Multivalent Carbohydrate Ligands Assembled on a Metal Template

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Multiple saccharide units are often involved in molecular recognition of biologically active carbohydrates. A simple mucin-type carbohydrate antigen, Tn, contains 2-acetamido-2-deoxy-α-D-galactopyranosyl serine or threonine residues and is reportedly a good marker of malignant transformation in several epithelial tissues.[1,2] Recent binding studies[3,4] of GalNAc-modified peptides with Tn-specific antibodies showed that repeating units of at least two GalNAc-Ser or -Thr residues were necessary for the specific antigen–antibody interactions. Tn-specific lectins, *Vicia villosa* B4 lectin[5,6] and *Salvia sclarea* lectin,[7] also appear to recognize multiple GalNAc residues in their binding to the antigen. Simple synthetic carbohydrates that mimic native structures of the cancer-specific antigens would be extremely useful in diagnostic and therapeutic applications.[8] The synthesis of native carbohydrate clusters, however, usually requires a tedious multistep synthetic strategy.[9–13] We have for the past several years been interested in self-assembly of biomolecules on a metal template.[14–16] Carbohydrate clusters could be assembled by the metal-assisted association of carbohydrate components modified with a metal-binding ligand. We report here the synthesis of bipyidine-modified GalNAc and its metal-assisted association to form tridentate GalNAc clusters (Figure 1).

Bipy-GalNAc[17,18,19] trimerizes to give a tridentate GalNAc cluster in the presence of Fe(II). Four isosbestic points (227, 250, 268, and 296 nm) were observed during the titration, consistent with the large third binding constant of $Fe(bipy)_3$ complexes relative to the first and second ones.[16] The stoichiometry of the complex formation was determined to be 3.2:1 for bipy-GalNAc:Fe(II). The absorption spectrum of the red iron complex ($Fe^{II}(bipy\text{-}GalNAc)_3$, λ_{max} = 303, 351, 481 (sh), and 516 nm) is comparable to that of the iron(II) tris(5,5′-dimethylbipyridine) complex $[Fe(Me_2bipy)_3]^{2+}$ (λ_{max} = 296, 348, 480 (sh), and 519 nm). Since the bipyridine moiety of bipy-GalNAc is unsymmetrical, four stereoisomers, *fac*-Λ, *fac*-Δ, *mer*-Λ, and *mer*-Δ, are possible at the Fe(II) center.[16] The structural differences between the *fac* and *mer* templates are significant; for example, in the *fac* isomers, the pyridyl carbons that are attached to the glycosidic oxygen of the GalNAc unit are positioned 7 Å apart, while in the *mer* isomers, two GalNAcs are 11 Å distant from one another and each is 7 Å away from the third GalNAc. Each GalNAc cluster would, therefore, exhibit considerably different affinity to a lectin or antibody that recognizes multiple GalNAc residues. All four isomers were separated by reverse-phase HPLC, using a linear gradient of acetonitrile and water containing 0.1% TFA on a Vydac C4 analytical column (Figure 2). The isomer ratio was 26:42:12:20, which deviates slightly from the expected statistical ratio, 37.5:37.5:12.5:12.5, for the two *mer* isomers and two *fac* isomers. The CD spectrum of $Fe^{II}(bipy\text{-}GalNAc)_3$ showed only weak peaks at 296 nm ($[\theta] = 7.5 \times 10^4$ deg cm²/dmol) and 311 nm ($[\theta] = -1.5 \times 10^5$ deg cm²/dmol), in accord with the formation of four diastereomers in almost equal amounts.[20]

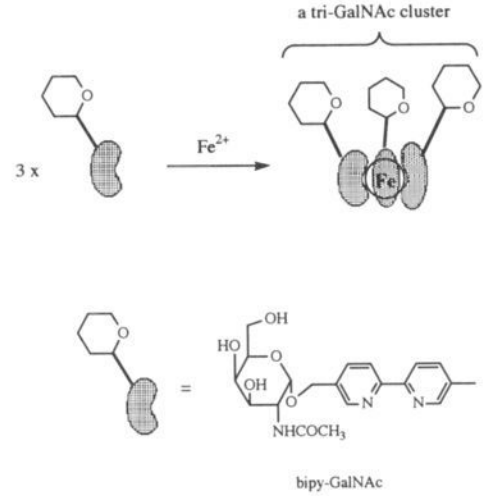

Figure 1. Schematic representation of the assembly of a tri-GalNAc cluster by metal-assisted self-association of bipy-GalNAc.

We studied the binding of bipy-GalNAc, $Fe^{II}(bipy\text{-}GalNAc)_3$ and asialoglycophorin A (MN) to *Vicia villosa* B_4 lectin. All synthetic carbohydrates inhibited the binding of peroxide-modified

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(1) Springer, G. F. *Science* **1984**, *224*, 1198–1206.
(2) Springer, G. F.; Tegtmeyer, H.; Taylor, C. R. *Proc. Am. Assoc. Cancer Res.* **1986**, *27*, 5–65.
(3) Nakada, H.; Numata, Y.; Inoue, M.; Tanaka, N.; Kitagawa, H.; Funakoshi, I.; Fukui, S.; Yamashina, I. *J. Biol. Chem.* **1991**, *266*, 12402–12405.
(4) Nakada, H.; Inoue, M.; Numata, Y.; Tanaka, N.; Funakoshi, I.; Fukui, S.; Mellors, A.; Yamashina, I. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 2495–2499.
(5) Tollefsen, S. E.; Kornfeld, R. *J. Biol. Chem.* **1983**, *258*, 5166–5171.
(6) Tollefsen, S. E.; Kornfeld, R. *J. Biol. Chem.* **1983**, *258*, 5172–5176.
(7) Piller, V.; Piller, F.; Cartron, J.-P. *Eur. J. Biochem.* **1990**, *191*, 461–466.
(8) Hakomori, S. *Curr. Opin. Immunol.* **1991**, *3*, 646–653.
(9) Ferrari, B.; Pavia, A. A. *Int. J. Pept. Protein Res.* **1983**, *22*, 549–559.
(10) Kunz, H.; Birnbach, S. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 360–361.
(11) Kunz, H.; Birnbach, S.; Wernig, P. *Carbohydr. Res.* **1990**, *202*, 207–223.
(12) Kunz, H.; Wernig, P.; Schilling, M.; Marz, J.; Unverzagt, C.; Birnbach, S.; Lang, U.; Waldmann, H. *Environ. Health Perspect.* **1990**, *88*, 247–249.
(13) Toyokuni, T.; Dean, B.; Hakomori, S. *Tetrahedron Lett.* **1990**, *31*, 2673–2676.
(14) Lieberman, M.; Sasaki, T. *J. Am. Chem. Soc.* **1991**, *113*, 1470–1471.
(15) Lieberman, M.; Tabet, M.; Tahmassebi, D.; Zhang, J.; Sasaki, T. *Nanotechnology* **1991**, *2*, 203–205.
(16) Sasaki, T.; Lieberman, M. *Tetrahedron* **1993**, *49*, 3677–3689.
(17) Fiandor, J.; Garcia-Lopez, M. T.; de las Heras, F. G.; Mendez-Castrillon, P. P. *Synthesis* **1985**, 1121–1123.
(18) The fully acetylated GalNAc was treated with ammonia in acetonitrile to selectively remove the acetyl group at the C-1 hydroxyl group.[17] The resulting tri-*O*-acetyl-*N*-acetylgalactosamine (Ac_3-GalNAc) was reacted with 5-(bromomethyl)-5′-methylbipyridine in dry DMF in the presence of Ag_2O. After deacetylation with $NaOCH_3$ in methanol, bipyridine-modified *N*-acetylgalactosamine (bipy-GalNAc) was purified with C4 reverse-phase HPLC. The small coupling constant of the C-1 proton (3.81 Hz) in ^1H NMR is consistent with the α-configuration[19] of bipy-GalNAc. Bipy-GalNAc: ^1H NMR (300 MHz, D_2O) δ 8.84 (br s, 1H), 8.70 (br s, 1H), 8.45 (br s, 2H), 8.31 (d, 1H, *J* = 8.4 Hz), 8.22 (d, 1H, *J* = 8.4 Hz), 5.04 (d, 1H, *J* = 3.8 Hz, H-1), 4.94 (d, 1H, *J* = 13.2 Hz, benzyl), 4.79 (d, 1H, *J* = 13.2 Hz, benzyl), 4.20 (dd, 1H, *J* = 10.5, 3.7 Hz), 4.05–3.96 (m, 3H), 3.76 (d, 2H, *J* = 6.0 Hz, H-6,6′), 2.60 (s, 3H, CH_3), 2.02 (s, 3H, $COCH_3$); MS(FAB) *m/e* = 404 $(M + H)^+$; UV (20 mM Tris-HCl, 150 mM NaCl, pH 7.2) λ_{max} 240 (ε = 15 000), 286 nm (19 500).
(19) Pavia, A. A.; Ferrari, B. *Int. J. Pept. Protein Res.* **1983**, *22*, 539–548.
(20) Δ-$[Fe(bipy)_3]^{2+}$ complex exhibits peaks at 285nm ($[\theta] \geq 4.95 \times 10^5$ deg cm²/dmol) and 300 nm ($[\theta] \leq -1.34 \times 10^6$ deg cm²/dmol) in its CD spectrum, see: Milder, S. J.; Gold, J. S.; Kliger, D. S. *J. Am. Chem. Soc.* **1986**, *108*, 8295–8296.

0002-7863/94/1516-1587$04.50/0

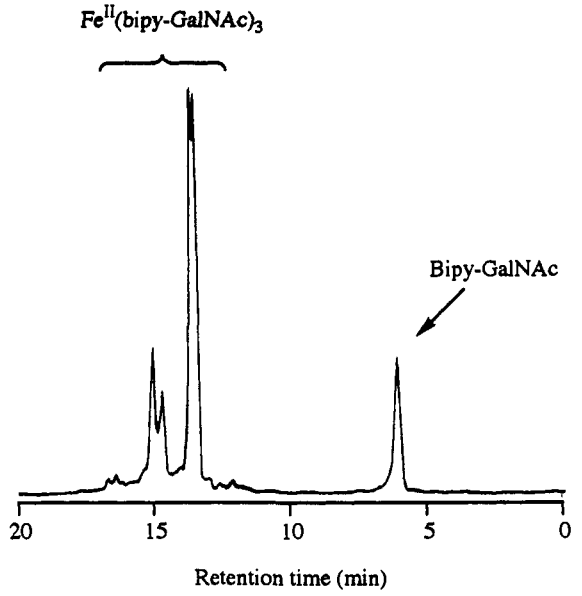

Figure 2. HPLC separation of four diastereomers of the Fe^{II}(bipy-GalNAc)$_3$ complex. The complex was analyzed with C4 reverse-phase HPLC, using a solvent system of water–CH_3CN containing 0.1% TFA. A linear gradient of 10%–30% CH_3CN over 15 min, with a flow rate of 1.0 mL/min, was employed to separate the four isomers. The chromatography was monitored at 296 nm, which is an isosbestic point for the formation of the Fe^{II}(bipy-GalNAc)$_3$ complex.

B_4 lectin to GalNAc-agarose by competition. Figure 3 shows the binding curves of the synthetic GalNAc derivatives to B_4 lectin. The calculated relative binding constants[21] for GalNAc, benzyl-α-GalNAc, bipy-GalNAc, and Fe^{II}(bipy-GalNAc)$_3$ are 1.0, 1.7 ± 0.1, 2.0 ± 0.1 and 15.3 ± 1.0, respectively. $Fe^{II}(Me_2bipy)_3$ neither binds to B_4 lectin nor enhances the binding of benzyl-α-GalNAc. The increased binding affinity of Fe^{II}(bipy-GalNAc)$_3$ over bipy-GalNAc toward B_4 lectin must, therefore, be due to the cluster formation of GalNAc residues on the metal template. Asialoglycophorin A (MN) showed a similar binding constant to Fe^{II}(bipy-GalNAc)$_3$. Asialoglycophorin A (MN) contains several repeating units of GalNAc-modified Ser or Thr residues. Recently, Pepe et al. reported[22] the conformational analysis of a pentapeptide taken from the sequence of asialoglycophorin. The energy-minimized structure of the pentapeptide, Ser-Ser*-Thr*-Thr*-Gly, where * indicates *O*-linked α-D-GalNAc residue, shows a cyclic conformation in which the distances between Cα carbons in this model Tn antigen are 5.3 and 6.7 Å, comparable to inter-GalNAc distances of Fe^{II}(bipy-GalNAc)$_3$ complexes.[23]

(21) Data presented in Figure 3 were fitted to the standard binding isotherm, % inhibition = 100 × [inhibitor]/([inhibitor] + IC_{50}). Dividing the IC_{50} value for GalNAc by IC_{50} values for other inhibitors gave the relative binding constants.

(22) Pepe, G.; Siri, D.; Oddon, Y.; Pavia, A. A.; Reboul, J.-P. *Carbohydr. Res.* **1991**, *209*, 67–81.

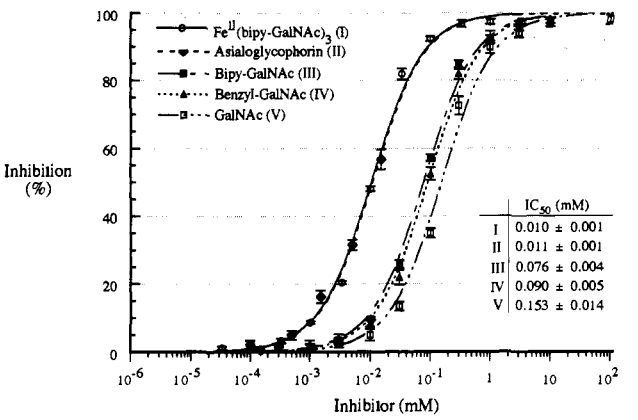

Figure 3. Binding of peroxidase-labeled B_4 lectin to GalNAc-modified agarose measured in the presence of synthetic GalNAc ligands and asialoglycophorin A as an inhibitor. All inhibition experiments were carried out at 22 °C in an aqueous buffer (20 mM Tris-HCl, 150 mM NaCl, 0.25 or 0.5% bovine serum albumin, pH 7.2). A mixture of lectin (5 μM), agarose-bound GalNAc (30 mg of packed gel), and an inhibitor of varying concentrations was gently shaken for 2 h to establish the binding equilibrium. The concentration of unbound lectin was determined by a colorimetric assay, using *o*-phenylenediamine and hydrogen peroxide as substrates for peroxidase. Each inhibition experiment was repeated more than three times to ensure reproducibility.

In conclusion, we have demonstrated a novel metal-assisted assembly process of a tridentate GalNAc cluster that is recognized by a Tn-specific lectin. Multivalent carbohydrate–protein interactions have been shown[24-27] in binding of several other lectins to their carbohydrate ligands. A similar metal-assisted assembly process could be used to synthesize analogues of these multivalent carbohydrate ligands. The approach could also be extended to construct heteromeric carbohydrate clusters, using a combination of various bipyridine-modified carbohydrates. A series of such compounds would be useful in generating a library of synthetic carbohydrate ligands for screening of specific inhibitors for various biologically important lectins and receptors.

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(23) Binding selectivity of four diasteromers of Fe^{II}(bipy-GalNAc)$_3$ was tested using agarose-bound B_4 lectin. Preliminary experiments showed that isomers nos. 1 and 4 bind to the agarose-bound B_4 lectin with much higher affinities than to other isomers. Structural characterizations of these isomers are currently underway.

(24) Glick, G. D.; Knowles, J. R. *J. Am. Chem. Soc.* **1991**, *113*, 4701–4703.

(25) Lee, R. T.; Lin, P.; Lee, Y. C. *Biochemistry* **1984**, *23*, 4255–4261.

(26) Lee, R. T.; Lee, Y. C. *Biochem. Biophys. Res. Commun.* **1988**, *155*, 1444–1451.

(27) Wu, A. M.; Sugii, S. *Adv. Exp. Med. Biol.* **1988**, *228*, 205–263.