Sequence Dependent Cyclization-Cleavage of Dipeptides from the Oxime Resin and Its Prevention

Norikazu NISHINO,* Ming XU, Hisakazu MIHARA, and Tsutomu FUJIMOTO
Department of Applied Chemistry, Faculty of Engineering, Kyushu Institute of Technology, Tobata-ku, Kitakyushu 804
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The rates of cyclization-cleavage of dipeptides with various sequences assembled on the Kaiser's oxime resin were determined. *cyclo*(-Ala-Ala-) was liberated from the H-Ala-Ala-oxime resin with the apparent first-order rate constant, k_{app}, 1.8×10^{-1} min^{-1} under basic conditions. While *cyclo*(-Ala-Pro-) was much more rapidly formed (k_{app}=1.5 min^{-1}) from the H-Ala-Pro-resin, *cyclo*(-Ala-Val-) was produced with k_{app}, 2.2×10^{-2} and 5.5×10^{-2} min^{-1} from H-Ala-Val-resin and H-Val-Ala-resin, respectively. During the coupling reaction for tripeptides by the dicyclohexylcarbodiimide/1-hydroxybenzotriazole or the symmetrical anhydride method was observed significant loss of dipeptides by cyclization, which was successfully prevented by the [(benzotriazol-1-yl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate (BOP) method except in the case of H-Ala-Pro-resin. In consequence of elucidating the defect and describing the method for the prevention, the reliability of the oxime resin was increased to be widely used in the practical synthesis of protected peptides.

The solid-phase peptide synthesis (SPS)[1] on the 4-nitrobenzophenone oxime resin is a highly promising method in the preparation of the intermediate protected peptides.[2] The first advantage is to afford protected peptides cleaved from the resin in mild conditions. These protected segments are used to assemble the larger polypeptides in solution or solid-phase method.[2d-g] The utility of the oxime resin originates in the anchoring linkage which is a kind of active ester. However, the resin has a defect also based on the lability of the oxime ester. Especially amplified is the known danger of the cleavage of the first dipeptide as a cyclic dipeptide during the coupling of the third Boc-amino acid (Fig. 1).[2a,g,h] DeGrado and Kaiser[2a] pointed out this danger but cleared by the use of the symmetrical anhydride method[3] for coupling. The faster coupling reagents are desirable to avoid the loss of dipeptide on the resin.

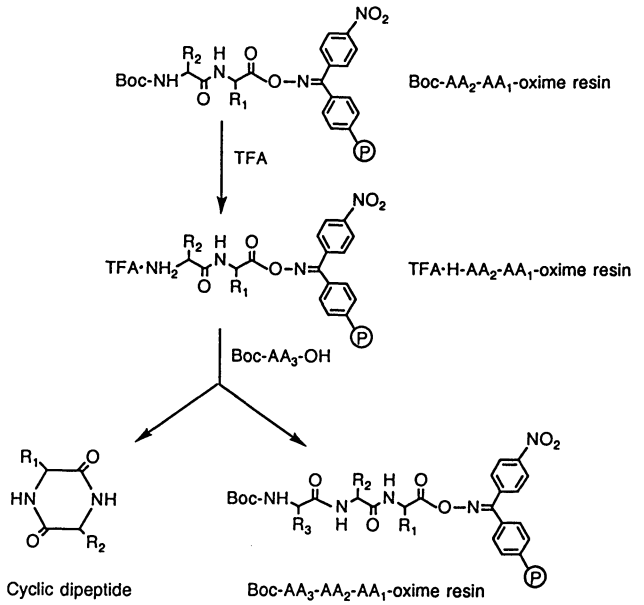

Fig. 1. The cyclization-cleavage of dipeptide during the incorporation of the third amino acid.

Nevertheless, we experienced that the cleavage is rather faster and unavoidable enough even by the use of the symmetrical anhydride method in the case of unhindered dipeptide sequence. Ösapay et al. recently described the peptide cyclization on the oxime resin.[2h] Although they showed some examples, the sequence dependence was not systematically studied to elucidate the danger hidden during the incorporation of the third amino acid. Either they were not concerned for the prevention of the undesired loss of dipeptides from the resin during the condensation for tripeptides. Therefore, we attempted to determine the rates of cyclic dipeptide formation by HPLC analysis and to examine the dependence on the amino acid sequences by employing 8 dipeptide-resins (Table 1) which may represent the features of whole 20 amino acids. Furthermore, we have found out the methods which prevent the autolysis at the coupling of the third amino acid by using the same analytical technique for the determination of the cleavage rates.

In addition, for the search for more stable oxime esters, new oxime resins containing 3-nitro-, 3,5-dinitro-, 3,5-dichloro-, and 4-trifluoromethyl-benzophenone groups were prepared instead of 4-nitrobenzophenone oxime resin. The properties of them in the utility in SPS were also examined.

Results and Discussion

Cyclization-Cleavage of Dipeptides from the Oxime Resin. Eight different dipeptide-oxime resins were prepared and examined for the rates of the cyclization-cleavage of dipeptides (Table 1). Alanine is not only often seen in the peptides but also to represent other less-hindered amino acid residues with or without the side chain protection, for instance, Leu, Met, Glu, Lys,

Table 1. Half Times and Rate Constants for the Formation of Cyclic Dipeptides from the Oxime Resin

Dipeptide resin	$t_{1/2}$/min[a]	$k_{app}\times10^2$/min^{-1}[b]
Ala-Gly-	4	23
Ala-Ala-	4	18
Ala-Pro-	<0.5	150
Ala-Val-	30	2.2
Gly-Ala-	4	18
D-Ala-Ala-	3	28
Pro-Ala-	8	8.4
Val-Ala-	16	5.5

a) Half time of the cleavage to give cyclic dipeptides in 50% yield. b) Apparent first-order rate constant.

and Arg. Valine has a β-branched side chain and more or less would be analogous in behavior with Ile and other somewhat hindered amino acids including aromatic ones. Glycine and proline are individual, as well accepted, in behaviors in reactivity and conformation. The first set of four dipeptide sequences in Table 1 were expected to elucidate the difference in the reactivities of the corresponding oxime esters. The next set may afford information on the nucleophilic reactivity of the amino groups with differently hindered side chains.

Boc-dipeptide-oxime resins were prepared with 3 equiv of Boc-amino acid by the DCC/HOBt method.[4] They were treated with 25% TFA in DCM for 30 min, then the resin was washed with DCM (2 times), isopropyl alcohol (1 time), DCM (2 times). Finally the resin was suspended in DMF, 2 equiv of Et_3N was added. An aliquot was withdrawn in appropriate intervals and analyzed by HPLC (see Experimental). The progress of the formation of cyclic dipeptides was plotted by using HPLC integral values. Figure 2 shows the time courses for the formation of *cyclo*(-Ala-Pro-), *cyclo*(-Ala-Ala-), and *cyclo*(-Ala-Val-) for the comparison. The apparent first-order rate constants, k_{app}, were calculated according to the method by Gisin and Merrifield[5] and summarized in Table 1 with the half times. The dipeptides consisting of unhindered amino acids produced the corresponding cyclic dipeptides fairly fast with similar rates. In the case of *cyclo*(-Ala-Gly-) and *cyclo*(-Ala-Ala-), the cyclization-cleavage of dipeptides was completed within 20 min with $t_{1/2}$, 4 min for both and k_{app}, 2.3×10^{-1} and 1.8×10^{-1} min^{-1}, respectively. In their earlier study, DeGrado and Kaiser estimated $t_{1/2}$ and k_{app} only for the formation of *cyclo*(-Glu(OBzl)-Ser(Bzl)-) (27 min and 2.6×10^{-2} min^{-1}, respectively).[2a] The benzyloxy group on β-carbon in Ser(Bzl) should have affected to slower the cleavage reaction. The steric behavior of Ser(Bzl) seems to mislead them to judge that the cleavage is generally not so fast. As a good support for this argument, Val, a representative of the hindered amino acids, was shown to decrease the rates of the cleavage (k_{app} for *cyclo*(-Ala-Val-) from Ala-Val-resin, 2.2×10^{-2} min^{-1}). The steric hindrance in cyclization was more severe when Val is at *C*-terminus. Proline at *C*-terminus extremely increased the cleaving rate (k_{app}=1.5 min^{-1}) due to the easiness in *cis*-peptide bond formation. It should be noted that the formation of *cyclo*(-Pro-Ala-) is rather slower among the dipeptides tested probably due to the poorer reactivity of the pyrrolidine NH than the amino group to the oxime ester. The D-amino acid residue at *N*-terminal did not significantly affect the rate of the cleavage. Since the rather fast cleavage of dipeptides with cyclization was clearly observed, its prevention was investigated in the next stage.

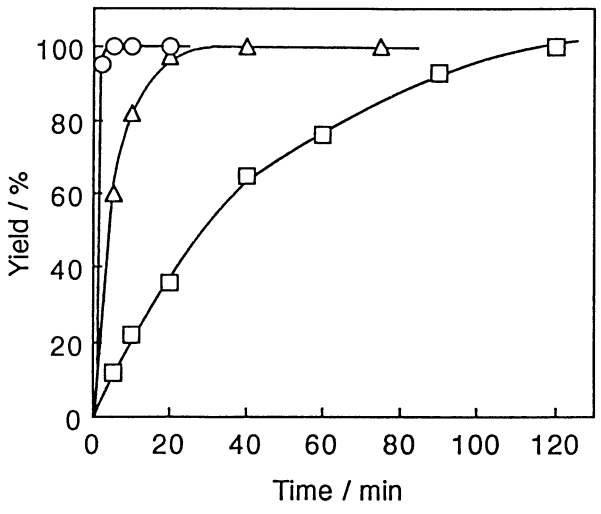

Fig. 2. Time courses for the formation of cyclic dipeptides from the oxime resin.
(○). *cyclo*(-Ala-Pro-); (△) *cyclo*(-Ala-Ala-); (□) *cyclo*(-Ala-Val-).

Prevention of the Formation of Cyclic Dipeptides. For the longer protected peptide assembly, the deletion of the first dipeptide produces the serious problem to give defective peptides.[2a,g] Therefore, we attempted to compare the several methods in efficiency on the coupling between Boc-Val-OH and dipeptide-oxime resin (Table 2). During the coupling reaction was monitored the appearance of cyclic dipeptide as mentioned above. We have found that the coupling method with the BOP reagent[6] is superior to the DCC/HOBt and symmetrical

Table 2. Yields of Tripeptides and Cyclic Dipeptides in the Coupling between Boc-Val-OH and Dipeptide Oxime Resins by Various Coupling Methods

Dipeptide resin	Coupling method	Yield/%	
		Tripeptide[a]	Cyclic dipeptide[b]
Ala-Ala-	None[c]	0	100
Ala-Ala-	DCC/HOBt	40	55
Ala-Ala-	Symmetrical anhydride	70	27
Ala-Ala-	BOP1	85	10
Ala-Ala-	BOP2	98	ND[d]
Pro-Ala-	BOP2	93	ND[d]
Ala-Pro-	BOP2	44	50

a) Determined by the picrate assay. b) Determined by HPLC. c) Only Et_3N was added. d) Not detected.

anhydride methods[3] and allowed little formation of cyclic peptides. Additionally, two different procedures of BOP methods were examined. One adopted the addition of Et_3N (6.5 equiv) to the mixture of BOP (3.0 equiv), Boc-Val-OH (3.0 equiv) and dipeptide-oxime resin (1.0 equiv) in DMF (BOP1 method), and another was that the mixture of BOP, Boc-Val-OH, and Et_3N was prepared at first, then added into the DMF suspension of dipeptide-oxime resin at once (BOP2 method). The reaction mixture was allowed to shake for 60 min at room temperature. The latter method gave the desired tripeptide-resin in quantitative yield with completely free from the formation of *cyclo*(-Ala-Ala-), while the former method in slightly lower yield. However, the formation of *cyclo*(-Ala-Pro-) could not be avoided even by the BOP2 method, while Pro-Ala caused no problem. These results were coincident with the results on the formation rates of cyclic dipeptides.

The Substituted Oxime Resins. To examine the influence of the 4-nitro group on the cyclization-cleavage of dipeptides, 4-nitrophenyl moiety in the oxime resin was replaced by 3-nitro-, 3,5-dinitro, 3,5-dichloro-, and 4-trifluoromethyl-phenyl groups to prepare the new oxime resins. The resins were successfully prepared by a similar manner to that of 4-nitrobenzophenone oxime resin and Boc-Ala-OH was introduced by the routine method to give sufficient substitution levels (0.5—0.65 mmol/g resin). DeGrado and Kaiser reported 4-H, 4-OMe, and 4-Cl substituted oxime resin as well as 4-nitrobenzophenone oxime resin in their first paper of the oxime resin.[2a] They, however, mentioned only briefly that the ester linkage on the 4-nitro-resin was stable toward 25% TFA. The new resins prepared in this work were judged to be stable to the exposure to 25% TFA for 30 min, because the picrate assay of the corresponding Boc-Ala-oxime resins showed proper values. The formation rates of *cyclo*(-Ala-Ala-) from the new resins were examined by the same method mentioned above. The rate constants were not much different from each other including the 4-nitrobenzophenone oxime resin (Table 3).

The Kaiser's oxime resin has high utility to prepare protected peptides with various modification at the *C*-terminus such as free acid, amides, or esters. The oxime resin approach, however, suffers from the same problem in other resins (for example, the Merrifield resin[3] and resins for the Fmoc strategy[7]) that the cleavage of X-Pro dipeptide with cyclization can not be avoided at the step of the third amino acid coupling. When the desired peptide contains Pro at *C*-terminus, the fragment coupling between a dipeptide and Pro-oxime resin is recommended. Otherwise, the cleavage of peptide segments with H-Pro-X (X; NHR or OR)[8] after the stepwise coupling on the resin can be also selected. The present study offered the quantitative understanding of the cleavage of dipeptides with cyclization and its prevention with the BOP reagent, which is also recommended in the literature.[2g] These findings finally added the high reliability to the oxime resin SPS and will benefit the resin users in avoiding the wrong protocol.

Table 3. Half Times and Rate Constants for the Formation of *Cyclo*(-Ala-Ala-) from the New Oxime Resins

Oxime resin	$t_{1/2}$/min[a]	$k_{app}\times10^2$/min^{-1}[b]
4-NO_2	4	18
3-NO_2	4	19
3,5-$(NO_2)_2$	3	22
3,5-Cl_2	4	19
4-CF_3	3	21

a) Half time of the cleavage to give the cyclic dipeptide in 50% yield. b) Apparent first-order rate constant.

Experimental

General. The 4-nitrobenzophenone oxime resin was prepared according to the reported method[2b] with slight modifications.[9] SPS was carried out manually in an SPS vessel. HPLC was carried out on a μBondasphere 5μ Si-100 Å (3.9 mm×15 cm) column with hexane-EtOH (85 : 15, v/v) detected at 220 nm by employing a Hitachi L-6200 intelligent pump equipped with a Hitachi L-4200 UV-VIS detector and a Hitachi D-2500 chromato-integrator.

Preparation of Substituted Oxime Resins. 3-Nitro-, 3,5-dinitro, 3,5-dichloro-, and 4-trifluoromethyl-benzophenone oxime resins were prepared by the same manner as 4-nitrobenzophenone oxime resin. To the suspension of Bio-Beads SX1 (poly(styrene-*co*-divinylbenzene) (99 : 1 w%) resin, Bio-Rad) (10 g) in DCM (170 ml) was added the corresponding benzoyl chloride (10 mmol) and $AlCl_3$ (1.9 g, 14 mmol). The suspension was stirred at room temperature for 36 h. The dark red suspension was filtered and washed with dioxane-4 M HCl aqueous (3 : 1, v/v, 300 ml), dioxane-H_2O (3 : 1, v/v, 300 ml), DMF (300 ml), MeOH (300 ml), DCM (150 ml), and MeOH (150 ml), and dried in vacuo; ca. 11 g of white benzophenone resin was obtained. To the suspension of the benzophenone resin in pyridine-EtOH (1 : 5, v/v, 90 ml) was added $NH_2OH\cdot HCl$ (11 g), and the mixture was refluxed for 20 h. The resin was filtered and washed with MeOH-H_2O (3 : 1, v/v, 150 ml), DMF (70 ml), DCM (70 ml), MeOH (70 ml), DCM (70 ml), and MeOH (70 ml), and dried in vacuo; ca. 11 g of the corresponding oxime resins was obtained. The IR spectra showed strong absorbance at 3530, 1520, and 1310 cm^{-1}, and the absorption at 1650 cm^{-1} for the carbonyl had disappeared. After the introduction of Boc-Ala-OH (to be described below), the picrate assay[10] revealed the substitution levels of the resins were 0.50—0.65 mmol/g resin.

Boc-Amino Acid-Oxime Resin. The introduction of the first amino acid to the oxime resin was carried out according to the literature.[2c] To the oxime resin (1.0 g) was added the solution of Boc-amino acid (1.0 mmol) and DCC (1.0 mmol) in DCM (15 ml) in a manual SPS vessel. The mixture was shaken for 15 h at room temperature. The solvent was filtered off and the resin was washed with DCM (×2), DCM-EtOH (1 : 1, v/v) (×4), and DCM (×2) (The solvent volume for each step was 15 ml/g resin.), and then dried in vacuo. An aliquot of the amino acid resin was withdrawn and subjected to the picrate assay to estimate the substitution level (normally 0.5—0.6 mmol/g resin).

Boc-Dipeptide-Oxime Resin. Boc-amino acid-oxime resin (1.0 g, 0.5 mmol/g resin) was placed in a reaction vessel, and the coupling was carried out manually as follows; (1) wash, DCM (×2); (2) prewash, 25% TFA/DCM (×1); (3) deprotect, 25% TFA/DCM (30 min ×1); (4) wash, DCM (×2); (5) wash, isopropyl alcohol (×1); (6) wash, DCM (×3); (7) wash DMF (×1) (8) couple, Boc-amino acid HOBt ester (3 equiv) and Et_3N (2 equiv) in DCM-DMF (1:1, v/v) (90 min ×1); (9) wash, DCM-DMF (1:1, v/v) (×4); (10) wash, DCM (×2). The solvent volume for each step was 15 ml/g resin. The coupling efficiency was checked by the Kaiser test.[11]

Determination of the Formation Rates of Cyclic Dipeptides from the Oxime Resin. The Boc group in Boc-dipeptide-resin (200 mg, 0.1 mmol peptide) was removed by the step (1)—(6) as described above. To the resulting TFA·dipeptide-resin suspended in DMF (5.0 ml) was added Et_3N (2 equiv) and the mixture was shaken for 24 h at room temperature. An aliquot was withdrawn at appropriate interval, concentrated to dryness in vacuo, dissolved in MeOH, and analyzed by HPLC. The retention times for *cyclo*(-Ala-Gly-), *cyclo*(-Ala-Ala-), *cyclo*(-Ala-D-Ala-), *cyclo*(-Ala-Pro-), and *cyclo*(-Ala-Val-) are 16.3, 10.6, 9.0, 11.0, and 8.8 min, respectively, in the HPLC conditions described in General section. The yields of cyclic peptides were calculated by using the integral values and the time courses were shown in Fig. 2.

Boc-Val-Ala-Ala-Oxime Resin (Comparison of Coupling Methods). Boc-Val-OH was coupled with H-Ala-Ala-oxime resin (200 mg, 0.1 mmol peptide) prepared by the steps mentioned above by various coupling methods as follows: (1) The DCC/HOBt method; Boc-Val-OBt (3 equiv) was prepared with Boc-Val-OH (3 equiv), DCC and HOBt (3 equiv each) at 0 °C for 30 min in DCM-DMF (1:1, v/v). The solution was added to TFA·H-Ala-Ala-resin after removal of *N,N'*-dicyclohexylurea and then to the suspension was added Et_3N (2 equiv). The mixture was shaken for 90 min at room temperature. (2) The symmetrical anhydride method; Boc-Val-symmetrical anhydride (3 equiv) was prepared with Boc-Val-OH (6 equiv) and DCC (3 equiv) at 0 °C for 30 min in DCM. The solution was added to TFA·H-Ala-Ala-resin after removal of *N,N'*-dicyclohexylurea and then to the suspension Et_3N (2 equiv) was added. The mixture was shaken for 60 min at room temperature. (3) The BOP1 method; To TFA·H-Ala-Ala-resin was added the solution of Boc-Val-OH and BOP (3 equiv each) in DMF, and then was added Et_3N (6.5 equiv). The mixture was shaken for 60 min at room temperature. (4) The BOP2 method; To the solution of Boc-Val-OH and BOP (3 equiv each) in DMF was added Et_3N (6.5 equiv) at first, and this solution was added to TFA·H-Ala-Ala-resin. The mixture was shaken for 60 min at room temperature. At the end of each coupling, an aliquot of the reaction solution was withdrawn and the amount of *cyclo*(-Ala-Ala-) liberated in the solution phase was determined by HPLC as mentioned above. After the washing steps (9) and (10) mentioned above, the tripeptide-resin was dried in vacuo. An aliquot was subjected to the picrate assay to determine the yields of tripeptide. Other tripeptides were also examined by the same manner. The results are shown in Table 2.

References

1) Abbreviations: AA, amino acid; BOP, [(benzotriazol-1-yl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate; Bzl, benzyl; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DMF, *N,N*-dimethylformamide; Et_3N, triethylamine; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; SPS, solid-phase synthesis; TFA, trifluoroacetic acid; Tos, *p*-tolylsulfonyl.

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