Further Studies of the Chemical Cleavage of the Tryptophyl Bond by Ozonization-hydrazine Treatment[1,2]

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The second step (hydrazine treatment) in the chemical cleavage of the tryptophyl bond according to the scheme proposed previously was further studied under mild conditions using a model *N'*-formylkynurenyl peptide, carbobenzoxy-L-alanyl-*N'*-formyl-L-kynurenyl-L-leucine. The reaction of the model compound with an excess of hydrazine acetate at 25—30°C for 24—48 hr gave rise to the selective cleavage of the kynurenyl bond together with a concomitant tetrahydropyridazone formation. The maximum cleavage was 80—90%. It was then suggested that the incomplete cleavage of the tryptophyl bond in synthetic oligopeptides by means of ozonization-hydrazine treatment was mainly due to insufficient conversion of the indole nucleus into *N'*-formylanthranyl grouping in the first step (ozonization).

A new principle for the chemical cleavage of the tryptophyl bond has previously been proposed.[3,4] According to the reaction scheme, the tryptophyl residue in the peptide chain is first oxidized selectively by ozone to the *N'*-formylkynurenyl residue, which serves the γ-carbonyl function: a subsequent reaction with hydrazine results in 2,3,4,5-tetrahydropyridazone formation, with a concomitant cleavage of the peptide bond acylated by the tryptophyl residue (Fig. 1). On the basis of the experiments performed on model compounds, the cleavage reaction of several synthetic tryptophyl peptide derivatives was carried out, and fairly good results were obtained.[1] However, on the reaction of the ozonized hen's egg-white lysozyme with hydrazine under the same conditions, the expected cleavage reaction was accompanied by a considerable non-selective hydrolysis of other peptide bonds.[5]

Sin e the oxidation step is free from such hydrolytic cleavage, it was quite obvious that the reaction temperature in the second step with hydrazine was too high to exclude undesirable hydrolysis. A similar disadvantage is expected in other chemical reactions reported in the literature aiming at effecting the cleavage of a kynurenyl bond.[6-8] For instance, the intramolecular participation of the γ-carbonyl or the γ-hydroxy group in the preferential hydrolysis of the peptide linkage involving the kynurenine is effectively accomplished only on heating at 100°C in a weakly alkaline or a diluted hydrochloric acid solution. Thus, it is desirable to establish milder conditions for the selective cleavage of the kynurenyl bond with hydrazine. In the present report, the authors will deal

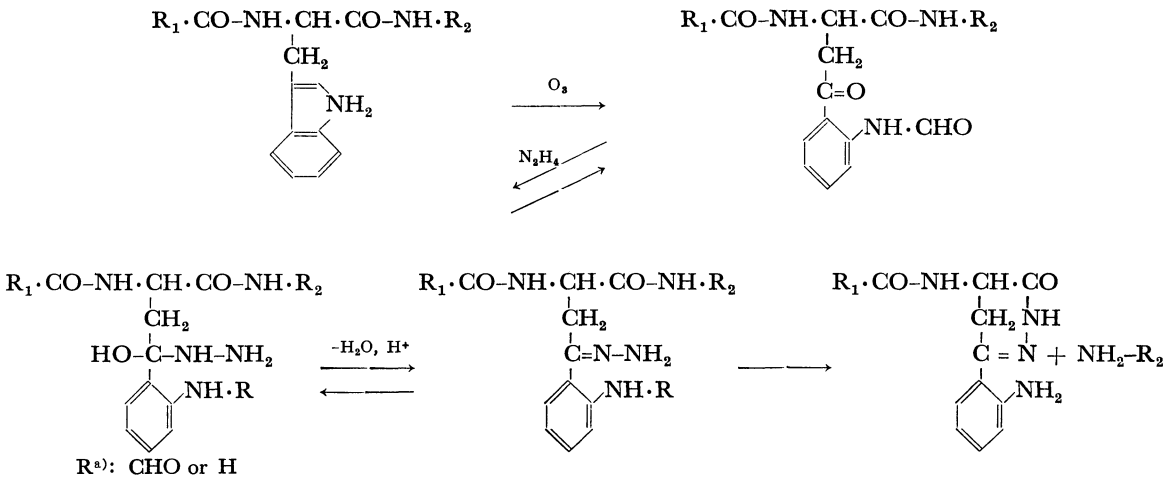

Fig. 1. The chemical cleavage of the tryptophyl bond *via* the *N'*-formylkynurenyl peptide derivative.
a) The stage at which the formyl group is eliminated has not been clarified yet in the direct reaction of the model peptide with hydrazine acetate.

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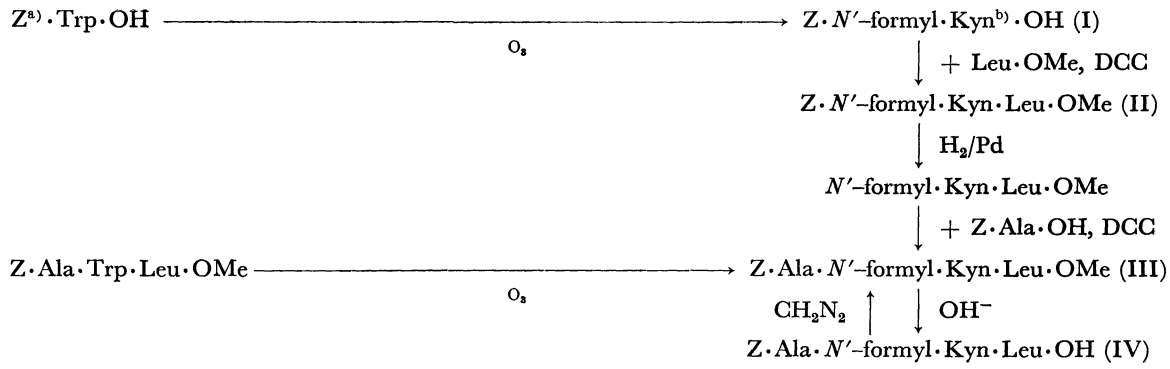

Fig. 2. Synthesis of carbobenzoxy-L-alanyl-*N'*-formyl-L-kynurenyl-L-leucine.
a) Z: carbobenzoxy b) Kyn: kynurenine

with the preparation of a model peptide derivative containing *N'*-formylkynurenine and its reaction with hydrazine, and with the application of the improved method to the cleavage of the tryptophyl bond in several oligopeptides and gramicidin A.

Results and Discussion

Synthesis of a Model Peptide Derivative. The synthesis of peptide derivatives containing *N'*-formylkynurenine has previously been reported by Previero and his colleagues,[9] who utilized the ozonolysis of the corresponding tryptophyl peptide derivatives in the presence of resorcinol.[10] As a model peptide derivative, the carbobenzoxy derivative of alanyl-*N'*-formylkynurenylleucine was chosen. Two routes of synthesizing the tripeptide derivative were examined; one was a step-by-step elongation of the peptide chain, starting from the carboxyl-terminal leucine, while the other was a direct ozonization of carbobenzoxyalanyltryptophylleucine or its methyl ester (Fig. 2).

For the step-by-step procedure, it was necessary to prepare the *N*-α-protected derivative of *N'*-formylkynurenine. Since a conventional *t*-butyloxycarbonyl substituent was not suitable for the present purpose,[11] the classical carbobenzoxy residue was used. The *N*-α-carbobenzoxy derivative of tryptophan was ozonized without resorcinol; in order to isolate crystalline carbobenzoxy-*N'*-formylkynurenine, the crude material had to be purified by chromatography on a silicic-acid column. The formation of the kynurenyl bond was then attempted either by the *p*-nitrophenyl-ester method or by the dicyclohexylcarbodiimide (DCC) method.[12] The conversion of carbobenzoxy-*N'*-formylkynurenine into its *p*-nitrophenyl-ester was performed by means of interesterification with trifluoracetyl *p*-nitrophenolate;[13] though the active ester thus obtained was subjected to reaction with the leucine methyl ester, no fruitful results were obtained. Then, the carbobenzoxy-*N'*-formylkynurenylleucine methyl ester was prepared by the DCC method without any considerable difficulties. The formation of the alanyl bond was similarly achieved by the coupling of carbobenzoxy alanine with the *N'*-formylkynurenylleucine methyl ester which had been prepared by the catalytic hydrogenation of its carbobenzoxy derivative. By a comparison of the melting points and by a study of the infrared spectrum and the chromatographic behavior, the carbobenzoxy tripeptide ester was found to be identical with that prepared by the direct ozonization of the carbobenzoxyalanyltryptophylleucine methyl ester.[14]

The protected tripeptide ester was then submitted to usual hydrolysis in aqueous alkaline media. However, the ester group was not quantitatively hydrolyzed, and the ultraviolet spectrum of the reaction mixture revealed an anomalous absorption at 332 mμ, indicating that a certain unexpected reaction occurred during this treatment.[15] On the other hand, the alkaline hydrolysis in an aqueous pyridine solution reduced such an unfavorable side reaction; finally, the repeated recrystallization of the hydrolysate yielded the pure carbobenzoxytripeptide, which reverted to the parental tripeptide methyl ester on esterification by diazomethane.

The direct ozonization of the carbobenzoxy derivative of alanyltryptophylleucine into the corresponding *N'*-formylkynurenyl derivative was a less favorable procedure for the present purpose because it was difficult to purify the ozonization product, as is also observed in the preparation of carbobenzoxy *N'*-formylkynurenine.

Reaction of Carbobenzoxy-L-Alanyl-N'-Formyl-L-Kynurenyl-L-Leucine with Hydrazine. The results of the experiments on the cleavage of the tryptophyl bond in lysozyme under the conditions reported in a previous paper[1] suggested that one of the major defects to be improved was the non-specific hydrolysis of the peptide bonds during the reaction of the ozonized protein with hydrazine. From this point of view, the cleavage reaction was again investigated at room temperature using

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10) A. Previero, A. Signor, and S. Bezzi, *Nature*, **204**, 687, (1964); A. Previero and E. Bordignon, *Gazz. Chim. Ital.*, **94**· 630 (1964).
11) The *t*-butyloxycarbonyl group could be removed by the action of formic acid in which the tryptophan residue was usually ozonized. B. Halpern and D. E. Nitecki, *Tetrahedron Lett.*, **1967**, 3031.
12) J. C. Sheehan and G. P. Hess, *J. Amer. Chem. Soc.*, **77**, 1067 (1955).
13) S. Sakakibara and N. Inukai, This Bulletin, **38**, 1979 (1965).
14) For preparation of the methyl ester of the model peptide, the ozonization method of the corresponding tryptophyl peptide derivative was more useful than the stepwise method involving the protected *N'*-formylkynurenine.
15) As one of possible side reactions, a heteroring formation such as 4-quinolone might be suggested from the spectroscopic data, but no further study was carried out.

the model peptide derivative. However, on the reaction of carbobenzoxy-L-alanyl-*N'*-formyl-L-kynurenyl-L-leucine in an aqueous hydrazine-acetate buffer at pH 4.0, neither leucine nor alanine was released, even after 72 hr. On the other hand, on the incubation of the above reaction mixture at 100°C for 3.5 hr, leucine was detected with an apparent increase in the absorption around 350 mμ due to the formation of the expected tetrahydropyridazone derivative.

When the reaction scheme presented in Fig. 1 is considered, it is rational to assume that, if the hydrazone is once formed, the intramolecular condensation of the amino group in it with the adjacent α-carbonyl function takes place and that the cleavage of the peptide linkage concerned is accomplished by a concomitant heterocyclic ring formation. Therefore, the formation of the hydrazone of the (*N'*-formyl-) kynurenine must be a key reaction in the present cleavage of the peptide bond. Since the hydrazone is formed by an equilibrium reaction through an intermediate tetrahedral hydroxyhydrazino (carbinolamine) derivative (Fig. 1), the higher the hydrazine concentration, the more efficient the formation of the hydrazone. In addition, it must be noted that the hydrazone formation consists of a successive reaction, addition of hydrazine, and subsequent elimination of water, which is usually catalyzed with either an acid or a base. Therefore, the reaction of the tripeptide derivative with 80% hydrazine hydrate was attempted in order to convert quantitatively its carbonyl function into hydrazone. In fact, a marked decrease in the characteristic absorption at 322 mμ due to the *N'*-formylkynurenyl residue was observed within five minutes of mixing the *N'*-formylkynurenyl peptide derivative and hydrazine base at room temperature. This fact indicates that the addition of hydrazine to the carbon-oxygen double bond in the kynurenyl residue takes place effectively.

When the hydrazine-treated tripeptide derivative was incubated in aqueous media at pH 4.0 at room temperature after the excess of hydrazine hydrate had been removed, leucine could be liberated in a 30% yield after 72 hr. During this reaction, white precipitates were separated and identified as 4-carbobenzoxyalanylamino-6-(*o*-aminophenyl)-2,3,4,5-tetrahydropyridaz-3-one. This fact shows that, in an aqueous solution, the hydroxyhydrazino (carbinolamine) intermediate was dehydrated to the hydrazone, and that the intramolecular hydrazinolysis of the peptide bond subsequently took place on the carboxyl side of the kynurenyl residue.

The release of leucine from the hydrazine-treated model peptide derivative under various conditions was determined; the results are summarized in Table 1. In the reaction in aqueous media, the extent of the cleavage was estimated to be 30—45%. When dioxane was added in the reaction media, more leucine was released and the course of the cleavage could also be followed by means of ultraviolet spectroscopy. On the other hand, when the reaction solvent was replaced by glacial acetic acid, the leucine released was similar in extent with that released in aqueous media.[16] Even after treatment with hydrazine hydrate, the cleavage reaction with a large excess of hydrazine was more effective than that without the cleaving reagent. This finding suggests that, under those conditions, the retroreactions of the hydroxyhydrazino (carbinolamine) derivative and the hydrazone by equilibrium are prevented, and that enough hydrazone for the effective cleavage of the kynurenyl bond is always produced during the reaction.

Table 1. Release of leucine by the reaction of carbobenzoxy-L-alanyl-*N'*-formyl-L-kynurenyl-L-leucine with hydrazine after pretreatment with the binucleophile

Concentration of the model peptide derivative (mM)	Concentration of hydrazine (M)	Reaction: Media	Reaction: Temp.	Reaction: Period (hr)	Yield of leucine released (%)	Yield of tetrahydropyridazone formed (%)
2.00[a]	0	aq. soln (pH. 3.8)	r.t.	72	30	—
2.00[a]	0	aq. soln (pH. 3.8)	r.t. then 100°C	72 3	47	—
2.00[a]	0.02	aq. soln (pH. 1.8)	r.t.	30	36	—
1.78[a]	0.05	aq. soln (pH. 3.6)	40°C	5 25 48	40 45 44	— — —
1.34[b,c]	0	aq. soln (pH. 3.65) - dioxane (4:1 by vol.)	r.t.	43 120	— 71	51 53
1.26[b,d]	0	gl. acetic acid	r.t.	120	51	—
1.38[b]	1.6	gl. acetic acid	r.t.	47 120	— 77	79 81
1.15[b,e]	1.6	gl. acetic acid	r.t.	47 120	— 55	45 56

a,b) The pre-treatmet with 80% hydrazine hydrate was carried out at room temperature for 2 min (a) or for half an hour (b).
c) The residual material after evaporation of hydrazine and water was allowed to stand overnight at room temperature.
d) Distinct absorption maximum at 353 mμ was not obtained, but a shoulder was observed.
e) After treatment with hydrazine, removal of the excess reagent was omitted.

The cleavage reaction was subsequently simplified into a single procedure because the hydrazone formation and subsequent cleavage of the peptide bond could then be achieved under the same circumstances as in the present case. When the model peptide derivative was subjected to a reaction with a large excess of hydrazine in glacial acetic acid at room temperature, the absorption at 322 mμ was gradually decreased and a new absorption at 353 mμ emerged. The ninhydrin assay of the reaction mixture showed the liberation of the amino acid, which increased parallel with the increase in the absorption at 353 mμ (Fig. 3). As is shown in Table 2, the extent of the cleavage reaction for 48—50 hr at room temperature was estimated to be 73—92% on the basis of the absorbance of the tetrahydropyridazone derivative formed. By amino acid analysis,

16) Incubation of the hydrazine-treated tripeptide derivative in glacial acetic acid brought about liberation of leucine without the heteroring formation (see Table 1). This liberation of the amino acid may be due to a different mechanism from the present scheme.

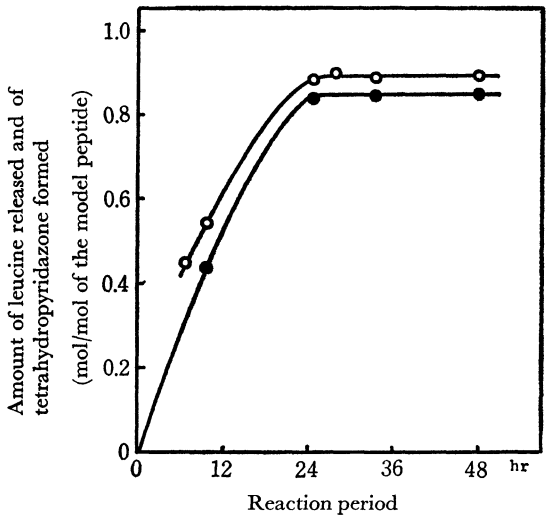

Fig. 3. Relationship between the tetrahydropyridazone formation (—○—) and liberation of leucine (—●—) by the reaction of the model *N'*-formyl-kynurenyl peptide (IV, 1.32 mM) with hydrazine (0.66M) in glacial acetic acid at room temperature.

TABLE 2. RELEASE OF LEUCINE BY THE DIRECT REACTION OF CARBOBENZOXY-L-ALANYL-*N'*-FORMYL-L-KYNURENYL-L-LEUCINE WITH HYDRAZINE ACETATE IN GLACIAL ACETIC ACID[a]

Concentration of		Yield of	
the model peptide derivative(mM)	hydrazine (M)	leucine released	tetrahydropyridazone derivative
6.18	5.0	—	74
2.41	2.0	—	91
1.60	1.6	81(81)	91(84)
1.19–1.39	0.5–1.2	78–87	82–90
0.59	1.6	78(80)	92(86)
0.57	0.5	—	84
0.27	1.6	113[b](86)	73(67)
0.12	0.1	—	no maximum

a) The formation of the tetrahydropyridazone derivative was estimated after 48—50 hr at room temperature and analysis of the amino acid was done after 120 hr. The yield after 24 hr was presented in parentheses.
b) See b) in Table 4.

it was also confirmed that leucine was the sole amino acid liberated during the cleavage reaction for 120 hr and that about 78—87% of the theoretical amount of the amino acid was present.[17] A study of the ultraviolet spectrum showed another reaction product to be identical with the expected 4-L-(*N*-carbobenzoxy-L-alanylamino)-6-(*o*-aminophenyl)-2,3,4,5-tetrahydropyridaz-3-one (Table 3). Table 4 shows the results of the cleavage reaction of the tryptophyl bond by ozonization and subsequent hydrazine treatment under the same conditions. The over-all reaction yielded the corresponding amino acid in a 30—50% yield on the basis of the parent peptide, but the second step directly causing the cleavage of the peptide chain could be

17) Removal of the β-benzoylpropionyl grouping as a substituent in the nucleotide synthesis was carried out with 0.5 M hydrazine in pyridine-water (R. L. Letsinger and P. S. Miller, *J. Amer. Chem. Soc.*, **91**, 3356 (1969)).

TABLE 3. SPECTRAL IDENTIFICATION OF THE TETRAHYDROPYRIDAZONE DERIVATIVE OBTAINED IN THE REACTION OF Z-ALA-*N'*-FORMYLKYN-LEU-OH WITH HYDRAZINE.

	Ratio of the absorbance against that at 283 mμ		
	250 mμ	283 mμ	353 mμ
4-L-(*N*-carbobenzoxy-L-alanylamino)-6-(*o*-aminophenyl)-2,3,4,5-tetrahydropyridaz-3-one[a]	1.50	1.0	0.76
A sample obtained by base treatment and subsequent reaction in aq. media[b]	1.46	1.0	0.67
A sample obtained by direct cleavage reaction in glacial acetic acid[c]	1.44	1.0	0.71

a) Part II, p. 530 in Ref. 1).
b) Compound V in Experimental part.
c) The sample was isolated on reaction of carbobenzoxy-L-alanyl-*N'*-formyl-L-kynurenyl-L-leucine (1.91 mM) with hydrazine acetate (1.2 M) in glacial acetic acid at room temperature for 48 hr.

TABLE 4. THE CHEMICAL CLEAVAGE OF THE TRYPTOPHYL BOND IN SEVERAL SYNTHETIC PEPTIDES AND GRAMICIDIN A[a]

Peptide derivative	Ozonization yield determined as		Yield of the over-all cleavage reaction determined by	
	N'-formyl-Kyn	Kyn	tetrahydropyridazone formation	amino acid analysis
Z·Trp·Gly·OH	47	41	42	34
Z·Trp·Leu·OH	45	51	50	65[b]
Z·Trp·Phe·OH	47	57	56	40
Z·Gly·Trp·Gly·OH	49	39	38	30
Z·Ala·Trp·Leu·OH	48	54	44	56[b]
Gramicidin A	86	43	46	—

a) The data presented were given by duplicate experiments except ozonization of gramicidin.
b) These yields were probably over-estimated according to low resolution between the leucine and a byproduct (possibly acetylhydrazine, eluted just before leucine in analysis on a Mitamura Amino Acid Analyzer (a column used for analysis, 0.9×50 cm, Aminex A-4)). Flow rate of the developing buffer (0.2 N citrate buffer, pH 3.25) was 50 ml/hr.

carried out to an extent similar to that in case of the model compound. From this result it must again be emphasized that, in the cleavage of the tryptophyl bond, the complete conversion of the tryptophan into the (*N'*-formyl-) kynurenine is necessary for the quantitative scission of the peptide linkage.

Propionic or butyric acid can be utilized as a reaction solvent in place of the glacial acetic acid, but formic acid, which is a good solvent for proteins, can not. Moreover, hydrazine acetate only must be used as the cleaving reagent; it is not replaceable by hydrazine dihydrochloride or sulfate.

In conclusion, the cleavage of the kynurenyl bond can be achieved by the aid of hydrazine under such mild conditions that no other peptide bond is affected. The hydrazine treatment can be carried out by two methods: one is the direct reaction of the kynurenyl

residue with hydrazine, while the other involves the preformation of the carbinolamine intermediate by treatment with the binucleophile in basic media. Though the cleavage of the tryptophyl bond in oligopeptides is possible to a similar extent by either procedure, the latter method may also be useful for that of the peptide bonds in proteins.

Experimental

All the melting points were uncorrected. The amino-acid analyses were performed on a Beckman amino acid analyzer model MS unless otherwise specified. The absorption spectra were measured by a Beckman spectrophotometer, type DB. The examination of the synthetic peptide by mass spectrometry was done by means of a Hitachi Mass Spectrograph, type RMU-6D. Ozone was generated from oxygen by a generator manufactured by the Nippon Ozone Co., Ltd., type 0-3-2. The formic acid (98—100%) was purchased from Merck AG and was used without purification.

The Cleavage Reaction of the N'-Formylkynurenyl Bond in the Model Peptide by Hydrazine. A typical procedure for the present reaction will be described below. About 2.8 mg (5 μmol) of carbobenzoxy-L-alanyl-*N'*-formyl-L-kynurenyl-L-leucine was dissolved in 80% or 100% hydrazine hydrate (0.2 m*l*); after this mixture had stood at room temperature for half an hour, the hydrazine and water were evaporated *in vacuo* by the same procedure as was used with lyophilization.[18] The residual substance was then dissolved in glacial acetic acid containing hydrazine acetate (1.6 M) (3 m*l*), and the reaction vessel, equipped with a stopper, was kept at 25—27°C on a water bath equipped with a thermostat. Portions (0.2 m*l*) of the acetic acid solution were withdrawn at suitable intervals and diluted with ethanol (3 m*l*). The ethanol solution was submitted to spectrometry at ranges from 450/400 mμ to 300 mμ or from 400 mμ to 230 mμ, with dilution to a suitable concentration, if necessary. The progress of the cleavage reaction could be followed in terms of the tetrahydropyridazone formation, and the extent of the cleavage reaction was determined from the absorbance at 353 mμ ($\varepsilon_{353m\mu}=8.13\times10^3$).

When the pretreatment by hydrazine was omitted, the model peptide was directly subjected to the reaction with hydrazine acetate in glacial acetic acid.

For the determination of the amino acid released, about 0.1 μmol (a theoretical amount when the reaction occurred quantitatively) was pipetted, diluted immediately with a 0.2 M citrate buffer at pH 2.2, frozen, and kept in a freezer (−20°C) until analysis has been performed by means of an amino-acid analyzer.

Isolation and Characterization of the 4-L-(N-Carbobenzoxy-L-alanylamino)-6-(o-aminophenyl)-2,3,4,5-tetrahydropyridaz-3-one (V) Formed on the Reaction of the Model Peptide with Hydrazine. The carbobenzoxy-L-alanyl-*N'*-formyl-L-kynurenyl-L-leucine (113 mg, 0.2 mmol), dissolved in 80% hydrazine hydrate (2 m*l*), was allowed to stand at room temperature for 5 min; then, the excess hydrazine was evaporated *in vacuo* to dryness over concentrated sulfuric acid (overnight). The residue was dissolved in water (15 m*l*), and the pH of the aqueous solution was adjusted to 3.8 with 4M acetic acid. At this stage, a large amount of an insoluble material was separated, but the suspension, after dilution to 100 m*l* with water, changed to an almost clear solution on standing at room temperature for 2 hr. After 24 hr, white precipitates which had separated were collected; they amounted to 23 mg. Additional precipitates (6 mg) were obtained after 72 hr. The white precipitates were combined and crystallized from methanol-water. Mp 200—204°C (lit,[1] 197.5—198°C). Molecular weight (mass spectrometry), Found: 409. Calcd for $C_{21}H_{23}O_4N_5$: 409. R_f (TLC, SiO_2) 0.69 (ethyl acetate: acetic acid=5:1 by volume). λ_{max}; 353 mμ, 283 mμ and 250 mμ (in ethanol).

Cleavage Reaction of the Tryptophyl Bond in Several Tryptophyl Peptide Derivatives and Gramicidin. The tryptophyl peptide derivative (10 μmol) was dissolved in 98—100% formic acid (2 m*l*) and oxidized by approximately 0.01% ozone at 7—8°C until the increase of the absorption at 320 mμ reached its maximum. An aliquot (0.1 m*l*) was withdrawn, evaporated to dryness *in vacuo* over sodium hydroxide pellets, and used for amino-acid analysis after acid hydrolysis. Another portion (0.5 m*l*) was similarly evaporated and then dissolved in a glacial acetic acid (2 m*l*) containing hydrazine acetate (1.6 M). The acetic acid solution was kept at 25—27°C on a water bath for 48—50 hr, and then the liberated amino acid was determined. The progress of the cleavage was also followed by the spectrometric method, which has been described previously.

Carbobenzoxy-N'-formyl-L-kynurenine (I). Carbobenzoxy-L-tryptophan (1.38 g) was dissolved in a 98—100% formic acid (50 m*l*), and ozone in oxygen was bubbled at 5—10°C into the formic acid solution until a maximal absorption at 320 mμ was obtained. The solvent was then replaced by chloroform, and the insoluble materials were filtered off. The clear chloroform solution was shaken with 1N sodium carbonate, and the aqueous alkaline solution was acidified to pH about 1 after mixing with chloroform. The acidified solution was shaken with chloroform, and the sirupy ozonization product (854 mg) obtained on the evaporation of the chloroform was purified by chromatography on a silicic-acid (20 g) column. On the concentration of the first fraction from the column (300 m*l*, developing with chloroform containing 5—10% methanol), an amorphous substance (669 mg)[19] resulted. The crude carbobenzoxy-*N'*-formyl-L-kynurenine was obtained by repeating extraction and back-extraction between chloroform and aqueous acid or an alkaline solution as has been described above. The pure sample (246 mg, 16%) was obtained by recrystallization from methanol; mp 145—146°C. $[\alpha]_D^{25}=+23.1$ (*c* 0.73, in methanol). R_f (TLC, SiO_2) 0.85 (ethyl acetate: acetic acid=10:1 by volume). Molecular weight (mass spectrometry), Found: 370. Calcd: 370. λ_{max} (ethanol): 320 mμ and 258 mμ. ν(KBr): 3320, 3200, 1748, 1685, 1642, 1598, 1580, 1520, 1442 and 1407 cm^{-1}.

Found: C, 61.46; H, 4.68; N, 7.73%. Calcd for $C_{19}H_{18}O_6N_2$: C, 61.61; H, 4.90; N, 7.56%.

Carbobenzoxy-N'-formyl-L-kynurenyl-L-leucine Methyl Ester (II). Carbobenzoxy-*N'*-formyl-L-kynurenine (222 mg) and L-leucine methyl ester (88 mg) were dissolved in chloroform (2 m*l*), and dicyclohexylcarbodiimide (124 mg) was added to the chloroform solution under cooling with ice water. After 2 hr at 0°C and then 1 hr at 20°C, the insoluble dicyclohexylurea was filtered off. A crystalline material (280 mg) resulted on the evaporation of the chloroform; it was recrystallized from methanol. Yield, 205 mg

18) If evaporation of the aqueous hydrazine was omitted, the cleavage took place less effectively.

19) In another experiment, a crystalline material (mp 177—184°C, browning with gas evolution) was isolated. λ_{max} (methanol) 263 mμ and 325 mμ. Found: C, 58.03: H, 4.16; N, 7.14; ash 8.1%. Since this compound showed identical properties with the *N'*-formylkynurenine derivative after alkali-acid treatment, the crystalline sample was throught to be an adduct with silicic-acid used for chromatography.

(69%); mp 146—147°C (sintered at 139°C). $[\alpha]_D^{25} = -15.2$ (*c* 0.94, in methanol). Molecular weight (mass spectrometry), Found; 497. Calcd; 497.

Found: C, 62.67; H, 6.46; N, 8.35%. Calcd for $C_{26}H_{31}O_7N_3$: C, 62.76; H, 6.28; N, 8.45%.

Carbobenzoxy-L-alanyl-N′-formyl-L-kynurenyl-L-leucine Methyl Ester (III). *i) By Step-by-step Synthesis from the Leucine Methyl Ester*: The carbobenzoxy-*N′*-formyl-L-kynurenyl-L-leucine methyl ester (876 mg) was dissolved in methanol (70 m*l*) containing glacial acetic acid (106 mg) and hydrogenated on palladium-charcoal for 5 hr at room temperature. After the filtration of the catalyst, the clear methanol solution was passed through a Dowex 1 (OH-form) column and the effluent was evaporated to dryness. The residue (676 mg), solidified in crystals, was coupled with carbobenzoxy-L-alanine (394 mg) by dicyclohexylcarbodiimide (363 mg) in chloroform (10 m*l*) for 2 hr under cooling with ice water. After the chloroform solution freed from the resulting urea derivative was washed with diluted alkali, diluted acid, and water successively, the crude tripeptide derivative (863 mg) was obtained on the evaporation of the solvent. The crude product was then purified by silicic-acid chromatography. The fraction (60 m*l*) eluted with 5% methanol in chloroform after the column had been washed with chloroform (300 m*l*) was evaporated. Pure crystals (320 mg, mp 142—144°C) resulted on treatment with methanol. From the mother liquor, an additional product (130 mg, mp 143°C) was isolated by chromatographic purification. Total yield, 450 mg; 45%. The sample for analysis was further purified by recrystallization from methanol; mp 143—144°C (sintered at 140°C). By admixing with the carbobenzoxy-*N′*-formyl-L-kynurenyl-L-leucine methyl ester (II), the melting temperature dropped to 134—144°C. $[\alpha]_D^{18} = +27.1$ (*c* 1.4, in chloroform). λ_{max} (ethanol), 322 mμ. Molecular weight (mass spectrometry), Found, 568. Calcd: 568.

Found: C, 61.47; H, 6.33; N, 9.76%. Calcd for $C_{29}H_{30}O_8N_4$: C, 61.25; H, 6.38; N, 9.85%.

ii) By the Ozonization of the Carbobenzoxy-L-alanyl-L-tryptophyl-L-leucine Methyl Ester. The carbobenzoxy-L-alanyl-L-tryptophyl-L-leucine methyl ester[1] (5.37 g) was dissolved in formic acid (30 m*l*) and oxidized by ozone for 70 min under cooling with ice water. Then the solvent was replaced with chloroform, and the chloroform solution was washed with a diluted sodium carbonate solution. On the evaporation of the solvent, a neutral substance (5.35 g) was obtained; it was similarly chromatographed on a silicic-acid column. From the fraction eluted with chloroform containing 2—3% methanol, the crystalline, protected tripeptide derivative (1.84 g) was obtained by the evaporation of the solvent and by subsequent treatment with methanol. An additional ozonization product was obtained by repeated chromatography of the mother liquor after the first crystalline materials had been collected. The total yield of the crude products (2.22 g, mp 139—141°C) was 39%. The sample for analysis was again recrystallized from methanol; mp 143—144°C (after sintered at 139°C). $[\alpha]_D^{18} = +26.8$ (*c* 1.4, in chloroform). No depression of the melting point of the pure sample was observed after admixing with the sample obtained by the (i) procedure.

Carbobenzoxy-L-alanyl-N′-formyl-L-kynurenyl-L-leucine (IV). The protected tripeptide ester (III) (142 mg) was dissolved in pyridine (3 m*l*), and after 1N sodium hydroxide (0.5 m*l*) had been added to the pyridine solution, the aqueous pyridine solution was stirred for 40 min under cooling with ice water. After the methyl ester (III) was no longer detected (thin layer chromatography), dry ice and water were added successively. The hydrolysate was then shaken twice with chloroform (30 m*l* each), and the chloroform solution was dried with anhydrous sodium sulfate and concentrated under reduced pressure. The resulting crystalline material was washed with ether and recrystallized from methanol-water. Yield, 100 mg (70%). Mp 138—140°C (sintered at 136°C). The ultraviolet spectrum showed a small shoulder at 332 mμ. Recrystallization from the same solvent was repeated. The pure crystals thus prepared showed no absorption at 332 mμ; mp 140°C (foam after sintered at 136°C). $[\alpha]_D^{25} = -29.7$ (*c* 0.77, in methanol). The crystals showed a higher melting point at 159—162°C (dec., sintered at 132°C) after having been dried at 50°C for 5 hr under reduced pressure (10 mmHg) for analysis.

Found: C, 59.40, 59.34; H, 6.20, 6.28; N, 9.98, 9.90%. Calcd for $C_{28}H_{34}O_8N_4 \cdot 1/2H_2O$; C, 59.67; H, 6.26; N, 9.94%. The protected *N′*-formylkynurenyl peptide (IV) (87 mg), dissolved in methanol (1 m*l*), was treated with an excess of diazomethane in an ethereal solution. The methylation was continued for half an hour at room temperature, and the solution was concentrated to a sirup giving a crystalline product by treatment with methanol. The pure esterification product (55 mg, mp 140—142°C) obtained by recrystallization from methanol showed no depression of the melting point after admixture with compound (III).

The infrared absorption spectrum of the esterification product also showed a good agreement with that of compound (III).

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