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The Non-enzymatic Cleavage of Peptide Bonds. I. Deacylation of γ-Oxoacyl Amino Acids and Peptide by Hydrazines[1—3]

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A few γ-oxoacyl amino compounds, such as *N*-levuloyl-L-phenylalanine, *N*-(3-benzoylpropionyl)-L-phenylalanine and -L-leucyl glycine, and *N*-α-*N*-δ-dibenzoylamino-γ-oxo-L-ornithyl-L-phenylalanine, were synthesized by the conventional method for peptide synthesis. The deacylation of the acyl group with hydrazines was studied in an attempt to establish the procedure for the second step in the newly-developed principle for the non-enzymatic cleavage of the *C*-tryptophyl and *C*-histidyl peptide bonds. The quantitative release of the amino group was accomplished by heating the γ-oxoacyl amino acid or peptide with the unsubstituted hydrazine in an acid solution. In this reaction the tetrahydropyridazone derivative was formed. The mechanism of the deacylation reaction was also considered.

In the study of the primary structure of proteins, pancreastic proteolytic enzymes (trypsin and chymotrypsin) have mainly been used to cleave specified peptide bonds for the first, rough fragmentation of the peptide chain. The selective cleavage[4] by a proper chemical reagent is also a useful technique for amino acid residues which can not participate in the specific hydrolysis of the peptide bond by enzymes. One of the most popular methods successfully utilized in the non-enzymatic reaction for such aromatic amino acid residues as tyrosine[5] and tryptophan[6] is NBS[7] oxidation. For the cleavage of the histidyl bond,[8] a similar method has been applied in acid media. However,

1) A part of this investigation was presented at the 19th Annual Meeting of the Chemical Society of Japan held in Tokyo (April, 1966). M. Morishita, F. Sakiyama and K. Narita, Proceedings of the 19th Annual Meeting of the Chemical Society of Japan, No. IV, p. 80 (1966).

2) Preliminary report was presented in this Bulletin. F. Sakiyama, M. Morishita, T. Sowa and K. Narita, This Bulletin, **39**, 631 (1966).

3) C-Histidyl and C-tryptophyl bonds denote the peptide bond at the carboxyl side of these residues in the polypeptide chain.

4) B. Witkop, *Adv. in Protein Chem.*, Vol. 16, p. 221 (1961), Academic Press, New York.

5) a) G. L. Schmir, L. A. Cohen and B. Witkop, *J. Am. Chem. Soc.*, **81**, 2228 (1959); b) E. J. Corey and L. F. Haefele, *ibid.*, **81**, 2225 (1959).

6) A. Patchornik, W. B. Lawson, E. Gross and B. Witkop, *ibid.*, **82**, 5923 (1960).

7) NBS: *N*-Bromosuccinimide

8) S. Shaltiel and A. Patchornik, *J. Am. Chem. Soc.*, **85**, 2799 (1963).

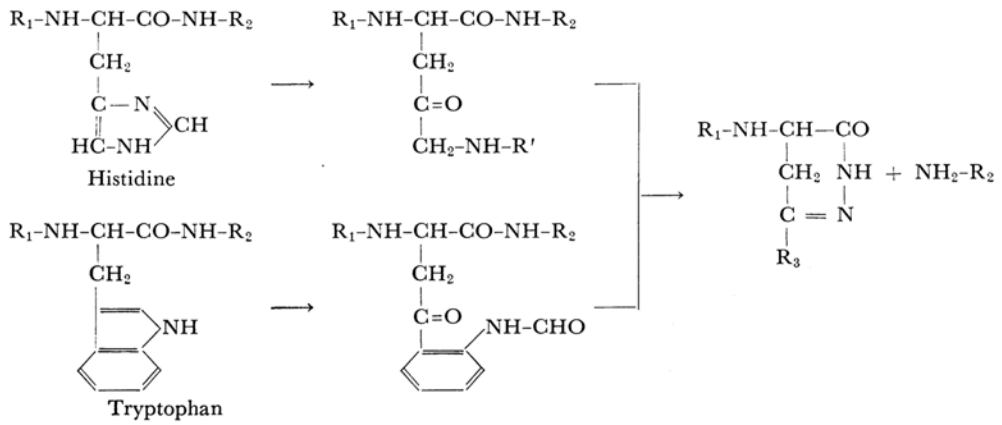

Fig. 1. The reaction scheme for the chemical cleavage of *C*-histidyl and *C*-tryptophyl bonds.

tryptophyl and tyrosyl residues are also affected by NBS, and this procedure can not be used to cleave histidyl bonds while leaving tryptophyl and tyrosyl residues intact.[9]

It is, therefore, desirable to establish an individual principle which is strictly responsible for the cleavage of the peptide bond at a specified amino acid residue. The γ-oxoacyl grouping could then be readily derived from the histidyl residue and the tryptophyl residue by an appropriate reaction. The γ-oxoornithyl residue[10] is the descendant of the histidyl residue after it has been subjected to Bamberger degradation[11,12] and subsequent mild acid hydrolysis. The tryptophyl residue can be converted to the kynurenine residue by ozone oxidation.[13] The second step is the utilization of the carbonyl group for selective cleavage of the adjacent peptide bond. The fact that levulinic acid was readily subjected to reaction with hydrazine to give a heterocyclic ring[14a] prompted us to apply this ring formation to the deacylation of the γ-oxoacyl amino acid (Fig. 1). In the present paper the authors will describe the synthesis of γ-oxoacyl amino derivatives and the reaction of the above compounds with hydrazines.

Results and Discussion

Several oxoacyl amino acids and peptide were synthesized as model compounds for the deacylation study. Though Wolff[15] reported preparation of the amide of levulinic acid by the reaction of angelicalactone with the corresponding amino component, we attempted to synthesize the γ-oxoacyl amino acid and peptide by the usual methods of peptide. The coupling of levulinic acid with L-phenylalanine methyl ester by *N*, *N'*-dicyclohexylcarbodiimide gave a sirupy acylation product, which could be isolated as crystalline dicyclohexylammonium salt of *N*-levuloyl-L-phenylalanine. *N*-(3-Benzoylpropionyl)-L-phenylalanine and -L-leucyl glycine were prepared from 3-benzoylpropionic acid and the respective amino component by a mixed anhydride method using isobutylchloroformate.[16] The condensation of *N*-α-*N*-δ-dibenzoyl-γ-oxo-L-ornithine and the amino reactant by the mixed anhydride method was unsuccessful, but coupling by dicyclohexylcarbodiimide afforded the protected γ-oxo peptide ester. In the synthesis of these γ-oxoacyl amino acids and peptide, the pure reaction products except *N*-(3-benzoylpropionyl)-L-phenylalanine could be obtained by repeated recrystallizations. Though, for the preparation of the γ-oxoacyl amino derivative, the carbodiimide and the mixed anhydride methods have been studied so far, neither is satisfactory. Moreover, the presence of the carbonyl grouping in the side chain prohibited the use of the azide method.[17] The attempted synthesis of *N*-α-*N*-γ-*N*-δ-tribenzoylamino-Δ^{γ}-pentenoyl-L-phenylalanine methyl ester as a precursor of the γ-oxoornithyl derivative by the conventional method for peptide synthesis was unsuccessful.

9) Differentiation of NBS oxidation between tryptophyl and tyrosyl residues could be possible. See, M. Funatsu, N. M. Green and B. Witkop, *ibid.*, **86**, 1846 (1964).

10) a) J. N. Ashly and C. R. Harington, *J. Chem. Soc.*, **1930**, 2586. b) H. Heath, A. Lawson and C. Rimington, *ibid.*, **1951**, 2215. c) B. Witkop and T. Beiler, *J. Am. Chem. Soc.*, **78**, 2882 (1956).

11) E. Bamberger and A. Berle, *Ann.*, **273**, 342 (1893).

12) The imidazole ring in the histidyl peptide could be opened by Bamberger degradation with carbobenzoxychloride and alkali. See F. Sakiyama, M. Morishita and K. Narita, p. 81 in Ref. 1.

13) a) B. Witkop, *Ann.*, **556**, 103 (1944). b) A. Previero, E. Scoffone, P. Pajetta and C. A. Benassi, *Gazz. Chim. Ital.*, **93**, 841 (1963). c) A. Previero, M. A. Colletti and L. Galzigna, *Biochem. Biophys. Res. Comm.*, **16**, 195 (1964). d) A. Previero and E. Bordignon, *Gazz. Chim. Ital.*, **95**, 630 (1965).

14) a) L. Wolff, *Ann.*, **394**, 98 (1912). b) S. Skraup and E. Schwanberger, *ibid.*, **462**, 135 (1928).

15) L. Wolff, *ibid.*, **229**, 256 (1885).

16) a) T. Wieland and H. Bernhard, *ibid.*, **572**, 190 (1951). c) J. R. Vaughan, Jr., *J. Am. Chem. Soc.*, **73**, 3547 (1951).

17) I. Smith, "Chromatographic Techniques," ed. by I. Smith, William Heineman, London (1959), p. 72.

The thin-layer chromatography of *N*-(3-benzoylpropionyl)-L-phenylalanine in chloroform-methanol-glacial acetic acid (95 : 5 : 3 by volume) showed two substances, R_f 0.45 and R_f 0.75, when the Ehrlich reagent was used. Each compound extracted was again examined by thin-layer chromatography in the same solvent. Both spots were still detected from each sample. The infrared spectrum in a chloroform solution showed a strong absorption at 1678 cm^{-1}, but in the solid state the amide I absorption (1658 cm^{-1}) and the carbonyl absorption (1684 cm^{-1}) were clearly observed. Cromwell *et al.*[18] reported that some 3-benzoylpropionic amides, such as methylamide, anilide, benzylamide and cyclohexylamide, existed as tautomeric mixtures of the 3-benzoylpropionylamide and the 5-hydroxy-5-phenylpyrrolidin-2-one in a dioxane solution. Therefore, the two substances observed by the thin-layer chromatography seem to correspond to a tautomeric mixture of the open-chain structure (I) and the cyclic pyrrolidinone (II).

The chemical behavior of the γ-oxoacyl amide has previously been studied.[18-20] Walton[19] reported that 3-benzoylpropionic amide was stable in an alkaline solution, but, in acid, was decomposed to 3-benzoylpropionic acid and the corresponding amine. α-Aminolevulinic acid was decomposed by a base to the γ-oxo-α, β-unsaturated acid, accompanied by a rapid elimination of ammonia, while *N*-glycyl-α-aminolevulinic acid eliminated glycine when warmed (70°C) in 0.1 M sodium carbonate.[21] Recently Previero *et al.*[22] reported a selective cleavage of the *C*-kynurenyl bond in weakly basic media at elevated temperatures. However, the amino acid could not be liberated from *N*-levuloyl-L-phenylalanine by either acid or alkaline treatment at room temperature.[23]

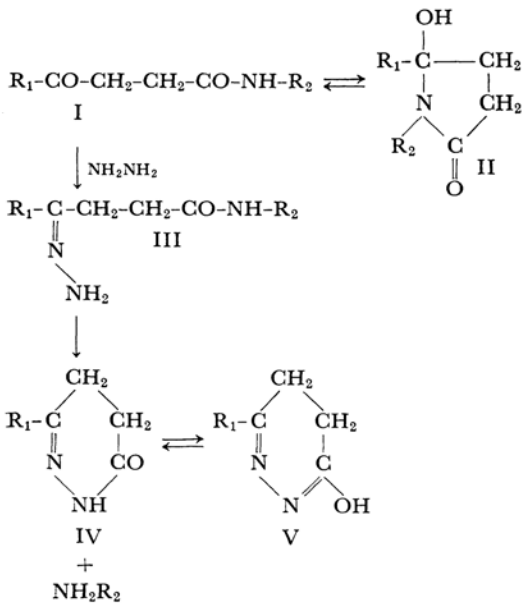

Fig. 2. Removal of the γ-oxoacyl group from the *N*-γ-oxoacylamino compound by hydrazine.

R_1: $-CH_3$, R_2: $-CH(CH_2C_6H_5)COOH$

When dicyclohexylammonium salt of *N*-levuloyl-L-phenylalanine (I) was treated with an excess of hydrazine hydrate at room temperature, the levuloyl derivative changed to a compound which developed a yellowish-orange color with the Ehrlich reagent. At this stage no amino acid was liberated; only the unchanged acyl amino acid was detected in addition to the above compound, which was purified on thin-layer plates.[24] When its aqueous solution, phenylalanine and another product (IV) could be detected on a paper chromatogram (Figs. 2 and 3). The compound IV was isolated and shown to be identical with 6-methyl-2, 3, 4, 5-tetrahydropyridaz-3-one[14a] by infrared spectroscopy. The intermediate compound was eventually regarded as the hydrazone (III) of the levuloyl derivative of L-phenylalanine.

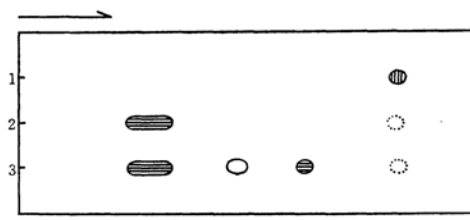

Fig. 3. Paperchromatogram of the reaction product of *N*-levuloyl-L-phenylalanine with hydrazine.

1. *N*-Levuloyl-L-phenylalanine
2. After the reaction with hydrazine at room temperature.
3. After heating of (2) in aqueous solution for 5 hr.

⊜ Orange with the Ehrlich reagent.
◍ Purple with the Ehrlich reagent.
○ Purple with the ninhydrin reagent.

The maximum liberation of phenylalanine was accomplished by heating the solution for 5—6 hr at 100°C (Fig. 4). Even after this reaction period the *N*-valeroyl derivative of L-phenylalanine, in which the carbonyl group is absent, was not hydrolyzed to any large extent. The increase in the

18) N. H. Cromwell and K. E. Cook, *J. Am. Chem. Soc.*, **80**, 4573 (1950).
19) E. Walton, *J. Chem. Soc.*, **1940**, 438.
20) R. Lukes and Z. Linhartova, *Coll. Czech. Chem. Comm.*, **25**, 502 (1960).
21) C. H. Hassel, D. I. John, T. G. Martin and J. A. Schofield, *J. Chem. Soc.*, **1963**, 3100.
22) A. Previero, M. A. Colletti Previero and P. Jolles, *Biochem. Biophys. Res. Comm.*, **22**, 17 (1966).
23) Unpublished data.
24) Silica Gel G. Merck AG. Darmstadt.

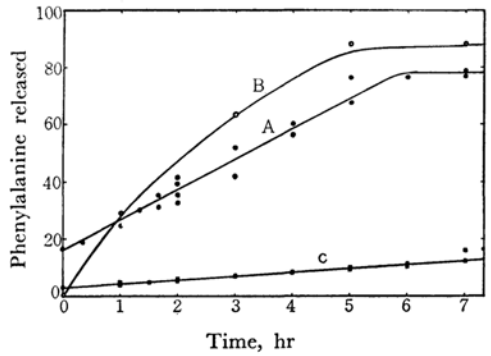

Fig. 4. The release of phenylalanine from *N*-levuloyl-L-phenylalanine.

Curve A: Phenylalanine by the ninhydrin method
Curve B: Phenylalanine by the DNP method
Curve C: Phenylalanine from *N*-valeroyl-phenylalanine (ninhydrin)

acidity of the reaction seemed to favor the hydrazone formation of the carbonyl compound. A qualitative study of the deacylation of the *N*-levuloyl amino acid at various pH's demonstrated that the rate of disappearance of the γ-oxoacyl derivative increased with the decrease in the pH of the reaction, as had been expected. A weakly acid medium (pH 3.6) was chosen for the cyclization reaction. As is shown in Fig. 5, phenylalanine was released when the solution was heated at pH 3.6 for half an hour. Lower temperatures were not effective in causing quantitative deacylation (Fig. 6). The

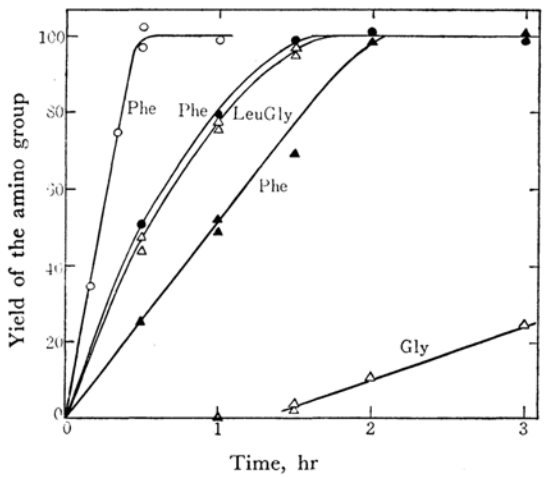

Fig. 5. The release of the amino group from the γ-oxoacyl amino acid and peptide.

○ *N*-Levuloyl-L-phenylalanine
● *N*-(3-Benzoylpropionyl)-L-phenylalanine
△ Leucylglycine and glycine from *N*-(3-benzoylpropionyl)-L-leucylglycine
▲ *N*-(*N*-α-*N*-δ-Dibenzoyl-γ-oxoornithyl)-L-phenylalanine

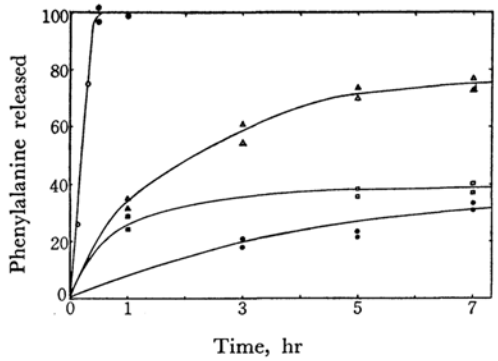

Fig. 6. Effect of temperature on the deacylation of *N*-levuloyl-L-phenylalanine.

○ 100°C
△ 50°C
□ 30°C
● *N*-Valeroyl-L-Phe (100°C)

hydrolysis of the *N*-valeroyl derivative was negligible under the present conditions.

In place of hydrazine, substituted hydrazines such as *N*-methyl, *N*-phenyl and *N*-(2, 4-dinitrophenyl)-hydrazines were also tested in the study of the deacylation of the *N*-levuloyl derivative. Both in the absence and in the presence of acetic acid, these hydrazines were less effective for the liberation of the amino group than were the unsubstituted hydrazine (Fig. 7). For example, the liberation of the amino group from *N*-levuloyl and *N*-(3-benzoylpropionyl)-L-phenylalanine with phenylhydrazine at pH 3.6 was 9% and 6% respectively, when a complete exposure of the amino group by the unsubstituted hydrazine could be accomplished.

Ten molar equivalents of hydrazine to the γ-oxoacyl-derivative was originally used, but a reduction of the reagent (5 molar equivalents) caused

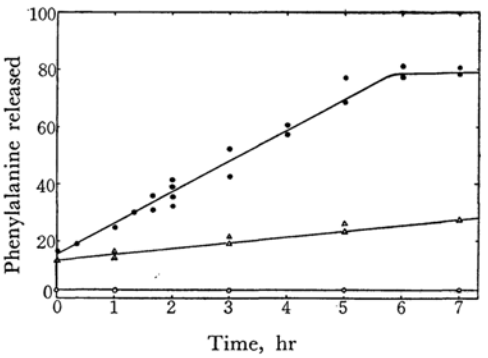

Fig. 7. Comparison of hydrazines as a deacylation reagent of *N*-levuloyl-L-phenylalanine in the absence of acetic acid.

● Hydrazine
△ *N*-Methylhydrazine
○ *N*-(2,4-Dinitrophenyl)- and *N*-phenylhydrazines

no appreciable difference in the rate of the deacylation in the present case. The rate of the release of the amino group from various γ-oxoacyl amino derivatives mainly depended on the nature of the acyl group (Fig. 5). The levuloyl group could be most readily removed, but the introduction of a couple of benzoylamino groupings at both the α- and δ-positions markedly decreased the rate of the deacylation. It required two hours to quantitatively liberate phenylalanine from the *N*-α-*N*-δ-dibenzoylamino-γ-oxo-L-ornithyl derivative. The benzoylpropionyl grouping, used as a model for the descendant of the tryptophan residue, could be removed within 1.5 hr, and the rate was not affected by the kind of amino acid.

In a preliminary study,[25] it was found that the *C*-tryptophyl bond in several dipeptide derivatives could be cleaved by the present procedure in yields of approximately 70% based on the kynurenine residue derived from the *N*-carbobenzoxy amino acid by ozone oxidation.[26]

Hydrazine as a bifunctional nucleophilic reagent was reactive enough to cyclize the γ-oxoacyl amide to the pyridazone derivative; this was accompanied by a liberation of the amino group. However, a high temperature had to be used for quantitative deacylation. In consequence, the partial acid hydrolysis of the peptide bond at the adjacent position of the aspartic acid residue in the polypeptide chain[27] seemed not to be excluded, though the hydrolysis of the *C*-leucyl bond in *N*-(3-benzoylpropionyl)-L-leucyl glycine was negligible under the present conditions (1.5 hr). This type of deacylation from the β-oxoacyl peptide has also been reported,[28] but no details have yet been presented.

Experimental

All melting points are uncorrected. The R_f value is given in the case of the paper chromatography (Toyo No. 51) in the solvent composed of *n*-butanol - water - acetic acid (4 : 1 : 1 by volume), unless otherwise mentioned. TLC denotes thin-layer chromatography on silicic acid (Silica Gel G, Merck AG.).

Release of Phenylalanine from *N*-Levuloyl-L-phenylalanine. Aliquots of an aqueous solution containing 0.5 μmol of *N*-levuloyl-L-phenylalanine were mixed with hydrazine hydrate (5 μmol). The final volume of the mixture was adjusted to 0.3 m*l* with water, and the solution was allowed to stand for three hours at room temperature. After lyophilization and the complete removal of the excess hydrazine *in vacuo* over concentrated sulfuric acid and sodium hydroxide pellets (6 days), the residue was dissolved in 0.25 m*l* of water and heated for the desired time on a boiling water bath. The phenylalanine released was identified on a paper chromatogram and estimated by the ninhydrin colorimetry[29] (curve A in Fig. 4). On the other hand, the phenylalanine was dinitrophenylated and chromatographed on a filter paper in a *t*-amyl alcohol - phthalate buffer (pH 6.0) in order to identify and determine the DNP-phenylalanine (curve B in Fig. 4). As a control experiment, *N*-valeroyl-L-phenylalanine was similarly treated with hydrazine, and the liberation of amino group was estimated (curve C in Fig. 4).

Comparison of the Deacylation Reaction of *N*-Levuloyl-L-phenylalanine with Several Hydrazine Derivatives. A similar procedure was carried out except for the replacement of hydrazine with *N*-methyl, *N*, *N'*-dimethyl, *N*-phenyl, and *N*-*p*-nitrophenyl hydrazine. Benzaldehyde was used for the removal of the excess phenylhydrazines.

Isolation of the Cyclization Product from *N*-Levuloyl-L-phenylalanine and Hydrazine. Dicyclohexylammonium salt of *N*-levuloyl-L-phenylalanine (438 mg) was dissolved in 10 m*l* of water, and then 80% hydrazine hydrate (0.17 m*l*) was added to the solution. Stirring was continued for 4 hr at room temperature, and then the mixture was lyophilized. A white powder (494 mg) was obtained upon the removal of the excess hydrazine. A paper chromatogram of this powder in a *n*-butanol - acetic acid - water mixture (4 : 1 : 1 by volume) showed two spots when the Ehrlich reagent was used; one was the hydrazone of the γ-oxoacyl amino acid, while the other the unchanged acyl amino acid (faint spot). The crude product (390 mg) was dissolved in 5 m*l* of water and heated to the boiling point for 5 hr. After the solvent had then been removed, the residue was treated with 10 m*l* of ethanol and an insoluble precipitate (phenylalanine) was removed by centrifugation. The supernatent was purified by thin-layer chromatography (first solvent, *n*-butanol - acetic acid - water, 4 : 1 : 1 by volume and second solvent, chloroform-methanol, 9 : 1 by volume). The substance (R_f 0.61 for the first development, and R_f 0.59 for the second) was scratched and eluted with ethanol. The ethanol solution was evaporated to dryness, and the small amount of white powder (R_f 0.61, orange with the Ehrlich reagent) thus obtained was submitted to infrared analysis without further purification. The infrared spectrum showed good agreement with that of an authentic sample (6-methyl-2, 3, 4, 5-tetrahydropyridaz-3-one) throughout.

Effect of pH on the Deacylation of *N*-Levuloyl-L-phenylalanine. Aliquots of an aqueous solution of the dicyclohexylammonium salt of *N*-levuloyl-L-phenylalanine were diluted with a variety of acetate buffers (listed in Table 1) to a final concentration of 10 μmol per m*l*. Each solution was then allowed to stand at room temperature, after which the process of deacylation was followed qualitatively by thin-layer chromatography in a *n*-butanol - acetic acid - water mixture (4 : 1 : 1 by volume). The starting material and the pyridazone derivative were detected by the Ehrlich reaction, and the phenylalanine released was stained by the ninhydrin reaction. The deacylation

25) See Ref. 1.
26) Ozone oxidation was performed in the presence of resorcinol. See Ref. 13d.
27) *Cf.* R. L. Hill, *Adv. in Protein Chem.*, **20**, 37 (1965).
28) F. D'Angeli, F. Filia E. Scoffone, *Tetrahedron Letters*, **1965**, 605.
29) E. W. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955).

Table 1. Buffers used for the deacylation reaction of *N*-levuloyl-L-phenylalanine at various pH's

pH	Final concn. of hydrazine, M	Final concn. of acetic acid, M
9.6	5×10^{-2}	0
7.4	5×10^{-2}	0.08
6.4	5×10^{-2}	0.10
5.5	5×10^{-2}	0.12
4.7	5×10^{-2}	0.20
3.6	5×10^{-2}	2.00

reaction at pH 3.6 was the most effective in the present experiment for the rapid release of phenylalanine and the disappearance of the γ-oxoacyl amino acid.

Effect of the Temperature on the Deacylation of *N*-Levuloyl-L-phenylalanine. An aqueous solution (0.25 m*l*) containing 0.5 μmol of *N*-levuloyl-L-phenylalanine and 5 μmol of hydrazine was adjusted to pH 3.6 with diluted acetic acid and then heated for a desired time at 30°C, 50°C or 100°C. After the addition of 2 N sodium bicarbonate and subsequent lyophilization, the residue was dissolved in water and the phenylalanine released was assayed by the ninhydrin method.[29]

Deacylation of γ-Oxoacyl Amino Acids and Peptide under Standardized Conditions. The deacylation of the γ-oxoacyl group from *N*-levuloyl-L-phenylalanine, *N*-(3-benzoylpropionyl)-L-phenylalanine and -L-leucyl glycine, and *N*-α-*N*-δ-dibenzoyl-γ-oxo-L-phenylalanine was performed in the following way. One m*l* of the sample solution was mixed with an equal volume of a 4 M acetic acid - 0.1 M hydrazine buffer (pH 3.6), after which the mixture was heated on a boiling water bath. The course of the deacylation was followed by densitometry[30] after paper chromatography (*n*-butanol - acetic acid - water, 4 : 1 : 1 by volume) and a subsequent Cd-ninhydrin reaction for the determination of phenylalanine and leucyl glycine. The cyclization product obtained from the γ-oxoacyl group was also identified on a paper chromatogram taken with authentic sample, but an attempt to estimate the pyridazone derivative by the Ehrlich reaction on filter paper was unsuccessful because of the instability of the color developed.

Dicyclohexylammonium Salt of *N*-Levuloyl-L-phenylalanine. Into a cold methylene chloride solution containing L-phenylalanine methyl ester prepared from its hydrochloride (6.75 g), levulinic acid (2.65 m*l*) and dicyclohexylcarbodiimide (6.43 g) were added; the reaction mixture was stirred for half an hour at 0°C and then overnight at room temperature. The filtrate, after the removal of dicyclohexylurea (2.1 g), was successively washed with 0.2 N hydrochloric acid, 5% sodium bicarbonate, and water. The oily acyl amino acid ester was obtained by evaporation, but attempts to crystallize it were unsuccessful. The oil was then dissolved in methanol and hydrolyzed with 2 N sodium hydroxide (15.6 m*l*). After the methanol and an insoluble material had been removed, the filtrate was acidified with 2 N hydrochloric acid. When the oily hydrolyzate, obtained by extraction with ethyl acetate and by subsequent concentration, was treated with dicyclohexylamine, white crystals (2.06 g, 47.7%) resulted. Mp 155—156°C. $[\alpha]_D^{20}$ +32.6 (*c* 1, glacial acetic acid). ν(KBr) 3250, 1716, 1660, 1625, 1550 cm^{-1}. R_f 0.81 (purple with the Ehrlich reagent).

Found: C, 70.08; H, 9.11; N, 6.23%. Calcd for $C_{26}H_{40}O_4N_2$: C, 70.23; H, 9.07; N, 6.30%.

***N*-α-*N*-δ-Dibenzoyl-γ-oxo-L-ornithyl-L-phenylalanine Methyl Ester.** *N*-α-*N*-δ-Dibenzoyl-γ-oxo-L-ornithine (1.13 g) was dissolved in tetrahydrofuran, and then into the solution there was added a tetrahydrofuran solution of L-phenylalanine methyl ester (prepared from its hydrochloride (647 mg) and triethylamine (0.6 m*l*)) under stirring and cooling. *N*, *N*'-Dicyclohexylcarbodiimide (0.74 g) in the same solvent was added in portions under cooling in an ice-water bath for half an hour. The reaction mixture was then allowed to stand overnight at room temperature. One drop of glacial acetic acid was added, and the precipitate was filtered off after an hour. The yellow mass obtained on the evaporation of the filtrate was dissolved in ethyl acetate, and the solution was washed successively with 0.1 N hydrochloric acid, 1% sodium bicarbonate, and water. The ethyl acetate solution was then dried over anhydrous sodium sulfate and concentrated under reduced pressure. The dilution of the concentrates with petroleum ether yielded yellow precipitates, which were crystallized from ethyl acetate - petroleum ether. Yield, 1.27—1.50 g (49—58%). Mp 120—155°C. A sample for analysis was carefully recrystallized from ethyl acetate - petroleum ether. Mp 150—154°C. R_f 0.97 (iodine vapor, TLC), ν(Nujol) 3289, 1739, 1715, 1692, 1670, 1642, 1541, 1539 cm^{-1}. On acid hydrolysis (6 N hydrochloric acid at 110°C overnight), both γ-oxo-ornithine and phenylalanine were detected on a paper chromatogram.

Found: C, 67.36; H, 5.67; N, 7.89%. Calcd for $C_{29}H_{29}O_6N_3$: C, 67.56; H, 5.67; N, 8.13%.

***N*-α-*N*-δ-Dibenzoyl-γ-oxo-L-ornithyl-L-phenylalanine.** Into a methanol solution (10 m*l*) of the crude ester of the acyl peptide (1.5 g), N sodium hydroxide (2.5 m*l*) was added; stirring was continued under cooling with ice-water for forty minutes and then for an additional twenty minutes at room temperature. After N hydrochloric acid (2.5 m*l*) had been added, the methanol was evaporated and the oily material resulting from the further addition of N hydrochloric acid (1 m*l*) was extracted with ethyl acetate. The alkaline hydrolyzate was taken into the aqueous sodium bicarbonate solution, and the oil precipitated by acidification was again extracted with ethyl acetate. The ethyl acetate solution was washed with water and dried over anhydrous sodium sulfate. White powder was obtained by scratching from the concentrate of the ethyl acetate solution. Yield, about 100 mg, mp 173—176°C. R_f 0.90 (iodine vapor, TLC) ν(Nujol) 3356, 1747, 1728, 1663, 1639, 1605, 1571, 1533 cm^{-1}.

Found: C, 66.65; H, 5.64; N, 7.88%. Calcd for $C_{28}H_{27}O_6N_3$: C, 67.05; H, 5.43; N, 8.38%.

***N*-(3-Benzoylpropionyl)-L-phenylalanine Methyl Ester.** 3-Benzoylpropionic acid (1.78 g) and triethylamine (1.4 m*l*) were dissolved in 100 m*l* of absolute

30) R. C. Canfield and C. B. Anfinsen, "The Proteins," Vol. I, ed. by H. Neurath, Academic Press, New York (1963), p. 318.

chloroform. Into the chloroform solution isobutylchloroformate (1.36 m*l*) in 20 m*l* of absolute chloroform was stirred in portions at −9—−11°C. After half an hour, a chloroform solution of the L-phenylalanine methyl ester (prepared from its hydrochloride (2.20 g) and triethylamine (1.54 m*l*)) was added. After stirring had continued for three hours, the reaction mixture was allowed to stand overnight at room temperature. The solvent was then replaced by 100m*l* of ethyl acetate and the insoluble crystals were filtered off. The filtrate was washed successively with 0.2 N hydrochloric acid, water, 5% sodium bicarbonate, and water. After drying over anhydrous sodium sulfate, the ethyl acetate solution was concentrated to an oil which was solidified by the addition of petroleum ether. Yield, 2.43 g (69.2%). Recrystallization from hot ethanol-water gave white needles (2.03 g, 60%), mp 94.5—95°C. $[\alpha]_D^{20}$ +46.8 (*c* 1, glacial acetic acid). R_f 0.98 (purple with the Ehrlich reagent). ν(Nujol) 3339, 1740, 1730, 1698, 1666, 1650, 1538 cm^{-1}.

Found: C, 71.10; H, 6.44; N, 4.24%. Calcd for $C_{20}H_{21}O_4N$: C, 70.78; H, 6.26; N, 4.13%.

***N*-(3-Benzoylpropionyl)-L-phenylalanine.** The *N*-(3-Benzoylpropionyl)-L-phenylalanine methyl ester (339 mg) was dissolved in 4 m*l* of methanol, and then N sodium hydroxide (1.05 m*l*) was stirred in. After half an hour at room temperature the solution was neutralized with N hydrochloric acid (1.05 m*l*) and the methanol was removed. The gelatinous product was extracted with ethyl acetate, and then the organic phase was washed with water, dried over anhydrous sodium sulfate, and concentrated. The white powder (344 mg) which separated was reprecipitated from ethyl acetate - petroleum ether. Yield, 280 mg (86.2%). Mp 115°C. R_f 0.90 (purple with the Ehrlich reagent). ν(Nujol) 1742(vs), 1684(m), 1666(sh), 1653(vs), 1538(m) cm^{-1}. $\nu(CHCl_3)$ 1724(w), 1692(sh), 1678(vs), 1600(s) $cm.^{-1}$

Found: C, 70.43; H, 5.85; N, 4.17%. Calcd for $C_{19}H_{19}O_4N$: C, 70.14; H, 5.89; N, 4.13%.

***N*-(3-Benzoylpropionyl)-L-leucyl Glycine Ethyl Ester.** By a mixed anhydride method, 3-benzoylpropionic acid (0.89 g) was coupled with L-leucyl glycine ethyl ester prepared from carbobenzoxy-L-leucyl glycine ethyl ester (1.93 g) by treatment with hydrogen bromide in glacial acetic acid. The gelatinous product was crystallized from chloroform - petroleum ether. Yield, 0.91 g (48.5%). Mp 111—112°C. R_f 0.82 (purple with the Ehrlich reagent). The dipeptide derivative was subjected to alkaline hydrolysis without purification.

Dicyclohexylammonium Salt of *N*-(3-Benzoylpropionyl)-L-leucyl Glycine. The *N*-(3-Benzoylpropionyl)-L-leucyl glycine ethyl ester (188 mg) was dissolved in methanol (5 m*l*), and then N sodium hydroxide (0.55 m*l*) was added. Stirring was continued for 70 min under cooling with ice water. The methanol was evaporated under reduced pressure after neutralization. The benzoylpropionyl dipeptide in the hydrolyzate was extracted with ethyl acetate after complete acidification with N hydrochloric acid. The acyl peptide was purified by extraction into a weakly alkaline layer and by counter-extraction into ethyl acetate after acidification. The ethyl acetate extract was then concentrated and treated with dicyclohexylamine. White crystals were immediately separated and recrystallized from methanol-ether, yielding pure salt (52%). Mp 209—210°C. R_f 0.50 (purple with the Ehrlich reagent).

Found: C, 67.77; H, 8.94; N, 7.93%. Calcd for $C_{30}H_{47}O_5N_3$: C, 68.02; H, 8.94; N, 7.93%.

***N*-Valeroyl-L-phenylalanine.** L-Phenylalanine methyl ester (prepared from its hydrochloride (2.59 g)) was coupled with valeric acid (1.02 g) in methylene chloride. After 5.5 hr the reaction mixture was washed with water and concentrated to a sirup. The sirup was submitted to alkaline hydrolysis (30 min at 0°C) with 2 N sodium hydroxide in methanol, and then the methanol was evaporated. After the acidification of the aqueous solution, the crystals (1.43 g, 57%) were collected. Mp 118—120°C. $[\alpha]_D^{20}$ +48.9 (*c* 1, glacial acetic acid).

Found: C, 67.47; H, 7.62; N, 5.42%. Calcd for $C_{14}H_{19}O_3N$: C, 67.44; H, 7.68; N, 5.62%.

The cyclohexylammonium salt was obtained from the above acyl phenylalanine and dicyclohexylamine (1.33 m*l*) in ethyl acetate. Mp 180°C.

Found: C, 72.10; H, 9.99; N, 6.32%. Calcd for $C_{26}H_{30}O_3N_2$: C, 72.52; H, 9.83; N, 6.51%.

6-Methyl-2, 3, 4, 5-tetrahydropyridaz-3-one. Into an ethanol solution (10 m*l*) of levulinic acid (1.16 g), 80% hydrazine hydrate (0.75 m*l*) was added and the mixture was stirred overnight at room temperature.

The yellow powder (450 mg) obtained by evaporation was washed with ethyl acetate and crystallized from ether - petroleum ether. The colorless needles obtained were dried under reduced pressure at 65°C. Mp 102.5—103°C (lit.,[14a] 104—105°C). R_f 0.69 (orange with the Ehrlich reagent).

Found: C, 53.49; H, 7.06; N, 24.62%. Calcd for C_5H_8ON: C, 53.55; H, 7.19; N, 24.99%.

6-Phenyl-2, 3, 4, 5-tetrahydropyridaz-3-one. 3-Benzoylpropionic acid (1.78 g) in 10 m*l* of ethanol was refluxed with hydrazine hydrate (0.75 m*l*) for forty minutes. After it had then been allowed to stand for two hours at room temperature, the white crystals which separated were collected. Recrystallization from ethanol gave colorless needles in a 95% yield. Mp 149.5—150°C. (lit.,[14b] 153°C). R_f 0.80 (orange with the Ehrlich reagent).

Found: C, 68.77; H, 5.67; N, 15.78%. Calcd for $C_{10}H_{10}ON_2$: C, 68.95; H, 5.79; N, 16.08%.

6-Benzoylaminomethyl-4-benzoylamino-2, 3, 4, 5-tetrahydropyridaz-3-one. By the same procedure as has been descrived above, the pyridazone derivative was prepared from *N*-α-*N*-δ-dibenzoyl-γ-oxo-L-ornithine methyl ester and hydrazine hydrate, yielding 97%. Mp 195—197°C. R_f 0.81 (orange with the Ehrlich reagent).

Found: C, 63.83; H, 5.26; N, 15.41%. Calcd for $C_{19}H_{18}O_3N_2 \cdot 1/2H_2O$: C, 63.49; H, 5.33; N, 15.99%.

2, 4, 5-Tribenzoylamino-Δ^4-L-pentenoic Acid Methyl Ester and *N*-α-*N*-δ-Dibenzoyl-γ-oxo-L-pentenoic Acid Methyl Ester. These two compounds were obtained according to the procedure of Heath *et al.*[10]

***N*-α-*N*-δ-Dibenzoyl-γ-oxo-L-ornithine.** The *N*-α-*N*-δ-dibenzoyl-γ-oxo-L-ornithine methyl ester (5.5 g) was suspended in 25 m*l* of methanol, and then 2 N sodium hydroxide (9 m*l*) was stirred in portions under cooling in an ice water bath. The stirring was continued for half an hour, and then the methanol was

evaporated under reduced pressure at room temperature. By neutralization with 2 N hydrochloric acid (9 ml), white crystals were obtained. Recrystallization from methanol-water gave a pure hemi-hydrate of dibenzoyl-γ-oxo-L-ornithine. Mp 126—128°C, lit.,[10] 116—118°C as hemi-methanolate.

Found: C, 62.87; H, 5.28; N, 7.49%. Calcd for $C_{19}H_{18}O_5N_2 \cdot 1/2H_2O$: C, 62.84; H, 5.27; N, 7.82%.

An anhydrous sample obtained by drying *in vacuo* at 50°C was submitted to analysis.

Found: C, 64.35; H, 5.39; N, 7.73%. Calcd for $C_{19}H_{18}O_5N_2$: C, 64.40; H, 5.12; N, 7.91%.

In another experiment, the monohydrate (mp 150—152°C, Found: C, 61.54; H, 5.41; N, 7.65%) of the diacyl-γ-oxoornithine derivative was isolated.