[CONTRIBUTION FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

Hydrolytic Behavior of Certain Branched Peptide Derivatives of Lysine

DIMITRI THEODOROPOULOS* AND LYMAN C. CRAIG

Received June 4, 1956

A number of peptide derivatives of lysine with both basic groups covered in peptidic linkage have been synthesized. Hydrolysis experiments have shown that the *epsilon* peptide linkage is unusually resistant to hydrolysis. Even an aspartyl residue on this position was difficult to hydrolyze.

INTRODUCTION

A recent paper[1] reported the synthesis of two optical isomers of the peptide isoleucylphenylalanine. One of these which had been isolated from an acid hydrolysate of bacitracin,[2] had been found to give an unexpectedly low color yield with the quantitative ninhydrin reagent. The other synthetic isomer gave a higher color yield than the first but still lower than that expected. The optical configuration, therefore, can play a role in the steric effects which influence the reactivity of a peptide.

Other unique linkages known to be present in bacitracin are also of interest not only because a number of them yield dextro amino acids on hydrolysis, but because they present other structural features not thus far found in proteins or naturally-occurring peptides. Thus an isoleucylcysteinyl sequence is present which gives one-half mole of alloisoleucine[3] on total hydrolysis. This has been satisfactorily explained by the finding that the simple peptide sequence is not present but instead the thiol group has cyclized with the keto group of the amide to form a thiazoline ring.[4] An attempt was made to synthesize isoleucylcysteine and cause it to cyclize to the corresponding thiazoline ring structure but all efforts to remove water from the dipeptide with formation of the thiazoline failed.

Still another unique linkage present in bacitracin involves the lysine residue. Both amino groups of this residue were found to be joined in peptide linkage. The lysine residue thus represents a point of cross linking in the peptide chain. These peptide linkages appeared to be unusually stable toward acid hydrolysis. When it was found that one of the basic groups had an isoleucyl residue attached to it part of the stability did not appear to be anomalous since isoleucyl peptides[5] are known in many cases to be difficult to hydrolyze. However, the other basic group of the lysine was found to be covered by an aspartyl residue. In this case marked resistance to hydrolysis was not expected since aspartic acid residues are thought to be among the first to be liberated on acid hydrolysis of proteins.

It appeared well worth while to undertake the synthesis of several of the sequences involving the lysine as well as certain analogs in order to study their hydrolytic behavior. This paper will report the synthesis of a number of such peptides.

EXPERIMENTAL

*Carboethoxy-*D,L*-isoleucine.* This compound was prepared from 1.3 g. of D,L-isoleucine and 2.16 g. of ethyl chlorocarbonate in a manner similar to that used by Fischer[6] in preparing other carboethoxy derivatives of amino acids. The ether extract of the derivative was dried over sodium sulfate, evaporated to dryness, and stored in a vacuum over P_2O_5. The residue, 1.80 g., was used for the next synthetic step without further purification.

*α-(Carboethoxy-*D,L*-isoleucyl), ε-(carbobenzoxy)-*L*-lysine methyl ester.* A solution containing 1.80 g. of carboethoxy-D,L-isoleucine, 10 ml. of dry chloroform, and 0.90 g. of dry triethylamine was cooled to 0° and 0.95 g. of ethyl chlorocarbonate was added. After ten minutes a chloroform solution of 2.95 g. of ε-(carbobenzoxy)-L-lysine methyl ester (prepared from lysine according to the direction of Bergmann and Zervas),[7] 0.91 g. of triethylamine, and 10 ml. of chloroform, was added. Coupling proceeded with evolution of CO_2. After standing at room temperature for 30 minutes the solution was washed successively with 1 *N* HCl, dilute sodium bicarbonate, and water. It was dried over sodium sulfate and evaporated to dryness. The last traces of chloroform were removed by addition of a few ml. of acetone and re-evaporation. The residue was heated to boiling in 50% aqueous ethanol, cooled in ice, and scratched with a glass rod. The crystals which separated were recrystallized from ethanol-water. The yield was 2.6 g.; 61% of the theory. The compound softened at 95° and melted at 106–107°.

Anal. Calc'd for $C_{24}H_{37}N_3O_7$: C, 60.10; H, 7.77. Found: C, 59.95; H, 7.83.

*α-(Carboethoxy-*D,L*-isoleucyl)-*L*-lysine methyl ester.* A solution of 2.3 g. of the above ester in absolute methanol was hydrogenated in the presence of 10% palladium on charcoal. After filtering off the catalyst, evaporation of the methanolic solution gave a syrup which weighed 1.4 g. No attempt was made to crystallize this syrup. It was used directly for coupling in the next step after standing in a vacuum over calcium chloride.

*α-(Carboethoxy-*D,L*-isoleucyl), ε-(carbobenzoxy-*L*-asparaginyl)-*L*-lysine methyl ester.* To a solution of 0.38 g. of dry triethylamine in 15 ml. of dry chloroform, 1 g. of carbo-

---

* Fellow of the Greek Fellowship Foundation.

(1) Theodoropoulos and Craig, *J. Org. Chem.*, **20**, 1169 (1955).

(2) Hausmann, Weisiger, and Craig, *J. Am. Chem. Soc.*, **77**, 723 (1955).

(3) Hausmann, Weisiger, and Craig, *J. Am. Chem. Soc.*, **77**, 721 (1955).

(4) Craig, Hausmann, and Weisiger, *J. Am. Chem. Soc.*, **76**, 2839 (1954).

(5) Harfenist, *J. Am. Chem. Soc.*, **75**, 5528 (1953).

(6) Fischer, *Ber.*, **39**, 2893 (1906).

(7) Bergmann, Zervas, and Ross, *J. Biol. Chem.*, **111**, 245 (1935).

benzoxy-L-asparagine[8] was added with shaking. The carbobenzoxy-L-asparagine at first was all in solution but upon cooling in ice a precipitate formed. After cooling the suspension in ice for 15 minutes, 0.40 g. of ethyl chlorocarbonate was added. This was followed by an additional 5-ml. portion of dry chloroform and continued shaking for 5 minutes at room temperature. This had brought the precipitate nearly into solution. A previously cooled solution of 1.4 g. of α-(carboethoxy-D,L-isoleucyl)-L-lysine methyl ester in 10 ml. of chloroform then was added. Coupling ensued with the usual evolution of CO_2. After standing at room temperature for 30 minutes twice the volume of chloroform was added. The solution was washed with dilute bicarbonate solution, normal hydrochloric acid, bicarbonate again, and finally water. After drying over sodium sulfate the chloroform was evaporated at 40° under reduced pressure. Acetone was added to the residue and this also removed by evaporation. When stirred with ethyl ether crystallization ensued. The yield was 0.56 g.; 25% of the theory. Final purification was accomplished by recrystallization from a mixture of chloroform and ethyl ether. The product was slightly soluble in ethanol and ethyl acetate. It melted at 196–197°.

Anal. Calc'd for $C_{23}H_{43}N_5O_9$: C, 56.64; H, 7.30; N, 11.79. Found: C, 56.93; H, 7.48; N, 11.58.

N-Tosyl-L-leucyl chloride. The following directions are given because they gave a better yield and are easier to carry out than those given in the literature.[9]

A mixture of 8 ml. of water, 4 ml. of tetrahydrofuran, 1.6 g. of triethylamine, and 0.65 g. of leucine was treated with 1.45 g. of tosyl chloride added in small portions with stirring over a half-hour period. After 45 minutes all the leucine had dissolved. The tetrahydrofuran was removed under a vacuum and 10 ml. of water was added. The solution was washed twice with ethyl ether and the acid was precipitated with hydrochloric acid. When recrystallized from aqueous ethanol the acid melted at 120–122°; yield 1 g.

The acid was converted to the chloride by treatment with PCl_5 in chloroform solution. The desired derivative was precipitated from this solution with pentane. The precipitate was used for the next step without further purification.

ε-(*Tosyl-L-leucyl*)-L-*lysine.* L-Lysine monohydrochloride (0.65 g.) was converted to the copper complex according to the directions of Neuberger and Sanger[10] in 5 ml. of H_2O. To this solution 5 ml. of 2 *N* sodium hydroxide and 4 ml. of tetrahydrofuran were added. The tosylleucylchloride (1.1 g.) was added in small portions with stirring during one hour at room temperature. The solution was maintained above *p*H 10 by addition of sodium hydroxide. After two additional hours 50 ml. of water was added to complete the precipitation of the dipeptide complex. The mixture was cooled and filtered. After washing the precipitate with water it was dried and powdered.

After an additional washing the complex was suspended in 50 ml. of water. Acetic acid (4–5 ml.) was added and H_2S was bubbled into the mixture for two hours. Then the mixture was boiled and treated again with H_2S before filtering off the copper sulfide. The filtrate was evaporated to dryness in a vacuum. Since paper electrophoresis showed a small amount of free lysine, the product was purified by a 30-transfer countercurrent distribution in a system made by equilibrating *n*-butanol with water. The major band at tubes 12–25 was recovered and freeze-dried from water. It weighed 0.62 g., 42% of the theory.

The residue was converted to the hydrochloride by addition of an equivalent of dilute hydrochloric acid and freeze-drying. The residue was soluble in hot acetone. It crystallized from this solution after cooling and with addition of a little dry ethyl ether. The melting point was 120–122° with previous softening at 85°. $[\alpha]_D^{25}$° −17° ± 2° (*c*, 1.18 in glacial acetic acid).

Anal. Calc'd for $C_{19}H_{32}ClN_3O_5S$: C, 50.71; H, 7.16; N, 9.33. Found: C, 51.10; H, 7.15; N, 9.60.

ε-(*Tosyl* D,L-*isoleucyl*), α-(*carbobenzoxy-L-asparaginyl*)-L-*lysine methyl ester.* The intermediate, ε-(tosyl-D,L-isoleucyl)-L-lysine was prepared exactly as was the leucyl derivative. The product was purified by a 30-transfer countercurrent distribution in 2-butanol–water and recovery of the solute in tubes 12–26. It melted at 192–195° after softening at 130°. The yield was 30% of the theoretical.

Anal. Calc'd for $C_{19}H_{32}ClN_3O_5S$: N, 9.33. Found: N, 9.45.

The above substance was converted to the methyl ester by repeated treatment with dry HCl in methanol. The hydrochloride obtained on evaporation of the methanol then was used for the coupling described below.

A solution containing 0.18 g. of triethylamine, 0.48 g. of carbobenzoxy-L-asparagine, and 15 ml. of dry chloroform was cooled to 0°. It became a thick suspension but in spite of this 0.19 g. of ethyl chlorocarbonate was added. After a few minutes stirring, the mixture was allowed to come to a somewhat higher temperature and a solution containing the above ester hydrochloride (0.5 g.) neutralized with triethylamine in 10 ml. of chloroform was added. Coupling ensued with evolution of CO_2. After 0.5 hour the solution was diluted to twice its volume with chloroform and successively washed with dilute bicarbonate solution, hydrochloric acid, bicarbonate solution again, and water. The chloroform solution was dried over sodium sulfate and evaporated to dryness. The residue solidified on rubbing with ethyl ether. It was taken up in 15 ml. of ethyl acetate, heated with a little Norit, and filtered. The filtrate was concentrated to about 4 ml. and treated with 2,3-dimethylbutane. The precipitate which melted at 125–127°, weighed 0.2 g.

Anal. Calc'd for $C_{32}H_{45}N_5O_9S$: C, 56.86; H, 6.71. Found: C, 56.73; H, 7.13.

ε-(*Tosylglycyl*)-L-*lysine.* This compound was prepared essentially as the leucyl derivative, but the copper complex of the dipeptide was decomposed with H_2S without previous acidification with acetic acid. It was purified by a 30-transfer countercurrent distribution in 2-butanol–water and recovery of the solute in tubes 7–20. It melted at 208–212°. The yield was 45% of the theoretical. $[\alpha]_D^{25}$° +12° ± 2° (*c*, 1.38 in glacial acetic acid).

Anal. Calc'd for $C_{15}H_{23}N_3O_5S$: C, 50.40; H, 6.48; N, 11.75. Found: C, 50.70; H, 6.45; N, 11.73.

DISCUSSION

Comparative hydrolytic experiments were made on a number of the peptide derivatives reported in this paper. The conditions of the hydrolysis were those customarily used for complete hydrolysis (24 hours at 110° in 6 *N* HCl in a sealed tube) and the conditions used for the partial hydrolysis of bacitracin[2] (concentrated hydrochloric acid at 80° for 3 hours). The hydrolysis products were investigated by a combination of paper electrophoresis and paper chromatography as was bacitracin.[2]

Under the conditions usually used for complete hydrolysis, ε-(tosylleucyl)lysine and the corresponding isoleucyl derivative were found to be incompletely hydrolyzed. Paper electrophoresis of the hydrolysate showed two spots, one in the position of lysine and the other in the neutral position. When the neutral spot was eluted and re-hydrolyzed a distinct lysine spot was again found. In contrast the corresponding glycine peptide derivative was completely hydrolyzed.

(8) Bergmann and Zervas, *Ber.*, **65**, 1192 (1932).

(9) McChesney and Swann, *J. Am. Chem. Soc.*, **59**, 1116 (1937).

(10) Neuberger and Sanger, *Biochem. J.*, **37**, 515 (1943).

Under the same conditions the tripeptide derivative, α-(carboethoxy-D, L-isoleucyl)-ε-(carbobenzoxy-L-asparaginyl)-L-lysine methyl ester was hydrolyzed completely. Following hydrolysis paper electrophoresis gave three spots corresponding to a strongly basic (lysine), a neutral (isoleucine), and an acidic (aspartic acid) amino acid. In order to be sure that the neutral spot did not contain unhydrolyzed peptide it was cut out, eluted, re-hydrolyzed and studied again. However, only the neutral spot again was found.

When the lysine peptide derivative with the positions reversed was studied greater stability was encountered. ε-(Tosyl-D,L-isoleucyl)-α-(carbobenzoxy-L-asparaginyl)-L-lysine methyl ester was found to be incompletely hydrolyzed. Evidence for the presence of isoleucyl-ε-lysine was obtained.

A sample of bacitracin A was also studied under the same hydrolytic conditions. Paper electrophoresis gave two basic spots, a neutral spot and two acidic spots. The most rapidly migrating of the basic spots contained lysine and ornithine. The other basic spot contained histidine. From previous experience[11] it was known that this spot also contained a basic peptide complex of unknown structure. When it was cut out, eluted, re-hydrolyzed, and studied again no isoleucine spot was found. Only spots corresponding to histidine (strong), lysine (weaker), and aspartic acid (weaker) were found. Thus the isoleucine-lysine bond in bacitracin is completely hydrolyzed under these conditions as far as the method can indicate but not the asparaginyl-lysine bond. This supports the sequence α-isoleucyl, ε-aspartyl-lysine suggested by Lockhart, Newton, and Abraham.[12] The basic complex resistant to hydrolysis which contained lysine and aspartic acid, however, was not found among the hydrolytic products from the synthetic peptides. This suggests that the second aspartic acid residue which in the case of bacitracin A must carry the single amide nitrogen, exerts a stabilizing influence.

When the two tripeptide derivatives above were studied under the conditions used for the partial hydrolysis of bacitracin (concentrated hydrochloric acid at 80° for 3 hrs.) a combination of paper chromatography and paper electrophoresis revealed spots corresponding to unhydrolyzed tripeptide, isoleucyllysine, and aspartic acid. No free lysine was found. Under these conditions ε-(tosylisoleucyl)lysine did not appear to yield any spots which would correspond to hydrolysis products.

The two tripeptides differed in the intensity of the basic spot corresponding to asparaginyllysine and that of aspartyllysine. The derivative with the aspartic in the ε-position showed a relatively strong spot whereas the isomer with the aspartic in the α-position showed a very weak spot. This finding again is in line with the hypothesis of an ε-aspartyl-α-isoleucyl-lysyl sequence. Additional direct evidence for this has recently been given by Lockhart and Abraham.[13]

The ε-peptide bond of lysine also appeared to be completely stable toward pepsin and ficin. ε-(Tosylglycyl)lysine and ε-(tosyl-leucyl)lysine were unchanged after incubation with pepsin at *p*H 2 for 24 hours at 37°. The latter was not attacked by ficin in citrate buffer at *p*H 5.3 when cysteine was used as an activator.

We wish to thank Mr. D. Rigakos for the microanalyses.

NEW YORK 21, NEW YORK

(11) Weisiger, Hausmann, and Craig, *J. Am. Chem. Soc.*, **77**, 731 (1955).

(12) Lockhart, Abraham, and Newton, *Biochem. J.*, **61**, 534 (1955).

(13) Lockhart and Abraham, *Biochem. J.*, **62**, 645 (1956).