Cyclic Glycopeptides

Glycotentacles: Synthesis of Cyclic Glycopeptides, Toward a Tailored Blocker of Influenza Virus Hemagglutinin**

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Influenza virus infection is initiated by the binding of hemagglutinin (HA), a viral carbohydrate-binding membrane protein, to sialic acid-containing oligosaccharides, such as GM3 trisaccharide [Neu5Acα(2,3)Galβ(1,4)Glc], on the host cell surfaces.[1] Since this molecular-recognition process leads to the host cell–virus adhesion stage, molecules with a high affinity for the viral HA would be potent candidates for blockers of the influenza virus. Various types of HA blockers carrying sialooligosaccharides have been synthesized over the last decade.[2] Enhanced blocking effects by the multidentate (multivalent) glycoligands were often found by clustering[3] the sialooligosaccharides on macromolecular scaffolds, such as synthetic polymers,[4] cyclodextrins,[5] and dendrimers,[6] although the mechanism of this multiple interaction has not yet been fully investigated.

In these macromolecular approaches, structural information on the spatial arrangement of the multiple binding sites of the protein is not usually accounted for in the design of ligands. Recently, it was found that ligands containing small oligodentate carbohydrate units, that are designed topologically to bind simultaneously to several binding sites of a single lectin, are very effective blockers.[7,8] The syntheses of the STARFISH dendrimer used to inhibit the adherence of verotoxins, is an excellent example of how detailed structure analysis has led to the creation of a strongly effective "multidentate" ligand.[7,2e]

Thus, on the basis of the HA structure, we created cyclic peptide scaffolds containing tridentate carbohydrate units as a potential HA blockers by using a convenient chemoenzymatic method. HA is a trimeric protein and each subunit contains one sialooligosaccharide binding site on the outer portion. From the three-dimensional structure of HA, as revealed by X-ray analysis,[9] these three binding sites are located on the apexes of an equilateral triangle, in which each binding pocket is separated by approximately 40–50 Å. To achieve a rational molecular design for an HA blocker, we focused on the use of cyclic peptides[10] as a scaffold because of the flexibility of the synthetic strategy and their biological compatibility in the human body.

Herein, we report a chemoenzymatic synthesis of a series of cyclic peptides presenting three sialotrisaccharide units [Neu5Acα(2,3)Galβ(1,4)Glc] by using transglutaminase and glycosyltransferase (Scheme 1). We also discuss the effect of peptide sequence on their biological activities.

Scheme 1 shows the synthetic route to cyclic glycopeptides by means of a combined chemical and enzymatic strategy. In this case, a cyclic peptide **1** prepared by employing the Fmoc-Asp-ODmab method[11] on a chlorotrityl resin[12] was enzymatically coupled with 6-aminohexyl lactoside (**4**) using transglutaminase from guinea pig liver.[13] The transamination reaction between three Gln residues of cyclic peptide **1** and **4** (large excess) gave a mixture of mono- (**1-m**), bi- (**1-b**), and tri-substituted (**1-t**) derivatives. After the introduction of sialic acids with recombinant rat α-2,3-(*N*)-sialyltransferase in the presence of CMP-Neu5Ac,[14] the crude product was subjected to purification by using lactose-binding lectin(RCA120)-based affinity chromatography, DEAE-Sepharose, Sephadex G-25, and reversed phase HPLC (ODS (octadecylsilyl)) to afford the desired cyclic glycopeptides **1-m** (8% yield based on peptide), **1-b** (10%), and **1-t** (12%). It should be noted that the lectin-based affinity chromatographic procedure greatly facilitated the purification of the sialylated cyclic glycopeptides, since intermediates still containing lactose branches are captured by this lectin-bearing adsorbent. Cyclic peptide **1** contains both negatively (Asp) and positively (His) charged residues. We also synthesized cyclic peptides **2** and **3**, which contain neutral amino acid residues (Ser, Gln, and Gly). After cleavage from the resin, the liner peptides were cyclized in the liquid phase to obtain cyclic peptides **2** and **3**. Following the same chemoenzymatic strategy as in Scheme 1, mono- (**2-m, 3-m**), bi- (**2-b, 3-b**), and tri- (**2-t, 3-t**) sialyllactose-attached cyclic peptides were obtained. All the compounds were identified by NMR spectroscopy (1D and 2D (COSY and NOESY)) and MALDI-TOF mass spectrometry.

Molecular modeling studies indicate that the maximum separation between sialic acid residues is approximately 50–70 Å for all the cyclic glycopeptides (**1-t, 2-t**, and **3-t**). Since the binding sites within the HA trimer are separated by 40–

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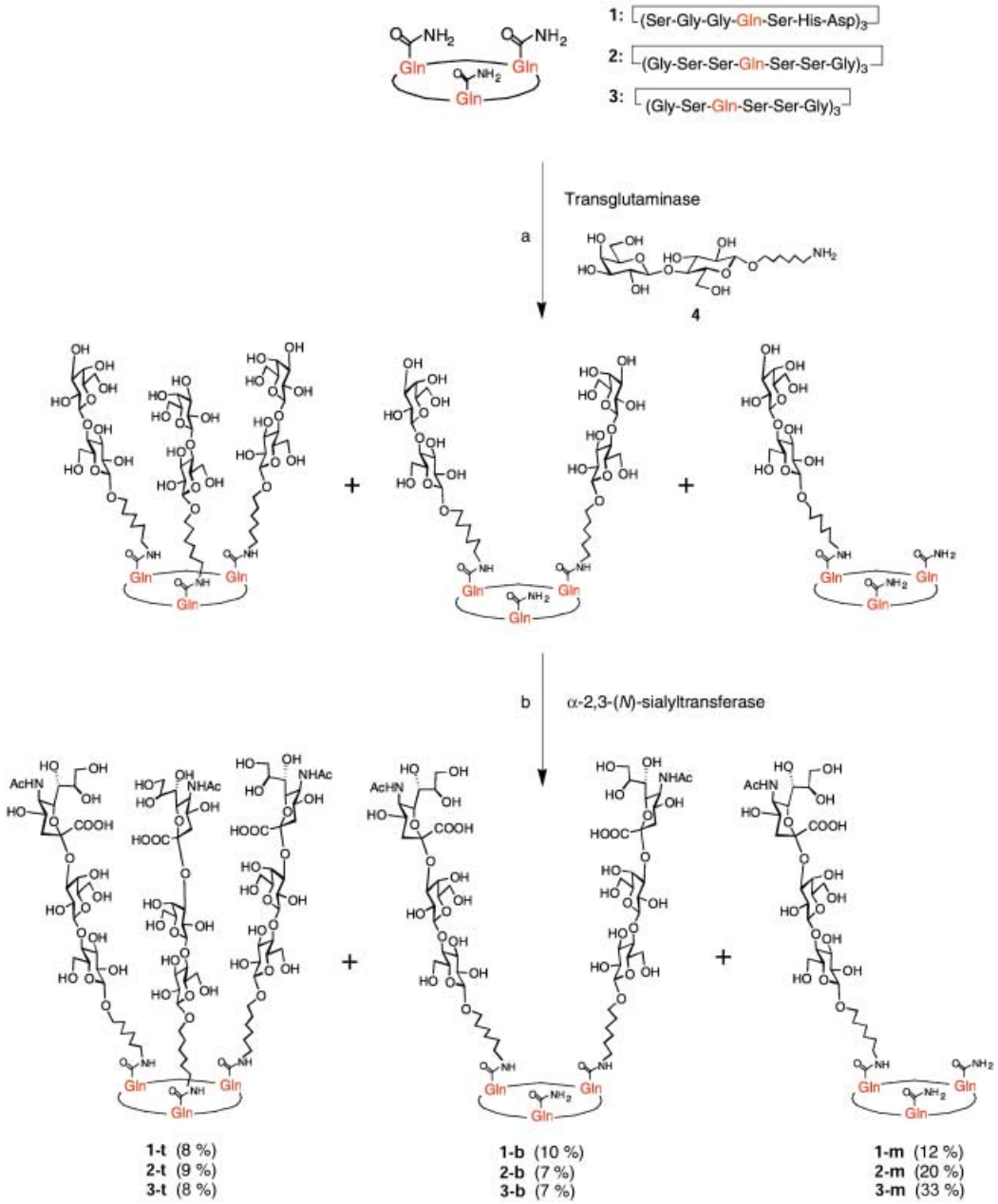

Scheme 1. Enzymatic synthesis of cyclic glycopeptide: a) **4**, transglutaminase (from guinea pig liver) (2 U), Tris/HCl buffer (0.2 M, 10 mM $CaCl_2$, pH 7.2), 37 °C 24 h; b) α-2,3-(*N*)-sialytransferase (0.1 U), sodiumcacodylate buffer (50 mM, 1.58 mM $MnCl_2$, pH 7.5), bovine serum albumin (2 mg mL^{-1}), triton CF-54 (0.1 %), CMP-Neu5Ac (11 mmol). The numbers in parenthesis indicate the two-step purified yields. CMP = cytidine-5′-monophospho-*N*-acetyl neuraminic acid sodium salt.

50 Å, all the tridentate ligands based on **1, 2** and **3** can simultaneously occupy the binding sites within the HA trimer (Figure 1).

The inhibitory effects of cyclic glycopeptides on virus-induced hemagglutination[15] were evaluated by using influenza virus, A/PR/8/34(H1N1), and the results are listed in Table 1. While potent inhibitory effects were observed for **1-t** and **1-b** at the concentrations tested, no inhibition of hemagglutination was observed for monodentate **1 m**. A simple monodentate sialoside has been reported to show a 2–3 mM inhibitory effect by HAI titre.[2e,f] Therefore, a comparison of the inhibitory effect of trivalent **1-t** and monodentate **1-m** indicates that the three oligosaccharides on the cyclic peptide occupy the three binding sites of HA simultaneously (multidentate effect). We used bovine fetuin as a positive control in the inhibitory assay. Bovine fetuin is a glycoprotein (ca. 50 kDa) containing nearly 30 % carbohydrate by weight and possessing six carbohydrate moieties; three N-linked carbohydrate chains (binary and ternary) with terminal sialic acid residues and three O-linked carbohydrate units.[16] On average, fetuin has about 10 sialic acid containing sugar chains on the surface. The minimum concentration of sialic acid residues for inhibition of hemagglutination is calculated as 0.026 mM (0.0026 mM as fetuin molecules). Therefore, the

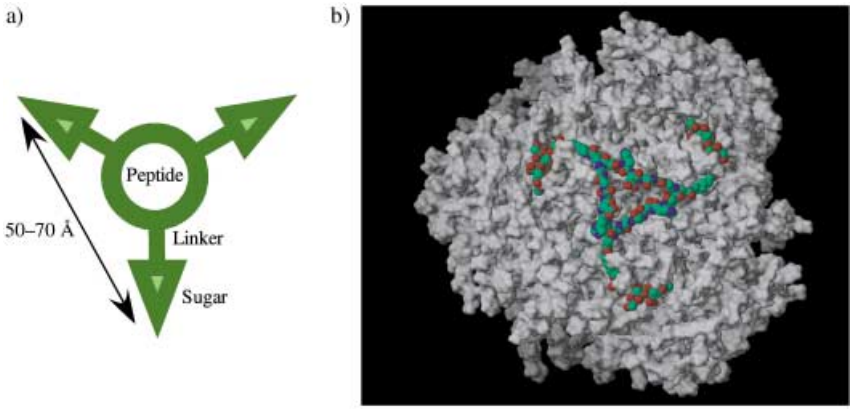

Figure 1. a) Schematic representation of a tridentate glycopeptide. b) The complex of HA and cyclic glycopeptide **1-t** based on computer calculations.[18]

Table 1: Inhibitory effect of cyclic glycopeptides on the hemagglutination of chicken erythrocytes induced by influenza virus and binding affinity against hemagglutinin.

Compound	HAI titre, dilution[a]	K_d [mM][c]
1	NA[b]	> 20
1-m	NA[b]	> 20
1-b	0.43 mg mL^{-1} (0.125 mM)	1.6
1-t	0.16 mg mL^{-1} (0.040 mM)	0.63
2	NA[b]	> 20
2-m	NA[b]	> 20
2-b	NA[b]	> 20
2-t	NA[b]	> 20
3	NA[b]	> 20
3-m	NA[b]	> 20
3-b	NA[b]	> 20
3-t	NA[b]	> 20
Fetuin	0.13 mg mL^{-1} (0.0026 mM)	

[a] In HAI titre, hemagglutinin (A/PR/8/34). [b] NA = no activity at a concentration of 0.25 mM or less. Details of experimental procedures are described in the Supporting Infomation. [c] Binding constants were obtained by SPR using hemagglutinin (A/Equine/La Plata/93).

sialic acid residues on the fetuin were only 4.6-times more effective than the cyclic glycopeptide **1-t** (0.12 mM as sialic acid concentration). This result means that the topological arrangement of the multiple binding sites in relation to the protein structure is of significant importance to the multidentate effect. Surprisingly, the glycopeptides attached on scaffold **2** or **3** (**2-t, 2-b, 2-m, 3-t, 3-b, 3-m**) had no inhibitory effect against hemagglutinin at a concentration of 0.25 mM or less. This observation suggests that the nature of the peptide scaffold also plays a significant role in determining the activity (see below).

The binding assay between the HA protein and cyclic peptide derivative was carried out using surface plasmon resonance (SPR; Biacore apparatus, see Supporting Information for details). HA (A/Equine/La Plata/93) was covalently attached to the carboxymethylated dextran-coated gold sensor surface (CM-dextran) using an amine-coupling procedure.[17] The solutions containing various concentrations (from 0 to 0.5 mM) of cyclic peptide or cyclic glycopeptides were injected over the sensor surface and the binding affinities of the compounds were determined. The respective dissociation constants (K_d) are shown in Table 1.

Interestingly, only **1-t** ($K_d = 0.63$ mM) and **1-b** ($K_d = 1.6$ mM) showed significant affinity to HA on the sensor surface and there was no binding of other cyclic peptide derivatives. This result is in close agreement with the result of the HAI titer experiment. In the SPR experiment, the HA was chemically immobilized on the dextran polymer; thus, the molecular orientation should have been random. Therefore, it is reasonable to consider that cyclic glycopeptides occupy the binding sites within a single HA molecule, rather than the intermolecular bridging of two HA molecules. These binding assay data imply that the amino acid sequence of the cyclic peptide has a marked influence on the direction and flexibility of the side chains of the glutamine residues.

The solution structures of cyclic peptides **1** and **3** were determined by molecular modeling using restraints derived from 2D NOESY data (see Supporting Information for details). Peak assignments were made from DQF-COSY, TOCSY, and NOESY NMR spectra acquired for the peptides at 293 K in H_2O. The 3D structures were calculated using simulated annealing and energy minimization protocols within X-PLOR. A total of 67 distance restraints and 12 dihedral-angle restraints were used to calculate family structures. Figure 2 shows the lowest energy and 30 convergent annealed structures for cyclic peptides **1** and **3**. In the active scaffold **1**, one of the three aspartic acid residues seems to interact with the histidine residue inside the cyclic peptide ring. Eventually, all three side chains of the glutamine residues are directed outwards from cyclic peptide ring

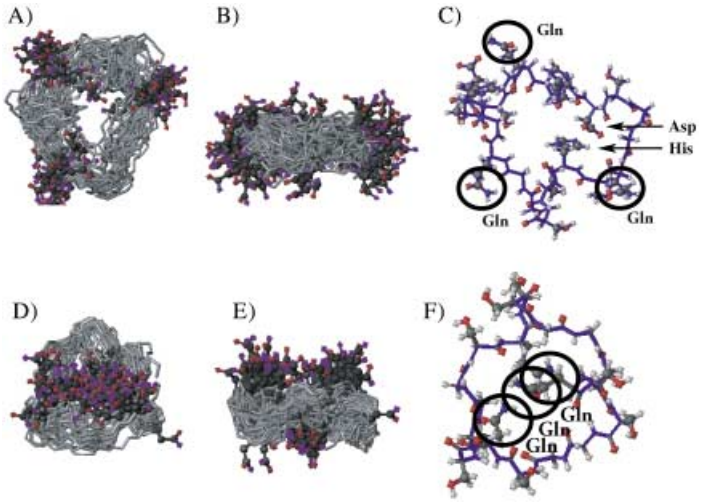

Figure 2. Conformation of cyclic peptide **1** (A, B, and C) and **3** (D, E, and F) calculated from NMR spectra: 30 convergent structure (A, B, D and E; top and side views), the lowest-energy structure (C and F).

(open formation), as expected. However, the side chains of the glutamines residues of the non-active scaffold **3** were directed inward from the cyclic peptide ring (close formation). Therefore, for scaffold **1**, the three attached sugars could reach the binding sites of HA simultaneously and a multidentate effect was obtained. However, in scaffold **3**, the attached sugars were orientated inwards and could not bind simultaneously to the HA binding sites, resulting in a low affinity owing to monodentate binding.

In conclusion, cyclic peptide templates provide an interesting framework for the manipulation of the multidentate presentation of carbohydrate units based on the structural data from hemagglutinin. The chemoenzymatic approach using transglutaminase and glycosyltransferase is effective in providing glycoconjugate linking between peptides and sugars. The biological activity of the hemagglutination assay is strongly depended on the amino acid sequence of the cyclic peptides, even though their circular sizes were identical. This result indicates that, in the addition to the size, the 3D spatial arrangement of multidentate ligands, which is determined by the scaffolds, is of significant importance in producing a "directed multidentate effect", thereby offering new insight into the design of carbohydrate-based drugs.

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[18] The stable conformation of **1-t** was optimized using the molecular dynamics (MD) method. The force field developed by Tripos, Inc was used and the partial charge of atoms was calculated by the Gastiger–Hückel method. The SYBYL program code developed by Tripos, Inc. was applied throughout these calculations. The cyclic glycopeptide **1-t** was docked with HA extracted from the protein data bank. The energy minimized 3D structure of the HA and **1-t** complex in water was determined by molecular mechanics (MM).

