Synthesis of the Fully Protected, Carboxyl-Terminal Tetradecapeptide Sequence of Staphylococcal Nuclease

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Abstract: The carboxyl-terminal nonapeptide sequence of the extracellular nuclease of *Staphylococcus aureus* has been synthesized in a fully protected form by the stepwise elongation of the peptide chain using classical methods of condensation. This compound was then coupled to a peptide fragment containing the preceding five residues of the nuclease sequence, prepared by a modified solid phase technique, to yield a fully protected tetradecapeptide suitable for subsequent elongation by further addition of fragments to its amino terminus.

The covalent structure of an extracellular nuclease produced by *Staphylococcus aureus* has recently been reported.[1] This enzyme is a globular protein containing a single chain devoid of half-cystine, and readily gives crystals suitable for X-ray analysis.[2] When nuclease is cleaved in a limited way by trypsin in the presence of deoxythymidine-3′,5′-diphosphate and Ca^{2+} three inactive fragments are produced: fragment P_1 (residues 1–5), P_2 (residues 6–48 or 49), and P_3 (49 or 50–149). When fragments P_2 and P_3 are combined, a noncovalently bonded complex, nuclease T, results which exhibits approximately 8% of the native enzymatic activity.[3] Chemical synthesis of fragment P_2 by solid phase[4] method has been reported[5] and the classical syntheses of both P_2 and P_3 are in progress.

The proposed plan for synthesis of fragment P_3 involves the successive coupling of protected peptide fragments to the free α-amino group of a growing, protected C-terminal sequence. Each peptide fragment must be fully protected except for a free terminal carboxyl group or its corresponding hydrazide which can be activated to the azide for coupling. We have reported two modifications of the solid phase method[4] for the syntheses of protected peptides.[6,7] The methods have been useful in the syntheses of a number of protected fragments of the nuclease sequence.

The present communication reports the synthesis of the tetradecapeptide (residues 135–148) derivative of the C-terminal portion of nuclease intended as an "anchor" peptide for the synthesis of the P_3 fragment. When the native nuclease was incubated with carboxypeptidase, the resulting polypeptide chain, devoid of the glutamine residue originally located at the C terminus, exhibited enzymatic activity at essentially the same level as that of the native enzyme.[8] Therefore, the C-terminal glutamine residue of the native enzyme has been omitted from the sequence of the protected peptide described below.

The choice of protecting groups for blocking reactive groups is particularly important in the synthesis of large peptides or proteins. *t*-Butyloxycarbonyl and trifluoroacetyl[9] groups were employed for selective blocking of the α-amino group and the ϵ-amino group of lysine, respectively. The benzyl group was selected for protection of ω-carboxyls of aspartic and glutamic acid residues and of hydroxyl groups of serine and threonine residues. The *t*-butyloxycarbonyl group can be easily removed with anhydrous trifluoroacetic acid. Benzyl and trifluoroacetyl groups will be removed by treatment with anhydrous liquid HF[10] and with 1 *M* aqueous piperidine,[11] respectively, after completion of assembly of the chain through successive coupling of peptide fragments. When the stability of native nuclease was tested under the conditions for removing the above protecting groups, its enzymatic activity was stable to trifluoroacetic acid and showed only a minor loss with both anhydrous liquid HF and 1 *M* aqueous piperidine.[5]

Figure 1 summarizes the synthesis of the tetradecapeptide derivative, *t*-butyloxycarbonyl-γ-benzylglutamyl-ϵ-trifluoroacetyllysylleucylasparaginylisoleucyltryptophyl-O-benzylseryl-γ-benzylglutamylasparaginyl-β-benzylaspartylalanyl-β-benzylaspartyl-O-benzylserylglycine benzyl ester (XXIV).[12]

The protected NH_2-terminal pentapeptide (residues 135–139), *t*-butyloxycarbonyl-γ-benzylglutamyl-ϵ-trifluoroacetyllysylleucylasparaginylisoleucine (XXIII) was synthesized by the modified solid phase method described previously.[6] The partially protected tetrapeptide (XXII), prepared by the solid phase method,[5] was allowed to react with 2 equiv of *t*-butyloxycarbonyl-γ-benzylglutamic acid N-hydroxysuccinimide ester in the presence of N-methylmorpholine. Final treatment of the protected pentapeptide-N-methylmorpholine salt with citric acid solution gave the crystalline protected pentapeptide (XXIII) in a yield of 64%.

The synthesis of the protected, C-terminal nonapeptide ester (residues 140–148), *t*-butyloxycarbonyltryptophyl-O-benzylseryl-γ-benzylglutamylasparaginyl-β-

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(12) The prefix L for each amino acid residue is omitted.

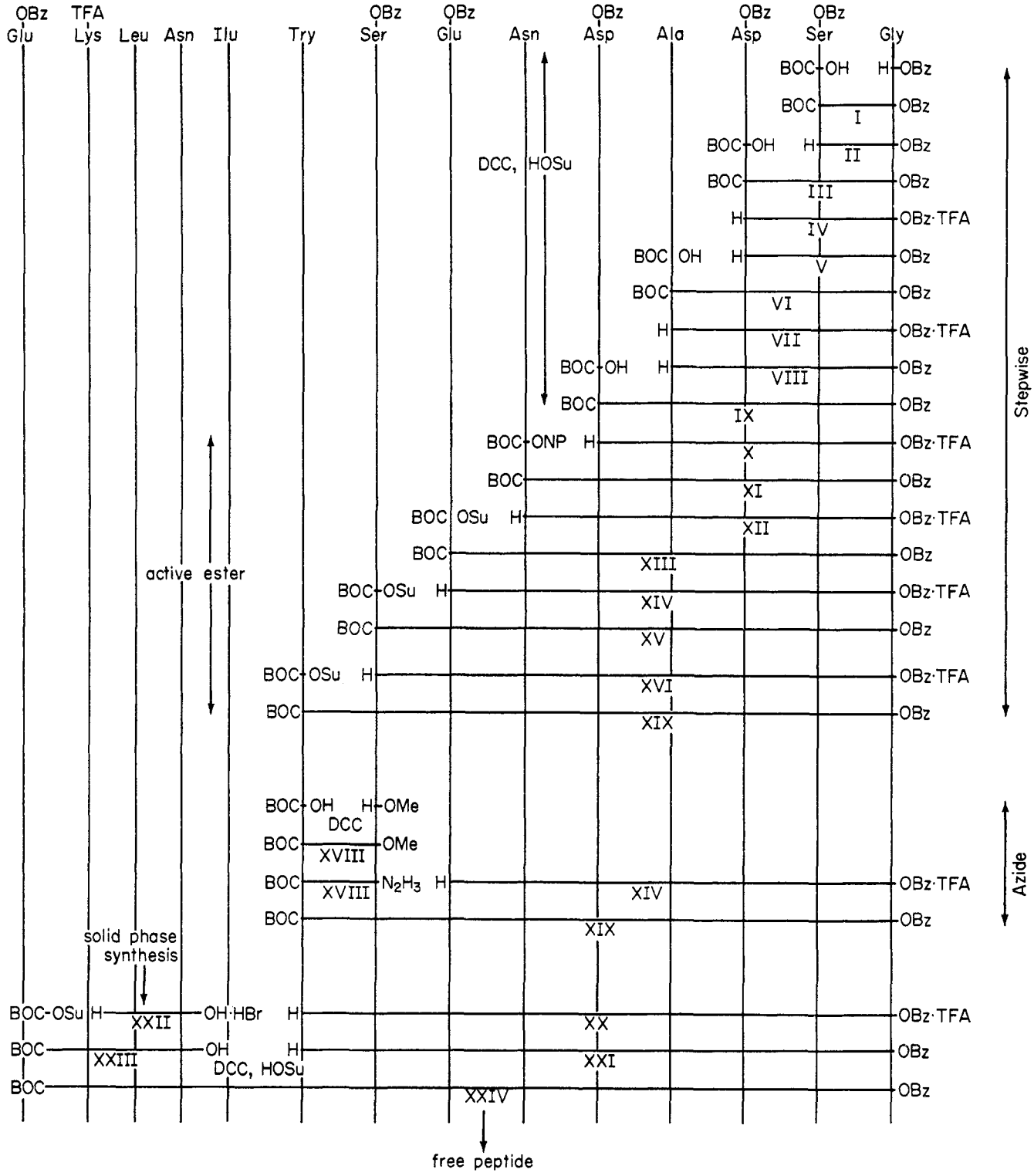

Figure 1. Scheme of the synthesis of C-terminal tetradecapeptide derivative.

benzylaspartylalanyl-β-benzylaspartyl-O-benzylserylglycine benzyl ester (XIX), was carried out by the conventional stepwise elongation procedure starting from glycine benzyl ester. The coupling procedure with N,N′-dicyclohexylcarbodiimide[13] as the condensing reagent in the presence of N-hydroxysuccinimide[14] was repeated as far as the pentapeptide (IX) stage. To simplify purification of products, 4–6% less than the equivalent amount of the appropriate *t*-butyloxycarbonyl amino acid was used for coupling to the free α-amino group of the growing C-terminal peptide ester up to the tetrapeptide level. This procedure was followed because the solubility of the unreacted portion of *t*-butyloxycarbonylamino acid N-hydroxysuccinimide ester formed during the reaction was similar to that of the product.

Active esters of the *t*-butyloxycarbonyl amino acids were employed, in the presence of base in dimethylform-

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(14) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *ibid.*, 86, 1839 (1964).

amide, for the elongation of peptide ester trifluoroacetates as long as or longer than hexapeptides. N-Hydroxysuccinimide esters[14] were used, except for the *p*-nitrophenyl ester of *t*-butyloxycarbonylasparagine. A 10–40% excess of the active esters was used in order to elevate the yields. Since the peptides were insoluble in a mixture of ether and petroleum ether (1:1 v/v), this solvent system could be used for removing the unreacted esters of BOC-amino acids by washing.

In an alternative synthesis of the protected nonapeptide, the azide derived from *t*-butyloxycarbonyltryptophyl-O-benzylserine hydrazide (XVIII) was allowed to react with the heptapeptide ester trifluoroacetate (XIV) in the presence of base. The crystalline, protected nonapeptide ester (XIX) was obtained in a yield of 76%. The fragment prepared by the azide procedure gave a slightly lower melting point than the product prepared by the stepwise elongation method. However, its countercurrent distribution, using the system of methanol–chloroform–cyclohexane–dimethylformamide–water (5:5:2:2:2 v/v), showed one peak of symmetrical shape ($K = 0.111$). Amino acid analyses were essentially theoretical.

The protected nonapeptide ester (XIX) obtained by the stepwise method was treated with anhydrous trifluoroacetic acid to afford an amorphous nonapeptide ester trifluoroacetate (XX). The ultraviolet absorption spectra of the nonapeptide derivatives before and after deprotection of the *t*-butyloxycarbonyl group with trifluoroacetic acid were indistinguishable, and it appears that the tryptophan residue is resistant to the trifluoroacetic acid treatment employed.

The protected tetradecapeptide ester (XXIV) was synthesized by the fragment condensation of the protected pentapeptide (XXIII) and the nonapeptide ester trifluoroacetate (XX) by means of the modified N,N′-dicyclohexylcarbodiimide method.[15,16] As suggested by the studies of Zimmerman and Anderson,[16] the protected pentapeptide (XXIII) was activated with N,N′-dicyclohexylcarbodiimide and N-hydroxysuccinimide to its N-hydroxysuccinimide ester prior to addition of the nonapeptide ester amine component. The crude product, contaminated with unreacted penta- and nonapeptides, was purified by silica gel column chromatography. When a mixture of methanol and ethyl acetate (1:2 v/v) was used as the developing solvent, the protected tetradecapeptide ester (XXIV) was retained at the top of the column and other materials were eluted. Extraction of the top of the silica gel column with dimethylformamide gave the tetradecapeptide (XXIV) in a yield of 48%.

Experimental Section

All melting points are uncorrected. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter. Commercially available silica gel plates (Brinkman) were used for thin layer chromatography. Peptide derivatives bearing free terminal amino groups were detected by spraying with 0.2% ninhydrin solution in 80% ethanol, followed by heating. Spots of *t*-butyloxycarbonylpeptide derivatives were stained after prior exposure to hydrogen chloride vapor for 15 min followed by heating for 5 min at 100°. Ehrlich reagent (0.5 g of *p*-dimethylaminobenzaldehyde in a mixture of 45 ml of acetone and 5 ml of concentrated hydrochloric acid) was used for detection of tryptophan-containing peptides. Amino acid analyses of peptide derivatives were carried out on samples that had been hydrolyzed with constant boiling hydrochloric acid for 20 hr in evacuated, sealed tubes at 110°.

R_f values refer to thin layer chromatography using the following solvent systems: (1) *n*-butyl alcohol–acetic acid–water (4:1:5 v/v, upper phase); (2) methanol–ethyl acetate (1:2 v/v); (3) *n*-butyl alcohol–acetic acid–pyridine–water (4:1:1:2 v/v).

***t*-Butyloxycarbonyl-O-benzylserylglycine Benzyl Ester (I).** To a solution of 5.32 g (15.8 mmol) of glycine benzyl ester *p*-toluenesulfonate in a mixture of 2.2 ml (15.8 mmol) of triethylamine and 60 ml of methylene chloride, 4.43 g (15 mmol) of *t*-butyloxycarbonyl-O-benzylserine and 0.86 g (7.5 mmol) of N-hydroxysuccinimide were added and the mixture was cooled to 0°. To this, 3.09 g (15 mmol) of N,N′-dicyclohexylcarbodiimide was added and the mixture was allowed to stir for 3 hr at 0° and then overnight at 4°. After removal of N,N′-dicyclohexylurea by filtration, the filtrate was evaporated to dryness and the residue was treated with water (40 ml) and ethyl acetate (150 ml). The ethyl acetate layer was separated and washed successively with a saturated sodium bicarbonate solution, 0.5 *M* citric acid and water, dried over sodium sulfate, and evaporated to dryness to give a pure oil: yield, 6.57 g (99%); R_f 0.92,[1] 0.97.[2]

O-Benzylserylglycine Benzyl Ester (Free Base) (II). Protected dipeptide ester (I, 6.5 g, 14.7 mmol) was dissolved in 40 ml of trifluoroacetic acid. After 20 min at room temperature, the solution was quickly evaporated nearly to dryness at room temperature. The residue was dissolved in 100 ml of methanol and 3.5 ml of triethylamine was added to neutralize the remaining trifluoroacetic acid. The solution was evaporated and the residue was treated with water (50 ml) and ethyl acetate (100 ml). The ethyl acetate layer was separated and washed with a saturated sodium bicarbonate solution and with water, dried over sodium sulfate, and evaporated to afford a light yellow oil: yield, 4.45 g (89%); R_f 0.31,[1] 0.63.[2]

***t*-Butyloxycarbonyl-β-benzylaspartyl-O-benzylserylglycine Benzyl Ester (III).** To a stirred and cooled (0°) solution of 4.04 g (12.5 mmol) of *t*-butyloxycarbonyl-β-benzylaspartate, 4.45 g (13 mmol) of II, and 0.81 g (7 mmol) of N-hydroxysuccinimide in 50 ml of methylene chloride, 2.57 g (12.5 mmol) of N,N′-dicyclohexylcarbodiimide was added and the mixture was stirred for 3 hr at 0° and then overnight at 4°. After removal of deposited N,N′-dicyclohexylurea, the filtrate was evaporated nearly to dryness. The residue was dissolved in ethyl acetate (100 ml) and the solution was washed successively with saturated sodium bicarbonate solution, 0.5 *M* citric acid, and water, dried over sodium sulfate, and evaporated to give a light yellow oil: yield, 8.29 g (92%); R_f 0.89,[1] 0.90.[2]

β-Benzylaspartyl-O-benzylserylglycine Benzyl Ester Trifluoroacetate (IV). Protected tripeptide ester (III, 8.2 g, 11.4 mmol) was dissolved in 60 ml of trifluoroacetic acid and the solution was allowed to stand for 20 min at room temperature. The solution was then evaporated to dryness (below 40°) and the residue was triturated with ether: yield, 6.84 g (86%); mp 158–159°; $[\alpha]^{20}D$ +9.9° (*c* 1.62, dimethylformamide); R_f 0.59.[1]

Anal. Calcd for $C_{32}H_{34}O_9N_3F_3$: N, 6.34. Found: N, 6.55.

β-Benzylaspartyl-O-benzylserylglycine Benzyl Ester (Free Base) (V). A solution of 6.68 g (9.6 mmol) of IV in a mixture of 1.6 ml of triethylamine and 80 ml of methanol was evaporated nearly to dryness at 30°. The residue was dissolved in ethyl acetate (100 ml) and the solution was washed with a saturated sodium bicarbonate solution and water, dried over sodium sulfate, and evaporated to dryness to afford a light yellow oil: yield 5.6 g (100%); R_f 0.38,[1] 0.68.[2]

***t*-Butyloxycarbonylalanyl-β-benzylaspartyl-O-benzylserylglycine Benzyl Ester (VI).** To a stirred and cooled solution of 1.70 g (9 mmol) of *t*-butyloxycarbonylalanine, 5.6 g (*ca.* 9.6 mmol) of V, and 0.58 g (5 mmol) of N-hydroxysuccinimide in 50 ml of methylene chloride, 1.85 g (9 mmol) of N,N′-dicyclohexylcarbodiimide was added and the mixture was allowed to stir for 3 hr at 0° and then overnight at 4°. After removal of N,N′-dicyclohexylurea by filtration, the filtrate was evaporated and the residue was dissolved in ethyl acetate (120 ml) with gentle warming. The solution was washed successively with a saturated sodium bicarbonate solution, 0.5 *M* citric acid and water, dried over sodium sulfate, and evaporated. The crystalline residue was triturated with ether and petroleum ether (bp 30–60°) (1:1) and filtered off (5.8 g). The recrystallization from ethyl acetate–ether gave a pure product: yield, 5.11 g (79%); mp 107–108°; $[\alpha]^{20}D$ −12.1° (*c* 1.13, dimethylformamide); R_f 0.88,[1] 0.88.[2]

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Anal. Calcd for $C_{38}H_{46}O_{10}N_4$: C, 63.49; H, 6.39; N, 7.79. Found: C, 63.46; H, 6.21; N, 7.71.

Alanyl-β-benzylaspartyl-O-benzylserylglycine Benzyl Ester Trifluoroacetate (VII). Protected tetrapeptide ester (VI, 4.17 g, 5.8 mmol) was treated with 20 ml of trifluoroacetic acid for 20 min at room temperature. After evaporation, the residue was triturated with ether and the crystals were collected by filtration: yield, 4.2 g (99%); mp 112–114°; $[\alpha]^{20}D$ −0.7° (*c* 1.34, dimethylformamide).

Anal. Calcd for $C_{35}H_{39}O_{10}N_4F_3$: N, 7.65. Found: N, 7.78.

Alanyl-β-benzylaspartyl-O-benzylserylglycine Benzyl Ester (Free Base) (VIII). Tetrapeptide benzyl ester trifluoroacetate (VII, 4.3 g, 5.7 mmol) was dissolved in 100 ml of methanol and 1.0 ml of triethylamine was added, and the solution was evaporated to dryness at 30°. The residue was dissolved in 150 ml of ethyl acetate and the solution was washed with a saturated sodium bicarbonate solution and water, dried over sodium sulfate, and evaporated. The residue crystallized quickly. The crystals were collected by filtration with ether and petroleum ether (1:1): yield, 3.26 g (93%); mp 116–118°; $[\alpha]^{20}D$ −7.0° (*c* 0.63, dimethylformamide); R_f 0.63,[1] 0.52.[2]

Anal. Calcd for $C_{33}H_{38}O_8N_4$: C, 64.07; H, 6.13; N, 9.04. Found: C, 63.74; H, 6.41; N, 8.94.

***t*-Butyloxycarbonyl-β-benzylaspartylalanyl-β-benzylaspartyl-O-benzylserylglycine Benzyl Ester (IX).** To a stirred and cooled solution of 1.82 g (5.61 mmol) of *t*-butyloxycarbonyl-β-benzylaspartate, 3.16 g (5.11 mmol) of VIII, and 0.35 g of N-hydroxysuccinimide in 60 ml of methylene chloride, 1.06 g (5.11 mmol) of N,N′-dicyclohexylcarbodiimide was added. The reaction and isolation were carried out by the same procedure as described in the preparation of VI: yield, 4.28 g (91%); mp 129–131°; $[\alpha]^{20}D$ −17.5 (*c* 1.00 MeOH); R_f 0.93,[1] 0.94.[2]

Anal. Calcd for $C_{49}H_{57}O_{13}N_5$: C, 63.69; H, 6.17; N, 7.57. Found: C, 63.75; H, 6.33; N, 7.47.

β-Benzylaspartylalanyl-β-benzylaspartyl-O-benzylserylglycine Benzyl Ester Trifluoroacetate (X). Protected pentapeptide ester (IX, 3.42 g, 3.70 mmol) was treated with 20 ml of trifluoroacetic acid for 20 min at room temperature. After evaporation the residue was triturated with ether–petroleum ether (6:4) and the crystals were filtered off: yield, 3.43 g (98%); mp 166–168°; $[\alpha]^{20}D$ −6.2° (*c* 1.11, dimethylformamide); R_f 0.45,[1] 0.70,[2] 0.76.[3]

Anal. Calcd for $C_{46}H_{50}O_{13}N_5F_3$: N, 7.46. Found: N, 7.71.

***t*-Butyloxycarbonylasparaginyl-β-benzylaspartylalanyl-β-benzylaspartyl-O-benzylserylglycine Benzyl Ester (XI).** To a cooled solution of 3.38 g (3.61 mmol) of X in a mixture of 0.52 ml (3.7 mmol) of triethylamine and 15 ml of dimethylformamide, 1.66 g (4.7 mmol) of *t*-butyloxycarbonylasparagine *p*-nitrophenyl ester was added and the reaction mixture was allowed to stir for 2 hr at 0° and then overnight at 4°. The solution was then diluted with ethyl acetate (200 ml) and was washed successively with a saturated citric acid solution (three times), a saturated sodium bicarbonate solution (three times), and water (to neutrality). During the washing a gelatinous precipitate appeared. The ethyl acetate layer, containing the precipitate, was separated and evaporated to give crystals which were collected by filtration and washed thoroughly with ether–petroleum ether (1:1, 3.41 g). A single spot was obtained on thin layer chromatography. Recrystallization from methanol–ether–petroleum ether gave 3.30 g (86%) of pure product: mp 197–199°; $[\alpha]^{20}D$ −15.6° (*c* 0.9, dimethylformamide); R_f 0.91,[1] 0.90.[2]

Anal. Calcd for $C_{53}H_{63}O_{15}N_7$: C, 61.32; H, 6.07; N, 9.45. Found: C, 61.44; H, 6.08; N, 9.63.

Asparaginyl-β-benzylaspartylalanyl-β-benzylaspartyl-O-benzylserylglycine Benzyl Ester Trifluoroacetate (XII). Protected hexapeptide ester (XI, 3.22 g, 3.12 mmol) was treated with 20 ml of trifluoroacetic acid for 20 min at room temperature. After evaporation the residue was triturated with ether–petroleum ether (6:4). The fine crystals were collected by filtration and washed with ether–petroleum ether (6:4): yield, 3.28 g (100%); mp 174–176°; $[\alpha]^{20}D$ −12.6° (*c* 1.14, dimethylformamide); R_f 0.46,[2] 0.75.[3]

Anal. Calcd for $C_{50}H_{56}O_{15}N_7F_3$: C, 57.09; H, 5.32; N, 9.31. Found: C, 57.07; H, 5.04; N, 9.58.

***t*-Butyloxycarbonyl-γ-benzylglutamylasparaginyl-β-benzylaspartylalanyl-β-benzylaspartyl-O-benzylserylglycine Benzyl Ester (XIII).** To a stirred and cooled solution of 3.60 g (3.1 mmol) of XII in a mixture of 0.49 ml (3.5 mmol) of triethylamine and 15 ml of dimethylformamide, 1.72 g (3.96 mmol) of *t*-butyloxycarbonyl-γ-benzylglutamic acid N-hydroxysuccinimide ester was added and the mixture was allowed to stir for 2 hr at 0° and then overnight at 4°. The reaction mixture was diluted with ethyl acetate (150 ml) and washed successively with a saturated citric acid solution (three times), a saturated sodium bicarbonate solution (three times), and water (to neutrality) in spite of the occurrence of precipitation during the washing. The ethyl acetate layer containing the precipitate was separated and evaporated, and the gelatinous residue was treated with ether–petroleum ether (1:1). The crystalline product was collected by filtration and washed thoroughly with ether–petroleum ether (1:1). The crystals on the filter were washed again with a citric acid solution, saturated sodium bicarbonate, and water (3.82 g). The product gave a single spot on thin layer chromatography. Recrystallization from methanol–ethyl acetate–ether afforded 3.53 g (85%) of the product: mp 211–212°; $[\alpha]^{20}D$ −14.4° (*c* 1.34, dimethylformamide); R_f 0.72,[1] 0.82.[2] Amino acid analysis gave the following ratio: glutamic acid 1.0, aspartic acid 3.0, alanine 1.0, serine 0.6, and glycine 1.0.

Anal. Calcd for $C_{65}H_{76}O_{18}N_8$: C, 62.09; H, 6.05; N, 8.91. Found: C, 62.01; H, 6.00; N, 8.76.

γ-Benzylglutamylasparaginyl-β-benzylaspartylalanyl-β-benzylaspartyl-O-benzylserylglycine Benzyl Ester Trifluoroacetate (XIV). Protected heptapeptide ester (XIII, 3.53 g, 2.81 mmol) was treated with 25 ml of trifluoroacetic acid for 15 min at room temperature. After evaporation the residue was triturated with ether–petroleum ether (7:3). The crystals were collected and washed with ether–petroleum ether (7:3): yield, 3.58 g (100%); mp 212–213°; $[\alpha]^{20}D$ −9.9° (*c* 1.34, dimethylformamide); R_f 0.53,[2] 0.75.[3]

Anal. Calcd for $C_{82}H_{89}O_{18}N_8F_3$: N, 8.81. Found: N, 8.92.

***t*-Butyloxycarbonyl-O-benzylseryl-γ-benzylglutamylasparaginyl-β-benzylaspartylalanyl-β-benzylaspartyl-O-benzylserylglycine Benzyl Ester (XV).** To a solution of 3.58 g (2.82 mmol) of XIV in a mixture of 0.42 ml (3 mmol) of triethylamine and 15 ml of dimethylformamide, 1.33 g (3.4 mmol) of *t*-butyloxycarbonyl-O-benzylserine N-hydroxysuccinimide ester was added and the mixture was stirred for 2 hr at 0° and then overnight at 4°. The solution was worked up by the procedure described for the preparation of XIII. The product was recrystallized from dimethylformamide–ether–petroleum ether: yield, 3.58 g (89%); mp 210–212°; $[\alpha]^{20}D$ −12.4° (*c* 1.29, dimethylformamide); R_f 0.71,[1] 0.70.[2]

Anal. Calcd for $C_{75}H_{87}O_{20}N_9$: C, 62.79; H, 6.07; N, 8.78. Found: C, 62.82; H, 6.29; N, 8.90.

O-Benzylseryl-γ-benzylglutamylasparaginyl-β-benzylaspartylalanyl-β-benzylaspartyl-O-benzylserylglycine Benzyl Ester Trifluoroacetate (XVI). Protected octapeptide ester XV, 3.48 g (2.43 mmol) was treated with 20 ml of trifluoroacetic acid for 15 min at room temperature. After evaporation, the residue was triturated with ether–petroleum ether (1:1) and crystals were collected by filtration and washed with ether–petroleum ether (1:1): yield, 3.40 g (97%); mp 202–204°; $[\alpha]^{20}D$ −10.4° (*c* 1.31, dimethylformamide).

Anal. Calcd for $C_{72}H_{80}O_{20}N_9F_3$: N, 8.70. Found: N, 8.56.

***t*-Butyloxycarbonyltryptophyl-O-benzylserine Methyl Ester (XVII).** To a stirred and cooled solution of 2.46 g (19 mmol) of O-benzylserine methyl ester hydrochloride in a mixture of 1.4 ml (10 mmol) of triethylamine and 40 ml of methylene chloride, 2.74 g (9 mmol) of *t*-butyloxycarbonyltryptophan was added followed by 1.85 g (9 mmol) of N,N′-dicyclohexylcarbodiimide. The mixture was allowed to stir for 2 hr at 0° and then overnight at 4°. After removal of N,N′-dicyclohexylurea by filtration the filtrate was evaporated and the residue was treated with water (40 ml) and ethyl acetate (60 ml). The ethyl acetate layer was washed with a saturated citric acid solution, a saturated sodium bicarbonate solution, and water, dried over sodium sulfate, and evaporated. The residue was triturated with petroleum ether (3.65 g). The product was used for the next reaction without further purification.

***t*-Butyloxycarbonyltryptophyl-O-benzylserine Hydrazide (XVIII).** To a solution of 2.70 g (5.45 mmol) of XVII in 15 ml of dimethylformamide, 3.5 ml (120 mmol) of hydrazine was added and the reaction mixture was allowed to stand overnight at room temperature. The mixture was then diluted with 250 ml of water and the milky solution was shaken with 100 ml of ethyl acetate. The ethyl acetate layer was separated and washed several times with water, dried over sodium sulfate, and evaporated. The residue was triturated with ether (2.0 g). Recrystallization from ethanol–ether–petroleum ether gave the pure product: yield, 1.84 g (68%); mp 163–165°; $[\alpha]^{20}D$ −0.5° (*c* 1.00, MeOH); R_f 0.73,[1] 0.88.[3]

Anal. Calcd for $C_{26}H_{33}O_5N_5$: C, 63.01; H, 6.66; N, 14.1. Found: C, 63.13; H, 6.58; N, 14.2.

***t*-Butyloxycarbonyltryptophyl-O-benzylseryl-N-benzylglutamylasparaginyl-β-benzylaspartylalanyl-β-benzylaspartyl-O-benzylserylglycine Benzyl Ester (XIX). a. Stepwise Approach.** To a cooled (0°) solution of 2.90 g (2 mmol) of XVI in a mixture of 0.31 ml (2.2 mmol) of triethylamine and 15 ml of dimethylformamide was

added 1.04 g (2.6 mmol) of *t*-butyloxycarbonyltryptophan N-hydroxysuccinimide ester and the reaction mixture was allowed to stir for 2 hr at 0° and then overnight at 4°. The solution was worked up by the procedure described for the preparation of XIII. The product was recrystallized from dimethylformamide–ether: yield, 2.43 g (75%); mp 217–218°; $[\alpha]^{20}D$ −12.3° (*c* 0.87, dimethylformamide).

The purity was checked by thin layer chromatography after deprotection of the *t*-butyloxycarbonyl group with trifluoroacetic acid, R_f 0.81[1] (single spot). Amino acid analysis gave the following ratios: serine 1.5, glutamic acid 1.0, aspartic acid 3.0, alanine 1.0, and glycine 1.0.

Anal. Calcd for $C_{86}H_{97}O_{21}N_{11}$: C, 63.73; H, 5.98; N, 9.48. Found: C, 63.15; H, 5.95; N, 9.59.

b. Azide Coupling (from XVIII and XIV). To a cooled (−10°) solution of 0.298 g (0.6 mmol) of XVIII in 8 ml of dimethylformamide, 2.4 ml (2.4 mmol) of 1 *N* hydrochloric acid was added, followed by 0.6 ml (0.6 mmol) of 1 *N* sodium nitrite. The solution was stirred for 7 min at −10° and then diluted with 40 ml of cold water. The azide deposited was extracted twice with 20 ml of ethyl acetate. The combined ethyl acetate extract was washed with a sodium bicarbonate solution and water and dried over sodium sulfate. The dried solution was added to a solution of 0.635 g (0.5 mmol) of XIV in a mixture of 0.1 ml of triethylamine and 9 ml of dimethylformamide. The reaction mixture was stirred for 2 hr at 0° and then overnight at 4°. The mixture, in which a gelatinous product deposited, was diluted with ethyl acetate (40 ml), and washed successively with citric acid solution, sodium bicarbonate solution, and water. The organic phase containing the gelatinous product was separated and evaporated nearly to dryness. The residue was triturated with petroleum ether (0.706 g), and then recrystallized from hot methanol–ether: yield, 0.61 g (76%); mp 208–210°; R_f 0.81,[1] 0.87.[2] Amino acid analysis gave the following ratio: Ser 1.2, Glu 1.0, Asp 3.1, Ala 1.0, Gly 1.0.

Anal. Calcd for $C_{86}H_{97}O_{21}N_{11}$: N, 9.48. Found: N, 9.60.

Tryptophyl-O-benzylseryl-γ-benzylglutamylasparaginyl-β-benzylaspartylalanyl-β-benzylaspartyl-O-benzylserylglycine Benzyl Ester Trifluoroacetate (XX). Blocked nonapeptide benzyl ester (XIX, 0.50 g, 0.308 mmol) was dissolved in cooled (0°) trifluoroacetic acid and the solution was kept for 10 min at this temperature and then for 10 min at room temperature. The solution was evaporated at low temperature and the oily residue was triturated with ether. The trifluoroacetate was collected by filtration and washed with ether: yield, 0.47 g (93%); mp 197–199°; $[\alpha]^{20}D$ −8.8 (*c* 0.8, dimethylformamide).

Anal. Calcd for $C_{83}H_{90}O_{21}N_{11}F_3$: C, 60.90; H, 5.49; N, 9.43; F, 3.49. Found: C, 60.45; H, 5.62; N, 9.84; F, 3.54.

Tryptophyl-O-benzyl-γ-benzylglutamylasparaginyl-β-benzylaspartylalanyl-β-benzylaspartyl-O-benzylserylglycine Benzyl Ester (Free Base) (XXI). Nonapeptide benzyl ester trifluoroacetate (0.30 g, 0.183 mmol) was dissolved in 3–4 ml of dimethylformamide and 0.28 ml (2 mmol) of triethylamine was added. The solution was further stirred for 5 min and then evaporated *in vacuo* in order to remove triethylamine at room temperature. The residual solution was diluted with a tenfold volume of water, and the precipitate was collected by filtration and washed with saturated sodium bicarbonate solution and with water (0.264 g). Recrystallization from dimethylformamide–ether gave 0.244 g (88%) of the free peptide ester: mp 192–194°; $[\alpha]^{20}D$ −16.0 (*c* 1.08, dimethylformamide).

Anal. Calcd for $C_{81}H_{89}O_{19}N_{11}$: C, 63.98; H, 5.85; N, 10.1. Found: C, 63.05; H, 6.14; N, 10.05.

Synthesis of ε-Trifluoroacetyllysylleucylasparaginylisoleucine Hydrobromide (XXII) by the Solid Phase Method of Merrifield. *t*-Butyloxycarbonylisoleucyl Resin. To 10.6 g of chloromethylated copolystyrene divinylbenzene resin (1.2 mequiv of Cl per g) in 40 ml of ethanol was added 2.18 g (9.8 mmol) of *t*-butyloxycarbonylisoleucine and 1.40 ml (9.8 mmol) of triethylamine. The suspension was refluxed for 72 hr. The resin was filtered, rinsed repeatedly with ethanol and CH_2Cl_2, and dried *in vacuo*. Acid hydrolysis at 110°, 20 hr in 50% dioxane–concentrated HCl showed the isoleucine content of the product to be 0.30 mmol/g.

***t*-Butyloxycarbonyl-ε-trifluoroacetyllysylleucylasparaginylisoleucyl Resin.** A 10-g sample (3.0 mmol) of the above resin was placed in a shaking vessel of the type described by Merrifield for repeated deblocking, rinsing, and coupling procedures. In a typical cycle the resin was rinsed three times with dioxane, shaken for 30 min in 4 *N* HCl/dioxane, rinsed three times with fresh dioxane, rinsed three times with $CHCl_3$, shaken for 10 min in 10% triethylamine/$CHCl_3$, and rinsed three times with $CHCl_3$ and three times with CH_2Cl_2. The appropriate *t*-butyloxycarbonylamino acid was added to a threefold molar excess (9.0 mmol) in 60 ml of CH_2Cl_2 and shaken with the resin for 10 min. A 9.0-mmol sample of N,N′-dicyclohexylcarbodiimide was then added in 5 ml of CH_2Cl_2, and shaking was continued for 2 hr. The coupling steps were terminated by rinsing three times with CH_2Cl_2, and the entire cycle was repeated. *t*-Butyloxycarbonylasparagine was coupled as the *p*-nitrophenyl ester in fourfold molar excess (12 mmol) with 60 ml of dimethylformamide as the solvent, and the reaction time was increased to 12 hr. Appropriate dimethylformamide rinses were added to the cycle before and after the coupling step, and N,N′-dicyclohexylcarbodiimide was omitted.

Cleavage of Peptide from Resin. After completion of the last coupling cycle the tetrapeptidyl resin was dried *in vacuo* and resuspended in 60 ml of anhydrous trifluoroacetic acid. HBr gas was bubbled slowly through the suspension for 120 min. The trifluoroacetic acid was then filtered off. The resin was resuspended and rinsed with three portions of trifluoroacetic acid and three portions of 50% trifluoroacetic acid in CH_2Cl_2. All filtrates were combined and evaporated to an oil which was triturated repeatedly with ethyl acetate to yield a white solid. The solid was recrystallized from methanol–ethyl acetate to yield 2.1 g of the hydrobromide, R_f 0.43,[3] 0.69 (pyridine–water, 2:1 v/v). Amino acid analysis gave the following ratio: Lys 1.0, Leu 1.0, Asp 1.0, and Ilu 1.0.

Synthesis of *t*-Butyloxycarbonyl-γ-benzylglutamyl-ε-trifluoroacetyllysylleucylasparaginylisoleucine (XXIII). To a solution of 2.1 g (3.3 mmol) of XXII in 40 ml of dimethylformamide was added 0.68 ml (6.6 mmol) of N-methylmorpholine and 2.86 g (6.6 mmol) of *t*-butyloxycarbonyl-γ-benzylglutamic acid N-hydroxysuccinimide ester. The solution was stirred at 25° for 8 hr until a thin layer chromatogram showed disappearance of the starting tetrapeptide. The volume was reduced to 10 ml by evaporation *in vacuo*. The product was precipitated by the addition of ether, then redissolved in methanol and precipitated by the addition of water. Final recrystallization from methanol by addition of 0.5 *M* citric acid, rinsing with water, and drying *in vacuo* yielded 1.9 g (2.1 mmol) of blocked pentapeptide: mp 218–220°; $\alpha^{20}D$ −19.3° (*c* 1.41, dimethylformamide); R_f 0.69,[1] 0.72.[3]

Anal. Calcd for $C_{41}H_{62}O_{17}N_7F_3$: C, 54.4; H, 6.85; N, 10.8; F, 6.3. Found: C, 54.7; H, 6.58; N, 11.0; F, 6.4.

***t*-Butyloxycarbonyl-γ-benzylglutamyl-ε-trifluoroacetyllysylleucylasparaginylisoleucyltryptophyl-O-benzylseryl-γ-benzylglutamylasparaginyl-γ-benzylaspartylalanyl-β-benzylaspartyl-O-benzylserylglycine Benzyl Ester (XXIV).** To a cooled (−5°) solution of 0.180 g (0.183 mmol) of XXIII and 0.035 g (0.305 mmol) of N-hydroxysuccinimide in 0.8 ml of dimethylformamide, 0.034 g (0.165 mmol) of N,N′-dicyclohexylcarbodiimide was added and the mixture was stirred for 2 hr at −5°. To this solution, a solution of 0.099 g (0.061 mmol) of XXI in a mixture of 0.020 ml (0.120 mmol) of triethylamine and 0.7 ml of dimethylformamide was added, the mixture was stirred for 2 hr at −5°, and then stored overnight in a refrigerator. The N,N′-dicyclohexylurea deposited was filtered off and the filtrate was evaporated *in vacuo*. The residue was treated with water, and the precipitate was collected by filtration and washed successively with a saturated citric acid solution, water (until neutral), and ether. A solution of the crude product (0.22 g) in 1.5 ml of dimethylformamide and 2.5 g of silica gel (fine powder) were mixed and dried overnight under reduced pressure. The dried silica gel was placed on the top of a fresh silica gel column (1.5 × 8 cm). The column was washed with a mixture of methanol and ethyl acetate (1:2 v/v) at a slow flow rate until the effluent became negative to the modified Ehrlich reagent.[17] Silica gel (*ca.* 3 g) from the top of the column was taken out and extracted with four 4-ml portions of dimethylformamide. The extract was evaporated *in vacuo* and the residue was triturated with water (0.072 g). Reprecipitation from dimethylformamide–ether gave a white powder, 0.060 g (40%).

Amino acid analysis of the product gave the following ratio: Glu 2.0, Lys 1.0, Leu 0.9, Asp 4.0, Ilu 0.9, Ser 1.3, Ala 1.1, and Gly 1.1: mp 243–245° with charring; $\alpha^{20}D$ −4.1° (*c* 0.155, dimethylformamide).

Anal. Calcd for $C_{122}H_{149}O_{35}N_{18}F_3$: C, 58.97; H, 6.04; N, 10.15. Found: C, 58.98; H, 6.29; N, 10.24.

A small sample of XXIV, in DMF, was deprotected by 5 hr hydrogenation in a Parr apparatus employing palladium–charcoal.

(17) A 0.5% solution of *p*-dimethylaminocinnamaldehyde in 0.5 *N* HCl.

The resulting product was treated with trifluoroacetic acid (10 min) to remove the BOC groups and 1 *M* aqueous piperidine at 0° (1 hr) to remove the ε-TFA group.

Amino acid analysis, after digestion with aminopeptidase-M,[6] gave the following ratio: Asp 2.0, Ser + Asn 3.2, Glu 1.9, Gly 1.0, Ala 0.9, Ile 1.2, and Leu 1.1.

Communications to the Editor

A Cyclobutanol Intermediate in the Decomposition of Aldehydes under Electron Impact. A Further Photochemical Analogy

Sir:

Loss of C_2H_4 from the molecular ion was reported in an early paper to be characteristic of aliphatic aldehydes in the mass spectrometer.[1] The assumption that this neutral product is derived from the terminal C_2H_5 group[1] seemed plausible, but it offered no clue to a possible driving force. As part of a more extensive study of the decomposition reactions of aldehydes under electron impact, we have obtained evidence pointing to a rather different process for the loss of C_2H_4. High-resolution mass measurement on heptanal identifies the $[M - 28]^+$ peak as a singlet and the ionic composition as $C_5H_{10}O^+$, in agreement with the conclusion of the earlier work,[1] based on ^{18}O labeling. However, the deuterium labels in heptanal-4-d_2, -5-d_2, and -6-d_2, hexanal-4-d_2, -5-d_2, and -6-d_3, and nonanal-1-d are retained, and those in heptanal-2-d_2 and -3-d_2 are lost almost quantitatively in this process. Thus, the ethylene clearly comprises the methylene groups originally in the α and β positions.

Especially in light of the close parallels between reactions of acyclic carbonyl compounds under electron impact and uv irradiation,[2,3] this finding suggested that the reaction may occur *via* a cyclobutanol intermediate, as in a photochemical reaction path of aliphatic ketones.[4-7] Subsequent rupture of the ring, characteristic of cyclobutane and its derivatives,[8-13] would then effect loss of the α- and β-methylene groups as ethylene. To test this surmise, we compared the mass spectrum of heptanal with that of the suspected intermediate, 2-propylcyclobutanol, which was prepared by irradiation[7,14] of heptanal in pentane solution with a medium-intensity Hanovia lamp. Carbonyl compounds were removed as water-soluble bisulfite salts, and 2-propylcyclobutanol was collected by gas chromatography.

The spectra of the two compounds are qualitatively similar, in accord with the proposal of such a common intermediate. They both contain metastable peaks corresponding to the primary loss of C_2H_4 and competing secondary processes by which CH_3 and H_2O are lost (Table I). Moreover, the intensity ratios listed in Table II (mass 86 refers to the normal peak due to the $[M - 28]^+$ ion) are nearly the same in the two spectra, as expected if the ions involved have the same structures and energy contents and distributions.[15,16]

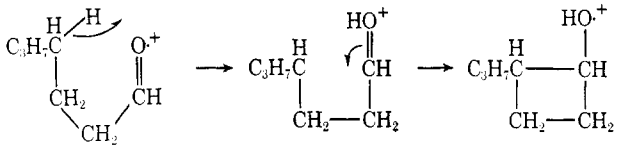
Table I. Metastable Peaks

Apparent Mass		Process	
64.9	$114^+ \longrightarrow 86^+ + 28$	$C_7H_{14}O^+ \longrightarrow C_5H_{10}O^+ + C_2H_4$
58.6	$86^+ \longrightarrow 71^+ + 15$	$C_5H_{10}O^+ \longrightarrow C_4H_7O^+ + CH_3$
53.8	$86^+ \longrightarrow 68^+ + 18$	$C_5H_{10}O^+ \longrightarrow C_5H_8^+{}_0 + H_2O$

Table II. Intensity Ratios at 70 eV

	Mass 64.9 : mass 86	Mass 53.8 : mass 58.6
Heptanal	0.0076 ± 0.0010	2.0 ± 0.2
2-Propylcyclobutanol	0.0081 ± 0.0008	1.9 ± 0.2

Analogy with the photochemical process, which has been shown conclusively to occur, at least in large part, by a stepwise mechanism,[7] suggests that isomerization to cyclobutanols under electron impact is also, again at least in large part, a stepwise, rather than a concerted, process.



Moreover, this is now the third known reaction of carbonyl compounds under electron impact that appears to involve γ-hydrogen migration as an essential part. The first is, of course, the well-known counterpart of the Norrish type II photolysis;[2] the second, ε cleavage of Δ^2-enones and -enoates,[3] is seemingly related to the photoisomerization of the same unsaturated types.[17] A growing body of evidence indicates that

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