ides to form in substantial yields di-*t*-alkyl or mixed *t*-alkyl aralkyl peroxides, provided that the hydrogen chloride formed is rapidly removed not by a base,[4] usually incorporated with the reactants, but by a vacuum in rotary evaporators. It is therefore self-evident that the above method can easily be extended to the solvolysis of acid chlorides with *t*-alkyl hydroperoxides, the subject of the present Note. This method not only obviates the need for using a base and its subsequent removal from the reaction products but it also leads to relatively high yields of peroxy esters and in some cases[5] to the formation of new types of peroxides not usually formed in the presence of a base. The experimental conditions described below are optimum for the yields reported.

Experimental

***t*-Butyl Peroxybenzoate.**—Benzoyl chloride was mixed in a round-bottomed ground-joined flask with a large excess of 99.2% *t*-butyl hydroperoxide[6] and the flask was attached to a rotary vacuum (60–70 mm.) evaporator. The reaction was initially exothermic with a rapid evolution of gas. It was then allowed to proceed for 24 hr. with occasional heating to 50–60° to complete the reaction, and then it was subjected to a vacuum of 2 mm. for several hours to remove the excess *t*-butyl hydroperoxide. A colorless residue was obtained which was nearly pure *t*-butyl peroxybenzoate contaminated with traces of benzoic acid. When an infrared spectrum of the residue was taken 10% in carbon tetrachloride and the intensity of the band at 1760 cm.$^{-1}$ compared with that of the infrared spectrum of an authentic sample of *t*-butyl peroxybenzoate, the estimated yield was nearly quantitative.

Di-*t*-butyl Diperoxysuccinate.—Succinyl chloride (5 g.) was mixed with 12 g. of 99.2% *t*-butyl hydroperoxide. The reaction was exothermic and had to be cooled under running cold water. As soon as the reaction subsided (3–4 min.) the flask was attached to a rotary vacuum (60–70 mm.) evaporator. A rapid evolution of gas took place and the reaction mixture almost solidified. In order to complete the reaction it was essential to heat it to 50–60° for 3 hr. longer under reduced pressure (60–70 mm.). Finally, the mixture was cooled to room temperature and dissolved in ethyl ether; the ethereal solution was shaken with a solution of sodium bicarbonate, dried with magnesium sulfate, and filtered; and the ether was removed *in vacuo*. A white cotton-like substance was obtained, yield 6.01 g. (71.2%), m.p. 53–54° (lit.[4a] m.p. 53–54°).

Di-*t*-butyl Diperoxyadipate.—Adipyl chloride (3 g.) was mixed with 9 g. of 99.2% *t*-butyl hydroperoxide. An exothermic reaction also occurred and had to be cooled as before. The mixture was treated and worked up in exactly the same manner as in the preceding case. Di-*t*-butyl diperoxyadipate was obtained as colorless needles, 4.06 g. (85.3%), m.p. 42–43° (lit.[4a] m.p. 42–45°).

Di-*t*-butyl Diperoxyazelate.—Azelayl chloride (5 g.) was mixed with 12 g. of 99.2% *t*-butyl hydroperoxide. A strong exothermic reaction took place and the mixture had to be cooled in cold running water so that the temperature was not allowed to rise above 35°. The reaction mixture became greenish yellow and when the reaction subsided (3–4 min.) the flask was attached to the rotary vacuum (60–70 mm.) evaporator and heated as before for 3 hr. at 50–60° to complete the reaction. The product was worked up as in the previous cases but it was obtained as a viscous oil which could not be crystallized, n^{22}D 1.4451, yield 6.9 g. (94.5%). The infrared spectrum showed two prominent bands at 1780 and 852 cm.$^{-1}$, respectively attributed to the *t*-butyl peroxy ester groups. Since this peroxy ester is new, it was subjected to elementary analysis. The active oxygen was determined by the method of Silbert and Swern.[7]

Anal. Calcd. for $C_{17}H_{32}O_6$: C, 61.42; H, 9.73; (O), 9.63. Found: C, 61.19; H, 9.75; (O), 9.63.

D-*t*-butyl Diperoxysebacate.—Sebacyl chloride (4.8 g.), precooled to 0°, was mixed with 10 g. of 99.2% *t*-butyl hydroperoxide. The reaction, as in the previous case, was highly exothermic and had to be cooled for a short time to about 35°. When the reaction had subsided (3–4 min.) the flask was attached to the rotary vacuum (60–70 mm.) and heated for 3 hr. at 50–60°. The reaction mixture was then worked up as in the previous cases. A colorless viscous oil, 6.5 g. (93.3%), n^{24}D 1.4454, was obtained which failed to crystallize. The infrared spectrum showed two prominent bands at 1780 and 852 cm.$^{-1}$, respectively attributed to the *t*-butyl peroxy ester groups. Since this peroxy ester was also a new product, it had to be analyzed.

Anal. Calcd. for $C_{13}H_{34}O_6$: C, 62.39; H, 9.89; (O), 9.24. Found: C, 62.15; H, 9.90; (O), 9.19.

Acknowledgment.—The authors wish to thank Dr. Nagy for the combustion analyses and the Lucidol Division of Wallace and Tiernan, Inc., and the Industrial Fund of Massachusetts Institute of Technology for financial support of this investigation.

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Solid Phase Peptide Synthesis. IV. The Synthesis of Methionyl-lysyl-bradykinin[1]

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A new plasma kinin was isolated recently by Elliott, *et al.*,[2] and identified by them as methionyl-lysyl-bradykinin. This undecapeptide was derived from ox blood by incubation of the pseudo-globulin fraction at pH 7.5, and was purified by ion-exchange chromatography. In continuation of our recent studies on solid phase peptide synthesis[3-6] the synthesis of this new biologically active peptide was undertaken. It was of interest, not only because of its kinin activity, but also because it contained two amino acids, lysine and methionine, which had not previously been introduced into peptides by the new method. In addition, it provided a test of the applicability of the method to the synthesis of a peptide containing eleven amino acid residues, which was longer than had been attempted before. While this work was in progress, a synthesis by classical methods was reported by Schröder.[7] The chemical and biological properties of the peptides made by the two different methods appear to be similar.

The synthesis followed exactly the general method described for the solid phase synthesis of bradykinin.[5,6] *t*-BOC-nitro-L-arginyl-L-prolyl-L-prolyl-glycyl-L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanyl-nitro-L-arginyl–copolystyrene–2% divinylben-

(1) Supported in part by Grant A 1260 from the U. S. Public Health Service.

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zene[8] was synthesized[6] in a stepwise manner from *t*-BOC-amino acids,[9-11] using dicyclohexylcarbodiimide[12] as the condensing agent. This protected nonapeptide, while still attached to the insoluble solid supporting resin, and while contained in the reaction vessel previously described,[4] was then lengthened by two amino acid residues in the following way. The *t*-BOC group was removed by 1 *N* HCl in acetic acid, and the resulting hydrochloride was neutralized with triethylamine in DMF. The nonapeptide derivative was then coupled by shaking with an excess of N^{α}-*t*-BOC-N^{ϵ}-Cbzo-L-lysine[10] and dicyclohexylcarbodiimide. Filtration and thorough washing of the totally insoluble product removed excess reagents and by-products. Again, the N-terminal *t*-BOC group was cleaved by HCl–acetic acid. The N^{ϵ}-Cbzo group, as well as the O-benzyl and guanidino-nitro substituents, was determined to be stable to this reagent. After neutralization, the decapeptide derivative was condensed with *t*-BOC-L-methionine[10,11] by the diimide reaction as before.

The *t*-BOC, O-benzyl, and N^{ϵ}-Cbzo groups and the substituted benzyl ester bond holding the peptide to the polystyrene resin were then all cleaved in one step by passing HBr through a suspension of protected undecapeptide resin in trifluoroacetic acid containing methyl ethyl sulfide.[13] The sulfide was necessary to prevent formation of the benzyl sulfonium derivative of methionine from the benzyl bromide derived from scission of O-benzyl and N^{ϵ}-Cbzo groups. Trifluoroacetic acid was used in preference to acetic acid to prevent acetylation of the serine residue. To remove the nitro substituents from the two arginine residues the product was hydrogenated with a freshly prepared palladium–black catalyst. The crude undecapeptide was then purified by chromatography on a column of IRC-50 resin (Fig. 1). The synthetic product was separated into one major component, comprising approximately 90% of the total Sakaguchi-positive material and traces of other substances which moved faster on the column. The undecapeptide was isolated from the main peak in an over-all yield of 65% calculated from the starting nonapeptide derivative. It was homogeneous by paper electrophoresis and paper chromatography. Analysis of an acid hydrolysate showed the calculated ratios for the constituent amino acids, and the elemental analysis was also satisfactory.

Leucine-aminopeptidase liberated approximately 1 mole of methionine and 1 mole of lysine, but did not attack the arginyl–prolyl bond. This is in agreement with the observation of Schröder[7] on this peptide, and of Meienhofer and Li[14] on a synthetic peptide of the ACTH series containing an arginyl–prolyl sequence,

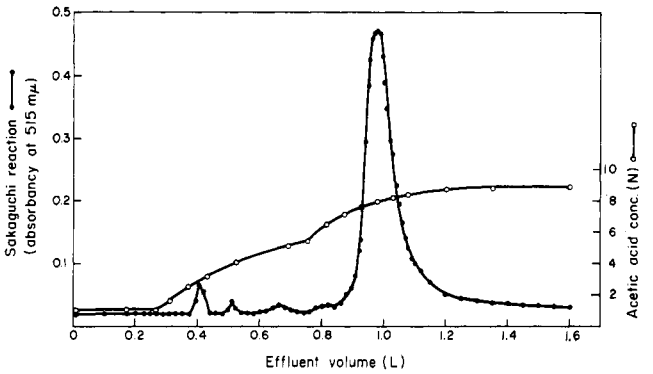

Fig. 1.—Chromatographic purification of synthetic methionyl-lysyl-bradykinin.

as well as with the early observation[15] on bradykinin itself. The high yields of these two amino acids produced by the enzyme indicated that racemization had not occurred during the addition of the lysine and methionine residues to the peptide chain.

The synthetic L-methionyl-L-lysyl-bradykinin was assayed for biological activity in the isolated rat uterus test.[16] The contraction caused by 3×10^{-9} g./ml. was approximately equivalent to that given by 1×10^{-9} g./ml. of bradykinin. Elliott, *et al.*,[2] reported an activity ratio of about 4:1 for bradykinin relative to the natural undecapeptide. Schröder[7] found a ratio of 3:1 for his synthetic preparation.

The present synthesis has demonstrated the applicability of the solid phase method to the rapid synthesis, in good yield, of a pure peptide containing as many as eleven amino acid residues, and has shown that methionine and lysine can be introduced into peptides in this way.

Experimental

N^{α}-*t*-BOC-N^{ϵ}-Cbzo-L-lysyl-nitro-L-arginyl-L-prolyl-L-prolyl-glycyl-L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanyl-nitro-L-arginyl–copolystyrene–2% Divinylbenzene.—A sample, 2.5 g., of *t*-BOC-nitro-L-arginyl-L-prolyl-L-prolyl-glycyl-L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanyl-nitro-L-arginyl–copolystyrene–2% divinylbenzene[6] (containing 0.15 mmole of peptide/g.) was placed in the reaction vessel described before[4] and suspended in 20 ml. of 1 *N* HCl in acetic acid. After a 30-min. shaking period the solvent was filtered through the fritted-glass disk at the bottom of the apparatus and the resin was washed three times each with acetic acid, ethanol, and dimethylformamide.[17] The resulting hydrochloride was neutralized by shaking for 10 min. with 20 ml. of DMF containing 2 ml. of triethylamine. The solvent was removed by suction and the product was freed of triethylamine by three 3-min. washes with DMF. A solution of 0.58 g. (1.5 mmoles) of N^{α}-*t*-BOC-N^{ϵ}-Cbzo-L-lysine[10] in 4 ml. of DMF was added. After shaking for 10 min., 0.62 ml. (1.5 mmoles) of a 0.5 g./ml. solution of N,N′-dicyclohexylcarbodiimide in DMF was added and the coupling was allowed to proceed for 2 hr. with shaking. The solvent was removed by suction and the resin was thoroughly washed with three 30-ml. portions each of DMF, ethanol, and acetic acid. In accord with the basic concept of solid phase peptide synthesis, this intermediate decapeptide derivative was not isolated or purified in any other way, but was used at once for the next step.

***t*-BOC-L-methionyl-N^{ϵ}-Cbzo-L-lysyl-nitro-L-arginyl-L-prolyl-L-prolyl-glycyl-L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanyl-nitro-L-arginyl–copolystyrene–2% Divinylbenzene.**—The peptide-resin from the previous step was deprotected with 1 *N*

(8) The following abbreviations are used: *t*-butyloxycarbonyl (*t*-BOC), benzyloxycarbonyl (Cbzo), and dimethylformamide (DMF). The designation—arginyl–copolystyrene–2% divinylbenzene—indicates the compound formed between the peptide and the resin in which the C-terminal arginine residue is bound to the resin by an ester linkage through hydroxymethyl side chains. The latter were derived by chloromethylation of the resin.

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HCl–acetic acid and converted to the free base with triethylamine as described above. A solution of 0.38 g. (1.5 mmoles) of *t*-BOC-L-methionine[10,11] in 4 ml. of DMF was added to the washed resin and the suspension was stirred for 10 min. This was followed by the addition of 0.62 ml. (1.5 mmoles) of a 0.5 g./ml. solution of N,N′-dicyclohexylcarbodiimide in DMF. After the mixture was shaken for 2 hr. the product was filtered and washed with DMF, ethanol, and acetic acid. A 50-mg. sample of the protected undecapeptide–resin was hydrolyzed and analyzed for amino acids by quantitative column chromatography.[18] The ratios were arg, 2.0; phe, 2.3; pro, 3.2; ser, 1.2; gly, 1.2; lys, 1.0; and met, 1.0. The average value of each of the eleven amino acid residues was 0.13 mmole/g.

L-Methionyl-L-lysyl-L-arginyl-L-prolyl-L-prolyl-glycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine.—The protected undecapeptide–resin, while still in the reaction vessel, was suspended in a mixture of 8 ml. of trifluoroacetic acid and 2 ml. of methyl ethyl sulfide, and a slow stream of hydrogen bromide was bubbled through for 90 min. The solution was withdrawn by suction and the resin was washed three times with 10 ml. of trifluoroacetic acid. The combined filtrates were evaporated to dryness under reduced pressure in the rotary evaporator and then in a desiccator over KOH. The solid was dissolved in acetic acid and lyophilized, and this step was repeated in order to free the product of volatile sulfur compounds.

The crude undecapeptide derivative was dissolved in 40 ml. of methanol containing 2 ml. of acetic acid and hydrogenated at 40 p.s.i. in the presence of palladium-black which had been prepared from 2 g. of $PdCl_2$.[19] The mixture was filtered and washed and the filtrate was evaporated to dryness. The undecapeptide was dissolved in acetic acid and lyophilized. For purification, 15% of the peptide was placed on a 2 × 98 cm. column of the cation-exchange resin, Amberlite IRC-50, and eluted at a rate of 15 ml./hr. with a gradient of acetic acid[6] (Fig. 1). The elution was followed by the Sakaguchi reaction,[20] carried out on 0.2-ml. aliquots of 7.5-ml. fractions. For isolation the fractions between 0.91 l. and 1.07 l. were combined, concentrated, and lyophilized to give 55 mg. of peptide. This was equivalent to an over-all yield of 366 mg. (65%) of purified undecapeptide from the 2.5 g. of starting *t*-BOC-nonapeptide–resin. The mobility relative to arginine (R_{arg}) was 0.75 (bradykinin, R_{arg} 0.62) by paper electrophoresis in 0.1 *M* (pH 5.0) pyridine acetate; and by paper chromatography, R_f 0.12 (propanol–H_2O, 2:1) and 0.19 (isoamyl alcohol–pyridine–H_2O, 35:35:30); $[\alpha]^{25}D$ −80° (*c* 0.5, *M* acetic acid). Amino acid ratios were arg, 2.00; phe, 2.11; pro, 2.89; gly, 1.09; ser, 1.05; lys, 1.05; and met, 0.95.

Anal.[21] Calcd. for $C_{61}H_{94}N_{18}O_{13}S\cdot 2CH_3COOH\cdot 2H_2O$: C, 52.9; H, 7.2; N, 17.1; CH_3COOH, 8.1. Found: C, 52.9; H, 7.1; N, 17.1; CH_3COOH, 8.5.

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A Stable Intermediate in the Hantzsch–Beyer Reaction

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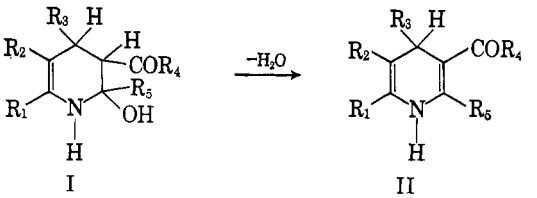
The occasional isolation of stable tetrahydropyridinols such as Ia and Ib, formed under Hantzsch–Beyer[1–3] reaction conditions, has been reported.[4] However, the structural assignments for these compounds are based upon little or no information other than combustion analysis. Hence, rather serious doubts have been expressed concerning the correctness of these structural assignments[5] since the compounds have not been clearly distinguished from the isomeric Michael adducts, their open-chain tautomers, or other isomeric ring structures.



I II

	R_1	R_2	R_3	R_4	R_5
Ia,	Me	CO_2Et	Ph	OEt	Ph
b,	Me	CN	Ph	OEt	Me
c,	Ph	CO_2Et	Ph	OEt	Me
IIa,	Me	CO_2Et	Ph	OEt	Ph
b,	Me	$COCH_3$	Ph	OEt	Ph

We have restudied one such reported compound, 2-hydroxy-2,4-diphenyl-3,5-dicarbethoxy-6-methyl-1,2,-3,4-tetrahydropyridine (Ia),[4] and have carefully examined its ultraviolet, infrared, and n.m.r. spectra for structural information.

Compound Ia was prepared in 78% yield by heating ethyl benzylidenebenzoylacetate and ethyl β-aminocrotonate for 3 days at 50–60° in absolute ethanol in the presence of a small amount of diethylamine. The ultraviolet spectrum showed a maximum at 278 mμ (log ε 4.21) which is near that for ethyl β-aminocrotonate [λ_{max} 276.5 mμ (log ε 4.21)] and therefore consistent with Ia. The infrared spectrum also supports the structure Ia, and pertinent band assignments are at 3410, NH; 1685, α,β-unsaturated ester carbonyl; 1720, hydrogen-bonded ester carbonyl[6]; and 1612 cm.$^{-1}$, carbonyl conjugated double bond. No free hydroxyl absorption was apparent, but the broadening of the NH band indicated overlap with hydrogen-bonded OH absorption.

The n.m.r. data are completely compatible with the structure Ia. The spectrum shows two ethyl ester groups, an olefinic methyl, two spin-coupled tertiary protons, a peak appropriate for exchanging OH and NH protons, and an aromatic resonance pattern, all with relative areas in accord with the given structure. The indirect spin–spin couplings and chemical shifts of the olefinic methyl and the two tertiary protons suggest the conformation illustrated in structure III. The doublet of closely spaced quartets located at 4.26 p.p.m. (benzyl hydrogen) has a chemical shift appropriate for the given structure, while the spin-coupling pattern indicates a large diaxial coupling to a single other proton and a small coupling typical of longer range couplings to three other protons. The olefinic methyl resonance at 2.32 p.p.m. is a doublet and shows a reciprocal coupling to the group at 4.26 p.p.m. A simple doublet

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