Bicyclic Peptide Based Lectinomimic

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───── THIS PAPER IS DEDICATED TO PROF. MLADEN ŽINIĆ ON THE OCCASION OF HIS 70TH BIRTHDAY ─────

Abstract: Peptide based lectin mimetics represent an attractive approach for the development of artificial carbohydrate receptors that might find application in bio-analytical and medicinal fields. Taking into consideration the structure of typical lectin binding site, we have designed a novel artificial receptor molecule possessing a rigid three-dimensional structure, hydrogen-bonding site and lipophilic binding pocket to promote hydrophobic interaction and hydrogen-bonding. A new solid-phase synthetic approach that allows complete synthesis of desired bicyclic peptide **1** on the solid support was developed. CD spectra of peptides **1** and **2** indicate that the structure of **1** is rather rigid and preorganised for the three-dimensional monosaccharide substrates binding. The binding affinities of bicyclic peptide receptor **1** toward various carbohydrate substrates at physiologically relevant conditions were estimated by UV/vis and fluorimetric titration experiments, and the observed values are in the millimolar range. With these results we have demonstrated that the bicyclic peptide **1** represent a promising basis for the design of new and more efficient carbohydrate receptors that may have broader application in bio-analytical or medicinal field.

Keywords: Aqueous media, artificial receptors, bicyclic peptides, lectinomimics, monosaccharide binding.

INTRODUCTION

CARBOHYDRATE recognition is one of the most sophisticated recognition processes in biological systems.[1] This process mediates many important aspects of cell-cell recognition, such as inflammation, cell differentiation, tumor cell colonization and metastasis. However, due to its complexity and weak binding affinities, carbohydrate recognition by natural systems is still poorly understood. On the other hand, studies on synthetic carbohydrate receptors could make significant contributions to a better understanding of this process and lead to the development of new agents for application in bio-analytical and medicinal fields. Considering the importance of the carbohydrate recognition, it is not surprising that design and synthesis of artificial receptors for these important biological substrates attracted a great deal of attention in biomimetic chemistry.[2] Current efforts are mainly focused on the design of receptors for complexation of mono- or disaccharides.[2a,3] This crude simplification has been based on the structural studies of the lectins, revealing that carbohydrate binding sites are typically shallow binding pockets where only binding of the terminal mono-saccharide moieties can occur.[4] Binding of an individual lectin to monosaccharide substrate is extremely weak; the association constant (K_a) for these complexes are typically in the $10^{-4} - 10^{-2}$ mol^{-1}dm^3 range. Binding was achieved through a combination of hydrogen bonding to the monosaccharide hydroxyl groups and van der Waals packing of the hydrophobic sugar face against aromatic amino acid side chains forming sandwich-type inter-actions.[4b,4d] Taking into consideration these binding principles, a variety of receptors for monosaccharide binding in organic solvent have been prepared.[2a,3a] However, construction of artificial receptors for binding of carbohydrates in water represent particularly challenging task, mainly due to the weak hydrogen-bonding interactions.

Carbohydrate binding protein mimicking molecules using the peptide-based systems (lectinomimics) represent particularly attractive approach for the development of

artificial carbohydrate receptors.[5] Given the number available L- and D-amino acid building blocks, even small peptides offer enormous diversity and potential for design and development of new, and more selective artificial carbohydrate receptors. Numerous efforts are underway to find carbohydrate specific peptides using the phage-display technique,[5a] or combinatorial chemistry.[5b] Importantly, these peptide based lectinomimics bind carbohydrate substrates in aqueous media with the affinities comparable to those reported for lectin/monosaccharide complexes.[4b,4c]

Considering the structure of typical lectin binding site, we have designed a novel artificial receptor molecule possessing a rigid three-dimensional structure, hydrogen-bonding site and lipophilic binding pocket to promote hydrophobic interaction and hydrogen-bonding. As a model system we chose a cyclic cationic peptide antibiotic polymyxin B (PMB),[6] which is known to bind the lipid A disaccharide moiety of lipopolysaccharide (LPS) with the high affinity.[7]

Herein, we report the design, synthesis and binding properties of polymyxin based bicyclic peptide **1,** Scheme 1, toward the various mono- and disaccharide substrates in aqueous media.

MATERIALS AND METHODS

Wang resin was purchased from Chem-Impex (Wood Dale, IL, USA) and Rink amide AM resin was purchased from Millipore-Sigma (Burlington, MA, USA). Fmoc-protected amino acids and coupling reagents (HOBt, HBTU, PyBOP) were purchased from Chem-Impex (Wood Dale, IL, USA). N-methylmorpholine, hydrazine, triethylamine and Pd(PPh$_3$)$_4$ was purchased from Sigma Aldrich (St. Louis, MO, USA). All solvents were purchased from Fisher-Scientific (Waltham, MA USA), and were analytical reagent grade or better. Individual peptides were synthesized on a PS3 automated peptide synthesizer (Gyros Protein Technologies, Tucson, AZ, USA). Mass spectrometry was performed on MALDI-TOF Voyager-DE STR (Applied Biosystems, Foster City, CA, USA) in reflector mode using α-cyano-4-hydroxycinnamic acid as a matrix and positive mode. Analytical RP-HPLC analyses and peptide purifications were performed on 1260 Infinity (Agilent Technologies, Santa Clara, CA, USA) liquid chromatography systems equipped with a UV/Vis detector. CD spectra were recorded on a Jasco-810 spectropolarimeter (Jasco, Easton, MD). Titration experiments were performed on Varian Cary 3 UV/vis spectrophotometer (Paolo Alto CA, USA) and Perkin Elmer LS50B Luminescence Spectrometer (Waltham, MA, USA).

Peptide Synthesis

All linear peptide precursors were synthesized in a 0.25 mmol scale on the automated peptide synthesizer using standard Fmoc chemistry. The level of the Asp residue

attachment to the resins was determined by the direct measurement of released dibenzofulvene after Fmoc deprotection using UV/vis spectroscopy.[8] ivDde, Mtt and Allyl protecting groups were removed according to the standard protocols.[9] Peptide cyclization was performed manually using PyBop/HOBt/NMM method over 10 h period. 2,2'-bipyridine-5,5'-dicarboxylic acid was coupled manually to Orn^5 residue after ivDde removal using standard PyBop/HOBt/NMM method over 10 h, followed by Mtt removal from Orn^3 residue and final macrocyclization using PyBop/HOBt/NMM method over 10 h. The resulting peptidyl resins were cleaved with a trifluoroacetic acid (TFA/TIS/CH$_2$Cl$_2$ (95:2.5:2.5). Synthesized peptides were purified by RP-HPLC (Phenomenex Jupiter C-18 column, 250 × 21.2 mm, 300 Å pore size, 10 μm particle size) using a linear gradient (100 % A → 100 % B; A = 0.1 % TFA in H$_2$O, B = 0.1 % TFA in MeCN:H$_2$O = 9:1) and a flow rate of 7 mL/min. Peptides purities were confirmed by analysis using analytical RP-HPLC (Grace Vydac C18 column, 250 × 4.6 mm, 120 Å pore size, 5 μm particle size), and MALDI TOF mass spectrometry. For structural comparison, cyclic peptide **2** possessing the same amino acid sequence as bicyclic peptide **1** was synthesized.

Circular Dichroism Spectroscopy

CD spectra were recorded in water using a quartz cell of 1 mm optical path length. Spectra were measured over a wavelength range 180−250 nm with an instrument scanning speed of 100 nm/min and a response time 1 s. The peptide concentrations were 1 mmol dm^{-3} for **1** and 1.2 mmol dm^{-3} for **2**, and the CD spectra are the result of eight averaged scans taken at 25 °C. All CD spectra are baseline-corrected for signal contribution due to the water. CD spectra were modeled using the CDPro suite of programs employing SDP42 database.
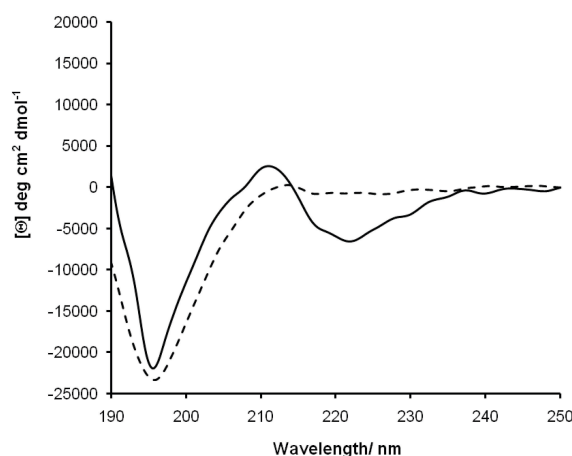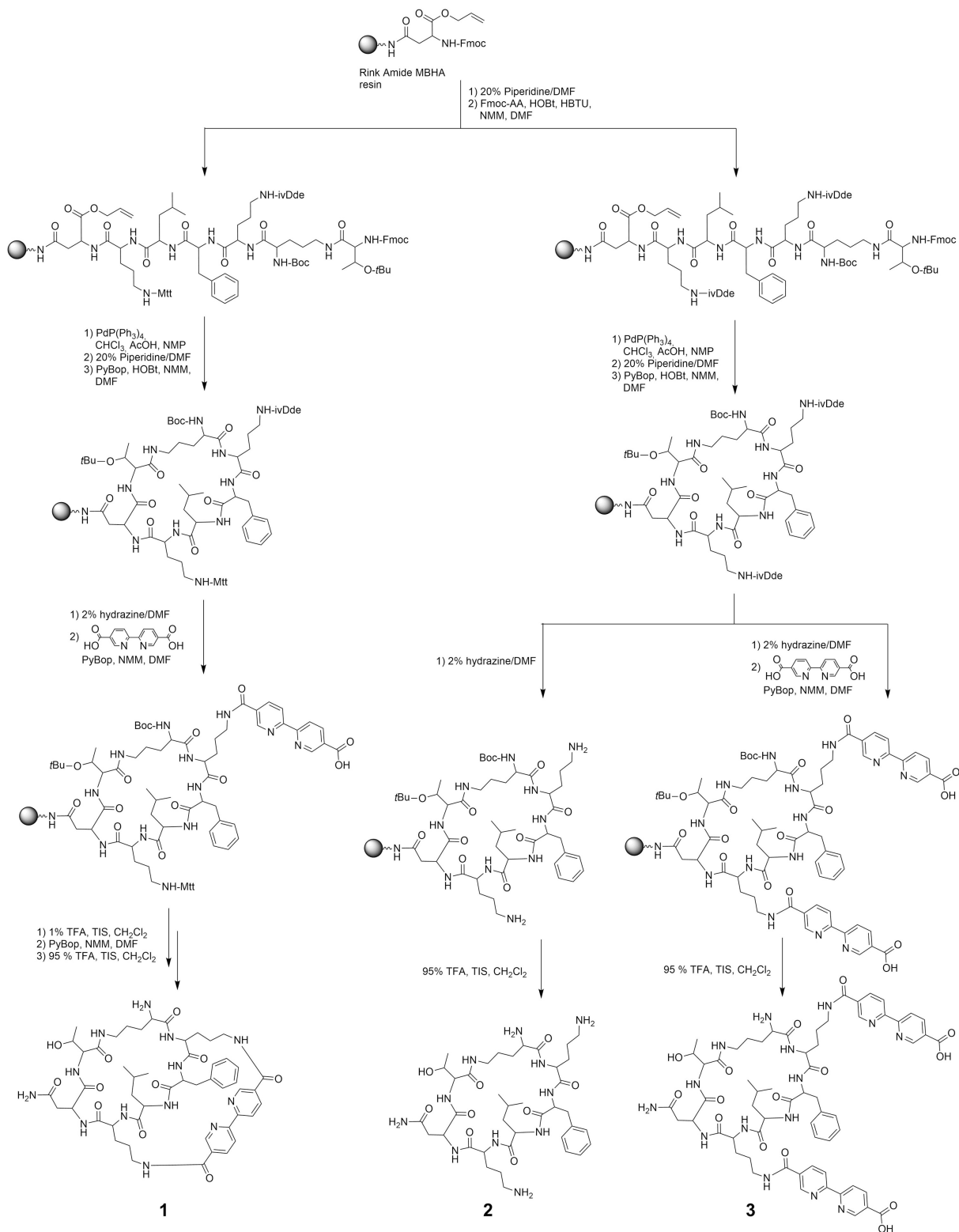

Figure 1. CD spectra of bicyclic peptide **1** (——), and cyclic peptide **2** (- - -).

Scheme 1. Solid-phase synthesis of bicyclic peptide **1**.

Fluorescence and UV/vis Titration Experiments

Titration experiments were performed at room temperature in aqueous sodium cacodylate buffer (50 mmol dm^{-3}, pH = 6.5). In all experiments concentration of receptor **1** was kept constant ($c_{receptor}$ = 2.5 × 10^{-5} mol dm^{-3} for fluorimetric and $c_{receptor}$ = 5 × 10^{-5} mol dm^{-3} for UV/vis titrations), whereas the concentration of monosaccharide substrates was varied from cca 1 × 10^{-5}−4 × 10^{-2} mol dm^{-3} in the case of UV/vis and 1 × 10^{-5}−1 × 10^{-3} mmol dm^{-3} in the case of fluorimetric titration experiments. The association constants (log K_a) were calculated using the SPECFIT global analysis software.[8] The stoichiometry for complexes of **1** with monosaccharides was determined by the mole ratio method (Job plot).[10]

RESULTS AND DISCUSSION

Bicyclic Peptide Design and Solid-phase Synthesis

The conformationally constrained cage-like molecules, such as bicyclic peptide receptor **1**, represent an interesting topology for designing artificial receptors capable of binding biologically important monosaccharides under physiological conditions. In this type of monosaccharide receptors, the three-dimensional lipophilic cavity is already preorganised for the three-dimensional monosaccharide substrates binding, meaning that there are less entropically unfavorable conformations formed in order to adopt the optimal complex geometry. In addition, incorporation of functionalities for hydrogen bonding interactions within receptor's three-dimensional structure will permit monosaccharide substrates not only to be complexed but to be encapsulated, allowing therefore monosaccharide binding in water.

Our synthetic receptor is modeled on cyclic decapeptide antibiotic polymyxin B.[6] Polymyxin B consists of a seven member ring containing four positively charged diaminobutyric acid (Dab) residues, one Thr residue, hydrophobic D-Phe and Leu residues, and the *N*-terminal lipophylic tail region composed of Dab-Thr-Dab-6-methyl-octanoic acid segment. However, previous reports showed that polymyxin derivative lacking the side chain terminal fatty acyl diaminobutyrate, so called polymyxin B nonapeptide or PMBN, can still bind LPS with relatively high affinity.[7a,11] The model of PMB/lipid A complex constructed by docking of PMBN NMR structure on lipid A suggested that the polar face of PMB peptide ring interacts with the disaccharide part of lipid A.[7a] Quite interestingly, the PMBN analogue in which the Dab residues were replaced by Orn has been shown to bind strongly lipid A even after losing its antibacterial activity.[11c] Taking into consideration the structure of typical lectin binding site and

above mentioned literature data, we based our peptide lectinomimic **1** on PMBN. In addition, since PMBN Orn analogue retains its binding affinity toward lipid A, and considering a greater selection of commercially available orthogonally protected Orn derivatives, we decided to incorporate Orn residues instead of Dab into the receptor's peptide sequence, Scheme 1.

Solid-phase synthesis of PMB[12] and its truncated analogue PMBN[11b,11c] has been reported previously. Described synthetic paths include solid-phase assembly of linear precursors using standard Fmoc-chemistry, followed by peptide cyclization in solution, or synthesis using a safety-catch sulfonamide linker and peptide cyclization with associated cleavage from the solid support. However, the same synthetic strategies cannot be applied for the synthesis of peptide receptor **1** due to its structural complexity (i.e. required bicyclic structure formed by incorporation of the bipyridine dicarboxylate moiety). Therefore, we have developed a new solid-phase synthetic approach that allows complete synthesis of desired bicyclic peptide **1** on the solid support. In general, our synthetic approach toward bicyclic peptide **1** was comprised of the resin attachment of the Asp residue *via* side chain, successful use of combination of six quasi-orthogonal removable protecting groups, stepwise Fmoc solid-phase synthesis of a linear precursor, on-resin head-to-tail cyclization, and in the final step on-resin attachment of the bipyridine dicarboxylate moiety. Anchoring of the first residue to the resin is one of the most critical steps in peptide synthesis. Choice of a wrong linker, loading method, or amino acid can have highly deleterious effects on the purity of synthesized peptides. In order to optimize this first step, two different types of resins were used: the hydroxymethyl-based Wang resins (substitution 0.95 mmol/g) yielding cyclic peptide with the *C*-terminal carboxylate moiety, and the aminomethyl-based Rink resins (substitution 0.43 mmol/g) yielding corresponding analogue with the *C*-terminal amide moiety. The level of the Asp residue attachment to the resins was determined by the spectroscopic (UV/vis) method as previously described in the literature.[8] The best results were obtained with the Rink amide resins (yields 75 %−80 %). The Fmoc chemistry was used throughout the linear peptidyl-resin precursor synthesis. After removal of *C*-terminal Allyl protecting group and *N*-terminal Fmoc protecting groups using standard deprotection methods,[9] linear peptide precursor was successfully cyclized on resin using PyBop/HOBt/NMM as we previously described.[13] Incorporation of bipyridine unit into peptide skeleton represented additional challenging synthetic task. For this purpose, Orn residues with ivDde protected side chain's amino groups were used. ivDde side chain protected Orn was chosen because ivDde can be removed by hydrazine

reagent without affecting remaining protecting groups present on the peptide. In this way, we have generated two primary amino groups available for incorporation of the bipyridine dicarboxylate bridge. It was expected that coupling of bipyridine dicarboxylate under pseudo-dilution conditions[14] will result exclusively in the desired bicyclic peptide **1**. However, after deprotection of Orn side chain amino groups with 2 % hydrazine in DMF and sub-sequential coupling of 1 eq of 2,2'-bipyridine-5,5'-dicarb-oxylic acid only undesired cyclic peptide **3** bearing two bipyridine dicarboxylate units was obtained (MALDI-TOF MS analysis: m/z = 1270 calculated; m/z = 1292 [M+Na]$^+$ observed), Scheme 1. This problem was solved by incorporating into the peptide precursor Orn residues with orthogonally protected side chain's amino groups. For this purpose, we chose Fmoc-Orn(Mtt)-OH and Fmoc-Orn(ivDde)-OH to incorporate in positions 2 and 5, respectively, of the cyclic peptidyl-resin precursor sequence. Selective removal of ivDde protecting group with 2 % hydrazine in DMF allowed incorporation of only one 2,2'-bipyridine-5,5'-dicarboxylic acid moiety. In the next step, Mtt protecting group was removed with 1 % TFA in CH$_2$Cl$_2$, followed by on-resin macrocyclization using PyBop/NMM. Progress and completion of the coupling reaction and macrocyclization was monitored by the ninhydrin colorimetric test.[15] Cleavage of the resulting bicyclic pep-tide product from the resins was carried out in 95 % of trifluoroacetic acid (TFA)/CH$_2$Cl$_2$ mixture in the presence of triisopropylsilane (TIS) as a scavenger. This time desired peptide **1** was synthesized in satisfactory yield (85 %, MALDI-TOF MS analysis: m/z = 1026.15 calculated; m/z = 1026.45 observed. RP-HPLC analysis: R_t = 23.2 min., linear gradient 100 % A → 100 % B in 30 min. at flow rate of 1 mL/min. As shown in Scheme 1, control cyclic peptide **2** was synthesized using the same solid-phase synthetic strategy (yield 90 %, MALDI-TOF MS analysis: m/z = 817,98, calculated; m/z = 818.41 [M+H]$^+$, 840.44 [M+Na]$^+$ observed.

RP-HPLC analysis: R_t = 20.18 min., linear gradient 100 % A → 100 % B in 40 min. at flow rate of 1 mL/min.

Conformational Characterization by CD Spectroscopy

Since the receptor preorganization plays a key role in the complexation process, we investigated an effect of bipyridine dicarboxylate bridge on receptor's conformation by CD spectroscopy. The CD spectra were recorded in cacodylate buffer (pH = 6.5, 50 mmol dm^{-3}). In both cases, the spectra minimums were obtained at λ = 196 nm characteristic of unordered peptide conformations, indic-ating absence of peptide conformational changes upon incorporation of the bipyridine bridge. In comparison to the control peptide **2**, the observed more pronounced negative band at λ = 223 and positive band at λ = 212 nm for bicyclic peptide **1** can be interpreted as a contribution of aromatic bipyridine bridge to the CD spectrum rather than the consequence of conformational change.[16] Obtained results are in good agreement with previously published CD data for PMBN also showing unordered conformation in aqueous solution.[11b,11c] However, previously reported ^{1}H NMR studies[17] suggested that conformation of free PMBN in aqueous solution is characterized by a type II' β-turn centered on the Dab^5-D-Phe^6-Leu^7-Dab^8 sequence and an inverse γ-turn at Thr^{10}. Based on our results and results of others, we can assume that receptor **1** has a rigid solution conformation and expect no significant conformational changes upon monosaccharide substrate binding, minimiz-ing therefore the entropic penalty of binding.

Binding Studies

Our preliminary binding studies indicate that the bicyclic peptide lectinomimic **1** is capable of binding monosac-charides with millimolar affinities in aqueous media. The binding affinities of bicyclic peptide **1** toward various monosaccharide substrates in cacodylate buffer (pH = 6.5,

Table 1. Association constants for 1 : 1 complexes between various carbohydrate substrates and **1** in aqueous media (pH = 6.5)

Carbohydrate substrates	log K_a	
	Fluorimetric titration	UV/Vis titration
Ribose	3.1 ± 0.21	3.45 ± 0.15
Xylose	3.8 ± 0.2	3.17 ± 0.05
Glucose	3.48 ± 0.15	2.96 ± 0.15
Galactose	3.67 ± 0.15	3.36 ± 0.05
Fructose	3.31 ± 0.1	2.85 ± 0.07
Glucose-6-phosphate	3.27 ± 0.09	3.11 ± 0.19
N-Acetylmuramic acid	3.21 ± 0.13	3.2 ± 0.13
Gentiobiose	ND	ND

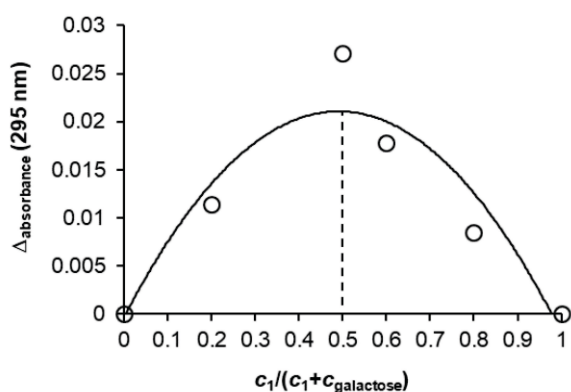ND=not determinable under applied experimental conditions

Figure 2. The Job plot indicating 1:1 stoichiometry for the complex between **1** and galactose.

50 mmol dm^{-3}) were estimated by UV/vis and fluorimetric titration experiments, and the observed values are shown in Table 1. The association constants (log K_a) were calculated using the SPECFIT global analysis software.[8] In all cases, a reasonably good agreement between the two methods was obtained. The stoichiometry for complexes of **1** with monosaccharides was determined by the mole ratio method (Job plot)[10] to be 1:1, Figure 2. As shown in Table 1, the selectivity of the peptide based receptor **1** with respect to a specific monosaccharide is, at best, modest. On another hand, affinity toward larger substrate such as gentiobiose, a disaccharide composed of two D-glucose units, are much lower and thus not determinable by fluorescence or UV/vis titration experiments under the same experimental conditions. The observed 1 : 1 stoichiometry for complexes of **1** with monosaccharide substrates and weak interaction with larger disaccharide substrate indicate that complexation occurs by insertion of the substrate molecule into receptor's lipophilic pocket, mimicking therefore similar process in lectins. The fact that the association constants for negatively charged mono-

saccharides are identical, within an experimental error, to those found for the neutral substrates suggests a significant contribution of hydrophobic interactions to the overall complex stability. Importantly, estimated association constants are similar to those reported in the literature for lectin/monosaccharide complexes, proving further our designing principle for the bio-mimetic monosaccharide receptors containing the three-dimensional lipophilic cavity.

CONCLUSION

Despite the lack of bicyclic peptide **1** selectivity toward various monosaccharide substrates, our binding studies successfully demonstrated that it is possible to construct peptide-based artificial receptor molecules capable of binding biologically important carbohydrates at physiologically relevant conditions. The design of this bio-mimetic receptor is based on the premises that the receptor molecule should be soluble in water, should possess a three-dimensional intermolecular binding site into which the three-dimensional monosaccharide substrates may be inserted, and that the conformation of the receptor molecule should be restricted to reduce the entropic penalty of binding.

With these results, we have also demonstrated that the bicyclic peptide **1** represents a promising scaffold for the design of new and more efficient carbohydrate receptors that may have broader application in bio-analytical field. Further optimization of bicyclic peptide receptor **1** using combinatorial chemistry approach is currently underway in order to improve its binding properties and selectivity.
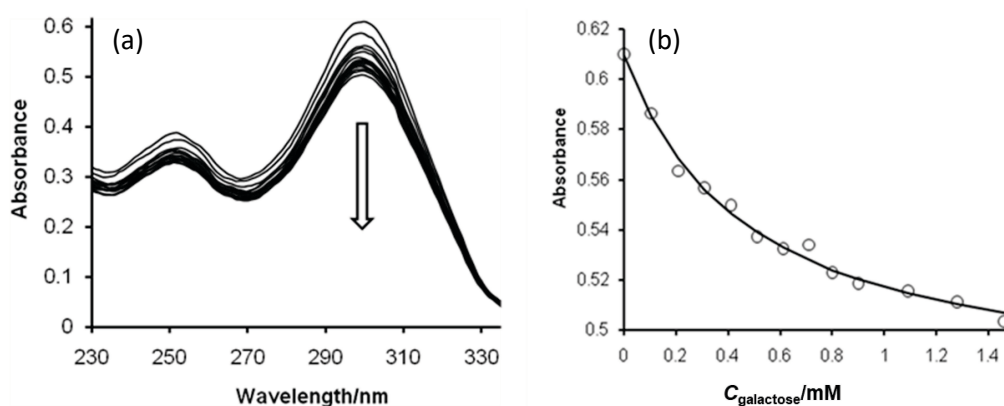
Figure 3. Representative titration experimant. (a) UV/vis titration of **1** with galactose ($c_{galactose}$ = 0.1–1.46 mmol dm^{-3}); (b) Experimantal (○) and calculated (—) absorbancies (λ_{max} = 295 nm) as a function of added galactose.

Abbreviations

TFA	Trifluoroacetic acid
PyBop	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
HOBt	Hydroxybenzotriazole
NMM	*N*-methylmorpholine
Fmoc	9-Fluorenylmethyloxycarbonyl protecting group
ivDde	1-(4,4-Dimethyl-2,6-dioxo-cyclohexylidene)-3-methyl-butyl protecting group
Mtt	4-Methyltrityl protecting group
DMF	*N*,*N*-dimethylformamide
TIS	Triisopropylsilane
RP-HPLC	Reverse phase-high performance liquid chromatography
MALDI-TOF	Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry
NMR	Nuclear magnetic resonance spectroscopy
CD	Circular dichroism spectroscopy

REFERENCES

[1]   a) A. Varki, *Biological roles of oligosaccharides: all of the theories are correct*, Vol. 3, **1993**; b) M. Fukuda, O. Hindsgaul, *Molecular and Cellular Glycobiology*, University Press, Oxford, **2000**.

[2]   a) D. B. Walker, G. Joshi, A. P. Davis, *Cell Mol Life Sci* **2009**, *66*, 3177.; b) M. Mazik, *ChemBioChem* **2008**, *9*, 1015.

[3]   a) A. P. Davis, R. S. Wareham, *Angew. Chem. Int. Ed. Engl.* **1999**, *38*, 2978.; b) T. D. James, S. Shinkai, in *Host-Guest Chemistry: Mimetic Approaches to Study Carbohydrate Recognition* (Ed.: S. Penadés), Springer Berlin Heidelberg, Berlin, Heidelberg, **2002**, pp. 159–200; c) A. Lützen, *Carbohydrate recognition by artificial receptors* in *Highlights in Bioorganic Chemistry*, (Ed.: C. Schmuck, Wennemers, H.), Wiley-VCH, Weinheim, **2004**, pp. 109–119; d) S. Striegler, *Curr. Org. Chem.* **2003**, *7*, 81.; e) A. A. Mensah, P. Cudic, *Curr. Org. Chem.* **2011**, *15*, 1097.; f) A. Jakas, M. Perc, J. Suć, M. C. Rodriguez, M. Cudic, P. Cudic, *J. Carbohydr. Chem.* **2016**, *35*, 69.

[4]   a) T. K. Dam, C. F. Brewer, *Chem. Rev.* **2002**, *102*, 387.; b) N. Sharon, H. Lis, *Adv. Exp. Med. Biol.* **2001**, *491*, 1.; c) W. S. Somers, J. Tang, G. D. Shaw, R. T. Camphausen, *Cell* **2000**, *103*, 467.; d) W. I. Weis, K. Drickamer, *Annu. Rev. Biochem.* **1996**, *65*, 441.

[5]   a) M. Kwon, S. Jeong, K. H. Lee, Y. K. Park, J. Yu, *J. Am. Chem. Soc.* **2002**, *124*, 13996.; b) N. Sugimoto, D. Miyoshi, J. Zou, *Chem. Comm.* **2000**, 2295.; c) T. Niidome, H. Murakami, M. Kawazoe, T. Hatakeyama, Y. Kobashigawa, M. Matsushita, Y. Kumaki, M. Demura, K. Nitta, H. Aoyagi, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1893.; d) J. Bitta, S. Kubik, *Org. Lett.* **2001**, *3*, 2637.; e) M. M. Benito JM, *QUSAR Comb. Sci.* **2004**, *23*, 117; f) T. Reenberg, N. Nyberg, J. O. Duus, J. L. J. van Dongen, M. Meldal, *Eur. J. Org. Chem.* **2007**, 5003.; g) M. C. Rodriguez, P. Cudic, *Chim. Oggi* **2011**, *29*, 36.

[6]   a) Z. Yuan, V. H. Tam, *Expert Opin. Inv. Drug* **2008**, *17*, 661.; b) A. P. Zavascki, L. Z. Goldani, J. Li, R. L. Nation, *J. Antimicrob. Chemoth.* **2007**, *60*, 1206.

[7]   a) D. S. Bhattacharjya, V. I. Mathan, P. Balaram, *Biopolymers* **1997**, *41*, 251.; b) J. C. Tsang, D. A. Weber, D. A. Brown, *J. Antibiot.* (Tokyo) **1976**, *29*, 735.; c) M. Vaara, *Fems. Microbiol. Lett.* **1983**, *18*, 117.

[8]   W. P. Chan WC, *Fmoc solid phase peptide synthesis. A practical approach*, University Press, Oxford, **2004**.

[9]   W. T. Greene, Wuts, P. G. M., *Protective groups in Organic Synthesis*, John Wiley and Sons, Inc., New York, **1999**.

[10]   D. Landy, F. Tetart, E. Truant, P. Blach, S. Fourmentin, G. Surpateanu, *J. Incl. Phenom. Macro.* **2007**, *57*, 409.

[11]   a) S. Srimal, N. Surolia, S. Balasubramanian, A. Surolia, *Biochem. J.* **1996**, *315*, 679.; b) H. Tsubery, I. Ofek, S. Cohen, M. Eisenstein, M. Fridkin, *Mol. Pharmacol.* **2002**, *62*, 1036.; c) H. Tsubery, I. Ofek, S. Cohen, M. Fridkin, *Biochemistry* **2000**, *39*, 11837.

[12]   a) S. K. Sharma, A. D. Wu, N. Chandramouli, C. Fotsch, G. Kardash, K. W. Bair, *J. Pept. Res.* **1999**, *53*, 501.; b) T. Kline, D. Holub, J. Therrien, T. Leung, D. Ryckman, *J. Pept. Res.* **2001**, *57*, 175.; c) P. C. de Visser, N. M. A. J. Kriek, P. A. V. van Hooft, A. Van Schepdael, D. V. Filippov, G. A. van der Marel, H. S. Overkleeft, J. H. van Boom, D. Noort, *J. Pept. Res.* **2003**, *61*, 298.

[13]   N. Bionda, M. Stawikowski, R. Stawikowska, M. Cudic, F. Lopez-Vallejo, D. Treitl, J. Medina-Franco, P. Cudic, *ChemMedChem* **2012**, *7*, 871.

[14]   M. Malesevic, U. Strijowski, D. Bächle, N. Sewald, *J. Biotech.* **2004**, *112*, 73.

[15]   E. Kaiser, R. L. Colescott, C. D. Bossinger, P. I. Cook, *Anal. Biochem.* **1970**, *34*, 595.

[16]   a) M. C. Manning, R. W. Woody, *Biochemistry* **1989**, *28*, 8609.; b) C. Krittanai, W. C. Johnson, *Anal. Biochem.* **1997**, *253*, 57.

[17]   P. Pristovsek, J. Kidric, *J. Med. Chem.* **1999**, *42*, 4604.