BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 43 2874—2877 (1970)

Studies of Unnatural Amino Acids and Their Peptides. IV. The Syntheses of DL-β-(5-Cytosinyl)-α-alanine Peptides

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(Received December 22, 1969)

DL-β-(5-Cytosinyl)-α-alanine and its dipeptides with glycine were synthesized. The benzyloxycarbonylation of this amino acid afforded the α-N-substituted derivative, but all attempts to obtain the diacylated derivative were unsuccessful. The condensation of the benzyloxycarbonyl derivative with glycine ethyl ester by the carbodiimide method afforded the expected dipeptide, though the yield was low (18%); it was accompanied by a large amount of a bicyclic compound, a lactam produced by an intramolecular acylation. The ethyl ester hydrochloride of this amino acid could not be obtained in a pure state, probably because of its ready cyclization to the same lactam structure under weakly acidic conditions. This complication could be avoided by using the tertiary butyl in place of the ethyl ester. Benzyloxycarbonylglycine *p*-nitrophenyl ester was condensed smoothly with this ester, affording the expected dipeptide in a 53% yield. The carbodiimide method could also be used, but it was less effective.

As a part of a series of studies of unnatural amino acids and their peptides, DL-β-(5-cytosinyl)-α-alanine derivatives were investigated. The present authors were interested in this amino acid not only biologically, because cytosine is one of the constituents of nucleic acid, but also chemically, because it had become clear that the usual method a synthesizing peptides was not always applicable to phenylalanine analogs containing such a heterocyclic ring as furan,[1)] thiazole,[2)] or pyridine.[3)]

This amino acid has already been prepared by Blank and Caldwell.[4)] The present authors followed substantially the same scheme, with only a few modifications; in place of 4-amino-5-bromomethyl-2-methylthiopyrimidine, the corresponding chloride was used. The starting material, 4-amino-5-ethoxycarbonyl-2-mercaptopyrimidine, was prepared according to the method described by Ulbricht and Price.[5)]

In order to synthesize N-benzyloxycarbonyl-DL-β-(5-cytosinyl)-α-alanyl-glycine ethyl ester, N-benzyloxycarbonyl-DL-β-(5-cytosinyl)-α-alanine (VII) was prepared from DL-β-(5-cytosinyl)-α-alanine (VI) and an equivalent amount of benzyloxycarbonyl chloride by the usual method. The analytical data of this substance showed that it contains only one benzyloxycarbonyl group introduced into the α-amino group, for this compound showed no ninhydrin reaction. The benzyloxycarbonyl derivative VII was coupled with glycine ethyl ester hydrochloride in dry pyridine in the presence of dicyclohexylcarbodiimide. The oily material thus obtained was then solidified by trituration with ethyl acetate, which was separated by extraction with a large amount of water into two components, *i.e.*, a water-insoluble, colorless, amorphous powder, IX, decomposing at 310—312°C, and a water-soluble, colorless, amorphous powder, VIII, decomposing at 254—258°C. When the compound VIII was hydrolyzed with concentrated hydrochloric acid, the hydrolysate gave two spots of cytosinylalanine and glycine on a paper chromatogram. This result, as well as that of its elementary analysis, supports that the compound VIII is a dipeptide derivative : N-benzyloxycarbonyl-cytosinylalanyl-glycine ethyl ester.

The molecular formula, $C_{15}H_{14}N_4O_4$, was assigned to the compound IX on the basis of the results of an elementary analysis (C, 57.23; H, 4.88; N, 17.58%) and the molecular weight, 329, determined by the method of Schwyzer and his coworkers.[6)] When hydrolyzed with hydrochloric

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1) H. Watanabe, S. Kuwata, S. Nakajima, K. Koshida and M. Hayashi, This Bulletin, **38**, 146 (1965).

2) H. Watanabe, S. Kuwata, T. Sakata and K. Matsumura, *ibid.*, **39**, 2473 (1966).

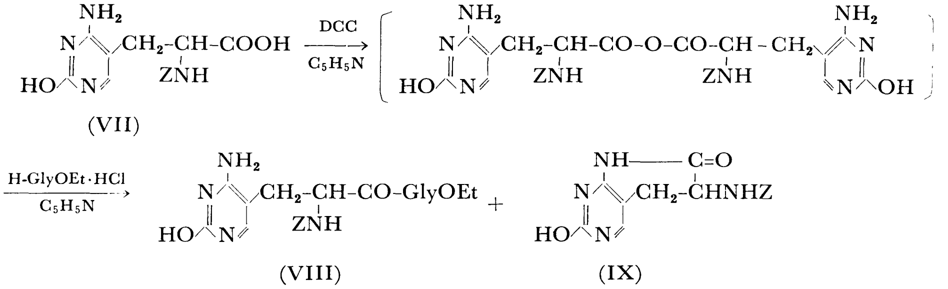
3) H. Watanabe, S. Kuwata, N. Naoe and Y. Nishida, *ibid.*, **41**, 1634 (1968).

4) B. Blank and W. T. Caldwell, *J. Org. Chem.*, **24**, 1138 (1959).

5) T. L. V. Ulbricht and C. C. Price, *ibid.*, **21**, 567 (1956).

6) R. Schwyzer, B. Iselin, W. Rittel and P. Sieber, *Helv. Chim. Acta*, **39**, 881 (1956).

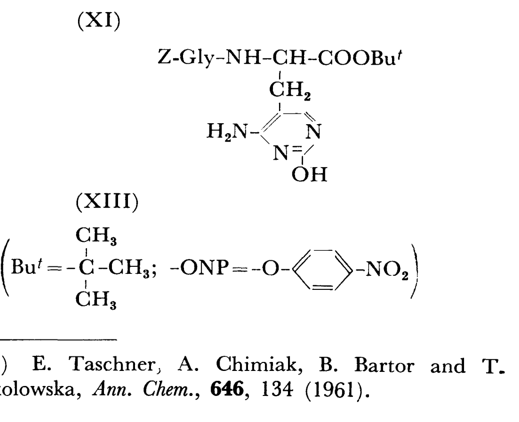
acid, IX gave only one ninhydrin-positive material, one which was identical with cytosinylalanine. On the basis of these facts, the structure of the compound IX was assumed to be 2-hydroxy-6-benzyloxycarbonylamino-5,6,7,8-tetrahydro-7-oxopyrido[2,3-*d*]pyrimidine; it is probably produced by lactam-formation between the 6-amino group of the pyrimidine and the carboxyl group activated by the carbodiimide. In order to confirm this, the benzyloxycarbonylated amino acid, VII, was treated with the carbodiimide under the same conditions as above except that ethyl glycinate hydrochloride was omitted. The substance thus obtained was found to be identical with IX by elementary analysis and by a comparison of the infrared spectra. Thus, the reactions producing these two compounds may be represented by the following equation:



(Z=benzyloxycarbonyl; DCC=dicyclohexylcarbodiimide; Gly=glycine)

To prevent the lactam formation of the 6-amino group, we attempted to provide protection by the benzyloxycarbonyl group; unfortunately, all the attempts to acylate it with a large excess of benzyloxycarbonyl chloride ended in failure. When, for instance, the further benzyloxycarbonylation of the monobenzyloxycarbonyl derivative was attempted in pyridine at 40°C, the compound IX was obtained in place of the expected one.

In order to esterify cytosinylalanine, hydrogen chloride gas was bubbled into a suspension of the amino acid in ethanol; the reaction was pursued by paper chromatography. When the spot of the free amino acid disappeared and a new spot (R_f=0.31[*3]) (probably of the desired ester) appeared, the reaction mixture was concentrated to dryness *in vacuo*; the colorless, hygroscopic crystalline residue was then left to stand over sodium hydroxide in a desiccator in order to remove the excess hydrogen chloride. In this step, paper chromatography gave only one spot. When the dried material was dissolved in hot ethanol for recrystallization, however, it gave another spot (R_f=0.41[*3]). This new spot can probably be attributed to 2-hydroxy-6-amino-5,6,7,8-tetrahydro-7-oxopyrido[2,3-*d*]pyrimidine produced by the cyclization of cytosinylalanine ethyl ester under weakly acidic conditions. This complication was avoided by using the tertiary butyl ester in place of the ethyl ester. It was found that *t*-butyl β-(5-cytosinyl)-α-alaninate could be prepared without any trouble by treating the acid with a large amount of *t*-butyl acetate in the presence of perchloric acid, according to the method of Taschner.[7] When we used five times as much catalyst as in the literature, the transesterification proceeded smoothly; after the raw product has been treated with sodium hydrogen carbonate, the ester was obtained in a 71% yield as perchlorate-monohydrate. The free ester was obtained as a colorless, amorphous powder by treating it with aqueous ammonia or triethylamine. Benzyloxycarbonylglycine *p*-nitrophenyl ester was condensed with *t*-butyl cytosinylalaninate in hexamethylphosphoramide. From



[*3] Circular paper chromatography. Toyo Roshi No. 51. Solvent, *n*-BuOH : AcOH : H_2O=4 : 1 : 2. (R_f of glycine = 0.34).

7) E. Taschner, A. Chimiak, B. Bartor and T. Sokolowska, *Ann. Chem.*, **646**, 134 (1961).

the reaction mixture, a colorless, amorphous powder decomposing at about 320°C was obtained in a 53% yield. Upon hydrolysis with concentrated hydrochloric acid, it gave cytosinylalanine and glycine. This result and the analytical data support that the substance obtained here is the desired product: benzyloxycarbonylglycyl-DL-β-(5-cytosinyl)-α-alanine *t*-butyl ester. The same compound was also obtained in a lower yield by the use of the carbodiimide method in a mixture of dimethylformamide and dimethyl sulfoxide.

Experimental

4-Amino-5-ethoxycarbonyl-2-mercaptopyrimidine (I). This was prepared in a 79% yield, according to the literature,[5] by condensing ethyl ethoxymethylenecyanoacetate and thiourea in the presence of sodium ethoxide.

4-Amino-5-ethoxycarbonyl-2-methylthiopyrimidine (II). The procedure described in the literature[5] was somewhat modified; into a suspension of I (100 g) in methanol (50—100 m*l*) was stirred aqueous potassium hydroxide (37 g/500 m*l*), and then dimethyl sulfate (67 g) over a 10—15-min period; the mixture was then stirred for 1 hr at room temperature. The pH of the reaction mixture was adjusted to 9.4—9.6 with aqueous potassium hydroxide; the separated crystals were collected by filtration, washed with dilute aqueous potassium hydroxide and then with water, and dried *in vacuo* on a water bath to give 89.5 g (84%) of II; mp 125—129°C. Recrystallization from 50% acetic acid gave tiny, glistening plates. The yield was 69 g (65%); mp 130—131.5°C (lit,[5] mp 130—131°C).

4-Amino-5-Hydroxymethyl-2-methylthiopyrimidine (III). Prepared, according to the literature,[4] in a 73% yield; mp 125—126°C (lit,[4] mp 126—127°C).

4-Amino-5-chloromethyl-2-methylthiopyrimidine Hydrochloride (IV). To a cooled and stirred suspension of III (24 g) in dry ether (600 m*l*), there was added thionyl chloride (40 m*l*) drop by drop. The mixture was refluxed for 3 hr with stirring and then evaporated to dryness. After this procedure had been repeated twice more, the residue was used for the next reaction without purification.

Ethyl 6-Acetamido-5,6,7,8-tetrahydro-2-methylthio-7-oxopyrido[2,3-*d*]pyrimidine-6-carboxylate (V). Prepared by condensing IV with diethyl acetamidomalonate in the presence of sodium ethoxide by a method similar to that in the literature.[4] The reaction period was, however, elongated to 6 hr. The yield was 57% (calculated from the alcohol (III)); mp 186—189°C (lit,[4] mp 187—188°C).

DL-β-(5-Cytosinyl)-α-alanine (VI). Prepared by the method in the literature.[4] The yield was 85%. Crude amino acid was purified by dissolving it in aqueous ammonia, decolorizing it, and acidifying it to pH *ca.* 6 with acetic acid. The yield was 65%; mp above 360°C (decomp.) (turned brown from about 220°C and darkened at 290°C) (lit,[4] mp above 300°C); R_f= 0.23[*3]; $\lambda_{max}^{0.1\,N\,HCl}$ 283 mμ (lit,[4] $\lambda_{max}^{0.1\,N\,HCl}$ 283 mμ).

***N*-Benzyloxycarbonyl-DL-β-(5-cytosinyl)-α-alanine (VII).** Benzyloxycarbonyl chloride (1.10 g) and 2 N sodium hydroxide (3.5 m*l*) were stirred, drop by drop, into a solution of VI (1.08 g) in 0.5 N sodium hydroxide (20 m*l*) over about a 1-hr period under ice-cooling. The solution was kept alkaline throughout the reaction. Stirring was continued further at room temperature until the solution became negative to the ninhydrin test (4—5 hr). After washing with ether, the solution was then acidified to Congo Red with glacial acetic acid. The white crystals thus formed were collected and washed thoroughly with water. The yield was 0.96 g (55%); mp about 310°C (decomp.). Recrystallization from a large amount of methanol gave a white, amorphous powder melting at about 315°C (decomp.). This compound dissolves sparingly in ethyl acetate, acetone, dioxane, methanol, ethanol, acetonitrile, tetrahydrofuran, or chloroform, and forms a viscous emulsion in dimethylformamide and pyridine.

Found: C, 51.32; H, 5.15: N, 15.57%. Calcd for $C_{15}H_{16}N_4O_5 \cdot H_2O$: C, 51.43; H, 5.18; N, 15.99%.

The Reaction of *N*-Benzyloxycarbonyl-DL-β-(5-cytosinyl)-α-alanine with Ethyl Glycinate.

***N*-Benzyloxycarbonyl-DL-β-(5-cytosinyl)-α-alanylglycine Ethyl Ester (VIII).** Glycine ethyl ester hydrochloride (0.35 g) was added to an emulsion of VII (0.84 g) in dry pyridine (20 m*l*). Into the mixture was then stirred a solution of dicyclohexylcarbodiimide (0.57 g) in dry pyridine (5 m*l*) over about a 15-min period under strong cooling (about −10°C). After an additional 2 hrs' stirring in the cold, the mixture was allowed to stand for three days at room temperature. The dicyclohexylurea which was thus separated was filtered off, and the filtrate was concentrated under reduced pressure. To the resulting oil was added ethyl acetate, and the mixture was allowed to stand at room temperature. Meanwhile, the oil solidified; this solid was thoroughly extracted with water. From the extracts the dipeptide derivative, VIII, was separated as an amorphous powder. The yield was 0.18 g (18%); mp 253—262°C (decomp.). Recrystallization from methanol gave a colorless, amorphous powder decomposing at 254—258°C.

Found: C, 54.40; H, 5.42; N, 16.55%. Calcd for $C_{19}H_{23}N_5O_6$: C, 54.67; H, 5.55; N, 16.78%.

2-Hydroxy-6-benzyloxycarbonylamino-5,6,7,8-tetrahydro-7-oxopyrido[2,3-*d*]pyrimidine (IX). The solid material, the residue of the extraction described above, melted at 308—314°C (decomp.). The yield of IX was 0.5 g (66%). Recrystallization from dimethylformamide-methanol gave an amorphous powder; mp 310—312°C (decomp.).

Found: C, 57.23; H, 4.88; N, 17.58%. Calcd for $C_{15}H_{14}N_4O_4$: C, 57.32; H, 4.49; N, 17.83%.

The Reaction of *N*-Benzyloxycarbonyl-DL-β-(5-cytosinyl)-α-alanine with Dicyclohexylcarbodiimide in Pyridine. Into an emulsion of VII (1.05 g) in dry pyridine (35 m*l*) was stirred a solution of dicyclohexylcarbodiimide (0.71 g) in the same solvent (6 m*l*) over about a 20-min period under cooling (about −5°C). The stirring was continued for 2 hr under the same conditions, and then the mixture was allowed to stand for 5 days at room temperature. The crystals which were thus separated were collected by filtration and washed with a large amount of boiling ethyl acetate. The yield of IX was 0.50 g. From the mother liquor some crystals were obtained by adding glacial acetic acid; this also gave an additional 0.21 g of IX. The total yield was 0.71 g (75%); mp about 316°C. For analysis, it was recrystallized from dimethylformamide.

Found: C, 57.64; H, 4.56; N, 17.46%. Calcd for $C_{15}H_{14}N_4O_4$: C, 57.32; H, 4.49; N, 17.83%.

The same compound was obtained by the action of 3 equivalents of benzyloxycarbonyl chloride on *N*-benzyloxycarbonyl-cytosinylalanine in dry pyridine at 40°C. The yield here was 0.60 g (56%).

DL-β-(5-Cytosinyl)-α-alanine *t*-Butyl Ester Perchlorate Monohydrate (X). Into a suspension of the amino acid, VI (1.08 g), in *t*-butyl acetate (116 m*l*), was stirred, drop by drop, 70% perchloric acid (3.66 g) at about 5°C; the mixture was then stirred at room temperature. After about 3 hr, the amino acid was almost dissolved into the solution and crystals began to separate. After the mixture had stood at room temperature overnight, the crystals were collected, dissolved in chilled water (10 m*l*), and kept in the cold after the pH had been adjusted to 7.6 with sodium hydrogencarbonate. Thus the crude crystalline *t*-butyl ester perchlorate was obtained. The yield was 1.42 g (71%); mp 175—180°C (decomp.). For further purification, it was dissolved in a mixture of acetone and chloroform (2 : 1) and freed from insoluble materials, while the solvent was removed under reduced pressure. To the residue was added a mixture of *t*-butyl alcohol and water (2 : 1), and the separated solid was recrystallized from the same mixture. The purified compound melted at 216—219°C (decomp.); R_f=0.57*3.

Found: C, 36.04; H, 5.71; N, 15.25%. Calcd for $C_{11}H_{18}N_4O_3 \cdot HClO_4 \cdot H_2O$: C, 35.44; H, 5.68; N, 15.03%.

DL-β-(5-Cytosinyl)-α-alanine *t*-Butyl Ester (XI). Into a solution of the perchlorate, X (1.00 g), in dimethylformamide (5 m*l*), was stirred triethylamine (0.3 g) at −10°C. After the solution had been stirred for 30 min at this temperature, a colorless solid separated; this was filtered, washed with cold water, and dried in a desiccator. The yield was 0.62 g (91%); mp above 320°C (decomp.). (It turned yellow from about 180°C and darkened from about 280°C.) Recrystallization from a mixture of dimethylformamide and dimethyl sulfoxide gave a colorless powder; mp above 320°C (it turned yellow from about 190°C and darkened from about 305°C).

***N*-Benzyloxycarbonyl-glycyl-DL-β-(5-cytosinyl)-α-alanine *t*-Butyl Ester (XII).** To a suspension of XI (0.25 g) in hexamethylphosphoramide (20 m*l*), there was added benzyloxycarbonylglycine *p*-nitrophenyl ester (0.33 g) and triethylamine (0.1 g); the mixture was then stirred at room temperature overnight. To the almost clear solution was added a large quantity of water, and then the solution was left to stand in a refrigerator overnight. The crystals which were thus separated were collected, washed with cold 0.1 N citric acid, water, 0.5 N aqueous sodium hydrogencarbonate, and then with water, and dried in a desiccator. The yield was 0.24 g (53%); mp above 320°C (decomp.). Recrystallization from a mixture of dimethyl sulfoxide and methyl ethyl ketone gave a colorless, amorphous substance; mp above 320°C (decomp.) (sintered at about 93°C). When hydrolyzed with hydrochloric acid, this compound gave glycine and cytosinylalanine, the presence of which was confirmed by paper chromatography.

Found: C, 55.01; H, 6.15; N, 14.93%. Calcd for $C_{21}H_{27}N_5O_6 \cdot H_2O$: C, 54.42; H, 6.31; N, 15.11%.

From XI (355 mg), benzyloxycarbonylglycine (881 mg), and dicyclohexylcarbodiimide (640 mg) in a mixture of dimethylformamide and dimethyl sulfoxide (1 : 1), the same dipeptide was obtained in a lower yield (58 mg; 9%).