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Review

How to improve guanidinium cations for oxoanion binding in aqueous solution? The design of artificial peptide receptors

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Dedicated to Prof. Siegfried Hünig on the occasion of his 85th birthday.

Contents

Abstract

Guanidinium cations are a prominent recognition motif for oxoanion binding both in Nature and in abiotic systems. However, simple ion pairing based on alkyl guanidinium cations is not strong enough to achieve an efficient complexation in aqueous solvents. Nature uses the less polar microenvironment of a protein to shield the ion pair from the solvent thereby increasing complex stability. For artificial supramolecular systems other ways to improve the binding affinity of guanidium cations have to be found. We will describe herein our use of modified acylguanidinium cations with additional H-bond donor sites to achieve oxoanion binding in aqueous solvents. The thermodynamic characterization of such systems is described as well as some applications from the field of bioorganic chemistry (e.g. artificial receptors for anionic biomolecules).

Keywords: Supramolecular chemistry; Self-assembly; Molecular recognition; Guanidinium cations; Carboxylate receptors

1. Introduction

In the biological world the guanidinium moiety is widely spread as a mediator of specific non-covalent binding [1] let it be with oxoanionic enzyme substrates (e.g. like lactate by the enzyme lactate dehydrogenase), the base pairing in nucleic acids (after all guanine is an acylated guanidine) or the dedicated interaction of biopolymers (e.g. the curling of DNA around the arginine-rich histone proteins to make up the nucleosome particle). In addition to these ground state recognition events guanidinium groups also play decisive roles in transition state binding as well, i.e. in catalysis, the hydrolysis of phosphate diesters being a very prominent example [2].

Based on this important role in Nature, the guanidinium group has also served as an excellent "working horse" for the abiotic supramolecular community for the last 30 years [3]. However, in the past most artificial model studies were restricted to organic solvents. The reason for this is that simple ion pairs between guanidinium cations and oxoanions are normally stable only in solvents of low polarity. In aqueous solutions, e.g. the com-

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peting solvation of both donor and acceptor sites by individual solvent molecules significantly decreases ion pair stability [4]. For Nature this normally does not pose any problems due to the rather hydrophobic interior of proteins where such ion pairing usually occurs [5]. However, for chemical receptors the weakness of simple ion pairs in polar solvents represents a severe limitation especially with regard to any potential application of such supramolecular systems.

Despite the power of natural evolution the guanidinium cation in the side chain of the amino acid arginine [6], a priori, might not be the best choice for oxoanion binding because higher reasons (e.g. the urge for economy in biosynthesis, the degradability and reuse, genetic coding, etc.) mandate its participation in spite of suboptimal binding properties. Indeed, in recent years abiotic guanidinium systems were developed that surpass the parent guanidinium cation in their binding properties [3a,7]. For example, carefully designed guanidinium derivatives now even bind oxoanions in water and the thermodynamics of the underlying ion pair formation can be studied in detail with modern techniques such as ITC revealing the importance of solvent effects [8]. Also computational methods have significantly improved over the last years allowing now a better theoretical understanding of the features and binding properties of supramolecular recognition events [9]. In this context, we wish to present here some examples of how to improve the oxoanion binding properties of guanidinium cations by adding additional binding sites to achieve efficient complexation of small oxoanionic biomolecules in water.

2. Oxoanion binding by simple guanidinium cations

As already mentioned, Nature often uses simple alkylguanidinium cations in form of the amino acid arginine for the binding of oxoanions: for example, in carboxypeptidase A [10], an enzyme that hydrolytically cleaves off an amino acid from the free C-terminus of a peptide chain, the peptidic carboxylate is essentially bound by an ion pair with the guanidinium group of arginine 145 and two additional H-bonds from asparagine 144 and tyrosine 248 (Fig. 1). The enzyme–substrate interaction is furthermore significantly facilitated by the overall hydrophobic character of the binding pocket which reduces competitive solvation of the binding sites by water molecules.

Unfortunately, without the hydrophobic shielding of an enzyme pocket, a guanidinium–carboxylate ion pair is only stable in solvents of low polarity such as chloroform or acetonitril. Even smallest amounts of more polar solvents such as DMSO, methanol or even water cause an immediate dissociation of these ion pairs. For example, the lactate–guanidinium ion pair has a stability of only $K \leq 6\,M^{-1}$ in water based on spectrapolarimetry measurements [11] and ion pairs formed between dicarboxylates and diammonium cations were shown to have a stability of only $K \leq 50\,M^{-1}$ in water, even though in some of these cases additional aromatic interactions still overlap with ion pair formation [12]. Based on a larger statistical analysis of a variety of data of organic and inorganic ions a single salt bridge was assigned a stability of $\leq 5\,kJ\,mol^{-1}$ in water, which corresponds to an association constant of $K \leq 7\,M^{-1}$ [13]. However, these

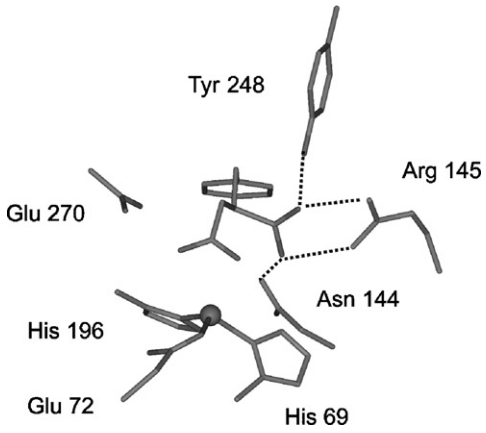


Fig. 1. Binding of an inhibitor (benzyl glutamic acid) within the active site of carboxypeptidase A. The carboxylate forms an ion pair with the guanidinium cation of Arg 145. Two additional H-bonds from Asn 144 and Tyr 248 further stabilize the complex.

latter data are all extrapolated to indefinite dilute solutions (zero ionic strength). Therefore, under real conditions (=millimolar salt concentrations), the binding is even much weaker than suggested by these numbers.

This is a general problem in supramolecular chemistry. Molecular recognition is based on non-covalent interactions but in contrast to covalent bonds their strength is highly depending on external parameters such as solvent composition, polarity or even temperature [14]. In this context water as a solvent is the most challenging one. On the one hand, electrostatic interactions (H-bonds or ion pairs), which are quite well understood and have been extensively used in artificial supramolecular systems due to their complementarity and directionality [15,16], are rather weak in this solvent. On the other hand, hydrophobic [17] or aromatic stacking interactions [18], which can be rather strong in aqueous solvents, are much more difficult to deliberately design and to use in artificial receptors.

Nevertheless, inspired by Nature's use of arginine a variety of beautiful and excellent abiotic host systems based on alkylguanidinium cations has been designed over the last three decades for oxoanion binding however mostly for organic solvents. This early work has been comprehensively summarized before and will therefore only be mentioned here very briefly (Fig. 2) [3]. Lehn and co-workers were the first to investigate the complexation of carboxylates by various simple bis- and tris-guanidinium salts in the late 1970s [19]. In the early 1980s, Schmidtchen and co-workers or deMendoza and co-workers used charged bicyclic guanidinium cations such as **1** for the complexation of anions in chloroform [3f,20]. All this work however was strictly limited to organic solvents. The best approach to achieve substrate binding in water by artificial hosts is to use not only one but several non-covalent interactions simultaneously. Though every individual contact between host and substrate by itself might be rather weak, their combined effect can still lead to high association constants ("Gulliver effect") [15]. Following this line, in the last decade Hamilton and co-workers **2** [21], Anslyn **3** [22] and Schmidtchen **4** [23] among others designed poly-guanidinium receptors that function even in more polar solvents such as aque-

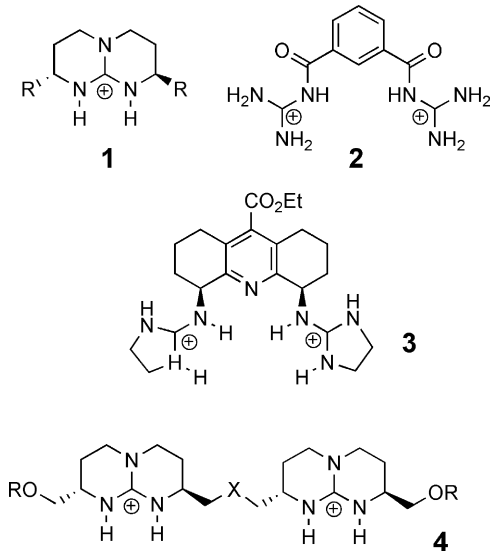


Fig. 2. Early examples for mono- and poly-guanidinium receptors for the complexation of anions in polar organic solvents.

ous DMSO or methanol due to the simultaneous formation of not only one but several guanidinium/carboxylate ion pairs within the complex. Despite the very strong electrostatic interaction of these poly-guanidinium complexes, the binding constants in aqueous solvents were still only moderate and the design and synthesis of such receptors remains a demanding problem.

Also the presence of other competing anions or salts in general significantly decreases the binding affinity in such complexes. As mentioned above, the latter is due to the increasing ionic strength of the solution which weakens Coulomb type interactions. For example, Anslyn and co-workers designed a very interesting citrate receptor **5** in recent years [24], which is based on a triple ion pair formation between the carboxylates of citrate (**6**) and guanidinium cations in the receptor **5** (Fig. 3). The binding affinity for citrate is very high with an association constant of $K = 6.9 \times 10^3\ M^{-1}$ in pure water allowing also for the selective detection of citrate in the presence of other similar anions such as tartrate. However, in buffer solution the affinity of **5** for citrate drops by more than two orders of magnitude ($K < 10^2\ M^{-1}$) due to the increased salt concentration in the buffer solution.

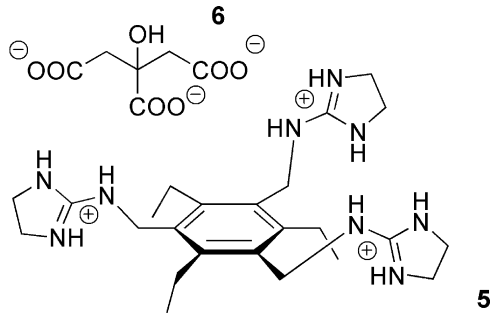


Fig. 3. Anslyn's guanidinium receptor **5** for the complexation of citrate **6**. Due to the triple ion pair formation stable complexes are formed even in pure water ($K = 6.9 \times 10^3\ M^{-1}$) but in the presence of buffer salts the complex stability significantly drops ($K < 10^2\ M^{-1}$).

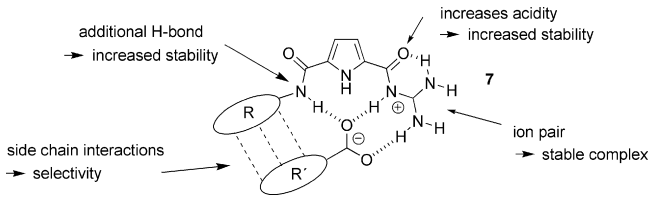


Fig. 4. Guanidiniocarbonyl pyrrole cations **7** efficiently bind carboxylates even in aqueous solvents due to a combination of ion pair formation and additional H-bonds.

Hence, in highly competitive solvents, simple 1:1 ion pairing of alkylguanidinium cations with carboxylates is normally not strong enough for the formation of sufficiently stable complexes. At least with regard to any future potential applications, artificial receptors for biomolecules have to be capable to efficiently bind their substrate under in vivo conditions and therefore to compete at least with a 120 mM aqueous sodium chloride solution. Obviously, this is rather difficult to achieve or nearly impossible with simple alkylguanidinium cations. As already pointed out, biological recognition events normally do not occur in the bulk aqueous phase but within the less polar microenvironment of a protein. Even then Nature often relies on additional H-bonds to further strengthen the ion pair as was shown above for substrate binding by carboxypeptidase A.

3. Guanidiniocarbonyl pyrroles: an improved binding motif for carboxylates

Hence, our idea for the design of an even more efficient carboxylate binding site (CBS) was to improve the binding affinity of the guanidinium cation itself by directly incorporating additional H-bond donors into the CBS. Based on theoretical calculations we therefore introduced cationic guanidiniocarbonyl pyrroles of type **7** as a new and easily accessible binding motif for carboxylate anions (Fig. 4) [25]. These acyl guanidinium receptors combine several advantages compared to simple guanidines, which makes them attractive candidates for the binding of carboxylates even in aqueous solvents [26]:

- Acyl guanidiniums have pK_a-values in the order of 7–8, whereas simple guanidiniums have pK_a around 13. This increased acidity favors the formation of hydrogen bonded ion pairs and hence increases the binding affinity.
- Additional prospective hydrogen bond donors such as the amide NH can further enhance the stability of the complex.
- The binding motif is planar and rather rigid and therefore ideally preorganised for the binding of planar anions such as carboxylates.
- Additional secondary interactions between the receptor side chain and the carboxylate can be easily introduced to achieve selectivity with respect to the bound substrate.

As anticipated from the theoretical predictions, guanidiniocarbonyl pyrrole receptors indeed strongly bind carboxylates even in aqueous solvents through a combination of ion pairing and multiple hydrogen bonding as could be shown by NMR-,

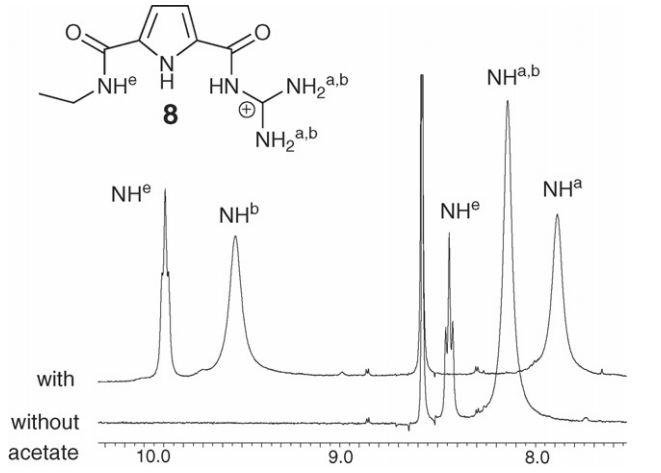


Fig. 5. ^{1}H NMR spectrum of a simple guanidiniocarbonyl pyrrole cation **8** (picrate salt) with (back) and without (front) acetate (NMe_4^+ salt) in [d_6]DMSO showing the CIS (= complexation induced shift) of the guanidinium NH protons and the amide NH (adapted from ref. [26]).

UV- and fluorescence-titrations (Fig. 5) [26]. For example, simple guanidiniocarbonyl pyrroles such as **8** bind carboxylates even in 50% water in dimethyl sulfoxide with association constants in the order of $K \approx 10^3\ M^{-1}$, whereas simple alkylguanidinium cations show no detectable complexation at all under these conditions (vide infra). Therefore, anion binding by guanidiniocarbonyl pyrrole cations in aqueous solvents is at least one to two orders of magnitude more efficient than with simple alkylguanidinium cations. The guanidiniocarbonyl pyrrole cation belongs to the most efficient monocationic binding sites for oxoanions reported so far [3]. Hence, this new recognition motif has already found versatile use in various fields of supramolecular [27] and bioorganic chemistry [7] and a few representative examples from the latter field will be described below.

The improved binding affinity of our CBS relative to the standard alkylguanidinium cation can be directly seen by comparing Anslyn's citrate receptor **5** [24] with a modified version which contains our guanidiniocarbonyl pyrrole binding motif instead of simple alkylguanidinium cations (Fig. 6) [28]. Our guanidiniocarbonyl pyrrole based receptor **9** binds citrate even in aqueous buffer in the presence of a 1000-fold excess of chloride anions with an association constant of $K \approx 10^5\ M^{-1}$. Hence, its affinity for citrate is several orders of magnitude larger due to the improved anion binding properties of the guanidiniocarbonyl pyrrole carboxylate binding site. The binding affinity in this highly competitive solvent is strong enough for potential applications. As other substrates with similar binding sites such as malate or tartrate are bound much less efficiently, this receptor can, for example, be used for the naked eye detection of citrate in aqueous solutions using on an indicator displacement assay with carboxyfluorescein (Fig. 7) [29].

4. Dissecting the guanidiniocarbonyl pyrrole binding motif using "knock-out" analogues

Which additional feature of the guanidiniocarbonyl pyrrole binding motif is responsible for the improved complex stabil-

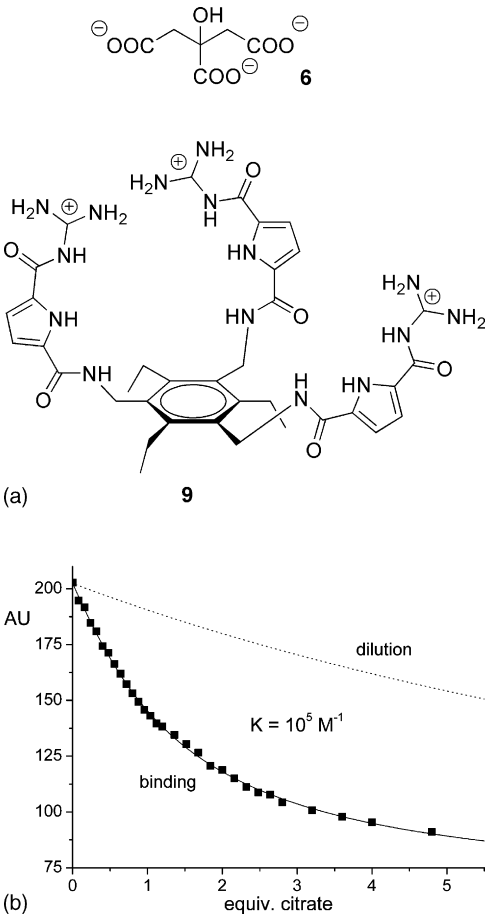


Fig. 6. (a and b) The improved anion binding properties of the guanidiniocarbonyl pyrrole cations in receptor **9** allow for an efficient complexation of citrate even in aqueous buffer solution with a 1000-fold excess of chloride as shown by the UV-titration binding isotherm ($\lambda = 300$ nm, [**9**] = 1.2×10^{-5} M, [**6**] = 2×10^{-4} M, buffered water, pH 6.3). The solid line represents the calculated curve fit for the experimental data (■), whereas the dotted line indicates the expected change in absorption due to simple dilution of the sample during the titration.

ity: the increased acidity of the acyl guanidinium cation or the additional H-bonds? Are the H-bonds all contributing equally to the complex stability? Unfortunately, it is impossible to experimentally determine the binding energy of an individual bond or type of interaction within an array of several non-covalent interactions as only the overall stability can be measured. Therefore, to get an idea about the individual energetic contributions of the different non-covalent interactions within this binding motif, we studied a series of systematically varied receptors (**8**, **10**–**13**, respectively) [26]. Within these series in each receptor one or more non-covalent interactions from the overall binding motif were missing (Fig. 8), so that the comparison of the relative binding properties of the receptors should allow at least to estimate the energetic contribution of this specific interaction.

The comparative binding study of these systematically varied guanidiniocarbonyl pyrrole receptors demonstrated that the energetic contributions of the individual non-covalent interactions within our binding motif are significantly different [26]: besides the ion pairing by the acylguanidinium mainly the amide NH in position 5 of the pyrrole ring is important for the effec-

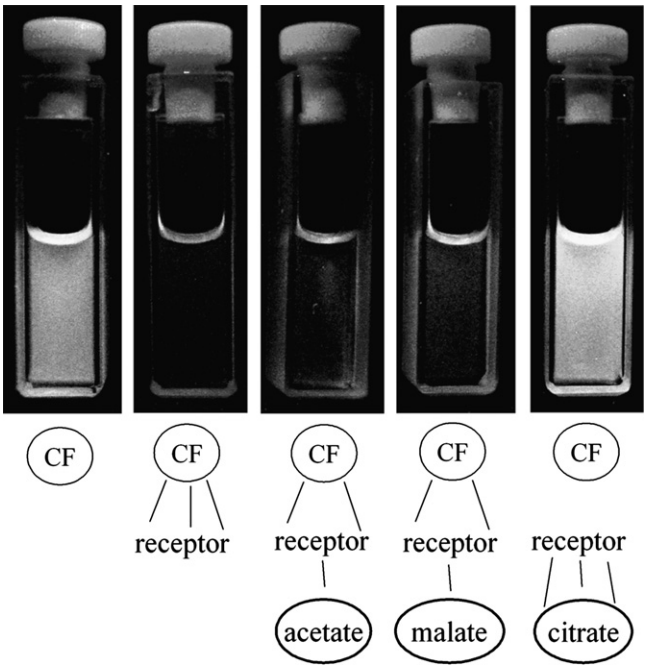


Fig. 7. Naked-eye detection of acetate, malate and citrate (all 10 mM) with a 1:1 mixture of receptor **9** and carboxyfluorescein (0.5 mM) in aqueous DMSO. Only citrate is capable to displace the carboxyfluorescein from its complex with **9** thereby restoring its fluorescence (adapted from ref. [29]; reproduced by permission of The Royal Society of Chemistry).

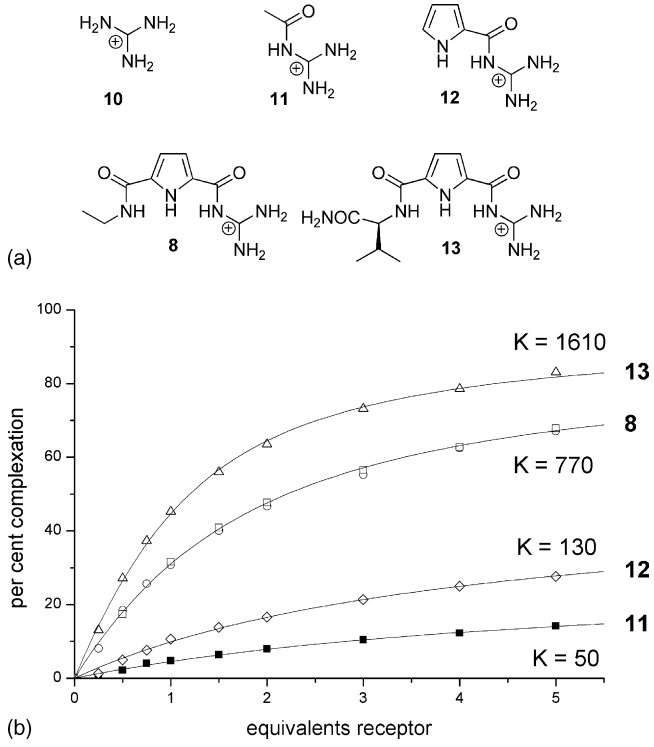


Fig. 8. (a and b) NMR-titration curves of receptors **8**, **10**–**13** (picrate salts) with *N*-acetyl alanyl carboxylate (NMe_4^+ salt, 1 mM) in 40% water in [d_6]DMSO and binding constants (M^{-1}) calculated thereof. Besides the ion pairing mainly the amide NH in position 5 of the pyrrole (receptor **8**) increases the complex stability significantly (adapted from ref. [26]).

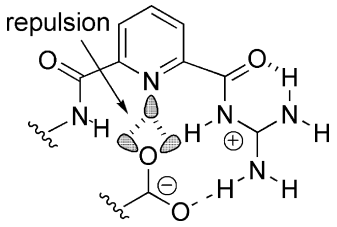


Fig. 9. The repulsion between the lone pairs of the pyridine nitrogen and the anionic carboxylate oxygen is responsible for the less efficient binding of pyridine based acylguanidinium cations compared to the pyrrole systems.

tive binding of the carboxylate substrate (receptor **8**), whereas the pyrrole NH seems to be of only minor importance (receptor **12**). Further studies showed that also the size and electronic structure of the aromatic ring are important [30]: pyrrole systems are superior to the analogous benzene derivatives which in turn show a higher binding affinity than pyridine or furane derivatives, in which the lone pair on the heteroatom exerts additional repulsive effects on the bound carboxylates (Fig. 9) [31].

Of course no exact quantitative determination of the influence of these individual interactions and structural factors is possible this way as the replacement of one interaction also affects the remaining ones (e.g. their individual strengths and also secondary interactions among them). If the changes in complex stability and hence binding energy associated with switching off one specific interaction are significantly large, at least its importance for the overall binding can be estimated. An exact quantitative thermodynamic analysis of the importance of an individual interaction within a multiple binding site motif is also possible but requires much more sophisticated studies such as the double mutant cycles advocated by Hunter and co-workers [32].

The importance of the ionic interaction in complexes between carboxylates and guanidiniocarbonyl pyrrole cations could be demonstrated by the comparison of an ionic zwitterion **14** with a neutral "knock-out" analogue **15** which possesses an identical hydrogen bonding pattern but no charges (Fig. 10) [33]. It turned out that the ionic interaction is indeed crucial for a complexation in polar solvents as the neutral analogue **15** only self-assembles in organic solvents of low polarity. The association constant for the zwitterion is approximately $K > 10^{10}\ M^{-1}$ in DMSO and still surprisingly high ($K = 170\ M^{-1}$) in pure water (Fig. 11). Therefore, compound **14** is one of the most efficient self-assembling systems relying solely on electrostatic interactions reported so far [34].

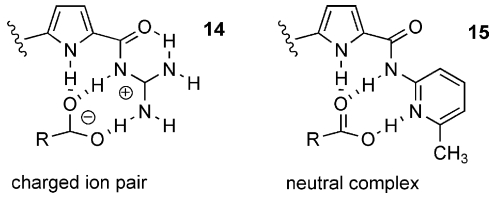


Fig. 10. Complexes between an amidopyridine pyrrole and a carboxylic acid (right) as a neutral "knock-out" analogue of the charged ion pair between our guanidiniocarbonyl pyrrole and a carboxylate (left): "switching off" the ionic interactions while keeping the hydrogen bond network constant.

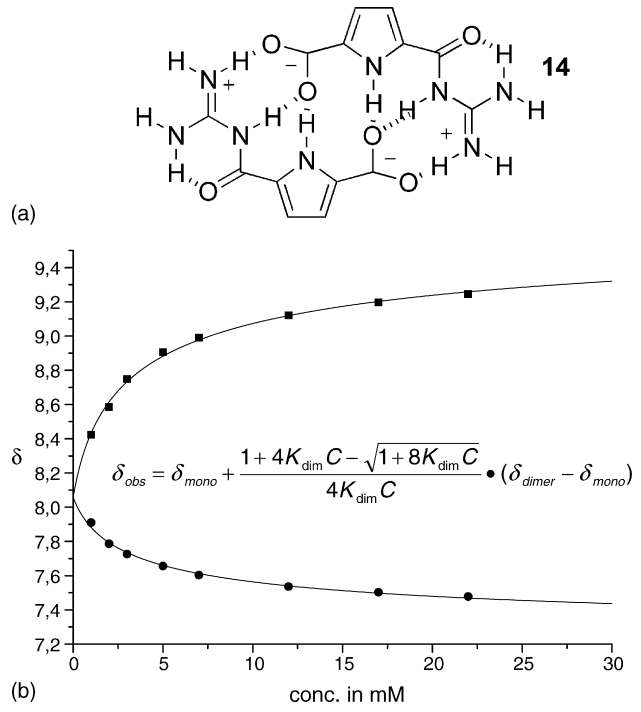


Fig. 11. (a and b) Zwitterion **14** dimerizes even in water with a surprisingly large association constant of $K = 170\ M^{-1}$ as derived from the binding isotherms of the guanidinium NH protons (adapted from ref. [33]).

The neutral binding motif in the "knock-out" analogue **15** has the same H-bond pattern like dimer **14** as could be shown by X-ray analysis. Nevertheless, the dimerization is several orders of magnitude less efficient (Fig. 12). Whereas **15** dimerizes in chloroform with $K > 10^4\ M^{-1}$ already the addition of >5% DMSO completely disrupts these dimers due to the competitive solvation of the H-bond donor by the polar solvent. Even the addition of only 0.5% DMSO reduces the complex stability by at least one order of magnitude!

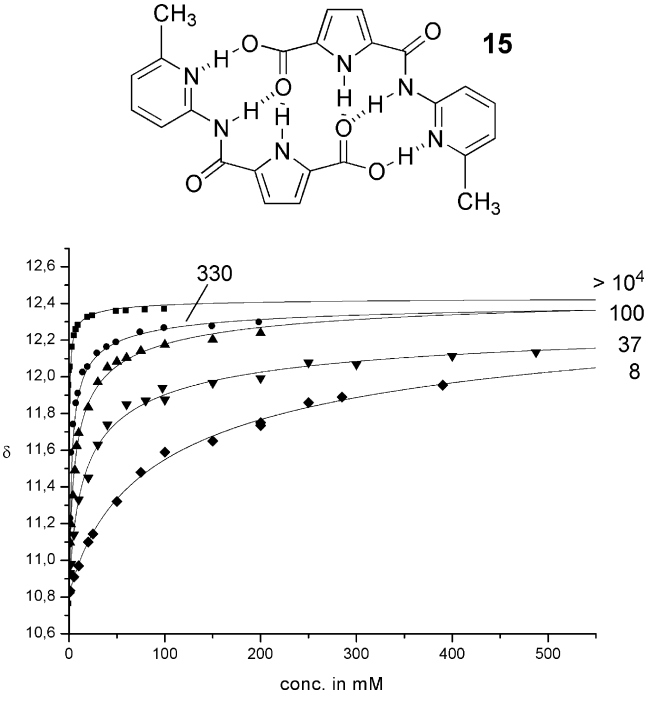


Fig. 12. The neutral analogue **15** strongly dimerizes in chloroform but the addition of even small amounts of DMSO completely disrupts the dimers (from top to bottom: 0, 0.5, 1, 2.5 and 5% DMSO in $CDCl_3$, K in M^{-1}) (adapted from ref. [33]).

This not only underlines the importance of the ion pair for complex formation but also demonstrates again the dramatic effect that the solvent can have on supramolecular complex formation. In another context we could also demonstrate that the solvent composition can change the complex stoichiometry [35]. With increasing polarity of the solvent, complex formation between an amino acid carboxylate and a guanidiniocarbonyl pyrrole bis-cation was shifted from a 2:1 to a 1:1 complex. Hence, it cannot be pointed out often enough that the solvent is an integral part of any supramolecular complex formation in solution. Especially in aqueous solvents polar interactions (such as ion pair formation) are often found to be actually endothermic [36]. Hence, binding in these cases is only due to an increase in entropy caused by the reorganisation of the solvent molecules upon complex formation. The individual binding partners are much more solvated than the resulting complex. The solvent molecules which are released from the solvation sphere when the complex forms have a larger mobility and are less ordered thereby increasing the entropy of the system. Normally, supramolecular chemists tend to think in static enthalpic pictures when "designing" receptors based on specific attractive interactions within a complex. Even though this simple picture often works as demonstrated by the large number of elegant host–guest systems that were designed this way, it might very well be for a completely wrong reason. With the increasing availability of modern techniques such as isothermal titration calorimetry (ITC) which allows one to determine both enthalpic and entropic contributions to complex stability our picture of supramolecular events in solution and especially the role of the solvent will hopefully improve. This knowledge should then help us to "design" even better host–guest systems in the future.

The analysis of the neutral knock-out analogue **15** showed that the ion pair formation is crucial for the efficient complexation of oxoanions in aqueous solvents [33]. However, oxoanion binding by guanidinium cations whether the parent one or modified derivatives such as our guanidiniocarbonyl pyrroles is not just a simple charge interaction. The bidentate H-bond assisted ion pair formed by a guanidinium cation is highly directional and much stronger than a simple Coulomb-charge interaction between two spherical ions [16]. To fully understand the binding properties of our guanidiniocarbonyl pyrrole cations one has therefore to account for the various H-bonds, their number and strength, the properties of the actual ion pair (e.g. the charge density) and further secondary electrostatic [37] and cooperative effects [38]. This is already evident from the experimental thermodynamic study within the series of systematically varied receptors mentioned above (e.g. the amide NH being more important than the pyrrole NH). For the dimerizing zwitterion we also performed a high-level theoretical DFT analysis to investigate this aspect further [39]. We theoretically studied several additional "knock-out" analogues in which single hydrogen bonds within these multiple point binding motif were

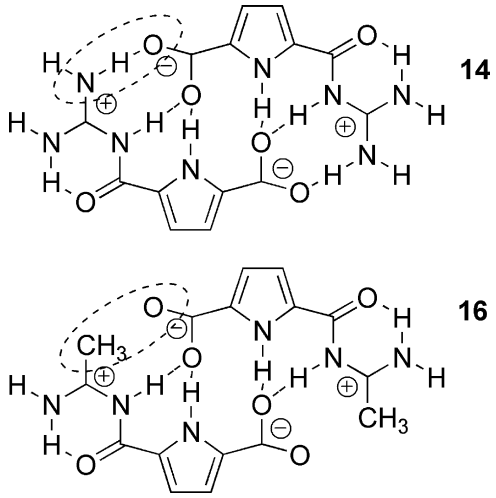


Fig. 13. The calculated dimerization energy of the acyl amidinium zwitterion **16** ($\Delta E = 13.6$ kcal/mol) shows that this dimer is only half as stable as the guanidiniocarbonyl pyrrole zwitterion ($\Delta E = 23$ kcal/mol). This underlines the importance of the H-bond assisted ion pair formation for oxoanion binding.

switched off by replacing N–H hydrogen donor groups with either methylene groups or an oxygen ether bridge. The calculated dimer stabilities revealed as a main conclusion that all simple models either based just on hydrogen bond counting or on the assumption that the charge interaction by itself is the main and dominant factor fail to explain the stability of such ion pairs. The computations showed that both the hydrogen bond network, the electrostatic attraction and also their mutual interactions are responsible for the high efficiency of the guanidiniocarbonyl pyrrole/carboxylate ion pair. For example, the amidinium derivative **16** shown in Fig. 13 also forms a zwitterionic dimer, but with one H-bond less within each ion pair. The calculated stability is only half that of the guanidinium zwitterion **14**. The charge interaction is therefore not sufficient but the individual H-bond pattern in each case is decisive. Hence, there is no simple explanation for the improved efficiency of oxoanion binding by our guanidiniocarbonyl pyrroles but their superior complexation properties stem from a subtle interplay of various factors.

5. Receptors for amino acid carboxylates

The design of artificial receptors, which efficiently bind amino acids under physiological conditions, still remains a challenging task [40]. Most amino acid receptors reported in the literature so far either need hydrophobic and/or strong metal–ligand interactions to achieve substrate binding in water. For example, often hydrophobic interactions of an aromatic side chain with, e.g. the cavity of a cyclodextrin or calixarene based receptor are mainly responsible for the binding. The improved binding properties of our guanidiniocarbonyl pyrrole cation compared to simple alkylguanidinium cations now allow for oxoanion binding in aqueous solvents solely based on electrostatic interactions without the need for hydrophobic cavities. Amino acid carboxylates can be bound side chain selectively and stereoselectively in aqueous DMSO even by simple guanidiniocarbonyl pyrrole cations. It was already described above that alanine carboxylate

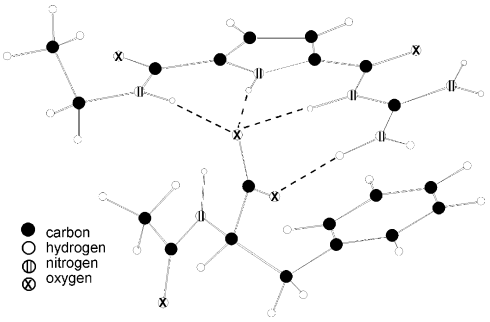


Fig. 14. Structure of receptor **8** with *N*-acetyl phenyl alanine carboxylate in water derived from molecular modelling (intermolecular hydrogen bonds are shown as broken lines). The aromatic ring forms an additional attractive cation–π interaction with the guanidiniocarbonyl pyrrole moiety (adapted from ref. [25]; reproduced by permission of The Royal Society of Chemistry).

is bound with $K \approx 10^3\ M^{-1}$ in 40% water in DMSO by guanidiniocarbonyl pyrrole receptors [26]. For different amino acids the complex stability is also depending on the side chain. For example, the association constant for the binding of D/L-phenyl alanine carboxylate by the ethyl amide substituted guanidiniocarbonyl pyrrole cation **8** is more than two times stronger ($K = 1700\ M^{-1}$) than for D/L-alanine ($K = 770\ M^{-1}$), whereas the association constant for D/L-lysine ($K = 360\ M^{-1}$) is about two times weaker [25]. The differences in complex stability among the various amino acid carboxylates must result from secondary interactions of their side chains with the receptor. In the case of phenyl alanine the aromatic ring probably π-stacks with the acyl guanidinium unit of **8** (Fig. 14). This cation–π interaction [41] further stabilizes the complex. In contrast to this, the positively charged ω-ammonium group in lysine decreases the binding affinity relative to alanine most likely due to unfavorable electrostatic interactions with the positively charged guanidinium group.

The chiral, L-valine derived receptor **13** shows not only side chain selectivity but also moderate stereoselectivity [26]. For example, in the case of alanine the L-enantiomer is bound better than the D-enantiomer. In the complex with the L-enantiomer the binding constant ($K = 1610\ M^{-1}$) is larger relative to the binding by the ethylamide receptor **8** ($K = 770\ M^{-1}$) probably due to an additional hydrogen bond from the terminal carbamoyl group (Fig. 15, top). With D-alanine there is an unfavorable steric repulsion between the methyl group of the amino acid and the isopropyl side chain of the receptor which is not present in the complex with the L-enantiomer where the methyl group points away from the isopropyl group. As the H-bond from the terminal carbamoyl group to the carboxylate is not strong enough to compensate for this increased steric strain, this H-bond is most likely lost in the complex with D-alanine (Fig. 15, bottom). Therefore, the remaining binding motif resembles the simple achiral amide substituted guanidiniocarbonyl pyrrole **8** which lacks the sterically demanding isopropyl group. In accordance with this, the binding constant for the D-enantiomer is also around the same ($K = 730\ M^{-1}$). Unfortunately, a further increase in the steric bulk between the amino acid carboxylate and the receptor as, for example, in a *tert*-leucine derived receptor caused a complete loss of stereoselectivity. In this case obviously the steric

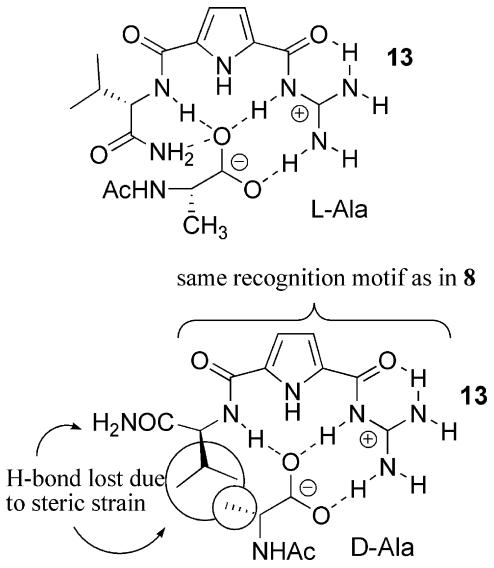


Fig. 15. An unfavorable steric interaction between the two side chains in the complex between D-alanine (bottom) and receptor **13** reduces the binding affinity relative to the L-enantiomer (top) due to the loss of a crucial H-bond between the terminal carbamoyl group of receptor **13** and the bound carboxylate.

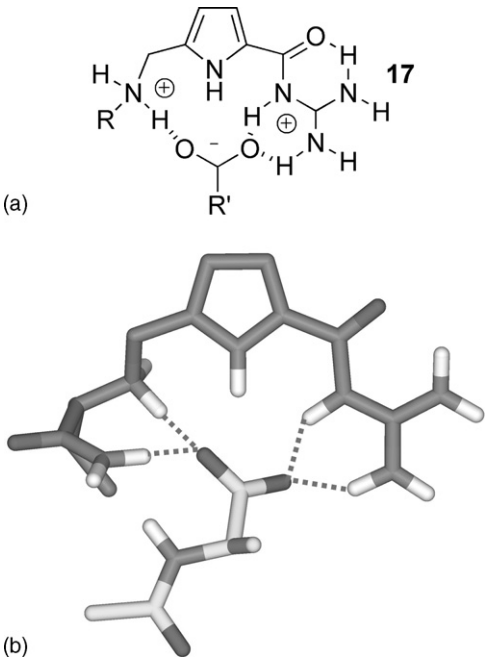


Fig. 16. (a) Bis-cation **17** shows improved anion binding features compared to the parent guanidiniocarbonyl pyrroles. In the calculated complex structure (b, R: –ValNH$_2$ and Ac–N–Ala) one of the carboxylate oxygen atoms is shifted towards the positively charged ammonium group (left side) which is a better H-bond donor than the neutral amide NH in the guanidiniocarbonyl pyrrole cations (adapted from ref. [35]).

interaction with the L-enantiomer is already too large and the H-bond to the terminal carbamoyl group is also lost so that only the interaction with the achiral guanidiniocarbonyl pyrrole moiety remains. Hence, no stereoselectivity is observed.

Despite the improved binding features, a simple guanidiniocarbonyl pyrrole monocation does not yet allow efficient oxoanion binding in pure water at high salt concentrations. We reasoned that the introduction of additional positive charges into the binding motif should stabilize the complex further. As the "knock-out" studies described above showed that the amide NH in position 5 of the pyrrole ring is rather important, this seemed to be a good position to try to improve the complex stability by increasing the donor capacity of this NH. Indeed, when the neutral amide NH was replaced by a positively charged secondary ammonium cation as in **17** (Fig. 16), the complexation properties improved even though only slightly [35]. Bis-cation **17** binds amino acid carboxylates around two times better than the monocations **7**. Even though the positively charged NH is a better donor than the amide NH the bis-cation is also much more heavily solvated than the monocation. This significantly counteracts the improved H-bond leading overall only to a slight increase in binding affinity.

An unexpected and significant further improvement was however achieved with the introduction of a third positive charge in form of a primary ammonium group in **18** [42]. The tris-cationic receptor **18** (Fig. 17) now binds amino acid carboxylates efficiently solely based on electrostatic interactions with $K \geq 10^3\ M^{-1}$ in nearly pure water (10% DMSO added for solubility reasons). Furthermore, **18** shows an unexpected cooperative 2:1 complex formation with *N*-acetyl glutamate—but not aspartate. This tris-cation **18** is hence capable to differentiate between glutamate and aspartate, which is remarkable regarding their structural similarity and flexibility.

This shows that a clustering of electrostatic interactions as in tris-cation **18** indeed allows the efficient complexation of amino acid carboxylates in water. Additional hydrophobic or metal/ligand interactions are not needed [43]. Furthermore, even small and flexible artificial receptors can show remarkable coop-

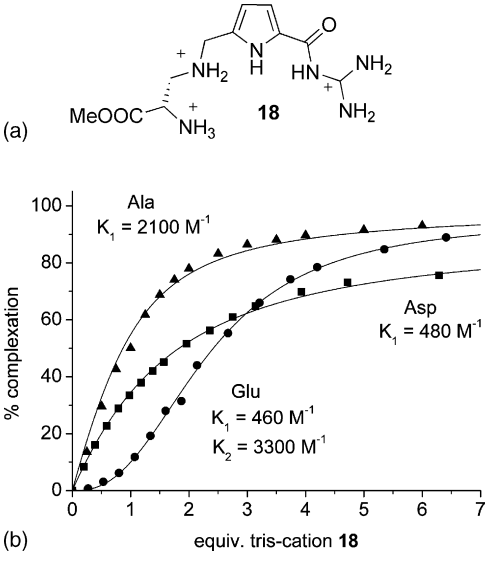


Fig. 17. (a and b) Binding isotherms for the amide-NH of various amino acid carboxylates ($c_0 = 1.5$ mM, NMe_4 salts) upon the addition of the tris-cationic guanidiniocarbonyl pyrrole receptor **18** (chloride salt) in 90% water/DMSO. The sigmoidal binding isotherm in the case of glutamate indicates the cooperative formation of a 2:1 complex (adapted from ref. [42]).

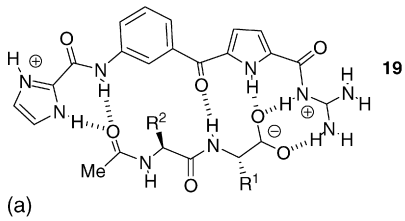
erativity thereby discriminating between structurally closely related guests.

6. Binding of larger substrates: de novo design of dipeptide receptors

Proceeding now from single amino acids to even larger substrates such as small oligopeptides requires further binding sites attached to the guanidiniocarbonyl pyrroles. These are needed for additional interactions with the other binding sites of the peptides, besides the carboxylate. In Nature the molecular recognition of C-terminal oligopeptides plays a central role in a variety of processes such as, for example, the mode of action of the antibiotic Vancomycin [44] or in Ras-protein induced oncogenesis [45]. The development of artificial receptors for the specific complexation of biologically important small oligopeptides under physiological conditions is thus of great importance for the design of sensors [46], the targeting of cellular processes [47] or the discovery of new therapeutics [48]. How should such a peptide receptor look like? In principle there are two distinct strategies one can follow [49]. One can try to rationally design a complete receptor de novo with the help of theoretical predictions. The larger the substrate is, the more difficult however this gets as calculations are not yet reliable enough to completely design a tailor made artificial host for a large substrate. Another possibility is to use a random trial and error approach and to identify suitable receptors with the help of combinatorial chemistry [50]. This combinatorial approach will be described later on. First we will focus on a receptor for dipeptides designed de novo with the help of theoretical predictions.

However, a realistic theoretical treatment of supramolecular complex formation is very difficult and time consuming and requires an expert in theoretical chemistry [9,39]. A real de novo design is therefore in most cases not possible. However, for rather small substrates more simple molecular mechanics calculations can be used to help design a receptor that, for example, should be capable to bind dipeptides with a free carboxylate. The results of such modelling predictions should however be treated with caution. Molecular mechanics is a helpful tool at least to check for structural complementarity between host and guest, but the predicted absolute complex stabilities are in most cases rather meaningless due to uncertainties in solvent treatment or the thermodynamics of non-covalent interactions such as dispersive forces. Hence, molecular mechanics at least performed by the non-expert should be regarded more as a modern version of a molecular tool kit. It can help to eliminate rather quickly potential receptor candidates which due to lacking structural complementarity or unfavorable steric interactions are perhaps not the best choice before even synthesizing them in the laboratory.

Using such an approach we designed receptor **19** (Fig. 18) to bind dipeptides [51]: the guanidiniocarbonyl pyrrole moiety is expected to form a hydrogen bonded ion pair with the carboxylate, whereas additional H-bonds between the dipeptide backbone and the receptor further stabilize the complex and also should provide the selectivity for dipeptides over simple anionic amino acids or other oxoanions. Indeed receptor **19** binds anionic



carboxylate	K [M^{-1}]
Gly-Gly	15.900
Ala-Ala	30.600
Val-Ala	43.800
Val-Val	54.300
Ala	7.400
Gly	5.200

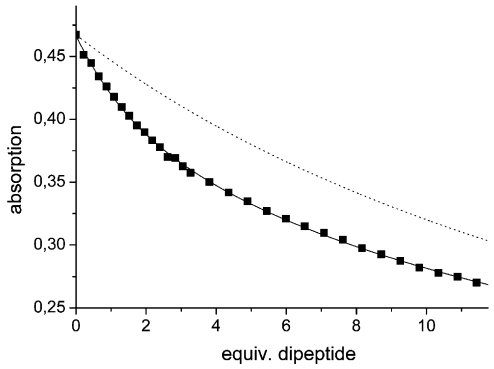


Fig. 18. (a and b) Binding of various dipeptide carboxylates by receptor **19** in buffered water. The change in the UV absorption of receptor **19** can be used to determine the binding constants for substrate binding (adapted from ref. [51]).

dipeptides very efficiently in aqueous buffer ($K \approx 5 \times 10^4\ M^{-1}$) as could be shown by NMR- and UV-titration experiments. Dipeptides are bound up to 10 times more efficiently than simple amino acids ($K \approx 5–7 \times 10^3\ M^{-1}$). For the latter the association constants are similar to the simple guanidiniocarbonyl pyrrole based carboxylate receptors presented before [26]. Hence, the increase in stability for the dipeptides must be due to the additional binding sites within the complex (the H-bonds between the backbone amides and interactions with the imidazol group). Within the series of dipeptides studied the complex stability increases depending on the side chains present in the order Gly < Ala < Val. This might be surprising at the first glance as there are no specific binding sites for side chain interactions present in **19**. However, the increase in stability in this order is in good agreement with both the decreasing flexibility of the peptide and the increasing hydrophobicity of the side chains. For example, valine is known to induce peptide conformations that favor the formation of β-sheets [52]. As the interactions within the complex with **19** are similar to those found in a β-sheet, it is not surprising that Val–Val is bound better than Ala–Ala or Gly–Gly, respectively. Furthermore, within the complex the isopropyl side chains effectively shield the H-bonds between the backbone amides from the surrounding solvent thereby increasing their strength. The complexation properties of **19** are superior to any other dipeptide receptor reported so far and again mostly rely on electrostatic interactions.

7. Oligopeptide binding using combinatorial receptor libraries

This modelling based de novo approach is difficult to extend to larger substrates such as tetrapeptides. However, as mentioned

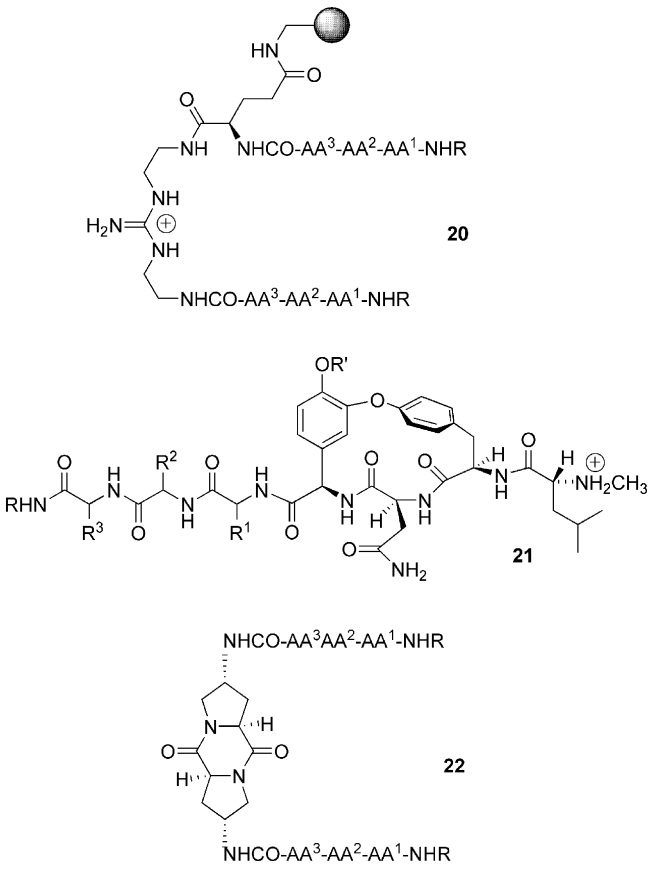


Fig. 19. Examples of peptide receptors used in combinatorial approaches with large, random libraries.

before, combinatorial chemistry is a powerful tool to identify supramolecular receptors for a specific target [50]. Most people especially in the beginning of combinatorial chemistry used rather large completely random libraries. This approach was pioneered by Still and co-workers [50d] for peptide binding in organic solvents in the early 1990s. In more recent years also peptide receptors which function in more polar solvents were identified this way (Fig. 19). For example, Kilburn and co-workers screened a library of 2197 guanidinium based flexible tweezer receptors **20** for tripeptide binding [53]. In aqueous solvent, efficient binding of a dye-labeled anionic tripeptide Glu(OtBu)–Ser(OtBu)–Val was observed ($K = 8 \times 10^4\ M^{-1}$). However, the deprotected substrate was not bound at all. This indicates that complex formation in this case is mainly driven by hydrophobic interactions most likely with the side chain protecting groups. Ellman and co-workers identified a synthetic Vancomycin analogue, a rigidified tripeptide composed of non-natural hydrophobic amino acids attached to Vancomycin's carboxylate binding site, from the screening of a library **21** with 39,304 members [54]. Complex formation heavily relied on hydrophobic interactions between the substrate and the non-natural amino acid building blocks incorporated into the library. More recently Wennemers et al. used tweezer receptors based on a diketopiperazine scaffold for peptide binding [55]. In this case individual receptors **22** were screened against a tripeptide substrate library with 24,389 members.

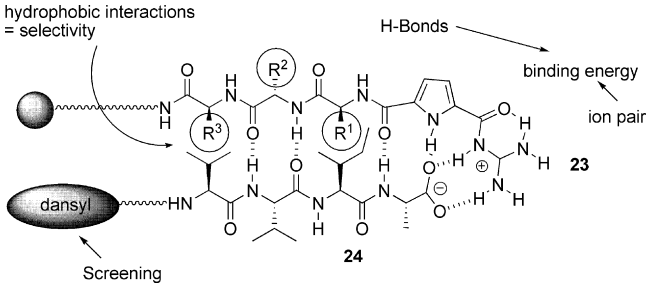
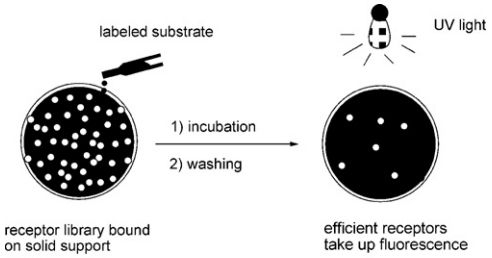


Fig. 20. A tripeptide based library of cationic guanidiniocarbonyl pyrrole receptors **23** designed for the binding of Ac-Val-Val-Ile-Ala-OH **24**, a tetrapeptide model representing the C-terminus of Aβ.

These examples show that combinatorial receptor libraries can be successfully used to identify potent receptors for oligopeptide binding. However, if completely random libraries are used the information obtained are rather limited compared to the time and effort needed to synthesize and screen the library. Only the most efficient hit-structures are detected from the qualitative screening and are then in the best case further investigated quantitatively in solution. However, all the information associated with the majority of the library members which do not bind or bind only weakly under the screening conditions is lost. The chances to find a hit-structure this way are limited. The focus in combinatorial chemistry in general is therefore shifting towards smaller libraries biased or privileged for a certain problem (e.g. by using structures derived from existing natural products for drug discovery as advocated independently, e.g. by Schreiber and co-workers [56] and Waldmann and co-workers [57]). We decided to also use small and specifically designed libraries in the context of supramolecular chemistry. In a focused combinatorial library such as **23** (Fig. 20) the chances to find a hit are much higher than in a complete random library as the structural diversity is already positively biased for a given problem, e.g. binding of a specific target. Hence, it is sufficient to use even small libraries with only a couple of hundreds different members.

Following this concept, we set out to find efficient receptors for tetrapeptidic substrates. As a first target the hydrophobic tetrapeptide, Ac-Val-Val-Ile-Ala-OH **24** was chosen [58]. This tetrapeptide represents the C-terminal sequence of the amyloid-β-peptide (Aβ) which is responsible for the formation of protein plaques within the brain of patients suffering from Alzheimer's disease [59]. This specific peptide sequence is thought to promote the formation of self-aggregated β-sheets of Aβ stabilized through a combination of H-bonds and hydrophobic interactions [60]. An artificial receptor which effectively binds to the model tetrapeptide Ac-Val-Val-Ile-Ala-OH **24** can therefore allow us to learn more about the molecular basis of the self-aggregation of the amyloid-peptide [61].

Our general design of a potential receptor **23** for this substrate is shown in Fig. 20. The ion pair between the carboxylate and the guanidiniocarbonyl pyrrole serves as a starting point for complex formation. An additional tripeptide unit attached to the pyrrole provides further binding sites for the formation of a hydrogen bonded antiparallel β-sheet with the backbone of the tetrapeptide



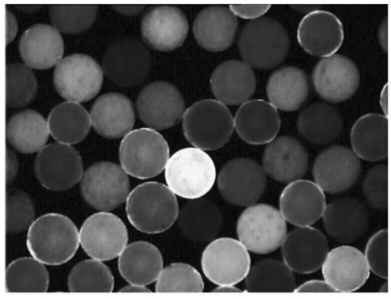


Fig. 21. (a and b) On-bead binding assay in water (5 μM bis–tris-buffer with pH 6). The strong fluorescence activity of some beads indicates selective binding of the tetrapeptide carboxylate **24** (2.5 μM) to some of the cationic receptors **23** within the library.

substrate **24**. In addition to these multiple electrostatic interactions, hydrophobic contacts between the amino acid side chains both in the substrate and the receptor should especially in aqueous solvents further stabilize the complex and also guarantee the necessary substrate selectivity. To identify which amino side chains in the receptor will be most efficient a solid phase bound library of 512 different but structurally related receptors **23** was synthesized using a standard Fmoc-protocol and a split-mix-approach [62]. In each of the three coupling steps eight different amino acids were used, specifically chosen to provide a large range of structurally varying hydrophobic or steric interactions. In the second step each of the various 512 different tripeptides thus obtained was coupled with our guanidiniocarbonyl pyrrole binding motif. The advantage of such a solid phase bound combinatorial receptor library is, besides the fast and time saving synthesis, that the whole library can be tested for a specific feature in a single experiment, in this case its binding properties towards the tetrapeptide substrate [50]. For this purpose a fluorescence label in form of a dansyl group was attached via a water soluble spacer to the N-terminus of the tetrapeptide substrate. After incubation of the library with this labeled substrate, a simple UV-assay can be used to identify efficient receptors (Fig. 21). Only those beads on which the attached receptor **23** is capable to bind the peptide **24** show the characteristic fluorescence of the dansyl group. All the other receptors which do not bind the peptide under the specific experimental conditions remain dark. Such binding assays showed that our one-armed cationic receptors **23** are indeed capable to efficiently bind the tetrapeptide even in water [58]. Interestingly, the non-charged methyl ester of the substrate shows only a weak and rather unselective binding to the receptor library, suggesting that side chain interactions alone are not strong enough to form a stable complex. However,

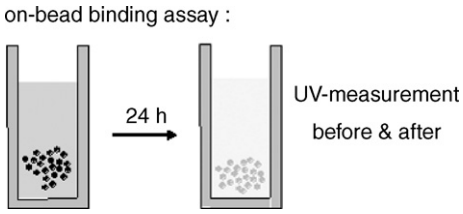


Fig. 22. Quantitative on-bead binding assay. By measuring the UV fluorescence before and after incubation the amount of substrate bound to the receptor on the bead and hence the binding constant can be calculated.

the negatively charged carboxylate substrate is selectively bound only by some and not all of the receptors, although the ion pairing with the guanidiniocarbonyl pyrrole unit is the same for all the different receptors within the library. Hence, the binding of the tetrapeptide by the receptors requires both electrostatic as well as hydrophobic interactions. Neither of these interactions alone is sufficiently strong to ensure complex formation in polar solution. The recognition of the tetrapeptide seems to be controlled by a fine balanced interplay between electrostatic and hydrophobic interactions.

Another advantage of using a small and focused combinatorial library is that the screening can also be performed quantitatively allowing a direct determination of the binding constants of all the various receptors on bead [63]. From the fluorescence intensity of the substrate in solution before and after incubation and the loading of the resin the association constants for each receptor can be calculated (Fig. 22). Even though such binding constants determined on a solid support are not the same and less accurate than data obtained in solution (for example, from NMR- or UV-titration experiments), a comparison of relative data within a series of related receptors can at least help rationalize aspects such as complex structure, stability and selectivity on a molecular basis. One can identify structural features that are associated with strong or weak binding. Which parts of our modular receptors are most important for binding or selectivity? What kind of binding sites, electrostatic or hydrophobic, in the various positions of the receptor is needed? In other words a supramolecular structure–binding relationship can be derived from binding data obtained on a solid support.

We have thus performed a detailed thermodynamic analysis for the binding of the tetrapeptide **24** in buffered water [58a]. As these data show, the binding is exceptionally strong with association constants of up to $10^4\,M^{-1}$ for the best receptors. Hence, these one-armed hosts belong to the most efficient peptide receptors in aqueous solvents reported so far. Furthermore, the selectivity of the receptors towards the tetrapeptide substrate is surprisingly high. The association constants for the various receptors differ by a factor of more than 100 among the library! Even small changes in the receptor structure have obviously pronounced effects on the binding properties. This also proves that even within such a small combinatorial library of only limited structurally diversity the binding selectivity can be rather high: a necessary prerequisite to also achieve selective binding of different tetrapeptides by this general receptor class.

A closer look at the binding data allows to correlate complex stability and receptor structure. For example, the quantitative

binding constants suggest that hydrophobic interactions of the receptor with the first amino acid residue of the substrate (Val) are mostly important. This is an excellent confirmation of previous studies of the Aβ self-aggregation which have shown that hydrophobic interactions with Val 39, which corresponds to the first amino acid of our tetrapeptide substrate, are especially important [64]. Furthermore, the best receptors identified in these screening experiments also inhibit the formation of amyloid plagues in vitro but only for Aβ (1–42) and not Aβ (1–40) which has the wrong C-terminal sequence [65]. For Aβ (1–42) with the correct C-terminus the receptors both retarded the formation of the amyloid plaques in vitro and also reduced the amount of fibrils formed significantly. This again underlines the feasibility of carefully chosen small bioorganic models for the analysis of more complex natural systems. Perhaps in the future new lead structures, for example, for drug development might result from such studies.

The binding of the amyloid tetrapeptide model **24** by these artificial receptors is based on an interplay of electrostatic and hydrophobic interactions. None of these two interactions alone is sufficient for substrate binding in water. Recently, we could show for the first time that also the binding of a non-hydrophobic tetrapeptide *N*-Ac-D-Glu-L-Lys-D-Ala-D-Ala-OH (EKAA) **26** in water is possible without the need for additional hydrophobic or metal–ligand interactions (Fig. 23) [66]. This tetrapeptide sequence is interesting in terms of its relevance to bacterial cell wall maturation [44]. During the synthesis of the bacterial cell wall, linear peptidoglycans are crosslinked via a transamidation reaction involving this tetrapeptide sequence which is also the point of attack of the glycopeptide antibiotic Vancomycin. This substrate is therefore not only challenging in terms of its highly flexible and polar character but also its biological relevance. Receptors such as **25** that selectively bind to this peptide sequence could be of interest to better understand the molecular basis of Vancomycin antibiotic activity and resistance.

Using again a quantitative on-bead screening of a medium-sized but focused combinatorial receptor library **25** with 512 members we were able to identify efficient receptors which bind the tetrapeptide **26** with $K > 10^4\ M^{-1}$ in buffered water according both to binding studies on bead and in free solution. Furthermore, from the quantitative analysis of all the 512 binding data from the on-bead screening a statistical quantitative structure activity relationship could be established. Using 49 physico-chemical parameters per variable amino acid position in the receptor a suitable mathematical QSAR model was set up (Fig. 24), which was then used first to analyze the non-covalent interactions within the complex. The data showed that at least in this case the binding is solely determined by electrostatic interactions. For example, a positively charged side chain especially at the position of the first amino acid in the receptor next to the carboxylate binding site has a strong positive effect on complex stability while a negatively charged side chain has a negative effect. Other properties such as hydrophobicity or van der Waals interactions, for example, had hardly any effect at all. They are of no importance here. Secondly, also based on the statistical QSAR model, a 15 times larger virtual library with 8000 members was set up and analyzed. Even within this much larger library no receptors with

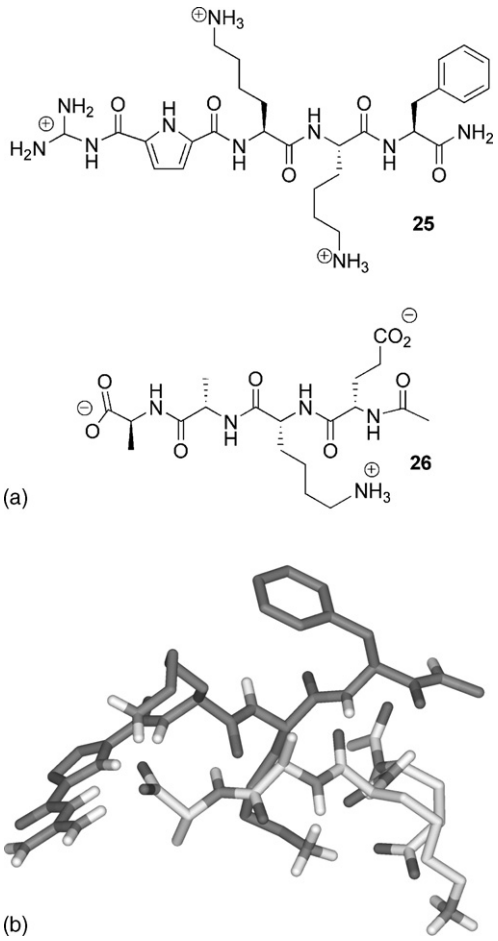


Fig. 23. (a and b) The polar tetrapeptide *N*-Ac-D-Glu-L-Lys-D-Ala-D-Ala-OH **26**, a model for a bacterial cell wall precursor and the calculated structure of its complex ($K = 17{,}100\ M^{-1}$ in buffered water) with a cationic guanidiniocarbonyl pyrrole receptor Gua-Lys-Lys-Phe-NH_2 **25** (Gua: guanidiniocarbonyl pyrrole cation) (adapted from ref. [67]).

significantly better binding properties than the best one identified from the experimental screening of our small and focused library are expected. Hence, the pure size of a library is not decisive but the library has to contain the correct diversity for a given problem. For example, in this case when electrostatic interac-

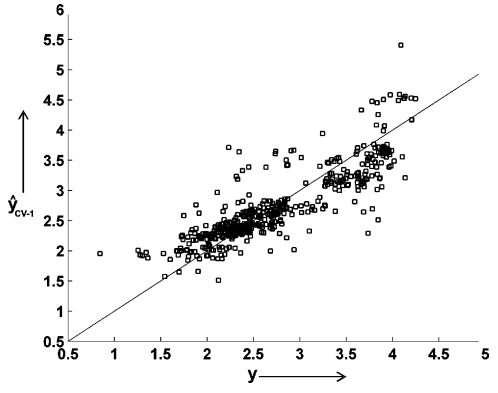


Fig. 24. Statistical analysis of all 512 quantitative binding data obtained from the on-bead screening of a combinatorial receptor library for binding of the polar tetrapeptide EKAA **26** in buffered water.

tions are responsible for the binding the library does not need to contain both aspartate and glutamate. One of them is sufficient to cover that specific property (negatively charged side chain). In this respect a carefully chosen subset of amino acids with varying structural and chemical properties is sufficient to set up a small but focused library. This is not only less time and cost consuming compared to a large but random library but also offers much more quantitative information as those small libraries can be thoroughly analyzed. The library output is then not restricted to a simple hit or non-hit output as in most qualitative screenings of large libraries but also allows detailed analyses in terms of structure–binding correlations which help to better understand and therefore also improve the receptor class under study.

We have also probed the substrate selectivity of this receptor library by screening a second tetrapeptide substrate, which has the inverse sequence AAKE **27** compared to our initial polar substrate EKAA **26** [67]. This inverse substrate is also bound by the guanidiniocarbonyl pyrrole receptor **25** with $K \approx 6000\,M^{-1}$ according to both on bead and solution studies. Even though the binding within the complex is mainly based on long ranging charge interactions and both substrate and receptor are rather flexible we observed a distinct substrate selectivity compared with tetrapeptide **26**. The inverse tetrapeptide **27** is bound in general two to three times less efficiently than the "normal" peptide **26**. Hence, these flexible one-armed cationic receptors **25** represent a very interesting and promising new class of artificial peptide receptors for the selective binding of polar tetrapeptides in water with significant potential for further development into peptide sensors, for example. First studies using an inverse approach (screening of a combinatorial substrate library with one receptor) indicate that also stereoselective binding of tetrapeptides in water is possible this way.

8. Conclusion and outlook

We have demonstrated here how ion pairing by guanidinium cations can be significantly improved by acylation and by incorporating additional H-bond donor sites into the recognition motif to allow complexation even in water. Thorough thermodynamic studies of "knock-out" analogues both experimentally and theoretically show that the improved performance relative to alkylguanidinium cations cannot be explained by simple models but is due to a subtle interplay of various factors (such as the acidity of the NH hydrogen atoms, the individual H-bond pattern and secondary interactions within the recognition motif). Such modified acylguanidinium cations can be used for various applications in the field of supramolecular (e.g. self-assembling zwitterions) or bioorganic chemistry (e.g. sensors, stereoselective recognition of amino acid carboxylates and peptides). To identify such receptors either rational approaches based on theoretical design can be used or combinatorial methods. If the use of designed binding motifs and combinatorial chemistry is combined then even small but focused libraries with a few hundred members can be successfully used to identify artificial receptors, e.g. for oligopeptide binding in water.

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