Studies on the Secondary Structure of Bradykinin in Aqueous Solution. Syntheses, Circular Dichroism Spectra, and Biological Activities of Bradykinin Amide and [D-Ala^4]-Bradykinin[1)]

Chikao HASHIMOTO,*,† Sukekatsu NOZAKI,†† and Ichiro MURAMATSU
Department of Chemistry, College of Science, Rikkyo University, Toshima-ku, Nishi-Ikebukuro, Tokyo 171
†† Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama 350-02
(Received July 9, 1991)

Bradykinin amide and [D-Ala^4]-bradykinin were synthesized by the 'hold-in-solution' method. The circular dichroism spectra of both peptides were measured and compared with that of bradykinin. These analogs exhibited weak activity (0.01% of bradykinin) in the increment of blood flow in a femoral artery of dog.

The secondary structure of bradykinin (BK), H–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg–OH, has been investigated by several research groups.[2–7)] It is known that the circular dichroism (CD) spectrum of BK shows troughs at about 200 and at 234 nm, and a peak at 221 nm. In our previous papers,[8,9)] on the basis of comparison of the CD spectra of several BK analogs containing a 5-aminovaleric acid residue, the presence of an intramolecular 3→1 hydrogen bond between the carbonyl oxygen of Ser^6 and the amide proton of Phe^8, an intramolecular 4→1 hydrogen bond between the carbonyl oxygen of Pro^2 and the amide proton of Phe^5, and a salt bridge between the guanidino group of Arg^1 and the carboxyl group of Arg^9 was emphasized for the secondary structure of BK in aqueous solution. Further, it was deduced that the intramolecular 3→1 hydrogen bond contributes to the trough at 234 nm and the intramolecular 4→1 hydrogen bond and the salt bridge contribute to the peak at 221 nm. Also, it was suggested that the trough at about 200 nm arises mainly from the sequences Arg^1–Pro^2–Pro^3–Gly^4 and Phe^5–Ser^6–Pro^7. The present study was carried out to investigate further the presence of the salt bridge and the intramolecular 4→1 hydrogen bond in the secondary structure of BK in aqueous solution. The syntheses, the CD spectra, and the biological activities of BK-amide and [D-Ala^4]-BK are described.

Results and Discussion

Synthesis. The side chains of Arg and Ser were protected by Mbs[10)] and Bzl, respectively. Protected BK and [D-Ala^4]-BK were synthesized by a stepwise elongation using H–Arg(Mbs)–ONBzl as a starting material, and Boc-amino acid derivatives as acylating reagents (Z derivative for the final acylation) by the 'hold-in-solution' method.[11–13)] The protected BK and [D-Ala^4]-BK were purified by gel chromatography on a Sephadex LH-20 column and a Sephadex LH-60 column, respectively. The protected BK was converted to the corresponding BK-amide by treatment with NH_3/MeOH. The protected BK-amide and [D-Ala^4]-BK were hydrogenolyzed and then treated with methanesulfonic acid.[14)] Both of the free peptides were converted to the acetate form by a Dowex 1×2 column, and further purified by ion-exchange chromatography on a carboxymethylcellulose column, gel chromatography on a Sephadex G-10 column, and finally, partition chromatography on a Sephadex G-25 column.[15)]

Circular Dichroism Spectra. The CD spectra were measured in the region of 190—270 nm. Free BK-amide and [D-Ala^4]-BK were dissolved in water. The CD data are reported as molar ellipticity, $[\theta]$, which is expressed in degrees square centimeters per decimole.

BK-amide can not form the salt bridge proposed by Ivanov et al.[5)] because of the lack of the C-terminal carboxyl group. Our previous study suggested that the salt bridge brings about the stabilization of the intramolecular 4→1 hydrogen bond and the increment of ellipticity at 221 nm.[8,9)] Therefore, the conversion of the C-terminal carboxyl group into an amide should have resulted in a change for this peak. The CD spectrum of BK-amide in Fig. 1 indicates a trough at 232 nm, a peak at 223 nm, and a deep trough at 202 nm. The peak at 223 nm was considerably lower compared with that of BK. This change can be postulated to be a result of a decrease in the stability of the intramolecular 4→1 hydrogen bond. In regard to the peak at 223 nm, the ellipticity of BK-amide was very similar to that of [5-Ava$^{1-2}$]-BK. [5-Ava$^{1-2}$]-BK also can not form the salt bridge because of the lack of the guanidino group of Arg^1. In spite of the modification of each site remote from others in the primary structure, the similarity of the ellipticities of both the peptides in this region strongly implies the presence of a salt bridge in the BK molecule.

Venkatachalam[16)] indicated that a type-II β-turn is stabilized by the introduction of a D-amino acid residue into the third position (i+2) of the turn. Therefore, the β-turn in the N-terminal part of BK generated by the 4→1 hydrogen bond should be stabilized by replacing the Gly^4 residue with a D-Ala residue. The CD spectrum of [D-Ala^4]-BK in Fig. 2 shows a trough at 202 nm,

† Present address: Department of Chemistry, The Jikei University School of Medicine, 8-3-1, Kokuryo-cho, Chofu-shi, Tokyo 182.

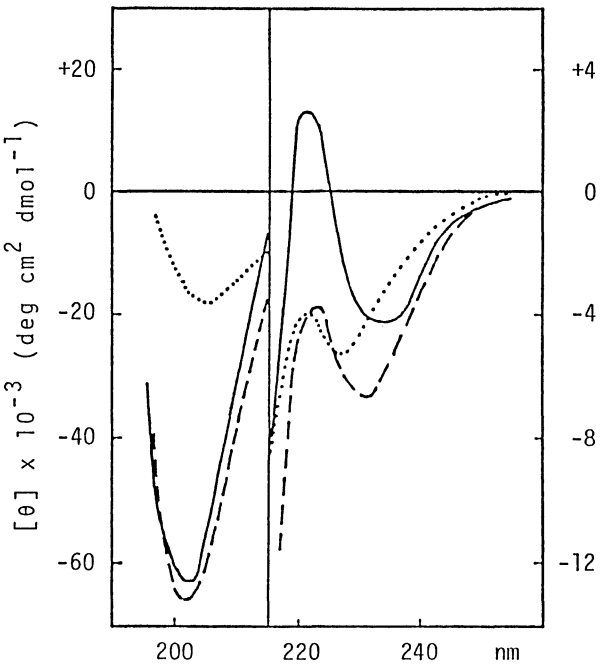

Fig. 1. CD spectra of BK-amide (----), [5-Ava[1-2]]-BK (·····),[8,9] and BK (——) in water.

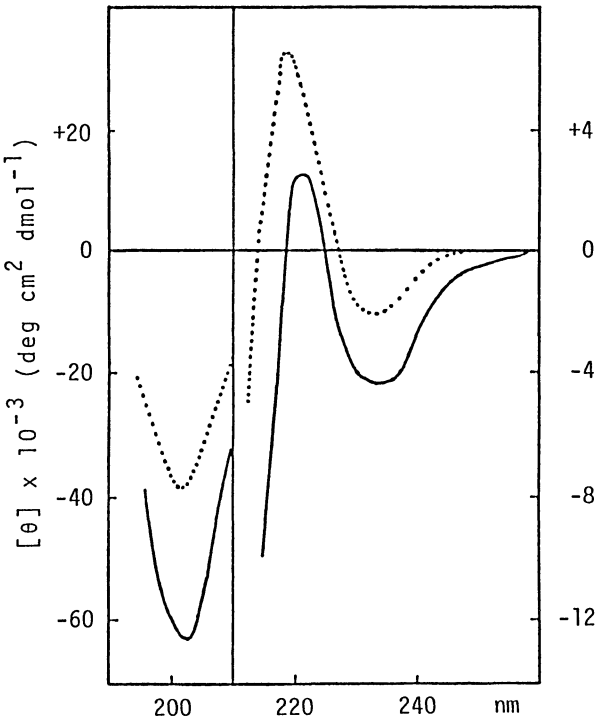

Fig. 2. CD spectra of [D-Ala[4]]-BK (·····) and BK (——) in water.

a high peak at 219 nm, and a trough at 234 nm. The increment of the ellipticity at 219 nm compared with BK seems to be based on stabilization of the intramolecular 4→1 hydrogen bond between the carbonyl oxygen of Pro[2] and the amide proton of Phe[5]; i.e., the β-turn. This hydrogen bond in BK has Pro and Gly residues at positions (i+1) and (i+2), respectively. It has been reported that the β-turn structure often involves Pro and Gly residues as preferable amino acids at positions (i+1) and (i+2) in the corner of the turn.[16,17] This indication does not conflict with our present conclusion.

As for the CD band near 234 nm, the troughs of BK-amide and [D-Ala[4]]-BK imply the presence of the intramolecular 3→1 hydrogen bond proposed by Cann et al.[4,6] in these peptides.

Regarding the trough near 200 nm, the spectrum of BK-amide was similar to that of BK. This result suggested that the C-terminal amide group does not contribute to the trough at about 200 nm. The trough of [D-Ala[4]]-BK was shallower than that of BK, that is, it may have been due to a conformational change of the amide bond of Pro[3]-Gly[4] caused by replacing the Gly[4] residue with a D-Ala residue. The present results are not in conflict with the previous conclusions that both of the sequences Arg[1]–Pro[2]–Pro[3]–Gly[4] and Phe[5]–Ser[6]–Pro[7] contribute to this band.[8,9]

Biological Activity. The biological activity of BK-amide and [D-Ala[4]]-BK was found to be 0.01% of that of BK in the increment of blood flow in a femoral artery of dog. The low activity of BK-amide was consistent with the fact that the addition of an amino acid residue to the carboxyl terminal of BK generally results in a lowering of biological activity. It showed that the carboxyl group of Arg[9] is important for biological activity.

The analogs of luteinizing hormone-releasing hormone,[18,19] enkephaline,[20] and motiline,[21] containing a D-Ala residue instead of the Gly residue in a sequence –Tyr–Gly– corresponding to positions (i+1) and (i+2) of the β-turn, showed very large activities compared with the corresponding native peptides. However, in the case of [D-Ala[4]]-BK, such a structure-activity correlation was not found.

Experimental

Melting points were measured by a capillary method and are given as uncorrected values. Optical rotations were determined with a Shimadzu polarimeter III or a DIP-370 digital polarimeter (JASCO). Amino acid analyses were performed on a JLC-6AS automatic analyzer (JEOL) or an L-8500 amino acid analyzer (Hitachi) after acid hydrolysis with 6 M (1 M=1 mol dm^{-3}) HCl in a sealed tube at 110 °C for 24 h. The theoretical values of amino acid ratios are shown in parentheses after each result.

Thin-layer chromatography was performed on TLC plates of silica gel 60 F_{254} (Merck and Co.) or cellulose (Avicel), using the following solvent systems (volume ratios): R_f^1, chloroform–methanol (95:5); R_f^2, chloroform–methanol (9:1); R_f^3, 1-butanol–acetic acid–water (4:1:1); R_f^4, 1-butanol–acetic acid–water (4:1:2); R_f^5, 1-butanol–pyridine–acetic acid–water (4:1:1:2); R_f^6, 1-butanol–pyridine–acetic acid–water (16:10:3:12); R_f^7, 1-butanol–pyridine–acetic acid–water (15:10:3:12).

The CD spectra were measured on a JASCO J-20 CD, ORD spectropolarimeter using a quartz cell with a path length of 0.50 mm. The measurements were carried out at room

temperature (24 °C) and at concentrations of 3.2×10^{-4} M and 1.5×10^{-3} M.

Biological activity was tested regarding the increment of blood flow in the femoral artery of dog according to a method similar to that in the previous paper.[9] The administration range of the analogs was $1\times(10^{-9}$—$10^{-4})$ g. The threshold dose of BK was 1×10^{-9} g, and its value was estimated as 100%. The activities of the analogs were expressed as relative activity compared with that of BK.

***Z*-Arg(Mbs)-Pro-Pro-Gly-Phe-Ser(Bzl)-Pro-Phe-Arg(Mbs)-ONBzl.** The protected BK was synthesized from H-Arg(Mbs)-ONBzl by the 'hold-in-solution' method, that is, by successive coupling with Boc-Phe-OH, Boc-Pro-OH, Boc-Ser(Bzl)-OH, Boc-Phe-OH, Boc-Gly-OH, Boc-Pro-OH, Boc-Pro-OH, and Z-Arg(Mbs)-OH. Each amino acid residue was introduced by repeating the following five steps. (1) Acylation of the amino component (1.0 mmol) in 1,2-dichloroethane (ca. 10 ml) at room temperature for several hours with a Boc-amino acid (Z-derivative for the final acylation) (2.0 mmol), EDC·HCl[22] (2.0 mmol), and HOBt[23] (2.0 mmol). (2) Washing with 0.1 M HCl, water, 5% Na_2CO_3, and water (removed by pipetting). (3) Cleavage of the Boc group by HCl/dioxane at room temperature for ca. 30 min. (4) Addition of a slight excess of 2 M Na_2CO_3 and removal of the aqueous phase. (5) Washing with water. The crude protected BK was purified by a Sephadex LH-20 column (1.6×116.5 cm) using DMF as eluent. Fractions 19—26 (4.0 g each) were combined and concentrated. The residue was triturated with diethyl ether; overall yield 1.047 g (59%). For analysis, a part of the product was further purified by a silica-gel column and recrystallization from chloroform–diethyl ether; mp 129—132 °C, $[\alpha]_D^{27.5}$ −35.6° (*c* 1.0, $CHCl_3$); R_f^1 0.22, R_f^2 0.54 (silica gel). Amino acid ratios in acid hydrolyzate: Ser 1.00 (1), Pro 3.13 (3), Gly 1.01 (1), Phe 1.92 (2), Arg 1.95 (2). Found: C, 56.99; H, 5.72; N, 12.78%. Calcd for $C_{86}H_{102}O_{21}N_{16}S_2\cdot2.5H_2O$: C, 57.23; H, 5.97; N, 12.42%. FAB mass: *m/z* 1759 (MH^+, $C_{86}H_{103}O_{21}N_{16}S_2$).

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-NH_2. The protected BK (300 mg) was dissolved in a mixture of MeOH (3.0 ml) and DMF (2.0 ml). The solution was saturated with NH_3 gas and then stirred at room temperature overnight. After removal of the solvent, the residue was solidified by the addition of diethyl ether. The product was dissolved in a mixture of DMF (8.0 ml), water (5.0 ml), and acetic acid (1.0 ml), and then hydrogenated over palladium black in a H_2 atmosphere for ca. 3 d. Water was added in several portions during the hydrogenation. After removal of the catalyst, the solvent was evaporated. The residue was treated with methanesulfonic acid (5.5 ml) containing anisole (0.20 ml) for 50 min at room temperature. Diethyl ether was added to this mixture. The solid was dissolved in water (2.5 ml) and the solution was passed through a Dowex 1×2 column (1.28×8.0 cm) (acetate form) with water as eluent. The eluate was concentrated and the residue was dissolved in ammonium acetate buffer (0.04 M, pH 6.0) (2.5 ml). The solution was applied to a carboxymethylcellulose column (1.7×22.5 cm: Whatman CM-52), and then eluted with ammonium acetate buffers (0.04—0.40 M, pH 6.0) by a linear gradient method. Fractions 68—82 (3.0 g each) were combined and concentrated. The residue was dissolved in 4% acetic acid (2.0 ml) and the solution was subjected to a Sephadex G-10 column (1.7×38.0 cm). Fractions 11—15 (2.5 g each) were combined and concentrated to give a first crop. Fractions 16—23 were also combined and concentrated. The residue was rechromatographed on a Sephadex G-10 column (1.7×38.5 cm) to obtain an additional crop. The combined crops were dissolved in 1-butanol–pyridine–acetic acid–water (16 : 10 : 3 : 12) (1.25 ml) and applied to a Sephadex G-25 column (1.7×23.9 cm). After elution by the same solvent, fractions 9—11 (3.0 g each) were collected and concentrated. The residue was lyophilized from water; yield 82.3 mg (36%); $[\alpha]_D^{24}$ −94.7° (*c* 0.10, H_2O); R_f^4 0.53, R_f^6 0.64, R_f^7 0.54 (cellulose). Amino acid ratios in acid hydrolyzate: Ser 1.18 (1), Pro 2.95 (3), Gly 1.01 (1), Phe 1.92 (2), Arg 1.94 (2), NH_3 1.07 (1). Found: C, 49.94; H, 6.57; N, 16.52%. Calcd for $C_{50}H_{74}O_{10}N_{16}\cdot3CH_3COOH\cdot6H_2O$: C, 49.92; H, 7.33; N, 16.63%.

Z-Arg(Mbs)-Pro-Pro-D-Ala-Phe-Ser(Bzl)-Pro-Phe-Arg(Mbs)-ONBzl. The protected [D-Ala⁴]-BK was synthesized from H-Arg(Mbs)-ONBzl (1.5 mmol) by successive coupling with Boc-Phe-OH, Boc-Pro-OH, Boc-Ser(Bzl)-OH, Boc-Phe-OH, Boc-D-Ala-OH, Boc-Pro-OH, Boc-Pro-OH, and Z-Arg(Mbs)-OH (3.0 mmol each) by the same method as described above. The residue was reprecipitated from methanol–diethyl ether (2.565 g). The crude protected [D-Ala⁴]-BK was purified by a Sephadex LH-60 column (1.7×135 cm) with DMF as eluent; overall yield 1.314 g (47%). For analysis, a part of the product was purified by a silica-gel column and recrystallization from methanol–ethyl acetate–diethyl ether; mp 121–123 °C; $[\alpha]_D^{27.5}$ −35.2° (*c* 1.0, $CHCl_3$); R_f^1 0.29, R_f^2 0.63 (silica gel). Amino acid ratios in acid hydrolyzate: Ser 0.98 (1), Pro 3.14 (3), Ala 1.01 (1), Phe 1.91 (2), Arg 1.97 (2). Found: C, 57.45; H, 6.11; N, 12.66%. Calcd for $C_{87}H_{104}O_{21}N_{16}S_2\cdot2.5H_2O$: C, 57.45; H, 6.04; N, 12.32%. FAB mass: *m/z* 1773 (MH^+, $C_{87}H_{105}O_{21}N_{16}S_2$).

H-Arg-Pro-Pro-D-Ala-Phe-Ser-Pro-Phe-Arg-OH. The protected [D-Ala⁴]-BK (350 mg) was dissolved in DMF–water–acetic acid (10, 5, and 1 ml) and then hydrogenated over palladium black for 4 d. After removal of the catalyst, the solvent was evaporated. The residue was treated with methanesulfonic acid (7.0 ml) containing anisole (0.5 ml) for 50 min at room temperature. The precipitates were obtained by the addition of diethyl ether. The solid was dissolved in water (3.0 ml) and the solution was passed through a Dowex 1×2 column (1.28×7.5 cm) (acetate form) with water as eluent. The eluate was combined and concentrated. The residue was dissolved in ammonium acetate buffer (0.04 M, pH 6.0) (3.0 ml) and the solution was chromatographed on a carboxymethylcellulose column (1.7×24.0 cm: Whatman CM-52) using ammonium acetate buffers (0.04—0.40 M, pH 6.0) by a linear gradient method. Fractions 68—76 (3.0 g each) were combined and concentrated. The residue was dissolved in 4% acetic acid (2.0 ml) and the solution was applied to a Sephadex G-10 column (1.7×39.8 cm) with the same solvent as eluent. Fractions of 2.5 g each were collected. Fractions 15—21 were combined and the solvent was removed. The residue was dissolved in 1-butanol–pyridine–acetic acid–water (4 : 1 : 1 : 2) (2.3 ml) and the solution was subjected to a Sephadex G-25 column (1.7×24.5 cm) with the same solvent as eluent. Fractions 28—34 (3.0 g each) were combined and the solvent was evaporated. The residue was further purified by the same chromatographic methods. The product was lyophilized from water; yield 53.24 mg (22%); $[\alpha]_D^{24}$ −63.7° (*c* 0.10, H_2O); R_f^3 0.59, R_f^5 0.65 (cellulose). Amino acid ratios in acid hydrolyzate: Ser 0.94 (1), Pro 2.92 (3), Ala 0.98 (1), Phe 2.03 (2), Arg 2.07 (2). Found: C, 52.23; H, 6.99; N, 15.82%. Calcd for $C_{51}H_{75}O_{11}N_{15}\cdot3CH_3COOH\cdot3H_2O$: C, 52.32; H, 7.16; N,

16.06%.

We are grateful to the Research Laboratory of Toyo Jozo Co., Ltd. for the elemental, amino acid, and FAB mass analyses, and to the Research Laboratory of Nihon Kayaku Co., Ltd. for the measurement of biological activity.

References

1) The amino acids used with the exception of glycine and D-alanine were in the L-configuration. The abbreviations used are those recommended by the IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN): *Eur. J. Biochem.*, **138**, 9 (1984). The following additional abbreviations are used: Mbs, *p*-methoxybenzenesulfonyl; Bzl, benzyl; ONBzl, *p*-nitrobenzyl ester; Boc, *t*-butoxycarbonyl; Z, benzyloxycarbonyl; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; DMF, *N,N*-dimethylformamide; FAB, Fast Atom Bombardment; 5-Ava, 5-aminovaleric acid.

2) A. Bodanszky, M. Bodanszky, E. J. Jorpes, V. Mutt, and M. A. Ondetti, *Experientia,* **26**, 948 (1970).

3) A. H. Brady, J. W. Ryan, and J. M. Stewart, *Biochem. J.*, **121**, 179 (1971).

4) J. R. Cann, J. M. Stewart, and G. R. Matsueda, *Biochemistry,* **12**, 3780 (1973).

5) V. T. Ivanov, M. P. Filatova, Z. Reissman, T. O. Reutova, E. S. Efremov, V. S. Pashkov, S. G. Galaktionov, G. L. Grigoryan, and Yu. A. Ovchinnikov, "Peptides," ed by R. Walter and J. Meienhofer, Ann Arbor Publishers, Michigan (1975), p. 151.

6) R. E. London, J. M. Stewart, J. R. Cann, and N. A. Matwiyoff, *Biochemistry,* **17**, 2270 (1978).

7) G. I. Chipens, F. K. Mutulis, N. V. Myshlyakova, I. P. Misina, R. O. Vitolina, V. J. Klusha, and B. S. Katayev, *Int. J. Pept. Protein Res.*, **26**, 460 (1985).

8) C. Hashimoto, M. Tamaki, H. Uchida, S. Nozaki, S. Sofuku, and I. Muramatsu, "Peptide Chemistry 1978," ed by N. Izumiya, Protein Research Foundation, Osaka (1979), p. 115.

9) C. Hashimoto, M. Tamaki, S. Sofuku, and I. Muramatsu, *Bull. Chem. Soc. Jpn.*, **64**, 1533 (1991).

10) O. Nishimura and M. Fujino, *Chem. Pharm. Bull.*, **24**, 1568 (1976).

11) I. Muramatsu, C. Hashimoto, A. Kimura, Y. Saika, and S. Nozaki, "Peptide Chemistry 1977," ed by T. Shiba, Protein Research Foundation, Osaka (1978), p. 61.

12) S. Nozaki, A. Kimura, and I. Muramatsu, *Chem. Lett.*, **1977**, 1057.

13) S. Nozaki and I. Muramatsu, *Bull. Chem. Soc. Jpn.*, **55**, 2165 (1982).

14) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii, and H. Irie, *Chem. Pharm. Bull.*, **23**, 1164 (1975).

15) D. Yamashiro, *Nature,* **201**, 76 (1964).

16) C. M. Venkatachalam, *Biopolymers,* **6**, 1425 (1968).

17) P. N. Lewis, F. A. Momany, and H. A. Scheraga, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 2293 (1971).

18) M. W. Monahan, M. S. Amoss, H. A. Anderson, and W. Vale, *Biochemistry,* **12**, 4616 (1973).

19) M. Fujino, I. Yamazaki, S. Kobayashi, T. Fukuda, S. Shinagawa, R. Nakayama, W. F. White, and R. H. Rippel, *Biochem. Biophys. Res. Commun.*, **57**, 1248 (1974).

20) D. H. Coy, A. J. Kastin, A. V. Schally, O. Morin, N. G. Caron, F. Labrie, J. M. Walker, R. Fertel, G. G. Berntson, and C. A. Sandman, *Biochem. Biophys. Res. Commun.*, **73**, 632 (1976).

21) S. Shinagawa, M. Fujino, H. Yajima, T. Segawa, and Y. Okuma, *Chem. Pharm. Bull.*, **26**, 880 (1978).

22) J. C. Sheehan, J. Preston, and P. A. Cruickshank, *J. Am. Chem. Soc.*, **87**, 2492 (1965).

23) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).