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Simple Synthetic Receptors that Bind Peptides in Water

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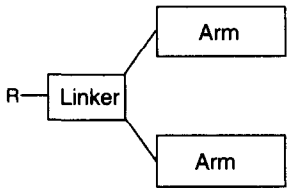
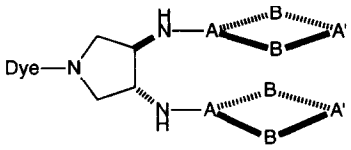
Abstract: *A simple two-armed receptor has been prepared that binds certain peptides sequence-selectively in water at pH 4.* © 1997 Elsevier Science Ltd.

Significant progress is being made in the design and preparation of sequence-selective synthetic receptors for peptides.[1] While the original peptide-binding receptors had complex, cage-like structures characteristic of typical host molecules, simpler structures have recently been found that also possess highly sequence-selective peptide-binding properties. The way we identify such structures involves the synthesis of prototypes of given receptor designs and then screening those prototypes for binding with a large combinatorial library of peptides. We are looking for those designs that not only bind peptides but also tend to bind them with high sequence-selectively. The ultimate objective is to identify receptor designs that can be prepared as large combinatorial libraries so that highly selective receptors for any given peptidic (or other) substrate can be selected from the library.

One class of simple peptide-binding molecules has been described as a "two-armed receptor" and its general structure is sketched below. In this structure, the *linker* is typically a conformationally restricted moiety that covalently bonds and directs two functionalized, substrate-binding *arms* toward one another to form a binding cleft.[2,3] While the general diagram below could well be applied to many well-known C_2-symmetric chiral reagents and tweezer-like host molecules,[4] there is no need that the two arms be identical and, indeed, one of the main attractions of this motif is its use in libraries of unsymmetrical two-armed receptors that could be assembled combinatorially from a diverse set of linkers and arms.



Two-Armed Receptor



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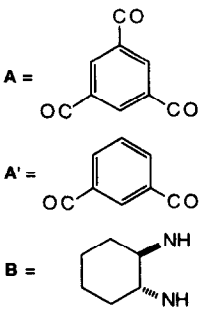
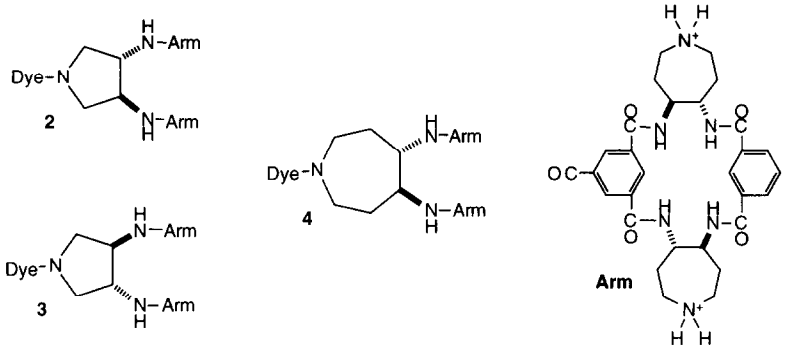


One two-armed receptor we have studied extensively is shown above as **1** and its arms are macrocyclic oligomers of isophthalic acids (**A, A'**) and *trans*-1,2-diaminocyclohexane (**B**). The **Dye** is used as a label to allow direct observation of the binding of **1** to substrates (*e.g.* peptides) attached to solid support particles (*e.g.* Merrifield synthesis beads). We have prepared a number of related molecules and

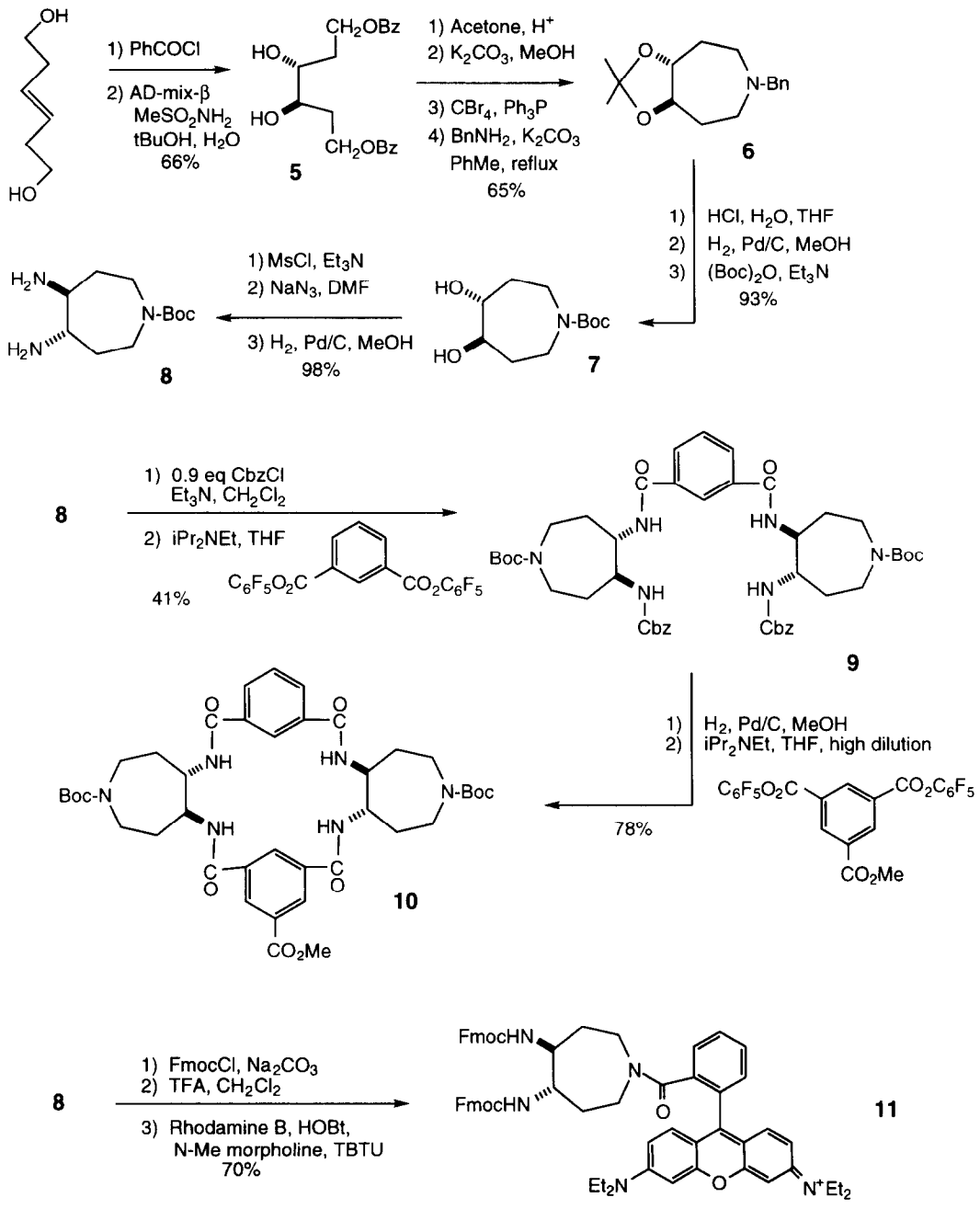
found that many of them bind peptides with remarkably high sequence-selectivity in organic solvents.[2] Compound **1** for example tightly binds only two sequences ((D)Pro(L)Val(D)Gln and (L)Lys(L)Val(D)Pro) out of a 3375-member tripeptide library in chloroform at a receptor concentration of 40 mM. Up to now, most of our work has involved binding peptides in low polarity organic solvents like chloroform; however, most biological applications of synthetic receptors for peptides require binding in water. In this paper, we turn our attention to the aqueous environment and describe the synthesis and binding properties of hydrophilic 2-armed receptor prototypes **2-4**. Like a related but structurally more complex hydrophilic cage-like host,[5] several of these simple receptors bind peptides sequence-selectively in aqueous solution.



In our new receptors **2-4**, hydrophobic cyclohexanoid **B** units are replaced by hydrophilic azepinoid **B**'s as indicated above. The required enantiomerically pure *trans*-4,5-diaminoazepine is prepared as shown in Scheme 1 by Sharpless chiral osmylation.[6] Because of the higher enantioselection with the Sharpless AD-mix-β reagent (91 %ee) than with the AD-mix-α ragent (84 %ee), we used the former reagent and a single recrystallization from ether provided **5** to >99 %ee. This reagent choice led to receptors **2-4** having the opposite chirality from that of **1** as originally prepared. Glycol **5** was then converted to protected 4(*S*),5(*S*)-diaminoazepine **8** by straightforward procedures analogous to those used to prepare the diaminopyrrolidine linker in **1** from diethyl tartrate.[7] Monoprotection and coupling to the *bis*(pentafluorophenyl) ester of isophthalic acid then yielded the macrocyclization precursor **9**. After hydrogenolysis of the Cbz protecting groups, the resulting diamine was both coupled at 0.4 mM concentration with the *bis*(pentafluorophenyl) ester of monomethyl trimesic acid and cyclized in a single step to provide the macrocyclic arm unit **10** in 78% yield after chromatography. It is worth noting that a variety of such macrocyclic dimers of isophthalic acid and 1,2-diamines can be prepared in high yields by analogous procedures. In most cases, high dilution conditions are not necessary.

With arm **10** in hand, dye-labeled pyrrolidine- and azepine-based linker units (*e.g.* **11**) were prepared as described previously and shown below. Coupling of arm and linker units to provide receptors **2-4** utilized the pentafluorophenyl ester of **10**. The dye we used to label these receptors was the brilliant red fluorescent dye Rhodamine S. This dye is hydrophilic and has been shown (unlike many other dyes) not to bind peptides with significant selectively in acidic water.[8]

Scheme 1. Synthesis of Receptor **4**.



We then tested the selectivity of the new receptors in binding peptides using a color-based solid phase binding assay. This assay has been described previously[1,5,7] and operates by equilibrating a dye-labeled receptor at low concentration with a combinatorial library of tripeptides on polymeric synthesis beads. Each bead in the library carries only one tripeptide sequence and binding between the receptor and the tripeptide is indicated when a bead picks up the color of the receptor-dye conjugate.

For the aqueous binding studies with **2-4**, we used a previously described[5] encoded tripeptide library on 150μ beads of TentaGel, a commercially available water-swellable block copolymer of polystyrene and poly(ethylene glycol).[9] Each member of the library had the following general structure:

$$AA3 - AA2 - AA1 - NHCH_2CH_2O\text{-TentaGel}$$

where AA1-3 represents an amino acid taken from the following set of 29 amino acids: (D and L) Ala, Phe, Val, Leu, Pro, Ser(*O*-tBu), Thr(*O*-tBu), Asp(*O*-tBu), Glu(*O*-tBu), Asn(*N*-Tr), Gln(*N*-Tr), Lys(*N*-Boc), His(*N*-Tr), Arg(*N*-Pmc) and Gly. Overall, the combinatorial library consisted of all possible combinations of these 29 different amino acids at the three different sequence positions and thus should contain 29^3 or 24,389 different tripeptides. The library was encoded with 15 electrophoric tags to allow identification of tripeptides on binding assay-selected individual beads.[10] The library was prepared in two forms: with N-terminal (AA3) acetylation and all sidechain protecting groups intact (the *protected* library), and with the N-terminal amino group free and with protecting groups removed (the *deprotected* library).

The binding assays were conducted by equilibrating a pH 4 aqueous solution of receptor and ~100 mg (3-4 copies of the library) of tripeptide library beads for 24 hours on a wrist action shaker. The concentration of the receptor was adjusted so that the smallest possible number of beads picked up the red color of the rhodamine-labeled receptor. These beads (carrying peptides that most tightly bound the receptor being tested) were picked and their sequences were determined using electron capture gas chromatography to analyze their associated molecular tags.[10]

With the protected tripeptide library and receptors **2** and **4** at ~5 μM in pH 4 water, ~1 bead per thousand of the library beads accumulated the red color of rhodamine-labeled receptors. These red beads were picked and the structures of their associated tripeptides are summarized below (*X* signifies any amino acid and *frequency* gives the percentage of the red beads having the indicated sequence):

Table 1. Receptors **2** and **4** binding to protected tripeptides in pH 4 water.

	Receptor 2						
AA3	AA2	AA1	Frequency	AA3	AA2	AA1	Frequency
(L)Asn	(D)Leu	(D)X	33%				
(D)Asn	(D)Leu	(D)X	19%				
(L)Gln	(D)Leu	X	10%	(L)Gln	(D)Leu	(D)X	14%
X	(L)Gln	(D)Leu	21%	X	(L)Gln	(D)Leu	80%

The most striking feature of these receptors is their high preference for binding peptides having a carboxamide-bearing amino acid (frequently (L)Gln) followed by (D)Leu. This pattern shows up frame-shifted both left and right in the selected sequences. **2** prefers peptides with an N-terminal Asn or Gln while **3** preferentially binds similar sequences that are frame-shifted to the right. Interestingly, these sequences are related to the (enantiomeric and sequence inverted) (L)Val-(D)Gln dipeptide sequence found to bind (enantiomeric) **1** in organic solvents.[11]

With the deprotected tripeptide library and 8 μM **2** and **4** in pH 4 water, ~2 beads per thousand of the library beads showed visible binding. As indicated in the table below, the majority of these beads carried various stereorandom aspartic and glutamic acid sequences and undoubtedly bound our receptors by nonspecific ionic association. Additionally, receptor **2** bound the tripeptide sequence Asp-Asp-Ser (stereorandomly) in ~10% of the red beads. Receptor **4** bound a different sequence (D)Asp-(D)Leu(or Phe)-(D)Asp also in ~5% of the read beads. This selectivity for a D hydrophobic amino acid is related to that observed with both **2** and **4** in the protected library. Interestingly, glutamic acid never occurred in these sequences carrying one nonacidic amino acid. The fact that the mixed triacidic Asp/Glu sequences dominate the red beads is not surprising because there are so many such sequences in the library.

Table 2. Receptors **2** and **4** binding to deprotected tripeptides in pH 4 water.

Receptor 2				**Receptor 4**			
AA3	AA2	AA1	Frequency	AA3	AA2	AA1	Frequency
Asp/Glu	Asp/Glu	Asp/Glu	83%	Asp/Glu	Asp/Glu	Asp/Glu	85%
Asp	Asp	Ser	11%				
				(D)Asp	(D)Phe/Leu	(D)Asp	6%

In contrast to our selective binding results with **2** and **4**, receptor **3** showed little evidence of binding the protected tripeptide library under the conditions described above. When **3**'s concentration was increased significantly in these assays, many beads turned red indicating both weak binding and very little selectivity. Because **2** and **3** are simple diastereomers, it appears that the chiralities of the linker and the arms work together to create an effective binding cleft.

Conclusion. The binding studies summarized above demonstrate that simple, two-armed receptors can bind peptides in water with significant sequence-selectivity. The selectivities found are closely related to those of structurally similar receptors (*e.g.* **1**) in organic solvents. Thus this work provides a second example of two structurally related receptors having similar peptide-binding properties in both water and chloroform.[12] While the particular sequences bound by **2** and **4** are interesting, we do not wish to speculate on detailed binding modes without additional study. It is likely that the hydrophobic sidechains of Leu or Phe bind in a cup-like hydrophobic cleft formed by the receptor arms (e.g. refs 7, 14) but the peptides are highly flexible with many opportunities for diverse and powerful intermolecular electrostatic interactions. More significant is the observation that simple two-armed receptors are able to bind peptidic substrates in water with a discrimination corresponding to approximately one tripeptide per thousand tripeptide sequences. This selectivity places **2** and **4** among the most selective synthetic receptors yet described.

EXPERIMENTAL SECTION

Glycol 5. To a solution of E-hex-3-en-1,6-diol[13] (6.76 g, 0.058 mol) in CH_2Cl_2 (40 mL) was added DMAP (0.1 g), Et_3N (23.7 mL, 0.17 mol) and BzCl (19.7 mL, 0.17 mol). The mixture was stirred at r.t. overnight under argon atmosphere. The reaction mixture was poured into $HCl_{aq.}$ (10%, 100 mL) and extracted with CH_2Cl_2 (2x50 mL). The organic phases were washed with a solution of $NaHCO_3$ (sat., 100 mL), dried ($MgSO_4$), filtered and concentrated. The residue was purified by flash chromatography on silica gel using 8% EtOAc/hexanes and vacuum distillation (Kügelrohr) to give 18.74 g of the corresponding dibenzoate.

In a 3 necked 2L round bottom flask, 80.7 g of AD-mix β (Aldrich) were dissolved in 290 mL of *t*-BuOH and 290 mL of water. Methanesulfonamide (5.45 g, 57.3 mmol) was added, and the mixture was stirred mechanically and cooled to 0 °C. The above dibenzoate (19 g, 58.6 mmol) was added in a single portion, and the heterogeneous slurry was stirred at 0 °C for 10 hrs. Solid sodium sulfite (60 g) was added and the mixture was allowed to warm to r.t. while stirred for 1 hr. After separation of the two layers, the aqueous phase was further extracted with EtOAc (2x200 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated. The crude residue was purified by flash chromatography on silica gel using 50% EtOAc/hexanes to give 0.55 g of recovered dibenzoate (3%) and 19.93 g of crude glycol **5** (95%, 55.6 mmol, 91 %ee by ^{1}H-NMR of the corresponding Mosher's diester). Recrystalization from Et_2O gave pure **5** as white needles (13.90 g, 38.8 mmol, 66%, >99 %ee):
MP(Et_2O) = 89.5 °C; R_f = 0.46 (60% EtOAc/hexane): $[\alpha]_D$ = +29° (c = 3, MeOH); ^{1}H-NMR (400 MHz, $CDCl_3$), δ: 8.03 (m, 4H); 7.57 (m, 2H); 7.44 (m, 4H); 4.65 (m, 2H); 4.46 (m, 2H), 3.66 (broad m, 2H), 2.91 (d, *J*=4.7 Hz, 2H); 2.06-1.92 (m, 4H); ^{13}C-NMR (300 MHz, $CDCl_3$), δ: 167.0, 133.1, 130.0, 129.6, 128.4, 71.1, 61.9, 33.0; IR (KBr, cm^{-1}): 3448, 3366, 3062, 2922, 2882, 1716, 1602, 1584, 1452, 1315, 1281, 1123, 713; MS(CI) m/z: 376 ($M+NH_4^+$, 100%), 359 ($M+H^+$, 18%), 272 (15%), 230 (45%); HRMS: calc. for $C_{20}H_{22}O_6$+ H 359.1494, found 359.1498; %C: calc. 67.01, found 66.90; %H: calc. 6.19, found 6.03.

Azepine Acetonide 6. A mixture of **5** (8.52 g, 23.8 mmol), Amberlyst 15 ion-exchange resin (Aldrich, 0.8 g) and acetone (70 mL) was stirred under argon for 4 hrs. The mixture was filtered over a short pad of $NaHCO_3$ and concentrated. The crude acetonide was isolated as an oil and used directly in following debenzoylation.

To a solution of crude acetonide above in MeOH (150 mL) and water (35 mL) was added solid K_2CO_3 (3.8 g). The mixture was stirred overnight at r.t.. After concentration to a small volume, the aqueous phase was saturated with NaCl and extracted with EtOAc (8x50 mL). The combined organic phases were dried ($MgSO_4$), filtered and concentrated to give a residue which was purified by flash chromatography on silica gel (EtOAc) to give the corresponding diol acetonide (4.32 g, 22.7 mmol, 96% from **5**): MP(iPrOH/EtOAc/Hexane 1:10:30) = 57 °C; R_f = 0.37 (10% MeOH/CH_2Cl_2); ^{1}H-NMR (400 MHz, $CDCl_3$), δ: 3.86 (m, 2H), 2.29 (broad s, 2H), 1.89-1.75 (m, 4H), 3.84 (t, *J*=5.6 Hz, 4H), 1.41 (s, 6H); ^{13}C-NMR (300 MHz, $CDCl_3$), δ: 108.8, 79.8, 60.5, 34.3, 27.2; IR (KBr, cm^{-1}): 3290, 2998, 2943, 2873, 1384, 1369, 1247, 1193, 1161, 1089, 1057, 1011, 996, 870; MS (CI) m/z: 208 ($M+NH_4^+$, 100%), 191

($M+H^+$, 42%), 190 (M^+, 6%), 150 (13%), 133 (18%); HRMS: calc. for $C_9H_{18}O_4$ + NH_4 208.1549, found 208.1555; %C: calc. 56.81, found 57.00; %H: calc. 9.54, found 9.72.

To a solution of the above diol acetonide (4.32 g, 22.7 mmol) and Ph_3P (14.39 g, 54.9 mmol) in CH_2Cl_2 (100 mL) at 0 °C was added under argon a solution of CBr_4 (18.16 g, 54.8 mmol) in CH_2Cl_2 (90 mL) during 20 min. The mixture was stirred at r.t. for 1 h. The volatiles were evaporated and the residue was purified by flash chromatography on silica gel with 5-10% EtOAc/hexanes to give the corresponding dibromide (6.70 g, 21.2 mmol, 93%) as an oil.

The above dibromide (6.70 g, 21.2 mmol), K_2CO_3 (12 g) and benzylamine (2.8 mL, 25.6 mmol) were mixed in toluene and refluxed under argon. At intervals of 12 h more benzylamine was added (3x3 mL, 82.3 mmol). After 2.5 days the mixture was allowed to warm to r.t., filtered and concentrated. The residue was purified by flash chromatography on silica gel using 10-15% EtOAc/hexanes to give azepine acetonide **6** as a yellow oil (4.04 g, 15.5 mmol, 73%): R_f = 0.46 (25% EtOAc/hexane); ^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.34-7.22 (m, 5H), 3.95 (m, 2H), 3.63 (AB, 2H), 2.68 (m, 4H), 2.12 (m, 2H), 1.59 (m, 2H), 1.41 (s, 6H); ^{13}C-NMR (300 MHz, $CDCl_3$), δ: 139.8, 128.4, 127.3, 126.9, 108.5, 80.0, 63.1, 53.1, 30.6, 27.3; IR (film, cm^{-1}): 3299, 3061, 3020, 2982, 2868, 1642, 1453, 1369, 1238, 1170, 1072, 700; MS (CI) m/z: 262 ($M+H^+$,100%), 261 (M^+, 5%); HRMS calc. for $C_{16}H_{23}O_2N$ 261.1729, found 261.1714.

Azepine Diol 7. To a solution of **6** (4.01 g, 15.34 mmol) in THF (60 mL) was added 10% aqueous HCl (30 mL) and the mixture was stirred for 30 min. Solid K_2CO_3 was added carefully until saturation. After concentrating to a small volume, the aqueous phase was extracted with EtOAc (7x50 mL). The combined organic extracts were dried ($MgSO_4$), filtered and concentrated to give the corresponding diol as a white solid (3.40 g, 15.36 mmol, 100%): MP(EtOAc/hexane) = 105-105.5 °C; R_f = 0.38 (25% MeOH, CH_2Cl_2); ^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.30 (m, 5H), 3.76 (m, 2H), 3.60 (AB, 2H), 2.89 (broad s, 2H), 2.66 (m, 4H), 1.98 (m, 2H), 1.73 (m, 2H); ^{13}C-NMR (300 MHz, $CDCl_3$), δ: 139.0, 128.7, 128.3, 127.1, 75.2, 63.1, 50.0, 32.0; IR (KBr, cm^{-1}): 3330, 3025, 2929, 2859, 2739, 2691, 2658, 1498, 1457, 1451, 1430, 1359, 1348, 1258, 1139, 1058, 761, 735, 702; MS (CI) m/z: 222 ($M+H^+$, 100%), 221 (M^+, 5%); HRMS: calc. for $C_{13}H_{19}O_2N$ 221.1416, found 221.1414; %C: calc. 70.54, found 70.59; %H: calc. 8.66, found 8.95; %N: calc. 6.33, found 6.29.

A mixture of the above diol (2.03 g, 9.17 mmol) and Pd/C (10%, 0.5 g) in MeOH (120 mL) was hydrogenated at atmospheric pressure for 30 min. The mixture was filtered over celite and the solids were washed with MeOH. The filtrate was concentrated to give crude debenzylated amine product which was used in next reaction without further purification.

To a solution of the preceding debenzylated amine in MeOH (80 mL) was added Et_3N (8 mL, 57 mmol) and $(Boc)_2O$ (2.2 g, 10.1 mmol). The mixture was stirred at r.t. for 2 h. After evaporation, the residue was purified by flash chromatography on silica gel using 80% EtOAc/hexanes to give the Boc azepine diol **7** as a white solid (1.97 g, 8.53 mmol, 93% from **6**): MP(EtOAc/hexane) = 86.5-87 °C; R_f = 0.30 (EtOAc); ^{1}H-NMR (400 MHz, $CDCl_3$), δ: 3.55 (m, 2H), 3.48 (m, 2H), 3.33 (m, 1H), 3.21 (m, 1H), 2.60 (s, 1H), 2.46 (s, 1H), 2.06 (m, 2H) 1.71 (m, 2H), 1.46 (s, 9H); ^{13}C-NMR (300 MHz, $CDCl_3$), δ: 155.4, 79.8, 75.8, 75.5, 41.4, 40.7, 32.6, 28.5; IR (KBr, cm^{-1}): 3455, 2974, 2940, 2924, 2885, 2713, 2682, 2636,

2594, 1677, 1487, 1431, 1365, 1242, 1164, 1122, 1047, 1016, 996; MS (CI) m/z: 249 ($M+NH_4^+$, 24%), 232 ($M+H^+$, 100%), 231 (M^+, 5%), 208 (22%), 193 (30%), 176 (28%); HRMS: calc. for $C_{11}H_{21}O_4N$ 231.1470, found 231.1461; %C: calc. 57.11, found 57.15; %H: calc. 9.16, found 9.19, %N: calc. 6.06, found 5.96.

Azepine Diamine 8. To a solution of azepine diol **7** (3.12 g, 13.49 mmol) in CH_2Cl_2 (45 mL) was added Et_3N (6.3 mL, 45 mmol) under argon at 0 °C. Then MsCl (2.46 mL, 31.77 mmol) was added slowly and the yellow solution was stirred at r.t. for 2 h. The reaction mixture was poured into a saturated solution of $NaHCO_3$ (150 mL) and extracted with CH_2Cl_2 (2x150 mL). The combined organic phase was washed with water (150 mL) and brine (2x100 mL), and dried ($MgSO_4$). After solvent removal at reduced pressure, the crude dimesylate was obtained as an yellow oil that solidified on standing.

To a solution of the above dimesylate in DMF (37 mL) was added sodium azide (3.7 g, 57 mmol) and the mixture was heated under argon in an oil bath at 85-90 °C for 20 h. After cooling to r.t., the mixture was poured into brine (200 mL) and extracted with Et_2O (2x200 mL). The combined organic phase was washed with brine (2x200 mL) and water (2x200 mL), and dried ($MgSO_4$). After solvent removal at reduced pressure, the corresponding diazide was obtained as a yellowish oil (3.71 g, 13.18 mmol, 98% in 2 steps): ^{1}H-NMR (400 MHz, $CDCl_3$), δ: 3.61-3.53 (m, 4H), 3.40 (m, 1H), 3.28 (m, 1H), 2.13 (m, 2H), 1.83 (m, 2H), 1.47 (s, 9H); ^{13}C-NMR (300 MHz, $CDCl_3$), δ: 155.0, 79.9, 64.7, 41.6, 40.8, 30.3, 28.4; MS (CI) m/z: 282 ($M+H^+$, 23%), 281 (M^+, 4%), 226 (84%), 182 (100%); HRMS: calc. for $C_{11}H_{19}O_2N_7$ 281.1600, found 281.1598.

A mixture of above diazide (3.65 g, 12.97 mmol), Pd/C (10%, 0.7 g) and MeOH (150 mL) was hydrogenated at atmospheric pressure for 8 h. The reaction mixture was filtered through celite and the solids were washed with MeOH. After solvent evaporation, azepine diamine **8** was obtained as as a pale oil (2.97 g, 12.95 mmol, 100%): R_f = 0.35 (5% Et_3N, 15% MeOH, 80% CH_2Cl_2); $[\alpha]_D$= -24° (c=3, MeOH); ^{1}H-NMR (400 MHz, $CDCl_3$), δ: 3.60 (m, 2H), 3.28 (m, 2H), 2.51 (m, 2H), 1.90 (m, 2H), 1.63 (m, 2H), 1.52 (s, 4H), 1.48 (s, 9H); ^{13}C-NMR (300 MHz, $CDCl_3$), δ: 155.2, 79.1, 57.8, 57.6, 41.7, 35.4, 28.3; IR (film, cm^-):3539, 3300, 2968, 2864, 1690, 1680, 1417, 1366, 1251, 1169; MS (CI) m/z: 230 ($M+H^+$, 100%), 229 (M^+, 4%); HRMS; calc. for $C_{11}H_{23}N_3O_2$ 229.1790, found 229.1791.

Cbz_2 Diamide 9. To a solution of azepine diamine **8** (139 mg, 0.606 mmol) in CH_2Cl_2 (10 mL) under argon was added Et_3N (110 μL, 0.8 mmol) and CbzCl (78 μL, 0.55 mmol). The mixture was stirred at r.t. for 8 h and then concentrated and purified by flash chromatography on silica gel using 12-15% MeOH in CH_2Cl_2 to afford the corresponding monoCbz azepine as a white hygroscopic foam (89.2 mg, 41%): R_f= 0.68 (Et_3N/MeOH/CH_2Cl_2 0.5:1:8.5%); ^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.35-7.27 (m, 5H), 5.36 (b s, 0.5H, NH), 5.21 (b s, 0.5H, NH), 5.09 (m, 2H), 3.73-3.11 (m, 7H), 2.88 (m, 1H), 2.07 (m, 2H), 1.67 (m, 2H), 1.44 (s, 9H); ^{13}C-NMR (300 MHz, $CDCl_3$), δ: 156.3, 155.2, 136.3, 128.4, 128.0, 128.0, 79.7, 66.8, 56.8, 55.2, 54.8, 42.3, 42.2, 41.3, 33.8, 33.4, 32.4, 28.4; IR (KBr, cm^{-1}): 3315, 2968, 2932, 1692, 1529, 1477, 1457, 1415, 1366, 1249, 1168, 1039, 752, 699; MS (CI) m/z: 364 ($M+H^+$, 80%), 321 (12%), 270

(20%), 256 (22%), 231 (15%), 230 (100%), 213 (35%); HRMS: calc. for $C_{19}H_{29}N_3O_4$ 363.2158, found 363.2172.

To a solution of the above monoCbz azepine (290 mg, 0.798 mmol) in THF (6 mL) under argon was added *bis*(pentafluorophenyl) isophthalate (179 mg, 0.36 mmol) and iPr_2NEt (174 μL, 1 mmol). The mixture was stirred overnight at r.t. and the volatiles were removed at reduced pressure. The residue was purified by flash chromatography on silica gel using 3.5% MeOH, 25% EtOAc in CH_2Cl_2 to give diamide **9** (308 mg, 100%) as a white solid (foam): R_f = 0.49 (10% MeOH/CH_2Cl_2); ^{1}H-NMR (400 MHz, $CDCl_3$), δ: 8.14 (m 1H), 7.90 (m, 2H), 7.45 (broad t, 1H), 7.24-7.13 (m, 11H), 6.86 (m, 1H), 5.20 (b d, 1H), 5.02-4.87 (m, 5H), 4.14-3.99 (m, 2H), 3.78 (m, 4H), 3.58-3.21 (m, 6H), 2.12 (m, 4H), 1.79 (m, 4H), 1.47 (s, 18H); ^{13}C-NMR (300 MHz, $CDCl_3$), δ: 166.5, 166.1, 157.3, 156.8, 155.2, 136.0, 135.9, 134.0, 129.9 (broad), 128.7, 128.2, 127.8, 127.4, 125.6, 79.8, 66.6, 66.4, 56.0, 55.8, 55.2, 54.8, 42.8, 42.6, 41.5, 41.1, 33.1, 32.8, 32.5, 32.3, 28.2; IR (KBr, cm^{-1}): 3407, 3061, 3020, 2968, 2929, 1691, 1664, 1534, 1519, 1417, 1367, 1321, 1252, 1169, 1042, 1016; MS (DCI) m/z: 875 (100%), 874 ($M+NH_4^+$, 100%) 757 (M^+-Boc, 85%); HRMS (FAB): calc. for $C_{46}H_{60}N_6O_{10}Na$ 879.4268, found 879.4268.

Macrocyclic tetraamide methyl ester 10. A mixture of diamide **9** (16.4 mg, 0.0191 mmol) and Pd/C (10%, 16 mg) in MeOH (5 mL) was hydrogenated at atmospheric pressure for 3 h. The mixture was filtered and the volatiles were removed at reduced pressure to give the corresponding diamine as an oil which was used in next reaction without further purification.

To a solution of crude diamine above in THF (40 mL) was added a solution of monomethyl bis(pentafluorophenyl) trimesate (10.6 mg, 0.0191 mmol) in THF (10 mL) and iPr_2NEt (10 μL, 0.057 mmol) and the mixture was stirred under argon at r.t. overnight. All volatiles were removed at reduced pressure and the residue was purified by flash chromatography on silica gel using 5-6% MeOH in CH_2Cl_2 to give macrocyclic **10** as an amorphous white solid (11.5 mg, 78%): R_f = 0.43 (10% MeOH / CH_2Cl_2); ^{1}H-NMR (400 MHz, CD_3OD), δ: 8.52 (broad s, 1H), 8.49 (m, 2H), 8.28 (broad s, 1H), 7.86 (broad d, 2H), 7.46 (t, J=7.8 Hz, 1H), 4.21 (m, 4H), 3.91 (s, 3H), 3.65 (m, 4H), 3.48 (broad q, 4H), 2.21-1.99 (m, 8H), 1.51 (s, 18H); ^{13}C-NMR (300 MHz, CD_3OD), δ: 169.3, 169.2, 169.1, 169.0, 168.3, 168.2, 168.1, 167.9, 166.8, 157.2, 136.4, 135.7, 132.4, 132.2, 132.0, 131.9, 131.4 (broad), 130.0, 128.2, 81.3, 56.2, 56.1, 55.9, 55.7, 55.5, 53.0, 43.9, 43.1, 33.6, 28.8; IR (KBr, cm^{-1}): 3424, 3061, 3020, 2968, 2939, 1664, 1535, 1417, 1368, 1257, 1169; MS (FAB) m/z: 799 ($M+Na^+$); HRMS: calc. for $C_{40}H_{52}N_6O_{10}Na$ 799.3643, found 799.3679.

Rhodamine-labeled Azepine 11. To a solution of azepine diamine **8** (39.4 mg, 0.172 mmol) in dioxane (4.5 mL) was added an aqueous solution of Na_2CO_3 (10%, 1 mL) and the mixture was cooled to 0 °C. FmocCl (98 mg, 0.37 mmol) was added and the mixture was stirred at r.t. for 18 h. Water (5 mL) was added and the mixture was extracted with EtOAc (2x30 mL). The combined organic phase was dried ($MgSO_4$) and, after solvent removal, the residue was purified by flash chromatography on silica gel using 40-50% EtOAc in hexanes to afford a di-Fmoc-protected derivative as a white solid (92.4 mg, 80%): MP(EtOAc/hexanes) =161 °C; R_f = 0.65 (10% MeOH/CH_2Cl_2); ^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.71 (d, *J*=7.32 Hz, 2H), 7.66 (d, *J*=7.61 Hz, 2H), 7.45 (m, 4H), 7.35 (m, 2H), 7.29 (m, 2H), 7.21 (m, 4H), 5.30 (m,

1H), 5.08 (m, 1H), 4.28 (m, 2H), 4.16 (m, 2H), 4.07 (m, 2H), 3.74 (m, 1H), 3.64 (m, 2H), 3.52 (m, 1H), 3.38 (m, 1H), 3.27 (m, 1H), 2.09 (m, 2H), 1.73 (m, 2H), 1.62 (s, 9H); ^{13}C-NMR (300 MHz, $CDCl_3$), δ: 156.7, 156.5, 155.2, 143.7, 143.6, 141.1, 127.6, 126.9, 124.9, 119.8, 79.8, 67.0, 67.0, 56.4, 55.7, 46.9, 42.8, 41.5, 33.3, 33.1, 28.4; IR (KBr, cm^{-1}): 3404, 3322, 3061, 3020, 2974, 2937, 1721, 1691, 1523, 1450, 1413, 1366, 1321, 1252, 1169, 1042, 759, 734; MS (CI) m/z: 691 ($M+NH_4^+$, 12%), 674 ($M+H^+$, 35%), 673 (M^+, 25%), 618(100%); HRMS: calcd for $C_{41}H_{43}N_3O_6$ + H^+ 674.3230, found 674.3252.; %C: calc. 73.08, found 72.83; %H: calc. 6.43, found 6.26; %N: calc. 6.24, found 6.18.

The remaining Boc group was then removed by treating a solution of the above protected derivative (30 mg, 0.0445 mmol) in CH_2Cl_2 (5 mL) under argon with 4 drops of anisole and TFA (2 mL). The mixture was stirred at r.t. for 3 h. After evaporation of the volatiles, crude amine salt was used directly in following coupling to rhodamine.

To a solution of the above amine salt in DMF (2 mL) was added under argon a mixture of HOBT (13.5 mg, 0.10 mmol), rhodamine B (21 mg, 0.044 mmol) and TBTU (Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillessen, D. *Tetrahedron Lett.* **1989**, *30*, 1989)(32.1 mg, 0.10 mmol). The mixture was stirred at 0 °C for 1 h. 4-Methylmorpholine (30 μL, 0.27 mmol) was added and the reaction mixture was stirred at r.t. overnight. After concentration, the residue was purified by flash chromatography using 6-7% MeOH and 20% EtOAc in CH_2Cl_2 and by gel filtration (MeOH, LH-20) to give the corresponding Fmoc-protected rhodamine-azepine conjugate **11** (40 mg, 88%) as a red amorphous solid: R_f = 0.41 (10% MeOH/CH_2Cl_2); ^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.73-6.95 (m, 24H), 6.77 (m, 2H), 6.13 (m, 1H), 5.74 (m, 1H), 4.31-4.05 (m, 6H), 3.81 (m, 1H), 3.68-3.39 (m, 12H), 3.04 (m, 1H), 2.17 (m, 1H), 2.00 (m, 1H), 1.89 (m, 1H), 1.60 (m, 1H), 1.30 (m, 6H), 1.24 (m, 6H); ^{13}C-NMR (300 MHz, $CDCl_3$), δ: 167.9, 157.7, 157.6, 156.7, 156.3, 155.6, 155.4, 143.8, 143.8, 141.1, 135.9, 131.9, 131.7, 131.0, 130.1, 129.8, 129.6, 127.5, 127.0, 125.4, 125.3, 125.1, 119.7, 114.2, 113.8, 113.5, 96.3, 96.2, 67.0, 66.7, 56.4, 55.3, 46.9, 46.3, 46.0, 45.9, 41.4, 32.7, 31.4, 12.5; IR (KBr, cm^{-1}): 3400, 3041, 2968, 2933, 1718, 1591, 1467, 1415, 1340, 1270, 1249, 1182, 1130, 1078, 744; MS (FAB, pos.) m/z: 998 (M^+); HRMS (FAB): calc. for $C_{64}H_{64}N_5O_6$ 998.4857, found 998.4845.

Receptor 4. To a solution of macrocyclic methyl ester **10** (76 mg, 0.0978 mmol) in THF (14 mL) and MeOH (7 mL) was added a solution of NaOH (1N, 1.4 mL). After stirring at r.t. for 4 h, the reaction mixture was neutralized with aqueous HCl (0.1N, 14 mL) and, after concentration, extracted with EtOAc (6x50 mL). The combined organic phase was dried ($MgSO_4$) and the volatiles were removed to give crude macrocyclic acid which was used directly in next reaction.

To a solution of the above macrocyclic acid in DMF (3 mL) was added a excess of pyridine (0.4 mL) and pentafluorophenyl trifluoracetate (0.4 mL). After stirring at r.t. under argon for 4 h, all volatiles were removed at reduced pressure and the residue was purified by flash chromatography on silica gel using EtOAc to yield the corresponding macrocyclic pentafluorophenyl ester as an amorphous white solid (79 mg, 87%): R_f = 0.47 (EtOAc); ^{1}H-NMR (400 MHz, CD_3OD), δ: 8.89 (m, 2H), 8.67 (m, 5H), 8.30 (broad. s, 1H), 7.87 (broad d, 2H), 7.46 (t, J=7.8 Hz, 1H), 4.23 (m, 4H), 3.64 (m, 4H), 3.48 (m, 4H), 2.20-2.00 (m, 8H), 1.50 (s, 18H); ^{13}C-NMR (300 MHz, CD_3OD), δ: 169.3, 169.2, 169.1, 168.9, 167.7, 167.5, 167.5,

167.3, 162.6, 157.1, 144.3, 144.2, 142.7, 141.0, 140.9, 140.8, 139.4, 137.9, 137.7, 137.5, 137.0, 135.7, 134.2, 133.0, 132.9, 132.7, 131.5, 131.4, 131.3, 130.0, 129.0, 128.2, 126.5, 126.4, 81.3, 56.3, 56.1, 55.8, 55.6, 55.4, 43.9, 43.0, 33.6, 28.7; IR (KBr, cm^{-1}): 3428, 3061, 2968, 2930, 2480, 1768, 1664, 1524, 1417, 1368, 1251, 1169, 1080, 997; MS (FAB) m/z: 951 ($M+Na^+$); HRMS (FAB): calc. for $C_{45}H_{49}N_6O_{10}F_5Na$ 951.3328, found 951.3364.

The rhodamine-azepine conjugate **11** was deprotected by treating it (8.8 mg, 8.5 μmol) in DMF (2 mL) under argon with piperidine (25 μmol) and stirring at r.t. for 20 min. All volatiles were then removed at reduced pressure and the resulting crude rhodamine-labeled diamine was suspended in dry THF (10 mL). The above macrocyclic pentafluorophenyl ester (17 mg, 18 μmol) and iPr_2NEt (10 μL) were added and the mixture was stirred at r.t. for 20 h, after which time the reaction mixture became homogeneous. The volatiles were removed at reduced pressure and the residue was purified by flash chromatography on silica gel using 8% MeOH in CH_2Cl_2 and by gel filtration (LH-20) using MeOH to afford a red residue which was washed with Et_2O to afford **Boc4** as a red amorphous solid (9.6 mg, 54%): R_f = 0.30 (10% MeOH/CH_2Cl_2); ^{1}H-NMR (400 MHz, CD_3OD), δ 8.35 (m, 2H), 8.19 (m, 6H), 7.82 (m, 4H), 7.74 (m, 3H), 7.46 (m, 3H), 7.28 (m, 2H), 7.04 (m, 2H), 6.93 (m, 2H), 4.25-3.35 (m, 38H), 2.20-1.94 (m, 20H), 1.50 (s, 36H), 1.20 (m, 12H); ^{13}C-NMR (300 MHz, CD_3OD), δ: 170.5, 169.4, 169.2, 169.2, 169.0, 168.6, 168.3, 168.2, 168.1, 159.3, 157.2, 137.2, 136.5, 136.3, 136.2, 135.9, 135.8, 133.3, 132.4, 132.2, 132.0, 131.8, 131.6, 131.4, 131.2, 131.0, 130.9, 130.5, 130.0, 129.8, 128.8, 128.2, 115.5, 115.3, 114.9, 97.3, 81.3, 56.3, 56.2, 56.0, 55.7, 55.3, 46.9, 44.0, 43.1, 33.7, 33.6, 28.8, 12.9; IR (KBr, cm^{-1}): 3423, 3061, 2978, 2930, 1663, 1591, 1530, 1477, 1415, 1339, 1275, 1249, 1178, 1134, 1081; MS (FAB, pos) m/z: 2044 (M^++1), 2043 (M^+); HRMS (FAB): calc. for $C_{112}H_{140}N_{17}O_{20}$ 2043.0460, found 2043.0520.

To a solution of **Boc4** (1.8 mg, 0.86 μmol) in CH_2Cl_2 (0.5 mL) was added CF_3COOH (0.15 mL) and the mixture was stirred at r.t. under argon for 2.5 hours. All volatiles were removed under vacuum, and the red receptor TFA salt **4** was washed with Et_2O, dried under vacuum overnight, and used in binding measurements without further purification: ^{1}H-NMR (400 MHz, CD_3OD), δ: 8.41 (m, 2H), 8.28-8.20 (m, 6H), 7.90 (dt, *J*= 7.5, 1.6 Hz, 4H), 7.75 (m, 3H), 7.52,(m, 3H), 7.29 (m, 2H), 7.05 (m, 2H), 6.94 (m, 2H), 4.39-4.08 (m, 12H), 3.66-3.32 (m, 26H), 2.37-2.12 (m, 20H), 1.23 (m, 12H); MS (FAB, pos.) m/z: 1643 ($C_{92}H_{108}O_{12}N_{17}^+$, M^+).

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