SIDE REACTIONS OF HYDROGEN FLUORIDE ON PEPTIDES CONTAINING GLUTAMIC ACID IN OR NEAR THE C-TERMINUS IN SOLID PHASE SYNTHESIS

Seiyo Sano and Shosuke Kawanishi

Department of Public Health, Faculty of Medicine,
Kyoto University, Kyoto, Japan

Received January 15,1973

SUMMARY: Various peptides containing glutamic acid in or near the C-terminus were synthesized by the solid phase method and treated with anhydrous hydrogen fluoride in the presence of anisole. Compounds, apparently resulting from conversion of glutamic acid to a cyclic derivative, and presumably containing anisole, appeared while glutamic acid disappeared.

In solid phase peptide synthesis, hydrogen fluoride (HF) has been widely used for the removal of the protecting group from the peptide as well as the cleavage of the peptide from the supporting resin (1,2). Side reactions such as N to O acyl migration (3), or degradation of methionyl bonds (4) have been observed but these occur at a very slow rate i.e. several days. During studies on the synthesis of undecapeptides which occur at the carboxyl terminal of cytochrome *c*, i.e. Boc-Leu-Ile-Ala-Tyr(O-Bzl)-Leu-Lys(Z)-Lys(Z)-Ala-Thr(O-Bzl)-Asn-Glu(γ-OBzl)-resin, it was discovered that glutamic acid was sensitive to anhydrous HF-anisole treatment. During purification of the peptide by Sephadex G 25 superfine, most of the material migrated more slowly than the original peptide, and an undecapeptide with glutamic acid missing was isolated in large amount. This suggested that some hitherto unknown reaction might take place with glutamic acid. Simple model compounds, Boc-Asn-Glu(γ-OBzl) or similar peptide resins appropriate for these studies have been utilized and we report here a side reaction of HF in the presence of anisole which causes a rearrangement of the glutamyl residue occurring in or near the C-terminus.

MATERIALS AND METHODS: Boc-amino acids were purchased from Kyoto Daiichi Kagaku Co. LTD. Peptide synthesis were carried out by a simplified solid phase procedure of Merrifield (5). Boc-γ-benzyloxy-glutamic acid was esterified to Merrifield resin (Bio Bead S-X2) by two different methods, one was by heating with triethylamine in benzene instead of alcohol to avoid trans-esterification according to the method of Bayer et al. (6), and the other by keeping with triethylamine in DMF at room temperature (7). The Boc-amino acid resin was washed 4 times with CH_2Cl_2, treated with trifluoroacetic acid (1:1 v/v) for 30 min., then washed 5 times with CH_2Cl_2, next twice neutralized with 15 % triethylamine-CH_2Cl_2, followed by washing with CH_2Cl_2 and DMF. Boc-L-asparagine p-nitrophenyl ester was added in 3-fold excess in DMF (12 hours). This coupling was repeated raising coupling yield to 100 %. Double couplings were mediated by N,N'-dicyclohexylcarbodiimide in CH_2Cl_2 for 2 hrs in the case of other Boc-amino acids used in this experiment. Hydrolysis of the peptide-resin was carried out in an anaerobic mixture of 0.5 ml of 12 N HCl and 0.5 ml of propionic acid (8) containing of 0.02 ml of 5 % phenol at 130° C for 2 hrs. Each coupling rate was checked by the Hitachi amino acid analyzer (Model KLA-3B). The peptide was removed from the resin by HF and anisole (2). Anisole was added in amounts from equimolar to 150 molar excess to peptide residues.

A typical experiment was as follows: Boc-Asn-Glu(γ-OBzl)-resin (100 mg, 0.325 mmole peptide per gram) was cleaved at 0° C in anhydrous HF (10 ml) containing anisole (0.025 to 0.5 ml), with or without trifluoroacetic acid (0.5 ml), for 1 to 1.5 hrs. HF was then removed by evaporation and dipeptide was extracted from the resin with 10 % acetic acid. The extract was lyophilyzed after washing with ether. Peptide was hydrolyzed by 6.1 N HCl after cleavage from the resin in the usual way. Descending paper chromatography of the peptide was utilized in a solvent system of n-butanol-pyridine-glacial acetic acid-H_2O (75:50:15:60 by volume).

RESULTS: Acid hydrolysis of peptide-resin gave the expected amino acid. Boc-Asn-Glu(γ-OBzl)-resin yielded equimolar amounts of aspartic and glutamic acid. By contrast, acid hydrolysis of dipeptide obtained from HF-anisole treatment resulted in a low recovery of glutamic acid (~20 % yield). Paper chromatography of the peptide yielded a minor and a major ninhydrin positive spots at positions with R_f values of 0.21 and 0.56 respectively (Fig. 1). The minor component accounted for approximately 20 % of the total material and was found to be the expected dipeptide, identified by amino acid analysis after acid hydrolysis. A major chromatographic component "X-compound" was obtained in ~80 % yield. Free peptide, Asn-Glu, obtained from the peptide-resin with either HBr-AcOH or HF-anisole treatment was further converted to the "X-compound" by HF-anisole treatment. Boc-Asp(β-OBzl)-Glu(γ-OBzl)-resin, Boc-Asn-Glu(γ-OBzl)-Ala-resin, Boc-Gly-Glu(γ-OBzl)-resin, Boc-Ala-Glu(γ-OBzl)-resin and Boc-Pro-Glu(γ-OBzl)-resin also yielded the corresponding "X-compound" under the same conditions. However HF-anisole treatment of protected dipeptide containing glutamic acid at the N-terminus, i.e. Boc-Glu(γ-OBzl)-Asn-Ala-resin, or other peptide resin without glutamic acid, i.e. Boc-Asn-Ala-Resin, did not give such "X-compounds" (Table 1).

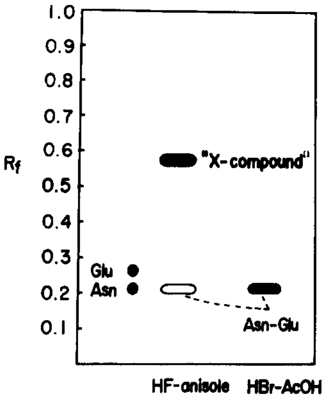

Fig. 1. Paper chromatograms of peptides isolated from Boc-Asn-Glu(γ-OBzl)-resin by HF-anisole or HBr-AcOH. n-butanol-pyridine-glacial acetic acid-H_2O (75:50:15:60 by volume).

Experiments were designed to isolate and identify the "X-compound" with R_f=0.56. It was extracted from the paper with 30 % acetic acid, diluted with water and then lyophilized. Further purification was carried out by column chromatography with Dowex 1 and elution with 0.2 N AcOH. Acid hydrolysis of the "X-compound" obtained from Boc-Asn-Glu(γ-OBzl)-resin gave the expected

Table 1. The effect of HF-anisole or HBr-AcOH treatment on various synthetic peptides containing glutamic acid.

	Yields of "X-compounds"	
	HF-anisole	HBr-AcOH
Boc-Glu(γ-OBzl)	0	0
Boc-Glu(γ-OBzl)-resin	0	0
Boc-Asn-Glu(γ-OBzl)-resin	80	0
"	20*	
"	trace**	
Asn-Glu	30	0
Boc-Asp(β-OBzl)-Glu(γ-OBzl)-resin	80	0
Boc-Asn-Glu(γ-OBzl)-Ala-resin	20	0
Boc-Gly-Glu(γ-OBzl)-resin	20	0
Boc-Ala-Glu(γ-OBzl)-resin	70∼80	0
Boc-Pro-Glu(γ-OBzl)-resin	30∼50	0
Boc-Asn-Ala-resin	0	0
Boc-Glu(γ-OBzl)-Asn-Ala-resin	0	0

HF-anisole treatment: anisole was added in amounts from 100 to 150 molar excess to peptide residues.
* equimolar ** without anisole.
HBr-AcOH treatment: cleavage by HBr-AcOH (25 %) for 16 hours at room temperature.

amount of aspartic acid and a trace amount of glutamic acid (1.5 %), serine and threonine. A small amount of the material was eluted slightly after the position of Glu(γ-OBzl) in a yield of 18 % by the amino acid analyzer. This compound was not converted to glutamic acid even by repeated acid hydrolysis. The amount of ammonia liberated during acid hydrolysis was in good agreement with that expected from deamination of asparagine.

As to the structure of the "X-compound" there exist possibilities of two cyclic compounds, one the imide type and the other a pyrrolidone type compound. Asparimide-glutamic acid, asparimide-glutamyl-alanine or asparaginyl-glutarimide-alanine can be expected as the former type. However, formation of imide type compounds from other peptides tested is difficult to consider. In alkaline hydrolysis, the imide type compound could regenerate the starting material (9) and following acid hydrolysis would give the corresponding amino acid. However the evidence that only aspartic acid, or aspartic acid and alanine, were obtained respectively by these alkaline and acid hydrolysis does not support the imide type structure. Edman degradation of "X-compound" gave PTH-asparagine from dipeptide and, PTH-asparagine and PTH-alanine from tripeptide. No PTH-glutamic acid was observed. This evidence also appears to eliminate the possibility of the imide compound formation.

Formation of a cyclic derivative of a five membered pyrrolidone type may be possible. However in alkaline and acid hydrolysis, pyrrolidone could regenerate the starting material. Formation of "X-compound" was increased in parallel with the amount of added anisole, whereas without anisole practically no side reaction was obtained (Table 1). The absorption spectrum showing strong 275 to 280 nm absorption, as well as mass spectroscopic observation, strongly support the presence of a benzene ring in the molecule. The formation of "X-compound" from free dipeptide, Asn-Glu, is also consistent with existence of a benzene ring in "X-compound" which might be anisole but not a benzyl group. Thus, glutamic acid may be converted to the pyrrolidone form of glutamic acid containing anisole in the peptide. The cleavage of the acyl-pyrrolidone bond is not observed based on the Edman degradation data obtained with the tripeptide.

It is interesting and important to note that the use of HBr-AcOH gave no such side reaction (Fig. 1).

DISCUSSION: When impurities occur in paper chromatography or electrophoresis of peptide obtained from solid phase synthesis, one may suppose that deblocking of Boc-group or coupling steps are inadequate. However, our results indicate that even when peptide synthesis proceeds satisfactory, anhydride HF-anisole treatment on glutamic acid-containing peptide can give side reactions resulting in a poor yield of glutamic acid. Side reactions occur particularly in peptides in which glutamic acid is located in or near the carboxyl terminal. Anisole commonly added as a scavenger of polymerized benzyl cation can initiate side reactions so far not reported in solid phase peptide synthesis. Anisole, probably anisole radical, seems to react with pyrrolidone form of glutamic acid. The structure of the product "X-compound" is still to be established. NMR and mass spectroscopic studies are now proceeding in our laboratory and detailed results will be reported at a later date.

ACKNOWLEDGEMENT: This work was supported by in part by Research Grant of Fujiwara Foundation of Kyoto University and Scientific Research Fund of the Ministry of Education of Japan. The authors thanks Drs. K. Hayashi and H. Ogoshi for their help for Edman degradation and mass spectroscopy. We express our thanks to Professor M. D. Kamen for his correcting of the manuscript.

REFERENCES:

1. Sakakibara, S., and Shimonishi, Y., Bull. Chem. Soc. Japan 38, 1412 (1965).
2. Lenard, J., and Robinson, A. B., J. Am. Chem. Soc. 89, 181 (1967).
3. Sakakibara, S., Shin, K. H., and Hess, G. P., J. Am. Chem. Soc. 84, 4921 (1962).
4. Lenard, J., Schally, A. V., and Hess, G. P., Biochem. Biophys. Res. Commun. 14, 498 (1964).
5. Merrifield, R. B., Biochemistry 3, 1385 (1964).
6. Bayer, E., Hagenmaier, H., Jung, G., and König, W., Peptides, 162p (1968).
7. Marglin, A., Tetrahedron Letters 33, 3145 (1971).
8. Scotchler, J., Lozier, R., and Robinson, A. B., J. Org. Chem. 35, 3151 (1970).
9. Battersby, A. R., and Robinson, J. C., J. Chem. Soc. (London), 259 (1955).