Modification of Peptides Containing Glutamic Acid by Hydrogen Fluoride–Anisole Mixtures. γ-Acylation of Anisole or the Glutamyl Nitrogen

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Received December 4, 1974

Abstract: Peptides containing glutamic acid can undergo two major side reactions when subjected to treatment with liquid hydrogen fluoride containing 10% anisole as a carbonium ion scavenger. In this medium, the side-chain γ-carboxyl group of glutamic acid was converted to an acyl carbonium ion, which then underwent either an intramolecular cyclization to pyrrolidone-containing peptides or an intermolecular Friedel–Crafts acylation of anisole. Formation of the acylium ion intermediate was highly temperature dependent. When cleaved at 0° for 0.5 hr the peptide-resin Bpoc-Leu-Lys(Z)-Glu(OBzl)-Ala-Val-Gly-resin gave the expected hexapeptide leucyllysylglutamylalanylvalylglycine (**1**), containing less than 1.5% of by-products, while cleavage at 24° for 2 hr gave a mixture containing only 10% of **1**, plus 11% of leucyllysylpyroglutamylalanylvalylglycine (**3**) and 79% of leucyllysyl-2-amino-4-(*p*-methoxybenzoyl)butyrylalanylvalylglycine (**2**). These cleavage conditions also caused partial fragmentation of the acylation by-product **2** into leucyllysine and the tetrapeptide, 2-*p*-methoxyphenyl-1-pyrroline-5-carbonylalanylvalylglycine (**5**), which was formed by condensation to the cyclic Schiff base. Hydrolyzates of peptides that had undergone anisole acylation were shown to contain 2-*p*-methoxyphenyl-1-pyrroline-5-carboxylic acid (**11**) in place of glutamic acid. The structures of these peptide by-products, which were isolated after electrophoretic separation, were established by amino acid analysis, ultraviolet absorption, and by tryptic digestion followed by comparison of fragments with synthetic standards. Cleavage in HF in the absence of anisole caused formation of 66% of the pyrrolidone by-product **3**. Formation of by-products was not dependent on the presence of a benzyl protecting group on the glutamic acid side chain nor on an endo position of the glutamyl residue. Cleavage of Boc-Glu(OBzl)-Ala-Val-Gly-resin in HF containing 10% anisole for 2 hr at 24° gave pyroglutamylalanylvalylglycine (**15**) and 2-*p*-methoxyphenyl-1-pyrroline-5-carbonylalanylvalylglycine (**5**) as by-products, both of which are ninhydrin negative. The aspartic acid containing hexapeptide resin, Boc-Leu-Lys(Z)-Asp(OBzl)-Ile-Val-Gly-resin, did not undergo acylation with anisole when cleaved at 24° for 2 hr, though 7% of cyclization to a succinimide derivative **18** was observed. Since formation of the acylium ion intermediate was temperature dependent, both of the observed side reactions of glutamyl peptides could be effectively minimized or eliminated by proper selection of reaction conditions.

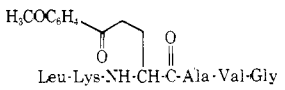
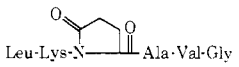
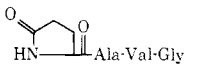
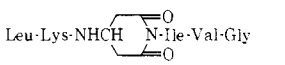
Several side reactions of glutamic acid, aspartic acid, and their amides have been reported to occur during peptide synthesis by classical and by solid-phase methods. The best documented of these is the α to β rearrangement of aspartyl peptides via the cyclic succinimide intermediate.[1] Although the analogous formation of glutarimide from glutamyl peptides and subsequent ring opening to give α to γ rearrangement product has not been observed, other as yet unexplained side reactions of glutamic acid residues have been reported. For example, in the solid-phase synthesis of an octadecapeptide containing two triglutamyl sequences, Polzhofer and Ney[2] observed drastic decreases in the number of reactive resin-bound chains after addition of each glutamic acid residue. As a possible explanation, they suggested partial loss of the benzyl ester side-chain protecting groups followed by coupling at the next step with the α-amino group to form the pyrrolidone, although glutamic acid benzyl esters are normally stable under the acid conditions they employed.[3] An alternative explanation might be nucleophilic displacement of the ester by the α-amino group, since N-terminal glutamine peptides undergo such cyclizations readily.[4] Similarly, no thorough explanation has been offered for the side reactions occurring in the syntheses of several γ-glutamyl[5-7] and α-glutamyl[8] oligopeptides where, in each case, the number of chromatographically and electrophoretically distinct by-products appeared to increase with each glutamic acid residue added to the peptide chain. In the syntheses of the peptide series $(Glu)_n$-$Phe(NO)_2$-Phe by the Scoffone group,[8] this phenomenon led to mixtures of increasing complexity until, when $n = 5$, the desired peptide could not be isolated.

The most obvious assumption is that such side reactions occurred during the coupling of the glutamic acid residues to the peptide chain; but, in fact, a number of other observations suggested that the by-products might have been formed during the removal of the peptide from the resin, rather than during its synthesis. For example, peptide-resin samples cleaved in liquid HF containing anisole as a scavenger gave more complex mixtures than those treated with HBr in trifluoroacetic acid.[8,9] Sano[9] had observed that peptides containing glutamic acid near or at the carboxy terminus underwent irreversible changes after treatment with HF–anisole. The altered ninhydrin-positive peptides, which he termed "X compounds", migrated differently on paper chromatography and their hydrolyzates no longer contained glutamic acid. He speculated that anisole could be incorporated into glutamic acid in the presence of HF, a suggestion supported by Hirschmann's observation[10] that treatment of native ribonuclease or a glutamic acid containing peptide with HF in the presence of radioactive anisole led to the incorporation of label into the product. Sano[9] did not observe formation of "X compounds" from peptides containing glutamic acid at the N terminus.

However, peptides with N-terminal glutamyl residues have been found, after treatment with HF, to give by-products which produced no reaction with ninhydrin. The peptide Glu-Glu-$Phe(NO_2)$-Phe[8] gave a product in 11% yield which was unreactive with trinitrobenzenesulfonyl chloride, indicating the absence of a free amino group, and Scoffone suggested, among other explanations, that formation of pyroglutamic acid at the N terminus might account for this finding.

Thus, one or more postsynthetic reactions of glutamic acid occurring during cleavage in liquid hydrogen fluoride seemed responsible for the numerous by-products found in polyglutamate peptide preparations, even though the assembly of the peptide chain had proceeded satisfactorily. On at least two occasions in this laboratory, syntheses of

Table I. Amino Acid Analyses[a]

Peptide	Description	Leu	Lys	Glu	Ala	Val	Gly
Resin I	Hexapeptide-resin	1.03	0.96	0.98	0.99	1.02	1.02
A	Hexapeptide cleaved from resin I in 10% anisole–HF, 0°, 0.5 hr	1.01	0.99	1.00	1.01	0.99	1.00
A[b]		1.04	1.03	0.97	0.97	1.00	0.98
Mixture B	Peptides cleaved from resin I in 10% anisole–HF, 24°, 2 hr	1.01	0.98	0.25	1.00	1.00	1.01
Mixture B[b]		1.06	0.97	0.35	0.99	0.97	1.00
Mixture B[b]		0.98	0.90	0.30	1.00	1.02	1.10
Mixture C	Peptides cleaved from resin I in HF (neat), 24°, 2 hr	0.99	0.97	1.02	1.02	0.99	1.01
1_B	Leu-Lys-Glu-Ala-Val-Gly	0.91	0.96	0.97	1.13	0.94	1.08
1_C		0.97	0.95	1.06	1.02	0.99	0.99
2_B		0.98	0.95	0.01	1.02	1.02	1.03
3_B		0.96	1.02	0.87	1.07	1.01	1.07
3_C		0.98	1.02	1.04	1.02	0.94	1.00
4_B		1.00	1.00				
$4_{(2B\text{-}Tryp)}$		1.00	1.00				
$4_{(1A\text{-}Tryp)}$	Leu-Lys	0.85	1.15				
$4_{(3C\text{-}Tryp)}$		0.98	1.02				
S4		1.00	1.00				
5_B				0.03	1.11	0.89	1.00
$5_{(2B\text{-}Tryp)}$				0.00	1.02	1.01	0.96
S5				0.00	0.94	1.00	1.06
$6_{(1A\text{-}Tryp)}$	Glu-Ala-Val-Gly			1.03	1.01	0.96	1.00
Mixture D	Tetrapeptide cleaved from resin II in 10% anisole–HF, 0°, 0.5 hr			0.97	1.01	0.96	1.00
Mixture E	Tetrapeptide cleaved from resin II in 10% anisole–HF, 24°, 2 hr			0.21	0.96	1.02	1.02
$15_{(3C\text{-}Tryp)}$				1.08	1.13	0.96	0.88
S15				0.96	0.95	1.10	0.99
$16_{(3C\text{-}Tryp)}$	Leu-Lys diketopiperazine	1.01	0.99				
		Leu	Lys	Asp	Ile	Val	Gly
Mixture F	Peptides cleaved from resin III in 10% anisole–HF, 25°, 2 hr	1.09	0.98	1.04	0.91	0.94	1.04
17_F	Leu-Lys-Asp-Ile-Val-Gly	0.95	1.02	0.99	0.93	0.78	1.04
18_F		0.96	1.05	1.07	0.89	0.90	1.12

[a] Peptide-resin hydrolyzate: ca. 6 mg of resin + anisole (0.5 ml) + 88% phenol (0.5 ml) + glacial acetic acid (1.0 ml) + concentrated HCl (1.0 ml) at 110° for 18–24 hr in evacuated tube. Peptide hydrolyzates: ca. 1 mg of peptide + 6 *N* HCl (2 ml) at 110° for 18–24 hr. [b] Peptide derived from a separate cleavage.

glutamyl peptides that appeared to have proceeded well at the coupling steps gave rise to by-products lacking glutamic acid that could be attributed to a side reaction during the HF–anisole cleavage step.

This paper defines two acid-catalyzed side reactions of peptides containing glutamic acid, identifies the resulting by-products, and describes conditions under which they are minimized or avoided.

Results and Discussion

Synthesis of a Model Peptide. In designing a model peptide useful for this investigation, several requirements were taken into consideration. The presence of several residues that could be accurately and reproducibly detected by amino acid analysis was desired to furnish an internal standard with which to compare any variation in the glutamic acid content of the peptide. The glutamyl residue was to be placed in the peptide sequence so as to remove it from proximity to the resin backbone, avoiding any effects this might have on the formation of by-products. Since peptides containing N-terminal glutamyl residues had been shown to react differently than those containing internal glutamic acid, a model containing a lysyl-glutamyl bond was desired, allowing a peptide with an internal glutamyl residue to be converted to a fragment with glutamic acid, or its rearrangement product, exposed at the amino terminus by a mild enzymatic hydrolysis.

To meet these criteria, Leu-Lys-Glu-Ala-Val-Gly was selected as a model hexapeptide and was synthesized by standard solid-phase techniques.[11] Direct hydrolysis of the Bpoc-Leu-Lys(Z)-Glu(OBzl)-Ala-Val-Gly-resin (I) and amino acid analysis of the hydrolyzate gave equimolar ratios for each residue (Table I), including glutamic acid, indicating that the assembly of the peptide had proceeded correctly. This conclusion was also supported by amino acid analysis of a hydrolyzate of the free hexapeptide Leu-Lys-Glu-Ala-Val-Gly (A) (see Figure 1), which had been cleaved from the resin by treatment for 30 min at 0° with

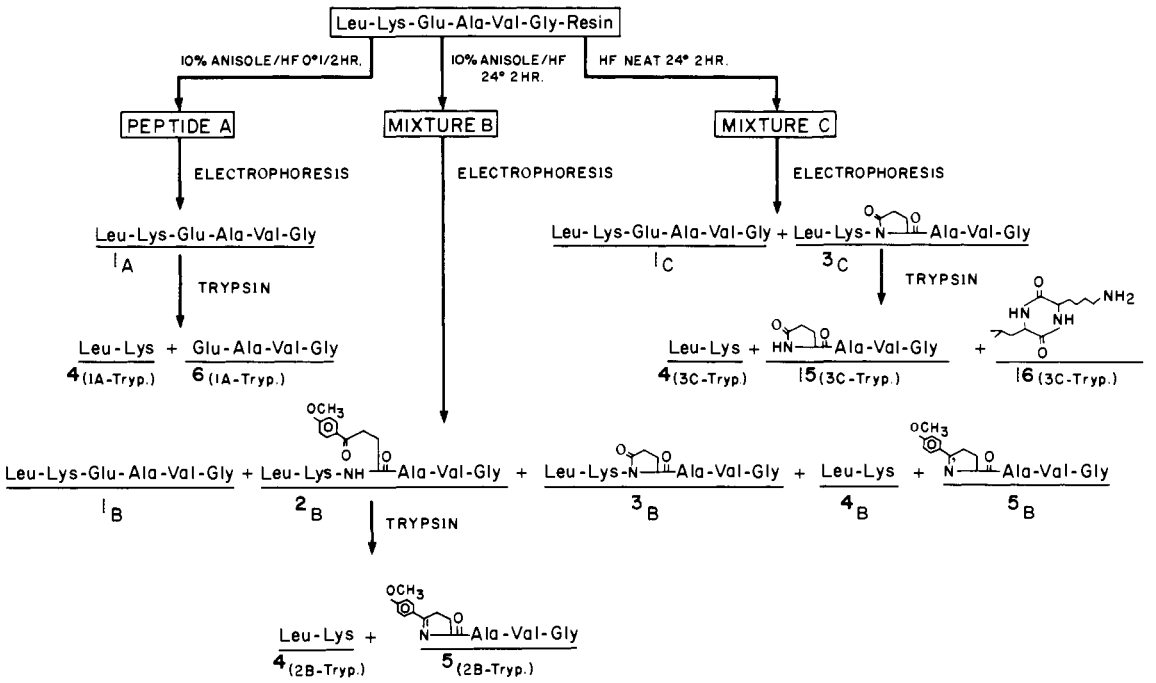


Figure 1. Modifications of a glutamic acid containing hexapeptide by HF–anisole under various conditions.

anhydrous hydrogen fluoride containing 10% anisole. Further evidence for the purity and identity of the peptide was provided by the observations that the peptide was homogeneous by high voltage electrophoresis, and a tryptic digest gave equal amounts of the expected peptides, Leu-Lys and Glu-Ala-Val-Gly.

Detection of Side Reactions. Under the favorable cleavage conditions just described, the desired hexapeptide was the dominant or exclusive product, but under other conditions several by-products resulting from two side reactions could be observed. By suitably altering the cleavage conditions, the proportions of the side reactions could be greatly exaggerated, and the by-products could then be readily isolated.

Thus, when the temperature of the HF–10% anisole cleavage reaction was raised to 24° and the time was extended to 2 hr, a peptide mixture (B) resulted which, in three separate samples, analyzed for only 0.24–0.35 equiv of glutamic acid per mole of peptide (Table I). Mixture B contained some of the expected hexapeptide $\mathbf{1}_B$, but as will be shown most of the mixture had been converted into the methoxyphenyl ketone hexapeptide derivative $\mathbf{2}_B$ (Figure 1) resulting from Friedel–Crafts acylation of anisole by the γ-carboxyl group of glutamic acid. This peptide possessed an altered electrophoretic mobility and aromatic absorption in the ultraviolet region but lacked glutamic acid. Mixture B also contained a second major by-product, the pyrrolidone-containing hexapeptide $\mathbf{3}_B$, which resulted from intramolecular acylation of the amide nitrogen atom of the glutamyl residue by the γ-carboxyl group. This hexapeptide $\mathbf{3}_B$ also showed an anomalous mobility under electrophoresis but, unlike by-product $\mathbf{2}_B$, it had no aromatic absorption and regenerated a full equivalent of glutamic acid after hydrolysis.

Another hexapeptide mixture (C) was produced by cleaving the hexapeptide-resin for 2 hr at 24° in liquid HF and omitting anisole as a carbonium ion trap. The acid hydrolyzate of mixture C contained a full mole of each of the component amino acids including glutamic acid (Table I), suggesting that no side reactions had occurred. Nevertheless, since the anisole-containing by-product could not be formed under these conditions, the intramolecular amide acylation had become the dominant side reaction. Mixture C thus contained both the unaltered hexapeptide $\mathbf{1}_C$ and the pyrrolidone by-product $\mathbf{3}_C$ (Figure 1).

Electrophoretic Separation of the Hexapeptide By-Products. Since acylation of either anisole or the amide nitrogen by the γ-carboxyl group of glutamic acid changes the net charge of the resulting hexapeptide derivative, paper electrophoresis was used to separate the mixtures of peptides resulting from cleavage reactions, A, B, and C into their individual components, with the results shown in Figure 2. In order to obtain sufficient material to determine the structure of the peptides producing each of the separate spots, approximately 1-mg portions of each crude peptide mixture were applied along the base line of the electropherograms, and the electrophoretically separated peptides were eluted from the paper after their locations were detected on guide strips by dipping in ninhydrin or treating with chlorine followed by starch–KI. Sufficient quantity of each of the electrophoretically separable species was thereby collected to perform an amino acid analysis and further degradative and analytical work.

Peptide A, produced by cleavage at 0° in 10% anisole–HF, was a homogeneous, neutral peptide by electrophoresis. This criterion as well as amino acid analysis indicated it was the pure unmodified hexapeptide, Leu-Lys-Glu-Ala-Val-Gly ($\mathbf{1}_A$). The electrophoretically purified $\mathbf{1}_A$ was cleanly cleaved with trypsin to the expected di- and tetrapeptides. Thus, electrophoresis of the digest ($\mathbf{1}_A$-Tryp in Figure 2) produced two ninhydrin-positive peptides **4** and **6**, which had the same mobility as synthetic samples of the expected peptides synthesized by independent routes.

Hexapeptide mixture B, which had been cleaved from the resin with 10% anisole–HF at 24° for 2 hr and analyzed low for glutamic acid, was electrophoretically resolved at pH 6.5 into a mixture of five distinct peptides (Figure 2), a more complex situation than suggested by the amino acid analysis data alone. Of the three major ninhydrin-positive peptides, the slowest moving one (R_{Lys} 0.17) was the unmodified neutral hexapeptide, Leu-Lys-Glu-Ala-Val-Gly ($\mathbf{1}_B$); by comparison, the mobilities of peptides $\mathbf{2}_B$ (R_{Lys} 0.45)

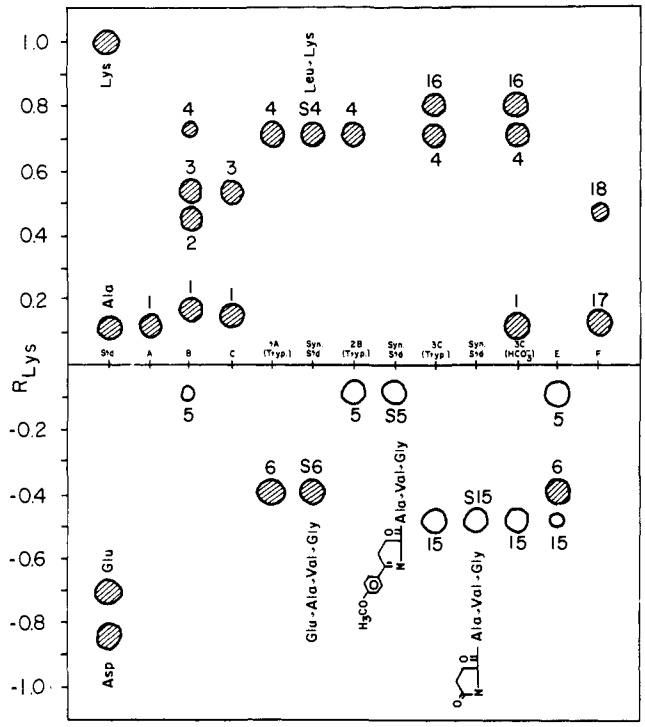


Figure 2. Electrophoretic separation of hexapeptide by-products: run on 3M paper, in pH 6.5 pyridine–acetate buffer, at 3000 V for 40 min at 10°. Under these conditions lysine migrated 242 mm toward the cathode. The mobilities of the other components are relative to lysine, without correction for endosmotic flow. ◍ indicates ninhydrin-positive spots; O indicates ninhydrin-negative, chlorine–starch–iodide positive spots.

and $\mathbf{3_B}$ (R_{Lys} 0.52) suggested that these peptides had lost the charge of a single carboxylate. The fourth ninhydrin-positive peptide, $\mathbf{4_B}$, with the highest cationic mobility (R_{Lys} 0.72) gave a considerably weaker spot than the others. The fifth peptide $\mathbf{5_B}$ was slightly acidic (R_{Lys} −0.1) and was unreactive with ninhydrin, but could be detected by exposure to chlorine followed by spraying with starch–KI.[12]

Electrophoresis of peptide mixture C, which had been cleaved from the resin at 24° without anisole and contained a full equivalent of glutamic acid, was resolved into the unmodified hexapeptide $\mathbf{1_C}$ and another ninhydrin-positive peptide $\mathbf{3_C}$ which had the same mobility as the component $\mathbf{3_B}$ of mixture B.

Structures of the Anisylated Peptides in Mixture B. The electrophoretic component $\mathbf{2_B}$ represented the fraction of the peptide mixture B in which the γ-carboxyl group of the glutamyl residue had undergone Friedel–Crafts condensation with anisole. Thus, the glutamyl residue in $\mathbf{2_B}$ had been altered to 4-*p*-methoxybenzoyl-2-aminobutyric acid (Figure 1), as shown by the following evidence.

(a) Amino Acid Analysis. The peptide isolated from the electropherogram analyzed for equal proportions of leucine, lysine, alanine, valine, and glycine but gave no glutamic acid (Table I).

(b) Electrophoretic Mobility. Its mobility (R_{Lys} 0.45) indicated that the molecule possessed a net positive charge, consistent with a modified carboxyl group.

(c) Chemical Derivatization. Peptide $\mathbf{2_B}$ formed an orange phenylhydrazone with 2,4-dinitrophenylhydrazine under conditions which did not derivatize the intact hexapeptide, indicating the presence of a keto group in the molecule.

(d) Spectrophotometric Evidence. Peptide $\mathbf{2_B}$ exhibited the same ultraviolet absorption spectrum as the parent mixture B; this spectrum is compared with the spectrum of anisole

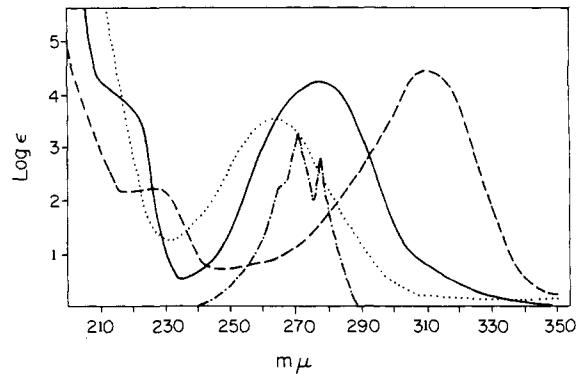
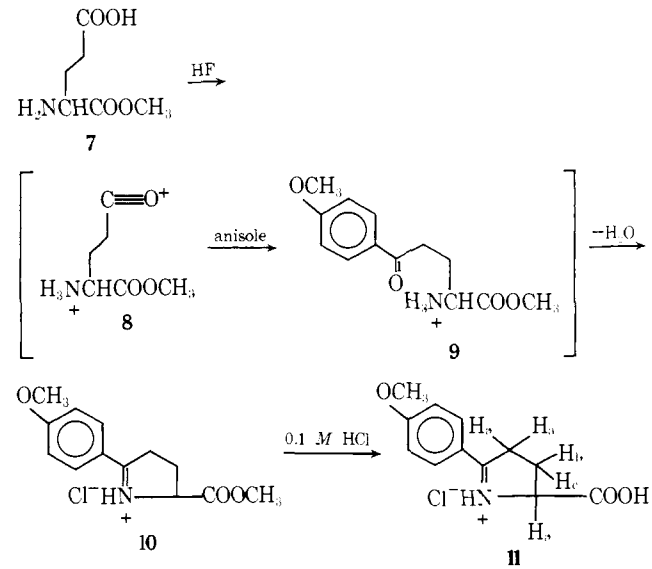


Figure 3. Ultraviolet absorption spectra of anisylated hexapeptide and its hydrolysis product: (·····) anisole; (———) anisylated hexapeptide mixture B and hexapeptide $\mathbf{2_B}$; (- - - -) hydrolyzate of hexapeptide mixture B and hexapeptide $\mathbf{2_B}$ in acid; (·····) hydrolyzate of hexapeptide mixture B and hexapeptide $\mathbf{2_B}$ in base. Extinctions do not apply to hexapeptide mixtures.

in Figure 3. The ultraviolet maximum and extinction coefficient of $\mathbf{2_B}$ in water (276 nm, ε 1.72×10^4) is, in fact, more comparable to those of *p*-methoxyacetophenone (276 nm, ε 1.6×10^4) than those of anisole (271 nm, ε 1.7×10^3) in ethanol.[13]

The ultraviolet spectrum of peptide $\mathbf{2_B}$ was not affected by changes of pH, as expected for a chromophore containing no ionizable functional group. When the peptide was hydrolyzed in acid, however, the absorption of the chromophore was altered significantly. As shown in Figure 3, the hydrolyzate produced a maximum at 262 nm when basic that changed to 310 nm (ε 2.75×10^4) after acidification. This indicated that the modified glutamic acid residue underwent a further change in structure during hydrolysis. In contrast to peptide $\mathbf{2_B}$, the anisylated glutamic acid formed on hydrolysis contained an ionizable chromophore sensitive to pH and with absorption maxima very different from the parent compound. It was also unreactive with ninhydrin, since glutamic acid was absent from amino acid analyses of peptide $\mathbf{2_B}$ and no new peak was observed.

(e) Comparison with Synthetic 2-*p*-Methoxyphenyl-1-pyrroline-5-carboxylic Acid. The structure of the glutamic acid by-product after acid hydrolysis of the hexapeptide $\mathbf{2_B}$ was established as pyrroline **11** by synthesis. α-Methyl glutamate (**7**) reacted readily with anisole in liquid HF to yield the pyrroline ester hydrochloride **10.** In accordance with the



cyclic structure, its infrared spectrum contained an ester carbonyl band at 1730 cm^{-1} and lacked a phenyl ketone band near 1695 cm^{-1}. Pyrroline ester **10** probably resulted from Friedel–Crafts acylation of anisole by the glutamate γ-acylium ion **8**, which could be formed in a strongly acid medium such as liquid hydrogen fluoride.[14] The resulting unstable methyl 4-*p*-methoxybenzoyl-2-aminobutyrate (**9**) was not isolated, for it evidently cyclized readily to the sterically favored Schiff base **10** which was obtained in analytically pure form as the hydrochloride after workup.

Acid hydrolysis of the ester **10** gave 2-*p*-methoxyphenyl-1-pyrroline-5-carboxylic acid (**11**). A 220-MHz NMR spectrum of **11** in D_2O showed H_b and H_c as two single-proton complex multiplets shifted 2.52 and 2.79 ppm downfield from β-trimethylsilylperdeuterio-*n*-propionic acid sodium salt (TSP). These methylene protons, adjacent to an asymmetric carbon, were not equivalent and split each other as well as the three adjacent protons. The two protons H_a were equivalent and, as the A_2 part of an A_2MX system, appeared as a triplet at 3.64 ppm. The most deshielded proton H_d was observed at 5.20 ppm split into a quartet by H_b and H_c. The methoxy protons gave a sharp singlet at 3.95 ppm, while the para-substituted phenyl protons were found as a pair of doublets at 7.20 and 8.04 ppm.

The hydrolyzate of hexapeptide mixture B had the same ultraviolet absorption spectrum as the pyrroline **11**, the presence of the basic nitrogen in the chromophore explaining its sensitivity to pH. It had not been detected in the hydrolyzate by amino acid analysis since, with the α-amino terminus incorporated in the pyrroline ring, it did not react with ninhydrin. However, it could be visualized on silica gel TLC plates by the sensitive chlorine–tolidine method.[15] By running a thin-layer chromatogram to compare hydrolyzates of hexapeptide A and hexapeptide mixture B with the pyrroline **11** (see Figure 4), it became clear that the hydrolyzate of peptide mixture B, low in glutamic acid, did contain a ninhydrin-negative material that co-chromatographed with **11**, while the hydrolyzate of A did not. It was concluded that the glutamic acid by-product after hydrolysis was 2-*p*-methoxyphenyl-1-pyrroline-5-carboxylic acid and that it had derived from the 4-*p*-methoxybenzoyl-2-aminobutyryl residue contained in the hexapeptide $\mathbf{2_B}$.

(f) Tryptic Hydrolysis. The fact that 2-*p*-methoxyphenyl-1-pyrroline-5-carboxylic acid (**11**), instead of 4-*p*-methoxybenzoyl-2-aminobutyric acid, is the product found after hydrolysis of anisole-modified peptide $\mathbf{2_B}$ indicates how strongly the formation of the cyclic Schiff base is favored. Whenever the α-amino group of a γ-anisylated glutamic acid was a free primary amine, this cyclization occurred, even when the amino group was generated under very mild conditions. Thus, when the modified hexapeptide $\mathbf{2_B}$ was subjected to tryptic hydrolysis of the peptide bond following lysine, the resulting tetrapeptide fragment $\mathbf{5_{(2B\text{-}Tryp)}}$ terminated in the substituted pyrroline form of the modified glutamyl residue (see Figure 1). Electrophoresis of the tryptic digest (2B-Tryp in Figure 2) gave a ninhydrin-positive peptide **4** identified as leucyllysine and a ninhydrin-negative peptide **5** that required visualization with Cl_2–starch–KI. This peptide (R_{Lys} −0.1) had a greater anionic mobility than the neutral hexapeptide **1** or the alanine standard since its N terminus is a weakly basic imine conjugated with the *p*-methoxyphenyl group rather than a more basic primary amine.

By contrast, most of the tetrapeptide fragment resulting from tryptic cleavage of peptide $\mathbf{2_B}$ dinitrophenylhydrazone was ninhydrin positive, with a neutral R_{Lys} on electrophoresis. As would be expected, derivatization of the $\mathbf{2_B}$ phenyl ketone strongly inhibited subsequent formation of the Schiff base after enzymatic hydrolysis, and the tryptic tetrapeptide therefore contained 4-*p*-methoxybenzoyl-2-aminobutyric acid dinitrophenylhydrazone as its N-terminal residue.

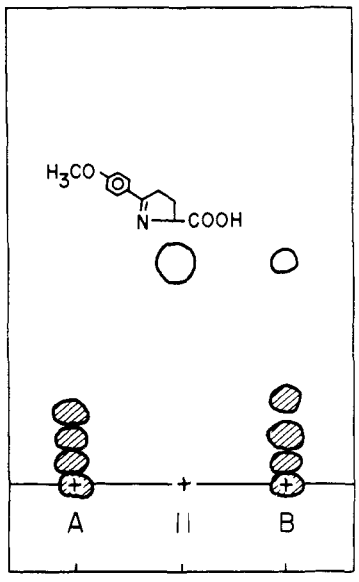
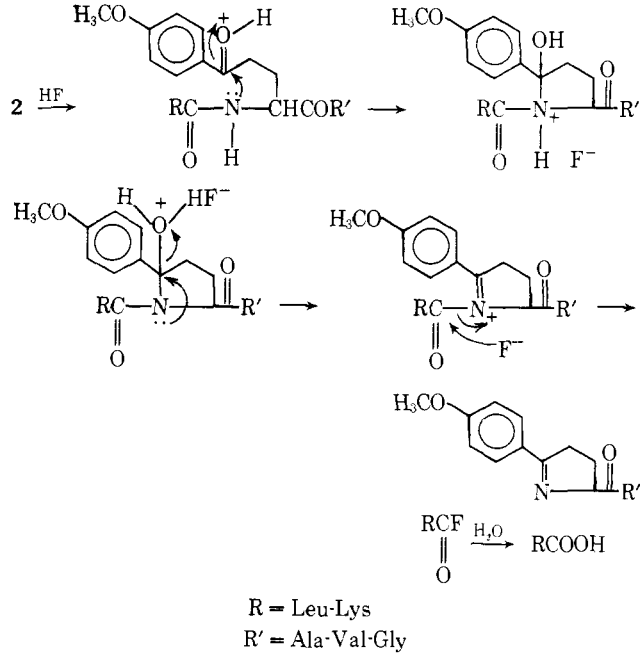


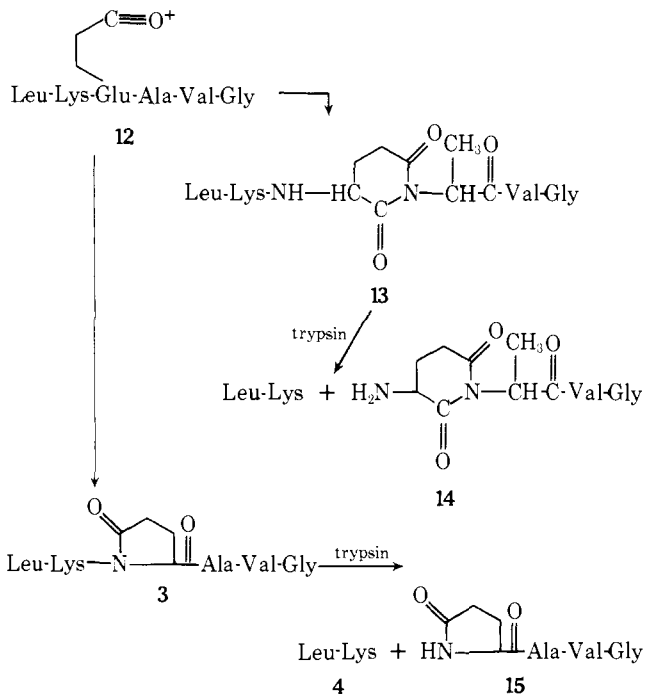
Figure 4. Thin-layer chromatogram of 2-*p*-methoxyphenyl-1-pyrroline-5-carboxylic acid (**11**) and hydrolyzates of peptide A and peptide mixture B. Support, silica gel on glass plates; solvent system, chloroform–methanol (4:1); shaded spots indicate ninhydrin positive; open spots indicate ninhydrin negative, chlorine–tolidine positive.

As final proof of structure, both tryptic fragments from hexapeptide $\mathbf{2_B}$ were shown to co-migrate during electrophoresis with authentic standards synthesized by independent routes. By comparison with these two synthetic standards, as well as from their own amino acid analyses, it is clear that the peptides producing the two weak spots $\mathbf{4_B}$ and $\mathbf{5_B}$ in the electrophoretic separation of mixture B resulted from a small amount of fragmentation of the anisylated hexapeptide $\mathbf{2_B}$ into these same two tryptic fragments leucyllysine ($\mathbf{4_B}$) and 2-*p*-methoxyphenyl-1-pyrroline-5-carbonylalanylvalylglycine ($\mathbf{5_B}$). A mechanism like the following, involving protonation of the ketone followed by intramolecular attack of the glutamic acid nitrogen and deacylation by fluoride, would explain the appearance of the observed products after work-up from aqueous medium.



Structure of the Pyrrolidone-Containing Hexapeptide. If acylium ion formation catalyzed by liquid hydrogen fluoride is the initial step in the formation of by-products of glutamic acid, there are several possible competitive pathways by which the ion can react. One is the acylation of the carbonium ion scavenger anisole, as we have seen. Another possibility is attack by the unshared pair of electrons from a neighboring nitrogen atom, forming a new amide bond with consequent loss of the glutamic acid side-chain carboxylic acid. Such a mechanism explains the presence of the second major hexapeptide by-product **3,** which occurred in both hexapeptide mixtures B and C. In mixture C, which had been cleaved in HF with anisole omitted, competition for the acylium ion intermediate by this scavenger was not possible and **3** was the sole by-product formed. A hexapeptide that had undergone an intramolecular amide acylation fitted with the observed electrophoretic mobility (R_{Lys} 0.52), indicating loss of a carboxyl group by comparison with hexapeptide **1,** yet which could yield a full equivalent of each of the six component amino acids, including glutamic acid, after hydrolysis (Table I).

Formation of a pyrrolidone is not the only intramolecular cyclization possible for the hexapeptide model compound **1.** There are three nitrogens within reasonable distance of the acylium ion **12** derived from the glutamic acid side-chain carboxyl group. The ϵ-amino group of the neighboring lysine residue is an unlikely acylation site since the product would be a peptide containing a hindered 12-membered

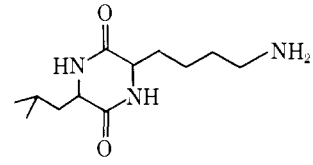


ring. This possibility was excluded because the resulting macrocycle would be neutral, not basic as observed. Far more likely would be attack of the acylium ion on one of the neighboring main-chain amide nitrogens. If the alanyl nitrogen were involved, the product would be the piperidinedione **13,** while attack on the glutamyl nitrogen would result in the pyrrolidone-containing peptide **3.**

These possibilities were readily differentiated by tryptic hydrolyses. Trypsinolysis of the hexapeptide piperidinedione **13** would be expected to cleave off leucyllysine, leaving the dione-containing tetrapeptide **14** which would be neutral and reactive with ninhydrin. The same treatment of a hexapeptide pyrrolidone **3** would also form leucyllysine (**4**), but the resulting tetrapeptide **15** terminating in a pyroglutamyl residue would be unreactive with ninhydrin and bear a net negative charge.

Trypsinolysis of the peptide $\mathbf{3}_C$ confirmed that it had the pyrrolidone structure. Electrophoresis of the tryptic mixture 3C-Tryp (see Figure 2) produced the ninhydrin-negative peptide **15,** whose mobility (R_{Lys} −0.48) showed the presence of a net negative charge. Peptide isolated from the spot gave equimolar amounts of glutamic acid, alanine, glycine, and valine, and a synthetic standard, pyroglutamylalanylvalylglycine **S15,** had the same analysis, electrophoretic mobility, and lack of reaction with ninhydrin.

Though it was expected to produce only one ninhydrin-positive product, the tryptic cleavage of $\mathbf{3}_C$ actually gave two ninhydrin-positive peptides, $\mathbf{4}_{(3C\text{-}Tryp)}$ and $\mathbf{16}_{(3C\text{-}Tryp)}$ (Figure 2), both of which were basic and analyzed for equimolar amounts of leucine and lysine. Peptide **4,** which had the same mobility as synthetic leucyllysine, was assigned the normal dipeptide structure. The other dipeptide (R_{Lys} 0.80) could have been either the leucyllysyl diketopipera-



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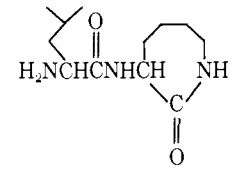

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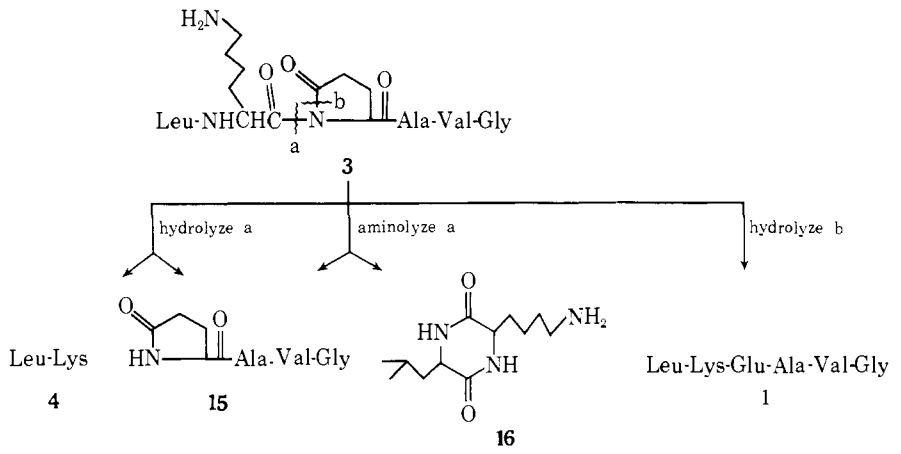
zine **16** or the dipeptide lactam **19.** The two possibilities were differentiated by dansylating the free amino groups and then hydrolyzing the peptide. Such a procedure applied to the peptide isolated from spot $\mathbf{16}_{(3C\text{-}Tryp)}$ gave only ϵ-dansyllysine and no dansylleucine, indicating that the peptide had the diketopiperazine structure **16.** As expected, the normal open-chain dipeptide $\mathbf{4}_{(3C\text{-}Tryp)}$ gave both dansylleucine and ϵ-dansyllysine under the same conditions.

The formation of the diketopiperazine **16** by intramolecular aminolysis of the first two residues of hexapeptide $\mathbf{3}_C$ is undoubtedly favored by the diacylimine structure. It is well known that such imines are readily hydrolyzed under mild alkaline conditions,[16] each acyl group labilizing the other to nucleophilic attack. Thus, the pyrrolidone hexapeptide $\mathbf{3}_C$ required only incubation in 0.5% aqueous NH_4HCO_3 for 0.5 hr at 37° for complete hydrolysis. Electrophoresis of the resulting hydrolyzate (3C-HCO_3 in Figure 2) gave the expected pattern of four spots, which can be rationalized as follows. Hydrolysis of the lysylpyroglutamyl peptide bond a formed the normal dipeptide leucyllysine (**4**) and the pyroglutamyl tetrapeptide **15.** Aminolysis of bond a by the terminal amino group of the hexapeptide yielded the same pyroglutamyl tetrapeptide **15** and the diketopiperazine **16.** Hydrolysis of the pyrrolidone ring at amide bond b, the other possible cleavage site, regenerated the unmodified hexapeptide **1.** This pattern of hydrolytic fragments indicated that the parent hexapeptide **3** must exist as a pyrrolidone and not as the alternative piperidinedione **13.** Hydrolysis of the latter would produce only hexapeptides, with the peptide backbone linked through either the α- or γ-carboxyl of the glutamic acid.

Quantitation of By-Product Formation. Three methods for determining the extent of by-product formation in glutamic acid containing peptides were considered: amino acid analysis, ion exchange chromatography, and spectrophotometry. The most obvious method was amino acid analysis of the peptide hydrolyzates to determine the percentage of glutamic acid missing by comparison with the other residues present. This method established a crude limiting value for the extent of anisole acylation. It was useless, however, when acylation fell below 5%, near the lower limit of reliability of most amino acid analyses, and provided no



data on formation of pyrrolidone by-product, which reverts to glutamic acid during normal hydrolytic procedures.

To obtain comparative data on all the ninhydrin-positive products formed during HF cleavage, direct ion-exchange chromatography on an amino acid analyzer was employed to separate the peptide mixtures. Since a part of the anisylated hexapeptide **2** cleaves to give leucyllysine (**4**) and the ninhydrin-negative tetrapeptide **5**, the total amount of anisylglutamic acid by-product measured by chromatography is equal to the sum of the anisylated hexapeptide **2** and the dipeptide **4**.

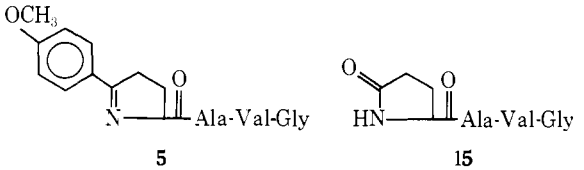
As with amino acid analysis, however, chromatography was not useful for detecting low levels of anisylated hexapeptide **2**. Because this by-product was so strongly adsorbed even on PA-35 resin, the observed peak width was very large (ca. 40 min) and small quantities of the anisylated hexapeptide did not produce a peak of significant height above the base line. When peptide **2** was present at levels below about 2%, a third technique was required to determine the amount of pyrroline **11** present, namely, the measurement of the ultraviolet absorption of the peptide hydrolyzate. This assay directly measures the total amount of glutamic acid in the sample that has reacted with anisole.

The results of the quantitative measurements of by-product formation are set forth in Table II. Only when the temperature was held at 0° during the cleavage period of 0.5 hr was by-product formation largely suppressed. Under these conditions in HF containing 10% anisole, the cleavage yield was 77%, and 98.8% of the product isolated was the unmodified neutral hexapeptide **1**, the remainder being divided between the pyrrolidone **3** and the anisylated by-product **2**. As the cleavage time was extended to 2 hr, the formation of the anisylated peptide **2** increased to about 8%, although cyclization to the pyrrolidone **3** did not increase significantly. Cleavage at room temperature (24°), however, caused massive conversion of the hexapeptide to by-products. In 1 hr 50% and in 2 hr up to 90% of the glutamic acid residue either cyclized to the pyrrolidone **3** or reacted with anisole, with the anisylation being six to seven times more prevalent than the cyclization. Part of the anisylated hexapeptide **2** also underwent breakdown, perhaps during work-up, to leucyllysine (**4**) and the pyrroline tetrapeptide **5**. In the absence of anisole, cleavage at room temperature yielded only about half as much peptide as with the scavenger present, and this was 66% converted to the pyrrolidone by-product **3**.

Attempts to minimize the side reactions by dilution of the liquid hydrogen fluoride with anisole to inhibit Friedel-Crafts catalysis were only partially successful. Cleavage with a mixture of 75% anisole in HF at 24° for 1 hr was required to suppress by-product formation to levels similar to those produced at 0° with 10% anisole. At such dilutions, anisole approaches molar equivalency with the HF and is presumably protonated by the hydrogen fluoride, which is then ineffective as either a catalyst or a cleavage reagent, and the yield of peptide obtained from the resin therefore fell to unacceptably low levels of 10–15%.

By-products were also formed if the glutamic acid was deprotected before the cleavage reaction. When a sample of hexapeptide **1** previously cleaved at 0° for 0.5 hr was retreated with HF–anisole at 24° for 2 hr, substantially the same quantities of by-products were observed as when the peptide had originally been cleaved at 24°. Therefore, glutamic acid by-product formation does not require an ester leaving group at the γ-carboxyl. This is in marked contrast to rearrangements of aspartic acid peptides through succinimido intermediates, which are suppressed when the β-carboxyl group is unprotected.[1e,f] This lends further weight to the probability that the mechanism of glutamic acid by-product formation proceeds through the acylium ion intermediate, as postulated above. It is also significant that cleavage with HBr in trifluoroacetic acid containing 12.5% anisole, a Friedel-Crafts catalyst weaker than HF, suppressed formation of all but 2.5% of the pyrrolidone by-product **3** and only 0.3% of the anisole acylation product **2**, even though the reaction was carried out at room temperature.

Glutamic Acid Modification in Other Peptides. Boc-Glu(OBzl)-Ala-Val-Gly-resin (II). In view of the work of Sano,[9] it was of interest to examine whether or not a peptide containing glutamic acid at its N terminus would also be affected by the conditions of its cleavage. Cleavage of Boc-Glu(OBzl)-Ala-Val-Gly-resin for 0.5 hr at 0° in 10% anisole–HF produced a tetrapeptide mixture D containing 0.4% of the anisole acylation product **5**. At 24° for 2 hr, the cleavage mixture E was 79% converted to **5** and a small percentage of the pyroglutamyl terminal tetrapeptide **15**,



which was not quantitated since it was ninhydrin negative and contained no near-ultraviolet chromophore. The remainder of the mixture was the unmodified tetrapeptide **6** (see Figure 2 and Table II). Peptides containing N-terminal glutamic acid, therefore, do undergo Friedel-Grafts acylation with anisole as readily as other glutamyl peptides, but the resulting aryl ketone condenses with the sterically available terminal amino group to form a five-membered cyclic Schiff base. To a smaller extent, cyclization to pyroglutamyl peptides also occurs, so that both by-products derived

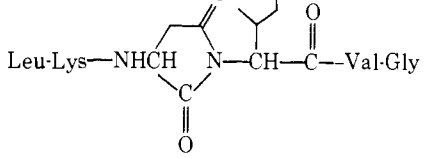
Table II. Effect of Cleavage Conditions on By-Product Formation in HF-Treated Glutamyl Peptides

Cleavage conditions[a]	Cleavage yield, %	Peptide,[b] mol %: By column chromatography: Intact Hexapep. 1	Anisylated Hexapep. 2	Pyrrolidone Hexapep. 3	Leu-Lys 4	Total anisyl-Glu 2 + 4	By AAA: Total anisyl-Glu
		Leu-Lys-Glu-Ala-Val-Gly-resin (I)					
10% anisole–HF, 0°, 0.5 hr[d]	63	98.8		0.5	0.0	0.65[c]	0
10% anisole–HF, 0°, 0.5 hr[d]	77	98.8		0.04	0.0	1.12[c]	3
10% anisole–HF, 0°, 1 hr[d]	37	94.5		0.4	0.0	5.12[c]	5
10% anisole–HF, 0°, 2 hr[d]	17	91.3	8.3	0.4	0.0	8.3	11
10% anisole–HF, 24°, 1 hr[d]	64	50.6	39.2	6.9	3.2	42.4	51
25% anisole–HF, 24°, 1 hr	66	64,4	28.6	5.5	1.5	30.1	38
50% anisole–HF, 24°, 1 hr	12	77.2	12.3	5.8	4.7		17
75% anisole–HF, 24°, 1 hr	16	98.7		0.51	0.0	0.75[c]	0
90% anisole–HF, 24°, 1 hr	11	91.0		8.1	0.01	0.88[c]	0
10% anisole–HF, 24°, 2 hr[d]	73	10.2	66.5	11.1	12.2	78.7	75
10% anisole–HF, 24°, 2 hr[d]	53	25.8	53.6	9.2	11.4	65.0	70
10% anisole–HF, 0°, 0.5 hr followed by retreatment, 24°, 2 hr	77	17.0	63.4	10.2	9.3	72.7	74
12% anisole–HBr–TFA, 24°, 1.5 hr	77	97.3	0.3	2.5	0.0	0.28[c]	0
HF (neat), 24°, 2 hr[d]	48	31.1		66.1	0.0		0
HF (neat), 24°, 2 hr[d]	26	51.7		46.6	0.0		0
		Glu-Ala-Val-Gly-resin (II)					
10% anisole–HF, 0°, 0.5 hr	67					0.4[c]	1
10% anisole–HF, 24°, 2 hr	57					79.4[c]	79

[a] 100–200 mg of peptide-resin substituted 0.12–0.18 mmol/g in 10 ml of HF mixture. [b] Structures of peptides are shown in Figure 1. [c] Determined by ultraviolet absorption of peptide hydrolyzate. [d] Two separate cleavages are quoted for these conditions.

from peptides containing N-terminal glutamic acid residues are ninhydrin negative.

Boc-Leu-Lys(Z)-Asp(OBzl)-Ile-Val-Gly-resin (III). The behavior of peptides containing aspartic acid instead of glutamic acid was also of interest. The hexapeptide-resin, Boc-Leu-Lys(Z)-Asp(OBzl)-Ile-Val-Gly-resin, was therefore synthesized, isoleucine being used to minimize formation of the aspartimidyl peptide, the intermediate in the α to β rearrangement. When this peptide resin was treated with 10% anisole–HF at 0° for 0.5 hr, only the unmodified hexapeptide Leu-Lys-Asp-Ile-Val-Gly **17** was isolated. Cleavage at 24° for 2 hr, however, produced a peptide mixture F, which was electrophoretically resolved into two components (Figure 2). The major product, unmodified hexapeptide $\mathbf{17}_F$, was accompanied by 7.0% of the aspartimide derivative



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$\mathbf{18}_F$. When this was dissolved in 0.5% $NaHCO_3$ and heated to 37° for 0.5 hr, it was converted only to peptides with an overall neutral charge. No leucyllysine or ninhydrin-negative tetrapeptide was produced, and no indication of any Friedel–Crafts acylation of anisole was detected by ultraviolet absorption, amino acid analysis, or electrophoresis.

Boc-Lys(Z)-Leu-Glu(OBzl)-Ala-Val-Gly-resin (IV). This hexapeptide-resin was examined because the lysine residue was removed from immediate proximity to glutamic acid to ensure that formation of the by-products observed in the work with the model peptide Leu-Lys-Glu-Ala-Val-Gly was not influenced by interaction with the lysine side-chain amino group. Cleavage of this resin in HF containing 10% anisole at 24° for 2 hr caused 51% of the peptide to undergo modification by anisole acylation and a smaller amount of cyclization to occur. It was concluded that the position of the lysine in the peptide was not important and the lysine side chain played no part in the mechanism of by-product formation.

Boc-Leu-Lys(Z)-Glu(OBzl)-*N*-Methylalanyl-Val-Gly-resin (V). To ensure that no intramolecular cyclization would occur by attack of the glutamic acid γ-carboxyl group on the nitrogen atom of the alanine residue in the model peptide Leu-Lys-Glu-Ala-Val-Gly, the alanine was replaced by an *N*-methylalanine residue. Cleaved in HF–10% anisole at 24° for 2 hr, this resin produced a mixture of products analogous to the alanine-containing model. Anisylated peptide comprised 52% and a smaller amount of a pyrrolidone by-product, cleavable into di- and tetrapeptide fragments by 0.5% NH_4HCO_3, was present.

Conclusion

It is clear that HF cleavage of peptides containing glutamic acid from solid supports should be very carefully controlled to avoid significant conversion to the pyrrolidone or anisole acylation by-products. Both by-products are probably generated by competing reactions from a common acylium ion precursor, whose formation is independent of the presence or absence of a γ-benzyl-protecting group on the glutamic acid and is only slightly affected by the position of the glutamic acid within the peptide sequence. The N-terminal glutamyl tetrapeptide investigated here, for example, underwent conversion to the anisole derivative with approximately the same facility as the hexapeptides possessing an interior glutamic acid.

Two general approaches for minimizing by-product formation can be taken. The first is to minimize the formation of the acylium ion precursor in the strong acid medium required for cleavage. At present, this can best be accomplished by cleaving peptides in liquid HF containing 10% anisole for 0.5 hr at 0°, with the cleavage vessel kept at ice bath temperatures thereafter until all HF has been evaporated. These conditions are sufficient for the removal of most of the commonly used side-chain protecting groups,[17] including the recently developed more stable 2,4-Cl_2Z group for lysine.[18] This short reaction time was sufficient to

cleave about 70% of the peptide from the resin and resulted in formation of 1.2% or less of by-products. This level of by-products before purification should be tolerable for most syntheses, especially as these by-products should be reasonably easy to remove by ion-exchange chromatography. However, it may still pose a problem for molecules containing many glutamic acid residues. An alternative approach is to use a cleavage medium of weaker acidity such as HBr in trifluoroacetic acid containing 12.5% anisole, although N^G-nitroarginine and N^G-tosylarginine are not deprotected under these conditions. Cleavage of the hexapeptide in this medium gave almost no anisole acylation but did cause 2.5% of the peptide to cyclize to the pyrrolidone.

Future research could focus on finding an inert diluent for the liquid HF that would reduce its ability to catalyze formation of the acyl carbonium ion intermediate without lowering the efficiency of the cleavage reaction, though these properties may well prove to be inseparable. A second approach would involve development of a new carbonium ion scavenger that could replace anisole in the cleavage reaction. It should be efficient enough to intercept most or all of the acylium ion before it has a chance to cyclize to the pyrrolidone, and the resulting by-product should then be reconvertible to the unmodified peptide by some mild chemical process.

Experimental Section

Instrumentation. Melting points were determined in a Thomas-Hoover melting point apparatus and are uncorrected. Routine amino acid analyses were performed on a Beckman 121 automatic amino acid analyzer, while samples requiring high dilution were analyzed on a Durrum Model D-500 analyzer. Peptide chromatography was performed on a 13 × 0.9 cm column of Beckman PA-35 resin on a Beckman 120B analyzer with buffer pumped at a rate of 66 ml/hr and overall flow rate of 99 ml/hr. Ultraviolet spectra were measured on a Cary Model 14 PM recording spectrophotometer and infrared spectra on a Perkin-Elmer Model 237B grating spectrophotometer. Nuclear magnetic resonance measurement was performed with a Varian 220-MHz instrument. Electrophoreses were run in a Savant apparatus under Varsol refrigerated to 10°. The pH 6.5 buffer used was composed of pyridine (200 ml), acetic acid (8 ml), and H_2O (1800 ml). Microanalyses were performed by Mr. Ted Bella, Microanalytical Laboratory, Rockefeller University.

Materials. Thin-layer chromatograms were run on Analtech silica gel G plates, 250 μ thick. *tert*-Butyloxycarbonyl (Boc) amino acids were purchased from Beckman Instruments, Palo Alto, and biphenylisopropyloxycarbonyl (Bpoc) amino acid amine salts were synthesized in this laboratory.[19] Bulk solvents used in solid-phase syntheses were reagent grade; DMF was stored over activated molecular sieves (Linde type 4A), CH_2Cl_2 was distilled from $CaCO_3$, diisopropylethylamine was distilled from lithium aluminum hydride, and trifluoroacetic acid was distilled before use.

Synthesis of Bpoc-Leu-Lys(Z)-Glu(OBzl)-Ala-Val-Gly-resin (I). Styrene–1% divinylbenzene resin (Bio-Beads, Bio-Rad Labs) was washed[11b] and then chloromethylated[20] to a substitution level of 0.18 mmol of Cl/g of resin. Chloride was quantitatively displaced by the cesium salt of biphenylisopropyloxycarbonylglycine,[21] and the resulting biphenylisopropyloxycarbonylglycine-resin (2.5 g) was subjected to five cycles of solid-phase synthesis,[11] summarized in Table III. N^{α}-Bpoc-protected amino acid derivatives[19] were used. The resulting N^{α}-biphenylisopropyloxycarbonylleucyl-N^{ϵ}-benzyloxycarbonyllysyl-O^{γ}-benzylglutamylalanylvalylglycyl-resin (I, yield 2.6 g) was subjected to direct hydrolysis by heating a sample of resin (6.0 mg) with liquefied (88%) phenol (0.5 ml), anisole (0.5 ml), glacial acetic acid (1.0 ml), and concentrated HCl (2 ml) in a sealed evacuated tube at 110° for 18 hr. The liquid was then separated from the resin by filtration, the resin was washed with glacial acetic acid (20 ml, in aliquots), and the combined filtrates were evaporated to dryness. The residue was dissolved in H_2O (20 ml) which was then extracted with $CHCl_3$ until the extracts were colorless, and the resulting yellow aqueous solution was reduced to an appropriate volume for amino acid analysis on a Beckman automatic analyzer, Model 121B. The molar ratios were Glu, 0.98; Gly, 1.02; Ala, 0.99; Val, 1.02; Leu, 1.03; Lys, 0.96.

Table III. Solid-Phase Peptide Synthesis Program for Addition of One Residue with Double Coupling[a]

Function	Solvent	Bpoc-amino acids: Applications	Bpoc-amino acids: Time, min	Boc-amino acids: Applications	Boc-amino acids: Time, min
Wash	CH_2Cl_2	2	1	2	1
Deprotection	TFA in CH_2Cl_2 3%	2	3		
	TFA in CH_2Cl_2 50%			1	5
	TFA in CH_2Cl_2 3%	1	10		
	TFA in CH_2Cl_2 50%			1	25
Wash	CH_2Cl_2	3	1	3	1
Neutralization	5% DIEA in CH_2Cl_2	2	1	2	1
		1	5	1	5
Wash	CH_2Cl_2	5	1	5	1
	2-Propanol	3	1	3	1
	CH_2Cl_2	5	1	5	1
Coupling	Amino acid	1	1	1	1
	DCC	1	30	1	30
Wash	CH_2Cl_2	5	1	5	1
	DMF	3	1	3	1
	CH_2Cl_2	3	1	3	1
Neutralization	5% DIEA in CH_2Cl_2	1	1	1	1
Wash	CH_2Cl_2	5	1	5	1
	2-Propanol	3	1	3	1
	CH_2Cl_2	5	1	5	1
Coupling	Amino acid	1	1	1	1
	DCC	1	30	1	30
Wash	CH_2Cl_2	5	1	5	1
	DMF	3	1	3	1
	CH_2Cl_2	5	1	5	1

[a] Volume, ca. 30 ml for 2.5 g of resin; 3 equiv of amino acid and DCC per coupling.

Other Peptide-Resins Synthesized. By the same techniques, the following peptide-resins were synthesized. N^{α}-*tert*-Butyloxycarbonyl-O^{γ}-benzylglutamylalanylvalylglycyl-resin (II): Glu, 0.97; Gly, 1.04; Ala, 1.01; Val, 0.98. N^{α}-*tert*-Butyloxycarbonylleucyl-N^{ϵ}-benzyloxycarbonyllysyl-O^{β}-benzylaspartylisoleucylvalylglycyl-resin (III): Asp, 1.02; Gly, 1.04; Val, 0.94; Ile, 0.92; Leu, 1.10; Lys, 0.97. N^{α}-*tert*-Butyloxycarbonyl-N^{ϵ}-benzyloxycarbonyllysyl-leucyl-O^{γ}-benzylglutamylalanylvalylglycyl-resin (IV): Glu, 0.98; Gly, 1.07; Ala, 1.09; Val, 1.07; Leu, 0.94; Lys, 0.85. N^{α}-*tert*-Butyloxycarbonylleucyl-N^{ϵ}-benzyloxycarbonyllysyl-O^{γ}-benzylglutamyl-N^{α}-methylalanylvalylglycyl-resin (V): Glu, 1.00; Gly, 1.04; Val, 0.09; Leu, 1.05; *N*-methyl-Ala, 0.97; Lys, 0.95. N^{α}-*tert*-Butyloxycarbonylalanylvalylglycyl-resin (VI): Gly, 1.00; Ala, 1.02; Val, 0.98.

Cleavage of Peptide-Resin I in Liquid HF Containing 10% Anisole at 0° for 0.5 Hr. Hexapeptide-resin I (0.20 g) was placed in a Teflon apparatus with anisole (1 ml), and liquid HF (9 ml) was condensed into the mixture by cooling with a Dry Ice–acetone bath. The Dry Ice was replaced with an ice–water bath, and stirring was maintained for 0.5 hr. The bulk of hydrogen fluoride was then evaporated under aspirator vacuum (ca. 20–30 min) without removing the ice bath, and evaporation was continued briefly under high vacuum until the cleaved suspension was colorless, indicating removal of the last traces of HF. The resulting residue and resin were removed from the Teflon apparatus by washing with glacial acetic acid onto a small filter funnel where the resin was thoroughly extracted with acetic acid (40 ml, in aliquots). The combined filtrate was then evaporated to dryness in vacuo at 30°. The residue was dissolved in H_2O (30 ml); the aqueous solution was extracted with ether (5 × 30 ml) and then frozen and lyophilized. Leucyllysylglutamylalanylvalylglycine (A) was isolated as a fluffy white solid (17 mg, 63% yield after correction for nonpeptide solids). For amino acid analysis, a sample of peptide (1 mg) was heated in a sealed evacuated tube with 6 *N* HCl (2 ml) for 18–24 hr, after which the liquid was evaporated in vacuo and the residue was diluted with distilled H_2O to the concentration required for amino acid analysis. Found: Glu, 1.00; Gly, 1.00; Ala, 1.01; Val, 0.99; Leu, 1.01; Lys, 0.99.

Cleavage of Peptide-Resin I in Liquid HF Containing 10% Anisole at 24° for 2 Hr. Hexapeptide-resin I (0.20 g) was placed in a Teflon apparatus with anisole (1 ml), and liquid HF (9 ml) was condensed into the mixture by cooling with a Dry Ice–acetone bath. This bath was replaced by a water bath maintained at 24° for 2 hr, after which the apparatus was immersed in ice water and the HF was removed under aspirator vacuum followed by high vacuum, as above. The peptide was worked up as in the previous cleavage. After lyophilization, peptide mixture B was isolated as white solids (20 mg, 73% yield after correction for nonpeptide solids). After anaerobic hydrolysis in 6 *N* HCl for 20 hr, amino acid analysis gave Glu, 0.25; Gly, 1.01; Ala, 1.00; Val, 1.00; Leu, 1.01; Lys, 0.96.

Cleavage of Peptide-Resin I in HBr–TFA Containing 12.5% Anisole. Hexapeptide-resin 1 was suspended in trifluoroacetic acid (7 ml) and anisole (1 ml), and HBr was bubbled through the liquid for 90 min at room temperature. The resin was separated by filtration and washed with TFA, and the combined filtrates were evaporated in vacuo. The residue was dissolved in H_2O (ca. 25 ml), extracted with ether (3 × 15 ml), and lyophilized. White fluffy peptide was recovered (24.1 mg, 77% yield after correction for nonpeptide solids). After hydrolysis, amino acid analysis gave Glu, 1.02; Gly, 0.99; Ala, 1.00; Val, 0.97; Leu, 1.01; Lys, 0.98.

Electrophoretic Separation of Peptide Mixture B. Mixture B (1 mg) was dissolved in H_2O (100 μl) and applied on 10 cm of a line drawn through the midpoint of a 57-cm long piece of Whatman 3M paper dampened with pH 6.5 pyridine–acetate buffer (pyridine 200 ml, acetic acid 8 ml, H_2O 1800 ml). Mixture B (0.05 mg in 20 μl of H_2O) was also spotted 2 cm on either side of this band, and a mixture of amino acids was spotted for reference. The paper was then immersed in a Savant electrophoresis apparatus containing Varsol refrigerated to 10°, and a potential of 3000 V was maintained for 40 min. The paper was then air-dried for 1 hr and cut into three strips. Strip 1 contained electropherograms of the amino acid standard solution and one spot of mixture B. Strip 2 contained the 10-cm banded mixture B electropherogram, and strip 3 contained the electropherogram of a single spot of mixture B. Strips 1 and 3 were further dried at 110° for 0.5 hr.

Strip 1 was developed by immersion in a ninhydrin solution consisting of ninhydrin (1 g) in acetone (200 ml) to which 24 ml of cadmium acetate solution [2 g of $Cd(OAc)_2 \cdot 2H_2O$ + 40 ml of HOAc + 200 ml of H_2O] was added, and the paper strip was then heated to 110° for 3 min. The spot made by the lysine standard had moved 242 mm from the point of origin toward the cathode, and this was made the reference to which the mobilities of all other spots on the electropherogram were compared, without correction for endosmotic flow. Four ninhydrin-positive peptides were detected, at the following comparative mobilities: $\mathbf{1}_B$ (R_{Lys} 0.17), $\mathbf{2}_B$ (R_{Lys} 0.45), $\mathbf{3}_B$ (R_{Lys} 0.52), $\mathbf{4}_B$ (R_{Lys} 0.71). Strip 3 was exposed to chlorine gas for 15–30 min, the excess chlorine was removed by blowing cool air across the paper for several minutes, and the spots were developed (dark blue on a light blue background) by spraying with a 1% solution of soluble starch containing 1% potassium iodide. Five peptides were visualized; four had the same mobilities as those developed by ninhydrin and the fifth peptide, $\mathbf{5}_B$, had R_{Lys} −0.10.

Strips 1 and 3 were taped in their original places on either side of undeveloped strip 2, and using the developed strips as locator guides, the paper bearing the five bands of peptide components of mixture B were cut from strip 2. Peptides were then removed from the paper by elution with 10% acetic acid, and the resulting solutions were evaporated in vacuo. The residues were dissolved in H_2O, and the aqueous solutions were frozen and lyophilized. The resulting five peptides were isolated as fluffy white solids. They were leucyllysylglutamylalanylvalylglycine ($\mathbf{1}_B$), leucyllysyl-2-amino-4-(*p*-methoxybenzoyl)butyrylalanylvalylglycine ($\mathbf{2}_B$), leucyllysylpyroglutamylalanylvalylglycine ($\mathbf{3}_B$), leucyllysine ($\mathbf{4}_B$), and 2-*p*-methoxyphenyl-1-pyrroline-5-carbonylalanylvalylglycine ($\mathbf{5}_B$). Their structures are found in Figure 1, their amino acid analyses in Table I, and the electrophoretic pattern in Figure 2.

Peptide Chromatography of Peptide Mixture B. Two chromatographic systems were used with a 13 × 0.9 cm column of Beckman PA-35 resin on a Beckman 120B amino acid analyzer, with buffer pumped at 66 ml/hr and ninhydrin at 33 ml/hr. In system 1, with the column temperature at 56°, 1.0 ml of the peptide mixture [(0.5–2 mg) in Beckman pH 5.26 buffer (3.0 ml)] was injected and the chromatography was run at pH 5.26. Elution times of the component peptides were $\mathbf{1}_B$, 17 min; $\mathbf{3}_B$, 53 min; $\mathbf{4}_B$, 93 min. Peptide $\mathbf{2}_B$ did not elute under these conditions. System 2 employed pH 7.00 buffer (made by adjusting Beckman pH 5.26 buffer with solid NaOH) with the column temperature at 70°. Peptide mixture B was injected in pH 7.00 buffer and elution times for chromatography at this pH were peptide $\mathbf{1}_B$, 15 min; $\mathbf{2}_B$, 94 min; $\mathbf{3}_B$, 23 min; $\mathbf{4}_B$, 29 min. To compare chromatograms of mixture B run under both systems, the area under peak $\mathbf{1}_B$ was normalized in each, and by assuming that the color values of each component were equal, the relative amount of each component of the mixture was evaluated. Results of this and other chromatographic analyses appear in Table II.

Tryptic Cleavage of Leucyllysylpyroglutamylalanylvalylglycine ($\mathbf{3}_C$). The pyrrolidone-containing hexapeptide $\mathbf{3}_C$, isolated after band electrophoresis of peptide mixture C, was dissolved in 0.5% NH_4HCO_3 (125 μl), 20 μl of a solution of Tpck-trypsin (1 mg/ml in 0.001 *M* HCl) was added, and the solution was incubated at 37° for 1.5 hr. The solution was then spotted on Whatman 3M paper dampened with pH 6.5 buffer and electrophoresis was carried out at 3000 V for 40 min. No trace of hexapeptide $\mathbf{3}_C$ (R_{Lys} 0.52) remained. Two ninhydrin-positive peptides, **4** (R_{Lys} 0.71) and **16** (R_{Lys} 0.80), and a ninhydrin-negative peptide **15** (R_{Lys} −0.48) appeared. Peptides were isolated from the paper as described above. Their amino acid analyses are given in Table I and their structures are in Figure 1. The electrophoretic pattern is shown in line $\mathbf{3}_C$(Tryp) in Figure 2. The peptides were leucyllysine (**4**), leucyllysyl diketopiperazine (**16**), and pyroglutamylalanylvalylglycine (**15**).

Hydrolysis of Leucyllysylpyroglutamylalanylvalylglycine ($\mathbf{3}_C$) by 0.5% NH_4HCO_3. The pyrrolidone-containing hexapeptide $\mathbf{3}_C$, isolated from a band electrophoresis of peptide mixture C, was dissolved in 0.5 NH_4HCO_3 (125 μl) and heated to 37° for 3 hr. The solution was then spotted on Whatman 3M paper wet with pH 6.5 buffer and electrophoresis was maintained at 3000 V for 40 min. Three ninhydrin-positive peptides were observed: **16** (R_{Lys} 0.81), **4** (R_{Lys} 0.71), **1** (R_{Lys} 0.13), and one ninhydrin-negative peptide, **15** (R_{Lys} −0.48). The electrophoretic pattern is given on line $\mathbf{3}_C(HCO_3)$ in Figure 2; amino acid analyses of peptides are in Table I and structures are given in Figure 1. The peptides were the same as in the tryptic cleavage above, with the addition of leucyllysylglutamylalanylvalylglycine (**1**).

Dansylation of Peptides Isolated from Spots 4 and 16 in Electropherogram of 0.5% NH_4HCO_3-Hydrolyzed Hexapeptide $\mathbf{3}_C$. In three 6 × 50 ml test tubes were placed ca. 0.1 mg of dipeptide isolated from spot **4**, from spot **16**, and leucyllysine (**S4**) from an independent preparation (described below). To each test tube was added 15 μl of a solution of dimethylaminonaphthalenesulfonyl chloride in acetone (2.5 mg/ml). The test tubes were covered with parafilm and heated to 37° for 1 hr, after which they were dried by evacuation in a desiccator. To the residue in each tube was added 6 *N* HCl (50 μl), and the tubes were sealed and heated to 110° for 20 hr. After cooling and opening the tubes, their contents were dried in vacuo and the residues were dissolved in acetone (10 μl). Acetone solutions from the tubes containing dipeptides **4** and **16** were each applied to the corner of 3-in. square polyamide thin-layer chromatography sheets coated on both sides, while the hydrolyzate from the leucyllysine control was applied on the obverse side of both sheets. The thin-layer chromatograms were developed first in 90% H_2O–formic acid (100:1.5 v/v) and then, after turning the chromatograms 90°, in *n*-heptane–1-butanol–acetic acid (3:3:1 v/v). The chromatograms were then viewed under ultraviolet light. The control gave fluorescent spots for dansylleucine and N^{ϵ}-dansyllysine, on which the two spots derived from peptide **4** were superposed. Peptide **16** gave only one fluorescent spot superposed on the control N^{ϵ}-dansyllysine.

Synthesis of 2-*p*-Methoxyphenyl-1-pyrroline-5-carboxylic Acid Methyl Ester Hydrochloride (10). Glutamic acid α-methyl ester (0.1 g, Fox Chemicals) was heated to 30° for 3 hr in a mixture of anisole (2 ml) and liquid HF (2 ml) in a Teflon apparatus. The HF was then evaporated under vacuum, the residue was neutralized with 0.5 *N* NaOH (ca. 12 ml), and the aqueous solution was extracted with ether (3 × 15 ml). The ether solution was dried ($MgSO_4$), filtered, and then shaken in an extraction funnel with H_2O (20 ml) whose pH was adjusted to 1 with 1 *N* HCl after the extraction and then reshaken with the ether. The aqueous layer was removed and back-washed with ether to remove traces of ani-

sole, and the solution was then frozen and lyophilized. The resulting oil was dissolved in several drops of methanol, ether was added to turbidity, and 2-*p*-methoxyphenyl-1-pyrroline-5-carboxylic acid methyl ester hydrochloride (**10**) was allowed to crystallize: yield 52.5 mg (30%). After recrystallization, the sample had mp 168–170°: ir (KBr) 1730 (C=O), 1650, 1600, 1260, 1175 cm^{-1}; uv (H_2O) 310 nm (ϵ 1.82 × 10^4); NMR ($CDCl_3$) (in parts per million downfield from tetramethylsilane) 2.30 (2 **H**, m), 3.07 (2 H, m), 3.80 (3 H, s), 3.86 (3 H, s), 4.91 (1 H, t), 6.93 (2 H, d), 7.86 (2 H, d). Anal. Calcd for $C_{12}H_{16}NO_3Cl$: C, 57.92; **H**, 5.92; N, 5.13. Found: C, 58.02; H, 5.96; N, 5.11.

Hydrolysis of Pyrroline Ester 10 to 2-*p*-Methoxyphenyl-1-pyrroline-5-carboxylic Acid (11). Pyrroline ester **10** (0.1 g) was dissolved in 0.1 *N* HCl (9 ml) and refluxed 5 hr. The aqueous solution was then evaporated in vacuo at 40° to a glass: ir (KBr) 3400 (OH), 1730 (C=O), 1630, 1590, 1355, 1270, 1180 cm^{-1}; uv (H_2O) (OH^-) 262 nm, (H^+) 310 nm (ϵ 2.75 × 10^4). For measurement of the NMR spectrum, the sample was dissolved in D_2O and dried in vacuo three times: NMR (D_2O) 2.52 (1 H, m), 2.79 (1 H, m), 3.64 (2 H, t), 3.95 (3 H, s), 5.20 (1 H, q), 7.20 (2 H, arom, d), 8.04 (2 H, arom, d, J = 8 Hz for phenyl protons). Peak positions are in parts per million downfield from sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$.

Synthesis of 2-*p*-Methoxyphenyl-1-pyrroline-5-carbonylalanylvalylglycine (S5). The 2-*p*-methoxyphenyl-1-pyrroline-5-carboxylic acid (**11**) was dissolved in DMF (2 ml) and diluted with methylene chloride (6 ml), and the resulting solution was divided in half to be used in the two coupling steps of solid-phase synthesis with N^{α}-*tert*-butyloxycarbonylalanylvalylglycyl-resin (VI) (0.35 g, substituted 0.12 mequiv/g of resin). The synthesis used the program set out in Table III. All washes were 10 ml. The weight of resin recovered after the synthesis was 0.34 g. The peptide-resin (0.10 g) was cleaved with anisole (1 ml) in liquid HF (9 ml) for 0.5 hr at 0°, evaporated at 0°, and extracted with glacial acetic acid. The resulting solution was evaporated in vacuo, the residue was dissolved in H_2O (10 ml), and the aqueous solution was extracted with ether (4 × 10 ml). The aqueous layer was then lyophilized, yielding 2-*p*-methoxyphenyl-1-pyrroline-5-carbonylalanylvalylglycine (**S5**), 11.2 mg (35%). Amino acid analysis: Glu, 0.00; Gly, 1.06; Ala, 0.94; Val, 1.00. The peptide gave a single ninhydrin-negative spot (R_{Lys} −0.11) after electrophoresis at pH 6.5, 3000 V, for 40 min.

Derivatization of Leucyllysyl-2-amino-4-(*p*-methoxybenzoyl)-butyrylalanylvalylglycine (2_B) with 2,4-Dinitrophenylhydrazine. Trypsinolysis of the Resulting Phenylhydrazone. The phenyl ketone containing hexapeptide 2_B (0.12 mg) isolated after band electrophoresis of peptide mixture B, and the unaltered hexapeptide **1** (0.12 mg) in small centrifuge tubes were each suspended in 95% ethanol (100 μl). A solution (1 mg of 2,4-dinitrophenylhydrazine in 1 ml of 95% ethanol containing 10 μl of 6 *N* HCl) of the phenylhydrazine (50 μl) was added to each, and both were heated to boiling for 2 min and then allowed to stand at room temperature for 15 min. The solution containing hexapeptide 2_B was orange, while **1** was light yellow. The ethanol was evaporated from each tube under vacuum, the residues were dissolved in H_2O (200 μl), and the resultant solutions were extracted with ether (6 × 0.5 ml). Both solutions were brought to a volume of 5 ml with H_2O. The solution containing hexapeptide 2_B, which appeared yellow, had an absorption maximum at 386 nm (ϵ 1 × 10^4). The solution containing hexapeptide **1** appeared colorless but contained a trace of unextracted dinitrophenylhydrazine (λ_{max} 365 nm).

After lyophilization of the solution containing 2_B dinitrophenylhydrazone, the yellow solid was dissolved in 0.5% NH_4HCO_3 (0.1 ml) and 25 μl of a Tpck-trypsin solution (1 mg/ml in 0.001 *N* HCl) was added. The solution was heated to 37° for 1.5 hr and was then spotted on Whatman 3M paper dampened with pH 6.5 buffer, and electrophoresis was carried out at 3000 V for 30 min. Three spots were found. Leucyllysine (**4**) (R_{Lys} 0.71) and 2-amino-4-(*p*-methoxybenzoyl-2,4-dinitrophenylhydrazone)butyrylalanylvalylglycine (R_{Lys} 0.14) were strong, ninhydrin-positive spots. A weak, ninhydrin-negative, Cl_2-starch-I positive spot (R_{Lys} −0.10) was 2-*p*-methoxyphenyl-1-pyrroline-5-carbonylalanylvalylglycine (**5**).

Synthesis of Pyroglutamylalanylvalylglycine (S15). N^{α}-*tert*-Butyloxycarbonylalanylvalylglycyl-resin (**VI**) (2.02 g, 0.12 mequiv/g of resin) was coupled with pyroglutamic acid (0.11 g/coupling, Schwarz-Mann) on the schedule in Table III. After two couplings, monitoring by the picrate method[22] indicated 1.7% free amino groups remained uncoupled. A further coupling step was performed, after which monitoring indicated the coupling reaction to be 99.3% complete. The peptide-resin (300 mg) was then cleaved in liquid HF (9 ml) and anisole (1 ml) at 0° for 0.5 hr. After evaporating the HF, the resin was extracted with acetic acid on a Buchner funnel, the filtrates were evaporated to dryness in vacuo, and the residue was dissolved in H_2O (ca. 15 ml). After extraction with ether (3 × 15 ml), the aqueous solution was frozen and lyophilized, yielding pyroglutamylalanylvalylglycine (**S15**), 10.8 mg (72%). Amino acid analysis: Glu, 0.96; Gly, 0.99; Ala, 0.95; Val, 1.10. The peptide gave a ninhydrin-negative spot (R_{Lys} −0.48) after electrophoresis at pH 6.5, 3000 V, for 40 min. There was a trace ninhydrin-positive spot (R_{Lys} 0.12) which was probably alanylvalylglycine.

Synthesis of Leucyllysine (S4). *tert*-Butyloxycarbonylleucine succinimide ester was prepared by the method of Anderson:[23] yield 2.7 g (82%); mp 109–111° (lit.[23] 116°).

N^{ϵ}-Benzyloxycarbonyllysine (0.14 g, Schwarz-Mann) was added to tetramethylguanidine (0.14 ml) in dimethyl sulfoxide (10 ml), the suspension was stirred and heated to 50°, and N^{α}-*tert*-butyloxycarbonylleucine succinimide ester (0.17 g) was added. All solids dissolved and the yellow solution was allowed to stand 2 hr at 50° and then 22 hr at ambient temperature. The solution was diluted with H_2O (60 ml), the pH was adjusted to ca. 2 with 0.1 *M* citric acid, and the solution was extracted with methylene chloride (4 × 20 ml). The CH_2Cl_2 extracts were then washed with pH 3.5 citrate buffer (2 × 20 ml) and H_2O (2 × 30 ml) and were dried ($MgSO_4$), filtered, and evaporated to dryness in vacuo. The resulting glass did not crystallize but ran as a single spot on silica gel thin-layer plates in butanol–pyridine–acetic acid–H_2O (15:10:3:12), R_f 0.73, and in chloroform–methanol (4:1), R_f 0.53.

The glass was refluxed for 0.5 hr in trifluoroacetic acid (30 ml). After evaporation of the TFA, the residue was dissolved in H_2O (7 ml) and extracted with ether (3 × 10 ml), and the aqueous layer was lyophilized. The resulting hygroscopic leucyllysine trifluoroacetate (0.16 g) was analyzed for Leu, 0.97; Lys, 1.03.

Acknowledgments. This work was supported in part by Grant AM 01260 from the U.S. Public Health Service and the Hoffmann-La Roche Foundation. The authors are indebted to Dr. Ta-Hsiu Liao for help with electrophoreses, Dr. Paul Fletcher for amino acid analyses on a Durrum high-pressure analyzer, and Dr. Bruce Erickson for many helpful discussions. Mr. Igor Uhrik's skilled technical assistance is gratefully acknowledged.

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Valence Electron Distribution in Perdeuterio-α-glycylglycine. A High-Resolution Study of the Peptide Bond

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Abstract: Carefully collected room temperature X-ray data on perdeuterio-α-glycylglycine have been combined with existing neutron data to give the time-averaged molecular valence and deformation electron densities with an estimated standard deviation of 0.08 e Å^{-3}. The maps presented give a clear indication of bonding effects in the glycylglycine molecule. The density in the peptide C–N bond is elongated perpendicular to the peptide plane, in agreement with the partial double bond character of this bond. Intramolecular effects may be responsible for the asymmetry of the density in the carboxyl group and the polarization of the charge cloud on the peptide oxygen toward the ammonio group. There is no build up of density at the midpoints of the hydrogen bonds, in agreement with the electrostatic model for this interaction. Net charges on the COO^- and NH_3^+ groups are about -0.4 to -0.5 and $+0.4$ electrons, respectively. A refinement on the shape of the atomic valence shell functions indicates the carbon atom to be contracted and the oxygen atom to be expanded relative to the isolated atom functions.

In recent years accurate diffraction techniques have been developed for the direct measurement of the electron distribution in solids. The most informative maps are obtained by a combination of X-ray and neutron diffraction data,[1,2] but qualitative information, especially in the bond regions of the molecules, can also be derived from modified treatments of the X-ray data alone.[3]

In the present study carefully collected room-temperature X-ray data on perdeuterio-α-glycylglycine are combined with the results of a rerefinement of the neutron measurements of Freeman et al.[4] The molecule has been the subject of two recent theoretical studies[5,6] and is of considerable importance because it contains the functional groups characteristic of all proteins. Of special interest are the electronic structure of the peptide bond, the amount and nature of the charge separation in the zwitterion, and the effect of hydrogen bonding on the electron distribution.

The geometry of the molecule has been discussed in earlier publications[4,7] and will therefore not be treated here.

Experimental Section

X-Ray Data Collection and Reduction. Crystals of perdeuterio-α-glycylglycine were kindly supplied by Drs. G. L. Paul and T. M. Sabine. A moderately well-formed crystal, roughly parallelepiped-shaped, 0.25 mm on edge was selected. The unit cell used by previous authors was transformed to a unit cell in space group $P2_1/c$ with a β angle of 107.656°. The cell constants, listed in Table I together with earlier values, were obtained by a least-squares refinement[8] of the setting angles of 18 reflections with $30° < 2\theta < 63°$. Data were collected on a Picker automated diffractometer using Zr filtered Mo Kα radiation. Three standard reflections were measured at regular intervals and used to scale the data. Integrated intensities were obtained by analysis of the reflection profiles using a technique described recently[9] and corrected for absorption (μ = 1.87 cm^{-1}) by Gaussian numerical integration. The 6382 measured reflections were symmetry-averaged to give 3590 independent reflections for the least-squares input; of these 2257 are at sin $\theta/\lambda > 0.65$ Å^{-1}. Discrepancies between symmetry-related reflections agree well with counting statistics and average less than 1.5% for the 1100 strongest reflections. All programs used for data reduction and refinement are part of the Integrated Crystallographic Computing Library at the State University of New York at Buffalo.

X-Ray Data Refinement. Neutron parameters were used as input in the full matrix least-squares minimization of $\Sigma w (F_{obsd}^2 - k^2F_{calcd}^2)^2$ in which $w = 1/\sigma^2$ and $\sigma^2 = \sigma^2_{counting} + (0.03F_{obsd}^2)^2$. Reflections for which $F_{obsd}^2 < 3\sigma(F_{obsd}^2)$ were included only if $F_{calcd}^2 > 3\sigma(F_{obsd}^2)$, in which case F_{obsd}^2 was taken as $3\sigma(F_{obsd}^2)$. Atomic scattering factors used were as listed in Volume 4 of the International Tables for X-ray Crystallography (C, N, O) and, for hydrogen, as given by Stewart et al.[10] A preferable refinement in which all measured reflections were included[11a] yielded positional and thermal parameters within one standard deviation from those listed in Table II. However, the direction of the differences is systematic as predicted by Hirshfeld and Rabinovich;[11a] the thermal parameters from the alternative refinement are larger than those from the previous treatment. In all refinements an isotropic extinction parameter was included as a variable.[11b] For a table of final X-ray structure factors, see ref 12. Agreement factors are summarized in Table III.

Neutron Data Refinement. In the neutron refinement by Freeman et al.[4] strong correlations were encountered between several parameters. In the rerefinement, these correlations were avoided by the transformation to the new cell, with a β angle closer to 90°. In this way correlation coefficients, except those between the scattering length and thermal parameters of the deuterium atoms, were reduced to values less than 0.5. The function minimized was $\Sigma w (F_{obsd} - kF_{calcd})^2$ with $w = 1/\sigma^2 = (4.0 + F_o + 0.013F_o^2)^{-1}$. The constants in this expression were chosen such that the first two terms dominated for weak reflections, while the third term prevented assignment of too high a weight to the strong reflections. A number of extinction-affected reflections were omitted from the

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