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Enzymatic Synthesis of 7-Deoxy-*N*-acetylneuraminic Acid and 7-*O*-Methyl-*N*-acetylneuraminic Acid

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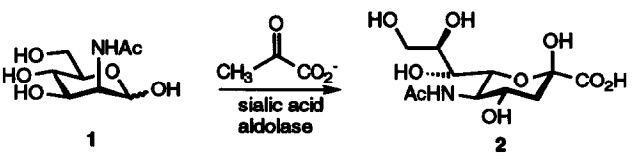
Abstract: 7-Deoxy-*N*-acetylneuraminic Acid and 7-*O*-methyl-*N*-acetylneuraminic acid were synthesized through the sialic acid aldolase-catalyzed aldol addition reactions of 4-deoxy-*N*-acetyl-D-mannosamine and 4-*O*-methyl-*N*-acetyl-D-mannosamine, respectively, with pyruvate. The obtained sialic acids will be used as probes for the investigation of the unusual mechanism of a novel sialidase from leech.

Introduction

Sialic acids are vital components of many cell-surface glycoproteins and glycolipids[1] and participate in biological recognition phenomena such as cell-cell communication, cell adhesion and receptor binding, among many others.[2] In reponse to the important role of these compounds in cellular biology, various naturally occuring and modified sialic acids have recently been synthesized by both chemical and enzymatic methods.[3] The enzymatic approach relies on the use of sialic acid aldolase (E.C. 4.1.3.3), which *in vivo* catalyzes the reversible aldol condensation between pyruvate and *N*-acetyl-D-mannosamine (**1**, ManNAc) to generate *N*-acetylneuraminic acid (**2**, NeuAc, a sialic acid), as shown in Scheme 1.[4] The aldolase displays a relaxed specificity towards the structure of the acceptor substrates,[3d-3h] thus allowing the straightforward synthesis of biologically interesting sialic acids from simple and affordable starting materials.

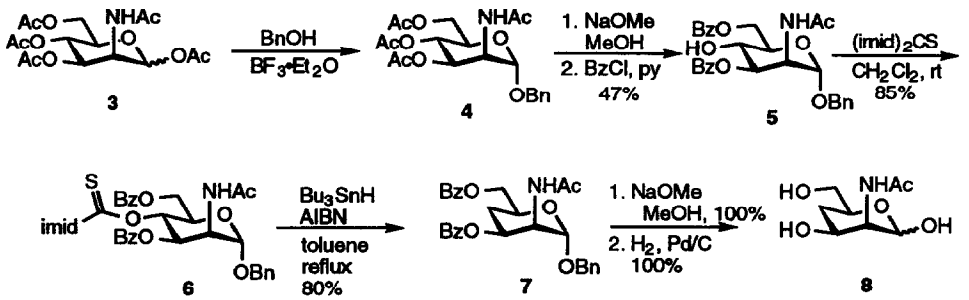


Scheme 1

As part of a program to study the substrate specificity of sialic acid aldolase, we were interested in the effect of modifications at the 4-position of ManNAc. The corresponding sialic acids, having modifications at the 7-position, can potentially be used to study the mechanism of a novel sialidase recently isolated from a species of leech.[5] This sialidase, rather than simply hydrolyzing the sialic acid glycoside, generates a bicyclic product in which the 7-OH has cyclized onto the putative oxonium intermediate.[6] We therefore enzymatically synthesized sialic acids with modifications at the 7-position for this purpose. 7-Deoxy-NeuAc[7] and 7-*O*-methyl-NeuAc[8] were chosen to study the effects of removal and capping of the 7-OH, respectively.

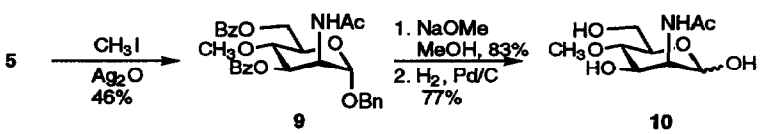
Results and Discussion

7-Deoxy- and 7-*O*-methyl-*N*-acetylneuraminic acid were synthesized from 4-deoxy-[9] and 4-*O*-methyl-*N*-acetyl-D-mannosamine,[8] respectively. The synthesis of 4-deoxy-*N*-acetyl-D-mannosamine (**8**) is outlined in Scheme 2. Compound **3**, prepared by acetylating ManNAc, was treated with benzyl alcohol and $BF_3 \cdot Et_2O$ to give the benzyl glycoside **4**.[10] Deacetylation and selective dibenzoylation[11] of **4** provided compound **5**. Deoxygenation of C-4 according to the procedure of Rasmussen[12] provided, *via* the imidazolide **6**, compound **7**. Finally, deprotection of all functionality afforded **8**.



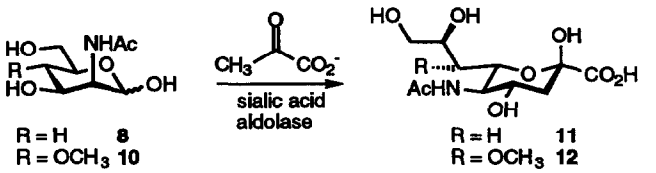
Scheme 2

The synthesis of 4-*O*-methyl-*N*-acetyl-D-mannosamine (**10**)[8] is shown in Scheme 3. The route utilized the common intermediate **5**.



Scheme 3

Finally, sialic acid aldolase-catalyzed aldol condensation of **8** and **10** with pyruvate provided **11** and **12** (Scheme 4). Both **8** and **10** were good substrates for the aldolase. Modifications at the 4-position of ManNAc appear to have very little or no effect on substrate binding and subsequent transformation. These results are in agreement with previously obtained data on the substrate specificity of sialic acid aldolase, which suggest that only the hydroxy group at C-3 of the acceptor substrate is of prime importance for binding to the enzyme.[3f,3h] The synthesis of the C-4-modified NeuAc derivatives illustrates the practicability of the enzymatic approach in the synthesis of biologically relevant sialic acids.



Scheme 4

Experimental

Phenylmethyl 3,4,6-Tri-*O*-acetyl-2-acetylamino-2-deoxy-α-D-mannopyranoside (4). A solution of **3** (8.85 g, 22.7 mmol), benzyl alcohol (8 mL) and BF_3•Et_2O (300 μL) in 50 mL CH_3NO_2 was heated to 80 °C for 1 h. The mixture was cooled to rt, added to 500 mL CH_2Cl_2, and washed with 200 mL saturated $NaHCO_3$. The organic layer was dried over $MgSO_4$, filtered, and concentrated under vacuum. The residue was chromatographed (silica gel, hexane/EtOAc 1:4 → EtOAc) to give 6.22 g (63%) of **4**. ^1H NMR (500 MHz, $CDCl_3$) δ 7.40-7.30 (m, 5 H, ArH), 5.73 (d, 1 H, J = 8.5 Hz, NH), 5.37 (dd, 1 H, J = 10.0, 4.5 Hz, H-3), 5.11 (t, 1 H, J = 10.0 Hz, H-4), 4.83 (d, 1 H, J = 1.5 Hz, H-1), 4.69 (app d, 1 H, J = 12.0 Hz, one of CH_2Ph), 4.66 (ddd, 1 H, J = 9.0, 4.5, 1.5 Hz, H-2), 4.55 (app d, 1 H, J = 12.0 Hz, one of CH_2Ph), 4.27 (dd, 1 H, J = 12.5, 5.0 Hz, H-6_a), 4.04-3.98 (m, 2 H, H-5, H-6_b), 2.13 (s, 3 H, Ac), 2.05 (s, 3 H, Ac), 2.04 (s, 3 H, Ac), 1.99 (s, 3 H, Ac); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.5, 170.0, 169.9, 135.8, 128.5, 128.2, 128.1, 98.0, 69.7, 69.1, 68.1, 65.9, 62.2, 50.2, 23.3, 20.7, 20.6; HRMS calcd for $C_{21}H_{28}NO_9$ (M + H^+): 438.1764, found: 438.1778.

Phenylmethyl 2-Acetylamino-3,6-di-*O*-benzoyl-2-deoxy-α-D-mannopyranoside (5). Compound **4** (6.15 g) and NaOMe (20 mg) were dissolved in MeOH (50 mL) and the resulting solution was stirred for 5 h. Dowex-50 (H^+) was added and the mixture was stirred for another 1 h. The mixture was filtered through Celite, and the filtrate was concentrated under vacuum to give phenylmethyl 2-acetylamino-2-deoxy-α-D-mannopyranoside (quantitative). ^1H NMR (500 MHz, $CDCl_3$) δ 7.40-7.25 (m, 5 H), 6.73 (m, 1 H), 4.81 (br s, 1 H), 4.66 (app d, 1 H, J = 12.0 Hz), 4.47 (app d, 1 H, J = 12.0 Hz), 4.46-4.32 (m, 1 H), 4.30 (br s, 1 H), 4.12-4.08 (m, 1 H), 4.07-4.99 (br s, 1 H), 3.95-3.90 (m, 1 H), 3.80-3.70 (m, 2 H), 3.66-3.61 (m, 1 H), 3.40-3.30 (br s, 1 H), 2.01 (s, 3 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.8, 136.7, 128.4, 127.9, 98.8, 72.2, 70.2, 69.3, 66.3, 60.7, 52.8, 22.9; HRMS calcd for $C_{15}H_{22}NO_6$ (M + H^+): 312.1447, found: 312.1460. Benzoyl chloride (2.8 mL, 2.2 eq) was added to a solution of the above triol (3.37 g, 10.8 mmol) in pyridine (10 mL) and CH_2Cl_2 (10 mL) at 0 °C. The reaction mixture was warmed to rt and stirred for

24 h, after which it was added to 150 mL CH_2Cl_2 and washed with 2 x 50 mL sat $NaHCO_3$. The organic layer was dried ($MgSO_4$), filtered, and concentrated under vacuum. The residue was chromatographed (SiO_2, hexane/EtOAc 4:6 to 25:75) to give 2.62 g (47%) of **5**. ^1H NMR (250 MHz, $CDCl_3$) δ 8.10-7.95 (m, 4 H, ArH), 7.65-7.20 (m, 11 H, ArH), 5.81 (d, 1 H, *J* = 9.4 Hz, NH), 5.50 (dd, 1 H, *J* = 4.4, 9.8 Hz, H-3), 4.84 (d, 1 H, *J* = 1.5 Hz, H-1), 4.82-4.69 (m, 2 H, H-2, H-6), 4.64 (AB, 2 H, *J* = 11.8 Hz, Δν = 57.0 Hz, OCH_2Ph), 4.63 (dd, 1 H, *J* = 2.3, 11.9 Hz, H-6'), 4.12 (ddd, 1 H, *J* = 2.3, 5.5, 9.8 Hz, H-5), 3.86 (dt, 1 H, *J* = 4.0, 9.8 Hz, H-4), 3.50 (d, 1 H, *J* = 4.3 Hz, OH), 1.98 (s, 3 H, Ac); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.8, 167.5, 166.6, 136.2, 133.4, 133.3, 129.9, 129.7, 129.3, 128.5, 128.4, 128.2, 128.1, 98.0, 73.4, 71.1, 69.4, 67.0, 63.6, 50.5, 23.3; HRMS calcd for $C_{29}H_{29}NO_8Na$ (M + Na^+): 542.1791, found: 542.1775.

Phenylmethyl 2-Acetylamino-3,6-di-*O*-benzoyl-2-deoxy-4-O-((1-imidazolido)-thiocarbonyl)-α-D-mannopyranoside (6). A solution of **5** (765 mg, 1.47 mmol) and thiocarbonyl-diimidazole (650 mg) in CH_2Cl_2 (15 mL was stirred at rt for 24 h. The reaction mixture was concentrated and chromatographed (SiO_2, hexane/EtOAc 4:6 to 3:7) to give 786 mg (85%) of **6**. ^1H NMR (250 MHz, $CDCl_3$) δ 8.26 (s, 1 H, imid-H), 8.06-8.00 (m, 2 H, ArH), 7.90-7.84 (m, 2 H, ArH), 7.61-7.27 (m, 12 H, ArH), 6.97 (s, 1 H, imid-H), 6.28 (t, 1 H, *J* = 9.9 Hz, H-4), 6.00 (d, 1 H, *J* = 9.2 Hz, NH), 5.89 (dd, 1 H, *J* = 4.2 Hz, H-3), 4.98-4.92 (m, 1 H, H-2), 4.94 (s, 1 H, H-1), 4.71 (AB, 2 H, *J* = 11.9 Hz, Δν = 51.8 Hz, OCH_2Ph), 4.59-4.42 (m, 3 H, H-5, H-6, H-6'), 2.00 (s, 3 H, Ac); ^{13}C NMR (125 MHz, $CDCl_3$) δ 183.4, 169.9, 165.9, 165.3, 135.77, 133.5, 133.4, 131.1, 129.7, 129.4, 129.39, 129.0, 128.7, 128.6, 128.4, 128.2, 117.9, 97.9, 75.6, 69.9, 69.8, 68.0, 62.8, 50.7, 23.2; HRMS calcd for $C_{33}H_{31}N_3O_8SCs$ (M + Cs^+): 762.0886, found: 762.0881.

Phenylmethyl 2-Acetylamino-3,6-di-*O*-benzoyl-2,4-dideoxy-α-D-*lyxo*-hexopyranoside (7). A solution of **6** (955 mg, 1.52 mmol), Bu_3SnH (670 mg), and AIBN (20 mg) in toluene (20 mL) was heated to reflux for 50 min. The mixture was cooled to rt, concentrated, and chromatographed (SiO_2, hexane/EtOAc1:1 to 4:6) to give 610 mg (80%) of **7**. ^1H NMR (250 MHz, $CDCl_3$) δ 8.10-8.05 (m, 2 H, ArH), 8.00-7.94 (m, 2 H, ArH), 7.63-7.27 (m, 11 H, ArH), 5.93 (d, 1 H, *J* = 9.4 Hz, NH), 5.59 (dt, 1 H, *J* = 4.5, 11.9 Hz, H-3), 4.91 (d, 1 H, *J* = 1.5 Hz, H-1), 4.67 (br d, 1 H, *J* = 3.8, 9.5 Hz, H-2), 4.64 (AB, 2 H, *J* = 11.8 Hz, Δν = 57.7 Hz, OCH_2Ph), 4.51-4.32 (m, 3 H, H-5, H-6, H-6'), 2.27-2.18 (m, 1 H, $H\text{-}4_{eq}$), 2.05 (s, 3 H, Ac), 1.41-1.25 (m, 1 H, $H\text{-}4_{ax}$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.7, 166.2, 165.6, 136.5, 133.3, 133.1, 129.9, 129.7, 129.6, 128.5, 128.4, 128.1, 128.0, 98.8, 69.2, 67.3, 66.1, 66.0, 48.5, 28.5, 23.5; HRMS calcd for $C_{29}H_{30}NO_7$ (M + H^+): 504.2022, found: 504.2025.

Phenylmethyl 2-Acetylamino-2,4-dideoxy-α-D-*lyxo*-hexopyranoside. A solution of **7** (610 mg, 1.21 mmol) and NaOMe (10 mg) was stirred at rt for 15 h. Solid NH_4Cl was added and the mixture was stirred for 30 min. The mixture was concentrated and chromatographed (SiO_2, $CHCl_3$/ MeOH 9:1 to 8:2) to give 357 mg (100%) of phenylmethyl 2-acetylamino-2,4-dideoxy-α-D-*lyxo*-hexopyranoside. ^1H NMR (300 MHz, $CDCl_3$) δ 7.39-7.26 (m, 5 H), 6.56 (d, 1 H, *J* = 8.4 Hz), 4.87 (s, 1 H), 4.57 (AB, 2 H, *J* = 11.9 Hz, Δν = 58.3 Hz), 4.38-4.28 (m, 2 H), 3.90 (m, 1 H), 3.77 -3.52 (m, 3 H), 3.02 (br s, 1 H), 2.12 (br s, 1 H), 2.04 (s, 3 H), 1.76-1.53 (m, 2 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.4, 136.9, 128.5, 128.0, 127.9, 98.2, 69.2, 65.0, 51.7, 30.0, 23.2; HRMS calcd for $C_{15}H_{22}NO_5$ (M + H^+): 296.1498, found: 296.1503.

2-Acetylamino-2,4-dideoxy-D-*lyxo*-hexopyranose (8). A solution of the above compound in MeOH (5 mL) was stirred with 5% Pd-C under 1 atm H_2 for 40 h. The mixture was filtered through Celite and

concentrated under vacuum to give 260 mg (quant) of **8**. ^{1}H NMR (300 MHz, D_2O) δ 4.90 (d, 0.5 H, J = 0.8 Hz, H-1$_\alpha$), 4.67 (d, 0.5 H, J = 1.6 Hz, H-1$_\beta$), 4.15-4.05 (m, 1 H), 4.00-3.83 (m, 1.5 H), 3.56-3.39 (m, 2.5 H), 1.89 (s, 1.5 H), 1.86 (s, 1.5 H), 1.62-1.50 (m, 1 H), 1.45-1.19 (m, 1 H).

Phenylmethyl 2-Acetylamino-3,6-di-*O*-benzoyl-2-deoxy-4-*O*-methyl-α-D-manno-pyranoside (9). A mixture of **5** (1.29 g, 2.49 mmol), CH_3I (1.1 mL), and Ag_2O (2.9 g) in DMF (12 mL) was stirred at rt for 24 h. The mixture was filtered through Celite, added to 150 mL EtOAc, and washed sequentially with 1 x 50 mL sat NaCl, 4 x 30 mL H_2O, and 1 x 30 mL sat NaCl. The organic layer was dried ($MgSO_4$), filtered, and concentrated under vacuum. The residue was chromatographed (SiO_2, hexane/EtOAc 1:1 to 3:7) to give 616 mg (46%) of **9**. $[\alpha]^{25}_D$ +65.2 ($CHCl_3$, c 1.15); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.12-8.07 (m, 2 H, ArH), 8.04-7.98 (m, 2 H, ArH), 7.65-7.28 (m, 11 H, ArH), 5.81 (d, 1 H, J = 9.3 Hz, NH), 5.61 (dd, 1 H, J = 4.1, 9.4 Hz, H-3), 4.86-4.79 (m, 2 H, H-1, H-2), 4.65 (AB, 2 H, J = 12.0 Hz, Δν = 57.3 Hz, OCH_2Ph), 4.64-4.58 (m, 2 H, H-6, H-6'), 4.12 (ddd, 1 H, J = 3.0, 4.8, 9.8 Hz, H-5), 3.57 (t, 1 H, J = 9.7 Hz, H-4), 3.50 (s, 3 H, OCH_3), 1.91 (s, 3 H, Ac); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.0, 166.0, 165.0, 133.4, 133.1, 129.5, 128.6, 128.5, 128.4, 128.1, 128.0, 97.8, 75.5, 72.6, 69.7, 69.3, 64.5, 60.6, 50.4, 23.3; HRMS calcd for $C_{30}H_{32}NO_8$ (M + H$^+$): 534.2128, found: 534.2119.

Phenylmethyl 2-Acetylamino-2-deoxy-4-*O*-methyl-α-D-mannopyranoside. A solution of **9** (6.07 mg, 1.14 mmol) and NaOMe (20 mg) in MeOH (12 mL) was stirred at rt for 24 h. Solid NH_4Cl was added and the mixture was stirred for 30 min, after which it was concentrated and chromatographed (SiO_2, $CHCl_3$/MeOH 9:1 to 85:15) to give 297 mg (83%) of phenylmethyl 2-acetylamino-2-deoxy-4-*O*-methyl-α-D-mannopyranoside. $[\alpha]^{25}_D$ +119 ($CHCl_3$, c 1.21); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40-7.28 (m, 5 H, ArH), 5.84 (d, 1 H, J = 8.2 Hz, NH), 4.86 (d, 1 H, J = 1.3 Hz, H-1), 4.58 (AB, 2 H, J = 11.9 Hz, Δν = 50.0 Hz, OCH_2Ph), 4.42 (dd, 1 H, J = 4.5, 9.4 Hz, H-3), 3.87-3.79 (m, 2 H, H-6, H-6'), 3.62 (dt, 1 H, J = 3.3, 9.9 Hz, H-5), 3.59 (s, 3 H, OCH_3), 3.29 (t, 1 H, J = 9.6 Hz, H-4), 2.06 (s, 3 H, Ac); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.2, 136.7, 136.7, 128.5, 128.0, 127.9, 98.2, 76.5, 71.3, 71.1, 69.3, 61.2, 60.8, 53.5, 23.3; HRMS calcd for $C_{16}H_{24}NO_6$ (M + H$^+$): 326.1604, found: 326.1599.

2-Acetylamino-2-deoxy-4-*O*-methyl-D-mannopyranose (10). A solution of the above compound (270 mg, 0.863 mmol) in MeOH (10 mL) was stirred with $Pd(OH)_2$ under 1 atm H_2 for 24 h. The mixture was filtered through Celite and concentrated. NMR analysis indicated the presence of a small amount of *N*-acetylglucosamine. Chromatography (SiO_2, $CHCl_3$/MeOH 8:2) afforded 157 mg (77%) of pure **10** (α/β 7:3). ^{1}H NMR (300 MHz, D_2O) δ 4.90 (d, 0.7 H, J = 1.5 Hz, H-1$_\alpha$), 4.80 (d, 0.3 H, J = 1.6 Hz, H-1$_\beta$), 4.23 (dd, 0.3 H, J = 1.4 Hz, 4.3 Hz, H-2$_\beta$), 4.11 (dd, 0.7 H, J = 1.6, 4.6 Hz, H-2$_\alpha$), 3.91 (dd, 0.7 H, J = 4.6, 9.7 Hz, H-3$_\alpha$), 3.71 (dd, 0.3 H, J = 4.5, 9.5 Hz, H-3$_\beta$), 3.70-3.59 (m, 3.3 H), 3.36 (s, 2.1 H, $OCH_{3\alpha}$), 3.35 (s, 0.9 H, $OCH_{3\beta}$), 3.23-3.06 (m, 1 H), 1.92 (s, 0.9 H, Ac$_\beta$), 1.88 (s, 2.1 H, Ac$_\alpha$).

Enzymatic Reactions. The aldolase reactions with **8** and **10** were carried out under the same condions as described previously[3d] using 7 equivalents of pyruvate. The resulting sialic acids **11** and **12** were obtained in 70% yield.

7-Deoxy-*N*-acetylneuraminic acid (11). ^{1}H NMR (500 MHz, D_2O) δ 3.95-3.88 (m, 2 H), 3.82-3.78 (m, 1 H), 3.54 (t, 1 H, J = 10.2 Hz), 3.51 (dd, 1 H, J = 11.7, 3.78 Hz), 3.38 (dd, 1 H, J = 11.7, 6.9 Hz), 2.22 (dd, 1 H, J = 13.1, 5.0 Hz), 1.99 (s, 3 H), 1.83 (dd, 1 H, J = 12.9, 11.8 Hz), 1.62-1.50 (m, 2 H); ^{13}C NMR (125 MHz, D_2O) δ 175.5, 175.2, 69.5, 68.6, 67.5, 66.5, 57.0, 39.9, 35.1, 22.9.

7-*O*-Methyl-*N*-acetylneuraminic acid (12). ^{1}H NMR (500 MHz, D_2O) δ 3.85 (app d, 1 H, J = 9.6 Hz), 3.80-3.71 (m, 2 H), 3.70-3.61 (m, 2 H), 3.45 (dd, 1 H, J = 11.7, 6.1 Hz), 3.27 (s, 3 H), 3.27-3.20 (m, 1 H), 2.01 (dd, 1 H, J = 13.4, 4.2 Hz), 1.89 (s, 3 H), 1.67 (t, 1 H, J = 11.7 Hz).

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