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Bile acids. LV. 2,2-Dimethoxypropane: an esterifying agent preferred to diazomethane for chenodeoxycholic acid

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Summary Artifacts formed by methylation of chenodeoxycholic acid with diazomethane are identical to the synthetic 3α- and 7α-monomethyl ethers of methyl chenodeoxycholate. The extent of formation of these artifacts with time has been studied. No detectable artifacts were found with 2,2-dimethoxypropane as the methylating agent.

Supplementary key words ethers of methyl chenodeoxycholate · gas–liquid chromatography · gas–liquid chromatography–mass spectrometry · high-pressure liquid chromatography · methyl 3α-methoxy-7α-hydroxy-5β-cholanate · methyl 7α-methoxy-3α-hydroxy-5β-cholanate · ethers from diazomethane

Extensive use has been made of diazomethane for esterification of free bile acids for analytical investigations. Additional products formed from chenodeoxycholic acid with this reagent, as observed in this laboratory[2] and reported by others (1, 2), were described as monomethyl ethers of methyl chenodeoxycholate. This paper reports the unequivocal identification of these side-products by comparison with the synthetic materials, and methods to reduce or eliminate their formation, thus providing an improved procedure for the derivatization of samples of chenodeoxycholic acid.

Methods and materials

Details of the following procedures or analytical methods have been reported (3–5): TLC and PLC on silica gel H; GLC on 3% OV-210 at 230°C; on 3% OV-17 at 260°C or 1% SE-30 at 230°C (relative retention time, RR_T, referred to methyl deoxycholate as 1.0); HPLC on a semi-preparative scale with two coupled Porasil A columns (Waters, ⅜ in × 2 ft) at a flow rate of 3 ml/min, or on an analytical column (μPorasil 0.25 in × 12 in) with 5% 2-propanol in hexane at a flow rate of 2 ml/min (relative retention volume, RR_V, referred to methyl deoxycholate as 1.0 with retention volume 44 ml); MS; PMR; and melting point determination. Gas–liquid chromatography–mass spectrometry (GLC–MS) was performed with a coiled glass column (0.25 in OD × 6 ft) containing 1% OV-17 on Gas Chrom Q at 250°C (flow rate of 20 ml/min) with an LKB Model 9000 mass spectrometer coupled to a Logos Model DS/1200 data acquisition system. Elemental analyses were determined by Galbraith Laboratories, Inc., Knoxville, TN. All intermediates were characterized by PMR, MS, melting point, and chromatographic means. Chenodeoxycholic acid (TLC-homogeneous) was taken from stock in this laboratory. Sodium hydride and lithium tri-*t*-butoxyaluminum hydride were obtained from Ventron Chemicals, Beverly, MA. Diazald and 2,2-dimethoxypropane were products of Aldrich Chem. Co., Milwaukee, WI. Dihydropyrane (Eastman Chem. Co., Rochester, NY) was distilled (bp 84–87°C) prior to use. Ethylene glycol,

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Abbreviations and trivial names: chenodeoxycholate, 3α,7α-dihydroxy-5β-cholanate; TLC, thin-layer chromatography; PLC, preparative-layer chromatography; HPLC, high-pressure liquid chromatography; PMR, proton magnetic resonance spectroscopy; GLC, gas–liquid chromatography; MS, mass spectrometry.

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[2] Shaw, R., and W. H. Elliott, unpublished observations.

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methyl iodide, *p*-toluenesulfonic acid, sodium borohydride, anhydrous ether, ethyl acetate, acetonitrile (distilled), 2-propanol and acetone (distilled prior to use) were obtained from Fisher Scientific Co., Pittsburgh, PA. Anhydrous methanol and silver nitrate originated from Mallinckrodt Chem. Co., St. Louis, MO. "Hexane" was routinely distilled from Quick Solv B (Chem Tech).

Methylation with diazomethane. Diazomethane in ether was prepared from Diazald as described by Aldrich Chem. Co. The yellow ethereal solution was added to a vial containing the bile acid (1 mg) and 0.1–0.2 ml of ether until a permanent yellow color developed. The vial was closed with a Teflon-lined cap and left standing at room temperature for 2 hr. The solvent was evaporated to dryness under nitrogen at room temperature, the residue was taken up in acetonitrile (50 μl), and an aliquot (2 μl) of the solution was used for GLC analysis.

Methylation with 2,2-dimethoxypropane. To a solution of bile acid (1 mg) in methanol (50 μl) were added 2,2-dimethoxypropane (50 μl) and conc. hydrochloric acid (10 μl). The mixture was capped as above and left to stand in the dark overnight at room temperature. If the vial remained in the presence of fluorescent light, a gradual darkening of the contents occurred. After the addition of excess solid sodium bicarbonate, the mixture was evaporated to dryness under nitrogen. The residue was extracted several times with ether and the extract was evaporated to dryness. The residue was treated as described above for GLC analysis.

Monomethyl ethers of methyl chenodeoxycholate. Methyl chenodeoxycholate was oxidized with silver carbonate–Celite as described by Fetizon and Golfier (6). After column chromatography through alumina, the purified 3-oxo-derivative (7) was converted to the 3,3-ethylenedioxy compound (8) by reaction with ethylene glycol and *p*-toluenesulfonic acid in dry benzene. The product was purified by PLC and was methylated with methyl iodide and sodium hydride by a modified procedure of Stoochnoff and Benoiton (9). The product was purified by PLC (acetone–benzene 1:9) and crystallized from methanol in 43% yield to afford methyl 3,3-ethylenedioxy-7α-methoxy-5β-cholanate, mp 165.5–167.5°C, homogeneous by TLC, GLC, and HPLC. Hydrolysis overnight at room temperature of the ketal in dichloromethane containing hydrochloric acid provided a product that was purified by PLC and then reduced with lithium tri-*t*-butoxyaluminum hydride to yield the desired methyl 7α-methoxy-3α-hydroxy-5β-cholanate. After purification by PLC (acetone–benzene 1:4) and by crystallization from aqueous methanol, crystalline material was obtained in 37% yield; mp 118–118.5°C; homogeneous by TLC, GLC and HPLC (**Table 1**); calc. C 74.24%, H 10.54%, found C 74.48%, H 10.57%.

Methyl 7α-hydroxy-3-oxo-5β-cholanate was derivatized with dihydropyrane (10) to provide a product in 72% yield after PLC and crystallization from hexane; mp 122–125°C. Treatment with sodium borohydride provided the 3α-hydroxy compound that was methylated with methyl iodide. Hydrolysis of the ketal by refluxing in a mixture of dichloromethane–methanol 2:1 in the presence of hydrochloric acid for 24 hr provided methyl 3α-methoxy-7α-hydroxy-5β-cholanate in 38% yield after purification by PLC (acetone–benzene 1:4) and crystallization from aqueous methanol; mp 86.5–87.5°C; homogeneous on TLC, GLC, and HPLC (Table 1); calcd. C 74.24%, H 10.54%; found C 74.36%, H 10.35%.

TABLE 1. Chromatographic mobilities of compounds A and B and synthesized methyl ethers

	Compound A	Me 3α-OH-7α-OMe[a]	Compound B	Me 7α-OH-3α-OMe[b]
GLC[c]				
RR_T (OV-210)	0.65	0.65	0.78	0.78
RR_T (OV-17)	0.71	0.71	0.87	0.87
TLC[d]				
R_f	0.44	0.44	0.49	0.49
HPLC[e]				
RR_V (μPorasil[e])	0.26	0.26	0.28	0.28

[a] Me 3α-OH-7α-OMe = methyl 3α-hydroxy-7α-methoxy-5β-cholanate.
[b] Me 7α-OH-3α-OMe = methyl 7α-hydroxy-3α-methoxy-5β-cholanate.
[c] Relative retention time (RR_T) and relative retention volume (RR_V) expressed in terms of methyl deoxycholate as standard.
[d] TLC in acetone–benzene 3:7; methyl chenodeoxycholate R_f 0.17.
[e] HPLC with 5% propan-2-ol in hexane.

Results and discussion

Side products from treatment of chenodeoxycholic acid with diazomethane. Chenodeoxycholic acid (2.5 g; mp 167–169°C, reported (1) mp 168°C) was crystallized from acetone–hexane, air-dried, and methylated with excess diazomethane in ether. After removal of solvent the product contained 0.5% of artifact A and 4.4% of artifact B as analyzed by GLC on OV-210. Fractional crystallization from ethyl acetate–*n*-heptane separated the components such that the final mother liquor became enriched in the side-products (methyl ethers) while the first and second crops of crystals contained little of these impurities, as judged by HPLC analysis. The enriched material (0.8 g) was chromatographed on two coupled Porasil A columns in 7% 2-propanol in hexane to remove methyl chenodeoxycholate, and then in 5% 2-propanol in hexane to yield a purified but unresolved mixture of the methyl ethers. Final separa-

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TABLE 2. Characteristic peaks in PMR spectra of methyl chenodeoxycholate and its monomethyl ethers

δ ppm	Me 3α,7α(OH)$_2$[a]	Compounds A + B	Me 3α-OH-7α-OMe[b]	Me 7α-OH-3α-OMe[c]
18-H	0.68	0.67	0.64	0.68
19-H	0.92	0.92	0.91	0.92
3β-H	3.42	3.04	3.38	3.04
3α-OCH_3		3.36		3.35
7α-OCH_3		3.27 (*ca.* 0.5H)	3.26	
ester CH_3	3.67	3.69	3.68	3.66
7β-H	3.85	3.85 (*ca.* 1H)	3.22	3.86

[a] Me 3α,7α(OH)$_2$ = methyl 3α,7α-dihydroxy-5β-cholanate (chenodeoxycholate).
[b] Me 3α-OH-7α-OMe = methyl 3α-hydroxy-7α-methoxy-5β-cholanate.
[c] Me 7α-OH-3α-OMe = methyl 7α-hydroxy-3α-methoxy-5β-cholanate.

tion was achieved by HPLC on a μPorasil column into components A and B which corresponded to methyl 7α-methoxy-3α-hydroxy- and 3α-methoxy-7α-hydroxy-5β-cholanate, respectively, in their HPLC, TLC, and GLC mobilities (Table 1). The chemical shifts in the PMR spectrum of the mixture (**Table 2**) also corresponded to the combined spectra of the individual methyl ethers. It is of interest to note that the methylation of a hydroxyl group leads to an upfield shift of 0.34 ppm for an adjacent axial proton (3β-H) and 0.64 ppm for an equatorial proton (7β-H), probably as a result of further ring deformation upon introduction of a bulky methyl group. The GLC–MS analysis of the synthetic ethers and of the isolated side-products (**Table 3**) further confirmed the assignment of the structures. Differences in the relative intensities (to base peak) of fragment ions *m/e* 402 (M − 18), 388 (M − 32), 387 [M − (18 + 15)], 287 [M-(18 + side chain)], 273 [M − (132 + side chain)] and 260 [M − (18 + side chain + C-17 + C-16)] were observed for the two methyl ethers as a result of the preferential fragmentation under electron impact with loss of the axial 7α-substituent over the equatorial 3α-substituent, in agreement with early studies reported from this laboratory (11) on analogous compounds and their TMSi ethers in which the oxygen atom at C-3 or C-7 was replaced with ^{18}O. Similar observations have been reported by Vouros and Harvey (12) with 3,7-disubstituted cholestanes.

By GLC analysis on OV-17 the level of production of artifacts during esterification with diazomethane amounts to as much as 1% for the 7α-ether and 4% for the 3α-ether. The appearance of these contaminants was observed within 3 min of reaction; a slight increase in the level of the 3α-ether took place upon prolonged treatment (**Fig. 1**). The experiences of van Straelen, de Jongh, and Jansen (1), which prompted them to search for better methods of crystallization of chenodeoxycholic acid to provide a product containing less than 0.3% of side-products, were extended in our hands by a comparison of the purity of the ester after crystallization of the acid from acetone–hexane or ethyl acetate–hexane. Crystallization of the acid from either of these solvent mixtures one to two days prior to formation of the ester with diazomethane

TABLE 3. Relative intensities of important fragment ions of compounds A and B and synthetic *O*-methyl ethers of methyl chenodeoxycholate

m/e	Compound A	Me 3α-OH-7α-OMe[a]	Compound B	Me 7α-OH-3α-OMe[b]	Interpretation
420	0.2	0.4	0.7	1.2	M^+
402	1.6	1.5	18.3	18.9	M − H_2O
388	10.8	13.2	3.6	3.0	M − CH_3OH
387	3.1	0	5.8	6.2	M − (H_2O + CH_3)
370	100.0	100.0	100.0	100.0	M − (H_2O + CH_3OH)
355	46.5	44.0	46.4	46.7	M − (H_2O + CH_3OH + CH_3)
341	0	8.8	15.0	17.2	M − 79
287	1.5	1.2	23.4	25.7	M − (H_2O + S.C.[c])
278	4.8	4.4	10.3	11.0	M − (S.C. + C-17 + C-16)
273	12.3	11.6	3.0	2.3	M − (CH_3OH + S.C.)
262	12.1	11.4	6.3	6.8	M − 158
260	3.4	3.3	10.0	9.7	M − (H_2O + S.C. + C-17 + C-16)
255	35.4	33.4	55.2	53.6	M − (CH_3OH + H_2O + S.C.)
246	10.2	9.4	14.5	15.7	M − (CH_3OH + S.C. + C-17 + C-16)
228	18.4	17.0	31.4	32.5	M − (CH_3OH + H_2O + S.C. + C-17 + C-16)
213	36.7	33.0	47.1	47.1	M − (CH_3OH + H_2O + S.C. + Ring D)
201	18.8	16.7	26.9	26.4	M − (CH_3OH + H_2O + Ring A+S.C.)

[a] Me 3α-OH-7α-OMe = methyl 3α-hydroxy-7α-methoxy-5β-cholanate.
[b] Me 7α-OH-3α-OMe = methyl 7α-hydroxy-3α-methoxy-5β-cholanate.
[c] S.C. = side chain.

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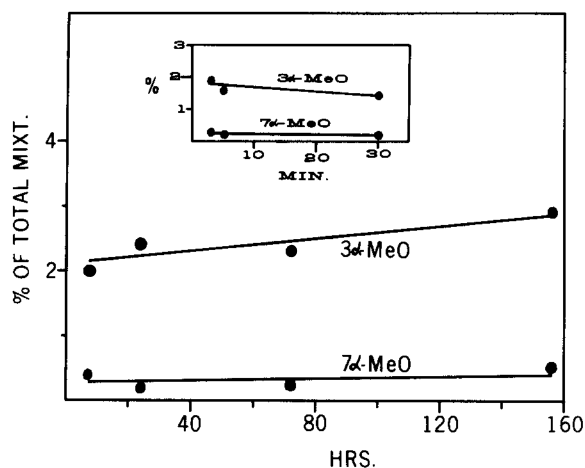

Fig. 1. Time course of methylation of chenodeoxycholic acid with diazomethane. The methyl ester was analyzed by GLC on 3% OV-210. 3α-MeO, methyl 3α-methoxy-7α-hydroxy-5β-cholanate; 7α-MeO, methyl 7α-methoxy-3α-hydroxy-5β-cholanate.

resulted in products with increased amounts of the ethers, in comparison to the products from these batches that were air dried up to 12 days, probably reflecting loss of volatile solvent. Mosbach, Nicolau, and Nichols (13) have shown conclusively that heptane is entrapped with crystalline acid in a fixed ratio with a resultant lower melting point (119°C) of the product. Acetone or ethyl acetate each reduced the production of artifactual ethers, but hexane enhanced the process. The residue remaining from distillation of hexane increased ether formation by a factor of 3.5. Addition of methanol depressed ether formation, but addition of methanol and solid sodium bicarbonate resulted in less than half the amount seen in Fig. 1. These observations correlate with those of van Straelen et al. (1) and suggest the presence of small amounts of acidic material in the solvent. The role of Lewis acids (14–16) in the catalysis of ether formation is well established. Finally, the formation of methyl ethers was demonstrated by treatment of purified [^{3}H-G]chenodeoxycholic acid [New England Nuclear, Boston, MA; 1.6 μCi/μmol, 0.81% of a more polar component present (**Fig. 2**)] with diazomethane for 2 hr; 0.7% of radioactive material remained at the origin in TLC, while 7.2% appeared in the zone for the methyl ethers.

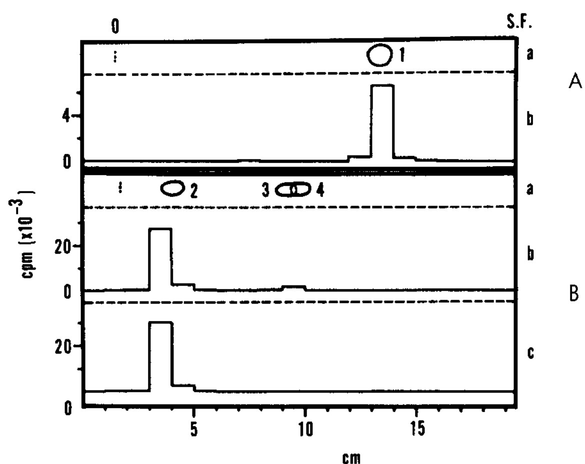

Fig. 2. Comparison of the TLC purities of the products of methylation of [^{3}H(G)]chenodeoxycholic acid with diazomethane and 2,2-dimethoxypropane. *A*. Purity of [^{3}H-(G)]chenodeoxycholic acid in eluent $CHCl_3$–CH_3OH–HOAc 80:12:3; *a*, standard. *B*. Methylation products in eluent acetone–benzene 3:7; *a*, standards; b, diazomethane treatment; and c, 2,2-dimethoxypropane treatment. 0, origin; S.F., solvent front; 1, chenodeoxycholic acid; 2, methyl chenodeoxycholate; 3, methyl chenodeoxycholate 3α-methyl ether; and 4, methyl chenodeoxycholate 7α-methyl ether.

The persistent presence of artifactual ethers even under modified conditions of reaction with diazomethane makes it desirable to use other methylating agents for chenodeoxycholic acid. Methanolic boron trifluoride also acts as a dehydrating agent. Silver oxide–methyl iodide is expensive, especially for large preparations. Hydrogen chloride–dry methanol has been shown to be quantitative with no by-product formation (2), but requires care and time. Rate studies on the reaction between radioactive chenodeoxycholic acid and a mixture of 2,2-dimethoxypropane, methanol, and conc. hydrochloric acid in the ratios 50 μl: 50 μl: 10 μl or 50 μl: 20 μl: 2 μl showed that they were similar and rapid, as illustrated in **Fig. 3.** Though the reaction was essentially complete in 1 hr, it was usually left overnight to ensure more complete methylation; about 1.6% of total recovered radioactivity was found at the origin (Fig. 2) where the unreacted acid should appear in that solvent system. Within the allowed reaction period, no methyl ether was detected (Fig. 2), although less polar material was gradually formed over several

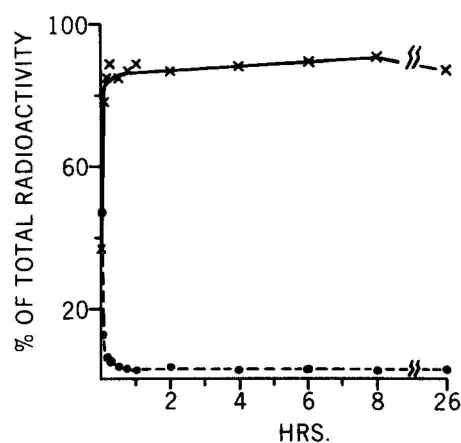

Fig. 3. Time course of methylation of [^{3}H-(G)]chenodeoxycholic acid with 2,2-dimethoxypropane. Reaction was monitored by TLC (acetone–benzene 1:1) and zones corresponding to the acid and methyl ester standards were scraped and counted directly in Bray's solution. ×, Methyl; ●, chenodeoxycholic acid.

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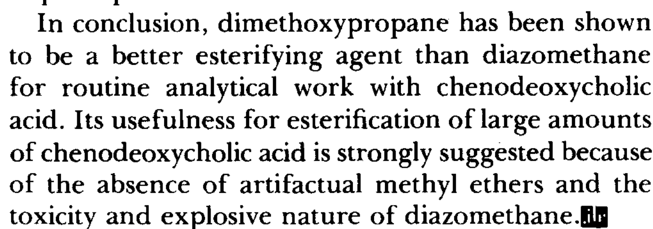
days along with significant darkening of the reaction mixture. Preliminary experiments indicate that this reagent is superior to or equal to diazomethane in methylating lithocholic and deoxycholic acids, and that its reaction with β-muricholic and hyocholic acids, which contain a *cis*-glycol functional group, generates acetonides in almost quantitative yields with no accompanying methyl ether. The identity and application of these acetonides will be discussed in a subsequent publication.



In conclusion, dimethoxypropane has been shown to be a better esterifying agent than diazomethane for routine analytical work with chenodeoxycholic acid. Its usefulness for esterification of large amounts of chenodeoxycholic acid is strongly suggested because of the absence of artifactual methyl ethers and the toxicity and explosive nature of diazomethane.

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