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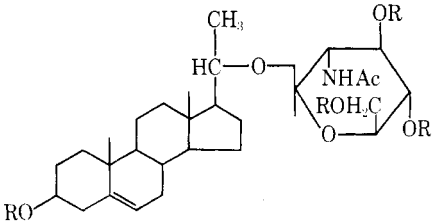
On the Configuration of Naturally Occurring Steroid *N*-Acetylglucosaminides*

Michio Matsui† and David K. Fukushima

ABSTRACT: Synthesis of 3β-hydroxy-Δ⁵-pregnen-20α-yl 2′-acetamido-2′-deoxy-β-D-glucopyranoside and the anomers of 3β-hydroxy-5α-pregnan-20α-yl 2′-acetamido-2′-deoxy-D-glucopyranoside has provided evidence for the assignment of the β configuration to C-1′ in 3β-hydroxy-Δ⁵-pregnen-20α-yl 2′-acetamido-2′-deoxy-D-glucopyranoside which was recently isolated from human urine by Arcos and Lieberman.

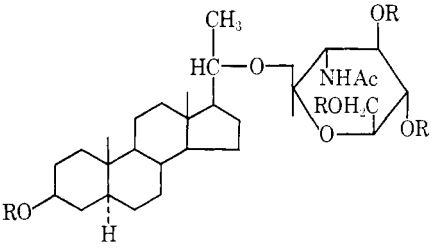
The synthesis of the anomeric pair of 2-acetamido-2-deoxy-D-glucopyranoside of C_{19} steroids conjugated at C-3 or at C-17 has been recently accomplished (Sauer *et al.*, 1969). Their physical properties were examined in order to determine whether the configuration of the glycoside linkage could be assigned from these studies. The results indicated that 3β-hydroxy-Δ⁵-pregnen-20α-yl 2′-acetamido-2′-deoxy-D-glucopyranoside isolated from human urine as the 3-sulfate ester by Arcos and Lieberman (1967) was not the α anomer as assigned by these authors but had the β-glycoside linkage. In order to confirm this conclusion the synthesis of 3β-hydroxy-Δ⁵-pregnen-20α-yl 2′-acetamido-2′-deoxy-β-D-glucopyranoside (Ib) was undertaken. Since the α anomer of this



Ia, R = Ac
Ib, R = H

conjugate could not be prepared by the method employed in the synthesis of C_{19} derivatives, the anomeric pair of the *N*-acetylglucosaminide of the saturated 5α-pregnane-3β,20α-diol was prepared for comparison purposes. The results firmly establish the β configuration of the *N*-acetylglucosaminide isolated from human urine.

It has been well established that the Koenigs–Knorr reaction leads almost exclusively to β-glycosides. *N*-Acetyl-β-D-glucosaminides of C_{19} steroids have recently been prepared by this method (Sauer *et al.*, 1969). Thus condensation of 5α-pregnane-3β,20α-diol 3-monoacetate and 1α-chloro-2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-D-glucopyranose with mercuric salts afforded 3β-acetoxy-5α-pregnan-20α-yl 2′-acetamido-2′-deoxy-3′,4′,6′-tri-*O*-acetyl-β-D-glucopyranoside (IIa). Transesterification with sodium methoxide readily



IIa, R = Ac
IIb, R = H

gave 3β-hydroxy-5α-pregnan-20α-yl 2′-acetamido-2′-deoxy-β-D-glucopyranoside (IIb). The β configuration of the glycoside linkage in these compounds was further established by their physical properties. The nuclear magnetic resonance spectrum (δ) of the tri-*O*-acetyl derivative IIa exhibited a doublet at 4.80, J = 8.5 cps for the anomeric H-1′ proton, and a broad multiplet at 4.17, $W_{1/2}$ = 8 cps. These values are consistent with those, 4.67–4.90 ppm, J = 8.5 cps, and 4.15–4.18 ppm, $W_{1/2}$ = 8 cps, found for the β anomer of the C_{19}-steroid conjugates. In addition, the molecular rotation difference between the conjugate and the steroid aglycone ΔM_D (β − steroid) was +9° for the free IIb and −1° for the tri-*O*-acetylated compound IIa. These values are in the range −91 to +51° calculated for the C_{19}-steroid *N*-acetyl-β-glucosaminides.

Anomerization of the tri-*O*-acetyl derivatives of the C_{19}-steroid *N*-acetyl-β-glucosaminides has been achieved in good

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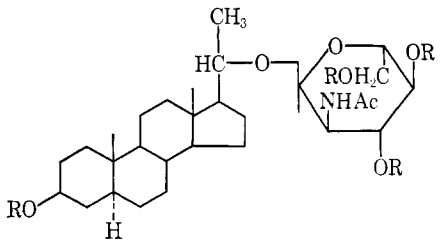
† Visiting Scientist, 1967–1969. On leave of absence from Tokyo Biochemical Research Institute, Japan.

yields with titanium tetrachloride. 3β-Acetoxy-5α-pregnan-20α-yl 2′-acetamido-2′-deoxy-3′,4′,6′-tri-*O*-acetyl-α-D-glucopyranoside (IIIa) was similarly prepared from its β anomer



IIIa, R = Ac
IIIb, R = H

IIa and transesterification afforded 3β-hydroxy-5α-pregnan-20α-yl 2′-acetamido-2′-deoxy-α-D-glucopyranoside (IIIb). The α configuration of the C-1′ was confirmed from nuclear magnetic resonance spectra and molecular rotations. In agreement with earlier studies with the C_{19} steroids, the H-1′ proton in the α anomer IIIa exhibited a doublet 4.95, J = 3.5 cps, further downfield and with a smaller coupling constant than in the β anomer IIa and the H-6′ methylene protons gave a narrower multiplet at 4.13, $W_{1/2}$ = 4 cps, than the β-glycoside. The α conjugates IIIa and IIIb had very high positive molecular rotations, +602 and +660°, much higher than that +21 and +61° for the β anomers IIa and IIb in accordance with Hudson's first principle. As a consequence the molecular rotation differences were very large positive values; ΔM_D (α − β) between the two anomers was +599° for the free and +580° for the acetylated derivative. These values were within the range found in the C_{19}-steroid *N*-acetylglucosaminides, +515 to +713° and +496 to +646°, respectively. In addition there was an intense band at 1128 and 1125 cm^{-1}, respectively, in the infrared spectra of IIIa and IIIb. The β anomers do not show this strong absorption in this region; these are replaced by several bands with less strong absorption. These differences in the infrared absorption spectra have been observed previously for the anomers of acetylated glycosides (Isbell *et al.*, 1957), glucosiduronates (Nitta *et al.*, 1961), and steroid *N*-acetylglucosaminides (Sauer *et al.*, 1969).

3β-Acetoxy-Δ⁵-pregnen-20α-yl 2′-acetamido-2′-deoxy-3′,4′,6′-tri-*O*-acetyl-β-D-glucopyranoside (Ia) was prepared from Δ⁵-pregnene-3β,20α-diol 3-monoacetate by the Koenigs–Knorr reaction. The nuclear magnetic resonance spectra of Ia had a doublet at 4.80 with a large coupling constant, J = 8.5 cps, and a broad multiplet at 4.17, $W_{1/2}$ = 7.5 cps, due to the H-1′ and H-6′ protons just as in the saturated 5α-pregnane *N*-acetyl-β-glucosaminide (IIa). Ia and its de-*O*-acetylated conjugate Ib had molecular rotation differences consistent with the β configuration of the glycosidic linkage, ΔM_D (β − steroid aglycone) was +3° and +22° for Ia and Ib, respectively. The α anomer could not be made by the titanium tetrachloride method and therefore no comparison between the anomers can be made. However the physical properties of Ia and Ib leave no doubt as to the assignment of the β configuration for these conjugates.

The assignment of the α configuration of the glycosidic bond in 3β-hydroxy-Δ⁵-pregnen-20α-yl 2′-acetamido-2′-deoxy-D-glucopyranoside isolated by Arcos and Lieberman (1967) was made on optical rotation measurements, the positive molecular rotation difference ΔM_D (conjugate − steroid) of +80°, and the absence of a Cotton effect in the optical rotatory dispersion spectrum with the curve becoming more positive at the lower wavelength. In the C_{19}-steroid series as well as in the present series, ΔM_D (β − steroid) generally are negative values but there are some with low positive values. The *N*-acetyl-β-glucosaminides of simple alcohols consistently have negative values (Zilliken *et al.*, 1955; Yoshimura *et al.*, 1964). It may be that the bulkier and more hindering steroid nucleus alters the conformation of the sugar ring to give a more positive rotation contribution to C-1′ so that the difference could result in a positive value. Since the ΔM_D for the conjugate isolated by Arcos and Lieberman was not as high a positive value as expected for an α anomer (around +600°), the conjugate must be the β anomer. The ΔM_D was not dissimilar to that of the synthetic 3β-hydroxy-Δ⁵-pregnen-20α-yl 2′-acetamido-2′-deoxy-β-D-glucopyranoside (Ib). The identity of the two compounds was confirmed by infrared spectrophotometry. Furthermore the optical rotatory dispersion spectra of the two compounds plotted as the molecular rotation difference between the conjugate and the steroid, ΔM_λ (β-steroid), were similar (Figure 1); the synthetic conjugate however exhibited a small negative Cotton effect with a trough at 230 mμ as demonstrated for methyl 2-acetamido-2-deoxy-β-D-glucopyranoside (Beychok and Kabat, 1965; Listowsky *et al.*, 1968). Since no direct comparison with the α anomer could be made, the ΔM_λ of the anomers of the saturated steroid conjugates IIb and IIIb was examined (Figure 1). The ΔM_λ (β − steroid) curve for the β anomer IIb was similar in shape to that of the Δ⁵-unsaturated conjugate Ib but was less positive; in fact most of the curve was negative. The ΔM_λ for the α anomer however had very high positive values and rose very sharply at the lower wavelength. Similar very high positive curves have been observed for the α anomers of the C_{19}-steroid *N*-acetylglucosaminide (unpublished data).

With the reassignment of configuration of 3β-hydroxy-Δ⁵-pregnen-20α-yl 2′-acetamido-2′-deoxy-β-D-glucopyranoside, all of the steroid *N*-acetylglucosaminides isolated from natural sources have the β-glycosidic linkage. In addition to the C_{21}-steroid conjugate, the *N*-acetyl-β-D-glucosaminide of two phenolic steroids from rabbit urine have been characterized; estradiol-17α conjugated at C-17 (Layne *et al.*, 1964; Collins *et al.*, 1967) and estriol-16α,17α conjugated at C-16 or -17 (Collins and Layne, 1968).

Experimental Section

Melting points were determined on a micro-hot stage and are corrected. Nuclear magnetic resonance spectra were obtained on a Varian A-60 instrument in $DCCl_3$ with $Si(CH_3)_4$ as internal standard; the chemical shifts are given in parts per million. Optical rotations were determined in methanol at 24°. The optical rotatory dispersion spectra were determined in methanol at 27° with a Cary Model 60 spectropolarimeter using a xenon arc, XBO-450 w/4 lamp. Cylindrical quartz cell with 1.0-mm path length was used. The slit width of the polarimeter was programmed for half-band-widths of less than 1.5 mμ through the entire spectral range. Infrared spectra were determined on a Beckman IR-9 spectrophotometer in potassium bromide dispersion; sh = shoulder, br = broad.

3β-Hydroxy-Δ⁵-pregnen-20α-yl 2′-Acetamido-2′-deoxy-β-D-glucopyranoside (Ib). A mixture of 500 mg of Δ⁵-pregnene-

3β,20α-diol 3-monoacetate (Hirschmann *et al.*, 1951) and 2.0 g each of 1α-chloro-2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-D-glucopyranose, mercuric cyanide, and mercuric bromide in 250 ml of anhydrous benzene was stirred at room temperature for 7 days. At 24-hr intervals additional 200 mg of the chloro sugar and of the mercuric salts was added. The solvent was evaporated from the reaction mixture. The residue was extracted with chloroform, washed with water, dried over sodium sulfate, and the solvent evaporated. The product was chromatographed on thin layer of silica gel GF in chloroform–acetone (10:1, v/v). The thin-layer chromatogram plates were sprayed with water to visualize the products. Elution of material with R_F 0.4 afforded 259 mg of Δ^5-pregnene-3β,-20α-diol 3-monoacetate. The fraction with R_F 0.1 yielded 976 mg containing the *O*-acetyl derivative Ia. Further purification by preparative thin-layer chromatography with ethyl acetate–cyclohexane (7:3, v/v) yielded 370 mg with R_F 0.3. Recrystallization from methanol gave 152 mg of *3β-acetoxy-Δ⁵-pregnen-20α-yl 2'-acetamido-2'-deoxy-3',4',6'-tri-O-acetyl-β-D-glucopyranoside* (*Ia*): mp 266–268°; $[\alpha]_D$ −25.8°; infrared spectrum 1695 (sh), 1658, 1545, 1173, 1132, 1120, and 1107 cm^{-1}. *Anal.* Calcd for $C_{37}H_{55}NO_{11} \cdot 0.5H_2O$: C, 63.57; H, 8.08; N, 2.00. Found: C, 63.64, H, 7.84; N, 1.99.

A solution of 86 mg of the tri-*O*-acetyl derivative Ia in 30 ml of 0.02 N sodium methoxide was allowed to stand at room temperature overnight. The solution was neutralized with the addition of Amberlite IR-120 (H^+) and filtered. The solvent was evaporated from the filtrate to give 66 mg of crystals. Recrystallization from aqueous methanol–ether gave *3β-hydroxy-Δ⁵-pregnen-20α-yl 2'-acetamido-2'-deoxy-β-D-glucopyranoside* (*Ib*): mp 276–280°; $[\alpha]_D$ −29.1°; infrared spectrum 1655, 1560, 1165, and 1115 cm^{-1}. All the absorption bands in its infrared spectrum coincided with that of the *N*-acetylglucosaminide isolated by Arcos and Lieberman (1967), mp 268–272°, $[\alpha]_D$ −7.0°. *Anal.* Calcd for $C_{29}H_{47}NO_7$: C, 66.77; H, 9.08; N, 2.68. Found: C, 66.92; H, 8.99; N, 2.63.

3β-Hydroxy-5α-pregnan-20α-yl 2'-Acetamido-2'-deoxy-β-D-glucopyranoside (*IIb*). A mixture of 1.0 g of 5α-pregnane-3β,20α-diol 3-monoacetate (Click and Hirschmann, 1962) and 4.0 g each of 1α-chloro-2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-D-glucopyranose and mercuric cyanide and mercuric bromide in 500 ml of anhydrous benzene was treated as above. The product was chromatographed on a column containing 500 g of silica gel. Elution with cyclohexane–ethyl acetate (1:1, v/v) afforded 526 mg of unreacted steroid. Further elution with cyclohexane–ethyl acetate (3:7, v/v) yielded the tetra-*O*-acetyl derivative IIa. Recrystallization from methanol gave 768 mg of *3β-acetoxy-5α-pregnan-20α-yl 2'-acetamido-2'-deoxy-3',4',6'-tri-O-acetyl-β-D-glucopyranoside* (*IIa*): mp 246–249°; $[\alpha]_D$ +3.0°; infrared spectrum 1690 (sh), 1665, 1540 (br), 1175, 1130, 1125, and 1108 cm^{-1}. *Anal.* Calcd for $C_{37}H_{57}NO_{11} \cdot 0.5H_2O$: C, 63.40; H, 8.34; N, 2.00. Found: C, 63.30; H, 8.21; N, 1.89.

3β-Hydroxy-5α-pregnan-20α-yl 2'-acetamido-2'-deoxy-β-D-glucopyranoside (*IIb*) was prepared from the acetyl derivative IIa by transesterification with sodium methoxide as above. Compound IIb melted at 284–288°; $[\alpha]_D$ +11.6°; infrared spectrum 1650, 1580, 1555 (sh), 1162, and 1108 cm^{-1}. *Anal* Calcd for $C_{29}H_{49}NO_7$: C, 66.51; H, 9.43; N, 2.67. Found: C, 66.21; H, 9.54; N, 2.57.

3β-Hydroxy-5α-pregnan-20α-yl 2'-Acetamido-2'-deoxy-α-D-glucopyranoside (*IIIb*). A solution of 200 mg of *3β-acetoxy-5α-pregnan-20α-yl 2'-acetamido-2'-deoxy-3',4',6'-tri-O-acetyl-β-D-glucopyranoside* (*IIa*) and 1.0 ml of titanium tetrachloride in 100 ml of chloroform was stored at room temperature for 30 hr. The chloroform solution was washed with dilute sodium bicarbonate and water, dried over sodium sulfate, and the solvent evaporated. The product was chomatographed on thin layer of silica gel GF with chloroform–acetone (10:1, v/v). The substance with R_F 0.3 was eluted and recrystallized from aqueous methanol to give 72 mg of *3β-acetoxy-5α-pregnan-20α-yl 2'-acetamido-2'-deoxy-3',4',6'-tri-O-acetyl-α-D-glucopyranoside* (*IIIa*): mp 110–115°; $[\alpha]_D$ +87°; infrared spectrum 1690, 1540, 1520 (sh), and 1128 cm^{-1}. *Anal.* Calcd for $C_{37}H_{57}NO_{11}$: C, 64.22; H, 8.31; N, 2.02. Found: C, 64.07; H, 8.15; H, 1.92.

3β-Hydroxy-5α-pregnan-20α-yl 2'-acetamido-2'-deoxy-α-D-glucopyranoside (*IIIb*) was obtained from the tetra-*O*-acetyl derivative IIIa by transesterification with sodium methoxide as above. Compound IIIb melted at 328–331°; $[\alpha]_D$ +122°; infrared spectrum 1650 (sh), 1625, 1568, 1545 (sh), and 1125 cm^{-1}. *Anal.* Calcd for $C_{29}H_{49}NO_7 \cdot H_2O$: C, 64.29; H, 9.49; N, 2.58. Found: C, 63.78; H, 9.23; N, 2.46.

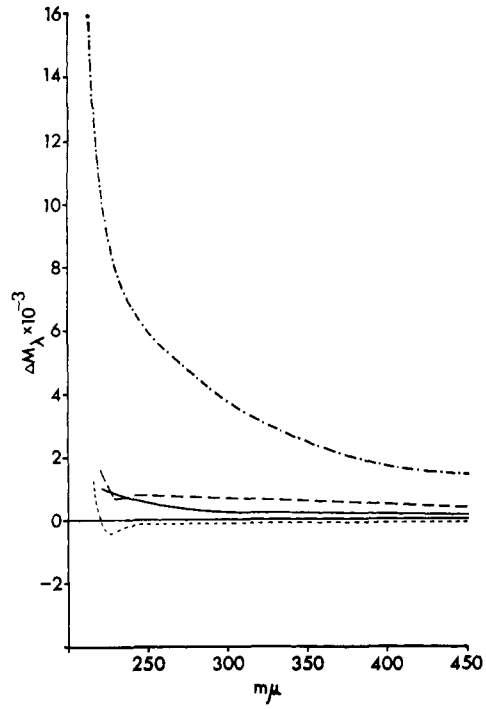


FIGURE 1: Molecular rotation difference curves, ΔM_λ, between the steroid *N*-acetylglucosaminide and the steroid. (– – –) 3β-Hydroxy-Δ⁵-pregnen-20α-yl 2'-acetamido-2'-deoxy-β-D-glucopyranoside (Ib); (—) 3β-hydroxy-Δ⁵-pregnen-20α-yl 2'-acetamido-2'-deoxy-D-glucopyranoside (Arcos and Lieberman, 1967); (- - -) 3β-hydroxy-5α-pregnan-20α-yl 2'-acetamido-2'-deoxy-β-D-glucopyranoside (IIb); (—·—·) 3β-hydroxy-5α-pregnan-20α-yl 2'-acetamido-2'-deoxy-α-D-glucopyranoside (IIIb).

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Hemoglobin Messenger Ribonucleic Acid. Synthesis of 9S and Ribosomal Ribonucleic Acid during Erythroid Cell Development*

Michael J. Evans† and Jerry B. Lingrel‡

ABSTRACT: When reticulocyte polysomes are dissociated with sodium dodecyl sulfate and analyzed by sucrose density gradient centrifugation, a ribonucleic acid of 9 S (9S ribonucleic acid) is observed. This ribonucleic acid exhibits many of the properties expected for the hemoglobin messenger ribonucleic acid, such as size, per cent of total ribonucleic acid, and involvement in polysomal structure.

Because of these unique features it was of interest to study its synthesis in relation to ribosomal ribonucleic acid synthesis during erythroid cell development. This was accomplished by injecting [^{3}H]uridine into anemic mice at various times prior to collecting the circulating reticulocytes. Cells obtained shortly after injection of the ribonucleic acid precursor are those reticulocytes originating from more mature nucleated erythroid cells while those reticulocytes obtained at longer times originate from more immature precursor cells. The data indicate that ribosomal ribonucleic acid is synthesized early in erythroid cell development while 9S ribonucleic acid synthesis is maximal in later cells.

Rabbit reticulocyte polysomes contain a 9S RNA exhibiting many of the properties expected for the hemoglobin mRNA (Marbaix and Burny, 1964; Burny and Marbaix, 1965; reviewed by Chantrenne *et al.*, 1967). Further support for this RNA being the hemoglobin mRNA has been obtained in our laboratory by showing that only one 9S RNA occurs per polysomal structure irrespective of the number of ribosomes in the polysome (Evans and Lingrel, 1969). Since polysomes are thought to be two or more ribosomes held together by a single mRNA molecule, the observation that various size polysomes contain a single 9S RNA is strong evidence in favor of this RNA being the hemoglobin mRNA.

The synthesis of 9S RNA in erythroid cells has been studied by Marbaix and Burny (1964). These workers have observed that the 9S RNA exhibits a specific activity higher than rRNA when rabbits were injected with ^{32}P 10–20 hr prior to removal of reticulocytes. This finding was interpreted to indicate that the 9S RNA was either rapidly turning over or that more 9S RNA than rRNA was synthesized after ^{32}P injection. As reticulocytes contain a stable mRNA (Marks *et al.*, 1962a,b; Burny and Chantrenne, 1964) it occurred to us that the 9S RNA was

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