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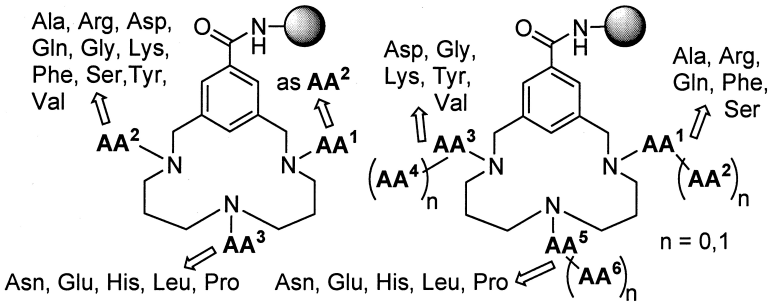

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Synthesis and Screening of Libraries of Synthetic Tripodal Receptor Molecules with Three Different Amino Acid or Peptide Arms: Identification of Iron Binders

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A novel selectively deprotectable triazacyclophane scaffold was used for the design and split−mix synthesis of two libraries of solid-phase bound tripodal synthetic receptors possessing three different amino acid or peptidic arms. In the synthesis of the first library, the two outer arms consisted of amino acid Ala, Arg, Asp, Gln, Gly, Lys, Phe, Ser, Tyr, or Val and the middle arm consisted of amino acid Asn, Glu, His, Leu, or Pro. The second library contained amino acid and/or (di)peptide arms. The arms were different in *all* library members. The first outer arm consisted of amino acid(s) Ala, Arg, Gln, Phe, or Ser, the second outer arm consisted of amino acid(s) Asp, Gly, Lys, Tyr, or Val, and the middle arm consisted of amino acid(s) Asn, Glu, His, Leu, or Pro, leading to a 27 000 member library of synthetic tripodal receptor molecules. In on-bead screening experiments, a remarkable selectivity of some library members for Fe^{3+} was observed and decoding of their structures by Edman degradation revealed consensus sequences with structural resemblance to non-heme iron proteins.

Introduction

In recent years, combinatorial synthesis of large numbers of artificial receptor molecules and parallel screening for their recognition properties have emerged as powerful tools for uncovering novel selective supramolecular interactions of possible biological relevance.[1,2] This approach, which may be regarded as the search for a suitable "lock" for a given "key", represents the reverse scenario of the familiar process of combinatorial preparation of libraries of ligands and subsequent testing of their binding ability to a particular receptor of biological origin.

In addition to use in research to gain insight in processes of biomolecular recognition, possible applications of artificial receptors include catalysis and sensing, sequestration of, for example, toxic components, selective transport through membranes, and disruption of undesired biological processes such as bacterial growth and docking of viruses to their host cells. Since artificial receptors are much smaller than antibodies, they probably do not trigger an immune response. In addition, they can withstand harsh conditions such as low pH or elevated temperature better than their natural counterparts.

Results and Discussion

A prominent class of artificial host molecules are the so-called tweezer receptors, which consist of two variable arms held together by a hinge.[2] The hinge is meant to align the binding arms in a parallel way and to create a binding site or cavity into which the guest molecule may fit. A considerable degree of preorganization of the receptor is required to avoid a large entropic penalty upon ligand binding.

Recently, we described the use of a macrocyclic tripodal scaffold for the construction of synthetic receptors having an additional arm compared to the molecular tweezers. These artificial receptors now consist of three identical peptide arms attached to the central triazacyclophane unit such as compound **1** depicted in Figure 1.[3] These were designed as mimetics of the glycopeptide antibiotic vancomycin and are capable of binding the C-terminal D-Ala-D-Ala and D-Ala-D-Lac sequences from the cell wall of pathogenic bacteria.

To significantly increase the structural diversity of our synthetic receptor molecules, we have developed a versatile, concise synthesis of the selectively deprotectable triazacyclophane **2**, which allows the attachment of *three* different substituents and selective elongation of three different oligopeptidic or peptidomimetic arms.[4]

The synthesis of scaffold **2** started from bis(aminopropyl)-amine, which was reacted with *o*-nitrobenzenesulfonyl chloride. The remaining primary amino group of the obtained sulfonamide was then selectively trifluoroacetylated with ethyl trifluoroacetate and water, yielding the TFA salt **3**.[5] Acylation of the remaining secondary amine with allyl chloroformate furnished the triply protected building block **4**, which in turn was cyclized with 3,5-bis(bromomethyl)-benzoic acid methyl ester[6] under basic conditions[7] to give triazacyclophane **5** in a yield of 47%. Finally, simultaneous basic hydrolysis of the trifluoroacetyl group and the methyl

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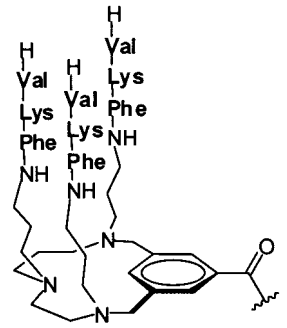


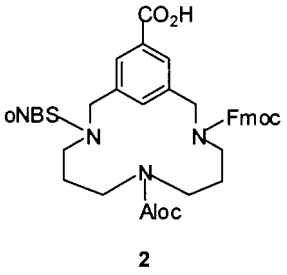


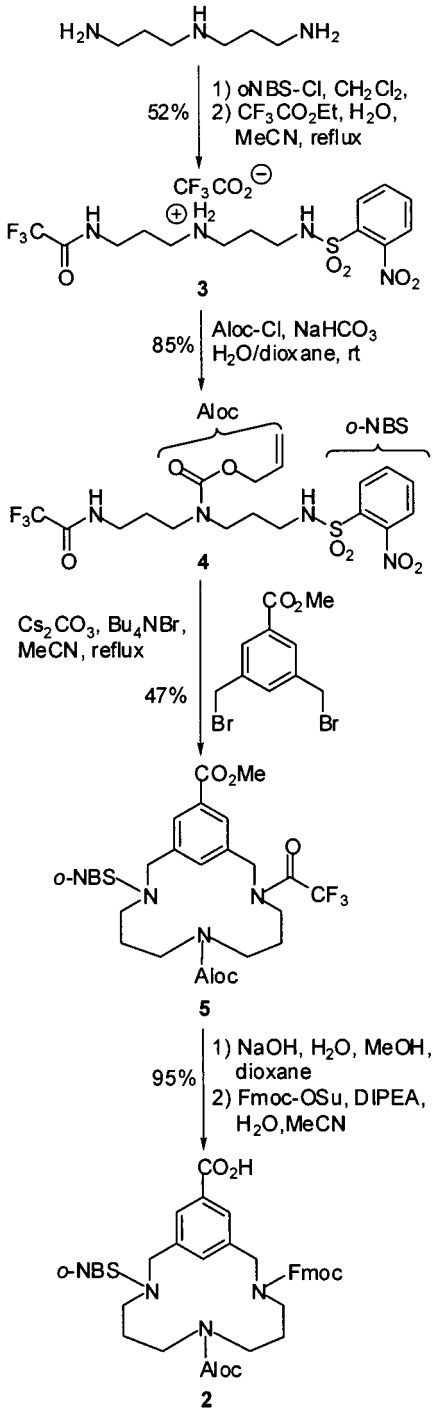
Figure 1. Triazacyclophanes.

ester and reprotection of the liberated amine in situ by reaction with Fmoc-*N*-hydroxysuccinimide gave triazacyclophane **2** in 98% yield.[4]

Combinatorial syntheses starting from this trivalent scaffold **2** (Scheme 1) clearly can give rise to huge libraries; for example, attachment of three tripeptide chains would lead to a library of about 2.56×10^{11} receptor molecules if all 20 proteinogenic amino acids were incorporated. One of the advantages of using only α-amino acids as building blocks or peptoid residues for the construction of the pendent arms is the possibility of simultaneous decoding of the sequences of all three arms by the Edman degradation protocol.[8] However, reduced sets of amino acids (e.g., a collection of five for each arm) have to be used to allow complete characterization without the need for resynthesis and comparison. To test the reliability of this method, a split−mix synthesis of a small library of receptors was carried out containing only one amino acid in each arm.[9] Since our scaffold molecule is symmetrical, the same set of 10 amino acids (Ala, Arg, Asp, Gln, Gly, Lys, Phe, Ser, Tyr, Val) could be used for the derivatization of both "benzylic" nitrogens and a "different" set of five amino acids (Asn, Glu, His, Leu, Pro) for the variation of the central nitrogen (Scheme 2).

Preparation of the library started with scaffold **2** linked to Argogel-NH_2. After removal of the Fmoc group, the resin was divided into 10 equal portions and the corresponding Boc-protected amino acid building blocks (vide supra; the functional amino acids carried acid-labile side chain protection (Arg(Pbf), Asp(O^{t}Bu), Gln(Trt), Lys(Boc), Ser(^{t}Bu), Tyr(^{t}Bu), Asn(Trt), Glu(O^{t}Bu), His(Trt))) were attached using HBTU as the coupling reagent in the presence of HOBt. After 16 h, a negative chloranil test for the presence of secondary amines confirmed completion of the coupling reactions in all cases.[10] The oNBS group was removed from the pooled resin portions by thiolysis,[11] and coupling of the amino acids

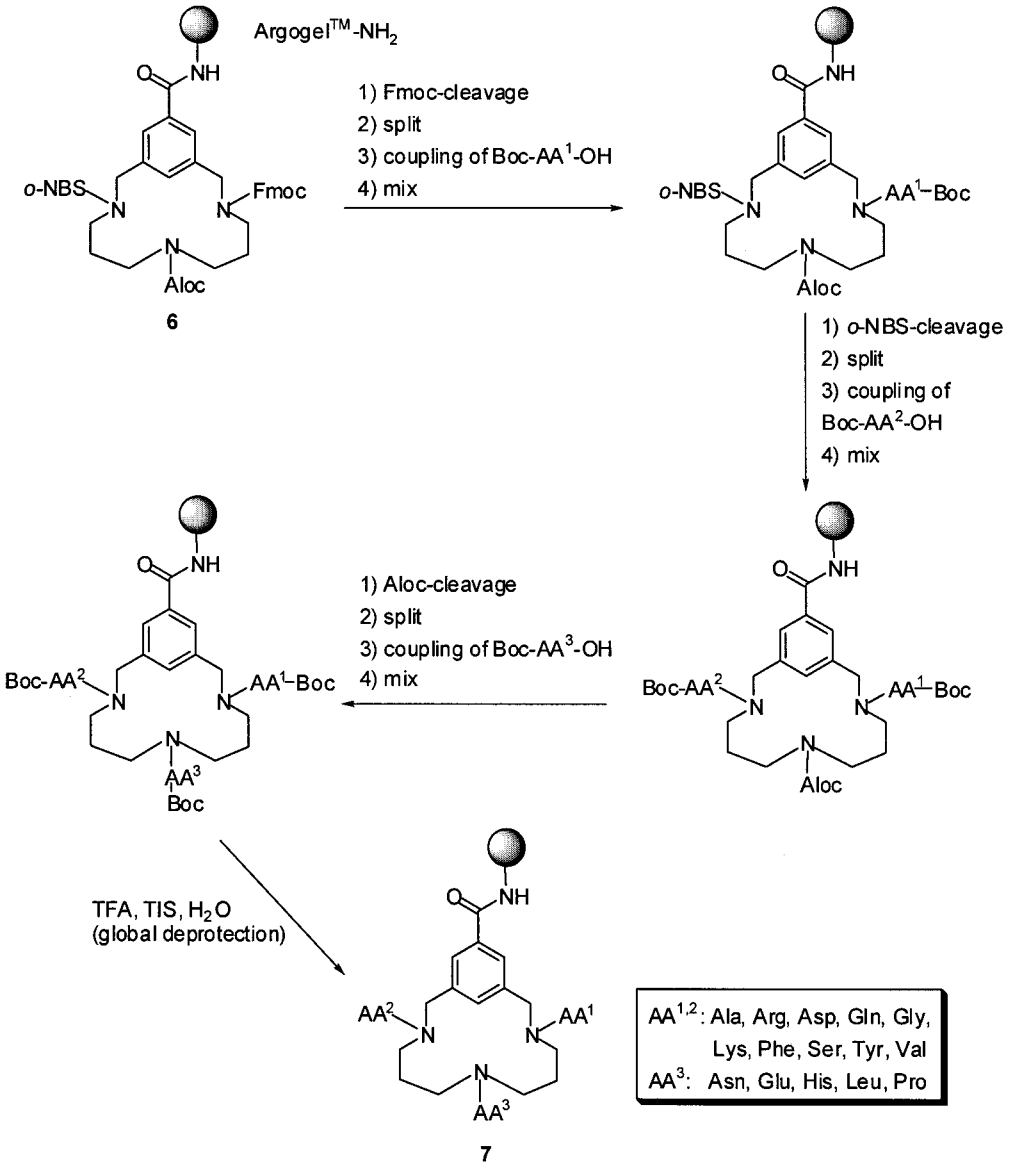
Scheme 1. Synthesis of Scaffold **2**



was performed under identical conditions. After the second pooling step, the Aloc group was removed by Pd-catalyzed allyl transfer to anilinium *p*-toluenesulfinate[4,12] and the last set of five amino acids was introduced likewise. Global deprotection of all side chain protecting groups as well as the Boc-protected N termini by TFA/^{i}Pr$_3$SiH/H_2O furnished the desired library, which should contain 275 different receptor molecules, 50 of which have two identical arms ($(10 \times 9 \times 5)/2 = 225$ receptors with three different arms and 10×5 C_2-symmetrical receptors with two identical arms).

To assess the recognition properties of the receptors, small portions of the library (∼1000 beads, corresponding to four copies per synthetic receptor with nonidentical arms and two

Scheme 2. Split−Mix Synthesis of Library **7**



copies per symmetrical synthetic receptor) were exposed to several metal salts,[13] organic ligands, or fluorescence-labeled cells.[14] Incubation of the beads with a 10 mM aqueous solution of $FeCl_3 \cdot 6H_2O$ and subsequent staining (after washing) with a 2% solution of potassium thiocyanate in water led to a surprising selectivity of the staining of only three beads showing the red color of the Fe(III) thiocyanate complex (Figure 2).

Sequence analysis by parallel Edman degradation revealed that the receptors on the selected beads all carried the same set of amino acids, namely, Asp/His/Asp (compound **7**{*1*}, Table 1, Figure 4). As a control, a colorless bead was picked and analyzed; the corresponding sequence was Ala/Pro/Tyr. A typical HPLC trace of a decoding cycle is shown in Figure 3.

We reasoned that in the iron complex of compound **7**{*1*} the two carboxylates from the aspartyl residues and the imidazole nitrogen might occupy three coordination sites of the ligand sphere of the Fe^{3+} ion, similar to the iron coordination complex present in heme diiron carboxylate proteins such as hemerythrin or the ribonucleotide reductase

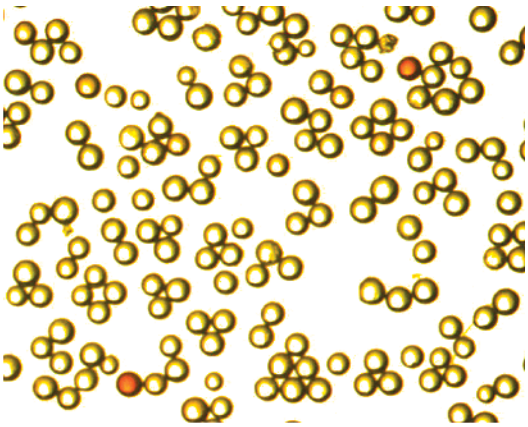


Figure 2. Colored beads from library **7** after staining with KSCN.

R2-type proteins such as methane monooxygenase hydroxylase (Figure 4).[15,16]

Having shown that a triazacyclophane library containing synthetic receptors with up to three different substituents (in this case amino acid residues) could be prepared and screened, we moved on to the preparation of an even more

Table 1. Sequences from Decoding of Active Beads from Library **7**

bead no.	outer arm (1)	Middle arm	outer arm (2)
1	Asp	His	Asp
2	Asp	His	Asp
3	Asp	His	Asp
4 (control)	Ala	Pro	Tyr

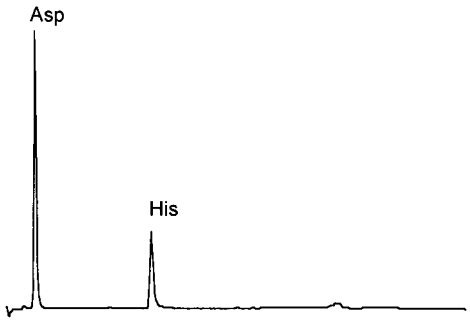


Figure 3. HPLC profile from decoding of bead 1 (library **7**), showing the presence of Asp in two arms.

diverse and larger synthetic receptor library with different peptide/amino acid arms. Possibly the increased length of (a) binding arm(s) may favorably influence the complexation behavior with, for example, iron. Thus, a library containing receptors with arms consisting of either one or two amino acids was prepared. Three different subsets of five amino acids (Ala, Arg, Gln, Phe, Ser/Asn, Glu, His, Leu, Pro/Asp, Gly, Lys, Tyr, Val) were used so that a unique decoding pattern for each library member will be obtained in the two cycles of the Edman degradation. The truncated sequences were included, and the the total number of different receptors was 27 000 ($5^6 = 15\,625$ molecules with three dipeptidic arms, $3 \times 5^5 = 9375$ receptors with two dipeptidic arms, $3 \times 5^4 = 1875$ receptors with one dipeptidic arm, and $5^3 = 125$ receptors with three arms consisting of only one amino acid; see Scheme 3).

The synthesis of this library was carried out by extending arm by arm in two cycles (using first Fmoc- and then Boc-protected building blocks) or, if a truncation was to be introduced, in one cycle (using Boc-protected amino acids; see Scheme 3). To achieve an equal representation of all sequences in the resulting library, the amount of resin which was reacted with one of the five Boc-protected amino acids in the first acylation cycle for each arm was a fifth of the amount coupled with each of the five Fmoc building blocks. Again, coupling periods of 16 h proved to be sufficient for complete acylations in all cases.

Incubation with 10 mM $FeCl_3 \cdot 6H_2O$ of a portion of about 55 000 beads (about two copies per receptor), and after two washing steps, staining with a 2% aqueous solution of potassium thiocyanate, followed by treatment with a solution of 1% 1,10-phenanthroline in methanol/water 1:1 was done to intensify the color. As was the case with library **7**, only a small percentage (<0.1%) of the beads turned intensely red.[17]

Seven dark beads (see Figure 5) as well as a colorless control sample from the screening with 10 mM Fe^{3+} were picked and decoded by parallel Edman degradation. Two

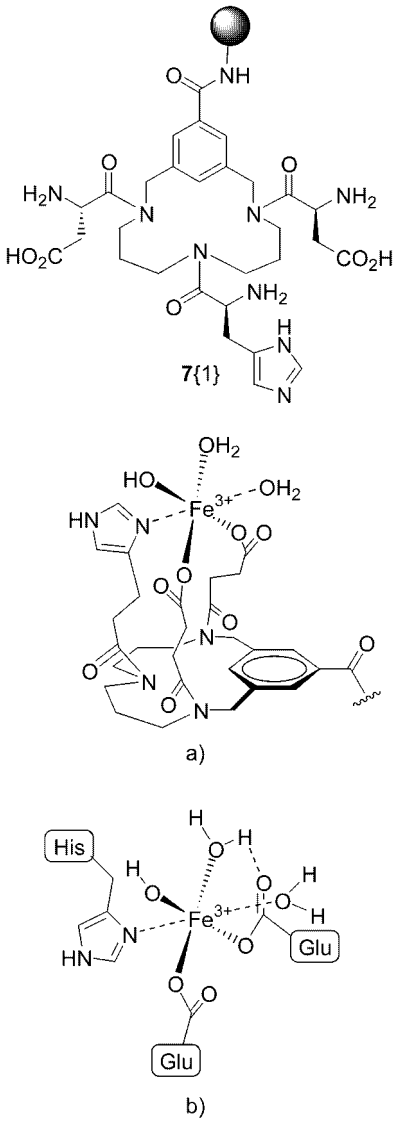


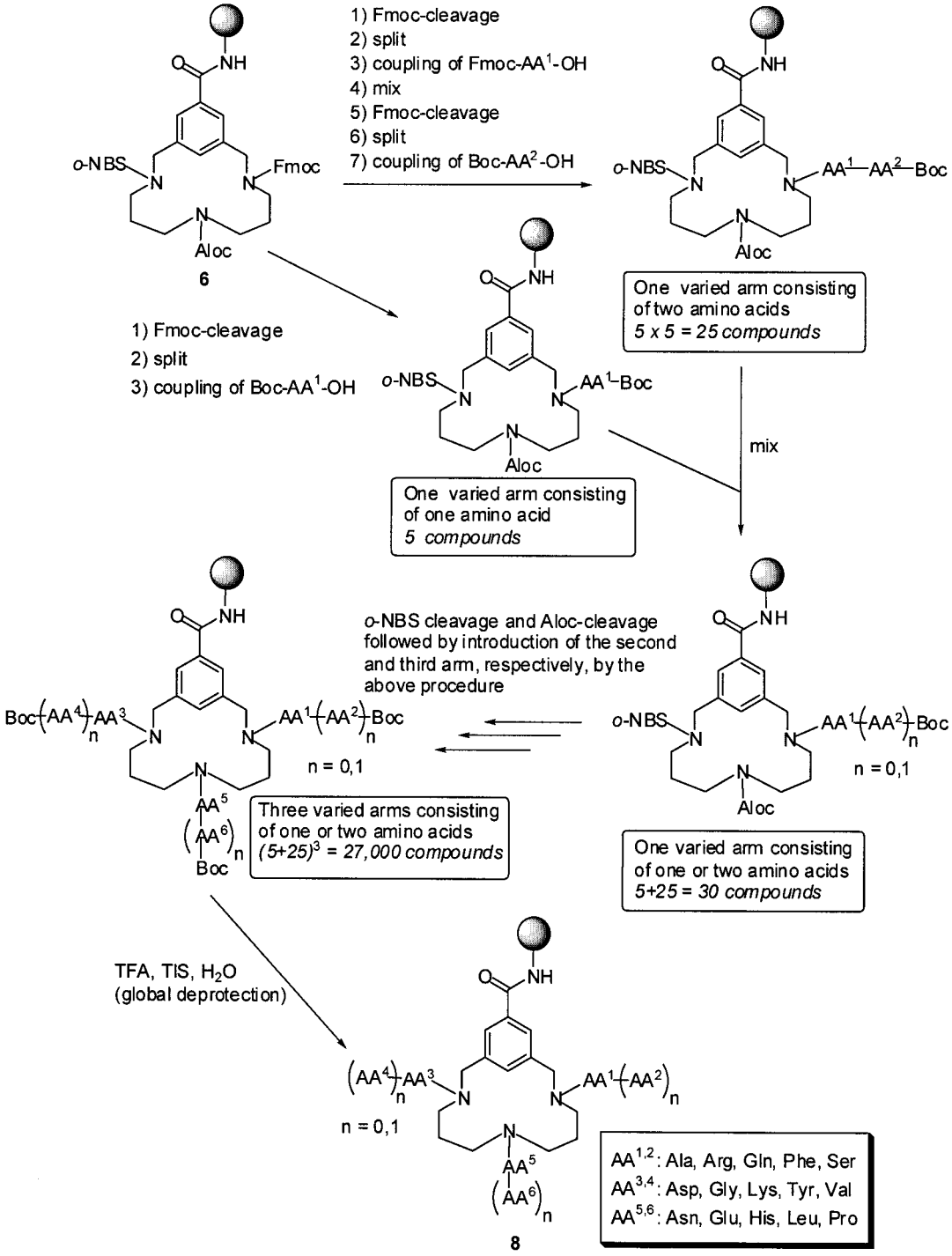
Figure 4. Structure of the active receptor **7**{*1*}: (a) possible chelation of iron by **7**{*1*} (α-amino groups omitted for clarity); (b) ligand sphere of one of the two iron centers in methane monooxygenase hydroxylase.[15]

typical HPLC traces are depicted in Figure 6; the corresponding sequences are listed in Table 2.

As can be seen, the common binding motif found in the active members of library **8** differs markedly from the one found in the hits identified in library **7**. It consists of two aspartic acids in the outer and at least one glutamic acid in the middle arm, giving these receptors a certain resemblance to the tricarboxylate pocket found in the ferritins, a family of globular iron storage proteins. This binding pocket, which accommodates one of the two ferric ions that form the so-called ferroxidase moiety (whose function is the oxidation of Fe^{2+} to Fe^{3+}), contains three glutamate side chains.[16,18] In contrast to the results obtained with library **7**, no histidine was found in the active receptors from library **8**.

In conclusion, the recently described tripodal scaffold **2** is excellently suited for the preparation of diverse and large libraries. Edman degradation can be very conveniently used to elucidate the structure of library members capable of binding ligands, in this case the Fe^{3+} ion. At present, approaches toward the construction of receptors for other

Scheme 3. Split−Mix Synthesis of Library **8**



biologically relevant ligands are in progress and will be reported in due course.[14]

Experimental Section

General. Acetonitrile used for the macrocyclization reaction was dried over molecular sieves prior to use. All other solvents were purchased from Biosolve or distilled and used without drying (dichloromethane, ethyl acetate, hexanes, diethyl ether). Argogel-NH_2 resin (65−125 mesh) was purchased from Argonaut Technologies. Solid-phase reactions and screening experiments were performed in glass-frit-containing vessels by bubbling with a gentle stream of dry nitrogen or in closed polyethylene syringes placed on a shaker. Anilinium *p*-toluenesulfinate was obtained by reaction of *p*-toluenesulfinic acid with aniline in dichloromethane and crystallized upon slow addition of hexane. *N*-Ethyldiisopropylamine was subsequently distilled from KOH and ninhydrin. All other chemicals were used without further purification. Solvents were evaporated in vacuo on a rotary evaporator at 40 °C. TLC was performed on 0.25 mm E. Merck silica gel 60 F_{254} plates and visualized by UV light (254 nm) or by staining with chlorine and *N*,*N*,*N*′,*N*′-tetramethyl-4,4′-diaminodiphenylmethane. Column chromatography was performed on ICN silica gel 60 with a particle

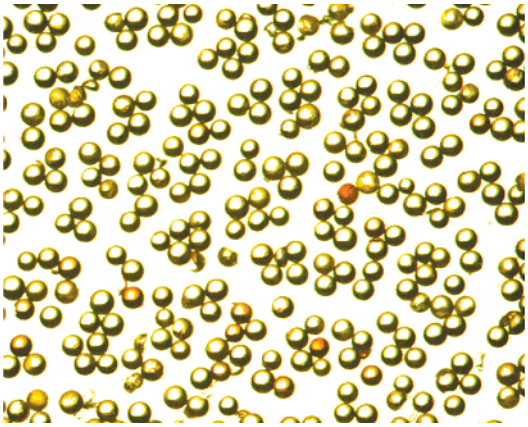


Figure 5. Colored bead from library **8** after staining with 1,10-phenanthroline.

Table 2. Sequences from Decoding of Active Beads from Library **8**[a]

bead no.	outer arm (1)	middle arm	outer arm (2)
1	Asp-Asp	Asn-Glu	Gln
2	Asp-Asp	Glu	Gln
3	Asp-Asp	Asn-Glu	Phe-Ser
4	Asp-Asp	Glu-Glu	Ser-Phe
5	Asp-Asp	Glu	Ser-Ala
6	Asp-Asp	Glu-Glu	Gln-Ala
7	Asp-Asp	Glu	Phe-Ser
8 (control)	Val-Val	Glu-His	Ala-Ser

[a] The notation of the sequences starts from the N termini.

size of 32−63 μm. Melting points were measured on a Gallenkamp melting point apparatus and are uncorrected. NMR spectra were recorded on a Varian Gemini 300 or a Varian Unity 500 spectrometer. ESI-MS spectra were recorded on a Shimadzu LCMS QP-8000 using 150 μL/min of 50% MeCN/H_2O + 0.2% formic acid. Edman degradations were performed on an Applied Biosystems ABI 476A protein sequencer. Polymer beads were visualized and manipulated under a Leica MZ FL III microscope with a CCD camera.

[3-(2-Nitrobenzenesulfonylamino)propyl][3-(trifluoroacetylamino)propyl]ammonium Trifluoroacetate 3. A solution of 12.5 g (56.4 mmol) *o*-nitrobenzenesulfonyl chloride in 200 mL of CH_2Cl_2 was slowly added to a stirred solution of 78.9 mL (564 mmol, 10 equiv) bis(aminopropyl)-amine in 250 mL of CH_2Cl_2 at 0 °C. The mixture was allowed to warm to room temperature under stirring for 45 min. The reaction mixture was washed with water (300 mL). Since considerable amounts of the desired product were also extracted in this washing step, a reextraction was performed: The aqueous phase was partially neutralized by addition of 100 mL of 1 N hydrochloric acid and was extracted with 150 mL of CH_2Cl_2. After addition of 50 g of NH_4Cl as a concentrated solution, the aqueous phase was again extracted with CH_2Cl_2 (2 × 100 mL). Concentration of the combined organic layers in vacuo and repeated coevaporation with acetonitrile gave 10.98 g (34.7 mmol, 61.5%) of the crude nitrobenzene sulfonamide as a yellow oil. This was dissolved in 100 mL of acetonitrile and refluxed with 12.4 mL (104 mmol, 3 equiv) of ethyl trifluoroacetate and 625 μL (34.7 mmol, 1 equiv) of water for 16 h. The reaction mixture was concentrated in vacuo and diluted with 250 mL of ethyl acetate, and crystallization of the product was achieved by slow addition of CH_2Cl_2.

Analysis (MS, NMR) of the product revealed the presence of the bis(*o*-nitrobenzenesulfonyl) derivative as an impurity that could be removed after Aloc protection of the remaining secondary amine.

Yield: 15.6 g (29.6 mmol, 52% over two steps) of **3** as yellow crystals. 300 MHz ^{1}H NMR (CD_3OD): δ 8.11−8.06 (m, 1H, aryl-H-3); 7.90−7.79 (m, 3H); 3.39 (t, 2H, J = 6.7 Hz, $CH_2NHCOCF_3$); 3.18−3.01 (m, 6H, 3 × CH_2N); 1.99−1.86 (m, 4H, 2 × β-CH_2). ^{1}H NMR (75 MHz APT, CD_3OD): δ 162.73 (q, $^2J_{(C,F)}$ = 35 Hz, $COCF_3$); 159.24 (q, $^2J_{(C,F)}$ = 37 Hz, $COCF_3$); 149.42 (aryl-C-2); 135.19, 133.76, 131.46 (aryl-C-4−6); 134.19 (aryl-C-1); 125.97 (aryl-C-3); 117.94 (q, $^1J_{(C,F)}$ = 292 Hz, CF_3); 117.34 (q, $^1J_{(C,F)}$ = 286 Hz, CF_3); 46.50, 46.39 (2 × CH_2NH_2R); 41.20 ($CH_2NHCOCF_3$); 37.64 (CH_2NHSO_2Ar); 27.73, 26.76 (2 × β-CH_2). MS (ESI) *m*/*z*: 524.2 (30%, [Di-oNBS + Na]$^+$); 501.9 (47%, [Di-oNBS + H]$^+$); 434.9 (31%, [M + Na]$^+$); 413.2 (100%, [M + H]$^+$). Anal. Calcd for $C_{16}H_{20}N_4SO_7F_6$: C, 36.51; H, 3.83; N, 10.64. Found: C, 36.36; H, 3.83, N, 10.57.

[3-(2-Nitrobenzenesulfonylamino)propyl][3-(trifluoroacetylamino)propyl]carbamic Acid Allyl Ester 4. A total of 10.0 g (19.0 mmol) of **3** were dissolved in a mixture of 100 mL of dioxane and 100 mL of water. After addition of a solution of 6.38 g (76.0 mmol, 4 equiv) of $NaHCO_3$ in 75 mL of water, the mixture was cooled in an ice bath. Under stirring, a solution of 2.4 mL (23 mmol, 1.2 equiv) of allyl chloroformate in 50 mL of dioxane was slowly added, and the ice bath was removed while stirring continued for another 4 h. To the reaction mixture was added 300 mL of water, and it was extracted with 300 mL of CH_2Cl_2 and another 150 mL of CH_2Cl_2. Drying of the combined organic layers

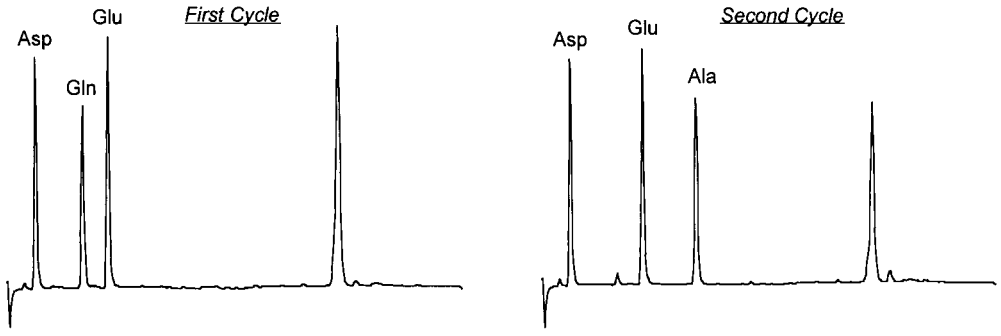


Figure 6. HPLC profiles (first and second cycle) of the Edman sequencing of bead 6, library **8**.

over Na_2SO_4 and concentration in vacuo gave crude **4**, which was purified by silica gel chromatography (hexanes/ethyl acetate 1:1) to yield 8.00 g (16.1 mmol, 85%) of **4** as a viscous yellow oil. 300 MHz ^{1}H NMR + COSY ($CDCl_3$): δ 8.14−8.08 (m, 1H, aryl-H-3); 8.01 (s, br, 0.55H, $ArSO_2$-NH, one rotamer); 7.85−7.81 (m, 1H); 7.78−7.74 (m, 2H); 7.06 (s, br, 0.24H, $ArSO_2NH$, other rotamer); 6.23 (s, br, 0.26H, $NHCOCF_3$, one rotamer); 5.91, (m, 1H, Aloc −CH═); 5.62 (s, br, 0.57H, $NHCOCF_3$, other rotamer); 5.28 (dd, 1H, $^3J_{trans}$ = 17.2 Hz, J_{gem}= 1.5 Hz); 5.22 (d, 1H, $^3J_{cis}$= 10.2 Hz); 4.59 (s, br, 2H, OCH_2); 3.32 (s, br, 6H, 3 × CH_2N); 3.13 (q, 2H, $J_{app} \approx$ 6.5 Hz, CH_2NHSO_2Ar); 1.78 (m, 4H, 2 × β-CH_2). 75 MHz ^{13}C NMR + APT ($CDCl_3$): δ 156.94 (Aloc −CO); 147.87 (aryl-C-2); 133.74, 132.76, 130.79 (aryl-C-4−6); 133.30 (aryl-C-1); 132.28 (Aloc −CH═ (?)); 125.25 (aryl-C-3); 118.16 (Aloc ═CH_2); 116.24 (q, $^1J_{(C,F)}$ = 290 Hz, CF_3); 66.46 (OCH_2); 43.75 (2 × Aloc-NCH_2); 40.92 ($CH_2NHCOCF_3$); 35.90* (CH_2NHSO_2Ar); 28.39*, 26.75* (2 × β-CH_2). The asterisk (∗) signals a split due to the presence of rotamers. MS (ESI) *m*/*z*: 519.2 (38%, $[M + Na]^+$); 497.1 (100%, $[M + H]^+$). TLC (EtOAc/hexanes 2:1): R_f = 0.44.

1-(2-Nitrobenzenesulfonyl)-5-trifluoroacetyl-9-allyloxycarbonyl-1,5,9-triaza-3(1,3)-benzenacyclododecaphane-3^5-carboxylic Acid Methyl Ester 5. A total of 3.63 g (11.26 mmol, 1 equiv) of tetrabutylammonium bromide, 3.63 g (11.27 mmol, 1 equiv) of 3,5-bis(bromomethyl)benzoic acid methyl ester, and 14.68 g (45.06 mmol, 4 equiv) of cesium carbonate were added to a solution of 5.59 g (11.26 mmol) of **4** in 1 L of refluxing dry acetonitrile. The reaction mixture was stirred under reflux until TLC monitoring indicated complete conversion of the starting material (after ca. 35 min). After rapid cooling to room temperature by immersion in a water bath, a total of 500 mL of acetonitrile was removed in vacuo and a solution of 9.2 g (67.6 mmol, 6 equiv) of potassium hydrogen sulfate in 300 mL of water was added. The mixture was extracted with 150 mL of CH_2Cl_2, and the extract was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. Purification of the product was achieved by silica gel column chromatography (hexanes/ethyl acetate/CH_2Cl_2 6:5:79 to 5:6:79) and crystallization from CH_2Cl_2/diethyl ether and furnished 3.45 g (5.25 mmol, 46.7%) of **5** as a colorless powder. 300 MHz ^{1}H NMR + COSY ($CDCl_3$): δ 8.08−8.02 (m, 2H); 7.96, 7.92 (2s, 1H); 7.82−7.67 (m, 4H); 5.84, (mc, 1H, Aloc −CH═); 5.25−5.15 (m, 2H, Aloc ═CH_2); 4.78 (s, 1H, benzyl); 4.66 (s, 1H, benzyl), 4.55−4.45 (m, 4H, benzyl + OCH_2); 3.95, 3.93 (2s, 3H, OMe); 3.55−3.23 (m, 4H, CH_2N); 3.13−3.00 (m, 3H, CH_2N); 2.93 (s, br, 1H, CH_2N); 1.64 (mc, 1.5H, β-CH_2); 1.36 (m, 2.5H, β-CH_2). 75 MHz ^{13}C NMR + APT ($CDCl_3$): δ 165.95, 165.80 (CO_2Me); 156.40 (q, $^2J_{(C,F)}$ = 36 Hz, $COCF_3$); 155.90 (Aloc-CO); 148.34, 148.28 (oNBS-C-2); 138.30 (broad), 137.85 (broad), 136.21 (very broad, benzoate-C3/5); 134.06, 133.98, 133.58, 132.73, 131.82, 131.13, 130.95, 130.35, 130.18, 129.97, 129.42 (oNBS-C-4−6, benzoate-C-2/4/6, Aloc −CH═); 131.98 (oNBS-C-1); 124.41, 124.35 (oNBS-C-3); 117.58 (Aloc ═CH_2); 116.53 (q, $^1J_{(C,F)}$ = 287 Hz, CF_3); 66.02, 65.99 (OCH_2); 54.26 (broad, benzyl CH_2 ?); 52.57, 52.50 (OMe); 51.30 (very broad, benzyl CH_2); 48.49, 48.27 (broad), 46.71 (very broad), 46.46 (very broad), 45.06 (4 × CH_2N); 29.68, 29.13, 28.55 (very broad), 27.96 (very broad) (2 × β-CH_2). MS (ESI) *m*/*z*: 1335.2 (13%, $[2M + Na]^+$); 1331.3 (13%, $[2M + H_3O]^+$); 1312.8 (10%, $[2M + H]^+$); 695.2 (11%, $[M + MeCN + H]^+$); 679.2 (44%, $[M + Na]^+$); 675.3 (25%, $[M + H_3O]^+$); 657.4 (100%, $[M + H]^+$). TLC (EtOAc/hexanes 2:1): R_f = 0.35. Mp: 176.5−177.5 °C. Anal. Calcd for $C_{28}H_{31}N_4O_9SF_3$: C, 51.22; H, 4.76; N, 8.53. Found: C, 51.29; H, 4.63, N, 8.42.

1-(2-Nitrobenzenesulfonyl)-5-(fluoren-9-ylmethoxycarbonyl)-9-allyloxycarbonyl-1,5,9-triaza-3(1,3)-benzenacyclododecaphane-3^5-carboxylic Acid 2. To a suspension of 2.00 g (3.05 mmol) of **5** in a mixture of 74.6 mL of dioxane and 26.6 mL of methanol, a total of 5.34 mL (21.4 mmol, 7 equiv) of a 4 N sodium hydroxide solution was added, and the reaction mixture was stirred at room temperature for 16 h. The homogeneous solution was neutralized by addition of 1 N HCl (ca. 17 mL), concentrated in vacuo, and resuspended in a mixture of 50 mL of water and 50 mL of acetonitrile. Upon addition of 1.06 mL (6.09 mmol, 2 equiv) of *N*-ethyldiisopropylamine, the mixture turned clear. A solution of 1.13 g (3.35 mmol, 1.1 equiv) of Fmoc-*N*-hydroxysuccinimide in 10 mL of acetonitrile was added, and the reaction mixture was stirred at room temperature for 1.5 h. After addition of 20 mL of 1 N HCl and 350 mL of water, the mixture was extracted with 250 mL and a second time with 100 mL of ethyl acetate. The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo, and the resulting crude product was purified by silica gel column chromatography (hexanes/ethyl acetate 1:2, 1% acetic acid). Remaining acetic acid was removed by repeated coevaporation with benzene and prolonged drying in vacuo. Yield: 2.23 g (2.90 mmol, 95.2%) of **2** as a colorless foam. 300 MHz ^{1}H NMR + COSY ($CDCl_3$): δ 9.44 (s, very broad, 1H, COOH); 8.10−7.84 (m, 3H); 7.77−7.56 (m, 7H); 7.40−7.21 (m); 5.84 (s, br, 1H, Aloc −CH═); 5.29−5.11 (m, 2H, Aloc ═CH_2); 4.80−4.59 (m, 2H); 4.55−4.20 (m, 7H); 3.38−3.19 (m, 2.6H, CH_2N); 3.06−2.77 (m, 4.1H, CH_2N); 2.48−2.26 (m, 1.3H, CH_2N);1.57−1.16 (m, 2.7H, β-CH_2); 1.10−0.82 (m, 1.3H, β-CH_2). 75 MHz APT + HSQC ($CDCl_3$): δ 169.81 (CO_2H); 156.98 (broad), 156.21, 155.74 (broad, urethane-CO); 148.19 (oNBS-C-2); 143.89 (Fmoc-C_q); 141.34 (Fmoc-C_q); 140.31 (very broad), 138.14 (very broad, benzoate-C3/5); 133.87, 131.75, 131.08 (oNBS-C4−6); 133.48 (broad, benzoate-C-4); 132.87 (Aloc −CH═); 132.10 (oNBS-C-1); 130.56 (benzoate C-1); 129.87, 129.41 (broad, benzoate C-2/6); 128.26 (benzene); 127.56, 127.13 (Fmoc-CH); 124.84, 124.48, 124.31 (Fmoc-CH, oNBS-C-3); 119.83 (Fmoc-CH); 117.34 (Aloc ═CH_2); 67.64 (broad), 67.02 (very broad, Fmoc-OCH_2); 65.86 (Aloc-OCH_2); 53.73 (broad), 52.91 (broad, benzyl CH_2); 48.31 (broad), 45.16 (very broad, 4 × CH_2N); 47.21 (Fmoc Ar_2CH); 28.33 (broad), 27.72 (very broad, 2 × β-CH_2). MS (ESI) *m*/*z*: 791.7 (17%, $[M + Na]^+$); 769.8 (100%, $[M + H]^+$); 547.4 (88%, $[M - Fmoc + H]^+$). TLC (EtOAc/hexanes 2:1 + 1% AcOH): R_f = 0.15. Anal. Calcd for $C_{40}H_{40}N_4O_{10}S$: C, 62.49; H, 5.24; N, 7.29. Found: C, 62.56; H, 5.20, N, 7.18.

Preparation of Library 7. To 1 g of Argogel-NH_2 resin (0.40 mmol/g, average bead diameter 178 μm) were added 308 mg (0.4 mmol, 1 equiv) of triazacyclophane **2**, 61 mg

(0.4 mmol, 1 equiv) of HOBt monohydrate, 152 mg (0.4 mmol, 1 equiv) of HBTU, and 5 mL of NMP. A gentle stream of dry nitrogen was bubbled through the mixture until all reagents were dissolved. After addition of 139 μL (0.8 mmol, 2 equiv) of DIPEA, N_2 bubbling was maintained for 18 h. The resin was washed twice with NMP and DCM, and a mixture of 1 mL of acetic anhydride, 3 mL of pyridine, and 10 mL of dioxane was added. After 5 min, the resin was washed with dioxane (twice), NMP (twice), MeCN, DCM (twice), and diethyl ether (3 times). Drying in vacuo gave 1.21 g of resin **6** with a loading of 0.236 mmol/g as determined by spectrophotometric Fmoc quantification.

The resin was divided into 10 equal portions (in PE syringes with PE frits), and the Fmoc group was removed by 20% piperidine in NMP (1 × 5 min, 1 × 15 min). After the portions were washed with NMP (twice), DCM (twice), and NMP (twice), solutions of 0.17 mmol (each, 6 equiv) of Boc-Ala-OH, Boc-Arg(Pbf)-OH, Boc-Asp(O^{t}Bu)-OH, Boc-Gln(Trt)-OH, Boc-Gly-OH, Boc-Phe-OH, Boc-Ser(^{t}Bu)-OH, Boc-Lys(Boc)-OH, Boc-Tyr(^{t}Bu)-OH, and Boc-Val-OH in 750 μL of NMP were added. To each of the syringes were added solutions of 25 mg (0.16 mmol, 5.7 equiv) of HOBt monohydrate in 300 μL of NMP and 61 mg (0.16 mmol, 5.7 equiv) of HBTU in 400 μL of NMP and 58 μL (0.33 mmol, 11.7 equiv) of DIPEA. The syringes were placed on a shaker, and after 18 h negative chloranil tests confirmed complete couplings in all cases. The resins were washed with NMP (twice) and pooled in a glass reactor. After they were washed with toluene, DMF (twice) and dry DMF (dried over molecular sieves), the oNBS group was cleaved by addition of 197 μL (2.8 mmol, 10 equiv) of 2-mercaptoethanol and 213 μL (1.4 mmol, 5 equiv) of DBU in 7 mL of dry DMF. After N_2 bubbling for 30 min, the green solution was replaced by a fresh mixture of identical composition and N_2 bubbling was maintained for another 30 min. The resin was washed with NMP (twice), DCM (twice), and NMP (twice) and was again divided into 10 equal portions. Coupling of the second amino acid was performed as was described for the first residue (vide supra), including the final washing steps and the pooling operation. Complete couplings (negative chloranil test) were achieved in all cases. For removal of the Aloc protecting group, to the resin was added a solution of 1.40 g (5.6 mmol, 20 equiv) of anilinium *p*-toluenesulfinate in 5 mL of NMP and, after N_2 bubbling for 5 min, 49 mg (42 μmol, 15 mol %) of tetrakis(triphenylphosphine)palladium-(0). The reaction vessel was kept in the dark, and N_2 bubbling was maintained for 1 h. The resin was washed with NMP (twice), a 0.1% solution of sodium diethyldithiocarbamate trihydrate in NMP, 20% piperidine in NMP, NMP, and DCM (twice), and again with NMP (twice).

The resin was then divided into five equal portions, and solutions of 0.34 mmol (each, 6 equiv) of Boc-Asn(Trt)-OH, Boc-Glu(O^{t}Bu)-OH, Boc-His(Trt)-OH, Boc-Leu-OH monohydrate, and Boc-Pro-OH in 1 mL of NMP were added. To each of the syringes were added solutions of 49 mg (0.32 mmol, 5.7 equiv) of HOBt monohydrate in 600 μL of NMP and 121 mg (0.32 mmol, 5.7 equiv) of HBTU in 860 μL of NMP and 114 μL (0.66 mmol, 11.7 equiv) of DIPEA. The syringes were placed on a shaker, and after 18 h negative chloranil tests confirmed complete couplings in all cases. The resin portions were washed with NMP (twice), MeCN, DCM (twice), and diethyl ether (3 times) and were dried in vacuo.

Aliquots of 15% of the total amount of resin in each syringe were pooled and subjected to global deprotection by reaction with 3 mL of TFA, 100 μL of ^{i}Pr$_3$SiH, and 100 μL of water for 4 h. The resin was washed with DCM (twice), MeCN, 20% triethylamine in NMP (to remove TFA bound to the receptors), NMP (twice), H_2O (twice), 5% aqueous NH_4Cl (to neutralize the resin), and H_2O (twice).

Screening of Library 7 with $FeCl_3 \cdot 6H_2O$. In a PE syringe with a PE frit, an amount corresponding to about 1000 beads of the deprotected library **7** was suspended in 1 mL of 50 mM Tris buffer (pH 7.4), and an amount of 2.7 mg (10 μmol) $FeCl_3 \cdot 6H_2O$ was added as a 0.1 M aqueous solution. The syringe was shaken for 18 h, and the resin was twice washed with water. The beads were plated on a Petri dish to give a monolayer, and after the monolayer was dried, a 2% aqueous solution of potassium thiocyanate was added for staining. After drying, three red beads were picked and analyzed by parallel Edman dgradation.

Preparation of Library 8. To 4 g of Argogel-NH_2 resin (0.38 mmol/g, average bead diameter 151 μm) were added 1.05 g (1.37 mmol, 0.9 equiv) of triazacyclophane **2**, 209 mg (1.37 mmol, 0.9 equiv) of HOBt monohydrate, 519 mg (1.37 mmol, 0.9 equiv) of HBTU, and 20 mL of DMF. A gentle stream of dry nitrogen was bubbled through the mixture until all reagents were dissolved. After addition of 477 μL (2.74 mmol, 1.8 equiv) of DIPEA, N_2 bubbling was maintained for 18 h. The resin was washed twice with DCM and dioxane, and a mixture of 1 mL of acetic anhydride, 3 mL of pyridine, and 15 mL of dioxane was added. After 5 min, the resin was washed with dioxane twice, DMF, DCM (twice), and diethyl ether (3 times). Drying in vacuo gave 4.66 g of resin **6** with a loading of 0.227 mmol/g as determined by spectrophotometric Fmoc quantification.

An amount of 2.33 g of the resin was divided into five equal portions (in PE syringes with PE frits), and the Fmoc group was removed by 20% piperidine in NMP (1 × 5 min, 1 × 15 min). After the portions were washed with DMF (twice), DCM (twice), and DMF (twice), solutions of 0.53 mmol (each 5 equiv) of Fmoc-Ala-OH hydrate, Fmoc-Arg-(Pbf)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ser(^{t}Bu)-OH, and Fmoc-Phe-OH in 1 mL of DMF were added to each portion. To each of the syringes were added solutions of 77 mg (0.5 mmol, 4.7 equiv) of HOBt monohydrate in 400 μL of DMF and 191 mg (0.5 mmol, 4.7 equiv) of HBTU in 1 mL of DMF and 180 μL (1.0 mmol, 9.7 equiv) of DIPEA. The syringes were placed on a shaker, and after 18 h negative chloranil tests confirmed complete couplings in all cases. The resins were washed with DMF, diethyl ether (twice), and toluene (twice) and were pooled in a glass reactor. After removal of the Fmoc group by 20% piperidine in NMP (1 × 5 min, 1 × 15 min) and being washed twice with DMF, DCM, and DMF, the resin was divided into five equal portions and Boc-Ala-OH, Boc-Arg(Pbf)-OH, Boc-Gln(Trt)-OH, Boc-Ser(^{t}Bu)-OH, and Boc-Phe-OH were coupled likewise.

A portion of 466 mg of resin **6** (20% of the amount used for coupling with the Fmoc building blocks) was divided into five equal portions and coupled with Boc-Ala-OH, Boc-Arg(Pbf)-OH, Boc-Gln(Trt)-OH, Boc-Ser(^{t}Bu)-OH, and Boc-Phe-OH according to the same coupling protocol (vide supra). All 10 resin portions (5 with 1 amino acid, 5 with 2 amino acids) were washed (2 × DMF, 2 × toluene, 1 × diethyl ether) and pooled. The oNBS group was cleaved by addition of 445 μL (6.3 mmol, 10 equiv) of 2-mercaptoethanol and 475 μL (6.3 mmol, 5 equiv) of DBU in 15 mL of dry DMF. After N_2 bubbling for 30 min, the green solution was replaced by a fresh portion of identical composition, and N_2 bubbling was maintained for another 30 min. The resin was washed with NMP (twice), DCM (twice), and NMP (twice), and the attachment and elongation of the second arm were carried out as described for construction of the first arm ($^5/_6$ of the total amount coupled with 5 Fmoc-protected and then with 5 Boc-protected building blocks, $^1/_6$ of the total amount was only coupled with 5 Boc-protected amino acids to introduce truncations) using Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, Fmoc-Tyr(^{t}Bu), and Fmoc-Val-OH or their Boc-protected analogues. For removal of the Aloc protection, to the resin was added a solution of 3.17 g (12.7 mmol, 20 equiv) of anilinium *p*-toluenesulfinate in 12 mL of NMP and, after N_2 bubbling for 5 min, 110 mg (95 μmol, 15 mol %) of tetrakis(triphenylphosphine)-palladium(0). The reaction vessel was kept in the dark, and N_2 bubbling was maintained for 1 h. The resin was washed with NMP (twice), a 0.1% solution of sodium diethyldithiocarbamate trihydrate in NMP, 20% piperidine in NMP, NMP, DCM (twice), and again with NMP (twice). For construction of the third arm, Fmoc-Asn(Trt)-OH, Fmoc-Glu(O^{t}Bu)-OH monohydrate, Fmoc-His(Trt)-OH, Fmoc-Leu-OH monohydrate, and Fmoc-Pro-OH monohydrate or their Boc-protected counterparts were used. After the final pooling step, the resin portions were washed with toluene, DMF, MeOH, H_2O/dioxane 1:1, MeOH (twice), DMF (3 times), and diethyl ether (twice) and were dried in vacuo to give a total amount of 3.45 g.

An amount of 1.73 g of this library was subjected to global deprotection by reaction with 23 mL of TFA, 1 mL of ^{i}Pr$_3$-SiH, and 1 mL of water for 4 h. The resin was washed with DCM (twice), MeCN, 20% triethylamine in NMP, NMP (twice), H_2O (twice), 5% aqueous NH_4Cl, and H_2O (twice). Drying in vacuo gave 1.68 g of the deprotected library **8**.

Screening of Library 8 with $FeCl_3 \cdot 6H_2O$. In a PE syringe with a PE frit, 75 mg of library **8** corresponding to about 55 000 beads was suspended in 1 mL of 50 mM Tris buffer (pH 7.4) and an amount of 2.7 mg (10 μmol) of $FeCl_3 \cdot 6H_2O$ was added as a 0.1 M aqueous solution. The syringe was shaken for 18 h, and the resin was washed with water (twice). The beads were plated on a Petri dish to give a monolayer, and after the monolayer was dried, a 2% aqueous solution of potassium thiocyanate was added for staining. Since the color of the positive beads was faint, a 1% solution of 1,10-phenanthroline in methanol/water 1:1 was added. After drying, the seven darkest beads were picked and analyzed by parallel Edman dgradation.

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