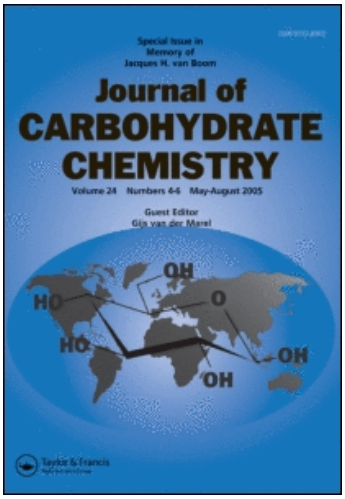

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Synthesis and Inhibitory Properties of 2-Acetamido-2-Deoxynojirimycin (2-Acetamido-5-amino-2,5-dideoxy-D-glucopyranose, 1) and 2-Acetamido-1,2-dideoxynojirimycin (2-Acetamido-1,5-imino-1,2,5-trideoxy-D-Glucitol, 2)

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SYNTHESIS AND INHIBITORY PROPERTIES OF 2-ACETAMIDO-2-DEOXYNOJIRIMYCIN (2-ACETAMIDO-5-AMINO-2,5-DIDEOXY-D-GLUCOPYRANOSE, 1) AND 2-ACETAMIDO-1,2-DIDEOXYNOJIRIMYCIN (2-ACETAMIDO-1,5-IMINO-1,2,5-TRIDEOXY-D-GLUCITOL, 2)

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Dedicated to Prof. Dr. Kurt Heyns on the occasion of his 80th birthday

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ABSTRACT

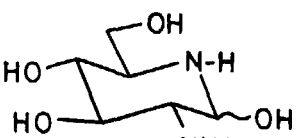
The title compounds were synthesized from N-acetyl-D-glucosamine by a 10-step route which featured oxidation at C-5 of a suitably protected 2-acetamido-2-deoxy-D-glucofuranoside. Reduction of its 5-oxime with Raney nickel gave the D-gluco epimer with ~90% stereoselectivity. Problems encountered in the preparation of 1 during cleavage of its precursor glycoside were circumvented by the use of trifluoromethanesulfonic acid.

N-Acetylglucosaminidases of fungal, plant, molluscan, and mammalian origin were inhibited by 1 and 2 in the nano- to micromolar range with 1 inhibiting >100-fold better than 2. The inhibition equilibrium with 1 was approached on a time-scale of minutes with all enzymes tested. Comparison with N-acetyl-D-glucosamine showed that replacement of the pyranose oxygen by nitrogen resulted in an up to 10^6-fold enhancement of the binding constants. N-Acetylglucosaminidases thus resemble the majority of other glycoside hydrolases. The enzymes from Aspergillus niger and Helix pomatia presented an exception in that their inhibition by 2 was only slightly better than by N-acetyl-D-glucosamine.

INTRODUCTION

N-Acetylglucosaminidases represent a group of enzymes of widespread occurrence in both plants and animals.[1] Their mechanism probably

resembles that of other glycoside hydrolases but detailed evidence from studies with non-covalent inhibitors is scanty compared to other glycosidases and no experiments with active site-directed inhibitors seem to have been reported. As important information on the active site and the mechanism of action of glycosidases has come from inhibition studies with 5-amino-5-deoxyhexoses and 1,5-dideoxy-1,5-iminohexitols[2-4] we have synthesized the corresponding analogues of N-acetyl-D-glucosamine 1 and 2 in order to test if mechanistic features deduced for a large number of glycosidases also apply for N-acetylglucosaminidases. In addition, it was expected that N-ω-carboxyalkyl derivatives of the iminohexitol would provide valuable ligands for the affinity chromatography of glucosaminidases and selective inhibition of these enzymes in living cells might permit new experiments on their biological function.



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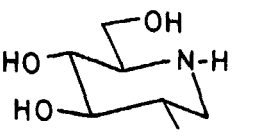
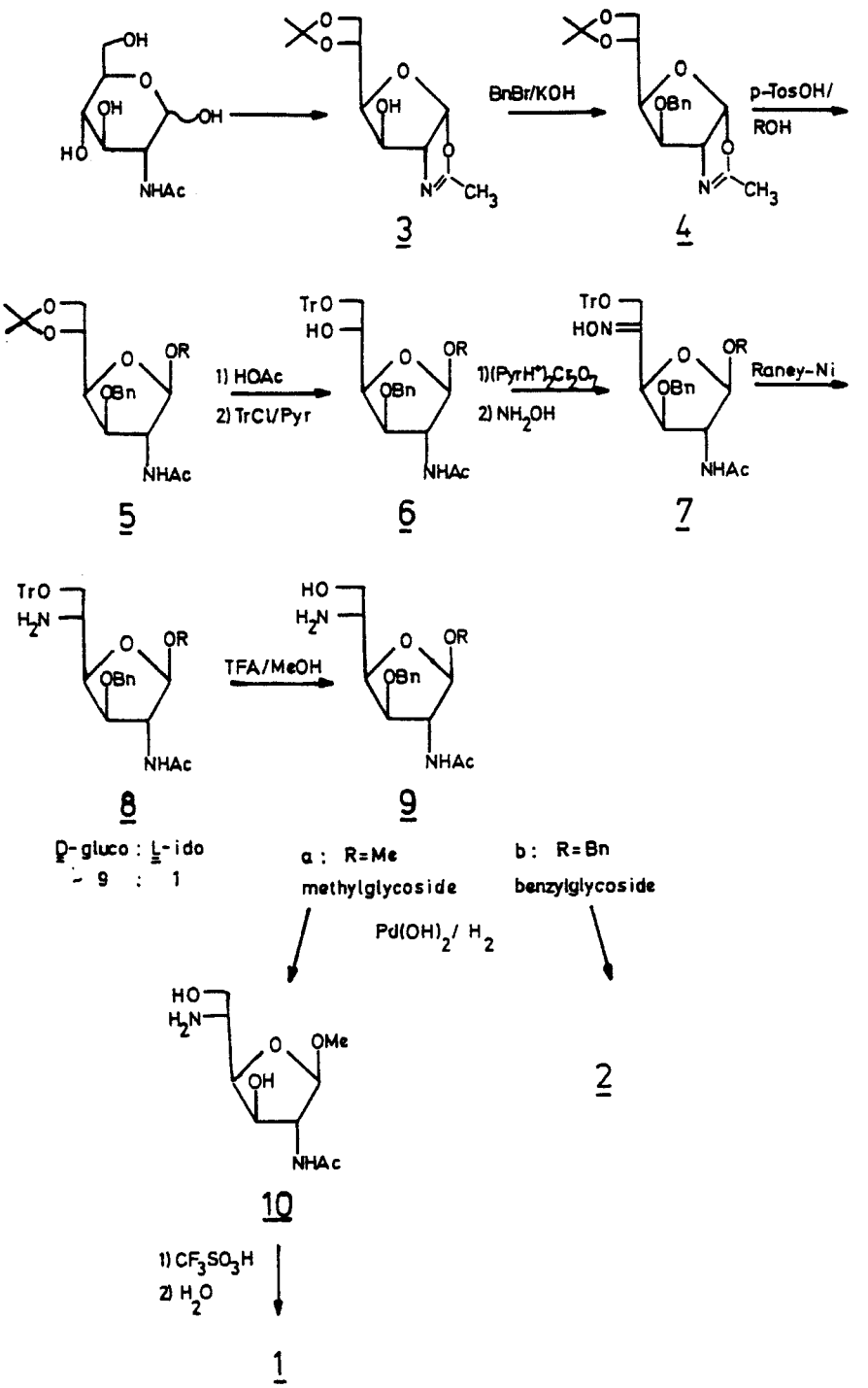


2

Since we started this work two publications have appeared which describe the synthesis of 2. Fleet an coworkers[5] used glucose as starting material which was converted into 2 by a 17-step procedure. Böshagen et al.[6] transformed 1-deoxynojirimycin into 2 by a 10-step route. The procedure described here takes 11 steps from N-acetyl-D-glucosamine for the preparation of 1 and also permits, with slight variations, the preparation of 2-acetamido-1,2-dideoxynojirimycin (2) in ten steps. We also report on the inhibition of N-acetylglucosaminidases of fungal, plant, molluscan, and mammalian origin by 1 and 2. The use of 2 for the affinity purification of N-acetylglucosaminidases will be published elsewhere.

RESULTS AND DISCUSSION

Syntheses. - The routes employed for the synthesis of 1 and 2 (Scheme 1) were modeled after routes which had been successfully applied previously for the preparation of the corresponding D-gluco,[7] D-manno-,[3] and D-galacto-isomers.[4] The methyl (for the synthesis of 1) and benzyl glycosides (for 2) of 2-acetamido-2-deoxy-3-O-benzyl-6-O-trityl-ß-D-glucofuranose (6) were oxidized at C-5. Conversion of the 5-keto deriva-



SCHEME 1

tives to the oximes (7) and reduction with Raney nickel gave the 5-amino furanosides (8). The stereoselectivity of the reduction step was 95% in favour of teh D-gluco epimer. This stereoselectivity was greatly reduced, however, when the trityl group had been removed prior to the reduction step in order to avoid the drastic conditions required for detritylation in the presence of the protonated amino group at C-5 which caused some deacetylation at C-2. About 30% of the L-ido epimer was formed using this latter approach (see below).

A more serious problem presented by the C-2 acetamido group was its stabilizing effect on the glycosidic bond in 10. Attempts to prepare the hydrogen sulfite adduct of 1 by the procedure used for the D-manno analogue,[3] i.e. acid hydrolysis in aqueous methanol saturated with SO_2, were unsuccessful. No hydrolysis was observed; under more drastic conditions (higher acid concentration and temperature) a large number of products without inhibitory potency was formed. Some success was achieved with glacial acetic acid as solvent. Inhibitory material could be isolated after chromatographic work-up which had K_i-values with the enzyme from jack beans in the sub-micromolar range. Somewhat better results (apparent K_i around 15 nM) were obtained with pyridine/hydrogen fluoride[8] or anhydrous hydrogen fluoride,[9] but product isolation was difficult due to solubility problems and to the formation of $(HF)_x$-adducts.

A solution to these problems was finally found in the use of neat trifluoromethanesulfonic acid which cleaves glycosides of N-acetylglucosamine at room temperature without effects on amide bonds.[10] Even though no information is available on the primary product formed from 10 we assume that it is the 1-O-trifluoromethanesulfonate, which is immediately converted into 1 on the addition of water and neutralization. Attempts to crystallize 1 as its hydrogen sulfite adduct[7,3,4] were unsuccessful. We therefore neutralized the primary product solution from 10 and trifluoromethanesulfonic acid with tributylamine and extracted the salts with chloroform. After addition of excess SO_2 the hydrogen sulfite adduct of 1 was recovered by lyophilisation. Identity of this material with the proposed structure followed from elemental analysis, mass spectrometry and reaction with 2-aminobenzaldehyde.

The preparation of 2 by hydrogenolysis of the O-benzyl groups of the amino glycoside 9b presented no problems. The melting point of 2 (224 °C) was in moderate agreement with published data (mp 227-228 °C[5,6]).

Its specific rotation ($[\alpha]_{578}^{20}$ + 14.8°) was close to the value given by Böshagen et al.[6] ($[\alpha]_{D}^{20}$ + 16.6°) whereas Fleet et al.[5] give $[\alpha]_{D}^{20}$ + 35°.

Potentiometric titration showed 2 to have pK_a 6.45. This is close to the pK_a of the gluco-analogue 1-deoxynojirimycin (pK_a 6.33).[11] The manno- and galacto-analogues, on the other hand, had pK_a-values of 7.2 and 7.1, respectively, which was ascribed to the effect of the axial hydroxyl groups of the latter in 1,3-position relative to the basic nitrogen.[3,4]

The by-product obtained in about 30% yield on reduction of detritylated 7b, which was also obtained in small amounts on detritylation of the reduction product from 7b, was tentatively identified as the L-ido epimer of 8b by its reaction with ninhydrin and its ^{1}H-NMR-spectrum. The latter contained signals for one N-acetyl and two benzyl groups whereas the signals for the hydrogen atoms at C-3, C-4, and C-5 were quite different from the corresponding signals of 9b.

Chemical quantification and stability of 1. - As enzymes like glucose oxidase or hexokinase/glucose-6-phosphate dehydrogenase for nojirimycin[12,13] or D-galactose oxidase for galacto-nojirimycin[4] are unknown for N-acetylglucosamine, we tested several chemical methods to determine the content of 1 in the lyophilized hydrogen sulfite adduct. Attempts to exploit the dissociation of the adduct followed by iodine oxidation of HSO_3^- [14] were unsuccessful because the dissociation of $1 \cdot HSO_3$ was much slower than that of the corresponding nojirimycin compound and accurate end point determinations were not possible. Sulfite content was, therefore, determined with sulfite oxidase/cytochrome c[15] and found to be 50% of the amount calculated for $1 \cdot HSO_3$.

5-Amino-5-deoxy-D-xylopyranose reacts with 2-aminobenzaldehyde to give an orange coloured dihydroquinazoline derivative.[16] Application of this reaction to the determination of 1 gave the same absorbance maximum (λ = 470 nm) as nojirimycin used as reference. With the molar absorbance of the nojirimycin product (1,900 M^{-1} cm^{-1}) the content of $1 \cdot HSO_3$ was found to be 90% of the calculated amount. It cannot be excluded, however, that this value is somewhat too high due to the presence of decomposition products also reacting with 2-aminobenzaldehyde. Paulsen et al.[16] report that the 1,2-dehydration product of 5-amino-5-deoxy-D-xylopyranose also gave the orange coloured product. It should be noted that maximal colour development in the reaction of 1 with 2-aminobenzaldehyde required 16 to

18 h, whereas that of nojirimycin was complete in 1.5 to 3 h. Colour stability, on the other hand, was much higher with the product from 1 than from nojirimycin. Part of the difference between the contents of 1 based on the sulfite determination (50%) and the reaction with 2-amino-benzaldehyde is probably due to a loss of SO_2 during lyophilisation because the hydrogen sulfite adducts of 5-amino-5-deoxyhexopyranoses are partially dissociated in aqueous solution.[14]

Stability determinations of stock solutions of 1 based on their inhibitory potency against N-acetylglucosaminidases (see below) gave the following results: no losses were observed in 0.25 M aqueous ammonia during 6 months at -18 °C; aqueous solutions freed from ammonia were stable for at least 4 weeks at 0 °C (galacto-nojirimycin showed 40% decomposition under the latter conditions[4]). On the other hand, solutions of 1 prepared in 25 mM phosphate buffer pH 7.0 were much less stable: 40% decomposition were observed after 2 weeks at 0 °C; at 25 °C the half-life of 1 was found to be 50 h.

Inhibition studies. - The results of inhibition studies with a number of N-acetylglucosaminidases of fungal, plant, molluscan, and mammalian origin with 1 and 2 as inhibitors and N-acetylglucosamine and N-acetylglucosylamine (2-acetamido-1-amino-1,2-dideoxy-D-glucose) as reference compounds are summarized in Tab. 1. The values given for 1 were based on its concentration determined with 2-aminobenzaldehyde; they thus represent an upper limit. The type of inhibition was purely competitive in all cases except for the bovine kidney enzyme which showed mixed competitive/non-competitive inhibition. The data show that most of the enzymes bind 1 and 2 10^3- to 10^6-fold better than the non-basic analogue N-acetylglucosamine and also, though to a lesser extent, than the glucosylamine derivative. The results thus resemble those found with the inhibition of other glycoside hydrolases by the corresponding 5-amino-5-deoxyhexoses and 1,5-dideoxy-1,5-iminohexitols.[2-4] The general features of ionic interactions between the cationic form of these basic hexose analogues and a carboxylate group at the catalytic site of the enzyme thus also hold for the N-acetylglucosaminidases studied here. The inhibition constants found with 2 are very similar to those reported by Fleet et al.[5] for the same compound with the enzymes from jack beans

TABLE 1. Inhibiton of ß-N-Acetylglucosamindases by 5-Amino-5-deoxy-analogues of N-Acetylglucosamine (1 and 2), by N-Acetylglucosamine (GlcNAc), and by 2-Acetamido-1-amino-1,2-dideoxy-D-glucose (GlcNAc-NH_2). Inhibition constants are given in µM. (see EXPERIMENTAL for other data)

Enzyme source	Inhibitors 1[a]	2	GlcNAc	GlcNAc-NH_2
Aspergillus niger	1.2	1 000	6 000	1 400
Jack beans	0.0012	0.14	4 000	12
Helix pomatia	0.5	80	800	34
Bovine kidney	0.003	0.6[c]	4 000	43
Human liver, A	n. d.	1.0[d]	4 000 to 8 000[e]	n. d.
" " , B	n. d.	1.0[d]		n. d.

[a] Slow approach to the inhibition equilibrium, K_i calculated from final rate (see EXPERIMENTAL)

[b] Crude preparation, data refer to enzyme with lower K_m and higer K_i

[c] Competitive component of mixed competitive/non-competitive inhibition

[d] Ref. 17; n. d. not determined

[e] Ref. 18

(0.23 µM), bovine kidney (0.6 µM), human placenta (0.9 µM), and Asp. niger (no inhibition at 0.3 mM of 2).

It should be noted that the inhibition equilibrium with 1 was approached slowly, i.e. on the time-sacle of minutes, a feature which has also been observed with the inhibition of other glycosidases by the corresponding 5-amino-5-deoxyhexopyranoses and -iminohexitols.[3,4,19]

Also, as with most other glycosidases, the basic hexose analogue 1 was a more than 100-fold better inhibitor than the iminohexitol 2. Two different explanations may be advanced for this observations: i) the unprotonated form inhibits several orders of magnitude better than the protonated one and ii) additional hydrogen bonds can be formed with the hydroxyl group at C-1 of the 5-aminohexopyranoses. For explanation i) the unprotonated form of the inhibitor should bind several orders of magnitude better than the cationic form. This would favour the inhibi-

tion by the 5-aminohexoses because their pK_a-values (5.1 to 5.3) are up to two units lower than the pK_as of the iminohexitols. Under the usual assay conditons (pH 5 or below) the proportion of free base is 50- to 100-fold larger for the nojirimycins, thus giving a correspondingly lower K_i when calculations are based the total inhibitor concentration. Examples for much better inhibition by the unprotonated form of the inhibitor are ß-glucosidase from almonds,[20] cytosolic ß-glucosidase from calf spleen,[21] and ß-galactosidase from Escherichia coli.[22]

Prime requirement for explanation ii) is restricted access of water to the catalytic site. If access of solvent water to this part of the enzyme inhibitor complex is greatly reduced one or two additional hydrogen bonds might well provide sufficient binding energy to account for the observed differences between 1 and 2.[23] Restricted access of solvent water to the catalytic site of glycosidases has been postulated to account for the strong interaction between hexose derivatives bearing a cationic group at C-1 and the active site carboxylate.[2-4] As the observed differences in the K_i-values for 1 and 2 are somewhat larger than the upper limit based on the different pK_a-values we favour explanation ii). Definitive conclusions would require studies with the permanently cationic N,N-dimethyl derivative of 2 and a close comparison of the pH-dependence of K_i and K_m.

The N-acetylglucosaminidases from Asp. niger and Helix pomatia differ from the other enzymes studied here in their poor inhibition by 2 which is only 6- to 10-fold better than by N-acetylglucosamine. As a relatively weak inhibition of the two enzymes was also found with the glycosylamine analogue GlcNAc-NH_2 their apparently anomalous inhibition is unlikely caused by an inability of active site functional groups to adjust themselves for optimal interaction with the basic or cationic centre of the putative oxocarbenium ion intermediate of substrate hydrolysis. On the other hand, both enzymes are inhibited by 1 more than 1000-fold better than by N-acetylglucosamine. As the weak inhibiton by 2 and by 2-acetamidoglucosylamine rules out electrostatic interactions with an active site carboxylate and both 1 and N-acetylglucosamine have an α/ß hydroxyl group at C-1, a different explanation has to be found for the the strong inhibition by 1. Replacement of the pyranose oxygen by an NH-group has two consequences: i) the ring becomes more flexible, thus permitting a better adaption to the half-chair conformation of the transiton state of substrate hydrolysis, and ii) this part of the hexose

ring has, in additon to its ability to function as a hydrogen bond acceptor, the potential to act as a hydrogen bond donor. Altered flexibility of the hexose ring alone cannot account for the tight binding of 1 because it would also hold for 2. Possibly, the combination of hydrogen bonds with the hydroxyl group at C-1 and features i) or ii) might provide an answer to this problem. Clearly, more experiments are needed, notably with 2-acetamido-2-deoxy-D-glucono-1,5-lactone and the corresponding lactam to be prepared from 1

In conclusion, we have shown that N-acetylglucosaminidases resemble other glycosidases with respect to their strong inhibition by hexose analogues bearing a basic nitrogen at C-1. This supports an arrangement of functional groups at the catalytic site as first proposed by Phillips and his group for lysozyme,[24] i.e. an acidic group near the glycosidic oxygen and a carboxylate near C-1 of the substrate on the α-face of the pyranose ring. As with other groups of glycoside hydrolases there are exceptions which do not follow this general inhibition pattern[25] and which require other approaches.

EXPERIMENTAL

General Procedures. - Melting points were determined on a hot-stage microscope and are uncorrected. Specific rotations were measured with a Perkin-Elmer Polarimeter 141. ^{1}H-NMR-Spectra were recorded on a Bruker AC 80. Mass spectra were taken with a Varian MAT 731 (FAB mode) or a Finnigan MAT 4510. TLC was performed on aluminum sheets precoated with silica gel F_{254} (Merck, Darmstadt) with detection by spraying with 10% H_2SO_4 (v/v) in glacial acetic acid or 1% ninhydrin in acetone.

Reagents. - Palladium hydroxide (20%) on charcoal was from Aldrich, 4-methylumbelliferyl 2-acetamido-2-deoxy-ß-D-glucopyranoside and 2-acetamido-1-amino-1,2-dideoxy-D-glucopyranose were from Serva, Heidelberg, all other chemicals were from Merck, Darmstadt, enzymes (except from human liver) were from Sigma, München. The oxazoline 3 was prepared according to Mack et al.[26] Nojirimycin hydrogen sulfite adduct was a gift from Dr. E. Truscheit (Bayer AG, Wuppertal).

2-Methyl-(3-O-benzyl-1,2-dideoxy-5,6-O-isopropylidene-α-D-glucofurano- 2,1-d -oxazoline (4). Powdered potassium hydroxide (46 g) and benzyl bromide (23 mL) were added to a rapidly stirred solution of 3

(24 g) in dry toluene (350 mL) and heated to 70-80 °C for 3 h. The solution was cooled, solids removed by decantation and thoroughly extracted with toluene. The combined toluene solutions were washed neutral with water, dried ($MgSO_4$) and concentrated to give crude 4 (45 g) as a brownish oil contaminated with ~ 5% acetone condensation products brought in with 3. A small sample was purified by preparative TLC (ether/diethylamine 99/1) to a colourless oil: IR (film) 1660 cm^{-1} (oxazoline); ^{1}H-NMR ($CDCl_3$) 1.36, 1.40 (2s, 6H, Me_2C); 2.05 (d, 3H, $^5J_{2,Me}$ = 1.6 Hz, Me (oxazoline); 3.84 (dd, 1H, $^3J_{3,4}$ = 2.9 Hz, $^3J_{4,5}$ = 7.2 Hz, H-4); 3.96-4,15 (m, 3H, H-3, H-6,6'); 4.15-4.58 (m, 2H, H-2, H-5); 4.70 (s, 2H, Ph-CH_2); 6.12 (d, 1H, $^3J_{1,2}$ = 5.2 Hz, H-1); 7.32 (s, 5H, Ph).

Methyl 2-Acetamido-3-O-benzyl-2-deoxy-5,6-O-isopropylidene-ß-D-glucofuranoside (5a) and Methyl 2-Acetamido-3-O-benzyl-2-deoxy-6-O-trityl-ß-D-glucofuranoside (6a). Crude 4 (45 g) was dissolved in anhydrous methanol (500 mL) containing 0.5 mM p-toluenesulfonic acid and left at 25 °C. Opening of the oxazoline ring was found to be complete after 8 h as shown by TLC (ether/methanol 10/1, R_f(5a) 0.7). Crude 5a was obtained after neutralisation of $KHCO_3$ in methanol and concentration to a syrup. Prolonged standing with traces of watercaused the appearance of a second spot with R_f 0.75 which was tentatively assumed to be the α-furanoside.

The isopropylidene group was removed by dissolving 5a in 60% aqueous acetic acid (400 mL). The reaction was complete after 15 to 20 h at 25 °C (TLC in ether/methanol 20/1, R_f 0.3; R_f(5a) 0.8). Evaporation of solvents and crystallisation of the residue from ethyl acetate/ether gave methyl 2-acetamido-3-O-benzyl-2-deoxy-ß-D-glucofuranoside (15 g; 47% based on 3), mp 123 °C, $[\alpha]_{578}^{20}$ -90° (methanol): ^{1}H-NMR ($CDCl_3$) 1.98 (s, 3H, AcN); 2.35 (br. t, 1H, J = 6.7 Hz, OH); 2.87 (d, 1H, J = 3.1 Hz, OH); 3.35 (s, 3H, OMe); 3.50-3.95 (m, 2H, H-6,-6'); 3.97-4.35 (m, 3H, H-3, H-4, H-5); 4.49 (d, 1H, $^3J_{2,NH}$ = 7.8 Hz, H-2); 4.79 (s, 1H, H-1); 4.59, 4.91 (q, 2H, J = 12 Hz, Ph-CH_2); 5.92 (br. d, 1H, NH); 7.33 (s, 5H, Ph).

For the preparation of 6a the preceding glucofuranoside (20 g) was dissolved in anhydrous pyridine (200 mL) and reacted with trityl chloride (20.7 g) for 2 d at 25 °C (TLC in toluene/methanol 10/1, R_f(6a) 0.3), poured into ice water (500 mL) and extracted with toluene. The combined extracts were washed with ice-cold aqueous 2 N acetic acid followed by saturated aqueous $NaHCO_3$ and dried ($MgSO_4$). Evaporation of solvents and

crystallisation of the residue from chloroform/ether gave 6a (29.7 g), mp 137 °C, $[\alpha]_{578}^{20}$ -62° (chloroform): ^{1}H-NMR ($CDCl_3$) 1.93 (s, 3H, AcN); 2.95 (d, $^3J_{5,OH}$ = 4 Hz, OH); 3.19 (s, 3H, OMe); 3.28, 3.32 (2s, 2H, H-6,6'); 3.89-4.48 (m, 4H, H-2 to H-5); 4.50-4.92 (m, 3H, H-1, Ph-CH_2); 7.19-7.56 (m, 20H, Ph).

Methyl 2-Acetamido-3-O-benzyl-2,5-dideoxy-5-oximino-6-O-trityl-ß-D-glucofuranoside (7a). A solution of 6a (37.7 g) in dichloromethane (100 mL) was added to a rapidly stirred suspension of pyridinium dichromate (25.2 g) in dichloromethane (150 mL) containing 31.6 mL acetic anhydride and heated under reflux for 2 h. After concentration to half its volume chromium salts were precipitated by addition of ethyl acetate (2 vol.) and removed by filtration. The dark filtrate was passed over silica gel (4 x 30 cm) equilibrated and eluted with ethyl acetate. The effluent was concentrated and freed from traces of pyridine and acetic acid by several additions of toluene and concentration to a greenish oil (32.7 g). The crude ketone was homogeneous by TLC in ether.

Potassium hydrogen carbonate (15.2 g) and hydroxylammonium chloride (12.2 g) were heated under reflux in methanol (300 mL) for 10 min, 20.1 g of the above ketone was added, and heating continued for 30 min when the reaction was found to be complete (TLC in toluene/methanol 10/1, R_f(ketone) 0.4, R_f(7a) 0.3 and 0.25 (syn- and anti-isomer, respectively). The mixture was filtered, concentrated to dryness, and the residue extracted several times with chloroform. The combined extracts were washed with water and concetrated to give 7a (20 g) as a glassy foam. The isomer of 7a with R_f 0.3 crystallized readily from chloroform/petroleum ether, mp 138 °C, $[\alpha]_{578}^{20}$ -50° (methanol): ^{1}H-NMR ($CDCl_3$) 1.92 (s, 3H, AcN); 2.91 (s, 3H, OMe); 4.03 (s, 2H, H-6-6'); 4.23 (d, 1H, $^3J_{3,4}$ = 5.0 Hz, H-3); 4.31 (d, 1H, $^3J_{2,NH}$ = 6.0, H-2); 4.51, 4.73 (AB, $^2J_{AB}$ = 12 Hz, 2H, Ph-CH_2), 4.68 (s, 1H, H-1); 5.41 (d, 1H, H-4); 5.42 (d, 1H, NH); 7.08-7.55 (m, 20H, Ph); 9.60 (br. s, 1H, =N-OH).

Methyl 2-Acetamido-5-amino-3-O-benzyl-2,5-dideoxy-6-O-trityl-ß-D-glucofuranoside (8a). A mixture of syn- and anti-oximes 7a (4.3 g) was reduced with Raney nickel as described for nojirimycin.[7] In order to prevent hydrolysis of the 2-acetamido group, the freshly prepared catalyst was washed under argon with water and methanol until neutral (pH ≤ 8) and reactivated by bubbling hydrogen through a slowly stirred suspension in methanol. Work-up as in [7] gave 8a (3.6 g) as a glassy foam contaminated

with 5-10% of the L-ido epimer (TLC in toluene/methanol 9/1, R_f(D-gluco) 0.37; R_f(L-ido) 0.29). Pure 8a was obtained by crystallisation from chloroform/light petroleum: mp 176 °C; $[\alpha]_{578}^{20}$ -79° (chloroform); ^{1}H-NMR ($CDCl_3$) 1.6 (s, 2H, NH_2); 1.95 (s, 3H, AcN); 3.11-3.57 (m, 6H, H-4, H-6,6', OMe); 3.83-4.00 (m, 2H, H-3, H-5); 4.42 (d, 1H, $^3J_{2,NH}$ = 7.5 Hz, H-2); 4.51 and 4.85 (AB, 2H, Ph-CH_2); 4.66 (br. s, 1H, H-1); 5.33 (br. d, 1H, NH-amide); 7.13-7.53 (m, 20 H, Ph).

Methyl 2-Acetamido-5-amino-3-O-benzyl-2,5-dideoxy-ß-D-glucofuranoside (9a). A solution of 8a (1.2 g) in trifluroacetic acid (9 mL) and methanol (3 mL) was left overnight at 25 °C. Some trityl trifluoroacetate, which had crystallized, was filtered off and the filtrate diluted with 2 vol. of toluene and quickly concentrated to a syrup. The syrup was dissolved in 30 mL of water and residual trityl cleavage products extracted with ether. The aqueous phase was adjusted to pH 8-9 with ammonia and extracted several times with n-butanol. Concentration of the butanol extracts gave 9a as a yellow oil which crystallized on addition of chloroform. Recrystallisation gave white crystals (0.54 g): mp 115-116 °C; $[\alpha]_{578}^{20}$ -92° (methanol); ^{1}H-NMR (pyridine-d_5) 2.10 (s, 3H, AcN); 3.33 (s, 3H, OMe); 4.15-4.55 (m, 3H, H-6,6', H-4); 4.68-4.52 (m, 6H, Ph-CH_2, H-1, H-2, H-3, H-5).

Methyl 2-Acetamido-5-amino-2,5-dideoxy-ß-D-glucofuranoside (10). To a solution of 9a (0.4 g) in methanol (3.4 ml) and acetic acid (1.2 mL) was added palladium hydroxyide on charcoal (30-40 mg) and the rapidly stirred mixture hydrogenated at 12 bar H_2 for 1-2 d. The catalyst was removed by filtration, washed with water, and the filtrates passed over Dowex 50 (H^+-form, 8 mL). Elution of the resin with 0.5 M ammonia, evaporation of solvents, and crystallisation from ethanol/chloroform gave 10 (0.23 g): mp 90-91 °C; $[\alpha]_{578}^{20}$ -52° (methanol); ^{1}H-NMR (pyridine-d_5) 2.06 (s, 3H, AcN); 3.38 (s, 3H, OMe); 3.60-4.98 (m, H-2 to H-5, H-6,6', 2 OH, NH_2); 5.19 (d, 1H, $^3J_{1,2}$ = 1.2 Hz, H-1); 8.83 (br. d, 1H, $^3J_{2,NH}$ = 6.5 Hz, NH-Ac).

2-Acetamido-2-deoxynojirimycin (1) and its Hydrogen Sulfite Adduct (11). 10 (100 mg) was dissolved in trifluormethanesulfonic acid (TFMS) 0.6 mL) and left at 25 °C overnight. Most of the TFMS was removed by precipitation with cold ether (10 mL) and washing the precipitate several times with the same solvent. The residue was dissolved in cold water (3 mL) and immediately brought to pH 7 by the addition of 1 M tributylamine in

ethanol (0.6-0.8 mL). The tributylammonium salts (partly precipitated) were removed by extracting the solution five times with an equal volume of chloroform. For the preparation of 11, methanol saturated with SO_2 (3 mL) was added to the aqueous phase and the mixture concentrated to about 1.5 mL by vacuum evaporation at 0 °C after it was left 15 min on ice. Lyophilisation gave crude 11 as a white solid, mp 108-111 °C.

Analysis of the lyophilized product for 1 by reaction with 2-aminobenzaldehyde[16] (see below) gave 94% based on the composition $C_8H_{15}N_2O_4HSO_3$. Sulfite content determined with sulfite oxidase/cytochrome c[15] corresponded to 50% of the amount calculated from the above formula. Analysis by mass spectrometry gave the following results: FAB mode, negative ions: m/z 283 (11 - H), m/z 241 (11 - CH_3CO). The presence of contaminating tributylammonium trifluoromethanesulfonate was indicated by prominent peaks with m/z 149 ($CF_3SO_3^-$) and, in the spectrum of the positive ions, with m/z 186 ($(C_4H_9)_3NH^+$). a Peak with m/z 221 was ascribed to protonated 1. In order to obtain a normal mass spectrum, 11 was treated with bis-trimethylsilyl trifluoroacetamide[27] for 30 min at 60 °C. Ionisation of the product at 70 eV gave peaks with m/z 490 and 562, indicating that 11 was trimethylsilylated to a pentakis- and hexakis-TMS-derivative, each of which looses HSO_3SiMe_3.

Stock solutions of 1 were prepared from 11 by dissolving the lyophilized product in 0.25 mL ammonia and leaving it for 3 h at 0 °C. If required, most of the ammonia was removed by partial evaporation under vacuum.

Determination of 5-Amino-5-deoxypyranoses (nojirimycins) with 2-Aminobenzaldehyde. Reagent solution: equal volumes of 4 mM 2-aminobenzaldehyde in ethanol and 100 mM sodium citrate buffer pH 3.6. Assay: 0.1 mL of an up to 2 mM aqueous solution of the nojirimycin was added to 0.9 mL of reagent solution and the absorbance measured at 470 nm at various time intervals (up to 18 h) to record maximal colour development. A_{470} was linear with the concentration of nojirimycin standardized with glucose oxidase/peroxidase.[28] Concentrations of 1 were calculated from maximal A_{470} values of the 2-aminobenzaldehyde reaction using the molar absorbance of the nojirimycin reaction product (ε_{470} 1,900 M^{-1} cm^{-1}) as calculated from the standard curve.

Benzyl 2-Acetamido-3-O-benzyl-2-deoxy-5,6-O-isopropylidene-ß-D-glucofuranoside (5b). To a solution of crude oxazoline 4 (from 24 g 3)

in chloroform (135 mL, freed from ethanol by passage over activated alumina) was added anhydrous benzyl alcohol (54 mL) and p-toluenesulfonic acid (45 mg). The mixture was left at 25-30 °C until TLC in ether/methanol 99/1 showed the reaction to be complete (usually after 1 to 3 d; R_f(4) 0.8; R_f(5b) 0.6). The solution was washed with saturated aqueous $NaHCO_3$, dried with $MgSO_4$ and chloroform removed by vacuum evaporation. Unreacted benzyl alcohol was removed by destillation at 0.1 mm Hg and 5b (18.7 g) crystallized by the addition of ether to the oily residue. Another 9.1 g of 5b was obtained from the mother liquor by chromatography on silica gel with chloroform as eluant: total yield 27.8 g (64% based on 3); mp 140-141 °C; $[\alpha]_{578}^{20}$ -89° (chloroform); ^{1}H-NMR ($CDCl_3$) 1.35, 2.45 (2s, 6H, Me_2C); 1.96 (s, 3H, AcN); 3.93-4.13 (m, 3H, H-4, H-6,6'); 4.28-4.95 (m, 6H, H-2, H-5, Ph-CH_2); 5.00 (s, 1 H, H-1); 5.63 (br. d, 1H, $^3J_{2,NH}$ = 7.5 Hz, NHAc); 7.29 (s, 10 H, Ph).

Benzyl 2-Acetamido-3-O-benzyl-2-deoxy-6-O-trityl-ß-D-glucofuranoside (6b). 25 g of 5b were dissolved in 60% acetic acid (300 ml) and left at 25 °C for 15 to 20 h when TLC in ether/methanol 95/5 showed that removal of teh isopropylidene group was complete R_f(5b) 0.8; R_f (deprotected compound) 0.2 . The solution was diluted with water, concentrated to a syrup that was then free from residual water and acetic acid by repeated coevaporation with toluene. The oily diol was reacted for 2 d at 25 °C with trityl chloride (20 g) in anhydrous pyridine [250 mL, control by TLC in chloroform /methanol 95/5, R_f (diol) 0.2; R_f (6b) 0.5] and poured into ice-water (400 mL). The product was extracted with chloroform, washed with ice-cold 2 N acetic acid and saturated $NaHCO_3$ solution and dried ($MgSO_4$). Concentration and crystallisation from ether/petroleum ether gave 6b (29.5 g): mp 143 °C; $[\alpha]_{578}^{20}$ -80° (chloroform); ^{1}H-NMR ($CDCl_3$) 1.92 (s, 3H, AcNH); 3.01 (d, 1H, $^3J_{5,OH}$ = 4.7 Hz, OH); 3.30, 3.35 (2s, 2H, H-6,6'); 4.03 (br. d, 1H, J 4.5 Hz, H-4); 4.15-4.95 (m, 8H, H-1 to H-3, H-5, Ph-CH_2); 5.56 (br. d, 1H, NH); 7.15-7.56 (m, 25 H, Ph).

Benzyl 2-Acetamido-3-O-benzyl-2,5-dideoxy-5-oximino-6-O-trityl-ß-D-glucofuranoside (7b). Oxidation of 6b (34 g) to the 5-keto derivative was carried out as described for 6a. The oily ketone (25.6 g) could be crystallized from methanol: mp 84-85 °C; $[\alpha]_{578}^{20}$ -41° (chloroform); ^{1}H-NMR ($CDCl_3$) 1.90 (s, 2H, AcN); 4.08, 4.10 (2s, 2H, H-6,6'); 4.23-5.03 (m, 6H, H-1 to H-4, 2 Ph-CH_2); 5.54 (br. d, 1H, NHAc); 7.13-7.46 (m, 25 H, 5 Ph). IR (CCl_4) 1,723 cm^{-1} (C=O); 1,670 (amide I).

The above ketone (18.5 g) was converted to the 5-oxime as described for 7a. Cystallisation of the glassy product from chloroform/petroleum ether gave the less polar oxime isomer (10.5 g): mp 152-155 °C (after phase transition at 104-112 °C); $[\alpha]_{578}^{20}$ -73° (methanol); ^{1}H-NMR ($CDCl_3$) 1.88 (s, 3H, AcN); 3.98-4.45 (m, 6H, H-2, H-3, H-6,6', Ph-CH_2); 4.55, 4.75 (AB, $^2J_{AB}$ = 11.8 Hz, Ph-CH_2); 4.85 (s, 1H, H-1); 5.43 (d, $^3J_{3,4}$ = 5.3 Hz, H-4); 5.45 (br. d, $^3J_{2,NH}$ = 7.3 Hz, 1H, NHAc); 6.97-7.52 (m, 25 H, 5 Ph); 9.73 (br. s, 1H =N-OH).

Benzyl 2-Acetamido-5-amino-3-O-benzyl-2,5-dideoxy-6-O-trityl-ß-D-glucofuranoside (8b). Reduction of 7b (10 g) as described for 8a gave 8b (9.5 g) as a white glassy foam. TLC in toluene/methanol 85/15 gave two spots reacting with ninhydrin with relative intensity 9 : 1 (R_f 0.5 and 0.4, respectively). The major spot was tentatively assigned to the D-gluco and the other to the L-ido epimer. Crystallisation from toluene gave 8b still contaminated with traces of the L-ido compound but was used for the subsequent steps without further purification: mp 158 °C; $[\alpha]_{578}^{20}$ -66° (methanol); ^{1}H-NMR ($CDCl_3$) 1.63 (s, 2H, NH_2); 1.93 (s, 3H, AcN); 3.0-3.7 (m, 3H, H-5, H-6,6'); 3.93-4.96 (m, 8H, H-1 to H-4, 2 Ph-CH_2); 5.46 (br. d, 1H, J = 7.8 Hz, NHAc); 7.18-7.5 (m, 25 H, 5 Ph).

Reduction of the mixture of oxime isomers from the mother liquor of the crystallisation of 7b gave the same proportion of D-gluco- and L-ido-amines as obtained from crystalline 7b.

Benzyl 2-Acetamido-5-amino-3-O-benzyl-2,5-dideoxy-ß-D-glucofuranoside (9b). Detritylation of 8b (3.75 g) was carried out as described for 9a. Part of 9b (1.3 g) crystallized as trifluoroacetate salt during the ether/water partition step following the toluene extraction. Another 0.54 g of 9b was crystallized with chloroform from the material extracted with butanol at pH 8-9: mp 182 °C; $[\alpha]_{578}^{20}$ -92° (methanol); ^{1}H-NMR (pyridine-d_5) 2.09 (s, 3H, AcN); 4.18-5.41 (m, 11 H, H-1 to H-6,6', 2 Ph-CH_2); 5.52 (s, 1H, OH); 7.14-7.63 (m, 10 H, 2 Ph); 8.5 (br. s, 2H, NH_2); 9.32 (br. d, 1H, $^3J_{2,NH}$ = 7 Hz, NHAc).

2-Acetamido-1,2-dideoxynojirimycin (2). Hydrogenation of 9b (0.9g) and work-up by ion exchange on Dowex 50 (H^+) was done as described for 9a. Crystallisation from methanol/ether of the material eluted with ammonia gave pure 2 (0.25 g): mp 224 °C (dec.); $[\alpha]_{578}^{20}$ +15° (water); potentiometric titration: pK_a 6.45.

Inhibition of N-Acetylglucosamindases. Enzyme activities were measured fluorimetrically at 25 °C with 4-methylumbelliferyl 2-acetamido-2-deoxy-ß-D-glucopyranoside as described[29] in 50 mM sodium citrate at the pH-optima of the enzymes as given by the supplier (Sigma, except for the enzymes from human liver). These were as follows: ß-N-acetylglucosaminidase from Asp. niger, pH 4.0; jack beans, pH 5.0; bovine kidney, pH 4.3; Helix pomatia (designated ß-glucuronidase H-2, crude), pH 5.0; human liver, pH 4.4. Kinetic constants K_m and K_i were evaluated from the slopes of Lineweaver-Burk plots in the absence and presence of inhibitor.[30] When 1 was used as inhibitor the final steady state rates of substrate hydrolysis were used for evaluation. Possible errors caused by substrate depletion in cases of a very slow approach to the inhibition equilibrium as with the enzymes from bovine kidney and jack beans were eliminated by preincubating the enzyme with inhibitor for 15 min. Reactions were started by the addition of substrate and the rates measured after a constant value had been reached.

Stock solutions of 1 were prepared from 11 as described above and stored at -18 °C. 2-Acetamido-2-deoxy-D-glucopyranosylamine was dissolved in anhydrous dimethylformamide. Small effects on enzyme activity cuased by this solvent were accounted for by control experiments.

The N-acetylglucosaminidase from the snail Helix pomatia was, in contrast to the other enzymes, a crude preparation (partially purified digestive juice). It contained, in addition to a number of other hydrolases, at least two or three different N-acetylglucosaminidases. The non-linear Lineweaver-Burk plot had asymptotes indicative of apparent K_m-values near 5.5 and 0.01 mM. Inhibition measurements were done with 0.03 mM substrate so that the activity of the high K_m enzyme was negligible. A Dixon plot (1/v vs. [I]) was also non-linear with asymptotes indicative for K_i-values of 80 µM and 30 µM. The data for the Helix pomatia enzyme listed in Tab. 1 are, therefore, only provisional.

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