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Carbohydrate Research 322 (1999) 201–208

CARBOHYDRATE RESEARCH

The synthesis and evaluation of novel sialic acid analogues bound to matrices for the purification of sialic acid-recognising proteins

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Received 31 March 1999; accepted 8 September 1999

Abstract

A novel *N*-acetylneuraminic acid analogue, 2-*S*-(5′-aminopentyl) 5-acetamido-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid, as well as the thiosialoside 2-*S*-(2′-aminoethyl) 5-acetamido-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid, have been synthesised and successfully coupled to CNBr-activated Sepharose 4B through the terminal amino group. The resultant affinity resins have proved efficient in purifying a number of sialic acid-recognising proteins such as *Vibrio cholerae* sialidase, sialidase-L from leech, *trans*-sialidase from *Trypanosoma cruzi*, and sialyltransferases from rat liver, all in high yield.

Keywords: Sialic acid-recognising proteins; Sialidases; Thiosialosides; Affinity chromatography

1. Introduction

In an effort to learn more about sialic acid-recognising proteins (SARPs) and their involvement in a large number of important biological processes [1,2], we have been interested over the past few years in developing new and rapid methods for the purification of such proteins. The ultimate aim of this investigation was to supply enough purified quantities of these important proteins for structure determination and functional studies. Affinity chromatography has always been considered the method of choice when a suitable adsorbent is available [3]. Our interest in sialidases and other SARPs has led us to develop the synthesis of a number of thiosialosides as potential inhibitors of SARPs [4–7]. Since thiosialosides are reported to be resistant to hydrolysis by sialidases [8], affinity matrices based on such ligands should, in principle, be refractory to these enzymes during purification.

We have recently reported [9] the synthesis of a novel thioketoside of *N*-acetylneuraminic acid and described its linkage to epoxy-activated Sepharose 6B. The resultant affinity adsorbent has proved efficient in purifying the overexpressed *Vibrio cholerae* sialidase (*Vc* sialidase) in a one-step purification process. The overall yield of the coupling of this ligand to the resin was not particularly satisfactory (about 15%). This led us to investigate other adsorbent supports such as CNBr-activated Sepharose 4B. In this paper, we describe the

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0008-6215/99/$ - see front matter
PII: S0008-6215(99)00249-9

synthesis and coupling of the novel thiosialosides, 2-*S*-(aminoalkyl) 5-acetamido-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acids (**1**) and (**3**). The evaluation of these affinity matrices for the purification of a range of SARPs is also presented.

2. Experimental

General methods.—Melting points were determined on a Mettler FP21 hot-stage melting point apparatus and are uncorrected. IR 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 either Bruker AM 300WB or 500WB multinuclear magnetic resonance spectrometers. Chemical shifts (δ) are reported in parts per million (ppm) relative to Me_4Si for $CDCl_3$ and external reference for D_2O. Where tentative assignments have been made, this has been indicated in the text as *. Mass spectra were obtained using either a Jeol JMS-DX 300 mass spectrometer (LRMS; thioglycerol–glycerol matrix and HRMS; polyethylene glycol matrix) or a Micromass Platform II spectrometer (LRMS). All solvents were distilled prior to use or were of analytical grade. pH 4 buffer was purchased from Activon and prepared as per manufacturer's instructions. Dowex-50W × 8 (H^+) resin was obtained from Aldrich Chemical Company, Inc. CNBr-activated Sepharose 4B was purchased from Pharmacia. Column chromatography was performed using E. 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} (E. Merck). High-performance liquid chromatography (HPLC) purification was performed on a Waters 600E system controller, using reversed-phase C_{18} Waters μBondapak™ columns (analytical: 8 × 100 mm, part no. WAT085721; preparative: 25 × 100 mm, part no. WAT038505), detecting at 230 nm with a Waters 484 tunable absorbance detector. 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**) [10], 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (MUN) [11], 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid (Neu5Ac2en) [12], and 2-*S*-(2′-aminoethyl) 5-acetamido-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (**3**) [9] were synthesised according to published procedures.

Synthesis of affinity ligands -N-*(*tert-*butyloxycarbonyl)aminopentanol.*—5-Aminopentanol (1.00 g, 9.7 mmol) was dissolved in water (6 mL), and was cooled to 0 °C. To this solution was added di-*tert*-butyl dicarbonate (2.04 g, 9.7 mmol) in CH_3CN (6 mL), and the pH of the solution was monitored and kept at pH 12 by the addition of Et_3N. The reaction was allowed to stir at room temperature (rt) for 12 h, then a further amount of di-*tert*-butyl dicarbonate (2.04 g, 9.7 mmol) was added. After stirring for a further 4 h at rt, the reaction mixture was concentrated under reduced pressure. The crude product was diluted with CH_2Cl_2 (25 mL), washed with 1 M HCl (25 mL), dried (Na_2SO_4), and concentrated under reduced pressure to give a clear oil (1.95 g, 99%), which was in a pure state according to ^{1}H NMR spectroscopy. (EtOAc; R_f 0.68); ν_{max} (NaCl) 3376, 2984, 2944, 1690 cm^{-1}; ^{1}H NMR (300 MHz; $CDCl_3$): δ 1.37 (s, 9 H, $C(CH_3)_3$), 1.26–1.58 (m, 6 H, H-2, 3, 4), 3.04, (t, 2 H, $J_{5,4}$ 6.9 Hz, H-5), 3.55 (t, 2 H, $J_{1,2}$ 6.5 Hz, H-1); ^{13}C NMR (75.5 MHz; $CDCl_3$): δ 23.0 (C-3), 28.5 ($C(CH_3)_3$), 29.8 (C-4), 32.2 (C-2), 40.7 (C-5), 62.4 (C-1), 79.2 ($C(CH_3)_3$), 156.3 (NC(O)O); LRMS: m/z 204 [$(M+1)^+$, 39%], 148(100), 104(100). HRMS (positive-ion): Calcd for $C_{10}H_{22}NO_3$ 204.15997; Found: 204.15894.

5-Bromo-N-*(*tert-*butyloxycarbonyl)-pentylamine* (**4**).—A solution of triphenylphosphine (1.84 g, 6.89 mmol) in CH_2Cl_2 (12 mL) was added to a stirred suspension of *N*-bromosuccinimide (1.31 g, 7.38 mmol) in CH_2Cl_2 (12 mL). After stirring for 5 min at rt, pyridine (0.24 mL, 2.95 mmol) was added dropwise, followed by slow addition of the alcohol (0.5 g, 2.46 mmol). After stirring for 8 h at rt, the reaction mixture was poured into saturated $NaHCO_3$ solution (25 mL), and the organic phase was washed with water (25 mL), brine

(25 mL), dried (Na_2SO_4) and concentrated under reduced pressure. Column chromatography on silica gel (1:2 EtOAc–hexane, R_f 0.63) gave **4** (0.34 g, 52%) as an amorphous pink mass; mp 26–27 °C (dec); ν_{max} (NaCl) 3368, 2984, 2940, 1688 cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$): δ 1.41 (s, 9 H, $C(CH_3)_3$), 1.41–1.53 (m, 4 H, H-4, 3), 1.84 (p, 2 H, $J_{2,1}$ 6.5 Hz, H-2), 3.09 (q, 2 H, $J_{1,2}$ 6.5 Hz, H-1), 3.38 (t, 2 H, $J_{5,4}$ 6.8 Hz, H-5), 4.63 (bs, 1 H, N*H*); ^{13}C NMR (75.5 MHz; $CDCl_3$): δ 25.5 (C-3), 28.5 ($C(CH_3)_3$), 29.4 (C-4), 32.4 (C-2), 33.8 (C-5), 40.4 (C-1), 79.2 ($C(CH_3)_3$), 156.1 (NC(O)O); LRMS: *m/z* 268 [(^{81}Br M + 1)$^+$, 38%], 266 [(^{79}Br M + 1)$^+$, 39%], 212 (43), 210 (47), 192 (32), 168 (15). HRMS (positive-ion): Calcd for $C_{10}H_{21}NO_3^{79}Br$ 266.07556; Found: 266.07574.

Methyl [2-S-(5′-N-tert-butyloxycarbonyl-aminopentyl) 5-acetamido-4,7,8,9-tetra-O-acetyl 3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid]onate (**5**).—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 **4** (0.34 g, 1.27 mmol) were dissolved in dry DMF (8 mL) at rt under N_2, Et_2NH (3.2 mL) was added, and the reaction mixture was stirred for 3 h at rt. The mixture was concentrated under reduced pressure and the residue was diluted with EtOAc (50 mL) and was washed with pH 4 buffer (50 mL), water (2 × 50 mL), dried (Na_2SO_4) and evaporated to dryness under reduced pressure. Column chromatography on silica gel (EtOAc, R_f 0.57) gave **5** (0.71 g, 80%) as an amorphous mass; mp 61–64 °C; $[\alpha]_D^{28}$ + 23.5° (*c* 1, $CHCl_3$); ν_{max} (KBr) 3408, 1740, 1688, 1228, 1030 cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$): δ 1.39 − 1.59 (m, 6 H, H-2′, 3′, 4′), 1.44 (s, 9 H, $C(CH_3)_3$), 1.88 (s, 3 H, AcN), 1.89–2.03 (m, 1 H, H-3a), 2.03, 2.04, 2.15, 2.17 (4 × s, 12 H, 4 × AcO), 2.49–2.59 (m, 1 H, H-1a′), 2.67–2.79 (m, 2 H, H-1b′, 3e), 3.11 (q, 2 H, $J_{5',4'}$ 6.2 Hz, H-5′), 3.80 (s, 3 H, CO_2Me), 3.83 (dd, 1 H, $J_{6,7}$ 2.0 Hz, H-6), 4.02–4.34 (m, 2 H, H-5, 9a), 4.32 (dd, 1 H, $J_{9b,9a}$ 9.9, $J_{9b,8}$ 2.7 Hz, H-9b), 4.76 (bs, 1 H, NHC(O)O), 4.87 (ddd, 1 H, $J_{4,3a}$ 11.0, $J_{4,5}$ 10.5, $J_{4,3e}$ 4.6 Hz, H-4), 5.11 (d, 1 H, $J_{NH,5}$ 10.0 Hz, NH), 5.31 (dd, 1 H, $J_{7,8}$ 8.6 Hz, H-7), 5.37 (ddd, 1 H, $J_{8,9a}$ 5.2 Hz, H-8); ^{13}C NMR (75.5 MHz; $CDCl_3$): δ 20.9, 21.0, 21.3 (4 × OC(O)*Me*), 23.3 (NC(O)*Me*), 26.1 (C-3′), 28.6 ($C(CH_3)_3$), 28.8 (C-4′), 29.1 (C-2′), 29.5 (C-1′), 38.3 (C-3), 41.1 (C-5′), 49.6 (C-5), 53.1 (CO_2Me), 62.5 (C-9), 67.7, 68.9 (C-7/C-8), 69.9 (C-4), 74.3 (C-6), 83.4 (C-2), 156.2 (NC(O)O), 168.7 (C-1), 170.4, 170.8, 171.1 (4 × O*C*(O)Me/N*C*(O)Me); LRMS: *m/z* 693 [(M + 1)$^+$, 10%], 593(40), 414(44).

Anal. Calcd for $C_{30}H_{48}N_2O_{14}S{\cdot}H_2O$: C, 50.69; H, 7.09; N, 3.94. Found: C, 50.26; H, 6.76; N, 3.94.

2-S-(5′-N-tert-butyloxycarbonyl-aminopentyl) 5-acetamido-2-thio-D-glycero-α-D-galacto-2-nonulopyranosidonic acid (**6**).—Compound **5** (0.61 g, 0.88 mmol) was treated with a soln of NaOMe (0.44 mmol) in anhyd MeOH (12 mL) at rt under N_2. After stirring for 2 h, the MeOH was removed under reduced pressure, water (12 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 lyophilised to afford the target compound **6** as an amorphous cream solid, which was in a pure state according to NMR spectroscopy (0.37 g, 83%); mp 148 °C (dec); (2:3:1 EtOAc–^{i}PrOH–H_2O, R_f 0.72); $[\alpha]_D^{28}$ + 21.7° (*c* 0.5, H_2O); ν_{max} (KBr) 3448, 1616, 1366, 1032 cm^{-1}; ^1H NMR (300 MHz; D_2O): δ 1.41–1.58 (m, 4 H, H-3′, 4′), 1.50 (s, 9 H, $C(CH_3)_3$), 1.63–1.74 (m, 2 H, H-2′), 1.81 (dd, 1 H, $J_{3a,3e}$ 12.0, $J_{3a,4}$ 11.9 Hz, H-3a), 2.11 (3 H, s, AcN), 2.64–2.82 (m, 2 H, H-1′), 2.87 (dd, 1 H, $J_{3e,4}$ 4.7 Hz, H-3e), 3.10–3.14 (t, 2 H, $J_{5',4'}$ 6.6 Hz, H-5′), 3.60–3.81 (m, 4 H, H-4, 6, 7, 9a), 3.84–3.95 (m, 3 H, H-5, 8, 9b); ^{13}C NMR (75.5 MHz; D_2O): δ 25.2 (NC(O)*Me*), 28.5 (C-3′), 31.0 ($C(CH_3)_3$), 31.6 (C-4′), 32.1 (C-2′), 32.5 (C-1′), 43.1, 44.3 (C-3, 5′), 54.9 (C-5), 65.7 (C-9), 71.3 (C-7*), 71.6 (C-8*), 74.1 (C-4), 77.9 (C-6*), 83.8 ($C(CH_3)_3$), 89.4 (C-2), 161.0 (N*C*(O)O), 177.2 (C-1), 178.1 (N*C*(O)Me); LRMS: *m/z* 533 [(M + Na)$^+$, 33%], 455(31).

2-S-(5′-aminopentyl) 5-acetamido-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosidonic acid (**1**).—Compound **6** (0.25 g, 0.49 mmol) in trifluoroacetic acid (8 mL) was stirred at rt for 10 min. The reaction mixture was diluted with toluene and concentrated

under reduced pressure. The crude product was taken up in toluene twice more to remove any residual trifluoroacetic acid. The crude product was purified by reverse-phase HPLC (3% MeCN in H_2O as eluant) to afford the target compound **1** (170.5 mg, 85%) as a white amorphous mass; mp 192–194 °C (dec); (3:1 ^{i}PrOH–H_2O, R_f 0.43); $[\alpha]_D^{28}$ +44.8° (*c* 0.56, H_2O); ν_{max} (KBr) 3412, 1626, 1374, 1124 cm^{-1}; ^1H NMR (500 MHz; D_2O): δ 1.41–1.52 (m, 2 H, H-3′), 1.61–1.79 (m, 5 H, H-2′, 3a, 4′), 2.10 (s, 3 H, AcN), 2.63–2.69 (m, 1 H, H-1a′), 2.73–2.79 (m, 1 H, H-1b′), 2.82 (1 H, dd, $J_{3e,3a}$ 13.0, $J_{3e,4}$ 4.8 Hz, H-3e), 3.0 (t, 2 H, $J_{5',4'}$ 7.5 Hz, H-5′), 3.58–3.72 (m, 4 H, H-4, 6, 7, 9a), 3.80–3.85 (m, 3 H, H-5, 8, 9b); ^{13}C NMR (75.5 MHz; D_2O): δ 23.1 (NC(O)*Me*), 27.2 (C-3′, 4′), 38.3 (C-2′), 39.6 (C-3), 40.4 (C-5′), 41.9 (C-1′), 52.8 (C-5), 64.8 (C-9), 69.2, 69.4 (C-4, 7*), 73.0 (C-6*), 75.9 (C-8), 86.9 (C-2), 174.9 (C-1), 176.1 (NH*C*(O)Me); LRMS: *m/z* 433 [$(M+Na)^+$, 10%], 411 [$(M+1)^+$, 23], 383 (100), 237 (38); HRMS (positive-ion): Calcd for $C_{16}H_{31}N_2O_8S$ 411.18011; Found: 411.18095.

Preparation of affinity matrix.—CNBr Sepharose 4B (3 g) was hydrated and swollen in 1 mM HCl as per the manufacturer's instructions. The ligand **1** or **3** (see Scheme 2) (2.2 or 2.5 mg, respectively, for each 1 mL of swollen matrix) was dissolved in enough coupling buffer, 0.1 M $NaHCO_3$ (pH 9), to allow adequate mixing and then combined with the swollen matrix. The suspension was mixed at rt for 2–16 h on a rotating platform. After coupling, the Sepharose was collected by filtration and uncoupled **1** or **3** was washed away with 0.1 M $NaHCO_3$ (pH 9.0). The residual active groups on the Sepharose were blocked with a large excess of 0.1 M Tris–HCl (pH 8.0) for 2 h at rt. The matrix was then washed with three cycles of 0.1 M Tris (pH 8) containing 0.5 M NaCl, followed by 0.1 M NaOAc (pH 4.0) + 0.5 M NaCl. The affinity matrix **7** or **8** (see Scheme 2) was then equilibrated with a suitable buffer 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 any SARPs.) The amount of **1** or **3** bound to the Sepharose was determined by measuring the absorbance at 220 nm against a standard curve of known amounts of **1** or **3** in 0.1 M $NaHCO_3$ (pH 9), and was estimated to be over 75% based on recovered starting material (**1** or **3**).

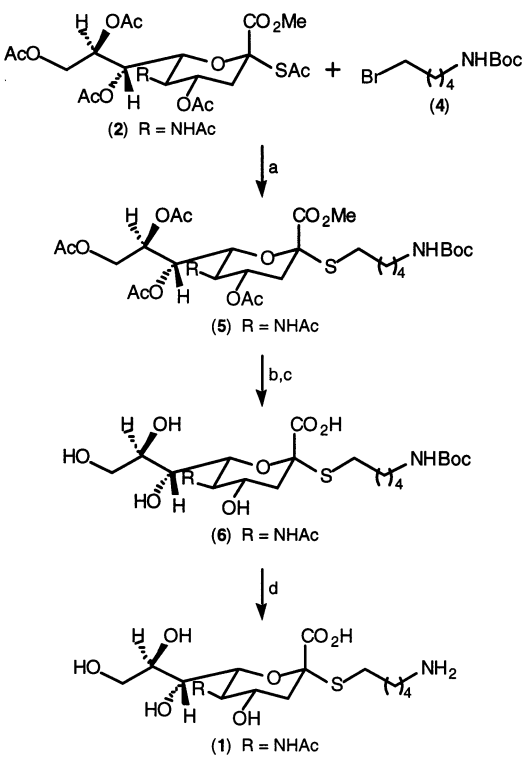
Affinity chromatography.—All purification procedures were carried out at 4 °C. For each affinity purification experiment 3 mL of affinity matrix was used. The *nanH* gene of *Vc* sialidase, kindly provided by E. Vimr (University of Illinois, Urbana, IL) was overexpressed and purified under similar conditions to those described before [9]. The recombinant sialidase-L [13], kindly supplied by Yu-Teh Li (Tulane University, School of Medicine, New Orleans, LA), was exchanged into 20 mM sodium phosphate buffer pH 6.8 containing 1 mM EDTA before being loaded onto the affinity column previously equilibrated with the same buffer. The enzyme was eluted with the same buffer containing 1 M NaCl. *trans*-Sialidase from *Trypanosoma cruzi* [14], kindly provided by M.E.A. Pereira (New England Medical Center Hospital, Department of Medicine, Boston, MA), was exchanged into 50 mM cacodylic acid (Na salt) buffer pH 6.9 and then loaded onto the affinity column equilibrated with the same buffer. The enzyme was then eluted with the equilibrating buffer in the presence of 1 M NaCl. Rat liver was used as the source for sialyltransferases where Golgi-enriched fractions were prepared according to Leelavathi et al. [15]. This crude enzyme preparation as well as the affinity column were equilibrated in 50 mM cacodylic acid (Na salt) buffer pH 6.5. A discontinuous gradient of varying concentrations (0.1–1 M) of NaCl was used to elute enzyme activity. For all of the above-mentioned enzymes, 4 mL fractions were collected at the rate of 0.75 mL/min. The protein concentration was followed by absorbance at 280 nm and the enzyme activities were assayed as described below. Fractions were pooled, concentrated and run on 12.5% SDS-PAGE. Proteins were visualised by silver staining.

Enzymes activity assays.—*Vibrio cholerae* sialidase was assayed as previously described [9] using MUN as substrate. MUN was also the substrate of choice for sialidase-L following a published assay [16]. For both enzymes,

activities were calculated from a 4-methylumbelliferone (MU) standard curve. One unit of activity was defined as the amount of enzyme that catalyses 1 μmol of MU cleaved/h. *trans*-Sialidase was assayed following a previously published method [14] using [^{14}C] *N*-acetyllactosamine (Sigma) as the acceptor substrate and 2,3-sialyllactose as the specific donor substrate for this enzyme. Sialyltransferases were assayed following the general procedure published by Harvey and Thomas [17]. The donor substrate for this assay was [^{14}C] CMPNeu5Ac (NEN Chemicals) and asialofetuin was used as the general acceptor substrate.

3. Results and discussion

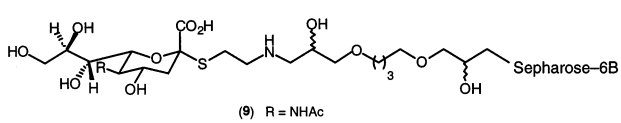
Synthesis of affinity ligands.—We have previously reported [9] a mild and efficient method for the synthesis of the thiosialoside **3**. This method involves the selective in situ thiodeacetylation of the 2-thioacetyl Neu5Ac precursor **2** using diethylamine, and coupling of the resultant thiolate with activated acceptors such as simple alkyl halides, activated carbohydrates and nucleosides [4–7,9]. In this report we treated the 2-thioacetyl derivative **2** in a solution of DMF and Et_2NH with the Boc-protected amine **4** to isolate the novel α-thiosialoside **5** in 80% yield (Scheme 1). Deacetylation and subsequent saponification of the α-thiosialoside **5** furnished **6** in 83% yield. Deprotection of the aglycon alkylamine **6** with $BF_3{\cdot}Et_2O$ in CH_3COOH gave the desired product **1**. However, difficulties were encountered in removing the triethylammonium acetate salt from the crude mixture after HPLC purification. In an attempt to overcome this problem, the Boc-protected α-thiosialoside **6** was treated with neat TFA to furnish the deprotected product **1**. The crude mixture was successfully purified by HPLC to isolate the pure product **1** in 80% yield.

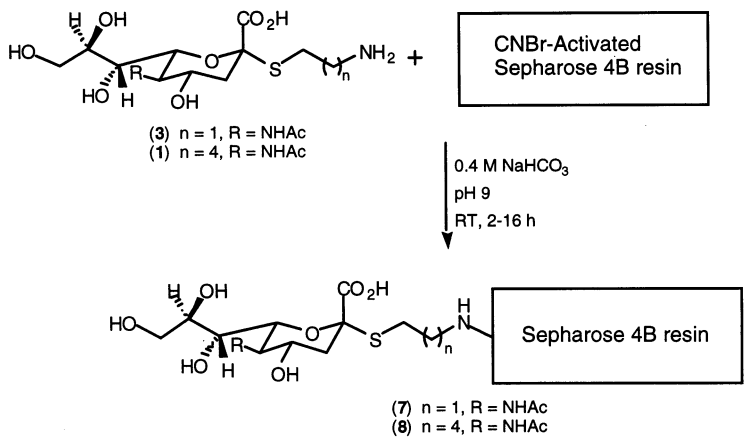


Scheme 1. Synthesis of the affinity ligand **1**: (a) DMF, Et_2NH, 80%; (b) NaOMe, MeOH; (c) H_2O, NaOH, 83%; (d) CF_3COOH, 85%.

Preparation of affinity matrices.—The coupling of the thiosialosides **1** or **3** to CNBr-activated Sepharose 4B (see Section 2) was achieved in excellent yields ($>75\%$ based on recovered starting material) (Scheme 2). The prepared affinity matrices **7** and **8** were found to be stable when stored at 4 °C in a convenient buffer containing 0.02% aqueous NaN_3. Both matrices were successfully reused several times without any significant changes in the efficiency of the purification process or the quality of the purified protein.

SARP purification.—Initially, we were interested in comparing the efficiency of the new affinity matrix **7** to affinity matrix **9** that was previously prepared by coupling **3** with epoxy-activated Sepharose 6B [9]. *Vc* sialidase was used as a benchmark for this comparative study. This study demonstrated that affinity matrix **7** not only has a higher capacity (more than 20-fold) to bind *Vc* sialidase than affinity matrix **9** [9], but is also significantly more efficient in purifying *Vc* sialidase in a one-step purification procedure. Surprisingly, and contrary to our earlier observations [9], no calcium (Ca^{2+}) was needed for the efficient binding of the enzyme to the matrix **7**. Identical results were obtained when matrix **8** was





Scheme 2. Synthesis of affinity matrices **7** and **8**.

employed. Why a requirement for Ca^{2+} has been influenced in a resin change is difficult to rationalise, although it is noteworthy that the epoxy-activated Sepharose 6B affinity matrix **9** has a long spacer arm (12 atoms), while the CNBr-activated Sepharose 4B affinity matrices **7** and **8** have no spacer arm. This fact may provide one possible explanation for this altered Ca^{2+} requirement, although it is equally likely that the high number of available sites on these new affinity adsorbents **7** and **8** (coupling efficiency $>75\%$) and presumably improved overall binding capacity have also influenced the requirement for Ca^{2+}.

Vc sialidase was readily purified by use of either matrix, **7** or **8**, free of other contaminating proteins, with a recovery of 65%. Only 4% of the activity was found in the wash (Table 1, only data for matrix **7** shown). SDS-PAGE visualised with silver stain showed one single protein band with an apparent molecular weight of approximately 83 kD, which is consistent with previous literature reports [9]. In an attempt to investigate the nature of the binding of *Vc* sialidase to the affinity adsorbent, the known competitive sialidase inhibitor Neu5Ac2en was used at micromolar concentrations to elute the enzyme from the column. Under these conditions Neu5Ac2en was able to elute the *Vc* sialidase, indicating that the nature of the binding to the matrix was indeed of an affinity type. These results demonstrate that both affinity matrices **7** and **8** are highly efficient in purifying *Vc* sialidase. The single difference between ligands **1** and **3** is that compound **1** has three extra carbons on the aglycon side chain, making the spacer in matrix **8** slightly longer than that in matrix **7**. This small difference in spacer arm length, on this occasion, does not appear to influence either the quality or the yield of the purified sialidase enzyme.

Based on the above results, we were interested in investigating the scope of these new matrices in the purification of other SARPs. Thus, we selected three other SARPs, namely sialyltransferase, sialidase-L and *trans*-sialidase from *T. cruzi*, which are the subject of our continuing research interest in this family of proteins and affinity matrix **7** to provide a starting point for these investigations. The results from this investigation are summarised in Table 1 and further discussed below.

Rat liver sialyltransferase.—Purification of a sialyltransferase activity from rat liver-derived Golgi-enriched fractions using affinity matrix **7** led to $>47\%$ of enzyme activity originally loaded onto the column being located in the eluted fractions (Table 1). Elution

Table 1
Activity (%) recovered in the fractions after purification by affinity matrix **7**

	Activity (%)	
	Eluted fractions	Wash fractions
Vc sialidase	65	4
Sialyltransferases	47	<5
Sialidase-L	50	42
trans-Sialidase	18	5

of this activity was achieved at a NaCl concentration of ~0.6 M and fractions were collected that had an enriched highly pure protein band as determined by SDS-PAGE. The apparent molecular weight of this band was around 44–46 kD, which is consistent with the size of an α-(2→3)-sialyltransferase purified from rat liver [18]. Only 5% of enzyme activity was found in the initial washings.

Sialidase-L from leech.—Recombinant sialidase-L from leech provided another useful example of the efficiency of these affinity adsorbents for other SARPs. Approximately 50% of the loaded activity was located in the eluted fractions (Table 1), and a single band of molecular weight 84 kD along with BSA, which was added to the enzyme as a stabilising agent, was detected by SDS-PAGE. It should be noted that from this particular experiment 42% of the activity was recovered in the initial wash fractions and from other experiments. We are confident that this was simply a direct result of column overloading. Further work is underway to optimise conditions for the purification of this enzyme. It should be noted that similar results were obtained when affinity matrix **8** was used (data not shown).

trans-Sialidase from T. cruzi.—In the case of *trans*-sialidase approximately 5% of the activity was found in the initial washings. The recovered activity in the eluted fractions was approximately 18% of the loaded activity (Table 1). This low recovery tends to suggest that a significant amount of enzyme activity has been lost during the purification procedure and is presumably due, in part, to the instability of this *trans*-sialidase. Indeed, it is necessary to add BSA to the buffer in order to stabilise the enzyme activity during storage. As noted for sialidase-L, a similar outcome was observed when matrix **8** was employed (data not shown). We have commenced an investigation into the effect of metal ions on *trans*-sialidase activity and stabilisation. Our preliminary findings lead us to suggest that metal ions such as Mn^{2+} may have a positive effect on enzyme activity and perhaps the stability of the enzyme itself (manuscript in preparation).

4. Conclusions

We have developed the synthesis of novel thiosialosides that contain a terminal amine group on the aglycon moiety. These novel compounds were successfully coupled through the amino group to CNBr-activated Sepharose 4B, producing two novel affinity matrices. These affinity media have demonstrated good efficiency in the purification of a number of SARPs in one step and in relatively high yield. Investigations aimed at producing affinity media with more specific ligands suitable for a variety of sialidases and sialyltransferases continue.

Acknowledgements

We thank Dr M.J. Kiefel for his helpful discussions and critical reading of the manuscript. M.v.I. gratefully acknowledges the financial support of the NIH (Grant number PO1 A1 38084) and the Australian Research Council.

References

[1] H. Kitagawa, J.C. Paulson, *J. Biol. Chem.*, 269 (1994) 17872–17878.
[2] R. Schauer, *Trends Biochem. Sci.*, 10 (1985) 357–360.
[3] S.R. Narayanan, *J. Chromatogr. A.*, 658 (1994) 237–258.
[4] S. Bennett, M. von Itzstein, M.J. Kiefel, *Carbohydr. Res.*, 259 (1994) 293–299.
[5] M.J. Kiefel, B. Beisner, S. Bennett, I.D. Holmes, M. von Itzstein, *J. Med. Chem.*, 39 (1996) 1314–1320.
[6] D.I. Angus, M. von Itzstein, *Carbohydr. Res.*, 274 (1995) 279–283.
[7] B. Smalec, M. von Itzstein, *Carbohydr. Res.*, 266 (1995) 269–272.
[8] Y. Suzuki, K. Sato, M. Kiso, A. Hasegawa, *Glycoconjugate J.*, 7 (1990) 349–356.
[9] S. Ciccotosto, M.J. Kiefel, S. Abo, W. Stewart, K. Quelch, M. von Itzstein, *Glycoconjugate J.*, 15 (1998) 663–669 and references therein.
[10] A. Hasegawa, J. Nakamura, M. Kiso, *J. Carbohydr. Chem.*, 5 (1986) 11–19.
[11] R.W. Myers, R.T. Lee, Y.C. Lee, G.H. Thomas, L.W. Reynolds, Y. Uchida, *Anal. Biochem.*, 101 (1980) 166–174.
[12] P. Meindl, H. Tuppy, *Monatsh. Chem.*, 100 (1969) 1295–1306.
[13] M.-Y. Chou, S.-C. Li, M. Kiso, A. Hasegawa, Y.-T. Li, *J. Biol. Chem.*, 269 (1994) 18821–18826.
[14] P. Scudder, J.P. Doom, M. Chuenkova, I.D. Manger, M.E.A. Pereira, *J. Biol. Chem.*, 268 (1993) 9886–9891.

[15] D.S. Leelavathi, L.W. Estes, D.S. Feingold, B. Lombardi, *Biochem. Biophys. Acta*, 211 (1970) 124–138.
[16] Y.-T. Li, H. Nakagawa, S.A. Ross, G.C. Hansson, S.-C. Li, *J. Biol. Chem.*, 265 (1990) 21629–21633.
[17] B.E. Harvey, P. Thomas, *Biochem. Biophys. Res. Commun.*, 190 (1993) 571–575.
[18] J. Weinstein, B. de Souza-e-Silva, J.C. Paulson, *J. Biol. Chem.*, 257 (1982) 13835–13844.