Synthesis of Peptides Containing Unnatural, Metal-Ligating Residues: Aminodiacetic Acid as a Peptide Side Chain

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Peptides possessing a pair of residues separated by one turn in the α-helical conformation and potentially capable of ligating a single metal ion in aqueous solution were designed. It was predicted that the resulting cross-link would shift toward α-helix the random coil/α-helix equilibrium. The syntheses of 10 peptides Ac-Ada^{l}Ala$_m$Ada^l(Ala$_4$GluLys)$_n$-NH$_2$ where Ada^l is an L-α-amino acid residue with an aminodiacetic acid bearing side chain, $-(CH_2)_lN(CH_2CO_2H)_2$ (with values of l, m, and n as follows: 1, 2, 3 (**1**); 1, 3, 1 (**2a**); 1, 3, 2 (**2b**); 1, 3, 3 (**2c**); 2, 2, 3 (**3**); 2, 3, 3 (**4**); 3, 2, 3 (**5**); 3, 3, 3 (**6**); 4, 2, 3 (**7**); 4, 3, 3 (**8**)) are described using Boc chemistry on *p*-methylbenzhydrylamine resin. The aminodiacetic acid bearing residues were incorporated with side chains protected as the dibenzyl esters. To avoid side reactions, residues Ada^l for l = 1 and 2 were incorporated by a block approach. Peptide structures were confirmed by observation of the predicted parent ions in the FAB MS. The circular dichroism spectra of several of these peptides that possess a pair of metal-ligating residues separated by two or three intervening residues have previously been shown to undergo changes on addition of metal ions consistent with appreciable enhancement of helix content.

Introduction

Contemporary studies in molecular recognition often commence with the design and synthesis of, by present standards, one or more large molecules, each possessing a putative binding cavity. Preorganization of this cavity is commonly achieved by incorporation of a mono- or polycyclic skeleton; affinity for some guest is then enhanced by the installation of judiciously chosen and positioned functional groups. This complex undertaking leaves the chemist envious of Mother Nature, who achieves molecular recognition with a generic class of, for the most part, acyclic molecules: the proteins. Following rules we do not as yet understand, these linear molecules fold into complex three-dimensional shapes capable of not only binding but also, in the case of enzymes, covalently modifying guest molecules.

What prevents the practitioner of molecular recognition from following this same approach? One especially appealing idea is the use of secondary structure elements found in proteins, such as the α-helix and β-sheet, as building blocks for macromolecules. A problem with this approach is that amino acid sequences that, when present in globular proteins, adopt a defined secondary structure do not generally do so as isolated peptides in aqueous solution, indicating that *tertiary* interactions are critical for the stabilization of *secondary* structure.[1] Consistent with this, aggregates of peptides, as in helical bundles[2] or coiled coils[3] of, for example, the proposed leucine zipper structure, do adopt well-defined secondary structure. A similar effect can be achieved by the so-called "template assisted" assembly approach.[4]

A conceptually simpler approach to assembling macromolecules using protein secondary structural elements as building blocks is to stabilize the structural elements in isolation, forgoing the benefit of stabilization by contact with some adjacent structural element. We have chosen as a goal the preparation of short peptides that in aqueous solution will adopt helical structure. Such stable helices have the potential to serve as well-defined structural members in rationally designed, conformationally defined macromolecules. We have recently achieved this goal by the installation into peptides of a pair of unnatural amino acid residues bearing side chains capable of ligation of metal ions.[5] Circular dichroism studies clearly reveal that a limited subset of these synthetic peptides undergoes remarkable conformational transitions on addition of metal ions.[5] We present here a full record of the design and synthesis of these peptides.

Design

There is ample precedent for the stabilization of the folded structure of macromolecules by cross-links. Globular proteins, for example, contain disulfide cross-links. In some cases, it has been shown that removal of these cross-links reduces the stability of the folded relative to unfolded state.[6] Conceptually analogous are proteins whose folded structures are stabilized by the presence of metal ions. One such example is the zinc finger motif, in which the folded form depends critically upon the presence of zinc, which "cross-links" histidine and cysteine residues by tetracoordination.[7] Another form of cross-link is the ionic interaction between lysine and glutamic acid residues, which has been shown to modestly stabilize the helical form of short peptides of de novo design.[1d,h] The physical basis for all of these phenomena may be similar: a cross-link is predicted to have limited impact on the conformational entropy of the folded form of a macromolecule. In contrast, the cross-link restricts the available conformational states of the random coil form. Cross-links are thus predicted to shift the equilibrium of random coil and

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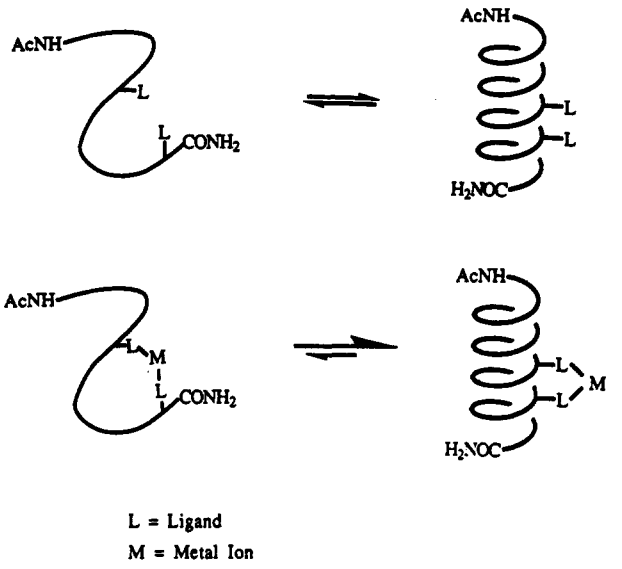


Figure 1. Predicted relative positions of the random coil/α-helix equilibrium for linear (upper) and cross-linked (lower) peptide in aqueous solution.

folded macromolecule toward the folded form by entropic destabilization of the unfolded form.[8]

We sought to design peptides in which the α-helical conformation would be stabilized by a cross-link formed by simultaneous coordination of two side-chain functional groups to a single metal ion. As shown in Figure 1, it was anticipated that this form of cross-linking would entropically destabilize the unfolded form and thus favor the folded form. It was recognized at the outset that the consequence of a poor design in which the cross-link disrupts the α-helical conformation would likely be destabilization of the helical form relative to the peptide in the absence of metal ions. Prosecution of this project required the selection of (a) specific ligands, (b) spacing of the pair of ligands in the peptide chain, and (c) a residue sequence and length for the complete peptides.

Ligand Selection. Naturally occurring amino acids that act as ligands for metals are available in the form of cysteine and histidine. While this work was in progress, it was demonstrated that these residues can be successfully employed in this helix-stabilizing strategy.[9] Because our goal was to broadly explore the structural requirements for helix stabilization, we opted instead to create a homologous family of ligands in which the spacing between the peptide backbone and the actual ligand could be varied. We furthermore sought a ligand that was expected to exhibit strong binding to a wide variety of metal ions. The aminodiacetic acid bearing side chains $-(CH_2)_l N(CH_2CO_2H)_2$ where l ranges from 1 to 4 satisfied these criteria. The analogous ligand EDTA binds a wide range of metal ions with stability constants in the range of 10^{15}–10^{20} M^{-1}. Peptides with this level of affinity for metal ions would at the solution concentrations commonly employed for conformational analysis (μM for circular dichroism, mM for ^{1}H NMR) be able to virtually completely capture even a single equivalent of added metal ion.

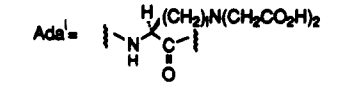
Spacing of Ligands. The appropriate spacing of ligands was dictated by the periodicity of the α-helix, 3.6 residues per turn.[6] As such, slightly less than one turn of helix is present between two residues with an i, $i + 3$ spacing and slightly more than one turn of helix separates a pair of residues with the i, $i + 4$ spacing. Both spacings were explored in this study.

Peptide Sequence and Length. Because there was no indication at the outset just how much helix stabilization could be expected, we elected to synthesize peptides that in the absence of metal ions would exhibit appreciable helicity. This ensured that circular dichroism analysis would then reveal even small changes in random coil/helix equilibrium position. We thus chose residues of known favorable helical potential, alanine, glutamic acid, and lysine. The charged nature of the latter pair near neutral pH assured water solubility; the distribution of these charged residues such that no face of the helix was hydrophobic was intended to avoid aggregation in dilute aqueous solution. To avoid charge–dipole interactions of charged termini with the helix macroscopic dipole,[10] we elected to mask the carboxyl and amino termini as amide and acetyl, respectively. A total length of ca. 20 residues was found to provide peptides of roughly 50% helix content in the absence of metal ions. For synthetic simplicity, the unnatural residues were incorporated near the amino terminus, allowing their installation late in the solid-phase synthesis.

Because peptides that in the absence of metal ions are already 50% helical can exhibit at best a doubling of helix content, we also studied peptides of lower initial helix content. This was easily achieved by shortening the peptides. The selection of which peptides to synthesize in shorter lengths was dictated by preliminary conformational studies on the longer peptides.

On the basis of all of these considerations, then, the family of peptides that became targets of synthesis was 1–8.

Ac-Ada^l-Ala$_m$-Ada^l-(Ala$_4$-Glu-Lys)$_n$-NH$_2$

Ada^l = 

	l	m	n		l	m	n
1	1	2	3	4	2	3	3
2a	1	3	1	5	3	2	3
2b	1	3	2	6	3	3	3
2c	1	3	3	7	4	2	3
3	2	2	3	8	4	3	3

Synthesis

Solid-phase peptide synthesis by the Boc approach was chosen,[11] using benzyl ester protection for Glu and Ada residues and carbo-*o*-chlorobenzyloxy protection for Lys. For reasons described in the following text, the methods of synthesis and incorporation of Ada^l residues that ultimately succeeded required that a variety of subtly different schemes be employed. The sections that follow first describe the syntheses of units bearing Ada^l, l = 1–4, which were found to be appropriate for incorporation into peptides. The peptide syntheses themselves follow.

Synthesis of Ada^l-Bearing Units. The syntheses of monomers of Ada^3 and Ada^4, which were ultimately in-

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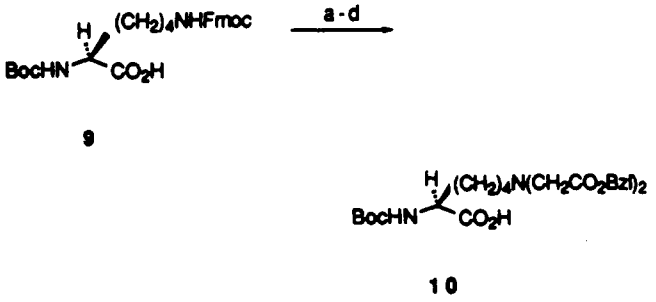
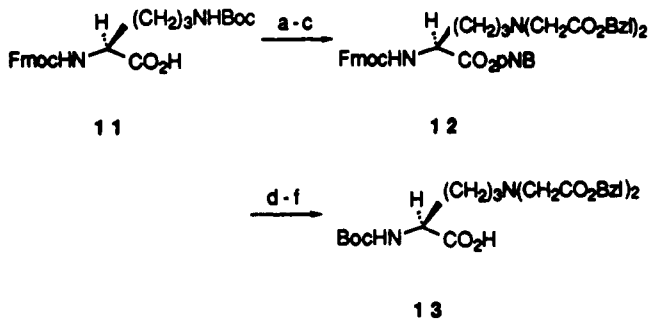
Scheme I[a]



9 **10**

[a] Key: (a) p-$NO_2C_6H_4CH_2Cl$, $NaHCO_3$, KI, DMF; (b) piperidine, DMF; (c) $BrCH_2CO_2CH_2C_6H_5$, KI, $NaHCO_3$, DMF; (d) Zn (dust), 90% HOAc/H_2O.

Scheme II[a]



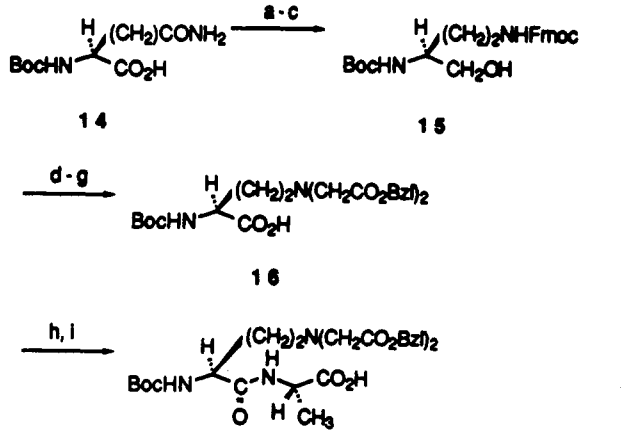
11 **12** **13**

[a] Key: (a) p-$NO_2C_6H_4CH_2Cl$, $NaHCO_3$, KI, DMF; (b) 50% TFA/CH_2Cl_2; (c) $BrCH_2CO_2CH_2C_6H_5$, KI, $NaHCO_3$, DMF; (d) piperidine, DMF; (e) Boc_2O, Et_3N, CH_2Cl_2; (f) Zn (dust), 90% HOAc/H_2O.

corporated into peptides, were similar to one another. For Ada[4] (Scheme I), the commercially available doubly N-protected carboxylic acid **9** was first protected by esterification.[12] Sequential selective removal of Fmoc,[13] bis-alkylation of the resulting primary amine, and deprotection of the carboxyl function afforded the desired Boc-protected carboxylic acid **10**. This same scheme did not work for the synthesis of the corresponding Ada[3] (**13**), apparently due to intramolecular attack of the exposed primary amine on the carboxyl function of the p-nitrobenzyl ester during removal of Fmoc. Instead, Scheme II was employed. The commercial doubly N-protected carboxylic acid **11** was protected by esterification, Boc was removed, and bis-alkylation then afforded **12**. Protecting-group reorganization by removal of Fmoc, installation of Boc and reductive removal of p-nitrobenzyl completed the synthesis, providing the Boc-protected amino acid **13** appropriate for Ada[3] installation.

The protection patterns successful for Ada[4] and Ada[3] synthesis failed for Ada[2]. The successful alternative (Scheme III) involved protection of the carboxyl function by reduction. Boc-Gln (**14**) was processed as shown to the doubly N-protected alcohol **15**,[14,15] which was in turn protected as its tetrahydropyranyl derivative.[16] Unmasking of the Fmoc-protected amine, bis-alkylation of the primary amine, and direct removal of the THP ether and oxidation of the alcohol to the carboxylic acid gave **16**.[15-17] Although the Boc-protected amino acid could in

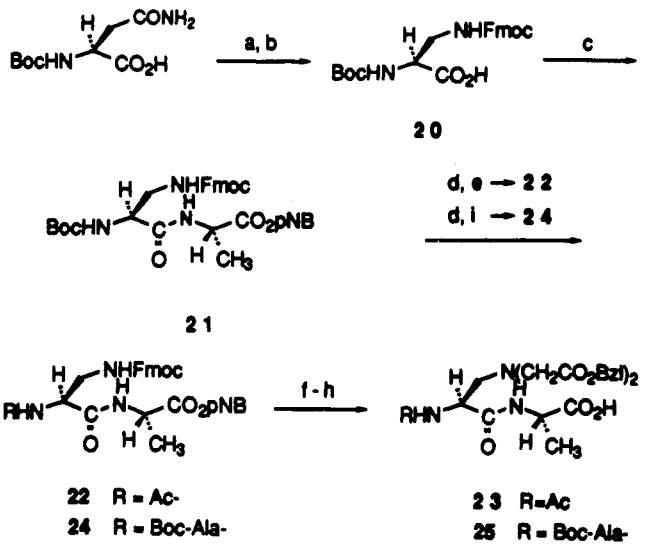
Scheme III[a]



14 **15** **16** **17**

[a] Key: (a) $C_6H_5I(OCOCF_3)_2$, pyr, DMF, H_2O; (b) FmocCl, $NaHCO_3$, dioxane, H_2O; (c) (i) $ClCO_2Et$, Et_3N, THF, (ii) $NaBH_4$, H_2O; (d) PPTS, dioxane, CH_2Cl_2; (e) piperidine, DMF; (f) $BrCH_2CO_2C$-$H_2C_6H_5$, KI, $NaHCO_3$, DMF; (g) $Na_2Cr_2O_7$, H_2SO_4, acetone; (h) AlaOPNB, DEPC, Et_3N, DMF; (i) Zn (dust), 90% HOAc/H_2O.

Scheme IV[a]



20 **21** **22** R = Ac- **23** R=Ac **24** R = Boc-Ala- **25** R = Boc-Ala-

[a] Key: (a) $C_6H_5I(OCOCF_3)_2$, pyr, DMF, H_2O; (b) FmocCl, $NaHCO_3$, dioxane, H_2O; (c) AlaOPNB, DEPC, Et_3N, DMF; (d) TFA, CH_2Cl_2; (e) Ac_2O, Et_3N, DMF; (f) piperidine, DMF; (g) $BrCH_2C$-$O_2CH_2C_6H_5$, KI, $NaHCO_3$, DMF; (h) Zn (dust), 50% HOAc/H_2O; (i) BocAlaOH, DEPC, Et_3N, DMF.

principle have been used directly for incorporation of Ada[2] into peptides, the results suggested the wisdom of one further step. It was found that the crude peptide synthesis products contained appreciable quantities of trifluoroacetyl-capped failure sequences apparently formed during attempted solid-phase coupling of either **10** or **13**. Meerifield has previously reported studies of this somewhat unexpected side reaction.[18] To avoid it in the case of Ada[2], we coupled acid **16** to carboxyl-protected Ala by solution methods and then exposed the carboxyl function of Ala to secure **17**. This approach avoided significant trifluoroacetylation-based termination during incorporation of Ada[2] into peptides by solid-phase synthesis.

Preparation of an intermediate appropriate for incorporation of Ada[1] into peptides required that one final hurdle be cleared. Preliminary studies uncovered a side

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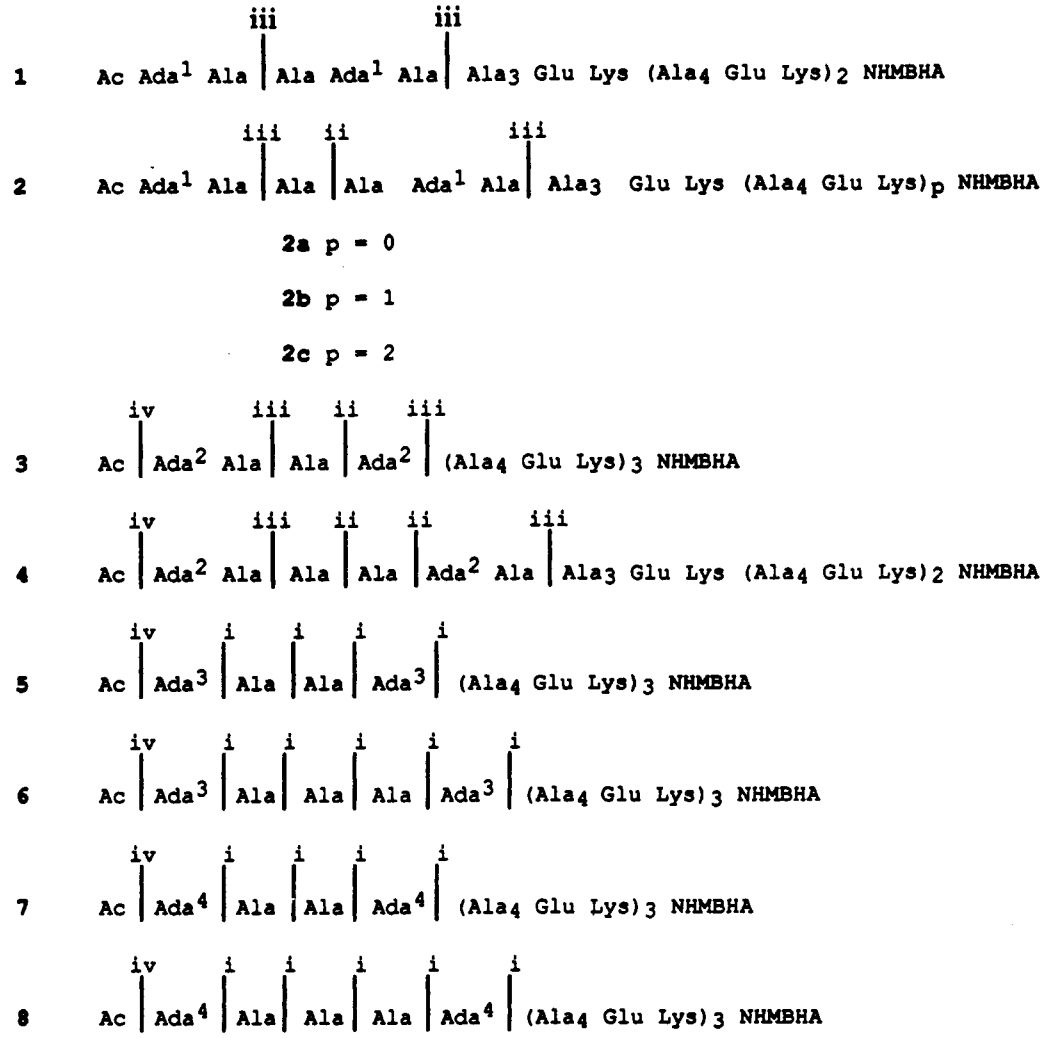

Figure 2. Manual coupling locations for solid-phase synthesis of peptides 1–8: (i) DCC; (ii) diisopropylcarbodiimide; (iii) DCC/HOBt; (iv) Ac_2O/Et_3N. Couplings between the MBHA resin and right-most manual coupling were preformed on an automated synthesizer (except for **2a**, prepared manually) using DCC. For further details, see Experimental Section.

reaction that resulted in chain termination during solid-phase peptide synthesis: the free amino group in synthetic intermediates of general structure **18** cyclize as shown to **19**. Evidence for this was the isolation of the major

18 **19**

product of an attempted synthesis of **1** that possessed a parent ion in the FAB MS consistent with cyclization-based chain termination following incorporation of the first unnatural residue. This problem was circumvented using the intermediates prepared as shown in Scheme IV. Boc-Asp was converted in two steps to the bis-N-protected carboxylic acid **20**, which was coupled to carboxyl-protected Ala **21**. Removal of Boc followed by acetylation to **22** and processing as shown gave **23**, an intermediate useful for installation of an N-terminal Ada^1 residue. Alternatively, coupling to Boc-Ala gave **24**, which was analogously converted to **25**, a key intermediate for installation of a central Ada^1 residue.

We have directly demonstrated that the enantiomeric purity of intermediates **10** and **13** (used in Ada^4 and Ada^3 incorporation) is insignificantly changed from the L starting materials. Both **10** and **13** were coupled to L- and DL-alanine methyl ester using the DEPC method.[19,20] For both **10** and **13**, the diastereoisomeric species resulting from coupling to the racemic alanine afforded doubling of numerous resonances in the 500-MHz ^1H NMR spectra. This doubling was absent in the products of coupling to L-Ala, indicating that **10** and **13** were originally enantiomerically pure. For intermediates **16**, **23**, and **25**, which already possess the potential to be diastereoisomeric mixtures due to the L-Ala residue present in each, the lack of significant loss of stereochemical purity is strongly suggested by the single set of resonances present in each case in the 500-MHz ^1H NMR spectra. It should be noted that in these cases we did not prepare authentic mixtures of the relevant diastereoisomers to verify that doubling resonances would necessarily be observable.

With the synthesis of intermediates appropriate for synthesis of peptides containing Ada^l, l = 1–4, complete, the stage was set for peptide synthesis.

Peptide Synthesis. The syntheses of peptides **1–8** were now straightforward and are outlined in Figure 2. Syntheses commenced by automated methods on MBHA resin and proceeded (right to left as shown in Figure 2) until one of the Ada-bearing residues was to be incorporated. At this stage, the resin was removed from the au-

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tomated synthesizer, and couplings were continued manually on an approximately 10-μmol scale. These couplings, indicated by vertical bars in Figure 2, were performed using DCC, DIC, or DCC/HOBt, as indicated in the figure legend. Following completion of each sequence, peptides were cleaved from the resin and protecting groups were removed by exposure to anhydrous HF. The resulting peptides were purified by gradient HPLC to a purity of at least 95%. FAB ionization of the purified peptides afforded in all cases a parent ion consistent with the indicated structure.

Conclusion

The syntheses described herein provide access to the first homologous series of peptides containing unnatural, metal-binding ligands. Studies of the metal-dependent conformational properties of these peptides are at a preliminary stage, but even now it is evident that the effort to synthesize the peptides was worthwhile; as reported elsewhere, addition of metal ions to virtually all of these peptides results in significant changes in their circular dichroism spectra in the region 190–250 nm, consistent with changes in their conformational equilibria.[5] Examples of changes in equilibrium position favoring as well as disfavoring the helical conformation have been observed. In a few cases, remarkable helical stabilization is observed, for example, peptides **2** with only 1 equiv of Cd^{2+}.[5] Peptide **2c** is especially remarkable, being converted from almost exclusively random coil to over 80% helix on addition of Cd^{2+} at 4 °C in aqueous solution at pH 7.9, representing at least a 20-fold change in equilibrium constant. Structural and thermochemical bases for these results are currently under investigation and will be reported in due course.

Experimental Section

General Procedures for Organic Synthesis. Moisture-sensitive reactions were conducted under a positive argon atmosphere. Organic solutions were concentrated on a Buchi rotary evaporator at reduced pressure using a water aspirator or vacuum pump. Commercial solvents and reagents were used without further purification, except for the following: (1) CH_2Cl_2 was distilled under argon from CaH_2; (2) DMF was dried over 4-Å sieves; (3) tetrahydrofuran was distilled from sodium and benzophenone ketyl. Column chromatography was performed under slight positive air pressure on Merck silica gel 60 (230–400 mesh); thin-layer chromatography (TLC) was performed on precoated silica gel 60 plates (0.25-mm thickness). Melting points were determined on a Fisher Johns melting point apparatus and are uncorrected. Unless otherwise noted, organic reaction products were extracted into an organic solvent, dried over $MgSO_4$, and concentrated in vacuo.

Boc-Lys(Fmoc) *p*-Nitrobenzyl Ester. To a suspension of Boc-Lys(Fmoc) (0.60 g, 1.3 mmol), $NaHCO_3$ (0.86 g, 1.0 mmol), and KI (0.21 g, 1.3 mmol) in 9 mL of DMF was added *p*-nitrobenzyl chloride (0.55 g, 3.2 mmol) in several portions at 25 °C. After being stirred for 5 h at 25 °C, the reaction mixture was poured into 30 mL of water and extracted with three 20-mL portions of CH_2Cl_2. The residue was purified by chromatography on silica gel (1.8 × 35 cm; 20% EtOAc/hexane then 40% EtOAc/hexane, and 50% EtOAc/hexane) to afford the title compound, 0.76 g (98%), as a white solid, mp 137–138 °C. ^1H NMR (200 MHz, $CDCl_3$): δ 1.24–1.80 (6 H, m, $(CH_2)_3CH_2NH$), 1.41 (9 H, s, *t*-C_4H_9), 3.16 (2 H, m, $(CH_2)_3CH_2NH$), 4.18 (1 H, t, J = 7 Hz, $CHCH_2O$), 4.35 (1 H, br s, α-H), 4.40 (2 H, d, J = 7 Hz, $CHCH_2O$), 4.75 and 5.01 (2 H, br s, 2 × NH), 5.22 (2 H, s, *p*-$NO_2C_6H_4CH_2$), 7.24–7.76 (8 H, m, aryl-H in Fmoc—), 7.48 (2 H, d, J = 9 Hz, *p*-$NO_2C_6H_4$ 2,6-H), 8.19 (2 H, d, J = 9 Hz, *p*-$NO_2C_6H_4$ 3,5-H). IR ($CHCl_3$): 3440, 3000–2850, 1710, 1605, 1500, 1350 cm^{-1}. FAB MS (3-nitrobenzyl alcohol matrix): calcd 604 (M^+ + H), found 604.

Boc-Ada^4(Bzl$_2$) *p*-Nitrobenzyl Ester. Piperidine (0.11 mL, 1.1 mmol) was added dropwise to a solution of Boc-Lys(Fmoc) *p*-nitrobenzyl ester (0.22 g, 0.37 mmol) in 10 mL of DMF at 25 °C over 40 min. The resulting mixture was concentrated in vacuo, dissolved in 10 mL of DMF, and then treated with KI (0.067 g, 0.40 mmol) and $NaHCO_3$ (0.46 g, 5.5 mmol). To this heterogeneous mixture was added dropwise benzyl 2-bromoacetate (0.46 mL, 2.9 mmol), and the resulting mixture was stirred 16 h at 25 °C. After being poured into 30 mL of water, the reaction mixture was extracted with three 10-mL portions of CH_2Cl_2. The residue was chromatographed on silica gel (1.2 × 43 cm, 10% EtOAc/hexane, then 40% EtOAc/hexane, then 50% EtOAc/hexane) to yield the title compound, 0.16 g (64%), as a pale yellow oil. ^1H NMR (200 MHz, $CDCl_3$): δ 1.32–1.75 (6 H, br m, $(CH_2)_3CH_2N$), 1.41 (9 H, s, *t*-C_4H_9), 2.66 (2 H, t, J = 7 Hz $(CH_2)_3CH_2N$), 3.55 (4 H, s, NCH_2CO_2), 4.29 (1 H, br m, α-H), 5.00 (1 H, d, J = 8 Hz, NH), 5.11 (4 H, s, $C_6H_5CH_2$), 5.23 (2 H, s, *p*-$NO_2C_6H_4CH_2$), 7.32 (10 H, m, $C_6H_5CH_2$), 7.49 (2 H, d, J = 9 Hz, *p*-$NO_2C_6H_4$ 2,6-H), 8.20 (2 H, d, J = 9 Hz, *p*-$NO_2C_6H_4$ 3,5-H). IR ($CHCl_3$): 3440, 3100–2900, 1740, 1710, 1600, 1520, 1500, 1350 cm^{-1}. FAB MS (3-nitrobenzyl alcohol matrix): calcd 678 (M^+ + H), found 678.

Boc-Ada^4(Bzl$_2$) (10). A solution of Boc-Ada^4(Bzl$_2$) *p*-nitrobenzyl ester (0.15 g, 0.22 mmol) in 19 mL of 90% aqueous acetic acid was treated with zinc dust (0.73 g, 11 mmol). The mixture was stirred at 25 °C for 2 h, passed through Celite, and dried in vacuo (over NaOH). The residue was taken up in 30 mL of ethyl acetate and washed sequentially with two 15-mL portions of 10% aqueous H_3PO_4 and 15 mL of water. The organic layer was dried ($MgSO_4$), filtered, and concentrated in vacuo. The resulting product was dissolved in $CHCl_3$ and concentrated in vacuo. This process was repeated until NMR analysis indicated that no acetic acid remained. The title compound was a yellow oil (0.11 g, 94%). ^1H NMR (200 MHz, $CDCl_3$): δ 1.30–1.80 (6 H, br m, $(CH_2)_3CH_2N$), 1.43 (9 H, s, *t*-C_4H_9), 2.69 (2 H, t, J = 6 Hz, $(CH_2)_3CH_2N$), 3.58 (4 H, s, NCH_2CO_2), 4.18 (1 H, br m, α-H), 5.11 (4 H, s, $C_6H_5CH_2$), 5.10 (1 H, br s, NH), 7.32 (10 H, m, $C_6H_5CH_2$). IR ($CHCl_3$): 3430, 3200–2300, 3100–2900, 1735, 1715, 1600, 1495, 1370 cm^{-1}. FAB MS (3-nitrobenzyl alcohol matrix): calcd 543 (M^+ + H), found 543. Optical rotation: $[\alpha]^{25}_D$ +4.5° (c = 45 mg/mL, $CHCl_3$).

Fmoc-Orn(Boc) *p*-Nitrobenzyl Ester. Fmoc-Orn(Boc) (1.0 g, 2.2 mmol) was converted to the title compound (1.1 g, 83%) as described in the previous text for the preparation of Boc-Lys(Fmoc) *p*-nitrobenzyl ester from Boc-Lys(Fmoc). The title compound was a white solid, mp 152–154 °C. ^1H NMR (200 MHz, $CDCl_3$): δ 1.35–1.88 (4 H, m, $(CH_2)_2CH_2NH$), 1.42 (9 H, s, *t*-C_4H_9), 3.12 (2 H, m, $(CH_2)_2CH_2NH$), 4.18 (1 H, t, J = 7 Hz, $CHCH_2O$), 4.36 (2H, d, J = 7 Hz, $CHCH_2O$), 4.50 (2 H, br, NH and α-H), 5.24 (2 H, s, *p*-$NO_2C_6H_4CH_2$), 5.44 (1 H, br m, NH), 7.24–7.76 (8 H, m, aryl-H in Fmoc—), 7.44 (2 H, d, J = 9 Hz, *p*-$NO_2C_6H_4$ 2,6-H), 8.18 (2 H, d, J = 9 Hz, *p*-$NO_2C_6H_4$ 3,5-H). IR ($CHCl_3$): 3430, 3100–2850, 1710, 1600, 1500, 1350 cm^{-1}. FAB MS (3-nitrobenzyl alcohol matrix): calcd 590 (M^+ + H), found 590.

Fmoc-Ada^3(Bzl$_2$) *p*-Nitrobenzyl Ester (12). A solution of Fmoc-Orn(Boc) *p*-nitrobenzyl ester (0.60 g, 1.0 mmol) in 15 mL of CH_2Cl_2 was treated with 30 mL of 50% CF_3CO_2H/CH_2Cl_2 at 25 °C. After being stirred at 25 °C for 3 h, the volatiles were removed in vacuo. The residue was dissolved in 15 mL of DMF and treated with KI (0.17 g, 1.0 mmol) and $NaHCO_3$ (3.5 g, 41 mmol). Benzyl 2-bromoacetate (2.4 mL, 15 mmol) was added dropwise to this heterogeneous mixture, and the mixture was stirred for 21 h at 25 °C. The mixture was poured into 100 mL of water and extracted with three 50-mL portions of CH_2Cl_2. The residue was chromatographed on silica gel (1.8 × 35 cm, 20% EtOAc/hexane and then 50% EtOAc/hexane) to afford the title compound **12**, 0.60 g (66%), as a pale yellow oil. ^1H NMR (500 MHz, $CDCl_3$): δ 1.55, 1.89 (4 H, br m, $(CH_2)_2CH_2N$), 2.77 (2 H, t, J = 7 Hz, $(CH_2)_2CH_2N$), 3.56 (4 H, s, NCH_2CO_2), 4.23 (1 H, br m, $CHCH_2O$), 4.43 (3 H, m, α-H and $CHCH_2O$), 5.15 (4 H, s, $C_6H_5CH_2$), 5.27 (2H, s, *p*-$NO_2C_6H_4CH_2$), 5.84 (1 H, d, J = 8 Hz, NH), 7.31–7.79 (8 H, m, aryl-H in Fmoc—), 7.36 (10 H, m, $C_6H_5CH_2$), 7.52 (2 H, d, J = 9 Hz, *p*-$NO_2C_6H_4$ 2,6-H), 8.21 (2 H, d, J = 9 Hz, p-$NO_2C_6H_4$ 3,5-H). IR ($CHCl_3$): 3430, 3100–2850, 1735, 1600, 1500, 1350 cm^{-1}. FAB MS (3-nitrobenzyl alcohol matrix): calcd 785 (M^+ + H), found 785.

Boc-Ada^3(Bzl$_2$) *p*-Nitrobenzyl Ester. A solution of **12** (0.30 g, 0.38 mmol) in 10 mL of DMF was treated dropwise with piperidine (0.10 mL, 1.0 mmol) at 25 °C. After being stirred for 0.5 h, the resulting yellow oil was dissolved in 5 mL of CH_2Cl_2

and then treated with triethylamine (71 μL, 0.51 mmol) and di-*tert*-butyl dicarbonate (0.21 mL, 0.51 mmol). The reaction mixture was stirred for 2 h at 25 °C, diluted with 10 mL of CH_2Cl_2, and washed with 5 mL of water. The residue was chromatographed on silica gel (1.2 × 35 cm, 10% EtOAc/hexane, 200 mL of 30% EtOAc/hexane, and then 40% EtOAc/hexane) and afforded the title compound, 0.170 g (67.5%), as a pale yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.34–1.87 (4 H, br m, $(CH_2)_2CH_2N$), 1.41 (9 H, s, *t*-C_4H_9), 2.71 (2 H, t, *J* = 7 Hz, $(CH_2)_2CH_2N$), 3.54 (4 H, s, NCH_2CO_2), 4.28 (1 H, br m, α-H), 5.10 (4 H, s, $C_6H_5CH_2$), 5.15 (1 H, br s, NH), 5.21 (2 H, s, *p*-$NO_2C_6H_4CH_2$), 7.32 (10 H, m, $C_6H_5CH_2$), 7.48 (2 H, d, *J* = 9 Hz, *p*-$NO_2C_6H_4$ 2,6-H), 8.19 (2 H, d, *J* = 9 Hz, *p*-$NO_2C_6H_4$ 3,5-H). IR ($CHCl_3$): 3430, 3100–2900, 1740, 1710, 1605, 1500, 1350 cm^{-1}; FAB MS (3-nitrobenzyl alcohol matrix): calcd 664 (M^+ + H), found 664.

Boc-Ada^3(Bzl$_2$) (13). Preparation of the title compound (0.164 g, 0.247 mmol) was carried out as described previously for the preparation of Boc-Ada^4(Bzl$_2$) (10), to afford the title compound, 0.117 g (89.6%), as a yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.30–1.80 (4 H, br m, $(CH_2)_2CH_2N$), 1.43 (9 H, s, *t*-C_4H_9), 2.69 (2 H, t, *J* = 6 Hz $(CH_2)_2CH_2N$), 3.58 (4 H, s, NCH_2CO_2), 4.18 (1 H, br m, α-H), 5.11 (4 H, s, $C_6H_5CH_2$), 5.10 (1 H, br s, NH), 7.32 (10 H, m, $C_6H_5CH_2$). IR ($CHCl_3$): 3430, 3200–2300, 3100–2900, 1725, 1600, 1495 cm^{-1}; FAB MS (3-nitrobenzyl alcohol matrix): calcd 529 (M^+ + H), found 529. Optical rotation: $[\alpha]^{25}_D$ +3.8° (*c* = 70 mg/mL, $CHCl_3$).

(2*S*)-*N*^{2}-Boc-*N*^{4}-Fmoc-2,4-diamino-1-butanol (15). Boc-Gln (2.12 g, 8.62 mmol) was added to a stirred solution of bis(trifluoroacetoxy)phenyliodine (5.56 g, 12.9 mmol) in 70 mL of aqueous DMF (1:1, v/v) at 25 °C, at which time TLC analysis indicated the reaction was complete. The solution was concentrated in vacuo and filtered, and the residue was dissolved in 60 mL of water. The aqueous solution was washed with four 50-mL portions of ether and concentrated in vacuo to afford 4.63 g of crude (2*S*)-*N*^{2}-Boc-2,4-diamino-1-butanoic acid as a liquid. This was dissolved in 10% aqueous Na_2CO_3 at 0 °C, to which was added dropwise Fmoc-Cl (2.25 g, 8.70 mmol) in 25 mL of dioxane at 0 °C. After being stirred for 1 h at 0 °C and 2 h at 25 °C, the reaction was quenched with 100 mL of water and washed with two 50-mL portions of ether. The aqueous layer was adjusted to pH ~1 with concentrated aqueous HCl and immediately extracted with three 50-mL portions of ethyl acetate. The residue was chromatographed on silica gel (180:8:2 CH_2Cl_2/CH_3OH/HOAc) to yield 1.11 g of a white foam. The crude (2*S*)-*N*^{2}-Boc-*N*^{4}-Fmoc-2,4-diaminobutanoic acid (1.11 g) was dissolved in 16 mL of THF, cooled to −5 °C, and treated sequentially with triethylamine (0.56 mL, 3.78 mmol) and ethyl chloroformate (0.365 mL, 2.87 mmol). The resulting slurry was stirred at −5 °C for 0.5 h and filtered. The filtrates were added dropwise to a slurry of $NaBH_4$ (0.476 g, 12.6 mmol) in 16 mL of water at 0 °C. After being stirred at 0 °C for 4 h, the mixture was diluted with 50 mL of saturated aqueous NaCl solution and extracted with three 30-mL portions of ethyl acetate. Chromatography of the residue on silica gel (20% acetone/CH_2Cl_2) afforded the title compound, 0.69 g (19% for the four steps), as a white solid, mp 140–142 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.44 (9 H, s, *t*-C_4H_9), 1.61, 1.72 (2 H, 2 × br s, $CHCH_2CH_2NH$), 2.11 (1 H, br s, OH), 3.07, 3.44, and 3.59 (3 H, 3 × br s, α-H and $CHCH_2OH$), 3.69 (2 H, br s, $CHCH_2CH_2NH$), 4.21 (1 H, t, $CHCH_2O$), 4.36 (2 H, br s, $CHCH_2O$), 4.85, 5.52 (2 H, br s, 2 × NH), 7.28–7.75 (8 H, m, aryl-H in Fmoc—). IR ($CHCl_3$): 3350, 3100–2900, 1700, 1500, 1350 cm^{-1}; FAB MS (3-nitrobenzyl alcohol matrix): calcd 427 (M^+ + H), found 427. Optical rotation: $[\alpha]^{25}_D$ −35° (*c* = 20 mg/mL, $CHCl_3$).

(2*S*)-*N*^{4},*N*^{4}-Bis[(benzyloxycarbonyl)methyl]-*N*^{2}-Boc-1-(2′-tetrahydropyranyloxy)-2,4-diaminobutane. A solution of (2*S*)-*N*^{2}-Boc-*N*^{4}-Fmoc-2,4-diamino-1-butanol (15) (0.4 g, 0.94 mmol), 3,4-dihydropyran (0.11 mL, 1.2 mmol), and ca. 3.5 mg of pyridinium *p*-toluenesulfonate in 12.5 mL of CH_2Cl_2 was stirred at 25 °C for 4 h. The reaction mixture was washed with 10 mL of saturated aqueous $NaHCO_3$ and 10 mL of saturated aqueous NaCl. The crude product (0.45 g), a white foam, was used without further purification. A solution of this THP ether in 15 mL of DMF was treated with piperidine (0.26 mL, 2.6 mmol) at 25 °C for 0.5 h. Concentration of this reaction mixture in vacuo afforded a white solid. To a heterogeneous mixture of this white solid, sodium bicarbonate (1.1 g, 13 mmol), and sodium iodide (0.16 g, 0.96 mmol) in 25 mL of DMF was added 2-bromobenzyl acetate, and the mixture was stirred at 25 °C for 19 h. This mixture was poured into 50 mL of water and extracted with three 50-mL portions of CH_2Cl_2. Chromatography of the residue on silica gel (25% ethyl acetate/hexane) afforded the title compound, 0.37 g (74% for three steps), as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.41 (9 H, s, *t*-C_4H_9, 1.4–1.6 (6 H, m, $CH_2CH_2CH_2$), 1.60–1.78 (2 H, m, $CHCH_2CH_2N$), 2.80 (2 H, m, $CHCH_2CH_2N$), 3.59 (4 H, s, NCH_2CO_2), 3.7 (1 H, m, α-H), 3.62–3.82 (4 H, m, 2 × OCH_2), 4.52 (1 H, m, OCHO), 5.06 (1 H, m, NH), 5.11 (4 H, s, $C_6H_5CH_2$), 7.32 (10 H, m, $C_6H_5CH_2$); FAB MS (3-nitrobenzyl alcohol matrix): calcd 585 (M^+ + H), found 585.

Boc-Ada^2(Bzl$_2$) (16). A solution of the (2*S*)-*N*^{4},*N*^{4}-bis[(benzyloxycarbonyl)methyl]-*N*^{2}-Boc-1-(2′-tetrahydropyranyloxy)-2,4-diaminobutane (0.34 g, 0.60 mmol) in 29 mL of acetone was cooled to 0 °C and treated dropwise with 1.3 mL (2.4 mmol) of Jones reagent (1.9 M). After 4 h, the mixture was diluted with 50 mL of water and extracted with three 25-mL portions of ethyl acetate. The combined organic extracts were washed with 20 mL of saturated aqueous NaCl. Purification of the residue by chromatography on silica gel (180:8:2, CH_2Cl_2/CH_3OH/HOAc) afforded the title compound, 0.18 g (60%), as a yellow oil after concentration twice from DMF (yield based on NMR analysis). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.42 (9 H, s, *t*-C_4H_9), 1.92 (2 H, m, β-H), 2.96 (2 H, m, CH_2N), 3.58 (4 H, s, NCH_2CO), 4.31 (1 H, br s, α-H), 5.13 (4 H, s, $C_6H_5CH_2$), 5.85 (1 H, br s, NH), 7.33 (10 H, m, $C_6H_5CH_2$). IR ($CHCl_3$): 3430, 3200–2300, 3100–2900, 1725, 1600, 1495, 1370 cm^{-1}. FAB MS (3-nitrobenzyl alcohol matrix): calcd 515 (M^+ + H), found 515. Optical rotation: $[\alpha]^{25}_D$ +3.1° (*c* = 21 mg/mL, $CHCl_3$).

Boc-Ada^2(Bzl$_2$)-Ala *p*-Nitrobenzyl Ester. A solution of Boc-Ala *p*-nitrobenzyl ester (0.10 g, 0.31 mmol) in 4.5 mL of CH_2Cl_2 was treated with 4.5 mL of trifluoroacetic acid at 25 °C under argon over 1 h. The volatiles were removed in vacuo, and the residues were dissolved in DMF and concentrated in vacuo to yield an oily residue. A solution of 16 (0.28 g, 0.14 mmol) in 3 mL of DMF was added to this oily residue. The mixture was cooled to 0 °C and treated with DEPC (0.099 mL, 0.65 mmol). After 5 min, triethylamine (0.11 mL, 0.79 mmol) was added, and the reaction mixture was stirred at 0 °C under argon for 1 h. The reaction mixture was diluted with 10 mL of water and extracted with three 10-mL portions of CH_2Cl_2. The residue was chromatographed on silica gel (50% ethyl acetate/hexane) to afford the title compound, 0.11 g (55%), as a pale yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.40 (3 H, d, *J* = ca. 7 Hz, $CHCH_3$), 1.43 (9 H, s, *t*-C_4H_9), 1.6–1.9 (2 H, br m, β-H), 2.89 (2 H, t, *J* = 6 Hz, CH_2CH_2N), 3.56, 3.67 (4 H, d, *J* = 4 Hz, NCH_2CO), 4.43, 4.62 (2 H, br s, and t, *J* = 7 Hz, 2 × α-H), 5.11 (4 H, s, $C_6H_5CH_2$), 5.18 (2 H, s, *p*-$NO_2C_6H_4CH_2$), 5.77, 7.63 (2 H, d, *J* = 8 Hz, 2 × NH), 7.33 (10 H, m, $C_6H_5CH_2$), 7.45 (2 H, d, *J* = 9 Hz, *p*-$NO_2C_6H_4$ 2,6-H), 8.19 (2 H, d, *J* = 9 Hz, *p*-$NO_2C_6H_4$ 3,5-H).

Boc-Ada^2-Ala(Bzl$_2$) (17). The selective deprotection of Boc-Ada^2-Ala *p*-nitrobenzyl ester (0.11 g, 0.15 mmol) was accomplished using the procedure described previously for the preparation of Boc-Ada^4(Bzl$_2$) (10) to yield the title dipeptide 17 as a yellow oil (79 mg, 88%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.40 (3 H, d, *J* = 7 Hz, $CHCH_3$), 1.44 (9 H, s, *t*-C_4H_9), 1.62–2.0 (2 H, br m, β-H), 2.95 (2 H, m, CH_2N), 3.60 (4 H, s, NCH_2CO), 4.45, 4.52 (2 H, t, *J* = 7 Hz, and br s, α-H), 5.13 (4 H, s, $C_6H_5CH_2$), 7.33 (10 H, m, $C_6H_5CH_2$). FAB MS (2,2′-dithiodiethanol): calcd 586 (M^+ + H), found 586.

Boc-Dap(Fmoc) (20). Boc-Asn (2.0 g, 8.6 mmol) was added to a stirred solution of bis(trifluoroacetoxy)phenyliodine (5.56 g, 12.9 mmol) in 70 mL of aqueous DMF (1:1, v/v) at 25 °C. After 15 min, pyridine (1.4 mL, 17 mmol) was added and the reaction mixture was stirred for 4 h at 25 °C, at which time TLC analysis indicated the reaction was complete. The solution was concentrated in vacuo and filtered, and the residue was dissolved in 60 mL of water. The aqueous solution was washed with four 50-mL portions of ether and the aqueous layer concentrated in vacuo to afford the crude *N*^{2}-Boc-L-2,4-diaminopropanoic acid, which was crystallized from ethyl ether (1.00 g, 57%), mp 185–187 °C dec. *N*^{2}-Boc-L-2,4-diaminopropanoic acid (0.36 g, 1.8 mmol) was dissolved in 5 mL of 10% aqueous Na_2CO_3 at 0 °C. To this solution was added dropwise Fmoc-Cl (0.55 g, 2.1 mmol) in 5 mL of dioxane at 0 °C. After being stirred for 1 h at 0 °C and 1 h

at 25 °C, the reaction was quenched with 50 mL of water and washed with two 25-mL portions of ether. The aqueous layer was adjusted to pH ~1 with concd HCl and immediately extracted with three 25-mL portions of ethyl acetate to afford N^2-Boc-N^3-Fmoc-L-2,3-diaminopropanoic acid (0.74 g) as a white solid. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.43 (9 H, s, t-C_4H_9), 3.62 (2 H, br m, $CHCH_2NH$), 4.2, 4.3 (2 H, m, α-H and $CHCH_2O$), 4.4 (2 H, d, J = 7 Hz, $CHCH_2O$), 5.5, 5.8 (2 H, br s, 2 × NH), 7.2–7.8 (8 H, m, aryl-H in Fmoc—).

Boc-Dap(Fmoc)-Ala *p*-Nitrobenzyl Ester (21). A solution of Boc-Ala *p*-nitrobenzyl ester (0.54 g, 1.7 mmol) in 25 mL of CH_2Cl_2 was treated with 25 mL of trifluoroacetic acid at 25 °C under argon over 1 h. The volatiles were removed in vacuo, and the residues were dissolved in DMF and concentrated in vacuo to yield an oily residue. A solution of Boc-Dap(Fmoc) (**20**) (0.59 g, 1.4 mmol) in 15 mL of DMF was added to this oily residue. The mixture was cooled to 0 °C and treated with DEPC (0.53 mL, 3.5 mmol). After 5 min, triethylamine (0.58 mL, 4.2 mmol) was added, and the reaction mixture was stirred at 0 °C for 1 h. The reaction mixture was diluted with 50 mL of water and extracted with three 50-mL portions of CH_2Cl_2. Chromatography on silica gel (50% ethyl acetate/hexane) afforded the title compound **21**, 0.34 g (39%), as a pale white solid, mp 185–187 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.41 (3 H, d, J = 6 Hz, $CHCH_3$), 1.43 (9 H, s, t-C_4H_9), 3.61 (2 H, br m, $CHCH_2NH$), 4.21 (3 H, m, α-H, α-H, and $CHCH_2O$), 4.40 (2 H, d, J = 7 Hz, $CHCH_2O$), 4.60 (1 H, t, J = 7 Hz, α-H), 5.45, 5.65 and 7.15 (3 H, br s, 3 × NH), 5.23 (2 H, s, p-$NO_2C_6H_4CH_2$), 7.24–7.77 (8 H, m, aryl-H in Fmoc—), 7.50 (2 H, d, J = 9 Hz, p-$NO_2C_6H_4$ 2,6-H), 8.17 (2 H, d, J = 9 Hz, p-$NO_2C_6H_4$ 3,5-H). FAB MS (3-nitrobenzyl alcohol matrix): calcd 634 (M^+ + H), found 634.

Ac-Dap(Fmoc)-Ala *p*-Nitrobenzyl Ester. Trifluoroacetic acid (7.5 mL) was added to a suspension of Boc-Dap(Fmoc)-Ala *p*-nitrobenzyl ester in 7.5 mL of CH_2Cl_2 at 25 °C, and the reaction mixture was stirred at 25 °C for 1 h. The volatiles were removed in vacuo, and the residue was dissolved in DMF and concentrated in vacuo to yield an oily residue. The solution of this oily residue in 10 mL of CH_2Cl_2 was stirred with acetic anhydride (0.12 mL, 1.3 mmol), triethylamine (0.14 mL, 1.0 mmol) was added, and the reaction was stirred at 25 °C for 1 h. Concentration in vacuo afforded the title compound, which was used directly in the next step. A sample of the title compound purified by chromatography on silica gel (50% acetone/CH_2Cl_2 gave the following ^{1}H NMR spectrum. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.45 (3 H, d, J = 7 Hz, $CHCH_3$), 2.03 (3 H, s, CH_3CO), 3.57 (2 H, br m, $CHCH_2NH$), 4.24, 4.49 (2 H, t, J = 7 Hz, α-H), 4.40 (3 H, m, $CHCH_2O$, and $CHCH_2O$), 5.25 (2 H, s, p-$NO_2C_6H_4CH_2$), 5.58, 6.88 (2 H, br s, 2 × NH), 7.24–7.77 (9 H, m, aryl-H in Fmoc, NH), 7.50 (2 H, d, J = 9 Hz, p-$NO_2C_6H_4$ 2,6-H), 8.17 (2 H, d, J = 9 Hz, p-$NO_2C_6H_4$ 3,5-H).

Ac-Ada^1(Bzl$_2$)-Ala *p*-Nitrobenzyl Ester. A solution Ac-Dap(Fmoc)-Ala *p*-nitrobenzyl ester in 4 mL of DMF was treated dropwise with piperidine (0.074 mL, mmol) and stirred at 25 °C for 40 min. The resulting mixture was concentrated to dryness in vacuo, dissolved in DMF, and dried in vacuo. The residue was dissolved in 8 mL of DMF and treated with KI (0.046 g, 0.27 mmol) and $NaHCO_3$ (0.32 g, 3.8 mmol). To this heterogeneous mixture was added benzyl 2-bromoacetate (0.32 mL, 2.0 mmol), and the mixture was stirred at 25 °C for 16 h. After being poured into 20 mL of water, the reaction mixture was extracted with three 10-mL portions of CH_2Cl_2. Chromatography of the residue on silica gel (15% acetone/CH_2Cl_2) afforded the title compound, 0.12 g (74%), as a pale yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.47 (3 H, d, J = 7 Hz, $CHCH_3$), 2.00 (3 H, s, CH_3CO), 2.63, 3.37 (2 H, dd, J = 4, 13 Hz, $CHCH_2N$), 3.62, 3.78 (4 H, d, J = 18 Hz, NCH_2CO_2), 4.26, 4.56 (2 H, m, α-H), 5.13 (4 H, d, J = 1.5 Hz, $C_6H_5CH_2$), 5.22 (2 H, s, p-$NO_2C_6H_4CH_2$), 6.80, 8.37 (2 H, d, J = 7 Hz, 2 × NH), 7.34 (10 H, m, 2 × $C_6H_5CH_2$), 7.48 (2 H, d, J = 9 Hz, p-$NO_2C_6H_4$ 2,6-H), 8.21 (2 H, d, J = 9 Hz, p-$NO_2C_6H_4$ 3,5-H. IR ($CHCl_3$): 3400, 3280, 3100–2800, 1700, 1650, 1510, 1490, 1350 cm^{-1}. FAB MS (3-nitrobenzyl alcohol matrix): calcd 649 (M^+ + H), found 649.

Ac-Ada^1(Bzl$_2$)-Ala (23). The deprotection of Boc-Ada^1-(Bzl$_2$)-Ala *p*-nitrobenzyl ester (0.11 g, 0.17 mmol) to yield **23** was accomplished using the procedure described previously for the preparation of compound **17** to yield the title dipeptide **23**, 85 mg (95%), as a yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.45 (3 H, d, J = 7 Hz, $CHCH_3$), 2.00 (3 H, s, CH_3CO), 2.71, 3.34 (2 H, dd, J = 4, 13 Hz, $CHCH_2N$), 3.62, 3.77 (4 H, d, J = 18 Hz, NCH_2CO_2), 4.29, 4.50 (2 H, m, 2 × α-H), 5.13 (4 H, s, $C_6H_5CH_2$), 7.03, 8.29 (2 H, d, J = 7 Hz, 2 × NH), 7.34 (10 H, m, $C_6H_5CH_2$). FAB MS (thioglycerol/DMSO/HCl): calcd 514 (M^+ + H), found 514. Optical rotation: $[\alpha]^{25}_D$ +51° (c = 34 mg/mL, $CHCl_3$).

Boc-Ala-Dap(Fmoc)-Ala *p*-Nitrobenzyl Ester (24). Trifluoroacetic acid (9.0 mL) was added to a suspension of Boc-Dap(Fmoc)-Ala *p*-nitrobenzyl ester (0.19 g, 0.30 mmol) in 9.0 mL of CH_2Cl_2 at 25 °C, and the reaction solution was stirred at 25 °C for 1 h. The volatiles were removed in vacuo, and the residue was dissolved in DMF and evaporated in vacuo to yield an oily residue. A solution of this oily residue and Boc-Ala (0.057 g, 0.30 mmol) in 2.2 mL of DMF at 0 °C was treated with DEPC (0.53 mL, 3.5 mmol). After 5 min, triethylamine (0.10 mL, 0.73 mmol) was added and the reaction mixture was stirred at 0 °C for 1 h. The reaction mixture was diluted with 10 mL of water, and extracted with three 10-mL portions of CH_2Cl_2. Chromatography of the residue on silica gel (20% acetone/CH_2Cl_2) afforded the title compound **24**, 0.18 g (84%), as a colorless glass. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.35 (9 H, s, t-C_4H_9), 1.37 (3 H, d, J = 7 Hz, $CHCH_3$), 1.44 (3 H, d, J = 7 Hz, $CHCH_3$), 3.61 (2 H, br m, $CHCH_2NH$), 4.16 (1 H, t, J = 7 Hz, $CHCH_2O$), 4.37 (2 H, d, J = 7 Hz, $CHCH_2O$), 4.27, 4.41, 4.57 (4 H, m, 3 α-H and 1 × NH), 4.95, 5.63 and 7.63 (3 H, m, 3 × NH), 5.24 (2 H, s, p-$NO_2C_6H_4CH_2$), 7.24–7.77 (8 H, m, aryl-H in Fmoc—), 7.50 (2 H, d, J = 9 Hz, p-$NO_2C_6H_4$ 2,6-H), 8.17 (2 H, d, J = 9 Hz, p-$NO_2C_6H_4$ 3,5-H). FAB MS (3-nitrobenzyl alcohol matrix): calcd 705 (M^+ + H), found 705.

Boc-Ala-Ada^1(Bzl$_2$)-Ala *p*-Nitrobenzyl Ester. A solution Boc-Ala-Dap(Fmoc)-Ala *p*-nitrobenzyl ester (0.15 g, 0.22 mmol) in 4 mL of DMF was treated dropwise with piperidine (0.066 mL, 0.67 mmol) and stirred at 25 °C for 40 min. The resulting mixture was concentrated to dryness in vacuo, dissolved in DMF, and dried in vacuo. The residue was dissolved in 6.8 mL of DMF and treated with KI (0.04 g, 0.24 mmol) and $NaHCO_3$ (0.27 g, 3.2 mmol). To this heterogeneous mixture was added benzyl 2-bromoacetate (0.27 mL, 1.7 mmol), and the mixture was stirred at 25 °C for 24 h. After being poured into 20 mL of water, the reaction mixture was extracted with three 10-mL portions of CH_2Cl_2. Chromatography of the residue on silica gel (10% acetone/CH_2Cl_2) afforded the title compound, 0.10 g (62%), as a colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.35 (3 H, d, J = 7 Hz, $CHCH_3$), 1.46 (3 H, d, J = 7 Hz, $CHCH_3$), 1.44 (9 H, s, t-C_4H_9), 2.71, 3.37 (2 H, dd, J = 4, 13 Hz, $CHCH_2N$), 3.61, 3.74 (4 H, d, J = 18 Hz, NCH_2CO_2), 4.17, 4.23, 4.56 (3 H, br m, 3 × α-H), 5.03, 5.15, 7.36 (3 H, br m, 3 × NH), 5.13 (4 H, d, J = 1.5 Hz, $C_6H_5CH_2$), 5.22 (2 H, s, p-$NO_2C_6H_4CH_2$), 7.34 (10 H, m, $C_6H_5CH_2$), 7.50 (2 H, d, J = 9 Hz, p-$NO_2C_6H_4$ 2,6-H), 8.20 (2 H, d, J = 9 Hz, p-$NO_2C_6H_4$ 3,5-H). IR ($CHCl_3$): 3400, 3300, 3100–2800, 1730, 1700, 1650, 1490, 1350 cm^{-1}. FAB MS (3-nitrobenzyl alcohol matrix): calcd 778 (M^+ + H), found 778.

Boc-Ala-Ada^1(Bzl$_2$)-Ala (25). The selective deprotection of Boc-Ala-Ada^1(Bzl$_2$)-Ala *p*-nitrobenzyl ester (0.075 g, 0.096 mmol) to yield the title compound was accomplished using the procedure described previously for the preparation of compound **17**. The title compound (0.067 g, 98%) was a yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.36 (3 H, d, J = 7 Hz, $CHCH_3$), 1.45 (3 H, d, J = 7 Hz, $CHCH_3$), 1.43 (9 H, s, t-C_4H_9), 2.80, 3.32 (2 H, dd, J = 4, 13 Hz, $CHCH_2N$), 3.62, 3.75 (4 H, J = 18 Hz, NCH_2CO_2), 4.25, 4.49 (3 H, m, α-H), 5.12 (4 H, m, $CH_2C_6H_5$), 5.20, 7.67, 8.20 (3 H, m, NH), 7.34 (10 H, m, $C_6H_5CH_2$). FAB MS (thioglycerol/DMSO/HCl): calcd 643 (M^+ + H), found 643. Optical rotation: $[\alpha]^{25}_D$ +28°.

Peptide Synthesis. Solvents were purified as follows: (1) CH_2Cl_2 was distilled under argon from calcium hydride; (2) DMF was dried over 4-Å sieves; (3) ethyl acetate was washed sequentially with 5% Na_2CO_3, saturated aqueous NaCl, and dried ($MgSO_4$). The following reagents were obtained commercially and used without purification: dicyclohexylcarbodiimide (DCC) in CH_2Cl_2 (0.5 M, Applied Biosystems), *N*-hydroxybenzotriazole (HOBt) in DMF (0.5 M, Applied Biosystems), diisopropylcarbodiimide (Aldrich), triethylamine (Baker), trifluoroacetic acid (Advanced Chem Tech), acetic anhydride (Baker), anisole (Aldrich), *p*-methyl BHA resin HCl salt (Applied Biosystems). Commercial amino

Table I. Observed and Calculated Most Abundant Ions in the FAB MS of Peptides 1–8

peptide	calculated formula	most abundant ion (m/e (M + H)$^+$) calcd[a]	obsvd
1	$C_{91}H_{152}N_{28}O_{37}$	2231	2230
2a	$C_{48}H_{79}N_{15}O_{22}$	1219	1218
2b	$C_{71}H_{118}N_{22}O_{30}$	1760	1755
2c	$C_{94}H_{157}N_{29}O_{38}$	2302	2301
3	$C_{93}H_{156}N_{28}O_{37}$	2259	2258
4	$C_{96}H_{161}N_{29}O_{38}$	2330	2329
5	$C_{95}H_{160}N_{28}O_{37}$	2287	2288
6	$C_{96}H_{165}N_{29}O_{38}$	2358	2357
7	$C_{97}H_{164}N_{28}O_{37}$	2315	2312
8	$C_{100}H_{169}N_{29}O_{38}$	2386	2385

[a] Except for 2a, in which only the most abundant isotope of each element is calculated to be present, the most abundant ion for all peptides is predicted to contain one atom of ^{13}C.

acids (Bachem or Applied Biosystems) were protected at the α-amino position with the Boc group, and the following side-chain protecting groups were used: Lys(2-Cl-Z), Glu(OBzl). Boc derivatives of the unnatural amino acids were synthesized using the procedures described previously. The peptide resins A and B (below) were synthesized on an Applied Biosystem peptide synthesizer using Boc chemistry. The peptide resin C was synthesized using manual stepwise SPPS (DCC as a coupling reagent).

A: Boc-[Ala_4-Glu(OBzl)-Lys(2-Cl-Z)]$_3$-MBHA resin

B: Boc-Ala_3-Glu(OBzl)-Lys(2-Cl-Z)-[Ala_4-Glu(OBzl)-Lys(2-Cl-Z)]$_2$-MBHA resin

C: Boc-Ala_3-Glu(OBzl)-Lys(2-Cl-Z)-MBHA resin

Substitution levels were ~0.32 (A) and 0.75 (B, C) mmol/g determined by picric acid analysis. Manual solid-phase peptide synthesis (10-mmol scale) was performed in a 2-mL reaction vessel with a screw cap top and a coarse glass frit in the bottom.

Syntheses followed the following general protocol. Peptide resin in a reaction vessel was allowed to swell in 1 mL of CH_2Cl_2 for 10 min three times. Attachment of each amino acid was accomplished as follows: (1) prewash, 50% TFA/CH_2Cl_2 (v/v), (1 × 1.5 min); (2) deprotect, 50% TFA/CH_2Cl_2 (1 × 30 min); (3) wash, CH_2Cl_2 (6 × 1.5 min); (4) neutralize, 10% TEA/CH_2Cl_2 (2 × 2 min); (5) wash, CH_2Cl_2 (6 × 1.5 min); (6) coupling as shown in Figure 2 and described in detail in the following text; (7) wash, CH_2Cl_2 (6 × 1.5 min). The solvent volume for all steps was 1 mL. Completion of deprotection and coupling steps was monitored by the analytical Kaiser test. The N-termini were acetylated using acetic anhydride dimenthylformamide/triethylamine (0.4:1.5:0.7) for 30 min at 25 °C. Coupling methods were as follows.

DCC or DIC. Boc-amino acid (3 equiv for unnatural amino acid, and 3–6 equiv for Boc-Ala-OH) was added to resin-bound peptide. After 1.5 min, a solution of DCC in CH_2Cl_2 (0.5 M, equimolar to the Boc-amino acid added) was added at 25 °C and the reaction mixture was shaken for 2 h.

DCC/HOBt. A solution of protected dipeptide or tripeptide (1.5–2.0 equiv) in DMF (1.8 mL/mmol peptide segment) and a solution of HOBt in DMF (0.5 M, 6 equiv) was added to the resin-bound peptide at 0 °C. After 1.5 min, a solution of DCC in CH_2Cl_2 (0.5 M, 6 equiv) was added, and the reaction mixture was shaken at 0 °C for 2 h and 25 °C for 16 h.

HF cleavage was accomplished by drying the resin-bound peptide at 25 °C (0.1 mm) in a Teflon vessel of an HF cleavage apparatus (Peninsula Laboratories, Inc.). Anisole (ca. 6 drops) was added, and anhydrous HF (ca. 5 mL, predried with CoF_3) was distilled into the cold (−78 °C) reaction vessel. After 1 h at 0 °C with stirring, the volatiles were removed in vacuo. The residue was washed with three 1-mL portions of ethyl acetate. The remaining residue was extracted with three 1-mL portions of 20% aqueous acetic acid, which were filtered from the resin. The combined aqueous acetic acid extracts were concentrated to dryness to afford the crude peptide. The crude cleaved peptide was dissolved in water and centrifuged to remove insoluble residues. Each crude peptide was purified by HPLC on an analytical (0.46 × 25 cm) Macrosphere 5 μ C18 column (Alltech), using a linear gradient of from 18 to 35% acetonitrile in H_2O (constant 0.1% TFA) over 50 min with detection at 214 nm. Purified peptides were at least 95% pure as analyzed by HPLC and afforded FAB MS (thioglycerol/DMSO/HCl as a matrix; shown in Table I) consistent with the assigned structures.

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Supplementary Material Available: ^1H NMR spectra of all title compounds in the Experimental Section, HPLC and FABMS for peptides 1, 2a, 2c, and 3–8, and amino acid analysis for all peptides (30 pages). Ordering information is given on any current masthead page.