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Combinatorial libraries of diamidopyridine-derived 'tweezer' receptors and sequence selective binding of peptides

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Abstract—A library of diamidopyridine derived tweezer receptors with peptidic side-arms has been prepared. Screening experiments with the library and a dye-labelled tripeptide L-Glu–L-Ser–L-Val–OH showed excellent selectivity (strong binding of the dye-labelled guest to ~0.2% of resin-bound library members was observed) and led to the identification of a tweezer receptor structure, which was shown to bind the tripeptide with good selectivity over related tripeptide sequences. Analogous screening experiments with the side-chain protected tripeptide L-Glu(O^{t}Bu)–L-Ser(^{t}Bu)–L-Val–OH, showed little selectivity for any particular library member. The results are discussed in relation to previous screening experiments, using the same tripeptide guests, with related tweezer receptor libraries, which differ by one methylene in the separation of the diamidopyridine unit from the peptidic side-arms of the tweezer receptors.

1. Introduction

Since the beginnings of supramolecular chemistry, chemists have sought to design and synthesise receptors capable of binding selectively to specific substrates.[1] In many cases receptors for biological substrates have been targeted, in part driven by the desire simply to mimic nature's own ability to produce highly selective receptors, but also to provide model systems and insights into natural molecular recognition systems, and because of the practical applications that such receptors could have—e.g. as sensors, therapeutics and catalysts. The ability to design a receptor rationally, based on our ever-increasing understanding of the non-covalent interactions responsible for molecular recognition, and using the huge range of sophisticated methodology now available to the synthetic chemist, has always been an intellectually satisfying challenge. Much of our own research over the last 10 years has focused on the development of receptors for peptides, particularly for peptides with a free carboxylic acid terminus. We have had considerable success in this area, developing individual macrocyclic receptors, which incorporate a specific recognition site for the carboxylic acids[2] or carboxylates,[3] which show good sequence selectivity (e.g. for the important Ala–Ala–OH dipeptide sequence[2b]). However, as most practitioners in the field have found, the rational design and synthesis of novel receptors—and a stepwise, iterative approach to producing increasingly selective receptors—can be both time-consuming and frustrating, and the selective recognition of many biological molecules remains a considerable challenge.

In the 1990s, an alternative to the iterative approach to the identification of peptide receptors was pioneered by Still and co-workers.[4] Mirroring developments in medicinal chemistry, the combinatorial approach was used to prepare large libraries of possible receptor structures, which could then be screened to identify a receptor for a given substrate. In practice, Still et al. used a steroidal core to attach two variable peptide strands to produce libraries, which were screened to identify receptors for enkephalin-like peptides.[4] Since the original publications by Still there have been a few reports of the approach being used by other research groups,[5] and the reverse process—i.e. screening a single receptor with a library of peptide guests to identify possible substrates for the receptor—has been used quite extensively.[6] The latter strategy has been applied particularly to the identification of peptide substrates for acyclic 'tweezer' receptors or 'two-armed' receptors.[7] The structure of such tweezer receptors generally consists of a 'head group' or 'hinge', which is typically a conformationally restricted moiety that directs two functionalised substrate-binding arms. The advantage of such structures is that they are relatively easy to synthesise (in comparison with more conformationally restricted macrocyclic structures) and the work of several groups has demonstrated that, despite their conformational flexibility, such structures are capable of peptide recognition, both in organic[4,5a–c,f,6c–h] and aqueous[5c,6b] solvents. Whereas in many of these systems the head group plays only a limited role in the binding of the guest, we became interested in developing tweezer receptors[5a,b,6b] which incorporated a head group that can

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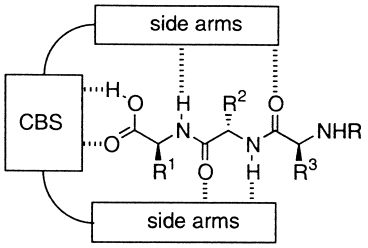


Figure 1. Schematic of tweezer receptor with a CBS.

bind specifically to carboxylic acid functionality (in analogy to our earlier macrocyclic peptide receptors), and with two peptidic arms. Such tweezers should be receptors for peptides with a carboxylic acid terminus, and it was anticipated that the peptidic arms might form (parallel) β-sheet-like interactions with the backbone of the peptidic guest (Fig. 1).

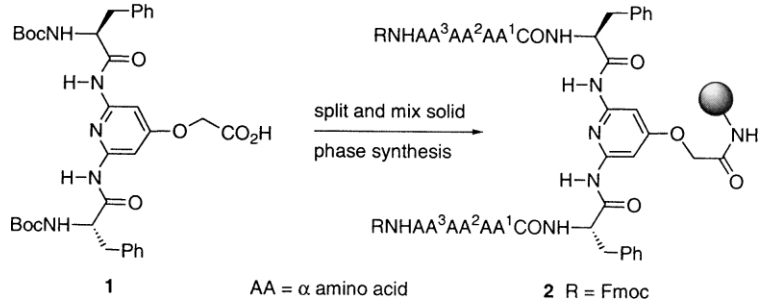
In pursuit of this idea, we have described the synthesis and screening of libraries of tweezer receptors, such as **2**, featuring a diamidopyridine as a carboxylic acid binding site (CBS).[5a,b] A diamidopyridine **1**, suitable for attachment to the solid phase, was prepared and incorporated two phenylalanine units as the starting point for the generation of the variable peptidic arms (Scheme 1).

The libraries were successfully screened with various dye-labelled tripeptides to identify tweezer receptors. Individual tweezers identified from the screening process could be resynthesised, and binding studies revealed that the tweezers bind peptides with binding constants 10^4–10^5 M^{-1} in $CHCl_3$. In order to investigate these systems in more detail, and to probe the effect of small structural changes to the library, we decided to create similar libraries, but using a diamidopyridine **5** with two β-alanine units, instead of phenylalanine units, as the starting point for the generation of the variable peptidic arms. By so doing the peptidic arms of the tweezer structure would be one methylene unit further removed from the CBS provided by the diamidopyridine. In this paper, we describe the results obtained in this new investigation.

2. Synthesis

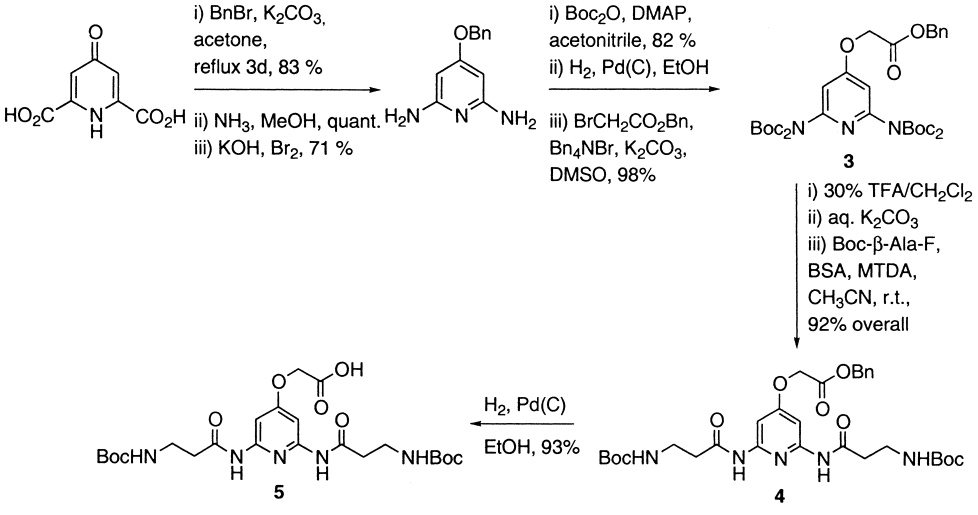
For the synthesis of the new tweezer libraries the previously described[5a] Boc protected diaminopyridine **3** was prepared in six steps from chelidamic acid (Scheme 2). After removal of the Boc protecting groups, the resulting diaminopyridine was preactivated with bis(trimethylsilyl)acetamide (BTSA) and then coupled with N-Boc β-alanine acid fluoride, using a methyl trimethylsilyl dimethylketene acetal (MTDA)[8] as a scavenger, to give the diamidopyridine **4** in excellent yield. Hydrogenolysis of the benzyl ester gave the corresponding acid **5**, suitable for attachment to the solid phase.

Acid **5** was attached to TentaGel resin (Rapp Polymere, 130 μm) prepared with a 10% loading of N-Boc phenylalanine



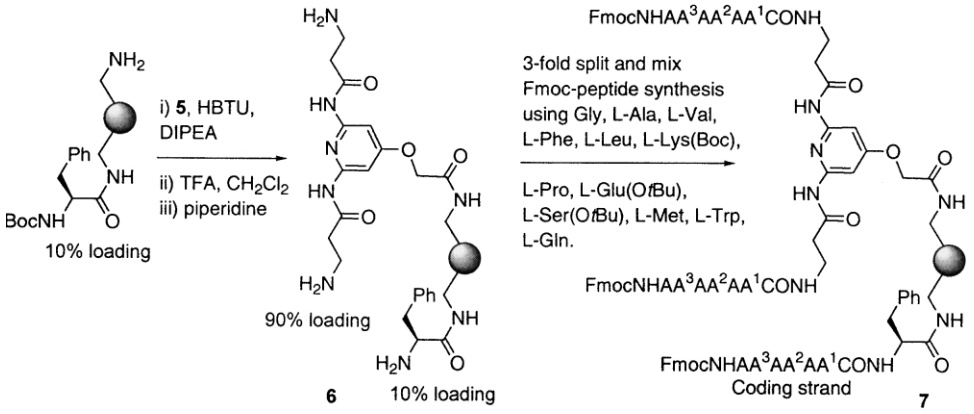
1 **2** R = Fmoc

Scheme 1.



3 **4** **5**

Scheme 2.



Scheme 3.

to serve as a coding strand.[9] Removal of the Boc protecting groups yielded a resin **6** ready for library generation.

A 1728 membered library of Fmoc protected tweezers **7** was prepared by a three-fold coupling of 12 Fmoc protected amino acids (Gly, L-Ala, L-Val, L-Phe, L-Leu, L-Lys(Boc), L-Pro, L-Glu(O^{t}Bu), L-Ser(^{t}Bu), L-Met, L-Trp, L-Gln) to the free amine groups of **6**, using the 'split and mix' strategy[10] (Scheme 3).

3. Screening experiments and binding studies

Screening experiments were carried out with the tweezer library using the dye-labelled peptide guests Red dye-spacer–L-Glu(O^{t}Bu)–L-Ser(^{t}Bu)–L-Val–OH **8**, and Red dye-spacer–L-Glu–L-Ser–L-Val–OH **9** (Fig. 2). These peptides were chosen as they had been used in our previous studies[5a,b] with the earlier libraries, and would allow a direct comparison of the screening results from both libraries **2** and **7**.

In a typical screening experiment a sample of ~10 mg (~9000 beads) of the library was equilibrated in a chosen solvent system for 24 h. Dye-labelled tripeptide, as a solution in the same solvent system, was added and incubated for a further 24 h. Beads were analysed in flat-bottomed glass pots under a Leica inverted DML microscope (magnification, 40×). The concentration of dye-labelled peptide guest could be increased to provide optimal selectivity as adjudged by the number of highly stained beads against a background of non- or lightly stained beads. Control experiments were carried out as in previous screening studies.[5a,b] Thus, we incubated the peptide guests with a simple peptide library directly attached to TentaGel resin (analogous to the coding strand). No evidence for any selective binding was observed in these experiments. Thus we conclude that any observed binding selectivity (of the dye-labelled guest peptide for the resin-bound library members) is not a consequence of interaction of the tripeptide guest simply with the peptide side arm of the tweezer receptor, or with the coding strand on the library beads. Furthermore, the simple acetylated red-dye **10** was incubated with the tweezer library, but again no selective binding to any of the resin-bound library

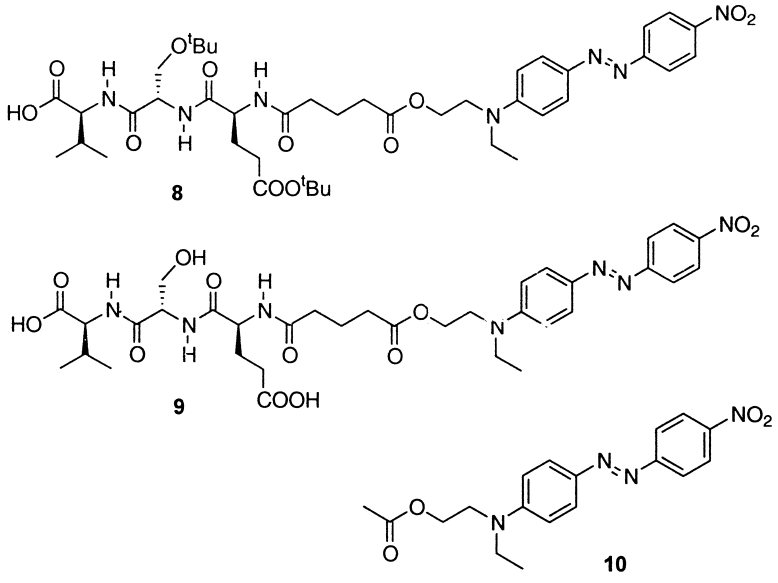


Figure 2. Peptides used in the screening experiments with library **2**.

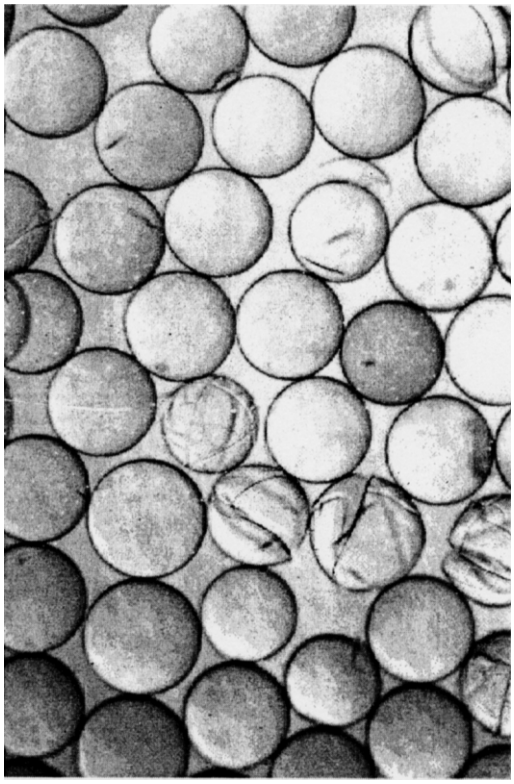


Figure 3. Portion of library **7**, after incubation with dye-labelled peptide **9**.

members was observed, confirming that the selectivity observed with the dye-labelled peptide guests are not a consequence of binding to the dye moiety alone.

When library **7** was incubated with the deprotected peptide red dye-spacer–L-Glu–L-Ser–L-Val–OH **9** in 1% DMSO:99% chloroform excellent binding selectivity was observed. Thus, at a peptide concentration of 18 μM, ~0.2% of beads were highly coloured after a 24 h incubation (Fig. 3). It was essential that the receptor library+peptide guest was left for >12 h to reach equilibrium, indicating that binding with the resin-bound receptors has relatively slow kinetics. The observed binding selectivity was also very solvent dependent as no beads remained highly stained after addition of 10% DMSO.

Seven highly coloured beads were taken from the screening experiment and sequenced by Edman degradation to identify the structures of the tweezer receptors on each bead. The sequencing results gave a high level of consensus with glutamine at the first position for five beads and at the third position for four beads, with the sequence Gln–*xxx*–Gln clearly favoured, and Val as the most common residue at the second position (Table 1).

Table 1. Sequencing data for seven dyed beads selected from screening experiment of Red dye-spacer–L-Glu–L-Ser–L-Val–OH **9** with library **7** in $CHCl_3$

Bead	AA^1	AA^2	AA^3
1	Gln	Val	Gln
2	Gln	Val	Gln
3	Gln	Val	Gln
4	Gln	Lys	Gln
5	Gln	Lys	Gln
6	Val	Ala	Glu(O^{t}Bu)
7	Val	Gln	Leu

Table 2. Sequencing data for five dyed beads selected from screening experiment of Red dye-spacer–L-Glu–L-Ser–L-Val–OH **9** with library **2** in $CHCl_3$

Bead	AA^1	AA^2	AA^3
1	Ala	Leu	Ala
2	Ala	Leu	Ala
3	Gln	Leu	Ala
4	Gln	Val	Ala
5	Met	Val	Ala

In comparison, previous screening experiments with peptide **9** and library **2** had given reasonable binding selectivity (~1% of beads were highly stained in the screening experiment),[5a] but not as high as that observed for peptide **9** with library **7**. The sequences identified from the former screening experiment (Table 2) have some similarities with the sequences identified from the latter (Table 1). Thus for peptide **9** and library **2**, Gln was found at the first position for two out of the five beads sequenced and Val, or structurally very similar Leu, was found consistently at the second position. At the third position, however, Ala was found for each bead. The similarity in sequences, at the first and second position, suggest that binding of the peptide **9**, by tweezers from libraries **2** and **7**, involve similar interactions with the tweezer side-arms.

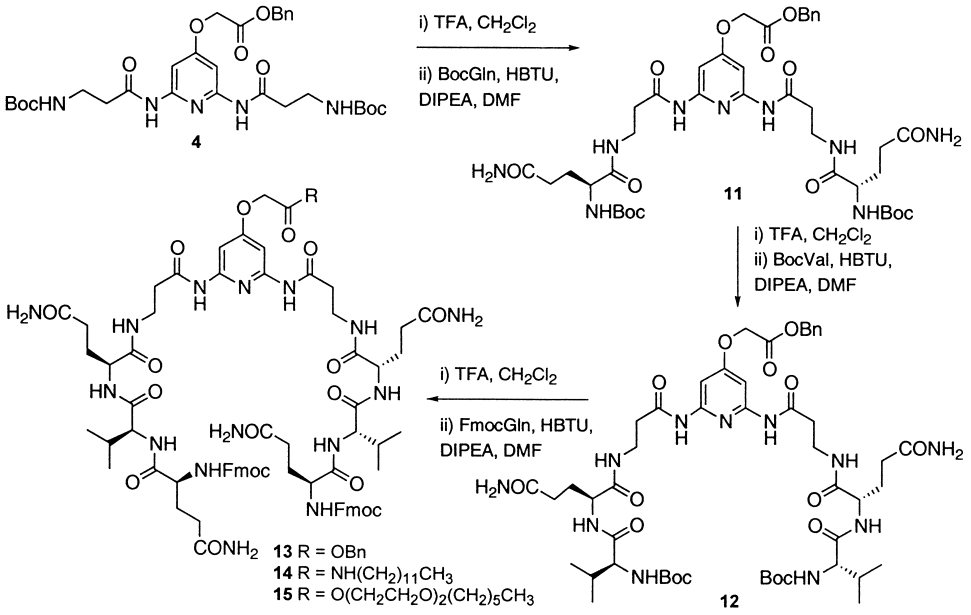
In contrast no binding selectivity was observed when library **7** was incubated with the protected peptide Red dye-spacer–L-Glu(O^{t}Bu)–L-Ser(^{t}Bu)–L-Val–OH **8** at a peptide concentration of 18 μM, and even at a peptide concentration of 100 μM only slight selectivity could be observed by inspection of the beads under the microscope.

These latter results are in marked contrast to the screening results obtained previously using the same peptide guest, **8**, with receptor library **2**.[5a] In that case excellent selectivity was observed and four beads were sequenced. Three beads gave identical results for the tweezer side-arm structure: Val–Ala–Pro; and the fourth bead yielded the closely related sequence Val–Met–Pro (Table 3).

As in previous studies, we sought to establish that the observed binding with resin bound tweezers was also operating in free solution. Thus tweezer **13** was prepared by solution-phase synthesis (Scheme 4), as a single compound, with tetrapeptide side-arms β-Ala–Gln–Val–Gln, corresponding to the most commonly found sequence in the screening experiments.

Table 3. Sequencing data for four dyed beads selected from screening experiment of Red dye-spacer–L-Glu(O^{t}Bu)–L-Ser(^{t}Bu)–L-Val–OH **8** with library **2** in $CHCl_3$

Bead	AA^1	AA^2	AA^3
1	Val	Ala	Pro
2	Val	Ala	Pro
3	Val	Ala	Pro
4	Val	Met	Pro

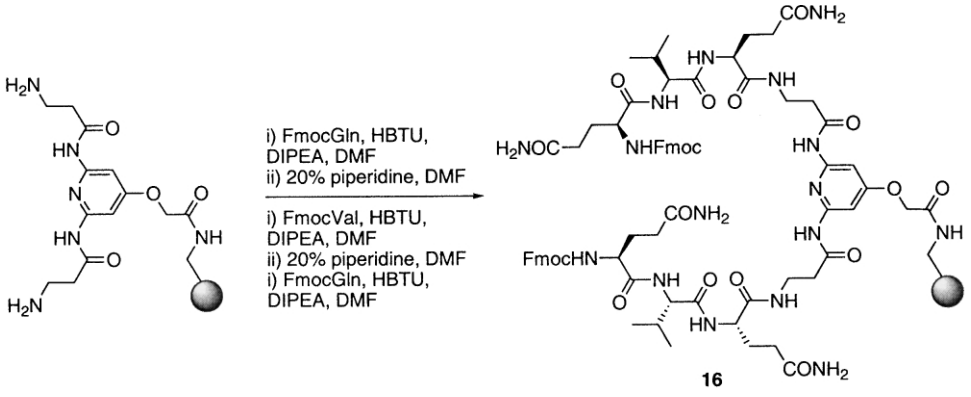


Scheme 4.

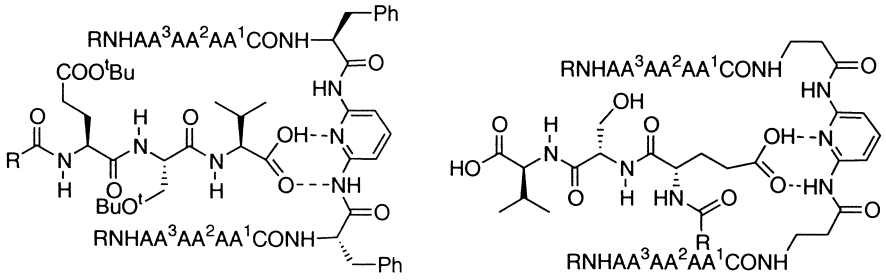
The synthesis of **13**, using sequential coupling of Boc-amino acids and TFA deprotection, was straightforward since each of the intermediates could be isolated cleanly by precipitation, without use of chromatography. However, and not too surprisingly, the final compound **13** proved to be completely insoluble in $CDCl_3$, which precluded any binding studies in this solvent. Tweezer **13** could be dissolved in DMSO, but there was no evidence for any binding in this solvent, which is consistent with the screening experiments where addition of as little as 10% DMSO to the chloroform solution resulted in a complete loss of binding. Derivatives **14** and **15** of the tweezer receptor were also prepared, incorporating a long (dodecyl)alkyl chain or a diethyleneglycol hexyl ether, but these derivatives were also insoluble in $CHCl_3$.

As an alternative to binding studies with the tweezer receptor in free solution, we established that the resin bound receptor **16**, with the same tetrapeptide side-arms, β-Ala–Gln–Val–Gln, as **13**, is genuinely selective. Thus portions of the resin-bound tweezer were added to solutions of the peptides **8** and **9**, and of the enantiomer Red dye-spacer–D-Glu–D-Ser–D-Val–OH, and the extent to which the peptides were absorbed onto the resin beads was determined.[11] Thus the receptor was resynthesised on solid phase, to give **16** with an estimated loading of 0.05 mmol of receptor/g resin (Scheme 5).

Addition of 3.0 mg of resin-bound tweezer receptor **16** (~0.15 μmol of receptor) to a solution of Red dye-spacer–L-Glu–L-Ser–L-Val–OH **9** (0.5 mL, 0.3 mM, ~0.15 μmol of peptide) in $CHCl_3$ led to absorption of 90% (±5%) of the peptide as adjudged by HPLC (using the acetylated red dye **10** as an internal standard) after 24 h incubation. (After only 2 h the amount of peptide absorbed by the resin was ~50%, again indicating the relatively slow rate at which binding equilibrium is reached with the resin-bound receptors). Since the addition of acetyl-capped tentagel resin beads led to no significant absorption of the peptides or acetylated red dye **10**, this provides an estimate[12] of the binding constant of $K_{ass} \sim 3 \times 10^5\ M^{-1}$. Addition of the same amount of resin-bound tweezer receptor (~0.15 μmol of receptor) to an equivalent solution of the side-chain protected peptide **8** (Red dye-spacer–L-Glu(O^{t}Bu)–L-Ser(^{t}Bu)–L-Val–OH,



Scheme 5.



(a) binding the sidechain protected peptide **8** with the C-terminal carboxylic acid

(b) binding the sidechain deprotected peptide **9** with the glutamic acid sidechain carboxylic acid

Figure 4.

0.5 mL, 0.3 mM, ~0.15 μmol of peptide) led to a absorption of ~20% (±3%) of the peptide, giving an estimated K_{ass}~1×10^3 M^{-1}. Thus it can be concluded that the resin-bound tweezer receptor **16** binds the peptide **9** >100 times more strongly than side-chain protected analogue **8**, which is consistent with the screening experiments. Identical experiments with the enantiomeric peptide Red dye-spacer–D-Glu–D-Ser–D-Val–OH, led to absorption of ~55% (±10%) of the peptide, giving an estimated K_{ass}~9×10^3 M^{-1}, suggesting that resin-bound tweezer receptor **13** binds the L-configured peptide **9** ~30 times more strongly than the enantiomer.

4. Discussion

The screening of the combinatorial library of diamidopyridine derived tweezer receptors **7** led to the identification of a relatively limited number of tweezer structures from a sizeable library, which appear to bind the chosen peptide guest **9**, while attached to the solid-phase. The selective binding properties of one of these receptors **16**, while attached to the solid-phase, is demonstrated by the ability to discriminate between peptide **9** and the side-chain protected analogue **8** by a factor of ~100:1, and by the ability to discriminate between peptide **9** and its enantiomer by a factor of ~30:1. The screening results using the same peptide guest **9**, with the two receptor libraries **2** and **7**, give sequences with clear similarities at the first and second positions, which suggest that there are similar interactions between the backbone of the peptide guest and the tweezer receptor side-arms. The side-chain protected peptide **8**, however, behaves very differently with the two libraries **2** and **7**, with excellent selectivity observed in the screening experiments with library **2** and no apparent selectivity in the screening experiments with library **7**. A possible explanation for these observations is that, for the side-chain protected peptide **8** with receptors derived from amidopyridine derivative **1** (library **2**), binding involves a strong amidopyridine–carboxylic acid interaction, which places the backbone of the peptide guest in a suitable position to bind with the tweezer side-arms of the receptor—possibly using parallel β-sheet-like interactions (Fig. 4a). In comparison, introduction of an additional methylene group to the side-arm of the receptors (i.e. using library **7** derived from amidopyridine derivative **5**), disrupts the alignment of the backbone of the peptide guest in relation to the side-arms of the receptor, and no selective binding is observed.

Binding of the deprotected peptide **9**, on the other hand, is complicated by the fact that *either* the terminal carboxylic acid moiety of the peptide *or* the side-chain carboxylic acid moiety from the glutamic acid may bind to the amidopyridine. If the glutamic acid side-chain carboxylic acid is used, the link from the carboxylic acid unit to the rest of the peptide backbone is both longer and more flexible, such that the increased separation of the amidopyridine unit and the receptor side-arms in library **7** may now be more appropriate for binding the peptide guest, but the increased flexibility of the side-chain carboxylic acid also allows binding with tweezers from library **2** (Fig. 4b). Unfortunately, although we have been able to prepare individual tweezer receptors identified from the screening experiments, the poor solubility of the receptors off-resin, and in the non-polar solvents suitable for binding (i.e. chloroform) has precluded any detailed studies (e.g. by NMR) on the structure of the receptor-substrate complex, so that the proposed differences in binding can not be confirmed experimentally. Indeed, in the present case the complete insolubility of tweezer receptor **13** in chloroform means that no binding in free solution can be demonstrated. This is clearly a limitation of this type of receptor and begs the question as to whether the binding properties of these systems, while resin-bound, is influenced by the environment created by the solid support.

5. Conclusions and outlook

Our studies described here further demonstrate the power of the combinatorial approach for identifying receptors for a chosen substrate, but also expose some of the limitations of the approach when using diamidopyridine derived tweezers. It is clear, from this and earlier work,[5a,b] that such diamidopyridine derived tweezers, despite their inherent flexibility, can act as receptors for specific peptide sequences, particularly when attached to the solid-phase. These receptors may therefore have applications, particularly in the development of chromatographic supports for separation or purification of peptides. However, the diamidopyridine–carboxylic acid interaction is probably too weak to promote peptide binding

by the tweezer receptors other than in relatively non-polar solvents, in which the receptors may have poor solubility—thus limiting their usefulness. There is also very little information regarding the actual mode of binding either in free solution or while the receptor is still attached to the solid-phase. Studies in our laboratory are aimed at answering the unresolved questions regarding the mode of binding of this class of tweezer receptors, and we are also evaluating the binding properties of monolayers of such receptors while attached to a surface. However, it is clearly desirable to extend the concept of screening receptor libraries to libraries suitable for peptide recognition in water. Successful realisation of this objective, and a clear understanding of the structure of resulting tweezer-substrate complexes formed in water, has considerable potential for providing model systems of peptide–peptide interactions and application to a range of therapeutic problems. Work in our laboratories is addressing this question, using libraries of tweezer receptors incorporating a guanidinium as the CBS,[6b] and results will be reported in the near future.

6. Experimental

6.1. Materials and general methods

Whenever possible all solvents and reagents were purified according to literature procedures. Solvents for peptide syntheses were purchased from Rathburn Chemicals, HPLC grade solvents from Riedel-de-Haën. TentaGel-*S*-NH_2 resin was used as solid support in screening experiments and in peptide syntheses and purchased from Rapp Polymere, Germany. Peptide and library syntheses on solid phase were performed in glass vessels with scinter frits or polypropylene filtration tubes with polyethylene frits on a Visiprep SPE Vacuum Manifold from Supelco. The reaction containers were agitated either on a shaker (Stuart Scientific Flash Shaker SF1) or on a blood tube rotator (Stuart Scientific Blood Tube Rotator SB1). Thin layer chromatography (TLC) was performed on aluminium-backed plates Merck silica gel 60 F_{254}. Sorbsil C60, 40–60 mesh silica was used for column chromatography. Infrared spectra were recorded on a Perkin–Elmer 1600 FT-IR spectrophotometer or on a Bio-Rad FT-IR spectrometer. Proton NMR spectra were obtained at 300 MHz on a Bruker AC 300 and at 400 MHz on a Bruker DPX 400. Carbon NMR spectra were recorded at 75 MHz on a Bruker AC 300 and at 100 MHz on a Bruker DPX 400. Chemical shifts are reported in ppm on the d-scale relatively to TMS as internal standard or to the solvent signal used. Coupling constants are given in Hz. The multiplicities of the signals were determined using the distortionless enhancement by phase transfer (DEPT) spectral editing technique. Mass spectra were obtained on a VG analytical 70-250-SE normal geometry double focussing mass spectrometer. All electrospray (ES) spectra were recorded on a Micromass Platform quadrupole mass analyser with an ES ion source using acetonitrile as solvent.

6.1.1. Benzyl 2-[(2,6-bis(*N-tert*-butoxycarbonyl-β-alanyl-amino)-4-pyridyloxy]acetate (4). TFA (6.4 mL) was added dropwise to a solution of benzyl 2-{(2,6-bis[bis(*tert*-butyloxycarbonyl)amino]-4-pyridyloxy}acetate **3** (1.5 g, 2.2 mmol) in CH_2Cl_2 (32 mL) and the resulting mixture was stirred for 2 h. The solution was concentrated and the trituration of the resulting oil with diethyl ether gave the bis-TFA salt as a white solid. The salt was suspended in an aqueous solution of potassium carbonate (10%) and extracted with dichloromethane to give the free 2,6 diamino pyridine. The diamine (0.74 g, 2.7 mmol) was dissolved in dry acetonitrile (63 mL). After addition of *N*,*O*-bis(trimethylsilyl)acetamide (0.84 mL, 2.7 mmol) the reaction mixture was strirred at room temperature for 2 h. *N-tert*-Butoxycarbonyl-β-alanyl fluoride (1.58 g, 13.5 mmol) and MTDA (1.68 mL, 13.5 mmol) were added and the reaction mixture was stirred for 20 h at room temperature. The solvent was removed under reduced pressure and the residue was redissolved in dichloromethane (300 mL). The organic layer was washed with an aqueous solution of sodium hydrogencarbonate (5%, 300 mL) and water (300 mL) and dried over magnesium sulfate. The solvent was evaporated and the resulting residue was purified by column chromatography (dichloromethane+2% methanol) to give diamidopyridine **4** as colourless foam (1.53 g, 92%). Analytical data: ^{1}H NMR (400 MHz, [D_6] DMSO):10.00 (s, 2H, NHCO), 7.39–7.33 (m, 7H, Ar*H*, C_4H_2N), 6.79 (br s, 2H, NHBoc), 5.21 (s, 2H, OCH_2Ar), 4.89 (s, 2H, OCH_2CO), 3.21 (q, *J*=6.5 Hz, 4H, CH_2NHBoc), 2.55, (t, *J*=6.5 Hz, 4H, $NHCOCH_2$), 1.36 (s, 18H, $(CH_3)_3C$); ^{13}C NMR (100 MHz, [D_6] DMSO) δ=170.5(0), 167.9(0), 166.3(0), 155.5(0), 151.3(0), 135.5(0), 128.4(1), 128.1(1), 128.0(1), 95.6(1), 79.2(0),77.6(0), 66.2(2), 64.5(2), 36.6(2), 36.2(2), 28.2(3); LRMS (ES^+): *m*/*z* (%)=616.2 (100) $[M+H]^+$, 638.2 (10) $[M+Na]^+$; HRMS calcd for $C_{30}H_{41}N_5O_9$ 615.29043, Found 615.29035; elemental analysis calcd (%) for $C_{30}H_{41}N_5O_9$·MeOH: C 57.48, H 7.00, N 10.81, O 23, Found: C 57.26, H 6.54, N 11.23.

6.1.2. 2-[2,6-Bis(*N-tert*-butoxycarbonyl-β-alaninyl-4-pyridyloxy)acetic acid (5). 10% Palladium on charcoal (1.1 g) was added to a solution of benzyl ester **4** (1.22 g, 2 mmol) in ethanol (25 mL). The reaction mixture was stirred vigorously for 18 h at room temperature under a hydrogen atmosphere. The catalyst was separated by filtration through a plug of celite. Evaporation of the solvent and drying of the residue at high vacuum yielded the corresponding carboxylic acid **5** as a colourless solid (1.05 g, 99%). Analytical data: mp 60°C; IR ν_{max}=3400, 1686.1, 1654.3, 1583, 1158.7, 848.5 cm^{-1}; ^{1}H NMR (400 MHz, [D_6] DMSO): δ=10.14 (s, 2H, NHCO), 7.32 (s, 2H, C_4H_2N), 6.81 (br s, 2H, NHBoc), 4.71 (s, 2H, OCH_2CO), 3.21 (4H, t, *J*=6.5 Hz, CH_2NHBoc), 2.55 (4H, t, *J*=6.5 Hz, $NHCOCH_2$), 1.36 (18H, s, $(CH_3)_3C$); ^{13}C NMR (100 MHz, [D_6] DMSO) δ=171.0(0), 169.4(0), 167.0(0), 155.7(0), 151.1(0), 95.6(1), 77.9(0), 64.6(2), 38.2(2), 36.4(2), 28.5(3); LRMS (ES^+): *m*/*z* (%)=526.3 (100) $[M+H]^+$, 548.2 (20) $[M+Na]^+$, 1051.4 (10) $[2M+H]^+$; HRMS calcd for $C_{23}H_{35}N_5O_9$: 525.24348, Found 525.24341

6.1.3. Synthesis of the tweezer receptors library. A solution of Boc–Phe (9.8 mg, 0.037 mmol), PyBOP (19.2 mg, 0.037 mmol), HOBt (6 mg, 0.037 mmol) in DMF (1 mL) was added to Tentagel–NH_2 resin (1.28 g, 0.37 mmol, NH_2), preswollen in DMF (2 mL) followed by DIPEA

(29 μL, 0.166 mmol), and the resulting suspension was shaken for 20 h. A solution of acid **5** (220 mg, 0.417 mmol), PyBOP (217 mg, 0.417 mmol), HOBt (56.3 mg, 0.417 mmol) in DMF (2 mL) was added to the resin, followed by DIPEA (303 μL, 1.74 mmol) and the suspension was shaken for 20 h. Any remaining amine residues were capped by treating the reisn with an excess of acetic anhydride. The resin was filtered and washed with CH_2Cl_2 (3×5 mL), DMF (3×5 mL), CH_2Cl_2 (3×5 mL). A qualitative ninhydrin test was negative. Subsequent Boc deprotection was achieved with a solution of TFA (30%) in DCM for 2 h. The resin was washed with CH_2Cl_2 (3×5 mL), DMF (3×5 mL), CH_2Cl_2 (3×5 mL) and washed with a solution of DIPEA (10%) in DCM, MeOH (3×5 mL) and Et_2O (5×5 mL) and dried in vacuo. The tweezer library was then prepared, using three cycles of split and mix synthesis. Thus the resulting resin was divided in 12 equal portions. To each resin portion one of the following Fmoc amino acids was added: L-Ala, L-Gln, L-Glu(O^{t}Bu), Gly, L-Leu, L-Lys(Boc), L-Met, L-Phe, L-Pro, L-Ser(^{t}Bu), L-Trp, L-Val (0.08 mmol amino acid per resin portion), along with HBTU (30 mg, 0.08 mmol) and DIPEA (47 μL, 0.27 mmol) in DMF (2 mL). The reaction mixtures were shaken for 18 h. Qualitative ninhydrin test were carried out to check all transformations were complete. The resin was mixed and the terminal Fmoc-groups removed with 20% piperidine in DMF. The resin was split again into 12 equal portions and the procedure repeated twice in order to build up the tweezer receptor library **7**.

6.1.4. Benzyl 2-(3,5-di{[3-({5-amino-2-[*tert*-butoxycarbonyl)amino]-5-oxopentanoyl}amino)propanoyl]amino}-phenoxy)acetate (11). TFA (2 mL) was added dropwise to a solution of the diamidopyridine **4** (0.16 g, 0.26 mmol) in CH_2Cl_2 (4 mL) and the resulting mixture was stirred for 2 h. The solution was concentrated and the trituration of the resulting oil with diethyl ether gave the bis TFA salt as a white solid, which was dissolved in DMF (2 mL). Boc–Gln (0.196 g, 0.76 mmol), HBTU (0.3 g, 0.76mmol) and DIPEA (0.23 mL, 1.32 mmol) were added and the resulting mixture stirred for 18 h. The precipitate formed was isolated by centrifugation to give the diamidopyridine **11** as a white solid (0.16 g, 70%). Analytical data: mp >240°C; ^{1}H NMR (400 MHz, [D$_6$] DMSO) δ=10.05 (s, 2H, NHpyr), 7.82 (br s, 2H, N*H*CH$_2$CH$_2$), 7.41–7.31 (m, 7H, ArH+pyrH), 7.22 (s, 2H, CON*H$_a$*H$_b$), 6.82 (d, *J*=7.5 Hz, 2H, N*H*Boc), 6.72 (s, 2H, CONH$_a$*H$_b$*), 5.21 (s, 2H, C*H*$_2$Ar), 4.88 (s, 2H, OCH$_2$CO), 3.84 (m, 2H, C*H*NHBoc), 3.38 (m, 4H, CH$_2$C*H*$_2$NH), 2.57 (m, 4H, C*H*$_2$CH$_2$NH), 2.07 (m, 4H, C*H*$_2$CONH$_2$), 1.81 (m, 2H, C*H$_a$*H$_b$CHNHBoc), 1.66 (m, 2H, CH$_a$*H$_b$*CHNHBoc), 1.35 (s, 18H, C(CH$_3$)$_3$). ^{13}C NMR (100 MHz, [D$_6$] DMSO) δ=173.2(0), 171.2(0), 170.1(0), 167.36(0), 165.7(0), 154.7(0), 150.8(0), 134.9(0), 127.8(1), 127.6(1), 127.4(1), 95.1(1), 77.5(0), 65.7(2), 63.9(2), 54.3(2), 53.5(1), 35.6(2), 34.2(2), 31.03(2), 27.6(3). LRMS (ES$^+$): *m*/*z* (%)=872.3 (80) [M+H]$^+$, 894.3 (10) [M+Na]$^+$.

6.1.5. Benzyl 2-(3,5-di[(3-{[5-amino-2-({2-[(*tert*-butoxycarbonyl)amino]-3-methylbutanoyl}amino)-5-oxopentanoyl]amino}propanoyl)amino]phenoxy}acetate (12). TFA (2 mL) was added dropwise to a solution of the diamidopyridine 2 (0.16 g, 0.18 mmol) in CH_2Cl_2 (4 mL) and the resulting mixture was stirred for 2 h. The solution was concentrated and the trituration of the resulting oil with diethyl ether gave the bis-TFA salt as a white solid, which was dissolved in DMF (2 mL). Boc–Val (0.12 g, 0.55 mmol), HBTU (0.21 g, 0.55 mmol) and DIPEA (0.16 mL, 0.93 mmol) were added and the resulting mixture stirred for 18 h. The precipitate formed was isolated by centrifugation to give the diamidopyridine **12** as a white solid (0.14 g, 73%). Analytical data: mp decompose >210°C; ^{1}H NMR (400 MHz, [D$_6$] DMSO) δ=10.04 (s, 2H, NHCO), 7.96 (br s, 2H, br CH$_2$CH$_2$N*H*CO), 7.82 (d, *J*=7 Hz, 2H, CHN*H*CO), 7.42–7.32 (m, 7H, ArH+pyrH), 7.19 (s, 2H, CON*H$_a$*H$_b$), 6.74 (m, 4H, N*H*Boc+CONH$_a$*H$_b$*), 5.21 (s, 2H, CH$_2$Ar), 4.89 (s, 2H, OCH$_2$CO), 4.22 (br s, 2H, C*H*CH(CH$_3$)$_2$), 3.79 (br s, 2H, C*H*CH$_2$CH$_2$CONH$_2$), 3.3 (m, 4H, NHCOCH$_2$C*H*$_2$), 2.57 (m, 4H, NHCOC*H*$_{2>}$CH$_2$), 2.05 (m, 4H, C*H*$_2$CONH$_2$), 1.94 (m, 2H, C*H*(CH$_3$)$_2$), 1.82 (m, 2H, C*H$_a$*H$_b$CH$_2$CONH$_2$), 1.72 (m, 2H, CH$_a$*H$_b$*CH$_2$CONH$_2$), 1.38 (s, 18H, C(CH$_3$)$_3$), 0.82 (d, *J*=6.5 Hz, 6H, CH(C*H*$_3$)$_2$), 0.8 (d, *J*=6.5 Hz, 6H, CH(C*H*$_3$)$_2$); ^{13}C NMR (100 MHz, [D$_6$] DMSO) δ=173.7(0), 171.2(0), 170.6(0), 168.0(0), 166.4(0), 162.4(0), 155.6(0), 151.4(0), 135.6(0), 128.5(1), 128.2(1), 128.1(1), 95.7(1), 78.2(0), 64.7(2), 62.9(2), 57.1(1), 51.8(1), 35.6(2), 34.3(2), 31.0(2), 30.3(1), 30.1(2), 28.2(3), 19.3(3), 18.1(3); MS (ES$^+$): *m*/*z* (%)= 1070.1 (100) [M+H]$^+$, 1091.9 (40) [M+Na]$^+$.

6.1.6. Benzyl 2-{3,5-di[(3-{[5-amino-2-({2-[(5-amino-2-{[(9*H*-9-fluorenylmethoxy)carbonyl]amino}-5-oxopentanoyl)amino]-3-methylbutanoyl}amino)-5-oxopentanoyl]-amino}propanoyl)amino]phenoxy}acetate (13). TFA (1 mL) was added dropwise to a solution of the diamidopyridine **12** (0.08 g, 0.075 mmol) in CH_2Cl_2 (3 mL) and the resulting mixture was stirred for 2 h. The solution was concentrated and the trituration of the resulting oil with diethyl ether gave the bis-TFA salt as a white solid, which was dissolved in DMF (2 mL). Fmoc–Gln (0.06 g, 0.16 mmol), HBTU (0.06 g, 0.16 mmol) and DIPEA (0.05 mL, 0.3 mmol) were added and the resulting mixture stirred for 18 h. The precipitate formed was isolated by centrifugation to give the diamidopyridine **13** as a white solid (0.08 g, 70%). Analytical data: mp decompose >240°C; ^{1}H NMR (400 MHz, 313 K, [D$_6$] DMSO) δ=9.94 (s, 2H, pyrN*H*), 7.96 (d, *J*=7.5 Hz, 2H, NH), 7.90 (d, 4H, *J*=7.5 Hz, fluorenylH), 7.85 (t, *J*=6 Hz, 2H, CH$_2$CH$_2$N*H*CO), 7.75–7.70 (m, 6H, NH+fluorenylH), 7.53 (d, 2H, NH), 7.45–7.32 (m, 15H, ArH+fluorenylH+pyrH), 7.24 (s, 2H, CON*H$_a$*H$_b$),7.16 (s, 2H, CON*H$_c$*H$_d$), 6.72 (s, 2H, CONH$_a$*H$_b$*), 6.67 (s, 2H, CONH$_c$*H$_d$*), 5.10 (s, 2H, OCH$_2$Ph), 4.79 (s, 2H, OCH$_2$CO), 4.16–4.07 (m, 10H, C*H*CH(CH$_3$)$_2$, C*H*CH$_2$CH$_2$CONH$_2$, C*H*C*H*$_2$fluorenyl), 3.94 (m, 2H, C*H*CH$_2$CH$_2$CONH$_2$), 3.40 (m, 4H, CH$_2$C*H*$_2$NH), 2.20–1.70 (m, 14H, C*H*$_2$CH$_2$NH+C*H*(CH$_3$)$_2$+C*H*$_2$C*H*$_2$CONH$_2$), 0.85 (d, 6H, CH$_3$), 0.83 (d, 6H, CH$_3$); ^{13}C NMR (100 MHz, [D$_6$] DMSO) δ=173.9(0), 173.7(0), 171.1(0), 170.7(0), 170.5(0), 167.9(0), 166.3(0), 157.3(0), 155.9(0), 151.3(0),143.8(0), 140.7(0), 135.5(0), 128.4(1), 128.1(1), 128.0(0), 127.6(0), 127.1(1), 125.3(1), 120.1(0), 95.7(1), 66.2(2), 65.7(2), 64.5(2), 57.5(1), 54.3(1), 52.3(1), 46.6(1), 36.1(2), 34.7(2), 31.6(2), 31.4(2), 30.5(1), 27.9(2), 27.7(2), 19.2(3), 17.8(3), MS (TOF LD+): *m*/*z* (%)= 1571.07 (100) [M+H]$^+$, 3301.65 (30) [2M+H]$^+$.

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