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Specific Recognition of Disaccharides in Water by an Artificial Bicyclic Carbohydrate Receptor

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We present the synthesis of a peptide-based bicyclic carbohydrate receptor capable of carbohydrate recognition in 10 % deuterated acetic acid in water. The binding constants were estimated by using NMR spectroscopy, and furthermore, a series of carbohydrate complexes were studied with the use of MS (ESI) of the complex.

The most abundant class of organic molecules on earth is that of the carbohydrates. They serve as structural materials in the form of cellulose or chitin, for example, and as energy storage to provide the fuels for biochemical processes. The class of carbohydrates covers protein and cell membranes, and carbohydrates act as signalling molecules that by interaction with receptors and lectins determine the fate of these. Carbohydrates are implicated in cell differentiation, in cancer immunology as well as in host defence against viruses, bacteria and parasites. By interaction with numerous receptors in membranes or with secondary receptor sites on functional macromolecules, carbohydrates determine the distribution and architecture of functional macromolecular assemblies and bring together growth hormones, receptors, proteases and so on.[1]

This important set of functionalities renders carbohydrate recognition an attractive target for artificial receptors. Selective artificial carbohydrate receptors could be used either diagnostically to determine the location and concentration of a particular carbohydrate structure, for example, the concentration of glucose in blood, or as a drug targeting compound functioning as an artificial antibody. In some instances an artificial receptor could even be a drug in itself by blockage of carbohydrate-mediated viral entry or bacterial adhesion.

Diabetes will, according to WHO, affect more than 360 million people in the world by 2030 (http://www.who.int/mediacentre/factsheets/fs312/en/index.html), and therefore the recognition, binding, metabolism and clearance of glucose is of great interest.

Many of these goals are not yet achievable and recognition of simple carbohydrates in biological fluid is still a major challenge. At the present time, most of the known synthetic carbohydrate receptor systems only function in nonpolar solvents[2] (usually chloroform), and the reported systems are based on the interaction between the OH groups on the carbohydrate and the polar groups on the receptor.

In aqueous media, host–guest systems relying solely on hydrogen bonding are inefficient due to competition from the solvent. Even Nature has problems in designing high-affinity carbohydrate receptors, although a few do exist.[3] Naturally occurring protein-based carbohydrate receptors have binding affinities in the range from 10^4 to 10^7 M^{-1}.[4]

To date, most synthetic carbohydrate receptors that are effective in water are those that rely on covalent boronate formation.[5] The noncovalent interactions available for binding carbohydrates are hydrogen bonding, CH–π interactions and "hydraphobic" effects.[6] The individual significance of these different effects is still not clear.[7] When looking at the crystal structures, hydrogen bonding is predominant; however, the polar groups involved are well solvated before the binding occurs.[8,9] This is supported by the exceptional role of entropy and entropy compensation in the recognition of carbohydrates. Artificial receptors should thus be designed to provide a combination of hydrophobic and hydrophilic interactions with the carbohydrate. The first receptors taking this into account were the water-soluble cyclic tetramers of resorcinol.[10]

Cyclodextrins have also been used as host molecules for carbohydrates;[11] unfortunately, this system is difficult to investigate with NMR spectroscopy owing to the overlap of the signals from the host and guest. The first peptide-based

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carbohydrate recognition was reported in 1993,[12] and later a similar combinatorial approach was investigated.[13] Recently a water-soluble bicyclic aromatic polyamide receptor was reported.[14,15]

In Nature, the protein–carbohydrate selectivity is obtained through a combination of hydrogen bonding to the hydroxy groups on the carbohydrate and the interaction of the CH groups of the carbohydrate and an aromatic segment on the protein.

In the present work, a cyclic peptide (Figure 1) was selected to constitute the hydrogen donor/acceptor of the complex, and in the centre of the cyclic peptide an aromatic bridge was introduced to provide the CH–π interactions. Cyclization lowers the flexibility of the peptide and thus provides a conformationally stable receptor molecule. The synthesis of the receptor is shown in Scheme 1, and its design was performed according to the following four criteria: (1) The receptor should be soluble in water and amphipathic to provide an organized solvation shell of water particularly around the naphthalene ring. (2) The hydrogen-bond interactions are of great importance for the specificity, so a polar cyclic peptide provides H-bond donors and acceptors for interaction with the carbohydrate hydroxy groups on the β face. (3) An aromatic surface sufficiently large for interaction with the carbohydrate α face was provided. (4) Receptor size was designed to facilitate a relatively tight fit to the glucose unit; the cyclic dodecapeptide model was derived through modelling.[16]

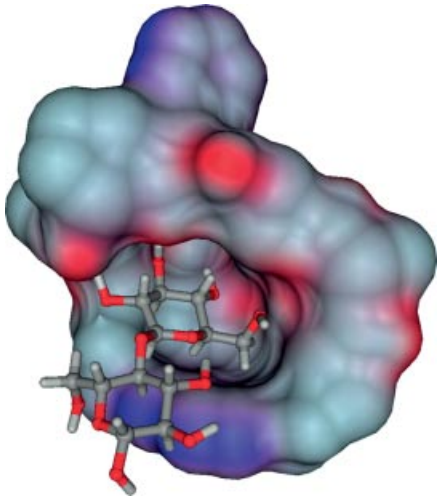


Figure 1. Structural representation of carbohydrate receptor **1**. 3D representation of receptor–cellobiose complex obtained through docking of the carbohydrate in water (top, water molecules have been removed for clarity).

In this arrangement, the entropic effect of releasing the organized water shell from the naphthalene bridge combined with the establishment of the CH–π interactions between the α face of the carbohydrate and the aromatic surface of the naphthalene serve as driving forces for the binding event between the carbohydrate and the receptor cavity. This allows a carbohydrate-specific H-bonding network of the hydroxy groups with the amide bonds of the peptide to be established.

The peptide was composed of polar amino acids except for a phenylalanine unit serving as an internal standard for the induced changes in the chemical shifts upon binding. This internal standard facilitated the differentiation between the changes in the chemical shifts derived from the direct interactions with the carbohydrate from the chemical shift changes due to the alteration of the solution properties induced by changes in the analyte concentration.

The chemical shifts of pure **1** was assigned by a combination of 2D HSQC,[17] H2BC,[28] HMBC, TOCSY and NOESY[27] NMR spectroscopic experiments and was performed in 10% deuterated acetic acid in H_2O at 5 °C. Starting from the easily discernible amide protons, the α carbons and the side chain groups were assigned. The chemical shifts of the naphthalene bridge was assigned starting from the NOESY cross peaks between the β protons of the cysteine units to the 2- and 6-methylene groups. From those, the chemical shifts of the naphthalenic aromatic protons 1, 3–5, 7 and 8 was determined, and they were found to fall in the narrow ranges of 7.40–7.76 ppm for ^{1}H and 127.4–129.0 ppm for ^{13}C (Figure 2 and Supporting Information).

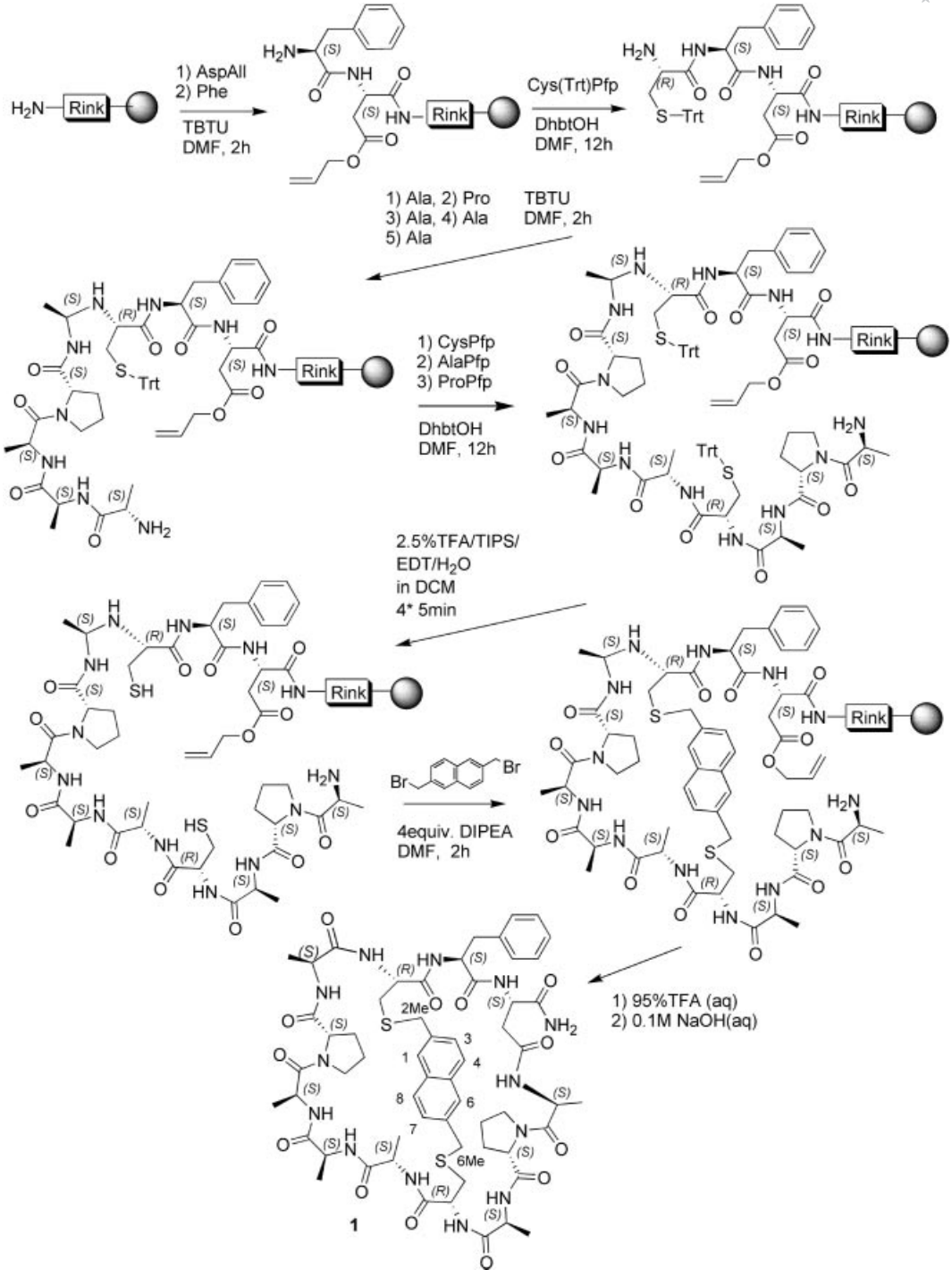
The changes in the chemical shifts following stepwise additions of cellobiose was studied by a series of HSQC NMR spectroscopic experiments (Figure 2 and spectra S26–S32 in the Supporting Information)

All the protons on the naphthalene bridge shifted downfield upon the addition of cellobiose, whereas the position of the aromatic signals from the phenylalanine varied considerably less (spectra in Supporting Information). For the 1-H proton, the pure compound has a ^{1}H chemical shift of 7.56 ppm and the change was 0.14 ppm for the highest concentration. Theses chemical shifts were fitted for a 1:1 complex for the determination of the bindings constant in water and yielded a K_a value of 7.7 M^{-1} ($\pm$0.4 M^{-1}) (Figure 3). The 1:1 binding was further substantiated by recording the MS of the complex.

In the last 5–10 years, exciting progress in mass spectrometry has enabled gas-phase studies of protein complexes[18] and other supramolecular aggregates.[19] Electrospray ionization has proven to be one of the most successful methods to achieve the delicate transfer of large molecules, including dendrimers from solution to the gas phase for mass spectrometry (MS).[20]

To evaluate the stability of molecular or complex ions in the gas phase, collision-induced dissociation (CID) is performed. Here, an ion is selected, accelerated and subsequently collided in a cell containing neutral gas atoms, for example argon.[21]

In the collision process a part of the kinetic energy of the ion is transformed into internal energy, which results in fragmentation. The extent of fragmentation depends on the internal energy of the excited ion, which in turn is related to the imposed acceleration. Use of CID in the analysis of the covalent structures is a common application;[22] however, by surveying the literature it appears to be more difficult to analyze the dissociation of noncovalent structures in the gas phase. Meijer et al. recently published a study of dendritic complexes.[23] In the MS (ESI) of noncovalent



Scheme 1. The synthesis of the water-soluble carbohydrate receptor. All couplings were performed on solid support ($PEGA_{1900}$) and the final cyclization of the preorganized peptide was performed in solution phase. Total isolated yield based on resin loading was 6.4%. Detailed synthesis and receptor characterizations are presented in the Supporting Information.

complexes, the relationship between the strength of the aggregate in solution (thermodynamically controlled) and in the gas phase (kinetically controlled) remains controversial.[24]

With the ambition to measure the binding constants of several carbohydrates to the bicyclic receptor, we attempted to use electrospray ionization MS. All the measurements were performed with a Q-TOF ultimate global instrument.

A mixture of **1** (10^{-6} M) and different carbohydrates (10^{-5} M) in water was injected into the mass spectrometer, and the complex was then, if present, isolated by using the quadrupole and collided with argon in the collision cell, which resulted in dissociation of the complex. The product of this dissociation was then analyzed by time-of-flight MS (Figure 4). Figure 5 shows a stacked plot of eight mass spectra obtained from a mixture of cellobiose and the re-

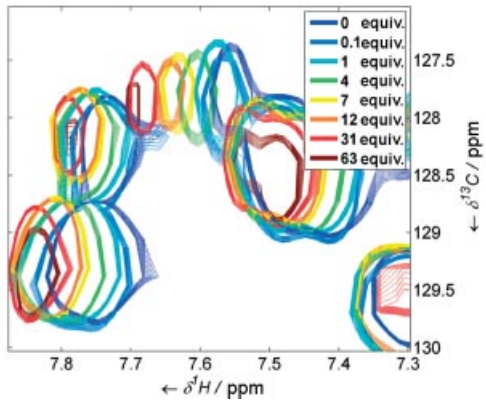


Figure 2. Details of overlaid HSQC spectra showing signals from the six aromatic protons in the naphthalene bridge of the bicyclic peptide at different cellobiose concentrations. For assignments see the Supporting Information. pH was kept constant at all times.

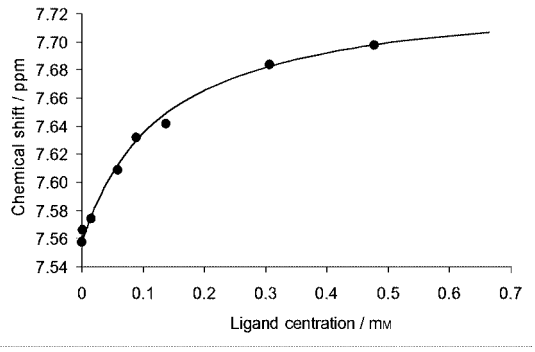


Figure 3. Change in ^{1}H chemical shifts for the 1-H naphthalene proton at δ = 7.56 ppm. K_A = 7.7 M^{-1} (±0.4 M^{-1}). Dots: measured shifts; solid line: simulated data with the use of K_A and chemical shift of the complex determined by dynamic fitting of the titration data (see Supporting Information).

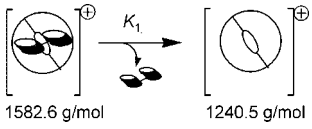


Figure 4. Schematic illustration of the complex dissociating in the collision cell. The cellobiose leaves the complex as a noncharged (and therefore "invisible") species.

ceptor with an increasing acceleration voltage in the collision cell.

Dissociation of cellobiose from the receptor was determined by observation of the decrease in intensity for the noncovalent complex peak at m/z = 1582 and the corresponding simultaneous increase in the empty receptor peak at m/z = 1240 with increasing collision energy.

To analyze complexes with different carbohydrates in a more quantitative manner, we compared the voltage required to reach an ion survival yield of 50% of the selected complex, the Ec_{50} value. To be able to compare complexes of different molecular weights the collision energy is transformed into the centre-of-mass energy.[25]

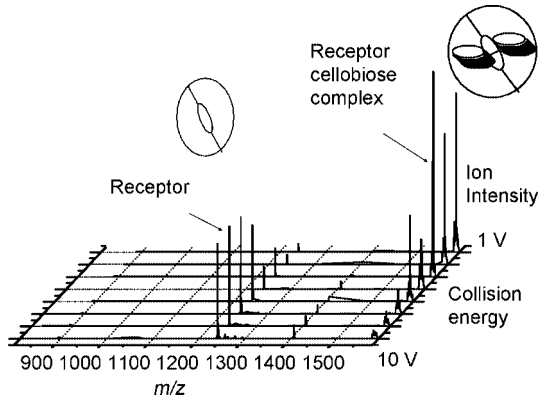


Figure 5. Stacked plots of mass spectra at different collision energies.

We are well aware that this is a relative measurement of the dissociation energy, but by assuming the similarities of the different complexes, it should be fair to compare the different values. Nesatyy et al.[24] performed an evaluation of the difference between solution and gas-phase binding constants of a series of noncovalent protein complexes. In three out of eight investigated complexes they found a quantitative correlation between the gas-phase and the solution-phase binding experiments. In the other five, the results indicated binding, but correlation was not quantitative. It is therefore important to keep in mind that the following data only provides proof of complex formation in solution, and that the Ec_{50} value is an approximate extrapolation from the gas-phase stability constant.

These values are plotted in Figure 6. All settings on the instrument (pressure in collision cell, etc.) were kept constant. As a result of the construction of the Q-TOF quadrupole, it was not possible to obtain a mass spectrum under very mild collision energies, which prohibited the measurement of very weak bindings of the monosaccharide. The use of helium or hydrogen as the collision gas could facilitate the low-energy measurements on monosaccharide complexes. All the data points were accumulated from 10 individual measurements. The peak for the empty receptor is inversely proportional to that of the complex. The results are presented in Table 1.

For the monosaccharides, a weak complex was observed in the analysis; however, it was not stable enough to measure an E_c50 value (indicating a low affinity). Binding of the trisaccharide raffinose [*O*-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-β-D-fructofuranoside] could not be observed. Thus, there is a specificity for the 1–4-linked disaccharides, which indicates that the receptor interacts with both the terminal sugar and the aglycon in a relatively specific manner.

We have presented the synthesis of a new carbohydrate receptor that is promising for use in combinatorial receptor studies on solid supports. The relatively low binding of the receptor to the disaccharides, as well as the specificity, can possibly be improved by appropriate combination of the amino acids in the bicyclic receptor scaffold. A focused

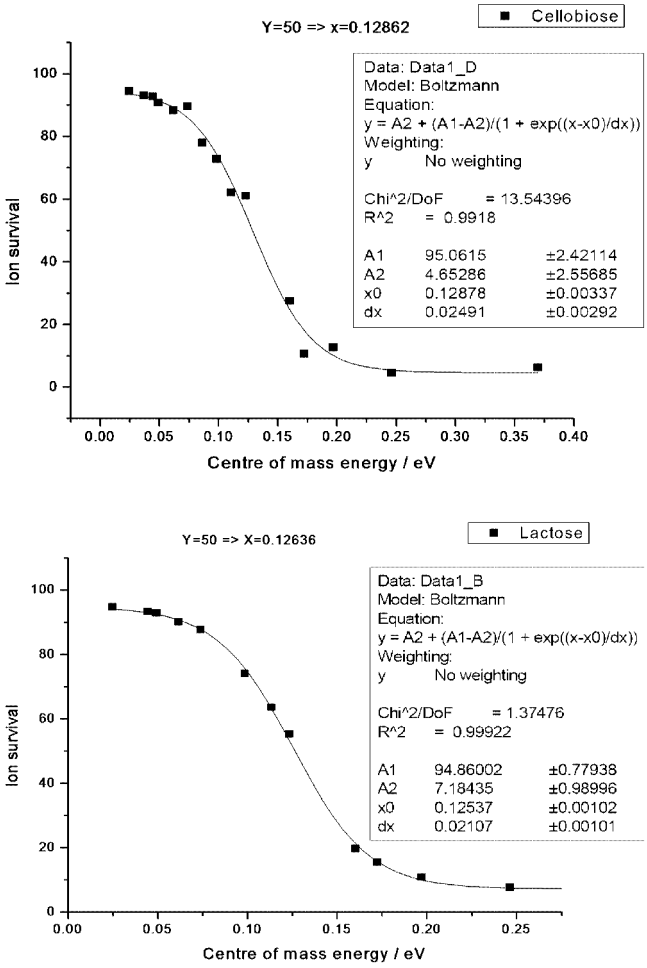


Figure 6. Ion survival plotted against the collision energy (corrected to centre of mass); cellobiose (top) and lactose (bottom).

Table 1. Ec_{50} values for the carbohydrate complexes investigated in this paper.

Carbohydrate	Ec_{50}/eV
Glucose	weak complex
Mannose	weak complex
Galactose	weak complex
Cellobiose	0.13
Maltose	0.12
Lactose	0.13
Raffinose	no complex formation

combinatorial investigation of the influence of the receptor composition by using encoded bead technology is in progress. We have used NMR spectroscopy and collision-induced dissociation mass spectrometry as a clean and fast method for determination of relatively weak binding constants between carbohydrates and noncovalent receptors.

Experimental Section

General: $PEGA_{1900}$ resin (loading 0.11 mmol/g) was purchased from Versamatrix A/S, Denmark. Solid-phase peptide chemistry and solid-phase organic chemistry were performed in plastic syringes equipped with sintered Teflon filters. All solvents were HPLC grade and used as received without further purification. Analytical HPLC was performed with a HP1100 HPLC system with the use of analytical Zorbax 300SB (4.5 × 50 mm) C18 columns with a flow rate of 1 mL/min. Detection was at 214 nm. All HPLC procedures were carried out by using buffers A (0.10% TFA in H_2O) and B (0.08% TFA in MeCN) with a linear gradient (98% A to 98% B in 25 min). All commercially available reagents were used as purchased without further purification; dry DMF was kept over molecular sieves (3 Å).

Synthesis of Artificial Receptor 1: $PEGA_{1900}$ resin (1.37 g, 0.15 mmol of $–NH_2$) was washed before use with aqueous NaOH (0.1 M, 1 × 1 h), H_2O, DMF (3 × 5 min), 30% TFA in DCM (1 × 1 h), DCM (3 × 5 min) and DMF (dry, 6 × 6 min). The resin was then coupled with preactivated Fmoc-Phe-OH (317.7 mg of Fmoc-Phe-OH/139 μL of NEM/252 mg of TBTU, 5 min) dissolved in dry DMF prior to linker attachment for opening up the resin. The mixture was added to the resin and was left for 2 h with occasional agitation. The resin was washed with DMF and the coupling was repeated. The resin was then washed with DMF (6 × 2 min) and Fmoc deprotection was performed with 20% piperidine in DMF (2 × 18 min), followed by washing with dry DMF (6 × 2 min).

The resin was then coupled twice with preactivated RINK amide linker, (441.9 mg/139 μL of NEM/252.4 mg of TBTU) in dry DMF as described above. The resin was then washed with DMF (6 × 2 min); Fmoc deprotection was performed with 20% piperidine in DMF (2 × 18 min), followed by washing with DMF (6 × 2 min). Fmoc-Asp(All)-OH, (323.8 mg/139 μL of NEM/252.4 mg of TBTU) was activated for 5 min in dry DMF and coupled and deprotected as described for Fmoc-Phe-OH above. Fmoc-Cys(Trt)-OPfp (615.7 mg) and Dhbt-OH (44.5 mg) was dissolved in dry DMF, and the mixture was added to the resin and left for 12 h with occasional agitation.

The resin was then washed with DMF and Fmoc deprotection was effected as above by using 20% piperidine in DMF. After washing with DMF (6 × 2 min) activated Fmoc-Ala-OH, (269.7 mg/139 μL of NEM/252.4 mg of TBTU, 5 min) dissolved in dry DMF was added to the resin and left for 2 h with occasional agitation. It was washed and deprotected as described for Fmoc-Phe-OH above. Activated Fmoc-Pro-OH (276 mg/139 μL of NEM/252.4 mg of TBTU, 5 min) dissolved in dry DMF was added to resin and left for 2 h with occasional agitation, followed by washings and deprotection as described above. Fmoc-Ala-OH, (270 mg) was activated (139 μL of NEM/252.4 mg of TBTU, 5 min) in dry DMF left with the resin for 2 h; the resin was then washed as described for Fmoc-Phe-OH above.

Two consecutive Fmoc-Ala-OH residues were coupled in a similar manner and Fmoc-Cys(Trt)-OPfp was coupled for 12 h as described for Cys above. Fmoc-Ala-OPfp (391 mg) and DhbtOH (44.5 mg) were dissolved in dry DMF and coupled for 12 h. The resin was then washed with dry DMF and the coupling was repeated. The resin was then washed with DMF (6 × 2 min), Fmoc deprotection was performed with 20% piperidine in DMF (2 × 18 min), followed by washing with DMF (6 × 2 min). Fmoc-Pro-OPfp (412 mg) and DhbtOH (44.5 mg) was dissolved in dry DMF and reacted, deprotected and washed in a similar manner. Finally, to complete the assembly of the cyclization precursor, coupling (12 h) with Fmoc-Ala-OPfp, (391 mg) and DhbtOH (44.5 mg) in dry DMF was performed and the Fmoc group was not removed.

Removal of the trityl groups from the cysteine residues was effected by treatment of the resin (under argon) with a mixture of DCM/TFA/TIPS/EDT/H_2O (90:2.5:2.5:2.5:2.5; 4 × 5 min). The resin was washed with DCM (4×), DMF (3×) and 5% DIPEA in DMF (3×). Cleavage of the Rink linker was insignificant under these conditions according to HPLC of the eluate from the resin. Freshly prepared cleavage cocktails seem to give the purest product. The resin was kept under an atmosphere of argon and the incorporation of the naphthalene bridge was performed immediately. 2,6-Bis(bromomethyl)naphthalene (129 mg, 1.5 equiv.) was dissolved into dry degassed DMF (140 μL), this solution was added dropwise to the resin over 10 min, with agitation under an atmosphere of argon. The mixture was left for 2 h, washed with dry degassed DMF and the coupling was repeated. Prior to peptide cyclization the linear peptide was cleaved from the Rink amide linker by treatment for 2 h with TFA/H_2O/TIPS (92.5:5:2.5). The peptide was simply isolated by evaporation of the solvent (speedvac at 0.1 Torr 35 °C overnight). RP-HPLC, general conditions 11.1 min. MS: m/z = 1298.3 $[M + H]^+$.

Initially the allyl ester of the aspartic acid was cleaved with aqueous NaOH to provide the linear peptide in one step and then the cyclization was performed in a following step with a standard TBTU peptide coupling. However, the cyclization of the linear peptide occurred spontaneously and unexpectedly in the first step when dissolving the crude material from the solid-phase synthesis in aqueous NaOH (0.5 M) for 1 h. This kind of aminolysis of esters is well known in the classical chemical literature,[26] but not easily predicted in such macrocyclization reactions. The solution was then neutralized with aqueous HCl (5 M) and freeze-dried, and the peptide was isolated as one major peak by HPLC. The yield of **1** based on initial loading (0.11 mmol/g) of the resin was 6.4%. Data for receptor **1**: HRMS: calcd. for $C_{59}H_{79}N_{13}O_{13}S_2$ $[M + 2H]^{2+}$ 620.7681; found 620.7686 (dev. 0.8 ppm).

NMR Spectroscopy: NMR spectra were recorded with a Bruker DRX-600 (proton frequency 600.13 MHz) equipped with a 5-mm triple-resonance inverse probe. Processing of spectra was made with the TopSpin software (Bruker). The sample (10 mg) was dissolved in 10% deuterioacetic acid in H_2O (0.45 mL) for analysis, and all spectra were acquired at 5 °C. Pulse widths were 7.7 and 12.3 μs for ^1H and ^{13}C, respectively. The latter was determined with the CALIS-sequence.[27] Chemical shifts are reported in ppm using acetic acid (CD_2HCOOH) as a reference (^1H 2.04 ppm and ^{13}C 20.0 ppm).

1D ^1H: 1D ^1H spectra were recorded by using a 1D NOESY pulse sequence with presaturation applied to the water signal at δ = 5.25 ppm during relaxation delay (2.2 s) and mixing time (80 ms). The spectral width was 6.8 kHz with 16 K data points recorded.

TOCSY and NOESY: $^1H/^1H$ TOCSY parameters: spectral width 6.8 kHz, 2 K data points, mixing time 60 ms and 8 transients for each of 512 t_1 increments. $^1H/^1H$ NOESY parameters: spectral width 6.8 kHz, 2 K data points, mixing time 100 ms and 48 transients for each of 512 t_1 increments. Water suppression was effected by using a 3–9–19 pulse sequence with gradients.[28]

HSQC and HMBC: Gradient enhanced $^1H/^{13}C$ HSQC parameters: spectral widths 6.8 kHz in f_2 and 25 kHz in f_1 (1 K data points, 256 t_1 increments). Gradient enhanced $^1H/^{13}C$ HMBC parameters: spectral width 6.8 kHz in f_2 and 33 kHz in f_1 (6 kHz for carbonyl band selective experiment), 4 K data points, 128 t_1 increments, and the delay time for evolution of long-range couplings was 70 ms. The low-pass J-filter was optimized to suppress couplings in the range 125–165 Hz.

H2BC: The multiplicity edited H2BC-experiment was used to deduce long-range couplings over two bonds.[29] The spectral widths were 6.8 and 25 kHz for f_2 and f_1, respectively. The number of data points in the direct dimension was 1 K with 148 transients for each of the 128 t_1 increments for two interleaved experiments. The final data matrix of 1024 × 512 was split into the two separate experiments and the linear combinations (a + b and a – b) was calculated before Fourier transform. The third-order low-pass J-filter was optimized to suppress couplings in the range 125–165 Hz.

Supporting Information (see footnote on the first page of this article): HPLC, mass spectra, fully assigned HSQC, H2BC, HMBC, TOCSY and NOESY spectra for compound **1**; binding titration of the complex with cellobiose; a source code written in Visual Basic (2005) for dynamic fitting of the binding data.

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