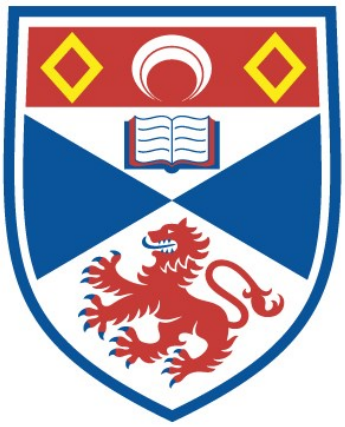
MECHANISTIC STUDIES ON MYROSINASE

Malcolm G. Taylor

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



1997

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Title of Thesis: "Mechanistic Studies on Myrosinase"

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Abstract

Myrosinase is the β-thioglucosidase enzyme which catalyses the hydrolysis of glucosinolates, a group of naturally occurring plant metabolites. Glucosinolates are *S*-glucosides which occur predominantly in the family Cruciferae, and are abundant in the *Brassica* vegetables. The hydrolysis reaction, specifically activated by L-ascorbic acid, gives β-D-glucose and an aglucone fragment, which then rearranges to give sulfate and an isothiocyanate. The aim of this study was the investigation of the chemical mechanism of myrosinase, in order for it to be compared with those of the much more widely studied β-glycosidases.

Myrosinase catalysed transglycosylation reactions were examined as a potential synthetic method. However, no transglycosylation was detected, even though a wide range of glycosyl acceptors was examined, including simple alcohols and thiols and examples with charged side chains to mimic the glucosinolate. Such reactions are commonly observed with β-glycosidases. However, the stability and activity of myrosinase was not substantially affected by the presence of the acceptors. A small amount of transglycosylation was observed using azide, a charged glycosyl acceptor. This is consistent with the other transglycosylation reactions failing due to the lack of a suitable basic residue at the active site to deprotonate the acceptor. A range of potential substrates was synthesised, although only the nitrophenyl-β-D-glucosides showed any substrate activity. All *S*-glucosides were inactive. Myrosinase therefore appears to be very specific. Inhibition studies revealed that D-glucono-γ-lactone, a potent competitive inhibitor and transition state mimic of β-glucosidases, was a poor noncompetitive inhibitor of myrosinase. It was proposed that this inhibitor binds at the L-ascorbic acid activator site. The inhibitory properties of a number of other compounds were examined, including reaction products and potential substrates (*S*-glucosides). [1-^{2}H]-Sinigrin, *p*-nitrophenyl- and 2,4-dinitrophenyl-β-D-[1-^{2}H]-glucoside were prepared to measure the secondary deuterium isotope effects. The isotope effect obtained for 2,4-dinitrophenyl-β-D-[1-^{2}H]-glucoside ($^D V = 1.26 \pm 0.11$) indicated an sp^2 hybridised intermediate *via* an S_N1-like mechanism. The solvent kinetic isotope effect for sinigrin hydrolysis was determined ($^D V = 1.54 \pm 0.07$, $^D V/K = 1.24 \pm 0.15$) implying that a proton transfer was partially rate limiting.

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Dedicated to Mum and Dad for all their love
and support during my university career

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Abbreviations

Allyl NCS	Allyl Isothiocyanate
DABCO	2,2,2-Diazabicyclooctane
1-DNM	1-Deoxynojirimycin
2,4-DNPG	2,4-Dinitrophenyl-β-D-glucopyranoside
EDTA	Ethylenediamine tetraacetic acid
HK	Hexokinase
2-MESNA	2-Mercaptoethanesulfonate
ONPG	*o*-Nitrophenyl-β-D-glucopyranoside
ONPTG	*o*-Nitrophenyl-1-thio-β-D-glucopyranoside
PCMB	*p*-Chloromercuribenzoate
PG	Phenyl-β-D-glucopyranoside
PIPES	1,4-Piperazinediethanesulfonic acid
PNP	*p*-Nitrophenol
PNPG	*p*-Nitrophenyl-β-D-glucopyranoside
PNPTG	*p*-Nitrophenyl-1-thio-β-D-glucopyranoside
PTG	Phenyl-1-thio-β-D-glucopyranoside
2-PYTG	2-Pyridyl-1-thio-β-D-glucopyranoside

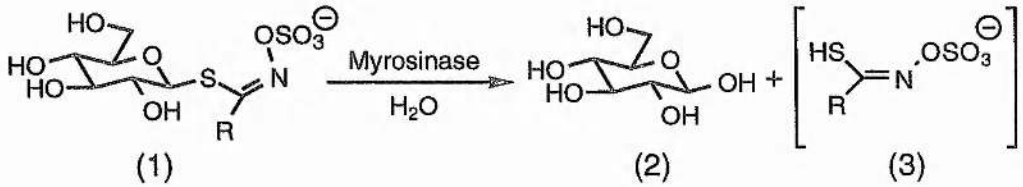
Chapter 1

INTRODUCTION

1.1 Myrosinase and Glucosinolates

1.1.1 The Myrosinase/Glucosinolate System

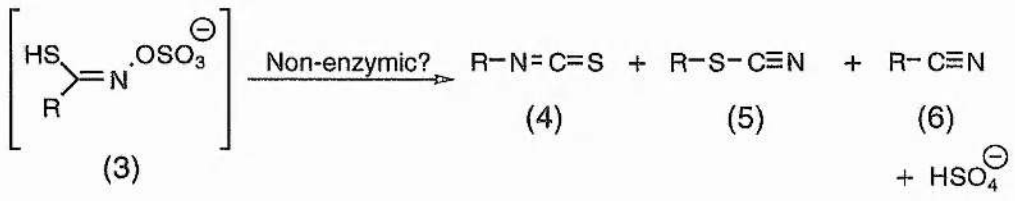
Myrosinase (thioglucoside glucohydrolase, E.C. 3.2.3.1) is the β-thioglucosidase enzyme responsible for the hydrolytic cleavage of glucosinolates (1), a group of naturally occurring plant metabolites also known as mustard oil glucosides. Glucosinolates are sulfur-containing glucosides which are present in all members of the Cruciferae, including the *Brassica* vegetables such as Brussels sprouts, cabbage, oilseed rape and mustard. The products of the hydrolysis reaction are β-D-glucose (2)[1] and an aglucone fragment (3) (Scheme 1).



(1) (2) (3)

Scheme 1: *Myrosinase Catalysed Hydrolysis of Glucosinolates*

This aglucone fragment (3) is unstable and undergoes a Lossen-type rearrangement[1] (Scheme 2) to form the isothiocyanate (4). The corresponding thiocyanate (5) and nitrile (6) are minor products and their relative amounts depend upon pH, temperature and plant species.[2] There is evidence to suggest that the aglucone rearrangement occurs spontaneously and is not enzyme catalysed, but it is not conclusive.[3]



(3) (4) (5) (6)

Scheme 2: *Rearrangement of the Aglucone Fragment*

Over 100 different glucosinolates have been isolated and characterised, and these vary only in the nature of the side chain R.[4] R in fact varies considerably from simple alkyl and alkenyl, hydroxylated and

polyhydroxylated alkyl to aryl and indolyl. The volatile products of the hydrolysis of glucosinolates - known as mustard oils - give rise to the characteristically pungent tastes and aroma of these vegetables. Due to the common occurrence of Cruciferae - and therefore glucosinolates - in the diet, the properties of glucosinolates and their breakdown products have been of great interest.[5]

Some breakdown products can have deleterious effects in the mammalian diet due to their pungency and in some cases goitrogenic and hepatoxic activity.[6,7] This is a particular problem with oilseed rape and its food and feed derivatives. E.C. recommendations concerning the maximum permitted levels of glucosinolates in rapeseed have resulted in major programmes to breed safer varieties. Additionally, glucosinolates are of great interest since some have breakdown products which are thought to be anticarcinogenic,[8] while others are thought to be procarcinogenic.[9]

Little is known about the chemical mechanism of myrosinase, and the main aim of this study is to further elucidate this mechanism. A comparison can then be made with the chemical mechanisms of β-glycosidases, which have been well studied in recent years (Section 1.2).

Also, the synthetic utility of myrosinase has not been explored, in contrast to the β-glycosidases, which have found use in organic synthesis (Section 1.3). Therefore, the use of myrosinase in synthesis was also investigated.

1.1.2 Historical Importance of the Myrosinase/Glucosinolate System

Man's affinity for foods possessing the pungent tastes and odour which are associated with the breakdown products of glucosinolates has been known since ancient times. The cultivation of glucosinolate-containing plants spread to many parts of the world due to the additional medicinal properties of extracts, juices and poultices obtained from these plants.[10,11]

Carbonised seed of *Brassica juncea* and *B. rapa* were discovered at Ban Po village in China and dated to approximately 4000 BC. In Pakistan, carbonised seed of *B. juncea* ("Rajika") was found and dated to the Harappan era (about 2300 to 1750 BC). The earliest reference to yellow sarson (*B. campestris*) occurs in Sanskrit manuscripts from about 1500 BC.[12] Mustard (*B. juncea* Coss.) was mentioned in Chinese manuscripts

of the Chou Dynasty (1122 to 247 BC).[13] The use of mustard as a condiment and a remedy for scorpion stings was recorded by Pythagoras in 530 BC and Hippocrates in 400 BC. The word "Brassica" presumably including cole crops, was used by the Roman authors Cato (234 to 149 BC), Culumella (first century AD) and Pliny (23 to 79 AD). It is clear from these and other writings that the Romans were familiar with, and cultivated various forms of cabbage, kales and possibly kohlrabi and broccoli.

The primary use of these crops in ancient times was for medicinal purposes. Their usefulness against gout, diarrhoea, celiac and stomach disorders, deafness and headache have all been documented.[10] The cultivation of mustard was introduced into Europe by the Moors and this condiment was carried around the Cape of Good Hope in 1497 by Vasco and Gama. The spread of cruciferous crops across Europe in the Middle Ages may be ascribed to a combination of factors including the Crusades, travel across and around the Mediterranean and the importance of the herb garden to both monastic and village life.

At the present time, the diets of people in many parts of the world include considerable amounts of cruciferous plants and crops, either raw, cooked or otherwise processed. These range from the consumption of processed radish and wasabi in the Far East to that of cabbage and traditional root vegetables in Europe and North America. Other crops, such as rapeseed, kale, swede and turnip may also contribute indirectly to the human food chain by virtue of their extensive use as animal feedstuffs.

Cabbage is the most important *Brassica* crop with a reported annual world production of 32 million tonnes. Other crops may be less widespread but are still important in particular parts of the world. For example, the annual consumption of radish in Japan is estimated at 5 million tonnes.[14]

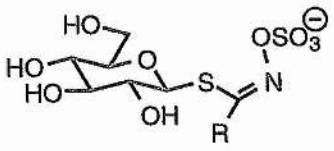
1.1.3 Glucosinolates

The occurrence of glucosinolates in plants appears limited to certain families of dicotyledonous angiosperms. These compounds occur predominantly throughout the order Capparales *sensu* Cronquist or Taktajan, constituting the Capparaceae, Cruciferae, Moringaceae, Resedaceae and Tovariaceae. Since the role of glucosinolates in human

foods and animal feedstuffs is predominantly of interest, emphasis is placed on their occurrence in cruciferous plants and crops. These include cole crops (*Brassica oleracea*), condiments (*B. nigra, B. hirta* or *Sinapis alba*), rapeseed (*B. campestris; B. napus*) and forages. For many cruciferous species, any single plant may contain as many as 15 different glucosinolates.[7]

The elucidation of the structures of the glucosinolates and their pungent breakdown products offers a fascinating insight into the development of scientific thought and practice over 3 centuries. The formation of a volatile oil from the distillation of mustard seed was reported in 1608 by Portas and later by Febure (1660). The properties of this malodorous oil were first described by Boerhaave in 1732. By the early 19th century it was known that mustard oil contained sulfur and was only formed after the seeds had been ground in water. In 1831, Robiquet and Boutron[15] isolated a crystalline mustard oil precursor, sinalbin, from white mustard seed (*Sinapis alba* L.). Subsequently Bussy[16] isolated a related compound, sinigrin, from the seeds of black mustard (*B. nigra* Koch.). These compounds were decomposed to the expected mustard oils under the influence of "myrosin", later called myrosinase, isolated from black mustard seed.[17] In 1844 it was suggested that the mustard oil from black mustard was C_3H_5CNS and 11 years later the product synthesised from 2-propenyl iodide and potassium thiocyanate was proved to be identical to that obtained from sinigrin. The conclusion that the compound was the 2-propenyl ester of thiocyanic acid was, however inconsistent with the behaviour of other such esters which had previously been prepared. In 1868, Hofmann[18] suggested that the mustard oil contained a 2-propenyl group attached to the nitrogen atom rather than to sulfur as would be the case for the thiocyanic ester. This finding was of great significance in the development of the concept of structural isomerism in organic chemistry.

The first structural formulae of sinigrin and sinalbin were proposed in 1897 by Gadamer,[19,20,21] and were based upon an analysis of their chemical decomposition products. Despite the fact that the proposed structures did not explain the formation of either nitriles or thiocyanates, the structures were generally accepted until 1956 when Ettlinger and Lundeen published a revision.[22] The new structure (1) (Figure 1) contained a β-thioglucose grouping, side chain R and sulfonated oxime moiety.



(1)

Figure 1: *General Structure of Glucosinolates as Proposed by Ettlinger and Lundeen in 1956*

Initial X-ray crystallographic analysis of sinigrin,[23] later refined,[24] confirmed this structure and also established the side chain and the sulfate groups to have the *anti* stereochemical configuration. The latter is of fundamental importance in the enzyme-induced breakdown of glucosinolates.

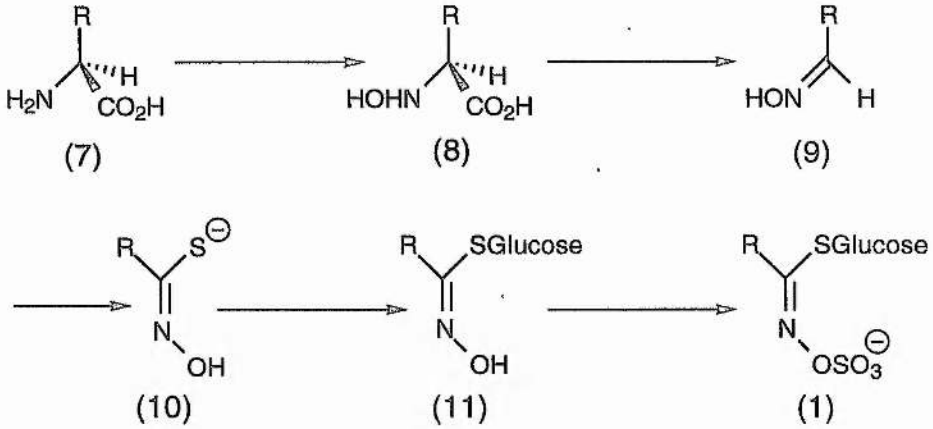
Although the two original glucosinolates were given classical names, a subsequent degree of uniformity of nomenclature was introduced. The glucosinolate took the prefix "gluco" followed by a suffix denoting the botanical species from which it had been isolated. Glucoerucin (isolated from *Eruca sativa*) and glucoiberin (from seeds of *Iberis amara*) exemplified this approach. With the isolation and characterisation of increasing numbers of glucosinolates, the obvious limitations of such terminology became apparent. To overcome this problem it was suggested[25] that the chemical structure of the aglucone chain be written as a prefix to the word glucosinolate. Thus sinalbin and sinigrin may be termed *p*-hydroxybenzyl and 2-propenyl (or allyl) glucosinolate, respectively.

Although over 100 different glucosinolates have been isolated and characterised, individual species have generally thought to contain relatively few, at least in high abundance. An investigation of the volatiles of horseradish using combined capillary gas chromatography-mass spectrometry (GC-MS),[26] however, suggested the presence of 30 glucosinolates although the evidence for the structures of some of the minor components must be regarded as tentative. The distribution of individual glucosinolates varies markedly; whereas, for example benzyl glucosinolate is distributed widely, methyl glucosinolate occurs rarely outside the Capparaceae.

The seeds of glucosinolate containing plants constitute an especially rich source of these compounds, but certain glucosinolates found in the seed may not be present or will only occur in trace amounts in the developing and mature plant. The total glucosinolate content may also depend upon variety, cultivation conditions, climate and agronomic practice.[27] Glucosinolates may also be concentrated in bulbous tissue and this is clearly important when considering such crops as rutabaga (swede), kohlrabi or radish.

1.1.4 Biosynthesis of Glucosinolates

Studies have shown that glucosinolates are derived from amino acids and that most lie on a common biosynthetic pathway, indole glucosinolates being apparent exceptions. The pathway, outlined in Scheme 3, has been proposed,[28] but few of the steps have been studied in any detail and little is known of the enzymology. It is, however, well established that amino acids (7), or their chain-extended homologues, provide the side chain for the glucosinolates. Initial *N*-hydroxylation to yield the *N*-hydroxy amino acid (8) is speculative, although the aldoxime (9) is a known intermediate. The mechanism of formation of the aldoxime (9), however, is only beginning to be identified. The next step involves conversion of the aldoxime (9) to the thiohydroximic acid (10), for which the nature of the sulfur donor is subject to speculation. However, some feeding experiments have suggested cysteine as the sulfur donor.[29,30] Glycosylation is known to be catalysed by UDPG thiohydroximate glucosyltransferase to give the desulfoglucosinolate (11), and the final step is known to involve 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as the sulfate donor to give the glucosinolate (1). The two enzymes from the final two steps have been purified and shown to be non-specific with respect to the nature of the side chain R.[31,32,33,34]



Scheme 3: *Biosynthetic Pathway of Glucosinolates*

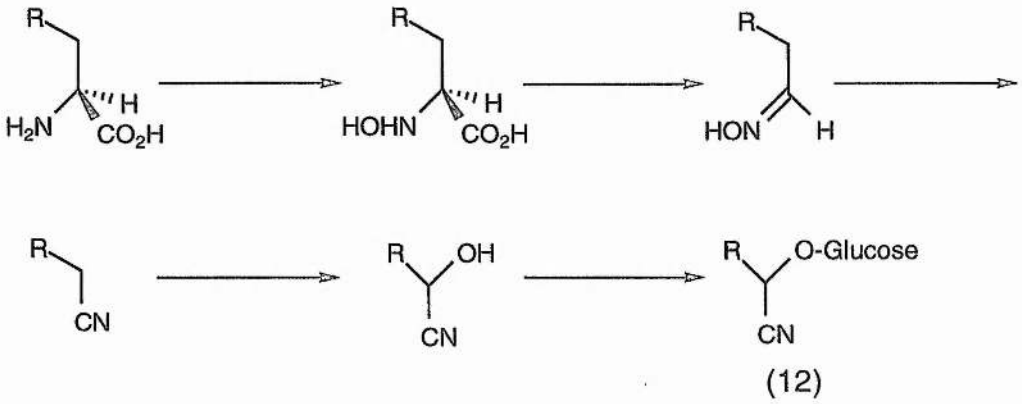
The nature of the enzymes in the early parts of the biosynthetic pathway, catalysing the conversion of amino acids (7) to aldoximes (9), has been the subject of much discussion. It has been established that in contrast to the non-specificity of the last two steps of the biosynthesis, the enzyme-catalysed oxidation to the aldoxime appears to be specific in relation to the side chain of the substrate amino acid. The involvement of flavin mono-oxygenases,[35,36] peroxidase type enzymes[37] and cytochrome-P450 enzymes has been suggested. Recently, it has been demonstrated that the conversion of tyrosine to *p*-hydroxyphenylacetaldoxime in *Sinapis alba* involves a cytochrome-P450 dependent mono-oxygenase, as evidenced by photoreversible carbon monoxide inhibition.[38] Also, the enzyme which catalyses the conversion of phenylalanine to phenylacetaldoxime in *Tropaeolum majus* has been shown to resemble the system in *Sinapis alba* and is also cytochrome-P450 dependent.[39] However, the monooxygenases present in *Brassica napus* (oilseed rape) and *Brassica campestris* (Chinese cabbage) resemble mammalian flavin-containing monooxygenases.[40]

Some glucosinolates are clearly derived directly from amino acids. For example, those possessing methyl, isopropyl and *p*-hydroxybenzyl side chains are derived from alanine, valine and tyrosine respectively. However, the majority possess side chains requiring some degree of structural modification, *e.g.* homologation, elimination or hydroxylation, prior to entering the biosynthetic pathway.[4] There is ample evidence to suggest that such processes can also occur at various points along the biosynthetic pathway. Additionally, the glucosinolate product is often

subjected to secondary modifications such as hydroxylations, methylations, *etc.*[41]

Indole glucosinolates are biosynthesised from L-tryptophan.[42] The points in the biosynthetic pathway where elaboration of the indole structure occurs are not fully known.

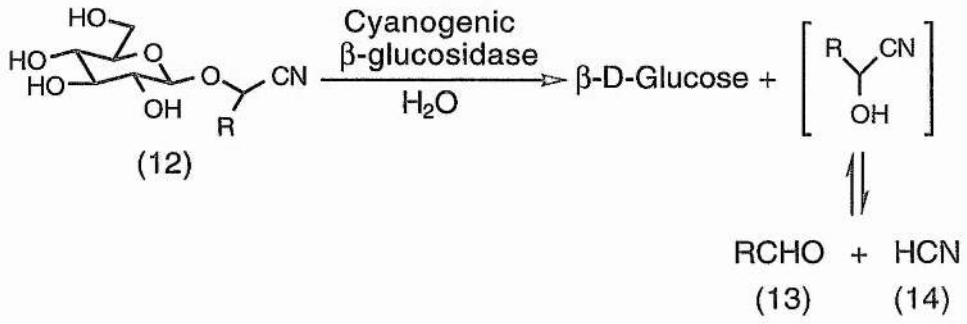
Cyanogenic glucosides (12) are structurally related to glucosinolates, are also derived from amino acids and have a biosynthetic pathway (Scheme 4) similar to that for glucosinolates.



(12)

Scheme 4: *Biosynthetic Pathway of Cyanogenic Glucosides*

The cyanogenic glucosides (12) are also cleaved hydrolytically by a β-glucosidase enzyme when plant tissues are damaged, and hydrogen cyanide (14) is released as one of the plant's defence mechanisms (Scheme 5).



(12) (13) (14)

Scheme 5: *Hydrolysis of Cyanogenic Glucosides as Catalysed by Cyanogenic β-Glucosidase*

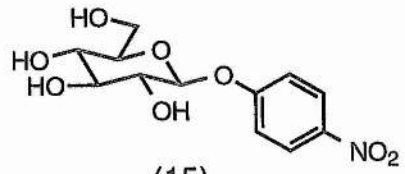
1.1.5 Myrosinase: The Group of β-Thioglucosidase Isoenzymes

Myrosinase is the trivial name for the group of isoenzymes which comprise β-thioglucoside glucohydrolase (E.C. 3.2.3.1). In plants, myrosinase occurs in association with one or more glucosinolates, but thioglucosidases have also been identified in fungi,[43] bacteria[44] and mammals.[45] Myrosinase has been isolated from a number of sources, and has a wide range of molecular weights. Myrosinases from *Aspergillus niger*, *A. sydowi* and *Enterobacter cloacae* have molecular weights of 90,000, 120,000 and 61,000 daltons respectively, similar to those reported for *Sinapis alba* (white mustard)[46] and *Brassica napus* (rapeseed)[46,47] of 120,000 to 150,000 daltons. These molecular weights are considerably less than that reported for *Wasabia japonica* (Japanese horseradish) of 580,000 daltons.[47,48]

Many investigations have shown that multiple forms of myrosinase occur in a large number of plants.[47] These isoenzymes may exhibit different properties. The size difference between the myrosinase isoenzymes for one particular species has been suggested to depend on different degrees of glycosylation.[49] For *Brassica napus* myrosinase, the calculated molecular sizes of the proteins encoded by the MA, MB and MC genes are 59,870, 60,686 and 60,754 Da respectively. The apparent sizes of these proteins, as determined by SDS-PAGE, are 75, 63 and 67 Da respectively. The differences appeared to be mainly accounted for by different degrees of glycosylation since treatment with glycosidases significantly reduced the molecular sizes of the myrosinases.

Recently, two myrosinases from cotyledons of 5-day old *Brassica napus* seedlings were separated.[50] The most striking difference between these two myrosinases was the degree of glycosylation. Myrosinase II was found to be considerably less glycosylated than myrosinase I.

The activity of the *Lepidium sativum* L. (light grown cress) enzyme was tested with 29 different glycosides, and only 4 were found to be good substrates.[51] These were, in order of decreasing activity, sinigrin ((1), R = allyl) (the natural substrate), glucotropaeolin ((1), R = benzyl), *p*-nitrophenyl-β-D-glucopyranoside (PNPG) (15) and *o*-nitrophenyl-β-D-glucopyranoside (ONPG) (16) (Figure 2).



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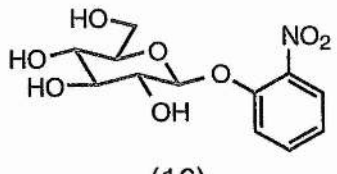


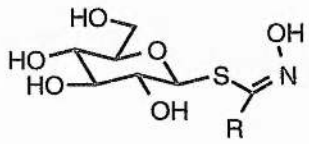
(16)

Figure 2: *Structures of PNPG and ONPG*

β-D-Glucose could not be replaced by any other sugar. α-Glucosides were inactive, as were unreactive β-glucosides such as methyl or phenyl. There appeared to be no discrimination between different glucosinolates, and all were hydrolysed at similar rates regardless of the nature of the side chain.

The two myrosinase isoenzymes from *Brassica napus* degraded different glucosinolates at different rates.[50] However, both isoenzymes showed highest activity against aliphatic glucosinolates and least activity against indole glucosinolates. From these results it is evident that members of a given class of glucosinolates are degraded at approximately the same rates *in vitro*, an exception being the significantly faster degradation of sinigrin.

The sulfate group of glucosinolates is important for binding at the active site of myrosinase, since desulfoglucosinolates ((17), Figure 3) were not hydrolysed.[52] These desulfoglucosinolates were, however, found to inhibit myrosinase.



(17)

Figure 3: *Desulfoglucosinolates: Inhibitors, not Substrates, for Myrosinase*

Two out of the three myrosinase isoenzymes of *Arabidopsis thaliana* are known to degrade 23 of the glucosinolates present in *A. thaliana*.[53]

In general, L-ascorbic acid exerts an activating effect on plant myrosinase, although the extent of this activation is variable. Studies have shown that the rate of hydrolysis of sinigrin, catalysed by the brown mustard seed enzyme, is increased more than 25-fold by 1 mM L-ascorbic acid[54] but is not increased significantly by any L-ascorbic acid analogues (Table 1).

Ascorbic Acid Analogue / 10^{-3} M.	Relative Activity / %[a]
None	100
L-Ascorbate	2560
Dehydro-L-ascorbate	122
D-Araboascorbate	140
Glucoascorbate	92
Ascorbyl palmitate[b]	170
Ascorbyl stearate[b]	85
Ascorbyl 2,6-dipalmitate[b]	47

[a] Enzymatic activities were measured by sulfate liberation

[b] Dissolved in 0.1 M dimethylformamide

Table 1: *Effects of L-Ascorbic Acid and its Analogues on Myrosinase Activity*

The rates of hydrolysis of sinigrin by myrosinase from *Sinapis alba* and *Brassica napus* were increased by limited amounts of L-ascorbic acid.[46] When larger amounts were added, L-ascorbic acid functioned as an inhibitor. The rate of hydrolysis of PNPG (15), catalysed by myrosinase, is not increased by L-ascorbic acid.[55] As for sinigrin, hydrolysis of PNPG is inhibited by larger amounts of L-ascorbic acid.

Figure 4 shows the effect of various amounts of L-ascorbic acid on enzyme activity for glucosinolate hydrolysis. The maximum activity was around 1 mM. When the L-ascorbic acid concentration was increased to 5 to 10 mM, the enzymatic activity was essentially absent.

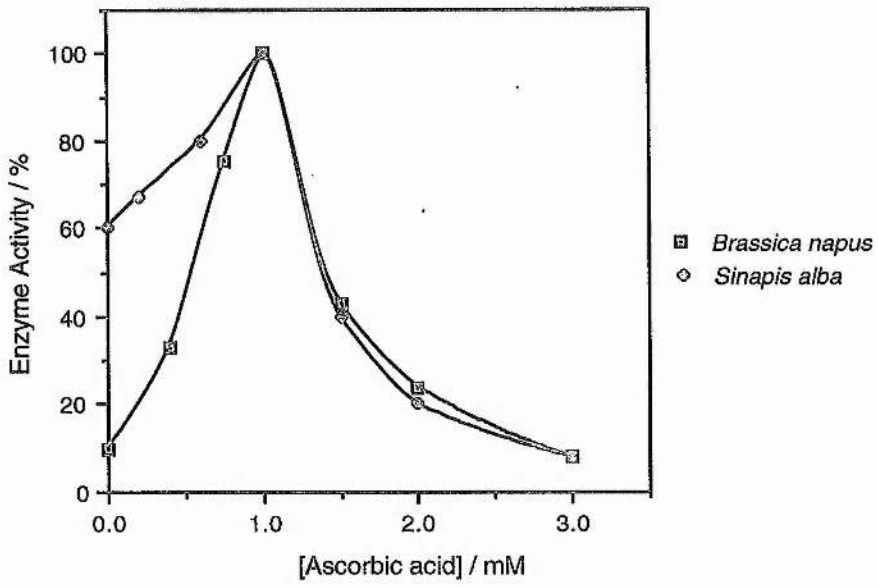


Figure 4: *Effect of L-Ascorbic Acid Concentration on the Activation of Myrosinases from Sinapis alba and Brassica napus*[46]

Other studies also showed that myrosinase was not activated by any other reducing agents (*e.g.* glutathione, cysteine, demercaprol and gallic acid), thus demonstrating that L-ascorbic acid does not act as a conventional reducing agent.[56]

In addition to increasing the reaction rate, the optimum temperature for hydrolysis is reduced from 55 °C to 35 °C by 1 mM L-ascorbic acid[54] (Figure 5).

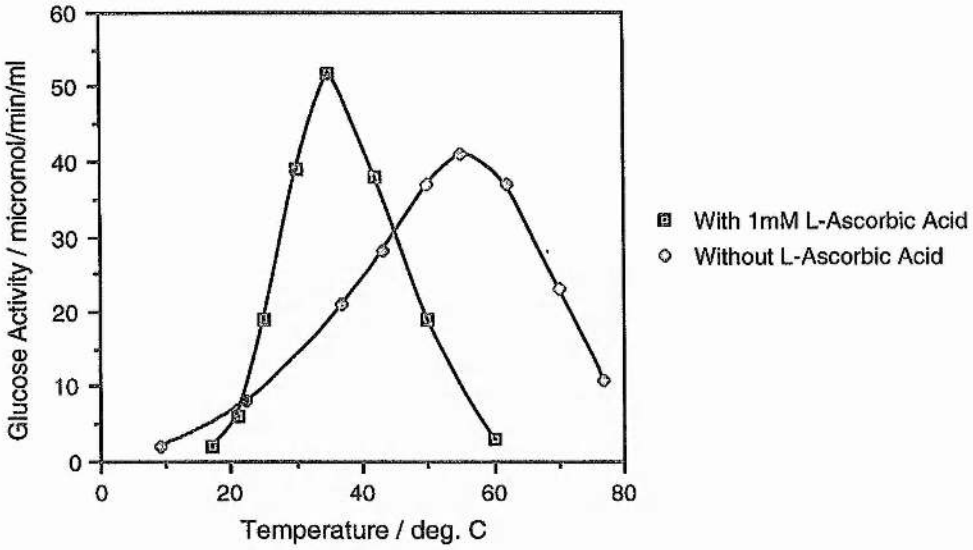
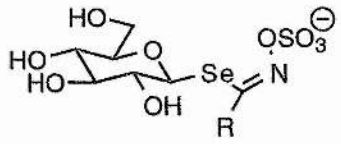


Figure 5: *Effect of Addition of 1 mM L-Ascorbic Acid on Optimum Temperature of Myrosinase* [54]

It was concluded that L-ascorbic acid is not itself involved in catalysis but changes the conformation of the enzyme active site. Differential UV spectral studies showed that some chromophores of the enzyme were changed by adding L-ascorbic acid. Approximately 1.5 amino acid residues appeared on the surface of the enzyme and about 2.3 tryptophan residues were buried in the molecule when 1 mM L-ascorbic acid was added.[54]

Glucosinolate analogues, in which the sulfur atom is replaced with a selenium, have been synthesised.[57] These analogues are termed selenoglucosinolates (18) (Figure 6) and have been shown to undergo enzymatic hydrolysis by myrosinase.



(18)

Figure 6: *General Structure of Selenoglucosinolates*

The products of hydrolysis were analogous to those obtained from glucosinolates, and the major product from benzylselenoglucosinolate was benzyl isoselenocyanate. L-Ascorbic acid caused a similar rate enhancement of the reaction to that of the hydrolysis of glucosinolates.

Other glucosinolate analogues which have been synthesised include a phosphate bio-isostere of glucotropaeolin (19)[58] and desulfo-aza analogues (20)[59] (Figure 7).

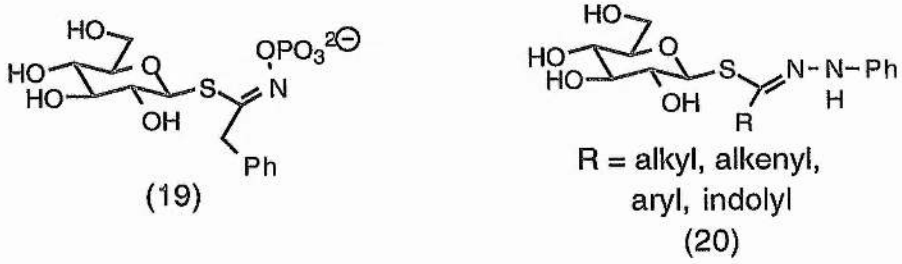


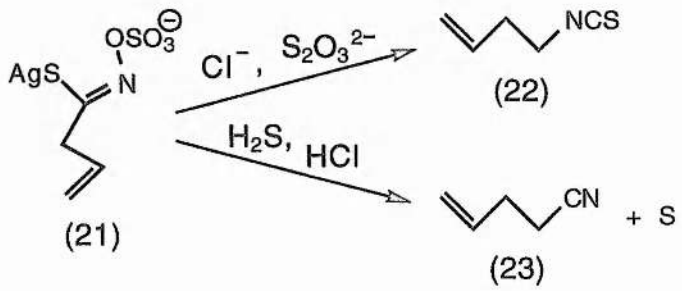
Figure 7: *Structures of Glucosinolate Analogues*

Preliminary studies of phospho-glucotropaeolin (19) with myrosinase[58] indicate that the analogue undergoes hydrolysis, albeit with modified kinetic parameters as compared with glucotropaeolin. The interaction of myrosinase with the desulfo-aza analogues (20) has not yet been reported.

In early studies on the hydrolysis of sinigrin with myrosinase and L-ascorbate at pH 7, the products allyl isothiocyanate, β-D-glucose, sulfate and acid were formed at approximately equivalent rates.[3] The velocity diminished below pH 6, but at pH 3.5 it was, with sufficient L-ascorbate, at least one tenth of optimal rate, and 50 times faster than without L-ascorbate. During the ascorbate-promoted neutral hydrolysis, studied by UV spectrophotometry, a temporary absorption appeared that was most obvious at 255 nm, where starting material and final product had almost equal extinction coefficients. The extra absorbance was up to 0.6 with a velocity of glucosinolate cleavage slightly below 0.003 mol dm^{-3} min^{-1}. When the transient was maximal, the rate of cleavage of sinigrin could be measured at 228 nm without interference. At fixed temperature, the ratio of largest absorption or amount of intermediate to concurrent speed was a constant, independent of enzyme or ascorbate concentrations or buffer (acetate, phosphate or carbonate, pH 5-9.4). The steady-state conditions

implied that the absorbing intermediate, formed by the enzymatic process, decomposed in a first-order reaction not involving enzyme, ascorbate or other ions.

Furthermore, these studies showed that silver sinigrate (21), a well known product[60,61] from the reaction of sinigrin with silver nitrate, is decomposed to allyl isothiocyanate (22) by neutral nucleophiles, *e.g.* thiosulfate, chloride, and to the nitrile (23) and sulfur by corresponding acidic reagents, *e.g.* hydrogen sulfide, hydrogen chloride (Scheme 6). This meant that the aglucone fragment rearranges without enzymatic participation, and it was concluded from these studies that the decomposition of the aglucone fragment in the myrosinase catalysed hydrolysis of glucosinolates occurs non-enzymatically.[3]



Scheme 6: *Non-Enzymatic Decomposition of Silver Sinigrate*

All myrosinase enzymes show pH optima between pH 6 and 7. Typical pH profiles are shown for *Wasabia japonica* myrosinase (Figure 8) and *Lepidium sativum* myrosinase (Figure 9).

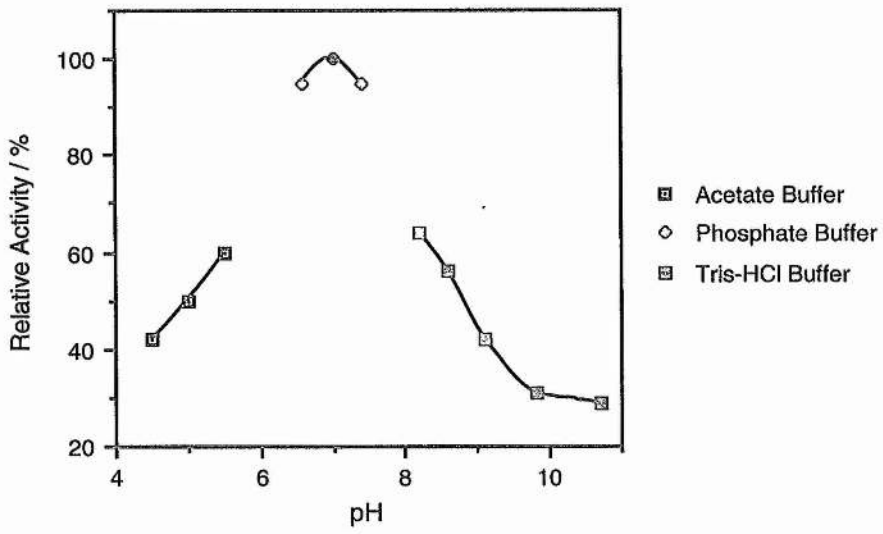


Figure 8: *Effect of pH on Activity of Wasabia japonica Myrosinase* [48]

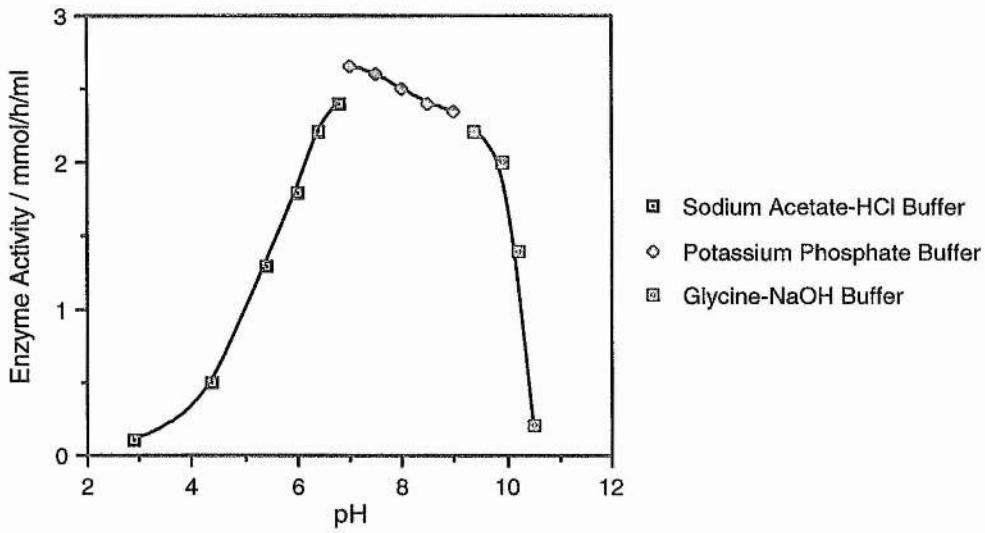


Figure 9: *Effect of pH on Activity of Lepidium sativum Myrosinase* [51]

Additionally, myrosinase shows optimum stability around pH 7[48] (Figure 10).

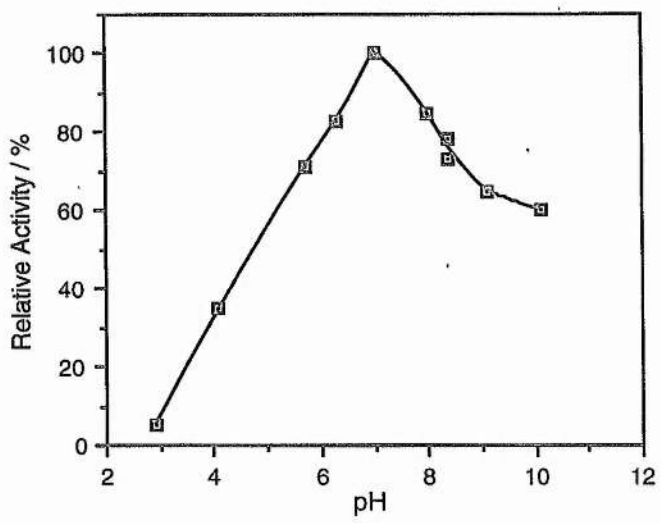


Figure 10: *pH-Stability Relationship for Wasabia japonica Myrosinase (stored at 5 °C for 41 h)* [48]

The temperature optimum for *Wasabia japonica* myrosinase is 37 °C.[48] The temperature-activity curve is shown in Figure 11.

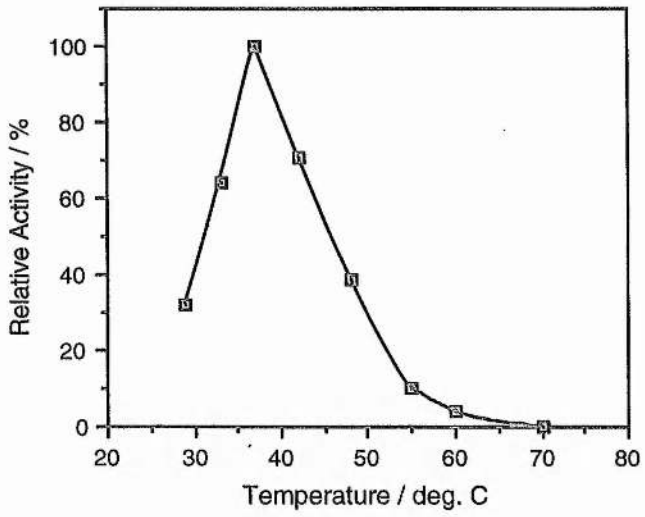


Figure 11: *Temperature-Activity Relationship for Wasabia japonica Myrosinase (pH 7, 20 min)* [48]

The optimum stability for *Wasabia japonica* myrosinase was found to be below 30 °C.[48] The temperature-stability curve is shown in Figure 12.

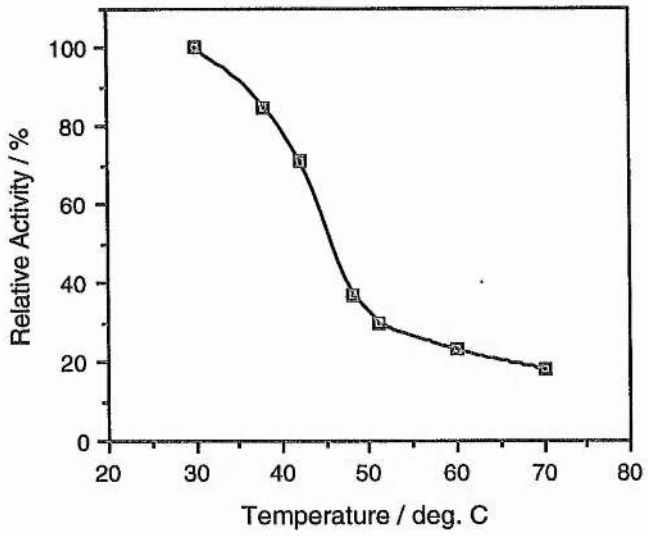


Figure 12: *Temperature-Stability Relationship for Wasabia japonica Myrosinase (pH 7, 20 min)* [48]

No metal ion requirement has been found for myrosinase, and this was confirmed by the failure of the metal chelators, such as EDTA (5 mM), diethyldithiocarbamate (5 mM), *o*-phenanthroline (1 mM) and 2,2'-dipyridyl (1 mM), to reduce enzyme activity.[51] However, the enzyme is severly inhibited by lead nitrate, mercury chloride and ferric chloride and moderately inhibited by copper sulfate and magnesium chloride (Table 2).[51]

Compound (all present at 1.0 mM concentration)	% Activity[a]
$ZnCl_2$	101
$CoSO_4$	99
NaCl	98
KCl	98
$CaCl_2$	97
$MnCl_2$	92
$MgCl_2$	69
$CuSO_4$	69
$FeCl_3$	26
$Pb(NO_3)_2$	1
$HgCl_2$	0

[a] Expressed as a percentage of enzyme activity in the absence of metal ion

Table 2: *Effect of Metal Ions on Lepidium sativum Myrosinase Activity* [51]

For most plant myrosinases, specific reagents which modify particular amino acids have implicated sulfhydryl groups as being essential for catalytic action, thus implying a cysteine residue is involved in catalysis. The *Brassica juncea* myrosinase activity was completely inhibited by 0.1 mM PCMB.[54,62]

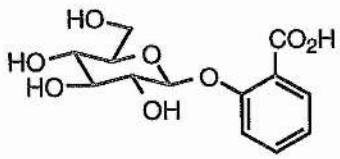
A data base search for amino acid sequence similarities between myrosinase and other sequenced proteins[63] did identify several proteins, all β-glycosidases or phospho-β-glycosidases. This is in accordance with the fact that myrosinases have weak β-glycosidase activity.[51,55] The highest degree of similarity was found with the two β-glycosidases from *Trifolium repens,* TRE104 and TRE361.[64] Interestingly, one of them, TRE104, is a cyanogenic β-glucosidase, an extracellular enzyme which is part of a defence system in which the enzyme hydrolyses cyanogenic glucosides (12) to liberate hydrogen cyanide (14) (Scheme 5). Myrosinases and the β-glycosidases from *T. repens* are in fact members of a large gene family of β-glycosidases, denoted the BGA family, which includes both β-glucosidases, β-galactosidases and phospho-β-glycosidases. An alignment of the deduced amino acid sequence of the

Brassica napus MYR1 and M10 as well as the partial *Sinapis alba* MA myrosinase clone, MA1, with that of the *T. repens* and *C. thermocellum* β-glucosidases showed that myrosinase is as similar to the other members of the family as the other BGA β-glycosidases are to each other. BGA enzymes have a 130 amino acid element that displays high similarity to a region in enzymes of a family of cellulases denoted A.[65]

Enzymatic cleavage of β-glycosidic bonds is believed to occur by general acid catalysis, *i.e.* protonation of the glycosyl oxygen by a Glu or a His residue in the active site of the enzyme. The resulting intermediate, containing a glycosyl carbonium ion, might be stabilised by an Asp or Glu residue. The 130 amino acid region includes a His followed by an Asp-Glu(-Pro) element, the Glu of which has been shown to be essential for catalysis in the *Bacillus polymyxa* and *B. subtilis* endo-β-1,4-glucanases.[66] Several Asp, Glu and His residues are conserved in all members of the BGA family, and among these are the His followed by the Asp-Glu element. It has therefore been suggested that the members of the BGA family use this Glu residue as a proton donor at the hydrolysis of their substrates, or that the proton would be donated by this particular His residue.

Comparison of the *T. repens* β-glucosidases with β-glucosidases of the BGA family showed that the *T. repens* glucosidases also share six out of seven conserved Asp, Glu and His residues, including the His/Asp-Glu motif. Myrosinases also have six out of seven conserved Asp, Glu and His residues, but not the Glu in the His/Asp-Glu motif. This indicates either that the reaction mechanism of myrosinase is partly different, or that another amino acid residue has taken over the role of this lacking Glu residue.

When the catalytic Glu170 residue of *Agrobacterium faecalis* β-glucosidase was replaced with Gly, the presence of an *o*-carboxyl group in the substrate caused a large increase in the rate of deglycosylation. Thus the *o*-carboxyl group of (24) (Figure 13) acted as the general acid-base catalyst, instead of the Glu residue, in a process known as substrate-assisted catalysis.[67]

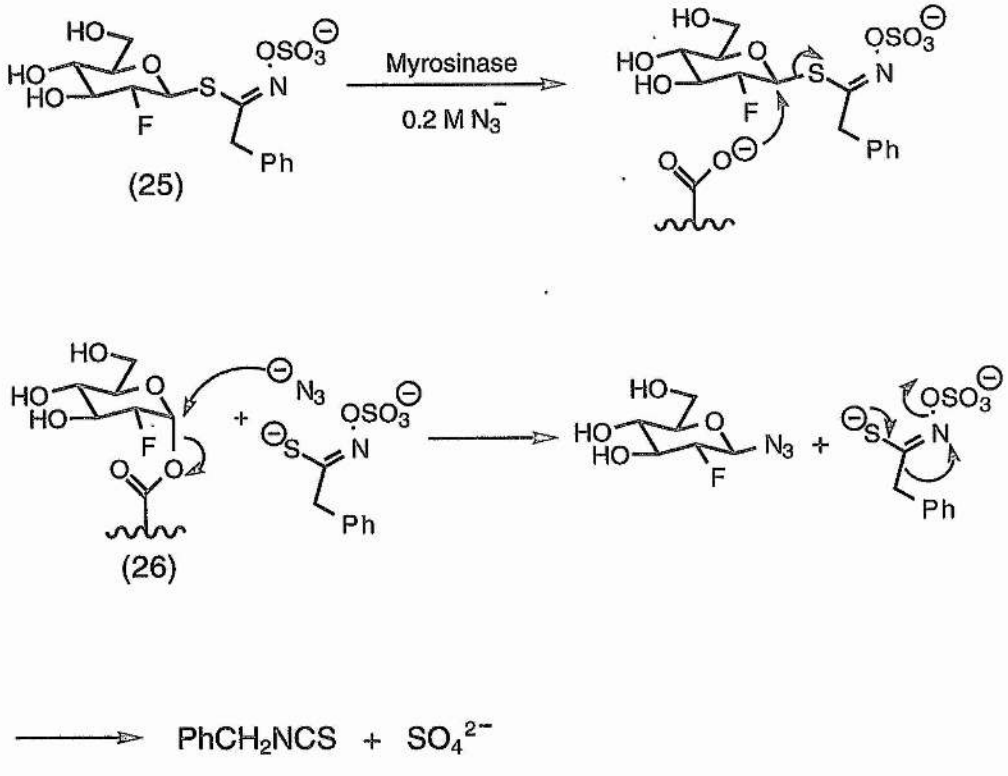


(24)

Figure 13: *o-Carboxyphenyl-β-D-Glucopyranoside: A Compound for Substrate-Assisted Catalysis in Agrobacterium faecalis β-Glucosidase*[67]

Interestingly, it is possible that substrate-assisted catalysis may have evolved naturally in the case of some glycosidases. Alignment of amino acid sequences for enzymes in β-glycosidase 1 showed[68] that in two members of this family, both of which are thioglucosidases,[69] the acid/base catalytic residue for the aglucone leaving group is replaced by a glutamine residue. This difference is likely to be an evolutionary consequence of the fact that the natural substrates for these enzymes, glucosinolates, contain an anionic sulfate group in their aglucone. Such substrates would not bind well to an enzyme containing a potentially anionic acid/base group, thus mutations to remove the charged residue from this position have occurred. It is possible that the sulfate group itself serves as the acid/base catalyst in these enzymes, in nature's own version of substrate-assisted catalysis.

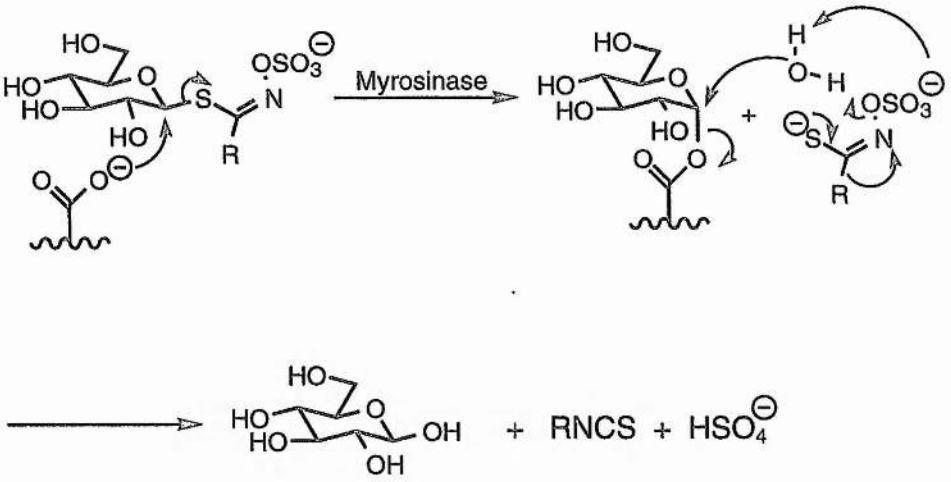
Work published during the period of this study has shown myrosinase (from *Sinapis alba*) to be inhibited by 2-deoxy-2-fluoro-glucotropaeolin (25).[70] This inhibition was a time-dependent inactivation of the enzyme as a consequence of accumulation of a long lived 2-deoxy-2-fluoro-glucopyranosyl-enzyme covalent intermediate (26), similar to those reported for two β-glucosidases[71,72,73,74] (Section 1.2.4). The K_I value for this inhibition was calculated to be 0.9 mM. Incubation in buffer only at 40 °C gave rise to reactivation of the enzyme at a slow rate, with a half-life of 46 hours. However, on the introduction of 0.2 M azide, the half-life of reactivation was reduced to 20 hours, implying that deprotonation is not required for attack of the glycosyl-enzyme intermediate. This inactivation and reactivation process is shown in Scheme 7.



Scheme 7: *Deactivation of Myrosinase Using 2-Deoxy-2-fluoro-glucotropaeolin and Reactivation Using Azide*[70]

The fact that deprotonation of the species which attacks the enzyme-glycosyl intermediate is not required fits well with the fact that the acid/base catalytic residue normally present in the *O*-glycosidases is replaced by a glutamine for myrosinase (described above),[68,69] thus making deprotonation of an attacking group not possible. Detailed analysis of residue conservation for myrosinase[70] also showed that a nucleophilic glutamate residue (Glu-426) is present, as in the *O*-glycosidases.

In relation to the residue studies and the azide reactivation observations for myrosinase, two mechanistic explanations have been proposed:[70] (i) the aglucone of glucosinolates is a sufficiently good leaving group that the nucleophilic attack of Glu-426 alone is sufficient to catalyse the hydrolysis reaction; or (ii) the sulfate group of the aglucone of glucosinolates acts as the acid/base catalyst to provide substrate-assisted catalysis (Scheme 8), as was suggested above.



Scheme 8: *Proposed Substrate-Assisted Catalysis Mechanism for the Myrosinase Catalysed Hydrolysis of Glucosinolates*[70]

Purification of myrosinase varies according to species origin, but a typical purification procedure is that carried out for *Lepidium sativum* myrosinase.[51] This involved Sephadex G-25 gel filtration followed by Red Dye and anion-exchange (FPLC Mono Q) chromatography then preparative isoelectric focusing. As judged by silver-stained SDS-PAGE gels, homogeneity was thereby achieved after an overall 13.7-fold purification of myrosinase with 36 % recovery of activity. Protein estimation was performed according to Bradford[75] using crystalline bovine serum albumin as standard.

An improved method of purification was developed which involved the use of concanavalin A-Sepharose affinity column chromatography.[76] Following extraction and dialysis, the dialysed-centrifuged extract was applied to a 1 x 10 cm column loaded with 1 ml of concanavalin A-Sepharose and the column thoroughly washed with buffer solution. Four different eluent solutions were tried, *i.e.* 0.25 M glucose, mannose, methyl-α-D-glucoside and methyl-α-D-mannoside. All produced high purification and yields, with the methyl-α-D-mannoside eluent being the most efficient, producing a 33-fold enzyme purification and a 91% yield.

The most common enzyme assay for myrosinase is a spectrophotometric one which measures the decomposition of the substrate (sinigrin) by following the decrease in its absorbance at 227 nm.[77] This is carried out using 1.0 ml of 0.1 mM sinigrin in 33.1 mM potassium phosphate buffer, pH

7.0 and 30 µl of concentrated enzyme solution in the same buffer. After stabilisation of the reaction solution at 37 ± 0.1 °C, the reaction is initiated by the addition of the enzyme solution. The rate of absorbance change is determined from the tangent to the beginning of the reaction curve. Initial velocities, in terms of disappearance of sinigrin concentration per minute, can then be calculated from these rates using $\varepsilon = 6784 \pm 25$ mol^{-1} dm^3 cm^{-1}, making this direct assay particularly convenient for steady-state kinetic studies of plant myrosinases.

Other assays include determination of released glucose[46,78] and titrimetric determination of formed bisulfate.[46] A typical glucose assay involves the use of a specific glucose reagent, Glox, containing glucose oxidase, peroxidase and O-dianisidine. The enzyme assay is carried out using a 1 ml reaction solution of 0.5 % sinigrin, 0.05 M citrate buffer, pH 5.5 and enzyme, which is incubated at 40 °C for 30 minutes. After boiling for 5 minutes the amount of free glucose is determined using the Glox reagent. The Glox reagent is inhibited by L-ascorbic acid and so a better assay is that of determination of formed bisulfate. This is done titrimetrically using a reaction solution of total volume 2 ml. The ionic strength is held constant with 0.2 M NaCl, and the titration is performed with 0.05 M NaOH. On the alkaline side the titration is carried out under nitrogen.

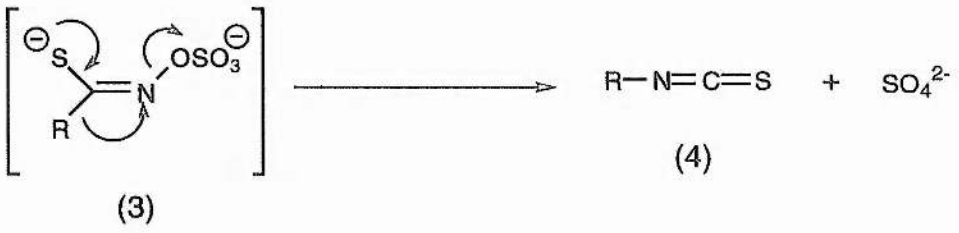
Myrosinase has also been studied in hydrated reverse micelles[79] and immobilised on a solid support.[80] When used in these systems, myrosinase can be (i) a bioreactor which produces isothiocyanates, nitriles, thiocyanates and thiones which in some cases cannot be easily synthesised by traditional organic synthesis methods, and (ii) a biosensor which is useful for rapidly determining the total glucosinolate content of aqueous cruciferous extracts. The pH profile of myrosinase activity in reverse micelles shows an increase in activity from pH 4 to 6. Up to pH 9, the activity remains constant, thus establishing a broad plateau as its maximum. An extraordinarily high optimum temperature of 60 °C is observed. The enzyme is activated by L-ascorbate, as in water.

Myrosinase was immobilised on common inexpensive solid matrices, *i.e.* trimethylchitosan, γ-alumina, γ-alumina activated by dodecadiamin, silanised sand, silanised silica and cellulose triacetate.[80] At pH 5.5 the enzyme bound to only some of the tested carriers. γ-Alumina and activated alumina appeared to be better than the organic supports, while silanised

alumina appeared to be better than the organic supports, while silanised silica and sand were completely ineffective. The properties of carrier-bound myrosinase were very similar to those typical of free myrosinase. The static and operational stabilities remained good.

1.1.6 Reactions of Glucosinolates

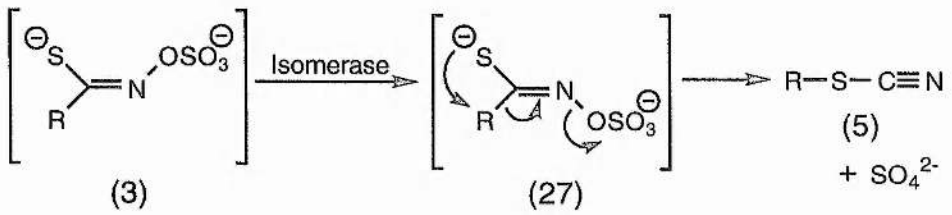
As mentioned before, the main final product of the myrosinase-catalysed hydrolysis of glucosinolates is an isothiocyanate. The formation of isothiocyanates from the unstable aglucone fragment is explained by the Lossen rearrangement, since the migrating R group and the leaving sulfate group are in the correct orientation, *i.e.* the aglucone has the *Z*-configuration. This is shown in Scheme 9.



(3) (4)

Scheme 9: *Lossen Rearrangement of the Z-Aglucone to form the Isothiocyanate*

The production of thiocyanates is less common, and has only been observed with allyl, 4-(methylthio)butyl and benzyl glucosinolates. For formation of benzyl thiocyanate in *Lepidium sativum*, it has been suggested[81] that a thiocyanate-producing factor is present. This factor is thought to be an isomerase, which causes *Z-E* isomerisation of the aglucone fragment. Lossen rearrangement to the isothiocyanate is then blocked by the sulfate, and the R group migrates instead to the sulfur atom (Scheme 10).

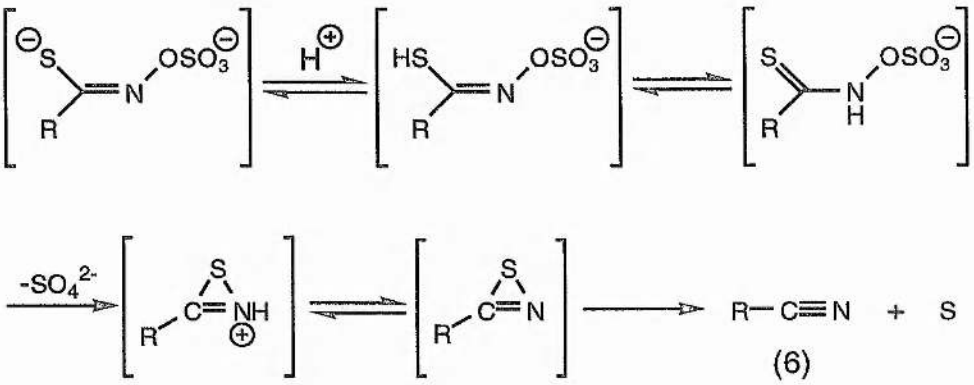


(3) (27) (5)

Scheme 10: *Rearrangement of the E-Aglucone to form the Thiocyanate*

The thiocyanate-producing factor of *Lepidium sativum* is inactivated by ascorbate,[82] is not pH dependent[83] and is quite distinct from the thioglucosidase activity.[81]

The nitrile is generally produced in greater quantities than the thiocyanate but it is still a minor product relative to the isothiocyanate. The enhanced production of nitrile is generally associated with lower pH and/or the presence of ferrous ions. Under acidic conditions (pH 3.5), formation of isothiocyanates is strongly inhibited,[84] and nitriles are formed preferentially. This can be attributed to the blockage of the Lossen rearrangement of the aglucone by the protonated sulfur.[85,86] This is represented in Scheme 11.

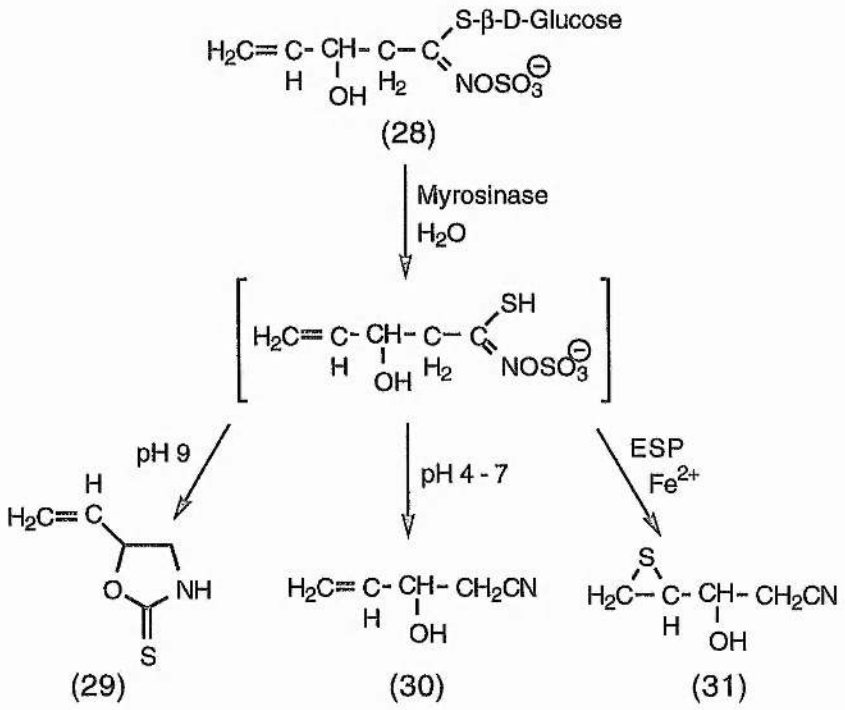


Scheme 11: *Formation of the Nitrile from the Breakdown of the Aglucone Under Acidic Conditions*

Studies of the effects of ferrous ion on the enzymatic degradation of sinigrin showed a decrease in allyl isothiocyanate formation in favour of the nitrile.[84] This effect did show dependence on pH, and 2.5 mM ferrous ion had maximum effect between pH 4.5 and 5.5. Its effect diminished at pH 6.5 and disappeared above pH 7.5.

Other minor products of the enzymatic degradation of glucosinolates include oxazolidine-2-thiones (29), hydroxynitriles (30) and epithionitriles (31). These products are formed from the hydrolysis of 2-hydroxy-3-butenyl glucosinolates (28), where the functional groups of the aglucone side chain react to form these alternative products (Scheme 12). Oxazolidine-2-thiones are predominantly formed at pH 9 and above, whilst at pH 4 to 7, the reaction yields predominantly hydroxynitrile.[87]

Epithionitrile formation is mediated by epithiospecifier protein (ESP).[88] This protein does not have myrosinase activity, but interacts with myrosinase to promote sulfur transfer from the S-glucose moiety to the terminal alkenyl moiety. ESP is unique in that it specifies the reaction products and not the substrate. Epithionitriles can be formed from the hydrolysis of any alkenyl glucosinolates with terminally unsaturated carbon. Ferrous ions are essential to epithionitrile formation, and ESP in *Brassica napus* was shown to be inactive in the absence of ferrous ions.[89]



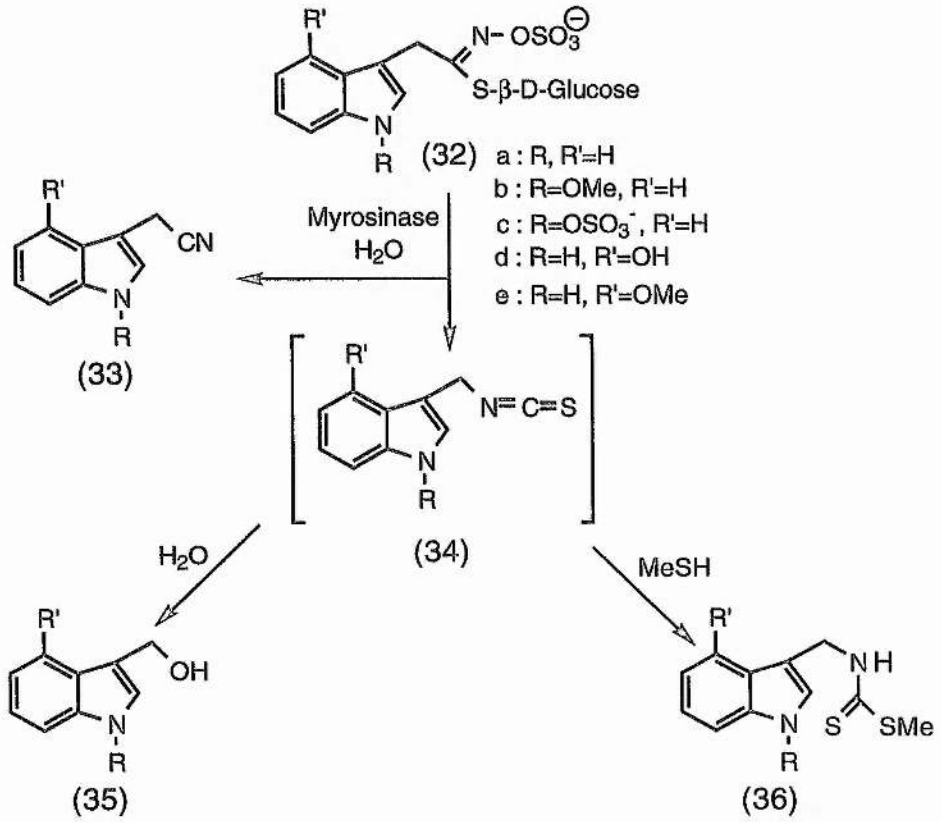
Scheme 12: *The Products of Myrosinase-Catalysed Hydrolysis of 2-Hydroxy-3-butenyl Glucosinolates*

The principal glucosinolates which give rise to the formation of oxazolidine-2-thiones, hydroxynitriles and epithionitriles are progoitrin/epiprogoitrin (side chain - 2-hydroxy-3-butenyl-) and gluconapoleiferin (side chain - 2-hydroxy-4-pentenyl-). As its name suggests, over-consumption of progoitrin leads to goitre, a disease of the thyroid gland. The feeding of the seed of cabbage and other *Brassicas* gave rise to anti-thyroid effects in experimental animals. This condition was little affected by increased iodine.[90] It is understood that the oxazolidine-2-thione produced acts by interfering with thyroxine synthesis thus causing hypothyroidism. Furthermore, thiocyanate ion (which is also

formed from the breakdown of progoitrin) acts as a pseudohalide and behaves as an iodine competitor.

Both hydroxynitriles[91] and epithionitriles[92] have been studied in feeding experiments and found to be toxic to the liver, kidneys and other organs of young rats. A number of natural isothiocyanates have been shown to be cytotoxic.[93]

Indole glucosinolates (32a-e), which occur throughout the family Cruciferae, give rise to a range of secondary products, whose nature depends on hydrolysis conditions. The isothiocyanates (34) which are formed as the primary products undergo further reaction to yield alcohols (35) or phytoalexins (36) (Scheme 13).[94] Phytoalexins are important antimicrobial compounds which are produced when plants are exposed to microorganisms. The nitriles (33) are also formed as primary products. The phytoalexins are understood to result from the addition of methanethiol - a known breakdown product in Cruciferous plants[95] - to the isothiocyanate.



Scheme 13: *Metabolism of Indole Glucosinolates as Catalysed by Myrosinase*

Glucosinolates have also been shown to degrade thermally, *i.e.* non-enzymatically, to yield two of the normal products of enzymatic degradation.[96] These are the nitrile and the isothiocyanate, which are formed from the thermal degradation on a gas chromatograph column of aqueous solutions of 3 glucosinolates. The products were identified by GC-MS. No thiocyanate was detected. For all 3 glucosinolates, the nitrile was the major product and the products formed at column temperatures of greater than 125 °C. The results of this study are shown in Table 3.

Column Temperature/°C	Allyl GSL[a] RCN[b]	RNCS[c]	Benzyl GSL RCN	RNCS	2-Phenethyl GSL RCN	RNCS
100	0	0	0	0	0	0
125	tr	0	tr	0	17±2	tr
150	14±1	0	30±1	0	22±1	10±1
175	32±1	17±1	51±4	0	31±4	14±1
200	44±2	32±2	63±6	13±4	36±1	18±2

[a] GSL = Glucosinolate

[b] RCN = Nitrile

[c] RNCS = Isothiocyanate

Table 3: *Molar Percentages of Products Formed on Injecting Aqueous Solutions Containing 0.2 ml of Allyl, Benzyl or 2-Phenethyl Glucosinolate into a G.C. at Different Column Temperatures*[96]

The most significant finding of this study is that the thermal degradation of glucosinolates yields products identical to those obtained from enzymatic degradation. Clearly, this need not have been the case since glucosinolates react chemically to yield a variety of other products.[97]

1.1.7 The Biological Role of Glucosinolates in Plants: 'The Myrosinase-Glucosinolate Bomb'

Once cellular disruption takes place and the myrosinase catalysed breakdown of glucosinolates occurs, the pungent volatile products which are released serve to act as one of the plant's defence mechanisms. The isothiocyanates and nitriles are toxic to insects and phytopathogens and therefore protect the plant against further insect attack. However, certain specialist pests and pathogens of crucifers, such as oilseed rape, have become adapted to glucosinolate containing plants and in fact use some of the isothiocyanates as host recognition cues.[98] Strategies to enhance the natural crop protection of oilseed rape and other important cruciferous crops require rather subtle changes to the glucosinolate content and the spectrum of components made and stored. Reducing the total glucosinolate content in vegetative tissues produces plants that are very susceptible to the generalist herbivores, yet simply increasing the total levels provides no extra defence against the specialist pests. Removing

the compounds recognised by the pests (and it has been shown that such insects only 'taste' a very few isothiocyanates, those derived from certain alkenyl glucosinolates), and perhaps introducing novel glucosinolates into crops, could have significant effects on pest and pathogen interactions with the crops. Such modified plants would have little requirement for agrochemical applications, and the production costs (of great significance for industrial and energy uses of rapeseed oil, for example) would be greatly reduced. It is thus very important that a fuller understanding of the biosynthesis of glucosinolates is achieved in order to breed improved varieties.

Attempts to determine the localisation of myrosinase in plants have been reported for more than 100 years and involve either morphological, anatomical and histochemical observations or cytochemical cell fractionation and biochemical studies. Using the former approach, several workers[99,101] have considered myrosinase to occur in myrosin cells, which stain specifically with Millon's reagent, orcein solution and concentrated hydrochloric acid, and lactophenol aniline blue. The combination of knowledge of myrosin cells, their distribution and specific antibodies finally enabled the localisation of myrosinases in myrosin cells.[101] Further immunogold-electron microscopy studies proved the subcellular localisation of myrosinase in the protein bodies/vacuoles of myrosin cells.[102]

The problem of the cellular localisation of glucosinolates still remains unsolved. Since glucosinolates are hydrolysed only after the plant is injured, due to *e.g.* cooking or attack by pests, at least the following three alternatives are possible for the location of substrate and enzyme respectively: (i) in different cells; (ii) in different compartments of the same cell; and (iii) inside the same compartment of the same cell, but in an inactive form.

Myrosinase, glucosinolates and ascorbic acid may reside in the same compartment, the myrosin grains/vacuoles of myrosin cells. High concentrations of ascorbic acid inhibit and low concentrations activate myrosinase activity.[46] Optimal ascorbic acid concentration for myrosinase from *Armoracia rusticana* has been reported to be 1.8 mM.[103] Together with the value for the total ascorbic acid concentration in *Armoracia rusticana* of 2.0 mM as reported by Grob and Matile,[104] this can be

considered to support the hypothesis suggested above. If all ascorbic acid is compartmentalised in vacuoles, disruption of the tonoplast membranes should give a maximum activation of the myrosinase and therefore a maximum response after wounding. Based on the results of Grob and Matile,[105] the ascorbic acid concentration in vacuoles of *Armoracia rusticana* must be considerably higher than 2.0 mM. At this concentration of ascorbic acid, the myrosinase enzyme system would be inactive. The co-localisation of myrosinase and glucosinolates would be most likely if this is a defence system. In this case, a co-localisation makes the system a 'toxic mine', which could be activated simply by disruption of the tonoplast membranes. After disruption, the ascorbic acid concentration will drop due to dilution and myrosinase will be activated.

A localisation of glucosinolates and myrosinase in different vacuoles in the same cell is not likely. The vacuoles of a myrosin cell do undergo considerable changes after sowing,[104] of which the fissions and fusions of the vacuoles in the cells are the most remarkable. To maintain the stability of such a system, a hitherto unknown mechanism for sorting and fusion of glucosinolates or myrosinase containing vacuoles is necessary. A more likely system would include compartmentation of glucosinolates in vacuoles of some cells and myrosinases in vacuoles of myrosin cells. The demolition of subcellular compartmentation by mechanical disruption or by micro or macro-organisms feeding on the plants would cause the necessary contact between enzyme and substrate.

Another possibility is that myrosinase is localised in myrosin cells and other components of the system in separate cells. To activate such a system, enzymes or substrate must be transported or the organisation of a tissue disrupted.

From the above mentioned, it can be concluded that the cellular organisation of the myrosinase-glucosinolate system is still unclear. Evidence shows that myrosinases, glucosinolates and ascorbic acid are localised in vacuoles, but with the exception of myrosinase which is localised in myrosin cells, the cellular localisation of the other components including myrosinase-associated proteins are unknown.

1.1.8 Biological Activity of Glucosinolates

In addition to the inhibitory effects upon pests, various other biological effects are exhibited by glucosinolates and their breakdown products. As mentioned above, some have anticarcinogenic properties.[8] Studies in rats have shown that the development of 7,12-dimethylbenz*(a)*anthracene-induced mammary tumours is greatly reduced on administration of benzyl isothiocyanate.[106] Benzyl thiocyanate also causes a reduction in mammary tumour development, but it is not as effective as benzyl isothiocyanate (Table 4).

		Mammary tumours[b]	
Material administered[a]	**No. of rats**	**No. of rats with tumours**	**% of rats with tumours**
Olive oil	14	14	100
Benzyl isothiocyanate	14	2	14
Benzyl thiocyanate	14	5	36

[a] Olive oil 1 ml or 0.33 mol of inhibitor in 1 ml olive oil were administered by oral intubation 4h before oral administration of 12 mg of 7,12-dimethylbenz*(a)*anthracene in 1 ml olive oil. Rats were 7 weeks old.

[b] Mammary tumours at 23 weeks.

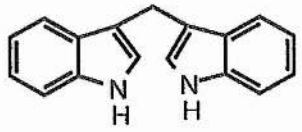
Table 4: *Effects of Benzyl Isothiocyanate and Benzyl Thiocyanate on 7,12-Dimethylbenz(a)anthracene-Induced Mammary Tumour Formation in Female Sprague-Dawley Rats*[106]

The possibility that the inhibitory effects are due to a metabolite, such as benzyl mercaptan or dibenzyl disulfide, cannot be discounted. It has also been reported that 2-phenylethyl- and phenyl isothiocyanates exhibit similar effects.[107,108]

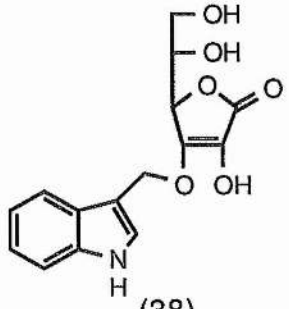
A possible protective effect of *Brassica* vegetables against colon cancer has also been reported,[109] and more recently a negative association between the incidence of rectum and colon cancer in Japan and the consumption of cabbage has been found.[110] Further studies are required to investigate which glucosinolates, and breakdown products, are

responsible for this. Also, an examination of the particular carcinogenic metabolites formed as a result of their action is required.

On enzymatic hydrolysis, indolyl glucosinolates (32) yielded non-volatile indole compounds. From glucobrassicin (32a), indole-3-acetonitrile (33) (R,R'=H), indole-3-carbinol (35) (R,R'=H) (both Scheme 13), diindolylmethane (37) and ascorbigen (38) (Figure 14) were all formed.



(37)



(38)

Figure 14: *Diindolylmethane (37) and Ascorbigen (38): Two Products of Indolyl Glucosinolate Hydrolysis Which React with Nitrite to Form Potentially Carcinogenic N-Nitroso Compounds*

The breakdown products (33) and (35) were shown to inhibit the neoplastic effects of carcinogens.[111] Besides the anticarcinogenic effects of indole-3-acetonitrile (33) (R,R'=H), it is also known to be a precursor of *N*-nitroso compounds. These *N*-nitroso compounds were isolated from nitrate-treated Chinese cabbage and exhibited direct mutagenic activity.[112] It was also shown that indole compounds were directly mutagenic to bacteria after nitrite treatment.[113,114] Approximately 75% of the nitrate ingested by humans is derived from vegatables,[115] and part of this load is converted to nitrite by bacteria in the oral cavity.[116] Since indole compounds reacted rapidly with nitrite, even at physiological feasible nitrite concentrations,[117] it is possible that endogenous nitrosation of indole compounds may occur. While it has been suggested that nitrosated indole-3-acetonitrile has tumour-initiating and tumour-promoting activity in rats,[118,119] the effects of such compounds in man are unknown.

Both intact and myrosinase-treated glucobrassicin and 4-hydroxyglucobrassicin formed significant amounts of *N*-nitroso compounds following treatment with nitrite. About 25% of both the intact and

myrosinase-treated glucobrassicin and 5% of both the intact and myrosinase-treated 4-hydroxyglucobrassicin were nitrosated.[120] It was thought that thiocyanate, a known product of myrosinase-catalysed hydrolysis of indole glucosinolates,[4] catalysed this reaction. Thiocyanate was previously reported to be a catalyst for nitrosation reactions.[121]

Isothiocyanates are also known to possess antifungal and antibacterial activities against a range of organisms.[122,123,124] For example, the behaviour of a number of such compounds has been reported[123] against *Staphylococcus aureus* and *Penicillium glaucum*.

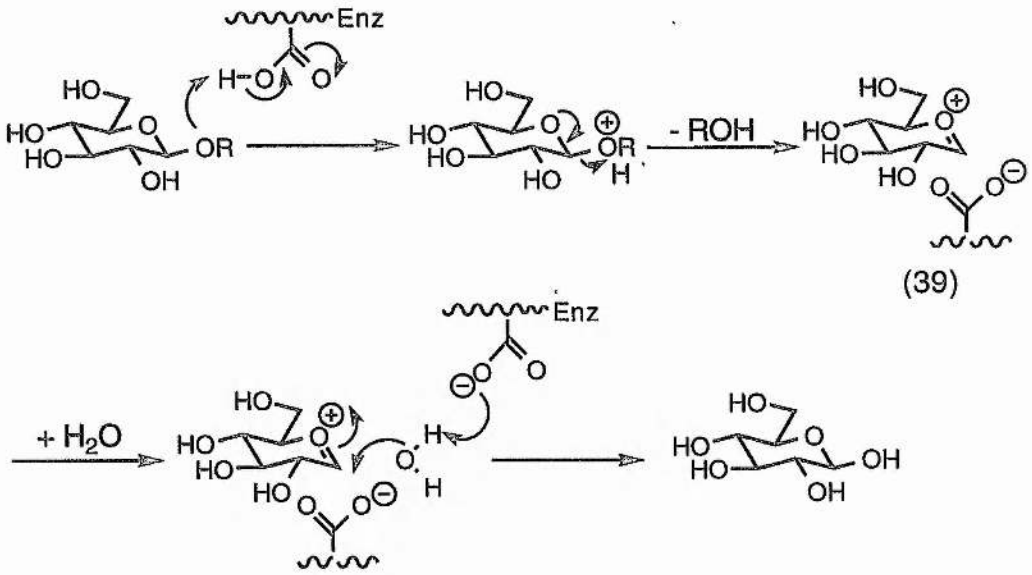
1.2 β-Glycosidases and Their Chemical Mechanisms

1.2.1 Introduction to β-Glycosidases and Their Chemical Mechanisms

In recent years, there has been a great increase in interest in β-glycosidase enzymes.[125] Inhibition of the enzymes which process carbohydrates has potential therapeutic application in the treatment of cancer, AIDS, other viral infections, diabetes and obesity. This high level of interest has resulted in the construction of a detailed picture of β-glycosidase action. This has been achieved by studies into pH/activity dependence; kinetic isotope effects; linear free energy relationships; nucleophilic competition; alternative substrates and inhibitors; affinity labels and suicide inactivators; and site-directed mutagenesis.

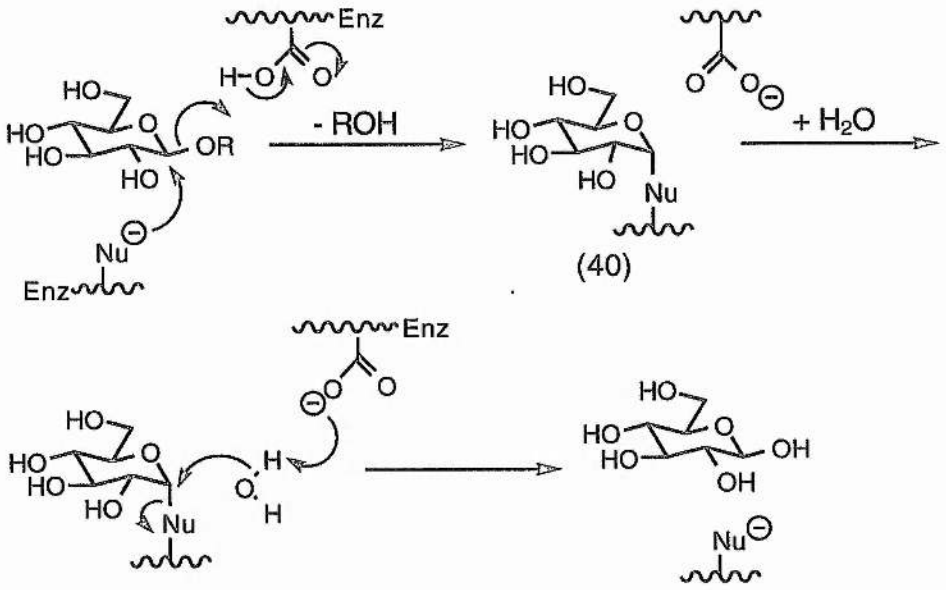
It is now understood that β-glycosidases which hydrolyse the glycoside linkage with retention of anomeric configuration utilise a double displacement mechanism. There are two possibilities for this mechanism, an S_N1-like or an S_N2-like pathway. Both involve an exocyclic cleavage in the glycosylation step, and the pyranose ring remains intact throughout the reaction.

The S_N1-like mechanism involves an ion-pair intermediate (39), in which the oxocarbonium cation is stabilised by an enzyme-bound carboxylate residue (Scheme 14). Since one side of the carbocation is shielded by the carboxylate, water attacks from only the upper face in the deglycosylation step, and the reaction thus proceeds with retention. For lysozyme, X-ray crystal data implicate Asp-52 as the stabilising residue, while Glu-35 is understood to act as the general acid for protonation of the glycosyl oxygen in the glycosylation step.[126] Kinetic isotope effect studies have been used to further establish this pathway.



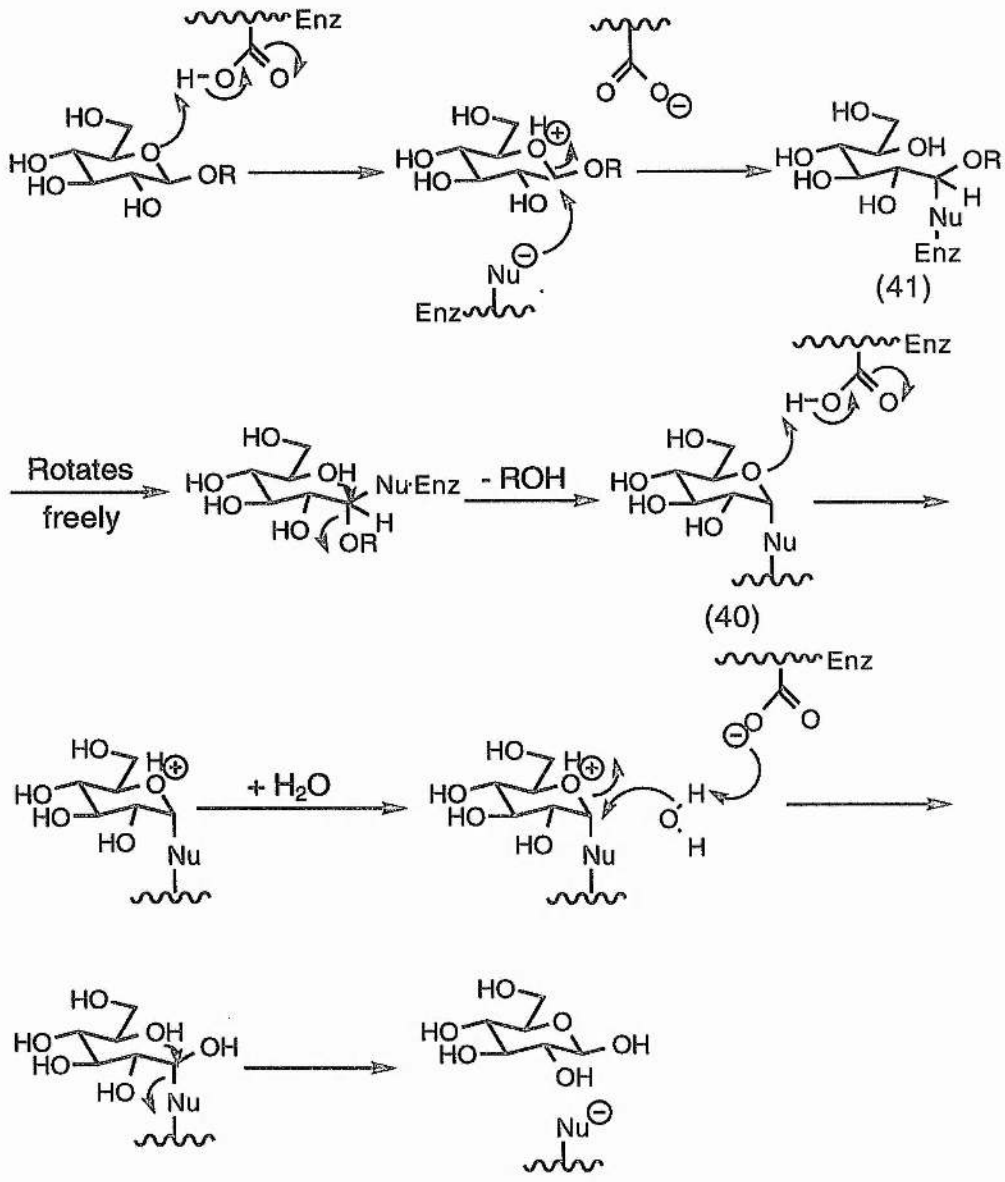
Scheme 14: *Proposed S_N1-Like Exocyclic Cleavage Mechanism for β-Glycosidases*

For the S_N2-like exocyclic cleavage mechanism, an enzymatic nucleophile is proposed to attack the anomeric carbon in the glycosylation step, resulting in a covalently bound intermediate (40) (Scheme 15). β-Glucosidase (from both *Aspergillus wentii* and sweet almonds) is thought to react *via* this mechanism, with an aspartate residue acting as the nucleophile.[127] Evidence for this mechanism was provided by trapping experiments and isotope effect studies.



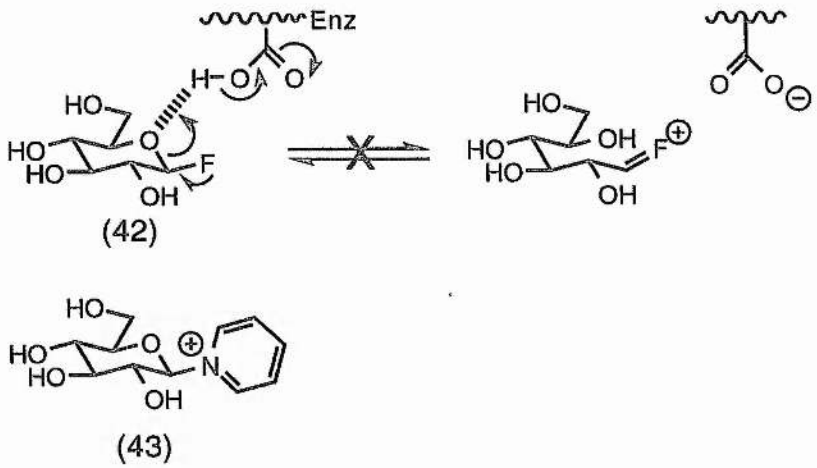
Scheme 15: *Proposed S_N2-Like Exocyclic Cleavage Mechanism for β-Glycosidases*

An alternative, although less well accepted mechanism for β-glycosidases is that of endocyclic cleavage. This mechanism involves the ring opening of the pyranose ring following the protonation of the ring oxygen (Scheme 16) to give an acyclic intermediate (41).[128] This then rearranges to give the same intermediate (40) as in the exocyclic cleavage pathway. Following this, attack by water and a further endocyclic cleavage leads to the product. Molecular dynamics calculations of lysozyme action were in agreement with this postulate.[129] Recently, literature data have been reassessed by Franck to provide further evidence for this little accepted mechanism.[130]



Scheme 16: *Proposed Endocyclic Cleavage Mechanism for β-Glycosidases*

However, there are strong arguments against this endocyclic cleavage mechanism. For example, the enzymatic hydrolysis of glucopyranosyl fluorides (42) or glucosylpyridinium (43) derivatives are not compatible with this mechanism,[131] since the back-donation of electrons to aid ring opening is not possible (Scheme 17).



Scheme 17: *The Hydrolysis of Glucopyranosyl Fluoride (42) and Glucosyl Pyridinium (43) Derivatives Does Not Follow the Endocyclic Cleavage Mechanism*[131]

1.2.2 Experimental Approaches for the Investigation of β-Glycosidase Mechanisms

As mentioned above, kinetic studies and isotope effect measurements have been used to provide evidence for these mechanisms. The mechanism shown in Scheme 15 has been proposed for β-glucosidase from *Agrobacterium faecalis*.[132]

For the pH dependence studies for this enzyme, kinetic parameters, k_{cat} and K_M, at a series of pH values between 5.1 and 9.0 were determined for two different substrates, *p*-nitrophenyl-β-D-glucopyranoside (PNPG) and phenyl-β-D-glucopyranoside (PG). Values of k_{cat} only were determined for a third substrate, 2,4-dinitrophenyl-β-D-glucopyranoside (2,4-DNPG), by measuring rates at a constant, high concentration of substrate (12 times its K_M value). Determination of K_M values at the two extremes of pH were carried out to ensure that the measured rate was not reduced beneath k_{cat} as a consequence of large increases in K_M values. The results obtained are plotted in logarithmic form in Figure 15.

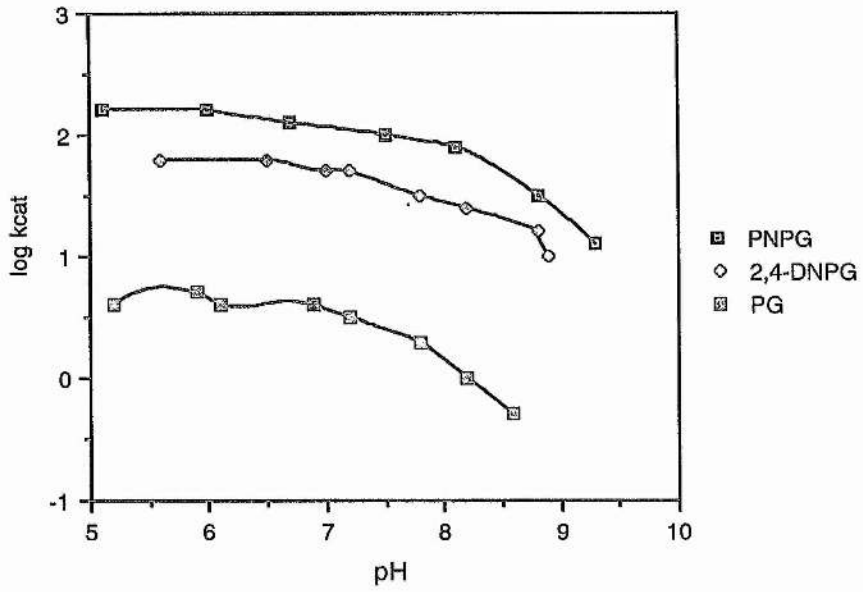


Figure 15: *Plot of log k_{cat} vs. pH for the pH Dependence of the Hydrolysis of PNPG, PG and 2,4-DNPG by Agrobacterium faecalis β-Glucosidase. (Lines Drawn Through Points are Simply Intended to Illustrate the Shapes of the Plots.)*

Values of k_{cat} for each substrate depend upon two ionisations as follows: 2,4-DNPG (pK_1 = 3.6, pK_2 = 8.1); PNPG (pK_1 = 3.9, pK_2 = 8.0); PG (pK_1 = 4.8, pK_2 = 7.6). The values of pK_1 are quite unreliable, but pK_2 values should be reasonably accurate.

Examination of the plots in Figure 15 reveals that essentially the same pH dependence is seen for each of a series of three different substrates, one of which has the glycosylation step rate limiting and two of which appear to have deglycosylation as their rate-limiting step. All reactions depend upon the apparent presence of a protonated group of pK_a 7.6 - 8.1 whose deprotonation results in loss of activity. It also appears that the deprotonation of a group of pK_a < 5 results in loss of activity, but unfortunately the relatively rapid irreversible inactivation of the enzyme at pH values less than this precludes accurate determination of this pK_a value.

Detailed interpretation of pH dependence data is a very risky pastime,[133,134] and in this case for *Agrobacterium faecalis* β-glucosidase, as also previously for sweet almond β-glucosidase,[134] is somewhat confusing and unsatisfying. Nonetheless, pH studies provide a useful way of characterising the enzyme and can serve a very valuable function in understanding the consequences of mutations (see Section 1.2.2) and in characterising mechanism-based inactivators.

Using a number of aryl-β-D-glucopyranosides, a biphasic Brønsted relationship was observed for *Agrobacterium faecalis* β-glucosidase,[132] implying a two-step mechanism with an intermediate. For substrates with relatively good leaving groups ($pK_a < 8$), deglycosylation was rate limiting, as the rate was in fact independent of leaving group ability. With poorer leaving groups ($pK_a > 8$), the rate was dependent on leaving group ability, implying that the glycosylation step was now rate limiting. The plot for this is shown in Figure 16.

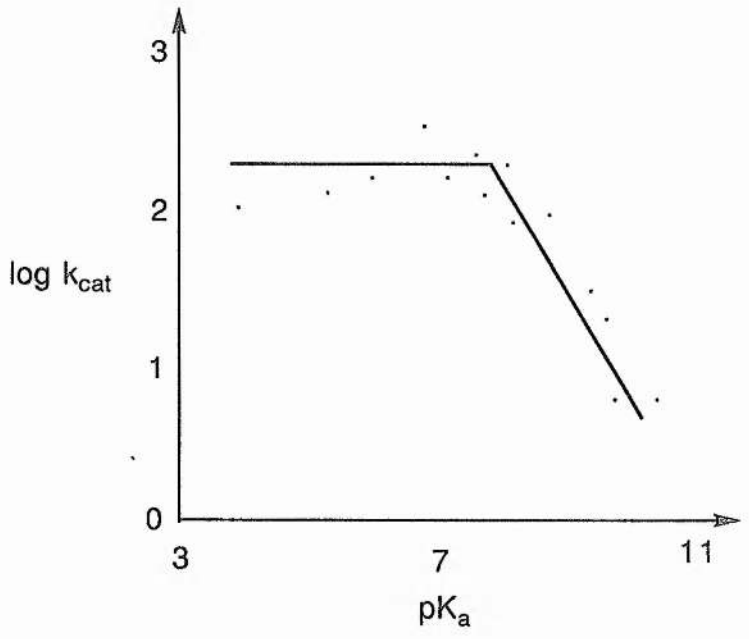


Figure 16: *Brønsted Plot of the Hydrolysis of Substituted Aryl-β-D-Glucopyronosides by Agrobacterium faecalis β-Glucosidase.*[132] *pK_a is the pK_a of the Aglucone Phenol.*

Although termed a Brønsted plot in this instance, a more correct name for the plot may be a Hammet plot. To avoid confusion, a more suitable term for the plot would simply be a linear free energy relationship.

A similar plot was also obtained for sweet almond β-glucosidase.[135]

For further investigation of β-glucosidase mechanisms, a secondary isotope effect can be measured, *i.e.* the α-deuterium kinetic isotope effect. This isotope effect is used to establish the changes in hybridisation at the anomeric centre as the reaction proceeds from the ground state to the rate limiting transition state. A secondary α-deuterium kinetic isotope effect originates from isotopic substitution at a bond that is not broken during the reaction and arises from changes in the zero-point energy of ordinary vibrations.

In distinguishing between S_N1 and S_N2-like mechanisms, the fact that the out-of-plane C-H bending frequency decreases in going from an sp^3 hybridised ground state carbon to an sp^2 hybridised transition state carbon (*i.e.* the C-H bond is made stonger) is extremely useful. Thus, when a deuterium is substituted for a hydrogen, the heavy atom will have a more pronounced effect on the energy of the transition state if it is sp^2 hybridised. Thus, for the S_N1-like mechanism in Scheme 13, a high secondary isotope value of up to 1.41[136] would be observed, whereas for the S_N2-like mechanism in Scheme 15, an almost negligible value of 1.00 to 1.06 is observed.

For studies on the *Agrobacterium faecalis* enzyme, a secondary deuterium isotope effect of 1.11 was observed for deglycosylation, and a value of 1.06 was obtained for the glycosylation step.[132] Thus the glycosylation step has a relatively small amount of oxocarbonium ion character at the transition state, although a large degree of bond cleavage was indicated by the Brønsted plot. The reaction must therefore have a late transition state with a considerable amount of preassociation of the enzymic nucleophile. Hence, the glycosylation step has substantial S_N2 character, while, in keeping with the larger secondary isotope effect, the deglycosylation step is much more S_N1-like. A distinction between stepwise and concerted enzyme-catalysed reactions can be made by measuring the effect of deuteration at one position in the substrate on the magnitude of a heavy atom isotope effect at another position.[137] If the isotope effects are indeed operating on the same step and the mechanism is concerted, deuteration will slow down the step and it will therefore

become more rate limiting. This can be done by combination of α-deuterium effects with primary ^{13}C isotope effects.

Primary kinetic isotope effects can also be used for investigation of β-glycosidase mechanisms, the ^{18}O kinetic isotope effect being the best known one. For reaction of 2,4-dinitrophenyl-β-D-galactopyranoside with β-galactosidase, ^{18}V was found to be only 1.002, because the second, deglycosylation, step was rate limiting.[138] However, $^{18}(V/K)$ was 1.030, showing that C-O bond cleavage was partly rate limiting for V/K.

Solvent kinetic isotope effects (SKIE) are also very useful since for a substrate substituted with a heavy atom, only one reaction step is sensitive to the change, whereas with a labelled solvent, the isotope effects are much more global in nature. When an enzyme-catalysed reaction is carried out in D_2O rather than H_2O, all the acidic hydrogens in both the enzyme and the substrate will be exchanged for deuterium. Since acid/base chemistry is very important in enzyme-catalysed reactions, these experiments can provide a lot of useful mechanistic information. Solvent kinetic isotope effects have thus been used extensively in the investigation of chemical mechanisms of β-glycosidases. A primary SKIE is observed if a hydrogen is transferred during the reaction. A secondary effect is observed if the hydrogen undergoes a change in its fractionation factor in the transition state relative to the reactants, but is not actually transferred.

For *Escherichia coli* (*lac*Z) β-galactosidase a negligable SKIE was observed,[139] implying that glycone-aglycone fission occurs without general acid catalysis, *i.e.* there is no proton transfer to the leaving group in, or before, the first irreversible step (even with poor substrates). Also, water is not involved in, or before, the first irreversible step (even with very good substrates). Similar results were found for sweet almond β-glucosidase.[135]

Partitioning experiments can be used provide evidence for a distinct intermediate which is normally attacked by water. Introduction of a competing attacking species which is more nucleophilic than water, *e.g.* methanol, leads to an increased rate of deglycosylation and possibly an increase in overall rate. An observed increase in rate, together with a decrease in the amount of free glucose formed indicates the presence of a

distinct intermediate. These studies provide further evidence on top of the findings of kinetic isotope effect studies.

1.2.3 Enzyme Active Site Mutation Studies

Further mechanistic evidence for *A. faecalis* β-glucosidase has been obtained by mutation experiments on the active site nucleophile glutamate 358 (Glu 358).[140] Glu 358 was identified previously as the catalytic nucleophile in *A. faecalis* β-glucosidase,[64] and in these studies was replaced with the amino acids asparagine (Asn), glutamine (Gln) and aspartate (Asp). Activities of the wild type and mutant enzymes against 2,4-DNPG were measured and are shown in Table 5.

Enzyme	k_{cat} / s^{-1}	K_M / mM	k_{cat}/K_M / $s^{-1}mM^{-1}$
Wild Type	88	0.031	2839
Glu358Asp	0.72	0.054	13.3
Glu358Asn[a]	9.5×10^{-5}	0.08	0.0012
Glu358Gln[a]	2.4×10^{-4}	0.07	0.0034

[a] At least part of these activities appears to be due to contaminating native enzyme or Asp mutant

Table 5: *Apparent Activities of Wild Type and Mutant Enzyme of Agrobacterium faecalis β-Glucosidase Against 2,4-DNPG*

The extremely low activities of the Asn and Gln mutants (approximately 10^6 times lower than the native enzyme) raise serious concerns as to the source of the activity. Experiments to probe this using 2-deoxy-2-fluoroglucopyranosides as an inactivator proved that for both mutants, the very low residual activity is almost certainly due to contaminating native enzyme or Asp mutant, probably rising from either translational misreading or spontaneous deamidation.

Studies with ground state (G.S.) and transition state (T.S.) inhibitors for the native and Asp mutant enzymes showed a dramatic reduction in binding ability of the T.S. inhibitors to the Asp mutant relative to the native enzyme. This effect is much less pronounced for the G.S. inhibitors. These observations are consistent with the minimal role of the Glu or Asp

residues in ground state binding and the fact that the transition state has been destabilised by the mutation.

Further studies on pH dependence, thermal stability and secondary deuterium kinetic isotope effects for both the native and Asp mutant enzymes provided additional information on the mechanistic consequences of Glu 358 mutation.

Overall, the Asp mutant enzyme has sufficient flexibility in its active site for the carboxylate to still function in stabilising the positive charge and to form the covalent intermediate, as for the native enzyme, although the glycosylation step is 2500 times slower for the Asp mutant enzyme due to the relatively poor placement of the catalytic groups.

1.2.4 Inhibition Studies

Inhibition studies for *Agrobacterium faecalis* β-glucosidase have provided further evidence for the proposed mechanism. One of the strongest inhibitors was found to be D-glucono-γ-lactone[132] (44) (Figure 17), a classical transition state analogue which has been shown to inhibit essentially all β-glucosidases.[141] According to X-ray analysis,[142,143] D-glucono-γ-lactone has been shown to adopt a distorted half-chair conformation in the solid state, thus mimicing the transition states for glycosylation and deglycosylation in enzymatic cleavage of β-D-glucopyranosides. D-Glucono-γ-lactone binds to *Agrobacterium faecalis* β-glucosidase with an affinity considerably greater than that shown for the free sugar, the K_I value being close to 1 μM, which means D-glucono-γ-lactone binds around 5000 times tighter than glucose.[144]

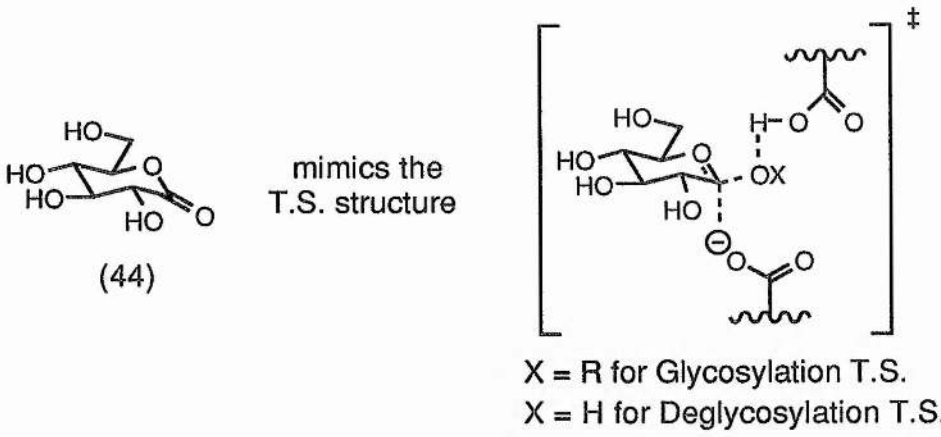
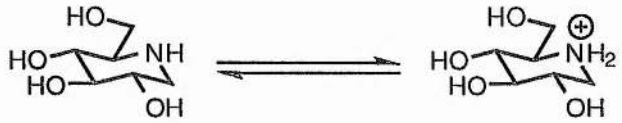


Figure 17: *D-Glucono-γ-lactone (44): A Classical Transition State Mimic Inhibitor for β-Glucosidases*

Relatively modest affinity (K_I = 0.05 mM) was seen for 1-deoxynojirimycin[132] (45) (Figure 18), a compound that is an extremely potent inhibitor of some glucosidases. This presumably reflects slightly different placements of the positively charged nitrogen on the inhibitor and the negative charge with which it interacts in the different enzyme systems.

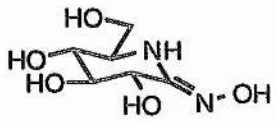


(45)

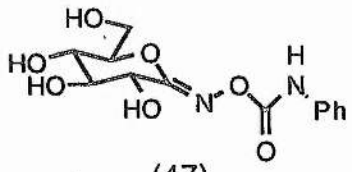
Figure 18: *1-Deoxynojirimycin: A Potent Inhibitor for some β-Glucosidases*

D-Glucono-γ-lactone and 1-deoxynojirimycin were shown to be potent competitive inhibitors of almond emulsin β-glucosidase, with K_I values of 0.3 μM and 0.015 μM respectively.[145]

Other inhibitors studied for *Agrobacterium faecalis* β-glucosidase included gluconohydroximolactone (46) and gluconophenylurethane (47) (Figure 19).[132] It is known that these two compounds adopt similar conformations in aqueous solution to D-glucono-γ-lactone, and therefore are expected to inhibit β-glucosidase enzymes.



(46)

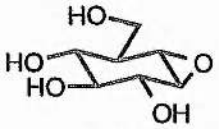


(47)

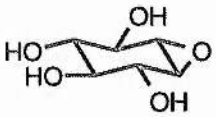
Figure 19: *Two Competitive Inhibitors for Agrobacterium faecalis β-Glucosidase*

These compounds were found to be competitive inhibitors with K_I values of 30 μM and 1.2 μM respectively. The fact that gluconophenylurethane (47) is a more potent inhibitor than D-glucono-γ-lactone (44) may be due to the fact that the urethane is more lipophilic and may therefore show more affinity for the enzyme.

The pH dependence of the inactivation of *Agrobacterium faecalis* β-glucosidase by the mechanism-based inactivator cyclophellitol (48) (Figure 20) was investigated.[132]



(48)



(49)

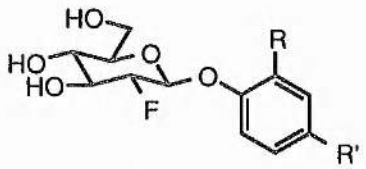
Figure 20: *Cyclophellitol (48) and Conduritol B Epoxide (49): Two Mechanism-Based Inactivators for β-Glucosidase*

The inactivation rate constant, k_I, and the equilibrium binding constant, K_I, were measured at a range of pH values. The pH dependence of the inactivation process was found to be essentially the same as that for hydrolysis of substrates. It is thus thought that the inactivation process occurs by binding of cyclophellitol (48) at the enzyme active site, followed by protonation in the usual fashion, generating a reactive protonated epoxide which is rapidly attacked by a nearby nucleophile, forming a stable ester derivative at the active site and thereby inactivating the enzyme.

A similar deactivation of β-glucosidase from *Aspergillus wentii* was observed with conduritol B epoxide (49) (Figure 20).[146]

Destabilisation of the proposed transition states shown in Figure 17 for *Agrobacterium faecalis* β-glucosidase has been shown to occur by substitution of the hydroxyl group at C-2 with an electronegative fluorine atom.[72,73] This destabilisation occurs because (i) the hydroxyl substituent at C-2, which is replaced by fluorine, plays a crucial role in transition state stabilisation by making key hydrogen bonding interactions with the enzyme active site[147] and (ii) the positive charge is destabilised by the inductive effect of the electronegative fluorine atom. The rates of both glycosylation and deglycosylation are thus decreased. The incorporation of a highly reactive leaving group as the aglucone into such deactivated substrates might increase the glycosylation rate sufficiently to permit trapping of the 2-deoxyfluoroglucosyl-enzyme intermediate, thereby inhibiting the enzyme in a temporary covalent fashion.

The design of these inactivators is based upon a perceived need for a very good leaving group on the 2-deoxy-2-fluoro-β-D-glucopyranoside in order to ensure that formation of the glycosyl-enzyme intermediate is faster than its decomposition. The need for this has been proved by study of the rates of inactivation with different aryl-2-deoxy-2-fluoro-β-D-glucopyranosides. 2,4-Dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside (50) (Figure 21) was found to be the most potent inactivator, whilst *p*-nitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside (51), with its much poorer leaving group, was the weakest, being four orders of magnitude slower.



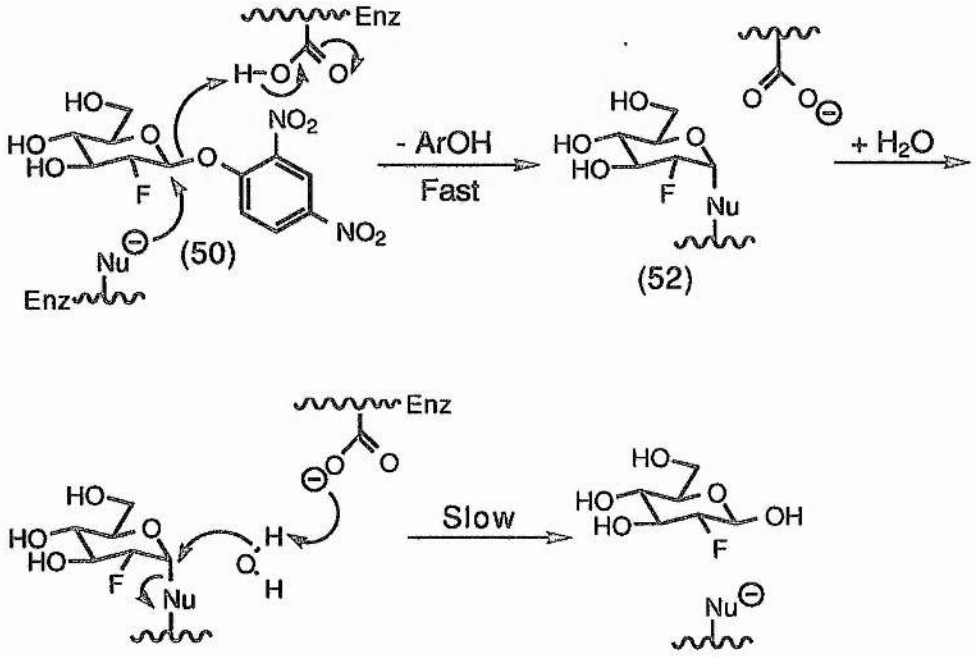
(50) R = NO_2, R' = NO_2

(51) R = H, R' = NO_2

Figure 21: *Aryl-2-deoxy-2-fluoro-β-D-glucopyranosides: Mechanism-Based Inactivators of Agrobacterium β-Glucosidase*

2,4-Dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside (50) was used chiefly for investigative studies since the good leaving group results in ready accumulation of the destabilised glycosyl-enzyme intermediate (52) (Scheme 18). This enabled identification of the active site nucleophile amino acid residue for both *Agrobacterium faecalis*[71] and *Aspergillus*

wentii[74] β-glucosidases. The amino acids were identified as glutamate and aspartate respectively.

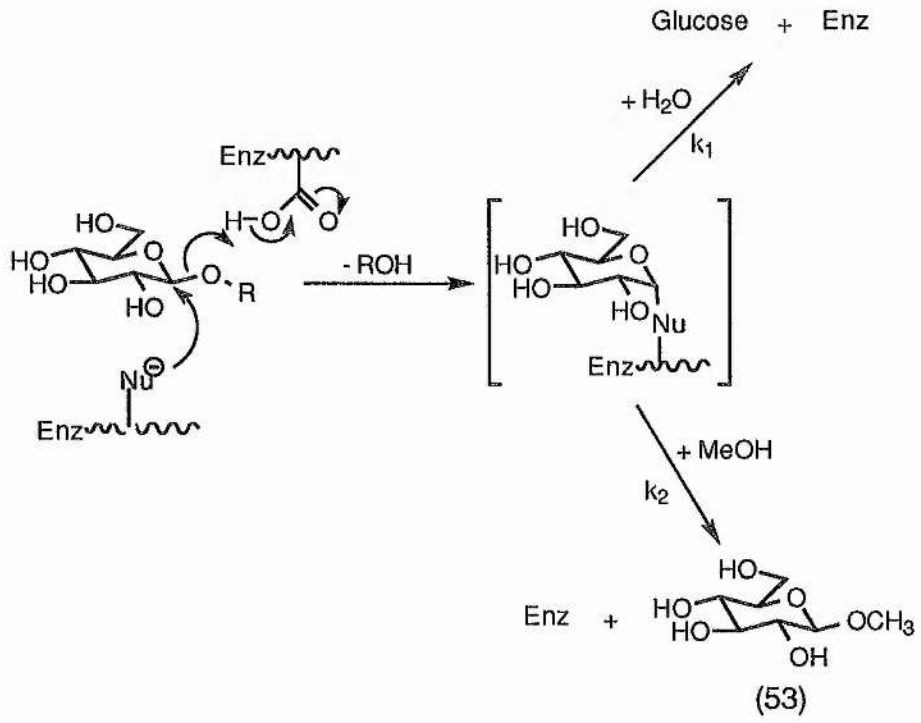


Scheme 18: *Inactivation Studies with β-Glucosidase Using 2,4-Dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside*[71,72,73]

Other mechanistic information from this type of deactivation can be obtained from ^{19}F N.M.R. studies of the inactivated enzyme. For *A. faecalis* β-glucosidase, this confirmed the formation of the covalent α-D-glucopyranosyl-enzyme intermediate (52).[73]

1.3 Use of β-Glycosidases in Organic Synthesis

It is well documented that many glycosidase enzymes are able to catalyse transglycosylation reactions, namely the transfer of residues from glycoside substrates to acceptor molecules other than water.[135,148] Synthetic organic chemists have frequently exploited this property and employed β-glycosidases in the synthesis of novel glycosides.[149] Transglycosylation occurs for almost all β-glycosidases where a more nucleophilic acceptor, *e.g.* methanol, traps out the glycosyl-enzyme intermediate faster than water (since $k_2 > k_1$) and methyl-β-D-glucopyranoside (53) is formed. For sweet almond β-glucosidase, this is represented in Scheme 19.



(53)

Scheme 19: *Pathway for Transglycosylation Reaction Catalysed by Sweet Almond β-Glucosidase*

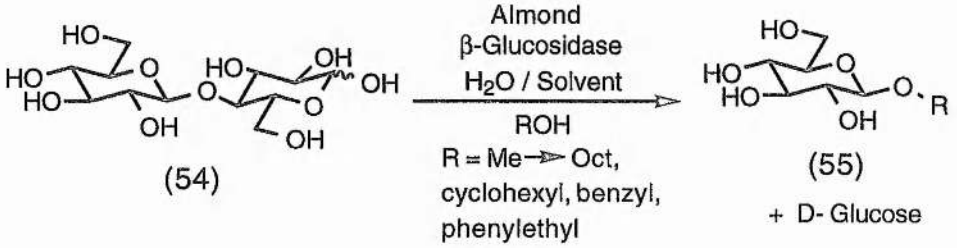
While under suitable conditions glycoside synthesis may be favoured kinetically, hydrolysis is favoured thermodynamically. The reactions are therefore carefully monitored and stopped when the glycosyl donor is consumed in order to minimise glycoside hydrolysis. Yields for these types of syntheses generally range from 20 to 40 %. These enzymatic syntheses

prove very useful since the corresponding reactions using classical synthetic methods are often very difficult.

The practical (*i.e.* multi-gram) preparation of carbohydrates by classical methods proved inadequate due to (i) the difficulty in the development of good general methods for construction of a single anomeric glycosyl linkage in high yields; (ii) the problem of regio- and stereoselectivity due to the presence of multiple functional groups (*e.g.* hydroxyl groups); (iii) complexity and diversity of linkages and (iv) the problem of solubility in organic solvents and the need for extensive use of aqueous media.

Enzymatic methods overcame many of these problems due to the selective nature of the reactions catalysed by the enzymes and the structure of their active sites. Also, mild methods without the requirement for activation, protection and deprotection reactions were possible. The number of chemical steps was often greatly reduced when using enzymatic methods.

Almond β-glucosidase (E.C. 3.2.1.21) has been utilised[150] to synthesise a wide range of glucosides (55) by the use of transglycosylation. The reaction was carried out by adding almond β-glucosidase (20 units ml^{-1}) to a solution of cellobiose (54) (0.05 M) with the appropriate alcohol (0.80 M) and stirred at 40 °C for 72 hours. The reaction medium was formed by mixing water and organic co-solvent (acetonitrile or *tert*-butanol). The reaction is represented in Scheme 20.

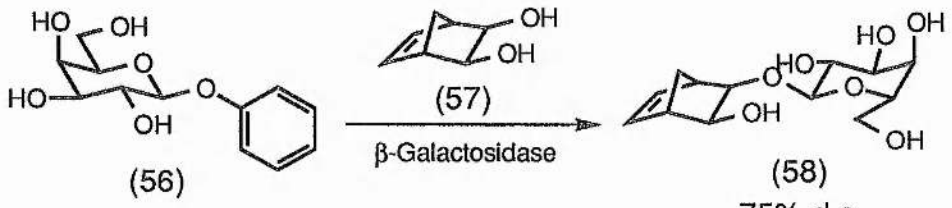


Scheme 20: *Use of Almond β-Glucosidase to Catalyse Trans-glycosylation in the Synthesis of Novel Glucosides*

This enzyme was particularly useful as it was very stable in these organic media, with transglycosylation showing a maximum rate at 80% (v/v) organic solvent.

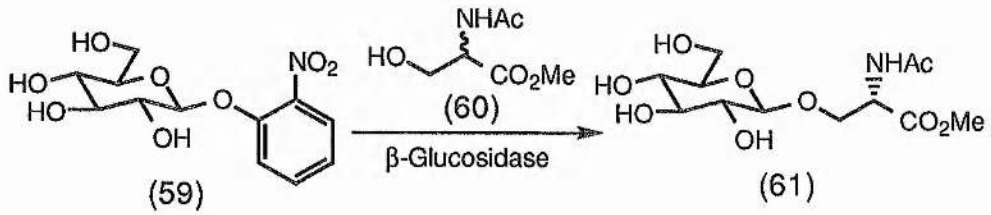
The glycosyl transfers to non-sugar acceptors were especially interesting with racemic chiral or *meso* alcoholic acceptors as some degree of diastereoselectivity might have been expected due to the asymmetric environment of the enzyme active site. Such stereoselectivity was indeed observed with diastereoselectivities ranging from moderate to exceptional.[151,152]

For a stereoselective reaction using β-galactosidase, where a chiral 1,2-diol (57) was used as the acceptor, a diastereomeric excess of 75% was observed (Scheme 21).



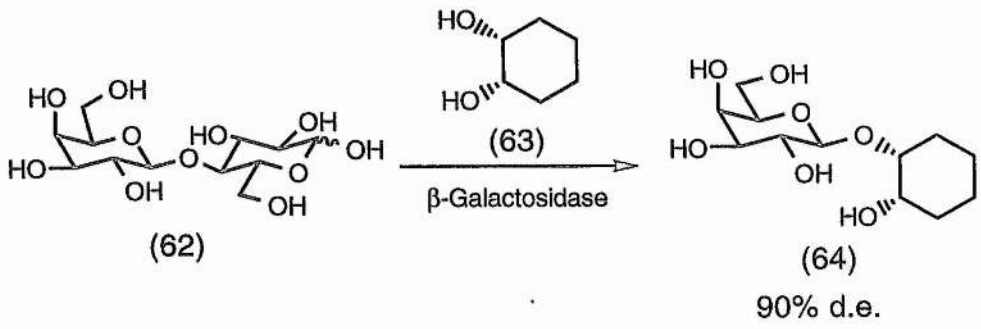
Scheme 21: *Stereoselective Synthesis of a Novel β-D-Galacto-pyranoside by the use of Transglycosylation*

For a β-glucosidase-catalysed reaction, diastereoselectivity in the glycosyl transfer step was observed when the racemic alcohol (60) (N-acetyl DL-serine methyl ester) was used as the acceptor.[153] The glucoside formed was shown to have a diastereomeric excess of 80% (Scheme 22).



Scheme 22: *A Highly Diastereoselective Transglycosylation Reaction Catalysed by β-Glucosidase*

A higher diastereomeric excess of 90% was observed for a reaction catalysed by β-galactosidase when the *cis* 1,2-diol (63) was used as the achiral acceptor (Scheme 23).



Scheme 23: *A Highly Diastereoselective Transglycosylation Reaction Catalysed by β-Galactosidase*

Chapter 2

RESULTS AND DISCUSSION

2.1 Investigation of the Transglycosylation Activity of Myrosinase

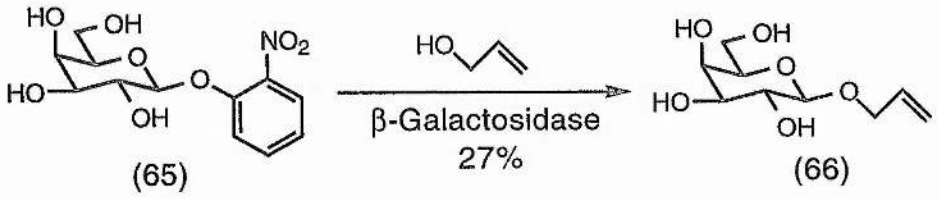
2.1.1 Introduction

In addition to the main investigation on the chemical mechanism of myrosinase, and to compare it with those of the β-glycosidases, the use of myrosinase in synthesis was also planned. As has been discussed (Section 1.3), β-glycosidase enzymes have found considerable use in organic synthesis. However, the synthetic utility of myrosinase had not been examined. An investigation into the catalysis of transglycosylation reactions by myrosinase was therefore carried out. Due to the fact that myrosinase is a readily available and fairly cheap enzyme, because it is stable (it rests in the plant cells in an active form ready to defend the plant), and it has fairly liberal active site requirements (the non-natural substrate PNPG is hydrolysed), it seemed that the enzyme would be a suitable candidate for use in organic synthesis.

The first step in the investigation of the synthetic utility of myrosinase involved preparative scale syntheses employing the transglycosylation (also known as partitioning) reaction, similar to that shown in Section 1.3. Firstly though, some practice reactions using β-galactosidase were carried out in order to provide a firm grounding to this work, before any studies with myrosinase were attempted.

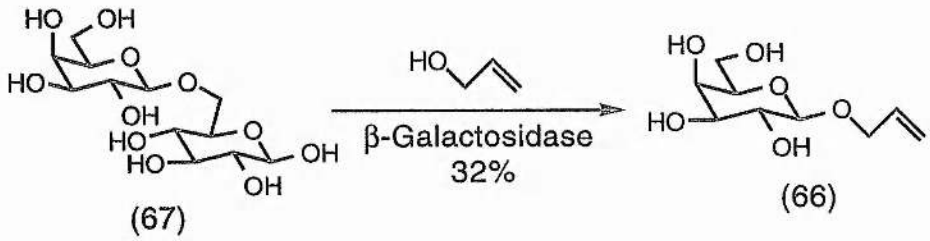
2.1.2 Use of β-Galactosidase in Synthesis

In order to gain experimental practice of enzymatic synthesis reactions using transglycosylation, two reactions were carried out using β-galactosidase. The first involved the use of *o*-nitrophenyl-β-D-galactopyranoside (65), β-galactosidase and allyl alcohol in the synthesis of allyl-β-D-galactopyranoside (66). This reaction was carried out using conditions previously reported.[154] Allyl alcohol (which was not used in the literature method) was chosen as it was planned that this alcohol would be used in the synthesis reactions using myrosinase. The reaction proceeded very smoothly and the product was purified by column chromatography giving a final yield of 27%. This reaction is shown in Scheme 24.



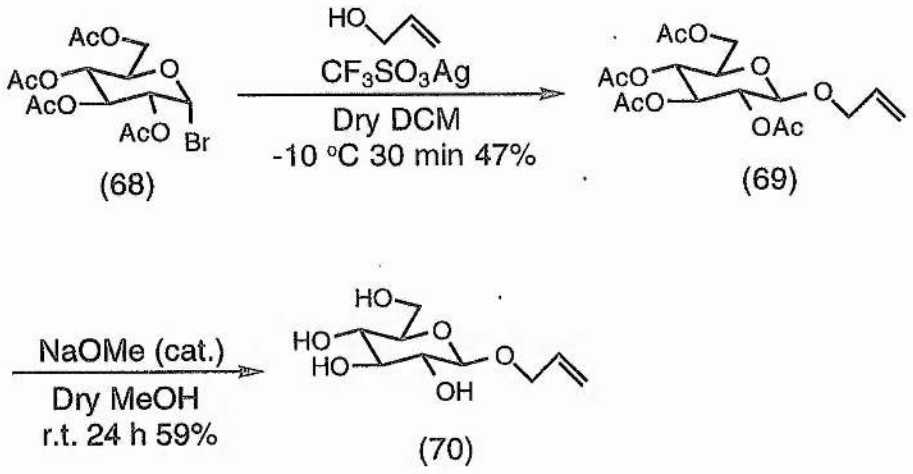
Scheme 24: *Synthesis of Allyl-β-D-galactopyranoside by Trans-glycosylation using β-Galactosidase*

The second method involved the use of lactose (67) as the glycosyl donor for the β-galactosidase catalysed reaction.[155] Allyl alcohol (which was not used in the literature method) was again used to give allyl-β-D-galactopyranoside (66), this time in 32% yield. This is shown in Scheme 25.



Scheme 25: *Synthesis of Allyl-β-D-galactopyranoside by Trans-glycosylation using β-Galactosidase*

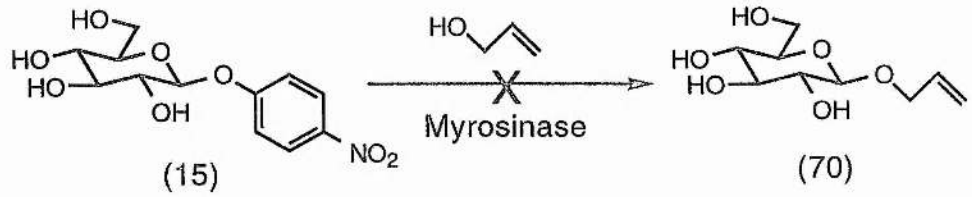
Allyl-β-D-glucopyranoside (70) was then synthesised using classical methods in order for it to be used as an authentic product for its attempted synthesis using myrosinase. This synthesis was carried out using the Koenigs-Knorr procedure using silver triflate as catalyst.[156] This is represented in Scheme 26.



Scheme 26: *Synthesis of Allyl-β-D-glucopyranoside using Classical Koenigs-Knorr Methodology*[156]

2.1.3 Use of Myrosinase in Synthesis

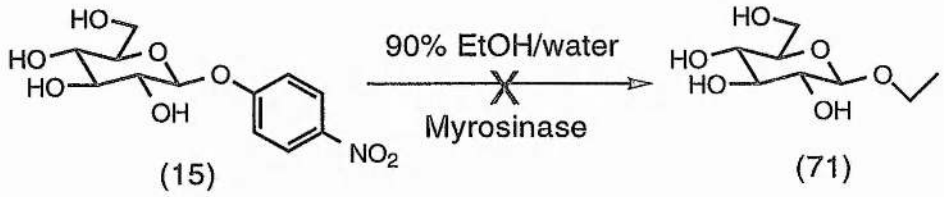
Attempts were made to use myrosinase in similar synthetic reactions to those shown in Section 2.1.2, using the same reaction conditions. In the first experiment, myrosinase was incubated with *p*-nitrophenyl-β-D-glucopyranoside (PNPG) (15) in the presence of allyl alcohol at 30 °C for 7 days. Allyl alcohol was used as it resembled the side chain of the glucosinolate sinigrin, and it was thus thought that this alcohol would have some chance of binding at the active site of myrosinase and acting as an acceptor. The reaction was monitored by t.l.c. (ethyl acetate/methanol/water (16:2:1)), however, no product spot was observed which corresponded to that of the authentic product. After work-up using the same method as for the β-galactosidase experiments, no product was isolated. This reaction is shown in Scheme 27.



Scheme 27: *Attempted Synthesis of Allyl-β-D-glucopyranoside using Myrosinase*

A second attempt to synthesise allyl-β-D-glucopyranoside using myrosinase was made. This time the reaction was done according to a method reported in previous work,[128] in which the production of *p*-nitrophenol was followed spectrophotometrically at 430 nm. After a period of $2^1/_2$ weeks at room temperature, the solution turned yellow due to the *p*-nitrophenol, and spectrophotometric measurements indicated that all of the PNPG had been consumed. An attempt was made to isolate the product using the same method as above, however no product was obtained.

It therefore seemed that the synthesis of allyl-β-D-glucopyranoside following adaptations of literature methods, in which only a small excess of the alcohol acceptor was used, was unsuccessful. It was thus decided to use a larger excess of alcohol. The synthesis of ethyl-β-D-glucopyranoside (71) was attempted using a large excess of ethanol. This meant that the reaction medium was almost entirely organic, in accordance with a method described earlier.[150] A 9:1 ethanol/water mixture was used as the solvent for this incubation and the reaction was followed spectrophotometrically at 430 nm to monitor the release of *p*-nitrophenol. After a 72 hour incubation, no increase in absorbance at 430 nm was observed, *i.e.* it appeared that no hydrolysis of PNPG occurred, and that myrosinase was thus inactive in the presence of such a large amount of ethanol. No attempt was therefore made to isolate any product. This reaction is shown in Scheme 28.



Scheme 28: *Attempted Synthesis of Ethyl-β-D-glucopyranoside using Myrosinase*

2.1.4 Discussion of Results

These early failures to observe any transglycosylation (partitioning) in the preparative scale experiments, using PNPG as the substrate for myrosinase, prompted a more detailed study of the activity of myrosinase in alcohol/water mixtures to investigate whether myrosinase was being destabilised or inactivated. Also, a more detailed investigation into the

transglycosylation reaction with myrosinase was required. These two lines of investigation would give an indication as to whether the synthesis reactions failed due to mechanistic reasons or simply because of reduced enzyme activity due to the increased amounts of alcohol.

2.1.5 Introduction to the Kinetics of Enzyme Catalysed Reactions

The most simple equation which represents enzyme catalysed reactions is the following:

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

where: E = free enzyme
S = substrate
ES = enzyme-substrate complex
P = product

It is assumed that the enzyme-substrate complex is in thermodynamic equilibrium with the free enzyme and substrate, *i.e.* $k_2 << k_{-1}$, and that the concentration of free enzyme is negligible compared with that of the substrate. The solution of the corresponding rate equations gives a quantitative expression (termed the Michaelis-Menten equation) (Equation 2).

$$v = \frac{[E_0] \cdot [S] \cdot k_{cat}}{K_M + [S]} \qquad (2)$$

where: v = initial rate of formation of product
$[E_0]$ = concentration of total enzyme = [E] + [ES]
$k_{cat} = k_2$ = first order rate constant (turnover number)
$K_M = k_{-1}/k_1$ = dissociation constant of the enzyme-substrate complex (Michaelis constant)

The mechanism proposed by Michaelis and Menten is in good agreement with the experimental behaviour of many enzymes. The concentration of the enzyme $[E_0]$ is directly proportional to v, and follows saturation kinetics with respect to the concentration of the substrate (Figure 22).

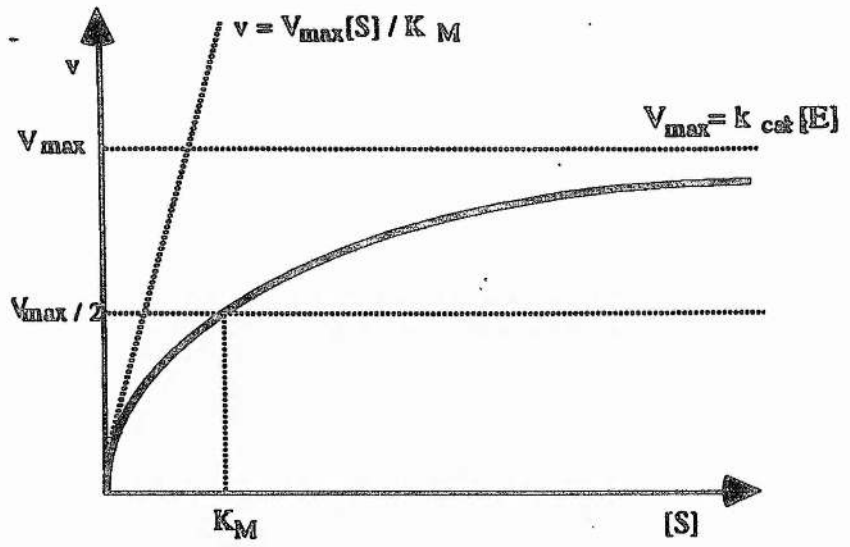


Figure 22: *Saturation Kinetics for Enzyme Catalysed Reactions*

The limiting value for v is termed V_{max}. The concentration of substrate at which $v = V_{max}/2$ is termed K_M. The specificity constant, k_{cat}/K_M is an apparent second order rate constant. It relates the reaction rate to the concentration of free, rather than total, enzyme, but only at low substrate concentration.

Experimentally, the data are usually plotted in linear form. The Lineweaver-Burk plot is derived by the transformation of Equation 2 into a double reciprocal (Equation 3).

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_M}{V_{max} \cdot [S]} \qquad (3)$$

This representation has the disadvantage of compressing the points at high substrate concentration into a small region and emphasising the points at lower concentrations. However, it is the most commonly used expression, since the values of v for a given value of substrate concentration are easy to read from it (Figure 23).

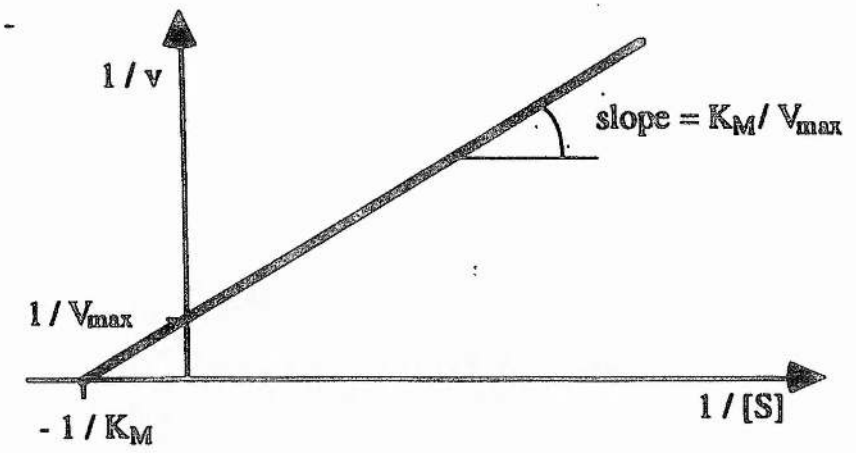


Figure 23: *Lineweaver-Burk Plot for Enzyme Catalysed Reactions*

2.1.6 Enzymatic Catalysis in Non-Aqueous Solvents

One of the central dogmas of biochemistry is the fact that enzymes require water to work. Nevertheless, high enzymatic activity has been observed in several organic systems.[157,158]

Enzymes generally require only a thin layer of water on the surface of the protein to retain a catalytically active conformation. Most non-aqueous media are hydrophobic solvents which do not displace these essential molecules of water from the enzyme surface. Organic solvents exert direct (*i.e.* binding or changing the dielectric constant of the reaction medium) and indirect (*i.e.* reversibly stripping water from the protein) effects. In general, enzymes are more stable in non-polar solvents than in polar solvents, with sensitivity varying among different enzymes.[159] The phenomenon of enzyme catalysis in organic media appears to be due to the structural rigidity of proteins in non-aqueous media resulting in high kinetic barriers that prevent the native-like conformation from unfolding.

The use of enzymes in organic solvents provides several advantages. An important functional characteristic of the enzyme, the thermostability, is greatly enhanced in non-aqueous solvents compared with water.[157] The same is true for storage stability. Strikingly different substrate specificity has been observed, as well as changes in stereoselectivity. Also, many reactions which are sensitive to water, or are thermodynamically impossible in water, become possible in organic systems.[160] Moreover, in

most cases, enzymes are insoluble in organic solvents and can therefore be recovered (by centrifugation or filtration) and used repeatedly.

Despite the advantages of enzyme transformations in organic solvents, some disadvantages are encountered. These are (i) organic solvents may not dissolve charged or polyfunctional species; (ii) adjusting the pH is difficult in large scale processes; (iii) enzymes are generally unstable and less active in non-aqueous media, particularly in hydrophilic solvents; (iv) loss of stereoselectivity may occur because of the reversible nature of the reaction and (v) severe substrate or product inhibition may occur for those enzymes which accept hydrophilic compounds (*e.g.* sugars) as natural substrates. Some of these problems can be overcome, for instance by immobilisation to improve stability, although many remain to be solved.

2.1.7 Investigation of Myrosinase Activity in Water/Organic Solvent Mixtures[161]

Myrosinase follows Michaelis-Menten kinetics for both substrates, sinigrin and *p*-nitrophenyl-β-D-glucopyranoside (PNPG). The determination of the kinetic parameters, k_{cat} and K_M, for the two substrates in water was carried out, and these were compared with literature values. These hydrolysis reactions were then examined in a series of water/organic solvent mixtures.

Information about the properties and reactions of the enzyme-substrate, enzyme-intermediate and enzyme-product complexes is given by k_{cat}. This is also called the turnover number because it represents the maximum number of substrate molecules converted to product per active site per unit time (the number of times the enzyme 'turns over' per unit time).

The measure of the amount of enzyme that is bound in any form whatsoever to the substrate is given by K_M. This is an apparent dissociation constant that may be treated as the overall dissociation constant of all the enzyme-bound species.

The reactions for both sinigrin and PNPG were followed by UV spectrophotometry. For sinigrin, the decrease in absorbance due to the substrate at 227 nm was monitored. For PNPG, the increase in absorbance due to the product, *p*-nitrophenol, was monitored at 430 nm. The kinetic constants were calculated by non-linear regression of the

experimental data. The Enzfitter program (see Appendix) was used for this purpose. K_M and V_{max} were expressed in mM and mol dm^{-3} min^{-1} respectively.

The standard assay for myrosinase involves measuring the hydrolysis of sinigrin in 33.1 mM potassium phosphate buffer, in 1.0 ml volume, at pH 7.0 at 37 ± 0.1 °C by following the decrease in its absorbance at 227 nm.[77] The rate of absorbance change is determined from the tangent to the beginning of the reaction curve. Initial velocities, in terms of disappearance of sinigrin concentration per minute, can then be calculated from these rates using $\varepsilon = 6784 \pm 25$ mol^{-1} dm^3 cm^{-1}, which is the literature value.[77] This value was assumed for the kinetic characterisation in all of the water/organic solvent mixtures. One unit of myrosinase was defined as the amount of enzyme necessary to convert 1 µmol of sinigrin per minute under standard assay conditions. All the kinetic values are corrected to one unit of enzyme.

For the myrosinase catalysed hydrolysis of PNPG, the extinction coefficients of the product, *p*-nitrophenol, in water and in the organic solvent mixtures were determined experimentally. Table 6 shows the values obtained.

Solvent system	ε_{430} / mol^{-1} dm^3 cm^{-1}
Water	7002 ± 84
20% Methanol	4293 ± 55
20% Ethanol	4293 ± 55
20% Acetonitrile	3338 ± 72
20% Dioxane	3559 ± 49
40% Methanol[a]	3845 ± 85

[a] Buffer used was 33.1 mM potassium phosphate, pH 7.0

Table 6: *Extinction Coefficient Values for p-Nitrophenol (In 50 mM Potassium Phosphate Buffer, pH 7.0, at 37 ± 0.1 °C)*

The range of concentrations examined for PNPG was between 1.0 and 25.0 mM. These substrate concentrations were higher then those for sinigrin since in this case the product absorption was being measured. This

was possible as only the first *ca.* 5% of reaction was measured and so the absorbance values were always small. The reactions were carried out at 37 ± 0.1 °C, in 50 mM potassium phosphate buffer (except in 40% methanol where 33.1 mM potassium phosphate buffer was used), at pH 7.0.

A range of water-miscible solvents with differing polarity was examined: methanol, ethanol, acetonitrile and dioxane. They were used at 20% ratio by volume (methanol was used up to 40% by volume). Sinigrin was studied at concentrations varying from 0.03 to 2.00 mM, thus giving a broad concentration range within reasonable absorbance limits. The reactions were carried out at 37 ± 0.1 °C, in 33.1 mM potassium phosphate buffer, at pH 7.0.

Before the sinigrin incubations with myrosinase were carried out, it was investigated whether β-glucosidase could catalyse the hydrolysis of sinigrin. This investigation was necessary to ensure that sinigrin was not being hydrolysed by contaminating β-glucosidase in myrosinase. Using β-glucosidase from almonds, incubations under both standard sinigrin conditions and literature conditions,[135] were carried out. It was found that no hydrolysis of sinigrin occurred, confirming that myrosinase is solely responsible for the hydrolysis of sinigrin.

Tables 7 and 8 show the results obtained for sinigrin and PNPG incubations with myrosinase in water and the water/organic solvent mixtures.

Solvent System	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
Water	0.42 ± 0.05	4.83 ± 0.22
20% Methanol	0.46 ± 0.08	3.04 ± 0.17
40% Methanol	0.57 ± 0.26	2.41 ± 0.40
20% Ethanol	0.49 ± 0.08	3.59 ± 0.19
20% Dioxane	0.87 ± 0.07	3.64 ± 0.12
20% Acetonitrile	0.55 ± 0.08	2.47 ± 0.14

Table 7: *Kinetic Parameters for the Myrosinase Catalysed Hydrolysis of Sinigrin (In 33.1 mM Potassium Phosphate Buffer, pH 7.0, at 37 ± 0.1 °C)*

Solvent System	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
Water	60.9 ± 11.2	0.15 ± 0.02
0.2 M Methanol	49.2 ± 19.6	0.10 ± 0.03
20% Methanol	30.5 ± 7.3	0.079 ± 0.012
40% Methanol[a]	51.9 ± 8.9	0.065 ± 0.008
0.2 M Ethanol	21.9 ± 3.9	0.066 ± 0.007
20% Ethanol	40.8 ± 6.0	0.11 ± 0.01
20% Dioxane	3.64 ± 0.77	0.022 ± 0.002
20% Acetonitrile	17.3 ± 3.1	0.11 ± 0.01

[a] Buffer used was 33.1 mM potassium phosphate, pH 7.0

Table 8: *Kinetic Parameters for the Myrosinase Catalysed Hydrolysis of PNPG (In 50 mM Potassium Phosphate Buffer, pH 7.0, at 37 ± 0.1 °C)*

The values of K_M for sinigrin and PNPG hydrolysis in water were in agreement with those quoted in the literature, although the literature incubations were carried out at different pH values using different myrosinases.[51,55,77] The values of K_M for sinigrin (at pH 5.5) and PNPG (at pH 6.5) with myrosinase from *Lepidium sativum* were 0.3 mM and 2 mM respectively.[51] The values of K_M for sinigrin and PNPG at pH 5.2 with myrosinase from *Brassica juncea* were 0.2 mM and 2 mM respectively.[55] For myrosinase from the same source, *i.e. Sinapis alba*, the K_M value for the hydrolysis of sinigrin at 30 °C was 0.156 mM.[77]

Some observations concerning the reaction can be made based on these results. It can be seen that the solvents used had a negligible effect on K_M for the hydrolysis of sinigrin. Only in dioxane was a significant change evident. V_{max}, which essentially represents the rate of the chemical transformations, decreased in all cases, in agreement with the common tendency of enzymes to catalyse reactions slower in organic solvents than in water. A more marked decrease was observed in acetonitrile.

The determination of V_{max} for PNPG hydrolysis was not as reliable as for sinigrin, since the rates are much slower and only small increases in absorbance are observed. In all the organic solvents used, K_M was lower than the value obtained for the reaction in water. It is notable that the

higher the methanol concentration, the lower the K_M value, *i.e.* the binding between PNPG and myrosinase is better. Again, as for sinigrin, V_{max} decreased in all cases.

Figures 24 and 25 show the reaction curves in water/methanol mixtures for sinigrin and PNPG respectively.

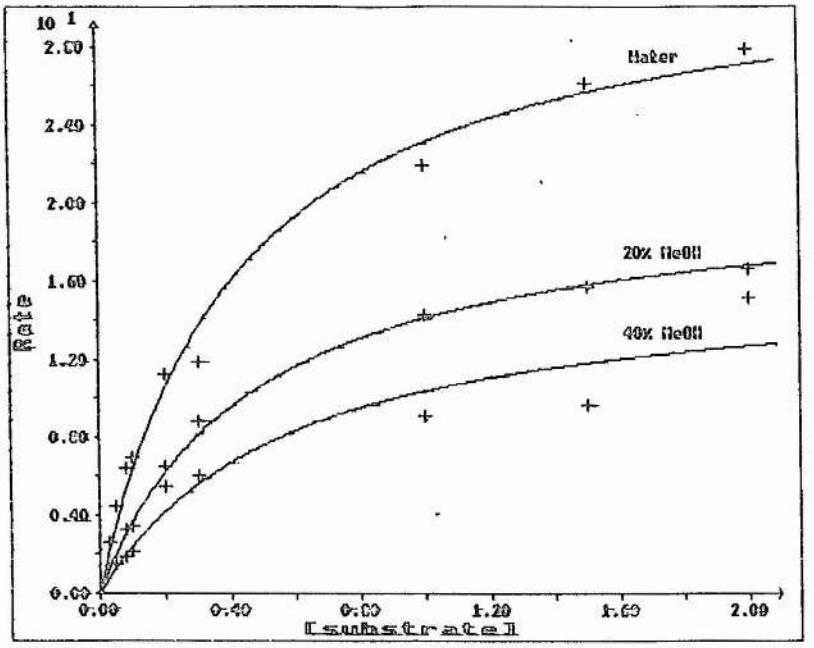


Figure 24: *Effect of Methanol on the Myrosinase Catalysed Hydrolysis of Sinigrin*

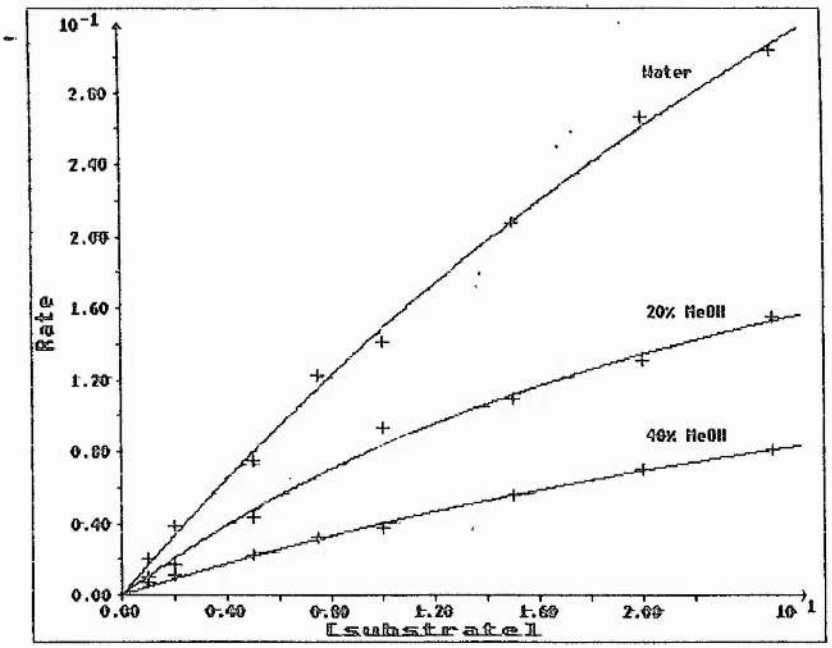


Figure 25: *Effect of Methanol on the Myrosinase Catalysed Hydrolysis of PNPG*

2.1.8 Discussion of Results

For sinigrin, the values of K_M remained constant as the organic solvents were added, and the observed rate decrease was all due to changes in V_{max}. Thus, binding of the substrate to the enzyme active site was unaffected by the change in the reaction medium, while the rate of the reaction decreased. Interestingly, even in 40% v/v methanol, there was an appreciable rate of reaction showing only a 50% decrease from the rate in aqueous solution. The results for PNPG hydrolysis showed similar trends.

The most informative parameter for these studies is K_M. For both sinigrin and PNPG, the absence of an increase in the values of K_M for the reactions carried out in organic solvents was observed. This suggests that the active site of myrosinase is not hydrophobic. If this were the case, then as the solvent becomes more hydrophobic (*i.e.* as the amount of organic solvent increases), then K_M should increase, showing poorer binding.

The decrease in V_{max} values is probably due to a disturbance on the protein structure caused by the organic solvent. This is translated in a reduction of the catalytic efficiency. Nevertheless, myrosinase does not

seem to be very sensitive to the variation of hydrophobicity of the system, and the enzyme can be considered fairly active in organic solvents. Certainly, there is sufficient enzymatic activity to allow myrosinase to be used for transglycosylation reactions (see Section 1.3).

2.1.9 Stability of Myrosinase in Methanol/Water Mixtures[161]

The stability of myrosinase was assayed by incubating the enzyme in various methanol/water mixtures. In all the solvent mixtures, 33.1 mM potassium phosphate buffer, pH 7.0 was used at 37 ± 0.1 °C. Aliquots of the enzyme solutions were removed at intervals and myrosinase activity was determined using the sinigrin assay described in Section 4.7.

The results showed that myrosinase appears to be a stable enzyme in methanol at 37 °C. In water alone, myrosinase activity did not decrease significantly over the time period measured. The half-life for enzymatic activity in 20% v/v methanol was found to be 40 hours, and in 40% v/v methanol it was reduced to 8 hours. The stability curves are shown in Figure 26.

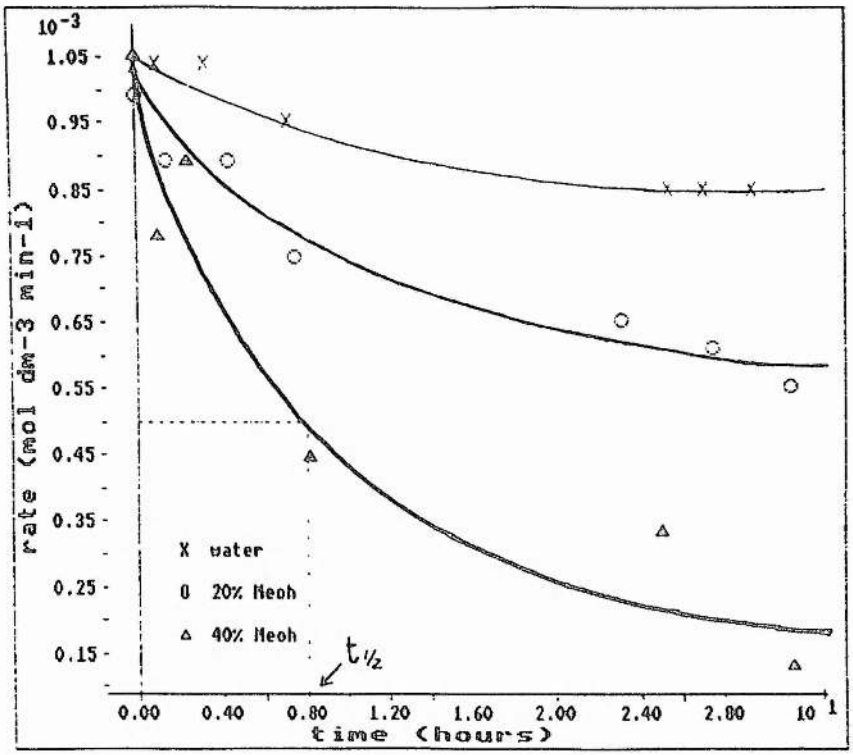


Figure 26: *Stability Curves for Myrosinase in Various Methanol/Water Mixtures*

If the reduced enzymatic activity (Section 2.1.7) is taken into account, and the fact that further aliquots of fresh enzyme solution can be regularly added to the system, myrosinase should still be suitable for use in transglycosylation reactions in organic solvents. In addition, it is worth noting that the enzyme solution, stored in a refrigerator (at 4 °C) when not in use, still exhibits the original activity after more than four weeks.

It is important to note that the role of myrosinase in the Cruciferae is to hydrolyse glucosinolates only when cell disruption takes place, *e.g.* from attack by pests when the plant needs to defend itself. This requires the enzyme to remain active in the cells for long periods, ready for immediate use. This may be the biological motivation for the plant to evolve such a stable enzyme.

2.1.10 Discussion of Results

The fact that the half-lives of myrosinase in different methanol/water mixtures are quite long implies that myrosinase does not denature in the alcoholic solvents, and that the enzyme is thus active and available for catalysis of transglycosylation reactions in alcohol/water mixtures.

The high stability of myrosinase in methanol may be due to the high carbohydrate content of the enzyme (*ca.* 5-20%) (see Section 1.1.4). A connection between the amount of carbohydrate in the protein structure and the behaviour in non-aqueous solvents has been observed for several glycoproteins. These glycoproteins generally show higher stability in organic solvents than other proteins.[162]

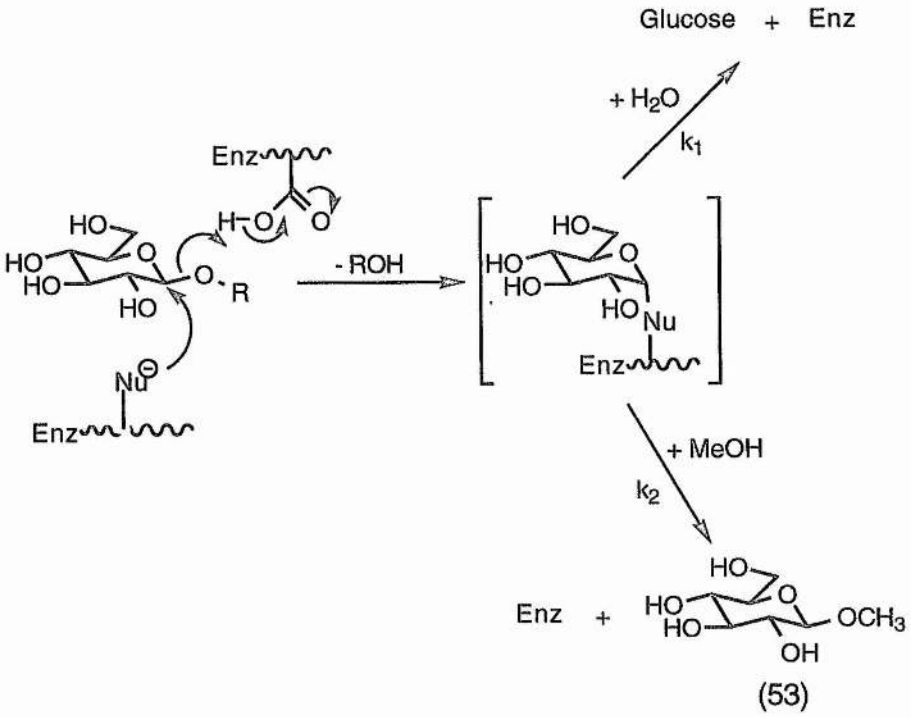
The fact that myrosinase (i) is not terribly sensitive to organic solvents and (ii) retains good stability in methanol/water mixtures implies that the reason why myrosinase does not catalyse transglycosylation reactions is due to mechanistic factors, rather than because of reduced enzyme activity due to the presence of the alcohols.[161] This prompted a more detailed study of transglycosylation (Section 2.1.11).

2.1.11 Further Investigation of the Transglycosylation Activity of Myrosinase[161]

To further investigate the apparent lack of observable transglycosylation with myrosinase, a further range of partitioning experiments was carried out using either PNPG or sinigrin as the glycosyl donor.

2.1.11.1 Transglycosylation Studies Using PNPG as the Glycosyl Donor

Initial transglycosylation experiments involved the incubation of 3 ml solutions each containing 20 mM PNPG, myrosinase solution (90 μl, 0.36 units), 50 mM potassium phosphate buffer in water, 20% methanol and 40% methanol, at pH 7.0 and 37 ± 0.1 °C. Aliquots of the solutions were removed at intervals and the amounts of D-glucose and *p*-nitrophenol were measured. D-Glucose concentrations were determined using the Glucose HK assay described in Section 4.16.1. *p*-Nitrophenol concentrations were determined by diluting a 10 μl aliquot of the reaction mixture into 0.1 M sodium hydroxide solution and measuring the absorbance due to the *p*-nitrophenolate ion at 400 nm (ε = 18,300 $mol^{-1}\ dm^3\ cm^{-1}$). A smaller amount of D-glucose produced relative to the amount of *p*-nitrophenol would imply the interception of the intermediate by the glycosyl acceptor and production of methyl-β-D-glucopyranoside, thus implying that transglycosylation had occurred (Scheme 18).



Scheme 19: *Pathway for Transglycosylation Reaction Catalysed by Sweet Almond β-Glucosidase*

Prior to these transglycosylation experiments, it was checked that methyl-β-D-glucopyranoside did not act as a substrate for myrosinase, to confirm a previous literature report.[50] A solution containing 20 mM methyl-β-D-glucopyranoside, 50 mM potassium phosphate buffer, pH 7.0, and 50 µl of myrosinase solution was incubated at 37 ± 0.1 °C for 3 days. The amount of D-glucose was then measured using the Glucose HK assay described in Section 4.16.1. No glucose was detected, and it was concluded that methyl-β-D-glucopyranoside does not act as a substrate for myrosinase.

The results of the transglycosylation reaction in 20% methanol are shown in Figure 29. It can be seen that exactly equal amounts of D-glucose and *p*-nitrophenol are produced in the early stages of the reaction, thus implying that no transglycosylation was taking place.

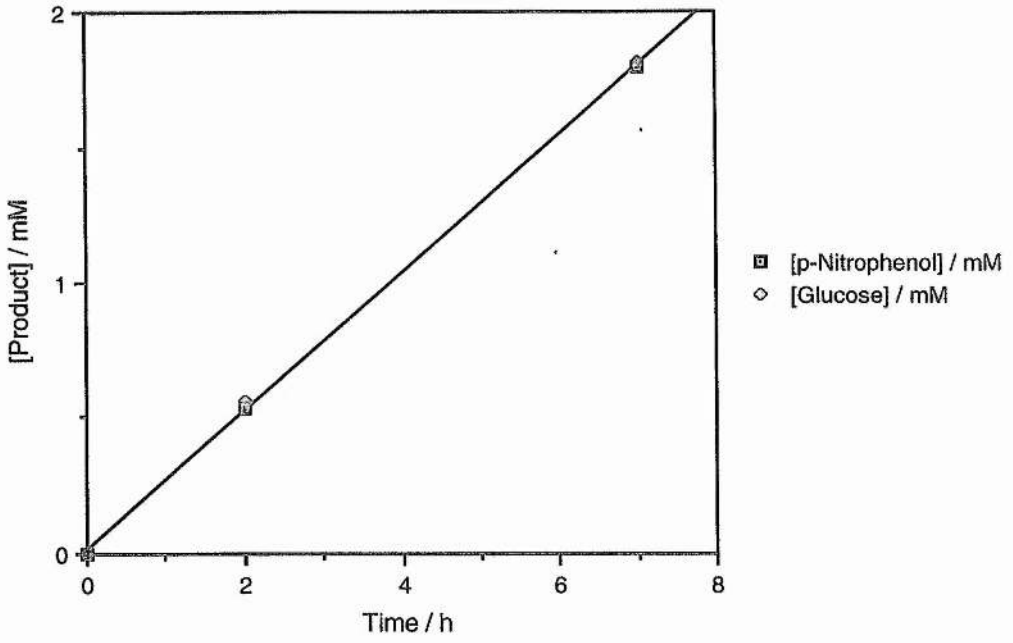
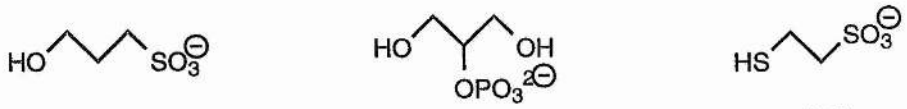


Figure 29: *Production of D-Glucose and p-Nitrophenol from the Transglycosylation Reaction in 20% Methanol using PNPG as the Glycosyl Donor*

A study was then carried out to examine the effect of changes in the structure of the glycosyl acceptor. Previous work had shown a correlation between the length of alkyl side chain and the degree of transglycosylation for cytosolic β-glucosidase.[163] Therefore, higher alcohols such as ethanol, propan-1-ol and butan-1-ol were examined.

Myrosinase is a thioglucosidase, cleaving an S-glycoside linkage. It might, therefore, be imagined that a thiol may be more appropriate as a glycosyl acceptor. 2-Mercaptoethanol was chosen as a readily available example.

Since the sulfate group of the glucosinolates is known to provide important binding interactions at the active site of myrosinase, it seemed feasible that glycosyl acceptors containing a sulfate or similar negatively charged group might have a better chance of binding at the active site and intercepting the glycosyl intermediate. Three commercially available compounds were examined, namely 3-hydroxy-1-propanesulfonate (72), glycerol-2-phosphate (73) and 2-mercaptoethanesulfonate (74), the last of which incorporated both a charged group for binding and a thiol group as the nucleophile. The structures of these compounds are shown in Figure 30.



(72) (73) (74)

Figure 30: *Structures of Potential Glycosyl Acceptors for Myrosinase*

In addition, the more strongly nucleophilic glycosyl acceptors azide, hydroxylamine and hydrazine were examined.

In the attempted transglycosylation reactions using PNPG as the glycosyl donor, the amounts of both D-glucose and *p*-nitrophenol were measured after 24 hour incubations. A high initial concentration of PNPG (20 mM), together with this long reaction time were employed, so that significant amounts of products were produced, thus minimising errors. These one point measurements of D-glucose and *p*-nitrophenol were a convenient and effective method for investigation of the transglycosylation reaction.

A full table of the results obtained from the transglycosylation experiments is shown in Table 10.

Glycosyl Acceptor[a]	[*p*-Nitrophenol][b]/mM	[Glucose][c]/mM
None	6.17 ± 0.09	6.46 ± 1.43
Methanol	6.28 ± 0.25	6.15 ± 0.34
Methanol (6.25 M)	4.21 ± 0.74	4.32 ± 0.45
Ethanol	5.58 ± 1.56	5.84 ± 2.93
Propan-1-ol	6.76 ± 0.14	7.58 ± 0.32
Butan-1-ol	5.42 ± 0.39	5.35 ± 1.77
2-Mercaptoethanol	6.37 ± 0.32	7.82 ± 0.23
3-Hydroxypropanesulfonate	4.69 ± 0.95	5.01 ± 1.09
Glycerol-2-phosphate	5.06 ± 0.04	7.06 ± 0.83
2-Mercaptoethanesulfonate	5.53 ± 1.03	6.83 ± 1.58
Sodium azide	6.36 ± 0.66	4.65 ± 1.93
Hyroxylamine hydrochloride	2.18 ± 0.22	0.33 ± 0.33
Hydrazine monohydrate	2.41 ± 0.03	0.82 ± 0.82

[a] All at 0.2 M concentration unless otherwise indicated
[b] Measured spectrophotometrically
[c] Measured using the Glucose-HK assay

Table 10: *Results from the Partitioning Experiments with Myrosinase using PNPG as the Glycosyl Donor*

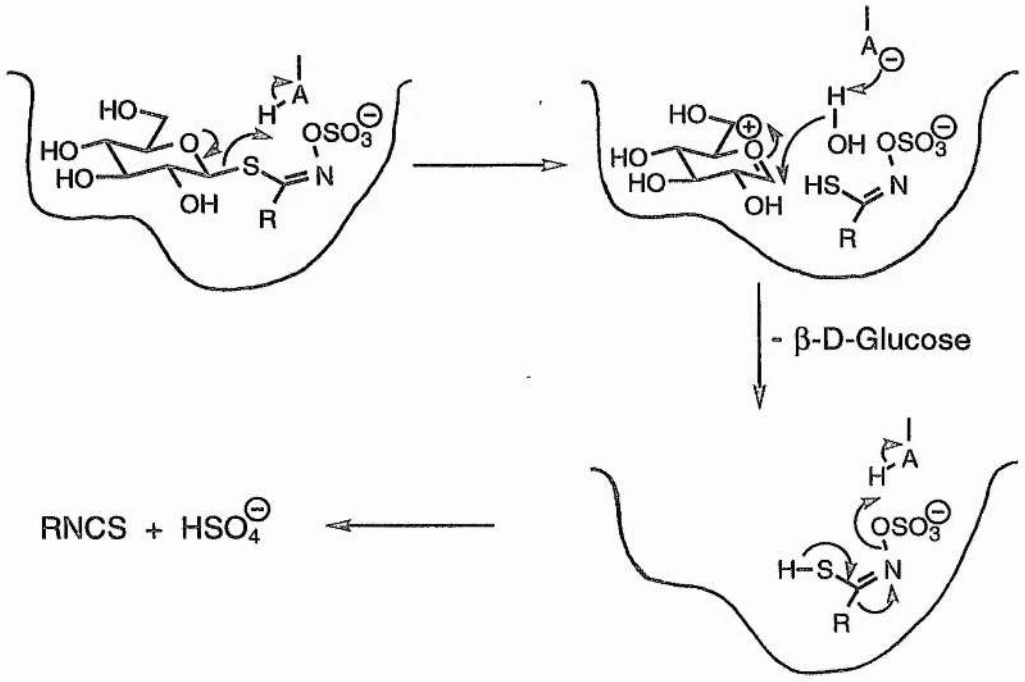
2.1.11.2 Discussion of Results

For the partitioning experiments with PNPG, no transglycosylation was observed with any of the higher alcohols used. In addition, the potential glycosyl acceptors which mimic the aglucone moiety of glucosinolates, *i.e.* the sulfur nucleophiles and the compounds with a sulfate (or similar charged) group, did not react to form any new glucosides.

Interestingly, it can also be observed that none of the alcohols or thiols acts as a particularly effective inhibitor of myrosinase. The amounts of D-glucose and *p*-nitrophenol produced in the presence of most of the acceptors are roughly the same as those where there is no acceptor. The yields are all approximately 30%, falling to 21% for 6.25 M methanol. The yields are in the region 22-25% for the three charged glycosyl acceptors (72), (73) and (74), reflecting only a small degree of inhibition. This inhibition is dealt with in more detail in Section 2.3. The fact that these

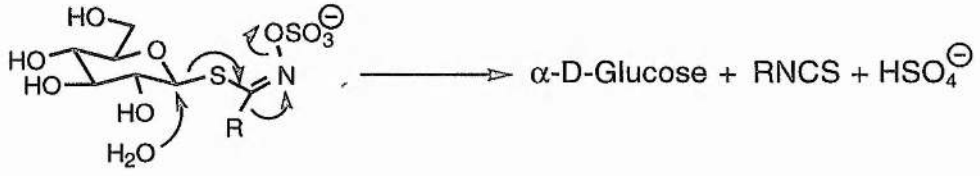
compounds do not inhibit the enzyme to any great degree would imply that they do not bind effectively at the active site of myrosinase.

The fact that there was no transglycosylation observed when these compounds are used as acceptors, nor any inhibition by the compounds with charged groups, could be due to two possible mechanistic factors: (i) the debinding order is such that glucose is released first, and thus there is no room at the active site for a competing acceptor to intercept the glycosyl intermediate since the aglucone fragment is still bound (Scheme 28).



Scheme 28: *Possible Mechanism for Myrosinase in Which Transglycosylation is Not Possible*

(ii) Myrosinase operates by a concerted mechanism in which the attack by water and the rearrangement of the aglucone fragment occur simultaneously, giving α-D-glucose as the initial product (Scheme 29).



Scheme 29: *Possible Concerted Mechanism for Myrosinase in Which Transglycosylation is not Possible*

The second mechanism has recently been disproven, since ^{1}H N.M.R. studies on the stereochemical course of the hydrolysis of sinigrin have shown that β-D-glucose is the first product formed.[70] Thus, myrosinase acts with retention of anomeric configuration. After 10 minutes hydrolysis, a doublet at 4.51 ppm (*J* 8 Hz), assignable to H-1 of β-D-glucose began to appear, together with peaks between 3.08 and 3.18 ppm, assignable to allyl isothiocyanate. After 60 minutes, the doublet at 4.51 ppm was intense, and a much smaller doublet at 5.10 ppm (*J* 3.7 Hz), corresponding to H-1 of α-D-glucose began to appear. After 3.5 hours, the ratio of β to α intensities was 5:1, thus proving that β-D-glucose is the initial product of glucosinolate hydrolysis. These ^{1}H N.M.R. studies are shown in Figure 31.

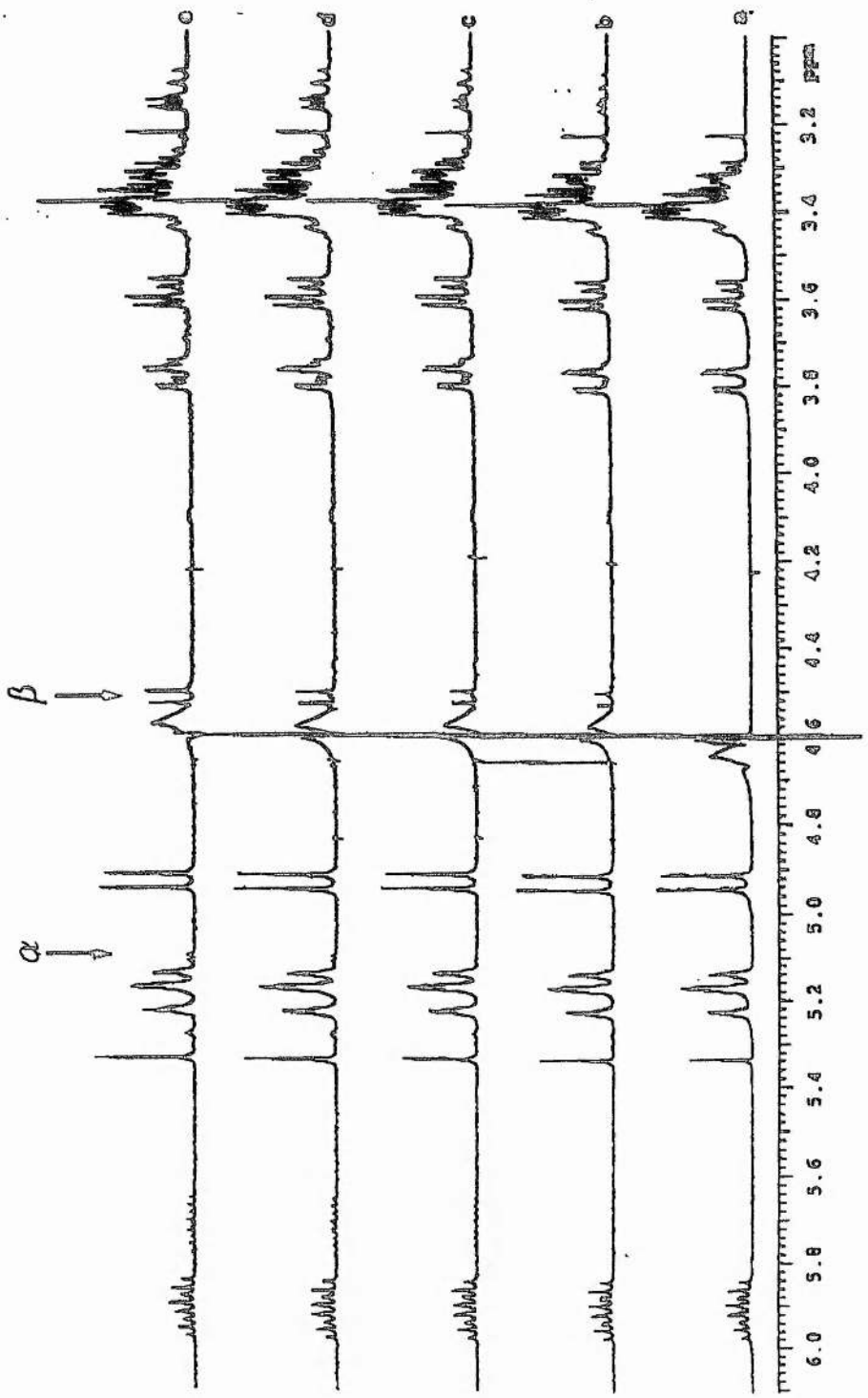
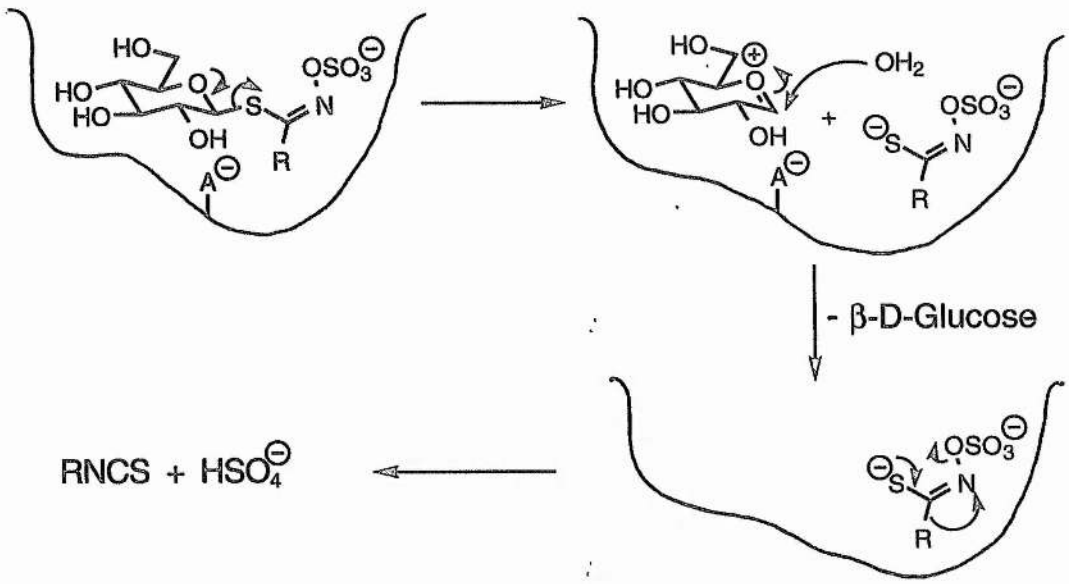


Figure 31: *Time Course Hydrolysis of Sinigrin Catalysed by Myrosinase, Followed by ^{1}H N.M.R.[70] (a) Spectrum Recorded Before Addition of Myrosinase; (b) - (e) Spectra Recorded 10,30,60 and 120 Minutes After Addition of Myrosinase*

When the reactive nitrogen nucleophiles azide, hydroxylamine and hydrazine were used as acceptors, smaller concentrations of D-glucose than *p*-nitrophenol were observed. This implies that transglycosylation had taken place. However, for hydroxylamine and hydrazine, enzymatic activity (either for myrosinase and/or the Glucose HK assay enzymes) seems to have been reduced significantly, since the yields of *p*-nitrophenol were very low (*ca.* 12%) relative to the standard reaction with no acceptor. Therefore the transglycosylation observed for hydroxylamine and hydrazine must be regarded as tenuous.

For azide, however, the yield of *p*-nitrophenol was no lower than that for the standard reaction. The amount of D-glucose produced was lower than that of *p*-nitrophenol by 27%, implying that 27% transglycosylation took place when azide was used as the acceptor. This transglycosylation appears to be much more genuine than that observed with hydroxylamine and hydrazine.

As was discussed in Section 1.1.5, azide has been shown to reactivate myrosinase following deactivation by 2-deoxy-2-fluoro-glucotropaeolin (Scheme 7),[70] while alcohols were ineffective. This fits with our results for transglycosylation reactions where azide was the only acceptor to give any. Also, it has been proposed that myrosinase has no acid/base residue at the active site for protonating the leaving group or deprotonating water (in the case of transglycosylation, deprotonating the glycosyl acceptor).[68,69] The fact that azide was the only acceptor which gave rise to any transglycosylation would fit with this proposal, since azide does not require deprotonation to react with the glycosyl intermediate. In this context, a third possibility for the chemical mechanism of myrosinase exists (Scheme 30).



Scheme 30: *Possible Mechanism for Myrosinase in Which Trans-glycosylation is Only Possible for Acceptors Which Do Not Require Deprotonation*

2.1.11.3 Transglycosylation Studies Using Sinigrin as the Glycosyl Donor

Since the myrosinase catalysed hydrolysis of sinigrin is very fast, it was impractical to determine D-glucose and isothiocyanate concentrations at discrete time intervals to monitor the transglycosylation reaction. Therefore, solutions of sinigrin (10 mM) were incubated at 37 ± 0.1 °C with myrosinase for three days, after which time it was assumed that the reaction had gone to completion. D-glucose concentrations were then measured to investigate whether any transglycosylation had occurred. For these preliminary experiments, solutions containing 20% and 40% methanol, 20% ethanol and 20% 2-mercaptoethanol were used.

Incubations with 20% and 40% methanol gave D-glucose yields of around 70%, whilst those containing 20% ethanol and 20% 2-mercaptoethanol gave 100% yields of D-glucose.

2.1.11.4 Discussion of Results

For the incubation solutions containing 20% and 40% methanol, 70% yields of D-glucose were observed. For these incubations, it could not be certain that there had indeed been 30% transglycosylation, since the yields

of D-glucose may have been lowered due to the non-completion of the reaction due to lower enzymatic activity or by denaturation of the enzyme. Therefore, there was no evidence for a significant amount of transglycosylation. No transglycosylation was observed for the incubation solutions containing 40% methanol, 20% ethanol and 20% 2-mercaptoethanol.

Even if the possible 30% partitioning observed for the 20% and 40% methanol incubations was genuine, it would still be very small compared with the 96% partitioning observed for other β-glycosidase catalysed reactions employing much lower methanol concentrations (0.2 M).[163]

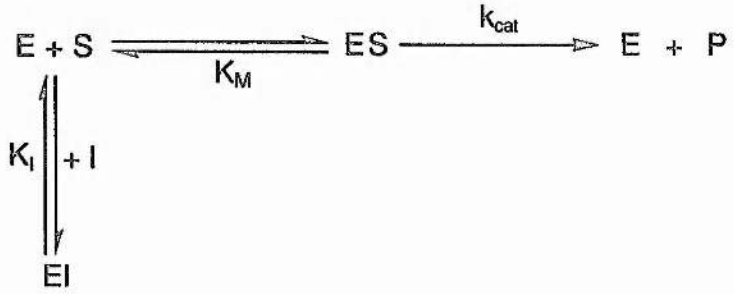
On the evidence of the stability studies and partitioning experiments for myrosinase, it appears that the enzyme does not catalyse transglycosylation reactions due to mechanistic reasons and not because of destabilisation in organic solvents.[161]

2.2 Inhibition of Enzyme Catalysed Reactions

As well as being irreversibly inactivated or inhibited by heat, chemical reagents or suicide (covalently binding) inhibitors, enzymes may be reversibly inhibited by the non-covalent binding of inhibitors. There are four main types of reversible inhibition, and these are described below.

2.2.1 Competitive Inhibition

If an inhibitor (I) binds reversibly to the active site of an enzyme and prevents the substrate S binding it is said to be a competitive inhibitor. In the case of the simple Michaelis-Menten mechanism the equilibrium (Equation 4) must be considered.



(4)

Solving the equilibrium and rate equations using

$$[E]_o = [ES] + [EI] + [E] \tag{5}$$

gives

$$V = \frac{[E]_o[S]k_{cat}}{[S] + K_M(1 + [I]/K_I)} \tag{6}$$

Competitive inhibition affects K_M only and not V_{max}, since infinitely high concentrations of S displace I from the enzyme. The Lineweaver-Burk plot for competitive inhibition is shown in Figure 32.

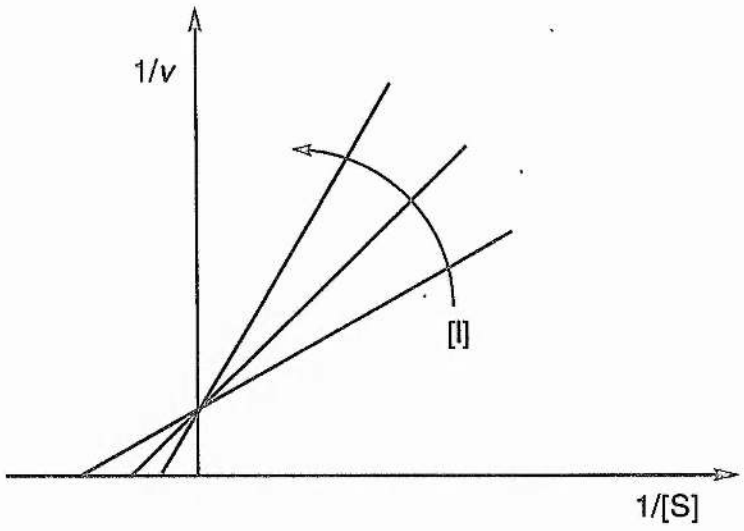
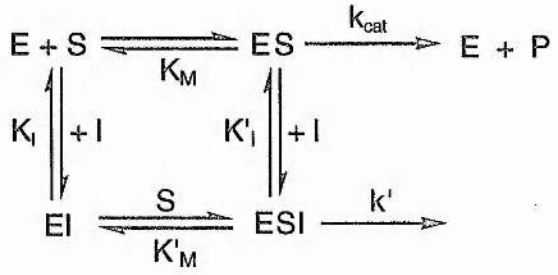


Figure 32: *Lineweaver-Burk Plot for Competitive Inhibition*

2.2.2 Noncompetitive, Uncompetitive and Mixed Inhibition

Different inhibition patterns occur if I and S bind simultaneously to the enzyme instead of competing for the same binding site (Equation 7).



(7)

It may be shown from the Michaelis-Menten mechanism - in the simplified case in which the dissociation constant of S from EIS is the same as that from ES (*i.e.* $K_M = K'_M$), but in which EIS does not react (*i.e.* k' = 0) - that

$$V = \frac{[E]_o[S]k_{cat} / (1 + [I]/K_I)}{[S] + K_M} \qquad (8)$$

Noncompetitive inhibition occurs when I binds to a different site on the enzyme to that which S binds. In this case K_M is unaffected, but V_{max} is lowered by a factor of $(1 + [I]/K_I)$. The Lineweaver-Burk plot for this is shown in Figure 33.

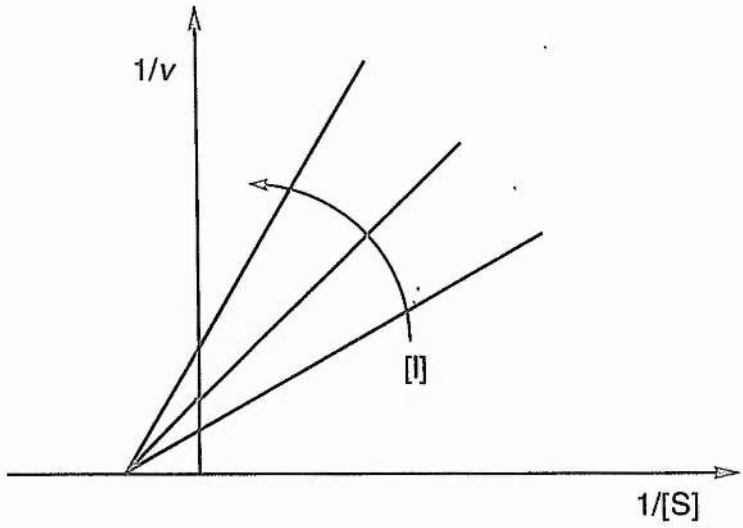


Figure 33: *Lineweaver-Burk Plot for Noncompetitive Inhibition*

Commonly, the dissociation constant of S from EIS is different than that from ES, *i.e.* $K_M \neq K'_M$. In this case, both K_M and k_{cat} are altered and the inhibition is termed mixed.

Uncompetitive inhibition occurs when I binds to ES but not to E (Equation 9).

$$E + S \underset{K_M}{\rightleftharpoons} ES \overset{I}{\underset{K_I}{\rightleftharpoons}} ESI \xrightarrow{k'}$$
$$\Big\downarrow k_{cat} \qquad (9)$$
$$E + P$$

The Lineweaver-Burk plot for uncompetitive inhibition is shown in Figure 34.

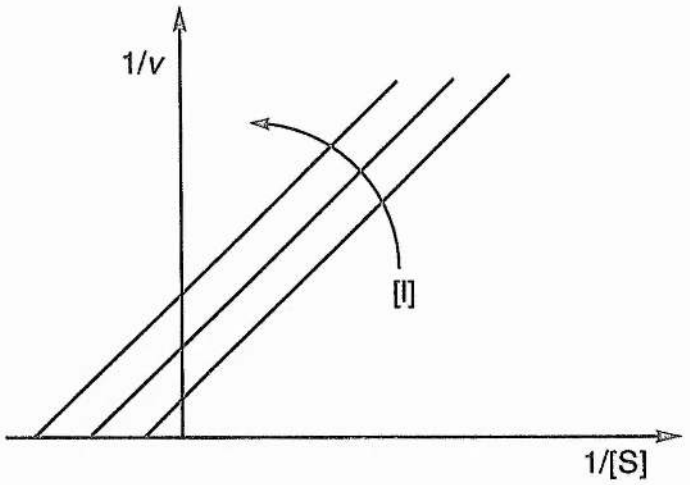


Figure 34: *Lineweaver-Burk Plot for Uncompetitive Inhibition*

2.2.3 Product Inhibition

Product inhibition studies give an insight into the chemical mechanism of an enzyme. The type of inhibition observed for each product upon the reaction gives an indication of the debinding order. For example, for the reaction:

$$A \xrightarrow{\text{Enzyme}} B + C \qquad (10)$$

where

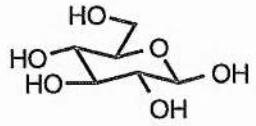
$$E + A \rightleftharpoons EA \rightleftharpoons EBC \overset{B}{\rightleftharpoons} EC \overset{C}{\rightleftharpoons} E + C \qquad (11)$$

product B is an uncompetitive inhibitor of A, whilst C is a competitive inhibitor.

2.3 Inhibition Studies on Myrosinase

For investigation of the chemical mechanism of myrosinase and for comparison of the mechanisms of β-glycosidases, the inhibitory properties of a number of compounds (many of which are inhibitors for β-glycosidases (see Section 1.2.4)) were examined. Studies of the inhibition of the myrosinase catalysed hydrolysis reactions of both sinigrin and *p*-nitrophenyl-β-D-glucopyranoside (PNPG) were made. Product inhibition studies would give an indication of the product debinding order.

2.3.1 Inhibition of Myrosinase by β-D-Glucose (2)



(2)

For investigation of the product inhibition of the myrosinase catalysed hydrolysis of sinigrin by β-D-glucose (2), concentrations between 1 and 20 mM β-D-glucose were used. As for the normal sinigrin incubations, these incubations were carried out in 33.1 mM potassium phosphate buffer, at pH 7.0, at 37 ± 0.1 °C. The results for these studies are shown in Table 11.

[β-D-Glucose] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	0.42 ± 0.05	4.83 ± 0.22
1	0.23 ± 0.02	3.38 ± 0.10
5	0.32 ± 0.04	3.97 ± 0.18
10	0.38 ± 0.05	4.16 ± 0.17
20	0.39 ± 0.02	4.02 ± 0.08

Table 11: *Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by β-D-Glucose*

2.3.2 Discussion of Results

Other studies[164] have shown that β-D-glucose is a competitive inhibitor of β-glucosidases, albeit with a very high K_I. From Table 11, it can be seen that at 1 mM concentration, β-D-glucose has some inhibitory effect since

the values of K_M and V_{max} are decreased. However, at higher concentrations, β-D-glucose exerts a poorer inhibitory effect since V_{max} actually begins to increase. The inhibition pattern shown in Table 11 resembles that for noncompetitive inhibition since with increasing inhibitor concentration, K_M remains roughly constant while V_{max} decreases. However, this inhibition pattern is too erratic and cannot be assigned as truly noncompetitive. Nor can the inhibition be assigned as competitive, as in the case of the β-glycosidases mentioned above. One possible explanation for the degree of noncompetitive inhibition may be due to the fact that β-D-glucose may bind at the L-ascorbic acid binding site, due to the similarity in structures between β-D-glucose and L-ascorbic acid. Most likely, the inhibition is a mixture of competitive and noncompetitive, and it can be concluded that β-D-glucose is a poor inhibitor of myrosinase exerting mixed inhibition. Therefore, these inhibition studies are too inconclusive to give an indication of the product debinding order.

2.3.3 Inhibition of Myrosinase by Sulfate

Concentrations between 1 and 25 mM sulfate were used for the study of the inhibition of the myrosinase catalysed hydrolysis of sinigrin. As for the normal sinigrin incubations, these incubations were carried out in 33.1 mM potassium phosphate buffer, at pH 7.0, at 37 ± 0.1 °C. The results for these studies are shown in Table 12.

[Sulfate] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	0.42 ± 0.05	4.83 ± 0.22
5	0.57 ± 0.10	3.12 ± 0.20
10	0.57 ± 0.11	3.22 ± 0.24
15	0.55 ± 0.02	2.39 ± 0.26
20	0.56 ± 0.11	2.79 ± 0.21
25	0.64 ± 0.10	2.19 ± 0.13

Table 12: *Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by Sulfate*

Similar concentrations of sulfate were used to study the inhibition of the myrosinase catalysed hydrolysis of PNPG, under normal PNPG incubation

conditions (50 mM potassium phosphate buffer, at pH 7.0, at 37 ± 0.1 °C). The results of this study are shown in Table 13.

[Sulfate] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	60.9 ± 11.2	0.15 ± 0.02
5	53.9 ± 7.8	0.14 ± 0.02
7.5	43.8 ± 12.4	0.12 ± 0.02
10	21.9 ± 8.2	0.070 ± 0.015
15	27.5 ± 3.7	0.058 ± 0.005
20	41.2 ± 7.9	0.097 ± 0.013
25	37.5 ± 1.5	0.093 ± 0.025

Table 13: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by Sulfate*

2.3.4 Discussion of Results

It can be seen from Tables 12 and 13 that sulfate is a poor inhibitor of myrosinase. As the sulfate concentration increases, V_{max} decreases, although the inhibitory effect appears to be less strong at higher concentrations since the values of V_{max} actually increase.

Sulfate is not only a product of glucosinolate hydrolysis but it is a charged molecule which may mimic the sulfate group of glucosinolates. Thus, sulfate may inhibit myrosinase by binding at the part of the active site where the sulfate group would normally bind. It was therefore decided to investigate the inhibitory effect of some other charged species (see Section 2.3.7) for comparison. This would give information as to whether sulfate inhibits myrosinase because it is a product of glucosinolate hydrolysis, or because it is an appropriately charged molecule which binds at the substrate active site.

Also, studies using higher phosphate buffer concentrations would give information as to whether sulfate was inhibiting the enzyme simply because of higher salt concentrations (Section 2.3.5).

2.3.5 Effect of Increased Phosphate Concentration on Myrosinase

The kinetic parameters for the myrosinase catalysed hydrolysis reactions of sinigrin and PNPG were measured in the presence of varying phosphate concentrations (20 to 100 mM) at pH 7.0 and 37 ± 0.1 °C. The results are shown in Table 14 and 15.

[Phosphate] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
20	0.44 ± 0.10	3.76 ± 0.31
33.1 (Standard)	0.42 ± 0.05	4.83 ± 0.22
50	0.43 ± 0.10	3.02 ± 0.23
100	0.62 ± 0.17	2.66 ± 0.29

Table 14: *Effect of Phosphate Concentration on the Myrosinase Catalysed Hydrolysis of Sinigrin*

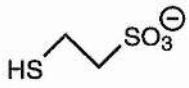
[Phosphate] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
20	56.4 ± 1.7	0.15 ± 0.03
50 (Standard)	60.9 ± 11.2	0.15 ± 0.02
75	38.8 ± 5.7	0.12 ± 0.01
100	47.8 ± 12.3	0.094 ± 0.02

Table 15: *Effect of Phosphate Concentration on the Myrosinase Catalysed Hydrolysis of PNPG*

2.3.6 Discussion of Results

It can be observed by comparing the results for sulfate inhibition (Tables 12 and 13) with those from the higher phosphate incubations (Tables 14 and 15) that the inhibitory effect of sulfate is not much greater than that due to increased phosphate levels. It may therefore be that the sulfate inhibition is a non-specific salt effect, or that phosphate is also capable of binding at the active site. Phosphoglucosinolates (mentioned in Section 1.1.5) have been synthesised, but it is not known whether they act as substrates for myrosinase. Certainly it can be concluded that sulfate inhibits myrosinase only weakly, and as a consequence, no definite type of inhibition could be assigned for determining the product debinding order.

2.3.7 Inhibition of Myrosinase by 2-Mercaptoethanesulfonate (2-MESNA) (74)

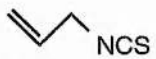


(74)

The inhibitory properties of the aglucone mimics used in the transglycosylation experiments (Section 2.1.11.1) were examined. Thus the hydrolysis of PNPG was carried out in the presence of 20 mM 2-mercaptoethanesulfonate (2-MESNA) (74). As for the normal PNPG incubations, these incubations were carried out in 50 mM potassium phosphate buffer, at pH 7.0, at 37 ± 0.1 °C. The values of the kinetic parameters for the hydrolysis reaction in the presence of 20 mM 2-MESNA were K_M = 93.8 ± 26.6 mM and V_{max} = 0.21 ± 0.05 x10^{-3} mol dm^{-3} min^{-1}.

It can be seen by comparing the kinetic parameters for the incubations using 20 mM 2-MESNA with those for PNPG that the value of V_{max} remained relatively unchanged within experimental error, whilst the value of K_M was increased from 60.9 ± 11.2 mM to 93.8 ± 26.6 mM. These results indicate that 2-MESNA acts as a competitive inhibitor of myrosinase, and the value of K_I was calculated as 37.0 ± 10 mM.

2.3.8 Inhibition of Myrosinase by Allyl Isothiocyanate (Allyl NCS) ((4), R = Allyl)



(4) R = Allyl

Since the isothiocyanate is the major ultimate product of glucosinolate hydrolysis, and that the commonly occurring glucosinolate sinigrin has an allyl side chain, the inhibition of myrosinase by allyl isothiocyanate was investigated. Due to the poor solubility of allyl isothiocyanate in aqueous media, the highest concentration available for inhibition studies in water was 5 mM. The hydrolysis reaction of PNPG was therefore carried out in the presence of 5 mM allyl isothiocyanate under standard conditions. The values of the kinetic parameters for the hydrolysis reaction in the presence

of 5 mM allyl isothiocyanate were K_M = 59.5 ± 20.8 mM and V_{max} = 0.15 ± 0.04 x10^{-3} mol dm^{-3} min^{-1}.

It can be observed that, for the concentration measured, allyl isothiocyanate has no inhibitory effect on the hydrolysis of PNPG as catalysed by myrosinase. This lack of inhibition prompted an investigation into the effect of allyl isothiocyanate upon myrosinase activity.

2.3.9 Investigation of the Effect of Allyl Isothiocyanate On Myrosinase Activity

Allyl isothiocyanate is an acylating reagent, capable of reacting with nucleophilic centres in proteins. Potentially, it could therefore irreversibly inactivate myrosinase on prolonged incubation. In order to examine this point the change in activity with time of a standard myrosinase solution was compared with that of a similar solution containing 5 mM allyl isothiocyanate. The solutions were incubated at 37 ± 0.1 °C in 33.1 mM potassium phosphate buffer at pH 7.0, and the activity of each solution was measured at various intervals using the standard sinigrin assay. The plot for these incubations is shown in Figure 35.

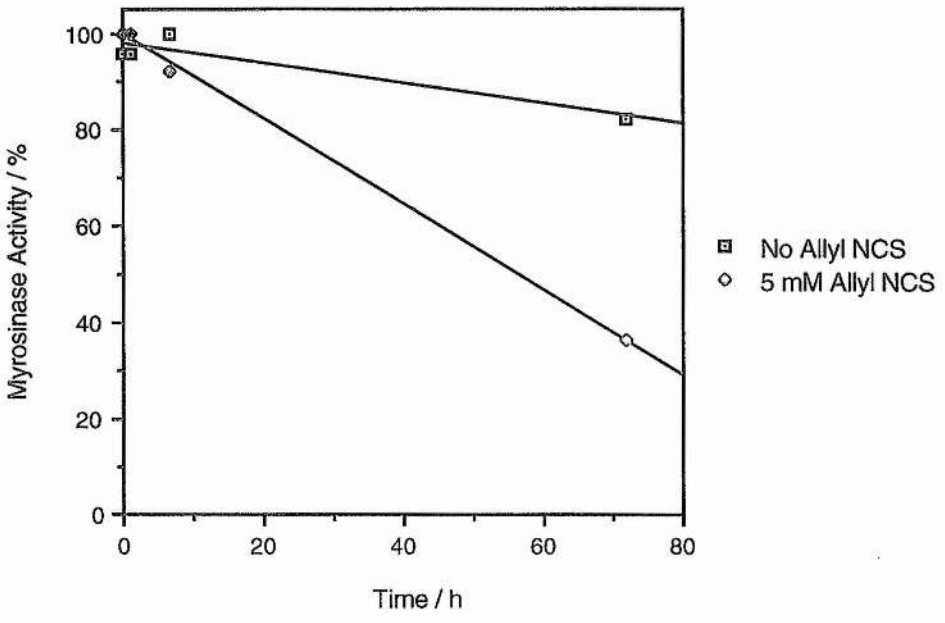
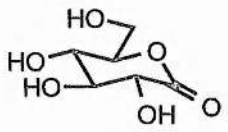


Figure 35: *Effect of Allyl Isothiocyanate Upon Myrosinase Activity*

From the plot in Figure 35, it can be seen that allyl isothiocyanate has only a small destabilising effect on myrosinase, the half-life being 57 hours. The enzyme retained 36% of activity after a 3 day incubation in the presence of 5 mM allyl isothiocyanate, compared to 82% without. Perhaps this should be expected as the enzyme ought to be stable in the presence of its product.

Overall, the combination of the poor mixed inhibition of myrosinase by β-D-glucose, the very weak inhibition by sulfate and the lack of inhibition by allyl isothiocyanate made identification of the product debinding order impossible.

2.3.10 Inhibition of Myrosinase by D-Glucono-γ-lactone (44)



(44)

As was discussed in Section 1.2.4, D-glucono-γ-lactone (44) has been found to be a potent competitive inhibitor of almost all β-glucosidases. The compound is a classical transition state mimic for the hydrolysis of β-glucosides. Since myrosinase had already exhibited mechanistic differences to β-glycosidases, in terms of its lack of transglycosylation activity, the inhbition by D-glucono-γ-lactone was examined. The degree and type of inhibition may shed further light on the mechanism and active site structure of myrosinase.

Concentrations in the range 1 to 20 mM D-glucono-γ-lactone were used for the study of the inhibition of the myrosinase catalysed hydrolysis of sinigrin. As for the normal sinigrin incubations, these incubations were carried out in 33.1 mM potassium phosphate buffer, at pH 7.0, at 37 ± 0.1 °C. Since D-glucono-γ-lactone can hydrolyse slowly in solution, fresh solutions of D-glucono-γ-lactone were always used immediately after preparation. The results for these studies are presented in Table 16 and Figure 36.

[D-Glucono-γ-lactone]/mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	0.42 ± 0.05	4.83 ± 0.22
1	0.50 ± 0.07	3.75 ± 0.19
5	0.51 ± 0.09	2.48 ± 0.17
10	0.51 ± 0.07	1.90 ± 0.09
20	0.51 ± 0.10	1.75 ± 0.12

Table 16: *Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by D-Glucono-γ-lactone*

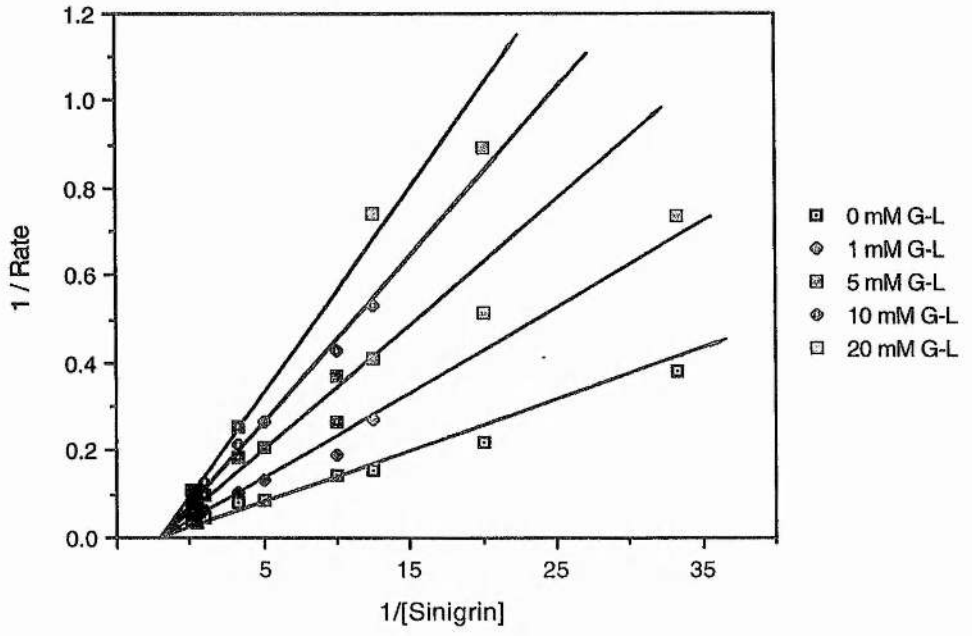


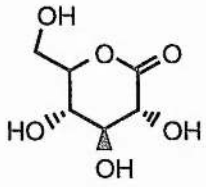
Figure 36: *Lineweaver-Burk Plot for Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by D-Glucono-γ-lactone*

2.3.11 Discussion of Results

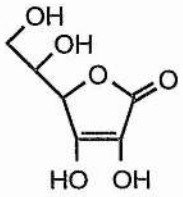
It can be observed from Table 16 that with increasing inhibitor concentration, K_M stays relatively constant whilst V_{max} decreases. This is indicative of noncompetitive inhibition, which is in complete contrast to the potent competitive inhibition exerted by D-glucono-γ-lactone on β-glucosidases (Section 1.2.4). By comparing the Lineweaver-Burk plots in Figures 33 and 36, it can be seen that D-glucono-γ-lactone indeed exhibits classical noncompetitive inhibition for myrosinase.

Using Equation 8 (Section 2.2.1), the value of K_I for D-glucono-γ-lactone in the absence of L-ascorbic acid was calculated to be 6.65 ± 4.0 mM. This is much higher than the K_I value reported for *Agrobacterium faecalis* β-glucosidase of 1 μM.[132]

The fact that D-glucono-γ-lactone inhibits myrosinase noncompetitively means that it must bind to the enzyme at a site other than that of the substrate as it can bind at the same time as the substrate. An alternative binding site does exist for myrosinase, namely that for L-ascorbic acid acting as a specific activator. The structures of D-glucono-γ-lactone (44) and L-ascorbic acid (75) are shown in Figure 37.



(44)



(75)

Figure 37: *Structures of D-Glucono-γ-lactone (44) and L-Ascorbic Acid (75)*

Therefore, to investigate this further, and to confirm or deny the competition of D-glucono-γ-lactone at the active site of L-ascorbic acid, studies of the inhibition of myrosinase by D-glucono-γ-lactone in the presence of L-ascorbic acid were carried out.

2.3.12 Inhibition of Myrosinase by D-Glucono-γ-lactone in the Presence of 1 mM L-Ascorbic Acid

Concentrations of 1 and 10 mM D-glucono-γ-lactone were used for the study of the inhibition of the myrosinase catalysed hydrolysis of sinigrin in the presence of 1 mM L-ascorbic acid. As for the normal sinigrin incubations, these incubations were carried out in 33.1 mM potassium phosphate buffer, at pH 7.0, at 37 ± 0.1 °C. The results for these studies are presented in Table 17.

[D-Glucono-γ-lactone]/mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	0.40 ± 0.07	7.12 ± 0.22
1	0.31 ± 0.10	5.30 ± 0.50
10	0.42 ± 0.06	1.52 ± 0.07

Table 17: *Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by D-Glucono-γ-lactone in the Presence of 1 mM L-Ascorbic Acid*

The reaction curves for these inhibition studies are shown in Figure 38.

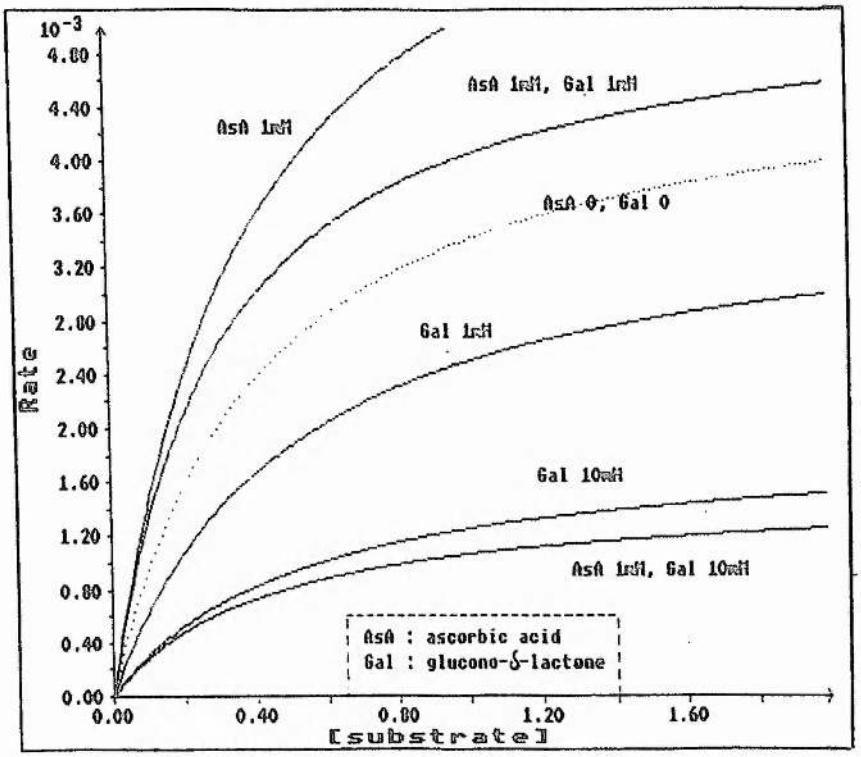


Figure 38: *Reaction Curves for Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin in the Presence of No or 1 mM L-Ascorbic Acid*

2.3.13 Discussion of Results

It can be seen from Table 17 that in the presence of 1 mM L-ascorbic acid, V_{max} is increased by a factor of 1.5, leaving K_M unaffected. These effects on the kinetic parameters are consistent with those obtained for *Wasabia japonica* myrosinase.[48] In the presence of 1 mM D-glucono-γ-lactone, however, there is an increase of 1.4-fold. Then at 10 mM D-glucono-γ-

lactone, there is essentially no increase at all, and in fact the rate is 20% lower than that without L-ascorbic acid. This is possibly the result of some small errors in measurement. It therefore appears that the D-glucono-γ-lactone competes out the activating effect of L-ascorbic acid. This would imply that they are binding at the same site on the enzyme. Determination of K_I for D-glucono-γ-lactone under these conditions (*i.e.* 1 mM L-ascorbic acid) in fact gives a lower value (2.82 ± 0.1 mM), essentially as a result of the greater overall decrease in rate observed.

2.3.14 Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by D-Glucono-γ-lactone

In order to make a comparison with the inhibition of the myrosinase catalysed hydrolysis of sinigrin, D-glucono-γ-lactone was studied for its inhibitory effects on the hydrolysis of PNPG. Since L-ascorbic acid does not increase the rate of hydrolysis of PNPG (discussed in Section 1.1.5), the inhibitory effect of D-glucono-γ-lactone on myrosinase in the presence of L-ascorbic acid could not be made in this instance.

Concentrations between 5 and 20 mM D-glucono-γ-lactone were used. As for the normal PNPG incubations, these incubations were carried out in 50 mM potassium phosphate buffer, at pH 7.0, at 37 ± 0.1 °C. The results for these studies are presented in Table 18 and Figure 38.

[D-Glucono-γ-lactone]/mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	60.9 ± 11.2	0.15 ± 0.02
5	63.4 ± 9.2	0.075 ± 0.008
10	113.6 ± 41.8	0.086 ± 0.027
20	106.1 ± 93.3	0.033 ± 0.025

Table 18: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by D-Glucono-γ-lactone*

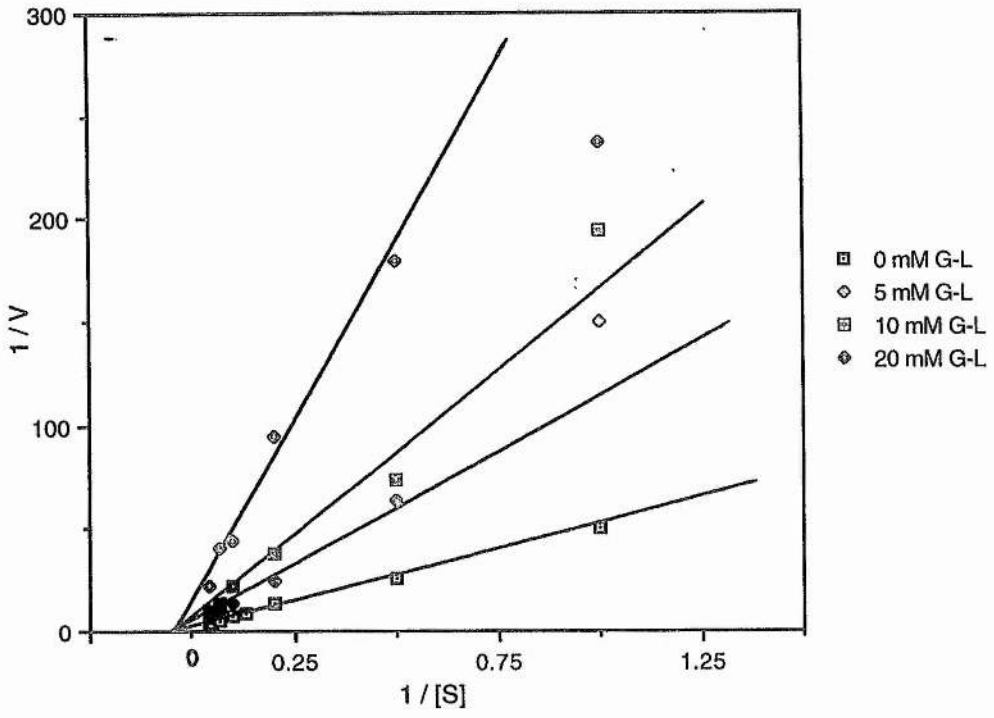
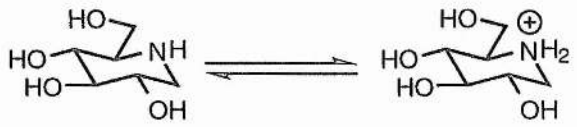


Figure 38: *Lineweaver-Burk Plot for Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by D-Glucono-γ-lactone*

2.3.15 Discussion of Results

It can be observed from the Lineweaver-Burk plot in Figure 38 that D-glucono-γ-lactone exhibits noncompetitive inhibition for PNPG also. This confirms the results for sinigrin inhibition. The value of K_I for the inhibition of the myrosinase catalysed hydrolysis of PNPG by D-glucono-γ-lactone, which was calculated from the factor $(1 + [I]/K_I)$, is 8.0 ± 4.2 mM, which shows a similar degree of inhibition as for sinigrin hydrolysis, implying that binding is occurring at the same site.

2.3.16 Inhibition of Myrosinase by 1-Deoxynojirimycin (45)



(45)

Another potent inhibitor of β-glycosidases was then investigated, *i.e.* 1-deoxynojirimycin (45). This compound has been found to inhibit a range of β-glycosidase enzymes (see Section 1.2.4).

Concentrations between 0.2 to 1.0 mM 1-deoxynojirimycin were used for the study of the inhibition of the myrosinase catalysed hydrolysis of sinigrin. Only a limited range of concentrations compared with that for D-glucono-γ-lactone was used, due to the high cost of 1-deoxynojirimycin. As for the normal sinigrin incubations, these incubations were carried out in 33.1 mM potassium phosphate buffer, at pH 7.0, at 37 ± 0.1 °C. The results for these studies are presented in Table 19.

[1-DNM] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	0.42 ± 0.05	4.83 ± 0.22
0.2	0.51 ± 0.10	3.08 ± 0.21
0.5	0.75 ± 0.18	3.31 ± 0.32
1.0	0.80 ± 0.11	3.36 ± 0.19

Table 19: *Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by 1-Deoxynojirimycin*

2.3.17 Discussion of Results

It can be seen from Table 19 that for the inhibition of the myrosinase catalysed hydrolysis of sinigrin by 1-deoxynojirimycin, V_{max} is reduced by 0.2 mM 1-deoxynojirimycin, but with increasing inhibitor concentration, V_{max} actually increases again. The values of K_M increase steadily with increasing inhibitor concentration.

These figures indicate that 1-deoxynojirimycin does not have a very strong inhibitory effect on myrosinase. This is in contrast to the strong competitive inhibition exhibited by 1-deoxynojirimycin on β-glucosidases (Section 1.2.4). This may be due to the fact that myrosinase has been proposed to have a glutamine residue instead of an acid/base catalytic residue for aglucone protonation/deprotonation (see Section 1.1.5). The tight-binding competitive inhibition by 1-deoxynojirimycin on β-glucosidases suggests an interaction of the positive charge of the protonated nitrogen on the sugar ring with a carboxylate anion at the enzyme active site (or alternatively an

interaction of the lone pair on the nitrogen with the proton of the acid). This interaction is shown in Figure 39.

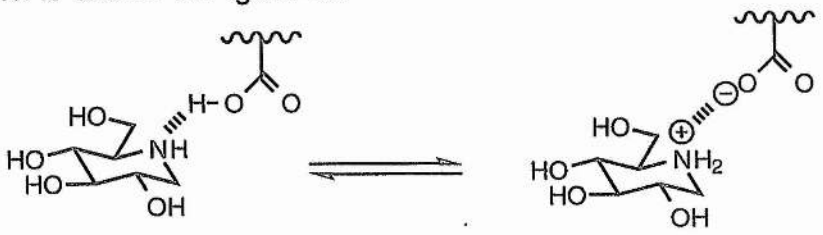
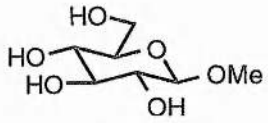


Figure 39: *Interaction Between 1-Deoxynojirimycin and the Amino Acid Carboxylate Group at the Active Site of β-Glucosidases*[132]

The fact that 1-deoxynojirimycin is such a poor inhibitor of myrosinase is in agreement with the proposition that the active site acid/base residue present in β-glucosidases is replaced by a glutamine residue in myrosinase, since no such interaction is possible.

2.3.18 Inhibition of Myrosinase by Methyl-β-D-glucopyranoside (53)



(53)

It has already been shown (Section 2.1.11.1) that methyl-β-D-glucopyranoside (53) does not act as a substrate for myrosinase. For the study of the inhibition of myrosinase catalysed hydrolysis of sinigrin, concentrations between 10 to 30 mM methyl-β-D-glucopyranoside were used. As for the normal sinigrin incubations, these incubations were carried out in 33.1 mM potassium phosphate buffer, at pH 7.0, at 37 ± 0.1 °C. The results for these studies are presented in Table 20.

[Me-β-D-Glucoside]/mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	0.42 ± 0.05	4.83 ± 0.22
10	0.41 ± 0.02	4.18 ± 0.08
20	0.38 ± 0.05	4.11 ± 0.18
30	0.31 ± 0.02	4.09 ± 0.10

Table 20: *Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by Methyl-β-D-glucopyranoside*

For the study of the inhibition of the myrosinase catalysed hydrolysis of PNPG, 5 mM methyl-β-D-glucopyranoside was used. As for the normal PNPG incubations, this incubation was carried out in 50 mM potassium phosphate buffer, at pH 7.0, at 37 ± 0.1 °C. The results for this study are presented in Table 21.

[Me-β-D-Glucoside]/mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	60.9 ± 11.2	0.15 ± 0.02
5	79.5 ± 66.2	0.21 ± 0.15

Table 21: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by Methyl-β-D-glucopyranoside*

2.3.19 Discussion of Results

It can be seen from Table 20 for the inhibition of the hydrolysis of sinigrin that with increasing methyl-β-D-glucopyranoside concentration, K_M decreases and V_{max} stays relatively constant. In Table 21 it can be seen that methyl-β-D-glucopyranoside does not inhibit PNPG hydrolysis. The result obtained is somewhat misleading, however, since only one concentration of inhibitor was used and the error factors are large. The results given for sinigrin hydrolysis appear to be more reliable in this instance.

The inhibition pattern for sinigrin hydrolysis is indicative of competitive inhibition. However, the inhibitory effect is very poor, and the K_I value for methyl-β-D-glucopyranoside was calculated to be 120 mM, demonstrating that the compound binds very weakly at the substrate active site of

myrosinase. Methyl-β-D-glucopyranoside is also a poor competitive inhibitor of β-glucosidases, with a K_I of around 400 mM.

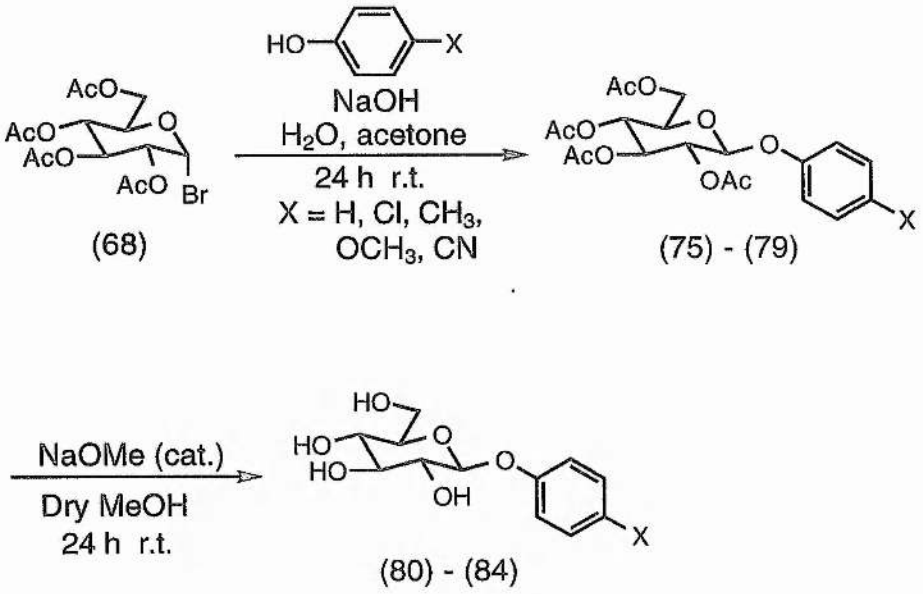
2.4 Investigation of the Electronic Requirements for Myrosinase Catalysed Reactions: The Brønsted Plot

2.4.1 Synthesis of Substituted Aryl-β-D-glucopyranosides for the Brønsted Plot

In order to gain information on the electronic requirements of the β-glucosidase action of myrosinase, and make comparisons with β-glucosidases, the construction of a Brønsted plot (as for *Agrobacterium faecalis* β-glucosidase (see Section 1.2.1)) was planned. A range of substituted aryl-β-D-glucopyranosides with varying degrees of aglucone leaving group ability (*i.e.* varying pK_as of the phenols) was synthesised in order for this to be carried out.

The synthesis of the required aryl-β-D-glucopyranosides (80) to (84) was carried out according to the method used for a similar range of aryl-β-D-galactopyranosides.[165] The more reactive compound, 2,4-dintrophenyl-β-D-glucopyranoside (2,4-DNPG) (88), was synthesised in an alternative way (Scheme 32) according to literature methods.[166,167,168] The *p* and *o*-nitrophenyl-β-D-glucopyranosides (15) and (16) were obtained commercially. The measurement of the kinetic parameters for (15) was described in Section 2.1.7.

For the synthesis of the aryl-β-D-glucopyranosides (80) to (84), the sodium salt of the appropriate phenol was condensed with 2,3,4,6-tetra-*O*-acetyl-1-bromo-α-D-glucopyranose (68) in aqueous acetone for 24 hours at room temperature. Subsequent deacetylation of the 2,3,4,6-tetra-*O*-acetyl-aryl-β-D-glucopyranoside compound using catalytic sodium methoxide in anhydrous methanol afforded the relevant aryl-β-D-glucopyranoside. These compounds were purified by recrystallisation from water. Both steps of this synthesis went cleanly without the production of any by-products. This synthesis is shown in Scheme 31.



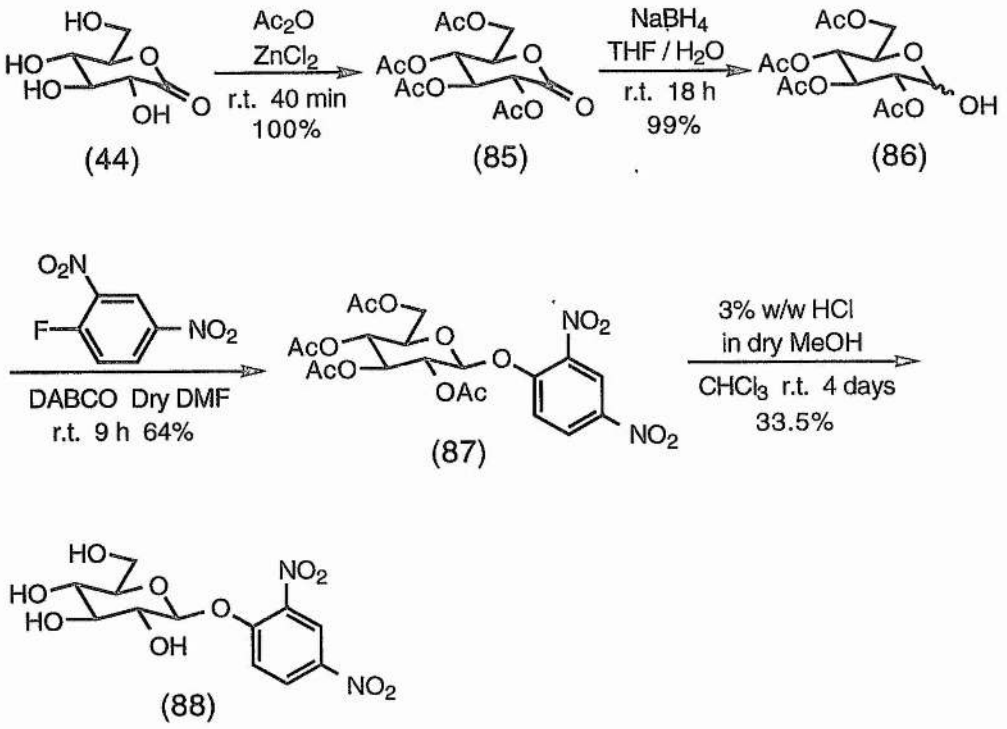
Scheme 31: *General Synthetic Method Used for the Range of Substituted Aryl-β-D-glucopyranosides for the Construction of the Brønsted Plot* [165]

The overall yields of these syntheses were in the region of 8 to 30%, and all of the products were pure according to t.l.c. (ethyl acetate/methanol/water (16:2:1)). The structures of these compounds were comfirmed from the agreement of their melting points with those from the literature, their microanalysis results and their mass spectra. The *p*- and *o*-nitrophenyl-β-D-glucopyranosides (15) and (16) of the series were obtained commercially.

In order to ensure the deacetylation reaction occurred smoothly, methanolic sodium methoxide was prepared fresh each time before use. Fresh anhydrous methanol was made at regular intervals.

2,4-Dinitrophenyl-β-D-glucopyranoside (2,4-DNPG) (88) was synthesised by firstly acetylating D-glucono-γ-lactone (44) using acetic anhydride in the presence of zinc chloride, according to a method reported in the literature.[166] This reaction was complete in 40 minutes and gave a quantitative yield of 2,3,4,6-tetra-*O*-acetyl-D-glucono-γ-lactone (85) as a colourless syrup. This compound was then reduced at C-1 using sodium borohydride in a tetrahydrofuran/water mixture in an analogous method to that used for production of the deuterated compound,[167] to give 2,3,4,6-

tetra-*O*-acetyl-α/β-D-glucopyranose (86) in 99% yield. ^{13}C N.M.R. studies revealed that this compound was present as an approximate 3 to 1 mixture of the α and β anomers, from the intensities of the resonances due to C-1. This compound was then reacted with 1-fluoro-2,4-dinitrobenzene in the presence of 2,2,2-diazabicyclooctane (DABCO) in dry dimethylformamide for 9 hours at room temperature, according to a literature method,[168] to afford 2,3,4,6-tetra-*O*-acetyl-2',4'-dinitrophenyl-β-D-glucopyranoside (87) (exclusively the β anomer) in 64% yield. Finally, this compound was deacetylated using 3% w/w HCl in dry methanol/chloroform, according to a literature method.[169] These conditions were used for the deacetylation reaction since sodium methoxide in dry methanol, *i.e.* strongly basic conditions, would result in removal of the aglucone moiety due to its good leaving ability. The crude deacetylated product was purified by silica chromatography using ethyl acetate/methanol/water (16:1:1) as the eluent. The yield for this step was 33.5%. The overall yield for the synthesis of 2,4-dinitrophenyl-β-D-glucopyranoside (88) from D-glucono-γ-lactone was 21%. The structure of 2,4-dinitrophenyl-β-D-glucopyranoside (88) was confimed its melting point (108-110 °C (lit.,[170] 100-1 °C)) and its ^{1}H and ^{13}C N.M.R. spectra. The synthesis of 2,4-dinitrophenyl-β-D-glucopyranoside is shown in Scheme 32.



Scheme 32: *Synthesis of 2,4-Dinitrophenyl-β-D-glucopyranoside (2,4-DNPG)*[166,167,168,169]

Approximately 0.5 g of each aryl-β-D-glucopyranoside (80) to (84) and (88) was prepared for the construction of the Brønsted plot. The overall yields and aglucone (phenol) pK_a values for these compounds are shown in Table 22.

Cpd. no.	Compound Name	Yield / %	Aglucone pK_a
88	2,4-Dinitrophenyl-β-D-glucopyranoside	21	4.10
15	*p*-Nitrophenyl-β-D-glucopyranoside	N/A	7.15
16	*o*-Nitrophenyl-β-D-glucopyranoside	N/A	7.17
80 (X=CN)	*p*-Cyanophenyl-β-D-glucopyranoside	9.5	8.49
81 (X=Cl)	*p*-Chlorophenyl-β-D-glucopyranoside	30	9.38
82 (X=H)	Phenyl-β-D-glucopyranoside	24.5	9.99
83 (X=OMe)	*p*-Methoxyphenyl-β-D-glucopyranoside	8	10.22
84 (X=Me)	*p*-Methylphenyl-β-D-glucopyranoside	12.5	10.24

Table 22: *Overall Yields and pK_a Values for the Range of Aryl-β-D-glucopyranosides for the Construction of the Brønsted Plot (PNPG and ONPG Were Obtained Commercially)*

2.4.2 Incubations of Aryl-β-D-glucopyranosides (16), (80) - (84) and (88) with Myrosinase for the Construction of the Brønsted Plot

The measurement of the kinetic parameters for PNPG (15) with myrosinase have been discussed in Section 2.1.7. Incubations of compounds (16), (80) to (84) and (88) with myrosinase were carried out using the same conditions as those used for PNPG, *i.e.* in 50 mM potassium phosphate buffer, pH 7.0, at 37 ± 0.1 °C.

For compounds (80) to (84), incubations were run for at least 24 hours. For these incubations, the region 250-350 nm was scanned at intervals for each reaction to monitor any increase in absorbance due to the release of the appropriate phenol. Literature[165] λ_{max} values for a similar range of phenols were all around the 270-290 nm region. In addition to monitoring the release of the appropriate phenol, the incubation samples were monitored for any glucose released using the Glucose HK assay described in Section 4.16.1.

No changes in absorbances in the region 250-350 nm were observed for any of the reactions. Also, none of the reactions produced any glucose. It was thus concluded that none of the aryl-β-D-glucopyranosides (80) to (84) is an active substrate for myrosinase. These findings rendered the Brønsted plot unobtainable.

o-Nitrophenyl-β-D-glucopyranoside (ONPG) (16) was found to act as a substrate for myrosinase, and thus incubations were carried out in identical manner to those described for PNPG in water, except that on measuring the increase in absorbance at 430 nm due to the product, *o*-nitrophenol, the extinction coefficient, $\varepsilon = 1783 \pm 144$ mol^{-1} dm^3 cm^{-1} (measured experimentally in the same way as for *p*-nitrophenol) was used. The results of these incubations are presented in Figure 40 and Table 23.

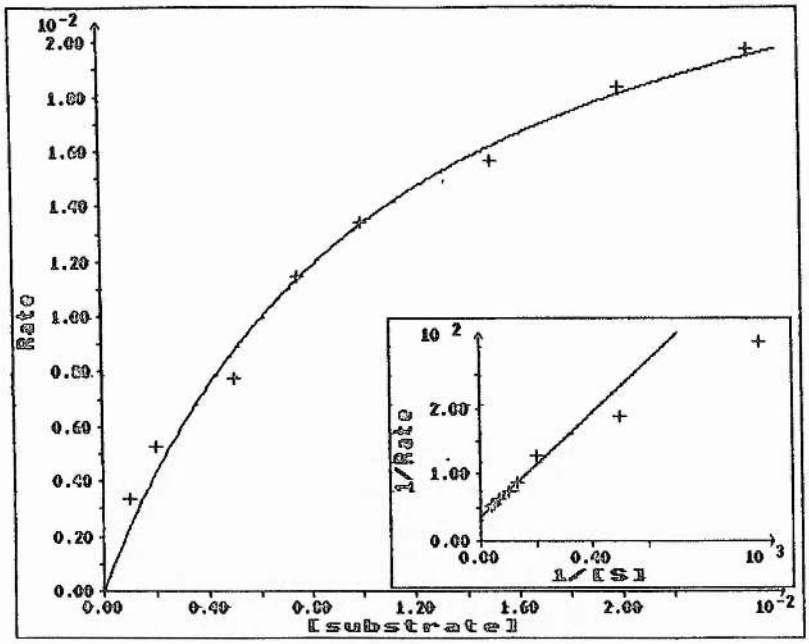


Figure 40: *Myrosinase Catalysed Hydrolysis of ONPG*

Substrate	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
ONPG	11.0 ± 1.7	0.016 ± 0.001

Table 23: *Kinetic Parameters for the Myrosinase Catalysed Hydrolysis of ONPG*

2,4-Dinitrophenyl-β-D-glucopyranoside (2,4-DNPG) (88) was also found to act as a substrate for myrosinase. The kinetic parameters for the myrosinase catalysed hydrolysis of 2,4-DNPG were measured by monitoring the release of 2,4-dinitrophenol at 361 nm ($\varepsilon = 15{,}480 \pm 3751$ mol^{-1} dm^3 cm^{-1}, measured experimentally in the same way as for *p*-nitrophenol). Incubations of concentrations 0.10 to 10 mM 2,4-DNPG (these concentrations were lower than those for PNPG in order to keep

absorbance values small due to the large extinction coefficient for 2,4-dinitrophenol) with myrosinase were carried out in 50 mM potassium phosphate buffer, at pH 7.0 at 37 ± 0.1 °C. The results for these incubations are shown in Figure 41 and Table 24.

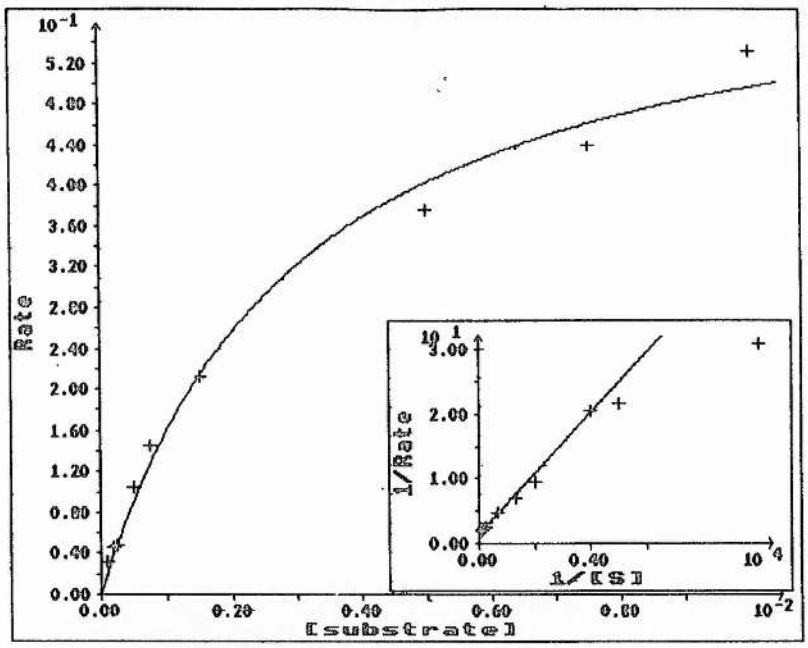


Figure 41: *Myrosinase Catalysed Hydrolysis of 2,4-DNPG*

Substrate	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
2,4-DNPG	3.30 ± 0.53	0.044 ± 0.003

Table 24: *Kinetic Parameters for the Myrosinase Catalysed Hydrolysis of 2,4-DNPG*

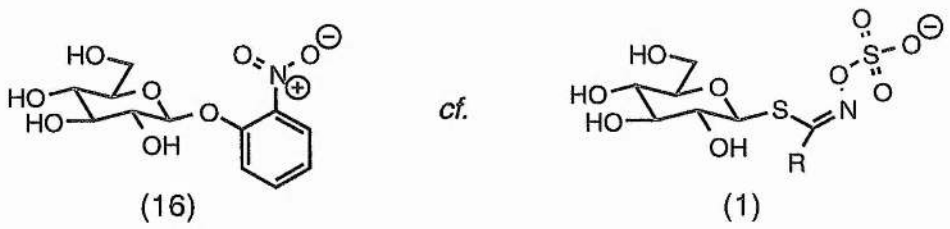
2.4.3 Discussion of Results

By comparing the fact that compounds (80) to (84) do not act as substrates for myrosinase whilst PNPG (15) and ONPG (16) do, points to the fact that these unnatural substrates have to be fairly reactive in order to be hydrolysed by myrosinase. It should be noted here that the pK_a value for the glucosinolate aglucone leaving group was estimated as 3.0.[1]

p-Cyanophenyl-β-D-glucopyranoside (84) has an aglucone pK_a value of 8.49 and is not hydrolysed, whereas ONPG (16), with its aglucone pK_a

value of 7.17, is. This means that aryl-β-D-glucopyranosides with an aglucone pK_a value lower than or equal to about 7.2 only act as substrates for myrosinase. These findings were further investigated by the study of a range of other potential substrates for myrosinase (Section 2.5).

By comparing the kinetic parameters for PNPG hydrolysis in Table 8 with those for ONPG in Table 23, it can be seen that V_{max} for ONPG is approximately one tenth of that for PNPG. This difference is very large considering that the pK_a values for *p*-nitrophenol and *o*-nitrophenol are 7.15 and 7.17 respectively, *i.e.* the leaving group abilities are fairly similar. Another important point to note is that the value of K_M is much lower for ONPG than for PNPG. This means that ONPG binds much tighter at the enzyme active site than PNPG. An explanation for this tighter binding may be due to the fact that the *ortho* nitro group of ONPG sits in the same position as the sulfate group of glucosinolates. The charged nitro group of ONPG may have similar binding interactions at the enzyme active site to the sulfate group of glucosinolates. This is shown in Figure 42.



(16) (1)

Figure 42: *The ortho Nitro Group of ONPG is in the Same Position as the Sulfate Group of Glucosinolates and May Have Similar Binding Interactions at the Active Site of Myrosinase*

The kinetic parameters for the myrosinase catalysed hydrolysis of 2,4-dinitrophenyl-β-D-glucopyranoside (2,4-DNPG) (Table 25) indicate that the substrate binds tightly at the active site relative to PNPG due to the low value for K_M. The substrate specificty values (*i.e.* V_{max}/K_M) for PNPG, ONPG and 2,4-DNPG were calculated as 0.0025, 0.0015 and 0.013 min^{-1} respectively, implying that 2,4-DNPG is the best substrate for myrosinase by a factor of about 10. A comparison of the kinetic parameters for 2,4-DNPG with those for the corresponding deuterated compound were made for the investigation of the α-deuterium kinetic isotope effect (Section 2.6.11).

Although a proper Brønsted plot cannot be drawn, there is a distinct variation in *V/K* for the three nitrophenyl-β-D-glucopyranoside substrates. An admittedly unsatisfactory plot of log *V/K* *v* pK_a with only three points can be drawn (Figure 43). This would give an estimate of β_{lg} as -0.27.

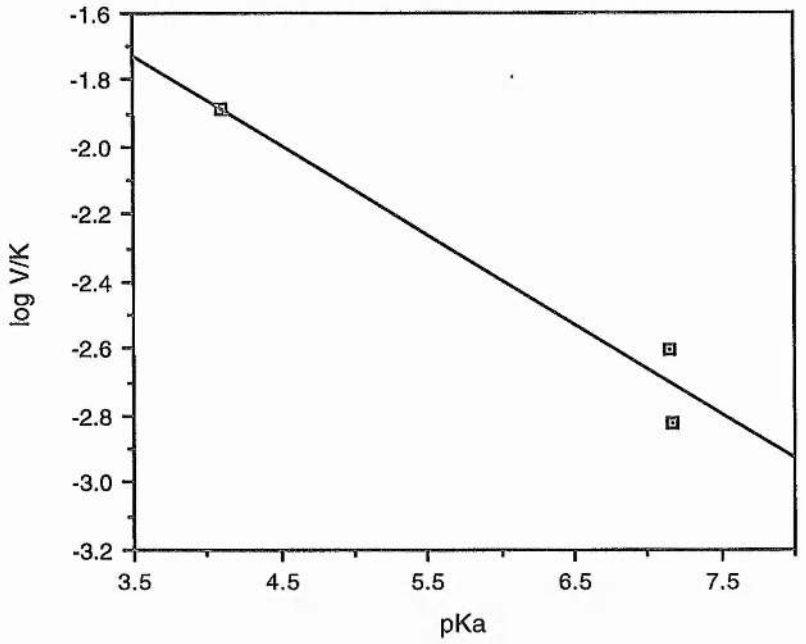


Figure 43: *Plot of log V/K vs pK_a for the Myrosinase Catalysed Hydrolysis of 2,4-DNPG, PNPG and ONPG*

Literature work with β-glucosidase from *Agrobacterium faecalis*[132] and sweet almond β-glucosidase[131] gave β_{lg} values of -0.7 in both cases, while the Mg^{2+} free *E. coli (lacZ)* β-galactosidase[131] gave β_{lg} = -0.5. The difference in these cases is that a biphasic relationship was observed. Thus for phenols with $pK_a < 8$, the rate was independent of pK_a ($\beta_{lg} = 0$), and a variation was only observed with phenols of $pK_a > 8$. With myrosinase, however, the rate does vary for leaving groups with $pK_a < 8$, but the others do not react at all.

Certainly, there is a major difference in behaviour between the β-glycosidases and myrosinase. Too much weight cannot be given to the value of β_{lg} obtained for myrosinase, but it may imply less cleavage of the glycosidic bond at the transition state than for the other β-glycosidases.[131,132]

2.5 Studies on Alternative Substrates for Myrosinase

Since the *O*-glucopyranosides were not readily hydrolysed by myrosinase, a range of *S*-glucopyranosides was synthesised to further examine the substrate specificity of the enzyme. Due to the fact that myrosinase is a thioglucosidase, it was thought that simple *S*-glucopyranosides may be more likely to act as substrates for myrosinase. Also, since it appeared that the pK_a for the leaving group had to be quite low for hydrolysis to take place, it was thought that the *S*-glucopyranosides (due to their low aglucone pK_as) would be more likely to act as substrates than their corresponding *O*-glucopyranosides.

Much interest in *S*-glucopyranosides rests in their biochemical action as competitive inhibitors for glycosidases[171] and their consequent use as ligands for affinity chromatography.[172] These compounds do not generally act as substrates for glycosidases due to the stronger carbon-sulfur bond, which more than compensates for the lower aglucone pK_as of the *S*-glucopyranosides relative to the *O*-glucopyranosides.

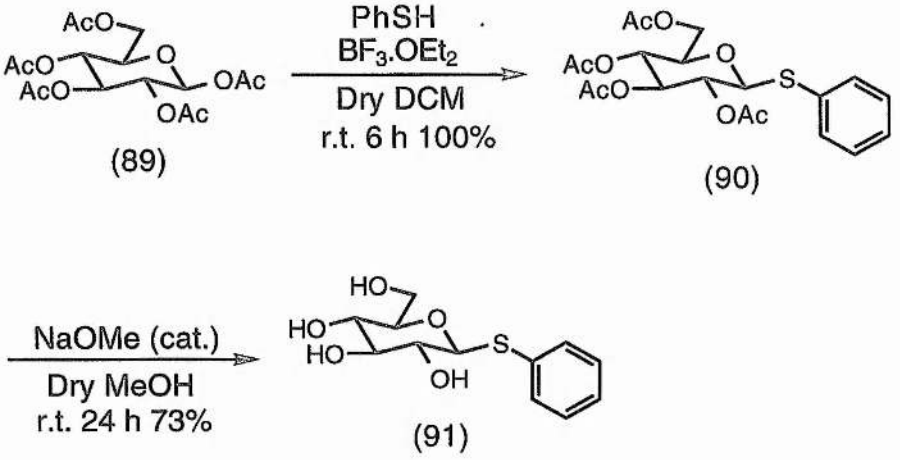
More reactive sulfoxides and sulfones have found use in carbohydrate synthesis for more efficient glycosylation of unreactive substrates.[173] Due to their higher reactivity, a sulfoxide and a sulfone were also synthesised to investigate whether they acted as substrates for myrosinase.

2.5.1 Synthesis of Aryl-1-thio-β-D-glucopyranosides

Several methods for the synthesis of aryl-1-thio-β-D-glucopyranosides can be found in the literature, two of which[174,175] were used for the synthesis of aryl-1-thio-β-D-glucopyranosides. The first method[174] involved reacting 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (89) with boron trifluoride etherate followed by the addition of the appropriate thiophenol. Subsequent deprotection in the usual way, using catalytic sodium methoxide in anhydrous methanol, gave the corresponding aryl-1-thio-β-D-glucopyranoside.

Syntheses using both thiophenol and *p*-nitrothiophenol were attempted using this method but initially both appeared unsuccessful. A further attempt using thiophenol and freshly distilled boron trifluoride etherate

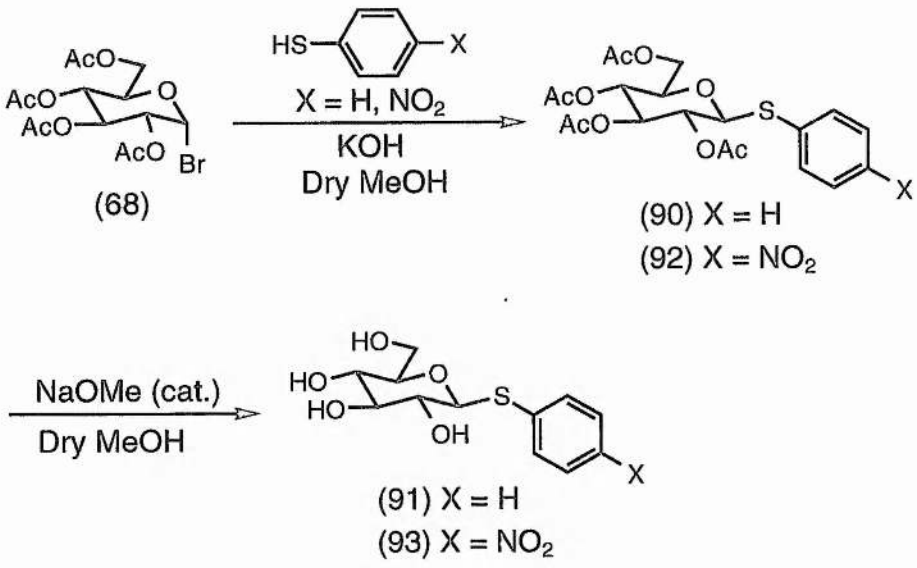
afforded pure phenyl-1-thio-β-D-glucopyranoside (PTG) (91) in good yield. The structure of this compound was confirmed from its melting point (123-6 °C (lit.,[176] 128-130 °C)) and its mass spectrum (*m/z* (CI) 290 ([*M* + NH_4]$^+$, 100%). The synthesis of phenyl-1-thio-β-D-glucopyranoside (PTG) (91) is shown in Scheme 33.



Scheme 33: *Synthesis of Phenyl-1-thio-β-D-glucopyranoside (PTG)*[174]

For the case of the reaction shown in Scheme 33 using *p*-nitrothiophenol, complications arose during the first stage of the reaction, and t.l.c. (hexane/ethyl acetate (2:1)) analysis showed that at least six compounds were present. It was noted at this stage that the commercial *p*-nitrothiophenol was only 80% pure and it was thought that this may have been the cause of the problems. Further attempts using recrystallised *p*-nitrothiophenol were made, but these too were unsuccessful.

The second method[175] is similar to that employed for the synthesis of the aryl-β-D-glucopyranosides (Scheme 32), in that 2,3,4,6-tetra-*O*-acetyl-1-bromo-α-D-glucopyranose (68) is condensed with the potassium salt of the appropriate thiophenol. Deprotection in the usual way using catalytic sodium methoxide in dry methanol affords the relevant aryl-1-thio-β-D-glucopyranoside (91) or (93). This is shown in Scheme 34.

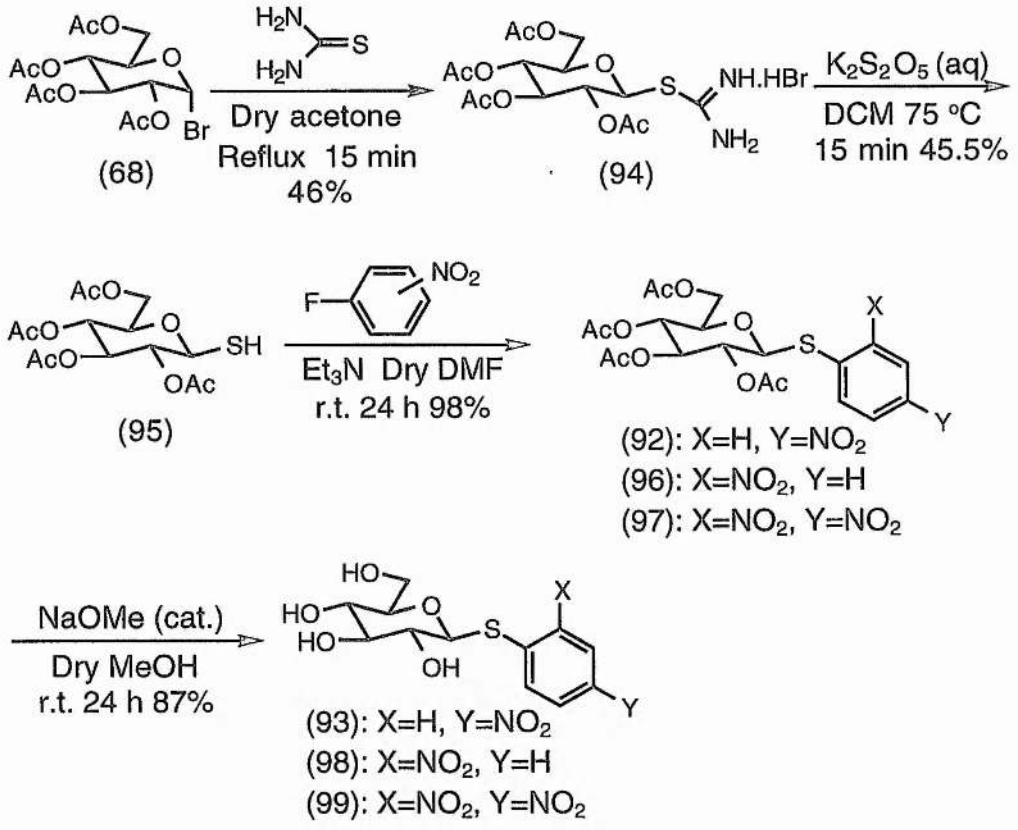


Scheme 34: *Attempted Synthesis of Aryl-1-thio-β-D-glucopyranosides*[175]

The synthesis of *p*-nitrophenyl-1-thio-β-D-glucopyranoside (PNPTG) (93) was attempted using this method, but proved unsuccessful. For the first stage of the reaction, it appeared that the reaction had occurred due to the colour change from red to yellow, signifying a decrease in concentration of the coloured thiolate anion, as described in the literature.[175] However, following deacetylation and recrystallisation, ^{1}H N.M.R. studies showed that only starting materials were present.

An alternative method involved firstly the reaction of 2,3,4,6-tetra-*O*-acetyl-1-bromo-α-D-glucopyranose (68) with thiourea in refluxing dry acetone to form 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylisothiouronium bromide (94), according to a literature method,[177] in 46% yield. This compound was hydrolysed in aqueous potassium metabisulfite at 75 °C to form 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (95) in 45.5% yield.[177] This thiol was then reacted by nucleophilic aromatic substitution, analogous to that reported in the literature,[178] with either 1-fluoro-2-nitrobenzene, 1-fluoro-4-nitrobenzene or 1-fluoro-2,4-dinitrobenzene to form the relevant 2,3,4,6-tetra-*O*-acetyl-aryl-1-thio-β-D-glucopyranoside (92), (96) or (97) in 98% yield. Subsequent deprotection in the usual way using catalytic sodium methoxide in dry methanol afforded the appropriate pure aryl-1-thio-β-D-glucopyranoside (93) (PNPTG), (98) (ONPTG) or (99) (2,4-DNPTG) in 87% yield. The structures of these compounds were confirmed from the agreement of their melting points and $[\alpha]_D$ values with

those from the literature. The synthesis of these compounds is shown in Scheme 35.



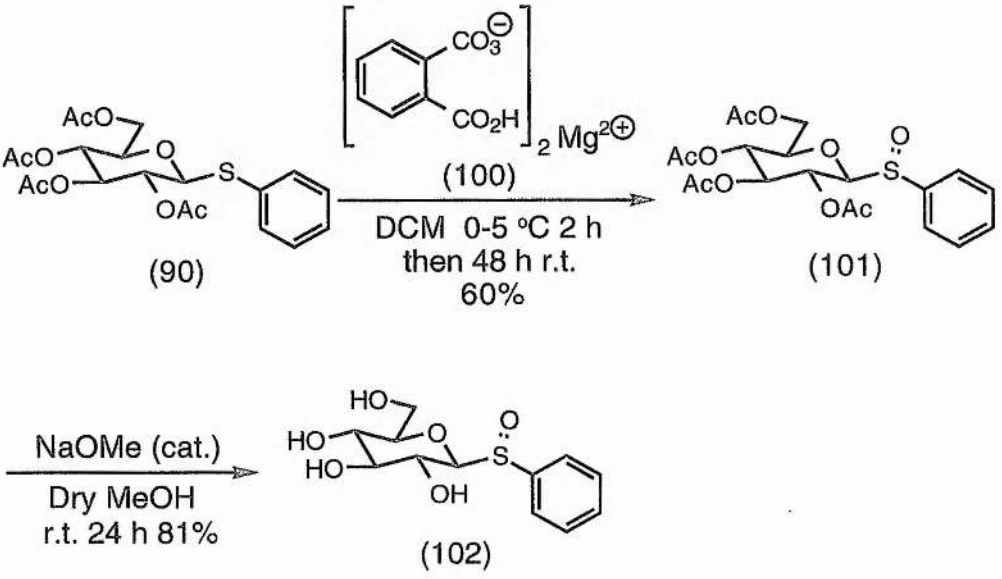
Scheme 35: *Synthesis of o- and p-Nitrophenyl- and 2,4-Dinitrophenyl-1-thio-β-D-glucopyranosides*[177,178]

2.5.2 Synthesis of Phenyl-1-thio-β-D-glucopyranoside sulfoxide (102) and sulfone (105)

These compounds were synthesised by firstly oxidising 2,3,4,6-tetra-*O*-acetyl-phenyl-1-thio-β-D-glucopyranoside (90) under controlled conditions (either adding one or two oxygens) then deacetylating using catalytic sodium methoxide in dry methanol.

The acetylated sulfoxide (101) was synthesised by firstly oxidising 2,3,4,6-tetra-*O*-acetyl-phenyl-1-thio-β-D-glucopyranoside (90) using the mild oxidising agent magnesium monoperoxyphthalate (100), which was added dropwise at 0-5 °C.[179] A small amount of acetylated sulfone (104) was detected by t.l.c. (light petroleum/ethyl acetate (1:1)), so the acetylated

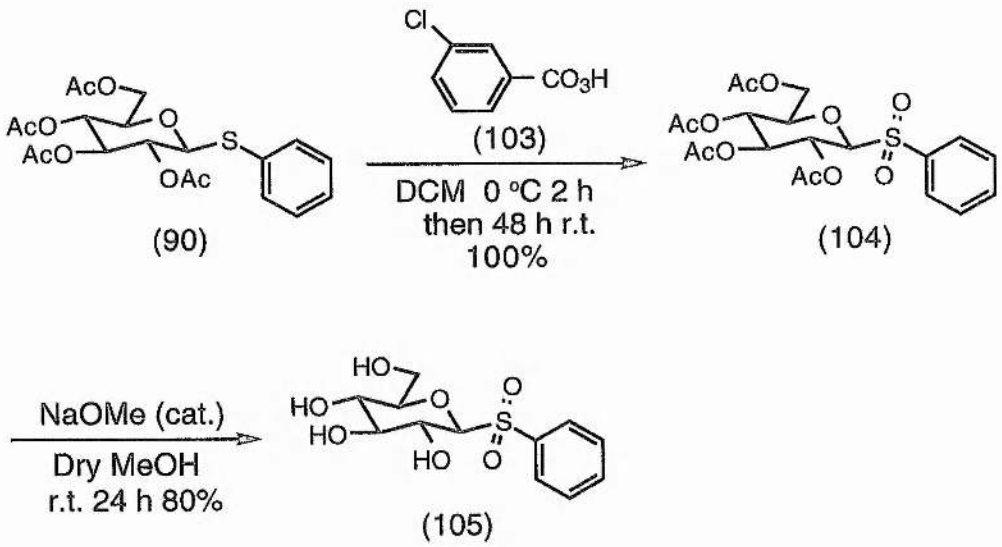
sulfoxide (101) was purified by silica chromatography using light petroleum/ethyl acetate (1:1) as the eluent. Deacetylation of (101) using catalytic sodium methoxide in dry methanol gave the sulfoxide (102), which was purified by silica chromatography using dichloromethane/methanol (4:1) as the eluent. This compound, which has a chiral centre at the sulfur atom, was present as approximately a 3:1 mixture of diastereomers. This was deduced from the ^{13}C N.M.R. spectrum, in which the intensities of the anomeric carbon peaks were in an approximate ratio of 3:1. The structure of phenyl-1-thio-β-D-glucopyranoside sulfoxide (102) was confirmed from its melting point (74-5 °C (lit.,[180] 78-80 °C)) and its N.M.R. spectra. The synthesis of phenyl-1-thio-β-D-glucopyranoside sulfoxide (102) is shown in Scheme 36.



Scheme 36: *Synthesis of Phenyl-1-thio-β-D-glucopyranoside sulfoxide*[179]

The acetylated sulfone (104) was synthesised by oxidising 2,3,4,6-tetra-*O*-acetyl-phenyl-1-thio-β-D-glucopyranoside (90) using 3 equivalents of *m*-chloroperoxybenzoic acid (*m*CPBA) (103).[173] This reaction was done by adding *m*CPBA at 0 °C and then stirring the reaction mixture at room temperature for 48 hours, after which time the reaction was complete according to t.l.c. (light petroleum/ethyl acetate (1:1)). Initial attempts of this reaction using 2 equivalents of *m*CPBA did not go to completion. A further equivalent of *m*CPBA added at the start of the reaction solved this problem. Deacetylation of (104) using catalytic sodium methoxide in dry methanol afforded the pure sulfone (105). The structure of phenyl-1-thio-β-D-

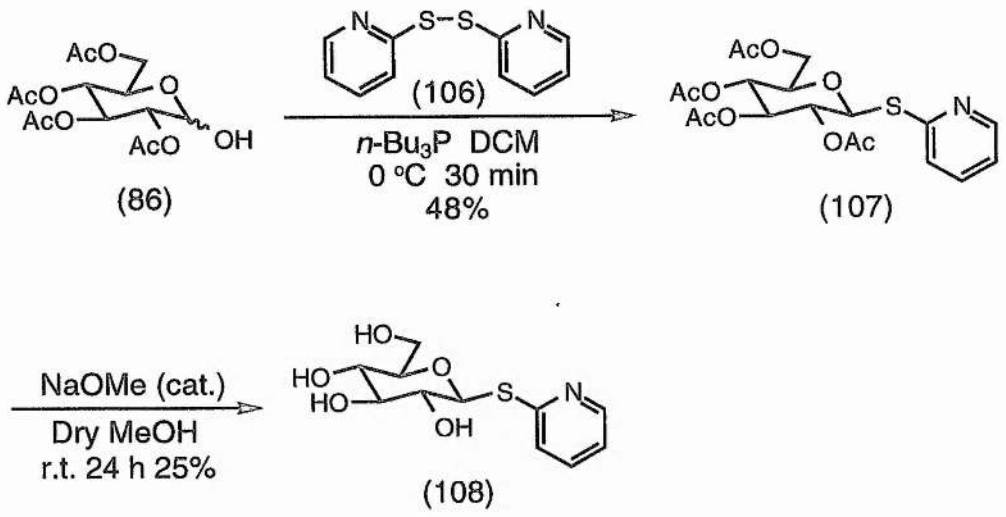
glucopyranoside sulfone was confirmed from its melting point (88-90 °C (lit.,[181] 91-92 °C)) and its ^{1}H and ^{13}C N.M.R. spectra. The synthesis of the sulfone (105) is shown in Scheme 37.



Scheme 37: *Synthesis of Phenyl-1-thio-β-D-glucopyranoside sulfone*[173]

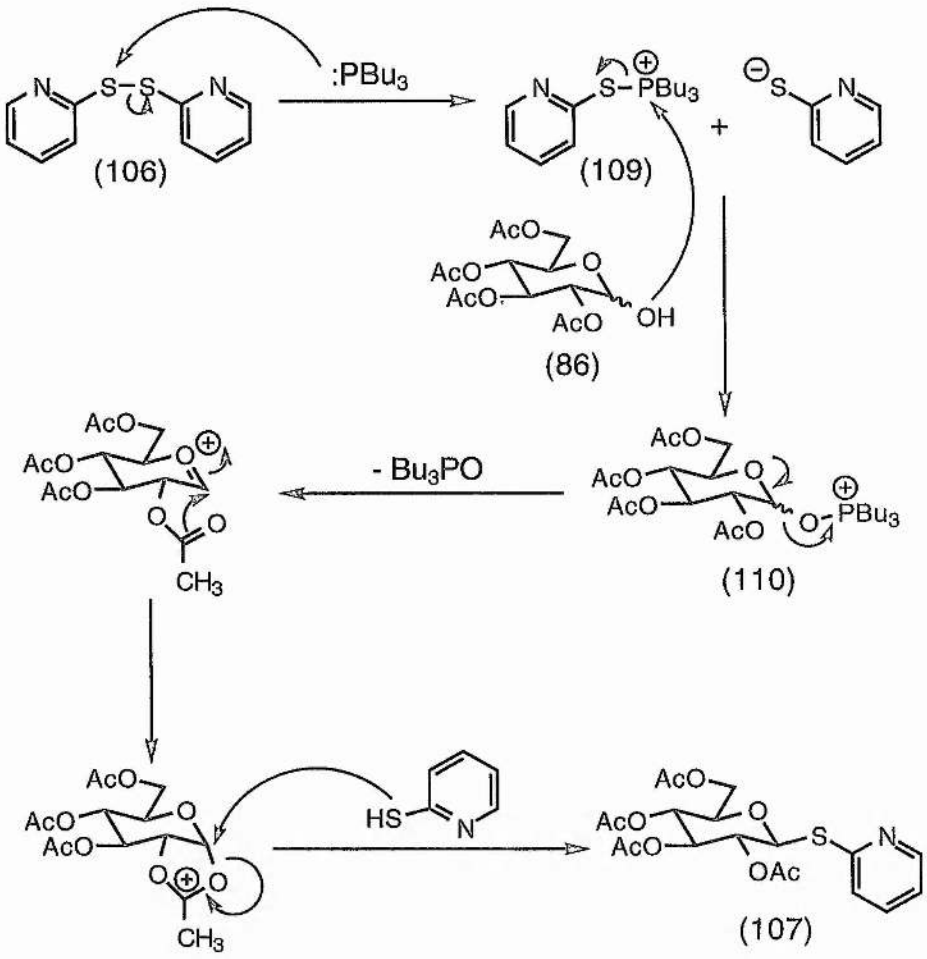
2.5.3 Synthesis of 2-Pyridyl-1-thio-β-D-glucopyranoside (2-PYTG)

This compound was synthesised in an analogous manner to that described in the literature[182] for another protected carbohydrate. This is shown in Scheme 38.



Scheme 38: *Synthesis of 2-Pyridyl-1-thio-β-D-glucopyranoside (2-PYTG)*[182]

The first stage of this synthesis involves initial attack of 2,2'-dipyridyldisulfide (106) by tri-*n*-butylphosphine to form an ylide-like species (109), then attack by 2,3,4,6-tetra-*O*-acetyl-α/β-D-glucopyranose (86) to form the phosphonium salt (110). This is then attacked by the anion to form the acetylated compound (107). The mechanism for this reaction is shown in Scheme 39.

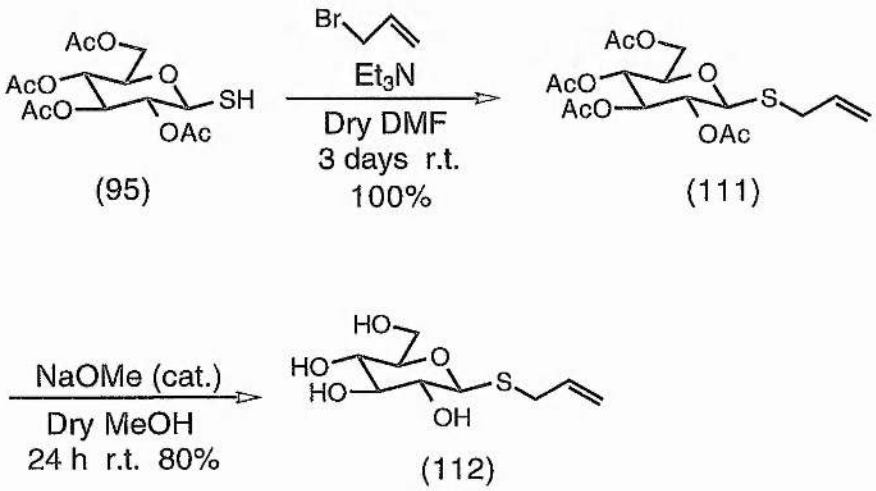


Scheme 39: *Mechanism for Synthesis of 2,3,4,6-Tetra-O-acetyl-2'-Pyridyl-1-thio-β-D-glucopyranoside*

In this stage of the synthesis, some starting material (2,3,4,6-tetra-*O*-acetyl-α/β-D-glucopyranose) was detected in the product by ^{1}H N.M.R. spectrophotometry. Due to the fact that the starting material and the product showed up as one spot by t.l.c. (light petroleum/ethyl acetate (3:2)), this impurity could not be removed by column chromatography. The product was therefore deacetylated using catalytic sodium methoxide in dry methanol, and due to the difference in polarity between D-glucose and the deacetylated product, the product was separated from glucose by silica chromatography using ethyl acetate/methanol (3:1) as the eluent. The pure product was obtained as a yellow oil, but on trituration with diethyl ether, the product crystallised to a yellow solid. This compound decomposed slowly over the course of a few days.

2.5.4 Synthesis of Allyl-1-thio-β-D-glucopyranoside (112)

Since the most commonly occurring glucosinolate, sinigrin, has an allyl side chain, it was thought that allyl-1-thio-β-D-glucopyranoside may have a chance of acting as a substrate for myrosinase. The synthesis of allyl-1-thio-β-D-glucopyranoside (112) was carried out by firstly condensing 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (95) (the synthesis of this compound was described in Section 2.5.1) with allyl bromide in dry dimethylformamide in the presence of triethylamine to give the acetylated product (111) in quantitative yield. This compound was deacetylated using catalytic sodium methoxide in dry methanol to give the crude product as a colouless oil. This product was purified by silica chromatography using ethyl acetate/methanol/water (16:2:1) as the eluent to give pure allyl-1-thio-β-D-glucopyranoside (112) as a colourless oil in 80% yield. This synthesis is shown in Scheme 40.



Scheme 40: *Synthesis of Allyl-1-thio-β-D-glucopyranoside*

2.5.5 Incubations of Synthesised Alternative Substrates with Myrosinase

All of the compounds synthesised in Sections 2.5.1, 2.5.2, 2.5.3 and 2.5.4 were incubated with myrosinase using the same conditions as those used for PNPG. In some cases, L-ascorbic acid, at 1 mM concentration, was also added to the solutions to investigate its effect. The reactions were

monitored by measuring the release of D-glucose using the Glucose HK assay described in Section 4.16.1.

It was found that only 2-pyridyl-1-thio-β-D-glucopyranoside (2-PYTG) (108) acted as a substrate for myrosinase. In all other cases no D-glucose was released, even on prolonged incubations with myrosinase.

The results of the glucose measurements from the incubations of 2-pyridyl-1-thio-β-D-glucopyranoside with myrosinase are shown in Table 26. It must be noted here that a significant background hydrolysis of 2-PYTG occurred, and thus the hydrolysis catalysed by myrosinase is not very fast.

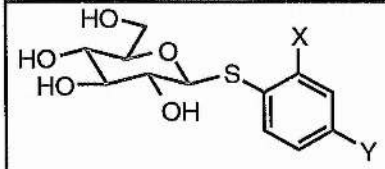
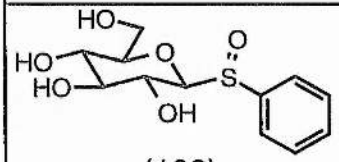
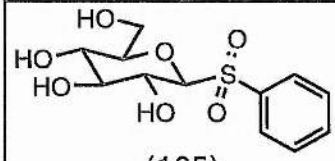
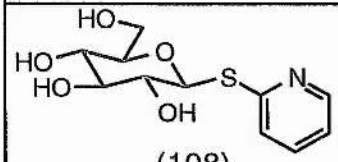
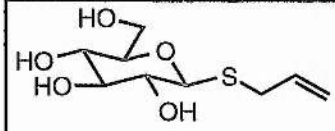
	[Glucose] / mM	
Solution[a]	**After 24 h**	**After 5 days**
10 mM 2-PYTG	0.57	0.54
10 mM 2-PYTG + myrosinase[b]	1.64	3.27

[a] Solutions in 50 mM potassium phosphate buffer, pH 7.0 at 37 ± 0.1 °C
[b] 60 µl of myrosinase solution (0.24 units myrosinase)

Table 26: *Measurements of D-Glucose Released from Incubations of 2-Pyridyl-1-thio-β-D-glucopyranoside with Myrosinase*

An attempt was made to measure the kinetic parameters for the hydrolysis of 2-PYTG. This was done by measuring the rate of increase in absorbance at 342 nm due to the release of 2-mercaptopyridine (ε = 8508 ± 870 mol^{-1} dm^3 cm^{-1} (measured experimentally as for *p*-nitrophenol)). However, a decrease in absorbance at 342 nm was observed, rendering the kinetic studies unworkable.

A summary of the synthesis of the *S*-glucopyranosides (91), (93), (98), (99), (102), (105), (108) and (112) and the specificity of myrosinase for them is shown in Table 27.

Compound	Yield / %	Substrate?
 (91): X,Y = H; (93): X=H, Y=NO_2 (98): X=NO_2, Y=H; (99) X,Y = NO_2	72.5; 43.5; 29; 86.5	No
 (102)	48.5	No
 (105)	80.5	No
 (108)	12	Yes[a]
 (112)	80	No

[a] Some background hydrolysis was also observed

Table 27: *Summary of the Synthesis of the S-Glucopyranosides and Their Activity With Myrosinase*

2.5.6 Discussion of Results

It can be seen from the results of the incubations that, as for the *O*-glucopyranosides, only the most reactive of the *S*-glucopyranosides acted as a substrate for myrosinase. This compound was 2-pyridyl-1-thio-β-D-glucopyranoside, which has an aglucone pK_a of -1.07.[183] This is a very low pK_a, and the compound is in fact hydrolysed in solution when no enzyme is present, albeit at a much lower rate. The pK_as of the thiophenol aglucone leaving groups are lower than those of the corresponding

phenols (*e.g.* the pK_as of thiophenol and *p*-nitrothiophenol are 6.43 and 4.50 respectively,[184] compared to 9.99 and 7.15 for phenol and *p*-nitrophenol), *i.e.* the thiophenols are better leaving groups. However, apart from the *S*-pyridyl derivative, the *S*-glucopyranosides are not hydrolysed by myrosinase. Therefore, even though myrosinase is a thioglucosidase, it does not catalyse the hydrolysis of a range of *S*-glucopyranosides.

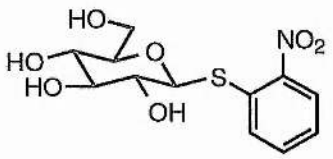
The fact that *S*-glucopyranosides are not hydrolysed by β-glucosidases (and also myrosinase) may be due to the fact that the carbon-sulfur bond is stronger than the carbon-oxygen bond. However, since myrosinase is a β-thioglucosidase, the fact that the *S*-glucopyranosides are not hydrolysed appears to be an unusual result.

Overall, these studies have shown that myrosinase is very specific, and only catalyses the hydrolysis of very reactive *O*-glucopyranosides, otherwise, the enzyme requires the complete glucosinolate structure.

2.5.7 Inhibition Studies with Synthesised Aryl-1-thio-β-D-glucopyranosides (91), (93) and (98)

Since the *S*-glucopyranosides were not hydrolysed by myrosinase, inhibition studies were carried out to investigate their binding properties at the enzyme active site. This was done by studying the inhibitory effects of *o*-nitrophenyl, *p*-nitrophenyl and phenyl-1-thio-β-D-glucopyranosides on the myrosinase catalysed hydrolysis of PNPG. Inhibition studies using sinigrin as the substrate could not be carried out since these aryl-1-thio-β-D-glucopyranosides absorb very strongly at 227 nm, the wavelength used for monitoring the hydrolysis of sinigrin.

2.5.7.1 Inhibition of Myrosinase by *o*-Nitrophenyl-1-thio-β-D-glucopyranoside (ONPTG) (98)



(98)

Concentrations of 5 to 20 mM *o*-nitrophenyl-1-thio-β-D-glucopyranoside (ONPTG) (98) were used for the study of the inhibition of the myrosinase catalysed hydrolysis of PNPG. As for the normal PNPG incubations, these incubations were carried out in 50 mM potassium phosphate buffer, at pH 7.0, at 37 ± 0.1 °C. The results of this study are shown in Table 28.

[ONPTG] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	60.9 ± 11.2	0.15 ± 0.02
5	76.4 ± 22.1	0.13 ± 0.03
10	73.1 ± 12.4	0.14 ± 0.02
15	43.4 ± 13.8	0.076 ± 0.02
20	68.1 ± 34.6	0.061 ± 0.02

Table 28: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by o-Nitrophenyl-1-thio-β-D-glucopyranoside (ONPTG)*

2.5.7.2 Discussion of Results

It can be seen from Table 28 that ONPTG has a poor inhibitory effect on the myrosinase catalysed hydrolysis of PNPG, and the data do not establish conclusively the type of inhibition. However, a Lineweaver-Burk plot for the inhibition of the myrosinase catalysed hydrolysis of PNPG by ONPTG was constructed and is shown in Figure 44. The data are not very good, but a pattern reminiscent of competitive inhibition can be drawn. This is most likely for such an *S*-glucopyranoside.

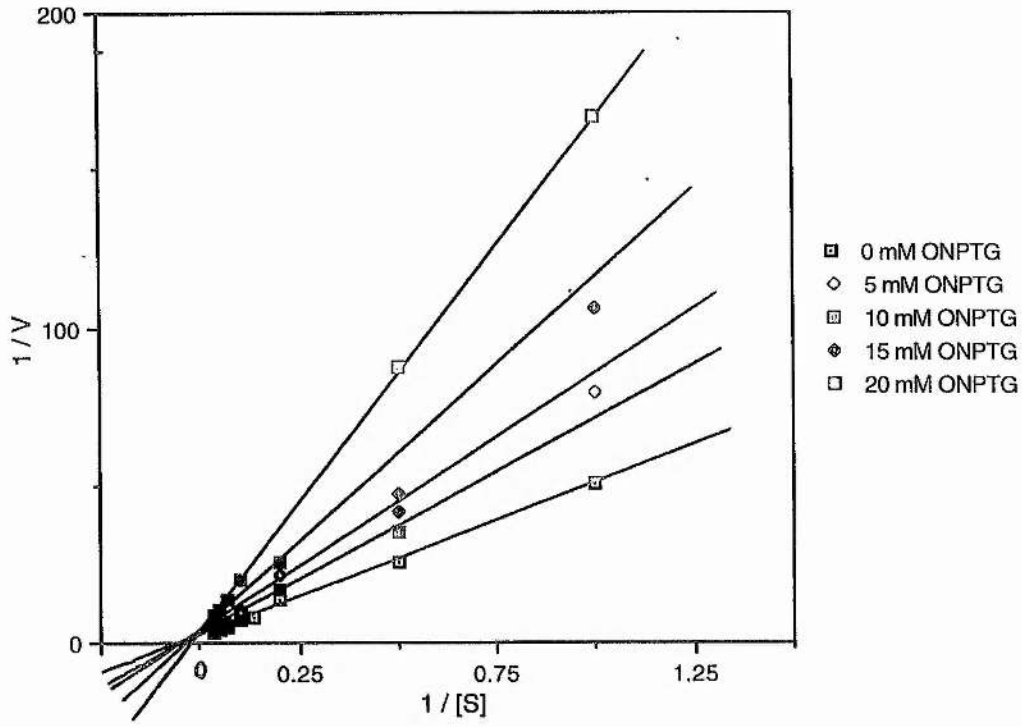
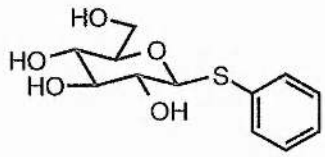


Figure 44: *Lineweaver-Burk Plot for the Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by ONPTG*

A rough value of K_I was calculated for ONPTG to be 350 mM, showing very poor binding.

2.5.7.3 Inhibition of Myrosinase by Phenyl-1-thio-β-D-glucopyranoside (PTG) (91)



(91)

Concentrations of 2.5 to 25 mM phenyl-1-thio-β-D-glucopyranoside (PTG) (91) were used for the study of the inhibition of the myrosinase catalysed hydrolysis of PNPG. As for the normal PNPG incubations, these incubations were carried out in 50 mM potassium phosphate buffer, at pH 7.0, at 37 ± 0.1 °C. The results of this study are shown in Table 29.

[PTG] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	60.9 ± 11.2	0.15 ± 0.02
2.5	74.1 ± 23.1	0.13 ± 0.03
5	45.3 ± 10.6	0.10 ± 0.02
10	49.5 ± 14.2	0.11 ± 0.02
15	81.8 ± 26.9	0.14 ± 0.04
20	57.5 ± 20.7	0.13 ± 0.04
25	104.3 ± 68.8	0.19 ± 0.10

Table 29: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by Phenyl-1-thio-β-D-glucopyranoside (PTG)*

2.5.7.4 Discussion of Results

It can be seen from Table 29 that phenyl-1-thio-β-D-glucopyranoside (PTG) does cause a decrease in V_{max} between 2.5 and 10 mM, but the inhibition is in fact lost at higher PTG concentrations. At these higher concentrations, K_M increases, implying that PTG is inhibiting competitively. Overall, the inhibitory pattern is very complicated and no specific type of inhibition can be assigned. The IC_{50} value (the concentration at which the inhibitor causes V_{max} to be half its original value) for the inhibition of myrosinase-catalysed hydrolysis of PNPG by PTG was calculated from the plot of rate v inhibitor concentration for a given (2 mM) PNPG concentration. This plot is shown in Figure 45.

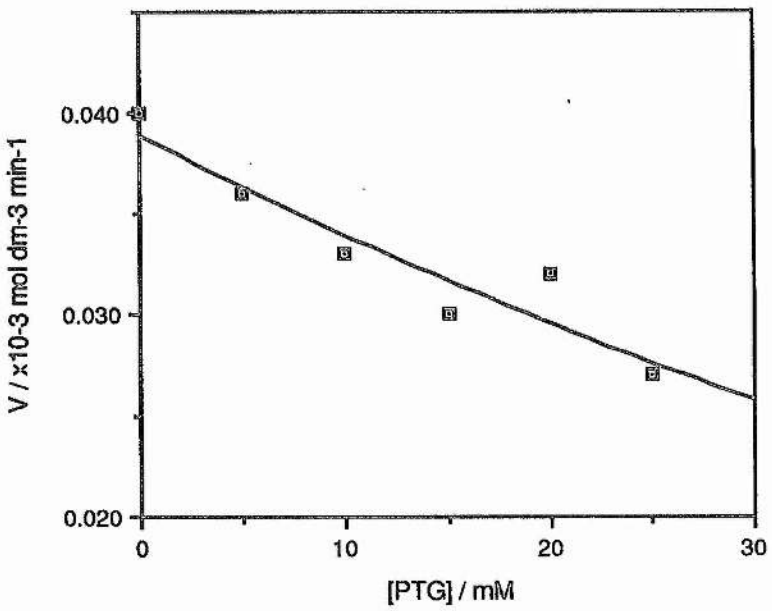
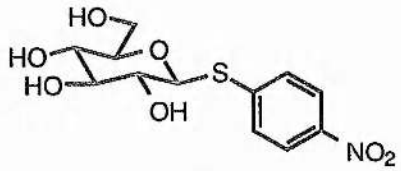


Figure 45: *Plot of Rate v [PTG] for the Inhibition of the Myrosinase Catalysed Hydrolysis of 2 mM PNPG*

From this plot, the IC_{50} value for the inhibition of myrosinase by PTG was calculated to be 51 mM, showing the very poor binding.

2.5.7.5 Inhibition of Myrosinase by *p*-Nitrophenyl-1-thio-β-D-glucopyranoside (PNPTG) (93)



(93)

Concentrations of 5 to 25 mM *p*-nitrophenyl-1-thio-β-D-glucopyranoside (PNPTG) (93) were used for the study of the inhibition of myrosinase-catalysed hydrolysis of PNPG. As for the normal PNPG incubations, these incubations were carried out in 50 mM potassium phosphate buffer, at pH 7.0, at 37 ± 0.1 °C. The results of this study are shown in Table 30.

[PNPTG] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	60.9 ± 11.2	0.15 ± 0.02
5	36.2 ± 10.8	0.095 ± 0.02
10	22.2 ± 10.1	0.074 ± 0.02
17.8	23.3 ± 9.5	0.075 ± 0.02
25	7.2 ± 2.4	0.039 ± 0.01

Table 30: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by p-Nitrophenyl-1-thio-β-D-glucopyranoside (PNPTG) (93)*

2.5.7.6 Discussion of Results

It can be seen from Table 30 that both K_M and V_{max} decrease as PNPTG concentration increases. However, the inhibition pattern is very complicated and the specific type of inhibition could not be assigned. The IC_{50} value for this compound was calculated from the plot of rate *v* inhibitor concentration for a given (20 mM) PNPG concentration. This plot is shown in Figure 46.

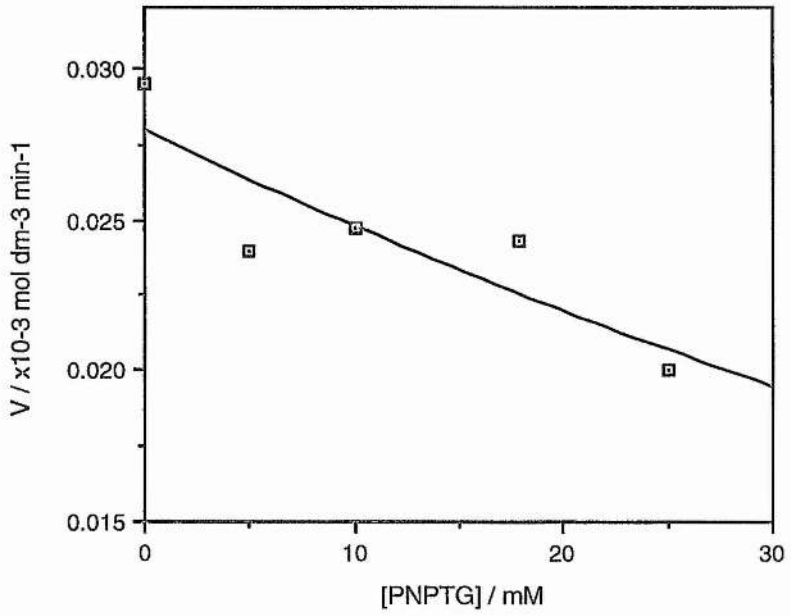


Figure 46: *Plot of Rate v [PNPTG] for Inhibition of the Myrosinase Catalysed Hydrolysis of 20 mM PNPG*

From this plot the IC_{50} value was calculated to be 55 mM, showing the very poor binding of PNPTG.

Overall, it can be seen that all three aryl-1-thio-β-D-glucopyranosides have a small inhibitory effect on myrosinase, however, these compounds do not act as effective competitive inhibitors as for the β-glucosidases. It therefore appears that the *S*-glucopyranosides can gain access to the active site of myrosinase, although this has not been proven conclusively.

2.6 α-Deuterium Kinetic Isotope Effect Studies

As was discussed in Section 1.2.2, α-deuterium kinetic isotope effect studies are very useful for the investigation of chemical mechansims of β-glycosidases, and have proved to be one of the key tools in the determination of the mechanisms proposed in Section 1.2. An α-deuterium kinetic isotope effect of between 1.20 and 1.41 gives an indication of an sp^2 hybridised intermediate, as in the S_N1-like mechanism for β-glycosidases, shown in Scheme 14, while a low value of between 1.00 and 1.06 gives an indication of an sp^3 hybridised intermediate, as in the S_N2-like mechanism for β-glycosidases, shown in Scheme 15.

In order to investigate the chemical mechanism of myrosinase, and to compare it with those of the β-glycosidases, the measurement of the α-deuterium kinetic isotope effects for the hydrolysis of the substrates sinigrin, PNPG and 2,4-DNPG was planned. A direct comparison of these values with those obtained for the β-glycosidases would give an indication of any similarities or differences which may exist between the mechanism of myrosinase and those of the more widely studied β-glycosidases.

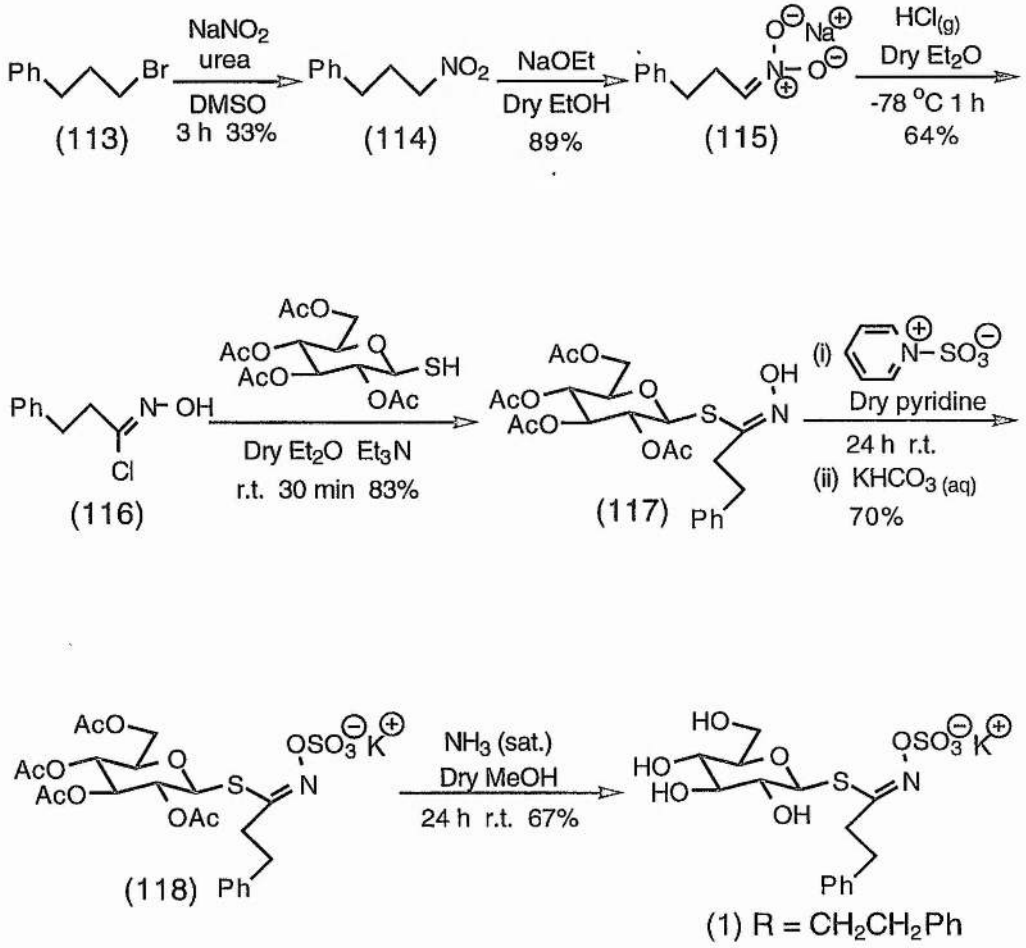
2.6.1 α-Deuterium Kinetic Isotope Effect Measurement for the Myrosinase Catalysed Hydrolysis of Sinigrin

For the measurement of the α-deuterium kinetic isotope effect for the myrosinase catalysed hydrolysis of sinigrin, the synthesis of [1-^{2}H]-sinigrin was required. In order for this to be achieved, the full synthesis of non-deuterated sinigrin was carried out first, according to literature methods.[185,186,188,189]

2.6.1.1 Literature Syntheses of Glucosinolates

The synthesis of sinigrin was based upon literature methods for the synthesis of sinigrin and 2-phenethyl glucosinolate.[185,186] The literature synthesis of sinigrin[185] was a dated one which was only a brief outline. However, data was given for the intermediate compounds formed after each step. The more recent synthesis of 2-phenethyl glucosinolate[186] was described comprehensively and each step was shown in detail. The only main difference in the chemistry between the two methods was the nature

of the glucosinolate side chain. The synthesis of 2-phenethyl glucosinolate (2.4 g of the final glucosinolate was prepared) is shown in Scheme 41.



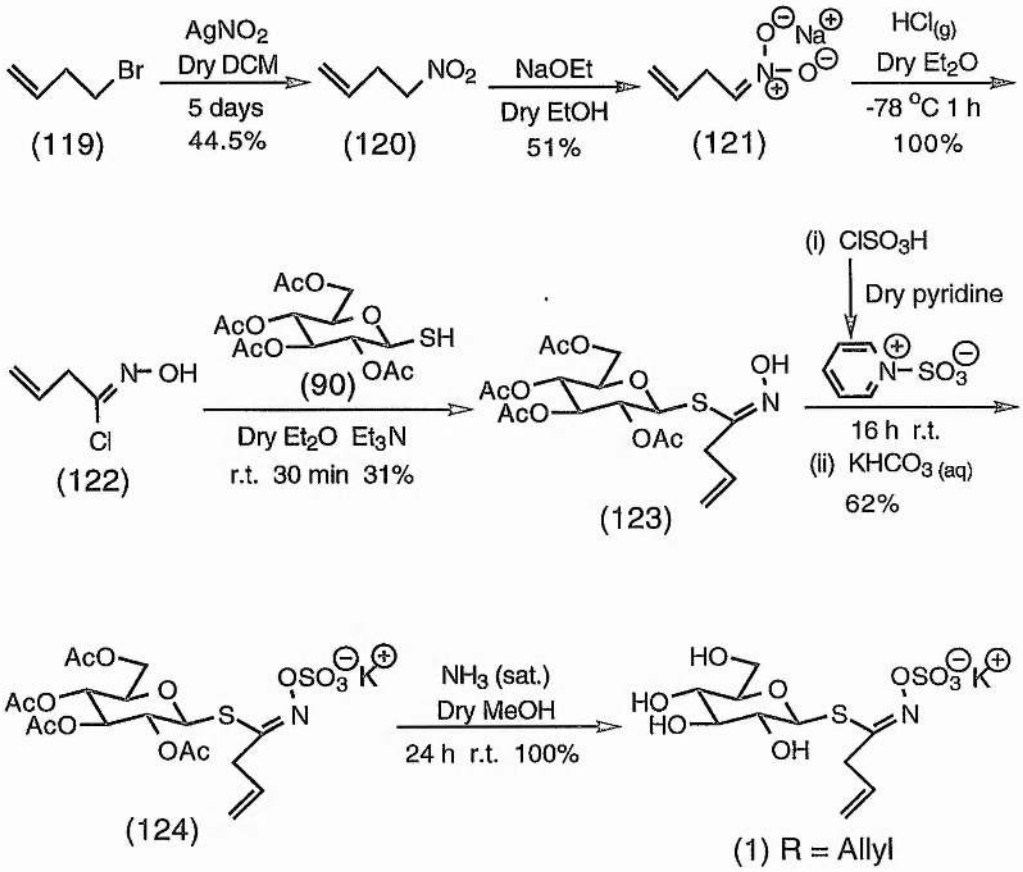
Scheme 41: *Synthesis of 2-Phenethyl Glucosinolate*[186]

2.6.1.2 Synthesis of Sinigrin

The synthesis of sinigrin (Scheme 42) was carried out on a slightly smaller scale than that for 2-phenethyl glucosinolate (Scheme 41), and the final yield of sinigrin was 0.50 g. The first step of the synthesis of sinigrin was the conversion of 4-bromobut-1-ene (119) to 4-nitrobut-1-ene (120). This step was initially attempted using sodium nitrite and urea in dimethylformamide, a method previously used for the conversion of an alkyl halide to the corresponding nitro compound,[187] which was similar to that used for the synthesis of 3-phenyl-1-nitropropane (114).[186] After stirring at room temperature under nitrogen for 3 hours - the specified reaction period - only starting material was recovered.

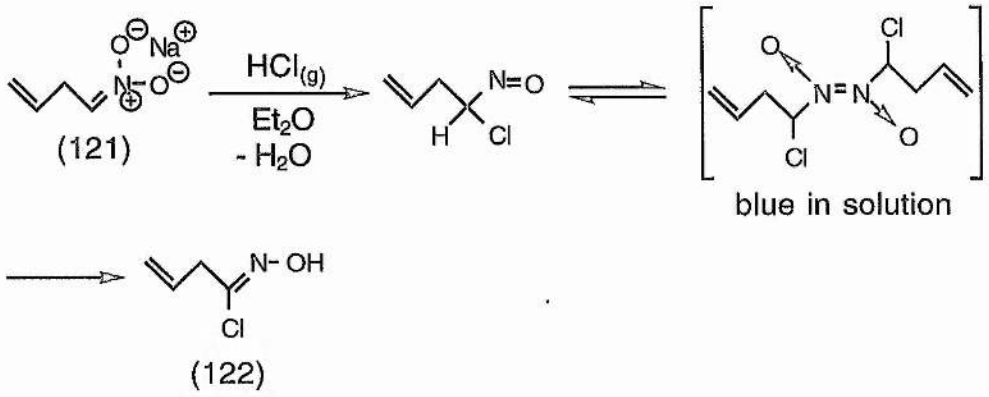
An alternative method, using silver nitrite as the nitrating agent,[188] was then attempted. A suspension of silver nitrite in dry dichloromethane was stirred in the dark with 4-bromobut-1-ene (119) under nitrogen for 5 days. On work-up, it was found that the halide had indeed been converted to the nitro compound (Scheme 42). The crude product, a red oil, was purified by distillation under reduced pressure to give pure 4-nitrobut-1-ene (120) as a colourless oil. The yield for this reaction was 44.5%. This product was identified from the ^{1}H N.M.R. spectrum (which showed that the multiplet for H-4^a,4^b had shifted to 4.49 ppm) and the mass spectrum (*m/z* (EI) 101 (M^+, 5%)).

The next step was the conversion of 4-nitrobut-1-ene (120) to its sodium salt (121), in a reaction analogous to that carried out for the synthesis of the sodium salt of 3-phenyl-1-nitropropane (115).[186] This was done by treating a solution of 4-nitrobut-1-ene (120) in dry ethanol with sodium ethoxide in dry ethanol (Scheme 42). The white sodium salt (121) immediately precipitated out of solution, and after diethyl ether was added, the solid was filtered off and dried. The yield for this step was 51%.



Scheme 42: *Synthesis of Sinigrin*[185,186,188,189]

The sodium salt of 4-nitrobut-1-ene (121) was then converted to but-3-enohydroximoyl chloride (122). This was achieved by bubbling dry hydrogen chloride gas through a suspension of the sodium salt in dry diethyl ether at -78 °C (Scheme 42), in a reaction analogous to that carried out for the synthesis of 1-chloro-3-phenylpropanal oxime (116).[186] The sodium salt of 4-nitrobut-1-ene (121) slowly dissolved as it was converted to but-3-enohydroximoyl chloride (122), and a precipitate of sodium chloride slowly formed. During this reaction, the solution turned a bright blue colour. This was due to the formation of a dimer of but-3-enohydroximoyl chloride in solution. The strong absorbance at 320 nm was due to the N=N, n -› n* transition in the dimer. The solution gradually became colourless as the dimer slowly dissociated to the monomer. The yield for this reaction was 100%. The structure of the product was confirmed by the loss of the triplet at 6.14 ppm due to H-4. This reaction is shown in Scheme 43.



Scheme 43: *Conversion of the Sodium Salt of 4-Nitrobut-1-ene (121) to But-3-enohydroximoyl Chloride (122)*

The key coupling step in the synthesis of sinigrin involved the reaction of but-3-enohydroximoyl chloride (122) with 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (95). The synthesis of (95) was carried out as before, shown in Scheme 35 (Section 2.5.1).[177] To a solution of (95) in dry diethyl ether was added but-3-enohydroximoyl chloride (122) followed by triethylamine (Scheme 42). Reaction occurred instantly, and after work-up, the crude product, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-but-3-enothiohydroximate (123) was obtained. This product was purified by recrystallisation from ethanol. The overall yield was 31%. The pure product was identified from its melting point (158-60 °C (lit.,[185] 164-5 °C) and its mass spectrum (*m/z* (FAB) 470 ([*M* + Na]$^+$, 100%)).

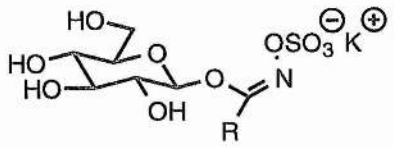
It was then necessary to sulfate 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-but-3-enothiohydroximate (123) to form sinigrin in its protected tetra-*O*-acetyl form (124). This step was attempted using commercially available pyridine-sulfur trioxide complex in dry pyridine, according to the method used for the synthesis of 2,3,4,6-tetra-*O*-acetyl-2-phenethyl glucosinolate (118).[186] Initial attempts of this reaction failed, and it was noted at this stage that sulfur-trioxide complex is very hygroscopic. Not only does the complex absorb water, but hydrolysis takes place to give pyridinium hydrogen sulfate. This species is inactive as a sulfating agent and so reaction does not take place. The problem was solved by synthesising the pyridine-sulfur trioxide complex *in situ*, according to a literature method.[189] This was done by adding chlorosulfonic acid to dry pyridine at 0 °C under nitrogen. A solution of (123) in dry dichloromethane was then added and the reaction mixture was stirred at room temperature under nitrogen for 16

hours. The reaction was quenched with an aqueous solution of potassium hydrogen carbonate, which converted the sulfated product to its potassium salt. For this reaction, an oil was formed instead of a salt, and three sets of peaks between 7.20 and 8.50 ppm in the ^{1}H N.M.R. spectrum revealed that the product was present as the pyridinium salt. This product was therefore stirred in an aqueous solution of potassium hydrogen carbonate to convert it to the potassium salt (Scheme 42). The product, 2,3,4,6-tetra-*O*-acetyl sinigrin (124) was purified by silica chromatography using ethyl acetate/methanol (4:1) as the eluent. The overall yield for this reaction was 62%. The pure product was identified from its melting point (187 °C (lit.,[185] 193-5 °C) and its mass spectrum (*m/z* (ES) 526 ([*M* - K]$^{+}$, 100%)).

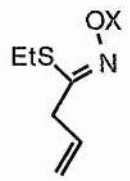
Finally, 2,3,4,6-tetra-*O*-acetyl sinigrin (124) was deacetylated using a saturated solution of ammonia in dry methanol, according to the method used for the deacetylation of 2,3,4,6-tetra-*O*-acetyl-2-phenethyl-glucosinolate (118).[186] This step gave a quantitative yield of sinigrin ((1), R = Allyl) (Scheme 42). However, the product formed was a hygroscopic oily solid. This product was crystallised by evaporation with ethanol, but the white crystals which were formed picked up water very rapidly. The structure of the product was confirmed by comparing its ^{1}H and ^{13}C N.M.R. spectra with those of the commercially available compound.

2.6.2 Synthesis of Glucosinolate Analogues

As an aside to the α-deuterium kinetic isotope effect studies, the synthesis of a number of glucosinolate analogues was attempted. Attempts were made to synthesise the *O*-glucosinolates (126), (130) and (134) (Figure 47), in order for these analogues to be used in incubation experiments to investigate further the specificity of myrosinase. Also, attempts were made to synthesise the aglucone analogues (135) and (136) (Figure 47), which would be used to investigate the involvement of the enzyme in the aglucone rearrangement.



(126) R = Allyl
(130) R = Benzyl
(134) R = *p*-Nitrobenzyl



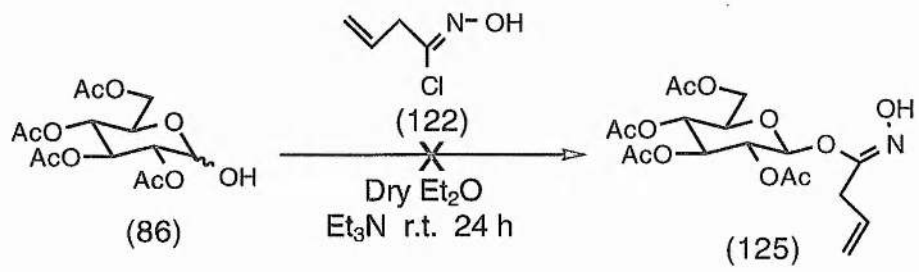
(135) X = H
(136) X = SO_3^-

Figure 47: *Structures of O-Glucosinolates (126), (130) and (134) and Aglucone Analogues (135) and (136)*

The attempted syntheses of these compounds, which met with varying success, are described below.

2.6.2.1 Attempted Synthesis of Allyl-*O*-Glucosinolate (126)

The coupling step of the synthesis of allyl-*O*-glucosinolate (the *O*- analogue of the commonly occurring glucosinolate sinigrin) was attempted in an identical manner to that described for 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-but-3-enothiohyroximate (123), except that 2,3,4,6-tetra-*O*-acetyl-α/β-D-glucopyranose (86) was used instead of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (95) (Scheme 44). However, after a 24 hour reaction period, only 2,3,4,6-tetra-*O*-acetyl-α/β-D-glucopyranose was recovered from the work-up.



(86) (122) (125)
Dry Et_2O
Et_3N r.t. 24 h

Scheme 44: *Attempt at the Coupling Step of the Synthesis of Allyl-O-Glucosinolate (126)*

An alternative attempt was made using dimethylformamide as the solvent and DABCO as the base, a method which was successful for the synthesis of 2,3,4,6-tetra-*O*-acetyl-2',4'-dinitrophenyl-β-D-glucopyranoside (Section

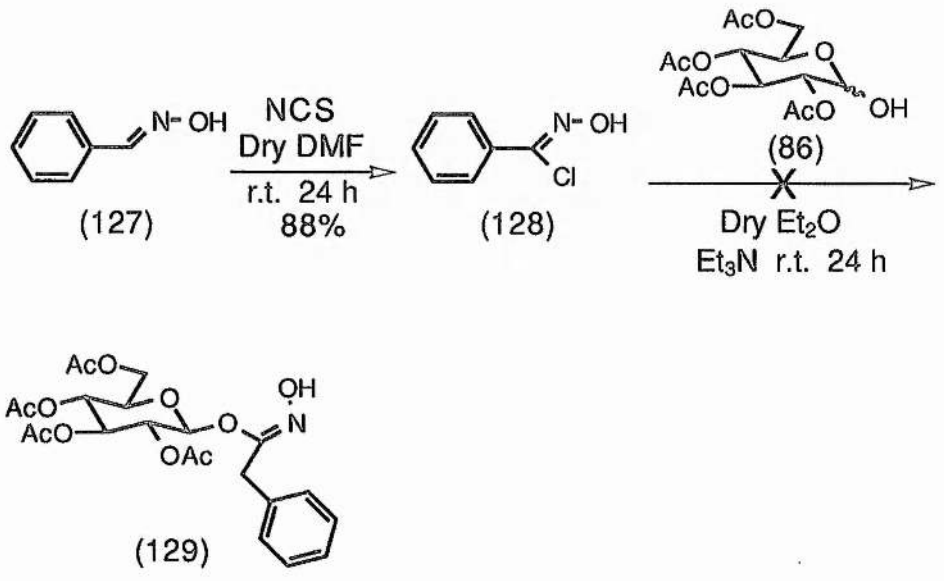
4.2.11). After a 24 hour reaction period and the usual work-up, none of the desired product was isolated.

Another attempt was made in dimethylformamide, using triethylamine as the base and silver nitrate to try to activate but-3-enohydroximoyl chloride (122). Again, none of the desired product was isolated.

The fact that these reactions did not work may have been due to the fact that oxygen is less nucleophilic than sulfur, and so in this case 2,3,4,6-tetra-*O*-acetyl-α/β-D-glucopyranose was not reactive enough. Further attempts at these reactions were made using alternative chloro-oximes, with the benzyl and *p*-nitrobenzyl side chains.

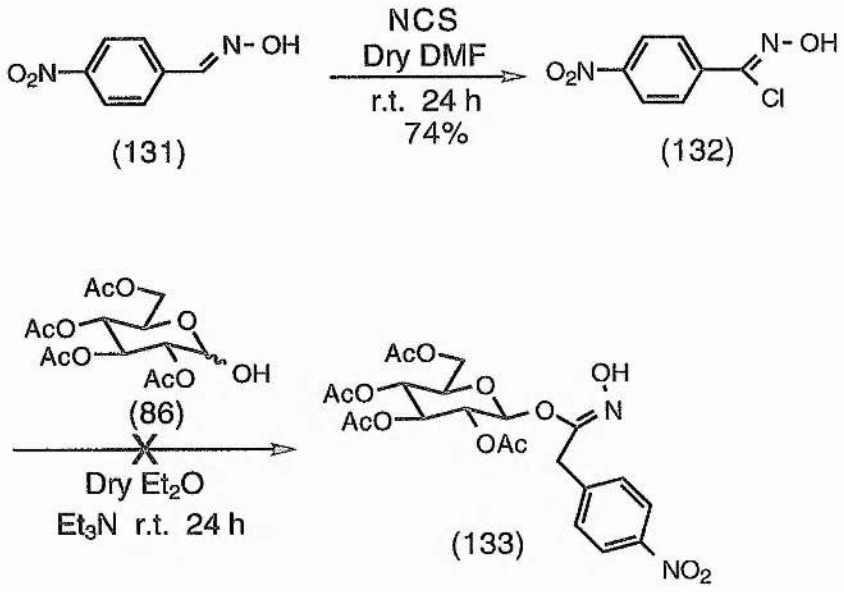
2.6.2.2 Attempted Synthesis of Benzyl-*O*-Glucosinolate (130)

The coupling step of the synthesis of (130) was attempted in an identical manner to that described in Section 2.6.2.1, using phenylacetohydroximoyl chloride (128) instead of but-3-enohydroximoyl chloride (122). Compound (128) was synthesised in 88% yield using *N*-chlorosuccinimide (NCS) according to a literature method,[57] and was used immediately in the reaction. Unfortunately, none of the desired product (129) was obtained using this method. This is shown in Scheme 45.



Scheme 45: *Attempt at the Coupling Step of the Synthesis of Benzyl-O-Glucosinolate (130)*

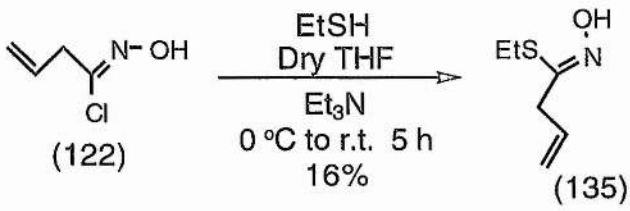
The coupling step of the synthesis of the *p*-nitro derivative (134) was attempted in an identical manner to that described in Section 2.6.2.2, using *p*-nitrophenylacetohydroximoyl chloride (132) instead of phenylacetohydroximoyl chloride (128). It was anticipated that the nitro group of the chloro-oxime may activate the compound, thus making reaction with 2,3,4,6-tetra-*O*-acetyl-α/β-D-glucopyranose (86) more likely. Compound (132) was synthesised in 74% yield using the same method as for (128). None of the desired product (133) was obtained using this method. This is shown in Scheme 46.



Scheme 46: *Attempt at the Coupling Step of the Synthesis of p-Nitrobenzyl-O-Glucosinolate*

2.6.2.3 Synthesis of Ethyl But-3-enothiohydroximate (135)

In order to investigate the role of myrosinase in the aglucone rearrangement, the aglucone mimic, ethyl but-3-enothiohydroximate (135), was prepared. The synthesis of this compound enabled the closer aglucone mimic, incorporating a sulfate group, to be synthesised. This is described in Section 2.6.2.4. The synthesis of (135) was carried out by reacting but-3-enohydroximoyl chloride (122) with ethanethiol in dry tetrahydrofuran in the presence of triethylamine (Scheme 47). After filtering off the triethylamine hydrochloride, the filtrate was evaporated at reduced pressure to give a red oil as the crude product.



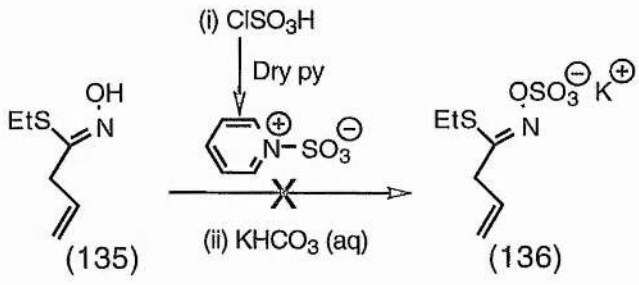
Scheme 47: *Synthesis of Ethyl But-3-enothiohydroximate (135)*

The crude product was distilled under reduced pressure to give pure ethyl but-3-enothiohydroximate (135) as a colourless oil in 16% yield. The structure of the product was confirmed from its ^{1}H and ^{13}C N.M.R. spectra and its mass spectrum ((EI) 145 (M^{+}, 85%) and 128 (55, $[M - OH]^{+}$).

Unfortunately, ethyl but-3-enothiohydroximate (135) was found to be insoluble in water, thus rendering any incubations with myrosinase impossible.

2.6.2.4 Attempted Sulfation of Ethyl But-3-enothiohydroximate (135) to give the Aglucone Analogue (136)

This reaction was attempted using the same method for the sulfation of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-but-3-enothiohydroximate (123) in the synthesis of sinigrin. This was done by firstly synthesising pyridine-sulfur trioxide complex *in situ*, then adding ethyl but-3-enothiohydroximate (135). Using the same reaction period as for the sulfation reaction in the synthesis of sinigrin, no reaction occurred and only starting material was recovered. A repeat attempt using a 4 day reaction period also gave only starting material. This is shown in Scheme 48.



Scheme 48: *Attempted Sulfation of Ethyl But-3-enothiohydroximate*

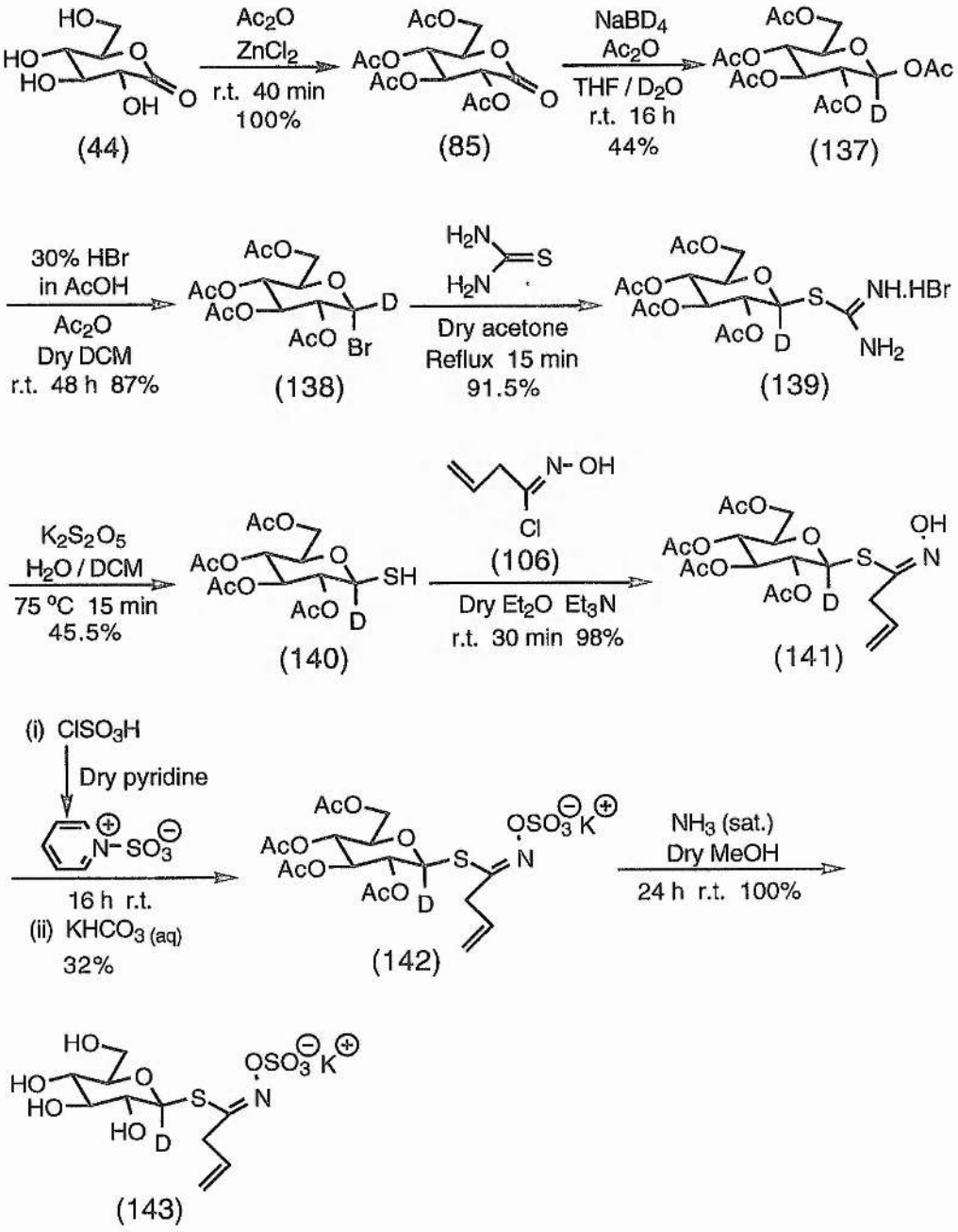
The fact that compound (136) could not be synthesised meant that no incubations of aglucone analogues with myrosinase were possible.

2.6.3 Synthesis of [1-^{2}H]-Sinigrin

The synthesis of [1-^{2}H]-sinigrin was carried out on a similar scale using the optimised method for sinigrin (Scheme 42), except that the deuterium at C-1 was incorporated by reducing 2,3,4,6-tetra-*O*-acetyl-D-glucono-γ-lactone (85) with sodium borodeuteride. D-Glucono-γ-lactone (44) was converted to 2,3,4,6-tetra-*O*-acetyl-D-glucono-γ-lactone (85) using the method described in Section 2.4.1.[166] The product was reduced using sodium borodeuteride in the presence of acetic anhydride to give 1,2,3,4,6-penta-*O*-acetyl-[1-^{2}H]-β-D-glucopyranose (137), using a literature method.[167] From the ^{1}H N.M.R. spectrum of this compound, it was found that there had been 99% incorporation of deuterium at C-1. The conversion of (137) to 2,3,4,6-tetra-*O*-acetyl-1-bromo-[1-^{2}H]-α-D-glucopyranose (138) was carried out after the reaction for the corresponding non-deuterated compound had been optimised first. Initial attempts of this reaction failed, and only starting material or 1,2,3,4,6-penta-*O*-acetyl-α-D-glucopyranose was recovered. The reaction was finally carried out successfully by using 3 equivalents of hydrogen bromide in acetic acid and lengthening the reaction time to 48 hours. This gave 2,3,4,6-tetra-*O*-acetyl-1-bromo-α-D-glucopyranose in 85.5% yield. The pure product was identified from its melting point (86-7 °C (lit.,[190] 88-9 °C)) and its mass spectrum (*m/z* (CI) 428 & 430 ([*M* + NH_4]$^+$, 18%)). The corresponding reaction for the deuterated compound gave 2,3,4,6-tetra-*O*-acetyl-1-bromo-[1-^{2}H]-α-D-glucopyranose (138) with a similar yield and purity. Compound (138) was fed into the synthesis already optimised for non-deuterated sinigrin, and the subsequent steps were carried out in an identical manner to those described for non-deuterated sinigrin. Pure 2,3,4,6-tetra-*O*-acetyl-[1-^{2}H]-β-D-glucopyranosyl isothiouronium bromide (139) was obtained in 91.5% yield, and its structure was confirmed from its microanalysis (Found: C, 37.05; H, 4.68; N, 5.92. Calc. for $C_{15}H_{22}DBrN_2O_9S$: C, 36.89; H, 4.75; N, 5.74%). Pure 2,3,4,6-tetra-*O*-acetyl-1-thio-[1-^{2}H]-β-D-glucopyranose (140) was obtained in 54% yield, and its structure was confirmed from its mass spectrum (*m/z* (CI) 383 ([*M* + NH_4]$^+$, 100%)). Pure 2,3,4,6-tetra-*O*-acetyl-[1-^{2}H]-β-D-glucopyranosyl-but-3-enothiohydroximate (141) was obtained in 98% yield and its structure was confirmed from its microanalysis (Found: C, 48.14; H, 5.82; N, 3.08. Calc. for $C_{18}H_{24}DNO_{10}S$: C, 48.21; H, 5.62; N,

3.12%) and its mass spectrum (*m/z* (EI) 332 ([*M* - C_4H_6NOS]$^+$, 37%)). Pure 2,3,4,6-tetra-*O*-acetyl-[1-^{2}H]-sinigrin (142) was obtained in 32% yield and its structure was confirmed from its melting point (194 °C) and its mass spectrum (*m/z* (EI) 527 ([*M* - K]$^+$, 17%)). The crude product of [1-^{2}H]-sinigrin (143) was purified by silica chromatography using propan-1-ol/ethyl acetate/0.1 M acetic acid (6:3:1) as the eluent. The structure of [1-^{2}H]-sinigrin was confirmed by comparing its ^{1}H and ^{13}C N.M.R. spectra with those of the commercially available non-deuterated compound, noting the absence of a doublet at 5.02 ppm for H-1 and a peak at 84.34 ppm for C-1 respectively. However, examination of the microanalysis results and the ^{1}H N.M.R. spectrum for [1-^{2}H]-sinigrin revealed that there was an impurity present. An investigation into the nature of this impurity found that an alternative literature method[191] for the synthesis of sinigrin reported that sinigrin could be contaminated with acetamide if ammonia-saturated methanol was used for the deacetylation reaction. Acetamide was the proposed by-product formed from the reaction of ammonia with acetate. The reported concentration of ammonia in methanol for a clean deacetylation reaction without the production of acetamide was within the range 0.01-0.18%.[191]

It was calculated that for every mole of [1-^{2}H]-sinigrin present, there were approximately 1.4 moles of acetamide. It was apparent that this impurity had not been removed by silica chromatography using propanol/ethyl acetate/0.1 M acetic acid (6:3:1) as the eluent. Authentic acetamide was tested against sinigrin by t.l.c. using this solvent system, and it was confirmed that acetamide and sinigrin had the same R.f. values. The overall synthesis of [1-^{2}H]-sinigrin (143) is shown in Scheme 49.



Scheme 49: *Synthesis of [1-^{2}H]-Sinigrin*[167,185,186,188,189]

The [1-^{2}H]-sinigrin formed was, as for sinigrin, a hygroscopic oily solid. After crystallisation by evaporation with ethanol, the white crystals which formed rapidly picked up water. This meant that the accurate weighing of [1-^{2}H]-sinigrin for preparation of the solutions for kinetic isotope studies was made impossible. Solutions were therefore prepared by dissolving [1-^{2}H]-sinigrin in 33.1 mM potassium phosphate buffer at pH 7.0 until the absorbance of the solution at 227 nm in a 0.1 cm pathlength cuvette was

the same as that for a 0.2 mM solution of sinigrin in a 1.0 cm pathlength cuvette. Thus, assuming that the extinction coefficient for [1-^{2}H]-sinigrin was the same as for sinigrin, *i.e.* 6784 mol^{-1} dm^3 cm^{-1}, the concentration of the solution of [1-^{2}H]-sinigrin was 2.00 mM. The lower concentration solutions of [1-^{2}H]-sinigrin were then prepared by diluting portions of this 2.00 mM solution with the appropriate volumes of 33.1 mM potassium phosphate buffer at pH 7.0, as for the sinigrin solutions before.

2.6.4 α-Deuterium Kinetic Isotope Effect Measurement for Sinigrin

These incubations were carried out in an identical manner to those described for non-deuterated sinigrin. The concentrations used were in the range 0.02 to 2.00 mM [1-^{2}H]-sinigrin. The kinetic parameters for the myrosinase catalysed hydrolysis of [1-^{2}H]-sinigrin were found to be very low, *i.e.* $K_M = 0.04 \pm 0.01$ mM and $V_{max} = 0.52 \pm 0.03 \times 10^{-3}$ mol dm^{-3} min^{-1}, as compared with the kinetic parameters for sinigrin (from Section 2.1.7), $K_M = 0.42 \pm 0.05$ mM and $V_{max} = 4.83 \pm 0.22 \times 10^{-3}$ mol dm^{-3} min^{-1}.

2.6.5 Discussion of Results

It can be seen that the values of V_{max} and K_M for [1-^{2}H]-sinigrin are very low, and there was obviously some problem with these measurements.

An investigation into the effect of acetamide on the kinetic measurements at 227 nm was carried out. Absorbance measurements of standard solutions of acetamide were made, and these measurements showed that acetamide does have a significant absorbance at 227 nm. This confirmed the fact that the contaminating acetamide's absorption at 227 nm caused the solution concentrations of [1-^{2}H]-sinigrin to be very low, thus giving the very low rates. Attempts were made to purify [1-^{2}H]-sinigrin by recrystallisation from methanol/ethanol mixtures, but the acetamide could not be removed.

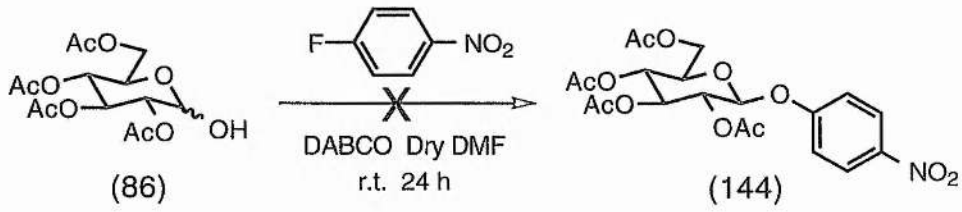
Acetamide (2.8 mM and 5.6 mM) was added to standard 2.00 mM sinigrin solutions in buffer to investigate whether it had any effect on the myrosinase catalysed hydrolysis reaction. It appeared from these incubations that acetamide did not affect enzyme activity.

The fact that the kinetics experments for sinigrin, measuring the decrease in absorbance due to the substrate at 227 nm, were so troublesome indicates that a more reliable method needs to be developed. This could either be the measurement of the release of D-glucose or the release of sulfate.

Since the α-deuterium kinetic isotope effect measurement for the myrosinase catalysed hydrolysis of sinigrin was considered unreliable, the measurement of the effects for the substrates PNPG and 2,4-DNPG were carried out in the knowledge that there would not be a back-up value for sinigrin.

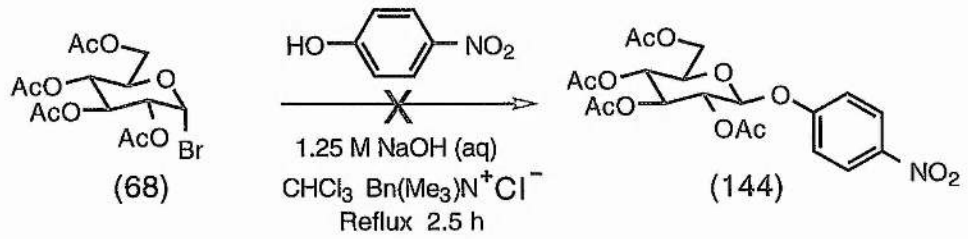
2.6.6 Synthesis of *p*-Nitrophenyl-β-D-glucopyranoside (PNPG)

The first step of the synthesis of PNPG was attempted using a similar method to that for 2,4-DNPG shown in Scheme 32. This involved reaction of *p*-fluoronitrobenzene with 2,3,4,6-tetra-O-acetyl-α/β-D-glucopyranose (86) in dry dimethylformamide. The reaction was monitored by t.l.c. (chloroform/methanol (96:4)). After 24 hours, no change had taken place, and work-up of the reaction mixture gave only starting materials. This is shown in Scheme 50.



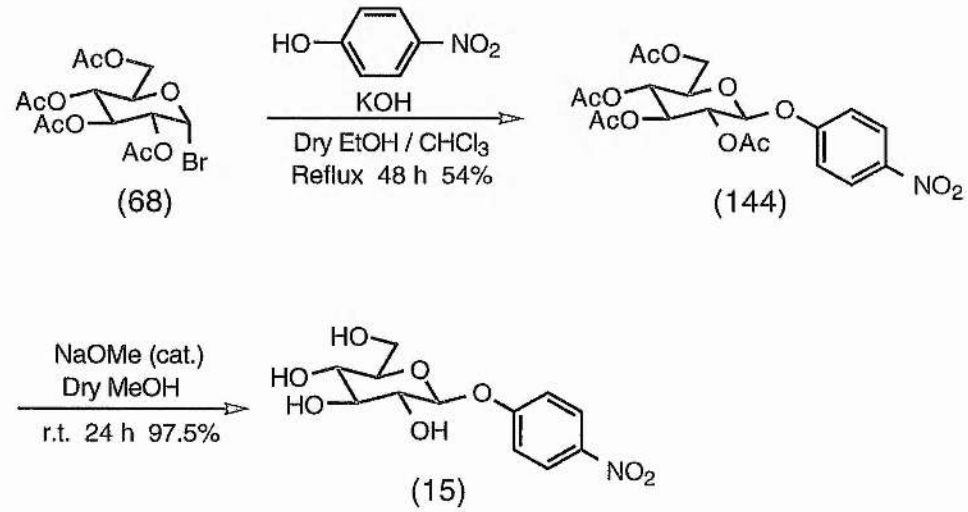
Scheme 50: *Attempted Synthesis of 2,3,4,6-Tetra-O-acetyl-p-nitrophenyl-β-D-glucopyranoside*

A second attempt to synthesise tetra-*O*-acetyl-PNPG (144) was made using a literature method.[192] This reaction involved the use of phase transfer catalysis using an aqueous sodium hydroxide/chloroform two-phase system. 2,3,4,6-Tetra-*O*-acetyl-1-bromo-α-D-glucopyranose (68) was stirred vigorously with *p*-nitrophenol under reflux in the presence of benzyl trimethylammonium chloride for 150 minutes. After work-up according to the method, none of the desired product was isolated. This is shown in Scheme 51.



Scheme 51: *Attempted Synthesis of 2,3,4,6-Tetra-O-acetyl-p-nitrophenyl-β-D-glucopyranoside*

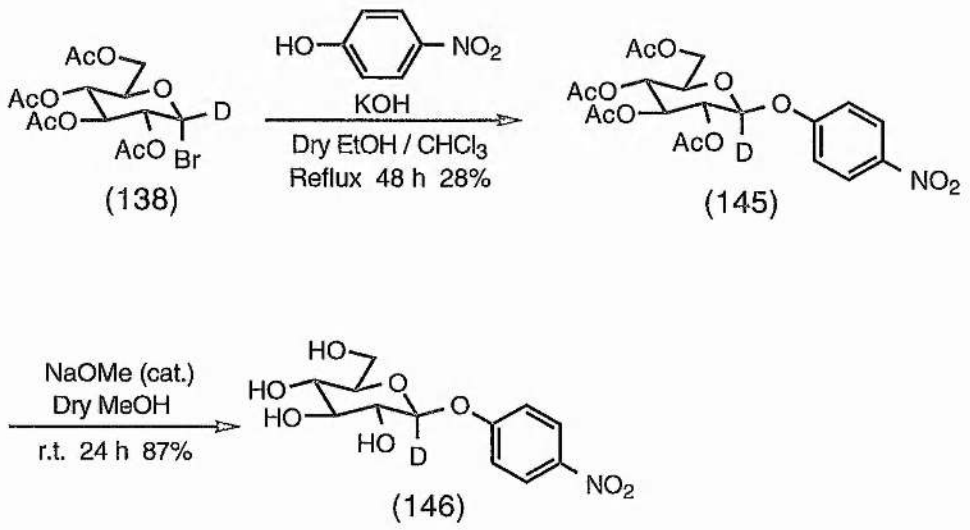
A literature method[193] involving the reaction of 2,3,4,6-tetra-*O*-acetyl-1-bromo-α-D-glucopyranose (68) with *p*-nitrophenol in a dry ethanol/chloroform mixture under reflux was then attempted. After a 48 hour reflux, work-up and purification by silica chromatography using light petroleum ether/ethyl acetate (3:2) as the eluent, pure 2,3,4,6-tetra-*O*-acetyl-*p*-nitrophenyl-β-D-glucopyranoside (144) was obtained in 54% yield. This product was deacetylated using catalytic sodium methoxide in dry methanol to give pure *p*-nitrophenyl-β-D-glucopyranoside (15) in 97.5% yield. The purity of PNPG was confirmed from its melting point (162-4 °C (lit.,[194] 165 °C)) and its $[\alpha]_D$ value (-72.1 ° (c 1.0 in H_2O) (lit.,[194] -79.6 ° (c 1.0 in MeOH))). The synthesis is shown in Scheme 52.



Scheme 52: *Synthesis of PNPG*

2.6.7 Synthesis of *p*-Nitrophenyl-β-D-[1-^{2}H]-glucopyranoside ([1-^{2}H]-PNPG)

p-Nitrophenyl-[1-^{2}H]-β-D-glucopyranoside ([1-^{2}H]-PNPG) (146) was synthesised in a similar way to PNPG, except that 2,3,4,6-tetra-*O*-acetyl-1-bromo-α-D-[1-^{2}H]-glucopyranose (138) (the synthesis of which was described in Section 2.6.1.3 for use in the synthesis of [1-^{2}H]-sinigrin) was used. The final product ([1-^{2}H]-PNPG (146)) was purified by silica chromatography using ethyl acetate/methanol/water (16:2:1) as the eluent. The purity of [1-^{2}H]-PNPG was confirmed from its melting point (160-2 °C) and its microanalysis (Found: C, 46.34; H, 5.15; N, 4.59. Calc. for $C_{12}H_{14}DNO_8$: C, 47.69; H, 5.00; N, 4.63%). The overall yield for [1-^{2}H]-PNPG (128) was 24.5%. The synthesis is shown in Scheme 53.



Scheme 53: *Synthesis of p-Nitrophenyl-β-D-[1-^{2}H]-glucopyranoside ([1-^{2}H]-PNPG) (146)*

2.6.8 α-Deuterium Kinetic Isotope Effect Measurement for PNPG

2.6.8.1 Measurement of Kinetic Parameters for PNPG Purified by Column Chromatography

Incubations of PNPG, which was subjected to silica chromatography to give a closer resemblance to synthesised [1-^{2}H]-PNPG, were carried out in an identical manner to those described for PNPG in water (Section 2.1.7). The

plots and parameters for these incubations are shown in Figure 48 and Table 31.

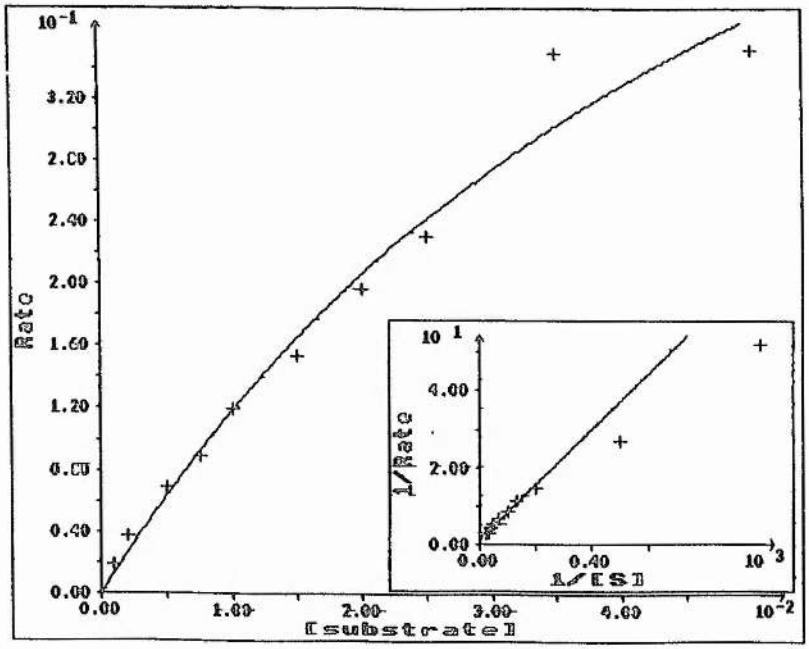


Figure 48: *Myrosinase Catalysed Hydrolysis of PNPG Purified by Column Chromatography*

2.6.8.2 Measurement of Kinetic Parameters for [1-^{2}H]-PNPG

Incubations for [1-^{2}H]-PNPG were carried out in an identical manner to those described for non-deuterated PNPG. The plots and parameters for these incubations are given in Figure 49 and Table 31. The kinetic parameters for PNPG (from Section 2.1.7) and PNPG purified by column chromatography are also shown in Table 31.

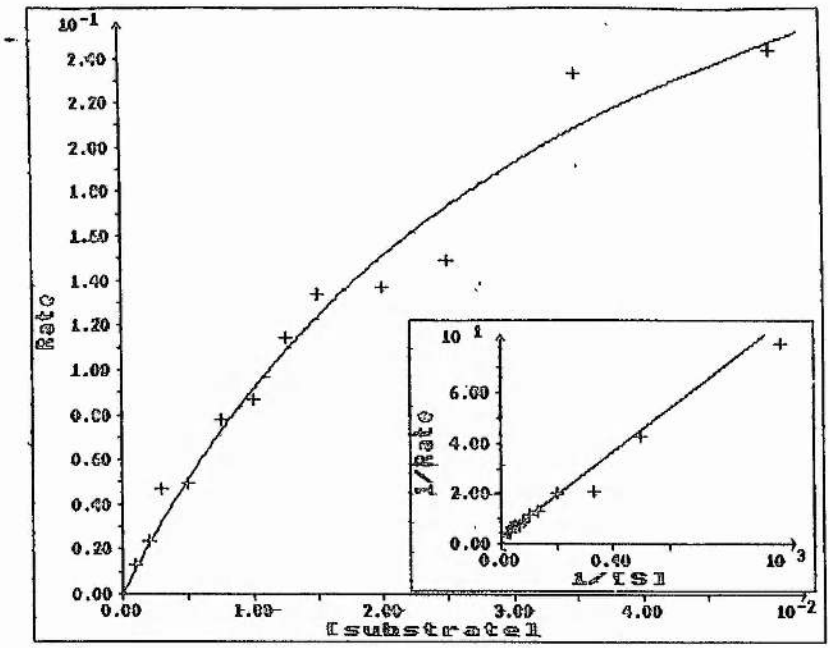


Figure 49: *Myrosinase Catalysed Hydrolysis of [1-^{2}H]-PNPG*

Substrate	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
PNPG[a]	60.9 ± 11.2	0.15 ± 0.02
Columned PNPG[b]	57.0 ± 16.9	0.114 ± 0.022
[1-^{2}H]-PNPG	36.6 ± 7.7	0.061 ± 0.008

[a] Commercially obtained PNPG

[b] Commercially obtained PNPG subjected to silica chromatography

Table 31: *Kinetic Parameters for the Myrosinase Catalysed Hydrolysis Reactions of PNPG, Chromatography Columned PNPG and [1-^{2}H]-PNPG*

Interestingly, the data for PNPG and the PNPG subjected to column chromatography are indeed very similar. The K_M values are identical within experimental error. However, there is a 30% decrease observed in V_{max}.

The isotope effects were calculated by dividing the parameter for non-deuterated PNPG by that for [1-^{2}H]-PNPG. These are given as follows:

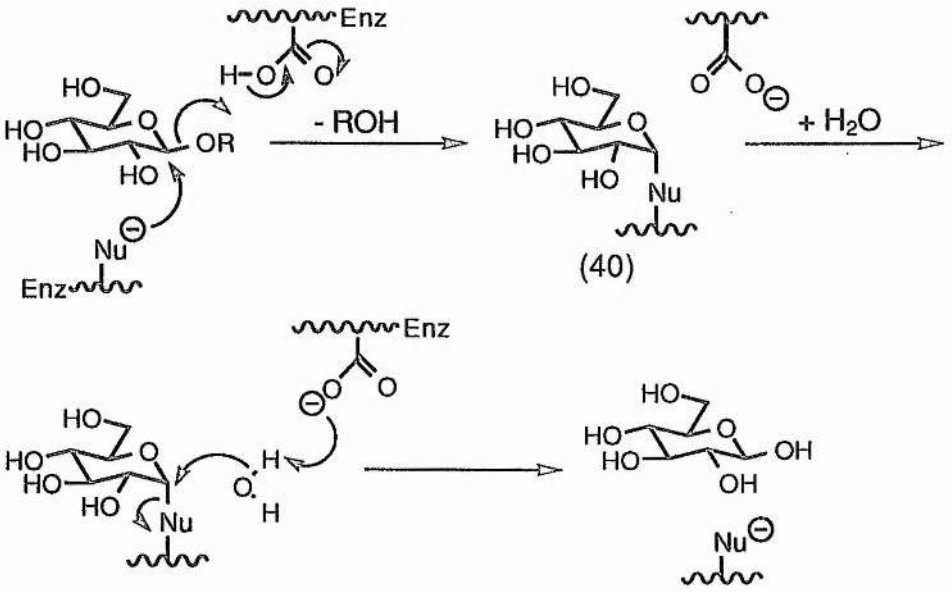
$^{D}V = 0.114 \pm 0.022 / 0.061 \pm 0.008 = \underline{1.87 \pm 0.36}$

$^{D}K = 57.0 \pm 16.9 / 36.6 \pm 7.7 = \underline{1.56 \pm 0.46}$

$^{D}V/K = 1.87 \pm 0.36 / 1.56 \pm 0.46 = \underline{1.20 \pm 0.35}$

2.6.9 Discussion of Results

It can be seen that the value for $^{H}V / {}^{D}V$, 1.87 ± 0.36 is much higher than the calculated upper limit for a secondary deuterium kinetic isotope effect of 1.41, shown in Section 1.2.2, therefore this is not a valid isotope effect. When α-deuterium kinetic isotope effect values were measured for five different substrates for *Agrobacterium faecalis* β-glucosidase,[132] these measurements were made by comparing the initial rates of hydrolysis of high (10 times the K_M value) concentrations of the non-deuterated and deuterated substrates. The values obtained for ^{D}V (*i.e.* $^{H}V / {}^{D}V$) were in the region 1.05 to 1.12. These low values gave an indication of an sp^3 hybridised intermediate, and an S_N2-like mechanism (such as that shown in Scheme 15) was proposed for this enzyme.



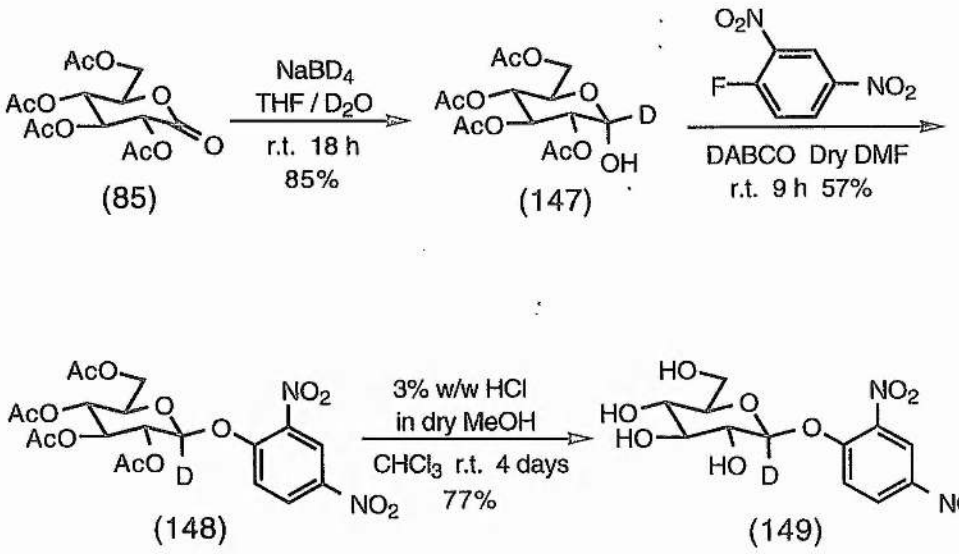
Scheme 15: *Proposed S_N2-Like Exocyclic Cleavage Mechanism for β-Glycosidases*

The problem with myrosinase is that the value of K_M for PNPG is 60.9 mM, and therefore in order to study a concentration 10 times K_M, 600 mM substrate would have to be used. The substrate would be insoluble at this concentration, and therefore there was no choice but to measure K_M and V_{max} as normal. However, since the myrosinase catalysed hydrolysis of PNPG is slow, there are problems in obtaining accurate values. The fact that in the literature this is seldom done is an indication of the problems involved.

Since the value of ^{D}V was so high for the myrosinase catalysed hydrolysis of PNPG and [1-^{2}H]-PNPG, no valuable mechanistic information could be drawn from this result.

2.6.10 Synthesis of 2,4-Dinitrophenyl-β-D-[1-^{2}H]-glucopyranoside ([1-^{2}H]-2,4-DNPG)

The synthesis of 2,4-dinitrophenyl-β-D-[1-^{2}H]-glucopyranoside ([1-^{2}H]-2,4-DNPG) (149) was carried out using the same methods as for the non-deuterated compound (88) (Scheme 35), except that 2,3,4,6-tetra-*O*-acetyl-α-D-[1-^{2}H]-glucopyranose (147) was used. The deuterium was incorporated at C-1 using sodium borodeuteride in a similar reaction to that shown in Scheme 32.[167] The deuterated product was present as exclusively the α-anomer, in contrast to the 3:1 α/β mixture for the non-deuterated compound. This was deduced from an examination of the ^{13}C N.M.R. spectra for the deuterated and non-deuterated compounds, which showed a correlation between the peaks for the major α anomer for the non-deuterated compound and the single set of peaks for the deuterated compound. The ^{1}H N.M.R. spectrum for (147) revealed that there was 99% incorporation of deuterium at C-1. 2,4-Dinitrophenyl-β-D-[1-^{2}H]-glucopyranoside ([1-^{2}H]-2,4-DNPG) (149) was purified by silica chromatography using ethyl acetate/methanol/water (16:1:1) as the eluent. The purity of this compound was confirmed from t.l.c. (ethyl acetate/methanol/water (16:1:1) and its ^{1}H and ^{13}C N.M.R. spectra. The overall yield for [1-^{2}H]-2,4-DNPG was 44%. The synthesis of [1-^{2}H]-2,4-DNPG (149) is shown in Scheme 54.



Scheme 54: *Synthesis of 2,4-Dinitrophenyl-β-D-[1-^{2}H]-glucopyranoside ([1-^{2}H]-2,4-DNPG) (149)*

2.6.11 α-Deuterium Kinetic Isotope Effect Measurement for 2,4-DNPG

Incubations for [1-^{2}H]-2,4-DNPG were carried out in an identical manner to those described for non-deuterated 2,4-DNPG in Section 2.4.2. The plot and parameters for these incubations are given in Figure 50 and Table 32.

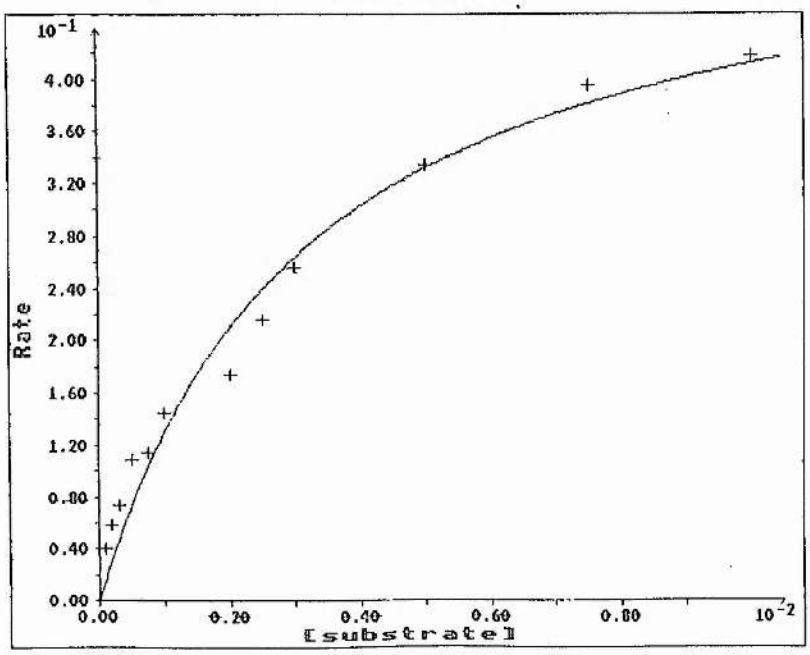


Figure 50: *Myrosinase Catalysed Hydrolysis of [1-^{2}H]-2,4-DNPG*

Substrate	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
2,4-DNPG	3.30 ± 0.53	0.044 ± 0.003
[1-^{2}H]-2,4-DNPG	3.15 ± 0.57	0.035 ± 0.003

Table 32: *Kinetic Parameters for the Myrosinase Catalysed Hydrolysis Reactions of 2,4-DNPG and [1-^{2}H]-2,4-DNPG*

The α-deuterium kinetic isotope effect values were calculated as follows:

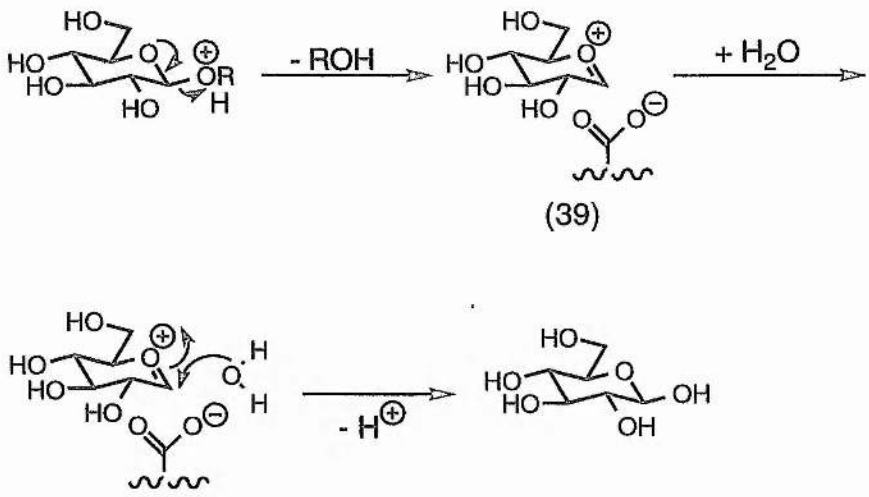
$^DV = 0.044 \pm 0.003 / 0.035 \pm 0.003 = \underline{1.26 \pm 0.11}$

$^DK = 3.30 \pm 0.53 / 3.15 \pm 0.57 = \underline{1.05 \pm 0.19}$

$^DV/K = 1.26 \pm 0.11 / 1.05 \pm 0.19 = \underline{1.20 \pm 0.22}$

2.6.12 Discussion of Results

More accurate values of K_M and V_{max} were obtained with 2,4-DNPG. This is because the low K_M means a better range of concentrations can be used to produce better plots and lower errors. It can thus be seen by comparing the value for 2,4-DNPG hydrolysis of $^DV = 1.26 \pm 0.11$ with the values for secondary deuterium kinetic isotope effects in Section 1.2.2 that this isotope effect gives an indication that myrosinase has a mechanism which has some S_N1-like character (similar to that shown in Scheme 14 in Section 1.2.2). Since the rates for myrosinase catalysed hydrolysis reactions show some dependence on pK_a (*i.e.* 2,4-DNPG is a better substrate than PNPG, and the aryl-β-D-glucopyranosides with higher aglucone pK_a values are not substrates), it is likely that cleavage of the glucoside is the rate limiting step and deglycosylation is very fast. Therefore for myrosinase, the rate limiting glycosylation step was proposed to be the step with S_N1-like character. Scheme 14 is shown again below.



Scheme 14: *Proposed S_N1-Like Exocyclic Cleavage Mechanism for Myrosinase*

For *Agrobacterium faecalis* β-glucosidase,[132] however, the biphasic Brønsted plot indicated that for phenols with $pK_a < 8$, the rate was independent of pK_a, thus implying that cleavage of the glucoside is fast and deglycosylation is the rate limiting step. Therefore, the value of $^{D}V = 1.10 \pm 0.02$ for the *Agrobacterium faecalis* β-glucosidase catalysed hydrolysis of 2,4-DNPG implies an sp^3 hybridised intermediate for the deglycosylation step.

2.7 Solvent Deuterium Kinetic Isotope Effect Studies

The solvent deuterium kinetic isotope effect for an enzyme catalysed reaction (discussed in Section 1.2.2) gives an indication of the importance of proton transfers in the reaction. The measurement of this isotope effect was also planned for myrosinase. This was done by carrying out the enzyme catalysed reaction in deuterium oxide (the enyzme was pre-equilibrated in this solvent before use). As for the α-deuterium kinetic isotope effect, a direct comparison of the value obtained for myrosinase with those reported for the β-glycosidases would be made in order to compare and contrast the mechanisms of these respective classes of enzymes.

2.7.1 Measurement of Solvent Deuterium Kinetic Isotope Effect for the Myrosinase Catalysed Hydrolysis of Sinigrin

Since the effect of D_2O on the structure and stability of enzymes has been an area of concern in the interpretation of solvent isotope effects, it was important that a study was done to compare the activities of myrosinase in H_2O and D_2O buffers. Problems may arise with the kinetics of enzyme catalysed reactions in D_2O due to the exchange of acidic hydrogens in both the enzyme and the substrate for deuterium. Possibly hundreds of atoms could be involved in such exchange reactions. There is also the possibility that a solvent kinetic isotope effect may arise because of differences in the stability of the protein in H_2O and D_2O.

A standard solution of myrosinase in D_2O buffer was equilibrated for 1 hour at room temperature. The activity of myrosinase in D_2O buffer was then compared with a standard myrosinase solution in H_2O buffer using the standard sinigrin assay in H_2O (described in Section 4.7). Three assays were carried out for each myrosinase solution.

It was observed that the rate of hydrolysis of sinigrin under standard assay conditions for myrosinase which had been equilibrated for one hour at room temperature in D_2O buffer was the same as that for myrosinase equilibrated in H_2O buffer. Therefore, the values for the solvent isotope effect can be used with confidence.

Incubations for the measurement of the solvent deuterium kinetic isotope effect were carried out the same way as for sinigrin, except that the buffer used was 33.1 mM deuterated potassium phosphate (KD_2PO_4). This buffer was prepared by lyophilising KH_2PO_4 from D_2O three times. To obtain neutral buffer, *i.e.* pD 7.0, a solution of 33.1 mM KD_2PO_4 was adjusted to pH 6.6 using 1 M NaOD. The hydrogens of the hydroxyl groups of the glucopyranose ring of sinigrin were exchanged for deuterium by lyophilising sinigrin from D_2O three times. After preparation, the myrosinase solution in D_2O buffer was equilibrated for 1 hour prior to use. The plot and parameters for these incubations are given in Figure 51 and Table 33.

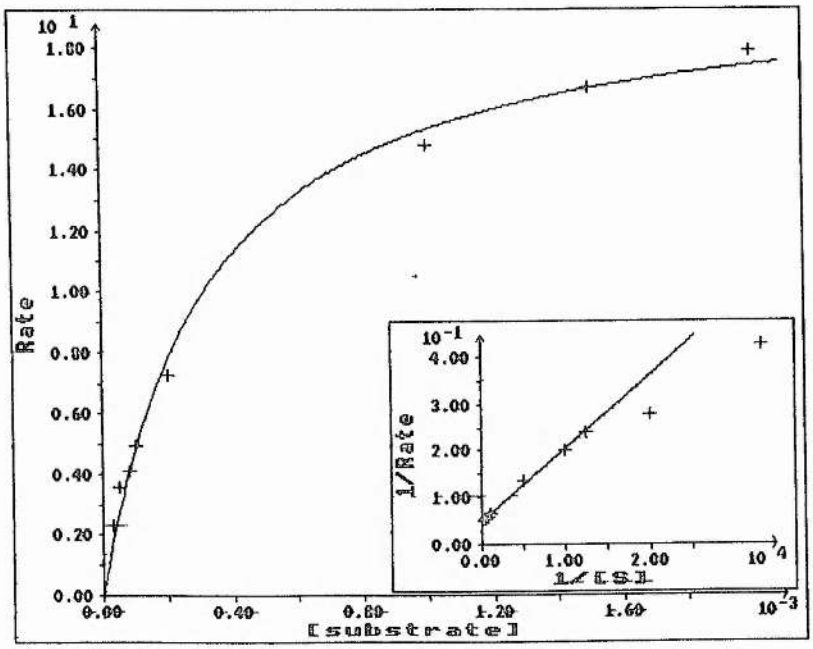


Figure 51: *Myrosinase Catalysed Hydrolysis of Sinigrin in D_2O*

Buffer	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
H_2O	0.42 ± 0.05	4.83 ± 0.22
D_2O	0.34 ± 0.04	3.14 ± 0.10

Table 33: *Kinetic Parameters for the Myrosinase Catalysed Hydrolysis of Sinigrin in H_2O and D_2O Buffers*

The solvent deuterium kinetic isotope effect values were calculated as follows:

$^{D}V = 4.83 \pm 0.22 / 3.14 \pm 0.10 = \underline{1.54 \pm 0.07}$

$^{D}K = 0.42 \pm 0.05 / 0.34 \pm 0.04 = \underline{1.24 \pm 0.15}$

$^{D}V/K = 1.54 \pm 0.07 / 1.24 \pm 0.15 = \underline{1.24 \pm 0.15}$

2.7.2 Discussion of Results

The value of the solvent isotope effect obtained for the myrosinase catalysed hydrolysis of sinigrin of $^{D}V = 1.54 \pm 0.07$ is similar to that obtained for the hydrolysis of 2,4-dinitrophenyl-β-D-glucopyranoside catalysed by sweet almond β-glucosidase of 1.5.[135] For sweet almond β-glucosidase, it was proposed that this value indicated that the rate-determining step was limited, at least partially, by the rate of one or more proton transfers.

The solvent effect of $^{D}V / K = 1.24 \pm 0.15$ for the myrosinase catalysed hydrolysis of sinigrin is a negligable one similar to that obtained for *Escherichia coli* (*lacZ*) β-galactosidase.[195] An effect of 1.7 on *V* was also obtained for this enzyme, and these values were proposed to arise from one transferred proton. The pH-independent solvent deuterium kinetic isotope effect on *V* for the hydrolysis of *p*-nitrophenyl-α-L-arabinofuranoside by the α-arabinofuranosidase III of *Monolinia fructigena* was 1.33, and was proposed to arise from a single transferred proton.[196]

The solvent deuterium kinetic isotope effect for myrosinase is therefore very similar to those observed for a number of other β-glycosidases. However, the identity of the proton transfer step for myrosinase is unknown. The obvious candidate would be protonation of the leaving group, and this is certainly possible for β-glycosidases. With myrosinase, however, other data would imply that there is no such protonation, *i.e.* (i) sequence analysis implies there is no suitable acidic residue present at the active site (Section 1.1.5); (ii) transglycosylation does not take place except where the acceptor does not require deprotonation (Section 2.1.11.1).

Further work is required to investigate this in more detail.

Overall, the kinetic isotope effect studies on myrosinase have met with variable success. Certainly the studies on 2,4-DNPG, which showed a relatively large effect on *V* of 1.26, implies a tendency towards an S_N1-like mechanism. Also, the solvent deuterium kinetic isotope effect of 1.54 ± 0.07 implies a proton transfer.

However, there were problems encountered with sinigrin due to the presence of an impurity. Further work is required to obtain a reliable and accurate value. There were also problems with the substrate PNPG. This was mainly due to the fact that the hydrolysis reaction is slow, and it was therefore difficult to obtain reproducible accurate data. Most literature methods employ a substrate concentration which is 10 times the value of K_M, but since K_M is so high for PNPG, such high concentrations are unobtainable.

Chapter 3

CONCLUSIONS AND FURTHER WORK

3.1 Conclusions

The main aim of this study was the investigation of the chemical mechanism of myrosinase, in order for it to be compared with those of the much more widely studied β-glycosidases. Superficially, the reaction catalysed by myrosinase resembles those of many β-glycosidases, with only two obvious differences. Firstly, the substrates for myrosinase are *S*-glycopyranosides rather than *O*-glycopyranosides, and secondly, there is a subsequent rearrangement of the aglucone fragment, which may or may not be enzyme catalysed. It appears from the results of this study that these differences may be only two of many which may exist between these two classes of enzyme.

Studies to investigate myrosinase catalysed transglycosylation reactions as a potential synthetic method were carried out. However, these preparative scale reactions failed to give any products, and this prompted a study of the activity and stability of myrosinase in water/organic solvent mixtures. It was found that myrosinase has both good activity and stability in water/organic solvent mixtures, and is certainly active and available for transglycosylation reactions. A more detailed investigation of the transglycosylation reaction, including the use of a range of glycosyl acceptors which mimic the glucosinolate aglucone structure, showed that myrosinase does not catalyse this reaction as many of the β-glycosidases do.[161] However, when azide was used as the glycosyl acceptor, 27% transglycosylation took place. This is the only example studied which does not require deprotonation in order to attack the glycosyl-enzyme intermediate. This result ties in with data which suggests that one of the active site acid/base residues present in β-glycosidases is missing in myrosinase, and thus deprotonation of the acceptor is not possible.[68]

A range of potential substrates for myrosinase was synthesised. However, only the nitrophenyl-β-D-glucopyranosides showed any substrate activity. All *S*-glucopyranosides were inactive, although inhibition studies did show that these compounds could gain access to the enzyme active site. Myrosinase therefore appears to be very specific in its action. Indeed, desulfoglucosinolates are not hydrolysed by myrosinase, and only act as simple competitive inhibitors.[52]

Inhibition studies revealed that D-glucono-γ-lactone, a potent competitive inhibitor and transition state mimic of β-glucosidases, is a poor noncompetitive inhibitor of myrosinase, with a K_I of *ca.* 5 mM. It was proposed that this inhibitor binds at the L-ascorbic acid activator site, and experimental evidence strongly indicated this. Another potent competitive inhibitor of β-glucosidases, 1-deoxynojirimycin, was also found to be a very weak inhibitor of myrosinase. It was proposed that this inhibitor binds very poorly at the active site of myrosinase because one of the acid/base residues, normally present in β-glucosidases, is missing in myrosinase. The protonated nitrogen of 1-deoxynojirimycin is proposed to interact with this residue in β-glucosidases, thus binding strongly to give competitive inhibition. Inhibition studies using the products, sulfate and allyl isothiocyanate, failed to give an indication of a product debinding order. This was due to the poor inhibition shown by these two compounds.

[1-^{2}H]-Sinigrin, *p*-nitrophenyl-β-D-[1-^{2}H]-glucopyranoside and 2,4-dinitrophenyl-β-D-[1-^{2}H]-glucopyranoside were prepared for α-deuterium kinetic isotope effect studies. Overall, these studies met with variable success. There were problems encountered with [1-^{2}H]-sinigrin due to the presence of an impurity, acetamide. Further work is required to obtain a reliable and accurate value. There were also problems with the substrate PNPG. This was mainly due to the fact that the hydrolysis reaction is slow, and it was therefore difficult to obtain reproducible, accurate data. Most literature methods employ a substrate concentration which is 10 times the value of K_M, but since K_M (61 mM) is so high for PNPG, such high concentrations are unobtainable. However, the isotope effect value obtained for 2,4-dinitrophenyl-β-D-[1-^{2}H]-glucopyranoside, $^DV = 1.26 \pm 0.11$, indicated an sp^2 hybridised intermediate *via* an S_N1-like mechanism.

The solvent deuterium kinetic isotope effect value, $^DV = 1.54 \pm 0.07$, obtained for myrosinase indicated the involvement of a proton transfer. This effect is very similar to those observed for a number of other β-glycosidases. However, the identity of the proton transfer step for myrosinase is unknown. The obvious candidate would be protonation of the leaving group, and this is certainly possible for β-glycosidases. With myrosinase, however, other data would imply that there is no such protonation, *i.e.* (i) sequence analysis[68] implies there is no suitable

acid/base residue at the active site; (ii) transglycosylation does not take place except where the acceptor does not require deprotonation.[161]

Overall, these studies have revealed significant mechanistic differences between myrosinase and β-glycosidases.

3.2 Further Work

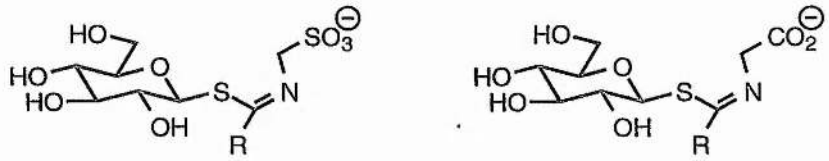
Additional mechanistic studies on myrosinase are required in several areas. Firstly, the α-deuterium kinetic isotope effects need to be further studied in order to obtain reasonable values for those substrates which have thus far proved problematic. This would enable a comparison of the values for glucosinolate and *p*-nitrophenyl-β-D-glucopyranoside hydrolysis with that already obtained for 2,4-dinitrophenyl-β-D-glucopyranoside hydrolysis.

Due to the problems encountered with [1-^{2}H]-sinigrin, an alternative method for the deacetylation step is required which will eliminate the formation of acetamide, an impurity which could not be easily removed. Also, the use of a more suitable glucosinolate with a different side chain may be required, since sinigrin was found to be very hygroscopic and difficult to crystallise, thus making accurate weighing for kinetic studies impossible.

Further to the secondary deuterium kinetic isotope effect studies, it would be interesting to examine the primary ^{13}C and ^{18}O isotope effects. Also, in addition to the solvent deuterium kinetic isotope effect studies already carried out, the variation of the effect with pH needs to be examined. Also, a proton inventory study would be interesting, to determine how many proton transfers give rise to the observed isotope effect.

Results obtained in relation to the role of the sulfate group of glucosinolates have so far been sketchy. It is still not known whether the sulfate group is important for binding only, or whether it is involved in substrate-assisted catalysis during the rearrangement.[70] The degree of enzymatic involvement in the rearrangement of the aglucone still needs to be established. It could be that the rearrangement reaction is the driving force for the overall reaction catalysed by myrosinase. Incubations of glucosinolate analogues which cannot rearrange with myrosinase may

give an insight into these questions. Therefore the synthesis of suitable analogues such as (150) and (151) (Figure 52), and then examination of their activity with myrosinase, is an important aim.



(150) (151)

Figure 52: *Structures of Glucosinolate Analogues Which Cannot Rearrange*

To gain further knowledge of the active site structure of myrosinase, the X-ray crystal structure is required. Since the enzyme is a glycoprotein, this is more difficult than for a conventional protein, however, studies are underway to obtain the X-ray crystal structure in a French laboratory.

It can thus be seen that there are indeed plenty of mechanistic studies still required to understand this interesting and unusual enzyme in detail.

Chapter 4

EXPERIMENTAL

N.M.R. spectra were recorded on either a Varian Gemini 200 F.T. spectrophotometer (^{1}H, 200 MHz; ^{13}C, 50.3 MHz) or a Bruker AM 300 F.T. spectrophotometer (^{1}H, 300 MHz; ^{13}C, 75.45 MHz). N.M.R. spectra are described in parts per million downfield shift from T.M.S. and are reported consecutively as position (δ_H or δ_C), relative integral, multiplicity (s.-singlet, d.-doublet, t.-triplet, q.-quartet, m.-multiplet, d.d.-double doublet and br.-broad), coupling constant ($J_{X,Y}$ Hz if applicable) and assignment. Infra-red spectra were recorded using a Perkin Elmer 1310 i.r. spectrophotometer. The samples were prepared as either thin films or nujol mulls between sodium chloride discs. Absorption maxima are given in wavenumbers (cm^{-1}) relative to a polystyrene standard. Melting points were taken on an electrothermal melting point apparatus and are uncorrected. Microanalyses were carried out by the University of St. Andrews Microanalysis Service. Optical rotations were measured on an Optical Activity AA-1000 polarimeter using 10 cm pathlength cells at room temperature. UV spectra were measured on Pye-Unicam SP8/500, Phillips PU8720, Shimadzu UV2101PC and Kontron Uvikon 932 scanning spectrophotometers. Mass spectra were recorded on a Kratos MS50, and obtained on an E.P.S.R.C. service based at the University of Swansea using a VG ZAB E. Major fragments are given as percentages of the base peak intensity. Flash chromatography was performed according to the method of Still[197] using Sorbsil C60 (40-60 mm mesh). Analytical thin layer chromatography was carried out on 0.25 m precoated silica gel plates (Whatman PE SIL G/UV254) and compounds were visualised using UV fluorescence, aqueous potassium permanganate, bromocresol blue or 5% sulfuric acid in aqueous ethanol/charring.

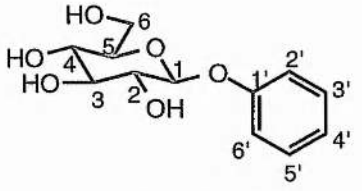
Materials

Myrosinase (thioglucoside glucohydrolase, E. C. 3.2.3.1) was obtained from Sigma. The source of the enzyme is *Sinapis alba* (white mustard seed). The specific activity of the enzyme preparation was 200 units/g. Sinigrin, potassium phosphate, methyl-β-D-glucopyranoside, D-glucono-γ-lactone, 3-hydroxy-1-propanesulfonate, 2-mercaptoethanesulfonate, and glycerol-2-phosphate were obtained from Sigma and were used without further purification. *p*-Nitrophenyl-β-D-glucopyranoside (PNPG), *o*-nitrophenyl-β-D-glucopyranoside (ONPG), L-ascorbic acid, lactose and alcohols were obtained from Aldrich and were used without further purification. Potassium sulfate was obtained from B.D.H. and was used without further purification. *o*- and *p*-Nitrophenol were obtained from B.D.H. and were purified by recrystallisation. β-Glucosidase (E.C. 3.2.1.21) was obtained from Sigma. The source of the enzyme was almonds. The specific activity of the enzyme preparation was 5.5 units/mg. β-Galactosidase (E. C. 3.2.1.23) was obtained from Sigma. The source of the enzyme was *Aspergillus oryzae*. The specific activity of the enzyme preparation was 5.1 units/mg. The enzymes for the analysis of D-glucose (*i.e.* hexokinase and glucose-6-phosphate dehydrogenase) were obtained from Sigma. Solvents (where required to be dry) were dried as follows: dichloromethane, dimethylformamide, pyridine and triethylamine were refluxed over, then distilled from calcium hydride; diethyl ether and tetrahydrofuran were refluxed over, then distilled from sodium/benzophenone; acetone was refluxed over then distilled from calcium chloride; methanol and ethanol were refluxed over, then distilled from magnesium/iodine (Grignard reaction). Methanolic sodium methoxide was always prepared using dry methanol immediately prior to use.

4.1 Synthesis of Compounds for the Brønsted Plot

The subsituted aryl-β-D-glucopyranosides (80) to (84) and (88) were synthesised to use in incubations with myrosinase for the Brønsted plot (see Section 4.16).

4.1.1 Synthesis of Phenyl-β-D-glucopyranoside (82)[165]

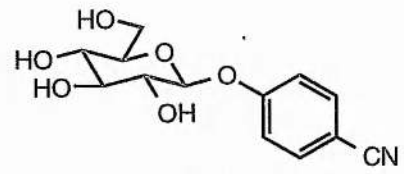


(82)

2,3,4,6-Tetra-*O*-acetyl-1-bromo-α-D-glucopyranose (5.00 g, 12.2 mmol) was dissolved in acetone (40 ml), and to the resultant solution was added a solution of phenol (1.88 g, 20.0 mmol) in 1.0 M sodium hydroxide solution (20 ml). The reaction mixture was stirred at room temperature for 24 hours. The acetone was then removed under reduced pressure and the remaining aqueous solution was diluted to 70 ml with water. This solution was extracted with dichloromethane (3 x 70 ml) and the combined organic extracts washed with 2 M sodium hydroxide solution (3 x 100 ml) and water (100 ml). The organic layer was then separated, dried ($MgSO_4$) and concentrated under reduced pressure to give an off-white solid (2.19 g) as the crude tetraacetate. This solid was dissolved in dry methanol (30 ml) under nitrogen and to the resulting solution was added 1.0 M sodium methoxide in dry methanol (2.0 ml). The reaction mixture was then stirred at room temperature under nitrogen for 24 hours. The solution was neutralised with glacial acetic acid and concentrated under reduced pressure to give an off-white solid (1.32 g). This product was recrystallised from water to give pure phenyl-β-D-glucopyranoside (80) as a white solid (0.76 g, 24.5%), m.p. 166-9 °C (lit.,[198] 175-6 °C); *m/z* (Found: M^+ 256.0995. $C_{12}H_{16}O_6$ requires 256.0947); $[\alpha]_D$ -58.7 ° (c 1.0 in H_2O) (lit.,[198] -71.9 ° (c 2.0 in H_2O)); ν_{max} (nujol)/cm^{-1} 3300 (OH), 1220 (CO) and 750 (aromatic); δ_H (200 MHz; ^2H_2O) 3.20-3.51 (4H, m., H-4,5,6^a,6^b), 3.57 (1H, d.d., $J_{1,2}$ 6.4 Hz, $J_{2,3}$ 12.4 Hz, H-2), 3.76 (1H, m., $J_{2,3}$ 12.4 Hz, H-3), 4.95 (1H, d., $J_{1,2}$ 6.4 Hz, H-1), 6.89-7.09 (3H, m., H-2',4',6'), 7.12-7.30 (2H, m., H-3',5'); δ_C (50.3 MHz; ^2H_2O) 63.33 (C-6), 72.23 (C-4), 75.75 (C-2), 78.36

(C-5), 78.91 (C-3), 102.92 (C-1), 119.33 (C-2',6'), 126.13 (C-4'), 132.77 (C-3',5') and 159.34 (C-1'); *m/z* (CI) 274 ([*M* + NH_4]$^+$, 65%) and 180 (100, [*M* - Ph]$^+$).

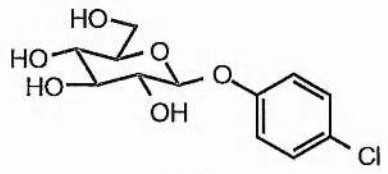
4.1.2 Synthesis of *p*-Cyanophenyl-β-D-glucopyranoside (80)[165]



(80)

This was synthesised in an identical manner to that described for phenyl-β-D-glucopyranoside (82) except that *p*-cyanophenol was used instead of phenol. The pure compound (80) was obtained as a white solid (0.46 g, 9.5%), m.p. 198-202 °C (lit.,[199] 193-4 °C); (Found: C, 55.42; H, 5.24; N, 4.91. Calc. for $C_{13}H_{15}NO_6$: C, 55.51; H, 5.38; N, 4.98%); $[\alpha]_D$ -97.7 ° (c 1.0 in H_2O); ν_{max} (nujol)/cm^{-1} 3300 (OH), 2200 (CN), 1240 (CO) and 815 (aromatic); δ_H (200 MHz; ^2H_2O) 3.30-3.90 (6H, m., H-2,3,4,5,6^a,6^b), 5.10 (1H, d., $J_{1,2}$ 7.5 Hz, H-1), 7.09 (2H, d., *J* 9.5 Hz, H-2',6'), 7.62 (2H, d., *J* 9.5 Hz, H-3',5'); δ_C (50.3 MHz; ^2H_2O) 63.16 (C-6), 72.04 (C-4), 75.49 (C-2), 78.15 (C-5), 78.97 (C-3), 102.06 (C-1), 107.73 (C-4'), 119.63 (C-2',6'), 122.43 (CN), 137.34 (C-3',5') and 162.79 (C-1'); *m/z* (FAB) 304 ([*M* + Na]$^+$, 37%) and 282 (8, [*M* + H]$^+$).

4.1.3 Synthesis of *p*-Chlorophenyl-β-D-glucopyranoside (81)[165]

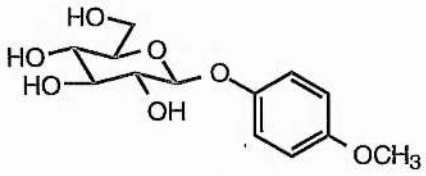


(81)

This was synthesised in an identical manner to that described for phenyl-β-D-glucopyranoside (82) except that *p*-chlorophenol was used instead of phenol. The pure compound (81) was obtained as an off-white solid (1.06 g, 30%), m.p. 167-170 °C (lit.,[199] 173-5 °C); $[\alpha]_D$ -67.6 ° (c 1.0 in H_2O) (lit.,[200] -69.5 ° (c 1.0 in H_2O)); ν_{max} (nujol)/cm^{-1} 3300 (OH), 1230 (CO) and 810 (aromatic); δ_H (200 MHz; ^2H_2O) 3.30-3.58 (4H, m., H-4,5,6^a,6^b), 3.64

(1H, d.d., $J_{1,2}$ 7.1 Hz, $J_{2,3}$ 12.3 Hz, H-2), 3.82 (1H, d.d., $J_{2,3}$ 12.3 Hz, $J_{3,4}$ 1.9 Hz, H-3), 4.99 (1H, d., $J_{1,2}$ 7.1 Hz, H-1), 7.00 (2H, d., J 10.0 Hz, H-2',6'), 7.27 (2H, d., J 10.0 Hz, H-3',5'); δ_C (50.3 MHz; ^2H_2O) 63.26 (C-6), 72.16 (C-4), 75.66 (C-2), 78.27 (C-5), 78.91 (C-3), 103.05 (C-1), 120.77 (C-2',6'), 130.23 ((C-4'), 132.38 (C-3',5') and 163.38 (C-1'); *m/z* (CI) 308 ([M + NH_4]$^+$, 100%) and 180 (68, [M - PhCl]$^+$).

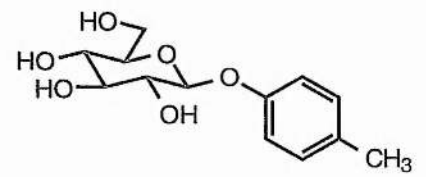
4.1.4 Synthesis of *p*-Methoxyphenyl-β-D-glucopyranoside (83)[165]



(83)

This was synthesised in an identical manner to that described for phenyl-β-D-glucopyranoside (82) except that *p*-methoxyphenol was used instead of phenol. The pure compound (83) was obtained as a white solid (0.27 g, 8%), m.p. 174-8 °C (lit.,[201] 174 °C); (Found: C, 54.65; H, 6.69. Calc. for $C_{13}H_{18}O_7$: C, 54.54; H, 6.34%); $[\alpha]_D$ -68.7 ° (c 1.0 in H_2O) (lit.,[201] -64 ° (c 1.0 in H_2O)); ν_{max} (nujol)/cm^{-1} 3300 (OH), 1220 (CO) and 810 (aromatic); δ_H (200 MHz; ^2H_2O) 3.29-3.73 (5H, m., H-2,4,5,6^a,6^b), 3.69 (3H, s., CH_3), 3.81 (1H, d.d., $J_{2,3}$ 13.5 Hz, H-3), 4.89 (1H, d., $J_{1,2}$ 7.5 Hz, H-1), 6.86 (2H, d., J 9.5 Hz, H-3',5'), 6.99 (2H, d., J 9.5 Hz, H-2',6'); δ_C (50.3 MHz; ^2H_2O) 58.52 ($\underline{C}H_3$), 63.31 (C-6), 72.21 (C-4), 75.76 (C-2), 78.34 (C-5), 78.87 (C-3), 103.97 (C-1), 117.79 (C-3'), 120.94 (C-2'), 153.68 (C-1') and 157.49 (C-4'); *m/z* (FAB) 325 ([M + K]$^+$, 12%), 286 (10, M^+), 255 (29, [M - OCH_3]$^+$) and 163 (41, [M - $OPhOCH_3$]$^+$).

4.1.5 Synthesis of *p*-Methylphenyl-β-D-glucopyranoside (84)[165]

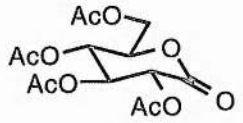


(84)

This was synthesised in an identical manner to that described for phenyl-β-D-glucopyranoside (82) except that *p*-methylphenol was used instead of

phenol. The pure compound (84) was obtained as a white solid (0.47 g, 12.5%), m.p. 177-180 °C (lit.,[176] 178-9 °C); (Found: C, 57.30; H, 6.90. Calc. for $C_{13}H_{18}O_6$: C, 57.77; H, 6.71%); $[\alpha]_D$ -66.4 ° (c 1.0 in H_2O) (lit.,[176] -68.1 ° (c 1.36 in H_2O)); ν_{max} (nujol)/cm^{-1} 3300 (OH) and 1225 (CO); δ_H (200 MHz; ^2H_2O) 2.20 (3H, s., CH_3), 3.30-3.56 (4H, m., H-4,5,6^a,6^b), 3.62 (1H, d.d., $J_{1,2}$ 6.5 Hz, $J_{2,3}$ 12.4 Hz, H-2), 3.81 (1H, d.d., $J_{2,3}$ 12.4 Hz, $J_{3,4}$ 1.8 Hz, H-3), 4.96 (1H, d., $J_{1,2}$ 6.5 Hz, H-1), 6.93 (2H, d., J 9.0 Hz, H-2',6'), 7.11 (2H, d., J 9.0 Hz, H-3',5'); δ_C (50.3 MHz; ^2H_2O) 22.14 ($\underline{C}H_3$), 63.33 (C-6), 72.23 (C-4), 75.74 (C-2), 78.35 (C-5), 78.67 (C-3), 103.25 (C-1), 119.36 (C-2',6'), 133.02 (C-3',5'), 135.05 (C-4') and 149.17 (C-1'); *m/z* (FAB) 293 ([*M* + Na]$^+$, 72%), 178 (27, [*M* - $PhCH_3$]$^+$) and 163 (77, [*M* - $OPhCH_3$]$^+$).

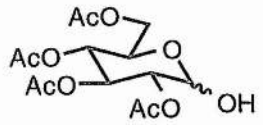
4.1.6 Synthesis of 2,3,4,6-Tetra-*O*-acetyl-D-glucono-γ-lactone (85)



(85)

Zinc chloride (5.0 g, 36.7 mmol) was dissolved in acetic anhydride (50 ml) under nitrogen, and to the resulting solution was slowly added D-glucono-γ-lactone (10.0 g, 56.1 mmol). The reaction mixture was stirred at room temperature under nitrogen for 40 minutes and then poured onto crushed ice (300 ml). After shaking, the homogeneous solution was extracted with dichloromethane (2 x 250 ml) and the combined organic extracts were washed with ice-cold water (2 x 250 ml), dried ($MgSO_4$) and concentrated under reduced pressure to give pure 2,3,4,6-tetra-*O*-acetyl-D-glucono-γ-lactone (85) as a gold syrup (19.43 g, 100%); (Found: C, 48.16; H, 5.29. Calc. for $C_{14}H_{18}O_{10}$: C, 48.56; H, 5.24%); $[\alpha]_D$ +74.1 ° (c 2.0 in $CHCl_3$) (lit.,[166] +79.7 ° (c 2.0 in $CHCl_3$)); ν_{max} (thin film)/cm^{-1} 2950 (CH) and 1715 (CO); δ_H (200 MHz; C^2HCl_3) 2.05-2.31 (12H, 4s., 4 x CH_3), 4.21 (1H, d.d., $J_{5,6a}$ 2.6 Hz, $J_{6a,6b}$ 12.8 Hz, H-6^a), 4.36 (1H, d.d., $J_{5,6b}$ 3.8 Hz, $J_{6a,6b}$ 12.8 Hz, H-6^b), 4.59 (1H, m., $J_{4,5}$ 8.9 Hz, H-5), 5.09 (1H, d., $J_{2,3}$ 9.4 Hz, H-2), 5.34 (1H, t., $J_{3,4}$ 9.1 Hz, H-4), 5.52 (1H, t., $J_{3,4}$ 9.1 Hz, H-3); δ_C (50.3 MHz; C^2HCl_3) 20.81, 20.96, 21.03 & 21.09 (4 x $\underline{C}H_3$), 61.77 (C-6), 66.80 (C-4), 70.73 (C-3,5), 76.21 (C-2), 165.14 (C-1), 169.67, 170.05, 170.45 & 170.78 (4 x $\underline{C}O$); *m/z* (EI) 347 (*M*$^+$, 22%), 304 (5.8, [*M* - Ac]$^+$), 287 (13.8, [*M* - OAc]$^+$), 244 (6, [*M* - OAc - Ac]$^+$), 202 (6, [*M* - OAc - 2Ac]$^+$) and 184 (20, [*M* - 2OAc - Ac]$^+$).

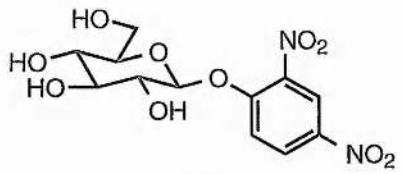
4.1.7 Synthesis of 2,3,4,6-Tetra-*O*-acetyl-α/β-D-glucopyranose (86)



(86)

2,3,4,6-Tetra-*O*-acetyl-D-glucono-γ-lactone (85) (3.83 g, 11.1 mmol) was dissolved in tetrahydrofuran (30 ml) and the resulting solution was cooled to 0 °C. A cold solution of sodium borohydride (0.21 g, 5.5 mmol) in water (1.5 ml) was then added dropwise. The reaction mixture was stirred at 0 °C for 2 hours before DOWEX-50W H^+ ion exchange resin (4 g) was added. The resulting suspension was then filtered and the filtrate was concentrated under reduced pressure to give an oily solid. This product was then washed with methanol to give pure 2,3,4,6-tetra-*O*-acetyl-α/β-D-glucopyranose (86) as a colourless syrup (3.82 g, 99%), $[\alpha]_D$ +11.2 ° (c 1.0 in $CHCl_3$) (lit.,[202] +9.8 ° (c 0.816 in $CHCl_3$); ν_{max} (nujol)/cm^{-1} 3320 (OH) and 1730 (CO); δ_H (200 MHz; C^2HCl_3) 1.90-2.21 (12H, 4s., 4 x CH_3), 3.71 (1H, m., H-5), 4.15 (2H, m., H-6^a,6^b), 4.75-5.74 (4H, m., H-1,2,3,4); δ_C (50.3 MHz; C^2HCl_3) 20.94, 21.15, 21.25 & 21.41 (4 x $\underline{C}H_3$), 62.44 (C-6α-isomer), 65.15 (C-6β-isomer), 67.59 (C-4α), 68.74 (C-4β), 68.92 (C-2α), 69.92 (C-2β), 70.33 (C-5α), 71.17 (C-5β), 71.55 (C-3α), 72.10 (C-3β), 90.56 (C-1α), 95.97 (C-1β) 170.05, 170.65, 170.70 & 171.27 (4 x $\underline{C}O$); *m/z* (CI) 366 ([*M* + NH_4]$^+$, 95%), 331 (100, [*M* - OH]$^+$), 271 (8, [*M* - $C_2H_5O_3$]$^+$) and 211 (3, [*M* - $C_4H_9O_5$]$^+$).

4.1.8 Synthesis of 2,4-Dinitrophenyl-β-D-glucopyranoside (2,4-DNPG) (88)



(88)

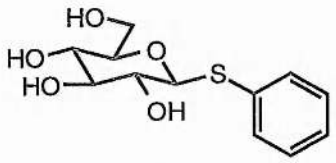
2,3,4,6-Tetra-*O*-acetyl-α/β-D-glucopyranose (86) (0.76 g, 2.18 mmol) was dissolved in dry dimethylformamide (7.0 ml) under nitrogen and to the resulting solution was added 2,2,2-diazabicyclooctane (DABCO) (0.73 g, 6.55 mmol) followed by 1-fluoro-2,4-dinitrobenzene (0.43 g, 2.29 mmol).

The reaction mixture was stirred at room temperature under nitrogen for 9 hours until the reaction was complete (t.l.c., light petroleum/ethyl acetate (3:2)). The solution was then concentrated under reduced pressure to give a dark red oil (1.68 g). This oil was dissolved in dichloromethane (150 ml) and the resulting solution was washed with 10% sodium hydrogen carbonate solution (100 ml) and water (2 x 100 ml), dried ($MgSO_4$) and concentrated under reduced pressure to give a yellow solid (0.75 g). This product was recrystallised from ethanol to give the pure tetraacetyl compound as an off-white solid (0.29 g). This product was dissolved in chloroform (2.90 ml) under nitrogen, and to the resulting solution was added 3% w/w hydrogen chloride in dry methanol (1.74 ml) under nitrogen. The reaction mixture was stirred at room temperature under nitrogen for 4 days after which time the reaction had gone to completion (t.l.c., ethyl acetate/methanol/water (16:1:1). The reaction solution was then concentrated under reduced pressure to give a red/brown oil (0.35 g). This product was purified by silica chromatography using ethyl acetate/methanol/water (16:1:1) as the eluent to give pure 2,4-dinitrophenyl-β-D-glucopyranoside (88) as a yellow solid (0.11 g, 21.5%), m.p. 108-10 °C (lit.,[170] 100-1 °C); $[\alpha]_D$ -94.2 ° (c 1.0 in MeOH) (lit.,[169] -102 ° (c 1.0 in MeOH)); ν_{max} (nujol)/cm^{-1} 3360 (OH) and 730 (aromatic); δ_H (200 MHz; ^2H_2O) 3.39-3.72 (5H, m., H-2,4,5,6^a,6^b), 3.84 (1H, d.d., $J_{2,3}$ 10.6 Hz, $J_{3,4}$ 2.8 Hz, H-3), 5.30 (1H, d., $J_{1,2}$ 7.1 Hz, H-1), 7.48 (1H, d., $J_{5',6'}$ 9.4 Hz, H-6'), 8.40 (1H, d., $J_{5',6'}$ 9.4 Hz, H-5'), 8.75 (1H, s., H-3'); δ_C (50.3 MHz; ^2H_2O) 63.18 (C-6), 71.88 (C-4), 75.33 (C-2), 78.18 (C-5), 79.34 (C-3), 103.02 (C-1), 120.52 (C-3'), 124.99 (C-6'), 132.65 (C-5'), 141.67 (C-2'), 144.20 (C-4') and 157.16 (C-1').

4.2 Synthesis of Alternative Substrates for Myrosinase

Compounds (91), (93) (98), (99), (102), (105) (108) and (112) were synthesised in order to be investigated as potential substrates for myrosinase.

4.2.1 Synthesis of Phenyl-1-thio-β-D-glucopyranoside (PTG) (91)[203]

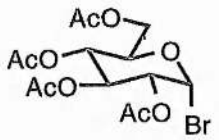


(91)

1,2,3,4,6-Penta-*O*-acetyl-β-D-glucopyranose (6.00 g, 15.4 mmol) was dissolved in dry dichloromethane (70 ml) and stirred under nitrogen. To the resulting solution was added thiophenol (2.04 g, 18.5 mmol) then boron trifluoride-diethyl ether complex (10.92 g, 76.9 mmol). The reaction mixture was stirred at room temperature under nitrogen for 6 hours until the reaction was complete (t.l.c., hexane/ethyl acetate (2:1)). The solution was then washed with saturated sodium carbonate solution (3 x 100 ml) and water (100 ml), dried (Na_2SO_4) and concentrated under reduced pressure to give a white solid (6.81 g). This solid was dissolved in dry methanol (150 ml) under nitrogen and to the resulting solution was added 1.0 M sodium methoxide in dry methanol (2.0 ml) under nitrogen. The reaction mixture was stirred at room temperature under nitrogen for 5 days. The solution was then neutralised with glacial acetic acid and concentrated under reduced pressure to give a white solid (3.52 g). This product was purified by silica chromatography using ethyl acetate/methanol/water (16:2:1) as the eluent to give pure phenyl-1-thio-β-D-glucopyranoside (91) as a white solid (3.04 g, 73%), m.p. 123-6 °C (lit.,[176] 128-130 °C); $[\alpha]_D$ -52.5 ° (c 1.0 in H_2O) (lit.,[176] -71.6 ° (c 1.0 in H_2O)); ν_{max} (nujol)/cm^{-1} 3350 (OH) and 730 (aromatic); δ_H (200 MHz; ^2H_2O) 3.22-3.53 (4H, m., H-4,5,6^a,6^b), 3.64 (1H, d.d., $J_{1,2}$ 5.0 Hz, $J_{2,3}$ 12.4 Hz, H-2), 3.83 (1H, d.d., $J_{2,3}$ 12.4 Hz, $J_{3,4}$ 1.9 Hz, H-3), 4.84 (1H, d., $J_{1,2}$ 5.0 Hz, H-1), 7.35 (3H, m., H-3',4',5'), 7.51 (2H, m., H-2',6'); δ_C (50.3 MHz; ^2H_2O) 63.60 (C-6), 72.16 (C-4), 74.53 (C-2), 80.04 (C-5), 82.71 (C-3), 90.11 (C-1), 130.92 (C-4'), 132.16

(C-3',5'), 134.43 (C-2',6') and 134.83 (C-1'); *m/z* (CI) 290 ([*M* + NH_4]$^+$, 100%) and 180 (12, [*M* - PhSH + NH_4]$^+$).

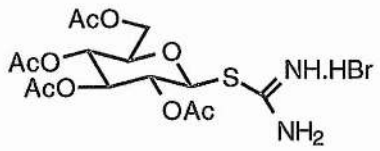
4.2.2 Synthesis of 2,3,4,6-Tetra-*O*-acetyl-1-bromo-α-D-glucopyranose (α-D-Acetobromoglucose) (68)[204]



(68)

To a solution of 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (0.50 g, 1.28 mmol) in dry dichloromethane (10 ml) was added acetic anhydride (1.0 ml) followed by 30% w/v hydrogen bromide in acetic acid (1.0 ml, 2.70 mmol) under nitrogen. The reaction mixture was stirred at room temperature under nitrogen for 48 hours until the reaction was complete (t.l.c., ethyl acetate/light petroleum (3:2)). Toluene (40 ml) was then added and the resulting solution was concentrated under reduced pressure. A further portion of toluene (20 ml) was added and the solution was again concentrated under reduced pressure to remove excess acetic acid. The residue was dissolved in dichloromethane (50 ml) and the solution washed with 10% sodium hydrogen carbonate solution (2 x 50 ml) and water (50 ml). The organic layer was separated, dried ($MgSO_4$) and concentrated under reduced pressure to give pure 2,3,4,6-tetra-*O*-acetyl-1-bromo-α-D-glucopyranose (68) as a pale gold syrup (0.45 g, 85.5%) which overnight turned to a white solid, m.p. 86-7 °C (lit.,[190] 88-9 °C); $[\alpha]_D$ +195.9 ° (c 2.42 in $CHCl_3$) (lit.,[205] +197.84 ° (c 2.42 in $CHCl_3$)); ν_{max} (nujol)/cm^{-1} 1730 (CO); δ_H (200 MHz; C^2HCl_3) 1.91-2.21 (12H, 4s., 4 x C$\underline{H}_3$), 4.11 (1H, m., H-5), 4.30 (2H, m., H-6^a,6^b), 4.82 (1H, d.d., $J_{1,2}$ 3.1 Hz, $J_{2,3}$ 9.6 Hz, H-2), 5.16 (1H, t., $J_{3,4}$ 9.6 Hz, $J_{4,5}$ 9.6 Hz, H-4), 5.55 (1H, t., $J_{2,3}$ 9.6 Hz, $J_{3,4}$ 9.6 Hz, H-3), 6.61 (1H, d., $J_{1,2}$ 3.1 Hz, H-1); δ_C (50.3 MHz; C^2HCl_3) 21.01 & 21.11 (4 x $\underline{C}H_3$), 61.39 (C-6), 67.60 (C-4), 70.59 (C-2), 71.00 (C-5), 72.57 (C-3), 86.56 (C-1), 169.91, 170.23, 170.29 & 170.94 (4 x $\underline{C}O$); *m/z* (CI) 428 & 430 ([*M* + NH_4]$^+$, 18%), 331 (8, [*M* - Br]$^+$) and 213 (29, [*M* - Br - 2OAc]$^+$).

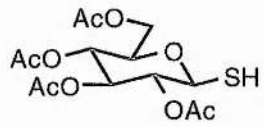
4.2.3 Synthesis of 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl Isothiouronium Bromide (94)[177]



(94)

2,3,4,6-Tetra-*O*-acetyl-1-bromo-α-D-glucopyranose (68) (6.75 g, 16.4 mmol) was dissolved in dry acetone (30 ml) and to the resulting solution was added thiourea (1.25 g, 16.4 mmol). Once dissolved, the reaction mixture was refluxed for 15 minutes then cooled to room temperature. A white precipitate formed, which was filtered off and washed with acetone to give a white solid (4.33 g). This product was recrystallised from acetone to give pure 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiouronium bromide (94) as a white solid (3.69 g, 46.1%), m.p. 196-7 °C (d.) (lit.,[206] 205 °C); (Found: C, 37.25; H, 4.59; N, 5.72. Calc. for $C_{15}H_{23}BrN_2O_9S$: C, 36.97; H, 4.76; N, 5.75%); $[\alpha]_D$ -20.2 ° (c 1.0 in MeOH) (lit.,[207] -17.3 ° (c 1.0 in MeOH)); ν_{max} (nujol)/cm^{-1} 3310-3160 (NH), 1750 (CO) and 1655 (NH); δ_H (200 MHz; ^2H_2O) 1.90-2.09 (12H, 4s., 4 x C$\underline{H}_3$), 4.12-4.25 (1H, m., H-5), 4.25-4.42 (2H, m., H-6^a,6^b), 5.12-5.50 (4H, m., H-1,2,3,4); δ_C (50.3 MHz; ^2H_2O) 14.89, 18.08, 18.11 & 18.21 (4 x $\underline{C}H_3$), 55.52 (C-1'), 59.92 (C-6), 65.65 (C-4), 67.24 (C-2), 71.43 (C-5), 73.93 (C-3), 79.18 (C-1), 170.46, 170.65, 170.99 & 171.65 (4 x $\underline{C}O$); *m/z* (EI) 331 ([*M* - CH_4BrN_2S]$^+$, 11%), 169 (35, $[C_8H_9O_4]^+$ and 127 (18, $[C_6H_7O_3]^+$).

4.2.4 Synthesis of 2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (95)[177]

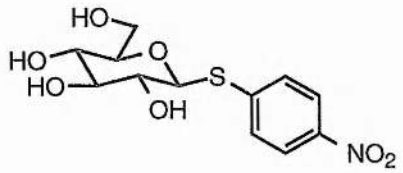


(95)

Potassium metabisulfite (1.68 g, 7.57 mmol) was dissolved in water (20 ml) and the resulting solution was heated to 75 °C. Dichloromethane (25 ml) was then added as the lower phase and 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiouronium bromide (94) (3.67 g, 7.57 mmol) was added

with stirring. The two-phase system was refluxed for 15 minutes with stirring and then allowed to cool to room temperature. The organic layer was separated and washed with water (3 x 25 ml), dried ($MgSO_4$) and concentrated under reduced pressure to give a white solid (1.42 g). This product was recrystallised from methanol to give pure 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (95) as a white solid (1.26 g, 46%), m.p. 72-5 °C (lit.,[177] 75 °C); (Found: C, 46.02; H, 5.41. Calc. for $C_{14}H_{20}O_9S$: C, 46.15; H, 5.53%); $[\alpha]_D$ -9.8 ° (c 1.5 in EtOH) (lit.,[208] -8.3 ° (c 1.5 in EtOH)); ν_{max} (nujol)/cm^{-1} 3460 (SH) and 1735 (CO); δ_H (200 MHz; C^2HCl_3) 1.85-2.35 (12H, 4s., 4 x $C\underline{H}_3$), 3.69 (1H, m., H-5), 4.13 (1H, d.d., $J_{5,6a}$ 2.5 Hz, $J_{6a,6b}$ 12.3 Hz, H-6^a), 4.26 (1H, d.d., $J_{5,6b}$ 4.8 Hz, $J_{6a,6b}$ 12.3 Hz, H-6^b), 4.54 (1H, t., $J_{1,2}$ 9.6 Hz, H-1), 4.98 (1H, t., $J_{1,2}$ 9.6 Hz, $J_{2,3}$ 9.6 Hz, H-2), 5.10 (1H, t., $J_{3,4}$ 9.6 Hz, $J_{4,5}$ 9.6 Hz, H-4), 5.20 (1H, t., $J_{2,3}$ 9.6 Hz, $J_{3,4}$ 9.6 Hz, H-3); δ_C (50.3 MHz; C^2HCl_3) 21.03 (2 x $\underline{C}H_3$), 21.20 (2 x $\underline{C}H_3$), 62.43 (C-6), 68.53 (C-4), 73.97 (C-2), 76.75 (C-5), 77.48 (C-3), 79.14 (C-1), 169.82 ($\underline{C}O$), 170.08 ($\underline{C}O$), 170.56 ($\underline{C}O$) and 171.11 ($\underline{C}O$); *m/z* (CI) 382 ([*M* + NH_4]$^+$, 100%), 331 (7, [*M* - SH]$^+$) and 322 (22, [*M* - OAc]$^+$).

4.2.5 Synthesis of *p*-Nitrophenyl-1-thio-β-D-glucopyranoside (PNPTG) (93)

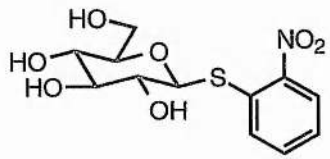


(93)

2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (95) (0.95 g, 2.6 mmol) was dissolved in dry dimethylformamide (5 ml) under nitrogen and to the resulting solution was added *p*-fluoronitrobenzene (0.37 g, 2.6 mmol) and triethylamine (0.29 g, 2.9 mmol) under nitrogen. The reaction mixture was stirred at room temperature under nitrogen for 24 hours. The mixture was then concentrated under reduced pressure to give a yellow solid. This solid was dissolved in dichloromethane (50 ml) and washed with water (3 x 50 ml), dried ($MgSO_4$) and concentrated under reduced pressure to give a yellow solid (1.29 g). This product was dissolved in dry methanol (130 ml) under nitrogen and to the resulting solution was added 1.0 M sodium methoxide in dry methanol (2.0 ml) under nitrogen. The reaction mixture was stirred at room temperature under nitrogen for 48 hours and then neutralised with glacial acetic acid. The resulting solution was

concentrated under reduced pressure to give a yellow solid (1.04 g). This product was recrystallised twice from water to give pure *p*-nitrophenyl-1-thio-β-D-glucopyranoside (93) as a yellow solid (0.34 g, 43.5%), m.p. 146-8 °C (lit.,[175] 148-9 °C); $[\alpha]_D$ -102.7 ° (c 5.0 in H_2O/EtOH (1:1)) (lit.,[209] -106.5 ° (c 5.0 in H_2O/EtOH (1:1))); ν_{max} (nujol)/cm^{-1} 3395 (OH); δ_H (200 MHz; ^2H_2O) 3.38-3.64 (4H, m., H-4,5,6^a,6^b), 3.70 (1H, d.d., $J_{1,2}$ 5.5 Hz, $J_{2,3}$ 12.3 Hz, H-2), 3.91 (1H, d.d., $J_{2,3}$ 12.3 Hz, $J_{3,4}$ 1.1 Hz, H-3), 5.01 (1H, d., $J_{1,2}$ 5.5 Hz, H-1), 7.59 (2H, d., *J* 9.5 Hz, H-3',5'), 8.13 (2H, d., *J* 9.5 Hz, H-2',6'); δ_C (50.3 MHz; ^2H_2O) 63.58 (C-6), 72.14 (C-4), 74.52 (C-2), 80.03 (C-5), 82.82 (C-3), 88.51 (C-1), 126.92 (C-3',5'), 132.81 (C-2',6'), 139.94 (C-1') and 148.74 (C-4').

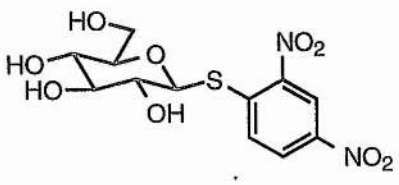
4.2.6 Synthesis of *o*-Nitrophenyl-1-thio-β-D-glucopyranoside (ONPTG) (98)



(98)

This was synthesised in an identical manner to that described for *p*-nitrophenyl-1-thio-β-D-glucopyranoside (93) except that *o*-nitrofluorobenzene was used instead of *p*-nitrofluorobenzene. The pure compound (98) was obtained as a yellow solid (0.26 g, 29%), m.p 142-6 °C; (Found: C, 45.55; H, 5.06; N, 4.20. Calc. for $C_{12}H_{15}NO_7S$: C, 45.42; H, 4.76; N, 4.41%); $[\alpha]_D$ -96.8 ° (c 5.0 in H_2O/EtOH (1:1)); ν_{max} (nujol)/cm^{-1} 3280 (OH) and 845, 815 & 690 (aromatic); δ_H (200 MHz; ^2H_2O) 3.40-3.57 (4H, m., H-4,5,6^a,6^b), 3.67 (1H, d.d., $J_{1,2}$ 8.4 Hz, $J_{2,3}$ 13.1 Hz, H-2), 3.89 (1H, d.d., $J_{2,3}$ 13.1 Hz, $J_{3,4}$ 1.1 Hz, H-3), 4.96 (1H, d., $J_{1,2}$ 8.4 Hz, H-1), 7.39 (1H, t., *J* 10.0 Hz, H-4'), 7.64 (1H, d., *J* 10.0 Hz, H-2'), 7.69 (1H, t., *J* 10.0 Hz, H-3'), 8.13 (1H, d., *J* 10.0 Hz, H-5'); δ_C (50.3 MHz; ^2H_2O) 63.63 (C-6), 72.12 (C-4), 74.50 (C-2), 80.13 (C-5), 82.71 (C-3), 87.75 (C-1), 128.84 (C-5'), 129.64 (C-4') 131.55 (C-2'), 137.56 (C-3') and 153.14 (C-6').

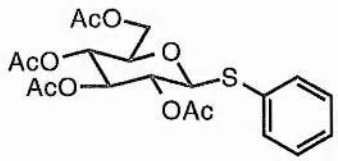
4.2.7 Synthesis of 2,4-Dinitrophenyl-1-thio-β-D-glucopyranoside (2,4-DNPTG) (99)



(99)

This was synthesised in an identical manner to that described for *p*-nitrophenyl-1-thio-β-D-glucopyranoside (93) except that 1-fluoro-2,4-dinitrobenzene was used instead of *p*-nitrofluorobenzene. The pure compound (99) was obtained as a yellow solid (0.86 g, 86.5%), m.p 178-180 °C (lit.,[207] 184-5 °C); $[\alpha]_D$ -239.1 ° (c 1.0 in MeOH) (lit.,[207] -247 ° (c 1.0 in MeOH)); ν_{max} (nujol)/cm^{-1} 3300 (OH); δ_H (200 MHz; ^2H_2O) 3.40-4.38 (6H, m., H-2,3,4,5,6^a,6^b), 5.11 (1H, d., $J_{1,2}$ 9.2 Hz, H-1), 7.89 (1H, t., $J_{5',6'}$ 9.1 Hz, H-6'), 8.41 (1H, d., $J_{5',6'}$ 9.1 Hz, H-5'), 9.02 (1H, s., H-3'); δ_C (50.3 MHz; $(C^2H_3)_2SO$) 69.72 (C-6), 72.47 (C-4), 78.36 (C-2), 81.26 (C-5), 84.00 (C-3), 115.41 (C-1), 121.41 (C-3'), 127.68 (C-5') 129.68 (C-6'), 129.90 (C-1') 144.30 (C-4') and 152.41 (C-2').

4.2.8 Synthesis of 2,3,4,6-Tetra-*O*-acetyl-phenyl-1-thio-β-D-glucopyranoside (90)

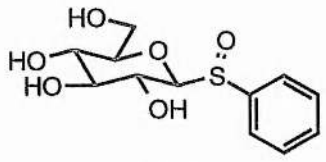


(90)

Phenyl-1-thio-β-D-glucopyranoside (91) (0.70 g, 2.57 mmol) was dissolved in pyridine (20 ml) and to the resulting solution was added acetic anhydride (1.60 ml, 17 mmol). The reaction mixture was then stirred at room temperature for 16 hours then diluted with dichloromethane (70 ml). The resulting solution was washed with 1 M hydrochloric acid (80 ml) and the organic extracts were then dried ($MgSO_4$) and concentrated under reduced pressure to give an off-white solid. This product was then co-evaporated with toluene (2 x 30 ml) under reduced pressure to give pure 2,3,4,6-tetra-*O*-acetyl-phenyl-1-thio-β-D-glucopyranoside (90) as an off-

white solid (1.13g, 100%), m.p. 115-6 °C (lit.,[203] 117-8 °C); $[\alpha]_D$ -14.8 ° (c 1.0 in MeOH) (lit.,[203] -16 ° (c 1.0 in MeOH)); ν_{max} (nujol)/cm^{-1} 1750 (CO) and 730 (aromatic); δ_H (200 MHz; C^2HCl_3) 1.95-2.15 (12H, 4s., 4 x CH_3), 3.72 (1H, m., H-5), 4.21 (2H, m., H-6^a,6^b), 4.71 (1H, d., $J_{1,2}$ 10.6 Hz, H-1), 4.90-5.31 (3H, m., H-2,3,4), 7.30 (3H, m., H-2',4',6'), 7.49 (2H, m., H-3',5').

4.2.9 Synthesis of Phenyl-β-D-glucopyranoside Sulfoxide (102)[173]

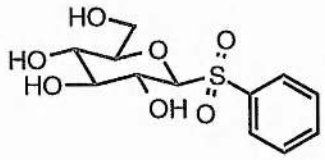


(102)

2,3,4,6-Tetra-*O*-acetyl-phenyl-1-thio-β-D-glucopyranoside (90) (0.22g, 0.50 mmol) was dissolved in methanol (40 ml) and the resulting solution was cooled to 0-5 °C. A solution of magnesium monoperoxyphthalate (0.15 g, 0.25 mmol) in methanol (20 ml) was added to the solution dropwise over 45 minutes. The reaction mixture was maintained below 5 °C for a further 2 hours, then allowed to warm to room temperature and stirred at room temperature for 48 hours, after which time the reaction was complete (t.l.c., light petroleum/ethyl acetate (1:1). The solution was then concentrated under reduced pressure to give a white solid. This solid was dissolved in dichloromethane (50 ml) and the solution was washed with a saturated solution of sodium hydrogen carbonate (2 x 50 ml) and water (50 ml), dried ($MgSO_4$) and concentrated under reduced pressure to give a white solid. This product was purified by silica chromatography using light petroleum/ethyl acetate (1:1) as the eluent to give the pure tetraacetyl compound. This product was dissolved in dry methanol (20 ml) under nitrogen and to the resulting solution was added 1.0 M sodium methoxide in dry methanol (1.0 ml) under nitrogen. The reaction mixture was stirred at room temperature under nitrogen for 24 hours, after which time the reaction was complete (t.l.c., light petroleum/ethyl acetate (1:1). Amberlite IR-120 H^+ ion exchange resin (1 g) was added, and after stirring at room temperature for 1 minute, the mixture was filtered and the filtrate was concentrated under reduced pressure to give a white solid. This product was purified by silica chromatography using dichloromethane/methanol (4:1) as the eluent to give pure phenyl-β-D-glucopyranoside sulfoxide (102) as a white solid (0.07 g, 49%), m.p. 74-5 °C (d.) (lit.,[180] 78-80 °C);

$[\alpha]_D$ +62.5 ° (c 0.6 in H_2O) (lit.,[180] +58.6 ° (c 0.6 in H_2O); ν_{max} (nujol)/cm^{-1} 3300 (OH), 1050 (S=O) and 755 & 740 (aromatic); δ_H (200 MHz; ^2H_2O) 3.00-3.79 (5H, m., H-2,4,5,6^a,6^b), 3.83 (1H, d.d., $J_{2,3}$ 13.0 Hz, $J_{3,4}$ 2.1 Hz, H-3), 4.25 (1H, d., $J_{1,2}$ 9.8 Hz, H-1), 7.61 (5H, m., H-2',3',4',5',6'); δ_C (50.3 MHz; ^2H_2O) Major isomer: 63.87 (C-6), 71.77 (C-4), 72.35 (C-2), 79.84 (C-5), 83.86 (C-3), 94.93 (C-1), 128.25 (C-4'), 129.48 (C-2',6'), 132.15 (C-3',5'), 135.72 (C-1'); Minor isomer: 62.97 (C-6), 71.27 (C-4), 71.34 (C-2), 79.74 (C-5), 82.95 (C-3), 95.48 (C-1), 128.25 (C-4'), 129.48 (C-2',6'), 132.23 (C-3',5'), 135.08 (C-1'); *m/z* (EI) 163 ($[C_6H_{11}O_5]^+$, 3%), 125 (42, $[C_6H_5SO]^+$), 109 (59, $[C_6H_5S]^+$) and 77 (25, $[C_6H_5]^+$).

4.2.10 Synthesis of Phenyl-β-D-glucopyranoside Sulfone (105)

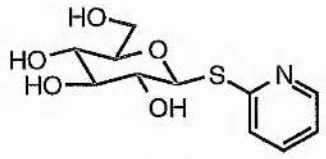


(105)

2,3,4,6-Tetra-*O*-acetyl-phenyl-1-thio-β-D-glucopyranoside (90) (0.20 g, 0.45 mmol) was dissolved in dichloromethane (100 ml). The resulting solution was cooled to 0 °C and *m*-chloroperoxybenzoic acid (50-55%) (0.45 g, 1.36 mmol) was added. The reaction mixture was maintained at 0 °C for 2 hours before being allowed to warm to room temperature. The reaction mixture was then stirred at room temperature for 3 days, after which time the reaction was complete (t.l.c., light petroleum/ethyl acetate (1:1). The reaction solution was then washed with a saturated solution of sodium hydrogencarbonate (2 x 80 ml) then water (80 ml), dried ($MgSO_4$) and concentrated under reduced pressure to give the tetraacetyl compound. This product was then dissolved in dry methanol (20 ml) under nitrogen and to the resulting solution was added 1.0 M sodium methoxide in dry methanol (1.0 ml) under nitrogen. The reaction mixture was then stirred at room temperature under nitrogen for 24 hours, after which time the reaction was complete (t.l.c., light petroleum/ethyl acetate (1:1). Amberlite IR-120 H^+ ion exchange resin (1 g) was then added, and after stirring at room temperature for 1 minute, the mixture was filtered and the filtrate was concentrated under reduced pressure to give pure phenyl-β-D-glucopyranoside sulfone (105) as a white solid (0.11 g, 80.5%), m.p. 88-90 °C (lit.,[181] 91-2 °C); $[\alpha]_D$ -11.9 ° (c 1.0 in H_2O) (lit.,[181] -13 ° (c 0.9 in H_2O);

ν_{max} (nujol)/cm^{-1} 3300 (OH), 1250 & 1110 (S=O) and 755 & 740 (aromatic); δ_H (200 MHz; ^2H_2O) 3.21-3.75 (6H, m., H-2,3,4,5,6^a,6^b), 4.63 (1H, d., $J_{1,2}$ 10.9 Hz, H-1), 7.61 (2H, t., J 9.8 Hz, H-3',5'), 7.72 (1H, d., J 9.8 Hz, H-4'), 7.90 (2H, d., J 9.8 Hz, H-2',6'); δ_C (50.3 MHz; ^2H_2O) 64.12 (C-6), 71.72 (C-4), 72.45 (C-2), 79.77 (C-5), 83.89 (C-3), 101.79 (C-1), 128.41 (C-4'), 132.58 (C-2',6'), 135.38 (C-3',5'), 144.63 (C-1').

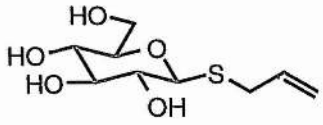
4.2.11 Synthesis of 2-Pyridyl-1-thio-β-D-glucopyranoside (108)[182]



(108)

2,3,4,6-Tetra-*O*-acetyl-α/β-D-glucopyranose (86) (1.80 g, 5.17 mmol) and 2,2'-dipyridyl disulfide (1.25 g, 5.69 mmol) were dissolved in dichloromethane (30 ml) at 0 °C. To the resulting solution was then added dropwise tri-*n*-butylphosphine (1.15 g, 5.69 mmol). The reaction mixture was stirred at 0 °C for 30 minutes and silica gel (3 g) was added and the mixture concentrated under reduced pressure. The residue was purified by silica chromatography using light petroleum/ethyl acetate (1:1) as the eluent to give the tetraacetyl compound as a yellow syrup. This product was dissolved in dry methanol (40 ml) under nitrogen and to the resulting solution was added 1.0 M sodium methoxide in dry methanol (1.0 ml). The reaction mixture was stirred at room temperature under nitrogen for 24 hours. Amberlite IR-120 H^+ ion exchange resin (1 g) was added, and after stirring at room temperature for 1 minute, the mixture was filtered and the filtrate was concentrated under reduced pressure to give 2-pyridyl-1-thio-β-D-glucopyranoside (108) as a gold oil (0.17 g, 12%). This product was crystallised to a pale yellow hygroscopic solid on trituration with diethyl ether. ν_{max} (nujol)/cm^{-1} 3300 (OH) and 750, & 705 (aromatic); δ_H (200 MHz; ^2H_2O) 3.40-3.65 (4H, m., H-4,5,6^a,6^b), 3.71 (1H, d.d., $J_{1,2}$ 7.9 Hz, $J_{2,3}$ 12.4 Hz, H-2), 3.90 (1H, d.d., $J_{2,3}$ 12.4 Hz, $J_{3,4}$ 1.9 Hz, H-3), 5.20 (1H, d., $J_{1,2}$ 7.9 Hz, H-1), 7.47 (1H, t., J 9.9 Hz, H-5'), 7.70 (1H, d., J 9.9 Hz, H-3'), 7.99 (1H, t., J 9.9 Hz, H-6'), 8.47 (1H, d., J 9.9 Hz, H-4'); δ_C (50.3 MHz; ^2H_2O) 63.49 (C-6), 72.67 (C-4), 74.82 (C-2), 79.94 (C-5), 82.33 (C-3), 87.84 (C-1), 126.04 (C-5'), 128.32 (C-3'), 144.73 (C-6'), 149.05 (C-4'), 156.30 (C-2').

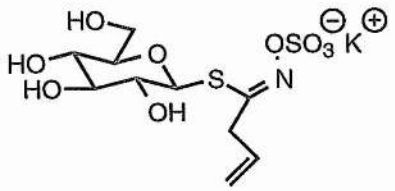
4.2.12 Synthesis of Allyl-1-thio-β-D-glucopyranoside (112)



(112)

2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (95) (1.00 g, 2.74 mmol) was dissolved in dry dimethylformamide (5 ml) under nitrogen and to the resulting solution was added allyl bromide (0.23 ml, 2.74 mmol) and triethylamine (0.24 ml, 3.27 mmol). The reaction mixture was stirred at room temperature under nitrogen for 3 days. The mixture was then filtered and the filtrate concentrated under reduced pressure. The resulting residue was then dissolved in dichloromethane (50 ml) and the solution washed with water (3 x 50 ml), dried ($MgSO_4$) and concentrated under reduced pressure to give a colourless syrup (1.11 g). This product was then dissolved in dry methanol (25 ml) under nitrogen and to the resulting solution was added 1.0 M sodium methoxide in dry methanol (2.0 ml) under nitrogen. The reaction mixture was stirred at room temperature under nitrogen for 4 days then neutralised with glacial acetic acid and concentrated under reduced pressure to give a colourless syrup. This product was purified by silica chromatography using ethyl acetate/methanol/water (16:2:1) as the eluent to give pure allyl-1-thio-β-D-glucopyranoside (112) as a colourless syrup (0.51g, 80%); $[\alpha]_D$ -12.7 ° (c 1.0 in H_2O) (lit.,[210] -11 ° (c 1.0 in H_2O); ν_{max} (thin film)/cm^{-1} 3400 (OH) and 3000 (CH); δ_H (200 MHz; ^2H_2O) 3.20-3.52 (6H, m., H-1'^{a},1'^{b},4,5,6^a,6^b), 3.67 (1H, d.d., $J_{1,2}$ 9.5 Hz, $J_{2,3}$ 12.9 Hz, H-2), 3.88 (1H, d., $J_{2,3}$ 12.9 Hz, H-3), 4.45 (1H, d., $J_{1,2}$ 9.5 Hz, H-1), 5.14 (1H, d., $J_{2',3'a}$ 9.5 Hz, H-3'^{a} (*cis*)), 5.19 (1H, d., $J_{2',3'b}$ 17.7 Hz, H-3'^{b} (*trans*)), 5.85 (1H, m., H-2'); δ_C (50.3 MHz; ^2H_2O) 35.54 (C-1'), 63.75 (C-6), 72.44 (C-4), 74.95 (C-2), 80.13 (C-5), 82.51 (C-3), 86.52 (C-1), 120.77 (C-3'), 136.59 (C-2'); *m/z* (CI) 275 ([*M* + K]$^+$, 7%), 237 (16, [*M* + H]$^+$), 219 (100, [*M* - H_2O]$^+$), 201 (21.5, [*M* - $(H_2O)_2$]$^+$), 184 (64, [*M* - $(H_2O)_2$ - OH]$^+$), 163 (73.5, [*M* - C_3H_5S]$^+$), 145 (99, [*M* - C_3H_5S - H_2O]$^+$) and 127 (27, [*M* - C_3H_5S - $(H_2O)_2$]$^+$).

4.3 Synthesis of Sinigrin (Allyl Glucosinolate) ((1), R = Allyl)[185]



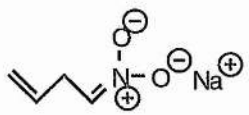
((1), R = Allyl)

4.3.1 Synthesis of 4-Nitrobut-1-ene (120)[188]

(120)

Silver nitrate (3.92 g, 25.4 mmol) was added to dry dichloromethane (5 ml) under nitrogen, and to this mixture was added 4-bromobut-1-ene (3.0 g, 4.0 ml) dropwise over 10 minutes, whilst the reaction mixture was maintained at 15 °C. The mixture was allowed to warm to room temperature and stirred in the dark under nitrogen for 5 days. The mixture was filtered over a bed of celite and washed well with dichloromethane. The filtrate was concentrated under reduced pressure to give an orange oil (2.46 g). This oil was purified by distillation under reduced pressure to give pure 4-nitrobut-1-ene (120) as a colourless oil (1.00 g, 44.5%), b.p. 38-56 °C/10 mmHg (lit.,[188] 55 °C/19 mmHg); ν_{max} (thin film)/cm^{-1} 2950 (CH) and 760 & 700 (C=C); δ_H (200 MHz; C^2HCl_3) 2.50 (1H, d.d., $J_{2,3a}$ 7.6 Hz, $J_{3a,4}$ 7.6 Hz, H-3^a), 2.78 (1H, d.d., $J_{2,3b}$ 7.6 Hz, $J_{3b,4}$ 7.6 Hz, H-3^b), 4.49 (2H, m., H-4^a,4^b), 5.16 (1H, d., $J_{1a,2}$ 12.5 Hz, (*trans*), H-1^a), 5.24 (1H, d., $J_{1b,2}$ 5.0 Hz, (*cis*), H-1^b), 5.80 (1H, m., H-2); δ_C (50.3 MHz; C^2HCl_3) 31.69 (C-3), 72.53 (C-4), 119.51 (C-1), 132.22 (C-2); *m/z* (EI) 101 (M^+, 5%), 55 (100, $[M - NO_2]^+$) and 41 (63, $[M - CH_2NO_2]^+$).

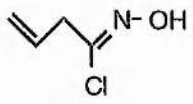
4.3.2 Synthesis of Sodium Salt of 4-Nitrobut-1-ene (121)[185,186]



(121)

Sodium ethoxide (1.0 M) in dry ethanol (9.0 ml, 9.0 mmol) was added to 4-nitrobut-1-ene (0.91 g, 9.0 mmol) under nitrogen. The white precipitate which immediately formed was diluted with dry ether (30 ml) under nitrogen. The resulting slurry was filtered and the white solid product was washed with ether. This product was dried under reduced pressure to give pure sodium salt of 4-nitrobut-1-ene (121) as a white solid (0.56 g, 51%), m.p. 208 °C (d.); ν_{max} (nujol)/cm^{-1} 720 (C=C); δ_H (200 MHz; ^2H_2O) 2.92 (2H, t., $J_{2,3}$ 6.5 Hz, $J_{3,4}$ 6.5 Hz, H-3^a,3^b), 5.08 (2H, d.d., $J_{1a,2}$ 12.5 Hz (*trans*), H-1^a $J_{1b,2}$ 5.0 Hz (*cis*), H-1^b), 5.78 (1H, m., $J_{2,3}$ 6.5 Hz, H-2), 6.14 (1H, t., $J_{3,4}$ 6.5 Hz, H-4); δ_C (50.3 MHz; ^2H_2O) 33.66 (C-3), 119.14 (C-4), 135.56 (C-1), 148.61 (C-2); *m/z* (EI) 123 (*M*$^+$, 49%), 69 (53, [*M* - NaO_2 + H]$^+$) and 55 (100, [*M* - NO_2Na + H]$^+$).

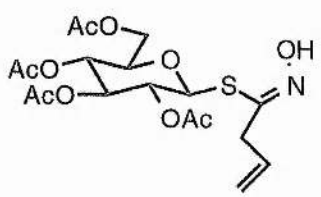
4.3.3 Synthesis of But-3-enohydroximoyl Chloride (122)[185,186]



(122)

The sodium salt of 4-nitrobut-1-ene (121) (0.30 g, 2.43 mmol) was suspended in dry ether (15 ml) under nitrogen and the suspension was cooled to -78 °C. Dry hydrogen chloride was bubbled through this suspension for 45 minutes, during which time the salt dissolved and a precipitate gradually appeared. The precipitate was filtered off and washed with cold dry ether. The filtrate was concentrated under reduced pressure to give pure but-3-enohydroximoyl chloride (122) as a gold oil (0.29 g, 100%); δ_H (200 MHz; C^2HCl_3) 3.28 (2H, d., $J_{2,3}$ 6.3 Hz, H-3^a,3^b), 5.24 (2H, m., H-1^a,1^b), 5.87 (1H, m., $J_{2,3}$ 6.3 Hz, H-2); δ_C (50.3 MHz; C^2HCl_3) 41.56 (C-3), 119.92 (C-1), 131.23 (C-2).

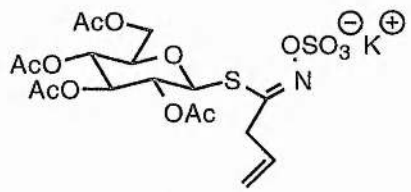
4.3.4 Synthesis of 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-but-3-enothiohyroximate (123)[185,186]



(123)

A solution of but-3-enohydroximoyl chloride (122) (0.16 g, 1.37 mmol) in dry ether (15 ml) was added to a stirred solution of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (95) (0.50 g, 1.37 mmol) in dry ether (30 ml) under nitrogen. Triethylamine (2.0 ml, 1.45 g, 14.3 mmol) was then added, and a white precipitate immediately formed. The reaction mixture was stirred at room temperature under nitrogen for a further 30 minutes. The mixture was then washed with 1N sulfuric acid (50 ml). Two-thirds of the organic layer was decanted and replaced with ethyl acetate (80 ml). The resulting mixture was shaken, separated and the organic layers combined, dried ($MgSO_4$) and concentrated under reduced pressure to give an off-white solid. This product was purified by recrystallisation from ethanol to give pure 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-but-3-enothiohyroximate (123) as a white solid (0.19 g, 31%), m.p. 158-160 °C (lit.,[185] 164-5 °C); (Found: C, 48.30; H, 5.87; N, 2.85. Calc. for $C_{18}H_{25}NO_{10}S$: C, 48.32; H, 5.63; N, 3.13%); $[\alpha]_D$ -12.3 ° (c 0.14 in $CHCl_3$) (lit.,[185] -13 ° (c 0.14 in $CHCl_3$)); ν_{max} (nujol)/cm^{-1} 3305 (OH), 1760 (CO) and 1710 (CO); δ_H (200 MHz; C^2HCl_3) 1.91-2.20 (12H, 4s., 4 x C<u>H</u>$_3$), 3.35 (2H, d., $J_{2',3'}$ 5.3 Hz, H-2'^{a},2'^{b}), 3.75 (1H, m., H-5), 4.18 (2H, d., $J_{5,6}$ 3.8 Hz, H-6^a,6^b), 5.09 (4H, m., H-1,2,3,4), 5.22 (2H, m., H-4'^{a},4'^{b}), 5.93 (1H, m., H-3'), 8.29 (1H, br., O<u>H</u>); δ_C (50.3 MHz; C^2HCl_3) 21.08 & 21.23 (4 x <u>C</u>H$_3$), 37.24 (C-2'), 62.81 (C-6), 68.58 (C-4), 70.50 (C-2), 74.22 (C-5), 76.30 (C-3), 79.90 (C-1), 118.63 (C-4'), 133.24 (C-3'), 151.56 (C-1'), 169.72, 169.91, 170.78 & 171.12 (4 x <u>C</u>O); *m/z* (FAB) 470 ([*M* + Na]$^+$, 7.5%), 447 (8.5, *M* $^+$) and 331 (100, [*M* - $C_4H_6NOS]^+$).

4.3.5 <u>Synthesis of 2,3,4,6-Tetra-*O*-acetyl Sinigrin (124)</u>[185,189]

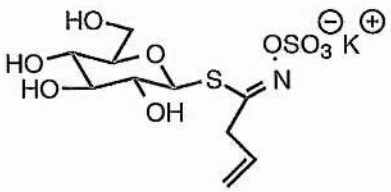


(124)

To a mixture of dry pyridine (16 ml) and dry dichloromethane (16 ml) at 0 °C under nitrogen was added dropwise a solution of chlorosulfonic acid (0.74 ml, 12 mmol) in dichloromethane (10 ml). A solution of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-but-3-enothiohyroximate (123) (0.43 g, 0.96 mmol) in dry dichloromethane (10 ml) was added. The reaction mixture

was stirred at room temperature under nitrogen for 24 hours after which time the reaction had gone to completion (t.l.c., ethyl acetate/methanol/water (16:1:1)). A solution of potassium hydrogen carbonate (1.78 g, 18 mmol) in water (25 ml) was added and the mixture was stirred for 30 minutes. The resulting mixture was concentrated under reduced pressure to give an oily solid. This residue was purified by silica chromatography using ethyl acetate/methanol (4:1) as the eluent to give pure 2,3,4,6-tetra-*O*-acetyl sinigrin (124) as a white solid (0.34 g, 62%), m.p. 187 °C (d.) (lit.,[185] 193-5 °C); $[\alpha]_D$ -14.6 ° (c 0.14 in H_2O) (lit.,[185] -16 ° (c 0.14 in H_2O)); ν_{max} (nujol)/cm^{-1} 1730 (CO); δ_H (200 MHz; ^2H_2O) 1.97-2.17 (12H, 4s., 4xC<u>H</u>$_3$), 3.42 (2H, d., $J_{2',3'}$ 5.5 Hz, H-2'^{a},2'^{b}), 4.07 (1H, m., H-5), 4.14 (1H, d.d., $J_{5,6a}$ 1.1 Hz, $J_{6a,6b}$ 12.9 Hz, H-6^a), 4.29 (1H, d.d., $J_{5,6a}$ 4.3 Hz, $J_{6a,6b}$ 12.9 Hz, H-6^b), 5.02-5.42 (6H, m., H-1,2,3,4,4'^{a},4'^{b}), 5.92 (1H, m., H-3'); δ_C (50.3 MHz; ^2H_2O) 22.93 (4xC<u>H</u>$_3$), 38.97 (C-2'), 64.91 (C-6), 70.73 (C-4), 72.83 (C-2), 76.77 (C-5), 78.00 (C-3), 82.26 (C-1), 121.41 (C-4'), 125.04 (C-1'), 134.66 (C-3'), 175.24, 175.57, 175.92 & 176.52 (4xC<u>O</u>); *m/z* (ES) 526 ([*M* - K]$^+$, 100%), 484 (13, [*M* - KOAc + H]$^+$) and 442 (1, [*M* - 2OAc - K + 2H]$^+$).

4.3.6 <u>Synthesis of Sinigrin (Allyl Glucosinolate) ((1), R = Allyl)</u>[185,186]



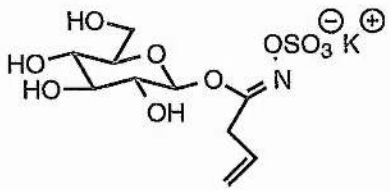
((1), R = Allyl)

To a saturated solution of ammonia in dry methanol (35 ml) under nitrogen was added 2,3,4,6-tetra-*O*-acetyl sinigrin (124) (0.50 g, 0.88 mmol). The reaction flask was then sealed and the mixture stirred at room temperature for 24 hours. The solution was concentrated under reduced pressure to give sinigrin ((1), R = allyl) as an oily hygroscopic solid (0.35 g, 100%). $[\alpha]_D$ -15.1 ° (c 0.2 in H_2O) (lit.,[185] -17 ° (c 0.2 in H_2O); ν_{max} (nujol)/cm^{-1} 3460 (OH); δ_H (200 MHz; ^2H_2O) 3.35-3.61 (6H, m., H-2,2'^{a},2'^{b},3,4,5), 3.69 (1H, d.d., $J_{5,6a}$ 5.9 Hz, $J_{6'a,6'b}$ 12.6 Hz, H-6^a), 3.89 (1H, d.d., $J_{5,6b}$ 2.6 Hz, $J_{6a,6b}$ 12.6 Hz, H-6^b), 5.02 (1H, d., $J_{1,2}$ 9.9 Hz, H-1), 5.26 (1H, d., $J_{3',4'a}$ 10.3 Hz, (*cis*), H-4'^{a}), 5.31 (1H, d., $J_{3',4'b}$ 17.3 Hz, (*trans*), H-4'^{b}), 6.00 (1H, m., H-3'); δ_C (50.3 MHz; ^2H_2O) 38.94 (C-2'), 63.42 (C-6), 71.92 (C-4), 74.74

(C-2), 79.85 (C-5), 82.80 (C-3), 84.34 (C-1), 121.25 (C-4'), 134.85 (C-3'), 165.89 (C-1').

4.4 Synthesis of Glucosinolate Analogues

4.4.1 Attempted Synthesis of Allyl-*O*-Glucosinolate (126)



(126)

4.4.1.1 Method 1

The coupling step of the synthesis of (126) was attempted in an identical manner to that described for 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-but-3-enothiohyroximate (123) (Section 4.3.4), except that 2,3,4,6-tetra-*O*-acetyl-α/β-D-glucopyranose (86) was used instead of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (95). None of the desired product was obtained by this method.

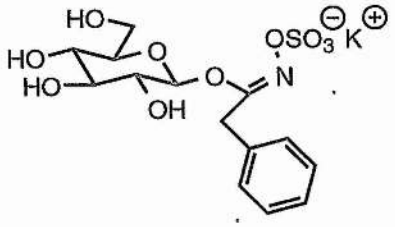
4.4.1.2 Method 2

The coupling step of the synthesis of (126) was attempted in an identical manner to that described for 2,3,4,6-tetra-*O*-acetyl-2',4'-dinitrophenyl-β-D-glucopyranoside (Section 4.2.12), except that but-3-enohydroximoyl chloride (122) was used instead of 1-fluoro-2,4-dinitrobenzene. None of the desired product was obtained by this method.

4.4.1.3 Method 3

The coupling step of the synthesis of (126) was attempted in an identical manner to that described in Section 4.4.1.2, except that triethylamine was used as the base and silver nitrate (1 eq) was added. None of the desired product was obtained by this method.

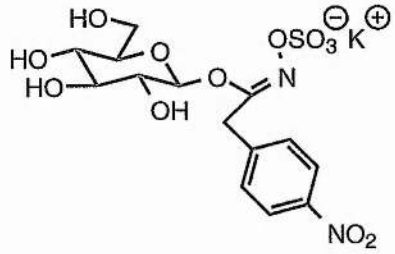
4.4.2 Attempted Synthesis of Benzyl-*O*-Glucosinolate (130)



(130)

The coupling step of the synthesis of (130) was attempted in an identical manner to that described in 4.4.1.1, except that phenylacetohydroximoyl chloride (128) was used instead of but-3-enohydroximoyl chloride (122). Compound (128) was synthesised from phenylacetaldoxime (127) using *N*-chlorosuccinimide in 88% yield according to a literature method,[57] and was added directly to the reaction mixture. None of the desired product was obtained by this method.

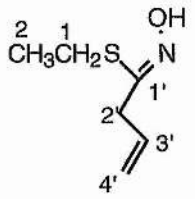
4.4.3 Attempted Synthesis of *p*-Nitrobenzyl-*O*-Glucosinolate (134)



(134)

The coupling step of the synthesis of (134) was attempted in an identical manner to that described in 4.4.2, except that *p*-nitrophenylacetohydroximoyl chloride (132) was used instead of phenylacetohydroximoyl chloride (128). Compound (132) was synthesised from *p*-nitrophenylacetaldoxime (131) in the same manner as (128) in 74% yield, and added directly to the reaction mixture. None of the desired product was obtained by this method.

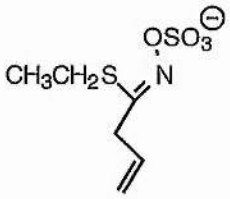
4.4.4 Synthesis of Ethyl But-3-enothiohydroximate (133)



(133)

But-3-enohydroximoyl chloride (122) (0.52 g, 4.35 mmol) was dissolved in tetrahydrofuran (20 ml) under nitrogen and the resulting solution was cooled to 0 °C. Triethylamine (3.68 ml, 26 mmol) and ethanethiol (0.32 ml, 4.39 mmol) were added, and the reaction mixture was allowed to warm to room temperature under nitrogen. The precipitate which formed was filtered off and the filtrate was concentrated under reduced pressure to give a gold oil. This oil was dissolved in dichloromethane (60 ml) and the resulting solution was washed with 1 N sulfuric acid (2 x 60 ml) and water (60 ml), dried ($MgSO_4$) and concentrated under reduced pressure to give a gold oil as the crude product. This oil was distilled under reduced pressure to give ethyl but-3-enothiohydroximate (133) as a colourless oil (0.10 g, 16%), b.p. 76-85 °C/0.1 mmHg; ν_{max} (thin film)/cm^{-1} 3300 (OH); δ_H (200 MHz; C^2HCl_3) 1.31 (3H, t., $J_{1,2}$ 7.6 Hz, H-2^a,2^b,2^c), 2.91 (2H, q., $J_{1,2}$ 7.6 Hz, H-1^a,1^b), 3.23 (2H, m., H-$2'^a$,$2'^b$), 5.20 (2H, m., H-$4'^a$,$4'^b$), 5.89 (1H, m., H-3'), 8.70 (1H, br., OH); δ_C (50.3 MHz; C^2HCl_3) 15.21 (C-2), 24.06 (C-1), 37.28 (C-2'), 118.49 (C-4'), 133.18 (C-3'), 155.29 (C-1'); *m/z* (EI) 145 (M^+, 85%), 128 (55, $[M - OH]^+$), 84 (52, $[M - C_2H_5S]^+$) and 61 (69, $[C_2H_5S]^+$).

4.4.5 Attempted Sulfation of Ethyl But-3-enothiohydroximate (133) to give the Aglucone Analogue (134)



(134)

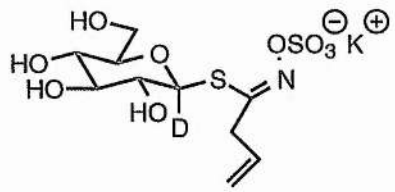
A solution of chlorosulfonic acid (3.5 ml, 53 mmol) in dry dichloromethane (8 ml) was added dropwise over 30 minutes to a mixture of dry pyridine (30 ml, 0.33 mol) and dry dichloromethane (10 ml) under nitrogen. A solution

of ethyl but-3-enothiohydroximate (133) (0.77 g, 5.30 mmol) in dry dichloromethane (5 ml) was then added. The reaction mixture was allowed to warm to room temperature and stirred for a further 24 hours under nitrogen. A solution of potassium hydrogen carbonate (5.3 g, 53 mmol) in water (25 ml) was added to the reaction mixture and the resulting mixture was stirred at room temperature for 30 minutes and concentrated under reduced pressure to give a red oil. This oil was purified by silica chromatography using ethyl acetate/methanol (4:1) as the eluent. Only starting material was recovered.

A repeat attempt with a 4 day reaction period also gave only starting material.

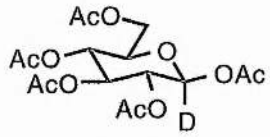
4.5 Synthesis of [1-^{2}H]-Sinigrin ([1-^{2}H]-Allyl Glucosinolate) (143)

[1-^{2}H]-Sinigrin (143) was synthesised for use in α-deuterium kinetic isotope effect studies (Section 4.19.1).



(143)

4.5.1 Synthesis of 1,2,3,4,6-Penta-*O*-acetyl-β-D-[1-^{2}H]-glucopyranose (137)[167]

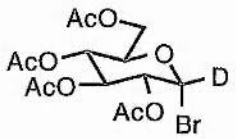


(137)

2,3,4,6-Tetra-*O*-acetyl-D-glucono-γ-lactone (85) (8.27 g, 23.9 mmol) was dissolved in dry tetrahydrofuran (80 ml) under nitrogen. The resulting solution was cooled to 0 °C and an ice-cold solution of sodium borodeuteride (0.50 g, 11.9 mmol) in deuterium oxide (3.0 ml) was added

dropwise under nitrogen. Ice-cold acetic anhydride (8.4 ml, 88.4 mmol) was added dropwise and the reaction mixture was stirred at room temperature for 16 hours under nitrogen. The mixture was then poured into ice-cold saturated sodium hydrogen carbonate (300 ml). The resulting mixture was stirred at 0 °C for 1 hour and extracted with dichloromethane (2 x 200 ml). The combined organic extracts were washed with water (2 x 250 ml), dried ($MgSO_4$) and concentrated under reduced pressure to give a white solid. This product was purified by recrystallisation from ethanol to give pure 1,2,3,4,6-penta-*O*-acetyl-β-D-[1-^{2}H]-glucopyranose (137) as a white solid (4.05 g, 44%), m.p. 123-4 °C (lit.,[193] 132 °C); (Found: C, 49.41; H, 5.58. Calc. for $C_{16}H_{21}DO_{11}$: C, 49.11; H, 5.67%); $[\alpha]_D$ +5.1 ° (c 1.0 in $CHCl_3$); ν_{max} (nujol)/cm^{-1} 1740 (CO); δ_H (200 MHz; C^2HCl_3) 1.95-2.20 (15H, 5s., 5 x C$\underline{H}_3$), 3.81 (1H, m., H-5), 4.07 (1H, d.d., $J_{5,6a}$ 2.2 Hz, $J_{6a,6b}$ 12.4 Hz, H-6^a), 4.28 (1H, d.d., $J_{5,6b}$ 4.6 Hz, $J_{6a,6b}$ 12.4 Hz, H-6^b),5.15-5.30 (3H, m., H-2,3,4); δ_C (50.3 MHz; C^2HCl_3) 20.82, 20.96, 21.07, 21.21 & 21.32 (5 x $\underline{C}H_3$), 61.86 (C-6), 68.11 (C-4), 70.55 (C-2), 73.10 (C-5), 73.20 (C-3), 169.47, 169.74, 169.88, 170.60 & 171.11 (5 x $\underline{C}O$); *m/z* (CI) 409 ([*M* + NH_4]$^+$, 100%), 332 (51, [*M* - OAc]$^+$), 273 (1, [*M* - 2OAc]$^+$) and 214 (14, [*M* - 3OAc]$^+$).

4.5.2 Synthesis of 2,3,4,6-Tetra-*O*-acetyl-1-bromo-α-D-[1-^{2}H]-glucopyranose (138)

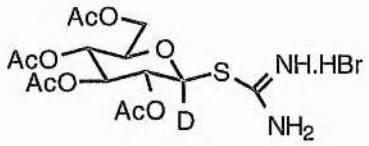


(138)

This was synthesised in an identical manner to that described for the non-deuterated compound (68), except that 1,2,3,4,6-penta-*O*-acetyl-β-D-[1-^{2}H]-glucopyranose (137) was used instead of 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose. The crude product was purified by silica chromatography using light petroleum/ethyl acetate (3:2) as the eluent to give pure 2,3,4,6-tetra-*O*-acetyl-1-bromo-α-D-[1-^{2}H]-glucopyranose (136) as a white solid (2.29 g, 87%), m.p. 84-6 °C; (Found: C, 40.29; H, 4.48. Calc. for $C_{14}H_{18}DBrO_9$: C, 40.79; H, 4.65%); $[\alpha]_D$ +192.4 ° (c 2.42 in $CHCl_3$); ν_{max} (nujol)/cm^{-1} 1730 (CO); δ_H (200 MHz; C^2HCl_3) 1.91-2.21 (12H, 4s., 4 x C$\underline{H}_3$), 4.11 (1H, m., H-5), 4.30 (2H, m., H-6^a,6^b), 4.82 (1H, d.,

$J_{2,3}$ 9.6 Hz, H-2), 5.16 (1H, t., $J_{3,4}$ 9.6 Hz, $J_{4,5}$ 9.6 Hz, H-4), 5.55 (1H, t., $J_{2,3}$ 9.6 Hz, $J_{3,4}$ 9.6 Hz, H-3); δ_C (50.3 MHz; C^2HCl_3) 21.01 & 21.11 (4 x $\underline{C}H_3$), 61.39 (C-6), 67.60 (C-4), 70.59 (C-2), 71.00 (C-5), 72.57 (C-3), 169.91, 170.23, 170.29 & 170.94 (4 x $\underline{C}O$); *m/z* (CI) 429 & 431 ([*M* + NH_4]$^+$, 13%), 351 (13, [*M* - Br + H + NH_4]$^+$), 332 (3, [*M* - Br]$^+$) and 214 (34, [*M* - Br - 2OAc]$^+$).

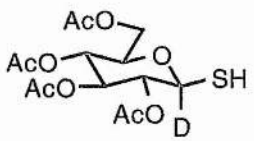
4.5.3 Synthesis of 2,3,4,6-Tetra-*O*-acetyl-β-D-[1-^{2}H]-glucopyranosyl Isothiouronium Bromide (139)



(139)

This was synthesised in an identical manner to that described for the non-deuterated compound (94) except that 2,3,4,6-tetra-*O*-acetyl-1-bromo-α-D-[1-^{2}H]-glucopyranose (138) was used instead of 2,3,4,6-tetra-*O*-acetyl-1-bromo-α-D-glucopyranose (68). The pure compound (139) was obtained as a white solid (1.84 g, 91.5%), m.p. 186 °C (d.); (Found: C, 37.05; H, 4.68; N, 5.92. Calc. for $C_{15}H_{22}DBrN_2O_9S$: C, 36.89; H, 4.75; N, 5.74%); $[\alpha]_D$ -14.8 ° (c 1.0 in MeOH); ν_{max} (nujol)/cm^{-1} 3310-3160 (NH), 1750 (CO) and 1655 (NH); δ_H (200 MHz; ^2H_2O) 2.00-2.15 (12H, 4s., 4 x $C\underline{H}_3$), 4.20 (1H, m., H-5), 4.24 (1H, d.d., $J_{5,6a}$ 2.0 Hz, $J_{6a,6b}$ 12.9 Hz, H-6^a), 4.37 (1H, d.d., $J_{5,6b}$ 4.9 Hz, $J_{6a,6b}$ 12.9 Hz, H-6^b), 5.12-5.50 (3H, m., H-2,3,4); δ_C (50.3 MHz; ^2H_2O) 14.89, 18.08, 18.11 & 18.21 (4 x $\underline{C}H_3$), 55.52 (C-1'), 59.92 (C-6), 65.65 (C-4), 67.24 (C-2), 71.43 (C-5), 73.93 (C-3), 170.46, 170.65, 170.99 & 171.65 (4 x $\underline{C}O$); *m/z* (EI) 365 ([*M* - CH_3BrN_2]$^+$, 6%), 332 (10, [*M* - CH_4BrN_2S]$^+$ and 170 (39, [$C_8H_8DO_4$]$^+$).

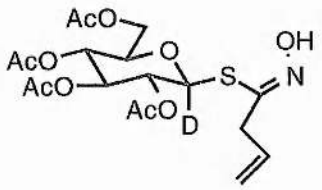
4.5.4 Synthesis of 2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-[1-^{2}H]-glucopyranose (140)



(140)

This was synthesised in an identical manner to that described for the non-deuterated compound (95) except that 2,3,4,6-tetra-*O*-acetyl-β-D-[1-^{2}H]-glucopyranosyl isothiouronium bromide (139) was used instead of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiouronium bromide (94). The pure compound (140) was obtained as a white solid (1.10 g, 54%), m.p. 75 °C; $[\alpha]_D$ -8.0 ° (c 1.5 in EtOH); ν_{max} (nujol)/cm^{-1} 3460 (SH) and 1735 (CO); δ_H (200 MHz; C^2HCl_3) 1.98-2.15 (12H, 4s., 4 x C$\underline{H}_3$), 2.30 (1H, s., S$\underline{H}$), 3.73 (1H, m., H-5), 4.11 (1H, d.d., $J_{5,6a}$ 2.5 Hz, $J_{6a,6b}$ 12.3 Hz, H-6^a), 4.26 (1H, d.d., $J_{5,6b}$ 4.8 Hz, $J_{6a,6b}$ 12.3 Hz, H-6^b), 4.97 (1H, d., $J_{2,3}$ 9.6 Hz, H-2), 5.09 (1H, t., $J_{3,4}$ 9.6 Hz, $J_{4,5}$ 9.6 Hz, H-4), 5.20 (1H, t., $J_{2,3}$ 9.6 Hz, $J_{3,4}$ 9.6 Hz, H-3); δ_C (50.3 MHz; C^2HCl_3) 21.03 & 21.21 (4 x $\underline{C}H_3$), 62.44 (C-6), 68.53 (C-4), 73.98 (C-2), 76.76 (C-5), 77.49 (C-3), 169.83, 170.09, 170.55 & 171.12 (4 x $\underline{C}O$); *m/z* (CI) 383 ([*M* + NH_4]$^+$, 100%), 332 (11, [*M* - SH]$^+$) and 323 (20, [*M* - OAc]$^+$).

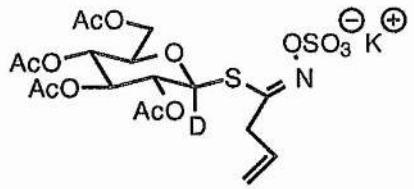
4.5.5 Synthesis of 2,3,4,6-Tetra-*O*-acetyl-β-D-[1-^{2}H]-glucopyranosyl-but-3-enothiohydroximate (141)



(141)

This was synthesised in an identical manner to that described for the non-deuterated compound (123) except that 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-[1-^{2}H]-glucopyranose (140) was used instead of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (95). The pure compound (141) was obtained as a white solid (1.23 g, 98%), m.p. 142-4 °C; (Found: C, 48.14; H, 5.82; N, 3.08. Calc. for $C_{18}H_{24}DNO_{10}S$: C, 48.21; H, 5.62; N, 3.12%); $[\alpha]_D$ -11.8 ° (c 0.14 in $CHCl_3$); ν_{max} (nujol)/cm^{-1} 3305 (OH), 1760 (CO) and 1710 (CO); δ_H (200 MHz; C^2HCl_3) 1.99-2.15 (12H, 4s., 4 x CH_3), 3.35 (2H, d., $J_{2',3'}$ 5.5 Hz, H-2'^{a},2'^{b}), 3.72 (1H, m., H-5), 4.18 (2H, d., $J_{5,6}$ 4.8 Hz, H-6^a,6^b), 5.09 (3H, m., H-2,3,4), 5.23 (2H, m., H-4'^{a},4'^{b}), 5.92 (1H, m., H-3'), 8.29 (1H, br., OH); δ_C (50.3 MHz; C^2HCl_3) 21.08 & 21.23 (4 x $\underline{C}H_3$), 37.24 (C-2'), 62.81 (C-6), 68.58 (C-4), 70.50 (C-2), 74.22 (C-5), 76.30 (C-3), 118.63 (C-4'), 133.24 (C-3'), 151.56 (C-1'), 169.72, 169.91, 170.78 & 171.12 (4 x $\underline{C}O$); *m/z* (EI) 332 ([*M* - C_4H_6NOS]$^+$, 37%).

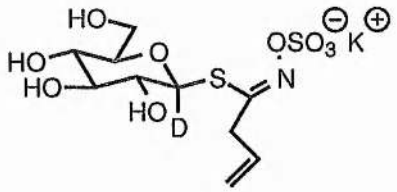
4.5.6 Synthesis of 2,3,4,6-Tetra-*O*-acetyl-[1-^{2}H]-sinigrin (142)



(142)

This was synthesised in an identical manner to that described for the non-deuterated compound (124) except that 2,3,4,6-tetra-*O*-acetyl-β-D-[1-^{2}H]-glucopyranosyl-but-3-enothiohydroximate (141) was used instead of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-but-3-enothiohydroximate (123). The pure compound (142) was obtained as a white solid (0.53 g, 32%), m.p. 194 °C (d.); $[\alpha]_D$ -13.7 ° (c 0.14 in H_2O); ν_{max} (nujol)/cm^{-1} 1730 (CO); δ_H (200 MHz; ^2H_2O) 1.90-2.14 (12H, 4s., 4 x CH_3), 3.41 (2H, d., $J_{2',3'}$ 5.5 Hz, H-2'^{a},2'^{b}), 4.06 (1H, m., H-5), 4.14 (1H, d.d., $J_{5,6a}$ 1.1 Hz, $J_{6a,6b}$ 12.9 Hz, H-6^a), 4.29 (1H, d.d., $J_{5,6b}$ 4.3 Hz, $J_{6a,6b}$ 12.9 Hz, H-6^b), 5.02-5.42 (5H, m., H-2,3,4,4'^{a},4'^{b}), 5.92 (1H, m., H-3'); δ_C (50.3 MHz; ^2H_2O) 22.86 (4 x $\underline{C}H_3$), 38.90 (C-2'), 64.71 (C-6), 70.40 (C-4), 72.66 (C-2), 77.18 (C-5), 77.85 (C-3), 121.30 (C-4'), 124.93 (C-1'), 134.53 (C-3'), 175.48 (4 x $\underline{C}O$); *m/z* (EI) 527 ([*M* - K]$^+$, 17%), 485 (2, [*M* - KOAc + H]$^+$) and 305 (17, [*M* - $C_6H_9KO_{10}S$]$^+$).

4.5.7 Synthesis of [1-^{2}H]-Sinigrin ([1-^{2}H]-Allyl Glucosinolate) (143)



(143)

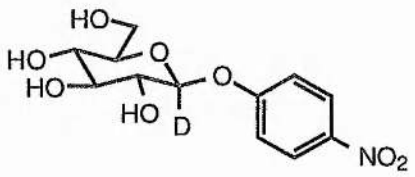
This was synthesised in an identical manner to that described for the non-deuterated compound ((1), R = allyl) except that 2,3,4,6-tetra-*O*-acetyl-[1-^{2}H]-sinigrin (142) was used instead of 2,3,4,6-tetra-*O*-acetyl sinigrin (124). The crude product was purified by silica chromatography using propanol/ethyl acetate/0.1 M acetic acid (6:3:1) as the eluent to give [1-^{2}H]-sinigrin (143) as an oily hygroscopic solid (0.37 g, 100%), $[\alpha]_D$ -13.2 ° (c 0.2 in H_2O); ν_{max} (nujol)/cm^{-1} 3460 (OH); δ_H (200 MHz; ^2H_2O) 3.35-3.61

(6H, m., H-2,2'^{a},2'^{b},3,4,5), 3.69 (1H, d.d., $J_{5,6a}$ 5.9 Hz, $J_{6a,6b}$ 12.6 Hz, H-6^{a}), 3.89 (1H, d.d., $J_{5,6b}$ 2.6 Hz, $J_{6a,6b}$ 12.6 Hz, H-6^{b}), 5.26 (1H, d., $J_{3',4'a}$ 10.3 Hz, (*cis*), H-4'^{a}), 5.31 (1H, d., $J_{3',4'b}$ 17.3 Hz, (*trans*), H-4'^{b}), 6.00 (1H, m., H-3'); δ_C (50.3 MHz; ^2H_2O) 38.95 (C-2'), 63.40 (C-6), 71.91 (C-4), 74.73 (C-2), 79.86 (C-5), 82.81 (C-3), 121.24 (C-4'), 134.85 (C-3'), 165.90 (C-1').

4.6 Synthesis of Other Deuterated Substrates

Compounds (146) and (149) were synthesised for use in α-deuterium kinetic isotope effect studies.

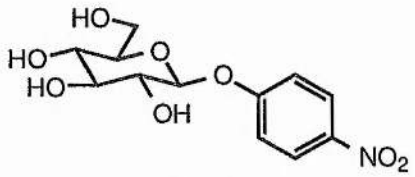
4.6.1 Synthesis of *p*-Nitrophenyl-[1-^{2}H]-β-D-glucopyranoside ([1-^{2}H]-PNPG) (144)



(146)

The non-deuterated compound (PNPG (15)) was synthesised first:

3.6.2 Synthesis of *p*-Nitrophenyl-β-D-glucopyranoside(PNPG) (15)[193]

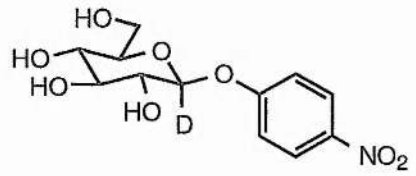


(15)

Potassium hydroxide (0.088 g, 1.57 mmol) was dissolved in dry ethanol (5.1 ml) under nitrogen, and to the resulting solution was added *p*-nitrophenol (0.186 g, 1.34 mmol). The reaction mixture was stirred at room temperature under nitrogen for 1 hour 30 minutes and a solution of 2,3,4,6-tetra-*O*-acetyl-1-bromo-α-D-glucopyranose (68) (0.50 g, 1.22 mmol) in chloroform (15 ml) was added. The reaction mixture was then refluxed for 48 hours under nitrogen and cooled to room temperature. The solution was diluted with chloroform (20 ml) and poured onto crushed ice (80 ml).

After shaking, the organic layer was separated, dried ($MgSO_4$) and concentrated down under reduced pressure to give a red syrup. This product was purified by silica chromatography using light petroleum/ethyl acetate (3:2) as the eluent to give a white solid (0.31 g). This solid was dissolved in dry methanol (30 ml) under nitrogen and to the resulting solution was added 1.0 M sodium methoxide in dry methanol (2.0 ml). The reaction mixture was stirred at room temperature under nitrogen for 24 hours. The solution was then neutralised with glacial acetic acid and concentrated under reduced pressure to give an off-white solid. This product was purified by silica chromatography using ethyl acetate/methanol/water (16:2:1) as the eluent to give pure *p*-nitrophenyl-β-D-glucopyranoside (15) as a white solid (0.05 g, 53%), m.p. 162-4 °C (lit.,[194] 165 °C); (Found: C, 47.01; H, 5.15; N, 4.48. Calc. for $C_{12}H_{15}NO_8$: C, 47.84; H, 5.02; N, 4.65%); $[\alpha]_D$ -74.1 ° (c 1.0 in H_2O) (lit.,[194] -79.6 ° (c 1.0 in MeOH)); ν_{max} (nujol)/cm^{-1} 3300 (OH) and 750 (aromatic); δ_H (200 MHz; ^2H_2O) 3.39-3.75 (5H, m., H-2,4,5,6^a,6^b), 3.88 (1H, d.d., $J_{2,3}$ 12.3 Hz, $J_{3,4}$ 2.3 Hz, H-3), 5.19 (1H, d., $J_{1,2}$ 7.6 Hz, H-1), 7.13 (2H, d., *J* 9.8 Hz, H-2',6'), 8.16 (2H, d., *J* 9.8 Hz, H-3',5'); δ_C (50.3 MHz; ^2H_2O) 63.45 (C-6), 72.40 (C-4), 75.95 (C-2), 79.30 (C-5), 80.05 (C-3), 102.70 (C-1), 119.45 (C-3',5'), 128.50 (C-2',6'), 144.55 (C-4') and 165.15 (C-1').

4.6.1 Synthesis of *p*-Nitrophenyl-[1-^{2}H]-β-D-glucopyranoside ([1-^{2}H]-PNPG) (146)

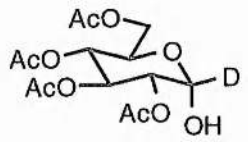


(146)

This was synthesised in an identical manner to that described for the non-deuterated compound (15) except that 2,3,4,6-tetra-*O*-acetyl-1-bromo-α-D-[1-^{2}H]-glucopyranose (138) was used instead of 2,3,4,6-tetra-*O*-acetyl-1-bromo-α-D-glucopyranose (68). The pure compound (146) was obtained as a white solid (0.48 g, 24.5%), m.p. 160-2 °C; (Found: C, 46.34; H, 5.15; N, 4.59. Calc. for $C_{12}H_{14}DNO_8$: C, 47.69; H, 5.00; N, 4.63%); $[\alpha]_D$ -73.5 ° (c 1.0 in H_2O); ν_{max} (nujol)/cm^{-1} 3360 (OH) and 730 (aromatic); δ_H (200 MHz; ^2H_2O) 3.39-3.75 (5H, m., H-2,4,5,6^a,6^b), 3.88 (1H, d.d., $J_{2,3}$ 12.3 Hz, $J_{3,4}$ 2.3 Hz, H-3), 7.13 (2H, d., *J* 9.8 Hz, H-2',6'), 8.14 (2H, d., *J* 9.8 Hz, H-3',5');

δ_C (50.3 MHz; ^2H_2O) 63.47 (C-6), 72.42 (C-4), 75.90 (C-2), 79.31 (C-5), 80.04 (C-3), 119.46 (C-3',5'), 128.48 (C-2',6'), 144.52 (C-4') and 165.16 (C-1').

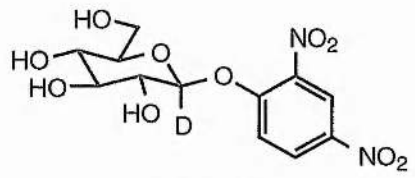
4.6.3 Synthesis of 2,3,4,6-Tetra-*O*-acetyl-α-D-[1-^{2}H]-glucopyranose (147)[167]



(147)

This was synthesised in an identical manner to that described of the non-deuterated compound (86), except that sodium borodeuteride was used instead of sodium borohydride. The pure compound (147) was obtained as a white solid (2.25 g, 85%), m.p. 108-112 °C; $[\alpha]_D$ +31.2 ° (c 1.0 in $CHCl_3$); ν_{max} (nujol)/cm^{-1} 3320 (OH) and 1730 (CO); δ_H (200 MHz; C^2HCl_3) 1.93-2.20 (12H, 4s., 4 x CH_3), 3.76 (1H, m., H-5), 4.13 (1H, d.d., $J_{5,6a}$ 2.3 Hz, $J_{6a,6b}$ 12.3 Hz, H-6^a), 4.24 (1H, d.d., $J_{5,6b}$ 4.6 Hz, $J_{6a,6b}$ 12.3 Hz, H-6^b), 4.88 (1H, d., $J_{2,3}$ 10.0 Hz, H-2), 5.02-5.30 (2H, m., H-3,4); δ_C (50.3 MHz; C^2HCl_3) 21.06 & 21.20 (4 x $\underline{C}H_3$), 62.46 (C-6), 68.90 (C-4), 72.49 (C-2), 72.74 (C-5),73.58 (C-3), 170.03, 170.67, 170.70 & 171.28 (4 x $\underline{C}O$); *m/z* (CI) 367 ([*M* + NH_4]$^+$, 100%), 332 (38, [*M* - OH]$^+$), 272 (8, [*M* - $C_2H_5O_3$]$^+$).

4.6.4 Synthesis of 2,4-Dinitrophenyl-[1-^{2}H]-β-D-glucopyranoside ([1-^{2}H]-2,4-DNPG) (149)



(149)

This was synthesised in an identical manner to that described for the non-deuterated compound (88), except that 2,3,4,6-tetra-*O*-acetyl-α-D-[1-^{2}H]-glucopyranose (147) was used instead of 2,3,4,6-tetra-*O*-acetyl-α/β-D-glucopyranose (86). The pure compound (149) was obtained as a yellow solid (0.25 g, 44%), m.p. 125-7 °C; $[\alpha]_D$ -107.1 ° (c 1.0 in MeOH); ν_{max}

(nujol)/cm^{-1} 3360 (OH) and 730 (aromatic); δ_H (200 MHz; ^2H_2O) 3.39-3.76 (5H, m., H-2,4,5,6^a,6^b), 3.89 (1H, d.d., $J_{2,3}$ 11.4 Hz, $J_{3,4}$ 2.4 Hz, H-3), 7.51 (1H, d., $J_{5',6'}$ 10.1 Hz, H-6'), 8.44 (1H, d., $J_{5',6'}$ 10.1 Hz, H-5'), 8.79 (1H, s., H-3'); δ_C (50.3 MHz; ^2H_2O) 63.22 (C-6), 71.90 (C-4), 75.26 (C-2), 78.19 (C-5), 79.33 (C-3), 120.53 (C-3'), 124.96 (C-6'), 132.66 (C-5'), 141.59 (C-2'), 144.20 (C-4') and 157.18 (C-1').

4.7 Myrosinase Assay

The enzyme solution was periodically constituted by dissolving 20 mg of myrosinase in 33.1 mM potassium phosphate buffer at pH 7.0 in a total volume of 1.0 ml. When not in use, the enzyme solution was stored at -18 °C. Myrosinase activity was assayed according to the method of Palmieri *et al.*[77] Assays were carried out using 33.1 mM potassium phosphate buffer at pH 7.0, containing 0.1 mM sinigrin, in a total volume of 1.0 ml. The assay solution was equilibrated at 37 ± 0.1 °C in a quartz cuvette of 1 ml volume/1 cm pathlength in the thermostatted cell holder of the UV spectrophotometer. Reaction was initiated by the addition of 30 µl of enzyme solution, and the decrease in absorbance at 227 nm, due to sinigrin, was monitored. The number of units in the enzyme solution was calculated by dividing the experimental initial rate by the theoretical rate (6.7 min^{-1}) calculated for 1 unit of enzyme. One unit of enzyme activity was defined as the amount required to catalyse the hydrolysis of 1 µmol of sinigrin per minute under standard assay conditions.

4.8 Determination of Kinetic Parameters for the Myrosinase Catalysed Hydrolysis of Sinigrin

Sinigrin incubations were carried out in 33.1 mM potassium phosphate buffer at pH 7.0. Solutions containing 9 different concentrations of sinigrin, ranging from 0.03 to 2.00 mM, were equilibrated at 37 ± 0.1 °C in varying sized quartz cuvettes in the thermostatted cell holders of the UV spectrophotometer. The reactions were initiated by the addition of a 20 µl aliquot of enzyme solution to each solution. The quartz cuvettes were as follows: 0.03-0.30 mM, 1 ml volume/1 cm pathlength; 0.50 mM, 1 ml volume/0.5 cm pathlength; 1.00-2.00 mM, 0.4 ml volume/0.1 cm pathlength. Initial rate measurements, in triplicate for each sinigrin

concentration, were made by monitoring the decrease in absorbance at 227 nm (ε = 6784 mol^{-1} dm^3 cm^{-1}) due to sinigrin. The reactions were linear over the time-course measured, up to approximately 5% of the overall reaction. The kinetic data were corrected for 1 unit of enzyme activity before being analysed by non-linear regression using the Enzfitter (see Appendix) program to obtain values of K_M and V_{max}. The data for these incubations are given in Table 34 and Figure 53.

[Sinigrin] / mM	Rate[a,b] / Abs min^{-1}
0.03	2.607
0.05	4.495
0.08	6.382
0.10	6.967
0.20	11.27
0.30	11.87
1.00	21.93
1.50	26.07
2.00	27.87

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 34: *Myrosinase Catalysed Hydrolysis of Sinigrin in Water*

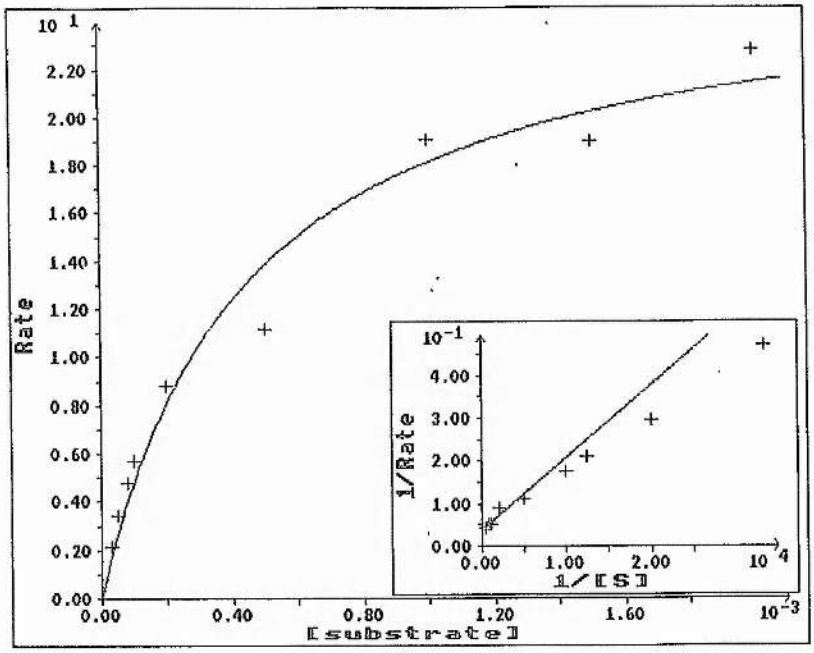


Figure 53: *Myrosinase Catalysed Hydrolysis of Sinigrin in Water*

V_{max} = (32.77 ± 1.48 / 6784) mol dm^{-3} min^{-1}

V_{max} = 4.83 ± 0.22 x10^{-3} mol dm^{-3} min^{-1}

K_M = 0.42 ± 0.05 mM

4.9 Incubations of Sinigrin with β-Glucosidase

4.9.1 Incubation of Sinigrin with β-Glucosidase Under Standard Sinigrin Conditions

This incubation was carried out in 33.1 mM potassium phosphate buffer at pH 7.0. A 1.0 ml solution containing 0.1 mM sinigrin was equilibrated at 37 ± 0.1 °C in a 1 ml volume/1 cm pathlength quartz cuvette in the thermostatted cell holder of the UV spectrophotometer. An aliquot of β-glucosidase solution (30 µl, 1 unit) was then added to the solution. The absorption at 227 nm due to sinigrin (ε = 6784 mol^{-1} dm^3 cm^{-1}) was monitored for 105 minutes. No change in absorption at 227 nm occurred.

4.9.2 Incubation of Sinigrin with β-Glucosidase at pH 5.6

This incubation was carried out in 10 mM PIPES/20 mM sodium acetate/0.1 mM EDTA buffer at pH 5.6.[135] A 1.0 ml solution containing 0.1 mM sinigrin was equilibrated at 37 ± 0.1 °C in a 1 ml volume/1 cm pathlength quartz cuvette in the thermostatted cell holder of the UV spectrophotometer. An aliquot of β-glucosidase solution (30 μl, 1 unit) was then added to the solution. The absorption at 227 nm due to sinigrin (ε = 6784 mol^{-1} dm^3 cm^{-1}) was monitored for 3 days. No change in absorption at 227 nm occurred. It was concluded that sinigrin does not act as a substrate for β-glucosidase.

4.10 Determination of Kinetic Parameters for the Myrosinase Catalysed Hydrolysis of Sinigrin in Water/Organic Solvent Mixtures

These incubations were carried out in an identical manner to those described for sinigrin in aqueous buffer solutions, except that the appropriate amount of the relevant solvent was added. The extinction coefficient value for sinigrin (ε = 6784 mol^{-1} dm^3 cm^{-1}) was assumed for all solvents.

4.10.1 Myrosinase Catalysed Hydrolysis of Sinigrin in 6.25 M (20% *v/v*) Methanol in 33.1 mM Potassium Phosphate Buffer, pH 7.0

The data for these incubations are given in Table 35.

[Sinigrin] / mM	Rate[a,b] / Abs min^{-1}
0.08	3.236
0.10	3.467
0.20	6.473
0.30	8.785
1.00	14.34
1.50	15.72
2.00	16.64

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 35: *Myrosinase Catalysed Hydrolysis of Sinigrin in 6.25 M Methanol*

V_{max} = (20.65 ± 1.18 / 6784) mol dm^{-3} min^{-1}

V_{max} = 3.04 ± 0.17 x10^{-3} mol dm^{-3} min^{-1}

K_M = 0.46 ± 0.08 mM

4.10.2 Myrosinase Catalysed Hydrolysis of Sinigrin in 12.5 M (40%*v/v*) Methanol in 33.1 mM Potassium Phosphate Buffer, pH 7.0

The data for these incubations are given in Table 36.

[Sinigrin] / mM	Rate[a,b] / Abs min^{-1}
0.05	1.517
0.08	1.820
0.10	2.124
0.20	5.461
0.30	6.068
1.00	9.104
1.50	9.708
2.00	15.17

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 36: *Myrosinase Catalysed Hydrolysis of Sinigrin in 12.5 M Methanol*

$V_{max} = (16.32 \pm 2.70 / 6784)$ mol dm^{-3} min^{-1}

$V_{max} = 2.41 \pm 0.40 \times 10^{-3}$ mol dm^{-3} min^{-1}

$K_M = 0.57 \pm 0.26$ mM

4.10.3 Myrosinase Catalysed Hydrolysis of Sinigrin in 4.35 M (20%*v/v*) Ethanol in 33.1 mM Potassium Phosphate Buffer, pH 7.0

The data for these incubations are given in Table 37.

[Sinigrin] / mM	Rate[a,b] / Abs min^{-1}
0.03	1.457
0.08	4.078
0.10	4.849
0.20	6.602
1.00	15.14
1.50	19.03
2.00	19.80

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 37: *Myrosinase Catalysed Hydrolysis of Sinigrin in 4.35 M Ethanol*

$V_{max} = (24.38 \pm 1.28 / 6784)$ mol dm^{-3} min^{-1}

$V_{max} = 3.59 \pm 0.19 \times 10^{-3}$ mol dm^{-3} min^{-1}

$K_M = 0.49 \pm 0.08$ mM

4.10.4 Myrosinase Catalysed Hydrolysis of Sinigrin in 20% *v/v* Dioxane in 33.1 mM Potassium PhosphateBuffer, pH 7.0

The data for these incubations are given in Table 38.

[Sinigrin] / mM	Rate[a,b] / Abs min^{-1}
0.20	4.855
0.30	6.838
0.50	8.602
1.00	13.24
2.00	16.83
3.00	19.53

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 38: *Myrosinase Catalysed Hydrolysis of Sinigrin in 20% v/v Dioxane*

V_{max} = (24.70 ± 0.83 / 6784) mol dm^{-3} min^{-1}

V_{max} = 3.64 ± 0.12 x10^{-3} mol dm^{-3} min^{-1}

K_M = 0.87 ± 0.07 mM

4.10.5 Myrosinase Catalysed Hydrolysis of Sinigrin in 20%*v/v* Acetonitrile in 33.1 mM Potassium Phosphate Buffer, pH 7.0

The data for these incubations are given in Table 39.

[Sinigrin] / mM	Rate[a,b] / Abs min^{-1}
0.05	1.940
0.08	2.424
0.10	3.006
0.20	4.727
0.30	4.946
0.50	8.154
1.00	11.26
1.50	11.65
2.00	13.59

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 39: *Myrosinase Catalysed Hydrolysis of Sinigrin in 20% v/v Acetonitrile*

V_{max} = (16.77 ± 0.95 / 6784) mol dm^{-3} min^{-1}

V_{max} = 2.47 ± 0.14 x10^{-3} mol dm^{-3} min^{-1}

K_M = 0.55 ± 0.08 mM

4.11 Extinction Coefficients for *p*-Nitrophenol

p-Nitrophenol was purified by recrystallisation until analytically pure. Various concentrations of *p*-nitrophenol in 50 mM potassium phosphate buffer at pH 7.0, ranging from 0.05 to 0.30 mM were then prepared. These solutions were equilibrated at 37 ± 0.1 °C in 1 ml volume/1 cm pathlength quartz cuvettes in the thermostatted cell holders of the UV spectrophotometer. The absorbances of these solutions at 430 nm were

then measured, and from this the extinction coefficient was calculated from Beer's Law.

The same procedure was followed for the determination of the extinction coefficient for *p*-nitrophenol in 20% and 40% methanol, 20% dioxane and 20% acetonitrile.

4.12 Determination of Kinetic Parameters for the Myrosinase Catalysed Hydrolysis of PNPG

PNPG incubations were carried out in 50.0 mM potassium phosphate buffer at pH 7.0. Solutions containing 8 different concentrations of PNPG, ranging from 1.0 to 25.0 mM, were equilibrated at 37 ± 0.1 °C in 3 ml volume/1 cm pathlength quartz cuvettes in the thermostatted cell holders of the UV spectrophotometer. The reactions were initiated by the addition of a 30 µl aliquot of enzyme solution to each solution. Initial rate measurements, made in triplicate for each PNPG concentration, were made by monitoring the increase in absorbance at 430 nm ($\varepsilon = 7002 \pm 84$ mol^{-1} dm^3 cm^{-1}) due to the product, *p*-nitrophenol. The reactions were linear over the time-course measured, up to approximately 5% of the overall reaction. The kinetic data were corrected for 1 unit of enzyme activity before being analysed by non-linear regression using the Enzfitter program to obtain values of K_M and V_{max}. The data for these incubations are given in Table 40 and Figure 54.

[PNPG] / mM	Rate[a,b] / Abs min^{-1}
1.0	0.020
2.0	0.039
5.0	0.075
7.5	0.122
10.0	0.141
15.0	0.208
20.0	0.267
25.0	0.304

[a] Corrected for 1 unit of enzyme activity

[b] Each rate is an average of three measured values

Table 40: *Myrosinase Catalysed Hydrolysis of PNPG*

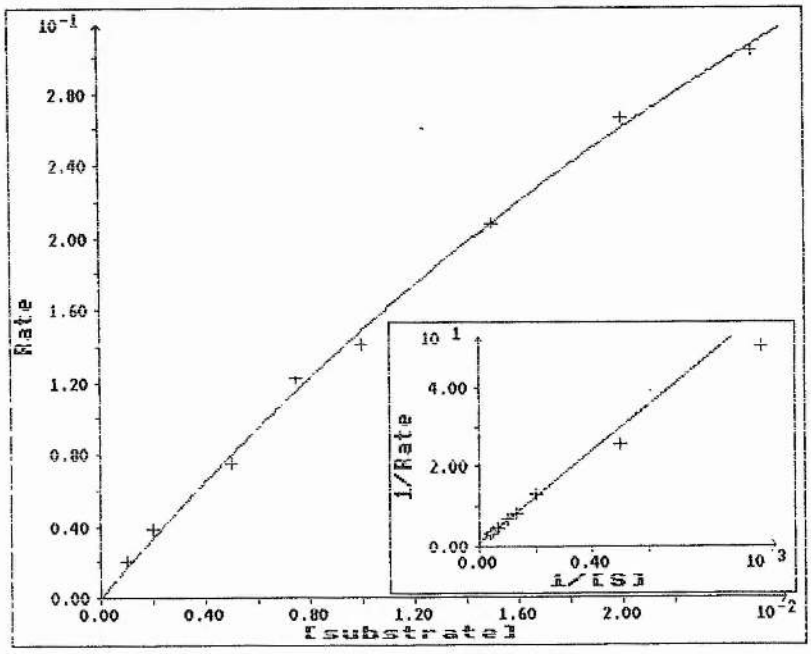


Figure 54: *Myrosinase Catalysed Hydrolysis of PNPG*

V_{max} = (1.054 ± 0.149 / 7002) mol dm^{-3} min^{-1}

V_{max} = 0.15 ± 0.02 x10^{-3} mol dm^{-3} min^{-1}

K_M = 60.9 ± 11.2 mM

4.13 Determination of Kinetic Parameters for the Myrosinase Catalysed Hydrolysis of PNPG in Water/Organic Solvent Mixtures

These incubations were carried out in an identical manner to that described for PNPG in aqueous buffer above, except that the appropriate amount of the relevant sovent was added. Due to the lack of solubility, the buffer used for 12.5 M (40% *v/v*) methanol was 33.1 mM potassium phosphate, pH 7.0.

4.13.1 Myrosinase Catalysed Hydrolysis of PNPG in 0.2 M Methanol in 50 mM Potassium Phosphate Buffer, pH 7.0

The data for these incubations are given in Table 41.

[PNPG] / mM	Rate[a,b] / Abs min^{-1}
1.0	0.018
2.0	0.039
5.0	0.054
7.5	0.100
10.0	0.117
15.0	0.156
20.0	0.206

[a] Corrected for 1 unit of enzyme activity

[b] Each rate is an average of three measured values

Table 41: *Myrosinase Catalysed Hydrolysis of PNPG in 0.2 M Methanol*

V_{max} = (0.697 ± 0.215 / 7002) mol dm^{-3} min^{-1}

V_{max} = 0.10 ± 0.03 x10^{-3} mol dm^{-3} min^{-1}

K_M = 49.2 ± 19.6 mM

4.13.2 Myrosinase Catalysed Hydrolysis of PNPG in 6.25 M (20% *v/v*) Methanol in 50 mM Potassium Phosphate Buffer, pH 7.0

The data for these incubations are given in Table 42.

[PNPG] / mM	Rate[a,b] / Abs min^{-1}
1.0	0.011
2.0	0.017
5.0	0.044
10.0	0.093
15.0	0.110
20.0	0.130
25.0	0.155

[a] Corrected for 1 unit of enzyme activity

[b] Each rate is an average of three measured values

Table 42: *Myrosinase Catalysed Hydrolysis of PNPG in 6.25 M Methanol*

V_{max} = (0.339 ± 0.051 / 4293) mol dm^{-3} min^{-1}

V_{max} = 0.079 ± 0.012 x10^{-3} mol dm^{-3} min^{-1}

K_M = 30.5 ± 7.3 mM

4.13.3 Myrosinase Catalysed Hydrolysis of PNPG in 12.5 M (40%*v/v*) Methanol in 33.1 mM Potassium Phosphate Buffer, pH 7.0

The data for these incubations are given in Table 43.

[PNPG] / mM	Rate[a,b] / Abs min^{-1}
1.0	0.007
2.0	0.011
5.0	0.023
7.5	0.032
10.0	0.038
15.0	0.056
20.0	0.069
25.0	0.082

[a] Corrected for 1 unit of enzyme activity

[b] Each rate is an average of three measured values

Table 43: *Myrosinase Catalysed Hydrolysis of PNPG in 12.5 M Methanol*

V_{max} = (0.249 ± 0.032 / 3845) mol dm^{-3} min^{-1}

V_{max} = 0.065 ± 0.008 x10^{-3} mol dm^{-3} min^{-1}

K_M = 51.9 ± 8.9 mM

4.13.4 Myrosinase Catalysed Hydrolysis of PNPG in 0.2 M Ethanol in 50 mM Potassium Phosphate Buffer, pH 7.0

The data for these incubations are given in Table 44.

[PNPG] / mM	Rate[a,b] / Abs min^{-1}
1.0	0.017
2.0	0.041
5.0	0.078
10.0	0.152
15.0	0.177
20.0	0.185

[a] Corrected for 1 unit of enzyme activity

[b] Each rate is an average of three measured values

Table 44: *Myrosinase Catalysed Hydrolysis of PNPG in 0.2.M Ethanol*

V_{max} = (0.463 ± 0.050 / 7002) mol dm^{-3} min^{-1}

V_{max} = 0.066 ± 0.007 x10^{-3} mol dm^{-3} min^{-1}

K_M = 21.9 ± 3.9 mM

4.13.5 Myrosinase Catalysed Hydrolysis of PNPG in 4.35 M (20%*v/v*) Ethanol in 50 mM Potassium Phosphate Buffer, pH 7.0

The data for these incubations are given in Table 45.

[PNPG] / mM	Rate[a,b] / Abs min^{-1}
1.0	0.009
2.0	0.016
5.0	0.052
7.5	0.072
10.0	0.093
15.0	0.119
20.0	0.147
25.0	0.175

[a] Corrected for 1 unit of enzyme activity

[b] Each rate is an average of three measured values

Table 45: *Myrosinase Catalysed Hydrolysis of PNPG in 4.35.M Ethanol*

V_{max} = (0.455 ± 0.047 / 4293) mol dm^{-3} min^{-1}

V_{max} = 0.11 ± 0.01 x10^{-3} mol dm^{-3} min^{-1}

K_M = 40.8 ± 6.0 mM

4.13.6 Myrosinase Catalysed Hydrolysis of PNPG in 20%*v/v* Dioxane in 50 mM Potassium Phosphate Buffer, pH 7.0

The data for these incubations are given in Table 46.

[PNPG] / mM	Rate[a,b] / Abs min^{-1}
2.0	0.0059
3.0	0.0090
5.0	0.0109
10.0	0.0124
15.0	0.0141
20.0	0.0164

[a] Corrected for 1 unit of enzyme activity

[b] Each rate is an average of three measured values

Table 46: *Myrosinase Catalysed Hydrolysis of PNPG in 20% v/v Dioxane*

V_{max} = (0.078 ± 0.005 / 3559) mol dm^{-3} min^{-1}

V_{max} = 0.022 ± 0.002 x10^{-3} mol dm^{-3} min^{-1}

K_M = 3.64 ± 0.77 mM

4.13.7 Myrosinase Catalysed Hydrolysis of PNPG in 20% *v/v* Acetonitrile in 50 mM Potassium Phosphate Buffer, pH 7.0

The data for these incubations are given in Table 47.

[PNPG] / mM	Rate[a,b] / Abs min^{-1}
2.0	0.0099
3.0	0.0123
5.0	0.0227
10.0	0.0308
15.0	0.0347
20.0	0.0474
30.0	0.0563

[a] Corrected for 1 unit of enzyme activity

[b] Each rate is an average of three measured values

Table 47: *Myrosinase Catalysed Hydrolysis of PNPG in 20% v/v Acetonitrile*

$V_{max} = (0.374 \pm 0.034 / 3338)$ mol dm^{-3} min^{-1}

$V_{max} = 0.11 \pm 0.01 \times 10^{-3}$ mol dm^{-3} min^{-1}

$K_M = 17.3 \pm 3.13$ mM

4.14 Determination of Kinetic Parameters for the Myrosinase Catalysed Hydrolysis of ONPG

This was carried out in an identical manner to that described for PNPG, measuring the increase in absorbance at 430 nm due to the product, *o*-nitrophenol. The extinction coefficient, $\varepsilon = 1783 \pm 144$ mol^{-1} dm^3 cm^{-1} was measured experimentally in the same way as for *p*-nitrophenol. The data for these incubations are given in Table 48 and Figure 55.

[ONPG] / mM	Rate[a,b] / Abs min^{-1}
1.0	0.0034
2.0	0.0053
5.0	0.0078
7.5	0.0114
10.0	0.0134
15.0	0.0157
20.0	0.0184
25.0	0.0198

[a] Corrected for 1 unit of enzyme activity

[b] Each rate is an average of three measured values

Table 48: *Myrosinase Catalysed Hydrolysis of ONPG*

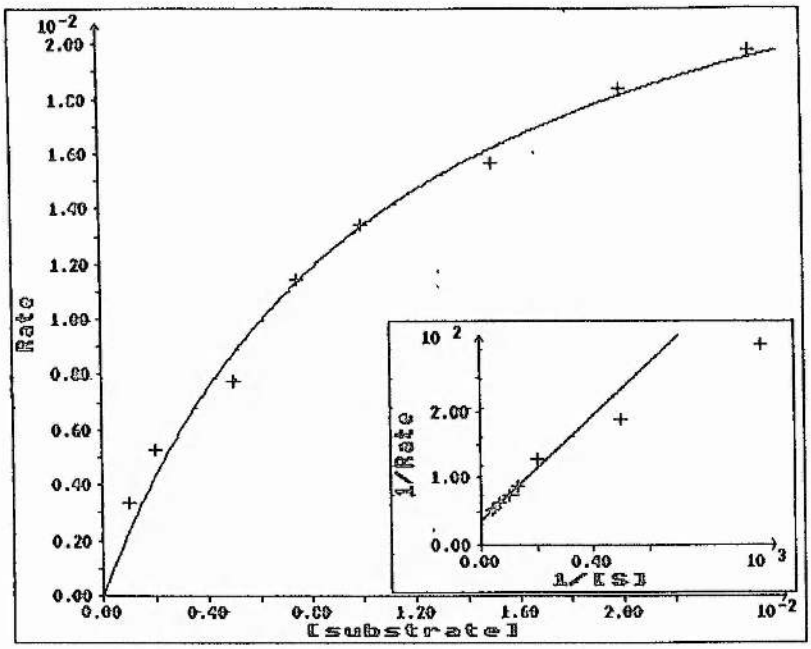


Figure 55: *Myrosinase Catalysed Hydrolysis of ONPG*

V_{max} = (0.028 ± 0.002 / 1783) mol dm^{-3} min^{-1}

V_{max} = 0.016 ± 0.001 x10^{-3} mol dm^{-3} min^{-1}

K_M = 11.0 ± 1.7 mM

4.15 Stability Experiments for Myrosinase

4.15.1 Stability of Myrosinase in Water/Methanol Mixtures

Three samples of myrosinase solution in 33.1 mM potassium phosphate buffer at pH 7.0 containing, respectively zero, 20% and 40% *v/v* methanol were constituted according to the procedure in Section 4.7. These samples were incubated at 37 ± 0.1 °C for 72 hours, during which time 20 µl aliquots were removed at various intervals. These aliquots were used to determine enzyme activity using the sinigrin assay described in Section 4.7. The rates for the sinigrin assays, measuring enzyme activities are given in Tables 49 to 51 below:

Sample A: No methanol (Table 49)

Time / h	Rate / mol dm^{-3} min^{-1}
0	1.039×10^{-3}
1.0	1.040×10^{-3}
3.5	1.040×10^{-3}
7.25	9.541×10^{-4}
25.5	8.519×10^{-4}
27.0	8.519×10^{-4}
29.5	8.519×10^{-4}
50.75	7.241×10^{-4}

Sample B: 20% *v/v* methanol (Table 50)

Time / h	Rate / mol dm^{-3} min^{-1}
0	9.920×10^{-4}
1.75	8.944×10^{-4}
4.58	8.944×10^{-4}
7.75	7.481×10^{-4}
23.08	6.515×10^{-4}
27.67	6.098×10^{-4}
31.25	5.529×10^{-4}

Sample C: 40% *v/v* methanol (Table 51)

Time / h	Rate / mol dm^{-3} min^{-1}
0	1.053×10^{-3}
1.0	7.826×10^{-4}
2.75	8.944×10^{-4}
8.25	4.472×10^{-4}
25.0	3.350×10^{-4}
31.5	1.342×10^{-4}

4.15.2 Stability of Myrosinase in the Presence of Allyl Isothiocyanate

These incubations were carried out in the same manner as those described in Section 4.14.1, except that samples containing only water or 5 mM allyl isothiocyanate (Allyl NCS) were used. The enzymatic activities for this study are presented in Table 52.

	Time / h			
[Allyl NCS] / mM	0	1	6.5	72
0	96%	96%	100%	82%
5	100%	100%	92%	36%

Table 52: *Enzymatic Activities for Myrosinase in the Presence of Allyl Isothiocyanate*

4.16 Incubations of Alternative Substrates with Myrosinase

4.16.1 Incubations of Aryl-β-D-glucopyranosides (80) to (84) with Myrosinase for the Brønsted Plot

These incubations were carried out using the same conditions as those for PNPG. The hydrolysis reactions were monitored by (i) monitoring the changes in absorbance in the region 250-350 nm and (ii) measuring the amount of D-glucose released using a hexokinase/glucose-6-phosphate dehydrogenase coupled assay, with the Glucose HK reagent obtained from Sigma. This reagent, when reconstituted according to the directions with 10 ml of distilled water, contains approximately the following concentrations of active ingredients:

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

The enzymatic reactions involved in this assay are as follows:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NAD} \xrightarrow{\text{G-6-PDH}} \text{6-PG} + \text{NADH}$$

Glucose is firstly phosphorylated by adenosine triphosphate (ATP), in a reaction catalysed by hexokinase (HK). The glucose-6-phosphate (G-6-P) produced is then oxidised to 6-phosphogluconate (6-PG) in the presence of nicotinamide adenine dinucleotide (NAD). This reaction is catalysed by glucose-6-phosphate dehydrogenase (G-6-PDH). During this oxidation, an equimolar amount of NAD is reduced to NADH. The increase in absorbance at 340 nm due to NADH is therefore directly proportional to the glucose concentration.

Each assay solution (1.0 ml) was equilibrated at 37 ± 0.1 °C in a 1 ml volume/1 cm pathlength quartz cuvette in the thermostatted cell holder of the UV spectrophotometer. A 10 µl aliquot of the reaction mixture was then added. After a stabilisation (after approximately 5 minutes incubation at 37 ± 0.1 °C), the absorbance value at 340 nm was measured. A blank sample of 10 µl of the reaction mixture in 1.0 ml distilled water was measured at 340 nm and 37 ± 0.1 °C for each sample. The glucose concentration was then calculated from the following equation:

$$[\text{Glucose}] / \text{mM} = \frac{(\text{Sample Abs.} - \text{Blank Abs.}) \times 293 \times 0.01}{180.16}$$

Standard D-glucose solutions in water were tested as follows:

Standard 1.0 mM D-Glucose Solution

Blank Absorbance: 0.106 A
Sample Absorbance: 0.173 A

[Glucose] = (0.173 - 0.106) x 293 x 0.01 / 180.16
[Glucose] = 1.09 mM

Standard 10.0 mM D-Glucose Solution

Blank Absorbance: 0.110 A
Sample Absorbance: 0.717 A

[Glucose] = (0.717 - 0.110) x 293 x 0.01 / 180.16
[Glucose] = 9.87 mM

No change in absorbances in the 250-350 region nor any glucose could be detected for the incubations of compounds (80), (81), (82), (83) and (84). Therefore none of these aryl-β-D-glucopyranosides acts as a substrate for myrosinase.

4.16.2 Incubation of 2,4-Dinitrophenyl-β-D-glucopyranoside (2,4-DNPG) (88) with Myrosinase

Measurement of the kinetic parameters for the myrosinase catalysed hydrolysis of 2,4-DNPG was carried out in an identical manner to that described for PNPG, except that the wavelength used for monitoring the product was 361 nm. The extinction coefficient for 2,4-dinitrophenol at λ_{max} = 361 nm was determined experimentally in the same way as that described for *p*-nitrophenol, and was found to be ε = 15,480 ± 3751 mol^{-1} dm^3 cm^{-1}. The data for these incubations are given in Table 53 and Figure 56.

[2,4-DNPG] / mM	Rate[a,b] / Abs min^{-1}
0.10	0.033
0.20	0.046
0.25	0.049
0.50	0.104
0.75	0.145
1.50	0.214
5.00	0.376
7.50	0.439
10.0	0.532

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 53: *Myrosinase Catalysed Hydrolysis of 2,4-DNPG*

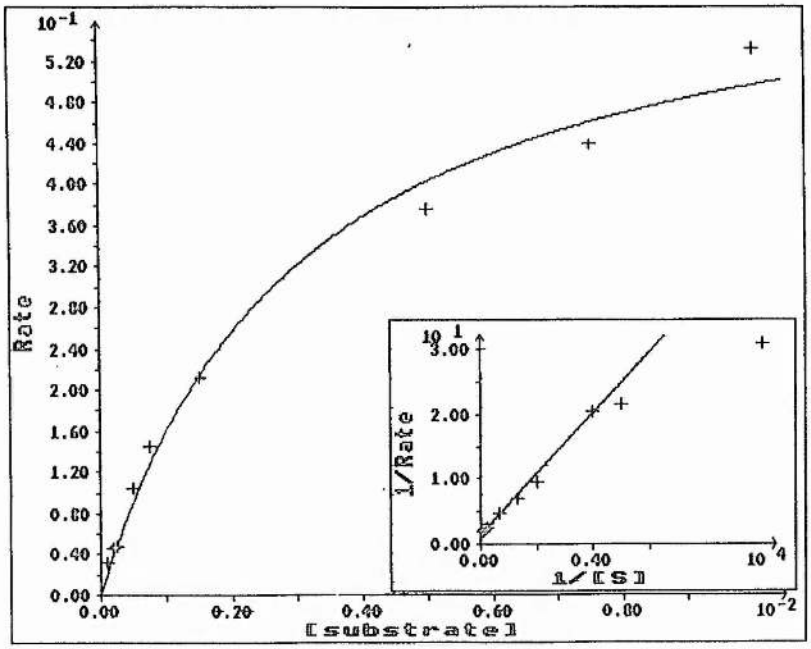


Figure 56: *Myrosinase Catalysed Hydrolysis of 2,4-DNPG*

V_{max} = (0.6771 ± 0.039 / 15,480) mol dm^{-3} min^{-1}

V_{max} = 0.044 ± 0.003 x10^{-3} mol dm^{-3} min^{-1}

K_M = 3.30 ± 0.53 mM

4.16.3 Incubations of Aryl-1-thio-β-D-glucopyranosides (91), (93), (98), (99), (102), (105) (108) and (112) with Myrosinase

These incubations were carried out using the same conditions as those for PNPG. The hydrolysis reactions were monitored by (i) monitoring the changes in absorbance in the region 250-350 nm and (ii) measuring the amount of glucose released using the Glucose HK assay described in Section 4.16.1. No change in absorbances in the 250-350 region nor any D-glucose could be detected after 24 hour incubations of compounds (91), (93), (98), (99), (102) (105) and (112) with myrosinase. It was therefore concluded that none of these compounds acts as a substrate for myrosinase.

Glucose was detected from the incubation of 2-pyridyl-1-thio-β-D-glucopyranoside (2-PYTG) (108) with myrosinase, and it was concluded that this compound does act as a substrate. However, there was also some background hydrolysis of this compound, at a slower rate. The glucose measurements for the incubations of this compound in water and with myrosinase are given in Section 2.5.6.

4.16.4 Incubation of Methyl-β-D-glucopyranoside (53) with Myrosinase

This incubation was carried out for 20 mM methyl-β-D-glucopyranoside and 50 μl of myrosinase solution using the same conditions as those for PNPG. The hydrolysis reaction was monitored by measuring the amount of glucose released using the Glucose HK assay described in Section 4.16.1. No glucose was detected after a 3 day incubation, and it was therefore concluded that methyl-β-D-glucopyranoside does not act as a substrate for myrosinase.

4.16.5 Incubation of Ethyl But-3-enothiohydroximate (141) with Myrosinase

Ethyl but-3-enothiohydroximate was found to be insoluble in aqueous media and therefore incubations with myrosinase could not be performed for this compound.

4.17 Transglycosylation Experiments

4.17.1 Transglycosylation Experiments with PNPG

Reactions were carried out using 2.0 ml solutions of 20.0 mM PNPG in 50 mM potassium phosphate buffer, pH 7.0. These solutions contained either no alcohol, 0.2 M, 20% or 40% methanol, 0.2 M ethanol, propan-1-ol, butan-1-ol, 2-mercaptoethanol, 3-hydroxy-1-propanesulfonate, glycerol-2-phosphate, 2-mercaptoethanesulfonate, sodium azide, hydroxylamine hydrochloride or hydrazine monohydrate. After equilibration of the solutions at 37 ± 0.1 °C, hydrolysis was initiated by the addition of 90 µl (0.36 units) of myrosinase solution, and samples were then incubated at 37 ± 0.1 °C for 24 hours. Following this, the concentrations of *p*-nitrophenol (PNP) and D-glucose were measured spectrophotometrically to investigate whether or not partitioning had taken place.

The *p*-nitrophenol concentrations were determined by removing a 10 µl aliquot from the reaction mixture and dissolving it in 0.99 ml of 0.1 M sodium hydroxide solution in a 1 ml volume/1 cm pathlength quartz cuvette. The absorbance due to the *p*-nitrophenolate ion at 400 nm (ε = 18,300 mol^{-1} dm^3 cm^{-1}) was then measured and the *p*-nitrophenol concentration calculated.

The D-glucose concentrations were determined using the Glucose HK assay described in Section 4.16.1.

4.17.2 Transglycosylation Experiments with Sinigrin

Solutions (1.0 ml) of 10 mM sinigrin in 33.1 mM potassium phosphate buffer, pH 7.0 were incubated at 37 ± 0.1 °C with 50 µl of myrosinase solution for 72 hours. It was assumed that reaction had gone to completion after this time. The concentration of released D-glucose for each sample was then determined using the Glucose HK assay described in Section 4.16.1. These incubations were carried out in duplicate in distilled water, 20% and 40% methanol and 20% ethanol.

4.18 Inhibition Studies

Inhibition studies were performed using sinigrin and PNPG as the substrate using the same procedures and conditions as for the kinetic parameter determinations described earlier. The following inhibitors were studied for the two substrates:

Sinigrin

D-Glucono-γ-lactone, β-D-glucose, methyl-β-D-glucopyranoside, sulfate and 1-deoxynojirimycin.

PNPG

D-Glucono-γ-lactone, β-D-glucose, methyl-β-D-glucopyranoside, sulfate, 2-mercaptoethanesulfonate (2-MESNA), allyl isothiocyanate (Allyl NCS), phenyl-1-thio-β-D-glucopyranoside (PTG), *o*-nitrophenyl-1-thio-β-D-glucopyranoside (ONPTG) and *p*-nitrophenyl-1-thio-β-D-glucopyranoside (PNPTG).

4.18.1 Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by D-Glucono-γ-lactone

The data for these inhibition studies are given in Tables 54 to 57 and Figure 57.

1.0 mM D-Glucono-γ-lactone

[Sinigrin] / mM	Rate[a,b] / Abs min^{-1}
0.08	3.641
0.10	5.228
0.20	7.469
0.30	9.335
1.00	15.92
1.50	18.67
2.00	21.47

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 54: *Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by 1 mM D-Glucono-γ-lactone*

V_{max} = (25.46 ± 1.31 / 6784) mol dm^{-3} min^{-1}

V_{max} = 3.75 ± 0.19 x10^{-3} mol dm^{-3} min^{-1}

K_M = 0.50 ± 0.07 mM

5.0 mM D-Glucono-γ-lactone

[Sinigrin] / mM	Rate[a,b] / Abs min^{-1}
0.03	1.359
0.05	1.942
0.08	2.427
0.10	3.786
0.20	4.855
0.30	5.437
1.00	10.10
1.50	13.20
2.00	13.59

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 55: *Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by 5 mM D-Glucono-γ-lactone*

V_{max} = (16.80 ± 1.12 / 6784) mol dm^{-3} min^{-1}

V_{max} = 2.48 ± 0.17 x10^{-3} mol dm^{-3} min^{-1}

K_M = 0.51 ± 0.09 mM

10.0 mM D-Glucono-γ-lactone

[Sinigrin] / mM	Rate[a,b] / Abs min^{-1}
0.08	1.867
0.10	2.334
0.20	3.734
0.30	4.667
1.00	7.842
1.50	10.08
2.00	10.27

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 56: *Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by 10 mM D-Glucono-γ-lactone*

V_{max} = (12.88 ± 0.63 / 6784) mol dm^{-3} min^{-1}

V_{max} = 1.90 ± 0.09 x10^{-3} mol dm^{-3} min^{-1}

K_M = 0.51 ± 0.07 mM

20.0 mM D-Glucono-γ-lactone

[Sinigrin] / mM	Rate[a,b] / Abs min^{-1}
0.05	1.123
0.08	1.345
0.10	2.700
0.30	3.955
1.50	8.989
2.00	9.348

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 57: *Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by 20 mM D-Glucono-γ-lactone*

V_{max} = (11.835 ± 0.790 / 6784) mol dm^{-3} min^{-1}

V_{max} = 1.75 ± 0.12 x10^{-3} mol dm^{-3} min^{-1}

K_M = 0.51 ± 0.10 mM

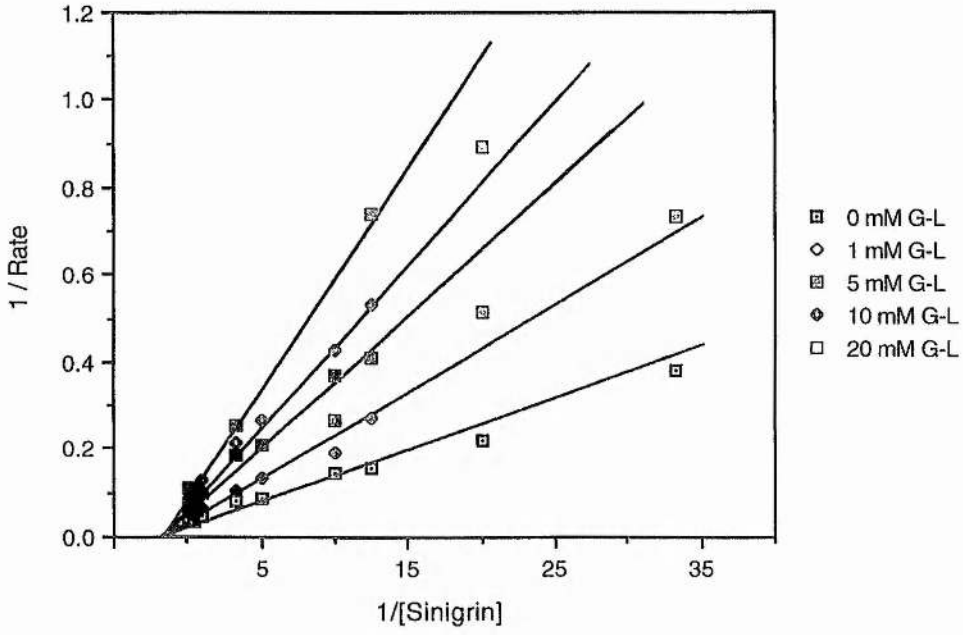


Figure 57: *Lineweaver-Burk Plot for Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by D-Glucono-γ-lactone*

4.18.2 Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by D-Glucono-γ-lactone in the Presence of L-Ascorbic Acid

The data for these inhibition studies are given in Tables 58 to 60.

No D-Glucono-γ-lactone / 1.0 mM L-Ascorbic Acid

[Sinigrin] / mM	Rate[a,b] / Abs min^{-1}
0.02	2.028
0.03	3.180
0.05	4.352
0.08	9.375
0.10	9.878
0.20	17.41
1.00	30.47
1.50	38.17
2.00	42.69

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 58: *Myrosinase Catalysed Hydrolysis of Sinigrin in the Presence of 1 mM L-Ascorbic Acid*

V_{max} = (48.31 ± 2.65 / 6784) mol dm^{-3} min^{-1}

V_{max} = 7.12 ± 0.39 x10^{-3} mol dm^{-3} min^{-1}

K_M = 0.40 ± 0.71 mM

1.0 mM D-Glucono-γ-lactone / 1.0 mM L-Ascorbic Acid

[Sinigrin] / mM	Rate[a,b] / Abs min^{-1}
0.10	8.541
0.20	14.02
0.30	19.78
1.00	23.37
1.50	28.76
2.00	34.52

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 59: *Inhibition of L-Ascorbate-Activated Myrosinase Catalysed Hydrolysis of Sinigrin by 1 mM D-Glucono-γ-lactone*

V_{max} = (35.94 ± 3.39 / 6784) mol dm^{-3} min^{-1}

V_{max} = 5.30 ± 0.50 x10^{-3} mol dm^{-3} min^{-1}

K_M = 0.31 ± 0.99 mM

10.0 mM D-Glucono-γ-lactone / 1.0 mM L-Ascorbic Acid

[Sinigrin] / mM	Rate[a,b] / Abs min^{-1}
0.08	1.798
0.10	2.248
0.20	3.416
0.30	3.955
1.00	7.503
1.50	7.551
2.00	8.989

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 60: *Inhibition of L-Ascorbate-Activated Myrosinase Catalysed Hydrolysis of Sinigrin by 10 mM D-Glucono-γ-lactone*

V_{max} = (10.30 ± 0.51 / 6784) mol dm^{-3} min^{-1}

V_{max} = 1.52 ± 0.08 x10^{-3} mol dm^{-3} min^{-1}

K_M = 0.43 ± 0.06 mM

4.18.3 Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by D-Glucono-γ-lactone

The data for these inhibition studies are given in Table 61 and Figure 58.

[D-Glucono-γ-lactone]/mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	60.9 ± 11.2	0.15 ± 0.02
5.0	63.4 ± 9.2	0.075 ± 0.008
10.0	113.6 ± 41.8	0.086 ± 0.027
20.0	106.1 ± 93.3	0.033 ± 0.025

Table 61: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by D-Glucono-γ-lactone*

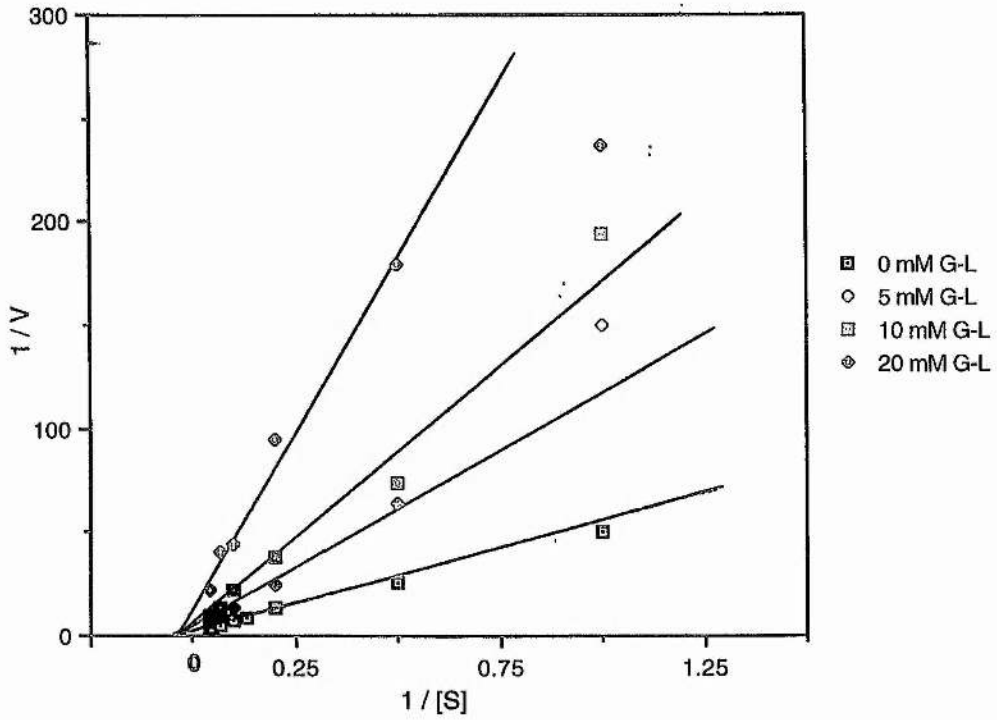


Figure 58: *Lineweaver-Burk Plot for Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by D-Glucono-γ-lactone*

4.18.4 Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by β-D-Glucose

The data for these inhibition studies are given in Table 62.

[β-D-Glucose] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	0.42 ± 0.05	4.83 ± 0.22
1.0	0.24 ± 0.02	3.38 ± 0.10
5.0	0.32 ± 0.04	4.00 ± 0.18
10.0	0.38 ± 0.05	4.16 ± 0.17
20.0	0.39 ± 0.02	4.02 ± 0.08

Table 62: *Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by β-D-Glucose*

4.18.5 Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by β-D-Glucose

The data for these inhibition studies are given in Table 63.

[β-D-Glucose] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	60.9 ± 11.2	0.15 ± 0.02
1.0	40.7 ± 21.2	0.12 ± 0.04
5.0	34.8 ± 4.62	0.12 ± 0.01

Table 63: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by β-D-Glucose*

4.18.6 Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by Sulfate

The data for these inhibition studies are given in Table 64.

[Sulfate] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	0.42 ± 0.05	4.83 ± 0.22
5.0	0.57 ± 0.10	3.12 ± 0.20
10.0	0.57 ± 0.11	3.22 ± 0.24
15.0	0.55 ± 0.02	2.39 ± 0.26
20.0	0.56 ± 0.11	2.79 ± 0.21
25.0	0.64 ± 0.10	2.19 ± 0.13

Table 64: *Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by Sulfate*

4.18.7 Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by Sulfate

The data for these inhibition studies are given in Table 65.

[Sulfate] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	60.9 ± 11.2	0.15 ± 0.02
5.0	53.9 ± 7.8	0.14 ± 0.02
7.5	43.8 ± 12.4	0.12 ± 0.02
10.0	21.9 ± 8.2	0.070 ± 0.015
15.0	27.5 ± 3.7	0.058 ± 0.005
20.0	41.2 ± 7.9	0.097 ± 0.013
25.0	37.5 ± 1.5	0.093 ± 0.025

Table 65: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by Sulfate*

4.18.8 Effect of Phosphate Buffer Concentration on the Myrosinase Catalysed Hydrolysis of Sinigrin

The data for these incubations are given in Table 66.

[Phosphate] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
20	0.44 ± 0.10	3.76 ± 0.31
33.1 (Standard)	0.42 ± 0.05	4.83 ± 0.22
50	0.43 ± 0.10	3.02 ± 0.23
100	0.62 ± 0.17	2.66 ± 0.29

Table 66: *Effect of Phosphate Concentration on the Myrosinase Catalysed Hydrolysis of Sinigrin*

4.18.9 Effect of Phosphate Buffer Concentration on the Myrosinase Catalysed Hydrolysis of PNPG

The data for these incubations are given in Table 67.

[Phosphate] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
20	56.4 ± 1.7	0.15 ± 0.04
50 (Standard)	60.9 ± 11.2	0.15 ± 0.02
75	38.8 ± 5.7	0.12 ± 0.01
100	47.8 ± 12.3	0.094 ± 0.017

Table 67: *Effect of Phosphate Concentration on the Myrosinase Catalysed Hydrolysis of PNPG*

4.18.10 Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by 2-Mercaptoethanesulfonate (2-MESNA)

The data for these inhibition studies are given in Table 68.

[2-MESNA] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	60.9 ± 11.2	0.15 ± 0.02
20.0	93.8 ± 26.6	0.21 ± 0.05

Table 68: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by 2-Mercaptoethanesulfonate (2-MESNA)*

4.18.11 Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by Allyl Isothiocyanate (Allyl NCS)

The data for these inhibition studies are given in Table 69.

[Allyl NCS] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	60.9 ± 11.2	0.15 ± 0.02
5.0	59.5 ± 20.8	0.15 ± 0.04

Table 69: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by Allyl Isothiocyanate*

4.18.12 Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by 1-Deoxynojirimycin (1-DNM)

The data for these inhibition studies are given in Table 70.

[1-DNM] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	0.42 ± 0.05	4.83 ± 0.22
0.2	0.51 ± 0.10	3.08 ± 0.21
0.5	0.75 ± 0.18	3.31 ± 0.32
1.0	0.80 ± 0.11	3.36 ± 0.19

Table 70: *Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by 1-Deoxynojirimycin*

4.18.13 Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by Methyl-β-D-glucopyranoside

The data for these inhibition studies are given in Table 71.

[Me-β-D-Glcsde] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	0.42 ± 0.05	4.83 ± 0.22
10.0	0.41 ± 0.02	4.18 ± 0.08
20.0	0.38 ± 0.05	4.11 ± 0.18
30.0	0.31 ± 0.02	4.09 ± 0.10

Table 71: *Inhibition of the Myrosinase Catalysed Hydrolysis of Sinigrin by Methyl-β-D-glucopyranoside*

4.18.14 Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by Methyl-β-D-glucopyranoside

The data for these inhibition studies are given in Table 72.

[Me-β-D-Glcsde] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	60.9 ± 11.2	0.15 ± 0.02
5.0	79.5 ± 66.2	0.21 ± 0.15

Table 72: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by Methyl-β-D-glucopyranoside*

4.18.15 Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by *o*-Nitrophenyl-1-thio-β-D-glucopyranoside (ONPTG)

The data for these studies are given in Tables 73 to 76 and Figure 59.

5.0 mM ONPTG

[PNPG] / mM	Rate[a,b] / Abs min^{-1}
1.0	0.013
2.0	0.024
5.0	0.060
10.0	0.095
15.0	0.150
20.0	0.187
25.0	0.219

[a] Corrected for 1 unit of enzyme activity

[b] Each rate is an average of three measured values

Table 73: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by 5 mM ONPTG*

V_{max} = (0.892 ± 0.206 / 7002) mol dm^{-3} min^{-1}

V_{max} = 0.13 ± 0.03 x10^{-3} mol dm^{-3} min^{-1}

K_M = 76.4 ± 22.1 mM

10.0 mM ONPTG

[PNPG] / mM	Rate[a,b] / Abs min^{-1}
2.0	0.028
5.0	0.060
10.0	0.121
15.0	0.177
20.0	0.213
25.0	0.258

[a] Corrected for 1 unit of enzyme activity

[b] Each rate is an average of three measured values

Table 74: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by 10 mM ONPTG*

V_{max} = (1.010 ± 0.136 / 7002) mol dm^{-3} min^{-1}

V_{max} = 0.14 ± 0.02 x10^{-3} mol dm^{-3} min^{-1}

K_M = 73.1 ± 12.4 mM

15.0 mM ONPTG

[PNPG] / mM	Rate[a,b] / Abs min^{-1}
1.0	0.009
2.0	0.021
5.0	0.045
10.0	0.101
15.0	0.149
20.0	0.165
25.0	0.192

[a] Corrected for 1 unit of enzyme activity

[b] Each rate is an average of three measured values

Table 75: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by 15 mM ONPTG*

V_{max} = (0.534 ± 0.118 / 7002) mol dm^{-3} min^{-1}

V_{max} = 0.076 ± 0.017 x10^{-3} mol dm^{-3} min^{-1}

K_M = 43.4 ± 13.8 mM

20.0 mM ONPTG

[PNPG] / mM	Rate[a,b] / Abs min^{-1}
1.0	0.006
2.0	0.011
5.0	0.039
10.0	0.051
15.0	0.073
20.0	0.095
25.0	0.116

[a] Corrected for 1 unit of enzyme activity

[b] Each rate is an average of three measured values

Table 76: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by 20 mM ONPTG*

V_{max} = (0.424 ± 0.168 / 7002) mol dm^{-3} min^{-1}

V_{max} = 0.061 ± 0.024 x10^{-3} mol dm^{-3} min^{-1}

K_M = 68.1 ± 34.6 mM

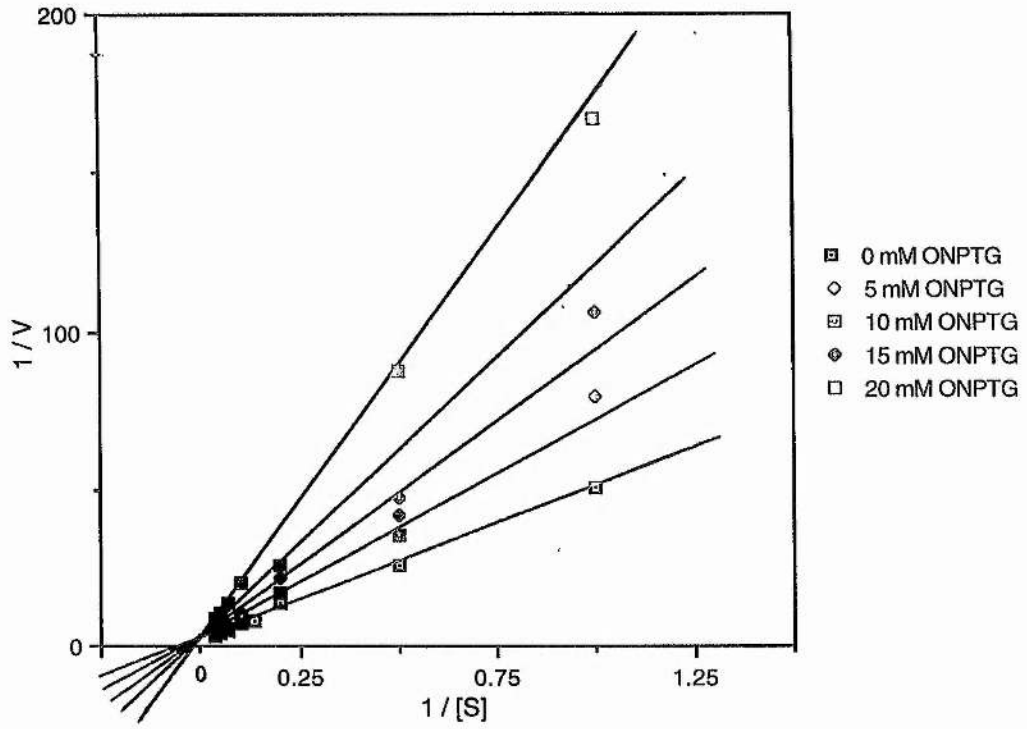


Figure 59: *Lineweaver-Burk Plot for Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by ONPTG*

4.18.16 Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by Phenyl-1-thio-β-D-glucopyranoside (PTG)

The data for these inhibition studies are given in Table 77.

[PTG] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	60.9 ± 11.2	0.15 ± 0.02
2.5	74.1 ± 23.1	0.13 ± 0.03
5.0	45.3 ± 10.6	0.10 ± 0.02
10.0	49.5 ± 14.2	0.11 ± 0.02
15.0	81.8 ± 26.9	0.14 ± 0.04
20.0	57.5 ± 20.7	0.13 ± 0.04
25.0	104.3 ± 68.8	0.19 ± 0.10

Table 77: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by Phenyl-1-thio-β-D-glucopyranoside (PTG)*

4.18.17 Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by *p*-Nitrophenyl-1-thio-β-D-glucopyranoside (PNPTG)

The data for these inhibition studies are given in Table 78.

[PNPTG] / mM	K_M / mM	V_{max} / 10^{-3} mol dm^{-3} min^{-1}
0	60.9 ± 11.2	0.15 ± 0.02
5.0	43.4 ± 5.5	0.11 ± 0.01
10.0	26.8 ± 6.5	0.083 ± 0.013
17.8	35.5 ± 10.2	0.096 ± 0.018
25.0	9.3 ± 2.9	0.042 ± 0.005

Table 78: *Inhibition of the Myrosinase Catalysed Hydrolysis of PNPG by p-Nitrophenyl-1-thio-β-D-glucopyranoside (PNPTG)*

4.19 α-Deuterium Kinetic Isotope Effect Studies

4.19.1 α-Deuterium Kinetic Isotope Effect Studies with Sinigrin

These incubations were carried out in an identical manner to those described for non-deuterated sinigrin. The data are given in Table 79.

[[1-^{2}H]-Sinigrin] / mM	Rate[a,b] / Abs min^{-1}
0.03	1.400
0.05	2.071
0.08	2.254
0.10	2.561
0.20	3.174
0.50	2.624
1.00	3.935
1.50	3.442
2.00	3.206

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 79: *Myrosinase Catalysed Hydrolysis of [1-^{2}H]-Sinigrin*

V_{max} = (3.49 ± 0.20 / 6784) mol dm^{-3} min^{-1}

V_{max} = 0.52 ± 0.03 x10^{-3} mol dm^{-3} min^{-1}

K_M = 0.04 ± 0.01 mM

4.19.2 α-Deuterium Kinetic Isotope Effect Studies with PNPG

4.19.2.1 Measurement of Kinetic Parameters for PNPG Purified by Column Chromatography

Incubations of PNPG, which was subjected to silica chromatography (to give a closer resemblence to synthesised [1-^{2}H]-PNPG), were carried out in an identical manner to those described for PNPG earlier. The data for these incubations are given in Table 80 and Figure 60.

[PNPG] / mM	Rate[a,b] / Abs min^{-1}
1.0	0.019
2.0	0.037
5.0	0.069
7.5	0.089
10.0	0.120
15.0	0.153
20.0	0.197
25.0	0.231
35.0	0.349
50.0	0.351

[a] Corrected for 1 unit of enzyme activity

[b] Each rate is an average of three measured values

Table 80: *Myrosinase Catalysed Hydrolysis of PNPG Purified by Column Chromatography*

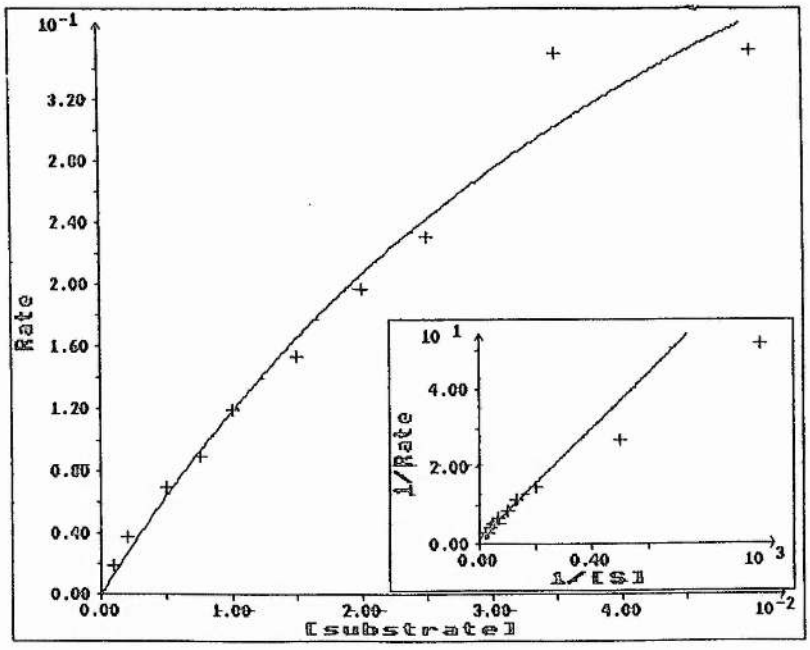


Figure 60: *Myrosinase Catalysed Hydrolysis of PNPG Purified by Column Chromatography*

V_{max} = (0.796 ± 0.154 / 7002) mol dm^{-3} min^{-1}

V_{max} = 0.114 ± 0.022 x10^{-3} mol dm^{-3} min^{-1}

K_M = 57.0 ± 16.9 mM

4.19.2.2 Measurement of the Kinetic Parameters for the Myrosinase Catalysed Hydrolysis of [1-^{2}H]-PNPG

Incubations for [1-^{2}H]-PNPG were carried out in an identical manner to those described for non-deuterated PNPG earlier. The data for these incubations are given in Table 81 and Figure 61.

[[1-^{2}H]-PNPG] / mM	Rate[a,b] / Abs min^{-1}
1.0	0.013
2.0	0.023
3.0	0.047
5.0	0.049
7.5	0.078
10.0	0.087
12.5	0.115
15.0	0.134
20.0	0.137
25.0	0.150
35.0	0.233
50.0	0.244

[a] Corrected for 1 unit of enzyme activity

[b] Each rate is an average of three measured values

Table 81: *Myrosinase Catalysed Hydrolysis of [1-^{2}H]-PNPG*

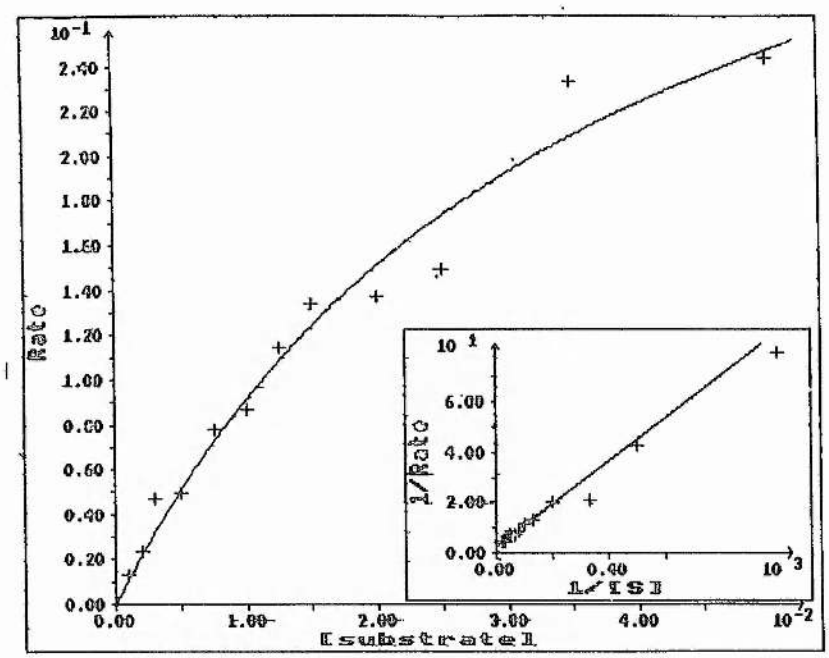


Figure 61: *Myrosinase Catalysed Hydrolysis of [1-^{2}H]-PNPG*

V_{max} = (0.428 ± 0.053 / 7002) mol dm^{-3} min^{-1}

V_{max} = 0.061 ± 0.008 x10^{-3} mol dm^{-3} min^{-1}

K_M = 36.6 ± 7.7 mM

The α-deuterium kinetic isotope effects for PNPG were calculated as follows:

^DV = 0.114/0.061 = 1.87 ^DK = 57.0/36.6 = 1.56

$^DV/K$ = 1.87/1.56 = 1.20

4.19.3 Measurement of the Kinetic Parameters for the Myrosinase Catalysed Hydrolysis of [1-^{2}H]-2,4-DNPG

Incubations for [1-^{2}H]-2,4-DNPG were carried out in an identical manner to those described for non-deuterated 2,4-DNPG earlier. The data for these incubations are given in Table 82 and Figure 62.

[[1-^{2}H]-2,4-DNPG] / mM	Rate^{a,b} / Abs·min^{-1}
0.10	0.041
0.20	0.059
0.30	0.074
0.50	0.109
0.75	0.114
1.00	0.145
2.00	0.174
2.50	0.216
3.00	0.256
5.00	0.334
7.50	0.395
10.0	0.418

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 82: *Myrosinase Catalysed Hydrolysis of [1-^{2}H]-2,4-DNPG*

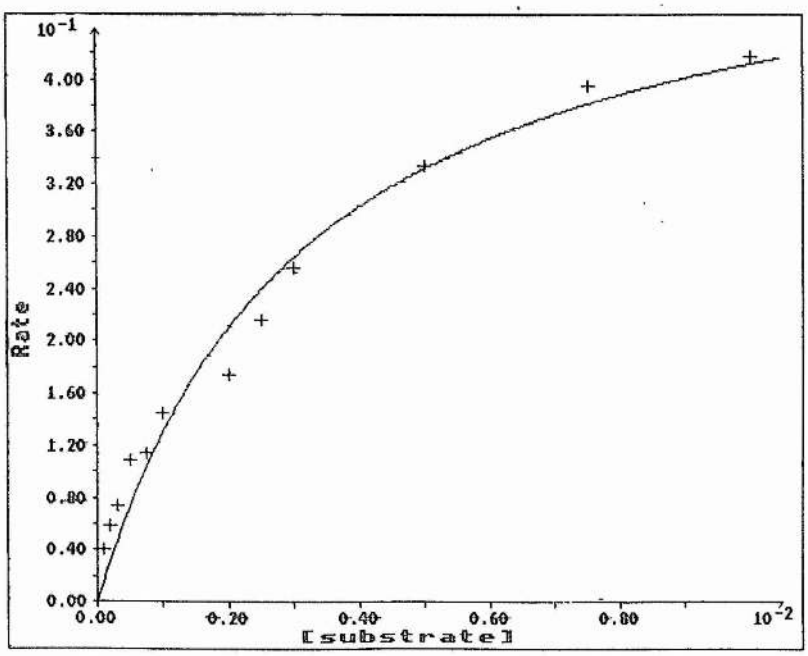


Figure 62: *Myrosinase Catalysed Hydrolysis of [1-^{2}H]-2,4-DNPG*

V_{max} = (0.542 ± 0.04 / 15,480) mol dm^{-3} min^{-1}

V_{max} = 0.035 ± 0.003 x10^{-3} mol dm^{-3} min^{-1}

K_M = 3.15 ± 0.57 mM

The α-deuterium kinetic isotope effects for 2,4-DNPG were calculated as follows:

^{D}V = 0.044/0.035 = 1.26 ^{D}K = 3.30/3.15 = 1.05

$^{D}V/K$ = 1.26/1.05 = 1.20

4.20 Solvent Deuterium Kinetic Isotope Effect Studies

4.20.1 Investigation of the Effect of D_2O on Myrosinase Activity

Since the effect of D_2O on the structure and stability of enzymes has been an area of concern in the interpretation of solvent isotope effects, a study was done to compare the activities of myrosinase in H_2O and D_2O buffers.

A standard solution of myrosinase in D_2O buffer was equilibrated for 1 hour at room temperature. The activity of myrosinase in D_2O buffer was then compared with a standard myrosinase solution in H_2O buffer using the standard sinigrin assay in H_2O buffer (described in Section 4.7). Three assays were carried out for each myrosinase solution.

The average activity of myrosinase in D_2O buffer (0.033) was found not to be significantly different from that of myrosinase in H_2O buffer (0.029).

4.20.2 Solvent Deuterium Kinetic Isotope Effect for Sinigrin

Incubations were carried out the same way as for sinigrin in Section 4.8, except that the buffer used was 33.1 mM deuterated potassium phosphate (KD_2PO_4) (prepared by freeze-drying KH_2PO_4 three times in D_2O) at pD 7.0 (pH 6.6). Also, the hydrogens of the hydroxyl groups of the glucopyranose ring of sinigrin were exchanged for deuterium by freeze-

drying sinigrin three times in D_2O. After preparation, the myrosinase solution in D_2O buffer was equilibrated for at least 1 hour prior to use. The data for these incubations are given in Table 83 and Figure 63.

[Sinigrin] / mM	Rate[a,b] / Abs min^{-1}
0.03	2.352
0.05	3.584
0.08	4.135
0.10	4.935
0.20	7.295
1.00	14.79
1.50	16.70
2.00	17.88

[a] Corrected for 1 unit of enzyme activity and cuvette pathlength/volume

[b] Each rate is an average of three measured values

Table 83: *Myrosinase Catalysed Hydrolysis of Sinigrin in D_2O Buffer*

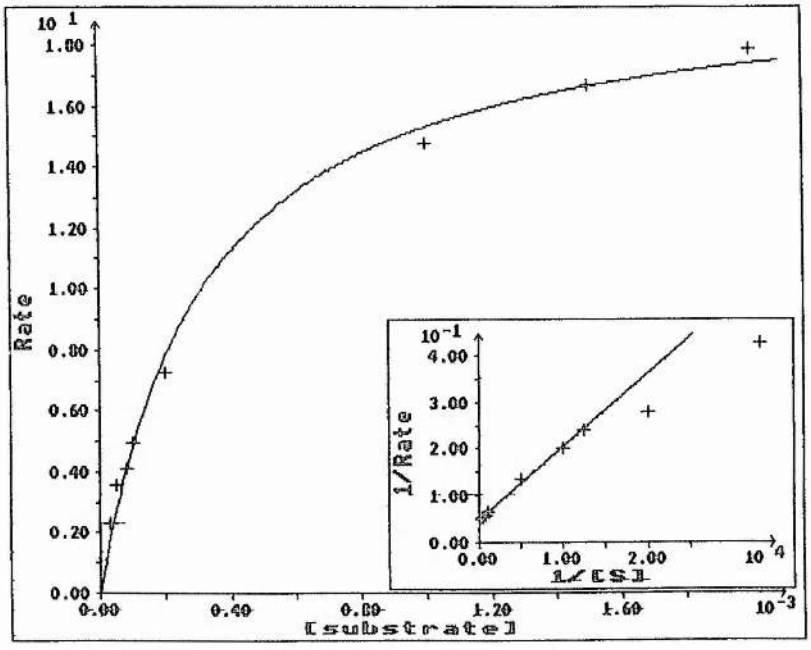


Figure 63: *Myrosinase Catalysed Hydrolysis of Sinigrin in D_2O Buffer*

V_{max} = (21.32 ± 0.67 / 6784) mol dm^{-3} min^{-1}

$\underline{V_{max} = 3.14 \pm 0.10 \times 10^{-3} \text{ mol dm}^{-3} \text{ min}^{-1}}$

$\underline{K_M = 0.34 \pm 0.04 \text{ mM}}$

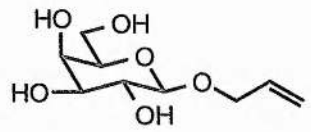
The solvent deuterium kinetic isotope effects were calculated as follows:

$^{D}V = 4.83/3.14 = \underline{1.54}$ $^{D}K = 0.42/0.34 = \underline{1.24}$

$^{D}V/K = 1.54/1.24 = \underline{0.98}$

4.21 Enzymatic Synthesis Experiments

4.21.1 Synthesis of Allyl-β-D-galactopyranoside (66) using β-Galactosidase (I)[154]

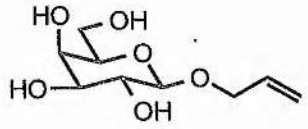


(66)

o-Nitrophenyl-β-D-galactopyranoside (65) (0.60 g, 2.0 mmol) was dissolved in sodium citrate/sodium hydroxide buffer, pH 5.0 (10 ml) and to the resulting solution was added allyl alcohol (1.16 g, 20 mmol) and β-galactosidase (200 mg, 1020 units). The reaction mixture was then shaken at 30 °C for 5 minutes and the reaction was stopped by boiling the solution for 1 minute. After cooling to 30-40 °C, the mixture was filtered over a bed of celite. The filtrate was then concentrated under reduced pressure to give a gold oil. This product was purified by silica chromatography using ethyl acetate/methanol/water (16:2:1) as the eluent to give pure allyl-β-D-galactopyranoside (66) as an off-white solid (0.12 g, 27%), m.p. 91-3 °C (lit.,[212] 101-2 °C); $[\alpha]_D$ -10.5 ° (c 2.0 in H_2O) (lit.,[212] -11 ° (c 2.0 in H_2O)); ν_{max} (nujol)/cm^{-1} 3295 (OH) and 1210 (C-O); δ_H (200 MHz; ^2H_2O) 3.34-3.72 (5H, m., H-2,4,5,6^a,6^b), 3.80 (1H, d., *J* 3.1 Hz, H-4), 4.04-4.31 (2H, m., H-1'^{a},1'^{b}), 4.32 (1H, d., $J_{1,2}$ 7.7 Hz, H-1), 5.10-5.33 (2H, m., $J_{2',3'a}$ 10.6 Hz (*cis*), $J_{2',3'b}$ 17.7 Hz (*trans*), H-3'^{a},3'^{b}), 5.88 (1H, m., H-2'); δ_C (50.3 MHz; ^2H_2O) 63.73 (C-6), 71.41 (C-4), 73.42 (C-1'), 73.53 (C-2),

75.61 (C-5), 77.90 (C-3), 104.53 (C-1), 121.50 (C-3'), 136.12 (C-2'); *m/z* (CI) 243 ([*M* + Na]$^+$, 100%) and 221 (91, [*M* + H]$^+$).

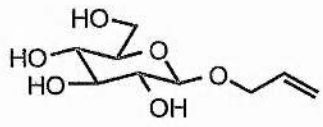
4.21.2 Synthesis of Allyl-β-D-galactopyranoside (66) using β-Galactosidase (II)[155]



(66)

Lactose (67) (2.36 g, 6.9 mmol) was dissolved in buffer containing 0.03 M sodium phophate/1 mM magnesium chloride/5mM 2-mercaptoethanol, at pH 7.8 (10 ml). A solution of β-galactosidase (200 mg, 1020 units) in buffer (0.4 ml) then allyl alcohol (1.00 g, 17 mmol) were added. The reaction mixture was incubated at 37 ± 0.1 °C for 108 hours. The reaction was then stopped by heating the mixture at 70 °C for 10 minutes. The solution was concentrated under reduced pressure to give an off-white solid. The product was purified by silica chromatography using dichloromethane/methanol/ethanol/water (6:3.5:1:0.8) as the eluent to give pure allyl-β-D-galactopyranoside (66) as an off-white solid (0.49 g, 32%), m.p. 90-2 °C (lit.,[212] 101-2 °C); $[\alpha]_D$ -10.1 ° (c 2.0 in H_2O) (lit.,[212] -11 ° (c 2.0 in H_2O)); ν_{max} (nujol)/cm^{-1} 3295 (OH) and 1210 (C-O); δ_H (200 MHz; ^2H_2O) 3.35-3.72 (5H, m., H-2,4,5,6^a,6^b), 3.80 (1H, d., *J* 3.1 Hz, H-4), 4.04-4.30 (2H, m., H-1'^{a},1'^{b}), 4.32 (1H, d., $J_{1,2}$ 7.7 Hz, H-1), 5.10-5.33 (2H, m., $J_{2',3'a}$ 10.6 Hz (*cis*), $J_{2',3'b}$ 17.7 Hz (*trans*), H-3'^{a},3'^{b}), 5.88 (1H, m., H-2'); δ_C (50.3 MHz; ^2H_2O) 63.72 (C-6), 71.39 (C-4), 73.41 (C-1'), 73.53 (C-2), 75.60 (C-5), 77.88 (C-3), 104.52 (C-1), 121.48 (C-3'), 136.12 (C-2'); *m/z* (CI) 243 ([*M* + Na]$^+$, 100%) and 221 (85, [*M* + H]$^+$).

4.21.3 Classical Synthesis of Allyl-β-D-glucopyranoside (70)[156]

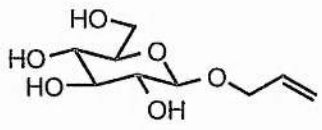


(70)

Allyl alcohol (0.40g, 6.9 mmol) was added to a mixture of silver triflate (1.50g, 5.9 mmol) (co-evaporated with toluene and then dried under

reduced pressure) and powdered activated 4Å molecular sieves (0.9 g) in dry dichloromethane (20 ml) at -10 °C under nitrogen. A solution of 2,3,4,6-tetra-*O*-acetyl-1-bromo-α-D-glucopyranose (2.00 g, 4.9 mmol) in dry dichloromethane (20 ml) was added slowly to the reaction mixture over 10 minutes. The reaction mixture was stirred at -10 °C for 30 minutes, and then a saturated solution of sodium hydrogen carbonate (50 ml) was added. The resulting mixture was diluted with dichloromethane (25 ml) and filtered. The filtrate was separated and the organic layer was washed with 10% sodium hydrogen carbonate solution (100 ml) and brine (100 ml), dried (Na_2SO_4) and concentrated under reduced pressure to give a colourless oil (1.61 g). This product was then purified by silica chromatography using ethyl acetate/light petroleum (3:2) as the eluent to give a white solid (0.89 g). This product was dissolved in dry methanol (15 ml) under nitrogen and to the resulting solution was added 1.0 M sodium methoxide in dry methanol (1.0 ml). The reaction mixture was stirred at room temperature under nitrogen for 60 hours, then neutralised with glacial acetic acid and concentrated under reduced pressure to give an off-white solid (0.30 g). This product was recrystallised from water to give pure allyl-β-D-glucopyranoside as a white solid (0.14 g, 13%), m.p. 96-8 °C; $[\alpha]_D$ -38.9 ° (c 1.0 in H_2O) (lit.,[213] -42 ° (c 1.0 in H_2O); ν_{max} (nujol)/cm^{-1} 3300 (OH) and 1220 (CO); δ_H (200 MHz; ^2H_2O) 3.10-3.53 (4H, m., H-4,5,6^a,6^b), 3.61 (1H, d.d., $J_{2,3}$ 12.5 Hz, $J_{1,2}$ 7.5 Hz, H-2), 3.83 (1H, d., $J_{2,3}$ 12.5 Hz, H-3), 4.17 (2H, m., H-1'^{a},1'^{b}), 4.39 (1H, d., $J_{1,2}$ 7.5 Hz, H-1), 5.13-5.34 (2H, d.d., $J_{2',3'a}$ 11.2 Hz (*cis*), $J_{2',3'b}$ 17.5 Hz (*trans*), H-3'^{a},3'^{b}), 5.93 (1H, m., H-2'); δ_C (50.3 MHz; ^2H_2O) 35.75 (C-1'), 63.69 (C-6), 72.47 (C-4), 74.99 (C-2), 80.19 (C-5), 82.72 (C-3), 85.42 (C-1), 120.80 (C-3'), 136.61 (C-2').

4.21.4 Attempted Synthesis of Allyl-β-D-glucopyranoside (70) using Myrosinase

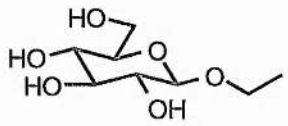


(70)

p-Nitrophenyl-β-D-glucopyranoside (PNPG) (15) (0.50 g, 1.66 mmol) and allyl alcohol (3.00 g, 51.7 mmol) were dissolved in 0.1 M potassium phosphate buffer, pH 7.0 (50 ml), and to the resulting solution was added

myrosinase (86 mg, 25 units). The reaction mixture was incubated at 37 ± 0.1 °C for 16 hours. A further portion of myrosinase (86 mg, 25 units) was added and the reaction mixture was incubated at 37 ± 0.1 °C for 17 days. The enzyme was then denatured by heating the reaction mixture to 70 °C for 10 minutes. After allowing to cool to 40 °C, the mixture was filtered over a bed of celite. The filtrate was then concentrated under reduced pressure to give an off-white solid. The product was purified by silica chromatography using ethyl acetate/methanol/water (16:2:1) as the eluent. A very small amount of starting material was recovered.

4.21.5 Attempted Synthesis of Ethyl-β-D-Glucopyranoside (71) using Myrosinase



(71)

p-Nitrophenyl-β-D-glucopyranoside (PNPG) (15) (0.10 g, 0.33 mmol) was dissolved in ethanol/water (9:1) (5.0 ml), and to the resulting solution was added myrosinase (34 mg, 10 units). The reaction mixture was then incubated at 37 ± 0.1 °C for 72 hours. An absorbance measurement was then taken at 430 nm to investigate the extent of the reaction. It was found that no reaction had taken place.

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6. Appendix

ENZFITTER

A Non-linear Regression
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Studies on the Mechanism of Myrosinase

INVESTIGATION OF THE EFFECT OF GLYCOSYL ACCEPTORS ON ENZYME ACTIVITY*

(Received for publication, March 20, 1995, and in revised form, May 24, 1995)

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Myrosinase (EC 3.2.3.1) is the β-thioglucosidase enzyme responsible for the hydrolysis of glucosinolates, a group of naturally occurring plant metabolites. The enzyme catalyzes the hydrolysis of these *S*-glucosides to give D-glucose and an aglycone fragment, which then rearranges to give sulfate and an isothiocyanate. As part of ongoing mechanistic studies on myrosinase, the ability of the enzyme to catalyze transglycosylation reactions has been examined. Enzyme activity and stability were both decreased in the presence of various organic solvents, including simple alcohols, but not sufficiently to prevent reaction taking place. However, in contrast to most other β-glycosidases, myrosinase did not catalyze transglycosylation reactions either with the alcohols or other suitable glycosyl acceptors. Although a wide range of potential acceptors were investigated, none proved to be effective. Even when appropriately charged side chains were included in the acceptor molecule to mimic the sulfonic acid in the glucosinolate structure, transglycosylation did not take place. The putative enzyme-glycosyl intermediate therefore appears to be unavailable for reaction, possibly because D-glucose is the first product released from the enzyme. The transition state analogue, glucono-δ-lactone, a potent competitive inhibitor of β-glucosidase, was found to be a poor noncompetitive inhibitor of myrosinase. Myrosinase is specifically activated by ascorbic acid, and it is proposed that the inhibitor is binding at this alternative site.

Myrosinase (EC 3.2.3.1) is the trivial name for the β-thioglucosidase enzyme responsible for the hydrolysis of glucosinolates (I) (Fig. 1), a group of sulfur-containing glycosides that occur in all members of the Cruciferae, including the brassica vegetables (1). Over 100 different examples have been isolated and characterized, containing a variety of substituents in the side chain R including allyl (sinigrin), benzyl, and indoyl (1). Enzymic hydrolysis usually occurs when cells are damaged as a result of plant injury or food processing giving as products β-D-glucose (II) and the aglycone fragment (III). The aglycone is unstable and reacts further giving the isothiocyanate (IV) by means of a Lossen-type rearrangement (Fig. 1). Some evidence implies that the rearrangement occurs spontaneously and is not enzyme catalyzed, but it is not conclusive (2).

Considerable work has been carried out in this area because of the agricultural and dietary importance of the Cruciferae. Products of glucosinolate hydrolysis are responsible for the distinctive flavor and aroma characteristics of crucifers. However, they can also have deleterious effects due to their pungency and in some cases goitrogenic activity and hepatoxicity (3, 4). This is a particular problem with oilseed rape and its food and feed derivatives. EC recommendations concerning the maximum permitted levels of glucosinolates in rapeseed have resulted in major programs to breed safer varieties. However, the chemistry of the system is still not fully understood and the mechanism of myrosinase has not been elucidated.

We were interested in studying the chemical mechanism of myrosinase and, in particular, comparing it with the much more widely studied β-glycosidases. In recent years, there has been a massive increase in interest in glycosidases (5). Inhibition of the enzymes that process carbohydrates has potential therapeutic applications for the treatment of cancer, AIDS, and other viral infections. Crystallographic studies, identification of active sites by mechanism-based labeling, and determination of transition state structure by kinetic analysis have allowed the construction of a detailed picture of glycosidase action. Myrosinase has been much less studied, and it is not known how the enzymatic hydrolysis of *S*-glycosides fits into the established picture of enzymatic *O*-glycoside hydrolysis.

Myrosinase has been isolated from a number of plant sources including *Lepidium sativum* L. (light grown cress) (6), *Sinapis alba* (white mustard) (7), and *Brassica napus* (rapeseed) seeds (7). The mustard and rapeseed (7) enzymes have molecular weights of 120,000–150,000. The activity of the *L. sativum* L. enzyme (6) was tested with 29 different glycosides, and only 4 were found to be good substrates. These were, in order of decreasing activity, sinigrin (the natural substrate), benzylglucosinolate, *p*-nitrophenyl-β-D-glucoside (PNPG),[1] and *o*-nitrophenyl-β-D-glucoside. β-D-Glucose could not be replaced by any other sugar. α-Glucosides were inactive, as were unreactive β-glucosides, such as methyl or phenyl. Interestingly, there appears to be no discrimination between different glucosinolates, and all are hydrolyzed at similar rates regardless of the nature of the side chain. Early work by Ettlinger *et al.* (2) demonstrated that the sulfate group was required for optimum activity as the desulfoglucosinolate did not act as a substrate with the *Sinapis* enzyme. The various isozymes have broad pH optima, from pH 5 to pH 7 (6, 7). No metal ion requirement has been found for the enzyme. For most plant enzymes, inhibition has been observed by –SH-directed reagents, implying that a cysteine residue is essential for catalysis. Myrosinase is specifically activated by ascorbic acid. The rate of hydrolysis of sinigrin, catalyzed by the mustard enzyme, is increased more than 25-fold by 1 mM ascorbic acid (8), although high concentrations begin to competitively inhibit reaction. Analogues of ascorbic acid are not effective. Hydrolysis of PNPG, by the same enzyme, however, is not increased by ascorbic acid (9). Spectro-

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[1] The abbreviation used is: PNPG, *p*-nitrophenyl-β-D-glucoside.

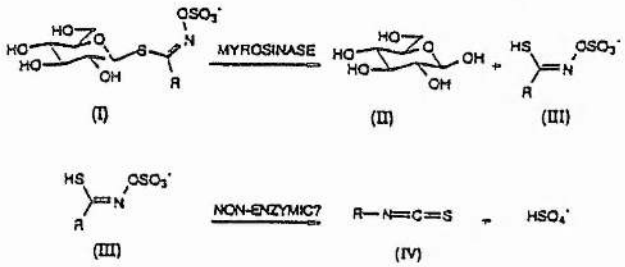

FIG. 1. Metabolism of glucosinolates as catalyzed by myrosinase.

scopic studies implied that there was a conformational change in the enzyme on binding ascorbic acid, and it was proposed that this brought about the rate enhancement.

We report the results of our initial studies on the chemical mechanism of myrosinase. These comprise examination of the activity of the enzyme in the presence of alternative glycosyl acceptors and inhibition studies.

EXPERIMENTAL PROCEDURES

Materials—Myrosinase (thioglucosidase, EC 3.2.3.1) was obtained from Sigma. The source of the enzyme is *S. alba* (white mustard seed). The specific activity of the enzyme preparation used was 200 units/g. Buffers and alcohols were obtained from Sigma or Aldrich. Sinigrin, methyl-β-D-glucoside, and glucono-δ-lactone were obtained from Sigma and PNPG from Aldrich and were used without further purification. The enzymes for analysis of D-glucose (*e.g.* hexokinase and glucose-6-phosphate dehydrogenase) were obtained from Sigma.

Enzyme Assay—Myrosinase activity was assayed according to the literature method of Palmieri *et al.* (10). Assays were carried out using 33 mM potassium phosphate buffer at pH 7.0, containing 0.1 mM sinigrin, in a total volume of 1 ml. The assay buffer was equilibrated at 37 ± 0.1 °C in a quartz cuvette (1-ml volume, 1-cm pathlength) in the thermostatted cell holder of the UV spectrophotometer. Reaction was initiated by the addition of 30 μl of enzyme solution, and the decrease in the absorbance at 227 nm (ϵ 6784 M^{-1} cm^{-1}), due to sinigrin, was monitored. 1 unit of enzyme was defined as the amount required to catalyze the hydrolysis of 1 μmol of sinigrin/min under standard assay conditions. Note that sinigrin is not hydrolyzed by β-glucosidases.

Determination of Kinetic Parameters—Sinigrin incubations were monitored as for the standard enzyme assay. Initial rate measurements were made using approximately 10 different sinigrin concentrations from 0.2 to 2.0 mM, and each measurement was repeated in triplicate. The reactions were linear over the time course measured, up to approximately 5% of the overall reaction. The kinetic data were then corrected for 1 unit of enzyme activity before being analyzed by non-linear regression using the Enzfitter program to obtain values of K_m and V_{max}.

For incubations involving PNPG, a similar procedure was employed, except that a larger concentration of potassium phosphate buffer, 50 mM, was employed. In this case, the absorbance due to the product, *p*-nitrophenol, was monitored at 430 nm (ϵ 7002 M^{-1} cm^{-1}). The extinction coefficient was found to change on addition of high concentrations of methanol, and it was necessary to determine the values. These were 4176 and 3193 M^{-1} cm^{-1} in 6.25 and 12.5 M methanol, respectively. Concentrations of PNPG from 1.0 to 30 mM were employed, and each measurement was repeated in triplicate. The reactions were linear over the time course measured, up to approximately 5% of the overall reaction. The kinetic data were then analyzed as above.

Partitioning Experiments—The glycosyl acceptor was added to the incubation mixture at a concentration of 0.2 M. Reactions were run under the same conditions as for the kinetic experiments. A high initial concentration of PNPG (20 mM) was employed, so that significant amounts of product were produced to minimize errors in measurement. Reactions were initiated by the addition of an aliquot (90 μl, 0.4 units) of enzyme solution. For the control reaction, with no added glycosyl acceptor, aliquots were taken at various time intervals, and the concentrations of *p*-nitrophenol and D-glucose were determined. The *p*-nitrophenol concentration was determined by diluting the aliquot into 1 ml of 0.1 N sodium hydroxide and measuring the absorbance of the resulting solution at 400 nm. Under these conditions, the extinction coefficient of *p*-nitrophenol is 18,300 M^{-1} cm^{-1}, so its concentration may be determined. The glucose concentration was determined using a coupled enzyme assay (11). In the presence of the glycosyl acceptors, incubations were run for a fixed time, and then the *p*-nitrophenol and glucose concentrations were determined.

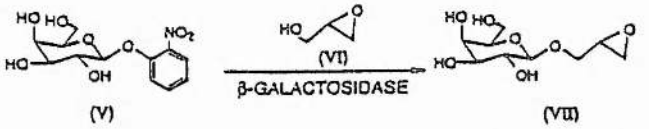

FIG. 2. The use of β-galactosidase-catalyzed transglycosylation in the synthesis of novel galactosides.

Inhibition Studies—Incubations were carried out in the presence of increasing inhibitor concentrations, as above. In each case, K_m and V_{max} values were determined as before. As glucono-δ-lactone is known to slowly hydrolyze in aqueous solution, incubations containing this compound were made up immediately prior to use.

RESULTS

It is well documented that many glycosidases are able to catalyze transglycosylation reactions, namely the transfer of residues from glycoside substrates to acceptor molecules other than water (12, 13). Synthetic organic chemists have frequently exploited this useful property and employed β-glycosidases in the synthesis of novel glycosides (14). For example, when *o*-nitrophenyl-β-D-galactoside (V) is hydrolyzed by β-galactosidase in the presence of the epoxy alcohol (VI), the galactosyl moiety is transferred to the acceptor hydroxyl group to give the new β-galactoside (VII) (15) (Fig. 2).

The ability of myrosinase to catalyze similar transglycosylation reactions had not been examined. Therefore, preliminary preparative scale experiments were carried out using myrosinase under similar conditions with PNPG as the substrate and allyl alcohol as the glycosyl acceptor. Allyl alcohol was chosen because it resembles the side chain of the glucosinolate, sinigrin, a natural substrate for myrosinase, and should have some chance of binding at the enzyme active site. However, no allyl-β-D-glucoside, the product of transglycosylation, was observed in any reaction. The same substrate was also hydrolyzed in a 9:1 ethanol:water mixture to produce the ethyl-β-D-glucoside. Again, no product was observed. These early failures to observe any transglycosylation prompted us to study the activity of myrosinase in alcohol:water mixtures in more detail and show whether the reactions failed due to mechanistic reasons or simply because of reduced enzyme activity.

Two myrosinase activities were monitored. The hydrolysis of sinigrin was followed by UV spectroscopy, monitoring the disappearance of the sinigrin absorbance at 227 nm. In separate experiments, PNPG hydrolysis was also followed by UV spectroscopy monitoring the appearance of the *p*-nitrophenol absorbance at 430 nm. The kinetics for the hydrolysis of sinigrin were determined under the standard assay conditions (pH 7.0, 33 mM phosphate buffer, 37 ± 0.1 °C). The K_m for sinigrin was found to be 0.42 ± 0.05 mM, in good agreement with previous workers (16), and V_{max} was also comparable (10) at (48 ± 0.2) × 10^{-3} mol dm^{-3} min^{-1}. The measurements were then repeated in the presence of increasing amounts of methanol, from 0.2 to 12.5 M (40% by volume) (Table I). The hydrolysis of PNPG was then examined under similar conditions but using 50 mM phosphate buffer. In aqueous solution, a high K_m of 89 ± 30 mM was obtained, as well as a V_{max} of (0.59 ± 0.2) × 10^{-3} mol dm^{-3} min^{-1}. Thus, the rate of PNPG was much lower than that of sinigrin as expected (16). The experiments were then repeated in methanol:water mixtures as for sinigrin (Table II).

For both activities, it can be seen that the rate of hydrolysis decreases as the amount of methanol increases. Values of K_m for sinigrin remained constant and the observed rate decrease was all due to changes in V_{max}. Thus, binding of the substrate to the enzyme active site was unaffected by the change in the reaction medium, while the rate of the reaction decreased. Even with 12.5 M (40% v/v) methanol, there was an appreciable

TABLE I
Kinetics of sinigrin hydrolysis by myrosinase in organic solvent mixtures

Incubations were carried out at 37 ± 0.1 °C at pH 7.0 in 33 mM potassium phosphate buffer containing the appropriate amount of the organic solvent. The hydrolysis was initiated by the addition of an aliquot (30 μl) of concentrated myrosinase solution in aqueous buffer (pH 7.0, 33 mM phosphate). The incubations were run in 3-ml cuvettes in the thermostatted cell housing of the UV spectrophotometer, and the rate was determined from the decrease in the absorbance due to sinigrin at 227 nm. Values of K_M and V_{max} were determined by non-linear regression methods. Values of V_{max} are corrected for 1 unit of myrosinase.

Solvent	K_M	V_{max}[a]
	mM	10^{-3} *mol dm*$^{-3}$ *min*$^{-1}$
Water	0.42 ± 0.05	4.83 ± 0.21
6.25 M methanol	0.42 ± 0.03	2.98 ± 0.06
12.5 M methanol	0.57 ± 0.26	2.40 ± 0.40
6.25 M ethanol	0.49 ± 0.08	3.59 ± 0.19
M dioxane	0.87 ± 0.07	3.64 ± 0.12
M acetonitrile	0.54 ± 0.08	2.47 ± 0.14

[a] Corrected for 1 unit of myrosinase activity under standard assay conditions.

TABLE II
Kinetics of p-nitrophenyl-β-D-glucoside hydrolysis by myrosinase in organic solvent mixtures

Incubations were carried out at 37 ± 0.1 °C at pH 7.0 in 50 mM potassium phosphate buffer containing the appropriate amount of the organic solvent. The hydrolysis was initiated by the addition of an aliquot (30 μl) of concentrated myrosinase solution in aqueous buffer (pH 7.0, 33 mM phosphate). The incubations were run in 3-ml cuvettes in the thermostatted cell housing of the UV spectrophotometer, and the rate was determined from the increase in the absorbance due to *p*-nitrophenol at 430 nm. Values of K_M and V_{max} were determined by non-linear regression methods. Values of V_{max} are corrected for 1 unit of myrosinase.

Solvent	K_M	V_{max}[a]
	mM	10^{-3} *mol dm*$^{-3}$ *min*$^{-1}$
Water	61 ± 11	0.15 ± 0.02
0.2 M methanol	49 ± 20	0.10 ± 0.03
6.25 M methanol	30 ± 7	0.05 ± 0.007
12.5 M methanol	12 ± 1	0.064 ± 0.003
6.25 M ethanol	51 ± 13	0.24 ± 0.05
M dioxane	4.0 ± 1.0	0.022 ± 0.001
M acetonitrile	17 ± 3	0.11 ± 0.01

[a] Corrected for 1 unit of myrosinase activity under standard assay conditions.

rate of reaction with only a 50% decrease from the rate in aqueous solution. Data for PNPG showed similar trends. Therefore, the enzyme is not terribly sensitive to the presence of the organic solvent, and so the chances of observing transglycosylation were reasonable. The measurements were also repeated in a range of other water-miscible organic solvents (ethanol, dioxan, acetonitrile) (Tables I and II), again showing reduced, although significant, activity.

The kinetic results were all initial rate measurements and so gave no information concerning the long term stability of myrosinase under the reaction conditions. Therefore, the stability of myrosinase was examined in the methanol:water mixtures by incubating the enzyme in buffer at 37 °C, removing aliquots at various time intervals, and measuring the myrosinase activity using the standard sinigrin assay (Fig. 3). If the enzyme proved to be very unstable, then the chances of transglycosylation reactions may be significantly reduced. However, myrosinase was in fact very stable. The half-life in the presence of 6.25 M (20%) methanol at 37 °C was 40 h but was reduced to 8 h with 12.5 M (40%) methanol. Myrosinase was generally found to be very stable. Concentrated stock solutions used for kinetic experiments, stored at 4 °C, did not lose appreciable amounts of activity even after 2–3 weeks.

Once the activity and stability of myrosinase in the presence

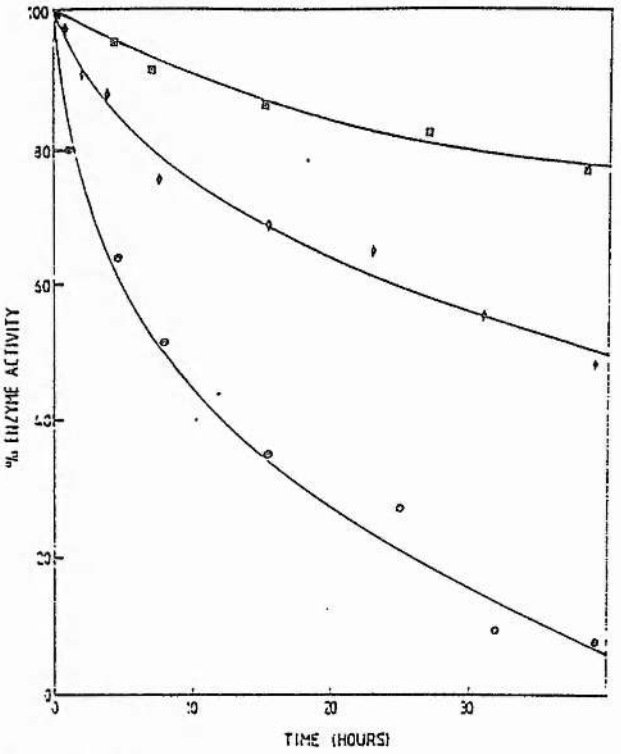

FIG. 3. Stability of myrosinase in methanol:water mixtures. Myrosinase (4 units) was incubated at 37 ± 0.1 °C at pH 7.0 in 33 mM potassium phosphate buffer (1 ml), containing the appropriate amount of methanol. Aliquots (30 μl) were withdrawn at various time intervals, and the enzyme activity was measured using the standard assay. The incubation conditions were as follows: ■, no methanol; ◆, 6.25 M (20%) methanol; ●, 12.5 M (40%) methanol.

of glycosyl acceptors had been examined, it was then necessary to determine the degree of transglycosylation that was taking place. This required measurement of the relative amounts of D-glucose and *p*-nitrophenol produced on hydrolysis of the PNPG under various conditions. A coupled enzyme assay was used to measure D-glucose concentrations, while the *p*-nitrophenol was determined spectrophotometrically. In the presence of a glycosyl acceptor, the amount of transglycosylation product can be calculated from the difference in these two values, *i.e.* less glucose will be produced than *p*-nitrophenol. Using methanol as the glycosyl acceptor, the transglycosylation product would be methyl-β-D-glucoside. A control experiment incubating this with myrosinase under the standard conditions resulted in no release of glucose. This demonstrated that methyl-β-D-glucoside was not a substrate for myrosinase and was stable under the reaction conditions. Indeed, the myrosinase preparation used in these studies has not been found to hydrolyze any of the alkyl *O*-glycosides or *S*-glycosides that have thus far been tested.[2] This also implies that there are no contaminating β-glucosidase enzymes, which would cause complications in the interpretation of our results.

For the transglycosylation experiments, incubations containing 20 mM PNPG were employed. To test the procedure, a reaction was run in aqueous solution, and, as expected, equal amounts of D-glucose and *p*-nitrophenol were produced. When the experiment was then repeated in 0.2, 6.25, and 12.5 M methanol, it was observed that equal amounts of D-glucose and *p*-nitrophenol were also obtained, implying that no observable transglycosylation was taking place. This was in agreement

[2] M. G. Taylor and N. P. Botting, unpublished results.

TABLE III
Attempts at transglycosylation

Incubations were carried out at 37 ± 0.1 °C at pH 7.0 in 50 mM potassium phosphate buffer containing the appropriate amount of the glycosyl acceptor.

Glycosyl acceptor[a]	[*p*-Nitrophenol][b]	[D-Glucose][c]
	mM	
None	6.17 ± 0.09	6.65 ± 1.2
Methanol	6.28 ± 0.25	6.15 ± 0.34
Methanol (6.25 M)	4.2 ± 0.7	4.3 ± 0.35
Ethanol	5.8 ± 0.2	7.3 ± 1.0
Propan-1-ol	6.8 ± 0.2	7.6 ± 0.3
Butan-1-ol	5.4 ± 0.4	5.35 ± 1.3
2-Mercaptoethanol	6.5 ± 0.4	8.0 ± 0.45
3-Hydroxy-1-propanesulfonate	4.9 ± 0.8	5.0 ± 1.3
Glycerol 2-phosphate	5.1 ± 0.06	7.1 ± 0.8
2-Mercaptoethanesulfonate	4.5 ± 0.1	5.4 ± 0.12

[a] All at 0.2 M concentrations unless indicated.
[b] Determined by UV.
[c] Determined using the coupled enzyme assay.

with the qualitative observations of the synthetic scale experiments but was still a very surprising result. For most of the β-glycosidases, significant amounts of transglycosylation have been observed under similar conditions (12, 13).

A range of other glycosyl acceptors were then investigated, all at 0.2 M concentrations. The amounts of D-glucose and *p*-nitrophenol produced after a 24-h incubation under standard conditions were determined in the presence of each potential glycosyl acceptor (Table III). The larger alcohols, ethanol, propan-1-ol, and butan-1-ol, proved to be ineffective in transglycosylation reactions. As the glucosinolates are actually *S*-glycosides, a thiol acceptor was also tried. However, mercaptoethanol did not appear to trap out any of the glucose. The other part of the glucosinolate structure that probably provides important binding interactions is the sulfate group. This charged moiety is presumably balanced by some positively charged residue at the enzyme active site. It therefore seemed feasible that glycosyl acceptors containing a negatively charged group might have a better chance of binding at the active site and intercepting the glycosyl moiety. Three examples were chosen, which were commercially available, namely, 3-hydroxy-1-propanesulfonate (VIII), glycerol 2-phosphate (IX), and 2-mercaptoethanesulfonate (X) (Fig. 4). When PNPG was incubated with myrosinase in the presence of these compounds at 0.2 M concentration, equal amounts of glucose and *p*-nitrophenol were produced, again implying that no transglycosylation had taken place. Interestingly, it can also be observed that none of these compounds seems to be a particularly effective inhibitor of myrosinase, as the amounts of D-glucose and *p*-nitrophenol produced do not seem to be very sensitive to the presence of the glycosyl acceptor. The yields are all approximately 30% after 24 h. In the case of 12.5 M methanol, this drops to 21%, as may be expected from the effect of high methanol concentrations on the enzyme activity. The three charged glycosyl acceptors also have a significant effect, giving 22–25% yields, reflecting some degree of inhibition of the hydrolysis reaction. The detailed inhibitory properties of these compounds are currently under investigation. Preliminary results using sinigrin as the glycosyl donor, rather than PNPG, have indicated that transglycosylation does not take place with this substrate either. Crude measurements of glucose yields on completion of sinigrin hydrolysis have been unaffected by the presence of glycosyl acceptors.

The above results call into question the existence of a long lived glycosyl-enzyme intermediate that can be trapped by nucleophilic species. In the case of the β-glycosidases, this intermediate has been thought to be either a stabilized oxocarbonium ion or a covalently bound glycosyl group, resulting from attack at the anomeric carbon by an active site nucleophile. In

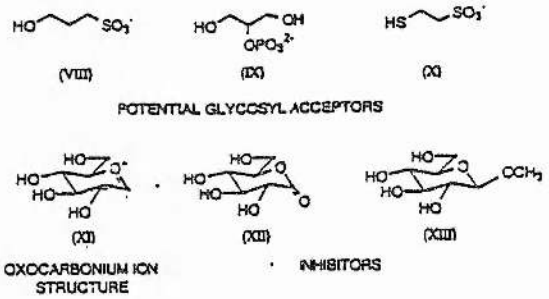

FIG. 4. Structures of inhibitors.

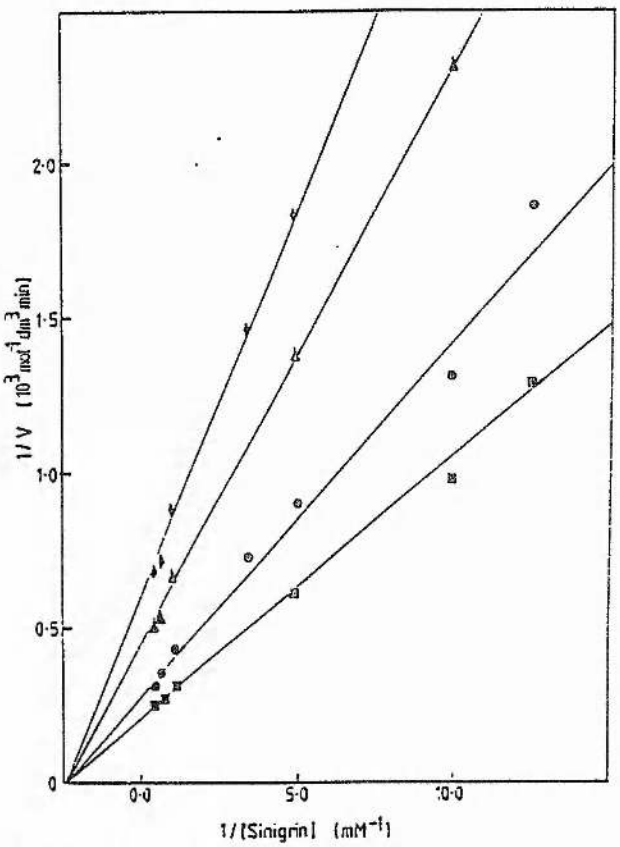

FIG. 5. Inhibition of myrosinase-catalyzed sinigrin hydrolysis by glucono-δ-lactone. Incubations were carried out under the standard conditions containing increasing concentrations of glucono-δ-lactone: ■, 0 mM; ●, 1 mM; ▲, 5 mM; ◆, 10 mM.

the former case, the oxocarbonium ion structure (XI) can been employed as a model for the transition state for the reaction. Enzyme inhibitors have then been designed that mimic this structure. One simple, readily available example is glucono-δ-lactone (XII) (Fig. 4). This molecule possesses a planar sp^2 hybridized carbon atom at the 1-position of the sugar, which mimics the planar structure of the transition state at that position. As a result, glucono-δ-lactone has been found to be a very tight binding competitive inhibitor for β-glucosidase, with a K_I of 0.2 ± 0.2 mM (18). We were interested in examining the interaction of myrosinase with this inhibitor to see if a similar type of inhibition was observed.

Thus, the rates of hydrolysis of both sinigrin and PNPG by myrosinase were measured in the presence of a range of concentrations of glucono-δ-lactone. The results are shown in Lineweaver-Burk form in Figs. 5 and 6. These show that for both substrates, glucono-δ-lactone acts as a noncompetitive

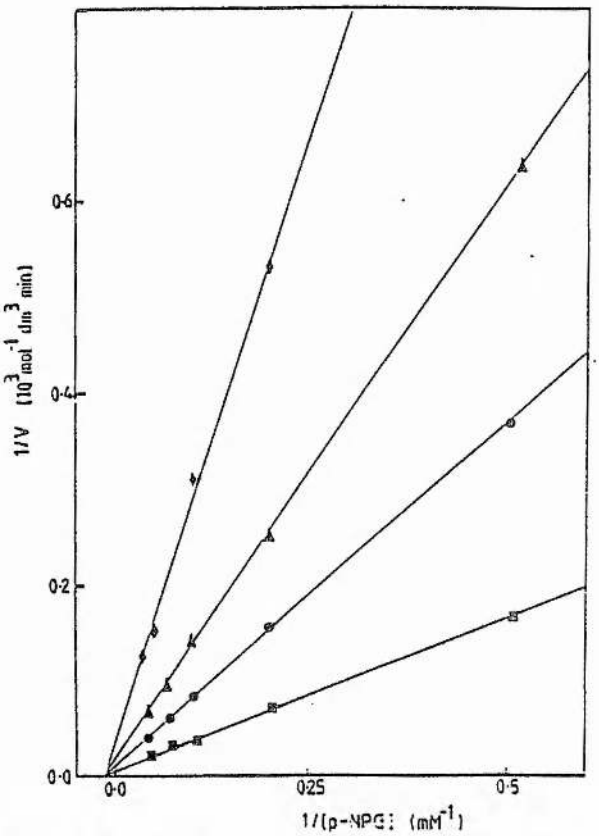

FIG. 6. **Inhibition of myrosinase-catalyzed *p*-nitrophenyl-β-D-glucoside hydrolysis by glucono-δ-lactone.** Incubations were carried out under the standard conditions containing increasing concentrations of glucono-δ-lactone: ■, 0 mM; ●, 5 mM; ▲, 10 mM; ◆, 20 mM.

inhibitor, that is, the K_m is unaffected, while V_{max} decreases with increasing inhibitor concentration. The variation of V_{max} with inhibitor concentration is not simple, but it can be estimated that K_i is approximately 5 mM for each substrate. Therefore, glucono-δ-lactone appears to bind much less tightly to myrosinase than to β-glucosidase.

The effect of the specific activator, L-ascorbic acid (8), on the degree of inhibition of myrosinase by glucono-δ-lactone was examined (Table IV). At a concentration of 1 mM, L-ascorbic acid exerts its maximum effect, increasing V_{max} for the hydrolysis of sinigrin by a factor of 1.5 but leaving K_m unchanged. When the measurements are repeated in the presence of glucono-δ-lactone, the degree of activation decreases as the concentration of the inhibitor increases. Thus, at 1 mM, glucono-δ-lactone V_{max} increases by a factor of 1.4, but at 10 mM, V_{max} actually decreases by a factor of 0.8. This raises the interesting possibility that the glucono-δ-lactone is actually binding at the activator site rather than at the substrate site, which would fit with the noncompetitive inhibition that is observed.

Methyl-β-D-glucoside (XIII) was found to be a simple competitive inhibitor of myrosinase, albeit with a rather poor K_i of 120 mM. The product, β-D-glucose, was also a very poor inhibitor of the enzyme. At a 1 mM concentration, it gave a 20% decrease in V_{max}, but as the concentration was increased, the rate also increased, approaching its original value.

DISCUSSION

The reaction catalyzed by myrosinase superficially resembles the hydrolysis of glycosides catalyzed by many β-glycosidase enzymes. It is obviously different in two respects. First, the substrate is a sulfur rather than an oxygen glycoside, and

TABLE IV

Effect of δ-gluconolactone on the activation of myrosinase by ascorbic acid

Incubations were carried out at 37 ± 0.1 °C at pH 7.0 in 33 mM potassium phosphate buffer, under standard conditions, containing the appropriate concentrations of ascorbic acid and glucono-δ-lactone. Values of V_{max} are corrected for 1 unit of myrosinase.

[Ascorbic acid]	[δ-Gluconolactone]	V_{max}
mM		$mol^{-1}\ dm^{-3}\ min^{-1}$
0.0	0.0	4.83 ± 0.21
0.0	1.0	3.75 ± 0.19
0.0	10.0	1.90 ± 0.09
1.0	0.0	7.12 ± 0.22
1.0	1.0	5.30 ± 0.50
1.0	10.0	1.52 ± 0.07

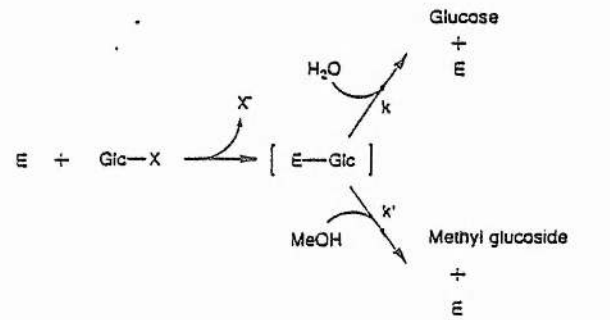

FIG. 7. **Reaction pathway for hydrolysis of β-D-glucosides in the presence of competing glycosyl acceptors.**

second, there is a subsequent rearrangement of the aglycone fragment, which may or may not be catalyzed by the enzyme. The initial results of our study of the chemical mechanism of myrosinase, reported here, have shown that the resemblance may indeed be only superficial, as there seem to be some important mechanistic differences. One interesting feature is that myrosinase is a very stable enzyme. This probably results from the role of the glucosinolate/myrosinase system as a defensive mechanism in the plant. In the cell, myrosinase is normally separated from the glucosinolates, and they only come together after cell damage, such as attack by pests or pathogens (4). Glucosinolate hydrolysis then releases isothiocyanates, which are noxious to pests and deter them from further attack. Immediate response is vital, and therefore there must always be active enzyme present in the cell. As a result, myrosinase must be stable and long lived. This is in contrast to many other enzymes, which are synthesized by the cell machinery when they are required, in response to the current needs of the cell.

Transglycosylation is commonly observed with β-glycosidases; however, we have been unable to obtain evidence for this reaction taking place with myrosinase. For example, Gopalan and co-workers (19) observed significant transglycosylation using mammalian cytosolic β-glucosidase in the presence of alcohol acceptors. The rate of hydrolysis of PNPG was increased 8-fold by the addition of 0.2 M *n*-butyl alcohol, and the ratio of *n*-butyl-β-D-glucoside to D-glucose was 24:1. The proposed reaction pathway for the transglycosylation is given in Fig. 7. In the case of the β-glucosidase, it was proposed that for PNPG, k_3 was rate-limiting. So on addition of butan-1-ol, which is more nucleophilic than water, an overall increase in rate was observed as $k_4 > k_3$.

However, the rate of *p*-nitrophenyl-β-D-glucoside hydrolysis catalyzed by myrosinase was not increased by the addition of alternative, and more reactive, glycosyl acceptors, and in fact decreased. Also, there was no decrease in the amount of D-glucose produced as a result of transglycosylation. A wide

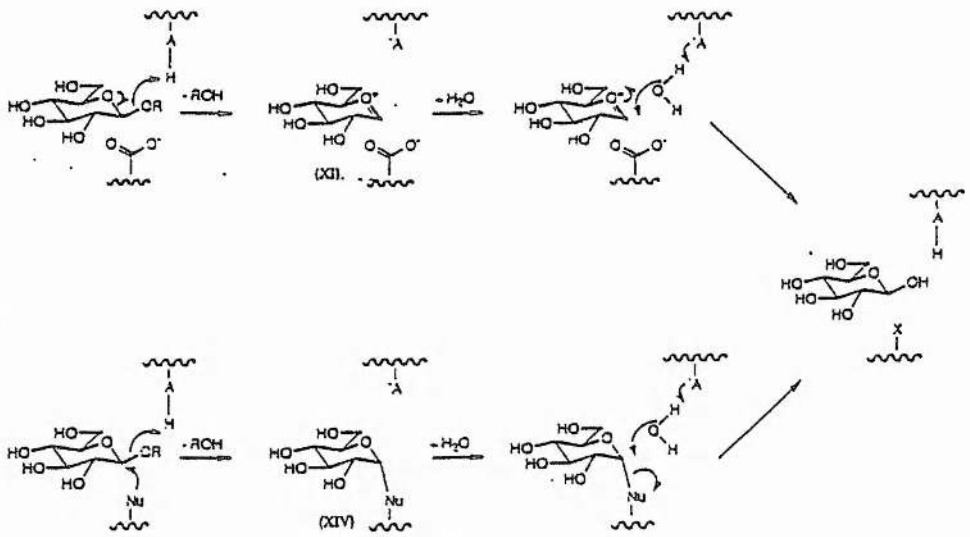

FIG. 8. **Proposed chemical mechanisms for β-glucosidases.**

range of potential glycosyl acceptors were employed, none of which were successful. Even the very nucleophilic thiols did not trap out the glycosyl moiety. It was reasoned that the acceptors simply were unable to gain access to the active site. The natural substrate contains a negatively charged group in the aglycone, and so acceptors containing similar charged side chains were employed. Again, these proved to be unsuccessful at transglycosylation.

The ease with which transglycosylation takes place with myrosinase is obviously much less than with β-glucosidase and β-galactosidase, despite the apparent similarities between the reactions that they catalyze. In the case of the β-glycosidases, reaction is thought to proceed either via an oxocarbonium ion (XI) or a covalent enzyme-glycosyl intermediate (XIV) (Fig. 8). This is formed following expulsion of the aglycone from the substrate. A similar pathway is feasible for myrosinase except that there is then a subsequent rearrangement of the aglycone.

Inhibition of myrosinase by glucono-δ-lactone, a tight binding competitive inhibitor of β-glucosidase that mimics the oxocarbonium ion intermediate, was examined. However, this compound was not a competitive inhibitor for myrosinase and instead showed noncompetitive inhibition with a poor K_I. This gives a second example of the unusual behavior of myrosinase. One possible reason for the observed noncompetitive inhibition is that the glucono-δ-lactone is not binding at the active site but at some other site on the enzyme. In the case of myrosinase, there exists a candidate for this alternative binding site. The enzyme is specifically activated by ascorbic acid (8), which apparently binds to the enzyme causing a change in its conformation. Therefore, it is possible that the glucono-δ-lactone binds at the ascorbic acid site, but in this case causes a decrease in the rate of reaction. Some preliminary experiments (Table IV) indicated that the degree of rate enhancement due to ascorbic acid is reduced in the presence of glucono-δ-lactone, decreasing as the inhibitor concentration increases. It certainly appears that the glucono-δ-lactone is not acting as a transition state analogue from its mode and degree of inhibitory activity. However, the simple glucoside, methyl-β-D-glucoside, was a competitive inhibitor, albeit with a poor K_I.

When discussing the implications of these findings on the chemical mechanism, it must always be remembered that myrosinase could also be involved in catalysis of the rearrangement of the aglycone. The early evidence for this reaction being spontaneous and not involving the enzyme is certainly not conclusive (2). A particularly interesting observation is that the desulfoglucosinolates are only inhibitors of myrosinase and are not hydrolyzed by myrosinase (17). This may imply that the driving force for the reaction is actually the rearrangement step. If the enzyme was indeed required to effect this rearrangement, then it is very possible that the aglycone is not the first product released during the catalytic cycle. If the other product, D-glucose, is released first, then the ease of transglycosylation would be expected to be greatly reduced, as there would be little room for the glycosyl acceptor to bind at the active site while the aglycone was still present. Determination of the product debinding order, using product inhibition studies, would allow this possibility to be examined. Unfortunately, D-glucose gave very poor inhibition, which could not be assigned as either competitive or uncompetitive. Work is now underway to prepare the aglycone fragment, and stable analogues thereof, to assess their inhibitory properties and address this possibility in more detail.

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