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Note

Synthesis of a glycosidic affinity ligand for purification of cytidine-5′-monophosphosialate synthase

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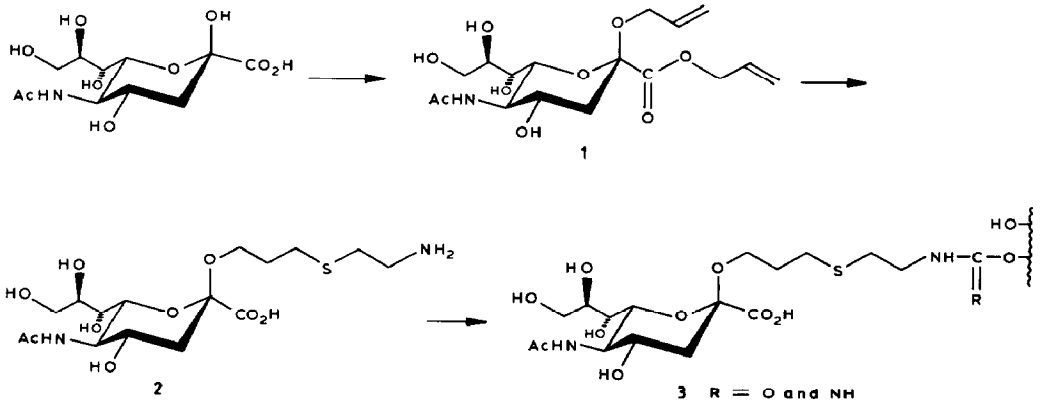
The enzyme cytidine-5′-monophosphosialate synthase (CTP:*N*-acylneuraminate cytidyltransferase; EC 2.7.7.43) catalyzes the formation of cytidine-5′-monophosphosialate[1], which is the activated neuraminic acid donor for the enzymic sialylation reaction[2]. Its first isolation by affinity chromatography was described by Brossmer *et al.*[3] who used cytidine-5′-diphosphohexanolamine as ligand. Zbiral *et al.*[4] have shown that the methyl β-glycoside of N-acetylneuraminic acid is an inhibitor (K_i 2.5mM) of the cytidine-5′-monophosphosialate synthase[4] because it competes with the enzyme for the native substrate. Therefore, [3-(2-aminoethylthio)propyl] β-glycoside of *N*-acetylneuraminic acid attached to Sepharose 4B should be an ideal ligand.

The synthesis of the 2-aminoethylthio-substituted ligand was based on a modified Fischer glycoside synthesis[5–8]. Starting from *N*-acetylneuraminic acid, acid-catalyzed allylation gave the allyl β-glycoside as the allyl ester **1**. After alkaline ester cleavage, irradiation of the material in the presence of an excess of cysteamine promoted the radical C–S bond formation to give the β-aminoethylthio-extended glycoside **2**. The affinity ligand was characterized by ^{1}H-n.m.r. spectroscopy. Compared to its diastereomeric counterpart, the α anomer of *N*-acetylneuramini acid, compound **2** showed a



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remarkable downfield shift of the ^{1}H-n.m.r. signal assigned to H-3*e* (δ 2.40; *cf.*[8] α anomer δ 2.75), thus proving the β configuration of the anomeric center[9]. This compound was coupled to cyanogen bromide-activated Sepharose 4 B to give the novel affinity material.

A solution of crude cytidine-5′-monophosphosialate synthase was prepared by homogenization and ultracentrifugation of calf brain. A standard method for semipurification by ammonium sulfate precipitation[6] and subsequent affinity chromatography yielded the pure enzyme. The stationary phase consisted of cyanogen bromide-activated Sepharose 4B coupled with 3-(2-aminoethylthio)propyl 5-acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-nonulopyranosidonic acid (**3**). Figure 1 illustrates the results of a typical analytical experiment using the *N*-acetylneuraminic acid-affinity adsorbent with elution of the protein by a potassium chloride gradient, yielding an enzyme sample having ~1.5 mU total activity (25% recovery). As compared to the crude extract, a sixfold purification was achieved in this step, and the final activity obtained on this analytical scale was 5 mU/mg.

The affinity material based on CDP-hexanolamine has proved its particular value in the isolation of sialyltransferase[2,10], and further studies showed its applicability for the purification of CMP-sialate synthase (200 mU/mg from calf brain[3]). However, a marked drawback of this phosphorylated material is its reduced stability during the purification and even during storage at 5°. The advantage of **3** is probably due to its

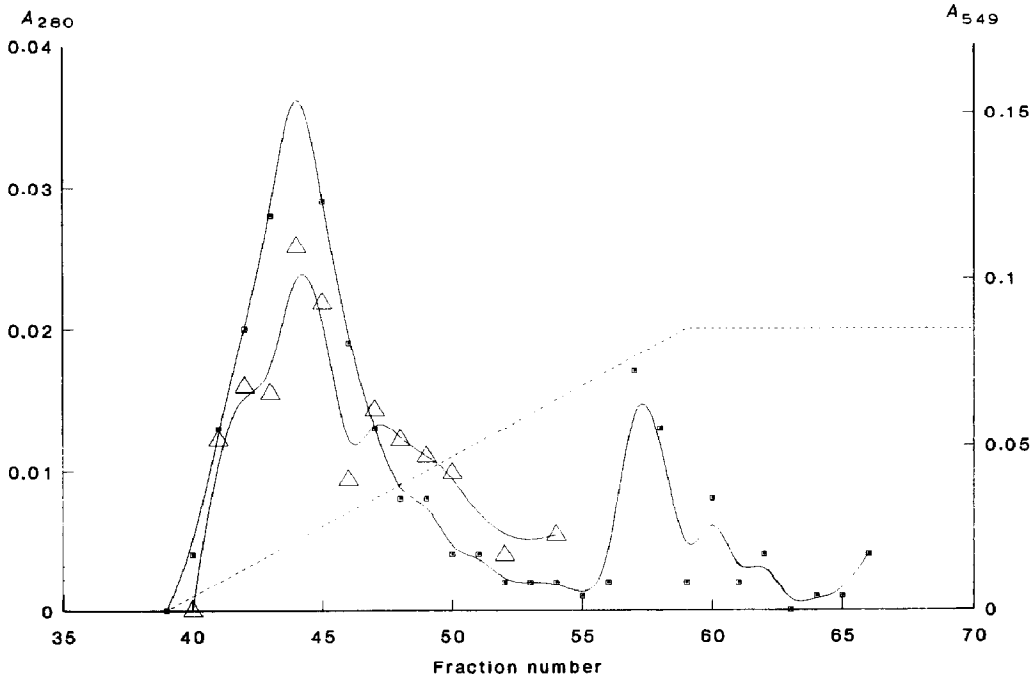

Fig. 1. Purification of CMP-Neu5Ac-synthase by affinity chromatography: Total protein (A_{280}, -□-□-□-), thiobarbituric assay (A_{549}, -△-△-△-), and KCl gradient (0 to 2M, - - - -).

resistance to neuraminidase, which does not cleave β anomers, and degradation by ubiquitous phosphatases is also avoided. The possibility that purification using **3** may operate *via* an ion-exchange mechanism has been indirectly disproved, as a corresponding *C*-glycosyl compound did not show any purification properties[11], even though the ion-exchange mechanism should have operated similarly.

Present efforts to transfer this method into a preparative scale turned out to be rather difficult, as the elution of the protein fraction has to be carried out with a salt gradient, resulting in denaturation.

EXPERIMENTAL

General. — Optical rotations were determined with a Perkin–Elmer 241 polarimeter. ^{1}H-N.m.r. spectra were recorded with a Bruker WH-400 (400 MHz) instrument. All reactions were monitored by t.l.c. on silica gel FG_{254} (Merck) with detection by u.v. light or by charring with H_2SO_4. Column chromatography was performed on Silica gel 60 (230–400 mesh, Merck) or Sephadex G-10 (Pharmacia). CHBr-activated Sepharose 4B was obtained from Pharmacia.

Fresh calf brain was collected on ice at a local slaughter house. The enzyme activity was determined by the thiobarbituric acid assay[1]. Solutions of enzyme were concentrated by ultrafiltration using Amicon concentrators with PH-10 Diaflo membranes at 0.35–0.5 MPa. Protein was determined by the method of Christian and Warburg[12] with a Shimadzu UV 2000 instrument using bovine serum albumin as the standard.

*Allyl (allyl 5-acetamido-3,5-dideoxy-β-*D*-glycero-*D*-galacto-2-nonulopyranosid)onate* (**1**). — Acetyl chloride (1 mL) was dissolved in allyl alcohol (50 mL) and kept at room tempeature for 15 min. After addition of *N*-acetylneuraminic acid (10 g, 3.2 mmol) the mixture was heated to 70° and stirred at this temperature for 2.5 h. After cooling to room temperature, the acid was neutralized with ammonia, and **1** was purified by flash chromatography in 5:1 ethyl acetate–methanol (yield 373.4 mg, 30%), $[\alpha]_D^{20}$ −21° (*c* 1.0, methanol); ^{1}H-n.m.r. (400 MHz, CD_3CN): δ 1.66 (dd, 1 H, H-3*a*), 1.97 (s, 3 H, $COCH_3$), 2.36 (dd, 1 H, H-3*e*), 3.41 (dd, 1 H, H-7), 3.55 (dd, 1 H, H-9a), 3.62–3.75 (m, 4 H, H-5,6,8,9b); 3.75–3.85 (m, 1 H, All), 4.01 (ddd, 1 H, H-4), 4.20–4.32 (m, 1 H, All), 4.63–4.70 (m, 2 H, All), 5.10–5.41 (m, 4 H, All), 5.80–6.05 (m, 2H, All), and 6.70 (d, 1 H, NH-5); $J_{3a,3e}$ 12.8, $J_{3e,4}$ 13.8, $J_{3a,4}$ 11.0, $J_{6,7}$ 0.8, $J_{7,8}$ 9.6, $J_{8,9a}$ 6.4, $J_{9a,9b}$ 12.0, $J_{4,5}$ 9.6, and $J_{5,NH}$ 6.0 Hz.

Anal. Calc. for $C_{17}H_{27}NO_9$ (389.4): C, 52.44; H, 6.99; N, 3.60. Found: C, 52.39; H, 6.92; N, 3.55.

*3-(2-Aminoethylthio)propyl 5-acetamido-3,5-dideoxy-β-*D*-glycero-*D*-galacto-2-nonulopyranosidonic acid* (**2**). — The allyl ester group was hydrolyzed by stirring in 0.1M NaOH (pH 12) for 1 h. After neutralization with 0.1M HCl, desalting on Sephadex G-10 with water, and lyophilization, the allyl glycoside (45 mg, 0.13 mmol) was dissolved in water (1 mL) containing cysteamine hydrochloride (700 mg, 6.2 mmol). The mixture was irradiated at 254 nm for 2 h, and then **2** was separated by gel filtration on Sephadex

G-10 with water (yield 40 mg, 69%); ^{1}H-n.m.r. (400 MHz, D_2O): δ 1.67 (dd, 1 H, H-3*a*), 1.92 (m, 2 H, $CH_2CH_2CH_2$), 1.99 (s, 3 H, $COCH_3$), 2.40 (dd, 1 H, H-3*e*), 2.73 (m, 2 H, SCH_2), 2.91 (m, 2 H, CH_2S), 3.27 (m, 2 H, NCH_2), 3.35–4.00 (m, 8 H, H-5,6,7,8,9*a*, 9*b*, OCH_2), and 4.05–4.20 (m, 1 H, H-4); $J_{3a,4}$ 10.0, $J_{3a,3e}$ 12.0, and $J_{3e,4}$ 4.0 Hz.

Anal. Calc. for $C_{16}H_{31}ClN_2O_9S$ (462.9): C, 41.51; H, 6.75; Cl, 7.66; N, 6.05; S, 6.93. Found: C, 41.79; H, 7.01; Cl, 7.52; N, 6.18; S, 6.77.

Compound **2** was coupled to activated Sepharose 4B by the standard method[13].

Affinity chromatography of cytidine-5'-monophosphosialate synthase. — A column of **2** coupled to CNBr-activated Sepharose 4B (**3**; 3 mL wet gel) was preequilibrated with mM NH_3 buffer (pH 9.0) containing mM 2-mercaptoethanol and NaN_3 (0.02%). The column was loaded with a solution of the semipurified[14] enzyme (1 mL, 10 mL/h) and washed with the afore mentioned NH_3 buffer. When A_{280} was $\sim$0, the enzyme was eluted with a KCl gradient (0→2.0M) in the NH_3 buffer (10 mL, 10 mL/h, 0.5-mL fractions). The active fractions were detected by the thiobarbituric acid assay[1] and combined.

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