to the acetyl derivatives of *dl*-phenylalanine, *dl*-norleucine, *dl*-leucine and *dl*-norvaline. Attempts to use the secondary halides, *s*-butyl bromide and isopropyl bromide, in similar alkylations were unsuccessful. *dl*-Glutamic acid is prepared from diethyl acetamidomalonate and methyl acrylate.

URBANA, ILL. RECEIVED NOVEMBER 20, 1944

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF GEORGE A. BREON & COMPANY]

3,12-Dihydroxy-7-ketocholanic Acid[1]

BY WILLARD M. HOEHN AND JACOB LINSK

Various investigators[2] have studied the partial oxidation of cholic acid. The results published to date have indicated varying degrees of success. This paper presents two methods which have been found to give good yields and which may be readily applied to large quantities, for the isolation of pure 3,12-dihydroxy-7-ketocholanic acid. Information is presented also to explain the melting points assigned to this acid by the investigators.

In an effort to ascertain the purity of the ethyl 3,12-dihydroxy-7-ketocholanate, a mixture was prepared with ethyl cholate. The melting point was observed to be higher than that for either compound. Crystallization of a mixture of various quantities of these esters from methanol gave a complex which contained one molecule of each compound. The melting point of the complex was approximately 10° higher than that of the ethyl 3,12-dihydroxy-7-ketocholanate. Among the derivatives prepared were ethyl 3-benzoxy-7-keto-12-hydroxycholanate, 3,12-diformoxy-7-ketocholanic acid[3] and a monobromo derivative of ethyl 3,12-diacetoxy-7-ketocholanate. The bromo derivative was recovered after treatment with dry pyridine or collidine at reflux temperatures. Further work is being carried out with this bromo compound which is tentatively considered to have the bromine at C_6.

The oxidation of non-crystalline ethyl 3-hydroxy-7-keto-12-acetoxycholanate produces a crystalline ethyl 3,7-diketo-12-acetoxycholanate (m. p. 164–165°; $[\alpha]^{25}D$ + 38° (dioxane)). Hydrolysis of this ester gave the corresponding acid (m. p. 168–169°; $[\alpha]^{25}D$ − 13° (dioxane)). The structure of the compound was confirmed by reducing it to the known 12-hydroxycholanic acid.[4] Oxidation gave the 12-ketocholanic acid which was directly compared with a sample prepared from desoxycholic acid. Methyl 12-hydroxycholanate prepared from the corresponding hydroxy acid was also compared with an authentic sample.

The authors wish to express their gratitude for technical assistance given by Jane Stickley and Mary B. Flint.

Experimental[5]

Ethyl 3,12-Dihydroxy-7-ketocholanate: (a) Bromine Oxidation of Cholic Acid.—In a three-necked flask 500 g. of cholic acid was dissolved in 3.0 liters of water containing 70 g. of sodium hydroxide and to this solution was added 275 g. of sodium bicarbouate. The solution (approximately 3.5 liters) was maintained at a temperature of −5 to −2° by means of an ice–salt-bath and with vigorous stirring a solution of 200 g. of technical bromine in 50 cc. of chloroform was added over a period of two hours. Stirring was continued for two hours longer and the mixture allowed to stand two days, when it was diluted to 6 liters with water and acidified to congo red with hydrochloric acid. The material which precipitated in a lump was washed free of mineral acid by kneading under a stream of cold tap water. The oxidized material was spread evenly on a glass tray and dried to constant weight. The solid was dissolved in 1.3 liters of standard denatured 2B alcohol to which 50 g. of sulfuric acid had been added. The solution was allowed to stand at room temperature overnight and filtered. The esterification mixture was taken into benzene and washed with water, dilute sodium bicarbonate solution and finally with water. The benzene solution was dried by distillation of the benzene and the solvent completely removed at 20 to 25 mm. pressure while the resinous mass was heated in a bath of boiling water. The residue was dissolved in 1 liter of methanol and on standing overnight the crystalline ester which formed was filtered by suction and washed with 50 cc. of methanol. The ethyl ester (m. p. 152–157°), after air drying, weighed 287 g. Recrystallization from 600 cc. of methanol gave 216 g. of ethyl 3,12-dihydroxy-7-ketocholanate (2c) (m. p. 158–160°; $[\alpha]^{25}D$ + 2 ± 2° (dioxane)). A second crop of ethyl ester was obtained on concentration of the mother liquor.

(b) Chromic Acid Oxidation of Ethyl Cholate.—To a solution of 88 g. of ethyl cholate in 500 cc. of 70% acetic acid solution, cooled to −5°, was added 85 cc. of *N* chromic acid solution over a period of one and a half hours. The mixture was stirred during the addition of the oxidant and after another half hour of stirring the reaction mixture was poured into water and extracted with benzene. The benzene layer was washed with water, dilute hydrochloric acid and again with water. The benzene was removed,

(1) Reported in part at the April, 1944, meeting of the American Chemical Society at Cleveland, Ohio.

(2) For instance: (a) Kaziro and Shimado, *Z. physiol. Chem.*, **249**, 220 (1937); (b) Charonnat and Horeau, U. S. Patent 2,244,328, June 3, 1941; (c) Haslewood, *Biochem. J.*, **109**, 107 (1943); (d) Gallagher and Long, *J. Biol. Chem.*, **147**, 133 (1943); (e) Hoehn, Schmidt and Hughes, *ibid.*, **156**, 59 (1944). (f) An article by Haslewood (*Biochem. J.*, **38**, 108 (1944)) has been brought to our attention since this paper was submitted. Haslewood described the 3,12-dihydroxy-7-ketocholanic acid (m. p. 196–197°), the 3,7-diketo-12-hydroxycholanic acid (m. p. 165–166°), the ethyl 3-benzoxy-12-hydroxy-7-ketocholanate (m. p. 138–139°) aud ethyl 3-benzoxy-7,12-diketocholanate (m. p. 168–169°).

(3) The authors are grateful to Dr. R. B. Moffett for the preparation of this compound: *cf.* "Preparation of Phenyl Ketones from Bile Acids," Moffett and Hoehn, presented at Org. Div. A. C. S. April, 1904.

(4) (a) Wieland and Schlichting, *Z. physiol. Chem.*, **150**, 270 (1925); (b) Wieland and Kapitel, *ibid.*, **212**, 276 (1932); (c) Barnett and Reichstein, *Helv. chim. acta*, **21**, 926 (1938).

(5) All specific rotations observed by J. Stickley; neutral and saponification equivalents determined by J. Stickley.

the residue dissolved in 150 cc. of methanol and the solution set aside overnight. The ester, which crystallized out, was filtered and after drying weighed 41.8 g. Recrystallized from 100 cc. of methanol, it gave 29.8 g. of ethyl 3,12-dihydroxy-7-ketocholanate (m. p. 157–159°). Another recrystallization from methanol raised the melting point to 160–162°. The specific rotation in dioxane was +1.5 ± 2°. The modified Pettenkofer reaction was negative.

The mother liquor from the first filtration was concentrated to 90 cc., mixed with 90 cc. of 2.5 *N* sodium methoxide solution and 20 cc. of 85% hydrazine hydrate and the mixture heated to 180–200° for three hours. The reaction was worked up as usual[2e] for desoxycholic acid which was isolated as the acetic acid–choleic acid (10.5 g.) (m. p. 139–141°). A trace of lithocholic acid (m. p. 184–185°) was isolated. Neither of these compounds gave depressions with the corresponding authentic specimens.

3,12-Dihydroxy-7-ketocholanic Acid.—Five grams of ethyl 3,12-dihydroxy-7-ketocholanate was heated to reflux with 10 cc. of methanol and 10 cc. of 2 *N* sodium hydroxide for five minutes. The reaction mixture was diluted with 100 cc. of water and poured with stirring into 300 cc. of 0.1 *N* hydrochloric acid. The crude acid crystallized within a few minutes after it was precipitated. The solid was filtered off and air-dried under a radiant lamp at a temperature of 70–80°. The acid melted at 87–89°, melted at 163–164° when dried at 80° and when dried at 100–110° was found to melt at 170–171°. The specific rotation was observed to be $[\alpha]^{25}D$ + 1.5 ± 1° (dioxane). This acid gave a negative test in the modified Pettenkofer test.

Anal.[6] Calcd. for $C_{24}H_{38}O_5$: C, 70.91; H, 9.44; neut. eq., 406. Found: C, 71.22; H, 9.56; neut. eq., 414.

Crystallization of this acid from ethyl acetate gave a product that melted at 199–200°. The modified Pettenkofer reaction was still negative. A neutral equivalent of 465 was observed on an air-dried sample (m. p. 87–89°) which calculated for 3 molecules of water of crystallization.

Ethyl 3-Benzoxy-7-keto-12-hydroxycholanate.—To a solution of 4.34 g. of ethyl 3,12-dihydroxy-7-ketocholanate in 25 cc. of dry benzene was added 1 cc. of dry pyridine and 1.2 cc. of benzoyl chloride. The reaction mixture was worked up for the neutral fraction, which, after removal of the benzene, was crystallized from methanol. The benzoate melted at 140–142° after two further crystallizations from methanol; $[\alpha]^{25}D$ + 29 ± 1° (dioxane).

Anal.[6] Calcd. for $C_{33}H_{44}O_6$: C, 73.66; H, 8.22; sapn. eq., 270. Found: C, 73.07; H, 8.07; sapn. eq., 270.

The oxidation of the benzoate led to a compound ((m. p. 167–168°), neut. eq. 269[5] (calcd. 269), $[\alpha]^{25}D$ +50°)), which, on hydrolysis, gave an acid that was crystallized from ethyl acetate and melted at 187–188°; mixed with reductodehydrocholic acid, it gave no depression in melting point.

Anal.[6] Calcd. for $C_{33}H_{42}O_6$: C, 73.96; H, 7.90. Found: C, 73.70; H, 8.29.

Ethyl 3,7-Diketo-12-acetoxycholanate.—Twenty grams of ethyl 3,12-dihydroxy-7-ketocholanate was dissolved in 45 cc. of acetic anhydride and refluxed for one hour. The acetic anhydride was removed by distillation at a pressure of 20–25 mm. and the residue dissolved in 50 cc. of benzene and the benzene removed by distillation *in vacuo*. The residue was dissolved in 100 cc. of 1.0% hydrogen chloride in methanol and allowed to stand overnight.[4e] The solution was mixed with 200 cc. of ether and 200 cc. of water. The ether layer was washed free of acidic substance and the ether removed. The gum was dissolved in 30 cc. of glacial acetic acid. This solution was cooled to 10° and 75 cc. of 2.3 *N* chromic acid in 90% acetic acid solution added. The reaction mixture stood about two hours, was diluted with 600 cc. of water and the crystalline solid filtered by suction. The solid was washed on the suction funnel with water, dried at 100–110° and crystallized from 40 cc. of ethyl acetate. The ethyl 3,7-diketo-12-acetoxycholanate melted at 164–165°; $[\alpha]^{25}D$ + 38 ± 2° (dioxane). This compound gave a negative modified Pettenkofer reaction and a positive Zimmermann reaction.

Anal.[6] Calcd. for $C_{28}H_{42}O_6 \cdot {}^1/_2H_2O$: C, 69.55; H, 8.98; neut. eq., 246. Found: C, 70.06; H, 9.06; neut. eq., 235.

A mixture of 4.2 g. of the ethyl ester, 10 cc. of methanol and 5 cc. of 20% sodium hydroxide solution was heated for ten minutes, diluted with 50 cc. of boiling water and filtered. The filtrate was cooled, acidified, ether extracted and the ether solution washed with distilled water. The ether was removed and the gummy residue dissolved in 15 cc. of ethyl acetate and recrystallized. The crystalline material was filtered, dried at 100° and 0.1 mm. of pressure and melted at 168–169°; $[\alpha]^{25}D$ − 13° ± 1°. The modified Pettenkofer test was negative and the Zimmermann test positive for this compound.

Anal.[6] Calcd. for $C_{24}H_{36}O_5$: C, 71.29; H, 8.97; neut. eq., 404. Found: C, 70.76; H, 9.01; neut. eq., 403, 412.

Methyl 12-Hydroxycholanate and 12-Ketocholanic Acid.—Five grams of ethyl 3,7-diketo-12-acetoxycholanate was mixed with 40 cc. of 6% sodium methoxide and 5 cc. of 85% hydrazine hydrate. This mixture was heated at 180–200° for three hours in a steel bomb. The contents of the tube were cooled, acidified and half of the gummy acidic material converted to the methyl ester by means of methanol and sulfuric acid. Recrystallized from 90% methanol, the ester melted at 118–119° and when mixed with methyl 12-hydroxycholanate, m. p. 118–119°, prepared in a similar manner from methyl 3-keto-12-acetoxycholanate,[4] showed no depression in melting point. The remainder of the product from the Wolff–Kishner reaction was dissolved in 10 cc. of acetic acid and oxidized with 15 cc. of *N* chromic acid in 90% acetic acid. The keto acid, which crystallized out of the oxidation mixture, was recrystallized from methanol and melted at 187–189°; $[\alpha]^{25}D$ + 90°. When mixed with a sample of 12-ketocholanic acid prepared in a similar manner from the reduction product, m. p. 187–189°, of methyl 3-keto-12-acetoxycholanate, no depression in melting point was observed.

Crystallizate of Ethyl Cholate and Ethyl 3,12-Dihydroxy-7-ketocholanate.—One gram of ethyl cholate and 1 g. of ethyl 3,12-dihydroxy-7-ketocholanate were crystallized from 10 cc. of methanol. The crystalline material was dried at 100° and 0.1 mm. pressure and was observed to melt at 167–168°. The Schmidt modification of the Gregory–Pascoe reaction[2e] was used to determine the cholic acid present in the form of ethyl cholate. The ethyl cholate used for the mixture was compared with a cholic acid standard and the crystallizate was found to consist of one molecule of ethyl cholate to one molecule of ethyl 3,12-dihydroxy-7-ketocholanate. The specific rotation of $[\alpha]^{25}D$ + 30 ± 0.5° (10 mg./cc. of dioxane) was observed for the ethyl cholate while the crystallizate had a specific rotation of $[\alpha]^{25}D$ + 15° (10 mg./cc. of dioxane).

Ethyl 3,12-Diacetoxy-6-bromo-7-ketocholanate.—Four grams of ethyl 3,12-dihydroxy-7-ketocholanate was heated to reflux for two hours in a solution of 20 cc. of acetic acid and 20 cc. of acetic anhydride. The mixture was heated thirty minutes longer with 50 cc. of methanol and the solvents removed *in vacuo*. Twenty cc. of acetic acid was used to dissolve the residue, the solution cooled to 15° and 19.2 cc. of *N* bromine–acetic solution added. The flask was stoppered and heated to 60–70° for one hour and the contents poured into 200 cc. of water, the solid filtered off and dried at 80–90°. The crude bromo compound was crystallized from 12 cc. of methanol. The melting point of the bromo compound was 129–130°.

Anal.[7] Calcd. for $C_{30}H_{45}O_7Br$: Br, 13.37. Found: Br, 13.3, 13.63.

Formate of 3,12-Dihydroxy-7-ketocholanic Acid.—One gram of 3,12-dihydroxy-7-ketocholanic acid in 2 cc. of formic acid (sp. gr. 1.20) was heated at 70–80° for five hours. The formic acid was partially removed *in vacuo* and the crystalline material filtered by suction and washed

(6) Carbon and hydrogen analyses by the Arlington Laboratories.

(7) Bromine determination by M. B. Flint.

with dilute formic acid on the filter. The dried sample melted at 204–208°.[3]

Anal.[6] $C_{26}H_{38}O_7$: C, 67.51; H, 8.28. Found: C, 67.14; H, 8.18.

Summary

The preparation of ethyl 3,12-dihydroxy-7-ketocholanate is described and subsequent hydrolysis of the ester yielded 3,12-dihydroxy-7-ketocholanic acid.

The various melting points described for the 3,12-dihydroxy-7-ketocholanic acid are discussed.

The hitherto unknown 3,7-diketo-12-hydroxycholanic acid is described.

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[CONTRIBUTION FROM THE WESTERN REGIONAL RESEARCH LABORATORY[1]]

Action of Aromatic Isocyanates on Proteins

BY HEINZ FRAENKEL-CONRAT, MITZI COOPER AND HAROLD S. OLCOTT

Aromatic isocyanates in anhydrous media are widely used for the characterization of alcohols and amines. However, since the amino group of amino acids reacts smoothly with isocyanates only in aqueous alkaline solution,[2] proteins were usually treated with isocyanates at *p*H 8–9. Hopkins and Wormall[3] showed that under mild conditions the reaction involves the amino groups almost exclusively, while others[4] have since observed that phenolic and thiol groups also may react.

It has been found in this Laboratory that in the absence of water and at elevated temperature the treatment of proteins with phenyl isocyanate causes the introduction of considerably more of the reagent than can be accounted for by the number of amino, thiol, and phenolic groups known to be present. Determination of the specific groups responsible for the unexpected extent of the reaction was the objective of this investigation.[5]

In general, the reaction was accomplished by heating a suspension of the dry protein in phenyl isocyanate and pyridine for twenty-four hours at 70°.[6] Four criteria were used to measure the extent of the interaction: (1) The weights of the protein samples were increased through addition of the isocyanate. The final weight gains after washing and extracting varied to a certain extent with the solubility characteristics of the original proteins, but, for any one protein, the extent of interaction was expressed clearly by the amount of reaction product isolated. (2) Chlorine analyses after treatment with chlorophenyl isocyanates furnished an alternate measure of the over-all extent of interaction, independent of other properties of the protein. On this basis hoof powder bound *o*- and *p*-chlorophenyl isocyanates to the extent of 39 and 45% of its weight, respectively. Such analyses were particularly useful in determining the small amount of combination of isocyanates with substances containing few reactive groups. (3) The decrease in hydrophilic property was found to be indicative of the extent of reaction.[5] (4) Protein-group analyses permitted a measure of the degree of substitution of specific polar groups. All types of acid and basic, primary amide, and, indirectly, peptide nitrogen groups were studied.

Reaction with Basic and Acid Groups.—The study of the nature of the reaction was facilitated by newly developed methods for the determination of total acid and basic groups applicable to insoluble proteins.[7] A comparison of the number of basic groups of treated and untreated proteins revealed the fact that these had been almost completely abolished by the treatment (Table I). Thus not only the primary amino groups of lysine, but also the guanidyl and imidazole residues of arginine and histidine, appeared to react with isocyanates under the conditions used. Since insulin is particularly rich in histidine, the loss of most of its basic groups was confirmatory evidence of the reactivity of the imidazole group.

In addition the number of acid groups of proteins was greatly decreased. The magnitude of this effect indicated that the carboxyl, as well as the thiol and phenolic groups, had reacted. The action of aromatic isocyanates on simple carboxylic acids has been repeatedly studied.[8] The reaction follows two paths, one resulting in the formation of acyl anilides and carbon dioxide and the other in the formation of anhydrides, N,N′-diphenyl ureas and carbon dioxide. Experiments carried out on amino acid derivatives in general confirmed these findings.

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture. Article not copyrighted.

(2) Paal, *Ber.*, **27**, 974 (1894); **38**, 2359 (1905).

(3) Hopkins and Wormall, *Biochem. J.*, **27**, 740 (1933); **28**, 2125 (1934).

(4) (a) Miller and Stanley, *J. Biol. Chem.*, **141**, 905 (1941); (b) Fraenkel-Conrat, *ibid.*, **152**, 385 (1944).

(5) The products of the anhydrous reaction of phenyl isocyanate and proteins were water-resistant to the extent that they appeared to have promise in the field of plastics. Data on this phase of the subject will be presented elsewhere.

(6) The use of pyridine was suggested by the work of Hearon, Hiatt and Fordyce (THIS JOURNAL, **65**, 829 (1944)) who studied the anhydrous reaction of phenyl isocyanate with cellulose. Control experiments, in connection with the work here reported, indicated that the polar groups of proteins were not affected by pyridine alone.

(7) Fraenkel-Conrat and Cooper, *J. Biol. Chem.*, **154**, 239 (1944).

(8) The literature has been reviewed by Naegeli and Tyabji, *Helv. Chim. Acta*, **17**, 931 (1934).