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Note

Synthesis of *p*-nitrophenyl 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosidonic acid, a chromogenic substrate for sialidases

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Aryl α-glycosides of sialic acid are useful chromogenic substrates for tracing and quantifying sialidase [EC 3.2.1.18] in biological materials, but methods for their stereospecific synthesis are limited. Privalova and Khorlin[1] described the silver carbonate-promoted glycosidation of *p*-nitrophenol with methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosyl chloride)onate (**1**) in chloroform in the presence of Drierite followed by *O*-deacetylation and saponification. In our hands, this procedure, which approaches the usual conditions of the Koenigs–Knorr reaction[2], resulted in elimination rather than substitution, and the major product was methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonate[3].

Zurabyan *et al.*[4] have described the reaction of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride with sodium *p*-nitrophenoxide in *N,N*-dimethylformamide, which gave *p*-nitrophenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside. Myers *et al.*[5] have extended this procedure to the synthesis of 4-methylcoumarin-7-yl 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosidonic acid.

When the glycosyl chloride (**1**) of methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-α-D-*glycero*-D-*galacto*-nonulopyranosonate reacted with sodium *p*-nitrophenoxide in *N,N*-dimethylformamide, 57% of the glycoside **2** was obtained after column chromatography. The $[\alpha]_D^{25}$ value [+46° (methanol)] for **2** contrasts with that (+4.2°) reported by Privalova and Khorlin[1]. Zemplén-deacetylation of **2** gave 71% of crystalline methyl (*p*-nitrophenyl 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosid)onate (**3**) which, on saponification, gave the title glycoside **4**. The $[\alpha]_D^{25}$ value [+69° (methanol)] indicated **4** to be α and this was supported by the chemical shift (δ 2.8) for the signal of H-3*e* which is diagnostic[6] for the α-configuration.

Incubation of **4** with *Vibrio cholerae* sialidase gave *N*-acetyl-D-neuraminic

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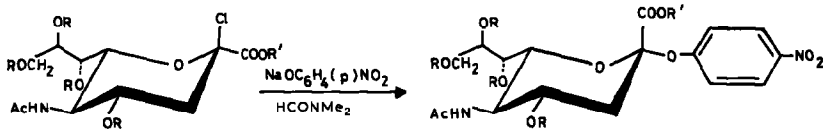

1 R = Ac, R' = Me

2 R = Ac, R' = Me
3 R = H, R' = Me
4 R = H, R' = H

acid and *p*-nitrophenol. The hydrolysis was complete within 12 h in agreement with the assigned α-configuration. The amount of *p*-nitrophenol released was consistent with the formula $C_{17}H_{22}N_2O_{11}$ for **4**. From a Lineweaver–Burk plot, a K_m value of 2.38×10^{-3} mol/L at pH 5.5 was obtained for the enzymic hydrolysis. The glycoside **4** was also a good substrate for the sialidases from *Clostridium perfringens* and *Arthrobacter ureafaciens*, and was cleaved quantitatively by each enzyme.

Most assays for sialidases use sialic acid α-glycosides and are based on the determination of the liberated *N*-acetyl-D-neuraminic acid, but for **4**, liberated *p*-nitrophenol can be conveniently determined spectrophotometrically.

EXPERIMENTAL

General. — Solutions were concentrated under reduced pressure at 40° (bath). Sodium *p*-nitrophenoxide dihydrate was dried overnight *in vacuo* at 100° over P_4O_{10} and stored over this drying reagent. *N,N*-Dimethylformamide was dried over molecular sieves.

Melting points (dec.) were determined with a Tottoli–Büchi apparatus and are uncorrected. Optical rotations were determined with a Perkin–Elmer 141 polarimeter (1-dm tube). I.r. spectra were recorded for KBr discs with a Beckman Model IR-8 spectrophotometer. U.v. analyses were performed with a Unicam SP 8000 spectrometer. A Varian XL-100 spectrometer was used to record ^{1}H-n.m.r. spectra.

T.l.c. was performed on Silica Gel 60 PF_{254} (Merck) with *A*, 1-butanol–1-propanol–0.1M HCl (1:2:1); *B*, chloroform–methanol (9:1); *C*, ether; and *D*, ethyl acetate; with detection by u.v. light or by charring with sulphuric acid.

In order to avoid decomposition of the *p*-nitrophenyl glycosides, all reactions and column chromatography were conducted in the dark.

Methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosyl chloride)onate (**1**) was synthesised according to a literature procedure[7].

*Methyl (*p*-nitrophenyl 5-acetamido-4,7,8,9-tetra*-O*-acetyl-3,5-dideoxy-*α-D-glycero-D-galacto-*2-nonulopyranosid)onate* (**2**). — A solution of freshly prepared **1** (1.9 g) and sodium *p*-nitrophenoxide (6 g) in dry *N,N*-dimethylformamide (50 mL) was stirred at room temperature for 12 h. T.l.c. then showed that **1** had been con-

sumed. The solution was concentrated and xylene was evaporated repeatedly from the residue to remove traces of *N,N*-dimethylformamide. The residue was extracted with ethyl acetate, and the combined extracts were concentrated to a small volume and added to a column of Silica Gel 60 (Merck). *p*-Nitrophenol was eluted with ether and **2** was eluted with ethyl acetate. The fractions containing only the chromogenic substance with R_F 0.33 (t.l.c., solvent *C*) were combined and concentrated, and the residual syrup (1.32 g, 57%) crystallised on treatment with ether–hexane to give **2** as a pale-yellow solid, m.p. 98–108° (dec.), which was homogeneous by t.l.c. (solvents *C* and *D*).

An analytical sample of **2** had m.p. 104–108° (dec.), $[\alpha]_D^{25}$ +46° (*c* 4.6, methanol) {lit.[1] colourless powder, $[\alpha]_D^{25}$ +4.2° (*c* 1.05, methanol)}, R_F 0.33 (solvent *C*); λ_{max}^{MeOH} (log ε) 288 nm (3.18); ν_{max} 1750 (C=O), 1600 and 1582 (aromatic), 1515 and 1345 (NO_2), 1240 and 1215 (acetate C–O–C), 859 and 750 cm^{-1}. ^{1}H-N.m.r. data ($CDCl_3$): δ 3.7 (s, Me), 5.8 (Me), 5.8 (d, *J* 5 Hz, NH), 7.2–8.2 (m, 4 H, aromatic protons).

Anal. Calc. for $C_{26}H_{32}N_2O_{15}$: C, 50.98; H, 5.27; N, 4.57. Found: C, 50.14; H, 5.45; N, 4.23.

*Methyl (*p*-nitrophenyl 5-acetamido-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosid)onate* (**3**). — To a solution of **2** (100 mg) in dry methanol (100 mL) was added methanolic M sodium methoxide (1 mL). After storage for 1 h at room temperature, the clear yellow solution was cooled to 0°, neutralised with methanol-washed Dowex 50W-X8 (H^+) resin, and concentrated. The residue crystallised from methanol–ether to afford **3** (51 mg, 71%). Recrystallisation gave material with m.p. 162–164°, $[\alpha]_D^{25}$ +48.5° (*c* 1.3, methanol); λ_{max}^{MeOH} (log ε) 288 nm (3.26); ν_{max} 3400 (OH), 3270 (NH), 1750 (C=O), 1610, 1585, and 1490 (aromatic), 1520 and 1350 (NO_2), 1250 and 1225 (C-O-C), 960, 880, 860, 790, 750 cm^{-1}. ^{1}H-N.m.r. data (CD_3CN): δ 2.0 (s, 3 H, NAc), 2.7–2.9 (m, 1 H, H-3*e*), 3.5–3.9 (m, 10 H, H-4,5,6,7,8,9,9′ and Me), 7.2–8.2 (m, 4 H, aromatic protons).

Anal. Calc. for $C_{18}H_{24}N_2O_{11}$: C, 48.64; H, 5.44; N, 6.30. Found: C, 48.43; H, 5.49; N, 6.05.

*p-Nitrophenyl 5-acetamido-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosidonic acid* (**4**). — A solution of **3** (100 mg) in 0.1M sodium hydroxide (100 mL) was stored at room temperature for 30 min, then cooled to 0°, freed from sodium ions using Dowex 50W-X8 (H^+) resin to pH 4, filtered, and concentrated. The residue was freeze-dried to give **4** as a chromatographically and electrophoretically homogeneous, colourless powder (84 mg, 87%). Crystallisation from methanol–ether gave material with m.p. 113–115°, $[\alpha]_D^{25}$ +69° (*c* 1.7, methanol) {lit.[1] $[\alpha]_D^{25}$ −1.33° (*c* 1, methanol)}; ν_{max} 3500–3300 (OH), 1600, 1520, and 1500 (aromatic), 1520 and 1360 (NO_2), 1050, and 870 cm^{-1}. ^{1}H-N.m.r. data (D_2O): δ 2.15 (s, 4 H, NAc and H-3*a*), 2.8–3.0 (m, 1 H, H-3*e*), 3.58–4.12 (m, 7 H, H-4,5,6,7,8,9,9′), 7.0–8.25 (m, 4 H, aromatic protons).

Anal. Calc. for $C_{17}H_{22}N_2O_{11}$: C, 47.44; H, 5.15; N, 6.51. Found: C, 46.90; H, 4.93; N, 6.22.

Enzyme hydrolyses. — Sialidases from *Vibrio cholerae* (Behringwerke AG, Marburg; 1 mL containing 500 U) *Clostridium perfringens* (1 U/mg), and *Arthrobacter ureafaciens* (0.02 U/mg) (Boehringer, Mannheim) were commercial products. Incubations were conducted at 37° in a total volume of 2 mL of sodium acetate buffer (0.1M, pH 5.5) containing sodium chloride (9 mg/mL) and calcium chloride (1 mg/mL). No sodium chloride was added in the assays using sialidases from *Cl. perfringens* or *A. ureafaciens*. At intervals, aliquots (0.1 mL) of each incubation mixture were treated with 0.1M sodium carbonate (2.9 mL), and the absorbance was measured at 400 nm.

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