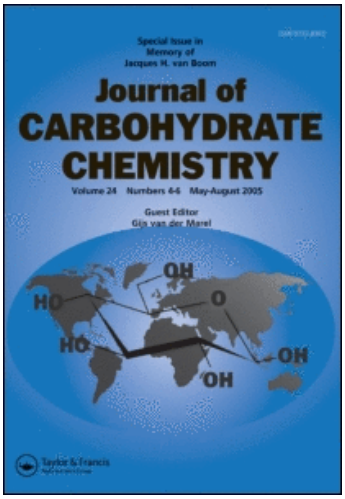

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SYNTHESIS OF AN AFFINITY ADSORBENT FOR SIALIDASE

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ABSTRACT

The reaction of the sodium salt of methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate (**1**) with styrene oxide, followed by deprotection, gave the diastereomeric mixture of (β-hydroxyphenethyl) 5-acetamido-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosidoic acid (**3**). The thioglycoside **3** was proved to be an inhibitor for *Clostridium perfringens* sialidase. An affinity adsorbent with immobilized sialic acid through a thioglycosidic linkage was prepared by treatment of **1** with epoxy-activated Sepharose 4B. The synthetic adsorbent was proved to adsorb *Cl. perfringens* sialidase specifically and to be capable of repeated operation.

INTRODUCTION

Several types of affinity adsorbents for sialidase have been described. Adsorbents with immobilized sialic acid[1-4] as well as synthetic inhibitors of sialidase[5,6] have been prepared. This paper describes an affinity adsorbent which has sialic acid immobilized through a thioglycosidic linkage.

RESULTS AND DISCUSSION

Treatment of the sodium salt of 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 (**1**)[7,8] with styrene oxide gave methyl [(β-hydroxyphenethyl) 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (**2**). Upon deacetylation

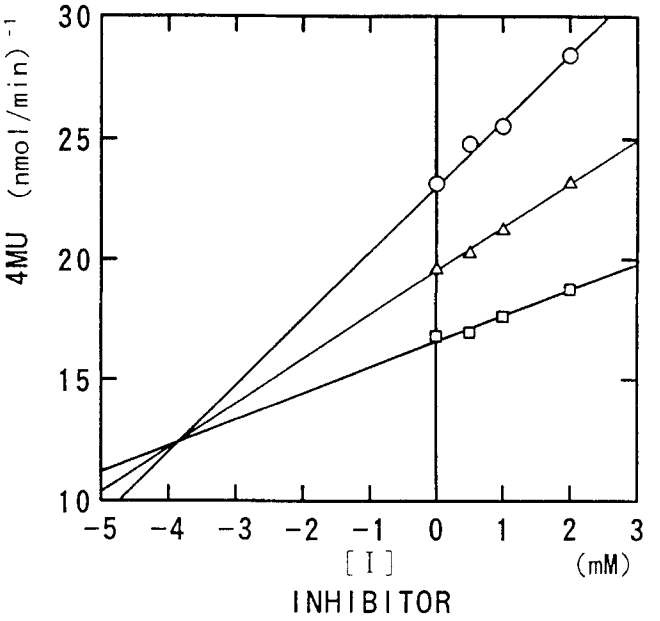


FIG. 1 Dixon Plots of the Effect of 3 on *Cl. perfringens* Sialidase Activity

and hydrolysis, **2** was converted into (β-hydroxyphenethyl) 5-acetamido-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosidoic acid (**3**). The chemical shifts of the protons at C-10 in the ^{1}H NMR spectra of **2** and **3** (δ 2.97~3.06) are clearly indicative of a thioglycosidic linkage.[7,9-11] Both **2** and **3** were obtained as diastereomeric mixtures as shown from ^{1}H NMR data. Other ^{1}H NMR data are consistent with the structures of **2** and **3**. Compound **3**, similar to known thioketosides,[12,13] was found to be an inhibitor against *Cl. perfringens* sialidase. The Ki value of 3.9 mM was calculated from the Dixon plots[14] shown in Fig. 1, and it was similar to those of other known thioketosides.[12,13]

This methodology was applied for preparation of an affinity adsorbent immobilized *N*-acetylneuraminic acid to the epoxy-activated Sepharose 4B. The structure of the affinity adsorbent is that shown in Fig. 2. The adsorbent thus prepared efficiently adsorbed the partially purified sialidase obtained from *Cl. perfringens* culture supernatant and separated from the bulk of protein. A chromatogram showing both sialidase activity and absorbance at 280 nm is shown in Fig. 3. The whole process for purifying *Cl. perfringens* sialidase from the culture supernatant is summarized in the Table. The enzyme was purified about 8,800-fold from the culture supernatant. The protein obtained by DEAE-Cellulose chromatography showed about 20 distinct bands on SDS-

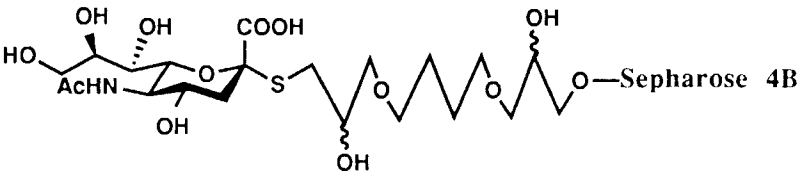


FIG. 2 An Affinity Adsorbent for Sialidase

polyacrylamide gel electrophoresis. The protein in the pooled eluate from the affinity column showed a single protein band with approximate molecular weight of 90,000 on SDS-polyacrylamide gel electrophoresis. Further applications for purification of the receptors recognizing *N*-acetylneuraminic acid and the mammalian sialidases are in progress.

EXPERIMENTAL

General Procedures. Melting points were determined with a Yanagimoto micro melting points apparatus. Specific rotations were determined with a Jasco DIP-140 polarimeter. Elemental analyses were obtained with a Yanaco CHN corder MT-3. IR spectra were recorded with a Jasco A-1 infrared spectrophotometer. ^{1}H NMR spectra were recorded with a JNM-EX270 spectrometer. TLC was performed on silica gel 60 and detection was carried out with diphenylamine-aniline-phosphoric acid reagent, followed by heating at 100 °C.[16] Plates were developed in the following solvent systems: (A) *n*-butyl alcohol-acetic acid-water, 3:1:1, v/v; (B) toluene-acetone, 7:3, v/v.

Methyl [(β-hydroxyphenethyl) 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (2). To a solution of **1**[7,8] (340 mg, 0.62 mmol) in dry methanol (8 mL) was added sodium methoxide (34 mg, 0.63 mmol in 6 mL methanol), and the mixture was stirred for 10 min at room temperature. After evaporation of the solvent, the residue was dissolved in dry *N*,*N*-dimethylformamide (DMF, 6 mL). To the solution was added styrene oxide (90 μL, 0.79 mmol), and the mixture was stirred under nitrogen gas overnight at room temperature. Evaporation of the solvent gave a solid, which was extracted with chloroform. The extract was washed with water, dried over sodium sulfate and filtered. The filtrate was concentrated to give a solid, which was subjected to chromatography on a column of silica gel (0.9 x 30 cm). Elution with chloroform-methanol (50:3) gave a crude solid. The solid was further chromatographed on a column of silica gel (0.9 x 30 cm) with

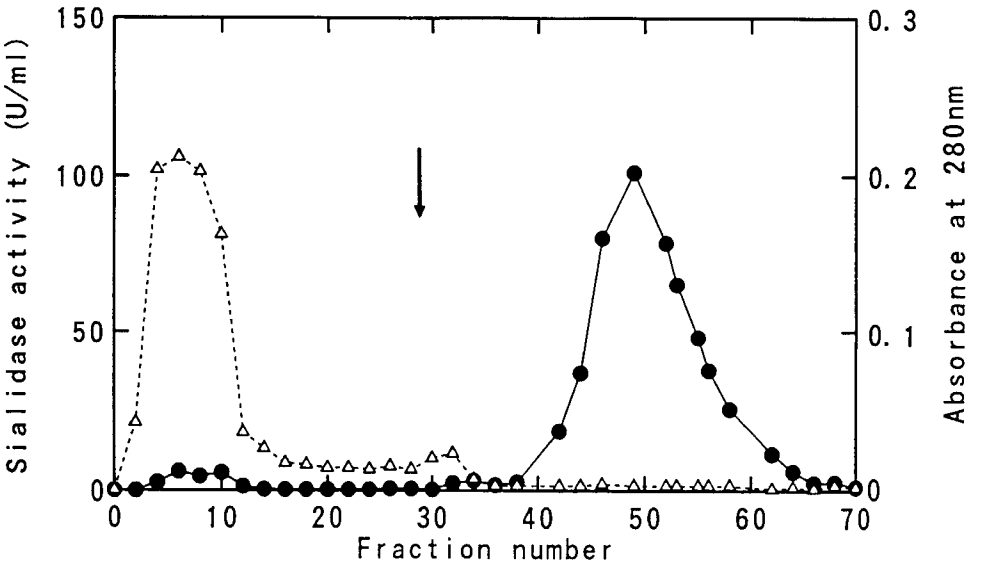
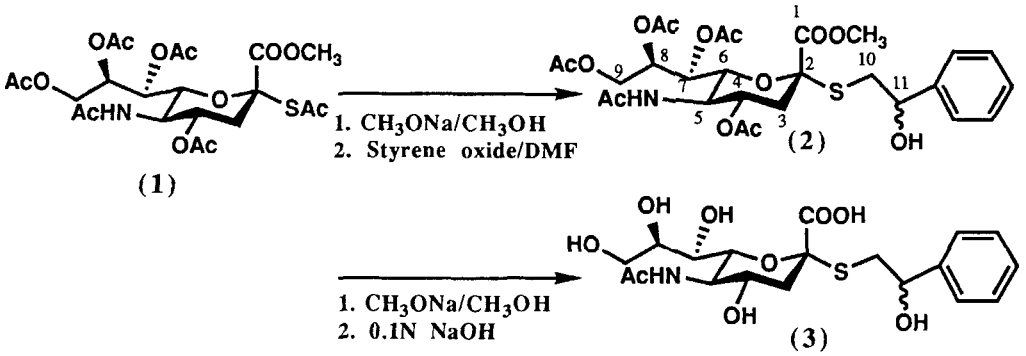


FIG. 3 Chromatography of Sialidase on an Affinity Adsorbent; Sialidase activity (●), Absorbance at 280 nm (△)

TABLE. Purification of *Cl. perfringens* Sialidase

	mL	Total protein[b] (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor (fold)	Yield (%)
Culture supernatant	410	3,000	18,800	6.3	1	100
Precipitates with ammonium sulfate[a]	20	270	16,000	59.3	9.4	85
Sephadex G-75	70	75	12,600	168	26.7	67
DEAE-Cellulose	42	4.8	8,500	1,770	281	45
Affinity	55	0.07	3,900	55,700	8,840	21

a. Contaminating impurities were removed by salting-out with ammonium sulfate at 50% saturation. Subsequently sialidase was precipitated by increasing the ammonium sulfate concentration to 85% saturation.
b. The amount of protein was estimated by the method of Bradford.[15]



toluene-acetone (7:3) to give a pure solid. The solid was crystallized from toluene/petroleum ether to give colorless crystals of **2** (110 mg, 25% yield): Rf=0.74 in solvent system A, Rf=0.18 in solvent system B; mp 78-80 °C; $[\alpha]_D^{25}$ +25.5° (*c* 0.48, methanol); IR (KBr) 3500-3350 (OH, NH), 1740 and 1230 (ester), 1665 and 1540 (amide), and 700 cm^{-1} (Ph); ^1H NMR ($CDCl_3$) δ 1.57, 1.88, 1.89, 1.94, 2.00, 2.02, 2.14, 2.15, 2.17 and 2.18 (each s, 27H, 8xOAc and NAc), 2.0-2.15 (1H overlapped with Ac-protons, H-3ax), 2.74 and 2.76 (each dd, 1H, $J_{3ax,3eq}$=12.5 and 12.5, and $J_{3eq,4}$=4.6 and 5.0 Hz, H-3eq), 2.95, 3.04 and 3.06 (each dd, 2H, $J_{10,10'}$=13.3, 13.3 and 13.5, and $J_{10,11}$=6.3, 5.9 and 9.9 Hz, respectively, H-10), 3.28 (d, 1H, $J_{11\text{-}OH}$=3.3 Hz, OH), 3.80 and 3.83 (each s, 3H, OCH_3), 3.89 and 3.91 (each dd, 1H, $J_{5,6}$=10.4 and 10.2, and $J_{6,7}$=2.3 and 2.3 Hz, respectively, H-6), 4.00 (t, 1H, J=~10.3 Hz, H-5), 4.11 (dd, 1H, $J_{9,9'}$=12.4 and $J_{8,9}$=5.7 Hz, H-9), 4.34 and 4.36 (each dd, 1H, $J_{9,9'}$=12.4 and 12.5, and $J_{8,9'}$=2.7 and 2.6 Hz, respectively, H-9'), 4.7-4.85 (m, 1H, H-11), 4.95 and 4.96 (each dt, 1H, $J_{4,5}$=$J_{3ax,4}$=10.2 and 10.4, and $J_{3eq,4}$=4.6 and 4.6 Hz, respectively, H-4), 5.15 (d, 1H, $J_{5,NH}$=9.6 Hz, NH), 5.33 and 5.37 (each dd, 1H, $J_{7,8}$=8.2 and 8.6, and $J_{6,9}$=2.3 and 2.3 Hz, respectively, H-7), 5.45 and 5.48 (each ddd, 1H, $J_{7,8}$=8.2 and 8.6, $J_{8,9}$=5.8 and 5.7, and $J_{8,9'}$=2.7 and 2.6 Hz, respectively, H-8) and 7.1-7.5 (m, 5H, phenyl protons).

Anal. Calcd for $C_{29}H_{37}NO_{13}S$: C, 54.45; H, 5.83; N, 2.19. Found: C, 54.49; H, 6.09; N, 2.28.

(β-Hydroxyphenethyl) 5-acetamido-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosidoic Acid (3). To a solution of **2** (63 mg, 0.10 mmol) in methanol (3 mL) was added sodium methoxide (26 mg, 0.48 mmol), and the mixture was stirred for 2 h at room temperature. Evaporation of the solvent gave a solid, which was dissolved in 0.1 M aqueous NaOH solution (5 mL). The mixture was stirred for 1 h at room temperature, and then neutralized with Dowex 50W-X8 resin. The resin was removed by

filtration and washed with water. The filtrate was concentrated to give a solid, which was subjected to chromatography on a column of silica gel (0.9 x 20 cm). Elution with 1-propanol-water (7:3) gave a solid, which was chromatographed on a column of Dowex 50W-X8 resin (0.4 x 20 cm). Elution with methanol gave a solid, which was further chromatographed on a column of Sephadex LH-20 (0.4 x 20 cm). Elution with methanol gave a colorless solid **3**. The solid was crystallized from a mixture of acetone and ethyl acetate to give colorless crystals (38 mg, quantitative): Rf=0.43 in solvent system A; mp 127-129 °C; $[\alpha]_D^{25}$ +52.2° (*c* 0.31, methanol), IR (KBr) 3450-3300 (OH, NH), 1700 (C=O), 1640 and 1560 (amide), and 700 cm^{-1} (Ph); ^1H NMR (D_2O) δ 1.75 and 1.79 (each dd, 1H, $J_{3ax,3eq}$=12.5 and $J_{3ax,4}$=11.2 Hz, H-3ax), 1.98 (s, 3H, NAc), 2.73 and 2.74 (each dd, 1H, $J_{3ax,3eq}$=12.5 and $J_{3eq,4}$=4.6 Hz, H-3eq), 3.00 and 3.10 (each dd, a part of 2H, $J_{10,10'}$=14.1 and 14.1, and $J_{10,11}$=8.11 and 5.0 Hz, a part of H-10 and H-10'), 3.11 (d, a part of 2H, J=6.6 Hz, a part of H-10 and H-10'), 3.43-3.83 (m, 6H, H-5, H-6, H-7, H-8, H-9 and H-9'), 3.67 and 3.68 (each dt, 1H, $J_{4,5}$=$J_{3ax,4}$=11.2 and $J_{3eq,4}$=4.6 Hz, H-4), 4.81 and 4.87 (t and dd, respectively, 1H, $J_{10,11}$=$J_{10',11}$=6.6, and $J_{10,11}$=8.1 and $J_{10',11}$=5.0 Hz, respectively, H-11) and 7.2-7.5 (m, 5H, phenyl protons).

Inhibition Constant. Incubation with *Cl. perfringens* sialidase was carried out in a total volume of 60 μL of 0.1 M aqueous potassium acetate buffer solution (pH 4.5) containing substrate, inhibitor **3** and the enzyme. Incubations were carried out in three concentrations of 4-methylumbelliferyl-*N*-acetylneuraminic acid as substrate (i.e., 0.067 mM, 0.133 mM and 0.267 mM) and inhibitor (i.e., 0 mM, 0.1 mM, 0.2 mM and 0.4 mM). The mixture was incubated at 37 °C for 10 min and then quenched by adding 0.085 M glycine-Na_2CO_3 buffer solution (2 mL) at pH 9.3. The released 4-methylumbelliferone was determined by fluorescence spectrophotometry at 360 nm excitation and 450 nm emission. The Ki value was obtained by using Dixon plots.[14]

Synthesis of Affinity Adsorbents for Sialidase. Sepharose 4B was activated with 1,4-butanediol diglycidyl ether according to the method of J. Porath.[17] To a solution of **1** (240 mg, 0.44 mmol) in dry methanol (3 mL) was added sodium methoxide (25 mg, 0.46 mmol), and the mixture was stirred for 10 min at room temperature. Evaporation of the solvent gave a solid, which was dissolved in dry DMF (20 mL). To the resulting solution was added the epoxide-activated Sepharose 4B (18 g) prepared by washing with dry DMF several times. The mixture was stirred under nitrogen gas at room temperature for 24 h, and then 0.1 M aqueous NaOH solution (20 mL) was added to the mixture. The mixture was stirred at room temperature for 5 h. After filtration, the resulting adsorbent was thoroughly washed with water. The adsorbent thus obtained was stored at 4 °C in 0.02 M sodium acetate buffer solution (pH 5.0) containing 0.1 M aqueous KCl solution and 0.02% aqueous NaN_3 solution.

Affinity Chromatography for *Cl. perfringens* Sialidase. Sialidase activity was assayed as follows. One unit of enzyme was defined as the amount of enzyme which catalyzed 1 nmol of 4-methylumbelliferone/min. All purification processes were carried out at 4 °C. *Cl. perfringens* sialidase (8500 U, 42 ml) partially purified by the DEAE-Cellulose chromatography according to the method of J. T. Cassidy *et al.*[18] was loaded on a column (0.9 x 22 cm) of the affinity adsorbent equilibrated with 0.02 M sodium acetate buffer solution (pH 5.0) containing 0.1 M aqueous KCl solution and 0.02% aqueous NaN_3 solution.[4] After the column was washed with the equilibrated buffer solution (100 mL) at a rate of 12 mL/h, sialidase was eluted with 0.2 M borate buffer solution (pH 8.5) containing 0.8 M aqueous KCl solution. SDS-polyacrylamide gel electrophoresis of the enzyme obtained was carried out on a 10% gel using the system of Laemmli.[19] The fractions were collected in 5 mL volumes. Protein was detected by silver staining.

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