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Artificial Ditopic Arg-Gly-Asp (RGD) Receptors

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Abstract: Covalent fusion of two artificial recognition motifs for arginine and aspartate resulted in a new class of ditopic RGD receptor molecules, **1**–**4**. The two binding sites for the oppositely charged amino acid residues are linked by either flexible linkers of different length (in **1**–**3**) or a rigid aromatic spacer (in **4**). These spacers are shown to be critical for the complexation efficiency of the artificial hosts. If the linkers are too flexible, as in **1**–**3**, an undesired intramolecular self-association occurs within the host and competes with, and thereby weakens, substrate binding. The rigid aromatic linker in **4** prevents any intramolecular self-association and hence efficient RGD binding is observed, even in buffered water (association constant of $K_a \approx 3000\,\text{M}^{-1}$). A further increase in hydrophobic contacts, as in host **16**, can complement the specific Coulomb attractions, thereby leading to an even more stable complex ($K_a = 5000\,\text{M}^{-1}$). The recognition events have been studied with NMR spectroscopy, UV/Vis spectroscopy, and fluorescence titrations.

Keywords: amino acids · molecular recognition · peptides · receptors · supramolecular chemistry

Introduction

The selective molecular recognition of a specific peptide sequence by an artificial receptor under competitive conditions is still a challenging task, despite the progress that has been achieved in recent years.[1] Both rational design[2] and combinatorial chemistry[3] have been successfully applied to find new peptide receptors, mainly for peptides of biological relevance, such as the L-Lys–D-Ala–D-Ala sequence, which plays an important role in bacterial cell-wall maturation.[4,5] Various parts of the amyloid β-peptide, which is responsible for plaque formation in Alzheimer's disease, have also been targeted by artificial receptors.[6] Some of them are even capable of interfering with plaque formation, at least in vitro. Another sequence of biological relevance is the RGD loop (arginine–glycine–aspartate), which is often found in proteins associated with cell–cell- and cell–matrix-adhesion processes.[7] Mutagenesis studies have shown that this RGD sequence is essential for the biological activity of these proteins as the loop is the primary binding site for their biological counterparts, for example, cell-surface-bound receptors such as the integrins.[8] The RGD–integrin interaction is responsible for a variety of biological events controlling the correct molecular function of proteins such as fibrinogen (blood coagulation), fibronectin (cell–matrix binding), osteopontin (bone formation), and certain growth factors (cell differentiation and angiogenesis).[9] Malfunction of these proteins can cause severe diseases. If, for example, fibrinogen, which controls the coagulation of blood platelets, does not function correctly, this can cause the formation of thrombi which are responsible for heart attacks or strokes. Human tumor cells deliberately alter the nature of integrins on their cell surface to facilitate their movement within the body, thereby attaching themselves to various RGD proteins on the intercellular matrix.[10] Furthermore, certain viruses, such as the yellow fever or the MKS virus, use the RGD sequence on their surface to attach themselves to surface-bound integrins on a host cell before infection can take place.[11] Hence, several RGD mimics, for example, based on conformationally restricted cyclopeptides, have been invented and studied for their medicinal effects.[12] We are interested in studying the supramolecular aspects of the opposite approach, that is, RGD recognition by artificial peptide receptors. This concept, which we introduced in 2002 in a pre-

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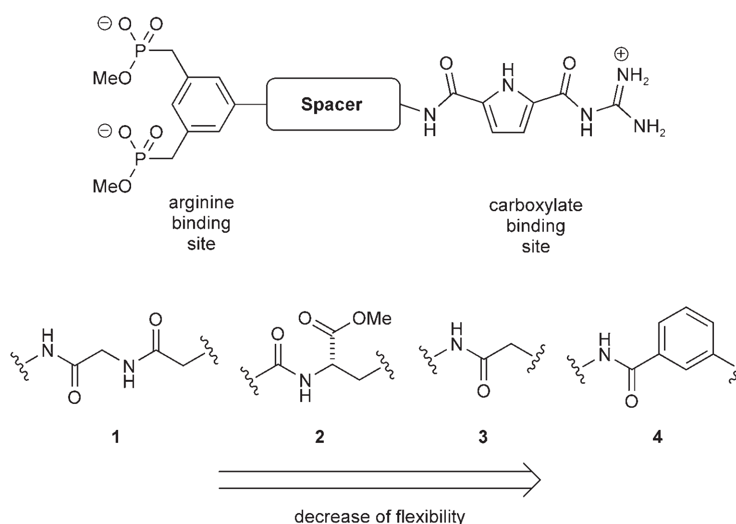
liminary work on this topic (see below), provides the opportunity to work with small model systems. The experimental and theoretical investigation of these systems might reveal critical factors governing this important biological recognition event. In this context, we report here a new class of artificial ditopic receptors, the best of which selectively binds the RGD tripeptide with an association constant (K_a) of up to $\approx 5000\,M^{-1}$, even in buffered water, whereas other tripeptides are not bound.

Results and Discussion

Design and synthesis of the receptors: In nature, molecular recognition of the RGD loop by proteins is primarily achieved through electrostatic interactions to the aspartate carboxylate anion and the arginine guanidinium cation. For example, a recent crystal structure shows, for the first time, an RGD peptide in its integrin binding site.[13] The only contacts between the protein and the RGD tripeptide are a chelate-like diaspartate guanidinium coordination and binding of the RGD aspartate by a manganese ion. Hence, for our biomimetic RGD receptor, we also chose to use electrostatic interactions to the two oppositely charged side chains of the RGD peptide. In preliminary work, we had already connected an arginine-selective trisphosphonate with a simple anilinium ion through a rigid benzylic spacer.[14] The resulting "primitive" RGD host indeed seemed to form a complex with RGD derivatives, for example, as indicated by complexation-induced shift changes in the NMR spectrum, even in polar solvents such as methanol or D_2O. However, no binding was detected in the presence of aqueous buffer, a result suggesting that either this host/guest system was not stable towards pH changes and proton transfer or that the electrostatic interactions used were not yet sufficient. This is not surprising as simple charge interactions in water, such as the ion pair formed between an ammonium cation and a carboxylate group, are rather weak due to the competitive solvation of both charges by the solvent. Furthermore, any salts present in the solution, such as buffers, further diminish the ion-pair stability relative to that in pure water.

We now decided to build a new generation of receptors, **1**–**4**, based on more efficient recognition motifs. Therefore, a *m*-xylylene bisphosphonate bisanion[15] was attached to a guanidiniocarbonyl pyrrole cation by a suitable spacer. The bisphosphonate moiety has been used as an arginine-binding site in solvents of medium polarity (DMSO, MeOH), while the guanidiniocarbonyl pyrrole cation[16] is highly efficient for binding carboxylate groups even in aqueous solvents.[17] We used both flexible peptidic bridges (in **1**–**3**) and a rigid aromatic spacer (in **4**) to connect the two recognition motifs (Scheme 1).

The general synthesis of these new receptors is based on



Scheme 1. The four new receptor molecules for the RGD sequence.

standard peptide synthesis as the *m*-xylylene bisphosphonate unit can be provided as a 5-aniline derivative[18] and the guanidiniocarbonyl pyrrole building block as a carboxylic acid.[19] Hence, both building blocks can be coupled to an amino acid or small peptide by using standard procedures. However, as both building blocks have a rather low reactivity (an aniline has low nucleophilicity, whereas pyrrole carboxylic acids have low electrophilicity), the choice of coupling reagents is restricted to the most reactive ones, such as *O*-(6-chlorobenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HCTU), *O*-(6-chlorobenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium tetrafluoroborate (TCTU), or benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP).

The synthesis of the two glycine receptors **1** and **3** is shown in Scheme 2. The aniline bisphosphonate **5** was treated with Z-Gly-OH (Z = benzyloxycarbonyl), which was previously activated with TCTU and 6-chloro-1-hydroxybenzotriazole (Cl-HOBt) in DMF. After removal of the Z group by using hydrogenolysis with H_2/Pd-C to give the free amine **6**, the Boc-protected (Boc = *tert*-butoxycarbonyl) guanidinocarbonyl pyrrole **8** was attached by PyBOP-mediated peptide coupling to give the protected receptor **9**. Consecutive acidic removal of the Boc-protecting group and cleavage of the bis(dimethyl)phosphonate to the bis(monomethyl)-phosphonate with LiBr provided receptor molecule **3** as the lithium phosphonate. The diglycine analogue **1** was synthesized with similar yields by the same procedure but with HCTU and Z-Gly-Gly-OH used in the first coupling step.

In these two RGD hosts, the pyrrole and the benzene moiety are separated by five (**3**, $n=1$) and eight (**1**, $n=2$) atoms, respectively. An intermediate spacer length of six

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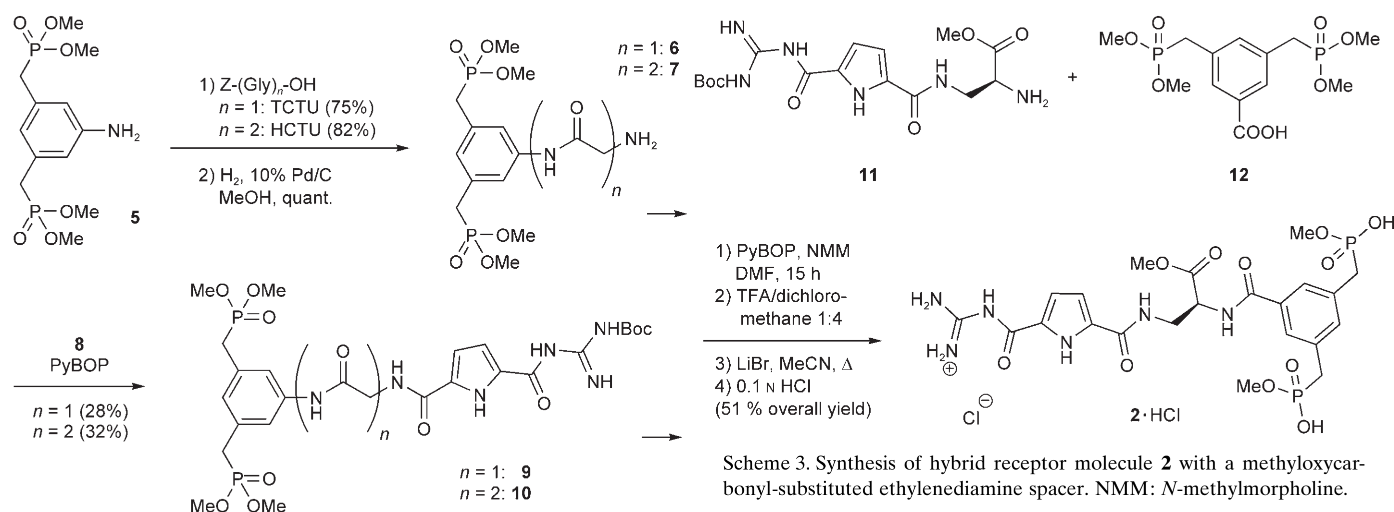
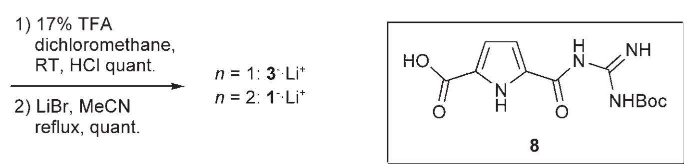


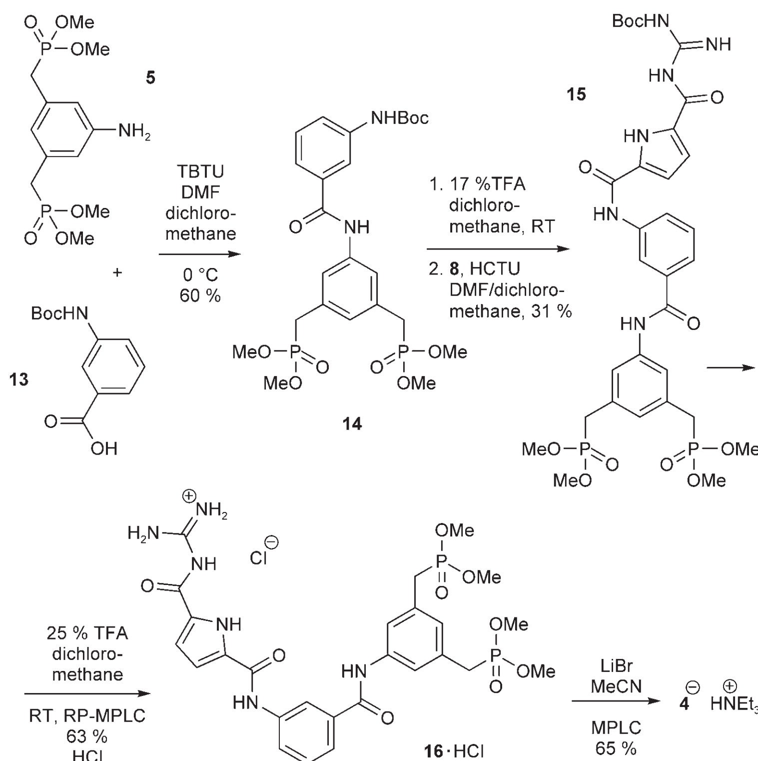
Scheme 3. Synthesis of hybrid receptor molecule **2** with a methyloxycarbonyl-substituted ethylenediamine spacer. NMM: *N*-methylmorpholine.



Scheme 2. Synthesis of hybrid receptor molecules **3** and **1** with a glycine or diglycine spacer. TFA: trifluoroacetic acid.

atoms with an additional potential H-bond acceptor site between both aromatic rings can be obtained with a methyloxycarbonyl-substituted ethylenediamine spacer, to give receptor **2**. The synthesis of **2** is shown in Scheme 3. The diamino propionic acid derived guanidinocarbonyl pyrrole building block **11** was synthesized according to a literature procedure reported earlier by one of us.[20] PyBOP was used again to effect amide formation with the benzoate bisphosphonate **12**. Boc removal and final methylphosphonate monodealkylation affords receptor **2** as the hydrochloride salt.

To complement these three hybrid molecules with flexible spacers of different lengths, we also synthesized **4**, in which the two binding motifs are linked with a rigid aromatic spacer. The Boc-protected aminobenzoic acid **13** was activated with TBTU and coupled with **5** (Scheme 4). The obtained **14** was deprotected with TFA and the resulting dark oil was purified by means of RP-MPLC to provide a white solid. This solid is highly hygroscopic and was, after twofold lyophilization with HCl to remove the TFA, therefore directly treated with **8** and HCTU to give the fully protected target compound **15**. After Boc deprotection, the product was purified again, this time with RP18-MPLC, to provide **16·TFA** in excellent purity. Monomethylphosphonate release was performed by exchanging the counterion of **16** with chloride and heating the bisphosphonate in acetonitrile with LiBr. After 10 days, the bis(monomethyl)phosphonate **4** was purified and isolated as the triethylammonium phosphonate by



Scheme 4. Synthesis of receptor molecule **4** with a rigid aromatic spacer.

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using semipreparative RP18-MPLC ($MeCN/H_2O$ + 0.1% NEt_3).

Binding studies: The newly synthesized ditopic guanidiniocarbonyl pyrrole bisphosphonate receptors **1**–**3** are soluble in water up to millimolar concentrations, but they are nearly completely insoluble in even polar organic media (DMSO, CH_3CN, acetone, methanol, and chloroform). Receptor **4** with the aromatic spacer is much less soluble in water, so completely dissolved solutions were only obtained at concentrations of <1 mM. The addition of up to 30% DMSO did not significantly improve the solubility. However, **4** is well soluble at concentrations in the μM range, as needed for UV spectroscopy and fluorescence titration studies.

Intermolecular self-association: As all four receptors contain, in principle, self-complementary binding sites, we first tested them for possible intermolecular self-association, which could interfere with peptide binding. For receptors **1**–**3**, NMR dilution studies were performed in water.[21] Receptor **3** with a single glycine spacer in between the two charged recognition motifs indeed showed concentration-dependent chemical-shift changes upon dilution. The dilution data can be interpreted as 1:1 dimer formation with an association constant of $K_{dimer} \approx 400\,M^{-1}$. Two molecules of **3** easily stack in an antiparallel fashion so that each cationic guanidiniocarbonyl pyrrole is interacting with a negatively charged bisphosphonate anion. The accompanying extensive π stacking interaction between the aromatic rings is especially powerful in aqueous solvents. A possible structure for such a dimer, as suggested by molecular mechanics calculations (Macromodel V. 7.2, Amber*, H_2O, 8000 steps),[22] is shown in Figure 1.

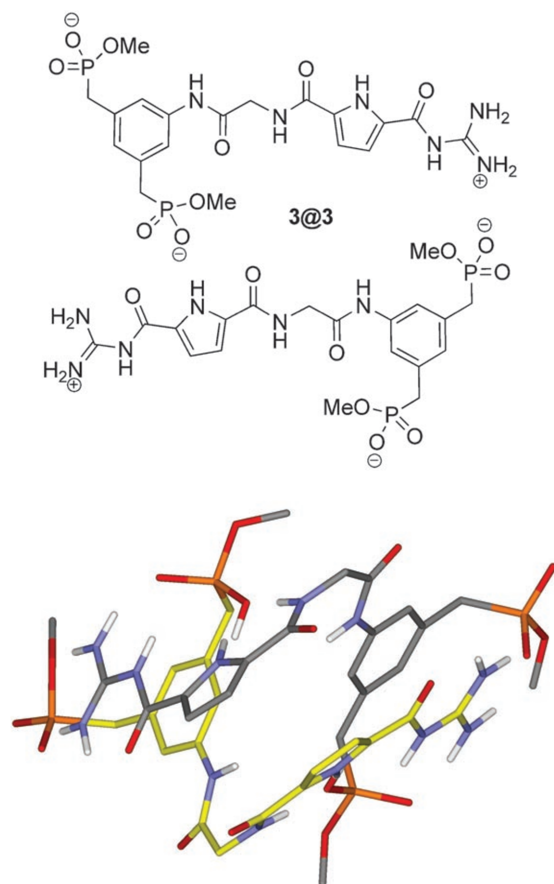

Figure 1. Possible formation of π-stacked dimers by intermolecular self-association of **3**.

Contrary to the results for **3**, no signs for any intermolecular self-complexation were found for receptors **1** and **2**. Receptor molecule **1** with the diglycine spacer was analyzed especially thoroughly in this respect. Upon dilution, monitored by NMR and UV/Vis spectroscopy as well as by microcalorimetry, **1** displays the normal behavior of a monomeric species with no indication of any aggregation, at least in the millimolar concentration range. As receptor **4** cannot be analyzed by NMR spectroscopy due to solubility problems, dilution experiments were performed in buffered water by using UV spectroscopy. In the μM concentration range, the absorbance of receptor **4** strictly followed Lambert–Beer behavior, thereby excluding any significant aggregation under these conditions.

Intramolecular self-association: A further aspect that has to be considered as it will interfere with substrate binding is the possibility of intramolecular self-association. In particluar, the more flexible receptors **1** and **2** might very well adopt looplike conformations in which the two oppositely charged binding motifs within one molecule can interact with each other. To probe the conformational flexibility of the receptors and hence the likelihood of intramolecular self-assembly, we performed molecular mechanics calculations. A Monte Carlo conformational search was conducted for compounds **1**–**4** in water (Macromodel V. 8.0, Amber* force field, GB/SA solvation model). The resulting energy-minimized conformations are shown in Figure 2. Even receptor **3** with the smallest spacer is capable of intramolecular self-complexation to some extent. However, only one, not both, of the two phosphonate anions can interact with the guanidinium cation and the overall structure is rather strained. The more flexible receptor **2** can adopt a conformation in which both phosphonates are in close proximity to the guanidinium cation, one forming a classical bidentate H-bonded ion pair while the other just allows undirected Coulomb interactions. For the most flexible molecule, **1** with the diglycine spacer, calculations suggest a full intramolecular association with H-bonded ion pairs between the guanidinium cation and both phosphonates, as well as cation–π interactions between the guanidinium cation and the benzene ring. The more rigid receptor **4**, however, can not adopt any conformation in which an intramolecular self-association can take place. Even when the two binding sites, the bisphosphonate and the guanidiniocarbonyl pyrrole cation, are preorientated for an intramolecular interaction in the starting conformation of the Monte Carlo search, the structure immediately relaxes to the extended conformation shown in Figure 2.

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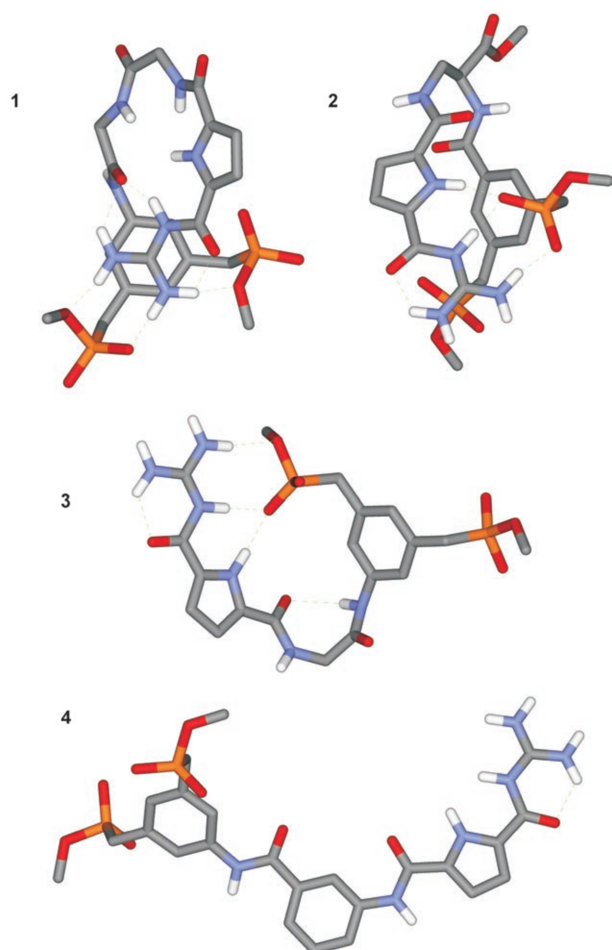

Figure 2. Calculated energy-minimized structures of **1**–**4** as obtained from a Monte Carlo conformational search (Macromodel Ver. 8.0, Amber*, GB/SA water solvation). Hydrogen bonds are displayed as dotted lines. Nonpolar hydrogen atoms are omitted for clarity.

These results are in good agreement with the experimentally observed dilution data. For receptor **3**, which can only self-associate intramolecularly in a rather strained conformation, the intermolecular formation of dimers (or higher aggregates), as indicated by the concentration-dependant chemical-shift changes in the NMR spectrum, can effectively compete at millimolar concentrations. For the two more flexible receptors **1** and **2**, intramolecular self-association can easily occur and is more likely (for entropic reasons) than intermolecular dimer formation. Hence, no changes upon dilution were observed. In receptor **4**, the rigid aromatic spacer effectively prevents any intramolecular interaction between the two binding motifs. In principle, intermolecular dimer formation is again possible but, at least in the μM concentration range used for the UV titrations, this is not observed. Therefore, receptor **4** seems to be the most promising candidate for strong substrate binding. For the more flexible receptors **1**–**3**, either intra- (in dilute solutions) or intermolecular self-association (at higher concentrations) is expected to interfere with substrate binding.

Binding properties of the flexible receptors 1–3: The binding properties of the three flexible receptors **1**–**3** were first investigated by using NMR titrations[21,23] in either phosphate buffer or bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris) buffer and in unbuffered water at pH ≈ 6.1 to ensure a completely protonated acylguanidinium moiety, while the phosphonate and the carboxylate units remain deprotonated. The substrates used were either the free H-RGD-OH tripeptide or, as a model for the internal RGD loop found in proteins, the N and C-protected tripeptide Ac-RGD-NH_2.

For receptor **3**, we were not able to detect any affinity to the RGD tripeptide in the NMR titration studies. Most likely, the dimer formation observed in the NMR dilution studies inhibits substrate binding. Furthermore, molecular mechanics calculations on a possible complex between **3** and the H-RGD-OH tripeptide suggest that monoglycine as a spacer is still too short to allow efficient substrate binding. The tripeptide is not able to get into tight contact with **3** in an extended conformation because the distances between the complementary recognition motifs in the receptor (the acylguanidine and arylic bisphosphonate) and the guest (the carboxylate and guanidine moieties) do not match (Figure 3).

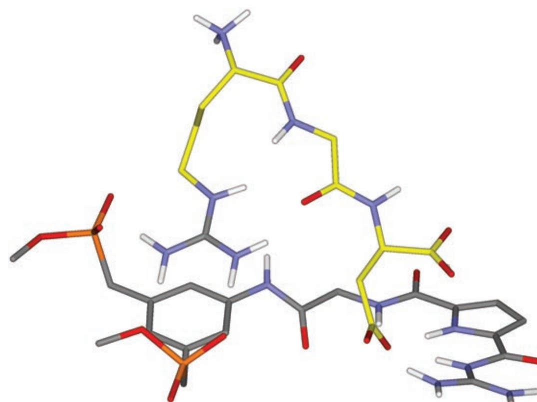

Figure 3. Calculated energy-minimized structure of a possible complex between host **3** and H-RGD-OH.

For receptors **1** and **2**, we observed chemical-shift changes during the NMR titration studies that indicate only weak association constants of $K_a \leq 10^2\,\text{M}^{-1}$ for the free H-RGD-OH tripeptide or the protected Ac-RGD-NH_2. The rather low affinity is most likely due to the efficient intramolecular self-association in both receptors, which competes with substrate binding. Before the RGD tripeptide can be bound, this intramolecular self-association has to be broken, which is energetically unfavorable. Hence, to increase the affinity of substrate binding, the receptor needs to be more rigid to prevent the competing intramolecular self-association. Furthermore, no binding of either tripeptide by any of the three receptors **1**–**3** was detected by UV titrations.[24]

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Binding properties of receptor 4: With the rigid receptor **4**, we performed UV titrations as its limited solubility did not allow for any NMR studies. As discussed above, receptor **4** does not intra- or intermolecularly self-associate under these conditions. First, we carried out UV titrations with the free H-RGD-OH tripeptide. Samples of receptor **4** were purified by preparative HPLC before use.[25] Titrations were then carried out by adding aliquots of a stock solution of the substrate to the receptor dissolved in a solution of Bis-Tris buffer ($c = 1–4 \times 10^{-3}$ M, pH 6) in water. The UV spectrum was recorded after each addition but no significant deviation of the absorbance from simple dilution changes was observed, a result showing that complex formation with the free RGD tripeptide is, at best, rather weak ($K_a < 1000\,\text{M}^{-1}$). However, it has been reported in several biological assay studies that the free RGD peptide often shows lower affinity than derivatives with an internal RGD sequence or a protected N terminus.[26] We therefore also studied the protected tripeptide Ac-RGD-NH_2 and investigated its affinity to **4**. In this case, a significant deviation of the absorbance from simple dilution was indeed observed and was indicative of complex formation. The corresponding binding isotherm at $\lambda = 307$ nm is shown in Figure 4. Job plots[27] and ESI-MS studies confirm the formation of a 1:1 complex. The nonlinear curve fitting of the titration data provided an association constant of $K_a = 2700\,\text{M}^{-1}$. Analysis of the whole spectral range from 260–340 nm by using SpecFit software gave essentially the same binding constant ($K_a = 2400\,\text{M}^{-1}$).

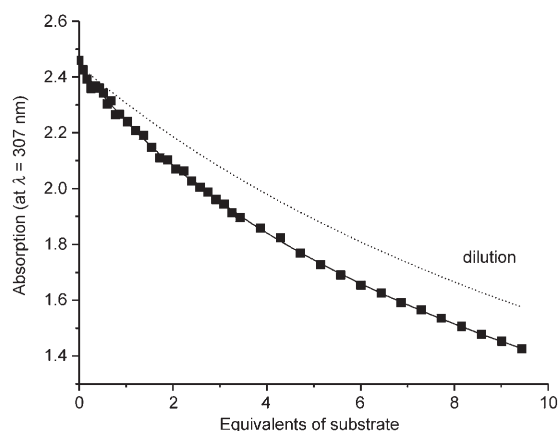

Figure 4. UV titration of host **4** ($c = 10^{-4}$ M) with Ac-RGD-NH_2 ($c = 1.74 \times 10^{-3}$ M) in buffered water (Bis-Tris buffer, $c = 2.4 \times 10^{-3}$ M, pH 6.1). The dotted line represents the expected change in UV absorption due to a simple dilution of the sample; the solid line represents the curve fitting for a 1:1 complex formation.

This binding constant was independently confirmed by fluorescence titration. Upon addition of Ac-RGD-NH_2 to a solution of **4**, a significant increase in the fluorescence intensity at $\lambda = 460$ nm was observed (Figure 5, synchronous excitation with $\Delta\lambda = 20$ nm). Analysis of the spectral changes by using either binding isotherms at 362 or 460 nm or the whole spectral range between 250–600 nm (with the SpecFit program) gave binding constants of $K_a = 2900$, 2300, and 3200 M^{-1}, respectively, results that are in good agreement with the value obtained from the UV titration (estimated error range for the spectroscopic titrations is ±25%).

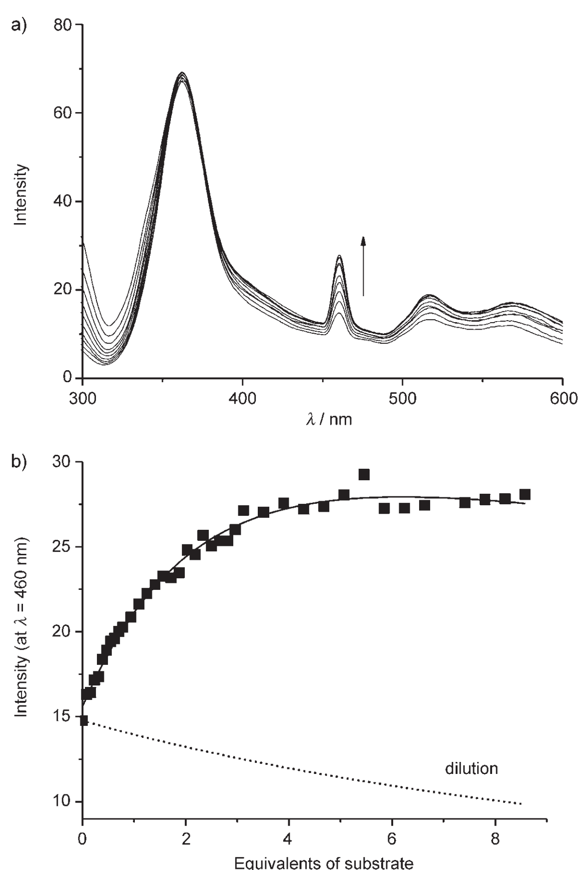

Figure 5. a) Fluorescence titration of receptor **4** with Ac-RGD-NH_2 in buffered water. b) Binding isotherm of the signal increase at $\lambda = 460$ nm.

Molecular modeling calculations show that receptor **4** can form a nearly perfect complex with Ac-RGD-NH_2 in terms of the distance between binding sites and the H-bond pattern (Figure 6). The guanidiniocarbonyl pyrrole cation binds the carboxylate moiety of the aspartate side chain in a similar way to that observed previously for simple amino acids,[16] while the bisphosphonate unit can interact with the arginine side chain, again as expected from earlier experiments. Furthermore, the glycine amide CO group can form an H-bond with the aninilium amide NH group in the receptor. Altogether, a network of ten inter- and two intramolecular hydrogen bonds is formed without introducing any strain into the whole complex.

As control experiments to probe the sequence specificity of receptor **4**, titrations were carried out with three other tripeptides as substrates: Ac-RGG-NH_2, Ac-GGD-NH_2, and Ac-GGG-NH_2. No complexation was detectable by UV titration for any of these three substrates, which indicates that the association constants were well below $K_a < 1000\,\text{M}^{-1}$. Hence, receptor **4** selectively binds the RGD tripeptide.

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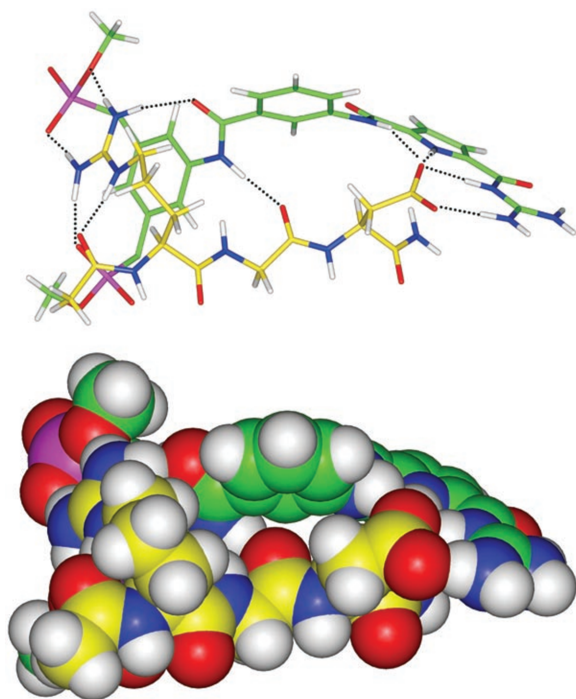

Figure 6. Calculated energy-minimized structure of the complex between receptor **4** and Ac-RGD-NH_2 (MacroModel Ver. 8.0, Amber*, GB/SA water solvation, Monte Carlo conformational search with 50000 steps). Nonpolar hydrogen atoms are omitted for clarity.

To get some information about the impact of the bisphosphonate motif on complex formation we also carried out titrations with the methyl ester precursor, **16**, as the host. Interestingly, we found a higher binding constant of $K_a = 4700\,\text{M}^{-1}$ for the complexation of Ac-RGD-NH_2 compared to that obtained with the bisphosphonate receptor **4**. This is counterintuitive at first glance but might reflect differences in the solvation behavior of both receptors (which are difficult to predict and to take into account when designing a receptor). Host **16** is only singly charged, whereas **4** is triply charged and therefore probably more solvated in water than **16**. Further work on this topic will be done in the future.

A recent theoretical ab initio analysis of related phosphonate hosts also investigated the importance of the electrostatic interactions of the bisphosphonate moiety for substrate binding. Gilson and co-workers suggested that, for the molecular recognition of peptides with an internal RGD sequence, Coulomb interactions between the phosphonate anion and the arginine guanidinium cation were of only limited importance and the complex stability and structure were often more dependent on hydrophobic contacts where possible.[28] The charged phosphonate groups, according to these calculations, seem to mostly interact with the solvent. If hydrophobic contacts with the aromatic ring of the bisphosphonate moiety are indeed an important factor for arginine complexation, this could explain why receptor **16**, which is overall less charged and more hydrophobic in this part of the molecule, binds the RGD substrate more efficiently than the free receptor **4**. Perhaps cation–π interactions between the arginine and the aromatic phosphonate moiety counterbalance the loss of electrostatic interactions in the complex of **16** and the RGD tripeptide, thereby giving rise to a similar affinity to that of host **4**.

Conclusion

In summary, the possibility of RGD recognition in water by artificial ditopic receptors with tailor-made binding sites for the two charged amino acid side chains (as suggested in earlier work by one of us[14]) has now been experimentally confirmed. The receptor design involves the covalent connection of two independent motifs for the guanidinium and carboxylate side chains of arginine and aspartate, respectively. The choice of an appropriate linker is essential for strong binding. Flexible linkers, as in **1**–**3**, induce intermolecular dimerization or intramolecular folding of the receptor and lead to weak RGD attraction. A rigid aromatic spacer efficiently prevents these unwanted complexation events and therefore receptor **4** shows strong affinity, even in buffered water. Most intriguingly, the transition to the neutral bisphosphonate tetraalkyl ester in host **16** additionally reinforces complex formation, most likely by facilitating desolvation of the singly charged receptor molecule prior to the formation of hydrophobic contacts. In the future, we will replace the bisphosphonate unit by even more powerful arginine-binding motifs discovered very recently and we will systematically exploit the use of hydrophobic contacts and π-stacking interactions.

Experimental Section

General remarks: Solvents were dried and distilled under argon before use. All other reagents were used as commercially obtained. ^{1}H and ^{13}C NMR shifts are reported relative to the signals of the deuterated solvents. Peak assignments are based on either DEPT analysis, 2D NMR studies, and/or comparison with literature data. IR spectra were recorded by using samples prepared as tablets (KBr). Melting points are uncorrected. All compound numbers in the Experimental Section refer to the overall uncharged compounds. If the compounds are obtained in other forms (for example, as a salt), this is explicitly mentioned.

General procedure for formation of peptide bonds in solution (GP01): The carboxylic acid (1.0 equiv) was suspended in CH_2Cl_2. DMF was added until complete dissolution was achieved. The solution was cooled to 0 °C and HOBt (2.5 equiv), coupling reagent (1.0 equiv; HCTU, TCTU, or TBTU), and *N*,*N*-diisopropylethylamine (DIEA; (3.0 equiv; 4.0 equiv if the amine is used as the hydrochloride) were added and the mixture was stirred for 15 min at 0 °C. The amine component (1.0 equiv) was then added and the solution was stirred for 1–3 h at room temperature. The progress of the reaction was monitored by TLC. After complete conversion, the reaction was quenched by adding water. If DMF was used as the solvent, CH_2Cl_2 was added to allow separation of the organic and inorganic phases for the aqueous workup. The organic phase was washed three times with 1 M $NaHSO_4$, three times with 1 M $NaHCO_3$ (pH 10), and three times with brine. After drying of the solution with Na_2SO_4, the solvent was removed in vacuo and the product was dried under high vacuum. Further purification can be performed with column chromatography. To increase the yield (due to the partial solubility of the

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phosphonic acid building blocks), the aqueous workup can be omitted. In these cases, column chromatography was performed directly after removal of the solvent.

General procedure for cleavage of the benzyloxycarbonyl (Z) protecting group (GP02): The protected amine was furnished with 5% (based on the mass of the protected amine) of 10% palladium on charcoal and suspended in methanol. This suspension was stirred under a hydrogen atmosphere at room temperature for 24 h. The catalyst was then filtered off and washed with abundant amounts of methanol. The solutions were combined and the solvent was removed in vacuo to obtain the deprotected amine without further purification.

General procedure for cleavage of the *tert*-butoxycarbonyl (Boc) protecting group (GP03): The protected compound was dissolved in CH_2Cl_2/TFA (5:1) and the mixture was stirred at room temperature for 3 h. After addition of abundant amounts of toluene, the solvent was removed in vacuo. The residue was suspended in toluene and the solvent was removed in vacuo. This process was repeated a second time. After drying under high vacuum, the deprotected compound was obtained as the trifluoroacetate salt.

General procedure for cleavage of bis(dimethyl)phosphonates to bis(monomethyl)phosphonates (GP04): The bis(dimethyl)phosphonate was dissolved in acetonitrile and dried lithium bromide (2.4 equiv) was added. The solution was heated to reflux for a minimum of 16 h. The formed bis(monomethyl)phosphonate precipitates as a white solid. After the mixture had cooled to room temperature, the solvent was removed by centrifugation. The white solid was then treated with Et_2O and reisolated by centrifugation three times. After drying under high vacuum, the bis(monomethyl)phosphonate was obtained as a white solid. For less reactive bis(dimethyl)phosphonates, elongated reaction times and up to 10 equivalents of lithium bromide were needed before complete conversion was detected by ^{31}P NMR spectroscopy.

Synthesis of Z-6: Z-Gly-OH (142 mg, 680 µmol), Cl-HOBt (283 mg, 1.68 mmol), and DIEA (350 µL, 2.04 mmol) were dissolved in DMF (10 mL). After addition of TCTU (240 mg, 680 µmol), the solution was stirred for 10 min at room temperature. The amine **5** (270 mg, 800 µmol) was added and the solution was stirred for 2 h at room temperature. Water (30 mL) was added and the aqueous phase was extracted with $CHCl_3$ (4×40 mL). The combined organic phases were washed with brine and dried with $NaSO_4$. After removal of the solvent in vacuo, the crude product was purified by column chromatography. Compound Z-**6** (268 mg, 510 µmol, 75%) was obtained as a white solid. R_f=0.20 (silica gel, ethyl acetate/ethanol 2:1); ^{1}H NMR (200 MHz, $CDCl_3$): δ=3.10 (d, $^2J_{H,P}$=22.2 Hz, 4H; PCH_2Ar), 3.66 (d, $^3J_{H,P}$=10.5 Hz, 12H; PO(OCH_3)$_2$), 3.99 (s, 2H; amido-CH_2), 5.12 (s, 2H; CO_2CH_2Ph), 6.94 (s, 1H; Ar-C*H*), 7.30–7.35 (m, 5H; Ar-C*H*), 7.42 (s, 2H; Ar-C*H*), 8.85 ppm (s, 1H; N*H*); ^{31}P NMR (81 MHz, $CDCl_3$): δ=29.1 ppm; MS (ESI): *m/z*: 551 [*M*+Na]$^+$; HRMS (ESI): calcd for $C_{22}H_{30}N_2NaO_9P_2^+$: 551.1319; found: 551.1289.

Deprotection of Z-6: Pd/C (approximately 50 mg) was added to a suspension of Z-**6** (87.1 mg, 165 µmol) in methanol and the suspension was stirred under a hydrogen atmosphere at room temperature for 24 h. The catalyst was filtered off and washed several times with methanol. The solutions were combined and the solvent was removed in vacuo to obtain the deprotected amine **6**, which was used directly without any further purification.

Synthesis of 9: The pyrrole carboxylic acid **8** (49 mg, 165 µmol) was dissolved in DMF, and NMM (55 µL, 495 µmol) and PyBOP (95 mg, 182 µmol) were subsequently added. The deprotected amine **6** (65 mg, 165 µmol) was suspended in CH_2Cl_2 (a few mL) and added to the solution of the activated acid. Upon stirring of the resulting mixture for 40 h, the suspension turned into a clear solution. Brine was added and the aqueous phase was extracted three times with $CHCl_3$. The organic phases were combined and the solvent was removed in vacuo. The crude product was purified by means of column chromatography. Compound **9** (31 mg, 46 µmol, 28%) was obtained as a white solid. R_f=0.12 (silica gel, ethyl acetate/ethanol 2:1); ^{1}H NMR (200 MHz, $CDCl_3$): δ=1.45 (s, 9H; C(CH_3)$_3$), 2.99 (d, $^2J_{H,P}$=20.3 Hz, 4H; PCH_2Ar), 3.59 (d, $^3J_{H,P}$=10.5 Hz, 12H; PO(OCH_3)$_2$), 4.09 (brs, 2H; amido-CH_2), 6.75 (brs, 1H; Py-C*H*), 6.81 (brs, 2H; Py-C*H*/Ar-C*H*), 7.21 (s, 1H; N*H*), 7.34 (s, 2H; Ar-C*H*), 7.61 (brs, 1H; N*H*), 7.68 (brs, 1H; N*H*), 8.42 (s, 1H; N*H*), 9.72 ppm (s, 1H; N*H*); ^{31}P NMR (81 MHz, $CDCl_3$): δ=29.2 ppm; MS (ESI): *m/z*: 711 [*M*+K]$^+$, 695 [*M*+Na]$^+$, 673 [*M*+H]$^+$.

Synthesis of 17 (Boc-deprotected 9): The Boc group in **9** was removed by following the general procedure GP03 described above. The resulting trifluoroacetate was dissolved in 0.1 N HCl and lyophilized. **17**·HCl was obtained quantitatively. ^{1}H NMR (400 MHz, D_2O): δ=3.29 (d, $^2J_{H,P}$=21.8 Hz, 4H; PCH_2Ar), 3.72 (d, $^3J_{H,P}$=10.9 Hz, 12H; PO(OCH_3)$_2$), 4.20 (brs, 2H; amido-CH_2), 6.88 (d, $^3J_{H,H}$=4.2 Hz, 1H; Py-C*H*), 7.01 (s, 1H; Ar-C*H*), 7.02 (d, $^3J_{H,H}$=4.2 Hz, 1H; Py-C*H*), 7.32 (s, 2H; Ar-C*H*), 6.88–7.61 ppm (m, 3H; N*H*); ^{31}P NMR (81 MHz, $CDCl_3$): δ=35.3 ppm; MS (ESI): *m/z*: 595 [*M*+Na]$^+$, 573 [*M*+H]$^+$.

Synthesis of 3: The bis(dimethyl)phosphonate **17**·HCl was cleaved to form the bis(monomethyl)phosphonate by following general procedure GP04, and Li^+·[**3**−H]$^-$ was obtained quantitatively as a white solid. M.p. >300 °C; ^{1}H NMR (200 MHz, D_2O): δ=2.97 (d, $^2J_{H,P}$=20.5 Hz, 4H; PCH_2Ar), 3.55 (d, $^3J_{H,P}$=10.5 Hz, 6H; PO(OCH_3)O$^-$), 4.12 (s, 2H; amido-CH_2), 6.85 (d, $^3J_{H,H}$=4.3 Hz, 1H; Py-C*H*), 6.93 (s, 1H; Ar-C*H*), 7.02 (d, $^3J_{H,H}$=4.3 Hz, 1H; Py-C*H*), 7.15 ppm (s, 2H; Ar-C*H*); ^{31}P NMR (81 MHz, D_2O): δ=27.0 ppm; MS (ESI): *m/z*: 567 [*M*+Na]$^+$, 545 [*M*+H]$^+$; HRMS (ESI): calcd for $C_{19}H_{26}N_6NaO_9P_2^+$: 567.1129; found: 567.1119. The ^{13}C NMR spectrum could not be obtained due to the limited solubility of **3**.

Synthesis of Z-7: Z-Gly-Gly-OH and **5** were coupled according to general procedure GP01 by using HCTU as the coupling reagent. Z-**7** was obtained as a white solid in 82% yield. M.p. 135 °C; R_f=0.33 (silica gel, ethyl acetate/methanol 2:1); ^{1}H NMR (200 MHz, $CDCl_3$): δ=3.06 (d, $^2J_{H,P}$=21.7 Hz, 4H; PCH_2Ar), 3.67 (d, $^3J_{H,P}$=10.8 Hz, 12H; PO(OCH_3)$_2$), 3.94 (d, $^3J_{H,H}$=3.0 Hz, 2H; amido-CH_2), 4.08 (d, $^3J_{H,H}$=4.0 Hz, 2H; amido-CH_2), 5.11 (s, 2H; CO_2CH_2Ph), 6.71 (brs, 1H; N*H*), 6.87 (s, 1H; Ar-C*H*), 7.29–7.34 (m, 5H; Ar-C*H*), 7.47 (s, 2H; Ar-C*H*), 9.37 ppm (s, 1H; N*H*); ^{13}C NMR (50 MHz, $CDCl_3$): δ=32.1 (d, $^1J_{C,P}$=91.0 Hz), 43.2, 44.3, 53.0, 67.0, 119.7, 126.4, 128.1, 128.2, 131.8, 136.2, 138.7, 157.2, 167.6, 170.6, 174.9 ppm; ^{31}P NMR (81 MHz, $CDCl_3$): δ=29.4 ppm; MS (ESI): *m/z*: 624 [*M*+K]$^+$, 608 [*M*+Na]$^+$; MS (field desorption): *m/z*: 608 [*M*+Na]$^+$, 585 [*M*]$^+$.

Synthesis of 7: Compound Z-**7** was deprotected by using general procedure GP02, to provide **7** in quantitative yield. ^{1}H NMR (300 MHz, [D$_3$]MeOD): δ=1.94 (brs, 2H; NH_2), 3.22 (d, $^2J_{H,P}$=21.6 Hz, 4H; PCH_2Ar), 3.27 (s, 2H; CH_2NH$_2$), 3.67 (d, $^3J_{H,P}$=10.6 Hz, 12H; PO(OCH_3)$_2$), 4.05 (s, 2H; amido-CH_2), 6.98 (s, 1H; Ar-C*H*), 7.22 (s, 1H; N*H*), 7.44 (s, 2H; Ar-C*H*), 7.65 ppm (s, 1H; N*H*); ^{13}C NMR (50 MHz, [D$_4$]MeOD): δ=32.5 (d, $^1J_{C,P}$=91.0 Hz), 41.6, 44.0, 53.8 (d, $^2J_{C,P}$=4.5 Hz), 121.1, 128.2, 133.6 (d, $^2J_{C,P}$=4.9 Hz), 139.9, 168.0, 169.4 ppm; ^{31}P NMR (81 MHz, [D$_4$]MeOD): δ=34.2 ppm; MS (ESI): *m/z*: 474 [*M*+Na]$^+$, 452 [*M*+H]$^+$.

Synthesis of 10: The pyrrole carboxylic acid **8** (49 mg, 165 µmol) was dissolved in DMF (a few mL). NMM (55 µL, 495 µmol) and PyBOP (95 mg, 182 µmol) were subsequently added. After the solution had been stirred for 30 min at room temperature, a suspension of **7** (75 mg, 166 µmol) in CH_2Cl_2 was added and the resulting mixture was stirred for 40 h at room temperature while it turned into a clear solution. Brine was added and the aqueous phase was extracted three times with $CHCl_3$. The organic phases were combined and the solvent was removed in vacuo. The resulting crude product was purified by means of column chromatography to provide **10** (38 mg, 52.1 µmol, 32%) as a white solid. R_f=0.17 (ethyl acetate/ethanol 2:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.48 (s, 9H; C(CH_3)$_3$), 3.06 (d, $^2J_{H,P}$=21.7 Hz, 4H; PCH_2Ar), 3.64 (d, $^3J_{H,P}$=10.7 Hz, 12H; PO(OCH_3)$_2$), 4.02 (s, 2H; amido-CH_2), 6.73 (s, 1H; Py-C*H*), 6.83 (s, 2H; Ar-C*H*/Py-C*H*), 7.16 (s, 1H; N*H*), 7.46 (s, 2H; Ar-C*H*), 7.60–7.71 (m, 1H; N*H*), 8.21 (s, 1H; N*H*), 8.55 (s, 1H; N*H*), 9.42 ppm (s, 1H; N*H*); ^{13}C NMR (50 MHz, $CDCl_4$): δ=27.9, 31.9 (d, $^1J_{C,P}$=90.2 Hz), 46.1, 46.2, 53.0 (d, $^2J_{C,P}$=4.5 Hz), 82.5, 113.4, 114.4, 118.4, 119.8, 126.0, 128.5, 131.9, 138.6, 158.8, 161.9, 164.1, 168.1, 170.9 ppm; ^{31}P NMR (81 MHz, $CDCl_3$): δ=29.6 ppm; MS (ESI): *m/z*: 768 [*M*+K]$^+$, 752 [*M*+Na]$^+$, 730 [*M*+H]$^+$, 668 [*M*−Boc+K]$^+$, 652 [*M*−Boc+Na]$^+$, 630 [*M*−Boc+H]$^+$.

Synthesis of 18 (Boc-deprotected 10): The Boc group in **10** was removed by following the general procedure GP03. The resulting trifluoroacetate

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was dissolved in 0.1 N HCl and lyophilized. **18** was obtained quantitatively as the chloride salt. ^{1}H NMR (200 MHz, D_2O): $\delta = 3.27$ (d, $^2J_{H,P} = 21.7$ Hz, 4H; PCH_2Ar), 3.65 (d, $^3J_{H,P} = 11.0$ Hz, 12H; PO(OCH_3)$_2$), 4.04 (s, 2H; amido-CH_2), 4.10 (s, 2H; amido-CH_2), 6.83 (d, $^3J_{H,H} = 4.3$ Hz, 1H; Py-C*H*), 7.01 (s, 1H; Ar-C*H*), 7.02 (d, $^3J_{H,H} = 4.2$ Hz, 1H; Py-C*H*), 7.27 (s, 2H; Ar-C*H*), 7.50–7.83 ppm (m, 2H; N*H*); ^{31}P NMR (81 MHz, $CDCl_3$): $\delta = 35.1$ ppm; MS (ESI): m/z: 652 [M+Na]$^+$, 630 [M+H]$^+$; HRMS (ESI): calcd for $C_{23}H_{34}N_7O_{10}P_2^+$: 630.1842; found: 630.1854.

Synthesis of 1: The bis(dimethyl)phosphonate in **18**·HCl was cleaved to form the bis(monomethyl)phosphonate by following general procedure GP04 and Li$^+$·[**1**−H]$^-$ was obtained quantitatively as a white solid. M.p. > 245 °C (decomp); ^{1}H NMR (400 MHz, D_2O): $\delta = 3.06$ (d, $^2J_{H,P} = 20.4$ Hz, 4H; PCH_2Ar), 3.58 (d, $^3J_{H,P} = 10.2$ Hz, 6H; PO(OCH_3)O$^-$), 4.13 (s, 2H; amido-CH_2), 4.20 (s, 2H; amido-CH_2), 6.96 (d, $^3J_{H,H} = 4.2$ Hz, 1H; Py-C*H*), 7.06 (s, 1H; Ar-C*H*), 7.09 (d, $^3J_{H,H} = 3.8$ Hz, 1H; Py-C*H*), 7.27 ppm (s, 2H; Ar-C*H*); ^{31}P NMR (81 MHz, D_2O): $\delta = 26.8$ ppm; MS (ESI): m/z: 630 [M−H+Li+Na]$^+$, 624 [M+Na]$^+$, 608 [M+Li]$^+$, 602 [M+H]$^+$; HRMS (ESI): calcd for $C_{21}H_{29}LiN_7O_{10}P_2^+$: 608.1606; found: 608.1601. The ^{13}C NMR spectrum could not be obtained due to the limited solubility of **1**.

Synthesis of 19 (Boc-protected bis(dimethyl)phosphonate derivative of 2): Amine **11** (20 mg, 50.5 µmol), carboxylic acid **12** (18.5 mg, 50.5 µmol), PyBOP (26.3 mg, 50.5 µmol), and NMM (17 µL, 152 µmol) were dissolved in CH_2Cl_2 (5 mL) and stirred for 15 h at room temperature. The solvent was removed in vacuo and the residue was purified by means of column chromatography to provide **19** (19 mg, 25.5 µmol, 51 %) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.50$ (s, 9H; C(CH_3)$_3$), 3.23 (d, $^2J_{H,P} = 21.7$ Hz, 4H; PCH_2Ar), 3.68 (d, $^3J_{H,P} = 10.8$ Hz, 6H; PO(OCH_3)$_2$), 3.70 (d, $^3J_{H,P} = 10.8$ Hz, 6H; PO(OCH_3)$_2$), 3.76 (s, 3H; CO$_2$CH_3), 3.88–4.10 (m, 2H; NCH_2), 4.67–4.74 (m, 1H; amido-C*H*), 6.78 (d, $^3J_{H,H} = 4.0$ Hz, 1H; Py-C*H*), 6.95 (d, $^4J_{H,H} = 2.7$ Hz, 1H; Ar-C*H*), 7.34 (s, 1H; N*H*), 7.71 (d, $^4J_{H,H} = 1.7$ Hz, 2H; Ar-C*H*), 8.12–8.18 (m, 1H; N*H*), 8.57 (d, $^3J_{H,H} = 5.7$ Hz, 1H; Py-C*H*), 10.96 ppm (brs, 1H; N*H*); ^{31}P NMR (81 MHz, $CDCl_3$): $\delta = 32.5$ ppm; MS (ESI): m/z: 767 [M+Na]$^+$.

Synthesis of 20 (bis(dimethyl)phosphonate derivative of 2): The Boc-protected compound **19** (19 mg, 24.5 µmol) was dissolved in CH_2Cl_2/TFA (4:1; 5 mL) and stirred for 3 h at room temperature. Toluene (10 mL) was added and the solvent was removed in vacuo; this procedure was repeated twice. Compound **20**·TFA (quantitative) was obtained as a white solid. The product was dissolved in 0.1 N HCl and the solvent was removed in vacuo to provide **20** as the chloride salt. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.23$ (d, $^2J_{H,P} = 21.7$ Hz, 4H; PCH_2Ar), 3.62–3.76 (m, 12H; PO(OCH_3)$_2$), 3.90 (s, 3H; CO$_2$CH_3), 4.48–4.49 (m, 2H; NCH_2), 4.67–4.75 (m, 1H; amido-C*H*), 6.78 (brs, 1H; Py-C*H*), 7.32–8.05 ppm (m, 6H; Ar-C*H*/Py-C*H*/N*H*); ^{31}P NMR (81 MHz, $CDCl_3$): $\delta = 32.4$ ppm; MS (ESI): m/z: 667 [M+Na]$^+$, 645 [M+H]$^+$; HRMS (ESI): calcd for $C_{24}H_{34}N_6NaO_{11}P_2^+$: 667.1653; found: 667.1664.

Synthesis of 2: Bis(dimethyl)phosphonate **20**·HCl (19 mg, 25 µmol) and LiBr (11 mg, 127 µmol) were suspended in acetonitrile (10 mL) and heated to reflux for 12 h. The precipitated product was separated from the solution by centrifugation. The crude product was treated with Et_2O and reisolated by centrifugation; this step was repeated three times. The product was dissolved in 1 N HCl (a few mL) and the solvent was removed in vacuo. Compound **2**·HCl (16 mg, 24.5, 98 %) was obtained as a white solid: ^{1}H NMR (300 MHz, [D$_4$]MeOD): $\delta = 2.95$ (d, $^2J_{H,P} = 20.5$ Hz, 4H; PCH_2Ar), 3.41 (d, $^3J_{H,P} = 10.5$ Hz, 6H; PO(OCH_3)OH), 3.63 (s, 3H; CO$_2$CH_3), 3.72–3.80 (m, 2H; NCH_2), 4.56 (dd, $^3J_{H,H} = 4.7$, $^3J_{H,H} = 6.2$ Hz, 1H; amido-C*H*), 6.63 (d, $^3J_{H,H} = 4.3$ Hz, 1H; Py-C*H*), 6.97 (d, $^3J_{H,H} = 4.3$ Hz, 1H; Py-C*H*), 7.25 (s, 1H; Ar-C*H*), 7.56 ppm (s, 2H; Ar-C*H*); ^{31}P NMR (81 MHz, [D$_4$]MeOD): $\delta = 28.5$ ppm; MS (negative ESI): m/z: 615 [M−H]$^-$; HRMS (ESI): calcd for $C_{22}H_{29}N_6O_{11}P_2^-$: 615.1375; found: 615.1351. The ^{13}C NMR spectrum could not be obtained due to the limited solubility of **2**.

Synthesis of 14: The carboxylic acid **13** (100 mg, 421 µmol) was coupled to the amine **5** according to the general procedure GP01 by using TBTU as the coupling reagent and DMF/CH_2Cl_2 (1:1) as the solvent, to provide **14** (140 mg, 252 µmol, 60 %). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.53$ (s, 9H; C(CH_3)$_3$), 3.15 (d, $^2J_{H,P} = 22.0$ Hz, 4H; PCH_2Ar), 3.69 (d, $^3J_{H,P} = 10.7$ Hz, 12H; PO(OCH_3)$_2$), 6.80 (s, 1H; Ar-C*H*), 7.00 (s, 1H; Ar-C*H*), 7.34–7.57 (m, 5H; Ar-C*H*), 7.93 (s, 1H; N*H*), 8.26 ppm (s, 1H; N*H*); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 28.4$, 32.7 (d, $^1J_{C,P} = 91.7$ Hz), 53.1 (d, $^2J_{C,P} = 3.7$ Hz), 81.2, 117.0, 120.4, 121.7, 127.1, 129.5, 131.5, 132.5, 137.0, 139.0, 153.0, 159.1 ppm; ^{31}P NMR (81 MHz, $CDCl_3$): $\delta = 29.0$ ppm; MS (ESI): m/z: 579 [M+Na]$^+$.

Synthesis of 15: The Boc-protected amine **14** (173 mg, 299 mmol) was deprotected according to general procedure GP03, purified by RP18 column chromatography (H_2O/MeOH (1:0→0:1+0.1 % TFA), and lyophilized with 0.1 N HCl. The resulting white solid was suspended with **8** (135 mg, 456 µmol) and HCTU (186 mg, 450 µmol) under argon in CH_2Cl_2 (10 mL) and DMF was added until a solution was achieved. NMM (98.4 µL, 895 µmol) was added and the solution was heated to reflux for 24 h. The resulting brown solution was diluted with ethyl acetate (100 mL) and washed with sat. $NaHCO_3$ (3×100 mL) and brine (2×100 mL). The organic phase was dried with Na_2SO_4 and the solvent was removed in vacuo. The crude product was purified by means of column chromatography to provide **15** (68 mg, 92.5 µmol, 31 %) as a white solid. $R_f = 0.23$ (silica gel (deact. with NEt_3), ethyl acetate/ethanol 9:1+0.1 % NEt_3); ^{1}H NMR (400 MHz, [D$_4$]MeOD): $\delta = 1.54$ (s, 9H; C(CH_3)$_3$), 3.28 (d, 4H, $^2J_{P,H} = 21.5$ Hz; PCH_2Ar), 3.73 (d, 12H, $^3J_{P,H} = 10.9$ Hz; POCH_3), 6.90 (d, 1H, $^3J_{H,H} = 4.0$ Hz; Py-C*H*), 7.05 (d, 1H, $^3J_{H,H} = 4.0$ Hz; Py-C*H*), 7.06 (s, 1H; Ar-C*H*), 7.49 (t, 1H, $^3J_{H,H} = 8.0$ Hz; Ar-C*H*), 7.61–7.63 (m, 2H; Ar-C*H*), 7.66 (d, 1H, $^3J_{H,H} = 7.7$ Hz; Ar-C*H*), 7.87 (d, 1H, $^3J_{H,H} = 8.0$ Hz; Ar-C*H*), 8.24 ppm (t, 1H, $^4J_{H,H} = 1.8$ Hz; Ar-C*H*); ^{13}C NMR (100 MHz, [D$_4$]MeOD): $\delta = 27.3$ (C(*C*H$_3$)$_3$), 32.7 (d, $^1J_{P,C} = 137.3$ Hz; P*C*H$_2$Ar), 53.8 (d, $^2J_{P,C} = 6.9$ Hz; PO*C*H$_3$), 83.9 (*C*(CH$_3$)$_3$), 113.8, 115.0, 121.1, 122.1, 124.2, 125.1, 128.4, 130.1 (Py-*C*H/Ar-*C*H), 133.6 (d, $^2J_{P,C} = 12.3$ Hz, Ar-C$_q$), 137.1, 140.1, 140.5 (Py-*C*$_q$/Ar-*C*$_q$), 160.7, 161.1, 168.7 ppm (gua-*C*N/carbonyl-*C*O); MS (ESI): m/z (%): 757 (100) [M+Na]$^+$, 683 (5), 657 (15) [M−Boc+Na]$^+$, 635 (7) [M−Boc+H]$^+$, 598 (50); HRMS (ESI): calcd for $C_{31}H_{40}N_6NaO_{11}P_2^+$: 757.2123; found: 757.2123; FTIR (KBr): $\tilde{\nu} = 3387$ (bs), 3264 (bs), 2955 (m), 2851 (w), 1729 (m), 1631 (s), 1550 (s), 1466 (m), 1438 (w), 1303 (s), 1241 (s), 1150 (s), 1033 (s), 808 (m), 753 cm^{-1} (w).

Synthesis of 16: Compound **15** (62.0 mg, 84.3 µmol) was suspended in CH_2Cl_2 (6 mL) and TFA (2 mL) and stirred at room temperature until complete disappearance of the starting material was confirmed by TLC. The solvent was removed in vacuo and the crude product was purified with repeated RP18-MPLC chromatography. Compound **16**·TFA (40.0 mg, 53.4 µmol, 63 %) was obtained as a white solid. M.p. > 230 °C (**16**·HCl after lyophilization with 0.1 N HCl); $R_f = 0.44$ (RP18, MeOH/H_2O 2:1+0.1 % TFA); MPLC (RediSep C18 Reverse Phase, 43 g): $\tau_R = 13.5$ min (2 min at H_2O+0.1 % TFA, then gradient over 16 min to MeOH+0.1 % TFA; flow rate: 40 mL min^{-1}); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 3.24$ (d, 4H, $^2J_{P,H} = 21.6$ Hz; PCH_2Ar), 3.63 (d, 12H, $^3J_{P,H} = 10.7$ Hz; POCH_3), 6.94 (s, 1H; Py-C*H*), 7.15 (s, 2H; Py-C*H*/Ar-C*H*), 7.53 (t, 1H, $^3J_{H,H} = 8.0$ Hz; Ar-C*H*), 7.62 (s, 2H; Ar-C*H*), 7.73 (d, 1H, $^3J_{H,H} = 7.9$ Hz; Ar-C*H*), 8.03 (d, 1H, $^3J_{H,H} = 8.1$ Hz; Ar-C*H*), 8.22 (t, 1H, $^4J_{H,H} = 1.9$ Hz; Ar-C*H*), 8.27 (brs, 4H; gua-NH_2), 10.33, 10.39, 11.14, 12.66 ppm (4×s, 4×1H; N*H*); ^{13}C NMR (62.5 MHz, [D$_4$]MeOD): $\delta = 32.6$ (d, $^1J_{P,C} = 137.4$ Hz; P*C*H$_2$Ar), 53.8 (d, $^2J_{P,C} = 6.6$ Hz; PO*C*H$_3$), 113.7, 116.0, 121.2, 122.1, 124.4, 125.0, 128.4, 130.2 (Py-*C*H/Ar-*C*H), 133.6, 137.2, 139.9, 140.4 (Py-*C*$_q$/Ar-*C*$_q$), 157.2, 160.2, 161.3, 168.7, 170.0 ppm (gua-*C*N/carbonyl-*C*O); ^{31}P NMR (162 MHz, [D$_6$]DMSO): $\delta = 30.0$ ppm (s, *P*O(OMe)$_2$); MS (ESI): m/z (%): 1292 (4) [2M+Na]$^+$, 1269 (5) [2M+H]$^+$, 657 (55) [M+Na]$^+$, 635 (100) [M+H]$^+$, 598 (46), 457 (7); HRMS (ESI): calcd for $C_{26}H_{33}N_6O_9P_2^+$: 635.1779; found: 635.1774; FTIR (KBr): $\tilde{\nu} = 3447$ (bs), 2959 (w), 2856 (w), 1701 (s), 1655 (s), 1551 (s), 1466 (m), 1295 (m), 1151 (s), 1205 (s), 1036 (s), 807 (m), 722 cm^{-1} (w); HPLC (Supelcosil LC-18, 25 cm×4.6 mm, 5 µm): $\tau_R = 17.7$ min (5 min at 100 % H_2O+0.1 % NEt_3, then gradient over 35 min to 100 % MeCN+0.1 % NEt_3; flow rate: 1.5 mL min^{-1}).

Synthesis of 4: Compound **16**·TFA (7.0 mg, 9.35 µmol) was dissolved in aqueous HCl (0.01 N, 30 mL) and lyophilized. The resulting **16**·HCl and carefully dried LiBr (12.3 mg, 143 µmol) were suspended under argon and heated to reflux for 10 days. The reaction mixture was cooled to room temperature and adjusted to pH ≈ 8 with NEt_3. RP18 silica gel was

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added and the solvent was removed in vacuo. The crude product was purified with MPLC. Compound **4**·NEt_3 (4.3 mg, 6.08 μmol, 65%) was obtained as a white solid. M.p.>230°C; MPLC (RediSep C18 Reverse Phase, 43 g): $\tau_R = 7.7$ min (4 min at H_2O+0.2% NEt_3, then gradient over 10 min to MeCN+0.2% NEt_3; flow rate: 40 mL min^{-1}); ^1H NMR (400 MHz, D_2O): $\delta = 1.22$ (t, 9H, $^3J_{H,H} = 7.3$ Hz; NCH_2CH_3), 3.10 (d, 4H, $^2J_{P,H} = 19.7$ Hz; PCH_2Ar), 3.25 (q, 6H, $^3J_{H,H} = 7.3$ Hz; NCH_2CH_3), 3.70 (d, 6H, $^3J_{P,H} = 10.4$ Hz; $POCH_3$), 6.85 (d, 1H, $^3J_{H,H} = 4.3$ Hz; Py-CH), 6.98 (d, 1H, $^3J_{H,H} = 4.3$ Hz; Py-CH), 7.02 (s, 1H; Ar-CH), 7.39 (s, 2H; Ar-CH), 7.42 (t, 1H, $^3J_{H,H} = 8.1$ Hz; Ar-CH), 7.55 (d, 1H, $^3J_{H,H} = 8.0$ Hz; Ar-CH), 7.59 (d, 1H, $^3J_{H,H} = 8.1$ Hz; Ar-CH), 7.69 ppm (s, 1H; Ar-CH); ^{31}P NMR (162 MHz, $[D_6]DMSO$): $\delta = 20.6$ ppm (brs, $PO(OMe)O^-$); MS (negative ESI): m/z (%): 2234 (10), 1634 (24), 1034 (36), 605 (61) $[M-H]^-$, 432 (17), 283 (100); HRMS (negative ESI): calcd for $C_{24}H_{27}N_6O_9P_2^-$ $[M-H]^-$: 605.1320; found: 605.1323.

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