Carbohydrate Research, 110 (1982) 11–18
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

REINVESTIGATION OF THE SYNTHESIS OF 4-METHYLCOUMARIN-7-YL 5-ACETAMIDO-3,5-DIDEOXY-α-D-*glycero*-D-*galacto*-2-NONULOPYRANOSIDONIC ACID, A FLUOROGENIC SUBSTRATE FOR NEURAMINIDASE

NEIL BAGGETT AND BRIAN J. MARSDEN

Department of Chemistry, University of Birmingham, P.O. Box 363, Edgbaston, Birmingham B15 2TT (Great Britain)

(Received March 11th, 1982; accepted for publication, March 26th, 1982)

ABSTRACT

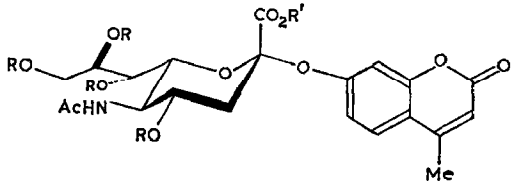
A crystalline tetrabutylammonium salt of 7-hydroxy-4-methylcoumarin was prepared and shown to contain two coumarin residues for each ammonium group. Condensation of this salt with the glycosyl chloride of methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate in dry acetonitrile at room temperature gave the corresponding α-glycoside in higher yield and purity than previously reported methods. Removal of the acetyl and methyl ester blocking-groups gave the free glycoside, which was shown to have the α configuration by n.m.r. spectroscopy. In contrast, the reaction of the free coumarin derivative with the chloro sugar in refluxing, dry toluene in the presence of cadmium carbonate as acid acceptor gave none of the above glycoside, but gave the corresponding glycal in good yield.

INTRODUCTION

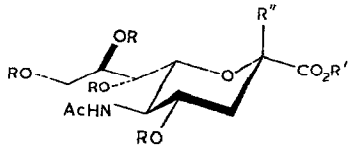
The objective of this work was to develop a reliable synthesis of 4-methylcoumarin-7-yl 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosidonic acid (**1**) as an artificial, fluorogenic substrate for neuraminidase (EC 3.2.1.18). Since the inception of the work, a number of syntheses of **1** have been reported[1–4], but there are significant discrepancies in the reported physical data. We now report a novel variation for synthesis of **1**, giving higher yields than the other methods, together with n.m.r. evidence for its configuration and our experience with some of the reported methods.

The first claimed[1] synthesis of **1** involved the reaction of the glycosyl chloride **2** of methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate with 7-hydroxy-4-methylcoumarin (**3**) in the presence of cadmium carbonate as the key coupling-step. This general strategy had been recommended[5] for synthesis of steroidal glucosiduronic acids. Subsequently, the method was further investigated[6] and it was found that yields are very dependent on batch variations in the catalyst.

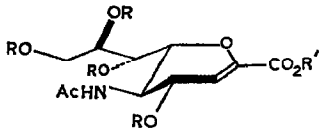
For synthesis of glycosides of *N*-acetylneuraminic acid, variations of the Koenigs–Knorr method have proved to be most satisfactory, using either **2** (ref. 7) or the corresponding free acid **4** (ref. 8). Higher stereoselectivity was obtained with the former reagent and this compensates for the extra synthetic steps involved. The halo sugar **2** was therefore used in this work.



1 R = R′ = H
9 R = Ac, R′ = Me



2 R = Ac, R′ = Me, R″ = Cl
4 R = Ac, R′ = H, R″ = Cl
5 R = H, R′ = Me, R″ = OH
6 R = Ac, R′ = Me, R″ = OH



7 R = Ac, R′ = Me
8 R = R′ = H

DISCUSSION

Methyl 5-acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate (**5**) was obtained in excellent yield by the literature route[7]. The observed m.p. was some 12° higher than the value reported, but the specific rotation corresponded to the reported value and the elemental analyses were as expected for a monohydrate[9].

Treatment of **5** with acetic anhydride and perchloric acid under conditions recommended[7] for formation of the penta-*O*-acetyl derivative gave a syrupy mixture from which the crystalline tetra-*O*-acetyl derivative **6** was obtained in 76% yield. The mother liquor contained another component, more mobile in t.l.c., which was presumed to be the penta-*O*-acetyl derivative. Acetylation of **5** with acetic anhydride in pyridine also gave the tetra-acetate **6**. The configuration of **6** was not previously assigned, but the small negative rotation is consistent with the β configuration. The halo sugar **2** was prepared from the tetra-acetate **6**, using a saturated solution of hydrogen chloride in acetyl chloride as described by Kuhn *et al.*[7], and the syrupy product was used directly for glycosidation.

When **2** was treated with a suspension of dry **3** in refluxing, dry toluene, as described by Thomas *et al.*[1], a syrupy product was obtained. T.l.c. revealed at least six components, but none of these was the required glycoside. The major product, isolated (82% yield) after chromatography, was the glycal **7**, the structure of which was deduced by n.m.r. spectroscopy and confirmed by elemental analysis. Significantly, there were no p.m.r. signals in the high-field region that could be attributed to H-3 in a glycoside, but there was a low-field doublet, at δ 5.96, for a vinylic H. In the ^{13}C-n.m.r. spectrum, signals at 145.2 and 108.1 p.p.m., which are diagnostic[10] of the α and β carbons of an α,β-unsaturated ester, can be assigned to C-2 and C-3 in **7**. Other signals correspond closely to those reported[11] for the parent glycal **8**, which was obtained by deacetylation and saponification of **7**.

Thus, in our hands, the attempted glycosidation with cadmium carbonate as acid acceptor, under the conditions reported by Thomas *et al.*[1], gave elimination rather than substitution. Dehydrohalogenation of tetra-*O*-acetyl-α-D-glucopyranosyl bromide with certain batches of cadmium carbonate has also been reported[6].

Glycosidation reactions may be considered as special cases of alkylation of the aglycon at its oxygen atom. During alkylation of phenols, phase-transfer catalysis has been recommended[12], in order to increase the nucleophilicity of the phenolic hydroxyl group, to promote *O- versus C*-alkylation, and to minimise the effect of traces of water. Crown ethers and ammonium salts have been used[13] to enhance the nucleophilicity of alcohols towards a halo sugar, and we have tried a similar approach, using the tetrabutylammonium salt of **3** to increase the solubility and enhance the nucleophilicity of the phenol. The crystalline tetrabutylammonium salt, prepared from tetrabutylammonium hydroxide and **3**, contained (elemental analysis and n.m.r. data) two coumarin residues per tetrabutylammonium group. Similar structures have been reported for other tetrabutylammonium salts[14].

When this tetrabutylammonium salt was allowed to react with the chloro sugar **2** in the presence of active silver carbonate and 3A molecular sieves in acetonitrile, a 63% yield of glycoside **9** was obtained after chromatography. The product was a glass which gave a solid, m.p. 98–100°, on trituration with toluene. This m.p. is the same as that cited by Myers *et al.*[4] and rather higher than the values found by Potier *et al.*[2] (82–83°) and Thomas *et al.*[1] (42–48°). The specific rotation (+52°, chloroform) is higher than the reported[2] value (+37°, chloroform). The elemental analysis and ^{1}H-n.m.r. spectrum support the assigned structure. The same compound was also made in lower yield and purity by the methods of Warner and O'Brien[3], and Potier *et al.*[2].

Deacetylation of **9** and removal of the methyl ester group were achieved by basic hydrolysis, and chromatography on silica gel gave a product having m.p. 171° (dec.), $[\alpha]_D^{22}$ +70° (*c* 0.8, water). The m.p. is higher than that (162°) reported by Myers *et al.*[4], but lower than that (200°) reported by Thomas *et al.*[1]; the specific rotation is also higher than reported[2,3] values (51 and 59.8°). The analysis figures for C and N are consistent with the presence of four molecules of water of crystallisa-

tion. The amount of **3** liberated on total enzyme hydrolysis is also consistent with the formula $C_{21}H_{25}NO_{11} \cdot 4\ H_2O$.

The high positive rotation suggests the α configuration and this is supported by two pieces of n.m.r. evidence. The chemical shift for H-3*e* at δ 2.9 is diagnostic of the α configuration[15]. In the undecoupled, ^{13}C-n.m.r. spectrum, the signal for the CO_2H group was a broadened doublet with $J_{C\text{-}1,H\text{-}3a} \sim 5$ Hz and $J_{C\text{-}1,H\text{-}3e} \leqslant 1$ Hz as expected[16] for the α-glycoside; two *J* values of $\leqslant 1$ Hz would be expected for the β-glycoside.

EXPERIMENTAL

General methods. — Concentrations were effected *in vacuo* below 40°. Melting points are uncorrected. T.l.c. was performed on silica gel (Merck 60 F_{254} plastic sheets) with detection by u.v. light or by charring with sulphuric acid. Column chromatography was conducted on silica gel (Merck 7734). Dry methanol refers to methanol distilled from magnesium methoxide[17]. Dry pyridine refers to pyridine distilled from phosphorus(V) oxide and stored over potassium hydroxide. Dry acetonitrile refers to acetonitrile that had been stirred for 3 h with calcium hydride, distilled from fresh calcium hydride, and then stored over Type 4A molecular sieves. Dry toluene refers to toluene dried over sodium wire. Optical rotations were determined with a Perkin–Elmer 141 automatic polarimeter (1-dm tube). A Perkin–Elmer R-14 or Varian XL-100 spectrometer was used to record ^{1}H-n.m.r. spectra, and a Jeol FX 60 FT spectrometer was used for ^{13}C-n.m.r. spectra. Coupling constants refer to observed splittings. For measurement of coupling constants in ^{13}C-n.m.r. spectra, the single-resonance spectra were recorded using 8000 data points over a spectral width of 1 kHz.

*Methyl 5-acetamido-3,5-dideoxy-β-*D*-glycero-*D*-galacto-2-nonulopyranosonate* (**5**). — This compound, prepared by the method of Kuhn *et al.*[7] in 92% yield, had m.p. 191–192°, $[\alpha]_D^{20}$ −28° (*c* 1, water); lit.[7] m.p. 179–180°, $[\alpha]_D^{20}$ −28° (water). P.m.r. data (D_2O): δ 4.12–3.48 (m, 10 H, H-4–H-9 and CO_2Me), 2.31 (q, 1 H, $J_{3,3}$ 14, $J_{3e,4}$ 4.5 Hz, H-3*e*), 2.04 (s, 3 H, NAc), and 1.89 (t, $J_{3,3}$ 14, $J_{3a,4}$ 14 Hz, H-3*a*).

Anal. Calc. for $C_{12}H_{21}NO_9 \cdot H_2O$: C, 42.2; H, 6.8; N, 4.1. Found: C, 42.7; H, 6.85; N, 4.3.

Acetylation of **5**. — (*a*) Treatment of **5** (3.84 g) with acetic anhydride (15 mL) and perchloric acid (0.1 mL; 60%) at 40° for 2.5 h, as described by Kuhn *et al.*[7], gave a syrup (5.2 g). Crystallisation from ether gave the tetra-acetate (**6**) as white needles (4.5 g), m.p. 174–175°, $[\alpha]_D$ −3.1° (*c* 1, chloroform); lit.[7] m.p. 174–175°, $[\alpha]_D^{20}$ −4.8 to −5.2° (chloroform). P.m.r. data ($CDCl_3$): δ 6.26 (d, 1 H, NH), 5.44–3.94 (m, 8 H, H-4,5,6,7,8,9,9′, OH), 3.84 (s, 3 H, OMe), and 2.24–1.89 (m, 17 H, H-3*e*,3*a*, 4 OAc, NAc); ^{13}C-n.m.r. data ($CDCl_3$): 171.7, 171.2, 170.8, 170.5, 170.3 (4 $OCOCH_3$, $NCOCH_3$), 169.1 (C-1), 95.0 (C-2), 72.3, 71.5, 69.3, 68.5 (C-

4,6,7,8), 62.9 (C-9), 53.3 (OCH_3), 49.2 (C-5), 36.3 (C-3), 23.1 ($NCOCH_3$), and 21.1–20.9 p.p.m. (4 $OCOCH_3$).

Anal. Calc. for $C_{20}H_{29}NO_{13}$: C, 48.9; H, 6.0; N, 2.85. Found: C, 49.0; H, 6.0; N, 2.7.

The mother liquor was evaporated to a syrup (0.63 g) which contained **5** (R_F 0.51; chloroform–methanol, 9:1) and a faster, major component (R_F 0.71), but could not be crystallised.

(*b*) Treatment of **5** (1 g) with acetic anhydride (5 mL) and pyridine (6 mL) for 16 h at room temperature gave the crystalline tetra-acetate **6** (0.69 g, 48%), and a syrup from the mother liquor which could not be crystallised and essentially comprised two components (R_F 0.51 and 0.71).

The glycosyl chloride (**2**) *of methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-β-*D*-glycero-*D*-galacto-2-nonulopyranosonate.* — This compound, prepared as described by Kuhn *et al.*[7], was a syrup, $[\alpha]_D^{20}$ −59° (*c* 1, chloroform); lit.[7] $[\alpha]_D^{20}$ −63° (*c* 1, chloroform).

*Attempted synthesis of methyl (4-methylcoumarin-7-yl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosid)onate* (**9**) *by the method of Thomas* et al.[1]. — A mixture of **3** (8 g), dry cadmium carbonate (4 g, Hopkin and Williams, GPR grade), and dry toluene (1.2 L) was heated to distil the toluene–water azeotrope whilst fresh dry toluene was added to maintain the original volume. After distillation of 1.4 L of toluene, a solution of **2** (2.1 g) in dry chloroform (50 mL) was added *via* a dropping funnel, and the funnel was then rinsed with more dry chloroform (2 × 25 mL). No pink colour was formed. The distillation was continued for a further 2.5 h, until the volume had been reduced to ~200 mL. The residual mixture was filtered, dry chloroform (200 mL) was added to the filtrate, the mixture was again filtered, and the filtrate was evaporated to dryness. Dry chloroform (100 mL) was added to the residue, the insoluble material was removed by filtration, and the filtrate was evaporated to dryness. The residue was chromatographed on a column (5 × 30 cm) of alumina (P. Spence, type H); no material was eluted with chloroform. Elution with chloroform–ethanol (9:1, 500 mL) gave a syrup (2.05 g) that contained (t.l.c.; chloroform–methanol, 9:1) at least six components, none of which was fluorescent. The syrup was re-chromatographed on a column of silica gel (100 g) with chloroform and then chloroform–methanol mixtures containing increasing amounts of methanol. Fractions (20 mL) were collected, and monitored by t.l.c.; those containing only the major product (R_F 0.52; chloroform–methanol, 9:1) were combined and evaporated to dryness, to give **7** as a syrup (1.6 g, 82%), $[\alpha]_D^{22}$ +61° (*c* 1, chloroform). P.m.r. data ($CDCl_3$): *inter alia*, δ 6.10 (broad, 1 H, NH), 5.93 (d, $J_{3,4}$ 3 Hz, H-3), 5.54–5.41 (m, 2 H), 5.39–5.23 (m, 1 H), 4.62 (q, 1 H, $J_{4,3}$ 3, $J_{4,5}$ 12 Hz, H-4), 4.42–4.32 (m, 2 H), 4.15 (q, $J_{5,4}$ 12, $J_{5,6}$ 6 Hz, H-5), 3.78 (s, CO_2Me), 2.10, 2.06, 2.04, and 1.91 (4 s, 15 H, 5 Ac); ^{13}C-n.m.r. data ($CDCl_3$): 170.9, 170.7, 170.4 ($COCH_3$), 161.7 (C-1), 145.2 (C-2), 108.1 (C-3), 76.7 (C-6), 71.0 (C-8), 68.3 (C-7), 67.7 (C-4), 62.0 (C-9), 52.7 (C-5), 46.3 (OMe), 23.0 ($NCOCH_3$), and 20.73 ($OCOCH_3$).

Anal. Calc. for $C_{20}H_{27}NO_{12}$: C, 50.7; H, 5.8; N, 3.0. Found: C, 51.0; H, 6.0; N, 2.9.

5-Acetamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-*non-2-enonic acid* (**8**). — A solution of **7** (0.4 g) in absolute methanol (2 mL) was made alkaline with ~0.2M methanolic sodium methoxide (0.1 mL). After 30 min at room temperature, the solution was evaporated to dryness, 0.2M sodium hydrogencarbonate (10 mL) was added to the residue, and the mixture was stirred overnight. Dowex 50 (H^+) resin was added to give pH 6.0, the mixture was filtered, and the filtrate was evaporated, to yield **8** as a powder (0.18 g, 73%), m.p. 128–134°, $[\alpha]_D^{22}$ +39° (*c* 1, water); lit.[18] m.p. 137–140°, $[\alpha]_D^{18}$ +41.6° (water).

Tetrabutylammonium salt of 7-hydroxy-4-methylcoumarin. — (*a*) A mixture of sodium hydroxide (1.3 g), **3** (5.9 g), and tetrabutylammonium bromide (10.7 g) in water (30 mL) was shaken and then extracted with chloroform (4 × 20 mL), the chloroform solution was evaporated to dryness, and the residue was recrystallised from chloroform, to give the title compound as pale-green needles (7.8 g), m.p. 170–172°. P.m.r. data ($CDCl_3$): δ 7.33–6.60 (m, 6 H, Ar), 5.80 (s, 2 H, vinyl H), 3.25–2.95 (m, 8 H, 4 N-CH_2-), 2.30 (s, 6 H, vinyl CH_3), 1.74–1.10 (m, 16 H, 8 -CH_2-), and 0.99–0.75 (m, 12 H, 4 CCH_3).

Anal. Calc. for $C_{36}H_{51}NO_6$: C, 72.8; H, 8.7; N, 2.4. Found: C, 72.5; H, 8.45; N, 2.1.

(*b*) The experiment was repeated as in (*a*), using 1.5 mol. equiv. of sodium hydroxide (2.0 g); the aqueous solution was dark red. Extraction and crystallisation gave colourless needles (6.4 g, 64%), m.p. 171–172°, identical to the product from (*a*) by n.m.r. spectroscopy.

Methyl (4-methylcoumarin-7-yl 5-acetamido-4,7,8,9-tetra-O-*acetyl-3,5-dideoxy*-α-D-glycero-D-galacto-*2-nonulopyranosid)onate* (**9**). — (*a*) A mixture of **2** (2.62 g), 3A molecular sieve (8 g), active silver carbonate (5.5 g), and the tetrabutylammonium salt of **3** (6 g) in dry acetonitrile (100 mL) was stirred magnetically in the dark at room temperature for 3 h. T.l.c. then showed that **2** had been consumed. The mixture was filtered, the precipitate was washed with chloroform (3 × 20 mL), and the combined filtrate and washings were evaporated to a brown syrup. Ethyl acetate (60 mL) was added, and the undissolved salt was filtered-off and washed with ethyl acetate. The combined ethyl acetate extract was evaporated to a brown syrup (3.56 g), which was chromatographed on a column of silica gel (220 g) by elution with mixtures of ether and acetone. The fractions were examined by t.l.c. and those containing only the fluorescent component with R_F 0.5 (chloroform–methanol 9:1) were combined and evaporated, to give **9** (2.1 g, 63%) as a syrup that was triturated with toluene to give a solid, m.p. 98–100°, $[\alpha]_D^{21}$ +52° (*c* 1, chloroform); lit.[2] m.p. 82–83°, $[\alpha]_D$ +37° (chloroform); lit.[4] m.p. 98–100°. P.m.r. data ($CDCl_3$): δ 7.57–7.02 (m, 3 H, aromatic), 6.20 (s, 1 H, coumarin H-3), 5.9 (broad d, 1 H, NH), 5.38 (s, 2 H), 5.16–4.86 (m, 1 H), 4.60–4.08 (m, 4 H), 3.72 (s, 3 H, OMe), 2.76 (q, 1 H, $J_{3,3}$ 13, $J_{3e,4}$ 4.5 Hz, H-3*e*), 2.44 (s, 3 H, CH_3), 2.16, 2.06, and 1.94 (3 s + m, 16 H, 5Ac and H-3*a*).

Anal. Calc. for $C_{30}H_{35}NO_{15}$: C, 55.5; H, 5.4; N, 2.2. Found: C, 55.2; H, 5.4; N, 2.1.

A further component was obtained as a syrup (0.26 g) which was shown by n.m.r. spectroscopy to be a 2:1 mixture of **7** and an acetylated methyl ester of neuraminic acid.

(*b*). The synthesis was carried out by a method similar to that reported by Warner and O'Brien[3], using the sodium salt of **3** (6.3 g), the chloro derivative **2** (3.85 g), 3A molecular sieve (12 g), and active silver carbonate (8 g) in acetonitrile (100 mL) at room temperature overnight. The mixture was processed and chromatographed essentially as in (*a*), to give **9** (2.31 g, 46%), R_F 0.5 (chloroform–methanol, 9:1).

(*c*). The synthesis was carried out as directed by Potier *et al.*[2], to give a product (51% yield), m.p. 65–73°, $[\alpha]_D^{22}$ +29° (*c* 1, chloroform).

*4-Methylcoumarin-7-yl 5-acetamido-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosidonic acid* (**1**). — The acetylated glycoside **5** (1.23 g) was dissolved in dry methanol (10 mL), methanolic sodium methoxide [prepared from sodium (25 mg) and dry methanol (25 mL)] was added, and the solution was stored at room temperature for 1.5 h. The methanol was then evaporated, water (15 mL) was added, and the solution was stored for 2 h. Sodium ions were removed by addition of Dowex 50 (H^+) resin to pH 4 followed by filtration. Ammonium hydroxide was added and the alkaline solution was evaporated to dryness, to yield a syrup (0.865 g) that was chromatographed on a column of silica gel (70 g) with an acetone–water gradient. The fractions were monitored by t.l.c. and those containing the product were combined and evaporated to dryness, to give **1** as a buff powder (0.63 g, 62%), m.p. 171° (dec.), $[\alpha]_D$ +70° (*c* 0.8, water); lit.[4] m.p. 162°; lit.[3] $[\alpha]_D^{22}$ +59.8° (water, pH 5); lit.[2] $[\alpha]_D$ +51° (Na salt in water); lit.[1] $[\alpha]_D^{25}$ −9.7° (water). P.m.r. data (D_2O): δ 7.60–7.06 (m, 3 H, aromatic), 6.09 (s, 1 H, vinyl H), 4.12–3.58 (m, 7 H, H-4,5,6,7,8,9,9′), 3.00–2.80 (m, 1 H, H-3*e*), 2.32 (s, 3 H, coumarin Me), 2.10 (s, 4 H, NAc and H-3*a*); ^{13}C-n.m.r. data (D_2O): 175.9 ($NHCOCH_3$), 173.4 (C-1), 164.8 (C-7′), 158.2 (C-2′), 156.6 (C-9′), 153.8 (C-4′), 126.8 (C-5′), 118.4 (C-6′), 116.3 (C-10′), 112.1 (C-3′), 108.4 (C-8′), 103.4 (C-2), 74.4 (C-6), 72.4 (C-8), 69.2 (C-7), 68.7 (C-4), 63.6 (C-9), 52.6 (C-5), 41.7 (C-3), 22.9 ($NCOCH_3$), and 18.7 p.p.m. (CH_3).

Anal. Calc. for $C_{21}H_{25}NO_{11} \cdot 4\ H_2O$: C, 46.7; H, 6.2; N, 2.6. Found: C, 46.9; H, 5.5; N, 2.4.

ACKNOWLEDGMENTS

We thank Professor S. A. Barker, Dr. P. B. Koch, and Mr. E. E. Vickers for their interest in this work, S.E.R.C. for financial support through a CASE studentship (to B.J.M.), and Mr. M. Tolley for recording the ^{13}C-n.m.r. spectra.

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