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The Biosynthesis of 1-Deoxynojirimycin in *Bacillus subtilis var niger*

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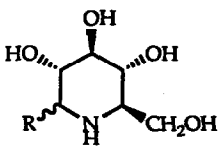
Abstract: *Bacillus subtilis var niger* ATCC 9372 when grown on a glucose containing soyabean medium produces the amino-sugar 1-deoxynojirimycin (DNJ). The C2 epimer 1-deoxymannojirimycin (DMJ) present in *Streptomyces subrutilus* ATCC 27467 is absent. ^{13}C and ^{2}H labelling studies confirm that glucose is the precursor of DNJ. Randomisation of the ^{13}C label from 1-[^{13}C]-D-glucose and the incorporation of 1-[^{13}C]-D,L-glyceraldehyde into DNJ also suggest that a minor part of the biosynthetic route involves cleavage of the glucose skeleton. Mannojirimycin (MJ) and nojirimycin (NJ) are postulated intermediates in the biosynthesis as a result of both enzyme assay and isotope labelling studies.

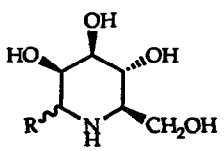
Introduction

1-Deoxynojirimycin (DNJ) is one of a range of polyhydroxylated piperidine alkaloids that have been the focus of recent research.[1,2,3,4] The reason for this interest can be attributed to their properties as glycosidase inhibitors. DNJ will inhibit α-glucosidase, the enzyme responsible for catalysing the hydrolysis of α-glucose residues within an oligosaccharide chain. Likewise, the related indolizidine alkaloid, castanospermine, will also inhibit α-glucosidase and both these compounds and their derivatives are known inhibitors of Human Immunodeficiency Virus (HIV) replication.[5] This property is thought to arise by altering the constitution of a glycoprotein on the surface of the virus and this is implicated in the binding of the HIV particle to the T4 cells of the immune system.[6,7,8]



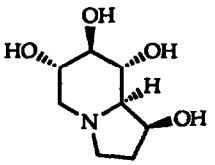
nojirimycin
NJ, R=OH

1-deoxynojirimycin
DNJ, R=H



mannojirimycin
MJ, R=OH

1-deoxymannojirimycin
DMJ, R=H



castanospermine

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The alkaloids have been isolated from natural sources. DNJ has been found in the root bark of a *Morus* species[9] (Mulberry tree) and is also produced by various strains of *Streptomyces* and *Bacilli* microorganisms.[10,11] Interest in the glycosidase inhibition mechanism then led to many efficient chemical syntheses of these alkaloids and their derivatives.[12,13,14] Little work has been published on the biosynthesis of these compounds[15], despite the fact that DNJ was conveniently produced by bacteria. Previous work in our laboratory[16] led to a biosynthetic scheme for DNJ and three other related alkaloids, 1-deoxymannojirimycin (DMJ), nojirimycin (NJ) and mannojirimycin (MJ) in *S. subrutilus* ATCC 27467. All four alkaloids originated from glucose by a head to tail inversion of the molecule and this led us to include glucose analogues in the fermentation medium to determine whether DNJ analogues would be produced by the microorganism. We also investigated the biosynthesis of DNJ in the unrelated *Bacilli* species. Previous work[11] using *B. subtilis* DSM 704 had shown that DNJ was produced from a wide range of carbon sources and that NJ was possibly present in the fermentation medium. No further details of the biosynthesis appear to have been published. We now report that DNJ originates from glucose in *B. subtilis var niger* ATCC 9372 by a similar mechanism to that found in *S. subrutilus* ATCC 27467, although differences exist between the two microorganisms.

Results and discussion

The biosynthesis of DNJ and DMJ from D-glucose in *S. subrutilus* is presented below, **Scheme 1**. This scheme describes a C2/C6 cyclisation of the original glucose molecule. This is effectively an inversion of glucose, as isotope label introduced at C1 of glucose finishes at C6 of DNJ during the biosynthesis. The first step in the pathway is isomerisation of the glucose (or a phosphorylated derivative) to fructose. In this way, deuterium at C2 of glucose is transferred to C1 of fructose whilst deuterium at C1 of glucose remains at C1 of fructose. The precise order of steps ii and iii in which C6 of glucose is oxidised to the aldehyde and the keto functionality is aminated were not finalised. The configuration at C2 is *R* following amination so that the first piperidine alkaloid formed is MJ. Both the cyclic and acyclic forms of MJ are shown in **Scheme 1**. The epimerisation to NJ then occurs (step iv) by oxidation of MJ at C2 followed by reduction back to the alcohol. Both intermediates dehydrate (step v) to give the cyclic imines which are further reduced from the α-face producing DNJ and DMJ.

Bacillus subtilis var niger ATCC 9372 when grown on a medium containing glucose (0.4% $^{w}/_{v}$), soyabean meal (1% $^{w}/_{v}$) and $CaCO_3$ (0.2% $^{w}/_{v}$) produced 0.02 mg mL^{-1} of DNJ by GC assay.[16] DMJ could not be detected in the fermentation medium at any stage, unlike that found for *S. subrutilus*. Significantly, NJ could not be detected by a glucosidase (trehalase) enzyme assay.[16] Previous results with *S. subrutilus* had indicated that NJ was a precursor to DNJ and indeed NJ was found in higher concentration to DNJ over a 7 day fermentation period using the same enzyme assay. In *B. subtilis var niger*, either NJ was at a low concentration or else it was not an intermediate. Isotope labelling studies with 1-[^{13}C]-D-glucose (16 atom % ^{13}C) gave DNJ with a ^{13}C isotope enrichment of 16.2 ± 1.2 atom % ^{13}C after purification. This level of enrichment differed from that when 1-[^{13}C]-D-glucose was used in a fermentation with *S. subrutilus*.[16] In this case only $^{2}/_{3}$ of the enrichment introduced in the glucose precursor was found in the isolated DNJ, as DNJ biosynthesis occurred irrespective of added glucose. ^{13}C NMR spectroscopy (**Figure 1**) showed that C6 was enriched as expected from the *S. subrutilus* pathway. Unlike previous results with *S. subrutilus*, C1 was also enriched from the NMR spectrum and this was confirmed from mass spectrometric studies. The C6/C1 enrichment ratio was approximately 4/1 from the ^{13}C NMR spectrum.

Scheme 1

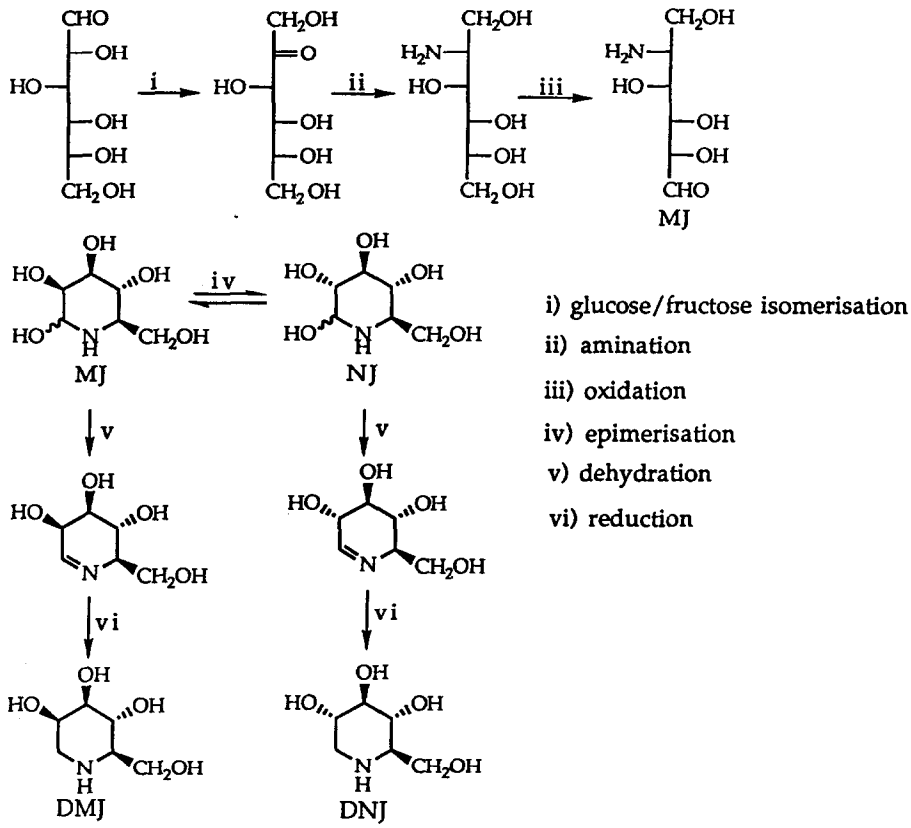
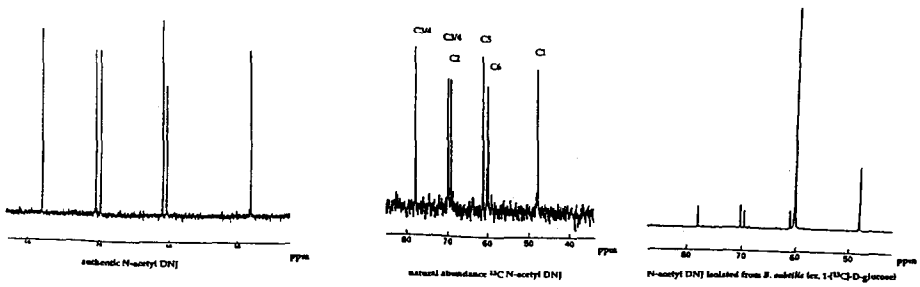


Figure 1



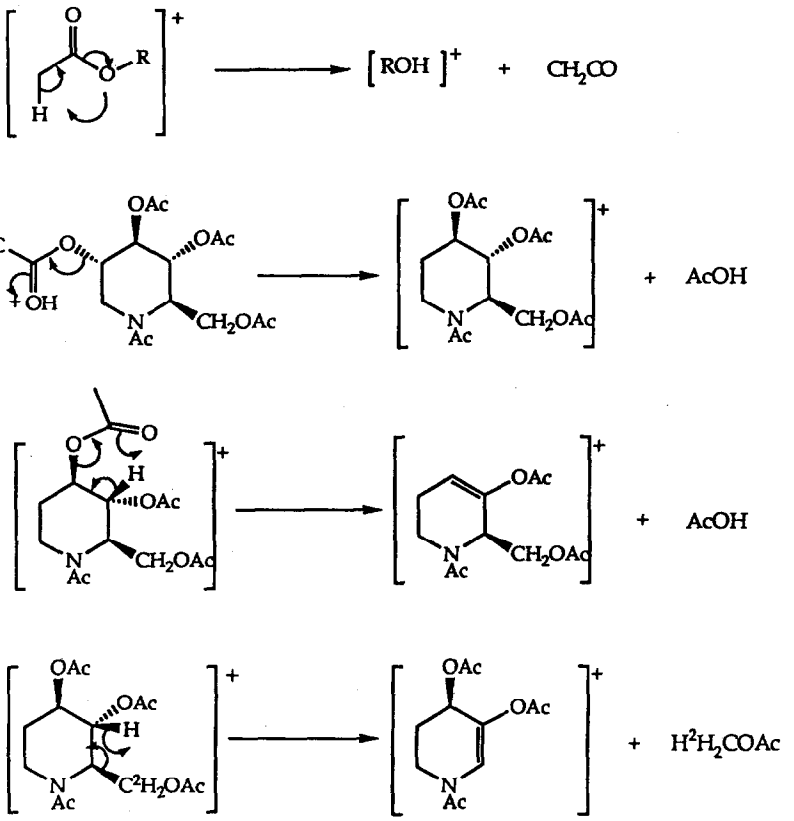
Peracetyl DNJ shows peaks in the CI mass spectrum corresponding to the loss of acetic acid and ketene (**Scheme 2**). These peaks are common fragments in the spectra of peracetylated glucoses.[17] Synthetic 6,6-[$^2H_2$]-peracetyl DNJ produced a mass spectrum with peaks 2 mass units higher than its protio analogue, except for m/z 240. This result was attributed to the loss of acetic acid followed by methyl acetate from the molecular ion, m/z 374 (protio species). As deuterium at H4 of DNJ was also lost in the m/z 240 fragment, we propose that a *cis* elimination of methyl acetate took place between C6 and H4. Correspondingly, any isotopic label at C6 is lost and this was found in the mass spectrum of peracetyl DNJ isolated after feeding 1-[^{13}C]-D-glucose to *B. subtilis var niger*. The enrichment of the molecular ion was found to be 16.2 ± 1.2 atom % ^{13}C, whereas for m/z 240, the enrichment was 6.3 ± 3.3 atom % ^{13}C. If all the ^{13}C label was located at C6, then m/z 240 would contain only natural abundance ^{13}C.

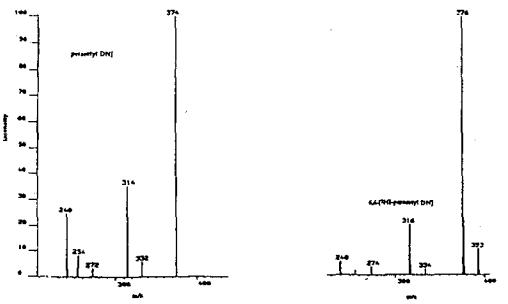
The randomisation of ^{13}C isotope on feeding 1-[^{13}C]-D-glucose to *B. subtilis* was inexplicable using the established pathway for *S. subrutilus*.[16] There was the possibility that a symmetrical intermediate was involved as the ^{13}C label was located at both ends of the DNJ. The relative difference in the enrichment at C1 and C6 was difficult to reconcile with such a symmetrical intermediate, so a further experiment with isotope label at C6 was undertaken. A fermentation with 6,6-[$^2H_2$]-D-glucose (90 atom % ^{2}H per H6) produced DNJ with a high deuterium enrichment of 48.4 ± 0.9 atom % ^{2}H. ^{2}H and ^{1}H NMR spectroscopy experiments confirmed that $H1_{(equatorial)}$ only was enriched with deuterium. No randomisation of the label occurred, a result identical with that from *S. subrutilus* studies. Presumably the decrease in enrichment of deuterium in the DNJ, from that fed in the original glucose, was due to a kinetic isotope effect in the oxidation of C6 (step iii, **Scheme 1**).

As the C1-C3 part of the glucose molecule appeared to behave differently to the C4-C6, we believe that the glucose molecule splits into two C3 fragments within the biosynthetic pathway. Two obvious fragments are glyceraldehyde-3-phosphate and dihydroxyacetone phosphate produced from fructose-1,6-diphosphate during glycolysis.[18,19] These two C3 fragments can interconvert rapidly, a reaction catalysed by triose phosphate isomerase, **Scheme 3**. At equilibrium, 96% of the triose phosphate is dihydroxyacetone phosphate, but the reaction proceeds to glyceraldehyde-3-phosphate because the latter is efficiently removed to continue in glycolysis. 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 via glycolysis, but as 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.

In the experiment with 6,6-[$^2H_2$]-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. Given the high enrichment of deuterium in the isolated DNJ and our detection limit of between 3-5 atom % ^{2}H by ^{2}H NMR, it is likely that randomisation of the deuterium would be detected.

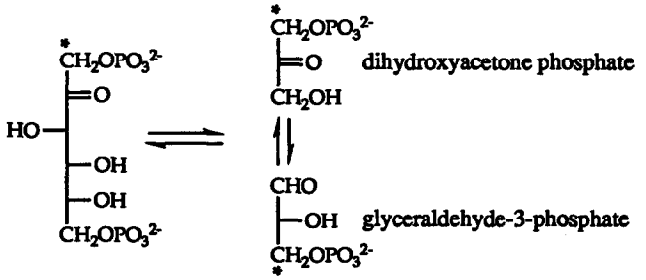
Scheme 2





Fragment	Assignment
m/z 374	Peracetyl DNJ molecular ion
m/z 314	m/z 374-AcOH
m/z 272	m/z 314-ketene or m/z 332-AcOH
m/z 254	m/z 314-AcOH
m/z 240	m/z 314-H_3COAc

Scheme 3



One possible reason is that 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 significantly different fates. Although the triose phosphate equilibrium is mainly on the side of dihydroxyacetone phosphate, the reaction is 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 smaller quantities, the isotope randomisation does not express itself to any significant extent in the dihydroxyacetone phosphate. Also, as the main direction of the aldol cleavage is not to produce a hexose, but the two trioses, it is likely that the level of deuterium at C1 of fructose-1,6-diphosphate from randomisation is low.

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 (50 atom % ^{13}C) was fed in a fermentation experiment. The purified DNJ contained 6.4 ± 0.7 atom % ^{13}C by mass spectrometry and the ^{13}C NMR spectrum showed that C3 or C4 of N-acetyl DNJ at δ 77.80 ppm was enriched. The peaks for C3 and C4 in N-acetyl DNJ appeared at δ 77.80 and 69.97 ppm, although these carbons could not be assigned specifically even employing heteronuclear correlation NMR spectroscopy at 61.4 MHz. By analogy with glucose,[20] where the C3 carbon resonates at higher frequency (δ 76.48 (β), 73.26 (α) ppm) compared to C4 (δ 70.20, 70.16 ppm), the C3 carbon of N-acetyl DNJ is labelled. 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.

Similar double labelling patterns have been observed in the biosynthesis of various 6-deoxyhexose sugars which originate from glucose.[21,22,23] When glucose labelled at C1 with ^{14}C was used in fermentation experiments, radioactivity was found predominantly at C1 in several 6-deoxyhexose sugars, although some enrichment was also found at C6. In one case, glucose labelled specifically at C1 with ^{14}C was re-isolated from a fermentation and found to contain ^{14}C at C6 in glucose. The postulated biosynthesis of these sugars did not involve cleavage of the glucose chain, although a minor pathway was recognised which suggested that the glucose molecule could split into two halves which were in equilibrium with each other. Following enzyme inhibition studies, whereby the inhibition of α-mannosidase was measured, we propose that MJ is present in the *B. subtilis var niger* fermentation. A large decrease in the inhibition of jack bean-α-D-mannnosidase (from in excess of 95%, down to 15%) occurred on removal of the MJ. Furthermore, addition of aliquots of unlabelled NJ, 58h after inoculation with *B. subtilis var niger*, gave three times as much DNJ by GC at the end of the fermentation when compared to the normal culture medium (0.06 mg mL^{-1} cf. 0.02 mg mL^{-1}). A similar

experiment with *S. subrutilus* had shown no such increase.[16] In a subsequent experiment, 6,6-[$^2H_2$]-nojirimycin (99 atom % ^2H per H6, 2 mg) was added to the fermentation, at the same point during the time course, and the isolated peracetyl DNJ contained 9.8 ± 1.8 atom % $^2H_2$. ^2H NMR spectroscopy confirmed that the label was located at δ 4.50 and 4.63 ppm (H6) in peracetyl DNJ. We conclude that NJ is a precursor to DNJ in *B. subtilis var niger*, but it is present in low concentration.

Feeding experiments with 2-[^2H]-D-glucose (39 atom % ^2H) gave a DNJ enrichment of 15 atom % ^2H by mass spectrometry and the ^2H NMR spectrum confirmed that H6 at δ 4.61 in peracetyl DNJ was labelled. This result follows from the fructose/glucose isomerisation where H2 of glucose is replaced at H1 of fructose. The randomisation observed with 1-[^{13}C]-D-glucose did not occur with 2-[^2H]-D-glucose, where enrichment of H1 might have been expected. This is possibly because any deuterium located at this position following the aldol cleavage and subsequent recombination is oxidised as in **Scheme 1**, step iii) in producing MJ. The relative amounts of label at C1 and C6 from the 1-[^{13}C]-D-glucose experiment meant that any deuterium at H1 in DNJ from 2-[^2H]-D-glucose should have been detected.

Experimental

General conditions

^1H NMR spectra were recorded at 250 MHz or 400 MHz using a Bruker ACF 250 or a Bruker WH400 spectrometer respectively. ^2H NMR spectra were recorded at 38.39 (ACF 250) or 61.42 MHz (WH400). ^{13}C NMR spectra were recorded at 61.42 (ACF 250) or 100.62 MHz (WH 400). The isolated DNJ was analysed either as the N-acetyl derivative in ^2H_2O or as the peracetyl derivative in pyridine. For ^1H and ^2H NMR spectroscopy, ^2HOH at δ 4.75 ppm and the α proton of pyridine at δ 8.70 ppm were used as reference signals.

Mass spectra were recorded on a Kratos MS80 spectrometer. Chemical ionisation (CI) mass spectra used ammonia as reagent gas. Peracetyl DNJ was used in all analyses to determine the isotopic enrichment and typically the mean was taken of 5 scans of a compound over a period of two or three days. In all cases, peracetyl DNJ with a natural isotopic enrichment was used as a standard. The experimental procedures for the enzyme and gas chromatography assays have been described previously.[16]

Growth of microorganisms and fermentation procedure

B. subtilis var niger ATCC 9372 was stored in glycerol solution (33%) at -10°C. Nutrient broth (Oxoid, 13 g L^{-1}, 50 mL) was inoculated with 100 μL of this bacterial suspension and incubated at 30°C/200 rpm for 24 h. A fermentation medium consisting of glucose (0.4% $^{w}/_{v}$), soyabean meal (1% $^{w}/_{v}$) and $CaCO_3$ (0.2% $^{w}/_{v}$) was made up to volume with tap water (final pH 6.8) and then this was divided into 30 mL aliquots in conical flasks (250 mL). The fermentation medium was inoculated with nutrient broth/bacteria suspension and incubated at 30°C/200 rpm for 7 days.

Full experimental details for the isolation of crude DNJ and its purification as the corresponding peracetyl derivative have recently been published.[16] The conversion of peracetyl DNJ into N-acetyl DNJ has also been reported.[16] The spectroscopic data for the isolated metabolites are:

Peracetyl DNJ. ^{1}H NMR (400 MHz, $C_5{}^2H_5N$, 90°C) 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, dd, H4, 3.8, 4.3), 5.39 (1H, dd, H3, 3.9, 3.4); MS (CI): found m/z 374.1450, calc. for $C_{16}H_{24}NO_9$ m/z 374.1451.
N-Acetyl DNJ. ^{1}H NMR (400 MHz, ^2H_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, dt, 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).

Isotopically labelled glucoses

1-[^{13}C]-D-Glucose

1-[^{13}C]-D-Glucose (98 atom % ^{13}C) was obtained from Aldrich Chemical Co. and diluted to 16 atom % ^{13}C with unlabelled D-glucose before use.

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

1,2-O-Isopropylidene-6,6-[$^2H_2$]-α-D-*gluco*-hexofuranose

6,6-[$^2H_2$]-D-Glucose (99 atom % ^{2}H per H6) was synthesised from 1,2-O-isopropylidene-α-D-glucuronolactone[24] by a modified route of Lemieux and Stevens.[25]

Anhydrous THF (50 mL) was added to a 250 mL round bottomed flask. $LiAl^2H_4$ (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 of $LiAl^2H_4$ was destroyed using a mixture of $Na_2SO_4.10H_2O$: 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 *in vacuo*. EtOH (40 mL) and NaB^2H_4 (0.7g, 17 mmol) were 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 1,2-O-isopropylidene-6,6-[$^2H_2$]-α-D-*gluco*-hexofuranose as a white solid.
^{1}H NMR (250 MHz, ^2H_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, ^2H_2O) δ 25.94 (Me), 26.36 (Me), 69.06, 74.38, 80.54, 85.22 (C2), 105.53 (C1), 113.46 (isopropylidene 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 (4.8g) was dissolved in water (40 mL) and H_2SO_4 (conc.) was added until the pH was less than 2 (meter). The solution was then heated at 90°C for 1h after which TLC (10% MeOH : EtOAc) showed that all the starting material had been 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 give 2.80g, (72%) of 6,6-[$^2H_2$]-D-glucose as a clear colourless syrup. TLC (BuOH : AcOH : H_2O : EtOAc, 1:1:1:1, v/v/v/v) showed only one sugar active component which co-eluted with authentic D-glucose.

^1H NMR (400 MHz, ^2H_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) Peracetyl derivative: m/z 410 $(M+NH_4)^+$, 393 $(M+H)^+$.

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

The method of Serianni *et al.*[26,27,28] was followed to yield 1.2g (90% over two steps) of racemic glyceraldehyde from glycolaldehyde. The enrichment was 50 atom % ^{13}C and this was not diluted further in the fermentation studies.

^{13}C NMR (62.4 MHz, ^2H_2O, natural abundance ^{13}C) δ 90.56 (C1), 74.88 (C2), 62.78 (C3).

The observed ^{13}C NMR spectrum was after equilibration in ^2H_2O for 36 h where hydrated glyceraldehyde was the main species. The 1-[^{13}C]-D,L-glyceraldehyde had one main peak at δ 90.58 and weaker resonances at higher frequency, including a free carbonyl signal at δ 205.58 ppm. These were attributed to the C1 resonances of dimers and oligomers of glyceraldehyde. The spectrum was identical to that reported in the literature.[26,27,28]

6,6-[$^2H_2$]-Nojirimycin

6,6-[$^2H_2$]-Nojirimycin (99 atom % ^2H per H6) was synthesised in 12% overall yield from 3,6-glucuronolactone as previously described.[16]

2-[^2H]-D-Glucose

2-[^2H]-D-Glucose (97 atom % ^2H) was synthesised from α-methylglucoside as previously described.[16]

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