Pergamon

Tetrahedron Letters, Vol. 37, No. 42, pp. 7557-7560, 1996

0040-4039/96 $15.00 + 0.00

PII: S0040-4039(96)01716-9

A Rapid Reaction Releasing the Carboxyl Terminal Residues of Peptides

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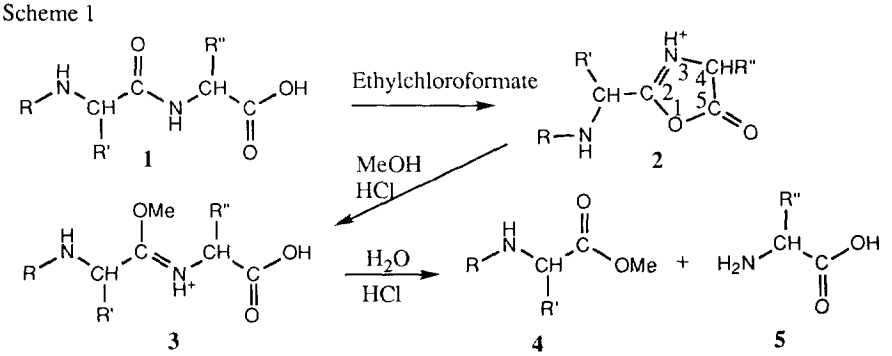
Abstract: Alcoholysis of peptide oxazolones in alcohols containing dilute acid, such as 0.02M HCl, gives the C-terminal amino acid and the ester of the shortened peptide within 10 minutes at room temperature in 30-70% yields. This together with the finding that the peptide oxazolones can be formed within 3 minutes with alkylchloroformates enables the rapid removal of peptide C-terminal residues. Copyright © 1996 Elsevier Science Ltd

Peptide sequencing is an important technique in biotechnology and related areas. It is mostly carried out by the Edman degradation which sequentially releases amino acids from the N-terminal of peptides. No method has yet been established for routine determination of the C-terminal residue, although it would greatly facilitate this research area. The thiohydantoin method [1,2] has seemed most promising and has been studied perseveringly. Degradation of peptide oxazole derivatives, such as N-peptidyl-2-oxazolidinones [3] and 5-alkoxyoxazoles [4], also has been a candidate for this purpose. One [5] of the procedures [5,6] for the C-terminal degradation by perfluoric acid vapor uses the peptide oxazolones as the intermediate. In this study we developed a procedure for splitting the peptide oxazolones according to the following scheme.

Scheme 1



The peptide **1** can be transformed to its oxazolone by various methods. The oxazolone in its protonated form **2** will give the imido ester **3** when attacked by alcohol (MeOH in the scheme) at the C2 carbon, although it will give the ester of the original peptide on the attack at the C5 carbon. The imido ester **3** in its protonated form will give the ester of the shortened peptide **4** and the C-terminal amino acid **5** when its C=N carbon is attacked by water. It is known that the C2 and C5 of protonated oxazolones are reactive to nucleophilic attacks [7] and that imido esters principally give the esters on acidic hydrolysis by expelling the amine (amino acid in this case), although they give amides (the original peptide in this case) by expelling the alcohol on alkaline hydrolysis [8].

In the standard experiments, oxazolones were prepared by adding 0.01 ml of 1 M ethylchloroformate (ECF) in tetrahydrofuran (THF) to 0.5 ml of 1 mM peptides [9] in THF containing 0.04 M N-methylmorpholine and 0.01M HCl at room temperature. After 3 minutes, acid alcoholysis of oxazolone (AAO) was carried out by diluting a portion of the oxazolone solution by more than 5-fold with alcohol (usually MeOH) containing 0.02 M HCl [10]. After more than 10 minutes, the products were subjected to HPLC and amino acid analysis. In the above

treatments HCl was added as an 1N etherial solution.The volume and the concentration of peptides can be reduced in practical applications without changing the concentrations of the reagents.

Figure 1 shows the nearly quantitative conversion of Bz-Gly-DL-Ala to the oxazolone [11]. The formation of the by-product assigned to the 5-ethoxycarbonyloxy oxazole was depressed under the present conditions. Figure 2 shows the acid alcoholysis of the oxazolone. About 60% of Bz-Gly-DL-Ala gave Bz-Gly-OMe.

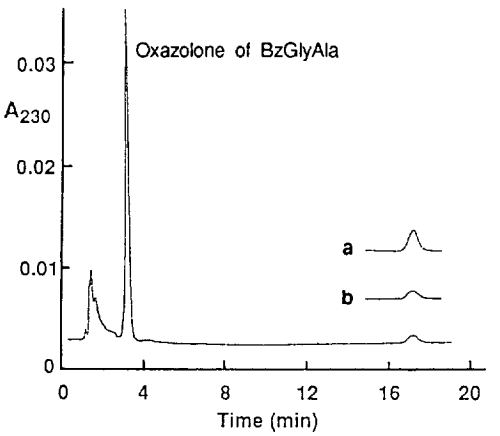


Figure 1. Oxazolone of Bz-Gly-DL-Ala.

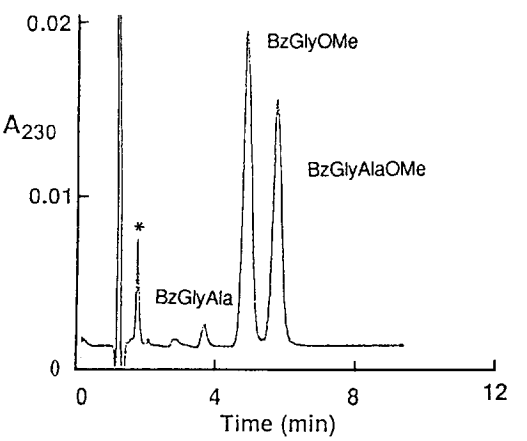


Figure 2. AAO of Bz-Gly-DL-Ala.

Fig.1. BzGlyAla treated with ECF under the standard condtions for 3 min was applied to HPLC after dilution with THF. Fig.1a. Oxazolone formation in the absence of HCl increased the by-product. Fig.1b. 10 min incubation under the standard conditions did not increase the by-product. Eluents: 40% acetonitrile / 0.02 M Na phosphate buffer pH 6.8. Fig. 2. The oxazolone was diluted with MeOH- 0.02M HCl. Eluents:30% MeOH / 0.1% aqueous trifluoroacetic acid. The peak with (*) is a reagent peak.

Figure 3 shows the AAO of acetyl leucine-enkephalin carried out using 0.3 ml of 0.1mM solution. The reaction was associated with nearly complete racemization of the C-terminal residue.

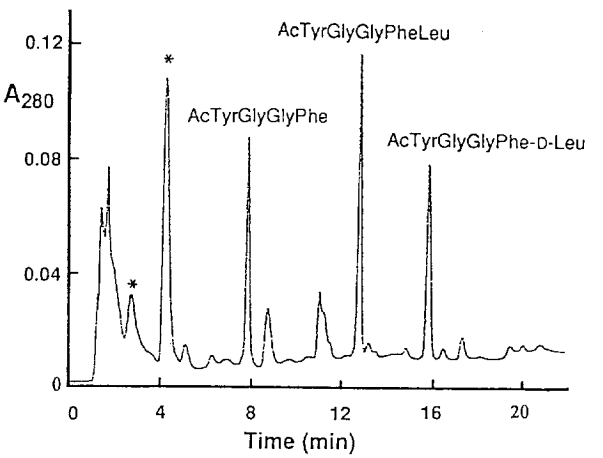


Figure 3. AAO of Acetyl Leucine-enkephalin.

HPLC was carried out after alkaline hydrolysis of the sample diluted in the acidic methanol by adding an equal volume of 0.1 M NaOH and neutralizing it with the equivalent amount of 1 M HCl after 15 min. It enabled the monitoring at 280nm reverting the modifications at the tyrosine hydroxyl. The peaks were assigned from FAB-MS and amino acid analysis. AcTyrGlyGlyPhe-D-Leu was assigned from the non-susceptibility to carboxypeptidase A. Eluent : a linear gradient of acetonitrile and 0.1% aqueous trifluoroacetic acid, 20%-35% / 16 min . (*) : peaks derived from the reagents.

Table 1 summarizes the results of AAO for various peptides. The yields given as the ratio of the shortened ester to the total esters are not affected by either the incomplete formation of the oxazolones, nor the hydrolysis of the oxazolones in the acid alcoholysis step. Therefore, these values exactly reflect the effects of the variation in the kind of peptides and alcohols.The yield was not greatly affected by the size of the amino acid side chains or by the kind of ordinary alcohols, while with the substituted ethanols with low pKa values (entries 11 to 13) the oxazolone did not split but gave the esters of Cbz-Leu-DL-Leu. Bz-Gly-Lys without prior acetylation (entry 15), split in a low yield giving the cyclic imide, but with prior acetylation (entry 16), it split normally releasing ε-acetyl lysine. Entries 17 and 18 for leucine enkephalins indicate that acetylation of the N-terminal amino group is also effective in promoting C-terminal splitting, probably preventing the formation of a cyclic peptide. Although not shown in the table, the addition of other solvents, such as dimethylformamide, acetonitrile, THF and t-amylalcohol up to levels equaling the acidic MeOH had little effect on the yield of AAO of Cbz-Leu-Leu. The yield increased slightly at a low temperature (65% yield with Cbz-Leu-Leu at -60°C).

Table 1 Acid Alcoholysis of Peptide Oxazolones

No	Peptides	Yields [a] %		Total esters [b] %	Alcohols
1	Bz-Gly-Ala	63	(51)	90	MeOH
2		51			Et OH
3		55			HOC_2H_4OH
4	Bz-Gly-Phe	69	(54)	85	MeOH
5		63			EtOH
6	Cbz-Leu-Gly	48	(45)	89	MeOH
7	Cbz-Leu-Leu [c]	50	(48)	91	MeOH
8		56			EtOH
9		48			nPrOH
10		51			$CH_3OC_2H_4OH$
11		8			CNC_2H_4OH
12		0			ClC_2H_4OH
13		0			CF_3CH_2OH
14	Bz-Gly-Arg	55	(47)	86	MeOH
15	Bz-Gly-Lys	4 [d]		n.d.	MeOH
16	Bz-Gly-(ε-Ac)Lys [e]	56	(48)	88	MeOH
17	Tyr-Gly-Gly-Phe-Leu		(16)	n.d.	MeOH
18	Ac-Tyr-Gly-Gly-Phe-Leu	32 [d]	(33)	n.d.	MeOH

Oxazolones prepared by the standard method were diluted 10-fold in the alcohols listed containing 0.02 M HCl at room temperature.

a. The ratio of the shortened ester to the total esters measured by the peak areas on HPLC. The values in parentheses are the yields based on the mole of amino acid released per mole of peptides.

b The sum of the shortened peptide ester and the original peptide esters (racemate). Only the values for MeOH were given, because the hydrolysis of the oxazolones in the acidic alcohols depends much on the moisture content of the alcohols, then the comparison of the total esters in different alcohols requires an exact control of the water content, which was not carried out in the present experiments.

c The reactions of Cbz-Leu-Leu were associated with nearly complete racemization of the C-terminal leucine as observed by the HPLC patterns showing the esters of Cbz-Leu-D-Leu.

d. The yields were measured as the ratios of the shortened peptides to total peptides after alkaline hydrolysis.

e. The acetylation was carried out *in situ* by adding acetic anhydride at 10 mM to the mixture for the oxazolone formation 10 minutes before the addition of ECF.

The proposed mechanism has not yet been proved. Other intermediates such as the orthoamide or orthoester may intervene the reaction. In any case, a reaction that can split the amide bonds in a few minutes, in other words at a rate comparable to that of the hydrolysis of acid anhydrides, is interesting in view of the reaction mechanism. In the present mechanism, the yield of splitting depends principally on the relative reactivity of the alcohol at the C=O and C=N carbons. The search for alcohols more selective to C=N carbons has not been successful as of yet. The role of the alcohol, however, can be replaced by other nucleophiles. Indeed, azide anion reacts mostly at the C=N carbon, although it gives tetrazole derivatives[12]. The reactions found by Tsugita et.al.[5,6] also can be interpreted as examples of organic acids replacing the alcohol in the scheme. A wider search for nucleophiles reacting at the C=N carbon is expected to find ways to split C-terminal residues more completely. The present yield of AAO is not enough for the sequential determination of C-terminal residues, but the reaction can be used as a method for the determination of C-terminal 1 residue after the check of its applicability to other amino acids with reactive side chains. The procedure taking only 15 minutes at room temperature is very convenient compared with other methods such as hydrazinolysis[13] and tritium labeling[14].

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9. Bz-Gly-DL-Ala was prepared by condensing Bz-Gly and DL-alanine by ECF ; analysis C,57.68;H,5.68; N,11.20 (calc. C,57.59; H, 5.64; N,11.19), mp.201°C (lit 200-201°C)[15]. Cbz-Leu-Gly and Cbz-Leu-Leu-OMe were purchased from Sigma Chem. Co.. Bz-Gly-Arg, Bz-Gly-Lys, Bz-Gly-Phe and leucine-enkephalin were from Peptide Institute Inc.(Osaka). Cbz-Leu-Leu was prepared by alkaline hydrolysis of Cbz-Leu-Leu-OMe analysis C,63.27; H,7.84; N,7.35 (calc. C,63,47;H,7.88;N,7.40), mp 88 °C (lit 90-92°C)[16]. Acetyl leucine enkephalin was prepared by treating leucine enkephalin with 0.01 M acetic anhydride in THF containing 0.04 M N-methylmorpholine and purified by HPLC. ODS columns (Toso 80TM) were used in the analysis and the preparation.The solvents were dried with Molecular Sieves 3A.
10. The reactions are usually completed in a few minutes and can be accelerated at higher HCl concentrations. 0.02M was chosen for the sake of the repetitive cycles which need the alkaline hydrolysis of the shortened peptide esters. The reaction requires water, but trace water in the solvents was enough for the reaction.
11. The oxazolone was identified from FAB MS (M+1=233) and an IR absorption[17] at 1825 cm^{-1}. Isobutyl-chloroformate formed oxazolones similarly, but dicyclohexyl carbodiimide and trifluoroacetic anhydride were less effective under the present conditions.The by-product was assigned to the 5-ethoxycarbonyloxy oxazole from FAB MS (M+1=305) formed by the acylation of the carbonyl oxygen by excess ECF. Murakami, M.; Iwanami, M. *Bull.Chem.Soc.Jap.* **1968**, 41, 726-727
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(*Received in Japan* 30 *July* 1996; *revised* 27 *August* 1996; *accepted* 2 *September* 1996)