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THE BIOSYNTHESIS OF NOJIRIMYCINS

By

David J. Hardick, B.Sc.

Submitted for the degree of Doctor of Philosophy

Department of Chemistry,

University of Warwick

October 1992

To

Mum and Dad

CONTENTS

Abbreviations

°C	degrees Celsius
ATCC	American Type Culture Collection
BOC	t-butyloxycarbonyl
CI	chemical ionisation
COSY	correlation spectroscopy
DMDP	dihydroxymethyldihydroxypyrrolidine
DMJ	1-deoxymannojirimycin
DNJ	1-deoxynojirimycin
EC	enzyme commission number
EI	electron impact
FAB	fast atom bombardment
GC	gas chromatography
Glc	glucose
GlcNAc	N-acetylglucosamine
gp120	glycoprotein 120
h	hours
HIV	Human Immunodeficiency Virus
IC_{50}	50% inhibitory concentration
IR	infra-red
K_i	inhibitory concentration
M	molar
M^+	molecular radical cation
M.Pt.	melting point
m/z	mass by charge
Man	mannose
MH^+	protonated molecular cation

min	minutes
MJ	mannojirimycin
M_r	relative molecular mass
MS	mass spectroscopy
NAD	nicotinamide adenosine dinucleotide
NJ	nojirimycin
NMR	nuclear magnetic resonance spectroscopy
ppm	parts per million
THF	tetrahydrofuran
Tlc	thin layer chromatography
TTP	thymidine triphosphate
v/v	volume by volume
w/v	weight by volume

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Acknowledgements.

I would especially like to thank my supervisor, Dr. David Hutchinson for his guidance and enthusiasm throughout my Ph.D studies. I am indebted to my co-worker, Sally Trew, for her expertise in the microbiology of *Streptomycetes* and I would also like to acknowledge the work of Jeremy Hastings and Inder Katyal for running the NMR and mass spectra. Amongst my colleagues in the chemistry department, I would especially like to thank Nicky, Cecile, Jane, Tim, Grace, Riz and Dave Chaplin for making my three years at Warwick so enjoyable. I would like to wish them all every success in their future careers. Lastly, I would like to give a special mention to Sharon, whose love and support has made this thesis possible.

Declaration

The work described in this thesis is the original work of the author except where acknowledgement is made to work and ideas previously published. It was carried out in the Department of Chemistry, University of Warwick, between February 1990 and October 1992, and has not been submitted previously for a degree at any institution.

Abstract

Streptomyces subrutilus ATCC 27467, when grown on a glucose-containing soyabean medium, produces both 1-deoxymannonojirimycin (DMJ) and 1-deoxynojirimycin (DNJ) in its culture medium. When 1- or 2-[^{2}H]-D-glucose is used, the deuterium label appears at C6 in both alkaloids and the labelling pattern suggests that the first step in the biosynthesis of both DNJ and DMJ is a glucose to fructose isomerisation. Studies with 5-[^{2}H]-D-glucose and 6,6-[$^2H_2$]-D-glucose indicate that oxidation of the 6-position of the glucose/fructose occurs during the biosynthesis and that mannonojirimycin is the first amino sugar to be formed. Mannonojirimycin can then undergo dehydration and reduction to DMJ. Alternatively, epimerisation of mannonojirimycin can occur at C2 to give nojirimycin which is then dehydrated and reduced to DNJ.

Studies with another microorganism, *B. subtilis var niger* ATCC 9372, indicate that a similar biosynthetic pathway is in operation. DMJ, however, is not produced by this microorganism and only low levels of NJ are postulated from enzyme inhibition and deuterium labelling studies. A minor biosynthetic route is also evident from labelling studies with 1-[^{13}C]-D-glucose and 1-[^{13}C]-D,L-glyceraldehyde. It is suggested that the fructose at the beginning of the biosynthesis can split into two C3 trioses which are in equilibrium with each other. These can then re-combine to continue in the usual biosynthetic pathway.

New chemical routes to 5-[^{2}H]-D-glucose and 2-[^{2}H]-D-glucose are described in Chapter 3 along with a method for introducing deuterium or tritium into DNJ or NJ. Other isotopically enriched glucoses or intermediates have also been prepared using literature methodology.

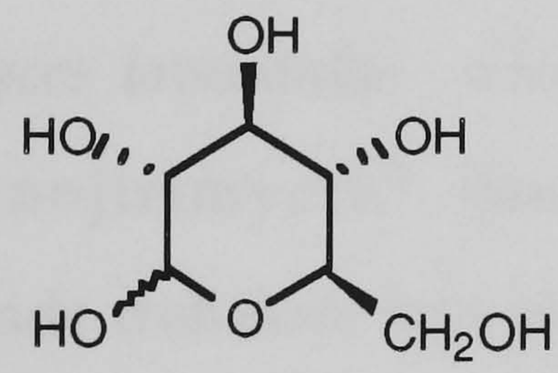
Studies have been undertaken to assess whether *S. subrutilus* can utilise glucose analogues in the biosynthetic pathway. To this end, new or modified routes to these glucose derivatives have been investigated and some work has focused on the chemical synthesis of DNJ analogues themselves.

CHAPTER ONE.

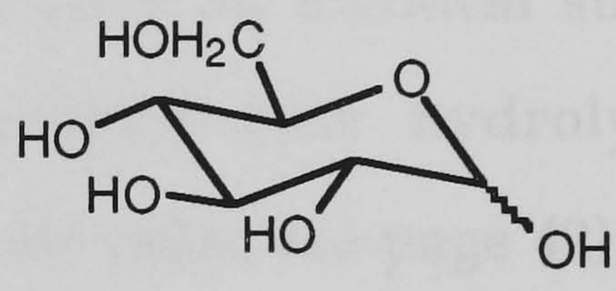
General Background

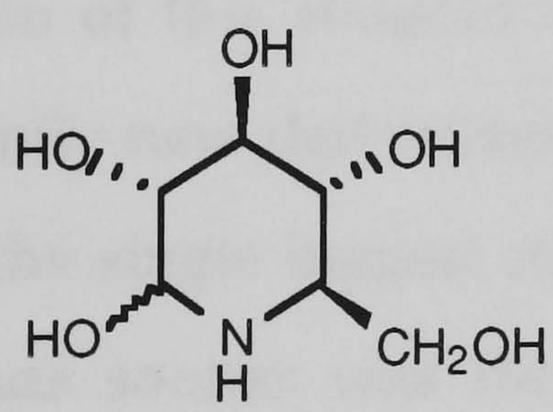
Nojirimycin is a piperidine alkaloid that was first isolated from strains of *Streptomyces* in 1967[1]. It aroused interest at this time because it acted as an antibiotic towards *Sarcina lutea* and various other microorganisms. Its similarity towards glucose was striking - it merely differed by having an amine group in place of the oxygen within the pyranose ring. As might be expected, it existed as the α and β anomers like glucose and adopted a chair conformation in aqueous solution.

Structure of nojirimycin and 1-deoxynojirimycin

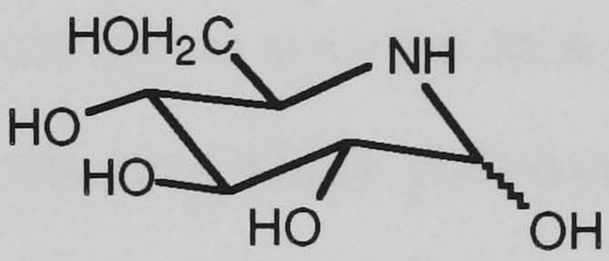


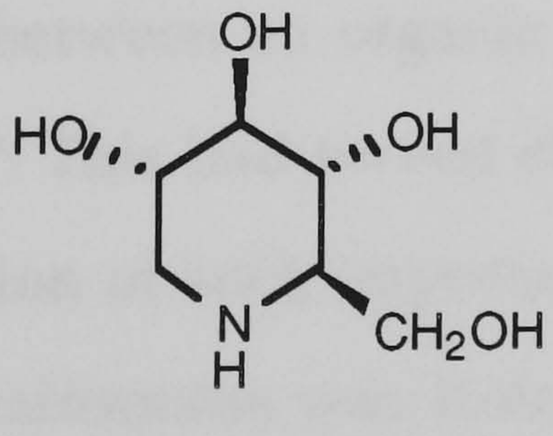
glucose



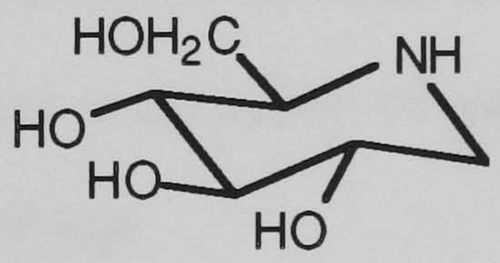


nojirimycin





1-deoxynojirimycin



The first chemical synthesis of nojirimycin was completed less than a year later[2], although Paulsen[3] had done extensive research, including NMR

and mass spectrometry studies, into carbohydrates containing nitrogen or sulphur in the "hemiacetal ring". The first synthesis of nojirimycin included detailed work on chemical and physical properties of the alkaloid, reinforcing the earlier observations that it was not particularly stable under neutral or acidic conditions. The compound could, however, be easily isolated as the bisulphite adduct and subsequently converted to the free base under alkaline conditions. From this comprehensive work by Inouye *et al*[2], a new deoxy derivative was synthesised, 1-deoxynojirimycin, that was much more acid stable than nojirimycin and which aroused much interest in future years.

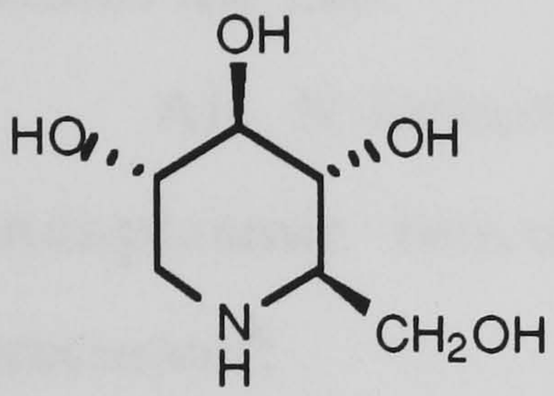
Several years elapsed until 1976 when 1-deoxynojirimycin was isolated as the natural product from the root bark of the mulberry[4]. Subsequently, four years later a trehalase inhibitor was purified from the fermentation broth of *Streptomyces lavendulae* which was found to have an identical structure to 1-deoxynojirimycin[5] (trehalase is a glycohydrolase hydrolysing the disaccharide trehalose into its constituent glucose units, see page 42).

The inhibition of glycohydrolases was to become the most important application of this alkaloid and related compounds for the next decade and consequently new derivatives were isolated from plant sources at a rapid rate. Perhaps the single biggest reason for not discovering these polyhydroxylated compounds sooner was the fact that they were water soluble. Extraction techniques using plant sources had traditionally relied upon partitioning the alkaloid between an organic solvent such as chloroform and a basic aqueous medium[6]. This had served chemists reliably for a number of years leading to the isolation of such important drugs as morphine.

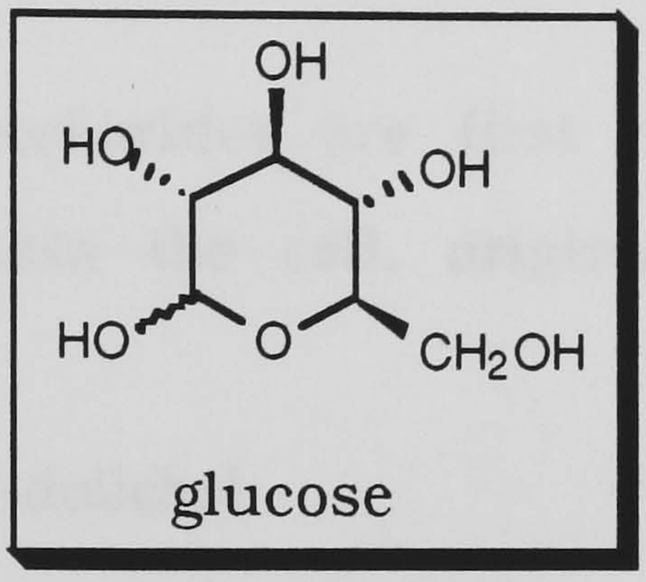
Swainsonine was isolated in 1979[7] and was found to be an inhibitor of mannosidases, those enzymes that hydrolyse oligosaccharides containing mannose residues. A further indolizidine alkaloid was discovered very soon afterwards in the Moreton Bay chestnut (*Castanospermum australe*)[8]. It was named castanospermine and bore a resemblance in the stereochemistry of its

hydroxyl groups to 1-deoxynojirimycin. Again it was a potent inhibitor of glucosidase enzymes. A few of the alkaloids which resemble pyranose sugars are shown below, **fig 1.01.**

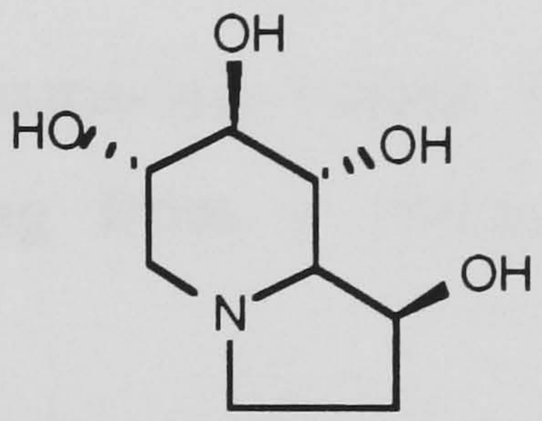
Figure 1.01



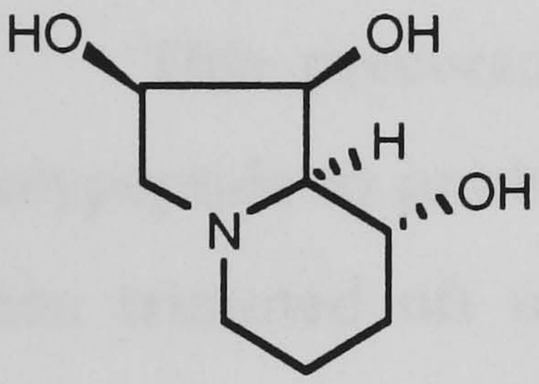
1-deoxynojirimycin
(*Streptomyces sp.*)
(*Bacillus sp.*)



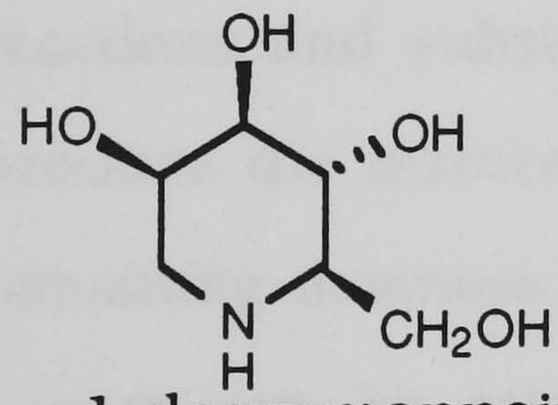
glucose



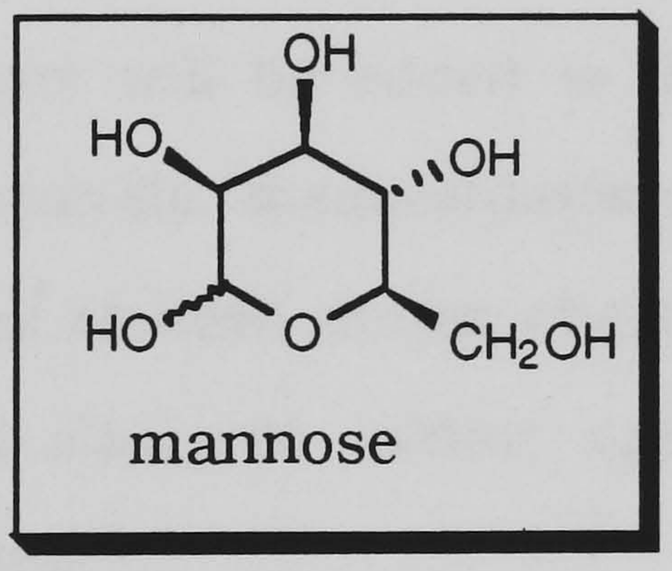
castanospermine
(*Castanospermum australe.*)



swainsonine
(*Swainsona sp.*)
(*Rhizoctonia leguminicola*)



1-deoxymannojirimycin
(*Streptomyces sp.*)



mannose

Inhibition of glycosidases by plant alkaloids

Before discussing a mechanism of inhibition by these plant alkaloids, it is important to consider in what context these compounds could become useful. To do this it is necessary to examine the biosynthesis of glycoproteins within the cell.

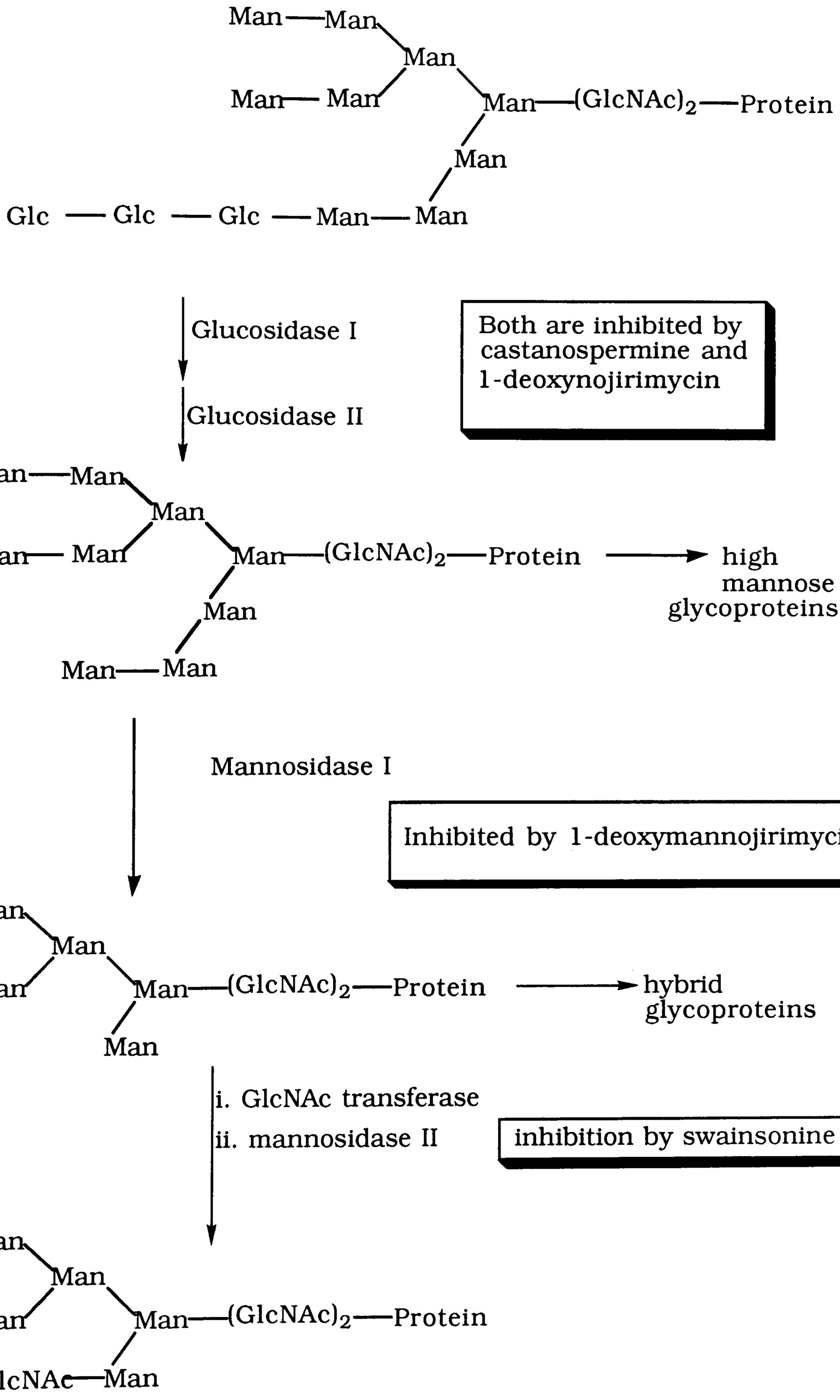
All N-linked oligosaccharides are first constructed inside the endoplasmic reticulum within the cell, originating from a common precursor[9]:

$Glc_3Man_9(GlcNAc)_2$-PP-dolichol

(where Glc=glucose, Man=mannose, GlcNAc=N-acetylglucosamine and dolichol=isoprene oligomer).

This precursor is then transferred to an asparagine residue on the polypeptide to produce $Glc_3Man_9(GlcNAc)_2$-protein. The glucose residues are then trimmed off using two α-glucosidases. Glucosidase I catalyses the hydrolysis of the first glucose unit whilst glucosidase II catalyses the removal of the remaining two. The high mannose glycoprotein that results from these reactions may remain in this state avoiding all further trimming reactions and subsequently new sugars will be added to this "stump" to produce the mature structure. Alternatively, mannosidases can act on the remaining mannose residues producing an even shorter chain to which new sugars can be attached. The plant alkaloids inhibit various enzymes throughout this process as outlined below[10].

Glycoprotein processing



Further glycosylation reactions can take place using the fully trimmed glycoprotein to build up the various types of oligosaccharide. For instance, galactose, sialic acid, N-acetyl glucosamine etc. can all be added to produce complex structures.

Recently, considerable effort has been directed towards evaluating the importance of glycoproteins in cell-cell interactions[11] and their function generally. What is required is to understand the contribution the oligosaccharide chains make to the activity of the glycoprotein and whether alterations to the sequence of sugars in the chains modifies this in any way[12]. In this respect plant alkaloids are ideal for this application. Many glycoproteins require their carbohydrate moiety for conformational stability as well as recognition, therefore it may be advantageous for the carbohydrate to be added to the protein in a single step and then study subsequent oligosaccharide modifications using a glycosidase inhibitor. A widely used inhibitor which blocks the formation of $Glc_3Man_9(GlcNAc)_2$-PP-dolichol is tunicamycin, but this compound suffers from the disadvantage that it completely prevents glycosylation and alterations at different points in the oligosaccharide chain cannot be made[10]. For instance, 1-deoxynojirimycin altered the composition of the α_1 -proteinase inhibitor in rats. Normal proteinase inhibitor contains high mannose-type glycoprotein, but on incubation with 1-deoxynojirimycin, glycoprotein containing significantly more glucose residues was found. The modification to the precursor oligosaccharide affected ultimately the type of glycoprotein produced and its secretion[13]. Care needs to be exercised in these experiments though, as it is important that the concentration of alkaloid is sufficient to keep the activity of inhibited enzyme to a low level. Furthermore, many of the plant alkaloids are not entirely specific to a particular enzyme (1-deoxynojirimycin, for example, is a better inhibitor of glucosidase II than glucosidase I)[11].

Other applications

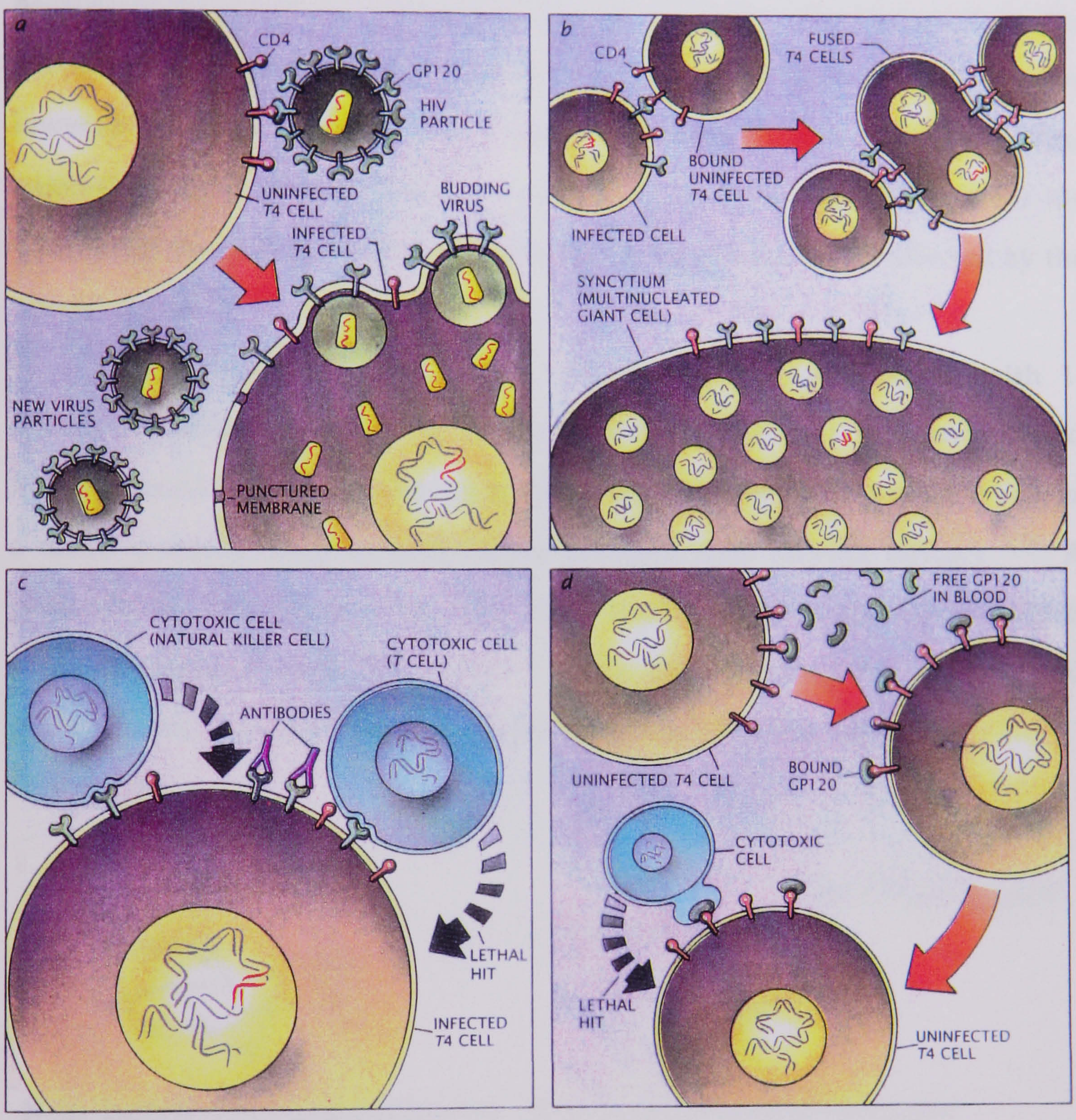
One of the main incentives for the study of alkaloid inhibitors has been the inhibition of HIV. Studies in 1987 showed that castanospermine, 1-deoxynojirimycin and various analogues of these all inhibited HIV *in vitro* to varying degrees[14]. Furthermore, the susceptibility of HIV to inhibition by these plant alkaloids was at a concentration which was not lethal to the host cell. Even at this early stage, it was suggested that they impaired the maturation of glycoproteins associated with the virus and it is now believed that one glycoprotein in particular, gp120, is involved.

The site of infection by HIV is the T4 lymphocyte, a white blood cell which recognises foreign antigens and activates other lymphocytes to produce antibodies to counteract any infection. There are receptors in the outer plasma membrane of the T4 cell, known as CD4 receptors, which bind to gp120. This glycoprotein is found on the surface of the HIV and once virus-cell binding has occurred, viral genetic material can be incorporated into the host cell. Here replication of the HIV takes place and further viral particles are produced. As illustrated in **figure 1.02**[138], the new particles can break the cell membrane as they bud off (case a), or alternatively, the viral protein, which is displayed on the surface of the infected cell, can interact with more T4 cells. This interaction causes the T4 cells to clump together and eventually merge with the original cell, causing the formation of syncytia (case b). Cell lysis in both cases kills the T4 cells.

HIV is also capable of eliciting normal immune defences against infected cells. An infected T4 cell, displaying gp120 on its surface, will be attacked by the natural killer cells (case c). A healthy T4 cell could also be destroyed in this way if unbound gp120 in the blood binds to the CD4 receptor (case d).

Figure 1.02.

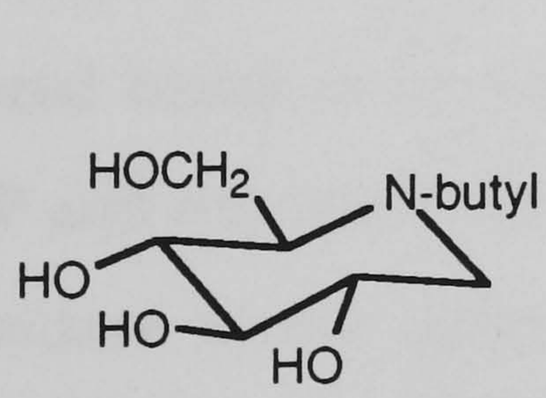
Mechanisms of HIV infection.



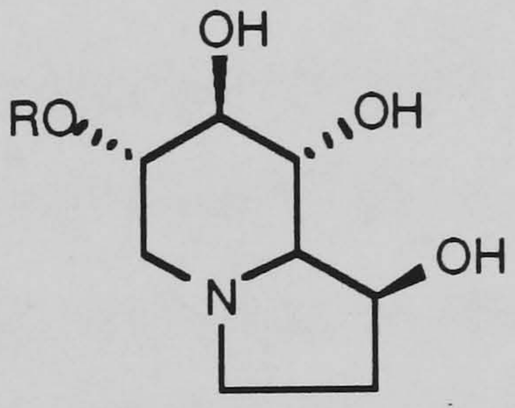
Research in 1987[15] indicated that the level of gp120 synthesis was not affected by the presence of glucosidase inhibitors. The infectivity of the virus and the formation of clumped T4 cells (syncitia) was, however, reduced because of the continued presence of glucose residues in the glycoprotein. The perturbed structure of the gp120 due to the plant alkaloids was said to have lowered the affinity for the CD4 receptor. Further research at the same time suggested, however, that the abnormal gp 120 had the same affinity for the CD4 receptors and that it was the formation of syncitia that was affected by the altered glycan[16,17].

Castanospermine was found to have the strongest effect, with 1-deoxymannojirimycin showing little decrease in infectivity. This encouraged many research groups into trying to find even better inhibitors of HIV and as a result a whole range of compounds emerged with varying efficacy[18,19]. A particularly promising drug was N-(n-butyl) 1-deoxynojirimycin which showed greater anti-HIV activity than castanospermine at a given concentration, but also had the benefit of lower cell toxicity, **fig 1.03**

Figure 1.03



N-(n-butyl)-1-deoxynojirimycin



R= $CH_3(CH_2)_2CO$

6-O-butyrylcastanospermine

Interestingly, the attachment of a fatty acid moiety to castanospermine at position 6 also gave a drug that was twenty times as effective at inhibiting HIV as the parent compound[20].

Many of the plant alkaloid drugs are non-specific and consequently cause side effects. 1-deoxynojirimycin and castanospermine, for example, both inhibit intestinal α-glucosidase which is responsible for the hydrolysis of

sucrose and starch[11]. Maintaining the concentration of the drug *in vivo* to produce inhibition of HIV represents a problem with existing alkaloids because of this lack of specificity, again reinforcing the need to examine new analogues[21].

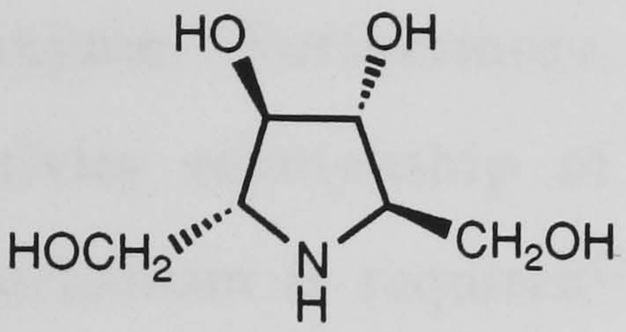
A few of the plant alkaloids have aroused interest as insecticides and insect anti-feedants, i.e. those drugs which deter the insect from feeding whilst not actually killing it. Two areas can be identified. Firstly, the inhibition of digestive enzymes in the insect (preferably without inhibiting enzymes from other species) and secondly, blocking receptor sites that inform the insect of the presence of food.

Dihydroxymethyldihydroxypyrrolidine (DMDP, **figure 1.04**) has featured predominantly in both respects[22,6,12]. For instance, this compound inhibits the enzyme trehalase, which is a glucosidase hydrolysing trehalose to glucose. Trehalose is an important carbohydrate reserve in insects, but it is not found in mammals so any specific inhibitor of the enzyme would obviously be useful. DMDP is relatively inactive against α-glucosidases in the mammalian gut (e.g. maltase, sucrase), but it does show inhibition of some other insect glucosidases, such as maltase in Bruchid beetles (a common pest of stored beans in tropical regions). Various organisms react differently to DMDP and it is impossible to say, without trial and error, whether a particular glycosidase in one species will behave similarly to that in another. This difference in specificity can be utilised to distinguish which glycosidases from various sources have the same mechanism of action.

The blocking of feeding receptor sites occurs in an entirely different way. The presence of sugars such as fructose provokes a response in insects to feed, presumably via sensory organs on the mouthparts of the insect. DMDP has the same shape and stereochemistry as fructose and this drug will prevent locusts from feeding, even if it is present in only trace quantities.

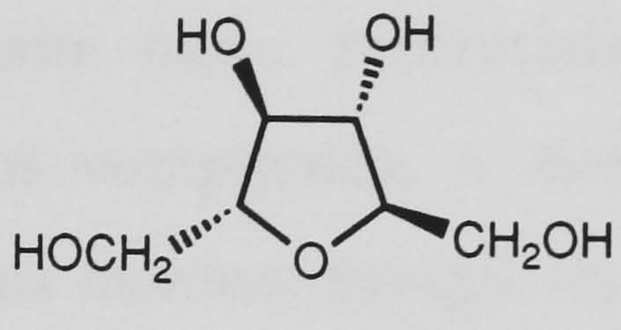
Figure 1.04.

The similarity between fructose and DMDP.



DMDP

Lonchocarpus sp.



fructose

The fructose analogue reversibly blocks the receptor sites which determine the presence of food, whilst it remains non-toxic to the insect. Other pests could, in theory, be controlled using natural plant alkaloids or synthetic derivatives, but again it is a matter of trial and error.

The plant alkaloids have other therapeutic properties which have perhaps not received as much attention due to the interest in HIV inhibition. For instance, castanospermine and swainsonine both have various effects on cancer cells. Studies[23] have shown that specific glycoproteins show increased branching of β-1,6 linked sugars during metastasis (the invasion of healthy tissue by cancer cells). If cancerous cells are incubated with swainsonine, the β-1,6 linked branched residues are reduced and a loss of metastasis is seen to occur.

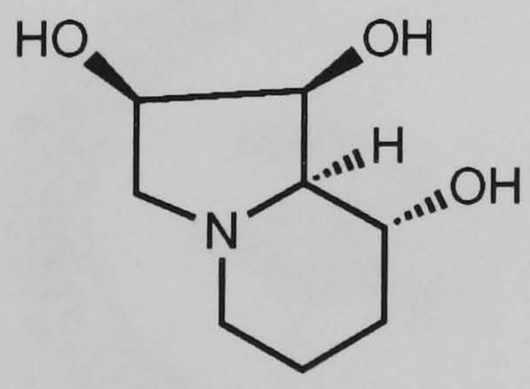
Likewise, swainsonine has also been reported to influence the effect natural killer cells have on cancerous cells[24]. Once again by altering the type of N-linked asparagine oligosaccharide present on the tumour cell, the susceptibility towards lysis by killer cells was increased *in vitro* . Normal cells would presumably suffer a similar fate, but cancer cells are often characterised by unrestrained multiplication and would be a prime target.

Mechanism(s) of glycosidase hydrolysis

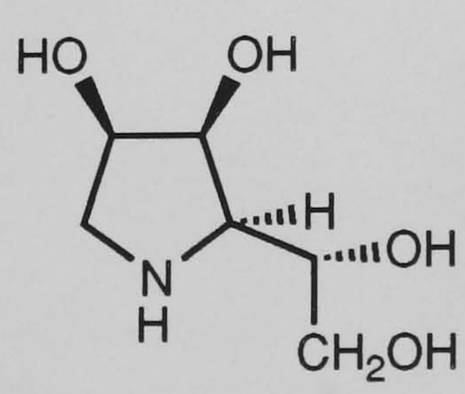
Being inhibitors of a range of glycosidases from a variety of sources, the many plant alkaloids are useful tools for exploring the active site(s) of this class of enzyme. Furthermore, to gain more information on the structure/activity relationship of various compounds, a model for the inhibition mechanism is required[21]. At this moment though, no one theory can satisfactorily explain the range of data available, but this can be overcome by increasing the volume of information for a given enzyme and its inhibitors.

On examination, many inhibitors are active against those enzymes that hydrolyse a substrate of similar size and stereochemistry. Thus, 1-deoxynojirimycin, related to glucose, inhibits the activity of several glucosidases[11]. Likewise, castanospermine, which can be thought of as an N-alkylated deoxynojirimycin, inhibits various glucosidases as well[25]. However, swainsonine, which is a potent inhibitor of mannosidase enzymes, bears no resemblance to the pyranose structure of mannose, although the pyrrolidine ring does contain aspects of the mannose stereochemistry. The synthetic pyrrolidine **(1)** also inhibits several mannosidases[26], suggesting that it is the five membered ring within the structure that is responsible for the activity of the inhibitor[27]. **fig 1.05**

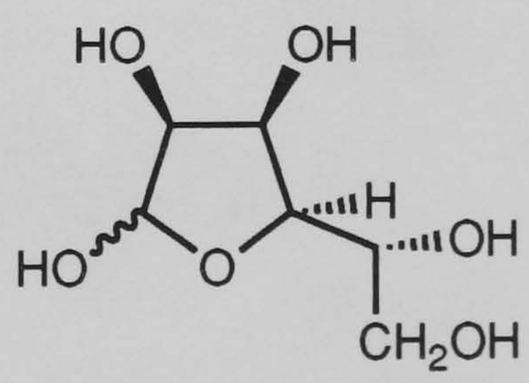
Figure 1.05



swainsonine



(1)

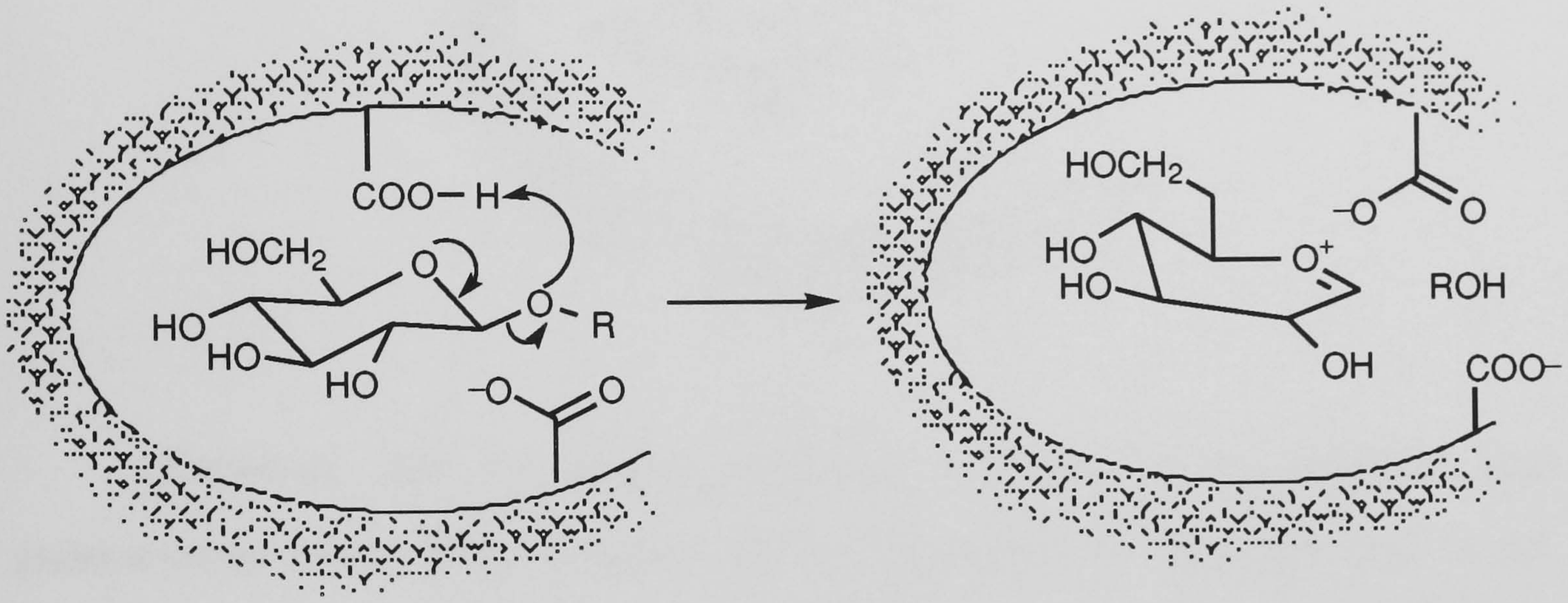


mannose
(furanose form)

The inhibition by these alkaloids is competitive and reversible and it

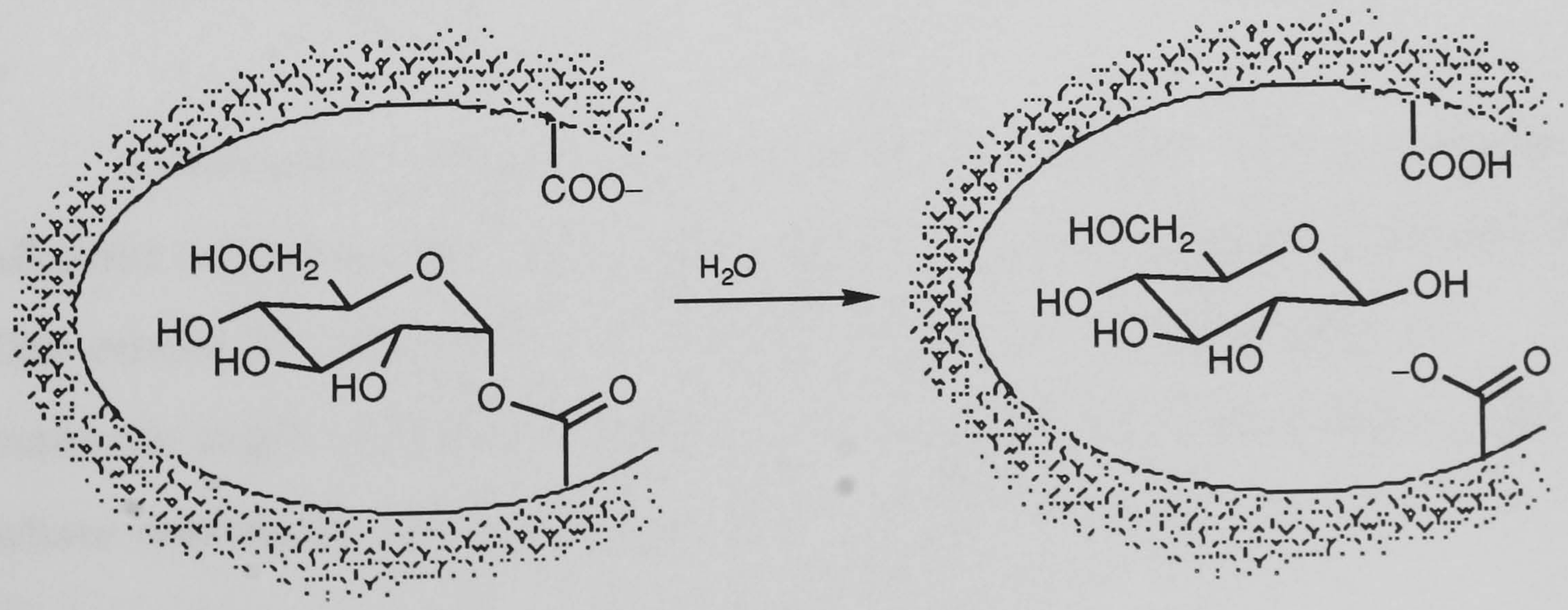
has been assumed that a glycopyranosyl cation is involved by protonation of the basic amine group (pKa~6)[28]. The natural substrate of the enzyme is likewise protonated at the glycosidic oxygen by what has been suggested to be an aspartate residue within the active site[29,30], **fig 1.06**

Figure 1.06



A half-chair oxonium ion species is then formed (possibly transient or covalently linked to another carboxylate group) which can then collapse on hydration. The stereochemistry at the anomeric carbon is retained at the end of this process, **fig 1.07.**

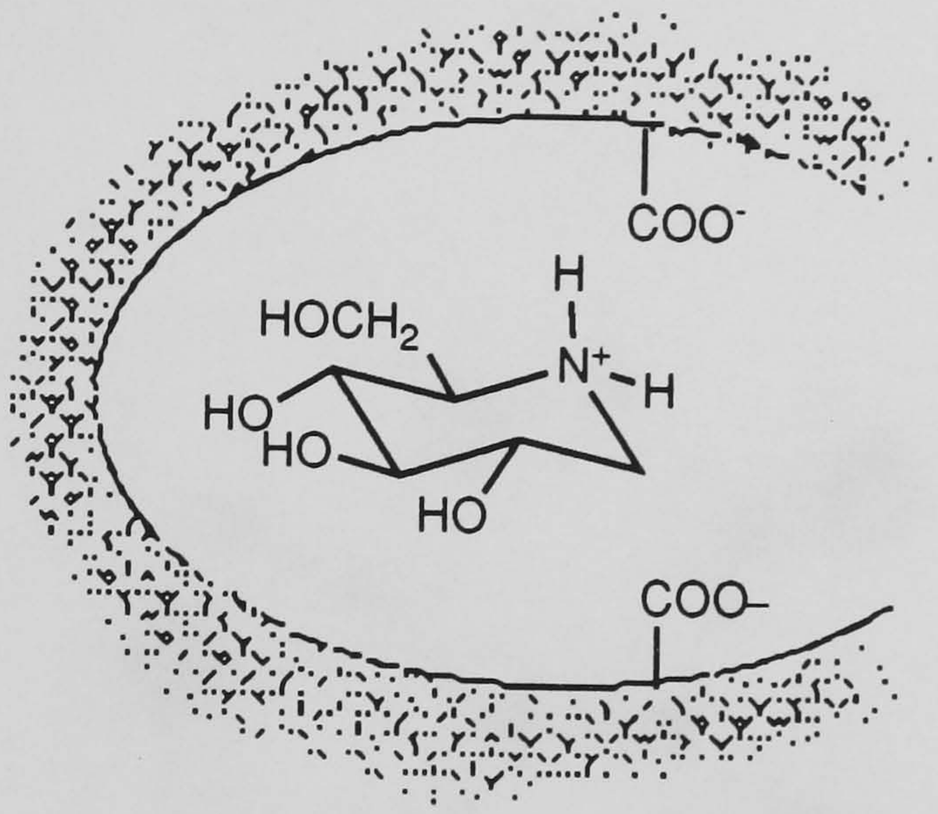
Figure 1.07



The protonated inhibitor is assumed to mimic this transient half-chair

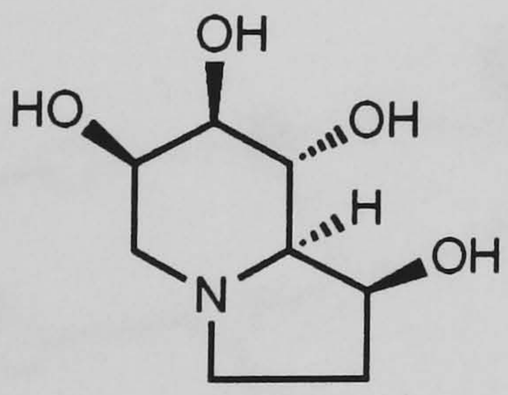
oxonium ion species, **fig 1.08**.

Figure 1.08

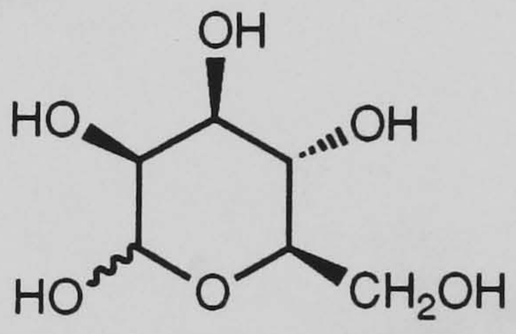


However, this hypothesis does not circumvent the problem that swainsonine bears little resemblance to mannose, or the fact that 6-*epi*-castanospermine resembles mannopyranose, yet is a poor inhibitor of mannosidases[31], **fig 1.09**

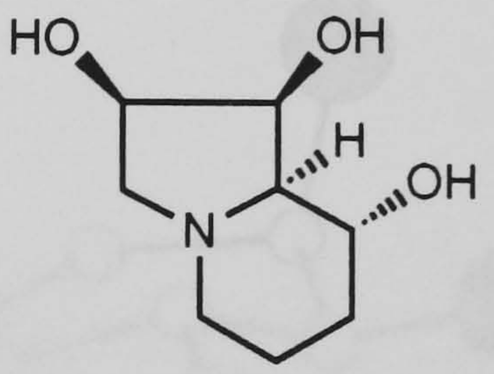
Figure 1.09



6-*epi*- castanospermine



mannose

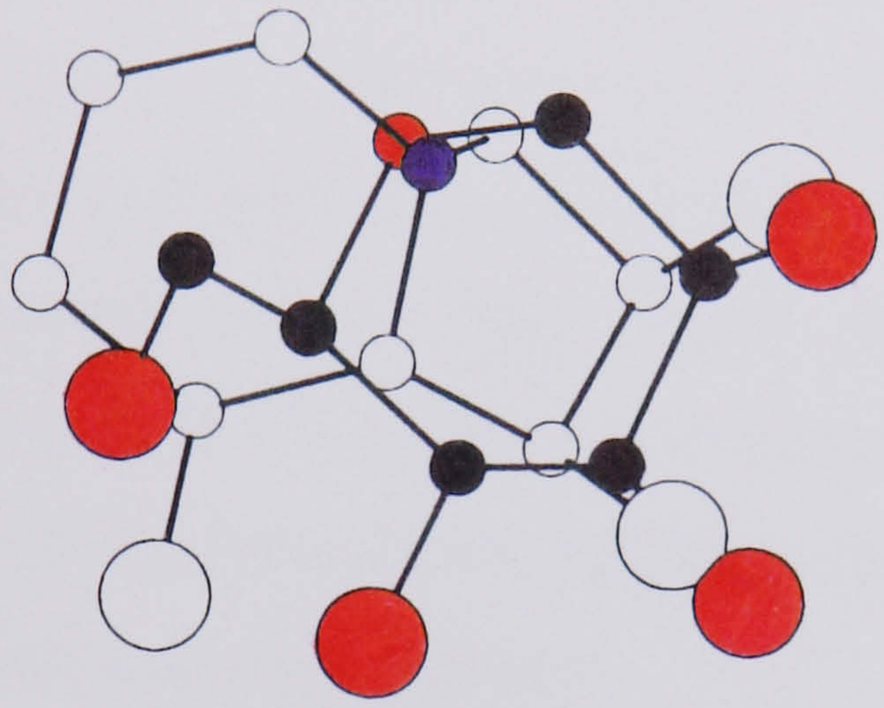


swainsonine

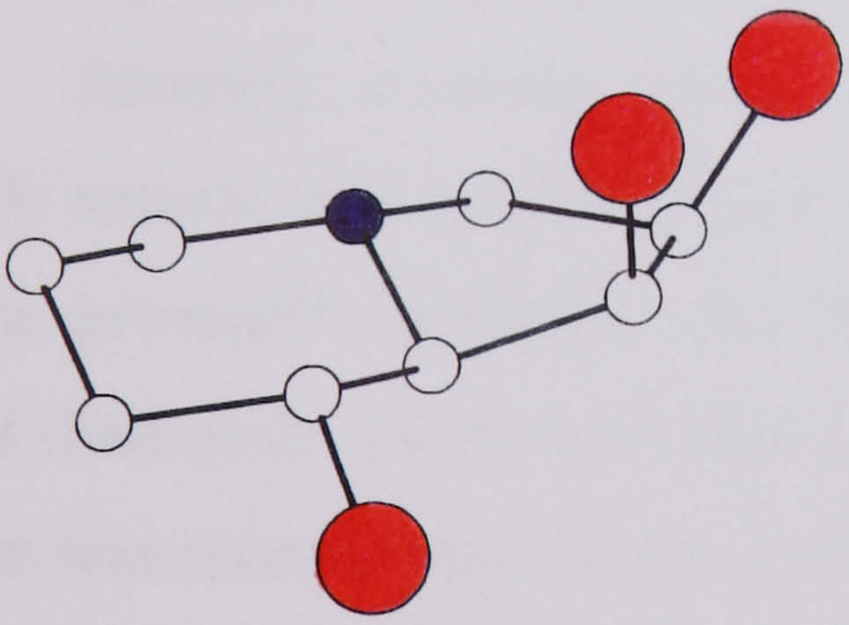
Literature modelling studies have been undertaken for a number of alkaloid inhibitors and their relationship to the mannopyranosyl cation[32,33]. Our results for computer aided minimisation of swainsonine and the mannose cation using the CHARMm program are presented in **figure 1.10,** where reasonable overlap between the two was acheived.

Figure 1.10

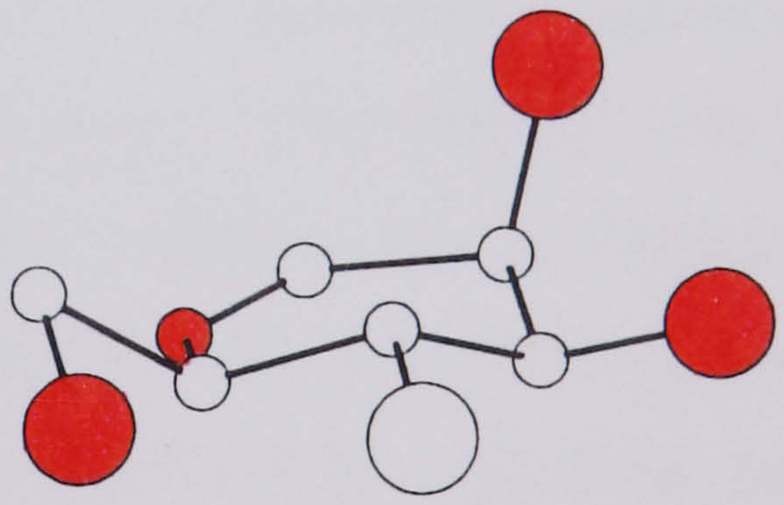
Two views of the minimised structures for swainsonine and the mannopyranosyl cation.



superimposition of swainsonine and mannose cation



swainsonine cation



mannopyranosyl cation

SCALE : 10.00 mm/A

If the ring heteroatoms in each molecule are superimposed, the C2 and C3 hydroxyl groups of the mannose cation line up with the two hydroxyl groups in the 5-membered ring of swainsonine. In previous work, the C6 hydroxyl of the mannose cation had also shown good overlap with the 6-membered ring

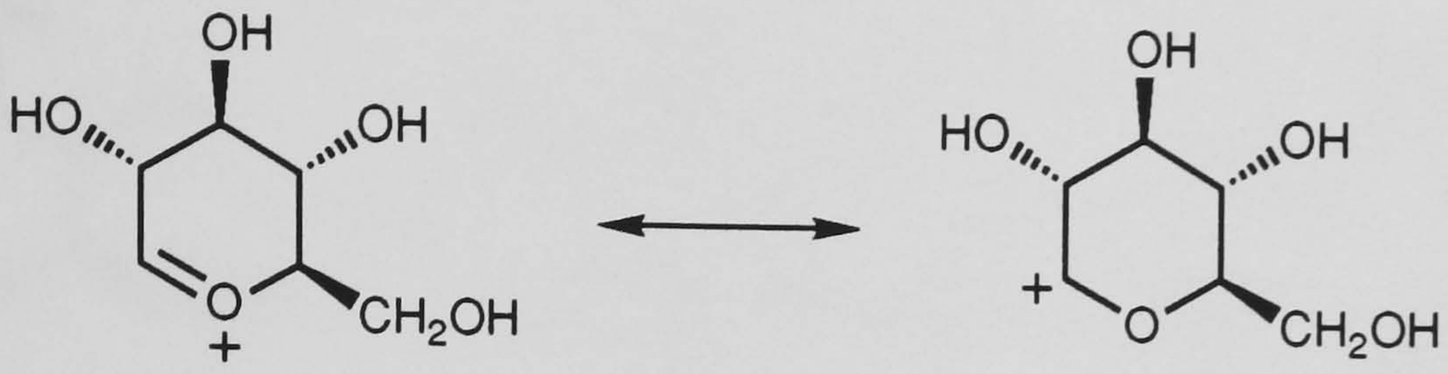
hydroxyl group of swainsonine. In our studies, however, they were not particularly close. The literature results also suggested that the C4 hydroxyl group of the mannose cation did not overlap with any atoms in swainsonine and this was confirmed in our studies as well.

It is interesting to note that previous computer aided minimisation of the mannose cation structure had resulted in a "flap up" half chair conformation. In this case, two of the C2, C3 and C4 hydroxyl groups are axial. Our minimisation resulted in the "flap down" conformation for the mannose cation, with one of the C2, C3 and C4 hydroxyl groups being axial. In either case, the C2 and C3 hydroxyl groups cannot be aligned exactly to those found in the 5-membered ring of swainsonine.

1-deoxymannojirimycin can be aligned via the hydroxyl groups with the mannopyranosyl cation, but in this case the ring heteroatoms do not lie in the same region of space. This mismatch may account for the lower activity of 1-deoxymannojirimycin compared to that of swainsonine. Likewise, 6-*epi*-castanospermine shows poor superimposition and poor inhibition.

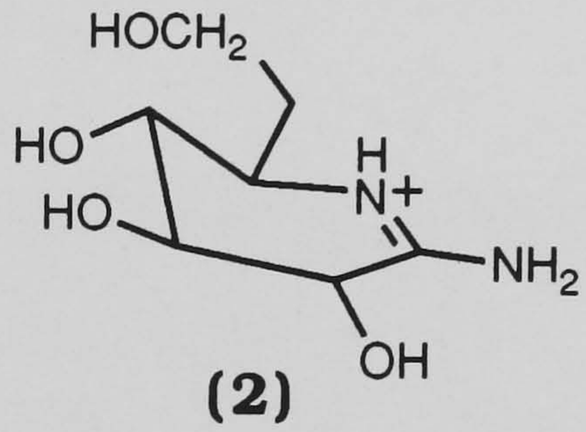
Recently, a similar study was undertaken on glucosidase inhibitors (both natural and synthetic) and similar conclusions were reached with an extra proviso[34]. Inhibitors could bind in either substrate-like or transition state (half-chair) forms and inhibition was noted for both types, although the latter was more potent. Hence, 1-deoxynojirimycin in a protonated form does not show close stereochemical similarity to glucopyranosyl cation and the positive charge on the nitrogen is not spread over the carbon and oxygen atoms as it is for the natural substrate (see **fig, 1.11**). Yet, 1-deoxynojirimycin is a potent inhibitor of α- and β-glucosidases (K_i =1.8 x 10^{-5} M for sweet almond β-glucosidase), almost as potent as the amidine,**(2)**, (K_i=8.0 x 10^{-6} M, same enzyme)[34],**fig, 1.12.**

Figure 1.11.



The amidine in the half-chair conformation has a similar charge distribution to the glucopyranosyl cation and the two structures can be superimposed very well[34].

Figure 1.12.

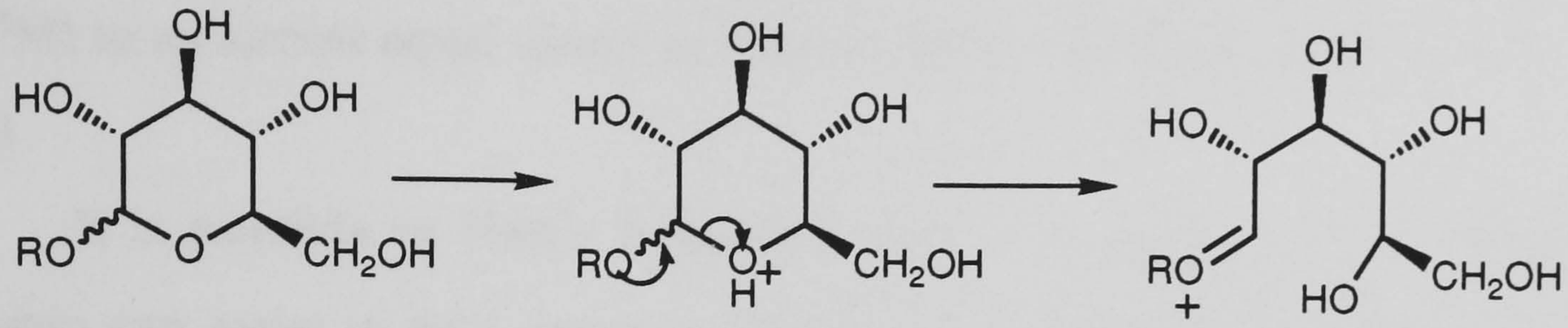


(2)

In a case like this the stereochemistry of the hydroxyl groups do not seem to play such an important role as the electrostatic interaction between the positive charge and the carboxylate group in the active site. For instance, **(2)** is as active against jackbean α-mannosidase and bovine β-galactosidase as against β-glucosidase[35]. The orientation of the hydroxyl groups is important for the recognition and binding of inhibitors which are more chair-like in character (1-deoxynojirimycin is only a weak inhibitor of jackbean α-mannosidase and shows no inhibition of β-galactosidase).[34]

Before this molecular modelling work had been published, Fleet proposed an alternative explanation that even now cannot be ruled out[26]. Instead of protonation occurring at the glycosidic oxygen, he suggested the oxygen heteroatom was protonated which then underwent cleavage to give an acyclic oxonium species, **scheme 1.01**

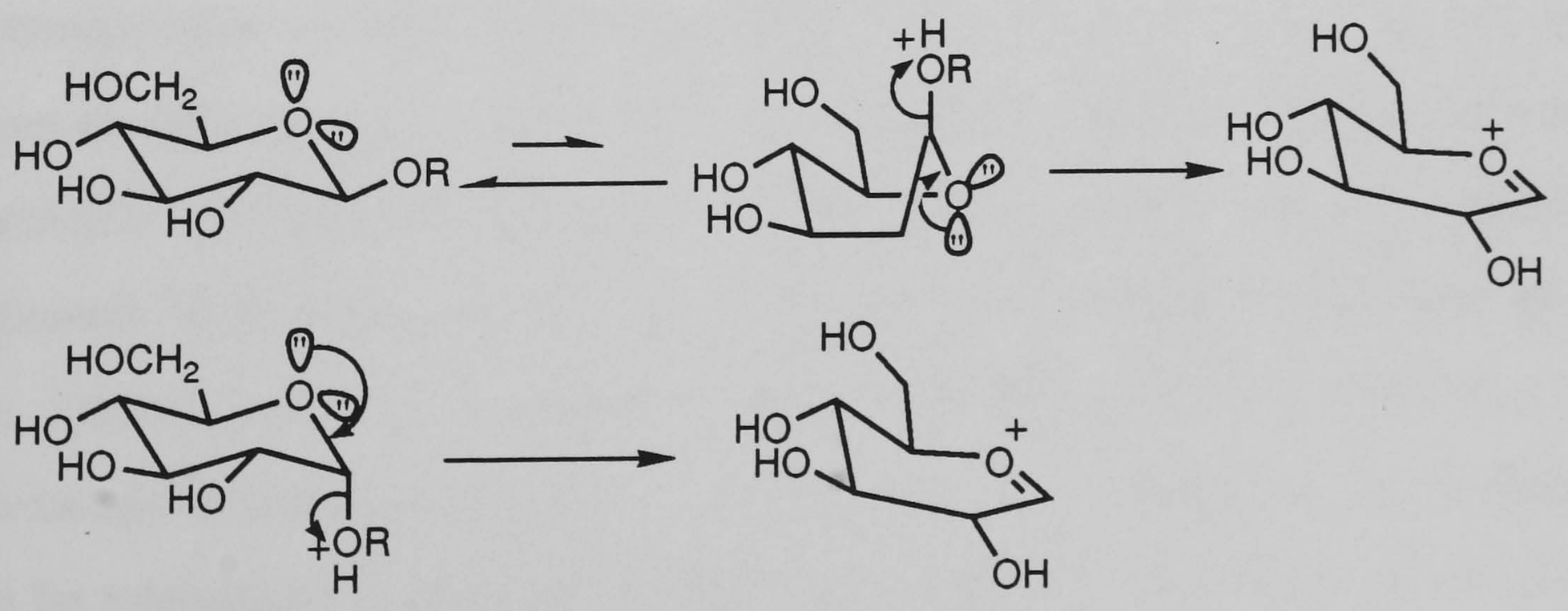
Scheme 1.01



This acyclic species could then be attacked by water to complete the hydrolysis. Protonated amine inhibitors would thus assume more of the shape of the intermediate protonated glucoside. It is possible that in some enzyme systems one process occurs instead of the other, or that the two are in competition.

Part of the problem with a glucopyranosyl cation is that the precise nature of the intermediate should be different for α- and β-glucosides. α–Glucosides should hydrolyse via the ground state chair conformation because the lone pair on the acetal oxygen, being anti-periplanar to the departing aglycon unit, can assist in oxonium ion formation. A β-glucoside would need to assume a boat conformation in order to fulfil this stereoelectronic requirement[36], **scheme 1.02**

Scheme 1.02

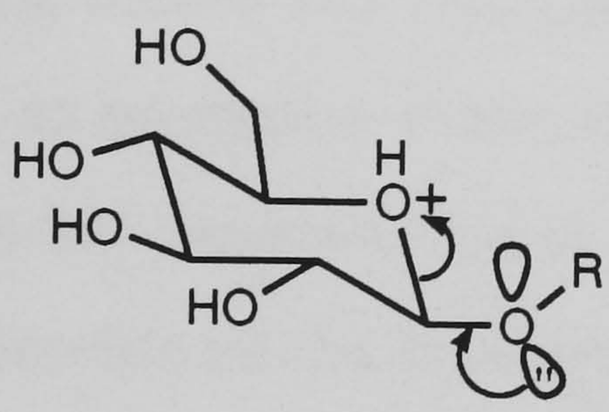


The two processes show different activation energies in model systems

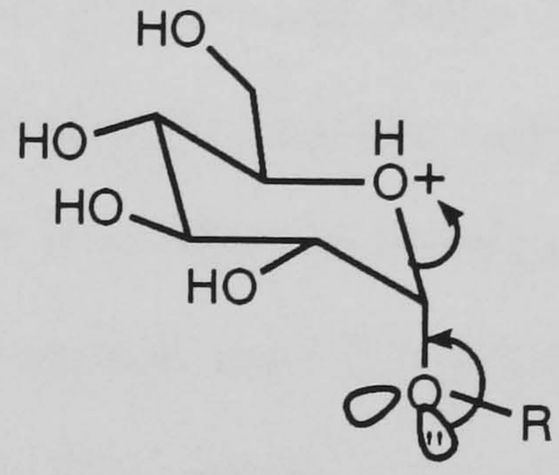
and presumably the same applies to enzymatic hydrolyses. 1-Deoxynojirimycin, however, inhibits sweet almond β-glucosidase (K_i = 1.8 x 10^{-5}M) to an almost equal extent as brewers yeast α-glucosidase (K_i = 8.7 x 10^{-6}M).

It is possible in Fleet's hypothesis, that a lone pair on the anomeric oxygen can assist in ring cleavage if both α-and β-glucosides are in a chair conformation, **fig, 1.13.**

Figure 1.13



β-glucoside



α-glucoside

Chemical syntheses of plant alkaloids

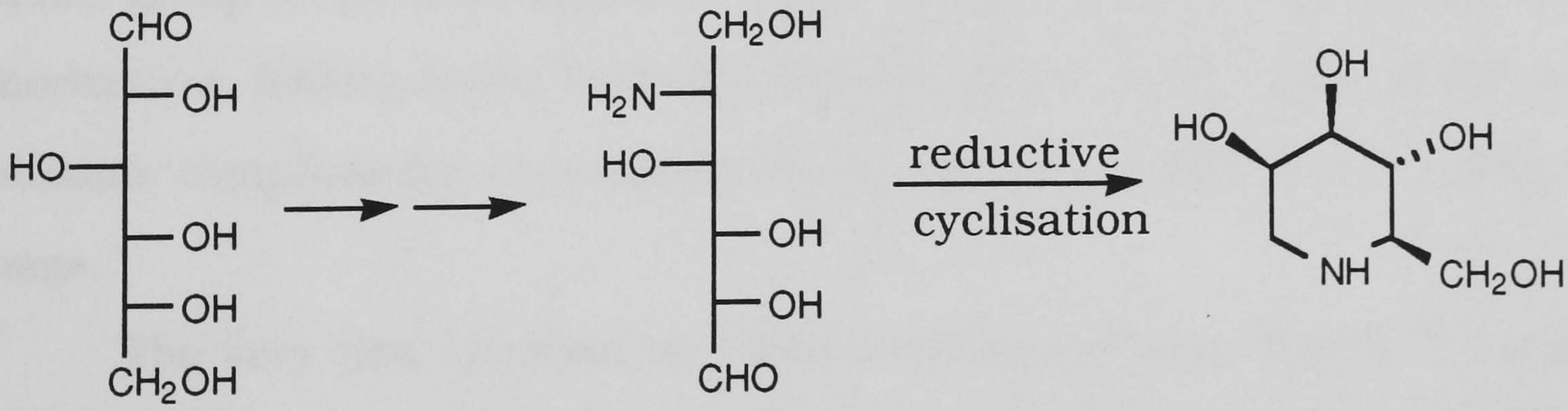
Since the first chemical synthesis of nojirimycin and its 1-deoxy analogue in 1968[2], there has been great interest in synthesising these and other alkaloids over the last ten years. It is interesting to examine the development in synthetic methodology over this period to the stage today where very efficient, short routes to 1-deoxynojirimycin are known. Retrosynthetic analysis of the construction of the piperidine ring can give clues as to possible biosynthetic routes, although often the biosynthesis of a particular compound can help in chemical synthesis via a biomimetic approach. A favoured strategy, and perhaps the most sensible, has been to use the "chiral pool" of available sugar derivatives[37,38,39,40]. This has the advantage of being cheap, and most importantly, a number of chiral centres can be introduced without resorting to specialist reactions. This is particularly applicable to the plant alkaloids, though, which have close similiarities to a

number of natural sugars. Other, non-carbohydrate strategies have also been employed, most recently the synthesis of nojirimycin and 1-deoxynojirimycin starting from diethyl tartrate[41].

Perhaps the biggest contribution to the synthesis of plant alkaloids and their analogues has been from the research group of Fleet[40,37,42]. His strategies illustrate the choices available and the flexibility which can be gained from using carbohydrate precursors.

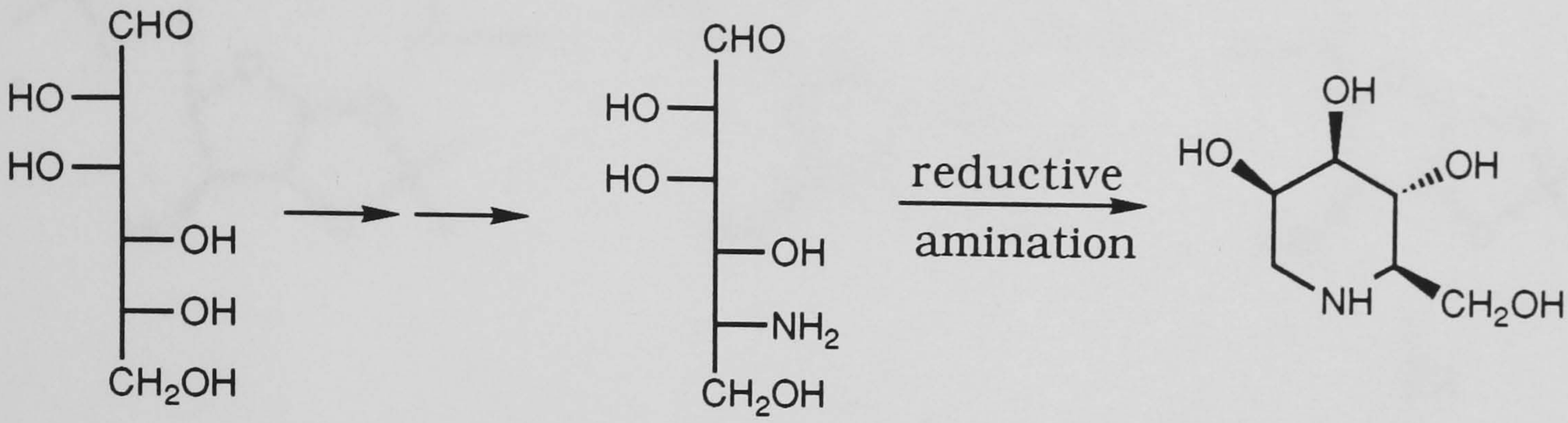
Essentially, two approaches are adopted for producing a piperidine ring from a starting hexose and examples will be given from both these classes[42]. For example, to synthesise 1-deoxymannojirimycin, glucose could be cyclised via a nitrogen link between C2 and C6 (with inversion of configuration at C2). This would provide all the necessary chiral centres and the synthetic problem then becomes one of protecting the sugar in the correct way to introduce the nitrogen functionality at C2 whilst also allowing oxidation at C6. By oxidising C6 to an aldehyde and using a reductive amination technique the 1-deoxy derivative would be produced,**scheme 1.03.**

Scheme 1.03.



Alternatively, mannose could be the starting hexose, but this time cyclisation via the nitrogen functionality would need to be via C1 and C5, **scheme 1.04.**

Scheme 1.04.

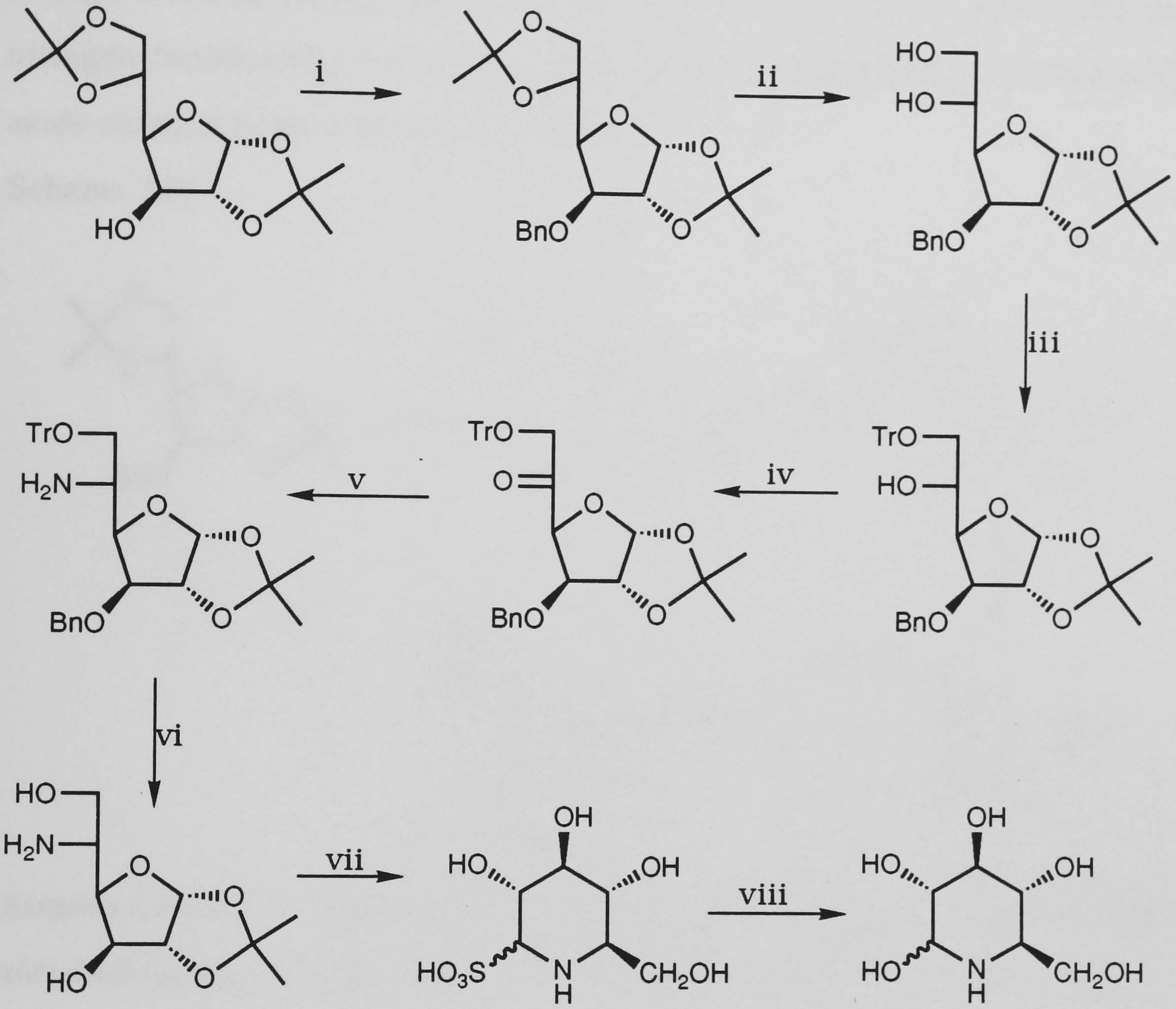


This time the nitrogen atom needs to be introduced with retention of configuration at C5. In either case, amination could be at one or other carbon centre, the only difference being that a different intermediate is involved in producing the 1-deoxy compound.

At first glance, the C2/C6 route as shown above seems complicated synthetically, i.e. the C6 carbon needs to be oxidised to the aldehyde and the C1 carbon needs to reduced to the primary alcohol. In practice, these complications are not apparent because the anomeric position is often in the form of an acetal and therefore protected for many synthetic steps. Furthermore, a reductive amination has been illustrated here, but equally the amine group on C2 could displace a good leaving group on C6 via an S_N2 type mechanism, leading to the 1-deoxy compound directly. This would avoid any possible complications in oxidising the C6 primary alcohol to the aldehyde stage.

The very first synthesis of 1-deoxynojirimycin[2] used the C1/C5 route with reductive amination, as below. Diacetone-D-glucose served as the chiral hexose, which has the advantage of being cheap whilst also being partially protected with the acetonide moiety. Protecting groups obviously have the purpose of allowing a particular functional group to be manipulated, but in this and subsequent examples, they have the additional benefit of making water soluble precursors soluble in organic solvents.

The first chemical synthesis of nojirimycin.

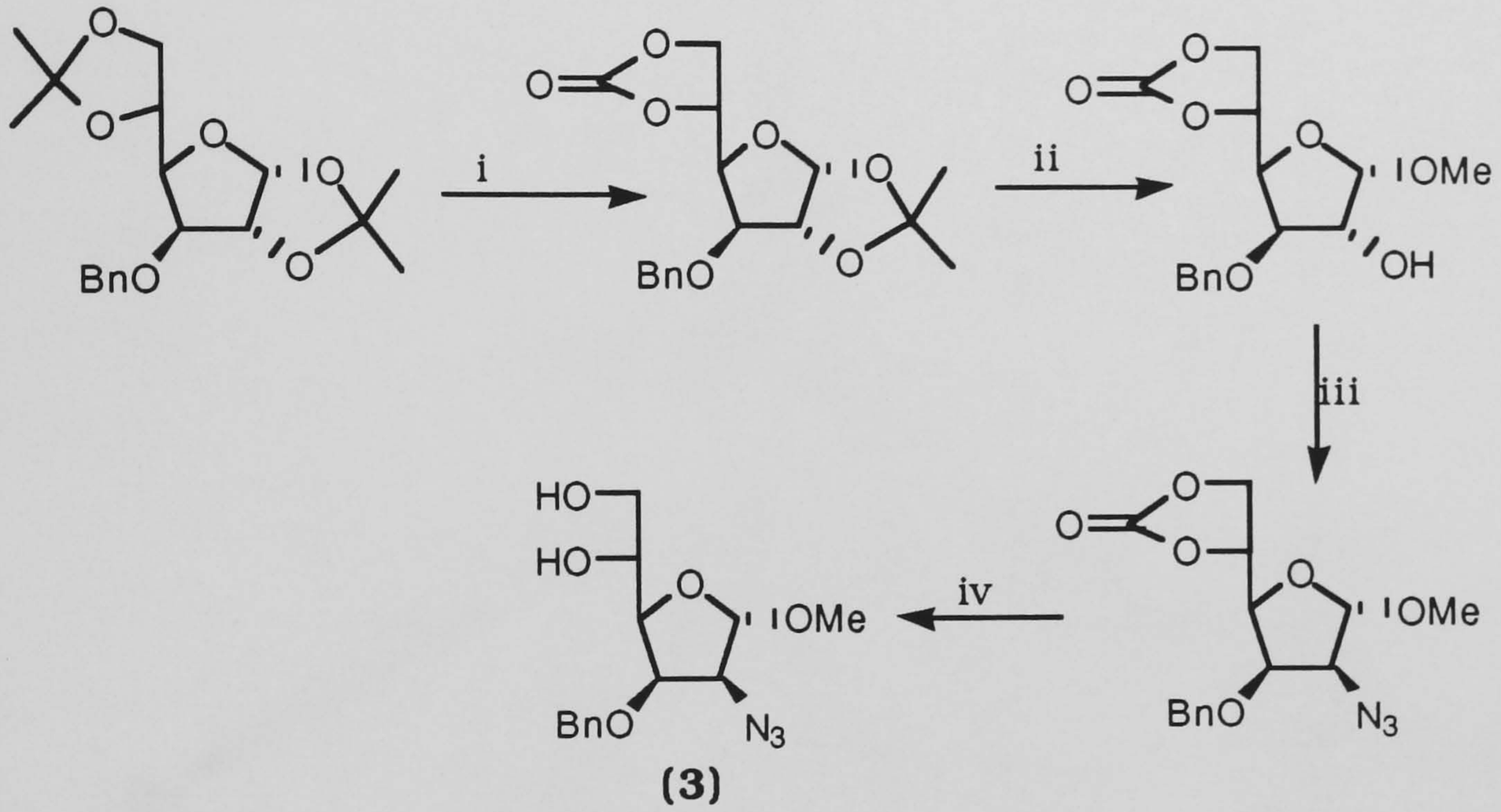


Reagents **i.** NaH/benzyl chloride **ii.** AcOH/H_2O, 40°C **iii.** TrCl/pyridine **iv.** Ac_2O/DMSO **v.** Raney Ni/MeOH/$NH_{3(g)}$ **vi.** Li/liquid NH_3/THF **vii.** gaseous SO_2/H_2O **viii.** Dowex 1X8(OH^-)

So nojirimycin was synthesised in eight steps. The 1-deoxy derivative can be made in one step from nojirimycin using a reductive amination reaction (Pt/H_2 in AcOH/H_2O). However, many sequences in this synthesis are protection/deprotection steps to manipulate the required functional groups and furthermore step **v** suffers from the disadvantage that diastereoisomers are produced. It would be an advantage to have a common intermediate from which several alkaloids could be made and also a synthesis that avoided complicated separations. To this end Fleet used the intermediate **(3)** to make many different alkaloids by simply modifying various steps[37,40]. In

contrast to the previous example, C2/C6 cyclisation with inversion of configuration at C2 proved to be the most profitable route, often with the nitrogen functionality being introduced by S_N2 displacement of triflate with azide anion, **scheme 1.05 and scheme 1.06.**

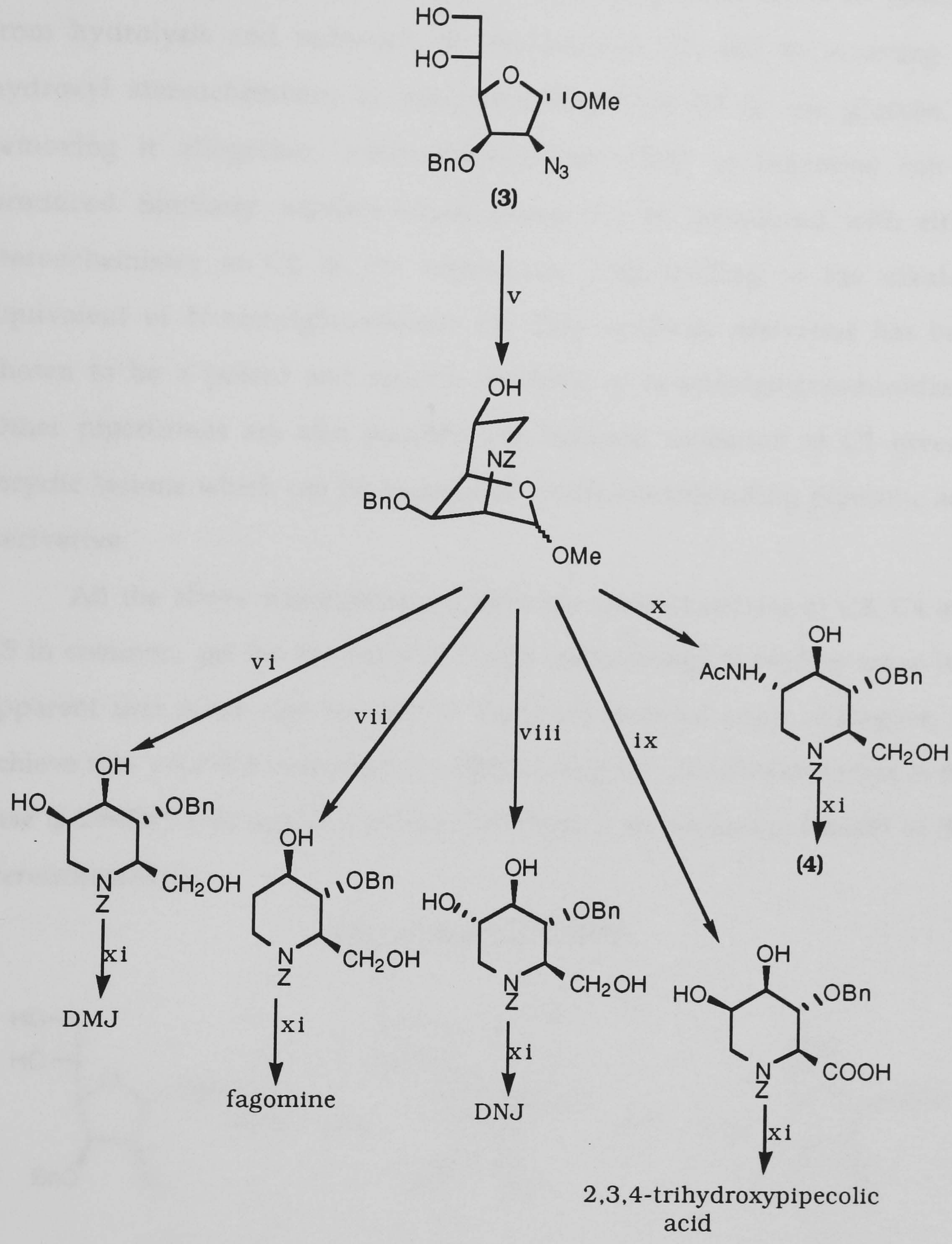
Scheme 1.05



(3)

Reagents i. 0.5% HCl/MeOH, $(MeO)_2CO$/NaOMe **ii.** Dowex 50-X8 (H^+)/MeOH **iii.** Triflic anhydride/pyridine/CH_2Cl_2, NaN_3/DMF **iv.** MeOH/NaOMe(trace)

Scheme 1.06.

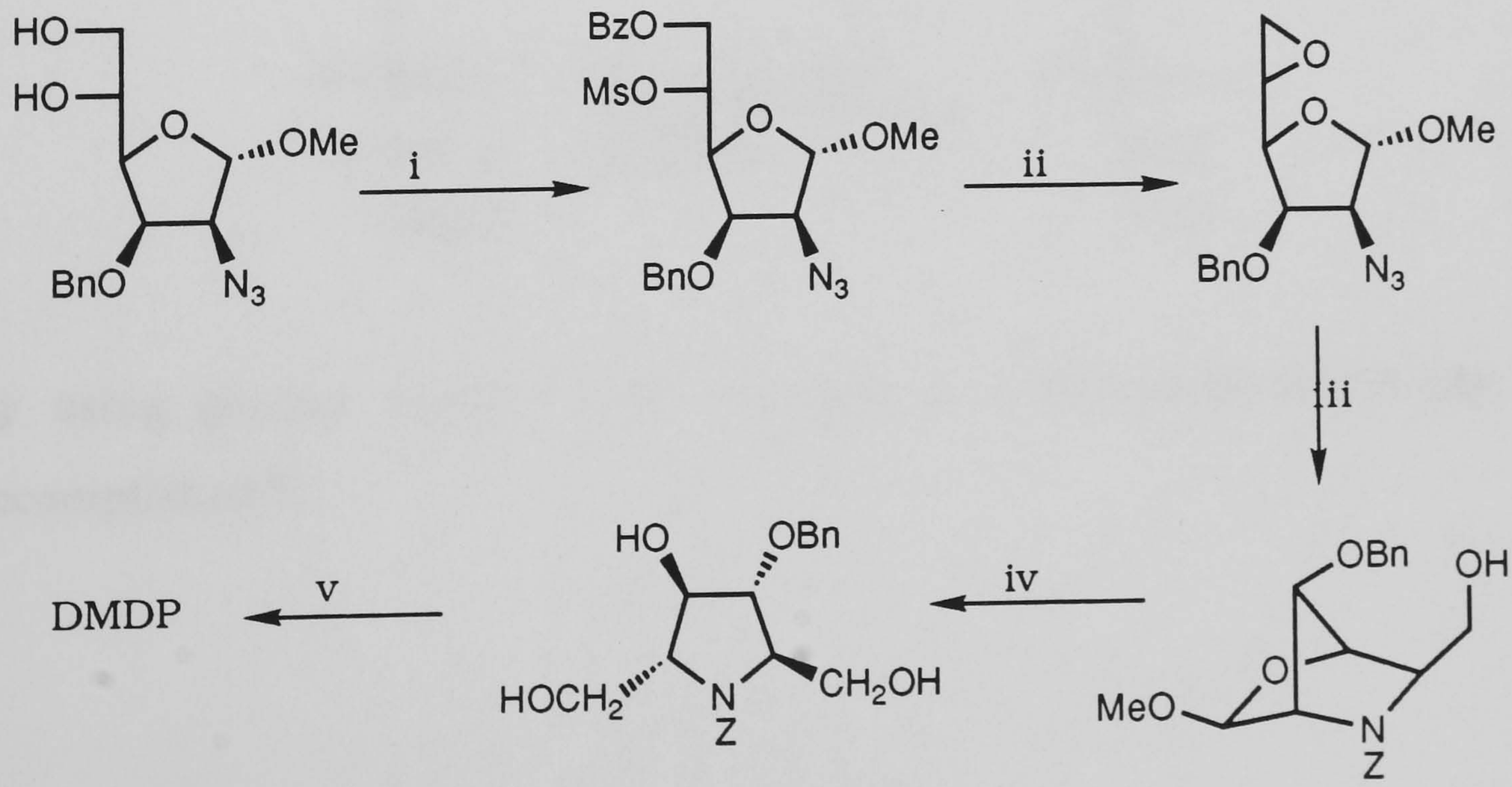


Reagents v. TsCl/pyridine, Pd on C/H_2/EtOH, $PhCH_2OCOCl$/ether/$NaHCO_{3(aq)}$ **vi** triflic acid/H_2O, $NaBH_4$/EtOH **vii.** triflic anhydride/pyridine, $LiBHEt_3$/THF **viii.** $Py_2Cr_2O_7$/CH_2Cl_2, $NaBH_4$/EtOH, triflic acid/H_2O then $NaBH_4$ **ix.** triflic acid/H_2O, $HOBr_{(aq.)}$ **x.** triflic anhydride/NaN_3/DMF, NaHTe/Ac_2O, triflic acid/H_2O then $NaBH_4$ **xi.** Pd on C/EtOH/H_2.

1-Deoxymannojirimycin (DMJ) is the obvious alkaloid to be obtained from hydrolysis and reduction of intermediate **(3)**, but by inverting the hydroxyl stereochemistry at what was originally C5 in the glucose, or removing it altogether, 1-deoxynojirimycin (DNJ) or fagomine can be produced. Similarly, another amino group can be introduced with either stereochemistry at C2 in the nojirimycin ring leading to the alkaloid equivalent of N-acetylglucosamine **(4)**. This synthetic derivative has been shown to be a potent and specific inhibitor of N-acetylglucosaminidases. Other piperidines are also possible, for instance, oxidation at C1 gives a bicyclic lactone which can be hydrolysed to the corresponding pipecolic acid derivative.

All the above transformations have the stereochemistry at C3, C4 and C5 in common, yet the flexibility of Fleet's methodology is evident when it is apparent that it can also be used to construct furanose sugar analogues. To achieve this, a C2/C5 cyclisation is required (step iii). An obvious target in this case is DMDP, although the synthetic strategy is by no means limited to this stereochemistry.

The synthesis of DMDP.



Reagents i. PhCOCl/pyridine, MsCl/pyridine **ii.** NaOMe/DMF **iii.** Pd on C/H_2/EtOH,

$PhCH_2OCOCl$/ether/$NaHCO_3$ **iv.** triflic acid/H_2O, $NaBH_4$/EtOH/H_2O **v.** $Pd(OH)_2/H_2$/EtOH

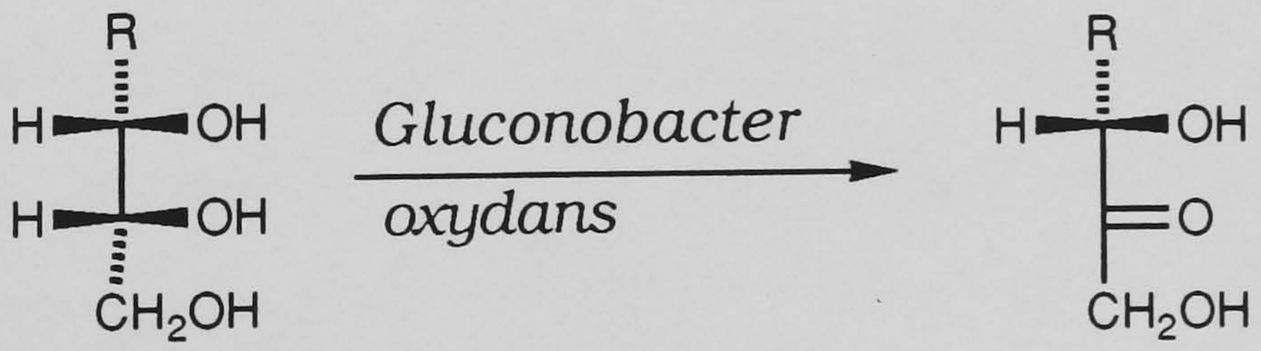
To reach DMDP, the stereochemistry at C5 needs to be retained so steps i-iii are concerned with introducing an epoxide function of opposite configuration so that in the cyclisation, a double inversion would have effectively taken place at C5.

Enzymatic syntheses

Recently, synthetic strategies have emerged whereby whole cells or isolated enzymes have been used to oxidise selectively a particular hydroxyl group, or couple together two simpler units to give the sugar derivative[43,44,38,45]. Despite being versatile, Fleet's methodology still requires significant protection/deprotection steps, yet using enzyme systems, this is kept to a minimum.

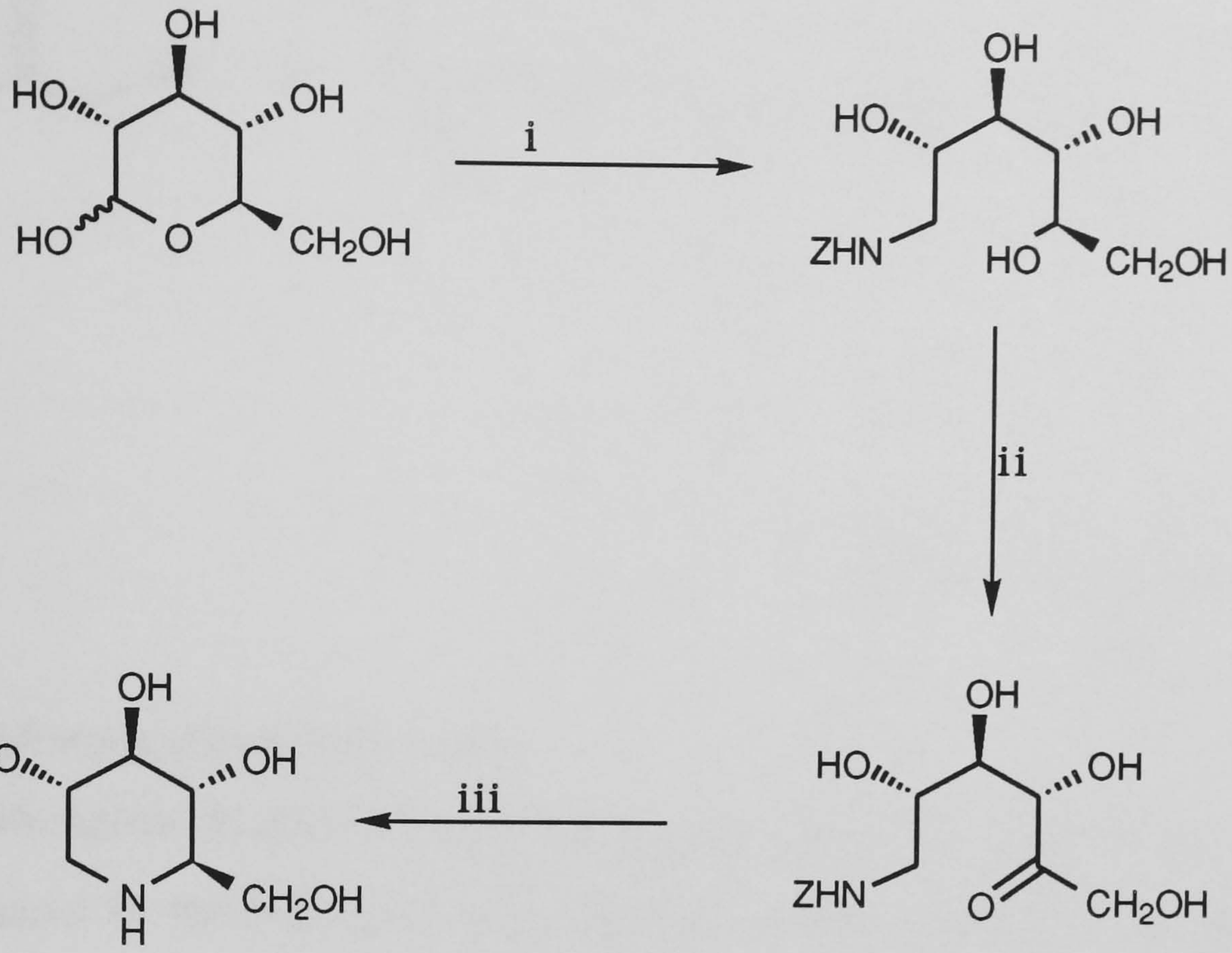
A well exploited transformation is the oxidation of the central hydroxyl group in D-*erthyro* configurated compounds by bacteria of the genus *Gluconobacter* , **scheme 1.07.**

Scheme 1.07.



By using glucose itself, a short synthesis of 1-deoxynojirimycin can be accomplished[38].

Enzymatic synthesis of 1-deoxynojirimycin.



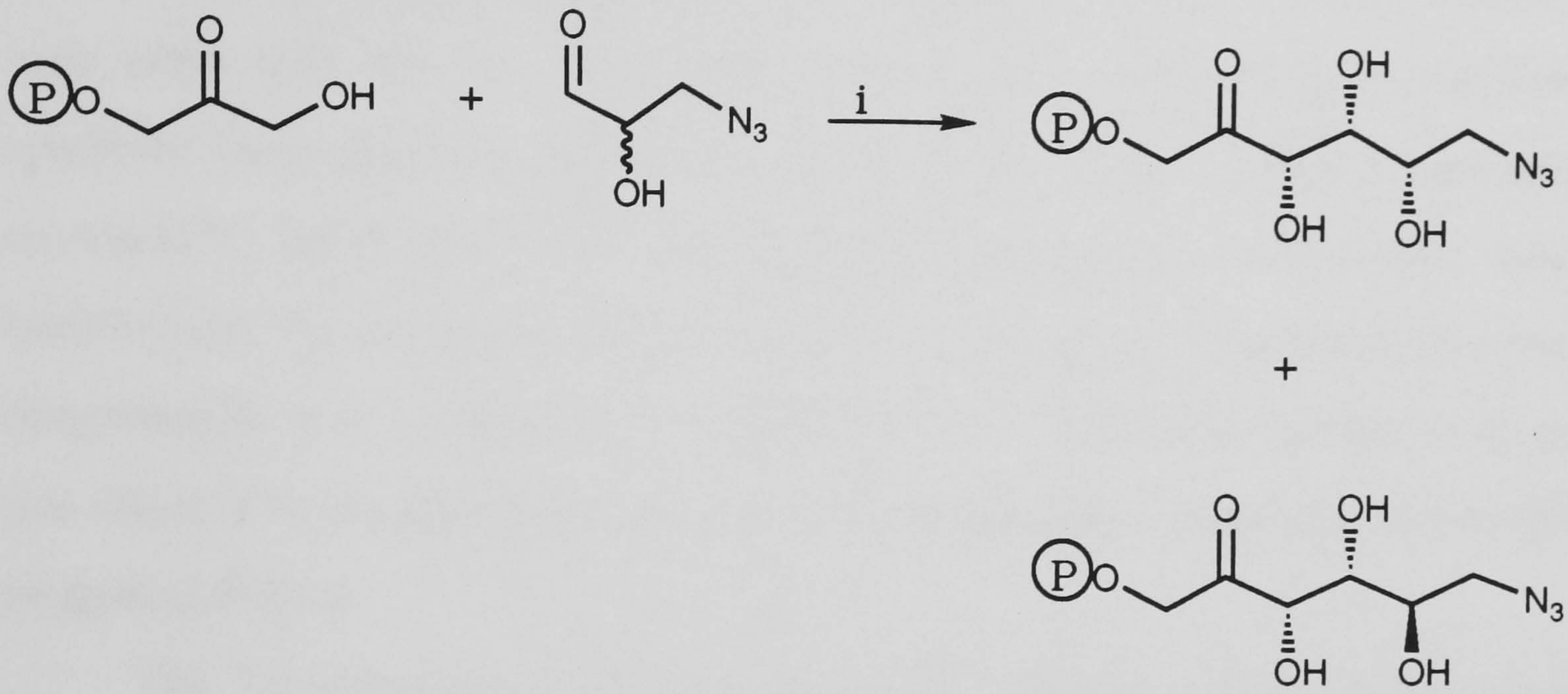
Reagents i. Pd on C/H_2/EtOH/NH_3 then $PhCH_2OCOCl$ **ii.** *Gluconobacter oxydans* **iii.** H_2/Pd on C/EtOH

(no 1,5-dideoxy-1,5-imino-L-iditol was observed in step **iii**)

Following reductive amination of the glucopyranose ring, a C1/C5 cyclisation is effected after regioselective oxidation of the C5 hydroxyl group.

A different approach, and one which has produced many synthetic derivatives of 1-deoxynojirimycin, uses an aldolase enzyme. Previous work had shown that fructose-1,6-diphosphate aldolase was very specific for dihydroxyacetone phosphate, but a wide variety of aldehydes were accepted[44,45] **scheme 1.08.**

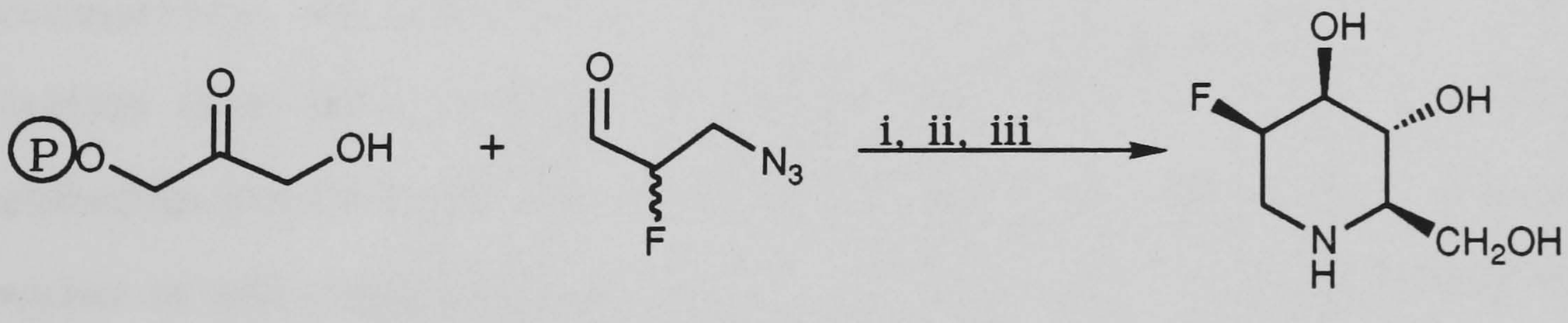
Scheme 1.08.



Reagents i) fructose diphosphate aldolase

Both azide diastereoisomers resulting from the aldol reaction above were reduced to the corresponding piperidine after removal of the phosphate group by an phosphatase. 1-Deoxynojirimycin and 1-deoxymannojirimycin were obtained in a ratio of about 1: 4, and these could be separated by ion exchange chromatography. The elegance of this synthesis is its simplicity and the only limit is the number of aldehydes that can be accepted by the aldolase enzyme. For instance, fluorinated azido-aldehyde gave the corresponding fluorinated 1-deoxymannojirimycin exclusively[34], **scheme 1.09.**

Scheme 1.09.



Reagents i. Fructose diphosphate aldolase **ii.** phosphatase **iii.** H_2/Pd on C/EtOH.

Biosynthetic considerations

Although a wide range of secondary metabolites have been discovered, very often they are derived from a few simple units which themselves originate from primary metabolic pathways essential for the organism's survival[46]. Such pathways might include glycolysis and amino acid metabolism, for instance. Before looking in detail at 1-deoxynojirimycin biosynthesis, it is worthwhile to examine why an organism would produce this alkaloid in the first place and any possible selection advantage that might be gained from it.

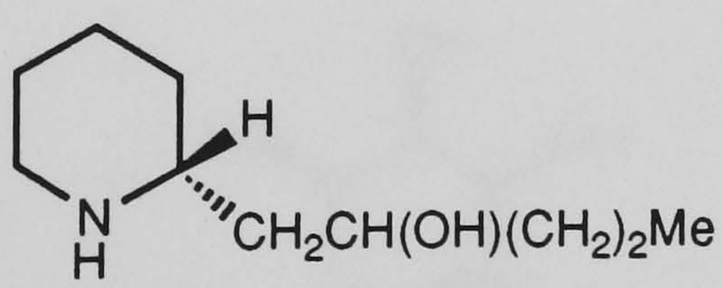
The biosynthesis of 1-deoxynojirimycin follows a very typical time course in that the alkaloid is produced only after the initial growth and replication steps have occurred (see chapter 2). The most important criterion for any living system is to grow and then reproduce and this takes precedence over any other process. The fact that secondary metabolism often takes place when nutrient supplies are low and this normal growth pattern is disrupted, has led many authors to suggest that secondary metabolism is an overflow process[47,48,46]. Intermediates from primary metabolism increase and these can then be used as substrates for producing secondary metabolites. In this way, enzymes that normally catalyse reactions essential for survival can be used in secondary metabolism as a means to keep these enzymes functioning until circumstances are right again for renewed primary metabolism. Natural selection then takes over and organisms that produce "useful" secondary metabolites are favoured over those that synthesise redundant ones. The vast number of soil organisms that produce antibiotics and the many plants that make toxic or unpalatable metabolites must be seen as acquired rather than pre-designed systems.

Although nojirimycin exhibits antibiotic properties, glucosidase inhibition could provide a microorganism with a mechanism to inhibit other organisms' glucosidase enzymes. This might be important when nutrient

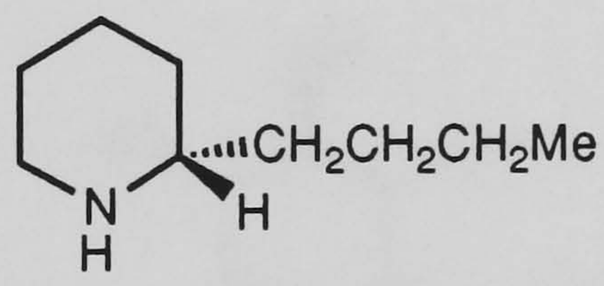
supplies were low, although the process relies on the producing microorganism being immune to its own alkaloid inhibitors. In addition, a plant could benefit from the insect anti-feedant properties of certain compounds, not to mention the inhibition of glycohydrolases essential to the digestive system of an insect. In the mulberry tree, 1-deoxynojirimycin is located in the roots[4] so either it is produced there or else translocated from another region and stored. It is in a place where no useful benefit is apparent, but is it coincidence that several *Streptomyces* species which produce this compound are also soil dwelling bacteria[4]? A possible link could be that originally one organism produced the alkaloid and then through infection, for instance, the other obtained the enzymes for its biosynthesis.

Usually, a good indication of the biosynthetic precursor(s) to a secondary metabolite can be gleaned from its structure, and also to a lesser extent, its source. There is a danger in this approach of assuming that compounds with similar structures are formed by similar metabolic pathways. Coniine, one of the hemlock alkaloids, has a similar structure to halosaline, yet the former is derived from acetate whilst the latter originates from lysine[49], **fig 1.14.**

Figure 1.14.



halosaline

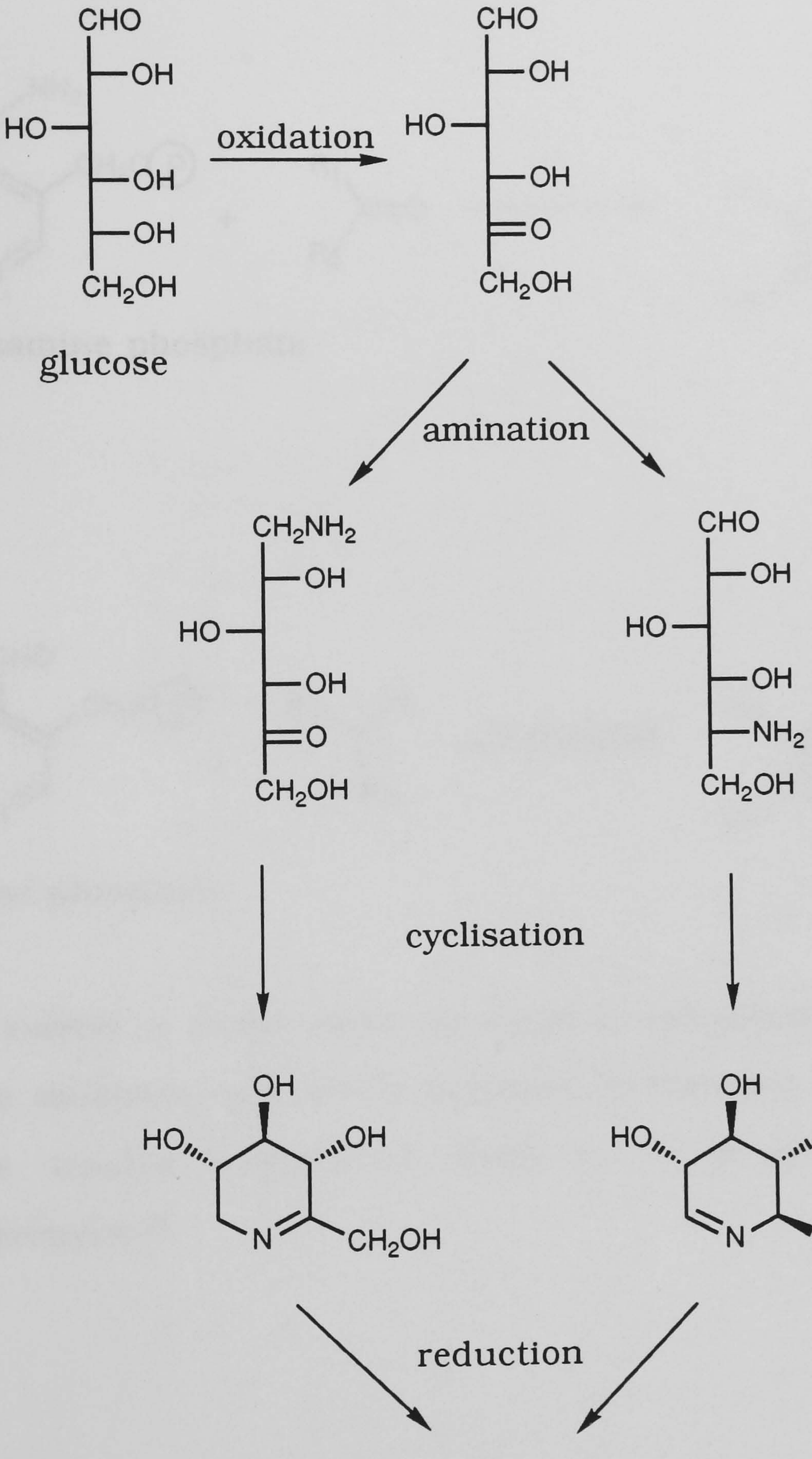


coniine

The extensive research into the chemical synthesis of 1-deoxynojirimycin suggests a carbohydrate as one of the main contenders for the biosynthetic source. Since glucose has all the required stereochemical centres, the simplest route might involve a C1/C5 cyclisation, yet as mentioned earlier, amination at either centre might then occur. The actual cyclisation mechanism might

also be different to the one shown here.

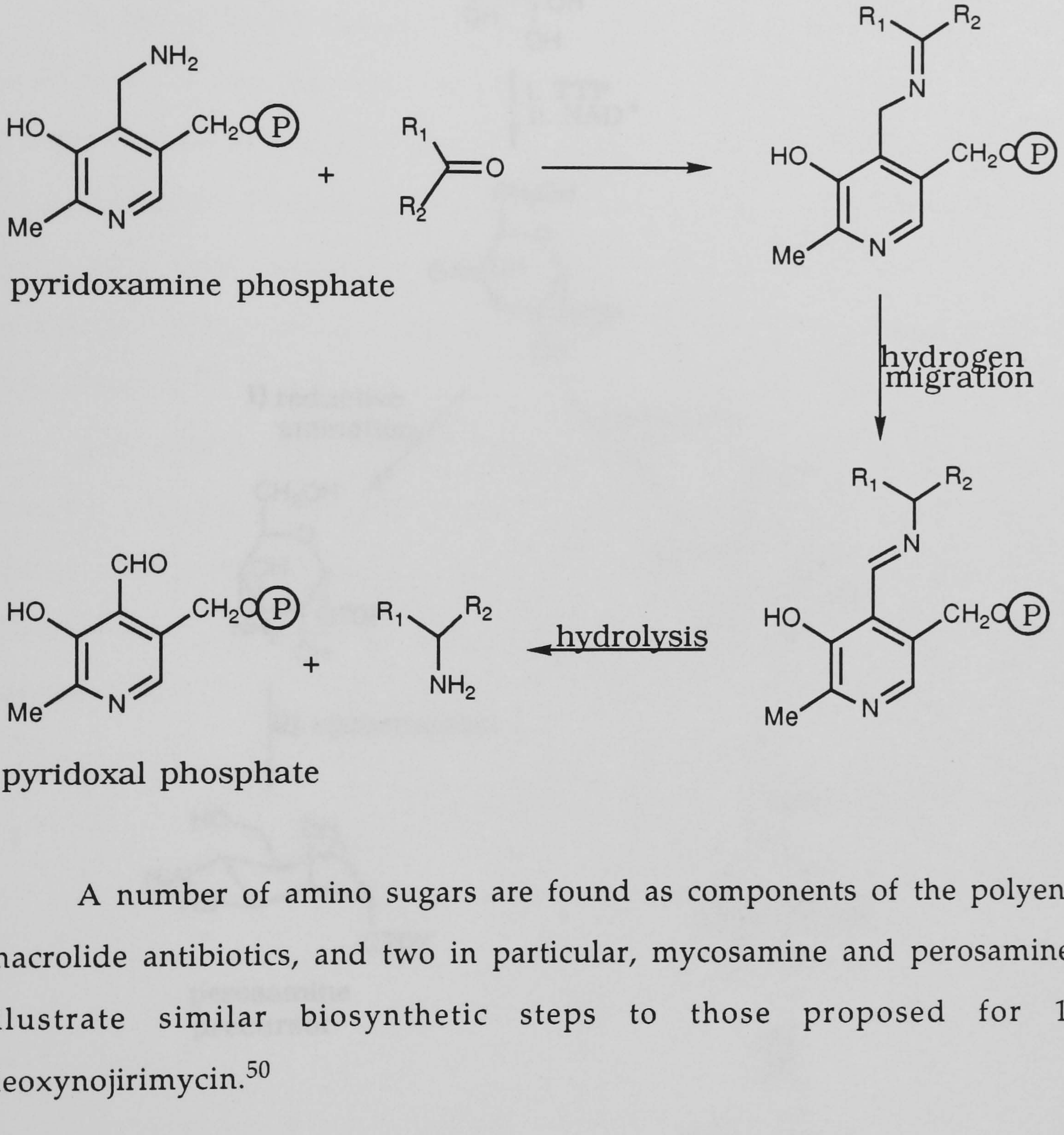
A proposed biosynthetic route for 1-deoxynojirimycin



Essentially, this is a biochemical version of the chemical route previously described in the first synthesis of nojirimycin. The oxidation and

reduction steps might involve NAD^+/NADH, and one possible method of reductive amination uses the co-enzyme pyridoxamine phosphate.

Reductive amination with pyridoxamine phosphate.

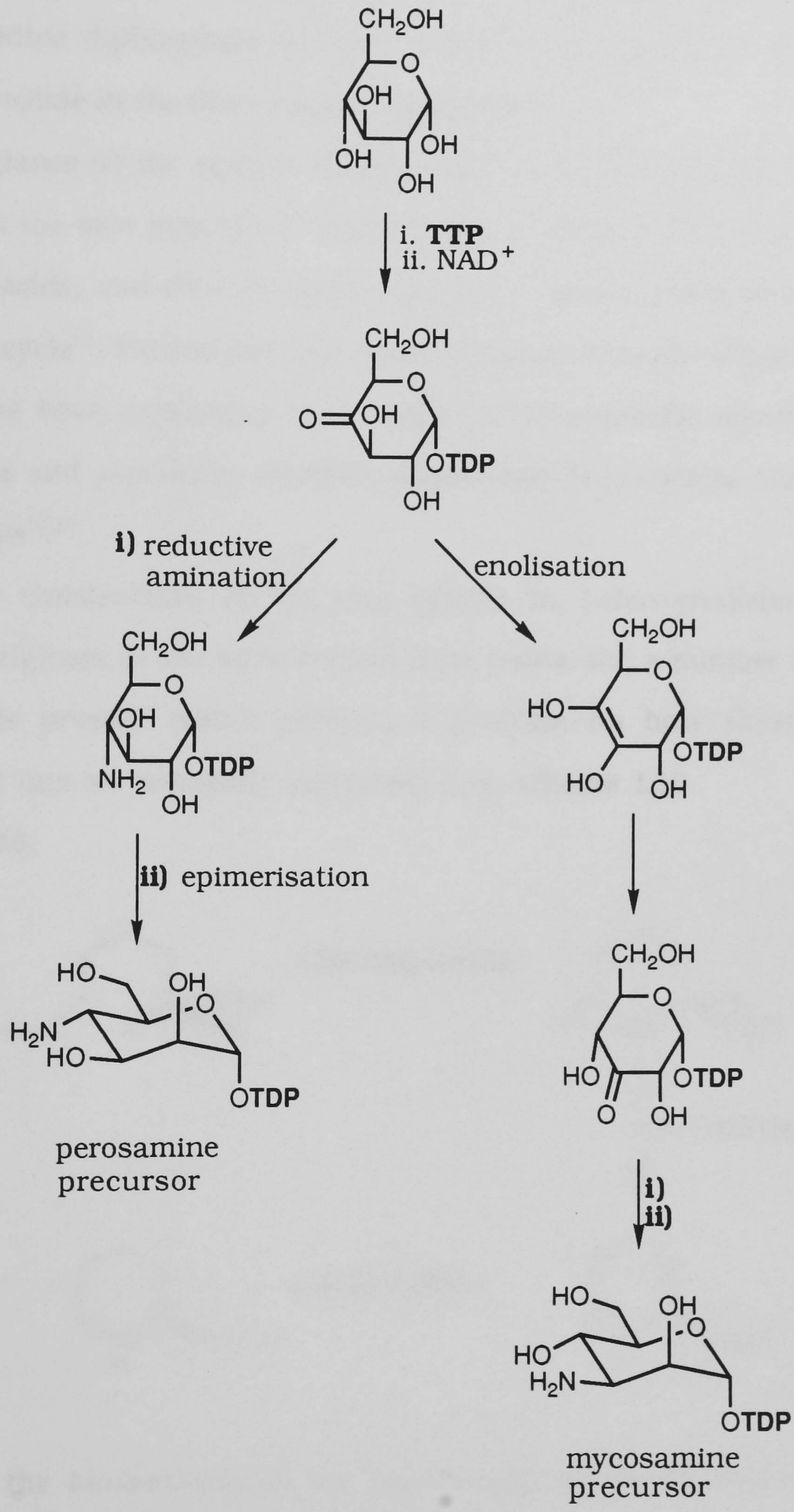


pyridoxamine phosphate

pyridoxal phosphate

A number of amino sugars are found as components of the polyene macrolide antibiotics, and two in particular, mycosamine and perosamine, illustrate similar biosynthetic steps to those proposed for 1-deoxynojirimycin.[50]

The proposed biosynthesis of mycosamine and perosamine.



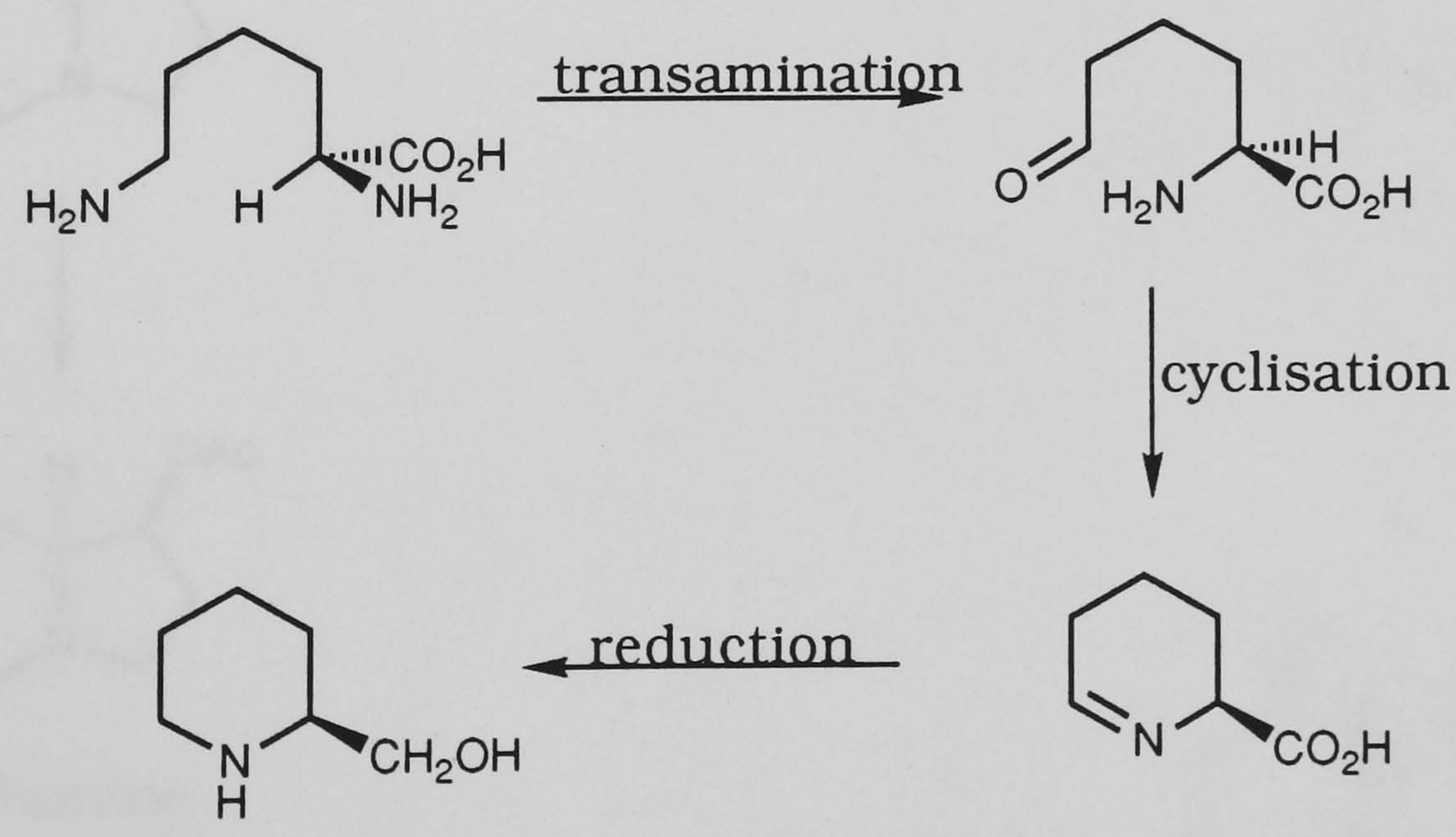
It is important to emphasise that all the transformations are carried out using an intact glucose ring structure, and although an amino sugar and not

an alkaloid is produced, it is a simple step to cyclise the amine function with the aldehyde (rather than a hydroxyl group) to produce a nitrogen heterocycle. The thymidine diphosphate serves to activate the sugar ring for attachment to the macrolide in the final stages of biosynthesis.

A glance at the studies already undertaken on alkaloid biosynthesis reveal that the vast majority of compounds are derived from the metabolism of amino acids, and thus in many cases from carbohydrate sources via the citric acid cycle[51]. Particularly, the aliphatic amino acids, (L)-ornithine and (L)-lysine have been implicated in a number of biosynthetic schemes towards pyrrolidine and piperidine alkaloids respectively (e.g. cocaine and anabasine biosynthesis[52,53].

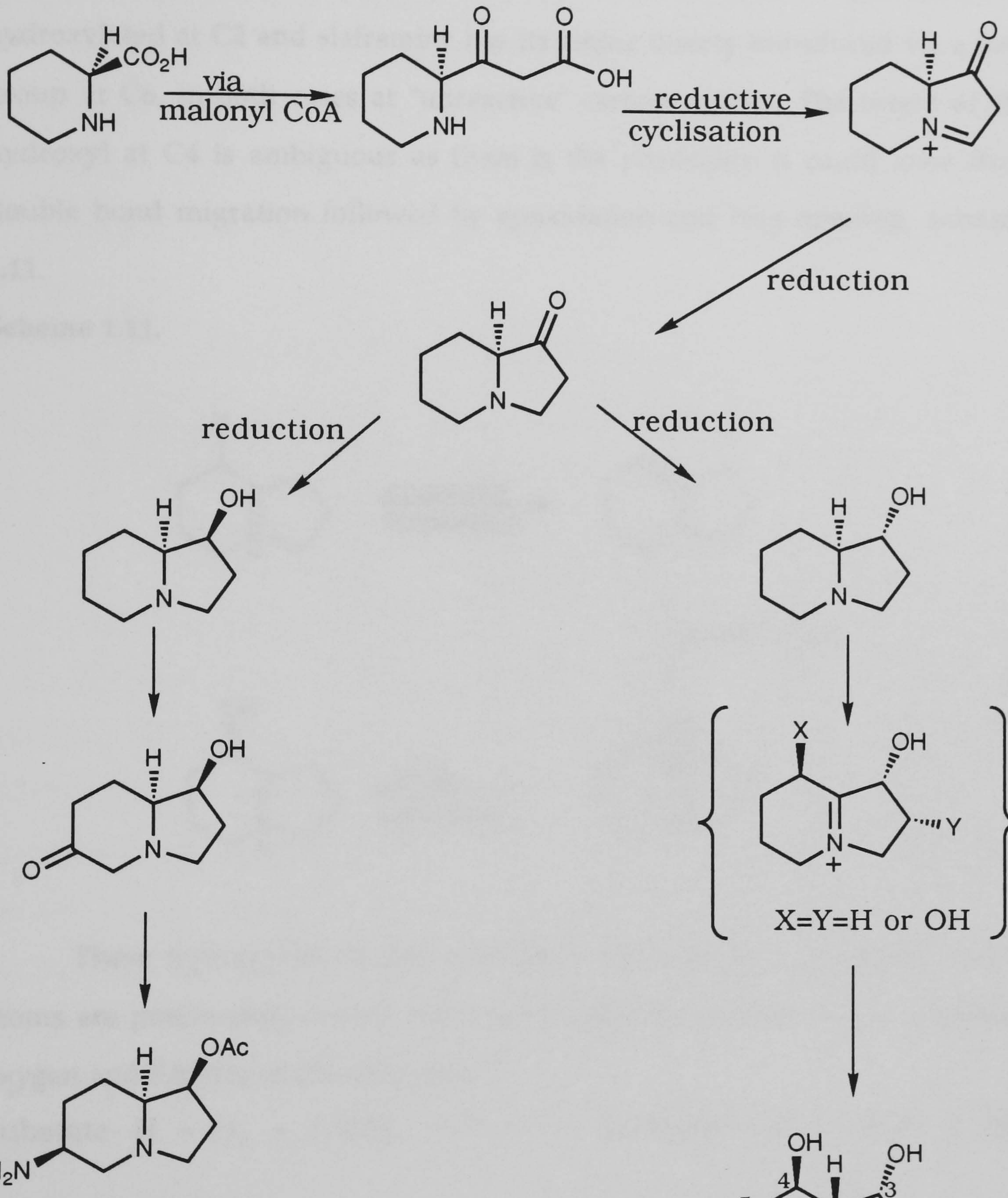
The construction of the ring system in 1-deoxynojirimycin could possibly originate in the same fashion from lysine, but a number of hydroxyl groups are present which presents a problem on how these might be introduced into an essentially unreactive ring, **scheme 1.10**

Scheme 1.10.



However, the biosynthesis of the indolizidine alkaloids swainsonine and slaframine in *Rhizoctonia leguminicola* both involve lysine and functionalisation of a saturated carbon centre[54].

The biosynthesis of swainsonine and slaframine in *Rhizoctonia leguminicola*.



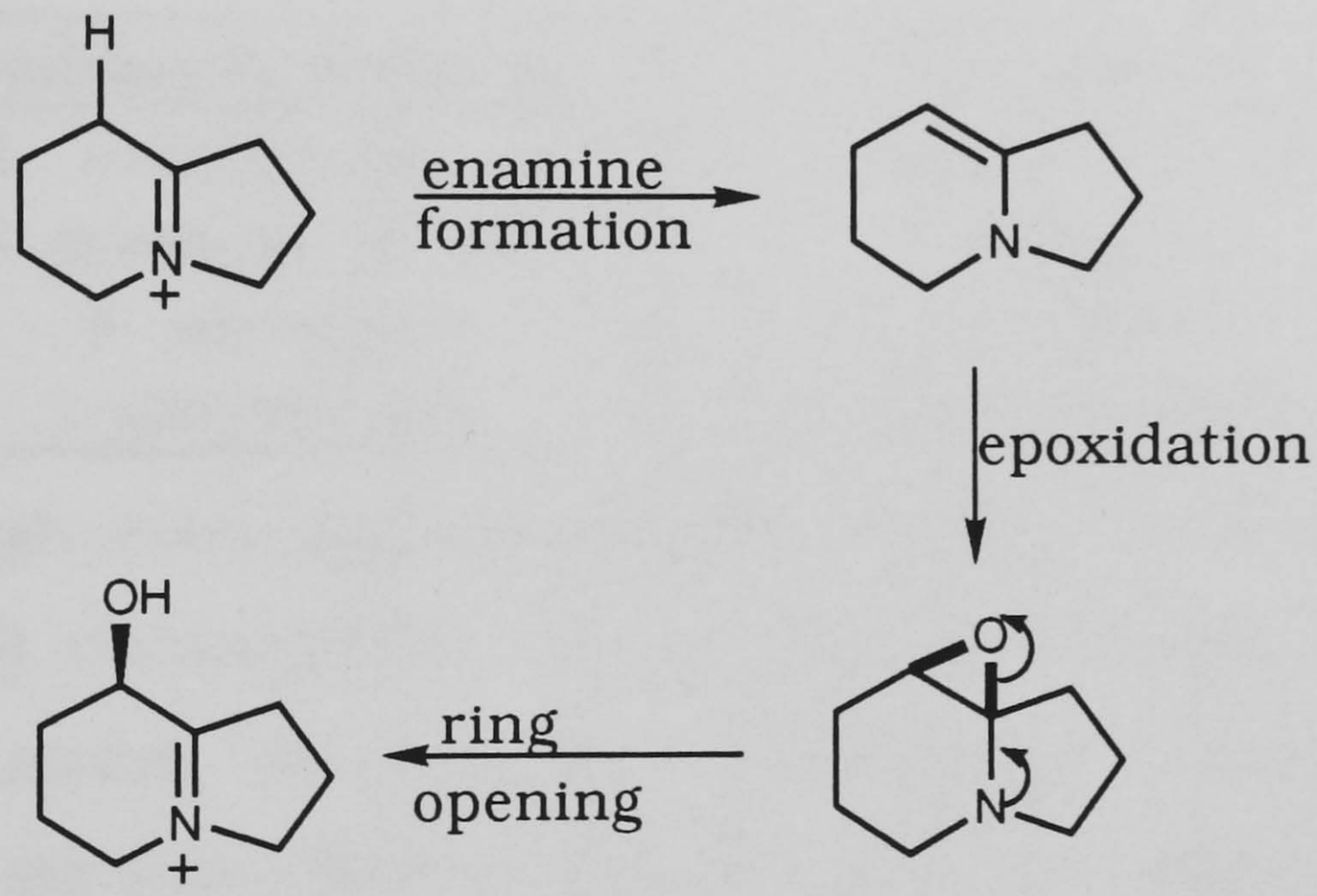
slaframine

swainsonine

The first stage involves the formation of pipecolic acid from (L)-lysine. The pipecolic acid, presumably as a thioester, then reacts with malonyl CoA

before reductive cyclisation affords the indolizidine ring structure. The two pathways then split and hydroxyl and amine groups are introduced, the exact timing of which still remains uncertain. Swainsonine, in particular, is hydroxylated at C2 and slaframine has its amine moiety introduced via a keto group at C6, in both cases at "unreactive" carbon centres. The origin of the hydroxyl at C4 is ambiguous as there is the possibility it could arise from double bond migration followed by epoxidation and ring opening, **scheme 1.11**.

Scheme 1.11.



These hydroxylations and oxidations of unreactive, saturated carbon atoms are presumably carried out by an oxygenase enzyme, using molecular oxygen and $FADH_2$ as the co-enzyme[55].

$$\text{substate--H} + O_2 + FADH_2 \longrightarrow \text{substrate--OH} + H_2O + FAD$$

However, it seems unlikely that three hydroxyl groups would be introduced successively into the basic piperidine ring structure to give 1-deoxynojirimycin and the carbohydrate route therefore looked the more plausible one before this work was undertaken.

CHAPTER TWO

Biosynthesis of nojirimycins in *S. subrutilus*.

Nojirimycin (NJ) and 1-deoxynojirimycin (DNJ) have been isolated from a wide range of microorganisms, and these cultures lend themselves to biosynthetic study by using isotopically labelled precursors. A few of the *Streptomyces* and *Bacillus* strains which produce nojirimycins are listed below.

Microorganisms which produce nojirimycins.

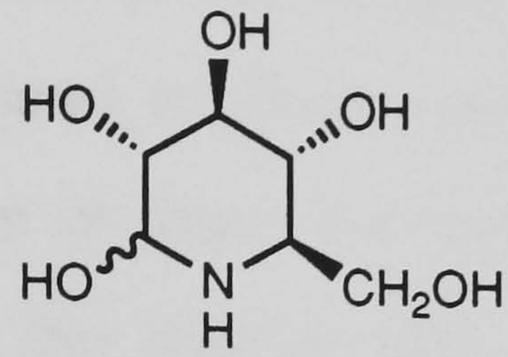
Nojirimycin producers	DNJ producers
S. roseochromogenes	*S. lavendulae* SF 425
S. lavendulae SF 425	*S. subrutilus*
S. nojiriensis	*B. subtilis*
S. subrutilus 445	*B. amyloliquefaciens*

Although several piperidine alkaloids have been isolated from plants, including DNJ, microorganisms offer a number of advantages over plants for biosynthetic studies. Usually it is a simple matter to feed the labelled precursor to the liquid medium from which the microorganism feeds, and this can be accommodated on virtually any scale of production. At the end of the fermentation, it is usually straightforward to remove the microorganism before final purification. In biosynthetic experiments involving plants, labelled precursors need to be introduced by means of a wick or by injection. The study is limited by the number of plants and in some cases by seasonal variations if the secondary metabolite of interest is produced in a fruit, for example. Usually mechanical methods are then required to extract the compound(s) from the leaves or roots before further purification can take place.

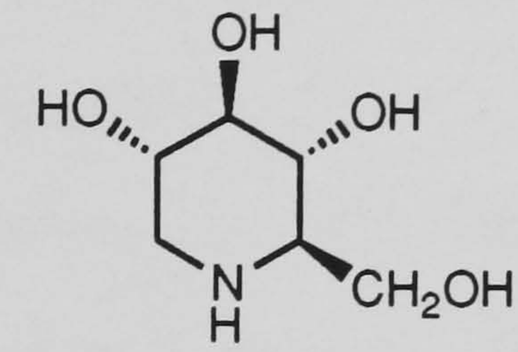
In this thesis, the production of four alkaloids, DNJ, NJ, 1-deoxymannojirimycin (DMJ) and mannojirimycin (MJ) has been evaluated in

S. subrutilus and *B. subtilis var niger*. This involved the synthesis of isotopically labelled precursors which are described in chapter 3.

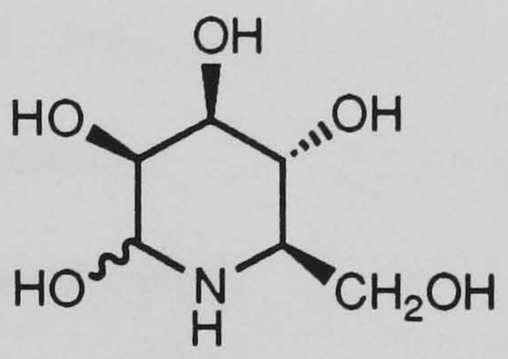
The stereochemical relationship between NJ, DNJ, MJ and DMJ.



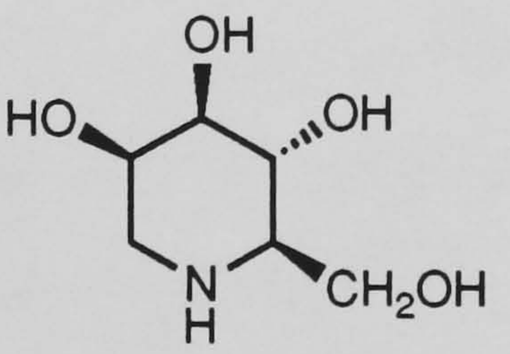
NJ



DNJ



MJ



DMJ

The influence of nutrients and culture conditions on the yield of DNJ in *S. subrutilus* and the development of the trehalase assay for time course studies was work carried out by S. J. Trew at Warwick University[56].

Fermentation medium

The complex medium used in all biosynthetic studies involving *S. subrutilus* is given below. This organism showed slightly higher titres of DNJ in a soyabean medium with starch as the carbon source when compared to the patented producer, *S. lavendulae* ATCC 31434.

Fermentation medium for *S. subrutilus*.

	% w/v
Glucose	0.4
KCl	0.05
$MgSO_4.7H_2O$	0.05
NaCl	0.5
$NaNO_3$	0.2
$CaCO_3$	0.35
Soyabean meal	1

The medium was made up to 500mL with distilled water and then split into 30mL aliquots before being autoclaved. After inoculation with *S. subrutilus*, the flasks were then incubated at 28-30°C/200 r.p.m for 7 days.

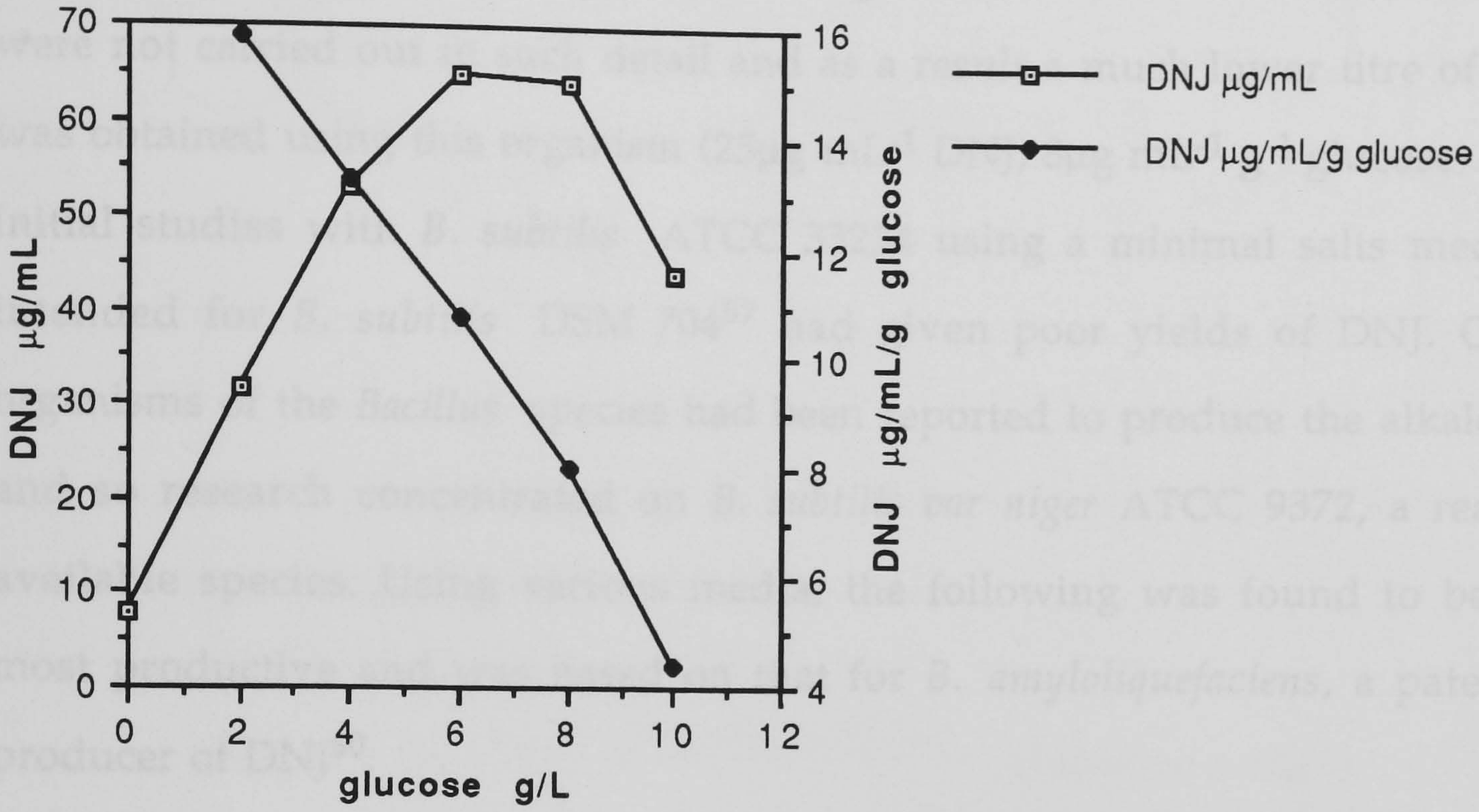
The distribution of the medium to several shake flasks improved the yield of DNJ compared to when a bulk reaction vessel was used. This was presumably because of improved aeration, so all subsequent fermentations were carried out in this manner, including those for *B. subtilis*. It was hoped that a defined medium could be used for the biosynthetic studies, in which the soyabean meal was replaced by a series of amino acids in known concentrations. However, *S. subrutilus* showed poor production of DNJ in this type of system so a complex medium as above was used as a base.

This complex medium was based on work by Ezure *et al*[4] who had investigated several carbon and nitrogen sources and the effect these had on the production of DNJ. They had found that soluble starch (2% $^{w}/_{v}$) was the best carbon source, giving a DNJ titre of 263µg ml^{-1} based on a sucrase assay. A carbohydrate biosynthetic route would originate from the starch whilst an amino acid pathway would utilise lysine from the soyabean meal.

To test this, the starch in the original medium needed to be replaced with an alternative carbohydrate source that could be easily labelled with an isotope. Glucose was found to be a good substitute, although mixtures of glucose and starch, which had been reported earlier[5], needed to be avoided. This was because the glucose might be used up rapidly in the growth of the cells and the reserve of starch might then contribute to secondary metabolism.

Extensive work by Trew[56] had revealed that the highest yield of DNJ was produced with 0.6% $^{w}/_{v}$ glucose in the medium (65µg mL^{-1} DNJ). In terms of alkaloid produced per weight of glucose fed, the medium with 0.2% $^{w}/_{v}$ carbon source proved to be more efficient (15.8µg mL^{-1} DNJ g^{-1} glucose) as shown below.

The relationship between glucose concentration and DNJ production[56].



A compromise was reached between efficiency of production and the quantity of fermentation medium that needed to be prepared in order to produce an isolable amount of alkaloid. This level was set at 0.4%$^{w/v}$ glucose which meant that with a 500mL fermentation, approximately 30mg of DNJ would be produced in total. Despite numerous experiments varying different nutrients and culture conditions, the basic medium of Ezure[4], without the starch, was utilised.

This yield of DNJ might appear to be low given that various strains in different species have been reported to produce very high levels of DNJ. Ezure has described UV mutant strains of *S. lavendulae* capable of producing up to 900 µg mL^{-1} of alkaloid. Similarly, a laboratory strain[57] of *B. subtilis* can produce DNJ from many different carbon sources up to a value of 1000 µg mL^{-1}. In both these cases, the inhibition of sucrase was used as an assay method, although this enzyme is inhibited by both DNJ and NJ, with K_i values of 2.2×10^{-8} M and 1.2×10^{-7} M respectively[58]. Hence, a sucrase assay may give optimistic concentrations of DNJ if appreciable amounts of NJ are

present, which is possible in the case of *S. lavendulae*. Ezure has described an HPLC method of analysis but no comparison of the two techniques was made.

Studies towards a high producing medium for *B. subtilis var niger* were not carried out in such detail and as a result a much lower titre of DNJ was obtained using this organism (25μg mL^{-1} DNJ, 8μg mL^{-1} g^{-1} glucose). Our initial studies with *B. subtilis* ATCC 33234 using a minimal salts medium intended for *B. subtilis* DSM 704[57] had given poor yields of DNJ. Other organisms of the *Bacillus* species had been reported to produce the alkaloid[59] and so research concentrated on *B. subtilis var niger* ATCC 9372, a readily available species. Using various media, the following was found to be the most productive and was based on that for *B. amyloliquefaciens*, a patented producer of DNJ[60].

Fermentation medium for *B. subtilis var niger*.

B. amyloliquefaciens	*B. subtilis var niger*
Glycerol(3%) $CaCO_3$(3%) Soyabean meal(3%) Diluted with tap water	Glucose(0.4%) $CaCO_3$ (0.2%) Soyabean meal(1%) Diluted with tap water pH=6.8

To compensate for the lower concentration of DNJ in this organism compared to *S. subrutilus* , most experiments were carried out using 750mL of fermentation medium, producing typically 10-12mg of DNJ.

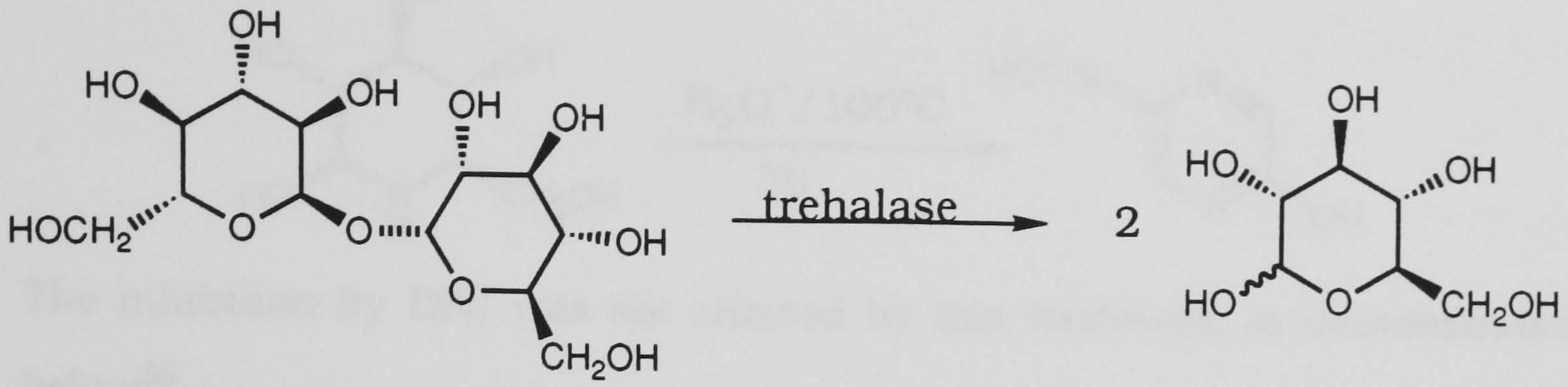
Assay techniques.

Two methods were used to quantify DNJ and NJ during the fermentation and purification processes.

Trehalase assay

DNJ and NJ both inhibit pig kidney trehalase (EC 3.2.1.28), an enzyme that hydrolyses trehalose into glucose, **scheme 2.01.**

Scheme 2.01



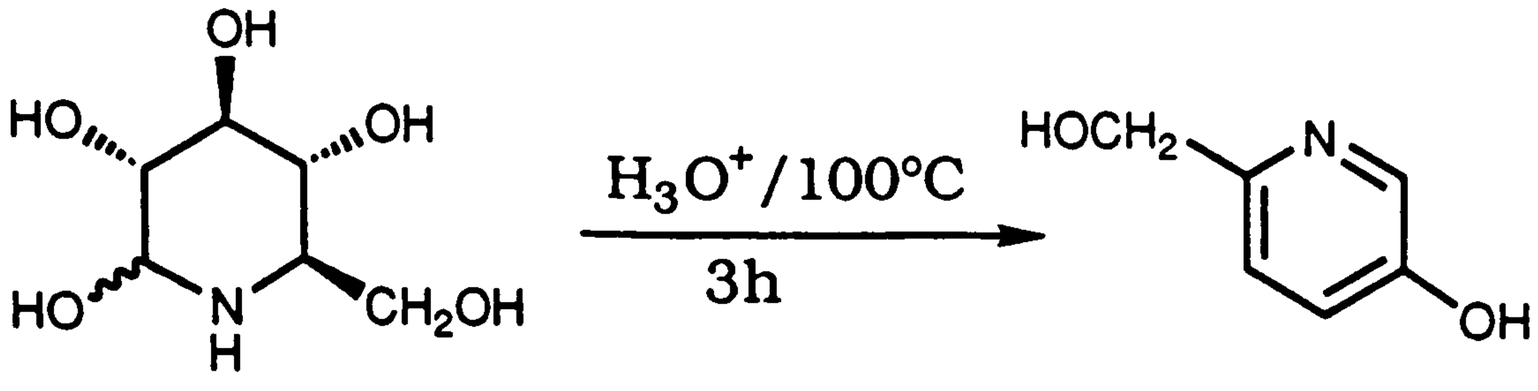
trehalose glucose

The amount of glucose released can be determined by a standard blood glucose assay using the enzyme glucose oxidase.

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconate} + H_2O_2$$

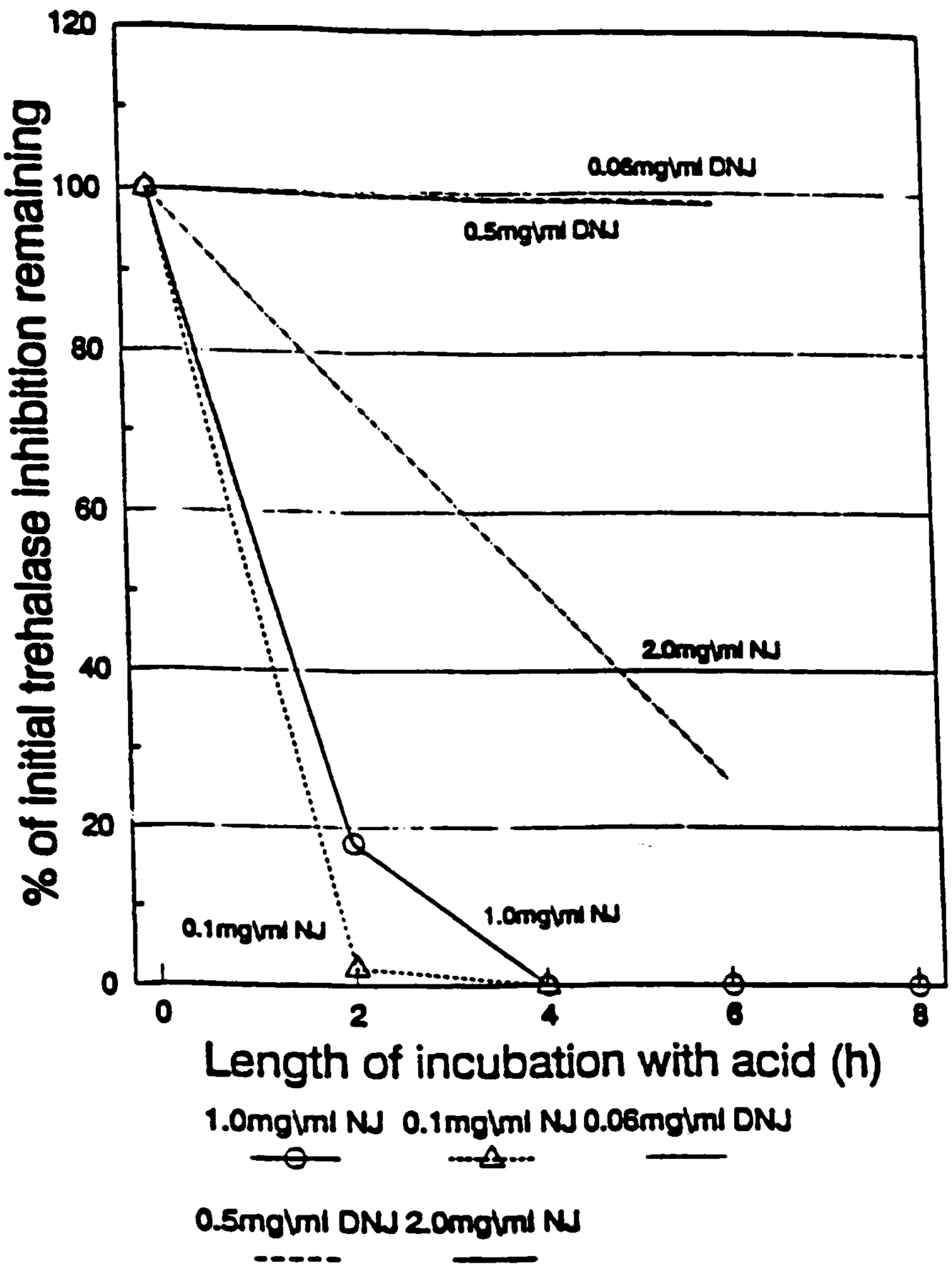
The assay comes in a kit form (Boehringer-Mannheim GmbH, Peridochrom®), where the hydrogen peroxide produced in the above reaction is used to oxidise phenol to benzoquinone. This reacts with 4-aminophenazone to give the red imino derivative which can be detected spectrophotometrically. The presence of NJ or DNJ inhibits trehalase and therefore the formation of glucose, so a weaker absorption was noted when these two alkaloids were present. Like sucrase, which has already been described, trehalase was affected by NJ and DNJ ($K_i = 2.6 \times 10^{-5}$ M and 3.4×10^{-6} M respectively)[56]. Although NJ was the weaker inhibitor, to get an accurate estimation of the concentration of DNJ, the former needed to be removed. This was conveniently done using a heat/acid treatment first described by Ishida[1]. The product(s) from this decomposition have been characterised by Inouye[2].

The decomposition of NJ under heat/acid conditions.



The inhibition by DNJ was not affected by this treatment, as demonstrated below[56].

Inhibition of trehalase by DNJ and NJ after incubation with an equal volume of 6M HCl at 90°C[56].



By preparing standard solutions of NJ and DNJ, an estimation of the amount of each alkaloid in a fermentation could be made following heat/acid treatment. The disadvantage with this method was that there was always the possibility that other compounds were present in the culture medium which inhibited this enzyme (see results for *B. subtilis* below). To ascertain whether the enzyme assay was correct, gas chromatography was also used for quantitation. This method was first described in 1986 for a range of polyhydroxyalkaloids and only necessitated derivatisation to the trimethylsilyl derivative[61]. By using an internal standard of methylglucoside and a standard calibration curve, the amount of DNJ in the fermentation could be determined. The disadvantage with this method was that NJ decomposed after silylation (or on the column) so only an estimate of the 1-deoxy derivative could be obtained.

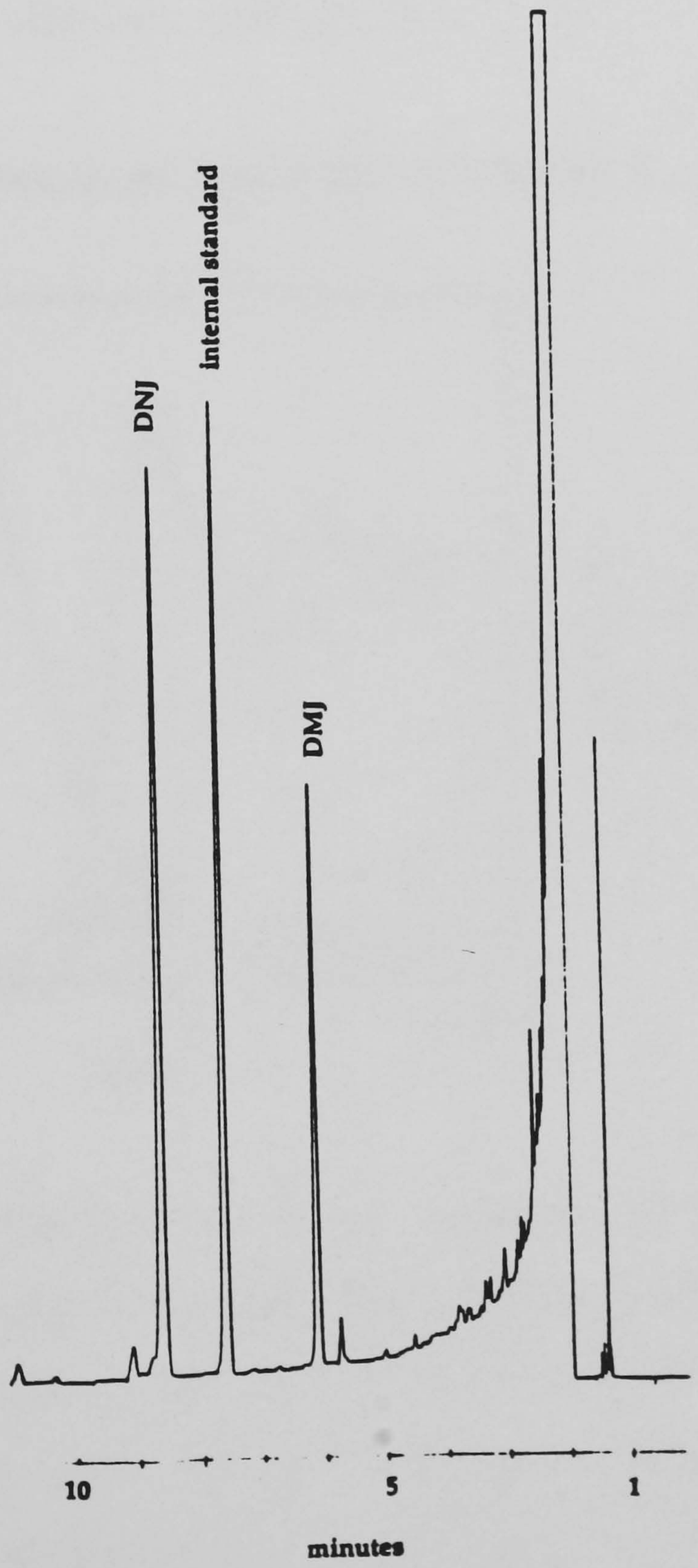
With *S. subrutilus,* the two techniques gave identical results for the quantity of DNJ. Neither technique was faster than the other, as the GC sample needed to be lyophilised prior to derivatisation, whilst the trehalase assay involved a heat/acid step. The enzyme assay did give extra information, however, in the form of the NJ concentration.

In the fermentations involving *B. subtilis var niger,* the agreement between the GC result and that from the trehalase assay was not good. The enzyme was inhibited to a higher degree after heat/acid treatment than could be accounted for by DNJ alone. After 7 days fermentation, a typical heat treated culture medium showed 70% inhibition of trehalase, corresponding to 0.05mg mL^{-1} DNJ. The GC assay consistently gave values of 0.025mg mL^{-1} for the same fermentation at the same point in time. One reason for the apparent low titre for DNJ might be the presence of an impurity co-eluting with the internal standard and therefore giving a larger area for this component. When the chromatogram was run without internal standard, no such impurity was found, so presumably at least one further inhibitor was present

which was not identified in this project.

From the GC chromatogram another alkaloid was seen to be present other than the DNJ in the *S. subrutilus* fermentation. This was always present in smaller amounts and later proved to be the *manno* epimer of DNJ, 1- deoxymannojirimycin (DMJ).

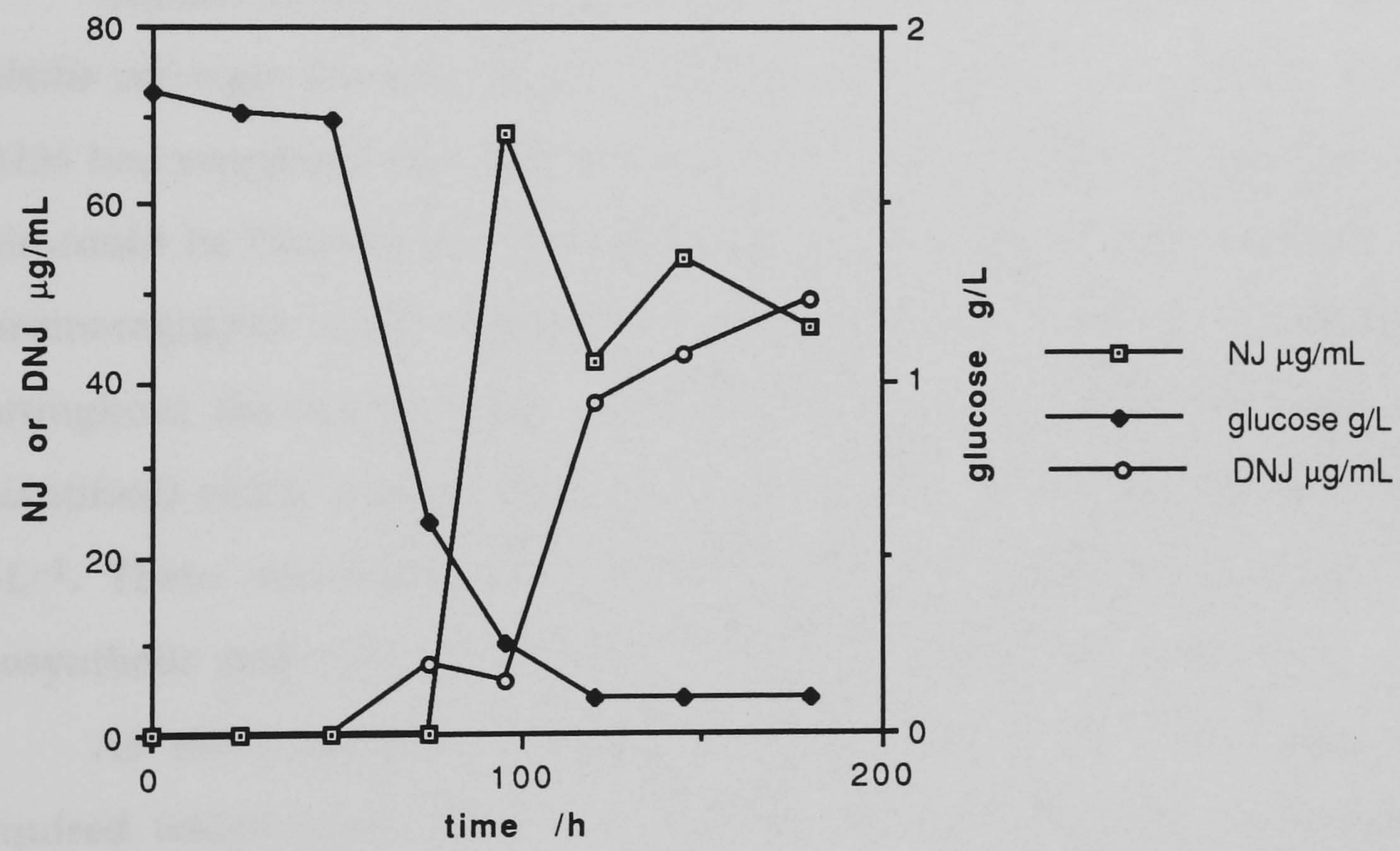
Gas chromatogram of the *S. subrutilus* fermentation after partial purification.



DMJ had not been reported in *S. subrutilus* before, although it had been isolated in 1988 from *S. lavendulae* GC-148, which also produced DNJ. Isolation of the two alkaloids confirmed the gas chromatography results that the level of DNJ was $2^{1}/2$ times higher than that of DNJ. Fortunately, DMJ was shown to have no effect on trehalase, even at concentrations up to 0.5mg mL^{-1}, well exceeding that found in the fermentation.

Time course studies by Trew[56] using *S. subrutilus* gave some indication of the interaction between nojirimycin and DNJ over the fermentation period, although DMJ had yet to be discovered in this organism at this time.

Time course studies on the production of DNJ and NJ in *S. subrutilus*.[56]



The biosynthesis of DNJ and NJ followed a typical path in that they were not produced until the glucose concentration had fallen to about 25% of its initial concentration. Growth of the organism before this time was the dominant factor. The amounts of NJ and DNJ produced then both increased together after 75h, with NJ showing a particularly large increase at this time. At all points until the end of the fermentation, the level of NJ was higher than that

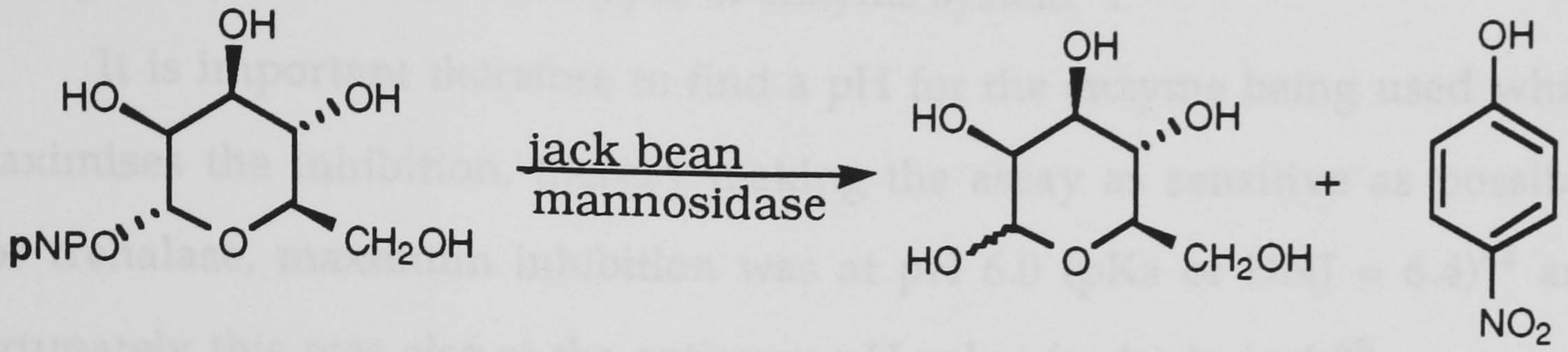
of DNJ. The time course studies only indicated that the two alkaloids were present in the fermentation at the same time and did not prove whether NJ was a precursor to DNJ, although this was a plausible hypothesis.

Time course studies with *B. subtilis var niger* appeared to follow a different pattern. Percentage inhibition of trehalase, although not an accurate method for estimating DNJ in this organism, can be used to detect the presence of NJ qualitatively. No decrease in inhibition, however, could be detected in heat/acid treated samples compared to the crude fermentation during 7 days incubation. This suggested that either NJ was not being produced or that it was at a much lower level compared to that in *S. subrutilus* and the enzyme assay was not sufficiently sensitive to detect it.

Another interesting difference between the two organisms was that *B. subtilis var niger* did not produce DMJ. Earlier work with *B. subtilis* ATCC 33234 had suggested that DMJ was not present after 7 days fermentation, but this could be because the compound was a precursor to DNJ itself. A gas chromatography study revealed that DMJ was not produced at any stage throughout the fermentation, although DNJ was produced after 48h (not quantified) rising steadily thereafter to give a final concentration of 0.02mg mL^{-1}. These observations were to have important consequences for the biosynthetic pathway in *B. subtilis var niger* and will be discussed later.

As more evidence emerged from labelling studies, an assay was required which could detect mannonojirimycin (MJ). Already it had been shown that the mannose analogue of DNJ was present in *S. subrutilus,* and investigations indicated that it was likely the manno epimer of NJ was there too. The inhibition of jack bean α-D-mannosidase (EC 3.2.1.24) was the basis for an enzyme assay, **scheme 2.02.**

Scheme 2.02.



pNP =*para*- nitrophenyl

Normally, the hydrolysis of *p*-nitrophenylmannoside gives mannose and *p*-nitrophenol, but in the presence of a mannosidase inhibitor, the concentration of the latter is reduced. Conversion to the yellow nitrophenolate anion allows the hydrolysis reaction to be monitored spectrophotometrically. As with the trehalase assay, inhibition of the enzyme decreases the absorbance due to nitrophenolate anion.

The compound of interest, MJ, was reported to lose its inhibitory properties after treatment at pH 5.5 and 50°C[62] (compare the heat/acid treatment of NJ). At this pH and temperature, the loss of inhibitory activity had a half life of ~4h so a test for MJ was feasible by comparing inhibition before and after 24h heat treatment. Unlike the NJ assay, where an authentic standard was available, a sample of MJ was not available and so the assay remained a qualitative rather than a quantitative one.

The importance of pH in inhibition studies using mannosidases.

The state of ionisation of the alkaloid inhibitor, i.e. whether it is protonated or not, and the degree of ionisation of carboxylate groups within the active site of the enzyme, are two important factors governing the effectiveness of inhibition. Ambiguities between the two can arise from experimental data[63]. For instance, an increase in inhibition with increasing pH can arise either because the inhibitor binds in an **unprotonated** form and subsequently protonates within the active site, or because there is more

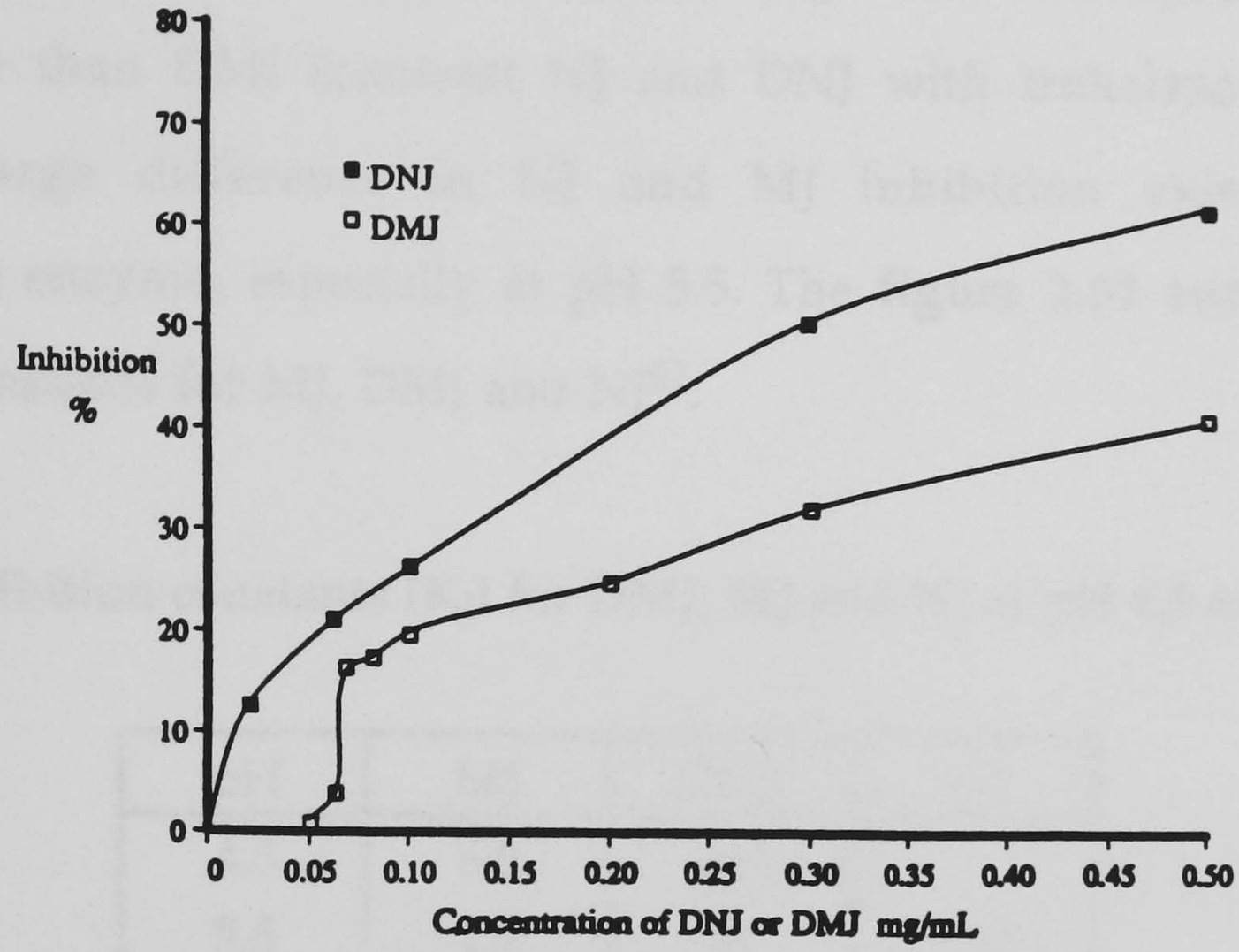
ionisation of carboxylate groups to bind the **protonated** form of the alkaloid. Examples are known of both types of enzyme system[63].

It is important therefore to find a pH for the enzyme being used which maximises the inhibition, thereby making the assay as sensitive as possible. For trehalase, maximum inhibition was at pH 6.0 (pKa of DNJ = 6.4)[64] and fortunately this was also at the optimum pH value for hydrolysis[65].

The mannosidase assay presented a dilemma. The inhibition of jack bean α-D-mannosidase by DMJ was reported to be higher at pH 5.5 than at pH 4.5 by a factor of 5.8, **figure 2.01**[62]. Studies by Li *et al* on the optimum hydrolytic pH for this enzyme gave a value of 4.2 as the ideal[66], with 75% of the activity of the enzyme being retained at the higher pH value of 5.5. However, in our studies, no inhibition occurred at pH 4.5, even using a standard concentration of 2.0×10^{-4} M DMJ. The IC_{50} of this alkaloid at this pH using jack bean α-D-mannosidase was reported to be 1.5×10^{-4}M, so approximately 50% inhibition of the enzyme should have taken place[67,68]. At pH 5.5, despite a slower hydrolysis rate, inhibition was observed and the experimental IC_{50} value of 4.5×10^{-4} M agreed with the literature value of 4.0×10^{-4} M at this pH[32]. This pH value was then used in our subsequent inhibition studies.

From the graph overleaf, it can be seen that jack bean α-D-mannosidase was also inhibited by DNJ.

The inhibition of α-jack bean mannosidase by DNJ and DMJ.



This contrasted with trehalase which was not inhibited by DMJ even at quite high levels (page 46). The difference between the two enzymes in terms of inhibition was also quite marked, with DNJ showing stronger inhibition of trehalase than DMJ with the mannosidase (DMJ $K_i = 6.8 \times 10^{-5}$M jack bean α-D-mannosidase, DNJ $K_i = 3.4 \times 10^{-6}$M pig kidney trehalase).

DNJ was present in the *S. subrutilus* fermentation at a level which would effect the mannosidase assay if DMJ was being quantified, but since the assay was really designed for MJ , this was not a great setback. The level of DMJ can be determined accurately using GC if required.

DMJ was not affected by the heat treatment already outlined so the decrease in inhibition following this step gave a direct means of establishing the presence MJ. More worrying was the fact that NJ also inhibited jack bean α-D-mannosidase, although 500 times less effectively than MJ ($K_i = 5.0 \times 10^{-4}$M and 1.2×10^{-6}M respectively). To circumvent this problem, the concentration of NJ in the fermentation could be assessed using the trehalase assay. Since NJ was available as a standard, the effect of this alkaloid on jack

bean α-D-mannosidase could then be determined.

It was fortunate in this study that MJ was a stronger inhibitor of mannosidase than DMJ (contrast NJ and DNJ with trehalase) and that a relatively large difference in NJ and MJ inhibition existed for the mannosidase enzyme, especially at pH 5.5. The **figure 2.01** summarises the inhibition constants for MJ, DMJ and NJ[62].

Figure 2.01

The inhibition constants (K_i) for DMJ, MJ and NJ at pH 4.5 and 5.5[62].

pH	MJ	DMJ	NJ
4.5	6.5	400	-
5.5	1.2	68	500

All K_i values are in μM for jack bean α-D-mannosidase.

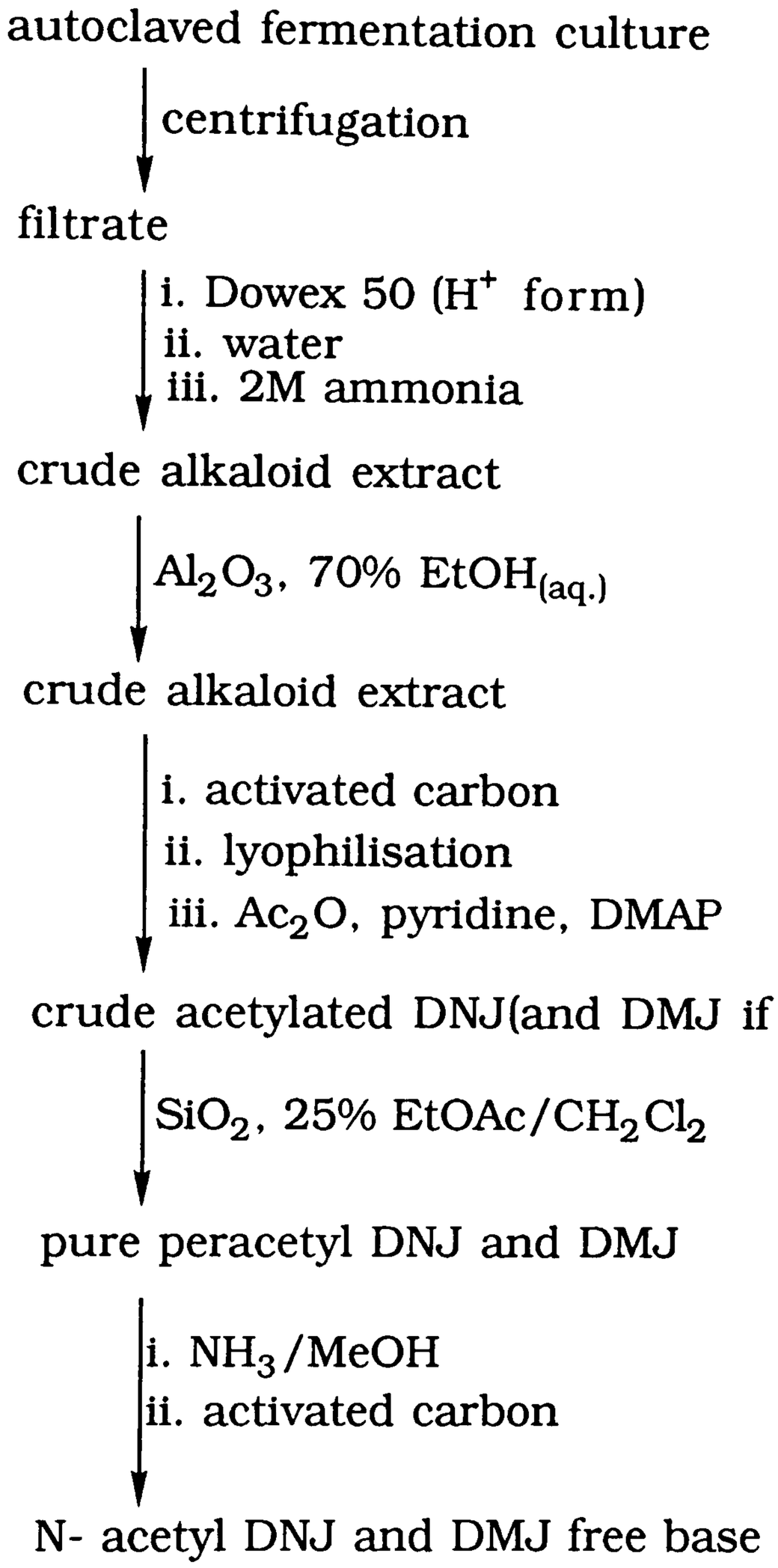
Purification of DNJ and DMJ from the fermentation medium

Nojirimycin was difficult to isolate from the fermentation. Inouye states, " isolation of nojirimycin in a pure state proved a difficult task owing to the instability of the antibiotic under neutral and acidic conditions, as indicated by a rapid decrease of the biopotency at room temperature". A widely used technique to semi-purify the crude culture medium was to pass the filtered or centrifuged fermentation liquor down a strongly acidic cation exchange resin. Protonated alkaloids adsorb to the column whilst neutral sugars and glycolytic byproducts pass through. This immediately presents a problem for NJ separation as the pH of the filtrate eluting from the column drops to about 2. This technique has been used to isolate NJ, although only from large scale fermentations[1].

DNJ and DMJ are not acid sensitive and can be easily adsorbed onto Dowex 50 (H^+ form). **Scheme 2.03** outlines the remaining steps to purify DNJ and DMJ from *S. subrutilus* and *B. subtilis*..

Scheme 2.03

The isolation of DNJ and DMJ from *S. subrutilus* and *B. subtilis*.



After adsorption to the cation exchange resin, the alkaloids were removed by eluting with 2M ammonia. Other workers had used 0.5M ammonia solution[5], but this was found to only remove some of the DNJ (e.g. 4mg out of 15mg). The eluate was then concentrated and taken up in 70% aqueous ethanol. At this stage the aqueous ethanol solution was a dark brown and not all the solid dissolved leaving suspended polymeric impurities. The alumina column removed most, but not all, of this dark coloration leaving a

very pale yellow solution. It was possible at this stage to separate DNJ from DMJ (in the free base forms) by careful chromatography on alumina, but there was considerable overlap of the two alkaloids and only a small amount of each could be obtained in a pure form. TLC (SiO_2) of the alumina column filtrate indicated one main spot with weak impurities present. DNJ and DMJ standards likewise co-eluted on a silica TLC plate.

The best way to separate the two epimers was by flash chromatography of the peracetyl derivatives. Using the solvent system in **scheme 2.03,** it was possible to separate most of each alkaloid, although there was some overlap between the two. To speed the purification process up, this was the only point where careful chromatography was carried out; the ion exchange and alumina columns essentially became filtration processes where fractions were not collected. The isolated peracetyl DNJ and DMJ were identical with the authentic acetylated alkaloids.

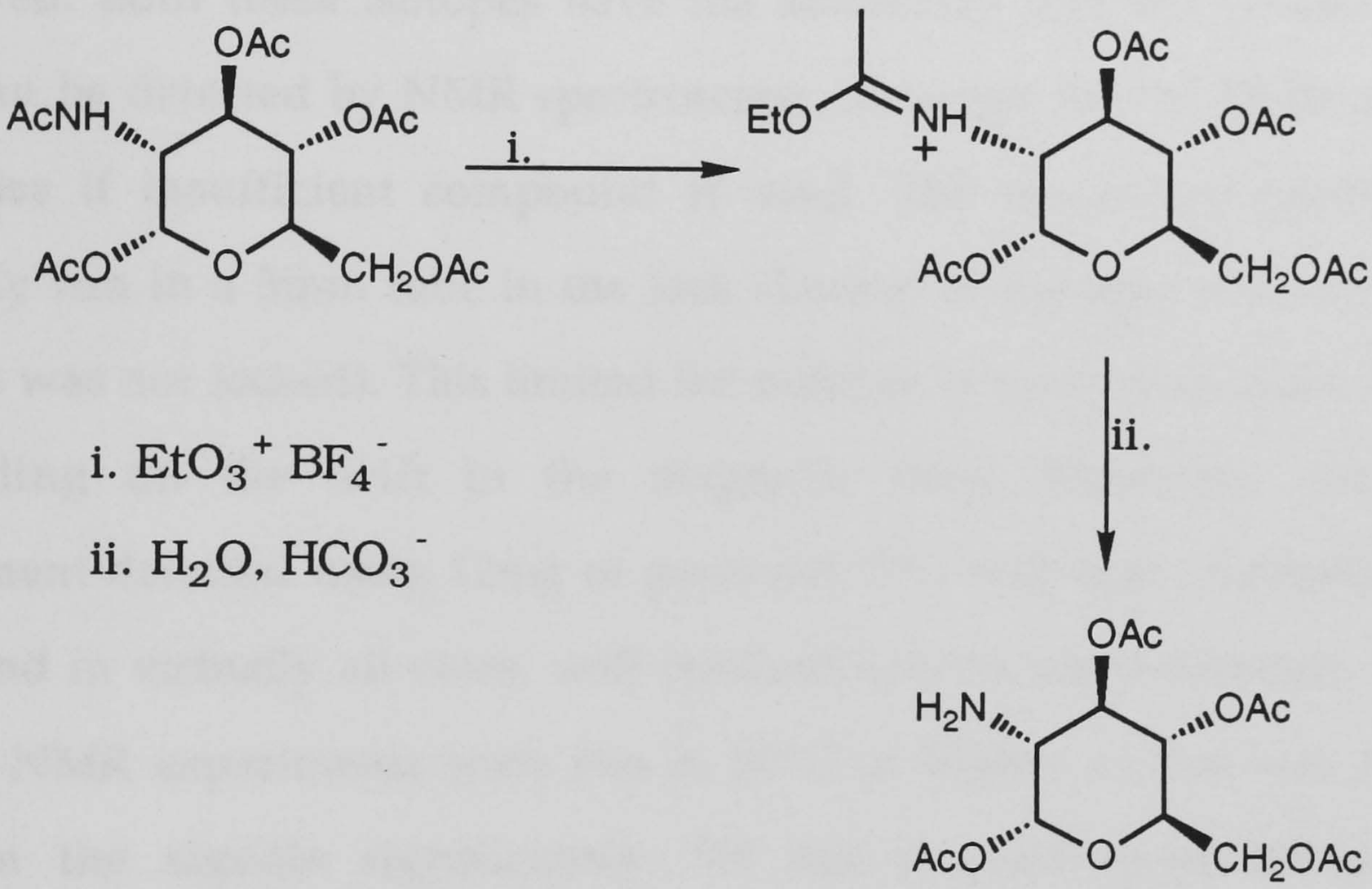
Unfortunately for NMR purposes, the peracetyl DNJ proved to be fluxional about the carbon/nitrogen amide bond, showing significant broadening in the ^1H NMR spectrum at room temperature. This made assignment of the signals impossible, although by warming the compound to 90°C in pyridine, the peaks in the spectrum coalesced so that the various proton positions could be identified. Peracetyl DMJ exhibited the same phenomenon, but this time the two rotamers about the carbon/nitrogen amide bond could be easily distinguished in the ^1H NMR spectrum. Under the same conditions as above, the peaks in this spectrum also coalesced, although some proton resonances remained slightly broadened.

There is a difference in the ease of deacetylation of peracetyl DNJ and DMJ. Treatment of peracetyl DMJ with concentrated methanolic ammonia removed all the O-acetyl and N-acetyl groups, whereas only the O-acetyl groups were removed from peracetyl DNJ under the same conditions. This contrasts to the observations of Inouye *et al* who claimed that all the acetyl

groups were removed by concentrated methanolic ammonia on the basis of IR evidence[2].

An attempt was made to remove the N-acetyl group in the peracetyl DNJ using Meerwein's salt, which had been reported[69] to hydrolyse the N-acetyl group in preference to the O-acetyl in peracetyl glucosamine, **scheme 2.04**. However, only N-acetyl DNJ could be recovered from the reaction.

Scheme 2.04.



The fully acetylated alkaloids and the N-acetyl DNJ were used to analyse the amount and position of various labels during biosynthetic studies. Occasionally, the DNJ/DMJ from *S. subrutilus* was analysed as a mixture directly after the alumina column without derivatisation, but this could only be carried out where labelled protons were not overlapped by other resonances. The assignment of proton and ^{13}C peaks for different derivatives are given later in this chapter and in the experimental section.

The recovery of DNJ (and DMJ where relevant) differed slightly between the two organisms studied. *S. subrutilus* was investigated first when the methods of isolation were being refined, and with practice it became routine to work with smaller quantities of alkaloid in larger volumes of

culture medium, as with *B. subtilis. var niger.*

The recovery of peracetyl DNJ and DMJ from *S. subrutilus* and *B. subtilis.*

	S. subrutilus	*B. subtilis var niger*
Production (after 7 days)	30mg in 350mL	12mg in550mL
Recovery (peracetyl)	12mg (DNJ)	14mg

Most of the labelling studies have been carried out using deuterium labelled precursors, although in certain cases, ^{13}C enriched compounds were employed. Both these isotopes have the advantage that the position of the label can be detected by NMR spectroscopy, although for ^{2}H NMR problems can arise if insufficient compound is used. The deuterium spectra were typically run in a 5mm tube in the lock channel of the spectrometer (i.e. the magnet was not locked). This limited the number of scans that could be taken, depending on the drift in the magnetic field. However, the lowest enrichment detected using 12mg of peracetyl DNJ was approximately 5 atom % D, and in virtually all cases, well resolved spectra were obtained. Usually, the ^{2}H NMR experiments were run at 50°C or higher as this was found to sharpen the signals significantly. ^{2}H has a quadrupole moment and intrinsically broadened peaks in the ^{2}H NMR spectrum at room temperature. Enrichment of precursors with tritium was less attractive, despite the fact that much lower levels could be detected, because the DNJ or acetyl derivatives resisted crystallisation. Furthermore, since only a small quantity of alkaloid was recovered, any degradation to locate the site of labelling would probably have presented practical difficulties.

In practice, some experiments using deuterium isotopes showed a high enough level of enrichment for the site of the label to be determined using ^{1}H NMR. The reduced integral height of labelled positions enabled the percentage enrichment to be compared with that obtained from mass spectrometry.

Mass spectrometry.

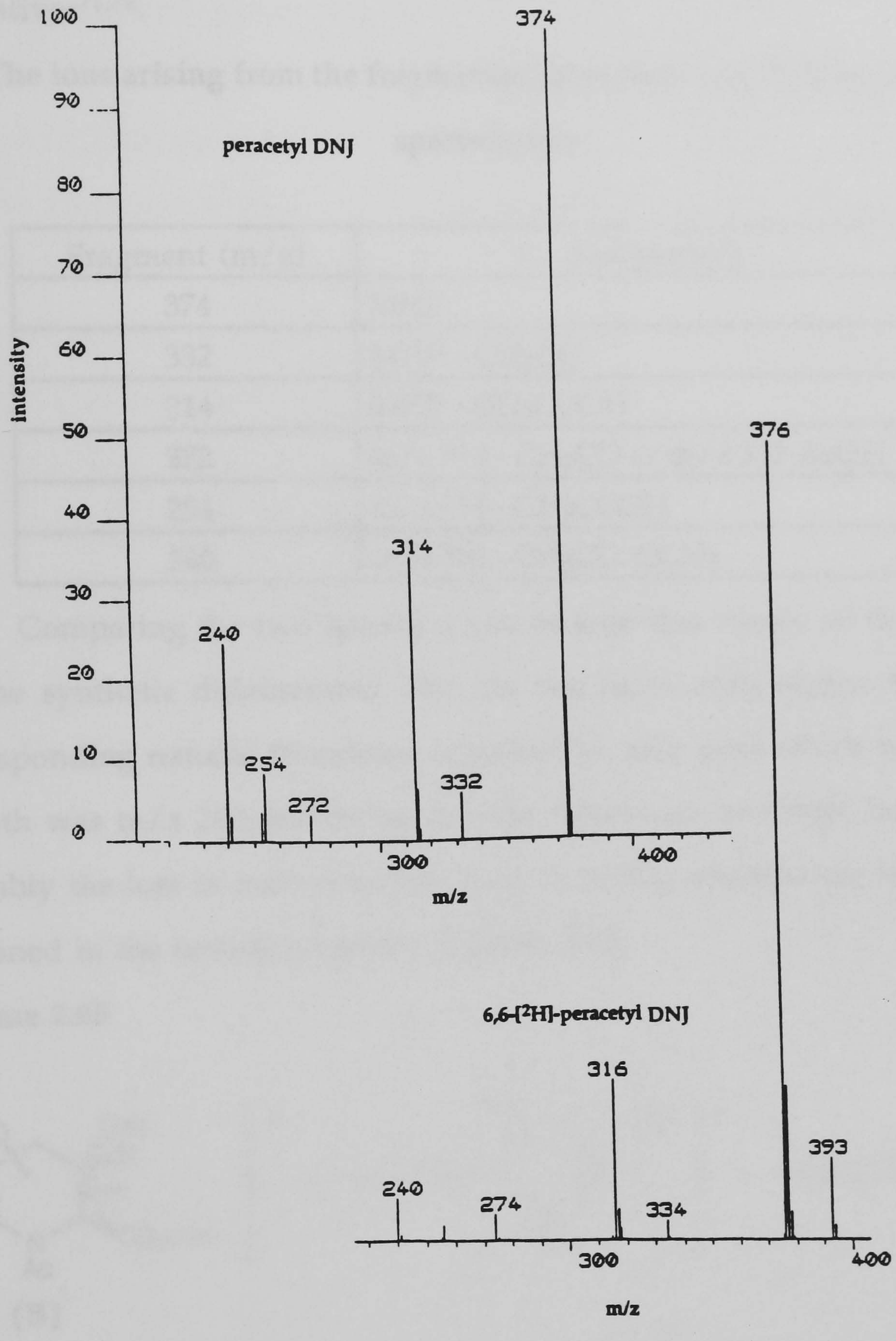
In conjunction with NMR studies, mass spectrometry not only revealed how much of a given isotope was present in the DNJ/DMJ, but also to a limited extent, where it was located. Spectra had been obtained for the free base for both DNJ and DMJ[70,5], but in this project the fully acetylated material was used. To minimise random errors, quantification of a particular isotope involved 5 scans of the isolated peracetyl alkaloid over two or three separate days. Natural abundance material was also analysed under identical conditions so that the contribution of natural abundance isotopes could be determined. Using ammonia chemical ionisation mass spectrometry, peracetyl DNJ gave an $(M + H)^+$ peak at m/z 374. After the contribution of the natural abundance isotopes to $((M + H)+1)^+$ had been subtracted, the percentage enrichment of a given isotope could be expressed as a percentage of the unlabelled material. Hence using the absolute intensity of each peak, the percentage enrichment was given by the following:

$$\frac{MH+1]^+ \text{ (corrected)} \times 100\%}{MH+1]^+ \text{ (corrected)} + MH]^+}$$

Figure 2.02 shows part of the mass spectrum for natural abundance peracetyl DNJ and a synthetic derivative, 6,6-dideuteroperacetyl DNJ. The molecular ion in each case can be seen at m/z 374 and m/z 376 respectively (m/z 393 is $(M + NH_4)^+$).

Figure 2.02

The partial mass spectrum for peracetyl DNJ and 6,6-dideuteroperacetyl DNJ.



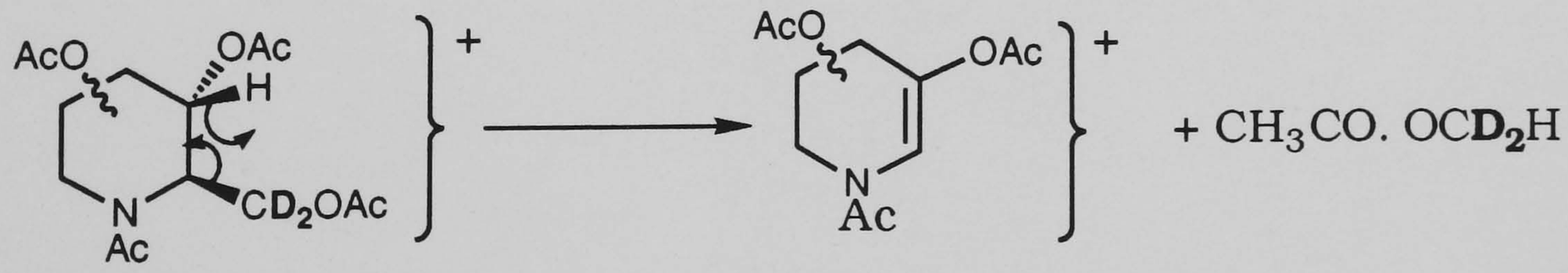
The fragmentation is dominated by the loss of acetic acid (M_r 60) and ketene (M_r 42), both of which are common fragments for acetylated sugar derivatives[71,72].

The ions arising from the fragmentation of peracetyl DNJ by CI mass spectrometry.

Fragment (m/z)	Assignment
374	$MH]^+$
332	$MH]^+ - CH_2CO$
314	$MH]^+ - CH_3COOH$
272	m/z 314 - CH_2CO or m/z 332-AcOH
254	m/z 314 - CH_3COOH
240	m/z 314 - $CH_3CO.OCH_3$

Comparing the two spectra it can be seen that nearly all the fragments for the synthetic dideuterated DNJ are two mass units higher than for the corresponding natural abundance sample. The only peak which was common to both was m/z 240, indicating that the deuterium had been lost. This was probably the loss of methyl acetate from m/z 314, whereby the isotopic label remained in the neutral fragment, **scheme 2.05**.

Scheme 2.05



(5)

(It is likely that protonation of an ester carbonyl occurs first, followed by the loss of acetic acid to give a charged fragment such as **(5)** shown in **scheme 2.05** above).

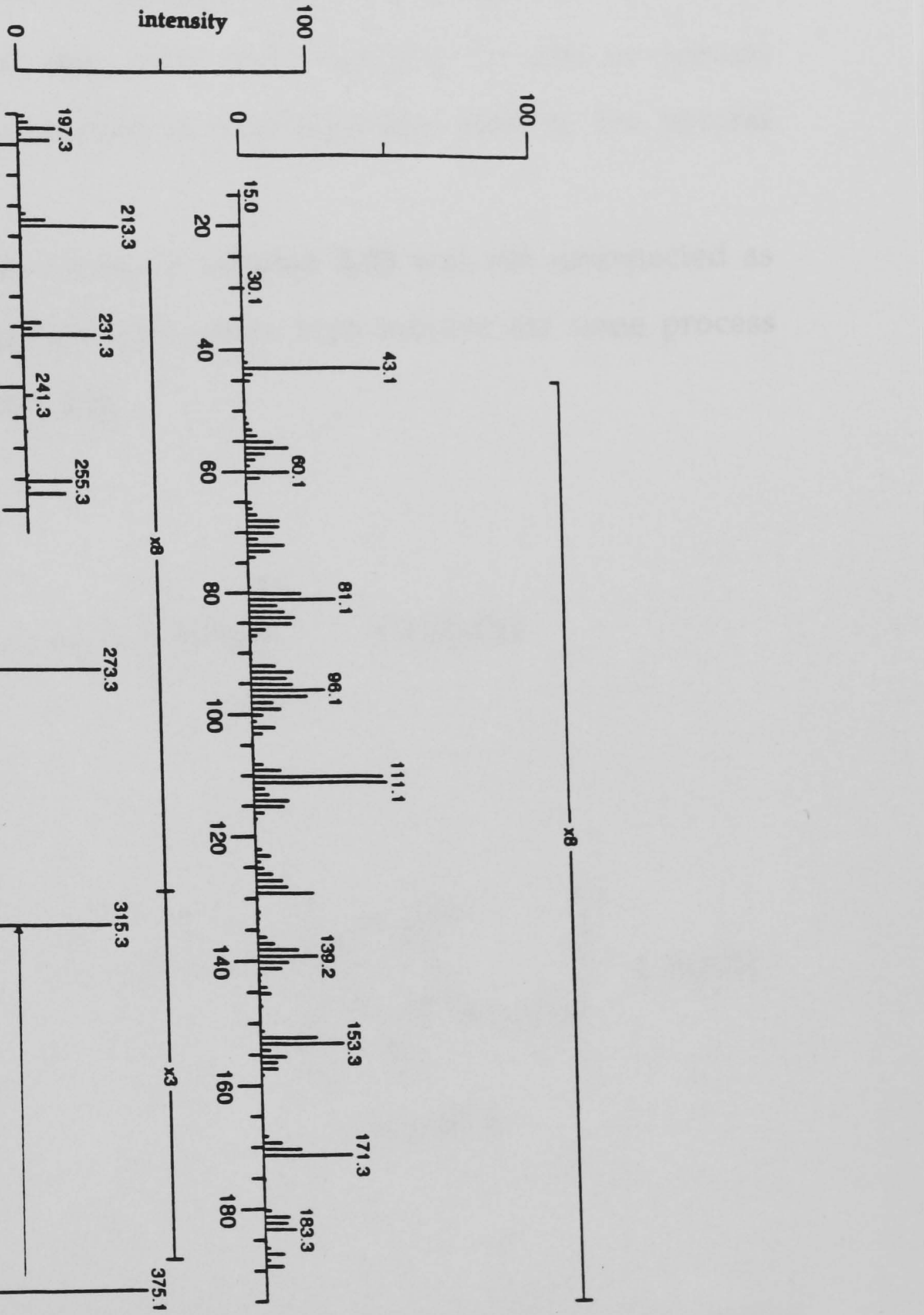
If the elimination occurred as in **scheme 2.05**, then peracetyl DNJ labelled at H4 with deuterium should also show peaks one mass unit higher than natural abundance material, although again m/z 240 should be found as

this label will be retained in methyl acetate. Experimentally, this was found to be the case.

Figure 2.03 shows the complete mass spectrum for peracetyl DNJ labelled at H4 with deuterium. Inset is the partial spectrum for the same compound labelled at H1 (m/z 200-260) These samples were run using a tandem mass spectrometer under FAB conditions where it was possible to select m/z 375 preferentially over m/z 374 (the derivatives were not 100 atom % D). All the fragments are one mass unit higher than the natural abundance material due to the retention of deuterium, including m/z 241 for H1 labelled peracetyl DNJ. The isotope at this position cannot be eliminated in methyl acetate, in contrast to when H4 is labelled.

Figure 2.03

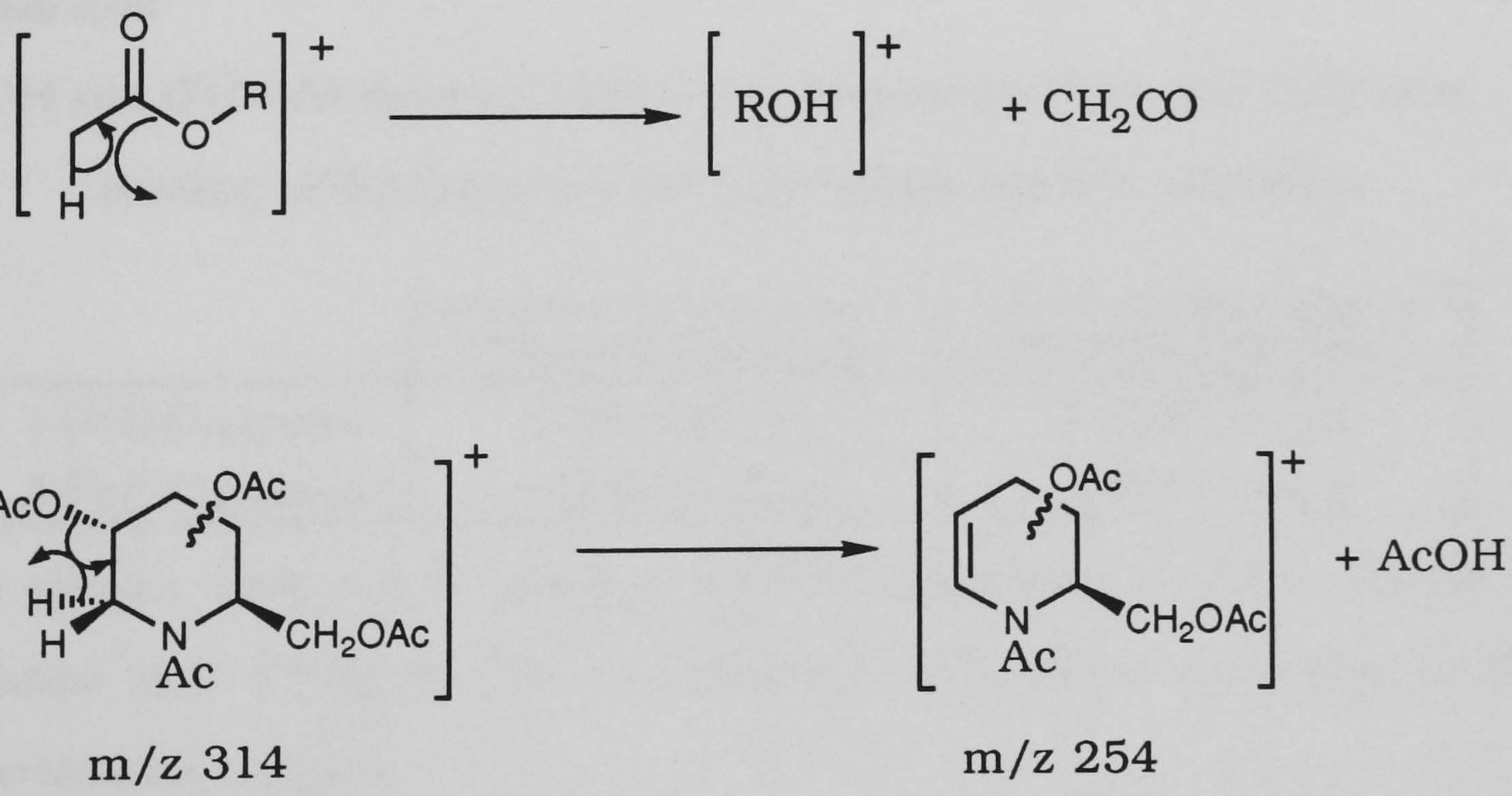
The tandem mass spectrum of peracetyl DNJ labelled at H4 with deuterium (FAB ionisation).



The lower mass fragments cannot be used as reliable evidence in this case because significant fragmentation of the parent molecule has taken place. By selecting m/z 375 in the spectrometer, the majority of the peak intensity is due to DNJ labelled with deuterium, but a proportion is also due to ^{13}C isotopes in DNJ **without** deuterium. Unfortunately, this natural abundance ^{13}C is randomised throughout the molecule. At lower masses where several neutral fragments have been lost, there will inevitably be loss of natural abundance ^{13}C and the appearance of ions normally seen in the natural abundance spectrum.

The type of elimination shown in **scheme 2.05** was not unexpected as the common fragments acetic acid and ketene both involve the same process of hydrogen migration, **scheme 2.06**.

Scheme 2.06



Isotope labelling studies.

The first two labelling experiments used glucose labelled separately at H1 and H2 with deuterium. When the DNJ and DMJ were isolated from the fermentation medium it was found that the H6 protons were enriched, but to very different levels according to which labelled sugar was used.

Furthermore, it was apparent that when 1-[^{2}H]-D-glucose was the carbon source, one of the prochiral protons was labelled exclusively, whereas with 2-[^{2}H]-D-glucose the other one was enriched. **Figure 2.04** summarises the percentage enrichment of the added glucoses and the isolated alkaloids.

Figure 2.04

The ^{2}H enrichment found in peracetyl DNJ and DMJ after feeding 1-[^{2}H]-glucose and 2-[^{2}H]-glucose to *S. subrutilus*.

	Peracetyl DNJ	Peracetyl DMJ
1-[^{2}H]-glucose (33 atom % D)	21.7 ± 2.4 atom % D	21.9 ± 1.7 atom % D
2-[^{2}H]-glucose (25 atom % D)	5.8 ± 0.3 atom % D	6.5 ± 0.3 atom % D

^{2}H NMR of the fully acetylated DNJ and DMJ and comparable shifts in the ^{1}H NMR spectrum (in brackets) are summarised below, **figure 2.05**.

Figure 2.05.

^{1}H and ^{2}H NMR chemical shift values for peracetyl DNJ and DMJ after feeding 1-[^{2}H]-D-glucose and 2-[^{2}H]-D-glucose to *S. subrutilus*.

	Peracetyl DNJ/ppm	Peracetyl DMJ/ppm
1-[^{2}H]-D-glucose	4.49(4.50), H_{6a}	4.47(4.50), H_{6a}
2-[^{2}H]-D-glucose	4.61(4.63), H_{6b}	4.79(4.81), H_{6b}

(All spectra were run in pyridine at 90°C, referenced to the α proton of pyridine at δ 8.70ppm. Full spectroscopic assignments are given in the experimental section).

The first conclusion was that glucose was a precursor to DNJ and DMJ in this fermentation. However, it was perplexing that both labelled glucoses should lead to alkaloids labelled exclusively at H6 when they were originally attached to two different carbon atoms. The deuterium NMR gave one peak for each epimer in the two labelling experiments, showing that randomisation of the label did not occur. Further confirmatory evidence was

required, as the chemical shift difference between a methylene adjacent to O-acetate (H6) and a methylene adjacent to N-acetate (H1) was not particularly large; for instance, $CH_3CH_2O.COR$ δ 3.83 ppm and CH_3CH_2NH COR δ 2.97 ppm[73]. The splitting pattern of both H1 and H6 protons might be similar - both would show a large geminal coupling as well as a much smaller vicinal coupling to either H5 (in the case of H6) or H2 (in the case of H1 protons). If the alkaloids had been isolated as the free base then it would have been straightforward to assign which proton was enriched with deuterium. The presence of the acetyl groups changed the shape of the pyranose ring and it is possible that the close proximity of an acetyl group might effect the chemical shift of H1 and/or H6.

Confirmatory evidence came from mass spectrometry. If deuterium was located at H6, then the fragment due to the loss of methyl acetate, m/z 240, should display an M+1/M isotope ratio equal to that found in the natural abundance sample. **figure 2.06**.

Figure 2.06.

The M+1/M ratio of three ions in the mass spectrum of peracetyl DNJ and DMJ after feeding 1-[^{2}H]-glucose and 2-[^{2}H]-glucose.

	m/z 374	m/z 314	m/z 240
AcDNJ(ex.1-[^{2}H]-glucose)	45.8 ± 2.8	42.1 ± 6.5	13.5 ±3.9
AcDNJ(ex.2-[^{2}H]-glucose)	21.4 ± 3.8	19.0	15.4 ± 2.9
AcDNJ natural abundance	17.5 ± 2.0	16.8 ± 4.6	14.3 ± 4.0
Calculated	18.4	16.1	12.7

(All values correspond to the M+1/M ratio of the particular ion in percent).

The peracetyl DNJ isolated from both labelling experiments had a very similar M+1/M ratio for m/z 240 compared to that from the natural abundance sample, indicating that the label was located at H6. This was particularly noticeable for the 1-[^{2}H]-D-glucose experiment, where the M+1/M ratio was large in m/z 374 and m/z 314, but much smaller in m/z 240. Since

the two protons in question coupled to each other in the ^{1}H NMR spectrum, their assignment from mass spectrometry after feeding 1-[^{2}H]-D-glucose confirmed the more ambiguous data from 2-[^{2}H]-D-glucose.

Further experiments were undertaken with 1-[^{13}C]-D-glucose where the movement of isotopic label was not expected, unlike the case with deuterium. Partial purification of the DNJ indicated that inversion of the glucose molecule had taken place and that C6 at δ 61.91ppm was enriched, **figure 2.07.**

Figure 2.07.

The ^{13}C chemical shift values of DNJ after feeding 1-[^{13}C]-D-glucose to *S. subrutilus*.

Assignment	C1	C5	C6	C2	C3/4	C3/4
Partially purified DNJ	49.23	61.20	61.91*	71.43	72.05	78.92
DNJ ex. Sigma	49.07	61.02	61.72	71.22	71.86	78.81

Since the proton positions in DNJ can be easily assigned and are documented in the literature, it was possible to assign the carbon NMR spectrum from heteronuclear COSY experiments.

It was noticeable in both the ^{13}C and ^{2}H experiments that the enrichment of the original glucose added to the fermentation was higher than that isolated in the alkaloids. 1-[^{13}C]-D-glucose had been added at a level of 50 atom % ^{13}C, but the isolated DNJ contained 33 ± 3 atom % ^{13}C. It was later discovered that the reason for this was because DNJ and DMJ could be produced in trace amounts even if glucose was not present in the medium. Either another glucose source, such as starch, was present in the soyabean medium, or less likely, another non-carbohydrate precursor was being utilised. The amount of DNJ produced without added glucose represented one third of the total when glucose was present (0.02mg mL^{-1} compared to 0.06mg mL^{-1}). This agreed with the values of enrichment when 1-[^{13}C]-D-glucose and 1-[^{2}H]-D-glucose were used as carbon sources. Using these precursors, approximately two thirds of the initial enrichment was recovered

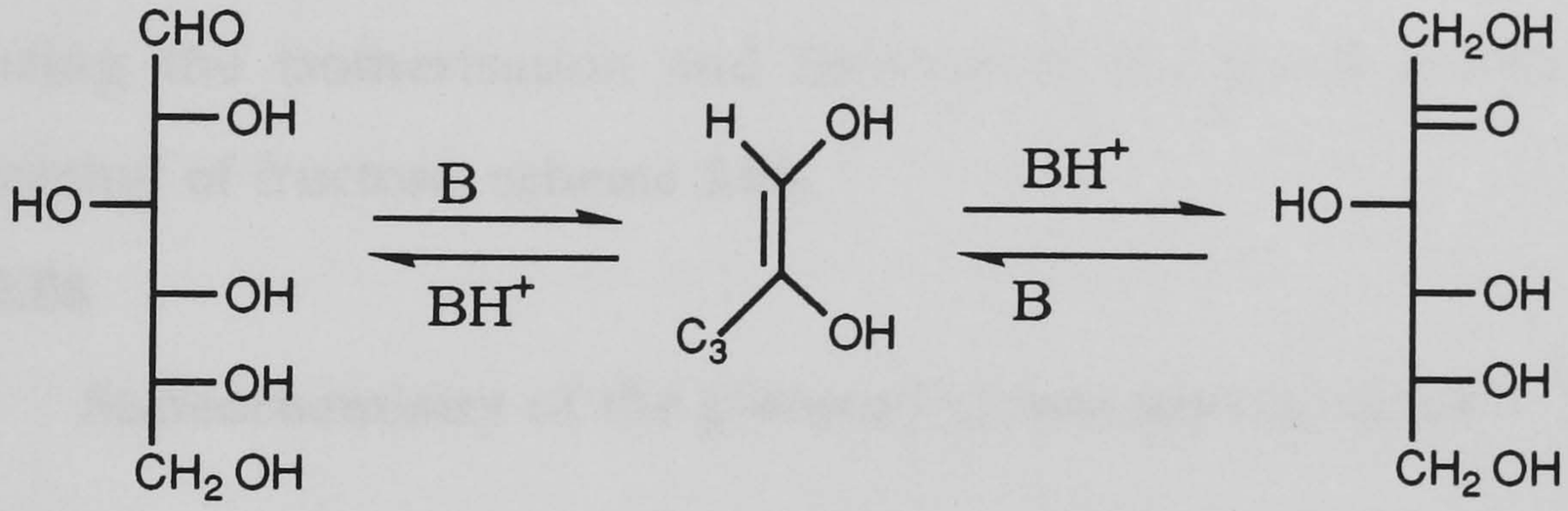
in the isolated alkaloids, whereas with 2-[^{2}H]-D-glucose the level of enrichment was much lower than might have been expected (two thirds of 25% = 16%). As the mechanism of biosynthesis became apparent, this could be explained.

The glucose/fructose isomerisation.

The inversion of the glucose molecule so that label introduced at C1 finished at C6 suggested the first step in the biosynthesis was the isomerisation of glucose to fructose, **scheme 2.07**.

Scheme 2.07

The fructose/glucose isomerisation.



glucose fructose

The reaction is reversible and two enzymes are known to catalyse the process, namely glucose-6-phosphate isomerase[74] and glucose isomerase[75]. At this stage in our experiments no inference was made as to whether the glucose was phosphorylated or not. This isomerisation meant that ^{2}H or ^{13}C at C1 of glucose would result in C6 of DNJ being labelled if cyclisation occurred between C2 and C6. This immediately ruled out the first possibility of a C1/C5 cyclisation which has already been outlined in the introduction, whereby the stereochemistry of DNJ was inherently present in the glucose molecule. A C2/C6 cyclisation gives a carbon backbone which does not contain the complete stereochemistry of DNJ and two chiral centres need to be inverted.

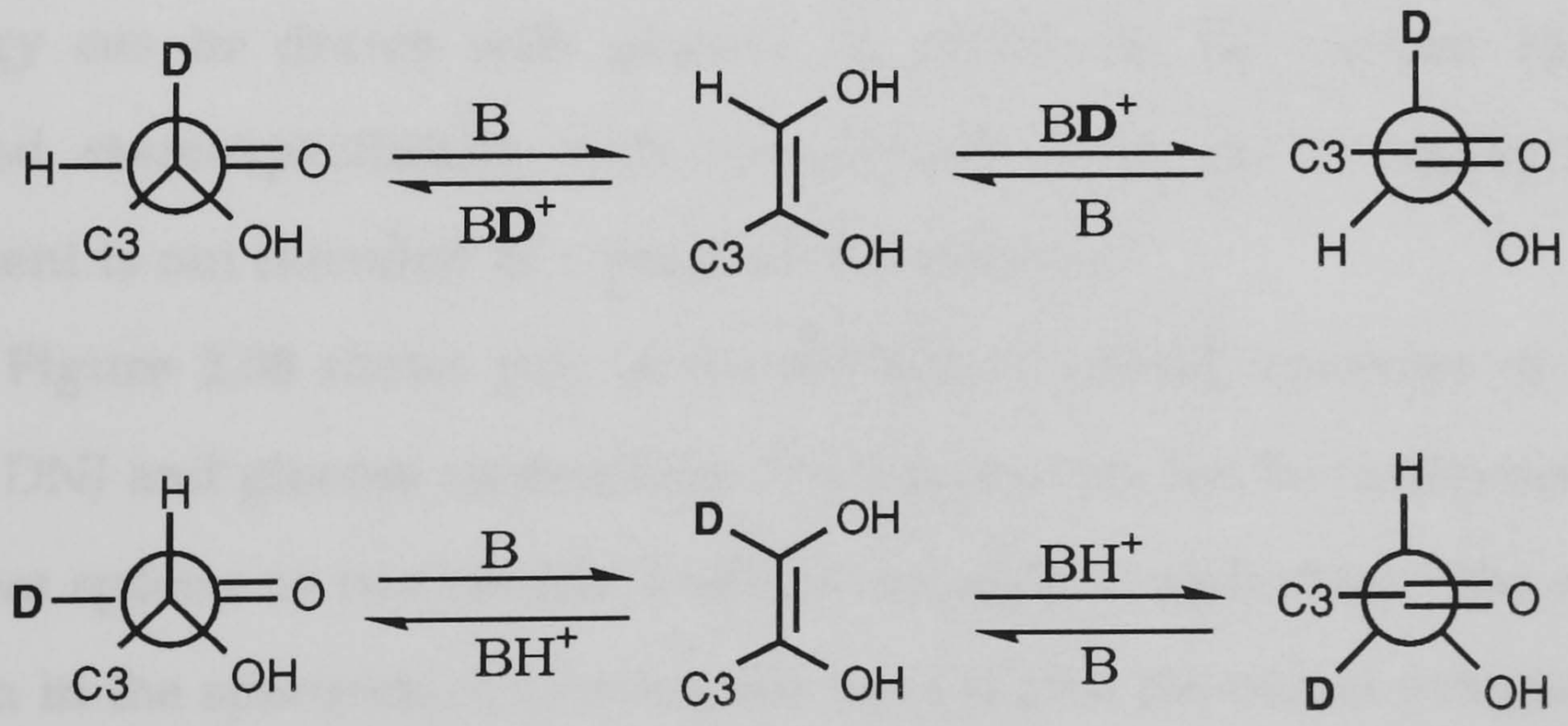
The mechanism of isomerisation catalysed by glucose-6-phosphate

isomerase has been studied in detail[76] and involves abstraction of H2 by a basic site (B in **scheme 2.07**) within the enzyme to form a *cis* ene diol. The involvement of a *trans* ene diol was discarded from evaluation of the radioactivity present in glyoxylate after isomerisation experiments with 1-[^{3}H]-D-glucose-6-phosphate[77]. More recently, deuterium NMR was used to evaluate the position of labelling in glycidyl acetate, which had been synthesised from glycerol following the glucose/fructose isomerisation[78].

Collapse of the ene diol and replacement of the original abstracted proton in a "windscreen wiper" type mechanism leads to fructose. Deuterium introduced at C2 in glucose finishes at the pro-R position in the hydroxymethyl of fructose. Deuterium introduced at C1 of glucose does not move during the isomerisation and finishes at the pro-S position in the hydroxymethyl of fructose, **scheme 2.08.**

Scheme 2.08

Stereochemistry of the glucose/fructose isomerisation



This mechanism satisfies why ^{2}H at H1 and H2 of glucose finishes at H6 in both cases. The lower enrichment of label when using 2-[^{2}H]-D-glucose could arise from two possibilities. First, exchange of the deuterium from the abstracting base with solvent water, so that when the ene diol collapses, protium is replaced at C1 to give fructose. This has been shown to occur by

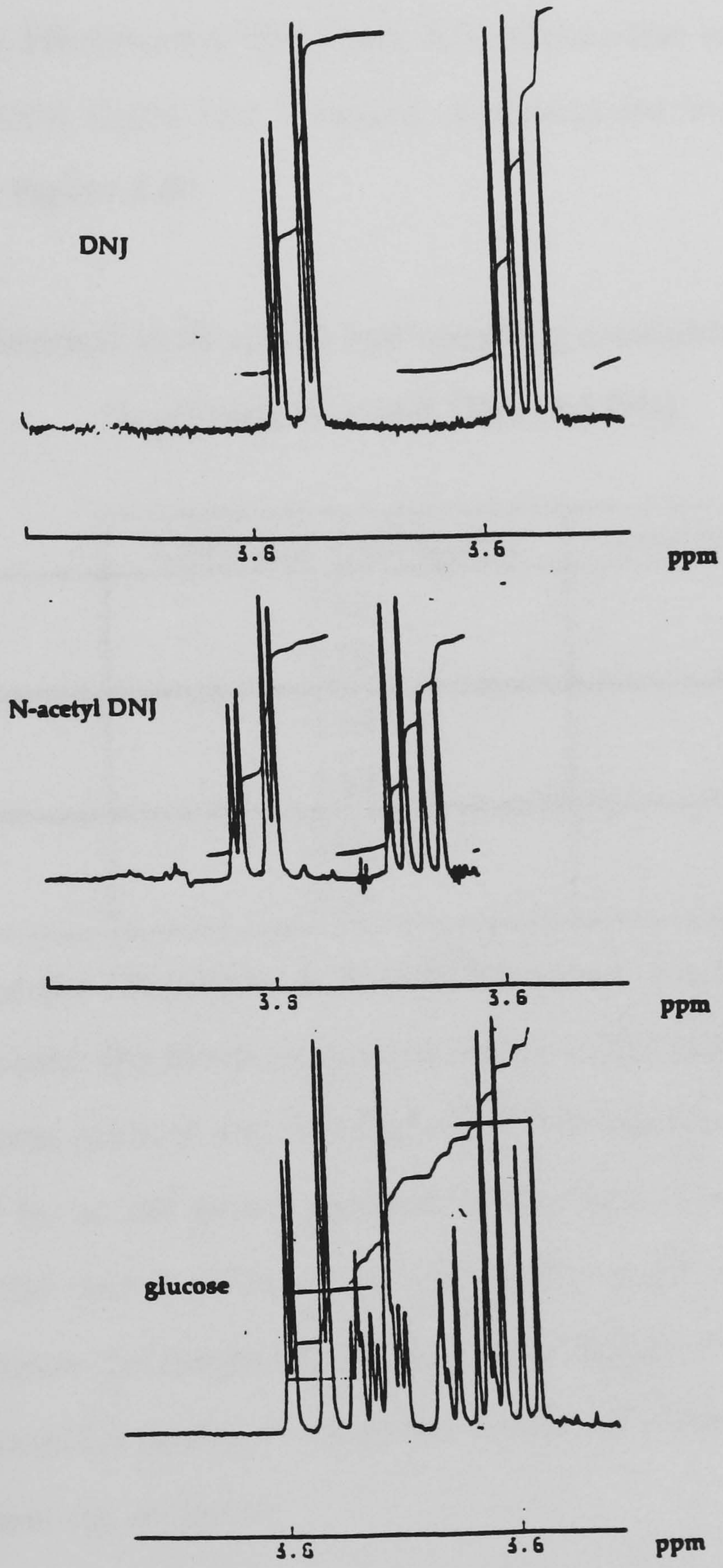
Rose *et al,* with glucose-6-phosphate isomerase and triose phosphate isomerase both catalysing the incorporation of tritium into the respective substrates from tritiated water. A second explanation is that a primary kinetic isotope effect operates and unlabelled glucose isomerises preferentially. In the current study, a fermentation with *S. subrutilus* carried out in deuterium oxide with unlabelled glucose showed that no isotope label was incorporated into the peracetyl DNJ or DMJ, so it seems likely that the isotope effect is operating. It is interesting to note at this stage that under equilibrium conditions, deuterium and tritium were incorporated from solvent using glucose-6-phosphate isomerase[79,76], yet under the same conditions at pH 7.0, essentially no deuterium was incorporated into glucose using glucose isomerase[75]. This highlights the difference between the two enzymes.

Different prochiral protons at H6 in DNJ and DMJ were labelled depending on which deutero-glucose was used in the fermentation. Without synthesising DNJ and DMJ labelled at pro-R and pro-S with deuterium, it is impossible to be absolutely certain which proton is which. However, an analogy can be drawn with glucose, in which the H6 protons have been labelled stereospecifically with deuterium[80]. It should be added that this treatment is not intended as a proof of the labelling.

Figure 2.08 shows part of the 400MHz ^{1}H NMR spectrum of DNJ, N-acetyl DNJ and glucose respectively. The H6 protons can be clearly seen in the first two spectra as two double doublets coupling to each other. The same can be seen in the spectrum of glucose, but there is also the added complication of the minor anomer, which can just be discerned as two smaller double doublets between the main signals.

Figure 2.08

The ^{1}H NMR spectrum of N-acetyl DNJ, glucose and DNJ in the region of the H6 protons.



N-acetyl DNJ adopts a very similar chair conformation to glucose and DNJ as can be seen from the coupling constants of the ring protons. When 1-[^{2}H]-D-glucose was used in the fermentation, the isolated N-acetyl DNJ was labelled at H6 which was at highest frequency (δ 3.82 ppm). Conversely, using 2-[^{2}H]-D-glucose, H6 at lowest frequency (δ 3.68ppm) was labelled.

The chemical shifts and coupling constants for the three compounds are very similar, **figure 2.09.**

Figure 2.09

The ^{1}H NMR chemical shift values and coupling constants for the H6 protons in glucose, N-acetyl DNJ and DNJ.

	Chemical shift (ppm)	Coupling constant(Hz)
Glucose	3.60 3.78	12.3, 5.7 12.3, 2.2
N-acetyl DNJ	3.68 3.82	12.4, 5.6 12.4, 3.0
DNJ	3.56 3.75	11.7, 6.2 11.7, 3.0

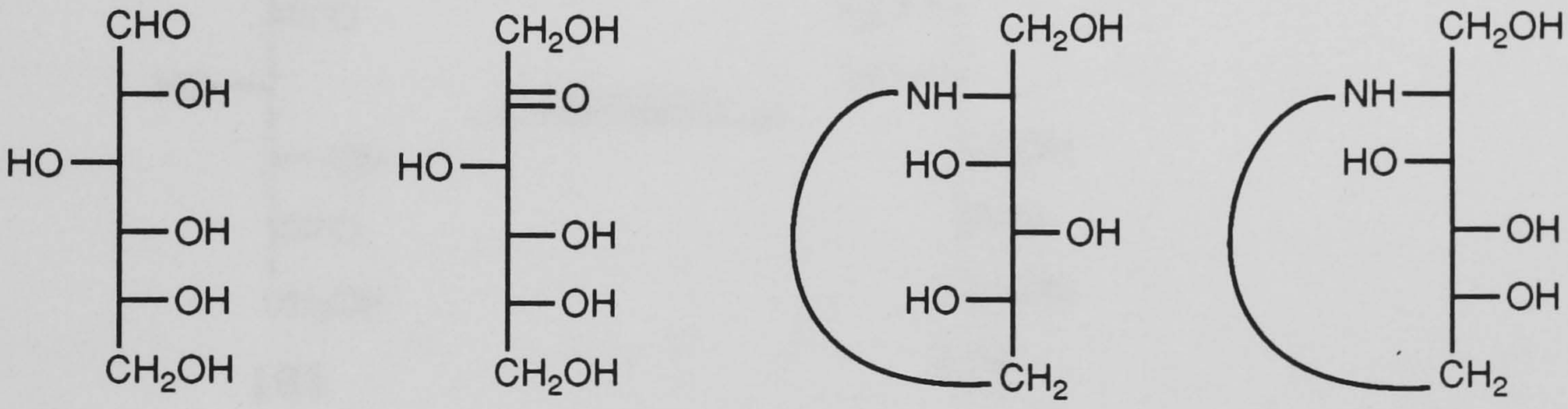
In glucose the H6 proton at highest frequency has been shown to be the pro-S proton, whilst the lower frequency signal is the pro-R. If an analogy can be drawn between glucose and N-acetyl DNJ, the label from feeding 1-[^{2}H]-D-glucose would be at the pro-S position whilst that from 2-[^{2}H]-D-glucose, would be at the pro-R. This is in agreement with the glucose/fructose isomerisation, since the migrating H2 proton is replaced in the pro-R position of the hydroxymethyl group of fructose, whilst H1 from the original glucose molecule, finishes up as pro-S.

Labelling at H5 and H6 of glucose

If a C2/C6 cyclisation is taking place to produce DNJ, the stereochemistry at the original C2 and C5 of glucose needs to be altered. If fructose, or a phosphorylated derivative, is the first intermediate in the biosynthesis, then amination of the ketone would need to give the R configuration at this centre, **figure 2.10**

Figure 2.10

The stereochemical relationship between glucose, fructose, DNJ and DMJ.

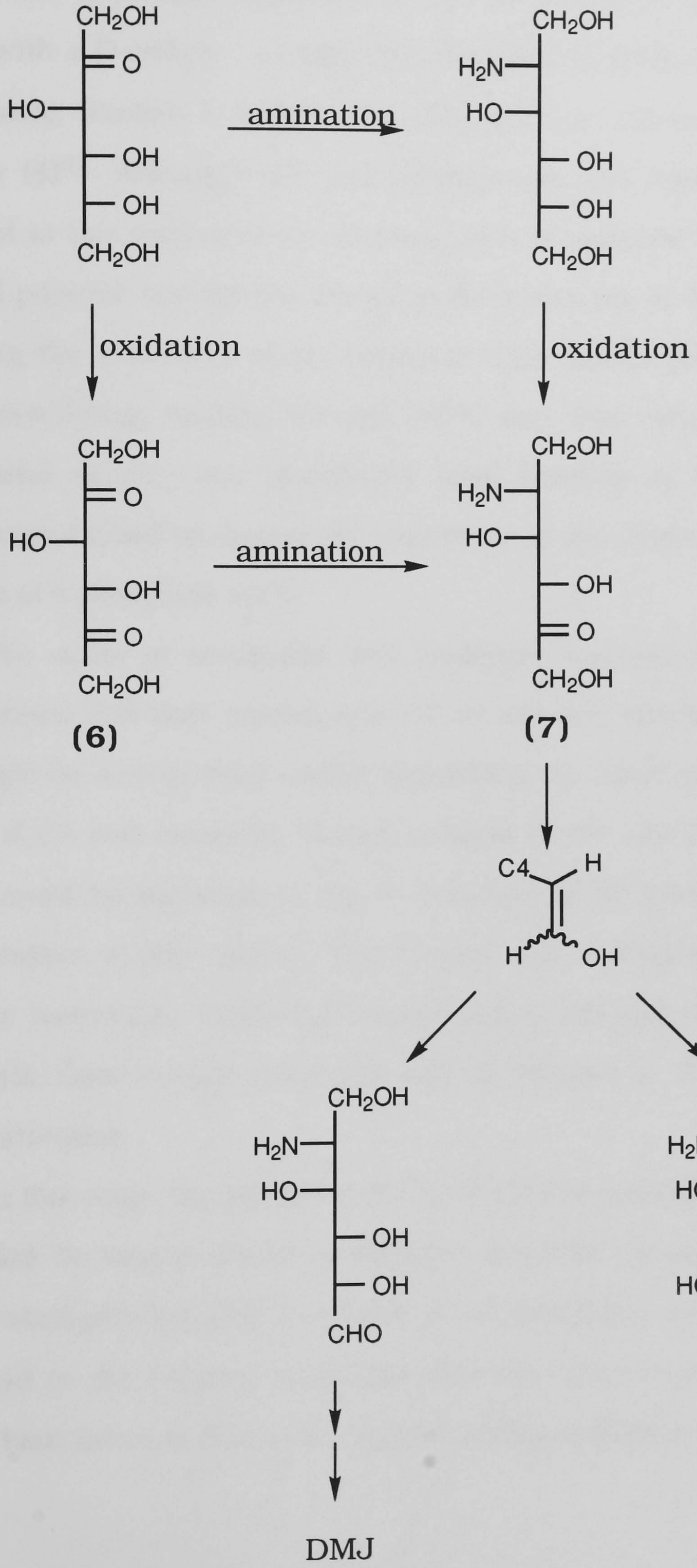


glucose fructose DNJ DMJ

Since DMJ was also produced in the fermentation and originated from fructose in the same way as DNJ, it was likely that the two alkaloids shared a common biosynthetic pathway. DMJ only needs to have one stereochemical centre altered, i.e. C2 of the original glucose.

The transamination reaction already outlined (page 32) allows an amine function to be introduced either side of the prochiral ketone in fructose. The epimerisation at the original C5 of glucose was then thought to involve a process similar to that occurring at C1/C2, **scheme 2.09.**

Scheme 2.09


CH2OH
O
HO
OH
OH
CH2OH
amination
CH2OH
H2N
HO
OH
OH
CH2OH
oxidation
oxidation
CH2OH
O
HO
OH
O
CH2OH
amination
CH2OH
H2N
HO
OH
O
CH2OH
(6)
(7)
C4
H
H
OH
CH2OH
H2N
HO
OH
OH
CHO
CH2OH
H2N
HO
HO
CHO
DMJ
DNJ

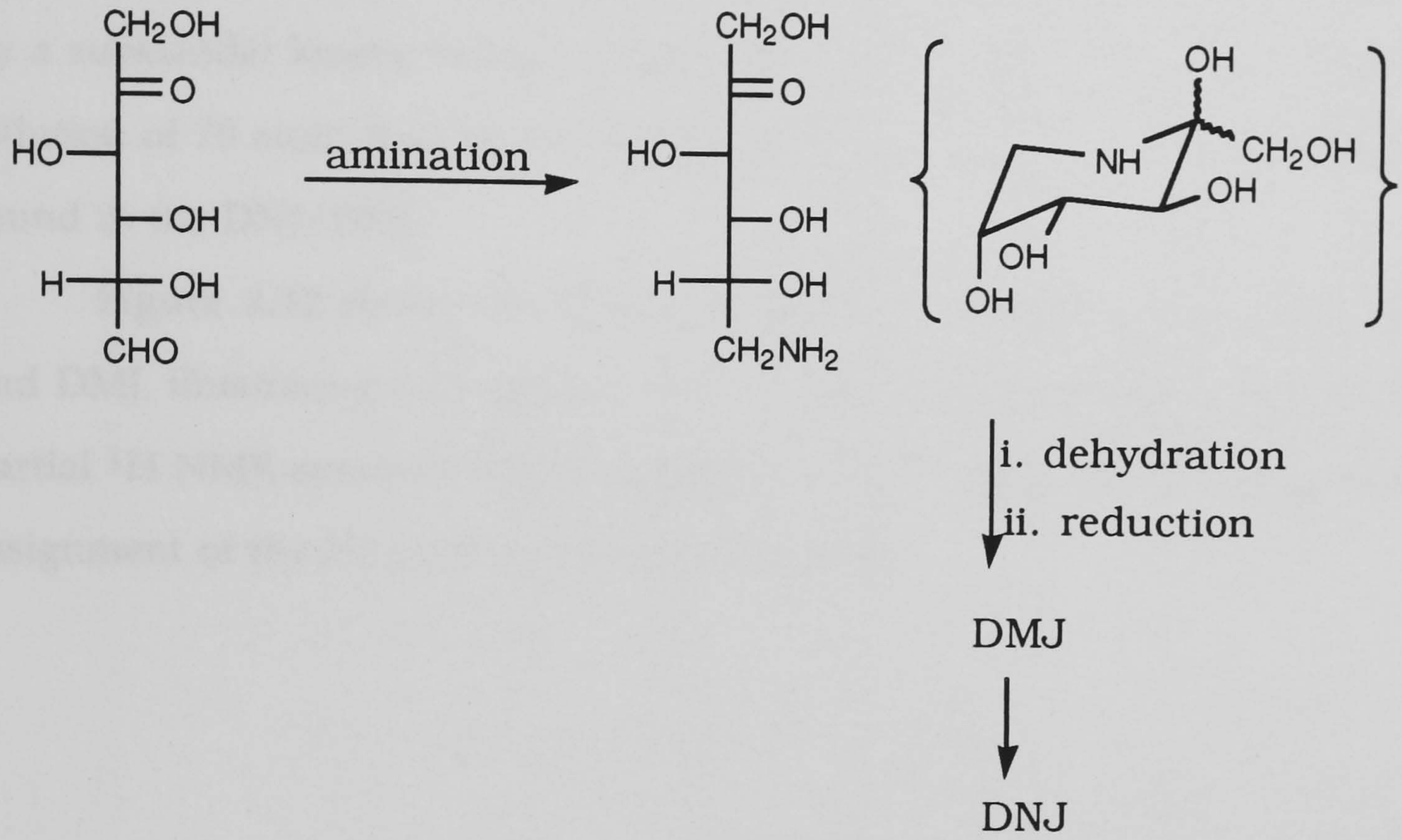
Oxidation of C5 of fructose leads either to a dicarbonyl hexose **(6)**, or if the fructose is already aminated, to the keto amine **(7)**. The oxidation of sugars with a D-*erthyro* configuration has been described in Chapter 1, and for instance, fructose is oxidised by *Gluconobacter suboxydans* to the 5-keto fructose **(6)**[81]. Although **(6)** was symmetrical and labelling experiments indicated at this stage that no randomisation of enriched isotope occurred, it was still possible that **(6)** was bound to the active site of the enzyme, thereby removing the symmetry of the molecule. This would prevent the molecule from dissociating, rotating through 180°C and then rebinding the opposite way round so that any introduced label finished at C1 and C6. Other possibilities existed to remove the symmetry of the intermediate, such as the presence of a phosphate at C6.

The order of amination and oxidation was not certain, but it was hypothesised that after oxidation at C5, an ene diol species could then form. This might be a *cis* or *trans* isomer depending on which prochiral methylene proton of C6 was removed, but on collapse of the ene diol intermediate, a proton could be replaced on the *re* or *si* face of C5 producing the desired epimerisation at this centre. The system was analogous to glucose and mannose isomerase. Following isomerisation, MJ and NJ (shown here in their acylic form) would dehydrate and be reduced to the corresponding 1-deoxy derivative.

At this stage, the amination of C6 of fructose instead of C2 could not be discounted. So long as the stereochemistry at C2 (of the original fructose) was in the R configuration after dehydration and reduction, an amine group at C6 could lead to the 1-deoxy compound directly. Compare the structure of the cyclised keto amine to that of the pyranose form of fructose, **scheme 2.10**.

Scheme 2.10

Amination of fructose at C6 to produce DMJ directly.



To test the hypothesis in **scheme 2.09**, glucose with both H6 protons labelled with deuterium was used in a fermentation. Deuterium was expected at H1 (the non-migrating proton) and also at H2 (the migrating proton) in DNJ. The fermentation in deuterium oxide had shown that deuterium was not incorporated into any part of the DNJ/DMJ molecule, so it was expected that transfer of label would occur rather than exchange with the solvent.

The results indicated that both DNJ and DMJ only had one deuterium label located at $H1_{(equatorial)}$ in both cases. No evidence of even slight enrichment by dideuterated material could be detected by mass spectrometry.

Figure 2.11 summarises the mass spectrometric data for the two alkaloids.

Figure 2.11

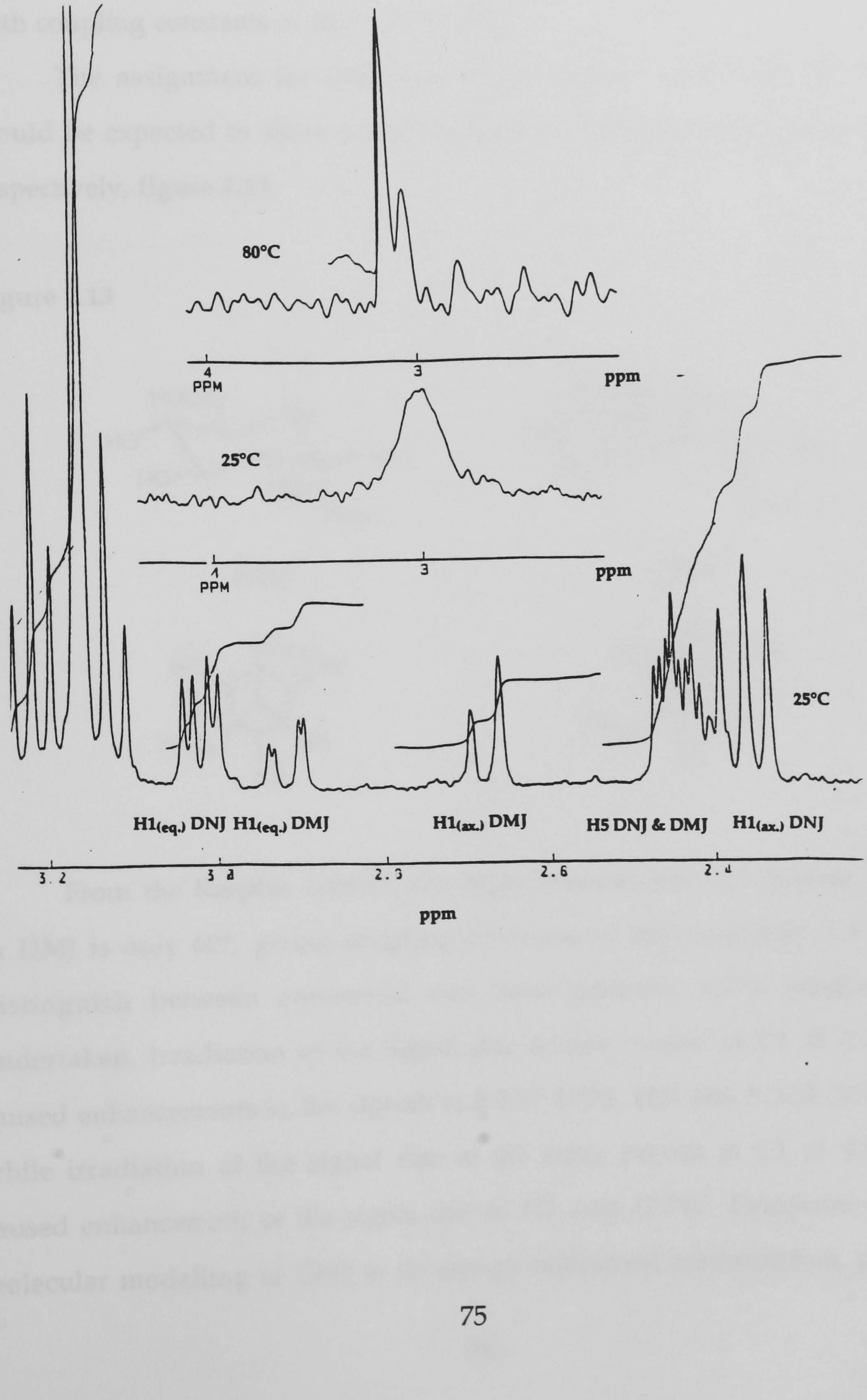
	Peracetyl DNJ	Peracetyl DMJ
6,6-[^{2}H]-D-glucose 70 atom % D per H6	43% (monodeuterated)	41% (monodeuterated)

Each C6 methylene proton in the glucose was enriched to 70 atom % D and the isolated alkaloids contained deuterium to 42 atom % D This suggested that the removal of one of the deuterium atoms was probably not influenced by a substantial kinetic isotope effect, unlike that found for 2-[^{2}H]-D-glucose. Dilution of 70 atom % D by one third would lead to the values of enrichment found in the DNJ/DMJ.

Figure 2.12 shows the deuterium NMR spectra for a mixture of DNJ and DMJ, illustrating how temperature can effect the resolution obtained. The partial ^{1}H NMR spectrum has been included for reference and to illustrate the assignment of the H1 protons for each compound.

Figure 2.12

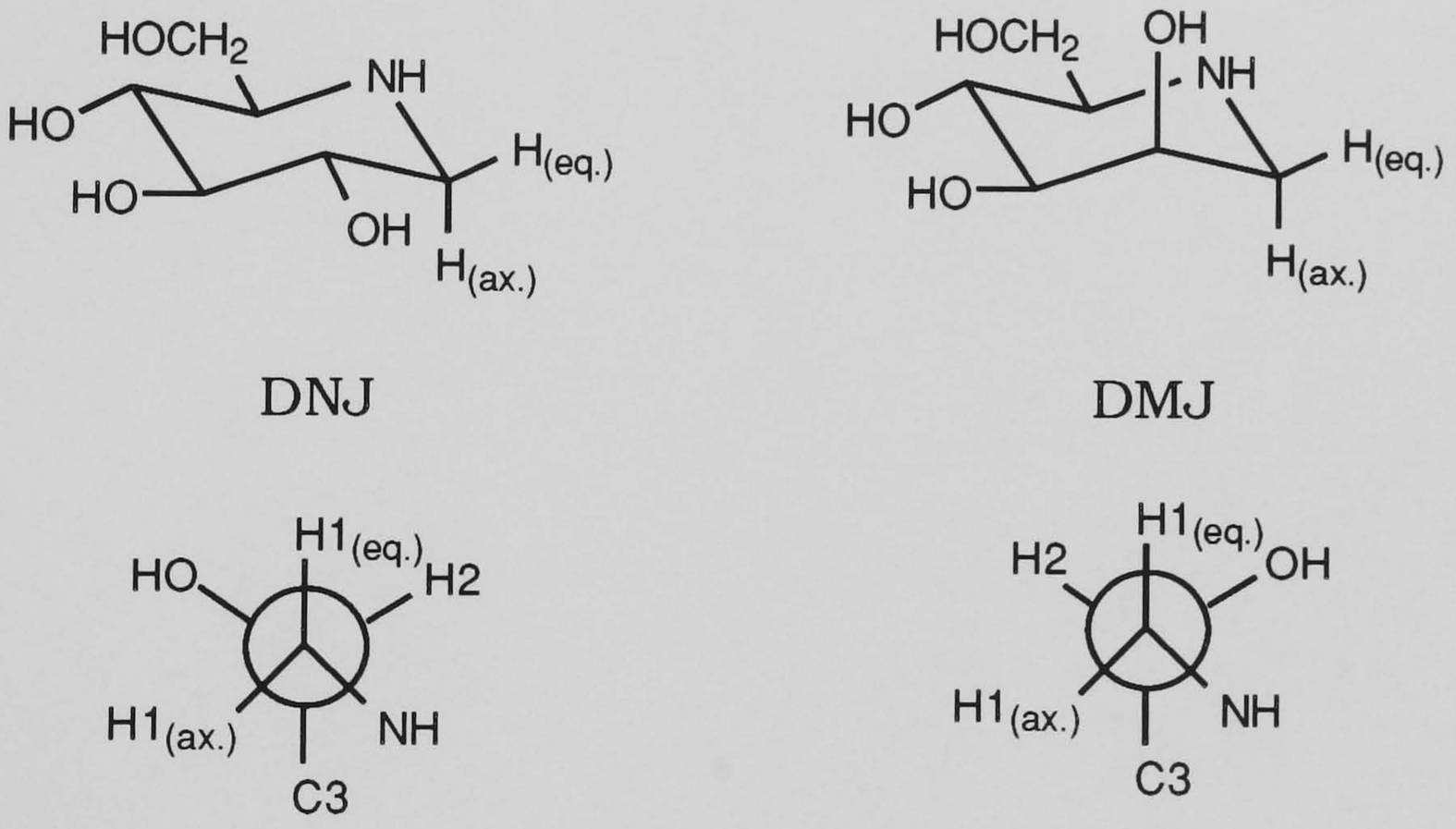
The ^2H NMR spectra for a mixture of DNJ and DMJ isolated from *S. subrutilus* after adding 6,6-[$^2H_2$]-D-glucose.



From the 400 MHz NMR spectrum of DNJ in the region of H1, it was possible to distinguish $H1_{(equatorial)}$ and $H1_{(axial)}$. $H1_{(axial)}$ shows two large axial coupling constants and appears as a large double doublet (almost a triplet) at δ 2.38 ppm, whilst $H1_{(equatorial)}$ shows a large axial coupling along with a much smaller equatorial one. This proton appears as a double doublet with coupling constants of 12.3 and 5.1 Hz.

The assignment for DMJ was not so simple, since both H1 protons would be expected to show a large and then a small coupling to H1 and H2 respectively, **figure 2.13**.

Figure 2.13

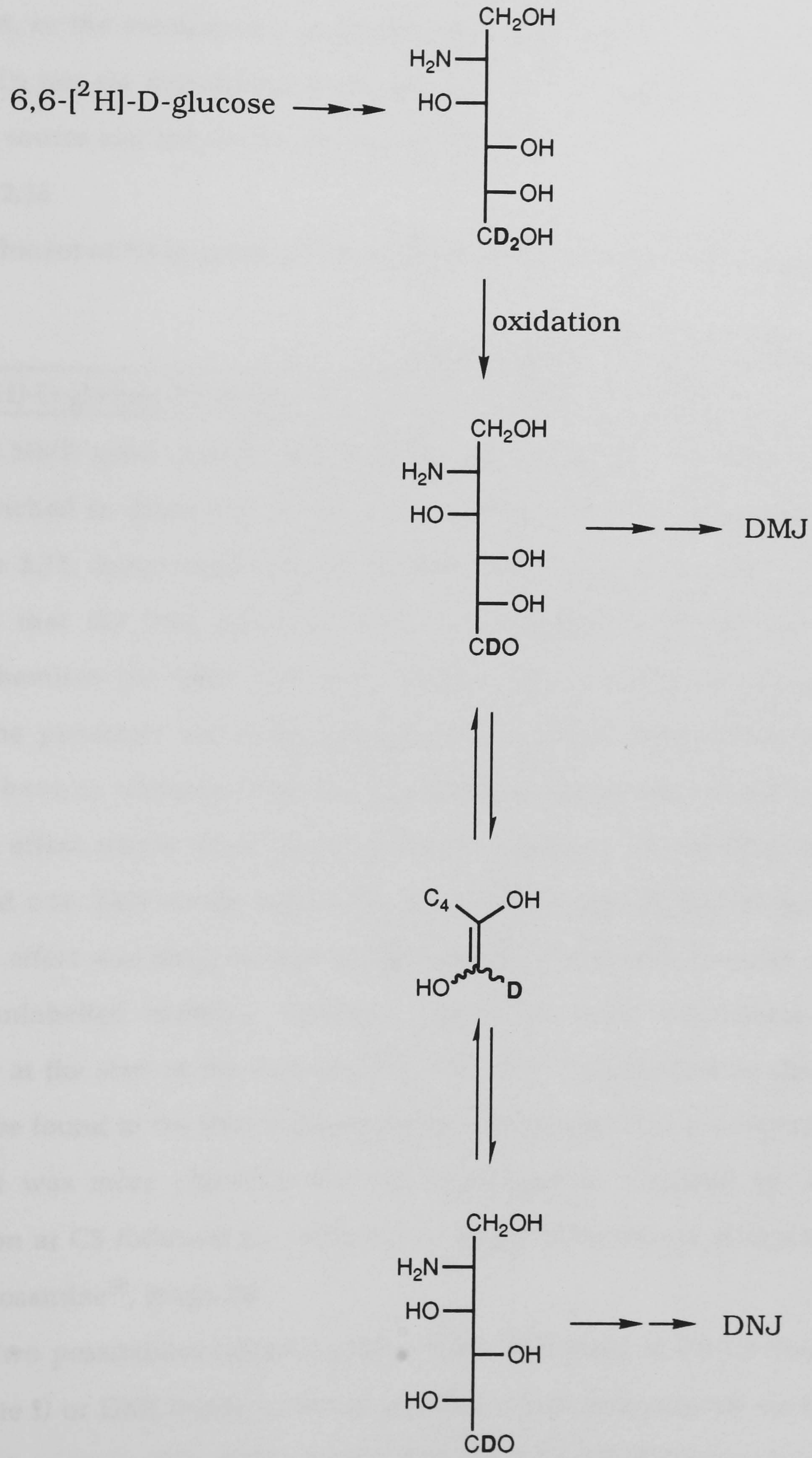


From the Karplus curve[82], the angle between both H1 protons and H2 in DMJ is only 60°, giving coupling constants of approximately 2.4 Hz. To distinguish between equatorial and axial protons, n.O.e studies were undertaken. Irradiation of the signal due to one proton at C1 (δ 2.65ppm) caused enhancements to the signals at δ 3.92 (7.9%, H2) and δ 3.50 (5.3%, H3), while irradiation of the signal due to the other proton at C1 (δ 2.90ppm) caused enhancement of the signal due to H2 only (7.7%). Computer assisted molecular modelling of DMJ in its energy minimised conformation, gave the

approximate distances H3-$H1_{(eq.)}$ as 3.8 Å and H3-$H1_{(ax.)}$ as 2.7 Å, suggesting that the proton at lower frequency (δ 2.65 ppm) was the axial one. The ^1H NMR spectrum of the hydrochloride salt of DMJ has been fully assigned in the literature[70] and this places the axial proton at lower frequency to the equatorial one as well. Protonation of the amine should not in theory alter the relative positions of $H1_{(eq.)}$ and $H1_{(ax.)}$, although it does increase their chemical shift value compared to the free base.

The evidence so far suggested that oxidation did not occur at C5, but at C6 and that a possible method of epimerisation at C5 might still involve a similar mechanism to the fructose/glucose isomerisation, **scheme 2.12**.

Scheme 2.11



Enolisation of the aldehyde could occur, but instead of isomerising to the 5-

keto derivative, the aldehyde reforms with the proton replaced to give the epimer at C5. An enzyme might be responsible for replacing the proton at this position, or the mechanism was possibly non-enzymatic.

To test the hypothesis in **scheme 2.11**, 5-[^{2}H]-D-glucose was used as a carbon source and the results are outlined below.

Figure 2.14

Enrichment of ^{2}H in peracetyl DNJ and DMJ after feeding 5-[^{2}H]-D-glucose.

	Peracetyl DMJ	Peracetyl DNJ
5-[^{2}H]-D-glucose (75 atom% D)	22.7 ± 1.7% D	none

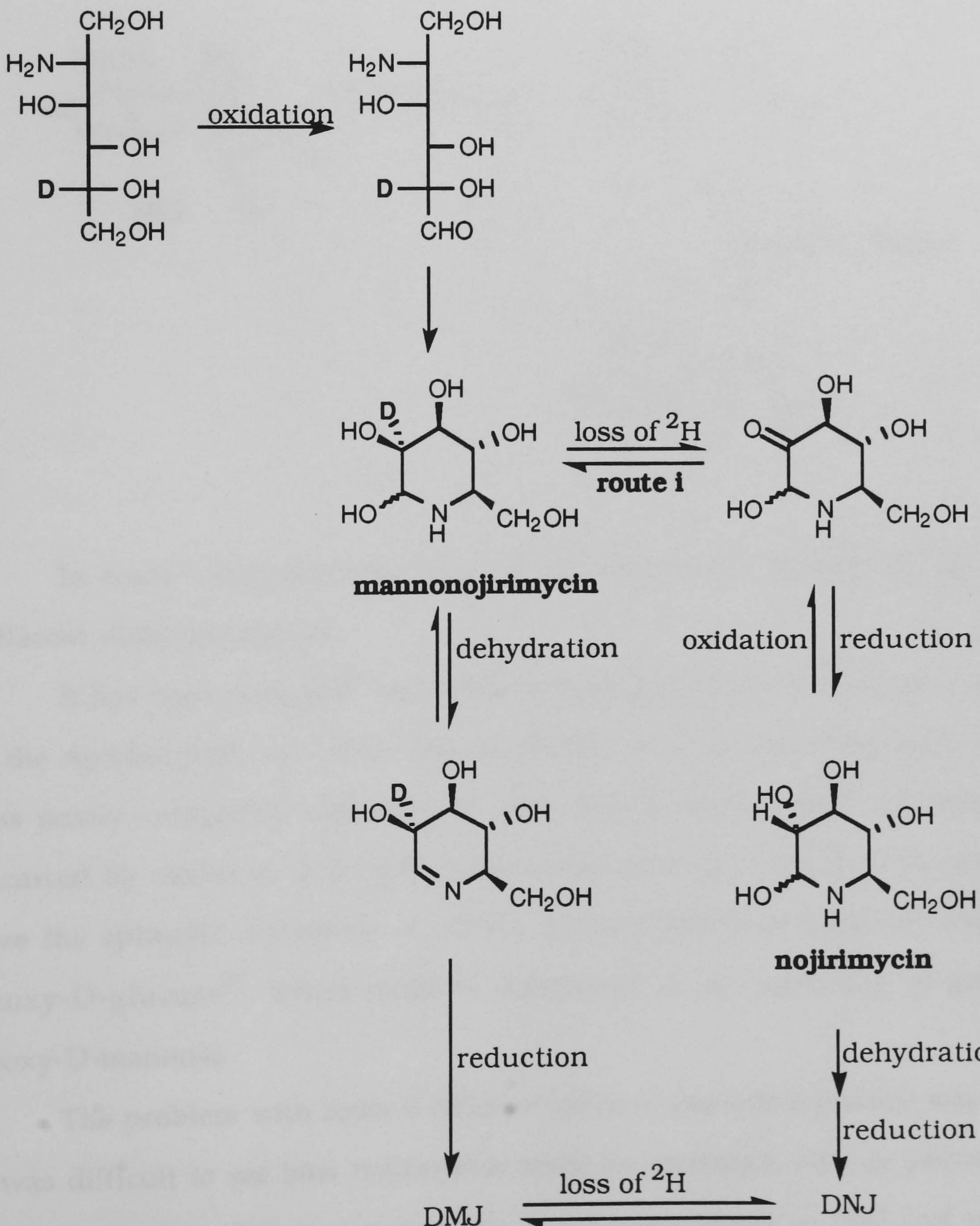
The ^{2}H NMR spectrum indicated that DMJ was labelled at H2 whilst DNJ was not enriched in deuterium at all. If the epimerisation was proceeding as in **scheme 2.11**, deuterium would be expected at H2 in DNJ as well. It could be argued that the first precursor formed in **scheme 2.11** had the correct stereochemistry for DMJ. The DMJ formed in the fermentation would result from the precursor remaining untouched by an epimerising enzyme. DNJ would have to originate from an epimerisation at this centre and a kinetic isotope effect would select for the natural abundance intermediate over the enriched one. This would explain the loss of deuterium in DNJ if the kinetic isotope effect was large enough to discriminate completely between labelled from unlabelled material. However, given the high enrichment of the glucose at the start of the fermentation, it is very unlikely that no deuterium would be found in the DNJ if the mechanism in **scheme 2.11** was operating.

It was more plausible that the epimerisation occurred by a direct oxidation at C5 followed by reduction as in the biosynthesis of mycosamine and perosamine[50], (page 33).

Two possibilities existed (**scheme 2.12**). Oxidation at the C2 position of MJ (route i) or DMJ (route ii) would produce a keto intermediate (in the case of MJ this may or may not be cyclic). Reduction of this derivative by NADH

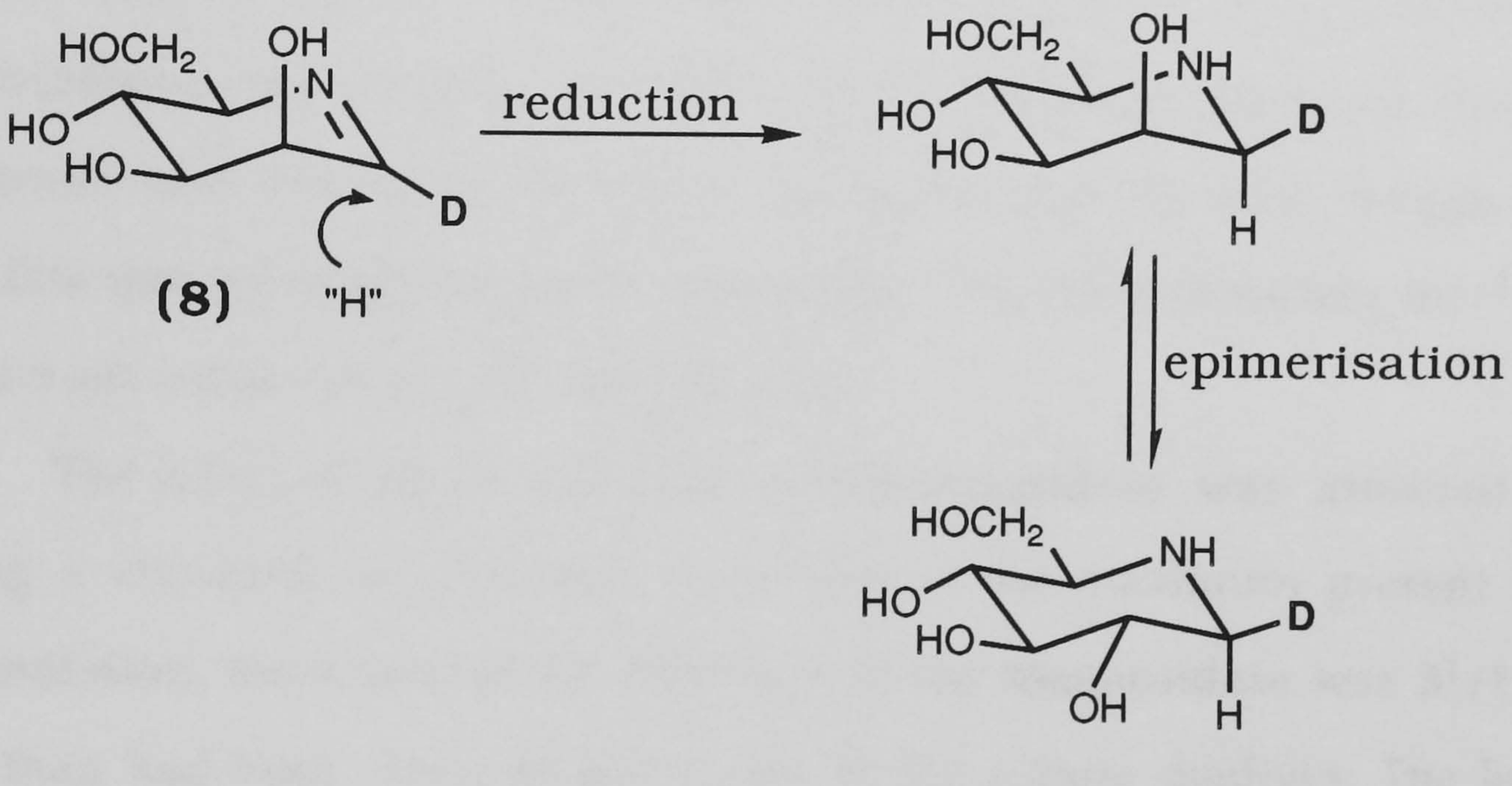
would give the NJ or DNJ alkaloid respectively. The deuterium would be retained in the MJ and DMJ if the rate at which the equilibrium between the two epimers was established was not fast. If the two diastereoisomers interconverted rapidly in either route i or route ii, the deuterium would probably be lost.

Scheme 2.12



From the studies undertaken with 6,6-[$^2H_2$]-D-glucose, the reduction of the unsaturated ring to produce label at $H1_{(equatorial)}$ in DNJ and DMJ must occur from the underside (α face) in both cases. A common intermediate for this reduction would seem sensible to give the same labelling pattern. On this basis, route ii was attractive because the intermediate **(8)** was the precursor to both DNJ and DMJ, **scheme 2.13.**

Scheme 2.13



In route i this reduction step would need to be carried out on two different diastereoisomers.

It had been reported[83] that DNJ could be epimerised to DMJ by a strain of the *Agrobacterium sp* . After 24h incubation with the microorganism, DNJ was nearly completely converted to DMJ. It was thought that this reaction occurred by oxidation to a cyclic 2-keto compound followed by reduction to give the epimeric derivative. A similar process had been observed with 1-deoxy-D-glucose[83], which could be epimerised in the same way to give 1-deoxy-D-mannose.

The problem with route ii (epimerisation at the 1-deoxy level) was that it was difficult to see how nojirimycin could be produced. NJ was present in the fermentation , but its involvement in the biosynthesis of DNJ had yet to

be proved.

The presence of mannojirimycin was suggested at this time from qualitative evidence using the mannosidase assay. At the end of the fermentation period, the level of NJ was estimated by the trehalase assay, and it was shown to be present at a concentration of 0.05mg mL^{-1}. This agreed well with previous time course studies. The effect of MJ on trehalase was not known, although DMJ had shown no inhibition of the enzyme at concentrations up to twenty times that present in the fermentation. Nonetheless, the decrease in inhibition of trehalase following heat/acid treatment was attributed entirely to the presence of NJ, even though it was possible that MJ might be partly responsible. The value of 0.05mg mL^{-1} was a maximum value for the NJ concentration.

The effect of NJ on jack bean α-D-mannosidase was assessed next. Using a standard concentration twice that of the maximum present in the fermentation, the extent of the inhibition of the mannosidase was $3^{1}/2$ times less than had been observed previously in the culture medium. The level of NJ was not sufficient to account for the observed decrease in inhibition of the mannosidase following heat treatment of the fermentation sample. Since the experiments concentrated on the difference in inhibition before and after heat/acid treatment, the DNJ and DMJ present would not effect these conclusions. Although MJ has not been isolated from *S. subrutilus*, it has been found in *S. lavendulae*, a closely related species[84,85]

At the time these studies were carried out, a gas chromatography assay also indicated that DMJ was produced before any DNJ. Forty eight hours after inoculating a fermentation, DMJ was easily detected in the culture broth (not quantified), whereas only a trace of DNJ was present. This agreed with the time course studies of DNJ production. After a further 26h, the same fermentation contained $2^{1}/2$ times the amount of DNJ as DMJ, a value that was consistently found in all the fermentations and which remained after the

full time course had been completed. This did not prove that DNJ was formed from DMJ, since if MJ was the common intermediate, dehydration and reduction need only occur to produce DMJ. With DNJ, an additional epimerisation step needed to be included and this possibly slowed the production of this alkaloid.

The investigations so far had not proved whether NJ was a precursor to DNJ or not. The addition of aliquots of unlabelled NJ to the fermentation at different time intervals was carried out to investigate whether the yield of DNJ increased. Using the trehalase assay, no significant change in the yield of DNJ was noted. NJ was present at a higher concentration than DNJ until the end of the fermentation, and it was possible that only a proportion of the NJ was finally converted to the DNJ. The latter, for instance, might inhibit the enzyme responsible for its manufacture from NJ. Labelled NJ needed to be introduced into the fermentation to see if it was incorporated into DNJ. The reported instability of NJ meant that initial studies concentrated on labelling with a tritium isotope at H6. This was in a position which was away from any possible dehydration and reduction processes and the tritium could still be detected even if the incorporation of NJ into DNJ was low.

Problems arose with this approach as it was found that if [^{3}H] labelled NJ was added to a fermentation just before sterilisation and purification, the isolated DNJ contained radioactivity. Impurities from nojirimycin decomposition appeared to be contaminating the DNJ, despite various attempts to purify or remove them.

NJ was then synthesised with two deuterium labels at H6 and when this was added to the fermentation after 70h and 119h, the DNJ and DMJ showed the following enrichment, **figure 2.15.**

Figure 2.15

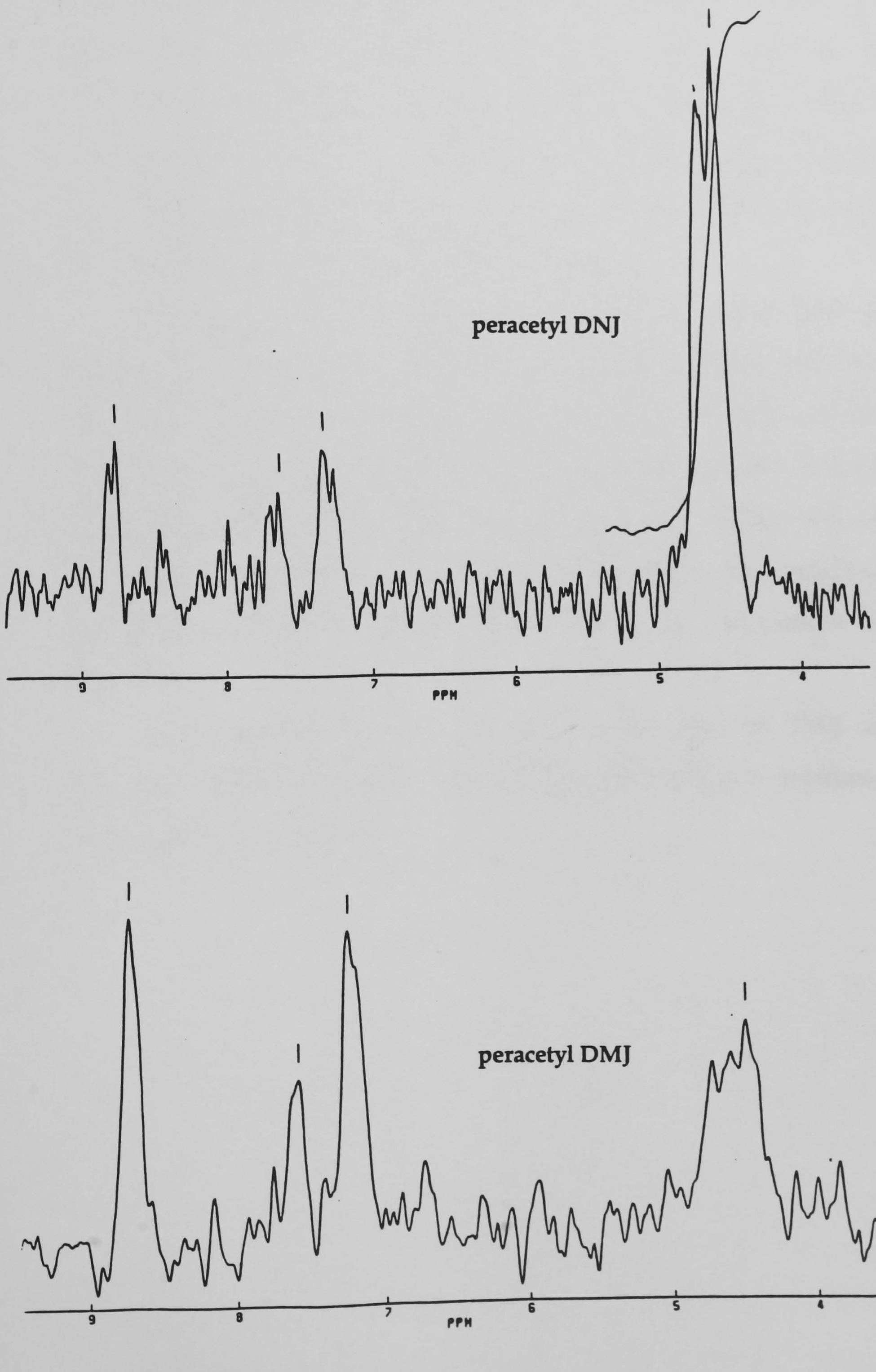
The ^{2}H enrichment in peracetyl DNJ and DMJ after feeding 6.6-[^{2}H]-nojirimycin to *S. subrutilus*.

	Peracetyl DNJ	Peracetyl DMJ
NJ (99 atom % D per H6) (29mg added overall)	10.9 ± 2.9 % (dideuterated)	4.4 ± 2.0 % (dideuterated)

Figure 2.16 shows the ^{2}H NMR spectrum of the peracetyl DNJ and DMJ. In each case, the natural abundance ^{2}H of the pyridine solvent can be seen between δ 7.00-9.00 ppm in addition to the H6 ^{2}H resonance of the alkaloid.

Figure 2.16

The ^2H NMR spectrum of peracetyl DNJ and DMJ after adding 6,6-[$^2H_2$]-nojirimycin to *S. subrutilus*.



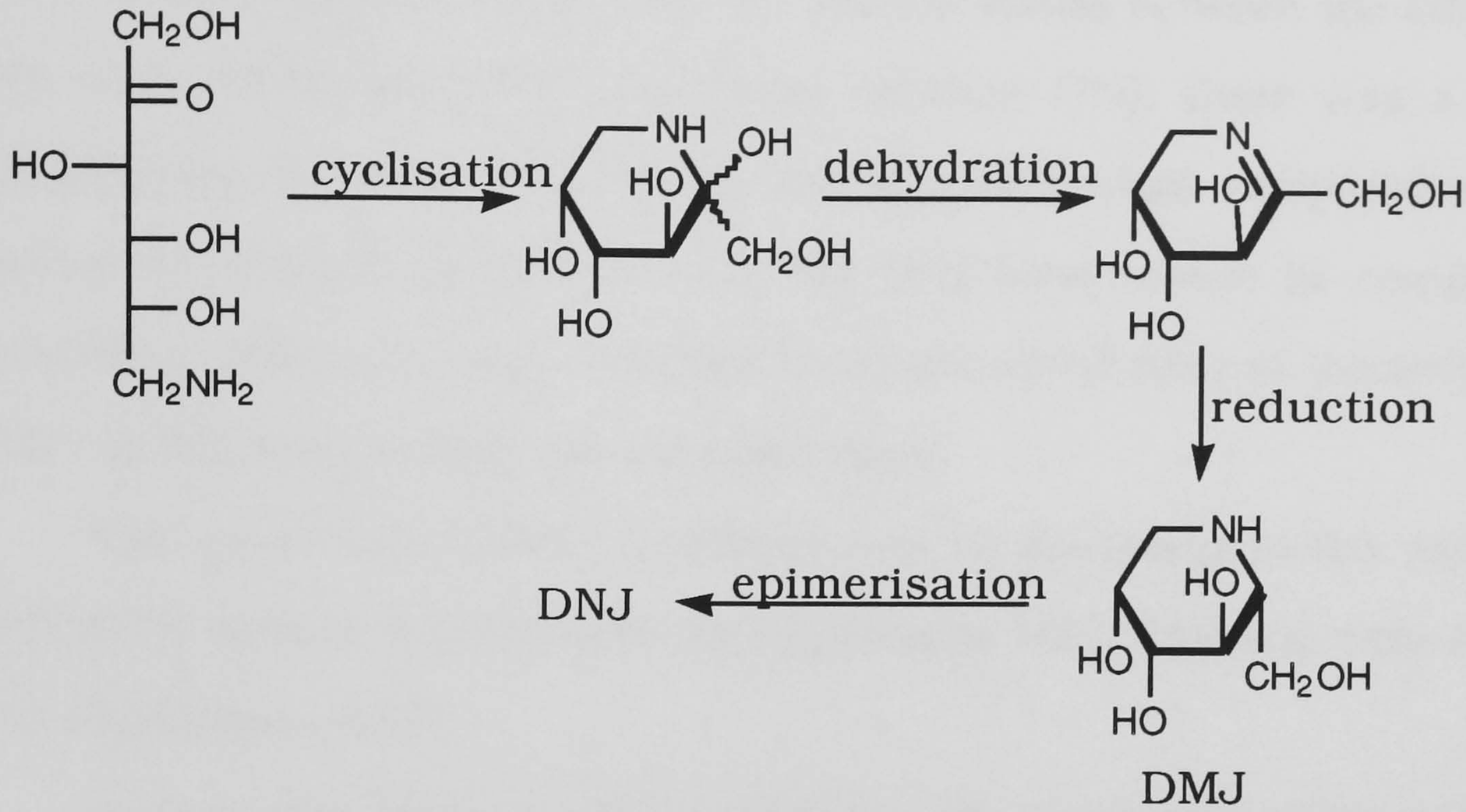
From the mass spectrometric data, only a small fraction of the DNJ and DMJ was enriched with deuterium considering that the maximum concentration of NJ in the fermentation at any stage was under 0.07mg mL^{-1} (600mL fermentation = 42mg). For the amount added, if all the labelled NJ had been incorporated, the total enrichment of DNJ and DMJ combined would be nearer 40%. Our ^{1}H NMR studies on the stability of NJ had shown that the compound was stable at pD 7.0 for at least 1 week, but this was not representative of the fermentation conditions.

The fact that the ^{2}H label was also found in the DMJ shows the epimerisation reaction in operation. The level of enrichment in the DNJ was nearly $2^{1}/2$ times that of the DMJ, which was the same ratio in which the two alkaloids were found in *S. subrutilus* cultures. The addition of deuterated NJ presumably upsets the equilibrium between NJ and MJ (or DNJ and DMJ if the NJ was converted to the deoxy derivative first). The restoration of this equilibrium was then reflected in the percentage enrichment of the two alkaloids.

The confirmation that NJ was a precursor to DNJ eliminated amination at C6 of fructose as this mechanism did not produce NJ as an intermediate, **scheme 2.14**.

Scheme 2.14

The formation of DNJ and DMJ by amination at C6 of fructose.



Confirmation of epimerisation.

The timing of the epimerisation of the two alkaloids was still uncertain. NJ had been shown to be a precursor for DMJ, but this could be via MJ or DNJ (route i or route ii in **scheme 2.12**). DNJ labelled at H6 was fed to the fermentation after 96h and after a further 67h, the DNJ and DMJ were isolated. The results are summarised below, **figure 2.17.**

Figure 2.17.

The ^{2}H enrichment in peracetyl DNJ and DMJ after feeding 6,6-[$^{2}H_2$]-1-deoxynojirimycin to *S. subrutilus*.

	Peracetyl DNJ	Peracetyl DMJ
DNJ(99 atom % D per H6) 10mg added overall	15.9 ± 1.3 % D (dideuterated)	1.6 ± 0.7 % D (dideuterated)

These results indicated that little or no epimerisation occurred at the 1-deoxy level (route ii was excluded). Although DMJ would be converted into DNJ in route ii, the epimerisation was an equilibrium process since labelled NJ was a precursor to DMJ. From this, the addition of labelled DNJ would shift the equilibrium to DMJ and label should be found in this alkaloid if

route ii was in operation.

The experiment with labelled NJ had only shown a small enrichment (4.5%) of DMJ with deuterium, but the relative values between the DNJ and DMJ were important. With introduced labelled DNJ, there was a large difference in the enrichment of the two alkaloids when compared to the labelled NJ studies. Epimerisation at the DNJ level cannot be completely excluded as there was some evidence for slight enrichment of deuterium in DMJ, but this was certainly not the major route.

The use of dideuterated precursors was an advantage in this study as the M+2/M ratio of the molecular ion of peracetyl DNJ/DMJ was only 3.4% ± 0.7% (calculated = 3.3%).

Assume that peracetyl DNJ or DMJ has 5% enrichment of the mono or dideuterated substrate and the intensity of the natural abundance molecular ion was 95 units:

a. If 5% monodeuterated, the intensity of M+1 will be (0.18 x 95) + 5.

b. If 5% dideuterated, the intensity of M+2 will be (0.034 x 95) + 5.

For natural abundance peracetyl DNJ, M+1/M = 18% and M+2/M = 3.4%

In case **a**, the enrichment only represents 22% of the total abundance at that mass, whereas in case **b**, the enrichment is nearly 61% of the M+2 abundance. It should be easier to detect the isotopic label in case **b** to case **a**.

The addition of labelled DNJ to the fermentation also allowed an estimate of the native DNJ concentration to be made by the isotope dilution method. Since 10mg of dideuterated DNJ was added to the fermentation, the observed enrichment of 16% in the isolated alkaloid indicated that 62mg of DNJ was present at the end. Gas chromatography analysis of the fermentation **after** the first purification step (cation exchange chromatography) showed that 52mg of DNJ was present. This did not take into account that, **a)** some labelled DNJ was perhaps epimerised to DMJ, or metabolised in some other way, or **b)** that some DNJ was lost during the first purification step. In retrospect, it was a

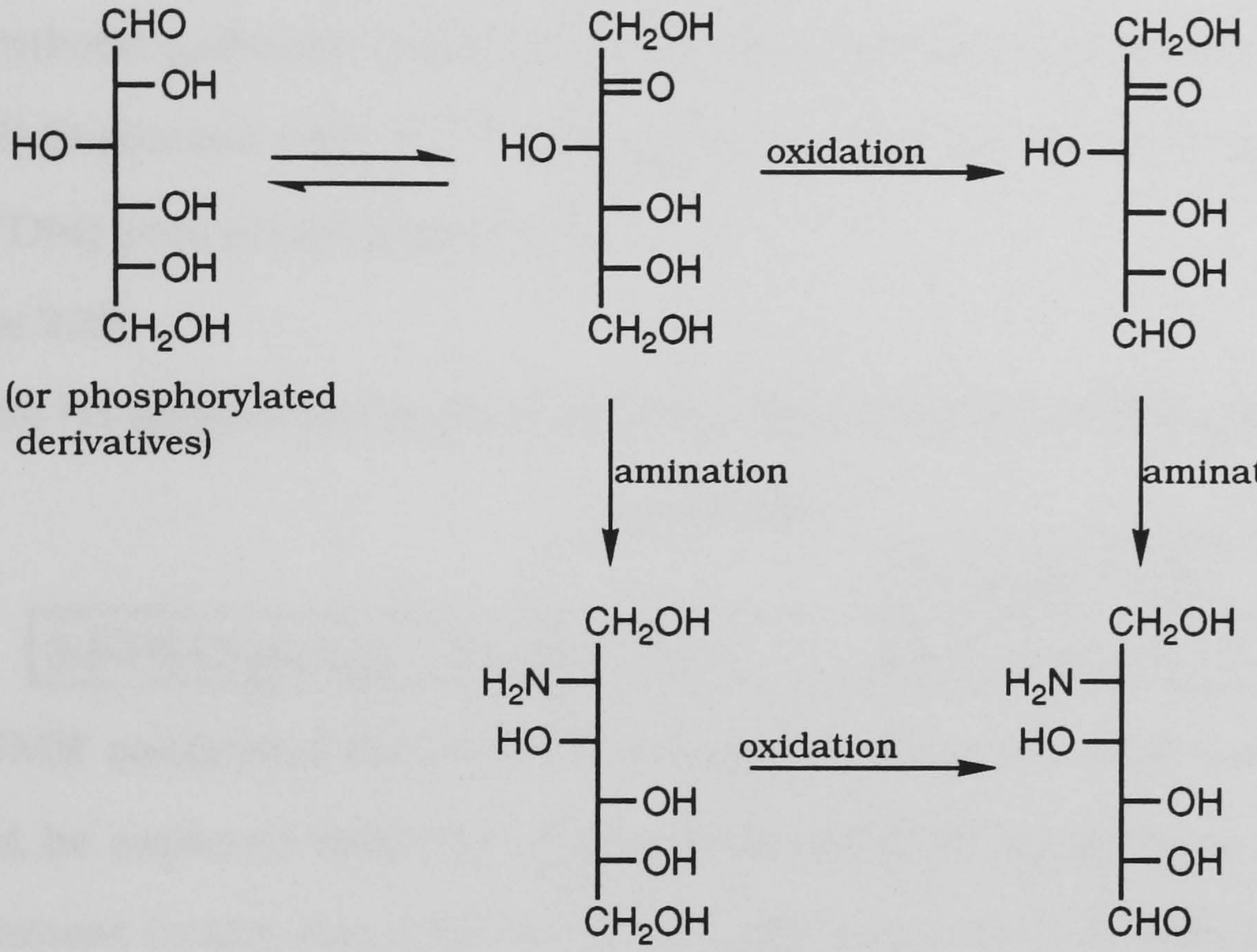
mistake not to analyse the fermentation directly by GC as this would have perhaps confirmed the slight enrichment found in the DMJ.

A summary of the proposed biosynthesis is as follows, **scheme 2.15**. This was analogous to Fleet's synthesis of DNJ and DMJ, involving C2/C6 cyclisation and nitrogen introduced at C2 with inversion of configuration

(page 23)

Scheme 2.15

The proposed biosynthesis of MJ, NJ, DNJ and DMJ in *S. subrutilus*.



Other experiments.

3-[^{2}H]-D-glucose was also fed to *S. subrutilus* to confirm the dilution of isotope label to two thirds of that originally introduced. In the proposed biosynthetic pathway, deuterium at C3 was not involved in any step and like 1-[^{2}H]-D-glucose and 1-[^{13}C]-D-glucose, it should be retained throughout DNJ/DMJ production, **figure 2.18.**

Figure 2.18

The ^{2}H enrichment in peracetyl DNJ after feeding 3-[^{2}H]-D-glucose to *S. subrutilus*.

	Peracetyl DNJ
3-[^{2}H]-D-glucose (35 atom % D)	23.6 ± 2.6 atom% D

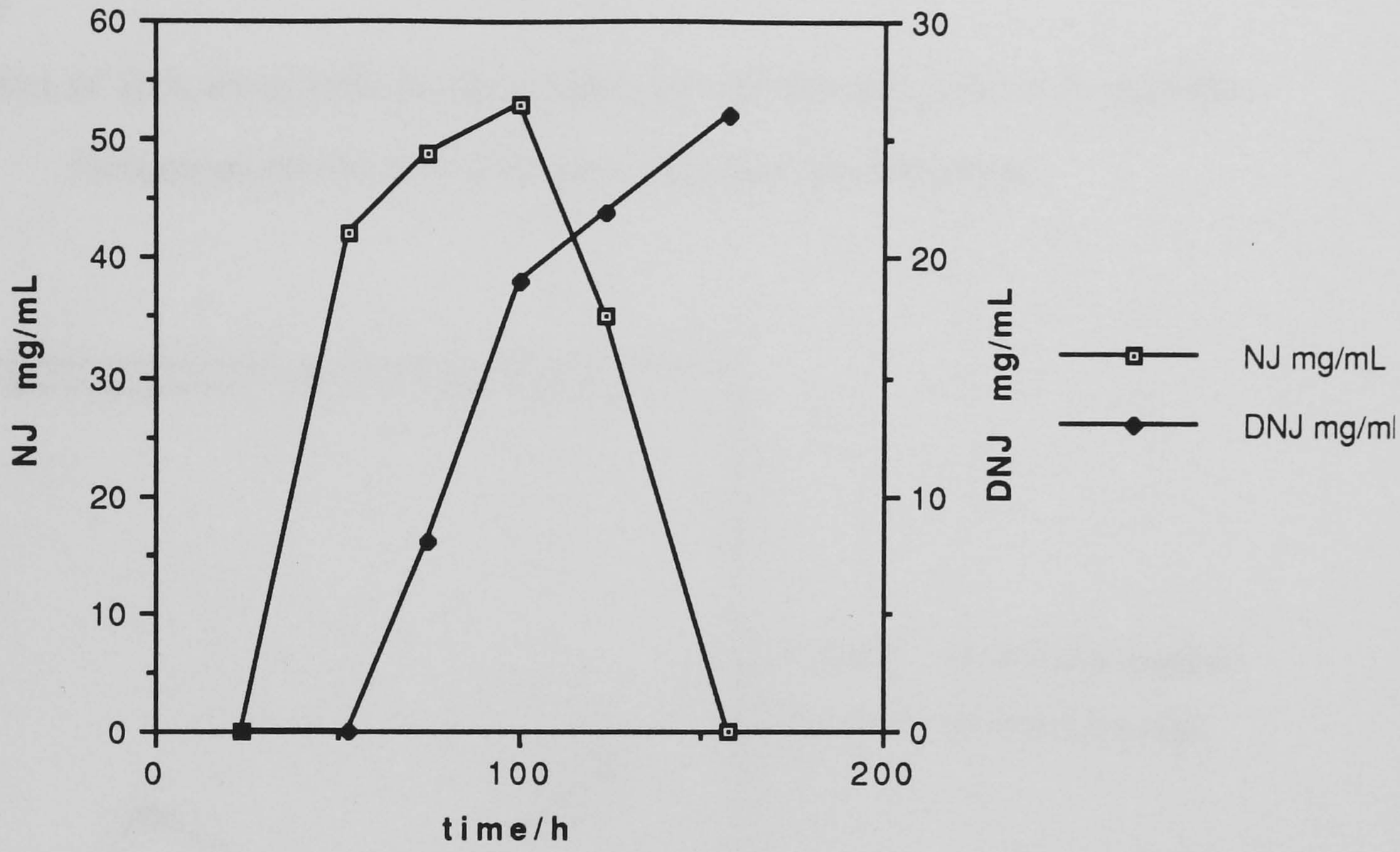
^{2}H NMR confirmed that the H4 position of peracetyl DNJ was labelled, as would be expected from the proposed inversion of the glucose molecule. The enrichment found was consistent with previous results in that 67% of the introduced isotope was retained in the final product.

The biosynthesis of DNJ and DMJ in *B. subtilis var niger*.

The biosynthesis of DNJ in this organism aroused interest for a number of reasons. It was perplexing that NJ could not be detected at any stage during the fermentation using the trehalase assay (page 47), and yet in *S. subrutilus* it had been shown to be a precursor to DNJ. Other *Streptomycetes* produced NJ, although not necessarily at the same time as *S. subrutilus* The following graph shows the relationship between DNJ and NJ in *S. lavendulae* [56], **figure 2.19.**

Figure 2.19

The production of NJ and DNJ in *S. lavendulae* [56]



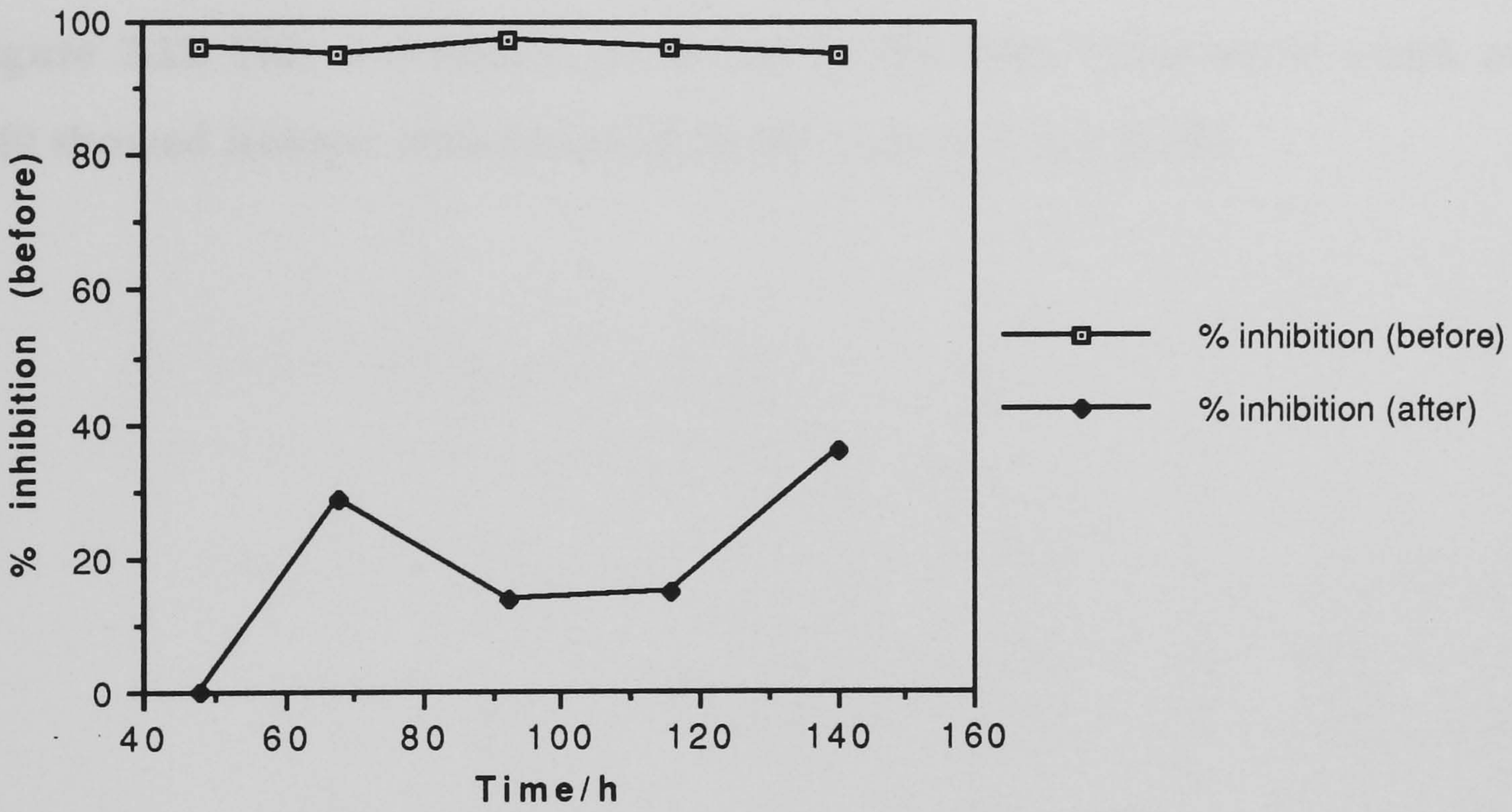
Unlike *S. subrutilus* , the production of NJ starts before that of DNJ and than rises to a maximum after about 100h. By the end of the fermentation, NJ can no longer be detected in the medium, presumably because it has all been converted to the 1-deoxy derivative. A pathway to DNJ without using NJ as a precursor has already been highlighted upon (page 73) if amination occurs at C6 of fructose.

Two other peculiarities existed which in themselves did not discount the biosynthesis as described for *S. subrutilus*.. DMJ was not produced in the medium at any stage and it was apparent that another inhibitor of trehalase was being produced other than DNJ (page 44 and 47). This was also recognised for the mannosidase assay as well. Using jack bean α-D-mannosidase, it was discovered that very high levels of inhibition occurred prior to heat treatment, but this decreased by a minimum of one third following heat/acid treatment, **figure 2.20**. The level of DNJ throughout the 7 day fermentation period did not increase above 0.02mg mL^{-1} which was insufficient to account

for the post heat/acid stage. DNJ only starts to inhibit this mannosidase at a concentration of 0.06mg mL^{-1} (3% inhibition). Again this suggested that an unidentified inhibitor(s) was(were) present making enzyme assays unreliable.

Figure 2.20

Inhibition of jack bean α-D-mannosidase by the filtrate from a *B. subtilis* fermentation before and after heat/acid treatment.



These results indicated that MJ was present, but it was difficult to reconcile the existence of this alkaloid with the production of DNJ. Without DMJ or NJ, how might MJ be processed into DNJ using the proposed *S. subrutilus* biosynthetic route? Route i and route ii in **scheme 2.12** both went via these intermediates. Clearly, either another biosynthetic scheme (or a variation of the *S. subrutilus* pathway) was in operation, or else the level of the intermediates was misleading.

1-[^{13}C]-D-glucose studies.

The results of feeding 1-[^{13}C]-D-glucose to *B. subtilis* are summarised below, **figure 2.21.**

Figure 2.21

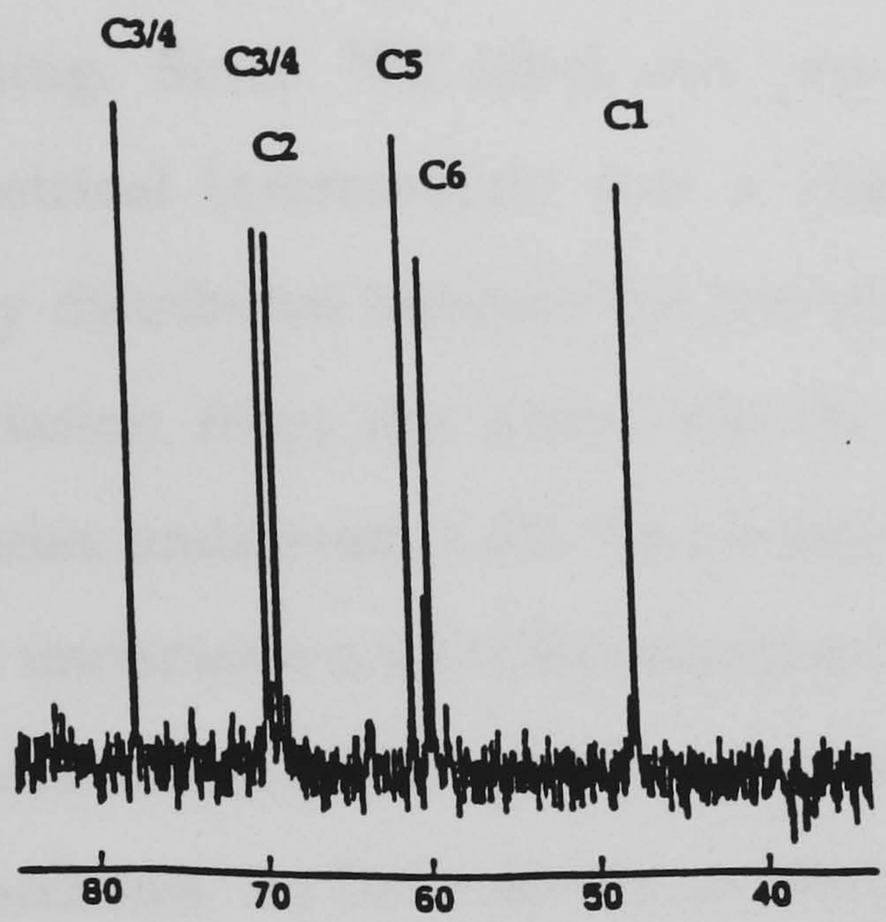
The ^{13}C enrichment in peracetyl DNJ after feeding 1-[^{13}C]-D-glucose to *B. subtilis var niger*.

	Peracetyl DNJ
1-[^{13}C]-D-glucose (16 atom % ^{13}C)	16.2 ± 1.2 atom % ^{13}C

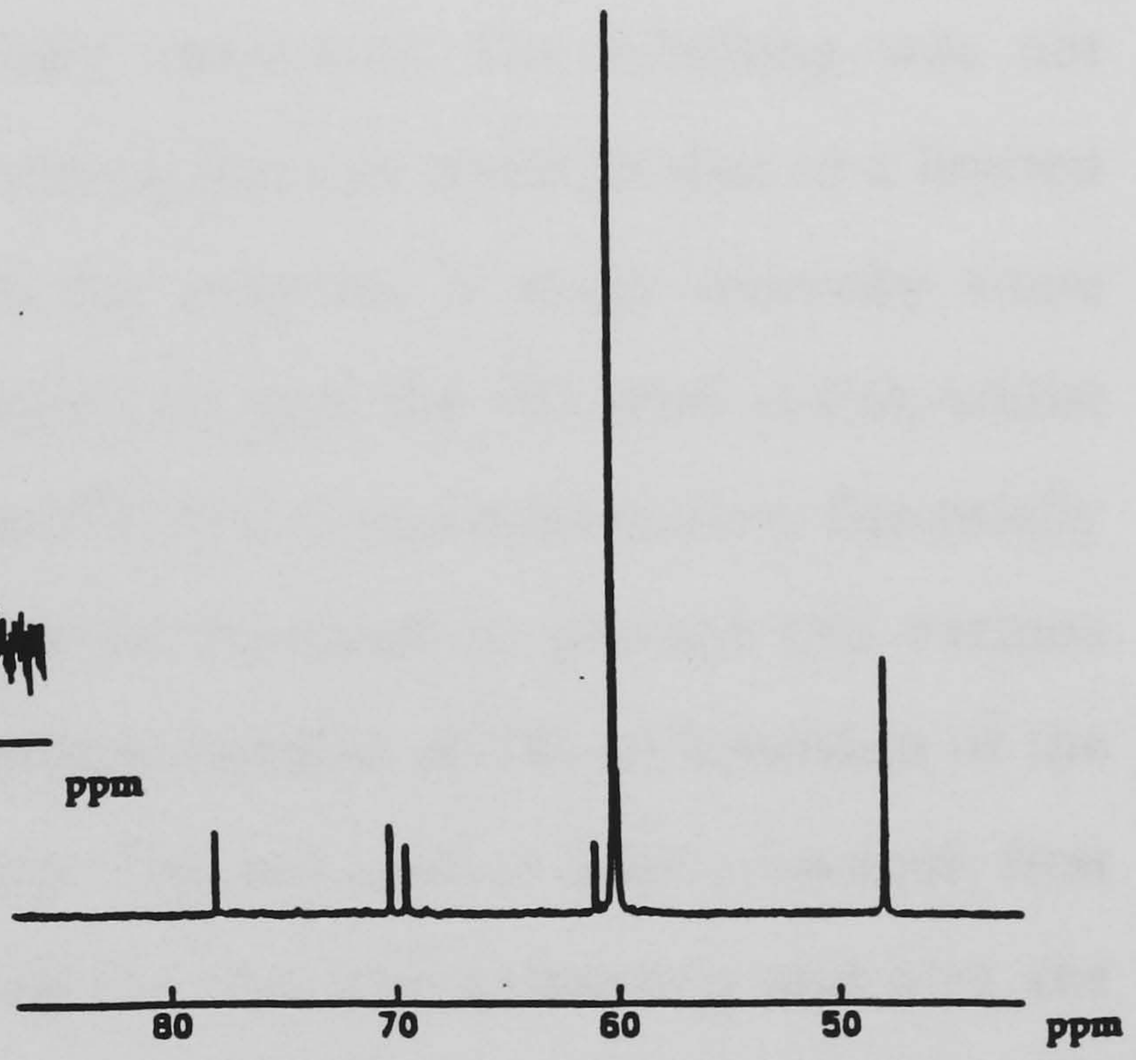
The ^{13}C NMR indicated that C6 was labelled (as previously), but also there was slight enrichment at C1 (approximately a 3:1 ratio between the two), **figure 2.22**. This was further confirmed by the mass spectrum in which m/z 240 showed isotopic enrichment of its M+1 ion (6.3 % ± 3.3%).

Figure 2.22

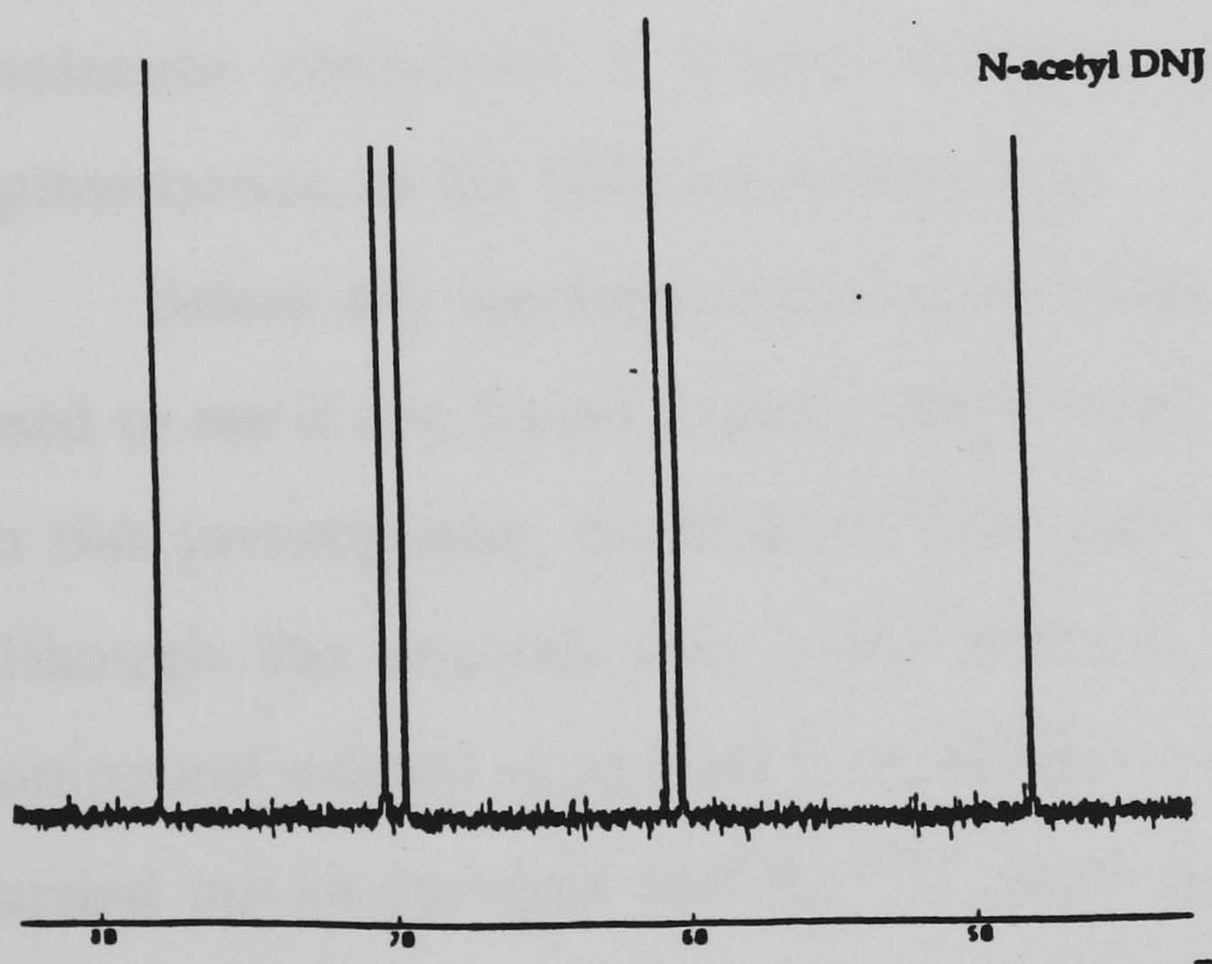
The ^{13}C NMR spectrum of natural abundance ^{13}C N-acetyl DNJ from *S. subrutilus* and that isolated from a fermentation using 1-[^{13}C]-D-glucose with *B. subtilis var niger*. Authentic N-acetyl DNJ is presented as a comparison.



natural abundance ^{13}C N-acetyl DNJ



N-acetyl DNJ isolated from *B. subtilis* (ex. 1-[^{13}C]-D-glucose)



authentic N-acetyl DNJ

In this instance, the enrichment of the glucose was reflected in the enrichment of the isolated peracetyl DNJ. Contrast this with *S. subrutilus* where a two thirds dilution occurred. In this case, DNJ was not coming from an alternative source, although a fermentation without glucose was not tried.

This result suggested that a different biosynthetic mechanism was operating. Since ^{13}C label was present at both ends of the molecule, a symmetrical intermediate was a likely candidate. The labelling was not equally distributed between the two atoms, but this could be due to a limited dissociation from the active site of the enzyme. A route whereby some molecules underwent a C2/C6 cyclisation (to give the ^{13}C label at C6), whilst others underwent a C1/C5 cyclisation (^{13}C at C1) was unattractive. Essentially two sets of enzymes would need to be involved to process the various intermediates. As the majority of labelling finished at C6, an inversion of the glucose molecule was still taking place. This led back to 5-keto fructose, first postulated for *S. subrutilus*. This had the required symmetry and also the molecule contained a 2-keto function which could be involved in epimerisation to the DNJ stereochemistry.

Before any labelling studies were undertaken, gas chromatography was used to see if any 5-keto fructose (5KF) could be detected in the fermentation. In this investigation, no silylated 5KF could be found over a 7 day period, although the analysis was made difficult by the fact that the silylated compound existed in at least four different forms by GC. The silylation was carried out in pyridine and the ^{13}C NMR spectrum in this solvent showed evidence for more than one species, including the acyclic form from a ^{13}C resonance at 206ppm. Previous studies on the hexose sugar in deuterium oxide indicated that the pyranose form was present predominantly[86].

Further experiments in which glucose and 5KF were added part way through a fermentation did not show any increase in the production of DNJ, in either case, when compared to a control.

To determine whether a symmetrical intermediate was involved, 6,6-[$^2H_2$]-D-glucose was used as a carbon source. The following results were obtained, **figure 2.23**.

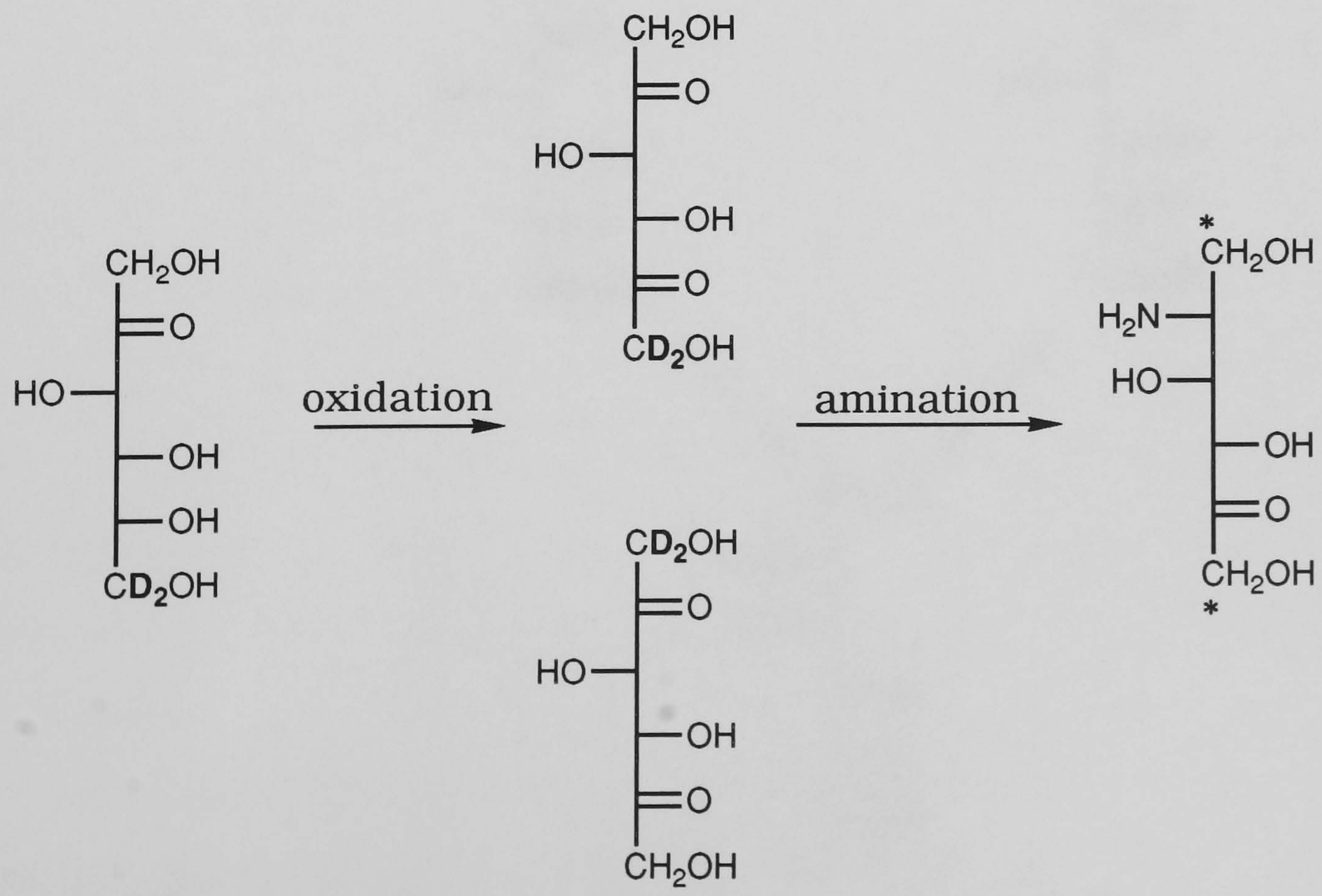
Figure 2.23

The ^2H enrichment found in peracetyl DNJ after adding 6,6-[$^2H_2$]-D-glucose to *B. subtilis var niger.*

	Peracetyl DNJ
6,6-[$^2H_2$]-D-glucose	48.4 ± 0.9 atom % D
(90 atom% D per H6)	(monodeuterated)

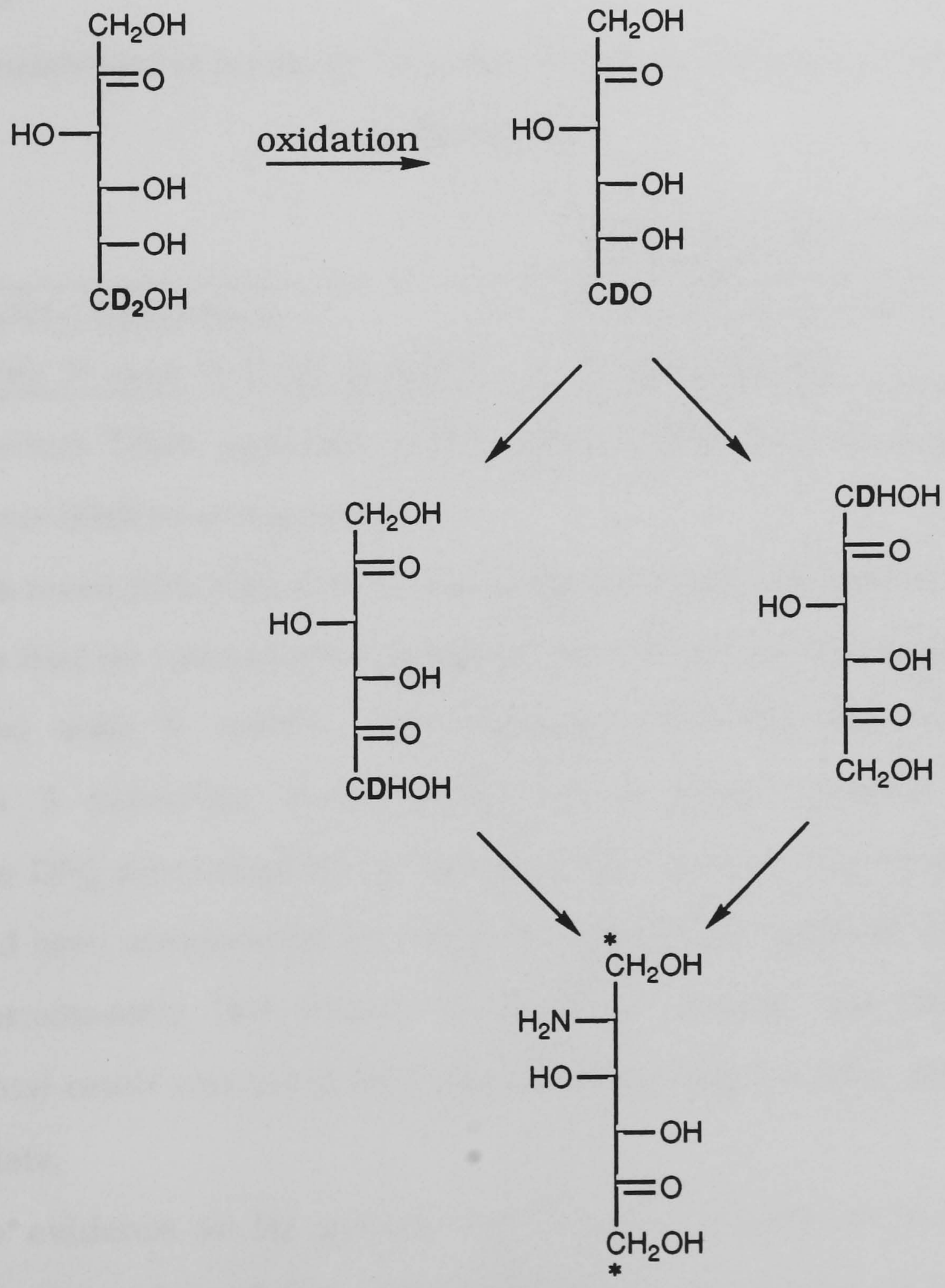
The ^2H NMR and ^1H NMR integrals indicated that $H1_{(eq.)}$ only was labelled in the recovered DNJ. This labelling pattern was identical to that for *S. subrutilus* , except that a lower enrichment of the isotope was found in *B. subtilis* These results ruled out a 5KF symmetrical intermediate, since if oxidation at C5 occurred first, deuterium should be found at C1 and C6, analogous to the ^{13}C experiment, **scheme 2.16.**

Scheme 2.16



If oxidation at C6 (**scheme 2.17**) was the initial step, again deuterium should be found at both ends of the DNJ. A kinetic isotope effect would favour protio glucose at this stage, which was before the molecule became symmetrical. The ^{13}C studies had also shown that only one quarter of the label introduced had swapped to the other end of the molecule, so these effects might successively dilute any label at H6 in the DNJ. However, the alkaloid was enriched by 50% in deuterium upon isolation and so any randomisation as observed by the ^{13}C experiment would be easily detectable.

Scheme 2.17.



As the 1-[^{13}C]-D-glucose and 6,6-[$^{2}H_2$]-D-glucose experiments contradicted each other in the results they gave, it was decided to work from the other end of the biosynthetic pathway. A fermentation was set up in which unlabelled NJ was added 58h after inoculation with the microorganism. After 6 days, GC assay indicated three times as much DNJ was present compared to the normal culture medium (0.06mg mL^{-1} compared to 0.02mg mL^{-1}). *S. subrutilus* had shown no such increase in an identical investigation. Labelled NJ was added in a further experiment at the same time and gave the following results, **figure 2.24**.

Figure 2.24

The ^{2}H enrichment in peracetyl DNJ after feeding 6,6-[$^{2}H_2$]-nojirimycin to *B. subtilis var niger*.

	Peracetyl DNJ
6,6-[$^{2}H_2$]-nojirimycin (2.5mg 99 atom % D per H atom)	9.8 ± 1.8 atom % D (dideuterated)

The deuterium NMR spectrum of the isolated DNJ showed that both H6 protons were labelled as expected.

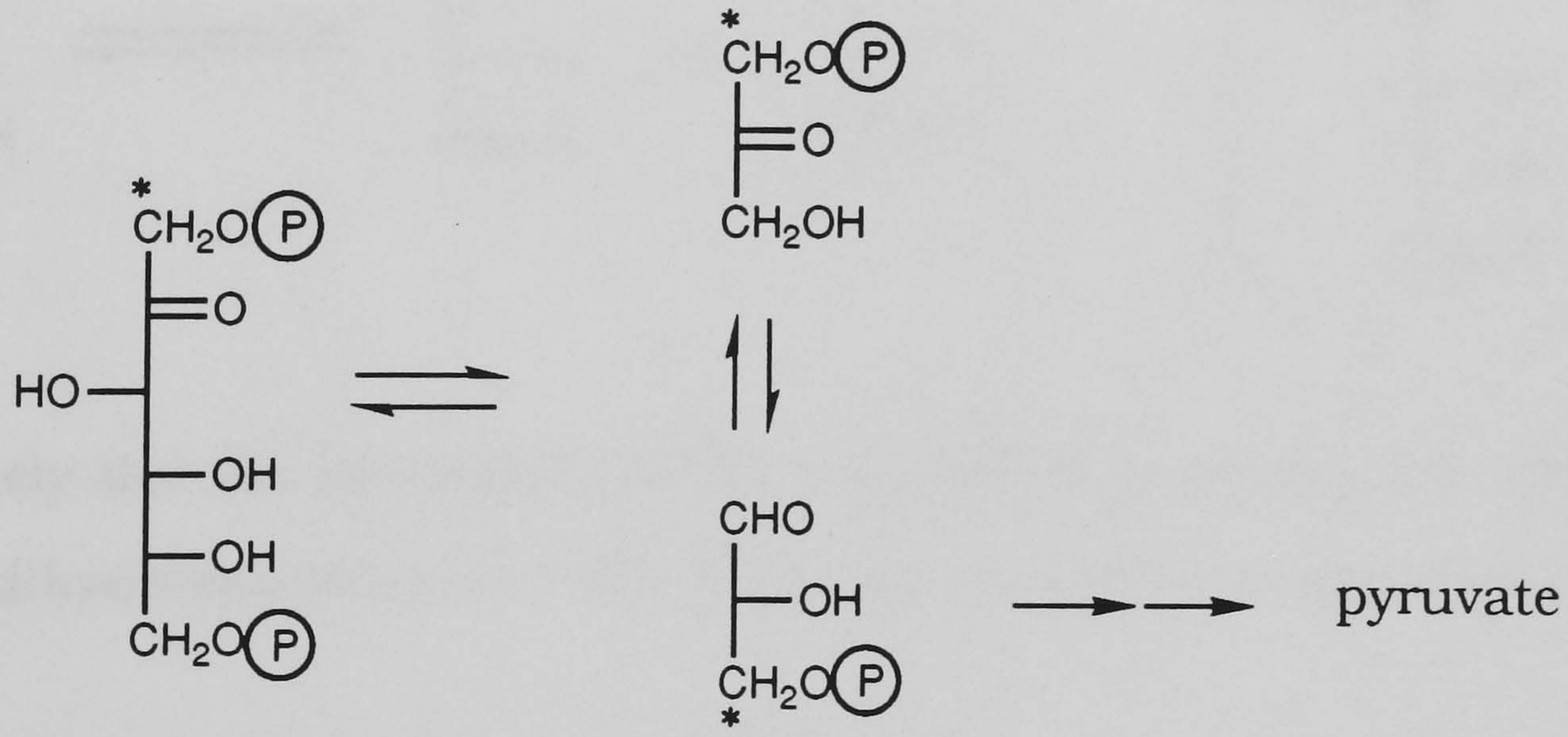
This result indicated that NJ was being produced and then siphoned off to DNJ so that its concentration remained low. In the natural abundance NJ experiment with *B. subtilis* , less alkaloid was added than that for the analogous *S. subrutilus* fermentation, yet the former showed a definite increase in DNJ production whilst the latter did not. If all the labelled NJ, as above, had been incorporated into DNJ, the expected enrichment would have been approximately 18% (based on 12mg of alkaloid per 600mL). The experimental result was lower than this, but there may be other fates for this intermediate.

The evidence so far pointed to a similar mechanism to that of *S. subrutilus*. Inversion of the glucose molecule was taking place to give ultimately mannojirimycin. This did not undergo dehydration and reduction

to give DMJ as had previously been the case, but the epimerisation step to NJ was presumably the same. The latter underwent dehydration and reduction to finally produce DNJ. The one anomaly at this stage was the 1-[^{13}C]-D-glucose result, where enrichment at C1 was seen. The "top" and "bottom" part of the glucose molecule appeared to behave differently, and this lead to the hypothesis that glucose split into two C3 fragments within the biosynthetic pathway.

The aldolase-catalysed reaction, whereby fructose-1,6-diphosphate is split into dihydroxyacetone and glyceraldehyde-3-phosphate, has been extensively studied as it is fundamental to energy production via glycolysis[87,88]. The two C3 fragments can inter-convert, a reaction catalysed by triose phosphate isomerase, **scheme 2.18.**

Scheme 2.18



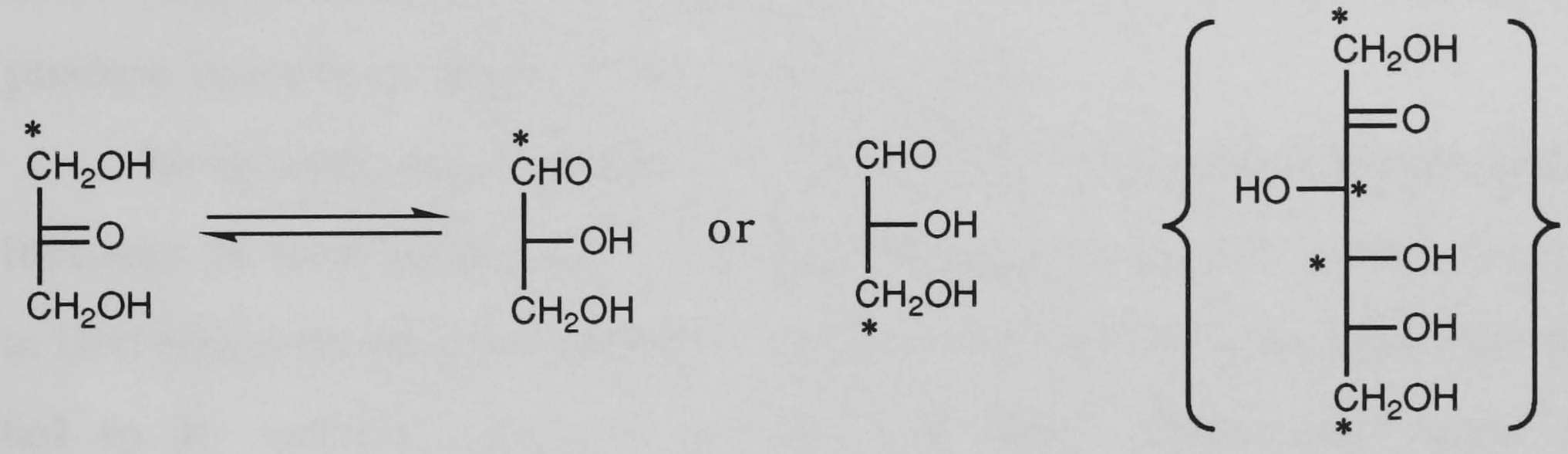
At equilibrium, 96% of the triose phosphate is dihydroxyacetone phosphate[87], but the reaction proceeds to glyceraldehyde-3-phosphate because the latter is efficiently removed to continue in glycolysis. The formation of glyceraldehyde-3-phosphate leads to its immediate removal as it is processed into other C3 metabolites, whereas the dihydroxyacetone essentially acts as a reserve for more glyceraldehyde-3-phosphate.

When the ^{13}C label is at C1 of glucose, this will become C1 of

dihydroxyacetone phosphate and consequently C3 of glyceraldehyde-3-phosphate. The latter will mostly continue into other C3 metabolites, but since the aldol reaction is reversible, it is possible that ^{13}C label will find its way into C6 of fructose-1,6-diphosphate. During the biosynthesis, if the "normal" C2/C6 cyclisation occurs from this intermediate to give DNJ, ^{13}C enrichment of C1 and C6 will be found. If the fructose derivative remains intact, label will be found at C6 only.

A number of assumptions are made here which need to be qualified. If dihydroxyacetone was produced then it is possible to find enrichment at four carbon atoms, C1, C3, C4 and C6 of fructose, **scheme 2.19**.

Scheme 2.19



It is likely that the phosphorylated form is utilised to remove the symmetry of the dihydroxyacetone and this is the usual substrate in glycolysis in any case.

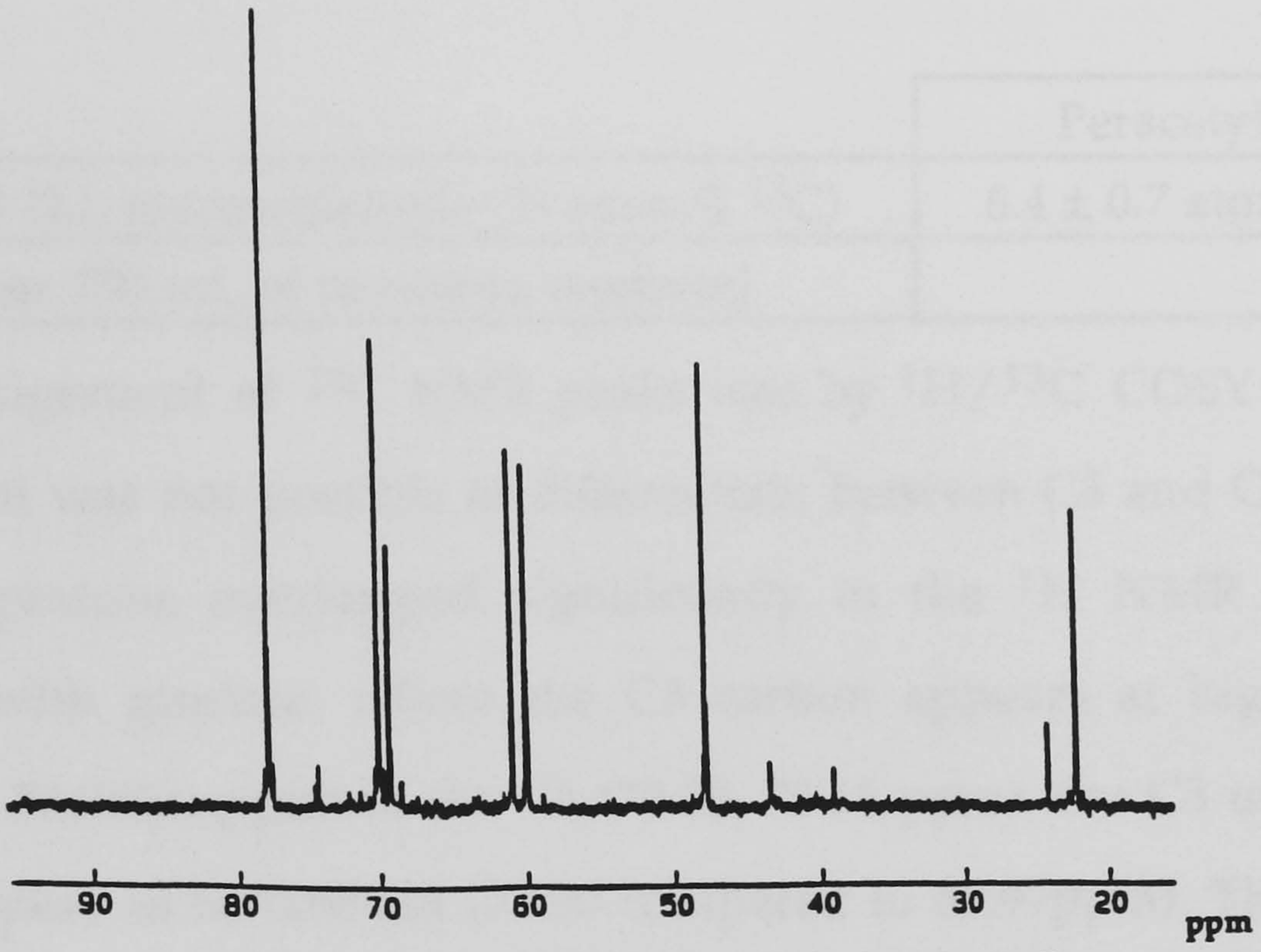
In the experiment with 6,6-[$^{2}H_2$]-D-glucose, it might be expected that glyceraldehyde-3-phosphate labelled at C3 with deuterium would be produced. From the isomerisation to dihydroxyacetone phosphate, it is plausible that the aldol equilibrium would lead to deuterium at C1 of fructose-1,6-diphosphate, eventually leading to the H6 protons of DNJ being labelled. DNJ isolated from this experiment did not show any deuterium at H6 which would arise from such a process. The reason was probably because the label from 1-[^{13}C]-D-glucose and 6,6-[$^{2}H_2$]-D-glucose lay in two different

intermediates following the aldol cleavage, and these suffered completely different fates. Although the triose phosphate equilibrium is mainly on the side of dihydroxyacetone phosphate, the reaction is very fast and significant randomisation of the label from dihydroxyacetone phosphate to glyceraldehyde-3-phosphate can occur. The same randomisation process occurs for glyceraldehyde-3-phosphate, but as it is present in very much smaller quantities, this isotope randomisation does not express itself to any significant extent in the dihydroxyacetone phosphate. Also, the main direction of the aldol cleavage is not to produce a C6 hexose, but the C3 trioses and it is very likely that randomisation of C6 deuterium cannot be detected. Presumably as the initial growth phase of the microorganism slowed and intermediates from glycolysis increased, the reverse of the aldol cleavage to produce fructose-1,6-diphosphate was encouraged.

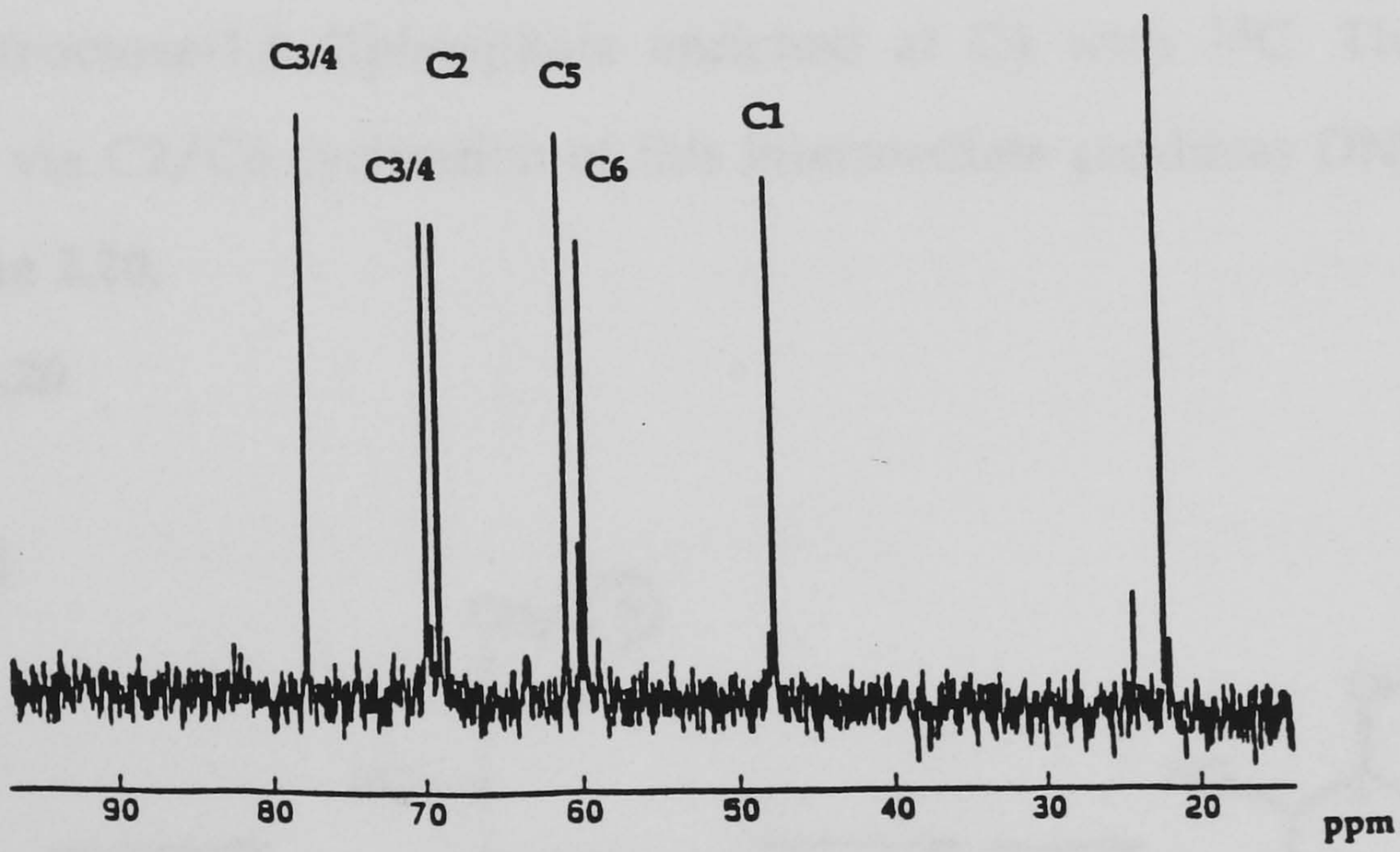
To investigate whether this fragmentation of fructose-1,6-diphosphate followed by isomerisation and then recombination of the C3 trioses occurred in DNJ biosynthesis, glyceraldehyde labelled at C1 with ^{13}C was used. This was fed to *B. subtilis* in one portion 24h after inoculation with the microorganism. The ^{13}C NMR spectrum indicated that C3 or C4 of the DNJ was enriched, but only to a slight extent over the natural abundance sample, **figure 2.25.**

Figure 2.25.

The ^{13}C NMR spectrum for N-acetyl DNJ isolated from *B. subtilis* after adding 1-[^{13}C]-D,L-glyceraldehyde.



N-acetyl DNJ after feeding 1-[^{13}C]-D,L-glyceraldehyde to *B. subtilis*



natural abundance ^{13}C N-acetyl DNJ

Mass spectrometry suggested slightly more enrichment than was evident from the ^{13}C NMR spectrum, **figure 2.26.**

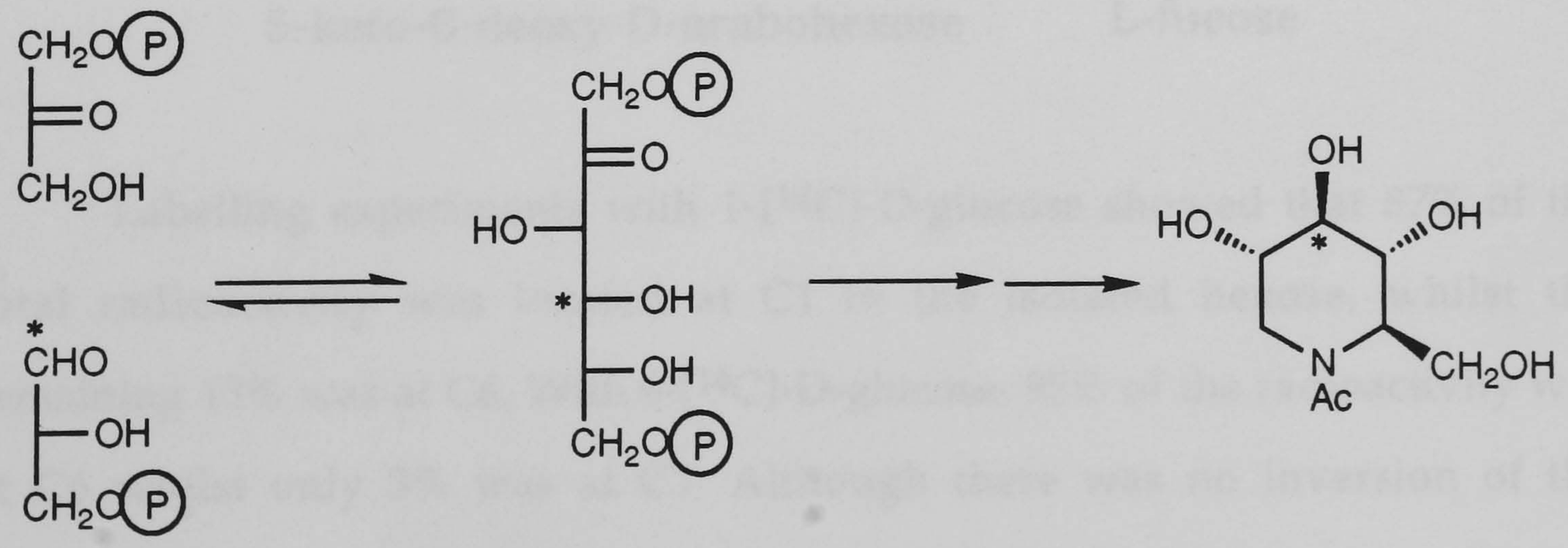
Figure 2.26.

The ^{13}C enrichment of peracetyl DNJ after feeding 1-[^{13}C]-D,L-glyceraldehyde to *B. subtilis var niger*.

	Peracetyl DNJ
1-[^{13}C]-D,L-glyceraldehyde (50 atom % ^{13}C)	6.4 ± 0.7 atom % ^{13}C
(1.2g per 750 mL of soyabean medium)	

Assignment of ^{13}C NMR peaks was by $^{1}H/^{13}C$ COSY spectroscopy, although it was not possible to differentiate between C3 and C4 since the H3 and H4 protons overlapped significantly in the ^{1}H NMR spectrum. By analogy with glucose, where the C3 carbon appears at higher frequency (76.48(β), 73.26(α) ppm) to the C4 (70.20, 70.16 ppm), the C3 of N-acetyl DNJ would appear to be labelled (77.80 compared to 69.97ppm). These results are consistent with the aldol reaction with dihydroxyacetone phosphate to produce fructose-1,6-diphosphate enriched at C4 with ^{13}C. The proposed inversion via C2/C6 cyclisation of this intermediate produces DNJ labelled at C3, **scheme 2.20.**

Scheme 2.20

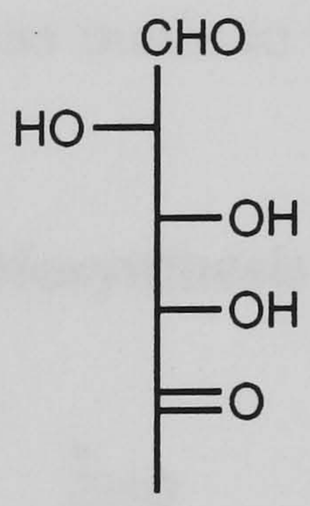


Other studies with *B. subtilis* had found that DNJ was produced on C3 carbon sources such as glycerol, but at approximately 1/50th of the

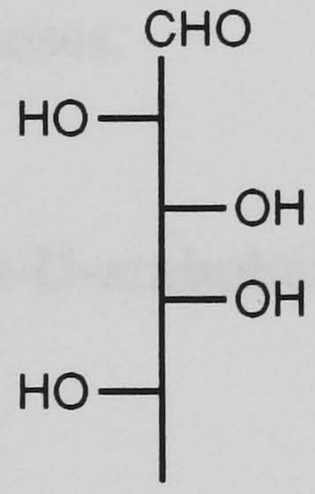
concentration when C6 sugars were used, such as fructose, glucose and sorbitol[57]. In hindsight, it would have been better to use a glucose labelled in two positions (e. g. C1 and C6) and to measure the ratio of the two labels in the glucose and in the isolated DNJ. There is always the danger in adding an intermediate such as glyceraldehyde that the equilibrium of a particular reaction is disturbed. The excess of glyceraldehyde may have pushed the reaction towards fructose-1,6-diphosphate thereby producing a false result. The high dilution of label from that introduced confirmed that this was not a major part of the biosynthetic route.

Similar labelling patterns have been observed in the biosynthesis of various 6-deoxy hexose sugars[89,90,91]. The antibiotic hygromycin A, produced by *S. hygroscopicus*, contains the deoxy sugar, 5-keto-6-deoxy-D-arabohexose. Biosynthetic studies have shown that the carbon backbone of this molecule originates from glucose. **figure 2.27.**

Figure 2.27



5-keto-6-deoxy-D-arabohexose



L-fucose

Labelling experiments with 1-[^{14}C]-D-glucose showed that 87% of the total radioactivity was located at C1 in the isolated hexose, whilst the remaining 13% was at C6. With 6-[^{14}C]-D-glucose, 95% of the radioactivity was at C6 whilst only 3% was at C1. Although there was no inversion of the glucose molecule, there was some randomisation of the label between the two ends of the molecule.

Studies on the biosynthesis of L-fucose by *Aerobacter aerogenes*

showed that glucose was a precursor. Again using 1-[^{14}C]-D-glucose, 63% of the total radioactivity of the isolated L-fucose was at C1 with 22% at C6. Similar results were also obtained with glucose enriched at C6 with ^{14}C. In this investigation the glucose was also re-isolated and shown to have the following isotope distribution, **figure 2.28**.

Figure 2.28

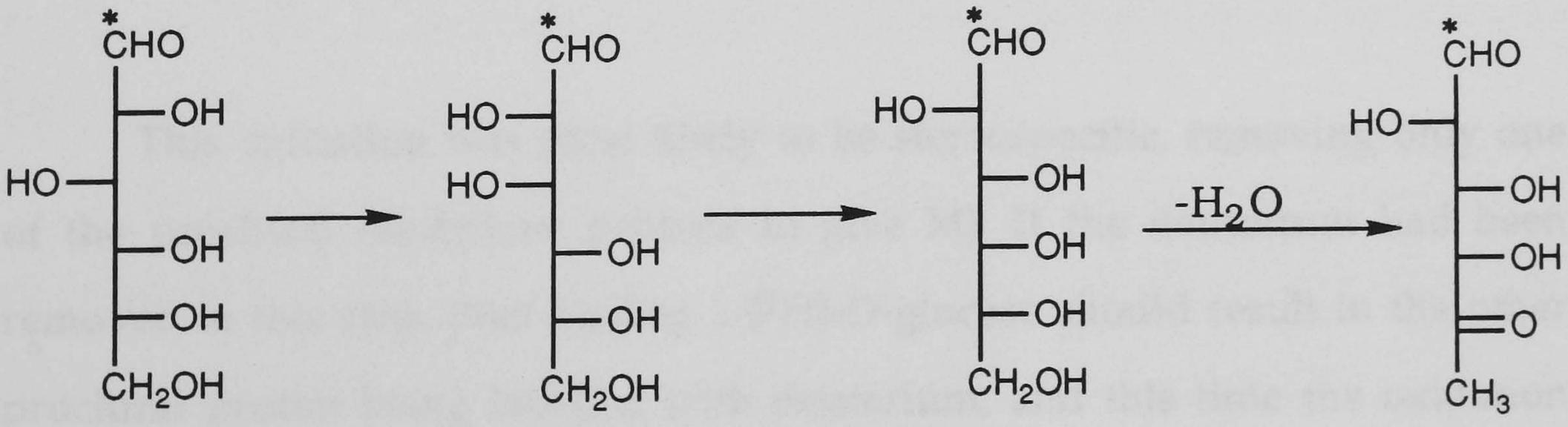
The location of ^{14}C in glucose re-isolated from *Aerobacter aerogenes*

	C1	C6
1-[^{14}C]-D-glucose	68%	23%
6-[^{14}C]-D-glucose	13%	94%

It was likely that the glucose molecule was incorporated intact into L-fucose (a possible mechanism for 5-keto-6-deoxyarabohexose is described below) and that the randomisation of the label occurred via a phosphorylated fructose intermediate. A mechanism in which the glucose could split into two halves which were in equilibrium with one another was postulated at the time, although no reference was made to the specific C3 trioses.

Scheme 2.21

The proposed biosynthesis of 5-keto-6-deoxy-D-arabohexose.



glucose mannose altrose

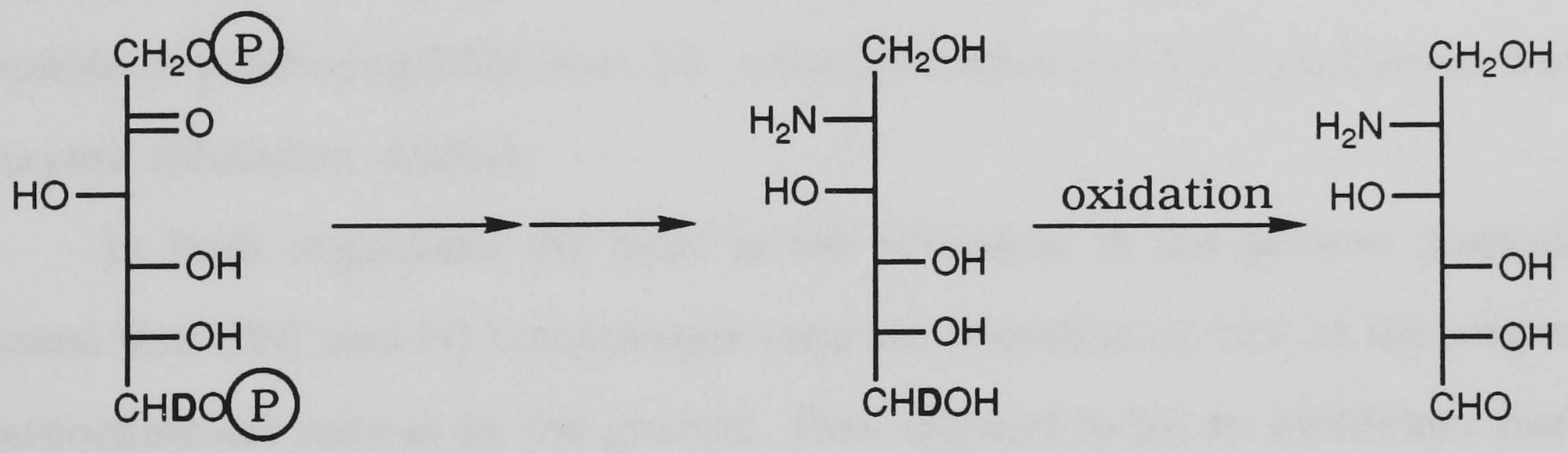
Given the similarity between the two hexoses, L-fucose might be derived from the reduction of the keto group.

In *B. subtilis* , isotopic label introduced into the top half of the

molecule should be slightly randomised in the production of DNJ by the minor pathway operating in **scheme 2.18**. 2-[^{2}H]-D-glucose was therefore investigated as a carbon source, in which the deuterium should be placed at H1 of fructose. Any randomisation of the label would be identifiable from enrichment at C1 as well as the expected C6.

After adding 2-[^{2}H]-D-glucose (39 atom % D) to a *B. subtilis* fermentation, the isolated peracetyl DNJ contained deuterium at H6 only (15 atom % D), a result identical with that from *S. subrutilus* . No evidence for randomisation of the deuterium existed and this was possibly because oxidation to MJ had involved the removal of the labelled proton, **scheme 2.22.**

Scheme 2.22



(fructose-1,6-diphosphate from randomisation)

This oxidation was most likely to be stereospecific, removing only one of the prochiral methylene protons to give MJ. If the deuterium had been removed in this step, then feeding 1-[^{2}H]-D-glucose should result in the other prochiral proton being labelled with deuterium, and this time the oxidation would remove a proton instead. Unfortunately, time constraints did not permit this investigation.

Conclusion.

In this project, the biosynthetic pathway to four different piperidine alkaloids in *S. subrutilus* has been discussed. Glucose was identified as the source of these secondary metabolites, and it is suggested that this carbohydrate is incorporated intact into the alkaloid structure with a novel head to tail inversion. Before this work was undertaken, DNJ and NJ were already known to be produced by *S. subrutilus*. Our studies identified DMJ as a further constituent of the fermentation medium and it is now believed that MJ is the common intermediate to the other three derivatives.

A similar biosynthetic route operates in *B. subtilis var niger* with only minor differences. DNJ was already known to be present in the culture medium and our investigations showed that NJ was a precursor to this alkaloid. This microorganism does not, however, contain the enzymes capable of producing DMJ from MJ, whose presence has been postulated from enzyme inhibition studies.

In both organisms the head to tail inversion of the glucose molecule means that DNJ and NJ biosynthesis requires alteration of two of the original stereochemical centres in the glucose. This appears to be an inefficient route when both alkaloids could be produced by oxidation and reductive amination of glucose itself. Epimerisation of one centre in each case would then provide DMJ and MJ.

Further work arising from this project might include the isolation of the enzymes or intermediates involved in the biosynthesis of these alkaloids. This would be of possible benefit to any commercial production of these compounds and might confirm the results already presented in this thesis. Other directions of research might include an investigation into the biosynthesis of the more complicated bicyclic polyhydroxylated alkaloids, or a screen for novel alkaloid enzyme inhibitors in existing fermentation cultures.

CHAPTER THREE

The synthesis of isotopically enriched precursors for biosynthetic experiments.

Introduction

To investigate the biosynthesis of DNJ and related alkaloids, glucose labelled specifically with ^{2}H or ^{13}C at various positions was required. The penalty for using spin active nuclei in this study was that high enrichment of the biosynthetic precursors was a necessity. Deuterated glucoses ideally needed to be prepared on a gram scale as the fermentations used 2g of glucose per 500mL of culture medium (3g/750mL for *B. subtilis*). A limited number of [^{2}H]- and [^{13}C]- enriched glucoses were available commercially, and some of these have been used in this investigation. However, even some of the cheaper isotopically enriched glucoses were expensive on the scale required.

Previous fermentation studies[4,57] had used high levels of glucose, typically 2% $^{w}/_{v}$ and yet our studies with *S. subrutilus* had shown that a decrease in DNJ production occurred with increasing glucose concentration. The smaller glucose concentrations used in this study (0.4%$^{w}/_{v}$) meant that ^{2}H or ^{13}C could be used as a label without synthesising large amounts of isotopically enriched precursors.

Synthetic routes were available for glucoses enriched at every carbon atom with deuterium, and these were based mainly on reductive methods using, for instance, metal hydrides. Another method of isotope incorporation which has also been used in this project is exchange of a proton with deuterium from solvent. This was the case in the synthesis of 2-[^{2}H]-D-glucose using deuterium oxide.

In the work described below, a new chemical route to 2-[^{2}H]-D-glucose has been developed, which complements the enzymatic synthesis from the

literature which is also described. A new strategy towards 5-[^{2}H]-D-glucose is presented which is superior to the two literature syntheses in that unwanted stereoisomers are avoided. Other isotopically enriched glucoses have been prepared according to the literature routes with little or no modification. Both NJ and DNJ have been prepared with deuterium enrichment by modifying two literature syntheses of these alkaloids, and the method has then been extended to include tritium labelling as well. In respect to the synthesis of glucose analogues for fermentation studies, a number of literature routes to these compounds have been examined, and the preferred methods, or new ones, have been outlined.

Synthetic methods.

1-[^{2}H]-D-glucose and 1-[^{13}C]-D-glucose.

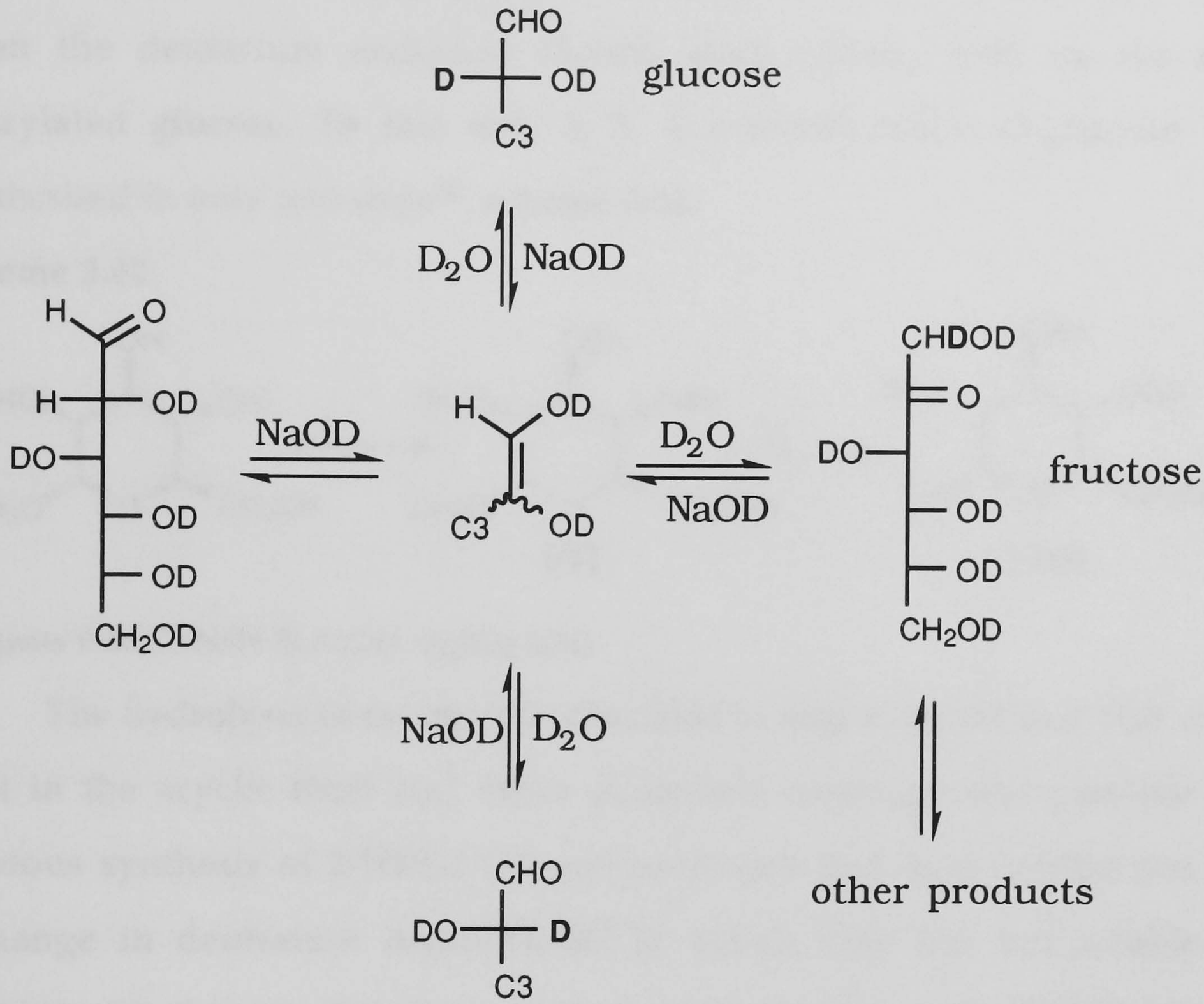
These two labelled precursors were the only ones purchased from a commercial supplier (Aldrich).

2-[^{2}H]-D-glucose

Since glucose can exist in an acyclic form in aqueous solution with H2 α to the aldehyde carbonyl, it might be expected that base exchange of this labile proton in deuterium oxide would lead to incorporation of label at the required position. Glucose, in the presence of alkali, had been reported[92] to produce fructose, mannose and various other intermediates from this proton abstraction and subsequent enolisation. Early studies, however, had not detected any incorporation of deuterium into the glucose molecule after reaction in alkaline deuterium oxide[93]. This was consistent with our studies where deuterium had not been incorporated into glucose in either acid or alkaline deuterium oxide by ^{1}H NMR. In contrast to this, other literature work suggested that a small incorporation of label did occur and that this varied depending on the intermediate isolated (~1 atom % D for glucose)[93,94].

Scheme 3.01

The isomerisation of glucose under alkaline conditions.



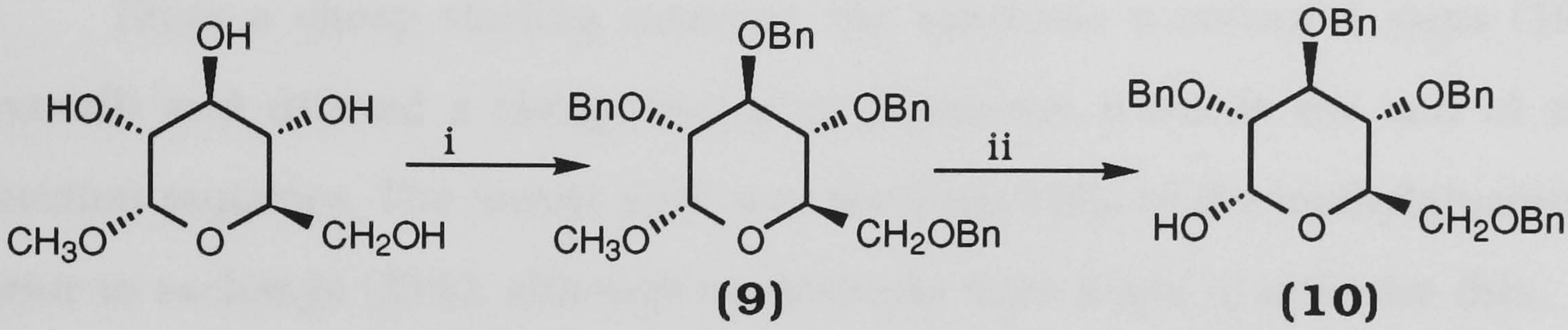
As only a small incorporation of deuterium was observed (presumably not detected by ^{1}H NMR in our experiment) and that several compounds were produced in the reaction, this synthetic route became impractical.

An alternative was based on a synthesis of deuterated arabinose[95]. By making the 2-O-benzyl ether of arabinose, the conversion to the corresponding ketose as above was precluded. In alkaline deuterium oxide, two C2 epimers of 2-O-benzylarabinose were produced, each with H2 enriched with deuterium. The desired *arabino* diastereoisomer was then recovered by re-crystallisation from ethyl acetate.

The corresponding 2-O-benzyl-α-D-glucose had already been

synthesised in 4 steps from glucose pentaacetate[96,97], although the H2 deuterated derivative had not been reported. It seemed unnecessary to spend several steps synthesising glucose protected solely at C2 with the benzyl ether when the deuterium exchange should work equally well on the fully benzylated glucose. To this end, 2, 3, 4, 6-tetrabenzyl-α-D-glucose was synthesised in only two steps[98], **scheme 3.02.**

Scheme 3.02



Reagents i) BnCl, NaH **ii)** AcOH, H_2SO_4 (2M)

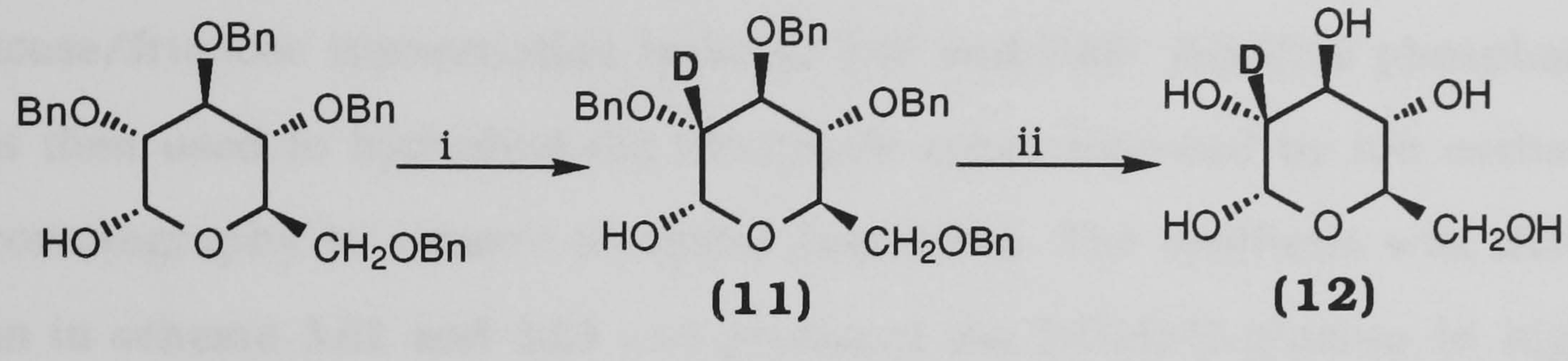
The hydrolysis of the methyl glucoside in step ii meant that **(10)** could exist in the acyclic form and hence deuterium exchange was possible The previous synthesis of 2-[^{2}H]-2-O-benzylarabinose had then carried out this exchange in deuterium oxide/NaOD in which **(10)** was not soluble. By carrying out this reaction in a mixture of deuterium oxide/NaOD/dioxan (anhydrous) instead, the C2 epimers of 2, 3, 4, 6-O-tetrabenzyl-α-D-glucose deuterated at C2 could be produced as a 1:1 mixture. The desired *gluco* epimer was then obtained by recrystallisation from methanol. In theory, the filtrate containing the *manno* epimer and some *gluco* could be re-equilibrated in NaOD/deuterium oxide/dioxan, although this was not carried out in practice.

It was straightforward to determine the amounts of each diastereoisomer after the deuterium exchange as the two anomeric proton signals were at different chemical shift values in the ^{1}H NMR spectrum (*gluco* δ 5.25 *manno* δ 5.31). In the protio-derivative, the anomeric proton of the α *gluco* diastereoisomer appeared as a doublet of 4.4Hz, whereas the deuterated analogue appeared just as a singlet.

The last remaining step was to deprotect the benzylated glucose by

hydrogenolysis, **scheme 3.03.**

Scheme 3.03



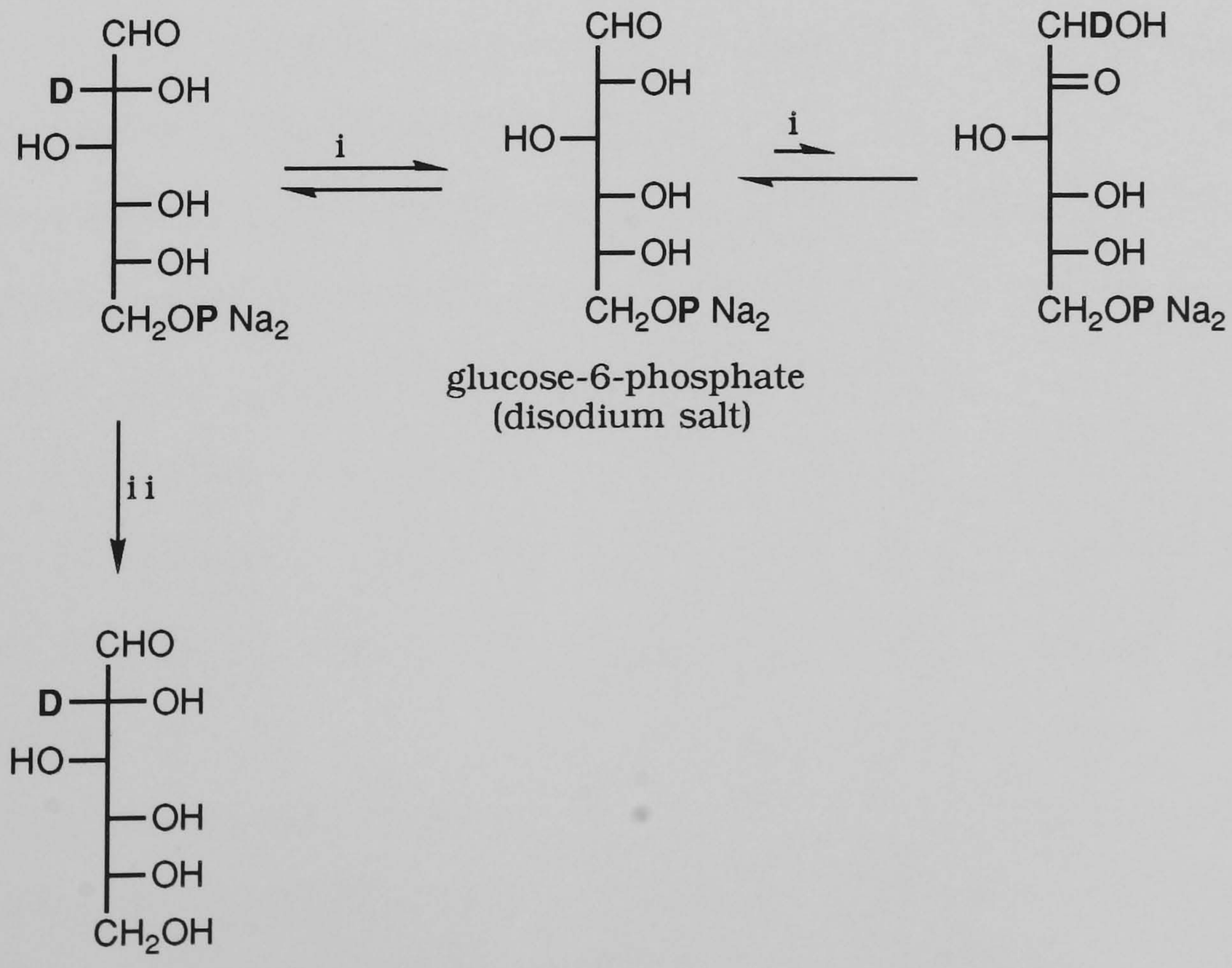
Reagents i) NaOD/deuterium oxide/dioxan, MeOH recrystallisation **ii)** H_2/Pd/AcOH

From a cheap starting material, the synthesis involved 4 steps (16% overall) and utilised a cheap source of deuterium towards the end of the reaction sequence. The lowest yield was the hydrolysis of the methylglucoside prior to exchange (33%), although no attempts were made to optimise this.

Enzymatic synthesis

An enzyme based method from the literature[75,79,99] was also employed. Incubation of either glucose or fructose-6-phosphate in deuterium oxide with glucose-6-phosphate isomerase led to a 9:1 mixture of 2-[^{2}H]-D-glucose-6-phosphate and 1-[^{2}H]-D-fructose-6-phosphate, **scheme 3.04.**

Scheme 3.04.



Reagents i) glucose-6-phosphate isomerase/deuterium oxide **ii)** alkaline phosphatase

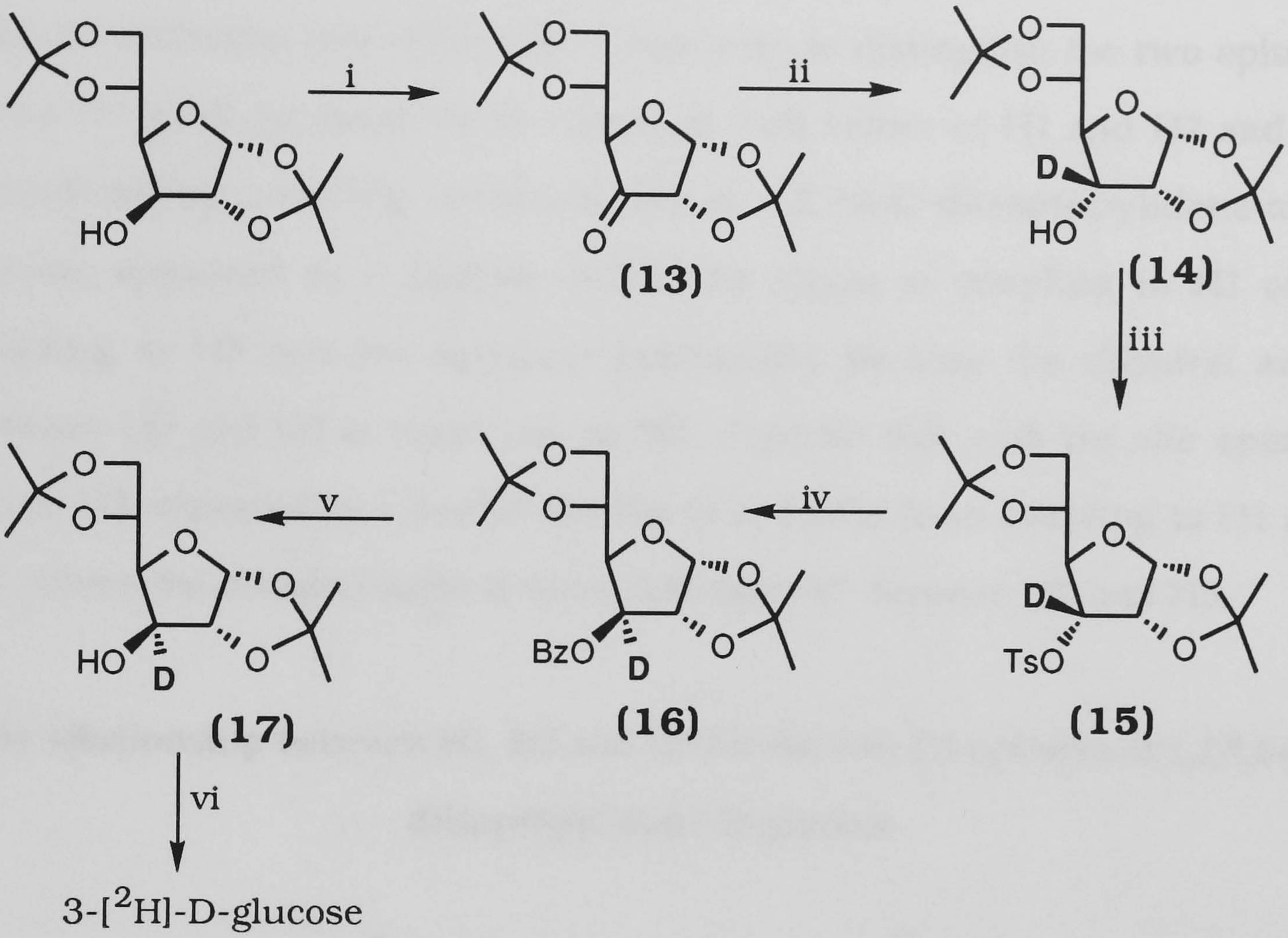
The reaction takes place under equilibrium conditions and the incorporation of isotope is via exchange of H2 with solvent water during the glucose/fructose isomerisation (**scheme 2.07 and 2.08**). Alkaline phosphatase was then used to hydrolyse the phosphate esters followed by ion exchange chromatography to remove inorganic impurities. The synthesis was shorter than in **scheme 3.02 and 3.03** and produced the 2-[^{2}H]-D-glucose in higher yield (60% overall). The stereospecificity of the enzyme mechanism meant that only the required stereoisomer was produced and hence this avoided any separation and wastage of labelled material.

Both methods were used to prepare 2-[^{2}H]-D-glucose for fermentation studies. The enzymatic route was preferred, although this had the disadvantage that traces of fructose were present in the recovered glucose. Attempts were made to purify the glucose by either recrystallisation or derivatisation followed by chromatography, but satisfactory results were not obtained. Mass spectrometry confirmed that the glucose was enriched to >98 atom % D.

3-[^{2}H]-D-glucose.

3-[^{2}H]-D-glucose was synthesised according to Koch and Perlin[100].

The synthesis of 3-[^{2}H]-D-glucose



3-[^{2}H]-D-glucose

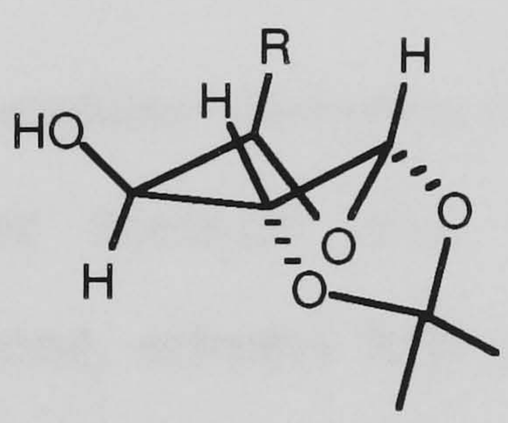
Reagents i) $Py_2Cr_2O_7$/molecular sieve/CH_2Cl_2 **ii)** $NaBH_4$/EtOH/D_2O **iii)** TsCl/pyridine **iv)** PhCOONa/DMF **v)** NaOMe/MeOH **vi)** H_2SO_4/H_2O

One modification was made. The literature route used DMSO/Ac_2O for the oxidation in step i, yet the analogous Swern oxidation[101] produced the ketone and other unidentified impurities by ^{1}H NMR and TLC. The best method of oxidation was with pyridinium dichromate in CH_2Cl_2 with the presence of powdered molecular sieves. This gave the ketone and the hydrated ketone in high yield without the need for chromatographic purification.

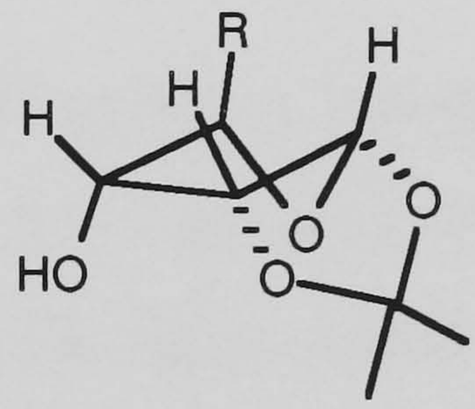
From the reaction sequence, the first two steps were concerned with introducing the deuterium label, whilst the remainder of the synthesis inverted the stereochemistry at C3. The hydride reduction with $NaBH_4$ was

highly stereospecific and led entirely to the 1,2,5,6-O-diisopropylidene-α-D-*allo*-hexofuranose by ^{1}H NMR analysis[102]. The corresponding reduction with $LiAlH_4$ gave a 7:3 *allo*/*gluco* mixture[103]. Previous studies had shown that the *allo* stereochemistry was produced from isolation of D-allose on hydrolysis after the reduction step. However, it was easy to distinguish the two epimers in the ^{1}H NMR spectrum by the chemical shift values of H1 and H2 and the corresponding coupling constants. H2 in 1,2,5,6-O-diisopropylidene-α-D-glucose appeared as a doublet (4.2Hz) by virtue of coupling to H1 only; coupling to H3 was not apparent presumably because the dihedral angle between H2 and H3 is very close to 90°. Contrast this with the *allo* epimer, where H2 appeared as a double doublet (4.2, 4.3Hz) from coupling to H1 and H3, where the dihedral angle is much less than 90° between H2 and H3.

The relationship between H1, H2 and H3 for the two C3 epimers of 1,2,5,6-O-diisopropylidene-D-glucose



gluco stereochemistry



allo stereochemistry

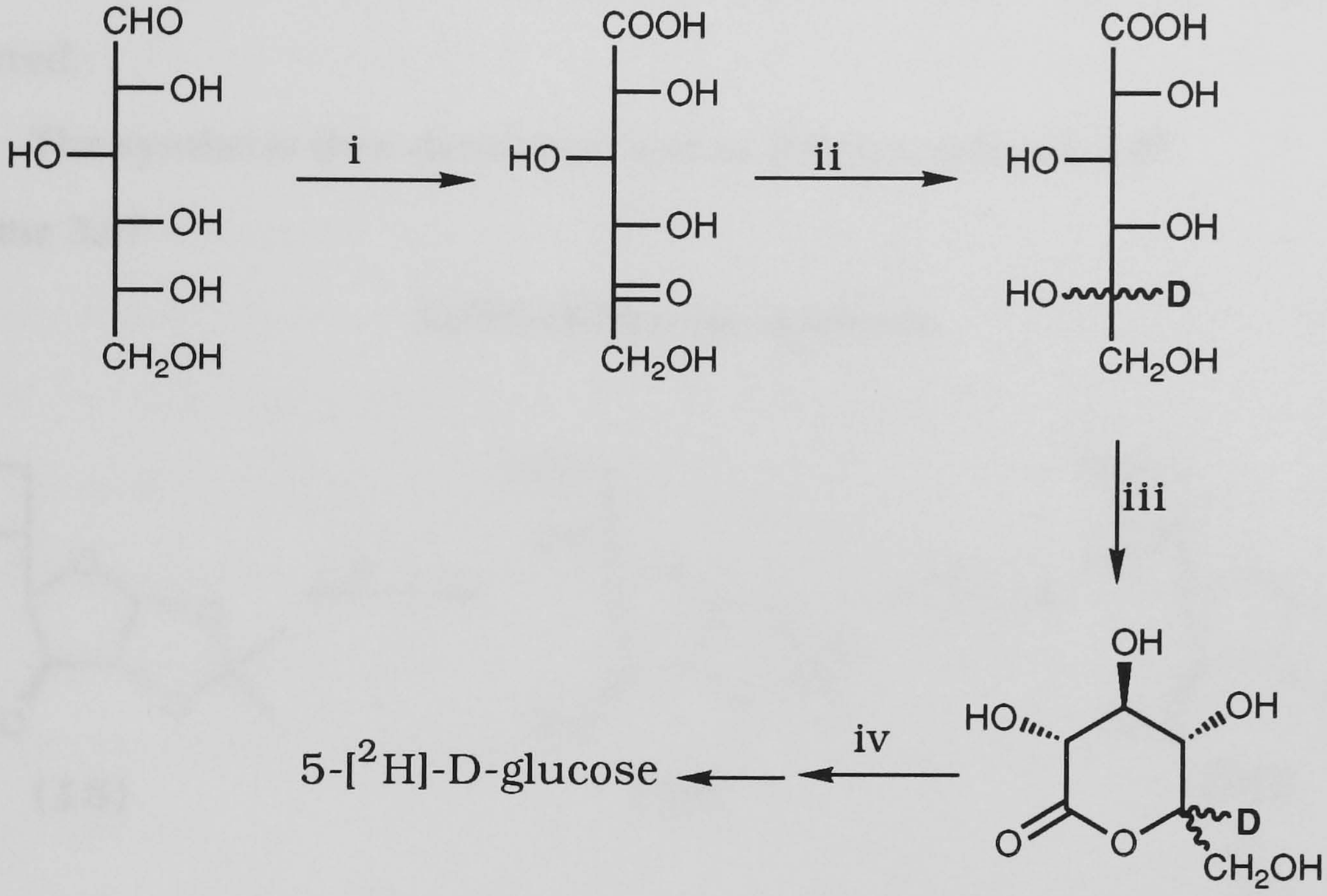
This stereospecificity in reduction was also observed in synthesising 5-[^{2}H]-D-glucose.

5-[^{2}H]-D-glucose

Previous syntheses of this deuterated glucose had not given satisfactory yields and/or stereospecificity[104,105]. One route involved the reduction of D-*xylo*-5-hexulosonic acid, produced from D-glucose using *Gluconobacter suboxydans*, **scheme 3.05**[106]. Repeating this procedure confirmed that an equimolar mixture of D-gluconic and L-idonic acids was produced[107].

Following lactonisation, reduction using boric acid/$NaBH_4$[108] gave only trace quantities of 5-[^{2}H]-D-glucose, a result also reported in the literature preparation.

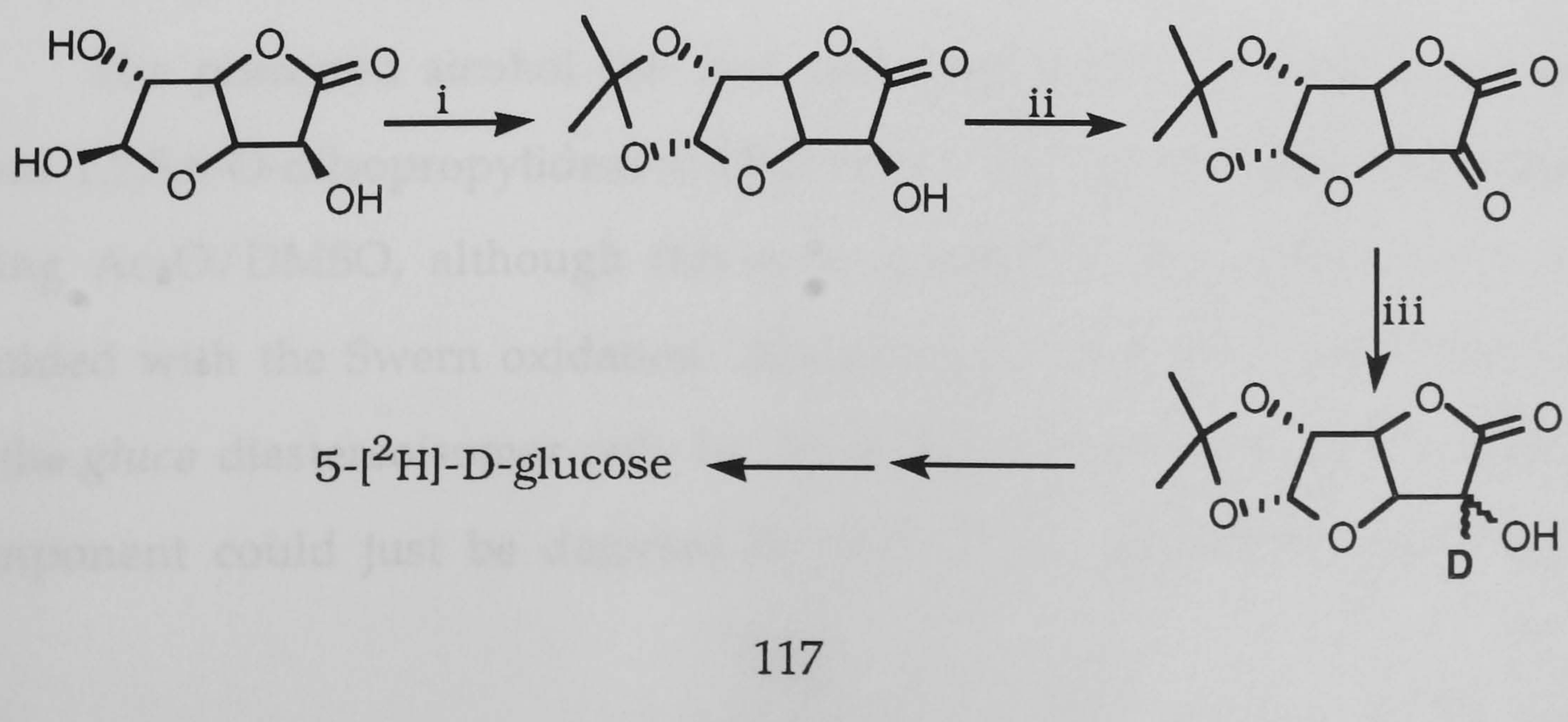
Scheme 3.05



Reagents i) *Gluconobacter suboxydans* **ii)** $NaBD_4$/EtOH **iii)** heat **iv)** $NaBH_4/H_2O$

Another method had employed 1,2-O-isopropylidene-α-D-6,3-glucuronolactone, **scheme 3.06**. Oxidation at C5 using chromium trioxide had given the keto lactone in only 17% yield, which was subsequently reduced with $NaBH_4$ to give the *gluco* and *ido* lactones in a 2:1 ratio.

Scheme 3.06



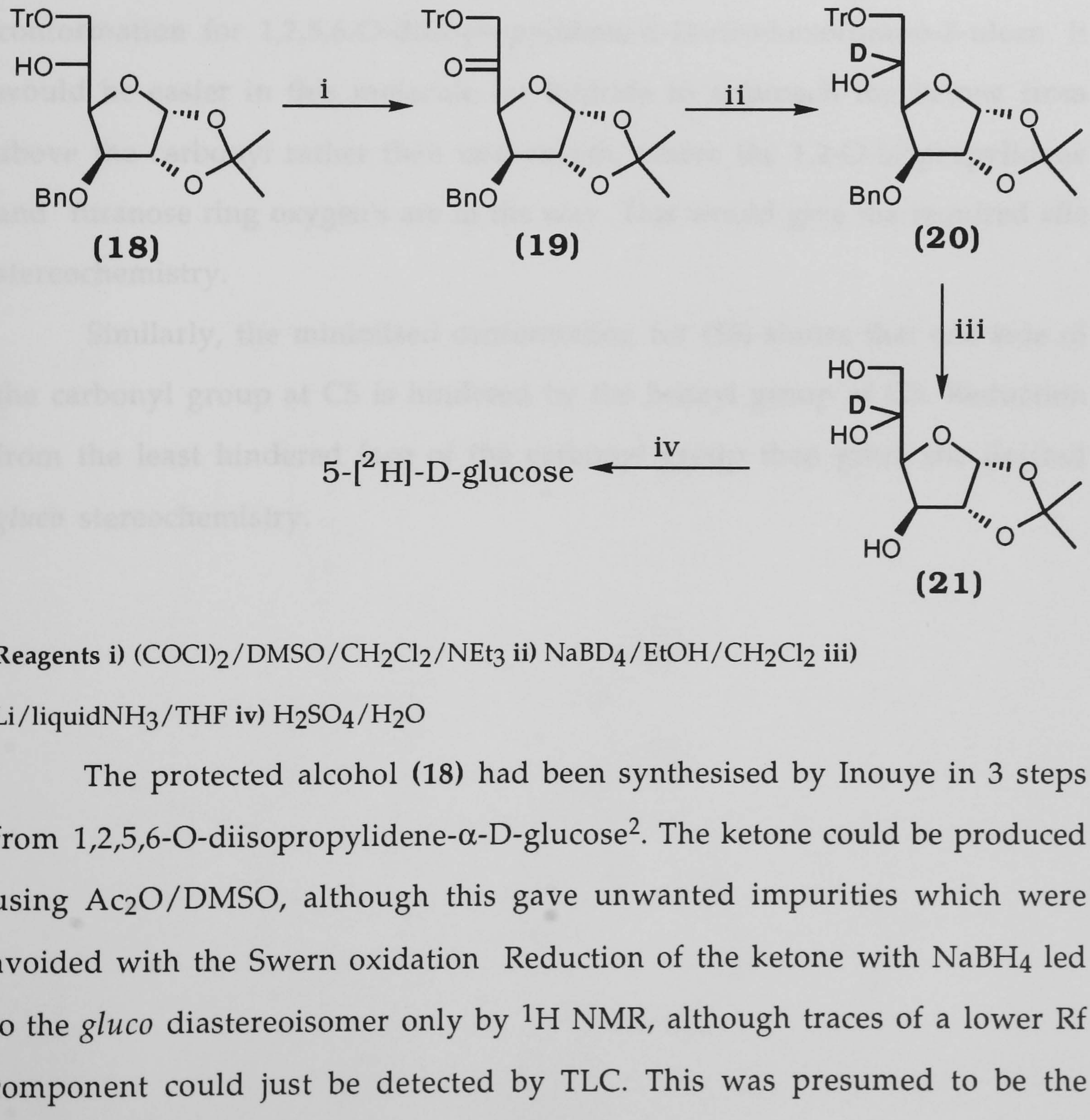
Reagents i) acetone/H^+ **ii)** CrO_3/AcOH **iii).** $NaBD_4$/EtOH

Various attempts to improve the oxidation at C5 were unsuccessful. It had been reported[109] that Swern oxidation produced the keto lactone conveniently in 90% yield, although this could not be repeated. TLC of the reaction indicated that oxidation appeared to be taking place, but on warming to room temperature and subsequent aqueous work up, decomposition occurred.

The synthesis then developed was as follows, **scheme 3.07**.

Scheme 3.07

5-[^{2}H]-D-Glucose synthesis



(18) **(19)** **(20)** **(21)**

Reagents i) $(COCl)_2$/DMSO/CH_2Cl_2/NEt_3 **ii)** $NaBD_4$/EtOH/CH_2Cl_2 **iii)** Li/liquidNH_3/THF **iv)** H_2SO_4/H_2O

The protected alcohol **(18)** had been synthesised by Inouye in 3 steps from 1,2,5,6-O-diisopropylidene-α-D-glucose[2]. The ketone could be produced using Ac_2O/DMSO, although this gave unwanted impurities which were avoided with the Swern oxidation Reduction of the ketone with $NaBH_4$ led to the *gluco* diastereoisomer only by ^{1}H NMR, although traces of a lower Rf component could just be detected by TLC. This was presumed to be the

alternative C5 epimer. Replacement of the $NaBH_4$ with $NaBD_4$ allowed easy incorporation of deuterium.

Attempted hydrogenolysis of the trityl and benzyl groups failed, a problem that also occurred when synthesising deoxyglucose analogues. The four phenyl groups possibly prevent the molecule from orienting correctly on the catalyst surface for reduction to take place. Dissolving metal reduction using lithium in liquid ammonia gave consistently high yields of the partially protected sugar.

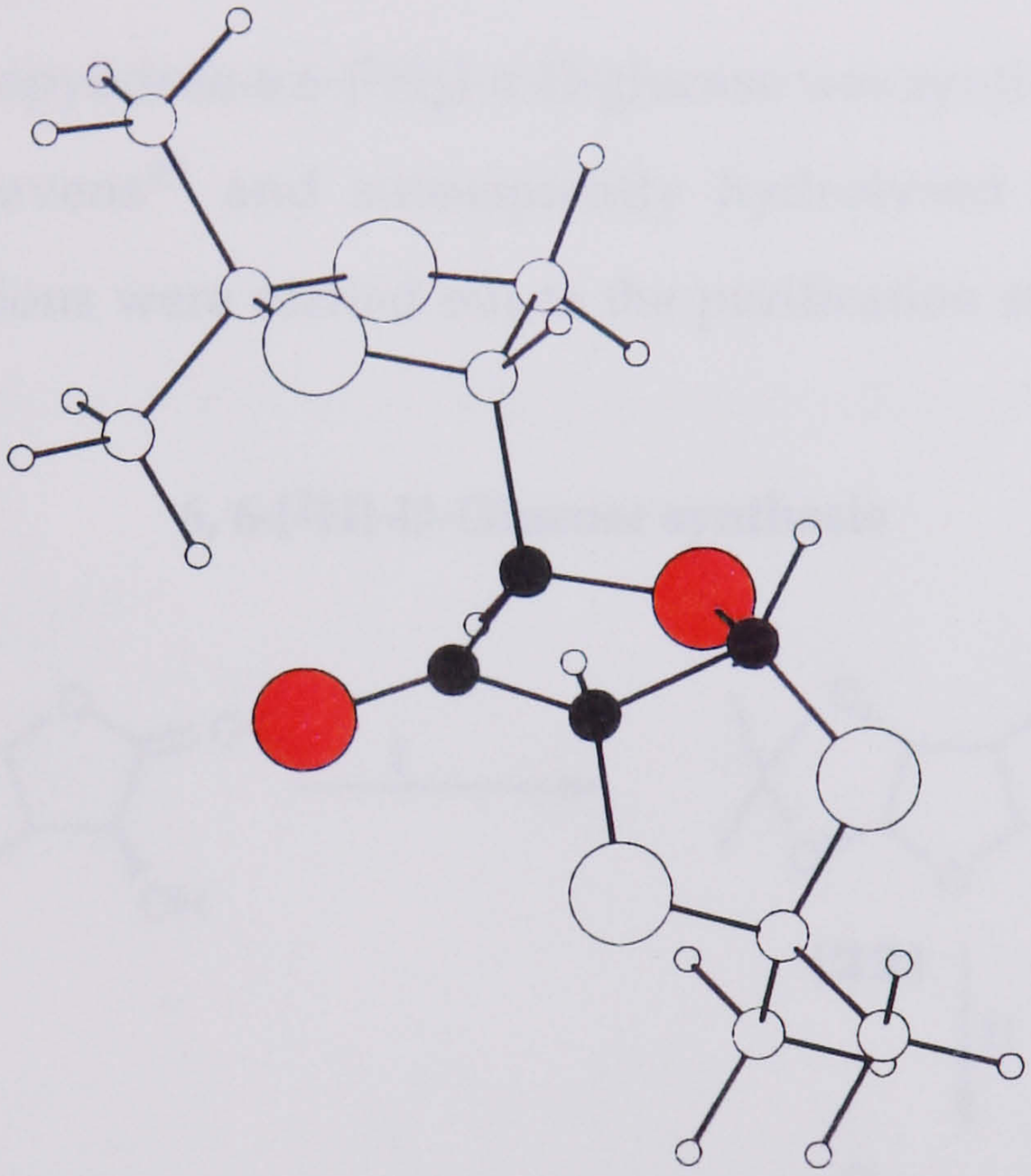
The stereospecificity observed in synthesising 3-[^{2}H]-D-glucose and 5-[^{2}H]-D-glucose can be attributed to attack of hydride from the least hindered side of the carbonyl in each case. **Figure 3.01** shows the energy minimised conformation for 1,2,5,6-O-diisopropylidene-α-D-*ribo*-hexofurano-3-ulose. It would be easier in this molecule for hydride to approach the ketone from above the carbonyl rather than underneath, where the 1,2-O-isopropylidene and furanose ring oxygen's are in the way. This would give the required *allo* stereochemistry.

Similarly, the minimised conformation for **(18)** shows that one side of the carbonyl group at C5 is hindered by the benzyl group at C3. Reduction from the least hindered face of the carbonyl group then gives the desired *gluco* stereochemistry.

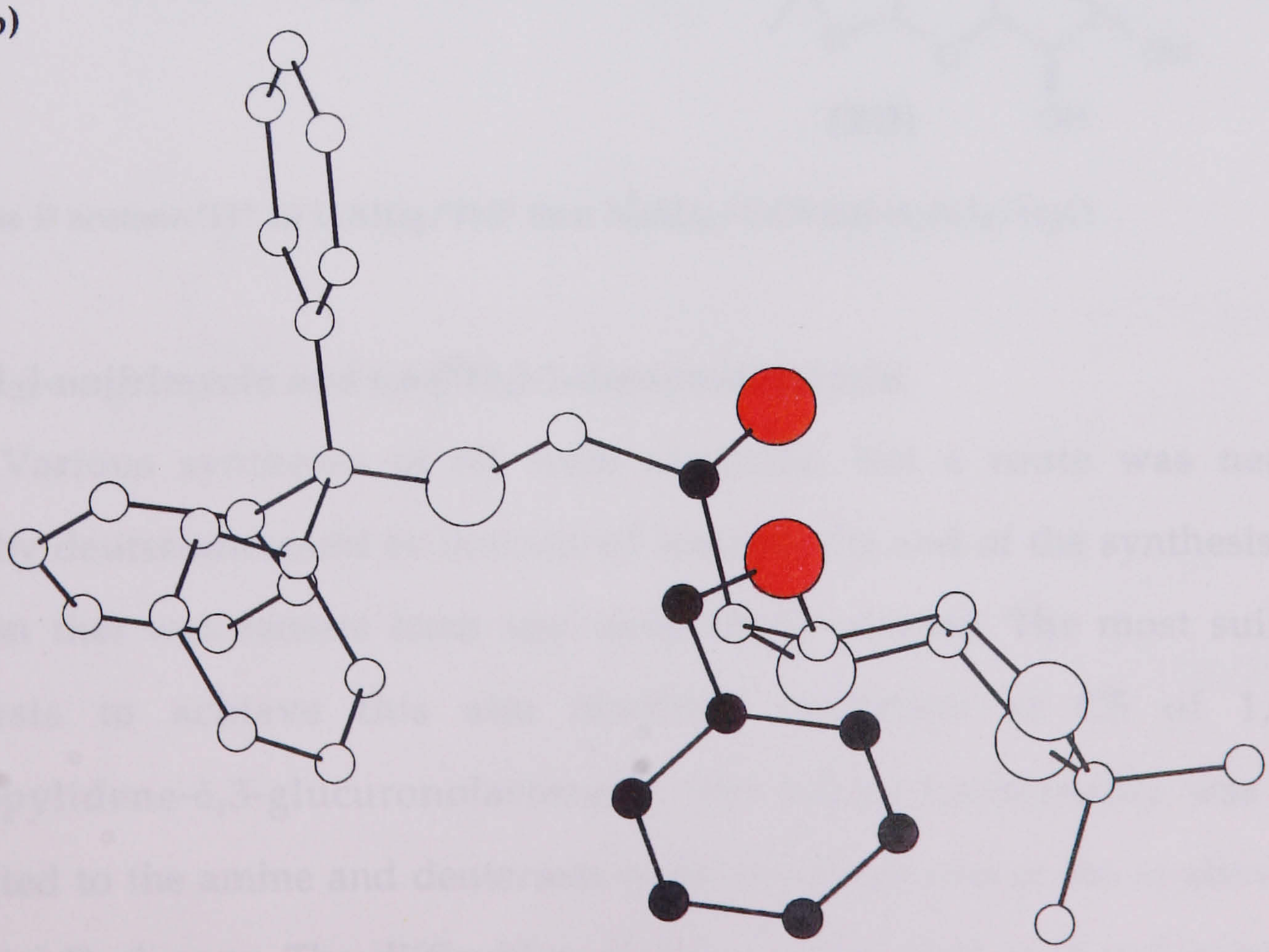
Figure 3.01

The energy minimised conformations for a) 1,2,5,6-O-diisopropylidene-α-D-*ribo*-hexofurano-3-ulose and b) 3-O-benzyl-6-O-triphenylmethyl-1,2-O-isopropylidene-α-D-*xylo* -hexofuranos-5-ulose.

a)



b)



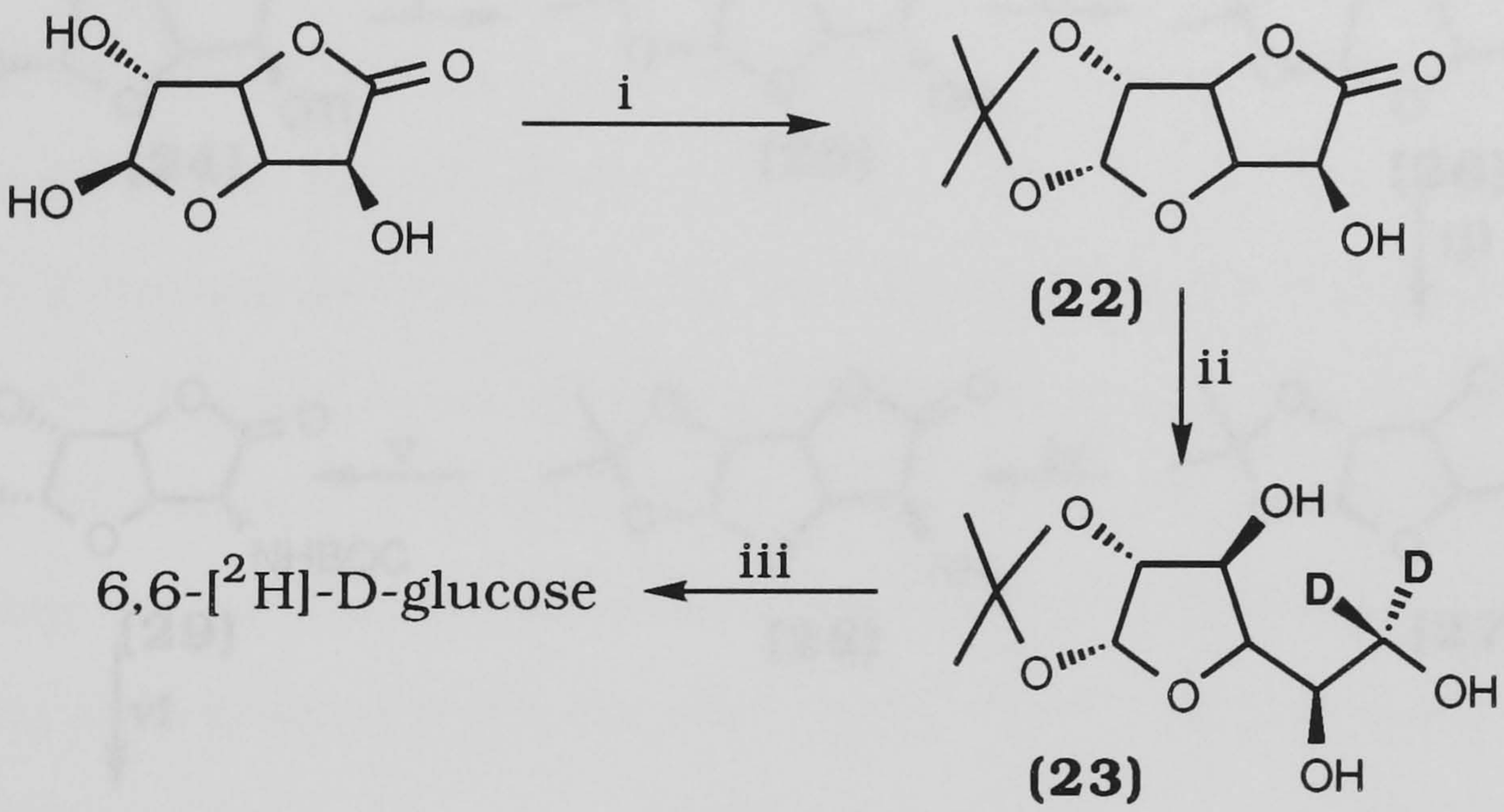
^{1}H and ^{13}C NMR confirmed the position of the label in the isolated glucose (no C5 epimer could be detected) and the mass spectrum of the peracetate showed an enrichment of 92 atom % D.

6, 6-[$^{2}H_2$]-D-Glucose

1,2-O-isopropylidene-6,6-[$^{2}H_2$]-α-D-glucose was synthesised according to Lemieux and Stevens[95] and subsequently hydrolysed to the deuterated hexose. Modifications were carried out to the purification stages only.

Scheme 3.08

6, 6-[^{2}H]-D-Glucose synthesis



Reagents i) acetone/H^{+} **ii)** $LiAlD_4$/THF then $NaBD_4$/EtOH**iii)** H_2SO_4/H_2O

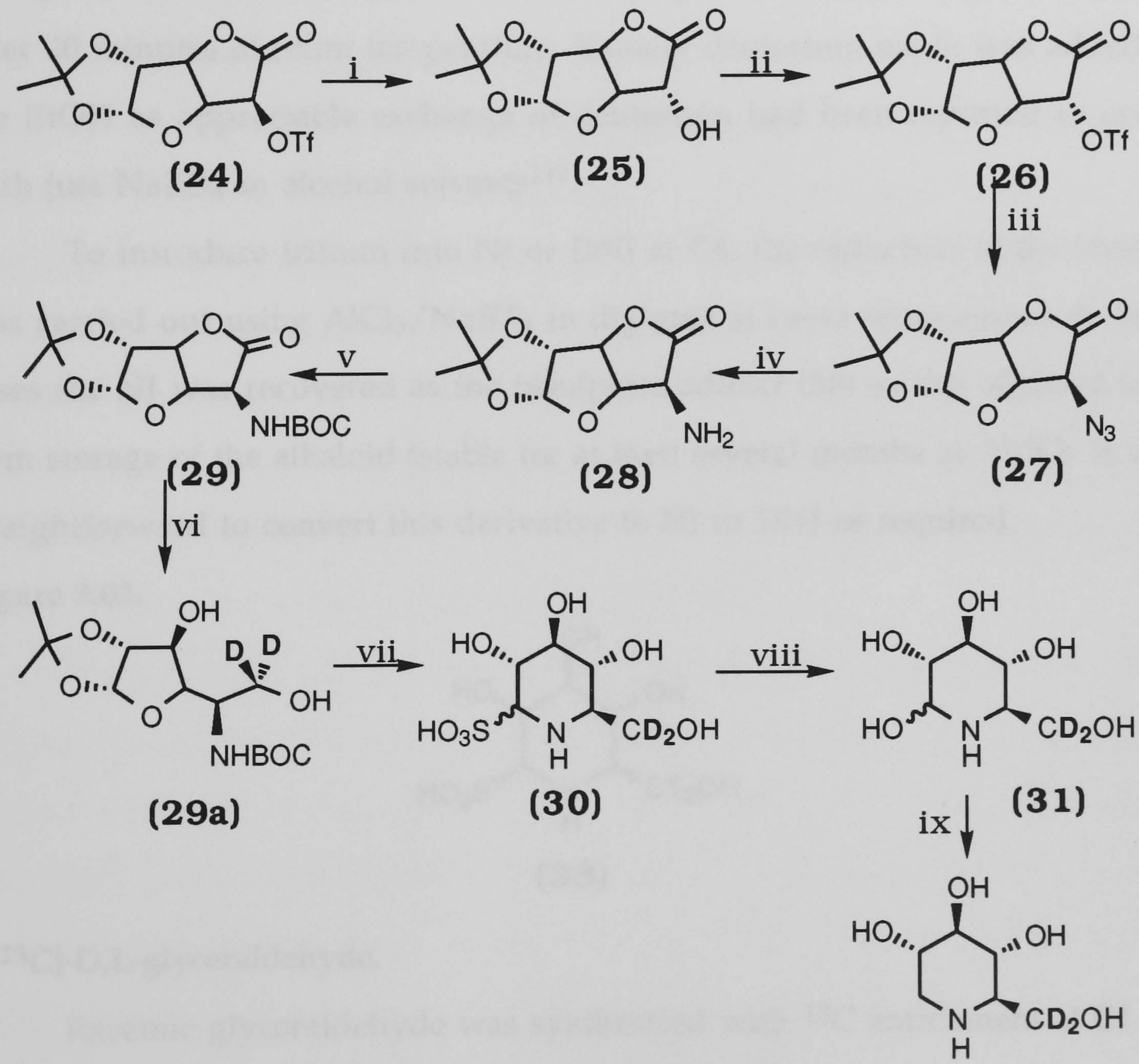
6,6-[$^{2}H_2$]-nojirimycin and 6,6-[$^{2}H_2$]-1-deoxynojirimycin

Various syntheses of NJ were available, but a route was needed whereby deuterium could be introduced towards the end of the synthesis in a position that was remote from any biosynthetic process. The most suitable synthesis to achieve this aim involved oxidation at C5 of 1,2-O-isopropylidene-6,3-glucuronolactone[109]. The ketone functionality was then converted to the amine and deuterium could be introduced at H6 as above for 6,6-[$^{2}H_2$]-D-glucose. The difficulties already encountered with oxidising the

lactone at C5 meant that an alternative route to the amine at C5 was required. Fleet[110,111] had synthesised the azido lactone **(27)** in 5 steps from 6,3-glucuronolactone and in our studies this was hydrogenated to the corresponding amine and protected as the BOC derivative. The original route of Anzeveno[109] with minor modifications then gave NJ which could be converted to DNJ by the method of Inouye[2]

Scheme 3.09

6,6-[$^2H_2$]-nojirimycin and 6,6 -[$^2H_2$] -1-deoxynojirimycin synthesis.

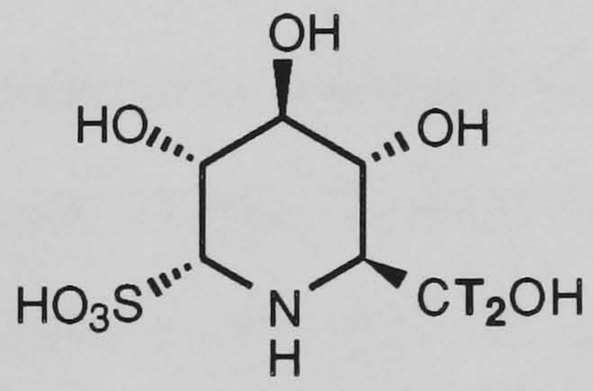


Reagents i) $CF_3COO^-Na^+$/MeOH **ii)** $(CF_3SO_2)_2O/CH_2Cl_2$/pyridine **iii)** NaN_3/DMF**iv)** H_2/Pd on C/EtOAc **v)** BOC anhydride/EtOAc **vi)** $LiAlD_4$/THF then $NaBD_4$/EtOH/deuterium oxide **vii)** SO_2/H_2O/35-45°C **viii)** Dowex 1x8(OH form) **ix)** H_2/PtO_2/AcOH/H_2O

The reduction of the lactone **(29)** to glycol was slow even with excess $LiAlD_4$. Typically the lactone would be reduced rapidly to a mixture of the lactol and glycol, but thereafter complete reduction to the latter took several hours, despite refluxing in THF. A faster procedure was to isolate the mixture of lactol and glycol and then complete the reduction with $NaBD_4$ in EtOH. Using this method, the reduction of the lactol had completely taken place after 20 minutes at room temperature. Usually deuterium oxide was added to the EtOH as appreciable exchange of deuterium had been reported to occur with just $NaBD_4$ in alcohol solvents[112].

To introduce tritium into NJ or DNJ at C6, the reduction of the lactone was carried out using $AlCl_3/NaBT_4$ in diglyme at room temperature. In both cases the NJ was recovered as the bisulphite adduct **(33)** as this allowed long term storage of the alkaloid (stable for at least several months at -10°C). It was straightforward to convert this derivative to NJ or DNJ as required.

Figure 3.02.

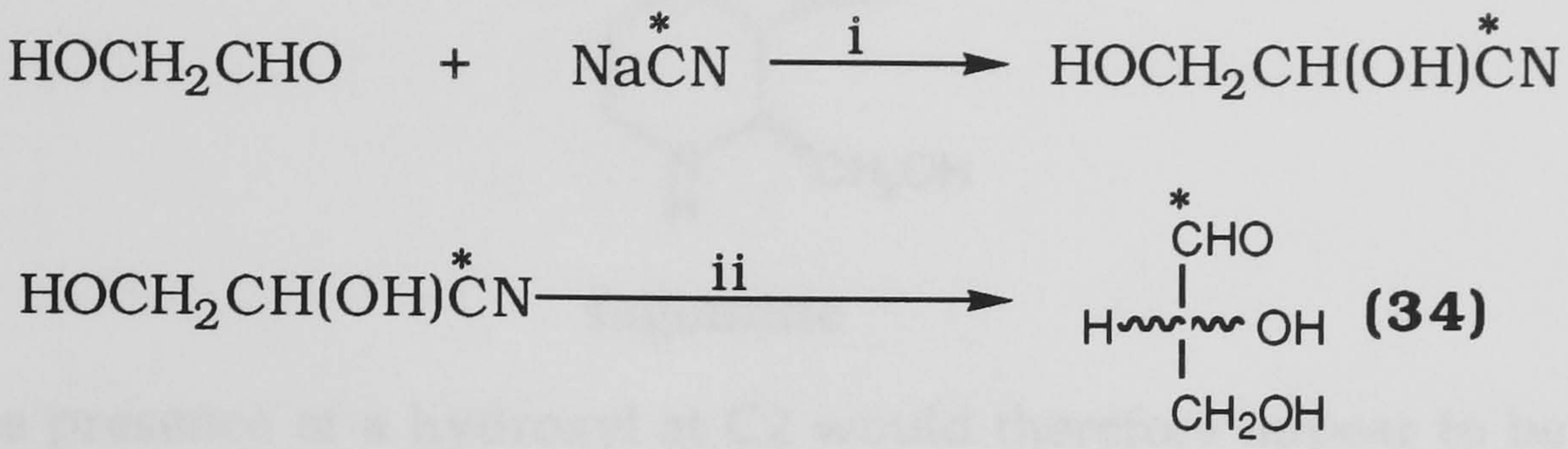


(33)

1-[^{13}C]-D,L-glyceraldehyde.

Racemic glyceraldehyde was synthesised with ^{13}C enrichment at C1 (50 atom % ^{13}C) by the method of Serianni *et al*[113,114,115].

1-[^{13}C]-D,L-Glyceraldehyde synthesis



(34)

Reagents i) NaCN/H_2O/pH 8 **ii)** H_2/Pd on C/H_2O/pH 1.7

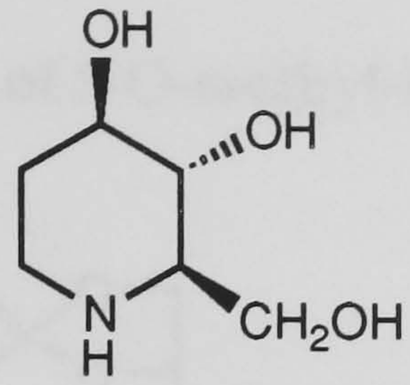
Detailed experimental procedures and ^{13}C NMR spectra were given.

Synthesis of glucose and DNJ analogues.

The aim of this section of the investigation was to synthesise analogues of glucose which could then replace the glucose in the usual fermentation medium. It was hoped that the corresponding DNJ analogue would be produced if subtle changes to the glucose molecule did not upset the biosynthesis mechanism. Recent interest had focused on analogues of those alkaloids normally found naturally, and this research effort had already revealed interesting results. For instance, the improvement in inhibition of HIV by acylating C6 of castanospermine has already been alluded to (page 9). In contrast, the removal of a hydroxyl at C1 destroyed the α-glucosidase activity when compared to the parent compound[116].

The discovery that a particular analogue shows weaker inhibition of an enzyme, or a class of enzymes, is not in itself a setback as the relationship between inhibition and structure is not clear. At the time of starting this study with DNJ, some knowledge of the relationship between structure and inhibition was known from natural alkaloid derivatives. Fagomine, the 2-deoxy analogue of DNJ, **figure 3.03**, failed to inhibit α- and β-glucosidases, α-mannosidase, α- and β-galactosidases and α-fucosidase[117]. DNJ and DMJ, in contrast, were known inhibitors of a number of these enzymes.

Figure 3.03



fagomine

The presence of a hydroxyl at C2 would therefore appear to be essential for a number of glycosidase enzymes, although the stereochemistry at this position is apparently less important. DNJ will inhibit α-mannosidase, although not as well as DMJ.

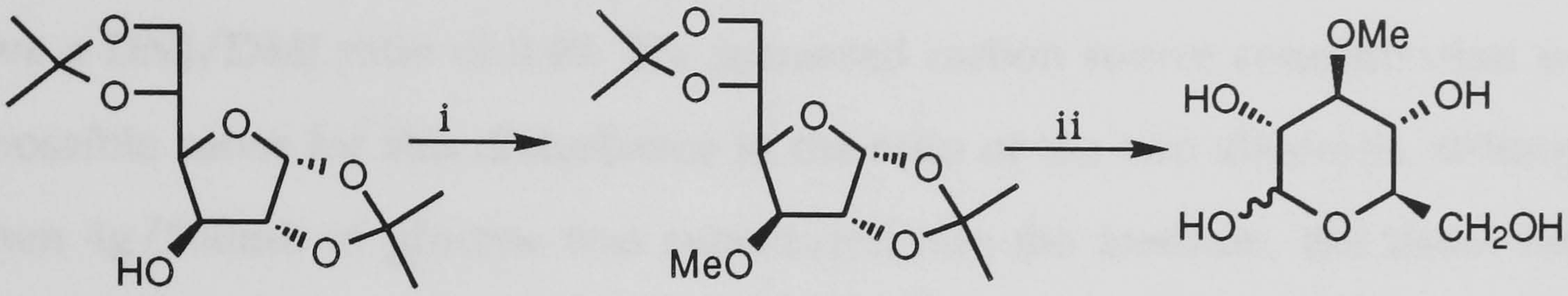
Three glucose analogues were synthesised and investigated to see if they could act as substrates in the biosynthetic pathway. They were 3-deoxy-D-glucose, 3-O-methyl-D-glucose and 5-deoxyglucose. These were chosen because all three were substrates for the glucose-fructose isomerisation reaction[75] (the first step in the biosynthesis) and the modified centre was in a position where it would not in theory interfere with any stage of the biosynthetic mechanism. If the analogues were substrates for metabolism, the first two derivatives would give 1,4-dideoxy NJ and 4-O-methyl DNJ respectively, whilst 5-deoxy-D-glucose would give fagomine. Although fagomine was a natural alkaloid, 5-deoxy-D-glucose was one of the best substrates for the fructose/glucose isomerisation. The lack of a hydroxyl at C5 meant that the normal epimerisation reaction in the biosynthesis would be avoided.

3-O-Methyl-D-glucose

3-O-Methyl-D-glucose was synthesised as below, **scheme 3.10.**

Scheme 3.10

Synthesis of 3-O-methyl-D-glucose.



(35) **(36)**

Reagents i) NaH/THF/MeI, RT **ii)** H_2SO_4/H_2O

When this glucose analogue was fed to *S. subrutilus*, glucose was also included in the fermentation medium as the microorganism needed an energy source for growth. It is unlikely that the 3-O-methyl-D-glucose would be able to fulfil this role. The final concentration was 2g of glucose and 2g of 3-O-methyl-D-glucose in 500mL of soyabean medium. After partial purification of the fermentation, mass spectrometry and gas chromatography indicated that DNJ and DMJ were present. No indication of the molecular ion (m/z 346) for the 4-O-methyl derivative could be found in the mass spectrum. Later work resulted in the synthesis of 3-O-methyl DNJ which gave the required peak in the mass spectrum. This confirmed that the molecular ion was not unstable for this derivative and it was likely the same applied to 4-O-methyl DNJ.

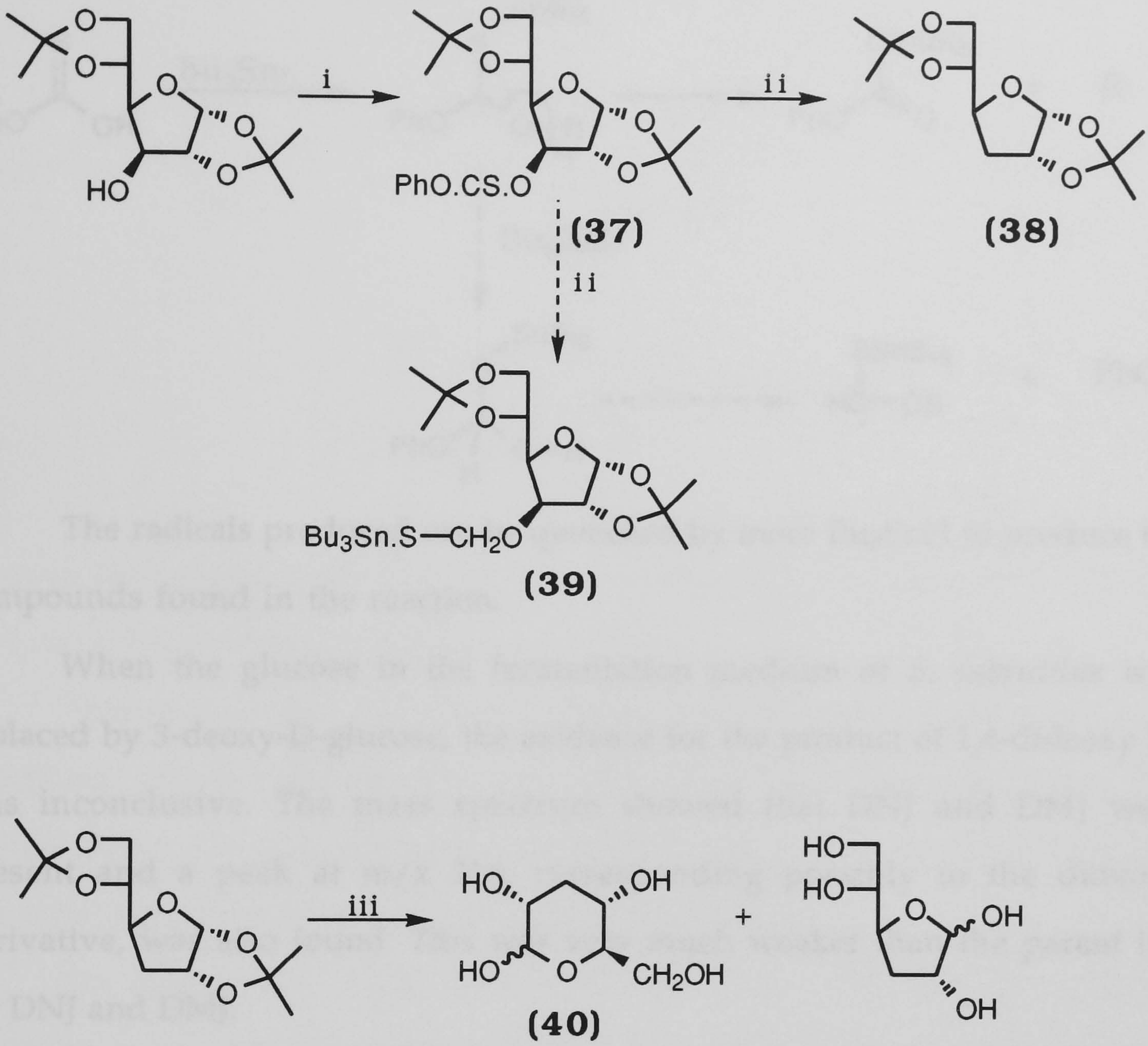
To test whether the glucose was being used preferentially for the biosynthesis, a second fermentation was tried which just contained 3-O-methyl-D-glucose (4g/500mL). Again DNJ and DMJ could be detected by GC and also as their peracetyl derivatives in the mass spectrum. These two alkaloids presumably originated from the carbon source that had been causing the dilution in the labelling studies. It was interesting that the ratio of DNJ to DMJ in these two fermentations was unlike that observed for fermentations in the biosynthetic studies. In previous work, DNJ was always found in excess

to DMJ (typically $2^{1}/2$ times as much). In both experiments with 3-O-methyl-D-glucose, DMJ was found in excess. For the 1:1 mixture of carbon sources, the DNJ/DMJ ratio was 0.78 (13mg of DNJ). Complete replacement of the glucose gave a DNJ/DMJ ratio of 0.49. The increased carbon source concentration was a possible cause for this disturbance in the ratio of the two alkaloids, although when 4g/500mL of glucose was substituted into the medium, the usual ratio was again observed. It seems likely that the glucose analogue is inhibiting one (or more) of the biosynthetic pathways leading to a decrease in the amount of DNJ. The fermentation with 2g of both glucose and 3-O-methyl-D-glucose had shown a lower titre of DNJ than when either 2g or 4g/500mL of glucose only was used. The unusual ratio observed was therefore due to less DNJ rather than more DMJ.

3-deoxy-D-glucose.

3-deoxy-D-glucose was synthesised by two different literature routes[118,119,120,121]. The best method was found to be as follows.

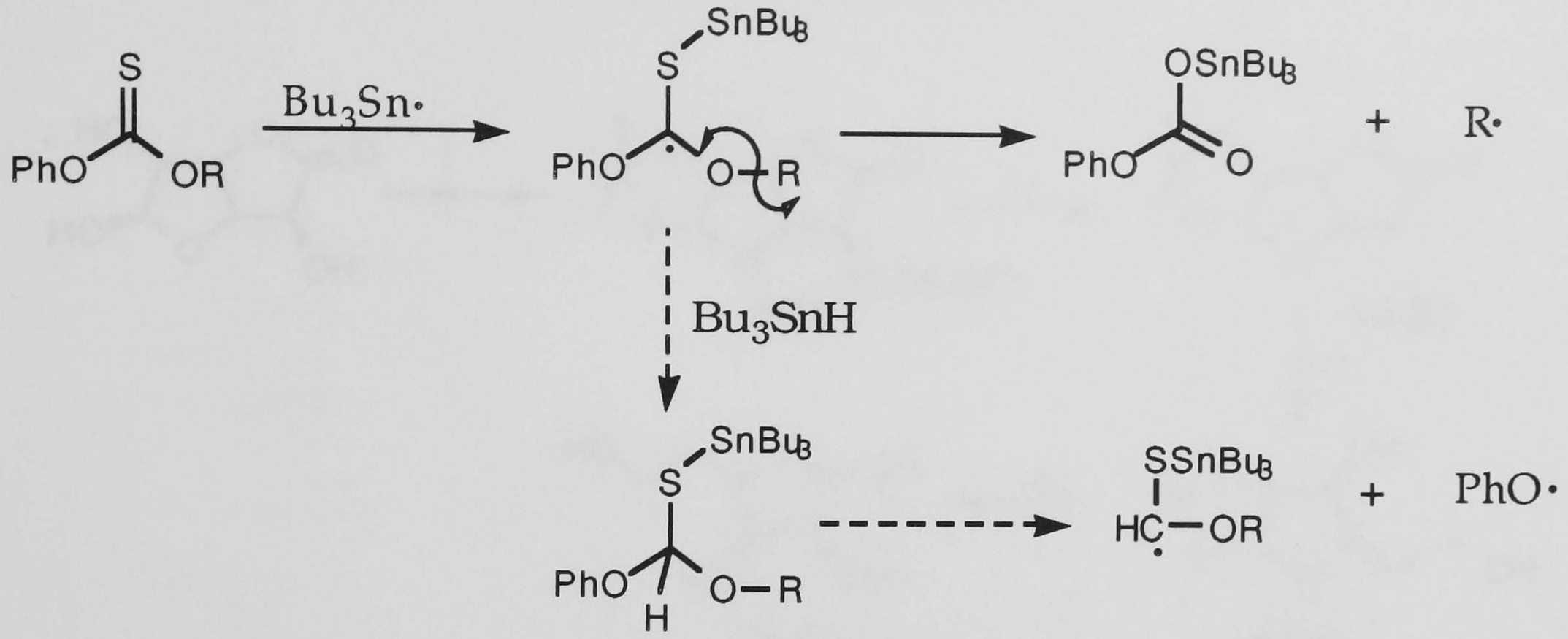
Synthesis of 3-deoxy-D-glucose



Reagents i) PhO.CS.Cl/CH_3CN/DMAP/pyridine **ii)** Bu_3SnH/AIBN/toluene 70°C **iii)** H_2SO_4/H_2O

The method above was based on the Barton deoxygenation procedure for secondary alcohols. The radical deoxygenation in step ii proceeded in 75-85% yield, giving in addition minor amounts of the characterised impurity **(39).** This had not been described in previous work, although the yield of 3-deoxy-1,2,5,6-O-diisopropylidene-α-D-*ribo*-hexofuranose was identical. Barton had found similar byproducts with aliphatic O-alkyl thioesters and the mechanism can be formulated as in **scheme 3.11.**

Scheme 3.11.



The radicals produced can be quenched by more Bu_3SnH to produce the compounds found in the reaction.

When the glucose in the fermentation medium of *S. subrutilus* was replaced by 3-deoxy-D-glucose, the evidence for the product of 1,4-dideoxy NJ was inconclusive. The mass spectrum showed that DNJ and DMJ were present and a peak at m/z 316, corresponding possibly to the dideoxy derivative, was also found. This was very much weaker than the parent ion for DNJ and DMJ.

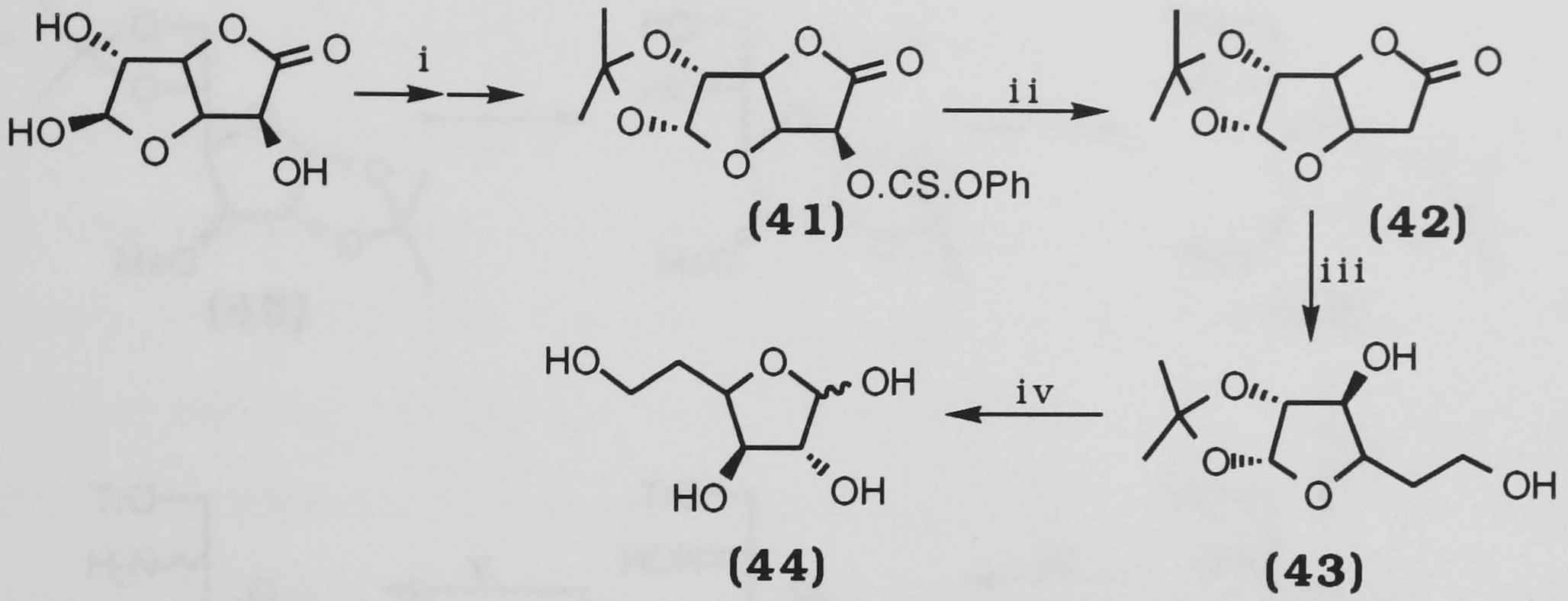
GC analysis of the fermentation indicated that DNJ was present at a concentration of 0.02mg/mL (400 mL) and that the usual ratio between DNJ and DMJ also existed.

5-deoxyglucose.

Several literature routes existed for this hexose[122,123,124,125,126,127], but in this project a short synthesis was developed based on the strategy of 3-deoxy-D-glucose, **scheme 3.12**.

Scheme 3.12

Synthesis of 5-deoxyglucose



Reagents i) H^+/acetone,thenPhO.CS.Cl/CH_3CN/DMAP/pyridine **ii)** Bu_3SnH/AIBN/toluene, 70°C **iii)** $LiAlH_4$/THF **iv)** H_2SO_4/H_2O

This strategy had the advantage that unwanted side products were not produced, which had been the case with some of the literature routes. The radical deoxygenation proceeded in almost quantitative yield without any other radical side reactions as noted for 3-deoxy-D-glucose.

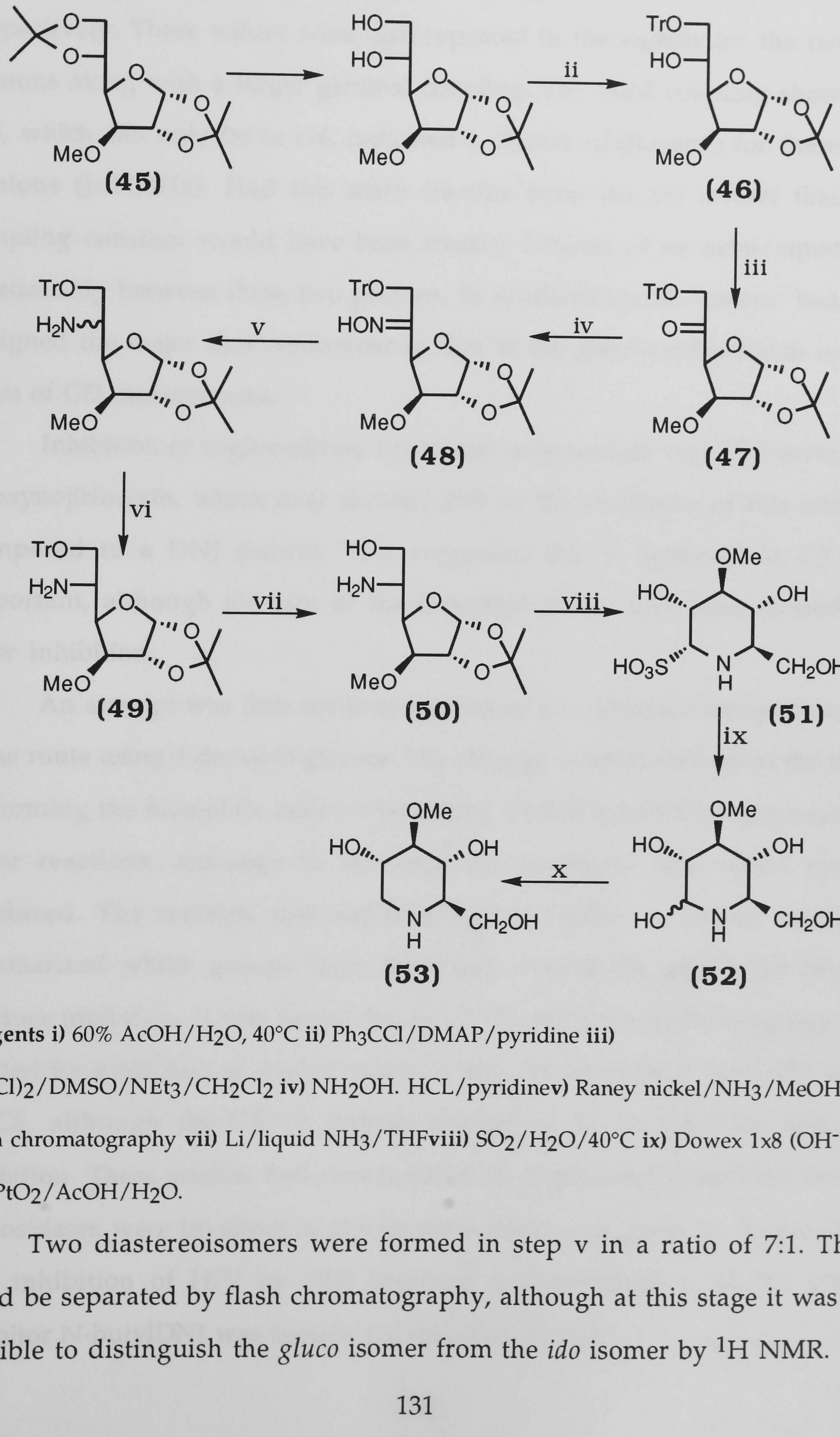
On feeding the 5-deoxyglucose (100%) to *S. subrutilus*, no fagomine could be detected by GC analysis using a standard sample. Furthermore, only traces of DNJ were present in the fermentation in contrast to the other two fermentation studies.

Chemical syntheses of DNJ analogues

As biochemical modification of the DNJ structure had been unsuccessful, chemical routes were also investigated. At the time this work was started, the synthesis of 3-O-methyl-1-deoxynojirimycin and 1,3-dideoxynojirimycin had not been published, yet the synthesis of 3-O-methyl-D-glucose and 3-deoxy-D-glucose meant that precursors to the desired alkaloids were available. Using methodology developed by Inouye[2], 3-O-methyl-1-deoxynojirimycin was synthesised as follows, **scheme 3.13**.

Scheme 3.13

Synthesis of 3-O-methyl-1-deoxynojirimycin.



Reagents i) 60% AcOH/H_2O, 40°C **ii)** Ph_3CCl/DMAP/pyridine **iii)** $(COCl)_2$/DMSO/NEt_3/CH_2Cl_2 **iv)** NH_2OH. HCL/pyridine**v)** Raney nickel/NH_3/MeOH **vi)** Flash chromatography **vii)** Li/liquid NH_3/THF**viii)** SO_2/H_2O/40°C **ix)** Dowex 1x8 (OH^-) **x)** H_2/PtO_2/AcOH/H_2O.

Two diastereoisomers were formed in step v in a ratio of 7:1. These could be separated by flash chromatography, although at this stage it was not possible to distinguish the *gluco* isomer from the *ido* isomer by ^1H NMR. The

main fraction was used to synthesise the bisulphite adduct **(51)** and this was shown to be the *gluco* derivative from the following spectroscopic evidence. H5 coupled to both H6 protons with coupling constants of 4.5 and 2.9 Hz respectively. These values were also repeated in the signals for the two H6 protons along with a larger geminal coupling. The third coupling shown by H5, which can only be to H4, indicated a diaxial relationship for these two protons (J=11.3Hz). Had this main fraction been the *ido* isomer then the coupling constant would have been smaller because of an axial/equatorial relationship between these two protons. In synthesising NJ, Inouye[2] had also assigned the major diastereoisomer to that of the *gluco* configuration on the basis of CD measurements.

Inhibition of α-glucosidase (trehalase) was weaker with 3-O-methyl-1-deoxynojirimycin, which only showed 20% of the inhibition of this enzyme compared to a DNJ control. This suggested that a hydroxyl at C3 was important, although the size of the O-methyl group may have caused the poor inhibition.

An attempt was then made to synthesise 1,3-dideoxynojirimycin by the same route using 3-deoxy-D-glucose. The strategy worked well up to the point of forming the bisulphite adduct. Saturating with SO_2 had been successful in other reactions, although in this case the sulphonic acid could not be produced. The reaction was not investigated further as recent results[128] summarised which groups were necessary around the piperidine ring to produce inhibition. It was found that at C2, C3 and C4 a hydroxyl group was needed for inhibition of α-glucosidase. It was not essential to have OH at C1 or C6, although the C5/C6 linkage needed to be in place for effective inhibition. These studies had concentrated on α-glucosidase because two α-glucosidases were involved in glycoprotein processing (page 5). It was likely that inhibition of HIV by DNJ involved α-glucosidase I, as the potent inhibitor N-butylDNJ was specific for this enzyme only.

Experimental

General conditions

^{1}H NMR spectra were recorded at 220, 250, or 400 MHz using a Perkin Elmer R34, a Bruker ACF 250 or a Bruker WH 400 spectrometer respectively. ^{2}H NMR spectra were recorded at 38.398 (ACF 250) or 61.424 MHz(WH 400). ^{13}C NMR spectra were recorded at 61.424 (ACF 250) or 100.623 MHz (WH400). In addition to TMS, the following reference signals were used for ^{1}H and ^{2}H NMR: HDO δ 4.75, pyridine α-H δ 8.70, $CHCl_3$ δ 7.23 ppm. All coupling constants are given in Hz.

Mass spectra were recorded on a Kratos MS80 spectrometer. Chemical ionisation (CI) mass spectra used ammonia as reagent gas. Peracetyl DNJ and DMJ were used in all analyses to determine isotopic content and typically the mean was taken of 5 scans of a compound obtained over a period of two or three days. For the analysis of fermentations carried out in D_2O, a Kratos concept four sector mass spectrometer was used with methane as the reagent gas for CI mass spectrometry. All other experiments with this instrument used FAB ionisation with a glycerol matrix.

Deuterated glucoses were analysed for deuterium enrichment by first acetylating the sugar (excess pyridine/Ac_2O) followed by mass spectroscopy. Comparison was with authentic glucose pentaacetate (Aldrich) in the same manner as described in chapter 2.

IR spectra were recorded using a Perkin Elmer 1720X FT spectrometer.

UV spectra were recorded using a Philips PU 8720 scanning spectrophotometer.

Liquid scintillation counting used Optiphase Safe® (10 mL, LKB) as scintillant and radioactivity was measured with a Packard 2000CA TriCarb liquid scintillation analyser.

GLC analysis was performed on a Carlo Erba Fractovap with an FID detector using a 30 m quartz J & W DB5/0.25μm column. The flow rate of the

carrier gas (N_2) was 2mL min^{-1} with a gas split ratio of 30:1. The injector/detector temperature was 250°C and the column temperature was 190°C

Anhydrous solvents were obtained by distillation from the following: THF, dioxan and toluene (Na/benzophenone); CH_2Cl_2 (CaH_2); pyridine (KOH); acetone (B_2O_3). DMSO and DMF were stored over freshly activated 4Å molecular sieves. Anhydrous MeOH and diglyme were purchased from Aldrich and CH_3CN was HPLC grade.

TLC was performed using glass backed SiO_2 TLC plates (Merck Kieselgel 60F-254) and compounds were visualised with either 10% H_2SO_4 (conc.) in EtOH for sugar derivatives or ninhydrin.(0.1% $^{w}/_{v}$ in n-butanol) for amines. In each case the TLC plate was heated with a hot air gun following spraying. Merck silica gel 60 (230-400 mesh ASTM) was used for flash chromatography.

Maintenance of microorganisms.

S. subrutilus ATCC 27467 and *B. subtilis* ATCC 9372 were first grown in nutrient broth upon receipt. The microorganisms were sub-cultured 2 or 3 times and then stored as follows.

S. subrutilus - a 100µl aliquot of the nutrient broth culture was transferred under sterile conditions to an oatmeal agar petri dish. After spreading across the agar, the microorganisms were incubated at 37°C for several days. On appearance of a white crust of spores, a sterile loop was used to scrape the spores into a small volume of sterile distilled water (2-3mL). This suspension was sterile filtered through cotton wool, whirlimixed and then centrifuged (3000rpm/20min.). The supernatant was discarded and the pellet was taken up in 20% aqueous glycerol (1000µL). To test for contamination and to quantify the viable spores, a 100µL aliquot was taken from this stock solution and diluted in a serial fashion. Contamination usually took the form of light coloured, soft colonies. *Streptomyces* colonies were pale brown, hard and appeared sub-divided. Serial dilution analysis

indicated typically 10^6-10^7 colonies per 100µL. The stock glycerol solution was then kept at -10°C.

B. subtilis - after sub-culturing, a 100µL aliquot of the nutrient broth culture was spread over a nutrient agar petri-dish. After incubation for 24h at 37°C, the *B. subtilis* colonies could be identified as soft, light brown spheres. Colonies that were not contaminated were transferred using a flamed loop to sterile nutrient broth and incubated for a further 24h at 30°C. During this time, the clear nutrient broth solution became turbid. A 500µL aliquot was removed and transferred to glycerol solution (500µL, 66%) for storage at -10°C. Inoculation of *Streptomyces* fermentations was with 50-100µL of the stored spore suspension. Growth was monitored by the formation of mycelia under the phase contrast microscope. For *B. subtilis* 100µL of the stored bacterial suspension was transferred to 50mL of sterile nutrient broth and incubated for 24h at 30°C. Inoculation of the main fermentation medium was with 300µL of this broth.

Media

Oatmeal agar

Oatmeal, finely ground, 30g

Yeast extract, 1g

Agar #1, 15g

Distilled water, 1L

The oats and yeast extract were boiled together for 3h. The agar was then added followed by vigorous stirring. The solution was then autoclaved and poured.

Nutrient broth (Oxoid) 13g L^{-1} Autoclaved (120°C/20 min.)

Nutrient agar (Oxoid) 28g L^{-1} Autoclaved (120°C/20 min.)

Fermentation conditions, isolation and characterisation of metabolites.

The constituents of the fermentation media are described in chapter 2.

In all feeding experiments, labelled glucoses were diluted with unlabelled D-glucose before use. Aliquots of media (30mL in a 250mL conical flask) were inoculated with the required microorganism and then shaken at 28-30°C for 7d at 200rpm. The concentration of DNJ was monitored during this period by either GC or with the trehalase assay. The medium was then autoclaved at 120°C for 20 min., centrifuged at 5000rpm for 30 min. and the supernatant added to a column of Dowex 50 ion exchange resin (H^+ form, 15mL per 100mL supernatant). The column was washed with distilled water (2 bed vol.) and then eluted with aqueous ammonia (2M, 2 bed vol.). This eluate was lyophilised or concentrated to 50mL for further GC analysis as required. The lyophilised residue was taken up in the minimum volume of 70% aqueous EtOH, filtered and applied to a column of Al_2O_3 (neutral, Brockmann grade I). This column was eluted with 70% aqueous EtOH (3 bed vol.) and concentrated *in vacuo*. The residue was dissolved in pyridine and treated with an excess of acetic anhydride in the presence of DMAP. The acylation mixture was evaporated to dryness *in vacuo* and the peracetylated alkaloid(s) were purified by flash chromatography on silica (elution with EtOAc : CH_2Cl_2, 30% $^v/_v$).

To deacetylate peracetyl DNJ or DMJ, the alkaloid was stirred with concentrated NH_3 (5mL, d 0.880) and methanol (5mL) overnight at room temperature. Evaporation of the reaction gave either N-acetyl DNJ or free DMJ in approx. 70% conversion.

Gas chromatography

For analysis of the fermentation culture directly, a 1mL aliquot was removed and centrifuged to remove suspended solids. This was then whirli-mixed with α-methylglucoside internal standard (100μL, 0.8 mg mL^{-1}) and lyophilised. Sigma SilA (100μL) was added and the reaction was set aside for 30 min. at 60°C. Aliquots (1μL) of this mixture were analysed by GLC for DNJ and DMJ.

Trehalase assay for NJ and DNJ[129]

To a solution of trehalase (Sigma Chemical Co., 20µL, 0.005µgmL^{-1}, 0.385 U mL^{-1}) in maleate buffer (pH 6.0) was added inhibitor solution (20 µL) and the mixture was incubated at 37°C for 15 min. Trehalose (20µL, 56 mM) in 0.1M maleate buffer (pH 6.0) was added and the reaction was incubated for a further 60 min. at 37°C. The reaction was stopped by the addition of GOD-PAP (Boehringer) glucose assay solution (1 mL) containing tris buffer and after a further incubation of 30 min., the absorbance at 510 nm was then determined. To eliminate interference by NJ, an equal volume of 6M HCl was added to the fermentation sample which was then heated in a boiling water bath for 6h. The solution was lyophilised, re-suspended to the original volume in maleate buffer (pH 6.0) and assayed as above.

Mannosidase assay for MJ and DMJ[130]

The inhibitor solution (150µL) was incubated with jack bean α-D-mannosidase (Sigma Chemical Co., 50µL, 0.02 mg mL^{-1}, 20U mg^{-1}) and sodium acetate buffer (250 µL, pH 5.5) at 25°C for 15 min. 4-Nitrophenyl-α-D-mannoside (200 µL, 30 mM) was added and the mixture was incubated at 25°C for 15 min. The reaction was stopped by the addition of glycine buffer (2 mL, 0.1 M, pH 10.7) and the absorbance of the solution at 400 nm was read immediately. As with the trehalase assay, determinations were carried out in triplicate against the appropriate blank reactions.

Spectroscopic data for isolated metabolites.

Peracetyl DNJ. ^{1}H NMR (400 MHz, C_5D_5N, 90°) δ 2.01 (3H, s, Me), 2.04 (6H, s, Me), 2.05 (3H, s, Me), 2.19 (3H, s, Me), 3.60-3.80 (1H, br.s, H1), 3.99-4.05 (2H, br.s, H1 & H5), 4.50 (1H, dd, H6, 11.5, 5.9), 4.63 (1H, dd, H6, 11.6, 7.8), 5.11 (1H, m, H2), 5.32 (1H, t, H4, 3.8, 4.3), 5.39 (1H, t, H3, 3.9, 3.4); **MS** (CI): found m/z 374.1450, calc. for $C_{16}H_{24}NO_9$ m/z 374.1451 $(M + H)^+$.

N-Acetyl DNJ. ^{1}H NMR (400MHz, D_2O) δ 1.84 (3H, s, Me), 2.73 (1H, t, $H1_{ax.}$, 12.0), 2.90 (1H, br.t, H5), 3.30 (1H, dd, $H1_{eq.}$, 12.4, 5.1), 3.34-3.44 (2H, m, H3 &

H4), 3.60 (1H, ddd, H2, 5.2, 8.8), 3.71 (1H, dd, H6, 12.4, 5.6), 3.82 (1H, dd, H6, 12.4, 3.0); **^{13}C NMR** (100.6 MHz, D_2O) δ 47.9 (C1), 60.2 (C6), 60.8 (C5), 69.6 (C2), 70.4 (C3 or C4), 78.0 (C3 or C4).

Peracetyl DMJ ^{1}H NMR (400 MHz, C_5D_5N, 90°C) δ 1.96 (3H, s, Me), 2.01 (3H, s, Me), 2.03 (3H, s, Me), 2.13 (3H, s, Me), 2.22 (3H, s, Me), 3.46 (1H, br.s, H1), 3.85 (2H, br.s, H1 & H5), 4.50 (1H, dd, H6, 11.4, 6.1), 4.81 (1H, br.dd, H6), 5.36 (2H, m, H3 or H4, H2), 5.70 (1H, t, H3 or H4, 3.3); **MS** (CI): found m/z 374.1450, calc. for $C_{16}H_{24}NO_9$ m/z 374.1451. **^{13}C NMR** (61.42 MHz, $CDCl_3$, 25°C) Two rotamers existed δ 20.6-21.7 (several peaks, Me), 34.40 (C1), 40.15 (C1), 49.96, 55.46, 59.96 (C6), 60.12 (C6), 64.83, 65.76, 66.90, 67.08, 67.63, 68.41, 168.8-170.7 (several peaks, CO)

DMJ ^{1}H NMR (400 MHz, D_2O) δ 2.85 (1H, m, H5), 3.00 (1H, dd, H1, 13.9, 1.3), 3.20 (1H, dd, H1, 13.9, 2.9), 3.58 (1H, dd, H3, 9.6, 3.1), 3.71 (1H, dd, H4, 10.0, 9.3), 3.75 (1H, dd, H6, 12.1, 6.0), 3.85 (1H, dd, H6, 12.4, 3.2), 4.10 (1H, M, H2). **^{13}C NMR** (100.6 MHz, D_2O) δ 48.3 (C1), 59.5, 61.0, 67.1, 67.5, 73.7ppm

Chemical syntheses.

2-[^{2}H]-D-Glucose

2,3,4,6-Tetra-O-benzyl-D-glucopyranoside **(10)** was synthesised in two steps (30% overall yield) according to the method of Tate and Bishop[98].

α-Methyl-2,3,4,6-tetra-O-benzyl-D-glucopyranoside (9). ^{1}H NMR (220 MHz, $CDCl_3$) δ 3.40 (3H, s, OMe), 3.55-4.10 (6H, m, H2-H6), 4.45-5.05 (9H, m, 4xCH_2, H1), 7.20-7.50 (20H, m, aromatic H). **MS** (CI) m/z 572 $(M + NH_4)^+$.

2,3,4,6-Tetra-O-benzyl-D-glucopyranose (10). ^{1}H NMR (220 MHz, $CDCl_3$) δ 3.50-4.10 (6H, m, H2-H6), 4.46-5.00 (8H, m, CH_2), 5.25 (1H, d, H1, 4.4), 7.10-7.50 (20H, m, aromatic H). **MS** (CI) m/z 558 $(M + NH_4)^+$, 540 $(M + NH_4 - H_2O)^+$.

2,3,4,6-Tetra-O-benzyl-2-[^{2}H]-D-glucopyranose (11).

Sodium (1g, 0.04 mol) was dissolved in deuterium oxide (54 mL) under N_2. This solution was then added to a solution of 2,3,4,6-tetra-O-benzyl-D-

glucopyranose (7.2g, 0.013 mol) in dry dioxan (235 mL) and the mixture was stirred under N_2 for three days at room temperature. The dioxan was evaporated *in vacuo* and the residual solid was dissolved in CH_2Cl_2. This solution was washed with water (3 x 50 mL) until the aqueous layer was neutral. The organic layer was dried ($MgSO_4$) and the solvent was removed *in vacuo* to yield a tan solid (8 g). ^1H NMR showed this to be an approximately equal mixture of *gluco*- and *manno*-isomers. Recrystallisation of this solid from methanol gave pure 2-[^2H]-2,3,4,6-tetra-O-benzyl-D-glucose (3 g, 83% of the available *gluco*-isomer). **M.Pt**. 136-138°C, lit[98]. 150-151°C (protio-derivative). **$[\alpha]^{23}_D$** = $^+$57.5° (c = 0.06 $CHCl_3$) lit. value[98] $^+$20.7°. **^1H NMR** (220 MHz, $CDCl_3$) δ 3.50-4.10 (5H, m, H3-H6), 4.46-5.00 (8H, m, CH_2), 5.25 (1H, s, H1), 7.10-7.45 (20H, m, aromatic H). **MS** (CI) found 559.2920 calc. for $C_{34}H_{39}DNO_6$ m/z 559.2919 $(M + NH_4)^+$.

2-[^2H]-D-Glucose (12).

To palladium black (11 mg) suspended in glacial HOAc (250 mL) was added 2-[^2H]-2,3,4,6-tetra-O-benzyl-D-glucose (2.45 g, 4.4 mmol) and the mixture was stirred under hydrogen (1 atm) for 24h after which time 455 mL of hydrogen was taken up. TLC (BuOH : AcOH : H_2O : EtOAc) showed that a mixture of glucose and acetylated derivatives were present. Water (20 mL) was added and the catalyst was removed by filtration through Celite. The filtrate was evaporated to dryness *in vacuo* and the residue was dissolved in MeOH to which a few drops of ammonium hydroxide (d 0.880) had been added. After 2h the solution was evaporated to dryness and the residue was dissolved in water (30 mL). The aqueous solution was extracted with CH_2Cl_2 (2 x 10 mL) and then lyophilised to yield 2-[^2H]-D-glucose as a clear, colourless syrup (0.548 g, 65%). **^1H NMR** (400 MHz, D_2O) δ 4.11-4.18 (4H, m, $H3_\beta$, $H4_\beta$, $H5_\beta$, $H4_\alpha$), 4.24-4.31 (3H, m, $H6_{\alpha\beta}$, $H3_\alpha$), 4.31-4.36 (3H, m, $H6_{\alpha\beta}$, $H5_\alpha$), 4.66 (1H, s, $H1_\beta$), 4.91 (1H, s, $H1_\alpha$). The spectrum compared well to that of authentic D-glucose, except that both H1 protons, normally seen as doublets, had collapsed

to two singlets. The lowest frequency signal in D-glucose (H2β, 8.0 Hz) was missing in the deuterated derivative. **^{13}C NMR** (100.6 MHz, D_2O) δ 96.42 (C1β), 92.61 (C1α), 76.48, 76.25 (C3β, C5β), 73.26 (C3α), 71.97 (C5α), 70.20, 70.16 (C4αβ), 61.31, 61.15 (C6αβ). Where α and β refer to the two anomers of glucose[131]. Authentic D-glucose had signals at δ 74.6 (H2β) and 71.9 (H2α), whereas the deuterated compound displayed two weak triplets at δ 74.24 and 71.61. **MS** (CI) m/z 409 $(M + NH_4)^+$, 391 $(M + H)^+$. Compared to authentic glucose pentaacetate, the level of deuterium enrichment was >97 atom % D. **^{2}H NMR** (61.4, D_2O) δ 3.13, 3.50 ppm. Authentic D-glucose exhibits a doubled doublet in its ^{1}H NMR spectrum due to H2β, δ 3.13 and H2α δ 3.46 ppm.

The method of Bock *et al*[75] was followed in preparing 2-[^{2}H]-D-glucose by the enzymatic route (60% overall yield). The ^{13}C and ^{1}H NMR spectra compared well to that of the 2-[^{2}H]-D-glucose synthesised by the chemical route.

3-[^{2}H]-D-Glucose.

This was synthesised according to Koch and Perlin[100], except that the initial oxidation of 1,2,5,6-O-diisopropylidene-α-D-*gluco*-hexofuranose was carried out using pyridinium dichromate[132] in 74% yield.

1,2,5,6-O-diisopropylidene-α-D-*ribo*-hexofurano-3-ulose (13).

CH_2Cl_2 (200 mL, dry) was stirred with $Py_2Cr_2O_7$ (43.5 g, 0.12 mol) and powdered molecular sieve (5 g) for 5 min. under N_2. The alcohol (15 g, 0.06 mol) was then added to the orange solution in one portion and the mixture was stirred for 48h at room temperature. After this time, TLC (20% EtOAc : CH_2Cl_2, Al_2O_3) indicated a slower moving component with no evidence of starting material. The reaction was filtered through Celite and the CH_2Cl_2 was evaporated *in vacuo* to give a crude brown oil. EtOAc (250 mL) was added and the suspension was sonicated for 5 min. Filtration through a SiO_2 plug using 30% EtOAc : CH_2Cl_2 gave a colourless oil that solidified overnight at 0°C. This solid was kept under vacuum until a constant mass was reached.

Yield 11.14 g (74%). The ^1H NMR spectrum in $CDCl_3$ showed that a mixture of ketone and hydrated ketone were present (30% hydrated). **^1H NMR** (400 MHz, $CDCl_3$, **hydrate**) δ 1.33 (3H, s, CH_3), 1.35 (3H, s, CH_3), 1.46 (3H, s, CH_3), 1.55 (3H, s, CH_3), 3.72 (1H, br.s, OH), 3.88 (1H, d, H4, 6.6), 4.15 (1H, br.s, OH), 4.24 (1H, d, H2, 3.8), 4.41 (1H, q, H5, 6.3), 5.82 (1H, d, H1, 3.8); (**ketone**) δ 1.31 (6H, s, CH_3), 1.41 (3H, s, Me), 1.43 (3H, s, Me), 4.00-4.05 (2H, m), 4.32-4.35 (2H, m), 4.36 (1H, d, H2, 4.5), 6.11 (1H, d, H1, 4.5). **MS** (CI, CH_4) m/z 259 $(M + H)^+$. **IR** (film) υ 3700-3100 cm^{-1} (OH, hydrate), 1776 (CO, strained), 1380 (doublet, **gem** dimethyl).

1,2,5,6-O-diisopropylidene-3-[^{2}H]-α-D-*allo*-hexofuranose (14). (95 %) **^1H NMR** (220 MHz, $CDCl_3$) δ 1.20-1.60 (12H, 3s, isopropylidene), 2.70 (1H, br.s, OH), 3.85 (1H, d, H4, 4.7), 4.08 (2H, m, H6), 4.35 (1H, m, H5), 4.65 (1H, d, H2, 4.2), 4.85 (1H, d, H1, 4.2). **MS** (CI) m/z 262 $(M + H)^+$.

1,2,5,6-O-diisopropylidene-α-D-*allo*-hexofuranose. $[\alpha]^{23}_D$ = $^+$36.4° (c = 0.4 H_2O) lit. value[102] $^+$37.7°. **^1H NMR** (220 MHz, $CDCl_3$) δ 1.30-1.65 (12H, 4s, isopropyl), 2.70 (1H, d, OH, 9.2), 3.85 (1H, dd, H4, 9.1, 4.5), 4.09 (3H, m, H3 & H6), 4.35 (1H, m, H5), 4.65 (1H, dd, H2, 4.2), 5.86 (1H, d, H1, 4.2). **MS** (CI) m/z 261 $(M + H)^+$, 245, 220.

3-Toluene-p-sulphonyl-1,2,5,6-O-diisopropylidene-3-[^{2}H]-α-D-*allo*-hexofuranose (15). (88%) **^1H NMR** (220 MHz, $CDCl_3$) δ 1.30-1.60 (12H, 4s, isopropylidene), 2.49 (3H, s, Me), 3.85 (1H, m, H5), 4.00 (1H, dd, H4, 6.5, 6.2), 4.70 (1H, d, H2, 2,8), 5.80 (1H, d, H1, 2.8), 7.35 (2H, d, aromatic, 9.7), 7.90-7.98 (2H, d, aromatic, 7.3).

3-Benzoyl-1,2,5,6-O-isopropylidene-3-[^{2}H]-α-D-*gluco*-hexofuranose (16). (72%) **^1H NMR** (220 MHz, $CDCl_3$) δ 1.28-1.57 (12H, s, isopropylidene), 4.10-4.20 (2H, m, H4-H6), 4.35-4.45 (2H, m, H4-H6), 4.68 (1H, d, H2, 4.3), 6.00 (1H, d, H1, 4.3), 7.45-7.70 (3H, m, aromatic H), 8.06-8.10 (2H, m, aromatic H), **MS** (CI) m/z 365 $(M + H)^+$, 349, 307.

1,2,5,6-O-diisopropylidene-3-[^{2}H]-α-D-*gluco*-hexofuranose (17). (60%) **^1H NMR** (220 MHz, $CDCl_3$) δ 1.20-1.51 (12H, 4s, isopropylidene), 2.78 (1H, br.s, OH), 4.40

(4H, m, H4-H6), 4.58 (1H, d, H2, 4.3), 6.00 (1H, d, H1, 4.3). **MS** (CI) m/z 262 $(M + H)^+$, 246. Compared to authentic 1,2,5,6-O-diisopropylidene-α-D-*gluco*-hexofuranose, the deuterated derivative was enriched to 97 atom % D.

3-[^{2}H]-D-Glucose (100%) **^{1}H NMR** (400 MHz, D_2O) δ 3.12 (1H, d, H2β, 8.0), 3.26-3.31 (2H, m, H4αβ), 3.36 (1H, ddd, H5β, 12.3, 5.7, 2.2), 3.41 (1H, d, H2α, 3.8), 3.61 (1H, dd, H6β, 12.3, 5.7), 3.64 (1H, dd, H6α, 12.4, 5.6), 3.70-3.75 (2H, m, H6α, H5α), 3.78 (1H, dd, H6β, 12.3, 2.2), 4.53 (1H, d, H1β, 8.0), 5.11 (1H, d, H1α, 3.8). The spectrum compared well to that of authentic D-glucose. H2α and H2β had both collapsed to doublets, and H3α and H3β (normally at δ 3.58 and 3.37 ppm respectively) were both absent. **^{2}H NMR** (61.4 MHz, D_2O) δ 3.36 and 3.58 ppm. The higher frequency signal was 2/3 the intensity of that at lower frequency. **MS** (CI) m/z 409 $(M + NH_4)^+$. Compared to authentic glucose pentaacetate, the deuterated derivative was enriched to 96 atom % D.

5-[^{2}H]-D-Glucose.

3-O-benzyl-6-O-triphenylmethyl-1,2-O-isopropylidene-α-D-*gluco*-hexofuranose **(18)** was prepared according to Gramera *et al*[122,123] in three steps, 35% yield overall.

3-O-benzyl-1,2,5,6-O-diisopropylidene-α-D-*gluco*-hexofuranose. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.30 (3H, s, Me), 1.37 (3H, s, Me), 1.42 (3H, s, Me), 1.49 (3H, s, Me), 3.98-4.02 (2H, m, H2 & H4), 4.09-4.15 (2H, dd, H6a & H6b, 8.6, 6.2, 3.1), 4.35 (1H, m, H5), 4.57 (1H, d, H3, 3.2), 4.62 (1H, d, CH_2 benzyl, 11.8), 4.67 (1H, d, CH_2 benzyl, 11.8), 5.89 (1H, d, H1, 3.7), 7.25-7.40 (5H, m, aromatic H). **MS** (CI) m/z 351 $(M + H)^+$, 335, 310

3-O-benzyl-1,2-O-isopropylidene-α-D-*gluco*-hexofuranose. ^{1}H NMR (220 MHz, $CDCl_3$) δ 1.33 (3H, s, Me), 1.50 (3H, s, Me), 2.20-2.90 (1H, br.s, OH), 3.72 (1H, dd, H2, 12.1, 5.4), 3.84 (1H, dd, H6, 12.1, 4.1), 4.04 (1H, m, H5), 4.15 (2H, m, H3 & H4), 4.60 (1H, d, CH_2 benzyl, 10.7), 4.66 (1H, d, H2, 4.3), 4.78 (1H, d, CH_2 benzyl, 10,7), 5.98 (1H, d, H1, 4.3), 7.40 (5H, m, aromatic H). **MS** (CI) m/z 328 $(M + NH_4)^+$, 311 $(M + H)^+$.

3-O-benzyl-6-O-triphenylmethyl-1,2-O-isopropylidene-α-D-*gluco*-hexofuranose ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.31 (3H, s, CH_3), 1.49 (3H, s, CH_3), 2.67 (1H, br.d, OH, 7.0), 3.23 (1H, dd, H6, 9.4, 5.5), 3.42 (1H, dd, H6, 9.4, 5.5), 4.03 (1H, d, H3, 3.1), 4.17 (1H, m, H5), 4.32 (1H, dd, H4, 7.6, 3.1), 4.45 (1H, d, CH_2 benzyl, 11.6), 4.56 (1H, d, H2, 3.8), 4.61 (1H, d, CH_2 benzyl, 11.6), 5.91 (1H, d, H1, 3.8), 7.20-7.45 (20H, m, aromatic H). **MS** (CI) m/z 553 $(M + H)^+$, 475, 461.

3-O-benzyl-6-O-triphenylmethyl-1,2-O-isopropylidene-α-D-*xylo*-hexofuranos-5-ulose (19).

CH_2Cl_2 (50 mL, dry) was added to a 250 mL round bottomed flask under N_2. Oxalyl chloride (2 mL, 23 mmol, freshly distilled) was added next before cooling to -78°C. This was followed by the addition of DMSO (3.4 mL, 48 mmol, dry) over 5 min. The two reagents were stirred together for approximately 10 min. before the alcohol (11.2 g, 20 mmol) in CH_2Cl_2 (20mL) was added dropwise. Immediately following the addition, the reaction was warmed to -30°C whereupon the previously cloudy solution cleared. The reaction was stirred at this temperature for a further 10 min. Et_3N (14.6 mL, 105 mmol, ex. CaH_2) was added next and the reaction was allowed to warm to room temperature slowly. A slight yellow tinge was apparent at this stage. Water (50 mL) and CH_2Cl_2 (50 mL) were added and the organic layer was separated. This was washed with more water (2 x 50 mL) and saturated brine (50 mL). The organic solution was dried ($MgSO_4$) and concentrated to yield 7.82 g of the ketone (70%). TLC (10% Et_2O : toluene) indicated that a higher Rf spot was present compared to the starting material. No alcohol was present by TLC. **M.Pt.** 160-162°C. Lit. 169-170°C[2]. **$[\alpha]^{23}_D$** = -9.7° (c = 0.12 $CHCl_3$) lit. value -18°. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 1.28 (3H, s, Me), 1.45 (3H, s, Me), 4.03 (1H, d, CH_2, 18.2), 4.09 (1H, d, CH_2, 18.2), 4.36-4.40 (2H, m, H3 or H4, H6), 4.47 (1H, d, H6, 11.7), 4.50 (1H, d, H3 or H4, 3.6), 4.85 (1H, d, H2, 3.7), 5.92 (1H, d, H1, 3.6), 7.14-7.44 (20H, m, aromatic H). **^{13}C NMR** (100.6 MHz, $CDCl_3$) δ 26.3 (Me), 26.8 (Me), 68.2, 69.1, 72.4, 81.6, 83.4, 84.5, 87.1, 105.6, 106.0, 112.2, 126-128, 136.8, 143.2,

146.8, 202.9 (CO). **MS** (CI) no molecular ion was seen m/z 307 $(M + H - CHPh_3)^+$ (base peak). **Analysis** : found; C 76.09, H 6.23% calc. for $C_{35}H_{34}O_6$; C 76.34, H 6.22%.

3-O-Benzyl-6-O-triphenylmethyl-1,2-O-isopropylidene-5-[^{2}H]-D-*gluco*-hexofuranose (20).

To a solution of 3-O-benzyl-6-O-triphenylmethyl-1,2-O-isopropylidene-α-D-*xylo*-hexofuranos-5-ulose (7.76 g, 14 mmol) dissolved in CH_2Cl_2 (60 mL) was added $NaBD_4$ (0.6 g, 14 mmol) dissolved in EtOH (75 mL). When TLC analysis (SiO_2, diethyl ether : toluene 10% v/v) showed that no starting material remained, the solvent was evaporated *in vacuo* and the residue was dissolved in CH_2Cl_2 (50 mL). This solution was washed with water (75 mL), dried ($MgSO_4$) and evaporated *in vacuo* to give the title compound 7.5 g, (96%). In identical experiments using $NaBH_4$, only the **gluco**-isomer and no **ido**-isomer could be detected in the product by ^{1}H NMR spectroscopy. **^{1}H NMR** (220 MHz, $CDCl_3$) δ 1.32 (3H, s, Me), 1.51 (3H, s, Me), 2.69 (1H, s, OH), 3.25 (1H, d, H6, 9.3), 3.45 (1H, d, H6, 9.3), 4.08 (1H, d, H3, 3.1), 4.37 (1H, d, H4, 3.3), 4.50 (1H, d, CH_2, 11.8), 4.60 (1H, d, H2, 4.0), 4.65 (1H, d, CH_2, 4.0), 6.98 (1H, d, H1, 4.0), 7.20-7.60 (20H, m, aromatic H). **MS** (CI) found m/z 476.219 calc. for $C_{29}H_{30}DO_6$ m/z 476. 2184 $(M + H - C_6H_6)^+$.

1,2-O-isopropylidene-5-[^{2}H]-α-D-*gluco*-hexofuranose (21).

This was prepared according to Inouye[2] with modification to the purification step.

Ammonia gas was condensed into a dry 250 mL round bottomed flask fitted with a NaOH drying tube. The total volume of the liquid was approx. 100 mL. The alcohol (3.75 g, 6.8 mmol) was added in dry THF (10 mL) followed by Li metal (200 mg, 28 mmol). The reaction became difficult to stir so an additional 15 mL of THF was added. As the Li dissolved, the solution went a deep red. After 45 min., NH_4Cl (3.0 g, 56 mmol) was added in portions

and the ammonia was left to evaporate overnight. The remaining residue was washed several times with THF, filtered and concentrated to give an off white oil. This was washed with cyclohexane (2 x 40 mL) with the aqueous layer being retained. Lyophilisation gave 1.3 g of a white foam. TLC (30% EtOAc : CH_2Cl_2) showed one baseline component with no starting material present. The products from previous reactions were pooled and freeze dried with SiO_2. This was applied to a short column of SiO_2 and this was then washed with 5% MeOH : EtOAc. 3-O-Benzyl-6-O-triphenylmethyl-1,2-O-isopropylidene-5-[^{2}H]-D-*gluco*-hexofuranose (7.5 g) gave the title compound as an amorphous solid (2.96 g, 97%). $[\alpha]^{23}_D = -4.3°$ (c = 0.09 H_2O). **^{1}H NMR** (220 MHz, D_3-MeOH) δ 1.20 (3H, s, Me), 1.36 (3H, s, Me), 3.50 (1H, d, H6, 11.8), 3.68 (1H, d, H6, 11.8), 3.94 (1H, d, H3 or H4), 4.14 (1H, d, H3 or H4), 4.40 (1H, d, H2), 5.81 (1H, d, H1).

5-[^{2}H]-D-Glucose.

1,2-O-isopropylidene-5-[^{2}H]-α–D-*gluco*-hexofuranose was dissolved in water (40 mL) and H_2SO_4 (conc.) was added until the pH was less than 2.0 (meter). The solution was then heated at 90°C for 1h after which time TLC (10% MeOH : EtOAc) showed that all the starting material had hydrolysed. The pH of the solution was adjusted to 6.6 (meter) with 1M NaOH and then the solution was applied to a short mixed bed ion-exchange column. After eluting with water, the recovered eluate was lyophilised to yield 1.54 g of the title compound as a clear colourless syrup.(64%). **^{1}H NMR** (400 MHz, D_2O) δ 3.19 (1H, t, H2β, 8.0), 3.35 (2H, m, H4αβ), 3.44 (1H, t, H3β, 9.3), 3.48 (1H, dd, H2α, 9.6, 3.8), 3.65 (1H, t, H3α, 9.6), 3.67 (1H, d, H6β, 12.3), 3.70 (1H, d, H6α, 12.3), 3.79 (1H, d, H6α, 12.3), 3.84 (1H, d, H6β, 12.3), 4.61 (1H, d, H1β, 8.0), 5.18 (1H, d, H1α, 3.8). The spectrum compared well with that of authentic D-glucose. H6 (both anomers) existed as a doublet in the ^{1}H NMR spectrum and H5 (both anomers) were missing. MS(CI) m/z 409 $(M + H)^+$ 332 $(M + H - AcOH)^+$. Compared to authentic glucose pentaacetate, the deuterated

derivative showed 92 atom % D.

6,6-[$^2H_2$]-D-Glucose.

1,2-O-isopropylidene-α-D-glucuronolactone (22).

This was prepared in 70% yield by the method of Kitihara *et al*[133]. **M.Pt** 117.5-118.5°C. Lit 120.5-121.5°C[110] **[α]$^{23}_D$** = $^+$48.5° (c = 0.1 $CHCl_3$) lit. value[134] 52.5°. **^{1}H NMR** (220 MHz, $CDCl_3$) δ 1.38 (3H, s, CH_3), 1.65 (3H, s, CH_3), 3.00 (1H, br.s, OH), 4.58 (1H, d, H5, 4.4), 4.90 (2H, m, H2 & H3), 5.00 (1H, dd, H4, 3.3, 2.7), 6.05 (1H, d, H1, 3.3). **MS** (CI) m/z 234 $((M + NH_4)^+$, 217 $(M + H)^+$.

6,6-[$^2H_2$]-1,2-O-isopropylidene-α-D-*gluco*-hexofuranose (23).

This was prepared according to Lemieux and Stevens with certain modifications[95].

THF (dry, 50 mL) was added to a 250 mL round bottomed 3-necked flask. $LiAlD_4$ (1g, 24 mmol) was added next followed by the dropwise addition of 1,2-O-isopropylidene-α-D-glucuronolactone (5.44g, 25 mmol) in dry THF (50 mL). The rate of addition was such so as to keep the temperature below 25°C. After all the lactone had been added, the mixture became gelatinous and a further 50 mL of THF was added. Stirring was continued overnight after which TLC (5% MeOH : EtOAc) showed mainly glycol with some lactol still remaining. No starting material was evident. The excess $LiAlH_4$ was destroyed using a mixture of Na_2SO_4. 10 H_2O : Celite (1 : 1) followed by a drop of water. The suspension was then filtered through a pad of Celite to give a clear yellow solution which was concentrated. EtOH (40 mL) and $NaBD_4$ (0.7g, 17 mmol) was added to the crude yellow solid to complete the reduction. The reaction was left for 45 min. without stirring after which TLC showed just glycol. MeOH (~ 40 mL aliquots) was continually added followed by distillation to remove borate. After 300 mL of MeOH had been evaporated, water (75 mL) was added followed by filtration to remove the precipitated aluminium hydroxide. The filtrate was clear and colourless (pH neutral) and this was lyophilised to give 4.82g (86%) of the title compound as a white solid.

$[\alpha]^{23}_D$ = -4.0° (c = 0.15 H_2O). **^{1}H NMR** (250 MHz, D_2O) δ 1.30 (3H, s, Me), 1.46 (3H, s, Me), 3.92 (1H, d, H5), 4.10 (1H, dd, H4), 4.38 (1H, d, H3), 4.75 (1H, d, H2), 6.04 (1H, d, H1). **^{13}C NMR** (61.4 MHz, D_2O) δ 25.94 (Me), 26.36 (Me), 69.06, 74.38, 80.54, 85.22 (C2), 105.53 (C1), 113.49 (3° C). 8 signals (9 carbons, 1 deuterated).

6,6-[$^2H_2$]-D-Glucose.

1,2-O-Isopropylidene-6,6-[$^2H_2$]-α-D-*gluco*-hexofuranose **(23)** was hydrolysed as for 5-[^{2}H]-D-glucose. After lyophilisation, 2.80g of 6,6-[$^2H_2$]-D-glucose was obtained as a clear, colourless syrup (72%). TLC (BuOH : AcOH : H_2O : EtOAc) showed only one sugar active component which co-eluted with authentic D-glucose. **^{1}H NMR** (400 MHz, D_2O) δ 3.18 (1H, t, H2β, 8.4), 3.31-3.45 (4H, m, H5β, H3β, H4α, H4β), 3.46 (1H, H2α, dd, 3.7, 9.9), 3.65 (1H, t, H3α, 9.5), 3.75 (1H, d, H5α, 10.1), 4.57 (1H, d, H1β, 7.9), 5.16 (1H, d, H1α, 3.7). **MS** (CI) m/z 410 $(M + NH_4)^+$, 409 $(M + H)^+$. Compared to authentic glucose pentaacetate, the deuterated derivative was enriched to 99% (corresponding to the incorporation of 2 atoms of deuterium).

Deuterated nojirimycins.

1,2-O-Isopropylidene-α-L-iduronolactone **(25)** was synthesised according to Bashyal *et al*[134] in 3 steps (59% yield overall). Data for 1,2-O-isopropylidene-α-D-glucuronolactone has already been given.

5-O-Trifluoromethanesulphonyl-1,2-O-isopropylidene-α-D-glucuronolactone (24). **^{1}H NMR** (220 MHz, $CDCl_3$) δ 1.40 (3H, s, CH_3), 1.58 (3H, s, CH_3), 4.95 (1H, d, H2, 3.6), 5.02 (1H, d, H3, 3.0), 5.15 (1H, dd, H4, 2.8, 4.3), 5.51 (1H, d, H5, 4.4), 6.16 (1H, d, H1, 3.6). **MS** (CI) m/z 366 $(M + NH_4)^+$, 349 $(M + H)^+$, 333 $(M + H - CH_4)^+$.

1,2-O-isopropylidene-α-L-iduronolactone (25). $[\alpha]^{23}_D$ = +105.4° (c = 0.03 acetone) lit. value[134] 101°. **^{1}H NMR** (220 MHz, $CDCl_3$ (sparingly soluble)) δ 1.38 (3H, s, Me), 1.54 (3H, s, Me), 3.35 (1H, br.s, OH), 4.38 (1H, s, H5), 4.83 (1H, d,

H3, 3.3), 4.89 (1H, d, H2, 3.9), 5.10 (1H, d, H4, 3.3), 6.00 (1H, d, H1, 3.9). **MS** (CI) m/z 234 $(M + H)^+$, 201 $(M + H - CH_4)^+$.

5-O-Trifluoromethanesulphonyl-1,2-O-isopropylidene-α-L-iduronolactone[134]. (26)

1,2-O-isopropylidene-α-L-iduronolactone (1g, 4.63 mmol) was stirred with CH_2Cl_2 (40 mL, dry) at $^-$40°C. Not all the solid dissolved. Anhydrous pyridine (1 mL, 12 mmol) was added followed by the dropwise addition of triflic anhydride (0.93 mL, 5.5 mmol). The reaction was stirred at $^-$40°C for 2h during which time all the lactone dissolved. TLC (30% EtOAc : CH_2Cl_2) indicated that no starting material remained after this time and that a faster moving component was present. The CH_2Cl_2 solution was extracted with HCl (1M, 2 x 25 mL) and then water (2 x 25 mL) . The organic solution was dried ($NaSO_4$) and concentrated to give a crude yellow solid (1.3g). Small quantities could be re-crystallised from EtOH, but larger amounts decomposed on heating to effect dissolution. The crude yellow solid was filtered through SiO_2 using 20% EtOAc : CH_2Cl_2 to give the title compound as a white solid after concentrating *in vacuo*. Yield 1.30g (81%). The alcohol and the triflate ester were not stable at room temperature for more than 6 weeks-2months. **^{1}H NMR** (220 MHz, $CDCl_3$) δ 1.39 (3H, s, CH_3), 1.56 (3H, s, CH_3), 4.92 (1H, d, H2, 3.6), 5.00 (1H, d, H3 or H4, 3.6), 5.17 (1H, d, H3 or H4, 3.3), 5.19 (1H, s, H5), 6.02 (1H, d, H1, 3.8). **MS** (CI) m/z 366 $(M + NH_4)^+$, 333 $(M + H - CH_4)^+$.

5-Azido-5-deoxy-1,2-O-isopropylidene-α-D-glucuronolactone[134] (27). 5-O-Trifluoromethanesulphonyl-1,2-O-isopropylidene-α-L-iduronolactone (1g, 4.63 mmol) was dissolved in dry DMF (10 mL). After cooling to $^-$20°C, NaN_3 (180 mg, 2.8 mmol) was added in one portion . The reaction was stirred for a further 90 min. at $^-$20°C and then it was poured into water (200 mL). The white emulsion was extracted with CH_2Cl_2 (3 x 30 mL) and the combined organic extracts were back extracted with water (2 x 30 mL). The organic component was dried (Na_2SO_4) and concentrated to give the title compound

as a white solid, 0.59g (95%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 1.38 (3H, s, Me), 1.55 (3H, s, Me), 4.10 (1H, d, H5, 4.3), 4.81 (1H, d, H2, 3.8), 4.82 (1H, d, H3, 3.0), 4.99 (1H, dd, H4, 2.8, 4.3), 5.98 (1H, d, H1, 3.6). **MS** (CI) m/z 259 $(M + NH_4)^+$, 231 $(M + NH_4 - N_2)^+$, 214 $(M + H - N_2)^+$.

5-amino-5-deoxy-1,2-O-isopropylidene-α-D-glucuronolactone (28).

To 5-azido-5-deoxy-1,2-O-isopropylidene-α-D-glucuronolactone (1.08g, 4.5 mmol) in EtOAc (50 mL) was added 10% palladium on carbon (55 mg) and the mixture was hydrogenated at 2 atm for 4h after which time the mixture was filtered through Celite. TLC (20% EtOAc : CH_2Cl_2)) indicated that no starting material remained Concentration *in vacuo* left the amine as a white foam, 0.96g (100%). **^{1}H NMR** (220 MHz, $CDCl_3$) δ 1.38 (3H, s, Me), 1.55 (3H, s, Me), 1.90-2.20 (1H, br.s, NH_2), 3.78 (1H, d, H5, 4.6), 4.85 (1H, d, H3, 3.1), 4.88 (1H, d, H2, 3.8), 4.98 (1H, dd, H4, 3.1, 4.8), 6.02 (1H, d, H1, 3.7). **IR** (nujol mull) υ 3381 cm^{-1} (br.,OH), 3326 (d, 1° amine), 1780 (CO, lactone).

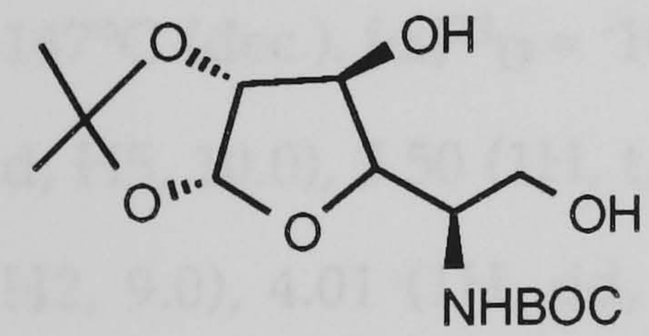
The reaction was repeated using 4.1g of 5-azido-5-deoxy-1,2-O-isopropylidene-α-D-glucuronolactone, except that after hydrogenation, di-t-butyl carbonate (4.08g, 20 mmol) was added to the filtrate which was then set aside overnight. The EtOAc was then removed *in vacuo* to yield a yellow solid (6.0g) which was purified by flash chromatography on silica (elution with 10% v/v EtOAc : CH_2Cl_2). 5-(N-t-Butyloxycarbonyl)amino-5-deoxy-1,2-O-isopropylidene -α-D-glucuronolactone **(29)** was obtained as a white solid (4.4g, 82%) after evaporation of the solvent. **$[\alpha]^{23}_D$** = $^+$58.8° (c = 0.1 $CHCl_3$). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 1.38 (3H, s, Me), 1.50 (9H, s, Me of t-butyl), 1.55 (3H, s, Me), 4.77 (1H, dd, H5, 9.0, 4.3), 4.80 (1H, d, H2, 3.7), 4.82 (1H, d, H3, 2.9), 4.93 (1H, dd, H4, 4.1, 3.0), 5.10 (1H, d, NH, 8.9), 5.91 (1H, d, H1, 3.6). **MS** (EI) found m/z 315.1309, calc. for $C_{14}H_{21}O_7N$, 315.1318 $(M^{+\cdot})$.

5-(N-t-Butyloxycarbonyl)amino-5-deoxy-1,2-O-isopropylidene-6,6-[$^2H_2$]-α-D-glucose (29a).

$LiAlD_4$ (50 mg, 1.2 mmol) was added to dry THF (5 mL) under N_2

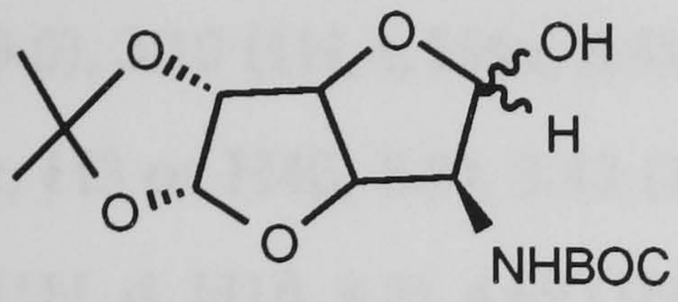
followed by 6,6-[$^2H_2$]-5-(N-t-butyloxycarbonyl)amino-5-deoxy-1,2-O-isopropylidene-α-D-glucuronolactone (168 mg, 0.5 mmol). The reaction was stirred overnight at room temperature and then an excess of EtOAc was added followed by a drop of water. After hydrolysis, a further amount of water (10 mL) was added and the mixture was extracted with EtOAc (3 x 10 mL) . The combined organic extracts were washed with water (2x 10 mL) and dried ($MgSO_4$). Removal of the solvent *in vacuo* left a clear oil (160mg) which TLC and ^1H NMR showed to contain a trace of lactol. The oil was dissolved in EtOH (5 mL) and deuterium oxide (2 mL) was added followed by a trace of $NaBD_4$. The reaction was left for 45 min. at room temperature after which time TLC and ^1H NMR showed that only glycol was present. The solvent was evaporated *in vacuo* and aliquots of MeOH (4 x 20 mL) were added and evaporated *in vacuo* to leave the title compound as a white solid (140 mg, 83%). **^1H NMR** (220 MHz, $CDCl_3$) δ 1.31 (3H, s, Me), 1.46 (9H, s, Me of t-butyl), 1.52 (3H, s, Me), 3.10 (1H, br.s, OH), 3.80 (1H, t, H5, 8.5), 4.10 (2H, m, H3 & H4), 4.62 (1H, d, H2, 3.7), 5.10 (1H, br.s, OH), 5.50 (1H, br.d, NH, 8.4), 6.00 (1H, d, H1, 3.7). **MS** (CI) found m/z 322.183 calc. for $C_{14}H_{24}DN_2O_7$ m/z 322.183 $(M + H)^+$.

5-(N-t-Butyloxycarbonyl)amino-5-deoxy-1,2-O-isopropylidene-α-D-glucose (protio).



^1H NMR (400 MHz, $CDCl_3$) δ 1.34 (3H, s, Me), 1.48 (9H, s, Me of t-butyl), 1.51 (3H, s, Me), 2.65 (1H, br.s, OH), 3.70-3.80 (2H, m, H5 & H6), 3.99-4.07 (3H, m, H6 & H3 & H4), 4.56 (1H, d, H2, 3.7), 5.04 (1H, br.s, OH), 5.35 (1H, br.d, NH, 8.4), 5.90 (1H, d, H1, 3.7). **MS** (CI) m/z 320 $(M + H)^+$.

5-(N-t-Butyloxycarbonyl)amino-5-deoxy-1,2-O-isopropylidene-α-D-glucuronolactol (protio).



^{1}H NMR (400 MHz, $CDCl_3$) δ 1.35 (3H, s, Me), 1.42 (9H, s, Me of t-butyl), 1.48 (3H, s, Me), 3.27 (1H, d, OH, 5.3), 4.10 (1H, m, H5), 4.61 (1H, d, H3, 4.5), 4.70 (1H, d, H2, 3.6), 4.83 (1H, t, H4, 4.7), 5.32 (2H, m, NH & H6), 5.98 (1H, d, H1, 3.6). **MS** (CI) m/z 318 $(M + H)^+$.

5-Amino-1,5-dideoxy-1-sulphonic acid-D-glucitol (nojirimycin bisulphite) (30).

Gaseous sulphur dioxide was bubbled through a suspension of 6,6-[$^2H_2$]-nojirimycin bisulphite (82 mg) in water (2 mL) for 10 min. The reaction was then set aside for 65h at 35-40°C. After this time TLC (BuOH : AcOH : H_2O : EtOAc) indicated that no starting material remained. One main fraction was present, with a minor faster moving component. The solution was then cooled to 0°C and methanol was added (20 mL). The crystalline bisulphite adduct (82 mg, 72%) was collected by filtration. **M.Pt.** 125-126°C (dec.) lit[2]. 145-147°C (dec.). **$[\alpha]^{23}_D$** = -10.4° (c = 0.02 H_2O). **^{1}H NMR** (400 MHz, D_2O) δ 3.08 (1H, d, H5, 10.0), 3.50 (1H, t, H3 or H4, 9.3), 3.55 (1H, t, H3 or H4, 9.4), 3.79 (1H, dd, H2, 9.0), 4.01 (1H, dd, H1, 10.3). The analogous protio compound compared well with the published spectrum[135]. **IR** (nujol mull) υ 3600-3000 cm^{-1} (br. strong, OH), 3120 (s, medium, NH), 1580 (weak, OH bend). The IR spectrum was identical to that given in the literature[2] (isolated from a natural source). **Analysis.** Found C 27.73, H 5.82, N 5.31, S 12.40%, calc. for $C_{16}H_{15}O_8NS$, C 27.59, H 5.79, S 12.48, O 5.36%.

The bisulphite adduct was converted to nojirimycin and 1-deoxynojirimycin in the usual way[2].

6,6-[$^2H_2$]-Nojirimycin (31). **^1H NMR** (400 MHz, D_2O). Two conformers were found in solution (α : β 2.25 : 1)[136]. δ 2.58 (1H, d, H5β, 9.0), 2.98 (1H, d, H5α, 9.0), 3.10 (1H, t, H4α, 8.9), 3.12 (1H, m, H2), 3.20 (1H, t, H3 or H4β, 8.8), 3.29 (1H, t, H3 or H4β, 8.8), 3.43 (1H, dd, H2α, 8.8, 3.4), 3.56 (1H, dd, H3α, 8.8, 8.2), 4.11 (1H, d, H1β, 8.2), 4.69 (1H, d, H1α, 3.4).

6,6-[$^1H_2$]-Nojirimycin. **^1H NMR** (400 MHz, D_2O). Assignments were made by homonuclear ^1H COSY spectroscopy. δ 2.61 (1H, ddd, H5β), 3.01 (1H, ddd, H5α), 3.11 (1H, t, H2β, 9.2), 3.15 (1H, t, H4α, 9.2), 3.21 (1H, t, H4β, 9.3), 3.30 (1H, t, H3β, 9.2), 3.45 (2H, m, H6α & H2α), 3.58 (2H, m, H3α & H6β), 3.77-3.84 (2H, m, H6α & H6β), 4.12 (1H, d, H1β, 8.2), 4.68 (1H, d, H1α, 3.4). The ^1H NMR spectrum compared well with the published spectrum[136] (no assignments made).

6,6-[$^2H_2$]-Deoxynojirimycin (32). (51% from bisulphite adduct) **$[\alpha]^{23}_D$ = +43.2°** (c = 0.1 H_2O) lit. value[2] +47°. **^1H NMR** (220 MHz, D_2O) δ 2.40 (1H, dd, $H1_{ax.}$ 10.7), 2.50 (1H, d, H5, 9.8), 3.10 (1H, dd, $H1_{eq.}$ 12.9, 5.3), 3.20 (1H, t, H3 or H4, 8.9), 3.30 (1H, t, H3 or H4, 8.9), 3.45 (1H, m, H2). The same protocol could also be used to prepare unlabelled DNJ which was identical with an authentic sample. **MS** (CI) m/z 376 $(M + H)^+$, 316 $(M + H - AcOH)^+$. Compared to authentic peracetyl DNJ, the deuterated derivative was enriched to 99 atom % (dideutero).

5-Amino-1,5-dideoxy-1-sulphonic acid-6,6-[$^3H_2$]-α-D-glucitol (33).

$NaBH_4$ (50 mg, 1.27 mmol) was added to anhydrous diglyme (2 mL, ex $NaBH_4$). $AlCl_3$ (60 mg, 0.45 mmol, anhydrous) was added next followed by 5-(N-t-butyloxycarbonyl)amino-5-deoxy-1,2-O-isopropylidene-α-D-glucuronolactone (100 mg, 0.32 mmol) as a solid. The reaction mixture was stirred at room temperature for 90 min. after which time water (1 drop) was added. After the $AlCl_3$ had been destroyed, more water was added (10 mL) and without delay, the reaction mixture was extracted with EtOAc (3 x 30 mL). The organic extracts were dried (Na_2SO_4) and concentrated to give 130 mg of crude solid (diglyme was still present by ^1H NMR). TLC (30% EtOAc : CH_2Cl_2) showed a mixture of lactol and glycol, although no starting material was present. EtOH (10 mL) was added followed by $NaBH_4$ (50 mg, 1.27 mmol) and the reaction was set aside for 30 min. After this time TLC showed that glycol only was present and the EtOH was removed by distillation. EtOAc (30 mL) was added next followed by extraction with water (2 x 20 mL). The solution was dried (Na_2SO_4) and concentrated to give 100 mg of the title compound as a white waxy solid. Traces of diglyme were still present by ^1H NMR and the yield was estimated to be 83%. The bisulphite adduct was then formed from this crude product in the usual way.

The shorter reaction time in this procedure was preferred to that when $NaBT_4$ was used.

$NaBT_4$ (Amersham, ~ 50 mCi/mmol) was substituted into the above reaction scheme except that it was reacted with the lactone for 3h before excess $NaBH_4$ (100 mg, 2.6 mmol) was added to complete the reaction. After stirring at room temperature overnight, the work-up was identical to that above except that the EtOH/$NaBH_4$ step was omitted. The glycol was converted directly to nojirimycin bisulphite, yield 9.5mg, 43 x 10^6 dpm.

1-[^{13}C]-D,L-Glyceraldehyde (34).

The method of Serianni *et al*[113,114,115] was followed exactly to yield 1.20g

(90% over 2 steps) of the title compound. The enrichment was 50 atom % ^{13}C and this was not diluted further in the fermentation studies. **^{13}C NMR** (62.4 MHz, D_2O, natural abundance) δ 90.56 (C1) 74.88 (C2) 62.78 (C3). Lit. δ 91.2, 75.5, 63.4. The observed ^{13}C NMR spectrum was after equilibration in D_2O for 36h where hydrated glyceraldehyde was the main species. The 1-[^{13}C]-D,L-Glyceraldehyde had one main peak at δ 90.58 ppm and weaker resonances at higher frequency, including a free carbonyl signal at δ 205.48ppm. These were attributed to the C1 resonances of dimers and oligomers of glyceraldehyde. The spectrum was identical to that reported in the literature.

3-O-Methyl-D-glucose.

3-O-Methyl-1,2,5,6-O-diisopropylidene-α-D-glucose (35).

Sodium hydride (2.8 g, 80% dispersion in mineral oil, 92 mmol) was added to dry THF (200 mL in a 500 mL round bottomed flask. After cooling to 0°C, 1,2,5,6-O-isopropylidene-α-D-glucose (20g. 70 mmol) was added as a solid in several portions. At the end of the addition, the reaction was stirred for a further 30 min. before MeI (6 mL, 94 mmol) was added dropwise. The reaction was left to stir overnight after which time TLC (20% EtOAc : CH_2Cl_2) indicated that only the methyl ether was present. EtOH (10 mL) was added to quench any excess NaH and the solution was concentrated to a turbid pale yellow oil. CH_2Cl_2 (100 mL) was added and this was washed twice with water (50 mL) and then brine (50 mL). Dried ($MgSO_4$) and concentrated to a colourless syrup (24.8g, >100%). **^1H NMR** (220 MHz, $CDCl_3$) δ 1.33-1.50 (12H, 4s, Me), 3.49 (3H, s, OCH_3), 3.80 (1H, d, H3, 2.8), 4.00-4.18 (3H, m, H4 & 2 x H6), 4.31 (1H, dt, H5, 7.8, 5.6), 4.60 (1H, d, H2, 3.9), 5.90 (1H, d, H1, 3.9). **MS** (CI) m/z 275 $(M + H)^+$, 259 $(M + H - CH_4)^+$.

This residue was not purified further and 12.4g (45 mmol) was hydrolysed in the usual manner to yield 7.29g (84%) of 3-O-methyl-D-glucopyranoside **(36)**. ^1H and ^{13}C NMR indicated that the pyranose form of the hexose was present only, with a β : α ratio of 1.29. **^1H NMR** (400 MHz,

D_2O) δ 3.20-3.40 (6H, m, H2-H4 (α and β)), 3.50 (1H, m, H5β), 3.54 (6H, s, OMe (α and β)), 3.60-3.70 (2H, m, H6 (α and β)), 3.75 (2H, m, H6α, H5α), 3.80 (1H, dd, H6β, 12.3, 1.9), 4.55 (1H, d, H1β, 7.7), 5.12 (1H, d, H1α, 3.6). **^{13}C NMR** (100.6 MHz, D_2O) δ 60.41, 60.69, 61.04, 61.22, 69.58, 69.70, 71.61, 72.09, 74.09, 76.42, 83.34, 85.97, 92.69 (C1), 96.47 (C1) 14 signals. **MS** (CI) m/z 380 $(M + NH_4)^+$, 303 $(M + H - AcOH)^+$.

3-Deoxy-D-glucose.

3-O-Phenoxythiocarbonyl-1,2,5,6-O-diisopropylidene-α-D-*gluco*-hexofuranose (37).

1,2,5,6-O-Isopropylidene-α-D-glucose (8g, 31 mmol) was dissolved in CH_3CN (100 mL) and pyridine (5 mL, anhydrous). To this was added phenylthionochloroformate (Lancaster Chemicals, 5.9g, 34 mmol) and DMAP (trace). The solution was left to stir at room temperature for 24h after which time TLC (30% EtOAc : toluene) showed a trace of starting material with a much stronger, faster migrating component. The reaction mixture was concentrated after 48h to give a yellow/red crude solid which was re-crystallised twice from EtOH. Yield of the title compound, 7.75g (64%). **M.Pt.** 101-103°C. **$[\alpha]^{23}_D$** = -46.9° (c = 0.16 $CHCl_3$). **H NMR** (250 MHz, $CDCl_3$) δ 1.57 (3H, s, Me), 1.46 (3H, s, Me), 1.36 (3H, s, Me), 1.39 (3H, s, Me), 4.10 (2H, m, 2 x H6), 4.35 (2H, br.m, H4 & H5), 4.80 (1H, d, H2, 3.7), 5.66 (1H, br.s, H3), 6.00 (1H, d, 3.7), 7.10-7.50 (5H, m, aromatic H). After 48h in $CDCl_3$ the product had decomposed slightly. **MS** (CI) m/z 397 $(M + H)^+$, 381 $(M + H - CH_4)^+$.

3-Deoxy-1,2,5,6-O-diisopropylidene-D-*ribo*-hexofuranose (38).

Bu_3SnH (8.1 mL, 30 mmol) was added to dry toluene (100 mL) under N_2. AIBN (trace) was added followed by heating to 70°C. 3-O-Phenoxythiocarbonyl-1,2,5,6-O-diisopropylidene-α-D-*gluco*-hexofuranose (7.7g, 20 mmol) in toluene (50 mL) was added dropwise at this temperature over 1h. Heating was continued for a further 3h after which time TLC (20% Et_2O : toluene) showed that a slower migrating component was present

compared to the starting material. An additional high Rf impurity was also identified. The solution was then evaporated and the residual colourless oil was purified by flash chromatography (20% Et_2O : petrol (40-60°C)) to yield the title compound as a clear colourless syrup (3.57g, 76%). **^{1}H NMR** (250 MHz, $CDCl_3$) δ 1.32 (3H, s, Me), 1.36 (3H, s, Me), 1.43 (3H, s, Me), 1.52 (3H, s, Me), 1.76 (1H, ddd, $H3_{exo}$ 13.2, 9.9, 4.5), 2.20 (1H, dd, $H3_{endo}$ 13.2, 4.1), 3.82 (1H, m, H5), 4.10-4.20 (3H, m, 2 x H6), 4.76 (1H, t, H2, 4.3), 5.82 (1H, d, H1, 3.7). **MS** (CI) m/z 245 $(M + H)^+$, 229 $(M + H - CH_4)^+$.

3-O-(Tri-n-butyltinmethylthioether)-1,2,5,6-O-diisopropylidene-α-D-*gluco*-hexofuranose (39).

This impurity was isolated from the above reaction. **^{1}H NMR** (250 MHz, $CDCl_3$) δ 0.84 (9H, t, CH_3, 7.2), 1.00-1.60 (30H, m, CH_2 (butyl), CH_3 (isopropylidene)), 3.97 (1H, dd, H4, 8.6, 5.2), 4.02-4.14 (2H, m), 4.15-4.25 (2H, m), 4.59 (1H, d, H2, 3.7), 4.81 (1H, d, OCH_2, 10.2), 4.88 (1H, d, OCH_2, 10.2), 5.76 (1H, d, H1, 3.6). **^{13}C NMR** (62.4 MHz, $CDCl_3$) δ 13.43 (Me, butyl), 25.20-28.39 (CH_2 (butyl), CH_3 (isopropylidene)), 67.06, 69.86, 72.31, 80.18, 80.89, 82.88, 104.9 (OCH_2), 108.8 (3° C), 111.5 (3° C). **MS** (CI) m/z 611, 613, 615 $(M + NH_4)^+$. **IR** (film) υ 2986-2855 cm^{-1} (CH aliphatic), 1458 (CH_2-S), 1373 (d, gem dimethyl).

3-Deoxy-D-glucose (40).

Acid hydrolysis of 3-Deoxy-1,2,5,6-O-diisopropylidene-D-*ribo*-hexofuranose in the usual fashion gave the title compound as a syrup, 1.9g (81%). ^{1}H and ^{13}C NMR showed that the compound existed as a mixture of pyranose and furanose forms (α and β anomers in each case). Pyranose (β : α 2.18). Furanose (β : α 3.0). Lit[137]. 51% β-pyranose, 26% α-pyranose, 17% β-furanose, 6% α-furanose. **^{1}H NMR** (400 MHz, D_2O) δ 1.46 (1H, q, $H3_{ax.}$ pyranose β, 11.5). 1.69 (1H, q, $H_{ax.}$ pyranose α, 11.4), 1.90-2.10 (3H, m, $H3_{eq.}$ pyranose α, $H3_{endo}$ and $H3_{exo}$ furanose), 2.30 (1H, dt, $H3_{eq.}$ pyranose β, 12.0, 4.9). The region between 3.30-4.20 ppm was complicated and could not be interpreted. δ 4.50 (1H, d, H1 pyranose β, 7.8), 5.05 (1H, d, H1 pyranose α, 3.5),

5.18 (1H, s, H1 furanose β), 5.24 (1H, d, H1 furanose α, 4.0). **^{13}C NMR** (100.6 MHz, D_2O) δ 31.49 (C3, αf), 33.39 (C3, βf), 34.28 (C3, αp), 38.86 (C3, βp), 61.21 (C6, αp), 61.47 (C6, βp), 63.13 (C6, αf), 63.43 (C6, βf), 64.79 (C4, αp), 65.00 (C4, βp), 67.08 (C2, αp), 69.30 (C2, βp), 71.25 (C5, αf), 72.76 (C5, αp), 73.52 (C2, αf), 74.71 (C5, βf), 76.18 (C2, βf), 77.50 (C4, αf), 79.76 (C4, βf), 80.33 (C5, βp), 91.50 (C1, αp), 97.04 (C1, αf), 98.49 (C1, βp), 102.29 (C1, βf). Where α and β refer to the two anomers of the furanose (f) and pyranose (p) forms[137]

5-Deoxyglucose.

5-O-(Phenoxythiocarbonyl)-1,2-O-isopropylidene-α-D-glucuronolactone (41).

1,2-O-Isopropylidene-α-D-glucuronolactone (8g 37 mmol) was dissolved in CH_3CN (100 mL) with pyridine (6 mL, 74 mmol, anhydrous) and DMAP (trace). Phenylchlorothionoformate (7.1g, 40 mmol) was added dropwise at room temperature and the reaction was stirred at this temperature overnight. After this time, TLC (20% EtOAc : CH_2Cl_2) indicated that only one sugar active component was present with no trace of starting material. The solvent was then evaporated and the yellow oil was crystallised from petrol (40-60°C) to give an off white solid. Recrystallisation from EtOH/petrol 40-60 gave the title compound as white needles, 10.26g (79%). **M.Pt.** 102-104°C. **[α]$^{23}_D$** = $^{+}$108.6° (c = 0.44 $CHCl_3$). **^{1}H NMR** (250 MHz, $CDCl_3$) δ 1.40 (3H, s, Me), 1.58 (3H, s, Me), 4.89 (1H, d, H2, 3.7), 4.98 (1H, d, H3, 3.0), 5.28 (1H, dd, H4, 4.3, 2.9), 6.12 (2H, dd, H1 & H5, 4.3, 3.6), 7.20 (2H, m, aromatic H), 7.30-7.50 (3H, m, aromatic H). **MS** (EI) m/z 352 $(M)^{+\cdot}$, 337 $(M - CH_3\cdot)^+$.

5-Deoxy-1,2-O-isopropylidene-α-glucuronolactone (42).

The method was identical to that when preparing 3-Deoxy-1,2,5,6-O-diisopropylidene-D-*ribo*-hexofuranose. The title compound was produced in 88% yield following crystallisation from petrol (40-60°C). **M.Pt** 86-87°C. **^{1}H NMR** (250 MHz, $CDCl_3$) δ 1.39 (3H, s, Me), 1.58 (3H, s, Me), 2.78 (2H, m, H5), 4.81 (1H, d, H3, 3.3), 4.84 (1H, d, H2, 4.1), 5.02 (1H, dt, H4, 3.3, 1.5), 6.00 (1H, d,

H1, 4.1). **MS** (CI) m/z 218 $(M + NH_4)^+$, 201 $(M + H)^+$.

5-Deoxy-1,2-O-isopropylidene-α-*xylo*-hexofuranose (43).

$LiAlH_4$ (0.7g, 18 mmol) was added to THF (50 mL, dry) in a 250 mL round bottomed flask. 5-Deoxy-1,2-O-isopropylidene-α-glucuronolactone (4.38g, 22 mmol) was dissolved in THF (50 mL, dry) and added dropwise to the $LiAlH_4$ suspension over 45 min. After 2h at room temperature, TLC (5% MeOH : EtOAc) showed that all the starting material had reacted to give a single product of lower Rf. Sufficient Na_2SO_4. $10H_2O$/Celite (1 : 1 v/v) was added to quench the excess $LiAlH_4$ and the solution was filtered to leave a clear colourless solution. Concentration *in vacuo* gave a white, waxy solid, 3.8g (85%). $[\alpha]^{23}_D = {}^+129.2°$ (c = 0.08 H_2O). **^{1}H NMR** (250 MHz, D_2O) δ 1.30 (3H, s, Me), 1.46 (3H, s, Me), 1.81 (2H, q, H5, 6.7), 3.62 (2H, m, H6), 4.09 (1H, d, H3, 2.6), 4.23 (1H, dt, H4, 6.7, 2.6), 4.61 (1H, d, H2, 3.8), 5.95 (1H, d, H1, 3.8). **MS** (CI) m/z 222 ($M + NH_4)^+$, 205 $(M + H)^+$.

This was hydrolysed in the usual way to give 3.3g, (100%) of 5-deoxy-*xylo*-hexofuranose **(44)**. Two anomers were present in approximately equal ratios. Only the furanose conformer could be detected by ^{1}H and ^{13}C NMR spectroscopy. **^{1}H NMR** (250 MHz, D_2O) δ 5.36 (1H, d, H1α, 4.7), 5.08 (1H, s, H1β). Below δ 4.75 ppm the spectrum could not be assigned. The ^{13}C NMR chemical shift values were identical with authentic 5-deoxy-*xylo*-hexofuranose prepared by a different route[75]. **^{13}C NMR** (62.4 MHz, D_2O) α anomer δ 31.98 (C5), 81.59 (C4), 96.51 (C1). β anomer δ 32.58 (C5), 76.20 (C3), 79.91 (C4), 102.52 (C1). Peaks were also present at δ 77.03 and 76.96 (C2 α and β, C3 α), 59.59 and 59.45 (C6 α and β).

3-O-Methyl-1-deoxynojirimycin.

This was synthesised from 3-O-methyl-1,2,5,6-O-isopropylidene-α-D-glucofuranose according to the strategy of Inouye *et al*, who had prepared 1-deoxynojirimycin from 1,2,5,6-O-isopropylidene-α-D-glucofuranose[2]. The procedures were the same unless otherwise stated.

3-O-Methyl-6-O-triphenylmethyl-1,2-O-isopropylidene-α-D-*gluco*-hexofuranose (46). (52% yield from 3-O-methyl-1,2,5,6-O-isopropylidene-α-D-*gluco*-hexofuranose **(45)**). **^{1}H NMR** (250 MHz, $CDCl_3$) δ 1.35 (3H, s, Me), 1.50 (3H, s, Me), 2.80 (1H, d, OH, 7.1), 3.24 (1H, dd, H6, 9.3, 4.5), 3.32 (3H, s, OMe), 3.40 (1H, dd, H6, 9.3, 5.6), 3.76 (1H, d, H3, 3.0), 4.18 (1H, m, H5), 4.28 (1H, dd, H4, 7.4, 3.0), 4.55 (1H, d, H2, 3.7), 5.90 (1H, d, H1, 4.1), 7.20-7.50 (15H, m, aromatic H). **MS** (CI) m/z 476 $(M)^{+\cdot}$, 399 $(M - C_6H_5\cdot)^+$. The compound was very hygroscopic.

3-O-Methyl-6-O-triphenylmethyl-1,2-O-isopropylidene-α-*xylo*-hexofuran-5-ulose (47). Swern[101] oxidation of the corresponding alcohol produced the title compound in 53% yield. **^{1}H NMR** (250 MHz, $CDCl_3$) δ 1.30 (3H, s, Me), 1.50 (3H, s, Me), 3.22 (3H, s, OMe), 3.96 (1H, d, H6, 18.2), 4.05 (1H, d, H6, 18.6), 4.14 (1H, d, H4, 3.4), 4.52 (1H, d, H2 or H3, 3.7), 4.90 (1H, d, H2 or H3, 3.7), 5.91 (1H, d, H1, 3.7), 7.20-7.50 (15H, m, aromatic H). **MS** (CI or EI) no MH^+ or $M^{+\cdot}$ (EI) m/z 444 $(M - CH_2O)^{+\cdot}$, 260 $(M - Ph_3COH)^{+\cdot}$.

The formation of the hydroxylamine oxime **(48)** from the title compound was modified from the literature as follows:

3-O-Methyl-6-O-triphenylmethyl-1,2-O-isopropylidene-α-*xylo*-hexofuran-5-ulose (2.8g, 5.9 mmol) was dissolved in pyridine (100 mL, dry) with NH_2OH. HCl (2.8g, 40 mmol). The solution was stirred at 70°C for 19h after which time TLC (20% EtOAc : pentane) showed the presence of two oxime isomers. The solution was concentrated *in vacuo* to leave a colourless oil. This was dissolved in $CH_2Cl_2/CHCl_3$ (50 mL, 1:1 v/v) and washed with water (3 x 50 mL) followed by brine (1 x 50 mL). After drying ($MgSO_4$) and evaporation, 2.82g of a white foam/glass was obtained (98%). Two geometric isomers were present in a ratio of 2:1 by ^{1}H and ^{13}C NMR spectroscopy. **^{1}H NMR** (250 MHz, $CDCl_3$) major isomer δ 1.18 (3H, s, Me), 1.37 (3H, s, Me), 1.80 (1H, s, OH), 3.20 (3H, s, OCH_3), 3.80 (1H, d, H6, 14.9), 3.85 (1H, d, H6, 14.9), 4.18 (1H, d, H3 or H4, 3.3), 4.37 (1H, d, H2, 3.7), 5.26 (1H, d, H3 or H4, 3.2), 5.65 (1H,

d, H1, 3.7), 7.04-7.40 (15H, m, aromatic H). Minor isomer δ 1.26 (3H, s, Me), 1.53 (3H, s, Me), 2.80 (1H, s, OH), 3.02 (3H, s, OCH_3), 3.73 (1H, d, H6, 14.9), 4.01 (1H, d, H3 or H4, 3.2), 4.47 (1H, d, H6, 14.9), 4.50 (1H, d, H2, 3.9), 5.34 (1H, d, H3 or H4, 3.1), 5.92 (1H, d, H1, 3.9), 7.04-7.40 (15H, m, aromatic H). **^{13}C NMR** (62.4 MHz, $CDCl_3$) major isomer δ 58.25, 62.57, 77.15, 81.94, 84.13, 87.18, 104.5, 111.8, 154.6 (C-N). Minor isomer δ 57.45, 58.80, 78.35, 81.45, 85.00, 87.37, 104.6, 11.6, 152.3 (C-N). In addition, 4 isopropylidene methyl peaks were present at δ 26.25, 26.32, 26.81, 26.87. Several aromatic resonances were observed between δ 123-150 ppm. **MS** no molecular ion could be observed under EI or CI conditions.

5-Amino-3-O-methyl-6-O-triphenylmethyl-1,2-O-isopropylidene-α-D-*gluco*-hexofuranose (49).

This was prepared according to Inouye[2], except that the crude product was purified by flash chromatography (7% MeOH : CH_2Cl_2, SiO_2). Crude yield (75%) as a white foam. Yield of *gluco* amine, 18%. **^{1}H NMR** (220 MHz, $CDCl_3$) δ 1.35 (3H, s, Me), 1.50 (3H, s, Me), 2.15-2.35 (2H, br.s, NH_2), 3.30-3.50 (6H, m, 2 x H6, H5, OMe), 3.84 (1H, d, H3, 3.2), 4.25 (1H, dd, H4, 8.0, 3.2), 4.62 (1H, d, H2, 4.0), 5.95 (1H, d, H1, 4.0), 7.25-7.65 (15H, m, trityl H). **MS** (CI) m/z 476 $(M + H)^+$, 413, 319.

A small amount of the *ido* amine was also recovered. **^{1}H NMR** (250 MHz, $CDCl_3$) δ 1.32 (3H, s, Me), 1.52 (3H, s, Me), 2.40 (2H, br.s, NH_2), 3.02 (3H, s, OCH_3), 3.20 (1H, d, H3, 3.2), 3.30 (1H, m, H5), 3.42 (1H, dd, H6, 12.6, 9.2), 4.25 (1H, dd, H4, 8.2, 3.2), 4.49 (1H, d, H2, 3.9), 5.90 (1H, d, H1, 3.9), 7.20-7.50 (15H, m, trityl H). **^{13}C NMR** (62.4 MHz, $CDCl_3$) δ 26.1, 26.5, 50.7, 57.1, 64.0, 81.5, 81.6, 83.6, 104.4, 111.3, 126-128, 143.6 ppm.

5-Amino-5-deoxy-3-O-methyl-1,2-O-isopropylidene-α-D-*gluco*-hexofuranose (50) (78%). **^{1}H NMR** (250 MHz, D_2O) δ 1.29 (3H, s, Me), 1.45 (3H, s, Me), 3.09 (1H, m, H5), 3.40 (3H, s, OMe), 3.51 (1H, dd, H6, 11.5, 6.3), 3.70 (1H, dd, H6, 11.5, 3.4), 3.89 (1H, d, H3, 3.0), 4.00 (1H, dd, H4, 8.7, 3.0), 4.83 (1H, d, H2, 3.7), 5.92 (1H,

d, H1, 3.7).

5-Amino-5-deoxy-3-O-methyl-D-glucose-1-β-sulphonic acid (51) (25%). **M.Pt.** 124-125°C. **$[\alpha]^{23}_D$** = $^{+}$0.3° (c = 0.18 H_2O). **^{1}H NMR** (400 MHz, D_2O) δ 3.21 (1H, ddd, H5, 11.3, 4.5, 2.9), 3.33 (1H, t, H3, 9.4), 3.61 (3H, s, OMe), 3.70 (1H, t, H4, 9.7), 3.87 (1H, dd, H6, 12.9, 4.5), 3.92 (1H, dd, H6, 12.9, 2.9), 3.94 (1H, t, H2, 9.4), 4.12 (1H, d, H1, 10.5). **^{13}C NMR** (100.62 MHz, D_2O) δ 58.00, 60.85, 61.21, 67.43, 69.66, 71.09, 86.20 (C1) 7 peaks. **Analysis** found C 33.4, H 6.0, N 5.0% calc. for $C_7H_{15}O_7NS$, C 32.7, H 5.9, N 5.4%.

5-Amino-5-deoxy-3-O-methyl-D-glucose (52). (70%).

Two anomers were present in aqueous solution α : β, 2.86 : 1. Compare this with nojirimycin, α : β, 2.25 : 1. **^{1}H NMR** (400 MHz, D_2O) α anomer δ 3.02 (1H, ddd, H5), 3.23 (1H, t, H4, 9.6), 3.33 (1H, t, H3, 9.6), 3.48 (2H, m, H6 & H2), 3.55 (3H, s, OMe), 3.80 (2H, m, H6α & H6β), 4.67 (1H, d, H1, 3.5). β anomer δ 2.61 (1H, ddd, H5), 3.09 (1H, t, H3, 9.2), 3.18 (1H, t, H2, 8.9), 3.30 (1H, t, H4, 9.6), 3.55 (3H, s, OMe), 3.58 (1H, m, H6), 3.80 (2H, m, H6α & H6β), 4.12 (1H, d, H1, 8.1). **^{13}C NMR** (100.6 MHz, D_2O) δ 53.97, 57.25, 60.32, 60.58, 61.69, 62.38, 71.07, 71.66, 72.32, 75.62, 79.25, 82.81, 84.35, 86.55 14 peaks, α and β anomers.

5-Amino-1,5-dideoxy-3-O-methyl-D-glucose (53) (82%). **^{1}H NMR** (250 MHz, D_2O) δ 2.36 (1H, dd, $H1_{ax.}$ 10.8, 12.5), 2.45 (1H, ddd, H5, 9.3, 6.1, 3.0), 3.00 (1H, dd, $H1_{eq.}$ 12.1, 5.2), 3.05 (1H, t, H3 or H4, 9.3), 3.21 (1H, t, H3 or H4, 9.3), 3.45 (1H, ddd, H2, 9.3, 10.8, 5.2), 3.55 (4H, m, OMe & H6), 3.72 (1H, dd, H6, 11.5, 3.0). **^{13}C NMR** (62.4 MHz, D_2O) δ 49.57, 60.77, 61.43, 61.98, 71.13, 71.72, 89.02 ppm (7 peaks). **MS** (CI) found m/z 346.1508 calc. for $C_{15}H_{24}O_8N$ m/z 346.1502 (M + H)$^{+}$.

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