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METHYL DERIVATIVES OF 2-ACETAMIDO-2-DEOXY-3-*O*-(β-D-GLUCOPYRANOSYLURONIC ACID)-D-GLUCOSE (HYALOBIOURONIC ACID) FROM METHYLATED HYALURONIC ACID*

ROGER W. JEANLOZ
Laboratory for Carbohydrate Research, Department of Biological Chemistry and Medicine, Harvard Medical School, and the Massachusetts General Hospital, Boston, MA 02114 (U.S.A.)
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ABSTRACT

Methanolysis of methylated hyaluronic acid, followed by acetylation, gave, in 70% yield, crystalline methyl 2-acetamido-2-deoxy-4,6-di-*O*-methyl-3-*O*-(methyl 4-*O*-acetyl-2,3-di-*O*-methyl-β-D-glucopyranosyluronate)-α-D-glucopyranoside. Removal of the *O*-acetyl and methyl ester groups gave compounds that are useful in the investigation, by ^{1}H-n.m.r. spectroscopy, of interaction within chains of hyaluronic acid in solution.

INTRODUCTION

Present studies by n.m.r. spectroscopy of the interaction between 2-acetamido-2-deoxy and uronic residues within the hyaluronic acid chain in solution[1,2], and by g.l.c.–m.s. of hyalobiuronic acid derivatives for the identification of hyaluronic acid, have renewed interest in derivatives of hyalobiouronic acid, the disaccharide corresponding to the repeating unit of hyaluronic acid. The methyl esters described herein were obtained about 30 years ago in a study of the structure of hyaluronic acid by methylation[3], which confirmed[4] the results of enzymic degradation obtained by Meyer and associates[5].

RESULTS AND DISCUSSION

Hyaluronic acid obtained from human umbilical cord[6] was methylated by the Haworth procedure[3,7]. Although this procedure is more time-consuming than the methods introduced later, which are based on methylsulfinyl sodium[8] or on solution in *N*,*N*-dimethylformamide[9], it presents the advantage of avoiding *N*-methylation

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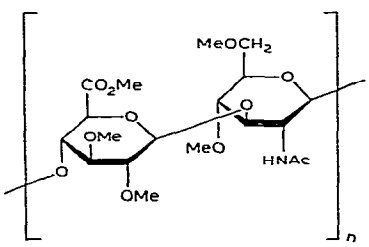
(which inhibits the methanolysis of methylated polysaccharides[8], and results in mixtures of *N*-methylated and *N*-acetylated compounds[10]) and degradation of the polymer[11]. The methyl ester of methylated hyaluronic acid (**1**) was methanolyzed with 5% hydrogen chloride in methanol for 24 h. Increase of the time of reaction to 72 h did not appreciably change the yields of the products obtained, probably because the concentration of hydrogen chloride decreased rapidly. The resulting syrupy product was acetylated with acetic anhydride and pyridine, and the acetates were chromatographed on a column of silica gel, to give a crystalline fraction in 84% yield. Several recrystallizations gave, in 45% yield, methyl 2-acetamido-2-deoxy-4,6-di-*O*-methyl-3-*O*-(methyl 4-*O*-acetyl-2,3-di-*O*-methyl-β-D-glucopyranosyluronate)-α-D-glucopyranoside (**2**) having a double m p. between 105 and 155°. The α-D configuration was attributed to the 2-acetamido-2-deoxy-D-glucose residue (*a*) on the basis of the stereoselectivity of the glycosylation of 2-acetamido-2-deoxy-D-glucose[12], (*b*) by analogy with the results of the methanolysis of the nonmethylated compound[13], and (*c*) on the basis of the positive optical rotation.



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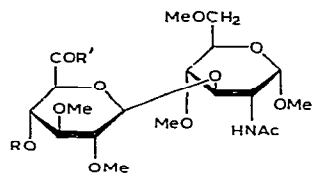
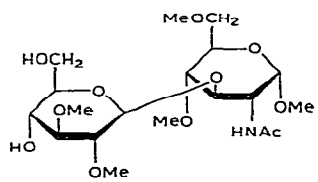


2 R = Ac, R' = OMe
3 R = H, R' = OMe
4 R = H, R' = OH
5 R = Me, R' = OMe
6 R = H, R' = NH_2
7 R = Me, R' = NH_2



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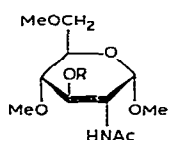
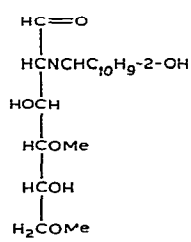


9 R = H
10 R = Ac



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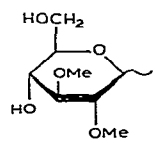

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Crude **1** was treated with barium methoxide in methanol at 0°, and then with diazomethane, to give, in 85% yield, a sharp-melting compound corresponding to methyl 2-acetamido-2-deoxy-4,6-di-*O*-methyl-3-*O*-(methyl 2,3-di-*O*-methyl-β-D-glucopyranosyluronate)-α-D-glucopyranoside (**3**). Treatment of **3** with barium methoxide,

TABLE I

^{1}H-N M R. DATA[a] FOR COMPOUNDS 2–4 AND 7

Compound	*Chemical shifts (δ) (J in Hz)*					
	H-1 ($J_{1,2}$)	*H-1′* ($J_{1',2'}$)	*NHAc*	*OMe*	*NHAc*	*Others*
2	4.76d (3 4)	4.40d (7 7)	5 63d	3 54s, 3.523s, 3 519s, 3 42s, and 3 33s	2.01s	3.71s (CO_2Me), and 2 08s (OAc)
3	4.78d (3.4)	4 43d (7.4)	5.71d	3.63s, 3.52s, 3 51s, 3 42s, and 3 33s	2 01s	3.81s (CO_2Me)
4	4 67d (3.5)	4.42d (7 7)	5 62d	3 65s, 3 52s, 3 50s, 3 43s, and 3 37s	2 04s	
7	4.68d (3 4)	4 45d (7 4)	6 05d	3 60s, 3 55s, 3 51s, 3 50s, 3.43s, and 3 37s	2 03s	6 94bs and and 5 60bs ($CONH_2$)

[a]At 270 MHz, for solutions in chloroform-*d*

but without treatment with diazomethane, gave, in 73% yield, the free acid **4**. Methylation of **3** with the Purdie reagents[14] gave, in good yield, the methyl ester of the fully methylated disaccharide **5**. Treatment of **3** with ammonia in methanol gave the crystalline amide **6**, and the same crystalline amide was obtained directly from crude **2** in 90% yield, thus confirming that **2** did not contain a mixture of 2-acetamido-2-deoxy-α- and -β-D-glucopyranosyl residues. Similarly, the fully methylated disaccharide **5** was characterized by the crystalline amide **7**.

Because the low yield of the recrystallization, and the double melting-point of **2**, might suggest[12] the presence of some β-D anomer in the crystalline product, or a mixture of compounds containing various interglycosidic linkages, compound **2** and the derivatives **3**, **4**, and **7** were re-investigated recently by ^{1}H-n.m.r. spectrometry (see Table I). In the spectra of the four compounds, only one signal, at δ 4 67–4.78, showed $J_{1,2}$ 3.4–3.5 Hz, and one signal, at δ 4.40–4.45, for H-1′ showed $J_{1',2'}$ 7.4–7.7 Hz, which were attributed to α- and β-D-glycoside bonds, respectively, and no β-D-glycoside bond at C-1 was observed.

In order to establish the structure of **2** and **3**, and, consequently, of hyaluronic acid, **2** or **3** was reduced with sodium borohydride, and then methanolyzed to give, in 57% yield, methyl 2-acetamido-2-deoxy-4,6-di-*O*-methyl-α-D-glucopyranoside (**9**), characterized by the crystalline 3-acetate[15] **10**. The same compound was obtained by prolonged methanolysis of **1**. Acid hydrolysis of **9** gave 2-amino-2-deoxy-4,6-di-*O*-methyl-D-glucose, characterized by the crystalline, 2-hydroxynaphthaldehyde Schiff base[15] **11**. Reduction of **3** with lithium borohydride in oxolane led, in 83% yield, to

crystalline methyl 2-acetamido-2-deoxy-4,6-di-*O*-methyl-3-*O*-(2,3-di-*O*-methyl-β-D-glucopyranosyl)-α-D-glucopyranoside (**8**), which was hydrolyzed with sulfuric acid to give 2,3-di-*O*-methyl-D-glucose (**12**), identified by electrophoresis[16].

The isolation of **3** in 60% yield, and of **2** in 70% yield, indicates that most, if not all, of the linkages in hyaluronic acid are alternatively β-D-(1→3) and -(1→4). The degree of methylation of the starting material **1** was only 4.55 methoxyl groups per disaccharide unit (theoretical value: 5.0 groups), suggesting a maximum yield of 55% of **2**. However, if underesterification of the carboxyl group is taken into consideration, and is estimated to be similar to that of hyaluronic acid[3] (calc. 7.9; found 5.35), a degree of methylation of 3.85 ether methoxyl groups per disaccharide unit (theoretical value: 4.0 groups) is calculated, corresponding to a yield of 85% of **2**. The yields of **2** and **3** obtained are well within the two extreme values of 55 and 85%, and suggest random undermethylation.

EXPERIMENTAL

General methods. — Melting points were determined on a hot stage equipped with a microscope, and correspond to "corrected melting points". Rotations were determined with a polarimeter equipped with a Rudolph photoelectric polarimeter attachment Model 200, or with a Perkin–Elmer No. 141 polarimeter. The chloroform used was A.R. grade and contained ~0.75% of ethanol. I.r. spectra were recorded, for potassium bromide discs, with a Perkin–Elmer spectrophotometer Model 237. N.m.r. spectra were recorded with a Bruker HX-270 spectrometer for solutions in chloroform-*d* containing tetramethylsilane as the internal standard. Column chromatography was performed on "Silica Gel Davison" (grade 950; 60–200 mesh), from the Davison Co., Baltimore, MD 21201, which was used without pretreatment. All mixtures of solvents are v/v. The microanalyses were performed by Dr. Alicino, New Jersey; Drs Ritter, Weiser, and Strauss, Zurich, Switzerland; and Dr. Manser, Zurich, Switzerland.

Methyl 2-acetamido-2-deoxy-4,6-di-O-methyl-3-O-(methyl 4-O-acetyl-2,3-di-O-methyl-β-D-glucopyranosyluronate)-α-D-glucopyranoside (**2**) *by methanolysis of methylated hyaluronic acid methyl ester* (**1**) — To methylated hyaluronic acid methyl ester[3] {0.88 g; OCH_3, 31.9%; $[\alpha]_D^{24}$ −51° (*c* 0.53, methanol)} was added 5% hydrogen chloride in methanol (40 mL), and the solution was boiled for 20 h under reflux, with protection from moisture. After evaporation, the residue was dried by several additions and evaporations of methanol, and then of toluene. The residue was treated overnight with pyridine (5 mL) and acetic anhydride (3 mL). The dark-red solution was poured into ice–water, extracted with chloroform (3 times), and the extract successively washed with dilute sulfuric acid and sodium hydrogencarbonate, dried (Na_2SO_4), and evaporated, to give a residue (1.26 g) which was chromatographed on silica gel. Elution with dichloroethane–ether, ether, ether–ethyl acetate, and 49:1 ethyl acetate–acetone gave syrupy fractions (0.17 g) that were not further investigated. Elution with 19:1, 9:1, 4:1, and 2:1 ethyl acetate–acetone gave crystalline fractions

(0.83 g, 84%). Elution with 1:1 ethyl acetate–acetone, acetone, and methanol gave syrupy fractions (0.07 g) that were not further investigated. Recrystallization from ether–pentane gave **2** as prisms (0.57 g, 45%), m.p. 95–115°, being transformed into long needles, m.p. 149–153°, $[\alpha]_D^{25}$ +65° (*c* 0.93, chloroform); ν_{max}^{KBr} 3295 (NH), 2855 (Me C-H), 1770, 1755 (ester C=O), 1655 (Amide I), 1570–1560 (Amide II), 1380 (Me C-H), and 1300 and 1240 cm^{-1} (C=O); for ^{1}H-n m r. data, see Table I.

Anal. Calc. for $C_{22}H_{37}NO_{13}$: C, 50.47; H, 7.12; N, 2 68; Ac, 16.44; OCH_3, 35.57; mol. wt. 523. Found: C, 50.68; H, 7.16; N, 2.37; Ac, 16.52; OCH_3, 35 74; mol. wt. (Rast) 542.

Methyl 2-acetamido-2-deoxy-4,6-di-O-methyl-3-O-(methyl 2,3-di-O-methyl-β-D-glucopyranosyluronate)-α-D-glucopyranoside (**3**). — To a solution of crude **2** (300 mg) in methanol (10 mL) was added 0.4M barium methoxide in methanol (2 mL). The solution was kept overnight at 0°, and then passed through a column of Amberlite IR-120 (H^+) cation-exchange resin. The resin was extensively eluted with 1:1 methanol–water, and the combined eluates were evaporated, to give a crystalline residue. In order to esterify any free carboxyl group that might have been formed by de-esterification during the processing, the residue was treated for 5 min with a 5% solution of diazomethane in ether. After evaporation, the residue (277 mg, 100%) was dissolved in ethyl acetate, and chromatographed on silica gel Elution with 19:1, 4:1, 3:1, 2:1, and 1:1 ethyl acetate–acetone gave crystalline fractions (230 mg, 85%) Recrystallization from acetone–ether gave **3** (165 mg, 60%), m p. 157–158°, $[\alpha]_D^{20}$ +74° (*c* 1.05, chloroform); ν_{max}^{KBr} 3445, 3400 (OH), 3280 (NH), 2855 (Me C-H), 1760, 1740 (ester C=O), 1665, 1640 (Amide I), 1565, 1485 (Amide II), 1380 (Me C-H), and 1300 cm^{-1} (C=O); for ^{1}H-n.m r. data, see Table I

Anal. Calc. for $C_{20}H_{35}NO_{12}$: C, 49.89; H, 7.33; N, 2.91; OCH_3, 38 67. Found· C, 49.70; H, 7.46; N, 2.87; OCH_3, 38.35.

Acetylation of **3** (m.p. 157–158°) with acetic anhydride–pyridine, and processing in the usual way, gave in nearly quantitative yield, compound **2** having an indefinite m.p. between 116° and 146°.

Methyl 2-acetamido-2-deoxy-4,6-di-O-methyl-3-O-(2,3-di-O-methyl-β-D-glucopyranosyluronic acid)-α-D-glucopyranoside (**4**). — A solution of **2** (395 mg) was treated with barium methoxide as described for the preparation of **3**. After passage through the column of cation-exchange resin, and evaporation, the crystalline residue was recrystallized from acetone–ether, to give thick prisms (258 mg, 75%), m p. 198–199°, $[\alpha]_D^{25}$ +54° (*c* 0.82, methanol); ν_{max}^{KBr} 3390 (OH), 3310 (NH), 2850 (Me C-H), 1690 (CO_2H), 1615–1600 (C=O), 1555 (Amide II), 1375 (Me C-H), and 1300, 1275, and 1255 cm^{-1} (C=O); for ^{1}H-n.m.r. data, see Table I.

Anal. Calc. for $C_{13}H_{33}NO_{12}$: C, 48.82; H, 7.12; OCH_3, 33.12. Found. C, 48.42; H, 7.45; OCH_3, 33.15.

Esterification of **4** (105 mg) with 1% diazomethane in methanol for 10 min, followed by evaporation, gave 92 mg (85%) of **3**, m.p. 157–158°, showing no depression of the m.p. with the compound described in the preceding paragraph

Methyl 2-acetamido-2-deoxy-4,6-di-O-methyl-3-O-(methyl 2,3,4-tri-O-methyl-

β-D-glucopyranosyluronate)-α-D-glucopyranoside (**5**). — Compound **3** (30 mg) was treated overnight under reflux with methyl iodide (5 mL) and silver oxide (100 mg). The mixture was filtered through a pad of charcoal (Darco G-60)–Celite, the solid residue washed with acetone, and the filtrates were combined, and evaporated. The crystalline residue was recrystallized from acetone–ether–pentane, to give short prisms (22 mg, 70%), m.p. 175–176°, $[\alpha]_D^{25}$ +70° (*c* 0.85, chloroform)

Anal. Calc. for $C_{21}H_{37}NO_{12}$: C, 50.90; H, 7.53; OCH_3, 43.84. Found: C, 50.93; H, 7.57, OCH_3, 43.20.

Methyl 2-acetamido-2-deoxy-3-O-(2,3-di-O-methyl-β-D-glucopyranosyluronamide)-4,6-di-O-methyl-α-D-glucopyranoside (**6**). — (*a*) *From* **3**. A solution of **3** (10.4 mg) in methanol (1 mL) was saturated at 0° with ammonia, and then kept for 48 h at room temperature. Evaporation gave a crystalline residue that was recrystallized from acetone–ether, to give **6** as elongated prisms (9.6 mg, 95%), m.p. 261–263°, $[\alpha]_D^{25}$ +75° (*c* 0.87, methanol)

Anal. Calc. for $C_{19}H_{34}N_2O_{11}$: C, 48.92; H, 7.35; N, 6.01. Found: C, 48.93; H, 7.23; N, 6.10.

(*b*) *From* **2**. An identical treatment of **2** (20 mg) with methanolic ammonia gave 16 mg (90%) of **6**, m.p 261–263°, mixed m p. with the compound described under (*a*) 260–262°.

Methyl 2-acetamido-2-deoxy-4,6-di-O-methyl-3-O-(2,3,4-tri-O-methyl-β-D-glucopyranosyluronamide)-α-D-glucopyranoside (**7**). — Treatment of **5** (27 mg) with methanolic ammonia, as described for the preparation of **6**, gave 24 mg (92%) of needles, m.p. 212–215°, resolidifying, and second m.p. 224–228°, $[\alpha]_D^{20}$ +43° (*c* 0 09, chloroform); ν_{max}^{KBr} 3420, 3290, 3205 (NH), 2850 (Me C-H), 1685, 1640 (Amide I), 1565 (Amide II), 1380 (Me C-H), and 1305 and 1240 cm^{-1} (C=O); for ^{1}H-n.m.r. data, see Table I.

Anal. Calc. for $C_{20}H_{36}N_2O_{11}$: C, 49.99; H, 7.55; N, 5.83. Found: C, 50.01; H, 7.49; N, 5.96.

Methyl 2-acetamido-2-deoxy-3-O-(2,3-di-O-methyl-β-D-glucopyranosyl)-4,6-di-O-methyl-α-D-glucopyranoside (**8**). — A solution of **3** (160 mg) in 0.5M lithium borohydride in oxolane (125 mL) was kept for 24 h at room temperature. Iced water was added, and the solution was acidified with 5% acetic acid, and passed through a column of Dowex 50 (H^+) cation-exchange resin. After evaporation of the eluate, the residue was dried by several additions and evaporations of absolute ethanol. The crystalline residue was recrystallized from acetone–ether, to give thick prisms (126 mg, 83%), m p. 148–149°, $[\alpha]_D^{23}$ +81° (*c* 0.91, chloroform).

Anal. Calc. for $C_{19}H_{35}NO_{11}$: C, 49.80; H, 7.78. Found: C, 50.19; H, 7.72.

Hydrolysis of **8** with M sulfuric acid for 10 h at 100°, followed by treatment with Dowex-1 X-8 (AcO^-) and Dowex-50 X-8 (H^+) ion-exchange resins, and evaporation, gave a residue that was analyzed by paper electrophoresis in borate buffer[16]; it migrated like an authentic specimen of 2,3-di-*O*-methyl-D-glucose (**12**).

Methyl 2-acetamido-2-deoxy-4,6-di-O-methyl-α-D-glucopyranoside (**9**). — A solution of **3** (320 mg) in water (5 mL) was treated with a solution of sodium boro-

hydride (200 mg) in water (5 mL) for 2 h at room temperature. After acidification to pH 6.2 with 0.1M acetic acid, the solution was diluted with water (50 mL), and passed through a column of Dowex 50 (H^+) cation-exchange resin. The eluate was evaporated, and the residue boiled under reflux for 2 h with a 5% solution of hydrogen chloride in methanol (5 mL). After neutralization of the acid with silver carbonate, filtration through Darco G-60 charcoal–Celite, and evaporation of the filtrate, the residue was chromatographed on silica gel. Elution with ethyl acetate–acetone gave fractions (55 mg) having m.p. >165°. Recrystallization from methanol–ether–pentane gave long needles, m.p. 205–206°, $[\alpha]_D^{25}$ +157° (*c* 0.17, methanol), showing no depression of m.p. in admixture with authentic[15] **9**; ν_{max}^{KBr} 3290, 2960, 2850, 1630 (Amide I), 1550, (Amide II), 1450, 1380, 1200, 1140–1125, 1050, 970, 955, 925, and 895 cm^{-1}, identical with the i.r. data for authentic[17] **9**.

Anal. Calc. for $C_{11}H_{21}NO_6$. C, 50.18; H, 8.04; N, 5.32; OCH_3, 35.36. Found: C, 50.20; H, 8.06; N, 5.28; OCH_3, 35.44

Compound **9** was acetylated with acetic anhydride and pyridine in the usual way, to give methyl 2-acetamido-3-*O*-acetyl-4,6-di-*O*-methyl-α-D-glucopyranoside (**10**), m.p. 108–109°, showing no depression of m.p. in admixture with an authentic sample[15].

Anal. Calc for $C_{13}H_{23}NO_7$· C, 51.14; H, 7.59. Found: C, 51.13; H, 7.67.

Hydrolysis of **2** (10 mg) with 5M hydrochloric acid for 8 h under reflux, followed by evaporation, and treatment with 2-hydroxynaphthaldehyde, gave, after chromatography on silica gel, with elution with acetone and 9:1 acetone–methanol, crystalline fractions that were recrystallized from methanol–ether–pentane, to give 5.7 mg (75%) of the 2′-hydroxynaphthylidene derivative **11**, m.p. 186–188°, showing no depression of the m.p. in admixture with an authentic sample[15].

ACKNOWLEDGMENTS

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