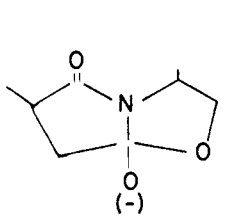
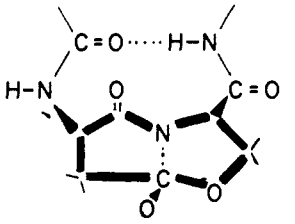
shown to act as a reactive nucleophile[24] due to its unusually high dissociation constant.[64] This high dissociation constant may also account for the intramolecular catalysis observed both with respect to ring closure and to ring opening. In the former case, the serinate ion would facilitate the ionization of the peptide nitrogen (A → B) by abstraction of the proton (general base catalysis) as shown in the following scheme (Fig. 11). In the latter case the β-hydroxyl would supply a proton to the ring nitrogen of the transition complex C′ (general acid catalysis), thus facilitating ring opening and stabilizing B′ relatively to C′. The structures A, B, C, D, A′, B′, C′ and D′ of Fig. 11 correspond to the respective structures of Fig. 10.

F. Speculation on the Chemistry of Proteolytic Enzyme Sites.—The unusual reactivity of the serine hydroxyl at the catalytic site of some proteolytic enzymes suggests the possibility that the hydroxyl group of serine in the sequence -Gly-Asp-Ser-Gly- is somehow chemically altered. One possibility of such a covalently altered structure, involving both the aspartic acid and the serine residue of the active site sequence, is the bicyclic structure VII. This structure, which is the intramolecular analog of structure D (Fig. 10), could be rapidly formed by a nucleophilic attack of the serine hydroxyl on the β-carbonyl of an existing imide ring, possibly aided by the presence of a general base catalyst such as imidazole. It might be established by hydrogen bonding between the carbonyl oxygen of the amino acid preceding the aspartyl residue and the peptide nitrogen of the residue following the serine, as indicated in VIII. In fact, a compact structure on the basis of VIII can be constructed from "space filling (Cal. Tech.) atoms" in which all the peptide bonds are coplanar and in the "*trans*" configuration (Fig. 12).

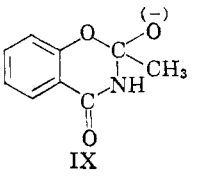


VII

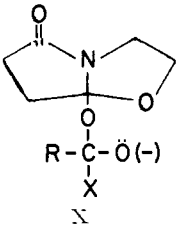


VIII

A bicyclic intermediate, IX (chemically related to VII), involved in acyl transfer reactions has been proposed by Brenner[32] for the reversible O–N isomerization of *O*-acetylsalicylamide to N-acetylsalicylimide.



IX



X

The nucleophilic oxygen at the bridgehead carbon atom in structure VIII could serve as a nucleophile in enzymic catalysis[66,67] and the conventional "Michaelis–Menten (enzyme–substrate) complex" could involve chemical bonding to substrate without concomitant breaking of any of the substrate bonds as in X.[66,67]

Although the above-mentioned mechanism of catalysis must be considered highly speculative at this time, it does suggest the possibility of treating enzyme reaction mechanisms in terms of the primary sequence of amino acids in the protein molecule, taking into consideration the possible existence of unconventional covalent structures of high reactivity. Two such possible structures, one involving arginine and aspartic acid[68] and the other involving serine oxazoline and aspartic acid,[34] have been recently described.

Acknowledgment.—This work was supported by a grant (B-5887) from the National Science Foundation, and a grant (A-3083) from the National Institutes of Health.

(64) T. C. Bruice, T. H. Fife, J. J. Bruno and N. E. Brandon, *J. Am. Chem. Soc.*, **84**, 1973 (1962).

(66) S. A. Bernhard, *J. Mol. Biol.*, in press (1962).

(67) S. A. Bernhard, *J. Cell. Compt. Phys.*, **54** (suppl. 1), 195 (1959).

(68) B. F. Erlanger, *Proc. Natl. Acad. Sci.*, **46**, 1430 (1960).

[CONTRIBUTION FROM THE HORMONE RESEARCH LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY 4, CALIF.]

Preparation of L-Lysyl-L-lysyl-L-arginyl-L-arginyl-L-proline

BY JOHANNES MEIENHOFER AND CHOH HAO LI

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The pentapeptide L-lysyl-L-lysyl-L-aringyl-L-arginyl-L-proline has been prepared. The optical purity of the product was established by microbiological assay of the amino acid content of the acid hydrolyzate of the peptide as well as by enzymic digestion. It was further demonstrated that the arginyl-proline bond in the pentapeptide is resistant to the action of leucine aminopeptidase and trypsin.

Introduction

In the course of synthetic work on a peptide sequence occurring in adrenocorticotropin (ACTH), the protected pentapeptide intermediate N^{α}-carbobenzoxy-N^{ϵ}-tosyl-L-lysyl-N^{ϵ}-tosyl-L-lysyl-N^{G}-tosyl-L-arginyl-N^{G}-tosyl-L-arginyl-L-proline had been synthesized.[1] It occurred to us that it would be of interest to prepare the free peptide, which contains an unusual sequence of amino acid residues. In addition, it would afford an opportunity to examine the optical purity of the constituent amino acids in the free pentapeptide.

(1) C. H. Li, J. Meienhofer, E. Schnabel, D. Chung, T. B. Lo and J. Ramachandran, *J. Am. Chem. Soc.*, **82**, 5760 (1960); **83**, 4449 (1961).

The protecting groups in the protected pentapeptide were removed by treatment with sodium in liquid ammonia.[2,3] For the complete removal of the protecting groups, it was found necessary to expose this peptide to excess sodium for a longer time than has been required in the case of protected amino acids[2,3] or polypeptides like oxytocin[4] and lysine-vasopressin.[5] The crude pentapeptide was purified by ion exchange chromatography on IRC-50 (XE-64) in ammonium acetate buffers of *p*H 7.0 to 8.0. The eluate fractions were analyzed by means of the Sakaguchi reaction for arginine, performed by the procedure described by Macpherson[6] and modified for the analysis of large numbers of fractions in test tubes. The chromatographically purified pentapeptide could then be crystallized with the use of methyl orange which forms rather insoluble helianthates.[7] Colorimetric determination showed that the methyl orange content of different helianthate preparations was between 4 and 5 equivalents. Various amounts of water were also found to be present. Attempts at preparation of a sample (pentapeptide helianthate) with a definite methyl orange content which would give a correct elementary analysis have so far proved unsuccessful; however, the crystallization and recrystallization procedures have proved to be an efficient means of purification. The free pentapeptide could be recovered from the helianthate by the use of the ion exchange resin IR-4B in the acetate form. The pentapeptide prepared in this way proved to be homogeneous as tested by paper chromatography. Amino acid analysis[8] gave the expected values; moreover, microbiological assay[9] of the acid hydrolysate suggested that all constituent amino acids were probably of an L-configuration.

The pentapeptide was submitted to hydrolysis with trypsin, leucine aminopeptidase (LAP) and their combination. Tryptic digestion released arginine and lysine but not completely. There were also indications of the presence of lysyllysine, lysylarginine and arginylproline. Hydrolysis with LAP released lysine in detectable amounts but left the pentapeptide essentially intact. The combined action of trypsin and LAP, however, resulted in extensive digestion, which produced only three products as revealed by chromatography on paper and in the Automatic Amino Acid Analyzer[8]; these were identified as lysine, arginine and arginylproline, in molar ratios of 2:1:1. Thus, it is apparent that the pentapeptide, L-Lys-L-Lys-L-Arg-L-Arg-L-Pro, is resistant to complete digestion by either trypsin or LAP, whereas a combination of these two enzymes can hydrolyze completely all the peptide bonds with the exception of the arginylprolyl linkage. These observations are consistent with earlier data obtained in similar studies with adrenocorticotropins[10-12] and synthetic α^{1-19}-ACTH.[1]

Experimental

The protected pentapeptide Nα-carbobenzoxy-Nε-tosyl-L-lysyl-Nε-tosyl-L-lysyl-N^G-tosyl-L-arginyl-N^G-tosyl-L-arginyl-L-proline[1] (717 mg.) was dissolved in liquid ammonia (400 ml.) which had been distilled from sodium. Sodium was added in small pieces until a blue color persisted which was maintained for two hours (115 mg. of sodium). Glacial acetic acid (0.4 ml.) was then added. Most of the ammonia was allowed to evaporate spontaneously to a volume of approximately 10 ml. which was removed from the material in a frozen state *in vacuo* with a water pump; a KOH drying jar was put between pump and flask. The residual loose powder (crude pentapeptide) was dissolved in 1.0 *M* acetic acid (10 ml.) and placed on an IRC-50 (XE-64) column (0.9 × 10 cm.) in the H^+ form for desalting.[13] The column was washed with 0.25% acetic acid (400 ml.) and the peptide was then eluted with a 30% pyridine–4% acetic acid solution. The elute was lyophylized to give a glassy hygroscopic material (425 mg., 85% based on a tetracetate).

Purification of L-lysyl-L-lysyl-L-arginyl-L-arginyl-L-proline by Ion Exchange Chromatography.—A column (measuring 0.9 × 105 cm.) of IRC-50 (XE-64) resin was equilibrated with 1 *M* ammonium acetate buffer of *p*H 7.0. Crude pentapeptide (400 mg.) was dissolved in 1 ml. of buffer and placed on the column. The chromatogram was developed with the same buffer at a flow rate of 1 ml. per hour. After 85 fractions (1.5 ml. each) had been collected, the buffer was changed and elution continued with 2 *M* ammonium acetate of *p*H 8.0. Aliquots (0.1 ml.) were taken for analysis by the Sakaguchi reaction. The material was placed in test-tube cuvettes measuring 19 × 150 mm.; 1% sodium hydroxide (2 ml.) was added to each tube followed by a solution (0.05 ml.) of 0.1% α-naphthol and 10% urea in 95% ethanol. The tubes were shaken thoroughly and cooled by placing the test tube rack in an ice bath. With vigorous shaking, a sodium hypobromite solution (0.5 ml. freshly prepared by adding 0.5 ml. of bromine to 100 ml. of 1 *N* sodium hydroxide) was added. A solution of 10% urea in water (0.5 ml.) was then added followed by another addition of 0.5 ml. of the hypobromite solution. After the solution had been allowed to stand for 15 minutes at room temperature,[14] the optical density was read at 540 mμ directly in the test tubes with a Coleman spectrophotometer. Four peaks were obtained, the first three representing material from which the protecting groups had been incompletely removed by sodium in liquid ammonia. The material isolated from the main peak (110 mg., 27%) was found to be the desired pentapeptide. The over-all recovery of material from the column was rather low (55%). The purified pentapeptide was found to be homogeneous when again submitted to chromatography on a small analytical XE-64 column (0.6 × 30 cm.), and on paper in two solvent systems: phenol-water (80:20), R_f = 0.75 and 1-butanol–pyridine–acetic acid–water (30:20:6:24), R_f = 0.08.

The peptide, approximately 2 mg., was hydrolyzed at 110° in a sealed evacuated tube with 1 ml. of constant boiling (5.7 *N*) HCl for 24 hr. The amino acid content in the hydrolyzate was determined by the method of Spackman, Stein and Moore[8] as well as by the microbiological procedure.[9] Results are summarized in Table I.

Purification by Helianthate Formation.—The crude pentapeptide, prepared from 290 mg. of Nα-carbobenzoxy-Nε-tosyl-L-lysyl-Nε-tosyl-L-lysyl-N^G-tosyl-arginyl-N^G-tosyl-arginyl-L-proline[1] by the procedure described above, was dissolved in 0.1 *N* acetic acid (15 ml.), and an aqueous solution of methyl orange (350 mg. in 20 ml. water of 60°) was

(2) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).

(3) J. du Vigneaud and O. K. Behrens, *ibid.*, **117**, 27 (1937).

(4) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis and S. Gordon, *J. Am. Chem. Soc.*, **75**, 4879 (1953).

(5) V. du Vigneaud, M. F. Bartlett and A. Jöhl, *ibid.*, **79**, 5572 (1957); J. Meienhofer and V. du Vigneaud, *ibid.*, **82**, 2279 (1960).

(6) H. T. Macpherson, *Biochem. J.*, **36**, 59 (1942).

(7) S. Gurin and C. F. Segal, *J. Am. Chem. Soc.*, **58**, 2107 (1936).

(8) D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(9) Microbiological assay of amino acids in the acid hydrolyzate of the peptide was carried out by Shankman Laboratories, Los Angeles, Calif. It should be pointed out that the microbiological assay of L-amino acids is at the best only 95% reliable.

(10) C. H. Li, I. I. Geschwind, R. D. Cole, I. D. Raacke, J. I. Harris and J. S. Dixon, *Nature*, **176**, 687 (1955).

(11) R. G. Shepherd, S. D. Wilson, K. S. Howard, P. H. Bell, D. S. Davis, S. B. Davis, E. A. Eigner and N. E. Shakespeare, *J. Am. Chem. Soc.*, **78**, 5067 (1956).

(12) C. H. Li, J. S. Dixon and D. Chung, *Biochem. et Biophys. Acta*, **46**, 324 (1961).

(13) H. B. F. Dixon and M. P. Stack-Dunne, *Biochem. J.*, **61**, 483 (1955).

(14) The color was found to be stable for more than 1 hr.

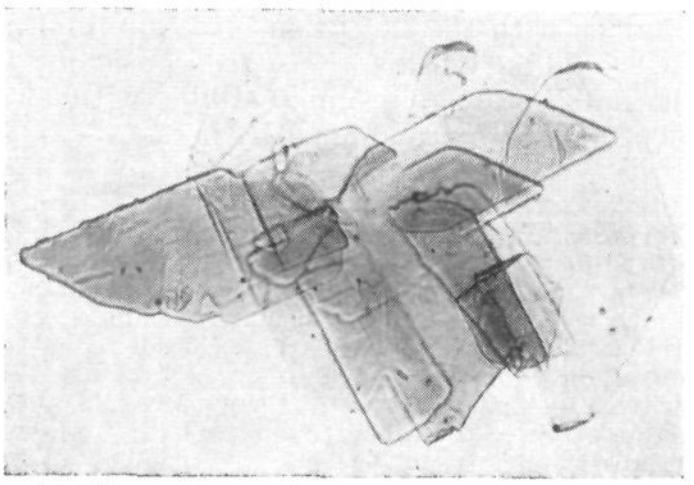

Fig. 1.—Crystalline L-lysyl-L-lysyl-L-arginyl-L-arginyl-L-proline in the form of helianthate (X1070).

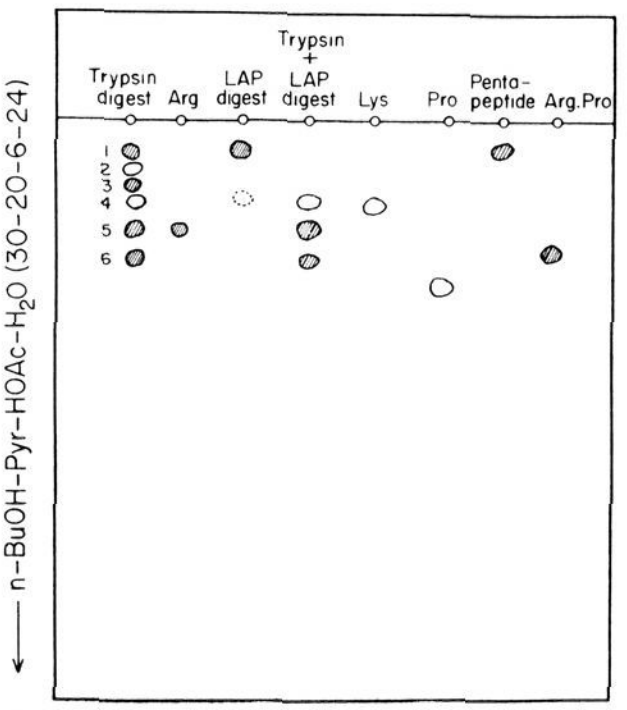

Fig. 2.—Paper chromatography of enzymic (trypsin and LAP) digests of the pentapeptide; solvent system, *n*-butanol–pyridine–acetic acid–water (30–20–6–24).

added with stirring. The precipitate was collected and recrystallized twice from methanol–water, (1:1, 100 ml.) to yield 415 mg. of brown crystalline material (Fig. 1); m.p. 213–215° dec.

TABLE I

AMINO ACID ANALYSIS OF L-LYSYL-L-LYSYL-L-ARGINYL-L-ARGINYL-L-PROLINE

Amino acid[a]	—Microbiological— assay[b] I	II	Chromatographic procedure[c]
Lysine	2.0	2.0	2.1
Arginine	1.8	1.9	2.0
Proline	1.0	1.0	1.0

[a] Values in mole/mole of the peptide. [b] Preparation I was obtained by chromatographic purification; II by helianthate formation. [c] Preparation I was employed.

For removal of the methyl orange the material (200 mg.) was dissolved in methanol–water (1:1, 150 ml.) and passed through an IR-4B column (1 × 10 cm.) in the acetate form.

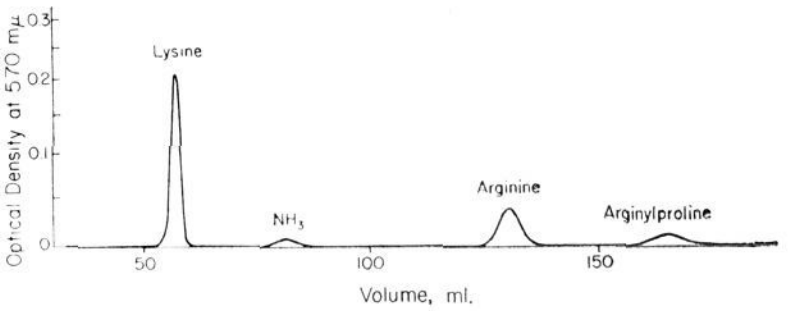

Fig. 3.—Analysis of an enzymic (combination of trypsin and LAP) hydrolyzate of the pentapeptide; 15 cm. column of Amberlite IR-120 of the Automatic Amino Acid Analyzer at 50° with *p*H 5.28 0.35 *N* Na citrate.

The colorless eluate was treated with charcoal; the methanol was then removed *in vacuo*. The remaining aqueous solution was lyophilized to give 70 mg. of a glassy hygroscopic material which was found to be homogeneous by the tests already described. A microbiological assay[9] of an acid hydrolyzate of the peptide gave the constituent amino acids as recorded in Table I.

Enzymic Studies.—One mg. of the pentapeptide, together with 1 mg. of crystalline trypsin (Armour Lot No. 904CL3) were dissolved in 0.9 ml. of a 2-amino-2-hydroxymethylpropane 1:3 diol (*Tris*) buffer of *p*H 8 and the reaction mixture was kept at 25°. An aliquot of 0.05 ml. was removed after 0, 1, 3, 7, 16 and 24 hr. and submitted to chromatography on paper in a solvent system consisting of 1-butanol–pyridine–acetic–water (30/20/6/24 by volume). Chromatograms were developed with ninhydrin, followed by the Sakaguchi reagent. Figure 2 shows the pattern for the 24-hour digest; it is evident that no free proline was present in the digest.

One mg. of the pentapeptide plus 0.14 mg. of LAP (Worthington Lot no. 5808B) were dissolved in 0.95 ml. of *p*H 8 *Tris* buffer containing 0.002 *M* $MgCl_2$, and the reaction mixture was kept at 25° for 16 hr. Chromatography on paper showed some liberation of lysine (See Fig. 2). Apparently, the pentapeptide was resistant to LAP under these conditions.

Five mg. of the pentapeptide plus 0.1 mg. of trypsin were then dissolved in 4.5 ml. of *p*H 8 *Tris* buffer containing 0.002 *M* $MgCl_2$, and the reaction mixture was kept at 25°; after 16 hr. of digestion, 0.25 ml. of an aqueous solution of LAP (0.7 mg. of the enzyme) was added and the mixture was allowed to stand at 25° for an additional 16 hr. The digest was submitted to chromatography on paper (Fig. 2) and analysis in the Amino Acid Analyzer (Fig. 3). Both analyses indicated that complete liberation of lysine and arginine had occurred, except that the arginylprolyl[15] bond remained intact under the combined action of trypsin and LAP.

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(15) The dipeptide, L-arginyl-L-proline, was prepared from the crystalline protected dipeptide.[1] Digestion of this dipeptide with a combination of trypsin and LAP again indicated that the bond between arginine and proline in the dipeptide is resistant to the action of these two enzymes.