Improved Solid-Phase Synthesis of Tryptophan-Containing Peptides. I. Use of Hydrogen Chloride in Formic Acid as a Reagent for the Cleavage of *t*-Butyloxycarbonyl Group[1]

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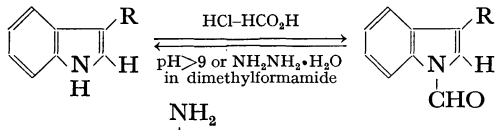
An improved method has been described for the solid-phase synthesis of tryptophan-containing peptides. Hydrogen chloride in formic acid has been introduced as a reagent for the cleavage of the *t*-butyloxycarbonyl group owing to the stableness of tryptophan and N^{i}-formyltryptophan in this system. The effectiveness of the new reagent has been demonstrated in the synthesis of the tryptophan-containing heptapeptide, lysylalanylglycylleucylglycyltryptophylleucine.

The usefulness of the solid-phase synthesis of polypeptides has been documented in the syntheses of a number of biologically active peptides[2] and of proteins.[3] This quick technique has also been becoming a valuable tool in the syntheses of enzymic fragments involved in noncovalently bonded, active complexes and their analogs as an approach to understanding of protein structure and function relationships, as exemplified in bovine pancreatic ribonuclease A[3a,4] and staphylococcal nuclease.[5] It is well known, however, that tryptophan in the peptide chain growing on the polymer support undergoes oxidation during treatment with hydrogen chloride in acetic acid or dioxane for the cleavage of the *t*-butyloxycarbonyl (Boc) group[2,3] that is usually employed as an α-amino protecting group in the solid-phase peptide synthesis. In such cases, sulfhydryl reagent such as β-mercaptoethanol (EtSH)[6] or dithiothreitol[3b] has been used as a scavenger to protect tryptophan from oxidative destruction.

Bubbling of hydrogen chloride into a solution of tryptophan[7] or its derivatives (including even enzymes) in formic acid results in formylation of the indole nitrogen (N^{i}) and this formyl group can be removed in a weakly alkaline medium.[8]



$R = -CH_2-\underset{\displaystyle NH_2}{\underset{|}{CH}}-CO_2H$ etc.

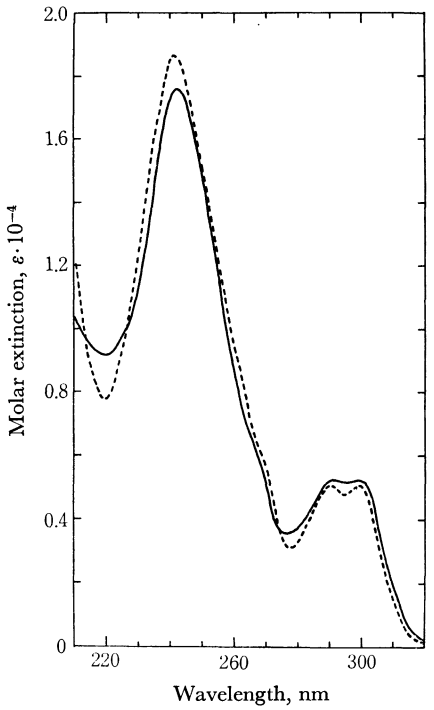


Fig. 1. Ultraviolet absorption spectra of N^{i}-formyltryptophan hydrochloride (——) in water and N^{α}-acetyl-N^{i}-formyltryptophan methyl ester (------) in methanol.

The UV absorption spectra characteristic of N^{i}-formyltryptophans are shown in Fig. 1. On the high-voltage paper electrophoresis (pH 2.08) of a solution of tryptophan dissolved in 0.1—1N hydrogen chlordie in formic acid, no band other than those of tryptophan and N^{i}-formyltryptophan has been detected even after a prolonged standing of the solution, showing the stability of the two indole derivatives in this system. The degree of transformation of tryptophan into N^{i}-formyltryptophan depends on concentration of hydrogen chloride and the time keeping in solution. No reaction occurred upon treatment of N^{i}-formyltryptophans with a large excess of anhydrous triethylamine in dimethylformamide. The formyl group, however, has been cleaved by dissolving N^{i}-formyltryptophans in dimethylformamide containing a 20—30 fold molar excess of hydrazine hydrate and standing for 48 hr. The thin-layer chromatographic studies using α-Boc-aspartic

1) A part of this work has been briefly communicated: M. Ohno, S. Tsukamoto, and N. Izumiya, *Chem. Commun.*, **1972**,, 663.

2) For example: R. B. Merrifield, *Biochemistry*, **3**, 1385 (1964); A. Marglin and R. B. Merrifield, *J. Amer. Chem. Soc.*, **88**, 5051 (1966).

3) For example: (a) B. Gutte and R. B. Merrifield, *J. Biol. Chem.*, **246**, 1922 (1971); (b) C. H. Li and D. Yamashiro, *J. Amer. Chem. Soc.* **92**, 7608 (1970).

4) M. C. Lin, B. Gutte, S. Moore, and R. B. Merrifield, *J. Biol. Chem.*, **245**, 5169 (1970).

5) D. A. Ontjes and C. B. Anfinsen, *ibid.*, **244**, 6316 (1969); I. M. Chaiken and C. B. Anfinsen, *ibid.*, **245**, 2337 (1970); *ibid.*, **245**, 4718 (1970); I. Parikh, L. Corley, and C. B. Anfinsen, *ibid.*, **246**, 7392 (1971).

6) J. Blake and C. H. Li, *J. Amer. Chem. Soc.*, **90**, 5882 (1968).

7) All amino acids in this report are of L-configuration except for glycine.

8) A. Previero, M. A. Coletti-Previero, and J.-C. Cavadore, *Biochim. Biophys. Acta*, **147**, 453 (1967).

acid β-benzyl ester showed that a 1.2—2.0 fold molar excess of hydrogen chloride (0.1N) in formic acid cleaves the Boc group as rapidly as with a 20-fold molar excess of hydrogen chloride (1N) in acetic acid. These observations led us to examine hydrogen chloride-formic acid as a reagent for cleavage of the Boc group in the solid-phase synthesis of tryptophan-containing peptides.

Since removability of the Boc group in the heterogeneous system such as solid-phase synthesis would not be expected to be the same level as in the homogeneous solution system and it is true, the more excess amount of hydrogen chloride than that required for complete cleavage in the solution system should be used.

In the preliminary experiments in which the syntheses of H–Lys–Ala–Gly–Trp–Leu–OH and H–Ala–Gly–Lys(ε-Tfa)–Gly–Trp–Leu–OH (Tfa: trifluoroacetyl) have been made by using hydrogen chloride-formic acid, it was clearly indicated through the UV absorption spectra and the electrophoretic behaviors of the products that the reagent was quite effective in protecting tryptophan from oxidative destruction. We report here, as a typical example, the details of the solid-phase synthesis of the heptapeptide, H–Lys–Ala–Gly–Leu–Gly–Trp–Leu–OH, using the new deblocking method.

The heptapeptide was built up by the usual manner[2,3] starting from Boc-leucyl resin and using a 6-fold molar excess of hydrogen chloride (0.1N) in formic acid. ε-Amino group of lysine was protected with benzyloxycarbonyl (Z) group. In parallel, two peptides with the same sequence were constructed on the resins using 1N hydrogen chloride in acetic acid in the presence and absence of EtSH (2%). Halves of the three protected peptidyl resins [**1a** (*via* HCl–HCOOH), **1b** (*via* HCl–AcOH–EtSH), and **1c** (*via* HCl–AcOH)] were cleaved by hydrazine hydrate in dimethylformamide as described.[9] In order to remove nonpeptide substances derived from the resin, the three products of Boc-Lys(ε-Z)–Ala–Gly–Leu–Gly–Trp*–Leu–$NHNH_2$[10] [**2a** (*via* HCl–HCOOH), **2b** (*via* HCl–AcOH–EtSH), and **2c** (*via* HCl–AcOH)] were reprecipitated from the methanolic solutions by addition of ethyl ether-petroleum ether (1:2 v/v). The other halves of **1a** and **1b** were treated with anhydrous hydrofluoric acid in the presence of anisole,[11,12] and the peptide produced *via* HCl–HCOOH was further treated with 0.1N aqueous piperidine to remove the N^{i}-formyl group attached to a part of tryptophyl residue since this group is resistant to anhydrous hydrofluoric acid (see Experimental section): the two completely deblocked heptapeptides [**3a** (*via* HCl–HCOOH) and **3b** (*via* HCl–AcOH–EtSH)] were obtained.

The UV absorption spectrum of **2a** (Fig. 2) was virtually identical with that of Z-tryptophan except that a trough in the 240—250 nm region showed somewhat greater absorption and was shifted to the red by 2 nm. This indicates that **2a** has been contaminated to only a minor extent by oxidized species. Extinction coefficients of **2b** and **2c** at 282 nm were respectively 1.34 and 1.45 times as large as that of **2a**. Such enhancement of the extinction at 282 nm should be ascribed to oxidation of the indole chromophore although what types of products formed has so far been unknown.

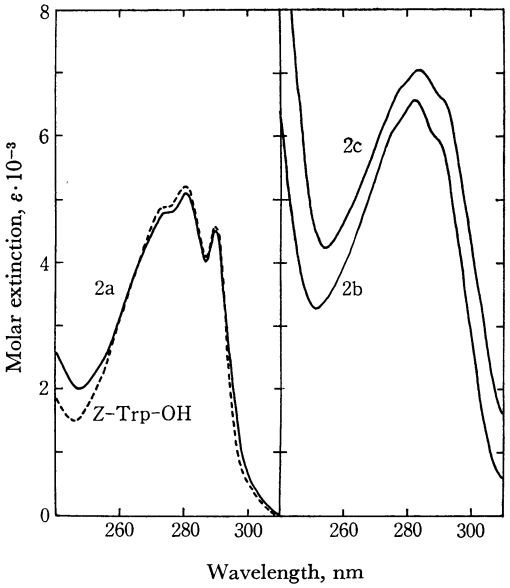


Fig. 2. Ultraviolet absorption spectra of the protected peptide hydrazides, **2a**, **2b**, and **2c**, and Z-tryptophan in methanol.

Figure 3 shows gel-chromatographic patterns of **3a** and **3b** on a Sephadex G-25 column. **3b** gave a complex profile owing to its heterogeneity. To ascertain whether the major peak from **3a** is due only to pure peptide, **3a** was treated with a 10-fold molar excess of 2-nitro-4-carboxyphenylsulfenyl chloride (NCPS–Cl)[13,14] in 80% formic acid and the modified peptide was chromatographed on the same column. Since the peptide containing 2-thio-(2-nitro-4-carboxyphenyl)-tryptophan[14] reveals an increased adsorptivity on the gel than the unmodified,[15] it is apparent from the delayed and shifted elution profile of the modified peptide that the original peptide in the major peak from **3a** involved only pure tryptophyl residue. This was further confirmed from the observation that the modified peptide from the major peak fractions gave exactly the same spectrum as that of 2-thio-(2-nitro-4-carboxyphenyl)-tryptophan and gave a single band upon thin-layer chromatography. Comparison of the two elution diagrams before and after reaction of **3b** with NCPS-Cl indicates that **3b** contains unchanged tryptophan to a small extent.

9) M. Ohno and C. B. Anfinsen, *J. Amer. Chem. Soc.*, **89**, 5994 (1967); M. Ohno, K. Kuromizu, H. Ogawa, and N. Izumiya, *ibid.*, **93**, 5251 (1971).

10) Asterisk signifies that the peptides may or may not involve pure tryptophyl residue.

11) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okuda, and H. Sugihara, This Bulletin, **40**, 2164 (1967).

12) J. Leonard and A. B. Robinson, *J. Amer. Chem. Soc.*, **89**, 181 (1967).

13) A. J. Havlik and N. Kharasch, *ibid.*, **77**, 1150 (1955).

14) F. M. Veronese, E. Boccu, and A. Fontana, *Ann. Chim.*, **58**, 1309 (1968).

15) M. Ohno, T. F. Spande, and B. Witkop, Unpublished results.

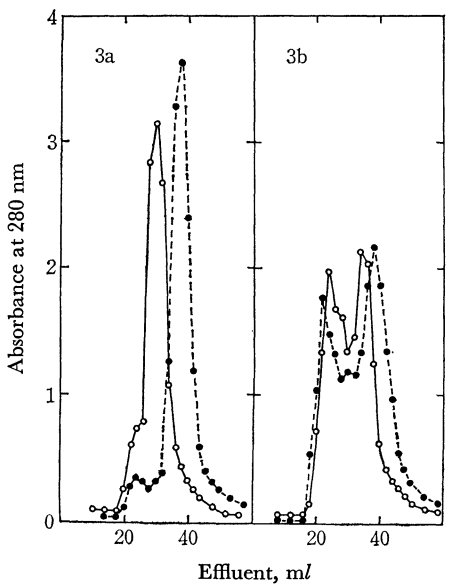


Fig. 3. Elution diagrams of the peptides, **3a** (6 mg) and **3b** (6 mg), on a Sephadex G-25 column (0.9×60 cm) before (○—○) and after (●----●) modification with NCPS-Cl; solvent, 10% acetic acid. The details are described in the Experimental section.

Use of hydrogen chloride-formic acid for the cleavage of the Boc group in the solid-phase synthesis is worthy of attention in such a remarkable suppression of tryptophan oxidation. This merit of the reagent is due possibly to the reductive nature of formic acid. Since formylation of the indole nitrogen stabilizes the indole nucleus against oxidation, use of N^{α}-Boc-N^{i}-formyltryptophan in the solid-phase synthesis is under investigation and will be the subject of future publication.

Experimental

Melting points are uncorrected. UV spectra were taken with a Hitachi spectrophotometer EPS-3T or a Shimadzu UV-200 spectrophotometer equipped with a U-125MU recorder. Optical rotations were measured with a Yanagimoto polarimeter OR-20. Thin-layer chromatographies were performed on Merck silica gel G using either ethyl acetate–methanol (2:1 v/v) (solvent 1) or *n*-butanol–acetic acid–water (4:1:5 v/v, upper layer) (solvent 2) as the developing solvent. Paper electrophoreses were carried out under the following condition: paper, Whatman 3MM (57 cm length); solvent, 0.24N formic acid (pH 2.08); voltage gradient, 48 V/cm; period, 90 min. In this condition all amino acids and peptides in the present experiments moved to the cathode. Substances were detected by spraying ninhydrin (0.2% solution in 80% ethanol) or a modified Ehrlich reagent (0.5% solution of *p*-dimethylaminocinnamaldehyde in 0.5N hydrochloric acid). Amino acid analyses of peptides were carried out on samples that had been hydrolyzed with constant boiling hydrochloric acid for 24 hr in evacuated, sealed tubes at 110°C, and were recorded on a Hitachi amino acid analyzer KLA-3A.

Boc-Leucine, glycine, alanine, valine, and tryptophan were prepared by the reaction of each amino acid with Boc-azide[16] or purchased from the Protein Research Foundation (Osaka). α-Boc-ε-Z-Lysine and α-Boc-ε-Tfa-lysine (mp 101—102°C, lit,[17] 103°C) were prepared by the reaction of ε-Z-lysine[18] and ε-Tfa-lysine[19] with Boc-azide. Z-Tryptophan was prepared from Z-Cl[20] and tryptophan by the Schotten-Baumann procedure. 2-Thio-(2-nitro-4-carboxyphenyl)-tryptophan [λ_{max} in 10% acetic acid 260 (log ε 4.29), 282 (log ε 4.33), and 355 nm (log ε 3.65)] was prepared from 2-nitro-4-carboxyphenylsulfenyl chloride and tryptophan as reported.[14] Chloromethylated copolystylene-divinylbenzene (2%) resin (1.3 mmol/g, 100—200 mesh) was obtained from the Protein Research Foundation (Osaka). Formic acid (analytical grade, 98—100%) was purchased from the Merck Co. and used without purification. Acetic acid was refluxed over potassium permanganate and distilled. Concentration of hydrogen chloride in formic acid or acetic acid was based on the weight of hydrogen chloride absorbed per unit volume of the solvent, or determined by the Volhard titration. The other solvents for the solid-phase synthesis were purified by the appropriate procedures.

Formylation of Tryptophans. Dried hydrogen chloride was bubbled into a solution of tryptophan (7.0 g) in formic acid (100 ml) at room temperature. At suitable intervals aliquotes of the solution were diluted with water for recording UV spectra. After the absorbance at 298 nm reached maximum (about 3 hr), the solvent was removed *in vacuo* with slight warming. N^{i}-Formyltryptophan hydrochloride crystallized by addition of ethyl ether to the syrup. Crystals were collected and washed with ethyl ether, 9.20 g (100%), mp 218°—220°C, $[\alpha]_D^{23}$ −4.7° (*c* 1.91, water).

Found: C, 53.60; H, 4.99; N, 10.42. Calcd for $C_{12}H_{12}O_3N_2 \cdot HCl$: C, 53.64; H, 4.88; N, 10.43%.

α-Acetyltryptophan methyl ester (0.30 g) was treated by the same way as described above, and the syrup was taken into ethyl acetate that was washed with water, dried over sodium sulfate and evaporated. Crystallization of the residue by addition of petroleum ether gave 0.28 g (85%) of N^{α}-acetyl-N^{i}-formyltryptophan methyl ester; mp 102—104°C, $[\alpha]_D^{23}$ +21.0° (*c* 1.64, methanol).

Found: C, 62.10; H, 5.76; N, 9.68. Calcd for $C_{15}H_{16}O_4N_2$: C, 62.49; H, 5.59; N, 9.72%.

N^{i}-Formyltryptophans are nonfluorescent under UV lamp and negative to the modified Ehrlich reagent.

Paper Electrophoresis of a Solution of Tryptophan Dissolved in 0.1—1N Hydrogen Chloride in Formic Acid. As a typical experiment, 30 mg of tryptophan was dissolved in 5 ml of 0.4N hydrogen chloride in formic acid and the solution was allowed to stand at room temperature. At suitable intervals aliquotes of the solution were taken and subjected to paper electrophoresis. No band other than those of tryptophan and N^{i}-formyltryptophan has been detected even after 100 hr. The ratio of the latter to the former increased with time. Under the lower or higher concentration of hydrogen chloride than that of the example, transformation of tryptophan into N^{i}-formyltryptophan took place in slower or faster rate. It should be noted that the mobilities of tryptophan and N^{i}-formyltryptophan under the condition mentioned above are 15 and 12 cm, respectively.

Stability of N^{i}-Formyltryptophans. a) Anhydrous Triethylamine: N^{α}-Acetyl-N^{i}-formyltryptophan methyl ester (10 mg) was dissolved in 1 ml of dimethylformamide containing

16) R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959).

17) C. B. Anfinsen, D. Ontjes, M. Ohno, L. Corley, and A. Eastlake, *Proc. Nat. Acad. Sci. U.S.A.*, **58**, 1806 (1967).

18) A. Neuberger and F. Sanger, *Biochem. J.*, **37**, 515 (1943).

19) E. E. Schallenberg and M. Calvin, *J. Amer. Chem. Soc.*, **77**, 2779 (1955).

20) M. Bergmann and L. Zervas, *Chem. Ber.*, **65**, 1192 (1932).

0.14 ml of anhydrous triethylamine. At suitable intervals aliquotes of the solution were taken and evaporated *in vacuo* to dryness. The residues were dissolved in ethanol and their UV spectra were recorded. No change has been observed in the starting spectrum even after standing for 24 hr.

b) Anhydrous Hydrofluoric Acid: In the reaction vessel of the type described by Sakakibara *et al.*,[11] N^{i}-formyltryptophan hydrochloride (10 mg) was treated with anhydrous hydrofluoric acid (2 ml) in the presence of anisole (0.1 ml) at 0°C for 1 hr.[11,12] After removal of anhydrous hydrofluoric acid *in vacuo* at room temperature, the residue was taken into 10% acetic acid. An aqueous solution was shaken with ethyl ether and lyophilized. The dried powder exhibited the typical N^{i}-formyltryptophan spectrum. On paper electrophoresis, mobility of a ninhydrin-positive but modified Ehrlich reagent-negative band was the same as that of the authentic N^{i}-formyltryptophan hydrochloride.

Hydrazinolytic Removal of Formyl Group from N^{i}-Formyltryptophans. N^{α}-Acetyl-N^{i}-formyltryptophan methyl ester (10 mg) was dissolved in 0.5 ml of dimethylformamide containing 0.05 ml of hydrazine hydrate and the solution was allowed to stand at room temperature. At suitable intervals aliquotes of the solution were diluted with ethanol for recording spectra. The complete indole spectrum was obtained after 48 hr.

Boc-Leucyl Resin. To 4.0 g of chloromethylated copolystylene-divinylbenzene (2%) resin (1.3 mmol of Cl/g), were added 34 ml of anhydrous ethanol containing 1.38 g (5.5 mmol) of Boc-leucine and 0.77 ml (5.5 mmol) of triethylamine. The mixture was refluxed for 72 hr with continuous stirring. The resin was filtered, rinsed repeatedly with ethanol and with methylene chloride, and dried *in vacuo*. Amino acid analysis of the resin indicated the leucine content to be approximately 0.20 mmol/g.

Boc-Lys(ε-Z)-Ala-Gly-Leu-Gly-Trp-Leu-resins (**1a**, **1b**, and **1c**).* Boc-leucyl resin (0.5 g) was placed in a reaction vessel of the type described by Merrifield for repeated deblocking, rinsing and coupling procedure. For the synthesis of **1a**, the following cycle was used for stepwise addition of the appropriate Boc-amino acid. The resin was rinsed with formic acid (three 6 ml portions), shaken for 30 min in 0.1N hydrogen chloride in formic acid (6 ml), rinsed with formic acid (three 6 ml portions), with anhydrous ethanol (three 6 ml portions) and with dimethylformamide (three 6 ml portions), shaken for 10 min in 10% triethylamine in dimethylformamide (two 6 ml portions), rinsed with dimethylformamide (three 6 ml portions) and with methylene chloride (three 6 ml portions). The resin was then suspended in 6 ml of methylene chloride containing a 4-fold molar excess of the appropriate Boc-amino acid and shaken for 15 min. Equimolar *N,N'*-dicyclohexylcarbodiimide was added in 1 ml of methylene chloride and shaking was continued overnight. The coupling steps were terminated by rinsing with methylene chloride (three 7 ml portions) and with anhydrous ethanol (three 6 ml portions). For the syntheses of **1b** and and **1c**, formic acid and 0.1N hydrogen chloride in formic acid were replaced by acetic acid and 1N hydrogen chloride in acetic acid, and EtSH (2% by volume) was added to the acid mixture in the case of **1b**.

Boc-Lys(ε-Z)-Ala-Gly-Leu-Gly-Trp-Leu-$NHNH_2$ (**2a**, **2b**, and **2c**).* To each suspension of **1a**, **1b**, and **1c** (0.40 g each) in 3.5 ml of dimethylformamide, were added 0.4 ml of 100% hydrazine hydrate and the mixture were shaken for 2 days at room temperature. The dimethyformamide solutions were separated by filtration and the resins were rinsed several times with dimethylformamide. The combined filtrates were evaporated *in vacuo* nearly to dryness. The hydrazides solidified upon the addition of water and were filtered and washed with water. The hydrazides were dissolved in methanol and the insoluble substances were filtered off. The filtrates were evaporated to a small volume and a mixture of ethyl ether and petroleum ether (1:2 v/v) was added. The crystalline products were filtered off: **2a**, 74 mg (86% yield of **2b**), mp 198—200°C, $[\alpha]_D^{20}$ −28° (*c* 0.165, methanol), $R_f(1)$ 0.91; **2b**, 86 mg, mp 189—191°C, $[\alpha]_D^{20}$ −22° (*c* 0.158, methanol), $R_f(1)$ 0.91;[21] and **2c**, 78 mg, mp 181—184°C, $[\alpha]_D^{20}$ −13° (*c* 0.205, methanol), $R_f(1)$ 0.91.[21] Amino acid analysis of **2a**: Lys, 0.97; Ala, 1.00; Gly, 1.85; Leu, 2.06. Amino acid analysis of **2b**: Lys, 1.00; Ala, 1.00; Gly, 2.09; Leu, 2.20. Amino acid analysis of **2c**: Lys, 1.12; Ala, 1.00; Gly, 2.14; Leu, 2.25. The UV absorption spectra were taken in the methanolic solutions (see Fig. 2). Elemental analysis was carried out only on **2a**.

Found: C, 57.98; H, 7.52; N, 15.13. Calcd for $C_{49}H_{73}N_{11}O_{11}\cdot H_2O$: C, 58.23; H, 7.29; N, 15.26%.

H-Lys-Ala-Gly-Leu-Gly-Trp-Leu-OH (**3a** and **3b**).* **1a** (0.2 g) was treated with 4—5 ml of anhydrous hydrofluoric acid for 1 hr at 0°C in the presence of anisole. After removal of anhydrous hydrofluoric acid *in vacuo*, the peptide cleaved was extracted with two 5 ml portions of 10% acetic acid. The aqueous solution was shaken with ethyl ether and lyophilized to afford 35 mg of a white powder. The UV spectrum of the product in 0.1N sodium hydroxide was virtually identical with that of tryptophan in the same solvent except for somewhat greater absorption in the 240—250 nm region. The above white powder was dissolved in 0.1N aqueous piperidine, and the solution was allowed to stand at room temperature for 10 min and lyophilized, to give **3a** as a white residue. **1b** (0.2 g) was treated by the same way as for **1a** except for treatment with 0.1N piperidine, to afford 49 mg of **3b** as a light brown powder. Its UV spectrum was similar to that of **2b** in Fig. 2. Amino acid analysis of **3a**: Lys, 0.98; Ala, 1.00; Gly, 1.90; Leu, 2.10. Amino acid analysis of **3b**: Lys, 0.96; Ala, 1.00; Gly, 2.10; Leu, 1.92.

Reaction of H-Lys-Ala-Gly-Leu-Gly-Trp-Leu-OH (**3a** and **3b**) with 2-Nitro-4-carboxyphenylsulfenyl Chloride (NCPS-Cl) and Sephadex G-25 Column Chromatographies of the Modified Peptides.* To a solution of 6 mg of **3a** in 2 ml of 80% formic acid, was added 16 mg of NCPS-Cl and the solution was stirred for 4 hr at room temperature and lyophilized. The dried residue was scratched with five 2 ml portions of acetone containing 2% concentrated hydrochloric acid in order to remove the excess reagent. The yellow peptide was separated by centrifugation from the acetone solution and dissolved in water, and the solution was lyophilized. The dried peptide was dissolved in 0.2 ml of 10% acetic acid and chromatographed on a Sephadex G-25 column (0.9×60 cm) equilibrated with the same solvent (Fig. 3). The elution was monitored by absorption at 280 nm. The spectra of the major peak fractions were exactly the same as that of 2-thio-(2-nitro-4-carboxyphenyl)-tryptophan. The unmodified peptide (**3a**) was chromatographed on the same column and its elution pattern was compared with that of the modified peptide (Fig. 3). The modified peptide recovered from the major peak fractions gave a single band on thin-layer chromatography, $R_f(2)$ 0.45, and on paper electrophoresis (mobility, 12.5 cm) and its amino acid analysis gave the ratio: Lys, 1.00; Ala, 1.00; Gly, 1.92; Leu, 1.98. **3b** (6 mg) was treated with 16 mg of NCPS-Cl and the modified peptide was chromatographed by the same manner as described above, and its elution diagram was compared with that of **3b** (Fig. 3).

21) When 47% hydrobromic acid was sprayed to cleave the Boc-group, the spot colored brown.