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Glucose is a Precursor of 1-Deoxynojirimycin and 1-Deoxymannonojirimycin in *Streptomyces subrutilus*

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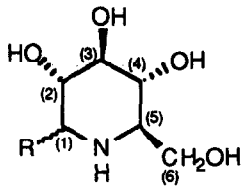
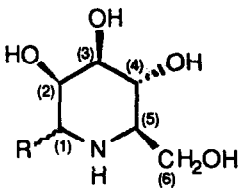
Abstract: *Streptomyces subrutilus* ATCC 27467, when grown on a glucose-containing soyabean medium, produces both 1-deoxymannonojirimycin (DMJ) and 1-deoxynojirimycin (DNJ) in its culture medium. When 1- or 2-[^{2}H]-D-glucose is used, the deuterium label appears at C6 in both alkaloids and the labelling 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]- 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 aminosugar 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.

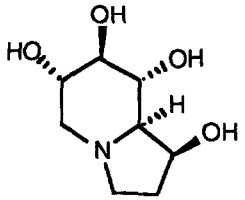
Certain sugar-like alkaloids e.g. 1-deoxynojirimycin (DNJ) and castanospermine can act as anti-HIV agents as they can inhibit the maturation of the envelope glycoprotein gp120 of HIV-1.[1] These alkaloids (Scheme 1) have been isolated from plants[2] and many chemical synthetic routes to DNJ and related alkaloids have been published.[3,4] Reports [5-7] that streptomycetes can produce nojirimycins led us to investigate their biosynthesis in these organisms with the view to the possibility of producing analogues by adding biosynthetic precursors to culture media. Little has been published on the biosynthesis of these compounds apart from a brief mention that glucose is a precursor of nojirimycin (NJ) in an unspecified organism (presumably a streptomycete) and that "the carbon chain of nojirimycin comes from that of D-glucose with the head C1 to tail C6 inversion and that an amino group has been introduced probably via 5-ketofructose".[8] Further details of this work do not appear to have been published.



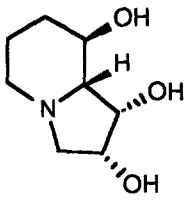
NJ, R =OH
DNJ, R = H



MJ, R = OH
DMJ, R = H



Castanospermine



Swainsonine

Scheme 1 Alkaloids related to nojirimycin

Two biosynthetic pathways to the nojirimycins can be envisaged (a) from glucose and (b) from pipecolic acid. The latter pathway is used in the biosynthesis in *Rhizoctonia leguminicola* of swainsonine, another alkaloid which inhibits glycosidases.[9] We now wish to report that NJ, mannonojirimycin (MJ), DNJ and DMJ are all produced by *Streptomyces subrutilus* ATCC 27467 and that glucose is the precursor of all these alkaloids. We initially suggested[10] that 5-ketofructose may be an intermediate in the biosynthetic pathway to DNJ but further studies with 5- and 6-deuterated glucoses as carbon sources in our fermentations have led us to correct this hypothesis. The proposed biosynthetic pathway to all four alkaloids is given below.

RESULTS AND DISCUSSION

The study of the biosynthetic pathways to nojirimycins required assays to be developed for MJ, DMJ,NJ and DNJ. Silylation of dried samples of culture broth of *S. subrutilus* followed by GLC proved to be an effective method of analysing for DMJ and DNJ but neither MJ nor NJ could be detected by this method as the silylated derivatives of these compounds were unstable on heating. MJ and DMJ can both inhibit mannosidases while NJ and DNJ can both inhibit glucosidases. Hence, we developed enzymatic assays which would allow us to distinguish between these pairs of compounds. Trehalase is inhibited by both NJ and DNJ. When NJ is heated in 6M hydrochloric acid at 90°C for 6 h, it decomposes to product(s) which no longer inhibit this enzyme but DNJ is unaffected by this treatment. DMJ did not inhibit trehalase even at a concentration of 0.1 mg/ml. Thus, a comparison of the inhibition of trehalase by a sample before and after acid treatment allowed the amounts of DNJ and NJ present to be determined. The enzymatic and GLC assays agreed to within ±5% for samples of DNJ. A similar assay based on α-mannosidase was developed to distinguish between MJ and DMJ although an authentic sample of MJ was not available to us. It has been reported[11] that heat treatment of MJ at

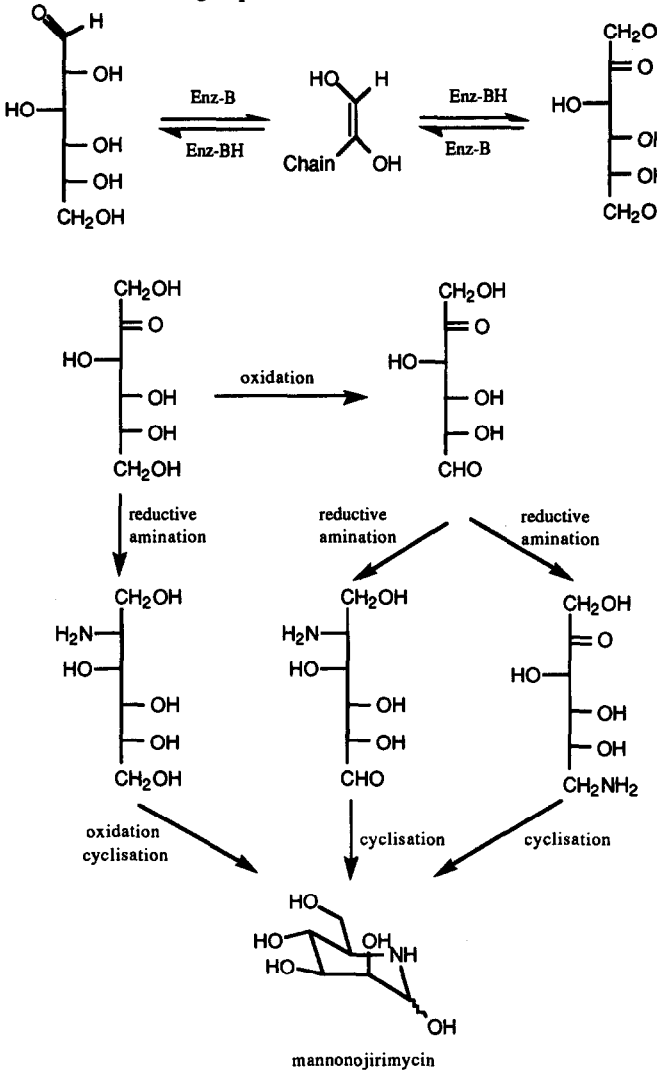
55°C at pH 5.5 gave a product with a decreased inhibitory effect on jack bean α-mannosidase and we have observed that the inhibitory effect of DMJ against α-mannosidase was unaffected by this treatment. In samples taken from our fementations, we found that there was a decrease of up to 50% in α-mannosidase inhibition after heat treatment depending on the time at which the sample was taken. This decrease could not be due to NJ as an authentic sample of NJ at a concentration of 0.1mg/ml caused only a 13% inhibition of α-mannosidase before heat treatment and the maximum amount of NJ in our fermentations as determined by the trehalase assay was 0.05mg/ml. As inhibitory effect of of DMJ and DNJ against α-mannosidase was unaffected by heat treatment, we suggest that the additional inhibitory activity against α-mannosidase was probably due to MJ.

Glucose was shown to be the precursor of both DNJ and DMJ in *S. subrutilus* as the deuterated alkaloids were produced when 1-[^{2}H]-D-glucose was used as a component of the standard soyabean culture medium. The alkaloids were isolated as their peracetylated derivatives and mass spectrometric analysis showed that the peracetyl [^{2}H]-DNJ and -DMJ derived from 1-[^{2}H]-D-glucose (33 atom % D) contained 21.7± 2.4 and 21.9 ± 1.7 atom % D respectively. The deuterium was located at C6 in both alkaloids as loss of deuterium label from C6 could be observed in the mass spectra of the peracetylated [^{2}H]-DNJ and -DMJ. A similar loss of deuterium from C6 was observed in the mass spectrum of authentic 6,6-[$^2H_2$]-DNJ. ^{1}H and ^{2}H NMR spectroscopic analysis of the peracetylated [^{2}H]-DNJ and -DMJ confirmed that deuterium had replaced one of the hydrogen atoms at C6. ^{1}H and ^{2}H NMR spectroscopic analysis of N-acetyl [^{2}H]-DNJ and free [^{2}H]-DMJ derived from the peracetylated alkaloids confirmed the location of deuterium at C6. When 1-[^{13}C]-D-glucose (50 atom % ^{13}C) was a component of the soyabean culture medium of *S. subrutilus* , the peracetylated [^{2}H]-DNJ and -DMJ obtained from this fermentation contained 33 ± 3 atom % ^{13}C. The location of the isotopic label at C6 in both alkaloids was deduced from the ^{13}C NMR spectrum of a partially purified mixture of [^{2}H]-DNJ and -DMJ before acetylation.

^{1}H and ^{2}H NMR spectroscopic analysis of peracetylated [^{2}H]-DNJ and -DMJ isolated from a culture medium of *S. subrutilus* which included 2-[^{2}H]-D-glucose showed that the deuterium label was located at C6. However, a deuterium atom had replaced a diastereotopic methylene proton other than that observed in [^{2}H]-DNJ and -DMJ which had been derived from 1-[^{2}H]-D-glucose. ^{1}H and ^{2}H NMR spectroscopic analysis of N-acetyl [^{2}H]-DNJ and free [^{2}H]-DMJ derived from the peracetylated alkaloids confirmed the position of the deuterium atom. Incorporation of deuterium from 2-[^{2}H]-D-glucose into the two alkaloids was low, the peracetyl [^{2}H]-DNJ contained 5.8 ± 0.3 atom % D and the peracetyl [^{2}H]-DMJ contained 6.5 ± 0.3 atom % D in contrast to incorporations of 16- 17% which were expected from the experiments with 1-[^{2}H]-glucose described above.

The chemical shifts and splittings of the signals in the ^{1}H NMR spectrum of N-acetyl DNJ are very similar to those attributable to the corresponding protons in glucose and we suggest that in aqueous solution N-acetyl DNJ adopts a conformation similar to that adopted by glucose. The signals due to the pro-*S* proton at C6 in the ^{1}H NMR spectra of N-acetyl [^{2}H]-DNJ and free [^{2}H]-DMJ derived from 1-[^{2}H]-D-glucose were diminished in intensity compared with the corresponding signals from their unlabelled analogues. The intensities of the signals due to the pro-*R* proton at C6 in N-acetyl [^{2}H]-DNJ and free [^{2}H]-DMJ were unaltered in comparison with the corresponding signals in the spectra of the unlabelled analogues indicating that no replacement of the pro-*R* proton by deuterium had occurred. Similar NMR evidence indicated that when 2-[^{2}H]-D-glucose was the carbon source in the fermentation, the pro-*R* protons at C6 in peracetylated [^{2}H]-DNJ and free [^{2}H]-DMJ were labelled with deuterium.

We suggest that in the biosynthetic pathway to DNJ and DMJ in *S. subrutilus* the first step is the isomerisation of glucose to fructose (Scheme 2) by a mechanism similar to that which has been deduced for glucose-6-phosphate isomerase.[12] A basic group at the active site of the isomerase removes the proton from C2



Scheme 2 Conversion of glucose to mannonojirimycin

of the glucose to generate an ene-diol. The protonated basic group in the enzyme then donates this proton to C1 of the ene-diol to generate fructose. The biosynthesis of the aminosugar mycosamine is believed to involve the participation of a similar ene-diol intermediate.[13] During this isomerisation, exchange of the mobile proton with solvent water can occur which accounts for the low incorporations of deuterium into DNJ and DMJ from 2-[^{2}H]-D-glucose compared with those observed from 1-[^{2}H]-D-glucose. This variation in labelling has

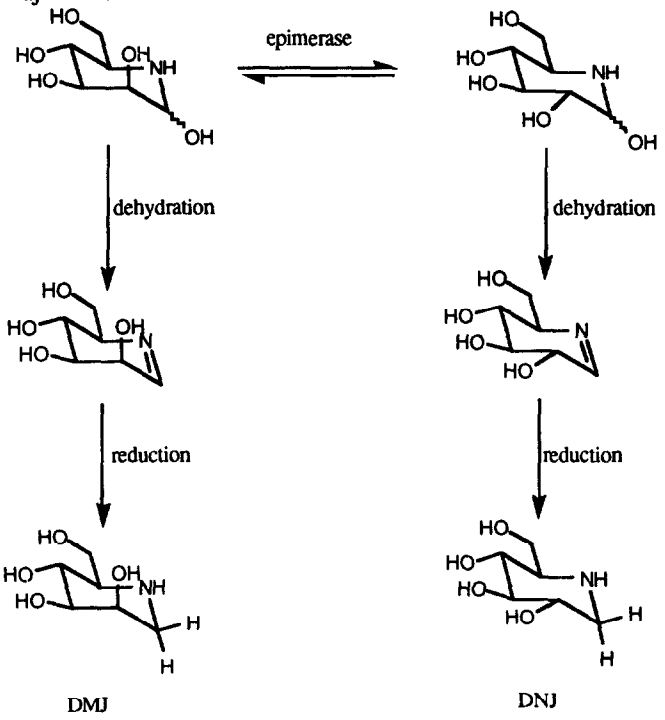
previously been observed in reactions catalysed by glucose-6-phosphate isomerase.[12] We have attempted unsuccessfully to observe incorporation of deuterium from solvent into C6 of DNJ or DMJ in fermentations carried out in deuterium oxide. The reason for the lack of incorporation of deuterium is unclear but may be due to adverse kinetic isotope effects. It may be noted that when D-glucose was treated with glucose isomerase at pH 7.0 in 100% deuterium oxide only 2% incorporation of deuterium was observed in the recovered glucose.[14]

Since both DNJ and DMJ are formed in the same fermentation, an isomerisation at C5 in the fructose must occur at some stage during the biosynthesis. We initially proposed[10] that oxidation at C5 of fructose to 5-ketofructose might occur followed by an isomerisation which was the reverse of that occurring at C1 and C2 giving rise to two epimers which lead to DNJ and DMJ. Subsequent fermentation experiments with 5-[^{2}H]- and 6,6-[$^2H_2$]-D-glucose showed that this was not the case. When 6,6-[$^2H_2$]-glucose (each of the hydrogens at C6 being labelled with 70 atom % D) was used, the mixture of [^{2}H]- DNJ and -DMJ in the fermentation contained only one deuterium atom (43 atom % D, corresponding 61% of the original label at one of the C6 positions). Comparison of the ^{1}H NMR spectrum of isolated [^{2}H]-DNJ with published spectra for unlabelled material showed that the deuterium atom retained in these experiments was in the equatorial position at C1. The location of the deuterium atom retained during the biosynthesis of [^{2}H]-DMJ was established using nOe difference spectroscopy. Irradiation of the signal due to one proton at C1 (δ 2.65 ppm) caused enhancements to the signals at δ 3.92 (7.9% H2) and 3.50 ppm (5.3%, H3), while irradiation of the signal due to the other proton at C1 (δ 2.90 ppm) caused enhancement of the signal due to H2 only (7.7%). Computer assisted molecular modelling of DMJ in its energy minimised conformation gives the approximate distances H3 - $H1_{eq}$ = 3.8Å and H3 - $H1_{ax}$ = 2.7Å suggesting that the signal at δ 2.65 ppm was due to the axial proton at C1 in DMJ. Literature assignment of the signals in the ^{1}H NMR spectrum of the hydrochloride of DMJ agrees with our deduction that the equatorial proton at C1 has been replaced by deuterium.[15]

This evidence suggests that the primary alcohol group at C6 in fructose (or an aminated fructose) is oxidised during the biosynthesis of DNJ and DMJ to an aldehydo-group with the loss of one hydrogen atom. The timing of the amination at C2 is unknown. At least three pathways are possible. Amination of fructose at C2 could be followed by oxidation at C6 and cyclisation. Alternatively, oxidation at C6 in fructose could be followed by amination at either C2 or C6. Cyclisation of the aldehyde function at C6 with the amino group at C2 or of the keto group at C2 with the amino group at C6 would give MJ. Elimination of water from the N-H group and the hydroxyl at C1 from MJ followed by reduction of the product from the underside (α-face) of the ring would give DMJ (Scheme 3). Confirmation for this scheme was obtained when 5-[^{2}H]-D-glucose (75 atom % D) was used in our fermentations. Only [^{2}H]-DMJ (22.7% $\pm$ 1.7 atom % D) could be isolated from the fermentation and ^{1}H and ^{2}H NMR spectroscopy showed that the deuterium was located at C2. The DNJ formed in this fermentation did not contain any deuterium. If MJ or DMJ were the initial products in the biosynthetic scheme and these underwent oxidative epimerisation to NJ or DNJ, loss of hydrogen isotope from C2 would be expected. In all our fermentations, the yield of DNJ was greater than that of DMJ. Thus, 48 h after inoculating a culture medium with growing cells of *S. subrutilus*, DMJ was easily detectable in the culture broth by GLC but only a trace of DNJ was present. After a further 26 h, the yield of DNJ was approximately 2.5 times that of DMJ suggesting that the latter is converted to DNJ during the late stages of the fermentation.

To determine whether the epimerisation took place at the NJ or DNJ level, aliquots of unlabelled NJ were added to a fermentation at different time intervals. No significant change in the amount of DNJ produced could

be detected using the trehalase assay. When 6,6-[$^2H_2$]-nojirimycin (99 atom % D) was added to the fermentation, both the [^{2}H]-DNJ and -DMJ could be isolated from the fermentation. In both compounds, both protons at C6 were labelled with deuterium. Mass spectrometric analysis showed that [^{2}H]-DNJ contained 10.9 ± 2.9 atom % D, while the [^{2}H]-DMJ contained 4.4 ± 2.0 atom % D. Thus epimerisation at C2 probably occurs at the NJ/MJ level. The difference in the levels of deuterium incorporation must reflect the position of the MJ = NJ equilibrium and the relative rates of conversion of MJ to DMJ and NJ to DNJ. Furthermore, DNJ and DMJ are produced in the fermentation from sources other than added glucose (see below). This hypothesis is supported by the report that both MJ and NJ can be isolated as their adducts from the culture medium of *S. lavendulae*, [16] a strain which is closely related to *S. subrutilus*.[17] On the other hand, when 6,6-[$^2H_2$]-DNJ (99 atom % D) was added to a fermentation after 96 h and DNJ and DMJ isolated after a further 67 h, mass spectrometric analysis showed that little deuterium had been incorporated into DMJ (1.6 ± 0.7 atom % D) while DNJ had retained 15.9 ± 1.3 atom % D. This suggests that some epimerisation can also occur at the DNJ/DMJ level but this is not the major route.



Scheme 3 Conversion of mannonojirimycin to DMJ and DNJ

In all our feeding experiments, the recovered alkaloids contain less ^{2}H or ^{13}C than the added glucose precursors suggesting the presence of a carbon source other than glucose in the culture broth, possibly the soyabean meal. The dilution of isotope was quantified using 3-[^{2}H]-glucose as carbon source as no migration or loss of isotope should occur with this compound during our proposed biosynthetic pathway. When 3-[^{2}H] glucose (35 atom % D) was added to a fermentation, the [^{2}H]-DNJ isolated contained 23.6 ± 2.6 atom % D

representing an incorporation of isotope of about 66%. This figure agrees with that obtained from experiments with [^{13}C]]-glucose.When no glucose was added to the fermentation medium, trace amounts of DNJ and DMJ could be detected in the broth confirming that synthesis of these alkaloids takes place irrespective of added glucose.

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(WH400). ^{13}C NMR spectra were recorded at 61.424 (ACF 250) or 100.623 MHz(WH 400). In addition to TMS, the following reference signals were used for ^{1}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 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.

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 2 ml min^{-1} with a gas split ratio of 30:1, and an injector temperature of 250^{o}C.

Fermentation Conditions, Isolation and Characterisation of Metabolites

1-[^{2}H]-D-Glucose (98 atom % D) and 1-[^{13}C]-D-glucose (98.7 atom % D) were obtained from Aldrich Chemical Co and diluted with unlabelled D-glucose before use. Full details for the fermentation of *Streptomyces subrutilus* ATCC 27467 will be given elsewhere.[18] The fermentation medium contained D-glucose or deuterated D-glucose(0.4%), soyabean meal (1.0%), KCl (0.05%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.5%), $NaNO_3$ (0.2%) and $CaCO_3$ (0.35%) made up in distilled water giving a final pH of 7.3-7.4. Aliquots of medium (30 ml) were inoculated with *S. subrutilus* and shaken at 28-30^{o}C for 7 d at 200 rpm. The concentration of DNJ by GLC analysis was 5mg per 100 ml culture broth. The fermentations were autoclaved at 120^{o}C for 20 min, centrifuged at 5,000 rpm for 30 min and the supernatant added to a column of Dowex 50 ion exchange resin (H^+ form, 15 ml per 100 ml supernatant). The column was washed with distilled water (2 bed vol) and then eluted with NH_4OH (2M, 2 bed vol). This eluate was concentrated to 50 ml and assayed by GLC. The concentrated eluate was lyophilised, the residue dissolved in EtOH : water (70% v/v) and applied to a column of Al_2O_3 (neutral, Brockmann grade I), the column was eluted with EtOH : water (3 bed vol, 70% v/v) . The eluate was evaporated to dryness *in vacuo*, the residue dissolved in pyridine and treated with excess acetic anhydride in the presence of DMAP. The acylation mixture was evaporated to dryness *in vacuo* and the peracetylated [^{2}H]-DNJ and -DMJ were purified by flash chromatography on silica (elution with EtOAc : CH_2Cl_2, 30% v/v) to yield 10 mg acylated products per 400 ml of culture.

Deacetylation of peracetyl-DNJ and -DMJ. Purified peracetyl-DNJ or -DMJ was stirred with conc. NH_4OH (5 ml, d 0.880) and methanol (5 ml) overnight at room temperature. Evaporation of the reaction gave either N-acetyl DNJ or free DMJ in approx 70% yield.

Gas chromatography. The concentrated eluate from the purification (100 µl) and β-methylglucoside (internal standard, 100 µl, 0.8 mg/ml) were combined and lyophilised, Sigma SilA (100µl) was added and the reaction 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.[19] To a solution of trehalase (Sigma Chemical Co., 20 µl, 0.005µg/ml, 0.385 U/ml) in maleate buffer (pH 6.0) was added inhibitor solution (20 µl) and the mixture incubated at 37°C for 15 min. Trehalose (20 µl, 56 mM) in 0.1M maleate buffer (pH 6.0) was added and the reaction incubated at 37°C for a further 60 min. The reaction was stopped by 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 sample which was heated in a boiling water bath for 6 h. The solution was lyophilised, resuspended in distilled water and assayed as above. Comparison of the GLC and trehalase assays showed that the values for DNJ agreed to ±5%.

Mannosidase assay for MJ and DMJ. The inhibitor solution (150 µl) was incubated with jack bean mannosidase (Sigma Chemical Co, 50 µl, 0.02 mg/ml) 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 incubated at 25°C for 15 min. The reaction was stopped by addition of glycine buffer (2 ml, 0.1M, 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°C) δ 2.01 (3Hs, Me), 2.04 (6Hs, Me), 2.05 (3Hs, Me), 2.19 (3Hs, Me) 3.60-3.80 (1H br.s, H1), 3.99-4.05 (2H br.s, H1 + H5), 4.50 (1Hdd, J = 11.5, 5.9, H6), 4.63 (1Hdd, J = 11.6, 7.8, H6), 5.11 (1Hm, H2), 5.32 (1Ht, J = 4.3, 3.8, H3 or H4), 5.39 ppm (1Ht, J = 3.9, 3.4, H4 or H3).

N-Acetyl DNJ. ^{1}H NMR (400 MHz, D_2O) δ 1.84, (3Hs, Me), 2.73 (1Ht, J = 12.0, $H1_{ax}$), 2.90 (1H br,t, H5), 3.30 (1Hdd, J = 5.1, 12.4, $H1_{eq}$), 3.34-3.44 (2Hm, H3 + H4), 3.60 (1Hddd, J = 5.2, 8.8, H2), 3.71 (1Hdd, J = 12.4, 5.6, H6), 3.82 ppm((1Hdd, J = 12.4, 3.0, H6).

DNJ (enriched with [^{13}C]-glucose). ^{13}C NMR δ 49.23 (C1), 61.20 (C5), 61.91 (C6), 71.43 (C2), 72.05 (C3 or C4), 78.92 ppm (C4 or C3).

Peracetyl-DMJ. ^{1}H NMR (400 MHz, C_5D_5N, 90°C) δ 1.96 (3Hs, Me), 2.01 (3Hs, Me), 2.03 (3Hs, Me), 2.13 (3Hs, Me), 2.22 (3Hs, Me), 3.46 (1H br.s, H1), 3.85 (2H br.s, H1 + H5), 4.50 (1Hdd, J = 11.4, 6.1, $H6_a$), 4.81 (1H br.dd, $H6_b$), 5.36 (2Hm, H3 or H4 + H2), 5.70 ppm (1Ht, J = 3.3,3.3, H3 or H4).

CI mass spectrum: found m/z 374.1450, calc. for $C_{16}H_{24}NO_9$ m/z 374.1451 $(M + H)^+$.

DMJ. ^{1}H NMR (400 MHz, D_2O) δ 3.00 (1Hdd, J = 1.2, 13.9, $H1_b$), 3.20 (1Hdd, J = 2.9, 13.9, $H1_a$), 3.58 (1Hdd, J = 9.6, 3.1, H3), 3.71 (1Hdd, J = 10.0, 9.3, H4), 3.75 (1Hdd, J = 6.0, 12.1, $H6_a$), 3.85 (1Hdd, J = 12.4, 3.18, $H6_b$), 4.10 ppm (1Hm, H2).

Chemical Syntheses

Deuterated Glucoses

3-[^{2}H]-D-glucose. This was synthesised (35 atom % D) according to Koch and Perlin.[20]

6,6-[$^2H_2$]-D-glucose. This was synthesised (99 atom % D) according to Lemieux and Stevens.[21]

α-Methyl 2,3,4,6-Tetra-O-benzyl-D-glucopyranoside. Sodium hydride (7 g, 0.29 mol, 80% dispersion in oil) was cautiously heated to 120°C in a solution of α-methyl D-glucopyranoside (5 g, 0.025 mol)

in benzyl chloride (125 ml). After 3 h, the reaction was cooled and ethanol was added to destroy excess sodium hydride. The organic phase was washed with water, dried ($MgSO_4$) and filtered. Benzyl chloride was removed *in vacuo* to leave α-methyl 2,3,4,6-tetra-O-benzyl-D-glucopyranoside (13.7 g) as a bright yellow oil which was used in the next stage without purification.

^1H NMR ($CDCl_3$, 220 MHz): δ 3.40 (3Hs, OMe), 3.55-4.10 (6Hm, H2-H6), 4.45-5.05 (9Hm, 4 x CH_2 + H1), 7.20-7.50 ppm (20Hm, aromatic H).

CI mass spectrum: m/z 572 $(M + NH_4)^+$

Glacial HOAc (240 ml) was added to the yellow oil and the solution heated to 90°C, H_2SO_4 (2M, 71 ml) was then added slowly and heating continued for a further 2 h. After this time, more H_2SO_4 (2M, 57 ml) was added and the reaction heated for 24 h. After cooling, the reaction mixture was poured into ice water. The orange precipitate was filtered off and washed with methanol to give colourless crystals (4.1 g, 30%) of 2,3,4,6-tetra-O-benzyl-D-glucopyranose.

^1H NMR ($CDCl_3$, 220 MHz) δ 3.50-4.10 (6Hm, H2-H6), 4.46-5.00 (8Hm, CH_2), 5.25 (1Hd, H1), 7.10-7.50 ppm (20Hm, aromatic H).

CI mass spectrum: m/z 558 $(M + NH_4)^+$, 540 $(M + NH_4 - H_2O)^+$

Sodium (1 g, 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-O-tetrabenzyl-D-glucopyranose (7.2 g, 0.013 mol) in dry dioxan (235 ml) and the mixture stirred under N_2 for 3 d at room temperature. The dioxan was evaporated *in vacuo* and the residual solid dissolved in CH_2Cl_2. This solution was washed with water (3 x 50ml) until the aqueous layer was neutral. The organic layer was dried ($MgSO_4$) and the solvent 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 (3gm, 42%).

^1H NMR ($CDCl_3$, 220 MHz) δ 3.50-4.10 (5Hm, H3-H6), 4.46-5.00 (8Hm, CH_2), 5.25 (1Hs, H1), 7.10-7.45 ppm (20Hm, aromatic H).

CI mass spectrum: found m/z 559.2920, calc for $C_{34}H_{39}DNO_6$ m/z 559.2919 $(M + NH_4)^+$.

2-[^{2}H]-D-glucose. 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) and the mixture stirred under hydrogen (1 atm) for 24 h after which time 455 ml of hydrogen had been taken up. TLC analysis showed that a mixture of glucose and acetylated derivatives were present. Water (20 ml) was added and the catalyst removed by filtration through Celite. The filtrate was evaporated to dryness *in vacuo* and the residue dissolved in methanol to which a few drops of ammonium hydroxide (d 0.880) had been added. After 2 h the solution was evaporated to dryness and the residue 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 (0.548 g, 65%).

^1H NMR (D_2O, 400 MHz) δ 4.11 - 4.18 (4Hm, $H3_b$, $H4_b$, $H5_b$, $H4_a$), 4.24-4.31 (3Hm, $H6_{ab}$, $H3_a$), 4.31-4.36 (3Hm, $H6_{ab}$, $H5_a$),4.66 (1Hs, $H1_b$), 4.91 ppm (1Hs, $H1_a$).

^2H NMR δ 3.13, 3.50 ppm. (D-glucose exhibits a doubled doublet in its ^1H NMR spectrum due to $H2_{ab}$ at δ 3.13, 3.46 ppm).

^{13}C NMR (D_2O, 100.623 MHz) 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 ppm (C6αβ). Where α and β refer to the two anomers of glucose.[9]

CI mass spectrum of peracetyl-2-[^{2}H]-D-glucose: m/z 409 $(M + NH_4)^+$, 391 $(M + H)^+$,

3-O-Benzyl-6-O-triphenylmethyl-1,2-O-isopropylidene-α-D-*xylo*-hexofuranos-5-ulose. was prepared in 70% yield by the oxidation[22] of 3-O-benzyl-6-O-triphenylmethyl-1,2-O-isopropylidene-α-D-glucofuranoside.[7]

^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.28 (3Hs, Me), 1.45 (3Hs, Me) 4.03 (1Hd, J = 18.2, CH_2), 4.09 (1Hd, J = 18.2, CH_2), 4.36-4.40 (2Hm, H3 or H4 and $H6_a$), 4.47 (1Hd, J = 11.7, H6), 4.50 (1Hd, J = 3.6, H3 or H4), 4.85 (1Hd, J = 3.7, H2), 5.92 (1Hd, J = 3.6, H1), 7.14-7.44 ppm (20Hm, aromatic H).

Analysis: found; C 76.09, H 6.23% calc. for $C_{35}H_{34}O_6$; C76.34, H 6.22%.

3-O-Benzyl-6-O-triphenylmethyl-1,2-O-isopropylidene-5-[^{2}H]-D-glucofuranoside. 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 ethanol (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 dissolved in CH_2Cl_2 (50 ml). This solution was washed with water (75 ml), dried ($MgSO_4$) and evaporated *in vacuo* to give 3-O-Benzyl-6-O-triphenylmethyl-1,2-O-isopropylidene-5-[^{2}H]-D-glucofuranoside (7.5 g, 96%). In identical experiments using $NaBH_4$, only the *gluco*-isomer and no *ido*-isomer and could be detected in the product by ^{1}H NMR spectroscopy.

^{1}H NMR ($CDCl_3$, 220 MHz) δ 1.32 (3Hs, Me), 1.51 (3Hs, Me), 2.69 (1Hs, OH), 3.25 (1Hd, J = 9.3, $H6_a$), 3.45 (1Hd, J = 9.3, $H6_b$), 4.08 (1Hd, J = 3.1, H3), 4.37 (1Hd, J = 3.3, H4), 4.50 (1Hd, J = 11.8, CH_2), 4.60 (1Hd. J = 4.0, H2), 4.65 (1Hd, J = 4.0, CH_2), 6.98 (1Hd, J = 4.0, H1), 7.20-7.60 ppm (20Hm, aromatic H).

CI Mass spectrum : found m/z 476.219 calc. for $C_{29}H_{30}DO_6$ m/z 476.2184 $(M + H - C_6H_6)^+$.

Deprotection[7] of 3-O-Benzyl-6-O-triphenylmethyl-1,2-O-isopropylidene-5-[^{2}H]-D-glucofuranoside gave **5-[^{2}H]-D-Glucose** (1.54 g 64%, 92 atom % D)

^{1}H NMR (D_2O, 400 MHZ) δ 3.19 (1Ht, J = 8.0, H2β), 3.35 (2Hm, H4αβ), 3.44 (1Ht, J = 9.3, H3β), 3.48 (1Hdd, J = 9.6, 3.8, H2α), 3.65 (1Ht, J = 9.6, H3α), 3.67 (1Hd, J = 12.3, H6β),3.70 (1Hd, J = H6α), 3.79 (1Hd, J = 12.3, H6α), 3.84 (1Hd, J = 12.3, H6β), 4.61 (1Hd, J = 8.0, H1β), 5.18 ppm (1Hd, J = 3.8, H1α). Where α and β refer to the two anomers of glucose.[20]

Deuterated Nojirimycins

5-Azido-5-deoxy-1,2-O-isopropylidene-D-glucuronolactone was prepared as described.[23]

5-(N-t-Butyloxycarbonyl)amino-5-deoxy-1,2-O-isopropylidene-D-glucuronolactone. To 5-azido-5-deoxy-1,2-O-isopropylidene-D-glucuronolactone (4.1 g, 17 mmol) in EtOAc (125 ml) was added 10% palladium on carbon (500 mg) and the mixture hydrogenated at 2 atm for 4 h after which time the mixture was filtered through Celite. Di-t-butyl carbonate (4.08 g, 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 (6.0 g) which was purified by flash chromatography on silica (elution with EtOAc:CH2Cl2, 10% v/v) to give 5-(N-t-Butyloxycarbonyl)amino-5-deoxy-1,2-O-isopropylidene-D-glucuronolactone (4.4 g, 82%).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.38 (3Hs, Me), 1.50 (9Hs, Me of t-butyl), 1.55 (3Hs, Me), 4.77 (1Hdd. J = 4.3, 9.0, H5), 4.80 (1Hd, J = 3.72, H2), 4.82 (1Hd, J = 2.9, H3), 4.93 (1Hdd, J = 3.0, 4.1, H4), 5.10 (1Hd, J = 8.9, NH), 5.91 ppm (1Hd, J = 3.6, H1).

EI Mass spectrum $M^{+\cdot}$ 315.1309, calc for $M^{+\cdot}$ $C_{14}H_{21}O_7N$ 315.1318.

6,6-[^{2}H]-5-(N-t-Butyloxycarbonyl)amino-5-deoxy-1,2-O-isopropylidene-D-glucose.

$LiAlD_4$ (50 mg, 1.2 mmol)was added to dry THF (5 ml) under N_2 followed by (N-t-Butyloxycarbonyl)amino-

5-deoxy-1,2-O-isopropylidene-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 to destroy unreacted $LiAlD_4$. When the reaction had subsided, a further amount of water (10 ml) was added and the mixture extracted with EtOAc (3 x 10 ml). The combined organic extracts were washed with water (2 x 10 ml) and dried ($MgSO_4$). Removal of the solvent *in vacuo* left a clear oil (160 mg) which TLC and ^1H NMR showed to contain a trace of lactol. The oil was dissolved in ethanol (5 ml) and deuterium oxide (2 ml) 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 methanol (4 x 20 ml) were added and evaporated in vacuo to leave 6,6-[^2H]-5-(N-t-Butyloxycarbonyl)amino-5-deoxy-1,2-O-isopropylidene-D-glucose (140 mg, 83%) as a white solid.

^1H NMR ($CDCl_3$, 220 MHz) δ 1.31 (3Hs, Me), 1.46 (9Hs, Me of t-butyl), 1.52 (3Hs, Me), 3.10 (1H br.s, OH), 3.80 (1Ht, J = 8.5, H5), 4.10 (2Hm, H3 + H4), 4.62 (1Hd, J = 3.7, H2), 5.10 (1H br.s,)H), 5.50 (1H, br.d, J = 8.4, NH), 6.00 ppm (1Hd, J = 3.7, H1).

CI mass spectrum: found m/z 322.183 calc for $C_{14}H_{24}DN_2O_7$ m/z 322.183 $(M + H)^+$.

6,6-[^2H]-Nojirimycin bisulfite. Gaseous sulfur dioxide was bubbled through a suspension of 6,6-[^2H]-5-(N-t-Butyloxycarbonyl)amino-5-deoxy-1,2-O-isopropylidene-D-glucose (140 mg) in water (2 ml) for 10 min.The reaction was then set aside for 65 h at 35-40°C. The solution was then cooled to 0°C and methanol was added (20 ml). The crystalline bisulfite adduct (82 mg, 72%) was collected by filtration.

^1H NMR δ 3.08 (1Hd, J = 10, H5), 3.50 (1Ht, J = 9.3, H3 or H4), 3.55 (1Ht, J = 9.4, H3 or H4), 3.79 (1Hdd, J = 9.0, H2), 4.01 ppm (1Hdd, J = 10.3, H1). This compared well with the published spectrum of the protio-compound .[24]

The IR spectrum was identical to that described in the literature[7] with bands at *inter alia* 3600 - 3000 (br. strong, OH), 3120 (s, medium, NH) and 1580 (weak, OH bend) cm^{-1}.

6,6-[^2H]-Nojirimycin. Dowex 1 x 8 resin (OH^- form, 1 ml) was added to a suspension of 6,6-[^2H]-nojirimycin bisulfite (82 mg) in water (5 ml).When the bisulfite adduct had dissolved, the aqueous suspension of the resin was applied to a Dowex 1 x 8 column (OH^- form, 5 ml) which was then eluted with water (20 ml). The eluate was lyophilised to give NJ which was either hydrogenated immediately to DNJ or added to fermentations.

6,6-[^1H]-nojirimycin. ^1H NMR (D_2O, 400 MHz) NB Two conformers were found in solution α:β (2.25:1), the assignments were made by homonuclear 1H COSY spectroscopy. δ 2.61 (1Hddd, H5β), 3.01 (1Hddd, H5α), 3.11 (1Ht, J = 9.2, H2β), 3.15 (1Ht, J = 9.2, H4α), 3.21 (1Ht, J = 9.3, H4β), 3.30 (1Ht, J = 9.2, H3β), 3.45 (2Hm, H6α + H2α), 3.58 (2Hm, H3α + H6β), 3.77-3.84 (2Hm, H6α + H6β), 4.12 (1Hd, J = 8.2, H1β), 4.68 ppm (1Hd, J = 3.4, H1α). The ^1H NMR spectrum compared well with the published spectrum.[25]

6,6-[^2H]-Deoxynojirimycin. The NJ obtained above was dissolved in water (5 ml), glacial HOAc (2 drops) was added followed by PtO_2 (trace) and the mixture was hydrogenated at 1 atm for 3 h. The catalyst was filtered off and the filtrate lyophilised to yield 6,6-[^2H]-1-deoxynojirimycin (26 mg, 51% from bisulfite adduct).This protocol could also be used to prepared unlabelled DNJ.

^1H NMR (D_2O, 220 MHz) δ 2.40 (1Hdd, J = 10.7, $H1_{ax}$), 2.50 (1Hd, J = 9.8, H5), 3.10 (1Hdd, J = 5.3, 12.9, $H1_{eq}$), 3.20 (1Ht, J = 8.9, H3 or H4), 3.30 (1Ht, J = 8.9, H3 or H4), 3.45 ppm (1Hm, H2).

CI mass spectrum of peracetylated 6,6-[^2H]-Deoxynojirimycin: m/z 376 $(M + H)^+$, 316 $(M + H - AcOH)^+$.

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