Vulpecholic acid (1α,3α,7α-trihydroxy-5β-cholan-24-oic acid): a novel bile acid of a marsupial, *Trichosurus vulpecula* (Lesson)[1]

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Abstract A novel trihydroxylated C_{24} bile acid was isolated from the gallbladder bile of the Australian opossum, *Trichosurus vulpecula* (Lesson). This acid, for which the name vulpecholic acid is proposed, was identified as 1α,3α,7α-trihydroxy-5β-cholan-24-oic. The structure proof included mass spectral and ^{1}H and ^{13}C nuclear magnetic resonance characterization of all crucial derivatives obtained by: *a*) oxidation of the methyl ester to a triketone with the enolizable 1,3-diketone function; *b*) methylation of this triketone to two isomeric methyl enol ethers; and *c*) reductive removal of oxygen functions from this triketone to give 5β-cholan-24-oic and 7-oxo-5β-cholan-24-oic acids. Vulpecholic acid was found in the bile in the unconjugated form; it accounted for more than 60% of the solid bile material. The marsupial *T. vulpecula* is the first example of a mammal secreting a 1α-hydroxylated bile acid as well as the first example of a mammal secreting the major bile acid in a free form. — **Lee, S. P., R. Lester, and J. St. Pyrek.** Vulpecholic acid (1α,3α,7α-trihydroxy-5β-cholan-24-oic acid): a novel bile acid of a marsupial, *Trichosurus vulpecula* (Lesson). *J. Lipid Res.* 1987. **28:** 19–31.

Supplementary key words gas–liquid chromatography–mass spectrometry • proton and carbon-13 nuclear magnetic resonance

Investigations of bile acids (BA) present in the bile of several monotremes and marsupials were conducted before 1962. Except for the observation of the exclusive occurrence of taurine conjugates, neither the apparent differences in BA composition compared to other mammals nor the presence of C_{27} BA were noted (1–3). These early observations evidently discouraged subsequent, more systematic attempts to study bile components of these primitive Australian and American mammals.

In the present report we describe the isolation of the principal bile acid from the bile of the vulpine phalanger, *Trichosurus vulpecula* (Lesson) (4), identified as 1α,3α,7α-trihydroxy-5β-cholan-24-oic acid (*1a*, **Fig. 1**). *T. vulpecula*, known also as Australian opossum, used for this study was of New Zealand origin where it is a common crop pest. Following the convention (2), we propose the name "vulpecholic acid" for this novel compound.

EXPERIMENTAL

General

Thin-layer chromatography (TLC) was performed with precoated silica gel plates (Whatman Chemical Separations, Inc., Clifton, NY) and high performance TLC plates (E. Merck, Darmstadt, West Germany) with the following solvent systems (v/v): A, benzene–MTBE 1:1; B, benzene–ethyl acetate 15:1; C, benzene–acetone 1:1; D, chloroform–isopropanol–acetic acid–water 48:48:2.4:0.6. Free BA were analyzed in solvent C (one or three developments at 0°C), methyl esters were analyzed in solvents A and C;

Abbreviations: NMR, nuclear magnetic resonance; SFORD, single frequency off-resonance decoupling; INEPT, insensitive nuclei enhanced by polarization transfer; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; MS, mass spectrometry; HRMS, high resolution mass spectrometry; EI, electron impact; RDA, retro-Diels-Alder fragment(ation); R_t, retention time; RRT, relative retention time; MU, methylene unit; SC, side chain; MTBE, methyl-*tert*-butyl ether; WKR, Wolff-Kishner reduction; DEAP-Lipidex, diethyl aminopropyl-Lipidex; BSTFA, *bis*-trimethylsilyl trifluoroacetamide; BA, bile acids; cholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic; hyocholic acid, 3α,6α,7α-trihydroxy-5β-cholan-24-oic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic; deoxycholic acid, 3α,12α-dihydroxy-5β-cholan-24-oic; ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholan-24-oic; lithocholic acid, 3α-hydroxy-5β-cholan-24-oic. Designations 1a–1h refer to structures indicated in Fig. 1.

[1]This paper is dedicated by one of the authors (J. St. P.) to Professor Ernest Wenkert for his sixtieth birthday; presented at Wenkert Symposium of Natural Products, October 14–15, 1985, Oxford, MS.

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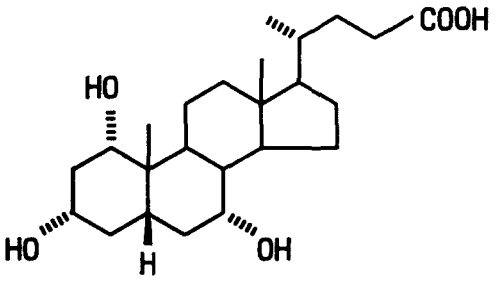

Fig. 1. Structure of vulpecholic acid and its derivatives: *1a,* free vupecholic acid; *1b,* methyl ester; *1c,* methyl ester, 1,3-diacetate; *1d,* methyl ester, 1,3,7-triacetate; *1e,* methyl ester, 1-TMS ether; *1f,* methyl ester, 3-TMS ether; *1g,* methyl ester, 1,3-*bis*-TMS ether; *1h,* methyl ester, 1,3,7-*tris*-TMS ether.

solvent B was used for methyl ester acetates. Analytical plates were visualized with Krowicki's reagent (5) at 100–110°C. Methyl-*tert*-butyl ether (MTBE) was freshly distilled from solid NaOH. DEAP-Lipidex (Packard, Downers Grove, IL) column was prepared according to the published method (6).

Methyl esters were obtained with diazomethane, acetates with acetic anhydride–acetic acid–perchloric acid at 0°C, 7:3:0.01 (v/v/v) (7), and oxidation to ketones was performed with Jones' reagent as previously described (8). Trimethylsilyl esters were obtained using BSTFA, and BSTFA-pyridine and ethyl acetate as solvent either at room temperature or at 110°C.

Capillary gas–liquid chromatography (GLC) was performed using a 15-m DB-5 fused silica column (J & W Scientific Inc., Rancho Cordova, CA) 0.25 mm id, 0.1 μ bonded stationary phase, and nitrogen as carrier gas. For gas–liquid chromatography–mass spectrometry (GLC–MS) the same 30-m column was used with the falling needle injector (Alltech Associates, Inc., Deerfield, IL) and split-splitless injector. The capillary column was directly introduced to the ion source as previously described (8). Temperature conditions were either 250°C and 280°C, isothermal, or programmed from 200°C, for 1 min, to 280°C, 2°C per min (conditions A). Electron impact mass spectra, in the GLC–MS mode, were measured at 22 eV with a Finnigan 3300 (Sunnyvale, CA) and at 70 eV with a Shimadzu QP-1000 (Shimadzu Corp., Kyoto, Japan) and Extrel EL-4000 (Extrel Corp., Pittsburgh, PA) quadrupole instruments. Probe mass spectra were obtained with an LKB-9000 magnetic sector spectrometer (Shimadzu Corp.) at 15 and 22 eV. High resolution mass spectra (HRMS) were obtained with JEOL HX110HF mass spectrometer (JEOL, USA Inc., Peabody, MA) by direct probe at 70 eV at 10,000 resolution.

Bile acid standards

All hydroxylated BA standards used for chromatographic comparison were of commercial origin (Steraloids, Inc., Wilton, NH); BA ketone derivatives were obtained by Jones' oxidation of corresponding methyl esters in acetone; methyl 7-oxo-5β-cholan-24-oate was from Dr. T. Iida; the mixture of methyl 5β- and 5α-cholan-24-oates was obtained by Wolff-Kishner reduction of 3,6-dioxo-5β-cholan-24-oic acid and was characterized by GLC–MS.

Animals and sample collection

Australian opossums were trapped in the bush near Auckland, New Zealand. Animals used for further experiments were kept in cages for 7–14 days and fed a vegetarian diet composed of apples, carrots, and lettuce. Before experiments, animals were fasted for 12 hr, anesthetized with urethane, and subjected to laparotomy. The cystic duct was ligated and the gallbladder contents were aspirated, immediately frozen in liquid nitrogen, lyophilized, and sealed under nitrogen before further investigation in Houston. The numbering of all animals used in the present report will be consistently followed in forthcoming papers.

Vulpecholic acid and its derivatives

1α,3α,7α-Trihydroxy-5β-cholan-24-oic acid (vulpecholic acid), (Fig. 1, 1a). A lyophilized bile sample (30 mg) obtained from animal No. 1 (female), dissolved in methanol, was applied to preparative TLC plates (20 × 20 × 0.025 cm) and developed in solvent D. The fast migrating band of free vulpecholic acid, *1a* (R_f 0.79), and the polar conjugates (R_f 0–0.11) were visualized, after spraying with water, as white spots on a gray, wet background. These spots were marked and scraped off when the gel was partially dried. The dry gel was eluted with methanol to give the noncrystalline free vulpecholic acid (18.6 mg, 62%) and polar compounds (9.5 mg), respectively. Two samples of vulpecholic acid *1a* (Fig. 1) obtained from animal No. 1 were used for all structural determinations described below.

Lyophilized bile samples (178 mg total) obtained from animals No. 8, 9, 10, and 11 (males and females) were separated by preparative TLC in solvent D. The combined band of free bile acids was additionally purified in solvent C to give vulpecholic acid (108 mg, 60%). The noncrystalline acid was dissolved in 72% ethanol and filtered through Amberlyst A-15 in H^+ form.[4] It crystallized out

[4]After the preparative TLC separation of free BA in solvent C, small amounts of high melting crystals (mp 289–296°C, with decomposition, 4.0 mg) was obtained from methanol. The position of C-24 signal in its ^{13}C NMR spectrum (δ 182.81, compare Table 5) and the elemental analysis (found C: 61.6%, H: 8.8%; calculated for the basic salt Ca(OH)($C_{24}H_{39}O_5$) C: 62.0%; H: 8.7%) indicated that a salt, probably calcium, crystallized out. Partial formation of salt either during TLC separation or during the elution from silica gel plates containing calcium sulfate as a binder was evidently responsible for the initial difficulties in the crystallization of small samples of vulpecholic acid purified by this method. It is noteworthy that both free vulpecholic acid as well as the above salt migrated in the same way when analyzed by TLC in solvent C.

from 50% ethanol (7 ml, at room temp, 81.4 mg) mp 150–152°C.

Lyophylized gallbladder bile (400 mg) obtained from animal No. 10 (female) was dissolved in 72% ethanol (5 ml) and filtered through Amberlyst A-15 in H^+ form. The eluate (20 ml) was directly applied on the column (22 × 1.5 cm) prepared from DEAP-Lipidex in the acetate form. The column was eluted with 72% ethanol (150 ml) and 0.1 N acetic acid in 72% ethanol (150 ml). Fifty fractions were analyzed by TLC in solvents C and D. Fractions 28–38, after evaporation, yielded unconjugated bile acids (340 mg, 85%). The same procedure was applied to the gallbladder bile obtained from animal No. 11 (male, 430 mg) to give unconjugated bile acids (360 mg, 84%).

A lyophilized bile sample (30 mg, animal No. 1, female) in aqueous NaOH (13% w/v) was heated at 120°C for 12 hr in a Teflon-stoppered glass screw-cap test tube under nitrogen atmosphere. The hydrolysate was extracted with MTBE (4 × 2 ml; neutral fraction). Subsequently, the pH was adjusted to 1 with 6 N HCl and extraction was repeated with ethyl acetate (3 × 2 ml; acid fraction). Both extracts were washed with water (1 ml), filtered through anhydrous sodium sulfate, and evaporated under nitrogen to dryness. Less than 0.6 mg of neutral and 28.3 mg (94%) of the acid fraction was obtained. MS (15 eV, probe), m/z (% int.) 408 (6, M^+), 390 (99, M-H_2O), 372 (100, M-2 × H_2O), 368 (40, M-(2 × H_2O+4H), variable intensity), 357 (14, M-2 × H_2O-Me), 354 (15, M-3 × H_2O), 302 (36, RDA-ion), 300 (10), 289 (11, M-H_2O-SC), 280 (10, M-SC-C16,17), 271 (9, M-2 × H_2O-SC), 262 (21, M-H_2O-SC-C16,17), 244 (10, M-2 × H_2O-SC-C16,17) (see **Fig. 2**). HRMS in **Table 1;** Anal. calc. for $C_{24}H_{40}O_5 \cdot H_2O$: C: 67.57; H: 9.92%. Found C: 67.31; H: 9.90%.

Methyl 1α,3α,7α-trihydroxy-5β-cholanoate (methyl vulpecholate) (Fig. 1, 1b). Vulpecholic acid *1a* was treated with diazomethane in methanol-MTBE to give noncrystalline ester *1b.* MS (22 eV, probe), m/z (% int.): 422 (6, M^+), 404 (88, M-H_2O), 389 (8, M-H_2O-Me), 386 (100, M-2 × H_2O), 371 (13, M-2 × H_2O-Me), 368 (21, M-3 × H_2O), 355 (11, M-2 × H_2O-MeO), 353 (7, M-3 × H_2O-Me), 327–332 (all ions ~6), 316 (56, RDA ion), 289 (35, M-H_2O-SC), 280 (6, M-SC-C16,17), 271 (33, M-2 × H_2O-SC), 262 (26, M-H_2O-SC-C16,17), 253 (10, M-3 × H_2O-SC), 244 (22, M-2 × H_2O-SC-C16,17), 217 (18), 211 (14), 208 (13) (see Fig. 2); HRMS in **Table 2;** NMR ($CDCl_3$, 90 MHz) δ 3.85 bs, 3.68 s (MeO), 3.30 bd, 3.5–2.5 unresolved br signal, 1.11 s (19-H_3), 0.90 bd (21-H_3), 0.76 s (18-H_3).

Methyl 1,3-diacetoxy (?) 1α,3α,7α-trihydroxy-5β-cholan-24-

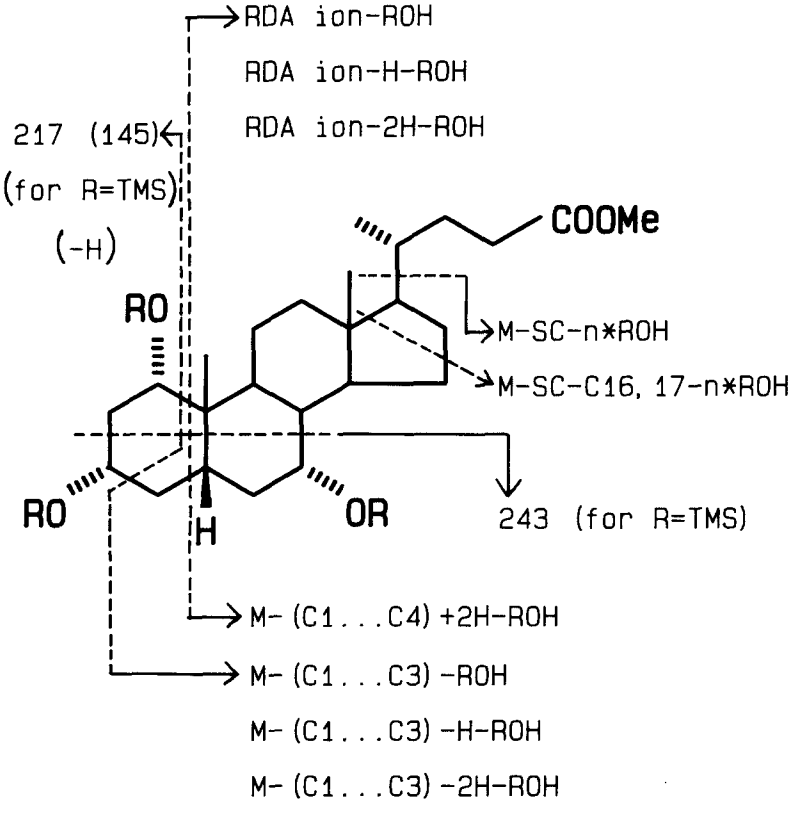

Fig. 2. Mass spectral fragmentation of vulpecholic acid methyl ester acetates and trimethylsilyl ethers.

TABLE 1. Vulpecholic acid (Fig. 1, *1a*): EI-HRMS at 70 eV

m/z	(% Rel. Int.)	Calc.	Composition	Probable Assignment
408.2896	(0.2)	408.2876	$C_{24}H_{40}O_5$	M^+
390.2722	(88)	392.2770	$C_{24}H_{38}O_4$	$M-H_2O$
375.2536	(1)	375.2535	$C_{23}H_{35}O_4$	$M-H_2O$-Me
372.2621	(100)	372.2665	$C_{24}H_{36}O_3$	$M-2 \times H_2O$
357.2310	(14)	357.2430	$C_{23}H_{33}O_3$	$M-2 \times H_2O$-Me
354.2502	(10)	354.2559	$C_{24}H_{34}O_2$	$M-3 \times H_2O$
339.2362	(5)	339.2324	$C_{23}H_{31}O_2$	$M-3 \times H_2O$-Me
328.2368	(5)	328.2402	$C_{22}H_{32}O_2$	
319.2625	(4)	319.2637	$C_{21}H_{35}O_2$	
317.2474	(4)	317.2481	$C_{21}H_{33}O_2$	$M-H_2O-CH_2CH_2COOH$
316.2386	(2)	316.2402	$C_{21}H_{32}O_2$	$M-H_2O$-(C1...C3)
315.2291	(6)	315.2324	$C_{21}H_{31}O_2$	$M-H_2O$-(C1...C3)-H
313.2127	(2)	313.2168	$C_{21}H_{29}O_2$	
304.2335	(8)	304.2402	$C_{20}H_{32}O_2$	$M-H_2O$-(C1...C4) + 2H
302.2246	(72)	302.2246	$C_{20}H_{30}O_2$	RDA ion-H_2O
301.2219	(6)	301.2168	$C_{20}H_{29}O_2$	RDA ion-H-H_2O
300.2072	(5)	300.2089	$C_{20}H_{28}O_2$	RDA ion-2H-H_2O
300.1967	(3)	300.2089	$C_{20}H_{28}O_2$	
289.2126	(4)	289.2168	$C_{19}H_{29}O_2$	$M-H_2O$-SC
287.2055	(8)	287.2011	$C_{19}H_{27}O_2$	$M-H_2O$-SC-2H(?)
285.1496	(2)	258.1491	$C_{18}H_{21}O_3$	
271.2081	(7)	271.2068	$C_{19}H_{27}O$	$M-2 \times H_2O$-SC
262.1950	(18)	262.1933	$C_{17}H_{26}O_2$	$M-H_2O$-SC-C16,17
261.1872	(6)	261.1855	$C_{17}H_{25}O_2$	$M-H_2O$-SC-C16,17-H
253.2000	(2)	253.1956	$C_{19}H_{25}$	$M-3 \times H_2O$-SC
244.1760	(24)	244.1827	$C_{17}H_{24}O$	$M-2 \times H_2O$-SC-C16,17
229.1524	(5)	229.1592	$C_{16}H_{21}O$	$M-2 \times H_2O$-SC-C16,17-Me
216.1567	(5)	217.1592	$C_{15}H_{21}O$	

oate (diacetoxy methyl vulpecholate) (Fig. 1, 1c). Vulpecholic acid methyl ester diacetate *1c* was observed by GLC and GLC-MS as an intermediate that disappeared upon longer acetylation time; MU = 36.11 (conditions A); MS (at 22 eV) m/z (% int.): 488 (~0.1, $M-H_2O$), 446 (0.6, M-AcOH), 428 (14, $M-H_2O$-AcOH), 413 (3, $M-H_2O$-AcOH-Me), 397 (3, $M-H_2O$-AcOH-MeO), 386 (11, M-2 × AcOH), 368 (100, M-2 × AcOH-H_2O), 353 (29,

TABLE 2. Vulpecholic acid methyl ester (Fig. 1, *1b*): EI-HRMS at 70 eV

m/z	(% Rel. Int.)	Calc.	Composition	Probable Assignment
404.2953	(81)	404.2927	$C_{25}H_{40}O_4$	$M-H_2O$
389.2747	(7)	389.2692	$C_{24}H_{37}O_4$	$M-H_2O$-Me
386.2869	(100)	386.2821	$C_{25}H_{38}O_3$	$M-2 \times H_2O$
371.2574	(12)	371.2586	$C_{24}H_{35}O_3$	$M-2 \times H_2O$-Me
368.2734	(13)	368.2715	$C_{25}H_{36}O_2$	$M-3 \times H_2O$
355.2661	(18)	355.2637	$C_{24}H_{35}O_2$	$M-2 \times H_2O$-Me
330.2528	(5)	330.2559	$C_{22}H_{35}O_2$	$M-H_2O$-(C1...C3)
329.2438	(7)	329.2481	$C_{22}H_{33}O_2$	$M-H_2O$-(C1...C3)-H
318.2551	(14)	318.2559	$C_{21}H_{34}O_2$	$M-H_2O$-(C1...C4) + 2H
316.2423	(58)	316.2402	$C_{21}H_{32}O_2$	RDA ion-H_2O
315.2370	(5)	315.2324	$C_{21}H_{31}O_2$	RDA ion-H-H_2O
314.2270	(5)	314.2246	$C_{21}H_{30}O_2$	RDA ion-2H-H_2O
289.2135	(28)	289.2168	$C_{19}H_{29}O_2$	$M-H_2O$-SC
271.2065	(26)	271.2062	$C_{19}H_{27}O$	$M-2 \times H_2O$-SC
262.1989	(20)	262.1933	$C_{17}H_{26}O_2$	$M-H_2O$-SC-C16,17
253.1987	(8)	253.1956	$C_{19}H_{25}$	$M-3 \times H_2O$-SC
244.1811	(13)	244.1827	$C_{17}H_{24}O$	$M-2 \times H_2O$-SC-C16,17
229.1640	(9)	229.1592	$C_{16}H_{21}O$	$M-2 \times H_2O$-SC-C16,17-Me
227.1808	(5)	227.1800	$C_{17}H_{23}$	
218.1702	(5)	218.1671	$C_{15}H_{22}O$	
217.1646	(5)	217.1592	$C_{15}H_{21}O$	
211.1490	(10)	211.1487	$C_{16}H_{19}$	
208.1514	(9)	208.1463	$C_{13}H_{20}O_2$	
201.1670	(5)	201.1643	$C_{15}H_{21}$	
199.1536	(11)	199.1487	$C_{15}H_{19}$	

M-2 × AcOH-H_2O-Me), 327 (35, BCD ion), 313 (84, M-AcOH-H_2O-SC), 271 (24), 253 (80, M-2 × AcOH-SC-H_2O), 244 (12, M-2 × AcOH-SC-C16,17), 226 (38, M-H_2O-2 × AcOH-SC-C16,17), 211 (53, M-H_2O-2 × AcOH-SC-C16,17-Me and/or M-H_2O-2 × AcOH-SC-C15...17-H) (see Fig. 2).

Methyl 1α,3α,7α-triacetoxy-5β-cholan-24-oate (triacetoxy methyl vulpecholate) (Fig. 1, 1d). Noncrystalline triacetate *1c* was obtained by preparative TLC separation of the methylated and acetylated total bile extract in solvent system B; MU = 35.82 (GLC conditions A). MS (22 eV, probe) m/z (% int.): 548 (0.4 M^+), 533 (0.4 M-Me), 506 (0.9 M-ketene), 488 (11, M-AcOH), 473 (0.5, M-AcOH-Me), 470 (0.5, M-AcOH-H_2O), 446 (1.6, M-AcOH-ketene), 445 (0.8, M-AcOH-43), 439 (1.4, M-AcOH-H_2O-MeO), 428 (100, M-2 × AcOH), 413 (5.2, M-2 × AcOH-Me), 373 (30, M-AcOH-SC), 368 (53, M-3 × AcOH), 353 (7, M-3 × AcOH-Me), 337 (1.6), 327 (8, BCD ion), 313 (47, M-2 × AcOH-SC), 253 (M-3 × AcOH-SC), 226 (18, M-3 × AcOH-SC-C16,17), 211 (10, M-3 × AcOH-C16,17-Me and/or M-3 × AcOH-SC-C15...17-H) (see Fig. 2). NMR ($CDCl_3$, 300 MHz) δ 4.85 q, (J = 3Hz, H-7β), 4.74 tt (J =10 and 5 Hz, 3β-H), 4.56 dd (J = 12.6 and 3.5 Hz, 1 β-H), 3.66 s (MeO), 2.1-2.4 m (23-H_2), 2.040 s, 2.037 s and 2.018 s (AcO), 0.982 s (19-H_3), 0.922 d (J = 6.3 Hz, 21-H_3) and 0.651 s (18-H_3).

Methyl vulpecholate trimethylsilyl ethers (Fig. 1, 1f, 1g, and 1h). The treatment of methyl vulpecholate *1b* with BSTFA-pyridine at 110°C for 20 hr formed *tris*-trimethylsilyl ether *1h* (Fig. 1) in ~98% yield which was analyzed by GLC-MS, 30 m DB-5 isothermal at 280°C, RRT = 1; MS (70 eV) m/z (% int): 548 (0.3, M-TMSOH), 533 (0.02, M-TMSOH-Me), 479 and 478 (0.1), 458 (M-2 × TMSOH), 443 (0.2, M-2 × TMSOH-Me), 432 (0.2, M-TMSOH-SC-H), 427 (0.3, M-2 × TMSOH-MeO), 368 (2.2, M-3 × TMSOH), 353 (0.4, M-3 × TMSOH-Me), 343 (0.3, M-2 × TMSOH-SC), 342 (0.3, M-2 × TMSOH-SC-H), 337 (0.2, M-3 × TMSOH-MeO), 327 (0.3, BCD ion), 316 (1.4, M-TMSOH-C1...C4, RDA ion), 314 (0.2), 301 (0.2), 284 (0.2), 271 (0.3), 253 (1.3, M-3 × TMSOH-SC), 243 (0.6, C3...C7 fragment TMSO(+)=CH-CH=CH-CH=CH-OTMS), 233 (0.7), 217 (100, TMSO(+)=CH-CH=CH-OTMS) (see Fig. 2). 1,3-*Bis*-trimethylsilyl ether *1g* (RRT = 1.30) was the second, minor product of the above reaction and was formed as the principal product when the reaction was carried out at room temperature; MS (70 eV), m/z (% int.), 548 (0.2, M-H_2O), 476 (0.5, M-TMSOH), 458 (1.6, M-H_2O-TMSOH), 443 (0.2, M-H_2O-TMSOH-Me), 427 (0.2, M-TMSOH-MeO), 406 (0.6), 386 (1.4, M-2 × TMSOH), 368 (1.4, M-2 × TMSOH-H_2O), 353 (0.6, M-2 × TMSOH-H_2O-Me), 337 (0.2, M-2 × TMSOH-H_2O-MeO), 327 (0.2, BCD ion), 316 (3.0, M-H_2O-Cl...C4, RDA ion), 314 (0.6), 301 (0.4), 271 (0.8, (M-2 × TMSOH-SC), 253 (1.6, M-2 × TMSOH-H_2O-SC), 233 (3.0), 232 (1.2), 217 (100, TMSO(+)=CH-CH=CH-OTMS) (see Fig. 2). Mild silylation, with BSTFA at room temperature, also produced the two monosilylated products; *1e* (RRT = 1.67) MS (70 eV), m/z (% int.): 476 (2, M-H_2O), 458 (9, M-2 × H_2O), 443 (1, M-2 × H_2O-Me), 432 (1), 427 (1, M-2 × H_2O-MeO), 407 (2), 406 (2), 404 (3, M-TMSOH), 386 (14, M-H_2O-TMSOH), 371 (4, M-H_2O-TMSOH-Me), 368 (6, M-2 × H_2O-TMSOH), 316 (33, M-H_2O-C1...C4, RDA ion), 314 (6), 301 (5), 271 (15, M-H_2O-TMSOH-SC), 253 (11, M-2 × H_2O-TMSOH-SC), 147 (37), 145 (100, TMSO(+)=CH-C(OH)=CH_2); and *1f* (RRT = 1.32) MS (70 eV) m/z (% int.): 476 (2, M-H_2O), 458 (15, M-2 × H_2O), 386 (24, M-H_2O-TMSOH), 371 (6, M-H_2O-TMSOH-Me), 368 (19, M-2 × H_2O-TMSOH), 355 (12, M-H_2O-MeO), 327 (11, BCD ion), 316 (29, M-H_2O-C1...C4, RDA ion), 301 (3), 271 (22, M-H_2O-TMSOH-SC), 253 (33, M-2 × H_2O-TMSOH-SC), 159 (54), 147 (62), 145 (100, HO-CH=CH-C((+)(OTMS))=CH_2) (see Fig. 2).

Methyl 1,3,7-trioxo-5β-cholan-24-oate, 3. A sample of the total acid fraction was oxidized with Jones' reagent in acetone to give three components detected by the TLC analysis in solvent A: R_f 0.70 (bile acid diketones, minor spot). 0.25 (*2*), and 0.15 (*3*, main spot) (**Fig. 3**). Standards of BA methyl ester-ketones showed the following values: methyl 3-oxo-5β-cholan-24-oate, 0.82; 3,7-dioxo-, 3,6-dioxo-, and 3,12-dioxo-5β-cholan-24-oate, 0.75; and 3,7,12-trioxo-5β-cholan-24-oate, 0.50. The principal product *3*, was purified, from a part of the reaction mixture, by preparative TLC using solvent A. NMR ($CDCl_3$-acetone-d_6, 300 MHz) was composed of broadened signals: δ 5.01 bs (H-2), 3.62 s (MeO), 2.84 b, 2.43 bt, 1.38 s (19-H_3), 0.93 d (21-H_3), 0.69 s (18-H_3) (the broadening due to the ketone-enol equilibration).

Methyl 1,7-dioxo-3-methoxy-5β-chol-2-en-24-oate, 4. The rest of the above mixture was treated with an excess of diazomethane. The TLC analysis demonstrated that the most polar spot, R_f 0.15, disappeared and two new major products, with R_f 0.30 (*5*) and 0.55 (*4*), were formed instead. The separation by preparative TLC in solvent system A gave the major product *4* (2.2 mg), MU = 33.99 (GLC conditions A); MS in **Fig. 4** and HRMS in **Table 3;** NMR ($CDCl_3$, 300 MHz) δ 5.31 d (J = 1.27 Hz, H-2), 3.67 s and 3.66 s (MeO), 2.81 dd (6β-H, J = 13.2 and 5.5 Hz), 2.52 m (5β-H), 2.44 t (8β-H, J = 11.1), 2.15-2.40 m (23-H_2), 2.13 dd (J = 13.2 and 2.2 Hz, 6α-H), 1.46 s (19-H_3), 0.89 d (J = 6.4 Hz, 21-H_3), 0.66 s (18-H_3).

Methyl 3,7-dioxo-1-methoxy-5β-chol-1-en-24-oate, 5. The second minor methylation product separated from the above reaction (1.3 mg), MU = 35.17, (GLC conditions A), R_f 0.30; MS in Fig. 4 and HRMS in **Table 4;** NMR ($CDCl_3$, 300 MHz) δ 5.35 s (H-2), 3.66 s and 3.68 s

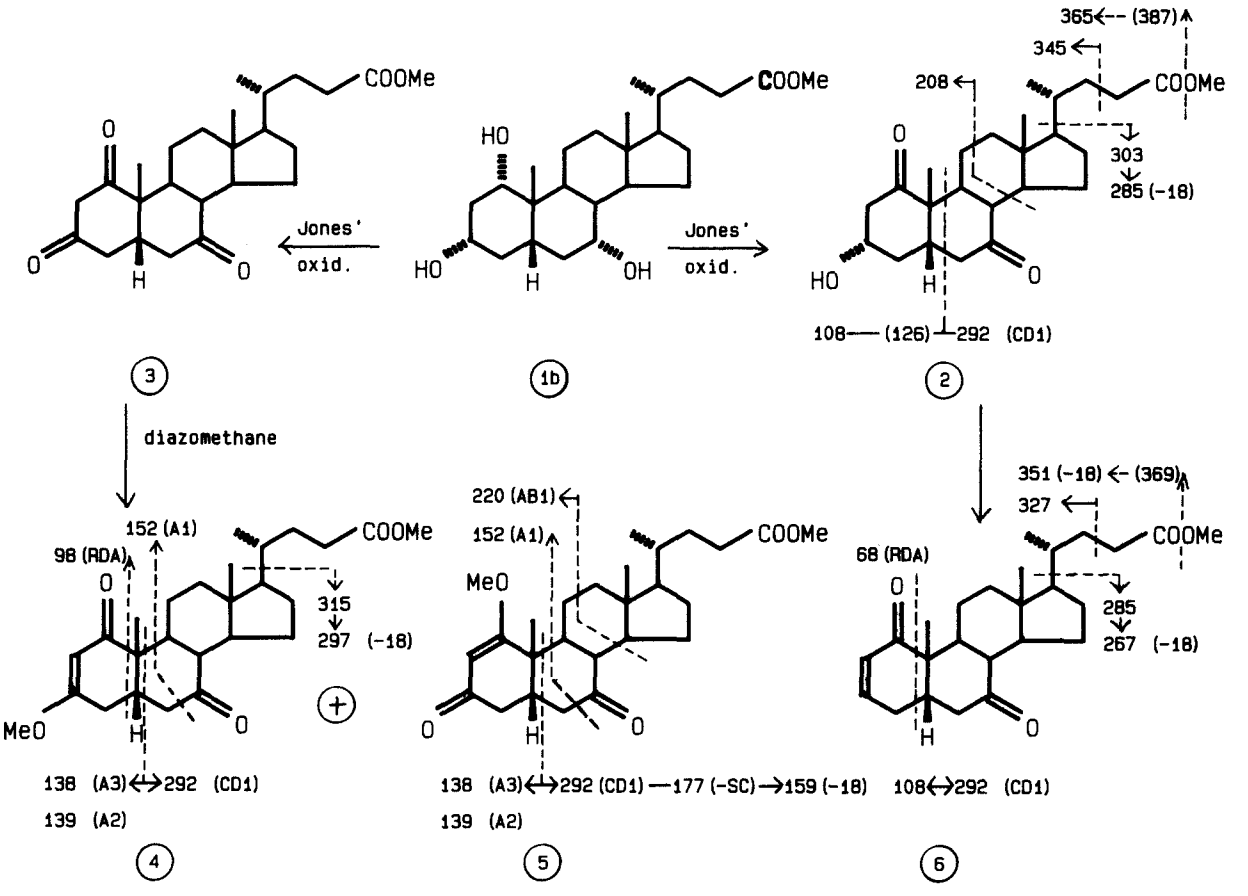

Fig. 3. Structure of products obtained from vulpecholic acid by Jones' oxidation and diazomethane methylation and their mass spectral fragmentation pattern.

(MeO), 2.92 d (J = 13.2 and 5.5 Hz, 6β-H), 2.57 bq (J = 3.4 Hz, 5β-H), 2.44 t (J = 11.2 Hz, 8β-H), 2.32 d (2H, J = 9.8 Hz, 4-H_2), 2.15-2.40 m (23-H_2), 2.08 dd (J = 13.2 and 2.2 Hz, 6α-H), 1.52 s (19-H_3), 0.91 d (J = 6.3 Hz, 21-H_3), 0.68 s (18-H_3).

Methyl 3α-hydroxy-1,7-dioxo-5β-cholan-24-oate, 2. The third minor oxidation product of the above reaction (0.5 mg), hydroxydiketone *2* (Fig. 3) was unchanged by the treatment with diazomethane; MU = 33.24 (conditions A), R_f 0.25; MS (22.2 eV, probe) m/z (% int.): 418 (89, M^+), 400 (29, M-H_2O), 369 (16, M-H_2O-MeO), 345 (42, M-CH_2COOMe), 303 (59, M-SC), 292 (39, CD ion), 285 (43, M-SC-H_2O), 208 (100, AB ion), 108 (20, A ion-H_2O). NMR ($CDCl_3$, 300 MHz) δ 3.85 br (3β-H), 3.66 s (MeO), 2.86 dd (J = 13.2 and 5.7 Hz 6β-H), 2.64 m (5β-H), 2.51 t (J = 12 Hz, 8β-H), 2.2-2.4 m (23-H_2), 1.38 s (19-H_3), 0.89 d (J = 6.5 Hz, 21-H_3), 0.65 s (18-H_3).

Methyl 1,7-dioxo-5β-chol-2-en-24-oate, 6. Dehydration of hydroxydiketone *2* under GLC and GLC-MS conditions formed the unsaturated diketone *6* (Fig. 3); MU = 31.24 (conditions A), m/z (% int.): 400 (1.5, M^+), 368 (0.5, M-MeOH), 351 (1.5, M-H_2O-MeO), 350 (0.5, M-H_2O-MeOH), 333-330 (all ~1), 327 (3, M-CH_2COOMe), 292 (2.5, CD ion), 285 (48, M-SC), 267 (12, M-SC-H_2O), 190 (11), 177 (6, CD ion-SC), 159 (4, CD ion-SC-H_2O), 115 (3, SC), 108 (100, A ion), 68 (4, RDA ion).

Wolff-Kishner reduction of methyl 1,3,7-trioxo-5β-cholan-24-oate, 3

The tri-ketone *3* (1.4 mg) in ethylene glycol (1 ml) was treated with potassium hydroxide (0.2 g), hydrazine sulfate (10 mg), and anhydrous hydrazine (0.3 ml) at 165°C for 20 hr. Products were recovered by dilution with water, acidification with 6 N HCl, extraction with MTBE, and methylation. The following compounds, identified by GLC-MS analysis, are listed below in the order of increasing R_t (**Fig. 5**).

Methyl A-nor-5β-cholan-24-oate, 9, MU = 27.39 (at 250°C), minor product (1.3%), m/z (% int.): 360 (36, M^+), 345 (21, M-Me), 329 (1, M-MeO), 264 (8, M-ring A and C-6), 218 (9) and 217 (10, ABC ion) 203 (100, ABC ion), 115 (1, SC) 74 (6, part of SC).

Methyl 5β,8α-cholan-24-oate, 8, MU = 27.73 (at

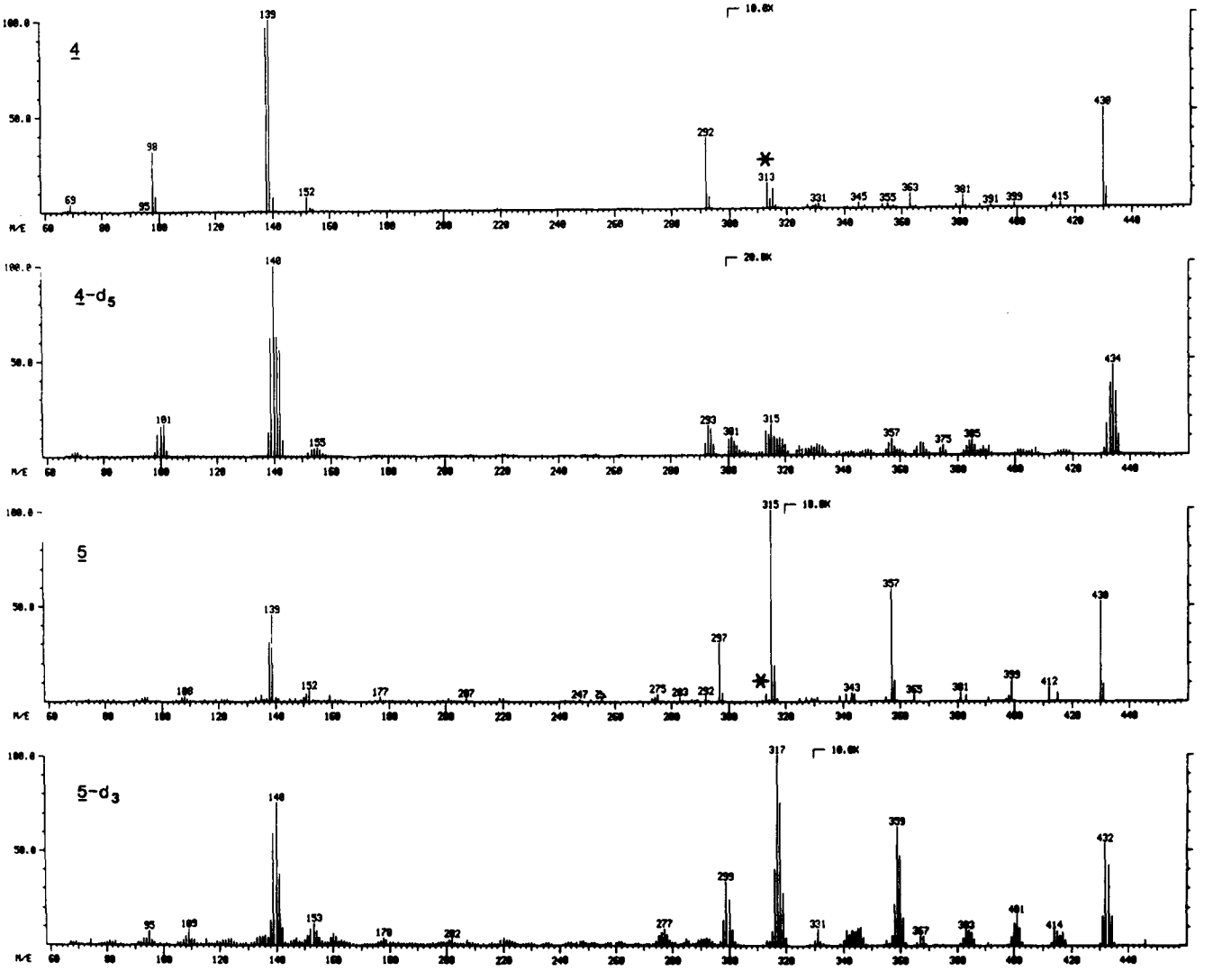

Fig. 4. Mass spectra of enol ethers *4* and *5* obtained at 22 eV by capillary GLC-MS. Ion at m/z 313 is from background (*).

TABLE 3. Methyl-3-methoxy-1,7-dioxo-5β-chol-2-en-24-oate, *4*: EI-HRMS at 70 eV

m/z	(% Rel. Int.)	Calc.	Composition	Probable Assignment
430.2698	(30)	430.2719	$C_{26}H_{38}O_5$	M^+
415.2440	(1)	415.2485	$C_{25}H_{35}O_5$	M-Me
399.2529	(3)	399.2535	$C_{25}H_{35}O_4$	M-MeO
363.2353	(1)	363.2324	$C_{25}H_{31}O_2$	M-2 × H_2O-MeO
353.2507	(2)	353.2481	$C_{24}H_{33}O_2$	M- H_2O-COOMe
297.1862	(1)	297.1855	$C_{20}H_{25}O_2$	M-H_2O-SC
292.2025	(13)	292.2039	$C_{18}H_{28}O_3$	CD1 ion
152.0813	(6)	152.0837	$C_9H_{12}O_2$	A1 ion
139.0743	(100)	139.0759	$C_8H_{11}O_2$	A2 ion
138.0695	(62)	138.0681	$C_8H_{10}O_2$	A3 ion
115.0790	(1)	115.0759	$C_6H_{11}O_2$	SC ion
99.0475	(5)	99.0446	$C_5H_7O_2$	
98.0388	(21)	98.0368	$C_5H_6O_2$	RDA ion
69.0374	(2)	69.0340	C_4H_5O	
68.0288	(3)	68.0262	C_4H_4O	

TABLE 4. Methyl 1-methoxy-3,7-dioxo-5β-chol-1-en-24-oate, *5*: EI-HRMS at 70 eV

m/z	(% Rel. Int.)	Calc.	Composition	Probable Assignment
430.2672	(58)	430.2719	$C_{26}H_{38}O_5$	M^+
399.2531	(7)	399.2535	$C_{25}H_{35}O_4$	M-MeO
398.2457	(4)	398.2457	$C_{25}H_{34}O_4$	M-MeOH
357.2419	(12)	357.2430	$C_{23}H_{33}O_3$	M-CH_2COOMe
344.2357	(4)	344.2351	$C_{22}H_{32}O_3$	M-CH_2=CHCOOMe
315.1937	(100)	315.1960	$C_{20}H_{27}O_3$	M-SC
297.1855	(16)	297.1854	$C_{20}H_{25}O_2$	M-SC-H_2O
275.1701	(6)	275.1647	$C_{17}H_{23}O_3$	M-SC-C15...17
274.1614	(4)	274.1569	$C_{17}H_{22}O_3$	M-SC-C15...17
273.1528	(5)	273.1491	$C_{17}H_{21}O_3$	M-SC-C15...17-H
220.1119	(6)	220.1099	$C_{13}H_{16}O_3$	AB1 ion
152.0817	(10)	152.0837	$C_9H_{12}O_2$	A1 ion
151.0771	(5)	151.0759	$C_9H_{11}O_2$	A1 ion-H
139.0795	(46)	139.0759	$C_8H_{11}O_2$	A2 ion
139.0550	(5)	139.0548	$C_{11}H_7$	
138.0725	(31)	138.0681	$C_8H_{10}O_2$	A3 ion
135.0880	(5)	135.081	$C_9H_{11}O$	

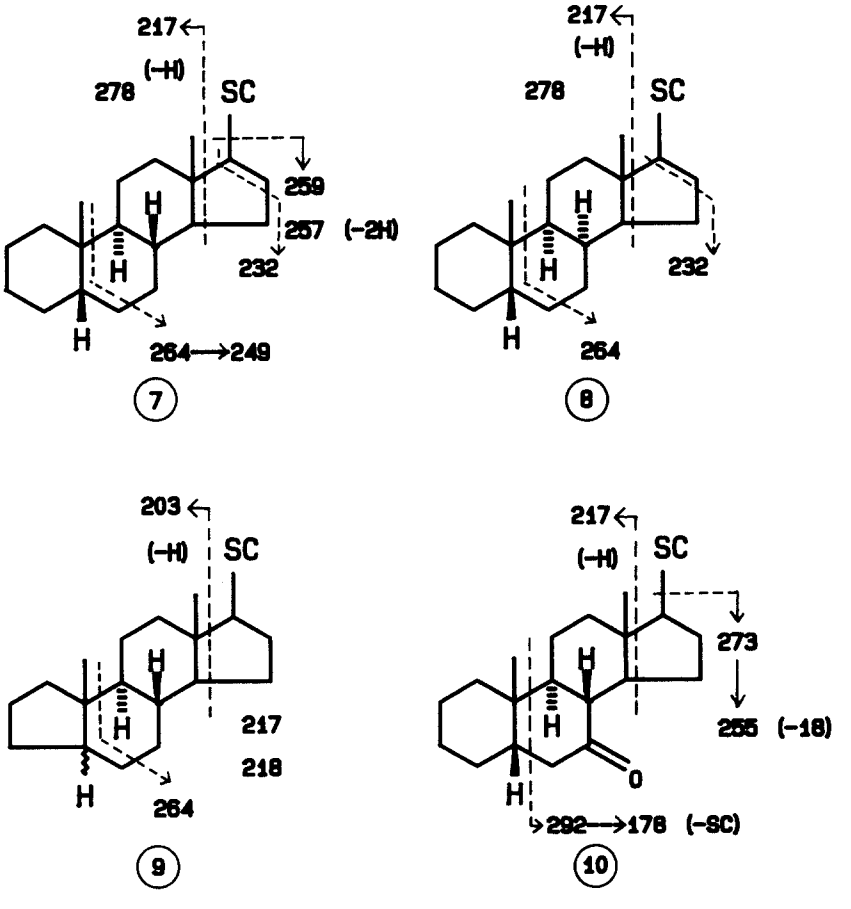

Fig. 5. Structure of products obtained from 1,3,7-trioxo-5β-cholan-24-oic acid by Wolff-Kishner reduction and their mass spectral fragmentation pattern.

250°C), minor product (0.8 %), m/z (% int.): 374 (32, M^+), 359 (23, M-Me), 343 (1, M-MeO), 278, (2, M-ring A), 264 (11, M-ring A and C-6), 232 (8, ABC ion), 218 (24), 217 (100, ABC ion), 203 (14), 115 (1, SC), 74 (9, part of SC).

Methyl 5β-cholan-24-oate, 7, MU = 28.65 (at 250°C) major product (94.5%) identical with standard by R_t and mass spectral comparison; m/z (% int.): 374 (79, M^+), 359 (100, M-Me), 343 (2, M-MeO), 325 (5, M-MeO-H_2O), 317 (4), 278 (5, M-ring A), 264 (12, M-ring A and C-6), 259 (1, M-SC), 257 (1, M-SC-2H), 249 (1, M-ring A and C-6-Me), 232 (15, ABC ion), 217 (100, ABC ion), 203 (11).

Methyl 7-oxo-5β-cholan-24-oate, 10, MU = 30.43 (at 250°C), minor product (3.5%) identical with standard by R_t and mass spectral comparison; m/z (% int.): 388 (50, M^+), 370 (21, M-H_2O), 356 (50, M-MeOH), 355 (28, M-Me-H_2O), 341 (10, M-MeOH-Me), 339 (16, M-MeO-H_2O), 323 (12, M-MeOH-H_2O-Me), 315 (97, M-part of SC), 297 (10, "315"-H_2O), 292 (100, M-ring A), 273 (35, M-SC), 178 (30, M-SC-ring A), 255 (75, M-SC-H_2O), 115 (12, SC fragment), 74 (20, SC fragment).

RESULTS

The TLC examination of gallbladder bile in solvents C and D demonstrated that the majority of BA were present in a free, unconjugated form. Subsequently, this fraction was separated either by the direct preparative TLC in solvents C and D or by ion exchange chromatography on DEAP-Lipidex. The unconjugated BA fraction constituted more than 80% of the bile solid material. The alkaline hydrolysis, when performed before separation, increased the amount of free BA to 94%.

The principal component showed the chromatographic behavior of a trihydroxylated BA. Thus, in TLC solvent C with three developments, the R_f value observed was 0.29. Under the same conditions BA standards showed the following R_fs: cholic acid, 0.10; hyocholic, 0.22; deoxycholic, 0.41; chenodeoxycholic, 0.48; ursodeoxycholic, 0.58; and lithocholic, 0.87. According to the capillary GLC and GLC-MS analysis, in the form of methyl ester acetate derivatives, the major component, referred to hereafter as vulpecholic acid, constituted more than 90% of the total acid fraction. Its MU value, 35.82, was distinctly higher than that of the corresponding derivatives of cholic and hyocholic acids (33.79 and 35.14, respectively). Moreover, mass spectral fragmentation of vulpecholic acid showed the absence of fragment ions at m/z 446 and 386, diagnostic for 3,6,7-trihydroxylated BA methyl ester acetates (9), thus excluding its identity with one of the isomers of hyocholic acid.

Elemental analysis of a crystalline sample of vulpecholic acid was consistent with the monohydrate of a trihydroxylated C_{24} BA. This composition was fully corroborated by the low and high resolution mass spectra of the free acid, its methyl ester (Tables 1 and 2), and low resolution spectra of di-, tri-acetate, *mono-, bis-,* and *tris*-trimethylsilyl ethers. In addition, ^{13}C NMR spectra discussed below demonstrated the presence of 24 carbon atoms in the steroidal skeleton.

Mass spectral data pointed to two hydroxyl groups located in ring A of the vulpecholic acid moiety. Thus, the odd-electron ion observed in the mass spectrum of free vulpecholic acid *1a* and its methyl ester *1b,* at m/z 302 and 316, respectively, was interpreted as the result of a retro-Diels-Alder (RDA) fragmentation of ring A (Fig. 2, HRMS in Tables 1 and 2). This fragment ion, at m/z 316, was also present in the spectra of vulpecholic acid methyl ester-trimethylsilyl ethers *1e-1h.* It was absent, however, in the spectra of di- and triacetates *1c* and *1d.*

The set of fragment ions at m/z 327-330 present in the mass spectra of vulpecholic acid methyl ester *1b* (Table 2) and its acetates and silyl ethers were tentatively assigned as corresponding to the BCD part of the molecule. Corresponding ions at m/z 315 and 316 were present in the spectrum of free vulpecholic acid (Table 1). The probable mode of their formation is shown in Fig. 2.

The silylation of methyl vulpecholate under mild conditions, with BSTFA pyridine at room temperature, resulted in a formation of three intermediate, partially substituted products, along with small amount of *tris*-trimethylsilyl ether *1h.* Full silylation, to give *1h,* was achieved only at 110°C in 20 hr. The latter ether showed a series of weak, high mass ions resulting from the successive losses of silanol, methyl group, and side chain (all below 2.2%) along with RDA and BCD ions discussed above. The base peak at m/z 217 constituting most of the ion current was assigned to the C1...C3 fragment (Fig. 2). The analogous ion is present in spectra of known 1β,3α-disubstituted BA (10). On the other hand, the low intensity fragment ion at m/z 243 indicated the presence of 3,7-*bis*-trimethylsilyl ether moiety, by analogy with other BA (11). This ion, however, was also present in 1,3-*bis*-trimethylsilyl ether *1g,* although its intensity was much lower.

The intermediate products of silylation were assigned the structures of 1-*mono*-trimethylsilyl, 3-*mono*-trimethylsilyl, and 1,3-*bis*-trimethylsilyl ethers of methyl vulpecholate *1e, 1f,* and *1g,* respectively. The structure assignment was based primarily on the expected relative reactivities and RRT (*tris*-TMS < 1,3-*bis*-TMS < 3-TMS <1-TMS ethers) and was confirmed by the mass spectral fragmentation. Thus, the *bis*-trimethylsilyl ether *1g* showed the base peak at m/z 217. Two other mono-silylated products *1e* and *1f* displayed much more intense fragment ions with high mass in addition to ions at m/z 145, analogous corresponding the ion at m/z 217 in the spectrum of *1h* (Fig. 2).

In order to confirm the position of hydroxyl groups in

the molecule of vulpecholic acid, its oxidation to a ketone derivative was performed. The removal of all ketone functions by Wolff-Kishner reduction was expected to give the parent 5β or 5α-cholan-24-oic acid, provided that no functionality at C-4 or C-6 was present.

Jones' oxidation of the total methylated acid fraction resulted in the formation of a product much more polar (TLC) than standards of 3-, 3,6-, 3,7-, 3,12-, and 3,7,12-substituted BA ketones. Reaction of this polar product with diazomethane afforded two derivatives and poitned to the formation of either a carboxylic acid, through ring cleavage, or an enolizable 1,3-diketone. Spectral data of the oxidation product and its two methylated derivatives definitively confirmed the formation of the enolizable 1,3-diketone. In the case of a cholane skeleton, a 1,3-diketone function could only be located in one of the following positions: 2,4-, 4,6-, and 1,3-. From a metabolic standpoint, as well as from the above spectral data, the last position was considered to be the most likely (Fig. 3).

The electron impact mass spectrum of the less polar, major dimethoxy-derivative, assigned structure *4,* showed a simple fragmentation pattern consisting of five major fragment ions at m/z 292, 152, 139, 138, and 98 (Figs. 3, 4, and Table 3). The set of fragment ions due to the elimination of side chain fragments was of much lower intensity. In contrast, in the case of the more polar dimethoxy-derivative, assigned structure *5,* ions due to the elimination of side chain at m/z 357, 315 and 297 dominated over fragment ions at m/z 292, 152, 139, and 138. As expected, the RDA fragment ion at m/z 98 was absent in the spectrum of *5.* By analogy with previously reported examples (10), as well as the known fragmentation mode of 1-keto-steroids (12) and 7-keto BA (11), the fragmentation pattern was interpreted as shown in Fig. 3 and was fully consistent with high resolution mass spectral data (Tables 3 and 4).

The low intensity fragment ion at m/z 152 (A1) was of special significance for the assignment of the position of the third ketone function. Its formation could be explained assuming the presence of 7-ketone and the α-cleavage of the 6-7 bond. The evidence for the operation of this mode of fragmentation was obtained as follows. The mixture of enol ethers *4* and *5* was submitted to deuterium exchange under alkaline conditions, followed by methylation. The mass spectrum of deuterated ether *4* (Fig. 4) demonstrated that up to three deuterium atoms were incorporated into the RDA ion (m/z 98), originating from the part of ring A. Furthermore, up to three deuterium atoms were incorporated into the A2 and A3 ions at m/z 138 and 139. The A1 fragment ion originally located at m/z 152, however, incorporated up to five deuterium atoms, indicating that the additional methylene group is located in the enolizable position, i.e., next to 7-ketone. Under the alkaline treatment, the molecular ion incorporated five, of six possible, deuterium atoms, evidently due to the difficulty toward the 7-8 enolization.

The second, minor enol ether *5* was less prone to enolization exchange as shown by the total incorporation of only two and three deuterium atoms to its molecular ion and A1, A2, and A3 fragment ions.

Jones' oxidation of vulpecholic acid afforded also the third, partially oxidized product which was assigned, chiefly based on its NMR and MS spectra, the structure *2* (Fig. 3). This compound was partially decomposed under GLC conditions and yielded the dehydrated diketone *6.* Mass spectral fragmentation of the latter compound *6* produced the CD1 ion at m/z 292 and the complementary A ion at m/z 108. The RDA ion, at m/z 68 and originating from the part of ring A, was also present.

The structure of the two crucial derivatives, methyl ethers *4* and *5,* was fully confirmed by their proton NMR spectra. In the case of the major, less polar ether *4* the olefinic signal of H-2 appeared at δ 5.305 as a doublet with J = 1.27 Hz due to its allylic coupling with H-4β. As expected, this coupling was absent in the spectrum of the second isomer *5* and H-2 signal was observed as a singlet at δ 5.347. Three hundred MHz spectra of both isomers displayed well resolved signals of 5-H and 6-H_2, whereas the signal of 2-H_2 was partially overlapped with the multiplet of 23-H_2. Coupling constants $J_{5\beta,6\alpha}$ = 2.7 and $J_{5\beta,6\beta}$ = 5.5 Hz, measured from signals of 6-methylene group, (*4*: 2.81 (6β), 2.13 (6α), *5*: 2.92 (6β), 2.08 (6α) all as double doublets) corroborated the 5β-H configuration since much higher magnitude would be expected for the axial-axial coupling ($J_{5\alpha,6\beta}$) in case of the 5α, allo-BA. The multiplet of 5β-H was located at δ 2.52 and 2.57 in spectra of *4* and *5,* respectively. The coupling pattern of all ring AB protons was fully confirmed by the two-dimensional proton-proton coupled spectra (data not shown).

The configuration of C-5 was independently established based on results of the Wolff-Kishner reduction of the triketone *3,* performed under Nagata's conditions (13). In anticipation of detection of partially reduced products, this reaction was performed at a considerably lower temperature (165°C) than that usually employed (~200-220°C). Apart from several minor, nitrogen-containing compounds, four reduction products were detected and characterized by capillary GLC-MS analysis of the methylated acid fraction (Fig. 5). The major product (94.5%, MU = 28.66) was identified as methyl 5β-cholan-24-oate *7,* by direct comparison with a reference substance. The 5α-isomer, with an expected longer R_t (MU = 28.98), could not be detected by capillary GLC even in trace quantities. In addition to the completely deoxygenated products, only one minor monoketone *10* was detected (~3.5%, MU = 30.43). The mass spectrum was consistent with the presence of a 7-keto group as indi-

cated by the formation of the prominent CD ion at m/z 292 resulting from the loss of ring A fragment (11). It was identical with the standard of methyl 7-oxo-5β-cholan-24-oate 10, kindly provided by Dr. T. Iida. Formation of ketone *10* independently demonstrated the 7-oxygenation of vulpecholic acid.

A trace (0.8%) of an isomeric methyl cholan-24-oate with relatively short R_t (MU = 27.73), assigned structure *8*, was also detected among the reaction products. Its mass spectrum was almost identical with that of the major product *7*. It is probable that this compound resulted from C-8 epimerization prior to the removal of 7-ketone. The fourth, minor reduction product (1.3%, MU = 27.39) detected in this mixture was identified based on its mass spectrum as methyl A-nor-5β-cholan-24-oate *9*, a probable decarbonylation product. The A-nor, rather than B-nor, skeleton was assigned for *9* based on the presence of the fragment ion at m/z 264. This ion, resulting from the elimination of ring A and C-6 methylene, was also present in spectra of products *7* and *8*, as well as the standard of methyl 5α-cholan-24-oate.

In order to establish the configuration of all hydroxyl groups, triacetoxy methyl vulpecholate was characterized by proton and carbon-13 NMR. The 300 MHz proton spectrum displayed three signals of axial protons adjacent to acetoxyl groups. In view of the established 1,3,7-substitution pattern, the two signals at δ 4.85, quartet J = 2.96 Hz, and septet J = 5.45 Hz were assigned by analogy with common BA to 3β-H and 7β-H, respectively, whereas a doublet of doublets at δ 4.57 ($J_{1\beta,2\alpha}$ = 12.65 and $J_{1\beta,2\beta}$ = 3.48 Hz) was assigned to 1β-H. Thus, all three hydroxyl groups of vulpecholic acid were found to be equatorial.

The spectrum showed also a well-resolved AB part of the ABXY system assigned to 23-H_2; coupling constants J_{AB} = 15.5, J_{AX} = 5.2, J_{AY} = 10.2, J_{BY} = 6.8, and J_{BX} = 9.7 Hz corresponding to the extended conformation of side chain. The same pattern was observed in high field spectra of standard C_{24} BA as well as derivatives *4* and *5*.

^{13}C NMR spectrum of triacetoxy methyl vulpecholate *1c* was assigned with the help of SFORD and INEPT (14) spectra (**Table 5**). Chemical shift differences, when com-

TABLE 5. 22.5 MHz ^{13}C NMR data of vulpecholic and chenodeoxycholic acid derivatives (in $CDCl_3$)[a]

	Vulpecholic Acid			Chenodeoxycholic Acid			Δδ(1α-OH)[e]		Δδ(1α-OAc)[e]
C #	Free,[b] *1a*	Me, *1b*	$MeAc_3$, *1c*	Free[c]	Me[d]	$MeAc_2$	(from *1a*)	(from *1b*)	
1	79.94	79.21	78.44	36.5	35.8	34.75	+43.4	+43.4 (α)	+43.69 (α)
2	40.17	40.08+	34.10	31.3	31.3x	26.75	+8.9	+8.8 (β)	+7.35 (β)
3	68.72	67.99x	70.58+	72.9	72.1	74.10	−4.2	−4.1 (γ_t)	−3.52 (γ_t)
4	39.82	39.20+	32.23	40.4	39.9	34.58	−0.6	−0.7 (δ)	−2.35 (δ)
5	41.37	39.20+	38.89	43.1	42.1	40.92	−1.7	−2.9 (γ_t)	−2.03 (γ_t)
6	35.19	34.15	30.65	35.9	35.5	31.28	−0.7	−1.4 (δ)	−0.63 (δ)
7	70.18	69.08x	70.44+	69.1	68.6	71.18	+1.1	+0.5	−0.74
8	41.12	40.57	38.32	40.2	39.9	37.86	+0.9	+0.7 (δ)	+0.46 (δ)
9	34.89	34.69	34.80	34.0	33.3	34.04	+0.9	+0.4 (γ_t)	+0.76 (γ_g)
10	41.1(?)	39.65	39.89	36.2	35.5	34.85		+4.2 (β)	+5.04 (β)
11	24.06	23.10	22.56	21.8	21.1	20.60	+2.3	+2.0 (1,3)	+1.96(1,3)
12	41.20	40.08	39.65	41.0	39.9	39.46	−0.5	+0.2	+0.19
13	43.21	42.25	42.25	43.7	43.0	42.66	−0.5	−0.8	−0.41
14	51.28	50.22	50.13	51.2	50.9	50.35	+0.2	−0.7	−0.22
15	24.89	24.00	23.75	24.6	24.0	23.48	+0.3	0.0	+0.27
16	28.88	27.90	27.70	29.2	28.5	27.95	−0.3	−0.6	−0.25
17	57.39	56.20	55.87	57.2	56.3	55.74	+0.2	−0.1	+0.13
18	12.17	11.74	11.67	12.2	12.1	11.64	0.0	−0.4	+0.03
19	19.31	18.73	18.09	24.6	23.2	22.64	−5.3	−4.5 (γ_g)	−4.55 (γ_g)
20	36.79	35.37	35.29	36.7	35.8	35.21	+0.1	−0.1	+0.08
21	18.76	18.29	18.19	18.8	18.4	18.22	0.0	−0.4	−0.03
22	32.47+	30.98	30.95	32.4	31.3x	30.93	+0.1	−0.3	+0.02
23	32.39+	30.98	30.95	32.3	31.1x	30.93	+0.1	−0.1	+0.02
24	178.90	174.88	174.59	178.2	175.2	174.57	+0.7	−0.3	+0.02
OMe		51.52	51.43		51.8	51.38		−0.3	+0.05
OAc (C=O)			170.02			170.53			
			170.10			170.31			
			170.18						
OAc (Me)				21.50			21.47		
			21.23			21.39			
			21.23						

[a]Central line of $CDCl_3$, δ 77.10 was used as reference line. Signals labeled with x or + can be interchanged.
[b]In d_4-methanol, 75.5 MHz.
[c]Data from reference 15.
[d]Data from reference 14; similar data were also reported for d_4-methanol solution (15).
[e]α, β, γ, δ substitution effects: γ_t, γ-trans; γ_g, γ-gauche; 1,3-diequatorial.

pared with the data obtained for diacetoxy methyl chenodeoxycholate, were fully consistent with the addition of 1α-acetoxy group to the 3α,7α-diacetoxy-5β-cholan-24-oate moiety. Especially diagnostic were shifts induced for signals of carbon atoms 9 and 11. The spectra of chenodeoxycholic acid derivatives were interpreted before (15, 16). For the present study, the spectrum of methyl chenodeoxycholate diacetate was remeasured using INEPT conditions with 5.5 and 3.4 ms pre-delay time in order to obtain unambiguous assignment of multiplicity as well as to obtain higher accuracy of the chemical shift comparison. Very close chemical shift differences were observed when published ^{13}C spectral data of chenodeoxycholic acid and its methyl ester were compared with those of vulpecholic acid *1a* and its methyl ester *1b* (Table 2).

DISCUSSION

Vulpecholic acid *1a* is the first example of a primary BA[5] with a 1α-hydroxyl group. Until now only 1β-hydroxylated BA were detected. Thus, microbial 1β-hydroxylation was demonstrated (10), and acids with a probable 1,3,7- and 1,3,7,12-substitution pattern were tentatively identified in urine (17–20) and human meconium (21). Recently, synthesis of respective standards and their GLC–MS comparison with meconium components supported their presence (22, 23). Moreover, the presence of 1β-hydroxycholate in urine was confirmed by its isolation (24). Recent synthesis of 1α,3α-dihydroxy-5β-cholan-24-oic acid (25) is also noteworthy.

Vulpecholic acid is present in the bile of *T. vulpecula* primarily in the unconjugated form.[5] This fact is exceptional, especially since other minor dihydroxylated BA are mostly conjugated. The occurrence of large quantities of free BA in the gallbladder bile, to the best of our knowledge, has no precedence in mammals, although free BA were identified in certain amphibians (26 and references therein). The early study of the bile of the American opossum, *Didelphys marsupialis virginiana* (3) demonstrated an entirely "normal" bile composition. Our TLC screening of bile obtained from this species also did not reveal the presence of unconjugated BA.

Because of the unique geography of Australia and history of evolution of the marsupials resulting from continental drift, the Australian and American opossums have distinctly different taxonomic and morphologic features. The difference in molecular structure of their BA may represent another marker of the different pathways in their evolution. To answer the question whether the bile of *T. vulpecula* is truly exceptional, more extensive studies of other known marsupials are required.[6] *T. vulpecula* may prove to be an extremely interesting and important model to add to the understanding of bile acid synthesis, bile secretion, and various elements of the enterohepatic circulation.

This work was supported in part by Medical Research Service of the Veterans Administration and by grants from the National Institutes of Health, HD-14198 and HL-15376, Robert A. Welch Foundation grant C-583, and March of Dimes grant 6-305. Authors acknowledge the help of Dr. William K. Wilson of Rice University, Houston, TX, in obtaining ^{13}C NMR spectra, Marie N. Monroe for preparation of the manuscript, and Dr. Takashi Iida of Nihon University, Koniyama, for providing the sample of methyl 7-oxo-5β-cholan-24-oate. HRMS measurements were performed in NIH/MSU Mass Spectrometry Facility, East Lansing, MI.

Manuscript received 3 February 1986 and in revised form 12 September 1986.

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[5]In a separate study, vulpecholic acid has been identified as a primary bile acid. Only a small fraction of this acid is present as a conjugate. Trace of cholesterol and no phospholipids were detected in the bile of *T. vulpecula* (to be published).

[6]Marsupialia constitute a relatively numerous group of mammals consisting of eight families, 79 genera and 234 species (4).

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