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Metal Ion Catalized Oxidation of Steroids. I. 15α-Hydroxylation of Deoxycholic Acid in Aqueous Solution by Ferrous Ion-Molecular Oxygen System[1]

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Hydroxylation reactions by Udenfriend's system[3] and the related systems were studied on the aqueous solutions of deoxycholic acid (I). No ascorbic acid and EDTA were necessary in the hydroxylation of I. A new bile acid (II), mp 322—325°, was obtained when an aqueous solution of $FeSO_4 \cdot 7H_2O$ (54 mmole) was added dropwise to a stirred buffer solution (0.1M Na_2HPO_4, pH 6.8) of I (2.25 mmole) under bubbling oxygen for 4 hr at 70°. The methyl ester of II gave triketo steroid (V), mp 176—177°, by chromate oxidation and the third oxygen function introduced was found to be situated at C_{15} as a result of Mass spectrum analysis (Fig. 3). From the smaller effect on the nuclear magnetic resonance signal peak of 18-CH_3 (Table II & III), the configuration of C_{15}-OH was concluded to be of alpha. Hydroboration-oxidation of methyl 3α,12α-dihydroxy-5β-chol-14-en-24-oate (VI) gave the methyl ester of II. The chemical structure of II was thus elucidated as 3α,12α,15α-trihydroxy-5β-cholan-24-oic acid.

Since a model system for the drug metabolizing enzyme has first been presented by Udenfriend, *et al.*,[3] the hydroxylation reaction has extensively studied by many investigators on a variety of aromatic substrates using this model system, Fe^{2+}/ethylenediamine tetraacetic acid (EDTA)/ascorbic acid/O_2, and several other similar systems.[4] Little work appears, on the contrary, to have been done on the hydroxylation of aliphatic substrates. As to the steroidal compounds, Keston, *et al.*[5] reported on the reactions of deoxycorticosterone acetate (21-hydroxy-pregn-4-en-3,20-dione 21-acetate) and 11-deoxy-17-hydroxycorticosterone (17,21-dihydroxy-pregn-4-en-3,20-dione) with Fe^{2+}/ascorbate/O_2 systen. Cier, *et al.*[6] studied on the 11β-hydroxylation of some corticosteroids and androstendione (androst-4-en-3,17-dione) by use of the Udenfriend's system and the modified system lacking ascorbate. Matkovics, *et al.*[7] demonstrated the transformation of deoxycholic acid (3α,12α-dihydroxy-cholan-24-oic acid) (I) into cholic acid (3α,7α,12α-trihydroxy-cholan-24-oic acid) by ascorbic acid-O_2 or -H_2O_2-system in the presence of ferrous ions and EDTA. In an incubation of I with Fe^{2+}/ascorbate/O_2 system, Kimura, *et al.*[1] obtained a new bile acid (II), instead of cholic acid. The present paper deals with the formation and structure elucidation of II, 3α,-12α,15α-trihydroxy-cholan-24-oic acid, which was formed through the reaction of I with the most simple aerobic hydroxylating model system, an aqueous solution of ferrous sulphate and molecular oxygen, at moderately temperature.

1) A preliminary account for this work has been published: M. Kimura, M. Kawata, M. Tohma, A. Fujino, and K. Yamasaki, *Tetrahedron Letters*, **1970**, 2021.
2) Location: *Nishi-6-chome, Kita-12-jo, Sapporo.*
3) S. Udenfriend, C.T. Clark, J. Axelrod, and B.B. Brodie, *J. Biol. Chem.*, **208**, 731 (1954).
4) G.A. Hamilton, *J. Am. Chem. Soc.*, **86**, 3390 (1964); V. Ullrich and Hj. Staudinger, *Z. Naturforsch.*, **24b**, 583 (1969).
5) A.S. Keston and R. Carsiotis, *Arch. Biochem. Biophys.*, **52**, 282 (1954).
6) A. Cier, C. Nofré, and A. Revol, *Compt. Rend. Acad. Sci. Paris*, **250**, 2638 (1960).
7) B. Matkovics, P. Pénzes, and G. Göndös, *Steroids*, **5**, 451 (1965).

Result and Discussion

Hydroxylation System

While the solubility of I (p*K*a=6.58) depends mostly on the pH of reaction mixture, the physiological conditions were desirable in an attempt to be a model system. Higher pH is, however, unfavourable due to precipitation of ferrous ions and nearly neutral pH of 6.8 was found most profitable. Buffer solution was not necessarily inevitable when the reaction mixture was kept at the defined pH with the occasional addition of alkaline solution during the course of oxygenation. Although the further oxidation tends to occur, the autoxidation in many cases has been carried out under rather higher temperatures. The temperature of 70°, far from the physiological conditions, was suitable for obtaining the new bile acid (II) in a preparative scale. Under absence of ferrous ions, no autoxidation occured in the aqueous solution of the substrate at pH 6.8 and 70° or higher temperature.

EDTA employed in the Udenfriend's system might be explained as a ligand for iron ions to keep a suitable oxidation potential, preventing the precipitation with phosphate anions as well. The large stability constant of ferrous EDTA complex might be higher than what would be suitable for the probable formation of ferrous-oxygen intermediary complex.[8] Carboxyl group in the substrate (I) may also be capable of forming the iron-complex. Hydroxylation of the substrate, as a matter of fact in this study, proceeded both in the presence and in the absence of EDTA, though cholic acid never obtained. The aromatic substrate such as acetanilide, on the contrary, was almost insensitive to the system lacking EDTA.[9]

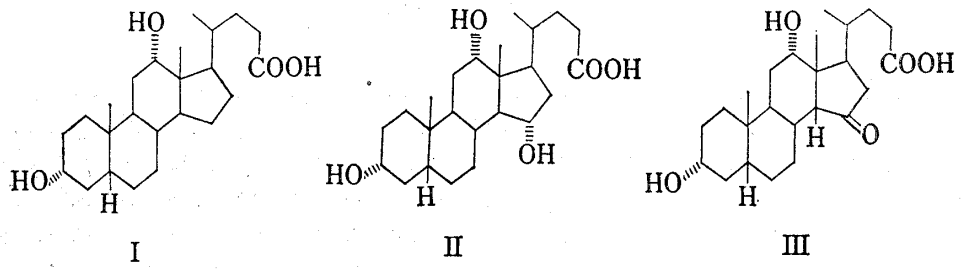

Chart 1

Ascorbic acid is not necessarily essential in the Udenfriend's system and can be replaced by many other endiols[3] and the related substances.[4,10] These reducing co-factors have been considered as the regenerator of ferrous ions,[11] on the one hand, and the complex formation might be essential with metal ions,[10] on the other hand. In the mere presence of oxalic acid which is one of the oxidation products of ascorbic acid, the hydroxylation of I also occured, if ferrous ions present. No hydroxylation of the substrate was observed in the buffered solution of ascorbic acid, when lacking iron ions. It is noteworthy that during the course of the autoxidation reaction the continuous addition of ferrous sulphate solution in a capillary stream gave actually higher yields of II even in the absence of ascorbic acid. Ferric ions were observed to be ineffective in the same conditions. The ferrous state thus seems to be most essential in this reaction. Consequently, it was realized that the Udenfriend's system can be reduced to the most simple constitution of ferrous ions and molecular oxygen, in order to obtain II from I as described in the experimental section. These facts and the results obtained by Matkovics[7] offer some interesting and complicated problems on

8) M.B. Dearden, C.R.E. Jefcoate, and J.R. Lindsay Smith, "Oxidation of Organic Compounds," Vol. 3, ed. by R.F. Gould, American Chemical Society, Washington D.C., 1968, p. 272.
9) Hj. Staudinger and V. Ullrich, *Z. Naturforsch.*, **19b**, 409 (1964).
10) G.A. Hamilton, *J. Am. Chem. Soc.*, **86**, 3391 (1964).
11) R.R. Grinstead, *J. Am. Chem. Soc.*, **82**, 3472 (1960).

the mechanism of the reaction which is likely to transform I into the different trihydroxy derivatives with different conditions. Further investigations on these hydroxylating systems are in progress.

Products Analysis

Thin-layer chromatography (TLC) using 4% acetic acid–ethyl acetate system for the free acidic products gave four main spots as shown in Fig. 1, two of which showed colour development with 2,4-dinitrophenyl hydrazine reagent. One of these carbonyl compounds was isolated by the column chromatography as described below and was identical with the authentic 3α,12α-dihydroxy-15-oxo-5β,14β-cholan-24-oic acid (III) prepared in a different way. The most polar spot and the last one were due to the new trihydroxy bile acid (II) and the intact substrate (I), respectively. There appeared some other unidentified minor spots; presumably most of them were those having carbonyl function or functions on the steroidal skeleton of the substrate as well as its hydroxylated products. Methyl esters of these acidic products gave the similar results in TLC using acetone–benzene (1:1) system. Semi-quantitative aspects were observable through the isolation of individual product by the column chromatography on silica gel as shown in Table I.

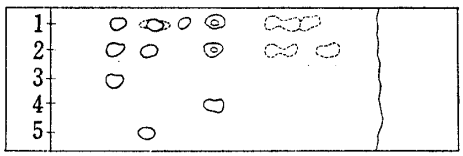

Fig. 1. Thin-layer Chromatography of Hydroxylation Products

1: products by Udenfriend's system
2: products by $Fe^{2+}-O_2$ system
3: new bile acid (II)
4: substrate (I)
5: C_{15}-keto derivative (III) of II
plate: silica gel (WAKOGEL B-5)
solvent system: AcOH–AcOEt (4: 96)
staining: conc. H_2SO_4 with heating

In spite of the numerous reexaminations in the Matkovics's conditions,[7] the results were thus entirely negative as to the transformation of I into cholic acid, but one of the main products was a new trihydroxy cholanic acid (II) revealing the *Rf* value similar to that of cholic acid in TLC and the melting point different from those of its isomers, cholic acid: mp 196°[7] and phythocholic acid (3α,12α,16α-trihydroxy-5β-cholan-24-oic acid): mp 187°.[12] Another main product was a 15-keto derivative (III) formed probably through further oxidation of II.

Table I. Column Chromatography of Methylated Products

Fraction No.	Solvent	Vol. (l)	Weight (g)	*Rf* value in TLC
1	AcOEt–benzene (25:75) AcOEt–benzene (30:70) AcOEt–benzene (50:50)	5.3	1.55	0.87, 0.79, 0.73
2	AcOEt–benzene (50:50)	5.1	1.55	0.56[a]
3	AcOEt–benzene (75:25)	2.8	0.50	0.41[b]
4	AcOEt	5.4	0.69	0.29[c]
5	MeOH–AcOEt (5:95)	1.8	0.53	(0.29), 0.000

a) methyl deoxycholate *b*) VII *c*) IV

Chemical Structure of the New Bile Acid (II)

The products were extracted with ethyl acetate from the reaction mixture acidified at pH 1.0 and the extracts were methylated. The methyl esters obtained were then submitted to chromatography on silica gel by eluting with the mixture of benzene and ethyl acetate (Table I). The eluates by ethyl acetate gave the colourless needles, mp 256—259°, methyl ester (IV) of II. The fragmentation pattern of mass spectrum of IV was considerably similar to that of methyl cholate as shown in Fig. 2.: *m/e* 404 (M^+-18), 386 (M^+-2×18), 368 (M^+-3×

12) G.A. Haslewood and V.M. Wooton, *Biochem. J.*, **49**, 67 (1951).

18), 271 (M^+-2×18−115) (base peak), 253 (M^+-3×18−115); 115 mass units may correspond to the intact side chain. The presence of three oxygen substituents in the steroidal ring may thus be elucidated.[13] Chromate oxidation of IV in acetic acid gave a triketo ester (V) and this is indicative of the secondary character of the third hydroxyl group. Fragmentation (2) of 12-ketosteroids as shown in Fig. 3 can be operative with hydrogen transfer[14] and methyl 3,12-diketocholan-24-oate derived from I gave the base peak of *m/e* 247 as expected. Fission of ring C occurs in 15-ketosteroids, affording the stable peak of ketonic ring D with the side chain,[15] to which the *m/e* 211 ion from V accompanied by hydrogen transfer may reasonably be corresponded. Free acid of V afforded the *m/e* 197 (=211−14) instead and also 247 ions, both in higher intensity. The fission (3) can not be operative in 16-ketosteroid.[16] The new oxygen function introduced in this hydroxylation reaction may, therefore, be situated at C_{15}.

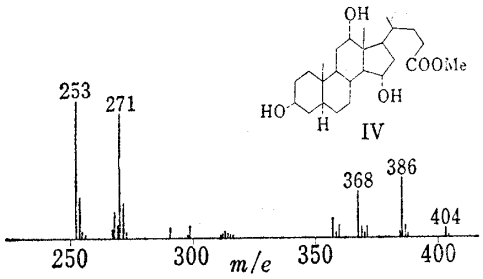

Fig. 2. Mass Spectrum of Methy 3α,-12α,15α-Trihydroxy-5β-cholan-24-oate (IV)

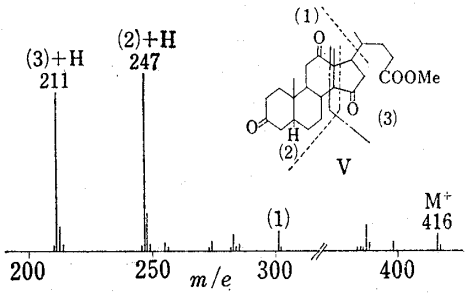

Fig. 3. Mass Spectrum of Methyl 3,-12,15-Trioxo-5β-cholan-24-oate (V)

In nuclear magnetic resonance studies on steroids, signal shifts of the angular methyl groups due to the hydroxyl groups in the various positions have been reported.[17] The chemical shifts in six known bile acids as well as IV and in some of those reported are shown in Table II. Contrary to the remarkable downfield shift (−0.45 ppm) of the signal peak of 18-CH_3 caused by C_{15}-βOH, the effect of C_{15}-αOH was as smaller as −0.10 ppm (Table III). The configuration of the third hydroxyl group in IV may thus be rather of alpha.

Table II. Chemical Shifts of Angular Methyl Protons

Methyl ester	Site of hydroxyl group	C_{18}-H (τ)	C_{19}-H (τ)
Cholanate	—	9.39	9.08
Lithocholate	3α	9.38	9.09
Chenodeoxycholate	3α, 7α	9.32	9.04
Ursodeoxycholate	3α, 7β	9.34	9.07
Deoxycholate	3α, 12α	9.30	9.07
Cholate	3α, 7α, 12α	9.23	9.03
Synthetic IV	3α, 12α, 15α	9.19	9.02
Compound IV	3α, 12α, ?	9.20	9.03

Spectra were taken in pyridine solution containing tetramethylsilane as an internal standard.

13) H. Budzikiewicz, C. Djerassi, and D.H. Williams, "Structure Elucidation of Natural Products by Mass Spectrometry," Vol. 11, Holden-Day, Inc., 1964, p. 104.
14) H. Budzikiewicz and C. Djerassi, *J. Am. Chem. Soc.*, **84**, 1430 (1962); P. Eneroth, B. Gordon, and J. Sjövall, *J. Lipid Res.*, **7**, 524 (1966).
15) C. Djerassi, G. von Mutzenbecher, J. Fajkos, D.H. Williams, and H. Budzikiewicz, *J. Am. Chem. Soc.*, **87**, 817 (1965).
16) C. Beard, J.M. Wilson, H. Budzikiewicz, and C. Djerassi, *J. Am. Chem. Soc.*, **86**, 269 (1964).
17) K. Tori and K. Aono, *Ann. Rept. Shionogi Res. Lab.*, **14**, 136 (1964); K. Tori, and E. Kondo, *Steroids*, **4**, 713 (1964).

Table III. Substituent Effect of Hydroxyl Group on the Chemical Shifts of Angular Methyl Protons

Site of hydroxyl group	Difference in ppm from parent compound	
	C_{18}-H	C_{19}-H
3α	−0.01 (−0.01)	+0.01 (−0.01)
7α	−0.06 (−0.06)	−0.05 (−0.09)
7β	−0.04 (−0.02)	−0.02 (−0.01)
12α	−0.08 (−0.12)	−0.02 (−0.03)
15α	−0.10 (−0.07)	−0.04 (−0.03)
15β	— (−0.45)	— (−0.07)

Numbers in the brackets are those reported.[17]

Hydroboration-oxidation[18] of methyl 3α,12α-dihydroxy-5β-chol-14-en-24-oate (VI), derived from methyl cholate by the method of Yamasaki, *et al.*,[19] yielded the trihydroxy derivative which was identical with IV giving the free acid (II) as a hydrolysate and the triketo ester (V) on chromate oxidation. Consequently, the chemical structure of the product (II) from I by the ferrous ion-oxygen system may reasonably be elucidated as 3α,12α,15α-trihydroxy-5β-cholan-24-oic acid.

Experimental[20]

Hydroxylation of 3α,12α-Dihydroxy-5β-cholan-24-oic Acid (I)——To a stirred buffer solution (0.1M Na_2HPO_4, pH 6.8, 2500 ml) of I (1.0 g, 2.25 mmole) under bubbling oxygen, was added dropwise an aqueous solution (500 ml) of $FeSO_4 \cdot 7H_2O$ (15 g, 54 mmole) during 4 hr at 70°. Oxygen was bubbled for another 2 hr at the same temperature. In the same procedure, 6.1 g of I in total amounts was treated. The reaction mixtures were collected and acidified with 10% H_2SO_4 to pH 1.0 and extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The brown residue (5.8 g) thus obtained was methylated with diazomethane in ether–MeOH and the products were extracted with ether. Evaporation of the solvent under reduced pressure gave 5.0 g of residue.

Isolation and Identification of Products

Thin-Layer Chromatography——TLC was carried on silica gel (WAKOGEL B-5) plate by the following solvent systems: AcOH–EtOAc (4:96) for free acidic products and acetone–benzene (1:1) for methyl esters. *Rf* values were given by staining with conc. H_2SO_4 and heating at 110°. The free acidic products and their methyl esters gave the chromatogram as shown in Fig. 1 and Table I, respectively.

Gas-Liquid Chromatography (GLC)——Trifluoroacetyl (TFA) and trimethylsilyl (TMS) derivatives were prepared according to the procedures of Van den Heuvel, *et al.*[21] and Sweeley, *et al.*,[22] respectively. The apparatus used was a Shimazu Model GC-4APF gas chromatograph equipped with a hydrogen flame ionization detector and a U-shaped stainless steel tube (3 mm i.d.) packed with 1% QF-1 (A) or 1.5% SE-30 (B) on Shimalite W (60—80 mesh). The column temperatures were kept at 230° for TFA on (A) and for TMS on (B) and at 215° for TFA on (B). N_2 was used as a carrier gas at a flow rates of 45 ml/min for (A) and 30 ml/min for (B). No peak of cholic acid was observed in GLC of hydroxylation products.

Methyl 3α,12α,15α-Trihydroxy-5β-cholan-24-oate (IV)——The methylated residue (5.0 g) from hydroxylation mixture described above was submitted to chromatography on silica gel (250 g) by eluting with the mixture of benzene and EtOAc as shown in Table I. Evaporation *in vacuo* of solvent from the fraction 4 left yellowish brown residue (0.69 g) which was recrystallized from MeOH–$CHCl_3$ into colourless needles, mp 256—259° (210/256—259° from EtOAc–MeOH). Total yield from hydroxylation mixture was *ca.* 10%.

18) H.C. Brown, "Hydroboration," W.A. Benjamin Inc. Publ., 1962; G. Zweitov, N.R. Ayyanger, and H.C. Brown, *J. Am. Chem. Soc.*, **85**, 2072 (1963).
19) K. Yamasaki, *Z. Physiol. Chem.*, **220**, 42 (1933); *idem*, *ibid.*, 233, 10 (1935).
20) Melting points were taken on a micro hot-stage apparatus and are uncorrected. Infrared (IR) spectral measurements were run on JASCO Model IR-S spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained by Hitachi Model H-6013 spectrometer at 60 Mc. Mass (MS) spectra were measured by Hitachi Model RMU-6E spectrometer.
21) W.J.A. Van den Heuvel, J. Sjövall, and E.C. Horning, *Biochim. Biophys. Acta*, **48**, 596 (1961).
22) C.C. Sweeley, R. Bentley, M. Makita, and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).

Anal. Calcd. for $C_{25}H_{42}O_5$: C, 71.06; H, 10.01. Found: C, 71.35; H, 10.15. IR ν_{max}^{Nujol} cm^{-1}: 3400, 1740. NMR (Table II). MS (Fig. 2). Yamasaki–Hammersten colour reaction[23]: negative. No depression of melting point was observed on admixture with the authentic specimen.

Methyl 3α,12α-Dihydroxy-15-oxo-5β,14β-chloan-24-oate (VII)——Evaporation of solvent *in vacuo* from the fraction 3 of silica gel chromatography described above (Table I) gave 0.50 g of residue which was recrystallized from acetone–MeOH to give colourless needles, mp 244—246°. Yield: *ca.* 8%. *Anal.* Calcd. for $C_{25}H_{40}O_5$: C, 71.39; H, 9.58. Found: C, 71.12; H, 9.46. IR ν_{max}^{Nujol} cm^{-1}: 3400, 1740, 1735 (sh). NMR ($CDCl_3$) τ: 9.01 (C_{19}-H), 9.01 (C_{18}-H), 6.35 ($COOCH_3$). Mass Spectrum *m/e*: 420 (M^+), 402 (M^+-18), 384 (M^+-2×18), 269 (M^+-2×18-115, base peak), 213. No depression of melting point was observed on admixture with the authentic specimen.

Synthesis of Standard Specimens

Methyl 3α,12α-Dihydroxy-5β-chol-8(14)-en-24-oate (VIII)[24]——To an acetone solution (100 ml) of cholic acid (10 g), was added $ZnCl_2$ (10 g) and the solvent was evaporated at 80° to give a yellow syrup,[19] to which was added 0.5% aq. AcOH (100 ml). The precipitates formed were collected and dried. The products (mixture of 7,8-, 8,14-, and 14,15-olefins) thus obtained were derived to their methyl esters by using MeOH–HCl mixture at room temperature overnight. Recrystallization of the olefinic esters thus formed (9.8 g) gave colourless needles (4.8 g), mp 76—80° (MeOH). *Anal.* Calcd. for $C_{25}H_{40}O_4$: C, 74.21; H, 9.97. Found: C, 74.09; H, 9.78. NMR ($CDCl_3$) τ: 9.18 (C_{19}-H), 9.13 (C_{18}-H). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400, 1760.

Free Acid (Apocholic acid)[24]: A mixture of the methyl ester (VIII) (1.2 g) and MeOH (6 ml) with 8 drops of 40% aq. KOH was refluxed for 20 min. After it was acidified with HCl the precipitates formed were collected. The filtrate was extracted with EtOAc and the combined mixture (1.08 g) of the precipitates and the extracts was recrystallized from EtOH–xylene to give colourless needles, mp 176—178°. *Anal.* Calcd. for $C_{24}H_{38}O_4$: C, 73.80; H, 9.81. Found: C, 73.73; H, 9.67. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400, 1690. Mass Spectrum *m/e*: 372 (M^+-18), 354 (M^+-2×18), 271, 253 (base peak).

Methyl 3α,12α-Dihydroxy-5β-chol-14-en-24-oate (VI)[24]——A $CHCl_3$ solution (140 ml) of methyl apocholate (VIII) (29 g) was saturated with dry HCl at 0° for 2 hr. After removing excess HCl with N_2 stream at room temperature, the solution was washed with 5% $NaHCO_3$ and water successively. The organic layer dried over anhydrous Na_2SO_4 left a residue (29.2 g), on evaporation of solvent *in vacuo*. The residue was submitted to the chromatography on silica gel (900 g) and the fraction eluted with 17.5% AcOEt–benzene gave the residue (10 g) which was then recrystallized from MeOH to give colourless needles, mp 76—78°. *Anal.* Calcd. for $C_{25}H_{40}O_4$: C, 74.21; H, 9.97. Found: C, 74.29; H, 10.02. Mass Spectrum *m/e*: 404 (M^+), 386, 368, 271, 253. NMR ($CDCl_3$) τ: 9.08 (C_{19}-H), 9.08 (C_{18}-H), 4.71 (C_{15}-H, vinyl proton).

Free Acid: Saponification of the ester (VI) (250 mg) with MeOH–KOH gave the free acid (233 mg), mp 256—257° (MeOH–AcOH–H_2O). *Anal.* Calcd. for $C_{24}H_{38}O_4$: C, 73.79; H, 9.82. Found: C, 73.77; H, 9.82.

Methyl 3α,12α,15α-Trihydroxy-5β-cholan-24-oate (IV)——To a stirred mixture of $NaBH_4$ (494 mg), diglyme (4 ml), tetrahydrofurane (16 ml) and cyclohexene (2 ml), was added dropwise tetrahydrofurane solution (20 ml) of BF_3-etherate (3 g) during 30 min at 0° under N_2 stream. After the mixture was allowed to stand for 3 hr and then to come to room temperature, B_2H_6 formed was blowed off with the violent bubbling of N_2. Tetrahydrofurane solution (15 ml) of VI (463 mg) was added dropwise to the reaction mixture at 45—50°, which was then allowed to stand for 1 hr. Dicyclohexyl borane remained was decomposed by adding water (10 ml) and then 3N NaOH (6 ml) and H_2O_2 (4 ml) were added to the mixture. After being allowed to stand overnight at room temperature, the reaction mixture was acidified with HCl and extracted with ether. The extract were submitted to chromatography on silica gel (34 g) and the fractions eluted with acetone–benzene (1:1) were collected. Evaporation of solvent *in vacuo* left the residue (155 mg) which was then recrystallized from MeOH–$CHCl_3$ to give colourless needles, mp 257—259°. *Anal.* Calcd. for $C_{25}H_{42}O_5$: C, 71.06; H, 10.01. Found: C, 70.84; H, 9.84. IR ν_{max}^{Nujol} cm^{-1}: 3400, 1740. NMR (Table II). MS (Fig. 2).

3α,12α,15α-Trihydroxy-5β-cholan-24-oic Acid (II)——MeOH solution (5 ml) of IV (110 mg) with 8 drops of 40% aq. KOH was refluxed for 20 min. The reaction mixture was acidified with HCl and the precipitates were collected, 67 mg. The aqueous layer was extracted with AcOEt and another 41 mg of the product was recovered. The hydrolyzates were combined and recrystallized from aq. EtOH to give crystalline powder, mp 322—325° (decomp.). IR ν_{max}^{Nujol} cm^{-1}: 3400, 1710 (COOH).

Methyl 3α,12α-Dihydroxy-14,15α-epoxy-5β,14α-cholan-24-oate (IX)——To an ether solution (10 ml) of VI (307 mg), was added ether solution (30 ml) of monoperphthalic acid (600 mg) and the reaction mixture was stirred at room temperature overnight. The mixture was then washed with water, 10% Na_2SO_3, 5% $NaHCO_3$ and finally with water again. Evaporation of solvent from ether solution dried over Na_2SO_4 left 335 mg of residue. Although recrystallization of the residue was in no success, NMR ($CDCl_3$, τ) spectroscopy indicated the presence of α-epoxide group[25]: 7.07 (C_{15}-βH), 9.07 (C_{19}-H), 9.13 (C_{18}-H).

23) O. Hammersten, *Z. Physiol. Chem.*, **61**, 495 (1909); K. Yamasaki, K. Takahashi, and C.H. Kim, *J. Biochem.* (Japan), **30**, 239 (1939).
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Methyl 3α,12α-Dihydroxy-15-oxo-5β,14β-cholan-24-oate (VII)——To an acetone solution (20 ml) of IX (300 mg) was added $HClO_4$–acetone mixture[26] (1 ml) and the reaction mixture was refluxed for 15 min. After the precipitates (83 mg) formed was filtered, the filtrate was diluted with water and extracted with ether. The extract (207 mg) was purified by chromatography on silica gel (10 g). The precipitates described above and the purified extracts were combined and the mixture (187 mg) was recrystallized from MeOH to colourless needles, mp 247—248°. *Anal.* Calcd. for $C_{25}H_{40}O_5$: C, 71.39; H, 9.58. Found: C, 71.49; H, 9.61. IR ν_{max}^{Nujol} cm^{-1}: 3450—3350, 1740, 1735 (sh). Mass Spectrum *m/e*: 420 (M^+), 402 (M^+-18), 348 ($M^+-2\times18$), 269 (M^+-$2\times18-115$, base peak), 213. NMR ($CDCl_3$)τ: 9.01 (C_{19}-H), 9.01 (C_{18}-H), 6.35 ($COOCH_3$).

3α,12α-Dihydroxy-15-oxo-5β,14β-cholan-24-oic Acid (III)——Alkaline hydrolysis of the ester (VII) (100 mg) by usual work-up gave 98 mg of the hydrolyzate which was then recrystallized from MeOH–$CHCl_3$. mp 269—270°. *Anal.* Calcd. for $C_{24}H_{38}O_5$: C, 70.90; H, 9.42. Found: C, 70.97; H, 9.44. IR ν_{max}^{Nujol} cm^{-1}: 1735, 1660. Mass Spectrum *m/e*: 406 (M^+), 388 (M^+-18), 370 (M^+-2×18), 269 (base peak), 213.

Methyl 3,12,15-Trioxo-5β-cholan-24-oate (V)——To a stirred acetic acid solution (5 ml) of IV (41 mg) was added gradually an aq. solution (0.4 ml) of K_2CrO_4 (1.48 g) at room temperature and the reaction mixture was allowed to stand overngiht. The mixture was then poured into water and the precipitates formed were collected and washed with water. The dried precipitates (25.7 mg) were recrystallized from acetone to give colourless needles, mp 176—177°. *Anal.* Calcd. for $C_{25}H_{36}O_5$: C, 72.08; H, 8.71. Found: C, 71.96; H, 8.61. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1739—1710. High MS (Fig. 3): Calcd. for $C_{25}H_{36}O_5$: 416.2562. Found: 416.2571. Calcd. for $C_{16}H_{22}O_2+H$: 247.1697. Found: 247.1755. Calcd. for $C_{12}H_{18}O_5+H$: 211.1322. Found: 211.1372.

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26) Prepared with 0.025 ml of 70% $HClO_4$ and 25 ml of acetone.