Hydrogen Fluoride–Anisole Catalyzed Reaction with Glutamic Acid Containing Peptides[1]

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Abstract: Various peptides containing glutamic acid in or near the carboxyl terminus were synthesized by both the solid-phase or conventional method and when treated with anhydrous hydrogen fluoride in the presence of anisole, the occurrence of a side reaction on glutamic acid residue resulted. From simple model compounds, *tert*-butyloxycarbonyl-L-asparaginyl-γ-benzyl-L-glutamyl-resin or *tert*-butyloxycarbonyl-L-asparaginyl-α,γ-dibenzyl-L-glutamate, L-asparaginyl-γ-*p*-methoxybenzoyl-α-aminobutyric acid was isolated as a side product and was characterized by proton and carbon-13 nuclear magnetic resonance spectra. The rate of formation of the side product from glutamic acid containing peptides was dependent on the composition and configuration of the peptide. Side reactions were absent when hydrogen bromide and acetic (or trifluoroacetic) acid were used to replace hydrogen fluoride and anisole.

During the synthetic work of cytochrome c peptide in the carboxyl terminal region by the solid-phase method, i.e., *tert*-butyloxycarbonyl-L-asparaginyl-γ-benzyl-L-glutamyl-resin, followed by cleavage of the peptide from the resin with anhydrous hydrogen fluoride (HF) and anisole, we observed that about 80% of the dipeptide changed to a modified derivative. Compounds apparently resulting from conversion of glutamic acid to a derivative presumably containing anisole appeared while glutamic acid disappeared. Similar results were obtained with other di- and tripeptides containing glutamic acid. The synthetic undecapeptide which occurs at the carboxyl terminal region of cytochrome c was also modified.

A preliminary paper from this laboratory dealt with hitherto unknown reactions which may take place with glutamic acid.[2] We wish to report the results of further investigations and, in addition, to elucidate the structure of the detectable product of this side reaction by using proton and carbon-13 nuclear magnetic resonance spectra.

Experimental Section

Amino acid analysis was determined using a Hitachi automatic amino acid analyzer (KLA-3B) on samples which had been hydrolyzed with 6 *N* HCl in the presence of 20 μl of 5% phenol at 110° for 24 hr. Elementary analysis was done at the Faculty of Pharmaceutical Sciences of Kyoto University. Optical rotations were measured using a Jasco automatic polarimeter (DIP-SL). Proton nuclear magnetic resonance spectrum (^{1}H NMR) was obtained using a Varian Associate (Palo Alto, Calif.) spectrophotometer, Model 220 system, operating at 220 Mcps. Spectra were scanned from low to high fields at rates of 12–60 cps/min. The sample was dissolved in D_2O at a concentration of 10 mg/0.6 ml. Tetramethylsilane (Me_4Si) was used as an internal reference. The data are expressed in parts per million referred to 10.00 (τ values), according to the procedure of Tiers.[3] The carbon-13 NMR spectrum (^{13}C NMR) at 25.14 MHz was recorded with JEOL JNM-PS/PET-100 FT-NMR system. The carbon chemical shifts are all given in parts per million downfield from Me_4Si. In order to increase the solubility of the sample, acetic acid at a concentration of 20 mg/0.6 ml was used to dissolution. The mass spectrum was measured with a Hitachi mass spectrometer Type RMU-7.

Solid-phase reactions were carried out by a simplified solid-phase procedure of Merrifield.[4] The procedures were performed either manually or automatically using Shimadzu APS-800. *tert*-Butyloxycarbonylamino acids were purchased from Kyoto Daiichi Kagaku Co. LTD. Direct hydrolysis of the peptide-resin was carried out in an anaerobic mixture of 1.0 ml of 12 *N* HCl and propionic acid (1:1 v/v) containing 20 μl of 5% phenol at 130° for 2 hr. The peptide was removed from the resin by HF and anisole.[5,6] Anisole was added in amounts from equimolar to 150 molar excess to peptide residues. Descending paper chromatography was performed on Whatman 3 MM paper in a solvent system of 1-butanol–pyridine–glacial acetic acid–water (75:50:15:60, by volume). Paper electrophoresis was carried out by Gilson apparatus, Model D, in a buffer composed of pyridine–acetic acid–water (1:10:28, by volume), pH 3.6. Peptides were isolated from the paper with 30% acetic acid and then lyophilized. Further purification of the peptide was carried out on a column (0.9 × 150.0 cm) of Dowex 1-X4 and the peptide was eluted with 0.2 *M* acetic acid.

Synthesis of Peptides. *tert*-Butyloxycarbonyl-di- or -tripeptide-resin (Solid-Phase Method). Glutamic acid was attached to the resin (Bio Bead S-X2) by esterification of Boc-L-Glu(γ-OBzl) ester by two different methods, one method being that of Bayer et al.,[7] that is by heating with triethylamine in benzene instead of alcohol to avoid transesterification, and the other method that of Marglin,[8] that is by keeping with triethylamine in dimethylformamide (DMF) at room temperature.

Starting with 32.5 μmol of Boc-L-Glu(γ-OBzl)-resin (substitution, 325 μmol/g), Boc-di- or -tripeptide-resin was prepared in the following way: (a) washed resin with CH_2Cl_2 3 × 2 min; (b) deprotection with TFA–CH_2Cl_2 (1:1 v/v) 1 × 30 min following washing with CH_2Cl_2 3 × 2 min; (c) neutralization with Et_3N–CH_2Cl_2 (15%) 1 × 10 min followed by washing with CH_2Cl_2 3 × 2 min and DMF 2 × 2 min; (d) coupling with threefold excess of *tert*-butyloxycarbonyl-L-asparagine *p*-nitrophenyl ester in DMF for 12 hr; (e) washing with alternating alcohol and DMF 3 × 2 min each; (f) repeated steps d and e. Under these conditions, deprotection and coupling were found to be almost quantitative. Double coupling was mediated by *N,N'*-dicyclohexylcarbodiimide in CH_2Cl_2 for 6 hr each in the case of other *tert*-butyloxycarbonylamino acids except asparagine. The following other peptide resins were prepared in a manner similar to that described above. Boc-Asp(β-OBzl)-Glu(γ-OBzl)-resin, Boc-Thr(OBzl)-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, Boc-Pro-Glu(γ-OBzl)-resin, Boc-Asn-Ala-resin, Boc-Glu(γ-OBzl)-Asn-Ala-resin, and Boc-Thr(OBzl)-Asn-Glu(γ-OBzl)-resin.

***tert*-Butyloxycarbonyl-L-asparaginyl-α,γ-dibenzyl-L-glutamate (Conventional Method).** *tert*-Butyloxycarbonyl-L-asparagine *p*-nitrophenyl ester (3.53 g, 0.01 mol) was added to the mixture of α,γ-dibenzyl-L-glutamate tosylate (5 g, 0.01 mol), triethylamine (1.97 ml, 0.014 mol), and tetrahydrofuran (10 ml). The reaction mixture was stirred for 5 days at room temperature and then evaporated to dryness under reduced pressure. The residue was dissolved in 50 ml of ethyl acetate and washed successively with water, 4% $NaHCO_3$ solution, 2 *N* HCl, and again water. The ethyl acetate solution was evaporated under reduced pressure, and recrystallization was carried out twice from aqueous acetic acid solution. A crystalline sample was obtained as white needles: yield 2.9 g (54%); mp 139–141°; $[\alpha]^{26}D$ −8.7° (*c* 1, AcOH).

Anal. Calcd for $C_{28}H_{35}N_3O_8$: C, 62.11; H, 6.47; N, 7.76. Found: C, 62.18; H, 6.56; N, 7.70.

HF–Anisole Treatment of Protected Peptide-Resin or Protected Peptide. Typical experiments were as follows. Boc-L-Asn-Glu(γ-

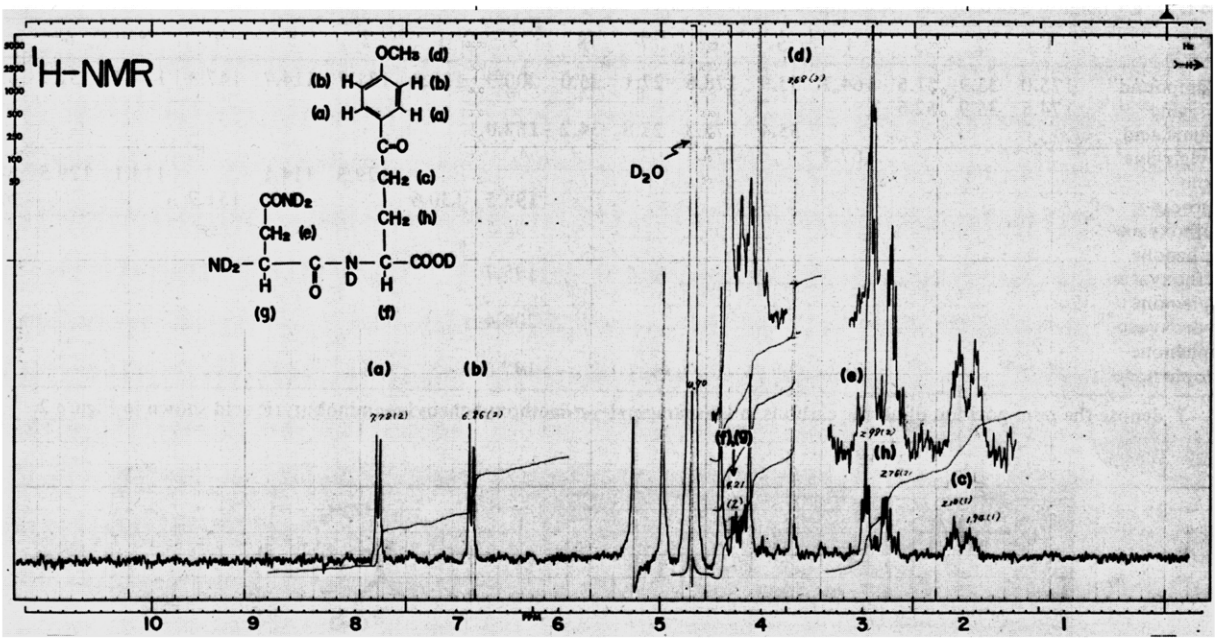


Figure 1. ^{1}H NMR spectrum of the "X compound" (L-asparaginyl-γ-*p*-methoxybenzoyl-α-aminobutyric acid).

OBzl)-resin (100 mg containing 39.5 μmol of peptide) was treated with 10–15 ml of HF in the presence of anisole which was added in different amounts (see Experimental Section). After 1–1.5 hr at 0° with stirring, excess HF was rapidly evaporated under reduced pressure, then completely distilled with a high vacuum pump, and dried over NaOH. The residue was suspended in 20 ml of 10% acetic acid for 1 hr at 0° and then filtered on a sintered glass filter. The same extraction procedure was repeated more than three times. The combined aqueous layer was washed with ether several times in order to remove anisole and was then lyophilized.

Boc-L-Asn-L-Glu(α- and γ-OBzl) ester (100 μmol, 54.1 mg) was treated in HF (15 ml) and anisole (1.6 ml) in a manner similar to that mentioned above.

Results and Discussion

Direct hydrolysis of Boc-L-Asn-L-Glu(γ-OBzl)-resin with the mixture of HCl–propionic acid yielded an equimolar amount of aspartic and glutamic acid with a theoretical yield. Cleavage of the dipeptide from the supporting resin with a conventional HBr–acetic acid (25%) for 16 hr at room temperature method gave the dipeptide, Asn-Glu, in high yield. Homogeneity was confirmed from the data on paper chromatography, electrophoresis, amino acid analysis, and Edman degradation. These results indicate that synthesis of glutamic acid containing peptide by the solid-phase technique, followed by cleavage of the peptide from the resin with HBr–acetic acid, proceeds satisfactorily. It was also demonstrated that Boc-L-Asn-L-Glu(α- and γ-OBzl) ester was converted to the expected dipeptide.

By contrast, treatment of dipeptide resin (solid-phase method) or protected dipeptide (conventional method) with HF in the presence of anisole gave a new derivative with high yield. In both cases, acid hydrolysis of the obtained dipeptide resulted in a low recovery of glutamic acid, while aspartic acid was obtained in the theoretical yield. Alkaline hydrolysis gave the same result. Paper chromatography of the dipeptide showed two ninhydrin-positive spots at positions of R_f values of 0.21 and 0.56 (see Figure 1, ref 2). The minor chromatography component (R_f 0.21) accounting for approximately 20% of the total material was found to be the expected dipeptide. The major chromatographic component (R_f 0.56) obtained in approximately 80% yield was found to be a new derivative ("X compound"). This compound was isolated from the paper, lyophilized, and furthur purified on a column of Dowex 1. A major peak was collected and then lyophilized.

Structure of the "X Compound". The ultraviolet spectrum of the "X compound" revealed an absorption maximum at 275–280 nm. This supports the fact that the benzene ring may be involved in the molecule. As the γ-benzyl ester of glutamic acid can be easily removed by HF treatment, the contamination of the γ-benzyl group in the "X compound" would be merely a trace if any. The most pertinent information leading to the establishment of the structure was obtained during examination of ^{1}H NMR and ^{13}C NMR spectra.

(a) ^{1}H NMR Spectrum of the "X Compound". The assignments of resonances to specific protons of the *p*-anisyl group and those of the asparagine and glutamic acid moiety in the "X compound" are shown with the data of their proton numbers in Figure 1.

The methoxy protons of the *p*-anisyl group are located at 3.68 ppm and the ortho- and meta-positioned protons appeared as a pair of doublets at 7.78 and 6.86 ppm, respectively. Two α-carbon protons of asparagine and glutamic acid overlapped at 4.21 ppm, and two β-methylene protons of asparagine and glutamic acid appeared at 2.98 and 2.76 ppm, respectively. Two γ-methylene protons of glutamic acid appeared in the region of 1.94 and 2.18 ppm with a fuzzy absorption.

From these ^{1}H NMR data, the structure of the "X compound" would appear to be L-asparaginyl-γ-*p*-methoxybenzoyl-α-aminobutyric acid; however, ambiguity in the ^{1}H NMR spectrum gave rise to the question of whether or not the compound contains pyrrolidine or 2-pyrroline type. Convincing proof of the proposed structure was obtained by ^{13}C NMR and other chemical techniques.

(b) ^{13}C NMR Spectrum of the "X Compound". The ^{13}C NMR spectrum and the observed ^{13}C chemical shifts of the "X compound" are shown in Figure 2 and Table I. Carbons in the anisyl group are unique and their signals are readily recognizable. The carbon resonances at 130.3, 131.7, 114.7, 147.3, and 55.9 ppm were assigned to be C-1′, C-2′ (6′), C-3′ (5′), C-4′, and C-7′ of the anisyl group, respectively. These assignments were compared with the set of data of anisole or anisole derivative (i.e., 3-fluoro-*p*-methoxyacetophenone and *p*-methoxyacetophenone) (see Table I). The

Table I. ^{13}C Chemical Shifts of the "X Compound" and Related Compounds[a]

	1	2	3	4	5	6	7	8	9	1′	2′	3′	4′	5′	6′	7′	Ref
"X compound"	175.0	35.9	51.6	164.7	53.9	178.6	27.1	35.0	200.3	130.3	131.7	114.7	147.3	114.7	131.7	55.9	
Asparagine	174.5	35.9	52.6														9
Glutamic acid					55.4	175.3	27.8	34.2	182.0								9
Glycylglycine				167.9		177.1											10
Anisole											129.5	114.1		114.1	129.5	54.7	10
3-Fluoro-*p*-methoxyacetophenone									195.5	130.6			151.9			56.2	10
p-Methoxyacetophenone									195.7								9
o-Hydroxyacetophenone									204.4								9
Acetophenone									197.6								9

[a] 1–7′ denote the ppm position of all the carbons in L-asparaginyl-γ-*p*-methoxybenzoyl-α-aminobutyric acid shown in Figure 2.

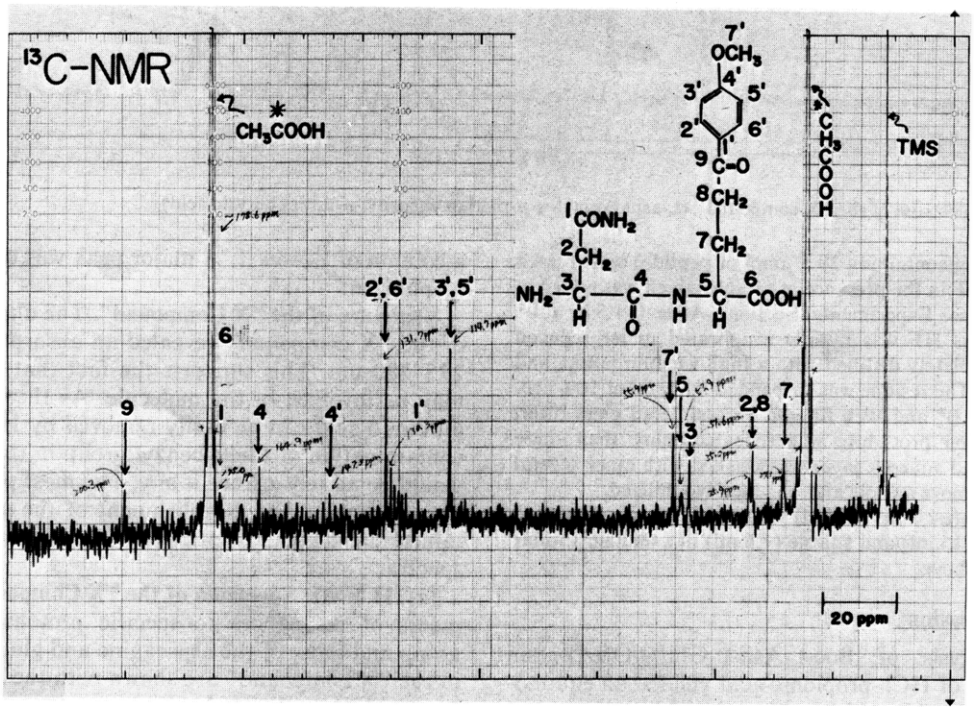


Figure 2. ^{13}C NMR spectrum of the "X compound" (L-asparaginyl-γ-*p*-methoxybenzoyl-α-aminobutyric acid).

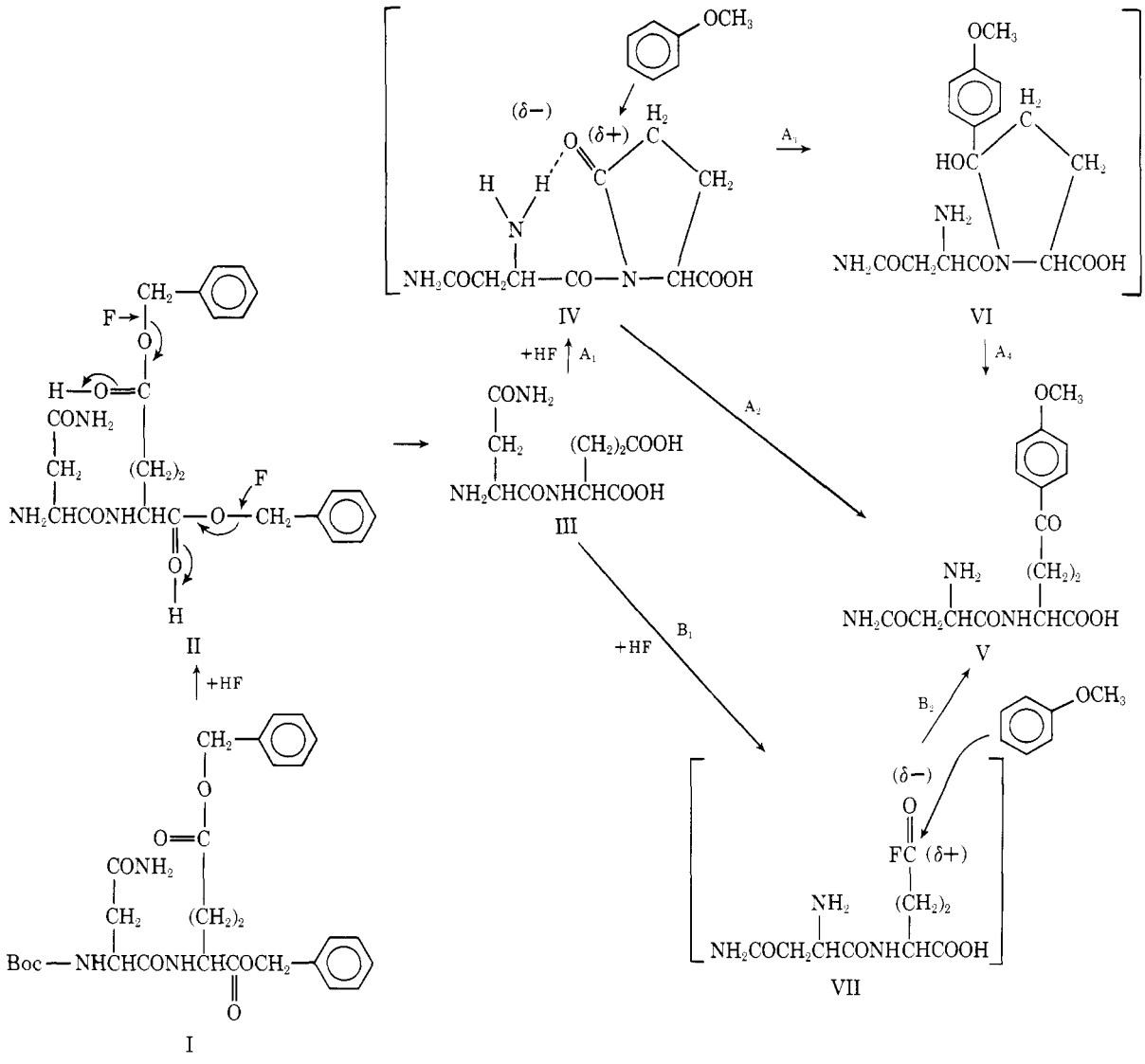
methoxy carbon (C-7′) of the anisyl group was characterized by its strong intensity resulting from the nuclear Overhauser effect.[9] Therefore, C-7′ could be easily differentiated from the resonance of α-carbon nuclei (C-3 or -5) of amino acids which appears in the same region.

In general, the carboxy carbons are most deshielded compared to other various constituents of carbon atoms. This is particularly true of the carbonyl carbons. The downfield peak at 200.3 ppm in Figure 2 is evident for the presence of keto group in the "X compound" that is C-9 (carbonyl carbon of side chain of glutamic acid). The highly deshielded pattern of C-9 eliminates any question of possible occurrence of the pyrrolidine or 2-pyrroline type compound in the molecule. The same deshielding is found for the keto group of *p*-methoxy- or *o*-hydroxyacetophenone. A minor influence on the carbonyl shielding in C-9 may be explained by the reason of the intramolecular hydrogen bonding as observed in the case of *o*-hydroxyacetophenone.[9] The resonance of the amide carbon (C-1) of asparagine at 175 ppm and that of the α-carboxyl carbon (C-6) of glutamic acid overlapped with that of acetic acid used for solvent. The chemical shift of the carbonyl carbon (C-4) of the peptide bond was often at 164.7 ppm, which was in a slightly higher field than that of the carboxyl carbon. Similar behavior was observed in the case of glycylglycine (see Table I). Carbon-13 resonance frequencies of all other substituent groups common to the amino acid, such as the α and β carbon of asparagine or α, β and γ carbons of glutamic acid are also recorded (Table I). From these ^{13}C NMR data, the structure of L-asparaginyl-γ-*p*-methoxybenzoyl-α-aminobutyric acid is clearly elucidated.

(c) Other Chemical Evidence for the "X Compound". Formation of the "X compound" was increased in parallel with the amount of added anisole, whereas without this agent no side reaction was obtained. The absorption spectrum showing strong 275–280-nm absorption, as well as mass spectroscopic observation of a fragment ion at *m/e* 135, supports the presence of a benzene ring in the molecule.

Acid hydrolysis of the "X compound" with the mixture of HCl–propionic acid at 130° for 2 hr or 6 *N* HCl at 110° for 24 hr followed by amino acid analysis gave aspartic acid and another ninhydrin-positive compound in a ratio of approximately 1:0.25. The latter compound was eluted slightly after the position of Glu(γ-OBzl) and was not converted to glutamic acid even by repeated acid hydrolysis. This was assumed to be γ-*p*-methoxybenzoyl-α-aminobutyric acid.

Scheme I. Proposed Mechanism of the Formation of L-Asparaginyl-γ-*p*-methoxybenzoyl-α-aminobutyric Acid from Boc-Asn-Glu-dibenzyl Ester by the Treatment of HF and Anisole



Formation of the "X compound" was observed from the free dipeptide, i.e., asparaginylglutamic acid. This is also consistent with existence of a group derived from anisole rather than from a benzyl-protecting group.

Boc-Asp(β-OBzl)-Glu(γ-OBzl)-resin, Boc-Asn-Glu(γ-OBzl)-Ala-resin, Boc-Thr(OBzl)-Asn-Glu(γ-OBzl)-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 as reported in Table I of our previous publication.[2] However, it is worthwhile to note that the additional reaction of anisole on either asparagine or aspartic acid was not observed and aspartic acid was quantitatively recovered after acid hydrolysis. HF–anisole treatment of Boc-Glu(γ-OBzl), Boc-Glu(γ-OBzl)-resin, or Boc-Glu(γ-OBzl)-Asn-Ala-resin did not give the "X compound" at 0°. Glutamic acid or Glu-Asn-Ala was quantitatively recovered. These results demonstrate that when glutamic acid is located at the N terminus of the peptide, this side reaction did not take place. Reactions occurred particularly in peptides where glutamic acid was located in or near the carboxyl terminus.

In Scheme I, we wish to present possible reaction mechanisms for the formation of L-asparaginyl-γ-*p*-methoxybenzoyl-α-aminobutyric acid from *tert*-butyloxycarbonyl-L-asparaginyl-L-glutamic acid dibenzyl ester by the treatment of HF and anisole.

One mechanism (A) is that HF removes the Boc group of asparagine and cleaves γ- and α-benzyl esters of glutamic acid forming Asn-Glu (III). An excess of HF probably makes a pyrrolidone type compound of glutamic acid via A_1. The α-amino group of asparagine adjacent to the glutamic acid could be hydrogen bonded with the keto group of pyrrolidone (IV). The presence of this bond is supported by stereochemical model configuration. Thus the methoxy substituent in anisole substantially enhances the nucleophilic attack on the electron-deficient fragment of the keto group, resulting in the formation of L-asparaginyl-γ-*p*-methoxybenzoyl-α-aminobutyric acid (V) via A_1 and A_2. The para attack is supported by the significant contribution of the methoxy group of anisole.

During the formation of V another possible cyclic intermediate such as L-asparaginyl-γ-*p*-anisyl-2-hydroxypyrrolidine-5-carboxylic acid (IV) has to be considered. The actual reaction may, however, proceed very rapidly through the migration of proton to the imino group thus forming a final product V via A_4.

Another possible mechanism (B) is the formation of the carbonyl fluoride intermediate VII. The anisole attacks the

electron-deficient group of carbonyl fluoride and the result is the formation of final compound V.

Both proposed mechanisms, both A and B, deal with the activation of the γ-carboxyl group of glutamic acid. A is a reaction via a pyrrolidone intermediate followed by intramolecular hydrogen bonding, whereas B is a reaction via a carbonyl fluoride intermediate. Accordingly, the nucleophilic attack of anisole on the carbonyl group could be facilitated in both cases. It is well known that an N-terminal glutamic acid (or glutamine) is cyclized by acid treatment forming pyrrolidonecarboxylic acid. HF did not produce such a cyclic compound from Boc-Glu(γ-OBzl) or Boc-Glu(γ-OBzl)-resin, but such is feasible where glutamic acid is located at the carboxyl terminal or middle position of the peptide. Mechanism A proposes the cyclic intermediate IV and emphasizes the appropriate stereochemical configuration for this reaction. When intramolecular hydrogen bonding was difficult to assume in the protein instead of in the peptide, no side reaction was observed. In fact, modification of glutamic acid by anisole was not observed in natural cytochrome c. Instead the "X compound" was formed from protected undecapeptide in the carboxyl terminal of cytochrome c.

Mechanism B is simpler, but an explanation of why a reaction does not take place with either the α-carboxyl group of glutamic acid containing peptides or with the β-carboxyl group of aspartate-containing peptides is difficult. Therefore the alternate pathway from IV to VII may be possible to consider.

When impurities occur in paper chromatography or electrophoresis of peptide from solid-phase synthesis, it is presumed that deblocking of the Boc group or that the coupling steps are inadequate. On the other hand, HF–anisole treatment is now widely applied as the most useful deblocking agent for peptide synthesis. In the present work even when peptide synthesis proceeded satisfactorily, the HF–anisole procedure on glutamic acid containing peptide gave a side reaction even under carefully controlled conditions at 0° temperature. When HBr–acetic (or trifluoroacetic) acid was employed, however, side reactions were absent.

Acknowledgment. The authors wish to thank Dr. H. Ogoshi, Faculty of Engineering Chemistry, Kyoto University, for his helpful discussion and Drs. I. Morishima, Faculty of Engineering, Kyoto University, and K. Matsushita, JEOL LTD, Japan, for recording ^{1}H NMR and ^{13}C NMR. We extend our thanks to Miss Mariko Ohara for assistance with the manuscript.

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(1) This investigation was supported in part by research grants from the Fujiwara Foundation of Kyoto University and the Scientific Research Fund of the Ministry of Education, Japan.
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