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Distinguishing mammalian sialidases by inhibition kinetics with novel derivatives of 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid, an unsaturated derivative of *N*-acetylneuraminic acid*

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ABSTRACT

Kinetic analysis of mammalian sialidases was carried out using analogs of the potent sialidase inhibitor, 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid (**1**). Substitutents at C-9 in place of the terminal hydroxyl group included a, 4-azido-2-nitrophenylthio group to give 5-acetamido-2,6-anhydro-9-*S*-(4-azido-2-nitrophenyl)-3,5,9-trideoxy-9-thio-D-*glycero*-D-*galacto*-non-2-enonic acid (**2**), and an azide group to give 5-acetamido-2,6-anhydro-9-azido-3,5,9-trideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid (**3**). Competitive inhibition kinetics were observed when **1**, **2**, and **3** were tested with the lysosomal sialidase (cultured fibroblasts) and the plasma membrane sialidase (adenovirus DNA-transformed, human embryonic kidney cells), giving a K_i of about 10μM for both enzymes with all three compounds. In contrast, only **1** was a potent inhibitor of the microsomal sialidase (rat muscle).

INTRODUCTION

Mammalian sialidases (EC 3.2.1.18) have been implicated in a number of important metabolic processes including the regulation of cell proliferation[1], clearance of plasma proteins[2], and the catabolism of gangliosides and glycoproteins[3]. Concordant with this functional diversity is the distribution of sialidase activity in several cellular organelles, such as the plasma membrane[4], lysosomes[5], and microsomes[6]. The lysosomal and microsomal proteins are the best understood of the mammalian enzymes. Although both have been highly purified, their characterization has been primarily

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limited to kinetic studies and the identification of sialylconjugate substrates[7–10]. Recently, a structural model for the lysosomal enzyme has been proposed[11]. As yet, the interrelationship, if any, between the different cellular forms of sialidase is not known. The primary or secondary structure of these proteins has not been determined and, as a result, it has not been possible to identify regions of sequence or structural homology, compare mechanisms of action, or establish a common genetic origin.

One feature shared by nearly all mammalian, microbial, and viral sialidases is potent inhibition by the sialic acid analog, 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic-acid (**1**) (for a review, see ref. 12). This molecule is a classical transition state analog, representing the optimal conformational state imposed on the sialic acid unit by the enzyme template during catalysis and cleavage of the glycosidic linkage. Thus, **1** serves as an excellent probe of the active sites of sialidases. We have recently modified this molecule by introducing a 4-azido-2-nitrophenylthio group in place of the terminal hydroxyl group at C-9[13]. Surprisingly, the resulting compound was a potent inhibitor of the human lysosomal sialidase in cultured skin fibroblasts, giving an inhibitory constant, K_i, of 10 μM, which was identical with that of unmodified **1**. Moreover, we have also shown that the fibroblast enzyme is partially inactivated when photolyzed with the aryl azide inhibitor, and several lysosomal proteins were identified by use of a radioactively-labeled probe[14,15]. We have interpreted these results to indicate that the unsaturated pyran portion of the molecule contains the primary contact points that influence protein recognition and binding. Apparently the terminal hydroxyl group does not play an important role in this regard. This was unexpected as earlier studies with the clostridium sialidase revealed that an intact glycerol side chain was required for high affinity binding[16]. We have postulated that modifications of this portion of **1** would provide derivatives that may be useful for characterizing the catalytic or binding sites of sialidases and for distinguishing these enzymes on the basis of their inhibition kinetics. We report herein the preparation of a second derivative of **1** containing an azide substituent at C-9 in place of the terminal hydroxyl group, namely, 5-acetamido-2,6-anhydro-9-azido-3,5,9-trideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid (**3**). In order to gain insight into the relative binding specificities of the various cellular forms of sialidase, we have carried out a comparative kinetic analysis testing **1**, **2**, and **3** as inhibitors of these enzymes.

RESULTS

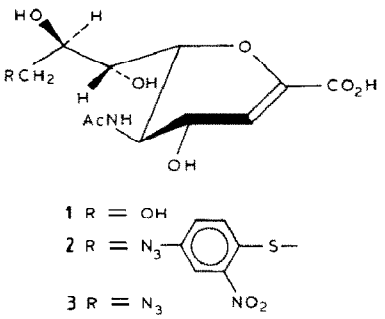
The spectral data and molecular mass for the novel sialic acid derivative, **3**, were consistent with its anticipated structure. Specific substitution of the azido group at C-9 was verified by ^{1}H-n.m.r. spectroscopy. The signals for the diastereomeric protons at this position appeared as a doublet of doublets at δ 3.525 and as an overlapping signal with the signal of H-7 at δ 3.665. In unmodified **1**, H_2-9 gave rise to signals downfield at δ 3.646 and 3.885, respectively. We have previously reported a similar finding for **2** where a sulfur atom substitutes the terminal hydroxyl group at C-9 and the resulting resonance signals for H_2-9 appear upfield[13] relative to those of **1**.

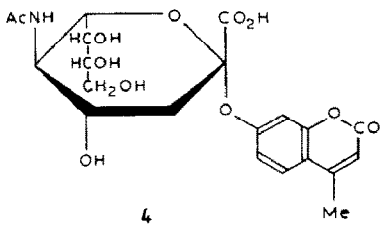
TABLE I

Inhibitory constants for derivatives of 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid (**1**)

Sialidase source	*Subcellular localization*	K_i (μM) *of compound*		
		1	**2**	**3**
Fibroblasts	Lysosomal	10	10	10
Adenovirus-transformed kidney cells	Plasma membrane	10	9	10
Rat muscle	Microsomal	9	[b]	50

[a] The derivatives were tested as inhibitors of sialidases from various sources by use of **4** as the substrate as described in the Experimental section. The apparent inhibitory rate constant was determined from Dixon plots of the data. [b] Not inhibited.





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Compounds **1**, **2**, and **3** were tested as inhibitors of the mammalian sialidase of several subcellular fractions (Table I). The sialidase of cultured fibroblasts, which is predominantly lysosomal, was inhibited by all three compounds, each having an identical inhibitory constant[13,14] of ~ 10μM. The plasma membrane enzyme from transformed human embryonic kidney cells showed a similar binding specificity, giving a nearly identical K_i for all three inhibitors. In contrast, the microsomal enzyme was strongly inhibited by **1**, weakly inhibited by **3**, and not inhibited by **2** over the range of concentrations tested.

DISCUSSION

A wide variety of the anhydro *N*-acetylneuraminic acid derivatives have been prepared and tested as competitive inhibitors of sialidases from both microbial and viral sources[17]. These studies have provided valuable information about the relative binding specificities of these proteins and the nature of their catalytic sites. However, a similar kinetic analysis using the mammalian enzyme has been lacking. Modifications of the inhibitor include substitution of the *N*-acetyl group at C-5 with carboxyl acids of varying size and chain length[17–19]. A trifluoroacetamido or propylamido group at C-5

can be accommodated by the microbial and viral enzymes, and inhibitory constants in the micromolar range were observed for these derivatives[19]. In contrast, modest alterations of the glycerol side chain of the molecule can result in a marked loss of inhibitor potency. Derivatives prepared by oxidative cleavage[19], dehydroxylation[20], and more subtle modifications such as epimerization[21] indicated that the presence of CHOH-8 and CH_2OH-9, and the conformational relationship between C-8 and C-7 are important features of the molecule that influence binding by the enzyme.

In contrast to the specificity of the viral and microbial enzymes, our results show that the lysosomal enzyme does not exhibit an extreme sensitivity to modifications of this part of the molecule. The azido or the large bulky arylazido group could be substituted for the terminal hydroxyl group with no effect on the apparent inhibitory constant. Remarkably, the plasma membrane sialidase, like the lysosomal enzyme, was inhibited to the same degree by all three derivatives. This broad specificity is shared by the two forms of the enzyme, and they differ uniquely from the microsomal sialidase. Interestingly, both enzymes also have other similar kinetic and physical properties. For example, both cleave sialyloligosaccharides as well as sialylgangliosides substrates, both proteins are membrane bound, and both have acidic pH optimums between 4 and 5. These considerations, as well as the kinetic inhibition data, led us to speculate that the plasma membrane enzyme and the lysosomal sialidase may have similar active sites, and that they may be related proteins.

The functional significance of the plasma membrane enzyme with an acid pH optimum residing at the cell surface where the pH is near neutrality raises intriguing questions. Recent studies have shown that acid phosphatase, a lysosomal membrane-associated protein, is rapidly exported directly from the Golgi–*trans*-Golgi apparatus to the cell surface[22]. The enzyme enters into the lysosomal compartment by leaving the plasma membrane and being internalized through an endocytotic process. It is possible that the cell surface sialidase undergoes similar translocating events and that it may have originally been destined for the lysosome, but it becomes misdirected or the translocation dynamics are altered upon viral transfection. Exploring intracellular trafficking as well as determining the interrelationship between the plasma membrane enzyme with the other cellular forms of sialidase will require more detailed characterization and complete purification of the protein. Additional derivatives of **1** may also provide more in-depth active site mapping, and reveal further differences in the binding specificities between these proteins.

EXPERIMENTAL

General. — Compounds **1** and **2** were prepared as previously described[13].

(4-Methylumbelliferyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosid)onic acid (**4**) (4-MU-Neu5Ac) was purchased from Sigma Chemical Co. (St Louis, MO). T.l.c. was carried out on precoated silicic acid, glass-backed plates (Whatman, Inc.), and spots were detected with resorcinol spray reagent[23]. Elemental composi-

tion was determined by high-resolution, fast-atom-bombardment mass spectrometry using a JEOL HX110HF mass spectrometer operating in the two sector mode with the sample suspended in a matrix of nitrobenzyl alcohol. The molecular ion of the matrix served as calibration standard. ^{1}H-N.m.r. spectra were recorded with a General Electric GN 500 NMR spectrometer operating at 500 MHz in the F.t. mode at ambient temperature. Aqueous samples were subjected to four freeze–thaw cycles in deuterium oxide. Acetone was the internal standard relative to external sodium 4,4-dimethyl-4-sila-(2,3-$^2H_4$)-pentanoate.

Sialidase from adenovirus-transformed, human embryonic kidney cells. — Human embryonic kidney cells, transformed with adenovirus DNA[24], were obtained from the Genentech cell repository and maintained in minimal essential medium containing 1% glutamine and penicillin–streptomycin–Fungizone (Gibco, Inc.), supplemented with 10% fetal calf serum. Cells were harvested at confluency by gently tapping the surface of the flask. After collection by centrifugation, the cells were washed once with phosphate-buffered saline solution at 4° and then disrupted by suspension in 10mM phosphate buffer, pH 7.5, containing 1% Triton X-100 (1 g of cell pellet in 2.5 mL buffer). The suspension was homogenized at 4° in a glass homogenizer fitted with a Teflon pestle. Unbroken cells and particulate matter were removed by centrifugation at 16 000*g* for 10 min. About 80% of the total activity was present in the supernatant fraction with a specific activity of about 52 nmol·h^{-1}·mg^{-1} protein. The plasma membrane form of the sialidase was separated from the lysosomal form by repetitive application of the supernatant (5 mL) to a column (1.0 × 2.0 cm) containing concanavalin A–Sepharose equilibrated in the extraction buffer. About 70% of the applied activity did not bind to the column after five applications. Since all lysosomal enzymes known bind tightly to this lectin, the unbound material served as a source of the plasma membrane sialidase, free of the lysosomal enzyme form, in the kinetic studies reported herein.

Sialidase from rat muscle. — Rat leg muscle tissue was removed from anesthetized animals and homogenized (1 g tissue/4 mL of buffer) in 100mM phosphate buffer, pH 6.1, containing 5mM EDTA at 4° with a Polytron homogenizer (Brinkman). The suspension was centrifuged at 40 000g for 15 min at 4°. The resulting supernatant was removed and served as a source of the enzyme with a specific activity of about 23.9 nmol·mg^{-1} of protein·h. Assays were carried out in 30mM phosphate, pH 6.1, 1.5mM EDTA with 1.3mM of **4** as substrate.

Enzyme assays. — Sialidase assays were carried out by use of the procedure described by Warner and O'Brien[25] employing **4** as substrate. With extracts of fibroblasts and transformed kidney cells, assays were conducted at pH 4.3, and with muscle extracts at pH 6.2. Initial experiments were carried out to evaluate the potency of the various inhibitors by use of standard enzyme assays at the K_m value for the substrate and by varying the inhibitor concentration within the range of 10 to 160μM. Additional kinetic analysis was carried out on those compounds that gave significant inhibition (> 20% of control) in these experiments. Inhibitory constants were determined by varying the concentration of the compound tested and assaying at the K_m value of the substrate, and at concentrations above and below the K_m value. The K_i value was obtained from Dixon plots of the data using linear regression analysis for curve fitting.

5-Acetamido-2,6-anhydro-9-azido-3,5,9-trideoxy-D-glycero-D-galacto-*non-2-enonic acid* (**3**). — Methyl 5-acetamido-4,7,8-tri-*O*-acetyl-2,6-anhydro-3,5-dideoxy-9-*O*-(4-tolylsulfonyl)-D-*glycero*-D-*galacto*-non-2-enonate[13] (18 mg, 31 μmol) was treated with NaN_3 (5.1 mg, 78 μmol) in dry dimethyl sulfoxide (0.3 mL) in a sealed tube under N_2 at 50° overnight. After being cooled to room temperature, the solution was suspended in chloroform (10 mL) and washed three times with saturated KCl and once with water. The organic phase was dried (Na_2SO_4), and after removal of the solvent, the residue was purified by reverse-phase h.p.l.c. using a column of octadecasilane on a silica support (Ultraphere, 4.6 × 25 cm, Beckman Inc.). The partially purified product was suspended in 3:7 (v/v) acetonitrile–water (0.5 mL), and applied to the column in the same solvent. Elution was accomplished with a linear gradient of increasing amounts of acetonitrile, up to 100%, in 25 min at a flow rate of 3 mL/min. The effluent was monitored at A_{243}. The appropriate fractions were pooled and the solvent removed under vaccum to give ~14 mg (51% yield) of the homogeneous peracetylated product. Removal of the *O*-acetyl and methyl ester groups was carried out with aq. NaOH, pH 12, for 2 h at 37°. Chromatography on a column of Dowex 50 cation-exchange resin, followed by solvent removal gave **3** (4.8 mg, 86% yield), R_t 0.51 in 4:2:2 (v/v) butanol–acetic acid–water, (R_t of **1** in this system 0.31), ν^{KBr} 2125 (max.) and 2110 cm^{-1} (shoulder), N_3; ^{1}H-n.m.r. (^2H_2O): δ 2.091 (s, 3H, $NCOCH_3$); 3.525 (d of d, 1 H, H-8), 3.665 (overlapping 2 H, H-7,9) 6.028 (d, 1 H, C=CH).

Anal. Calc. for $C_{11}H_{16}N_4O_7 + H^+$: 317.1097. Found: 317.1104.

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REFERENCES

1 S. Usuki, P. Hoops, and C. C. Sweeley, *J. Biol. Chem.*, 263 (1988) 10 595–10 599.
2 G. Ashwell and A. Morell, *Adv. Enzymol.*, 41 (1974) 99–128.
3 S. Usuki, S. Lyu, and C. C. Sweeley, *J. Biol. Chem.*, 263 (1988) 6874–6853.
4 C. Schengrund, A. Rosenberg, and M. A. Repman, *J. Cell Biol.*, 79 (1976) 555–561.
5 D. R. P. Tulsiani and R. Carubelli, *J. Biol. Chem.*, 245 (1970) 1821–1827.
6 T. Miyagi and S. Tsuiki, *J. Biol. Chem.*, 260 (1985) 6710–6716.
7 F. W. Verheijen, S. Palmeri, and H. Galjaard, *Eur. J. Biochem.*, 162 (1987) 63–67.
8 M. Hiriawa, Y. Uda, M. Nishizawa, and T. Miyatake, *J. Biochem. (Tokyo)*, 101 (1987) 1273–1279.
9 T. J. G. Van Der Horst, N. J. Galjart, A. Azzo, H. Galjaard, and F. W. Verheijen, *J. Biol. Chem.*, 264 (1989) 1317–1322.
10 M. Hiraiwa, M. Nishizawa, Y. Uda, T. Nakajima, T. Miyatake, *J. Biochem. (Tokyo)*, 103 (1988) 86–90.
11 M. Potier, L. Michaud, J. Tranchemontange, and L. Thauvett, *Biochem. J.*, 267 (1990) 197–202.
12 R. Schauer (Ed.), *Sialic Acids: Chemistry, Metabolism, and Function*, Springer-Verlag, New York, 1982.
13 T. G. Warner, *Biochem. Biophys. Res. Commun.*, 148 (1987) 1323–1329.
14 T. G. Warner and S. K. Loftin, *Enzyme*, 42 (1989) 103–109.

15 T. G. Warner and M. Potier, FASEB J., 4 (1990) 1367.
16 P. Meindl and H. Tuppy, *Monatsh. Chem.*, 101 (1970) 639–647.
17 P. Meindl, G. Bodo, P. Palese, J. Schulman, and H. Tuppy, *Virology*, 58 (1974) 457–463.
18 P. Meindl and H. Tuppy, *Monatsh. Chem.*, 100 (1969) 1295–1306.
19 P. Meindl and H. Tuppy, *Z. Physiol. Chem.*, 350 (1969) 1088–1092.
20 E. Zbiral, H. H. Brandstetter, R. Christian, and R. Schauer, *Justus Liebigs Ann. Chem.*, (1987) 159–165.
21 E. Zbiral, H. H. Brandstetter, R. Christian, and R. Schauer, *Justus Liebigs Ann. Chem.*, (1987) 781–786.
22 M. Braun, A. Waheed, and K. von Figura, *EMBO*, 8 (1989) 3633–3640.
23 L. Svennerholm, *J. Neurochem.*, 10 (1963) 613–620.
24 F. L. Graham, J. Smiley, W. C. Russell, and R. Nairn, *J. Gen. Virol.*, 36 (1977) 59–72.
25 T. G. Warner and J. S. O'Brien, *Biochemistry*, 18 (1979) 2783–2787.