Peptides. IV. Racemization Suppression by the Use of Ethyl 2-Hydroximino-2-cyanoacetate and Its Amide

Masumi ITOH

Research Laboratories, Fujisawa Pharmaceutical Co., Ltd. Kashima-cho, Higashiyodogawa-ku, Osaka 532

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Racemization suppression by the addition of ethyl 2-hydroximino-2-cyanoacetate (IIIa) or 2-hydroximino-2-cyanoacetamide (IIIb), during coupling stages in peptide synthesis, was studied. The effectiveness of IIIa was compared with these of known additives by the help of Bodanszky's test, which is a simple and accurate method for racemization detection.

There are so many literatures reported about racemization suppression during coupling stages in peptide synthesis.[1,2] These have recommended the use of restricted amount of base,[3] the use of weaker bases which have sufficient basicity to proceed the reaction,[4-6] or the addition of some acidic and nucleophilic compounds for racemization suppression.[7-11] A combination of dicyclohexylcarbodiimide (DCC) with *N*-hydroxysuccinimide (I) practically seems to be an attractive coupling reagent, but it has also known that I is not so stable and that the combination of DCC with I occasionally leads to some side reactions.[12,13] Later 1-hydroxybenzotriazole (II) and its derivatives have been proposed as superior additives than I.[10] In the DCC method using additives, Anderson has concluded that a major factor for racemization suppression was not neutralization of basicity of DCC by an additive, but its nucleophilic reactivity.[14] Some oximes reported as alcoholic components of active esters[15-18] seem to be good nucleophiles, but the reactivity of these esters is somewhat lower than those of other types of active esters. It was expected, therefore, that strongly acidic and nucleophilic oximes, which possess electron-withdrawing groups in the molecule, might be suitable as additives. The author intended to search for more promising additives and alcoholic components of active esters, and now wishes to report the use of strongly acidic oximes, ethyl 2-hydroximino-2-cyanoacetate (IIIa) and 2-hydroximino-2-cyanoacetamide (IIIb), as suitable additives for the same purpose.

Results

The pK_a values of several oximes prepared were compared (Table 1), and acidic oximes, IIIa and IIIb, were chosen as possible additives among them. At the same time several acylamino acid oxime esters shown in Table 2 were prepared and examined their reactivities with ethyl glycinate by the help of thin layer chromatography. It was also shown that those esters with IIIa and IIIb were more reactive than others tested. Both of IIIa and IIIb are stable, acidic compounds and are used in organic syntheses.

TABLE 1. APPARENT pK_a VALUES OF OXIMES, $HON{=}C\langle^{R_1}_{R_2}$

R_1	R_2	pK_a
CN	COOEt	4.6
CN	$CONH_2$	5.2
$COCH_3$	COOEt	7.0
COOEt	COOEt	7.1
$COCH_3$	$COCH_3$	7.4
H	$COCH_3$	8.4
H	CH_3	>11

Racemization with these additives were examined first by Anderson's test.[19] The coupling reaction of *N*-benzyloxycarbonylglycyl-L-phenylalanine with ethyl glycinate gave no racemate by the use of DCC with IIIa or IIIb in tetrahydrofuran at room temperature, while the control experiment, without additive, gave 8% of racemate. The effectiveness of IIIa was further examined by Young's test.[20] The crude coupling product, ethyl *N*-benzoylleucylglycinate, showed 93.6% optical purity calculated from the optical rotation, which was raised to 97% by recrystallization. This method is not satisfactory for the detection of small

1) E. Schröder and K. Lübke, "The Peptides," Vol. I, Academic Press Inc., New York, N.Y. (1966), p. 325.
2) M. Bodanszky and M. A. Ondetti, "Peptide Synthesis," Interscience Publishers, New York, N.Y. (1966), p. 137.
3) G. W. Anderson, F. M. Callahan, and J. E. Zimmerman, *Acta Chim. Acad. Sci. Hung.*, **44**, 51 (1965).
4) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **88**, 1338 (1966); *ibid.*, **89**, 5012 (1967).
5) S. Sakakibara and M. Itoh, This Bulletin, **40**, 656 (1967).
6) M. Bodanszky and A. Bodanszky, *Chem. Commun.*, **1967**, 591.
7) J. Kovacs, L. Kisfaludy, and M. Q. Ceprini, *J. Amer. Chem. Soc.*, **89**, 183 (1967).
8) F. Weygand, D. Hoffmann, and E. Wünsch, *Z. Naturforsch., B*, **21**, 426 (1966).
9) E. Wünsch and F. Dress, *Chem. Ber.*, **99**, 110 (1966).
10) W. König and R. Geiger, *ibid.*, **103**, 788, 2024, 2034 (1970).
11) G. C. Windridge and E. C. Jorgensen, *J. Amer. Chem. Soc.*, **93**, 6318 (1971).
12) H. Gross and L. Bilk, *Tetrahedron*, **24**, 6935 (1968).
13) F. Weygand, W. Steglich, and N. Chytil, *Z. Naturforsch., B*, **23**, 1391 (1968).
14) G. W. Anderson, "Peptides, Chemistry and Biochemistry," ed. by B. Weinstein and S. Lande, Marcel Dekker, Inc., New York, N.Y. (1970), p. 255.
15) S. Bittner, Y. Knobler, and M. Frankel, *Tetrahedron Lett.*, **1965**, 95.
16) G. Losse, K. H. Hoffmann, and G. Hetzer, *Ann. Chem.*, **684**, 236 (1965).
17) M. Fujino and O. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **17**, 1937 (1969).
18) B. O. Handford, J. H. Jones, G. T. Young, and T. F. N. Johnson, *J. Chem. Soc.*, **1965**, 6814.
19) G. W. Anderson and F. M. Callahan, *J. Amer. Chem. Soc.*, **80**, 2902 (1958).
20) M. W. Williams and G. T. Young, *J. Chem. Soc.*, **1963**, 881.

Table 2. Some esters of acylamino acids with oximes

Esters[a]	Yield (%)	Mp °C	Analysis (Calcd) C %	H %	N %
Cbz–Gly–ON=CHCOCH$_3$	83	60.5— 62[b]	56.34	5.16	10.31
			(56.11	5.07	10.07)
Cbz–Gly–ON=C$\langle^{COCH_3}_{COCH_3}$	76	83 — 85[c]	56.43	5.22	8.56
			(56.24	5.03	8.75)
Cbz–Gly–ON=C$\langle^{CN}_{COOEt}$	72	119 —121[c]	53.86	4.32	12.54
			(54.05	4.54	12.61)
Cbz–Gly–ON=C$\langle^{CN}_{CONH_2}$	60	141 —143[d]	51.52	3.86	18.33
			(51.31	3.98	18.42)

a) Cbz–Gly=*N*-Benzyloxycarbonylglycyl residue.
b) Recrystallized from ether–petroleum ether.
c) Recrystallized from ethyl acetate–petroleum ether.
d) Recrystallized from 2-propanol–petroleum ether.

Table 3. Comparison of effectiveness of the additives

Acetyl-L-isoleucine	Ethyl glycinate hydrochloride	Triethylamine	Additive	Extent of racemization (%)[a]
1.0 mmol	1.0 mmol	1.0 mmol	none	35
1.0	1.0	1.0	I 1.2 mmol	2.7
1.0	1.0	1.0	II 1.2	8.8
1.0	1.0	1.0	IIIa 1.2	1.8

a) Racemization during hydrolysis was not subtracted.

quantity of racemate, because it does not give accurate percentage of racemization if the protected dipeptide is contaminated with unconcerned impurities. Therefore, Bodanszky's test[21] was chosen as a more simple and sensitive procedure for racemization detection. For a comparison of additives, I, II, and IIIa, coupling of *N*-acetyl-L-isoleucine and ethyl gylcinate with DCC was carried out in dimethylformamide (DMF), in which significant racemization occurs. The crude product, ethyl *N*-acetylisoleucylglycinate, was hydrolyzed by 6M hydrochloric acid at 110 °C and the hydrolyzate was subjected to amino acid analysis to detect D-alloisoleucine. About 0.7% of racemization occured during hydrolysis of *N*-acetyl-L-isoleucine itself under the same condition. The results obtained are summarized in Table 3, which shows that all of additives examined suppress racemization potently. Unexpectedly, II is less effective than I. Although this is incompatible with previously reported results by König and Geiger,[10] it is difficult to discuss because the racemate detection systems are completely different.

Apart from such a problem the combination of DCC with IIIa seems to be a promising approach in coupling reactions.

Experimental

Capillary melting points were observed on a Hoover "Uni-Melt" apparatus and are uncorrected.

Measurement of pK_a *Values of Several Oximes in Aqueous Solution.* A solution of small amount of sample (2—7 mg) in 0.02 M potassium hydroxide (6.0 ml) was titrated by the use of an automatic titrator, Metrohm E 336. The pK_a values measured are listed in Table 1. Oximes were prepared according to the literatures: ethyl 2-hydroximino-2-cyanoacetate (IIIa),[22] 2-hydroximino-2-cyanoacetamide (IIIb),[22] ethyl 2-hydroximino-2-acetoacetate,[23] diethyl 2-hydroximinomalonate,[24] 3-hydroximinopentan-2,4-dione,[25] and 3-hydroximinopropan-2-one.[26]

Preparation of Esters of Acylamino Acids with Oximes. A typical example is as follows: DCC (1.05 g) was added to a solution of *N*-benzyloxycarbonyl-L-proline (1.25 g) and IIIb (0.56 g) in dioxane (10 ml) at 0 °C. The mixture was allowed to react for 2 hr at the same temperature and to stand for 16 hr at room temperature. After filtration the filtrate was evaporated to dryness. The product was filtered with small amount of 2-propanol and petroleum ether, and was recrystallized from same solvent system; yield 1.5 g (85%); mp 136—138 °C.

Found: C, 55.74; H, 4.71; N, 15.76%. Calcd for $C_{16}H_{18}O_4N_4$: C, 55.81; H, 4.68; N, 16.27%.

The other esters prepared are listed in Table 2.

Investigation of the Effectiveness of IIIa and IIIb for Racemization Suppression. a) Anderson's Test:[19] *N*-Benzyloxycarbonylglycyl-L-phenylalanine (1.78 g, 5 mmol), ethyl glycinate (0.52 g, 5 mmol) and IIIb (0.68 g, 6 mmol) were dissolved in dry tetrahydrofuran (25 ml). DCC (1.20 g, 5.8 mmol) was added to the solution at room temperature and the mixture was stirred for 4 hr. After the addition of acetic acid (0.05 ml), the mixture was filtered. The filtrate was evaporated completely, and the residue was taken up in ethyl acetate, washed with 1 M sodium bicarbonate solution, water, 1 M hydrochloric acid and water, and dried over mag-

21) M. Bodanszky and L. E. Conklin, *Chem. Commun.*, **1967**, 773.
22) M. Conrad and A. Schulze, *Ber.*, **42**, 735 (1909).
23) M. Z. Jovitchitch, *ibid.*, **35**, 151 (1902).
24) V. Cerchez, *Bull. Soc. Chim. Fr.*, **47**, 1279 (1930).
25) L. Wolff, *Ann. Chem.*, **325**, 139 (1902).
26) P. Fréon, *Ann. Chim.* (Rome), **11**, 453 (1939); *Chem. Abstr.*, **33**, 7733 (1939).

nesium sulfate. After evaporation of the solvent crude ethyl *N*-benzyloxycarbonylglycyl-L-phenylalanylglycinate was obtained. Fractional recrystallization from ethanol gave no racemate; yield of pure L isomer, 2.03 g (92%); mp 116—118 °C.

The use of IIIa (0.71 g) in place of IIIb gave similar result. In control experiment, without additive, 8% of racemate, ethyl *N*-benzyloxycarbonylglycyl-DL-phenylalanylglycinate (mp 130—132 °C), was obtained from crude protected tripeptide by fractional recrystallization.

b) Young's Test:[20] DCC (0.52 g, 2.52 mmol) was added to a solution of *N*-benzoyl-L-leucine (0.60 g, 2.55 mmol), free ethyl glycinate (0.30 g, 2.91 mmol) and IIIa (0.35 g, 2.46 mmol) in ethyl acetate (5 ml) at 0 °C. The mixture was stirred for 3 hr at the same temperature, and was filtered. The filtrate was diluted with ethyl acetate, washed with 1 M sodium bicarbonate solution, water, 1 M hydrochloric acid and water, and dried over magnesium sulfate. After evaporation of the solvent the residue was filtered with ether–petroleum ether mixture; yield, 0.73 g; mp 145—152 °C; $[\alpha]_D^{25}$ −30.9° (*c* 3, EtOH). Recrystallization from ethyl acetate–petroleum ether gave 0.61 g of product; mp 151—153 °C; $[\alpha]_D^{25}$ −33.0° (*c* 3, EtOH).

c) Bodanszky's Test:[21] A typical example is as follows: Ethyl glycinate hydrochloride (140 mg, 1.0 mmol) was dissolved in dry DMF (3.0 ml) and neutralized with triethylamine (0.14 ml, 1.0 mmol) under ice-cooling. Then IIIa (170 mg, 1.2 mmol) and *N*-acetyl-L-isoleucine[27] (173 mg, 1.0 mmol) were dissolved in the above solution. A solution of DCC (206 mg, 1.0 mmol) in dry DMF (2.0 ml) was added into the solution, and the mixture was allowed to react for 3 hr at +5 °C. After standing overnight DMF was evaporated completely under nitrogen gas, and the residue was extracted with ethyl acetate. The filtered extract was washed with water, 1 M sodium bicarbonate solution (4 times), water, 1 M hydrochloric acid and water, and dried over magnesium sulfate. After evaporation of the solvent the residue was filtered with small amount of ether–petroleum ether mixture; yield of crude ethyl *N*-acetylisoleucylglycinate, 178 mg (69%). A small amount of the sample was hydrolyzed by 6 M hydrochloric acid at 110 °C in a sealed tube for 16 hr. The hydrolyzate was subjected to an amino acid analyzer, and 1.8% of racemization was detected. The extent of racemization was calculated according to the following formula:

$$\text{Racemization (\%)} = \frac{\text{alloisoleucine} \times 100}{\text{alloisoleucine} + \text{isoleucine}}$$

During hydrolysis of *N*-acetyl-L-isoleucine under the same condition, very small amount of alloisoleucine (0.7%) was formed and detected by following amino acid analysis.

The results obtained are summarized in Table 3.

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27) J. P. Greenstein, L. Levintov, C. G. Baker, and J. White, *J. Biol. Chem.*, **188**, 647 (1951).