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An Enantioselective Fluorescence Sensor for Glucose Based on a Cyclic Tetrapeptide Containing Two Boronic Acid Binding Sites

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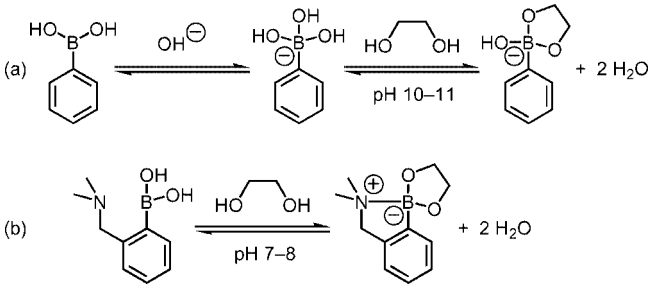
The synthesis and binding properties of carbohydrate receptor **1d**, which contains two boronic acid binding sites facing each other at opposing sides of a cyclotetrapeptide cavity, are presented. According to electrospray mass spectrometry, ^{1}H NMR spectroscopy, and fluorescence spectroscopy, this receptor forms stable 1:1 complexes with D-glucose (K_a = 24800 ± 1200 M^{-1}) and L-glucose (K_a = 11900 ± 1600 M^{-1}) in water/methanol (1:1) at pH = 11.7. Complexes with D-galactose, D-mannose, and D-allose are significantly less stable, while D-fructose, D-ribose, and D-xylose cannot be bound by cooperative action of both boronic acid binding sites. Thus, **1d** possesses high affinity and selectivity in glucose recognition combined with good enantioselectivity. Moreover, fluorescence of **1d** is quenched upon substrate binding, which allows the use of this receptor as an optical sensor for glucose in aqueous solution.

Introduction

The specific recognition of cell-surface carbohydrates by proteins initiates important biochemical processes such as cell–cell interactions or the immune response. Carbohydrate–protein interactions are also important for carbohydrate metabolism and for the transport of carbohydrates across cell membranes. Important insights into the principles of the underlying non-covalent interactions have come from crystal structures of carbohydrate binding proteins with the substrate or an inhibitor bound inside the active centre.[1–3] In addition, synthetic carbohydrate receptors have contributed significantly to our current understanding of the factors that control affinity as well as selectivity in carbohydrate recognition.[4] In spite of the significant progress that has undoubtedly been made in the development of artificial carbohydrate receptors, including the first systems active in aqueous solution,[5–9] practical applications for such receptors, for example as chemosensors to detect the presence and concentration of biologically important sugars in aqueous solution, are not yet in sight. These applications, examples of which are monitoring the glucose level in blood or the sugar concentration during fermentation processes, require a receptor to bind to a given substrate and signal complex formation in water, yet most of the systems developed so far exhibit appreciable activity only in non-competitive organic media.[4] Alternative approaches towards the development of artificial carbohydrate receptors with a high long-term stability and, if necessary, a specificity for substrates that cannot be targeted with natural systems are therefore actively being pursued. The most promising strategy relies on the reversible formation of cyclic esters between boronic acids and the hydroxy groups of sugar molecules.

Boronic acids are known to form five- or six-membered cyclic esters with 1,2-diols and 1,3-diols, respectively, in basic aqueous solution.[10–12] The underlying equilibrium is depicted in Scheme 1 (a).



Scheme 1.

Equilibrium constants determined for the reactions between phenylboronic acid and several monosaccharides show that the stability of the corresponding esters decreases in the order fructose > galactose > mannose > glucose (at 25 °C).[13] This order seems to be retained by all monoboronic acids and therefore reflects the intrinsic carbohydrate selectivity of a receptor containing one boronic acid

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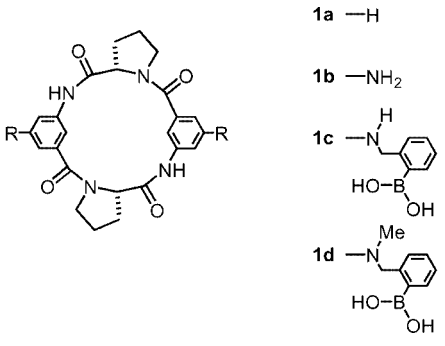
binding site. The selectivity order changes when a receptor contains two or more boronic acid groups that can interact with the substrate in a cooperative fashion. In this case, selectivity depends mainly on the mutual arrangement of the binding sites, which, in turn, is controlled by the structure of the receptor. Thus, attaching two boronic acids to a suitable molecular scaffold represents an efficient modular strategy for the development of selective receptors[10–12] not only for monosaccharides but also for sugar acids[14,15] and sugar alcohols.[16] Enantiodifferentiation in carbohydrate recognition can be achieved by using chiral scaffolds,[14–17] while scaffolds containing chromophores yield fluorescence or colour sensors that signal the binding event by a change in their optical properties.[18–21]

The structural complexity of most boronic acid based carbohydrate receptors developed so far is relatively low, which is a clear advantage in terms of synthesis.[10–12] One can speculate, however, that the use of structurally more elaborate scaffolds based on, for example, (chiral) macrocyclic compounds could have a positive effect on binding selectivity. The tetraboronic acid resorcinarene system described by Strongin represents a step in this direction.[22] Another potentially useful scaffold is the cyclic tetrapeptide **1a**, which contains alternating L-proline and 3-aminobenzoic acid residues and has been developed in our group.[23] A conformational analysis revealed that this peptide can adopt conformations in solution with converging aromatic subunits; the angle between these subunits is variable, however. The attachment of binding sites to the aromatic residues should therefore yield receptors in which the peptide ring functions as a molecular hinge that allows the distance and the orientation of the binding sites to adapt to the size of a bound substrate. Molecular modelling indicated that the angle between the aromatic rings in certain tetrapeptide conformations is well suited for the inclusion of a monosaccharide and we therefore expected a derivative of **1a** containing two boronic acid groups to exhibit the characteristic binding properties of a bis(boronic acid) based carbohydrate receptor.[10–12] In addition, the restricted conformational flexibility and the chirality of **1a** could have the advantage of imposing a high degree of selectivity in complex formation.



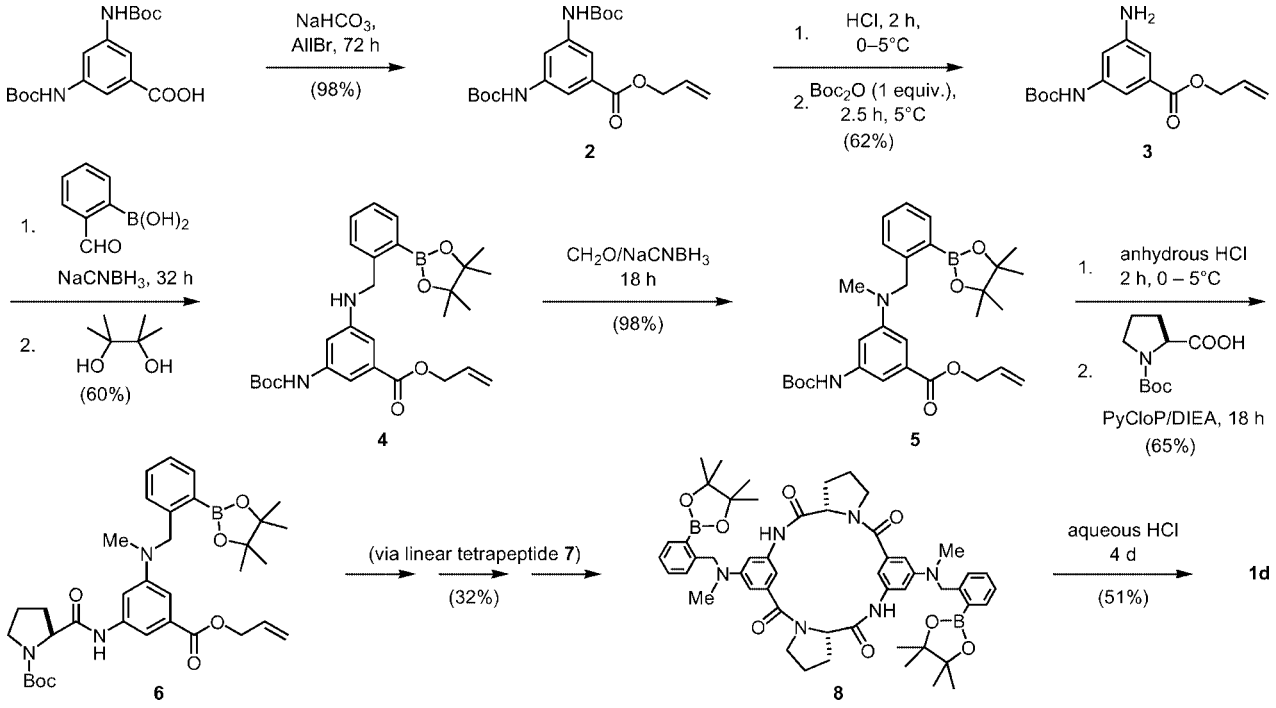
In this article, we describe the synthesis and binding properties of tetrapeptide **1d**, and show that **1d** binds strongly to glucose in water/methanol (1:1), albeit only at high pH. The binding affinity towards D-glucose is significantly higher than towards L-glucose and a change in the optical properties of **1d** upon binding allows complex formation to be monitored by means of fluorescence spectroscopy. Interestingly, electrospray ionisation mass spectrometry and fluorescence spectroscopy indicate that only glucose among the series of monosaccharides tested forms 1:1 complexes with **1d** of appreciable stability. Tetrapeptide **1d** therefore represents a new type of selective fluorescence sensor for the detection of glucose in aqueous solution.

Results

Synthesis

We initially chose **1c** as our first target because we expected that this compound would be easily accessible from the previously described tetrapeptide **1b**, which contains free amino groups in the 5-position of the aromatic rings.[23] Compound **1c** could also allow for N–B interactions between the boron centres and the neighbouring amino groups. Such interactions have been shown to induce the formation of tetrahedral boron centres also at neutral pH, thus shifting the working pH of a boronic acid based carbohydrate receptor from basic solution to an almost physiological environment [Scheme 1 (b)].[10–12,24] It should be noted that a detailed analysis has recently shown that the N–B dative bond depicted in Scheme 1 (b) is usually only present in aprotic solvents; in protic media a solvent insertion pathway dominates that leads to the formation of a hydrogen-bonded zwitterionic species with little or no N–B interaction.[25]

Unfortunately, our attempts to synthesise **1c** by reductive amination of **1b** with commercial 2-formylphenylboronic acid in the presence of sodium cyanoborohydride only yielded inseparable product mixtures consisting mainly of mono- and disubstituted products. We therefore decided to assemble the bis(boronic acid) containing cyclic tetrapeptide stepwise from appropriately protected preformed subunits. The corresponding synthesis started with the reductive amination of protected 3,5-diaminobenzoic acid derivative **3** (Scheme 2). The product formed was converted directly into the corresponding pinacol ester **4**. Because the secondary amino group in **4** can potentially interfere during the following peptide coupling reactions, it was transformed into a tertiary amine by another reductive amination prior to peptide synthesis. After acidic cleavage of the Boc group, **5** was coupled with Boc-protected L-proline. The resulting dipeptide **6** was chain-elongated to the corresponding tetrapeptide **7** by conventional deprotection and coupling methods. Compound **7** was then deprotected at the N- and the C-terminus, and the resulting product was cyclised under pseudo-high-dilution conditions. Chromatographic workup and acidic cleavage of the pinacol esters in **8** finally afforded analytically pure receptor **1d** in amounts sufficient for the following binding studies.



Scheme 2.

ESI Mass Spectrometry

To obtain information about the nature and the number of products formed during the reaction of **1d** with carbohydrates, and to see whether the two boronic acids in the receptor are indeed able to cooperatively bind to a given substrate to form 1:1 complexes, we investigated solutions of **1d** containing various monosaccharides by means of electrospray ionisation mass spectrometry (ESI-MS). The samples were prepared by adding 40 equiv. of a substrate to a 0.2 mM solution of **1d** in water/methanol (1:1) adjusted to pH = 11.7 with NaOH. After 2 h at room temperature, the ESI mass spectrum of each mixture was recorded. A large substrate excess was chosen to estimate the relative stability of a potential 1:1 complex between the receptor and a monosaccharide with respect to the 1:2 complex in which the two boronic acids act as individual binding sites. Only if the 1:1 complex is sufficiently stable should significant amounts of this complex be detectable in solution in the presence of an excess of substrate. As substrates we used D-glucose, L-glucose, D-galactose, D-mannose, D-allose, D-fructose, D-ribose, and D-xylose. The ESI mass spectrum of the **1d**/D-glucose mixture is depicted in Figure 1.

Six major peaks are visible in the spectrum, the most prominent of which at m/z = 865.61 can be assigned to the

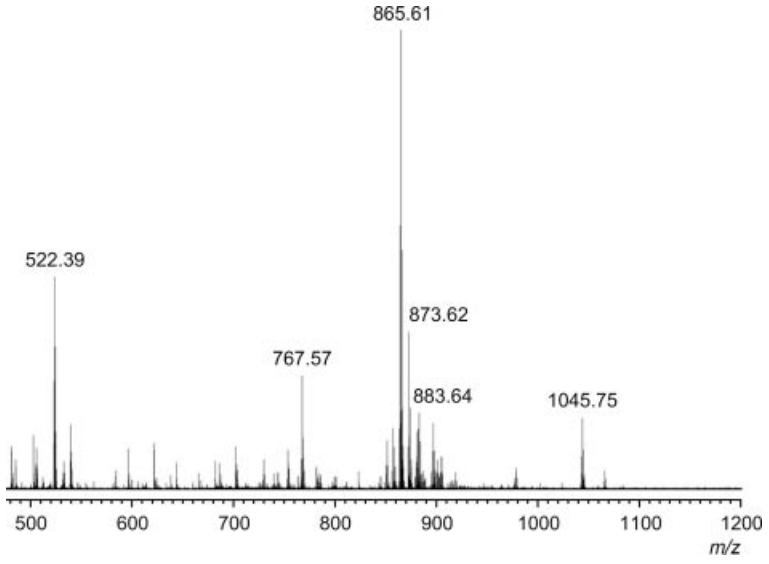


Figure 1. ESI mass spectrum (negative mode) of a solution of **1d** (0.2 mM) and D-glucose (8 mM) in water/methanol (1:1; pH = 11.7).

deprotonated 1:1 complex between **1d** and D-glucose [**1d**·D-glucose – $4H_2O$ – H^+]. The peak at *m/z* = 883.64 also represents a 1:1 complex in which only three ester bonds are formed [**1d**·D-glucose – $3H_2O$ – H^+]. Peaks of the 1:2 complex are visible at *m/z* = 1045.75 [**1d**·2 D-glucose – $4H_2O$ – H^+] and 522.39 [**1d**·2 D-glucose – $4H_2O$ – $2H^+$]. The peak at *m/z* = 767.57 can be assigned to a dehydrated dimethyl ester of the free receptor [**1d** + $2CH_3OH$ – $3H_2O$ – H^+] and the one at *m/z* = 873.62 to a compound in which one boronic acid group of **1d** is esterified with glucose and the other is replaced by a hydroxy group [{**1d** – $B(OH)_2$ + OH}·D-glucose – $2H_2O$ – H^+].

The results obtained for the **1d**/L-glucose mixture were very similar (see Supporting Information). Interestingly, significant amounts of the 1:1 complexes could only be observed for these two monosaccharides. The other substrates tested can be divided into two groups. Only minor amounts of 1:1 complexes were detected in the spectra of samples containing D-galactose, D-mannose, or D-allose; the more prominent peaks in these spectra represent 1:2 complexes, free receptor, or decomposition products of **1d** (see Supporting Information). The second group of substrates consists of D-fructose, D-ribose, and D-xylose. Here, no 1:1 complex formation could be observed and the peaks in the spectra were those of 1:2 complexes and decomposition products in which either one or both boronic acid groups of **1d** are substituted by hydroxy groups (see Supporting Information).

NMR Spectroscopy

Additional information on the binding equilibria between **1d** and D-glucose came from ^{1}H NMR spectroscopy. Figure 2 shows the ^{1}H NMR spectrum of free receptor (1 mM) in D_2O/CD_3OD (1:1) at pD = 11.7 and the effect of the addition of increasing amounts (0.5–25 equiv.) of D-glucose.

The free receptor exhibits a ^{1}H NMR spectrum with broad peaks, probably caused by slow conformational interconversion and/or intra- or intermolecular interactions between the boronic acid groups. Upon addition of D-glucose, a new set of much sharper signals appears in the ^{1}H NMR spectrum, which indicates the formation of a structurally defined complex which, on the NMR timescale, is in slow equilibrium with free **1d**. Complex formation is almost complete in the presence of 1 equiv. of D-glucose since a further increase in substrate concentration has practically no effect on the spectrum. Only when the substrate is present in large excess are minor signals visible in the ^{1}H NMR spectrum; these may be caused by the presence of additional species in solution.

Fluorescence Spectroscopy

The fact that **1d** is strongly fluorescent and that fluorescence is quenched upon complex formation with glucose allowed us to gain insight into the rate of complex formation and to quantitatively determine complex stability.

Upon excitation of a solution of **1d** in water/methanol (1:1; pH = 11.7) at 285 nm a strong emission band is observed in the fluorescence spectrum of **1d** with a maximum at 480 nm. Addition of an excess of D-glucose to the solution causes the fluorescence intensity of this band to decrease. This effect, which demonstrates the usefulness of **1d** as a chemosensor for glucose, is shown in Figure 3.

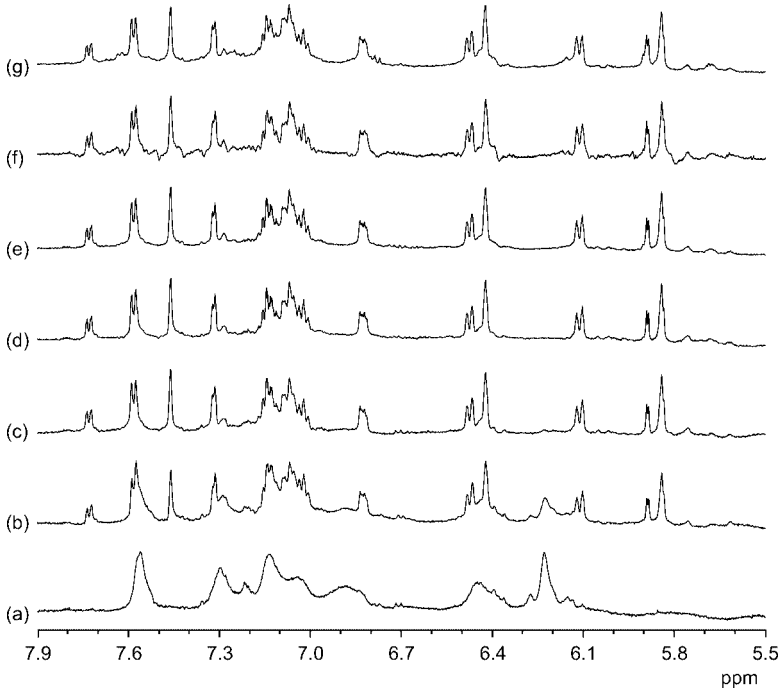


Figure 2. Section of the ^{1}H NMR spectrum of **1d** (1 mM) in D_2O/CD_3OD (1:1) at pD = 11.7 (a) and the effect of the addition of 0.5 (b), 1 (c), 2 (d), 5 (e), 10 (f), and 25 (g) equiv. of D-glucose.

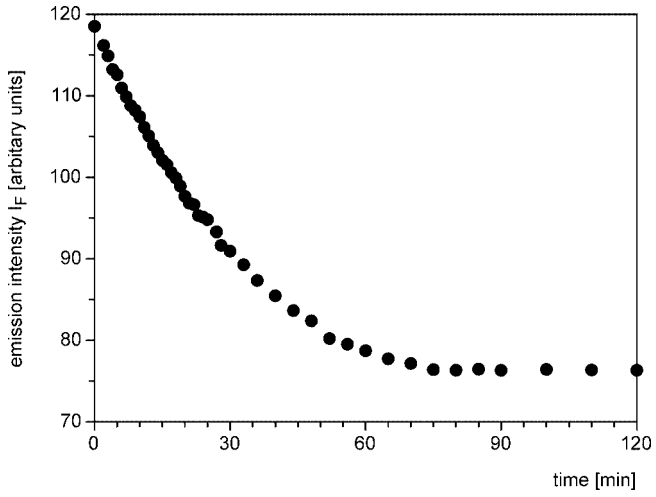


Figure 3. Time-dependent decrease of the fluorescence intensity of a solution of **1d** (10 μM) in water/methanol (1:1; pH = 11.7) after the addition of 10 equiv. of D-glucose.

Figure 3 shows that under the chosen conditions, equilibrium is reached after about 90 min. We therefore prepared each solution exactly 2 h prior to a spectroscopic or mass spectrometric analysis to make sure that all samples had reached thermodynamic equilibrium before the measurement. No decrease in fluorescence intensity was observed upon addition of D-glucose to a solution of **1d** in water/methanol (1:1) at neutral pH, thus indicating that formation of the tetrahedral boronate shown in Scheme 1 (a) is a prerequisite for complex formation.

Figure 4 shows a series of fluorescence spectra of solutions of **1d** (5 μM) in water/methanol (1:1; pH = 11.7) containing 0–40 equiv. of D-glucose. The arrow indicates the decrease in fluorescence intensity upon increasing the glucose concentration. Plotting the fluorescence intensity at

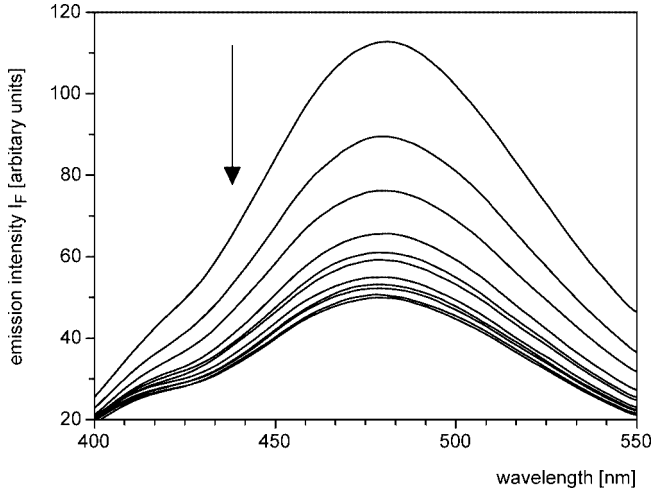


Figure 4. Fluorescence spectra of solutions of **1d** (5 μM) in water/methanol (1:1; pH = 11.7) containing 0–40 equiv. of D-glucose (excitation wavelength 285 nm). The arrow indicates the decrease in fluorescence intensity upon increasing the glucose concentration.

480 nm against the glucose/receptor ratio yields the saturation curve depicted in Figure 5 (circles), from which the stability of the complex between **1d** and D-glucose can be calculated by nonlinear regression using the mathematical treatment for 1:1 binding equilibria.[26,27] The excellent agreement between experimental and calculated results also shows that the assumption of 1:1 binding is valid.[28]

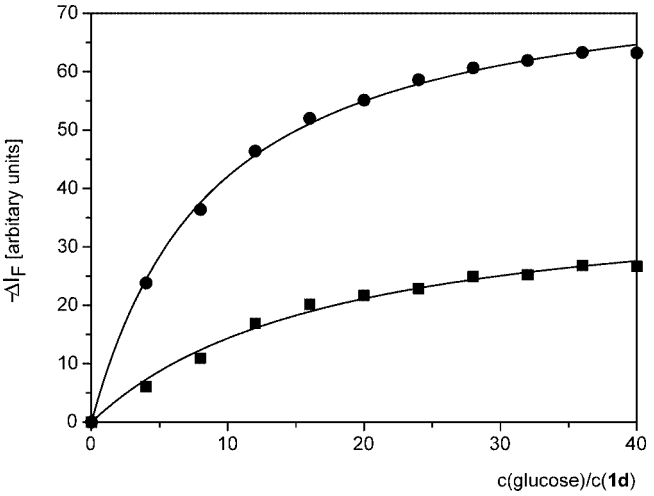


Figure 5. Saturation curves obtained by plotting the fluorescence intensity at 480 nm of solutions of **1d** (5 μM) in water/methanol (1:1; pH = 11.7) containing increasing amounts of D-glucose (circles) and L-glucose (squares) against the glucose/receptor ratio. The lines represent the best fit of the nonlinear regression to the experimental data.

The stability constant, K_a, determined from three individual measurements for the complex between **1d** and D-glucose amounts to $24800 \pm 1200\ M^{-1}$. A similar fluorescence titration using L-glucose as substrate yielded a K_a value of $11900 \pm 1600\ M^{-1}$, which shows that **1d** binds D-glucose ca. two times better than L-glucose. In other words, the enantioselectivity of **1d** in glucose recognition, as expressed by the ratio of the stability constants $K_a(D)/K_a(L)$, is 2.1. A large scattering of the data or a straight line were obtained during attempts to perform similar fluorescence titrations for complexes of **1d** with other monosaccharides, which is further evidence for the significantly lower stability of these complexes with respect to those of glucose.[29]

Discussion

The mass spectrometric analyses of the products formed during reactions between **1d** and various monosaccharides clearly demonstrate that the two boronic acids in the receptor are able to cooperatively bind to some of the investigated substrates. According to the ESI mass spectra, the monosaccharides investigated can be divided into three groups. Significant amounts of 1:1 complexes with respect to 1:2 complexes were only detected in mixtures of **1d** containing D- or L-glucose. Considering the large substrate excess used for the measurements, this result is strong evi-

dence that both monosaccharides form rather stable 1:1 complexes with the receptor. Significantly smaller amounts of 1:1 complexes were detected in the mass spectra of samples containing D-galactose, D-mannose, or D-allose, thus indicating that the complexes of these monosaccharides are considerably less stable. Finally, D-fructose, D-ribose, and D-xylose seem to be unable to form 1:1 complexes with the receptor.

The observed selectivity order significantly deviates from that of monoboronic acid carbohydrate receptors,[13] which clearly shows that the structure and conformation of the tetrapeptide scaffold in **1d** have a decisive effect on binding selectivity (including enantioselectivity). Moreover, the fact that only two of the eight sugars bind strongly to **1d** demonstrates that, as predicted, the use of structurally well-defined and not too flexible scaffolds can have a beneficial effect on binding selectivity.

It must also be noted that at the high pH used for the binding studies, nucleophilic replacement of one or both boronic acid groups by hydroxy groups causes the slow decomposition of the receptor. This is, of course, disadvantageous with respect to long-term stability, and improvement of the properties of **1d** must therefore also address strategies to increase stability or to shift the working pH of this receptor to neutral media. Interestingly, complex formation seems to have a protective effect against decomposition since large quantities of substitution products were only detected in solutions containing monosaccharides that interact only weakly with **1d**.

Support for the mass spectrometric results came from NMR spectroscopic investigations. The low resolution of the spectrum of the free receptor and the number of peaks indicate that, in the absence of suitable guests, several (presumably non-symmetrical) conformations of **1d**, some of which are possibly stabilized by interactions between the boronic acid groups, slowly interconvert in solution. No break of symmetry could therefore be detected in the ^{1}H NMR spectrum of **1d** upon complex formation. Nevertheless, the pronounced changes in the spectrum caused by the presence of D-glucose clearly demonstrate that **1d** forms a structurally well-defined complex with this monosaccharide which, in the presence of sub-stoichiometric amounts of the substrate, is in slow equilibrium with free receptor. Moreover, the fact that the spectrum of the complex is almost unaffected by increasing the substrate concentration beyond 1 equiv. is further evidence that the most stable complex species in solution is the 1:1 complex. Unfortunately, the complexity of the spectrum prevents a more detailed structural assignment. Also, quantitative information with respect to complex stability could not be derived from the NMR spectroscopic investigations because of the low resolution of the spectrum of the free receptor and the extensive signal overlap in the spectra containing free receptor and complex.

Complex stability could, however, be determined from fluorescence titrations. These measurements rely on the fact that the fluorescence of **1d**, which is due to the 3,5-diaminobenzoic acid subunits, is quenched upon complex formation. A variety of boronic acid based fluorescence sensors for carbohydrates have been described previously, of which many have in common that fluorescence is enhanced upon complex formation.[18] In some receptors, a photoinduced electron transfer quenching process is inhibited upon carbohydrate binding, whereas other receptors are conformationally rigidified upon complex formation, which also causes an increase in fluorescence intensity.[10–12] To explain the opposite effect in the system described here, we assume that fluorescence quenching upon glucose binding is due to photoinduced electron transfer from the analyte to the sensor or to structural constraints in the complex that prevent the efficient conjugation of the tertiary amino groups with the aromatic subunits in the peptide ring.[30]

The stability constants determined for the complexes of **1d** with D- and L-glucose by fluorescence titration are quite large, thereby indicating a good structural complementarity between the receptor and these substrates. A comparison of the glucose affinity of **1d** with that of known boronic acid based carbohydrate receptors has to consider factors such as number of boronic acid binding sites, pH (complex stability is pH-dependent and substantially increases with increasing pH[31]), and solvent composition. Although this comparison is not straightforward, it should be noted that bis(boronic acid) receptors with similar glucose affinity in protic media at high pH have been reported.[32,33] Also, the enantioselectivity of **1d** in glucose recognition is comparable to that of other chiral bis(boronic acid) receptors. The binol receptor reported by Shinkai and James, for example, binds D-glucose about 1.6 times better than L-glucose.[17] The enantioselectivity of the same receptor in fructose binding is even larger [K_a(D-fructose)/K_a(L-fructose) = 3.2]. Moreover, James and co-workers have recently described a chiral bis(boronic acid) receptor whose (*R*) enantiomer binds D-mannitol more than 2000 times better than the (*S*) enantiomer.[16] Hence, enantioselectivity in glucose recognition of **1d** can be considered moderate while affinity, and in particular carbohydrate selectivity, are significantly better.

We were, of course, disappointed to observe complex formation between **1d** and glucose only at high pH even though the tertiary amines in the receptor should, in principle, be able to induce binding also in a neutral environment by interacting with the boron centres.[10–12,24] One reason for this result could be that the basicity of the aromatic amino groups of **1d** is too low to allow for N–B interactions, although colour sensors are known in which the coordination of an aromatic amine to the boronic acid binding site does permit complex formation at pH = 8.2.[21] Geometrical features in the glucose complex of **1d** are therefore probably more important in preventing the formation of intramolecularly stabilized zwitterions[25] or coordinative N–B interactions. The results of molecular modelling studies support this assumption. The corresponding calculations were performed at a semi-empirical AM1 level with the program MacSpartan (*Spartan 04 for Macintosh*, Wavefunction, Inc., Irvine, CA) and were based on the crystal structure of tetrapeptide **1a**[23] assuming that the boronic acids in **1d** preferentially form cyclic esters with the hydroxy

groups in the 1,2- and in 4,6-positions of the α-anomer of the substrate.[33] The optimised structure of the complex between **1d** and D-glucose is depicted in Figure 6.

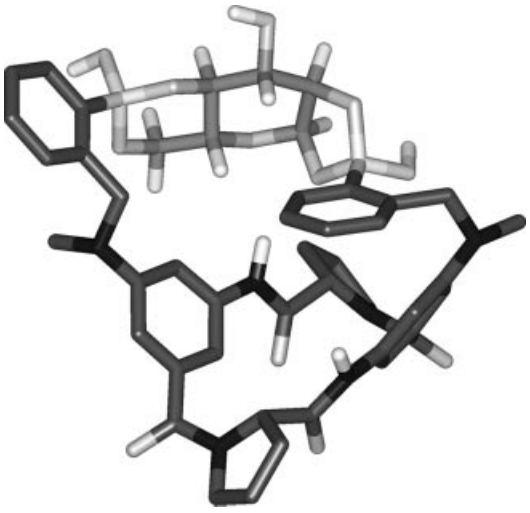


Figure 6. Calculated structure of the complex between **1d** and α-D-glucose. Calculations were performed at a semi-empirical AM1 level with the program MacSpartan. Hydrogen atoms at the C atoms of the receptor have been omitted for clarity.

This figure shows that the structure of the tetrapeptide in the complex does not deviate significantly from the ones previously reported for other structurally related cyclic tetrapeptides.[23] Thus, complex formation most probably does not induce much strain in the ring. The bound glucose adopts the energetically favourable $^4C_1$ conformation, which also contributes to the high stability of the complex. The calculated structure demonstrates, however, that in order to be oriented in a convergent fashion to allow for simultaneous interactions with the substrate, the boron centres of the cyclic esters must turn away from the neighbouring aromatic amino groups. This orientation prevents intramolecular N–H···O–B hydrogen bonds or N–B interactions and explains why complex formation of **1d** requires the formation of boronates and, as a consequence, high pH.

It should be pointed out that we have currently no experimental evidence whether the calculated structure in Figure 6 reflects the true structure of the D-glucose complex of **1d** in solution. In particular, we have no information about which anomer of glucose is bound or which cyclic form. It has been proposed that other bis(boronic acid) receptors bind to the pyranose form of D-glucose in a similar fashion,[34,35] although subsequent NMR investigations showed that the pyranose form is only bound initially and rapidly rearranges to the furanose form in aqueous solution.[36] We cannot rule out that a similar process also occurs in the system described here. Yet, independent of the exact structure of the substrate, a prerequisite for the cooperative action of both boronic acids in monosaccharide binding is a convergent arrangement which, according to our calculations, cannot simultaneously permit interactions of the aromatic amino groups with the boronic acid binding sites.

Conclusions

We have demonstrated that introducing boronic acid binding sites in the periphery of a chiral macrocyclic scaffold is a promising approach towards new selective carbohydrate sensors. Our first receptor, the cyclic tetrapeptide **1d**, possesses high affinity and selectivity in glucose recognition combined with good enantioselectivity. Moreover, fluorescence of **1d** is quenched upon substrate binding, which allows the use of this compound as an optical sensor for glucose in aqueous solution. Molecular modelling indicates that interactions between the boron centres and the neighbouring tertiary amines, which are required to shift the working pH of the receptor to neutral media, are prevented by the convergent arrangement of the binding sites in the 1:1 complex. As a consequence, complex formation of **1d** requires high pH, which limits the use of this compound in practical applications. Future work has to address this issue, for example by the introduction of additional Lewis basic substituents suitably arranged with respect to the receptor's boronic acid groups.

Experimental Section

General Details: Melting points: Büchi 510 apparatus. Optical rotation: Perkin–Elmer 241 MC digital polarimeter (d = 10 cm). NMR: Bruker DRX 500 equipped with an automatic sampler. FT-IR: Finnigan MAT 8200. ESI-MS: Micromass Q-Tof UltimaTM API. Fluorescence spectroscopy: JASCO FP-6200. Elemental analysis: Pharmaceutical Institute of the Heinrich Heine University, Düsseldorf. RP chromatography: Merck LiChroprep RP-8 (40–63 μm) prepacked column size B (310-25). Abbreviations: All: allyl; Boc: *tert*-butoxycarbonyl; DIEA: *N*-ethyldiisopropylamine; PyCloP: chlorotripyrrolidinophosphonium hexafluorophosphate; TBTU: *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; Pro: L-proline; AB: 3,5-diaminobenzoic acid; BMAB: 3-amino-5-[(2-boronobenzyl)(methyl)amino]benzoic acid; BMAB-(pin): 3-amino-5-{methyl[2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl]amino}benzoic acid.

Syntheses: All solvents were dried according to standard procedures prior to use. DMF p.A. was purchased from Merck and used without further purification. PyCloP was prepared according to a literature procedure.[37] TBTU and 2-formylphenylboronic acid are commercially available.

General Procedure for the Cleavage of *tert*-Butoxycarbonyl Groups: The starting material was suspended in 1,4-dioxane (30 mL). The resulting suspension was cooled with an ice bath, and a 6 M solution of HCl in 1,4-dioxane (60 mL) was added dropwise. The reaction mixture was stirred at 0–5 °C for 2 h and then concentrated to dryness in vacuo.

General Procedure for the Cleavage of Allyl Esters: The ester was dissolved in THF (20 mL per mmol) under inert conditions. [$Pd(PPh_3)_4$] (10 mg) and morpholine (2 equiv.) were added, and the reaction mixture was stirred at room temperature for 30 min. Completion of the reaction was checked by TLC. The solvent was evaporated in vacuo, the residue was redissolved in ethyl acetate, and the organic layer was extracted three times with aqueous 3% $KHSO_4$ and three times with water. After drying, the solvent was removed in vacuo.

Allyl 3,5-Bis(*tert*-butoxycarbonylamino)benzoate (2): A mixture of 3,5-bis(*tert*-butoxycarbonylamino)benzoic acid[38] (17.6 g, 50.0 mmol) and $NaHCO_3$ (12.6 g, 150.0 mmol) was suspended in DMF (150 mL). Allyl bromide (13.0 mL, 150.0 mmol) was added, and the reaction mixture was stirred at room temperature for 72 h. After addition of 10% aqueous Na_2CO_3 (450 mL), the resulting solution was extracted three times with ethyl acetate. The combined organic layers were washed with water, and dried. The solvent was removed in vacuo, and the product was isolated from the residue by column chromatography (hexane/ethyl acetate, 1:1). It was finally recrystallised from hexane/ethyl acetate. Yield: 19.2 g (98%); m.p. 148–157 °C. ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 1.48 (s, 18 H, Boc-CH$_3$), 4.78 (d, $^3J_{H,H}$ = 5.3 Hz, 2 H, All-CH$_2$), 5.29 (dd, $^3J_{H,H}$ = 10.6, $^2J_{H,H}$ = 1.6 Hz, 1 H, All-H$^{3(Z)}$), 5.41 (dd, $^3J_{H,H}$ = 17.3, $^2J_{H,H}$ = 1.6 Hz, 1 H, All-H$^{3(E)}$), 5.99–6.08 (m, 1 H, All-H^2), 7.74 (s, 1 H, AB-H), 7.75 (s, 1 H, AB-H), 7.93 (m, 1 H, AB-H), 9.56 (s, 2 H, AB-NH) ppm. $C_{20}H_{28}N_2O_6$ (392.5): calcd. C 61.21, H 7.19, N 7.14; found C 61.45, H 7.30, N 6.89.

Allyl 3-Amino-5-(*tert*-butoxycarbonylamino)benzoate (3): Allyl 3,5-bis(*tert*-butoxycarbonylamino)benzoate (**2**; 17.7 g, 45.0 mmol) was first deprotected at the amino groups according to the general procedure and isolated as the bis(hydrochloride) salt. Without further purification, the product obtained was suspended in dichloromethane (250 mL). After the addition of DIEA (17.1 mL, 99.0 mmol), the mixture was cooled in an ice bath. A solution of di-*tert*-butyl dicarbonate (9.8 g, 45.0 mmol) in dichloromethane (100 mL) was added dropwise whilst stirring over the course of 30 min. Stirring was continued at about 5 °C for 2 h, and at room temperature overnight. Afterwards, the solvent was removed in vacuo and the product was isolated from the residue by chromatographic workup (hexane/ethyl acetate, 1:1). Allyl 3,5-bis(*tert*-butoxycarbonylamino)benzoate (**2**) eluted first and then the desired monoprotected product. Fractions containing pure **3** were pooled and the solvents evaporated to dryness. The residue was triturated with hexane, filtered off and dried with P_4O_{10} in vacuo. Yield: 8.2 g (62%); m.p. 104 °C. ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 1.54 (s, 9 H, Boc-CH$_3$), 4.81 (d, $^3J_{H,H}$ = 5.4 Hz, 2 H, All-CH$_2$), 5.34 (dd, $^3J_{H,H}$ = 10.4, $^2J_{H,H}$ = 1.6 Hz, 1 H, All-H$^{3(Z)}$), 5,42 (s, 2 H, NH$_2$), 5.46 (dd, $^3J_{H,H}$ = 17.3, $^2J_{H,H}$ = 1.6 Hz, 1 H, All-H$^{3(E)}$), 6.05–6.13 (m, 1 H, All-H^2), 6.92 (t, $^4J_{H,H}$ = 1.6 Hz, 1 H, AB-H), 7.08 (s, 1 H, AB-H), 7.35 (t, $^4J_{H,H}$ = 1.6 Hz, 1 H, AB-H), 9.34 (s, 1 H, AB-NH) ppm. $C_{15}H_{20}N_2O_4$ (292.3): calcd. C 61.63, H 6.90, N 9.58; found C 61.77, H 7.05, N 9.45.

Allyl 3-(*tert*-Butoxycarbonylamino)-5-[2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzylamino]benzoate (4): Compound **3** (7.3 g, 25.0 mmol) and 2-formylphenylboronic acid (5.2 g, 35.0 mmol) were dissolved in ethanol (150 mL) and the mixture was stirred at room temperature. Sodium cyanoborohydride (3.9 g, 62.5 mmol) was added in three portions followed by glacial acetic acid (9.5 mL). After stirring overnight, another 4 mL of glacial acetic acid was added to the reaction mixture and stirring was continued for 24 h. The solvent was evaporated in vacuo and the residue was suspended in toluene (150 mL). After the addition of 2,3-dimethylbutane-2,3-diol (3.0 g, 25.0 mmol), the resulting mixture was heated to reflux in a Dean–Stark trap until TLC indicated complete conversion (hexane/ethyl acetate, 6:1; R_f = 0.26). The solvent was removed in vacuo, and the residue was dissolved in ethyl acetate. The organic layer was extracted once with water, three times with 10% aqueous Na_2CO_3, and three times with water. After drying with sodium sulfate, the solvent was evaporated and the product was isolated from the residue by column chromatography (hexane/ethyl acetate, 6:1; R_f = 0.26). Yield: 7.6 g (60%); m.p. 118–120 °C. ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 1.29 (s, 12 H, Pin-CH$_3$), 1.45 (s, 9 H, Boc-CH$_3$), 4.47 (d, $^3J_{H,H}$ = 6.0 Hz, 2 H, BM-CH$_2$), 4.72 (dt, $^3J_{H,H}$ = 5.4 Hz, 2 H, All-CH$_2$), 5.24 (dd, $^3J_{H,H}$ = 12.0, $^2J_{H,H}$ = 1.6 Hz, 1 H, All-H$^{3(Z)}$), 5.34 (dd, $^3J_{H,H}$ = 17.0, $^2J_{H,H}$ = 1.6 Hz, 1 H, All-H$^{3(E)}$), 5.94–6.04 (m, 1 H, All-H^2), 6.40 (t, $^3J_{H,H}$ = 5.7 Hz, 1 H, NH), 6.84 (t, $^4J_{H,H}$ = 1.9 Hz, 1 H, AB-H), 7.00 (s, 1 H, AB-H), 7.23 (td, $^3J_{H,H}$ = 7.3, $^4J_{H,H}$ = 1.0 Hz, 1 H, BM-H), 7.30–7.42 (m, 3 H, AB-H/BM-H), 7.68 (dd, $^3J_{H,H}$ = 7.6, $^4J_{H,H}$ = 1.2 Hz, 1 H, BM-H), 9.27 (s, 1 H, AB-NH) ppm. $C_{28}H_{37}BN_2O_6$ (508.4): calcd. C 66.15, H 7.34, N 5.51; found C 65.89, H 7.41, N 5.35. MS (EI): *m*/*z* (%) = 508 (41) [M$^+$].

Allyl 3-(*tert*-Butoxycarbonylamino)-5-{methyl[2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl]amino}benzoate (5): Compound **4** (7.2 g, 14.0 mmol) was dissolved in ethanol (100 mL). Aqueous formaldehyde (37%, 12 mL, 140.0 mmol) was added and the mixture was stirred at room temperature. Sodium cyanoborohydride (1.4 g, 22.4 mmol) was added in three portions followed by glacial acetic acid (8 mL). After stirring overnight, the solvent was removed in vacuo. The residue was dissolved in ethyl acetate, and the organic layer was extracted once with water, three times with 10% aqueous Na_2CO_3, and three times with water. After drying with sodium sulfate, the solvent was evaporated, and the product was isolated from the residue by column chromatography (hexane/ethyl acetate, 6:1; R_f = 0.32). Yield: 7.2 g (98%); m.p. 148–150 °C. ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 1.30 (s, 12 H, Pin-CH$_3$), 1.44 (s, 9 H, Boc-CH$_3$), 3.00 (s, 3 H, N–CH$_3$), 4.72 (dt, $^3J_{H,H}$ = 5.3 Hz, 2 H, All-CH$_2$), 4.77 (s, 2 H, BM-CH$_2$), 5.19 (dd, $^3J_{H,H}$ = 10.5, $^2J_{H,H}$ = 1.7 Hz, 1 H, All-H$^{3(Z)}$), 5.28 (dd, $^3J_{H,H}$ = 17.3, $^2J_{H,H}$ = 1.7 Hz, 1 H, All-H$^{3(E)}$), 5.88–6.10 (m, 1 H, All-H^2), 6.88 (s, 1 H, AB-H), 7.00 (d, $^3J_{H,H}$ = 7.3 Hz, 1 H, BM-H), 7.06 (s, 1 H, AB-H), 7.17–7.43 (m, 2 H, BM-H), 7.51 (s, 1 H, AB-H), 7.73 (dd, $^3J_{H,H}$ = 7.3, $^4J_{H,H}$ = 1.2 Hz, 1 H, BM-H), 9.31 (s, 1 H, AB-NH) ppm. $C_{29}H_{39}BN_2O_6$ (522.5): calcd. C 66.67, H 7.52, N 5.36; found C 66.40, H 7.42, N 5.32. MS (EI): *m*/*z* (%) = 522 (48) [M$^+$].

Dipeptide Boc-Pro-BMAB(pin)-OAll (6): Before the coupling reaction, compound **5** (6.3 g, 12.0 mmol) was deprotected at the terminal amino group according to the general procedure. The product, Boc-L-proline (3.1 g, 14.4 mmol), and PyCloP (6.1 g, 14.4 mmol) were dissolved in CH_2Cl_2 (240 mL). DIEA (9.1 mL, 52.8 mmol) was then added dropwise at room temperature and the reaction mixture was stirred overnight. Afterwards, the solvent was evaporated in vacuo, and the product was isolated chromatographically from the residue (hexane/ethyl acetate, 2:1; R_f = 0.42). Yield: 4.8 g (65%); m.p. 95 °C (dec.) (softening from 80 °C). $[\alpha]_D^{25}$ = –64.3 (c = 2, $CHCl_3$). ^{1}H NMR (500 MHz, [D$_6$]DMSO, 100 °C): δ = 1.30 (s, 12 H, Pin-CH$_3$), 1.32 (s, 9 H, Boc-CH$_3$), 1.73–1.98 (m, 3 H, Pro-H$^\beta$/Pro-H$^\gamma$), 2.05–2.23 (m, 1 H, Pro-H$^\beta$), 2.94 (s, 3 H, N–CH$_3$), 3.26–3.48 (m, 2 H, Pro-H$^\delta$), 4.21 (dd, $^3J_{H^a,H^e}$ = 4.0, $^3J_{H^a,H^a}$ = 8.0 Hz, 1 H, ProH$^\alpha$), 4.70–4.77 (m, 4 H, All-CH$_2$/BM-CH$_2$), 5.22 (dd, $^3J_{H,H}$ = 10.6, $^2J_{H,H}$ = 1.5 Hz, 1 H, All-H$^{3(Z)}$), 5.33 (dd, $^3J_{H,H}$ = 17.1, $^2J_{H,H}$ = 1.5 Hz, 1 H, All-H$^{3(E)}$), 5.86–6.13 (m, 1 H, All-H^2), 7.02–7.41 (m, 5 H, AB-H/BM-H), 7.57 (t, $^4J_{H,H}$ = 1.5 Hz, 1 H, AB-H), 7.71 (d, $^4J_{H,H}$ = 1.5 Hz, 1 H, BM-H), 9.57 (s, 1 H, AB-NH) ppm. $C_{34}H_{46}BN_3O_7$ (619.6): calcd. C 65.91, H 7.48, N 6.78; found C 66.02, H 7.65, N 6.52. MS (EI): *m*/*z* (%) = 619 (52) [M$^+$].

Tetrapeptide Boc-[Pro-BMAB(pin)]$_2$-OAll (7): Prior to coupling, equimolar amounts of **6** (2.2 g, 3.5 mmol) were deprotected at the amino group and at the carboxyl group as described in the general procedures for the deprotection of Boc groups and of allyl esters, respectively. Both products were dissolved in CH_2Cl_2 (80 mL). PyCloP (1.8 g, 4.2 mmol) and DIEA (2.7 mL, 15.4 mmol) were added, and the solution was stirred overnight. The solvent was evaporated in vacuo, and the residue was purified chromatographically (hex-

ane/ethyl acetate, 1:2; R_f = 0.36). Yield: 2.3 g (62%). MS (FAB): *m/z* (%) = 1103 (100) [M + Na^+], 1003 (65) [M – Boc + Na^+].

Cyclopeptide Cyclo[Pro-BMAB(pin)]$_2$ (8): The tetrapeptide **7** (1.1 g, 1.0 mmol) was first deprotected at the carboxyl group and subsequently at the terminal amino group according to the general procedures. For the cyclisation, the deprotected tetrapeptide was dissolved in a mixture of degassed DMF (40 mL) and DIEA (1.0 mL, 6 mmol). The resulting solution was added dropwise to a solution of TBTU (1.60 g, 5 mmol) and DIEA (0.4 mL, 2.5 mmol) in degassed DMF (200 mL) at 80 °C over the course of 4 h. If necessary, the pH of the reaction mixture was adjusted afterwards to about 9 by adding more DIEA, and stirring was continued at 80 °C for 1 h. The solvent was then evaporated in vacuo, and the product was isolated from the residue by two subsequent chromatographic separations using first chloroform/acetone (1:1; R_f = 0.27) and then 2-propanol/ethyl acetate (1:5; R_f = 0.46) as eluents. Yield: 0.48 g (52%); m.p. 210 °C (dec.). $[\alpha]_D^{25}$ = –193.0 (*c* = 1, DMF). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 1.30 (s, 24 H, Pin-CH_3), 1.74–1.97 (m, 6 H, Pro-H$^\beta$/Pro-H$^\gamma$), 2.01–2.17 (m, 2 H, Pro-H$^\gamma$), 2.96 (s, 6 H, N–CH_3), 3.46–3.62 (m, 4 H, Pro-H$^\delta$), 3.85 (d, $^3J_{H,H}$ = 8.5 Hz, 2 H, Pro-H$^\alpha$); 4.63 (d, $^2J_{H,H}$ = 17.7 Hz, 2 H, BM-CH_2), 4.79 (d, $^2J_{H,H}$ = 17.7 Hz, 2 H, BM-CH_2), 6.07 (s, 2 H, AB-H), 6.29 (s, 2 H, AB-H), 7.03 (d, $^3J_{H,H}$ = 7.6 Hz, 2 H, BM-H), 7.24 (dd, $^3J_{H,H}$ = 7.3 Hz, 2 H, BM-H), 7.28 (s, 2 H, AB-H), 7.40 (dd, $^3J_{H,H}$ = 7.6 Hz, 2 H, BM-H), 7.72 (d, $^3J_{H,H}$ = 7.3 Hz, 2 H, BM-H), 9.49 (s, 2 H, AB-NH) ppm. ^{13}C NMR (125 MHz, [D$_6$]DMSO): δ = 23.50 (Pro-C$^\gamma$), 25.60 (Pin-CH_3), 32.13 (Pro-C$^\beta$), 39.45 (N–CH_3), 47.51 (Pro-C$^\delta$), 56.22 (Pro-C$^\alpha$), 62.69 (BM-CH_2), 84.54 (Pin-C), 103.67 (AB-C), 104.89 (AB-C), 107.05 (AB-C), 126.34 (BM-C), 126.95 (BM-C), 132.25 (BM-C), 137.09 (BM-C), 139.81 (AB-C), 145.92 (AB-C), 150.59 (AB-C), 170.74 (AB-CO), 172.43 (Pro-CO) ppm. $C_{52}H_{64}B_2N_6O_8 \cdot 1.5H_2O$ (949.8): calcd. C 65.76, H 7.11, N 8.85; found C 65.88, H 6.99, N 8.88. MS (FAB): *m/z* (%) = 923 (100) [M + H^+], 945 (19) [M + Na^+].

Cyclopeptide *cyclo*[Pro-BMAB]$_2$ (1d): The cyclopeptide **8** (200 mg, 220 μmol) was dissolved in acetone/water (4:1; 220 mL), 1 M HCl was added (22 mL), and the solution was stirred at room temperature for 4 d. Afterwards, the solvent was evaporated in vacuo, and the material recovered was purified on an RP-8 column. For this, it was dissolved in a small amount of acetone/water (1:1) and applied to a column conditioned with acetone/water (1:10). The eluent composition was gradually changed until the pure product eluted (acetone/water, 2:3). Fractions containing pure product were pooled and the solvents evaporated to dryness. Finally, the product was dried with P_4O_{10} in vacuo. Yield: 85 mg (51%); m.p. >250 °C (softening from 175 °C). $[\alpha]_D^{25}$ = –103.1 (*c* = 1, DMF). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 1.79–1.97 (m, 6 H, Pro-H$^\beta$/Pro-H$^\gamma$), 2.03–2.16 (m, 2 H, Pro-H$^\beta$), 2.89 (s, 6 H, N–CH_3), 3.41–3.61 (m, 4 H, Pro-H$^\delta$), 3.88 (d, $^3J_{H,H}$ = 7.7 Hz, 2 H, Pro-H$^\alpha$), 4.62 (d, $^2J_{H,H}$ = 17.7 Hz, 2 H, BM-CH_2), 4.65 (d, $^2J_{H,H}$ = 17.7 Hz, 2 H, BM-CH_2), 6.14 (s, 2 H, AB-H), 6.32 (s, 2 H, AB-H), 6.96 (d, $^3J_{H,H}$ = 7.6 Hz, 2 H, BM-H), 7.20 (dd, $^3J_{H,H}$ = 7.6 Hz, 2 H, BM-H), 7.25–7.33 (m, 4 H, AB-H/BM-H), 7.54 (d, $^3J_{H,H}$ = 7.0 Hz, 2 H, BM-H), 9.52 (s, 2 H, AB-NH) ppm. ^{13}C NMR (125 MHz, [D$_6$]DMSO): δ = 23.59 (Pro-C$^\gamma$), 32.16 (Pro-C$^\beta$), 39.32 (N–CH_3), 47.57 (Pro-C$^\delta$), 56.91 (Pro-C$^\alpha$), 62.75 (BM-CH_2), 104.16 (AB-C), 105.34 (AB-C), 107.26 (AB-C), 125.95 (BM-C), 126.67 (BM-C), 130.04 (BM-C), 134.89 (BM-C), 139.82 (AB-C), 143.14 (AB-C), 150.95 (AB-C), 170.85 (AB-CO), 172.53 (Pro-CO) ppm. $C_{40}H_{44}B_2N_6O_8 \cdot 3.5H_2O$ (821.5): calcd. C 58.48, H 6.26, N 10.23; found C 58.47, H 6.05, N 10.08. MS (FAB): *m/z* (%) = 740 (2) [M^+ – H_2O]. MS (ESI): *m/z* (%) = 721 (100) [M – 2 H_2O – H^+].

Fluorescence Titrations: Stock solutions of the guest (0.6 mM) and of receptor **1d** (10 μM) were prepared in methanol/water (1:1) adjusted to pH = 11.7 with NaOH. In total, 11 samples were set up by adding increasing amounts of the guest solution (0–1000 μL in 100-μL steps) to 1.5 mL of the host solution. All samples were made up to a volume of 3 mL with the solvent mixture (methanol/water, 1:1; pH = 11.7). The fluorescence emission intensity at 480 nm was measured for each sample after 2 h upon excitation at 285 nm. K_a was calculated from the resulting saturation curves by a nonlinear least-squares fitting method and using the mathematical treatment for 1:1 complex formation.[26,27]

Supporting Information (see footnote on the first page of this article): ESI mass spectra of mixtures of **1d** with various monosaccharides.

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containing a small molar fraction of **1d** could not be determined with sufficiently high accuracy and reproducibility.
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