VIP Molecular Recognition

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Dynamic Peptides as Biomimetic Carbohydrate Receptors**

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The cell surface displays many carbohydrates, which act as recognition sites for proteins in cell–cell and cell–matrix interactions. For example, the human blood types originate from the presence of different oligosaccharides at the erythrocyte surface. Also the immune system is based on proteins (antibodies) that recognize carbohydrates on "foreign" cells, viruses, and bacteria. Furthermore, carbohydrates are recognized by the binding pocket of bacterial proteins involved in chemotaxis. Owing to the large number of carbohydrate stereoisomers, a nearly unlimited variety of ligands is conceivable. The structural basis for carbohydrate recognition by proteins has been investigated by X-ray crystallography.[1] Proteins interact with carbohydrates primarily through hydrogen bonding. In addition, the protein–carbohydrate complex is often stabilized by hydrophobic interactions, C–H/π interactions and/or coordination of metal ions such as Ca^{2+} and Mg^{2+}.

The development of artificial carbohydrate receptors or "synthetic lectins"—which would be valuable as drugs, in diagnostics, and in sensing—poses a tremendous challenge for supramolecular chemistry. A useful carbohydrate receptor must compete with the strong hydration of carbohydrates in water and in addition discriminate closely related isomers. In the past, chemists have designed synthetic receptors for carbohydrates that function in organic solvents (where the competition with water is circumvented) and can discriminate very similar carbohydrates, including epimers.[2] However, nearly all these receptors fail in polar solvents, and they are generally useless in water. A more versatile approach towards the recognition of carbohydrates in water is based on the coordination of carbohydrates and boronic acids,[3] including peptides functionalized with boronic acids,[4] but it should be emphasized that these innovative materials are covalent carbohydrate binders rather than noncovalent biomimetic receptors. It is very difficult to design a "synthetic lectin" de novo, as exemplified by the work of Davis and co-workers, who recently synthesized a cagelike receptor with two aromatic subunits that recognizes β-O-methyl GlcNAc ($K = 630\,\text{M}^{-1}$) in a mixture of monosaccharides in water.[5]

Herein we describe a dynamic combinatorial approach to the identification of biomimetic carbohydrate receptors.[6] We explore a dynamic combinatorial library (DCL) of cyclic peptides to select receptors that are assembled from tripeptides under thermodynamic equilibrium. Although carbohydrates[7] and peptides[8] have been used as building blocks in dynamic combinatorial chemistry, there are no reports on the selection of carbohydrate receptors by this methodology. To this end, we used the reversible disulfide exchange pioneered by the groups of Otto and Sanders[9] to create DCLs from a set of tripeptides under physiological conditions (Scheme 1). The tripeptides were synthesized by inverse peptide coupling (see the Supporting Information). N- and C-terminal Cys residues mediate the disulfide exchange reaction. Arg, Asp, Glu, Gln, His, Ser, and Thr were selected because of their potential hydrogen bonding with carbohydrates; GABA, Phe, Trp, and Tyr provide hydrophobic and/or aromatic moieties; and Gly was introduced as an inert residue.

According to the established principle of dynamic combinatorial chemistry, the introduction of a carbohydrate should shift the equilibrium composition of the DCL towards oligopeptides that interact with the carbohydrate and are therefore stabilized through noncovalent interactions ("thermodynamic templating").[6] In this way, the best carbohydrate receptors can be selected from a large set of linear and cyclic oligopeptides. We introduced a range of monosaccharides and disaccharides as templates for the tripeptide DCLs. The templates include the isomeric methylglycosides and disaccharides shown in Scheme 2 as well as the neurotransmitter *N*-acetyl neuraminic acid (NANA).

We first verified whether the disulfide exchange results in an equilibrium mixture of oligopeptides. Three methyl ester protected tripeptides (HisOMe, GlyOMe, and GluOMe) were mixed in NH_4HCO_3 buffer at pH 7.8 in a different order, and the formation of oligomers was monitored by HPLC/MS using hydrophilic interaction liquid chromatography and electrospray mass spectrometry (HILIC/ESI-MS). If the resulting DCL is at thermodynamic equilibrium, the order of addition of the tripeptides should not matter. Since the UV absorption of most tripeptides is low, the selected ion monitoring (SIM) mode was used to analyze the DCL. In this mode, only predefined peptide masses are monitored such that each chromatogram corresponds to detection of a single peptide. The disulfide exchange reaction ends if insufficient thiolate anion is available. Ellman reagent was used to measure the concentration of thiolate in the DCL (see the Supporting Information). No free thiolate could be detected with the Ellman reagent after 24 h. The presence of thiolate was also monitored by MS. In this case, no thiolate

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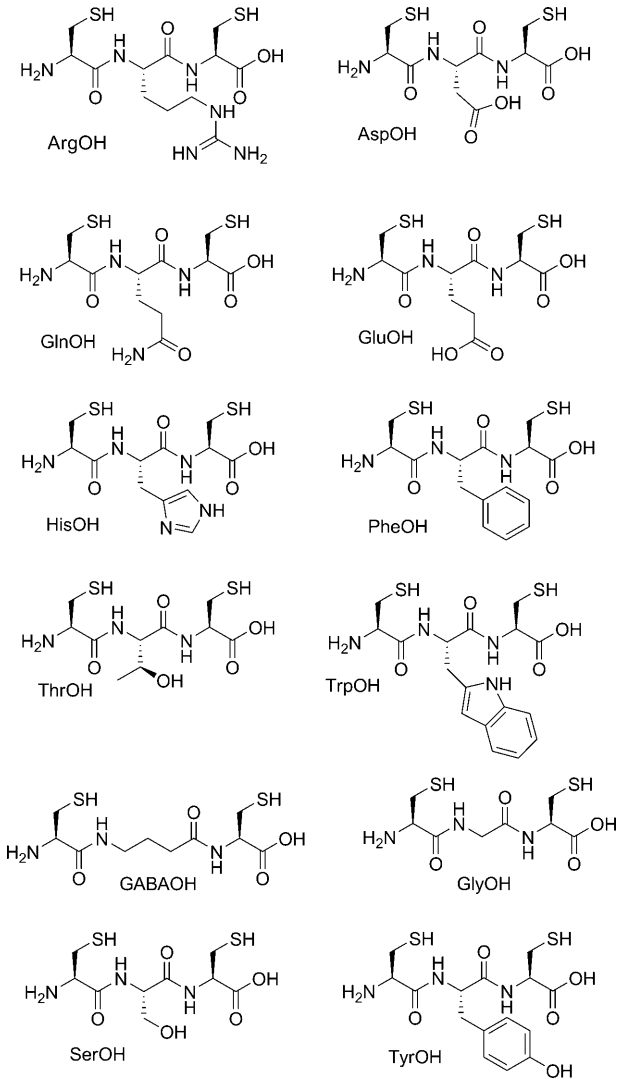
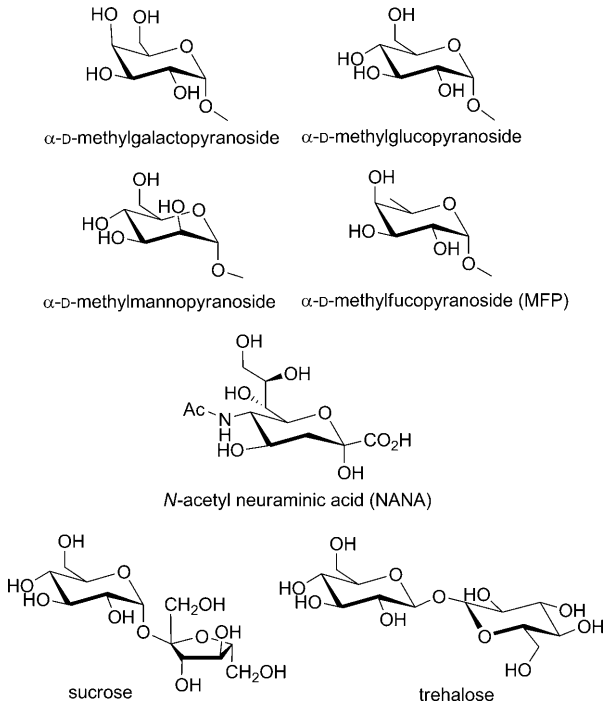


Scheme 1. Tripeptide building blocks for DCLs. ArgOH = Cys-Arg-Cys, AspOH = Cys-Asp-Cys, GABAOH = Cys-GABA-Cys (GABA = γ-amino-butyric acid), GlnOH = Cys-Gln-Cys, GluOH = Cys-Glu-Cys, GlyOH = Cys-Gly-Cys, HisOH = Cys-His-Cys, PheOH = Cys-Phe-Cys, SerOH = Cys-Ser-Cys, ThrOH = Cys-Thr-Cys, TrpOH = Cys-Trp-Cys, TyrOH = Cys-Tyr-Cys.



Scheme 2. Carbohydrate templates for tripeptide DCLs.

could be detected after 48 h, in accordance with the lower limit of detection of mass spectrometry.

Representative chromatograms of three cyclic heterodimers of tripeptides (i.e. cyclic hexapeptides) present in the DCL are shown in Figure 1. The chromatograms display two major peaks for each dimer, which can be attributed to the formation of constitutional isomers (cyclic C→N N→C and cyclic C→N C→N). Additional (minor) peaks may arise from racemization during equilibration. Importantly, it is evident from Figure 1 that each tripeptide dimer is formed to the same extent irrespective of the order of addition of the tripeptides to the DCL. The methyl esters of the peptides are readily hydrolyzed because of the slightly basic conditions and the catalytic activity of the thiolates so that only peptides with a deprotected C terminus remain after 48 h.

All possible cyclic homo- and heterodimers of tripeptides but no linear or higher oligomers could be detected by HILIC/ESI-MS in the DCL after 48 h at pH 7.8. The residual amount of tripeptides (detected as cyclic disulfides) is only 5.5% of the sum of integrated peak areas for all DCL members. In contrast, a mixture of tripeptides at pH 2 does not form any dimers or oligomers because no disulfide exchange occurs at this pH.

To identify possible interactions between the tripeptide dimers and a carbohydrate, the neurotransmitter NANA was added to a mixture of HisOMe, AspOMe, and GlnOMe. After 48 h, the composition of the DCL with and without NANA was compared using HILIC/ESI-MS in the SIM mode (Figure 2a and Table 1). All possible cyclic tripeptide homo- and heterodimers but no linear or higher oligomers could be detected. The residual amount of tripeptides (detected as cyclic disulfides) was only 7.0%. Also in this case, the methyl esters of the peptides were hydrolyzed during equilibration of the DCL. A number of significant changes to the composition of the DCL in the presence of NANA were observed. Most strikingly, the cyclic homodimer HisHis was amplified by a factor of 2.0.

Encouraged by the amplification of cyclic HisHis in the presence of NANA, we investigated this interaction in more detail (Figure 3). HisHis could be easily obtained by stirring the tripeptide HisOMe for 2 days in 100 mM phosphate buffer at pH 7.4. The amount of cyclic dimer was determined by HPLC/MS to be 99.5%. We did not attempt to separate the two constitutional isomers. An ITC titration of 2.0 mM HisHis with 40 mM NANA at pH 7.4 indicated an exothermic interaction with a stoichiometry of 1:2 (Figure 3a); in other words, one molecule of the cyclic HisHis complexes two

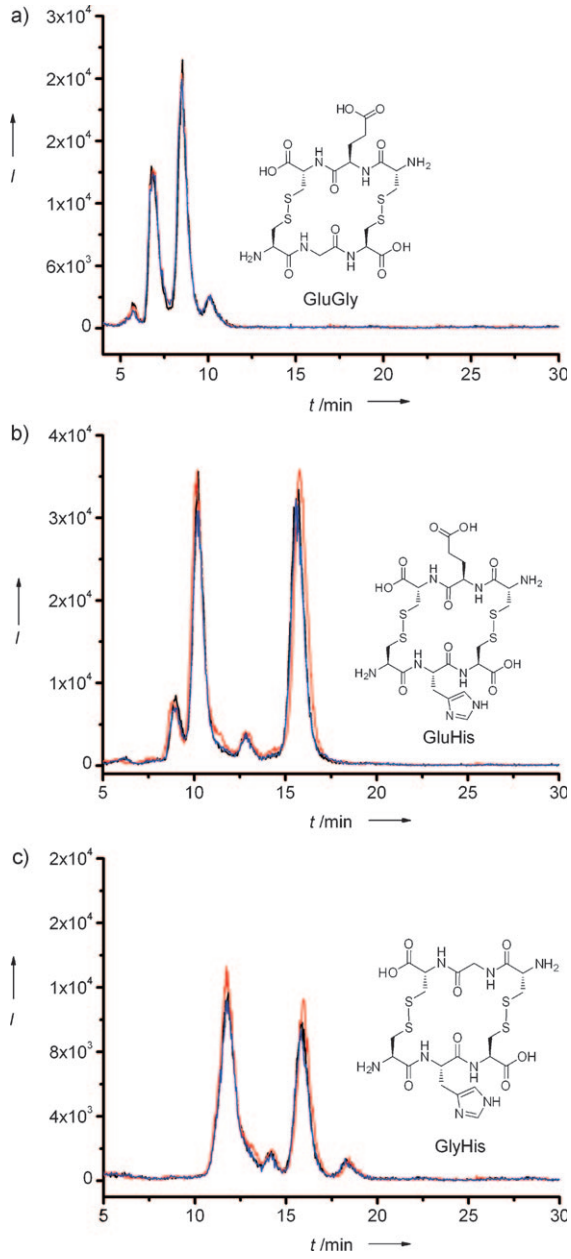


Figure 1. Chromatograms of three cyclic peptides formed in DCLs with different starting points: black: (HisOMe + GlyOMe) + GluOMe; red: (HisOMe + GluOMe) + GlyOMe; blue: (GluOMe + GlyOMe) + HisOMe. HPLC conditions: ZiC-HILIC column, NH_4OAc (20 mM, pH 3.2)/acetonitrile, 32 °C, flow rate 0.15 mL min^{-1}. One of two possible isomers of each peptide is shown.

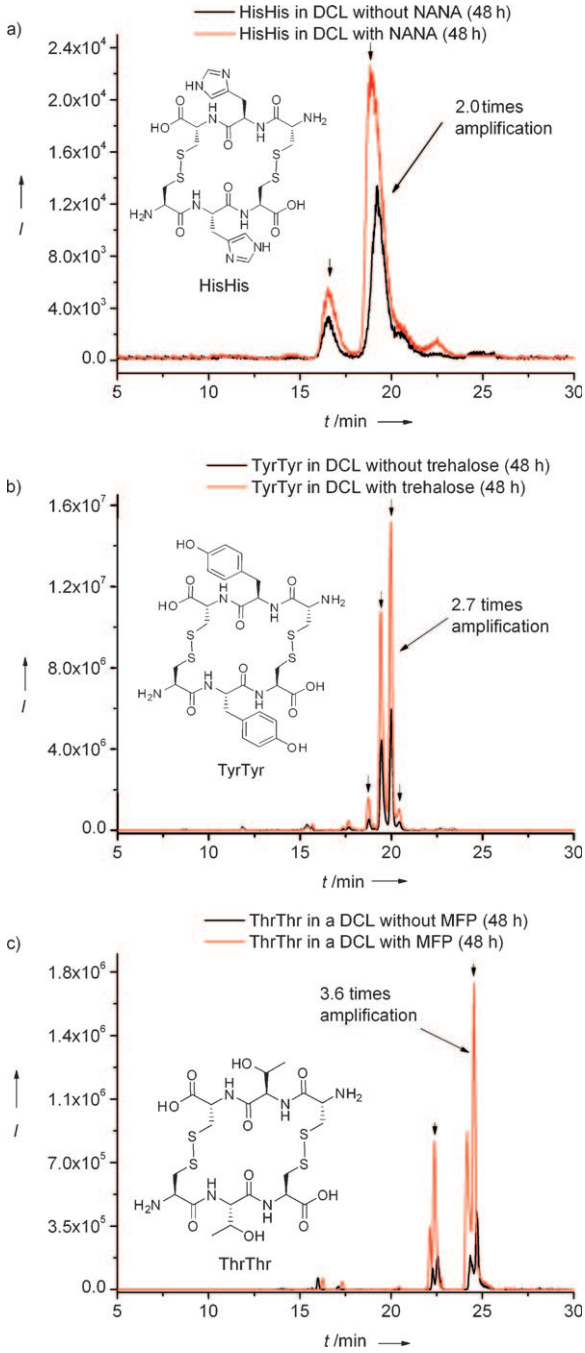


Figure 2. Amplification of cyclic peptides by the carbohydrate templates NANA (a), trehalose (b), and MFP (c). HPLC conditions: ZiC-HILIC column, NH_4OAc (20 mM, pH 3.2 (for a) and 6.8 (for b and c))/acetonitrile, 32 °C, flow rate 0.15 mL min^{-1}. One of two possible isomers of each peptide is shown.

molecules of NANA. The interaction of HisHis and NANA is also entropically favored. Modeling of the ITC titration (Figure 3b) gives two independent binding constants, $K_1 = 72.7\,\text{M}^{-1}$ and $K_2 = 7.76 \times 10^3\,\text{M}^{-1}$. In other words, HisHis binds two molecules of NANA in a cooperative fashion, since the second NANA is bound much more tightly than the first.

The interpretation of the ITC data was confirmed by a ^{1}H NMR titration experiment (Figure 3c), which showed pronounced shifts of the signals of the imidazole protons of HisHis upon addition of NANA at pH 7.6. The minimum of the Job plot of the NMR data (Figure 3d) indicates that the stoichiometry of the complex is 1:2 rather than 1:1. Data fitting provided two independent binding constants, $K_1 = 70.4\,\text{M}^{-1}$ and $K_2 = 5.52 \times 10^3\,\text{M}^{-1}$. The thermodynamic data are summarized in Table 2.

We note that the complexation of HisHis to NANA involves a strong interaction of a small peptide and a monosaccharide in water. In fact, the stability constants

Table 1: Integrated equilibrium SIM peak intensity *I* (in counts × 10^6; standard deviation < 10% ($n = 2$)) in a DCL composed of AspOMe, GlnOMe, and HisOMe with and without NANA.

Oligomer	*M* [g mol^{-1}]	*I* (DCL)	*I* (DCL+NANA)	Amplification
HisHis	719.6	0.84	1.65	2.0
HisGln	710.6	2.60	3.10	1.2
HisAsp	697.5	4.57	2.28	0.5
GlnAsp	688.5	4.54	5.35	1.2
GlnGln	701.4	2.88	1.39	0.5
AspAsp	674.8	2.26	2.86	1.3
AspOH	351.4	0.46	0.39	0.9
HisOH	374.1	0.22	0.16	0.7
GlnOH	364.4	0.65	0.49	0.8

approach those reported for the best synthetic lectins for NANA in DMSO/water (9:1).[10] These synthetic lectins are tripods of (cationic) N-heterocycles, and it is likely that HisHis binds NANA in a similar way as a result of a combination of hydrogen-bonding and C–H/π interactions.[11] It should be emphasized that an ITC titration of HisHis with acetic acid and with the methyl ester of NANA as well as an ITC titration of L-histidine with NANA showed no significant interaction. In addition, the NMR titration of HisHis with the methyl ester of NANA showed no significant shifts. These negative controls indicate that the complexation of NANA by HisHis requires the cooperative interaction of the peptide receptor and the carbohydrate ligand, rather than the additive interactions of the constituent elements.

To explore the potential of identifying carbohydrate receptors by use of a peptide DCL, each of the closely related methylglycosides and isomeric disaccharides shown in Scheme 2 was each added in separate experiments to a DCL containing six tripeptides (GlnOH, GluOH, HisOH, PheOH, TrpOH, and TyrOH) and to a DCL containing five tripeptides (ArgOH, AspOH, GABAOH, SerOH, and ThrOH). After 48 h, the composition of each DCL with and without each carbohydrate was compared using HILIC/ESI-MS. Both DCLs were dominated by cyclic dimers of tripeptides. It was found that some carbohydrates—but not all!—induce significant changes in the composition of the DCL, and that the changes are different for each carbohydrate and each peptide. Two striking amplifications are highlighted below.

In the DCL composed of six tripeptides the cyclic homodimer TyrTyr is amplified by a factor of 2.7 in the presence of trehalose (Figure 2b) but not in the presence of sucrose. The interaction of cyclic TyrTyr and trehalose was investigated in more detail by ITC and NMR and fluorescence spectroscopy (see Table 2 and the Supporting Information). TyrTyr was obtained by stirring the tripeptide TyrOH for 2 days at pH 7.4. The amount of cyclic dimer (two isomers) was determined by HPLC/MS to be 99.9%. An ITC titration of 1.0 mM TyrTyr with 12 mM trehalose indicated an exothermic interaction with a stoichiometry of 1:1. The ITC titration is readily fitted to a 1:1 model, giving a binding constant $K = 2.85 \times 10^3\ \text{M}^{-1}$. The interaction of TyrTyr and trehalose is also entropically favored.

This interpretation of the ITC data was confirmed by an NMR titration of TyrTyr with trehalose, which showed a shift

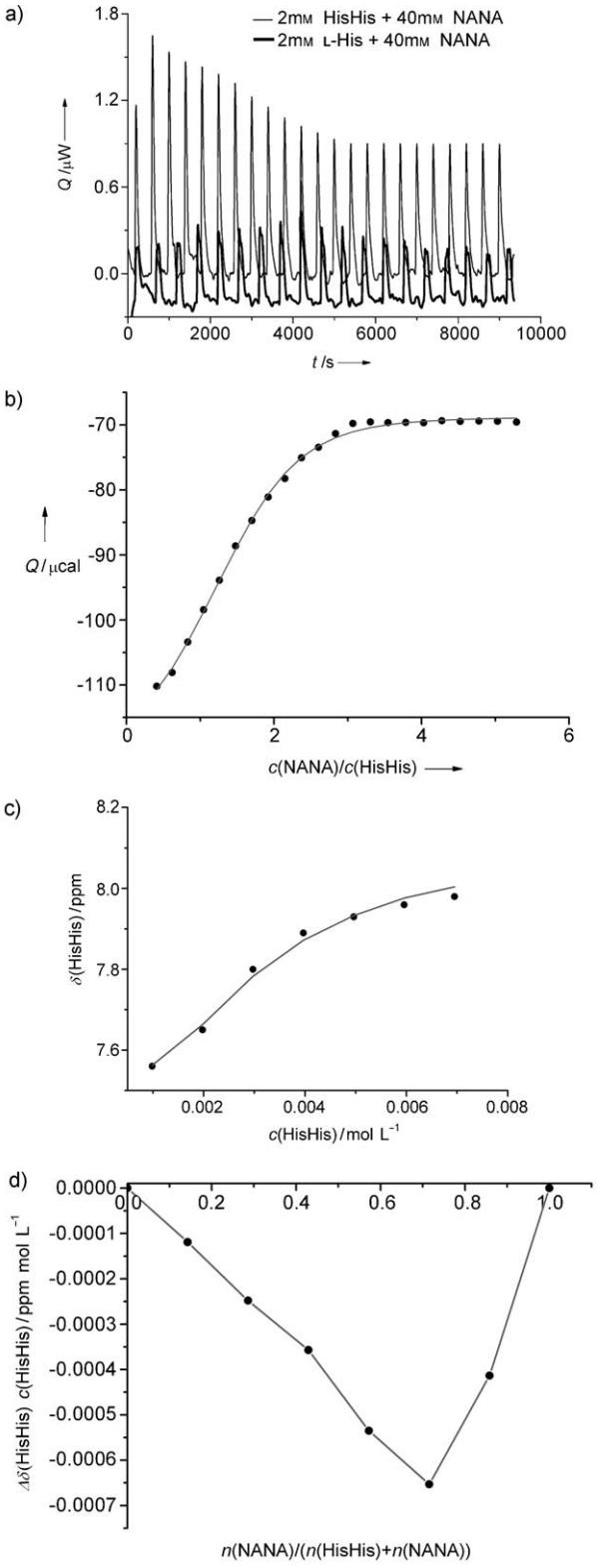


Figure 3. ITC and NMR data for the interaction of HisHis with NANA. a) ITC data. b) Fit of ITC data. c) NMR data. d) Job plot for the NMR titration. The ITC titration of L-histidine with NANA is shown for comparison (thick black curve in (a)). In the ITC fit (c), the first data point is omitted.

of the signals of the aromatic protons of the tyrosine moieties. A Job plot displays a maximum at 0.50 confirming that the

Table 2: Thermodynamic data for the complexation of carbohydrates by cyclic peptides in water.

Peptide	Carbohydrate	n[a]	K [M^{-1}]	ΔG [kJ mol^{-1}]	ΔH [kJ mol^{-1}]	ΔS [J mol^{-1} K^{-1}]	Method
HisHis	NANA	2	$K_1 = 72.7$, $K_2 = 7.76\times10^3$	$\Delta G_1 = -10.6$, $\Delta G_2 = -22.2$	$\Delta H_1 = -6.27$, $\Delta H_2 = -1.54$	$\Delta S_1 = 14.6$, $\Delta S_2 = 69.4$	ITC
		2	$K_1 = 70.4$, $K_2 = 5.22\times10^3$	$\Delta G_1 = -10.6$, $\Delta G_2 = -21.2$	–, –	–, –	NMR
TyrTyr	trehalose	1	2.85×10^3	−19.7	−2.89	56.5	ITC
		1	2.00×10^3	−18.4	–	–	NMR
		1	1.67×10^3	−18.8	–	–	fluorescence
ThrThr	MFP	1	4.00×10^3	−20.6	–	–	NMR

[a] n: stoichiometry.

stoichiometry of the complex is 1:1. A fit of the NMR data with a 1:1 model gives the binding constant $K = 2.00\times10^3\,M^{-1}$. Furthermore, a fluorescence titration showed a significant decrease of TyrTyr fluorescence intensity upon addition of trehalose. Also the fluorescence titration is best fitted to a 1:1 model, providing a binding constant of $K = 1.67\times10^3\,M^{-1}$. Thus, ITC, NMR, and fluorescence measurements are consistent and demonstrate a rather strong interaction of a peptide and a disaccharide in water. The complex must result from a combination of hydrogen-bonding and C−H/π interactions.[2,5,11] Furthermore, we emphasize that the interaction is highly selective since only trehalose but not sucrose (or any of the methylglucosides) amplifies TyrTyr, and (conversely) trehalose amplifies TyrTyr but not PhePhe.

In the DCL composed of five tripeptides the cyclic homodimer ThrThr is amplified by a factor of 3.6 in the presence of α-D-methylfucopyranoside (MFP; Figure 2c) but not in the presence of the similar methylglycosides α-D-methylglucopyranoside, α-D-methylmannopyranoside, and α-D-methylgalactopyranoside. The interaction of cyclic ThrThr and MFP was investigated in more detail (Table 2 and the Supporting Information). ThrThr was obtained by stirring the tripeptide ThrOH for 2 days in at pH 7.4. The amount of cyclic dimer (two isomers) was determined by HPLC/MS to be 99.9%. An NMR titration of ThrThr with MFP showed a significant shift of the signal of the anomeric proton of the carbohydrate. The NMR data is best fitted to a 1:1 model, giving the binding constant $K = 4.00\times10^3\,M^{-1}$. A Job plot displays a maximum at 0.50 confirming that the stoichiometry of the complex is 1:1.

An ITC titration of ThrThr with MFP was inconclusive owing to the very small heat effects that were observed. However, this result implies that the interaction of ThrThr and MFP is entropy driven. In any case, we have identified another example of a rather strong interaction of a peptide and a carbohydrate in water. The complex must result from hydrogen bonding and some type of hydrophobic interaction.[2,5,11] The methyl group of MFP is essential since none of the other methylglucosides (or disaccharides) amplifies ThrThr. Moreover, MFP amplifies ThrThr but not SerSer.

In conclusion, we have prepared equilibrated DCLs of tripeptides through disulfide-exchange reactions under physiological conditions. In these DCLs, all possible cyclic dimers (hexapeptides) are present. In three cases, the composition of the DCL changes significantly upon addition of a carbohydrate template. In a DCL composed of three tripeptides, an interaction between the cyclic dimer HisHis and neurotransmitter NANA was identified: HisHis and NANA form a cooperative 1:2 complex. In a DCL of six tripeptides, a selective 1:1 interaction of the cyclic dimer TyrTyr with trehalose was found, and in a DCL of five tripeptides, a selective 1:1 interaction of the cyclic dimer ThrThr with α-D-methylfucopyranoside was identified. We believe that these findings are a valuable proof-of-concept for the selection of synthetic lectins from a DCL of peptides. Moreover, the receptors identified here are among the strongest biomimetic carbohydrate receptors reported to date.

Experimental Section

The DCLs were created by mixing the peptides (5 mM per peptide when three tripeptides were used or 2.5 mM per peptide when five or six tripeptides were used) in NH_4HCO_3 buffer (total volume: 1.25 mL, 100 mM, pH 7.8) and stirring the solution in an open vial at room temperature. A 750 μL aliquot of a 15 mM carbohydrate stock solution in buffer was added directly afterwards to the stirred solution. 100 μL samples were collected after 48 h for HPLC/MS measurements and acidified with formic acid to pH 3 to stop the disulfide-exchange reaction. The samples were prepared by mixing a 110 μL sample with 90 μL acetonitrile (for DCLs of three tripeptides) or by diluting a 40 μL sample with 160 μL acetonitrile (for DCLs of five of six tripeptides).

The LC/MS setup comprised a Shimadzu HPLC system and a quadrupole ion trap (Q-TRAP) mass spectrometer (Applied Biosystems), equipped with a Turbo IonSpray (pneumatically assisted ESI) source. The LC system consisted of two LC10-AD_{VP} pumps, a DGC-14 A degasser, a SIL-HT_A autosampler, a CTO-$10AV_{VP}$ column oven, and a SPD-$10AV_{VP}$ UV detector. The software used for controlling LC and MS was Analyst 1.4.1 (Applied BioSystems). The separation was carried out using a ZIC-HILIC column (SeQuant GmbH) with the following dimensions: 150 mm × 2.1 mm i.d., 3.5 μm particle size, and 200 Å pore size. A ZIC-HILIC precolumn (20 mm × 2.1 mm i.d., 5 μm particle size, 200 Å pore size, SeQuant) was used to prevent contamination of the analytical column. The flow rate was 0.15 mL min^{-1}, the oven was tempered at 32 °C, and the UV absorption was monitored at 220 nm with a sampling frequency of 10 Hz. The injection volume was 10 μL. Acetonitrile and NH_4OAc (20 mM, pH 3.2) were used as the mobile phase with the following gradient (% acetonitrile, t [min]): 55, 0.01; 45, 15.00; 55, 19.0.

Mass spectra were recorded using ESI(+)MS in the SIM mode with an ion-spray voltage of 4200 V, 30 psi nebulizer gas, 30 psi dry gas, a declustering potential of 30 V, an entrance potential of 10 V, and an ESI source temperature of 300 °C.

DCLs containing five or six tripeptides were measured with a LC/MS setup comprising a Thermo Fischer Scientific Accela HPLC system and a LTQ Orbitrap XL mass spectrometer. For controlling the LC and MS Xcalibur (version 2.0.7, Thermo Fischer Scientific) was used. The separation was carried out using the ZIC-HILIC column described above. The flow rate was 0.15 mL min^{-1}, the oven was tempered at 32 °C, and the injection volume was 10 μL. Acetonitrile and NH_4OAc (20 mM, pH 6.8) were used as the mobile phase with the following gradient (% acetonitrile, t [min]): 70, 0.00; 55, 2.00; 45, 15.00; 55, 19.00.

Mass spectra were recorded using ESI(+)MS with an ion-spray voltage of 3970 V, 25 psi sheath gas, 10 psi auxiliary gas, 5 psi sweep gas, a tube lens voltage of 145 V, and a capillary temperature of 275 °C.

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