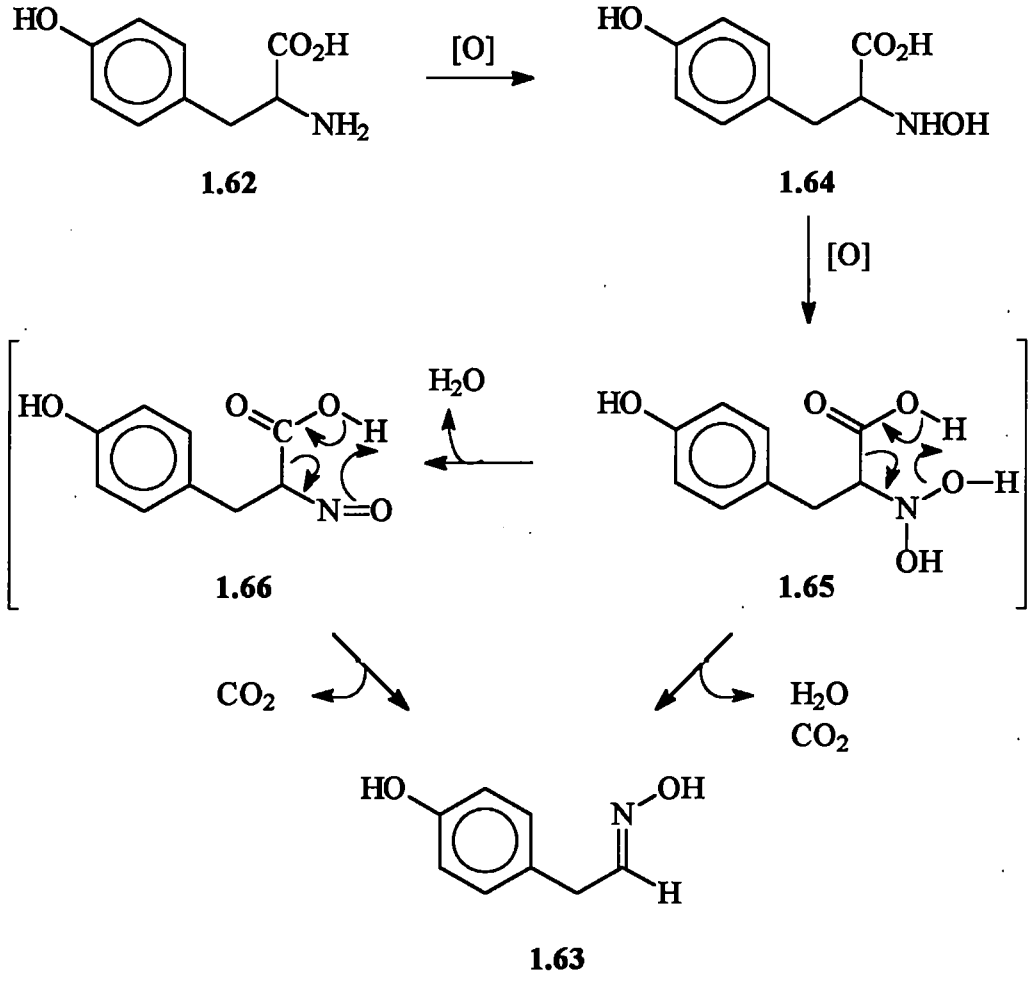

There are a number of similarities between glucosinolates and cyanogenic glycosides, based on function, catabolism and biosynthesis. Both groups of compounds act as plant defence compounds against herbivores, and both accomplish this by enzymic cleavage of a sugar to give an unstable intermediate, which rearranges to give the toxiphore. The two biosynthetic pathways also show marked similarities, with both groups being derived from amino acids via an aldoxime intermediate. The biosynthetic pathway for cyanogenic glycosides is shown in Scheme 1.3.




**Scheme 1.3** Biosynthesis of Cyanogenic Glycosides

This apparent similarity with the glucosinolate biosynthetic pathway has led to the idea that the two pathways which share common intermediates, may also share common enzymes for the first part of the pathways, before divergence after aldoxime formation. The enzymes responsible for the formation of aldoximes in cyanogenic glycosides have been identified as membrane bound cytochromes P450[87]. The enzyme responsible for the transformation of tyrosine to *p*-hydroxyphenylacetaldoxime in *Sorghum bicolor* (L.) Moench has recently been purified and characterised[88,89], and the gene encoding for it has been sequenced[90] and expressed in *E. coli*[91]. The mechanism for the oxidative decarboxylation of tyrosine (**1.62**) to its corresponding aldoxime (**1.63**) has been shown to proceed via two successive *N*-hydroxylation reactions[89,90]. These occur at the same

active site on the enzyme. Formation of *N*-hydroxytyrosine (**1.64**) is followed by a second *N*-hydroxylation reaction to form **1.65**. This is followed by loss of water and carbon dioxide to form the aldoxime (**1.63**), possibly via the nitroso acid (**1.66**), although the mechanism could involve a concerted reaction from **1.65**.




Early studies showed that the hydrogen at C-2 is retained during the oxidative decarboxylation reaction, but this does not give any information as to whether the reaction takes place via **1.65** or **1.66**[70].

It has been reasoned that similar enzymes may be involved in glucosinolate biosynthesis. This assumption is based on the common starting point and the common intermediates of the *N*-hydroxyamino acids and the aldoximes. To date their has been no

data to support this hypothesis other than the shared intermediates. However, the recent work on the early steps in the biosynthetic pathway for the cyanogenic glycosides, particularly dhurrin in *Sorghum bicolor* has identified intermediates involved in that pathway and this may serve as a useful starting point for examining the early steps in glucosinolate biosynthesis[70-72].

## 1.8 *Strategies for Pest Control in* B. napus

This thesis is part of a major project at IACR-Rothamsted which aims to improve strategies for pest control in oilseed rape, based on semiochemicals and other natural control mechanisms, such as insect pathogenic fungi and parasites[92]. The strategies will be based on knowledge obtained about the basic biology and chemically mediated behaviour of the insect pest species of oilseed rape, and integrated with the natural control mechanisms mentioned. The overall strategy will be based on a stimulo-deterrent diversionary strategy (SDDS) or "push-pull" strategy[12]. Here the aim is to repel the insect away from the main part of the crop and attract it to a small "trap" part of the crop where the pest can be controlled more effectively using either conventional pest control agents, or natural control strategies developed from other work in the project. The "push" and the "pull" part of the strategy will be provided mainly by behaviour controlling chemicals identified in other parts of the project. This work has shown a wide range of chemicals to be of interest, but particularly the isothiocyanates, which have shown activity at electrophysiological, behavioural and field levels[93-95]. The results show that specialist insect pests of oilseed rape, which only feed on crucifers and not other crop plants, are attracted by isothiocyanates, but non-specialist generalist pests are repelled[96-98]. Also of interest is

the discovery that a number of insects possess cells which contain specific isothiocyanate receptors and cells which respond to particular classes of isothiocyanates (alkenyl or aromatic)[23]. This opens up the possibility that the different classes of isothiocyanates play different behavioural roles for the insect. Exploitation of this possible difference may allow development of oilseed rape cultivars which are either considerably more, or less, attractive to the insect and allow the development of a "push-pull" strategy. This would require oilseed rape cultivars with altered glucosinolate profiles, which may be achieved either with conventional plant breeding techniques, or by use of genetic engineering to manipulate the genes of interest[99,100]. Genetic engineering has the advantage of being a highly targeted method with a higher likelihood of success. The use of genetic engineering requires detailed knowledge of the biosynthetic pathway, and its regulation, to allow identification of the genes of interest.

## 1.9 *Objectives*

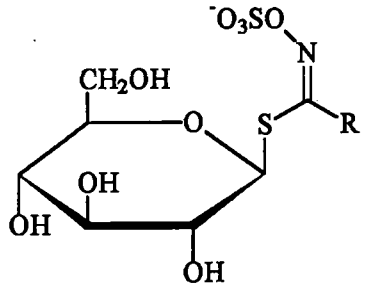
The main objectives of this study are to provide the chemical expertise to examine the role of glucosinolates involved in the insect-plant interactions, and to develop the chemistry required to allow detailed study of the biosynthetic pathway of these glucosinolates in *B. napus*. This will mainly focus on the early part of the biosynthetic pathway as the area most likely to be involved in regulation of the pathway. It will involve chemical synthesis, particularly of radiolabelled substrates, to facilitate study of the enzymes involved in the early part of the pathway, as well as synthesis of a wide range of possible intermediates and other compounds of interest for use in determining the activity of the enzymes involved. Analysis of products from enzymic reactions using

chromatographic and spectroscopic techniques will be used to determine and confirm the presence or absence of various intermediates. This work should then facilitate the identification of the enzymes involved in the key steps of glucosinolate biosynthesis and lead to the identification of the genes involved in their expression. Synthesis of glucosinolate catabolites will also be required to test their effects in the laboratory, and in field experiments. This should then allow the development of oilseed rape cultivars suitable for use in an alternative crop protection strategy using the ideas outlined for alternative methods of pest control.

# 2 RESULTS AND DISCUSSION

# 2.1 *Chemical Studies on Aldoxime Formation*

The glucosinolates (**2.1**) comprise three distinct groups, based on the nature of the aglycone, R, which is determined by the amino acid from which the glucosinolate is derived. The three distinct groups are aliphatic, derived from methionine homologues, aromatic from phenylalanine homologues and derivatives, and indolyl from tryptophan and derivatives.




R = Aliphatic
Aromatic
Indolyl

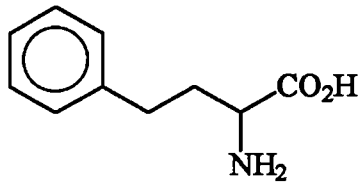
**2.1**

The precursor amino acid may be modified before entering the biosynthetic pathway, and this modification typically takes the form of chain extension of the amino acid[84]. For this study two amino acids were chosen, one aliphatic and one aromatic, because of evidence suggesting that glucosinolates, and more particularly the isothiocyanate catabolites, with different R groups have different biological activities in both plant-insect and plant-pathogen interactions[23,24,101-104]. This provides interesting possibilities for genetic manipulation of the plants, if the different classes of glucosinolates have different enzymes responsible for their biosynthesis[99].

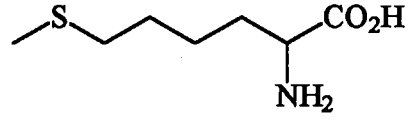
The aromatic amino acid chosen was homophenylalanine (2-amino-4-phenylbutanoic acid)[105] (**2.2**) which gives the 2-phenylethylglucosinolate (gluconasturtiin) (**2.1**, R = $PhCH_2CH_2$-)[83]. The first step in the biosynthesis of 2-phenylethylglucosinolate is a one

carbon homologation of phenylalanine to homophenylalanine. For studies on the aliphatic system the amino acid chosen was dihomomethionine (2-amino-6-methylthiohexanoic acid) (**2.3**), which is the biogenetic precursor of 3-butenylglucosinolate (gluconapin) (**1.51**) and (*R*)-2-hydroxy-3-butenylglucosinolate (progoitrin) (**1.39**)[64,86]. Dihomomethionine is derived from methionine in what is thought to be two separate one carbon homologation reactions.

The stereochemistry at C-2 of the amino acid is thought to be the natural L-form. Problems associated with the requirement for a single enantiomer will be discussed later. Since homophenylalanine (**2.2**) and dihomomethionine (**2.3**) are both non-protein amino acids any interference from primary amino acid metabolism is minimised. The glucosinolate products are also present in large amounts in *B napus*, and have been shown to accumulate rapidly in young, expanding, green leaves[54]. This should make the isolation of enzymes associated with their biogenesis should prove more straightforward than with other less common glucosinolates.

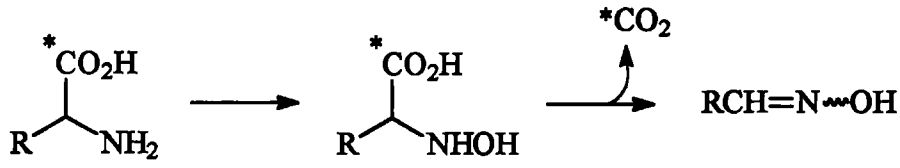



**2.2**




**2.3**

After the chain extension process, the amino acid (**2.4**) undergoes oxidation to the *N*-hydroxyamino acid (**2.5**), followed by oxidative decarboxylation to give the aldoxime (**2.6**). The oxidative decarboxylation results in the loss of the C-1 carbon of the parent amino acid as carbon dioxide.



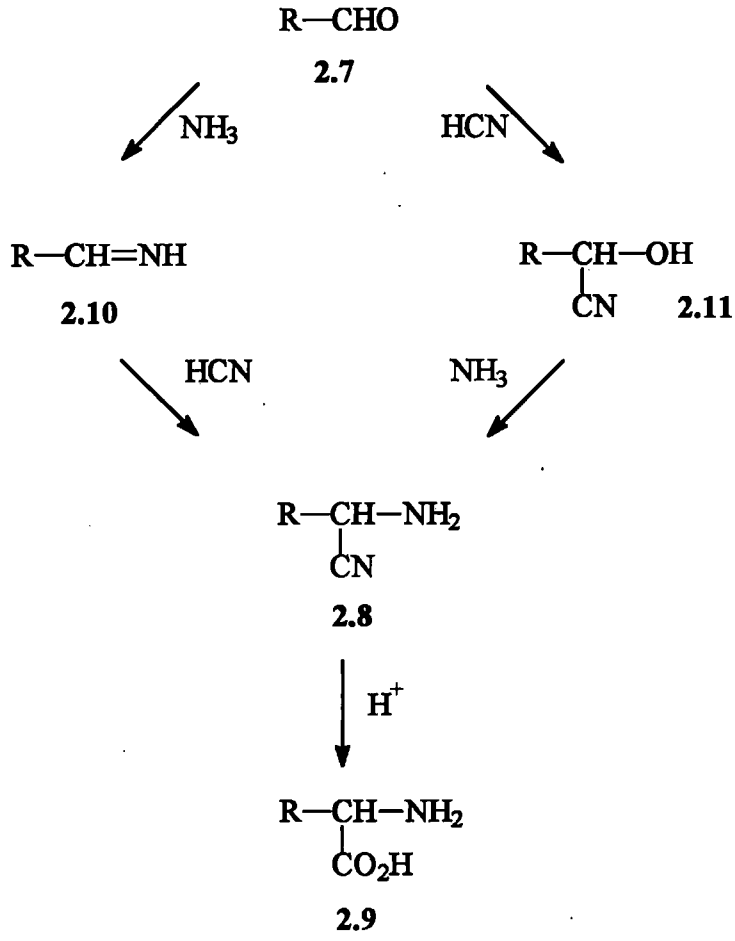

**2.4** **2.5** **2.6**

This is analogous to the initial stages in the biosynthesis of cyanogenic glycosides, where amino acids are also oxidatively decarboxylated to give aldoximes. There are other analogous reactions whereby amino acids can be oxidatively decarboxylated chemically with a variety of oxidising agents, usually to give acids, but sometimes oximes[106]. In the biosynthetic system the loss of carbon dioxide could be measured as radioactive $^{14}CO_2$, from the correspondingly labelled [$^{14}$C-1]amino acid, and used to assay for enzyme activity. Incubation of the amino acids with cell free preparations from *B. napus* leaves would work as a part of allow a collaborative investigation into the enzymes involved in the oxidative decarboxylation of amino acids to aldoximes. Therefore non-protein [$^{14}$C-1]amino acids were synthesised as potential substrates to assay for the enzymes likely to be involved.

## 2.1.1 *Synthesis of [$^{14}$C-1]Amino Acids*

For the initial investigations involving the biosynthesis of aromatic and aliphatic glucosinolates, both unlabelled and [$^{14}$C-1] labelled amino acids were required. Many routes are available for synthesis of amino acids, but the route chosen was the Strecker amino acid synthesis. This utilises cyanide as the source of the C-1 carbon and thereby provides a convenient method of introducing the $^{14}$C label. Other advantages are that the

route has applicability to a wide range of amino acids, and that the main starting material is the aldehyde which can also be used in the synthesis of the corresponding aldoxime. The Strecker synthesis was first developed in 1850, but has been modified from the original method to give a versatile and efficient route from an aldehyde (**2.7**) starting material. The aldehyde reacts with ammonia and cyanide to give the aminonitrile (**2.8**) which is then hydrolysed in dilute acid to give the amino acid (**2.9**). It is not known whether the pathway proceeds via the imine (**2.10**) or the cyanohydrin (**2.11**)[107].




One obvious problem with the synthesis is that the resultant amino acid is racemic and the enzymes associated with glucosinolate biosynthesis are likely to require the L-enantiomer. This problem can be overcome or avoided in a number of ways.

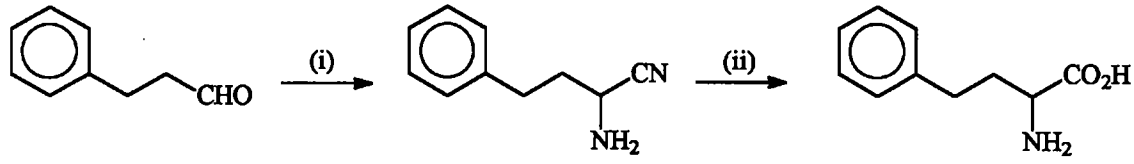
1. The Strecker approach can be adapted to give stereospecific products[108]. However, this may cause problems on the small scale required for the radiolabelled synthesis. Also the e.e.'s obtained may not be sufficiently high to justify the effort involved.

2. The resulting racemic mixture from the Strecker synthesis can be resolved into *(R)* and *(S)* enantiomers using stereospecific enzyme reactions which have been used to separate a wide range of amino acids[109-111]. This would give the separate enantiomers for separate assays.

3. The problem may be by-passed if the unnatural form shows no inhibitory activity to the enzymes involved. Although this would result in the non-utilisation of half the $^{14}C$ label, but would require no potentially difficult separation steps. It would also be possible to determine which isomer was being utilised in the pathway with both isomers present, by using D- and L- amino acid oxidases. Incubation of the racemic mixture with one of the amino acid oxidases effectively destroys one or other of the isomers. Then this mixture now containing only one amino acid enantiomer can be used to assay for activity, thus determining which enantiomer is required by the enzyme.

It was decided to use the racemic Strecker synthesis, due to the likely problems in terms of yield losses associated with 1. and 2., as this should maximise yields, particularly for the radiolabelled syntheses. This could then be adapted if any problems were found with inhibition by one or other of the isomers. D- and L- Amino acid oxidases would then be used to determine the required stereospecificity.

The aromatic series required the synthesis of 2-amino-4-phenylbutanoic acid (homophenylalanine, HPhe) **(2.2)**, from the aldehyde 3-phenylpropanal (hydrocinnamaldehyde) **(2.12)**, which is readily available. This was used in the Strecker amino acid synthesis as follows.

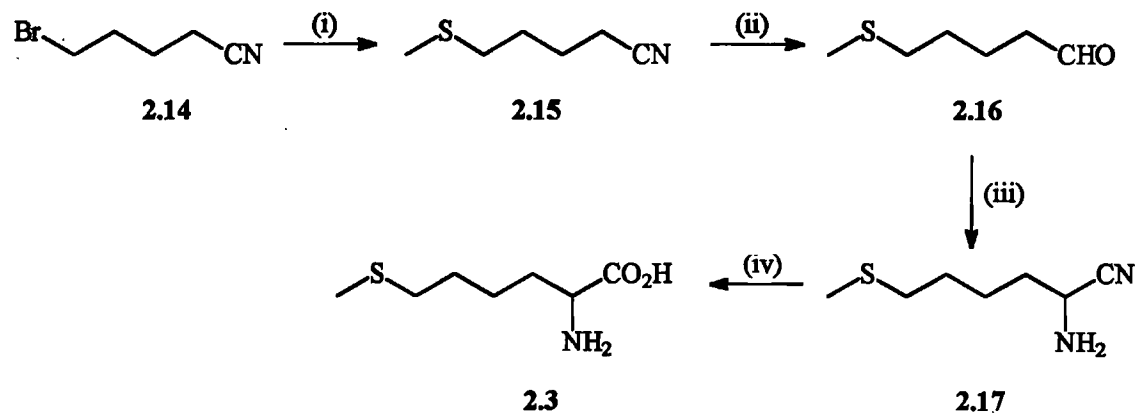



**2.12** **2.13** **2.2**

(i) KCN, $NH_4Cl$, $NH_3$(aq), EtOH, $H_2O$, (ii) a) HCl (conc.), b) $H^+$(aq).

This reaction demonstrates the general nature of the Strecker synthesis and it is widely used throughout this thesis. Nucleophilic attack of ammonia and cyanide ion on the aldehyde generates the aminonitrile (**2.13**). The aminonitrile was then purified by extraction into an organic solvent, extraction into acid, basification of the acid fraction and back extraction into diethyl ether. This gave the pure aminonitrile (**2.13**) in reasonable yield (63%). Hydrolysis of the nitrile to give the amino acid (**2.2**) was carried out by treatment with conc. hydrochloric acid, usually overnight, followed by addition of water, and heating to reflux for 5-6 hours. The hydrolysis step gave almost quantitative yields. If further purification was required this was carried out using an quaternary ammonium salt ion exchange resin, which bound the carboxylic acid group. This resin was then eluted with dilute hydrochloric acid and the eluate concentrated to give the amino acid (**2.2**) as its hydrochloride. It was not usually necessary to isolate the zwitterionic form of the amino acid, as the amino acids required were for assays in buffer, with a low concentration of the amino acid. The free amino acid could be isolated (if required) by treatment of a solution of the amino acid hydrochloride with aqueous ammonia to pH 7, and subsequent filtration of the precipitate.

The aliphatic series required the amino acid, 2-amino-6-methylthiohexanoic acid (dihomomethionine, DHMet) (**2.3**). The corresponding aliphatic aldehyde was not commercially available and was made from the readily available ω-bromonitrile (**2.14**) by the route below, and then used in the Strecker synthesis.



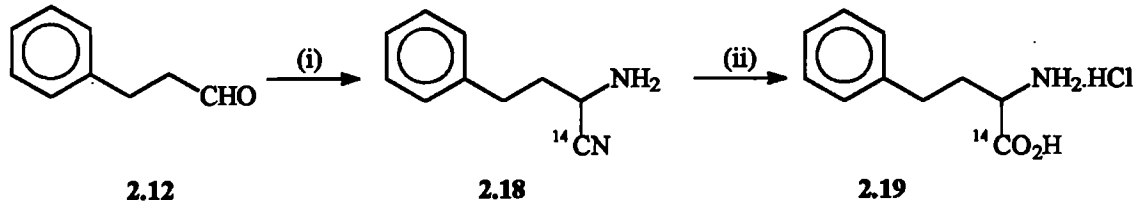

**2.14** **2.15** **2.16** **2.17** **2.3**

(i) $MeS^-$ $Na^+$, MeOH, (ii) a) DIBAL, $Et_2O$, -78°C, b) $H^+$(aq), (iii) KCN, $NH_4Cl$, $NH_3$(aq), EtOH, $H_2O$, (iv) a) HCl (conc.), b) $H^+$(aq) c) pyr.

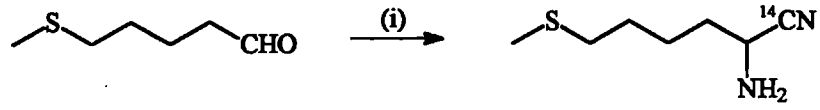
This route to synthesise the required aldehyde shows a large degree of flexibility and allows modifications at all stages, such as chain length of the starting material, and in the choice of nucleophile. The addition of methanethiol to the ω-bromonitrile (**2.14**) proceeded in good yield (75%) using a slight excess of the nucleophile. The resulting 5-methylthiopentanenitrile (**2.15**) was reduced to the corresponding aldehyde (**2.16**) in reasonable yield (64%) using DIBAL with acidic workup. The aldehyde was used immediately, or distilled for storage. Pure aldehyde was stable indefinitely at low temperature, however, impure aldehyde quickly decomposed. Once the pure aldehyde was available the Strecker synthesis was carried out, firstly giving the aminonitrile (**2.17**), which was then hydrolysed to give the amino acid (**2.3**) as its hydrochloride in the same manner as for homophenylalanine. The yield obtained for the two steps from the aminonitrile was 70%.

The two syntheses were then adapted for the small scale synthesis of the corresponding [$^{14}$C-1]amino acids. Typically this involved the use of less than 5mg of aldehyde and 1-2mg of potassium cyanide. The introduction of the label was accomplished using $^{14}$C potassium cyanide with a high specific activity (50-60mCi/mmol). The [$^{14}$C-

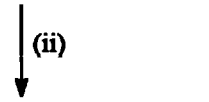
1]aminonitriles (**2.18, 2.20**) were purified by acid/base extraction as before, then hydrolysed to give the [$^{14}$C-1]amino acids. These were then purified by ion exchange chromatography to give the [$^{14}$C-1]amino acids as their hydrochlorides. The synthesis of [$^{14}$C-1]homophenylalanine (**2.19**) was accomplished with a radiochemical yield, based on potassium cyanide, of 44% and the synthesis of [$^{14}$C-1]dihomomethionine (**2.21**) gave a radiochemical yield of 19%.

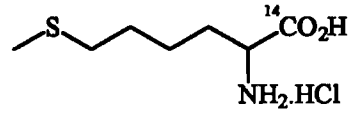



**2.12** **2.18** **2.19**




**2.16** **2.20**




**2.21**

(i) K$^{14}$CN, $NH_4Cl$, $NH_3$(aq), EtOH, $H_2O$, (ii) a) HCl (conc.), b) $H^+$(aq).

This gave [$^{14}$C-1]amino acids which were used as highly sensitive probes to search for the activity of the enzymes responsible for the initial stages of aromatic and aliphatic glucosinolate biosynthesis. The aldoxime forming enzyme systems involved in cyanogenic glycoside biosynthesis in cassava and sorghum, and a different enzyme responsible for indolacetaldoxime synthesis in Chinese cabbage, are all membrane bound[87,112,113].

Microsomes were prepared to search for comparable enzyme systems that might be involved in glucosinolate biosynthesis in *B. napus*. Using [$^{14}$C-1]homophenylalanine (**2.19**) and [$^{14}$C-1]dihomomethionine (**2.21**) as probes, cell free microsomal preparations were obtained from young *B. napus* leaves which catalysed the oxidative decarboxylation of these two non-protein amino acids[95,114]. These assays showed a release of 200-400 nmol of $CO_2$ released per hour per gram fresh weight of leaf material. The enzymes responsible were shown to be NADPH dependent, and did not require other cofactors such as ATP, Mg.ATP, CoA, pyridoxal phosphate or NADH. Competition assays using labelled and unlabelled substrates are given in Table 2.1. [$^{14}$C-1]Amino acid was incubated with a large excess of the other unlabelled amino acid, and the effect on the release of $^{14}CO_2$ monitored. There was no effect on the release of $^{14}CO_2$ by addition of the other unlabelled amino acid. These data clearly showed that there were two separate enzyme systems responsible for the aromatic and aliphatic systems.

**Table 2.1** The effect of HPhe and DHMet on $^{14}CO_2$ release from [$^{14}$C-1]dihomomethionine and from [$^{14}$C-1]homophenylalanine

| Addition | HPhe Activity % | DHMet Activity % |
|---|---|---|
| None | 100 | 100 |
| HPhe | - | 104 |
| DHMet | 119 | - |

The stereochemistry of the amino acid substrate in the biosynthetic pathway was determined by preincubation of the amino acid with D- and L-amino acid oxidases, as shown in Table 2.2.

**Table 2.2** The effect of D- and L- amino acid oxidases on $^{14}CO_2$ release from [$^{14}$C-1]dihomomethionine

| Preincubation treatment | Activity % |
|---|---|
| None | 100 |
| L-Amino acid oxidase | 5 |
| D-amino acid oxidase | 101 |

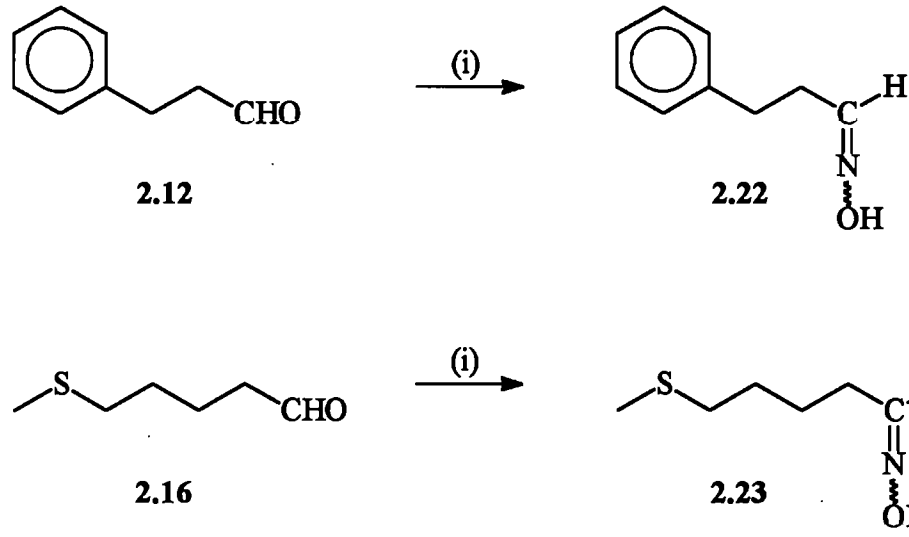
Preincubation of [$^{14}$C-1]dihomomethionine (**2.21**) with D-amino acid oxidase or L-amino acid oxidase and subsequent incubation with rape leaf microsomes showed loss of activity only with L-amino acid oxidase. This showed that the enzyme(s) involved for the oxidative decarboxylation are specific for L-amino acids, as would be expected. Also there is no increase in activity following treatment with D-amino acid oxidase, suggesting that the D-amino acid does not act as a competitive inhibitor for the release of $^{14}CO_2$ from L-[$^{14}$C-1]dihomomethionine. This means that the use of the racemic Strecker synthesis should have no adverse effects in further experiments as there appeared to be no inhibition from the inactive enantiomer, unless there was inhibition with subsequent enzymes in the pathway, although this is unlikely.

## 2.1.2 *Aldoxime Isolation and Characterisation*

The next step after isolation of the microsomal preparation was to identify the products of the oxidative decarboxylation of the two amino acids. These should be the aldoximes corresponding to the starting aldehyde used in the Strecker amino acid synthesis.

Aldoximes have been suggested as intermediates in the glucosinolate biosynthetic pathway[65,66], but not properly isolated and fully characterised, and thus confirmed as

intermediates on the pathway. Characterisation required the synthesis of the two aldoximes, which could be conveniently obtained by the reaction of the corresponding aldehydes with hydroxylamine hydrochloride in aqueous ethanol. The aromatic aldoxime, 3-phenylpropanaldoxime (**2.22**) was obtained in good yield (81%) from 3-phenylpropanal, and similarly the aliphatic aldoxime, 5-methylthiopentanaldoxime (**2.23**) was obtained in good (77%) yield from the aldehyde (**2.16**) synthesised earlier.




(i) $NH_2OH.HCl$, $NaHCO_3$, EtOH, $H_2O$.

For both the aromatic (**2.22**) and aliphatic (**2.23**) systems this gave a mixture of both the (*E*)- and the (*Z*)-isomers of the aldoxime, which were separable by capillary GC, and identifiable by $^1H$ and $^{13}C$ NMR and mass spectrometry. GC analysis of the aldoximes required careful control of the analysis conditions. This was because the aldoximes are unstable at the high temperatures typically found in GC injector systems and undergo loss of water to give the corresponding nitrile. The degree of decomposition on a split/splitless injector is temperature dependent, as shown in Table 2.3, so a low inlet temperature was chosen to minimise decomposition while still allowing the aldoxime to get onto the

column.

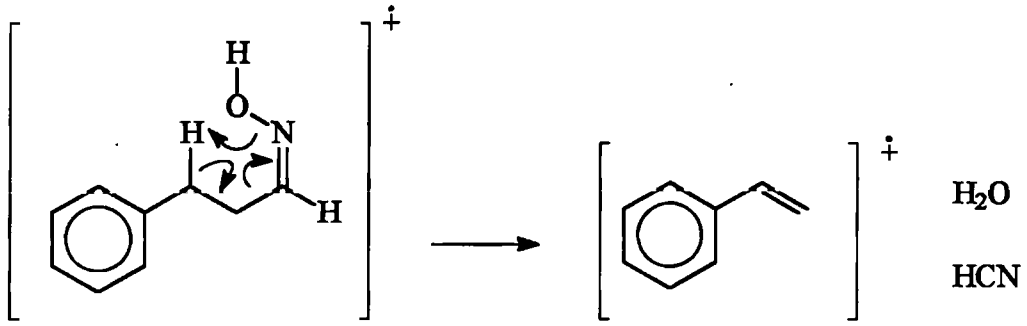
**Table 2.3** Effect of temperature on decomposition of 3-phenylpropanaldoxime on a split/splitless injection system

| Inlet Temperature °C | Nitrile Area % | (*E*)-Aldoxime Area % | (*Z*)-Aldoxime Area % |
|---|---|---|---|
| 100 | 0 | 8.2 | 91.8 |
| 125 | 0 | 8.1 | 91.9 |
| 150 | 2.9 | 9.2 | 87.9 |
| 200 | 5.7 | 7.7 | 86.6 |
| 250 | 15.9 | 3.7 | 76.9 |

The use of a temperature programmed or cold on-column injector overcomes this decomposition problem altogether, but another problem does arise, that of injecting impure extracts directly onto the GC column. The use of a short length of guard column does help alleviate this problem but this does result in extra GC maintenance, requiring regular changing of the guard column. The two isomers could be readily separated on both non-polar (eg. HP-1) and medium polarity (eg. SPB-35) columns. In this study both GC injection methods and both types of columns were used in analysing the results of incubations of amino acids with microsomal preparations from *B. napus*.

The (*Z*)-isomer of 3-phenylpropanaldoxime (**2.22**) was isolated by slow recrystallisation. This was the later eluting peak by GC and was identified by comparison with $^{1}H$ and $^{13}C$ NMR data from similar oxime ethers. In the $^{1}H$ NMR spectrum the $\alpha$-H of the (*E*)-isomer was shifted 0.7ppm downfield compared to the (*Z*)-isomer. The $^{13}C$ NMR spectrum shows the shift of C-2 in the (*E*)-isomer was also downfield, by 4.8ppm compared to the (*Z*)-isomer. These shifts are due to steric compression in the (*Z*)-

isomer[115,116]. This assignment agreed with the mass spectrum which showed a greater intensity at m/z 104 in the (*Z*)-isomer. This was due to a stereochemically favoured rearrangement, forming the styrene radical ion, which occurred to a lesser extent in the (*E*)-isomer. This isomer underwent normal homolytic cleavage to give the predominant ion m/z 105. Both isomers gave spectra which included loss of water ($M^+$-$H_2O$) to give the nitrile radical ion (m/z 131), with the base peak from the tropylium radical ion at m/z 91.




m/z 149 m/z 104

The aliphatic aldoxime, 5-methylthiopentanaldoxime (**2.23**) gave similar $^1H$ and $^{13}C$ NMR results to the aromatic system. There was a 0.7ppm shift of the $\alpha$-H in the $^1H$ NMR spectrum and a shift of ~4ppm of C-2 in the $^{13}C$ NMR spectrum. The mass spectra of the two isomers were very similar with no equivalent rearrangement to that seen in the aromatic system. These data showed a small $M^+$ ion (m/z 147) as well as a loss of water ($M^+$-$H_2O$) to give the nitrile radical ion (m/z 129), with the base peak at m/z 61 from $CH_3-S^+=CH_2$.

The full characterisation of the aldoximes was required for unequivocal confirmation of the presence of any of the aldoximes in the microsomal preparations after feeding with unlabelled amino acid precursors. To analyse for aldoxime formation from an amino acid, the unlabelled homophenylalanine (**2.2**) or dihomomethionine (**2.3**) was incubated with the microsomal preparation which had been shown to catalyse the NADPH

dependent loss of $^{14}CO_2$ from [$^{14}$C-1]homophenylalanine and [$^{14}$C-1]dihomomethionine. After a fixed time period (30-90 minutes) the incubation was stopped by the addition of dilute acid and extraction with diethyl ether/hexane (50/50). This was chosen as an extraction system as tests showed that this solvent system could extract the aldoxime efficiently while minimising the problem of emulsification found with more polar solvent systems, such as dichloromethane or 100% diethyl ether. The organic layer was then dried and concentrated slowly under a stream of dry nitrogen to minimise any losses from evaporation of the small quantities of aldoxime present, and to minimise the risk of oxidation of the samples. This was then analysed by GC and GC-MS. GC analysis was chosen in preference to HPLC because of the increased sensitivity and resolving power obtainable in these systems.

Incubation of homophenylalanine with the microsomal preparation in the presence of NADPH gave two peaks with retention times corresponding to the two aldoxime isomers. Identification was confirmed using GC-MS analysis and coinjection of the sample with synthetic 3-phenylpropanaldoxime (**2.22**) gave enhancement of the GC peaks with no peak broadening. This was the first unequivocal identification of an aldoxime from a glucosinolate producing plant, and as such is important in understanding glucosinolate biosynthesis. The aldoxime was isolated as a mixture of the two isomers but this was likely to be because of chemical equilibration during the incubation, extraction or analysis procedures, rather than because the plant produced a mixture of the two isomers. Glucosinolates only exist as a single isomer, so it is likely that a similar situation exists for the aldoximes, their biogenetic precursors.

Incubation of dihomomethionine (**2.3**) with the microsomal preparation did not show any production of the 5-methylthiopentanaldoxime (**2.23**). GC-MS mass fragmentography studies did show the base peak m/z 61 giving a peak at the correct retention time for the (*E*)-isomer, but this alone was not enough evidence for aldoxime

formation. Addition of the aldoxime to the microsomal preparation did show a greater than 80% loss over 60 minutes. Other possible metabolites of the aldoximes such as the corresponding *S*-oxidised sulfoxides and sulfones were searched for using the GC-MS data, but none were found. Together, these data suggest that although the aldoxime may be formed, its detection was unlikely under the experimental conditions used. This could have been due to oxidation at sulfur, nominal loss of methanethiol to form the alkene, or that the aldoxime underwent further steps on the biosynthetic pathway, and was therefore lost to the system. Isolation of this aldoxime from the aliphatic system is dependent on further purification of the enzymes in the microsomal preparation.

## 2.1.3 *Conclusions*

The synthesis of [$^{14}$C-1]amino acids using the Strecker route has allowed the study of a microsomal preparation from young expanding leaves of *B. napus* which catalyses the NADPH dependent oxidative decarboxylation of homophenylalanine and dihomomethionine. The decarboxylation activities have been shown to be separate, and to involve only the L-amino acids. The product of the oxidative decarboxylation of homophenylalanine has been identified from the microsomal preparation as 3-phenylpropanaldoxime by GC and GC-MS. No product was isolated from dihomomethionine, but the proposed aldoxime product was found to be not readily extractable from the microsomal preparation.

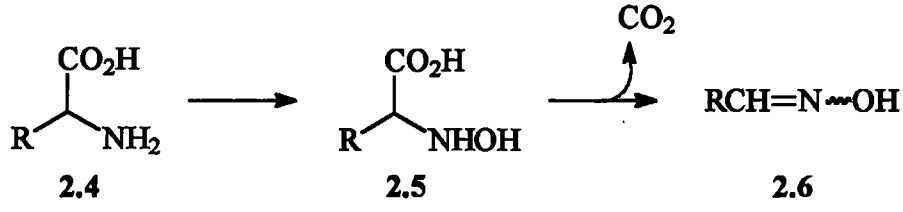
## 2.2 *Enzyme Characterisation*

As part of a major project at IACR-Rothamsted with the eventual aim of reducing pesticide inputs on oilseed rape, one goal is the genetic manipulation of the glucosinolate biosynthetic pathway to give the specific glucosinolates required for the application of a "push-pull" pest management strategy. This will require the purification and characterisation of the enzymes involved in the regulation of the pathway, and the identification and subsequent sequencing of the genes encoding for these enzymes. The gene sequence can then be used to alter glucosinolate profiles in a rational way, using conventional molecular biology techniques.

The glucosinolate pathway appears to be non-specific following aldoxime formation, which suggests that the regulation of the pathway takes place at the step involving aldoxime formation. This suggests that the enzymes involved in the oxidative decarboxylation of amino acids are the point at which the pathway could be controlled, requiring the gene sequence encoding for the enzyme responsible. One method of obtaining this sequence is by purifying the enzymes and then amino acid sequence determination. This would then lead to the gene sequence and the possibility of genetic modification, which could be used to either increase or decrease the concentration of a particular class of glucosinolates. This would be a feasible proposition as we have determined that there are separate enzymes responsible for the biosynthesis of aliphatic and aromatic glucosinolates. Decreasing a particular class of glucosinolates would involve incorporating a gene in the antisense form, which would make the plant unable to translate a particular gene, by creation of two complementary strands of mRNA. Increasing a class of glucosinolates would involve inserting more copies and increasing the expression of the genes encoding for the enzymes. Other studies under this project at Rothamsted should

determine which of the classes would need to be reduced and which increased, and how the genetically modified plants would be integrated into an overall crop protection strategy[92].

The conventional method of sequencing the genes for these enzymes would be from the protein amino acid sequences themselves. This would require further purification of the microsomal preparation to give the pure enzymes. The activity found for the oxidative decarboxylation of [$^{14}$C-1]amino acids is contained in a microsomal fraction, which means that the enzymes are membrane bound. Further purification requires the disruption of the membrane and isolation of the enzymes from it. This poses a number of problems. The assay which has been used to probe for enzyme activity involves determining release of $^{14}CO_2$ from [$^{14}$C-1]amino acids. This is a summated assay for two steps in the pathway; the oxidation of the amino acid (**2.4**) to the *N*-hydroxyamino acid (**2.5**) and the oxidative decarboxylation to form the aldoxime (**2.6**).




**2.4** **2.5** **2.6**

In the plant these two oxidative processes could be seen to happen in a number of ways.

1. There could be two separate enzymes, either close together or far apart on the membrane.
2. There could be a membrane bound enzyme complex which performs both reactions.
3. There could be one membrane bound enzyme which oxidises the amino acid to the *N*-hydroxyamino acid, and then the *N*-hydroxyamino acid could chemically decompose to give the aldoxime.

4. There could be one membrane bound enzyme which catalyses both oxidation reactions, followed by spontaneous decarboxylation.

These distinct possibilities mean that if either case 1 or 2 is the actual situation in the plant, then disruption of the membrane could mean loss of activity from the assay. Disruption of the membrane could also affect the tertiary or quaternary structure of the enzyme thereby losing activity of the enzyme *per se*. As a part of the overall project at IACR-Rothamsted a significant chemical contribution was required in the further studies on the enzymes identified. The biochemical results obtained in the Biochemistry and Physiology Dept. at IACR-Rothamsted are reported in brief here, as a integral part of the overall project, together with the chemical synthesis required to obtain the data reported.

The possibility of further purification of the microsomal preparation was examined by treatment of the microsomal preparation with two different detergents known to disrupt membranes, CHAPS and Triton X-100, see Table 2.1. These detergents were incorporated into the resuspension buffer at varying concentrations. Both detergents showed loss of activity at a concentration of 0.1% level, and total loss of activity at 1% when included in the buffer used to prepare the microsomes.

**Table 2.1** The effect of detergents on release of $^{14}CO_2$ from [$^{14}$C-1]amino acids

| Addition to buffer | Concentration % (w/v) | HPhe Activity % | DHMet Activity % |
|---|---|---|---|
| None | | 100 | 100 |
| CHAPS | 0.1 | 57 | 68 |
| CHAPS | 1.0 | 0 | 0 |
| Triton | 0.1 | 43 | 53 |
| Triton | 1.0 | 0 | 0 |

The loss of activity meant that further purification of the enzymes using this assay was unlikely to be successful, as membrane disruption leads to loss of activity. Further progress towards enzyme purification therefore required either the development of a new assay or use of different purification techniques. For the enzyme involved in the early stages of cyanogenic glycoside biosynthesis very careful purification and resuspension techniques were used to purify the membrane bound enzyme[88], suggesting that enzyme purification may be possible, if the glucosinolate enzyme is as stable as the cyanogenic glycoside enzyme. This may be explored further at a later stage.

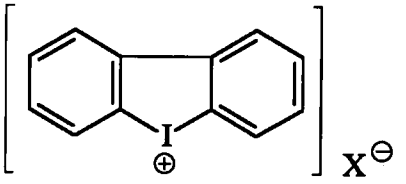
A new assay for the early stages of the glucosinolate pathway would either involve looking for production of the *N*-hydroxyamino acid, or using the *N*-hydroxyamino acid as substrate and looking for the products of the oxidative decarboxylation reaction; either carbon dioxide, or the aldoxime. An alternative approach could utilise the knowledge gained about the class of the enzyme during studies with different effectors. It may then be possible to use molecular biology techniques, such as probing a cDNA library from *B. napus* with probes from published genes sequences encoding for similar enzymes from different systems, to search for the genes encoding for the glucosinolate enzymes. These genes could be expressed in a system such as yeast or *E. coli*, where the enzymes could be expressed at high concentrations and then studied in detail. This approach required detailed studies on the class of the enzymes responsible for the early stages of glucosinolate biosynthesis, to enable probes from enzymes with sufficiently homologous gene sequences to be used.

The enzymes in the microsomal preparation could be characterised using the [$^{14}$C-1]amino acid assay, and monitoring the change in $^{14}CO_2$ release from [$^{14}$C-1]homophenylalanine and [$^{14}$C-1]dihomomethionine upon the addition of different effectors. These results could then be compared with other similar studies and the enzymes characterised on this basis. In cyanogenic glycoside biosynthesis the enzymes responsible

for the formation of aldoximes from amino acids are known to be cytochromes P450[87], and so this made an obvious starting point for examining this system.

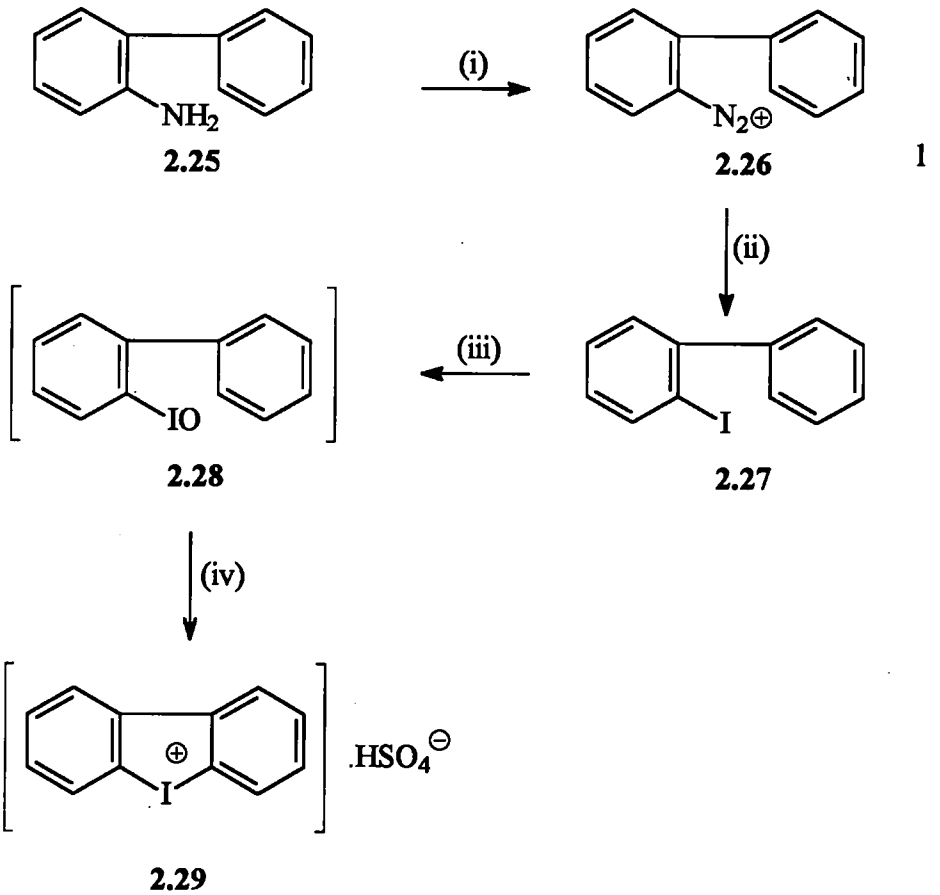
A range of inhibitors known to inhibit cytochromes P450 were tested for inhibition, but these showed limited activity. Inhibitors known to inhibit heme containing enzymes were also examined, but again only limited inhibition was found. Inhibition was found with diethyl pyrocarbonate and copper ions [Cu(I) and Cu(II)], but not with a wide range of other ions. Various studies to characterise the enzymes were carried out, looking at pH optima, chelating agents, and amino acid modifiers, results of which are reported elsewhere[114,117]. Some increase in release of $^{14}CO_2$ was seen with addition of flavins during early studies before the extraction conditions were optimised[114]. The extraction conditions used in the early studies could have caused a loss of enzyme bound flavin, which was replaced by the addition of FAD and FMN. The optimised conditions appeared to avoid this problem. However, this stimulation did suggest the possibility of flavin containing enzymes being involved in the reaction. These characterisation results, together with the effector studies and the requirement for NADPH, suggested that the possibility that the enzymes may be flavin linked monooxygenases (FMOs). *N*-Hydroxylation reactions are already known for flavin containing monooxygenases found in mammalian liver and lung tissue. However, the substrates are for this class of enzyme are normally xenobiotics, and amino acids are not thought to be accepted as substrates.[118]

Flavin containing enzymes are known to be inhibited by diphenyleneiodonium salts (DPI salts)[119]. Therefore these salts should have acted as inhibitors for the release of $^{14}CO_2$ from the microsomal preparation, if the enzymes contained flavin prosthetic groups.



**2.24**

Synthesis of **2.24** was from 2-aminobiphenyl (**2.25**) which involved diazotisation to give **2.26** and then reaction with potassium iodide to form 2-iodobiphenyl (**2.27**). This was then oxidised with peracetic acid to form the iodoso compound (**2.28**). Treatment with concentrated sulfuric acid in acetic anhydride caused ring closure and formation of the iodonium salt as its hydrogensulfate (**2.29**) in good yield (62% overall)[120].



(i) $HNO_2$, $H_2O$, (ii) KI, (iii) AcOOH, AcOH, (iv) c. $H_2SO_4$.

The iodonium salt (**2.29**) was added to the microsomal preparation in DMSO and found to inhibit $^{14}CO_2$ release from [$^{14}$C-1]amino acids at concentrations of 0.25mM and above, as shown in Table 2.5. This suggests that the enzymes may contain a flavin prosthetic group.

**Table 2.5** The effect of **2.29** on $^{14}CO_2$ release from [$^{14}$C-1]dihomomethionine and from [$^{14}$C-1]homophenylalanine

| Concentration mM | DHMet % Activity | HPhe % Activity |
|---|---|---|
| 0 | 100 | 100 |
| 0.25 | 71 | 67 |
| 0.50 | 49 | 43 |
| 1.0 | 12 | 7 |
| 2.0 | 0 | 0 |

## 2.2.1 *Conclusions*

The characterisation of the enzymes in the microsomal preparation gave a number of results.

1. The microsomes prepared from young expanding leaves of *B. napus* were shown to contain enzymes that catalysed the NADPH dependent oxidative decarboxylation of homophenylalanine and dihomomethionine.

2. These enzymes, did not show definite inhibition with a number of cytochromes P450 inhibitors, unlike those found in similar microsomes in cyanogenic glycoside biosynthesis catalysing similar transformations, which are cytochromes P450.

3. Characterisation studies suggested that the enzymes were possibly flavin linked

monooxygenases, similar to enzymes found in a number of mammalian tissues. However, substrate specificity for the mammalian enzymes suggest that they do not accept amino acids as substrates.

The characterisation work has allowed work in the overall project to use probes from mammalian flavin linked monooxygenases to look for similar gene sequences in a cDNA library generated from *B. napus*. Any similar genes identified will be sequenced and then expressed to determine the function of the gene. This is an alternative technique to the continued purification of the microsomal preparation. However, the likelihood of finding sufficient homology between the mammalian and plant systems is not high, and further purification work may still be required.

Very recent work on glucosinolate biosynthesis in *Sinapis alba* (white mustard) has identified a cytochrome P450 dependent enzyme which catalyses the oxidative decarboxylation of tyrosine to (4-hydroxyphenyl)acetaldoxime[121]. Since it is highly unlikely that two different systems would have evolved to biosynthesise the same products (aldoximes) in closely related plants it seems likely that the enzymes found in oilseed rape are also cytochromes P450. This recent result necessitates further studies on the biochemistry of the enzymes from *B. napus*.

## 2.3 *Oxidative Decarboxylation Mechanism*

Studies on the mechanism of the enzymes involved in the oxidative decarboxylation of amino acids to aldoximes were possible despite the difficulties in the further purification of the enzymes involved. The microsomal preparation could be used to give a wide range of information, and also to examine the enzyme mechanism. The earlier studies had shown that there were two distinct systems responsible for the conversion of the aromatic and aliphatic amino acids to their corresponding aldoximes. It would therefore be possible to examine the enzyme mechanism for both pathways. However, as this mechanism was likely to be the same in both pathways, with just small changes in the enzyme active site responsible for enzyme specificity, it was decided to focus on the aromatic pathway, because of the greater ease of synthesis of the target molecules. Three approaches were used to examine the mechanism.

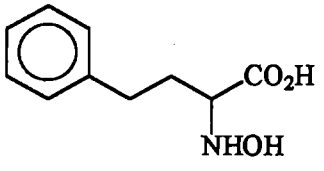
1. Competition assays where [$^{14}$C-1]homophenylalanine (**2.19**) was incubated with a possible unlabelled intermediate and $^{14}CO_2$ release was compared with a control which had no additional possible intermediate.

2. Studies were made using stable and radio labelled substrates to examine the retention, or loss, of the C-2 hydrogen in the oxidative decarboxylation reaction. This would give evidence as to a possible mechanism for the reaction, because certain intermediates can only be formed with retention of this hydrogen, and others can only be formed through its loss.

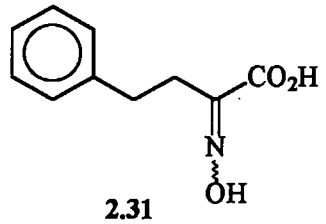
3. Studies were made using $^{19}$F NMR to examine intermediates formed *in situ*. This sensitive technique can examine the generation of intermediates, using the microsomal preparation in an NMR tube, incubated with a potential substrate with $^{19}$F substitution.

## 2.3.1 *Potential Intermediates*

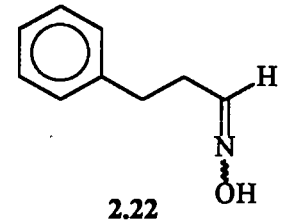
Although the glucosinolate enzymes may belong to a different class of enzyme from those found in the early stages of cyanogenic glycoside biosynthesis, potential intermediates between the amino acid and the aldoxime might be similar. Early studies on the glucosinolates had implicated *N*-hydroxyamino acids (**2.30**) as likely intermediates[67], and these have also been shown to be involved in cyanogenic glycoside biosynthesis[71,72]. Other possible intermediates between the *N*-hydroxyamino acids (**2.30**) and the aldoxime (**2.22**) include the corresponding 2-oximino acid (**2.31**), the nitro acid (**2.32**) and the nitroalkane(**2.33**)[72]. Examination of the aldoxime as a potential feedback inhibitor of the enzyme would also give useful information. Synthesis of these compounds was required for testing them in a competition assay with [$^{14}$C-1]homophenylalanine (**2.19**).



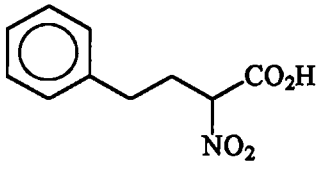

**2.30**



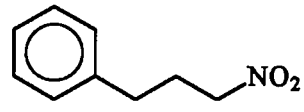

**2.31**




**2.22**



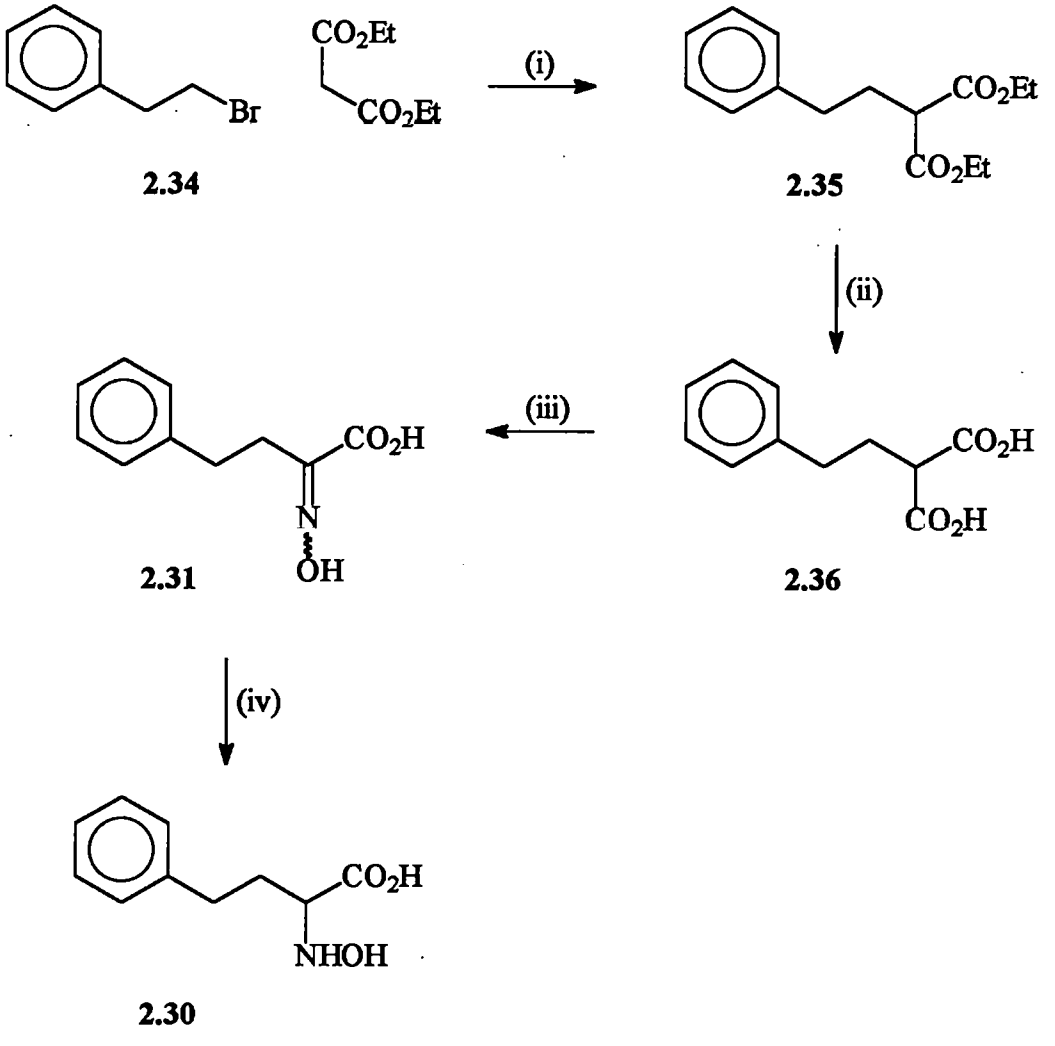

**2.32**




**2.33**

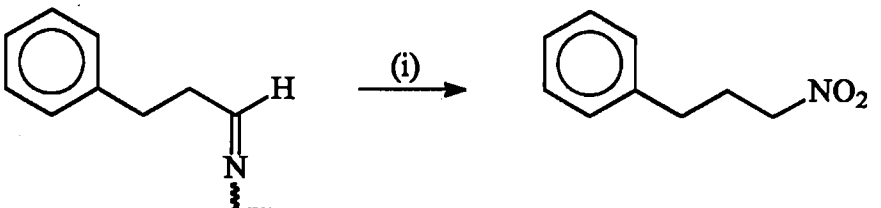
The synthesis of *N*-hydroxyhomophenylalanine (**2.30**) was carried out using the pathway shown in the scheme following, which also generated the corresponding 2-oximino acid (**2.31**)[122-124]. Phenylethyl bromide (**2.34**) was condensed with diethyl malonate and then the substituted malonate (**2.35**) was hydrolysed with hydrochloric acid to give the malonic acid (**2.36**) in moderate yield (52%). This was treated with *n*-propylnitrite and

then HCl gas to give the 2-oximino acid (**2.31**), as a single isomer about the C=N bond, in high yield (80%) following spontaneous loss of $CO_2$. Reduction of the 2-oximino acid (**2.31**) with sodium cyanoborohydride in acetic acid/water gave the *N*-hydroxyamino acid (**2.30**) which was purified using an ion exchange resin. This scheme gave both the *N*-hydroxyamino acid (**2.30**) and the 2-oximino acid (**2.31**) for use in competition assays with [$^{14}$C-1]homophenylalanine (**2.19**).




(i) $K^+t$-$BuO^-$, glyme, Δ, (ii) HCl (aq), (iii) *n*-propyl nitrite, HCl (g), (iv) $NaBH_3CN$, $AcOH/H_2O$.

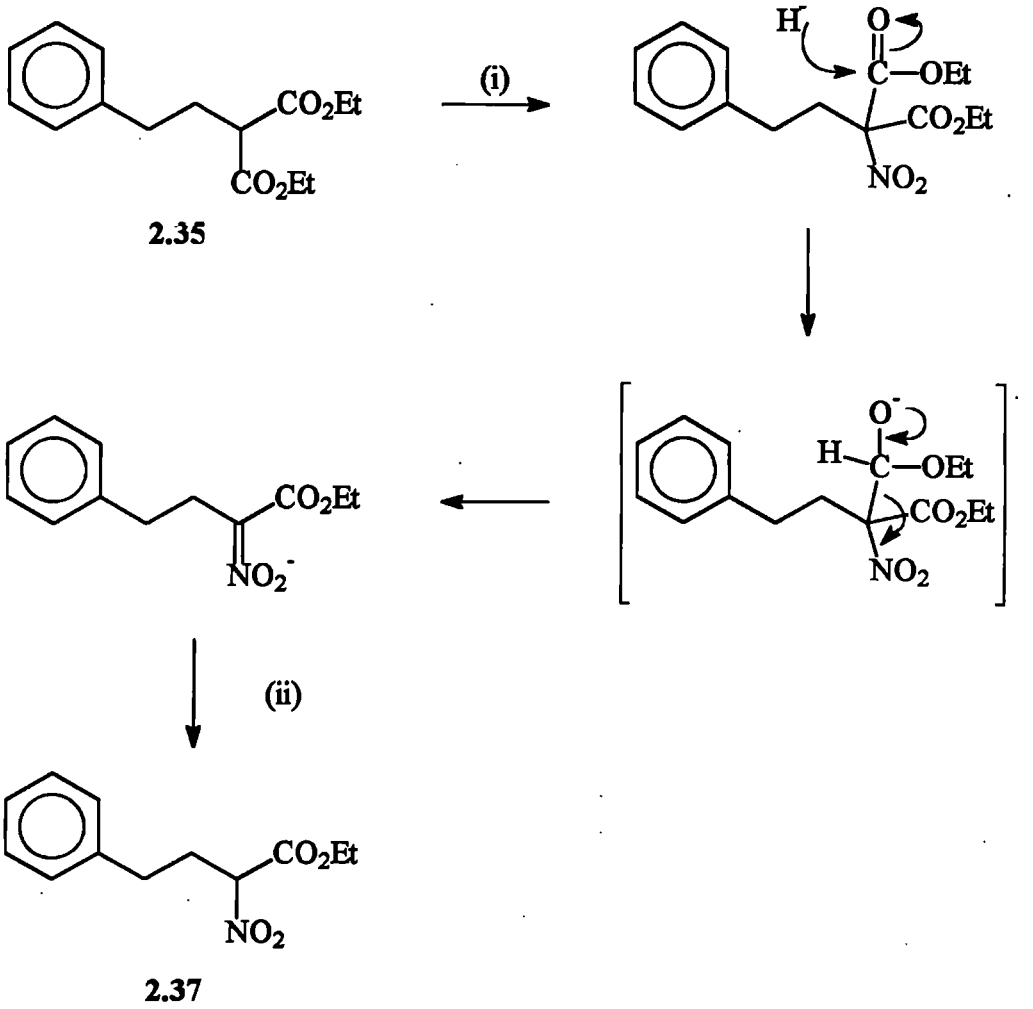
The 3-phenylpropanaldoxime (**2.22**) was already available from the earlier studies, and this was used to synthesise the nitroalkane (**2.33**) by oxidation of the aldoxime with urea-hydrogen peroxide complex and trifluoroacetic anhydride in buffered acetonitrile[125].




**2.22** **2.33**

(i) UHP, $(CF_3CO)_2O$ , MeCN, $Na_2HPO_4$.

$\alpha$-Nitro acids are unstable, readily decomposing on warming to give the nitroalkane and carbon dioxide, so instead of synthesising the $\alpha$-nitro acid (**2.32**) the corresponding stable ethyl ester (**2.37**) was synthesised. The synthesis utilised the substituted malonate ester (**2.35**) from the *N*-hydroxyhomophenylalanine synthesis. This was treated with sodium hydride and then acetone cyanohydrin nitrate to give the $\alpha$-nitro ester (**2.37**) in poor yield (30%) via a base catalysed decarboxylation reaction[126,127].




**2.35**

**2.37**

(i) a) NaH, glyme, Δ, b) Acetone cyanohydrin nitrate, Δ, (ii) $H^+$.

Studies on the microsomal preparation showed that there was significant esterase activity present, probably from cytosolic contamination of the microsomes[128]. This suggested that the use of the α-nitro ester (**2.37**) was a reasonable strategy for the introduction of the unstable α-nitro acid (**2.32**) into the microsomal preparation.

The potential intermediates could then be tested in competition assays with [$^{14}$C-1]homophenylalanine (**2.19**). In a competition assay the [$^{14}$C-1]amino acid is incubated with the microsomal preparation, with and without the presence of the potential intermediate (effector) to be tested. The release of $^{14}CO_2$ is monitored and the release for

the control reaction is compared to the reactions where the effector has been added. The results are expressed as a percentage of the control reaction. A result of 100% means that the compound of interest did not inhibit the release of $^{14}CO_2$ and so is unlikely to be acting at the enzyme active site. Figures of less than 100% mean that the compound did inhibit the release of $^{14}CO_2$ from the [$^{14}$C-1]amino acid and so acted as an inhibitor, either at the active site, or possibly another site on the enzyme.

Using this technique the potential intermediates except for the nitroalkane (**2.33**) were then tested in competition assays with [$^{14}$C-1]homophenylalanine (**2.19**), at two concentrations, both of which were considerably higher than the concentration of [$^{14}$C-1]homophenylalanine. The $\alpha$-nitro ester (**2.37**) was preincubated with microsomes for 15 minutes before the addition of NADPH to start the oxidative decarboxylation reaction. Release of $^{14}CO_2$ was monitored and compared to a control where no potential intermediate had been added. All the potential intermediates tested in this competition assay except the $\alpha$-nitro ester (**2.37**) showed a degree of inhibition, but no one compound showed significantly higher inhibition than any other, as shown in Table 2.6. This showed that the $\alpha$-nitro acid (**2.32**) is unlikely to be an intermediate on the pathway between the amino acid (**2.2**) and the aldoxime (**2.22**). The other compounds tested gave no significant information as to the likely intermediates, and only showed that the potential intermediates except for the $\alpha$-nitro acid could act equally well as inhibitors. The inhibition shown by the aldoxime (**2.22**) suggested that there might be a degree of feedback inhibition of the enzyme by its product.

**Table 2.6** The effect of potential intermediates on $^{14}CO_2$ release from [$^{14}$C-1]homophenylalanine [13.6$\mu$M]

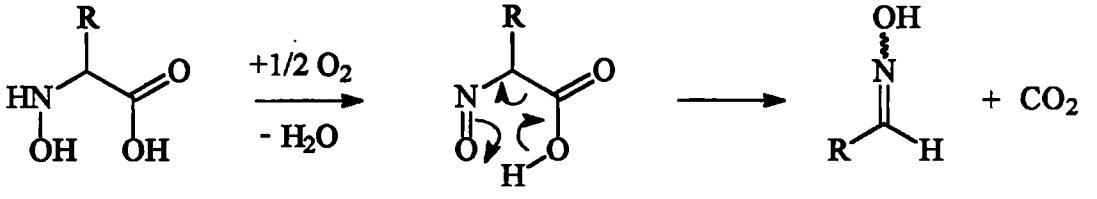
| Addition | Concentration | Activity |
|---|---|---|
| | $\mu$M | % |
| *N*-Hydroxyamino acid | 100 | 53 |
| **(2.30)** | 250 | 32 |
| 2-Oximino acid | 100 | 48 |
| **(2.31)** | 250 | 19 |
| Aldoxime | 100 | 52 |
| **(2.22)** | 250 | 30 |
| $\alpha$-Nitro ester | 100 | 81 |
| **(2.37)** | 250 | 80 |

100% Activity is with no addition of potential inhibitor.

The involvement of the nitroalkane **(2.33)** in the pathway was investigated by GC-MS analysis of a sample known to produce the aldoxime **(2.22)**. The sample was searched for the presence of the nitroalkane **(2.33)** using as diagnostic ions the parent *m/z* 165, loss of water giving *m/z* 147, and other abundant ions at *m/z* 135, 117 and 91 (base ion). No trace of the nitroalkane **(2.33)** was found, suggesting that it is not an intermediate on the pathway. In the cyanogenic glycoside studies the nitroalkane was only found when feeding with a large excess of the appropriate *N*-hydroxyamino acid[72], and it was later shown to be only a side product of the pathway[89].

Further studies were carried out to examine *N*-hydroxyhomophenylalanine **(2.30)** as a possible intermediate on the glucosinolate pathway. *N*-Hydroxyhomophenylalanine **(2.30)** was incubated with the microsomal preparation, and then this was examined for aldoxime formation by GC, as for the amino acids. The aldoxime was found in relatively high amounts in the samples examined. However, further studies showed that the aldoxime was also formed in control reactions where the *N*-hydroxyhomophenylalanine was

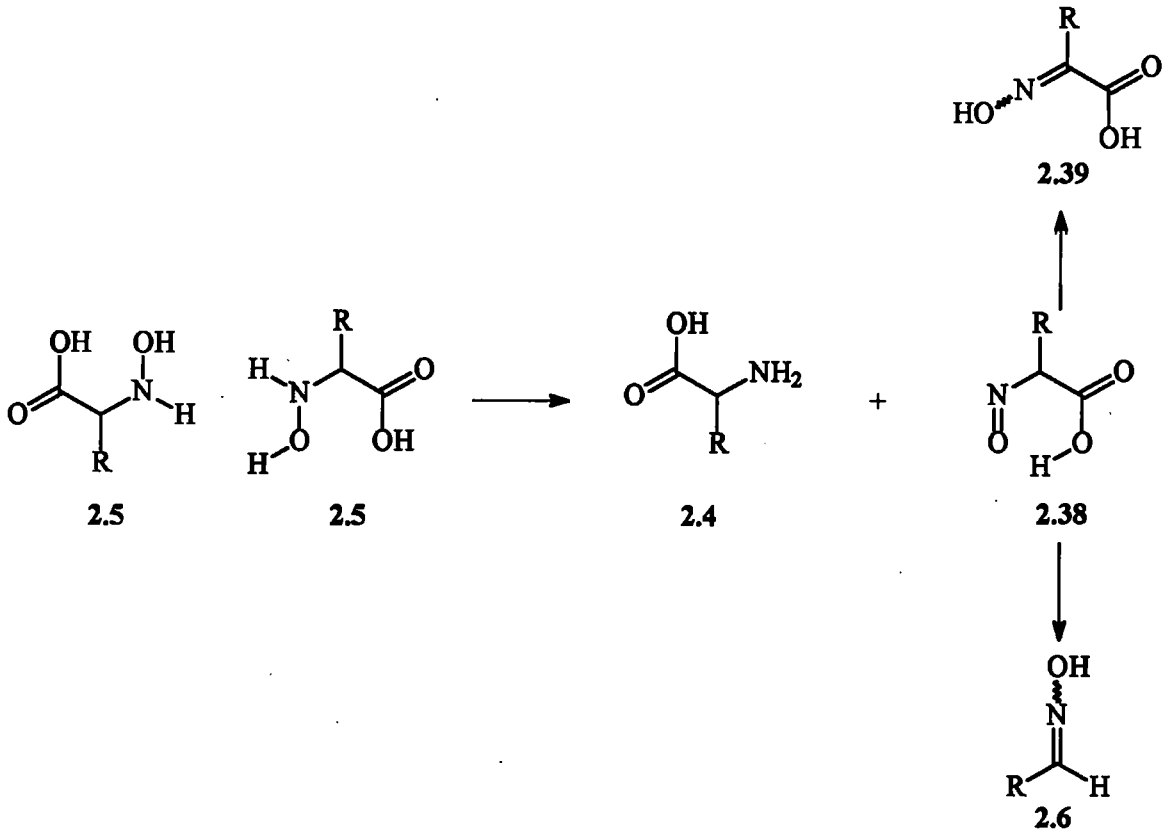
incubated with buffer, with no microsomes present, and then extracted with organic solvent and analysed as before. In hindsight this was not a surprising result, given that *N*-hydroxyamino acids (**2.5**) have been shown to undergo a pH dependent, chemical, oxidative decarboxylation reaction which is thought to proceed via the α-nitroso acid (**2.38**). In the presence of oxygen the products are the aldoxime and carbon dioxide (**2.6**)[122,129].




**2.5** **2.38** **2.6**

However, in an inert atmosphere a disproportionation reaction occurs giving the amino acid (**2.4**) and the α-nitroso acid (**2.38**). The α-nitroso acid can then either decompose to give the aldoxime (**2.6**) and carbon dioxide, as before, or rearrange to give the 2-oximino acid (**2.39**). Both these routes in the presence and absence of oxygen show that *N*-hydroxyamino acids (**2.5**) can decompose chemically to give aldoximes (**2.6**)[129].

One possible way around this problem was to look for increased rates of aldoxime production in the microsomal preparation and compare these to those found with just the buffer[67]. However, in this system the figures obtained for aldoxime formation, with and without microsomes, were too similar to be able to see any increase in the rate of aldoxime formation with microsomes present. To pursue this further a more reliable assay would have been required.

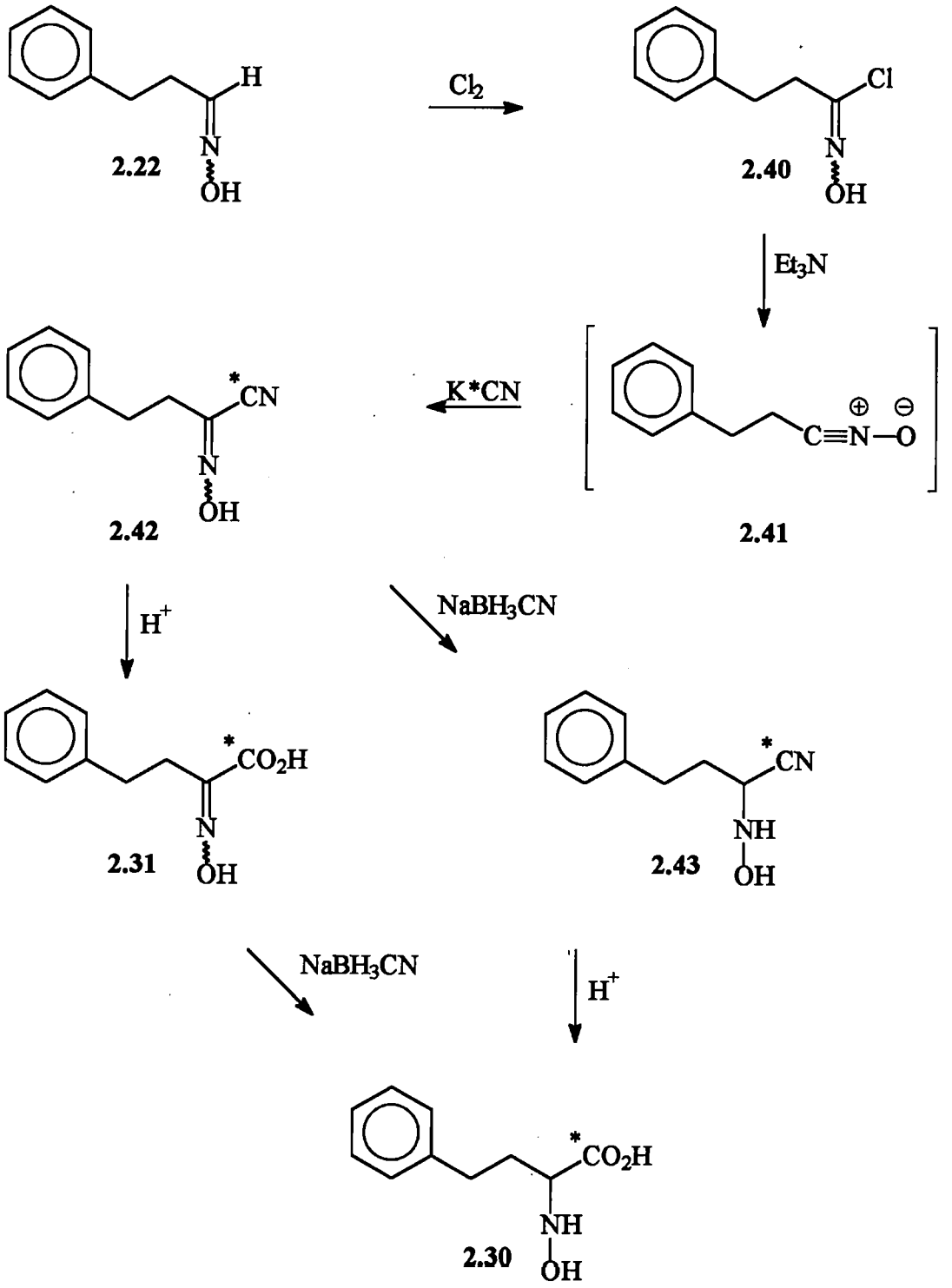



The possibility of using $^{14}C$ labelled *N*-hydroxyhomophenylalanine was also investigated, requiring the development of a synthetic route to introduce the label. This label could either be in the C-1 or C-2 position. Introduction of the label at C-1 of *N*-hydroxyhomophenylalanine would allow the conversion to aldoxime to be monitored by measuring release of $^{14}CO_2$ in the same way as for the C-1 labelled amino acids. Introduction of the label at C-2 would allow detection of the products if a suitably reliable separation system for the reaction products such as HPLC could be developed.

The route used to prepare the unlabelled *N*-hydroxyhomophenylalanine would be suitable to prepare the labelled compound, using either [$^{14}$C-1] or [$^{14}$C-2]diethyl malonate to introduce the label. Use of [$^{14}$C-2]diethyl malonate would successfully put the label at C-2 of the *N*-hydroxyhomophenylalanine. However, the use of [$^{14}$C-1]diethyl malonate would result in the loss of 50% of the label during the synthesis, giving a maximum theoretical radiochemical yield of 50%, which was clearly unacceptable for the synthesis

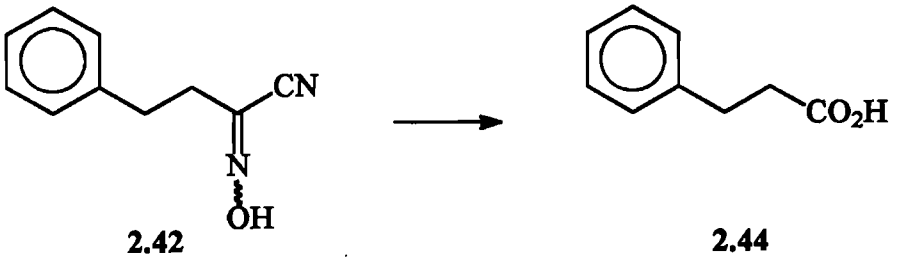
of [$^{14}$C-1]*N*-hydroxyhomophenylalanine. The [$^{14}$C-1]*N*-hydroxyhomophenylalanine would also be the preferable synthetic target, as the assay for monitoring this reaction is straightforward and reliable, and would possibly allow further enzyme purification, which is one of the major aims of this study. Due to the prohibitive cost of $^{14}$C labelled diethyl malonate, and the low maximum theoretical yield, this route to [$^{14}$C-1]*N*-hydroxyhomophenylalanine was discarded and alternative routes were sought The route also had the problem of requiring distillation to purify products on the pathway which is not desirable in the small scale synthesis of radiolabelled compounds. No further routes to [$^{14}$C-2]*N*-hydroxyhomophenylalanine were considered due to the difficulty of devising a simple assay for the products, with the resources available.

A number of alternative routes for introducing the label at C-1 of the *N*-hydroxyamino acid were examined[130,131]. One possible route utilised potassium cyanide as the source of the label from the 1,3 addition to a nitrile oxide (**2.41**) to form the 2-oximinonitrile (**2.42**). The highly reactive nitrile oxide could be generated *in situ* from the aldoxime via action of triethylamine on the corresponding hydroximoyl chloride (**2.40**). The 2-oximinonitrile (**2.42**) formed would then require reduction of the oxime group to form the *N*-hydroxy functionality, and hydrolysis of the nitrile to give the acid. The favoured sequence for these last two steps would be hydrolysis to the 2-oximino acid (**2.31**) and then reduction to the *N*-hydroxyamino acid (**2.30**), as for the standard *N*-hydroxyamino acid synthesis. This sequence would be preferable because if the last step were the hydrolysis of an *N*-hydroxyaminonitrile (**2.43**) this would mean that the *N*-hydroxyamino acid would be formed in an acidic or basic system where spontaneous decarboxylation or disproportionation was likely.




The hydroximoyl chloride (**2.40**) was generated in moderate yield (47%) by the action of chlorine gas on a solution of the aldoxime (**2.22**) in the same manner as seen in the coupling stage of glucosinolate synthesis[49]. This was then used without further purification and treated with potassium cyanide and then triethylamine dropwise, to

generate the nitrile oxide (**2.41**) *in situ*[132]. The 1,3 addition reaction proceeded to give the desired 2-oximinonitrile (**2.42**). Hydrolysis of the nitrile group was attempted under a variety of acidic or basic conditions. These either resulted in no reaction taking place, or in complete hydrolysis to give 3-phenylpropanoic acid (**2.44**).

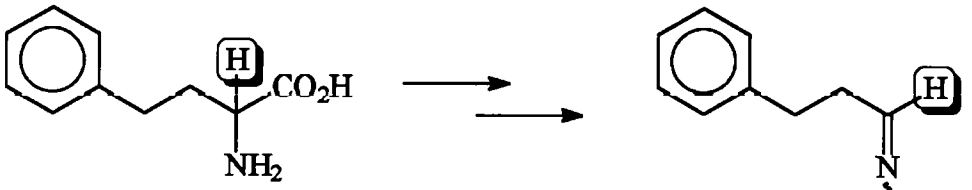



**2.42** **2.44**

Reduction of the oxime group was also attempted, although for the reasons stated earlier this was not the best route to choose, due to the likely decomposition of the product under the conditions necessary to hydrolyse the nitrile. A variety of reducing agents were used, but none were effective in bringing about the desired transformation. These results led to the abandonment of this pathway as a possible route to [$^{14}$C-1]*N*-hydroxyhomophenylalanine. However, further studies may be able to find suitable conditions for the hydrolysis reaction, which does not also hydrolyse the oxime group.

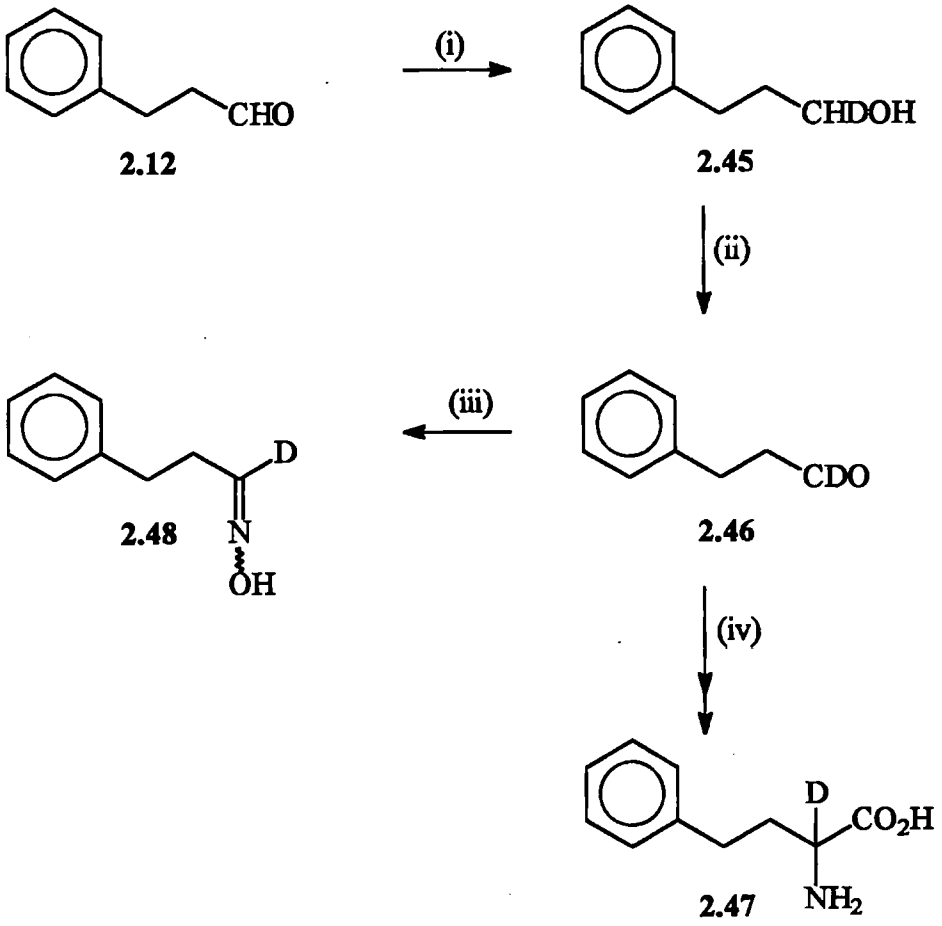
## 2.3.2 *Deuterium and Tritium Labelling Studies*

The proton at C-2 is very important in determining the possible mechanism of the oxidative decarboxylation reaction. This is because certain intermediates can only be formed with loss of this proton and others will retain this proton and therefore it will be present in the aldoxime product.




**2.2** **2.22**

For example the 2-oximino acid (**2.31**) can only be formed with loss of this proton, whereas the $\alpha$-nitro acid (**2.32**) could retain the proton in its formation. Feeding of homophenylalanine labelled at C-2 with deuterium or tritium and examination of the aldoxime product should demonstrate either retention or loss of this proton. This feeding experiment required the synthesis of the labelled amino acid and the labelled aldoxime, both of which were obtainable from the labelled aldehyde. Use of lithium aluminium deuteride gave the deuterated product as shown.



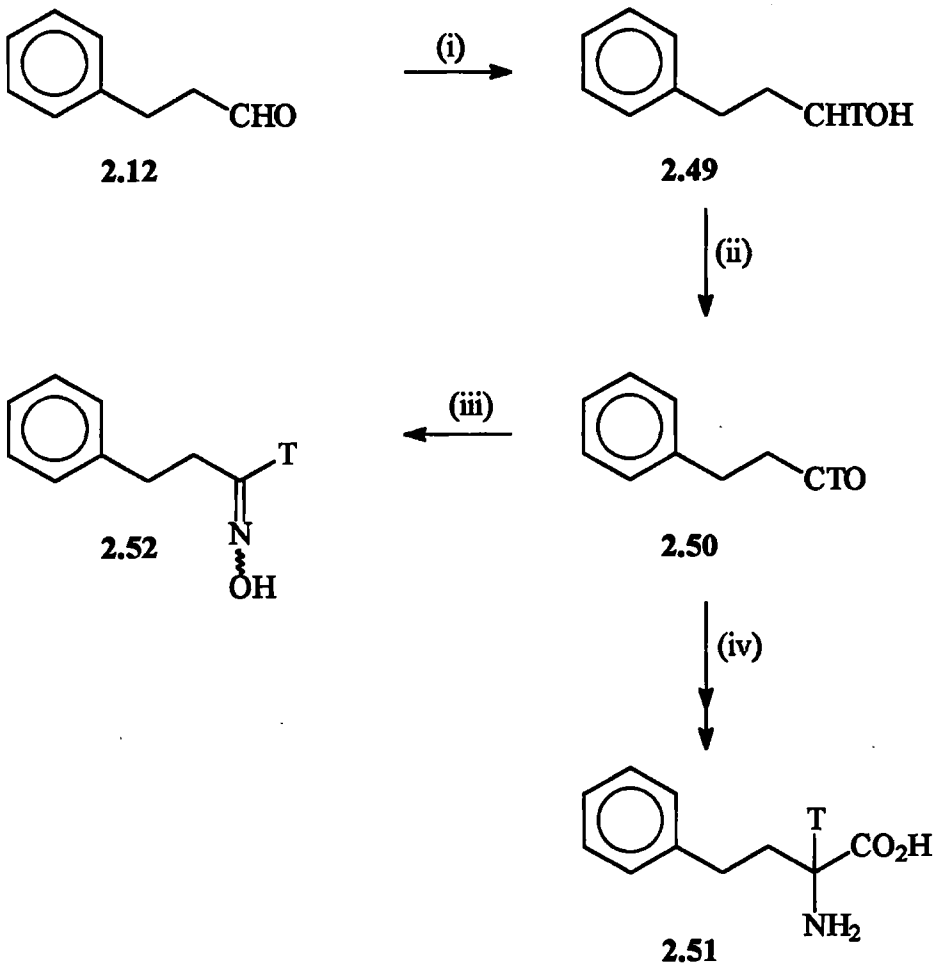

(i) a) $LiAlD_4$ , $Et_2O$, b) $H^+(aq)$, (ii) PCC, $CH_2Cl_2$ , (iii) $NH_2OH.HCl$, $NaHCO_3$ , EtOH, $H_2O$, (iv) a) KCN, $NH_4Cl$, $NH_3$ , EtOH, $H_2O$, b) c.HCl, c) $H^+(aq)$.

The reduction to give the deuterated alcohol (**2.45**) went in high yield (87%). This was then oxidised to the aldehyde (**2.46**) by PCC in moderate yield (48%) after distillation. This was then used in the Strecker synthesis of [$^2$H-2]homophenylalanine (**2.47**) in 40% yield from the aldehyde. The corresponding labelled aldoxime (**2.48**) was also synthesised using hydroxylamine hydrochloride as before. Although the theoretical yield of deuterium at C-2 of the amino acid was only 50%, the figure obtained in practice, as measured by $^1$H and $^{13}$C NMR was ~85%. This was due to a strong isotope effect during the PCC oxidation of the alcohol (**2.45**) to the aldehyde (**2.46**) resulting in an enrichment of the deuterium content[133]. However, despite this enrichment, a more suitable approach

would have used the ester as a starting material which would give the di-deuterated alcohol, and hence 100% deuterium in the aldehyde and final products, assuming no D/H exchange took place at any stage in the pathway. However the degree of incorporation obtained should be sufficient for the feeding experiments.

As mass spectrometry was to be the preferred method of analysis for retention or loss of the deuterium label the mass spectra of the two labelled aldoxime isomers **(2.48)** were recorded. These were then compared with the equivalent spectra for the unlabelled aldoxime isomers. Both isomers of the labelled aldoxime showed a small peak at m/z 150 for the molecular ion, as well as the greater intensity at m/z 104 in the (*Z*)-isomer, from the stereochemically favoured rearrangement, forming styrene, as found in the unlabelled aldoxime. Both isomers gave spectra which included loss of OH (m/z 133), compared with the unlabelled compound which had loss of water ($M^+$-$H_2O$) to give the nitrile (m/z 131). This difference was presumably due to the increased strength of the C-D bond which limited the loss of water from the labelled compounds. Both isomers showed the expected base peak from the tropylium ion at m/z 91. Another significant ion was found at m/z 118 ($M^+$-32), compared with m/z 117 in the unlabelled isomers, which appeared to come from loss of $H_2NO$. These differences in spectra between the labelled and unlabelled compounds would allow the presence or absence of the label to be detected by GC-MS.

A similar synthetic approach was used in the synthesis of the tritiated amino acid **(2.51)** and aldoxime, except that the reducing agent used was sodium borotritiide. The reduction to the alcohol **(2.49)** proceeded in 48% radiochemical yield. This was then oxidised to the labelled aldehyde **(2.50)** with PCC with a high radiochemical yield (60%). This is higher than would be expected from a purely theoretical calculation, again because of an isotope effect in the oxidation of the alcohol to the aldehyde[133]. The remaining steps to form the amino acid **(2.51)** proceeded in reasonable yield (71%), as expected. The corresponding aldoxime **(2.52)** was also synthesised in very high yield (96%).




2.12 2.49 2.50 2.52 2.51

(i) a) $NaBT_4$ , $Et_2O$, b) $H^+(aq)$, (ii) PCC, $CH_2Cl_2$ , (iii) $NH_2OH.HCl$, $NaHCO_3$, EtOH, $H_2O$, (iv) a) KCN, $NH_4Cl$, $NH_3$ , EtOH, $H_2O$, b) c.HCl, c) $H^+(aq)$.

The feeding experiments utilising the two labelled amino acids (**2.47, 2.51**) were designed to be essentially identical except for the method of analysis. Labelled amino acid was used as a substrate for the microsomal preparation, and then after a fixed time period the preparation was diluted with water and extracted with diethyl ether/hexane. This was then analysed by GC for the presence of aldoxime, as for the unlabelled material. For the deuterium labelled material the presence or absence of the label was detected by GC-MS analysis. For the tritium labelled material the presence or absence of the label was to be detected by GC with the effluent from the column split to both a radiochemical detector

and FID.

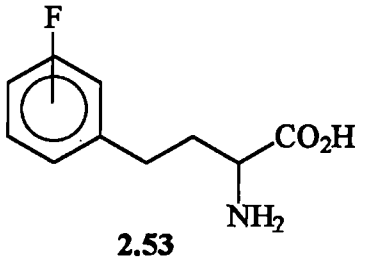
Feeding experiments were carried out using the deuterium labelled amino acid (**2.47**). Initial experiments looked at the labelled amino acid, but were unable to detect formation of any aldoxime by GC and GC-MS analysis. To examine this result further labelled amino acid was tested in parallel experiments with the unlabelled material (**2.2**), and a 50/50 mixture of labelled and unlabelled amino acids. GC analysis of the organic extracts showed the presence of aldoxime in the two control experiments, but not in the experiment with only the labelled amino acid as a substrate. This suggested the possibility of an isotope effect with the labelled material which slowed down the enzymic reaction. Since the reaction is NADPH dependent and the microsomal preparation contained other enzymes likely to utilise NADPH, the reduction in the rate of the reaction could have been sufficient, such that only a small amount of aldoxime was formed before all the NADPH present in the preparation was utilised. This would imply that the $\alpha$-C-H bond was being broken during the reaction, which limits the choice of the likely intermediates in the oxidative decarboxylation reaction. However, this conclusion was based on what is effectively a negative result, as under the conditions used in these experiments no aldoxime could be found from the preparations utilising the deuterated amino acid (**2.47**) as a substrate, and as such should be treated with caution. Isolation of aldoxime and confirmation of the retention or loss of the $\alpha$-H is essential to determine the true mechanism of decarboxylation.

The use of the tritiated amino acid (**2.51**) has been delayed until the GC-radiochemical detector is functioning properly.

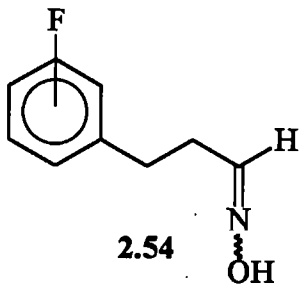
## 2.3.3 *Fluorine Labelling Studies*

The use of fluorine as a probe to study enzymic reactions is a very interesting development[134,135]. The technique relies on the detection of fluorine by $^{19}F$ NMR, and the absence of fluorine from most natural biological systems. As a result, any fluorine detected in the sample must originate from introduced material. The range of chemical shift values obtained in $^{19}F$ NMR is very wide, and this means that a small change in the molecule can have a significant change in $^{19}F$ chemical shift, even for changes a significant distance from the fluorine atom. This would allow detection of small amounts of fluorine labelled product as it should be able to be detected with no interference from the fluorine labelled substrate. Fluorine is a well known isosteric replacement for hydrogen, and as such compounds containing fluorine often retain their biological activity. Because of the absence of naturally occurring fluorine in biological samples relatively crude preparations can be used to conduct experiments, providing the concentration of the intermediates and products is sufficient to be detected by $^{19}F$ NMR. The whole experiment can be conducted in an NMR tube and any changes in the substrate monitored over time. This time course experiment depends on the concentrations of substrates, intermediates and products being sufficiently high enough to give short NMR sampling periods and hence accurate time dependent results. However, even at low concentrations it would be possible to obtain results showing product formation.

The aromatic system was chosen for this series of experiments, which required the synthesis of fluorine substituted homophenylalanines (**2.53**) and associated aldoximes (**2.54**). As the enzymes involved in the oxidative decarboxylation reaction had been shown to be substrate specific, fluorine substitution was placed on the aromatic ring, so that any electronic effects affecting the amino acid end of the molecule were minimised.

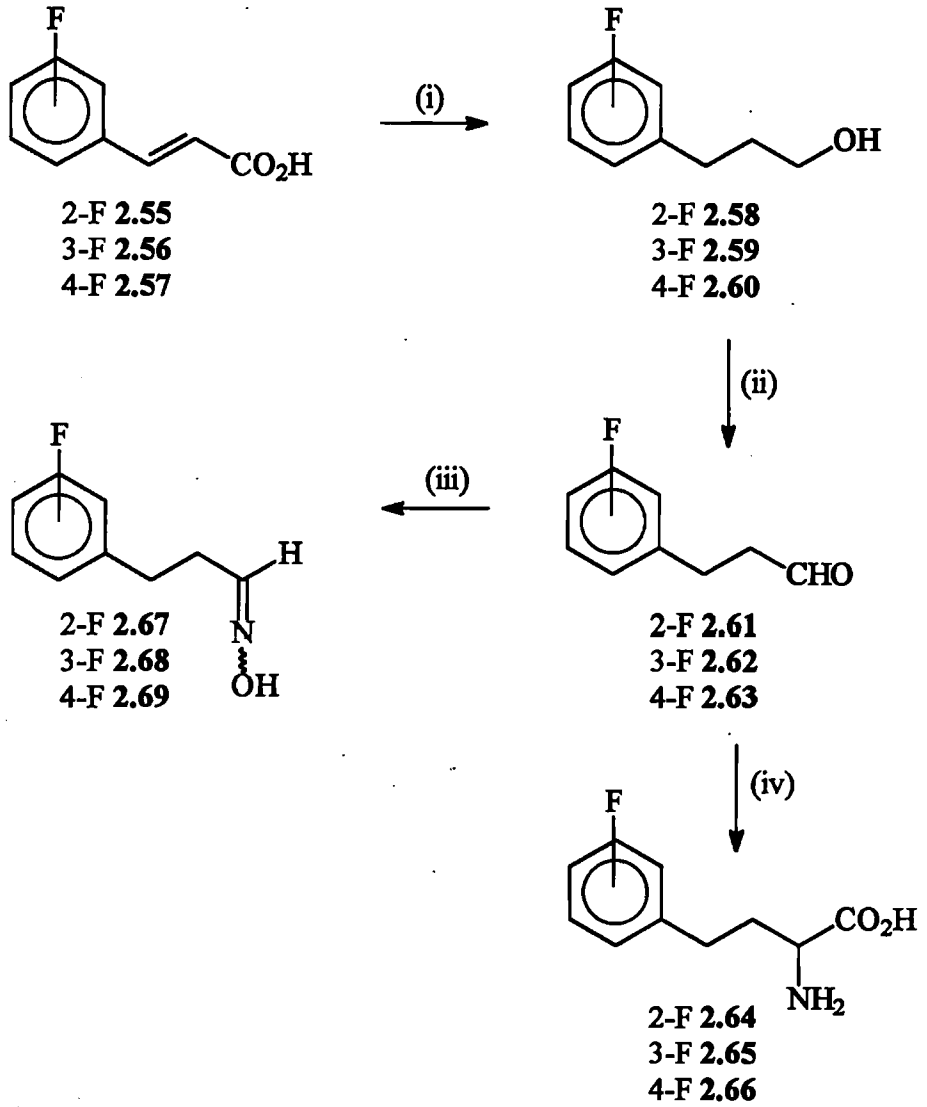



2.53




2.54

The synthetic route was based on readily available fluorocinnamic acids (**2.55-2.57**). Treatment of the three isomers of fluorocinnamic acid with lithium aluminium hydride reduced both the double bond and the acid, giving the corresponding fluorophenylpropanols (**2.58-2.60**) in high yield (73-87%). The alcohols were then oxidised back to the aldehydes (**2.61-2.63**) with PCC in moderate yield (37-62%). These aldehydes were then used to synthesise both the fluorine substituted homophenylalanine analogues (**2.64-2.66**) using the standard Strecker synthesis for homophenylalanine. The corresponding aldoximes (**2.67-2.69**) were also synthesised from the aldehydes, but surprisingly, low yields were obtained (24-36%).




2-F **2.55**
3-F **2.56**
4-F **2.57**

2-F **2.58**
3-F **2.59**
4-F **2.60**

2-F **2.61**
3-F **2.62**
4-F **2.63**

2-F **2.64**
3-F **2.65**
4-F **2.66**

2-F **2.67**
3-F **2.68**
4-F **2.69**

(i) a) $LiAlH_4$ , $Et_2O$, b) $H^+(aq)$, (ii) PCC, $CH_2Cl_2$ , (iii) $NH_2OH.HCl$, $NaHCO_3$ , EtOH, $H_2O$, (iv) a) KCN, $NH_4Cl$, $NH_3$ , EtOH, $H_2O$, b) c.HCl, c) $H^+(aq)$.

This gave the 2-, 3-, and 4- fluorine substituted homophenylalanines (**2.64-2.66**) and the corresponding aldoximes (**2.67-2.69**). The amino acids were then tested as potential inhibitors in the competition bioassay with [$^{14}$C-1]homophenylalanine. If the fluorinated analogues were to act as substrates they should have been good competitive inhibitors in this bioassay. The results of the competition studies showed that all the analogues were reasonable competitive inhibitors, suggesting that the fluorine substituent

was not affecting the biological activity greatly (Table 2.7).

**Table 2.7** The effect of potential intermediates on $^{14}CO_2$ release from [$^{14}$C-1]homophenylalanine [13.6$\mu$M]

| Addition | Concentration | Activity |
|---|---|---|
| | $\mu$M | % |
| *o*-Fluoro HPhe | 200 | 71 |
| **(2.64)** | 500 | 59 |
| *m*-Fluoro HPhe | 200 | 81 |
| **(2.65)** | 500 | 66 |
| *p*-Fluoro HPhe | 200 | 89 |
| **(2.66)** | 500 | 80 |

100% Activity is with no addition of potential inhibitor.

Feeding experiments looking for the production of fluorinated aldoxime from microsomes incubated with fluorinated amino acid failed to show the production of any aldoxime, for any of the three analogues, using GC analysis. This may have been due to low turnover of the fluorinated amino acids in comparison to the unfluorinated compound. However, this result did not preclude the $^{19}$F NMR experiments which could still show the formation of the aldoxime, and/or the binding of the amino acid to the active site of the enzyme. However, initial studies with microsomal preparations incubated with $^{19}$F substrates failed to show any turnover of substrate, or product formation, probably due to the low levels of the enzyme in the preparation. This work requires further studies.

## 2.3.4 *Conclusions*

A number of potential intermediates for the homophenylalanine pathway have been synthesised and tested in a competition assay with [$^{14}$C-1]homophenylalanine. The results

showed inhibition for all the potential intermediates tested, giving no specific indication of the likely mechanism.

Studies looking at the retention or loss of the α-H from homophenylalanine during the oxidative decarboxylation reaction, using synthetic [$^{2}$H-2] and [$^{3}$H-2]homophenylalanine as substrates have yet to be completed, but initial results suggest that the C-H bond may be cleaved during aldoxime formation.

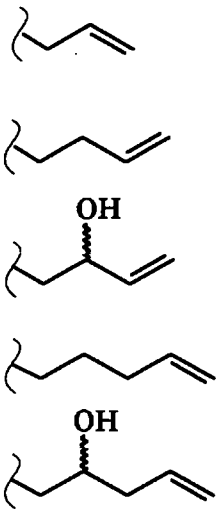
$^{19}$F NMR showed promise as a technique for studying enzyme bound intermediates, but the concentration of the enzyme in the microsomal preparation was too low to obtain any meaningful results. Further work depends on further purification of the enzymes involved, to allow higher concentrations of substrate to be used giving higher concentrations of product.

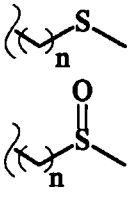
# 2.4 *Specificity of Aliphatic Amino Acid Decarboxylation*

It has already been established that the enzymes responsible for the oxidative decarboxylation of homophenylalanine (**2.2**) and dihomomethionine (**2.3**) are separate systems. There are a wide range of different aliphatic glucosinolates found in brassicas, and in *B. napus* there are a number of glucosinolates with different aliphatic chain lengths. This raises the question as to whether there is a single enzyme system responsible for the oxidative decarboxylation reaction to eventually form the different aliphatic glucosinolates in *B. napus*, or if each glucosinolate has its own specific enzyme system. In *B. napus* the two most common chain lengths found are the 3-butenyl and 4-pentenylglucosinolates[54]. In other glucosinolate containing species, such as *Arabidopsis thaliana,* a wider range of aliphatic glucosinolates is found with much longer chain lengths for the aliphatic side chain, and with retention of the sulfur atom in the side chain[136]. The sulfur atom can be in oxidised or unoxidised forms.

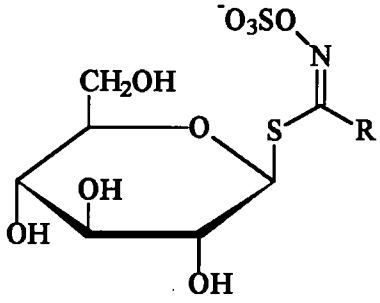
*B. napus* *A. thaliana*

R =





n = 3-8



**2.1**

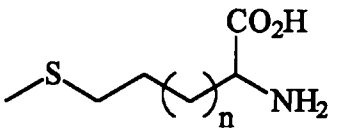
There are a number of questions which can be posed about aliphatic glucosinolate biosynthesis in *B. napus* and the specificity of the enzymes involved.

1. Is there a separate enzyme system for each aliphatic glucosinolate, or one system which can accept substrates of different lengths?
2. If there is only one enzyme system, are there limits (maximum and minimum) in the chain length of the starting amino acid?
3. Can there be modifications in the chain of the amino acid prior to the oxidative decarboxylation reaction? For example oxidation at sulfur, or isosteric or isoelectronic substitution of sulfur.

These questions could be answered by first looking at the methionine homologues corresponding to the aliphatic glucosinolates found in *B. napus*, and also at longer and shorter homologues.

## 2.4.1 *Methionine Homologues*

The studies carried out so far on the aliphatic glucosinolate system had utilised dihomomethionine (**2.3**) as the amino acid. The studies on methionine homologues required the synthesis of a range of chain extended amino acids from homomethionine (2-amino-5-methylthiopentanoic acid) (**2.70**) to tetrahomomethionine (2-amino-8-methylthiooctanoic acid) (**2.72**). This would include amino acids with both shorter and longer chain lengths than would be expected to be utilised by *B. napus* in glucosinolate biosynthesis.

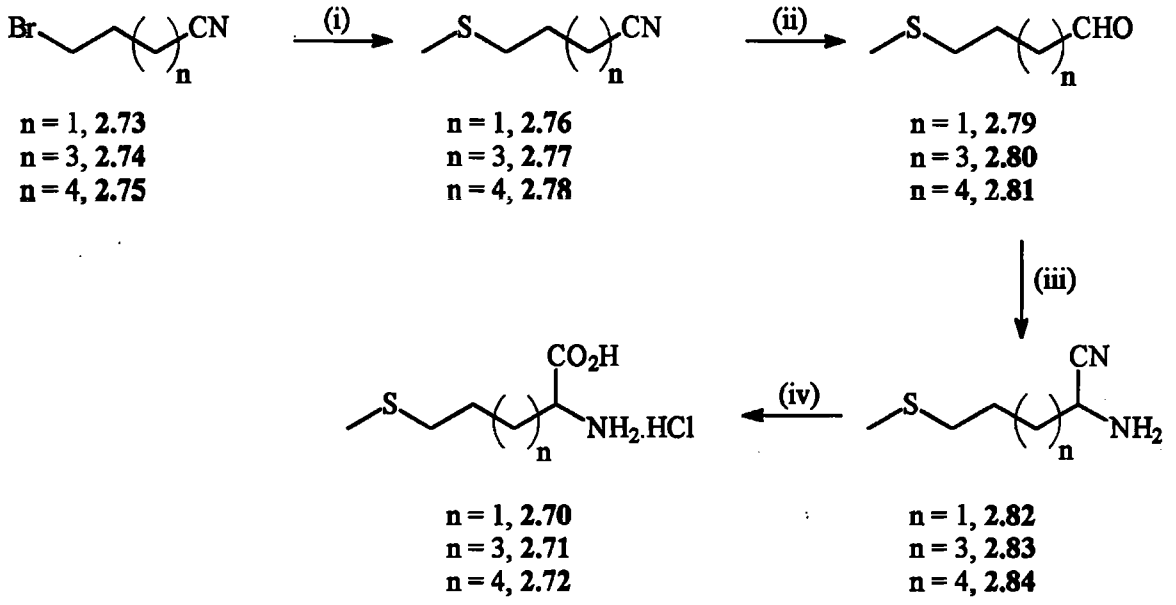



n = 1-4

n = 1, **2.70**
n = 2, **2.3**
n = 3, **2.71**
n = 4, **2.72**

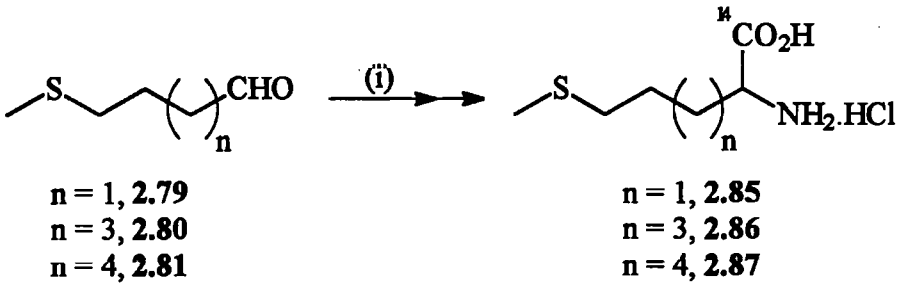
These amino acids were required in both [$^{14}$C-1]labelled and unlabelled forms. The [$^{14}$C-1]labelled forms would be used to look for intrinsic activity of the amino acid based on release of $^{14}CO_2$. The unlabelled forms would be required to carry out competition assays based on the levels of release of $^{14}CO_2$ from the [$^{14}$C-1]labelled amino acids, incubated with and without the unlabelled forms. These competition assays would be similar to those used for examining the intermediates in the oxidative decarboxylation of homophenylalanine.

The synthetic route chosen for both labelled and unlabelled forms was identical to that for the synthesis of the [$^{14}$C-1]labelled and unlabelled dihomomethionine, except for a change in the chain length of the starting ω-bromonitrile, underlining the general nature of this route.




(i) $MeS^-\ Na^+$, MeOH, (ii) a) DIBAL, $Et_2O$, -78°C, b) $H^+$(aq), (iii) KCN, $NH_4Cl$, $NH_3$(aq), EtOH, $H_2O$, (iv) a) HCl (conc.), b) $H^+$(aq).

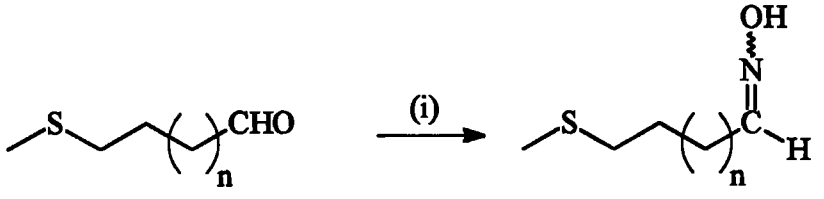
Synthesis of the aldehydes (**2.79-2.81**) required was accomplished in satisfactory yield (18-45% for two steps from ω-bromonitrile). These were then used to obtain the three methionine homologues (**2.70-2.72**) using the Strecker synthesis in moderate yields (30-70% from the aldehyde), and high purity. There seemed to be no correlation between yield and chain length, or any other parameters.




(i) a) $K^{14}CN$, $NH_4Cl$, $NH_3$, EtOH, $H_2O$, b) HCl (conc.), c) $H^+$(aq).

The route was then adapted to for the radiolabelled synthesis of the [$^{14}$C-1]methionine homologues. This incorporated K$^{14}$CN giving the three [$^{14}$C-1]labelled methionine homologues (**2.85-2.87**)(8-40% yield based on K$^{14}$CN). Again there was no correlation of yield with chain length. However, the lowest yield was always obtained in the reactions associated with the synthesis of unlabelled and labelled homomethionine (**2.70, 2.85**).

The aldehyde intermediates (**2.79-2.81**) were also used to synthesise the corresponding aldoximes (**2.88-2.90**) as before, using hydroxylamine hydrochloride in aqueous ethanol.




n = 1, **2.79**
n = 3, **2.80**
n = 4, **2.81**

n = 1, **2.88**
n = 3, **2.89**
n = 4, **2.90**

(i) $NH_2OH.HCl$, $NaHCO_3$ , EtOH, $H_2O$.

The [$^{14}$C-1]methionine homologues (**2.85-2.87**) were then tested to see if they released $^{14}CO_2$ in a similar manner and under similar conditions as [$^{14}$C-1]dihomomethionine (**2.21**) when incubated with a microsomal preparation from *B. napus*.

**Table 2.8** The release of $^{14}CO_2$ from [$^{14}$C-1]methionine homologues

| [$^{14}$C-1]substrate | Activity range nmol $g^{-1}$ $h^{-1}$ | $K_m$ $\mu$M |
|---|---|---|
| DL-HMet (**2.85**) | 0 | |
| DL-DHMet (**2.3**) | 220-280 | 45 |
| DL-THMet (**2.86**) | 170-200 | 60 |
| DL-TetHMet (**2.87**) | 85-130 | 90 |

The results in Table 2.8 clearly show that there was no apparent activity for [$^{14}$C-1]homomethionine (**2.85**). This was not as expected, as homomethionine is the proposed biogenetic precursor for 2-propenylglucosinolate (sinigrin). Although this glucosinolate is only found in very small amounts in *B. napus* it would be expected that there would be some activity for the substrate in this system. This result obviously raised a number of questions as to the biogenetic precursor of 2-propenylglucosinolate in *B. napus*, and other plants which produce large amounts of this glucosinolate.

The results from the other methionine homologues showed maximum release of $^{14}CO_2$ for [$^{14}$C-1]dihomomethionine (**2.21**), and a reduction in $^{14}CO_2$ release with increasing chain length (**2.86, 2.87**). It was interesting to note that there was release of $^{14}CO_2$ from [$^{14}$C-1]tetrahomomethionine (**2.87**) even though there is no glucosinolate corresponding to this amino acid found in *B. napus*. The $K_m$ values also show a similar trend with increasing values found with increasing chain length, suggesting that the longer methionine homologues bind to the enzyme less well than dihomomethionine. This suggests an enzyme active site where a minimum chain length is essential, but there is a degree of flexibility to allow longer chain amino acids than those normally encountered by the natural system.

These four [$^{14}$C-1]amino acids were also used to characterise further the enzymes found in the microsomal preparation which are responsible for the oxidative

decarboxylation reaction. A range of chemicals and enzymes known to remove various oxygen species were used to determine the oxygen species required for the oxidative decarboxylation reaction[117]. The addition of free radical scavengers had no effect on activity, nor did the addition of catalase, which destroys hydrogen peroxide. However, the combined addition of glucose and glucose oxidase, which had the effect of removing molecular oxygen from the preparation, resulted in a significant loss of activity. These combined data suggested that the oxygen species required for the oxidative decarboxylation of amino acids was molecular oxygen. This result needs further confirmation by the use of $^{18}O_2$ and the analysis of aldoxime formed for the presence of $^{18}O$. The requirement for molecular oxygen does not differentiate between the enzymes being flavin monooxygenases or cytochromes P450.

The activities shown for the trihomomethionine and tetrahomomethionine showed identical enzyme properties to those already found for dihomomethionine[114]. These included pH maxima at pH 7.5, and similar effects from the range of potential inhibitors used for dihomomethionine. The results of these experiments suggested that there was a single enzyme system responsible for the oxidative decarboxylation of the longer chain methionine homologues, which showed maximum activity with dihomomethionine.

Further confirmation of this hypothesis was obtained from a series of competition assays, as already used in examining the mechanism of the oxidative decarboxylation reaction. Homologues showing release of $^{14}CO_2$ from [$^{14}$C-1]amino acids were incubated with the full sequence of unlabelled methionine homologues from methionine to tetrahomomethionine, shown in Table 2.9.

**Table 2.9** The effect of unlabelled Met homologues on $^{14}CO_2$ release from [$^{14}$C-1]Met homologues

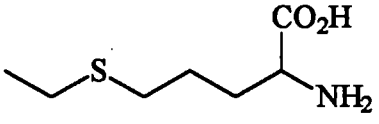
| Addition | Concentration | [$^{14}$C-1]Methionine homologue | | |
|---|---|---|---|---|
| | | DHMet (2.21) | THMet (2.86) | TetHMet (2.87) |
| | $\mu$M | % | % | % |
| L-Met | 250 | 96 | 94 | 100 |
| | 500 | 93 | 90 | 97 |
| DL-HMet | 250 | 118 | 98 | 100 |
| (2.70) | 500 | 91 | 100 | 100 |
| DL-DHMet | 250 | | 51 | 39 |
| (2.3) | 500 | | 22 | 18 |
| DL-THMet | 250 | 30 | | 29 |
| (2.71) | 500 | 14 | | 12 |
| DL-TetHMet | 250 | 47 | 53 | |
| (2.72) | 500 | 19 | 19 | |

100% Activity is with no addition of potential inhibitor.

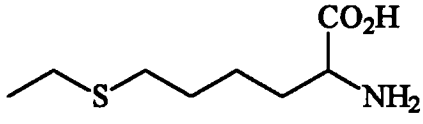
The absence of any inhibition by homomethionine (**2.70**) of $^{14}CO_2$ release from longer [$^{14}$C-1]methionine homologues confirmed the result found with [$^{14}$C-1]homomethionine, namely that it was not accepted as a substrate or inhibitor by the enzyme system found in microsomes from *B. napus*. The cross competitive inhibition seen with the longer methionine homologues again suggested a single enzyme system to be responsible for the oxidative decarboxylation of the amino acids. The specificity shown by the enzyme system raised further questions as to why homomethionine is not accepted as a substrate, and why it did not act as a competitive inhibitor, for the other methionine analogues. These questions were explored by looking at some other analogues of dihomomethionine.

## 2.4.2 *Dihomomethionine Analogues*

The lack of activity obtained with homomethionine either as a substrate or an inhibitor, raised questions as to whether it was not accepted as a substrate or inhibitor because of the total length of the molecule, or the length of the side chain, or because of the distance between the amino acid functionality and the sulfur in the side chain. To examine these different hypotheses homoethionine (**2.91**) and dihomoethionine (**2.92**) were required. These are similar to the methionine homologues, but with the S-methyl group being replaced by an S-ethyl group.

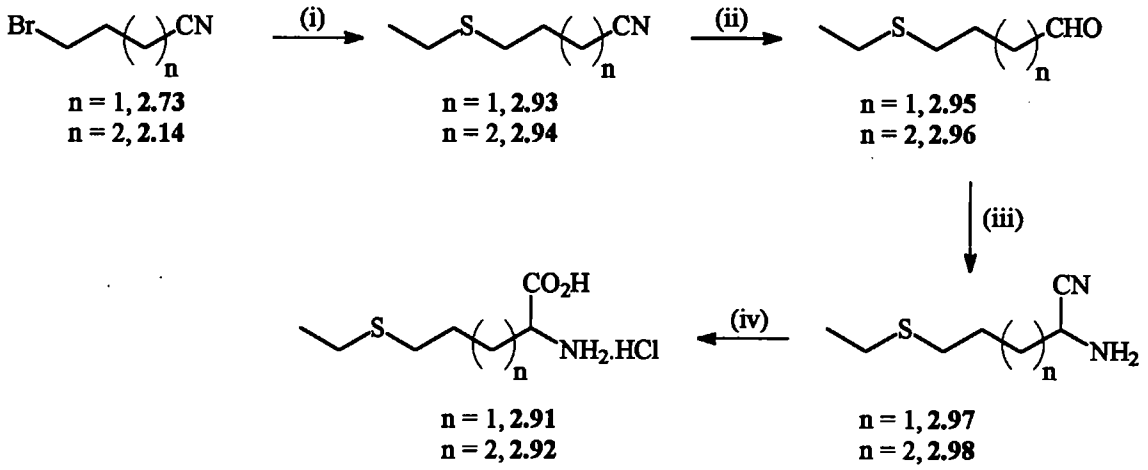



**2.91**




**2.92**

Homoethionine (**2.91**) has the same overall chain length as dihomomethionine (**2.3**), but with the sulfur atom moved one position closer to the amino acid end of the molecule. This should show whether the correct overall chain length or the correct distance between the sulfur atom and the amino acid group was required for activity. If the sulfur to amino acid distance was crucial, results obtained from dihomoethionine (**2.92**) would show if the size of the group attached to the sulfur was important, or if the enzyme system allows some degree of flexibility at this point. The two amino acids were synthesised using the standard route for the methionine homologues, but with substitution of methanethiol with ethanethiol.




(i) $EtS^- Na^+$, MeOH, (ii) a) DIBAL, $Et_2O$, -78°C, b) $H^+(aq)$, (iii) KCN, $NH_4Cl$, $NH_3(aq)$, EtOH, $H_2O$, (iv) a) HCl (conc.), b) $H^+(aq)$.

The synthesis of the two ethanethioalkanenitriles (**2.93, 2.94**) proceeded in high yield (83-89%). However, the reduction reactions to the aldehydes (**2.95, 2.96**) gave poorer yields than expected (11-33%), partly because of the need to distil the aldehydes before they decomposed. The two ethionine homologues (**2.91, 2.92**) were both obtained in moderate yield (15-35%) from the corresponding aldehydes (**2.95, 2.96**). These were then tested with [$^{14}$C-1]dihomomethionine (**2.21**) and [$^{14}$C-1]trihomomethionine (**2.86**) as potential competitive inhibitors of $^{14}CO_2$ release.

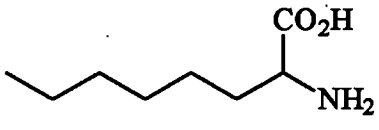
**Table 2.10** The effect of unlabelled DHMet analogues on $^{14}CO_2$ release from [$^{14}$C-1]Met homologues

| | [$^{14}$C-1]Methionine homologue | |
|---|---|---|
| | DHMet (**2.21**) | THMet (**2.86**) |
| Analogue | % Activity | % Activity |
| HEt (**2.91**) | 59 | 57 |
| DHEt (**2.92**) | 12 | 9 |

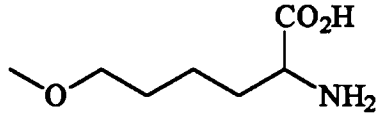
100% Activity is with no addition of potential inhibitor.

The results for both ethionine homologues (**2.91, 2.92**) were similar for testing against both [$^{14}$C-1]dihomomethionine (**2.21**) and [$^{14}$C-1]trihomomethionine (**2.86**), as shown in Table 2.10. This again suggested that there was a single enzyme system responsible for the oxidative decarboxylation of methionine homologues in *B.napus*. Homoethionine (**2.91**) showed a significant degree of inhibition, although not sufficient enough to say unequivocally that the overall chain length was crucial for activity and the sulfur amino acid distance is unimportant. Dihomoethionine (**2.92**) showed a very high degree of activity as an inhibitor at the concentrations tested, which suggested that the replacement of a methyl group with an ethyl group in the side chain was unimportant. As the overall chain length of this analogue was the same as for trihomomethionine (**2.71**), and the competition assays gave almost identical results, this also suggested that overall chain length was more important than the distance from the sulfur atom to the amino acid functionality.

The importance of the sulfur atom in the side chain was examined by isosteric replacement of the sulfur with a methylene group to give **2.99** and isoelectronic replacement with oxygen to give **2.100**, as shown.




**2.99**

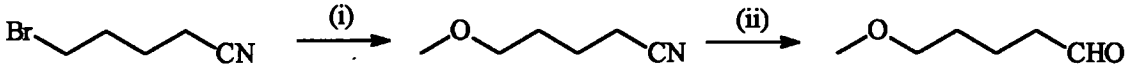



**2.100**

The synthesis of the methylene replacement (**2.99**) was readily accomplished by using readily available heptanal as the starting material in the Strecker amino acid synthesis. However, the yields obtained were low when compared to other amino acids synthesised using this route (7% from the aldehyde). This may have been due to the nature of the product which was relatively insoluble in dilute acid, or base, possibly due to the

hydrophobic nature of the aliphatic chain.

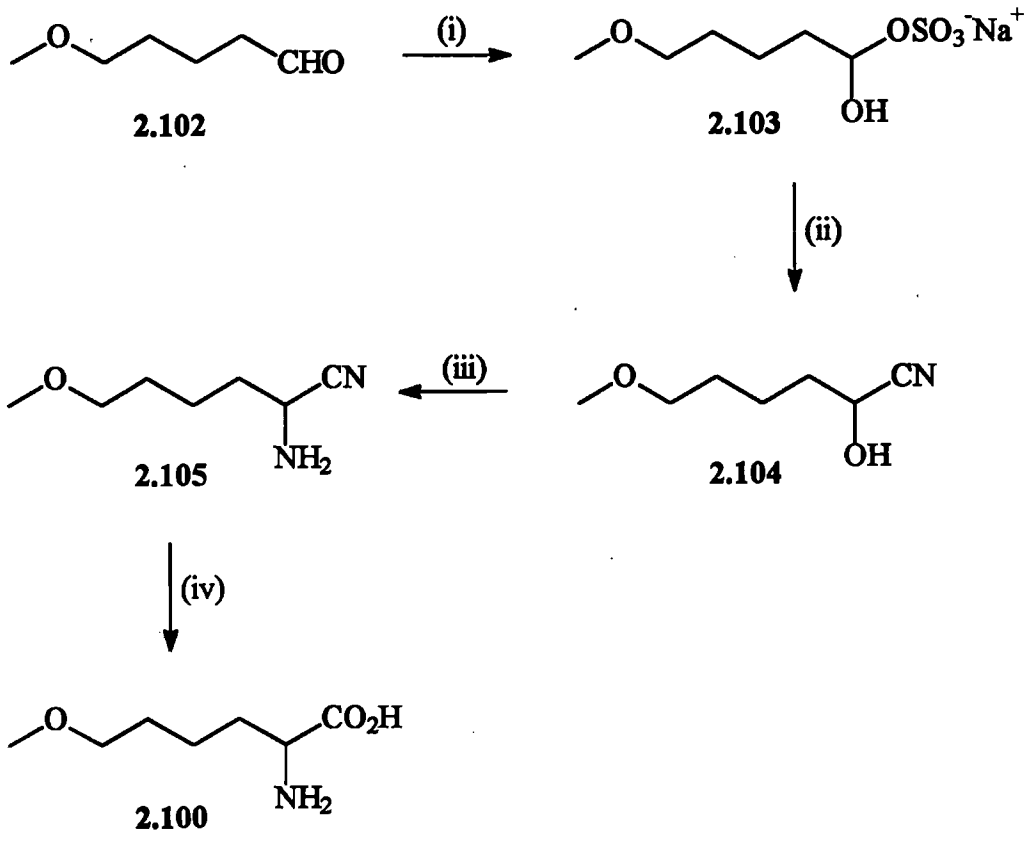
The synthesis of the amino acid with replacement of sulfur by oxygen was more difficult than predicted. The obvious route was just an extension of the standard method used for the methionine homologues with methanol used instead of methanethiol as the nucleophile to attack the starting ω-bromonitrile (**2.14**). The first two steps to form the aldehyde (**2.102**) via the nitrile (**2.101**) worked in reasonable yield as would be expected (31% for two steps). However, there were problems in the work up of all the reactions, with difficulties in extracting the product from the aqueous layer.




**2.14** **2.101** **2.102**

(i) $MeO^-\ Na^+$, MeOH, (ii) a) DIBAL, $Et_2O$, -78°C, b) $H^+(aq)$.

Attempts at using the standard Strecker conditions to synthesise the amino acid met with failure, giving only a complex mixture with only traces of the required product. To overcome this problem the conditions were modified. Firstly, the aldehyde (**2.102**) was treated with sodium bisulfite, to give the aldehyde-bisulfite addition complex (**2.103**). This was followed by treatment with potassium cyanide which gave the cyanohydrin (**2.104**) in high purity, but low yield (27%). The cyanohydrin was then converted to the aminonitrile (2.105) with dry methanolic ammonia which gave the crude product in excellent yield (94%). This was used without further purification. Hydrolysis of the aminonitrile (**2.105**) with hydrochloric acid gave the crude product which was purified using cationic and anionic exchange resins to give the pure product (**2.100**) as its ammonium salt (35%).




2.102 2.103 2.104 2.105 2.100

(i) $NaHSO_3$ , $H_2O$, (ii) KCN, $H_2O$, (iii) $NH_3$ , MeOH, (iv) a) HCl (conc.), b) $H^+$(aq).

This gave the two sulfur replacement amino acids (**2.99, 2.100**) to test in the competition assay with [$^{14}$C-1]dihomomethionine (**2.21**) and [$^{14}$C-1]trihomomethionine (**2.86**).

**Table 2.11** The effect of unlabelled DHMet analogues on $^{14}CO_2$ release from [$^{14}$C-1]Met homologues

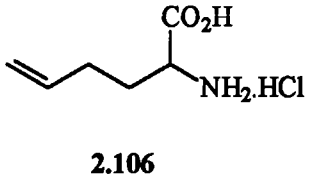
| | [$^{14}$C-1]Methionine homologue | |
|---|---|---|
| | DHMet (**2.21**) | THMet (**2.86**) |
| Analogue | % Activity | % Activity |
| 'O' DHMet (**2.100**) | 100 | 97 |
| '$CH_2$' DHMet (**2.99**) | 17 | 15 |

100% Activity is with no addition of potential inhibitor.

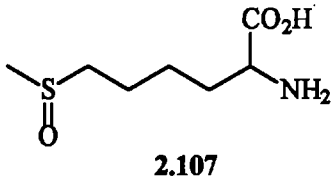
Again, as shown in Table 2.11, there were no significant differences between the results obtained with [$^{14}$C-1]dihomomethionine (**2.21**) and those obtained with [$^{14}$C-1]trihomomethionine (**2.86**). The results showed very clearly that the oxygen analogue (**2.100**) did not act as a competitive inhibitor of $^{14}CO_2$ release from either [$^{14}$C-1]dihomomethionine (**2.21**) or [$^{14}$C-1]trihomomethionine (**2.86**). This suggested that the sulfur atom was important in binding at the active site, as isoelectronic replacement by oxygen, gave an inactive molecule in this assay. However, oxygen has a higher electron density than sulfur, and this may interfere with binding of the side chain. Replacement of sulfur by methylene gave a potent inhibitor (**2.99**). However, problems associated with its solubility meant that the pH of the buffer containing the amino acid had to be significantly adjusted in order to get it to dissolve. This would have had a slight effect on the overall pH of the assay mixture, and as the enzyme system has been shown to be pH sensitive[114], the activity of the compound may be explained by this pH change. Alternatively there may have been some non-specific binding interactions caused by the hydrophobic side chain of the amino acid. However, the possibility that the methylene analogue was simply acting as a good competitive inhibitor could not be ruled out. This would mean that the enzyme active site for the aliphatic system required a hydrophobic side chain for good binding.

Other interesting compounds to examine in this competition assay were the amino acids corresponding to known modifications in the side chain found in the final glucosinolate. These modifications include hydroxylation, oxidation at sulfur, and the nominal loss of methanethiol to give the terminal double bond. The hydroxylation reaction to give glucosinolates such as (*R*)-2-hydroxy-3-butenylglucosinolate (progoitrin) is known to take place after glucosinolate biosynthesis[85,86], so the corresponding amino acid was not examined at this point. However, it is not known when the oxidation at sulfur takes place, or the nominal loss of methanethiol to give aliphatic glucosinolates with terminal double bonds in the side chain, although both are thought to be post glucosinolate biosynthesis

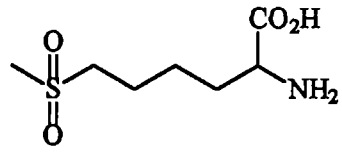
modifications[137]. To examine this further, the amino acids shown (**2.106-2.108**) were examined for activity as inhibitors.




**2.106**

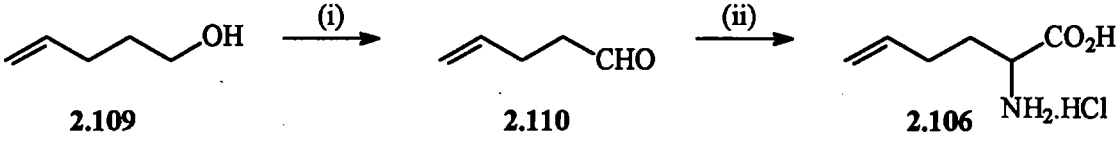



**2.107**




**2.108**

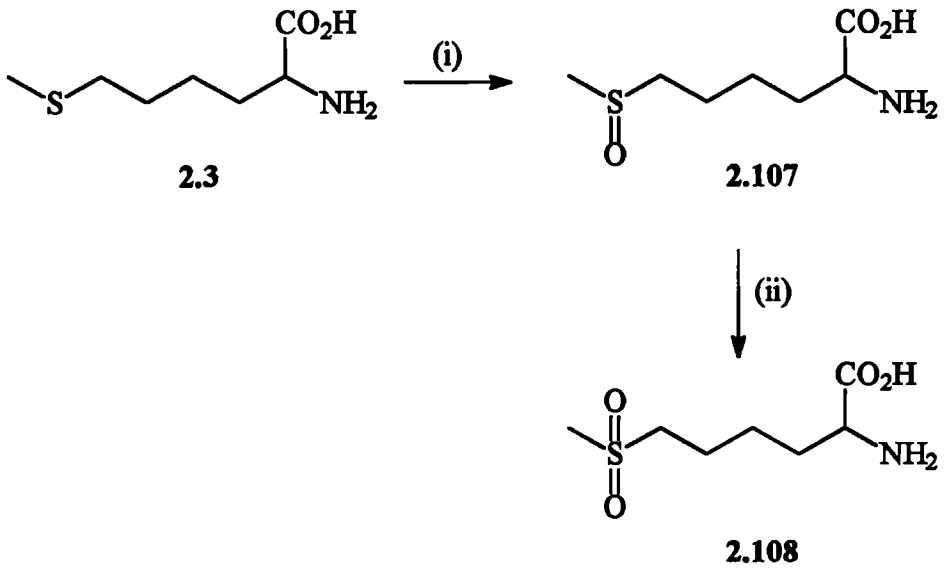
The alkenyl amino acid (2-amino-5-hexenoic acid) (**2.106**) was synthesised using standard Strecker conditions in poor yield (17% from the aldehyde). The unsaturated aldehyde (**2.110**) for the Strecker synthesis was obtained by PCC oxidation of the alcohol (**2.109**).




**2.109** **2.110** **2.106**

(i) PCC, $CH_2Cl_2$ , (ii) a) KCN, $NH_4Cl$, $NH_3$(aq), EtOH, $H_2O$, b) HCl (conc.), c) $H^+$(aq).

The dihomomethionine sulfoxide (**2.107**) and sulfone (**2.108**) were obtained by oxidation of the free amino acid (**2.3**) with peracetic acid using the method of Lepp and Dunn[138]. The sulfoxide was obtained by use of one mole equivalent and the sulfone by use of two mole equivalents with heating.




(i) AcOOH (1 eq.), AcOH, (ii) AcOOH (1 eq.), AcOH, Δ.

The three amino acids (**2.106-2.108**) were then used in the competition bioassay with [$^{14}$C-1]dihomomethionine (**2.21**) and [$^{14}$C-1]trihomomethionine (**2.86**). As shown in Table 2.12, none of the three amino acids tested showed any significant activity as inhibitors of $^{14}CO_2$ release, suggesting that the sulfur oxidation and the nominal loss of methanethiol are post glucosinolate biosynthesis modifications. This result was as expected, but further work needs to be carried out on the nominal loss of methanethiol, or an oxidised equivalent, to form the alkenyl glucosinolates found in *B. napus*.

**Table 2.12** The effect of unlabelled DHMet analogues on $^{14}CO_2$ release from [$^{14}$C-1]Met homologues

| | [$^{14}$C-1]Methionine homologue | |
|---|---|---|
| | DHMet (**2.21**) | THMet (**2.86**) |
| Analogue | % Activity | % Activity |
| DHMet SO (**2.107**) | 96 | 93 |
| DHMet $SO_2$ (**2.108**) | 89 | 100 |
| Alkenyl (**2.106**) | 100 | 100 |

100% Activity is with no addition of potential inhibitor.

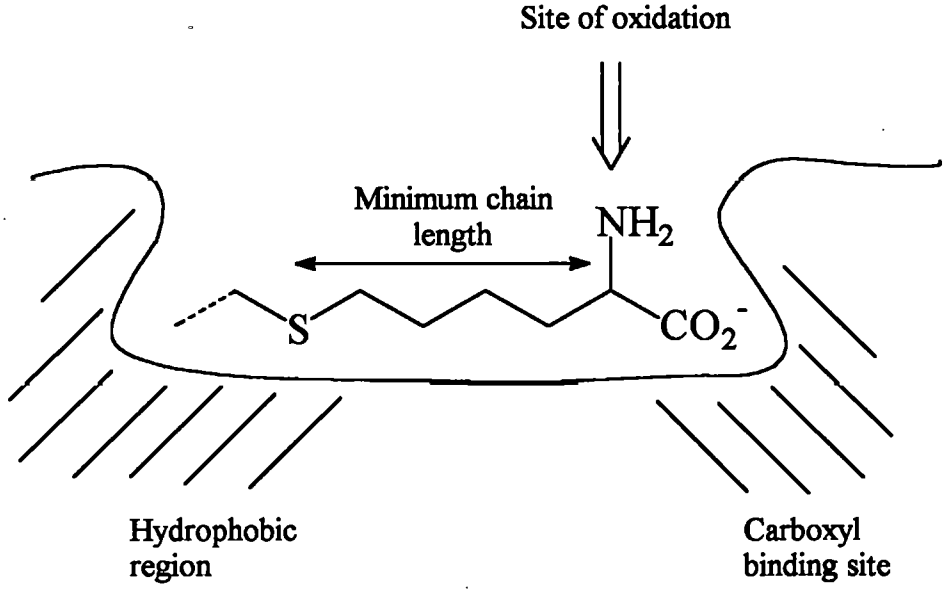
## 2.4.3 *Enzyme Active Site Model*

The information obtained from the competition assays with methionine homologues and dihomomethionine analogues was used to propose a model of the proposed active site of the enzyme system responsible for the oxidative decarboxylation reaction. This model was based on the results obtained to date, and as such is not complete, but it gives a good idea of likely active site of the enzyme.

There were a number important points to be noted from the results obtained from the assays looking at loss of $^{14}CO_2$ from [$^{14}$C-1]amino acids, and competition assays looking at inhibition of $^{14}CO_2$ release following addition of unlabelled amino acids.

1. Homomethionine was not accepted as a substrate, nor was it active as a competitive inhibitor for any of the activities found. This implied that there was minimum chain length required for activity.
2. Activity of methionine homologues in terms of decreasing release of $^{14}CO_2$ and increasing $K_m$ values, decreased with increasing chain length of the methionine homologues.
3. Moving the sulfur atom one position closer to the amino acid group in dihomomethionine reduced activity, but did not abolish it.
4. Replacement of the terminal methyl group with ethyl had little effect on activity.
5. Replacement of sulfur by oxygen abolished activity as an inhibitor, although replacement by methylene was possible. This second result needs further investigation due to the physico-chemical properties of the molecule involved.
6. Oxidation of sulfur, or removal of methanethiol to form the terminal double bond found in the final glucosinolate, abolished all activity.

When all these results were taken into consideration a picture of the active site could be built up. There was likely to be binding at both ends of the molecule. The loss of activity from analogues with sulfur replacement and sulfur oxidation, implied binding at this end of the molecule. The requirement to keep the amino group of the amino acid in the correct position for oxidation meant that there was likely to be binding of the carboxyl group. These two binding sites needed to be a minimum distance apart, as shown by the lack of activity with homomethionine, and the decrease in activity as an inhibitor when the sulfur was moved by one position in the chain. Oxidation of the sulfur, or its replacement by oxygen resulted in total loss of activity, suggesting possibly a sulfur specific binding site, although replacement by methylene was possible. This may mean that the side chain need only be hydrophobic. With longer chain homologues activity was retained suggesting a degree of flexibility in the active site, with the aliphatic chain being able to bend and still be accommodated in the active site, while still binding at the carboxyl group and the sulfur. These ideas are summarised in a diagram showing the proposed active site of the enzyme involved in the oxidative decarboxylation of methionine homologues.



## 2.4.4 *Conclusions*

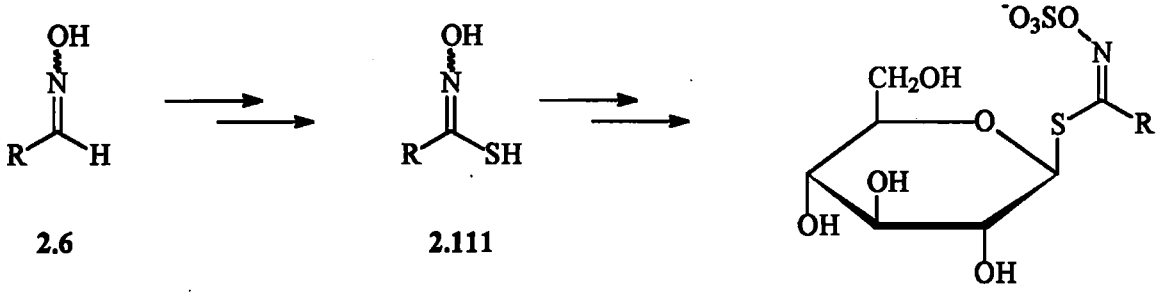
A series of methionine homologues have been synthesised in [$^{14}$C-1] and unlabelled forms using the Strecker amino acid synthesis. This route has generally proved very successful for the wide range of amino acids synthesised, although some problems were encountered with the oxygen analogue of dihomomethionine. The yields varied from poor to good, although no attempt was made to maximise yields as the amino acids were only required in relatively small quantities for testing.

These amino acids have been used to show that there is likely to be a single enzyme system responsible for the oxidative decarboxylation of longer chain methionine homologues in *B. napus*. However, no activity was found for methionine or homomethionine. This was unexpected for homomethionine, since there is a known glucosinolate, found in small amounts in *B. napus*, expected to have homomethionine as its biogenetic precursor.

A range of analogues has been synthesised to gain a better understanding of the enzyme active site. This has allowed the development of an active site model which incorporates a hydrophobic binding pocket for the aliphatic chain, which must be of a minimum length, and a binding site for the carboxyl group of the amino acid.

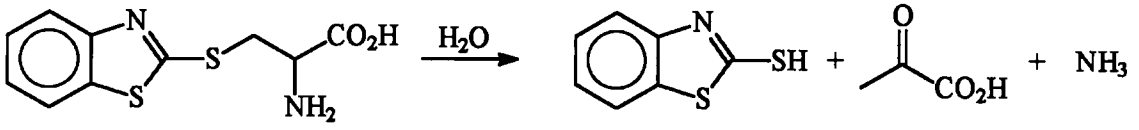
## 2.5 *Sulfur Addition*

After formation of the aldoxime (**2.6**), the next step in the glucosinolate biosynthetic pathway is the addition of sulfur to form a thiohydroximic acid (**2.111**)[43]. The thiohydroximic acid is then glucosylated and finally addition of sulfate forms the glucosinolate (**2.1**). The source of sulfur in the thiohydroximic acid formation is thought to be cysteine, although some incorporation from methionine has also been shown[79].




**2.6** **2.111** **2.1**

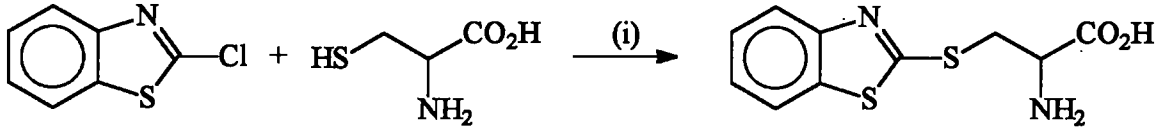
If the sulfur donor is cysteine, then there must be cleavage of the cysteine C-S bond at some point in the transformation from the aldoxime (**2.6**) to the thiohydroximic acid (**2.111**). This allowed the use of an artificial substrate, *S*-(benzothiazolyl)cysteine (**2.112**), to assay for the enzyme activity involved in cleavage of this C-S bond[139]. This artificial cysteine conjugate is cleaved at the C-S bond by $\beta$-lyase enzymes to give 2-mercaptobenzothiazole (**2.113**), pyruvic acid and ammonia. An assay can then be used either to look for the production of 2-mercaptobenzothiazole (**2.113**) using a UV based method[139], or at the production of pyruvic acid with derivatisation and monitoring by UV.



**2.112** **2.113**

The *S*-(benzothiazolyl)cysteine (**2.112**) was synthesised by the reaction of 2-chlorobenzothiazole (**2.114**) with the disodium salt of cysteine (**2.115**) in liquid ammonia[139]. This gave the product in moderate yield (38%), although when scaling up the reaction it was important to ensure that all the sodium had dissolved in liquid ammonia before addition of the cysteine.

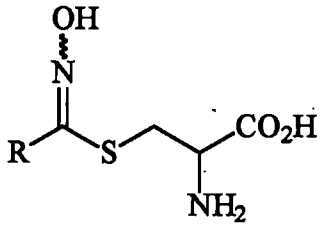
(i) Na, $NH_3$ (l)



**2.114** **2.115** **2.112**

The cysteine conjugate (**2.112**) thus formed could then be used to assay for activity in tissues from *B. napus* known to produce glucosinolates. Using an assay based on production of pyruvic acid and analysis by UV it was possible to find C-S lyase activity in preparations from young expanding leaves of *B. napus*[140].

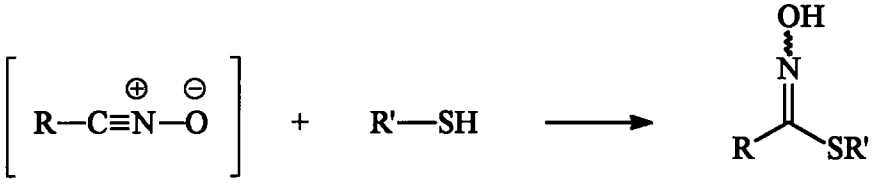
Although the artificial cysteine conjugate (**2.112**) is useful for assaying for C-S lyase activity it is a non-specific probe. There are C-S lyases found in different aspects of plant metabolism. Thus, another more specific, probe was required to study further the C-S lyase activity which had been identified. This specific probe chosen was the proposed conjugate formed as an intermediate during glucosinolate biosynthesis, a thiohydroximate with cysteine elaborated through sulfur at C-1 of the thiohydroximate (**2.116**). This has been suggested as an intermediate in glucosinolate biosynthesis[80].



**2.116**

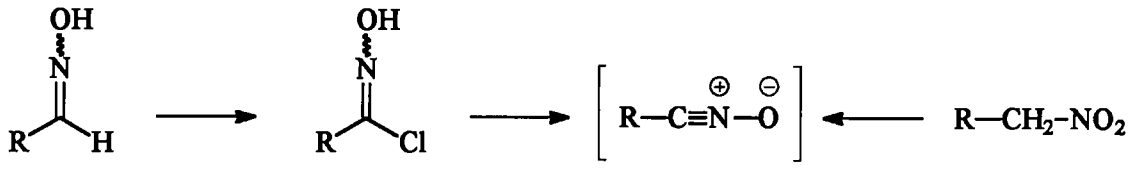
The cysteine thiohydroximate conjugate (**2.116**) could then be used as a more specific probe for C-S lyase activity, and also as confirmation that the sulfur donor in thiohydroximate formation is cysteine. The assay would again look for production of pyruvic acid, with monitoring by UV following derivatisation with 2,4-dinitrophenylhydrazine to give a suitable chromophore.

The synthetic route chosen to form **2.116** was based on nitrile oxide methodology. 1,3-Dipolar addition of thiols (**2.118**) is known to take place across nitrile oxides (**2.117**), to give thiohydroximates (**2.119**), and this was used as a starting point for the synthesis[132,141].



**2.117** **2.118** **2.119**

There are two main routes for the generation of the unstable nitrile oxides (**2.117**). The first, and most common, is the dehydrogenation of the corresponding aldoxime (**2.6**), often via dehydrochlorination of the corresponding hydroximoyl chloride (**2.120**)[132]. The second, less used route is by dehydration of the primary nitroalkane (**2.121**) with isocyanates[142].

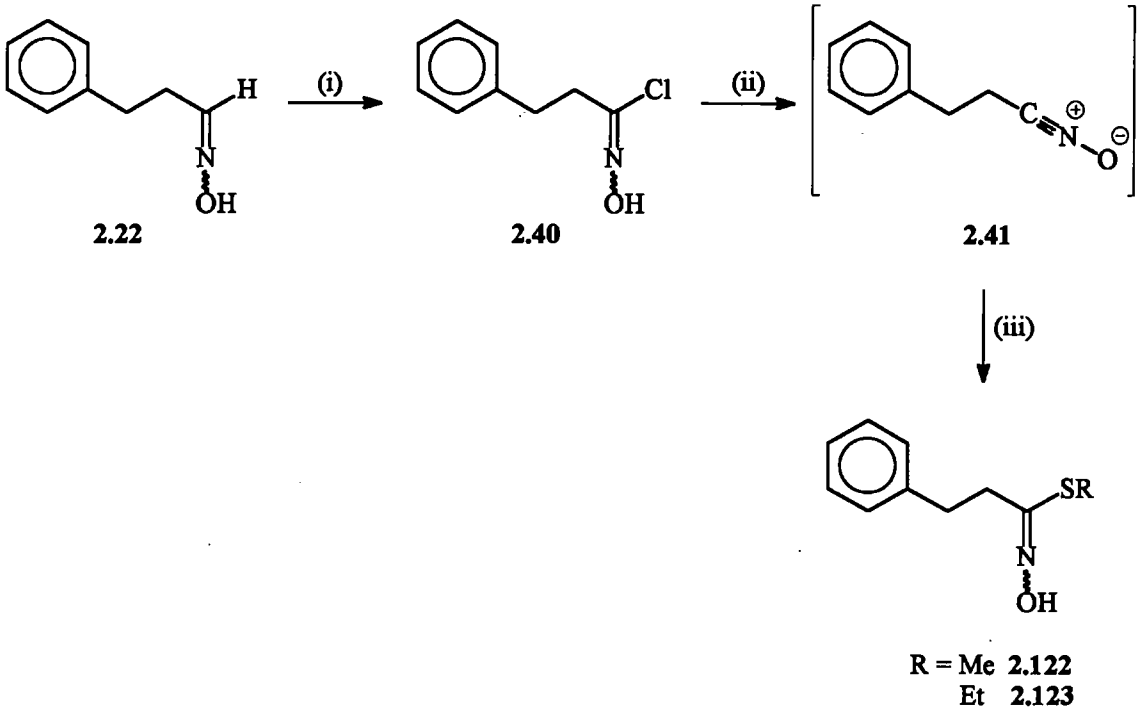


**2.6** **2.120** **2.117** **2.121**

Since a range of aldoximes was already available, it was decided to use the approach from the aldoxime. The hydroximoyl chloride (**2.120**) can be synthesised from the aldoxime (**2.6**) using a variety of chlorinating agents[143-147]. The most common reagent used is chlorine gas[49,146], but this can be difficult to regulate accurately and can result in chlorination of other parts of the molecule. Other chlorinating agents that have been used include *N*-chlorosuccinimide[144], nitrosyl chloride[132], *tert*-butyl hypochlorite[145], or anhydrous hydrogen chloride with oxone® in DMF[143].

In order to explore a simple one-pot procedure for the synthesis of the thiohydroximates chlorine gas was employed as the chlorinating agent. This facilitated removal of the excess chlorinating agent from the reaction mixture. Methyl and ethyl thiohydroximates (**2.122, 2.123**) were chosen as model compounds to develop the synthetic route to the proposed cysteine conjugate. Early methods for generating the hydroximoyl chlorides used in glucosinolate synthesis used chlorine gas with diethyl ether as the solvent and reaction temperatures of -60°C[49]. Attempts to reproduce this work using 3-phenylpropanaldoxime (**2.22**) met with failure due to the insolubility of this particular aldoxime in diethyl ether at the concentrations and temperatures reported[49]. For this reason it was decided to change the solvent to THF. The aldoxime (**2.22**) was treated with dry chlorine gas[148] at -78°C, and the formation of the hydroximoyl chloride (**2.40**) was apparent with the generation of a characteristic sky blue colour, which is actually the nitroso compound in equilibrium with the hydroximoyl chloride[49]. This was then treated with methanethiol or ethanethiol, and followed by dropwise addition of an excess of triethylamine to generate the nitrile oxide (**2.41**) *in situ*. This underwent 1,3 addition with

the alkanethiol to give the desired thiohydroximates. The yields obtained for the two model compounds were poor (25-37%), but products were obtained in high purity after recrystallisation.



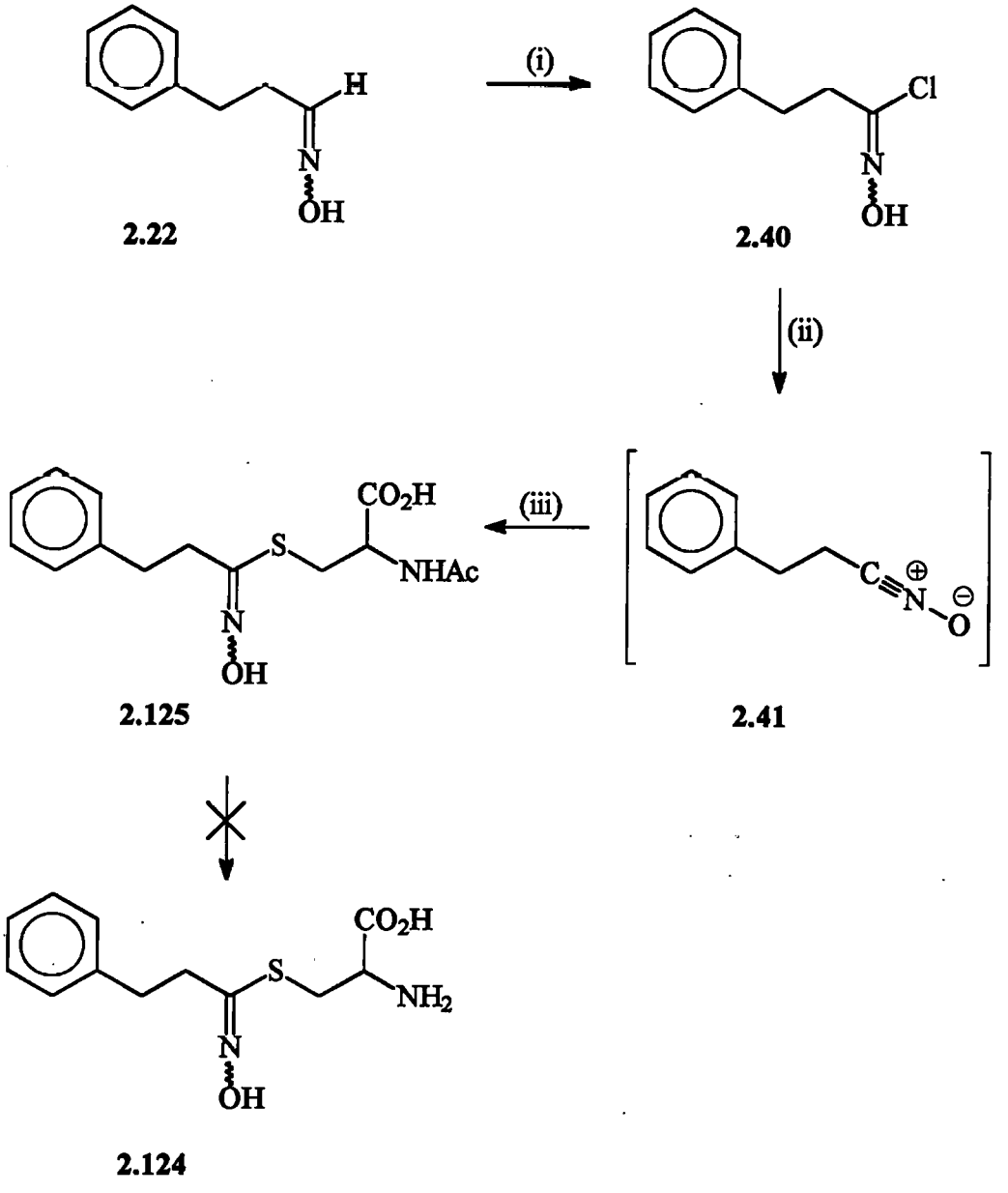

**2.22** **2.40** **2.41**

R = Me **2.122**
Et **2.123**

(i) $Cl_2$ (g), THF, -78°C, (ii) $Et_3N$, (iii) RSH.

The poor yields obtained may have resulted from incomplete chlorination of the aldoxime, or due to the relative instability of alkylhydroximoyl chlorides, which are more unstable than the corresponding arylhydroximoyl chlorides. However, the products were obtained in high purity, so it was decided to attempt the synthesis of the proposed cysteine conjugate (**2.124**) using this route. Use of unmodified cysteine as the sulfur source was impractical due to the insolubility of cysteine in organic solvents. For this reason it was decided to use the readily available *N*-acetylcysteine, which is more soluble, and then to remove the acetyl group chemically or enzymically after the formation of the cysteine

elaborated thiohydroximate (**2.125**).



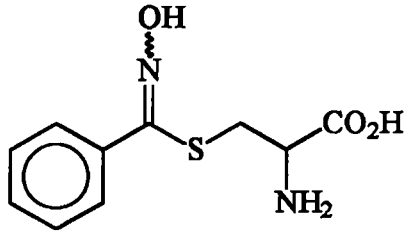

**2.22** **2.40** **2.125** **2.41** **2.124**

(i) $Cl_2$ (g), THF, -78°C, (ii) $Et_3N$, (iii) *N*-AcCysteine

Using this route the *N*-acetylcysteine conjugate (**2.125**) was synthesised in poor yield (20%). Attempted chemical deacetylation to form the free amino acid (**2.124**) using a range of conditions either resulted in no reaction, or in hydrolysis to form 3-phenylpropanoic acid. Enzymic deacetylation was attempted using acylase 1 from porcine

kidney[111]. This appeared to have a low hydrolysis rate for this substrate, but further attempts at deacetylation with acylase 1 from *Aspergillus* sp.[111] were not attempted due to a lack of material. Further attempts at synthesis of more acetylated conjugate (**2.125**) proved unsuccessful by this route.

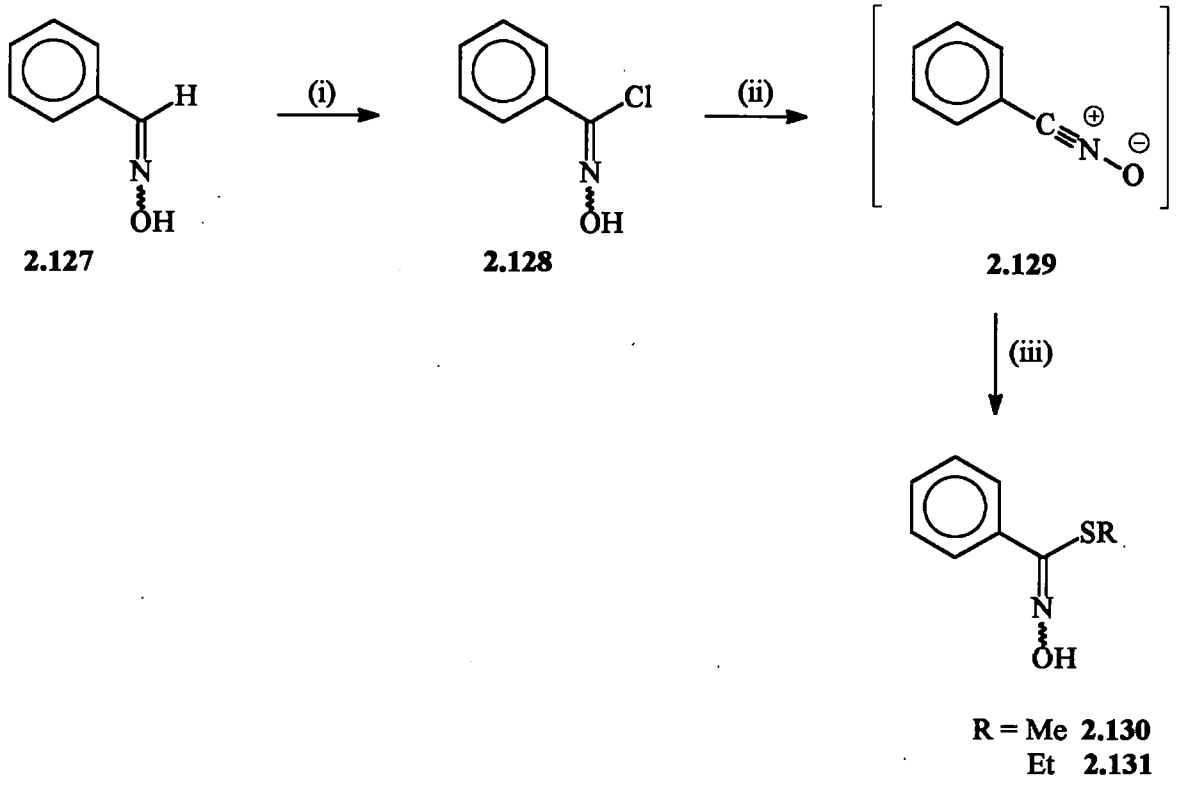
The glucosinolate biosynthetic pathway is thought to be non-specific after aldoxime formation. This meant that it was possible to use benzaldoxime as the starting material for the synthesis instead of 3-phenylpropanaldoxime. This would give a different conjugate (**2.126**) but this should still be accepted by the C-S lyase responsible for the C-S bond cleavage in glucosinolate biosynthesis.




**2.126**

The synthesis of this compound (**2.126**) should be more straightforward than for the corresponding phenylethyl compound due to the increased stability of arylhydroximoyl chlorides and nitrile oxides, compared to their alkyl equivalents[132].

The route was investigated first with using methanethiol and ethanethiol, as the source of sulfur, in order to optimise the reaction conditions. Because of the increased stability, and hence decreased reactivity of the aryl nitrile oxides compared to their aliphatic counterparts, the reaction temperature was raised to 0°C. Yields obtained were slightly better than those obtained for the phenylethylthiohydroximate (39-56%). However, attempts to use this method to couple *N*-acetylcysteine with the nitrile oxide proved unsuccessful. It was then decided to abandon the one-pot approach, and to isolate the hydroximoyl chloride before proceeding with generation of the nitrile oxide.

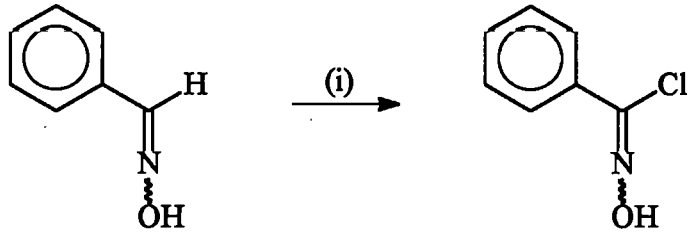



**2.127** **2.128** **2.129**

R = Me **2.130**
Et **2.131**

(i) $Cl_2$ (g), THF, 0°C, (ii) $Et_3N$, (iii) RSH.

For the next approach a different chlorinating agent could be used. Literature reports had suggested the use of *N*-chlorosuccinimide in DMF[144]. Synthesis of benzohydroximoyl chloride (**2.128**) was accomplished in excellent yield (95%) using this reagent.

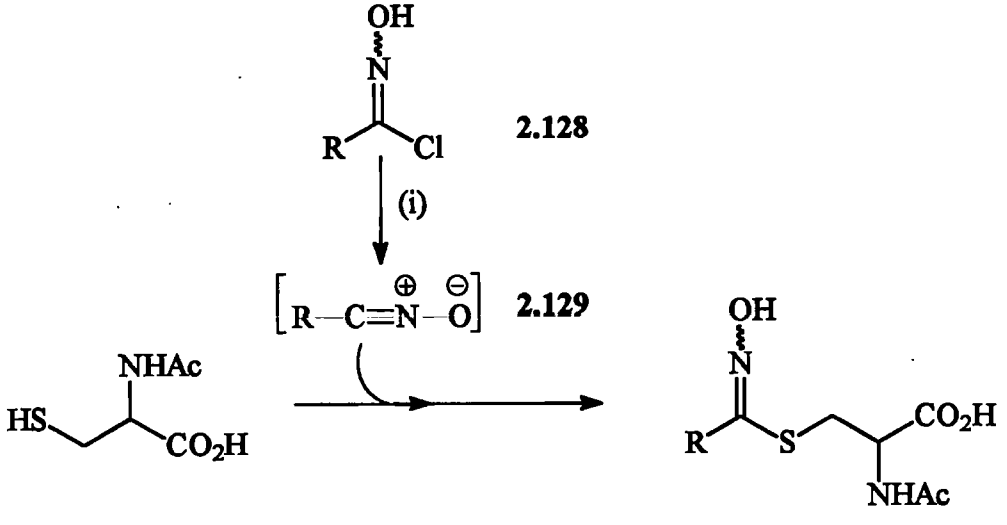



**2.127** **2.128**

(i) NCS, DMF.

The product (**2.128**) was then used without further purification to generate the nitrile oxide (**2.129**) as before, by the dropwise addition of triethylamine, followed by reaction with ethanethiol. This gave the thiohydroximate (**2.131**) in 85% yield, or 80% yield for the whole reaction, which is a considerable improvement on the one-pot approach with chlorine gas as the chlorinating agent. This two step approach was then used in the coupling reaction of the nitrile oxide with *N*-acetyl cysteine.

Although excellent yields had been obtained for the model reaction, with cysteine providing the sulfur nucleophile no reaction was seen. This problem was overcome by the use of an inverse addition procedure. *N*-Acetylcysteine (**2.132**) was stirred with a four fold excess of triethylamine in dry THF, converting the sulfur of the cysteine to $S^-$ which is more nucleophilic than SH. The benzohydroximoyl chloride (**2.128**) was then added dropwise to the reaction mixture, so that the nitrile oxide (**2.129**) was only ever present at low concentrations in the reaction mixture, with an effective excess of the activated cysteine. Acidic workup then gave the desired compound (**2.133**) in good yield (73%), as a white crystalline solid which precipitated out of solution.




**2.132**

R = Ph- **2.133**

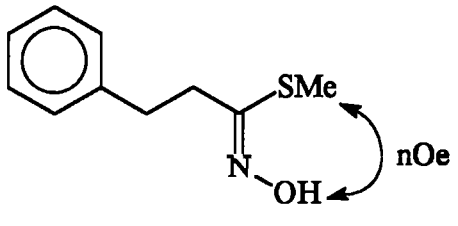
(i) $Et_3N$, THF.

This method of isolating the hydroximoyl chloride first, followed by an inverse addition reaction was also used to synthesise the original *N*-acetylated target (**2.125**), from 3-phenylpropanaldoxime (**2.22**), where R = $PhCH_2CH_2$-. The hydroximoyl chloride (**2.40**) was isolated in high (90%) yield, and was used immediately, before it decomposed. A model reaction of the nitrile oxide (**2.41**) with ethanethiol gave the ethylthiohydroximate (**2.123**) in 86% yield. The inverse addition approach was then used to give the *N*-acetyl cysteine thiohydroximate (**2.125**) in good yield (68%).

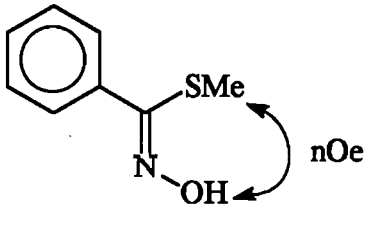
As with all the thiohydroximates synthesised this reaction gave almost exclusively one isomer about the C=N bond. This contrasts with the aldoximes synthesised which always gave a mixture of isomers before purification. The geometry about the C=N bond was examined using 2D NMR techniques. Previous studies on **2.130** had suggested that the single isomer to be formed would be the (*Z*)-isomer[149,150]. Interconversion between the two isomers is possible using UV light, but the (*E*)-isomer naturally converts back to the more thermodynamically stable (*Z*)-isomer[149]. 2D NOESY experiments were performed to look for nOe's between the -OH of the thiohydroximate and any protons on either side of the double bond. Experimental conditions had to be modified to look at a wide frequency window as the -OH proton appeared at $\delta$ 10.8-11.8 in the $^1$H NMR spectrum. Deuterochloroform was not a suitable solvent as this did not give sufficiently sharp peaks for the acidic protons, even at elevated temperatures. However, $d_6$-dimethylsulfoxide gave very sharp peaks for the -OH proton and hence was used as the NMR solvent for this range of experiments.

Initial studies with the *N*-acetylated proposed intermediates (**2.125** and **2.133**) did not show sufficiently strong nOe signals to give conclusive answers as to the exact nature of the stereochemistry around the C=N double bond. Therefore it was decided to look at the S-methylated thiohydroximates (**2.122** and **2.130**). These both gave clear results with the -OH proton giving an nOe with the methyl protons and not with protons on the other

side of the C=N double bond.




**2.122**




**2.130**

This defines the stereochemistry as the (*Z*)-isomer for both compounds, which agrees with earlier reports which used dipole moments to assign the stereochemistry for **2.130**[150]. The (*Z*) stereochemistry is also that found in allylglucosinolate (sinigrin) which is the only glucosinolate which has had its stereochemistry defined by X-ray crystallography[45]. Although it was not possible to show similar results for the *N*-acetylated proposed intermediates it seems reasonable to extrapolate the results for the S-methylated thiohydroximates to include these compounds.

The acetylated compounds were enzymically deactylated before the assay for C-S lyase activity, to give the free amino acid. These two cysteine thiohydroximates (**2.125** and **2.130**) are currently being used to assay for C-S lyase activity, results of which will be reported elsewhere.

## 2.5.1 *Conclusions*

An artificial cysteine conjugate has been synthesised to assay for C-S lyase activity in *B. napus*. The C-S lyase activity identified using this assay was further studied using the proposed intermediate involved in the conversion of aldoximes to thiohydroximic acids

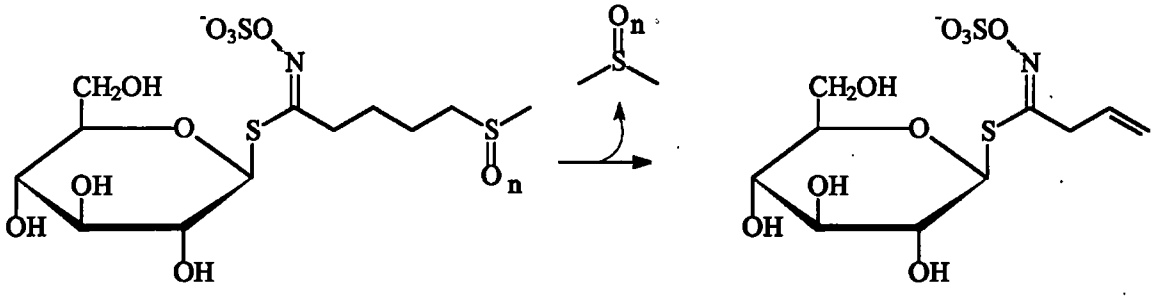
in glucosinolate biosynthesis. The synthesis of these intermediates was accomplished using nitrile oxide methodology. The nitrile oxides were first generated from the hydroximoyl chloride via the direct chlorination of the aldoxime with chlorine gas, and subsequent dehydrochlorination with triethylamine to give the nitrile oxide *in situ*. Addition of simple thiols was successful using this method, but to synthesise the proposed intermediate a different approach had to be adopted. This involved chlorination of the aldoxime with NCS and isolation of the hydroximoyl chloride. This was then used in an inverse addition reaction with *N*-acetylcysteine to give the proposed intermediate in high yield. This was then used to study further the C-S lyase activity already identified.

2D NOESY studies were used to confirm the stereochemistry about the C=N double bond as the expected (*Z*)-isomer for the S-methylated thiohydroximates.

# 2.6 *Retained Label Studies*

Studies on biosynthetic pathways are often enhanced by experiments in which an isotopic label is retained from one intermediate to the next. Retained labelled studies are the most reliable method of proving that a particular intermediate is actually on the main pathway of interest, and is not a by product relating to the particular assay conditions used. When a labelled substrate is fed to a whole organism, an enzyme or an enzyme preparation, and a labelled product is obtained, this is confirmation that there is a transformation of substrate to product, which cannot always be confirmed by studies with non-labelled substrates.

A number of questions can be raised about the results obtained so far on the glucosinolate biosynthetic pathway which can be answered by the use of substrates where the label is retained in the product. The most interesting question relates to the nature of the biogenetic precursor of 2-propenylglucosinolate, which has always been thought to be derived from homomethionine. However, the results obtained from the studies with [$^{14}$C-1]homomethionine, and the competition assays with unlabelled homomethionine, suggest that this may not be the case. There is a possibility that the terminal double bond in 2-propenylglucosinolate (**2.134**) is not derived from the nominal loss of methanethiol from the homomethionine moiety, but from loss of dimethyl sulfide, or an oxidised equivalent, such as dimethyl sulfoxide from a dihomomethionine product (**2.135**). Thus, for this




n = 0, 1, 2. **2.135** **2.134**

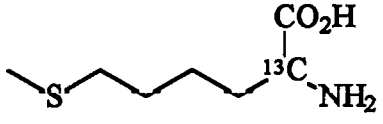
situation to occur, the starting amino acid would have to be dihomomethionine.

To examine this hypothesis a retained label form of dihomomethionine was required to see it was incorporated into 2-propenylglucosinolate.

## 2.6.1 *Labelled Dihomomethionine*

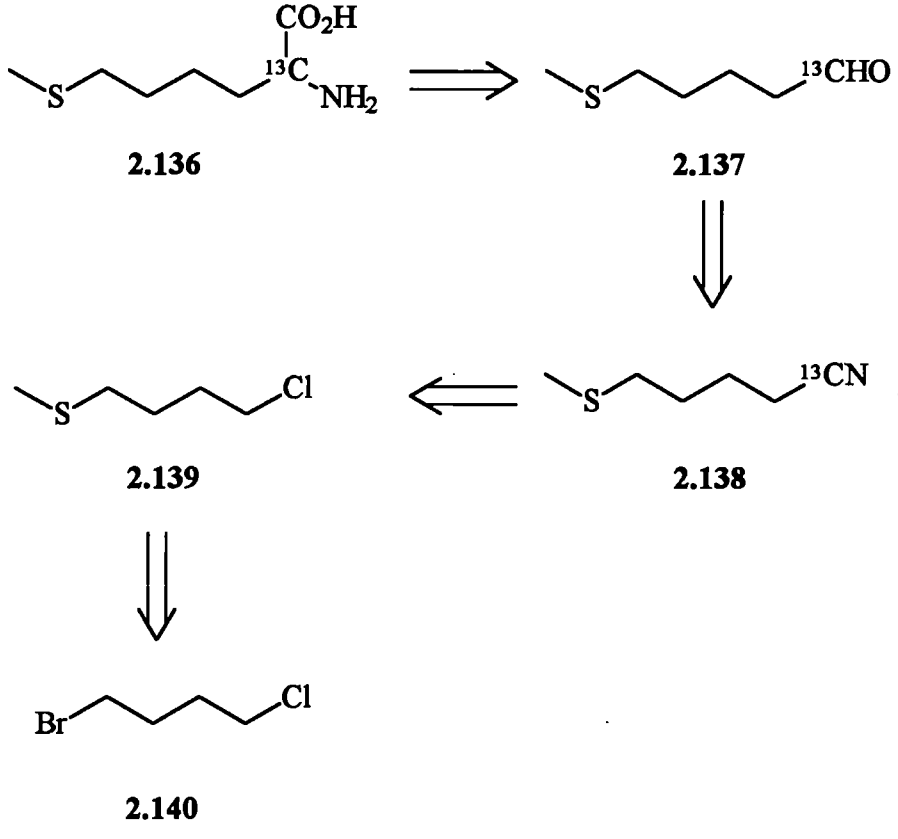
The label to be introduced as a retained label can be a number of different isotopes for both carbon and hydrogen. For example, $^{13}C$ or $^{14}C$ for a carbon label, or $^{2}H$ or $^{3}H$ for a hydrogen label. Radioactive labels have the advantage of a much higher sensitivity over stable isotope labels, but bring with them the increased problems associated with small scale radiolabelled synthesis, handling, and the need for dedicated equipment for product analysis. The stable isotopes have the advantage that NMR and MS techniques can readily be employed to follow the biosynthetic pathway and for these reasons $^{13}C$ was chosen for this study. The objective was to feed a plant known to produce high amounts of 2-propenylglucosinolate such as *Brassica nigra* (black mustard) or *B. juncea* (oriental mustard) with the $^{13}C$ labelled dihomomethionine, and then isolate the 2-propenylglucosinolate formed as its desulfo analogue using HPLC and examine this product for incorporation of the label. The HPLC separation should ensure that pure desulfo-2-propenylglucosinolate is isolated for analysis by both $^{13}C$ NMR and mass spectrometry.

The label would need to be in the aliphatic chain of the dihomoethionine in order to be retained, thereby giving the synthetic target [$^{13}C$-2]dihomomethionine (**2.136**).




**2.136**

A retrosynthetic route was devised based on the introduction of the label as $^{13}C$ cyanide. A four carbon mixed halide (**2.140**) was chosen to react with methanethiol. The other halogen could then be displaced by labelled cyanide to form the nitrile (**2.138**). Reduction of the labelled nitrile would form the aldehyde (**2.137**), followed by a Strecker synthesis to give the amino acid (**2.136**).

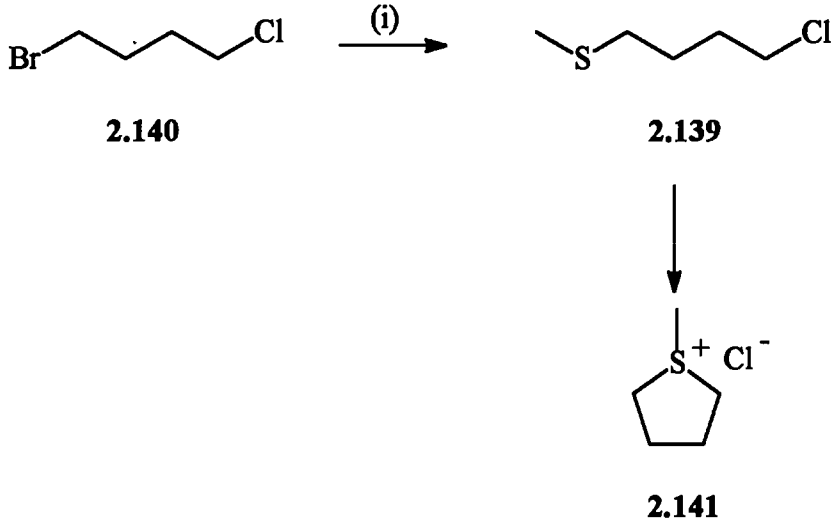



**2.136** **2.137**

**2.139** **2.138**

**2.140**

Preliminary experiments were conducted with unlabelled material to establish the best reaction conditions. However, problems were found with the first reaction in the sequence. On formation, the 1-chloro-4-methylthiobutane (**2.139**) cyclised to give the corresponding sulfonium salt[151,152].

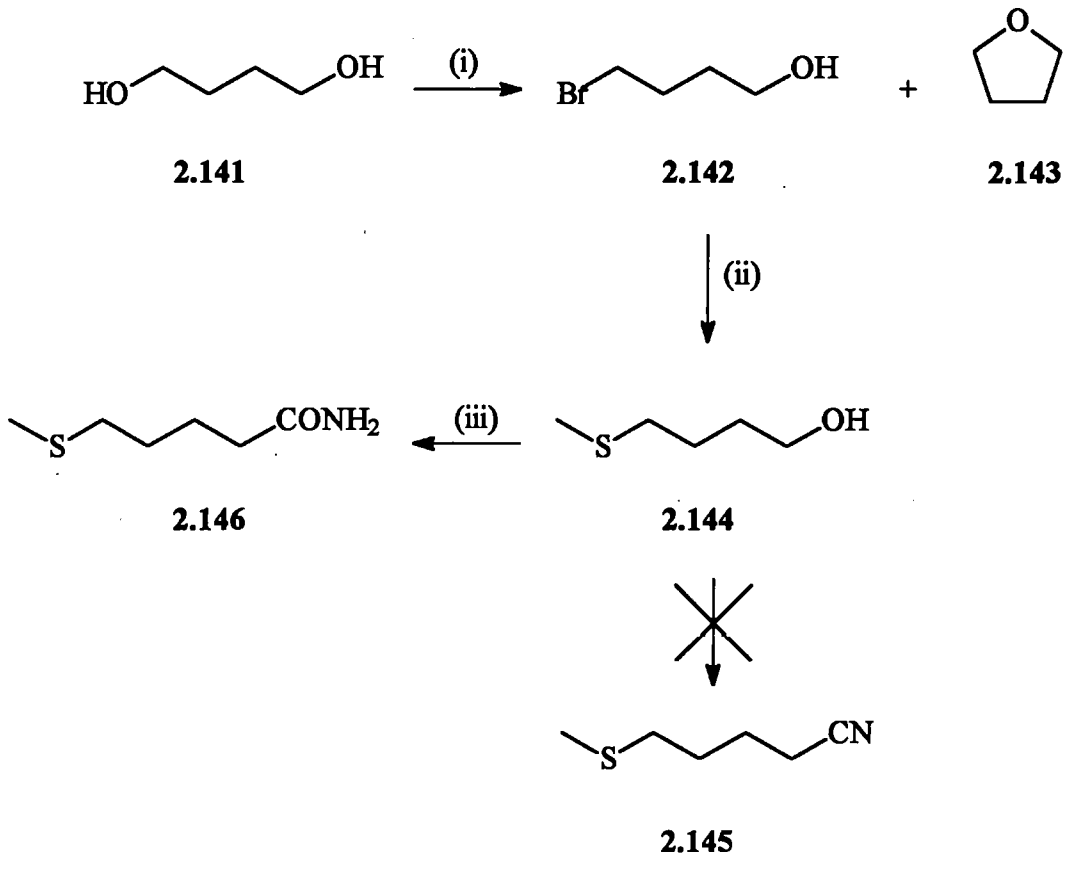



**2.140** **2.139**

**2.141**

(i) $MeS^-Na^+$, MeOH.

This cyclisation would obviously have led to problems with the important next step, which was the addition of the labelled cyanide, so alternative approaches were sought. Attempts at using a different leaving group, such as tosyl instead of chloride, led to even quicker formation of the sulfonium salt. The direct substitution of an alcohol by cyanide has been reported[153]. This required the synthesis of 4-methylthiobutanol (**2.144**), which was obtained from the corresponding bromoalcohol (**2.142**). The bromoalcohol was not readily available and had to be synthesised from 1,4-butanediol (**2.141**), using hydrobromic acid with continuous extraction. This reaction gave a poor yield (16%) of 4-bromobutanol (**2.142**), due to the concurrent formation of tetrahydrofuran (**2.143**) before extraction could take place. This bromoalcohol (**2.142**) was then converted the methylthio compound (**2.144**) with the sodium salt of methanethiol in reasonable yield (76%). However, using 4-methylthiobutanol (**2.144**) as the starting material in the cyanide displacement reaction, the product obtained was the amide (**2.146**) instead of the nitrile (**2.145**), with some unreacted starting material (**2.144**).




**2.141** **2.142** **2.143**

**2.146** **2.144**

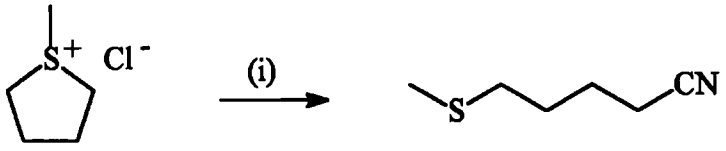
**2.145**

(i) HBr, (ii) $MeS^-Na^+$, MeOH, (iii) TMSCl, KI (cat), KCN, DMF, MeCN.

Other routes to [$^{13}$C-1]dihomomethionine (**2.136**) were also considered, such as the introduction of the nitrile group using radical chemistry[154,155]. However, all the possibilities examined required a large (5-6 fold) excess of cyanide, which is obviously inappropriate in a synthesis using labelled cyanide, where the most important reagent is the labelled cyanide. For this reason it was decided to reexamine the original route, and see if it could be modified in any way to give the desired product in satisfactory yield.

An attempt was made to see if the sulfonium salt (**2.141**) would react with potassium cyanide to form the desired nitrile (**2.145**). The reaction was successful, although the yield obtained was poor (30%) and the product contained a number of by-

products.



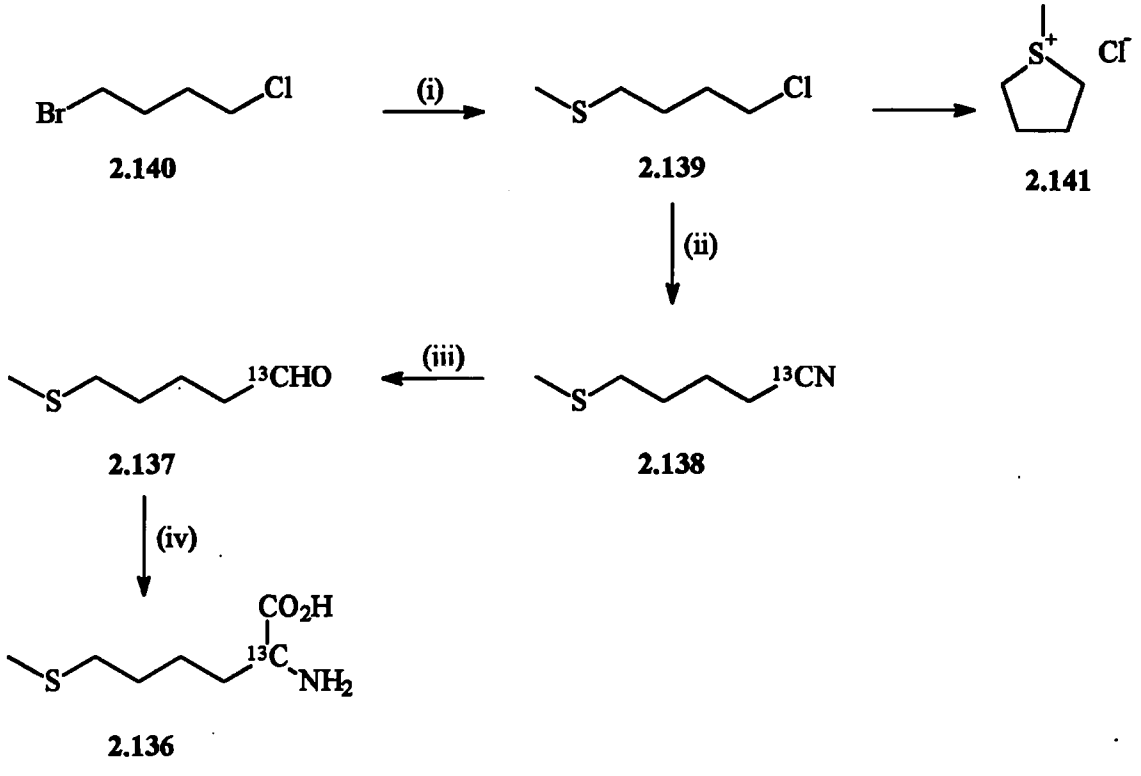

**2.141** **2.145**

(i) KCN, 18-crown-6, MeCN, Δ.

This led to strategies for limiting the cyclisation reaction to **2.141**, and reacting the cyanide with uncyclised precursor (**2.139**). Various reaction conditions were used, but eventually conditions were found which maximised the yield based on the label. It had been noticed that the sulfonium salt (**2.141**) only formed after a number of hours after isolation of the 1-chloro-4-methylthiobutane (**2.139**), so it was possible to use the uncyclised compound in the reaction with labelled cyanide, provided that the reaction was carried out immediately after isolation of the 1-chloro-4-methylthiobutane. Excess 1-chloro-4-methylthiobutane (**2.139**) was used in the reaction with cyanide, so that any unreacted material cyclised to the sulfonium salt (**2.141**), which was water soluble, and hence was easy to remove in the work up of the reaction. The conditions which gave the best yield of the required 5-methylthiopentanenitrile (**2.145**) involved refluxing an excess of 1-chloro-4-methylthiobutane with cyanide in aqueous ethanol.

With the reaction conditions optimised using unlabelled material, the reactions to synthesise [$^{13}$C-2]dihomomethionine (**2.136**) were carried out. A slight excess of methanethiol was used in reaction with 1-bromo-4-chlorobutane (**2.140**), to ensure that no dihalogenated compound remained which could react with the cyanide, wasting valuable label. Any bis-*S*-methylated compound would be removed at a later stage in the synthesis. The 1-chloro-4-methylthiobutane (**2.139**) was then used immediately, reacting with the $^{13}$C cyanide to give a moderate (36%) yield of the nitrile (**2.138**) with 100% incorporation of

the label. This was then reduced to the aldehyde (**2.137**) with DIBAL, in moderate yield (34%). Transformation to the amino acid using standard Strecker conditions, with purification of the amino nitrile by acid/base extraction, and subsequent purification of the amino acid by ion exchange chromatography gave [$^{13}$C-2]dihomomethionine (**2.136**) in 33% yield for the two steps. All the reaction products to the nitrile, were examined by GC, as well as NMR, as this gave an accurate figure on the purity of the product. This thorough examination of all the reaction conditions in the pathway allowed a seemingly unpromising route to be developed for the synthesis of [$^{13}$C-2]dihomomethionine (**2.136**).

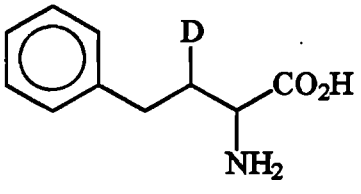



i) $MeS^-Na^+$, MeOH, (ii) $K^{13}CN$, EtOH, $H_2O$, Δ, (iii) a) DIBAL, $Et_2O$, n-hexane, -78°C, b) $H^+$(aq), (iv) a) KCN, $NH_4Cl$, $NH_3$ , EtOH, $H_2O$, b) c. HCl, c) $H^+$(aq).

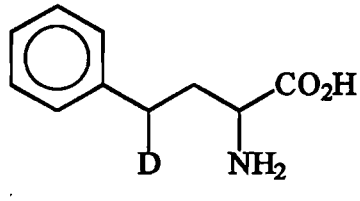
The [$^{13}$C-2]dihomomethionine will be used in feeding studies to investigate incorporation into 2-propenylglucosinolate.

## 2.6.2 *Labelled Homophenylalanine*

Earlier studies had shown that the product of feeding homophenylalanine to the microsomal preparation from *B. napus* was 3-phenylpropanaldoxime. However, this result was not unequivocal proof that the aldoxime came from the homophenylalanine that had been added to the system. To confirm that the added homophenylalanine gave the aldoxime a retained label substrate needed to be used. The earlier success with detection of the aldoxime by GC and GC-MS suggested that this would provide a good detection system for retained label studies. It was decided to use $^{2}H$ as the label, as this could easily be introduced into the substrate, at both C-3 (**2.147**) and C-4 (**2.148**).

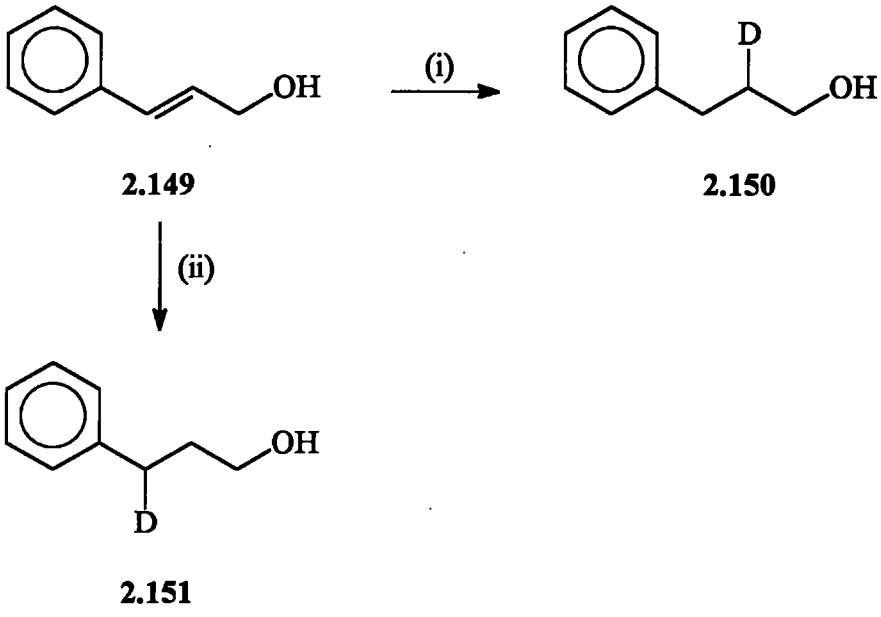



**2.147**




**2.148**

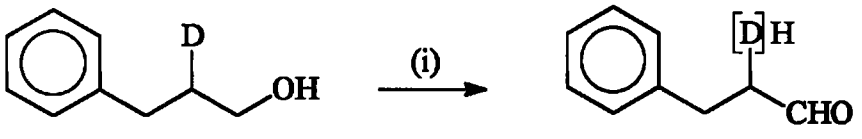
The label could be introduced into both positions by utilising a variation of the reduction reaction used in the fluorinated homophenylalanine synthesis. Cinnamyl alcohol (**2.149**) can be reduced to 3-phenylpropan-1-ol by reduction with lithium aluminium hydride and work up with water[156,157]. Substitution of lithium aluminium deuteride for the hydride would give selective labelling at C-2 of the alcohol (**2.150**). Substitution of the water for $D_2O$ would give selective labelling at C-3 of the alcohol (**2.151**). This selectivity is accomplished due to the lithium complex which forms during the reduction process[157].



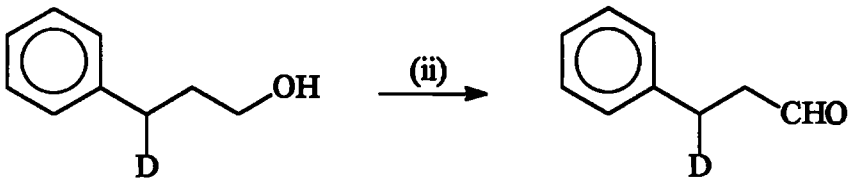

**2.149** **2.150** **2.151**

(i) a) $LiAlD_4$ , $Et_2O$, b) $H_2O$, $H^+(aq)$. (ii) a) $LiAlH_4$ , $Et_2O$, b) $D_2O$, $H^+(aq)$.

This route gave the two alcohols (**2.150, 2.151**) labelled regioselectively with deuterium in excellent yield (85-86%). Analysis of the products by $^1H$ and $^{13}C$ NMR showed 100% incorporation of the label. The next step was oxidation to the aldehyde, which was accomplished using PCC in dichloromethane. However, when oxidising [$^2H$-2]3-phenylpropan-1ol (**2.150**), a degree of hydrogen exchange was seen, with the product (**2.152**) containing 25% $^1H$. This was due to the deuterium occupying an enolisable position in the final aldehyde (**2.152**). To avoid this problem the conditions were changed to a buffered PCC oxidation, which limited the acidity of the reaction mixture, and hence the degree of enolisation[158]. Using these buffered conditions the aldehyde (**2.152**) was obtained in reasonable yield (63%) and the degree of exchange was limited to less than 10%. The synthesis of [$^2H$-3]3-phenylpropanal (**2.153**) from the alcohol (**2.151**) proceeded in good yield (79%) as would be expected.



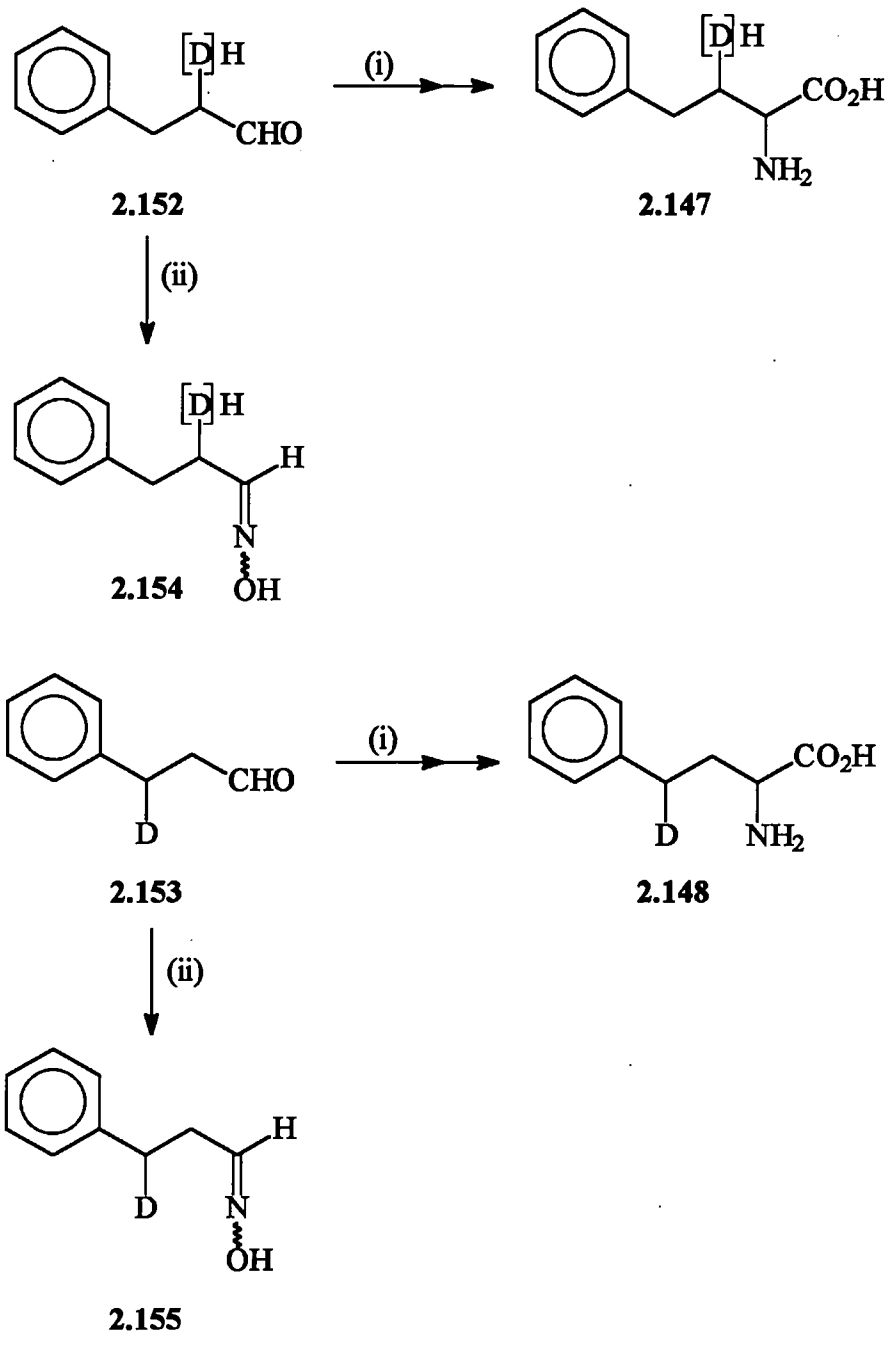

**2.150** **2.152**




**2.151** **2.153**

(i) PCC, NaOAc, $CH_2Cl_2$ , 4Å ms. (ii) PCC, $CH_2Cl_2$.

The two labelled aldehydes (**2.152, 2.153**) were then converted to the amino acids (**2.147, 2.148**) using standard Strecker conditions in moderate yield (30-42% for two steps). The aldehydes were also used to synthesise the corresponding aldoximes (**2.154, 2.155**) as standards for GC-MS analysis of the products of the microsomal preparations. The regioselectively deuterated amino acids (**2.147, 2.148**) could then be used in feeding experiments with microsomal preparations from *B. napus*.




(i) a) KCN, $NH_4Cl$, $NH_3$ , EtOH, $H_2O$, b) c. HCl, c) $H^+$(aq). (ii) $NH_2OH.HCl$, $NaHCO_3$, EtOH, $H_2O$.

Initial feeding studies with the two $^2H$ labelled homophenylalanines (**2.147, 2.148**) failed to show any production of aldoxime, probably due to inadequacies in the biochemical protocol adopted by the collaborators in this aspect of the project. Further

work in this area is planned to overcome these problems.

## 2.6.3 *Conclusions*

[$^{13}$C-2]Dihomomethionine (**2.136**) has been synthesised in satisfactory overall yield for use in feeding experiments to examine the biosynthesis of 2-propenylglucosinolate.

Two regioselectively deuterated homophenylalanines (**2.147, 2.148**) and the corresponding aldoximes (**2.154, 2.155**) have been synthesised and used in preliminary, but as yet inconclusive, biosynthesis studies to confirm the production of 3-phenylpropanaldoxime from homophenylalanine.

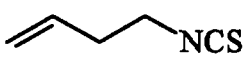
# 2.7 *Behaviour Controlling Chemicals From* B. Napus

As part of an extensive project aimed at reducing pesticide inputs in oilseed rape (*B. napus*) cultivation, studies have been carried out to identify those chemicals from the plant which influence pest behaviour and colonisation. A variety of techniques have been employed which isolate, and then identify, those chemicals which are detected neurophysiologically by insect pests of *B. napus*[23,24,94]. These are then tested in behavioural bioassays to examine the behavioural effect of each chemical[25,93].

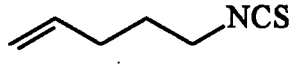
The isolation studies utilise air entrainment techniques, which is a form of dynamic headspace sampling[159]. Dry purified air is drawn through a chamber containing *B. napus* plants, and then through a porous cross linked polystyrene polymer (Porapak Q) which adsorbs the organic compounds from the air above the plants. The polymer trap is then eluted with an organic solvent to give a solution of the volatile compounds released by the plant. This solution can then be analysed by GC, and typically gives 100-300 separate peaks for a plant sample[23,94]. In order to determine which of the 100-300 compounds are important to the insect in finding and colonising the crop, part of the effluent from the GC column is passed over a neurophysiological preparation comprising chemosensory organs of the insect of interest. The preparation consists of a live insect with micro-electrodes inserted into one of its antenna, which record any electrical impulses when individual compounds from the GC effluent pass over the antenna. Using this electrophysiological technique it is possible to determine which compounds can be detected by the insect, and so which peaks need to be identified from the GC trace[160]. This identification is usually performed using GC-MS, and co-injection of authentic samples with the unknown compound of interest. This leads to a requirement for authentic, synthetic compounds which in turn has required the synthesis of a number of compounds.

Any compounds shown to be active in the electrophysiological assay are then tested for activity in behavioural assays and possibly in full scale field trials. Compounds required for field trials are usually required on a large scale, and in high purity, in order to get statistically significant results.

Two of the compounds required were 3-butenyl and 4-pentenyl isothiocyanates, the catabolites of 3-butenyl and 4-pentenylglucosinolates, which were identified as having electrophysiological activity for a wide range of insects[24,96].

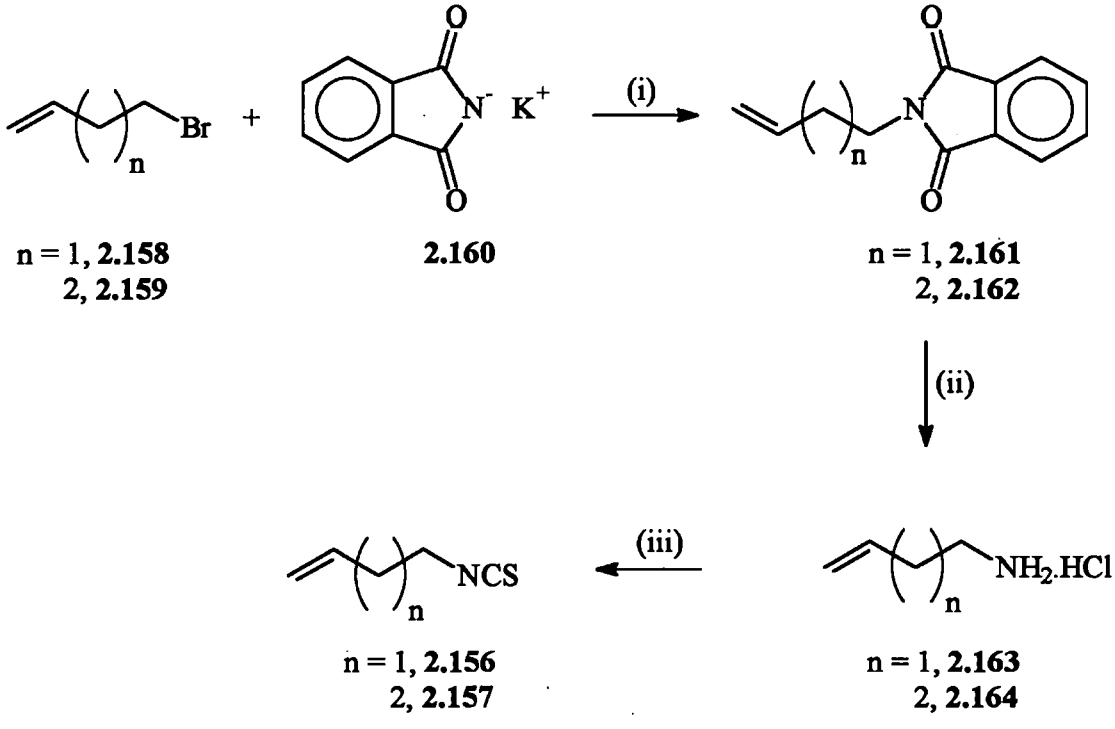



**2.157**




**2.158**

These isothiocyanates are readily synthesised from the corresponding amine[161], which is obtained from the alkenyl halide using the Gabriel synthesis of primary amines via the *N*-alkenyl phthalimide[162,163].




n = 1, **2.158**
2, **2.159**

**2.160**

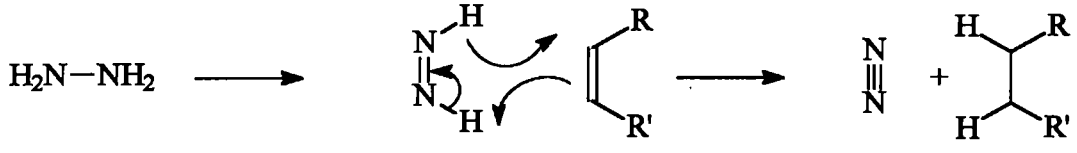
n = 1, **2.161**
2, **2.162**

n = 1, **2.156**
2, **2.157**

n = 1, **2.163**
2, **2.164**

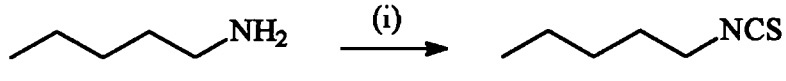
(i) KI (cat), DMF, 130°C, (ii) $NH_2NH_2.H_2O$, EtOH, (iii) $CSCl_2$ , NaOH (3 eq), $Et_2O$, $H_2O$, 0°C.

The alkenyl halides (**2.158, 2.159**) were coupled with potassium phthalimide (**2.160**) to give the *N*-alkenyl phthalimides (**2.161, 2.162**) in excellent yield (94%). These then underwent hydrazinolysis to give the two amines (**2.163, 2.164**) in reasonable yield (68-74%), which were isolated as their hydrochlorides. Occasional problems were encountered when a slight excess hydrazine (**2.165**) was used, as diimide (**2.166**) was generated *in situ* which reduced the terminal double bond of the alkene (**2.167**) to form the saturated amine (**2.168**)[107]. The reduced compound never formed more than 10% of the product, even in the worst case. With precise control of the reaction conditions the reduction of the double bond was limited to 2-3%.



**2.165** **2.166** **2.167** **2.168**

The two amine hydrochlorides (**2.163, 2.164**) were then treated with thiophosgene and three equivalents of sodium hydroxide in a two phase system of ice water/diethyl ether. This reaction gave the two crude alkenyl isothiocyanates (**2.156, 2.157**) in high yield, which were then purified by distillation at reduced pressure to give compounds sufficiently pure for biological testing in reasonable yield (41-54%). The saturated pentyl (amyl) isothiocyanate (**2.170**) was also required, and this was synthesised from the readily available amine (**2.169**) in reasonable yield (63%).



**2.169** **2.170**

(i) $CSCl_2$ , NaOH (3 eq), $Et_2O$, $H_2O$, 0°C.

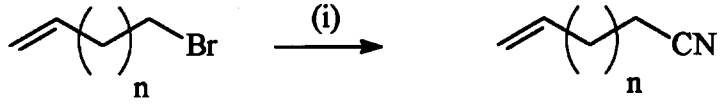
The two alkenyl isothiocyantes (**2.156, 2.157**) have been used in a number of assays, including electrophysiological testing, behavioural bioassays and field testing[23-25,94]. They are currently being evaluated as components of a bait for monitoring traps for a number of insect pests of *B. napus*.

Other compounds of interest for biological testing are the nitriles also corresponding to those obtained from the catabolism of 3-butenylglucosinolate and 4-pentenylglucosinolate.


**2.171**


**2.172**

These were readily obtained by the reaction of potassium cyanide with the corresponding alkenyl halide (**2.158, 2.159**) in DMSO. The 4-pentenenitrile (**2.171**) was obtained in reasonable yield (51%), and the 5-hexenenitrile (**2.172**) in excellent yield (86%).



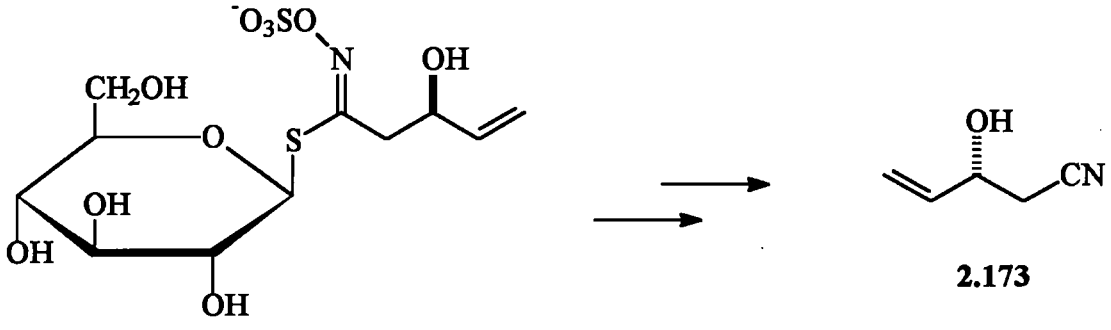

n = 1, **2.158**
2, **2.159**

n = 1, **2.171**
2, **2.172**

(i) KCN, DMSO, Δ.

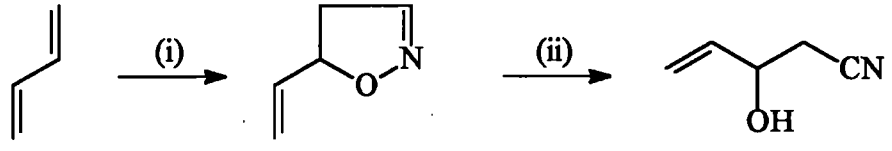
These two nitriles (**2.171, 2.172**) were then tested for electrophysiological activity with two pests of *B. napus*, the cabbage seed weevil (*Ceutorhynchus assimilis* Payk.) and the cabbage stem flea beetle (*Psylliodes chrysocephala* L.). Neither compound was found to show significant electrophysiological activity at the concentrations tested.

Another compound of interest was 3-hydroxy-4-pentenylnitrile (**2.173**). This is formed from the catabolism of 2-hydroxy-3-butenylglucosinolate , which is one of the major glucosinolates found in *B. napus*, under conditions favouring nitrile formation over isothiocyanate formation[164].



**1.39** **2.173**

This compound was available from the base catalysed thermal decomposition of 5-vinyl-2-isoxazoline (**2.175**), which was synthesised by the reaction of butadiene (**2.174**) with the trimethylsilylester of *aci*-nitromethane[165].



**2.174** **2.175** **2.173**

(i) a) $MeNO_2$ , TMSCl, $Et_3N$, $C_6H_6$ , MeCN, b) TFA, (ii) $Et_3N$, Δ.

This route gave first the substituted isoxazoline (**2.175**) in reasonable yield (53%), and then the racemic hydroxynitrile (**2.173**) in 41% yield. This could then be used in electrophysiological assays against *C. assimilis* and *P. chrysocephala*. The compound showed some activity against *P. chrysocephala* but not at statistically significant levels[164].

## *2.7.1 Conclusions*

A range of glucosinolate catabolites have been synthesised to test in electrophysiological assay against insect pests of *B. napus*. Those which were shown to be electrophysiologically active in other parts of this project have been synthesised on a large scale for behavioural assays and field trials. This is an essential part of the project to determine the particular compounds which may be modified using a rational genetic approach once the genes involved in glucosinolate biosynthesis have been sequenced.

## 2.8 *Summary and Future Work*

Synthesis of both $^{14}C$ labelled and unlabelled non-protein amino acids with use of $K^{14}CN$ to introduce the label at C-1 of the amino acid has allowed the first identification of a cell free plant preparation which catalyses the first step on the glucosinolate biosynthetic pathway in *B. napus*. The pathways for aliphatic and aromatic glucoisnolate biosynthesis have been shown to utilise different enzymes for this step. This is an oxidative decarboxylation of an amino acid which results in the formation of an aldoxime, which has been isolated and fully characterised for the system which utilises homophenylalanine as a substrate.

Use of the [$^{14}C$-1]amino acids has allowed limited characterisation of the enzymes involved in the oxidative decarboxylation. Preliminary results suggest that they may be flavin linked monooxygenases[114], although more recent studies suggest that the enzymes may, in fact, be cytochromes P-450[121]. Further work is required on the purification of the enzyme preparation to allow more detailed studies on enzyme characterisation.

The intermediates between the amino acid and the aldoxime have been studied by synthesis of a range of potential intermediates, and then competition assays with monitoring of $^{14}CO_2$ release when the [$^{14}C$-1]amino acid is incubated with the potential intermediate. These results did not give much positive information although they did appear to rule out the involvement of the $\alpha$-nitro acid as a potential intermediate. Synthesis of [$^{2}H$-2]homophenylalanine has allowed preliminary investigations into the intermediates by examination of the retention or loss of the label in the aldoxime product. The use of $^{19}F$ NMR as a technique to examine the intermediates as they are formed *in situ* has been investigated. A range of fluorine substituted amino acids and aldoximes was synthesised, but any further studies require purification of the cell free preparation to give a higher

concentration of the enzymes involved. A purer enzyme system could be obtained, either from conventional enzyme purification techniques or using molecular biology techniques to identify the gene encoding for the enzyme responsible for the oxidative decarboxylation. Both these approaches should give genes which can be overexpressed in an expression system such as yeast to provide large quantities of the enzyme. This could then be used to complete the studies on the reaction mechanism of the oxidative decarboxylation reaction. The availability of pure enzyme would allow much more detailed studies on the enzyme and its mechanism using techniques such as $^{19}F$ NMR and electrospray mass spectrometry to examine enzyme bound intermediates.

Synthesis of a range of unlabelled and [$^{14}C$-1]methionine homologues has allowed investigation of the enzymes involved in aliphatic glucosinolate biosynthesis. There appears to be a single enzyme which catalyses the transformation of methionine homologues from dihomomethionine and longer. This enzyme does not accept homomethionine as a substrate, which is surprising given that this is thought to be the biogenetic precursor of 2-propenylglucosinolate, which is found in a wide range of glucosinolate producing plants. The synthesis of a range of dihomomethionine analogues, and subsequent competition assays have outlined the substrate specificity of the aliphatic enzyme, and allowed the development of a crude active site model. This active site model could be further developed using a wider range substrates. For example, longer chain methionine homologues, conformationally constrained by the introduction of a double bond or ring into the alkyl chain, would examine the degree of flexibility allowed in chain length. Longer chain homologues than those already examined could be synthesised to examine the amino acids utilised by *A. thaliana* to make long chain glucosinolates. A similar study on homophenylalanine could look at different homologues and also a wide range of substitution on the aromatic ring.

The biosynthesis of thiohydroximates from aldoximes has been investigated, by

examining the C-S bond cleavage which takes place during thiohydroximate formation. Synthesis of an artificial substrate has allowed the identification of C-S lyase enzymes in *B. napus*. Investigation of these enzymes required the synthesis of the proposed intermediate, a cysteine thiohydroximate conjugate. This was accomplished using nitrile oxide methodology, with an inverse addition procedure essential to obtain the cysteine conjugate. Further work will examine the conversion of these potential intermediates into desulfoglucosinolates by coupling the C-S lyase enzyme with the glucosyltransferase responsible for desulfoglucosinolate biosynthesis. Synthesis of deuterium labelled intermediates will be carried out by combining the methods used for deuterium labelled homophenylalanine and the nitrile oxide methodology used to obtain the thiohydroximates. These will be used to demonstrate the true conversion of the thiohydroximates into desulfoglucosinolates and confirm their role in the biosynthetic pathway. Analysis of the products will involve isolation and purification by HPLC and analysis by mass spectrometry.

Retained label substrates to examine the glucosinolate biosynthetic pathway have been synthesised. Use of regiospecific deuteration has allowed the synthesis of deuterated homophenylalanines, which will allow further investigation of the pathway. The possibility of incorporation of dihomomethionine into 2-propenylglucosinolate will be investigated by feeding [$^{13}$C-2]dihomomethionine. This was synthesised in reasonable yield, despite a number of unforseen problems in the synthesis. [$^{13}$C-2]Dihomomethionine will be fed to plants known to produce high levels of 2-propenylglucosinolate to examine its role as a potential starting material. If this experiment shows conversion of dihomomethionine to 2-propenylglucosinolate then further work will be needed to examine the formation of the terminal double bond and the role of homomethionine in the biosynthesis.

Glucosinolate catabolites have been synthesised in order to study their biological activity at electrophysiological, behavioural and field levels. These compounds are an

essential part of the overall project at IACR-Rothamsted, in determining which secondary metabolites in *B. napus* need to be genetically manipulated in a rational way, in order to develop a novel crop protection strategy not dependent on broad spectrum irradicant pesticides. Further work may be required on identification and synthesis of compounds from air entrainment studies which are shown to have electrophysiological activity with pests of *B. napus*.

# 3 EXPERIMENTAL

## *Spectroscopic and Analytical Techniques*

NMR spectra were recorded in either deuterochloroform ($CDCl_3$), $d_6$-acetone or $d_6$-DMSO with tetramethylsilane (TMS) as an internal standard ($\delta$ 0.0ppm), or in $D_2O$ with 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt (DSS), as an internal standard, on a JEOL GNX 400 spectrometer at 400 MHz for $^1H$ spectra and at 100 MHz for $^{13}C$ spectra. *J* values are given in Hz to the nearest 0.5Hz.

IR spectra were recorded as either, a thin film on NaCl discs or as a KBr disc on a Nicolet 205 FT-IR using 32 scans at a resolution of $4cm^{-1}$ from 400-$4000cm^{-1}$.

Microanalyses were performed by Butterworth Laboratories Ltd, Teddington.

Accurate mass measurements were recorded on a VG-Autospec.

Melting points were recorded on a Townson and Mercer melting point apparatus and are uncorrected.

## *Chromatographic and Purification Techniques*

Thin layer chromatography (tlc) was carried out using prepared plates (Merck, D.C. Kieselgel 60 F254) of 0.2mm thickness on plastic sheets.

"Flash" chromatography was carried out using Merck Kieselgel 60 with a variety of solvent systems[166].

Ion exchange chromatography was carried out using Amberlite CG-400 ($Cl^-$ form) which was washed with acid, water, aqueous sodium hydroxide, and more water before use.

Gas chromatography (GC) was carried out using a Hewlett Packard 5890 series 2 GC, with either a temperature programmed on-column injector (3°C above column

temperature) or a split/splitless injector operating in the splitless mode ( at 150°C) with a flame ionisation detector (FID). The column was either a 50m, 320$\mu$ i.d. capillary column with an HP-1 stationary phase, or a 30m, 320$\mu$ i.d. capillary column with an SPB-35 stationary phase. The oven temperature programme was 40°C for 1 min, then 10°C/min to 250°C, then isothermal for 25 minutes.

Gas chromatography-mass spectroscopy (GC-MS) was carried out using the following equipment and conditions.

GC: Hewlett Packard 5890 GC, with a cold on-column injector.

Column: 30m, 320$\mu$ i.d., DB-1 (J. & W. Scientific, Folsom).

Temperature programme: 30°C for 5 min, 5°C/min to 250°C, isothermal.

MS: directly coupled 70-250 (VG Analytical, Manchester) 70 eV, 250°C.

Distillation at reduced pressure was carried out using a Kugelrohr distillation oven with dry ice used as the bulb coolant, with the pressure and oven temperature recorded.

## *Radioactivity Measurements*

Radioactivity measurements were taken using Drummond microcaps of known volume. Aliquots were dissolved in water (300$\mu$l) and Cocktail T (2.7ml) and measured in plastic inserts using a Kontron Betamatic liquid scintillation counter. All measurements were done in duplicate and were repeated when samples differed by $\pm$10%.

## *Solvents and Reagents*

Solvent extracts were dried using anhydrous magnesium sulfate. All solvent

evaporations were carried out using a Büchi rotary evaporator under water pump reduced pressure. The following solvents were dried and/or distilled before use:

Tetrahydrofuran (THF) - refluxed and distilled from sodium benzophenone ketyl.

1,2-Dimethoxyethane (glyme) - refluxed and distilled from sodium benzophenone ketyl.

Dimethylsulfoxide (DMSO) - distilled under reduced pressure.

All other solvents were used as supplied except when specified. Reagents were purified using standard laboratory procedures[148,167].

## *Bioassays*

All microsomal preparation and enzyme bioassays were carried out by Richard Bennett, Guy Kiddle and Agnes Donald, of the Biochemistry and Physiology Department, IACR-Rothamsted.

## *Plant Material*

Oilseed rape (*Brassica napus* L., cv Bienvenu) seeds were obtained from Rothamsted farm stocks. Plants were grown in compost in a controlled environment room under the following conditions: 12 hour light period (a combination of tungsten and fluorescent lamps, average PAR 350 $\mu E\ m^{-2}\ s^{-1}$), temperature day/night 17/14°C, relative humidity day/night 80/90%.

## *Microsomal Preparation*

All operations were carried out on ice in a cold room (4°C), and the pestle and mortar and all buffer solutions were pre-chilled. A mixture of leaves five and six from 48-day old plants were used in all experiments, as these gave the highest enzyme activity. Twenty grams (fresh weight) of leaves were chopped and then homogenised in a pestle and mortar with acid-washed sand (10g) and homogenisation buffer (100ml; 0.25M sucrose, 50mM sodium bisulfite, 10mM ascorbic acid, 0.1M potassium dihydrogen phosphate, adjusted to pH 7.0 with potassium hydroxide). The resulting homogenate was slurried with polyvinylpyrrolidone (10g) and Amberlite XAD-4 (2g) for two minutes, then filtered through four layers of muslin. The filtrates were combined and centrifuged at 27,000 x g for 20 min. The supernatant was centrifuged at 100,000 x g for 1 hour, and the resulting pellet resuspended in suspension buffer (2 x 500$\mu$l, 20% (v/v) glycerol, 0.1M potassium dihydrogen phosphate, adjusted to pH 7.5 with potassium hydroxide). The suspension was transferred to Eppendorf tubes and centrifuged in a Biofuge for 10 min. The combined supernatants were made up to 5ml with resuspension buffer, and the resulting solution comprised the crude microsome preparation used in all assays.

## *Enzyme Assays*

Metabolism of homophenylalanine or dihomomethionine by the microsome preparations was monitored by following the release of $^{14}CO_2$ from amino acid substrates with the label at C-1. Both substrates were synthesised at a specific activity of 800MBq $mmol^{-1}$ and were diluted to 222MBq $mmol^{-1}$. Glass vials (5 x 2cm) were used for the assays, with a smaller glass vial placed within containing potassium hydroxide (1ml, 1M)

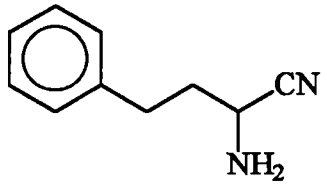
plus a wick of Whatman No. 1 filter paper. Assays consisted of microsomes (200$\mu$l) in a final volume of 500$\mu$l. Reactions were started by the addition of substrate (25$\mu$l, 6.8nmol), and the vials were capped with a Subaseal prior to incubation at 30°C. The reaction was stopped by addition of hydrochloric acid (30$\mu$l, 40%(v/v)) to the assay mix. After overnight incubation at 30°C (to allow $^{14}CO_2$ released from the acidified assay mix to be trapped in the potassium hydroxide solution), an aliquot (500$\mu$l) of potassium hydroxide solution was added to Cocktail T (5ml). Radioactivity was determined using a Packard 2500T scintillation counter. All assays were run in triplicate, and compared to control assays containing microsomes, substrate and buffer.

The assay for oxime determination was run using unlabelled substrate (200$\mu$l, 10mM), incubated at 30°C for 0, 30, 60 or 90 mins using microsomes (400$\mu$l) in resuspension buffer (200$\mu$l) containing NADPH (200$\mu$l, 12.5mM). The assay was diluted to 5ml with water and then extracted with diethyl ether/hexane (5ml, 50/50). The organic layer was separated, dried over magnesium sulfate and concentrated to 50$\mu$l. This was then analysed by GC and GC-MS using the conditions stated earlier.

# *Chemical Synthesis*

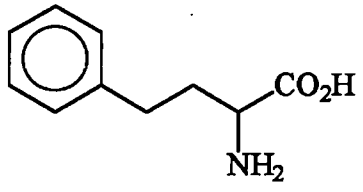
## *3.1 Chemical Studies on Aldoxime Formation*

### *2-Amino-4-phenylbutanenitrile* (**2.13**)



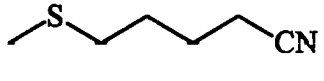

Potassium cyanide (6.60g, 0.10mol), ammonium chloride (5.5g, 0.10mol), and ammonia (20ml, d. 0.88) were added to a mixture of 3-phenylpropanal (**2.12**) (90% tech. 15.0g, 0.11mol) in ethanol (150ml) and water (50ml) and stirred for 72 hours at room temperature. The mixture was treated with water (150ml), then extracted with diethyl ether (3 x 100ml), and then the combined organic layers were extracted with dilute hydrochloric acid (3 x 100ml, 0.5N). The acid layer was basified with sodium hydroxide solution (~150ml, 1.0M) and back extracted with diethyl ether (3 x 100ml). These ether layers were combined, dried and concentrated *in vacuo* to give the product (**2.13**) as an oil (11.2g, 63%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.60 (2H, br s, $NH_2$), 2.03 (2H, q, *J* 7.0Hz, $CH_2CH$), 2.79 (1H, dt, *J* 7.0, 14.0, $ArCH_aH_b$), 2.87 (1H, dt, *J* 7.0, 14.0, $ArCH_aH_b$), 3.59 (1H, *J* 7.0, $CH_2CH$), 7.19-7.32 (5H, m, 5 x Ar*H*). $^{13}C$: $\delta$ 31.6 ($CH_2$), 36.8 ($CH_2$), 42.6 (*C*H), 122.1 (*C*N), 126.5 (*C*H), 128.4 (2 x *C*H), 128.7 (2 x *C*H), 139.8 ($C_q$). $\upsilon_{max}$ (film, $cm^{-1}$): 3380, 3030, 2930, 2240, 1605, 1495, 1455, 755, 700.

*D,L-2-Amino-4-phenylbutanoic acid (D,L-Homophenylalanine, HPhe)* **(2.2)**[105,168,169]




2-Amino-4-phenylbutanenitrile (**2.13**) (9.60g, 0.060mol) was treated with concentrated hydrochloric acid (20ml), with cooling, and left at room temperature overnight. Addition of water (20ml) was followed by heating to reflux for 6 hours. The reaction mixture was then cooled and washed with diethyl ether (50ml). The aqueous layer was treated with ammonia (to pH7) and then left at 4°C overnight. The precipitate was filtered off, washed with diethyl ether and dried to give the product (**2.2**) as a white powder (7.6g, 71%). **m.p.** 204-206°C (dec). **NMR** ($D_2O$/NaOD): $^1H$: $\delta$ 1.81 (1H, dt, *J* 6.0, 8.0, $CH_2CH_aH_b$), 1.89 (1H, dt, *J* 6.0, 8.0, $CH_2CH_aH_b$), 2.63 (2H, t, *J* 8.0, $ArCH_2$), 3.25 (1H, t, *J* 6.0, $CH_2CH$), 7.26-7.40 (5H, m, 5 x Ar*H*). $^{13}C$: $\delta$ 31.1 ($CH_2$), 36.6 ($CH_2$), 55.3 (*C*H), 125.8 (*C*H), 128.2 (2 x *C*H), 128.4 (2 x *C*H), 141.9 ($C_q$), 183.0 ($CO_2H$). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): 3350, 3040, 2860, 1730, 1600, 1575, 1530, 1455, 1275, 1215, 700. **Elemental analysis** Found: C, 67.3; H, 7.2; N, 7.8. $C_{10}H_{13}NO_2$ requires C, 67.0; H, 7.3; N, 7.8.

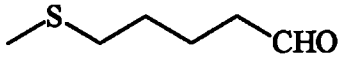
*5-Methylthiopentanenitrile* **(2.15)**[170]




A solution of sodium methoxide was formed from sodium (0.77g, 0.033mol) and methanol (50ml) was treated with gaseous methanethiol (1.8g, 0.037mol). To this solution

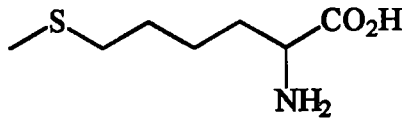
was added 5-bromopentanenitrile (**2.14**) (5.4g, 0.033mol). After 3.0 hours diethyl ether (100ml) and water (200ml) were added. The aqueous phase was re-extracted with diethyl ether (100ml) and the combined organic phases washed with water, dried and concentrated. The residue was distilled at reduced pressure to give the pure product (**2.15**) (74°C/0.7mm Hg, 3.2g, 75%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.78 (4H, m, 2 x $CH_2$), 2.10 (3H, s, $CH_3S$), 2.40 (2H, t, $J$ 6.5, $CH_2CH_2$), 2.53 (2H, t, $J$ 6.0, $CH_2CH_2$). $^{13}C$: $\delta$ 15.4 ($CH_3S$), 16.8 ($SCH_2$), 24.2 ($CH_2$), 27.7 ($CH_2$), 33.1 ($CH_2$), 119.5 ($CN$).

## *5-Methylthiopentanal* (**2.16**)[171]



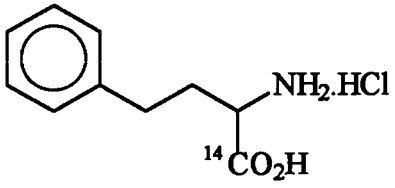

5-Methylthiopentanenitrile (**2.15**) (6.45g, 0.050mol) in hexane (50ml) and diethyl ether (50ml) at -78°C under nitrogen was treated with DIBAL (7.92g, 0.056mol) in hexane (50ml) dropwise over 5 mins. After 30 mins the reaction was allowed to warm up to room temperature and left stirring for 2 hours. Then ethyl acetate (5ml) was added followed by ammonium chloride solution (sat. 50ml) with stirring for 20 mins. After the addition of sulfuric acid (10% v/v, 50ml) and further stirring for 30 mins, the reaction mixture was partitioned and the aqueous phase extracted with diethyl ether (2 x 50ml). The organic layers were combined, washed with brine and then water, dried, and then concentrated to leave the crude product (**2.16**) as an oil. This was purified by distillation at reduced pressure (60°C/0.6 mm Hg, 4.20g, 64%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.64 (2H, m, $CH_2$), 1.73 (2H, m, $CH_2$), 2.10 (3H, s, $CH_3S$), 2.48 (2H, m, $CH_2CHO$), 2.51 (2H, t, $J$ 7.0, $SCH_2CH_2$), 9.77 (1H, t, $J$ 1.5, $CHO$). $^{13}C$: $\delta$ 15.3 ($CH_3S$), 20.8 ($SCH_2$), 28.2 ($CH_2$), 33.5 ($CH_2$), 43.1 ($CH_2CHO$), 201.9 ($CHO$). $\nu_{max}$ (film, $cm^{-1}$): 2940, 2915, 2860, 1725, 1125.

*D,L-2-Amino-6-methylthiohexanoic acid (D,L-Dihomomethionine, DHMet)* **(2.3)**[64]



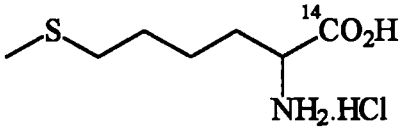

5-Methylthiopentanal (**2.16**) (2.64g, 0.020mol), potassium cyanide (1.35g, 0.021mol) and ammonium chloride (1.10g, 0.021mol) in ethanol (30ml) and water (20ml), were treated with ammonia (10ml, 0.2mol) and stirred for 48 hours. Then water (50ml) was added and the reaction mixture was extracted with diethyl ether (3 x 50ml). The combined organic layers were extracted with hydrochloric acid (2 x 75ml, 0.5N). The combined acid layers were then treated with sodium hydroxide solution (2N, to pH9) and back extracted with diethyl ether (3 x 75ml). The combined organic phases were dried, and concentrated *in vacuo* to give the aminonitrile (**2.17**) as an oil. This was treated with hydrochloric acid (10ml, 12N) and left for 24 hours at room temperature. Then water (10ml) was added and the mixture heated to reflux for 8 hours. The reaction mixture was then taken to dryness *in vacuo* to give the amino acid hydrochloride as a solid. This was dissolved in water, treated with pyridine to ~pH 7 and the precipitated amino acid filtered off and dried *in vacuo* to leave the product as a light yellow solid (**2.3**) (2.48g, 70%). **m.p.** 221-224°C (dec). **NMR** ($D_2O$/DCl): $^1$H: $\delta$ 1.55 (2H, m, $CH_2$), 1.67 (2H, m, $CH_2$), 1.98 (2H, m, $CH_2$), 2.10 (3H, s $CH_3S$), 2.57 (2H, t, $J$ 7.0, $CH_2CH$), 4.14 (1H, t, $J$ 6.5, $CH_2CH$). $^{13}$C: $\delta$ 16.6 ($CH_3S$), 25.7 ($CH_2$), 30.1 ($CH_2$), 31.6 ($CH_2$), 35.0 ($CH_2$), 55.1 ($CH$), 174.1 ($CO_2H$). $\upsilon_{max}$ (KBr disc, cm$^{-1}$): 3030, 2935, 2915, 2860, 1700, 1585, 1485, 1415, 1405. **Elemental analysis** Found: C, 47.6; H, 8.4; N, 7.9. $C_7H_{15}NO_2S$ requires C, 48.2; H, 8.7; N, 8.0. **Accurate mass** Found: 177.08269. $C_7H_{15}NO_2S$ requires 177.08235.

*D,L-[$^{14}$C-1]-2-Amino-4-phenylbutanoic acid ([$^{14}$C-1]-Homophenylalanine)* **(2.19)**




As for D,L-2-amino-4-phenylbutanoic acid (**2.2**). 3-Phenylpropanal (**2.12**) (3.13mg, 0.023mmol) in ethanol (580$\mu$l), ammonium chloride solution (120$\mu$l, 10.5mg/ml), potassium cyanide (800MBq/mmol, 1.50mg, 0.023mmol, Sigma) in water (200$\mu$l), and ammonia (120$\mu$l) were stirred together for 2 days. Addition of water (4ml), then partitioning between diethyl ether (15ml total) and water, extraction of the aminonitrile (**2.18**) into acid (0.5N, 3 x 5ml), back extraction after aqueous base treatment (1.0M, 10ml), followed by acid hydrolysis (all as **2.2**) gave the product (**2.19**). This was purified using an ion exchange resin (Amberlite CG-400) to give a radiochemical yield of 6.0MBq (32%).

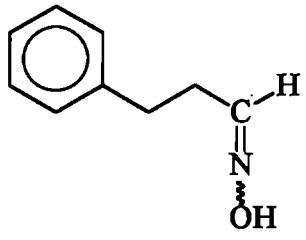
*D,L-[$^{14}$C-1]-2-Amino-6-methylthiohexanoic acid* **(2.21)**




As for D,L-2-amino-6-methylthiohexanoic acid (**2.3**). 5-Methylthiopentanal (**2.16**) (3.0mg, 0.023mmol) in ethanol (300$\mu$l), ammonium chloride solution (120$\mu$l, 10.5mg/ml), potassium cyanide (800MBq/mmol, 1.50mg, 0.023mmol, Sigma) in water (200$\mu$l) and ammonia (120$\mu$l) were stirred for 2 days. Addition of water (4ml), then partitioning between diethyl ether (15ml total) and water, extraction of the aminonitrile (**2.20**) into acid

(0.5N, 3 x 5ml) back extraction after aqueous base treatment (1.0M, 10ml), followed by acid hydrolysis (all as **2.3**) gave the product (**2.21**) as its hydrochloride. This was purified using an ion exchange resin (Amberlite CG-400) to give a radiochemical yield of 3.52MBq (19%).

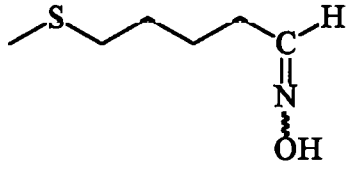
### *3-Phenylpropanaldoxime* (**2.22**)[172]




Hydroxylamine hydrochloride (7.63g, 0.11mol) was added over 15 minutes to a solution of 3-phenylpropanal (**2.12**) (13.40g, 0.10mol) and sodium bicarbonate (8.5g, 0.10mol) in water (100ml) and ethanol (100ml). The mixture was refluxed for 2.5 hours and then the ethanol was removed *in vacuo* to precipitate the crude aldoxime (**2.22**). This was removed by filtration and dried. (12.1g, 81%). Recrystallisation from diethyl ether/hexane gave a pure mixture of the (*E*)- and (*Z*)-isomers in a 3:2 ratio. This ratio could be changed by varying the recrystallisation conditions. Further slow recrystallisation (over 3 days) from diethyl ether/hexane gave exclusively the (*Z*)-isomer. **m.p.** 91-3°C. **NMR** ($d_6$-acetone): (*E*)-isomer $^1$H: $\delta$ 2.45 (2H, dt, *J* 6.0, 8.0, $CH_2$CH=NOH), 2.79 (2H, t, *J* 8.0, Ar$CH_2$), 7.16-7.31 (5H, m, 5 x Ar*H*), 7.39 (1H, t, *J* 6.0, $CH_2CH$=NOH), 9.8 (1H, s, CH=NO*H*). $^{13}$C: $\delta$ 32.0 ($CH_2$), 33.5 ($CH_2$), 126.8 (*C*H), 129.1 (2 x *C*H), 129.2 (2 x *C*H), 142.1 ($C_q$), 150.0 (*C*=NOH). **MS**: *m/z* 149 ($M^+$, 8%), 132, (32), 131 (78), 117 (60), 105 (28), 104 (19), 91 (100), 77 (30), 65 (58). (*Z*)-isomer $^1$H: $\delta$ 2.65 (2H, dt, *J* 5.0, 7.5, $CH_2$CH=NOH), 2.81 (2H, t, *J* 7.5, Ar$CH_2$), 6.67 (1H, t, *J* 5.0, $CH_2CH$=NOH), 7.16-7.31 (5H, m, 5 x Ar*H*), 10.0 (1H, s, CH=NO*H*). $^{13}$C: $\delta$ 27.2 ($CH_2$), 32.7 ($CH_2$),

126.8 (*C*H), 129.1 (2 x *C*H), 129.2 (2 x *C*H), 142.2 ($C_q$), 150.7 (*C*=NOH). **MS**: *m/z* 149 ($M^+$, 8%), 132, (25), 131 (69), 117 (50), 105 (18), 104 (58), 91 (100), 77 (31), 65 (58). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): (both isomers): 3185, 3025, 2860, 1455, 1430, 1310, 940, 910, 760, 725, 700. **Elemental analysis** Found: C, 72.6; H, 7.4; N, 9.3. $C_9H_{11}NO$ requires C, 72.5; H, 7.4; N, 9.4. **Accurate mass** Found: 149.08450. $C_9H_{11}NO$ requires 149.08406.

*5-Methylthiopentanaldoxime* (**2.23**)[173]

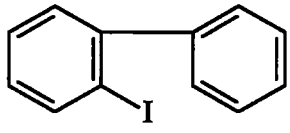



5-Methylthiopentanal (**2.16**) (330mg, 2.5mmol), hydroxylamine hydrochloride (250mg, 3.5mmol) and sodium hydrogen carbonate (300mg, 3.5mmol) were stirred in ethanol (10ml) and water (5ml) at room temperature for 4 hours. The solution was diluted with water (50ml) and partitioned with diethyl ether (50ml). The organic phase was washed with water, saturated brine, dried, filtered and concentrated to leave the product (**2.23**) as a mixture (3:2) of (*E*)- and (*Z*)- isomers, unchanged in ratio after recrystallisation from diethyl ether/hexane (0.28g, 77%). **m.p.** 46-48°C. **NMR** ($CDCl_3$): (*E*)-isomer $^1$H: δ 1.60 (4H, m, 2 x $CH_2$), 2.09 (3H, s, $CH_3S$), 2.23 (2H, m, $CH_2CH$=NOH), 2.50 (2H, t, *J* 7.0, $SCH_2CH_2$), 7.42 (1H, t, *J* 6.5, $CH_2CH$=NOH), 8.9 (1H, s, CH=NO*H*). $^{13}$C: δ 15.5 ($CH_3S$), 25.6 ($CH_2$), 28.4 ($CH_2$), 29.0 ($CH_2$), 33.8 ($CH_2$), 151.7 (*C*=NOH). (*Z*)-isomer $^1$H: δ 1.60 (4H, m, 2 x $CH_2$), 2.10 (3H, s, $CH_3S$), 2.41 (2H, q, *J* 7.5, $CH_2CH$=NOH), 2.52 (2H, t, *J* 7.0, $SCH_2CH_2$), 6.74 (1H, t, *J* 7.5, $CH_2CH$=NOH), 9.3 (1H, s, CH=NO*H*). $^{13}$C: δ 15.5 ($CH_3S$), 24.6 ($CH_2$), 25.1 ($CH_2$), 28.7 ($CH_2$), 33.8 ($CH_2$),

152.3 (*C*=NOH). **MS**: (both isomers) *m/z* 147 ($M^+$, 1.5%), 129, (31), 82 (30), 61 (100), 55 (25), 48 (21), 47 (15), 45 (17) 41 (19). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): (both isomers): 3205, 3090, 2910, 2935, 1455, 1440, 925, 910. **Elemental analysis** Found: C, 48.8; H, 8.9; N, 9.4. $C_6H_{13}NOS$ requires C, 48.9; H, 8.9; N, 9.5. **Accurate mass** Found: 147.0721 $C_6H_{13}NOS$ requires 147.0718.

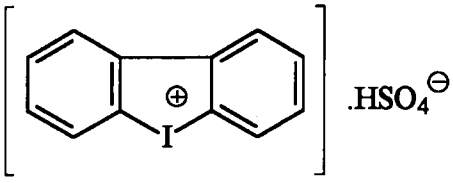
# *3.2 Enzyme Characterisation*

## *2-Iodobiphenyl* (**2.27**)[119]




To a mixture of concentrated hydrochloric acid (100ml) and 2-aminobiphenyl (**2.25**) (30.0g, 0.177mol), was added ice (70g), followed by cooling to 0°C using an ice/salt bath. The reaction mixture was then treated with sodium nitrite (12.5g, 0.181mol) in water (50ml) dropwise, with the reaction temperature kept between 0-5°C. This gave the diazo species (**2.26**) which was used without purification. Stirring for 30 minutes was followed by filtration and then addition of potassium iodide (100g, 0.60mol), in water (100ml) to the filtrate. This was left at room temperature for 24 hours, then extracted with toluene (8 x 200ml). The combined organic layers were washed with sodium hydroxide solution (10% w/v), water, sodium bisulfite solution (5% w/v), and more water. The combined organic layers were dried and concentrated *in vacuo* to leave the product (**2.27**) (45.0g, 90%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 6.99 (1H, dt, *J* 1.5, 7.5, Ar*H*), 7.25-7.42 (7H, m, 7 x Ar*H*), 7.92 (1H, dd, *J* 1.0, 8.0, Ar*H*). $^{13}C$: $\delta$ 98.6 ($C_q$), 127.6 (*C*H), 127.9 (*C*H), 127.9 (*C*H), 128.0 (*C*H), 128.7 (*C*H), 129.2 (*C*H), 129.2 (*C*H), 130.0 (*C*H), 139.4 (*C*H), 144.0 ($C_q$), 146.5 ($C_q$).

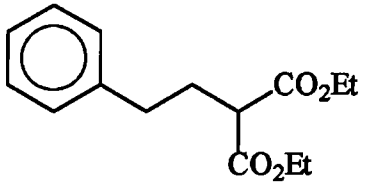
*Diphenyleneiodonium hydrogensulfate* (**2.29**)[119]



To a cold mixture of glacial acetic acid (25ml) and concentrated sulfuric acid (0.6ml) was slowly added 30% hydrogen peroxide (5ml). This was stirred at room temperature for 16 hours, then treated with 2-iodobiphenyl (**2.27**) (4.5g, 0.016mol), in acetic acid (10ml) with stirring for a further 6 hours to give the iodoso compound (**2.28**). After addition of concentrated sulfuric acid (5ml) the reaction mixture was left for a further 16 hours and then treated with cold water (100ml), with further cooling to give a white precipitate which was removed by filtration. Recrystallisation of the precipitate from hot water gave the product (**2.29**) as a white crystalline solid (3.6g, 69%). **m.p.** 272-3°C (dec). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): 1440, 1120, 1080, 1010, 750, 730, 620, 605. **Accurate mass** Found: 375.92804. $C_{12}H_9IO_4S$ requires 375.92663.

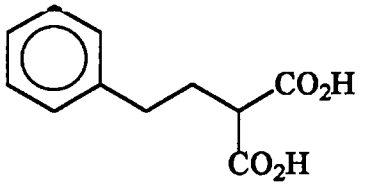
# *3.3 Oxidative Decarboxylation Mechanism*

## *2-(2-Phenethyl)diethylmalonate* (**2.35**)[168]



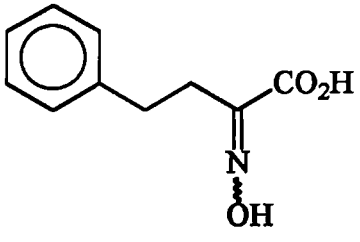

Potassium *t*-butoxide (11.2g, 0.10mol) was added to a solution of diethyl malonate (16.0g, 0.10mol) in dry glyme (200ml). The mixture was stirred at room temperature for 30 minutes, then 2-phenethyl bromide (**2.34**) (16.4g, 0.089mol) was added and the mixture heated to reflux for 1 hour. After cooling, the solvent was removed *in vacuo*, and the residue partitioned between water (200ml) and ethyl acetate (3 x 200ml). The combined organic layers were dried and concentrated *in vacuo* to give the crude product (**2.35**) as an oil. This was purified by distillation at reduced pressure (0.25mm Hg, 130°C) to give the pure product (**2.35**) (15.2g, 65%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.25 (6H, t, *J* 7.0, 2 x $CH_2CH_3$), 2.21 (2H, dt, *J* 7.5, 8.0, $CH_2CH$), 2.65 (2H, t, *J* 8.0, $ArCH_2$), 3.34 (1H, t, *J* 7.5, $CH_2CH$), 4.18 (4H, q, *J* 7.0, 2 x $CH_2CH_3$), 7.18-7.29 (5H, m, 5 x ArH). $^{13}C$: $\delta$ 14.1 (2 x $CH_3$), 30.4 ($CH_2$), 33.3 ($CH_2$), 51.2 (CH), 61.3 (2 x $CO_2CH_2$), 126.2 (CH), 128.5 (2 x CH), 128.6 (2 x CH), 140.7 ($C_q$), 169.3 (2 x $CO_2CH_2$).

## *2-(2-Phenylethyl)malonic acid* (**2.36**)[168]



To a solution of 2-(2-phenylethyl)diethylmalonate (**2.35**) (15.0g, 0.057mol) in ethanol (100ml) and water (100ml), was added sodium hydroxide (20g, 0.50mol), and the mixture was heated to reflux for 2 hours. Then the mixture was cooled, the ethanol removed *in vacuo*, and the residue extracted with diethyl ether (100ml). The aqueous phase was then cooled with ice and acidified to pH 2 with concentrated hydrochloric acid and extracted with ethyl acetate (2 x 100ml). The combined ethyl acetate extracts were washed with water and saturated brine, then dried and concentrated to leave the product (**2.36**). The solid residue was recrystallised from benzene to leave the pure product (10.8g, 91%). **NMR** ($d_6$-DMSO): $^1$H: $\delta$ 2.01 (2H, m, C*H*$_2$CH), 2.59 (2H, t, *J* 7.5, ArC*H*$_2$), 3.20 (1H, t, *J* 8.5, CH$_2$C*H*), 7.18-7.31 (5H, m, 5 x Ar*H*), 12.8 (2H, br s, 2 x COO*H*). $^{13}$C: $\delta$ 30.2 (*C*H$_2$), 32.6 (*C*H$_2$), 50.9 (*C*H), 125.9 (*C*H), 128.2 (2 x *C*H), 128.3 (2 x *C*H), 140.9 (C$_q$), 170.6 (2 x *C*O$_2$H).

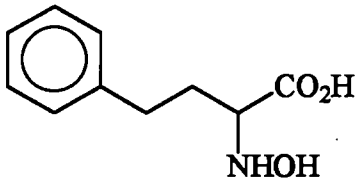
*2-Oximino-4-phenylbutanoic acid* (**2.31**)[174]




A solution of 2-(2-phenethyl)malonic acid (**2.36**) (5.2g, 0.025mol) and *n*-propylnitrite (2.5g, 0.028mol) in glyme was cooled in an ice/salt bath to -10°C and treated with hydrogen chloride gas until the solution became brownish-yellow. The mixture was left at room temperature for 48 hours, then concentrated *in vacuo* and the residue recrystallised from benzene/ethyl acetate to give the pure product (**2.31**) (3.8g, 80%). **NMR** ($d_6$-DMSO): $^1$H: $\delta$ 2.71 (4H, s, 2 x C*H*$_2$), 7.14-7.28 (5H, m, 5 x Ar*H*), 12.20 (1H, br s, O*H*), 12.70 (1H, br s, O*H*). $^{13}$C: $\delta$ 26.3 (*C*H$_2$), 31.5 (*C*H$_2$), 126.2 (*C*H), 128.5 (2

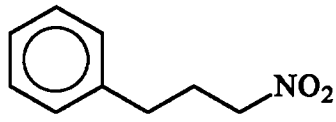
x *C*H), 128.7 (2 x *C*H), 141.2 ($C_q$), 151.4 (*C*=NOH), 165.4 (*C*$O_2$H). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): 3220, 3085, 3025, 2935, 1695, 1475, 1195, 1030, 1025, 695. **Accurate mass** Found: 193.0440. $C_{10}H_{11}NO_3$ requires 193.07389.

*2-Hydroximino-4-phenylbutanoic acid (*N*-Hydroxyhomophenylalanine)* (**2.30**)



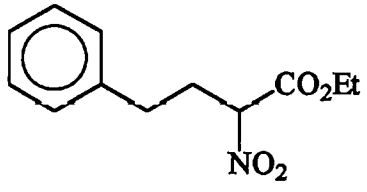

2-Oximino-4-phenylbutanoic acid (**2.31**) (1.93g, 0.010mol) in glacial acetic acid (10ml) and water (10ml) was treated with sodium cyanoborohydride (1.1g, 0.020mol) and stirred at room temperature for 16 hours. Hydrochloric acid (5ml, 12N) was added and stirring continued for a further 1 hour. The mixture was evaporated to dryness, dissolved in water, and again taken to dryness. Then the residue was dissolved in water and applied to a sulfonic acid resin column (Amberlyst 15, $H^+$ form, 10g). This was washed with water (500ml) and the product was eluted with aqueous ammonia (2% w/v). The ammoniacal eluate was taken to dryness to give the product (**2.30**) as its ammonium salt. **m.p.** 137-139°C (dec). **NMR** ($d_6$-DMSO): $^1$H: $\delta$ 1.80 (2H, m, C$H_2$CH), 2.66 (2H, m, ArC$H_2$), 3.25 (1H, t, *J* 7.0, $CH_2$C*H*), 4.50 (3H, br s, 3 x *H*), 7.14-7.30 (5H, m, 5 x Ar*H*). $^{13}$C: $\delta$ 31.3 ($CH_2$), 31.9 ($CH_2$), 65.4 (*C*H), 125.6 (*C*H), 128.3 (2 x *C*H), 128.4 (2 x *C*H), 142.4 ($C_q$), 176.1 (*C*$O_2$H). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): 3025, 2945, 2925, 1585, 1560, 1455, 1430, 1395, 965. **Accurate mass** Found: 195.08945. $C_{10}H_{13}NO_3$ requires 195.08954.

*3-Nitropropylbenzene* (**2.33**)[175,176]



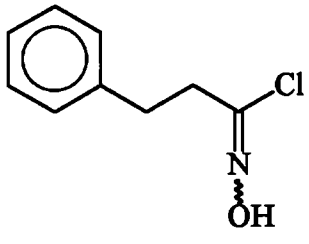

Trifluoroacetic anhydride (10.60g, 0.050mol) in acetonitrile (10ml) was added to a stirred mixture of urea-hydrogen peroxide complex (5.82g, 0.060mol) in acetonitrile (30ml) dropwise at 0°C over 30 minutes. This solution was then added dropwise to a stirred mixture of 3-phenylpropanaldoxime (**2.22**) (1.00g, 6.7mmol) and disodium hydrogen phosphate (21g, 0.150mol) in acetonitrile (50ml) at 0°C. After stirring for 2 hours at room temperature the solvent was removed *in vacuo*. The residue was treated with saturated sodium hydrogencarbonate solution (100ml) and extracted with diethyl ether (2 x 100ml). The ether layers were combined, washed with sodium bisulfite solution (100ml, 5% w/v) and dried. Removal of the solvent *in vacuo* gave the crude product. This was purified by "flash" chromatography (Merck Kieselgel 60, hexane/ethyl acetate; 80/20) to give the pure product (**2.33**) as a light brown oil (252mg, 23%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 2.31 (2H, quin, *J* 7.0, $CH_2CH_2CH_2$), 2.71 (2H, t, *J* 7.0, $ArCH_2$), 4.34 (2H, t, *J* 7.0, $CH_2NO_2$), 7.16-7.32 (5H, m, 5 x Ar*H*). $^{13}C$: $\delta$ 28.8 ($CH_2$), 32.2 ($CH_2$), 74.6 ($CH_2NO_2$), 126.6 (*C*H), 128.4 (2 x *C*H), 128.7 (2 x *C*H), 139.5 ($C_q$). $\upsilon_{max}$ (film, $cm^{-1}$): 3020, 2920, 1550, 1495, 1455, 1435, 1385, 750, 700. **MS**: *m/z* 165 ($M^+$, 6%), 147, (33), 135 (62), 131 (30), 117 (25), 107 (17), 104 (20), 91 (100), 65 (23).

*Ethyl(2-nitro-4-phenylbutanoate)* (**2.37**)



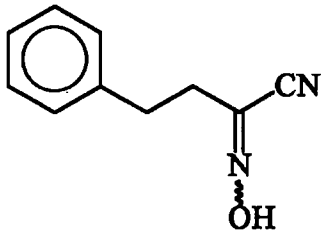
To sodium hydride (60% dispersion in oil, 912mg, 0.023mol) in THF (50ml) was added 2-(2-phenethyl)diethylmalonate (**2.35**) (2.59g, 0.010mol), followed by heating to reflux for 1 hour. After cooling, acetone cyanohydrin nitrate (1.95g, 0.015mol) in THF (10 ml) was added slowly, followed by further refluxing for 2.5 hours. The reaction mixture was poured onto crushed ice (50g) and hydrochloric acid (6M, 5ml) and extracted with diethyl ether (2 x 100ml). The combined organic layers were dried and concentrated to leave the crude product. This was purified using "flash" chromatography (ethyl acetate/pet. ether (40°-60°C), 20/80) to leave the pure product (**2.37**) as an oil (720mg, 30%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.27 (3H, t, *J* 7.0, $CH_2CH_3$), 2.44 (1H, m, $CH_aH_bCH$), 2.64 (2H, m, $ArCH_2$), 2.76 (1H, m, $CH_aH_bCH$), 4.25 (2H, q, *J* 7.0, $CH_2CH_3$), 5.06 (1H, dd, *J* 5.0, 9.0, $CH_2CH$), 7.16-7.32 (5H, m, 5 x Ar*H*). $^{13}C$: $\delta$ 13.9 ($CH_3$), 31.5 ($CH_2$), 31.9 ($CH_2$), 63.1 ($CO_2CH_2$), 87.1 (*C*H), 126.8 (*C*H), 128.5 (2 x *C*H), 128.8 (2 x *C*H), 138.9 ($C_q$), 164.5 ($CO_2CH_2$). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): 3030, 2970, 2630, 1750, 1560, 1455, 1375, 1255, 1205, 1175, 1030, 700.

### *3-Phenylpropanohydroximoyl chloride* (**2.40**)[49]



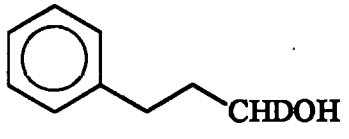

Dry chlorine gas was bubbled through a solution of 3-phenylpropanaldoxime (**2.22**) (2.98g, 0.020mol) in dry diethyl ether (50ml) at -60°C. The reaction mixture turned a deep blue colour and a white precipitate formed. After fifteen minutes the precipitate was filtered and washed with dry diethyl ether to leave the product (**2.40**) as a white crystalline solid (1.73g, 47%).

*2-Oximino-4-phenylbutanenitrile* **(2.42)**




18-Crown-6 (2.64g, 0.010mol) was dissolved in dry acetonitrile (100ml) and then potassium cyanide (0.65g, 0.010mol) was added. After cooling to 0°C the hydroximoyl chloride (**2.40**) (1.73g, 0.0094mol) was added, and then the reaction mixture was treated with triethylamine (1.01g, 0.010mol) in acetonitrile (5ml) dropwise over 30 minutes. After stirring for 1 hour the solvent was removed *in vacuo* and the residue partitioned between dichloromethane and water. The organic layer was washed with water, brine, and then dried. The solvent was removed *in vacuo* to leave an oil containing a mixture of product (**2.42**) and 18-crown-6. **NMR** ($CDCl_3$): $^1H$: $\delta$ 2.74 (2H, t, *J* 8.0, $CH_2$), 2.94 (2H, t, *J* 8.0, $CH_2$), 3.66 (s, $CH_2$, 18-crown-6), 7.17-7.31 (5H, m, 5 x Ar*H*), 8.7 (1H, br s, C=NO*H*). $^{13}C$: $\delta$ 32.4 ($CH_2$), 33.5 ($CH_2$), 70.5 ($CH_2$, 18-crown-6), 110.7 (*C*N), 126.5 (*C*H), 128.4 (2 x *C*H), 128.6 (2 x *C*H), 131.7 (*C*=NOH), 139.3 ($C_q$).

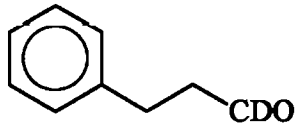
*[$H^2$-1]3-Phenylpropanol* **(2.45)**




To a suspension of lithium aluminium deuteride (1.00g, 0.024mol), in dry diethyl ether (50ml) was added 3-phenylpropanal (**2.12**) (3.20g, 0.024mol), in dry diethyl ether (20ml). After heating to reflux for 3 hours the reaction mixture was cooled to room temperature, and treated (with further cooling) with water (10ml), and dilute sulfuric acid

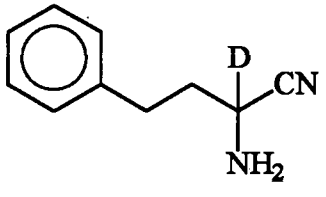
(10% v/v, 20ml). The organic layer was removed and the aqueous layer extracted with diethyl ether (3 x 30ml). The combined organic layers were dried and concentrated *in vacuo* to give the product as an oil (**2.45**) (2.84g, 87%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.85 (2H, m, $CH_2CHD$), 2.15 (1H, br s, O*H*), 2.68 (2H, t, *J* 8.0, Ar$CH_2$), 3.61 (1H, t, *J* 7.0, *C*HDOH), 7.15-7.29 (5H, m, 5 x Ar*H*). $^{13}C$: $\delta$ 32.0 ($CH_2$), 34.0 ($CH_2$), 61.7 (*C*HDOH, t, *J* 22), 125.8 (*C*H), 128.4 (2 x *C*H), 128.4 (2 x *C*H), 141.8 ($C_q$).

### *[$H^2$-1]3-Phenylpropanal* (**2.46**)[177]



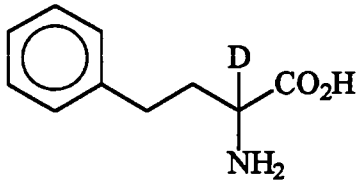

To a mixture of PCC (6.60g, 0.030mol), in dichloromethane (100ml), was added [$H^2$-1]3-phenylpropanol (**2.45**) (2.84g, 0.021mol). After stirring for 4 hours at room temperature this was treated with diethyl ether (100ml) and passed through a short florisil column. The filtrate was concentrated *in vacuo* to give the crude product as an oil. This was purified using Kugelrohr distillation (0.5mm Hg, 100°C oven temp.) to give the pure product (**2.46**) (1.34g, 48%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 2.71 (2H, m, $CH_2$), 2.91 (2H, m, $CH_2$), 7.15-7.29 (5H, m, 5 x Ar*H*). $^{13}C$: $\delta$ 28.0 ($CH_2$), 45.0 ($CH_2CDO$, t, *J* 3.5), 126.2 (*C*H), 128.3 (2 x *C*H), 128.5 (2 x *C*H), 140.3 ($C_q$), 201.4 (*C*DO, t, *J* 26.5).

### *[$H^2$-2]2-Amino-4-phenylbutanenitrile*



To a solution of [$H^2$-1]3-phenylpropanal (**2.46**) (810mg, 6.0mmol), in ethanol (20ml), and water (10ml) was added ammonia (d. 0.88, 10ml), ammonium chloride (360mg, 6.7mmol) and potassium cyanide (420mg, 6.5mmol). This was stirred at room temperature for 48 hours, then water (30ml) was added and the mixture extracted with diethyl ether (3 x 50ml). The combined ether layers were extracted with dilute hydrochloric acid ( ~ 1N, 3 x 50ml), which was then basified with dilute sodium hydroxide solution, and back extracted with diethyl ether (3 x 50ml). The combined back extracts were dried and concentrated *in vacuo* to give the product as an oil (490mg, 51%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.7 (2H, br s, $NH_2$), 2.04 (2H, t, *J* 8.0, $CH_2CD$), 2.79 (2H, m, $ArCH_2$), 7.14-7.28 (5H, m, 5 x Ar*H*). $^{13}C$: $\delta$ 31.5 ($CH_2$), 36.6 ($CH_2$), 42.3 (*C*H, t, *J* 22.0), 122.1 (*C*N), 126.2 (*C*H), 128.4 (2 x *C*H), 128.7 (2 x *C*H), 139.8 ($C_q$).

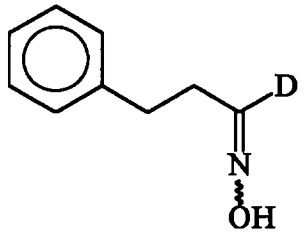
## *[$H^2$-2]2-Amino-4-phenylbutanoic acid* (**2.47**)




[$H^2$-2]2-Amino-4-phenylbutanenitrile (490mg, 3.04mmol), was treated with concentrated hydrochloric acid (3ml), and left at room temperature for 48 hours. Addition of water (20ml) with heating to reflux for 4 hours and removal of the solvent *in vacuo* gave the crude product. This was purified using ion-exchange chromatography (Amberlite CG-400) to give the pure product (**2.47**) as its hydrochloride (530mg, 80%). **m.p.** 225-228°C (dec). **NMR** ($D_2O$/NaOD): $^1H$: $\delta$ 2.11 (2H, t, *J* 8.0, $CH_2CD$), 2.65 (1H, m, $ArCH_aH_b$), 2.80, (1H, m, $ArCH_aH_b$) 7.15-7.29 (5H, m, 5 x Ar*H*). $^{13}C$: $\delta$ 30.3 ($CH_2$), 31.7 ($CH_2$), 51.3 (*C*H, m) 126.0 (*C*H), 128.2 (2 x *C*H), 128.4 (2 x *C*H), 140.4 ($C_q$) 170.6

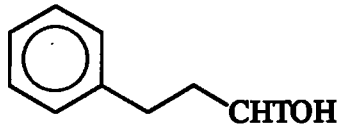
($CO_2H$). $\nu_{max}$ (KBr disc, $cm^{-1}$): 3160, 3020, 2950, 2865, 1735, 1600, 1525, 1455, 1405, 1290, 700. **Accurate mass** Found: 180.10159. $C_{10}H_{12}{}^2HNO_2$ requires 180.10096.

## *[H²-1]3-Phenylpropanaldoxime* (**2.48**)



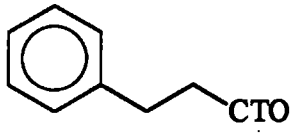

To a solution of [H²-1]3-phenylpropanal (**2.46**) (310mg, 2.3mmol), in ethanol (15ml), and water (5ml) was added sodium hydrogen carbonate (200mg, 2.4mmol), and hydroxylamine hydrochloride (170mg, 2.45mmol). This was heated to reflux for 4 hours, cooled to room temperature, and then water (20ml) was added. Extraction with diethyl ether (3 x 25ml), followed by drying and concentration *in vacuo* gave the crude product. This was recrystallised from diethyl ether/hexane to give the pure product (**2.48**) as a white crystalline solid (135mg, 39%). **m.p.** 86-87°C. GC analysis 84% *Z*-isomer, 16% *E*-isomer. **NMR** ($CDCl_3$): $^1H$: (both isomers) $\delta$ 2.71(2H, t, *J* 7.5, $CH_2$), 2.83 (2H, t, *J* 7.5, $CH_2$), 7.19-7.32 (5H, m, 5 x Ar*H*). $^{13}C$: (*Z*-isomer) $\delta$ 26.3 ($CH_2$), 31.9 ($CH_2$), 126.3 (*C*H), 128.4 (2 x *C*H), 128.5 (2 x *C*H), 140.6 ($C_q$), 151 (*C*=NOH, m). $^{13}C$: (*E*-isomer) $\delta$ 31.1 ($CH_2$), 32.8 ($CH_2$), 126.3 (*C*H), 128.4 (2 x *C*H), 128.5 (2 x *C*H), 140.6 ($C_q$), 151 (*C*=NOH, m). **MS**: *Z*-isomer *m/z* 150 ($M^+$, 3%), 133, (8), 131 (7), 118 (20), 105 (11), 104 (15), 91 (100), 77 (10), 65 (14). *E*-isomer *m/z* 150 ($M^+$, 3%), 133, (10), 131 (8), 118 (20), 105 (11), 104 (10), 91 (100), 77 (10), 65 (15). $\nu_{max}$ (KBr disc, $cm^{-1}$): (both isomers): 3130, 3030, 2855, 1460, 1420, 750, 720, 700. **Accurate mass** Found: 150.09024. $C_9H_{10}{}^2HNO$ requires 150.09034.

## *[$^3$H-1]-3-Phenylpropan-1-ol* (2.49)



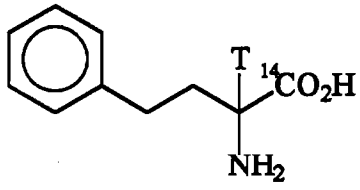

To a solution of 3-phenylpropanal (**2.12**) (6.7mg, 0.05mmol) in ethanol (500$\mu$l) was added sodium borotritiide (0.47mg, 0.0125mmol, 500mCi/mmol) in ethanol (300$\mu$l). After 45 minutes this was diluted with water (25ml) and extracted with diethyl ether (25ml total). The organic layer was dried and concentrated to leave the product as an oil (**2.49**) (3.05mCi) with a radiochemical yield of 49%.

## *[$^3$H-1]-3-Phenylpropanal* (2.50)[178]



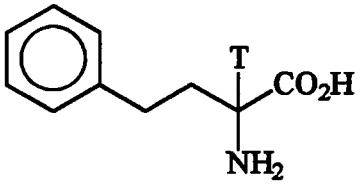

[$^3$H-1]-3-Phenylpropan-1-ol (**2.49**) (3.05mCi) was dissolved in dichloromethane (3ml) and then treated with PCC (10mg, 0.046mmol). After stirring for 2 hours at room temperature the reaction mixture was filtered through florisil (0.5g). The florisil was washed with dichloromethane to give a total volume of 10ml. The solvent was removed *in vacuo* to leave the product (**2.50**) as an oil (1.84mCi) with a radiochemical yield of 60%. GC showed the product to be 88% pure. This was dissolved in ethanol (300$\mu$l) and split into three equal samples.

*D,L,[$^{14}$C-1,$^{3}$H-1]-Homophenylalanine*




As for D,L-homophenylalanine (**2.2**). [$^{3}$H-1]-3-Phenylpropanal (**2.50**) (0.61mCi) in ethanol (100$\mu$l), ammonium chloride solution (120$\mu$l, 10mg/ml), potassium cyanide (800MBq/mmol, 1.36mg, 0.021mmol) (Sigma) in water (100$\mu$l), and ammonia (d. 0.88, 10$\mu$l) were stirred together for 12 days. Treatment with water, then partitioning between diethyl ether and water, extraction of the amino-nitrile into acid, back extraction after aqueous base treatment, followed by acid hydrolysis (as for **2.2**) gave the product. This was purified using an ion exchange resin (Amberlite CG-400) to give a radiochemical yield of 10.4MBq (38%) for $^{3}$H and 6.8MBq (38%) for $^{14}$C.

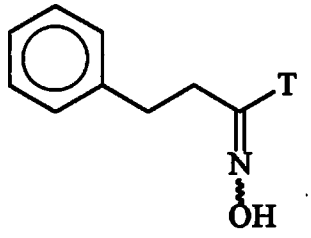
*D,L,[$^{3}$H-1]-Homophenylalanine* (**2.51**)




As for D,L-homophenylalanine (**2.2**). [$^{3}$H-1]-3-Phenylpropanal (**2.50**) (0.61mCi) in ethanol (100$\mu$l), ammonium chloride solution (120$\mu$l, 10mg/ml), potassium cyanide (800MBq/mmol, 1.36mg, 0.021mmol) (Sigma) in water (100$\mu$l), and ammonia (10$\mu$l) were stirred together for 12 days. Treatment with water, then partitioning between diethyl ether and water, extraction of the amino-nitrile into acid, back extraction after aqueous base treatment, followed by acid hydrolysis (as for **2.2**) gave the product (**2.51**). This was

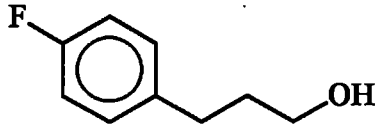
purified using an ion exchange resin (Amberlite CG-400) to give a radiochemical yield of 17.3MBq (71%).

### *[$^3$H-1]-3-Phenylpropanaldoxime* (**2.52**)




[$^3$H-1]-3-Phenylpropanal (**2.50**) (0.61mCi) in ethanol (100$\mu$l) was treated with sodium hydrogen carbonate (2.1mg, 0.025mmol) in water (210$\mu$l) and hydroxylamine hydrochloride (1.6mg, 0.023mmol) in water (160$\mu$l). After addition of ethanol (500$\mu$l) the reaction mixture was left sealed for 19 days. Addition of water (10ml) was followed by extraction with diethyl ether (2 x 10ml). The organic layer was dried and concentrated to leave the product (**2.52**) as a solid with a radiochemical yield of 25.5MBq (95%).

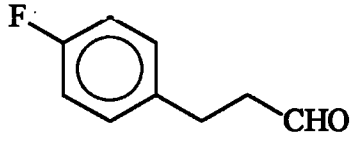
### *3-(4-Fluorophenyl)propan-1-ol* (**2.60**)[179]




To a slurry of lithium aluminium hydride (1.96g, 0.051mol) in dry diethyl ether (50ml) was added 4-fluorocinnamic acid (**2.57**) (3.32g, 0.020mol) with diethyl ether (50ml) over 10 minutes. The reaction mixture was heated to reflux for 1 hour, then allowed to cool to room temperature. Treatment with water (30ml) and dilute sulfuric acid (10% v/v, 30ml) was followed by separation of the organic layer and extraction of the aqueous phase with diethyl ether (2 x 50ml). The combined organic layers were dried and

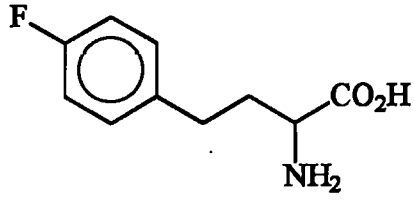
concentrated to leave the crude product (**2.60**) as an oil. This was purified by distillation at reduced pressure (0.1mm Hg, 120°C, 1.40g, 45%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.84 (2H, m, $CH_2CH_2OH$), 2.36 (1H, br s, O*H*), 2.65 (2H, t, *J* 7.5, Ar$CH_2$), 3.63 (2H, t, *J* 6.5, $CH_2OH$), 6.95 (2H, m, 2 x Ar*H*), 7.12 (2H, m, 2 x Ar*H*). $^{13}C$: $\delta$ 31.2 ($CH_2$), 34.3 ($CH_2$), 61.9 ($CH_2OH$), 115.1 (2 x *C*H, d, *J* 21.0), 129.7 (2 x *C*H, d, *J* 7.5), 137.4 ($C_q$, d, *J* 3.0), 161.3 (*C*F, d, *J* 243.5).

### *3-(4-Fluorophenyl)propan-1-al* (**2.63**)[180]



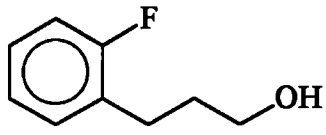

To a solution of 3-(4-fluorophenyl)propan-1-ol (**2.60**) (3.08g, 0.020mol) in dichloromethane (100ml) was added PCC (4.40g, 0.021mol). After stirring for 16 hours at room temperature pet. ether (40-60°C, 50ml) was added and the reaction mixture was filtered through silica (50g). The eluate was concentrated to leave the crude product (**2.63**) which was purified distillation at reduced pressure (0.2mm Hg, 125°C, 1.13g, 37%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 2.75 (2H, t, *J* 7.5, Ar$CH_2$), 2.92 (2H, t, *J* 7.5, $CH_2CHO$), 6.96 (2H, m, 2 x Ar*H*), 7.14 (2H, m, 2 x Ar*H*), 9.78 (1H, s, C*H*O). $^{13}C$: $\delta$ 27.2 ($CH_2$), 45.3 ($CH_2CHO$), 115.2 (2 x *C*H, d, *J* 21.0), 129.6 (2 x *C*H, d, *J* 8.0), 135.9 ($C_q$, d, *J* 3.0), 161.4 (*C*F, d, *J* 244.5), 201.3 (*C*HO).

*D,L-2-Amino-4-(4-fluorophenyl)butanoic acid (4-Fluorohomophenylalanine)* **(2.66)**



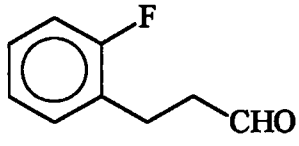

To a solution of 3-(4-fluorophenyl)propan-1-al (**2.63**) (460mg, 3.0mmol) in ethanol (20ml) and water (10ml), was added ammonium chloride (165mg, 3.0mmol), potassium cyanide (200mg, 3.0mmol), and ammonia (d. 0.88, 5ml). This was stirred at room temperature for 48 hours, then water (30ml) was added and the reaction mixture extracted with diethyl ether (2 x 50ml). The combined organic extracts were extracted with dilute hydrochloric acid (1 M, 2 x 50ml), then basified with dilute sodium hydroxide solution (2 M, 60ml) and back extracted with diethyl ether (3 x 50ml). The combined organic extracts were dried and concentrated to leave the amino nitrile as an oil. This was treated with concentrated hydrochloric acid (5ml) for 48 hours at room temperature and then water (20ml) was added and the reaction mixture heated to reflux for 6 hours. The solvent was removed *in vacuo* and the crude product purified using an ion exchange resin (Amberlite CG-400). Washing with water (100ml), elution with dilute hydrochloric acid (1 M, 100ml) and evaporation of the eluate to dryness gave the product (**2.66**) as a white solid (325mg, 46%). **m.p.** 210-212°C (dec). **NMR** ($d_6$-DMSO): $^1$H: $\delta$ 2.09 (2H, m, C$H_2$CH), 2.67 (1H, m, ArC$H_a$H$_b$), 2.77 (1H, m, ArCH$_a$$H_b$), 3.85 (1H, m, CH$_2$C$H$), 7.14 (2H, m, 2 x Ar$H$), 7.28 (2H, m, 2 x Ar$H$), 8.6 (4H, br s, 4 x $H^+$). $^{13}$C: $\delta$ 29.4 ($CH_2$), 31.8 ($CH_2$), 51.4 ($CH$), 115.1 (2 x $CH$, d, $J$ 21.0), 130.0 (2 x $CH$, d, $J$ 8.0), 136.4 ($C_q$, d, $J$ 3.5), 160.2 ($CF$, d, $J$ 241.5), 170.6 ($CO_2H$). $\upsilon_{max}$ (KBr disc, cm$^{-1}$): 3240, 2850, 1735, 1510, 1280, 1245, 1230. **Accurate mass** Found: 197.08521. $C_{10}H_{12}FNO_2$ requires 197.08521.

*3-(2-Fluorophenyl)propan-1-ol* (**2.58**)[181]




To a slurry of lithium aluminium hydride (3.99g, 0.105mol) in dry diethyl ether (150ml) was added 2-fluorocinnamic acid (**2.55**) (4.98g, 0.030mol). The reaction mixture was heated to reflux for 4 hours, then cooled to 0°C. Treatment with water (50ml) and dilute sulfuric acid (10% v/v, 100ml) was followed by separation of the organic layer and extraction of the aqueous phase with diethyl ether (2 x 50ml). The combined organic layers were dried and concentrated to leave the product (**2.58**) as an oil (4.15g, 88%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.85 (2H, m, $CH_2CH_2OH$), 2.70 (1H, br s, $OH$), 2.71 (2H, t, $J$ 7.5, $ArCH_2$), 3.64 (2H, t, $J$ 6.5, $CH_2OH$), 7.03 (2H, m, 2 x $ArH$), 7.17 (2H, m, 2 x $ArH$). $^{13}C$: $\delta$ 25.3 ($ArCH_2$, d, $J$ 2.0), 32.9 ($CH_2$), 61.9 ($CH_2OH$), 115.2 ($CH$, d, $J$ 23.0), 124.0 ($CH$, d, $J$ 3.5), 127.6 ($CH$, d, $J$ 8.0), 128.6 ($C_q$, d, $J$ 15.5), 130.7 ($CH$, d, $J$ 5.5), 161.2 ($CF$, d, $J$ 244.5).

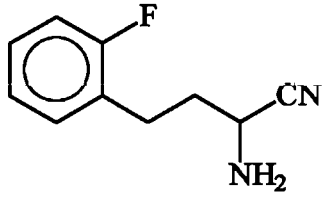
*3-(2-Fluorophenyl)propan-1-al* (**2.61**)[180]




To a solution of 3-(2-fluorophenyl)propan-1-ol (**2.58**) (3.08g, 0.020mol) in dichloromethane (100ml) was added PCC (4.50g, 0.022mol). After stirring for 4 hours at room temperature, diethyl ether (50ml) was added and the reaction mixture was filtered through silica (50g). The eluate was concentrated to leave the crude product (**2.61**) which was purified by distillation at reduced pressure (0.5mm Hg, 100°C, 1.87g, 62%). **NMR**

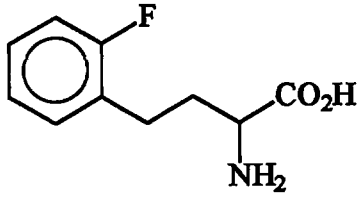
($CDCl_3$): $^1H$: $\delta$ 2.77 (2H, dt, $J$ 1.0, 7.5, $CH_2CHO$), 2.97 (2H, t, $J$ 7.5, $ArCH_2$), 7.03 (2H, m, 2 x Ar*H*), 7.18 (2H, m, 2 x Ar*H*), 9.80 (1H, s, C*H*O). $^{13}C$: $\delta$ 21.8 ($ArCH_2$, d, $J$ 2.3.0), 43.9 ($CH_2CHO$), 115.4 (*C*H, d, $J$ 22.0), 124.1 (*C*H, d, $J$ 3.5), 127.2 ($C_q$, d, $J$ 15.5), 128.1 (*C*H, d, $J$ 8.5), 130.6 (*C*H, d, $J$ 4.5), 161.2 (*C*F, d, $J$ 245.5).

*2-Amino-4-(2-fluorophenyl)butanenitrile*



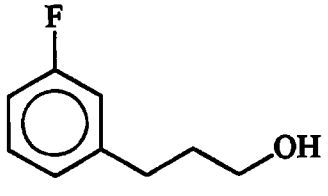

To a solution of the 3-(2-fluorophenyl)propan-1-al (**2.61**) (1.08g, 7.1mmol) in ethanol (20ml) and water (10ml) was added ammonium chloride (380mg, 7.1mmol), potassium cyanide (460mg, 7.1mmol), and ammonia (d. 0.88, 10ml). This was stirred at room temperature for 96 hours, then water (50ml) was added and the reaction mixture extracted with diethyl ether (3 x 50ml). The combined organic extracts were extracted with dilute hydrochloric acid (1 M, 2 x 50ml), then basified with dilute sodium hydroxide solution (2 M, 60ml) and back extracted with diethyl ether (3 x 50ml). The combined organic extracts were dried and concentrated to leave the aminonitrile as an oil (460mg, 36%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.70 (2H, br s, $NH_2$), 2.04 (2H, m, $CH_2CH$), 2.86 (2H, m, $ArCH_2$), 3.63 (1H, t, $J$ 7.5, $CH_2CH$), 7.05 (2H, m, 2 x Ar*H*), 7.21 (2H, m, 2 x Ar*H*). $^{13}C$: $\delta$ 25.3 ($ArCH_2$, d, $J$ 3.0), 35.5 ($CH_2$), 42.7 (*C*H), 115.5 (*C*H, d, $J$ 22.0), 122.0 (*C*N), 124.2 (*C*H, d, $J$ 3.0), 126.7 ($C_q$, d, $J$ 15.5), 128.3 (*C*H, d, $J$ 8.5), 130.7 (*C*H, d, $J$ 5.5), 161.1 (*C*F, d, $J$ 245.5).

*D,L-2-Amino-4-(2-fluorophenyl)butanoic acid (2-Fluorohomophenylalanine)* **(2.64)**




2-Amino-4-(2-fluorophenyl)butanenitrile (430mg, 2.4mmol) was treated with concentrated hydrochloric acid (5ml) for 96 hours at room temperature and then water (30ml) was added and the reaction mixture heated to reflux for 6 hours. The solvent was removed *in vacuo* to leave the product (**2.64**) as a white solid (450mg, 80%). **m.p.** 188-190°C. **NMR** ($d_6$-DMSO): $^1$H: $\delta$ 2.14 (2H, q, *J* 7.5, C$H_2$CH), 2.75 (1H, m, ArC$H_a$H$_b$), 2.86 (1H, m, ArCH$_a$$H_b$), 3.95 (1H, m, CH$_2$C*H*), 7.15 (2H, m, 2 x Ar*H*), 7.30 (2H, m, 2 x Ar*H*), 8.8 (4H, br s, 4 x $H^+$). $^{13}$C: $\delta$ 23.8 (ArCH$_2$, d, *J* 2.0), 30.2 (*C*H$_2$), 51.6 (*C*H), 115.1 (*C*H, d, *J* 22.0), 124.4 (*C*H, d, *J* 3.5), 126.9 ($C_q$, d, *J* 15.5), 128.3 (*C*H, d, *J* 8.5), 130.5, (*C*H, d, *J* 4.5), 160.3 (*C*F, d, *J* 243.5), 170.5 (*C*O$_2$H). $\upsilon_{max}$ (KBr disc, cm$^{-1}$): 3135, 3050, 2950, 1735, 1495, 1405, 1235, 750. **Accurate mass** Found: 197.08524. $C_{10}H_{12}FNO_2$ requires 197.08521.

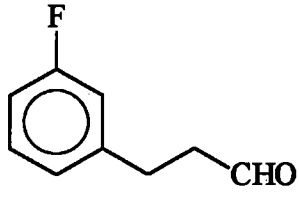
*3-(3-Fluorophenyl)propan-1-ol* **(2.59)**[182]




To a slurry of lithium aluminium hydride (4.04g, 0.106mol) in dry diethyl ether (150ml) was added 3-fluorocinnamic acid (**2.56**) (4.98g, 0.030mol). The reaction mixture

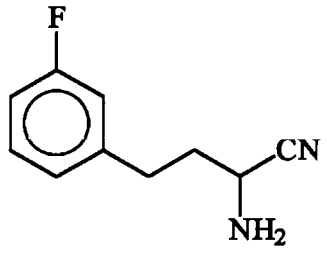
was heated to reflux for 4 hours, then cooled to 0°C. Treatment with water (30ml) and dilute sulfuric acid (10% v/v, 100ml) was followed by separation of the organic layer and extraction of the aqueous phase with diethyl ether (2 x 50ml). The combined organic layers were dried and concentrated to leave the product (**2.59**) as an oil (4.13g, 87%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.85 (2H, m, $CH_2CH_2OH$), 2.67 (2H, t, $J$ 8.0, $ArCH_2$), 2.70 (1H, br s, $OH$), 3.62 (2H, t, $J$ 6.5, $CH_2CH$), 6.90 (3H, m, 3 x $ArH$), 7.20 (1H, m, $ArH$). $^{13}C$: $\delta$ 31.8 ($CH_2$), 33.8 ($CH_2$), 61.8 ($CH_2OH$), 112.7 ($CH$, d, $J$ 21.0), 115.2 ($CH$, d, $J$ 21.0), 124.1 ($CH$, d, $J$ 3.0), 129.8 ($CH$, d, $J$ 8.5), 144.5 ($C_q$, d, $J$ 7.5), 162.9 ($CF$, d, $J$ 244.5).

### *3-(3-Fluorophenyl)propan-1-al* (**2.62**)



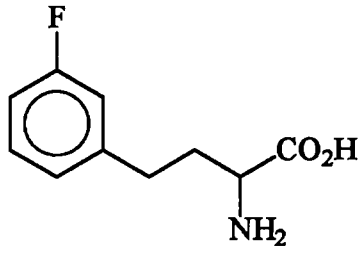

To a solution of 3-(3-fluorophenyl)propan-1-ol (**2.59**) (3.08g, 0.020mol) in dichloromethane (100ml) was added PCC (6.40g, 0.029mol). After stirring for 4 hours at room temperature, diethyl ether (50ml) was added and the reaction mixture was filtered through silica (50g). The eluate was concentrated to leave the crude product (**2.62**) which was purified by distillation at reduced pressure (0.5mm Hg, 100°C, 1.70g, 56%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 2.77 (2H, m, $CH_2CHO$), 2.93 (2H, t, $J$ 7.5, $ArCH_2$), 6.90 (3H, m, 3 x $ArH$), 7.20 (1H, m, $ArH$), 9.79 (1H, t, $J$ 1.0, $CHO$). $^{13}C$: $\delta$ 27.8 ($ArCH_2$, d, $J$ 1.5), 44.9 ($CH_2CHO$), 113.2 ($CH$, d, $J$ 20.5), 115.3 ($CH$, m), 124.0 ($CH$, d, $J$ 3.5), 130.1 ($CH$, m), 143.0 ($C_q$, d, $J$ 7.5), 163.0 ($CF$, d, $J$ 245.5), 201.1 ($CHO$).

*2-Amino-4-(3-fluorophenyl)butanenitrile*




To a solution of the 3-(3-fluorophenyl)propan-1-al (**2.62**) (1.08g, 7.1mmol) in ethanol (20ml) and water (10ml) was added ammonium chloride (390mg, 7.3mmol), potassium cyanide (470mg, 7.2mmol), and ammonia (d. 0.88, 5ml). This was stirred at room temperature for 96 hours, then water (40ml) was added and the reaction mixture extracted with diethyl ether (3 x 50ml). The combined organic extracts were extracted with dilute hydrochloric acid (1 M, 3 x 50ml), then basified with dilute sodium hydroxide solution (2 M, 60ml) and back extracted with diethyl ether (3 x 50ml). The combined organic extracts were dried and concentrated to leave the amino nitrile as an oil (700mg, 55%). NMR ($CDCl_3$): $^1H$: $\delta$ 1.70 (2H, br s, $NH_2$), 2.03 (2H, m, $CH_2CH$), 2.83 (2H, m, $ArCH_2$), 3.60 (1H, t, *J* 7.0, $CH_2CH$), 6.91 (3H, m, 3 x ArH), 7.25 (1H, m, ArH). $^{13}C$: $\delta$ 31.3 ($CH_2$), 36.5 ($CH_2$), 42.5 (CH), 113.4 (CH, d, *J* 21.0), 115.3 (CH, d, *J* 21.0), 122.0 (CN), 124.1 (CH, d, *J* 2.5), 130.1 (CH, d, *J* 8.5), 142.4 ($C_q$, d, *J* 7.5), 162.9 (CF, d, *J* 246.5).

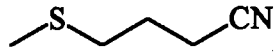
*D,L-2-Amino-4-(3-fluorophenyl)butanoic acid (3-Fluorohomophenylalanine)* **(2.65)**




2-Amino-4-(3-fluorophenyl)butanenitrile (700mg, 3.9mmol) was treated with concentrated hydrochloric acid (5ml) for 40 hours at room temperature and then water (20ml) was added and the reaction mixture heated to reflux for 4 hours. The solvent was removed *in vacuo* to leave the product (**2.65**) as a white solid (890mg, 97%). **m.p.** 190-194°C (dec). **NMR** ($d_6$-DMSO): $^1$H: $\delta$ 2.15 (2H, q, *J* 7.5, C$H_2$CH), 2.72 (1H, m, ArC$H_a$H$_b$), 2.87 (1H, m, ArCH$_a$$H_b$), 3.86 (1H, m, CH$_2$C*H*), 7.07 (1H, m, Ar*H*), 7.37 (3H, m, 3 x Ar*H*), 8.8 (4H, br s , $H^+$). $^{13}$C: $\delta$ 30.1 (*C*H$_2$), 31.6 (*C*H$_2$), 51.6 (*C*H), 113.1 (*C*H, d, *J* 21.0), 115.1 (*C*H, d, *J* 21.0), 124.6 (*C*H, d, *J* 3.0), 130.4 (*C*H, d, *J* 8.5), 143.5 ($C_q$, d, *J* 8.5), 162.3 (*C*F, d, *J* 243.5), 170.7 (*C*O$_2$H). $\nu_{max}$ (KBr disc, cm$^{-1}$): 3130, 3050, 2950, 1735, 1490, 1405, 1190. **Accurate mass** Found: 197.08603. $C_{10}H_{12}FNO_2$ requires 197.08521.

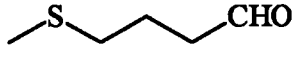
# *3.4 Specificity of Aliphatic Amino Acid Decarboxylation*

## *4-Methylthiobutanenitrile* (**2.76**)[183]



A solution of sodium methoxide formed from sodium (1.40g, 0.061mol) and methanol (50ml) was treated with gaseous methanethiol (4.0g, 0.083mol). To this solution was added 4-bromobutanenitrile (**2.73**) (7.4g, 0.050mol). After 2.5 hours diethyl ether (100ml) and water (150ml) were added. The aqueous phase was extracted with diethyl ether (2 x 100ml) and the combined organic phases were washed with water, dried and concentrated to leave the product (**2.76**) as an oil (4.30g, 75%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.94 (2H, quin, *J* 7.0, $CH_2CH_2CN$), 2.10 (3H, s, $CH_3S$), 2.52 (2H, t, *J* 7.0, $CH_2$), 2.62 (2H, t, *J* 7.0, $CH_2$). $^{13}C$: $\delta$ 15.2 ($CH_3S$), 15.8 ($SCH_2$), 24.6 ($CH_2$), 32.6 ($CH_2$), 119.4 (*C*N).

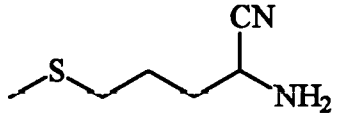
## *4-Methylthiobutanal* (**2.79**)



4-Methylthiobutanenitrile (**2.76**) (2.40g, 0.021mol) in hexane (100ml) and diethyl ether (50ml) at -70°C under nitrogen was treated with DIBAL (3.30g, 0.023mol) in hexane (50ml), dropwise over 5 minutes. After 30 minutes the reaction was allowed to warm up to room temperature and left for 5 hours. Then ethyl acetate (5ml) was added, followed by ammonium chloride solution (sat. 100ml) with stirring for 30 mins. After the addition of sulfuric acid (2N, 100ml) and further stirring for 1 hour the reaction mixture was partitioned and the aqueous phase extracted with diethyl ether (2 x 100ml). The organic layers were combined, washed with brine and then water, dried and then concentrated to

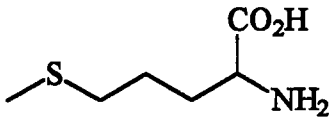
leave the product (**2.79**) as an oil, which was purified by distillation at reduced pressure (50°C/0.6 mm Hg, 0.510g, 21%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.94 (2H, m, $CH_2CH_2$), 2.09 (3H, s, $CH_3S$), 2.54 (2H, t, $J$ 7.0, $SCH_2$), 2.60 (2H, dt, $J$ 1.5, 7.0, $CH_2CHO$), 9.81 (1H, t, $J$ 1.5, CHO). $^{13}C$: $\delta$ 15.2 ($CH_3S$), 21.5 ($CH_2$), 33.4 ($CH_2$), 42.5 ($CH_2CHO$), 201.7 (CHO). $\upsilon_{max}$ (film, $cm^{-1}$): 2915, 2830, 1725, 1435, 1425.

## *2-Amino-5-methylthiopentanenitrile* (**2.82**)



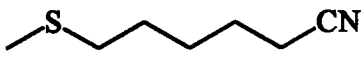

4-Methylthiobutanal (**2.79**) (2.15g, 0.018mol) in ethanol (20ml) and water (10ml) was treated with ammonium chloride (1.01g, 0.019mol), ammonia (d. 0.88, 5ml) and potassium cyanide (1.19g, 0.018mol) and stirred at room temperature for 72 hours. Water (50ml) was added and the reaction mixture extracted with diethyl ether (3 x 50ml). The combined organic layers were extracted with dilute hydrochloric acid (1N, 2 x 100ml), which was the treated with diluted sodium hydroxide solution (2M to pH9). This was then back extracted with diethyl ether (3 x 100ml). The combined organic extracts were dried and concentrated *in vacuo* to leave the product (**2.82**) as an oil (0.860g, 33%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.75 (2H, br s, $NH_2$), 1.84 (4H, m, 2 x $CH_2$), 2.11 (3H, s, $CH_3S$), 2.56 (2H, t, $J$ 6.5, $SCH_2$), 3.73 (1H, t, $J$ 6.5, CH). $^{13}C$: $\delta$ 15.4 ($CH_3S$), 24.7 ($CH_2$), 33.3 ($CH_2$), 34.1 ($CH_2$), 43.0 (CH), 122.1 (CN).

*D,L-2-Amino-5-methylthiopentanoic acid (D,L-Homomethionine, HMet)* **(2.70)**[62,184]



2-Amino-5-methylthiopentanenitrile **(2.82)** (860mg, 5.97mmol) was treated with concentrated hydrochloric acid (12N, 5ml) and left overnight at room temperature. Water (25ml) was added and the reaction mixture heated to reflux for 6 hours. The mixture was taken to dryness *in vacuo* and the residue dissolved in aqueous sodium hydroxide solution (2N, 2ml). This was applied to an ion exchange resin (Amberlite CG-400) which was washed with water (50ml) then eluted with dilute hydrochloric acid (1N, 50ml). The eluate was taken to dryness to leave the product **(2.70)** as its hydrochloride (560mg, 50%). **m.p.** 168-172°C (dec). **NMR** ($d_6$-DMSO): $^1$H: $\delta$ 1.61 (1H, m, C$H_a$CH$_b$), 1.73 (1H, m, CH$_a$C$H_b$), 1.90 (2H, m, CH$_2$), 2.04, (3H, s, C$H_3$S), 2.48 (2H, t, *J* 7.0, SC$H_2$), 3.89 (1H, m, C*H*), 8.6 (3H, br s, $H^+$). $^{13}$C: $\delta$ 14.8 (*C*H$_3$S), 24.2 (*C*H$_2$), 29.3 (*C*H$_2$), 32.8 (*C*H$_2$), 52.0 (*C*H), 171.0 (*C*O$_2$H). $\upsilon_{max}$ (KBr disc, cm$^{-1}$): 3065, 2915, 1740, 1715, 1495, 1455, 1420, 1225, 1175.

*6-Methylthiohexanenitrile* **(2.77)**[185]



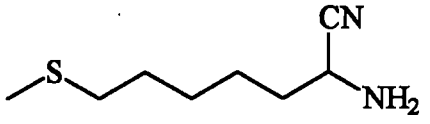
A solution of sodium methoxide formed from sodium (1.50g, 0.065mol) and methanol (100ml) was treated with gaseous methanethiol (4.52g, 0.094mol). To this solution was added 6-bromohexanenitrile **(2.74)** (11.0g, 0.062mol). After stirring for 6 hours at room temperature the solvent was removed *in vacuo* and the residue partitioned

between water (100ml) and diethyl ether (3 x 100ml). The combined organic layers were dried and concentrated to leave the product (**2.77**) as an oil (5.94g, 66%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.53-1.72 (6H, m, 3 x $CH_2$), 2.09, (3H, s, $CH_3S$), 2.37 (2H, t, *J* 7.0, $CH_2$), 2.51 (2H, t, *J* 7.0, $CH_2$). $^{13}C$: $\delta$ 15.5 ($CH_3S$), 17.1 ($SCH_2$), 25.0 ($CH_2$), 27.7 ($CH_2$), 28.2 ($CH_2$), 33.7 ($CH_2$), 119.7 (*C*N).

## *6-Methylthiohexanal* (**2.80**)

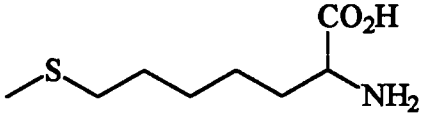

6-Methylthiohexanenitrile (**2.77**) (5.00g, 0.035mol) in hexane (50ml) and diethyl ether (50ml) at -70°C under nitrogen was treated with DIBAL (5.50g, 0.039mol) in hexane (50ml) dropwise over 10 minutes. After stirring for 1 hour the reaction was allowed to warm up to room temperature and stirred for a further 5 hours. Then ethyl acetate (5ml) was added, followed by ammonium chloride solution (sat. 50ml) with stirring for 30 minutes. After the addition of sulfuric acid (2N, 50ml) and further stirring for 30 minutes the reaction mixture was partitioned and the aqueous phase extracted with diethyl ether (2 x 100ml). The organic layers were combined, washed with brine and then water, dried and then concentrated to leave the product (**2.80**) as an oil. This was purified by distillation at reduced pressure (125°C/0.8 mm Hg, 2.67g, 52%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.46 (2H, m, $CH_2$), 1.64 (4H, m, 2 x $CH_2$), 2.11 (3H, s, $CH_3S$), 2.49 (4H, m, 2 x $CH_2$), 9.77 (1H, s, CHO). $^{13}C$: $\delta$ 15.5 ($CH_3S$), 21.6 ($CH_2$), 28.2 ($CH_2$), 28.8 ($CH_2$), 33.9 ($CH_2$), 43.7 ($CH_2CHO$), 202.5 (*C*HO). $\upsilon_{max}$ (film, $cm^{-1}$): 2920, 2860, 1725.

*2-Amino-7-methylthioheptanenitrile* (**2.83**)




6-Methylthiohexanal (**2.80**) (1.46g, 0.010mol) in ethanol (30ml) and water (10ml) was treated with ammonium chloride (0.555g, 0.010mol), ammonia (d. 0.88, 10ml) and potassium cyanide (0.660g, 0.010mol) with stirring at room temperature for 48 hours. Water (50ml) was added and the reaction mixture extracted with diethyl ether (3 x 50ml). The combined organic layers were extracted with dilute hydrochloric acid (1N, 2 x 100ml), which was then treated with diluted sodium hydroxide solution (2M to ph9). This was then back extracted with diethyl ether (3 x 100ml). The combined organic extracts were dried and concentrated *in vacuo* to leave the product (**2.83**) as an oil (1.16g, 67%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.41-1.78 (10H, m, 4 x $CH_2$, $NH_2$), 2.09 (3H, s, $CH_3S$), 2.50 (2H, t, *J* 7.0, $SCH_2$), 3.68 (1H, t, *J* 7.0, *CH*). $^{13}C$: $\delta$ 15.5 ($CH_3S$), 25.1 ($CH_2$), 28.1 ($CH_2$), 28.8 ($CH_2$), 34.0 ($CH_2$), 35.2 ($CH_2$), 43.3 (*CH*), 122.2 (*CN*).

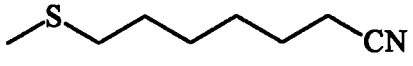
*D,L-2-Amino-7-methylthioheptanoic acid (D,L-Trihomomethionine, TriHMet)* (**2.71**)




2-Amino-7-methylthioheptanenitrile (**2.83**) (1.10g, 0.0064mol) was treated with hydrochloric acid (12N, 5ml) and left overnight at room temperature. Water (20ml) was added and the reaction mixture heated to reflux for 6 hours. The mixture was taken to dryness *in vacuo* to leave the product as its hydrochloride (**2.71**) as a white solid (1.37g,

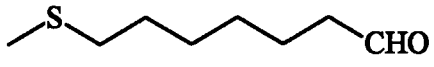
94%). **m.p.** 149-151°C (dec). **NMR** ($d_6$-DMSO): $^1$H: $\delta$ 1.47 (6H, m, 3 x C$H_2$), 1.84 (2H, m, C$H_2$), 2.07, (3H, s, C$H_3$S), 2.49 (2H, t, *J* 7.0, SC$H_2$), 3.86 (1H, m, C*H*), 8.55 (3H, br s, $H^+$). $^{13}$C: $\delta$ 14.8 (*C*$H_3$S), 23.8 (*C*$H_2$), 27.6 (*C*$H_2$), 28.2 (*C*$H_2$), 29.8 (*C*$H_2$), 33.1 (*C*$H_2$), 51.9 (*C*H), 170.9 (*C*$O_2$H). $\upsilon_{max}$ (KBr disc, cm$^{-1}$): 3025, 2925, 2855, 1730, 1485, 1405, 1220, 1210. **Elemental analysis** Found: C, 50.5; H, 9.0; N, 7.6. $C_8H_{17}NO_2S$ requires C, 51.0; H, 9.1; N, 7.4. **Accurate mass** Found: 191.0994. $C_8H_{17}NO_2S$ requires 191.09800.

## *7-Methylthioheptanenitrile* (**2.78**)[186]



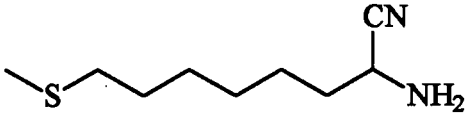

A solution of sodium methoxide formed from sodium (1.70g, 0.074mol) and methanol (100ml) was treated with gaseous methanethiol (4.5g, 0.094mol). To this solution was added 7-bromoheptanenitrile (**2.75**) (14.0g, 0.073mol). After stirring for 4 hours at room temperature, water (100ml) was added and the reaction mixture was extracted with diethyl ether (3 x 100ml). The combined organic layers were dried and concentrated to leave the product (**2.78**) as an oil (11.05g, 95%). **NMR** ($CDCl_3$): $^1$H: $\delta$ 1.46 (4H, m, 2 x C$H_2$), 1.64 (4H, m, 2 x C$H_2$), 2.10 (3H, s, C$H_3$S), 2.36 (2H, t, *J* 7.0, C$H_2$CN), 2.50 (2H, t, *J* 7.0, SC$H_2$). $^{13}$C: $\delta$ 15.5 (*C*$H_3$S), 17.1 (S*C*$H_2$), 25.3 (*C*$H_2$), 27.9 (*C*$H_2$), 28.3 (*C*$H_2$), 28.8 (*C*$H_2$), 34.0 (*C*$H_2$), 119.8 (*C*N).

*7-Methylthioheptanal* (**2.81**)




7-Methylthioheptanenitrile (**2.78**) (5.00g, 0.032mol) in hexane (50ml) and diethyl ether (50ml) at -70°C under nitrogen was treated with DIBAL (4.52g, 0.032mol) in hexane (50ml) dropwise over 10 minutes. After stirring for 1.5 hours the reaction was allowed to warm up to room temperature and stirred overnight. Then ethyl acetate (2ml) was added, followed by ammonium chloride solution (sat. 50ml) with stirring for 30 minutes. After the addition of sulfuric acid (2N, 50ml) and further stirring for 30 minutes the reaction mixture was partitioned and the aqueous phase extracted with diethyl ether (2 x 100ml). The organic layers were combined, washed with brine and then water, dried and then concentrated to leave the product (**2.81**) as an oil. This was purified by distillation (150°C/0.5 mm Hg, 2.34g, 46%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.42 (4H, m, 2 x $CH_2$), 1.63 (4H, m, 2 x $CH_2$), 2.09, (3H, s, $CH_3S$), 2.45 (4H, m, 2 x $CH_2$), 9.76 (1H, t, $J$ 1.5, CHO). $^{13}C$: $\delta$ 15.5 ($CH_3S$), 21.9 ($CH_2$), 28.5 ($CH_2$), 28.7 ($CH_2$), 28.9 ($CH_2$), 34.1 ($CH_2$), 43.8 ($CH_2CHO$), 202.7 (CHO). $\upsilon_{max}$ (film, $cm^{-1}$): 2925, 2860, 1730, 1125.

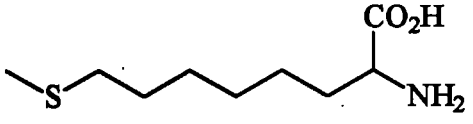
*2-Amino-8-methylthiooctanenitrile* (**2.84**)




7-Methylthioheptanal (**2.81**) (1.60g, 0.010mol) in ethanol (20ml) and water (10ml) was treated with ammonium chloride (0.540g, 0.010mol), ammonia (d. 0.88, 8ml) and potassium cyanide (0.65g, 0.010mol) and stirred at room temperature for 48 hours. Water (50ml) was added and the reaction mixture extracted with diethyl ether (3 x 50ml). The

combined organic layers were extracted with dilute hydrochloric acid (1N, 2 x 100ml), which was then treated with diluted sodium hydroxide solution (2M to pH9). This was then back extracted with diethyl ether (3 x 100ml). The combined organic extracts were dried and concentrated *in vacuo* to leave the product (**2.84**) as an oil (1.08g, 58%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.34-1.75 (12H, m, 5 x C$H_2$, N$H_2$), 2.09 (3H, s, C$H_3$S), 2.49 (2H, t, *J* 7.0, SC$H_2$), 3.68 (1H, t, *J* 7.0, C*H*). $^{13}C$: $\delta$ 15.5 (*C*$H_3$S), 25.3 (*C*$H_2$), 28.4 (*C*$H_2$), 28.7 (*C*$H_2$), 28.9 (*C*$H_2$), 34.1 (*C*$H_2$), 35.2 (*C*$H_2$), 43.3 (*C*H), 122.2 (*C*N).

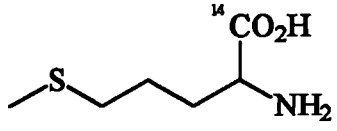
## *D,L-2-Amino-8-methylthiooctanoic acid (D,L-Tetrahomomethionine, TetHMet)* (2.72)




2-Amino-8-methylthiooctanenitrile (**2.84**) (1.08g, 5.8mmol) was treated with concentrated hydrochloric acid (12N, 5ml) and left overnight at room temperature. Water (20ml) was added and the reaction mixture heated to reflux for 6 hours. The mixture was taken to dryness *in vacuo* and the residue dissolved in aqueous sodium hydroxide solution (2N, 2ml). This was applied to an ion exchange resin (Amberlite CG-400) which was washed with water (50ml) then eluted with dilute hydrochloric acid (1N, 50ml). The eluate was taken to dryness to leave the product (**2.72**) as its hydrochloride (920mg, 66%). **m.p.** 118-121°C (dec). **NMR** ($d_6$-DMSO): $^1H$: $\delta$ 1.30-1.53 (6H, m, 3 x C$H_2$), 1.78 (2H, m, C$H_2$), 2.03 (3H, s, C$H_3$S), 2.45 (2H, t, *J* 7.5, SC$H_2$), 3.85 (1H, m, C*H*), 8.6 (3H, br s, $H^+$). $^{13}C$: $\delta$ 14.8 (*C*$H_3$S), 24.1 (*C*$H_2$), 27.8 (*C*$H_2$), 28.2 (*C*$H_2$), 28.4 (*C*$H_2$), 29.9 (*C*$H_2$), 33.2 (*C*$H_2$), 51.9 (*C*H), 171.1 (*C*$O_2$H). $\nu_{max}$ (KBr disc, $cm^{-1}$): 3015, 2930, 2855, 1725, 1675, 1480, 1220, 1210. **Elemental analysis** Found: C, 52.8; H, 9.4; N, 6.8. $C_9H_{19}NO_2S$

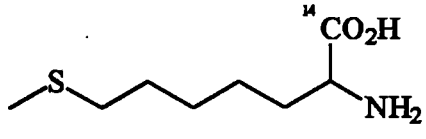
requires C, 53.4; H, 9.5; N, 6.9. **Accurate mass** Found: 205.11426. $C_9H_{19}NO_2S$ requires 205.11365.

### *D,L-[$^{14}$C-1]-2-Amino-5-methylthiopentanoic acid* (**2.85**)




As for 2-amino-5-methylthiopentanoic acid (**2.70**). 4-Methylthiopentanal (**2.79**) (1.38mg, 11.7μmol) in ethanol (370μl), ammonium chloride solution (65μl, 10mg/ml, 11.9μl), ammonia (d. 0.88, 50μl), [$^{14}$C] potassium cyanide (0.76mg, 11.7μmol, 50.6mCi/mmol, Sigma) were mixed and left at room temperature for 48 hours. Addition of water (15ml), partitioning with diethyl ether, extraction into acid, back extraction into diethyl ether after aqueous base treatment gave the aminonitrile. This was followed by acid hydrolysis and purification of the product using an ion exchange resin (Amberlite CG-400) to give the product (**2.85**) as its hydrochloride with a radiochemical yield of 47μCi (8%).

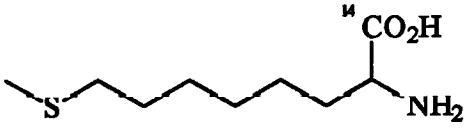
### *[$^{14}$C-1]-2-Amino-7-methylthioheptanoic acid* (**2.86**)




As for 2-amino-7-methylthioheptanoic acid (**2.71**). 6-Methylthiohexanal (**2.80**) (3.60mg, 24.7μmol) in ethanol (480μl), ammonium chloride solution (140μl, 10mg/ml, 26.1μl), ammonia (d. 0.88, 100μl), [$^{14}$C] potassium cyanide (1.60mg, 24.6μmol, 50.6mCi/mmol, Sigma) were mixed and left at room temperature for 5 days. Addition of water (15ml), partitioning with diethyl ether, extraction into acid, back extraction into

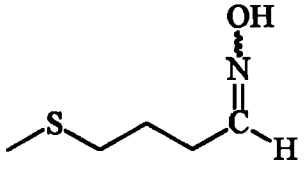
diethyl ether after aqueous base treatment gave the aminonitrile. This was followed by acid hydrolysis and purification of the product using an ion exchange resin (Amberlite CG-400) to give the product (**2.86**) as its hydrochloride with a radiochemical yield of 200$\mu$Ci (16%).

### *[$^{14}$C-1]-2-Amino-8-methylthiooctanoic acid* (**2.87**)



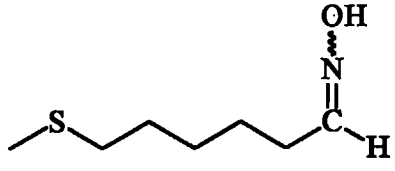

As for 2-amino-8-methylthiooctanoic acid (**2.72**). 7-Methylthioheptanal (**2.81**) (1.80mg, 11.9$\mu$mol) in ethanol (395$\mu$l), ammonium chloride solution (65$\mu$l, 10mg/ml, 11.9$\mu$l), ammonia (d. 0.88, 50$\mu$l), [$^{14}$C] potassium cyanide (0.76mg, 11.7$\mu$mol, 50.6mCi/mmol, Sigma) were mixed and left at room temperature for 4 days. Addition of water (15ml), partitioning with diethyl ether, extraction into acid, back extraction into diethyl ether after aqueous base treatment gave the aminonitrile. This was followed by acid hydrolysis and purification of the product using an ion exchange resin (Amberlite CG-400) to give the product (**2.87**) as its hydrochloride with a radiochemical yield of 237$\mu$Ci (40%).

### *4-Methylthiobutanaldoxime* (**2.88**)[187]



4-Methylthiobutanal (**2.79**) (400mg, 3.4mmol), hydroxylamine hydrochloride (240mg, 3.4mmol) and sodium hydrogen carbonate (290mg, 3.4mmol) were stirred in ethanol (20ml) and water (10ml) at room temperature for 16 hours. The solution was diluted with water (50ml) and extracted with diethyl ether (3 x 50ml). The combined organic layers were washed with water, saturated brine, dried, and concentrated to leave the product (**2.88**) as an oil, as a 55/45 mixture of (*E*)- and (*Z*)- isomers (280mg, 77%). NMR ($CDCl_3$): (*E*)-isomer $^1H$: $\delta$ 1.80 (2H, m, C$H_2$), 2.10 (3H, m, C$H_3$S), 2.33 (2H, m, C$H_2$), 2.52 (2H, m, C$H_2$), 7.44 (1H, t, *J* 6.0, C*H*=NOH), 9.0 (1H, br s, CH=NO*H*). $^{13}C$: $\delta$ 15.4 (*C*$H_3$S), 25.9 (*C*$H_2$), 28.5 (*C*$H_2$), 33.8 (*C*$H_2$), 151.3 (*C*=NOH). (*Z*)-isomer $^1H$: $\delta$ 1.80 (2H, m, C$H_2$), 2.10 (3H, s, C$H_3$S), 2.52 (4H, m, 2 x C$H_2$), 6.75 (1H, t, *J* 5.5, C*H*=NOH), 9.4 (1H, br s, CH=NO*H*). $^{13}C$: $\delta$ 15.4 (*C*$H_3$S), 24.2 (*C*$H_2$), 25.3 (*C*$H_2$), 33.4 (*C*$H_2$), 151.7 (*C*=NOH). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): (both isomers): 3300, 2915, 2860, 1440, 925.

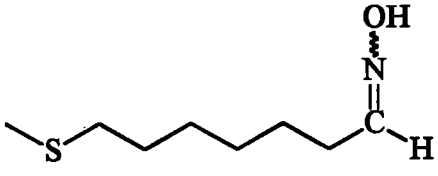
### *6-Methylthiohexanaldoxime* (**2.89**)




6-Methylthiohexanal (**2.80**) (1.09g, 7.5mmol), hydroxylamine hydrochloride (520mg, 7.5mmol) and sodium hydrogen carbonate (640mg, 7.6mmol) were stirred in ethanol (20ml) and water (10ml) at room temperature for 16 hours. The solution was diluted with water (50ml) and extracted with diethyl ether (3 x 75ml). The combined organic layers were washed with water, saturated brine, dried, and concentrated to leave the product as an oil which crystallised on standing. Recrystallisation from diethyl

ether/hexane gave the pure product (**2.89**) as a mixture (2:1) of (*E*)- and (*Z*)- isomers (1.10g, 91%). **m.p.** 54-55°C. **NMR** ($CDCl_3$): (*E*)-isomer $^1$H: δ 1.49 (4H, m, 2 x C*H*$_2$), 1.61 (2H, m, C*H*$_2$), 2.10 (3H, s, C*H*$_3$S), 2.20 (2H, m, C*H*$_2$CH=NOH), 2.50 (2H, t, *J* 7.0, SC*H*$_2$), 7.43 (1H, t, *J* 6.0, C*H*=NOH), 8.4 (1H, br s, CH=NO*H*). $^{13}$C: δ 15.5 (*C*H$_3$S), 26.1 (*C*H$_2$), 28.2 (*C*H$_2$), 28.8 (*C*H$_2$), 29.4 (*C*H$_2$), 34.0 (*C*H$_2$), 152.0 (*C*=NOH). (*Z*)-isomer $^1$H: δ 1.49 (4H, m, 2 x C*H*$_2$), 1.61 (2H, m, C*H*$_2$), 2.10 (3H, s, C*H*$_3$), 2.39 (2H, dt, *J* 5.5, 7.0, C*H*$_2$CH=NOH), 2.50 (2H, t, *J* 7.5, SC*H*$_2$), 6.72 (1H, t, *J* 5.5, C*H*=NOH), 8.9 (1H, br s, CH=NO*H*). $^{13}$C: δ 15.5 (*C*H$_3$S), 24.8 (*C*H$_2$), 25.7 (*C*H$_2$), 28.5 (*C*H$_2$), 28.8 (*C*H$_2$), 34.0 (*C*H$_2$), 152.6 (*C*=NOH). $\nu_{max}$ (KBr disc, cm$^{-1}$): (both isomers): 3200, 3080, 2935, 2910, 2840, 1460, 1440, 925, 910. **Elemental analysis** Found: C, 52.1; H, 9.3; N, 8.6. $C_7H_{15}NOS$ requires C, 52.1; H, 9.4; N, 8.7. **Accurate mass** Found: 161.0844. $C_7H_{15}NOS$ requires 161.08744.

## *7-Methylthioheptanaldoxime* (**2.90**)




7-Methylthioheptanal (**2.81**) (650mg, 4.0mmol), hydroxylamine hydrochloride (285mg, 4.1mmol) and sodium hydrogen carbonate (340mg, 4.0mmol) were stirred in ethanol (20ml) and water (5ml) at room temperature for 16 hours. The solution was diluted with water (50ml) and extracted with diethyl ether (3 x 50ml). The combined organic layers were washed with water, saturated brine, dried, and concentrated to leave the crude product. This was purified by flash chromatography (pet. ether 40-60°C/ethyl acetate:

85/15) to give the pure product (**2.90**) as a mixture (2:1) of (*E*)- and (*Z*)- isomers (276mg, 39%). **NMR** ($CDCl_3$): (*E*)-isomer $^1H$: $\delta$ 1.39 (4H, m, 2 x $CH_2$), 1.50 (2H, m, $CH_2$), 1.60 (2H, m, $CH_2$), 2.10 (3H, s, $CH_3S$), 2.20 (2H, m, $CH_2$), 2.49 (2H, t, *J* 6.5, $SCH_2$), 7.42 (1H, t, *J* 6.0, CH=NOH), 8.8 (1H, br s, CH=NO*H*). $^{13}C$: $\delta$ 15.5 ($CH_3S$), 26.4 ($CH_2$), 28.4 ($CH_2$), 28.6 ($CH_2$), 28.9 ($CH_2$), 29.0 ($CH_2$), 34.2 ($CH_2$), 152.1 (*C*=NOH). (*Z*)-isomer $^1H$: $\delta$ 1.39 (4H, m, 2 x $CH_2$), 1.50 (2H, m, $CH_2$), 1.60 (2H, m, $CH_2$), 2.10 (3H, s, $CH_3S$), 2.38 (2H, m, $CH_2$), 2.49 (2H, t, *J* 7.0, $SCH_2$), 6.72 (1H, t, *J* 5.5, CH=NOH), 9.2 (1H, br s, CH=NO*H*). $^{13}C$: $\delta$ 15.5 ($CH_3S$), 24.9 ($CH_2$), 25.9 ($CH_2$), 28.4 ($CH_2$), 28.4 ($CH_2$), 29.0 ($CH_2$), 34.2 ($CH_2$), 152.7 (*C*=NOH).

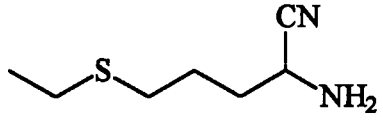
### *4-Ethylthiobutanenitrile* (**2.93**)[188]


A solution of sodium methoxide formed from sodium (0.85g, 0.037mol) and methanol (100ml) was treated with ethanethiol (2.3g, 0.037mol). To this solution was added 4-bromobutanenitrile (**2.73**) (5.5g, 0.037mol). After stirring for 5 hours at room temperature the solvent was removed *in vacuo* and the residue partitioned between water (100ml) and diethyl ether (3 x 100ml). The combined organic layers were dried and concentrated to leave the product (**2.93**) as an oil (3.99g, 83%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.26 (3H, t, *J* 7.5, $CH_3CH_2S$), 1.94 (2H, m, $CH_2$), 2.52 (2H, t, *J* 7.0, $SCH_2CH_2$), 2.54 (2H, q, *J* 7.5, $CH_3CH_2S$), 2.66 (2H, t, *J* 7.0, $CH_2CN$). $^{13}C$: $\delta$ 14.7 ($CH_3$), 16.0 ($CH_2$), 25.2 ($CH_2$), 25.8 ($CH_2$), 30.1 ($CH_2$), 119.3 (*C*N).

*4-Ethylthiobutanal* (**2.95**)


4-Ethylthiobutanenitrile (**2.93**) (3.85g, 0.030mol) in hexane (50ml) and diethyl ether (50ml) at -70°C under nitrogen was treated with DIBAL (4.40g, 0.031mol) in hexane (50ml) dropwise over 10 minutes. After stirring for 30 minutes the reaction was allowed to warm up to room temperature and stirred for 3 hours. Then ethyl acetate (2ml) was added, followed by ammonium chloride solution (sat. 50ml) with stirring for 15 minutes. After the addition of sulfuric acid (2N, 50ml) and further stirring for 1 hour, the reaction mixture was partitioned and the aqueous phase extracted with diethyl ether (2 x 100ml). The organic layers were combined, washed with brine and then water, dried and then concentrated to leave the product (**2.95**) as an oil. This was purified by distillation at reduced pressure (100°C/0.6 mm Hg, 1.31g, 33%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.25 (3H, t, *J* 7.0, $CH_3CH_2S$), 1.93 (2H, quin, *J* 7.0, $CH_2CH_2CH_2$), 2.58 (6H, m, 2 x $CH_2$), 9.81 (1H, s, CHO). $^{13}C$: $\delta$ 14.7 ($CH_3$), 21.8 ($CH_2$), 25.7 ($CH_2$), 30.9 ($CH_2$), 42.7 ($CH_2CHO$), 201.7 (CHO).

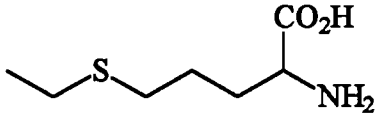
*2-Amino-5-ethylthiopentanenitrile* (**2.97**)




4-Ethylthiobutanal (**2.95**) (1.21g, 9.2mmol) in ethanol (20ml) and water (5ml) was treated with ammonium chloride (500mg, 9.2mmol), ammonia (d. 0.88, 3ml) and potassium cyanide (600mg, 9.2mmol) and stirred at room temperature for 72 hours. Water (50ml) was added and the reaction mixture extracted with diethyl ether (3 x 50ml). The

combined organic layers were extracted with dilute hydrochloric acid (1N, 2 x 100ml), which was the treated with diluted sodium hydroxide solution (2M to pH9). This was then back extracted with diethyl ether (3 x 100ml). The combined organic extracts were dried and concentrated *in vacuo* to leave the product (**2.97**) as an oil (550mg, 38%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.23 (3H, t, *J* 7.0, $CH_3CH_2S$), 1.27 (2H, m, $CH_2$), 1.84 (2H, m, $CH_2$), 2.20 (2H, br s, $NH_2$), 2.56 (4H, m, 2 x $CH_2$), 3.72 (1H, t, *J* 7.0, C*H*). $^{13}C$: $\delta$ 14.7 ($CH_3$), 25.3 ($CH_2$), 25.8 ($CH_2$), 30.8 ($CH_2$), 34.3 ($CH_2$), 43.0 (*C*H), 122.0 (*C*N).

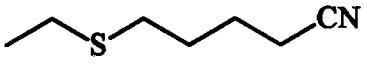
*D,L-2-Amino-5-ethylthiopentanoic acid* (**2.91**)[189]




2-Amino-5-ethylthiopentanenitrile (**2.97**) (550mg, 3.48mmol) was treated with concentrated hydrochloric acid (12N, 3ml) and left for 72 hours at room temperature. Water (20ml) was added and the reaction mixture heated to reflux for 6 hours. The mixture was taken to dryness *in vacuo* and the residue dissolved in aqueous sodium hydroxide solution (2N, 2ml). This was applied to an ion exchange resin (Amberlite CG-400) which was washed with water (50ml) then eluted with dilute hydrochloric acid (1N, 50ml). The eluate was taken to dryness to leave the product (**2.91**) as its hydrochloride (280mg, 40%). **m.p.** 128-132°C (dec). **NMR** ($d_6$-DMSO): $^1H$: $\delta$ 1.17 (3H, t, *J* 7.0, $CH_3CH_2S$), 1.57 (1H, m, $CH_aH_b$), 1.70 (1H, m, $CH_aH_b$), 1.88 (2H, m, $CH_2$), 2.49 (2H, q, *J* 7.0, $CH_3CH_2S$), 2.51 (2H, t, *J* 7.0, $SCH_2$), 3.88 (1H, t, *J* 6.0, C*H*), 8.5 (3H, br s, $H^+$). $^{13}C$: $\delta$ 14.8 ($CH_3$), 24.5 ($CH_2$), 24.7 ($CH_2$), 29.1 ($CH_2$), 29.9 ($CH_2$), 51.7 (*C*H),

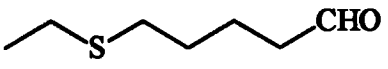
170.9 ($CO_2H$). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): 3430, 3030, 2925, 1740, 1220. **Accurate mass** Found: 177.08224. $C_7H_{15}NO_2S$ requires 177.08235.

## *5-Ethylthiopentanenitrile* (**2.94**)




A solution of sodium methoxide formed from sodium (1.25g, 0.054mol) and methanol (100ml) was treated with ethanethiol (3.40g, 0.055mol). To this solution was added 5-bromopentanenitrile (**2.14**) (8.80g, 0.054mol). After stirring for 3 hours at room temperature the solvent was removed *in vacuo* and the residue partitioned between water (100ml) and diethyl ether (3 x 100ml). The combined organic layers were dried and concentrated to leave the product (**2.94**) as an oil (6.91g, 89%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.26 (3H, t, *J* 7.5, $CH_3CH_2S$), 1.77 (4H, m, 2 x $CH_2$), 2.39 (2H, t, *J* 6.5, $CH_2CN$), 2.54 (4H, m, 2 x $CH_2S$). $^{13}C$: $\delta$ 14.7 ($CH_3$), 16.8 ($CH_2$), 24.4 ($CH_2$), 25.9 ($CH_2$), 28.3 ($CH_2$), 30.6 ($CH_2$), 119.5 (*C*N).

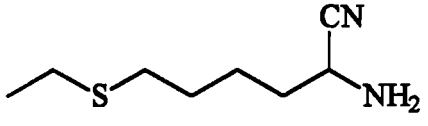
## *5-Ethylthiopentanal* (**2.96**)




4-Ethylthiopentanenitrile (**2.94**) (6.24g, 0.044mol) in hexane (50ml) and diethyl ether (50ml) at -70°C under nitrogen was treated with DIBAL (7.06g, 0.049mol) in hexane (50ml) dropwise over 10 minutes. After stirring for 30 minutes the reaction was allowed to warm up to room temperature and stirred for 2 hours. Then ethyl acetate (2ml) was added, followed by ammonium chloride solution (sat. 50ml) with stirring for 15 minutes. After the addition of sulfuric acid (2N, 50ml) and further stirring for 30 minutes the

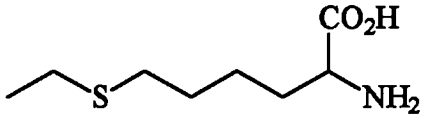
reaction mixture was partitioned and the aqueous phase extracted with diethyl ether (3 x 50ml). The organic layers were combined, washed with brine and then water, dried and then concentrated to leave the product (**2.96**) as an oil. This was purified by distillation at reduced pressure (150°C/0.5 mm Hg, 695mg, 11%). **NMR** ($CDCl_3$): $^1$H: $\delta$ 1.25 (3H, t, *J* 7.5, $CH_3CH_2S$), 1.63 (2H, m, $CH_2$), 1.74 (2H, m, $CH_2$), 2.48 (2H, dt, *J* 1.5, 7.0, $CH_2CHO$), 2.52 (2H, t, *J* 7.0, $SCH_2$), 2.54 (2H, q, *J* 7.5, $CH_3CH_2S$), 9.77 (1H, t, *J* 1.5, CHO). $^{13}$C: $\delta$ 14.8 ($CH_3$), 21.2 ($CH_2$), 25.9 ($CH_2$), 28.9 ($CH_2$), 31.2 ($CH_2$), 43.4 ($CH_2CHO$), 202.2 (CHO).

## *2-Amino-6-ethylthiohexanenitrile* (**2.98**)



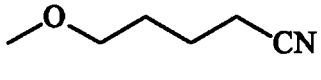

5-Ethylthiopentanal (**2.96**) (620mg, 4.2mmol) in ethanol (20ml) and water (10ml) was treated with ammonium chloride (240mg, 4.5mmol), ammonia (d. 0.88, 5ml) and potassium cyanide (280mg, 4.3mmol) and stirred at room temperature for 48 hours. Water (20ml) was added and the reaction mixture extracted with diethyl ether (3 x 50ml). The combined organic layers were extracted with dilute hydrochloric acid (1N, 2 x 100ml), which was the treated with diluted sodium hydroxide solution (2M to pH9). This was then back extracted with diethyl ether (3 x 100ml). The combined organic extracts were dried and concentrated *in vacuo* to leave the product (**2.98**) as an oil (400mg, 55%). **NMR** ($CDCl_3$): $^1$H: $\delta$ 1.26 (3H, t, *J* 7.5, $CH_3CH_2S$), 1.64 (4H, m, 2 x $CH_2$), 1.70 (2H, br s, NH), 1.77 (2H, m, $CH_2$), 2.54 (4H, m, 2 x $SCH_2$), 3.68 (1H, m, CH). $^{13}$C: $\delta$ 14.7 ($CH_3$), 24.6 ($CH_2$), 25.9 ($CH_2$), 28.8 ($CH_2$), 31.2 ($CH_2$), 34.9 ($CH_2$), 43.2 (CH), 122.0 (CN).

*D,L-2-Amino-6-ethylthiohexanoic acid* (**2.92**)



2-Amino-5-ethylthiohexanenitrile (**2.98**) (320mg, 1.86mmol) was treated with concentrated hydrochloric acid (12N, 5ml) and left for 72 hours at room temperature. Water (20ml) was added and the reaction mixture heated to reflux for 5 hours. The mixture was taken to dryness *in vacuo* and the residue dissolved in aqueous sodium hydroxide solution (2N, 2ml). This was applied to an ion exchange resin (Amberlite CG-400) which was washed with water (50ml) then eluted with dilute hydrochloric acid (1N, 50ml). The eluate was taken to dryness to leave the product (**2.92**) as its hydrochloride (280mg, 66%). **m.p.** 176-180°C (dec). **NMR** ($d_6$-DMSO): $^1$H: $\delta$ 1.17 (3H, t, *J* 7.5, $CH_3CH_2S$), 1.52 (4H, m, 2 x $CH_2$), 1.82 (2H, m, $CH_2$), 2.50 (4H, m, 2 x $SCH_2$), 3.83 (1H, m, CH), 8.6 (3H, br s, $H^+$). $^{13}$C: $\delta$ 14.8 ($CH_3$), 23.6 ($CH_2$), 24.9 ($CH_2$), 28.7, 29.5 ($CH_2$), 30.3 ($CH_2$), 51.8 (CH), 170.8 ($CO_2H$). $\nu_{max}$ (KBr disc, cm$^{-1}$): 3430, 3025, 2925, 2870, 1735, 1495, 1405, 1210, 1195. **Accurate mass** Found: 191.09918. $C_8H_{17}NO_2S$ requires 191.09800.

*5-Methoxypentanenitrile* (**2.101**)[190]



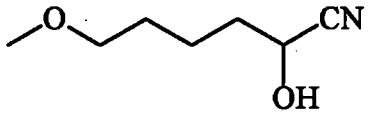
A solution of sodium methoxide was formed from sodium (2.50g, 0.109mol) and methanol (100ml). To this solution was added 5-bromopentanenitrile (**2.14**) (16.2g, 0.100mol). After stirring for 4 hours at room temperature, sat. ammonium chloride

solution (30ml) was added, then the solvent was removed *in vacuo* and the residue partitioned between water (50ml) and diethyl ether (3 x 50ml). The combined organic layers were dried and concentrated to leave the product (**2.101**) as an oil (8.88g, 79%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.74 (4H, m, 2 x $CH_2$), 2.39 (2H, t, $J$ 7.0, $CH_2CN$), 3.32 (3H, s, $CH_3O$), 3.41 (2H, t, $J$ 6.0, $OCH_2$). $^{13}C$: $\delta$ 17.0 ($CH_2$), 22.5 ($CH_2$), 28.5 ($CH_2$), 58.6 ($CH_3O$), 71.5 ($OCH_2$), 119.8 ($CN$).

*5-Methoxypentanal* (**2.102**)[191]

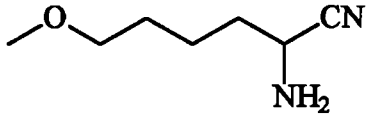

5-Methoxypentanenitrile (**2.101**) (8.78g, 0.078mol) in pentane (50ml) and diethyl ether (50ml) at -70°C under nitrogen was treated with DIBAL (11.80g, 0.083mol) in pentane (50ml) dropwise over 10 minutes. After stirring for 1 hour the reaction was allowed to warm up to -10°C. Then ammonium chloride solution (sat. 50ml) was added slowly, not allowing the temperature to rise above 15°C, with stirring for 30 minutes. After the slow addition of sulfuric acid (2N, 50ml) and further stirring for 30 minutes, the reaction mixture was partitioned and the aqueous phase extracted with diethyl ether (2 x 50ml). The organic layers were combined, washed with brine and then water, dried and then concentrated to leave the product (**2.102**) as an oil (3.75g, 42%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.63 (2H, m, $CH_2$), 1.72 (2H, m, $CH_2$), 2.47 (2H, dt, $J$ 1.5, 7.0, $CH_2CHO$), 3.32 (3H, s, $CH_3O$), 3.39 (2H, t, $J$ 6.0, $OCH_2$), 9.77 (1H, t, $J$ 1.5, $CHO$). $^{13}C$: $\delta$ 15.9 ($CH_2$), 29.0 ($CH_2$), 43.6 ($CH_2CHO$), 58.6 ($CH_3O$), 72.2 ($OCH_2$), 202.5 ($CHO$). $\upsilon_{max}$ (film, $cm^{-1}$): 2935, 2870, 1725, 1120.

*2-Hydroxy-5-methoxypentanenitrile* (**2.104**)



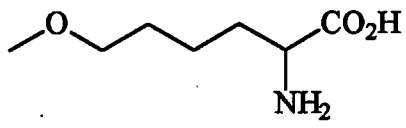

5-Methoxypentanal (**2.102**) (348mg, 3.0mmol) was stirred overnight in sat. sodium bisulfite solution (20ml). The white precipitate formed was filtered off and the filtrate treated with potassium cyanide (190mg, 3.0mmol) and stirred overnight. The reaction mixture was then extracted with diethyl ether (3 x 20ml), which was dried and concentrated to leave the product (**2.104**) as an oil (105mg, 27%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.65 (4H, m, 2 x $CH_2$), 1.88 (2H, m, $CH_2$), 3.35 (3H, s, $CH_3O$), 3.43 (2H, t, $J$ 6.0, $OCH_2$), 3.8 (1H, br s, OH), 4.48 (1H, t, $J$ 6.5, CH). $^{13}C$: $\delta$ 21.6 ($CH_2$), 28.7 ($CH_2$), 34.9 ($CH_2$), 58.6 ($CH_3O$), 61.1 (CH), 72.4 ($OCH_2$), 120.1 (CN).

*2-Amino-5-methoxypentanenitrile* (**2.105**)



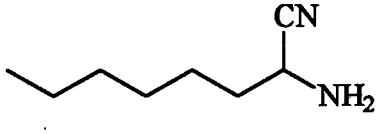

2-Hydroxy-5-methoxypentanenitrile (**2.104**) (80mg, 0.56mmol) was treated with dry ammonia in methanol (5% w/v, 5ml) for 48 hours. Then the solvent was removed *in vacuo* to leave the product (**2.105**) as an oil (75mg, 94%). This was used without further purification. **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.61 (4H, m, 2 x $CH_2$), 1.77 (2H, m, $CH_2$), 3.33 (3H, s, $CH_3O$), 3.40 (2H, t, $J$ 6.0, $OCH_2$), 3.69 (1H, t, $J$ 7.0, CH). $^{13}C$: $\delta$ 22.3 ($CH_2$), 29.0 ($CH_2$), 35.1 ($CH_2$), 43.3 (CH), 58.6 ($CH_3O$), 72.2 ($OCH_2$), 122.2 (CN).

*D,L-2-Amino-5-methoxypentanoic acid* (**2.100**)[192]




2-Amino-5-methoxypentanenitrile (**2.105**) (75mg, 0.58mmol) was treated with concentrated hydrochloric acid (12N, 2ml) and left for 72 hours at room temperature. Water (10ml) was added and the reaction mixture heated to reflux for 6 hours. The mixture was taken to dryness *in vacuo* and the residue dissolved in aqueous sodium hydroxide solution (2N, 2ml). This was applied to an ion exchange resin (Amberlite CG-400) which was washed with water (50ml) then eluted with dilute hydrochloric acid (1N, 50ml). The eluate was taken to dryness to leave the crude product as its hydrochloride. This was then dissolved in dilute hydrochloric acid (1N, 2ml) and applied to another ion exchange resin (Amberlyst 15) which was washed with water (30ml), then eluted with dilute ammonia solution (2N, 50ml). The eluate was taken to dryness to leave the product (**2.100**) as its ammonium salt (30mg). **m.p.** 192-197°C (dec). **NMR** ($D_2O$): $^1H$: $\delta$ 1.40 (2H, m, $CH_2$), 1.60 (2H, m, $CH_2$), 1.83 (2H, m, $CH_2$), 3.32 (3H, s, $CH_3O$), 3.48 (2H, t, $J$ 6.5, $OCH_2$), 3.70 (1H, t, $J$ 3.5, $CH$). $^{13}C$: $\delta$ 21.9 ($CH_2$), 29.1 ($CH_2$), 31.2 ($CH_2$), 55.6 ($CH$), 58.6 ($CH_3O$), 72.9 ($OCH_2$), 175.9 ($CO_2H$). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): 3020, 2940, 2830, 1655, 1580, 1420, 1125.

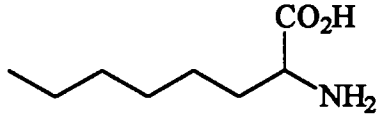
*2-Aminooctanenitrile*




Freshly distilled 1-heptanal (2.30g, 0.020mol) in ethanol (75ml) and water (20ml)

was treated with ammonium chloride (1.07g, 0.020mol), ammonia (d. 0.88, 5ml) and potassium cyanide (1.30g, 0.020mol) and stirred at room temperature for 4 days. Water (100ml) was added and the reaction mixture extracted with diethyl ether (2 x 150ml). The combined organic layers were extracted with dilute hydrochloric acid (1N, 2 x 100ml), which was the treated with diluted sodium hydroxide solution (2M to pH9). This was then back extracted with diethyl ether (3 x 100ml). The combined organic extracts were dried and concentrated *in vacuo* to leave the product as an oil (0.45g, 16%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 0.89 (3H, t, *J* 7.0, $CH_3CH_2$), 1.32 (6H, m, $CH_2$), 1.48 (2H, m, $CH_2$), 1.72 (4H, m, $CH_2$, $NH_2$), 3.68 (1H, t, *J* 7.0, CH). $^{13}C$: $\delta$ 14.0 ($CH_3$), 22.5 ($CH_2$), 25.4 ($CH_2$), 28.7 ($CH_2$), 31.5 ($CH_2$), 35.3 ($CH_2$), 43.4 (CH), 122.3 (CN).

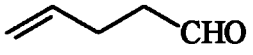
## *D,L-2-Aminooctanoic acid* (**2.99**)[193]




2-Aminooctanenitrile (450mg, 3.2mmol) was treated with concentrated hydrochloric acid (12N, 5ml) and left overnight at room temperature. Water (20ml) was added and the reaction mixture heated to reflux for 5 hours. The mixture was taken to dryness *in vacuo* and the residue dissolved in aqueous sodium hydroxide solution (2N, 2ml). This was applied to an ion exchange resin (Amberlite CG-400) which was washed with water (50ml) then eluted with dilute hydrochloric acid (1N, 50ml). The eluate was taken to dryness to leave the product as its hydrochloride (230mg, 37%). **m.p.** 165-185°C (dec). **NMR** ($d_6$-DMSO): $^1H$: $\delta$ 0.87 (3H, m, $CH_3CH_2$), 1.25 (6H, m, 3 x $CH_2$), 1.40 (2H, m, $CH_2$), 1.78 (2H, m, $CH_2$), 3.83 (1H, m, CH), 8.6 (3H, br s, $H^+$). $^{13}C$: $\delta$ 14.0 ($CH_3$), 22.0 ($CH_2$), 24.1 ($CH_2$), 28.2 ($CH_2$), 29.9 ($CH_2$), 30.9 ($CH_2$), 52.0 (CH), 171.0 ($CO_2H$). $\upsilon_{max}$ (KBr

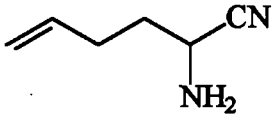
disc, $cm^{-1}$): 3425, 3015, 2955, 2925, 2860, 1680, 1485. **Accurate mass** Found: 159.12737. $C_8H_{17}NO_2$ requires 159.12593.

*4-Penten-1-al* (**2.110**)




To a solution of 4-penten-1-ol (**2.109**) (4.3g, 0.050mol) in dichloromethane (100ml) was added PCC (11.0g, 0.051mol). This was stirred at room temperature for 6 hours, then treated with diethyl ether (100ml) and filtered through silica. The filtrate was concentrated *in vacuo* to leave the product (**2.110**) (1.40g, 33%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 2.40 (2H, m, $CH_2$), 2.54 (2H, m, $CH_2$), 5.03 (2H, m, $CH_2$=CH), 5.81 (1H, m, $CH_2$=CH), 9.78 (1H, t, *J* 1.5, CHO). $^{13}C$: $\delta$ 26.1 ($CH_2$), 42.7 ($CH_2$CHO), 115.7 ($CH_2$=CH), 136.4 ($CH_2$=CH), 202.0 (CHO).

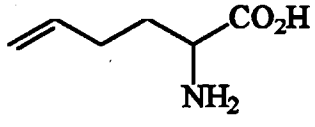
*2-Amino-5-hexenenitrile*




4-penten-1-al (**2.110**) (840mg, 10.0mmol) in ethanol (20ml) and water (10ml) was treated with ammonium chloride (540mg, 10.0mmol), ammonia (d. 0.88, 5ml) and potassium cyanide (660mg, 10.0mmol) and stirred at room temperature for 72 hours. Water (30ml) was added and the reaction mixture extracted with diethyl ether (3 x 50ml). The combined organic layers were extracted with dilute hydrochloric acid (1N, 2 x 50ml),

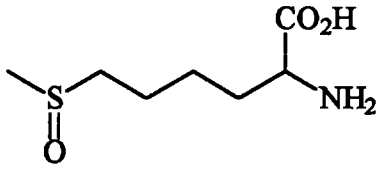
which was then treated with diluted sodium hydroxide solution (2M to pH9). This was then back extracted with diethyl ether (3 x 50ml). The combined organic extracts were dried and concentrated *in vacuo* to leave the product as an oil (0.41g, 37%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.75 (2H, br s, $NH_2$), 1.84 (2H, q, *J* 7.0, $CH_2CH$), 2.28 (2H, m, $CH_2$), 3.70 (1H, t, *J* 7.0, *CH*) 5.09 (2H, m, $CH_2$=CH), 5.79 (1H, m, $CH_2$=*CH*). $^{13}C$: $\delta$ 29.6 ($CH_2$), 34.3 ($CH_2$), 42.7 (*CH*), 116.4 ($CH_2$=CH), 122.1 (*CN*), 136.2 ($CH_2$=*CH*).

## *2-Amino-5-hexenoic acid* (2.106)[194]



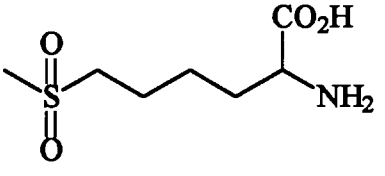

2-Amino-5-hexenenitrile (380mg, 3.45mmol) was treated with concentrated hydrochloric acid (12N, 5ml) and left for 72 hours at room temperature. Water (20ml) was added and the reaction mixture heated to reflux for 4 hours. The mixture was taken to dryness *in vacuo* and the residue dissolved in aqueous sodium hydroxide solution (2N, 2ml). This was applied to an ion exchange resin (Amberlite CG-400) which was washed with water (50ml) then eluted with dilute hydrochloric acid (1N, 50ml). The eluate was taken to dryness to leave the product (**2.106**) as its hydrochloride (250mg, 44%). **m.p.** 143-148°C (dec). **NMR** ($d_6$-DMSO): $^1H$: $\delta$ 1.91 (2H, m, $CH_2$), 2.11 (1H, m, $CH_aH_b$), 2.21 (1H, m, $CH_aH_b$), 3.83 (1H, m, *CH*), 5.06 (2H, m, $CH_2$=CH), 5.80 (1H, m, $CH_2$=*CH*), 8.60 (4H, br s, $H^+$). $^{13}C$: $\delta$ 28.3 ($CH_2$), 28.9 ($CH_2$), 51.3 (*CH*), 115.8 ($CH_2$=CH), 136.7 ($CH_2$=*CH*), 170.6 ($CO_2H$). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): 3010, 2920, 1735, 1490, 1205, 920, 830.

*D,L-2-Amino-6-methylsulfinylhexanoic acid (Dihomomethionine sulfoxide)* **(2.107)**



To a slurry of dihomomethionine (**2.3**) (344mg, 2.0mmol) in glacial acetic acid (10ml) was added hydrogen peroxide (29.7%, 220$\mu$l, 2.08mmol) with stirring at room temperature for 4 hours. The solvent was removed *in vacuo* and the residue recrystallised from ethanol/water to give the product (**2.107**) as a white crystalline solid (130mg, 35%). **m.p.** 232-234°C (dec). **NMR** ($D_2O$): mixture of diastereomers $^1H$: $\delta$ 1.55 (2H, m, C$H_2$), 1.78 (2H, m, C$H_2$), 1.90 (2H, m, C$H_2$), 2.69 (3H, s, C$H_3$SO), 2.91 (2H, m, SOC$H_2$), 3.75 (1H, m, C$H$). $^{13}C$: $\delta$ 24.3 (*C*$H_2$), 24.3 (*C*$H_2$), 26.1 (*C*$H_2$), 26.2 (*C*$H_2$), 32.6 (*C*$H_2$), 32.6 (*C*$H_2$), 39.1 (*C*$H_3$SO), 39.1 (*C*$H_3$SO), 54.7 (SO*C*$H_2$), 54.7(SO*C*$H_2$), 57.2 (*C*H), 57.2 (*C*H), 177.2 (*C*$O_2$H), 177.2 (*C*$O_2$H). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): 2920, 1625, 1580, 1415, 1345, 1015.

*D,L-2-Amino-6-methylsulfonylhexanoic acid (Dihomomethionine sulfone)* **(2.108)**

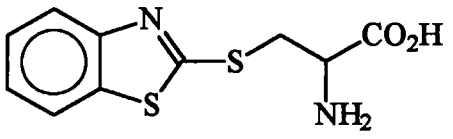


To a slurry of dihomomethionine (**2.3**) (177mg, 1.0mmol) in glacial acetic acid (10ml) was added hydrogen peroxide (29.7%, 500$\mu$l, 4.2mmol) with stirring at 50-60°C

for 5 hours. The solvent was removed *in vacuo* and the residue recrystallised from ethanol/water to give the product (**2.108**) as a white crystalline solid (133mg, 63%). **m.p.** 240-242°C (dec). **NMR** ($D_2O$): $^1H$: $\delta$ 1.56 (2H, m, $CH_2$), 1.87 (4H, m, 2 x $CH_2$), 3.08 (3H, s, $CH_3SO_2$), 3.29 (2H, t, *J* 7.5, $SO_2CH_2$), 3.74 (1H, t, *J* 5.9, CH). $^{13}C$: $\delta$ 23.9 ($CH_2$), 25.9 ($CH_2$), 32.5 ($CH_2$), 42.3 ($CH_3SO_2$), 55.9 ($SO_2CH_2$), 57.2 (CH), 177.2 ($CO_2H$). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): 3025, 2935, 2910, 2870, 1655, 1625, 1605, 1580, 1415, 1345, 1310, 1300, 1280, 1135, 1100.

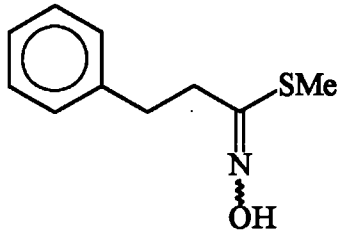
# *3.5 Sulfur Addition*

## *Benzthiazole-2-cysteine* (**2.112**)[139]



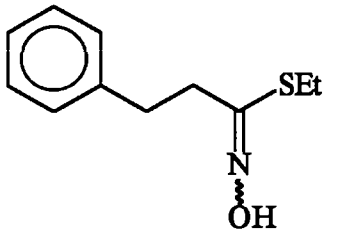

Liquid ammonia (100ml) was condensed into a 3-necked flask, fitted with a dry ice/acetone condenser and treated with sodium (1.52g, 0.066mol). The resulting deep blue solution was treated with L-cysteine (**2.115**) (4.0g, 0.033mol), and then 2-chlorobenzthiazole (**2.114**) (5.6g, 0.033mol) was added dropwise over 30 minutes. The reaction mixture was allowed to warm up to room temperature and then the ammonia was allowed to evaporate overnight. The solid residue was treated carefully with water (60ml), then washed with diethyl ether (2 x 50ml). The aqueous phase was acidified to pH 5 with glacial acetic acid, treated with ethanol (50ml) and left at 4°C for 24 hours. The white precipitate formed was filtered off and redissolved in hot water (60°C, 100ml), containing a trace of ammonia. This was treated with charcoal, filtered, and the pH adjusted to pH 5 with glacial acetic acid. After 48 hours at 4°C the precipitate was filtered off and dried *in vacuo* to leave the product (**2.112**) as a white solid (3.20g, 38%). **m.p.** 143-145°C. **NMR** ($D_2O$/NaOD): $^1H$: $\delta$ 3.50 (2H, m, $CH_2CH$), 3.94 (1H, m, $CH_2CH$), 7.30 (1H, d, *J* 7.5, Ar*H*), 7.41(1H, d, *J* 7.5, Ar*H*), 7.64 (1H, d, *J* 8.0, Ar*H*), 7.70 (1H, d, *J* 7.5, Ar*H*). $^{13}C$: $\delta$ 51.7 ($CH_2$), 58.0 (*C*H), 123.0 (*C*H), 124.1 (*C*H), 127.4 (*C*H), 129.2 (*C*H), 137.0 ($C_q$), 154.6 ($C_q$), 171.8 ($CO_2H$), 182.5 ($C_q$). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): 3530, 3460, 3060, 1670, 1595, 1535, 1460, 1425, 13995, 1340, 1000, 750, 725.

## S-*Methyl-3-phenylpropanothiohydroximate* (**2.122**)



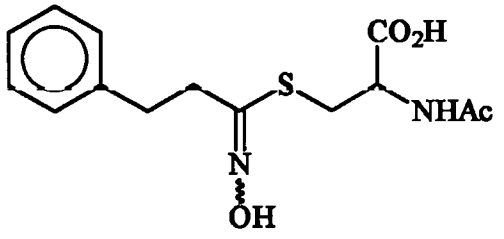

A solution of 3-phenylpropanaldoxime (**2.22**) (1.49g, 0.10mol), in dry THF (75ml) at -78°C, under $N_2$ was treated with dry chlorine gas over 20 minutes. Into the resulting blue solution was bubbled methanethiol (2.10g, 0.044mol), and then triethylamine (5.0g, 0.050mol), in dry THF (20ml), was added dropwise over 20 minutes. The reaction mixture was allowed to warm up to room temperature over 30 minutes then filtered through Celite® and concentrated *in vacuo* to give the crude product. This was washed with the minimum amount of cold diethyl ether to leave the pure product (**2.122**) (0.73g, 37%). **m.p.** 113-115°C. **NMR** ($CDCl_3$): $^1$H: $\delta$ 2.35 (3H, s, C*H*$_3$S), 2.72 (2H, t, *J* 8.0, C*H*$_2$CH$_2$), 2.93 (2H, t, *J* 8.0, CH$_2$C*H*$_2$), 7.22 (3H, m, 3 x Ar*H*), 7.29 (2H, m, 2 x Ar*H*), 9.2 (1H, br s, C=NO*H*). $^{13}$C: $\delta$ 12.7 (*C*H$_3$S), 33.5 (*C*H$_2$), 34.1 (*C*H$_2$), 126.4 (*C*H), 128.4 (2 x *C*H), 128.6 (2 x *C*H), 140.5 (*C*$_q$), 156.1 (*C*=NOH). $\upsilon_{max}$ (KBr disc, cm$^{-1}$): 3240, 3180, 3085, 3030, 1625, 1120, 970, 945, 760, 710, 695. **Elemental analysis** Found: C, 61.9; H, 6.6; N, 7.0. $C_{10}H_{13}NOS$ requires C, 61.5; H, 6.7; N, 7.2. **Accurate mass** Found: 195.07188. $C_{10}H_{13}NOS$ requires 195.07179.

## S-*Ethyl-3-phenylpropanothiohydroximate* (**2.123**)



A solution of 3-phenylpropanaldoxime (**2.22**) (1.49g, 0.010mol), in dry THF (75ml) at -78°C, under $N_2$ was treated with dry chlorine gas over 20 minutes. The resulting blue solution was treated with ethanethiol (700mg, 0.011mol), and then triethylamine (5.05g, 0.050mol), in dry THF (20ml), was added dropwise over 20 minutes, with stirring for a further 30 minutes. The reaction mixture was allowed to warm up to room temperature over 30 minutes then filtered through Celite® and concentrated *in vacuo* to give the crude product. This was purified by "flash" chromatography (pet. ether/diethyl ether, 70/30), followed by recrystallisation from diethyl ether/hexane to give the product (**2.123**) as a white crystalline solid (0.52g, 25%). **m.p.** 83-84°C. **NMR** ($CDCl_3$): $^1$H: $\delta$ 1.32 (3H, t, *J* 7.5, $CH_3CH_2$), 2.72 (2H, m, $CH_2$), 2.89 (2H, q, *J* 7.5, $SCH_2CH_3$), 2.93 (2H, m, $CH_2$), 7.20-7.32 (5H, m, 5 x Ar*H*), 9.0 (1H, br s, C=NO*H*). $^{13}$C: $\delta$ 14.9 ($CH_3$), 23.8 ($CH_2S$), 33.6 ($CH_2$), 34.7 ($CH_2$), 126.4 (*C*H), 128.4 (2 x *C*H), 128.6 (2 x *C*H), 140.6 ($C_q$), 155.5 (*C*=NOH). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): 3210, 3120, 2930, 1125, 950, 695. **Elemental analysis** Found: C, 63.0; H, 7.2; N, 6.6. $C_{11}H_{15}NOS$ requires C, 63.1; H, 7.2; N, 6.7. **Accurate mass** Found: 208.0794. $C_{11}H_{15}NOS$ requires 208.07961.

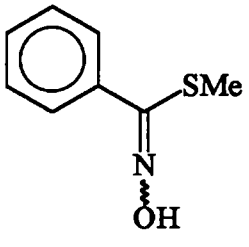
## S-*(3-Phenylpropanohydroximoyl)*-N-*acetyl-cysteine* (**2.125**)




A solution of 3-phenylpropanaldoxime (**2.22**) (2.98g, 0.020mol), in dry THF (75ml) at -78°C, under $N_2$ was treated with dry chlorine gas over 20 minutes. The resulting blue solution was allowed to warm up to room temperature, then treated with *N*-

acetyl-L-cysteine (1.63g, 0.010mol), and then triethylamine (2.52g, 0.025mol), in dry THF (10ml), was added dropwise over 30 minutes, with stirring for a further 30 minutes. Addition of dilute sulfuric acid (~2 N 100ml), was followed by removal of the THF *in vacuo* and extraction of the aqueous layer with dichloromethane (3 x 50ml). The combined organic layers were dried and concentrated *in vacuo* to give the crude product as an oil. This was purified by recrystallisation from methanol/ethanol to give the pure product (**2.125**) as a white solid (0.63g, 20%). **m.p.** 176-177°C. **NMR** ($D_2O$/NaOD): $^1H$: $\delta$ 2.02 (3H, s, $CH_3CO$), 2.71 (2H, m, $CH_2CH_2$), 2.84 (2H, m, $CH_2CH_2$), 3.19 (2H, m, $CH_2CH$), 4.41 (1H, dd, *J* 4.5, 6.0, $CH_2CH$) 7.25-7.39 (5H, m). $^{13}C$: $\delta$ 24.6 ($COCH_3$), 33.5 ($CH_2$), 35.9 ($CH_2$), 37.0 ($CH_2$), 57.5 (*C*H), 128.9 (*C*H), 131.3 (2 x *C*H), 131.4 (2 x *C*H), 143.8 ($C_q$), 154.4 (*C*=NOH), 176.2 (*C*O), 179.2 (*C*O). **Accurate mass** Found: 310.09845. $C_{14}H_{18}N_2O_4S$ requires 310.09873.

## *S-Methylphenylthiohydroximate* (**2.130**)[141]



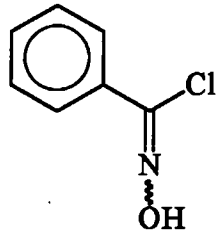

A solution of benzaldoxime (**2.127**) (2.42g, 0.020mol), in dry THF (75ml) at 0°C, under $N_2$ was treated with dry chlorine gas over 30 minutes. Into the resulting blue solution was bubbled methanethiol (2.40g, 0.050mol), and then triethylamine (8.08g, 0.080mol), in dry THF (20ml), was added dropwise over 30 minutes. After stirring for 30 minutes at room temperature the reaction mixture was filtered through Celite® and concentrated *in vacuo* to give the crude product as an oil. This was recrystallised from

diethyl ether/hexane to give the pure product (**2.130**) as a white crystalline solid (1.30g, 39%). **m.p.** 81-83°C. **NMR** ($CDCl_3$): $^1H$: $\delta$ 2.07 (3H, s, $CH_3S$), 7.42 (5H, s, 5 x Ar*H*), 9.65 (1H, s, C=NO*H*). $^{13}C$: $\delta$ 14.8 ($CH_3S$), 128.6 (2 x *C*H), 128.8 (2 x *C*H), 129.6 (*C*H), 132.6 ($C_q$), 157.2 (*C*=NOH). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): 3220, 1240, 1120, 770, 700. **Accurate mass** Found: 167.04030. $C_8H_9NOS$ requires 167.04049.

## *S-Ethylphenylthiohydroximate* (**2.131**)[141]

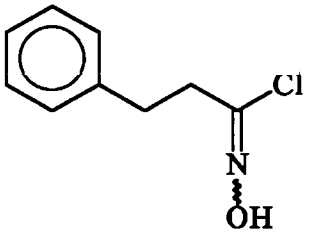

A solution of benzaldoxime (**2.127**) (2.42g, 0.020mol), in dry THF (75ml) at 0°C, under $N_2$ was treated with dry chlorine gas over 30 minutes. Into the resulting blue solution was added ethanethiol (2.0g, 0.032mol), and then triethylamine (8.08g, 0.080mol), in dry THF (20ml), was added dropwise over 30 minutes. After stirring for 2 hours at room temperature the reaction mixture was filtered through Celite® and concentrated *in vacuo* to give the product (**2.131**) as an oil (2.03g, 56%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.11 (3H, t, *J* 7.5, $CH_3CH_2S$), 2.61 (2H, q, *J* 7.5, $CH_3CH_2S$), 7.38-7.52 (5H, m, 5 x Ar*H*). $^{13}C$: $\delta$ 15.2 ($CH_3$), 26.1 ($CH_2S$), 128.6 (2 x *C*H), 128.8 (2 x *C*H), 129.6 (*C*H), 133.4 ($C_q$), 155.8 (*C*=NOH). $\upsilon_{max}$ (film, $cm^{-1}$): 3250, 3185, 2970, 1445, 1240, 990, 925, 765, 700.

*Benzohydroximoyl chloride* (**2.128**) (using NCS)[146]




To a solution of benzaldoxime (**2.127**) (12.1g, 0.10mol) in DMF was added *N*-chlorosuccinimide (~2g). Then hydrogen chloride gas (5ml) was bubbled through the reaction mixture which resulted in a temperature rise to 35°C. Further NCS was added (13.3g total, 0.10mol) slowly, to keep the reaction temperature at 35°C. After stirring for 30 minutes at room temperature, the reaction mixture was poured into water (400ml) and extracted with diethyl ether (3 x 100ml). The combined ether layers were washed with water (3 x 100ml), dried and concentrated *in vacuo* to leave the product (**2.128**) as an oil (14.8g, 95%) which crystallised on standing. This was used without further purification. **NMR** ($CDCl_3$): $^1H$: $\delta$ 7.40 (3H, m, 3 x Ar*H*), 7.81 (2H, m, 2 x Ar*H*), 9.20 (1H, s, C=NO*H*). $^{13}C$: $\delta$ 127.5 (2 x *C*H), 128.8 (2 x *C*H), 131.1 (*C*H), 132.5 ($C_q$), 140.8 ($C_q$).

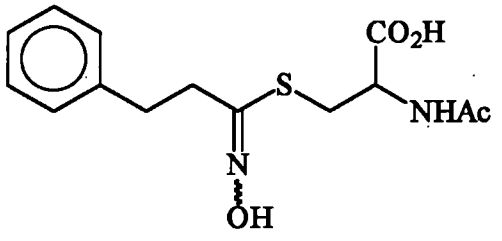
*3-Phenylpropanohydroximoyl chloride* (**2.40**) (using NCS)[49]




To a solution of 3-phenylpropanaldoxime (**2.22**) (1.49g, 0.010mol) in DMF (20ml) was added *N*-chlorosuccinimide (1.35g, 0.010mol). The reaction mixture was then treated with hydrogen chloride gas (10ml) and stirred for 2 hours during which time the reaction

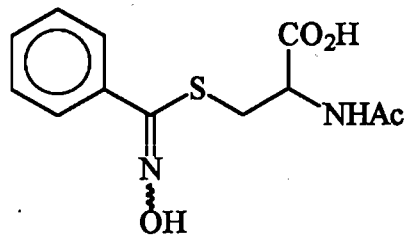
temperature reached a maximum of 30°C. Addition of water, extraction with diethyl ether, washing with water (2 x 50ml), drying and concentration *in vacuo* gave the product (**2.40**) as an oil which crystallised on standing (1.66g, 90%). This was used immediately, without further purification. **NMR** ($CDCl_3$): $^1H$: $\delta$ 2.81 (2H, t, *J* 8.0, $CH_2CH_2$), 2.97 (2H, t, *J* 8.0, $CH_2CH_2$), 7.18-7.32 (5H, m, 5 x Ar*H*), 7.90 (1H, s, C=NO*H*). $^{13}C$: $\delta$ 32.4 ($CH_2$), 38.4 ($CH_2$), 126.5 (*C*H), 128.4 (2 x *C*H), 128.5 (2 x *C*H), 139.6 ($C_q$), 141.2 ($C_q$).

S-*(3-Phenylpropanohydroximoyl)*-N-*acetyl-cysteine* (**2.125**) (via **2.40** using NCS)




To a solution of *N*-acetyl-cysteine (1.63g, 0.010mol) in THF (30ml) with triethylamine (4.04g, 0.040mol) at 0°C was added the hydroximoyl chloride (**2.40**) (1.40g, 0.0076mol) in THF dropwise over 1 hour. After stirring for a further 30 minutes the mixture was filtered through Celite®, and concentrated *in vacuo*. The residue was dissolved in dichloromethane, and treated with dilute sulfuric acid (10% v/v) resulting in an oil dropping out of solution. This quickly crystallised to give the product (**2.125**) as a white crystalline solid which was isolated by filtration (1.61g, 68%). Spectroscopic and physical data as before.

S-*(Benzohydroximoyl)*-N-*acetyl-cysteine* **(2.133)** (via **2.128** using NCS)



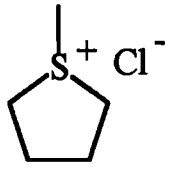

To a solution of *N*-acetyl-cysteine (1.63g, 0.010mol) in THF (30ml) with triethylamine (4.04g, 0.040mol) at 0°C was added the hydroximoyl chloride (**2.128**) (1.55g, 0.010mol) in THF dropwise over 1 hour. This was stirred for a further 30 minutes, then treated with dilute sulfuric acid (5% v/v, 100ml). The THF was removed *in vacuo* and the aqueous layer left at room temperature for 48 hours. The product precipitated as a white crystalline solid which was isolated by filtration. This was washed with diethyl ether and dried to give the pure product (2.06g, 73%). **NMR** ($D_2O$, NaOD): $^1$H: $\delta$ 1.95 (3H, s, C*H*$_3$CO), 2.98 (1H, dd, *J* 5.0, 14.0, C*H*$_a$H$_b$CH), 3.00 (1H, dd, *J* 5.0, 14.0, CH$_a$*H*$_b$CH), 4.17 (1H, t, *J* 5.0, CH$_a$H$_b$C*H*) 7.40-7.49 (5H, m, 5 x Ar*H*), $^{13}$C: $\delta$ 24.8 (CO*C*H$_3$), 35.1 (*C*H$_2$), 57.6 (*C*H), 131.4 (2 x *C*H), 131.5 (*C*H), 131.6 (2 x *C*H), 137.0 (*C*$_q$), 155.3 (*C*=NOH), 175.8 (*C*O), 179.2 (*C*O). **Accurate mass** Found: 282.06937. $C_{12}H_{14}N_2O_4S$ requires 282.06743.

# *3.6 Retained Label Studies*

## *1-Chloro-4-methylthiobutane* (**2.139**)[152]

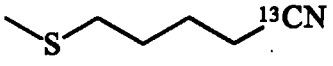

To a solution of sodium methoxide made from sodium (1.00g, 0.043mol), and methanol (100ml) at 0°C was added methanethiol (3.20g, 0.067mol). Then 1-bromo-4-chlorobutane (**2.140**) (7.65g, 0.044mol) was added and the reaction mixture was stirred for 6 hours. Addition of water (100ml), extraction with diethyl ether (3 x 75ml), drying and concentration *in vacuo* gave the crude product as an oil. This was purified by Kugelrohr distillation (0.2mm Hg, 40°C oven temp) to give the pure product (**2.139**) (3.49g, 57%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.75 (2H, m, $CH_2$), 1.89 (2H, m, $CH_2$), 2.10 (3H, m, $CH_3S$), 2.52 (2H, t, *J* 7.0, $SCH_2$), 3.56 (2H, t, *J* 6.5, $CH_2Cl$). $^{13}C$: $\delta$ 15.4 ($CH_3S$), 26.2 ($CH_2$), 31.4 ($CH_2$), 33.4 ($CH_2$), 44.6 ($CH_2Cl$).

On standing the product crystallised as the sulfonium salt (**2.141**).



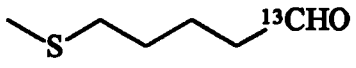

**NMR** ($D_2O$): $^1H$: $\delta$ 2.28 (2H, m, 2 x $CH_aH_b$), 2.38 (2H, m, 2 x $CH_aH_b$), 2.78 (3H, s, $CH_3S^+$), 3.36 (2H, m, 2 x $CH_cH_dS^+$), 3.56 (2H, m, 2 x $CH_cH_dS^+$). $^{13}C$: $\delta$ 27.2 (2 x $CH_2$), 30.7 (2 x $CH_2$), 47.5 ($CH_3S^+$).

## *[$^{13}$C-1]5-Methylthiopentanenitrile* (2.138)




To a solution of freshly made 1-chloro-4-methylthiobutane (**2.139**) (12.2g, 0.089mol) in ethanol was added potassium cyanide (99% $^{13}$C, Aldrich, 1.00g, 0.015mol) in water (10ml). This was heated to reflux for 16 hours, then the ethanol was removed *in vacuo* and the residue partitioned between water (50ml) and diethyl ether (3 x 50ml). The combined organic layers were dried and concentrated *in vacuo* to leave the crude product (**2.138**) as an oil (0.700g, 36%). GC analysis showed 75% purity. NMR ($CDCl_3$): $^1$H: $\delta$ 1.78 (4H, m, 2 x $CH_2$), 2.10 (3H, s, $CH_3S$), 2.40 (2H, dt, $J$ 3.0, 9.5, $CH_2{}^{13}CN$), 2.54 (2H, t, $J$ 6.5, $SCH_2$). $^{13}$C: $\delta$ 15.4 ($CH_3S$), 16.8 ($CH_2{}^{13}CN$, d, $J$ 56.0), 24.2 ($CH_2$, d, $J$ 3.0), 27.2 ($CH_2$, d, $J$ 3.0), 33.2 ($CH_2$), 119.5 ($CN$, $^{13}$C).

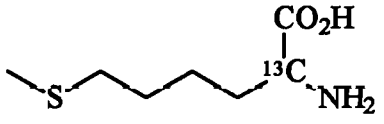
## *[$^{13}$C-1]5-Methylthiopentanal* (2.137)




To a solution of crude [$^{13}$C-1]5-methylthiopentanenitrile (**2.138**) (700mg, 5.4mmol) in hexane (10ml) and diethyl ether (20ml) under $N_2$ at -78°C was added DIBAL (900mg, 6.3mmol) in hexane (20ml). This was stirred at -78°C for 30 minutes, then allowed to warm up to room temperature over 2 hours. After treatment with ethyl acetate (5ml), sat. ammonium chloride (30ml) and dilute sulfuric acid (10% v/v, 30ml) and subsequent stirring for 30 minutes the organic layer was partitioned off and the aqueous layer extracted with diethyl ether (3 x 30ml). The combined organic layers were washed with water, brine, then dried and concentrated *in vacuo* to leave the crude product as an oil. This was purified using Kugelrohr distillation (0.6mm Hg, 100°C oven temp) to give the

product (**2.137**) as an oil (245mg, 34%). GC analysis showed 78% purity. **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.63 (2H, m, $CH_2$), 1.74 (2H, m, $CH_2$), 2.10 (3H, s, $CH_3S$), 2.47 (2H, m, $CH_2{}^{13}CHO$), 2.51 (2H, t, $J$ 7.0, $SCH_2$), 9.78 (1H, dt, $J$ 1.5, 171.0, $CH_2{}^{13}CHO$). $^{13}C$: $\delta$ 15.5 ($CH_3S$), 21.1 ($CH_2$), 28.4 ($CH_2CH_2{}^{13}CHO$, d, $J$ 2.5), 33.8 ($CH_2$), 43.4 ($CH_2{}^{13}CHO$, d, $J$ 9.0), 202.3 ($CHO$, $^{13}C$).

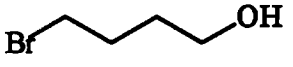
## *[$^{13}$C-1]2-Amino-5-methylthiopentanoic acid* (**2.136**)




To a solution of [$^{13}$C-1]5-methylthiopentanal (**2.137**) (245mg, 1.84mmol) in ethanol (20ml) and water (10ml) was added ammonia (d. 0.88, 1.5ml), ammonium chloride (110mg, 2.0mmol) and potassium cyanide (135mg, 2.08mmol). This was stirred at room temperature for 48 hours then treated with water (50ml) and extracted with diethyl ether (4 x 25ml). The combined ether layers were extracted with dilute hydrochloric acid (~0.6N, 3 x 20ml), which was then basified with dilute sodium hydroxide solution (1M, 40ml), and back extracted with diethyl ether (4 x 25ml). The combined back extracted ether layers were dried and concentrated *in vacuo* to leave the aminonitrile as an oil (120mg). This was treated with concentrated hydrochloric acid (3ml), for 48 hours, then water was added and the mixture was heated to reflux for 5 hours. Removal of the solvent *in vacuo* gave the crude product as its hydrochloride. This was purified using ion-exchange chromatography (Amberlite CG-400) to give the pure product (**2.136**) as its hydrochloride (130mg, 33%). **NMR** ($D_2O$): $^1H$: $\delta$ 1.46 (2H, m, $CH_2$), 1.59 (2H, m, $CH_2$), 1.90 (2H, m, $CH_2$), 2.02 (3H, m, $CH_3S$), 2.49 (2H, t, $J$ 7.0, $SCH_2$), 4.01 (1H, dt, $J$ 6.0, 146.5, $^{13}CH$). $^{13}C$: $\delta$ 16.8 ($CH_3S$), 25.9 ($CH_2$), 30.3 ($CH_2CH_2{}^{13}CH$, d, $J$ 3.5), 31.9 ($CH_2{}^{13}CH$, d, $J$ 33.0), 33.3

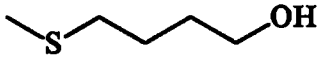
($CH_2$), 55.6 ($CH$, $^{13}C$), 174.9 ($^{13}CHCO_2H$, d, *J* 59.0). **Accurate mass** Found: 178.08426. $C_6{}^{13}CH_{15}NO_2S$ requires 178.08571.

*4-Bromo-1-butanol* (**2.142**)




In a continuous liquid/liquid extraction vessel was heated hydrobromic acid (250ml) and 1,4-butanediol (**2.141**) (45.0g, 0.50mol), with cyclohexane as the extraction solvent. The vessel was heated to 70°C for 16 hours, with the cyclohexane heated separately to reflux. Then the cyclohexane was cooled, washed with water (3 x 50ml) and sat. sodium bicarbonate solution (50ml), dried and concentrated *in vacuo* to leave the product (**2.142**) as a light brown oil (12.1g, 16%). **NMR** ($CDCl_3$): $^1H$: δ 1.72, (2H, m, $CH_2$), 1.96 (2H, m, $CH_2$), 3.46 (2H, t, *J* 6.5, $CH_2Br$), 3.68 (2H, t, *J* 6.5, $CH_2OH$). 4.3 (1H, br s, *OH*). $^{13}C$: δ 29.2 ($CH_2$), 30.9 ($CH_2$), 33.7 ($CH_2$), 61.7 ($CH_2OH$).

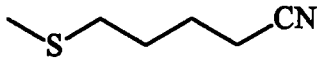
*4-Methylthio-1-butanol* (**2.144**)[195]




To a solution of sodium methoxide made from sodium (250mg, 10.9mmol) and dry methanol (75ml) at 0°C was added methanethiol (2.0g, 42mmol). This was treated with 4-bromobutanol (**2.142**) (1.53g, 10mmol), with stirring for 2 hours. Then water (50ml) was added and the methanol was removed *in vacuo*. The aqueous layer was extracted with diethyl ether (3 x 50ml) and the combined ether layers were dried and concentrated *in vacuo* to leave the product as an oil (**2.144**) (680mg, 57%). **NMR** ($CDCl_3$): $^1H$: δ 1.67, (4H, m, 2 x $CH_2$), 2.10 (3H, s, $CH_3S$), 2.53 (2H, t, *J* 7.0, $SCH_2$), 3.65 (2H, t, *J* 6.0,

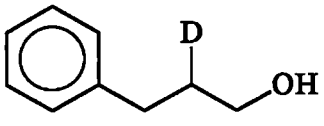
$CH_2OH$), 4.4 (1H, br s, $CH_2OH$). $^{13}C$: $\delta$ 15.5 ($CH_3S$), 25.4 ($CH_2$), 31.7 ($CH_2$), 34.0 ($CH_2$), 62.2 ($CH_2OH$).

*5-Methylthiopentanenitrile* (**2.145**) (from **2.141**)[170]




To a solution of the sulfonium salt (**2.141**) (140mg, 1.0mmol) in dry DMSO (10ml) was added potassium cyanide (130mg, 2.0mmol) and 18-crown-6 (500mg, 2.1mmol). This was heated to 90°C for 4 hours, then cooled to room temperature. Addition of water (40ml) and extraction with diethyl ether (3 x 30ml) followed by drying and concentration *in vacuo* gave the crude product (**2.145**) as an oil (40mg, 30%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.78 (4H, m, 2 x $CH_2$), 2.10 (3H, s, $CH_3S$), 2.40 (2H, t, *J* 3.0, $CH_2CN$), 2.54 (2H, t, *J* 6.5, $SCH_2$). $^{13}C$: $\delta$ 15.4 ($CH_3S$), 16.9 ($CH_2CN$), 24.2 ($CH_2$), 27.3 ($CH_2$), 33.1 ($CH_2$), 119.5 ($CN$).

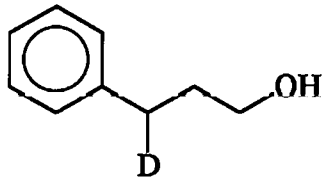
*[$^2$H-2]-3-Phenylpropan-1-ol* (**2.150**)




To a suspension of lithium aluminium deuteride (1.08g, 0.026mol), in dry diethyl ether (30ml) was added dropwise cinnamyl alcohol (**2.149**) (1.40g, 0.0105mol), in dry diethyl ether (10ml). After addition the mixture was heated to reflux for 1.5 hours then cooled to 0°C. Dropwise addition of water (20ml) and then dilute sulfuric acid (10% v/v, 50ml) to dissolve the white precipitate was followed by partitioning of the organic layer. The aqueous layer was reextracted with diethyl ether (3 x 25ml) and the combined organic

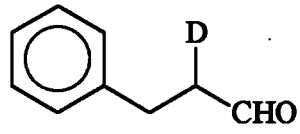
layers were dried and concentrated *in vacuo* to leave the product (**2.150**) as an oil (1.28g, 89%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.80 (1H, br s, O*H*), 1.86 (1H, m, C*H*D), 2.68 (2H, d, *J* 7.5, Ar$CH_2$), 3.64 (2H, d, *J* 6.5, $CH_2$OH), 7.15-7.29 (5H, m, 5 x Ar*H*). $^{13}C$: $\delta$ 32.0 (Ar$CH_2$), 33.8 (*C*HDCH$_2$OH, t, *J* 19.0), 62.1 (*C*H$_2$OH), 125.8 (*C*H), 128.4 (2 x *C*H), 128.4 (2 x *C*H), 141.8 ($C_q$).

## *[$^2$H-3]-3-Phenylpropan-1-ol* (**2.151**)



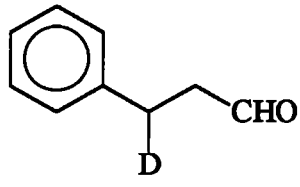

To a suspension of lithium aluminium hydride (1.10g, 0.029mol), in dry diethyl ether (30ml) was added dropwise cinnamyl alcohol (**2.149**) (1.50g, 0.011mol), in dry diethyl ether (10ml). This was stirred for 4 hours at room temperature before treatment with $D_2O$ (20ml) dropwise over 30 minutes. Addition of dilute sulfuric acid (10% v/v, 50ml) to dissolve the white precipitate was followed by partitioning of the organic layer. The aqueous layer was reextracted with diethyl ether (2 x 25ml) and the combined organic layers were dried and concentrated *in vacuo* to leave the product as an oil (1.30g, 85%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.78 (1H, s, O*H*), 1.87 (2H, q, *J* 7.0, $CH_2CH_2$OH), 2.68 (1H, m, ArC*H*D), 3.66 (2H, t, *J* 6.5, $CH_2$OH), 7.17-7.31 (5H, m, 5 x Ar*H*). $^{13}C$: $\delta$ 31.7, (ArCHD, t, *J* 20.0), 34.1 ($CH_2CH_2$OH), 62.2 ($CH_2$OH), 125.8 (*C*H), 128.4 (2 x *C*H), 128.4 (2 x *C*H), 141.8 ($C_q$).

*[$^2$H-2]-3-Phenylpropan-1-al* (**2.152**)[196]




To a stirred suspension of powdered 4Å molecular sieve (1.0g), PCC (2.5g, 11.6mmol), and sodium acetate (180mg, 2.2mmol), in dry dichloromethane was added a solution of [$^2$H-2]-3-phenylpropan-1-ol (**2.150**) (1.25g, 9.1mmol) in dry dichloromethane (10ml) dropwise over 30 minutes. This was stirred at room temperature for 3 hours and then filtered through Florisil. The filtrate was concentrated *in vacuo* to leave the product (**2.152**) as an oil which was used without further purification (780mg, 63%). **NMR** ($CDCl_3$): $^1$H: $\delta$ 2.76 (1H, m, C*H*DCHO), 2.95 (2H, d, *J* 7.5, ArC$H_2$), 7.18-7.31 (5H, m, 5 x Ar*H*), 9.82 (1H, d, *J* 1.5, CHDC*H*O). $^{13}$C: $\delta$ 28.0 (Ar*C*H$_2$), 44.9 (*C*HDCHO, t, *J* 19.5), 126.3 (*C*H), 128.4 (2 x *C*H), 128.6 (2 x *C*H), 140.3 ($C_q$), 201.8 (*C*HO).

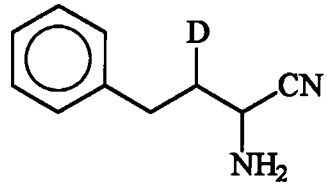
*[$^2$H-3]-3-Phenylpropan-1-al* (**2.153**)[196]




To a solution of [$^2$H-3]-3-phenylpropan-1-ol (**2.151**) (1.28g, 0.0093mol) in dichloromethane (50ml) was added PCC (2.7g, 0.0125mol). This was stirred at room temperature for 8 hours and then diethyl ether (50ml) was added and the reaction mixture filtered through Florisil. The filtrate was concentrated *in vacuo* to leave the crude product (**2.153**) as an oil which was used without further purification (1.00g, 79%). **NMR** ($CDCl_3$): $^1$H: $\delta$ 2.75 (2H, d, *J* 7.5, C$H_2$CHO), 2.91 (1H, m, ArC*H*D), 7.17-7.31 (5H, m,

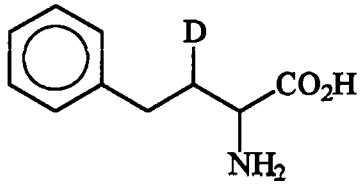
5 x Ar*H*), 9.79 (1H, t, *J* 1.0, $CH_2CHO$). $^{13}C$: δ 27.7 (ArCDH, t, *J* 20.0), 45.1 ($CH_2CHO$), 126.2 (*C*H), 128.2 (2 x *C*H), 128.5 (2 x *C*H), 140.2 ($C_q$), 201.6 (*C*HO).

*[$^2$H-3]-2-Amino-4-phenylbutanenitrile*



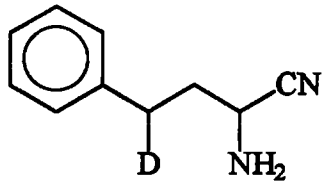

To a solution of crude [$^2$H-2]-3-phenylpropan-1-al (**2.152**) (405mg, 3.0mmol), in ethanol (10ml) and water (5ml), was added ammonia (d. 0.88, 2ml), ammonium chloride (170mg, 3.2mmol), and potassium cyanide (230mg, 3.5mmol). This was stirred at room temperature for 48 hours, then water (25ml) was added and the reaction mixture extracted with diethyl ether (3 x 20ml). The combined organic fractions were extracted with dilute hydrochloric acid (0.5N, 3 x 20ml) and these acid fractions were basified with sodium hydroxide solution and back extracted with diethyl ether (3 x 20ml). These combined back extracts were dried and concentrated *in vacuo* to leave the product as an oil (170mg, 35%). **NMR** ($CDCl_3$): $^1H$: δ 1.6 (2H, br s, N$H_2$), 2.03 (1H, m, C*H*DCH), 2.84 (2H, m, Ar$CH_2$), 3.59 (1H, d, *J* 7.0, CHDC*H*). $^{13}C$: δ 31.5 (Ar$CH_2$), 36.4 (*C*HDCH, t, *J* 20.0), 42.6 (*C*H), 122.0 (*C*N), 126.5 (*C*H), 128.4 (2 x *C*H), 128.7 (2 x *C*H), 139.8 ($C_q$).

*[$^2$H-3]-2-Amino-4-phenylbutanoic acid* (**2.147**)



To [$^2$H-3]-2-amino-4-phenylbutanenitrile (170mg, 1.05mmol), was added concentrated hydrochloric acid (3ml). This was left at room temperature for 16 hours then water (10ml) was added and the mixture was heated to reflux for 6 hours. The solvent was then removed *in vacuo* to leave the product (**2.147**) as a white solid (210mg, 92%). **NMR** ($d_6$-DMSO): $^1$H: $\delta$ 2.08 (1H, m, C*H*DCH), 2.65 (1H, m, ArC$H_aH_b$), 2.76 (1H, m, ArCH$_a$*H$_b$*), 3.83 (1H, m, C*H*), 7.19-7.32 (5H, m 5 x Ar*H*), 7.4 (1H, br s, $H^+$), 7.6 (1H, br s, $H^+$), 8.7 (2H, br s, 2 x $H^+$). $^{13}$C: $\delta$ 30.2 (Ar$CH_2$), 31.5 (*C*HDCH, t, *J* 20.0), 51.5 (*C*H), 126.0 (*C*H), 128.2 (2 x *C*H), 128.4 (2 x *C*H), 140.4 ($C_q$), 170.7 ($CO_2$H). $\upsilon_{max}$ (KBr disc, cm$^{-1}$): 3150, 3030, 2820, 1730, 1600, 1525, 1450, 1405, 1240, 745, 700. **Accurate mass** Found: 180.09894. $C_{10}H_{12}{}^2HNO_2$ requires 180.10091.

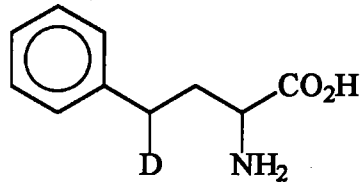
### *[$^2$H-4]-2-Amino-4-phenylbutanenitrile*




To a solution of crude [$^2$H-3]-3-phenylpropan-1-al (**2.153**) (540mg, 4.0mmol), in ethanol (15ml) and water (5ml), was added ammonia (d. 0.88, 2ml), ammonium chloride (240mg, 4.4mmol), and potassium cyanide (280mg, 4.3mmol). This was stirred at room temperature for 72 hours, then water (30ml) was added and the reaction mixture extracted with diethyl ether (3 x 20ml). The combined organic fractions were extracted with dilute hydrochloric acid (0.5N, 3 x 20ml) and these acid fractions were basified with sodium hydroxide solution and back extracted with diethyl ether (3 x 20ml). These combined back extracts were dried and concentrated *in vacuo* to leave the product as an oil (250mg, 39%). **NMR** ($CDCl_3$): $^1$H: $\delta$ 1.62 (2H, br s, N$H_2$), 2.03 (2H, t, *J* 7.5, C$H_2$CH), 2.80 (1H,

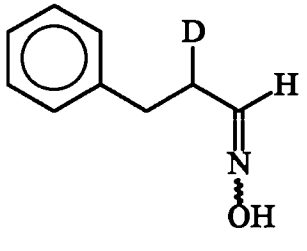
dt, *J* 7.5, 28.0, ArC*H*D), 3.59 (1H, t, *J* 7.0, $CH_2CH$), 7.19-7.31 (5H, m, 5 x Ar*H*). $^{13}C$: $\delta$ 31.2 (ArCHD, t, *J* 20.0), 36.7 ($CH_2$), 42.6 (*C*H), 122.1 (*C*N), 126.5 (*C*H), 128.4 (2 x *C*H), 128.7 (2 x *C*H), 139.8 ($C_q$).

### *[$^2$H-4]-2-Amino-4-phenylbutanoic acid* (2.148)




To [$^2$H-4]-2-amino-4-phenylbutanenitrile (230mg, 1.43mmol) was added concentrated hydrochloric acid (3ml). This was left at room temperature for 16 hours then water (5ml) was added and the mixture was heated to reflux for 8 hours. The solvent was then removed *in vacuo* to leave the product (**2.148**) as a white solid (280mg, 91%). **NMR** ($d_6$-DMSO): $^1H$: $\delta$ 2.11 (2H, t, *J* 7.0, $CH_2CH$), 2.70 (1H, m, ArC*H*D), 3.86 (1H, m, $CH_2CH$), 7.19-7.35 (5H, m, 5 x Ar*H*), 8.7 (3H, br s, $H^+$). $^{13}C$: $\delta$ 30.0 (ArCHD, t), 31.7 ($CH_2$), 51.5 (*C*H), 126.0 (*C*H), 128.2 (2 x *C*H), 128.4 (2 x *C*H), 140.3 ($C_q$), 170.6 ($CO_2H$). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): 3180, 3120, 3080, 2820, 1730, 1600, 1525, 1450, 1405, 700. **Accurate mass** Found: 180.10046. $C_{10}H_{12}{}^2HNO_2$ requires 180.10091.

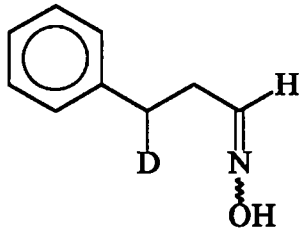
### *[$^2$H-2]-3-Phenylpropan-1-aldoxime* (2.154)




To a solution of crude [$^2$H-2]-3-phenylpropan-1-al (**2.152**) (270mg, 2.0mmol), in

ethanol (10ml) and water (5ml), was added sodium hydrogen carbonate (200mg, 2.4mmol), and hydroxylamine hydrochloride (170mg, 2.4mmol). After stirring for 48 hours at room temperature, water (30ml) was added and the reaction mixture was extracted with diethyl ether (3 x 20ml). The combined organic fractions were dried and concentrated *in vacuo* to leave the product (**2.154**) as semi crystalline solid. This was recrystallised from hexane/diethyl ether to leave the product, exclusively as the (*Z*)-isomer as a white crystalline solid (130mg, 53%). **NMR** ($CDCl_3$): (*Z*)-isomer $^1H$: $\delta$ 2.69 (1H, m, C*H*DCH), 2.81 (2H, d, *J* 7.5, Ar$CH_2$), 6.75 (1H, d, *J* 5.0, C*H*=NOH), 7.18-7.31 (5H, m, 5 x Ar*H*), 9.04 (1H, s, CH=NO*H*). $^{13}C$: $\delta$ 26.1 (*C*HDC=NOH, t, *J* 20.0), 31.8 (Ar$CH_2$), 126.2 (*C*H), 128.3 (2 x *C*H), 128.5 (2 x *C*H), 140.6 ($C_q$), 151.7 (*C*=NOH). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): (*Z*)-isomer: 3210, 3030, 2865, 1500, 1460, 1420, 1315, 935, 910, 750, 700. **Accurate mass** Found: 150.09006. $C_9H_{10}{}^2HNO$ requires 150.09034.

## *[$^2$H-3]-3-Phenylpropan-1-aldoxime* (**2.155**)

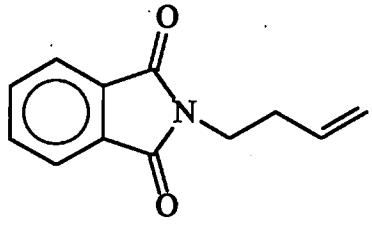



To a solution of crude [$^2$H-3]-3-phenylpropan-1-al (**2.153**) (405mg, 3.0mmol), in ethanol (10ml) and water (5ml), was added sodium hydrogen carbonate (260mg, 3.1mmol), and hydroxylamine hydrochloride (230mg, 3.3mmol). After stirring for 72 hours at room temperature water (30ml) was added and the reaction mixture was extracted with diethyl ether (3 x 25ml). The combined organic fractions were dried and concentrated *in vacuo* to leave the product as semi crystalline solid. This was recrystallised from hexane/diethyl ether to leave the product (**2.155**) as a white crystalline solid (160mg,

36%). **NMR** ($CDCl_3$): (*E*)-isomer $^1H$: $\delta$ 2.52 (2H, m, $CH_2CH{=}NOH$), 2.79 (1H, m, ArC*H*D), 7.18-7.31 (5H, m, 5 x Ar*H*), 7.46 (1H, t, *J* 6.0, C*H*=NOH), 8.3 (1H, br s, CH=NO*H*). $^{13}C$: $\delta$ 31.1 ($CH_2C{=}NOH$), 32.4 (Ar*C*HD, t, *J* 19.0), 126.3 (*C*H), 128.3 (2 x *C*H), 128.5 (2 x *C*H), 140.4 ($C_q$), 151.8 (*C*=NOH). (*Z*)-isomer $^1H$: $\delta$ 2.71 (2H, m, $CH_2CH{=}NOH$), 2.79 (1H, m, ArC*H*D), 6.75 (1H, t, *J* 5.0, C*H*=NOH), 7.18-7.31 (5H, m, 5 x Ar*H*), 8.8 (1H, br s, CH=NO*H*). $^{13}C$: $\delta$ 26.3 ($CH_2C{=}NOH$), 31.6 (Ar*C*HD, t, *J* 19.0), 126.3 (*C*H), 128.3 (2 x *C*H), 128.5 (2 x *C*H), 140.6 ($C_q$), 151.4 (*C*=NOH). $\upsilon_{max}$ (KBr disc, $cm^{-1}$): (both isomers): 3180, 3060, 3030, 2855, 1430, 720, 700. **Elemental analysis** Found: C, 72.0; H, 7.4; N, 9.3. $C_9H_{11}NO$ requires C, 72.5; H, 7.4; N, 9.4. **Accurate mass** Found: 150.09048. $C_9H_{10}{}^2HNO$ requires 150.09034.

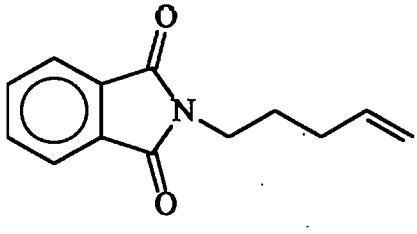
# 2.7 *Behaviour Controlling Chemicals From* B. Napus

## N-*(3-Butenyl)phthalimide* (**2.161**)[163]



4-Bromo-1-butene (**2.158**) (17.9g, 0.133mol) was mixed with potassium phthalimide (**2.160**) (26.17g, 0.141mol) and potassium iodide (0.8g) in dry DMF (200ml). This was heated at 130°C for 2 hours under $N_2$ and then poured onto crushed ice (200g). Extraction with diethyl ether (4 x 200ml) was followed by washing with dilute sodium hydroxide (200ml, 1M), water, and dilute hydrochloric acid (200ml, 0.5M). The organic layer was dried and the solvent removed *in vacuo* to leave the product as a yellow oil (25.5g, 94%), which solidified on standing and was recrystallised from diethyl ether/hexane to give the pure product (**2.161**) as a white crystalline solid. **m.p.** 50-51°C. **NMR** ($CDCl_3$): $^1H$: $\delta$ 2.45 (2H, q, *J* 7.0, $CH_2CH{=}CH_2$), 3.77 (2H, t, *J* 7.0, $NCH_2$), 5.05 (2H, m, $CH{=}CH_2$), 5.79 (1H, m, $CH{=}CH_2$), 7.70-7.85 (4H, m, 4 x Ar*H*). $^{13}C$: $\delta$ 32.8 ($CH_2$), 37.3 ($CH_2$), 117.5 ($CH{=}CH_2$), 123.2 (2 x *C*H), 132.1 (2 x $C_q$), 133.9 (2 x *C*H), 134.5 ($CH{=}CH_2$), 168.3 (2 x *C*O).

## N-*(4-Pentenyl)phthalimide* (**2.162**)[197]

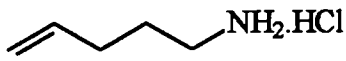


5-Bromo-1-butene (**2.159**) (15.0g, 0.100mol) was mixed with potassium phthalimide (**2.160**) (21.0g, 0.114mol) and potassium iodide (1.0g) in dry DMF (250ml). This was heated at 130°C for 3 hours under $N_2$ and then poured onto crushed ice (400g). Extraction with diethyl ether (3 x 200ml) was followed by washing with dilute sodium hydroxide (100ml, 1M), water, and dilute hydrochloric acid (100ml, 0.5M). The organic layer was dried and the solvent removed *in vacuo* to leave the product (**2.162**) as a yellow oil (20.2g, 94%), which crystallised on standing. **NMR** ($CDCl_3$): $^1$H: $\delta$ 1.79 (2H, quin, *J* 7.5, $CH_2CH_2CH_2$), 2.12 (q, *J* 7.0, $CH_2CH{=}CH_2$), 3.72 (2H, t, *J* 7.5, $NCH_2$), 5.05 (2H, m, $CH{=}CH_2$), 5.82 (1H, m, $CH{=}CH_2$), 7.70-7.85 (4H, m, 4 x Ar*H*). $^{13}$C: $\delta$ 27.6 ($CH_2$), 31.0 ($CH_2$), 37.5 ($CH_2$), 115.3 ($CH{=}CH_2$), 123.1 (2 x *C*H), 132.1 (2 x $C_q$), 133.9 (2 x *C*H), 137.3 ($CH{=}CH_2$), 168.4 (2 x *C*O).

### *3-Butenylamine hydrochloride* (**2.163**)[163]

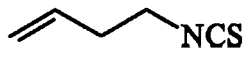

*N*-(3-Butenyl)phthalimide (**2.161**) (10.05g, 0.050mol) was dissolved in ethanol (200ml) and refluxed with hydrazine monohydrate (2.4ml, 0.050mol), for 3 hours. After cooling, water (100ml) and dilute hydrochloric acid (to pH 4) were added. Filtration of the white precipitate gave a clear yellow filtrate which was concentrated to leave the product (**2.163**) as a yellow hygroscopic solid (4.00g, 74%). **NMR** ($d_6$-DMSO): $^1$H: $\delta$ 2.39 (2H, q, *J* 7.0, $CH_2CH{=}CH_2$), 2.84 (2H, t, *J* 7.0, $CH_2NH_2$), 5.12 (2H, m, $CH{=}CH_2$), 5.82 (1H, m, $CH{=}CH_2$), 8.3 (3H, br s, $NH_3^+$). $^{13}$C: $\delta$ 30.9 ($CH_2$), 37.9 ($CH_2NH_2$), 117.4 ($CH{=}CH_2$), 134.0 ($CH{=}CH_2$).

*4-Pentenylamine hydrochloride* (**2.164**)[197]




*N*-(4-Pentenyl)phthalimide (**2.162**) (20.2g, 0.094mol) was dissolved in ethanol (200ml) and refluxed with hydrazine monohydrate (5.5ml, 0.094mol), for 4 hours. After cooling, water (100ml) and dilute hydrochloric acid (to pH 4) were added. Filtration of the white precipitate gave a clear yellow filtrate which was concentrated to leave the product (**2.164**) as a yellow hygroscopic solid (7.73g, 68%). **NMR** ($d_6$-DMSO): $^1$H: $\delta$ 1.67 (2H, m, $CH_2$), 2.09 (2H, q, *J* 7.0, $CH_2CH{=}CH_2$), 2.73 (2H, t, *J* 7.5, $CH_2NH_2$), 5.04 (2H, m, $CH{=}CH_2$), 5.80 (1H, m, $CH{=}CH_2$), 8.2 (3H, br s, $NH_3^+$). $^{13}$C: $\delta$ 25.9 ($CH_2$), 29.8 ($CH_2$), 38.1 ($CH_2NH_2$), 115.4 ($CH{=}CH_2$), 137.3 ($CH{=}CH_2$).

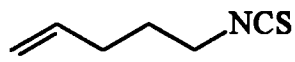
*3-Butenyl isothiocyanate* (**2.156**)[198]




3-Butenylamine hydrochloride (**2.163**) (26.5g, 0.246mol) in a two phase system of diethyl ether/ice water (300ml/300ml) was treated with thiophosgene (19.0ml, 0.249mol), and then sodium hydroxide (30.0g, 0.750mol) in water (100ml), dropwise over 30 minutes. After stirring for 3 hours the organic layer was separated and the aqueous layer extracted with diethyl ether (3 x 200ml). The combined organic layers were dried and concentrated to leave the crude product as a brown oil. This was purified by distillation at reduced pressure (0.5mm Hg, 39°C) to give the product (**2.156**) as a colourless oil (14.9g, 54%). **NMR** ($CDCl_3$): $^1$H: $\delta$ 2.45 (2H, q, *J* 6.5, $CH_2CH{=}CH_2$), 3.53 (2H, t, *J* 6.5, $CH_2NCS$), 5.20 (2H, m, $CH{=}CH_2$), 5.80 (1H, m, $CH{=}CH_2$). $^{13}$C: $\delta$ 34.3 ($CH_2$), 44.5 ($CH_2NCS$), 118.7 ($CH{=}CH_2$), 130.5 (NCS), 133.2 ($CH{=}CH_2$). $\upsilon_{max}$ (film, $cm^{-1}$):

2185, 2105, 1620, 1345, 925.

*4-Pentenyl isothiocyanate* (**2.157**)[197]




4-Pentenylamine hydrochloride (**2.164**) (12.1g, 0.100mol) in a two phase system of diethyl ether/ice water (200ml/200ml) was treated with thiophosgene (7.6ml, 0.100mol), and then sodium hydroxide (12.0g, 0.300mol) in water (100ml) dropwise over 30 minutes. After stirring for 3 hours the organic layer was separated and the aqueous layer extracted with diethyl ether (3 x 150ml). The combined organic layers were dried and concentrated to leave the product as a brown oil. This was purified by distillation at reduced pressure (0.5mm Hg, 55°C) to give the product (**2.157**) as a colourless oil (5.2g, 41%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.80 (2H, quin, *J* 7.0, $CH_2CH_2CH_2$), 2.19 (2H, q, *J* 7.0, $CH_2CH{=}CH_2$), 3.53 (2H, t, *J* 6.5, $CH_2NCS$), 5.07 (2H, m, $CH{=}CH_2$), 5.75 (1H, m, $CH{=}CH_2$). $^{13}C$: $\delta$ 29.0 ($CH_2$), 30.5 ($CH_2$), 44.2 ($CH_2NCS$), 116.4 ($CH{=}CH_2$), 129.9 (NCS), 136.2 ($CH{=}CH_2$). $\upsilon_{max}$ (film, $cm^{-1}$): 2945, 2930, 2185, 2105, 1640, 1450, 1345, 995, 915.

*Pentyl isothiocyanate* (**2.170**)

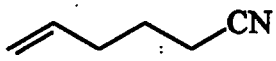

Pentylamine (**2.169**) (4.35g, 0.050mol) in a two phase system of diethyl ether/ice water (150ml/150ml) was treated with thiophosgene (4.0ml, 0.050mol), and then sodium hydroxide (4.0g, 0.100mol) in water (40ml) dropwise over 30 minutes. After stirring for 2 hours the organic layer was separated and the aqueous layer extracted with diethyl ether (3 x 100ml). The combined organic layers were dried and concentrated to leave the

product as a brown oil. This was purified by distillation at reduced pressure (0.5mm Hg, 35°C) to give the product (**2.170**) as a colourless oil (4.09g, 63%). **NMR** ($CDCl_3$): H: $\delta$ 0.93 (3H, t, *J* 7.0, $CH_3$), 1.38 (4H, m, 2 x $CH_2$), 1.71 (2H, quin, *J* 7.0, $CH_2CH_2CH_2$), 3.52 (2H, t, *J* 6.5, $CH_2NCS$). $^{13}C$: $\delta$ 13.9 ($CH_3$), 22.0 ($CH_2$), 28.7 ($CH_2$), 29.7 ($CH_2$), 45.1 ($CH_2NCS$), 129.5 (NCS). $\upsilon_{max}$ (film, $cm^{-1}$): 2960, 2935, 2875, 2860, 2180, 2105, 1465, 1455, 1345.

## *4-Pentenenitrile* (**2.171**)

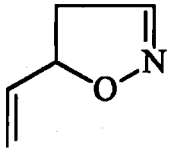

A mixture of 4-bromo-1-butene (**2.158**) (13.5g, 0.10mol), potassium cyanide (25g, 0.38mol), 18-crown-6 (2.1g, 8.0mmol), and acetonitrile (100ml) was refluxed for 72 hours. After cooling the solids were removed by filtration and the filtrate treated with diethyl ether (100ml), followed by washing with water (2 x 100ml), brine (2 x 100ml) and then drying and concentration *in vacuo* to leave the crude product as an oil. This was purified by distillation (0.5mm Hg, 40°C) to give the pure product (**2.171**) (4.1g, 51%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 2.41 (4H, m, 2 x $CH_2$), 5.16 (2H, m, $CH=CH_2$), 5.84 (1H, m, $CH=CH_2$). $^{13}C$: $\delta$ 16.9 ($CH_2CN$), 29.3 ($CH_2$), 117.6 ($CH=CH_2$), 119.3 (CN), 134.3 ($CH=CH_2$). $\upsilon_{max}$ (film, $cm^{-1}$): 3080, 2990, 2960, 2250, 1640, 1460, 1435, 1420, 1000, 920.

*5-Hexenenitrile* **(2.172)**




To a suspension of sodium cyanide (5.0g, 0.10mol), in freshly distilled DMSO (70ml), under $N_2$ was added 5-bromo-1-pentene (**2.159**) (5.0g, 0.034mol). After stirring for 2 hours at 100°C, the reaction mixture was cooled by the addition of water (150ml), and then extracted with pet. ether (40-60°C, 3 x 75ml). The combined organic layers were washed with water, dried and concentrated *in vacuo* to leave the crude product (**2.172**) as an oil. This was purified by distillation at reduced pressure (0.5mm Hg, 50°C) to give the pure product (2.75g, 86%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 1.76 (2H, quin, *J* 7.0, $CH_2CH_2CH_2$), 2.21 (2H, q, *J* 7.0, $CH_2CH{=}CH_2$), 2.35 (2H, t, *J* 7.0, $CH_2CN$), 5.07 (2H, m, $CH{=}CH_2$), 5.74 (1H, m, $CH{=}CH_2$). $^{13}C$: $\delta$ 16.3 ($CH_2CN$), 24.5 ($CH_2$), 32.4 ($CH_2$), 116.6 ($CH{=}CH_2$), 119.7 ($CN$), 136.1 ($CH{=}CH_2$).

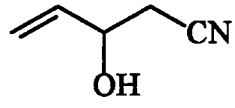
*5-Vinyl-2-isoxazoline* **(2.175)**[165]




To a solution of butadiene (**2.174**) (5.9g, 0.109mol), in benzene (16ml), acetonitrile (8ml), at 0°C, was added nitromethane (4.4g, 0.072mol) triethylamine (7.0g, 0.070mol), and chlorotrimethylsilane (7.8g, 0.071mol). This was stirred at 0°C for 3 hours then allowed to warm to room temperature and stirred for a further 72 hours. Addition of trifluoroacetic acid (0.5ml) was followed by stirring for a further 24 hours. The precipitate was removed by filtration and washed with benzene (50ml). The filtrate was washed with

water, then dried and concentrated *in vacuo* to leave the product (**2.175**) as an orange oil (3.75g, 53%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 2.78 (1H, ddd, *J* 1.0, 8.5, 17.0 $CH_aH_b$), 3.15 (1H, ddd, *J* 1.0, 6.5, 17.0 $CH_aH_b$), 4.91 (1H, m, *J* 8.5, OC*H*), 5.21 (1H, m, CH=$CH_cH_d$), 5.34 (1H, m, CH=$CH_cH_d$), 5.84 (1H, m, C*H*=$CH_cH_d$), 7.35 (1H, t, *J* 1.0, C*H*=N). $^{13}C$: $\delta$ 40.7 ($CH_2$), 79.0 (*C*H), 117.4 (CH=$CH_2$), 135.6 (*C*H=$CH_2$), 145.8 (*C*=N).

## *3-Hydroxy-4-pentenenitrile* (**2.173**)[165]




5-Vinyl-2-isoxazoline (**2.175**) (1.82g, 0.020mol) was heated to reflux with triethylamine (4.0g, 0.040mol) for 1 hour. Then the triethylamine was removed *in vacuo* and the crude product was passed through a silica column with chloroform as the eluent. Concentration and then distillation *in vacuo* (0.6mm Hg, 60-62°C) gave the pure product (**2.173**) as an oil (0.80g, 41%). **NMR** ($CDCl_3$): $^1H$: $\delta$ 2.61 (2H, m, $CH_2$CN), 3.32 (1H, s, O*H*), 4.45 (1H, q, *J* 6.0, C*H*OH), 5.29 (1H, m, CH=$CH_aH_b$), 5.40 (1H, m, CH=$CH_aH_b$), 5.91 (1H, m, C*H*=$CH_aH_b$). $^{13}C$: $\delta$ 27.1 ($CH_2$CN), 69.5 (*C*HOH), 117.3 (*C*N), 117.4 (CH=$CH_2$), 138.4 (*C*H=$CH_2$). $\upsilon_{max}$ (film, $cm^{-1}$): 3430, 2255, 1415, 1230, 1055, 1030, 995, 935.

# 4 REFERENCES

(1) Bennett, R. N.; Wallsgrove, R. M. *New Phytologist.* **1994**, *127*, 617-633.

(2) Jones, C. G.; Firn, R. D. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **1991**, *333*, 273-280.

(3) Wani, M. C.; Taylor, H. L.; Wall, M. E.; Coggon, P.; McPhail, A. T. *Journal of the American Chemical Society* **1971**, *93*, 2325-2327.

(4) Asakawa, Y.; Dawson, G. W.; Griffiths, D. C.; Lallemmand, J.-Y.; Ley, S. V.; Mori, K.; Mudd, A.; Pezechk-Leclaire, M.; Pickett, J. A.; Watanabe, H.; Woodcock, C. M.; Zhong-Ning, Z. *Journal of Chemical Ecology* **1988**, *14*, 1845-1855.

(5) Morgan, E. D.; Thornton, M. D. *Phytochemistry* **1973**, *12*, 391-392.

(6) *Pyrethrum: The Natural Insecticide*; Casida, J. E., Ed.; Academic Press: New York, 1973.

(7) *Secondary Plant Products*; Conn, E. E., Ed.; Academic Press: New York, 1981; Vol. 7.

(8) Luckner, M. *Secondary Metabolism in Microorganisms, Plants and Animals*; 2nd ed.; Springer-Verlag: Berlin, 1984.

(9) Robinson, T. *The Organic Constituents of Higher Plants*; 4th ed.; Cordus Press: North Amherst, 1980.

(10) Torssell, K. B. G. *Natural Product Chemistry*; 1st ed.; John Wiley and Sons: Cichester, 1983.

(11) Mann, J. *Secondary Metabolism*; 2nd ed.; Clarendon Press: Oxford, 1987.

(12) Griffiths, D. C. *Brighton Crop Protection Conference* **1990**, 487-495.

(13) Butenandt, A.; Beckmann, R.; Stamm, D.; Hecker, E. *Zeitschrift Naturforschung B* **1959**, *14B*, 283-284.

(14) Dawson, G. W.; Griffiths, D. C.; Janes, N. F.; Mudd, A.; Pickett, J. A.; Wadhams, L. J.; Woodcock, C. M. *Nature* **1987**, *325*, 614-616.

(15) Pickett, J. A.; Wadhams, L. J.; Woodcock, C. M.; Hardie, J. *Annual*

*Reviews of Entomology* **1992**, *1992*, 67-90.

(16) Dawson, G. W.; Griffiths, D. C.; Merritt, L. A.; Mudd, A.; Pickett, J. A.; Wadhams, L. J.; Woodcock, C. M. *Journal of Chemical Ecology* **1990**, *16*, 3019-3030.

(17) Campbell, C. A. M.; Dawson, G. W.; Griffiths, D. C.; Pettersson, J.; Pickett, J. A.; Wadhams, L. J.; Woodcock, C. M. *Journal of Chemical Ecology* **1990**, *16*, 3455-3465.

(18) Mori, K. *Pure and Applied Chemistry* **1994**, *66*, 1991-1998.

(19) *Pheromone Biochemistry*; Prestwich, G. D.; Blomquist, G. J., Eds.; Academic Press: Orlando, 1987.

(20) Pickett, J. A.; Griffiths, D. C. *Journal of Chemical Ecology* **1980**, *6*, 349-359.

(21) Blight, M. M.; Pickett, J. A.; Smith, M. C.; Wadhams, L. J. *Naturwissenschaften* **1984**, *71*, 480-481.

(22) Laurence, B. R.; Mori, K.; Otsuka, T.; Pickett, J. A.; Wadhams, L. J. *Journal of Chemical Ecology* **1985**, *11*, 643-648.

(23) Blight, M. M.; Pickett, J. A.; Wadhams, L. J.; Woodcock, C. M. *Journal of Chemical Ecology* **1995**, *21*, 1649-1664.

(24) Blight, M. M.; Pickett, J. A.; Wadhams, L. J.; Woodcock, C. *Aspects of Applied Biology* **1989**, *23*, 329-334.

(25) Bartlet, E.; Blight, M. M.; Hick, A. J.; Williams, I. H. *Entomologia experimentalis et applicata* **1993**, *68*, 295-302.

(26) Khrimian, A. P.; DeMilo, A. B.; Waters, R. M.; Cunniingham, R. T.; Leonhardt, B. A. *Journal of Chemical Ecology* **1993**, *19*, 2935-2946.

(27) West, T. F.; Hardy, J. E. *Chemical Control of Insects*; 2nd ed.; Chapman and Hall: London, 1961.

(28) Robinson, J.; Richardson, A.; Crabtree, A. N.; Coulson, J. C.; Potts, G.

R. *Nature* **1967**, *214*, 1307-1311.

(29) Cremlyn, R. J. *Agrochemicals: Preparation and Mode of Action*; 1st ed.; John Wiley and Sons: Chichester, 1991.

(30) Elliott, M.; Janes, N. F. *Chemical Society Reviews* **1978**, *7*, 473-505.

(31) Pillmore, R. E. In *Pyrethrum: The Natural Insecticide*; Casida, J. E., Ed.; Academic Press: New York, 1973, p 143-165.

(32) Wall, C. In *Behavior-Modifying Chemicals for Insect Management: Applications of Pheromones and Other Attractants*; Ridgway, R. L., Silverstein, R. M., Inscoe, M. N., Eds.; Marcel Dekker: Ney York, 1990, p 9-23.

(33) Inscoe, M. N.; Leonhardt, B. A.; Ridgway, R. L. In *Behavior-Modifying Chemicals for Insect Management: Applications of Pheromones and Other Attractants*; Ridgway, R. L., Silverstein, R. M., Inscoe, M. N., Eds.; Marcel Dekker: Ney York, 1990, p 631-715.

(34) Silverstein, R. M. In *Behavior-Modifying Chemicals for Insect Management: Applications of Pheromones and Other Attractants*; Ridgway, R. L., Silverstein, R. M., Inscoe, M. N., Eds.; Marcel Dekker: Ney York, 1990, p 1-8.

(35) Vale, G.; Hargrove, J. W.; Cockbill, G. F.; Phelps, R. J. *Bulletin of Entomological Research* **1986**, *76*, 179-193.

(36) Dawson, G. W.; Griffiths, D. C.; Pickett, J. A.; Plumb, R. T.; Woodcock, C. M.; Zhong-Ning, Z. *Pesticide Science* **1988**, *22*, 17-30.

(37) Anon "Agriculture in the United Kingdom," HMSO, 1994.

(38) *Brassica Oilseeds. Production and Utilization*; Kimber, D. S.; McGregor, D. I., Eds.; CAB International: Wallingford, 1995.

(39) Walker, K. C. In *Oilseeds for Food and Factory*; Semundo/BASF: Cambridge, 1994, p 40-47.

(40) Duncan, A. J. In *Toxic Substances in Crop Plants*; D'Mello, J. P. F.,

Duffus, C. M., Duffus, J. H., Eds.; Royal Society of Chemistry: Cambridge, 1991, p 126-147.

(41) Poulton, J. E.; Moller, B. L. In *Enzymes of Secondary Metabolism*; Lea, P. J., Ed.; Academic Press: London, 1993; Vol. 9, p 209-237.

(42) Daxenbichler, M. E.; Spencer, G. F.; Carlson, D. G.; Rose, G. B.; Brinkler, A. M.; Powell, R. G. *Phytochemistry* **1991**, *30*, 2623-2638.

(43) Larsen, P. O. In *Secondary Plant Products*; Conn, E. E., Ed.; Academic Press: New York, 1981; Vol. 7, p 501-525.

(44) Ettlinger, M. G.; Lundeen, A. J. *Journal of the American Chemical Society* **1956**, *78*, 4172-4173.

(45) Marsh, R. E.; Waser, J. *Acta Crystalographica* **1970**, *B 26*, 1030-1037.

(46) Ettlinger, M. G.; Dateo, G. P.; Harrison, B. W.; Mabry, T. J.; Thompson, C. P. *Proceedings of the National Academy of Sciences, USA* **1961**, *47*, 1875-1880.

(47) Benn, M. H. *Pure and Applied Chemistry* **1977**, *49*, 197-210.

(48) Ettlinger, M. G.; Lundeen, A. J. *Journal of the American Chemical Society* **1957**, *79*, 1764-1765.

(49) Gil, V.; MacLeod, A. J. *Tetrahedron* **1980**, *36*, 779-783.

(50) Benn, M. H. *Journal of the Chemical Society* **1964**, 4072-4074.

(51) Viaud, M. C.; Rollin, P. *Tetrahedron Letters* **1990**, *31*, 1417-1418.

(52) Heaney, R. K.; Fenwick, G. R. In *Methods in Plant Biochemistry*; Waterman, P. G., Ed.; Academic Press: London, 1993; Vol. 8, p 531-550.

(53) Milford, G. F. J.; Fieldsend, J. E.; Spink, J.; Child, R. D. *Aspects of Applied Biology* **1989**, *23*, 173-176.

(54) Milford, G. F. J.; Fieldsend, J. K.; Porter, A. J. R.; Rawlinson, C. J.; Evans, E. J.; Bilsborrow, P. E. *Aspects of Applied Biology*. **1989**, *23*, 83-90.

(55) Fenwick, G. R.; Heaney, R. K.; Mullin, W. J. In *Critical Reviews in Food*

*Science and Nutrition* 1983; Vol. 18, p 123-201.

(56) Hanley, A. B.; Parsley, K. R.; Lewis, J. A.; Fenwick, G. R. *Journal of the Chemical Society, Perkin Transaction 1* **1990**, *29*, 2273-2276.

(57) Hanley, A. B.; Parsley, K. R. *Phytochemistry* **1990**, *29*, 769-771.

(58) Zhang, Y.; Kensler, T. W.; Cho, C.-G.; Posner, G. H.; Talalay, P. *Proceedings of the National Academy of Sciences, USA* **1994**, *91*, 3147-3150.

(59) Posner, G. H.; Cho, C.-G.; Green, J. V.; Zhang, Y.; Talalay, P. *Journal of MedicinalChemistry* **1994**, *37*, 170-176.

(60) Benn, M. H. *Chemistry and Industry* **1962**, 1907.

(61) Underhill, E. W.; Chisholm, M. D.; Wetter, L. R. *Canadian Journal of Biochemistry and Physiology* **1962**, *40*, 1505-1514.

(62) Chisholm, M. D.; Wetter, L. R. *Canadian Journal of Biochemistry* **1966**, *44*, 1625-1632.

(63) Underhill, E. W.; Chisholm, M. D. *Biochemical and Biophysical Research Communications* **1964**, *14*, 425-430.

(64) Lee, C.-J.; Serif, G. S. *Biochemistry* **1970**, *9*, 2068-2071.

(65) Tapper, B. A.; Butler, G. W. *Archives of Biochemistry and Biophysics* **1967**, *120*, 719-721.

(66) Underhill, E. W. *European Journal of Biochem.* **1967**, *2*, 61-63.

(67) Kindl, H.; Underhill, E. W. *Phytochemistry* **1968**, *7*, 745-756.

(68) Conn, E. E. In *Secondary Plant Products*; Conn, E. E., Ed.; Academic Press: New York, 1981; Vol. 7, p 479-500.

(69) Conn, E. E. In *Biologically Active Natural Products: Potential Use in Agriculture*; Cutler, H. G., Ed.; American Chemical Society: Washington, D.C., 1988, p 143-154.

(70) Halkier, B. A.; Olsen, C. E.; Moller, B. L. *The Journal of Biological*

*Chemistry* **1989**, *264*, 19487-19494.

(71) Halkier, B. A.; Moller, B. L. *The Journal of Biological Chemistry* **1990**, *265*, 21114-21121.

(72) Halkier, B. A.; Lykkesfeldt, J.; Moller, B. L. *Proceedings of the National Academy of Sciences, USA* **1991**, *88*, 487-491.

(73) Underhill, E. W.; Wetter, L. R. *Plant Physiology* **1969**, *44*, 584-590.

(74) Matsuo, M.; Underhill, E. W. *Phytochemistry* **1971**, *10*, 2279-2286.

(75) Matsuo, M.; Underhill, E. W. *Biochemical and Biophysical Research Communications* **1969**, *36*, 18-23.

(76) Jain, J. C.; Reed, D. W.; GrootWassink, J. W. D.; Underhill, E. W. *Analytical Biochemistry* **1989**, *178*, 137-140.

(77) Jain, J. C.; Michayluk, M. R.; GrootWassink, J. W. D.; Underhill, E. W. *Plant Science* **1989**, *64*, 25-29.

(78) Jain, J. C.; GrootWassink, J. W. D.; Reed, D. W.; Underhill, E. W. *Journal of Plant Physiology* **1990**, *136*, 356-361.

(79) Wetter, L. R.; Chisholm, M. D. *Canadian Journal of Biochemistry* **1968**, *46*, 931-935.

(80) Matsuo, M.; Kirkland, D. F.; Underhill, E. W. *Phytochemistry* **1972**, *11*, 697-701.

(81) Glendening, T. M.; Poulton, J. E. *Plant Physiology* **1990**, *94*, 811-818.

(82) Glendening, T. M.; Poulton, J. E. *Plant Physiology* **1988**, *86*, 319-321.

(83) Dornemann, D.; Loffelhardt, W.; Kindl, H. *Canadian Journal of Biochemistry* **1974**, *52*, 916-921.

(84) Underhill, E. W. *Canadian Journal of Biochemistry* **1968**, *46*, 401-405.

(85) Rossiter, J. T.; James, D. C. *Journal of the Chemical Society, Perkin Transactions 1* **1990**, 1909-1913.

(86) Rossiter, J. T.; James, D. C.; Atkins, N. *Phytochemistry* **1990**, *29*, 2509-2512.

(87) Halkier, B. A.; Moller, B. L. *Plant Physiology* **1991**, *96*, 10-17.

(88) Sibbesen, O.; Koch, B.; Halkier, B. A.; Moller, B. L. *Proceedings of the National Acadamy of Sciences USA* **1994**, *91*, 9740-9744.

(89) Sibbesen, O.; Koch, B.; Halkier, B. A.; Moller, B. L. *The Journal of Biological Chemistry* **1995**, *270*, 3506-3511.

(90) Koch, B. M.; Sibbesen, O.; Halkier, B. A.; Svendsen, I.; Moller, B. L. *Archives of Biochemistry and Biophysics* **1995**, *323*, 177-186.

(91) Halkier, B. A.; Nielsen, H. L.; Koch, B.; Moller, B. L. *Archives of Biochemistry and Biophysics* **1995**, *322*, 369-377.

(92) Pickett, J. A. "Towards Zero Pesticide Residues: the Biomanagement of Pests and Diseases of Oilseed Rape," Institute of Arable Crops Research, Annual Report, 1989.

(93) Bartlet, E.; Williams, I. H.; Blight, M. M.; Hick, A. J. In *8th International Symposium on Insect-Plant Relationships*; Menken, S. B. J., Visser, J. H., Harrewijn, P., Eds.; Kluwer Academic Publishers: Dordecht, 1991, p 103-104.

(94) Blight, M. M.; Hick, A. J.; Pickett, J. A.; Smart, L. E.; Wadhams, L. J.; Woodcock, C. M. In *8th International Symposium on Insect-Plant Relationships*; Menken, S. B. J., Visser, J. H., Harrewijn, P., Eds.; Kluwer Academic Publishers: Dordecht, 1992, p 105-106.

(95) Dawson, G. W.; Hick, A. J.; Bennett, R. N.; Donald, A.; Pickett, J. A.; Wallsgrove, R. M. *The Journal of Biological Chemistry* **1993**, *268*, 27154-27159.

(96) Isaacs, R.; Hardie, J.; Hick, A. J.; Smart, L. E.; Wadhams, L. J. In *8th International Symposium on Insect-Plant Relationships*; Menken, S. B. J., Visser, J. H., Harrewijn, P., Eds.; Kluwer Academic Publishers: Dordecht, 1992, p 112-114.

(97) Isaacs, R.; Hardie, J.; Hick, A. J.; Pye, B.; Smart, L. E.; Wadhams, L. J.; Woodcock, C. M. *Pesticide Science* **1993**, *39*, 349-355.

(98) Nottingham, S. F.; Hardie, J.; Dawson, G. W.; Hick, A. J.; Pickett, J. A.; Wadhams, L. J.; Woodcock, C. M. *Journal of Chemical Ecology* **1991**, *17*, 1231-1242.

(99) Hallahan, D. L.; Pickett, J. A.; Wadhams, L. J.; Wallsgrove, R. M.; Woodcock, C. In *Plant Genetic Manipulation for Crop Protection*; Gatehouse, A. M. R., Hilder, V. A., Boulter, D., Eds.; C.A.B. International: Wallingford, 1992, p 215-248.

(100) Boulter, D. *Phytochemistry* **1993**, *34*, 1453-1466.

(101) Mithen, R. F.; Lewis, B. G.; Fenwick, G. R. *Transactions of the British Mycological Society* **1986**, *87*, 433-440.

(102) Bartlet, E.; Parsons, D.; Williams, I. H.; Clark, S. J. *Entomologia experimentalis et applicata* **1994**, *73*, 77-83.

(103) Chew, F. S. In *Biologically Active Natural Products: Potential Use in Agriculture*; Cutler, H. G., Ed.; American Chemical Society: Washington, D.C., 1988, p 155-181.

(104) Mithen, R. *Euphytica* **1992**, *63*, 71-83.

(105) Weller, H. N.; Gordon, E. M. *Journal of Organic Chemistry* **1982**, *47*, 4160-4161.

(106) Paradkar, V. M.; Latham, T. B.; Denko, D. M. *SYNLETT* **1995**, 1059-1060.

(107) March, J. *Advanced Organic Chemistry*; 3rd ed.; John Wiley and Sons: New York, 1985.

(108) Davis, F. A.; Reddy, R. E.; Portonovo, P. S. *Tetrahedron Letters* **1994**, *35*, 9351-9354.

(109) Greenstein, J. P. *Methods in Enzymology* **1957**, *3*, 554-570.

(110) Vriesema, B. K.; ten Hoeve, W.; Wynberg, H.; Kellog, R. M.; Boesten,

W. H. J.; Meijer, E. M.; Schoemaker, H. E. *Tetrahedron Letters* **1986**, *26*, 2045-2048.

(111) Chenault, H. K.; Dahmer, J.; Whitesides, G. M. *Journal of the American Chemical Society* **1989**, *111*, 6354-6364.

(112) Koch, B.; Nielson, V. S.; Halkier, B. A.; Olsen, C. E.; Moller, B. L. *Archives of Biochemistry and Biophysics* **1992**, *292*, 141-150.

(113) Ludwig-Muller, J.; Rausch, T.; Lang, S.; Hilgenberg, W. *Phytochemistry* **1990**, *29*, 1397-1400.

(114) Bennett, R.; Donald, A.; Dawson, G.; Hick, A.; Wallsgrove, R. *Plant Physiology* **1993**, *102*, 1307-1312.

(115) Karabatsos, G. J.; Taller, R. A.; Vane, F. M. *Journal of the American Chemical Society* **1963**, *85*, 2327-2328.

(116) Karabatsos, G. J.; Taller, R. A.; Vane, F. M. *Journal of the American Chemical Society* **1963**, *85*, 2326-2327.

(117) Bennett, R. N.; Hick, A. J.; Dawson, G. W.; Wallsgrove, R. M. *Plant Physiology* **1995**, *109*, 299-305.

(118) Ziegler, D. M. *Drug Metabolism Reviews* **1988**, *19*, 1-32.

(119) Stuehr, D. J.; Fasehun, O. A.; Kwon, N. S.; Gross, S. S.; Gonzalez, J. A.; Levi, R.; Nathan, C. F. *The FASEB Journal* **1991**, *5*, 98-103.

(120) Collette, J.; McGreer, D.; Crawford, R.; Chubb, F.; Sandin, R. B. *Journal of the American Chemical Society* **1956**, *78*, 3819-3820.

(121) Du, L.; Lykkesfeldt, J.; Olsen, C. E.; Halkier, B. A. *Proceedings of the National Acadamy of Sciences USA* **1995**, *92*, 12505-12509.

(122) Moller, B. L.; McFarlane, I. J.; Conn, E. E. *Acta Chemica Scandinavica* **1977**, *B 31*, 343-344.

(123) Ahmad, A. *Bulletin of the Chemical Society of Japan.* **1974**, *47*, 1819-1820.

(124) Gribble, G. W.; Nutaitis, C. F. *Organic Preparations and Procedures*

*International* **1985**, *17*, 317-384.

(125) Ballini, R.; Marcantoni, E.; Petrini, M. *Tetrahedron Letters* **1992**, *33*, 4835-4838.

(126) Emmons, W. D.; Freeman, J. P. *Journal of the American Chemical Society* **1955**, *77*, 4387-4390.

(127) Emmons, W. D.; Freeman, J. P. *Journal of the American Chemical Society* **1955**, *77*, 4391-4393.

(128) Oldfield, M. F.; Bennett, R. N., personal communication.

(129) Ottenheijm, H. C. J.; Herscheid, J. D. M. *Chemical Reviews* **1986**, *86*, 697-707.

(130) Buehler, E.; Brown, G. B. *Journal of Organic Chemistry* **1967**, *32*, 265-267.

(131) Moller, B. L. *Journal of Labelled Compounds and Radiopharmaceuticals* **1978**, *14*, 663-671.

(132) Grundmann, C.; Grunanger, P. *The Nitrile Oxides*; Springer-Verlag: Berlin, 1971.

(133) Laidler, K. J. In *Chemical Kinetics*; Tata McGraw Hilll: New Delhi, 1983, p 90-98.

(134) Dwek, R. A. In *Carbon-Fluorine Compounds - Chemistry, Biochemistry and Biological Activities*; Elsevier: Amsterdam, 1972, p 239-279.

(135) Percival, M. D.; Withers, S. G. *Biochemistry* **1992**, *31*, 505-512.

(136) Haughn, G. W.; Davin, L.; Giblin, M.; Underhill, E. W. *Plant Physiology* **1991**, *97*, 217-226.

(137) Parry, R. J.; Naidu, M. V. *Journal of the American Chemical Society* **1982**, *104*, 3217-3219.

(138) Lepp, A.; Dunn, M. S. *Biochemical Preparations* **1955**, 80-86.

(139) Dohn, D. R.; Anders, M. W. *Analytical Biochemistry* **1982**, *120*, 379-386.

(140) Kiddle, G. A.; Bennett, R. N., personal communication.

(141) Benn, M. H. *Canadian Journal of Chemistry* **1964**, *42*, 2393-2397.

(142) Mukaiyama, T.; Hoshino, T. *Journal of the American Chemical Society* **1960**, *82*, 5339-5342.

(143) Kim, J. N.; Ryu, E. K. *Journal of Organic Chemistry* **1992**, *57*, 6649-6650.

(144) Liu, K.-C.; Shelton, B. R.; Howe, R. K. *Journal of Organic Chemistry* **1980**, *45*, 3916-3918.

(145) Peake, C. J.; Strickland, J. H. *Synthetic Communications* **1986**, *16*, 763-765.

(146) Chiang, Y. H. *Journal of Organic Chemistry* **1971**, *36*, 2146-2155.

(147) Kumaran, G.; Kulkarni, G. H. *Tetrahedron Letters* **1994**, *35*, 9099-9100.

(148) Vogel, A. I. *Vogel's Textbook of Practical Organic Chemistry*; 5th ed.; Longman Scientific and Technical: Harlow, 1989.

(149) Davies, J. H.; Davies, R. H.; Kirby, P. *Journal of the Chemical Society (C)* **1968**, 431-435.

(150) Exner, O.; Benn, M. H.; Willis, F. *Canadian Journal of Chemistry* **1968**, *46*, 1873-1880.

(151) Anklam, E.; Mohan, H.; Asmus, K.-D. *Journal of the Chemical Society Perkin Transactions 2* **1988**, 1297-1302.

(152) Anklam, E. *Synthesis* **1987**, 841-843.

(153) Davis, R.; Untch, K. G. *Journal of Organic Chemistry* **1981**, *46*, 2985-2987.

(154) Barton, D. H. R.; Jaszberenyi, J. C.; Theodorakis, E. A. *Tetrahedron Letters* **1991**, *32*, 3321-3324.

(155) Barton, D. H. R.; Ozbalik, N.; Vacher, B. *Tetrahedron* **1988**, *44*,

3501-3512.

(156) Nystrom, R. F.; Brown, W. G. *Journal of the American Chemical Society* **1947**, *69*, 2548-2549.

(157) Hochstein, F. A.; Brown, W. G. *Journal of the American Chemical Society* **1948**, *70*, 3484-3486.

(158) Batty, D. , PhD Thesis, University College London, 1991.

(159) Blight, M. In *Chromatography and the Isolation of Insect Hormones and Pheromones*; McCaffrey, A. R., Wilson, I. D., Eds.; Plenum Press: New York, 1990, p 281-288.

(160) Wadhams, L. J. In *Chromatography and Isolation of Insect Hormones and Pheromones*; McCaffrey, A. R., Wilson, I. D., Eds.; Plenum Press: New York, 1990, p 289-298.

(161) Dawson, G. W. ,PhD Thesis CNAA, 1988.

(162) Gibson, M.; Bradshaw, R. *Angewte Chemie International Editon in English* **1968**, *7*, 919-930.

(163) Lavery, A.; Nelson, S. *Journal of the Chemical Society Dalton Transactions*. **1984**, 615-620.

(164) Woodcock, C. M., personal communication.

(165) Das, N. B.; Torssell, K. B. G. *Tetrahedron* **1983**, *39*, 2247-2253.

(166) Still, W. C.; Kahn, M.; Mitra, A. *Journal of Organic Chemistry* **1978**, *43*, 2923-2925.

(167) Perrin, D. D.; Armarego, W. L. F. *Purification of Laboratory Chemicals*; 3rd ed.; Pergamon Press: Oxford, 1988.

(168) Fischer, E.; Schmitz, W. *Chemische Berichte* **1906**, *39*, 2208-2215.

(169) Underhill, E. W.; Kirkland, D. F. *Phytochemistry* **1972**, *11*, 1973-1979.

(170) Kjaer, A.; Larsen, I.; Gmelin, R. *Acta Chemica Scandinavica* **1955**,

1311-1316.

(171) Campi, E.; Jackson, W. R.; Perlmutter, P.; Tasdelen, E. E. *Australian Journal of Chemistry* **1993**, *46*, 995-1007.

(172) Gawley, R. E.; Nagy, T. *Tetrahedron Letters* **1984**, *25*, 263-264.

(173) Lee, C.-J.; Serif, G. S. *Biochimica et Biophysica Acta* **1971**, *230*, 462-467.

(174) Auricchio, S.; Ricca, A. *Tetrahedron* **1987**, *43*, 3983-3986.

(175) Kornblum, N.; Eicher, J. H. *Journal of the American Chemical Society* **1956**, *78*, 1494-1497.

(176) Kornblum, N.; Larsen, H. O.; Blackwood, R. K.; Mooberry, D. D.; Oliveto, E. P.; Graham, G. E. *Journal of the American Chemical Society* **1956**, *78*, 1497-1501.

(177) Meyers, A. I.; A., T. T.; Comins, D. L. *Tetrahedron Letters* **1978**, *47*, 4657-4660.

(178) Herbert, R. B.; Knagg, E. *Tetrahedron Letters* **1986**, *27*, 1099-1102.

(179) Zhou, J.-Q.; Alper, H. *Journal of the Chemical Society. Chemical Communications* **1991**, 233-234.

(180) Bream, J. B.; Picard, C. W.; White, T. G.; Lauener, H. *Journal of Medicinal Chemistry* **1970**, *13*, 1051-1057.

(181) Houghton, R. P.; Voyle, M.; Price, R. *Journal of Organometallic Chemistry* **1983**, *259*, 183-189.

(182) Parlow, J. J. *Tetrahedron* **1994**, *50*, 3297-3314.

(183) Truce, W. E.; Bannister, W. W.; Knospe, R. H. *Journal of Organic Chemistry* **1962**, *27*, 2821-2828.

(184) Kjaer, A.; Wagner, S. *Acta Chemica Scandinavica* **1955**, *9*, 721-726.

(185) Kondo, H.; Yamauchi, M.; Nakamura, T.; Nozaki, H. *Agricultural and Biological Chemistry* **1985**, *49*, 3587-3589.

(186) Kjaer, A.; Christiansen, B. *Acta Chemica Scandinavica* **1957**, *11*, 1298-1307.

(187) Matsuo, M. *Tetrahedron Letters* **1968**, 4101-4104.

(188) Lynn, J. W.; Roberts, R. L.; Kilsheimer, J. R. *Journal of Organic Chemistry* **1961**, *26*, 4300-4303.

(189) Rakshinda, M. A.; Khan, N. H. *Indian Journal of Chemistry* **1978**, *16B*, 634-635.

(190) Kirrmann, A.; Geiger-Berschandy, S. *Bulletin Societe Chimique France* **1955**, 991-994.

(191) Hurd, C. D.; Saunders, W. H. *Journal of the American Chemical Society* **1952**, *74*, 5324-5329.

(192) Wormser, H. C.; Abramson, H. N. *Journal of Pharmaceutical Sciences* **1975**, *64*, 1514-1517.

(193) Albertson, N. F. *Journal of the American Chemical Society* **1946**, *68*, 450-453.

(194) Kennewell, P. D.; Matharu, S. S.; Taylor, J. B.; Westwood, R.; Sammes, P. G. *Journal of the Chemical Society. Perkin Transactions 1* **1982**, 2553-2562.

(195) Schluter, M.; Gmelin, R. *Phytochemistry* **1972**, *11*, 3427-3431.

(196) Keinan, E.; Gleize, P. A. *Tetrahedron Letters* **1982**, *23*, 477-480.

(197) Kjaer, A.; Jensen, R. B. *Acta Chemica Scandinavica* **1956**, *10*, 1365-1371.

(198) Kjaer, A.; Rubinstein, K.; Jensen, K. A. *Acta Chemica Scandinavica* **1953**, *7*, 518-527.