Characterization of Synthetic Peptides. Purified peptide products were characterized by C18 RP HPLC for homogeneity, and by amino acid analysis following gas-phase hydrolysis in 5.7 N HCl.[65] In all cases a single peak was obtained and the amino acid compositions were consistent with the calculated molar ratios. Certain peptides were also subjected to sequence analysis, either by gas-phase methodology[66] with the phenylthiohydantoin derivatives quantitated by RP HPLC or, in the case of N- and C-terminally blocked peptides, by FAB mass spectrometry.[58,67]

FTIR Spectrometry. KBr disks were prepared with dried peptidoresin (100 mg of KBr:3 mg of peptidoresin) and scanned from 4000 to 400 cm^{-1} (resolution 1.0 cm^{-1}) with a Bruker IFS48 spectrometer (Rheinstetten, BRD).

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Supplementary Material Available: Sequences, cumulative $\langle P_c^* \rangle$ values for the predictive method, and synthetic details of the 101 syntheses (986 aminoacylations) used as the database for the histograms shown in Figure 4 (34 pages). Ordering information is given on any current masthead page.

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Multicyclic Polypeptide Model Compounds. 1. Synthesis of a Tricyclic Amphiphilic α-Helical Peptide Using an Oxime Resin, Segment-Condensation Approach[1]

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Abstract: An idealized model amphiphilic α-helical peptide, *cyclo*(3-7,10-14,17-21)H-[LysLeuLysGluLeuLysGlu]$_3$-OH (peptide **1-1-1**), comprising three repeats of a Lys^3-Glu^7 side-chain bridged heptapeptide, has been synthesized by a generally applicable segment-condensation approach that involves a novel solid-phase cyclization reaction. The linear heptapeptide, Boc-Lys-(2Cl-Z)LeuLys(Trt)Glu(OBzl)LeuLys(2Cl-Z)Glu(oxime resin)-OPac, was built on a *p*-nitrobenzophenone oxime derivatized polystyrene solid support by standard methods. After selective detritylation with TFA, the Lys^3 ϵ-amino group was liberated with DIEA, and then intrachain cyclization in the presence of AcOH released the protected cyclic heptapeptide precursor to peptide **1-1-1** into the solvent in 61% yield and high purity. Selective N$^\alpha$- and C$^\alpha$-group deprotection, followed by two solution-phase segment-condensation reactions and then complete deprotection with trimethylsilyl triflate, yielded peptide **1-1-1**. Circular dichroism spectra indicated that peptide **1-1-1** adopted mostly disordered conformations in aqueous solution, but a high α-helix content was induced in 50% TFE and upon adsorption of peptide **1-1-1** from aqueous solution onto siliconized quartz slides.

Introduction

Many biologically active peptides have flexible structures and exist in multiple disordered conformational states in aqueous solution. Studies of peptide models indicate that the conformations induced in these peptides by their functional environment, which is generally an interface, will often be a folded structure not present in solution and may include segments of amphiphilic secondary structure.[2] For several such peptides, including the serum apolipoprotein A-I, the bee venom peptide toxin melittin, and the peptide hormones β-endorphin and calcitonin, the functional requirement for amphiphilic α-helical structures has been demonstrated through the design and study of analogues incorporating minimally homologous models of these structures that are able to reproduce all of the functional properties of the native sequences.[2,3] However, similar studies of other peptide hormones, including glucagon,[4] calcitonin gene-related peptide,[5] and neuropeptide Y,[6] are less conclusive, because multiple substitutions in the amino acid sequence result in greatly reduced potencies. In these cases, which may represent the majority of intermediate-sized peptide hormones acting at cell-surface receptors, it is likely that functional requirements for both primary and secondary structural features are superimposed in the same peptide segments. For example, when highly conservative, helix-stabilizing substitutions in a potential amphiphilic α-helical segment of glucagon were limited to only three residue positions, enhanced receptor-binding potency was achieved.[7] In order to investigate structure-activity relationships in such peptides, the study of conformationally constrained analogues,[8] where primary structure is largely conserved, may be more appropriate than the use of minimally homologous model peptides. However, meaningful conclusions concerning potential folded conformations that may involve 10-20 residues or more will require the introduction of multiple conformational constraints, each of which is compatible with the conformation in question and limits the conformational freedom of a peptide segment several residues long. With this

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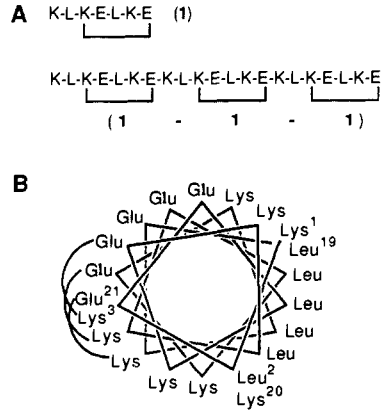


Figure 1. (A) Structures of peptide **1** and peptide **1–1–1**. (B) Axial projection of peptide **1–1–1** in the α-helical conformation.

goal in mind, we are developing a new synthetic approach to the synthesis of peptides that incorporate multiple lactam bridges.

Monocyclic and bicyclic amide-bridged structures that constrain peptide segments 2–10 residues long have often been used successfully to explore the functional conformations of small bioactive peptides.[9,10] More specifically, in relation to our present goal, incorporation of lactam-bridged Asp^i, Lys^{i+4} side chains stabilized an amphiphilic α-helical structure in the solution conformation of growth hormone releasing factor 1–29 amide and enhanced its receptor-binding potency.[11] The synthesis of such amide-bridged peptides has been achieved through selective deprotection and coupling of the amine and carboxyl groups, either in solution[12] or during a polymer-supported synthesis.[10,11,13] In solution, this coupling must be performed at high dilution, in order to favor the desired intramolecular reaction. In the solid-phase approach, in contrast, there is little control over the interchain reactions that would result in polymeric byproducts, and the desired monomeric structure can only be purified after complete assembly of the peptide chain and its deprotection and cleavage from the solid support. The solid-phase approach generally has the advantage of being more rapid, but it may not always be applicable to the synthesis of peptides incorporating multiple lactam bridges, where the problem of interchain coupling reactions will be compounded.

We are developing a general approach to the synthesis of peptides incorporating multiple lactam bridges that combines the advantages for this purpose of both solution- and solid-phase methods. A *p*-nitrobenzophenone oxime polymer[14] (oxime resin) is employed as the solid support for assembly and release into solution of fully protected, lactam-bridged peptide segments that

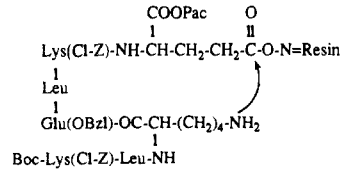
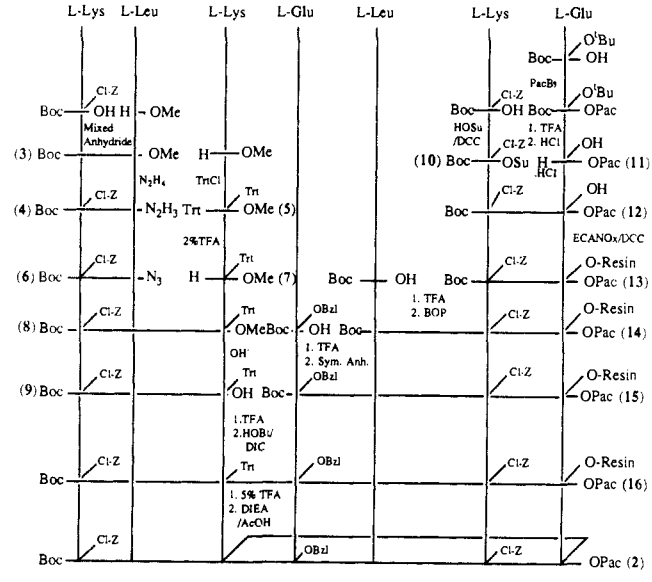


Figure 2. Intrachain cyclization reaction on oxime resin.

Scheme I. Synthetic Route to Boc-(1–7)-OPac (**2**)



are then used in segment-condensation syntheses. The key step is the peptide cyclization, which takes advantage of the active ester character of the peptidyl oxime resin linkage to perform the cyclization reaction with concomitant cleavage of the protected peptide segment from the solid support. We now describe its application to the synthesis of a multicyclic analogue of a well-characterized linear peptide model of the amphiphilic α-helical structures proposed for serum apolipoprotein A-I.[15]

Results

Peptide Design. A Lys^3–Glu^7 side-chain bridged heptapeptide (**1**) was designed so that the condensation of three such peptide units would result in peptide **1–1–1**, a 21-residue, amphiphilic α-helical peptide with three Lys^i, Glu^{i+4} side-chain linked amide bridges that are compatible with the α-helical conformation (Figure 1). This peptide retains the general characteristics in the α-helical conformation of the linear 22-residue peptide studied previously, as well as those of the native sequences from apolipoprotein A-I on which that peptide was based.[15] These characteristics include segregation of the hydrophobic residues in a single domain lying parallel to the helix axis and covering about one-third of its surface, and distribution of the charged, hydrophilic residues on the helix surface such that positively charged side chains lie adjacent to the hydrophobic domain and negatively charged side chains lie on the opposite side of the helix from that domain. This arrangement was expected to be optimal for binding of the α-helical structures to phospholipid surfaces.[16] The amide bridges in peptide **1–1–1** were positioned such that they would lie in the middle of the hydrophilic face of the potential α-helical structure, where their direct effects on the amphiphilic character of that structure and its interface-binding properties were expected to be minimal.

Peptide Synthesis. Peptide **2** (fully protected **1**) was assembled by using the oxime resin, as described in Scheme I. Dipeptide Boc-Lys(2Cl-Z)Glu-OPac (**12**) was prepared in solution and coupled to the oxime resin by using EACNOx[17,18] and DCC (yield

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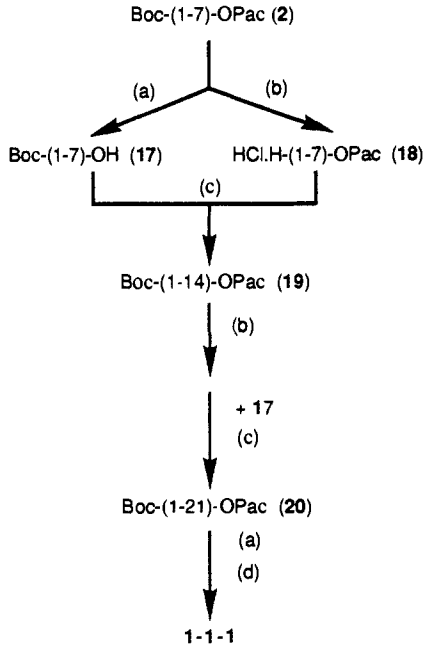
Scheme II. Synthesis of **1-1-1** by Segment Condensation[a]



[a] (a) Zn/AcOH; (b) TFA followed by HCl/dioxane; (c) HOBt/DCC; (d) TMSOTf + thioanisole in TFA.

89%; substitution level 0.066 mmol/g). The peptidyl resin **13** was then subjected to two cycles of the standard oxime resin peptide synthesis protocol,[14] using the BOP reagent to couple Boc-Leu-OH in the first cycle and the symmetric anhydride method to couple Boc-Glu(OBzl)-OH in the second cycle, to obtain peptidyl resin **15**. The tripeptide Boc-Lys(2Cl-Z)LeuLys(Trt)-OH (**9**) was prepared separately in solution and then coupled to **15** by using HO-Bt/DCC, yielding the fully protected, linear heptapeptidyl resin **16**. A solution of 5% (v/v) TFA in TFE/DCM (1/1, v/v) was used for selective removal of the N^{ϵ}-Trt protecting group of **16** in the presence of the N^{α}-Boc group. After liberation of the free ϵ-amino group from its TFA salt with DIEA, the intrachain cyclization shown in Figure 2 was then allowed to proceed in DCM, in the presence of AcOH as a catalyst. After 3 days, the cyclic heptapeptide Boc-(1-7)-OPac **2** was washed from the resin and purified by flash chromatography. The desired HPLC-pure product **2** was obtained from its peptidyl resin precursor **16** in 61% yield. Trial cyclization reactions performed without addition of AcOH yielded no product.

Condensations of the cyclic heptapeptide derivatives to produce peptide **1-1-1** were carried out in solution phase (Scheme II). The partially deprotected peptides Boc-(1-7)-OH **17** and H-(1-7)-OPac **18** were generated from **2** by reduction with Zn in aqueous AcOH (yield 84%) and by acidolysis with TFA (quantitative), respectively. These two peptides were then condensed by using HO-Bt/DCC, and the crude product, Boc-(1-14)-OPac **19**, was purified by flash chromatography and HPLC (yield 66%). Boc deprotection of **18**, followed by a repeat condensation with **2** using the HO-Bt/DCC procedure, gave Boc-(1-21)-OPac **20** in 27% yield. This crude, 21-residue, fully protected peptide was purified by gel permeation chromatography on Sephadex LH-60 eluted with DMF. The Pac group was then cleaved from **20** by Zn in aqueous AcOH and a final deprotection was carried out by the TMSOTf/thioanisole/TFA procedure.[19] Gel permeation chromatography and reversed-phase HPLC resulted in the desired HPLC-pure peptide **1-1-1**.

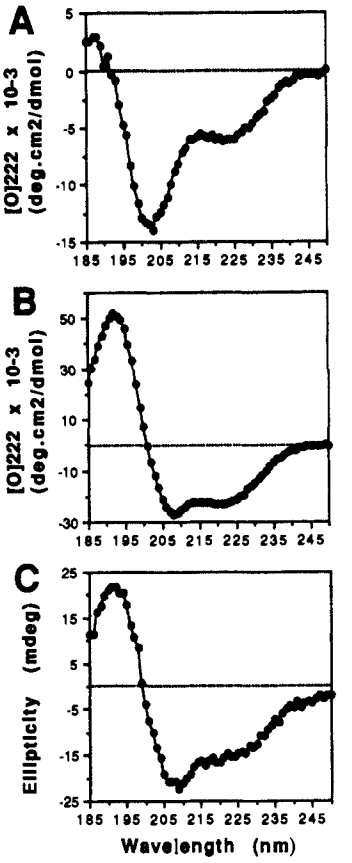


Figure 3. Circular dichroism spectra of peptide **1-1-1** (43 μM) in aqueous buffer (A), in 50% TFE/buffer (B), and adsorbed from aqueous buffer onto siliconized quartz slides[11] (C).

Optical Purity. The optical purities of peptide **1-1-1** and the synthetic intermediate Boc-(1-7)-OH **17** were determined, after hydrolysis in 6 N HCl at 110 °C for 24 h, by HPLC analysis of the diastereomers obtained upon derivatization of the component amino acids with 1-fluoro-(2,4-dinitrophenyl)-5-L-alanine amide (Marfay's reagent).[20] The amounts of D-Glu, D-Leu, and D-Lys in the acid hydrolysates, expressed as percent of the total for each amino acid, were D-$Glu_{4.8}$, D-$Leu_{1.0}$, and D-$Lys_{0.4}$ for peptide **1-1-1** and D-$Leu_{1.3}$ and D-$Lys_{4.7}$ for peptide **17**. This compares to values of D-$Glu_{5.5}$, D-$Leu_{0.3}$, and D-$Lys_{1.7}$ obtained for the earlier apo A-I model peptide, which had been resynthesized by a standard N^{α}-Boc-protected symmetric anhydride coupling protocol on Merrifield resin[21] and provided a control for the known racemization of amino acids during the acid hydrolysis of peptides. Within the limitations of this analysis, therefore, peptides **1-1-1** and **17** appeared to be optically pure.

Circular Dichroism Studies. The chiroptical properties of peptide **1-1-1** were studied in 0.01 M KH_2PO_4/NaOH aqueous buffer (pH 7.0) at several peptide concentrations (0.52–97 μM peptide) and in TFE/buffer (1/1, v/v) solution (20 μM peptide) (Figure 3). In the aqueous buffer, concentration-independent circular dichroism spectra indicative of a mixture of α-helical and disordered structure were obtained. For comparative purposes, we estimated α-helix contents for peptide **1-1-1** using the same approximation [based on standard spectra for the disordered and α-helical conformations of poly(L-lysine)[22]] as was used previously for the linear apo A-I model peptide. This gave values of 23%

(17) Abbreviations used are those recommended by the IUPAC-IUB Joint Commission on Biochemical Nomenclature: BOP, (benzotriazol-1-yloxy)-tris(dimethylamino)phosphonium hexafluorophosphate; 2Cl-Z, 2-chlorobenzyloxycarbonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethane; DIC, *N,N'*-diisopropylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; EACNOx, ethyl 2-(hydroxyimino)-2-cyanoacetate; HO-Bt, 1-hydroxybenzotriazole; Pac, phenacyl; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TMSOTf, trimethylsilyl trifluoromethanesulfonate; Trt, trityl (=triphenylmethyl).

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helix ($[\theta]_{222}$ = −5960 deg·cm²/dmol) for peptide **1-1-1** in the aqueous buffer and 67% helix ($[\theta]_{222}$ = −23 000 deg·cm²/dmol) in 50% TFE/buffer solution. The linear apo A-I model peptide was reported to be slightly less helical as a monomer in 0.1 M phosphate buffer, pH 7.0, (19% helix) and in 50% TFE/buffer solution (61% helix), but self-associated in the aqueous buffer in the 1–50 μM concentration range, forming a discrete tetrameric structure in which the α-helical conformation was stabilized (50% helix).[15]

Peptide **1-1-1** was also adsorbed onto siliconized quartz slides from an aqueous buffer consisting of 0.02 M KH_2PO_4/NaOH in 0.16 M KCl, pH 7.5, and the CD spectrum of the resultant surface-adsorbed peptide film (Figure 3) was consistent with induction of a strongly α-helical structure at this interface,[23,24] assuming that the helices are oriented with their axes parallel to the plane of the interface, as expected from their amphiphilic character.[25,26]

Discussion

Peptide **1-1-1**, a multicyclic 21-residue peptide incorporating three lactam bridges compatible with the α-helical conformation, has been synthesized by a segment-condensation approach. The novel method used to prepare the fully protected lactam-bridged intermediate peptide segment **2** (Scheme I), has significant advantages over equivalent solution- and solid-phase methods for the synthesis of monocyclic and multicyclic peptides: the linear precursor to **2** is rapidly assembled on the oxime resin by standard solid-phase methods, but the cyclization reaction results in release of the desired monomeric product into the solution phase in high yield and essentially free of polymeric byproducts. The labor-intensive procedures of solution-phase methods and their requirement for dilute conditions for the cyclization reaction have thus been avoided, and the subsequent incorporation of these cyclic peptide segments into larger peptide chains by solution- or solid-phase methods[27] should result in purer final products than the equivalent direct solid-phase methods, especially when multicyclic peptides are desired. The protecting group strategy that we have employed is applicable to all peptide sequences.

The CD spectra of peptide **1-1-1** in aqueous solution and in 50% TFE (Figure 3) indicate that the lactam-bridged Lys^i, Glu^{i+4} side chains are fully compatible with the α-helical conformation, but their helix-stabilizing effect is small. In its monomeric form in aqueous solution, and also in 50% TFE, the linear peptide model of apo A-I on which the structure of peptide **1-1-1** is based was reported to have only slightly less α-helical structure.[15] Sequence differences between the earlier linear model peptide and peptide **1-1-1** may contribute to the conformational properties of these two peptides, and a definitive measure of the helix-stabilizing effects of the Lys^i, Glu^{i+4} side-chain bridges will have to await the synthesis and study of a strict linear analogue of peptide **1-1-1**, which is currently underway. However, this preliminary result suggests that the helix-stabilizing effects recently found for Asp^i Lys^{i+4} lactam bridges incorporated into GRF analogues are stronger.[11] More important to our goal of understanding the functional importance of amphiphilic α-helical structures in biologically active peptides is the demonstration that multiple Lys^i, Glu^{i+4} lactam bridges are compatible with induction of the amphiphilic α-helical conformation at interfaces, as indicated by the CD spectrum of peptide **1-1-1** adsorbed from aqueous solution onto siliconized slides.

Although we have not determined an apparent molecular weight for peptide **1-1-1** directly, the lack of any measurable differences in its CD spectrum in aqueous solution at concentrations ranging from 520 nM to 97 μM, and its relatively low α-helix content in this concentration range compared to the tetrameric form of the earlier apo A-I model peptide,[15] strongly suggest that it is monomeric in this concentration range despite its amphiphilic character in the α-helical conformation. The apparent failure of peptide **1-1-1** to self-associate in the same concentration range as the earlier model peptide (1–50 μM) may, in part, be a consequence of differences in the arrangement of the leucine side chains on the surfaces of the α-helical structures that these peptides form. The arrangement in peptide **1-1-1** is expected to favor dimerization as parallel coiled coils of the type found in tropomyosin and leucine-zipper peptides and to be less favorable for formation of antiparallel four-helix bundles of the type probably formed by the linear apo A-I model peptide.[28,29] Studies of tropomyosin model peptides that consist of similar seven-residue repeats have indicated a minimum requirement of four repeats for dimerization,[29] and the leucine-zipper motifs are also about 30 residues long. We are currently extending our syntheses of multicyclic amphiphilic α-helical peptides to include 28-residue analogues incorporating four lactam bridges, in order to address this question.

Experimental Section

Protected amino acids were purchased from either Bachem or Peninsula Laboratories. All amino acids were of the L configuration. *p*-Nitrobenzophenone oxime resin[14] and EACNOx[18] were prepared according to the literature. ACS grade DCM, *i*-PrOH, EtOAc, $CHCl_3$, and MeOH were purchased from Fisher Scientific and dried over sodium aluminosilicate molecular sieves (4 Å nominal pore diameter) from Sigma. TFA (Halocarbon), TFE (Aldrich), and AcOH (Baker) were used without further purification. DIEA (Aldrich) was dried over KOH and distilled from ninhydrin. Silica gel for flash chromatography was purchased from Baker; HO-Bt from Pierce; BOP from Richelieu Biotechnologies (QC, Canada); DIC, thioanisole, and TMSOTf from Aldrich; and DCC from Fluka AG.

Melting points are the uncorrected values measured in a capillary melting point apparatus. Optical rotations were measured with a Perkin-Elmer Model 241 polarimeter at 22 °C. Crude and purified peptides were analyzed on precoated silica gel F-254, 0.25-mm plates (Aldrich) using (A) acetone/hexane/AcOH, 19/20/1; (B) $CHCl_3$/MeOH/AcOH, 44/4/1; (C) EtOAc/hexane, 1/1; (D) $CHCl_3$/MeOH/AcOH, 18/1/1; (E) $CHCl_3$/MeOH/AcOH, 17/2/1; (F) $CHCl_3$/MeOH, 9/1; (G) *n*-BuOH/AcOH/H_2O, 4/1/1; or (H) EtOAc/hexane, 1/5. RP-HPLC analyses were performed on a Vydac C_4 analytical (0.46 × 25 cm) column using 0.1% TFA or AcOH in acetonitrile/water (1.2 mL/min.) as the eluant. Mass spectrometric analyses were performed under the direction of Dr. Brian Chait in the Rockefeller University Mass Spectrometry Laboratory, using the ^{252}Cf fission fragment method.[30] Carboxyl end-group titration[31] was carried out in MeOH with 0.1 N NaOH/MeOH using Thymol Blue as indicator.

Amino Acid Analysis. Peptides were hydrolyzed in 6 N HCl at 110 °C for 24 h, and peptidyl resins were hydrolyzed in propionic acid/concentrated HCl (1/1) at 130 °C for 24 h. Hydrolysates were then analyzed for their amino acid content with a Dionex Model D-300 analyzer. Hydrolysates were additionally derivatized by use of 1-fluoro-(2,4-dinitrophenyl)-5-L-alanine amide (Pierce, Rockford, IL), and the resultant diastereomers were separated by HPLC on a Vydac C_{18} analytical reversed-phase column using linear gradients of acetonitrile in aqueous buffer, according to protocols similar to those described elsewhere.[20] A_{340} peaks were quantitated with a Hewlett-Packard Model 3390A integrator connected to the HPLC detector.

Boc-Lys(2Cl-Z)Leu-N_2H_3 (4). Boc-Lys(2Cl-Z)Leu-OMe (**3**) was prepared by the mixed-anhydride peptide coupling method,[32] and it was recrystallized in EtOAc/hexane: yield 90.4%; mp 79–81 °C; R_f(C) 0.60, $[\alpha]_D$ −16.5 ± 0.30 (*c* = 2, $CHCl_3$). **3** (6.5 g, 12 mmol) was converted into the corresponding hydrazide in MeOH (18 mL) with hydrazine

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hydrate (2 mL) in the usual manner[33] giving the product: yield 5.7 g, 87.6%; mp 132–133 °C; R_f(A) 0.77; $[\alpha]_D$ −25.2 ± 0.4° (c = 1.1, AcOH).

Boc-Lys(2Cl-Z)LeuLys(Trt)-OMe (8). Trt-Lys(Trt)-OMe[34] (6.45 g, 10 mmol) was dissolved in DCM (25 mL) and cooled to −5 °C, and a chilled solution of TFA (1.5 mL) in DCM (75 mL) was poured into it. After being stirred at 4 °C for 4 days, the solution was washed at the same temperature with 10% $NaHCO_3$ solution and brine. The DCM was partly removed in vacuo at 0 °C, then diluted with $CHCl_3$ (50 mL), and reconcentrated to about 30 mL. At the same time, $NaNO_2$ (0.73 g, 10.6 mmol) in water (5 mL) at 0 °C was added to a cooled solution of **4** (5.4 g, 10 mmol) in AcOH (12 mL), 5 N HCl (5 mL), and water (50 mL). After being stirred for 5 min, the precipitated azide was extracted with ether (2 × 40 mL). The combined organic phase was washed with chilled (0 °C) water and dried over Na_2SO_4 in the refrigerator. This azide solution was poured into the solution of ε-trityllysine methyl ester. The reaction mixture was stirred at 4 °C for 48 h and then evaporated. The oily residue was partitioned between ethyl acetate (100 mL) and precooled 5% citric acid (30 mL). The organic phase was washed with 5% $NaHCO_3$ (30 mL) and brine and then dried (Na_2SO_4). Evaporation left a light yellow oil, which was purified by flash chromatography (5 × 23 cm silica gel, eluted with EtOH/hexane, 1/1). Fractions containing the desired compound were pooled and evaporated. The product was recrystallized from EtOH/hexane: yield 3.8 g, 42%; mp 71–74 °C; R_f(A) 0.74; R_f(B) 0.70; R_f(C) 0.55; $[\alpha]_D$ −14.8 ± 0.3° (c = 2.6, $CHCl_3$); amino acid analysis, $Leu_{0.96}Lys_{2.00}$.

Boc-Lys(2Cl-Z)LeuLys(Trt)-OH (9). Compound **8** (4.0 g, 4.38 mmol) was dissolved in MeOH (4.4 mL) and mixed with 2 N NaOH in MeOH (4.88 mL). This solution was stirred at 20 °C for 30 min. After being diluted with water (130 mL, pH 7), the solution was cooled to 0 °C, acidified with 30% AcOH to pH 4, and stirred for 1 h. The precipitate was collected, washed with water, and dried. The crude product was recrystallized from EtOH/water: yield 3.7 g, 94%; mp 78–82 °C; R_f(A) 0.54; $[\alpha]_D$ −14.8 ± 0.7° (c = 0.5, EtOH); M_r (titration) = 895 ± 4.5 (calcd 898.5); racemization test, 1.3% D-Leu, 4.7% D-Lys.

Glutamic Acid α-Phenacyl Ester Hydrochloride (11). Boc-Glu(O'Bu)-OH (6 g, 20 mmol) was esterified in EtOAc (40 mL) with 2-bromoacetophenone (Pac-Br, Aldrich; 3.98 g, 20 mmol) in the presence of TEA (2.8 mL, 20 mmol), following the literature method:[35] yield 7.5 g, 89%; mp 123–124 °C; R_f(C) 0.81; $[\alpha]_D$ −27.0 ± 0.6° (c = 0.9, EtOH). This product (4.95 g, 11.7 mmol) was dissolved in TFA (10 mL) and the resultant mixture stirred for 1 h at 20 °C. Then 4 N HCl/dioxane (4 mL) was added to the reaction mixture at 0 °C, and after 5 min of stirring, the precipitated salt was collected by filtration, washed with absolute ether, and dried: yield 3.43 g, 97.2%; mp 188–189 °C; R_f(G) 0.65; $[\alpha]_D$ +25.9 ± 0.4° (c = 1.1, 0.1 N HCl).

Boc-Lys(2Cl-Z)Glu(OH)-OPac (12). Glutamic acid α-phenacyl ester hydrochloride (**11**; 2.32 g, 7.7 mmol) was dissolved in water (45 mL) by heating, then the solution was cooled to 0 °C, and solid $NaHCO_3$ (1.3 g 15.4 mmol) was added. This mixture was poured into a stirred solution of Boc-Lys(2Cl-Z)-OSu[36] (**10**; 3.58 g, 7 mmol) in dioxane (60 mL) at 5° C, followed by the addition of more solid $NaHCO_3$ (0.58 g, 7 mmol) and cold dioxane (200 mL). This solution was stirred at 4 °C for 48 h. Filtration followed by evaporation gave a residue, which was dissolved in water (120 mL), cooled to 0° C, and then acidified to pH 3 with 0.5 N HCl. The precipitated product was filtered, dissolved in EtOH (60 mL), washed with water and then brine, dried over Na_2SO_4, and concentrated in vacuo. The crude product was recrystallized from 5% MeOH/ether: yield 3.79 g, 74.3%; mp 148–153 °C; R_f(A) 0.51; $[\alpha]_D$ −22.1 ± 0.3° (c = 1.0, AcOH); M_r (titration) = 657 ± 5 (calcd. 662). For the DCHA salt derivative: mp 84–87 °C; $[\alpha]_D$ −20.6 ± 0.6° (c = 1.2, $CHCl_3$).

Boc-Glu(OBzl)LeuLys(2Cl-Z)Glu(O-resin)-OPac (15). Boc-Lys(2Cl-Z)Glu(OH)-OPac (**12**; 1.55 g, 2.34 mmol) was attached to *p*-nitrobenzophenone oxime resin (25 g) with EACNOx (0.665 g, 4.68 mmol) and DCC (0.53 g, 2.57 mmol) in DCM (300 mL) at −10 °C for 30 min and at 20 °C overnight in a solid-phase peptide synthesis reaction vessel. After the vessel was drained, the resin was washed with DCM (2×), EtOH/DCM (1/1, 2×), and DCM (2×) and then acetylated with a mixture of acetic anhydride (37.5 mL) and DIEA (12.5 mL) in DCM (250 mL) for 2.5 h. The product was obtained after washing with DCM (3×), EtOH/DCM (1/1, 1×), and EtOH (2×) and was dried in vacuo:[15] yield 1.73 mmol of peptide (74%); substitution level 0.066 mmol/g, based on picric acid titration.[37]

The peptidyl resin was deprotected and washed according to the standard oxime resin protocol.[14] A mixture of Boc-Leu-OH·H_2O (1.42 g, 5.7 mmol) and BOP (2.52 g, 5.7 mmol) in DMF (30 mL) was added to the peptidyl resin in DCM (70 mL), followed by DIEA (0.99 mL, 5.7 mmol). After 2 h of shaking, a Kaiser ninhydrin test[38] indicated the coupling reaction was incomplete and a recoupling was carried out with 1 mol equiv of reagents. The resin was then washed with DMF (2×), EtOH/DCM (1/1, 1×), DMF (2×), and DCM (2×) and acetylated with 7 mmol acetic anhydride and DIEA in DCM for 1 h. After deprotection and washing as before,[14] the resin was neutralized with 5% DIEA/DCM (2 × 2 min) and washed again with DCM (2×). Then 4 equiv (6.92 mmol) of the freshly prepared symmetric anhydride of Boc-Glu(OBzl)-OH in DCM (110 mL) was used for the next coupling in the presence of DIEA (0.35 mL, 2 mmol). After 2 h of shaking, the resin was washed with DCM (2×), EtOH/DCM (1/1, 1×), and EtOH (2×) and dried: yield 1.72 mmol of peptide (99.3% for the two coupling steps); substitution level 0.065 mmol/g, based on Leu. Amino acid analysis: $Glu_{2.25}Leu_{1.00}Lys_{0.80}$.

Boc-Lys(2Cl-Z)LeuLys(Trt)Glu(OBzl)LeuLys(2Cl-Z)Glu(O-resin)-OPac (16). Peptidyl resin **15** (1.72 mmol of peptide on 25 g of resin) was deprotected, washed, and neutralized as usual[14] and then coupled with peptide **9** (1.84 g, 2.05 mmol) by using HO-Bt (0.63 g, 4.1 mmol) and DIC (0.32 mL, 2.05 mmol) in DMF (125 mL) and shaking the mixture at room temperature over 3 days. On the second day, further quantities of DIC (2 mmol) and DIEA (2 mmol) were added to the reaction mixture. The product was then washed and dried: yield 1.1 mmol of peptide (64.0%);[39] substitution level 0.041 mmol/g, based on Leu; amino acid analysis, $Glu_{2.68}Leu_{2.00}Lys_{3.00}$.

Cyclization Reaction: *cyclo*(1–5)Boc-Lys(2Cl-Z)LeuLysGlu(OBzl)-LeuLys(2Cl-Z)Glu-OPac; Boc-(1–7)-OPac (2). Peptidyl resin **16** (900 mg, 36.9 μmol of peptide on resin) was swollen in DCM (10 mL) in a solid-phase peptide synthesis vessel. The Trt group was removed from the Lys ε-amino group with 5% TFA in DCM/TFE (1/1, 4 × 15 mL), shaking the reaction vessel at room temperature for 4 × 15 min. The peptidyl resin was then drained and washed (10 mL/wash) with DCM (2×), *i*-PrOH (1×), DCM (2×), *i*-PrOH (1×), and DCM (2×). The Lys ε-amino group was neutralized by treating the peptidyl resin with 5% DIEA in DCM (2 × 1.5 min) and then washing with DCM (4×). The cyclization reaction was then carried out by shaking the peptidyl resin in DCM (14 mL) in the presence of 10 equiv of AcOH at room temperature for 72 h. The cyclic peptide product was collected from the reacting vessel by draining and then washing the resin with DCM (3×). These solutions were combined and evaporated to a reduced volume and then washed at 0 °C with water, 0.1 N HCl, 5% $NaHCO_3$, and brine. The solvent was then evaporated and the crude product was purified by silica gel flash chromatography (2 × 20 cm; eluant $CHCl_3$/MeOH/AcOH, 18/1/1). The appropriate fractions were pooled and the solvent was evaporated. The pure solidified product was recrystallized from methanol/ether: yield 33.9 mg, 60.5 %; mp 90–94 °C; R_f(A) 0.41; R_f(D) 0.48; $[\alpha]_D$ −15.3 ± 0.3° (c = 1.7, $CHCl_3$); amino acid analysis, $Glu_{2.05}Leu_{1.92}Lys_{3.00}$; MS (^{252}Cf fission fragment) m/e = 1514.3 (theoretical 1514.6 for $(M + Na)^+$, Δ = −0.3).

***cyclo*(3–7)Boc-Lys(2Cl-Z)LeuLysGlu(OBzl)LeuLys(2Cl-Z)Glu-OH; Boc-(1–7)-OH (17).** Protected peptide ester **2** (150 mg, 1 mmol) was dissolved in 90% AcOH (2.5 mL), and Zn dust (2 × 120 mg) was added to the vigorously stirred solution. The reaction was followed by TLC (solvent mixture D). After a 1-h reaction time at room temperature, the Zn was removed by filtration and the solution was concentrated in vacuo. Filtration was repeated with the DCM solution of the product, followed by evaporation. The residue was subjected to flash chromatography (2 × 20 cm silica gel; eluent $CHCl_3$/MeOH/AcOH, 90/7/3; product in the 140–300-mL fraction) and the product was recrystallized from MeOH/ether: yield 117 mg, 83.9%; mp 112–116 °C; R_f(D) 0.25; R_f(E) 0.70; $[\alpha]_D$ −27.9 ± 0.8° (c = 0.5, AcOH); amino acid analysis, $Glu_{2.14}Leu_{1.98}Lys_{3.00}$.

***cyclo*(3–7,10–14)Boc-[Lys(2Cl-Z)LeuLysGlu(OBzl)LeuLys(2Cl-Z)-Glu]$_2$-OPac; Boc-(1–14)-OPac (19).** Boc-(1–7)-OPac (**2**; 90.8 mg, 60 μmol) was treated with TFA (4 mL) at 20 °C for 15 min. The product (**18**) was precipitated with ether after partial removal of TFA in vacuo, then filtered, washed with ether, and dried over NaOH. This material was dissolved in DMF (2 mL) together with Boc-(1–7)-OH (**17**; 83.4 mg,

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(36) Boc-Lys(2Cl-Z)-OSu was prepared according to the literature method: Daoust, H.; St.-Pierre, S. *J. Chem. Soc., Perkin Trans. I* **1976**, 1453–1457. Yield 79%; mp 101–103 °C; R_f(A) 0.69; $[\alpha]_D$ −9.2 ± 0.2° (c = 1.8, $CHCl_3$).

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(39) By use of the mother liquor of the coupling reaction, a further 15% of **12** could be coupled to 2.5 g of fresh oxime resin with DCC during 24 h.

60 μmol) and HO-Bt (18.4 mg, 120 μmol). The solution was cooled to −5 °C, and then DCC (13.6 mg, 66 μmol) and DIEA (11.5 μL, 66 μmol) were added. After being stirred at 0 °C for 1 h and at 20 °C overnight, the mixture was concentrated in vacuo, dissolved in DCM (25 mL), filtered, and then washed with water, 5% citric acid, 5% $NaHCO_3$, and brine (each 10 mL at 0 °C). The DCM solution was concentrated and subjected to flash chromatography (2 × 25 cm silica gel; eluent $CHCl_3$/MeOH/AcOH, 18/1/1) to afford a chromatographically pure white solid: yield 110 mg, 65.6%; mp 68–72 °C; $[\alpha]_D$ −8.5 ± 0.3° (*c* = 2, MeOH); amino acid analysis, $Glu_{4.00}Leu_{3.98}Lys_{6.00}$; MS ($^{252}Cf$ fission fragment) *m/e* = 2,793.4 (theoretical 2792.8 for $(M + Na)^+$, Δ = +0.6).

***cyclo*(3–7,10–14,17–21)Boc-[Lys(2Cl-Z)LeuLysGlu(OBzl)LeuLys-(2Cl-Z)Glu]$_2$-OPac; Boc-(1–21)-OPac (20).** Boc-(1–14)-OPac (**18**; 100 mg, 35.6 μmol) was deprotected with TFA (4 mL), and TFA salt **21** was obtained after ether precipitation: yield 88.5 mg, 88%; mp 65–70 °C; R_f(G) 0.92; $[\alpha]_D$ −4.5 ± 0.2° (*c* = 6, DMF). Compound **21** (88.5 mg, 31.5 μmol) was dissolved in DMF (2 mL) together with Boc-(1–7)-OH (**17**; 46 mg, 33 μmol) and HO-Bt (10.1 mg, 66 μmol). At −5 °C, DCC (7.4 mg, 36 μmol) and DIEA (6.27 μL, 36 μmol) were added. After being stirred at 0 °C for 1 h and at 20 °C overnight, the mixture was concentrated in vacuo, diluted with DCM (25 mL), filtered, and then washed with water, 5% citric acid, 5% $NaHCO_3$, and brine (each 10 mL at 0 °C). The peptide solution was concentrated in vacuo and subjected to gel permeation chromatography (3 × 115 cm Sephadex LH-60 eluted with DMF). Fractions containing the major peptide peak were collected and evaporated: yield 35.1 mg, 27.3%; R_f(F) 0.53; amino acid analysis, $Glu_{6.18}Leu_{6.00}Lys_{8.73}$; MS ($^{252}Cf$ fission fragment) *m/e* = 4,070.5 (theoretical 4071.2 for $(M + Na)^+$, Δ = −0.7).

***cyclo*(3–7,10–14,17–21)(LysLeuLysGluLeuLysGlu)$_3$-OH (1-1-1).** Boc-(1–21)-OPac (**20**; 25 mg, 6.1 μmol) was dissolved in 90% AcOH (3 mL), and Zn dust (100 mg) was added to the vigorously stirred mixture. After 1 h, the solution was filtered to remove the Zn and concentrated in vacuo. The residue was dissolved in DCM, filtered again, and the solvent evaporated. This residue was then subjected to flash chromatography (2 × 25 cm silica gel, eluent $CHCl_3$/MeOH/AcOH, 90/10/5). Fractions (7 mL) 20–42 were pooled and concentrated in vacuo: yield 24 mg, 98.8%; R_f(E) 0.55. This intermediate was then completely deprotected as follows. It was dissolved in TFA (1.5 mL) at 0 °C and then thioanisole (282 μL, 2.4 mmol) and TMSOTf (462 μL, 2.4 mmol) were added. After 1 h of stirring at 4 °C, the solution was diluted with ether. The precipitated material was separated by centrifugation and washed with ether. It was then treated for 1 h at 4 °C with a 1 M NH_4F solution (1 mL) adjusted to pH 8 with 5% NH_4OH. This peptide solution was subjected to gel permeation chromatography (0.7 × 18 cm Sephadex G-25 eluted with 0.1 M AcOH). The peptide fractions were pooled and lyophilized. Final purification of the product was carried out by RP-HPLC on a Vydac C-18 column (1.0 × 25 cm) eluted with 0.1% TFA in acetonitrile/water. A linear gradient from 25 to 35% acetonitrile over 15 min, with a flow rate of 4 mL/min, was employed. The product was eluted at 30.5% acetonitrile and lyophilized to give a solid product: yield 2.6 mg of peptide, 16.8%; amino acid analysis, $Glu_{6.00}Leu_{5.82}Lys_{9.00}$; racemization assay 4.8% D-Glu, 1.0% D-Leu, 0.4% D-Lys; MS (^{252}Cf fission fragment) *m/e* = 2,571.5 (theoretical 2571.2 for $(M + Na)^+$, Δ = +0.3).

Circular Dichroism Studies. CD spectra were measured at 25 °C, using an Aviv Model 62ds spectropolarimeter fitted with a fused-silica modulator. Stock peptide concentrations were determined by amino acid analysis after hydrolysis in 6 N HCl at 110 °C for 24 h, using crystalline alanine (Sigma Chemical Co., St. Louis, MO) as an internal standard. Solution spectra were measured in 0.1, 0.5, or 2.0 cm path length cells, as appropriate for the peptide concentration under study, using a time constant of 2.0 s and averaging the data from five scans. Data collected at dynode voltages greater than 450 V were discarded. The CD spectrum of peptide **1-1-1** adsorbed onto siliconized circular quartz slides (22 × 1 mm, Hellma Cells Inc., Jamaica, NY) was determined by the method described previously.[23] Four siliconized slides were immersed for 10 min each in peptide solution (10 μM) in 0.02 M NaH_2PO_4/NaOH buffer, pH 7.5, containing 0.16 M KCl, then rinsed in H_2O, and allowed to air-dry. These slides were then placed in an adapted cell holder, designed to hold the slides vertically in the light path at alternating angles of +5 and −5° from the horizontal direction perpendicular to the light path. Data were collected for eight equally spaced orientations of the slides about the light path, in order to eliminate linear dichroism artefacts,[25] and are presented in Figure 3C as the sum of these spectra after blank subtraction, without further manipulation. This spectrum is thus equivalent in signal intensity to that resulting from a total of 32 coated slides or 64 surfaces.

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Reduction of the Oxy Form in Hemoproteins to the Ferryl Form

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Abstract: One-electron reduction of the oxy form in hemoproteins by reducing radicals such as $NAD^•$, $CO_2^{-•}$, and benzoate electron adduct has been investigated by the pulse radiolysis technique. These radicals reacted with oxymyoglobin to form the stable product, of which spectrum is assigned to the corresponding ferryl form. On the other hand, only benzoate electron adduct reacted with the oxy form of diacetyldeuteroperoxidase to form compound I of the enzyme, though the reaction by other radicals could not be detected. From these results, it was demonstrated that the one-electron reduction state in the oxy form of hemoproteins is the higher oxidation state.

The reduction of a ferrous–dioxygen complex [Fe(II)–O_2] in hemoproteins has received considerable attention, since this reaction appears in the hydroxylation of substrate by cytochrome P-450.[1] The resulting reducing species in P-450 is generally believed to be structurally similar to the high-valent intermediates such as compound I formed during the catalytic cycle of the peroxidase enzymes, though the high-valent intermediate for P-450 has not yet been isolated. This is supported by the fact that P-450 has been shown to catalyze monoxygenation reactions by utilizing oxidizing reagents such as hydroperoxide.[2] In the case of hor-

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