Vol. 92, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 29, 1980 Pages 606-609

TRYPTIC CLEAVAGE OF A PEPTIDE AT MODIFIED ASPARTIC ACID+

F. Eugene DeBons and Gordon Marc Loudon*

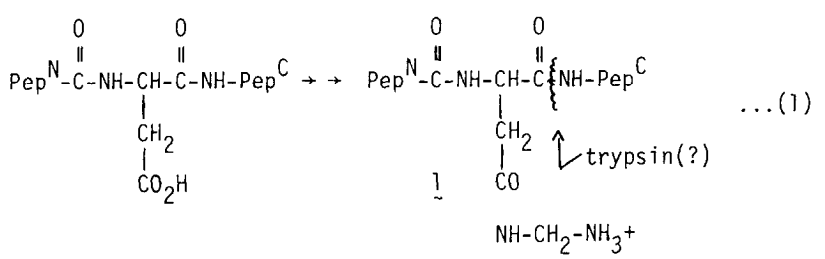
Department of Medicinal Chemistry and Pharmacognosy
School of Pharmacy and Pharmacal Sciences
Purdue University
West Lafayette, IN 47907

Received December 7,1979

SUMMARY. Conversion of the carboxylic acid side chain of aspartic acid in the peptide pyroGlu·Asp·Phe·amide to a carboxamidomethylamine side chain (structure 1) results in the tryptic hydrolysis of the peptide at the Asp-Phe bond.

The specific cleavage of peptides at defined loci is an important tool of the peptide sequencer (1). One of the best known methods involves cleavage of peptides at lysine and arginine by trypsin. Equally attractive would be a complementary method which effects cleavage at the acidic amino acid residues. This has been realized for glutamic acid in the discovery of staphylococcal protease (2). A cleavage at aspartic acid would be highly useful in the same sense. Although specific Asp-X linkages (Asp-Gly, Asp-Pro) can be selectively cleaved in some cases (3), no general method exists.

The modification of amino acid side chains in peptides to yield residues susceptible to trypsin cleavage has been used successfully with cysteine (4). A similar strategy, shown in eq. 1, suggests itself for aspartic acid.



...(1)

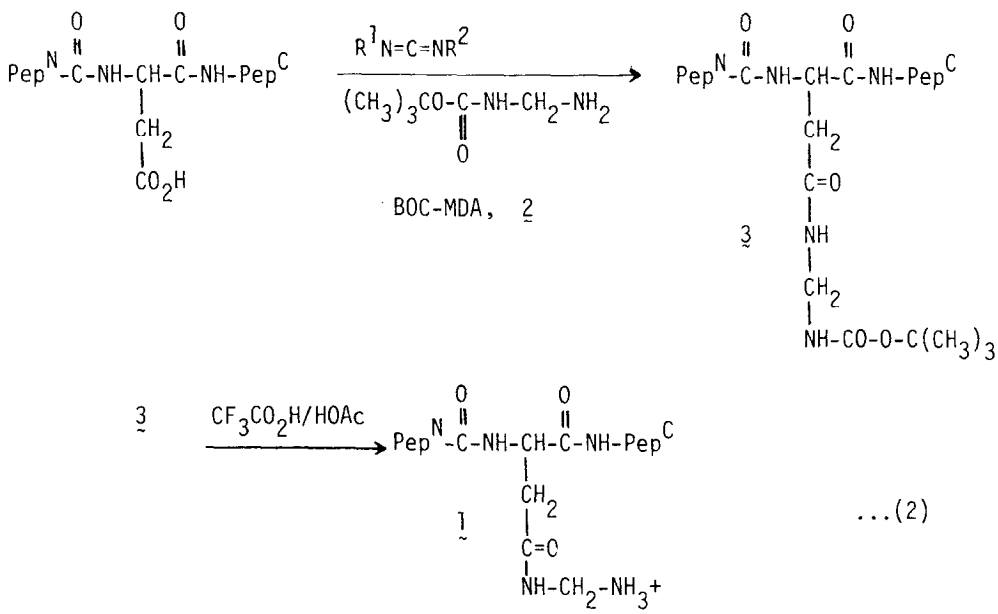
1

+ABBREVIATIONS. BOC-MDA, N-aminomethylcarbamic acid tert-butyl ester, compound 2; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide·HCl; BAW butanol-acetic acid-water; TFA, trifluoroacetic acid.

0006-291X/80/020606-04$01.00/0

Both hydrazides (5) and N-2-aminoethyl amides (6) of aspartyl side chains have been prepared, and weak trypsin cleavage at these residues has been reported. However, the chain length requirement of trypsin (7) suggests that the derivative 1 would be superior as a close analog of lysine. Some important questions surround the preparation of such a derivative. (1) Is such a derivative easily prepared and stable to aqueous solution? (2) Will trypsin be "fooled" by such a derivative and thereby effect cleavage at such a modified Asp? (3) How specific is the trypsin cleavage? In this preliminary report, we answer affirmatively the first two questions.

The direct modification of Asp in peptides with methylenediamine (H_2N-CH_2-NH_2) is not reasonable, despite one report to the contrary (8). This compound, although known as the dihydrochloride (9), decomposes instantaneously in water. The key to the synthesis of derivatives such as 1 is the fact that acylation of one nitrogen of methylenediamine yields a stable compound (10,11) The basic chemistry which we have used is shown in eq. 2.



MATERIALS AND METHODS. The peptide pyroglutamylaspartylphenylalanyl amide, PyroGlu·Asp·Phe-amide (9.47 mg, 23.19 μmol) was dissolved in 2 mL of water, and BOC-MDA HCl (10), 2, was added (51 mg, 0.28 mmol). The pH was adjusted to 4.2 and 0.12 mL of a 0.814 M solution of EDC was added. The

pH was maintained at 4.3-4.5 by periodic addition of 1 M NaOH, and equal volume EDC aliquots were added at 15, 30, and 45 minutes. At 30 minutes, a second portion of BOC-MDA (25 mg, 0.137 mmol) was added to raise the concentration to 0.186 M. At 75 minutes the solution was extracted with ether (3 x 3 mL) in a test tube using a Vortex mixer, and the aqueous phase was passed via syringe through a Waters Associates RP-18 (reversed phase) Sep-Pak. The reaction vessel and the test tube were rinsed with 2 mL of water, and this was also passed through the Sep-Pak. The Sep-Pak was then eluted with 3 x 1 mL portions of CH_3OH. The modified peptide was isolated from the CH_3OH eluant and further purified on a 1.5 x 50 cm, RP-18 mplc column using methanol water gradients (30:70 v/v methanol to 70:30). The purified peptide was obtained in 16% yield. The peptide (700 mmol) was deprotected with 70:30 TFA: acetic acid (15 min) and the acids removed by evaporation at 30° in vacuo. The peptide became positive to fluorescamine only after deprotection. Amino acid analysis of the peptide both before and after deprotection gave the theoretical amino acid ratios.

The peptide was dissolved in 0.046 M Tris buffer, pH 7.5, which was 0.011 M in $CaCl_2$ to a final concentration of 1.23×10^{-3} M (determined by amino acid analysis). The solution was digested with trypsin (5×10^{-5} M) for 3 hr at 37°, and 200 µL of the digest was applied to a silica gel plate and eluted with BAW 4/0.6/1. The plate was sprayed with fluorescamine, the Phe-amide band was scraped off, placed in a cm filter, and eluted with 6N HCl (4 x 0.5 mL) directly into a hydrolysis tube containing a norleucine standard. Hydrolysis and amino acid analysis enabled quantitation of the Phe-amide release by trypsin.

Treatment of the blocked peptide 3 in a similar fashion gave no cleavage by trypsin. Furthermore, the quality of the analytical method was assessed by subjecting a known amount of Phe-amide to the same recovery technique; 101% recovery was obtained.

RESULTS AND DISCUSSION. BOC-MDA, 2, was synthesized and characterized (10) and was found to have a half-life in aqueous solution of about 50 minutes. Conversion of pyroGlu·Asp·Phe-amide to derivative 3 was accomplished as in eq. 1, using EDC as the carbodiimide. As a control, trypsin was found to have no effect on 3, including no release of Phe-amide, under conditions used for the digestion of 1. Deprotection of 3 with TFA to yield 1 gave a peptide which was positive to fluorescamine and which was digested by trypsin in 3 hr to give a 44-47% cleavage yield of Phe-amide.

This work shows that it is possible to prepare a modified derivative of Asp which is stable and which is accepted as a substrate by trypsin (11). These results indicate that further work is in order to clarify the optimum procedure(s) for producing such a derivative in peptides, as well as the specificity of trypsin for cleavage at the C-terminus of such modified residues. This work is in progress, and has the potential to develop into a specific cleavage for peptides at aspartic acid.

ACKNOWLEDGEMENT. This work was supported by grants from the National Institute of General Medical Sciences and the National Science Foundation.

REFERENCES

1. For a review, see, for example, Spande, T.F., Witkop, B., Degani, Y., and Patchornik, A. (1970), Adv. Protein. Chem., 24, 97-260.
2. a. Drapeau, G.R. (1978), J. Biol. Chem., 253, 5899-5901.
b. Drapeau, G.R. (1977), Methods Enzymology, 47, 89-91.
3. a. Hill, R.L. (1975), Adv. Protein Chem., 20, 37-107.
b. Austen, B.M., Nyc, J.F., and Smith, E.L. (1977), J. Biol. Chem., 252, 8160-73.
c. Piszkiewicz, D., Landon, M., and Smith, E.L. (1970), Biochem. Biophys. Res. Comm., 40, 1173-8.
4. Lindley, H. (1956), Nature, 178, 647-8.
5. Ebata, M. (1961), J. Biochem., 49, 110-15.
6. Bertrand, R., Derancourt, J., and Roseau, G. (1976), Biochemie, 58, 385-9.
7. Seely, J.H., and Benoiton, N.L. (1970), Can. J. Biochem., 48, 1122-31.
8. Wang, T.-T., and Young, N.M. (1978), Anal. Biochem., 91, 696-9.
9. Knudsen, P. (1914), Chem. Ber., 47, 2698-2701.
10. DeBons, F.E., and Loudon, G.M., manuscript submitted. The synthesis and properties of BOC-MDA will be described in this paper.
11. Loudon, G.M., and Jacob, J., manuscript submitted. A complete study of the hydrolytic mechanism of compounds of the form R-CO-NH-CHR'-NH_2 has been carried out. The findings of this study relevant to this work are that the stability of such compounds is highly dependent on the nature of R. Although compound 2 has a half-life of only 50 minutes, the modified Asp side chain is estimated to have a half-life at 25° of about 400 hr at pH 7.5. A further finding relevant to the tryptic cleavage is that the pK_a of the protonated amine in such compounds is about 7.2.