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Suppression of Diketopiperazine Formation in Solid Phase Peptide Synthesis[1]

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When N-methylmorpholine salt of Boc-Ile was coupled with HCl·Pro-Pro-resin suspended in methylenechloride by dicyclohexylcarbodiimide, diketopiperazine formation of the dipeptide-resin was suppressed almost completely and the isoleucine residue in the synthesized Boc-Ile-Pro-Pro-resin was not racemized. Generalization of this coupling procedure in a schedule for solid phase peptide synthesis is suggested.

The polymer-supported dipeptide ester, aminoacyl-prolyl-resin, was found to undergo intramolecular aminolysis, which was catalyzed by carboxyl group, resulting in the formation of the diketopiperazine and the side reaction suppressing approach was reported by Gisin, *et al.*[3] A similar side reaction was also reported in the case of aminoacyl-iminoacid-resin by Bumpus, *et al.*[4] and by contraries it was revealed that the cleavage of the dipeptide from the polymer, as the diketopiperazine, caused during neutralization of the α-amine hydrochloride salt of the dipeptide ester. Very recently, quite similar side reaction was reported independently by Rothe, *et al.*[5]

This study dealing with suppression of such of a side reaction in solid phase peptide synthesis stemmed from the observation made during conventional peptide synthesis in which Z–Pro–Lys(Tos)–Ala–OMe was prepared in high yield when H–Lys(Tos)–Ala–OMe hydrochloride was mixed with Z–Pro–ONp followed by triethylamine, however, the yield of the tripeptide derivative was poorer and the formation of the diketopiperazine of H–Lys(Tos)–Ala–OH was detected when H–Lys(Tos)–Ala–OMe hydrobromide was mixed first with triethylamine followed by Z–Pro–ONp.[6]

In order to monitor a suppression of the loss of dipeptide from a resin, Boc–Pro–Pro–resin was used as a model compound because it was reported that the dipeptide-resin was sensitive to form the diketopiperazine among many dipeptide-resins tested.[3,4a,5] Boc-Ile-OH[7] was coupled with the dipeptide-resin in four different procedures, those were, standard procedure,[8] the new approaches namely A and B, and a slightly modified active ester procedure.[9] Standard procedure schedule for the coupling of Boc–Ile–OH on Boc–Pro–Pro–resin is shown in Table I. For the procedure A, steps 5 to 7 were eliminated and in step 10 three equivalent N-methylmorpholine salt of Boc–Ile–OH in methylenechloride was added followed by step

1) Symbols for amino acid derivatives and peptides used in this text are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature; *Biochem. J.*, **104**, 17 (1967); *ibid.*, **126**, 773 (1972). Other abbreviations: DMF=dimethylformamide, DCC=dicyclohexylcarbodiimide, ONo=*o*-nitrophenyl ester.
2) Location: *Komatsushima, Sendai, 983, Japan.*
3) B.F. Gisin and R.B. Merrifield, *J. Am. Chem. Soc.*, **94**, 3102 (1972).
4) *a*) M.C. Khosla, R.R. Smeby and F.M. Bumpus, *J. Am. Chem. Soc.*, **94**, 4721 (1972); *b*) *Idem*, "Chemistry and Biology of Peptides, Proceedings of the Third American Peptide Symposium," ed. by J. Meienhofer, Ann Arbor Science, Michigan, 1972, p. 227.
5) M. Rothe and J. Mazánek, *Ann.*, **1974**, 439.
6) P.G. Katsoyannis and K. Suzuki, *J. Am. Chem. Soc.*, **84**, 1420 (1962).
7) T. Nagasawa, K. Kuroiwa, K. Narita and Y. Isowa, *Bull. Chem. Soc. Japan*, **46**, 1269 (1973).
8) R.B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963); J.M. Stewart and J.D. Young, "Solid Phase Peptide Synthesis," W.H. Freeman and Co., San Francisco, 1969.
9) M. Bodanszky and K.W. Funk, *J. Org. Chem.*, **38**, 1296 (1973).

Table I. Standard Schedule for Solid Phase Peptide Synthesis

Step	Reagent and purpose	Time (min)
1	dioxane, wash (3×)	2
2	4N HCl–dioxane, prewash (1×)	5
3	4N HCl–dioxane, deblock (1×)	30
4	dioxane, wash (3×)	2
5	DMF, wash (3×)	2
6	10% Et_3N–DMF, prewash (1×)	5
7	10% Et_3N–DMF, neutralization (1×)	10
8	DMF, wash (3×)	2
9	CH_2Cl_2, wash (3×)	2
10	Boc–amino acid–CH_2Cl_2 (1×)	5
11	DCC–CH_2Cl_2, coupling (1×)	300
12	DMF, wash (3×)	2
13	Ac_2O–Et_3N–DMF, acetylation (1×)	60
14	DMF, wash (3×)	2
15	EtOH, wash (1×)	2
16	AcOH, wash (3×)	2
17	EtOH, wash (3×)	2

11. For the procedure B, step 10 and 11 in the procedure A were reversed, namely DCC in methylenechloride was first added followed by N-methylmorpholine salt of Boc–Ile–OH in methylenechloride. For the active ester procedure, steps 6 to 9 and 11 were eliminated and in step 10 three eqivalent of Boc–Ile–ONo[10] in DMF was added followed by 1.1 equivalent of N-methylmorpholine in DMF. The rate of the suppression was monitored by comparison of amino acid content in the acid hydrolysates[11] of the expected Boc–tripeptide polymer esters as shown in Table II. The data in Table II indicate that the yield of the tripeptide-resin, Boc–Ile–Pro–pro-resin, was drastically improved by the new procedure A and B as compared with those of either standard or active ester procedure.

Table II. Amino Acid Content in the Acid Hydrolysates of Peptide Polymer Esters after Each Step[a]

Procedure	Expected peptide polymer ester	mmole of amino acid/g of polymer	
		Ile	Pro
	Boc–Pro–P[b]		0.269
	Boc–Pro–Pro–P		0.537
	Boc–Ile–Pro–Pro–P		
Standard		0.088	0.310
Procedure A		0.237	0.522
Procedure B		0.248	0.535
Active ester		0.060	0.424

a) Samples corresponding to about 0.25 μmole of each amino acid residues were hydrolyzed and analyzed.
b) P=–OCH_2-polystyrene-2%-divinylbenzene copolymer

Applying the system of Bodanszky and Conklin,[12] known as one of convenient and sensitive tests for racemization, we have monitored the degree of recemization which may occur during the coupling reaction. Samples taken for analysis were about 0.75 μmole as

10) M. Bodanszky, K.W. Funk and M.L. Fink, *J. Org. Chem.*, **38**, 3565 (1973).
11) J. Scotchler, R. Lozier and A.B. Robinson, *J. Org. Chem.*, **35**, 3151 (1970).
12) M. Bodanszky and L.E. Conklin, *Chem. Commun.*, **1967**, 773.

content of isoleucine, since in the range of sample about 1% racemate, D-alloisoleucine, could be detectable and determined on the amino acid analyzer,[13] Hitachi Model KLA-3B. Isoleucine as starting material and isoleucine prepared from Boc–Ile–OH by deblocking with trifluoroacetic acid contained no racemate. In a preliminary experiment, the hydrolysates of Boc–Ile–Ala–resin prepared from Boc–Ile–OH and Boc–Ala–resin by standard procedure, procedure A and B with a mixture of conc. HCl and propionic acid at 130° for 2 hr, according to the procedure given by Robinson, *et al.*,[11] contained less than 1.5% racemate in every samples. The hydrolysate of Boc–Ile–OH contained also less than 1.5% racemate. Similar analyses of Boc–Ile–Pro–Pro–resin prepared by standard procedure, procedure A and B respectively revealed that content of racemate in the acid hydrolysates was similarly less than 1.5% in every samples. When Boc–Ile–Pro–Pro–resin prepared by procedure A and B was hydrolyzed with constant boiling HCl in sealed tube at 110° for 24 hr and the hydrolysates were analyzed, the recovery of amino acids was about 10% as compared with that of Robinson's procedure and the content of racemate was similarly less than 1.5% as reported by Bodanszky, *et al.*[12] These findings indicate that no racemization occur during the coupling reaction by procedure A and B. Incidentally, Gisin, *et al.*[3] did not report the racemization test during the coupling step in their suppression procedure of diketopiperazine formation. On the other hand, Bumpus, *et al.*[4b] reported that racemization of histidine residue was observed during their suppression procedure.

In conclusion, procedure A and B described above provide a satisfactory approach for the suppression of the loss of dipeptide from the resin during solid phase peptide synthesis and these findings suggest further that steps 5 to 8 in a standard schedule listed in Table I could be eliminated when procedure A or B would be applied for general solid phase peptide synthesis, resulting in saving operation time and solvents.

Experimental

Boc–Pro–resin and Boc–Ala–resin were prepared from the Boc–amino acids and dry chloromethylated polystyrene 2% divinylbenzene in DMF according to the procedure described by Sakakibara[14] and Marglin.[14] The amino acid contents in the resins so obtained were 0.269 mmole Pro/g and 0.470 mmole Ala/g respectively.

Syntheses of Boc-peptide-resins——a) Standard Procedure: The syntheses followed the schedule listed in Table I. Boc–Pro–Pro–resin for a model compound was prepared by this procedure.

b) Procedure A: Steps 5 to 7 in Table I were eliminated and in step 10 three equivalent N-methylmorpholine salt of Boc–Ile–OH in methylenechloride was added followed by step 11.

c) Procedure B: Step 10 and 11 in the procedure A were reversed.

d) Active Ester Procedure: Steps 6 to 9 and 11 in Table I were eliminated and in step 10 three equivalent of Boc–Ile–ONo in DMF was added followed by 1.1 equivalent of N-methylmorpholine in DMF.

Amino Acid Analyses——The synthetic Boc–peptide–resins were dried in vacuum to constant weight and hydrolyzed at 130° for 2 hr in a same oven according to Robinson's procedure.[11] For measurement of the suppression rate of diketopiperazine formation as shown in Table II, samples corresponding to about 0.25 μmole of each amino acid residues were hydrolyzed and analyzed on Hitachi Model KLA-3B amino acid analyzer according to the directions given by Moore, *et al.*[13] Samples taken for quantitative analysis of the racemate, D-alloisoleucine, were about 0.75 μmole as content of isoleucine.

13) D.H. Spackmann, W.H. Stein and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

14) A. Kishi, Y. Kishida and S. Sakakibara, "Proceedings of the 7th Symposium on Peptide Chemistry," ed. by S. Akabori, Protein Research Foundation, Osaka, 1969, p. 36; A. Marglin, *Tetrahedron Letters*, **1971**, 3145.