*Studies on the Synthesis of Peptides Containing Glutamine as the C-Terminal. I. Protection of Amide-nitrogen with Xanthyl Group during Peptide Synthesis**

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To date reports on the synthesis of glutaminyl peptides have been rare, because the chemically unstable amide group in glutamine makes preparation of these peptides difficult. Therefore the development of a practical method for synthesizing these peptides would be of particular interest in the study of the synthesis of peptides. The authors have attempted to find a new method especially for the synthesis of *C*-terminal glutaminyl peptides, as well as to improve earlier methods.

It is known that troublesome side reactions[1] occur during the synthesis of asparagine- and glutamine-peptides because of the instability of the β- and γ-amide groups respectively. For example, Sondheimer and Holley[2] synthesized peptides such as L-glutaminyl-L-leucine by the carbobenzoxy azide method, but their method could not be used to prepare peptides containing *C*-terminal glutamine. They reported also[3] that carbobenzoxyglutamine and its isomer, namely carbobenzoxyisoglutamine, could be obtained by normal alkaline saponification of carbobenzoxyglutamine methyl ester. During this treatment α-carbobenzoxyaminoglutarimide was found to be an intermediate; consequently the product was a mixture of α- and γ-amides. Similar reactions took place in the synthesis of asparagine series. It was also reported[4] that peptide derivatives, when treated in the same way, gave rise to a mixture of α- and γ-peptide derivatives.

Glutamine or glutaminyl peptides are labile even in neutral solution and are cyclized to pyrrolidonecarboxylic acid or to pyrroglutamyl peptides, respectively, merely by heating their aqueous solutions[5,6] or by passing them through a strongly acidic ion-exchange resin[7]. It has been found[8-10] that the application of tetraethylpyrrophosphite or *N,N'*-dicyclohexylcarbodiimide as a condensing reagent gave the cyano-compound as one of the products by intramolecular dehydration of the amide group during the formation of asparaginyl peptide bonds and, to a lesser extent, during the formation of glutaminyl peptide bonds.

Hofmann et al.[11] prepared L-methionyl-L-glutamine, intermediate for melanocite stimmulating hormone, from carbobenzoxy-L-methionine and L-glutamine by the mixed anhydride procedure. By the same method, Shiba et al.[5,12] and Rudinger et al.[13] synthesized L-pyrroglutamyl-L-glutaminyl-L-glutamine (fastigiatin) from carbobenzoxy-L-glutamic acid γ-methyl ester and L-glutaminyl-L-glutamine, and L-glutaminyl-L-glutamine (its intermediate) starting with carbobenzoxy-L-glutamic acid γ-methyl ester and L-glutamine. Vigneaud et al.[14] synthesized tosyl-L-isoleucyl-L-glutamine from tosyl-L-isoleucyl chloride and L-glutamine, but this procedure has not been used generally. These methods are limited to the reaction in which unprotected glutamine can be used.

In developing a method of preparation of these peptides which does not involve side reactions, the following considerations must be investigated. It is desirable to protect the amide nitrogen by a protective group which is easily removable after the formation of peptide bonds. This protective group should not be attacked by the reagents used for synthesis of the peptide. The optical activity of the amino acids or peptides should be maintained during the introduction and removal of the protective group.

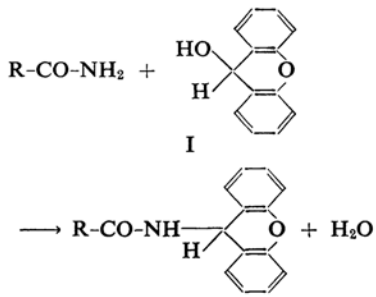
The author first tried the benzyl group as a

* Preliminary report, This Bulletin, **34**, 739 (1961).

1) J. Rudinger, *Angew. Chem.*, **71**, 742 (1959).
2) E. Sondheimer and R. W. Holley, *J. Am. Chem. Soc.*, **76**, 2816 (1954).
3) E. Sondheimer and R. W. Holley, ibid., **76**, 2467 (1954).
4) V. Bruckner, J. Kovacs and K. Medzihradszky, *Naturwiss.*, **42**, 96 (1955).
5) T. Shiba, S. Imai and T. Kaneko, This Bulletin, **31**, 244 (1958).
6) J. Melville, *Biochem. J.*, **29**, 179 (1935).
7) K. Narita, *Biochem. et Biophys. Acta*, **30**, 352 (1958).
8) D. T. Gish, P. G. Katsoyannis, G. P. Hess and R. J. Stedmann, *J. Am. Chem. Soc.*, **78**, 5954 (1956).
9) C. Ressler, ibid., **78**, 5956 (1956).
10) P. G. Katsoyannis, D. T. Gish, G. P. Hess and V. du Vigneaud, ibid., **80**, 2558 (1958).
11) K. Hofmann, T. A. Thompson and E. T. Schwarz, ibid., **79**, 6087 (1957).
12) T. Shiba and S. Imai, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **80**, 497 (1959).
13) J. Rudinger and Z. Pravoa, *Chem. Listy*, **52**, 120 (1958).
14) P. G. Katsoyannis and V. du Vigneaud, *J. Am. Chem. Soc.*, **76**, 3113 (1954).

protective group and prepared N^{α}-carbobenzoxy-N^{γ}-benzyl-L-glutamine from carbobenzoxy-L-glutamic acid γ-hydrazide, but the benzyl group could not be removed. Frankel et al.[15] also reported that they prepared poly-N^{β}-benzyl-asparagine in order to obtain poly-asparagine, but that the removal of the benzyl group was very difficult because of its unexpected stability.

Phillips et al.[16] reported that unsubstituted acyl amide reacted with xanthydrol (I) to give acyl xanthyl amide. The present authors applied the reaction to carbobenzoxy-L-glutamine (II) and N^{α}-carbobenzoxy-N^{γ}-xanthyl-L-glutamine (III) was obtained in a good yield.



The authors found that the N^{γ}-xanthyl group was comparatively stable in caustic alkalis, but very unstable in mineral acids. Both decarbobenzoxylation and dexanthylation took place, when III was treated with glacial acetic acid containing hydrogen bromide. From the reaction mixture, L-glutamine was recovered in a 55% yield by the usual procedures, and it had the same optical activity as glutamine which had been obtained directly from II. By catalytic reduction with Pd-charcoal only decarbobenzoxylation occurred, and N^{γ}-xanthyl-L-glutamine (IV) was obtained. Moreover, IV could not be prepared from glutamine and I in acetic acid. The xanthyl derivative III, obtained from carbobenzoxy-L-glutamine (II) with xanthydrol (I), was esterified to N^{α}-carbobenzoxy-N^{γ}-xanthyl-L-glutamine methyl ester (V) by means of diazomethane. After catalytic hydrogenation of V by Pd-charcoal in the presence of acetic acid, the acetate ester (VI) obtained was coupled with carbobenzoxy-glycine *p*-nitrophenyl ester[17] (VII, R=H) to form carbobenzoxy-glycyl-N^{γ}-xanthyl-L-glutamine methyl ester (VIII). Glycyl-L-glutamine

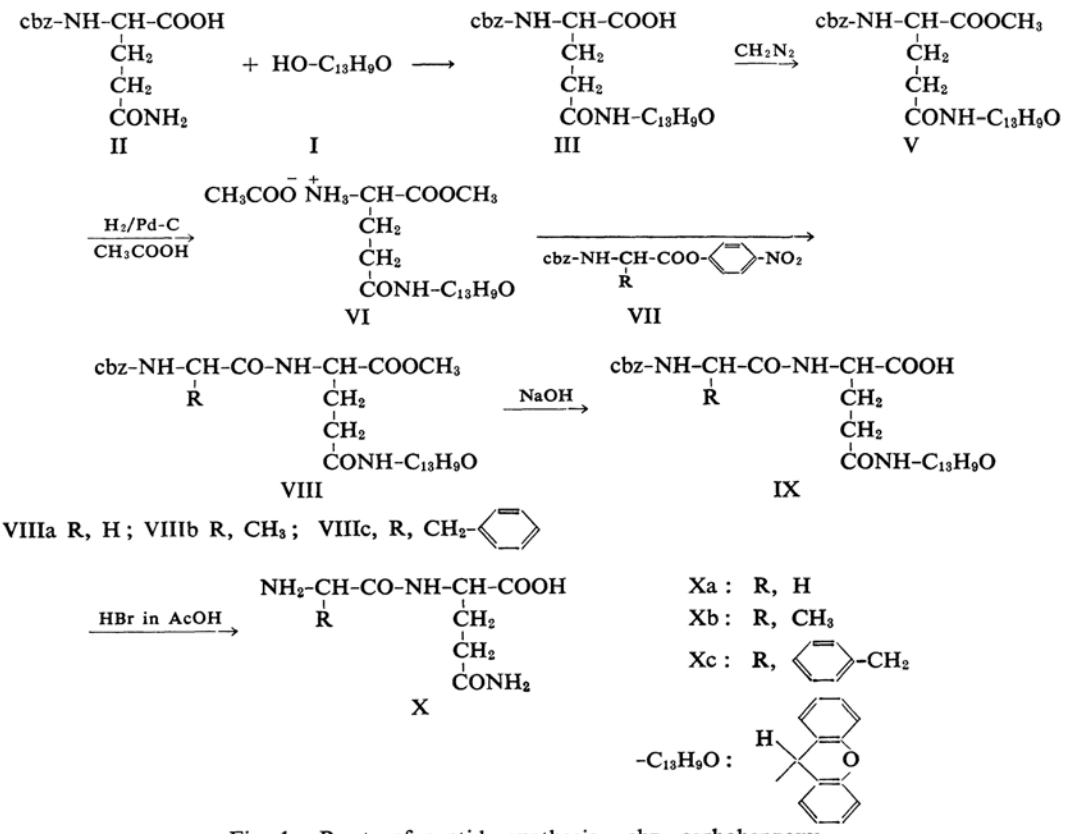


Fig. 1. Route of peptide synthesis. cbz=carbobenzoxy

15) M. Frankel, Y. Liwschitz and A. Zilkha, ibid., 75, 3270 (1953).

16) R. F. Phillips et al., *J. Org. Chem.*, 8, 1355 (1943).

17) R. Schwyzer and P. Sieber, *Angew. Chem.*, 68, 518 (1956).

(Xa) was obtained by treatment of carbobenzoxy-glycyl-N^{γ}-xanthyl-L-glutamine (IX, R=H) with hydrogen bromide, which had been prepared by saponification of VIII (R=H). The yield at each step of these reactions was more than 70%. The above synthetic route is shown in Fig. 1. Carbobenzoxy-L-alanyl- (VIIIb), -L-phenylalanyl (VIIIc) N^{γ}-xanthyl-L-glutamine methyl ester were synthesized from N^{γ}-xanthyl-L-glutamine methyl ester acetate (VI) and the respective carbobenzoxyamino acid *p*-nitrophenyl esters (VII) prepared by the procedure of Bodanszky et al.[18] After saponification of VIII, followed by treatment with hydrogen bromide, L-alanyl-L-glutamine (Xb) and L-phenylalanyl-L-glutamine (Xc) were obtained. This method, using the xanthyl derivatives, was superior to the methods used in earlier work in that there was less possibility of the occurrence of side reactions. A weak point is that the solubility of xanthyl derivatives in organic solvents is low. Therefore, it was difficult to observe the rotation of the xanthyl derivatives of peptides. An attempt was made to apply the protection resulting from the xanthyl group to asparagine derivative; however, N^{β}-xanthyl-L-asparagine methyl ester acetate, a starting material in peptide synthesis, could not be obtained from the catalytic hydrogenation of carbobenzoxy-N^{β}-xanthyl-L-asparagine methyl ester, which had been previously obtained by the reaction of carbobenzoxy-N^{β}-xanthyl-L-asparagine with diazomethane.

Experimental*

N^{α}-Carbobenzoxy-N^{γ}-xanthyl-L-glutamine.—Xanthydrol[19] (9.8 g., 0.050 mol.) and carbobenzoxy-L-glutamine (13.9 g., 0.050 mol.) were dissolved in 200 ml. of glacial acetic acid and the solution was allowed to stand for 2 days at room temperature. The gelatinous solid product was drained, filtered and dried in an alkali desiccator. The resulting white powder was recrystallized from tetrahydrofran to give colorless needles with a melting point at 182~183°C. The yield was 18.5 g. (80% of the theoretical amount). $[\alpha]_D^{17}=-5.7°$ (*c* 5.7, dimethylformamide).

Found: C, 66.36; H, 5.55; N, 6.13. Calcd. for $C_{26}H_{24}O_6N_2 \cdot 1/2H_2O$ C, 66.52; H, 5.37; N, 5.97%.

N^{α}-Carbobenzoxy-N^{γ}-xanthyl-L-glutamine Methyl Ester.—*Preparation of an Ethereal Solution of Diazomethane*[20]. — In a 200 ml. round bottomed flask were placed 12 ml. of 50% aqueous potassium hydroxide solution and 35 ml. of ether. The mixture was cooled to 5°C in an ice-bath, and 4.0 g. of nitrosomethylurea was slowly added with shaking. The upper layer was colored yellow by diazomethane. After the complete decomposition of nitrosomethylurea by alkali to diazomethane, the upper layer was decanted and dried over pellets of potassium hydroxide for two hours. A measured portion of this solution was allowed to react at 0°C with a solution of an accurately weighed excess of pure benzoic acid dissolved in absolute ether. The unreacted benzoic acid was titrated with standard N alkali. The yield of diazomethane was found to be about 70 per cent.

Preparation of the Ester (V).—The dry ethereal solution of diazomethane was slowly dropped, with stirring, into a suspension of 6.0 g. (0.013 mol.) of N^{α}-carbobenzoxy-N^{γ}-xanthyl-L-glutamine in 100 ml. of dioxane at 0~10°C. With the evolution of nitrogen gas the reaction proceeded smoothly. The mixture was kept overnight at room temperature in a flask, to which was attached a tube of mercury seal. The precipitate was filtered by suction, washed with ether and dried on sodium hydroxide in a vacuum desiccator. After recrystallization of the crude product (5.2 g., m. p. 235°C) from 150 ml. of dioxane, colorless needles were obtained, m. p. 235~235.5°C. The yield was 81% of the theoretical (5 g.). Titration by sodium methoxide solution showed the absence of free carboxylic acid.

Found: C, 68.21; H, 5.50; N, 5.94. Calcd. for $C_{27}H_{26}O_6N_2$: C, 68.34; H, 5.52; N, 5.90%.

N^{γ}-Xanthyl-L-glutamine. — N^{α}-Carbobenzoxy-N^{γ}-xanthyl-L-glutamine (4.6 g., 0.01 mol.) was hydrogenated in 150 ml. of ethanol under 70~80 atm. of H_2 at 75~80°C in the presence of Pd-charcoal catalyst, which was prepared from 10% palladium chloride in dilute hydrochloric acid (15 ml.) and charcoal (3 g.). The hydrogenated product was deposited on the catalyst. After 3 hr. the reaction mixture was cooled to room temperature. The mixture was filtered and washed three times with 20 ml. of hot ethanol. The residue on the filter paper was suspended in 60 ml. of water containing 1.5 ml. of triethylamine to dissolve the product deposited on the charcoal. After filtration, the filtrate and aqueous washings were combined and brought to pH 4.5~5.0 with 2 N hydrochloric acid. The precipitate was separated by centrifugation and washed with methanol and then with ether. 2.2 g. of N^{γ}-xanthyl-L-glutamine was obtained after drying in a desiccator, and purified by reprecipitation from 2 N ammonium hydroxide and 2 N hydrochloric acid. This compound melted at 218~220°C (60% of the theoretical).

Found: C, 65.17; H, 5.60; N, 8.94. Calcd. for $C_{18}H_{18}O_4N_2$: C, 66.24; H, 5.56; N, 8.58%.

N^{γ}-Xanthyl-L-glutamine Methyl Ester Acetate.—Five grams (0.01 mol.) of N^{α}-carbobenzoxy-N^{γ}-xanthyl-L-glutamine methyl ester were suspended in 130 ml. of methanol and palladium-charcoal catalyst, prepared freshly from 25 ml. of 1% palladium chloride solution, and 5 g. of charcoal were added. Three milliliters of glacial acetic acid were dropped into the above mixture. Hydrogen gas was allowed to bubble through the mixture for

18) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

* Melting points are uncorrected.

19) R. Meyer and E. Saul, *Ber.*, **26**, 1276 (1893).

20) F. Arndt, "Orgnic Syntheses", Coll. Vol. II (1948), pp. 165—167.

6 hr. at room temperature. The filtrate was concentrated under reduced pressure, at low temperature. The residue was dissolved in a small amount of methanol and the undissolved part was filtered off. Ether was added to the filtrate and a colorless crystalline product was obtained in a 67% yield (2.8 g.) which melted at 148~148.5°C. This material was ninhydrin positive and had an R_f value of 0.85 on paper chromatography in a solvent system of *n*-butanol : acetic acid : water (4 : 1 : 1).

Found: C, 62.72; H, 6.07; N, 6.90. Calcd. for $C_{21}H_{24}O_6N_2$: C, 62.99; H, 6.04; N, 7.00%.

Carbobenzoxy-glycyl-N^{γ}-xanthyl-L-glutamine Methyl Ester.—Carbobenzoxy-glycine *p*-nitrophenyl ester (3.5 g., 0.011 mol.) was added to an anhydrous solution of N^{γ}-xanthyl-L-glutamine methyl ester acetate (3.5 g., 0.0087 mol.) and triethylamine (1.2 ml., 0.0087 mol.) in chloroform according to the description of du Vigneaud[18]. The clear pale yellow solution was allowed to stand overnight at room temperature. The crystalline precipitate was filtered and washed with chloroform. After recrystallization of crude material (4.0 g.) from dioxane, fine colorless needles, which melted at 184°C, (3.5 g.) were obtained in a 76% yield.

Found: C, 65.49; H, 5.64; N, 7.89. Calcd. for $C_{29}H_{29}O_7N_3$: C, 65.52; H, 5.50; N, 7.91%.

Carbobenzoxy-L-alanyl-N^{γ}-xanthyl-L-glutamine Methyl Ester. — N^{γ}-Xanthyl-L-glutamine methyl ester, prepared from its acetate (2.0 g., 0.0050 mol.), was treated with carbobenzoxy-L-alanine *p*-nitrophenyl ester (2.4 g., 0.0070 mol.). Carbobenzoxy-L-alanyl-N^{γ}-xanthyl-L-glutamine methyl ester (2.4 g.) was obtained as a precipitate. Recrystallization from ethanol yielded colorless needles (1.4 g., 52% of the theoretical yield) with a melting point at 208°C (decomp.).

Found: C, 65.17; H, 5.69; N, 7.79. Calcd. for $C_{30}H_{31}O_7N_3$: C, 66.04; H, 5.73; N, 7.70%.

Carbobenzoxy-L-phenylalanyl-N^{γ}-xanthyl-L-glutamine Methyl Ester. — By the same treatment as above, crystalline precipitates (5.9 g.) were obtained in an 80% yield from N^{γ}-xanthyl-L-glutamine methyl ester acetate (4.8 g., 0.012 mol.) and carbobenzoxy-L-phenylalanine *p*-nitrophenyl ester (6.0 g., 0.014 mol.). M. p. 215~216°C.

Found: C, 69.60; H, 5.59; N, 6.59. Calcd. for $C_{36}H_{35}O_7N_3$: C, 69.55; H, 5.68; N, 6.76%

Carbobenzoxy-glycyl-N^{γ}-xanthyl-L-glutamine.— To a solution of 1.6 g. (0.003 mol.) of carbobenzoxy-glycyl-N^{γ}-xanthyl-L-glutamine methyl ester in 100 ml. of dioxane 30 ml. of 0.1 N sodium hydroxide was added (1.0 equiv.). After standing for an hour at room temperature (25°C), the solution was acidified to about pH 3.5 with N hydrochloric acid and concentrated under reduced pressure at low temperature. The residue was suspended in water and filtered. The resulting precipitate was dried in a desiccator and then dissolved in a warm mixture of methanol and toluene (4 : 1) and the insoluble material was filtered off. A small quantity of water was slowly added to the filtrate whereby IX (R=H) deposited in colorless needles. M. p. 198°C. The yield was 77% of the theoretical.

Found: C, 65.17; H, 5.39; N, 8.04. Neut. eq. 516. Calcd. for $C_{28}H_{27}O_7N_3$: C, 64.98; H, 5.26; N, 8.12%; mol. wt. 517.5.

Glycyl-L-glutamine. — Carbobenzoxyglycyl-N^{γ}-xanthyl-L-glutamine (1.5 g., 0.0029 mol.) was treated with 4.2 g. (5.0 equiv.) of 28.1 per cent glacial acetic acid containing hydrogen bromide. This solution was kept for about an hour at room temperature and dry ether was added where a colored material precipitated. Then the precipitate was washed with ether and the ether was decanted. The residue was dissolved in water and the undissolved material was filtered. The filtrate was passed through a weakly basic resin (Amberlite IR-4B) column, and the product was washed out of the column with a sufficient volume of water. The effluent was lyophilized, and the residue was dissolved in a small amount of water. On addition of ethanol to the solution, the final product was obtained as fine colorless crystals of the monohydrate (0.44 g., 70%), m. p. 260°C (decomp.). $[\alpha]_D^{15}=-1.8\pm0.5°$ (*c* 3.8, water). R_f value was 0.17 in solvent system (*n*-BuOH : AcOH : H_2O=4 : 1 : 1).

Found: C, 38.02; H, 7.02; N, 19.12. Calcd. for $C_7H_{13}O_4N_3\cdot H_2O$: C, 38.00; H, 6.84; N, 19.00%.

Thierfelder reported; m. p. 199~200°C. $[\alpha]_D^{15}=-2.47°$ (*c* 4.20, water).

L-Alanyl-L-glutamine. — One gram (1.83×10^{-3} mol.) of carbobenzoxy-L-alanyl-N^{γ}-xanthyl-L-glutamine methyl ester was saponified with 2.8 ml. (1.5 equiv.) of N sodium hydroxide in 100 ml. of dioxane for about 4 hr. at room temperature (15~20°C). The reaction mixture was concentrated to dryness after neutralization with dilute hydrochloric acid. The residue was crystallized from dioxane to yield a product (0.7 g.) having m. p. 138°C. This product was treated with 2.7 g. of 20% glacial acetic acid containing hydrogen bromide. This mixture was kept for 2 hr. at room temperature, and then dry ether (100 ml.) was added. A precipitate was obtained and the upper solution was decanted. The precipitate was dissolved in a small amount of water and the undissolved material was filtered. The filtrate was passed through an Amberlite IR-4B (OH-form) column. The effluent was lyophilized and the residue was recrystallized from water-ethanol as fine needles. One hundred milligrams of L-alanyl-L-glutamine were obtained as the monohydrate in about a 30% yield which melted at 214~215°C with decomposition. On paper chromatography it had an R_f value of 0.14 in the solvent system (*n*-BuOH : AcOH : H_2O=4 : 1 : 1). $[\alpha]_D^{14}=+11.4\pm0.5°$ (*c* 3.7, water).

Found: C, 40.42; H, 7.52; N, 17.82. Calcd. for $C_8H_{15}O_4N_3\cdot H_2O$: C, 40.84; H, 7.28; N, 17.86%.

Carbobenzoxy-L-phenylalanyl-N^{γ}-xanthyl-L-glutamine. — Carbobenzoxy-L-phenylalanyl-N^{γ}-xanthyl-L-glutamine methyl ester (2.6 g., 0.0042 mol.) was dissolved in 130 ml. of dimethylformamide and 6.3 ml. (1.5 equiv.) of N sodium hydroxide were added. After standing for 3 hr. at room temperature, the solution was neutralized with aqueous hydrochloric acid and then concentrated under reduced pressure. The residue was suspended in water, finely crushed with a rod, filtered by suction and dried in vacuo. Recrystallization of crude material (2.4 g., 188~

189.5° with decomposition) from methanol-water gave a crystalline product (1.7 g.) in a 68% yield. M. p. 188~189.5°C (decomp.). Neut. eq. 601, (mol. wt. 604).

L-Phenylalanyl-L-glutamine. — Carbobenzoxy-L-phenylalanyl-N^{γ}-xanthyl-L-glutamine (1.6 g., 0.0027 mol.) was treated with 5.0 g. (5 equiv.) of 21.4% glacial acetic acid solution containing hydrogen bromide. After the mixture was allowed to stand for 2 hr. at room temperature, a precipitate was obtained by adding 150 ml. of dry ether. The upper layer was decanted, and the residue was washed with dry ether and dissolved in a small amount of water. After filtration, the filtrate was passed through an Amberlite IR-4B (OH-form) column. The effluent was lyophilized and the residue was dissolved in water. Fine crystals were obtained in a 35% yield 0.26 g.) by adding ethanol. These melted at 209~210°C. $[\alpha]_D^{17}=+51.9\pm0.5°$ (*c* 4.0, in water).

Found: C, 57.55; H, 6.78; N, 14.45. Calcd. for $C_{14}H_{19}O_4N_3$: C, 57.32; H, 6.53; N, 14.33%.

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