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Bioorganic & Medicinal Chemistry Letters 13 (2003) 301–304

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis and Evaluation of Phosphoramidate Amino Acid-Based Inhibitors of Sialyltransferases

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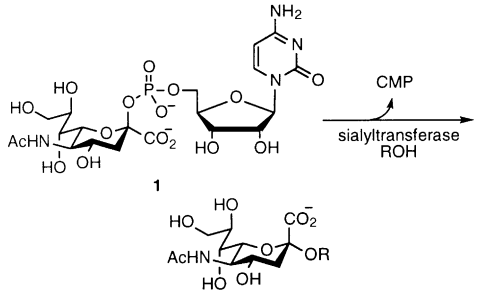
Received 31 May 2002; accepted 12 August 2002

Abstract—Several phosphoramidate analogues of CMP-*N*-acetylneuraminic acid were prepared for evaluation as inhibitors of α-2,3- and α-2,6-sialyltransferase. Central to the synthesis was the oxidative coupling of an amino acid ester with an *H*-phosphonate to construct the phosphoramidate linkage. All compounds synthesized were weak inhibitors of both of the sialyltransferases as determined by an HPLC-based inhibition assay.

The sialyltransferases are a family of membrane-bound enzymes located in the endoplasmic reticulum and Golgi apparatus of cells. As a subset of the glycosyltransferases, sialyltransferases catalyze the transfer of sialic acid (*N*-acetylneuraminic acid, NeuAc) from cytidinyl-5′-monophospho-β-*N*-acetylneuraminic acid (**1**, CMP-NeuAc) to acceptor hydroxyl groups located on the carbohydrate regions of glycoproteins and glycolipids (Scheme 1).[1] The presence of sialic acid-containing oligosaccharides has been linked to biologically relevant processes such as cell adhesion and inflammation.[2] Furthermore, studies have shown a link between sialyltransferase activity and the growth and metastasis of certain tumor cells.[3] As a result, there is considerable interest in the synthesis and evaluation of inhibitors of the sialyltransferases.



Scheme 1. Reaction catalyzed by the sialyltransferases.

Although **1** appears to be the common donor of sialic acid for all of the sialyltransferases, each enzyme varies in regioselectivity and to some degree in acceptor specificity. We chose to evaluate two commercially available enzymes, the α-2,3- and α-2,6-sialyltransferases. Limited information has been collected on the structure and active site composition of the sialyltransferases, although they appear to share a common topography.[4] However, one well-known distinction between the two enzymes is the acceptor sequence recognized by each. The α-2,6-sialyltransferase from rat liver (α-2,6-ST) prefers the *N*-acetyllactosamine (LacNAc) sequence, while the α-2,3-sialyltransferase from rat liver (α-2,3-ST) recognizes either LacNAc or lactose.[4b,5] Although potential inhibitors of the α-2,6-ST have been prepared,[6] the α-2,3-ST remains much less studied. Most efforts to inhibit the α-2,6-ST are centered on the synthesis of analogues of **1** itself or of a proposed transition state conformation of **1**.

Our design for inhibitors of sialyltransferases included analogues of **1** in which the natural phosphodiester linkage is replaced with a phosphoramidate (Fig. 1). The nitrogen of the phosphoramidate originates from

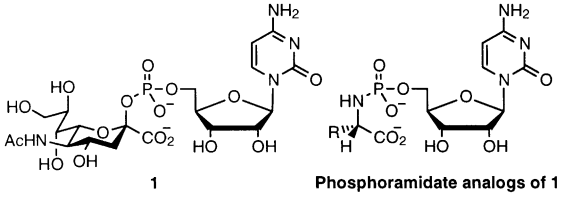


Figure 1. CMP-NeuAc (**1**), the natural substrate of the sialyltransferases, and phosphoramidate analogues of **1**.

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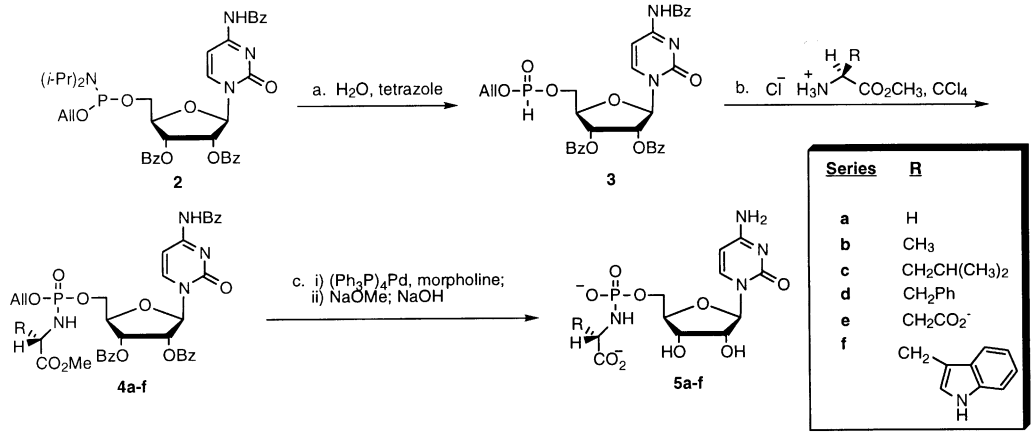
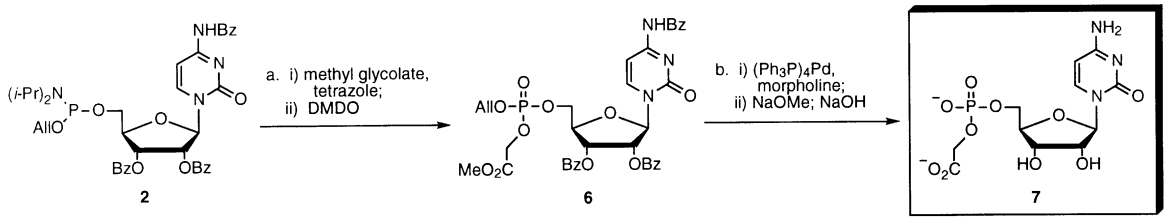
PII: S0960-894X(02)00735-7



Scheme 2. (a) H_2O, CH_3CN, tetrazole, rt (66%); (b) amino acid ester hydrochloride, CCl_4, Et_3N, CH_2Cl_2, rt (30–78%); (c) (i) $(Ph_3P)_4Pd$, Ph_3P, morpholine, CH_2Cl_2, rt; (ii) NaOMe, MeOH, 0.1 M NaOH, rt; NH_4HCO_3 (80%).



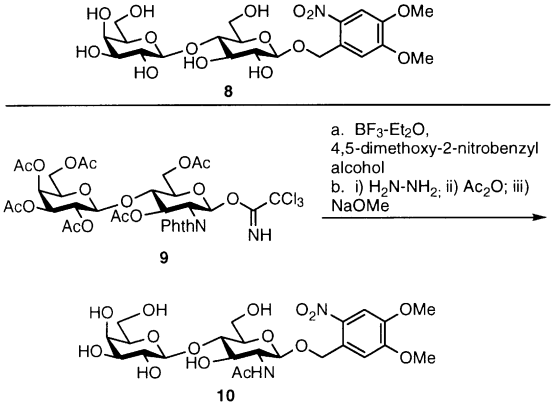
Scheme 3. (a) (i) Methyl glycolate, tetrazole, CH_3CN, 0 °C; (ii) Dimethyl dioxirane (DMDO), CH_2Cl_2, rt (78%); (b) (i) $(Ph_3P)_4Pd$, Ph_3P, morpholine, CH_2Cl_2, rt; (ii) NaOMe, MeOH, 0.1 M NaOH, rt; NH_4HCO_3 (66%).

an amino acid, thereby allowing access to a diverse array of side chains with different functional groups. It was hypothesized that a side chain could be found that has favorable interactions with amino acid residues in the active site of the sialyltransferases.

The synthesis of the phosphoramidate conjugates began with cytidine *O*-allyl phosphoramidite **2**[7] as a 1:1 mixture of diastereomers at the phosphorus stereocenter (Scheme 2). Hydrolysis of **2** to *H*-phosphonate **3** as a 1:1 mixture of diastereomers was accomplished using tetrazole as a catalyst. Coupling of a variety of amino acid esters to **3** using the Atherton–Todd reaction[8] furnished protected phosphoramidates **4a–f**. In the syntheses of compounds **4a–e**, a 1:1 ratio of diastereomers at phosphorus was obtained in each case; however, in the synthesis of **4f** a 2.2:1 ratio of diastereomers resulted. It is interesting to note that coupling reactions attempted with L-histidine methyl ester and *N*-ε-Fmoc-L-lysine methyl ester were not successful. A deprotection sequence of deallylation, debenzoylation, and methyl ester saponification completed the synthesis of phosphoramidates **5a–f**.

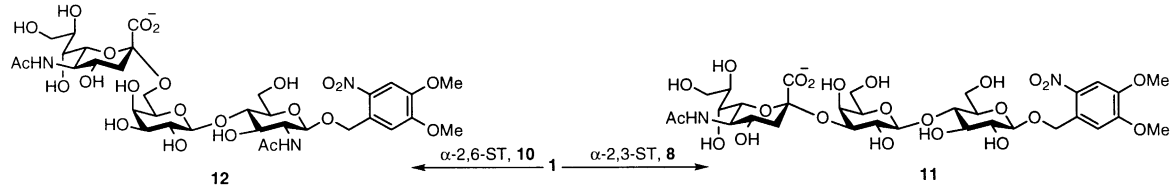
An additional potential inhibitor, the CMP–glycolate conjugate **7,** was also prepared from **2** (Scheme 3). Coupling of methyl glycolate to **2** produced a mixture of phosphite and phosphate triesters which was then oxidized[9] with dimethyldioxirane (DMDO) to yield phosphate triester **6** as a 1:1 mixture of diastereomers. Deprotection of the allyl and benzoyl esters and benzamide followed by saponification of the methyl ester furnished compound **7**.

An assay reported by Schmidt[6d] and coworkers was used as the basis for inhibition studies. For this HPLC-based enzyme inhibition assay, the modified disaccharide acceptors **8** and **10** (Scheme 4) that bear UV-chromophores were needed. Lactose-based acceptor **8**, for use in the α-2,3-ST assay, was prepared from lactose as previously reported.[7] LacNAc-based acceptor **10**, for use in the α-2,6-ST assay, was prepared from trichloroacetimidate **9**.[10] Standard borontrifluoride-catalyzed glycosylation[11] of **9** with 4,5-dimethoxy-2-nitrobenzyl alcohol afforded exclusively the β-anomer. Deprotection of the phthalimide, acetamide formation, and deacylation of the alcohols gave the acceptor **10**.



Scheme 4. (a) (i) BF_3-Et_2O, 4,5-dimethoxy-2-nitrobenzyl alcohol, CH_2Cl_2, rt (82%); (b) (i) H_2N–NH_2–H_2O, MeOH, reflux; (ii) Ac_2O, pyridine, rt; (iii) NaOMe, MeOH, reflux (60%).

Both **8** and **10** have a λ_{max} value at 348 nm ($\varepsilon_{348} = 6000\ M^{-1}\ cm^{-1}$), which is far removed from the



Scheme 5. Reactions of α-2,3-ST and α-2,6-ST with their specific acceptors **8** and **10** bearing UV-chromophores.

λ_{max} of the cytosine chromophore ($\lambda_{max} = 272$ nm). The transfer of sialic acid to either **8** or **10** was cleanly resolved with reverse-phase HPLC. The identities of the sialylated products (Scheme 5), compounds **11**[7] and **12**, were confirmed by ^{1}H NMR and mass spectrometry of preparative-scale reaction products.

The evaluation studies of **5a–f** and **7** were performed in an aqueous solution containing MES buffer (200 mM, pH 6.0), sodium chloride (100 mM), disodium-EDTA (0.5 mM), Triton X-100 (0.01%), and **8** or **10** (20 μM). Once enzyme was added, the reactions were incubated in a water bath at 37 °C until a detectable amount of product **11** (α-2,3-ST) or **12** (α-2,6-ST) was produced. At this time they were frozen in liquid nitrogen and stored at −20 °C until HPLC analysis, which was never more than 1 or 2 days. Storing the reactions for a longer period of time affected the reproducibility of results. Detection and integration of either **8** and **11** or **10** and **12** permitted the calculation of percent product and thus initial velocity. A Michaelis–Menten curve was used to determine the K_m of **1** for the α-2,3-ST and the α-2,6-ST under the reaction conditions. The average K_m determined from three runs was 0.3 mM for the α-2,3-ST, in agreement with the value previously reported by this laboratory.[7] The average K_m determined for the α-2,6-ST was 0.1 mM, which is reasonably close to the value of 0.046 mM reported by Schmidt and coworkers.[6d]

Inhibition assays were carried out under identical reaction conditions, except that the inhibitor was added to the reaction; the concentrations of **5a–f** and **7** ranged from 0.1 to 10 mM in the experiments. A fixed concentration of 20 μM acceptor **8** or **10** was used. Lineweaver–Burk plots were used to classify compounds **5a–f** and **7** as competitive inhibitors. For the α-2,3-ST, K_m and V_{max} in conjunction with the Michaelis–Menten equation for competitive inhibition were used to determine the K_i (Table 1). For the α-2,6-ST, Dixon plots were used to determine the K_i values (Table 1). The K_i for CMP, a known inhibitor of the sialyltransferases,[12] was determined as a standard. The values of 65 μM and 64 μM are within agreement of the reported K_i of 50 μM for human serum sialyltransferase.[12b] In a typical K_i experiment, four different concentrations of **1** and four different concentrations of inhibitor were used, resulting in a total of 16 reactions per inhibition experiment.

As seen in Table 1, none of the phosphoramidate analogues inhibits the α-2,3-ST as strongly as CMP; they have inhibition constants that are at least an order of magnitude higher. The K_i of **5f** is similar to the K_m of **1**. While the binding constants for **7** and **5c** appear higher than those of **5f** and **1**, they are less than 5-fold higher,

Table 1. K_i values for compounds **5a–f** and **7**

Compd	α-2,3-ST K_i (mM)[a]	α-2,6-ST K_i (mM)[a]
CMP	0.065 (0.007)	0.064 (0.019)
5a	5.3 (1.0)	2.4 (0.14)
5b	2.3 (1.1)	1.7 (0.57)
5c	0.90 (0.30)	2.5 (0.99)
5d	2.3 (2.7)	3.8 (1.1)
5e	5.1 (1.9)	2.2 (0.35)
5f	0.30 (0.13)	0.73 (0.45)
7	0.71 (0.48)	0.37 (0.25)

[a]Values are means of three experiments, three to four determinations each; standard deviation is given in parentheses.

a small difference. Compounds **5a**, **5b**, **5d**, and **5e** had significantly higher K_i's and were relatively poor inhibitors. Based on the similarity of **5a** to **7**, it is apparent that a phosphoramidate is not a completely adequate mimic of a phosphodiester. The data for compound **5f** suggest that aromatic groups can have favorable interactions in the active site; however, the simple presence of an aromatic group is not entirely sufficient since compound **5d** has a larger K_i.

Similar results were obtained with inhibition assays of the α-2,6-ST; compounds **5f** and **7** again possess the lowest K_i values, reinforcing the significance of the phosphodiester linkage and the importance of aromatic groups. Again, **5d** has a larger K_i than **5f**, suggesting that the indole ring of **5f** has a more favorable interaction in the active site than the phenyl ring of **5d**.

To summarize, we synthesized a series of phosphoramidate analogues of **1** and evaluated their ability to inhibit two members of the sialyltransferase family of enzymes using an HPLC-based assay. All of the phosphoramidates were weak inhibitors of the α-2,3-ST and the α-2,6-ST. However, two trends emerged in the analysis. First, the importance of the phosphate ester linkage in **1** was demonstrated through the comparison of compounds **5a** and **7**. Second, the relevance of aromatic side chains in the phosphoramidates was shown through compounds **5f** and **5d**. However, the location and type of these aromatic side chains has a large impact on the inhibitory effects. We hope to use these findings to design stronger second-generation inhibitors.

Acknowledgements

This research was supported by the NIH (GM55852). R.L.H. also thanks the National Science Foundation (Career Award), the Camille and Henry Dreyfus

Foundation (Camille Dreyfus Teacher-Scholar Award), Pfizer (Junior Faculty Award), and Novartis (Young Investigator Award) for support. This work was greatly facilitated by a 500 MHz NMR spectrometer that was purchased partly with funds from an NSF Shared Instrumentation Grant (CHE-9523034). L.J.W. thanks the National Science Foundation for a predoctoral fellowship.

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