Use of Polymers as Chemical Reagents. II. Synthesis of Bradykinin

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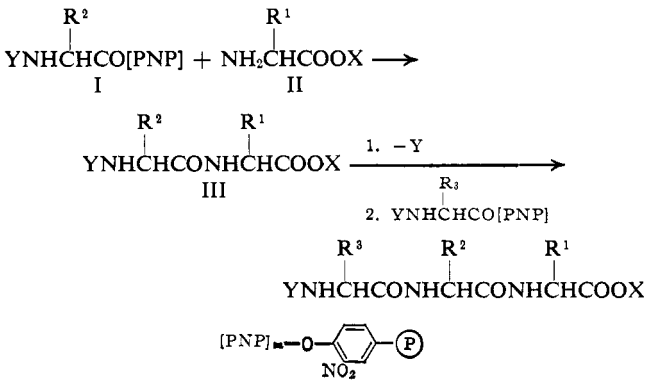
Abstract: Coupling of cross-linked poly-4-hydroxy-3-nitrostyrene by the dicyclohexylcarbodiimide method with the following N-blocked amino acids or peptide: benzyloxycarbonyl-L-phenylalanine, benzyloxycarbonyl-L-proline, N-*o*-nitrophenylsulfenyl-O-benzyl-L-serine, N-*o*-nitrophenylsulfenyl-L-phenylalanine, N-*o*-nitrophenylsulfenylglycine, N-*o*-nitrophenylsulfenyl-L-prolyl-L-proline, and benzyloxycarbonyl-nitro-L-arginine, yielded the corresponding polymeric insoluble active esters. When these were treated in dimethylformamide with a soluble amino acid or peptide ester, containing a free α-amino group, blocked peptides were formed. Removal of the N-blocking groups from the newly formed peptides and repetition of the coupling reaction with the appropriate polymeric active ester enabled the stepwise elongation of the peptide chain. The use of the insoluble active amino acid esters in excess ascertained the synthesis of the desired peptides in high yield and enabled the removal of excess of polymeric reagent by filtration or centrifugation. By the method developed it was possible to synthesize the blocked nonapeptide: benzyloxycarbonylnitro-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester in 65% yield from nitro-L-arginine *p*-nitrobenzyl ester and the polymeric active esters listed above. Removal of the blocking groups by treatment with liquid HF followed by catalytic reduction yielded fully biologically active bradykinin (L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine) in 39% yield.

The possible use of polymeric active esters of N-blocked amino acids in the synthesis of peptides has been illustrated in a previous communication.[1] An N-blocked amino acid ester of cross-linked poly-4-hydroxy-3-nitrostyrene (I) was treated in dimethylformamide with a soluble amino acid or peptide ester (II) containing a free α-amino group to give the blocked peptide III. Upon removal of the N-blocking group from the newly formed peptide it could be allowed to react with an insoluble active ester of another N-blocked amino acid. Repetition of this set of reactions led, by stepwise elongation of the peptide chain, to the formation of a peptide with the desired amino acid sequence (Scheme I). The use of the insoluble active amino acid

Scheme I



ester in excess ascertained the synthesis of the desired peptides in high yield (85–100%). The excess of the polymeric reagent could be quantitatively removed by filtration or centrifugation. The C- and N-blocked peptide (III) in the filtrate could be readily purified by extraction of the unreacted amino acid or peptide ester (II) with aqueous acid, after evaporation of solvent and dissolution in ethyl acetate. In our previous communication[1] we described the synthesis by the new method of several dipeptides and tripeptides. In the present article we describe the use of polymeric active esters of N-blocked amino acids in the synthesis of the nonapeptide hormone, bradykinin.

Bradykinin has been synthesized by classical techniques[2–5] as well as by the recently developed solid-phase method.[6] Our synthesis of the nonapeptide with the aid of the corresponding active amino acid esters of the insoluble poly-4-hydroxy-3-nitrostyrene is summarized in Scheme II. Nitro-L-arginine *p*-nitrobenzyl ester was coupled in dimethylformamide with excess polymeric insoluble active ester of benzyloxycarbonyl-L-phenylalanine (Z-Phe-[PNP]) to yield the dimethylformamide-soluble benzyloxycarbonyl-L-phenylalaninenitro-L-arginine *p*-nitrobenzyl ester (IV). Removal of its benzyloxycarbonyl-protecting group with HBr in glacial acetic acid and neutralization with triethylamine yielded the dipeptide ester L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester (IVb). Compound IVb was coupled with Z-Pro-[PNP] to give the dimethylformamide-soluble protected tripeptide benzyloxycarbonyl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester (V). Removal of the benzyloxycarbonyl group and neutralization with triethylamine as above yielded the tripeptide ester L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester (Vb). Compound Vb was coupled in dimethylformamide with excess polymeric insoluble active ester of *o*-nitrophenylsulfenyl-O-benzyl-L-serine (NPS-Ser(Bzl)-[PNP]) to give *o*-nitrophenylsulfenyl-O-benzyl-L-seryl-L-prolyl-L-

(1) M. Fridkin, A. Patchornik, and E. Katchalski, *J. Am. Chem. Soc.*, **88**, 3164 (1966).

(2) R. A. Boissonnas, S. Guttmann, and P. A. Jaquenoud, *Helv. Chim. Acta*, **43**, 1349 (1960).

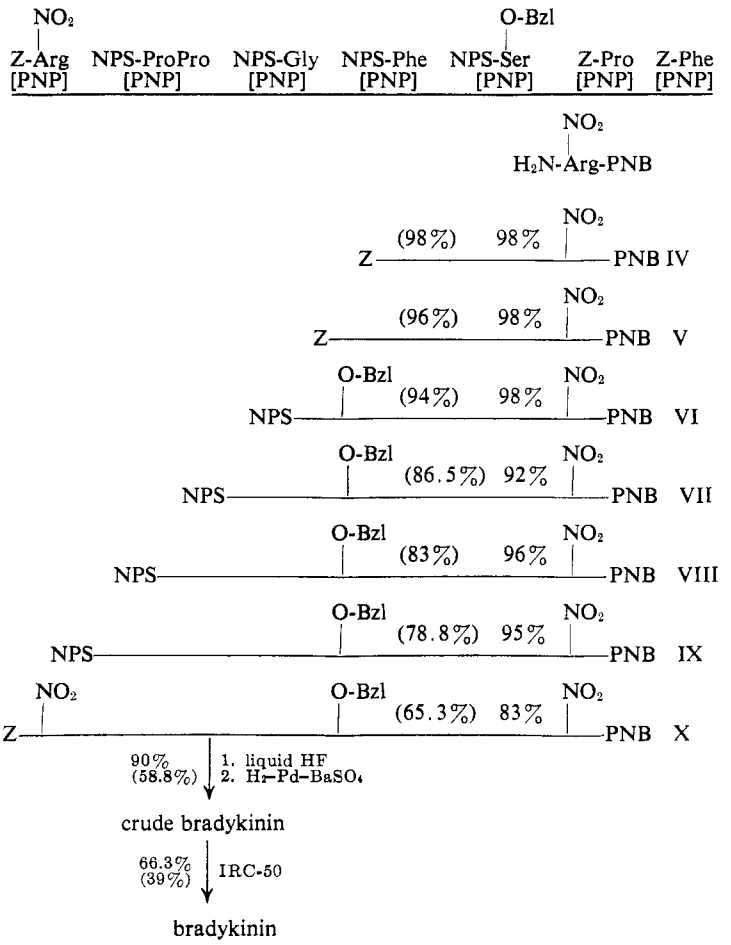
(3) E. D. Nicolaides and H. A. Dewald, *J. Org. Chem.*, **26**, 3872 (1961).

(4) S. Guttmann, J. Pless, and R. A. Boissannas, *Helv. Chim. Acta*, **45**, 170 (1962).

(5) S. Sakakibara and N. Inukai, *Bull. Chem. Soc. Japan*, **39**, 1567 (1966).

(6) R. B. Merrifield, *Biochemistry*, **3**, 1385 (1964).

Scheme II



phenylalanylnitro-L-arginine *p*-nitrobenzyl ester (VI). Removal of the *o*-nitrophenylsulfenyl-protecting group with 1 *N* HCl in methanol and neutralization gave the tetrapeptide ester O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester (VIb). Coupling of VIb with NPS-Phe-[PNP], isolation of the dimethylformamide-soluble blocked pentapeptide VII, and removal of the *o*-nitrophenylsulfenyl-protecting group gave the pentapeptide ester L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester (VIIb). Coupling of VIIb with NPS-Gly-[PNP] and treatment of the product liberated as above yielded glycyl-L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester (VIIIb). The hexapeptide ester VIIIb was treated with NPS-Pro-Pro-[PNP] to give *o*-nitrophenylsulfenyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester (IX). The *o*-nitrophenylsulfenyl-protecting group was removed as usual and the free ester was treated with Z-Arg(NO_2)-[PNP] to give the blocked derivative of bradykinin, benzyloxycarbonylnitro-L-arginyl-L-prolyl-L-prolyl-glycyl-L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester (X), in an over-all yield of 65%. Removal of the benzyloxycarbonyl-, nitro- and benzyl-protecting groups was effected with liquid HF.[7] The remaining protecting ester group (*p*-nitrobenzyl) was subsequently removed by catalytic hydrogenation. The crude nonapeptide thus obtained was purified on an IRC-50 ion-exchange resin to yield a bradykinin preparation possessing full biological activity in 39% yield.[8,9] The material obtained showed chromatographic and electrophoretic behavior identical with that of an authentic sample of bradykinin.

In the stepwise synthesis of bradykinin described above, terminal *o*-amino groups were blocked in the first two steps by benzyloxycarbonyl groups and thereafter by *o*-nitrophenylsulfenyl groups. The guanidino group of arginine was masked by a nitro group, whereas the OH of serine was blocked by a benzyl group. The *p*-nitrobenzyl group was used as a protecting group of the terminal carboxyl. The use of *o*-nitrophenylsulfenyl as a reversible masking group in the different polymeric active amino acid esters, starting with O-benzyl-L-serine (see Scheme II), was found necessary since removal of the benzyloxycarbonyl group with HBr in glacial acetic acid leads to partial debenzylation of O-benzyl-L-serine accompanied by acetylation.[10] The hydroxyl of the side chain of serine was blocked with a benzyl group since preliminary experiments have shown that when synthesis with polymeric active esters is carried out with an unmasked serine, marked losses in serine content occur during the removal of the *o*-nitrophenylsulfenyl-protecting groups with methanolic hydrochloric acid. The nitro group was chosen as a protecting group of the guanidine moiety of arginine because of its quantitative

(7) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).

(8) G. P. Lewis, *Nature*, **192**, 596 (1961).

(9) H. Edery, *Brit. J. Pharmacol.*, **24**, 485 (1965).

(10) S. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, **41**, 1852 (1958).

removal by treatment with liquid HF.[7] The *p*-nitrobenzyl masking group of the terminal carboxyl was retained on removal of the benzyloxycarbonyl- and the *o*-nitrophenylsulfenyl-protecting groups with HBr in glacial acetic acid and methanolic hydrochloric acid, respectively. Since treatment with liquid HF did not remove the *p*-nitrobenzyl group from the blocked nonapeptide it was necessary to unmask the carboxyl by catalytic hydrogenation to obtain the final product.

All of the polymeric active esters used in the synthesis of bradykinin, listed in Table I, were prepared by coupling the corresponding N-blocked amino acids or N-blocked peptide (NPS-Pro-Pro) with cross-linked poly-4-hydroxy-3-nitrostyrene, after activation by the dicyclohexylcarbodiimide (DCC) method.[11,12] The insoluble polymeric reagents obtained contained 1.0–2.0 mmoles of activated amino acid/g of polyester. Dimethylformamide and acetonitrile were found to be most useful solvents for the coupling reaction as they cause marked swelling of the polymer carrier. The amount of covalently bound amino acid or peptide in a polymeric active ester could be readily assayed by: (a) the increase in weight of the insoluble polymer as a result of the coupling reaction; (b) spectrophotometric determination of the amount of N-blocked amino acid liberated on short treatment with sodium methoxide, or on exhaustive acid hydrolysis; and (c) elemental analysis. Sulfur analysis was of particular use for the *o*-nitrophenylsulfenyl derivatives, as well as for the polymeric reagents containing cysteine and methionine. All three methods yielded similar results within the limits of experimental error.

The coupling reactions with polymeric active esters summarized in Scheme II were carried out in dimethylformamide for 8–12 hr, at room temperature. A fourfold molar excess of the polymeric reagent was used throughout. The purity of all of the intermediate peptides synthesized was checked on thin layer chromatography using at least two different solvent systems. The intermediate peptides were obtained in high yield (83–98%). Amino acid analysis of the peptides synthesized was carried out after exhaustive acid hydrolysis. A molar residue ratio close to the theoretical was found in all cases.

A comparison of the method for peptide synthesis described here with that of Merrifield[13] reveals that whereas in the "solid-phase peptide synthesis" it is the peptide which is bound to the insoluble carrier and the N-blocked amino acid active ester is added while in solution, in our case a solution of free peptide ester is added to an insoluble N-blocked amino acid active ester. Furthermore, purification of the intermediate peptides during synthesis can be readily effected in our method since these peptides are liberated into solution. In the Merrifield synthesis, on the other hand, peptide purification can be carried out only after detachment of the final product from the polymeric carrier.

Finally it is pertinent to note that in the new technique described, one can carry out the reaction between peptide ester and active amino acid ester in the presence of a large excess of insoluble active ester without markedly contaminating the reaction mixture, as the excess of the insoluble active ester can be readily removed by filtration or centrifugation. It is the excess of the polymeric amino acid active ester which ascertains the high yields of the peptides synthesized. The use of the amino acid polymer reagents in peptide synthesis obviously requires that the reacting amino acid or peptide esters be soluble in the solvents used. One might, therefore, expect that the new method developed will be of particular use in the synthesis of peptides, the blocked derivatives of which dissolve readily in the suitable organic solvents.

Experimental Section[14]

Synthesis of Cross-Linked Poly-4-hydroxy-3-nitrostyrene. 4-Acetoxystyrene and divinylbenzene, at a molar ratio of 100:4, were copolymerized according to the literature,[16] and the cross-linked polymer obtained (10 g) was nitrated by stirring with concentrated nitric acid (density 1.42, 200 ml) for 5 hr at 25°. The yellow suspension was poured into cold, distilled water (500 ml), and the nitrated polymer was filtered and washed with distilled water until the washings were neutral. The wet polymer was washed in addition with methanol and ether and dried *in vacuo* over sodium hydroxide pellets; yield, 12 g. The dried polymer gave on analysis N, 8.9; CH_3CO, 4.3. Removal of the remaining acetyl groups was effected by treatment with ammonia. The polymer (12 g) was suspended in dimethylformamide (100 ml) and concentrated aqueous ammonia (13.7 *M*, 5 ml) was added to the gel formed. The reaction mixture was kept at room temperature for 2 hr and poured into cold water with stirring, and the suspension formed was acidified with 3 *N* hydrochloric acid. The cross-linked nitro polymer was finally filtered, washed with water, methanol, and ether, and dried *in vacuo* over sodium hydroxide pellets; yield of brown polymer, 11.9 g.

Anal. Calcd for a 4% cross-linked (divinylbenzene) poly-4-hydroxy-3-nitrostyrene: N, 8.2; CH_3CO, 0.0. Found: N, 9.0; CH_3CO, 0.29.

N-Benzyloxycarbonyl-L-phenylalanyl Polymer (Z-Phe-[PNP]). Benzyloxycarbonyl-L-phenylalanine (12 g, 40 mmoles) in dimethylformamide (15 ml) or in acetonitrile (25 ml) was added to a suspension of cross-linked poly-4-hydroxy-3-nitrostyrene (5 g, containing 5.9 mmoles of OH groups/g) in dimethylformamide (50 ml) or in acetonitrile (30 ml). The reaction mixture was stirred for 10 min at 0°, whereupon DCC (8.24 g, 40 mmoles) in dimethylformamide (15 ml) or in acetonitrile (20 ml) was added. The reaction mixture was stirred for 1 hr at 0°, followed by 5 hr at room temperature. The Z-Phe-[PNP] formed was filtered and washed with three portions of hot methanol (250 ml each) and with ether; yield after drying *in vacuo*, 8.75 g. The Z-Phe-[PNP] synthesized contained 1.50 mmoles of N-benzyloxycarbonyl-L-phenylalanine/g, as determined by the various assays described below.

Polymer-Active Esters of Other N-Blocked Amino Acids. High molecular weight insoluble active esters of different N-blocked amino acids and peptides used in the synthesis of bradykinin were obtained by the coupling procedure given above for the preparation of Z-Phe-[PNP]. These are listed in Table I. It should be noted that under the experimental conditions of coupling used, the polymer was found to bind also *o*-nitrophenylsulfenyl-L-prolyl-L-proline.

Assay of the Content of Acyl Groups in the Insoluble Polymer-Active Esters Synthesized. The content of the various acyl residues in the high molecular weight active esters given in Table I could be determined by the following methods: (a) determination of the increase in weight of the cross-linked poly-4-hydroxy-3-nitrostyrene as a result of the coupling reaction; (b) determination of the amounts of free amino acids liberated on exhaustive acid hydrolysis

(11) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

(12) M. Fridkin, A. Patchornik, and E. Katchalski, *J. Am. Chem. Soc.*, **87**, 4646 (1965).

(13) R. B. Merrifield, *ibid.*, **85**, 2149 (1963).

(14) All melting points were taken on a capillary melting point apparatus and are uncorrected. Dimethylformamide was dried over molecular sieves (Fischer Scientific Co., type 5A) and fractionally distilled *in vacuo*. Thin-layer chromatography of the peptides synthesized was carried out on Merck's silica gel G. The following solvent system were used: system I, chloroform–methanol (9:1); system II, *n*-butyl alcohol–acetic acid–water (4:1:1); system III, *n*-butyl alcohol–pyridine–acetic acid: water (15:10:3:12); system IV, *n*-propyl alcohol–aqueous NH_4OH (13.7 *M*) (67:33). R_f values are uncorrected. The chlorine method[15] was used for the detection of N-blocked peptides.

(15) H. Zahn and E. Rexroth, *Z. Anal. Chem.*, **148**, 181 (1955).

(16) D. I. Packham, *J. Chem. Soc.*, 2617 (1964).

Table I. N-Blocked Amino Acid Esters of Cross-Linked Poly-4-hydroxy-3-nitrostyrene

Compound bound to polymer[a]	mmoles of amino acid or peptide bound per g of polyester[b]
Z-Phe	1.5
NPS-Phe	1.5
NPS-Gly	1.4
Z-Pro	1.4
NPS-Ser | Bzl	1.3
NPS-Pro-Pro	1.0
Z-Arg | NO_2	0.9

[a] Binding was effected in dimethylformamide by the DCC method.[11,12] [b] Assayed by the analytical methods described in the Experimental Section.

(The insoluble active ester (20–30 mg) was suspended in a mixture of acetic acid (1 ml) and 12 *N* hydrochloric acid (1 ml), and the suspension was refluxed for 24 hr. The insoluble polymer was filtered, and the amino acid content in the filtrate was determined by quantitative paper chromatography or with the aid of the amino acid analyzer, Beckman–Spinco Model 120C); (c) spectrophotometric determination of the benzyloxycarbonyl group in an alkaline hydrolysate (The insoluble active ester (20–30 mg) was suspended in ethanolic sodium hydroxide (15 ml, consisting of 5 ml of aqueous 1 *N* NaOH and 10 ml of ethanol), and the mixture shaken for 2 min at room temperature. The insoluble polymer was filtered and the amount of benzyloxycarbonyl in the filtrate was derived from optical density measurements at 257 mμ. The molar extinction coefficient of this group is ϵ_{257} 200); and (d) sulfur content. The content of *o*-nitrosophenylsulfenyl groups or of sulfur-containing amino acids could be derived from elemental sulfur analysis.[17]

General Procedure for the Preparation of Blocked Peptides. The required amino acid ester or peptide ester (0.5–1.0 mmole) in dimethylformamide (5 ml) was added to one of the acyl polymers listed in Table I (2 g, containing 2.0–4.0 mmoles of the acyl derivative) suspended in dimethylformamide (20 ml) in the form of highly swollen particles, and the reaction mixture was stirred for 8 hr at room temperature. The polymer was filtered and washed twice with 30-ml portions of dimethylformamide, and the filtrate and washings were combined and evaporated at low pressure (2 mm). The residue left was dissolved in wet ethyl acetate (150 ml), and the solution was washed with 1 *N* hydrochloric acid, 5% aqueous $NaHCO_3$, and water. Drying was effected with anhydrous sodium sulfate. The required, solid, pure product was obtained as a rule after evaporation of solvent *in vacuo*.

Benzyloxycarbonyl-L-phenylalanylnitro-L-arginine *p*-Nitrobenzyl Ester (IV). Nitro-L-arginine *p*-nitrobenzyl ester (354 mg, 1 mmole) in dimethylformamide (5 ml) was added to a suspension of Z-Phe-[PNP] (2.66 g containing 4 mmoles of phenylalanine in dimethylformamide (30 ml) and the reaction was stirred for 8 hr at room temperature. The mixture was then treated according to the general procedure above for the preparation of blocked peptides. Benzyloxycarbonyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester (620 mg, 98%) was obtained as a colorless powder after trituration with petroleum ether (bp 30–60°): mp 169°, $[\alpha]^{25}D$ −11° (*c* 0.4, methanol); lit.[17] mp 174°, $[\alpha]^{22}D$ −9.9 ± 1° (*c* 1.0 methanol); R_{fII} 0.83, R_{fIII} 0.79.

Benzyloxycarbonyl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-Nitrobenzyl Ester (V). L-Phenylalanylnitro-L-arginine *p*-nitrobenzyl ester hydrobromide was obtained on treatment of IV (620 mg) with 35% HBr in glacial acetic acid (2 ml) for 30 min at room temperature and precipitation with ether (750 ml). The hydrobromide was dissolved in dimethylformamide (5 ml) and neutralized with triethylamine, and the solution was added to a suspension of Z-Pro-[PNP] (2.85 g containing 4 mmoles of proline in dimethylformamide (30 ml). The mixture was stirred for 8 hr at room temperature and treated as above. Benzyloxycarbonyl-L-propyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester (700 mg) was obtained in 98% yield: mp 112° dec; lit.[2] mp 115° dec; R_{fII} 0.90, R_{fIII} 0.89, R_{fIII} 0.90. Compound V yielded L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester hydrobromide (Va) on treatment with HBr in glacial acetic acid as above: R_{fII} 0.61, R_{fIV} 0.85. The tripeptide ester gave on acid hydrolysis (6 *N* HCl, 22 hr) a molar amino acid ratio of Arg, 0.90; Phe, 1.00; and Pro, 1.10.

N-*o*-Nitrophenylsulfenyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-Nitrobenzyl Ester (VI). The coupling of L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester, derived from Va on neutralization with triethylamine, with NPS-Ser-(Bzl)-[PNP] (3.1 g containing 4 mmoles of serine) and treatment of the product formed were carried out according to the general procedure described. Compound VI was obtained in semisolid form: yield, 870 mg (98%); R_{fII} 0.86, R_{fIII} 0.91, R_{fIII} 0.93. O-Benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester hydrochloride (VIa) was obtained on removal of the *o*-nitrophenylsulfenyl-protecting group with 1 *N* HCl in methanol (3 ml, 5–10 min at room temperature) and precipitation with ether (750 ml); R_{fII} 0.63, R_{fIV} 0.76. A molar amino acid ratio of Arg, 1.00; Phe, 1.04; Pro, 1.00; and Ser, 0.85 was found after acid hydrolysis (6 *N* HCl, 22 hr).

***o*-Nitrophenylsulfenyl-L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-Nitrobenzyl Ester (VII).** The coupling of O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester, derived from VIa on neutralization with triethylamine, with NPS-Phe-[PNP] (2.66 g, containing 4 mmoles of phenylalanine) and treatment of the product formed was carried out as usual. Compound VII was obtained as a yellow powder: yield, 930 mg (92%); R_{fI} 0.84, R_{fII} 0.89, R_{fIII} 0.91. L-Phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester hydrochloride (VIIa) was obtained on removal of the *o*-nitrophenylsulfenyl-protecting group with 1 *N* HCl in methanol (3 ml) and precipitation with ether: R_{fII} 0.59, R_{fIII} 0.67. The molar amino acid ratio was Arg, 1.00; Phe, 2.36; Pro, 0.96; and Ser, 1.00.

***o*-Nitrophenylsulfenylglycyl-L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl Ester (VIII).** The coupling of L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester, derived from VIIa, with NPS-Gly-[PNP] (2.85 g, containing 4 mmoles of glycine) and purification of the product formed was carried out as usual. Compound VIII was obtained as a yellow powder: yield, 938 mg (96%); R_{fI} 0.80, R_{fII} 0.82, R_{fIII} 0.84. Glycyl-L-phenylalanyl-O-benzyl-L seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester hydrochloride (VIIIa) was obtained on removal of the *o*-nitrophenylsulfenyl-protecting group: R_{fII} 0.61, R_{fIII} 0.63.

***o*-Nitrophenylsulfenyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-Nitrobenzyl Ester (IX).** The coupling of glycyl-L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester, derived from VIIIa, with NPS-Pro-Pro-[PNP] (4 g, containing 4 mmoles of prolylproline) and purification of the product formed was carried out as usual: yield of oily yellow product, 1040 mg (95%); R_{fI} 0.88, R_{fII} 0.79, R_{fIII} 0.85; after crystallization from acetonitrile–ether, mp 118–125°, $[\alpha]^{25}D$ −72.1° (*c* 1.17, dimethylformamide). L-Prolyl-L-prolylglycyl-L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester hydrochloride (IXa) was obtained as a solid powder after removal of the *o*-nitrophenylsulfenyl-protecting group with 1 *N* HCl in methanol (3 ml), precipitation with ether, and trituration with isopropyl alcohol and ether: R_{fI} 0.61, R_{fIII} 0.68. The molar amino acid ratio was Arg, 1.00; Phe, 2.00; Pro, 3.00; Ser, 0.92; and Gly, 1.10.

Benzyloxycarbonylnitro-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-Nitrobenzyl Ester (X). The coupling of L-prolyl-L-prolylglycyl-L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine *p*-nitrobenzyl ester, derived from IXa, with Z-Arg-(NO_2)-[PNP] (4.4 g, containing 4 mmoles of arginine) and purification of the product formed were carried out as usual. The oily product obtained was crystallized from acetonitrile–ether: yield, 985 mg (83%); R_{fII} 0.75, R_{fIII} 0.85; mp 110–125°; $[\alpha]^{25}D$ −54.1° (*c* 1.08, dimethylformamide). Compound X gave on acid hydrolysis (6 *N* HCl, 22 hr) a molar amino acid ratio of Arg, 2.07; Phe, 2.00; Pro, 3.00; Ser, 0.94; and Gly, 1.00.

L-Arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine (Bradykinin). The benzyloxycarbonyl-, nitro-, and benzyl-protecting groups of X were removed with anhydrous hydrofloric acid according to Sakakibara.[7] Compound X (50 mg) was placed in a polyethylene test tube to which anisole

(17) W. Schöniger, *Z. Anal. Chem.*, **181**, 28 (1961).

(0.05 ml) and anhydrous HF (1.5 ml) were added. The colorless solution turned brown-red within several minutes. It was kept at room temperature for 60 min whereupon the hydrogen fluoride was evaporated in a stream of dry nitrogen gas. The residue was triturated with dry ether and the solvent was removed by centrifugation. The powder which had sedimented was dried under vacuum (1 mm).

Final removal of the *p*-nitrobenzyl-protecting group was effected by catalytic hydrogenation. The dry powder was dissolved in acetic acid (90%, 5 ml), 100 mg of 5% Pd on $BaSO_4$ was added, and the hydrogenation was carried out for 2 days at atmospheric pressure. An additional amount of catalyst (100 mg) was added and the hydrogenation was continued for another 2 days. The yellow solution became gradually colorless during the reduction. The catalyst was then filtered and washed with 85% acetic acid. The filtrate and washings were combined and evaporated to dryness under vacuum (1 mm). The residue was dissolved in a few milliliters of water and lyophilized to give a colorless solid preparation of crude bradykinin (48 mg). The crude nonapeptide yielded on acid hydrolysis (6 *N* HCl, 22 hr) a molar amino acid ratio of Arg, 2.05; Phe, 1.98; Pro, 2.98; Ser, 0.96; and Gly, 1.00. The homogeneity of the product obtained was checked by paper electrophoresis (pH 1.5, 3.5, and 6.5) and by paper chromatography using *n*-butyl alcohol–acetic acid–water (4:1:1), 80% pyridine, or 1-propyl alcohol–water (2:1) as developers. Comparison of the patterns obtained, using ninhydrin and Sagakuchi reagents, respectively, with the corresponding patterns given by an authentic sample of bradykinin (kindly supplied by Dr. Sakakibara) revealed that the crude bradykinin synthesized contained traces of arginine and *p*-toluidine and probably traces of unreduced nonapeptide ester. Final purification was effected on IRC-50 ion-exchange resin which had been equilibrated with 1 *M* acetic acid according to Merrifield.[6]

The pure bradykinin obtained (31 mg) yielded on acid hydrolysis: Arg, 2.12; Phe, 1.85; Pro, 3.05; Ser, 0.90; and Gly, 1.10. Its biological activity as assayed by the guinea pig ileum contraction test,[8] or by the Edery sensitization test,[9] was found identical with that of an authentic sample of the biologically active nonapeptide.

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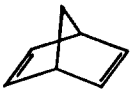
Communications to the Editor

Mechanisms of Photochemical Reactions in Solution. LIV.[1] A New Mechanism for Photosensitization

Sir:

In this and the accompanying report[2] we report what we believe to be the first clearly documented cases of photosensitized reactions in which the act of energy transfer apparently involves conversion of electronic to vibrational energy. The work is closely related to our studies of quenching of the fluorescence of aromatic compounds by substances having no low-lying excited singlet states.[3]

Since we have found that conjugated dienes are effective quenchers, a logical extension of the study was to look for reactivity in bicycloheptadiene (**1**). The compound contains two centers of unsaturation which interact strongly even though they are not in direct conjugation.



1

2

We find that **1** quenches the fluorescence of naphthalene with a rate constant of 1.3×10^7 l. mole^{-1} sec^{-1}. This rate is slightly slower than quenching by piperylene (3.4×10^7 l. mole^{-1} sec^{-1}) but is faster than quenching by cyclohexene or cyclopentene ($\sim 5 \times 10^6$ l. mole^{-1} sec^{-1}). In the course of the study we checked to determine whether or not quenching was accompanied by formation of quadricyclene (**2**) since the isomerization **1** → **2** has been accomplished by both direct excitation[4] and triplet energy transfer.[5] No detectable reaction occurred.

To our considerable surprise, we find that **2** *is a very reactive quencher and that quenching is accompanied by isomerization of* **2** *to* **1** *with an efficiency of about 50%.* The rate constant for the quenching reaction was measured by observing the reduction in the intensity of naphthalene fluorescence in the presence of varying concentrations of quadricyclene.

$$\frac{\tau_0}{\tau} = 1 + k_q\tau[Q] \qquad (1)$$

The value of k_q is 3.2×10^9 l. mole^{-1} sec^{-1}. With the concentration of **2** of 0.6 *M* the quantum yield for the production of **1** is 0.52 ± 0.01.

We visualize the mechanisms of quenching and isomerization are as shown in eq 2–6. $A^{*(1)}$ is an exciplex[6]

$$S \xrightarrow{h\nu} S^{*(1)} \qquad (2)$$

$$S^{*(1)} \xrightarrow{k_i} S^{*(3)} \qquad (3)$$

$$S^{*(1)} + 2 \xrightarrow{k_q} A^{*(1)} \qquad (4)$$

$$A^{*(1)} \xrightarrow{k_1} S + 1 \qquad (5)$$

$$A^{*(1)} \xrightarrow{k_2} S + 2 \qquad (6)$$

(1) Part LIII: H. Gotthardt, R. Steinmetz, and G. S. Hammond, submitted for publication.

(2) R. S. Cooke and G. S. Hammond, *J. Am. Chem. Soc.*, **90**, 2958 (1968).

(3) (a) L. M. Stephenson, D. G. Whitten, G. F. Vesley, and G. S. Hammond, *ibid.*, **88**, 3665 (1966); (b) L. M. Stephenson and G. S. Hammond, *Pure Appl. Chem.*, in press.

(4) W. G. Dauben and R. L. Cargill, *Tetrahedron*, **15**, 197 (1961).

(5) G. S. Hammond, N. J. Turro, and A. Fischer, *J. Am. Chem. Soc.*, **83**, 4674 (1961).

(6) The term "exciplex" is intended to be nonspecific as to the kind of binding forces involved. We are inclined to believe that weak attractions are generated by exciton interaction and possibly by charge transfer, since we expect the ground state of the complex to be essentially nonbonding. However, we have no way to rule out the possibility that localized bonds between the partners may be formed and eventually broken again. Specifically, a Schenck mechanism[7] cannot be entirely ruled out.