MICROBIOLOGICAL DEGRADATION OF BILE ACIDS
VI. ON THE STRUCTURE OF THE UNSATURATED C-22 ACID TRANSFORMED FROM CHOLIC ACID BY S. GELATICUS 1164

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In the previous papers (1-3), it was demonstrated that a new unsaturated C-22 acid of m.p. 280-282° (decomp.) was obtained from cholic acid by the action of S. gelaticus 1164. The new compound was presumed to be 7α-hydroxy-3,12-diketo-Δ⁴,9(11)-bisnorcholedienic acid, but there was no definite proof on the positions of the two double bonds in the cholane nucleus. Recently the ultraviolet and infrared absorption characteristics of the new compound and a degradation product of it were investigated, and the results obtained showed that the new acid may be 7α-hydroxy-3,12-diketo-Δ⁴-bisnorcholenic acid.

EXPERIMENTAL

Acid with M. P. 280-282° (Decomp.) and Its Methyl Ester

Sodium cholate (0.3 g./dl.) was incubated with S. gelaticus 1164 by a shaking culture as described in the previous paper (4), and the new acid of m.p. 280-282° (decomp.) was isolated by the same method as described in the first paper (1) of this series. The infrared spectrum (Fig. 1-A) of this acid showed the absorption bands (in Nujol) at the following wavelengths: 3.00, 5.83, 6.05, 6.21 and 6.25 μ. As this acid was insoluble in chloroform, the measurement of the infrared spectrum in chloroform was impossible. The infrared spectrum (Fig. 1-B) of the methyl ester, m.p. 244-246° (decomp.), of this acid (1) showed the following absorption bands (in Nujol): 2.99, 5.75, 5.87, 5.99, 6.175 and 6.24 μ.

Dehydration Product of the Above Acid and Its Methyl Ester

Formation of Acid by Caustic Alkali—Four hundred and eighty milli-723
grams of the methyl ester, m. p. 244–246° (decomp.), was dissolved in 100 ml. of warm methanol. To this solution 4.5 g. of potassium hydroxide dissolved in 50 ml. of water was added and the mixture was refluxed on a boiling water bath for 6 hours. The resulting deep red-brown solution was cooled, neutralized with dilute hydrochloric acid, concentrated in vacuum to about 40 ml. and acidified by dropwise addition of dilute hydrochloric acid. After cooling, the crystalline precipitate was collected and washed. Crystallization from ethyl acetate gave 150 mg. of yellow needles, m. p. 240–250° (decomp.). Twice recrystallizations from acetone gave pale yellow needles sintering at 240° and melting at 250–252° with decomposition, $\lambda_{\text{max}}^\text{calc.}$ 280.5 mÊ (log $\varepsilon$ 4.43). The infrared spectrum (Fig. 1-C) of this acid for carboxyl and ketone groups showed the following two bands (in Nujol): 5.805 and 5.88 mÊ, and there was no hydroxyl band. Furthermore, the spectrum (in Nujol) for $\Delta^{4,6}$-3-ketone group in this acid showed only two bands (6.11 and 6.19 mÊ) and an absorption at around 6.3 mÊ was not clear, but showed characteristic $\Delta^{4,6}$-3-ketone absorption at the fingerprint region.

**Analysis.**

Calcd. for C$_{22}$H$_{28}$O$_4$: C, 74.13; H, 7.92.

Found: C, 74.03; H, 8.13.

The dienone acid above obtained was also obtained by refluxing the above original acid, m. p. 280–282° (decomp.), with aqueous caustic alkali. The saponification and dehydration of the methyl ester, m. p. 244–246° (decomp.), by alcoholic potassium carbonate was incomplete in one hour and gave a mixture of this dienone acid, m. p. 250–252° (decomp.), and the unsaponified original ester.

The methyl ester of this dienone acid, prepared by esterification with diazomethane in ether by the usual method, was crystallized from methanol in pale yellow, prismatic needles, m. p. 182–184° without decomposition, $\lambda_{\text{max}}^\text{calc.}$ 280.5 mÊ (log $\varepsilon$ 4.45). The infrared spectrum showed the following absorption bands (in Nujol): ester and ketone, 5.79 and 5.84 mÊ; $\Delta^{4,6}$-3-ketone; 6.04, 6.18 and 6.33 mÊ. This spectrum (Fig. 1-D) showed no hydroxyl absorption, but characteristic $\Delta^{4,6}$-3-ketone absorption both at around 6 mÊ and at the fingerprint region.

**Analysis.**

Calcd. for C$_{23}$H$_{30}$O$_4$: C, 74.56; H, 8.16.

Found: C, 74.49; H, 8.30.

**Formation of Ester by Sulfuric Acid**—A suspension of 200 mg. of the C-22 acid, m. p. 280–282° (decomp.), in 4 ml. of methanol containing
two drops of concentrated sulfuric acid was refluxed on a boiling water bath for 30 minutes. Soon the acid dissolved into the reaction mixture. The resulting green solution was diluted with dilute sodium bicarbonate solution and the crystalline precipitate which was separated on standing, was collected, washed with water and dried. The filtrate and washings were combined and stored for the later experiment. Crystallization of the dried precipitate from ethyl acetate afforded 70 mg. of pale yellow needles of m.p. 179-181°. This methyl ester gave no depression of melting point with the methyl ester, m. p. 182-184°, of the dienone acid, m. p. 250-252° (decomp.), obtained from the C-22 acid by refluxing with methanolic potassium hydroxide and further an identification of the both esters was confirmed by infrared spectrum.

*Analysis.*
Calcd. for C_{23}H_{30}O_{4}:  C, 74.56; H, 8.16.
Found:  C, 74.46; H, 8.53.

The combined filtrates were acidified with dilute hydrochloric acid and kept at O° for several hours. The resulting precipitate was collected, washed with a small volume of water and dried. Crystallization of the dried substance from acetone gave 12 mg. of pale yellow needles sintering at 240° and melting at 249-251° with decomposition. This acid gave no depression of melting point with the acid, m. p. 250-252° (decomp.), obtained from the C-22 acid with potassium hydroxide and infrared analysis confirmed the identity of these acids.

*Analysis.*
Calcd. for C_{22}H_{28}O_{4}:  C, 74.13; H, 7.92.
Found:  C, 74.44; H, 8.18.

For this dehydration reaction which was catalyzed with proton, the following procedure was adopted and the same result as described above was obtained. A suspension of 100 mg. of methyl ester, m. p. 244-246° (decomp.), of the C-22 acid in 5 ml. of methanol containing three drops of concentrated sulfuric acid was left to stand at room temperature. The ester dissolved into the reaction mixture within 24 hours. After four days, the solution was diluted with dilute sodium bicarbonate solution and the crystalline precipitate was collected, washed with water and dried. By acidifying the filtrate and washings no precipitate occurred. Crystallization of the dried substance afforded 80 mg. of colorless needles of m. p. 181-183°. This ester gave no depression of melting point with the ester obtained from the C-22 acid by sulfuric acid or potassium hydroxide and infrared analysis confirmed
the identity of the three esters.

*Analysis.*

Calcd. for C_{23}H_{30}O_{4}:  C, 74.56;  H, 8.16.
Found:  C, 74.46;  H, 8.37.

*Infrared Spectra*

The infrared spectra of this experiment were measured kindly by Mr. Y. Matsui of Research Laboratory, Shionogi & Co., Ltd. with a Perkin-Elmer Spectrophotometer, model 12 C.

![Infrared spectra in Nujol mull: A; C-22 acid, B; methyl ester of the C-22 acid, C; dehydration product (acid) of the C-22 acid, D; dehydration product (methyl ester) of the C-22 acid, E; 3,12-diketo-Δ4,8-choladienic acid (synthetic sample), F; methyl 3,12-diketo-Δ4,8-choladionate (synthetic sample).]
DISCUSSION

Ultraviolet absorption analysis played an important role in substantiating the structures of steroids. Dorfman (5) in a noteworthy paper on the ultraviolet absorption spectra of steroids states that a \( \Delta^4 \)-3-ketone generally absorbs selectively at 241 m\( \mu \) with an average molecular extinction coefficient of 16,600 (e.g., methyl 12\( \alpha \)-hydroxy-3-keto-\( \Delta^4 \)-bisanorholenate, \( \lambda_{\text{max}} \) 241 m\( \mu \), \( \varepsilon \) 14,000), whereas a \( \Delta^9(11) \)-12-ketone absorbs selectively at 240 m\( \mu \) with an average molecular extinction coefficient of 11,200 (e.g., methyl 3\( \alpha \)-hydroxy-12-keto-\( \Delta^9(11) \)-cholenate, \( \lambda_{\text{max}} \) 240 m\( \mu \), \( \varepsilon \) 12,000). Further, it is well known that \( \Delta^4,16 \)-pregnadiene-3,20-dione (6) which contains two isolated \( \alpha, \beta \)-unsaturated ketonic chromophores, namely, \( \Delta^4 \)-3-ketone and \( \Delta^9(11) \)-12-ketone absorbs selectively at 241 m\( \mu \), with a molecular extinction coefficient of 25,200.

As described in the previous papers (1-3), the new unsaturated C-22 acid, m. p. 280-282\( ^\circ \) (decomp.), was presumed to be 7\( \alpha \)-hydroxy-3,12-diketo-\( \Delta^4(9(11)) \)-bisanorcholadienic acid and the ultraviolet absorption spectrum of the acid showed a maximum at 240.3 m\( \mu \) (\( \varepsilon \) 11,500) in alcohol. The presence of a bile acid containing two chromophores of \( \Delta^4 \)-3-ketone and \( \Delta^9(11) \)-12-ketone is not yet known, but the molecular extinction coefficient of the C-22 acid is about half compared with that of \( \Delta^4,16 \)-pregnadiene-3,20-dione which has two chromophores in the molecule. Thus the \( \Delta^4,9(11) \)-3,12-diketone structure postulated for the C-22 acid is now doubtful. So the infrared absorption spectra of this acid and its methyl ester were determined in order to confirm the presence of two chromophores of \( \beta \)-3-ketone and \( \Delta^9(11) \)-12-ketone in these molecules. The spectrum (Fig. 1-A) of the acid showed the following absorption bands: hydroxyl, 3.00 m\( \mu \); carboxyl and six-membered cyclic ketone, 5.83 m\( \mu \); conjugated ketone, 6.05 m\( \mu \); conjugated double bond, 6.21 and 6.25 m\( \mu \), and the spectrum (Fig. 1-B) of the methyl ester showed the following absorption bands which is similar to the original acid: hydroxyl, 2.99 m\( \mu \); ester and ketone, 5.75 and 5.87 m\( \mu \); conjugated ketone, 5.99 m\( \mu \); conjugated double bond, 6.175 and 6.24 m\( \mu \). The band systems centered around 6 m\( \mu \) of both acid and methyl ester indicated the presence of a non-conjugated ketone in those molecules and displayed bands at approximately 6.00, 6.17 and 6.24 m\( \mu \), this triad being characteristic of the \( \Delta^1,4 \)-3-ketone grouping (7, 8). So the possibility of the structure of \( \Delta^4(9(11)) \)-3,12-diketone postulated previously for the C-22 acid was eliminated.
From a consideration of the above-stated absorption characteristic it seems most reasonable to conclude that the C-22 acid is 7α-hydroxy-3,12-diketo-Δ4,14-bisnorcholadienic acid, because it was confirmed synthetically (3) that the hydrogenated C-22 acid was 7α-hydroxy-3,12-diketobisnorcholanic acid. Recently, Jones et al. (9) reported the infrared spectra of keto-steroids at the region of longer wave length (7–15 μ). The band systems at the fingerprint region of the C-22 acid and its methyl ester as shown in Fig. 1-A and 1-B does not coincide with the band systems of various Δ4,14-3-keto-steroids examined by Jones et al. (9), but that resemble with the spectrum of Δ4-3-ketone rather than that of Δ4,14-3-ketone. Further, since a partial synthesis of 7α-hydroxy-3,12-diketo-Δ4,14-bisnorpholadienic acid from cholic acid is rather cumbersome, it was thought advisable to investigate a degradation product of this C-22 acid for establishing a constitution of the C-22 acid.

Fieser et al. (10) reported that methyl 3α,7α-diacetoxy-12-keto-Δ9(11)-choenate affords 3α-hydroxy-12-keto-Δ7,9(11)-choladienic acid by heating with caustic alkali and stated that the facile dehydration reaction of C7α-hydroxyl (or acetoxyl) with C8-hydrogen atom is attributable to activation of the C8-hydrogen atom by 9,11-double bond. Also Greenhalgh et al. (11) reported that the only product isolated from the hydrolysis of 7α-hydropyranyloxy-Δ4-cholestene-3-one was Δ4,6-cholestadiene-3-one, the 7α-hydroxy-Δ4-cholestene-3-one, presumably formed initially, having undergone dehydration in the acidic medium. These dehydration reactions were applied for the elimination of the C7α-hydroxyl groups of the C-22 acid and its methyl ester, and the dehydration products were examined. The dehydration product of the C-22 acid and its methyl ester by caustic alkali was obtained in crystalline form, and the data of the microanalysis gave a formula C22H28O4, its ultraviolet absorption spectrum showed the maximum at 280.5 mμ (log ε 4.43), as exhibited by Δ4,6-3-ketones and the infrared spectrum (Fig. 1-C) showed characteristic Δ4,6-3-ketone absorption although an absorption at around 6.3 μ was not clear. Furthermore, the methyl ester of the above-described dehydration product by alkali was identical with the methyl esters obtained from the C-22 acid and its methyl ester through the dehydration reaction by sulfuric acid and the both esters coincided with a formula C23H30O4, its ultraviolet absorption spectrum showed a maximum at 280.5 mμ (log ε 4.45) and the infrared spectrum showed the following characteristic Δ4,6-3-ketone absorption both at around 6 μ and at fingerprint region as described in Fig. 1-D: ester and C12-ketone, 5.79 and 5.84 μ; Δ4,6-3-ketone, 6.04, 6.18 and 6.33 μ.
From these microanalysis data and ultraviolet and infrared absorption characteristics (5, 8, 12), it was suggested that the 7α-hydroxyl group in the C-22 acid was easily dehydrated to occur 3,12-diketo-$\Delta^{4,6}$-bisnorcholadienic acid by catalytic action of hydroxyl ion or proton. In order to confirm the presence of $\Delta^{4,6}$-3,12-diketone group in the dehydration product, 3,12-diketo-$\Delta^{4,6}$-choladienic acid and its methyl ester were prepared from cholic acid (13), and the infrared absorption spectra (Fig. 1-E and 1-F) of the compounds were determined. The comparison with the absorption spectra of the dehydration products of the C-22 acid and its methyl ester and that of the authentic 3,12-diketo-$\Delta^{4,6}$-choladienic acid and its methyl ester indicated that $\Delta^{4,6}$-3-ketone group is present in the dehydration product. So the possibility of the structure of $\Delta^{4,4}$-3,12-diketone postulated above for the C-22 acid was eliminated.

In view of the above-stated facts the most reasonable conclusion to be drawn from available data is that the new unsaturated C-22 acid is 7α-hydroxy-3,12-diketo-$\Delta^{4}$-bisnorchenolic acid and 7α-hydroxyl group in the acid is probably dehydrated to produce 3,12-diketo-$\Delta^{4,6}$-bisnorcholadienic acid by the action of caustic alkali or sulfuric acid as shown in the following Fig. 2.

**SUMMARY**

*S. gelaticus* 1164 converts cholic acid to a new unsaturated C-22 acid with m. p. 280–282° (decomp.) which was previously postulated as 7α-hydroxy-3,12-diketo-$\Delta^{4,9(11)}$-bisnorcholadienic acid. Further investigations demonstrated that the new acid may be 7α-hydroxy-3,12-diketo-$\Delta^{4}$-bisnorcholenic acid.
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