preparation of **6**) at room temperature was added the metal substrates. Thus 1.77 g (3.75 mmol) of *cis*-$Cl_2Pt(NCPh)$[17] was added neat, 0.324 g (1.66 mmol) of $AgBF_4$ was added as a solution in 5 mL of Me_2CO, 0.548 g (1.50 mmol) of $Ni(ClO_4)_2 \cdot 6H_2O$ was added after being dissolved in 20 mL of $MeC(OMe)_2Me$ and stirred for 2 h, and 0.549 g (1.5 mmol) of $Co(ClO_4)_2 \cdot 6H_2O$ was added after being dissolved in 15 mL of Me_2CO and 15 mL of $MeC(OMe)_2Me$ and stirred for 2 h. After the reaction solutions of **1** to which the metal compounds had been added were stirred for 1 h, the precipitates (colorless for the first two metals and yellow for the latter two) were filtered and the filtrates evaporated to dryness. All attempts to recrystallize the precipitates and the filtrate residues using a variety of solvents produced either insoluble materials or solutions which displayed complicated ^1H and ^{31}P NMR spectra.

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Registry No. 6, 10022-55-6; **7**(P-5 entry), 98777-08-3; **7**(P-3 entry), 98777-09-4; **8**(P-5 entry), 98760-10-2; **8**(P-3 entry), 98760-13-5; **9**(P-5 entry), 98760-11-3; 9(P-3 entry), 98760-14-6; **10**, 98760-12-4; $[Co(\mathbf{6})_5](ClO_4)_2$, 98799-14-5; $[Zn(\mathbf{6})_5](ClO_4)_2$, 98799-16-7; $P(NMe_2)_3$, 1608-26-0; $N(CH_2CH_2OH)_3$, 102-71-6.

The Use of Polymer-Bound Oximes for the Synthesis of Large Peptides Usable in Segment Condensation: Synthesis of a 44 Amino Acid Amphiphilic Peptide Model of Apolipoprotein A-I

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Abstract: A 44-peptide was synthesized by using the polymer-bound oxime method and subsequent segment condensation. The peptide was designed to model the surface properties of apolipoprotein A-I, and it has the following sequence: H-(Pro-Lys-Leu-Glu-Glu-Leu-Lys-Glu-Lys-Leu-Lys-Glu-Leu-Leu-Glu-Lys-Leu-Lys-Glu-Lys-Leu-Ala)$_2$-OH. The peptide readily forms α-helical secondary structure in 50% trifluoroethanol, at the air–water interface, and by self-association in water. The peptide's amphiphilic properties equal those of the apolipoprotein A-I itself, whereas the 22-peptide corresponding to one-half of the 44-peptide was significantly less amphiphilic. Thus, the optimal structural unit responsible for the surface properties of apolipoprotein A-I consists of at least two amphiphilic 22-peptides linked by helix breakers. The synthesis of the 44-peptide demonstrates the advantages of the oxime method for the synthesis of long peptide chains.

The most widely used method for the synthesis of polypeptides is the stepwise addition of carboxyl activated amino acids to a growing chain covalently linked to a solid-phase support.[1,2] In our previous papers[3-5] we reported the usefulness of *p*-nitrobenzophenone oxime bound to polystyrene–1% divinylbenzene copolymer as a solid support in peptide synthesis. The polymer was used for the stepwise synthesis of oligopeptides from the carboxyl termini, and the subsequent cleavage of the peptides from the polymer support gave peptide segments with appropriate protecting groups which were suitable for the further elongation of peptide chains.

In the synthesis of long peptide chains it is strategically important to be able to introduce at some locations activated peptides instead of individual amino acids. This is often necessary for regions of the peptide which present difficulties of synthesis or which contain chemical functions which are not conducive to normal stepwise synthesis.

The advantage of the use of the oxime method is the facile cleavage of the peptides from the solid support under mild conditions, which leave intact most common protecting groups such as *tert*-butyl or benzyl groups. We demonstrated this by cleavage of protected peptides from the oxime resin with amino acid esters in the presence of acetic acid as a catalyst to give peptide esters[4] or with 1-hydroxypiperidine to give 1-piperidyl esters.[5] Protected peptide 1-piperidyl esters are subsequently converted to the corresponding free carboxylic acids by reduction with zinc in acetic acid. No significant racemization accompanied these procedures. The protected oligopeptide segments thus obtained then can be purified and assembled for the synthesis of larger peptides either on the solid support or in solution.

In order to demonstrate the feasibility of segment condensations of peptides activated by the oxime method, we chose to synthesize a 44 amino acid peptide, **2** (tetratetracontapeptide or 44-peptide[6]), designed to model the amphiphilic secondary structure of apolipoprotein A-I. We had already synthesized a 22-peptide segment, **1**, of this model peptide by the oxime method in our laboratory. The synthesis of a 44-peptide containing two repeating 22-peptide segments presented in this paper not only demonstrates the usefulness of the chemical method but it has also provided us with a peptide with which the consequences of the covalent attachment of two secondary amphiphilic domains could be investigated.

Results

The amino acid sequence[7] designed for the target 44-peptide, **2**, is shown in Figure 1. This peptide is composed of two identical

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(7) All amino acids are of L-configuration. Standard abbreviations for amino acids, peptides, and protecting groups follow the recommendations of the IUPAC–IUB Commission on biochemical nomenclature. Abbreviations used in the text are the following: apo A-I, apolipoprotein A-I; DCC, *N,N'*-dicyclohexylcarbodiimide; DIEA, *N,N'*-diisopropylethylamine; DMF, *N,N'*-dimethylformamide; HOBt, 1-hydroxybenzotriazole; HOPip, 1-hydroxypiperidine; NMM, *N*-methylmorpholine; TFA, trifluoroacetic acid; CD, circular dichroism; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

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```
 1                                   10
Pro-Lys-Leu-Glu-Glu-Leu-Lys-Glu-Lys-Leu-Lys-

                                20      22
Glu-Leu-Leu-Glu-Lys-Leu-Lys-Glu-Lys-Leu-Ala

                    1
```

```
 1                                   10
Pro-Lys-Leu-Glu-Glu-Leu-Lys-Glu-Lys-Leu-Lys-

                                20      22
Glu-Leu-Leu-Glu-Lys-Leu-Lys-Glu-Lys-Leu-Ala-

 23                         30
Pro-Lys-Leu-Glu-Glu-Leu-Lys-Glu-Lys-Leu-Lys-

                        40              44
 Glu-Leu-Leu-Glu-Lys-Leu-Lys-Glu-Lys-Leu-Ala

                    2
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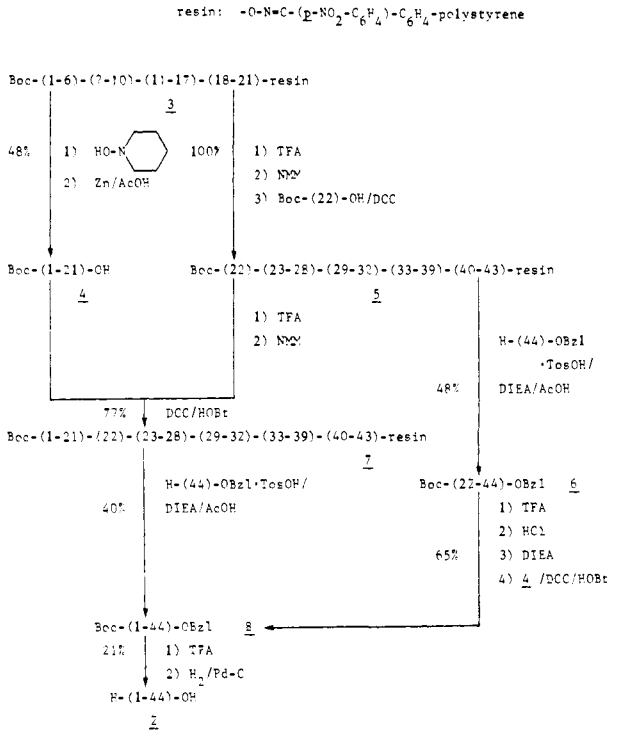
Figure 1. Amino acid sequences of peptide model **1** and model **2** of human plasma apo A-1.

Scheme I



22-peptide domains, which are the same as the human apolipoprotein A-1 model peptide, **1**, described previously.[8] The strategy of the synthesis took advantage of the fact that the oxime method allows one to introduce readily an additional amino acid residue in the C-terminal position after cleavage from the resin. Thus, our strategy for the synthesis of model compound **2**, shown in Scheme I, consisted of using as a starting material a 21-peptide resin which lacks Ala-22. The peptide which ultimately will constitute the N-terminal domain is first cleaved from the resin and transformed into the free carboxylic acid. Then, the peptidyl resin which will yield the C-terminal domain is extended one more step toward the N-terminus, thus introducing Ala which will become Ala-22 in the final peptide. This resin bound 22-peptide is then condensed with the 21-peptide containing the free α-carboxyl group. In the cleavage of the resulting 43-peptide from the resin, the Ala-44 residue is introduced in the final step. We also proposed to show that the condensation of two segments is equally feasible by segment condensation in solution. For the latter purpose, we first cleaved the resin bound 22-peptide containing alanine in the N-terminal position from the resin by using alanine benzyl ester which then yielded the C-terminal 23-peptide in solution. From this peptide—still fully protected—the N^{α}-protecting group was removed, and the product of this reaction was condensed with the N-terminal segment.

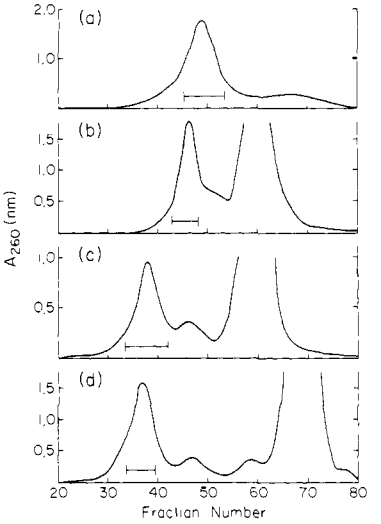


Figure 2. Gel filtration of crude protected peptides using a Sephadex LH-60 column (2 × 57 cm) with 9:1 MeOH/$CHCl_3$ at a flow rate of 11 mL/h. The fractions were 2.2 mL in volume: (a) Boc-(1-21)-OH (**4**); (b) Boc-(22-44)-OBzl (**6**); (c) Boc-(1-44)-OBzl (**8**) obtained by segment condensation on the solid phase; (d) Boc-(1-44)-OBzl (**8**) obtained by segment condensation in solution.

As the starting material we used the protected 21-peptide *p*-nitrobenzophenone oxime resin ester (**3**), synthesized as described in a previous paper.[5] For the N-terminal segment an aliquot of the resin peptide ester was treated with 1-hydroxypiperidine in methylene chloride to cleave it from the resin as the 1-piperidyl ester. The crude peptide ester was converted to its free α-carboxylic acid by reduction with zinc dust in 90% acetic acid. After purification by passage through a column of Sephadex LH-60 with a solvent system of 9:1 methanol/chloroform (v/v) (Figure 2a), an analytically pure compound (**4**) was obtained in 48% yield from the resin ester (**3**).

For the C-terminal segment an aliquot of the same 21-peptide resin ester (**3**) was elongated toward the N-terminus with alanine to yield a protected 22-peptide resin ester (**5**). For the condensation reaction a portion of this resin ester was treated with the benzyl ester of the C-terminal amino acid (position 44), alanine, in the presence of acetic acid to remove the protected peptide segment from the solid support. The crude product was purified by repeated gel-filtration with a Sephadex LH-60 column (Figure 2b) to give compound **6** in 48% yield. This compound corresponding to the C-terminal protected 23-peptide segment of the target 44-peptide was employed in segment condensation in solution.

A portion of the protected 22-peptide resin ester (**5**) was directly used for segment condensation with the N-terminal protected 21-peptide acid (**4**). The coupling reaction between the 21-peptide and the resin-linked 22-peptide was carried out by the dicyclohexylcarbodiimide-1-hydroxybenzotriazole procedure.[9] Only a slight excess of the carboxyl component (11% molar excess) was used. From the leucine content, the coupling yield was estimated to be 77%. The protected 43-peptide resin ester (**7**) thus obtained was treated with alanine benzyl ester to cleave the peptide from the solid support and to introduce the C-terminal residue. After

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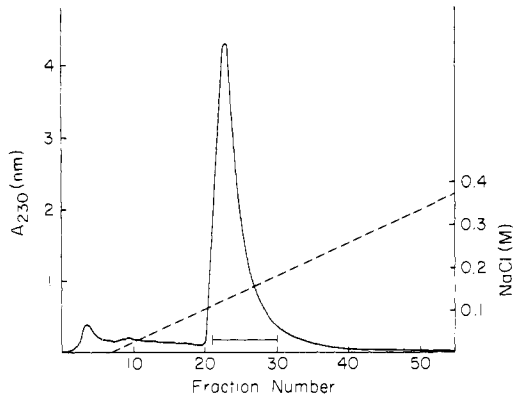

Figure 3. Ion exchange chromatography of crude peptide **2** using a CM-cellulose column equilibrated with 0.05 M NH_4OAc (pH 5.6) and eluted with a linear gradient of NaCl (concentration 0 to 0.4 M) in the original buffer solution.

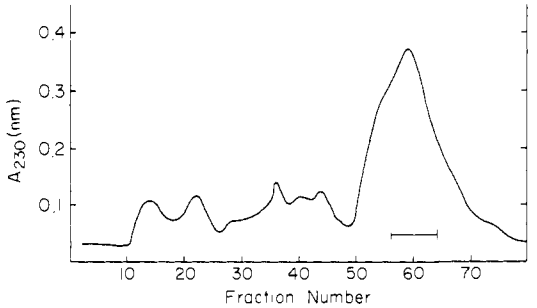

Figure 4. Partition chromatography of partially purified peptide **2** using a Sephadex G-25 column and a solvent system of 6:4:1:10 1-butanol/pyridine/acetic acid/water (v/v/v/v).

purification with use of a column of LH-60 (Figure 2c), the fully protected 44-peptide (**8**) was obtained in 40% yield.

The same compound was also synthesized by segment condensation in solution. The N^{α}-*tert*-butyloxycarbonyl group of the fully protected 23-peptide (**6**) was removed with 1:1 trifluoroacetic acid/methylene chloride (v/v), and the resulting trifluoroacetate salt was converted to the hydrochloride. After neutralization with diisopropylethylamine, the α-amino group of this peptide was reacted with the free carboxylic acid of the protected 21-peptide (**4**) by using the dicyclohexylcarbodiimide-1-hydroxybenzotriazole procedure and purified by gel-filtration with use of LH-60 column (Figure 2d) to give the product **8** (44-peptide) in 65% yield.

The fully protected 44-peptide was treated with 1:1 trifluoroacetic acid/methylene chloride (v/v) to remove the N^{α}-*tert*-butyloxycarbonyl group. Subsequently, all other benzyl-type protecting groups were removed by catalytic hydrogenation with 10% palladium on activated charcoal as a catalyst. Ammonium formate was added to the reaction mixture to enhance the reaction rate by catalytic transfer hydrogenation.[10] The free peptide **2** was purified by a combination of CM-cellulose ion-exchange chromatography (Figure 3) and partition chromatography[11] by using Sephadex G-25 (Figure 4). The purity of the final peptide was ascertained by analytical high-performance liquid chromatography (Figure 5) and amino acid analysis.[12] The combined yield of the removal of protecting groups and the subsequent purification was 21%.

From the circular dichroism studies the α-helicity of compound **2** was estimated[13] to be 54% in neutral buffered solution over the peptide concentration range between 6×10^{-5} and 6×10^{-7} M. The value was increased to 65% in a mixture of 1:1 trifluoroethanol/buffered solution (v/v) (Figure 6).

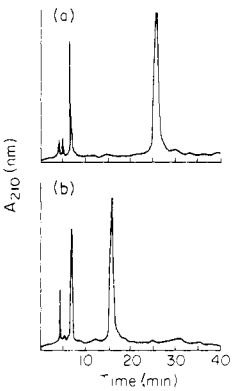

Figure 5. HPLC of peptide models for apo A-1 using a C-18 column. The solvent system was a linear gradient from 32% to 52% acetonitrile in 0.2 M phosphoric acid sodium salt (pH 2.5) over a period of 40 min, flow rate = 40 mL/h: (a) compound **2** (44-peptide) and (b) compound **1** (22-peptide).

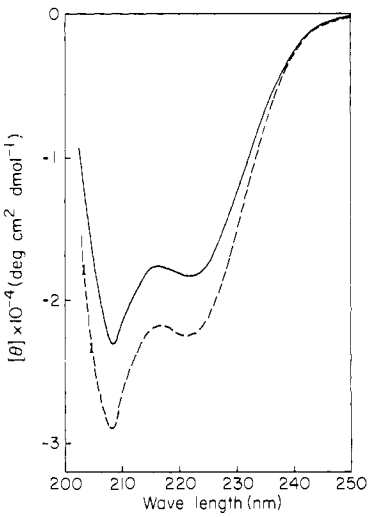

Figure 6. CD spectra of model peptide **2**. Solid line, spectrum measured with peptide dissolved in 0.02 M sodium phosphate (pH 7.0) containing 0.15 M NaCl. Dotted line, spectrum measured with peptide dissolved in 1:1 trifluoroethanol/buffer solution (v/v).

Monomolecular layers of compound **2** were prepared at the interface of air and pH 7.4 buffer containing 0.16 M KCl and 0.01 M Tris·HCl. The surface isotherms were analyzed according to the semiempirical equation[14] $\pi = nkT/(A - A_0)$, where π is the surface pressure, A is the area available for a molecule, k is the Boltzmann constant, T is the temperature, and A_0 is the limiting area per molecule. The experimental force–area curve was fitted to this equation by using a nonlinear least-squares fit to determine the best values of the parameters. From the value of nkT and the known quantity of materials spread on the surface, we found a molecular weight of 4300 ± 1400, clearly showing that the peptide exists as a monomer at the air–water interface. We also found that the limiting area of the peptide is $A_0 = 16.1 \pm 1.4/A^2$ per amino acid. This latter value indicates a compact structure of the peptide at the interface and is in full agreement with the assumption that the peptide assumes an amphiphilic α-helical secondary structure[15] at amphiphilic surfaces. The collapse point was also ascertained by measuring the stability of the peptide monolayer at various surface pressures. Indeed, at the pressure indicated by the inflection point of the surface isotherm, the stability of the monolayer increased discontinuously.

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Table I. Comparison of the Physical Properties of Apo A-I and of Its Model Peptides

property	Apo A-I (144–165)	Apo A-I (121–164)	model A-I (**1**)	model A-I (**2**)	Apo A-I
amino acid residue	22	44	22	44	243
α-helicity (%) in water	15 (monomer)[a]	18 (monomer)[b]	30 (monomer)[c]		
in water			50 (tetramer)[c]	54 (aggregate)	61 (aggregate)[b]
in 50% trifluoroethanol	40[a]	60[b]	61[c]	65	73[b]
air–water interface					
collapse pressure (dyn/cm^2)	8[b]	12[b]	22[c]	24	22[c]
A_∞ (Å^2/amino acid)	20.8[b]	18.8[b]	23[c]	16.1	16.3[d]
binding to lecithin-coated beads					
$K_d \times 10^8$ (M)			280 (±170)[e]	44 (±9)[e]	8.5 (±4.2)[e]
saturating area (Å^2/amino acid)			22.0[e]	13.8[e]	14.8[e]

[a] From ref 24. [b] From ref 25. [c] From ref 8. [d] From ref 26. [e] From ref 18.

By both methods we found the collapse pressure to be 24 ± 1 dyn/cm^2.

The binding of this peptide to single bilayer phospholipid vesicles could not be investigated because the peptide vesicle complexes were not temporally stable. Such a behavior is not surprising in the light of the vesicle fusions observed with several strongly amphiphilic peptides such as melittin[16] and some β-endorphin[17] models. Nevertheless, current work[18] in our laboratory has shown that peptide **2** is an excellent surfactant as measured by its adsorption to phospholipid-coated polystyrene beads (see Table I). The hydrodynamic behavior of the peptide in solution was investigated by gel-permeation chromatography with a column (1.45 × 63 cm) of Sephadex G-50 (fine) equilibrated with 0.02 M phosphate buffer (pH 7.0) containing 0.15 M NaCl. The column was calibrated with a series of standard globular proteins of known molecular weight. When compound **2** was applied at the initial concentration of 5×10^{-5} M, a single peptide peak eluted at the position corresponding to an apparent molecular weight of 14 500. We feel that since peptide **2** is an asymmetric molecule when it is in the α-helical form this gel-permeation behavior must reflect the increased Stokes radius due to a high axial ratio. When compound **2** was applied at the high initial concentration of 9×10^{-4} M, we found that the peptide eluted at the void volume. Thus, peptide **2** has, indeed, a high tendency to self-associate in aqueous solution, a property which is characteristic of amphiphilic peptides able to form peptide micelles.

Discussion

The present work was undertaken to demonstrate the usefulness of the oxime method for segment condensation whereby the segment to be introduced into the growing chain is first synthesized by the oxime method and cleaved from the resin with the concomitant generation of a C-terminal carboxyl which can be activated for further coupling. A peptide with 44 amino acid residues was successfully synthesized utilizing the *p*-nitrobenzophenone oxime polymer. Though our previous report[5] on the synthesis of a 22-peptide demonstrated the versatility of this polymeric support in peptide synthesis, the present study confirmed that the method is workable for the synthesis of larger peptides. Detachment of the protected 21-peptide from the *p*-nitrobenzophenone oxime polymer by the use of 1-hydroxypiperidine, as well as the subsequent reduction by zinc in 90% acetic acid to give the free C-terminal carboxylic acid, proceeded without any difficulties. Detachment of the protected 22-peptide or 43-peptide from the polymer support by the reaction with an amino acid ester gave the fully protected 23-peptide or 44-peptide, respectively, also without any difficulties. The protected peptide segments thus obtained can be purified by conventional procedures and then be used as the building units for the synthesis of larger peptides either on solid-phase or in solution or can be deprotected to obtain the free peptide.

An advantage of the method employed in the present work is that it avoids the microheterogeneity of the peptides synthesized by the stepwise solid-phase method.[1,2] The protected oligopeptides synthesized by using the oxime resin (in this case containing 4–7 amino acid residues) can be purified by crystallization, by gel filtration on a Sephadex LH-20 column, by adsorption chromatography on silica gel, including preparative high-performance liquid chromatography, or by a combination of some of these procedures. Several of the purified segments are then assembled on the polymer, detached from the polymer, and purified by gel filtration on an LH-60 column with use of an appropriate organic solvent as the eluant. Since unreacted amino groups after each segment condensation were acetylated to terminate further elongation, it is not difficult to obtain the segment in good purity. The medium-sized protected peptides thus purified (ca. 20–25 amino acid residues in length) can be used further as segments for the synthesis of larger peptides.

Another advantage of this method is that mild conditions can be employed for the removal of peptides from the polymeric support. Detachment of peptides from the resin by treatment with anhydrous hydrogen fluoride,[19] generally used in solid-phase peptide synthesis, is not required, unless protecting groups are employed which are cleavable only by hydrogen fluoride. In the present synthesis, the N$^\alpha$-*tert*-butyloxycarbonyl group was removed by treatment with trifluoroacetic acid and all other protecting groups, including the benzyloxycarbonyl and benzyl ester groups, were removed by catalytic hydrogenation. As judged by thin-layer chromatography of the product, the addition of ammonium formate as the hydrogen donor[10] in the catalytic hydrogenolysis had a considerable accelerating effect on the rate of deprotection. However, we found that without introducing hydrogen gas the hydrogenolysis did not proceed well in our case. Probably a more powerful catalyst, such as 20% palladium hydroxide on carbon,[20] would be advantageous for the complete hydrogenolysis of a peptide like ours which contains 27 benzyl-type protecting groups per molecule.

The reason we did not use the N-terminal protected 22-peptide as the carboxyl component is that a proline residue would then have been present at the N-terminal position of the amine component. A preliminary experiment in which we attempted to condense such segments on the resin was not successful. Condensation between the N-terminal 23-peptide and the C-terminal 21-peptide might provide a very good alternative approach to the synthesis of our target 44-peptide since the use of a C-terminal proline in the carboxyl component would have eliminated the possibility of racemization. However, this strategy would require a considerable change in the synthetic scheme which was originally designed for the preparation of the monomer **1**. Therefore, this route was not attempted in the present work.

The amino acid sequence of apolipoprotein A-I[21] shows the presence of several highly homologous 22-peptide segments with a high amphiphilic helical potential.[22] These segments are punctuated by Pro or Gly residues. We have first demonstrated that indeed these 22-peptides possess the physical properties which

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are deemed to be essential for the physiological role of the apolipoprotein.[8] Nevertheless, the physical properties of a 22-peptide segment (both with idealized amphiphilicity[8,23] and with the natural sequence[24]) were less than optimal, and we were able to show previously[25] that a 44-peptide segment containing the exact amino acid sequence from 121–164 of apolipoprotein A-1 mimicked more closely the surface properties of apolipoprotein A-1 than the 22-peptide segment. With this 44-peptide the question remained open, however, whether the improved surface properties were due to the mere increase in size of the peptide or whether the second 22-peptide had an amino acid sequence more conducive to the exaltation of the surface properties. In order to demonstrate conclusively the role of peptide size in surface properties we had to synthesize a 44-peptide composed of two identical 22-peptide segments. The peptide synthesized in this present paper satisfies this criterion, and it was of great interest to us to compare its physical properties to those of the previously studied apolipoprotein A-1 homologues. As shown in Table I, the covalent linkage of the two segments resulted indeed in a considerable increase in the amphiphilicity of the peptide. As compared to the 22-peptide most notable is the increase in helicity for the 44-peptide as measured in 50% trifluoroethanol and the much higher tendency of the peptide to form aggregates, i.e., peptide micelles, in aqueous solution. That these aggregates are in fact genuine micelles is borne out by the fact that aggregation is uniformly accompanied by an increase in helicity as measured by circular dichroism. The most important observation we made with our peptide is the determination of the limiting molecular areas of the peptide adsorbed at amphiphilic interfaces: air–water interface as well as phospholipid coated polystyrene beads. As shown in Table I, the model 22-peptide occupies some 22 Å^2 per amino acid at the interface.[8] Thus, the peptide is not fully helical and must contain some random coil segments, presumably at the terminus. On the other hand, the 44-peptide occupies 14–16 Å^2 per amino acid on both surfaces in total agreement with the value observed for the apolipoprotein A-1 itself, 15–16 Å^2 per amino acid.[26] Thus, it appears that the true structural unit of the apolipoprotein is not the 22-peptide segment but is in fact the 44-peptide segment which is punctuated in the middle by a helix breaker, either Gly or Pro.

It is notable that the strongly amphiphilic cytotoxic peptide melittin is also composed of two amphiphilic helical segments linked by a Pro residue.[27] The analogy must not be fortuitous between melittin and apolipoprotein A-1 since the break introduced by the Pro keeps the hydrophobic faces of the helices in phase, and in both cases the Pro is located in the middle of the hydrophilic region, thereby producing a slightly concave hydrophobic surface. Such a concavity is ideally suited for the adsorption of a 44-peptide segment to the highly curved surface of human plasma high density lipoprotein-3 (radius 40–50 Å). In fact, simple geometric calculations using the X-ray crystallographic data for melittin[28] and the length of the α-helices show that the concavity of the Pro-puncuated 44-peptide segment matches closely the curvature of the surface of high-density lipoprotein. Because of these considerations we feel very strongly that it is the 44-peptide segment which is the paradigm of apolipoprotein A-1, as far as its biological function is concerned. It is tempting to speculate that because of its structural and functional role the Pro-containing α-helices are a new protein structural unit with physical and biological properties distinct from a single α-helix, and we are currently investigating the properties of these. Our preliminary observations concerning the temperature dependence of the solubility and self-aggregation of our 44-peptide also suggest that the amphiphilic properties of the peptide are strictly controlled by a cis–trans proline–amide bond transition. Such a control would account for much of the hitherto unexplainable behavior of the apolipoprotein[29] and the temperature dependency of the lytic ability of melittin which we are currently investigating.

Experimental Section

Equipment, Materials, and Methods. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. Amino acid analyses[12] were performed with a Beckman Model 121 amino acid analyzer. Peptides were hydrolyzed with 6 N HCl in evacuated and sealed tubes at 110 °C for 24 h. Peptidyl resins were hydrolyzed with a 1:1 propionic acid/concentrated HCl (v/v) mixture[30] at 110 °C for 18 h. CD spectra were measured with a Cary 60 spectropolarimeter. HPLC was performed with a Waters Associates μ-Bondapak C-18 column (0.4 × 30 cm). TLC was performed on precoated silica gel 60F-254 plates, 0.25 mm (E. Merck) in the following solvent systems: (A) 95:5:3 chloroform/methanol/acetic acid (v/v/v); (B) 88:10:2 chloroform/methanol/acetic acid (v/v/v); (C) 85:10:5 chloroform/methanol/acetic acid (v/v/v); (D) 15:3:10:12 1-butanol/acetic acid/pyridine/water (v/v/v/v). Spots were visualized by spraying with 2 N HCl and 0.5% ninhydrin solution in ethanol and heating. A Sephadex LH-60 column (2 × 57 cm) was used for the purification of the protected peptides with a solvent system consisting of 9:1 methanol/chloroform (v/v) at a flow rate of ca. 11 mL/h. Fractions of 2.2 mL volume were collected, and the UV-absorbance at 260 nm was measured to detect the peptides. The purity of the peptide in each fraction of the peak corresponding to the desired product was monitored by TLC. Reaction vessels for the manual solid-phase synthesis performed were constructed at the University of Chicago glass-blowing shop.

DMF was distilled from ninhydrin under reduced pressure and stored over molecular sieves. DIEA, NMM, and pyridine were also distilled from ninhydrin. Amino acid derivatives were purchased from Chemical Dynamics Co. Sephadex G-15, G-25, G-50, and LH-60 were products of Pharmacia. CM-Cellulose (microgranular form) was purchased from Sigma. HOPip from Aldrich was recrystallized from *n*-hexane.

Boc-Pro-Lys(Z)-Leu-Glu(OBzl)-Glu(OBzl)-Leu-Lys(Z)-Glu(OBzl)-Lyz(Z)-Leu-Lys(Z)-Glu(OBzl)-Leu-Leu-Glu(OBzl)-Lys(Z)-Leu-Lys(Z)-Glu(OBzl)-Lys(Z)-Leu-OH,Boc-(1-21)-OH (4). Fully protected Boc-(1–6)-(7–10)-(11–17)-(18–21)-resin (**3**) was synthesized according to the procedure described in our previous publication.[5] The level of substitution on the resin was 0.102 mmol/g based on the leucine content. The peptide resin **3** (250 mg, 26 μmol estimated in the form of the peptide, ca. a total of 50 μmol as oxime esters) was suspended in CH_2Cl_2 (2.5 mL), and the mixture was stirred with HOPip (20 mg, 200 μmol) at room temperature for 24 h. The resin was filtered and washed with CH_2Cl_2, 2:1 CH_2Cl_2/EtOH, and EtOH. The combined filtrate was evaporated to a solid residue, which was suspended in 1:2 Et_2O/*n*-hexane and filtered: crude yield of 1-piperidyl ester, 92 mg. This was dissolved in 90% AcOH (5 mL), and the solution was stirred vigorously with Zn dust (100 mg) at room temperature for 1 h. Zinc was removed by filtration and washed with 90% AcOH. The combined filtrate was evaporated, and the residue was taken into a mixture of EtOAc (20 mL) and 5% citric acid (20 mL). The organic layer was further washed with 5% citric acid twice, with saturated NaCl twice, and once with water and then dried over Na_2SO_4. The dried solution was evaporated, and the residue was dissolved in 9:1 MeOH/$CHCl_3$ (4 mL). The solution was subjected to gel filtration with use of a column of LH-60 (Figure 2a). Fractions under the major peak (no. 45–53) were pooled, evaporated, freeze-dried from AcOH, and dried over NaOH and P_2O_5: yield 53 mg (48%); TLC R_f(B) 0.30, R_f(C) 0.42; $[\alpha]^{22}_D$ −11.3° (*c* 0.9, AcOH); amino acid analysis $Glu_{6.11}Pro_{1.02}Leu_{7.00}Lys_{6.98}$.

Anal. Calcd for $C_{222}H_{298}N_{28}O_{50}$ (4159.0): C, 64.11; H, 7.22; N, 9.43. Found: C, 63.86; H, 7.37; N, 9.28.

Boc-(22)-(23–28)-(29–32)-(33–39)-(40-43)-resin (5). Protected Boc-(1–6)-(7–10)-(11–17)-(18–21)-resin (**3**) (600 mg, 61 μmol as the peptide) was placed in a solid-phase reaction vessel, and the synthesis was carried out according to the following protocol: (1) wash, CH_2Cl_2 (2×); (2) acetylate, AcOH (6 equiv) and DCC (6 equiv) in CH_2Cl_2 (6 mL) (1 × 30 min); (3) wash, CH_2Cl_2 (2×); (4) wash, 2:1 CH_2Cl_2/EtOH (3×); (5) wash, CH_2Cl_2 (4×); (6) prewash, 25% TFA/CH_2Cl_2(v/v) (1 × 1 min); (7) deprotect, 25% TFA/CH_2Cl_2 (v/v) (1 × 30 min); (8) wash, CH_2Cl_2 (2×); (9) wash, *i*-PrOH (2×); (10) wash, CH_2Cl_2 (3×); (11) neutralize, 1.1% NMM/CH_2Cl_2 (3 × 1 min); (12) wash, CH_2Cl_2

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(4×); (13) couple, Boc-Ala-OH (95 mg, 0.5 mmol) with DCC (0.5 mmol) in CH_2Cl_2 (6 mL) (1 × 30 min); (14) wash, CH_2Cl_2 (2×); (15) wash, 2:1 CH_2Cl_2/EtOH (3×); (16) wash, EtOH (3×); (17) dry over P_2O_5. The solvent volume for all steps except steps 2 and 13 was 15 mL, and the time for the washing steps was 1 min; yield 596 mg (0.098 mmol/g based on Leu); amino acid analysis $Ala_{0.99}Leu_{7.00}$.

Boc-Ala-Pro-Lys(Z)-Leu-Glu(OBzl)-Glu(OBzl)-Leu-Lys(Z)-Glu-(OBzl)-Lys(Z)-Leu-Lys(Z)-Glu(OBzl)-Leu-Leu-Glu(OBzl)-Lys(Z)-Leu-Lys(Z)-Glu(OBzl)-Lys(Z)-Leu-Ala-OBzl, Boc-(22–44)-OBzl (6). Protected peptide resin ester **5** (200 mg, 19.5 μmol as peptide, ca. 40 μmol estimated as oxime esters) was stirred with H-Ala-OBzl·TosOH (53 mg, 0.16 mmol), DIEA (28 μL, 0.16 mmol), and AcOH (10 μL, 0.16 mmol) in CH_2Cl_2 (2.3 mL) at room temperature for 24 h. The resin was collected by filtration and washed with CH_2Cl_2, 2:1 CH_2Cl_2/EtOH, and EtOH. The combined filtrate was evaporated, and the resulting residue was dissolved in 9:1 MeOH/$CHCl_3$ (2 mL). The solution was subjected to gel filtration with use of a column of LH-60 (Figure 2b). Fractions under the main peptide peak (no. 43–48) were pooled, evaporated, and once more applied to the same column under the same conditions. Fractions comprising the symmetrical peak obtained (no. 42–50) were pooled, evaporated, and freeze-dried from AcOH: yield 41 mg (48%): TLC R_f (A) 0.11; $[\alpha]^{22}_D$ −12.7° (*c* 1, AcOH); amino acid analysis $Glu_{5.99}Pro_{1.03}Ala_{1.99}Leu_{7.00}Lys_{7.12}$.

Anal. Calcd for $C_{235}H_{314}N_{30}O_{52}$ (4391.3): C, 64.28; H, 7.21; N, 9.57. Found: C, 64.50; H, 7.35; N, 9.69.

Boc-(1–21)-(22)-(23–28)-(29–32)-(33–39)-(40–43)-resin (7). Protected peptide resin ester **5** (120 mg, 11.7 μmol) was placed in the reaction vessel for manual solid-phase synthesis, and the procedures described in the synthesis of compound **5** were followed, except for the coupling step 13. The coupling reaction was carried out as described below. A solution of Boc-(1–21)-OH (**4**) (54.1 mg, 13 μmol) and HOBt·H_2O (4.0 mg, 26 μmol) in CH_2Cl_2 (0.8 mL) and DMF (0.4 mL) was added after step 12, and the mixture was cooled to −15 °C with a MeOH–ice bath. A solution of 1 M DCC/CH_2Cl_2 (15 μL, 15 μmol) was stirred into the mixture, and the stirring was continued at −15 °C for 30 min and then at room temperature for a day. After step 15, the peptide resin was acetylated according to the procedure of steps 1 to 4, washed with EtOH (3×), and dried over P_2O_5: yield 139 mg (77%, 0.054 mmol/g based on Leu).

Boc-[Pro-Lys(Z)-Leu-Glu(OBzl)-Glu(OBzl)-Leu-Lys(Z)-Glu-(OBzl)-Lys(Z)-Leu-Lys(Z)-Glu(OBzl)-Leu-Leu-Glu(OBzl)-Lys(Z)-Leu-Lys(Z)-Glu(OBzl)-Lys(Z)-Leu-Ala]$_2$-OBzl, Boc-(1-44)-OBzl (8). **(A) By Solid-Phase Synthesis.** Protected peptide resin ester **7** (130 mg, 7 μmol as peptide, ca. 23 μmol estimated as oxime ester) was stirred with H-Ala-OBzl·TosOH (33.3 mg, 100 μmmol), DIEA (100 μmol), and AcOH (110 μmol) in CH_2Cl_2 (1.3 mL) at room temperature for 63 h. The reaction mixture was worked up as described for the synthesis of compound **6**. Fractions under the major peptide peak (no. 33–42) seen on chromatography with use of a column of LH-60 (Figure 2c) were collected, and the gel filtration was repeated to give a single symmetrical peak. Fractions no. 34–41 were pooled, evaporated, and freeze-dried from AcOH: yield 23.5 mg (40%); TLC R_f(B) 0.48, R_f(C) 0.74; $[\alpha]_D^{22}$ −8.0° (*c* 0.5, AcOH); amino acid analysis $Glu_{11.92}Pro_{1.92}Ala_{2.04}Leu_{14.00}Lys_{14.00}$.

Anal. Calcd for $C_{452}H_{602}N_{58}O_{99}$ (8432.2): C, 64.38; H, 7.20; N, 9.63. Found: C, 64.24; H, 7.40; N, 9.42.

(B) By Solution Synthesis. Compound **6** (35 mg, 8 μmol) was treated with 1:1 TFA/CH_2Cl_2 (2 mL) at room temperature for 45 min. The solvent was evaporated and dried over NaOH, and the residual solid was dissolved in AcOH (5 mL). To this 0.1 M HCl (100 μL, 10 μmol) was added, and the solution was freeze-dried immediately. The lyophillizate was dried over NaOH overnight. The dried HCl·H-(22–44)-OBzl was dissolved in DMF (0.2 mL) together with Boc-(1–21)-OH (*4*) (33.3 mg, 8 μmol) and 1 M HOBt·H_2O/DMF (25 μL, 25 μmol). The solution was cooled to −15 °C, and 0.32 M DIEA/CH_2Cl_2 (25 μL, 8 μmol) and 1 M DCC/CH_2Cl_2 (10 μL, 10 μmol) were added. The mixture was stirred at −15 °C for 30 min and then at room temperature for 22 h, and subsequently it was diluted with 9:1 MeOH/$CHCl_3$ (2 mL). Insoluble material was removed by filtration, and the filtrate was applied to a column of LH-60 (Figure 2d). Fractions of the major peptide peak (no. 34–40) were pooled, evaporated, and freeze-dried from AcOH: yield 44 mg (65%); TLC R_f(B) 0.48; amino acid analysis $Glu_{12.18}Pro_{2.12}Ala_{1.99}Leu_{14.00}Lys_{14.05}$.

H-(Pro-Lys-Leu-Glu-Glu-Leu-Lys-Glu-Lys-Leu-Lys-Glu-Leu-Leu-Glu-Lys-Leu-Lys-Glu-Lys-Leu-Ala)$_2$-OH, H-(1–44)-OH (2). Compound **8** (37.5 mg, 4.4 μmol) was stirred with 1:1 TFA/CH_2Cl_2 (4 mL) at room temperature for 45 min. The solvent was evaporated, and the residue was dried over NaOH and then dissolved in a mixture of AcOH (2.5 mL) and MeOH (2.5 mL). To this a solution of ammonium formate (30 mg) in water (0.5 mL) and a suspension of 10% Pd on activated carbon (20 mg) in AcOH (2.5 mL) were added. Hydrogen gas was bubbled through the mixture gently with stirring at room temperature for 90 min. Then the mixture was kept in a refrigerator overnight. The catalyst was removed by filtration of the mixture through a layer of Celite and washed with 50% AcOH. The combined filtrate was evaporated to a small volume and then diluted with water (4 mL). The solution was passed through a column of Sephadex G-15 (1.4 × 52 cm) eluting with 0.2 M AcOH. Fractions under the major peak at the void volume, monitored by the UV-absorbance at 230 nm, were pooled and freeze-dried: yield 25 mg. The crude lyophilizate (24.5 mg) was dissolved in 0.05 M ammonium acetate (pH 5.6, 4 mL). The solution was applied to a column of CM-cellulose (1.0 × 10 cm) previously equilibrated with the same buffer solution. The column was first washed with the same buffer solution (15 mL) and then eluted at a flow rate of 9 mL/h with a linear gradient of NaCl over a concentration range from 0–0.4 M (total gradient volume 200 mL). The UV-absorbance at 230 nm was monitored to detect the peptide (Figure 3). Fractions corresponding to the major peak (NaCl concentration 0.12 M) were pooled, freeze-dried, and desalted by passage through a column of Sephadex G-15 employing 0.2 M AcOH as the eluant. The peptide fractions were pooled and freeze-dried: yield 16.5 mg. A portion of the peptide (15 mg) was further purified by partition chromatography with use of Sephadex G-25[11] fine (1.0 × 18.3 cm). The solvent system used was 6:4:1:10 1-butanol/pyridine/acetic acid/water (v/v/v/v). Fractions of 0.6 mL volume were collected, and the flow rate was 2.4 mL/h. The solvent in each fraction was evaporated, and the residue was dissolved in H_2O (2 mL). The peptide content of every second tube was monitored by measuring the UV-absorbance at 230 nm (Figure 4). The purity of the peptide corresponding to the major peak (R_f 0.16–0.12) was examined by HPLC by using an aliquot of each fraction under the peak. The solvent system used for HPLC was a linear gradient from 32% to 52% acetonitrile in 0.2 M phosphoric acid sodium salt (pH 2.5) employed over a time period of 40 min. The flow rate was 40 mL/h. Under these conditions compound **2** was eluted at 26 min, whereas compound **1** was eluted at 15 min (Figure 5). The analytically pure fractions of **2** (R_f 0.15–0.13) were pooled and freeze-dried (3.9 mg). More product (0.8 mg) was obtained by repeating the partition chromatography step on analytically impure fractions of the peptide: yield 4.7 mg (21% from **8**); TLC R_f(D) 0.13; $[\alpha]^{22}_D$ −34.2° (*c* 0.24, 0.2 M AcOH); amino acid analysis $Glu_{12.08}Pro_{1.93}Ala_{1.99}Leu_{14.00}Lys_{13.99}$.

Circular Dichroism Studies. CD spectra of compound **2** over the concentration range between 6 × 10^{-5} and 6 × 10^{-7} M were measured from 250 to 205 nm at room temperature (23 °C), in 0.02 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl. Quartz cells of 1 and 10 mm path length were used. The mean residue ellipticity at 222 nm ($[\theta]_{222nm}$, deg·cm^2/dmol) was used to estimate the α-helicity.[13]

Studies of Peptide Monolayers at the Air–Water Interface. The procedure followed corresponded to that of the previous paper,[31] except that a peptide solution of 37.5 μg in 50 μL of the 0.01 M Tris·HCl/0.16 M KCl, pH 7.4, was spread on the surface of the subphase, which consisted of the same buffer.

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