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A Study of Unnatural Amino Acids and Their Peptides. III. The Syntheses of DL-β-(2-Pyridyl)-α-alanine and Its Peptides*1

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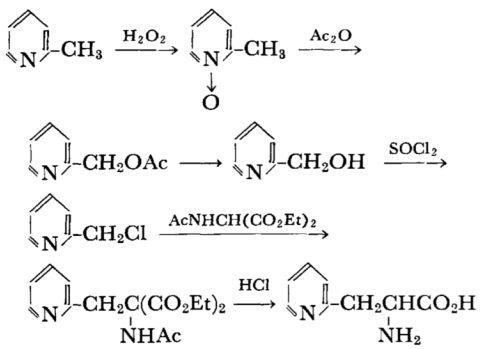
DL-β-(2-Pyridyl)-α-alanine has been synthesized in a good yield by the condensation of α-picolyl chloride and diethyl acetamidomalonate and by the subsequent hydrolysis of the product. The *N*-carbobenzoxy derivative of this amino acid, quite different from 3-pyridylalanine reported by Griffith and Harwood, could easily be prepared in a good yield. Only the carbodiimide method and the *N*-bromosuccinimide method proved to be successful in bringing the *N*-carbobenzoxylated amino acid into a peptide-coupling reaction with an amine component. Other methods, *i. e.*, the mixed anhydride method, the azide method and the *p*-nitrophenyl ester method, were unsuccessful. It is noteworthy that, in the former cases, the activation of the carboxyl group occurs *in situ* in the presence of an amine component. No difficulties were encountered in the coupling reaction of the alkyl ester of this amino acid with usual *N*-protected amino acids. The alkaline hydrolysis of the protected dipeptide ester afforded the *N*-protected peptide in a good yield. The catalytic hydrogenation could be used to remove the benzyl ester group as well as the carbobenzoxy group without any damage to the pyridine ring. Decarbobenzoxylation also proceeded smoothly with hydrogen bromide in acetic acid.

In connection with the previous reports[1] of this series, the syntheses of DL-β-(2-pyridyl)-α-alanine and its peptides will be reported in this paper. This amino acid had already been synthesized in several ways.[2-5] In this experiment it was prepared by the condensation of diethyl acetamidomalonate and α-picolyl chloride, easily obtainable from α-picoline, and by the subsequent hydrolysis of the condensation product. Both the condensation and the hydrolysis proceeded smoothly, and the hydrochloride of the amino acid was obtained in a good yield (Scheme I). By treating the hydrochloride with an ion exchange resin, there was obtained free amino acid, which gave a characteristic yellow-brown color in a ninhydrin test on paper.



Scheme I

The carbobenzoxylation of this free amino acid with carbobenzoxy chloride proceeded smoothly in water, with sodium hydroxide as the base, and gave the expected carbobenzoxy derivative in a 77% yield. According to Griffith and Harwood,[6] 3-pyridylalanine is carbobenzoxylated only in a low yield (34%) by usual methods; they attributed this low yield to "the ability of carbobenzoxy-3-pyridylalanine, once formed, to rapidly promote the destruction of unreacted carbobenzoxy chloride." They also examined the carbobenzoxylation of phenylalanine in the presence of an equivalent of pyridine or a one-third equivalent of carbobenzoxy-3-pyridylalanine and observed that "the yield of carbobenzoxyphenylalanine fell essentially to zero." They concluded from these facts that organic soluble pyridine derivatives facilitate the decomposition of carbobenzoxy chloride in aqueous alkali. Therefore, we attempted the carbobenzoxylation of phenylalanine under similar conditions, using 2-pyridylalanine instead of 3-pyridylalanine, with

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1) Part II: This Bulletin, **39**, 2473 (1966).
2) J. Overhoff, J. Boeke and A. Gorter, *Rec. trav. chim.*, **55**, 293 (1936).
3) C. Niemann, R. N. Lewis and J. T. Hays, *J. Am. Chem. Soc.*, **64**, 1678 (1942).
4) F. Zymalkowsky, *Arch. Pharm.*, **291**, 436 (1958).
5) S. J. Norton, C. G. Skinner and W. Shive, *J. Org. Chem.*, **26**, 1495 (1961).
6) R. K. Griffith and H. J. Harwood, *ibid.*, **29**, 2658 (1964).

a view to finding if there were any characteristics similar to those claimed for the latter amino acid derivative. The reaction, however, proceeded normally, and carbobenzoxyphenylalanine was isolated from the reaction mixture in a 90% yield; no unfavorable effect of this amino acid was observed during the carbobenzoxylation. A similar carbobenzoxylation of phenylalanine in the presence of picolinic acid afforded carbobenzoxyphenylalanine in a 78% yield. In the presence of an equivalent of pyridine, the yield of carbobenzoxyphenylalanine dropped to 42% (raw yield), but not to zero, in spite of the description of Griffith and Harwood.

The esterification of this amino acid proceeded smoothly in the presence of *p*-toluenesulfonic acid, giving 2-pyridylalanine benzyl ester in a 83% yield as *p*-toluenesulfonate.

To examine the applicability of common peptide synthetic methods to this amino acid, the coupling of carbobenzoxy-2-pyridylalanine and glycine benzyl ester was tried by the following four methods: mixed anhydride method, dicyclohexylcarbodiimide method, azide method, and *p*-nitrophenyl ester method.

(1) The mixed anhydride method: When ethyl chloroformate was added, drop by drop, to a dichloromethane solution of carbobenzoxy-2-pyridylalanine and an equivalent triethylamine, intense coloration (dark brown) occurred, even under strong cooling, and after the addition of the glycine ester, no expected dipeptide could be isolated from the reaction mixture. The replacement of triethylamine with a weaker base, *N*-ethylmorpholine, reduced the coloration of the reaction mixture, but the dipeptide could not be isolated in this case either.

(2) The dicyclohexylcarbodiimide method: When dicyclohexylcarbodiimide was stirred into a dichloromethane solution of carbobenzoxy-2-pyridylalanine, glycine benzyl ester *p*-toluenesulfonate, and an equivalent of triethylamine under ice-cooling, dicyclohexylurea soon began to be separated. When the reaction was carried out at room temperature, a strong coloration was observed, as in the preceding method, but under good cooling the color of the reaction mixture remained a pale yellow. After the usual treatment, the expected carbobenzoxy-dipeptide benzyl ester was isolated in a 75% yield.

(3) The azide method: Carbobenzoxy-2-pyridylalanylhydrazide could be obtained in a good yield from carbobenzoxy-2-pyridylalanine through its ester in a usual way. Into a solution of the hydrazide in hydrochloric acid, covered with ethyl acetate, there was stirred, drop by drop and under sufficient cooling, a cold concentrated solution of sodium nitrite and, after a few minutes, a 50% potassium carbonate solution. At this stage, a large quantity of an amorphous material, soluble in neither ethyl acetate nor an aquoeus alkaline solution, was separated out. When the ethyl acetate extract was made to react on the glycine ester, with or without this amorphous material, no expected dipeptide could be isolated. The amorphous material, obtained here during the preparation of the azide, melted at about 120°C. It afforded 2-pyridylalanine by hydrolysis, and its molecular weight was found to be 318; thus it was assumed to be carbobenzoxy-2-pyridylalanine amide, formed by the decomposition of carbobenzoxy-2-pyridylalanyl azide.

(4) The *p*-nitrophenyl ester method: To synthesize carbobenzoxy-2-pyridylalanine *p*-nitrophenyl ester, dicyclohexylcarbodiimide was added to a dichloromethane solution of carbobenzoxy-2-pyridylalanine and *p*-nitrophenol. After the removal of the precipitated dicyclohexylurea, the filtrate was, with or without purification, made to react on glycine benzyl ester, but no expected dipeptide could be isolated. This result can be attributed to the lack of success in the active ester synthesis rather than to that in the coupling reaction, because the attempts to obtain carbobenzoxy-2-pyridylalanine *p*-nitrophenyl ester in a crystalline state were all unsuccessful. Another attempt to prepare the *p*-nitrophenyl ester by the reaction of carbobenzoxy-2-pyridylalanine and *p*-nitrophenylsulfite in pyridine at 50°C afforded only a black, tarry material.

From these experiments, we concluded that, in the case of 2-pyridylalanine, it is very difficult to obtain the pyridylalanyl peptide by such methods as the previous activation of the carboxyl group of this amino acid and the subsequent addition of an amine component. On the contrary, only the activation of the acid component *in situ* in the presence of an amine component seems to give the desired peptide, as is the case with the dicyclohexylcarbodiimide method. To confirm these assumptions, another coupling method, the *N*-bromosuccinimide method, was attempted; this method permits the activation of an acid component in the presence of an amine component. According to the instructions of Wolman and his coworkers[7] *N*-bromosuccinimide was added in small portions to a mixture of carbobenzoxy-2-pyridylalanine, glycine benzyl ester *p*-toluenesulfonate, and three equivalents of triethylamine in tetrahydrofuran. From the reaction mixture, the desired dipeptide could be isolated, though the yield was very poor (about 6%).

When pyridylalanine was used as an amine component, no difficulty was encountered in coupling reactions with other ordinary *N*-protected amino acids. As an example, tritylglycine was

7) Y. Wolman, P. M. Gallop and A. Patchornik, *J. Am. Chem. Soc.*, **83**, 1263 (1961); Y. Wolman, P. M. Gallop, A. Patchornik and A. Berger, *ibid.*, **84**, 1889 (1962).

coupled with 2-pyridylalanine benzyl ester by the mixed anhydride method; tritylglycyl-2-pyridylalanine benzyl ester was thus obtained in a 48% yield.

The alkaline hydrolysis of dipeptide esters proceeds smoothly, on whichever side the pyridylalanine residue may be situated, thus enabling carbobenzoxy-pyridylalanylglycine or tritylglycyl-2-pyridylalanine to be obtained from its esters in a good yield.

In the case of peptide synthesis with this amino acid, another problem remains to be clarified: the stability of the pyridine ring during hydrogenation. In order to study this problem, tritylglycyl-2-pyridylalanine benzyl ester was hydrogenated in a quantitative hydrogenation apparatus, using palladium black as a catalyst. One mole equivalent of hydrogen was absorbed very quickly, but then the rate of hydrogenation decreased markedly; even after ten hours, the total volume of the absorbed hydrogen did not amount to two mole equivalents. When the incompletely hydrogenated contents were treated with hot aqueous acetic acid triphenylcarbinol was released, and from the filtrate glycylpyridylalanine was isolated in a chromatographycally-pure state. When carbobenzoxy-2-pyridylalanine was hydrogenated in the presence of sodium hydroxide in the same manner as has previously been reported,[1] one mole equivalent of hydrogen was absorbed very quickly, but then the rate of hydrogenation dropped markedly and only a small quantity of hydrogen was absorbed even after a longer period. All these facts show that the carbobenzoxy- and the benzyl ester-groups can be removed very easily by hydrogenolysis, but that the hydrogenolytic removal of the trityl group proceeds with difficulty, while the pyridine ring is preserved under these conditions. In fact, the simultaneous hydrogenolytic removal of the protecting groups from carbobenzoxy-2-pyridylalanylglycine benzyl ester proved to produce a good yield, accompanied by no reduction of the pyridine ring.

The removal of the carbobenzoxy group with hydrogen bromide in acetic acid proceeded smoothly, without any recognizable side reactions.

Experimental

2-Picolyl Chloride (I). According to the method of Itai and Ogura,[8] 2-pyridylmethanol,[9] obtained from α-picoline through its *N*-oxide and acetate of 2-pyridylmethanol, was converted into 2-picolyl chloride (I) by thionyl chloride. Bp 85—90°C/15 mmHg (lit.[8] bp 70—80°C/5 mmHg). Strongly-irritating pink liquid. Because of its strong resinifying tendency, it was used for the next step immediately after distillation.

Diethyl (2-Pyridylmethyl)-acetamidomalonate (II). To a solution of acetamidomalonate (50 g) and metallic sodium (5 g) in ethanol (300 m*l*), the above chloride (I) (28 g) was added, and then the mixture was stirred for 6 hr under reflux. After the removal of the precipitated sodium chloride by filtration, the filtrate was concentrated and water was added. The crystals separated were recrystallized from ethanol or acetone. Yield, 91%. Mp 92.5—93°C (lit.[4] 90—91°C).

DL-β-(2-Pyridyl)-α-alanine (III). The above malonate (II) (20 g) was boiled with 6 N hydrochloric acid for 8 hr. The solution was concentrated to dryness, and the residue was dissolved in water and treated with Amberlite CG-120 to obtain the free amino acid. Yield, 70%. Mp 200—208°C (lit.[4] 200—210°C). R_f*4= 0.30 (free amino acid); 0.21 (hydrochloride).

Found: C, 57.58; H, 6.06; N, 17.27%. Calcd for $C_8H_{10}O_2N_2$: C, 57.82; H, 6.07; N, 16.86%.

Carbobenzoxy-DL-β-(2-pyridyl)-α-alanine (IV). To a solution of (III) (16.6 g) and sodium hydroxide (4 g) in water (50 m*l*), carbobenzoxy chloride (19 g) and sodium hydroxide solution (sodium hydroxide 4 g; water 20 m*l*) were added under ice-cooling. When the reaction had finished, the mixture was adjusted to pH 4 with acetic acid. A white precipitate was collected by filtration, washed with water thoroughly, and recrystallized from aqueous alcohol to give colorless needles. Yield, 77%. Mp 145—147°C.

Found: C, 64.25; H, 5.45; N, 9.13%. Calcd for $C_{16}H_{16}O_4N_2$: C, 63.99; H, 5.37; N, 9.33%.

DL-β-(2-Pyridyl)-α-alanine Benzyl Ester Di-*p*-toluenesulfonate (V). A mixture of pyridylalanine (III) (16.6 g), *p*-toluenesulfonic acid monohydrate (45 g), benzyl alcohol (20 g), and benzene (200 m*l*) was boiled with occasional shaking and the continuous azeotropical removal of water. After 8.5 hr the solution was cooled, whereupon the crystals which had separated out were collected by filtration, washed thoroughly with ether, and recrystallized from alcohol-petroleum ether. Yield, 83%. Mp 158—159°C.

Found: C, 57.65; H, 5.27; N, 4.60%. Calcd for $C_{29}H_{32}O_8N_2S_2$: C, 57.98; H, 5.37; N, 4.66%.

Carbobenzoxy-DL-β-(2-pyridyl)-α-alanylhydrazide (VI). According to the method of Izumiya and his co-workers,[10] IV (40 g) was converted into the ethyl ester (yield 38.5 g); it was obtained as a yellow syrup and did not crystallize even after having been stored for a long time. To a methanol (120 m*l*) solution of the ester, 90% hydrazine hydrate (10 g) was added and the mixture was allowed to stand for 2 days at room temperature. When the clear solution had been cooled well and triturated, voluminous crystals were formed. Yield, 32.3 g (77.1% from IV). After recrystallization from ethyl acetate, it melted at 131—132°C into a turbid liquid, which became clear at 137°C.

Found: C, 61.14; H, 6.00; N, 17.78%. Calcd for $C_{16}H_{18}O_3N_4$: C, 61.13; H, 5.77; N, 17.83%.

8) T. Itai and H. Ogura, *Yakugaku Zasshi (J. Pharm. Soc. Japan)*, **75**, 296 (1955).
9) G. Kobayashi and S. Furukawa, *Chem. Pharm. Bull. Japan*, **1**, 347 (1953); V. Boekelheide and W. J. Linn, *J. Am. Chem. Soc.*, **76**, 1286 (1954).

*4 Circular paper chromatography. Solvent, *n*-BuOH : AcOH : H_2O=4 : 5 : 1.
10) T. Kato, K. Makisumi, M. Ono and N. Izumiya, *Nippon Kagaku Zasshi (J. Chem. Soc. Japan, Pure Chem. Sect.)*, **83**, 1151 (1962).

Carbobenzoxy-DL-β-(2-pyridyl)-α-alanyl-glycine Benzyl Ester (VII). *a) The Dicyclohexylcarbodiimide Method.* Into a cold solution of IV (3 g), glycine benzyl ester *p*-toluenesulfonate (3.8 g), and triethylamine (1.5 m*l*) in dichloromethane (80 m*l*), a solution of dicyclohexylcarbodiimide (2.1 g) in dichloromethane (2 m*l*) was stirred, drop by drop. After 2 hrs' stirring under ice-cooling, the mixture was allowed to stand overnight at room temperature. The dicyclohexylurea was then filtered off and washed with dichloromethane. The pale yellow filtrate and the washings were combined and washed successively with 0.5 N hydrochloric acid, N sodium hydroxide, and water, and dried over sodium sulfate. After the removal of the dicyclohexylurea which had separated during the evaporation of the solvent by filtration, the solution was concentrated to dryness. The viscous syrup which remained soon solidified completely. Yield, 3.2 g (72%). Mp 105—107°C (from ethanol).

Found: C, 67.01; H, 5.70; N, 9.26%. Calcd for $C_{25}H_{25}O_5N_3$: C, 67.10; H, 5.63; N, 9.39%.

b) The N-Bromosuccinimide Method. Carbobenzoxypyridylalanylhydrazide (1.6 g), glycine benzyl ester *p*-toluenesulfonate (1.7 g), and triethylamine (1.5 g) were dissolved in 30 m*l* of tetrahydrofuran. Into the cold solution solid *N*-bromosuccinimide (1.8 g) was then stirred in small portions. A remarkable evolution of gas was observed, and the color of the reaction mixture gradually changed to yellow brown. After the addition, the mixture was stirred for an additional 20 min, then diluted with water and extracted with ethyl acetate. The extract was washed successively with water, hydrochloric acid, a potassium carbonate solution, and water, and then dried over sodium sulfate and concentrated. The residual red-brown syrup (0.74 g) was dissolved in alcohol, from which mixture crystals were separated by letting it stand in a refrigerator overnight. Yield, 0.17 g (7.6%). Mp 105—107°C (from alcohol). The crystals showed no depression of the melting point when mixed with the preceding specimen.

c) An Attempted Coupling Reaction by the Azide Method. A solution of VI (942 mg) in 1 N hydrochloric acid (9 m*l*) was covered with ethyl acetate (12 m*l*), and then into the mixture there was stirred a solution of sodium nitrite (210 mg) in water (2 m*l*) at 0°C. After 2 min a 50% potassium carbonate solution (3.6 m*l*) was added, and the organic layer was separated. The aqueous layer was extracted with ether. At this stage a large amount of a solid material soluble in neither ethyl acetate and ether nor water was separated. This solid, insoluble also in aqueous alkali, melted at about 120°C. A paper chromatogram of the hydrolysate with hydrochloric acid showed one spot identical with that of an authentic sample of the hydrochloride of pyridylalanine. The molecular weight was determined to be 318 by a cryoscopic method, using 2,4,6-tribromophenol as the solvent. From these evidences, this substance was assumed to be carbobenzoxy-DL-β-(2-pyridyl)-α-alanine amide (molecular weight 299). To the organic layer mentioned above, after it had been dried over sodium sulfate, there was added an ether solution of the glycine benzyl ester (obtained from 1.18 g of glycine benzyl ester *p*-toluenesulfonate), but from the mixture no dipeptide derivative could be obtained.

Carbobenzoxy-DL-β-(2-pyridyl)-α-alanyl-glycine (VIII). VII (2.3 g) was dissolved in methanol (60 m*l*) and hydrolyzed with N sodium hydroxide (6 m*l*) for 1 hr at room temperature. The solution was then diluted with water and treated with active charcoal. The clear solution was acidified with acetic acid and allowed to stand overnight in a refrigerator. The crystals which thus separated were collected and washed with water. Yield, 1.3 g (72%). Mp 178—179°C.

Found: C, 60.36; H, 5.35; N, 11.62%. Calcd for $C_{18}H_{19}O_5N_3$: C, 60.49; H, 5.36; N, 11.76%.

DL-β-(2-Pyridyl)-α-alanyl-glycine (IX). *a) By the Hydrogenolysis of VII.* Hydrogen gas was allowed to bubble through a solution of VII (4.0 g) in methanol (150 m*l*) for 4 hr in the presence of a Pd-catalyst (0.4 g). The catalyst was then filtered off, and a large amount of water was added to the filtrate. After treatment with active charcoal, the clear solution was concentrated *in vacuo* to a small volume, and then ethanol was added. The hydrated dipeptide was separated as needles (sintered at about 80°C), which became anhydrous upon being dried at 110°C *in vacuo*. Yield, 1.5 g (75%). Mp 179—180°C. A paper chromatogram showed only one spot (R_f*4=0.55).

Found: C, 53.23; H, 5.88; N, 18.78%. Calcd for $C_{10}H_{13}O_3N_3$: C, 53.80; H, 5.87; N, 18.83%

b) By the Action of Hydrogen Bromide in Acetic Acid on VIII. A mixture of VIII (0.36 g) and 20% hydrogen bromide in an acetic acid solution (*ca.* 2 m*l*) was kept for 5 min at about 25°C. Ether was then added, and the crystals were collected and washed several times with ether. Yield, 0.42 g (quantitative). Mp 169—171°C (decomp.). A paper chromatogram showed only one spot. The free dipeptide obtained by the passage of the hydrobromide through the column of an ion exchange resin showed a chromatogram identical with that of the sample obtained by the method *a*. The hydrobromide of the dipeptide obtained by the method *a* melted at 165—167°C (decomp.) and showed no depression of melting point when mixed with the hydrobromide obtained by the method *b*.

Tritylglycyl-DL-β-(2-pyridyl)-α-alanine Benzyl Ester (X). To a stirred solution of tritylglycine (4.7 g) and triethylamine (2.1 m*l*) in dichloromethane (100 m*l*), ethyl chlorocarbonate (1.43 m*l*) was added, drop by drop, under strong cooling. To this mixture, after an additional 30 mins' stirring, there was added a solution of V (9 g) and triethylamine (4.2 m*l*) in dichloromethane (400 m*l*). The solution was left to stand overnight at room temperature, then washed with an aqueous citric acid, an aqueous sodium carbonate, and water, and dried over sodium sulfate. When the solvent was evaporated off, there remained a viscous syrup (6 g) which crystallized when triturated with ethyl acetate and petroleum ether. Yield, 4 g (48%). Mp 119—121°C (large cubes from ethyl acetate).

Found: C, 77.75; H, 6.32; N, 7.22%. Calcd for $C_{36}H_{33}O_3N_3$: C, 77.81; H, 5.99; N, 7.56%.

Tritylglycyl-DL-β-(2-pyridyl)-α-alanine (XI). X (666 mg) was hydrolyzed with N sodium hydroxide (1.5 m*l*) in methanol (20 m*l*) for about 2 hr at room temperature and then treated as usual. Amorphous powder. Yield, 97%. Mp 93—95°C.

Found: C, 74.47; H, 5.69; N, 8.47%. Calcd for $C_{29}H_{27}O_3N_3$: C, 74.82; H, 5.85; N, 9.03%.

Glycyl-DL-β-(2-pyridyl)-α-alanine (XII). *a) From X.* X (2.23 g) was hydrogenated in aqueous

methanol (methanol 100 m*l*; water 20 m*l*) in the presence of a Pd-catalyst (0.2 g) with a quantitative hydrogenating apparatus. In about 50 min 97 m*l* of hydrogen was absorbed, but thereafter the rate of the absorption became so slow that even after 4 hr the volume of hydrogen absorbed amounted only to 34 m*l*. The solution, freed from the catalyst, was evaporated under reduced pressure. The residue was treated with 50% acetic acid on a boiling-water bath for 2 min; water was then added, and the triphenylcarbinol thus separated was filtered off. The filtrate decolorized with active charcoal was concentrated to dryness under reduced pressure. Yield, 0.33 g (37%). Mp 196—200°C (from water - ethanol). A paper chromatogram showed only one spot, $R_f^{*4}=0.43$.

Found: C, 49.51; H, 6.31; N, 17.56%. Calcd for $C_{10}H_{13}O_3N_3 \cdot H_2O$: C, 49.78; H, 6.27; N, 17.42%.

b) From XI. **XI** (200 mg) was dissolved in 70% acetic acid (5 m*l*) and warmed to about 70°C for 10 min. Water was then added to the reaction mixture, and it was treated as above. Yield, 82 mg (58%). A paper chromatogram afforded an R_f value identical with that of the preceding specimen.