Some Further Studies on the Synthesis of Glycopeptide Derivatives: 2-Acetamido-2-deoxy- β -D-glucopyranosylamine Derivatives

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1. The isolation, characterization and properties of two by-products in the preparation of 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy- β -D-glucopyranosylamine are described. They are bis(2-acetamido-2-deoxy-D-glucopyranosyl)amines. 2. An independent synthesis of the bis-glycopyranosylamines is reported and conditions are given for their preparation in high yield. 3. Further improvements are given for the synthesis of 2-acetamido-1-N-(β -L-aspartyl)-2-deoxy- β -D-glucopyranosylamine and the α -L-aspartyl isomer. 4. The synthesis of 2-acetamido-1-N-acetyl-2-deoxy- β -D-glucopyranosylamine is described.

The glycopeptide linkage between the carbohydrate and amino acid moieties of ovalbumin involves a unit of 2-acetamido-1-N-(β -L-aspartyl)-2-deoxy- β -D-glucopyranosylamine (Johansen, Marshall & Neuberger, 1961; Nuenke & Cunningham, 1961; Lee & Montgomery, 1962; Yamashina & Makino, 1962; Bogdanov, Kaverzneva & Andrejeva, 1962). The synthesis of model compounds containing this type of linkage has been reported by Bolton & Jeanloz (1963), Marks, Marshall & Neuberger (1963), Marshall & Neuberger (1964), Tsukamoto, Yamamoto & Miyashita (1964) and Yamamoto, Miyashita & Tsukamoto (1965b). All of these syntheses proceed via 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy- β -D-glucopyranosylamine (II; R=H), prepared by the hydrogenation of the corresponding β -azide (I). Yamamoto, Miyashita & Tsukamoto (1965a) have isolated a further product from this hydrogenation, to which they tentatively assigned a bisglycosylamine structure.

Our preliminary studies have revealed that the by-products of this reaction contain two 2-acetamido-2-deoxy-D-glucopyranosyl units linked through an amine bridge (Bolton, Hough & Khan, 1966). They could be further separated into two of the three possible anomeric forms which were synthesized independently, and neither of which reacted with α -benzyl N-benzyloxycarbonyl-L-aspartate. The bis-derivatives were present in several preparations of glycopeptides and could be excluded only by using very carefully controlled conditions of synthesis.

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EXPERIMENTAL

All melting points were determined on a Kofler hot-stage apparatus. Unless stated otherwise, all evaporations were carried out under reduced pressure. Optical rotations were measured manually on a Zeiss polarimeter. All reactions were followed by thin-layer chromatography at room temperature on silica gel G (Merck) or microcrystalline cellulose (Avicel). Column separations were carried out by chromatography on silica gel (Davison, grade 950, 60–200 mesh).

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy- β -D-glucopyranosyl azide (I). This was prepared by the method of Bolton & Jeanloz (1963). The yield was 52%, and the product had m.p. 166–168° and $[\alpha]_D^{24} - 60^\circ$ (c 2.0 in chloroform). The use of sodium azide instead of the dangerously explosive silver azide, and of either acetonitrile or *NN*-dimethylformamide as solvent, or acetochloroglucosamine (Leaback & Walker, 1957) instead of the bromine analogue (Inouye, Onodera, Kitaoka & Ochiai, 1957), all gave much inferior yields and slower reactions. Nuclear magnetic resonance (60 Mc/sec.) of a solution of the 1-azide in deuteriochloroform confirmed the β -anomeric configuration, since $H_{(1)}$ gave a doublet with $J_{1,2}$ 9 c/sec.

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy- β -D-glucopyranosylamine (II; R=H). A solution of (I) (1 g.) in ethanol (50 ml.) was hydrogenated at room temperature and atmospheric pressure with Adams platinum oxide catalyst (0.1 g.) for 4 hr. After removal of the catalyst, the solution was concentrated at room temperature to a crystalline residue, which, after recrystallization from a cold mixture of chloroform and ether, gave (II) (R=H) (0.73 g., 78%), m.p. 150° (decomp.), $[\alpha]_D^{23} - 25.5^\circ$ (c 2.0 in chloroform) (Found: C, 48.7; H, 6.5; N, 8.2. Calc. for $C_{14}H_{22}N_2O_8$: C, 48.55; H, 6.4; N, 8.1%). This compound was characterized by *N*-acetylation, by using acetic anhydride in methanol, to give 2-acetamido-3,4,6-tri-O-acetyl-1-N-acetyl-2-deoxy- β -D-glucopyranosylamine (II; R=Ac), m.p. 236–237°, $[\alpha]_D^{24} + 18.5^\circ$ (c 2.0 in chloroform).

(Found: C, 49.3; H, 6.1; N, 7.3; $C_{16}H_{24}N_2O_9$ requires C, 49.5; H, 6.2; N, 7.2%), and *N*-benzoylation, by using benzoic anhydride in methanol, to give 2-acetamido-3,4,6-tri-*O*-acetyl-1-*N*-benzoyl-2-deoxy- β -D-glucopyranosylamine, m.p. 246–247°, $[\alpha]_D^{24.5} = -16^\circ$ (c 2.0 in chloroform) (Found: C, 55.9; H, 5.8; N, 6.1. Calc. for $C_{21}H_{26}N_2O_9$: C, 56.0; H, 5.8; N, 6.2%). Bolton & Jeanloz (1963) reported m.p. 250.5–251.5° and $[\alpha]_D^{25} = -14^\circ$ (c 1.1 in chloroform) for the 1-benzamido derivative.

2-Acetamido-1-*N*-acetyl-2-deoxy- β -D-glucopyranosylamine and its 3,4,6-tri-*O*-methanesulphonate. The pentaacetate (II; R=Ac) (0.5 g.) was dissolved in dry methanol and 1% (w/v) sodium methoxide in methanol (0.2 ml.) was added; the mixture was stirred for 1 hr. at room temperature and then evaporated to dryness at 40°. The residue was dissolved in water (30 ml.) and deionized with Amberlite IR-120 resin (H^+ form). The eluate and washings were concentrated to dryness and the residue was recrystallized from methanol to give 2-acetamido-1-*N*-acetyl-2-deoxy- β -D-glucopyranosylamine (0.3 g., 88.5%), m.p. 232–233°, $[\alpha]_D^{19} = -24^\circ$ (c 2.0 in water) (Found: C, 46.0; H, 7.0; N, 10.5; $C_{10}H_{18}N_2O_6$ requires C, 45.8; H, 6.9; N, 10.7%).

A suspension of this diacetamido derivative (0.79 g.) in pyridine (18 ml.) was treated with methanesulphonyl chloride (1.1 ml.) and stirred overnight at 0°. The reaction mixture was then poured into ice-water with stirring, when the *trimesylate* precipitated. It was filtered off, washed well with water and recrystallized from a mixture of acetone and ethanol (1:2 g., 79%), m.p. 190–191° (Found: C, 31.6; H, 5.0; N, 5.6; S, 19.4; $C_{13}H_{24}N_2O_{12}S_3$ requires C, 31.4; H, 4.9; N, 5.6; S, 19.4%).

Isolation of two further products, A_1 and A_2 , from the hydrogenation of the azide (I). The hydrogenation was carried out as above. After removal of the catalyst, however, the solution was concentrated to dryness on a hot-water bath (> 40°). The residue was examined by thin-layer chromatography on silica gel with chloroform-methanol (15:1, v/v) as mobile phase and contained at least three products (R_F 0.21, 0.43, 0.62). The compound of low R_F was identical with the previous amine (II; R=H). On recrystallization of the reaction product from hot ethyl acetate, two fractions were obtained: *A* (0.15 g.), m.p. 244–247° (decomp. after darkening from 220°), and *B* (0.42 g.), m.p. 150° (decomp.). *B* was identical with the amine (II; R=H) and *A* contained the two faster-moving products (A_1 and A_2) which had co-crystallized.

Separation of A_1 and A_2 was achieved by careful crystallization from a mixture of either chloroform or ethyl acetate and light petroleum which afforded A_1 , m.p. 260° (decomp.). The mother liquor was then chromatographed on a column of silica gel by using 5% methanol in benzene as mobile phase, yielding A_2 , which, after recrystallization from a mixture of ethyl acetate and light petroleum, had m.p. 245–246° (decomp. after darkening from 225°). The optical rotations of A_1 and A_2 were $[\alpha]_D^{23} = -32^\circ$ (c 2.0 in chloroform) and $[\alpha]_D^{23} + 44^\circ$ (c 2.0 in chloroform) respectively. Addition of one drop of dilute hydrochloric acid to the rotation solutions produced a change to $[\alpha]_D^{23} + 30^\circ$ after 2 hr. in both cases. Examination of both final rotation solutions by thin-layer chromatography revealed identical mixtures of A_1 and A_2 , thus indicating some form of anomerization or interconversion. The two compounds were isomeric from analysis (A_1 : Found: C, 49.8; H, 6.4; N, 6.3; total acetyl, 50.3. A_2 : Found: C, 49.7; H, 6.4; N,

6.3; total acetyl, 50.3; $C_{28}H_{41}N_3O_{16}$ requires C, 49.8; H, 6.1; N, 6.2; total acetyl, 50.9%) and molecular-weight determinations (Mechrolab Vapor Pressure Osmometer, model 30/A), which on both samples gave 680 ± 5 in agreement with their formulation as bis-(2-acetamido-2-deoxy-D-glucopyranosyl)amine derivatives (mol.wt. 676).

1-*N*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-D-glucopyranosyl)-2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-D-glucopyranosylamines. A dry solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- α -D-glucopyranosyl bromide (VII) (1 g.) in chloroform (prepared by the method of Inouye *et al.* 1957) was added to a stirred suspension of the β -amine (II; R=H) (0.84 g.) and mercuric cyanide (1 g.) in nitromethane (20 ml.) and left overnight (Flowers & Jeanloz, 1963). Insoluble material was removed by filtration and the filtrate was concentrated to dryness. Examination by thin-layer chromatography revealed two components, having the same mobility as A_1 and A_2 . The two components were separated on a column of silica gel, and after recrystallization were found to be identical with A_1 and A_2 by comparison of melting points, infrared spectra and optical rotations.

Attempts to form derivatives of the secondary amine function in A_1 and A_2 by several methods proved to be unsuccessful. Examination of Courtauld space-filling models revealed that the group was too sterically hindered to allow introduction of bulky alkyl or aryl function.

1-*N*-(2-Acetamido-2-deoxy-D-glucopyranosyl)-2-acetamido-2-deoxy-D-glucopyranosylamine. A dry solution of A_1 , or A_2 (0.1 g.) in methanol, was cooled in an ice-salt bath, and a rapid stream of dry ammonia gas was passed through for 45 min. After allowing the solution to remain at 10° for 20 hr., it was concentrated to a syrup to remove excess of ammonia. Dissolution in methanol and addition of ether precipitated the bis-(2-acetamido-2-deoxy-D-glucopyranosyl)amine (0.057 g., 64%), m.p. 169–170°, $[\alpha]_D^{26} + 71^\circ$ (c 1.0 in methanol) (Found: C, 45.1; H, 7.1; N, 9.7; $C_{16}H_{29}N_3O_{10}$ requires C, 45.4; H, 6.9; N, 9.8%). The same product was obtained by using either A_1 or A_2 , or a mixture of the two as starting material, and sodium methoxide or lithium hydroxide for de-*O*-acetylation. The product gave only one spot on thin-layer chromatography in a variety of solvents.

1-*N*-(2-Acetamido-3,4,6-tri-*O*-benzoyl-2-deoxy-D-glucopyranosyl)-2-acetamido-3,4,6-tri-*O*-benzoyl-2-deoxy-D-glucopyranosylamines. 1-*N*-(2-Acetamido-2-deoxy-D-glucopyranosyl)-2-acetamido-2-deoxy-D-glucopyranosylamine (0.1 g.) was dissolved in dry pyridine (0.5 ml.), benzoyl chloride (0.36 g.) was then added and the mixture stirred at room temperature for 48 hr. A few drops of water were added and the mixture was poured with stirring into a cold dilute solution of sodium hydrogen carbonate. The syrupy product was extracted with chloroform (three times), washed consecutively with cold 2*N*-sulphuric acid, water, sodium hydrogen carbonate solution and water. The solution was dried (Na_2SO_4), filtered and evaporated to dryness. The residue (0.34 g.), on examination by silica-gel thin-layer chromatography (benzene-methanol; 200:1, v/v), showed two components. After separation by preparative thin-layer chromatography the *benzoates* had respectively m.p. 178–180°, $[\alpha]_D^{22.5} = -10^\circ$ (c 2.0 in chloroform), and m.p. 122–124°, $[\alpha]_D^{22.5} + 10^\circ$ (c 2.0 in chloroform) (Found: C, 66.8; H, 5.3; N, 3.9; and C, 66.6; H, 5.2; N, 3.9; $C_{58}H_{55}N_3O_{16}$ requires C, 66.5; H, 5.1; N, 4.0%).

Separation of α - and β -benzyl esters of *N*-benzyloxycarbonyl-L-aspartate. The mixture of benzyl esters produced on condensation of benzyl alcohol with *N*-benzyloxycarbonyl-L-aspartic anhydride was separated by counter-current distribution between aqueous 0.15% sodium carbonate and ethyl acetate. As reported by Marshall & Neuberger (1964), four compounds were isolatable, namely the free acid, the α - and β -monobenzyl esters and the $\alpha\beta$ -dibenzyl ester.

2-Acetamido-3,4,6-tri-O-acetyl-1-N-(α -benzyl-N-benzyloxycarbonyl- β -L-aspartyl)-2-deoxy- β -D-glucopyranosylamine (IIIa). This derivative was synthesized by combination of the amine (II, R=H) with the α -benzyl aspartyl ester derivative, according to the method of Marks *et al.* (1963) with the following modification. After removal of the bulk of the dicyclohexylurea by filtration, the filtrate was concentrated to dryness and the residue was washed repeatedly with ether, followed by column chromatography on silica gel. Elution with chloroform-acetone (2:1, v/v) gave (IIIa), which was recrystallized from a mixture of chloroform and ether (yield 43%), m.p. 220–221°, $[\alpha]_D^{25} + 17^\circ$ (c 2.0 in chloroform). These purification procedures were found to be absolutely necessary to ensure that the product was completely free from impurities. The corresponding β -benzyl-N-benzyloxycarbonyl- α -L-aspartyl derivative (IIIb) could be readily obtained by condensing the glycosylamine derivative (II; R=H) with β -benzyl-N-benzyloxycarbonyl-L-aspartate under identical conditions. It had m.p. 188–190°, $[\alpha]_D^{25} + 15^\circ$ (c 2.0 in chloroform) (Found: C, 58.0; H, 5.8; N, 6.2; C₃₃H₃₉N₃O₁₃ requires C, 57.8; H, 5.7; N, 6.1%).

2-Acetamido-1-N-(α - and β -L-aspartyl)-2-deoxy- β -D-glucopyranosylamine (Va; R=H; Vb). Method A. A solution of (IIIa) (0.13g.) in dry methanol (25ml.), to which had been added methanolic 1% sodium methoxide (0.1ml.), was stirred for 2 hr. at room temperature and then concentrated to dryness at room temperature. An aqueous solution of the residue was deionized by passage through a column of Amberlite IR-120 resin (H⁺ form). The eluate and washings were concentrated to about 1ml. at 40°, when 2-acetamido-1-N-(N-benzyloxycarbonyl- β -L-aspartyl)-2-deoxy- β -D-glucopyranosylamine (IVa) separated. On recrystallization from aqueous acetone, the monohydrate of (IVa) (0.06g., 69%) was obtained with m.p. 172–174° (decomp.) (Found: C, 49.2; H, 6.1; N, 8.85; loss at 100°, 3.6. C₂₀H₂₇N₃O₁₀.H₂O requires C, 49.3; H, 6.0; N, 8.6; H₂O, 3.6%). The use of either magnesium methoxide or lithium hydroxide for deesterification affected neither the yield nor the nature of the product.

An aqueous solution of the benzyloxycarbonyl derivative (IVa) was hydrogenated over 10% palladized charcoal for 2 hr. at room temperature. The catalyst was removed by filtration and the filtrate was evaporated to about 1ml. at 40°. Addition of ethanol (15–20ml.) caused the precipitation of the glycopeptide (Va; R=H), which on recrystallization from aqueous ethanol yielded the monohydrate (83%), m.p. 219–221° (decomp.), $[\alpha]_D^{25} + 24^\circ$ (c 1.0 in water) (Found: C, 40.9; H, 6.85; N, 11.6; loss at 100°, 5.0. Calc. for C₁₂H₂₁N₃O₈.H₂O: C, 40.8; H, 6.5; N, 11.9; H₂O, 5.1%).

Examination of this material by paper and thin-layer chromatography (butanol-acetic acid-water; 12:3:5, by vol.) revealed that a small amount of an impurity, staining blue with ninhydrin, was contaminating the major product, which gave a brown colour with ninhydrin. A pure product

was obtained by hydrogenation followed by de-O-acetylation (method B).

The analogous derivatives from β -benzyl-N-benzyloxycarbonyl-L-aspartate were similarly obtained and in corresponding yields. Thus 2-acetamido-1-N-(N-benzyloxycarbonyl- α -L-aspartyl)-2-deoxy- β -D-glucopyranosylamine (IVb) had m.p. 173–175° (decomp.), $[\alpha]_D^{25} + 5.2^\circ$ (c 1.0 in water) (Found: C, 49.4; H, 5.9; N, 8.5; loss at 100°, 3.5. C₂₀H₂₇N₃O₁₀.H₂O requires C, 49.3; H, 6.0; N, 8.6; H₂O, 3.6%), and 2-acetamido-1-N-(α -L-aspartyl)-2-deoxy- β -D-glucopyranosylamine (Vb) had m.p. 218–220° (decomp.), $[\alpha]_D^{25} + 25^\circ$ (c 1.0 in water) (Found: C, 40.7; H, 6.4; N, 11.6; loss at 100°, 4.9%; C₁₂H₂₁N₃O₈.H₂O requires C, 40.8; H, 6.5; N, 11.9; H₂O, 5.09%).

Method B. A solution of (IIIa) (0.38g.) in 90% acetic acid (50ml.) was hydrogenated over 10% palladized charcoal (0.08g.) for 2 hr. at room temperature. The catalyst was removed by filtration and the filtrate evaporated at 40° to dryness. The residue was taken up in a little water and again evaporated to dryness. This process was repeated several times to remove as much acetic acid as possible. The syrup thus obtained, when dried *in vacuo* at room temperature over phosphoric oxide and potassium hydroxide pellets, solidified, giving 2-acetamido-3,4,6-tri-O-acetyl-1-N-(β -L-aspartyl)-2-deoxy- β -D-glucopyranosylamine (VIa) (0.23g., 89%), which after crystallization had m.p. 212–214°, $[\alpha]_D^{21} + 11^\circ$ (c 1.0 in water) (Found: C, 46.65; H, 6.2; N, 9.1. Calc. for C₁₈H₂₇N₃O₁₁: C, 46.8; H, 5.9; N, 9.2%). Marshall & Neuberger (1964) reported m.p. 212–214°, $[\alpha]_D^{20} + 6^\circ$ (c 1.0 in water).

De-O-acetylation as described by Marshall & Neuberger (1964) yielded the monohydrate of (Va) (R=H) (0.145g., 95%), which had m.p. 219–221° (decomp.), $[\alpha]_D^{25} + 24^\circ$ (c 1.0 in water) (Found: C, 40.8; H, 6.7; N, 11.8; loss at 100° 4.9. Calc. for C₁₂H₂₁N₃O₈.H₂O: C, 40.8; H, 6.5; N, 11.9; H₂O, 5.1%).

The analogous derivatives from β -benzyl-N-benzyloxycarbonyl-L-aspartate were similarly obtained. Thus 2-acetamido-3,4,6-tri-O-acetyl-(α -L-aspartyl)-2-deoxy- β -D-glucopyranosylamine (VIb) had m.p. 207–209°, and $[\alpha]_D^{21} + 5^\circ$ (c 0.7 in water) (Found: C, 46.6; H, 6.15; N, 9.1; C₁₈H₂₇N₃O₁₁ requires C, 46.8; H, 5.9; N, 9.2%), and 2-acetamido-1-(α -L-aspartyl)-2-deoxy- β -D-glucopyranosylamine (Vb) had m.p. 218–220° (decomp.), $[\alpha]_D^{25} + 25^\circ$ (c 1.0 in water) (Found: C, 40.7; H, 6.4; N, 11.6; loss at 100°, 4.95. Calc. for C₁₂H₂₁N₃O₈: C, 40.8; H, 6.5; N, 11.9; H₂O, 5.1%).

The α -aspartyl (Vb) and β -aspartyl compounds (Va), on examination by paper chromatography and silica-gel thin-layer chromatography (butanol-acetic acid-water; 12:3:5 and 2:4:1, by vol.), were found to be homogeneous, giving only one spot, whereas method A showed an impurity staining blue with ninhydrin. The purity of glycopeptides (Va) and (Vb) (prepared by method B) was also confirmed by thin-layer (0.3mm.) electrophoresis on Avicel cellulose at 280v for 1–1½ hr. with a variety of buffers, namely: citrate, pH 3.4 (0.1M-citric acid adjusted to pH 3.4 with 0.1M-sodium citrate); acetate, pH 4.5 (0.2M-sodium acetate adjusted to pH 4.5 with 0.2N-acetic acid); phosphate, pH 7.4 (0.2M-Na₂HPO₄ adjusted to pH 7.4 with 0.2M-NaH₂PO₄); 5N-acetic acid. In each case only one spot was observed, brown for the β -aspartyl (Va) and purple for the α -aspartyl derivative (Vb), except in 5N-acetic acid when the latter gave a yellow colour.

2-Acetamido-1-N-(N-acetyl- β -L-aspartyl)-2-deoxy- β -D-glucopyranosylamine (Va; R=Ac). Compound (Va) (0.1 g.) was added to a fresh mixture of dry methanol and acetic anhydride (3:1, v/v; 25 ml.). Silver acetate (about 5 mg.) was added and the mixture stirred at room temperature for 24 hr. Silver salts were filtered off and the filtrate was evaporated to dryness at $<40^\circ$. The residue was left over potassium hydroxide pellets *in vacuo* to remove acetic anhydride, and the monohydrate of the N-acetyl derivative (Va; R=Ac) crystallized from a mixture of methanol and ether (0.06 g., 55.4%), m.p. $214\text{--}216^\circ$, $[\alpha]_D^{25} + 30^\circ$ (c 0.7 in methanol) (Found: C, 42.5; H, 6.7; N, 10.6; loss at 100° , 4.5. $C_{14}H_{23}N_3O_9 \cdot H_2O$ requires C, 42.5; H, 6.4; N, 10.6; H_2O , 4.55%).

RESULTS AND DISCUSSION

The synthesis of a model compound containing a unit of D-glucosamine and of L-aspartic acid linked through a β -aspartylglycosylamine bond involves several experimental problems, not least of which is the synthesis of pure 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy- β -D-glucopyranosylamine (II; R=H). Although this synthesis has been reported several times (Bolton & Jeanloz, 1963; Marks *et al.* 1963; Marshall & Neuberger, 1964; Tsukamoto *et al.* 1964; Yamamoto *et al.* 1965b) the reported physical properties differ considerably. The reason for these discrepancies could stem from either the use of impure starting material (namely, 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy- β -D-glucopyranosyl azide, I) or decomposition of the product by anomerization or some similar mechanism. Although replacement of the α -bromo group in 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy- α -D-glucopyranosyl bromide (VII) by the azide nucleophile is almost certain to give the β -azido anomer as product, no direct proof of this has so far been given. Examination of the nuclear-magnetic-resonance spectrum revealed a coupling constant between $H_{(1)}$ and $H_{(2)}$ of 9 cyc./sec., which is characteristic of a *trans* di-axial arrangement of hydrogen substituents at $C_{(1)}$ and $C_{(2)}$ of the preferred 'chair' conformation, thus revealing that the azide derivative formed is the β -anomer and therefore any impurity arising in the preparation of the amine (II; R=H) must have arisen at a later stage.

The preparation of the azido derivative has been studied under a variety of conditions. The use of silver azide in chloroform has led to serious accidents

(Marks *et al.* 1963). A study of the use of sodium azide in a variety of solvents was therefore undertaken. We have found that the use of acetonitrile or *NN*-dimethylformamide, with varying times of reaction, led to inferior yields of 1-azido derivative, as did the employment of the more stable glycosyl chloride (Leaback & Walker, 1957) instead of bromide (Inouye *et al.* 1957). In view of the considerably lower yields obtained by variation of the conditions, silver azide was finally resorted to, special care being taken in its preparation (Bertho & Revesz, 1955). Yamamoto *et al.* (1965a) have recently reported the use of sodium azide in formamide which gives a high yield (84%) of a material having m.p. $160\text{--}161^\circ$ and $[\alpha]_D^{30} - 40^\circ$ (c 1.0 in chloroform). The product obtained under our conditions, in a yield of 52%, had m.p. $166\text{--}168^\circ$, $[\alpha]_D^{24} - 60^\circ$ (c 2.0 in chloroform).

There have been several reports on the hydrogenation of the 1-azido derivative (I) to give the glycosylamine (II; R=H), with considerable diversity in the physical properties reported. Bolton & Jeanloz (1963) gave m.p. $225\text{--}230^\circ$ and $[\alpha]_D^{25} - 5.2^\circ$ (c 1.27 in chloroform); Marks *et al.* (1963) gave m.p. $152\text{--}153^\circ$ and $[\alpha]_D - 13^\circ$ (c 1.0 in chloroform); Marshall & Neuberger (1964) gave melting points ranging from 151° to 238° and $[\alpha]_D^{20}$ from -19.6° to -23.3° (c 1.5 in chloroform). Yamamoto *et al.* (1965a) have given m.p. $147\text{--}149^\circ$ and $[\alpha]_D^{30} - 14^\circ$ (c 1.0 in chloroform).

We have carried out extensive studies of this reaction. If the hydrogenation was carried out at room temperature and atmospheric pressure, in ethanolic solution, a high yield (78%) of the glycosylamine (II; R=H) was obtained, with m.p. 150° and $[\alpha]_D^{25} - 25.5^\circ$ (c 2.0 in chloroform). Examination of the reaction mixture by thin-layer chromatography on silica gel G (Merck) (detection by spraying with 10% sulphuric acid in ethanol and heating to 120°) revealed that the reaction was complete within 4 hr. If, during the concentration of the reaction mixture, the temperature was allowed to rise above a maximum of about 30° , further reaction of the amine took place, giving rise to two faster-moving spots on thin-layer chromatography. This conversion was optimum if the reaction mixture was heated, and, indeed, if the hydrogenation was carried out in ethyl acetate crystals of the converted material separated out from the hydrogenation mixture. Separation of the two new compounds could be readily achieved by careful fractional crystallization and silica column chromatography. These two products (A_1 and A_2) had different melting points (respectively 260° and $245\text{--}246^\circ$) and widely differing optical rotations: $[\alpha]_D^{23} - 32^\circ$ and $+44^\circ$ (c 2.0 in chloroform). Treatment of the rotation solutions with a trace of hydrogen chloride caused both compounds to mutarotate to

a final steady value $+30^\circ$ (after 2 hr.). Their analyses and infrared spectra were identical, thus indicating that they were anomers. The action of heat on the pure 1-amino derivative (II; R=H) in ethyl acetate, chloroform, methylene chloride etc. caused the evolution of ammonia, and the formation of A_1 and A_2 . The evidence therefore favoured their formulation as bisglycosylamines.

This supposition was confirmed by the unequivocal synthesis from 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- α -D-glucopyranosyl bromide (VII) and 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- β -D-glucopyranosylamine (II; R=H), by using mercuric cyanide as catalyst, in nitromethane solution (Flowers & Jeanloz, 1963). This reaction yielded A_1 and A_2 , separable as before, and identical in every way with the previously obtained products. Final confirmation of this structure came from a molecular-weight determination, which gave a value 680 ± 5 (calculated 676).

The configurations at the anomeric centres of the bisglycosylamines were examined by comparison of their molecular rotations [A_1 , $[M]_D - 21600$ (chloroform); A_2 , $[M]_D + 29700$ (chloroform)] with those calculated from Hudson's rules of isorotation (cf. Stanek, Cerny & Pacak, 1965), by using methyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- α -D-glucopyranoside, $[M]_D + 36172$ (chloroform) (Kuhn, Zilliken & Gauhe, 1953), and its β -anomer, $[M]_D - 8664$ (chloroform) (Inouye *et al.* 1957), and 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- β -D-glucopyranosylamine (II; R=H), $[M]_D - 8664$ (chloroform), as reference substances. The $\alpha\beta$ -isomers gave the contribution to the molecular rotation of each pyranosyl unit minus the anomeric centre (i.e. $2G = +27508$) and the glycosylamine (II; R=H) gave the contribution of the anomeric centres ($2A = +45154$).

Thus the molecular rotations were calculated for the $\alpha\alpha$ -isomer $= 2G + 2A = 72662$, the $\alpha\beta$ -isomer $= 2G = +27508$ and the $\beta\beta$ -isomer $= 2G - 2A = -17646$.

The values suggest that A_1 is the $\beta\beta$ -isomer (VIII) and that A_2 is the $\alpha\beta$ -isomer (IX), which is in agreement with conformational analysis since unfavourable non-bonded interactions in the $\alpha\alpha$ -form, due to interaction of the equatorial 2-acet-

amido group with the axial 1-glycosylamine group, are absent from the $\beta\beta$ -isomer.

De-*O*-acetylation of the $\beta\beta$ -isomer and the $\alpha\beta$ -isomer with sodium methoxide or methanolic ammonia gave what appeared to be only one product, but re-acetylation with acetic anhydride in pyridine or benzoylation with benzoyl chloride in pyridine gave both isomers. Anomerization of these bisglycosylamines and also of glycopeptides is an important and difficult matter that requires further study.

The basicity of the secondary amine function in such derivatives would be expected to be much reduced owing to the combined electron-withdrawing effects of the two ring oxygen atoms. In addition to this, examination of Courtault space-filling models reveals that the proton attached to the secondary nitrogen is very sterically hindered. A combination of the above two facts would suggest that the reactions of this nitrogen atom would be severely limited. Experimental proof of this derives from its inability to form any amide derivatives, including even the formamido compound. This lack of reactivity obviously means that any glycosylamine preparation containing a significant amount of bisglycosylamine would give a lower yield of glycopeptide derivative on reaction with α -benzyl-*N*-benzyloxycarbonyl-L-aspartate. This probably explains the low yield of glycopeptide obtained by the earlier procedures (cf. Bolton & Jeanloz, 1963).

Condensation of 2-acetamido-2-deoxy-D-glucosylamine and L-aspartic acid derivatives. In addition to the difficulty in isolating a pure glycosylamine derivative, the preparation of α -benzyl-*N*-benzyloxycarbonyl-L-aspartate has previously presented

some problems. The potentialities of counter-current distribution for such a separation were realized by Marshall & Neuberger (1964), who used a system employing ether and 0.1M-phosphate buffer. We have applied the same technique equally successfully by using ethyl acetate and aqueous 0.15% sodium carbonate solution. All possible products were isolated, namely the free acid, the α - and β -monobenzyl esters and, from the organic layer, the $\alpha\beta$ -dibenzyl ester.

Condensation of the pure glycosylamine (II; R=H) with α -benzyl *N*-benzyloxycarbonyl-L-aspartate was effected in dry methylene chloride in the presence of dicyclohexylcarbodi-imide. The normal methods for removal of the dicyclohexyl-urea side product involve simply filtration. This, we have found, never completely removes all the urea derivative. It was necessary always to wash the product with ether, and finally to submit the mixture to column chromatography to remove all the urea. Only after rigorous purification in this way could the pure glycopeptide (Va; R=H) be isolated.

Methods for the de-esterification of the fully substituted glycopeptide (IIIa) involve treatment with alkali at some stage. Under these conditions, acyl migrations are possible and often likely. Removal of protective groups in (IIIa) and (IIIb) by de-O-acetylation followed by hydrogenation gave an impure product (method A), but reversal of these procedures gave the pure glycopeptide (Va; R=H) (method B). Rearrangement of the α -benzyl ester derivative (IIIa) to the corresponding β -ester (IIIb) is conceivable under such conditions (Battersby & Robinson, 1955), though Marks *et al.* (1963) gave some chromatographic evidence that the β -ester (Vb) did not occur in their preparation, although some impurity was present. Marshall & Neuberger (1964) have now claimed to have isolated their glycopeptide (Va) free from contamination. We have now confirmed by an independent synthesis of 2-acetamido-1-*N*-(α -L-aspartyl)-2-deoxy- β -D-glucopyranosylamine (Vb) that it is not formed under the experimental conditions used and is not therefore the contaminating material. Yamamoto *et al.* (1965b) have given evidence which is at variance with our results.

We have also noted that the pure compound and the impurity in the glycopeptide preparation

(method A) (staining brown and purple respectively with ninhydrin) have similar electrophoretic mobilities and ninhydrin colours on paper to spots occurring in an ovalbumin glycopeptide preparation (Clamp & Hough, 1963, 1965).

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