Synthesis of Protected Peptide Segments and Their Assembly on a Polymer-Bound Oxime: Application to the Synthesis of a Peptide Model for Plasma Apolipoprotein A-I

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A new method for the synthesis of peptide segments with protecting groups suitable for subsequent segment condensations was developed by using polystyrene-bound *p*-nitrobenzophenone oxime esters. The protected peptide segment prepared in a stepwise manner with the C-terminal amino acid anchored as an oxime ester on the solid support was removed by displacement with 1-hydroxypiperidine. The protected peptide 1-piperidyl ester was subsequently converted to the corresponding C-terminal free carboxylic acid by reduction with zinc in acetic acid. This procedure causes no significant racemization and preserves N^{α}-*tert*-butyloxycarbonyl- or benzyl-type side chain protecting groups intact. The oxime resin method was successfully applied to the synthesis of a peptide model for human plasma apolipoprotein A-I (22 amino acid residues) previously synthesized by the stepwise method on the Merrifield resin. Tetra-, hexa-, and heptapeptides containing N^{α}-*tert*-butyloxycarbonyl, N^{ϵ}-benzyloxycarbonyl, and C^{γ}-benzyl ester protecting groups were prepared by using the oxime resin. These peptide segments were then assembled on this polymeric support by dicyclohexylcarbodiimide-mediated condensation employing an additive, either 1-hydroxybenzotriazole or ethyl 2-(hydroxyimino)-2-cyanoacetate. The fully protected docosapeptide was obtained from the uncosapeptide bound to the oxime resin by aminolysis with the C-terminal amino acid ester in the presence of acetic acid as the catalyst. The subsequent removal of the protecting groups under mild conditions facilitated the purification of the final product which was found to be identical in all respects with the model docosapeptide synthesized earlier by the stepwise solid-phase procedure.

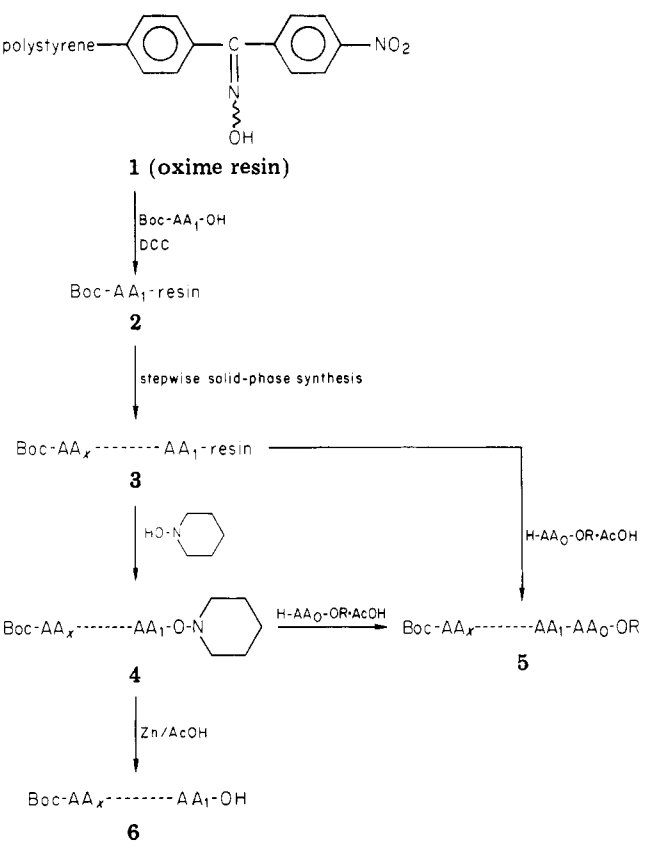
p-Nitrobenzophenone oxime bound to polystyrene–1% divinylbenzene copolymer (oxime resin; **1**) was recently developed in our laboratory[1] as a new type of solid support for peptide synthesis. The oxime ester linkage anchoring the N-protected amino acid to the polymer has been shown to be sufficiently stable to allow the elongation of peptide chains under controlled conditions but to be active enough to permit the cleavage of the protected peptide product from the polymer support under mild conditions, including brief hydrazinolysis at a low hydrazine concentration or aminolysis by amino acid esters with acetic acid as the catalyst.[2]

In our continuing investigation of the versatility of this polymer support in peptide synthesis, we have now developed a unique solid-phase synthesis procedure that provides protected peptides with their C-termini in the free carboxylic acid form under mild conditions without the accompaniment of significant racemization.

Preliminary experiments in our laboratory[3] have shown that the oxime ester linkage between the carboxylic acid group of an N-protected amino acid or peptide and the polymeric support can be cleaved efficiently by *N,N'*-dialkylhydroxylamines without detectable racemization. Several reports on the use of esters of *N,N'*-dialkylhydroxylamines as active esters in peptide synthesis have been published.[4-7] Young's group has investigated extensively esters of 1-hydroxypiperidine and has shown that these esters have remarkable enantiomeric stability and high selectivity as acylating agents. Weygand et al.[8,9] have also reported that N-acylated peptide 1-piperidyl esters condense with amino acid esters or peptide esters without racemization. Though the 1-piperidyl ester method has been shown to be useful for the synthesis of relatively small peptides in the presence of acetic acid as a catalyst,[5] these esters have been reported to be insufficiently reactive for use in the cases of sterically hindered amino acids or larger peptides.[6]

For this reason we have focused on the use of 1-piperidyl esters of peptides, which are readily available by synthesis employing the oxime resin, not as active esters but rather

Scheme I. Synthetic Route Leading to Protected Peptide Segment Employing the *p*-Nitrobenzophenone Oxime Resin



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Pro-Lys-Leu-Glu-Glu-Leu-Lys-Glu-Lys-Leu-Lys-Glu-Leu-
Leu-Glu-Lys-Leu-Lys-Glu-Lys-Leu-Ala

Figure 1. Amino acid sequence of peptide model of human plasma apolipoprotein A-I (12).

as precursors for the corresponding protected peptide carboxylic acids, which can be versatile intermediates for segment-condensation methods. Along these lines, we sought a convenient method for the selective cleavage of 1-piperidyl esters to free carboxylic acids and have utilized reductive cleavage with zinc in aqueous acetic acid (Scheme I). The extent of racemization by the use of this method has been tested by employing model peptides.

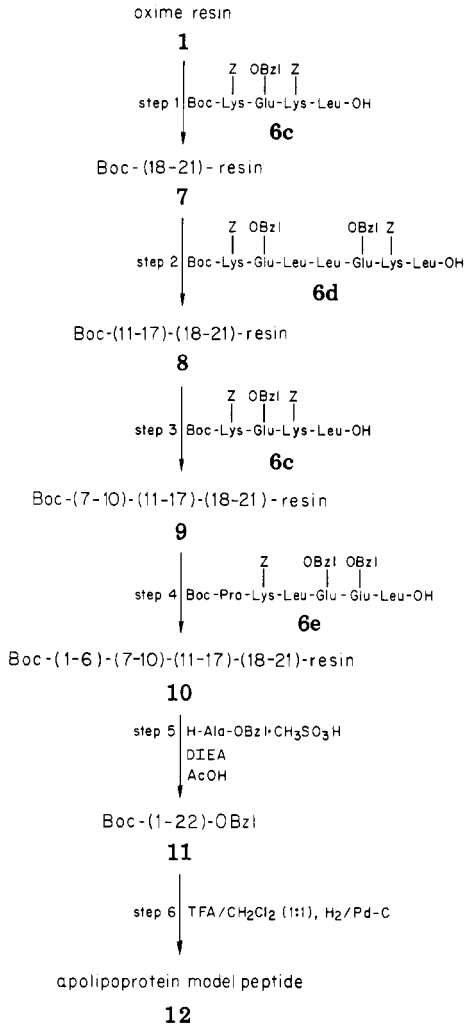
As illustrated in Scheme II, the synthesis of protected peptide carboxylic acids, using the oxime resin in combination with peptide segment condensation on the resin, was applied to the synthesis of the docosapeptide **12** (Figure 1), a model peptide possessing the properties of human plasma apolipoprotein A-I and previously synthesized[10] in a stepwise fashion on Merrifield's chloromethyl resin.[11] This peptide (**12**) was designed to idealize the amphiphilic α-helical structure[14] of a domain corresponding to six highly homologous 22 amino acid segments of the apolipoprotein A-I molecule[12,13] but to have an amino acid sequence as different as possible from any of these segments. The model peptide was found to show the essential surface properties of the intact apolipoprotein A-I and to activate lecithin:cholesterol acyltransferase in a similar way.[15] Therefore, the development of synthetic routes for the preparation of the model peptide is of interest because of its use in investigations of fundamental features of lipid–protein interactions.

In the present report on the preparation of protected peptide segments, we have utilized the *tert*-butyloxycarbonyl group for α-amino protection, the benzyloxycarbonyl group for ϵ-amino protection of lysine residues, and the benzyl ester group for the protection of the γ-carboxyls of glutamic acid residues. The condensation of these segments has been performed on the oxime resin (**1**) to investigate the potential for the use of this resin in the synthesis of large peptides. The protected peptide consisting of 21 amino acid residues bound to the oxime resin by attachment at the amino acid residue penultimate to the C-terminus of the desired product was removed from the solid support by aminolysis of the C-terminal amino acid ester function as shown in Scheme II. The protecting groups of the fully protected docosapeptide were removed under mild conditions to yield the final product. The effects of 1-hydroxybenzotriazole[16] and ethyl 2-(hydroximino)-2-cyanoacetate[17] as additives and the effects of changes in the solvent were investigated by using small model peptides to develop a procedure for minimizing the racemization due to the direct attachment of the C-terminal segment to the oxime resin by the dicyclohexylcarbodiimide method.

Results

The resistance to racemization in the cleavage of the protected peptide from the oxime resin with 1-hydroxypiperidine (HOPip)[18] was first tested by Izumiya's method,[19] involving coupling to form Gly-Ala-Leu where the amino and carboxyl groups were protected in the initial product. Boc-Gly-Ala-resin (**3a**) prepared in a stepwise fashion was stirred with 3 equiv of HOPip in CH_2Cl_2 for 3 h. The crude cleavage product (**4a**) was directly coupled with 1.2 equiv of Leu-O-*t*-Bu·AcOH in CH_2Cl_2. After removal of the protecting groups with trifluoroacetic acid (TFA), a portion of the resulting reaction mixture was directly introduced on an amino acid analyzer for the analysis of the content of peptide diastereoisomers. Another portion of the mixture was hydrolyzed with 6 N HCl for amino acid analysis. By combining these analyses, it was concluded that the yield of Boc-Gly-Ala cleaved from the oxime resin as the 1-piperidyl ester was 85%, the yield of the subsequent condensation with Leu-O-*t*-Bu was 79%, and the diastereoisomer (Gly-D-Ala-Leu) was not detected (less than 0.1%).

Scheme II. Synthetic Route to Model Peptide for Apolipoprotein A-I by the Solid-Phase Segment-Condensation Approach



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(18) All amino acids except glycine are of the L configuration unless otherwise described. Standard abbreviations for amino acids, peptides, and protecting groups are followed according to the recommendations of the IUPAC-IUB commission on biochemical nomenclature. Abbreviations used in the text: DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DIEA, diisopropylethylamine; EACNOx, ethyl 2-(hydroxyimino)-2-cyanoacetate; HOBt, 1-hydroxybenzotriazole; HOPip, 1-hydroxypiperidine; LAP, leucine aminopeptidase; NMM, *N*-methylmorpholine; TEA, triethylamine; TFA, trifluoroacetic acid.
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Table I. Effects of Additives and Solvents in Experiments Designed to Find Conditions for Minimizing Racemization in the DCC-Mediated Condensation Reactions of Protected Peptides on the Oxime Resin[a]

acyl component	amt used, equiv	additive	solvent	yield,[b] %	D isomer found, %
Boc-Gly-Ala-OH (6a)	1.35	HOBt	DMF/CH_2Cl_2 (1:1)	56.6	4.8
		EACNOx	DMF/CH_2Cl_2 (1:1)	26.6	3.0
		EACNOx	CH_2Cl_2	85.4	0.1
Boc-Gly-Ile-OH (6b)	1.35	HOBt	DMF/CH_2Cl_2 (1:1)	13.9	27.6
		EACNOx	DMF/CH_2Cl_2 (1:1)	5.5	34.8
		EACNOx	CH_2Cl_2	63.7	1.2
Boc-Lys(Z)-Glu(OBzl)-Lys(Z)-Leu-OH (6c)	1.43	HOBt	DMF/CH_2Cl_2 (1:1)	52.6	
	+0.53	HOBt		70.6	10
	1.25	EACNOx	CH_2Cl_2	93.5	3

[a] See Experimental Section for reaction conditions. [b] Based on C-terminal amino acid as determined by amino acid analysis.

Another test was carried out with the isoleucine-containing peptide Boc-Gly-Ile-OH. Boc-Gly-Ile-resin (**3b**) was prepared in the usual way, and the protected peptide was cleaved from the oxime resin with 3 equiv of HOPip in CH_2Cl_2 for 18 h. Cleavage of the peptide from the resin proceeded in 95% yield, and 0.15% of D-aIle was detected after acid hydrolysis with 6 N HCl and amino acid analysis.[20] The crude product (**4b**) was treated with Zn dust in 90% AcOH. After removal of Zn and extraction with ethyl acetate from acidic solution, an aliquot of the crude product (**6b**) was subjected to amino acid analysis to show that 80% recovery of peptide had occurred and that there was 0.17% of D-aIle. In a control experiment, Boc-Ile-OH was hydrolyzed under the same conditions, and 0.23% of D-aIle was detected. Therefore, no detectable racemization occurred during the preparation of Boc-Gly-Ile-OH (**6b**).

Because in the syntheses of the peptides described above it was shown that each step of the preparative procedure was not accompanied by any significant racemization and gave a good yield, the oxime resin method was then applied to the synthesis of a peptide model of plasma apolipoprotein A-I (**12**). Leucine was chosen as the C-terminal amino acid in each segment anchoring the growing peptide chain to the oxime resin. Segments comprising the tetrapeptide **6c** (positions 7–10 and 18–21, the same sequence), heptapeptide **6d** (position 11–17), and hexapeptide **6e** (position 1–6) were synthesized individually. After the assembly of these segments by coupling them on the oxime resin, the fully protected peptide product was removed from the resin by using as the attacking nucleophile the amino group of Ala, which became the C-terminal residue.

Boc-Leu-resin (0.584 mmol/g) was used throughout the segment syntheses. Boc groups were deprotected with 25% TFA/CH_2Cl_2 (v/v) for 30 min, and peptide chains were elongated in a stepwise manner by acylation with 3 equiv of the symmetrical anhydrides[21,22] of the Boc-amino acids in the presence of 2 equiv of diisopropylethylamine (DIEA). The resulting protected peptide resins were treated with 3 equiv of HOPip in CH_2Cl_2 for 16 h. The crude products (mostly 1-piperidyl esters, but partly free C-terminal carboxylic acids according to the TLC behavior) were subjected to reduction with Zn in 90% AcOH for 15–30 min. The protected peptide segments containing free C-terminal carboxylic acid groups thus obtained were purified by a combination of column chromatography on silica gel and gel filtration on Sephadex LH-20 with an organic solvent in yields of 54% (**6c**), 33% (**6d**), and 55% (**6e**), based on the Boc-Leu-resin used.

The three peptide segments were then assembled sequentially on the oxime resin proceeding from the C-terminal to the N-terminal direction. The C-terminal segment (**6c**) was directly condensed with the oxime resin (**1**) by using the dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBt) method. The oxime resin (**1**) was acylated with 1.4 equiv of **6c** in 1:1 dimethylformamide (DMF)/CH_2Cl_2 (v/v) at −10 °C for 30 min and then at room temperature for 17 h. The reaction was repeated with 0.53 equiv of **6c**. The yield of the segment incorporated on the oxime resin was 70%.

For determination of the degree of racemization due to the use of this method, a portion of the peptide resin was treated with HOPip to cleave the peptide from the resin, and subsequently, the protecting groups were removed by treatment with TFA and then by catalytic hydrogenation. A portion of the free peptide was then hydrolyzed with 6 N HCl, and another portion was hydrolyzed by leucine aminopeptidase (LAP), with use of the same internal standard alanine, before each hydrolysis. From comparison of the two amino acid analyses, 10% of the D isomer of the C-terminal amino acid was estimated to have formed.

Ito's[17] strongly acidic oxime additive, ethyl 2-(hydroxyimino)-2-cyanoacetate (EACNOx), was tried as an additive to suppress racemization in the condensation of the peptide segment on the oxime resin. An additional advantage to using this additive as compared to HOBt was the good solubility of this reagent in CH_2Cl_2. The oxime resin (**1**) was acylated with **6c** (1.25 equiv) in CH_2Cl_2 by employing DCC (1.25 equiv) in the presence of EACNOx (2.5 equiv) at −10 °C for 30 min and at room temperature for 23 h. The yield of acylation of the oxime resin was increased to 93%, and the amount of the D isomer was suppressed to 3%.

Once again, two test peptides were prepared to determine unequivocally whether this improvement was effected by the acidic oxime additive or by the solvent. The oxime resin (**1**) was acylated with Boc-Gly-Ala-OH (**6a**) by using DCC–HOBt in 1:1 DMF/CH_2Cl_2, DCC-EACNOx in 1:1 DMF/CH_2Cl_2, and DCC-EACNOx in CH_2Cl_2. Each of the Boc-Gly-Ala-resin samples were brought into reaction with Leu-O-*t*-Bu·AcOH in CH_2Cl_2, since it is known that displacement with the latter amino acid ester does not cause racemization.[1,2] After deprotection the resulting crude peptide products were analyzed to determine their content of the optical isomers, as described before. The oxime resin was also acylated with Boc-Gly-Ile-OH (**6b**) under the same conditions as used for Boc-Gly-Ala-OH (**6a**). The peptide products were removed from the resin by the HOPip method and subjected to amino acid analyses after acid

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Table II. Yields and Reaction Conditions in the Syntheses of 12 by Segment Condensation

	DCC-HOBt					DCC-EACNOx				
step[a]	acyl component, equiv	solvent	reaction time, h	yield[b] %	yield[b] overall %	acyl component, equiv	solvent	reaction time, h	yield[b] %	yield[b] overall %
1	1.43 +0.53	DMF/CH_2Cl_2 (1:1)	17 17	70.6	100	1.25	CH_2Cl_2	23	93.5	100
2	1.25	DMF/CH_2Cl_2 (1:2)	18	(88.5)		1.2	DMF/CH_2Cl_2 (1:3)	21	87.4	87.4
3	1.46	DMF/CH_2Cl_2 (1:2)	26	(100)	88.5	1.33	CH_2Cl_2	21	100	87.4
4	1.26	DMF/CH_2Cl_2 (1:1)	18	84.5	74.8	1.35	DMF/CH_2Cl_2 (1:3)	23	92.3	80.7
5		DMF/CH_2Cl_2	24	73.4	54.9		CH_2Cl_2	22	63.0	50.8
6		(1:1)		43.2	23.7				30.8	15.6

[a] See Scheme II. [b] Based on Leu by amino acid analysis.

hydrolyses. The results are summarized in Table I. Apparently, the use of the acidic oxime in CH_2Cl_2 improved the yield and suppressed racemization.

The protected tetrapeptide oxime resin ester (7) was further elongated by consecutive addition of the three segments **6d**, **6c**, and **6e** in the order shown in Scheme II. Unreacted hydroxyl or amino groups were blocked by acetylation with acetic anhydride and *N*-methylmorpholine (NMM) prior to the removal of the Boc groups. The Boc groups were removed by treatment with 30% TFA/CH_2Cl_2 (v/v). Protected peptide segments were then coupled to the peptide resin by treatment with DCC using an additive at −10 °C for 30 min and then at room temperature for a day. A slight excess of the acyl component (1.2–1.5 equiv) was used in each coupling. The fully protected docosapeptide was obtained by aminolysis with Ala-OBzl in the presence of AcOH. The yields and reaction conditions are summarized in Table II.

The protected docosapeptides obtained by use of coupling procedures involving either HOBt or EACNOx were purified by passage through a column of Sephadex LH-60 with DMF or 9:1 $MeOH/CHCl_3$ (v/v) as solvent. Following deprotection by treatment with 50% TFA/CH_2Cl_2 and catalytic hydrogenation using Pd on charcoal a crude product was obtained, which was purified by a combination of an ion-exchange chromatography on CM-cellulose, partition chromatography on Sephadex G-50, and desalting on Sephadex G-25 or G-15 to give analytically pure peptide **12** in overall yields of 24% and 16% by using DCC–HOBt and DCC–EACNOx, respectively. On HPLC and TLC both preparations showed behavior identical with the preparations synthesized previously on Merrifield's chloromethyl resin in a stepwise manner.[10]

Discussion

The removal of protected peptides from the oxime resin with HOPip takes place efficiently under extremely mild conditions. The conditions employed in this work allow most of the side chain functional groups or protecting groups of amino acids to remain intact.[23] However, the subsequent reduction by Zn in aqueous AcOH to give the protected peptides with their C-terminal carboxyl groups free will restrict the applications of this approach to cases where Zn-labile groups such as nitro, phenacyl, or disulfides are absent.

Methylene chloride was used exclusively as a solvent in the HOPip-mediated reaction cleaving the protected peptides from the resin because of the good solubilities of the peptides and of the ideal swelling properties of the resin in this solvent. For the peptides with less solubility in CH_2Cl_2, DMF or a mixture of DMF and CH_2Cl_2 are also effective.

Isolation of a peptide as its 1-piperidyl ester after its cleavage from the resin with HOPip is also possible. Protected peptide esters, in most cases, have better solubility in the organic solvents employed than do the corresponding acids. Thus, for a peptide with poor solubility, purification of a segment as a 1-piperidyl ester might be a useful alternative procedure.

However, the present synthetic studies showed that the purification of the protected peptide 1-piperidyl esters from the reaction mixture resulting from treatment of the resin-bound peptides with HOPip required considerable effort and gave relatively low yields, partly because of the degradation of the esters during the purification procedure. Although 1-piperidyl esters are quite stable once purified, the crude mixtures containing the esters tend gradually to form the corresponding free carboxylic acids. For this reason the crude peptide esters were converted to their carboxylic acids without further purification. It had been known that the 1-piperidyl ester group can be removed by treatment with hydrogen bromide in acetic acid or by catalytic hydrogenation in the presence of palladium.[5] We sought a more selective and milder procedure to remove the ester group and concluded that a suitable method was reduction by Zn in aqueous AcOH. This method had already been used by Stevenson and Young[24] for the removal of the piperidinoxycarbonyl amino-protecting group. Although this method is selective and mild enough for our present purposes, alternative cleavage methods will have to be employed if Zn-labile groups are present in the segments.

The 1-piperidyl ester function was first introduced in peptide synthesis as an active ester group with extraordinary resistance toward racemization.[4-9] However, attempts to synthesize acylamino acid or peptide 1-piperidyl esters without causing racemization were not particularly successful. Among the several methods tried, the use of Woodward's "reagent K" proved to be a fairly good method, but even this procedure was not free from the danger

(23) Besides the Boc-, Z-, and -OBzl groups used in the model peptide synthesis, the stabilities of the following amino acid derivatives were tested: Boc-Arg(Tos)-OH, Boc-Cys(acetoamidomethyl)-OH, Boc-Cys(methoxybenzyl)-OH, Boc-His(Tos)-OH, Boc-Lys(TFA)-OH, Boc-Met-OH, Boc-Ser(Bzl)-OH, Boc-Trp(For)-OH, Boc-Tyr(2,6-dichlorobenzyl)-OH. By the criteria of thin-layer chromatography all compounds were shown to be unchanged on incubation at room temperature for a day at a concentration of 0.02 M in 0.1 M hydroxypiperidine in methylene chloride or dimethylformamide.

(24) D. Stevenson and G. T. Young, *J. Chem. Soc., Chem. Commun.*, 900 (1967); *J. Chem. Soc. C*, 2389 (1969).

of racemization. Thus, optically pure Bz-Leu-OPip was prepared through its acid azide.[5] Optically pure Z-Tyr-Leu-OPip was also prepared by the same route.[25] The present work using the oxime resin method appears to provide a racemization-free route for the synthesis of 1-piperidyl esters of acylamino acids or protected peptides that could be prepared otherwise only by a lengthy process.

Despite the optical stability of the 1-piperidyl esters toward the condensation reactions, the reactivity of the ester function has been shown to be insufficient for the syntheses involving sterically hindered amino acids or oligopeptides, and therefore, this ester group has not been used extensively recently. We also recognized the insufficient reactivity of the ester for its use as an active ester.[26] For that reason we employed the 1-piperidyl esters solely as intermediates for the preparation of the corresponding free carboxylic acids in the present work. However, the possibility remains that the 1-piperidyl-active ester method might be applied to the coupling of peptide segments if catalysts better than acetic acid could be found in further investigations.

As the C-terminal amino acid anchoring the growing peptide chain to the oxime resin in the synthesis of each segment, leucine was chosen. This amino acid has been considered to be relatively less prone to racemization than many other amino acids in the subsequent segment condensations by the DCC–HOBt method, according to recent reports by Benoiton et al.[27-29]

The segment condensations involved in the synthesis of the apolipoprotein A-I model peptide could be performed, in principle, in solution or on the solid phase by using various types of resins. We chose to employ solid-phase segment condensation on the *p*-nitrobenzophenone oxime resin (1) to test the applicability of this resin for the synthesis of relatively large peptide segments. The length of the present model peptide (22 amino acid residues) is considered to be appropriate to serve as a unit for the synthesis of a larger peptide or protein, if there are no problems with the solubility of the protected peptide. Indeed, the model here represents only one helix while there are thought to be potentially at least six helices in apolipoprotein A-I. For studies on models of the apolipoproteins it will be worthwhile to assemble systems involving more than one helical region, and the oxime resin method appears to be well suited for this purpose.

The main problem encountered in the present work occurred in the attachment of the first segment to the oxime resin. Generally, solid-phase sytheses utilizing segments start with the condensation of a segment to a single amino acid attached to the resin or with a short peptide chain that has been elongated on the resin in a stepwise manner.[30-32] Another approach involves attachment to the resin through a side chain functional group of a segment.[33] The direct attachment of a peptide segment through an α-carboxyl group is limited to the use of a racemization-free procedure such as esterification by the reaction of the α-carboxylic acid salt with the bromo- or chloromethylated resin.[34] Recently, Tam et al.[35] have developed multidetachable resins. According to this approach, a segment is synthesized on a resin with a spacer which allows reattachment of the segment to an aminomethyl resin without the problem of racemization.

In the racemization tests using Boc-Gly-Ala-OH or Boc-Gly-Ile-OH, the effect of the solvent was greater than the effect of additives. Methylene chloride was found to be a much superior solvent than 1:1 DMF/CH_2Cl_2. The alanine peptide gave a better yield and less racemization than the sterically hindered isoleucine peptide. However, regardless of the improvement in the coupling yield in CH_2Cl_2 in the preparation of the apo A-I model (**12**), suppression of racemization in the attachment of the C-terminal tetrapeptide to the oxime resin was not as great as expected. Unlike the poor solubility of HOBt in CH_2Cl_2, Ito's acidic oxime (EACNOx) is very soluble in this solvent. Therefore, the use of this additive for the reattachment of a soluble C-terminal segment to the oxime resin by DCC-mediated condensation is advantageous for the suppression of racemization.

Although in the present synthesis the final product was purified by partition chromatography and this procedure removed the optical isomers effectively, for the synthesis of larger peptides, additional developments such as improvements in the suppression of racemization or in the separation of diastereoisomers of the protected peptide by HPLC or other methods will be required.

The segment condensations of three peptide components added to the tetrapeptide–oxime resin by the DCC–HOBt or DCC–EACNOx method proceeded in reasonable yields (more than 85% in each step). This result indicates that the acidic oxime EACNOx is also useful as an additive in solid-phase segment condensations.

Both the uncosapeptides assembled on the oxime resin were removed from the solid support by aminolysis with the C-terminal amino acid ester in the presence of acetic acid in good yield without any difficulties. Although the synthesis of peptide segments by similar aminolysis reactions were reported in previous papers,[1,2] the present application of this process to the preparation of larger peptides confirmed the potential of the oxime resin in solid-phase peptide synthesis.

Experimental Section

Equipment, Materials, and Methods. Melting points were taken on a Thomas-Hoover apparatus and are not corrected. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. Stepwise solid-phase syntheses were carried out with a Beckman Model 990 peptide synthesizer. Amino acid analyses[36] 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 20–24 h. Peptidyl resins were hydrolyzed with 1:1 propionic acid/concentrated HCl (v/v) mixture[37] at 110 °C for 15–18 h. High-performance liquid

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(26) Analytically pure Boc-Leu-Arg(Tos)-Arg(Tos)-Ala-OPip was condensed with H-Ser(Bzl)-Leu-Gly-OBzl in DMF in the presence of 1 equiv of AcOH as the catalyst to give the heptapeptide in approximately 10% yield after 2 days.
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chromatography was performed by employing a Waters Associates apparatus, with two Model 6000A solvent delivery systems and a Model 450 variable-wavelength UV detector, on a μ-Bondapak C18 column (0.4 × 30 cm). Solvents were glass distilled, filtered through Millipore (0.45 μm), and deaerated just before use. Thin-layer chromatography was performed on precoated silica gel 60 F-254 plates, 0.25 mm (E. Merck) in the following solvent systems: (A) 95:5:3 chloroform/methanol/acetic acid (v/v/v); (B) 85:10:5 chloroform/methanol/acetic acid (v/v/v); (C) 15:3:10:12 1-butanol/acetic acid/pyridine/water (v/v/v/v). Spots were visualized with a UV light (254 nm) and/or by spraying with a ninhydrin solution and heating.

Methylene chloride was distilled from phosphorus pentoxide. Dimethylformamide was distilled from ninhydrin under reduced pressure and stored over molecular sieves. *N,N'*-Diisopropylethylamine, *N*-methylmorpholine, and pyridine were also distilled from ninhydrin. Dicyclohexylcarbodiimide was distilled under vacuum. Trifluoroacetic acid and 1-butanol were distilled.

All amino acid derivatives were purchased from Chemical Dynamics Corp. Trypsin (bovine pancreas type I), leucine aminopeptidase (porcine kidney microsomes type VI-S), and CM-cellulose (microgranular forms) were purchased from Sigma. Sephadex G-15, G-25, G-50, LH-20, and LH-60 were products of Pharmacia. Silica gel for column chromatography was a product of Baker. 1-Hydroxybenzotriazole monohydrate was obtained from Aldrich. 1-Hydroxypiperidine from Aldrich was recrystallized from *n*-hexane before use. Palladium (10%) on powdered charcoal was a product of Matheson Coleman and Bell. Ethyl 2-(hydroxyimino)-2-cyanoacetate was synthesized according to the literature.[38] *p*-Nitrobenzophenone oxime resin was prepared according to a previous report from this laboratory.[1] Two substitution levels of the resin (0.368 and 0.758 mmol/g, respectively, determined by nitrogen analysis) were used throughout this work.

General Procedure for Attachment of *tert*-Butyloxycarbonyl Amino Acid to *p*-Nitrobenzophenone Oxime Resin (2). Boc-amino acid (1 mmol) and DCC (1 mmol) were added to the resin 1 (1 g) in CH_2Cl_2 (10 mL). The mixture was shaken at room temperature for 1 day and filtered. The resin was washed with CH_2Cl_2, 2:1 CH_2Cl_2/EtOH (v/v), and EtOH and dried under vacuum. The substitution level for the amino acid bound to the polymeric support was determined by an amino acid analysis after hydrolysis of an aliquot of the resin.

General Procedure for Stepwise Peptide Synthesis on *p*-Nitrobenzophenone Oxime Resin (3). Boc-amino acid oxime resin ester **2** was placed in a reaction vessel, and the synthesis was carried out automatically by the Beckman synthesizer programmed as follows: (1) wash, CH_2Cl_2 (2×); (2) prewash, 25% TFA/CH_2Cl_2 (v/v) (1 × 1 min); (3) deprotect, 25% TFA/CH_2Cl_2 (1 × 30 min); (4) wash, CH_2Cl_2 (2×); (5) wash, *i*-PrOH (1×); (6) wash, CH_2Cl_2 (2×); (7) wash, *i*-PrOH (1×); (8) wash, CH_2Cl_2 (4×); (9) couple, 3 equiv of Boc-amino acid symmetric anhydride[21,22]/CH_2Cl_2 and 2 equiv of DIEA (1 × 45–120 min); (10) wash, CH_2Cl_2 (4×); (11) wash, 2:1 CH_2Cl_2/EtOH (2×); (12) wash, CH_2Cl_2 (2×). The solvent volume for all wash steps was 15 mL/g resin, and the time was 1 min. After the last cycle, the protected peptide resin was washed with EtOH and dried over P_2O_5 under vacuum.

General Procedure for Removal of Protected Peptide Segment from the Oxime Resin by 1-Hydroxypiperidine and Subsequent Reduction with Zinc Dust in 90% Acetic Acid (4 and 6). Protected peptide oxime resin ester **3** was suspended in CH_2Cl_2 (1 g/10 mL), and the mixture was shaken with 3 equiv of HOPip at room temperature for 3–16 h. The resin was filtered and washed with CH_2Cl_2 and/or DMF and then with MeOH. The combined filtrate was evaporated under vacuum. The residue was triturated with Et_2O or hexane to obtain a crude peptide 1-piperidyl ester (**4**). The crude ester was then dissolved in 90% AcOH (ca. 15 mL/g of peptide), and Zn dust (ca. 30 equiv) was stirred into the solution. After vigorous stirring at room temperature for 15–30 min, Zn was removed by filtration and washed with 90% AcOH. The combined filtrate was evaporated under vacuum or freeze-dried. The residue (not very dry) was dissolved in an appropriate mixture of $CHCl_3$/MeOH/AcOH and applied on a column of silica gel and chromatographed. This procedure was effective for the removal of residual Zn and of coproducts originating from HOPip. The partially purified peptide acid (**6**) was further purified by gel filtration on Sephadex LH-20 with an organic solvent, MeOH, 9:1 MeOH/$CHCl_3$, or DMF. The peptide content of the various fractions was monitored by the UV absorption at 270 nm and by TLC. The fractions corresponding to the desired product were pooled, evaporated, and triturated with Et_2O and/or crystallized.

Test for Racemization in the Cleavage of Protected Peptide from Oxime Resin by 1-Hydroxypiperidine. Gly-Ala-Leu. The oxime resin 1 (10.00 g, 3.68 mmol) was acylated by employing Boc-Ala-OH (1.89 g, 10 mmol) and DCC (10 mmol) in CH_2Cl_2 (100 mL) for 22 h to yield Boc-Ala-resin (**2a**): 10.63 g; substitution level 0.317 mmol/g. Boc-Gly-OH was coupled to **2a** (5.00 g, 1.585 mmol) for 1 h to yield Boc-Gly-Ala-resin (**3a**): 5.05 g (92.6%, 0.288 mmol/g based on Ala); amino acid analysis $Gly_{1.03}Ala_{1.00}$. To **3a** (1.00 g, 0.288 mmol) in CH_2Cl_2 (10 mL) was added HOPip (87 mg, 0.86 mmol), and the mixture was stirred for 3 h and worked up to yield crude Boc-Gly-Ala-OPip (**4a**); yield 0.282 mmol (97.9% based on Ala); $Gly_{1.03}Ala_{1.00}$; TLC R_f (A) 0.52 (major), 0.61 (trace), 0.37 (HOPip). A solution of **4a** (0.141 mmol) in CH_2Cl_2 (1.5 mL) was stirred with H-Leu-O-*t*-Bu·AcOH[2] (43 mg, 0.173 mmol) for 19 h. The crude Boc-Gly-Ala-Leu-O-*t*-Bu (**5a**) thus obtained was deprotected by the addition of TFA (1.5 mL). After 60 min the reaction mixture was evaporated and dried over NaOH and P_2O_5 under vacuum. A portion of the mixture was hydrolyzed by 6 N HCl and subjected to amino acid analysis. Another portion was directly subjected to ion-exchange chromatography[19] by using the amino acid analyzer: coupling yield (Gly-Ala-Leu) 83.7%; no D isomer was detected (less than 0.1%).

Boc-Gly-Ile-OH (6b). The oxime resin 1 (5.00 g, 1.83 mmol) was acylated with Boc-Ile-OH·$^1/_2H_2O$ (1.20 g, 5 mmol) for 26 h to yield Boc-Ile-resin **2b**: 5.43 g; substitution level 0.341 mmol/g. Boc-Gly-OH was coupled to **2b** (4.00 g, 1.364 mmol) for 50 min to yield Boc-Gly-Ile-resin **3b**: 4.04 g (90.4%, 0.302 mmol/g based on Ile); $Gly_{1.02}Ile_{1.00}$. **3b** (1.00 g, 0.302 mmol) was treated with HOPip (91 mg, 0.9 mmol) in CH_2Cl_2 (10 mL) for 18 h to yield crude Boc-Gly-Ile-OPip (**4b**): yield 0.287 mmol (94.9% based on Ile); TLC R_f (A) 0.59 (major), 0.20 (minor; Boc-Gly-Ile-OH), 0.38 (HOPip). The amount of D-aIle detected by amino acid analysis was 0.15%. Crude **4b** was dissolved in 90% AcOH (5 mL) and stirred vigorously with Zn dust (0.50 g) at room temperature for 20 min. The reaction was not complete when checked by TLC. Another portion of Zn (0.10 g) was added. After 15 min, Zn was removed by filtration, and the filtrate was evaporated to a small volume, which was taken up in EtOAc and H_2O. The organic layer was washed with H_2O, 5% citric acid, and saturated NaCl solution: crude yield 0.229 mmol (79.9% from **4b** based on Ile); TLC R_f(A) 0.20. The amount of D-aIle detected was 0.17%. In a control experiment Boc-Ile-OH was hydrolyzed under the same conditions (6 N HCl, 110 °C, 20 h) and subjected to amino acid analysis. The amount of D-aIle detected was 0.23%.

Syntheses of Protected Peptide Segments for the Preparation of Human Plasma Apolipoprotein A-I Model Peptide. Boc-Leu-resin (2c). The oxime resin 1 (15.00 g, 11.37 mmol) was acylated with Boc-Leu-OH·H_2O (3.73 g, 15 mmol) for 13 h: 17.24 g (89%); substitution level 0.584 mmol/g.

Boc-Lys(Z)-Glu(OBzl)-Lys(Z)-Leu-resin (3c). Stepwise solid-phase synthesis with Boc-Lys(Z)-OH, Boc-Glu(OBzl)-OH, and Boc-Lys(Z)-OH was carried out by starting from the Boc-Leu-resin **2c** (3.425 g, 2.0 mmol): 4.608 g (92.9%, 0.379 mmol/g based on Leu); amino acid analysis $Glu_{1.06}Leu_{1.00}Lys_{1.70}$.

Boc-Lys(Z)-Glu(OBzl)-Lys(Z)-Leu-OH (6c). The peptide resin ester **3c** (4.40 g, 1.667 mmol) was treated with HOPip (0.67 g, 6.6 mmol) for 6 h in CH_2Cl_2 (44 mL) to give crude yield of **4c**: 1.331 g (78.0%); TLC R_f (A) 0.75 (major), 0.44 (minor). The ester intermediate was treated with Zn (2.33 g) in 90% AcOH (20 mL) for 15 min. The freeze-dried powder was purified by chromatography on a silica gel column (2 × 17 cm) with a solvent system of 95:5:3 $CHCl_3$/MeOH/AcOH (v/v/v) and then by gel filtration on a Sephadex LH-20 column (2.8 × 98 cm) with MeOH. The peptide was triturated with Et_2O and then recrystallized from MeOH/Et_2O: 0.945 g (58.1% from **3c**); mp 99–100 °C; $[\alpha]^{23}_D$ −16.0° (*c* 2, DMF); TLC R_f (A) 0.49; amino acid analysis $Glu_{1.01}Leu_{1.00}Lys_{1.98}$.

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Anal. Calcd for $C_{51}H_{70}N_6O_{13}$ (975.16): C, 62.82; H, 7.24; N, 8.62. Found: C, 62.62; H, 7.48; N, 8.58.

Boc-Lys(Z)-Glu(OBzl)-Leu-Leu-Glu(OBzl)-Lys(Z)-Leu-resin (3d). Stepwise solid-phase synthesis was carried out starting from the Boc-Leu-resin **2c** (3.423 g, 2.0 mmol): 5.191 g (82.5%, 0.285 mmol/g based on Leu); amino acid analysis $Glu_{2.22}Leu_{3.00}Lys_{1.76}$.

Boc-Lys(Z)-Glu(OBzl)-Leu-Leu-Glu(OBzl)-Lys(Z)-Leu-OH (6d). The peptide resin ester **3d** (5.00 g, 1.425 mmol) was treated with HOPip (0.45 g, 4.45 mmol) for 16 h to give a crude yield of ester **4d**: 1.782 g (83.5%); TLC R_f (A) 0.65 (major). The ester was treated with Zn (2.1 g) in 90% AcOH (25 mL) for 30 min. The freeze-dried powder was purified by chromatography on a silica gel column (2 × 21 cm) with a solvent system of 100:3:1.5 $CHCl_3$/MeOH/AcOH (v/v/v) and then by repeated gel filtration on an LH-20 column with 9:1 MeOH/$CHCl_3$ (v/v). The final product was obtained by precipitation from 95% EtOH: 0.809 g (40.0% from **3d**); mp > 260 °C; $[\alpha]^{23}_D$ −24.1° (*c* 2, DMF); TLC R_f (A) 0.28; amino acid analysis $Glu_{2.04}Leu_{3.00}Lys_{2.00}$.

Anal. Calcd for $C_{75}H_{105}N_9O_{18}$ (1420.72): C, 63.41; H, 7.45; N, 8.87. Found: C, 63.40; H, 7.50; N, 8.87.

Boc-Pro-Lys(Z)-Leu-Glu(OBzl)-Glu(OBzl)-Leu-resin (3e). Stepwise solid-phase synthesis was carried out by starting from Boc-Leu-resin **2c** (2.66 g, 1.553 mmol): 3.833 g (85.5%, 0.326 mmol/g based on Leu); amino acid analysis $Glu_{1.95}Pro_{1.13}Leu_{2.00}Lys_{1.07}$.

Boc-Pro-Lys(Z)-Leu-Glu(OBzl)-Glu(OBzl)-Leu-OH (6e). The peptide resin ester **3e** (1.853 g, 0.605 mmol) was treated with HOPip (0.188 g, 1.86 mmol) in CH_2Cl_2 (19 mL) for 16 h to give a crude yield of the ester **4e**: 0.667 g (87.9%); TLC R_f (A) 0.55. The ester was treated with Zn (1.0 g) in 90% AcOH (10 mL) for 30 min. The peptide acid was purified by silica gel column (1.8 × 17 cm) chromatography with a solvent system of 95:5:1 $CHCl_3$/MeOH/AcOH (v/v/v) and then gel filtration on an LH-20 column with 9:1 MeOH/$CHCl_3$ (v/v); yield 0.445 g (64.4% from **3e**); mp 249–251 °C dec; $[\alpha]^{23}_D$ −31.8° (*c* 2, DMF); TLC R_f(A) 0.35; amino acid analysis $Glu_{2.02}Pro_{0.96}Leu_{2.00}Lys_{1.05}$.

Anal. Calcd for $C_{60}H_{83}N_7O_{15}$ (1142.37): C, 63.08; H, 7.32; N, 8.58. Found: C, 62.96; H, 7.18; N, 8.37.

Racemization Test for the Reattachment of a Protected Peptide Segment to the Oxime Resin. Boc-Gly-Ala-resin. A solution of DCC in CH_2Cl_2 (0.4 M, 0.125 mL, 0.05 mmol) was stirred into a mixture of the oxime resin 1 (0.10 g, 0.037 mmol), Boc-Gly-Ala-OH (0.05 mmol, prepared from **4a** by treatment with Zn in 90% AcOH), and HOBt·H_2O (15.3 mg, 0.1 mM) in 1 mL of 1:1 DMF/CH_2Cl_2 (v/v) or EACNOx (14.2 mg, 0.1 mmol) in 1 mL of 1:1 DMF/CH_2Cl_2, or 1 mL of CH_2Cl_2 at −10 °C. Stirring was continued at −10 °C for 30 min and then at room temperature for 22 h. Each reaction mixture was filtered, and the resin washed with CH_2Cl_2, 2:1 CH_2Cl_2/EtOH, and MeOH and then dried under vacuum. A portion of each peptide resin was subjected to amino acid analysis to determine the coupling yield. Another portion of each peptide resin (50 mg) was suspended in CH_2Cl_2 (0.5 mL) and stirred with 3 equiv of H-Leu-O-*t*-Bu·AcOH for 18 h. After removal of the resin by filtration, the filtrate was evaporated and deprotected with 50% TFA/CH_2Cl_2 (2 mL) for 45 min. Each free tripeptide obtained after evaporation and drying over NaOH under vacuum was subjected to ion-exchange chromatography on the Beckman amino acid analyzer for the determination of the degree of racemization. The results are shown in Table I.

Boc-Gly-Ile-resin. Boc-Gly-Ile-OH (**6b**, 0.05 mmol) was used for the acylation of the resin 1 under three different sets of conditions, as described previously. Boc-Gly-Ile-resin (50 mg each) was treated with HOPip (5 mg) in CH_2Cl_2 (0.5 mL) for 18 h. The resin in each reaction mixture was collected by filtration and washed with CH_2Cl_2 and then with MeOH. The solvent was removed from the filtrate by evaporation, and the residue was hydrolyzed for amino acid analysis to determine the degree of racemization of Ile to D-aIle. The results are shown in Table I.

Reattachment of the C-Terminal Tetrapeptide Segment to the Oxime Resin (7). DCC–HOBt Method. A solution containing DCC (0.5 M in CH_2Cl_2, 0.8 mL, 0.4 mmol) was stirred into a mixture of the oxime resin 1 (0.763 g, 0.28 mmol), protected tetrapeptide segment **6c** (0.39 g, 0.4 mmol), and HOBt·H_2O (0.086 g, 0.56 mmol) in 1:1 DMF/CH_2Cl_2 (6 mL) at −10 °C. After stirring at −10 °C for 30 min and at room temperature for 17 h, the peptide resin ester was collected by filtration, washed with CH_2Cl_2, 2:1 CH_2Cl_2/EtOH, EtOH, and MeOH, and dried over P_2O_5 under vacuum; 0.904 g (53% based on weight gain). The condensation reaction was repeated with **6c** (0.145 g, 0.15 mmol), HOBt·H_2O (0.30 mmol), and DCC (0.15 mmol); 0.948 g (70.6%, 0.192 mmol/g based on Leu). A portion of the peptide resin (8 mg, 1.54 μmol) was stirred with HOPip (2 mg, 20 μmol) in CH_2Cl_2 (2 mL) for a day. The peptide cleaved from the resin was treated with 50% TFA/CH_2Cl_2 (2 mL) for 1 h, and the solvent was evaporated. The residue was dissolved in 50% AcOH, and the mixture was subjected for 2 h to catalytic hydrogenation using 10% Pd on carbon (10 mg) as the catalyst. After removal of the catalyst and the solvent, the peptide was freeze-dried from water. A portion of this residue was hydrolyzed with 6 N HCl, and another portion (ca. 0.5 μmol of peptide) was hydrolyzed with LAP (0.1 mg) in 0.2 M TEA·AcOH (pH 8.2) at 37 °C for 15 h. Amino acid analysis: (HCl hydrolysis) $Glu_{1.01}Leu_{1.00}Lys_{2.01}$; (LAP hydrolysis) $Glu_{0.99}Leu_{0.88}Lys_{1.87}$. Recovery from the LAP digestion was 98% (calculated for the internal standard, Ala, used). Therefore, approximately 10% of the C-terminal Lys–Leu bond was not hydrolyzed by the enzyme.

DCC–EACNOx Method. A solution containing DCC (0.4 M in CH_2Cl_2, 0.125 mL, 0.05 mmol) was stirred into a mixture of the resin 1 (110 mg, 0.04 mmol), protected peptide segment **6c** (49 mg, 0.05 mmol), and EACNOx (14.2 mg, 0.1 mmol) in CH_2Cl_2 (1 mL) at −10 °C. The reaction mixture was stirred at −10 °C for 30 min and at room temperature for 23 h and worked up as described before: 146 mg (93.5%, 0.254 mmol/g based on Leu). A part of the peptide bound to the resin (11.4 mg, 2.9 μmol) was cleaved from the resin and deprotected. The free tetrapeptide was hydrolyzed with 6 N HCl and LAP, and the hydrolysates were subjected to amino acid analyses: (HCl hydrolysis) $Glu_{1.02}Leu_{1.00}Lys_{2.04}$; (LAP hydrolysis) $Glu_{1.00}Leu_{0.95}Lys_{1.97}$. Recovery from the enzyme digestion was 98%. Therefore, the C-terminal amino acid was estimated to contain 3% of the D isomer.

Segment Condensations on the Oxime Resin by the Dicyclohexylcarbodiimide–1-Hydroxybenzotriazole Method (8–10). General Procedure. Protected peptide resin ester was placed in a reaction vessel, and the synthesis was carried out manually according to the following protocol: (1) wash, CH_2Cl_2 (2×); (2) acetylate, Ac_2O (1.5 equiv) and NMM (1.5 equiv) in CH_2Cl_2 (1 × 60 min); (3) wash, CH_2Cl_2 (4×); (4) prewash, 30% TFA/CH_2Cl_2 (v/v) (1 × 1 min); (5) deprotect, 30% TFA/CH_2Cl_2 (1 × 30 min); (6) wash, CH_2Cl_2 (3×); (7) wash, *i*-PrOH (2×); (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 (5×); (13) couple, protected peptide acid (1.2–1.5 equiv) and HOBt·H_2O (2.4–3.0 equiv) in DMF/CH_2Cl_2, DCC (1.2–1.5 equiv), −10 °C, 30 min, room temperature, 1 day; (14) wash, 1:1 DMF/CH_2Cl_2 (3×); (15) wash, CH_2Cl_2 (3×); (16) wash, 2:1 CH_2Cl_2/EtOH (3×); (17) wash, EtOH (3×); (18) dry over P_2O_5 under vacuum. The solvent volume for all wash steps was 20 mL/g, and the time was 1 min.

Boc-(11–17)-(18–21)-resin (8). Boc-Lys(Z)-Glu(OBzl)-Lys(Z)-Leu-resin (**7**, 0.833 g, 0.16 mmol) was acetylated (0.24 mmol), deprotected, neutralized, and coupled with Boc-Lys(Z)-Glu(OBzl)-Leu-Leu-Glu(OBzl)-Lys(Z)-Leu-OH (**6d**, 284 mg, 0.2 mmol) by employing DCC (41.3 mg, 0.2 mmol) in the presence of HOBt·H_2O (61.3 mg, 0.4 mmol) in 1:2 DMF/CH_2Cl_2 (6 mL) for 18 h; 1.045 g.

Boc-(7–10)-(11–17)-(18–21)-resin (9). The peptide resin **8** (1.003 g) was acetylated, deprotected, neutralized, and coupled with Boc-Lys(Z)-Glu(OBzl)-Lys(Z)-Leu-OH (**6c**, 194 mg, 0.2 mmol) by DCC (0.2 mmol) in the presence of HOBt·H_2O (0.4 mmol) in 1:2 DMF/CH_2Cl_2 (6 mL) for 26 h; 1.045 g (88.5% from **7**, 0.121 mmol/g based on Leu).

Boc-(1–6)-(7–10)-(11–17)-(18–21)-resin (10). The peptide resin **9** (0.980 g, 0.119 mmol) was acetylated, deprotected, neutralized, and coupled with Boc-Pro-Lys(Z)-Leu-Glu(OBzl)-Glu(OBzl)-Leu-OH (**6e**, 171 mg, 0.15 mmol) by DCC (0.15 mmol) in the presence of HOBt·H_2O (0.3 mmol) in 1:2 DMF/CH_2Cl_2 (6 mL) for 18 h; wt 1.020 g (84.5% from **9**, 0.091 mmol/g based on Leu).

Removal of the Protected Docosapeptide from the Resin by Aminolysis with C-Terminal Amino Acid Ester (11). The peptide resin **10** (0.770 g, 0.07 mmol as peptide, 0.172 mmol as oxime) was suspended in a mixture of DMF (3.5 mL) and CH_2Cl_2

(4 mL). To this, H-Ala-OBzl·CH_3SO_3H (165 mg, 0.6 mmol), DIEA (0.105 mL, 0.6 mmol), and AcOH (36 μL, 0.6 mmol) were added in that order. The mixture was stirred at room temperature for 24 h. The resin was collected by filtration, washed with DMF, CH_2Cl_2, EtOH, and MeOH, and dried. The weight of the resin recovered was 0.446 g. The filtrate was evaporated to give a residue, which was dissolved in DMF (3 mL). The resultant solution was applied to a column of Sephadex LH-60 (2 × 57 cm) with DMF. Fractions of 2.25-mL volume were collected, and the UV absorbance at 270 nm was measured. Fractions comprising the major peak (no. 38-47) were pooled. The solvent was evaporated under vacuum, and the residue was redissolved in DMF (2 mL). Rechromatography on the same column was carried out to yield a single symmetrical peak. After evaporation of the solvent, the peptide was freeze-dried from AcOH: yield 222 mg (73.4%); $[\alpha]^{23}_D$ −5.7° (*c* 1, DMF): TLC R_f(A) 0.23, R_f(B) 0.68; amino acid analysis $Glu_{6.01}Pro_{0.94}Ala_{0.97}Leu_{7.00}Lys_{7.07}$.

Anal. Calcd for $C_{232}H_{309}N_{29}O_{51}$ (4320.17): C, 64.50; H, 7.21; N, 9.40. Found: C, 64.14; H, 7.28; N, 9.25.

H-Pro-Lys-Leu-Glu-Glu-Leu-Lys-Glu-Lys-Leu-Lys-Glu-Leu-Leu-Glu-Lys-Leu-Lys-Glu-Lys-Leu-Ala-OH (12). Fully protected docosapeptide 11 (200 mg, 46.3 μmol) was stirred with 50% TFA/CH_2Cl_2 (10 mL) at room temperature for 45 min. The solvent was removed by evaporation, the residue was triturated with Et_2O, and the precipitate was dried over NaOH under vacuum. The dried residue was suspended in a 1:1:1 mixture of AcOH/MeOH/H_2O (v/v/v, 45 mL), together with 10% Pd on carbon (200 mg), and H_2 gas was bubbled gently into the mixture with stirring at room temperature for 5 h. The peptide dissolved during the first 30 min. The catalyst was removed by filtration through a layer of Celite and washed with the same solvent mixture. The combined filtrate was evaporated to a small volume, which was diluted with water and freeze-dried. The crude lyophilisate was dissolved in 0.05 M NH_4OAc (pH 5.59, 6 mL). The solution was applied on a column of CM-cellulose (1.9 × 16.5 cm) previously equilibrated with the same buffer. The column was first washed with the same buffer (100 mL) and then eluted with a linear gradient of NaCl with a concentration from 0 to 0.4 M (total gradient volume 600 mL). The flow rate was 33 mL/h. The UV absorbance at 230 nm was monitored to detect the peptide. Fractions corresponding to the major peak were pooled, freeze-dried, and desalted by passage through a column of Sephadex G-25 with 0.2 M AcOH. The peptide fractions were pooled and freeze-dried; yield 84.6 mg. A portion of the peptide (33 mg) was further purified by partition chromatography[39] on Sephadex G-50 (1.8 × 30 cm). The solvent system used was 6:4:1:10 1-BuOH/pyridine/AcOH/H_2O (v/v/v/v). Fractions of 1.87-mL volume were collected, and the peptide was detected by the Folin–Lowry procedure.[40] The purity of the peptide was checked by HPLC using a solvent system of 36% CH_3CN/0.2 M phosphoric acid sodium salt (pH 2.5) at a flow rate of 1.5 mL/min. The analytically pure fractions no. 90–120 (R_f 0.15–0.11) were pooled, evaporated, passed through a column of Sephadex G-15 with 0.2 M AcOH, and then freeze-dried: 22.2 mg (recovery 67%); yield 43.2% from 11; $[\alpha]^{23}_D$ −53.5° (*c* 0.47, 0.2 M AcOH); TLC R_f(C) 0.21. Amino acid analysis: (acid hydrolysis) $Glu_{5.84}Pro_{0.96}Ala_{1.00}Leu_{6.98}Lys_{6.92}$; (enzymatic hydrolysis) $Glu_{6.00}Pro_{0.93}Ala_{1.00}Leu_{7.02}Lys_{6.81}$. For the enzymatic hydrolysis, peptide (200 μg) was incubated with trypsin (20 μg) and LAP (100 μg) in the presence of $CaCl_2$ (10 μg) in 0.1 M TEA·AcOH (pH 8.2, 0.32 mL) at 37 °C for 4 h (recovery 100%).

Synthesis of 12 by the Dicyclohexylcarbodiimide/Ethyl 2-(Hydroxyimino)-2-cyanoacetate Method. Boc-Lys(Z)-Glu(OBzl)-Lys(Z)-Leu-resin (7) obtained by the DCC–EACNOx method (122 mg, 31 μmol) was used as the starting material, and three segment condensations were carried out under conditions similar to those described above by employing EACNOx as an additive instead of HOBt. A summary of the reaction conditions and the coupling yields for each step are shown in Table II. The protected uncosapeptide resin ester was allowed to react with the C-terminal amino acid ester as discussed previously, leading to formation of the fully protected docosapeptide, which was purified by gel filtration on Sephadex LH-60 with 9:1 MeOH/$CHCl_3$; yield 52 mg (50.8% from 7); TLC R_f(A) 0.23, R_f(B) 0.68; amino acid analysis $Glu_{6.16}Pro_{1.04}Ala_{1.04}Leu_{7.00}Lys_{7.00}$. A portion of this material was deprotected in the same manner as described above, and the final product was purified by partition chromatography on Sephadex G-50, CM-cellulose ion-exchange chromatography, and gel filtration to give a yield of 30.8% from 11. The purity was shown by analytical HPLC to be the same as the compound synthesized by the DCC–HOBt method and the compound synthesized in our laboratory previously by the stepwise Merrifield method.[10] Amino acid analysis: (acid hydrolysis) $Glu_{6.05}Pro_{0.96}Ala_{1.00}Leu_{6.98}Lys_{7.04}$; (enzymatic hydrolysis) $Glu_{6.04}Pro_{1.03}Ala_{1.00}Leu_{7.00}Lys_{7.01}$.

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Registry No. **4a**, 84195-21-1; **4b**, 84195-22-2; **4c**, 84195-24-4; **4d**, 84195-26-6; **4e**, 84215-04-3; **5a**, 60872-51-7; **6c**, 84195-23-3; **6d**, 84195-25-5; **6e**, 84195-27-7; **11**, 84393-73-7; **12**, 71269-73-3; Boc-Ala-OH, 15761-38-3; Boc-Gly-OH, 4530-20-5; HOPip, 4801-58-5; H-Leu-O-*t*-Bu·AcOH, 38024-17-8; Gly-Ala-Leu, 22849-49-6; Boc-Ile-OH, 13139-16-7; Boc-Gly-Ile-OH, 53481-49-5; Boc-Leu-OH, 13139-15-6; Boc-Lys(Z)-OH, 2389-45-9; Boc-Glu(OBzl)-OH, 13574-13-5; Boc-Gly-Ala-OH, 42291-52-1; H-Ala-OBzl·CH_3SO_3H, 74279-66-6.

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