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Design, Synthesis and Evaluation of Synthetic Receptors for the Recognition of Aspartate Pairs in an α-Helical Conformation†

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Abstract—The specific targeting of protein surface functional groups remains a largely unexplored aspect in molecular recognition. In this study, a series of zwitterionic, 16-mer peptides serve as models for the recognition of carboxylate pairs in proteins. A receptor is described that contains two guanidinium groups separated by 4–5 Å by a rigid bicyclo[3.3.0]octane spacer. Modeling studies indicate that such a receptor would be suitable for binding with two aspartate carboxylates when the amino acids are separated by two (i+3) or three (i+4) other amino acids in an α-helical peptide. Studies employing circular dichroism spectroscopy demonstrated that the addition of the receptor to the i+3 peptide substrate caused a 23% enhancement of helical structure in 15% water/methanol at 25 °C. Other substrate peptides [(i+1), (i+4), (i+7), (i+10)] showed lower helical induction. Similar, but weaker binding and helical induction were observed under buffered conditions (10 mM Tris–Mes, pH 7.0). These results, along with studies employing a series of related di-cationic receptors, suggest a 1:1 binding model composed of specific hydrogen interactions between each receptor guanidinium with each substrate carboxylate when the peptide adopts a helical conformation. © 1997 Elsevier Science Ltd.

Introduction

In comparison to the progress that has been realized in the recognition of enzyme active sites,[1] little attention has been directed toward the design of molecules that recognize functional groups at a protein surface. Such targets are important because often the functional role of a protein is carried out via surface interactions rather than through a discrete active site. Numerous examples of this include proteins involved in multicomponent complexes, DNA targeting, and cell signaling events.[2] Protein surface recognition is complex in part because the solvent-exposed periphery is usually lined with a multitude of charged or polar functional groups that are highly solvated or involved in intramolecular interactions. In contrast to DNA, protein surfaces generally lack regular, easily predictable structural features.

As an extension of our development of synthetic receptors that bind designated substrates in competitive media,[3] we have initiated a program aimed at the design of small molecules that may influence protein–protein interactions by complexing with protein surfaces. We are presently focusing on a simple protein model that consists of a short peptide (16 amino acids) in which the carboxylate side chains from two aspartates are targeted. Short peptides are expected to have a substantial degree of conformational flexibility in solution, resulting in a heterogeneous mixture of target conformers. In the presence of the receptor, however, the peptide conformational equilibrium would be expected to shift to favor those forms that facilitate complexation. In the case illustrated in Figure 1, when the target peptide adopts an α-helical conformation, the carboxylates assume positions that are compatible with receptor binding. Correspondingly, the helical structure in the substrate peptide would be expected to be stabilized in the presence of a receptor that binds to, and pre-organizes, the carboxylates with the same relative positioning.

One approach to stabilizing the helical structure in short peptides is through the formation of covalent linkages between adjacent turns of the chain. Such linkages have been constructed from lactam bridges between aspartate and lysine side chains[4] and through the formation of side-chain disulfides.[5] Similarly, non-covalent helix-stabilizing linkages have been described based on the intrahelical formation of salt bridging,[6] metal coordination,[7] and hydrophobic interactions.[8] Most relevant for the present work are the few examples of helical stabilization based on the design of complementary molecules that bind to peptide or protein surface regions. In work by Voyer et al., short peptides having two crown ether-bearing amino acids increased in helical stability due to the presence of bis-ammonium guests which bound to and organized the modified residues in chloroform solution.[9] In a related approach, Mallick et al. have used two mercury-coordinating macrocycles that are connected by a tether to bind pairs of imidazoles that served as protein models.[10] In a third example, Tabet et al. described a 14-amino acid, tetra-anionic peptide that shows an increase in helicity from 11 to 24% (in aq buffer containing 30% trifluoroethanol) on addition of spermine.[11] Helical induction in this case is attributed to interactions between the spermine ammonium groups and the glutamate carboxylates. In the present report, we

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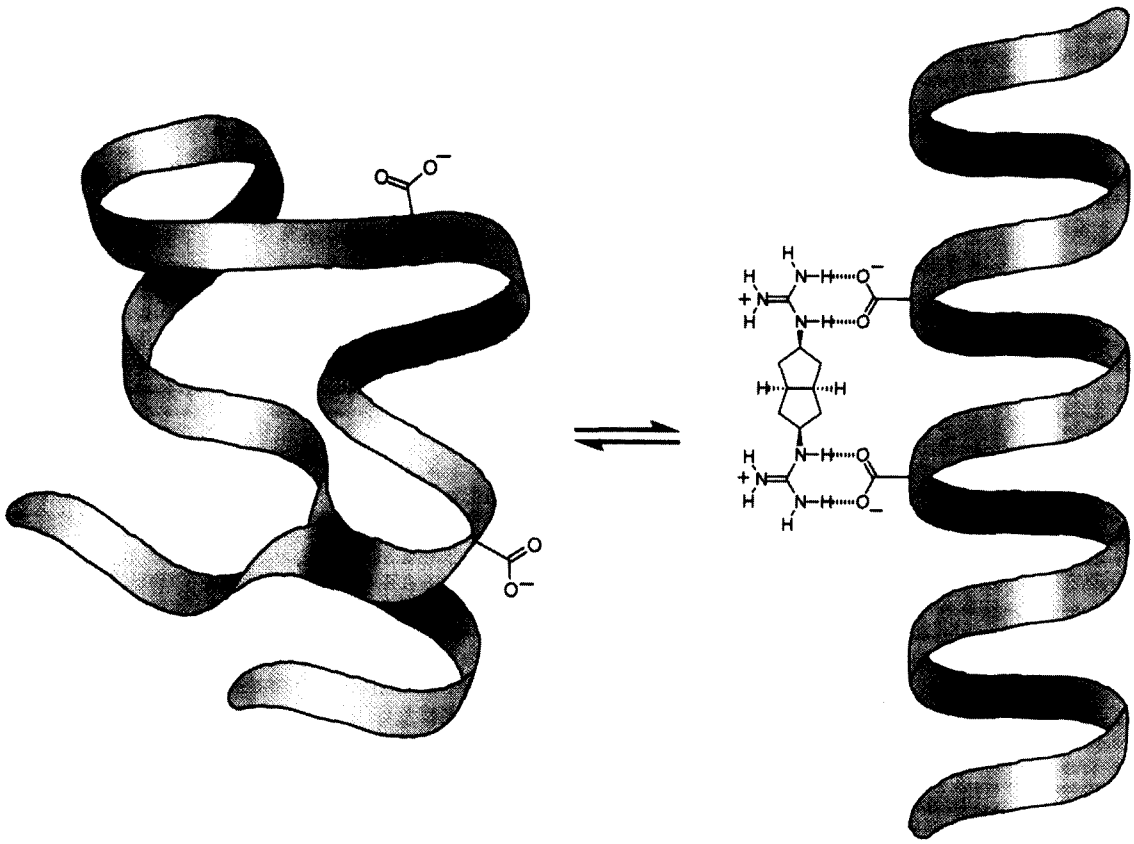

Figure 1. Schematic representation showing the binding of a rigid receptor to an α-helical conformation of a substrate peptide. Intermolecular stabilizing interactions are shown as hashed bonds.

describe the design of a family of rigidified bis-guanidinium receptors and show that they both bind to, and cause helical induction in, a series of zwitterionic 16-mer peptides.

The strong interaction of guanidinium groups with electron-rich or anionic target sites in aq media has been demonstrated in many examples with proteins[12] and nucleic acids.[13] The use of electrostatic[14] and hydrophobic[15] forces separately, or in combination, to direct binding interactions in model compounds has also received considerable attention. In particular, several synthetic receptors that employ guanidinium groups have been used for the targeting of dicarboxylate substrates in competitive solvents.[3,14(c,d),16] Previous work in our laboratory has demonstrated the strong and selective binding of simple dicarboxylate substrates using a family of bis-guanidinium receptors in highly competitive media. In receptor **1** (Fig. 2), two guanidinium groups are separated by a rigid bicyclo[3.3.0]octane spacer. This receptor was previously shown to selectively bind glutarate with an association constant of 3900 M^{-1} in 10% water/methanol at 25 °C.[3] Subsequently, we reported that **1** binds selectively to a dianionic alanine-based peptide containing two targeted aspartate residues.[17] In 10% water–methanol, binding affinities were 700–2000 M^{-1}, and the strongest binding was with a peptide that had the target aspartate carboxylates separated by two amino acid residues

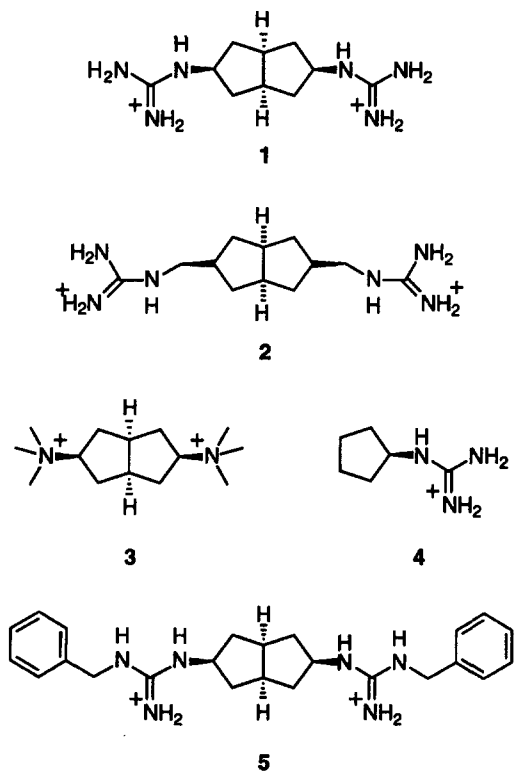

Figure 2. Structures of receptors.

(i+3 disposition). Substrate binding showed modest specificity for the helical conformation and induced a 5–10% increase in helicity.

In the present report, we describe the next stage in these studies using a broader series of receptors and peptide substrates under more competitive solvent conditions. We have prepared a series of zwitterionic, bis-aspartate-containing peptide substrates and investigated their binding to a family of bis-cationic receptors containing different types and spacings of recognition groups in 15% water–methanol under unbuffered and buffered (Tris–Mes, 10 mM, pH 7.0) conditions. Under our experimental conditions, each of the peptides will exist in a conformational equilibrium that samples a multitude of random coil states as well as helical states. Consequently, receptor binding can also occur to any peptide conformation that allows appropriate positioning of the target carboxylates. If a receptor binds to a helix-oriented array of carboxylates with preference over the mean distribution of all possible conformations, then increasing receptor concentration should result in a shift in the conformational equilibrium to favor the helical state. Comparisons using substrate peptides with different arrangements of the targeted aspartates (in the primary sequence) are used to analyze structural specificity.

Design of peptide substrates

Each of the peptide substrates in this study (Fig. 3) has two target aspartates, separated by a varying number of other residues. The carboxylates are expected to be the principle interacting sites with the receptor guanidiniums. The strength of association between the receptor and each peptide substrate will be largely determined by the ability of the peptide to adopt a conformation that places the target aspartates in positions that are complementary for binding with the receptor guanidiniums. Peptides were designed such that zero, two, three, six, or nine residues separate the aspartates, leading to the sequences referred to as i+1, i+3, i+4, i+7, and i+10. The peptides are variants of a sequence family originally studied by Huyghues-Despointes et al.[18] and employ several features commonly used to promote helix formation. Each peptide sequence has a high proportion of alanine and the destabilizing effects of the helix macrodipole are reduced by *N*-capping (acetylation) and *C*-capping (amidation). These characteristics increase the propensity for helical stabilization, although the peptides would still be expected to exist predominantly as a random coil ensemble under our experimental conditions.[18] To aid solubility, two lysines and two glutamines were incorporated at conserved positions. In order to minimize intramolecular side-chain interactions with the aspartate carboxylates, the lysines were placed at the (normally frayed) helix termini. A single *C*-terminal tyrosine was included to facilitate concentration determination.

Design of receptors

In receptor **1**, two guanidinium groups are separated by a rigid bicyclo[3.3.0]octane spacer designed to bind to a pair of carboxylates separated by a distance of 4–5 Å. Complexation of the substrate peptides was also studied with a series of related receptors to probe the nature of the bound complex. To assess the sensitivity to receptor rigidity and preorganization, receptor **2** has an additional methylene between each guanidinium group. It therefore represents an elongated and more flexible analogue of receptor **1**. The role of hydrogen bonding interactions in complexation was assessed by comparisons with receptor **3**, which cannot act as a hydrogen bond donor because the guanidinium groups have been replaced with quaternary ammoniums. The half-receptor **4** serves as a control for effects from nonspecific binding and ionic strength changes. The addition of benzyl groups in receptor **5** assesses the generality of this framework for the targeting of suitably positioned dicarboxylates and serves as a further test of structural models.

Each peptide substrate must adopt a conformation where the aspartate carboxylates are accessible and separated by a distance of about 4–5 Å in order to simultaneously interact with both guanidiniums in the rigidified receptor **1**. In a perfectly α-helical conformation, the peptide backbone has a right-handed twist with 3.6 residues per turn and a translation along the long helix axis of 5.41 Å. As illustrated in Figure 4, from these considerations the location of the aspartates in the primary sequence can be used to estimate the relative positioning of the target carboxylates when the peptide adopts a helical geometry. From the geometrical factors, the binding of receptor **1** is possible for helical conformations of peptides i+3, i+4, and i+7. For α-helical conformations of peptides i+3 and i+4, the aspartate carboxylates are located on adjacent turns, whereas for the i+7 peptide they are separated by one helical turn. Despite this difference, side-chain flexibility may allow the aspartates to assume similar positions for the i+7 peptide as for the i+3 and i+4 peptides with minimal backbone distortion. In comparison, the

i+1: Ac-Lys-Ala-Ala-Gln-Ala-Ala-Ala-Ala-**Asp**-**Asp**-Ala-Gln-Ala-Ala-Lys-Tyr-$CONH_2$
i+3: Ac-Lys-Ala-Ala-Gln-Ala-Ala-**Asp**-Ala-Ala-**Asp**-Ala-Gln-Ala-Ala-Lys-Tyr-$CONH_2$
i+4: Ac-Lys-Ala-Ala-Gln-Ala-**Asp**-Ala-Ala-Ala-**Asp**-Ala-Gln-Ala-Ala-Lys-Tyr-$CONH_2$
i+7: Ac-Lys-Ala-Ala-Gln-Ala-**Asp**-Ala-Ala-Ala-Ala-Ala-Gln-**Asp**-Ala-Lys-Tyr-$CONH_2$
i+10: Ac-Lys-Ala-**Asp**-Gln-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Gln-**Asp**-Ala-Lys-Tyr-$CONH_2$

Figure 3. Substrate peptide sequences; target aspartates are shown in bold.

binding of receptor **1** to α-helical conformations of peptide $i+1$ would not be expected to be favorable because this conformation causes the carboxylates to project from different faces along the helix surface. Additionally, if receptor **1** did bind to helical conformations of peptide $i+1$, the resultant restriction of the adjacent aspartates would not be expected to enhance helical stability as much as the same restriction would for further separated residues. Receptor **1** binding cannot occur with α-helical conformations of peptide $i+10$ because of the very long distance between the aspartates.

Results

CD titrations

In the absence of a receptor, each of the substrate peptides displayed a CD (circular dichroism) spectrum with a maximum at about 190 nm and minima at ~208 and ~222 nm (Fig. 5), consistent with a significant degree of helical structure. The intensity of the CD effect at 222 nm was monitored for each peptide in the presence of increasing receptor concentration in 15% water in methanol. In this way, binding affinities and the change in helical stability were evaluated. The magnitude of the CD effect at 222 nm has typically been correlated with the extent of helix formation.[19] The shapes of the CD spectra did not change as a result of receptor addition, indicating that no substantial alterations of peptide structure (other than changes in the degree of helical stability) occurred due to the presence of the receptors. To verify that the observed CD changes resulted from peptide interactions with receptor **1**, rather than as a result of medium effects such as ionic strength, analogous titrations were carried out under the same solvent conditions in the presence of 10 mM Tris–Mes buffer (pH 7.0). Features of receptor **1** that are essential for its binding were assessed in a series of titrations using the receptor analogues **2–5**.

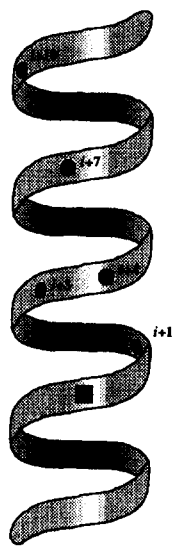

Figure 4. Schematic illustration of the relative spacing between pairs of amino acids along an α-helical peptide. Circles indicate the position ($i+1$, $i+3$, $i+4$, $i+7$, and $i+10$) of an amino acid relative to another amino acid position indicated by the square. Note that because of differences in the primary sequence, this figure is not intended to represent actual positions for the substrate peptides in Figure 3.

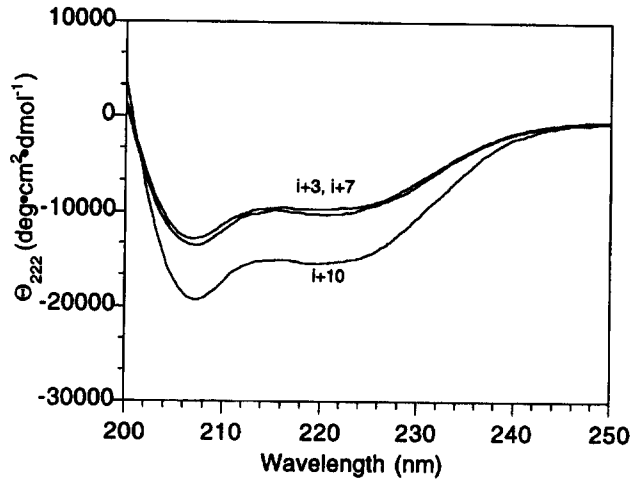

Figure 5. CD spectra of peptides $i+3$, $i+7$, and $i+10$ (spectra of peptides $i+3$ and $i+7$ are nearly coincident) at 500 μM concentration, 15% water/methanol, 25 °C. Peptides $i+1$ and $i+4$ are partially aggregated under these conditions and are not shown here.

Synthesis of receptors

The synthesis of receptor **1** is illustrated in Figure 6. Reductive amination of 3,7-bicyclo[3.3.0]octandione (**6**) with benzylamine gave **7** as the major product; smaller amounts of the undesired *endo,exo* isomer were removed by column chromatography. Benzyl deprotection and guanidinylation with *N,N'*-bis(Boc)-1-amidinopyrazole[20] afforded the fully protected bis-guanidine (**9**), which after flash chromatography, deprotection, and ion exchange gave the bis-guanidinium dihydrochloride product. The corresponding half-receptor **4** was similarly obtained starting from cyclopentylamine.

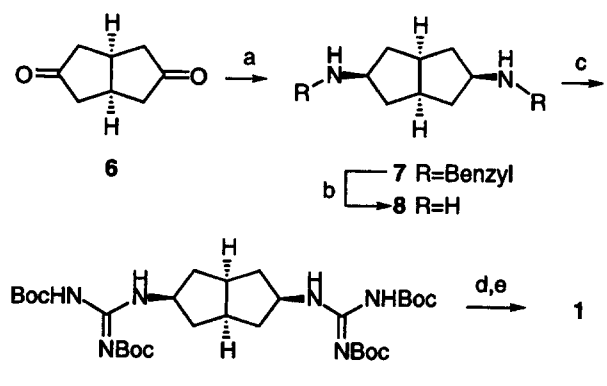

Figure 6. Synthesis of **1**. (a) Benzylamine hydrochloride, $NaBH_3CN$, methanol; (b) NH_4OAc, Pd/C, methanol; (c) *N,N'*-bis(Boc)-1-amidinopyrazole, THF; (d) trifluoroacetic acid; (e) Dowex Cl^- 1X80-50.

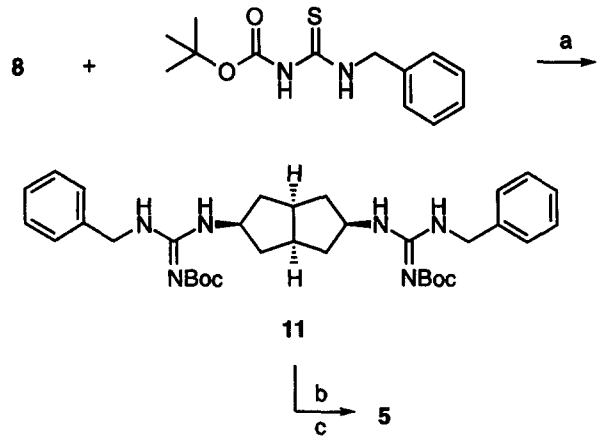

Figure 7. Synthesis of **5**. (a) EDCI, TEA, DCM; (b) trifluoroacetic acid; (c) Dowex Cl⁻ 1X80-50.

An alternate route for the preparation of **1** was developed to provide access to protected or modified bis-guanidinium adducts. Such adducts will facilitate the examination of substituent effects and may provide a tethering point for the further addition of interacting groups. The preparation of *N,N'*-dialkyl guanidinium derivatives has been carried out using alkylated pyrazole adducts such as *N,N'*-bis-(Boc)-*N*-benzyl-1-amidinopyrazole[20] or *S*-alkylisothiouronium salts.[21] However, these proved to be insufficiently reactive with diamine **8**. Alternatively, reaction of **8** with *N*-Boc-*N'*-benzylthiourea[22] in the presence of EDCI (1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide) afforded the orthogonally protected, bis-trialkyl guanidine **11**. Bis-dialkyl guanidinium **5** was obtained after Boc-deprotection and ion exchange (Fig. 7).

The extended receptor **2** was synthesized (Fig. 8) by alkylation of **6** with trimethylsilyl cyanide. Elimination of the TMS-cyanohydrin via phosphorous oxychloride afforded a mixture of isomeric olefins (**13**). Receptor **2** was obtained after stereoselective olefin reduction, nitrile reduction, and guanidinylation with *N,N'*-bis-(Boc)-1-amidinopyrazole.[20] Receptor **3** was synthesized by reductive alkylation of **6** with dimethylamine in the presence of $NaBH_3CN$ followed by methylation using methyl iodide.

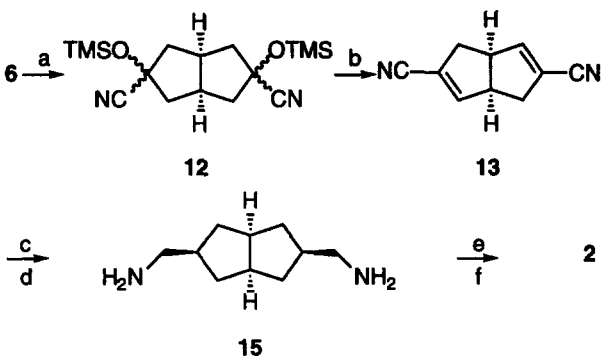

Figure 8. Synthesis of **2**. (a) Trimethylsilyl cyanide, ZnI, DCM; (b) $POCl_3$, pyridine (affords a mixture of olefin isomers); (c) H_2, Pd/C; (d) BH_3, THF; (e) *N,N'*-bis(Boc)-1-amidinopyrazole, TEA, DCM; (f) HCl, diethyl ether.

Discussion

Protein surface recognition is complicated by the multitude of functional groups present, their poorly defined arrangements, and the potential for conformational flexibility. Furthermore, charged and polar groups at the protein exterior are highly solvated and therefore especially difficult to target in aq media. As part of our continuing efforts to design synthetic host molecules that bind specific substrates in highly polar media, we have begun a stepwise approach to the utilization of simple rigidified receptors that bind to key protein functional groups.

Short peptides serve as protein models in which two aspartate carboxylates are targeted. The rigidified receptor **1** (Fig. 2) was designed to interact optimally with carboxylates pairs that are separated by 4–5 Å. Modeling studies indicated that the side-chain carboxylates from two aspartates, separated by two (i+3), three (i+4), or possibly six (i+7) other amino acids could favorably attain this distance when folded in an α-helical conformation. All of the peptide substrates in this study are 16 amino acids in length and have identical compositions but differ in the spacing between the targeted aspartates (Fig. 3). Peptides of this length are expected to exist in a conformational equilibrium that samples the random coil state as well as defined helical states.[23] Under the experimental conditions (15% water/methanol, 25 °C), each of the peptides has a significant propensity for helicity (20–40%). The position of the coil–helix equilibrium can therefore be estimated from CD spectra (Fig. 9). Through the analysis of the CD spectra of the substrates as the receptor is added, we can estimate the degree to which receptor addition causes a shift in the coil–helix

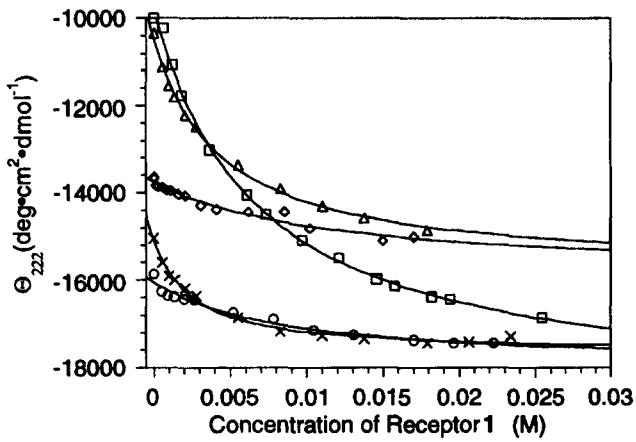

Figure 9. Ellipticity at 222 nm for each peptide substrate in the presence of increasing concentration of **1** with each peptide substrate (15% water in methanol, 500 μM peptide, 25 °C); i+1 (○), i+3 (□), i+4 (◇), i+7 (△), i+10 (×).

equilibrium. Because it is assumed that the system follows a two-state coil–helix transition, it should be noted that this technique is interpreted to primarily measure binding to the helical state. For example, strong binding may occur to one of the multitude of populated conformations in the random coil state, yet this may have a very small effect on the CD spectral characteristics.

Peptide binding in unbuffered 15% water–methanol

In 15% water–methanol, the peptide substrates (at 500 μM) displayed varying degrees of intrinsic helical stability (Table 1). Peptides $i+1$, $i+4$, and $i+10$ were the most stabilized, characterized as being 30–38% helical. These also were least sensitive to the addition of **1**, with helicity increasing by only about 10% in the presence of saturating amounts (10 equiv) of receptor. Concentration dependence titrations of peptides $i+1$ and $i+4$ (in the absence of any receptor) indicated that they were significantly aggregated in the range studied. Peptides $i+3$ and $i+7$ had the lowest level of intrinsic helical stability (about 18%), but were affected most by the addition of receptor **1**, resulting in 23% and 7% increases in helical stability. This corresponds to an overall increase in the population of the helical conformation (ΔΔ) of 120% and 35%, respectively.

As shown in Table 1, the addition of receptor **1** causes the most efficient helical stabilization for peptide $i+3$, followed by peptide $i+7$. Substantially weaker helical induction is observed for peptides $i+1$, $i+4$, and $i+10$. For peptides $i+3$ and $i+7$, helical conformations place the target carboxylates approximately along the same face on the helix (Fig. 4). Unlike the $i+3$ peptide, helical conformations of the $i+7$ peptide separate the carboxylates by a full helical turn. However, when peptide $i+7$ adopts a helical conformation, the length and flexibility that remain present in the aspartate side chains may be sufficient to allow the carboxylates to bind the receptor in a similar manner as proposed for peptide $i+3$. This is consistent with the lower affinity and degree of helical stabilization for peptide $i+7$ relative to peptide $i+3$ in the presence of receptor **1**.

The substantially weaker degree of helical induction for peptide $i+4$ with receptor **1** was unexpected. Modeling studies suggested that α-helical conformations of peptide $i+4$ should be well suited to allow binding with **1**. Unlike peptides $i+3$ and $i+7$, this peptide is not entirely monomeric under the assay conditions; however, we do not yet have a full rationale for the dramatic differences between peptides $i+3$ and $i+4$.

Under the unbuffered conditions, peptides $i+1$ and $i+10$ showed very little change in helical stability as the receptor is added. Modeling analysis suggests that helical conformations of both peptides place the target carboxylates distant from each other such that the receptors cannot simultaneously bind both sites. Electrostatic neutralization of the di-anionic substrate peptide by the receptor is likely to be a significant component in complexation, particularly when binding requires the close positioning of the carboxylates. Because the aspartates are well separated in α-helical conformations of the $i+1$ and $i+10$ peptides, they would be expected to be more thermodynamically stabilized (in the absence of a receptor) than other peptides for which the α-helical arrangement of residues places the aspartates in proximity. Note that strong binding of **1** is evident for the peptide $i+10$ even though there is little effect in helical stability; this could result from receptor binding interactions with nonhelical conformations. When ligand binding results in such small changes, it is clearly not possible to make detailed interpretations other than for comparison with peptides $i+3$ and $i+7$.

Peptide binding in buffered 15% water–methanol

It is likely that receptor binding interactions include components from specific interactions (of the guanidiniums with the substrate carboxylates) as well as from nonspecific interactions (including charge screening

Table 1. Binding affinities of peptide substrates with receptor **1**, and peptide helical stability following the addition of receptor

	15% Water in methanol					15% Water in methanol, 10 mM Tris–Mes, pH 7.0				
Peptide	K_a (M^{-1})	H_i[a]	H_f[b]	Δ[c]	ΔΔ[f]	K_a (M^{-1})	H_i[a]	H_f[b]	Δ[c]	ΔΔ[f]
$i+1$[d]	120±18	38±5	41±6	3	8	40±6	23±3	25±4	2	9
$i+3$	290±44	17±3	40±6	23	120	90±14	23±3	31±5	8	35
$i+4$[e]	90±14	32±5	35±5	3	10	50±8	32±5	36±5	4	13
$i+7$	170±25	20±3	27±4	7	35	90±14	29±4	33±5	4	14
$i+10$	390±58	37±6	41±6	4	11	340±51	36±5	39±6	3	8

[a]Percentage of peptide helicity in the absence of receptor.
[b]Percentage of peptide helicity in the presence of 10 equiv of receptor.
[c]Change in percentage of peptide helicity after addition of 10 equiv of receptor.
[d]Peptide is partially aggregated in the unbuffered solvent system.
[e]Peptide is partially aggregated in both the buffered and unbuffered solvent systems.
[f]Overall change in percentage of peptide helicity relative to starting helicity.

and ionic strength changes). To help distinguish among these, analogous titrations were carried out in the same solvent mixture with the addition of Tris–Mes buffer (10 mM, pH 7.0). Consideration of intrinsic helical destabilization (in the absence of receptor) may be relevant in understanding the effects of added buffer. On changing to the buffered conditions, the $i+3$ and $i+7$ peptides each show a moderate increase in intrinsic helical stability (Table 1). This is contrary to the typical effect where helix stability is decreased when hydrogen bonding competitors are added. The increase in stability may reflect charge-screening effects that reduce repulsions between the aspartate carboxylates that would be in proximity for helical conformations. For the $i+4$ and $i+10$ peptides, intrinsic helical stability was similar for buffered and unbuffered conditions. Although this certainly originates from the balancing of many component forces, it suggests that these peptides adopt helical conformations without substantial electrostatic penalties. While this is easy to rationalize for the distant aspartates in the $i+10$ peptide, it may also be true for the $i+4$ peptide which, according to molecular modeling, can fold into an α-helix with the aspartate carboxylates in opposed directions. An additional complexity for this peptide is that it begins to aggregate under buffered and unbuffered conditions, and is therefore not completely monomeric. The $i+1$ peptide is unique in that its intrinsic helicity drops substantially under the buffered conditions. We attribute the high helicity to substantial aggregation that takes place only in unbuffered conditions; on addition of the buffer, aggregation and concomitant helicity is reduced.

Since polar interactions primarily mediate the binding of substrate peptides with receptor **1**, it is expected that changing to buffered conditions would reduce the productive binding due to electrostatic screening and competition effects. Consistent with this proposal, the degree to which helicity is enhanced for the $i+3$ (23%) and the $i+7$ (7%) peptides decreases when the titrations are carried out under buffered conditions ($i+3$: 8%; $i+7$: 4%). In contrast, however, the $i+1$, $i+4$, and $i+10$ peptides showed a smaller difference in helical induction due to the presence of receptor **1** on changing between buffered and unbuffered conditions. As discussed above, these peptides also displayed only a small change in intrinsic helical stability in buffered and unbuffered conditions. These observations suggest that helicity in peptides $i+1$, $i+4$, and $i+10$ is not strongly electrostatically disfavored, and further that (unlike peptides $i+3$ and $i+7$) helical conformations do not optimally position the targeted aspartate carboxylates for binding to receptor **1**.

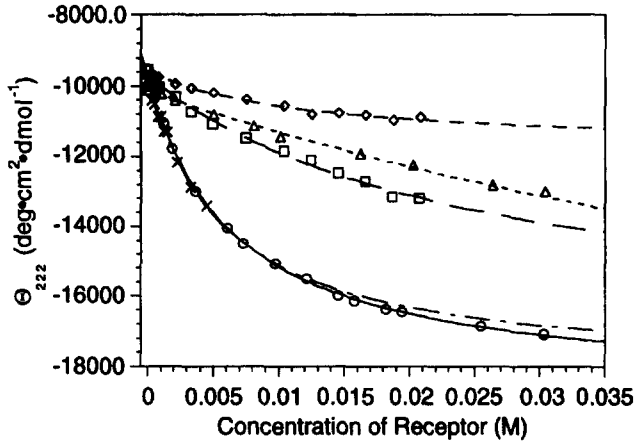

Figure 10. Ellipticity at 222 nm of peptide $i+3$ with each receptor (15% water/methanol, 500 μM peptide, 25 °C). Because of the high UV absorbance of receptor **5**, CD titrations could be carried out only over a limited receptor concentration range.; **1** (○), **2** (□), **3** (◇), **4** (△), **5** (×).

Peptide binding with receptor analogues of 1

To further explore the mechanistic basis and the conformational requirements for receptor binding to the peptide substrates, a series of titrations was carried out with receptors **2–5** and peptide $i+3$ (Fig. 10). Peptide $i+3$ was selected for its affinity with **1**, its high degree of receptor-induced helical stabilization, and the lack of any propensity for aggregation. In receptor **2**, each guanidinium is further separated from the rigid bicyclic spacer by a methylene unit. As indicated in Table 2, titrations demonstrated a greater than seven-

Table 2. Binding affinities of various peptide receptors with peptide $i+3$, and peptide helical induction following receptor binding

	15% Water in methanol				15% Water in methanol, 10 mM Tris–Mes pH 7.0			
Receptor	K_a (M^{-1})	H_f[a]	Δ[b]	ΔΔ[c]	K_a (M^{-1})	H_f[a]	Δ[b]	ΔΔ[c]
1	290±44	40±6	23	120	90±14	31±5	8	35
2	40±5	32±5	15	88	60±9	27±4	4	18
3	50±3	23±3	6	38	100±15	25±4	2	9
4	10±3	21±3	4	57	10±2	26±4	3	18
5[c]	170±26[d]	ND[d]	ND[d]	ND[d]	ND	ND	ND	ND

[a]Percentage of peptide helicity in the presence of 10 equiv of receptor, except for **4** where 30 equiv were used. Percent helicity in the absence of receptor is given in Table 1.
[b]Change in percent helicity after addition of receptor.
[c]Because of the high UV absorbance of receptor **5**, CD titrations could be carried out only over a limited receptor concentration range.
[d]Because 10 equiv were not reached in titrations with receptor **5**, H_f was not determined.
[e]Overall change in percentage of peptide helicity relative to starting helicity.

fold reduction in binding strength with receptor **2** in comparison to receptor **1**. Additionally, the degree of receptor-induced helicity for **2** (15%) was reduced in comparison to **1** (23%).

Optimal interactions of **1** with the substrate peptides require that the target carboxylates be separated by 4–5 Å in the bound conformation. The observation that **1** can recognize helical conformations of the $i+3$ and $i+7$ peptide substrates suggests that the peptides have sufficient flexibility within the helical motif to accommodate binding to the same receptor. This further suggests that a critical feature of receptor **1** is its conformational rigidity. By comparison, the flexibility incurred by the freely rotatable methylenes in **2** must add to the already substantial entropic penalty of helix folding and carboxylate positioning in the bimolecular complex.

Receptor **1** may associate with the substrate dicarboxylates of peptides $i+3$ and $i+7$ through electrostatic and/or hydrogen bonding interactions. Although **1** is structurally and electrostatically similar to the bis-trimethylammonium analogue **3**, the latter lacks hydrogen bonding donors and can interact only electrostatically with the substrate peptides. For receptor **3**, this results in a substantial loss of binding affinity with peptide $i+3$ and nearly abolishes helical induction. This suggests that the formation of specific hydrogen bonds is important for complex formation.

Receptor **4** lacks a second guanidinium group and cannot participate in bidentate binding interactions as proposed for **1**. Although identical electrostatic/hydrogen bonding interactions can take place for receptors **1** and **4**, receptor **1** would not be expected to help organize the substrate peptide into any particular conformation. We have previously shown through NMR studies that 1:1 complexation takes place with receptor **1** and a related peptide substrate that had a −2 net charge.[17] In that case, the interaction was shown to simultaneously involve both aspartate carboxylates and both receptor guanidiniums. The same behavior is implicated here for the interaction of **4** with these zwitterionic peptides. This is supported by the dramatic decrease in binding affinity for the peptide $i+3$ with the half-receptor **4**. The monofunctional receptor **4** binds much less efficiently to the substrate peptide even at concentrations that are equivalent in ionic strength (corresponding to a threefold-higher molar concentration of **4** in comparison to **1**).

Binding titrations with the benzylated adduct (**5**) showed virtually identical binding interactions as **1** with peptide $i+3$ (Fig. 10). This suggests that the *endo*-NHs of the guanidiniums may have general utility in the recognition of similarly spaced bis-carboxylate substrates, and further, that the outer guanidinium NHs may be useful tethering points for the addition of functionality to enhance substrate binding and selectivity.

Conclusions

These results are consistent with a model for the $i+3$ peptide in which the helix-oriented aspartate carboxylates are positioned in proximity to allow simultaneous binding with the two guanidiniums from receptor **1** (Fig. 11). The requirement for a simultaneous 1:1 interaction of both carboxylates with both guanidiniums is supported by (1) the substantial decrease in affinity for the more flexible receptor **2** and (2) the low affinity and relative inefficiency of the half-receptor **4** to induce helicity. These data support earlier NMR results with a related di-cationic target peptide that similarly showed a 1:1 association with the receptor **1**.[17]

Taken together, these results suggest that the bicyclooctane spacer effectively serves as a rigid scaffold that organizes the guanidinium groups in a manner that is complementary to a specific arrangement of aspartate carboxylates. The low intrinsic degree of conformational helicity in the substrate peptides allows for a sensitive CD assay for the effects of potential receptors on each peptide substrate. However, this flexibility also effectively reduces the binding affinity for any one peptide conformation, due to the high entropy penalty and due to competition from the multitude of non-helical substrate conformations. Despite these effects, receptor **1** binds preferentially to helical conformations of the $i+3$ peptide with affinities that would be expected for the proposed binding interactions.[3] Compared to protein recognition events in nature, the ligand binding energies observed here are small. We note, however, that analogous interactions in natural examples would almost certainly have a substantial hydrophobic binding element. The system demonstrates some of the highest degrees of helical stabilization for zwitterionic peptides of this length in the absence of covalent or metal-coordinating interactions. We are exploring further approaches for the enhancement of affinity and selectivity by (1) incorporating into the receptors the potential for hydrophobic interactions in addition to polar interactions, and (2) analyzing high affinity sites for these receptors in larger and more conformationally stable proteins.

Experimental

Chemicals and instrumentation

All chemicals were obtained from the Aldrich Chemical Company except where noted. Peptides were synthesized on a 0.10 mmol scale as *C*-terminal amides using Fmoc-PAL-PEG resin (Millipore) with a Millipore 9050 peptide synthesizer and conventional Fmoc solid-phase chemistry. Four equivalents of each *N*-protected amino acid (Millipore) were used with BOP/HOBt (benzotriazole-1-oxytris-(dimethlyamino)-phosphonium hexafluorophosphate/1-hydroxybenzotriazole hydrate) activation and a 1 h coupling time. Peptides were *N*-terminal acetylated by treatment of the resin with a solution of acetic anhydride (0.5 M) and pyridine (0.5

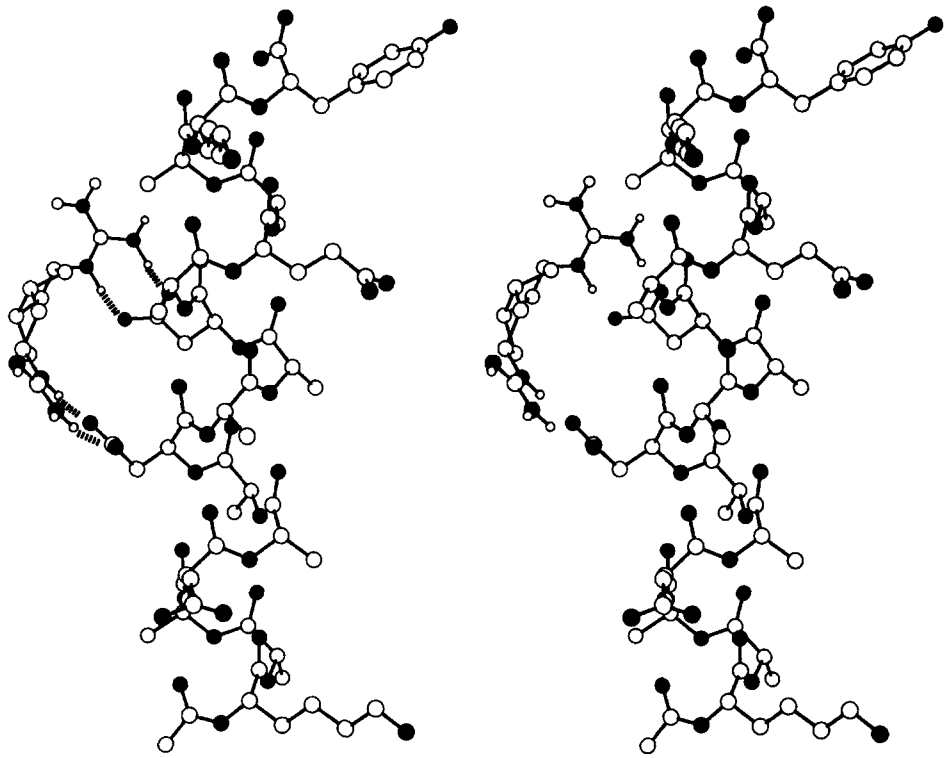

Figure 11. Stereoview of an energy minimized model of receptor **1** complexed with peptide $i+3$. Intramolecular hydrogen bonds are indicated with hashed bonds; all other hydrogens have been omitted for clarity.

M) in DMF (dimethylformamide) for 1 h. Peptide cleavage and deprotection were effected by treatment of the peptide-bound resin with a solution of trifluoroacetic acid (9.0 mL), thioanisole (0.5 mL), ethanedithiol (0.3 mL), and anisole (0.2 mL) for 2 h. After precipitation and washing with 1:1 diethyl ether: hexanes, peptides were purified using a Waters 600E HPLC system with a 490E detector, and a 25 × 10 cm Delta-Pak C18 300 Å cartridge column inside a Waters radial compression module. An eluent gradient of 5–70% CH_3CN in water (with 0.1% TFA) over 40 min was used to afford 50–90 mg of purified (>95%) peptide. Purity was determined by analytical HPLC which was performed using a Rainin HP controller and a Rainin UV-C detector with a Rainin 250 × 4.6 mm 5 μM Microsorb C18 column and a gradient of 10–90% CH_3CN in water (with 0.1% TFA) over 20 min. All peptides were satisfactorily analyzed by FABMS and amino acid analysis. Unless otherwise noted, reactions were performed at ambient temperature using anhydrous solvents under an argon atmosphere. NMR spectra were acquired using Bruker AM-300 and AM-500 spectrometers. Molecular modeling studies were carried out on a Silicon Graphics 4D-35 workstation using Macromodel v3.5a.[24]

Circular dichroism measurements

Peptide concentrations were initially determined by quantitative amino acid analysis and thereafter by UV in 6.0 M guanidine hydrochloride, 100 mM phosphate, pH 7.0, with ε_{274} = 1450 M^{-1} cm^{-1}.[25] CD spectra were determined using a Jasco 710 circular dichroism spectropolarimeter, which was calibrated using D-(−)-pantoyl-lactone (ellipticity = 19.0 mdeg at 0.15 mg/mL in water, 219 nm, 0.10 cm path) and (1*S*)-(+)-camphorsulfonic acid (ellipticity = 18.8 mdeg at 0.60 mg/mL in water, 290 nm, 0.10 cm path). A water-jacketed cylindrical cell (0.1 cm, Helma) was used at 25.0 °C with a Neslab programmable thermal bath. HPLC-grade water was obtained from Baxter Scientific. The pH of buffered samples was adjusted to 7.00 (by minimum addition of NaOH or HCl) before dilution with methanol, and no further corrections were made. The solvent mixture is composed of 15% (by volume) water and 85% spectrograde methanol. Binding and helicity data were collected by monitoring the change in magnitude of the 222 nm absorption which was observed on increasing addition of the receptors. Titration samples were prepared by combining aliquots of a solution containing the substrate peptide and receptor with a second solution containing only the peptide. In this manner, the peptide concentration was held constant at 500±50 μM and the receptor concentration was varied from 0 to approximately 30 mM (except for receptor **4**, which was varied from 0 to 120 mM and receptor **5**, which was varied from 0 to 5 mM). None of the receptors has an intrinsic contribution to the CD spectra at 222 nm over the concentration range studied, however, the total (achiral) UV absorbance due to the receptors imposes an upper concentration limit.

The degree of helicity was determined by monitoring the ellipticity at 222 nm using the average of 301 data

points collected over 60 s. Ellipticity is reported as the mean residue ellipticity (Θ_{222}) using $\Theta_{222} = (100\Theta_{obs})/(LNC)$, where Θ_{obs} is the measured ellipticity (mdeg), L is the path length (0.1 cm), N is the number of amino acids in the peptide (16), and C is the peptide concentration (in millimolars). Percentage helicity (f) was determined from $f = ((\Theta_{Obs}-\Theta_0)/(\Theta_{100}-\Theta_0))\cdot 100$ where Θ_{Obs}, Θ_{100}, and Θ_0 represent the measured ellipticity, the ellipticity corresponding to the fully helical state, and the ellipticity corresponding to the fully random coil state, respectively. The molar ellipticity at 100% helical content (Θ_{100}) was estimated to be $-34{,}444$ deg cm^2 dmol^{-1} according to the approximation of Chakrabartty et al.[26] The molar ellipticity at 0% helical content (Θ_0) was estimated to be -4400 deg cm^2 dmol^{-1} by thermal denaturation of the peptides in 100% water. Association constants were determined by nonlinear curve-fitting analysis of the change in helicity as a function of added substrate in three or more independent titrations. Data analysis was performed using HOSTEST II.[27] Because of the hygroscopic nature of the receptors, the preparation of their stock solutions is the main contributor to experimental error; accuracies in determined values are estimated to be ±15%. To assess the potential for peptide aggregation under the experimental conditions, the mean residue ellipticity of all peptides was measured over a concentration range between 50 and 800 μM. The peptides for which the helicity changed by less than 5% over the concentration range were considered to be monomeric.

3,7-*endo,endo*-Di(benzylamino)bicyclo[3.3.0]octane (7). $NaBH_3CN$ (0.89 g, 14.2 mmol) was added to a stirred suspension of bicyclo[3.3.0]-3,7-octandione[28] (**6**) (1.50 g, 10.9 mmol), benzylamine (8.04 g, 75.0 mmol), benzylamine hydrochloride (10.8 g, 75.0 mmol), and methanol (20 mL). The reaction mixture was stirred under a drying tube for 72 h at room temperature, after which it was carefully acidified to pH 2, extracted with diethyl ether (3 × 100 mL) and basified to pH 12. The organic layer was extracted with DCM (3 × 100 mL), dried (Na_2SO_4), and the solvent was removed under reduced pressure. The remaining benzylamine was removed by distillation under reduced pressure. This material was purified by flash chromatography (0–15% MeOH/1% triethylamine/DCM). The *endo,endo* isomer eluted after the *endo,exo* isomer and constituted approximately 70% of the mixture. The *exo,exo* isomer was occasionally observed as a minor impurity in the *endo,exo* fractions. The desired product was recovered as a yellow oil (1.14 g, 3.56 mmol, 33%). TLC R_f 0.27 (3:2 acetone/hexanes +2% NH_4OH, ninhydrin visualization); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.30 (10H, m), 3.77 (4H, s), 3.09 (2H, m), 2.31 (2H, m), 2.21 (4H, m), 1.46 (2H, br s), 1.19 (4H, m); ^{13}C NMR (75 MHz, $CDCl_3$) 128.2, 127.9, 126.8, 62.1, 52.8, 41.2, 40.0; HRMS (EI) calcd for $C_{15}H_{21}N_2$ 229.1705, found 229.1709.

3,7-*endo,endo*-Diaminobicyclo[3.3.0]octane (8). After dissolving compound **7** (1.14 g, 3.56 mmol) in methanol (100 mL) the reaction flask was thoroughly purged with argon and then charged with $NH_4^+HCO_3^-$ (3.00 g, 47.6 mmol) and 10% Pd/C (1.10 g). The reaction mixture was heated under reflux for 2 h and then filtered through celite. The celite was washed with methanol (100 mL), and the solvent was removed under reduced pressure. The product was recovered as a colorless oil which solidified after storage at −20 °C (410 mg, 2.93 mmol, 82%), and was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.21 (2H, m), 2.34 (2H, m), 2.12 (4H, m), 1.07 (4H, m); ^{13}C NMR (75 MHz, $CDCl_3$) 56.6, 44.6, 40.8.

3,7-*endo,endo*-Di(N^2,N^3-bis(Boc)guanidino)bicyclo[3.3.0]-octane (9). Compound **8** (160 mg, 1.14 mmol) was added to a solution of *N,N'*-bis(Boc)-1-amidinopyrazole[20] (707 mg, 2.27 mmol) in THF (3 mL) and was stirred overnight. The solvent was removed under reduced pressure and the residue was dissolved in DCM (40 mL), and washed with 0.01 M HCl (20 mL). The aq layer was extracted with DCM (2 × 20 mL). The combined organic layers were dried (Na_2SO_4) and concentrated under reduced pressure. The crude product was purified by flash chromatography (4:1 hexanes:EtOAc), affording the product as a white solid (520 mg, 0.83 mmol, 73%). TLC R_f 0.41 (4:1 EtOAc: hexanes, visualized under UV); mp >300 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.48 (2H, s), 8.40 (2H, d, J = 8.1 Hz), 4.42 (2H, m), 2.30 (6H, m), 1.22 (4H, m); ^{13}C NMR (75 MHz, $CDCl_3$) 163.7, 155.6, 153.2, 82.9, 79.1, 53.7, 40.4, 39.3, 28.3, 28.0; LRMS (FAB) 625 (M+H). Anal. calcd for $C_{30}H_{52}N_6O_8$: C, 57.66; H, 8.39; N, 13.46; found: C, 57.38; H, 8.28; N, 13.39.

3,7-*endo,endo*-Diguanidinobicyclo[3.3.0]octane dihydrochloride (1). Compound **9** (400 mg, 0.64 mmol) was treated with trifluoroacetic acid (2.0 mL) and water (100 μL) at 0 °C for 1.5 h. The reaction was concentrated and dried in vacuo to give a quantitative yield of the TFA salt of 3,7-*endo,endo*-diguanidinobicyclo[3.3.0]octane. The TFA salt was converted to the chloride salt by overnight treatment with Dowex 1 X 8-50 anion exchange resin (chloride form) in 1:1 water: methanol (10 mL). After filtering and washing the resin, the methanol was removed under reduced pressure. The remaining aq solution was lyophilized to give the product as a white, hygroscopic solid (187 mg, 0.63 mmol, 98%). Mp 110–115 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 3.83 (2H, m), 2.47 (2H, m), 2.35 (4H, m), 1.32 (4H, m); ^{13}C NMR (75 MHz, CD_3OD)158.0, 56.3, 40.6, 40.3.

N^2,N^3-Bis(Boc)guanidinocyclopentane (10). A solution of cyclopentylamine (150 μL, 1.52 mmol) and *N,N'*-bis(Boc)-1-amidinopyrazole[20] (450 mg, 1.45 mmol) was stirred for 18 h in THF (2 mL). The solvent was removed under reduced pressure and replaced with DCM (100 mL). This solution was extracted with water (50 mL), dried (Na_2SO_4), and concentrated under reduced pressure to give a white solid. Purification by chromatography (silica gel, 5:1 hexanes:EtOAc) afforded the product as a white solid (365 mg, 1.12 mmol, 73%). Mp 145–147 °C (dec); ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.50 (1H, s), 8.34 (1H, d, J = 6.9 Hz), 4.44

(1H, m), 2.00 (2H, m), 1.67 (2H, m), 1.58 (2H, m), 1.49 (9H, s), 1.48 (9H, s), 1.44 (2H, m); ^{13}C NMR (75 MHz, $CDCl_3$) 163.9, 155.6, 153.3, 82.9, 79.1, 52.0, 33.1, 28.4, 28.1, 23.6; LRMS (FAB) 327 (M^+).

***N*-Cyclopentylguanidinium chloride (4).** A solution of **10** (365 mg, 1.11 mmol) in trifluoroacetic acid (3.0 mL) and water (150 μL) was stirred at 0 °C for 1.5 h. The mixture was concentrated under reduced pressure and dried in vacuo to give a quantitative yield of *N*-cyclopentylguanidinium trifluoroacetate. The TFA salt was converted to the chloride salt by treatment with Dowex 1 X 8-50 anion exchange resin in 1:1 water: methanol (10 mL). After the removal of the resin, the solvent was partially concentrated under reduced pressure and dried by lyophilization to afford the product as a colorless, glassy solid (157 mg, 0.96 mmol, 86%). ^{1}H NMR (300 MHz, CD_3OD) δ 7.47 (1H, br s), 3.84 (1H, m), 2.01 (2H, m), 1.73 (2H, m), 1.66 (2H, m), 1.54 (2H, m); ^{13}C NMR (75 MHz, CD_3OD) 158.1 (s), 54.3 (d), 33.6 (t), 24.4 (t); LRMS (EI) 127 (M^+); HRMS (EI) calcd for $C_6H_{13}N_3$ 127.1109, found 127.0999.

3,7-*endo*,*endo*-Bis(N^2-Boc-N^3-benzylguanidino)bicyclo-[3.3.0]octane (11). A solution of **8** (0.051 g, 0.36 mmol), *N*-Boc-*N'*-benzylthiourea[22] (0.191 g, 0.72 mmol) and TEA (0.10 mL, 0.72 mmol) was stirred in DCM (4 mL). To this solution was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI; 0.206 g, 1.07 mmol) and stirred for approximately 48 h. The reaction mixture was diluted with DCM (100 mL), then washed with water (2 (100 mL) and 0.1 mM HCl (2 × 100 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The resulting foam was purified by flash chromotography using an elution gradient of 50–100% EtOAc in hexanes. The pure fractions were pooled and the solvent removed under reduced pressure to afford the product as a clear foam (0.08 g, 36%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 (m, 10H), 4.46 (br s, 4H), 2.22 (m, 2H), 2.19 (m, 4H), 1.49 (s, 18H), 1.20 (m, 4H).

3,7-*endo*,*endo*-Di(benzylguanidino)bicyclo[3.3.0]octane dihydrochloride (5). A solution of **11** (70 mg, 0.115 mmol), trifluoroacetic acid (1.0 mL), and water (0.05 mL) was stirred for 4 h. The solvent was removed under reduced pressure and the sample was dissolved in 10% methanol in water. Dowex Cl^- anion exchange was added and the mixture stirred overnight. After filtration of the resin, methanol was removed under reduced pressure, and the resulting aq solution was lyophilized to give the product as a white solid in quantitative yield (0.055 g, 0.115 mmol). Mp 153 °C (dec); ^{1}H NMR (300 MHz, methanol-d_4) δ 7.33 (m, 10H), 4.42 (s, 4H), 3.88 (m, 2H), 2.37 (m, 2H), 2.33 (m, 4H), 1.33 (m, 4H). ^{13}C NMR (75 MHz, methanol-d_4) δ 157.1, 137.8, 129.9, 129.0, 128.3, 56.4, 45.9, 40.3 (2); LRMS (FAB) for $C_{22}H_{34}N_6$ 405 ($M+Na^+$).

3,7-Dicyano-3,7-bis(trimethylsilyloxy)-*cis*-bicyclo[3.3.0]-octane (12). This synthetic route was described by Camps et al.[29] but full experimental details were not provided. Trimethylsilyl cyanide (5.0 mL, 37.49 mmol) and zinc iodide (20 mg, 0.062 mmol) were added to a solution of 3,7-bicyclo[3.3.0]octanedione (**6**) (2.00 g, 14.47 mmol) in $CHCl_3$ (10 mL). After stirring for 48 h, DCM (50 mL) was added, and the solution was extracted with 0.5 M, pH 7.0 phosphate buffer (3 × 50 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure to a yellow oil and crystallized from Et_2O at −20 °C. Second and third crops were obtained by crystallization from hexanes at −20 °C. The product was obtained as a mixture of isomers (resulting from the direction of the addition of trimethylsilyl chloride) as flat crystals (4.53 g, 13.37 mmol, 92%). TLC R_f 0.86 (30% EtOAc in hexanes, visualized with phosphomolybdic acid); mp 59–62 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.86 (m, 2H), 2.35 (dd, J = 13.0 Hz, J = 8.6Hz, 4H), 2.04 (dd, J = 13.1 Hz, J = 5.7 Hz, 4H), 0.24 (s, 18H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 121.6, 75.8, 46.8, 38.5, 0.95; LRMS (EI) 336 (M^+).

***cis*-3,7-Dicyanobicyclo[3.3.0]octa-2,6-diene and *cis*-3,7-dicyanobicyclo[3.3.0]octa-2,7-diene (isomeric mixture) (13).** A solution of **12** (4.40 g, 12.99 mmol) and phosphorous oxychloride (12 mL, 128.74 mmol) was heated under reflux in pyridine (30 mL) for 3.5 h. A solution of 10:1 THF:water was cautiously added to the black, ice-cooled mixture to quench excess phosphorous oxychloride. After concentration under reduced pressure, the mixture was diluted with 0.1 M HCl (50 mL) and extracted with DCM (3 × 50 mL). The combined organic layers were washed with 0.1 M HCl (50 mL), dried ($MgSO_4$), filtered, and concentrated. After adding hexanes (10 mL) and storing overnight at −20 °C, the precipitate was recovered by filtration and dried under vacuum to afford the isomeric product mixture as a white precipitate (1.69 g, 10.85 mmol, 84%). Mp 119–122 °C; R_f 0.40 (30% EtOAc/hexanes, visualized using permanganate solution); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.53 (m, 4H), 6.44 (m, 6H), 4.11 (m, 2H), 3.70 (m, 6H), 3.26 (m, 2H), 3.05–2.88 (m, 10H), 2.58 (m, 3H), 2.50 (m, 5H), 2.44 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 149.9, 145.5, 115.7, 115.4, 113.4, 59.7, 48.5, 41.9, 38.4; HRMS (EI) calcd for $C_{10}H_8N_2$ 156.0681, found 156.0687.

3,7-*endo*, *endo*-Dicyanobicyclo[3.3.0]octane (14). A mixture of **13** (1.59 g, 10.23 mmol), 10% Pd/C, and glacial acetic acid (1 mL) was stirred in methanol (50 mL) for 18 h under hydrogen (1 atm). The mixture was filtered through celite and concentrated under reduced pressure to a white precipitate, which was redissolved in water (10 mL) and lyophilized to afford the product as a white powder (1.60 g, 9.99 mmol, 98%). Mp 59–62 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.79 (m, 2H), 2.61 (m, 2H), 2.33 (m, 4H), 1.82 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 121.6, 43.3, 37.5, 30.1. HRMS (EI) calcd for $C_9H_{11}N$ 133.0891, found 133.0898.

3,7-*endo*,*endo*-Di(aminomethyl)bicyclo[3.3.0]octane dihydrochloride (15). A solution of **14** (1.54 g, 9.61 mmol) in THF (10 mL) was added to a solution of 1.0 M diborane in THF (30 mL). After stirring at room temperature for 1 h, the solution was heated to reflux

for 5 h. The reaction was then cooled and quenched by the cautious addition of methanol. A saturated solution of HCl gas in methanol (50 mL) was added to the mixture resulting in the formation of a white precipitate. After stirring at room temperature for 20 min, the solution was heated to reflux for 2 h, and then stirred overnight at room temperature. The mixture was concentrated under reduced pressure and dried under high vacuum affording the product as a white precipitate (2.26 g, 9.38 mmol 97%). Mp >250 °C; ^{1}H NMR (300 MHz, D_2O) δ 2.69 (d, 7.1H, 4H), 2.25 (m, 2H), 1.97 (m, 2H), 1.83 (m, 4H), 0.69 (m, 4H); ^{13}C NMR (75 MHz, D_2O) δ 43.53, 43.17, 41.72, 37.29.

3,7-*endo,endo*-Di(N^2,N^3-bis(Boc)guanidinomethyl)bicyclo-[3.3.0]octane (16). A mixture of **15** (0.500 g, 2.07 mmol), TEA (0.610 mL, 4.38 mmol) and *N,N'*-bis(Boc)-1-amidinopyrazole[20] (1.36 g, 4.38 mmol) in 10 mL of dry DCM was stirred at room temperature for 48 h. The reaction mixture remained cloudy throughout the duration. The mixture was diluted with DCM (100 mL) and washed with 0.1 M HCl (2 × 100 mL) and water (2 × 100 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give a white powder. The powder was pre-adsorbed onto silica gel and purified by flash chromatography using 2:1 hexanes:EtOAc as the eluent. Pure fractions were pooled and the solvent removed under reduced pressure to afford the Boc-protected guanidine as a white powder (0.870 g, 1.33 mmol, 64%). Mp >250 °C; R_f 0.48 (2:1 hexane:ethyl acetate, visualized under UV); ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.49 (s, 2H), 8.36 (s, 2H), 3.40 (t, J = 6Hz, 4H), 2.48 (m, 2H), 2.17 (m, 2H), 2.08 (m, 4H), 1.50 (s, 18H), 1.49 (s, 18H), 0.96 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.3, 155.7, 153.1, 82.7, 78.9, 45.3, 43.7, 43.6, 38.3, 28.0, 27.8. Anal. calcd for $C_{32}H_{56}N_6O_8$: C, 58.87; H, 8.65 N, 12.87; O, 19.61; found: C, 59.12; H, 8.49; N, 12.84.

3,7-*endo,endo*-Di(guanidinomethyl)bicylo[3.3.0]octane dihydrochloride (2). The Boc-protected guanidine, **16**, (304 mg, 0.466 mmol) was stirred in HCl satd diethyl ether (50 mL). After 4 h, a white precipitate began to form. Periodic NMR analysis of reaction aliquots indicated that the reaction was complete after approximately 36 h. During the course of the reaction, three additional portions of HCl satd diethyl ether (20 mL) had been added. At the completion of the reaction the solvent was removed under reduced pressure to afford the pure product as a white powder (150 mg, 0.462 mmol, 99%). Mp 227–230 °C (dec); ^{1}H NMR (300 MHz, D_2O) δ 2.92 (d, 4H), 2.24 (br s, 2H), 1.97 (m, 2H), 1.79 (br t, 4H), 0.72 (m, 4H); ^{13}C NMR (75 MHz, D_2O) δ 156.5, 45.5, 43.3, 43.1, 37.6; LRMS (FAB) for $C_{12}H_{26}N_6Cl$ 289 ($M-Cl^-$).

3,7-*endo,endo*-Bis(dimethylamino)bicyclo[3.3.0]octane (17). A mixture of **6**[28] (0.675 g, 4.9 mmol), dimethylammonium hydrochloride (2.36 g, 29 mmol), and sodium cyanoborohydride (0.410 g, 6.5 mmol) was stirred in a 1.5 M solution of dimethylamine in methanol (20 mL) for 72 h. After concentration under reduced pressure, the residue was dissolved in water (50 mL) and the solution was acidified to pH <1 with concd HCl and extracted with diethyl ether (3 × 50 mL). The aq layer was basified to pH >10 with solid NaOH. The aq layer was then extracted with DCM (5 × 40 mL) and the combined DCM layers were dried over Na_2SO_4 and concentrated. The resulting oil was purified by flash chromotography and eluted DCM:methanol:TEA (5–15% methanol, 1% TEA) to give the pure *endo,endo* isomer (0.130 g, 14%). TLC R_f 0.22 (3:2 acetone/hexanes +2% NH_4OH, ninhydrin visualization); mp 61–63 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.34 (m, 2H), 2.31 (m, 2H), 2.18 (s, 12H), 2.08 (m, 4H), 1.18 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 70.7, 44.6, 40.1, 38.9; HRMS (EI) for $C_{12}H_{25}N_2$: 181.1705, found 181.1707.

3,7-*endo,endo*-Bis(trimethylammonium)bicyclo[3.3.0]-octane dihydrochloride (3). A solution of **17** (0.10 g, 0.51 mmol) and methyl iodide (0.128 ml, 2.0 mmol) in ethanol (5 mL) was stirred overnight. The product bis-ammonium salt precipitated from the solution and was recovered by filtration as a pale-yellow solid. The solid was dissolved in 5:1 methanol:water and Dowex chloride ion exchange resin was added. After 24 h with periodic swirling, the resin was removed and the filtrate lyophilized to afford the product as a white powder (0.10 g, 66%). Mp 201 °C (dec); ^{1}H NMR (300 MHz, methanol-d_4) δ 3.80 (m, 2H), 3.15 (s, 24H), 2.61 (m, 2H), 2.39 (m, 4H); 1.62 (m, 4H); ^{13}C NMR (75 MHz, methanol-d_4) δ 77.5, 52.5, 38.4, 33.3; LRMS (FAB) for $C_{14}H_{30}N_2Cl$ ($M+Cl^-$) 261.

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