Studies on the Solid-Phase Synthesis of Peptide Fragments of Apolipoproteins A-I and A-II

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We report the solid-phase synthesis of four peptides chosen from a region of human serum high-density apolipoprotein A-I, which appears to be composed of repeating structural units of 22 amino acids, further divided into two similar 11-residue units. These repeat units have sequences that are predicted to give rise to amphipathic helices separated by small sections of random or β-turn structure, usually including a proline residue. The sequences of the peptides we have prepared correspond to: I, residues 158–168 of A-I; II, an analogue of I with Arg substituted for Asp_{160}; III, residues 147–168 of A-I; and IV, residues 114–133 of A-I. Additionally, a peptide (V) corresponding to residues 22–31 of A-II has been synthesized. The circular dichroism (CD) spectra of peptides I and V between 250 and 200 nm suggest that these peptides have predominantly random structures. While the longer peptides III and IV seem to have mostly random structure in the range pH 4–9, upon addition of trifluoroethanol the CD spectra undergo marked changes. In 50% trifluoroethanol, peptide III is calculated to have a 40% α-helical structure while peptide IV has a 33% helical structure. The lipid binding and surface properties of the synthetic peptides are now under examination.

The apoproteins of serum lipoproteins exhibit a number of unusual properties.[1] The manner in which these proteins bind to lipids is of particular interest. At the present time, the three-dimensional structures of the lipoproteins have not been unequivocally defined, and no x-ray crystallographic data for any apoprotein have been obtained. Amino acid sequences of several of the human apoproteins have been determined,[2–6] however, and estimates of their secondary structure can be made on the basis of circular dichroism (CD) measurements and predictions by the method of Chou and Fasman.[7] (See for example the structural predictions in ref 1.) As judged from CD measurements, the major protein components of high-density lipoproteins, A-I and A-II, have α-helix contents of approximately 55 and 35%, respectively; these values increase by 25–30% upon relipidation.[8] Examination of models of sections of these proteins in a helical conformation reveals that they have a polar surface containing pairs of acidic and basic amino acids and a nonpolar surface made up of hydrophobic residues. The C proteins, found mainly in very low density lipoproteins, also contain regions of potential helices of similar structure. It has been proposed that these two-sided or "amphipathic" helices are responsible for the lipid binding properties of the apolipoprotein.[9] We are currently engaged in a program to determine what structural features of the apolipoproteins are responsible for their physical properties and interactions with lipids.

One approach to assessing the functional importance of various regions of the apolipoproteins is to study the chemical and physical properties of synthetic peptides corresponding to various segments of the proteins. One such study of peptide fragments of the C-III protein has been reported.[10] The advantages of using synthetic peptides rather than chemically or enzymatically produced fragments derived from natural protein for studies of structure–activity relationships are that relatively large amounts of material can be obtained, the size and sequence of the fragments can be readily selected, and peptides with structural replacements or modifications can be prepared. In the investigation described in this report, the focus of our research was on the synthesis of peptides corresponding to segments of apolipoproteins which should have a high potential for forming amphipathic helices. Our attention was centered on the portion of human apolipoprotein A-I including residues 80–222, due to the observation made by Fitch[11] and, independently, McLachlan[12] that this region is composed of repeating structural units of 22 amino acids which are further divided into two similar 11-residue units. Interestingly, these repeat units correspond to sequences that are predicted to be amphipathic helices. The helices are separated by small sections of random or β-turn structure, usually including a proline residue. The presumption that these units of repeating structures arose from duplication of an ancestral gene suggests the possibility that one of the units alone could exhibit many of the properties of the complete protein. In this report the solid-phase synthesis of several of these repeating units of A-I apolipoprotein to be used in investigating their contributions to the properties of the protein is described. The results of work initiated on the synthesis of peptide fragments of human A-II lipoprotein are briefly summarized.

Results and Discussion

The following peptides have been synthesized: I, corresponding to residues 158–168 of A-I; II, an analogue of I with Arg substituted for Asp_{160}; III, corresponding to residues 147–168 of A-I; IV, corresponding to residues 114–133 of A-I; and V, corresponding to residues 22–31 of A-II (see Figure 1). Peptides I and III are representative 11 and 22 amino acid repeat units. Peptide IV contains segments of two predicted amphipathic helices separated by a bend or kink introduced by Pro_{124}. In the native lipoprotein complex the phospholipids could conceivably be bound within the loop formed by such structures. Peptide IV was acetylated at the N-terminal to give a structure more similar to that of the native protein. To investigate whether the correct amino acid sequence of the peptide, in particular, the location of the charged residues in the structure as described by Segrest et al.,[9] is necessary for imparting any special properties to these peptides, peptide II in which Asp is replaced by an Arg residue was synthesized. In addition to these peptide fragments of apolipoprotein A-I, a portion of A-II, peptide V, was synthesized. This peptide includes the single methionine residue of the apo A-II monomer. The lipid binding properties of the cyanogen bromide fragments of apo A-II have been studied by Assman and Brewer.[13] The fact that the Met residue is in the middle of a predicted amphipathic helix may explain why the lipid-binding capacity of the fragments was much less than that of the intact protein. For this reason we chose to make peptide V which includes the methionine residue as a starting point for our work on A-II synthetic fragments.

The peptides were synthesized by variations of the solid-phase method developed by Merrifield.[14,15] The benzyl ester linkage of the growing peptide chain to the polymer support was employed. Low substitution levels of peptide and 1% cross-linked resin were used to aid in attaining rapid, complete reactions and to minimize termination of the growing peptide

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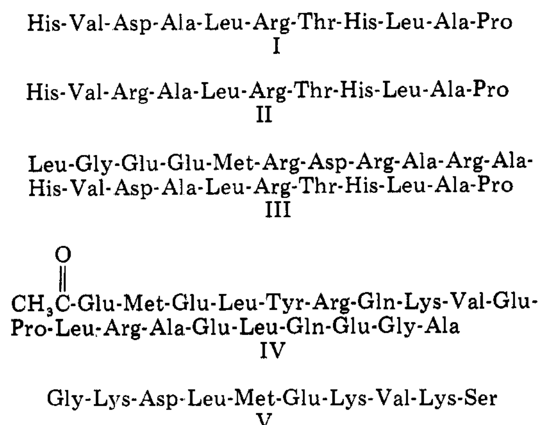

Figure 1. Sequences of synthetic peptides prepared in this work.

chain. Amino acid side chain protecting groups that are stable under the synthesis conditions were chosen to minimize chain branching. All of the protecting groups are known to be removable by HF treatment.[15] The completeness of the coupling reaction was monitored by the sensitive ninhydrin test described by Kaiser et al.[16] When a positive test was observed, the amino acid derivative was recoupled. Recoupling was found to be necessary in the case of the Val residue of peptide I, the second His residue of peptide II, the first Lys residue of peptide V, and the Gly moiety of peptide IV. Under the reaction conditions used, only the sterically hindered His-Val-Asp sequence and the first coupling step presented difficulty in achieving complete reaction.

In the synthesis of peptide III the symmetric anhydride method of coupling was employed.[17,18] It is claimed that using the preformed symmetric anhydride leads to a reduction in the formation of termination peptides.[19] We found that this method gave complete coupling in much shorter reaction times (45–75 min) than the DCC method.

The glutamine residues of peptide IV were introduced by coupling using the *p*-nitrophenyl ester. An equivalent of 1-hydroxybenzotriazole, which has been shown to catalyze the coupling reaction and also to minimize racemization,[20a,b,21] was added to the reaction mixture. After the incorporation of methionine into peptides III, IV, and V, anisole was added to the trifluoroacetic acid deprotection reagent to help prevent alkylation of the sulfur.

The amounts of the Boc-amino acid resins that were used in the syntheses and their substitution levels were: I, 4.87 g, 0.21 mmol Pro/g; II, 1.65 g, 0.24 mmol Pro/g; III, 5.00 g, 0.13 mmol Pro/g; IV, 5.5 g, 0.15 mmol Ala/g; V, 5.25 g, 0.05 mmol Ser/g. The analysis value for Ser may be low due to decomposition under the hydrolysis conditions employed. Yields of protected peptides calculated from the weight gain of the resins ranged from about 65% for peptide III and IV to 85% for peptide II, which indicates an average yield of about 98% for each amino acid incorporated. Some of the losses were due to the removal of samples for the ninhydrin test after each coupling step and amino acid analysis at several points in the synthesis. Another likely major loss of peptide resulted from diketopiperazine formation, particularly in the cases of those peptides which had proline at their C termini.[22,23] In the course of the synthesis of peptide III, an amino acid analysis on a hydrolyzate produced after incorporation of the fourth residue, threonine, on the polymer indicated that losses due to diketopiperazine formation had occurred. The substitution level of Pro at that point was calculated as being 0.102 mmol/g of polymer backbone which is a reduction of 0.029 mmol/g from the value at the start of the synthesis. At the end of the synthesis only a slight further decrease of the substitution level of Pro was noted.

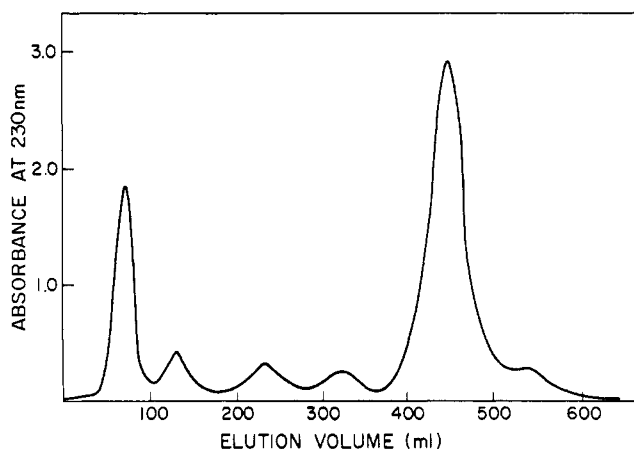

Figure 2. Ion-exchange chromatography of peptide I; 1.8 × 32 cm CM-Sephadex C-25 column employed using 0.02 M phosphate at pH 6.4 with 0–0.25 M NaCl gradient.

The peptides were cleaved from the solid support by treatment with hydrofluoric acid under conditions that were designed to remove all of the protecting groups, yet cause minimal side reactions. The crude peptide mixtures were extracted from the resin with 10% acetic acid. Lyophilization yielded the deprotected peptides in 70–82% yield for this step.

Purification of the Peptides. Because of incomplete reactions, loss of side-chain protecting groups, deprotection of side chains leading to chain branching, and various side reactions,[15] careful purification of peptides synthesized by the solid-phase method is an essential part of the synthetic procedure. The crude peptides from the HF cleavage process were gel filtered to remove small termination peptides, as well as side products resulting from the HF cleavage reaction. The peptides were purified further by ion-exchange chromatography.

Peptide I was chromatographed on a CM-Sephadex cation exchange resin with a sodium chloride gradient. One major peak and several smaller peaks were observed in the elution profile (Figure 2). After desalting, isolation of the major product gave an amount of peptide that corresponded to 35% of the material that had been loaded on to the column. Therefore, the overall yield based on the starting substitution level of the solid support was 18%. The purity of the product from the ion-exchange chromatography step was assessed by thin-layer chromatography. A good separation of the components of the starting peptide mixture was found using cellulose TLC plates developed with 1:1:1 *n*-butyl alcohol–pyridine–water. The purified peptide gave a single spot, R_f 0.45. Amino acid analysis of peptide I showed the expected ratios of amino acids (Table I). Sequencing of peptide I by the automated Edman degradation method established that the sequence was the desired one and confirmed the purity of the material (Table II).

Gel filtration of peptide II resulted in a 76% recovery of high molecular weight peptide. Cation exchange chromatography of this material with a sodium chloride gradient gave the major product in 21% yield after desalting. N-terminal analysis of this product by the Edman degradation method showed that it contained an impurity that had valine rather than histidine at the N-terminus. A problem in achieving complete coupling of the final histidine in the synthesis of peptide II had been indicated by the application of the ninhydrin test. Thus, peptide II was purified further by cation exchange chromatography with a pH step gradient of increasing pH. One minor and another very minor component eluted from the column before the major product. A center cut of the major component peak yielded 25 mg of purified peptide, 62% of the amount

Table I. Amino Acid Analyses of Synthetic Peptides

Amino acid	I	II	III	IV	V
Lys				1.19	3.16
His	2.09	2.01	2.16		
Arg	0.96	1.95	4.03	1.85	
Asp	1.09		2.04		1.02
Thr	0.94	0.96	0.98		
Ser					0.93
Glx			1.85	7.02	1.00
Pro	1.11	1.00	1.00	1.01	
Gly			0.87	1.29	1.08
Ala	2.20	2.10	4.02	2.34	
Val	0.93	0.72	0.97	0.73	0.91
Met			0.92	0.78	0.94
Leu	2.05	2.08	3.12	3.02	1.00
Tyr				0.70	

placed on the column. End-group analysis of this product by the dansylation method demonstrated that the only free amino group present was that due to histidine. Amino acid analysis of the purified peptide agreed with the theoretical values (Table I). On TLC peptide II had R_f values of 0.76 with 1:1:1 n-butyl alcohol–pyridine–water on cellulose and 0.66 with 50:30:15 pyridine–acetic acid–water on silica gel, and on paper electrophoresis it displayed a single spot, R_f 0.93, relative to lysine (3000 V for 45 min, pH 3.5).

In the case of peptide III, amino acid analysis of the peptide bound to the resin at the completion of the synthesis gave values of 0.10 mmol/g resin backbone for Pro, 0.082 mmol/g for Ala, and 0.057 mmol/g for Glu. Since proline is found only at the C-terminus, alanine throughout the peptide, and glutamic acid only near the N-terminal region of the peptide, considerable chain termination had occurred. Before gel filtering the crude peptide, it was dissolved in a solution of dithiothreitol to reduce any methionine sulfoxide back to methionine.[24] 2-Mercaptoethanol was added to the eluents in the chromatographic purification to prevent oxidation. Part of the product from the gel filtration step in the purification of the crude peptide was purified further by anion exchange chromatography, first with a concentration gradient of ammonium bicarbonate, then with a pH gradient of Tris buffer. A number of peaks were observed in the first ion-exchange column (Figure 3), but there was one major component. The material isolated from this peak was 35% of the total. Amino acid analysis of this product indicated that it was the desired peptide. Further purification of the peptide on the pH gradient column gave a 55% yield of peptide III. The purified peptide contained a single component as evidenced by TLC in the following systems: 15:10:3:12 n-butyl alcohol–pyridine–acetic acid–water on cellulose, R_f 0.68; 4:1:1 n-butyl alcohol–acetic acid–water on cellulose, R_f 0.39; 50:30:15 pyridine–acetic acid–water on silica, R_f 0.35. A single band was observed on high voltage paper electrophoresis of the product (2500 V for 1 h at pH 6.4, migration was toward the cathode; R_f 0.49 relative to arginine). Amino acid analysis of the final product agreed very well with the values expected for peptide III (Table I). Edman degradation of the purified peptide was fully consistent with the amino acid sequence (Table II).

Crude peptide IV was obtained in 77% yield from the HF cleavage reaction. Gel chromatography of this material on Sephadex G-10 resulted in the separation of three low molecular weight components; one of these components had an absorbance at 280 nm and the other two only at 230 nm. The product obtained from the void volume peak was 85% of the total. Ion-exchange chromatography of this material with an ammonium bicarbonate gradient resulted in an elution profile showing two major peaks which were unsymmetrical and not

Table II. Edman Degradation Results[a]

Cycle	Amino acids detected	Amount
	Peptide I	
1	His	Spot test
2	Val	900 nmol
3	Asp	800 nmol
4	Ala	750 nmol
5	Leu	700 nmol
6	Arg	Spot test
7	Thr	500 nmol
8	His	Spot test
9	Leu	350 nmol
10	Ala	50 nmol
11	Pro	Small amount
	Peptide III	
1	Leu	1950 nmol
2	Gly	1600 nmol
3	Glu	1890 nmol
4	Glu	1800 nmol
5	Met	1800 nmol
6	Arg	Spot test
7	Asp	1720 nmol
8	Arg	Spot test
9	Ala	1600 nmol
10	Arg	Spot test
11	Ala	1300 nmol
12	His	Spot test
13	Val	825 nmol
14	Asp	810 nmol
15	Ala	800 nmol
16	Leu	820 nmol
17	Arg	Spot test
18	Thr	350 nmol
19	His	Spot test
20	Leu	300 nmol
21	Ala	200 nmol
22	Pro	50 nmol
	Cyanogen Bromide Fragment of Peptide IVa	
1	Glu	500 nmol
2	Leu	500 nmol
3	Tyr	475 nmol
4	Arg	Spot test
5	Gln,Glu	200, 100 nmol
6	Lys	300 nmol
7	Val	350 nmol
8	Glu	310 nmol
9	Pro	300 nmol
10	Leu	300 nmol
11	Arg	Spot test
12	Ala	250 nmol
13	Glu	250 nmol
14	Leu	260 nmol
15	Gln,Glu	120, 70 nmol
16	Glu	100 nmol
17	Gly	70 nmol
18	Ala	40 nmol
	Peptide V	
1	Gly	2.25 μmol
2	Lys	1.75 μmol
3	Asp	2.00 μmol
4	Leu	1.75 μmol
5	Met	1.50 μmol
6	Glu	1.10 μmol
7	Lys	1.20 μmol
8	Val	1.20 μmol
9	Lys	0.85 μmol
10	Ser	0.20 μmol

[a] Degradation experiments carried out by P. Keim and R. Heinrikson.

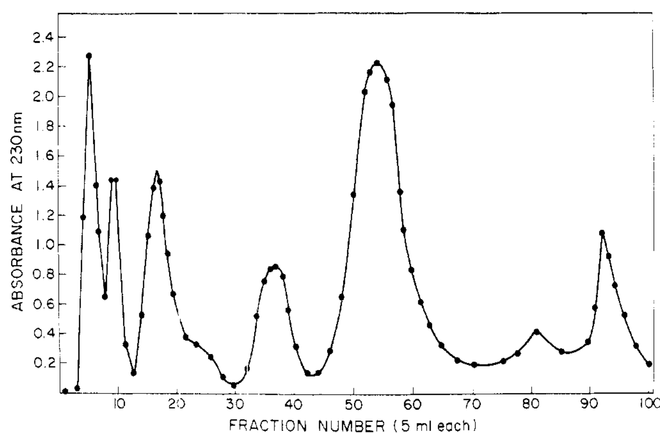

Figure 3. Ion-exchange chromatography of peptide III; DEAE Sephadex A-25 column, using 0.01 M–0.40 M $NH_4^+HCO_3^-$.

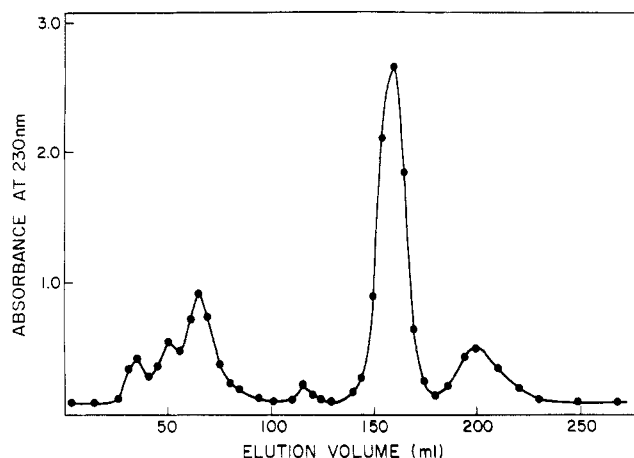

Figure 4. Ion-exchange chromatography of peptide V; 1.8 × 32 cm CM-Sephadex C-25 column employed, using 0.02 M phosphate at pH 7.0 with 0–0.4 M NaCl gradient.

well separated. The products isolated from these two fractions had essentially the same amino acid analysis. Analysis after basic hydrolysis established that neither product contained methionine sulfoxide, a possible side product of the synthesis. The component eluting from the column first (IVa) was found to be appreciably more soluble in dilute acetic acid than the other product (IVb), accounting for the partial solubility that was observed in the gel chromatography of the crude peptide. Peptide IVa was further purified by partition chromatography on Sephadex LH-20. The major peptide fraction (86%) eluted near the void volume and was followed by several smaller peaks. Edman sequence analysis indicated that this product was not homogeneous (previews began appearing at cycle 9), but most of it had the correct amino acid sequence. It should be noted that since the amino terminus of the peptide was acetylated, the reaction with phenyl isothiocyanate could not be performed directly on the peptide; instead, the peptide was cleaved at the methionine residue with cyanogen bromide and the 18-residue fragment was sequenced. A final purification of peptide IVa was effected with a sodium chloride gradient ion-exchange chromatography in which two minor impurities were separated from the main product. Peptide IVb separated into several components under the same column conditions implying that IVb is a mixture of side products. The purified peptide IVa exhibited a single band in high voltage paper electrophoresis (pH 6.4, 3000 V for 50 min) with an R_f of 0.14 relative to aspartic acid. On TLC on silica gel, the peptide showed one ninhydrin positive spot with R_f values of 0.41 in 15:10:3:12 *n*-butyl alcohol–pyridine–acetic acid–H_2O and 0.032 in 4:1:1 *n*-butyl alcohol–acetic acid–water. The Edman degradation analysis of the cyanogen bromide fragment of IVa (Table II) confirmed its purity and covalent structure. No previews were observed and the only extra amino acid detected was Glu at cycles 5 and 15 due to the decomposition of Gln under the reaction conditions. The amino acid analysis of peptide IVa is shown in Table I.

Peptide V was purified by gel filtration and ion-exchange chromatography. In preliminary pH step chromatography, the main component of the product isolated from the gel filtration step was found to be eluted at around pH 7.5. Based on this observation, a cation exchange column at pH 7.0 employing sodium chloride gradient elution was chosen to purify peptide V. The resulting elution profile is shown in Figure 4. The isolated yield of the main component was 42% of the amount of material which was applied to the chromatographic column. This product displayed a single spot on TLC with several solvent systems: 4:1:1 *n*-butyl alcohol–acetic acid–water on cellulose, R_f 0.11; 15:10:3:12 *n*-butyl alcohol–pyridine–acetic acid–water, R_f 0.35; and 50:30:15 pyridine–acetic acid–water on silica gel, R_f 0.69. Only two fluorescent spots, corresponding to the dansyl derivatives of Gly and ϵ-Lys, were observed in dansyl end group analysis of purified peptide V. The amino acid analysis of the product is shown in Table I. Finally, an Edman sequence determination established the structure and homogeneity of peptide V (Table II).

Circular Dichroism Studies. As mentioned in the introductory section, it has been proposed that amphipathic helices are responsible for the lipid binding properties of apolipoproteins. Peptides I–V were chosen for synthesis because of their correspondence to regions that were predicted to have this type of structure. Accordingly, it was desirable to determine if they existed in a helical conformation in solution. Greenfield and Fasman have shown that circular dichroism can be used to evaluate protein conformation.[25a,b] Using synthetic polypeptides, they observed that α-helical structures display characteristic negative extrema at 208 and 222 nm in their CD spectra and they developed a method for calculating an approximate α-helical content using the mean residue ellipticities at these two wavelengths. Although polypeptides may not provide the best models for analyzing the CD spectra of proteins,[26,27] due in part to the smaller length of helices in proteins, the chain length dependent factor discussed in ref 26 introduces only a small correction for helices of over 20 amino acids. The percentages of helical structure of the peptides described in the present report were estimated from their $[\theta]_{222nm}$ values using the equation: % α helix = $([\theta]_{222nm} + 3000)/(36000 + 3000)$.

The circular dichroism spectra of peptides I, III, IV, and V were measured in the pH range 4–9 and in the presence of organic solvents. Organic solvents such as chloroethanol and trifluoroethanol are known to induce α-helix conformation preferentially.[28a,b] The spectra of peptides I and V between 250 and 200 nm were typical of a peptide that had predominantly random structure. In the presence of a high concentration of chloroethanol they appeared to have only a slight amount of helical structure. Perhaps these two peptides are too small to attain a significantly ordered structure, particularly since they have free end groups, and solvation may interfere with α-helix formation. Peptides III and IV also display mostly random structure throughout the pH 4–9 range; however, a helical contribution of 10–15% was calculated from their CD spectra. Upon addition of trifluoroethanol the spectra changed dramatically (definite troughs at 208 and 222 appeared (Figure 5)). Calculation of the percentage of α helix of peptide IV in 50% trifluoroethanol gave a value of 33%. Peptide III contained 35% helical structure in 25% trifluoroethanol and 40% helical structure in 50% of this solvent. The

Table III

Step	Reagent	Applications	Time, min
1	CH_2Cl_2 wash	2 × 40 mL	1
2	1:1 TFA–CH_2Cl_2	1 × 25 mL	1
3	1:1 TFA–CH_2Cl_2	1 × 40 mL	30
4	CH_2Cl_2 wash	3 × 40 mL	1
5	2-Butanol wash	1 × 25 mL	1
6	CH_2Cl_2 wash	2 × 25 mL	1
7	10% TEA or DIEA in $CHCl_3$	1 × 40 mL	4
8	CH_2Cl_2 wash	4 × 25 mL	1
9	Boc-amino acid in CH_2Cl_2 [a]		1
10	DCC in CH_2Cl_2 [b]		120–360
11	CH_2Cl_2 wash	2 × 25 mL	1
12	DMF wash	1 × 25 mL	1
13	CH_2Cl_2 wash	3 × 25 mL	1

[a] A 2.5-fold molar excess of amino acid derivative in about 15 mL of CH_2Cl_2 was used. The derivatives of lysine and arginine were dissolved in a small amount of DMF and then diluted with CH_2Cl_2. [b] A 2.5-fold molar excess in 5 mL of CH_2Cl_2 was employed.

two longer peptides are capable of forming an α helix, and possibly, in the presence of lipids, an increase in the percent helicity will be observed as is seen for the whole protein.

Conclusion. This report has described the solid-phase synthesis and purification of five peptide fragments of apolipoproteins for use in studies to determine the structural characteristics which impart to these proteins their surface properties, as well as their ability to bind lipids. The synthetic methods employed produced crude peptides in good yields, but for the preparation of peptides containing about 20 amino acid residues, extensive purification was necessary to isolate products substantially free of impurities. In the synthesis of larger peptide segments of the apolipoproteins it may be advisable to use methods involving the condensation of purified peptide fragments to obtain products of sufficient purity for structure-activity investigations. Work is continuing in this laboratory on the preparation of other peptide fragments of apolipoproteins A-I and A-II and on defining the physical and chemical properties of the synthetic peptides.

Experimental Section

Materials and Methods. Dichloromethane and chloroform from Burdick and Jackson Laboratories were distilled from phosphorus pentoxide. Triethylamine (Eastman) and *N,N*-diisopropylethylamine (Aldrich) were distilled from ninhydrin and then redistilled from calcium hydride. Trifluoroacetic acid obtained from Aldrich was distilled within 4 days of being used. Dimethylformamide (DMF) (Fisher Scientific) was purified by codistillation with dry benzene,[29] followed by distillation from ninhydrin under reduced pressure. The purified DMF was stored under nitrogen at 4 °C over molecular sieves. *sec*-Butyl alcohol from Matheson, Coleman and Bell was fractionally distilled. Dicyclohexylcarbodiimide from Aldrich was vacuum distilled.

Chloromethylated styrene–divinylbenzene copolymer (1% cross-linked) was obtained from Pierce and Bachem. *tert*-Butoxycarbonyl-L-amino acid derivatives were purchased from Bachem. These derivatives were as follows: L-alanine, N^g-nitro or tosyl-L-arginine, L-aspartic acid β-benzyl ester, N^{im}-tosyl-L-histidine, *O*-benzyl-L-serine, *O*-benzyl-L-threonine, *O*-2,6-dichlorobenzyl-L-tyrosine, ϵ-2-chlorobenzyloxycarbonyl-L-lysine, L-glutamic acid γ-benzyl ester, L-glutamine *p*-nitrophenyl ester, glycine, L-leucine, L-methionine, L-proline, and L-valine.

Sephadex G-10, DEAE-Sephadex A-25, CM-Sephadex C-25, and Sephadex LH-20 were purchased from Pharmacia.

Ninhydrin, 1-hydroxybenzotriazole, cyanogen bromide, trifluoroethanol, and 2-mercaptoethanol were obtained from Aldrich. Dithiothreitol was purchased from Sigma. Solvents for the peptide synthesis were stored over Linde 4A molecular sieves (Union Carbide). Buffers were prepared from tris(hydroxymethyl)aminomethane (Eastman) and from sodium phosphate (Fisher Scientific). Boiled, deionized water was used to prepare buffer solutions.

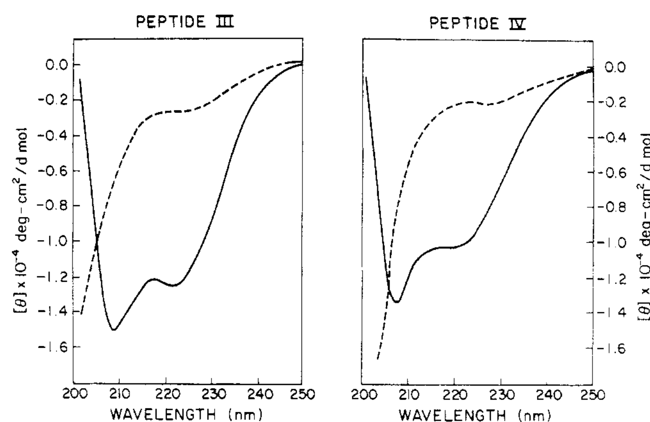

Figure 5. Circular dichroism spectra of peptides III and IV. Solid line represents spectra in 50% trifluoroethanol. For peptide III the dashed line shows the spectrum at pH 7.0 and for peptide IV it illustrates the spectrum at pH 7.5.

The optical densities of column fractions were measured with a Gilford Spectrophotometer. Circular dichroism spectra were recorded with a Cary 60 spectropolarimeter. Amino acid analyses were performed on a Beckman Spinco Model 121 amino acid analyzer. Measurements of pH were made with a Beckman Model 3500 digital pH meter.

The amino acid sequences of the peptides were determined by the method of Edman and Begg[30] using automatic techniques similar to those described by Niall[31] with a Beckman Model 890-C protein-peptide sequencer. The phenylthiohydantoins were identified and quantitated by gas chromatography. Peptide IV was reacted with a large excess of cyanogen bromide in 70% formic acid for 24 h prior to sequencing. At the end of this period the reaction mixture was added to a large volume of water and lyophilized, and the residue was gel filtered to obtain the cleaved peptide.

Amino end group analyses by dansylation were done using the procedures of Gray.[32] The dansyl derivative products were chromatographed on polyamide TLC sheets (Cheng Chin) developed with 100:1.5 water–formic acid in the first dimension, 9:1 benzene–acetic acid in the second dimension, and in the same direction with 20:1:1 ethyl acetate–acetic acid–methanol.

The purity of the peptides was also assessed by thin-layer chromatography using silica gel and cellulose TLC sheets obtained from Eastman Chemicals.

High-voltage paper electrophoresis experiments were carried out with a Savant Flat plate electrophoresis apparatus and 5000 V power supply. The buffers used were pyridine–acetic acid, pH 3.5 and 6.4. The samples were spotted on Whatman 3M chromatography paper along with standard amino acids.

The peptides described in this article were synthesized with the aid of a Beckman Model 990 automated peptide synthesizer. Chloromethylated styrene–divinylbenzene copolymer was esterified with the appropriate Boc-amino acid derivative by the method of Marglin.[33] Substitution levels were determined by amino acid analysis after hydrolysis with 1:1 12 N HCl–propionic acid[34] and were found to be in the range 0.05–0.23 mmol/g. The amino acid substituted resin was placed in the reaction vessel of the peptide synthesizer which executed the programmed sequence of reaction and washing steps shown in Table III. For active ester coupling, a fourfold molar excess of glutamine *p*-nitrophenyl ester and an equivalent of 1-hydroxybenzotriazole in DMF solution were added at the coupling step, the addition being preceded by washing the resin twice with DMF. Addition of DCC was omitted. In the symmetric anhydride coupling procedure, step 10 was also omitted and in step 9 a freshly prepared solution of Boc-amino acid anhydride (threefold excess, prepared as in ref 35) was added. The coupling was allowed to proceed for 45 min, then 1.5 equiv of DIEA was added and stirring was continued for another 15 min, followed by washing as usual. The completeness of the coupling reaction was determined by the use of the ninhydrin color test.[16] When a positive test was observed, the coupling reaction (step 9–13) was repeated. After completion of the synthesis, the peptide resin was washed with methanol and dichloromethane and finally vacuum dried.

The peptides were cleaved from the polymer support by reaction with hydrofluoric acid.[36] The apparatus and procedure for the cleavage have been described previously.[37] After washing the cleaved peptide and resin mixture with ethyl acetate to remove HF and ani-

sole, the peptide was extracted with about 50 mL of 10% acetic acid and the extracts were lyophilized to obtain the crude peptide.

Purification of Peptides. Peptide I. The crude peptide obtained from HF cleavage was gel filtered through a 2.0 × 41 cm Sephadex G-10 column, eluted with 0.2 M aqueous acetic acid. The fractions with a high-UV absorbance at 230 nm just following the void volume were pooled and lyophilized to give 616 mg of solid material. A portion of this product (138 mg) was loaded onto a 1.8 × 32 cm column of CM-Sephadex C-25 cation exchange resin equilibrated with 0.02 M phosphate buffer at pH 6.4. Elution was begun with this buffer at a rate of 50 mL/h; 5-mL fractions were collected. After 40 fractions had been collected, a sodium chloride gradient of 0–0.25 M over a volume of 400 mL was begun. The fractions were analyzed by their optical density at 230 nm. An elution profile is shown in Figure 2. Fractions 82–94 were combined, reduced in volume by rotary evaporation, and desalted on the Sephadex G-10 column. Lyophilization of the salt-free, peptide-containing fractions yielded 48 mg of fluffy white solid.

Peptide II. Lyophilization of the acetic acid extracts of the product of the HF cleavage reaction yielded 308 mg of solid. This material was passed through a 2.0 × 40 cm column of Sephadex G-10 with 0.2 N acetic acid. The fractions containing the major portion of the UV-absorbing material were lyophilized to give 234 mg of peptide. Part of this product (121 mg) was dissolved in a few milliliters of 0.02 M phosphate buffer, pH 7.5, and applied to a CM-Sephadex C-25 cation exchange resin column (1.8 × 32 cm), equilibrated with the same buffer. The column was eluted with 100 mL of the buffer. Then, a sodium chloride gradient solution was begun using a mixing chamber containing 300 mL of the 0.02 M phosphate buffer and a reservoir containing 300 mL of the same buffer but which was 0.25 M in NaCl. Fractions of 5 mL were collected and their absorbance at 230 nm was measured. The major component came off the column in fractions 120–143. The contents of the tubes containing these fractions were combined and reduced to about 6 mL in volume by rotary evaporation. This solution was desalted with a 2.5 × 42 cm column of Sephadex G-10. The lyophilized product which weighed 25 mg was combined with 15 mg of material obtained from another similar column chromatographic run, and the combined material was further purified by chromatography on a 1.8 × 32 cm column of CM-Sephadex C-25 with a pH step elution. After application of the peptide, the column was eluted with 100 mL each of 0.04 M sodium phosphate buffers at pH 6.5, 7.4, and 8.0, followed by elution with 0.10 M phosphate at pH 8.0. Five-milliliter fractions were collected. The contents of tubes 85–95 yielded 24.8 mg of peptide after desalting with a Sephadex G-10 column and lyophilization.

Peptide III. The amount of material obtained from the HF cleavage reaction was approximately 800 mg. The crude peptide was dissolved in 10 mL of water containing 0.12 g of dithiothreitol, and the solution was allowed to stand at 4 °C overnight. The solution then was applied to a 3.8 × 50 cm column of Sephadex G-10 and eluted with 0.2 M acetic acid containing 0.004 M 2-mercaptoethanol. The eluate with an optical density greater than 1.0 at 240 nm was lyophilized, yielding 747 mg of an off-white powder. Two portions of this material (282-mg total) were chromatographed on a 1.8 × 34 cm column of DEAE-Sephadex-A-25 anion exchange resin with an ammonium bicarbonate gradient of 0.01–0.40 M/600 mL. The slope of the gradient was increased after 400 mL of eluate had been collected. Those fractions of both column runs which contained the major peaks were combined, reduced in volume, and desalted on a Sephadex G-10 column. Lyophilization of the peptide fraction produced 97.4 mg of solid. This product (96.6 mg) was dissolved in a few milliliters of 0.05 M Tris buffer at pH 8.2 and added to a 1.8 × 33 cm DEAE-Sephadex A-25 column that had been equilibrated with the same buffer. A pH gradient elution was applied using a mixing chamber containing 250 mL of 0.05 M Tris buffer at pH 8.2 and a reservoir containing 250 mL of 0.05 M Tris buffer at pH 7.0. From a center cut of the main peak fraction, 53 mg of peptide was obtained after desalting.

Peptide IV. The crude, cleaved peptide (957 mg) was divided into two portions for gel chromatography. The crude material was found to be only partially soluble in dilute acetic acid, but it dissolved readily when the solvent was made slightly basic with ammonium hydroxide. The peptide was gel filtered through a 2.8 × 40 cm column of Sephadex G-10, eluting with this solvent and analyzing the fractions by their absorbances at 280 and 230 nm. Approximately 225 mg of the product of the gel-filtration step was chromatographed on a 1.8 × 42 cm column of DEAE-Sephadex A-25, using a gradient elution of 0.10–1.0 M ammonium bicarbonate. The fractions corresponding to the two largest peaks of the elution profile (A_{280}) were lyophilized. The peptide fraction from the earlier eluting material (73 mg) was subjected to partition chromatography on a 2.4 × 54 cm column of Sephadex LH-20 using 50% aqueous acetone containing 0.05 M pyridine as the eluent. Fractions of 4 mL were collected and analyzed for their peptide content by the ninhydrin reaction. Peptide IV was further purified by a sodium chloride gradient ion-exchange chromatography. A 1.8 × 31 cm column of DEAE-Sephadex A-25 was equilibrated with 0.05 M Tris at pH 8.4 and eluted using a mixing chamber containing 300 mL of the same buffer and a reservoir containing 300 mL of the buffer that was 0.60 M in sodium chloride. The fractions corresponding to the major peak of the elution profile were combined, reduced in volume, and desalted on a Sephadex G-10 column using 10% acetic acid as the eluent. Lyophilization of the peptide fractions yielded 48.8 mg of product.

Peptide V. Crude peptide V was gel filtered on a 2.5 × 42 cm column of Sephadex G-10 with 0.1 M acetic acid containing 4 mM 2-mercaptoethanol as the eluent. Several fractions eluting after the void volume were collected and lyophilized to obtain the peptide. Purification of a portion of this material (70.8 mg) was accomplished by chromatography on a 1.8 × 32 cm CM-Sephadex C-25 column eluted at pH 7.0 (0.02 M phosphate) with a sodium chloride gradient of 0–0.4 M over 300 mL. The fractions containing most of the main product were pooled, reduced in volume by rotary evaporation, and eluted through the Sephadex G-10 column. Lyophilization of the desalted peptide solution yielded 29.7 mg of white solid.

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Registry No.—I, 65452-43-9; II, 65392-53-2; III, 65392-52-1; IV, 65452-62-2; V, 65392-51-0.

References and Notes

(1) For a review on the properties of serum lipoproteins see A. M. Scanu, C. Edelstein, and P. Keim in "The Plasma Proteins: Structure, Function and Genetic Control", Vol. 1, F. Putnam, Ed., Academic Press, New York, N.Y., 1975, pp 317–391.
(2) H. N. Baker, A. M. Gotto, and R. L. Jackson, *J. Biol. Chem.*, **250**, 2725 (1975).
(3) H. B. Brewer, Jr., S. E. Lux, R. Ronan, and K. M. John, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 1304 (1972).
(4) R. Shulman, P. Herbert, K. Wehrly, B. Chesebro, R. I. Levy, and D. S. Fredrickson, *Circulation*, **45(2)**, 246 (1972).
(5) H. B. Brewer, Jr., R. Shulman, P. Herbert, R. Ronan, and K. Wehrly, *J. Biol. Chem.*, **249**, 4975 (1974).
(6) R. L. Jackson, H. N. Baker, E. B. Gilliam, and A. M. Gotto, Jr., *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 1942 (1977).
(7) P. Y. Chou and G. D. Fasman, *Biochemistry*, **12**, 211, 222 (1974).
(8) S. E. Lux, R. Hirz, R. I. Shrager, and A. M. Gotto, *J. Biol. Chem.*, **247**, 2598 (1972).
(9) J. P. Segrest, R. L. Jackson, J. D. Morrisett, and A. M. Gotto, Jr., *FEBS Lett.*, **38**, 247 (1974).
(10) J. T. Sparrow, A. M. Gotto, Jr., and T. D. Morrisett, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2124 (1974).
(11) W. Fitch, *Genetics*, in press.
(12) A. D. McLachlan, *Nature (London)*, **267**, 465 (1977).
(13) G. Assman and H. B. Brewer, Jr., *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 989 (1974).
(14) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
(15) For a recent comprehensive review see: B. W. Erickson and R. B. Merrifield in "The Proteins", Vol. II, 3rd ed, H. Neurath, R. Hill, and C. L. Boeder, Ed., Academic Press, New York, N.Y., 1976.
(16) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).
(17) F. Flor, C. Birr, and T. Wieland, *Justus Liebigs Ann. Chem.*, 1601 (1974).
(18) F. Weygand, P. Haber, and K. Weiss, *Z. Naturforsch., B*, **22**, 1084 (1967).
(19) D. Yamashiro and R. L. Noble, as cited in J. Blake and C. H. Li, *Int. J. Peptide Protein Res.*, **8**, 589 (1976).
(20) (a) W. Konig and R. Geiger, *Chem. Ber.*, **103**, 788 (1970); (b) *ibid.*, **103**, 2024 (1970).
(21) G. C. Windridge and E. C. Jorgensen, *J. Am. Chem. Soc.*, **93**, 6318 (1971).
(22) M. Rothe and J. Mazanek, *Justus Liebigs Ann. Chem.*, 439 (1974).
(23) B. F. Gisin and R. B. Merrifield, *J. Am. Chem. Soc.*, **94**, 3102 (1972).
(24) W. W. Cleland, *Biochemistry*, **3**, 480 (1964).
(25) (a) N. Greenfield and G. D. Fasman, *Biochemistry*, **8**, 4108 (1969); (b) A.

J. Adler N. J. Greenfield, and G. D. Fasman, *Methods Enzymol.*, **27**, 675 (1974).
(26) Y.-H. Chen, J. T. Yang, and K. H. Chau, *Biochemistry*, **13**, 3350 (1974).
(27) C. C. Baker and I. Isenberg, *Biochemistry*, **15**, 629 (1976).
(28) (a) M. Goodman, F. Naider, and R. Rapp, *Bioorg. Chem.*, **1**, 310 (1971); (b) see also: P. Marche, J.-L. Morgat and P. Fromageot, *Eur. J. Biochem.*, **40**, 513 (1973).
(29) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis", W. H. Freeman, San Francisco, Calif., 1969.
(30) P. Edman and G. Begg, *Eur. J. Biochem.*, **1**, 80 (1967).
(31) H. D. Niall, *Methods Enzymol.*, **27**, 942 (1973).
(32) W. R. Gray, *Methods Enzymol.*, **25**, 121 (1972).
(33) A. Margin, *Tetrahedron Lett.*, **33**, 3145 (1971).
(34) F. C. Westall, J. Scotchler, and A. D. Robinson, *J. Org. Chem.*, **37**, 3363 (1972).
(35) D. Yamashiro and C. H. Li, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 4945 (1974).
(36) These cleavages were performed by Mr. Jim Geever of the Armour Pharmaceutical Co.
(37) N. H. Tan and E. T. Kaiser, *J. Org. Chem.*, **41**, 2787 (1976).

Examples of Amino Acid Transaminations with *o*-Formylbenzoic Acid

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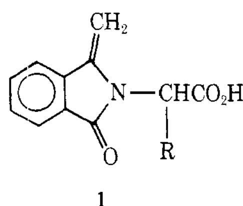
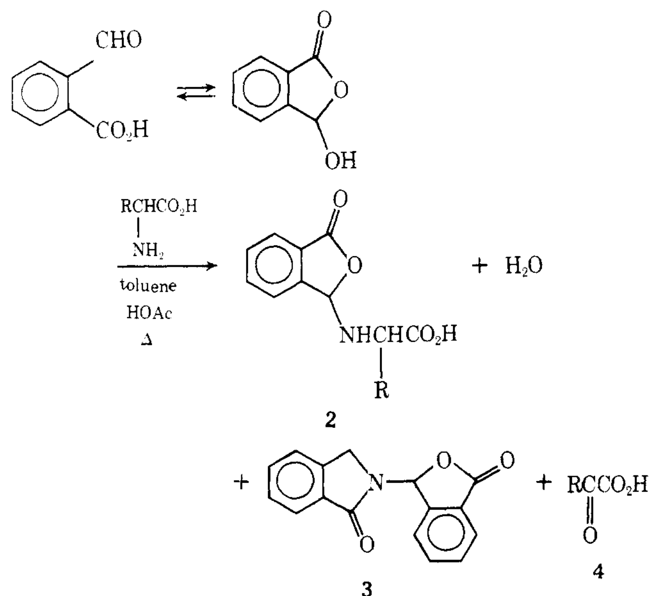
The present paper describes an unusual example of a nonenzymatic transamination of two amino acids by a non-pyridoxal-type carbonyl compound. During the course of some recent research on the interaction of certain *o*-carbonylbenzoic acids with amino acids, *o*-formylbenzoic acid was found to undergo transamination reactions with L-glutamic acid and L-alanine in the presence of acetic acid and toluene. The products of this novel reaction are an α-ketocarboxylic acid (**4**) and *N*-(3-phthalidyl)phthalimidine (**3**). Structural proof of the products was based on derivative formation, spectral and elemental analyses, and synthesis. α-Amino-*o*-toluic acid (**6**) was a probable intermediate in the transformation. Apparently, *o*-formylbenzoic acid condensed with **6** or with phthalimidine (**5**) to form product **3**. With L-glutamic acid, an additional product (the anhydride of **2**, where R = $CH_2CH_2CO_2H$) was obtained which resulted from a simple condensation of starting materials.

In a recent publication,[2] *o*-acetylbenzoic acid was reported to condense readily with various amino acids and produce 3-methylenephthalidylamino acids (**1**).

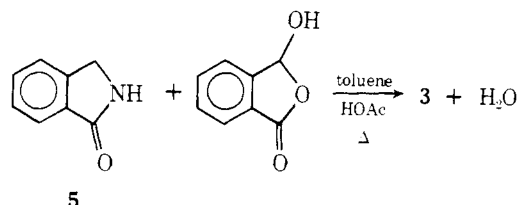


1

When *o*-formylbenzoic acid was treated with L-glutamic acid, the expected product, **2** (R = $CH_2CH_2CO_2H$), was formed, but only in 19% yield. However, other products were obtained which indicated that transamination had occurred: **3** in 19% yield and **4** (R = $CH_2CH_2CO_2H$) in 17% yield as the semicarbazone derivative. The yield of product **3** was increased to 46% when dioxane was used in place of toluene. Transamination was apparently the only reaction when L-alanine and L-phenylalanine were each treated with *o*-formylbenzoic acid. None of the condensation product, **2**, was obtained with these amino acids. In the reaction of L-alanine with *o*-formylbenzoic acid, product **3** was formed in 66% yield and a crystalline phenylhydrazone derivative of pyruvic acid (**4**, R = CH_3) was isolated in 19% yield.



The structure of the base-insoluble product (**3**) was established by an unambiguous synthesis from phthalimidine[3] (**5**) and *o*-formylbenzoic acid under conditions identical to those used with the latter compound and an amino acid. The product from this experiment was isolated in 50% yield and was identical to that obtained from the transamination reaction with respect to the thin-layer chromatogram R_f value, infrared spectrum, and melting point. Similar reactions of amides with *o*-formylbenzoic acid are in the literature.[4]



The formation of α-amino-*o*-toluic acid (**6**) and the keto acid (**4**) may be rationalized by analogy with the well-established mechanism for the transamination with pyridoxal phosphate. Experiment has shown that no reaction occurs in the absence of acetic acid. In fact, a large excess of the reagent was found to afford optimum yields of products. Apparently, α-amino-*o*-toluic acid (**6**) reacts with *o*-formylbenzoic acid or its tautomer as soon as it is formed and two molecules of water are eliminated. Two equally plausible routes are possible for this transformation. In the first, **6** cyclizes to phthalimidine (**5**) which then condenses with *o*-formylbenzoic acid, a reaction which has already been demonstrated (see above). The

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