then at room temperature for 60 min, solvent was removed under reduced pressure and the residue recrystallized from MeOH-EtOAc-hexane to give 0.41 g (83%) of analytically pure product: mp 195-7 °C; ^{1}H NMR (D_2O) δ = 2.82 (3 H, s), 2.88 (3 H, s), 3.32 (2 H, m), 3.45 (2 H, m), 5.34 (2 H, s), 8.05 (1 H, d), 8.23 (1 H, d of d), 8.57 (1 H, d). Anal. ($C_{12}H_{19}N_5O_6S$·HCl) C, H, N.

Nitrite Release from Electrolytic Reduction of 4. A 0.01 mM solution of 4 in 0.1 M tetrabutylammonium perchlorate-DMF solution (5 mL) was electrolyzed with dc polarography at a Hg electrode (vs Ag/AgCl) under Ar. Immediately after electrolysis for 4-5 min, 1 mL of the resulting solution was added to the supporting electrolyte, 4 mL of an aqueous solution (pH 2-3) of diphenylamine, NaSCN, and $HClO_4$. Released nitrite was determined by differential pulse polarography under conditions where the test compound did not release nitrite. Nitrite concentration was determined by comparison of peak current with calibration curves. The method of standard additions was also used to insure accuracy.

Biological Assays. In vitro studies were performed with exponentially growing cultures of EMT6 mouse mammary tumor cells incubated under aerobic and hypoxic conditions. The methodology used was essentially the same as that previously reported.[16] In cytotoxicity studies (Figure 1) cells were treated with drug for 2 h under fully hypoxic conditions. In radiation/drug interaction studies, cells were treated with drug for a total of 75 min and were irradiated (or sham irradiated) during the final minutes of drug treatment with 250-kV X-rays at a dose rate of ~1 Gy/min. In all cases, points shown are geometric mean ± SEM's of survivals determined in three or more independent experiments. Survival curves, D_0's, and n's were fitted by regression analyses.[1,17,18] Cells treated with radiation alone and with radiation plus misonidazole were included in the studies, as were drug- and solvent-treated controls. The oxygen enhancement ratios, calculated from the ratios of the D_0's for cells irradiated in hypoxia and air, were 3.2 and 2.9 for the experiments shown in Figures 3 and 4, respectively, showing that good, radiobiological hypoxia was achieved. Sensitizer enhancement ratios (SERs) were calculated analogously, as the ratios of the D_0's in the absence and presence of drug.

In vivo experiments were performed with EMT6 mouse mammary tumors in BALB/c mice. In these studies, the response of the tumors to treatment was assessed by euthanizing the animals after treatment, removing the tumors, preparing a single cell suspension from the tumors, and assaying the ability of the suspended cells to form colonies in vitro.[16]

Stability of 11 at pH 7.4 and in Rat Plasma. Stability of 11 in both pH 7.4 buffer at 23 °C and rat plasma at pH 7.4 and 37 °C was studied. A 10.5 mM solution of the compound in 0.085% H_3PO_4 was prepared. One hundred microliters of this solution was added to 1600 μL of rat plasma and 400 μL of pH 7.4 phosphate buffer to yield an assay concentration of 0.5 mM. The pH was monitored with an Orion Research pH meter and maintained by the addition of 0.1 N HCl. Temperature was maintained by using a Haake circulating water bath.

For the plasma sample, 50-μL aliquots were removed periodically and quenched in 950 μL of 7% $HClO_4$. The quenched solutions were centrifuged (Beckman Microfuge) and the supernate analyzed on a Hewlett-Packard HPLC (50-μL injection, 280 nm, gradient elution of 5-95% MeCN with 0.085% H_3PO_4 over 10 min at a flow rate of 3 mL/min, 10-cm VYDAC C18 protein and peptide column).

The pH 7.4 buffer experiment was prepared by using the same 10.5 mM stock prepared previously and diluting 20-fold into pH 7.4 buffer. Five-microliter aliquots were taken directly by the HPLC autosampler without dilution (the temperature of the autosampler was 23 °C).

For the plasma sample, 100% of the starting material was recovered after 2 h. For the pH 7.4 buffer sample 82.2% of the starting material was recovered after 96 h.

Acknowledgment. We thank C. Irvin, M. Kelley, and M. Homiski for their assistance with the cell culture and tumor studies. Experiments at Yale were supported in part by Grant PDT 145 from the American Cancer Society, USPHS Grant CA-43659 from the NCI (ACS), and by a grant from Merck Sharp & Dohme Research Laboratories. We are indebted to Y. Lee for the electrolysis experiments and pK_a values, J. Moreau for elemental analyses, M. Zrada and S. Smith for partition coefficients and buffer and plasma stability determinations, and J. Kaysen for manuscript preparation. We also thank P. Anderson, A. Oliff, and D. Heimbrook for their support and assistance in arranging for evaluation of the compounds.

Registry No. 2, 135685-01-7; 3, 135685-02-8; 4, 126813-40-9; 5, 126813-46-5; 6, 135685-03-9; 7, 126813-47-6; 8, 126813-48-7; 9, 126813-49-8; 11, 126813-43-2; 11 (free base), 135685-04-0; 12, 119-32-4; 13, 135685-05-1; 14, 135685-06-2; 15, 135685-07-3; 16, 130129-52-1; 17, 135685-08-4; 18, 135685-09-5; 19, 135685-10-8; 20, 135685-11-9; 20 (free base), 135685-12-0; $Me_2NCH_2CH_2NHMe$, 142-25-6; $MeNHCH_2CH_2NMeBOC$, 112257-19-9; $LiMe_2CNO_2$, 3958-63-2; PhthOH, 524-38-9; 4-chloro-3-nitrobenzenesulfonyl chloride, 97-08-5; mono-BOC-ethylenediamine, 57260-73-8; 4-nitrophenol, 100-02-7.

Communications to the Editor

Monovalent Sialosides That Bind Tightly to Influenza A Virus

The first step in the cascade of events that leads to infection by influenza virus is adsorption of virus particles to the surface of susceptible cells.[1] This process occurs through recognition and binding of cell-surface oligosaccharides that terminate in sialic acid (SA) residues.[2] Previous studies have shown that SA is the only component of these carbohydrate chains that is recognized by the virus[3] and that the viral entity responsible for this interaction is the protein hemagglutinin (HA).[4] One approach to combating cellular infection may be to inhibit this adsorption process by using SA analogues that compete with the cell surface sialyl oligosaccharides, for viral HA. However, the binding affinity for HA of all known monovalent α-sialosides, such as *N*-acetyl-2-*O*-methyl-α-D-neuraminic acid (Neu5Acα2Me, 1) is weak ($K_d \approx 2$ mM),[5] and the virus presumably attaches to cells at many

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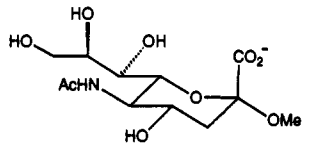


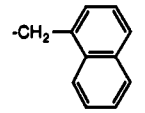
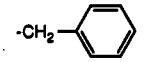
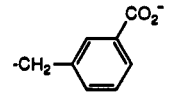
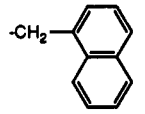
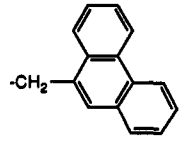
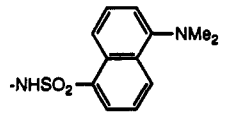
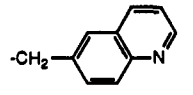
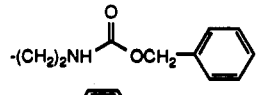
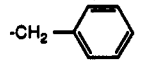
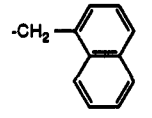
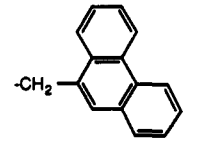
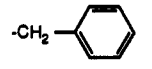
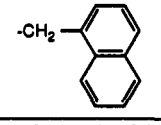
1

loci. To compete with these polyvalent interactions, an inhibitor of viral adsorption will need to have a much higher affinity for HA than that associated with typical monovalent α-sialoside ligands.

The crystal structure of sialyllactose bound to BHA (HA that has been cleaved from the viral membrane by the protease bromelain) shows that the SA binding site is a shallow depression at the end of the protein furthest from the membrane.[6] Chemical modification of Neu5Acα2Me, as well as screening of naturally occurring oligosaccharides that contain single SA residues, has failed to provide compounds with values of K_d less than about 2 mM.[6,7] These findings suggest that each feature of SA that contacts the protein is necessary for binding to HA and that modifications to the SA structure may not afford molecules with the required binding properties.

In the crystal structure of the complex of BHA with α(2,3)-sialyllactose, the sialoside aglycon protrudes from the SA binding site out into solution.[6] The electron density for these atoms is low, suggesting a disordered structure for the sugars that are attached to the sialic acid when the ligand is bound to BHA. Nevertheless, the ability of different mutant strains of influenza A virus to distinguish (even if only weakly) between α(2,3)-sialyllactose and α(2,6)-sialyllactose suggests the possibility of contacts between HA and the sialoside aglycon. Consideration of these facts, and examination of molecular models, led us to explore the possibility of building sialoside chains capable of making extended contacts with the protein, thus enhancing the binding of monovalent sialoside ligands to HA. During this search we have identified a number of sialosides with hydrophobic aglycons that bind to HA with significantly higher affinity than that of Neu5Acα2Me (1). We here describe monovalent inhibitors of hemagglutination that are better than any yet reported. All the compounds were synthesized by a common route, shown in Scheme I. Acids **2a–d** were prepared from methyl 4,7,8,9-tetra-*O*-acetyl-*N*-acetyl-2-chloro-2-deoxy-β-D-neuraminate[8] using Koenigs–Knorr reaction conditions.[9] The glycosyl chloride was stirred with mercuric cyanide and 3-Å molecular sieves at room temperature under argon, in the appropriate ω-alkenol as solvent,[10] until there was no starting material remaining by thin-layer chromatography. Using acids **2a–d**, a variety of different amides were prepared by standard peptide-coupling methodology, from which the acetate and ester protecting groups were then removed hydrolytically. The affinity of these compounds for HA was measured by their ability to inhibit viral hemagglutination of chicken erythrocytes.[11]

Table I. Relative Affinity of Monovalent Sialosides for Hemagglutinin[a]

no.	aglycon chain length (n)[b]	aglycon substituent (R)[b]	relative affinity[c]
3	4	$-CH_3$	1
4	2		8
5	4		8
6	4		16
7	4		32
8	4		32
9	4		4
10	4		8
11	4		4
12	6		16
13	6		64
14	6		64
15	8		<8[d]
16	8		16

[a] Binding affinities were determined in hemagglutination inhibition experiments with influenza A strain X-31 and adult chicken red blood cells. [b] All compounds have the general structure 3 (Scheme I). [c] The relative affinity of Neu5Acα2Me (1; K_d = 2.8 mM) is arbitrarily defined as 1. [d] That is, K_i >80 μM.

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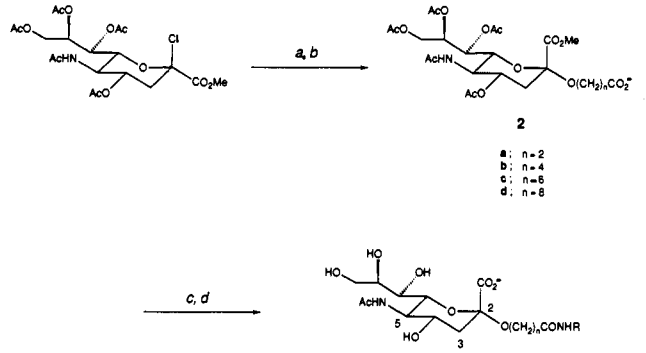
(9) Okamoto, K.; Goto, T. Glycosidation of Sialic Acid. *Tetrahedron* **1990**, *46*, 5835–5857.

(10) Oct-7-enol was prepared according to the procedure of Bestmann et al.[12] The other alkenols that were used are commercially available.

(11) WHO Tech. Rep. Sec. **1953**, *64*, 1.

Scheme I. General Route to Monovalent Sialosides[a]



[a] (a) $HO(CH_2)_nCH{=}CH_2$ (as solvent), $Hg(CN)_2$ (0.1 equiv), 3-Å molecular sieves, room temperature; (b) $KMnO_4$ (3 equiv), AcOH(aq), 4 °C, 2 h; (c) 1-hydroxybenzotriazole (1 equiv), dicyclohexylcarbodiimide (1 equiv), diisopropylethylamine (1 equiv), RNH_2 (1 equiv), CH_2Cl_2; (d) methanol, NaOH(aq), room temperature, 2 h.

In several cases, binding to BHA was also measured by the NMR titration assay.[5] This method affords relative binding affinities that are in agreement with those obtained from hemagglutination inhibition experiments.

From Table I it can be seen that every compound that binds to HA better than Neu5Acα2Me (1) carries an aromatic group attached to the nitrogen of the aglycon amide linkage. This finding suggests that the aromatic group contributes to binding and that its positioning is critical for tight association with HA. The best inhibitors (13 and 14), with K_i = 40 μM, bind to HA more than 60-fold better than Neu5Acα2Me (K_i = 2.8 mM).[5] These are the tightest binding monovalent inhibitors of hemagglutination that have been described. It is interesting to note that the binding affinity is related to both the nature of the aglycon substituent (R) and the chain length (n). When R is the 1-naphthylmethyl group, the tightest binding inhibitor has the chain length n = 6. Examination of inhibitors modeled into the SA binding site of BHA does not reveal an obvious hydrophobic binding locus on the protein surface, and a proper understanding of the reason for the observed increases in binding affinity will have to await the solution of the crystal structure of a protein–ligand complex.

The identification of tight-binding sialosides containing aromatic substituents also suggests the possibility of synthesizing fluorescent ligands for BHA. This idea is currently being developed to provide a convenient fluorescence assay for the binding of ligands to influenza A hemagglutinin.

Acknowledgment. This work was supported by the National Institutes of Health, by the Science and Engineering Research Council, and by the Schweizerischen Nationalfonds. We thank Dr. J. J. Skehel for supplying the influenza A virus and Don Wiley, Elmar Weinhold, and Gregory Priebe for helpful discussions.

Registry No. **2a**, 136067-24-8; **2b**, 136097-52-4; **2c**, 136067-25-9; **2d**, 119054-28-3; **3**, 136067-12-4; **4**, 136088-26-1; **5**, 136067-13-5; **6**, 136097-51-3; **7**, 136067-14-6; **8**, 136067-15-7; **9**, 136067-16-8; **10**, 136067-17-9; **11**, 136067-18-0; **12**, 136067-19-1; **13**, 136067-20-4; **14**, 136067-21-5; **15**, 136067-22-6; **16**, 136067-23-7; $MeNH_2$, 74-89-5; $PhCH_2NH_2$, 100-46-9; 3-$(H_2NCH_2)C_6H_4COOH$, 2393-20-6; $PhCH_2OCONHCH_2CH_2NH_2$, 72080-83-2; (1-naphthylmethyl)amine, 118-31-0; (9-phenanthrylmethyl)amine, 15398-91-1; (5-dimethylamino-1-naphthyl)sulfonylhydrazine, 33008-06-9; (6-quinolinylmethyl)amine, 99071-54-2; methyl 4,7,8,9-tetra-*O*-acetyl-*N*-acetyl-2-chloro-2-deoxy-β-D-neuraminate, 67670-69-3.

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Peptide to Glycopeptide: Glycosylated Oligopeptide Renin Inhibitors with Attenuated in Vivo Clearance Properties

Given the remarkable breadth of biological activity vested by Nature in the assembled amino acids, the value of peptides as preeminent templates for therapeutic drug design is self-evident. It is in recognition of this fact that considerable resources are expended, on a continuing basis, on the task of identifying chemical strategies that preserve the information content of the peptide and abolish its intrinsic shortcomings as a practical drug.[1] Of these shortcomings three are particularly vexing. The typical oligopeptide is susceptible to proteolytic degradation and is poorly orally absorbed, and of that modest fraction which is absorbed, there exists an efficient liver transport system[2] that extracts the oligopeptide from the blood and excretes it through the bile. It is within the context of this latter problem—liver/biliary clearance—that we report a distinct contrast in disposition between an oligopeptide and its glycosylated derivative.

A practical illustration of the difficulty posed by the therapeutic oligopeptide is provided by the inhibitors[3] of the aspartyl protease renin. This enzyme is an attractive target for the amelioration of hypertension, as a result of its specific proteolytic release of the oligopeptide precursor of the vasoconstrictive hormone angiotensinogen II. Di-

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