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Synthetic Approaches to Biologically Active Peptides and Proteins Including Enzymes

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The advent of modern molecular biology, together with the explosion of structural information available from protein X-ray crystallography, has had very invigorating effects on the field of protein chemistry in recent years. Many fundamental problems relating structure to activity in enzymes, for instance, can be attacked by judicious application of site-directed mutagenesis. With these powerful methods of molecular biology in hand, it is important to ask whether there is a need to explore other approaches, such as the use of peptide synthetic methods to prepare both peptides and proteins. For peptides that are of the length of 30 or 40 amino acids, the justification for using a peptide synthesis approach to obtain the molecules of interest is easily made. The development of the Merrifield solid-phase method,[1] together with enormous improvements in peptide purification including principally the use of high-performance liquid chromatography (HPLC), has revolutionized the field. In general, one can readily prepare peptides that are 30–40 amino acids in length in a good state of purity, as our own work on the modeling of apolipoproteins, peptide toxins, and peptide hormones has demonstrated.[2,3]

In the usual situation, if one wishes to make a large number of analogues of a peptide of this length, the solid-phase methodology is more rapid and easier to apply than the molecular biological methods. Indeed, for peptides that are in the 30–40 amino acid length range, the principal preparative uses for molecular biological methods are likely to be large-scale production. At this point, while solid-phase synthesis can certainly be used to make multigram quantities of peptides, the production of multikilogram-scale materials may be more amenable to molecular biological approaches. Since, however, basic research laboratories are likely to be dealing with relatively small amounts of peptides and their analogues, solid-phase peptide synthesis is usually the method of choice for preparing peptides 30–40 amino acids in length.

The situation is not so clear, however, when one turns to peptides that run from about 50 amino acids to 100 amino acids in length. A major advantage of stepwise solid-phase synthesis is that one does not have to isolate and purify the growing peptide intermediates at each stage of reaction. However, because of incomplete coupling and deprotection reactions, impurities that will be generated at each stage of the synthesis accumulate, and eventually the crude peptide product obtained at the end of the synthesis may be quite impure. Purification of the final product by HPLC can often be successfully accomplished for the relatively shorter peptides, but when one starts to produce peptides greater than 50 amino acids in length, the separation of the pure product from impurities differing by only one or two amino acids becomes extremely difficult.

Dr. Emil T. Kaiser was born in Budapest, Hungary, in 1938. He earned the B.S. degree from the University of Chicago in 1956 and the Ph.D. degree in chemistry from Harvard University in 1959. Following postdoctoral work at Harvard and Northwestern University, he was named assistant professor at Washington University in St. Louis in 1961. He then joined the faculty of the University of Chicago in 1963, where he became professor in 1970 and Louis Bock Professor in 1981. He was appointed a professor at The Rockefeller University in 1982 and organized the University's laboratory of bioorganic chemistry and biochemistry. He became the University's first Patrick E. and Beatrice M. Haggerty Professor in 1984. Dr. Kaiser's distinguished research career focused on the structure and function of enzymes and other biologically active peptides and proteins. He served on numerous scientific panels and advisory boards and also consulted extensively in industry. He was a member of the American Academy of Arts and Sciences and the National Academy of Sciences.

[†] Deceased, July 18, 1988.

[‡] Minor modifications were made to this manuscript by the editorial staff after the death of Professor Kaiser.

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1                5                10                15
TYR-GLY-GLY-PHE-MET-THR-SER-GLU-LYS-SER-GLN-THR-PRO-LEU-VAL-

                 20               25                30
THR-LEU-PHE-LYS-ASN-ALA-ILE-ILE-LYS-ASN-ALA-TYR-LYS-LYS-GLY-GLU
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Figure 1. Amino acid sequence of β-endorphin.

There have been reports of the synthesis of peptides that are 100 amino acids or more in length,[4] but it has been very difficult to establish that materials of the highest purity have been obtained. Furthermore, even if it were possible to purify to a high degree of homogeneity a peptide of 100 amino acids in length, it seems likely that the purification procedure would be very arduous. If one were trying to make a large number of structural analogues, the effort involved would be prohibitive.

Despite these difficulties, we feel that very strong arguments can be made in favor of the development of improved synthetic methods as an alternative to molecular biological approaches. In particular, it is not always straightforward to express peptides 50–100 amino acids in length even if one has the appropriate gene in hand, whether from cloning or from polynucleotide synthesis. In appropriate production systems such as *Escherichia coli* or yeast, it is often found that expression of quantities of a peptide may be limited by a number of factors, including often the difficult problem of proteolytic breakdown by the enzymes present in the *E. coli* or yeast system. Furthermore, even if a large amount of peptide can be generated in the appropriate host system, there can be advantages to preparing the same peptide by peptide synthesis. For example, if physical studies are to be undertaken on the peptide product or on its analogues, it is often important to introduce spectroscopic labels such as ^{13}C- or ^{15}N-labeled amino acids at very specific locations in the peptide molecule. In principle, this can be readily accomplished by total synthesis. Usually, by the molecular biological approaches that would be employed, such specific labeling of a single amino acid in a protein may not be feasible unless it does not occur elsewhere in the molecule. Another point that must be considered is that the synthetic approach has a great deal of flexibility. An important example of this flexibility is that D-amino acids or non-peptidic regions can be introduced into peptides and proteins. These sorts of changes cannot be made by the usual molecular biological techniques.

Two examples illustrating this point can be taken from our work on β-endorphin. We have postulated that this opioid hormone has three structural regions which are important for receptor binding.[5,6] First, there is the N-terminal "active site" region corresponding to [Met^5]enkephalin (Figure 1). This is followed by the sequence from residues 6–13, which is a hydrophilic spacer region, and then, an amphiphilic helix is formed from residue 13 to residue 29, almost at the C-terminus. In the case of the spacer region, we have shown that the residues from 6 through 12 can be replaced by a repeating pattern of four γ-amino-γ-(hydroxymethyl)-butyric acid residues linked by amide linkages.[7] A model peptide containing this non-peptidic spacer unit exhibits high activity in various assays for β-endorphin-like characteristics, including analgesic activity.

Another drastic change that we have been able to accomplish by the use of peptide synthesis is the replacement of the C-terminal region of β-endorphin, which is presumed to be in a right-handed amphiphilic helical structure, by a designed left-handed amphiphilic helical structure.[8] The peptide with the left-handed helix is in a sense diastereomeric with the β-endorphin structure. However, if one examines a Corey–Pauling–Koltun space-filling model of the latter peptide, one can see that, with the flexibility due to the spacer region from residues 6–12, the left-handed model does not appear very different from β-endorphin. We have seen that, indeed, the peptide that contains the left-handed helix behaves in a very β-endorphin-like fashion in receptor assays and other tests of biological activity, including analgesia.[8] It will be interesting to carry out similar substitutions in other small proteins on the order of 100 amino acids in length.

Development of Polymer-Bound Oxime for the Synthesis of Protected Peptide Segments

In our quest for organic chemical methodology for the preparation of peptides and small proteins containing 50–100 amino acids, we have turned to an approach employing segment synthesis–condensation. We have focused on the development of a rapid and convenient method for the synthesis of protected peptide segments on a solid support and on subsequent coupling of such segments either in solution or on solid supports. Several years ago, we showed that a *p*-nitrobenzoyl oxime substituted polystyrene polymer cross-linked with 1% divinylbenzene could be used for the synthesis of protected peptide segments.[9,10] As outlined in Figure 2, the segments are prepared in a stepwise manner with the C-terminal amino acid anchored as an oxime ester on the solid support. We have shown that the oxime ester linkage anchoring the N-protected amino acid to the polymer is sufficiently stable to allow the elongation of peptide chains under convenient conditions, but it is active enough to permit the cleavage of the protected peptide product from the polymeric support under rather mild conditions. For instance, the peptide chain can be removed by a treatment with *N*-hydroxypiperidine (HOPip), an α-nucleophile, giving the protected peptide ester of HOPip. By treatment with zinc and acetic acid, the peptide ester can be converted into the protected peptide with a free carboxyl group. This method is quite general except for cases of peptides that have sulfur functions like methionine. Additionally,

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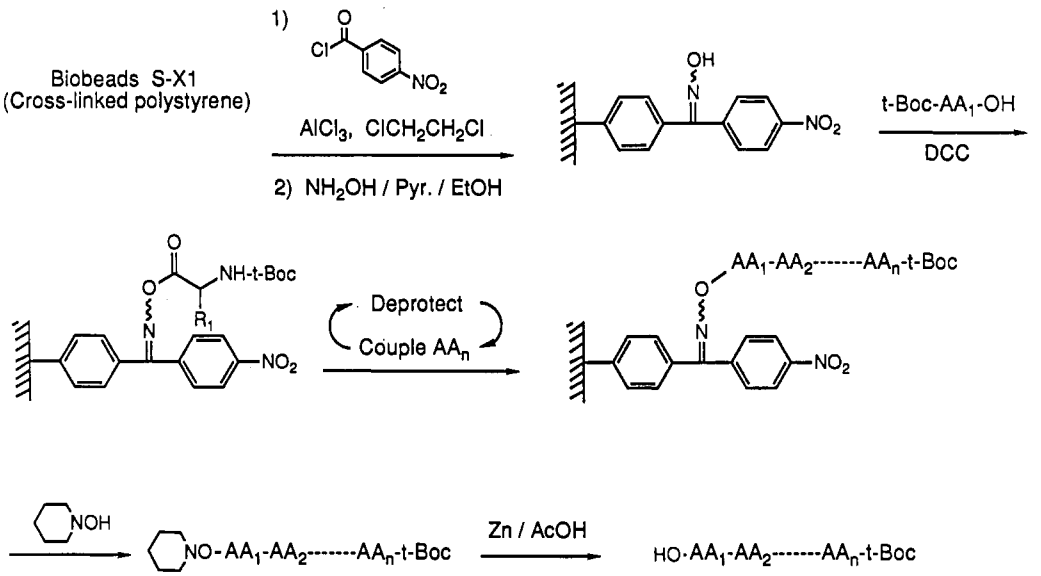

Figure 2. Steps in the synthesis of protected peptide segments by the oxime resin approach.

aminolysis by amino acid esters with acetic acid as the catalyst or treatment with the tetra-*N*-butylammonium salt of an amino acid[11] can be used to remove the protected peptides from the resin. Either the amino acid ester or the amino acid is inserted at the C-terminus of the final protected peptide product by the displacement reactions.

Early experiments in our laboratory showed 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.[9,10] Prior to our work, the use of esters of *N,N'*-dialkylhydroxylamines as active esters in peptide synthesis had been described.[12,13] Extensive investigations of esters of HOPip have shown that these esters have high enantiomeric stability and marked selectivity as acylating agents. N-Acylated peptide HOPip esters condense with amino acid esters or peptide esters without racemization.[14] However, while the HOPip ester method has been demonstrated to be useful for the synthesis of relatively small peptides in the presence of acetic acid as a catalyst, such esters have been reported to be insufficiently reactive for condensations of sterically hindered amino acids or for the synthesis of larger peptides.

Because of these limitations, we have concentrated on the use of peptide esters of HOPip that are readily available by synthesis employing the oxime resin (Figure 2), not as active esters but rather as precursors for the corresponding protected peptide carboxylic acids.[15] These protected peptide segments are versatile intermediates for segment condensation methods. As already mentioned, we have frequently utilized reductive cleavage with zinc in an aqueous acetic acid for the selective cleavage of HOPip esters to free carboxylic acids. An important point that was addressed early in our work was the question of possible racemization by the use of this method. This has been examined in the syntheses of model peptides.[15,16]

We first tested the resistance to racemization in the cleavage of a protected peptide from the oxime resin with HOPip by the method of Izumiya[17] which involves coupling to form Gly-Ala-Leu where the amino and carboxyl groups were protected in the initial product. We prepared Boc-Gly-Ala–oxime resin in a stepwise fashion on the oxime polymer by the route of Figure 2 and stirred this resin with 3 equiv of HOPip in CH_2Cl_2 for 3 h. The crude cleavage product was directly coupled with 1.2 equiv of Leu-*O*-*t*-Bu·AcOH in CH_2Cl_2. After removal of the protecting groups by treatment with trifluoroacetic acid, a portion of the resulting reaction mixture was introduced directly onto an amino acid analyzer for the analysis of the content of the peptide diastereoisomers. Another part of the mixture was hydrolyzed with 6 N HCl and subjected to amino acid analysis. Through a combination of these analyses, we found that the yield of Boc-Gly-Ala cleaved from the oxime resin as the *N*-piperidyl ester was 85%, the yield of Boc-Gly-Ala cleaved from the oxime resin as the *N*-piperidyl ester was 85%, the yield of the subsequent condensation with Leu-*O*-*t*-Bu was 79%, and an undetectable amount of the diastereoisomer (Gly-D-Ala-Leu) was formed (less than 0.1%).

In another test of racemization,[18] Boc-Gly-Ile–resin was prepared in the usual way, and using 3 equiv of HOPip in CH_2Cl_2, the protected peptide was cleaved from the oxime resin, employing an 18-h time period. We found that the peptide was cleaved from the resin in 95% yield and that 0.15% of D-aIle was obtained after acid hydrolysis with 6 N HCl and subsequent amino acid analysis. The crude product was treated with Zn dust in 90% acetic acid. Removal of zinc and extraction with ethyl acetate from the acidic solution gave crude product, which when subjected to amino acid analysis showed that 80% recovery of the peptide had occurred and that there was 0.17% of D-aIle. When we performed a control experiment, Boc-Ile-OH was hydrolyzed under the same conditions and 0.23% of

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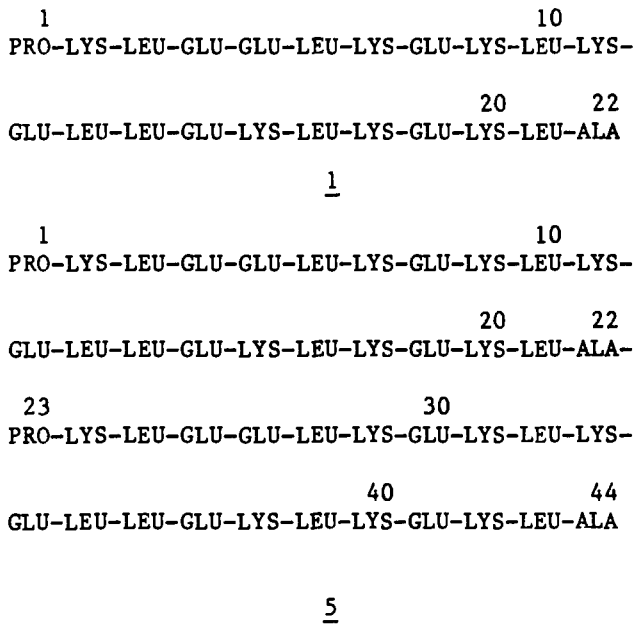

Figure 3. Amino acid sequences of peptides 1 and 5, models for apolipoprotein A-I.

D-aIle was detected. Therefore, we could conclude that no detectable racemization occurred in the preparation of Boc-Gly-Ile-OH.

Synthesis of Apolipoprotein A-I Models through the Preparation of Peptide Segments and Their Condensation

Having explored the synthesis of protected peptide segments by the oxime method extensively, we next decided to try to apply our synthetic approach to the preparation of a docosapeptide, 1[15] (Figure 3). This compound, a model peptide mimicking the properties of human plasma apolipoprotein A-I (apo A-I),[19] had been previously synthesized in a stepwise fashion by the Merrifield procedure on a chloromethyl resin. Apo A-I which contains 243 amino acids[20] is the principal protein constituent of high-density lipoprotein. Peptide 1 was designed to idealize the amphiphilic α-helical structure of a domain corresponding to the putative six to seven amphiphilic α-helical 22 amino acid segments[21] of the apolipoprotein A-I molecule. An important feature of peptide 1 is that it has an amino acid sequence for which the homology to any of the helical regions of naturally occurring apo A-I has been reduced as much as possible. We had shown that the model peptide exhibited the essential surface properties of the intact apolipoprotein A-I and activated catalysis by the important enzyme lecithin:cholesterol acyl transferase in a similar way. Since the model peptide and related peptides have proven to be very useful in investigations of fundamental features of lipid–protein interactions, we felt that as an initial target for the development of our segment synthesis–condensation method this was a very appropriate system.

In our synthesis of peptide 1 (Figure 4),[15] we employed three protected peptide segments, the tetrapeptide 2 (corresponding to positions 7–10 and 18–21 in the peptide 1 sequence), heptapeptide 3 (positions 11–17), and the hexapeptide 4 (positions 1–6). In each segment, leucine was chosen as the C-terminal amino acid anchoring the growing peptide chain to the oxime resin.

The three peptide segments were assembled sequentially on the oxime resin proceeding from the C-terminus to the N-terminus. The C-terminal segment, peptide 2, was directly condensed with the oxime resin either by the use of dicyclohexylcarbodiimide (DCC)/*N*-hydroxybenzotriazole (HOBt) or DCC/ethyl 2-(hydroxyamino)-2-cyanoacetate (EACNOx). The additive EACNOx was found to be more effective than HOBt in suppressing racemization in the condensation of the C-terminal segment on the oxime resin (only 3% D isomer formed under the conditions used). Assembly of the protected peptide was performed by consecutive addition of the three segments, peptides 3, 2, and 4 as shown in Figure 4. The fully protected docosapeptide was obtained by aminolysis with Ala-OBzl in the presence of acetic acid. After purification on Sephadex LH-60 of the protected docosapeptides obtained by the use of coupling procedures involving either HOBt or EACNOx, catalytic deprotection was carried out by treatment with 50% TFA/50% CH_2Cl_2 and catalytic hydrogenation using Pd on charcoal. The crude product was purified by a combination of ion-exchange chromatography on CM-cellulose, partition chromatography on Sephadex G-50, and desalting on Sephadex G-25 or G-15, giving the analytically pure peptide 1 in overall yields of 24% and 16% through the use of DCC/HOBt and DCC/EACNOx, respectively. The chromatographic and physical properties of both preparations were identical with those of a sample of peptide 1 synthesized previously on a chloromethyl resin in a stepwise manner.[19]

While the construction of the amphiphilic α-helical docosapeptide model of apo A-I by segment synthesis–condensation clearly was accomplished in a satisfactory manner, we had shown already that this peptide could be readily prepared by the stepwise solid-phase method and could be obtained quite pure by HPLC. The important question raised at this stage was whether the segment synthesis–condensation approach could be applied to the preparation of peptides that were unlikely to be easily obtained by stepwise solid-phase synthesis and that would probably pose considerable purification problems if they were synthesized by that method. To start to answer this question, we chose to synthesize a 44 amino acid peptide, 5, containing two repeating 22-peptide segments corresponding to the docosapeptide, 1 (Figure 3). The synthesis of a 44-peptide of this sort would not only demonstrate the usefulness of our segment approach but also provide us with a peptide in which the consequences of the covalent attachment of two amphiphilic secondary structural domains could be investigated.

As outlined in Figure 5, we synthesized the 44-peptide both by coupling large segments on the oxime resin and by coupling large segments in solution.[16] In the former approach, we used as a starting material the 21-peptide resin that had been an intermediate in the synthesis of peptide 1. This 21-peptide, which ultimately constituted the N-terminal domain of peptide 5, was first cleaved from the resin and transformed into the free

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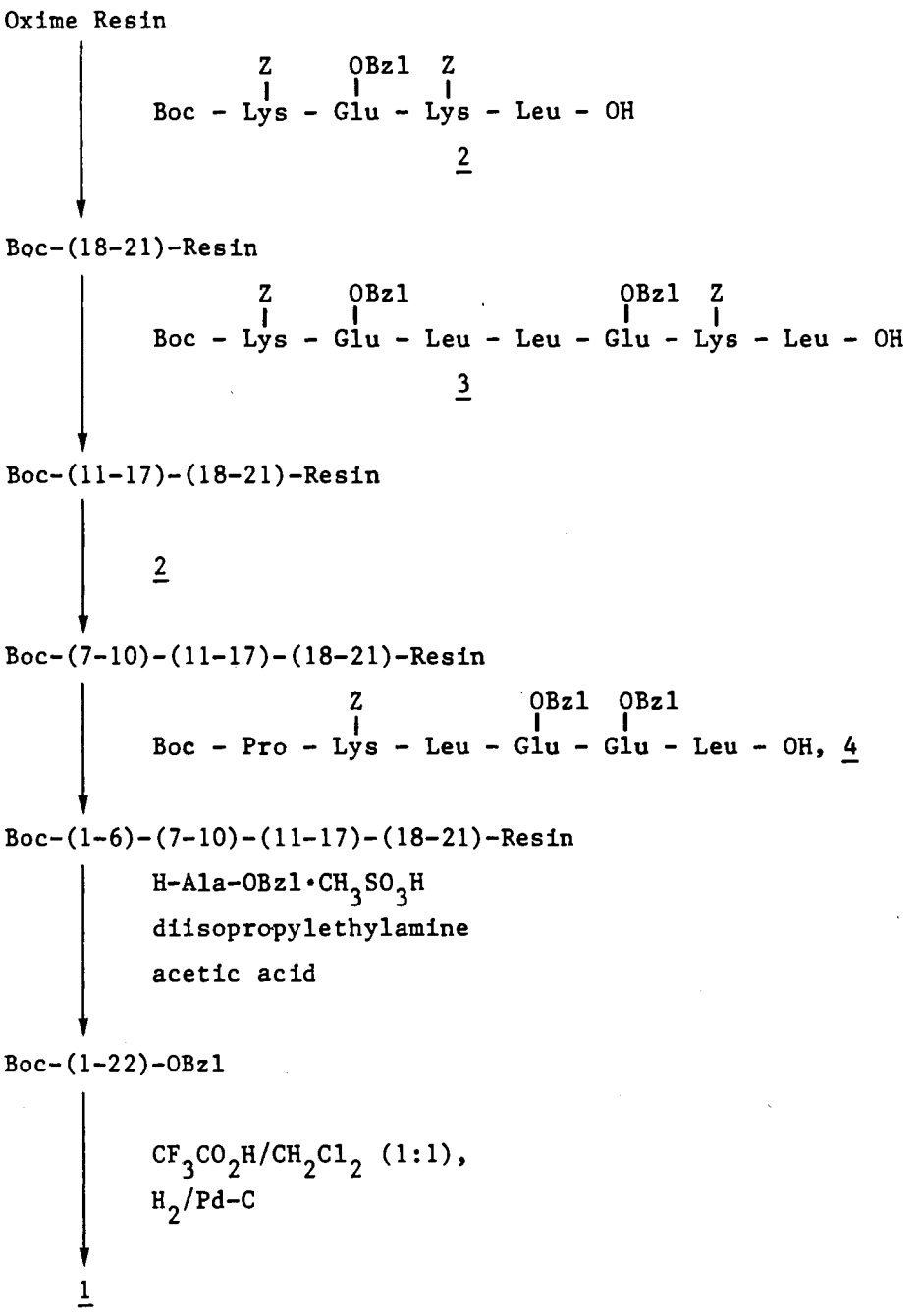

Figure 4. Synthesis of peptide 1 using the oxime ester methodology.

carboxylic acid. Then another portion of the 21-peptide resin which would yield the C-terminal domain was extended one more step toward the N-terminus, with introduction of the Ala residue which would become Ala-22 in the final peptide. The resin-bound 22-peptide was then condensed with the 21-peptide containing the free α-carboxyl group. The Ala-44 residue was introduced in the final step of the cleavage of the resulting 43-peptide from the resin. Alternatively, we condensed two major segments in solution. In the latter case, we first cleaved the resin-bound 22-peptide containing Ala in the N-terminal position from the resin by using Ala benzyl ester, which then yielded the C-terminal 23-peptide in solution. From this peptide, which was still fully protected, was removed the N^{α}-protecting group, and the product of this reaction was condensed with the 21-peptide containing the free α-carboxyl group. The protected 44-peptide obtained either by the condensation of the two major segments on the solid support or by condensation in solution was treated with 50% TFA/50% CH_2Cl_2 (v/v) to remove the N^{α}-*tert*-butyloxycarbonyl group. Then, the remaining protecting groups were removed by catalytic hydrogenation with 10% Pd on activated charcoal as a catalyst. The reaction rate was enhanced by the addition of ammonium formate.

The free 44-peptide, **5**, was purified through the use of a combination of CM-cellulose ion-exchange chromatography and partition chromatography on Sephadex G-25. The purity of the final peptide was confirmed by analytical HPLC and by amino acid analysis. In the final step involving the removal of protecting groups and purification, the overall yield was 21%. Although peptides of this size are accessible by stepwise solid-phase synthesis, they are of a length such that heterogeneities introduced in the stepwise synthesis may be quite difficult to remove, and purification procedures may be rather tedious. Furthermore, while

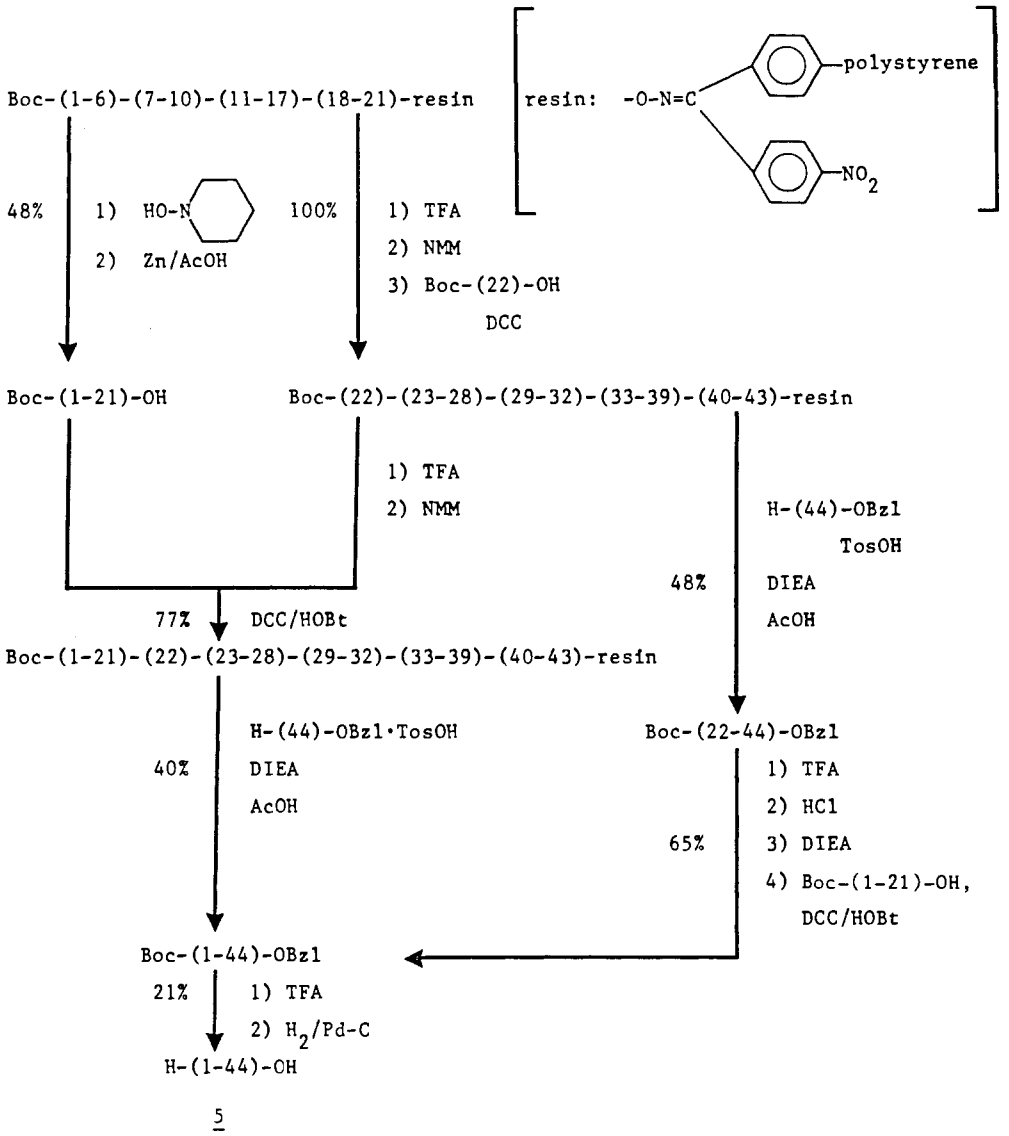

Figure 5. Synthesis of peptide **5** using the oxime ester methodology.

we have not applied our approach to the preparation of a covalent trimer of the 22-peptide, **1**, in principle, this should not be very difficult by the method that we have developed. The stepwise solid-phase synthesis, obtaining a pure peptide of such length would be a very major undertaking.

As mentioned earlier, we had demonstrated that the model 22-peptide, **1**, possesses many of the physical properties that are deemed to be essential for the physiological role of apo A-I.[3,19] Having prepared the 44-peptide containing two potential α-helices, we found that the covalent linkage of the two segments resulted in a considerable increase in the amphiphilicity of the peptide. For example, the 44-peptide exhibited a considerably greater α-helicity in 50% trifluoroethanol and a much higher tendency to form peptide micelles in aqueous solution. Furthermore, measurements of the limiting molecular area of peptide **5** adsorbed at amphiphilic surfaces such as the air–water interface, as well as phospholipid-coated polystyrene beads, showed that the 44-peptide was a considerably more effective model of apo A-I than was the 22-peptide. The model 22-peptide occupies approximately 22 Å^2 per amino acid at the interface,[19] and this indicates that the peptide is not fully helical. It must contain some random coil segments, presumably at the termini. On the other hand, in the case of the 44-peptide, 14–16 Å^2 is the area occupied per amino acid at both the air–water interface and on the phospholipid-coated polystyrene beads,[16] and these values are in excellent agreement with the value observed for apo A-I itself, 15–16 Å^2 per amino acid.[22] Our results suggest that the basic structural unit of the apolipoprotein is not the 22-peptide segment but rather is the 44-peptide segment which is punctuated in the middle by a helix breaker, Pro in the case of our synthetic peptide. In apo A-I, the putative amphiphilic α-helical segments are punctuated by either Gly or Pro residues as helix breakers. We have performed simple calculations using X-ray crystallographic data for the amphiphilic α-helical peptide melittin,[23] which contains a Pro residue flanked by two α-helical segments, and these show that the concavity of the Pro-punctuated 44-peptide segment matches closely the curvature of the surface of high-density lipoprotein. We have concluded that it is the 44-peptide segment that is the paradigm of apo A-I in terms of biological and physical properties. Our synthesis and subsequent study of the 44-peptide

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model of apo A-I exemplify the wealth of information that can be derived from the construction of peptides accessible by the segment synthesis–condensation method.

Construction of Large Peptides and Small Proteins by Segment Synthesis–Condensation

Having demonstrated with the preparation of the 44-peptide model of apo A-I that we could obtain a sizable peptide in good yield and high purity by means of our segment synthesis–condensation approach, we turned next to systems that would be accessible only with very great difficulty by the usual stepwise solid-phase methodology. One of our major targets has been the enzyme ribonuclease T_1, a guanosine-specific enzyme that cleaves the 3′,5′-phosphodiester linkage of single-stranded RNA. The X-ray crystal structure of an RNase T_1–2′-GMP complex has been reported.[24-26] The native enzyme and several of its mutants have been obtained by gene synthesis and expression of the protein in *E. coli*.[27] From consideration of the X-ray data together with the results of mutations at the His-40 and His-92 residues, a mechanism for RNase T_1 action related to that for RNase A has been proposed in which the His-40 residue acts as a base assisting formation of a cyclic 2′,3′-phosphate intermediate, and the His-92 residue acts as a general acid. In the breakdown of the cyclic phosphate intermediate, the His-92 residue acts as a base and the His-40 residue acts as a general acid.[28]

In view of the considerable structural and mechanistic information available for RNase T_1, we felt that this would be an excellent target to test whether our synthetic methodology could be readily applied to the preparation of a small enzyme. Besides testing our methodology for synthesis, construction of the enzyme offered some very interesting possibilities for protein-engineering studies. In particular, there is a helical region corresponding to residues 12–29 in the N-terminal region which has considerable amphiphilic character.[24-26] In our previous work on models for apolipoproteins, peptide toxins, and peptide hormones,[2,3] we had designed molecular regions where we could focus on secondary structural features and neglect to a first approximation the tertiary structural characteristics.

A very major problem which retards progress in protein engineering is the current inability to predict tertiary structure from primary amino acid sequence. In our work on RNase T_1, we have sought to answer the question whether we could apply the design principles that we had used earlier for preparation of amphiphilic secondary structural models to the construction of a mutant α-helix in the enzyme, allowing it to fold properly and to show catalytic activity. If, indeed, we could substitute a mutant helix with little homology to the sequence of the helix in the native enzyme, this would be an important first step in an empirical approach for the construction of tertiary structure from primary sequence. In addition to our objective of replacing the amphiphilic helix in RNase T_1, we wish eventually to replace portions of the enzyme molecule by non-peptidic linkages and to introduce spectroscopic labels at specific residues for further studies of the enzyme's mechanism.

Our strategy in the synthesis of RNase T_1[29] has been to divide the molecule into three major segments chosen not only for their synthetic accessibility but also because they contain structural features of the enzyme such as the amphiphilic helix on which we want to concentrate our attention. The major segments were assembled from smaller segments that had been prepared by the oxime ester method and that had been purified and characterized by a variety of techniques including NMR, mass spectroscopy, and amino acid analysis. The importance of ^{252}Cf fission fragment ionization mass spectroscopy to the characterization of our protected peptide segments cannot be overemphasized. When protected peptide segments have been purified, even if their amino acid analyses indicate that the composition is satisfactory, the question may remain as to whether all of the protecting groups are still intact. In principle, this could be addressed by a quantitative examination of NMR spectra, but such an approach could be rather tedious, and it might not be very easy to determine exactly where the loss of a protected group had occurred. In contrast, the mass spectral approach has provided us with a quick picture of the extent to which the peptide side chain functional groups are protected.

The coupling of the small segments has been carried out sequentially on a polymeric support to give the larger segments. Generally, we have used dicyclohexylcarbodiimide as the coupling agent in the presence of *N*-hydroxybenzotriazole, which is added to suppress racemization. The points of coupling have been chosen so as to reduce the problem of racemization, but we have not been able to avoid totally the possibility of this problem. After assembly of the peptide segments, we have taken the major segments off the resin and purified them extensively. The final coupling of the three major segments Z-(1–34)-OH (which contains the amphiphilic α-helix), H_2N-(35–71)-OH, and H_2N-(72–104)-OBn has been carried out in solution. After deprotection by the low HF–high HF procedure[29] and purification by column chromatography, material was obtained which was enzymatically active against yeast RNA.[30]

Since the final purification of this material has not yet been obtained, we do not know, as yet, whether we have obtained fully active enzyme. However, we do have a strong indication that the answer to the protein-engineering question that we have posed in connection with our work on RNase T_1 is that a secondary structural unit can be replaced by a designed unit to give proper folding. By a route related to that for the synthesis of RNase T_1 we can make a mutant species in which the N-terminal segment has been altered to contain a designed amphiphilic α-helix in which 11 of

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the 18 residues present in the native helix have been changed. When the protein containing the mutant helix is isolated, it shows catalytic activity against yeast RNA.[30] Because the mutant material has also not been purified fully, we cannot state what its kinetic parameters are, compared to the native enzyme, but it does appear clearly that it is possible to make a major alteration in the sequence of the protein and still retain quite appreciable catalytic activity. We believe that this is an important first step in demonstrating that through the redesign of secondary structural units in folded proteins it is possible to develop an empirical approach which may allow one to go from a designed primary to a predicted folded structure.

In conclusion, it can be seen that the methodology for stitching proteins together by segment synthesis–condensation is proceeding well, based on the use of a rapid method for segment synthesis. Major improvements will be needed in the steps involving coupling of very large segments (e.g., of the size of the roughly 30 amino acid containing segments which are the major parts of the RNase T_1 molecule). The possibilities that we are investigating for improved peptide segment coupling include the use of mutant[31] and native proteolytic enzymes. The enzymatic methods have the potential to avoid the possible problems of racemization that can occur with chemical coupling and should involve much less protection of functional groups in the peptide segments than is needed for the use of the chemical coupling approach. As the methodology for stitching proteins together improves, this becomes an increasingly viable alternative to molecular biological approaches for the preparation of the many important peptides and small proteins in the vicinity of 50–100 amino acids in length.

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