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Peptides. III. A Novel Amino-activation by the Carbamate Formation from Amino Acids and Peptides in Aqueous Solution[1,2]

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A novel amino-activation by the carbamate formation of amino acids and peptides in aqueous solution are described. The coupling reaction of acylamino acid active esters with carbamates is several times faster than with corresponding amine components.

Since Bailey[4] noticed the formation of 3-methylpiperazine-2,5-dione from the salt of ethyl N-carboxy-DL-alanylglycinate with ethyl glycinate in water, some interesting reactions, in which carbamate formation plays important role, have been reported; for example, smooth hydrogenolytic debenzyloxycarbonylation of N-benzyloxycarbonyl-L-methionine[5] and hydrolysis of amino acid *p*-nitrophenyl esters in the presence of carbon dioxide.[6] It is known that amino acid carbamates are stable[7-9] unless weaker bases are used,[4] therefore Bailey's observations[4] support the applicability of amino-activation by the carbamate formation to peptide synthesis.

In the present paper a novel method of amino-activation by the formation of carbamates of amino acids and peptides in aqueous solution is described. The reactions of amino acid carbamates,[7-9] prepared by the addition of carbon dioxide to aqueous solution of amino acids in the presence of two equivalents of base, with *p*-nitrophenyl N-benzyloxycarbonyl-L-phenylalaninate (IIIa) in aqueous dioxane were traced by the measurement of ultraviolet (UV) absorption at 320 mμ. The time-courses of *p*-nitrophenol formation in the reactions of triethylammonium glycinate (Ia) and its carbamate, bis-triethylammonium N-carboxyglycinate (IIa), with IIIa are shown in Fig. 1. It is clear that the reaction of IIIa with IIa is quasi first-order to the concentration of IIIa and about seven times as fast as with Ia. The same acceleration was observed in the comparison of the reactions of IIIa with sodium

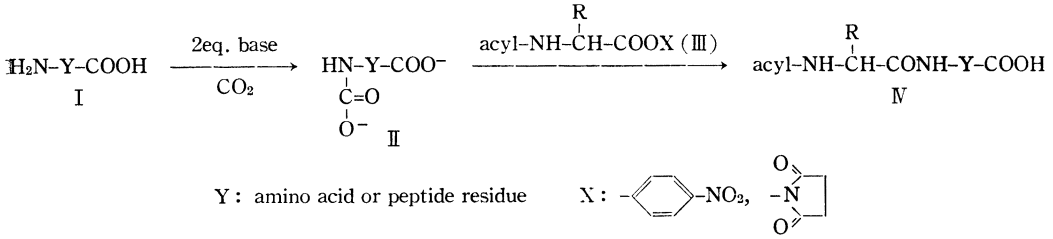

Chart 1

1) Part II: M. Itoh, *Chem. Pharm. Bull.* (Tokyo), **18**, 784 (1970).
2) Abbreviation used for protective groups, amino acids and peptides are those recommended by IUPAC-IUB commission on biochemical nomenclature: *Biochemistry*, **5**, 2485 (1966).
3) Location: *1, Kashima-cho, Higashiyodaogawa-ku, Osaka, 532, Japan.*
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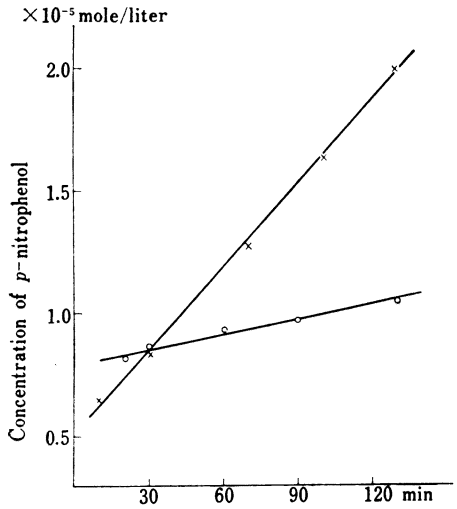

Fig. 1. Aminolysis of *p*-Nitrophenyl N-Benzyloxycarbonyl-L-phenylalaninate (IIIa) with Gycinates in 60% aqueous dioxane at 23°

—○—: with triethylammonium glycinate (Ia)
—×—: with bistriethylammonium N-carboxyglycinate (IIb)

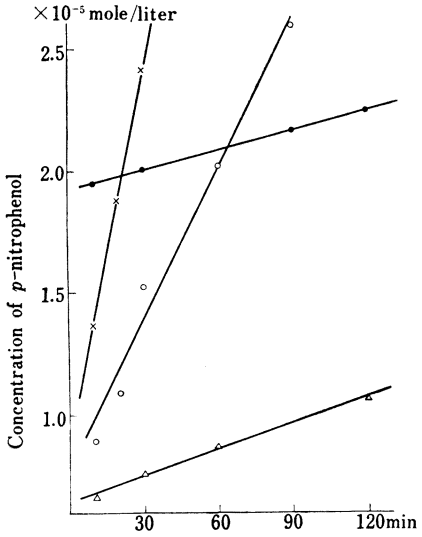

Fig. 2. Aminolysis of *p*-Nitrophenyl N-Benzyloxycarbonyl-L-phenylalaninate (IIIa) with Various Carbamates in 60% aqueous dioxane at 28°

—×—: disodium N-carboxyglycinate (IIb)
—○—: bistriethylammonium N-carboxyglycinate
—△—: bis-N-ethylmorpholinium N-carboxyglycinate
—●—: sodium glycinate (Ib)

glycinate (Ib) and disodium N-carboxyglycinate (IIb).[8] However, with weak bases such as N-ethylmorpholine, pyridine and N,N-dimethylaniline no acceleration of the reaction was found at all (Fig. 2). The time-course of *p*-nitrophenol formation shown in Fig. 1 or 2 consists of coupling reaction of IIIa with IIa or Ia and competitive hydrolysis of IIIa by hydroxy ions, and the two reactions are not measured independently by the UV method. Therefore, suppression of hydrolysis by carbamate formation was measured in the similar manner at room temperature. Hydrolysis of IIIa was significantly suppressed because the pH of the reaction mixture is kept at about 8 by the addition of carbon dioxide (Fig. 3). These facts suggest that stronger bases, for example, triethylamine, sodium bicarbonate and sodium hydroxide, are required for the formation of carbamates. While there is no doubt about the stability of disodium N-carboxyglycinate (IIb),[8] the stability of bis-triethylammonium N-carboxyglycinate (IIa) is questionable, because if IIa decomposes to Ia, triethylamine and carbon dioxide, the excess triethylamine will accelerate the coupling reaction.[10] It is difficult to isolate IIa, because only free glycine remains on the lyophilization of its aqueous solution. Therefore, nuclear magnetic resonance (NMR) spectrum of IIa, prepared by the addition of dry ice to a solution of glycine and two equivalent of triethylamine in deuterium oxide and standing for two hours at room temperature, was compared with that of Ia with excess triethylamine in deuterium oxide.[11] IIa showed a triplet at (ppm) 1.24 (methyl group of triethylamine, 18H, $J=7.5$ cps), a quartet at 3.14 (methylene group of triethyl-

10) R. Schwyzer, M. Feurer, and B. Iselin, *Helv. Chim. Acta*, **38**, 83 (1955).
11) NMR spectrum was measured with a Varian A-60 spectrometer using sodium 2,2-dimethyl-2-silicapentane-5-sulfonate as an internal standard.

amine, 12H, J=7.5 cps) and a singlet at 3.54 (methylene group of glycine, 2H). Ia with excess triethylamine showed a triplet at (ppm) 1.22 (methyl group of triethylamine, 9H, J=7.5 cps), a quartet at 3.08 (methylene group of triethylamine, 6H, J=7.5 cps) and a singlet at 3.24 (methylene group of glycine, 2H) for Ia, and a triplet at 0.65 (J=7.0 cps) and a quartet at 2.13 (J=7.0 cps) for excess triethylamine. These data show that IIa does not decompose to Ia, triethylamine and carbon dioxide.

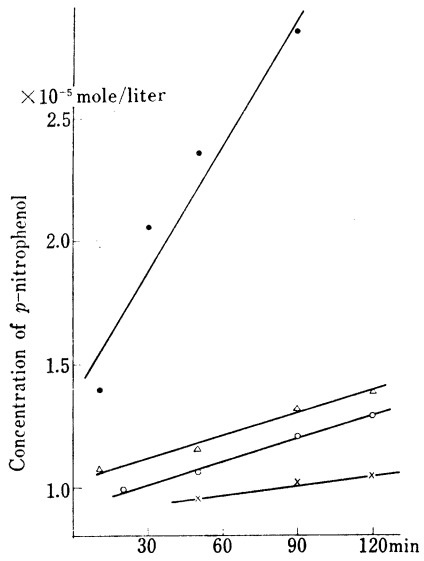

Fig. 3. Suppression of Hydrolysis of *p*-Nitrophenyl N-Benzyloxycarbonyl-L-phenylalaninate with triethylamine by the Treatment with Dry Ice at 23°

—△—: hydrolysis with equimolar amount of triethylamine in 67% aqueous dioxane (A)
—×—: dry ice treatment of A
—●—: hydrolysis with equimoarlar amount of triethylamine in 33% aqueous dioxane (B)
—○—: dry ice treatment of B

When the coupling reaction of IIIa with Ia is carried out in the presence of an equimolar amount of triethylamine, the rate of *p*-nitrophenol formation is higher than in the carbamate formation procedure. However, considerable hydrolysis of IIIa was recognized by thin-layer chromatography, and therefore further examination of this reaction was discontinued.

These preliminary experiments were carried out in highly diluted solution for convenience of spectral measurements, but it seemed to be promising to apply this process in peptide synthesis. Practical peptide synthesis in usual concentration of the reactants was followed by thin-layer chromatography on Silica gel G. The coupling reaction of IIIa with IIa in 60% aqueous dioxane was complete within 2 hours, while the similar reaction of IIIa with Ia required more than 5 hours. In a second example, the reaction of N-benzyloxycarbonyl-L-phenylalanine N-hydroxysuccinimido ester with bistriethylammonium N-carboxy-L-prolinate in 60% aqueous dioxane was complete within 15 minutes, in contrast to more than 1 hour under the usual coupling condition.

On the other hand, in the reaction of benzhydryl pentachlorophenylcarbonate,[12] *tert*-butyl pentachlorophenylcarbonate,[12] *p*-methoxybenzyl pentachlorophenylcarbonate[12] or *tert*-butyl carbazide[13] with carbamates of amino acids, in the reaction of IIIa with the carbamate of ethyl glycinate, and in the reaction of pentachlorophenyl N-benzyloxycarbonylglycinate[14] with IIa, no acceleration effect due to the carbamate formation could be demonstrated. These results suggest the importance of hydrophilic character of both active esters and amine components. In actual synthesis with triethylammonium carbamates of amino acids or peptides in aqueous dioxane or aqueous tetrahydrofuran it is desirable to use clear solutions of the carbamates. If the carbamate separates, the reaction does not proceed smoothly. *p*-Nitrophenyl and N-hydroxysuccinimido esters are suitable as active esters. N-Hydroxysuccinimido esters are especially favorable because of their high reactivity and the ease in the removal of N-hydroxysuccinimide. Amino-activation by carbamate formation possesses two advantages: the increase in the nucleophilic reactivity of amino group, and the suppression of the competitive hydrolysis of active esters.

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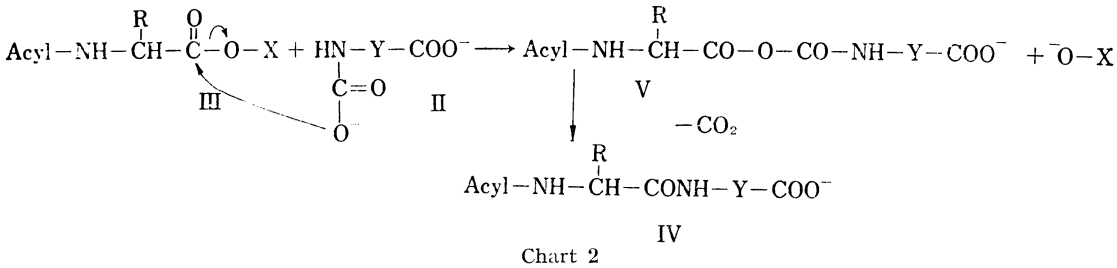

Chart 2

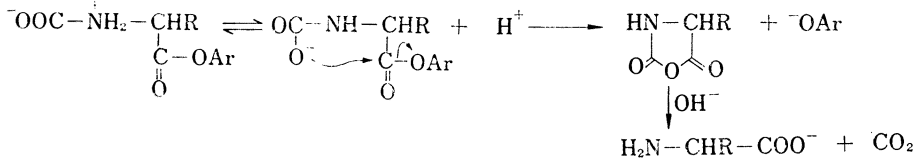

Chart 3

The proposed reaction mechanism is shown in Chart 2. Nucleophilic attack of carboxyl carbon atom by the carboxylate anion followed by decarboxylation accounts for the acceleration by the carbamate formation. It is known that decarboxylation of V type intermediate, which is prepared from N-benzyloxycarbonylamino acid and ethyl N-carbonylglycinate, proceeds easily in polar solvent.[15] Hay and Main[6] proposed the mechanism for the hydrolysis of amino acid *p*-nitrophenyl esters in the presence of carbon dioxide (Chart 3); carbon dioxide presumably reacts with the amino group to give the carbamate, which is hydrolyzed by intramolecular nucleophilic attack by the carboxylate group. Additional support can be found in the rearrangement of 6-aminopenicillanic acid to 8-hydroxypenillic acid.[16]

Thus prepared acylpeptides are listed in the Table I. Application of this procedure is limited to the synthesis of peptides with a free C-terminal carboxyl group, but the method could be useful when the carboxyl protection is undesirable.

Experimental

Ultraviolet absorption was measured with a Hitachi Perkin Elmer 139 UV-VIS spectrophotometer. Optical rotations were determined with a JASCO optical rotatory dispersion recorder Model ORD/UV-5. Melting points were observed on a Hoover "Uni-Melt" apparatus and are reported uncorrected. For the measurement of *p*-nitrophenol formation by ultraviolet absorption analytically pure *p*-nitrophenyl esters of protected amino acids, freshly distilled triethylamine, dioxane and water were used. Reaction mixtures were examined on thin-layer chromatograms of Silica gel G and the solvent system of chloroform–acetic acid–methanol (95 : 3 : 5). Spots of N-benzyloxycarbonyl derivatives were revealed by the combination of hydrogen bromide and ninhydrin.

Rate Determination by Spectrophotometrical Measurement of *p*-Nitrophenol Concentration——Reagents: Glycine in distilled water (2.22×10^{-3} mole/liter); triethylamine in distilled water (1.96×10^{-3} mole/liter); *p*-nitrophenyl N-benzyloxycarbonyl-L-phenylalaninate (IIIa) in dioxane (1.0×10^{-4} mole/liter). i) Each 5.0 ml of aqueous glycine (1.11×10^{-5} mole) and triethylamine (0.98×10^{-5} mole) solutions were combined with 10.0 ml of IIIa (1.0×10^{-6} mole) solution. Aliquots of the solution were allowed to react in photometric cell at 23° and ultraviolet absorption was measured. The rate of liberation of *p*-nitrophenol was calculated from an experimentally determined value 10100 as $\varepsilon_{320\,m\mu}$ at pH 8. ii) 5.0 ml solutions of aqueous glycine (1.11×10^{-5} mole) and of triethylamine (1.96×10^{-5} mole) were combined and treated with dry ice to pH 8. The resulting solution was mixed with a 10.0 ml solution of IIIa (1.0×10^{-6} mole) and the mixture was allowed to react as described in i) at 23°.

The influence of various bases and the suppression of hydrolysis by the carbamate formation process were determined in a similar manners with the same concentration of the reactants (see Fig. 2 and Fig. 3).

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16) D.A. Johnson and G.A. Hardcastle, Jr., *J. Am. Chem. Soc.*, **83**, 3534 (1961).

Table I. Acylpeptides Prepared by the Carbamate Method

Acylamino acid acylpeptides active esters		Reaction time (min)	Yield (%)	mp (°C) Found/Lit.	Recrystd. from[a]	$[\alpha]_D^t$ (c, solvent) Found/Lit.
Z-Asp(OBu^t)-OHsu	Z-Asp(OBu^t)-Ser-OH	20	78.0	133 —135 135	A–B	+ 6.2[23] (1.4, MeOH) + 8.6[28] (1.41, MeOH)[17]
Z-Lle-ONp	Z-Ile-Phe-OH	120	73.9	177 —179 175 —176	A–B	−19.4[23] (1.0, MeOH) −25.5[28] (1.0, MeOH)[18]
Z-Phe-ONp	Z-Phe-Gly-OH	120	84.3	151 —152 152 —153	C	− 9.5[23] (1.5, AcOH) − 9.8[18] (1.5, AcOH)[19]
Z-Phe-OHsu	Z-Phe-Glu $(OH)_2$	20	80.9	173 —175 180[20]	C	− 4.0[23] (1.6, EtOAc)
Z-Phe-OHsu	Z-Phe-Pro-OH	20	72.5	104 —106 109 —111	A–D	−42.1[23] (2.0, MeOH) −33.7[22] (2.0, MeOH)[21]
Z-Pro-OH[b]	Z-Pro-Leu-OH	30	60.7	134 —135 137.5—139	E–F, G–D	−75.0[23] (6.0, $CHCl_3$) −75.8[20] (6.0, $CHCl_3$)[22]
Z-Pro-OHsu	Z-Pro-Phe-OH	15	87.0	127 —128 126 —127	H–B	−48.9[23] (2.0, $CHCl_3$) −49±2[22] (2.46, $CHCl_3$)[23]
Z-Val-OHsu	Z-Val-Phe-OH	60	68.6	174 —175	A–B	+3.9[23] (1.3, DMF)
Z-Phe-ONp	Z-Phe-Gly-Gly-OH 1/2 H_2O	30	82.5	99 —101 76 —80[24]	C	−6.8[23] (1.0, EtOH)
Z-Pro-OHsu	Z-Pro-Gly-Gly-OH	15	86.5	140 —142	H–A–B	−34.2[23] (1.4, DMF)
Z-Pro-OHsu	Z-Pro-Leu-Gly-OH	30	89.5	159 —161 163.5—164	I–A–B	−84.6[23] (2.0, EtOH) −85.2[23.5] (2.0, EtOH)[25]

a) A: EtOAc; B: petroleum ether; C: aqueous EtOH; D: hexane; E: AcOH; F: H_2O; G: acetone; H: EtOH; I: MeCN
b) Mixed anhydride method using isobutyl chloroformate

Comparison of the Reaction Rates by Thin-Layer Chromatography——a) *p*-Nitrophenyl N-benzyloxycarbonyl-L-phenylalaninate (IIIa; 140 mg, 3 mmole) was added to a solution of glycine (25 mg, 3 mmole) and triethylamine (0.047 ml, 3 mmole) in a mixture of water (1.0 ml) and dioxane (1.5 ml) with stirring at room temperature. b) A solution of glycine (25 mg, 3 mmole) and triethylamine (0.094 ml, 6 mmole) in a mixture of water (1.0 ml) and dioxane (1.5 ml) was adjusted to pH 8 by the addition of dry ice with stirring. To the resulting solution IIIa (140 mg, 3 mmole) was added at room temperature. Aliquots of the reaction mixtures a) and b) were checked by thin-layer chromatography to determine the presence of IIIa from time to time. In the reaction b) a spot due to IIIa was disappeared completely within 2 hours, while in the reaction a) more than 5 hours were required.

A General Procedure for Coupling through the Carbamate Formation——A solution of the amino acid or peptide (2 mmole) and triethylamine (4 mmole) in the mixture of water (5 ml) and dioxane or tetrahydrofuran (5—10 ml) was adjusted to pH 8 by the addition of dry ice with stirring. Acylamino acid acitve ester (2 mmole) was added to the resulting solution at room temperature with stirring. Completion of the reaction generally required 0.5—2 hours in the case of *p*-nitrophenyl esters, and 15—30 minutes in the case of N-hydroxysuccinimido esters. After the acidification with 1N HCl, product was taken up in ethyl acetate, the organic layer was washed with water, dried over $MgSO_4$ and evaporated to give crude product, which was recrystallized from suitable solvent cited in Table I.

Acknowledgement The author expresses his deep gratitude to Prof. K. Okada of Kwansei Gakuin University, and Dr. H. Nakano, director of this laboratory, for their kind advices and encouragements.

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