Potential Bile Acid Metabolites. 2.[1] 3,7,12-Trisubstituted 5β-Cholanic Acids[2]

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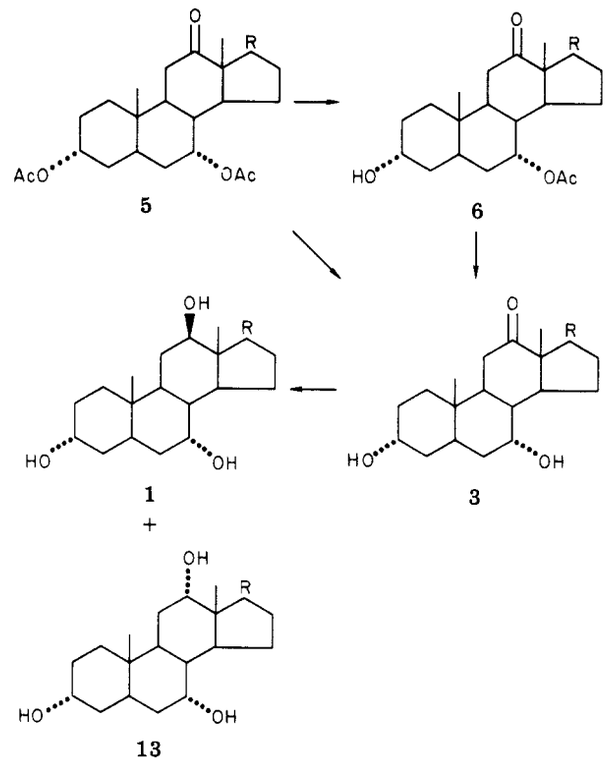
Possible implication of bile acids in the development of colon cancer has stimulated interest in the identity of unidentified metabolites of the primary acids. This paper reports the preparation of two new 12β-hydroxy stereoisomers of cholic acid and several related derivatives. A mild method of inversion of axially substituted C-3 hydroxy bile acid derivatives was uncovered when the 3β,7α-dihydroxy keto compound (**4**), catalytically hydrogenated with Raney nickel, unexpectedly gave rise to a 3α-hydroxyl product. ^{1}H NMR and high pressure LC were of key importance in characterizing the compounds and determining their purity.

The involvement of bile acids as possible cocarcinogens or as promoters of carcinogenesis in the colon[3] has stimulated interest in the identity of unknown bile acid derivatives usually referred to in metabolic studies as "unidentified bile acids".[4]

Our laboratory has initiated a program to synthesize bile acid derivatives which are potential metabolites of the primary acids, the selection of compounds being based on the metabolic changes of bile acids already known to occur in mammalian systems. We have chosen as initial targets the potential 3,7,12-substituted metabolites more directly obtainable from the one feasible starting material, cholic acid, the major bile acid in humans, which fortunately is available in ample supply from animal sources.

This report covers the synthesis of the 3α,7α,12β- and 3β,7α,12β-trihydroxy acids (**1a** and **2a**), two stereoisomers of cholic acid, and several related potential metabolites. On the basis of previous work[5] the 12β-acids were expected to be straightforwardly available through Raney nickel hydrogenation of the corresponding 12-keto derivatives, **3** and **4**. The 3α-compound **3** had been synthesized years ago by a reaction sequence which necessitates hydrolysis of the diacetate **5**[6] to the free acid **3a** and subsequent reesterification to **3**.[7] By treatment of **6** with methanolic sodium methylate a direct route to **3** was found[8] (Scheme I).

Scheme I



R = $CH(CH_3)CH_2CH_2COOCH_3$. The corresponding acids [R = $CH(CH_3)CH_2CH_2COOH$] are designated "a".

Inversion at C-3

Since synthesis of ketone **4** from cholic acid as starting material required inversion at C-3 at some intermediate stage regardless of the choice of routes to **4**, we considered several known methods for the inversion. Reduction of the 3-ketone to obtain axial alcohols by use of an appropriate catalyst has been successful with many steroids.[9,10] While selective oxidation of a 3-hydroxy group to the 3-ketone by Oppenauer conditions on cholanic acid derivatives having more than one hydroxyl substituent is feasible, the proportion of axial (3β) hydroxy epimer formed in the catalytic reduction of these compounds decreases with increasing number of hydroxyls in the molecule; with methyl 7α,12α-dihydroxy-3-oxocholanate, the yield of 3β-epimer was 5%.[10] Furthermore, separation of the epimers is often tedious.

A promising inversion reaction adapted to steroidal alcohols by Bose et al.[11] has the virtue of not requiring prior conversion of the alcohol to a derivative with a leaving group suitable for inversion. However, our attempts to make use of the reaction were disappointing. With methyl cholate, and formic acid as the acid reagent, we were able to obtain the 3β-formate in about 15% yield only after a tedious chromatographic separation; with benzoic acid as the acid no steroidal benzoate was found. Methyl cholate 7-acetate (**7**) with formic acid and methyl 3α-hydroxy-7-

(1) Part 1 (preliminary communication): F. C. Chang, *Tetrahedron Lett.*, **23**, 2085 (1979).

(2) All cholanic derivatives mentioned in this work are of the 5β series; the 5β designations are omitted in their names. The older name cholanic acid is used throughout in place of the newer IUPAC-suggested "cholanoic acid".

(3) M. Lipkin, *Cancer Bull.*, **30**, 196 (1978).

(4) E.g., P. Eneroth, B. Gordon, R. Ryhage and J. Sjövall, *J. Lipid Res.*, **7**, 511 (1966).

(5) (a) F. C. Chang, N. F. Wood, and W. F. Holton, *J. Org. Chem.*, **30**, 1718 (1965). (b) R. C. Ebersole and F. C. Chang, *ibid.*, **38**, 2713 (1973).

(6) L. F. Fieser and S. Rajagopalan, *J. Am. Chem. Soc.*, **72**, 5530 (1950).

(7) E. Berner, A. Lardon, and T. Reichstein, *Helv. Chim. Acta*, **30**, 1542 (1947).

(8) While the acylates at C-3 are easily hydrolyzed (MeOH–HCl; Al_2O_3), the acetates at C-7 require considerably stronger base for hydrolysis; in some instances the methyl ester at C-24 is hydrolyzed before the acetate at C-7.

(9) J. T. Edward and J. M. Ferland, *Can. J. Chem.*, **44**, 1311 (1966).

(10) H. Danielsson, P. Eneroth, K. Hellström and J. Sjövall, *J. Biol. Chem.*, **237**, 3657 (1962).

(11) A. K. Bose, B. Lai, W. A. Hoffman, and M. S. Manhas, *Tetrahedron Lett.*, 1619 (1973).

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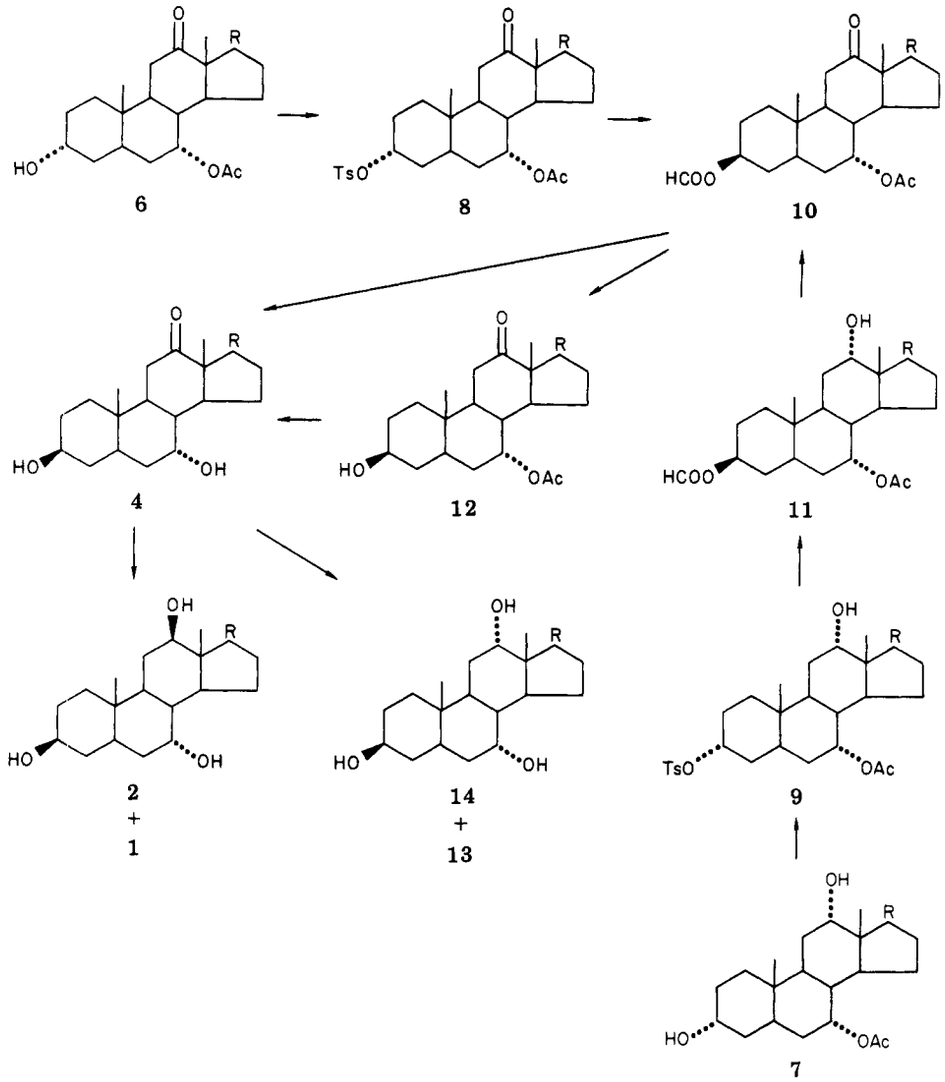
Scheme II



R = $CH(CH_3)CH_2CH_2COOCH_3$. The corresponding acids [R = $CH(CH_3)CH_2CH_2COOH$] are designated "a".

acetoxy-12-oxocholanate (6) with either formic or benzoic acid failed to yield the desired inverted acylates.[12]

Thus, we had to fall back on methods which involve inversion of displaceable substituents such as tosylates. To evaluate several known procedures of this type, we compared the reaction of methyl cholate 3-tosylate[33] with: (1) tetra-*n*-butylammonium hydroxide in Me_2SO;[13] (2) tetra-*n*-butylammonium formate in acetone;[14] (3) potassium acetate in aqueous DMF;[15] (4) DMF.[5,16] Of the four methods compared[17] we have found the last to be the most satisfactory because of its simplicity of reagent and reaction conditions and the ease of isolation of inverted product in good yield, although the reaction is slow. The two factors which govern success of the reaction are purity of the starting tosylate and proper temperature control.[18] The formates crystallizing on dilution with water contain little olefinic product. In exploratory experiments where higher proportions of olefin were obtained, hydrolysis to the methyl ester mixture gave a product which was easily separated into the olefinic ester and the hydroxy ester by column chromatography. The 3β-formates **10**, **11** and **15** (see Experimental Section) were prepared by the DMF inversion method (Scheme II).

Raney Nickel Hydrogenation

The initial hydrogenation experiments carried out on the diacetate **5**, monitored by TLC, indicated that the rate of hydrolysis at C-3 is higher than the rate of hydrogenation at C-12; thus, the hydrogenation reaction is largely that of the 7-monoacetate **6**.[19,20] Accordingly, the product

(12) The other main products of the reaction, triphenylphosphine oxide and diethyl hydrazinodicarboxylate, were difficult to separate from the formate. The reactions were carried out according to the procedure of the original publication; for the keto compound an additional mole of triphenylphosphine was used as suggested.[11]

(13) D. B. Cowell, A. K. Davis, D. W. Mathieson, and P. D. Nicklin, *J. Chem. Soc., Perkin Trans. 1*, 1505 (1974).

(14) E. J. Corey and S. Terashima, *Tetrahedron Lett.*, 111 (1972).

(15) P. Ziegler and K. R. Bharucha, *Chem. Ind. (London)*, 1351 (1955).

(16) F. C. Chang and R. T. Blickenstaff, *J. Am. Chem. Soc.*, **80**, 2906 (1958).

(17) (1) After 1 h at 70 °C the tosylate had been consumed, and the product was soluble in the diluted aqueous medium and appears to be mainly the olefinic bile acid salt. (2) After 1 h no formylation took place. Blickenstaff et al. [*J. Org. Chem.*, **36**, 1271 (1971)] reported that reaction of the tosylate with tetrabutylammonium acetate in acetone after 29 h at reflux and 3 days at room temperature yielded the 3β-acetate in 35% yield. (3) At 75 °C after 7 h the product was a mixture of olefin, methyl 3β,7α,12α-trihydroxycholanate (**14**), the 3-formate, and the 3-acetate of **14**.

(18) A temperature of 80 ± 1 °C appears to be optimum; higher temperatures favor formation of olefin (Δ^3). Reagent grade DMF (Fisher, H_2O content 0.08%) seems to be equally effective without prior drying with molecular sieves.

(19) The Raney nickel (no. 28) is basic when received; although it is first shaken and rinsed with three portions of methanol, apparently it is still basic enough to cause hydrolysis of the 3-acetate.

is a complex mixture, and although the mixture could be converted into the epimers 1 and 13, by inspection of its NMR spectrum the proportion of 12β to 12α compound is clearly seen to be low.[21]

Hydrogenation of the unacetylated ester 3 afforded the highest proportion (ca. 1:1) of the desired 3α,7α,12β-trihydroxy ester 1 and also, as expected, gave the cleanest product. The epimers 1 and 13 were separated without difficulty by column chromatography (Scheme I).

We expected that similar hydrogenation of the 3β-formoxy-7-acetoxy-12-oxo ester 10 also would give a mixture, but perhaps a simpler one, because hydrolysis of the 3-formate should be fast, and the hydrogenation would essentially be that of the 3β-hydroxy derivative 12. Instead, the product mixture was more complex; the reason for this only became obvious after the hydrogenation of the dihydroxy-12-oxo ester 4 had been studied (Scheme II).

The latter reaction by analogy with the hydrogenation of 3 was expected to yield the epimeric 3β,7α,12β- and 3β,7α,12α-trihydroxy esters 2 and 14. The first indication that the reaction was not straightforward appeared when the reaction product on standing in methanol deposited a dense crystalline product which was identified as methyl cholate (13). A rationalization that the crystalline starting compound contained the 3α epimer in a molecular complex (such complexing among steroids of related structure is common) proved to be unfounded.

TLC and high-pressure analyses showed that the product of hydrogenation consisted of four main components, of which two had R_f values and retention times identical with those of the hydrogenation products of 3α compound 3. Careful column chromatography effected isolation of the four components in homogeneous form. Two of these were identified as the 3α-trihydroxy esters 1 and 13, and the remaining two were characterized as the expected products of the hydrogenation, the desired 3β,7α,12β compound (2) and its 12α epimer (14). The methyl ester 14 like methyl cholate (13) is crystalline, whereas the two 12β stereoisomers have resisted crystallization attempts. However, all four acids are crystalline.

Obviously, under the hydrogenation conditions epimerization had taken place at C-3. Racemization of steroidal alcohols at C-3 has been long known;[22] the reactions requiring alkoxides and elevated temperatures proceed through a transient ketone mechanism.[23] Raney nickel in boiling cymene has been reported to catalyze oxidation to ketones, epimerization (hydrogen) of steroids at C-5, and reduction of ketones when hydrogen acceptors are present.[24] Extensive equilibration studies by Wicker[25] and by Eliel[26] with Raney nickel on cyclohexane alcohols, although performed under either elevated-temperature or elevated-pressure conditions, should have forecasted the results of our hydrogenations.

The suggested mechanism, as for the alkoxide reactions,[23] that equilibration of the alcohols with Raney nickel proceeds through a transient ketone intermediate, with the point of equilibrium depending on the relative stability of the epimers, is reasonable and applicable to our reaction. The slow reduction of the ketone 4 and the high ratio of catalyst to substrate are conditions which promote the inversion of the axial to the more stable equatorial epimer. In contrast, the hydrogenation of epimeric ketone 3, which undergoes reduction at a faster rate and has the more stable equatorial hydroxyl at C-3, produces no detectable inverted alcohol; evidently the equilibrium point is far on the side of the more stable epimer.

This explanation also resolves the apparent anomaly pertaining to the Raney nickel reduction of methyl 3β-hydroxy-12-oxocholanate reported previously[5a] in which no 3α-hydroxy product was found. In that reaction the hydrogenation was complete in 4 h, whereas the reduction of 4 in the present work required over 40 h. Apparently because the inversion step is much slower than the hydrogenation, in the faster reaction no perceptible inverted product had been formed before the reduction was complete.

An observation was made in an earlier publication[5a] that the considerable variation in both the rate of hydrogenation of the 12-ketone and the ratio of 12β- to 12α-alcohol produced depended on the structure of the compounds. The present results bear this out. That steric factors are involved in both the rate of hydrogenation and the β:α ratio is logical; substituents which interfere with the approach of the catalyst would lower the rate of hydrogenation and would also determine whether the β or α catalytic reaction is favored. This theory is generally consistent with experiment with respect to the slower reactions of the trihydroxy compounds but does not satisfactorily explain a number of other results. For instance, the axial hydroxyl at C-7 can understandably be the cause for the dihydroxy derivatives 3 and 4 to be reduced more slowly than their unsubstituted (at C-7) counterparts, but in the reactions of 3 and 4, why the configuration of the hydroxyl at C-3 should be such a decisive factor with respect to both the rate of hydrogenation and the ratio of 12β:12α compound formed is unclear. Molecular models of 3 and 4 show that the 3α and 3β groups are approximately equidistant from the 12-ketone; the bulk of ring A (cis) should be the significant steric factor.[27]

Additionally, a steric factor must be involved in the selective inversion at C-3 which does not take place at C-7 or C-12. All three hydroxyls of 3β,7α,12β-trihydroxy ester 2 have axial configurations, yet only the more spatially accessible group at C-3 is inverted.

Thus, this Raney nickel catalyzed reaction constitutes the mildest method for selective inversion of an axial C-3 steroidal alcohol and has the advantage of not involving an intermediary derivative before or after the inversion step.[11]

Characterization and Analyses of Compounds

Melting points generally are not reliable criteria of purity among most bile acid derivatives, as is well-known, and the group of compounds described in this work provides no exception to the generalization. Many of the com-

(20) A separate hydrogenation of the C-7 acetate 7 confirmed this deduction; essentially the same mixture was obtained.

(21) The chemical shifts of the sharp C-18 methyl signals of the epimers differ sufficiently for an estimate to be made of the proportion of the 12β to 12α products based on their peak heights.

(22) A. Windaus and C. Uibrig, *Ber. Dtsch. Chem. Ges.*, **48**, 857 (1915).

(23) W. von E. Doering and T. C. Aschner, *J. Am. Chem. Soc.*, 71, 838 (1949).

(24) M. N. Mitra and W. H. Elliott, *J. Org. Chem.*, **33**, 175 (1968); S. K. Banerjee, D. Chakravati, R. N. Chakravati, and M. N. Mitra, *Tetrahedron*, **24**, 6459 (1968); E. C. Kleiderer and E. C. Kornfeld, *J. Org. Chem.*, **13**, 455 (1948).

(25) R. J. Wicker, *J. Chem. Soc.*, 2165 (1956).

(26) E. L. Eliel and S. H. Schroeter, *J. Am. Chem. Soc.*, **87**, 5031 (1965).

(27) The hydrogenation experiments reported here were carried out under essentially identical conditions. The same batch of catalyst was used, but weights of catalyst were not quantitative (net dry weight not determined). In the work reported in ref 5a and 5b, different batches of catalyst were used. Experiments under precisely controlled conditions need to be performed before valid conclusions can be drawn.

(28) In a preliminary experiment, the trihydroxy ester 2 under the same conditions was epimerized to ester 1, which shows (1) that the 12-ketone is not an obligatory accessory to the inversion reaction and (2) that neither inversion at C-7 nor inversion at C-12 takes place.

Table I. ^{1}H NMR Reference Data[a]

methyl cholanate derivative	CHOR[b] conf[c]	CHOR[b] δ[d]	CHOR[b] $w/2$[e]	Me shifts, δ C-19	Me shifts, δ C-18	other, δ
3α-OH	ax	3.6[f]	16	0.93	0.65	
3β-OH	eq	4.07	8	0.96	0.65	
7α-OH	eq	4.08	9	0.92	0.67	
12α-OH	eq	4.03	9	0.90	0.68	
12β-OH	ax	3.44	16	0.94	0.72	
3α-OTs	ax	4.65	14	0.87	0.62	7.30, 7.75 ($ArCH_3$)
3β-OCHO	eq	5.25	8	0.97	0.67	8.1 (OCHO)
7α-OAc	eq	4.90	9	0.93	0.66	2.0 (OAc)
12-oxo				1.02	1.02	

[a] Signals are derived from spectra of monosubstituted methyl cholanates pertinent to characterizations of compounds in this work (solvent $CDCl_3$; Me_4Si as internal standard). [b] Proton signal geminal to substituent at C-3, C-7, or C-12. [c] Conformation of H geminal to substituent: axial or equatorial. [d] Approximate center of multiplet. [e] Width of multiplet at half-height (in Hz). [f] Partly merged with OMe signal.

pounds are solvated and are difficult to convert to the unsolvated form; melting points vary considerably, depending on the crystallizing solvent and also on the conditions of the determination.[5a]

Fortunately other criteria are available for characterization and assessment of purity.

By NMR. Authentic monosubstituted derivatives related to the products of the reactions provide reliable reference data (see Table I). Chemical shifts of the sharp C-18 and C-19 methyl proton signals and of the protons geminal to the hydroxyl (or acyloxy) group and the shapes and bandwidths of the latter proton signals serve as excellent criteria for assignment of structure as well as for evaluation of purity. These data (see Experimental Section) supplement literature values, in particular the ones described by Bhacca and Williams and the extensive compilation of Zurcher derived from various classes of steroids,[29] but since our data are derived only from substituted methyl cholanates, they are specific for bile acid derivatives.

By High-Pressure LC. The methyl esters of the four stereoisomers, **1**, **2**, **13**, and **14**, and a number of their derivatives are well resolved by high-pressure liquid chromatography. The technique has been especially useful in identifying the fractions from column chromatography of the products of Raney nickel hydrogenation and in determining their purity.[30]

Experimental Section

General Methods. Melting points were determined on an electrical micro hot stage and are uncorrected. Infrared spectra were obtained on a Perkin-Elmer infrared spectrophotometer. NMR spectra were determined with either a Varian A-60 or a Perkin-Elmer R-32 instrument, with deuteriochloroform containing Me_4Si as solvent except where otherwise indicated. Optical rotations were obtained in a Zeiss photoelectric precision polarimeter with $CHCl_3$ as solvent except as indicated. Solvent was evaporated on a Rotavap at 50 °C.

General Procedure for Hydrolysis to Free Acid. The methyl ester is refluxed in 5% methanolic potassium hydroxide (100 mg of ester/3 mL of base) for 1 h. Solvent was evaporated, and the residue was dissolved in water, cooled in an ice bath, and neutralized with 3 N H_2SO_4. The precipitated solid was dissolved in aqueous ethanol for crystallization.

(29) R. F. Zurcher, *Helv. Chim. Acta*, **46**, 2054 (1963); N. S. Bhacca and D. H. Williams, "Applications of NMR Spectroscopy in Organic Chemistry", Holden-Day, San Francisco, 1964, pp 47, 77.

(30) The high-pressure LC instrument used was a Waters Associates assembly with a septumless injector, refraction index detector, and a Bondapak C-18 reverse-phase column. The solvent system used was methanol–water (70:30) at a flow rate of 2.0 mL/min for the separation of the four stereoisomeric esters. The compounds emerged in the following order: $\beta\alpha\beta$ ester (**2**), $\alpha\alpha\beta$ ester (**1**), $\beta\alpha\alpha$ ester (**14**), and $\alpha\alpha\alpha$ ester (**13**). A more detailed report will be published.

(1) Methyl 3α,7α-Dihydroxy-12-oxocholanate (3). The diacetate[6] **5** (500 mg) was dissolved in 5 mL of 10% $NaOCH_3$ in methanol and kept at ca. 25 °C for 24 h. The reaction flask was cooled in an ice bath while the product was neutralized with concentrated HCl. Removal of the solvent in an evaporator at reduced pressure, redissolving the solid with methanol, and dilution with water caused crystallization (quantitative yield). Recrystallization of **3** from aqueous methanol yielded thin plates: mp 154.5–161.5 °C (lit.[7] 158–160 °C); NMR δ 1.03 (6 H, s, C-18 and C-19 Me), 3.50 (1 H, br m, CHOH), 3.70 (3 H, s, OMe), 3.93 (1 H, m, CHOH); identical, according to TLC, NMR, and IR comparisons, with the ester prepared by complete hydrolysis of **5** and reesterification to the methyl ester **3**.[7]

(2) Methyl 3α-Hydroxy-7α-acetoxy-12-oxocholanate (6). To the diacetate[6] **5** (6.35 g) dissolved in 300 mL of methanol was added 7.5 mL of concentrated HCl, and the mixture was kept at 25 °C for 24 h. Dilution of the clear solution to near turbidity with H_2O caused separation of well-shaped crystals in two crops (total 4.65 g, 80% yield) mp 183.0–183.5 °C, identical according to melting point, TLC, and NMR with the product prepared by oxidation of methyl cholate 7-acetate (**7**).[6]

(3) Methyl 3α-Tosyloxy-7α-acetoxy-12-oxocholanate (8). To compound **6** (4.5 g), dried by azeotropic distillation with benzene (a few milliliters of methanol was needed to effect complete solution), dissolved in 25 mL of anhydrous pyridine was added 4.4 g of freshly recrystallized tosyl chloride[31] in 8 mL of pyridine in one portion; very slight warming of the solution resulted. When the solution was allowed to stand overnight, needles of pyridinium chloride separated. To the flask immersed in an ice bath were added ice chips which dissolved the chloride. More water was added until the clear, light yellow solution became slightly turbid. Refrigeration and standing caused crystallization of the tosylate **8** which was washed with cold 0.1 N HCl: yield in two crops, 5.14 g (air-dried weight, 85%); recrystallized from benzene–hexane, mp 152–154 °C; IR 1704 (C=O), 1724 (OCOMe, COOMe), 1176 (OTs) cm^{-1}; NMR δ 0.98 (6 H, s, C-18 and C-19 Me), 1.98 (3 H, s, OCOMe), 2.43 (3 H, s, $C_6H_4CH_3$), 3.64 (3 H, s, COOMe), 4.3 (1 H, br m, CHOTs), 4.97 (1 H, m, CHOAc), 7.30 and 7.75 (each 2 H, d, J = 9 Hz, para disubstituted phenyl).

Anal. Calcd for $C_{34}H_{47}O_8S\cdot0.5CH_3OH$: C, 65.48; H, 7.97. Found: C, 65.71; H, 7.99.

(4) Methyl cholate 3-tosylate 7-acetate (9) was prepared by tosylation of methyl cholate 7-acetate (**7**) according to the procedure used for tosylate **8**. Crystalline **9** was obtained similarly: crude yield 100%; recrystallized from benzene–hexane as fine needles, mp 161.5–163.5 °C; IR 1724 (COOMe), 1176 (OTs) cm^{-1}; NMR δ 0.67 (3 H, s, C-18 Me), 0.88 (3 H, s, C-19 Me), 2.0 (3 H, s, OCOMe), 2.45 (3 H, s, $ArCH_3$), 3.65 (3 H, s, OMe), 7.31 and 7.75 (each 2 H, d, J = 9 Hz, para disubstituted phenyl).

Anal. Calcd for $C_{34}H_{50}O_8S\cdot0.5C_6H_6$: C, 67.55; H, 8.12. Found: C, 67.91; H, 8.04.

(5) Methyl 3β-Formoxy-7α-acetoxy-12-oxocholanate (10). (a) By DMF Inversion of Tosylate. The tosyloxy acetoxy ester **8** (3.2 g), after azeotropic distillation in benzene, was dissolved in 120 mL of DMF (reagent grade, dried with molecular sieves)

(31) S. W. Pelletier, *Chem. Ind. (London)*, 1034 (1953).

and kept at a temperature of 80 ± 1 °C (thermistor controlled). As monitored by TLC, the tosylate had been consumed after 66 h. The straw-colored solution was diluted with water to near turbidity, and on standing, crystals separated. A second crop was obtained on further dilution of the mother liquor and refrigeration: total of the two crops 2.18 g (86%); crystallized from methanol as thin flakes, mp 155.0–158.0 °C; IR (CS_2) 1720 (C=O), 1735, 1750 cm^{-1} (OCOH, $OCOCH_3$, COOMe); NMR δ 1.03 (3 H, s, C-18 Me), 1.06 (3 H, s, C-19 Me), 1.98 (3 H, s, OCOMe), 3.64 (3 H, s, OMe), 5.0 (1 H, m, CHOAc), 5.15 (1 H, m, CHOCHO), 8.1 (1 H, s, OCHO).

Anal. Calcd for $C_{28}H_{42}O_7$: C, 68.54; H, 8.63. Found: C, 68.96; H, 9.03.

(b) By Oxidation of Compound 11. The 12-hydroxy ester analogue of **10** was oxidized in acetic acid–potassium chromate by the procedure described by Fieser[6] for the oxidation of methyl cholate 7-acetate (**7**). The crystalline product obtained was identical with compound **10** according to melting point, TLC, and NMR comparisons.

(6) Methyl 3β-formoxy-7α-acetoxy-12α-hydroxycholanate (11) was from the tosylate **9** by the procedure used in section 5a. Recrystallization from MeOH–H_2O yielded fine needles: mp 136.0–138.5 °C; IR 1730 cm^{-1} (br); NMR δ 0.68 (3 H, s, C-18 Me), 0.96 (3 H, s, C-19 Me), 2.02 (3 H, s, $OCOCH_3$), 3.66 (3 H, s, OMe), 4.0 (1 H, m, C-12, CHOH), 4.95 (1 H, m, CHOAc), 5.16 (1 H, m, CHOCHO), 8.05 (1 H, s, OCHO).

Anal. Calcd for $C_{28}H_{44}O_7 \cdot CH_3OH$: C, 66.38; H, 9.22. Found: C, 66.55; H, 9.10.

(7) Methyl 3β-Hydroxy-7α-acetoxy-12-oxocholanate (12). (a) By Alumina Reaction. Compound **10** dissolved in benzene solution was placed on a column of neutral alumina (50:1 ratio), left for 18 h and eluted with CH_2Cl_2–hexane (98:2). On concentration of the eluant compound **12** crystallized spontaneously: mp 197–199 °C; IR 1703 (C=O), 1724 cm^{-1} (OCOMe, COOMe); NMR δ 1.03 (3 H, s, C-18 Me), 1.05 (3 H, s, C-19 Me), 2.00 (3 H, s, OCOMe), 3.66 (3 H, s, OMe), 4.08 (1 H, m, CHOH), 5.00 (1 H, m, CHOAc).

Anal. Calcd for $C_{27}H_{42}O_6$: C, 70.10; H, 9.15. Found: C, 69.85; H, 9.36.

(b) By HCl. The formate **10** by treatment with concentrated HCl in methanol solution processed as in part 2 yielded crystalline product identical with compound **12** prepared in section 7a, according to TLC, IR, and NMR comparisons.

(8) Methyl 3β,7α-Dihydroxy-12-oxocholanate (4). Compound **10** was partially hydrolyzed to the methyl ester **4** by treatment with 10% $NaOCH_3$ in methanol as in section 1 (preparation of **3**). Recrystallization from aqueous methanol yielded colorless needles: mp 170.0–170.5 °C; $[\alpha]_D$ +93.2°; IR (CS_2) 1720 cm^{-1} (C=O); NMR δ 1.03 (6 H, s, C-18 Me and C-19 Me), 3.65 (3 H, s, OMe), 3.95 (1 H, m, CHOH), 4.05 (1 H, m, CHOH).

Anal. Calcd for $C_{25}H_{40}O_5$: C, 71.39; H, 9.59. Found: C, 71.31; H, 10.04.

(9) 3β,7α-Dihydroxy-12-oxocholanic (4a) was obtained quantitatively from either the ester **4** or the corresponding formate acetate **10** by the general hydrolysis procedure described at the beginning of the Experimental Section. The acid **4a** crystallized from aqueous ethanol as colorless thin plates: mp 205.0–206.0 °C; IR (KBr) 1695 (C=O), 1715 cm^{-1} (COOH); NMR (CD_3COCD_3–Me_2SO-d_6) δ 1.07 (6 H, s, C-18 and C-19 Me), 3.50 (1 H, br m, CHOH), 3.91 (4 H, m, CHOH and H_2O).

Anal. Calcd for $C_{23}H_{34}O_5$: C, 70.90; H, 9.42. Found: C, 71.28; H, 9.58.

(10) Methyl 3α,7α,12β-Trihydroxycholanate (1). The oxo ester **3** (2.73 g) dissolved in 125 mL of methanol, to which was added ca. 27 g of Raney nickel,[19] was shaken at a hydrogen pressure of 45 lb/sq in. As monitored by TLC, the ketone was completely reduced at 26 h. The supernatant solution, decanted after settling of the catalyst, was combined with two 50-mL methanol washings of the nickel residue and evaporated to an oil. The oil (2.90 g) was incompletely dissolved in CH_2Cl_2; the soluble part examined by NMR indicated that the proportion of 12α- to 12β-hydroxy product was approximately 1:1, according to the relative heights of the sharp C-18 methyl signals at δ 0.068 and 0.72, respectively.[21] The CH_2Cl_2-insoluble part of the methanol extracts was water soluble and apparently is inorganic matter.

The CH_2Cl_2-soluble part was evaporated and redissolved in a few milliliters of methanol. From the solution large dense crystals separated, which by comparison with authentic material were identified as essentially pure methyl cholate (**13**). The mother liquor (979 mg) was azeotropically distilled with benzene and chromatographed on a Florisil column (50:1 ratio). Those fractions eluted with CH_2Cl_2–MeOH (98:2) and found to be homogeneous by TLC were combined, total 580 mg. Although homogeneous by TLC and high-pressure LC, this material resisted crystallization attempts and was characterized as the 12β-hydroxy compound **1** mainly by NMR through the chemical shift of the C-18 methyl signal and the broad 2-proton signal due to the overlapping of the axial protons at C-3 and C-12 (see text): $[\alpha]_D$ +14.0°; IR 1724 cm^{-1} (ester C=O); NMR δ 0.72 (3 H, s, C-18 Me), 0.90 (3 H, s, C-19 Me), 3.4 (2 H, br, 12α-H), 3.54 (3 H, s, OMe), 3.86 (1 H, m, CHOH); mass spectrum, *m/e* 422 (M^+), 420 (M – 2), 404 (M – 18).

Anal. Calcd for $C_{25}H_{42}O_5 \cdot 0.5CH_3OH$: C, 69.82; H, 10.11. Found: C, 69.50; H, 10.12.

The final fractions eluted from the column with CH_2Cl_2–MeOH (90:10) were mixtures of the epimers **1** and **13**, with increasing proportions of **13**.

(11) 3α,7α,12β-Trihydroxycholanic acid (1a) was obtained from methyl ester **1** by the general hydrolysis method described above: well-shaped needles of **1a** crystallized out of aqueous ethanol, mp 200.0–201.5 °C;[34] $[\alpha]_D$ +30.5° (EtOH); NMR (CD_3COCD_3–D_2O) δ 0.71 (3 H, s, C-18 Me), 0.91 (3 H, s, C-19 Me), 3.40 (2 H, br m, 2 CHOH overlapping signals; C-7 CHOH obscured by H_2O). According to TLC **1a** is completely free of cholic acid.

(12) Methyl 3β,7α,12β-Trihydroxycholanate (2). The dihydroxy keto ester **4** (1 g) in 100 mL of methanol was shaken in a Parr hydrogenator with ca. 18 g of Raney nickel[19] at 47 lb/sq in. of hydrogen. After 40 h an aliquot (fraction I) examined by high-pressure LC and NMR showed that a small amount of ketone remained. At 62 h, when reduction of ketone was complete, the supernatant solution was decanted after the catalyst had settled, combined with two 50-mL methanol washings of the nickel solid, and evaporated to an oil (fraction II, 980 mg). The nickel residue was further extracted with 50 mL of methanol by occasional stirring and shaking of the mixture over a 24-h period (not in a hydrogen atmosphere) to give fraction III (8.8 mg). The NMR spectra of the three successive fractions differed principally in the increasing complexity of the signals in the regions due to the angular methyl protons and the proton at C-3 (see text).

The CH_2Cl_2-soluble part of fraction II (833 mg) (the CH_2Cl_2-insoluble part was soluble in water as found with the analogous extract of the nickel residue of section 10) was chromatographed on a Florisil column 50:1; small fractions eluted slowly by CH_2Cl_2–MeOH (98:2) were collected. Examination of the fractions revealed the presence of four essentially separated components.[32] The homogeneous fractions of each were combined, evaporated, and weighed: component I, 225 mg; II, 296 mg; III, 92 mg; IV, 86 mg. (Intermediate mixed fractions contained little material and are not included in the weights given.) The four components were characterized as follows.

(I) Methyl 3β,7α,12β-trihydroxycholanate (2), although homogeneous by TLC, high-pressure LC, and NMR, resisted crystallization attempts: $[\alpha]_D$ +17.0°; IR 1721 cm^{-1} (ester C=O); NMR δ 0.73 (3 H, s, C-18 Me), 0.95 (3 H, s, C-19 Me), 3.45 (1 H, br q, CHOH), 3.66 (3 H, s, OMe), 3.88 (1 H, m, CHOH), 4.04 (1 H, m, CHOH); mass spectrum, *m/e* 422 (M^+), 420 (M – 2), 404 (M – 18).

Anal. Calcd for $C_{25}H_{42}O_5$: C, 71.05; H, 10.02. Found: C, 70.87; H, 10.41.

(II) Methyl 3α,7α,12α-trihydroxycholanate (1) was identical with **1** prepared by Raney nickel reduction (section 10) according to TLC, NMR, and high-pressure LC comparisons.

(32) After the third component had emerged, the fourth component consisting of nearly homogeneous methyl cholate was eluted with CH_2Cl_2–MeOH (95:5).

(33) J. Barnett and T. Reichstein, *Helv. Chim. Acta*, **21**, 926 (1938); R. T. Blickenstaff, K. Atkinson, D. Breaux, E. Foster, Y. Kim, and G. C. Wolf, *J. Org. Chem.*, **36**, 1271 (1971).

(34) The melting point for this compound given in the preliminary report (ref 1) was erroneous.

(III) Methyl 3β,7α,12α-trihydroxycholanate (14) crystallized as needles from ethyl acetate: mp 184.5–187.5 °C; identical according to TLC, high-pressure LC, and NMR with authentic 14 (below).

(IV) Methyl 3α,7α,12α-trihydroxycholanate (13), dense prisms from MeOH, was identical with authentic methyl cholate.

(13) 3β,7α,12β-Trihydroxycholanic acid (2a) obtained from ester 1 by the general hydrolysis procedure (above) crystallized from aqueous ethanol as colorless prisms: mp, 225–226 °C;[34] $[\alpha]_D$ +23.4° (EtOH); NMR (CD_3COCD_3–D_2O) δ 0.73 (3 H, s, C-18 Me), 0.95 (3 H, s, C-19 Me), 3.43 (1 H, br m, CHOH), 3.82 (1 H, m, CHOH), 3.97 (1 H, m, CHOH).

Anal. Calcd for $C_{24}H_{40}O_5 \cdot 0.5H_2O$: C, 69.51; H, 9.96. Found: C, 69.16; H, 10.04.

(14) Methyl 3β,7α,12α-trihydroxycholanate 3-formate (15), prepared by inversion of methyl cholate 3-tosylate[33] by reaction with DMF as in the preparations of the tosylates **10** and **11**, crystallized out of aqueous methanol: mp 157.0–158.5 °C (lit.[11] mp 110–112 °C); IR 1721 cm^{-1} (formate and COOMe); NMR δ 0.68 (3 H, s, C-18 Me), 0.93 (3 H, s, C-19 Me), 3.85 (1 H, m, CHOH), 3.96 (1 H, m, CHOH), 5.14 (1 H, m, CHOCHO), 8.03 (1 H, s, OCHO).

(15) Methyl 3β,7α,12α-trihydroxycholanate (14) was obtained by hydrolysis of the formate group of **15** either by treatment with MeOH–HCl or by contact with alumina as in the analogous preparations of **12** (section 7). The product was crystallized from ethyl acetate: mp 185.0–187.0 °C (lit.[10] mp 176 °C, from acetone); NMR δ 0.68 (3 H, s, C-18 Me), 0.91 (3 H, s, C-19 Me), 3.64 (3 H, s, OMe), 3.86 (1 H, m, CHOH), 4.01 (2 H, m, merging of 2 CHOH signals).

(16) 3β,7α,12α-Trihydroxycholanic acid (14a) was obtained from any of its derivatives **11**, **14**, or **15** by the general method of hydrolysis to the free acids described at the beginning of the Experimental Section and was crystallized out of aqueous EtOH as needles: mp 196.5–197.0 °C[34] (lit.[6] mp 200–202 °C from EtOAc).

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Registry No. 1, 71883-63-1; **1a**, 71883-64-2; **2**, 71883-65-3; **2a**, 71883-66-4; **3**, 10538-64-4; **4**, 71837-86-0; **4a**, 18069-63-1; **5**, 28535-81-1; **6**, 71837-87-1; **7**, 7432-44-2; **8**, 71837-88-2; **9**, 71837-89-3; **10**, 71837-90-6; **11**, 71837-91-7; **12**, 71837-92-8; **13**, 1448-36-8; **14**, 28050-54-6; **14a**, 3338-16-7; **15**, 42921-40-4; methyl 3α-hydroxycholanate, 1249-75-8; methyl 3β-hydroxycholanate, 5405-42-5; methyl 7α-hydroxycholanate, 28050-19-3; methyl 12α-hydroxycholanate, 1249-70-3; methyl 12β-hydroxycholanate, 28050-18-2; methyl 3α-tosyloxycholanate, 1261-92-3; methyl 3β-formyloxycholanate, 71837-93-9; methyl 7α-acetoxycholanate, 19684-60-7; methyl 12-oxocholanate, 1173-30-4.

Mass Spectra of Nitrate Esters of Cholic Acid Derivatives

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Steroidal nitrate esters preferentially eject HNO_3, HNO_2, and NO_2· over NO_3· and NO·. It is proposed that oxidative elimination of HNO_2 results in the formation of an oxo group. No direct ionization of the nitroxy group itself occurs in the mass spectrometer, but the observed ejections are triggered by movement of charge to the vicinity of the nitroxy group. The electron impact induced loss of HNO_3 and NO_2· from steroidal nitrates has a strong mechanistic similarity to the loss of HOAc and Ac·, respectively, from steroidal acetates, but the former losses usually produce less intense mass spectral peaks. An example of the facile ejection of NO_2· from a carbonium ion to regenerate an ion radical is provided.

Virtually no definitive mass spectral summary of steroidal nitrates has yet been published. Observations, generalizations, and complexities associated with the mass spectra of steroid nitrate esters are therefore described in this paper. Since the highly electronegative nitrate functional group is expected to possess an ionization energy higher than that of any other functional group, except fluorine, one anticipates that the possible ejection of HNO_3, HNO_2, NO_3·, NO_2·, or NO· from a nitrate-containing molecule will result from ionization and subsequent alteration of the molecular region remote or adjacent to the nitrate group. Thus, the study of mass spectral breakdown of nitrate esters should uniquely provide further insight on fundamental principles which govern mass spectral fragmentation processes, e.g., charge localization, migration, and induction of fragmentations. In addition, intramolecular oxidation and the formation of radical ions from carbonium ions due to ejection of reasonably stable NO_2· or NO· radicals from even-electron ions are processes that need to be fully defined.

Results and Discussion

Except for the 12-eV spectra, the partial, monoisotopic mass spectra in Table I are cited in terms of relative percent of the most intense mass peak above m/z 200; the true base peak was usually a peak below m/z 200 with a relative intensity of approximately 2–7 times the cited 100% peak. Two problems complicate the interpretion of the mass spectra of steroid nitrates: (1) thermal decomposition of the sample on the direct-inlet probe, and (2) the low relative intensity of some of the characteristic peaks which could be indiscernible with prominent peaks that have their origin from impurities. Nevertheless, multiple mass spectral scans with increasing probe temperature and successive scans at ionization voltages of 70 and 12 eV gave fairly reproducible spectra except for the trinitrates; the propensity for decomposition of a steroidal nitrate increased with the degree of nitration of the molecule. Trinitrates **5c** and **6b** decomposed slowly enough that most of the characteristic peaks could be identified in the initial spectra taken in the first few scans at the lowest probe temperature giving significant ion current above background. However, trinitrates **6a** and **6c** decomposed more rapidly and only a few significant mass peaks persisted sufficiently in several of the initial successive spectral scans to be reliably reported in Table I. The mass spectra of nitrates **7a**, **7b**, **8a**, and **8c** have been reported[1] and are summarized again in Table I but

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