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2,3-Didehydro-2-deoxysialic acids structurally varied at C-5 and their behaviour towards the sialidase from *Vibrio cholerae*[*,†]

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ABSTRACT

2,3-Didehydro-2-deoxy-*N*-trifluoroacctylncuraminic acid (5-trifluoroacetyl-Neu2en) (**3**) has been synthesised from Neu5Ac2en (**1**) by hydrazinolysis, to give Neu2en (**2**), followed by *N*-trifluoroacetylation. 2,3-Didehydro-2,3-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (Kdn2en, **8**) and 5-azido-2,3-didehydro-2,3,5-trideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (5-azido-5-deoxy-Kdn2en, **9**) have been prepared from the acetylated methyl esters of Kdn (**4**) and 5-azido-5-deoxy-Kdn (**5**) via Zemplén saponification. The behaviour of the above 2,3-didehydro-2-deoxysialic acids towards *Vibrio cholerae* sialidase has been investigated

INTRODUCTION

The best inhibitors of sialidases (EC 3.2.1.18) from *Vibrio cholerae* and other bacterial, viral, protozoal, and animal sources are 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid (Neu5Ac2en) and some of its analogues[2–9]. The inhibitors described by Tuppy and co-workers[3,10] have various 5-acyl groups, and the best enzyme–substrate interaction was achieved if *N*-acetyl was replaced by *N*-trifluoroacetyl[9]. The *N*-trifluoroacetyl derivative was synthesised by the Kuhn–Baschang procedure[11], which is not a stereochemically defined reaction, and we now report the synthesis of 2,3-didehydro-2-deoxy-*N*-trifluoroacetylneuraminic acid (5-trifluoroacetyl-Neu2en, **3**) by an enantioselective route. In the context of the inhibition of *Vibrio cholerae* sialidase, the effect of replacing the *N*-acetyl group in **1** by the azido, hydroxy, and the ammonio group has been investigated.

RESULTS AND DISCUSSION

The reaction sequence (**1**→**2**→**3**) for the synthesis of **3** is shown in the formulae scheme. An attempt to obtain **2** via the reaction of **9** with triphenylphosphine gave a

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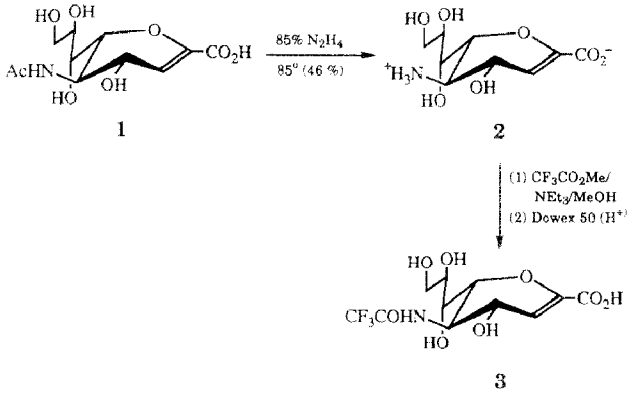
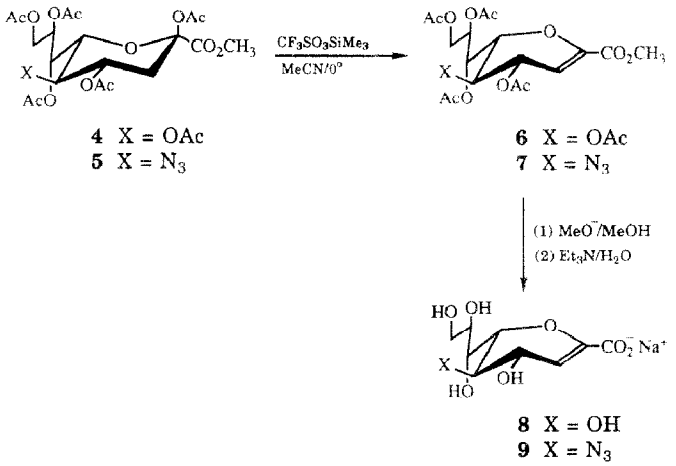
poor yield. Hydrazinolysis[12] of **1** gave a moderate yield (46%) of **2**, which, with methyl trifluoroacetate[13], gave **3**. Compound **3** has $[\alpha]_D^{20}$ +24 (*cf.* the reported[10] value of +40.4°). The 400-MHz n.m.r. spectrum of **3** was in complete accordance with expectations. The typical pattern of the resonances of H-3,4,6 in **3** corresponded to those of **2**, **8**, and **9** (see Experimental). The small couplings caused by the neighbouring protons (H-3,4,7) appeared as well resolved signals. These characteristic patterns for **2**, **3**, **8**, and **9** indicate H-4,5,6 to be axial.



1 **2** **3**

Starting from the known[14] acetylated sialic acid derivatives **4** and **5**, reaction with trimethylsilyl trifluoromethanesulfonate[15] in a modified version[16] afforded the acetylated 2,3-didehydro-2-deoxy derivatives **6** and **7**, respectively. Zemplén saponification of **6** and **7** and hydrolysis of the methyl ester in the products with aqueous triethylamine yielded Kdn2en (**8**) and 5-deoxy-Kdn2en (**9**), respectively.



4 X = OAc
5 X = N_3

6 X = OAc
7 X = N_3

8 X = OH
9 X = N_3

The results in Table I demonstrate that Neu5Ac2en and its derivatives **2**, **3**, and **9**, modified at C-5, are inhibitors of the *Vibrio cholerae* sialidase-catalysed hydrolysis of the methylumbelliferyl α-glycoside of *N*-acetylneuraminic acid but with different po-

TABLE I

Inhibition of the sialidase from *Vibrio cholerae* by Neu5Ac2en and the analogues **1–3**, **8**, and **9**

Inhibitor	K_i[mM]
Neu5Ac2en (**1**)	2.5×10^{-2}
Kdn2en (**8**)	[a]
5-Azido-5-deoxy-Kdn2en (**9**)	2.5
Neu2en (**2**)	2.9×10^{1}
5-Trifluoroacetyl-Neu2en (**3**)	1.8×10^{-3}

[a] No inhibition detected up to 0.8mM.

tencies, as expected. The *N*-trifluoroacetyl derivative **3** is the best inhibitor so far tested.

The inhibition constant of 1.8μM for **3** was changed to 1.5μM when measured under the conditions of Meindl *et al.*[3], and agrees well with that (1.3μM) calculated on the basis of the relative inhibition data. This finding is surprising in view of the other stereoisomers probably present in the earlier preparation of **3** and the different $[\alpha]_D$ values. Kdn2en (**8**) did not inhibit the sialidase, but, at 0.8mM, it reduced the value of K_m by 23%, thereby activating the enzyme. This result rules out the binding of **8** at the active site. The decrease of K_i by three orders of magnitude on going from Neu5Ac2en (**1**) to Neu2en (**2**) and the absence of any inhibition by Kdn2en (**8**) highlight the need for a carboxamido function. The decrease of the K_i by two orders of magnitude for 5-azido-5-deoxy-Kdn2en (**9**) points to the fact that the π-system of the azido group is not a substitute for that of the acetamido group. The methylumbelliferyl α-glycoside of 5-azido-5-deoxy-Kdn was hydrolysed by *Vibrio cholerae* sialidase with a K_m of 2.3mM and a v_{max} that was only 2.4% of that obtained with the corresponding derivative of Neu5Ac, and indication of a different mode of binding. The minimal structure for tight binding seems to be the *N*-acetyl group, since, with an *N*-formyl group, the concentration needed[3] for 50% inhibition was increased by a factor of 27.

EXPERIMENTAL

General methods. — Solvents were distilled before use. *N*-Acetylneuraminic acid (Neu5Ac) was prepared either from meconium[17] or from bird's-nest glycoprotein[18]. Solutions were concentrated in a rotatory evaporator at $<40°$. T.l.c. was performed on Silica Gel 60 F_{254} (Merck) and detection with 2% $Ce(NO_3)_4$ in 2M H_2SO_4, followed by heating at 200°. Flash chromatography[19] was performed on Silica Gel 60 (0.040–0.063 mm). ^{1}H-N.m.r. spectra were obtained with Bruker WM 250 (250 MHz) and AM 400 (400 MHz) spectrometers, i.r.spectra with a Perkin–Elmer 377 spectrometer, and mass spectra with a Varian CH-7-instrument.

5-Ammonio-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-*non-2-enonate (Neu2en,* **2**). — A solution of Neu5Ac2en (**1**; 500 mg, 1.7 mmol) in 100% hydrazinium hydroxide (10 mL) was kept at 85° for 72 h, then concentrated *in vacuo*, and a solution of the residue in a few mL of water was added to a column of Dowex 50 (H^+) resin (20 g).

Elution with water gave **1** and with 1.5M ammonium hydroxide gave **2**. Fractions that contained **2** were combined and concentrated, and the residue was put on a column of Dowex 2-X8 (HO^-) resin (20 g). Hydrazinium hydroxide was eluted with water, and **2** with 4M acetic acid. Fractions that contained **2** were combined and concentrated. In order to remove residual acetic acid, the residue was twice dissolved in water (2 mL) and the solution was concentrated. Lyophilisation then gave **2** (195 mg, 46%), $[\alpha]_D^{20} -10°$ (*c* 0.26 water), R_f 0.14 (t.l.c.; 1-propanol–water–acetic acid, 15:4:0.5). ^{1}H-N.m.r. data (250 MHz, D_2O–HDO): δ 3.48 (dd, 1 H, $J_{4,5}$ 8.6, $J_{5,6}$ 10.2 Hz, H-5), 3.60 (dd, 1 H, $J_{8,9A}$ 5.8, $J_{9A,9B}$ −11.7 Hz, H-9A), 3.74 (d, 1 H, $J_{7,8}$ 8.0 Hz, H-7), 3.87 (dd, 1 H, $J_{8,9B}$ 2.3 Hz, H-9B), 3.98 (ddd, 1 H, H-8), 4.55 (d, 1 H, H-6), 4.44 (d, 1 H, H-4), 5.71 (s, 1 H, H-3).

2,6-Anhydro-3,5-dideoxy-5-trifluoroacetamido-D-glycero-D-galacto-*non-2-enonic acid (5-trifluoroacetyl-Neu2en*, **3**). — To a dispersion of dried **2** (200 mg, 0.8 mmol) in dry methanol (6 mL) at −10° were added triethylamine (0.16 mL, 1.2 mmol) and methyl trifluoroacetate (0.24 mL, 2.4 mmol) with vigorous stirring, and the mixture was then warmed to 0° and stirred thereat for 10 h. The solvent was then evaporated, and a solution of the residue in cold water (2 mL) was filtered through Dowex 50 (H^+) resin (2 g) and washed through a column of Dowex 2-X8 (AcO^-) resin (15 g) as rapidly as possible. Elution with 4M acetic acid gave **3**, the appropriate fractions were combined, and the solvent was evaporated. In order to remove residual acetic acid, the residue was twice dissolved in water (2 mL) and the solution was concentrated. Lyophilisation gave **3** (135 mg, 49%), $[\alpha]_D^{20} +24°$ (*c* 0.78, water), R_f 0.51 (t.l.c.; 1-propanol–water–acetic acid, 15:4:0.5). ^{1}H-N.m.r. data (400 MHz, D_2O–HDO, δ 4.65): δ 3.46 (dd, 1 H, $J_{5,6}$ 1.0, $J_{7,8}$ 8.8 Hz, H-7), 3.49 (dd, 1 H, $J_{8,9A}$ 6.2, $J_{9A,9B}$ −11.8 Hz, H-9A), 3.73 (dd, 1 H, $J_{8,9B}$ 2.9, $J_{9A,9B}$ −11.8 Hz, H-9B), 3.79 (ddd, 1 H, H-8), 4.11 (dd, 1 H, $J_{4,5}$ 8.9, $J_{5,6}$ 10.8 Hz, H-5), 4.29 (dd, 1 H, H-6), 4.44 (dd, 1 H, $J_{3,4}$ 2.3 Hz, H-4), 5.89 (d, 1 H, $J_{3,4}$ 2.3 Hz, H-3).

Methyl 4,5,7,8,9-penta-O-*acetyl-2,6-anhydro-3-deoxy*-D-glycero-D-galacto-*non-2-enonate* (**6**). — A solution of **4** (82 mg) in dry acetonitrile (2 mL) was treated with 1 equiv. of trimethylsilyl trifluoromethansulfonate at 0°. The progress of the reaction was monitored by t.l.c. (ethyl acetate–hexane, 1:1). For work-up, dry K_2CO_3 (1.5 equiv.) was added, the mixture was stirred for 2 min at 0° and then filtered through Celite, the solvent was evaporated, and the resulting residue was purified by chromatography on silica gel (15 g) with ethyl acetate–hexane (1:1), to give **6** (46 mg, 63%), $[\alpha]_D^{20} +26.3°$ (*c* 0.24, chloroform), R_f 0.22 (t.l.c.; ethyl acetate–hexane, 1:1). ^{1}H-N.m.r. data (250 MHz, $CDCl_3$–Me_4Si): δ 2.07, 2.09, 2.12, 2.16, 2.18 (5 s, each 3 H, 5 Ac), 3.81 (s, 3 H, CO_2Me), 4.13 (dd, 1 H, $J_{8,9A}$ 6.1, $J_{9A,9B}$ −12.5 Hz, H-9A), 4.26 (dd, 1 H, $J_{8,9B}$ 2.5 Hz, H-9B), 4.50 (dd, 1 H, $J_{5,6}$ 12.0, $J_{6,7}$ 2.7 Hz, H-6), 5.14 (dd, 1 H, $J_{4,5}$ 9.0 Hz, H-5), 5.32 (ddd, 1 H, $J_{7,8}$ 6.7 Hz, H-8), 5.42 (dd, 1 H, H-7), 5.51 (dd, 1 H, $J_{3,4}$ 2.5 Hz, H-4), 5.91 (d, 1 H, H-3). Mass spectrum (70 eV, 150°): *m/z* 474 (0.17%) [M^+].

Methyl 4,7,8,9-tetra-O-*acetyl-2,6-anhydro-5-azido-3,5-dideoxy*-D-glycero-D-galacto-*non-2-enonate* (**7**). — Treatment of **5** (115 mg), as described above for **4**, gave **7** (73 mg, 72%), $[\alpha]_D^{20} +41°$ (*c* 0.2, chloroform), R_f 0.35 (t.l.c.; ethyl acetate–hexane, 1:1); ν_{max} (CH_2Cl_2) 2105 cm^{-1} (N_3). ^{1}H-N.m.r. data (250 MHz, $CDCl_3$–Me_4Si): δ 2.08, 2.14, 2.19 (3 s, 12 H, 4 Ac), 3.69 (dd, 1 H, $J_{4,5}$ 8.6, $J_{5,6}$ 10.7 Hz, H-5), 3.81 (s, 3 H, CO_2Me), 4.07 (dd, 1

H, $J_{6,7}$ 1.9 Hz, H-6), 4.22 (dd, 1 H, $J_{8,9A}$ 6.3, $J_{9A,9B}$ 12.3 Hz, H-9A), 4.56 (dd, 1 H, $J_{8,9B}$ 2.5 Hz, H-9B), 5.37 (ddd, 1 H, $J_{7,8}$ 6.3 Hz, H-8), 5.58 (dd, 1 H, $J_{3,4}$ 2.3 Hz, H-4), 5.61 (dd, 1 H, H-7), 5.98 (d, 1 H, H-3). Mass spectrum (70 eV, 140°): *m/z* 457 (0.24%) [M^+].

Sodium 2,6-anhydro-3-deoxy-D-glycero-D-galacto-*non-2-enonate* (*Kdn2en*, **8**). — Zemplén saponification of **6** (40 mg) with 0.25 equiv. of sodium methoxide in dry methanol (5 mL) was monitored by t.l.c. (dichloromethane–methanol, 2:1). When the reaction was complete, solid CO_2 was added, the solvent was evaporated *in vacuo*, and a solution of the residue in dichloromethane–methanol (2:1) was filtered through Florisil (2 g, 200 mesh) with the same solvent. The eluate was concentrated and the residual pure methyl ester was cleaved with triethylamine–water. The product was washed through Florisil (2 g) with dichloromethane–methanol–0.1M triethlylamine in water (4:4:1), and **8** (21 mg, 92%), obtained by treatment of the product with Dowex 50 (Na^+) resin followed by lyophilisation, had $[\alpha]_D^{20}$ +136° (*c* 0.28, water); R_F 0.18 (t.l.c.; 1-propanol–water–acetic acid (15:4:0.5). ^{1}H-N.m.r. data (400 MHz, D_2O–HDO): δ 3.73 (dd, 1 H, H-9A), 3.80 (dd, 1 H, $J_{5,6}$ 10.6 Hz, H-5), 3.93 (dd, 1 H, $J_{7,8}$ 9.2 Hz, H-7), 3.95 (dd, 1 H, $J_{9A,9B}$ −12 Hz, H-9B), 3.98 (ddd, 1 H, $J_{8,9A}$ 2.6, $J_{8,9B}$ 6.3 Hz, H-8), 4.16 (dd, 1 H, $J_{6,7}$ 1 Hz, H-6), 4.44 (dd, 1 H, $J_{4,5}$ 7.9 Hz, H-4), 5.65 (d, 1H, $J_{3,4}$ 2.3 Hz, H-3); the couplings of H-3, H-4, H-5, H-6, and H-9A are first order, and the shift values and couplings of other protons are iterated ones.

Sodium 2,6-anhydro-5-azido-3,5-dideoxy-D-glycero-D-galacto-*non-2-enonate* (*5-azido-5-deoxy-Kdn2en*, **9**). — Treatment of **7** (65 mg), as described above for **6**, gave **9** (38 mg, 92%), $[\alpha]_D^{20}$ −1.28° (*c* 0.47, water), R_F 0.52 (t.l.c.; 1-propanol–water–acetic acid, 15:4:0.5); ν_{max}^{Nujuol} 2110 cm^{-1} (N_3). ^{1}H-N.m.r. data (250 MHz, D_2O–HDO): δ 3.46–3.58 (m, 2 H), 3.63–3.86 (m, 3 H), 3.95 (d, 1 H, $J_{5,6}$ 10.8 Hz, H-6), 4.36 (d, 1 H, $J_{4,5}$ 8.5 Hz, H-4), 5.48 (s, 1 H, H-3).

Inhibition experiments. — Incubations with sialidase were performed essentially as described[6]. 4-Methylumbelliferyl-2α-Neu5Ac was used at 0.1 and 2mM. In preliminary experiments with only one concentration of inhibitor, approximate K_i values were determined assuming competitive inhibition. For the more precise determination of the K_i of **3**, four concentrations of inhibitor were chosen at 0.1-, 1.2-, 4-, and 20-fold the approximate K_i. Each set of concentrations was measured 5 times. The kinetic model was selected with the help of Lineweaver–Burk plots after non-linear regression analysis with the Enzfitter program (Biosoft, Oxford). The constants were calculated by fitting the data with a non-linear regression program[19] to a model of partially mixed inhibition. The K_i values in Table I represent the competitive part of that model in order to facilitate comparison with older data. The other inhibitors were tested with 4 concentrations of substrate between 0.1 and 1mM at inhibitor concentrations of 0.8mM for **8**, 10mM for **2**, and 0.3 and 1mM for **9**. Three replicates were measured. The values of K_m and v_{max} were determined with the Enzfitter program, and the inhibition constants for competitive inhibition were calculated by including the constants of the uninhibited reaction.

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