obtained from oxidations allowed to proceed one hour at room temperature.

Methyl 3-Keto-12-hydroxycholanate (V) from 3-Keto-12-acetoxycholanic Acid (XXIII).—Saponification of 0.7 g. of 3-keto-12-acetoxycholanic acid gave 3-keto-12-hydroxycholanic acid. This was dissolved in 10 ml. of methanol to which 1 ml. of acetyl chloride was added. The solution was allowed to stand overnight, poured into water and extracted with ether. The ether solution was washed with water, 5% aqueous sodium carbonate, and evaporated *in vacuo*, leaving an orange resin which was crystallized from an alcohol–water mixture, then an acetone and petroleum ether mixture. The yield of methyl 3-keto-12-hydroxycholanate (m. p. 140–141°) was 0.28 g.

Methyl 3-Keto-12-hydroxy-*nor*-cholanate (VI) from 3-Hydroxy-12-acetoxy-*nor*-cholanic Acid (XXII).—A solution of 2.4 g. of chromium trioxide in 2 ml. of water and 15 ml. of glacial acetic acid was added in four portions at five-minute intervals to a solution of 2.84 g. of 3-hydroxy-12-acetoxy-*nor*-cholanic acid in 25 ml. of glacial acetic acid. The reaction mixture was kept at room temperature, and allowed to stand for two hours, then was poured into 200 ml. of water and extracted with ether. The ether solution was washed with 3 *N* hydrochloric acid, water, and then extracted with 5% aqueous sodium carbonate. Acidifying the alkaline extract gave 2.17 g. of crude 3-keto-12-acetoxy-*nor*-cholanic acid (XXIV), which was dissolved in 80 ml. of 5% aqueous potassium hydroxide and refluxed for two hours. After acidifying the solution 1.67 g. of crude 3-keto-12-hydroxy-*nor*-cholanic acid was obtained.

A 1.17-g. sample of the crude acid was methylated as described above. The product was crystallized from acetone plus petroleum ether, then from 25 ml. of 50% ethanol to give 0.55 g. of methyl 3-keto-12-hydroxy-*nor*-cholanate melting at 146.5–147.5°. The mixture melting point with methyl 3-keto-12-hydroxy-*nor*-cholanate (m. p. 143–145°) prepared from methyl *nor*-desoxycholate by the Oppenauer reaction was 144–144.5°.

3-Keto-12-hydroxy-*bisnor*-4-cholenic Acid.—Saponification of 0.10 g. of methyl 3-keto-12-hydroxy-*bisnor*-4-cholenate (VII) gave 0.09 g. of 3-keto-12-hydroxy-*bisnor*-4-cholenic acid melting at 210–220°.

Anal. Calcd. for $C_{22}H_{32}O_4$: C, 73.29; H, 8.95. Found: C, 73.09; H, 8.67.

Summary

1. Methyl 3-keto-12-hydroxy-4-cholenate, and the *nor*, *bisnor*, and *etio* homologs have been prepared from the corresponding desoxycholic acids.

2. The methyl 3-keto-12-hydroxycholanates used as intermediates were made by selective oxidation of the 3-hydroxyl group by means of the Oppenauer reaction.

3. Proof of the selective oxidation was obtained for two of these compounds by preparing them using a procedure involving partial hydrolysis.

4. The absorption spectra of methyl 3-keto-12-hydroxy-4-cholenate, and the *nor*, *bisnor*, and the *etio* homologs gave similar curves typical of conjugated unsaturation.

EVANSTON, ILLINOIS RECEIVED MARCH 2, 1944

[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL AND COLLOIDAL CHEMISTRY, THE HEBREW UNIVERSITY]

Preparation of *l*-Leucyl-*l*-glutamic Acid Anhydride and its Behavior toward Proteinases

BY N. LICHTENSTEIN

When *d,l*-α-bromoisocaproyl bromide and *l*-glutamic acid are coupled, a reaction product is recovered in over 70% yield. When this product is aminated with aqueous ammonia and the evaporated amination mixture treated with water and alcohol, *l*-leucyl-*l*-glutamic acid separates, whereas the diastereoisomer, *d*-leucyl-*l*-glutamic acid, is apparently retained, together with ammonium bromide, in the aqueous alcoholic phase. The separated dipeptide possesses a specific optical rotation corresponding to that characteristic of the product prepared by E. Fischer[1] from *l*-glutamic acid and the chloride of *l*-α-bromoisocaproic acid. To confirm the configuration, our dipeptide was hydrolyzed by heating with hydrochloric acid and compared as to optical activity with an equimolecular mixture of *l*-leucine and *l*-glutamic acid similarly heated with hydrochloric acid. The specific rotation of the amino acid hydrochloride mixtures obtained from the two solutions by evaporation was one and the same.

The dipeptide is readily converted into its anhydride by treatment with hot β-naphthol. As has previously been reported[2] a number of dipeptides can be converted into corresponding diketopiperazines by solution in β-naphthol at 135–150°. It has been found that *l*-leucyl-*l*-glutamic acid, in distinction, does not dissolve in β-naphthol at the mentioned temperature. At 170–180°, however, solution is effected and anhydride formation occurs. The C, H and N content and the carboxyl titer of the product correspond in value with the theoretical expectation from a diketopiperazine composed of glutamic acid and leucine. Titration according to Linderstrøm-Lang failed to reveal the presence of a free amino group. Hydrolysis with hydrochloric acid yielded, after evaporation to dryness, a product of corresponding specific rotation to that obtained when solutions of the original dipeptide or equimolecular mixtures of its components are similarly treated. The anhydration product is therefore *l*-leucyl-*l*-glutamic acid anhydride.

According to K. Shibata,[3] leucylglutamic acid anhydride ought to be cleaved by trypsin and papain, which are "carboxy-cyclopeptidases."

(1) E. Fischer, "Untersuchungen über Aminosäuren, etc.," **2**, 465, 466 (1923).

(2) N. Lichtenstein, THIS JOURNAL, **60**, 560 (1938).

(3) K. Shibata, *Acta Phytochim.*, **8**, 173 (1934).

However, in agreement with the finding, now corroborated by different authors,[4,5,6,7] that glycylglutamic acid anhydride resists hydrolysis by papain and by trypsin, we found that *l*-leucyl-*l*-glutamic acid anhydride is not hydrolyzed by a glycerol extract of pancreatin, purified pancreatic proteinase, or papain.

Experimental

Preparation of *l*-Leucyl-*l*-glutamic Acid.—*l*-Glutamic acid is coupled with *d,l*-α-bromoisocaproyl bromide in the usual manner, the bromide being present in excess (1.25 equivalents), in aqueous alkaline solution which is cooled in a freezing mixture of salt and ice. After acidification with hydrochloric acid, the solution is repeatedly extracted with ether, the ether extract is dried with sodium sulfate, and then evaporated. The residual sirup when rubbed up with successive portions of petroleum ether, solidifies either immediately or after standing for some time in a vacuum desiccator over sulfuric acid. The dry material is pulverized, washed with petroleum ether, and dried in a vacuum desiccator. The yield, calculated on glutamic acid, is over 70%; 76.2 mg. of the product dissolved in water consumed 4.65 cc. of 0.1 *N* sodium hydroxide (phenolphthalein as indicator); calculation for bromoisocaproylglutamic acid, 4.70 cc. For amination, bromoisocaproylglutamic acid is treated in 10-g. portions with 50 cc. of concentrated aqueous ammonia at 37° for three days. The solution is evaporated *in vacuo*, the residual sirup is taken up in a little water, and the solution evaporated over steam with repeated additions of alcohol. The product, rubbed up with water to give a thin paste, is then treated with absolute alcohol. The crystalline residue is separated by suction, washed with absolute alcohol, and dried; yield, 2 g. To purify, the crude dipeptide is dissolved in 40 parts of hot water and three volumes of absolute alcohol added. The dipeptide then separates in a crop of brightly glittering crystals. By concentrating the filtrate and further addition of alcohol, a second crop of crystals is obtained, bringing the total yield to nearly 90% of the raw product. The substance was dried in a vacuum desiccator over sulfuric acid: calcd. 10.76, found 10.68% N; $[\alpha]^{14}D$ +10.2° in *N* HCl (according to Fischer, $[\alpha]^{20}D$ +10.4° and +10.5° in *N* HCl). To confirm the identity, the substance was boiled in 30 parts of about 4 *N* hydrochloric acid for fifteen hours. An equimolar mixture of *l*-leucine and *l*-glutamic acid was similarly treated. Both solutions were evaporated and the residue dried in a vacuum desiccator over sulfuric acid and soda lime. The specific rotations of the two amino acid hydrochloride mixtures were found, respectively, $[\alpha]^{19}D$ +15.7° and $[\alpha]^{19.5}D$ +15.7°.

Preparation of *l*-Leucyl-*l*-glutamic Acid Anhydride.—Five grams of dipeptide is mixed with 50 g. of β-naphthol and the mixture heated in an oil-bath at 170–180° for an hour. The limpid hot solution is poured into a mortar where it solidifies. The solid is broken up, and left to stand in a vacuum desiccator over sulfuric acid for a few days. The powdered mixture is then suspended in ether, the ether drawn off by suction, and the residue washed with ether and dried; yield 2–2.1 g. The material was purified by recrystallizing from hot absolute alcohol as follows: 2 g. of the raw substance is dissolved in 70 cc. of hot alcohol, boiled with carbo animalis and filtered. A crystalline deposit is obtained by cooling. It is separated by suction, washed with alcohol and dried. A second crop is obtained from the filtrate by reduction of its volume by evaporation. The yield is three-fourths of the raw material. For further purification, the substance is recrystallized from 40 parts of hot water. It separates on cooling as a colorless crystalline mass. The latter is separated by suction, washed with water, and dried *in vacuo;* yield, 60% of the alcohol recrystallized material. For analyses, the substance is dried *in vacuo* over phosphorus pentoxide in a drying apparatus heated with boiling xylene. *Anal.* Calcd. for $C_{11}H_{18}O_4N_2$: C, 54.50; H, 7.49; N, 11.57. Found: C, 54.40; H, 7.41; N, 11.63. 53.6 mg. consumed 2.17 cc. of 0.1 *N* alcoholic KOH (Willstätter–Waldschmidt-Leitz method), calcd. 2.21 cc. The Linderstrøm–Lang titration value was 0; m. p. 213–215° (uncor.); α for 71.8 mg. dissolved in 10 cc. of 0.1 *N* ammonia in a decimeter tube at 18° was found to be −0.34°; $[\alpha]^{18}D$ −47.4° (in 0.1 *N* ammonia). Hydrolysis with hydrochloric acid in the conditions employed previously to split the dipeptide yielded an amino acid hydrochloride mixture of $[\alpha]^{24}D$ +15.4°.

Enzyme Experiments.—(a) The diketopiperazine was suspended in water, dissolved in dilute sodium hydroxide (added dropwise) and incubated in phosphate buffer (*p*H 8.1) with a glycerol extract of pancreatin[8] at 37° for two days. Formol titrations showed that cleavage had not occurred. (b) Solutions of the diketopiperazine were incubated in phosphate buffer (*p*H 8) with purified pancreatic proteinase (prepared according to a modification of the method of L. Weil[9]) at 37° for three days. Van Slyke amino determinations showed that cleavage had not occurred. (c) Suspensions of the substrate in citrate buffer *p*H 5 were incubated with papain[10] (activated by hydrocyanic acid) at 37° for two days. Samples were removed, brought to solution with sodium hydroxide, and examined in the Van Slyke apparatus. No cleavage was found.

Summary

l-Leucyl-*l*-glutamic acid is obtained in optically pure form by treating the aminated product of the coupling of *d,l*-α-bromoisocaproyl bromide and *l*-glutamic acid with water and alcohol.

The dipeptide is converted into the corresponding diketopiperazine, *l*-leucyl-*l*-glutamic acid anhydride, on solution in β-naphthol at 170–180°.

l-Leucyl-*l*-glutamic acid anhydride is not hydrolyzed by a glycerol extract of pancreatin, purified pancreatic proteinase, or papain.

JERUSALEM, PALESTINE RECEIVED JANUARY 7, 1944

(4) M. Bergmann, L. Zervas and J. S. Fruton, *J. Biol. Chem.*, **111**, 225 (1935).

(5) E. Waldschmidt-Leitz and M. Gaertner, *Z. physiol. Chem.*, **244**, 221 (1936).

(6) S. Akabori and S. Takase, *Proc. Imp. Acad. Tokyo*, **12**, 242 (1936); S. Akabori and S. Maeda, *ibid.*, **13**, 213 (1937).

(7) E. Abderhalden, K. Weichert, H. Schumann and E. Haase, *Fermentforschung*, **16**, 182 (1940); *C. A.*, **36**, 3194 (1942).

(8) Parke, Davis & Co.

(9) N. Lichtenstein, *Enzymologia*, **6**, 108 (1939).

(10) B. D. H.