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Synthesis and evaluation of *N*-acetylneuraminic acid-based affinity matrices for the purification of sialic acid-recognizing proteins

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The synthesis of 2-*S*-(2-aminoethyl) 5-acetamido-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (1) has been successfully achieved from the precursors methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate (2) and 2-bromo-*N*-(*tert*-butoxycarbonyl)-ethylamine (5). Compounds 1 and 2 were coupled, *via* amino and thioglycosidic linkages, respectively, to epoxy-activated Sepharose 6B. The resultant affinity adsorbents have proved efficient in purifying the sialic acid-recognizing enzyme *Vibrio cholerae* sialidase, in a one-step process with yields in the order of 60%.

***Keywords*: affinity chromatography, sialic acid-recognizing proteins, *Vibrio cholerae* sialidase, thiosialosides**

Introduction

Sialidases are a class of enzymes which cleave terminal sialic acid residues from glycoproteins and glycolipids and as such are involved in a large number of important biological processes [1–4]. Information, however, concerning their structure and many of their physiological functions is still limited, due primarily to the instability of these enzymes and the difficulty of their purification. In fact, most of the purification protocols employed to date are complicated and multi-step processes, typically requiring initial precipitation (with ammonium sulfate) of the enzyme followed by at least two individual chromatographic steps [5–10]. As part of our research program on sialic acid-recognizing proteins (SARP's), in particular sialidases, we required a quick and efficient purification method of a large amount of these important proteins. Affinity chromatography has long been considered to be the most specific method for protein purification, since it is based on the unique specificity inherent in a ligand-biomacrocmolecule interaction [11]. Several affinity adsorbents for sialidases have been described [5–10, 12–19], and many of these are based on immobilizing some form of the natural substrate (i.e., synthetic *N*-acetylneuraminic acid derivatives [5–7, 12–14] or glycoproteins which contain *N*-acetylneuraminic acid residues [8, 15–17]) onto a chromatographic support. However, a number of these affinity adsorbents suffer from either a lack of specificity or from the hydrolysis of the immobilized ligand [9, 10, 12, 16, 18, 19]. Suzuki *et al.* [5] have reported a method which goes some way to overcoming these limitations. These workers report the preparation of an affinity adsorbent made by the attachment of *N*-acetylneuraminic acid (Neu5Ac) to epoxy-activated Sepharose 4B through a thioglycosidic linkage [5]. Since thiosialosides are thought to be resistant to hydrolysis by sialidases [20] (Pegg MS, Wilson JC, Kiefel MJ, Laver WG, von Itzstein M, unpublished results), affinity matrices based on such ligands should be stable to the enzymes being purified. Indeed, the affinity matrix prepared by Suzuki and coworkers was able to adsorb the overexpressed *Clostridium perfringens* sialidase, and gave a purification of 21% over three steps [5].

Our interest in affinity chromatography stems from our continued investigations into the chemistry and biochemistry of sialic acids and their associated proteins [21, 22], as well as our studies into the synthesis of novel α-thioketosides of *N*-acetylneuraminic acid [23–26]. In particular, we have been interested in the influence of spacer arm length on the efficacy of the matrix in the purification of a sialidase. This paper describes the synthesis of the α-thiosialoside, 2-*S*-(2-aminoethyl) 5-acetamido-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (**1**) and its linkage, through the amino group, to epoxyactivated Sepharose 6B. In addition, the preparation of an affinity matrix which contains *N*-acetylneuraminic acid directly attached, through a thioglycosidic linkage, to epoxy-activated

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Sepharose 6B is also described. The efficient purification of the sialic acid-recognizing protein, *Vibrio cholerae* sialidase, has been achieved using both of these affinity matrices.

Materials and methods

General methods

Melting points were determined on a Mettler FP21 hot-stage melting point apparatus and are uncorrected. Infrared spectra were recorded on a Hitachi 270-30 infrared spectrophotometer. Optical rotations were measured using a Jasco DIP-370 polarimeter. ^{1}H and ^{13}C (JMOD) NMR spectra were recorded using a Brüker AM 300WB or 500WB multinuclear magnetic resonance spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to Me_4Si for $CDCl_3$ and external reference for D_2O. Mass spectra were obtained using either a Jeol JMS-DX 300 mass spectrometer (LRFABMS; thioglycerol-glycerol matrix and HRFABMS; polyethylene glycol matrix) or a Micromass Platform II spectrometer (LRESIMS). All solvents were distilled prior to use or were of analytical grade. Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-*galacto*-2-nonulopyranosonate (**2**) was synthesized according to published procedure [27]. Dowex-50W × 8 (H^+) resin was obtained from Aldrich Chemical Company, Inc. Epoxy-activated Sepharose 6B was purchased from Pharmacia. Column chromatography was performed using Merck silica gel 60 (0.040–0.063 mm). Thin layer chromatography (TLC) was performed on aluminium plates coated with silica gel 60 F_{254} (Merck). 4-Methylumbelliferyl-α-D-*N*-acetylneuraminic acid (MUN) was synthesized according to published procedure [28].

Synthesis of affinity ligands

2-Bromo-N-*(*tert-*Butyloxycarbonyl)-ethylamine (**5**)*

2-Bromoethylamine hydrobromide (2.16 g 11 mmol) was dissolved in water (10 ml), and the pH of the solution was adjusted to 9 with Et_3N. To this solution at 0 °C was added di-*tert*-butyl dicarbonate (2.26 g, 11 mmol) in CH_3CN (10 ml) and the pH of the solution monitored and kept at 9 by the addition of Et_3N. The reaction was allowed to stir at room temperature for 24 h, before being concentrated under reduced pressure. The crude product was diluted with CH_2Cl_2 (50 ml), washed with 1 M HCl (50 ml), dried (Na_2SO_4) and concentrated under reduced pressure. Column chromatography on silica gel (EtOAc : hexane; 1 : 3; R_f 0.75) gave the title compound **5** (1.72 g, 73%) as an amorphous mass; mp 25–26°C (dec); ν_{max} (NaCl) 3360, 2984, 2940, 550 cm^{-1}; ^{1}H NMR (300 MHz; $CDCl_3$) : δ 1.43 (9H, s, $C(CH_3)_3$), 3.41–3.52 (4 H, m, H-1′, H-2′), 5.03, (1 H, bs, N*H*); ^{13}C NMR (75.5 MHz; $CDCl_3$) : δ 28.5 ($C(CH_3)_3$), 32.6 (C-2), 42.5 (C-1), 79.9 ($C(CH_3)_3$), 155.8 (NC(O)O); LRFABMS; *m/z* 226 [(^{81}Br M + 1)$^+$, 38%], 224 [(^{79}Br M + 1)$^+$, 39], 198 (43), 196 (47), 170 (100), 168 (100), 126 (34), 124 (37).

*Methyl [2-*S*-(2-*N*-tert-butyloxycarbonyl-aminoethyl) 5-acetamido-4,7,8,9-tetra-*O*-acetyl 3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (**6**)*

Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate (**2**) (0.7 g, 1.27 mmol) and **5** (0.27 g, 1.27 mmol) were dissolved in dry *N*,*N*-DMF (8 ml) at room temperature under N_2, Et_2NH (3.2 ml) was added and the reaction stirred for 3 h at room temperature. The mixture was concentrated under reduced pressure and the residue diluted with EtOAc (50 ml) and washed with pH 4 buffer (50 ml), H_2O (2 × 50 ml), dried (Na_2SO_4) and evaporated to dryness under reduced pressure. Column chromatography on silica gel (EtOAc; R_f 0.31) gave **6** (0.64 g, 77%) as an amorphous mass; mp 69–70 °C; $[\alpha]_D^{28}$ + 13.5° (*c* 1, $CHCl_3$); ν_{max} (KBr) 3456, 1744, 1690, 1608, 1226, 1032 cm^{-1}; ^{1}H NMR (300 MHz; $CDCl_3$) : δ 1.45 (9 H, s, $C(CH_3)_3$), 1.88 (3 H, s, AcN), 2.03, 2.05, 2.16, 2.19 (12 H, 4 × s, 4 × AcO), 2.64–2.82 (3 H, m, H-3e/H-2′), 3.25–3.32 (2 H, m, H-1′), 3.80 (3 H, s, CO_2Me), 3.83 (1H, dd, $J_{6,7}$ 1.9 Hz, H-6), 4.02 (1 H, ddd, $J_{5,4} = J_{5,6} = J_{5,NH} = 10.2$ Hz, H-5), 4.05 (1 H, dd, $J_{9a,9b}$ 12.2, $J_{9a,8}$ 5.4 Hz, H-9a), 4.44 (1 H, dd, $J_{9b,8}$ 2.7 Hz, H-9b), 4.87 (1 H, ddd, $J_{4,3a}$ 11.2, $J_{4,3e}$ 4.7 Hz, H-4), 5.13 (1 H, d, NH), 5.30 (1 H, dd, $J_{7,8}$ 9.1 Hz, H-7), 5.37 (1 H, ddd, H-8); ^{13}C NMR (75.5 MHz; $CDCl_3$) : δ 20.8, 20.9, 21.3 (4 × OC(O)*Me*), 23.2 (NC(O)*Me*), 28.4 ($C(CH_3)_3$), 28.9 (C-2′), 38.2 (C-3), 41.1 (C-1′), 49.3 (C-5), 53.1 (CO_2Me), 62.6 (C-9), 67.2, 68.3 (C-7/C-8), 69.7 (C-4), 74.2 (C-6), 79.2 ($C(CH_3)_3$), 83.1 (C-2), 155.9 (NC(O)O), 168.8 (C-1), 170.2, 170.3, 170.4, 170.9 (4 × O*C*(O)Me/N*C*(O)Me); LRFABMS : *m/z* 651 [(M + 1)$^+$, 33%], 551 (100), 414 (100).

Analytical data. Calculated for $C_{27}H_{42}N_2O_{14}S{\cdot}H_2O$: C, 48.50; H, 6.63; N, 4.19. Found : C, 48.14; H, 6.51; N, 3.76%.

*2-*S*-(2-*N*-tert-butyloxycarbonyl-aminoethyl) 5-acetamido-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (**7**)*

Compound **6** (0.2 g, 0.32 mmol) was treated with a solution of NaOMe (0.16 mmol) in anhydrous MeOH (10 ml) at room temperature under N_2. After stirring for 2 h, the MeOH was removed under reduced pressure, H_2O (10 ml) added and the pH adjusted to 12 with 0.1 M NaOH. After stirring overnight, the pH of the reaction mixture was adjusted to 7.0–7.5 with Dowex-50W × 8 (H^+) resin. After filtration, the filtrate was lyophilized to afford the target compound **7** as an amorphous white solid which was in a pure state according to NMR spectroscopy (0.15 g, 99%); mp 175 °C (dec); R_f 0.62 (EtOAc: ^{i}PrOH : H_2O; 2 : 3 : 1); $[\alpha]_D^{28}$ − 5.0° (*c* 0.6, H_2O); ν_{max} (KBr) 3416, 1692, 1276, 1030 cm^{-1}; ^{1}H NMR (300 MHz; D_2O): δ 1.56 (9 H, s, $C(CH_3)_3$), 1.89 (1 H, dd, $J_{3a,3e}$ 12.3, $J_{3a,4}$ 11.9 Hz, H-3a), 2.18 (3 H, s, AcN), 2.87–3.03 (3 H, m, H-3e/H-2′), 3.39–3.45 (2H, m, H-2′), 3.69–3.85 (4 H, m, H-4/H-6/H-7/H-9a), 3.91–4.01 (3 H, m, H-5/H-8/H-9b); ^{13}C NMR (75.5 MHz; D_2O): δ 24.7 (NC(O)*Me*), 30.3 ($C(CH_3)_3$), 32.3 (*C*-2′), 42.7, 43.6 (C-3/C-1′), 54.4 (C-5), 65.2 (C-9), 70.7 (C-7*), 71.2 (C-8*),

74.5 (C-4), 77.5 (C-6*), 83.9 ($C(CH_3)_3$), 88.7 (C-2), 160.9 (N*C*(O)O), 176.8 (C-1), 177.7 (N*C*(O)Me); LRFABMS; *m/z* 491 [$(M + Na)^+$, 38%], 469 [$(M + 1)^+$, 15], 369 (31), 201 (100).

*2-*S*-(2-Aminoethyl) 5-acetamido-3,5-dideoxy-2-thio-*D*-*glycero*-α-*D*-galacto-2-nonulopyranosidonic acid (**1**)*

To a solution of **7** (0.15 g, 0.32 mmol) in CH_3COOH (0.9 ml, 15.7 mmol) was added $BF_3 \cdot OEt_2$ (10 µl, 0.79 mmol), and the mixture stirred for 4 h at room temperature under N_2. The solution was diluted with H_2O (20 ml) and the pH was adjusted to 7 with Et_3N. The solution was then lyophilized and purified by reverse phase HPLC (H_2O as eluent) to afford the target compound **1** (76 mg, 64%) as a white amorphous mass; mp 210 °C (dec); R_f 0.12 (EtOAc : ^{i}PrOH : H_2O; 2 : 3 : 1); $[\alpha]_D^{28}$ + 52.6° (*c* 0.5, H_2O); ν_{max} (KBr) 3404, 1620, 1374, 1078 cm^{-1}; ^1H NMR (500 MHz; D_2O): δ 1.75 (1 H, t, $J_{3a,3e} = J_{3a,4} = 12.0$ Hz, H-3a), 1.98 (3 H, s, AcN), 2.76 (1 H, dd, $J_{3e,4}$ 5.0 Hz, H-3e), 2.88–2.97 (2 H, m, H-1′), 3.13–3.23 (2 H, m, H-2′), 3.52–3.56 (2 H, m, H-6/H-7), 3.58 (1 H, dd, $J_{9a,9b}$ 12.0, $J_{9a,8}$ 6.5 Hz, H-9a), 3.65 (1 H, ddd, $J_{4,5}$ 10.5 Hz, H-4), 3.72 (1 H, ddd, $J_{8,7}$ 7.4, $J_{8,9b}$ 1.5 Hz, H-8), 3.77 (1 H, t, $J_{5,6}$ 10.5 Hz, H-5), 3.81 (1 H, dd, H-9b); ^{13}C NMR (75.5 MHz; D_2O): δ 25.8 (NC(O)*Me*), 31.2 (C-2′), 43.5 (C-1′), 45.1 (C-3), 55.9 (C-5), 66.8 (C-9), 72.2 (C-4/C-7*), 76.1 (C-6*), 79.2 (C-8), 90.7 (C-2), 177.4 (C-1), 178.9 (NH*C*(O)Me); LRFABMS: *m/z* 391 [$(M + Na)^+$, 4%], 369 [$(M + 1)^+$, 32], 338 (17), 312 (29);

Analytical data. Calculated for $C_{13}H_{24}N_2O_8SNa \cdot 4H_2O$: C, 33.76; H, 6.76; N, 6.06. Found: C, 33.82; H, 6.64; N, 6.04%; HRFABMS: $C_{13}H_{25}N_2O_8S$ requires 369.13315, found 369.13564.

*Methyl [2-*S*-(2-hydroxy-3-phenoxypropyl) 5-acetamido-4, 7, 8, 9-tetra-*O*-acetyl 3,5-dideoxy-2-thio-*D*-glycero-α-galacto-2-nonulopyranosid]onate (**9**)*

To a solution of **2** (100 mg, 0.18 mmol) in dry *N*,*N*-DMF (2 ml) at 0 °C under N_2 was added 1,2-epoxy-3-phenoxypropane (30 µl, 0.22 mmol) and then Et_2NH (1 ml). The reaction was stirred for 0.5 h at 0 °C before removal of the Et_2NH (water pump). The residue was poured into 1 M HCl (10 ml), extracted with EtOAc (3 × 10 ml), the combined EtOAc extracts washed with H_2O (2 × 20 ml), dried (Na_2SO_4) and concentrated under reduced pressure. Chromatography on silica gel ($CHCl_3$: MeOH; 25 : 1 R_f 0.3) gave **9** (100 mg, 83%) as a mixture of diastereomers; ν_{max} (KBr) 3400 (br), 1740, 1664, 1549, 1440, 1369, 1228, 1033 cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$; other diastereomer given in parentheses where visible): δ 1.87 (1.88) (3 H, s, AcN), 2.01 (2.02), 2.04 (2.05), 2.13 (2.14), 2.18 (2.19) (12 H, 4 × s, 4 × AcO), 2.74 (2.83) (1 H, dd, $J_{3e,3a}$ 12.7, $J_{3e,4}$ 4.6 Hz, H-3e), 2.95–3.01 (2 H, m, H-1′), 3.79 (3.80) (3H, s, CO_2Me), 3.83–3.86 (1 H, m, H-6), 4.00–4.12 (5H, m, H-5/H-9a/H-2′/H-3′), 4.28 (1 H, dd, $J_{9b,9a}$ 12.5, $J_{9b,8}$ 2.5 Hz, H-9b), 4.85–4.92 (1H, m, H-4), 5.18 (1H, d, $J_{NH,5}$ 9.9 Hz, NH), 5.31 (1H, dd, $J_{7,8}$ 8.8, $J_{7,6}$ 1.8 Hz, H-7), 5.41 (1H, ddd, $J_{8,9}$ 5.3 Hz, H-8), 6.93–6.97 (3H, m, Ph), 7.26–7.29 (2H, m, Ph); ^{13}C NMR (75.5 MHz; $CDCl_3$): δ 20.6, 20.7, 21.1, 21.2 (4 × OC(O)*Me*), 23.1 (NC(O)*Me*), 31.9 (C-1′), 37.8 (37.9) (C-3), 49.0 (49.2) (C-5), 53.1 (53.3) (CO_2Me), 62.1 (62.2) (C-9), 66.9, (67.0), 67.5 (68.0), 69.2 (69.3), 69.4 (69.7), 74.0 (75.0) (C-2′/C-4/C-6/C-7/C-8), 70.0, (70.1) (C-3′), 82.4 (82.8) (C-2), 114.6, 120.9, 129.3 (CH, Ph), 158.5 (158.6) (ipso Ph) 168.5 (168.8) (C-1), 170.0, 170.1, 170.4 (170.5), 170.6 (170.7), 170.8 (4 × O*C*(O)Me/N*C*(O)Me); LRESIMS: *m/z* 680 [$(M + Na)^+$, 100%], 658 [$(M + 1)^+$, 75] 530 (48), 508 (70), 414 (37), HRESIMS: $C_{29}H_{40}NO_{14}S$ requires 658.21695, found 658.21791.

Preparation of affinity matrices

*Amine linkage (**8**)*

Epoxy-activated Sepharose 6B was hydrated and washed with distilled H_2O as per manufacturers instructions. The ligand **1** (40 mg for each 1 ml of swollen matrix) was dissolved in enough 0.4 M Na_2CO_3 (pH 13.0) to allow adequate mixing and then combined with the swollen matrix. The pH was checked and adjusted if needed. The suspension was mixed at 21 ± 2 °C for 24 h on a rotating platform. After coupling, the Sepharose was collected by filtration and uncoupled **1** was washed away with 0.4 M Na_2CO_3 (pH 13.0) followed by distilled H_2O[§]. The excess epoxy groups on the Sepharose were blocked with ethanolamine (1.0 M, pH 8.0) for 16 h at 35 °C, and then the matrix washed with three cycles of 0.1 M Tris (pH = 8 by adding HCl) containing 0.5 M NaCl followed by 0.1 M NaOAc (pH 4.0) + 0.5 M NaCl. It was then equilibrated with 50 mM MES buffer (pH 5.6) containing 0.02% aq. NaN_3 and stored at 4 °C until use. (This cycle of washing was also used to regenerate the column after use in the purification of *V. cholerae* sialidase.)

*Thioglycoside linkage (**10**)*

Epoxy-activated Sepharose 6B (12 g) was washed with dry *N*,*N*-DMF (5 × 50 ml), before being added to **2** (200 mg, 0.36 mmol) in *N*,*N*-DMF (15 ml) under N_2. The mixture was cooled to 0 °C, Et_2NH (2 ml) added and the mixture shaken for 1 h at 0 °C and 1 h at 20 °C. The Sepharose was collected by filtration[¶], washed with *N*,*N*-DMF (30 ml)[¶] and H_2O (500 ml). The resulting adsorbent was then treated with 0.1 M NaOH (15 ml) in *N*,*N*-DMF (20 ml) for 5 h at room temperature. After filtration and washing with H_2O (500 ml) the

* Tentative assignments.

[§] The binding of **1** onto the Sepharose was determined by measuring the absorbance at 220 nm against a standard curve of known amounts of **1** in 0.4 M Na_2CO_3 (pH 13.0), and was estimated to be 15%.

[¶] The *N*,*N*-DMF filtrate (from the coupling reaction and washing) was poured into 1 M HCl (50 ml) and extracted with EtOAc (3 × 50 ml), and the combined EtOAc extracts washed with H_2O (2 × 50 ml), dried (Na_2SO_4) and concentrated. Chromatographic analysis of this EtOAc extract revealed the presence of methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate (64 mg, 0.13 mmol), which corresponds to a coupling efficiency of about 65%.

adsorbent was treated with ethanolamine and washed as described above for **8**.

Expression of *Vibrio cholerae* sialidase

The *nanH* gene of *V. cholerae* sialidase, kindly supplied by E. Vimr (University of Illinois, Urbana, Illinois), was expressed in *E. coli* as described by Vimr *et al.* [29] and Taylor *et al.* [30]. Briefly, the *E. coli* cell line HB101 transformed with pCVD364 was grown overnight in 2YT culture at 37 °C with vigorous shaking. The expressed sialidase was extracted by the osmotic shock procedure. The final pellet, containing the sialidase activity, was resuspended in 50 mM NaOAc (pH 5.2, 4 ml), $CaCl_2$ (9 mM), NaCl (154 mM) and 0.05% NaN_3 for storage at 4 °C.

Affinity chromatography

All purification procedures were carried out at 4 °C. The *V. cholerae* sialidase was exchanged into 50 mM MES buffer (pH 5.6) + $CaCl_2$ (6 mM) and loaded onto affinity column **8** equilibrated with the same buffer. Unbound proteins were eluted with 50 mM MES buffer (pH 5.6) + $CaCl_2$ (6 mM) until the protein concentration came down to base line. The bound sialidase was then eluted with 50 mM MES (pH 5.6) + 1.0 M NaCl + 0.01% Triton X-100 + 1 mM EDTA. Five ml fractions were collected at the rate of 0.75 ml min^{-1}. The protein concentration was followed by absorbance at 280 nm and the sialidase activity was assayed using the fluorescence substrate MUN as described below. Fractions were pooled, concentrated and run on 12.5% SDS-PAGE. protein were visualised by silver staining. A parallel experiment was performed without adding $CaCl_2$ to the loading buffer.

Sialidase assay

Sialidase activity was assayed using a modification [21] of a fluorometric method previously described [31] and MUN was used as substrate in NaOAc buffer (pH 5.6). $CaCl_2$ at 24 mM was added when the buffer system contained EDTA (1 mM), and at 6 mM when the buffer system did not contain EDTA. Results were calculated from a 4-methylumbelliferone (MU) standard curve. One unit of activity was defined as the amount of enzyme which catalyse 1 μmol of MU cleaved per hour.

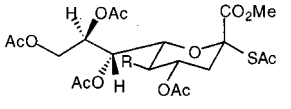
K_m and K_i determinations

The K_m of *V. cholerae* sialidase for the substrate MUN was determined using the sialidase assay mentioned above with a varying amount of substrate. Substrate concentrations ranged from 0 to 150 mM MUN. The K_m was calculated using a double reciprocal Lineweaver-Burk plot. The inhibition constant (K_i) for **1** against *V. cholerae* sialidase was determined by Dixon plot at two different MUN concentrations, 100 mM and 350 mM MUN and various inhibitor concentrations [32].

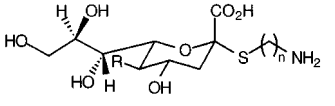
Results and discussion

Synthesis of affinity ligands

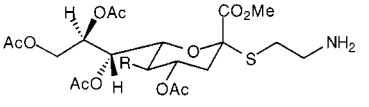
We have previously reported [23–26] a mild and efficient method for the synthesis of thioglycosides of *N*-acetylneuraminic acid. In its simplest terms, this method involves the selective *in situ* thiodeacetylation of the 2-thioacetyl Neu5Ac derivative **2** using diethylamine, and coupling of the resultant thiolate with activated acceptors such as simple alkyl halides, activated carbohydrates and nucleosides [23–26]. For the preparation of affinity matrices suitable for the purification of sialidases, we initially decided to synthesise α-thiosialosides such as **3**, since the terminal amine substituent should allow efficient coupling of the thiosialoside to a chromatographic support. In this regard we attempted an Et_2NH promoted [23] coupling between methyl 5-acetamido-4, 7, 8, 9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate (**2**) (prepared according to published procedure [27]) and commercially available 2-bromoethylamine hydrobromide. However this reaction failed to furnish any of the α-thiosialoside **4**, presumably due to the presence of the hydrobromide salt.



(2) R = NHAc



(3) R = NHAc



(4) R = NHAc

In an attempt to overcome this problem, the amino group was protected by reaction of an aqueous solution of 2-bromoethylamine hydrobromide with di-*tert*-butyl dicarbonate in CH_3CN at pH 9 to give the Boc protected amine **5** in 73% yield after purification. Exposure of an *N*,*N*-DMF solution of the 2-thioacetyl Neu5Ac derivative **2** and the Boc protected amine **5** to Et_2NH resulted in smooth formation of the novel α-thiosialoside **6** in 77% yield (Figure 1). The ^1H n.m.r. spectrum of **6** is consistent with the structure shown. In particular, the presence of a two proton multiplet centred at δ 2.82 (H-1′) is indicative of a CH_2 attached to sulfur [33]. Deacetylation and subsequent saponification of the α-thiosialoside **6** furnished **7** in near quantitative yield (99%). Deprotection of the aglycon alkylamine in **7** was achieved with $BF_3{\cdot}Et_2O$ in CH_3COOH and gave the desired α-thiosialoside **1** (Figure 1) in 64% yield after HPLC purification. The thiosialoside **1**, which contains the

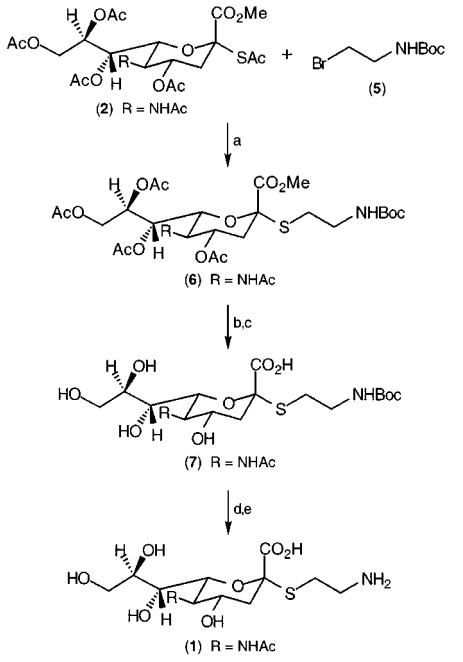


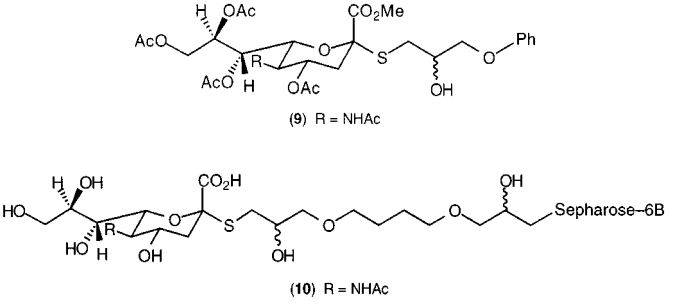
Figure 1. Synthesis of the affinity ligand **1**. (a) DMF, Et_2NH, 77%; (b) NaOMe, MeOH; (c) H_2O, NaOH, 99%; (d) CH_3COOH, $BF_3 \cdot Et_2O$; (e) Et_3N, pH = 7, 64%.

appropriate functionality to facilitate attachment to a chromatographic support, is formed in an overall 49% yield from readily accessible starting materials.

Preparation of affinity matrices

Coupling of the Neu5Ac-α-thioketoside **1** to epoxy-activated Sepharose 6B was achieved under alkaline conditions, using a procedure similar to that recommended by the manufacturer (see Materials and Methods), to give the affinity matrix **8** (Figure 2). The affinity matrix **8** is stable when stored at 4 °C in a convenient buffer containing 0.02% aq. NaN_3, and can be easily regenerated for repetitive use by three cycles of washing with 0.1 M Tris (pH 8.0) containing 0.5 M NaCl followed by 0.1 M NaOAc (pH 4.0) containing 0.5 M NaCl.

The report by Suzuki *et al.* [5] involving the direct attachment of Neu5Ac to a chromatographic support *via* a thioglycosidic linkage, prompted us to explore the possibility of using our Et_2NH promoted [23] coupling of 2-thioacetyl Neu5Ac derivatives (e.g., **2**) to epoxy-activated Sepharose 6B. Of particular interest was the opportunity to further expand the scope of this Et_2NH promoted coupling reaction in the synthesis of novel sialic acid derivatives as potential biological probes. In contemplating such an approach, consideration had to be given to the reactivity of the thiolate generated from **2** towards epoxides. While Suzuki *et al.* successfully employed the sodium methoxide promoted [27, 34] coupling of **2** to epoxy-activated Sepharose 4B [5], the question remained as to the reactivity of the Et_2NH generated thiolate of **2** towards epoxides. In an attempt to address this issue, the 2-thioacetyl Neu5Ac derivative **2** was exposed to 1, 2-epoxy-3-phenoxypropane in the presence of Et_2NH and *N*,*N*-DMF. After only 0.5 h at 0 °C TLC analysis of the reaction mixture showed the coupling was complete, and the α-thiosialoside **9** was obtained in 83% yield after purification. Secure in the knowledge that the Et_2NH promoted coupling of **2** with epoxides was possible, the coupling of **2** with epoxy-activated Sepharose 6B was performed under conditions similar to those employed for the synthesis of **9** (see Materials and Methods). After coupling, the ester groups on Neu5Ac were deprotected using 0.1 M



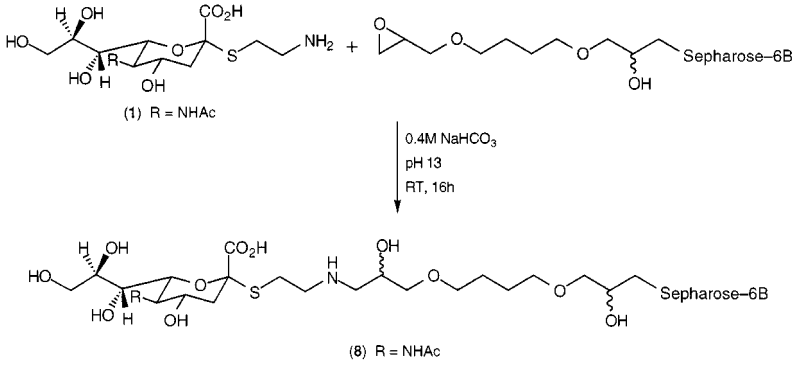


Figure 2. Synthesis of affinity matrix **8**.

NaOH, and then any unreacted epoxy groups on the Sepharose were blocked by exposure to ethanolamine (1.0 M). The resulting affinity matrix **10** shows characteristics similar to those of the matrix **8** (*vide infra*), including long term storage and repeated use.

Purification of *Vibrio cholerae* sialidase using affinity matrices

The K_m of *Vibrio cholerae* sialidase to 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid (MUN) was calculated to be 80 μM from the double reciprocal Lineweaver-Burk plot. The K_i of the Neu5Ac α-thioketoside of **1** to *V. cholerae* sialidase was in the order of 4×10^{-5} M. This good binding affinity suggests that **1** is a suitable candidate for an affinity adsorbent to purify *V. cholerae* sialidase.

It has been demonstrated [35] that *V. cholerae* sialidase requires Ca^{2+} for enzyme activity and has been suggested that the divalent metal cation is involved in the stabilization of the enzyme-substrate complex. We have found that the binding of *V. cholerae* sialidase onto affinity matrix **8** was dependent upon the presence of Ca^{2+} in the buffer system. In the absence of $CaCl_2$, the sialidase was only weakly bound on this column, and when eluted with 1 M NaCl/buffer, was contaminated with the bulk of *E. coli* proteins (Figure 3A). The addition of 6 mM $CaCl_2$ to the equilibrated buffer increased the binding of the *V. cholerae* sialidase to the affinity column **8**, with only 7% of the enzyme activity lost in the wash step. Elution of *V. cholerae* sialidase from the column required the addition of 1 mM EDTA to the elution buffer in order to chelate the Ca^{2+} away from the enzyme-substrate complex. Fractions containing 1 mM EDTA were assayed in the presence of 24 mM $CaCl_2$ to reverse the inhibitory effect of EDTA on the enzyme activity. The results obtained from affinity column **8**, presented in Figure 3B, show that *V. cholerae* sialidase is

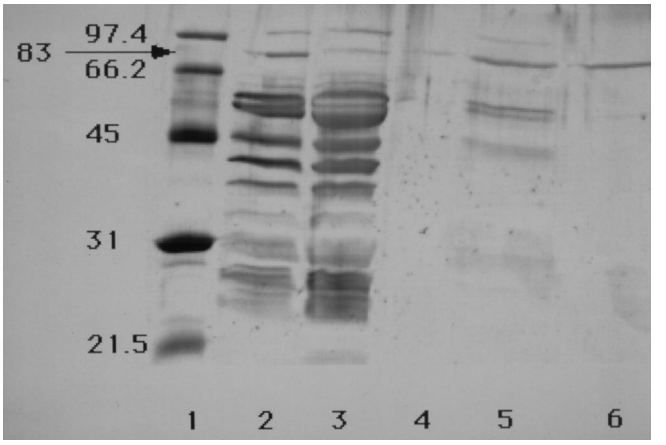


Figure 4. 12.5% SDS-PAGE of the results obtained using affinity adsorbent **8**. lane 1: MW standard (in kDa); lane 2: Load on the column; lane 3: flow through; lane 4: wash step; lane 5: fractions 1 and 2 eluted with 1 M NaCl containing 1 mM EDTA; lane 6: fractions 3 to 8 eluted with 1 M NaCl containing 1 mM EDTA. 83 kDa is the MW of purified protein corresponding to the *V. cholerae* sialidase activity recovered after elution.

obtained free of bulk protein, with a recovery rate of 60%. SDS-PAGE visualized with silver stain (Figure 4) showed one single band (lane 6) with an apparent molecular weight at approximately 83 kDa. Similar recovery rate and purity of sialidase were obtained when affinity matrix **10**, which contains Neu5Ac thio-linked directly to Sepharose 6B, was used for the purification of *V. cholerae* sialidase. Affinity matrices **8** and **10** can be used repeatedly without any overall loss of efficiency, which suggests that the immobilized ligands are not cleaved by *V. cholerae* sialidase during the purification process.

Conclusion

The one step purification and efficient recovery of highly purified *V. cholerae* sialidase using these chromatographic

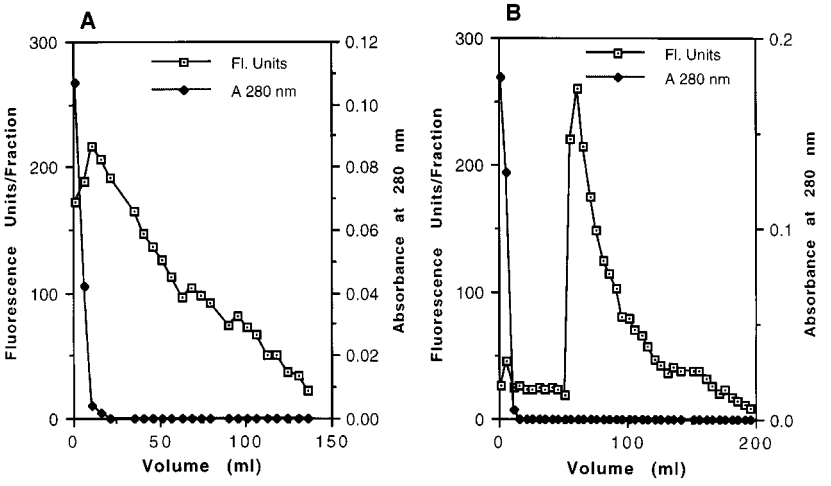


Figure 3. Chromatography of *V. cholerae* sialidase on the affinity adsorbent **8**, (5 ml fractions).(**A**) without $CaCl_2$; (**B**) the loading buffer contained 6 mM $CaCl_2$ and the elution buffer contained 1 mM EDTA.

supports demonstrates the efficiency of using such affinity matrices in purifying sialidases. The high specificity of sialic acid-recognizing proteins, including sialidases, towards sialic acid derivatives suggests that affinity matrices such as those described herein will become increasingly important in studies involving sialic acid-recognizing proteins. We are currently testing the matrices described here in the purification of other sialidases, including mammalian, as well as sialyltransferases. The results presented show no significant differences in the specificity of the matrices **8** and **10** towards *V. cholerae* sialidase, despite small differences in the length of spacer between the immobilized ligands and the Sepharose. We are however continuing to explore the effects of the length of the spacer between the immobilized ligand and the chromatographic support, with a view to establishing the most efficient type of affinity matrix for sialidases and other sialic acid-recognizing proteins.

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