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Improved Solid-Phase Synthesis of Tryptophan-Containing Peptides. II. Use of N^{α}-*t*-Butyloxycarbonyl-N^{i}-formyltryptophan[1]

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N^{i}-Formyltryptophan has proved to be more resistant than tryptophan against oxidation mediated by hydrogen chloride in acetic acid. N^{α}-*t*-Butyloxycarbonyl-N^{i}-formyltryptophan has been synthesized *via* nonaqueous *acylation* reaction and used for the solid-phase synthesis of tryptophan-containing peptides, in combination with hydrogen chloride in formic acid as the reagent for cleavage of the *t*-butyloxycarbonyl group. The validity of the method has been demonstrated, on the basis of several criteria, in the synthesis of the tryptophan-containing octapeptide, Lys-Gly-Val-Leu-Ala-Gly-Trp-Leu.

In solid-phase synthesis of tryptophan-containing peptide, repeated deprotection of the Boc[2] group with hydrogen chloride in acetic acid brings about more or less oxidative destruction of tryptophan even in the presence of 2-mercaptoethanol as a scavenger.[3] Because of such well-known acid lability of tryptophan, it has been of special value to develop a new reagent for cleavage of the Boc group which would not produce the undesirable oxidation and to find out an acid-stable tryptophan derivative suitable for peptide synthesis. Hydrogen chloride in formic acid has recently been introduced as a reagent for cleavage of the Boc group in the solid-phase synthesis of the tryptophan-containing heptapeptide. Its effectiveness has been demonstrated on the basis of relevant chemical and physical properties of the resulting heptapeptide and its derivatives.[3] The merit of the reagent is ascribed to its greater ability to prevent oxidation of tryptophan owing to the reductive nature of formic acid.

Tryptophan undergoes formylation at its indole nitrogen in hydrogen chloride–formic acid as described by Previero *et al.*[4] and in our previous reports.[3] This formyl group is removed easily in a weakly alkaline medium or by dissolving formylated derivatives in dimethylformamide containing hydrazine hydrate without any serious influence on other amino acids.[3] It seemed of interest to examine whether N^{i}-formyltryptophan is more resistant than tryptophan against oxidation mediated by acids, since it would satisfy the prerequisite that the group introduced for stabilization of indole nucleus should be removed under mild conditions without affecting other residues. Thus, the acid-stability of N^{i}-formyltryptophan was tested using 1 M hydrogen chloride in acetic acid and compared with tryptophan as a reference.

Changes of N^{i}-formyltryptophan[3] and tryptophan in 1 M hydrogen chloride in acetic acid were followed

1) A part of this paper was presented at the 10th Symposium on Peptide Chemistry, Sapporo, September 26 and 27, 1972.

2) All amino acids in this report are of the L-configuration. Abbreviations used are: Boc, *t*-butyloxycarbonyl; Z, benzyloxycarbonyl; Trp, trytophan; Trp(CHO), N^{i}-formyltryptophan; NCPS-Cl, 2-nitro-4-carboxyphenylsulfenyl chloride; Trp(NCPS), 2-thio-(2-nitro-4-carboxyphenyl)-tryptophan; βME, 2-mercaptoethanol; HCl, hydrogen chloride; AcOH, acetic acid; Et_3N, triethylamine; DMF, dimethylformamide; EtOH, ethanol; HF, anhydrous hydrofluoric acid.

3) M. Ohno, S. Tsukamoto, and N. Izumiya, *Chem. Commun.*, **1972**, 663; M. Ohno, S. Tsukamoto, S. Makisumi, and N. Izumiya, This Bulletin, **45**, 2852 (1972).

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

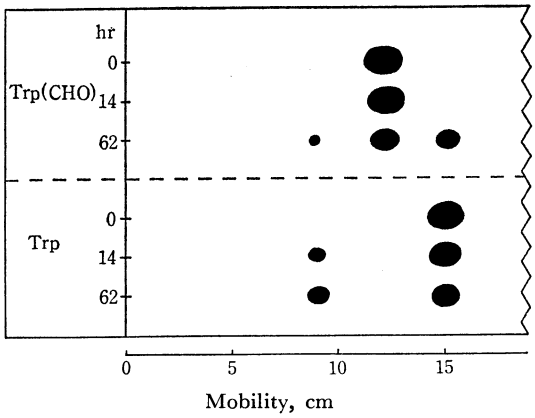

Fig. 1. Electrophoreograms of the solutions of N^i-formyltryptophan and tryptophan in 1 M hydrogen chloride in acetic acid after 14 and 62 hr of standing. Fresh solutions of these amino acids in 0.1 M hydrochloric acid were used as references. The spots were developed with ninhydrin. The substance at 9 cm from the original line was Ehrlich-negative and is noted as "unidentified substance" in the text.

by paper electrophoresis and UV absorption spectra at appropriate time intervals. In Fig. 1 are shown electrophoreograms for the two solutions after 14 and 62 hr of standing. After 14 hr, an Ehrlich-negative, less mobile unidentified substance was detected for the test solution of tryptophan in an appreciable amount. On the other hand, N^i-formyltryptophan remained unchanged. After 62 hr of standing, however, about one-half of N^i-formyltryptophan had been deformylated to produce tryptophan and the unidentified substance was also present although in a very small quantity. At that time the amount of the unidentified substance in the test solution of tryptophan had increased markedly. These phenomena indicate that N^i-formyltryptophan in 1 M hydrogen chloride in acetic acid is first deformylated and tryptophan thus formed is successively oxidized to produce the unidentified substance. It should be noted that there was a long time lag for the conversion of N^i-formyltryptophan into tryptophan. It is concluded that N^i-formyltryptophan is more resistant than tryptophan against oxidation mediated by the acid at least within a limited period. Thus, use of N^i-formyltryptophan has been undertaken for the solid-phase synthesis of tryptophan peptide.

t-Butyloxycarbonylation of α-amino group of N^i-formyltryptophan with Boc-chloride[5] or Boc-azide[6] in an aqueous alkaline medium (pH 9.0) was unsuccessfull because of alkali lability and insolubility at a near-neutral pH of N^i-formyltryptophan. *Acylation*, therefore, was carried out in nonaqueous, triethylamine-buffered dimethylformamide. The method gave an yield as high as 89% for N^α-Boc-N^i-formyltryptophan. This type of nonaqueous *t*-butyloxycarbonylation might be widely applicable for hydrophobic amino acid with suitable solubility in dimethylformamide.

5) S. Sakakibara, I. Honda, K. Takada, M. Miyoshi, T. Ohnishi, and K. Okumura, This Bulletin, **42**, 809 (1969).

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

This paper describes the use of N^α-Boc-N^i-formyltryptophan and hydrogen chloride in formic acid for the Merrifield type solid-phase synthesis of tryptophan peptide. The method has been compared with two other methods: one making use of Boc-tryptophan and hydrogen chloride in formic acid, and the other Boc-tryptophan and hydrogen chloride in acetic acid containing βME. The latter method is usually applied to the solid-phase synthesis of tryptophan peptide.[7] Arbitrarily sequenced octapeptide consisting of amino acids which do not absorb near-ultraviolet light except for tryptophan, Lys–Gly–Val–Leu–Ala–Gly–Trp–Leu, was selected as a model peptide to test the methods.

The three octapeptides were built up as usual, in parallel, starting from Boc-leucyl resin and using either a 6—7 fold molar excess of hydrogen chloride (0.4 M) in formic acid or a 15-fold molar excess of hydrogen chloride (1 M) in acetic acid containing 2% βME. ε-Amino group of lysine was protected with the Z group. At every neutralization step, chlorides were determined on the combined dimethylformamide washings. As seen in Fig. 2, levels of chloride titrated for hydrogen chloride–formic acid systems were 83—84% of that for hydrogen chloride–acetic acid system. Such phenomena have always been observed in all other comparable experiments although the reason has not yet been clarified.

The larger halves of the three protected octapeptide resins [**1a** (*via* Boc–Trp(CHO)—HCl–HCOOH), **1b** (*via* Boc–Trp—HCl–HCOOH), and **1c** (*via* Boc–Trp—HCl–AcOH–βME)] were cleaved by hydrazine hydrate in dimethylformamide.[3,8] In order to remove non-peptide substances derived from the resin, the three products for Boc–Lys(Z)–Gly–Val–Leu–Ala–Gly–Trp*–Leu–$NHNH_2$[9] [**2a** (*via* Boc–Trp(CHO)—HCl–HC-

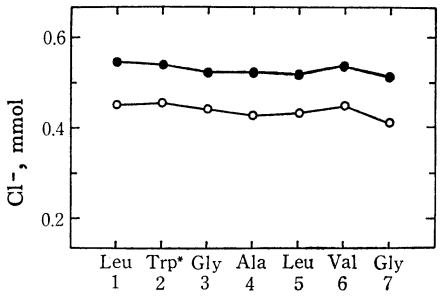

Fig. 2. Millimoles of chlorides determined at neutralization steps in the solid-phase syntheses using HCl–HCOOH (-○-) and HCl–AcOH (-●-). Values presented are those for 1.00 g each of Boc-leucyl resin used. Two runs using HCl–HCOOH gave the same titration values. Details are described in the 'Experimental" section.

7) G. R. Marshall, "Pharmacology of Hormonal Polypeptides and Proteins," ed. by N. Back, R. Paoletti, and L. Martini, Plenum Press, New York, N. Y., (1968); J. Blake and C. H. Li, *J. Amer. Chem. Soc.*, **90**, 5882 (1968); H. Matsuo, A. Arimura, R. M. G. Nair, and A. V. Schally, *Biochem. Biophys. Res. Commun.*, **45**, 822 (1971).

8) 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).

9) Asterisk signifies that Trp may or may not denote pure tryptophan. In some cases the denotation is extended to derivatives.

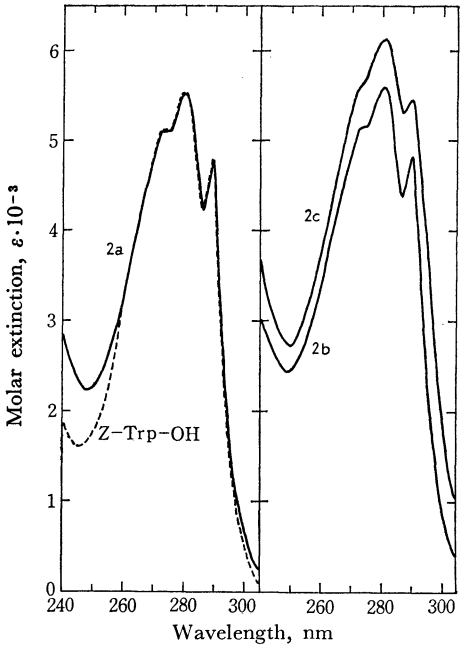

Fig. 3. Ultraviolet absorption spectra of the protected octapeptide hydrazides, **2a**, **2b**, and **2c**, and Z-Trp in methanol.

OOH), **2b** (*via* Boc-Trp—HCl-HCOOH), and **2c** (*via* Boc-Trp—HCl-AcOH-βME)] were reprecipitated (see Experimental). Yields were in parallel with levels of chloride titrated in the neutralization steps. The UV absorption spectra of **2a** and **2b** were 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—3 nm (Fig. 3). Such phenomena suggest that **2a** and **2b** are contaminated to only a minor extent by oxidized species, but it seems likely that an increase of absorption in the 240—250 nm region owes to some extent to the additional peptide absorption as discussed by Beaven and Holiday.[10] On the other hand, extinction of **2c** at 280 nm, for example, was about 20% greater than those of **2a** and **2b**, reflecting oxidative destruction of indole chromophore to a considerable extent.

Small portions of **1a**, **1b**, and **1c** were cleaved with anhydrous hydrofluoric acid in the presence of anisole,[11] to produce **3a**, **3b**, and **4c**, respectively. The formyl group is still attached more or less to tryptophan residues of **3a** and **3b** since it is resistant to anhydrous hydrofluoric acid as well as anhydrous triethylamine in dimethylformamide in the neutralization step. Hydrogen chloride-acetic acid never acetylates tryptophan. **3a** and **3b** were chromatographed on a Sephadex G-25 column (Fig. 4): the peptides recovered from the major peaks are denoted by **3a**† and **3b**†, respectively. **3a**† gave a spectrum typical of N^i-formyltryptophan,[12] indicating that a N^i-formyltryptophan residue is kept intact. The spectrum of **3b**† showed that about one-half of tryptophan involved is formylated. **3a** and **3b** were then treated with 0.1 M aqueous triethylamine for removing the formyl group to produce completely deprotected peptides, **4a** and **4b**.

Figure 5 shows gel-chromatographic patterns of **4a**, **4b**, and **4c** on a Sephadex G-25 column: the peptides from the major peaks for **4a** and **4b** are denoted by **4a**† and **4b**†, respectively. In contrast to **4a** and **4b**, **4c** gave a complex profile owing to its heterogeneity. The peptide from peak II (denoted by **4c**-II) exhibited

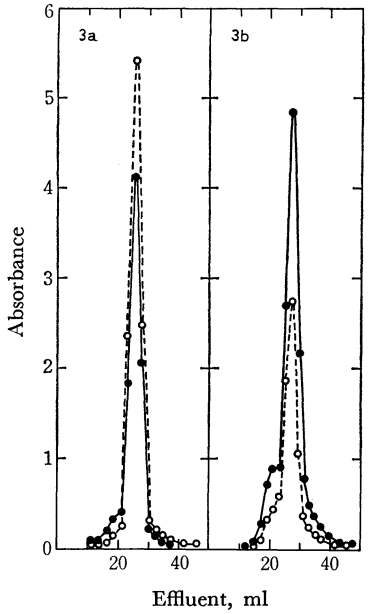

Fig. 4. Chromatographies on a Sephadex G-25 column (0.9×61 cm) of the octapeptides, **3a** and **3b** (7 mg each), which were obtained by cleavages of the peptide resins, **1a** and **1b**, with HF. Elutions were conducted with 5% AcOH and monitored by absorptions at 280 (-●-) and 300 (···○···) nm.

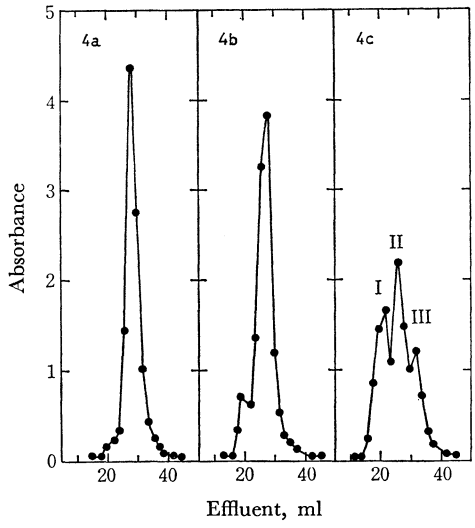

Fig. 5. Chromatographies on a Sephadex G-25 column (0.9×61 cm) of the completely deblocked peptides, **4a**, **4b**, and **4c** (5 mg each). Elutions were conducted with 5% AcOH and monitored by absorption at 280 nm.

10) G. G. Beaven and E. R. Holiday, *Advan. Protein Chem.*, **7**, 319 (1952).

11) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okuda, and H. Sugihara, This Bulletin, **40**, 2164 (1967); J. Lenard and A. B. Robinson, *J. Amer. Chem. Soc.*, **89**, 181 (1967).

12) Spectra of N^i-formyltryptophans have been presented in a previous paper.[3]

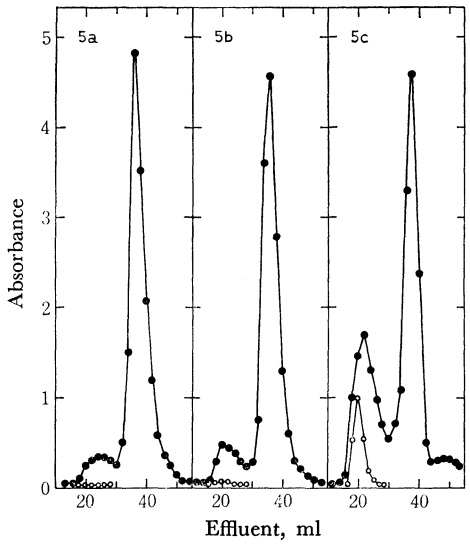

Fig. 6. Chromatographies on a Sephadex G-25 column (0.9×61 cm) of the peptides, **5a**, **5b**, and **5c**, which were obtained by reactions of **4a**, **4b**, and **4c** (7 mg each) with NCPS-Cl. Elutions were conducted with 5% AcOH and monitored by absorptions at 280 (-●-) and 585 (-○-) nm.

a similar spectrum to that given by **4a**† and **4b**† but was contaminated by a minor component of less mobility on paper electrophoresis. **4c**-I and **4c**-III consisted of peptides involving oxidized tryptophan. For further inspection of purity, **4a**, **4b**, and **4c** were treated with NCPS-Cl[13,14] as described[3] and the modified peptides (denoted by **5a**, **5b**, and **5c**, respectively) were examined chromatographically and spectrally. During the modification, the reaction mixture for **4c** became dark blue, giving an absorption maximum at 585 nm. The mixture for **4b** was yellow slightly tinged with blue but that for **4a** was yellow. If only intact tryptophan is involved, the reaction mixture should be yellow. It is extremely likely that oxidized tryptophans form blue substances upon reaction with NCPS-Cl. Chromatography of the modified peptides was carried out on the same column as for the unmodified ones, elutions being monitored by absorptions at 280 and 585 nm (Fig. 6). The peptides from the major peaks for **5a** and **5b**, denoted by **5a**† and **5b**†, were pure yellow and showed an increased adsorptivity on the gel compared with **4a**† and **4b**† (Figs. 5 and 6).[15] Such shifts suggest that **4a**† and **4b**† involve only pure tryptophan peptide. This was confirmed by the fact that **5a**† and **5b**† exhibited the same spectra as 2-thio-(2-nitro-4-carboxyphenyl)-tryptophan[14] (Table 1) and gave a single band by paper chromatography. Although **5c** gave a major peak at almost the same elution volume as for **5a**† and **5b**†, the peptide from the major peak for **5c**, denoted by **5c**†, was brownish yellow and

13) A. J. Havlik and N. Kharasch, *J. Amer. Chem. Soc.*, **77**, 1150 (1955).

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

15) The peptide containing Trp(NCPS) showed an increased adsorptivity on the gel compared with the corresponding tryptophan peptide.

Table 1. Spectral characteristics of **5a**†, **5b**†, and **5c**†

Substance	λ_{max} (nm)			$A_{282}/A_{350-356}$
Trp(NCPS)	260(s)[a]	282	355	4.80
5a†	260(s)	282	356	4.94
5b†	260(s)	282	356	4.94
5c†	257(s)	282.5	350	4.02

a) (s), shoulder.

contaminated by two additional minor impurities. The spectrum of **5c**† was rather different from that of 2-thio-(2-nitro-4-carboxyphenyl)-tryptophan.

Octapeptide samples were hydrolyzed in the presence of 3% thioglycolic acid[16] and the contents of tryptophan were analyzed on amino acid analyzer. Yields of tryptophan from the protected peptide hydrazides **2a**, **2b**, and **2c** were distinctly lower than those of the corresponding unprotected peptides, *e.g.*, **3a**, **3b**, and **4c**. The peptides **2c** and **4c** synthesized by use of HCl-AcOH-βME gave about 20—30% lower yields of tryptophan compared with the peptides prepared by use of HCl-HCOOH. Yield of tryptophan for **3b** was lower to some degree than that for **3a**, indicating that **3b** involves somewhat more oxidized peptide. As seen in the elution diagrams for **3a** and **3b** as well as **4a** and **4b** (Figs. 4 and 5), the oxidized peptides were eluted in part of shoulder prior to the major peak and shoulders for **3a** and **4a** were smaller than those for **3b** and **4b**, respectively. Considering these observations together with differences in physical properties between **2a** and **2b**, the peptides *via* Boc-Trp(CHO)—HCl-HCOOH are superior in quality to those *via* Boc-Trp—HCl-HCOOH. However, when both the crude unprotected peptides were purified on a Sephadex G-25 column, the resulting purified ones showed the same behaviors in terms of electrophoresis, chromatography, and chemical modification by NCPS-Cl and gave the same yields for tryptophan on amino acid analysis. The results demonstrate usefulness of hydrogen chloride in formic acid for the solid-phase synthesis of tryptophan peptide, and show pre-introduction of the formyl group to indole nucleus to be so effective as regards the protection of tryptophan from oxidation under solid-phase synthesis conditions. N^{α}-Boc-N^{i}-Formyltryptophan will be a useful derivative for synthesis of longer, complex tryptophan-containing peptides which may resist purification.

Experimental

General experimental and analytical procedures used were those described in the previous paper.[3] Thin-layer chromatography was performed using methanol-ethyl acetate (3 : 1 v/v) (solvent 1) and paper chromatography *n*-butanol-acetic acid-water (4 : 1 : 5 v/v, upper layer) (solvent 2). Paper electrophoresis was carried out under the following conditions: paper, Whatman 3 MM (57 cm length); solvent, 0.24 M formic acid (pH 2.08); voltage gradient, 48 V/cm; period, 90 min. All peptides in the present experiments moved towards the cathode. Amino acids and peptides were detected by a spray of ninhydrin (0.2% solution in 80%

16) H. Matsubara and R. H. Sasaki, *Biochem. Biophys. Res. Commun.*, **35**, 175 (1969).

ethanol). Modified Ehrlich reagent (0.5% solution of *p*-dimethylaminocinnamaldehyde in 0.5 M hydrochloric acid) was also used for the detection of tryptophan and its peptides. For amino acid analysis, peptides were hydrolyzed with constant boiling hydrochloric acid containing 3% thioglycolic acid in an evacuated, sealed tube at 110 °C for 24 hr. Formic acid (analytical grade, 98—100%, Merck) was used without purification. Acetic acid was refluxed over potassium permanganate, dehydrated with boron triacetate and distilled.[17] Concentration of hydrogen chloride in formic acid or acetic acid was determined by the modified Volhard method.[18]

Stability Test of N^{i}*-Formyltryptophan and Tryptophan in 1 M Hydrogen Chloride in Acetic Acid.* Trp(CHO)·HCl[3] and tryptophan (40 mg each) were respectively dissolved in 5 ml of 1 M HCl in AcOH and the solutions were allowed to stand at room temperature. After 14 and 62 hr of standing, aliquots of the solutions were subjected to paper electrophoresis, and 0.5 ml portions were taken and dried by lyophilization. The residues were dissolved in water and UV spectra were taken. After 14 hr no spectral change was observed for the test solution of tryptophan, when compared with untreated tryptophan, except for somewhat greater absorption in the 240—250 nm region, but an Ehrlich-negative, less mobile unidentified substance was detected in an appreciable amount on paper electrophoresis. On the other hand, Trp(CHO) was unchanged on paper electrophoresis although the test solution became tinged with yellow and the fine structure of the spectrum in the 280—290 nm region was only slightly disturbed. After 62 hr of standing, however, about one-half of Trp (CHO) had been deformylated to produce tryptophan and the unidentified substance was also present although in a minute quantity. Amount of the unidentified substance in the test solution for tryptophan had increased markedly but the spectrum was virtually the same as before.

N^{α}*-Boc-*N^{i}*-Formyltryptophan.* To a solution of 2.68 g (10 mmol) of Trp(CHO)·HCl in 60 ml of DMF was added 4.2 ml (30 mmol) of Et_3N followed by 3.0 ml of Boc-azide. The mixture was stirred at room temperature for 2 days. Gelatinous stuff appeared at the beginning but gradually dissolved with the progress of reaction except for Et_3N·HCl. The mixture was poured into 300 ml of 0.5 M citric acid and an oil deposited was extracted with 150 ml of ethyl acetate. The ethyl acetate layer was washed four times with water, dried over anhydrous sodium sulfate and evaporated below 40 °C nearly to dryness. The syrup was crystallized by adding petroleum ether and by scratching. Crystals were filtered and washed with petroleum ether, 2.98 g (89%); mp 102—104 °C; $[\alpha]_D^{21}$ +22.3° (*c* 1.61, EtOH); λ_{max} (EtOH) 242 (log ε 4.23), 293 (log ε 3.67), 301 nm (log ε 3.67).

Found: C, 61.24; H, 6.26; N, 8.35. Calcd for $C_{17}H_{20}O_5N_2$: C, 61.43; H, 6.07; N, 8.43%.

Boc-Leucyl Resin. Chloromethylated copolystylene-divinylbenzene (2%) resin (1.3 mmol Cl/g) obtained from the Protein Research Foundation, Osaka, was esterified with Boc-Leu.[3] Amino acid analysis indicated the leucine content to be 0.56 mmol/g.

Boc-Lys(Z)-Gly-Val-Leu-Ala-Gly-Trp-Leu-resins (**1a**, **1b**, and **1c**).* Boc-Leucyl resin (1.00 g each) was placed in three reaction vessels of the type described by Merrifield for repeated deblocking, rinsing and coupling procedures. The procedures used for the stepwise addition of the appropriate Boc-amino acids were almost the same as described[3] except that either a 6—7 fold molar excess of HCl (0.4 M) in HCOOH (8 ml) or a 15-fold molar excess of HCl (1 M) in AcOH (8 ml) was used for the cleavage of the Boc group. About 8—9 ml portions of the appropriate solvents were used for rinsing and coupling procedures. βME (2% by volume) was added to HCl-AcOH and also to the AcOH washes after deprotection. The Boc group on valine amino group was not completely cleaved by 30 min shaking in 0.4 M HCl in formic acid. The acid treatment was repeated twice for all runs. Yields: **1a**, 1.42; **1b**, 1.44; and **1c**, 1.48 g.

At every neutralization step, Et_3N-DMF for neutralization and DMF washings were pooled, chlorides being determined by the modified Volhard method (Fig. 2).

Boc-Lys(Z)-Gly-Val-Leu-Ala-Gly-Trp-Leu-*$NHNH_2$ *(**2a**, **2b**, and **2c**).* To suspensions of **1a**, **1b**, and **1c** (0.80 g each) in 3 ml of DMF was added 1.0 ml each of 100% hydrazine hydrate and the mixtures were shaken for 3 days at room temperature. The DMF solutions were separated by filtration and the resins were rinsed several times with DMF. The combined filtrates were evaporated *in vacuo* nearly to dryness. The hydrazides quickly solidified upon addition of water and were filtered and washed with water. The crystalline products were dissolved in hot methanol and insoluble materials were filtered off. The filtrates were evaporated *in vacuo* nearly to dryness and the residues were dissolved in a small volume of DMF. To these solutions were added larger volumes of water and the crystalline precipitates were filtered: **2a**, 159 mg (76% of **2c**), mp 235—236 °C, $[\alpha]_D^{21}$ −28.7° (*c* 0.223, DMF), $R_f(1)$ 0.90; **2b**, 169 mg (81% of **2c**), mp 233—234 °C, $[\alpha]_D^{21}$ −27.7° (*c* 0.235, DMF), $R_f(1)$ 0.89; and **2c**, 209 mg, mp 217—219 °C, $[\alpha]_D^{21}$ −25.7° (*c* 0.296. DMF), $R_f(1)$ 0.89.[19] UV Spectra were taken in the methanolic solutions (Fig. 3), A_{281}/A_{248}[20] for **2a**, 2.42; A_{281}/A_{249} for **2b**, 2.24; and A_{282}/A_{250} for **2c**, 2.09. Amino acid analysis data are presented as 1.00 for lysine. **2a**: Lys 1.00, Trp 0.75, Gly 1.92, Ala 0.95, Val 0.97, Leu 1.92. **2b**: Lys 1.00, Trp 0.72, Gly 1.99, Ala 0.98, Val 0.94, Leu 1.91. **2c**: Lys 1.00, Trp 0.45, Gly 2.12, Ala 1.05, Val 1.08, Leu 1.95.

Found **2a**: C, 58.39; H, 7.65; N, 15.13. **2b**: C, 58.63; H, 7.76; N, 15.15. **2c**: C, 58.12; H, 7.63; N, 14.96. Calcd for $C_{54}H_{82}N_{12}O_{12}\cdot H_2O$: C, 58.46; H, 7.63; N, 15.15%.

Lys-Gly-Val-Leu-Ala-Gly-Trp(CHO)-Leu (**3a** and **3b**) and Lys-Gly-Val-Leu-Ala-Gly-Trp*-Leu (**4a**, **4b**, and **4c**).* **1a**, **1b**, and **1c** (0.140 g each) were respectively treated with 3—4 ml of HF for 1 hr at 0 °C in the presence of anisole (0.2 ml). After removal of HF *in vacuo*, the peptides released were extracted with four 3 ml portions of 20% AcOH. The aqueous solutions were shaken with ethyl ether and lyophilized. Yields: **3a**, 30 mg; **3b**, 32 mg; and **4c**, 39 mg. Amino acid analyses of the samples gave the following ratios. **3a**: Lys 1.00, Trp 0.91,[21] Gly 2.05, Ala 1.03, Val 1.00, Leu 1.95. **3b**: Lys 1.00, Trp 0.80, Gly 2.07, Ala 1.04, Val 0.97, Leu 1.97. **4c**: Lys 1.00, Trp 0.58, Gly 2.01, Ala 1.12, Val 1.07, Leu 2.12. **3a** and **3b** (7 mg each) were chromatographed on a Sephadex G-25 column (0.9×61 cm) using 5% AcOH (Fig. 4). Fractions of the major peaks were lyophilized to give **3a**† and **3b**†. Their spectra were taken in 5% AcOH and compared with those of Trp(CHO) and the mixtures of

17) L. F. Fieser, "Experiments in Organic Chemistry," 3rd Ed., D. C. Heath and Co., New York (1955), p. 281.

18) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis," W. H. Freeman and Co., San Francisco (1969), p. 55.

19) When 47% hydrobromic acid was sprayed to cleave the Boc group, a spot turned purple.

20) *A* signifies absorbance at the indicated wave lengths. Such ratios are presented as a sort of measure for the extent of oxidation for tryptophan.

21) The formyl group of Trp(CHO) was spontaneously removed by heating in constant boiling hydrochloric acid at 110 °C with concomitant formation of tryptophan. Thioglycolic acid produced no side reaction.

Trp(CHO) and tryptophan in varying ratios. Amino acid analyses gave the following ratios. **3a**†: Lys 1.00, Trp 0.87. **3b**†: Lys 1.00, Trp 0.86. **3a** and **3b** (15 mg each) were dissolved in 1.5 ml of 0.1 M aqueous Et_3N and the solutions were allowed to stand at 0 °C for 10 min. Lyophilization of the solutions gave **4a** and **4b** as white fluffy powders.

*Chromatographies of the Octapeptides (**4a**, **4b**, and **4c**) on a Sephadex G-25 Column.* **4a**, and **4b**, and **4c** (5 mg each) were chromatographed on a Sephadex G-25 column (0.9× 61 cm) using 5% AcOH. The elutions were monitored by absorption at 280 nm (Fig. 5). Fractions of the major peaks for **4a** and **4b** were lyophilized to give **4a**† and **4b**†. Fractions of three peaks for **4c** were lyophilized. The resulting peptides were subjected to paper electrophoresis and their spectra were taken in 5% AcOH. Mobilities on paper electrophoresis (cm): **4a**†, 13.2; **4b**†, 13.2; **4c**-I, 14.5 (broad); **4c**-II, 13.2 (major) and 24.2 (minor); and **4c**-III, 11.7 (broad). Absorbance ratios: A_{280}/A_{247} for **4a**†, 2.17; and A_{280}/A_{247} for **4b**†, 2.20; A_{282}/A_{254} for **4c**-I, 1.45; A_{280}/A_{249} for **4c**-II, 2.05; and A_{280}/A_{251} for **4c**-III, 1.98. R_f's (2): **4a**†, 0.69; **4b**†, 0.69; **4c**-I, 0.53; **4c**-II, 0.69 (major) and 0.13 (minor); and **4c**-III, 0.75. Amino acid analyses gave the following ratios. **4a**†: Lys 1.00, Trp 0.88. **4b**†: Lys 1.00, Trp 0.86.

*Reactions of the Octapeptides (**4a**, **4b**, and **4c**) with NCPS-Cl and Chromatographies of the Modified Peptides (**5a**, **5b**, and **5c**) on a Sephadex G-25 Column.* To solutions of **4a**, **4b**, and and **4c** (7.0 mg each) in 1.2 ml of 80% HCOOH were added 19.5 mg each of NCPS-Cl and the solutions were stirred for 5 hr at room temperature and lyophilized. Color changes observed during the course of reactions have been described in the text. The dried residues were scratched with five 2 ml portions of acetone containing 2% concentrated hydrochloric acid in order to remove the excess reagent. The peptides were separated by centrifugation from the acetone solutions and finally dissolved in water, and the solutions were lyophilized. The dried peptides were dissolved in 0.6 ml of 5% AcOH and chromatographed on a Sephadex G-25 column (0.9×61 cm). The elutions were monitored by absorptions at 280 and 585 nm (Fig. 6). Fractions of the major peaks for **5a**, **5b**, and **5c** were lyophilized to give **5a**†, **5b**†, and **5c**†, respectively. Their spectra were taken in 5% AcOH and compared with that of Trp(NCPS) (Table 1). **5a**† and **5b**† (both pure yellow) gave a single band on paper chromatography, R_f (2) 0.80. On paper electrophoresis they did not move from the original line owing to extreme adhesiveness to the paper. **5c**† (brownish yellow) was shown on paper electrophoresis to contain two impure peptides in addition to the yellow peptide identical with **5a**† and **5b**†. Major and minor impurities moved 0.5 and 7.7 cm, respectively.