Since (2*R*)-2-phosphoglycerate is the specific product, proton addition at C-2 must be from the *si* face[16] of the plane of PEP.

Having established the stereochemistry of specifically deuterated PEP, the way is open to the steric analysis of additions to C-3 that occur in many enzymatic reactions.[17,18]

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Partial Enzymic Deprotection in the Synthesis of a Protected Octapeptide Bearing a Free Terminal Carboxyl Group

Motonori Ohno and Christian B. Anfinsen

Laboratory of Chemical Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014.
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Abstract: A method is described for the preparation of protected oligopeptides terminating in free carboxyl groups. The desired peptide is built up by the stepwise addition of protected monomers to a small "supporting" peptide ester which may be removed by enzymatic digestion with the bacterial protease, thermolysin.

The preparation of fully protected peptides suitable for successive coupling to a growing COOH-terminal fragment may be of value in the syntheses of long-chained polypeptides or proteins. We have described a modified solid phase method in which peptides were synthesized by the solid phase method of Merrifield and then coupled with the protected NH_2-terminal amino acid N-hydroxysuccinimide ester in the presence of base.[1] It was of advantage to select, as an NH_2-terminal residue, α-N-protected aspartic or glutamic acid bearing a benzyl or *t*-butyl ester group at the ω position, thus circumventing the removal of these protecting groups on the acidic amino acids during the action of HF or of HBr in trifluoroacetic acid that are used for detachment of the peptides from the supporting resin. We subsequently reported an alternative procedure involving cleavage of the protected peptide from the Merrifield resin by hydrazinolysis.[2] This method is effective for preparation of protected peptide hydrazides which do not contain aspartic or glutamic acid residues with an ester group at the ω position, or other protecting groups labile to hydrazine.

In the syntheses of protected peptides containing ω-substituted aspartic or glutamic acid, alkaline hydrolysis of COOH-terminal ester groups in order to obtain a free terminal COOH group causes a transpeptidation reaction, *via* intermediary imide formation, to yield a mixture of α- and ω-peptides.[3] This occurs even when the *t*-butyl ester group, which is resistant to alkali, is used for protection of ω-carboxyl groups.[4] When phthaloyl[5] or trifluoroacetyl[6] is employed for protection of the amino group, alkaline hydrolysis is also not feasible since these protecting groups are not stable in strong alkaline medium.

We describe in this article an approach to the preparation of protected peptides bearing a free terminal group through enzymatic cleavage of an appropriate peptide bond. The studies by Matsubara, *et al.*, have clarified the specificities of thermolysin, a protease prepared from cultures of *Bacillus thermoproteolyticus* Rokko.[7] This enzyme hydrolyzes peptide bonds on the amino side of hydrophobic amino acid residues, such as leucine, isoleucine, phenylalanine, and valine. Thus, thermolysin appeared to be a suitable enzyme for the preparation of protected peptide carboxylates by cleavage from a "supporting" peptide in which the NH_2-terminal residue is one of these hydrophobic amino acids. The principle of enzymatic cleavage of protected peptides from solid phase supports has been discussed in an earlier report.[8]

This method was applied to the preparation of *t*-butyloxycarbonylglutaminylthreonyl-ε-benzyloxy-carbonyllysylhistidylprolyl-ε-trifluoroacetyllysyl-ε-trifluoroacetyllysylglycine (XIV) which comprises an analog of

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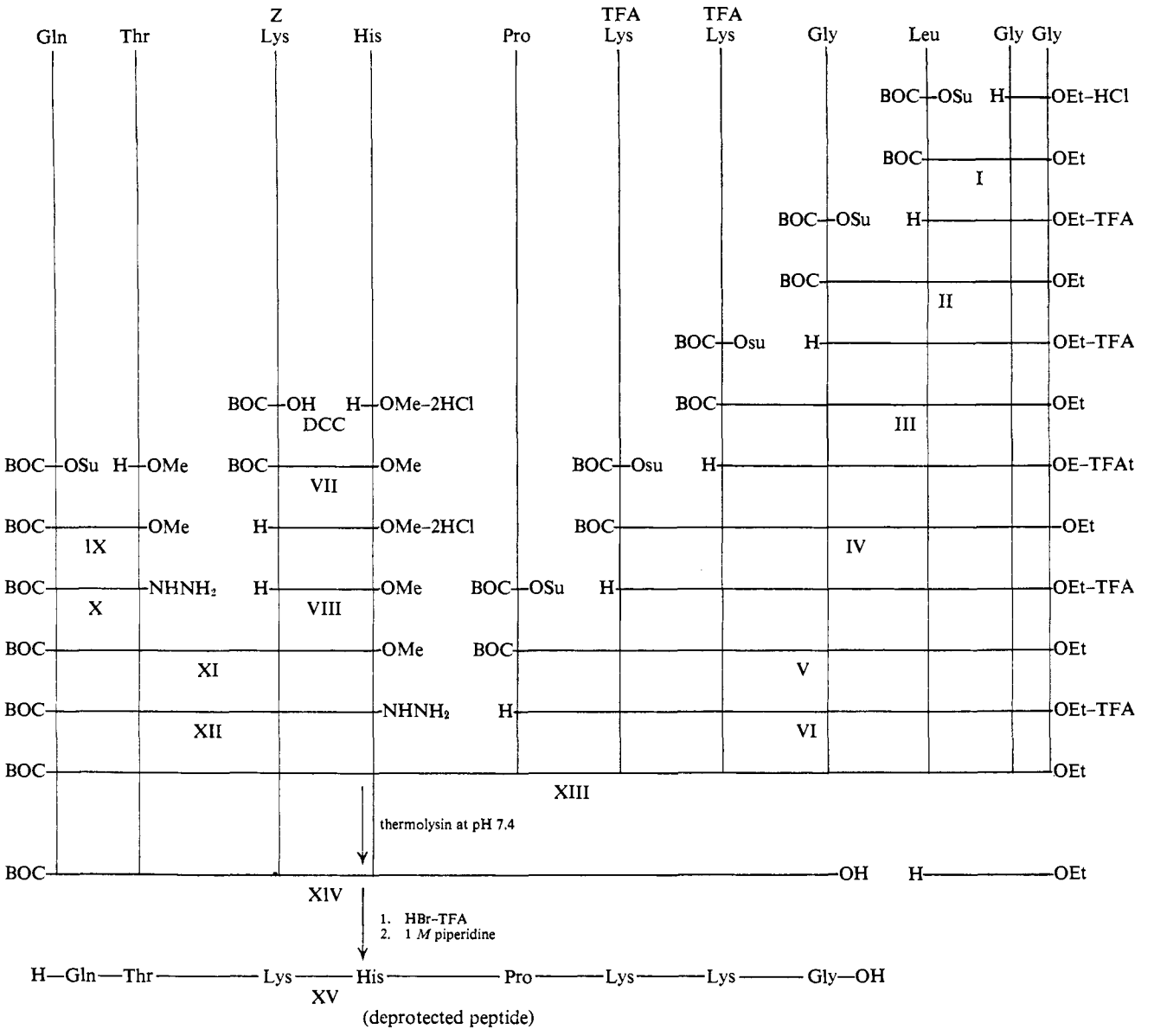

Figure 1. Schematic diagram of synthesis of the protected undecapeptide ester (XIII) and its hydrolysis by thermolysin: BOC, *t*-butyloxycarbonyl; Z, benzyloxycarbonyl; TFA, trifluoroacetyl or trifluoroacetic acid; OSu, N-hydroxysuccinimide ester. The azide couplings were performed by the Rudinger modification.[12]

the 43–50 sequence of staphylococcal nuclease.[9] Leucylglycylglycine ethyl ester was employed as a supporting peptide.

The protected undecapeptide ester, *t*-butyloxycarbonylglutaminylthreonyl-ε-benzyloxycarbonyllysylhistidylprolyl-ε-trifluoroacetyllysyl-ε-trifluoroacetyllysylglycylleucylglycylglycine ethyl ester (XIII), was synthesized as summarized in Figure 1. The heptapeptide derivative (V) of the C-terminal moiety was synthesized, by the stepwise elongation procedure, from glycylglycine ethyl ester with *t*-butyloxycarbonyl amino acid N-hydroxysuccinimide esters.[10] In this sequence, two lysine ε-amino groups were protected by the trifluoroacetyl group, which may be considered fairly hydrophilic and which is resistant to acid but easily removed by treatment with 1 *M* piperidine at 0°.[11] Deprotection of the *t*-butyloxycarbonyl group in each cycle of elongation was carried out with anhydrous trifluoroacetic acid. The NH_2-terminal tetrapeptide was synthesized from *t*-butyloxycarbonylglutaminylthreonine hydrazide (X) and ε-benzyloxycarbonyllysylhistidine methyl ester (VIII) by the Rudinger modification[12] of the azide procedure. The protected tetrapeptide ester (XI) thus obtained was converted to the corresponding hydrazide (XII). The coupling of XII and heptapeptide ester (VI) by Rudinger's azide method[12] gave the pure protected undecapeptide ester (XIII) in a yield of 64%.

The two intermediates, *t*-butyloxycarbonyl-ε-trifluoroacetyllysyl-ε-trifluoroacetyllysylglycylleucylglycylglycine ethyl ester (IV) and *t*-butyloxycarbonylprolyl-ε-trifluoroacetyllysyl-ε-trifluoroacetyllysylglycylleucylglycylglycine ethyl ester (V), were independently incubated with thermolysin in a 20% alcoholic solution of pH 6.5 containing calcium ion and the reactions

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were followed by alkali titration using a pH stat. Very rapid consumption of alkali was observed and the glycyl–leucine bond was completely hydrolyzed as judged by thin-layer chromatography of the hydrolysates and from amino acid analyses of the products. No detachment of trifluoroacetyl groups on ε-amino groups of lysine occurred under these conditions. Alkali uptake by the enzyme alone was negligible at this pH.

The hydrolysis of 70 mg of the protected undecapeptide ester (XIII), using one-hundredth the amount of thermolysin by weight, was carried out by the same procedure as described for the two shorter peptide derivatives. The solution was slightly cloudy at the beginning of the reaction but the reaction proceeded rapidly with clearing, and alkali uptake ended within 15 min. The uptake of base was approximately that to be expected if the pK of the amino group of the liberated tripeptide ethyl ester were in the neighborhood of 8.0. When alkali uptake ceases, the reaction mixture was lyophilized. The dried materials were transferred into the first tube of a countercurrent distribution apparatus and distributed using the system *sec*-butyl alcohol–0.05 M pyridinum acetate buffer (pH 6.5). After 60 transfers, fractions containing the protected octapeptide were pooled and lyophilized. Crystallization of the dried material from methanol–ether yielded 37 mg of the pure protected octapeptide (XIV).

This thermolysin-type synthesis is particularly suitable for the preparation of "water-soluble" protected peptides which contain mainly hydrophilic amino acid residues and which may be difficult to synthesize by conventional methods. On the other hand, the hydrophobic peptides *t*-butyloxycarbonyl-γ-benzylglutamyl-ε-trifluoroacetyllysyl-O-benzyltyrosylglycyl (52–55 sequence of the nuclease) leucylglycylglycine ethyl ester and *t*-butyloxycarbonyl-β-benzylaspartyl-ε-trifluoroacetyllysyl-O-benzyltyrosylglycyl (83–86 sequence of the nuclease) leucylglycylglycine ethyl ester have been synthesized by the stepwise elongation of glycylglycine ethyl ester and attempts were made to hydrolyze these with thermolysin. No detectable hydrolysis occurred since these protected peptide esters were completely insoluble in a 20% alcoholic solution.

It should be emphasized that the method described in this paper is not applicable to the synthesis of peptides containing thermolysin-sensitive bonds other than that contributed by the "supporting" peptide. Furthermore, the protected peptide must be sufficiently hydrophilic to impart at least moderate solubility in aqueous ethanol for the enzymatic cleavage.

Experimental Section

All melting points are uncorrected. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter. Brinkmann silica gel plates were used for thin-layer chromatography. Peptides bearing a free amino group were detected with 0.2% ninhydrin solution in 80% ethanol, followed by heating. Spots of *t*-butyloxycarbonyl peptide derivatives were detected after exposure to HCl vapor (15 min over concentrated hydrochloric acid) followed by heating for 5 min at 100° and spraying with ninhydrin solution. Amino acid analyses of peptide derivatives were carried out on samples which had been hydrolyzed with constant boiling hydrochloric acid for 20 hr in an evacuated, sealed tube at 110°.

***t*-Butyloxycarbonylleucylglycylglycine Ethyl Ester (I).** To a solution of 1.08 g (5.5 mmol) of glycylglycine ethyl ester hydrochloride[13] in a mixture of 0.77 ml (5.5 mmol) of triethylamine and 20 ml of acetonitrile, 1.64 g (5 mmol) of *t*-butyloxycarbonylleucine N-hydroxysuccinimide ester[10] was added at 4° and the reaction mixture was allowed to stir overnight at the same temperature. The solution was evaporated *in vacuo*, the residue was treated with 30 ml of ethyl acetate, and the ethyl acetate solution was washed successively with a saturated sodium bicarbonate solution, 0.5 M citric acid, and water, dried over sodium sulfate, and evaporated to dryness. An oily residue resulted, 1.76 g (94%); R_f 0.92.[14]

***t*-Butyloxycarbonylglycylleucylglycylglycine Ethyl Ester (II).** Compound I (1.76 g, 4.72 mmol) was dissolved in 7 ml of trifluoroacetic acid and the solution was kept for 20 min at room temperature. Evaporation of the solution gave an oily residue which was washed several times with ether by decantation. The residue was dissolved in 20 ml of methanol, 2 ml of triethylamine was added, and the solution was evaporated to dryness *in vacuo* in order to remove excess triethylamine. The residue was dissolved in 15 ml of dimethoxyethane and 1.28 g (4.7 mmol) of *t*-butyloxycarbonylglycine N-hydroxysuccinimide ester[10] was added. The reaction mixture was allowed to stir overnight at 4°. After removal of the solvent by evaporation the residue was dissolved in 30 ml of ethyl acetate and the solution was washed successively with a saturated sodium bicarbonate solution, 0.5 M citric acid, and water, dried (Na_2SO_4), and evaporated to dryness. An oil was obtained, 1.79 g (88%).

***t*-Butyloxycarbonyl-ε-trifluoroacetyllysylglycylleucylglycylglycine Ethyl Ester (III).** Compound II (1.70 g, 4 mmol) was dissolved in 8 ml of trifluoroacetic acid and the solution was allowed to stand for 20 min at room temperature. The solution was then evaporated *in vacuo*. The residue was dissolved in 20 ml of water and shaken with ethyl acetate, and the aqueous phase was lyophilized. An oily residue remained (tetrapeptide ethyl ester trifluoroacetate), 1.69 g (96%). The trifluoroacetate was dissolved in 15 ml of methanol and 1 ml of triethylamine was added. The solution was then evaporated *in vacuo* at 30° and the residue was kept over sulfuric acid in a desiccator *in vacuo*. The residue was dissolved in a mixture of 10 ml of dimethoxyethane and 10 ml of methylene chloride and 1.67 g (3.8 mmol) of *t*-butyloxycarbonyl-ε-trifluoroacetyllysine N-hydroxysuccinimide ester[1] was added. The reaction mixture was allowed to stir overnight at 4°. The solution was evaporated and the residue was dissolved in 40 ml of ethyl acetate, washed successively with a saturated sodium bicarbonate solution, 0.5 M citric acid, and water, dried (Na_2SO_4), and evaporated. The residue crystallized quickly. Crystals were filtered off with petroleum ether (2.05 g). Recrystallization from ethyl acetate–petroleum ether yielded 1.87 g (75% from the trifluoroacetate) of the product, mp 153–154°; $[\alpha]^{20}D$ −11.8° (c 0.57, dimethylformamide).

Anal. Calcd for $C_{27}H_{45}O_9N_6F_3$: C, 49.53; H, 6.92; N, 12.83. Found: C, 50.35; H, 7.32; N, 12.63.

***t*-Butyloxycarbonyl-ε-trifluoroacetyllysyl-ε-trifluoroacetyllysylglycylleucylglycylglycine Ethyl Ester (IV).** The deprotection of 1.75 g (2.67 mmol) of III with trifluoroacetic acid and neutralization with triethylamine were carried out as described in the preceding paragraph. The residue (1.79 g) thus obtained was dissolved in 15 ml of dimethoxyethane and 1.18 g (2.7 mmol) of *t*-butyloxycarbonyl-ε-trifluoroacetyllysine N-hydroxysuccinimide ester was added. The reaction mixture was allowed to stir overnight at 4°, and the product was isolated as described above for III. The residue was triturated with ether and petroleum ether, 1.94 g (82%), mp 159–160°; $[\alpha]^{20}D$ −12.1° (c 0.33, dimethylformamide).

Anal. Calcd for $C_{33}H_{56}O_{11}N_8F_6$: C, 47.82; H, 6.42; N, 12.75. Found: C, 47.65; H, 6.18; N, 12.59.

***t*-Butyloxycarbonylprolyl-ε-trifluoroacetyllysyl-ε-trifluoroacetyllysylglycylleucylglycylglycine Ethyl Ester (V).** The deprotection of 1.80 g (2.05 mmol) of IV and neutralization were carried out in the manner described for the preparation of II. The residue was dissolved in 10 ml of methylene chloride, 0.69 g (2.2 mmol) of *t*-butyloxycarbonylproline N-hydroxysuccinimide ester[10] was added, and the reaction mixture was stirred overnight at 4°. The solution was evaporated and the residue was purified as described for III. The crystalline residue was triturated with 8 ml of ether and 30 ml of petroleum ether (1.92 g). Recrystallization from ethyl acetate–petroleum ether gave 1.80 g (90%) of crystals, mp 174–176°; $[\alpha]^{20}D$ −24.6° (c 1.05, dimethylformamide); R_f 0.86.[15]

Anal. Calcd for $C_{40}H_{62}O_{12}N_9F_6$: C, 49.37; H, 6.41; N, 12.92; F, 11.69. Found: C, 50.03; H, 6.82; N, 12.03; F, 11.10.

(13) E. Fischer and E. Forneau, *Ber.*, 34, 2868 (1901).

(14) The R_f of thin-layer chromatograms refers to the system *n*-butyl alcohol–acetic acid–water (4:1:5, v/v).

(15) The R_f of the thin-layer chromatography refers to the system *n*-butyl alcohol–acetic acid–pyridine–water (4:1:1:2, v/v).

Prolyl-ε-trifluoroacetyllysyl-ε-trifluoroacetyllysylglycylleucylglycylglycine Ethyl Ester Trifluoroacetate (VI). A sample of compound V (0.46 g, 0.46 mmol) was dissolved in 8 ml of trifluoroacetic acid and the solution was allowed to stand for 15 min at room temperature. The solution was evaporated to dryness *in vacuo*. The residue was triturated with ether, and crystals were collected by filtration and washed with ether, 0.435 g (87%), mp 137–139°; $[\alpha]^{20}D$ −18.2° (*c* 1.13, dimethylformamide).

Anal. Calcd for $C_{37}H_{55}O_{12}N_9F_9$: C, 44.93; H, 5.60; N, 12.74; F, 17.29. Found: C, 44.69; H, 5.89; N, 12.59; F, 17.07.

***t*-Butyloxycarbonyl-ε-benzyloxycarbonyllysylhistidine Methyl Ester (VII).** To a suspension of 2.90 g (12 mmol) of histidine methyl ester dihydrochloride[16] in 30 ml of dimethylformamide, 3.36 ml (24 mmol) of triethylamine was added and the mixture was allowed to stir for 2 hr at room temperature.

To a stirred suspension of 5.62 g (10 mmol) of *t*-butyloxycarbonyl-ε-benzyloxycarbonyllysine dicyclohexylamine salt[17] in 40 ml of water, 3.15 g (15 mmol) of citric acid was added, followed by 40 ml of ethyl acetate. After stirring for 20 min, the ethyl acetate layer was separated and an aqueous layer was shaken with two 20-ml portions of ethyl acetate. The combined ethyl acetate extract was dried (Na_2SO_4) and evaporated. The residue was dissolved in 10 ml of dimethylformamide and this solution was added to the solution containing histidine methyl ester prepared above. The mixture was then cooled to 0° and 2.06 g (10 mmol) of N,N′-dicyclohexylcarbodiimide and 0.575 g (5 mmol) of N-hydroxysuccinimide were added. The reaction mixture was allowed to stir overnight at 4° and then for 4 hr at room temperature. After removal of insoluble material by filtration, the solution was evaporated nearly to dryness *in vacuo* and the residue was treated with 40 ml of ethyl acetate and 40 ml of a saturated sodium bicarbonate solution. The ethyl acetate layer was separated and an aqueous layer was shaken with two 40-ml portions of ethyl acetate. The combined ethyl acetate solution was washed with 20 ml of water, dried (Na_2SO_4), and evaporated. The oily residue was triturated with petroleum ether and resulting crystals were collected by filtration (4.89 g). Recrystallization from ethyl acetate–ether yielded 3.80 g (71%) of the product, mp 95–97°; $[\alpha]^{20}D$ −6.1° (*c* 2.38, ethanol).

Anal. Calcd for $C_{24}H_{37}O_7N_5$: C, 58.74; H, 7.02; N, 13.18. Found: C, 58.87; H, 6.92; N, 12.95.

ε-Benzyloxycarbonyllysylhistidine Methyl Ester (Free Base) (VIII). To a solution of 3.19 g (6 mmol) of VII in 20 ml of ethyl acetate, 40 ml of 2.5 *N* HCl in ethyl acetate was added and the solution was allowed to stand for 15 min at room temperature. After evaporation at room temperature *in vacuo*, the residue was triturated with ether to give the dihydrochloride (3.01 g). To a solution of the dihydrochloride in 15 ml of water, 2.3 ml (16 mmol) of triethylamine was added, the mixture was stirred for 5 min, and then 40 ml of methylene chloride was added. After stirring for several minutes, the methylene chloride layer was separated and an aqueous layer was shaken with two 25-ml portions of methylene chloride. The combined methylene chloride extract was dried (Na_2SO_4) and evaporated to afford colorless crystals, which were collected by filtration with petroleum ether (2.06 g). Recrystallization from methanol–ether yielded 1.98 g (75%) of the free base, mp 107–109°; $[\alpha]^{20}D$ −11.7° (*c* 1.69, dimethylformamide); R_f 0.43,[13] 0.16.[18]

Anal. Calcd for $C_{21}H_{29}O_5N_5$: C, 58.45; H, 6.78; N, 16.23. Found: C, 58.22; H, 6.56; N, 15.87.

***t*-Butyloxycarbonylglutaminylthreonine Methyl Ester (IX).** To a cooled (4°) solution of 1.60 g (12 mmol) of threonine methyl ester[19] in 40 ml of methylene chloride, 3.09 g (9 mmol) of *t*-butyloxycarbonylglutamine N-hydroxysuccinimide ester[20] was added and the mixture was stirred overnight at 4°. The crystals deposited were collected by filtration and washed with ether (1.92 g). The filtrate was evaporated to dryness and the residue was dissolved in 30 ml of ethyl acetate. The solution was washed successively with each 10-ml portion of a saturated sodium bicarbonate solution, 0.5 *M* citric acid, and water. The combined washings were then shaken with 20 ml of ethyl acetate. The combined ethyl acetate extract was dried (Na_2SO_4) and evaporated to dryness, and the residue was triturated with ether and petroleum ether (1.08 g). The first and second crystals were combined and recrystallized from ethyl acetate–ether, 2.70 g (83%), mp 122–124°; $[\alpha]^{20}D$ −14.9° (*c* 1.45, ethanol); R_f 0.77,[14] 0.91.[15]

Anal. Calcd for $C_{15}H_{27}O_7N_3$: C, 49.85; H, 7.53; N, 11.62. Found: C, 49.73; H, 7.27; N, 11.35.

***t*-Butyloxycarbonylglutaminylthreonine Hydrazide (X).** To a solution of 1.60 g (4.43 mmol) of IX in 16 ml of ethanol, 0.65 ml (20 mmol) of anhydrous hydrazine was added and the reaction mixture, which was allowed to stand overnight at room temperature, solidified. The crystals were collected by filtration with ether (1.58 g). Recrystallization from methanol–ether yielded 1.51 g (94%) of the hydrazide, mp 183–185°; $[\alpha]^{20}D$ −11.8° (*c* 1.68, dimethylformamide).

Anal. Calcd for $C_{14}H_{27}O_6N_5$: C, 46.52; H, 7.53; N, 19.38. Found: C, 46.53; H, 7.30; N, 19.54.

***t*-Butyloxycarbonylglutaminylthreonyl-ε-benzyloxycarbonyllysylhistidine Methyl Ester (XI).** To a cooled (−25°) solution of 1.63 g (4.5 mmol) of X in a mixture of 17 ml of dimethylformamide and 4.5 ml (18 mmol) of 4 *N* HCl in dioxane, 0.54 ml (4.5 mmol) of *t*-butyl nitrite[21] was added and the mixture was stirred for 20 min at −25 to −40°. The mixture was then cooled to −65° and 2.52 ml (18 mmol) of triethylamine was added, followed by a solution of 1.98 g (4.5 mmol) of VIII in 10 ml of dimethylformamide. The reaction mixture was allowed to stir for 1 hr at −25° and overnight at 4°. After removal of insoluble triethylamine hydrochloride by filtration the filtrate was evaporated to dryness *in vacuo*. The residue was treated with 15 ml of water and 15 ml of methylene chloride, and the mixture was kept for 2 hr at 0°. The crystals deposited were collected by filtration (1.15 g). The filtrate was shaken with two 30-ml portions of ethyl acetate, and the combined extract was dried (Na_2SO_4) and evaporated to dryness. The resulting crystals were collected by filtration with the aid of ether and petroleum ether (0.89 g). First and second crops were combined and recrystallized from methanol–ether, 1.67 g (39%), mp 160–162° dec; $[\alpha]^{20}D$ −15° (*c* 1.17, dimethylformamide); R_f 0.45,[14] 0.51.[18] The product was used for the next reaction without further purification.

The aqueous phase from the extraction was lyophilized, and the residue was dissolved in 20 ml of a chloroform–acetic acid mixture (95:5, v/v). The solution was applied onto the silica gel column (5 × 20 cm) and eluted with methanol–ethyl acetate (1:2, v/v). Fractions of 6 ml each were collected. Fractions 12–30, containing the product, were pooled and evaporated to dryness. The crystalline residue was filtered off with the aid of ether and petroleum ether, 0.51 g (12%). Thin-layer chromatography showed that this product was slightly contaminated by a Pauly-positive material to a minor extent. The material was not pooled with the main product.

***t*-Butyloxycarbonylglutaminylthreonyl-ε-benzyloxycarbonyllysylhistidine Hydrazide (XII).** To a solution of 0.844 g (0.97 mmol) of XI in 8 ml of methanol, 0.2 ml (6.2 mmol) of anhydrous hydrazine was added and the mixture was allowed to stand for 36 hr at room temperature. The solution was concentrated *in vacuo* nearly to dryness and the crystalline residue was triturated with water. Crystals were filtered off and washed with a small volume of water (0.45 g). The filtrate and washings were combined and lyophilized. Treatment of the dried residue with water gave the second crop (0.25 g). The first and second products were combined and recrystallized from dimethylformamide–ether, 0.52 g, mp 182–183°; $[\alpha]^{20}D$ −8.0° (*c* 1.06, dimethylformamide); R_f 0.33,[14] 0.78.[15]

Anal. Calcd for $C_{34}H_{52}O_{10}N_{10}$: C, 53.75; H, 6.84; N, 18.41. Found: C, 53.48; H, 6.58; N, 18.39.

***t*-Butyloxycarbonylglutaminylthreonyl-ε-benzyloxycarbonyllysylhistidylprolyl-ε-trifluoroacetyllysyl-ε-trifluoroacetyllysylglycylleucylglycylglycine Ethyl Ester (XIII).** To a chilled (−25°) solution of 0.39 g (0.45 mmol) of XIII in 4.5 ml of dimethylformamide, 0.9 ml (1.8 mmol) of 2 *N* HCl in dioxane was added, followed by 0.9 ml (0.45 mmol) of 0.5 *N* *t*-butyl nitrite in dimethylformamide, and the mixture was stirred for 20 min at −25°. The mixture was then cooled to −65° and 0.26 ml (1.8 mmol) of triethylamine was added. To this solution containing the azide, a solution of 0.489 g (0.5 mmol) of VI in 5 ml of 0.1 *N* triethylamine in dimethylform-

(16) M. Brenner and W. Huber, *Helv. Chim. Acta*, **36**, 1109 (1953).

(17) An oily *t*-butyloxycarbonyl-ε-benzyloxycarbonyllysine (G. W. Anderson and A. C. McGregor, *J. Amer. Chem. Soc.*, **79**, 6180 (1957)) has been converted to the crystalline dicyclohexylamine salt.

(18) The R_f of the thin-layer chromatography refers to the system methanol–ethyl acetate (1:2, v/v).

(19) J. Degraw, L. Goodman, B. Weinstein, and B. R. Baker, *J. Org. Chem.*, **27**, 576 (1962).

(20) The ester was prepared by the action of N-hydroxysuccinimide on the mixed carboxylic–carbonic anhydride from *t*-butyloxycarbonylglutamine and isobutyl chloroformate as recommended by Dr. G. W. Anderson, American Cyanimid Co., Pearl River, N. Y.

(21) C. S. Coe and T. F. Doumani, *J. Amer. Chem. Soc.*, **70**, 1516 (1948).

amide was added and the mixture was allowed to stir for 1.5 hr at −25° and overnight at 4°. The mixture, after removal of insoluble triethylamine hydrochloride, was evaporated to dryness *in vacuo*. The residue was treated with 15 ml of water and 25 ml of ethyl acetate. An organic phase was separated, washed with water containing sodium bicarbonate, dried (Na_2SO_4), and evaporated. The residue was triturated with ether to give 0.462 g (64%) of the pure product. The combined aqueous phase was shaken with three 20-ml portions of ethyl acetate. The combined ethyl acetate extract was dried (Na_2SO_4) and evaporated. Trituration of the residue with ether afforded 0.096 g (13%) of the slightly contaminated product, which was not pooled with the original batch.

For analysis a small amount of the first product was recrystallized from methanol–ether, mp 140–142°; $[\alpha]^{20}D$ −21° (*c* 0.3, dimethylformamide); R_f 0.62.[14]

Anal. Calcd for $C_{69}H_{102}O_{20}N_{17}F_6 \cdot 2H_2O$: C, 50.54; H, 6.47; N, 14.53; F, 6.97. Found: C, 50.34; H, 6.27; N, 14.24; F, 7.37.

Hydrolysis of *t*-Butyloxycarbonyl-ε-trifluoroacetyllysyl-ε-trifluoroacetyllysylglycylleucylglycylglycine Ethyl Ester (IV) with Thermolysin. To 4.5 ml of water in a reaction vessel kept at 37°, a solution of 8.8 mg (10 μmol) of IV dissolved in 0.5 ml of ethanol was added, followed by 0.25 ml of 0.1 *M* $CaCl_2$. The pH of the solution was adjusted to 6.5 and 5 μl of thermolysin solution (50 mg of the enzyme/ml) was added. The hydrolysis was followed by titration with 0.025 *N* NaOH by using a pH stat. When alkali uptake had ceased, the solution was acidified to pH 3.0 with citric acid solution and extracted with two 5-ml portions of ethyl acetate. The combined ethyl acetate extract was dried and evaporated to dryness to give a crystalline residue (*ca.* 4 mg). Thin-layer chromatography showed a single spot with R_f 0.85.[15] Amino acid analysis gave the molar ratio, Lys 2.0 and Gly 1.0.

Hydrolysis of *t*-Butyloxycarbonylprolyl-ε-trifluoroacetyllysyl-ε-trifluoroacetyllysylglycylleucylglycylglycine Ethyl Ester (V) with Thermolysin. Hydrolysis of 9.6 mg (10 μmol) of V with thermolysin was carried out by the same procedure as described in the preceding paragraph. When the titration was complete, the solution was brought to pH 3.5 by addition of citric acid solution and shaken with two 4-ml portions of ethyl acetate. The combined ethyl acetate extract was dried and evaporated to dryness to give 5.5 mg of crystalline residue (single spot on thin-layer chromatography, R_f 0.88[15]). Amino acid analysis gave the ratio: Pro 1.0, Lys 2.0, and Gly 1.0. Thin-layer chromatography of an aqueous phase showed a single spot with the same R_f value (0.87[15]) as leucylglycylglycine ethyl ester.

Synthesis of *t*-Butyloxycarbonylglutaminylthreonyl-ε-benzyloxycarbonyllysylhistidylprolyl-ε-trifluoroacetyllysyl-ε-trifluoroacetyllysylglycine (XIV) by Hydrolysis of XIII with Thermolysin. To 26,5 ml of 0.01 *M* $CaCl_2$, a solution of 70 mg of XIII in 3 ml of ethanol was added and the pH was adjusted to 7.4. To this, 30 μl of thermolysin solution (5.0 mg/0.2 ml) was added and the hydrolysis (at room temperature) was followed by titration with 0.05 *N* NaOH at pH 7.4 using a pH stat. After completion of the reaction, the solution was brought to pH 3.5 and shaken with ethyl acetate in order to remove a trace of unhydrolyzed undecapeptide derivative (XIII). The aqueous phase was lyophilized and the dried material was subjected to countercurrent distribution involving 60 transfers using *sec*-butyl alcohol–0.05 *M* pyridinium acetate buffer (pH 6.5) (upper phase 10 ml, lower phase 10 ml). An aliquot of each tube was treated by the method of Lowry, *et al.*[22] The optical densities at 750 mμ were measured. The fractions 48–59 (K = 9.0, tube number of maximum optical density = 54) were pooled and lyophilized. The dried material was dissolved in methanol and a small amount of insoluble material was filtered off. After evaporation of the filtrate, the residue was crystallized with ether, 37 mg, mp 173–175° dec; $[\alpha]^{20}D$ −38° (*c* 0.11, dimethylformamide); R_f 0.43.[14] Amino acid analysis gave the molar ratio: Glu 0.9, Thr 0.7 (uncorrected for acid destruction), Pro 1.0, Gly 1.2, Lys 3.1, and His 1.3.

Anal. of an air-dried sample. Calcd for $C_{57}H_{81}O_{17}N_{14}F_6 \cdot 6H_2O$: C, 47.00; H, 6.43; N, 13.46. Found: C, 46.78; H, 6.50; N, 13.78.

Into a solution of 6 mg of XIV in 2 ml of trifluoroacetic acid, a stream of HBr was bubbled for 1.5 hr at 0° and then for 0.5 hr at room temperature. The solution was evaporated *in vacuo*, the residue was dissolved in 2 ml of 1 *M* piperidine, and the solution was allowed to stand for 2 hr at 0° and lyophilized. Paper electrophoresis (2000 V/60 cm) of the deprotected peptide (XV), using 0.05 *M* acetic acid–pyridine buffer (pH 3.7), showed a single band positive to the ninhydrin and Pauly reactions (in addition to the band due to the remaining piperidine). Amino acid analysis of XV gave the ratios: Glu 1.1, Thr 0.9, Pro 0.9, Gly 1.1, Lys 3.0, and His 1.1.

XV was hydrolyzed by leucine aminopeptidase M[1], and amino acid analysis of an aliquot of the hydrolysate yielded the following results (expressed in μmoles): Gln, 0.25; Thr, 0.25; Lys, 0.40; His, 0.10; Pro, 0.08; Gly, 0.04. If we assume that the first lysine residue in the octapeptide was liberated in stoichiometric amounts, the results suggest that the exopeptidase was unable to digest beyond the His–Pro bond with full efficiency.

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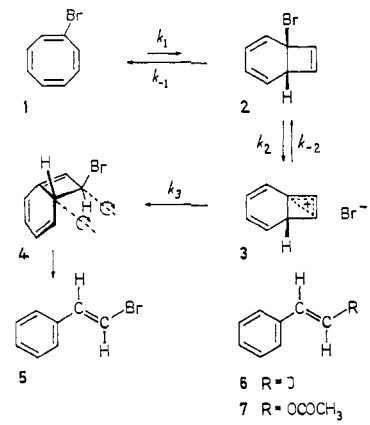
Communications to the Editor

Kinetics and Mechanism of the Rearrangement of Bromocyclooctatetraene to *trans*-β-Bromostyrene

Sir:

Cope and Burg[1] have described the conversion of bromocyclooctatetraene (**1**) at 100° to β-bromostyrene (**5**). Bromo- and chlorocyclooctatetraene recently became readily accessible by *cis* addition of halogen to cyclooctatetraene in dichloromethane at −55°[2,3] with subsequent *in situ* elimination of hydrogen halide by potassium *t*-butoxide.[4] We have now established the reaction path by which bromocyclooctatetraene rearranges to β-bromostyrene. Thus, valence tautomerization of **1** to 1-bromobicyclo[4.2.0]octatriene (**2**) is followed by ionization to the homocyclopropenium salt **3**. Ion recombination produces the cyclo-



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(4) G. E. Gream and R. Huisgen, unpublished.