Combined Use of Subtilisin and *N*-Acetylneuraminic Acid Aldolase for the Synthesis of a Fluorescent Sialic Acid

Wolfgang Fitz and Chi-Huey Wong*

Department of Chemistry, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037

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Sialic acids, derivatives of *N*-acetylneuraminic acid (Neu5Ac), are important constituents of glycoproteins, gangliosides and oligosaccharides where they usually occur in the terminal position at the nonreducing end, bound in a α (2,3) or α (2,6) linkage to D-galactose or *N*-acetyl-D-galactosamine.[1] In mammalian brain tissues[2] and in capsular polysaccharides in *E. coli*[3] Neu5Ac forms homopolymers via α (2,8) linkages. Sialic acids participate in a variety of biological and pathological processes such as cell adhesion[4] and cell-virus interaction[5] and have been shown to occur at an elevated level in certain types of cancer cells.[6]

Fluorescence labeling has proven to be a useful tool in studies directed toward the elucidation of complex recognition phenomena in which sialic acids are involved. For example, fluorescent gangliosides have been used in monitoring the molecular organisation of glycolipids in membranes[7] and a sensitive fluorescence polarization assay for the binding of ligands to the viral surface protein hemagglutinin has been developed.[8] The standard procedure[7,9] for introducing a fluorescence probe into sialosides involves mild periodate oxidation of these compounds resulting in cleavage of the C(7)–C(8) bond of Neu5Ac and subsequent derivatisation of the newly formed aldehydo group at C(7) with a fluorescence marker.

Our approach, however, was to attach a fluorescent reporter group at an intact sialic acid which could then be be used as a building block in chemoenzymatic oligosaccharide synthesis.[10] The design of sialic acid **5** was based on the following considerations: The position of the attachment of the fluorophore was chosen to be the C(9) of Neu5Ac, because previous work[11] has shown that CMP-NeuAc synthetase, the enzyme needed for activation prior to transfer, and α (2,6) sialyl transferases tolerate modifications at this position. The dansyl group was used because its fluorescence intensity depends on the polarity of the environment which is of interest with regard to future fluorescence assays for the binding of sialic acid containing ligands to their receptors. Finally, the glycine linkage should give some flexibility to the large dansyl group.

We now wish to report a facile three step chemoenzymatic synthesis of fluorescent sialic acid **5** starting from *N*-acetyl-D-mannosamine **2** (Scheme 1). First, the BOC protected glycine linker was transferred onto ManNAc **2** in a transesterification reaction catalyzed by subtilisin BNP'. The reaction was performed in a mixture of 97% DMF and 3% aquous Tris buffer with four equivalents of the cyanomethylester **1** as the acylating agent. Acylation occurred in 65% yield regioselectively at the primary hydroxy group in **1** as indicated by the downfield shift of H-6 and H-6' in the ^{1}H NMR spectrum of the product **3**.[12]

Asymmetric aldol addition between ManNAc derivative **3** and pyruvate mediated by *N*-acetylneuraminic acid (Neu5Ac) aldolase (E.C. 4.1.3.3) afforded the Neu5Ac derivative **4** with the BOC-glycyl group at the desired C(9) position in 62% yield. Earlier work[11a,13] had already shown that the enzyme accepts 6-*O*-acetyl ManNAc and 6-*O*-lactyl ManNAc as the carbonyl component in the aldol reaction. However, **3** is the ManNAc derivative with the sterically most demanding substitution at C(6) which has to date been reported to be a substrate for Neu5Ac aldolase.

Finally, the *N*-protective group was removed by treating **4** with dilute acid and the resulting ammonium salt was sulfonylated with dansyl chloride after *in situ* deprotonation. Attempts to perform the second step in basic aquous solution with organic cosolvents[14] were not successful, since the ester functionality proved not to be compatible with these conditions. The best results were obtained when the sulfonylation was carried out with Na_2CO_3 in anhydrous DMF. Under these conditions the fluorescent sialic acid **5** was isolated in 47% yield after Bio Gel-P2 chromatography.

Overall, the described synthesis illustrates the advantages offered by the enzymatic approach in the synthesis of modified carbohydrates. Protection/deprotection steps are limited to a minimum and the generation of new chiral centers with defined sterochemistry allows an economical use of simple starting materials. We are currently evaluating the enzymatic activation and transfer of **5** onto oligosaccharides.

Experimental Section

General. BOC-glycine was purchased from Advanced ChemTech. ManNAc, sodium pyruvate and subtilisin BNP' (protease N, type XXVII) were obtained from Sigma. *N*-acetylneuraminic acid aldolase (E.C. 4.1.3.3) was purchased from Toyobo. Other chemicals and solvents were obtained from Aldrich. All ^{1}H NMR spectra were recorded at 400 MHz using TMS (in $CDCl_3$) or HDO (in D_2O, $\delta = 4.80$ ppm) as internal reference. ^{13}C NMR spectra

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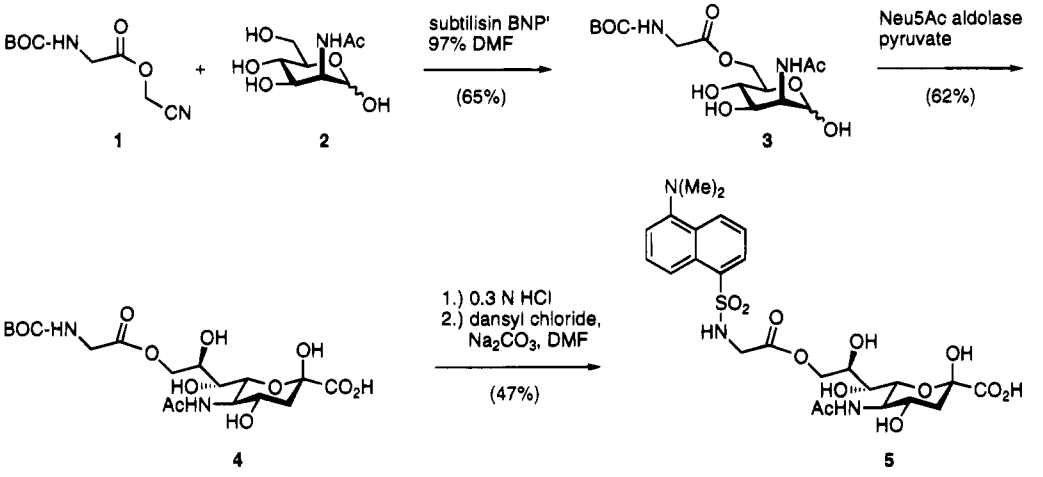
0022-3263/94/1959-8279$04.50/0

Scheme 1



were run at 100 MHz with $CDCl_3$ ($\delta = 77.00$) or CH_3CN (in D_2O, $\delta = 1.60$) as internal reference. J values are given in Hz. Flash chromatography was carried out with silica gel 60 (230–400 mesh) from Mallinckrodt.

***N*-(*tert*-Butoxycarbonyl)glycine Cyanomethyl Ester 1.** Compound **1** was synthesized following the procedure of Schwyzer et al.[15] ^{1}H NMR ($CDCl_3$) δ 5.17 (br s, 1 H), 4.80 (s, 2 H), 4.00 (br d, 2 H, $J = 5.9$), 1.46 (s, 9 H). ^{13}C NMR ($CDCl_3$) δ 169.15, 155.61, 113.92, 80.38, 48.75, 41.92. HRMS: calcd for $C_9H_{14}N_2O_4Na$ ($M + Na^+$) 237.0851; found: 237.0856.

2-Acetamido-6-*O*-(*N*-(*tert*-butoxycarbonyl)glycyl)-2-deoxy-D-mannopyranose 3. ManNAc **2** (0.97 g, 4.1 mmol) and cyanomethyl ester **1** (3.5 g, 16 mmol, 4 equiv) were dissolved in a mixture of 97% DMF and 3% aquous Tris buffer (0.5 M, pH 8.5). Subtilisin BNP′ (350 mg) was added and the suspension was vigorously stirred at rt for 3 d. The reaction mixture was concentrated under reduced pressure and chromatographed on silica gel (eluting with 12% MeOH in CH_2Cl_2 followed by 20% MeOH in CH_2Cl_2) to give compound **3** (1.0 g, 65%) as a white amorphous solid. ^{1}H NMR (D_2O) δ 5.16 (br s, 1 H_α), 5.07 (br d, 1 H_β, $J = 1.4$), 4.55–4.25 (m, 3 H), 4.45 (d, 1 H_β, $J = 5.1$), 4.35 (d, 1 H_α, $J = 4.6$), 4.11 (dd, 1 H_α, $J = 9.8, 4.8$), 3.99 (s, 2 H_β), 3.98 (s, 2 H_α), 3.88 (dd, 1 H_β, $J = 9.7, 4.5$), 3.69 (t, 1 H_α, $J = 9.8$), 3.56 (t, 1 H_β, $J = 9.8$), 2.15 (s, 3 H_β), 2.11 (s, 3 H_α), 1.49 (s, 9 H). ^{13}C NMR (D_2O) δ 176.48, 175.56, 173.16, 159.00, 93.88, 93.79, 82.48, 74.70, 72.55, 70.53, 69.26, 67.78, 67.64, 65.01, 54.69, 54.04, 42.77, 28.34, 22.87, 22.71. ratio α anomer:β anomer = 7:3. HRMS: calcd for $C_{15}H_{26}N_2O_9Na$ ($M + Na^+$) 401.1536; found 401.1547.

9-*O*-(*N*-(*tert*-butoxycarbonyl)glycyl)-*N*-acetylneuraminic Acid 4. To a solution of **3** (317 mg, 0.84 mmol) and sodium pyruvate (924 mg, 8.4 mmol) in 0.05 M potassium phosphate containing 1 mM dithiothreitol (pH 7.2, 8 ml) were added 10 units of Neu5Ac aldolase. The solution was allowed to stand at 37 °C for 4 d and then directly applied to a Bio Gel-P2 column (3 × 50 cm). Elution with water at 4 °C at a flow rate of 5 mL/h gave title compound **4** (184 mg, 47%). The mix fractions were purified by another gel filtration step to yield a second batch of pure **4** (55 mg, 14%). ^{1}H NMR (D_2O) δ 4.46 (br d, 1 H, $J = 11.6$), 4.27 (br dd, 1 H, $J = 11.4, 5.5$), 4.1–3.85 (m, 4 H), 3.93 (s, 2H), 3.58 (d, 1 H, $J = 9.3$), 2.23 (dd, 1 H, $J = 12.9, 4.8$), 2.08 (s, 3 H), 1.85 (t, 1 H, $J = 12.8$), 1.45 (s, 9 H). ^{13}C NMR (D_2O) δ 177.14, 175.44, 173.35, 158.99, 97.04, 82.42, 70.76, 69.13, 68.46, 67.99, 67.81, 52.97, 42.73, 40.09, 28.32, 22.88. HRMS: calcd for $C_{18}H_{30}N_2O_{12}Na$ ($M + Na^+$) 489.1696; found 489.1686.

9-*O*-(*N*-((5-(Dimethylamino)naphth-1-yl)sulfonyl)glycyl)-*N*-acetylneuraminic Acid 5. A solution of **4** (35 mg, 0.075 mmol) in 5 mL 0.3 N HCl was stirred at rt for 2.5 h and applied to a Bio Gel-P2 column (3 × 50 cm, eluting at 4 °C at a flow rate of 5 mL/h). The fractions containing the product were freeze dried and the residue was dissolved in DMF (2 mL). At 4 °C Na_2CO_3 (16 mg, 0.15 mmol) and dansyl chloride (20 mg, 0.075 mmol) were added. The reaction mixture was vigorously stirred at 4 °C for 12 h. Then the solvent was evaporated under reduced pressure and the residue was dissolved in 0.5 M sodium phosphate buffer (pH 6.8, 1 mL) and chromatographed on Bio Gel-P2 (3 × 50 cm) at 4 °C at a flow rate of 5 mL/h to afford title compound **5** (21 mg, 47%) as a green fluorescent solid. ^{1}H NMR (D_2O) δ 8.52 (d, 1 H, $J = 8.6$), 8.34 (d, 1 H, $J = 8.6$), 8.27 (d, 1 H, $J = 7.2$), 7.73 (t, 1 H, $J = 7.9$), 7.69 (t, 1 H, $J = 8.4$), 7.46 (d, 1 H, $J = 7.7$), 4.01 (ddd, 1 H, $J = 11.4, 9.3, 4.9$), 3.95–3.8 (m, 4 H), 3.90 (s, 2 H), 3.74 (ddd, $J = 9.0, 5.8, 2.7$), 3.44 (d, 1 H, $J = 9.3$), 2.90 (s, 6 H), 2.21 (dd, 1 H, $J = 12.9, 4.9$), 2.02 (s, 3 H), 1.83 (br t, 1 H, $J = 12.7$). ^{13}C NMR (D_2O) δ 177.36, 175.46, 171.48, 151.37, 134.31, 131.05, 130.47, 129.73, 129.59, 129.53, 124.70, 116.82, 97.10, 70.95, 70.68, 69.02, 68.22, 68.02, 67.68, 52.97, 45.69, 40.12, 22.86. HRMS: calcd for $C_{25}H_{33}N_3O_{12}SCs$ ($M + Cs^+$) 732.0839; found 732.0850.

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Supplementary Material Available: Copies of ^{1}H NMR spectra of **1**, **3**, **4**, and **5** (4 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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