papers and notes on methodology

Synthesis of the putative metabolites of plant sterols: (24R)- and (24S)-24-methyl-5β-cholestane-3α,7α,12α,25-tetrols and 24-ethyl-5β-cholestane-3α,7α,12α,24ξ-tetrol[1]

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Abstract This report describes the syntheses of (24R)- and (24S)-24-methyl-5β-cholestane-3α,7α,12α,25-tetrols and 24-ethyl-5β-cholestane-3α,7α,12α,24ξ-tetrol starting from cholic acid. The bile alcohols epimeric at C-24 were resolved by analytical and preparative thin-layer chromatography and characterized by gas–liquid chromatography and mass spectrometry. These epimeric bile alcohols may be useful for studying the transformation of β-sitosterol to cholic acid.—**Dayal, B., G. Salen, G. S. Tint, A. K. Batta, and S. Shefer.** Synthesis of the putative metabolites of plant sterols: (24R)- and (24S)-24-methyl-5β-cholestane-3α,7α,12α,25-tetrols and 24-ethyl-5β-cholestane-3α,7α,12α,24ξ-tetrol. *J. Lipid Res.* 1984. **25:** 865–870.

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β-Sitosterol, (24R)-24-ethyl-cholest-5-en-3β-ol is present in small amounts in both human and animal tissues (1–4). It originates from plants and about 5% of dietary β-sitosterol is absorbed from the mammalian intestine. Salen, Ahrens, and Grundy (5) have suggested that β-sitosterol, like cholesterol, is converted into primary bile acids, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid) and cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid). Shulman and co-workers (6) have also suggested similar results. In the rat, however, bile acids formed from intravenously injected β-sitosterol were not identical with any of the natural rat biliary bile acids (7). The difference in the metabolism of β-sitosterol may be dependent upon the ability of the species to dealkylate β-sitosterol to cholesterol and desmosterol. Werbin, Chaikoff, and Jones (8) stated that the cleavage of β-sitosterol side chain is most probably identical as in the case of cholesterol in the guinea pig. However, the mechanism of the side chain breakdown of β-sitosterol in the formation of bile acids is not known. In order to investigate the individual biochemical steps in the conversion of β-sitosterol to cholic acid, we required the synthesis of the hypothetical intermediates, isomeric (24R)- and (24S)-24-methyl-5β-cholestane-3α,7α,12α,25-tetrols and 24-ethyl-5β-cholestane-3α,7α,12α,24-tetrol (**Fig. 1,** Compounds V and VII). The structures of epimeric (24R)- and (24S)-24-methyl-5β-cholestane-3α,7α,12α,25-tetrols are shown below. The synthesis and characterization of these reference compounds required for this study are described.

Abbreviations: CTX, cerebrotendinous xanthomatosis; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; TMS, trimethylsilyl; RRT, relative retention time; EI–MS, electron ionization–mass spectrometry; S.C., side chain. Systematic names of steroids referred to in the text by trivial names are: cholesterol, cholest-5-en-3β-ol; cholestanol, 5α-cholestan-3β-ol; campesterol, (24R)-24-methyl-cholest-5-en-3β-ol; dihydrobrassicasterol, (24S)-24-methyl-cholest-5-en-3β-ol; brassicasterol, (24R)-24-methyl-5,22E-cholestadien-3β-ol; crinosterol, (24S)-24-methyl-5,22E-cholestadien-3β-ol; β-sitosterol, (24R)-24-ethyl-cholest-5-en-3β-ol; fucosterol (also called avenasterol), 24E-ethylidene-cholest-5-en-3β-ol; isofucosterol, 24Z-ethylidene-cholest-5-en-3β-ol; stigmasterol, (24R)-24-ethyl-5,22-cholestadien-3β-ol.

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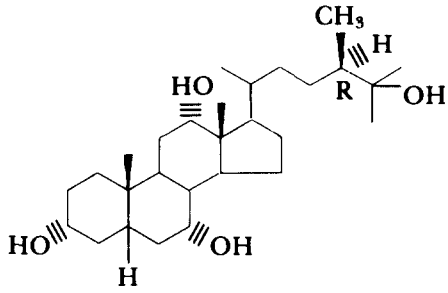
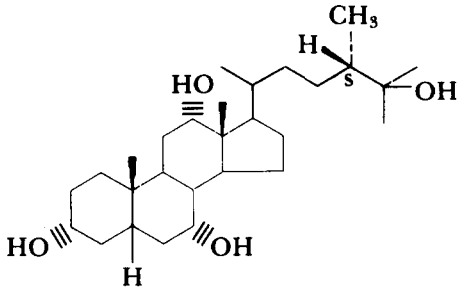


(24R)-24-methyl-5β-cholestane-3α,7α,12α,25-tetrol



(24S)-24-methyl-5β-cholestane-3α,7α,12α,25-tetrol

METHODS

Physical measurements

Melting points were determined on a Thermolyne apparatus (Thermolyne Corp., Dubuque, IA, model MP-12600) and are uncorrected.

GLC. The bile alcohols, as the TMS derivatives, were analyzed on a 180 cm × 4 mm column packed with 3% OV-17 on 80/100 mesh Gas Chrom Q; column temperature 270°C (Hewlett-Packard model 7610 gas chromatograph) (Hewlett-Packard, Palo Alto, CA).

Mass spectra of the bile alcohols were obtained with a Varian MAT-5 and Varian MAT-111 gas chromatograph–mass spectrometer (Varian Associates, Palo Alto, CA) at an ion source pressure of 2–3 × 10^{-6} mm and an electron energy of 70 eV, as described previously (9).

TLC. The bile alcohols were separated on silica gel G plates (Brinkmann Instruments, Westbury, NJ, 0.25 mm thickness), or on precoated plastic sheets (Brinkmann, 0.25 mm silica gel without gypsum) with the solvent system chloroform–acetone–methanol 70:50:10 (v/v/v). The spots were made visible either with iodine or with water.

RESULTS

Synthesis of (24R)- and (24S)-24-methyl-5β-cholestane-3α,7α,12α,25-tetrols (Fig. 1, V)

Preparation of 3α,7α,12α-triformyloxy-25-diazo-24-oxo-27-nor-5β-cholestane (Fig. 1, III). Triformyloxy cholanoic acid chloride (II) (prepared from 0.9 g of triformyloxy-cholic acid and 2 ml of oxalyl chloride (10, 11) was dissolved in 5 ml of anhydrous benzene (dried over sodium) and poured over 50 ml of a cold ethereal solution of diazoethane [prepared from 5 g of nitrosoethylurea, synthesized as described for nitrosomethylurea (12)]. The reaction mixture was left overnight at 0°C and the solvent was evaporated under a current of dry N_2. The yellow oily product obtained showed a major spot on TLC, R_f 0.6, indicative of diazoketone (III) (solvent system, benzene–ethyl acetate 95:5, v/v) in addition to a minor spot, R_f 0.80. The mass spectrum of the diazoketone, 3α,7α,12α-trihydroxy-25-diazo-24-oxo-27-nor-5β-cholestane exhibited a molecular ion at m/z 446 (4% intensity) and prominent peaks at m/z 428 (7%, $M^+ - H_2O$), 418 (5%, $M^+ - N_2$), 410 (1%, $M^+ - 2H_2O$), 400 (27%, $M^+ - H_2O - N_2$), 385 (38%, $M^+ - H_2O - CH_3 - N_2$), 382 (14%, $M^+ - 2H_2O - N_2$), 355 (26%, $M^+ - 2H_2O$ − 55), 299 (25%, $M^+ - 2H_2O$ − 111), 271 (100%, M^+ $- 2H_2O$ − S.C.), 253 (76%, $M^+ - 3H_2O$ − S.C.).

Preparation of methyl-24-nor-3α,7α,12α-trihydroxy-5β-cholestanoate (Fig. 1, IV). Diazoketone (III, 125 mg) obtained above was subjected to a modified Arndt-Eistert synthesis and the reaction product was worked up as described previously (10). The crude trihydroxy-24-methyl-substituted 25-homocholic acid (160 mg) was then directly treated with methanol/*p*-toluene sulfonic acid (15 mg) at room temperature overnight (13). Standard workup yielded 56 mg of methyl-24-nor-3α,7α,12α-trihydroxy-5β-cholestanoate.

Preparation of (24R)- and (24S)-24-methyl-5β-cholestane-3α,7α,12α,25-tetrols (Fig. 1, Va and Vb). The crude methyl ester of 24-nor-5β-cholestanoic acid (56 mg) was dissolved in 20 ml of anhydrous ether–benzene 13:7 (v/v) and added to 5 ml of methylmagnesium iodide and worked up as previously reported (10, 11). The reaction yielded 34 mg of crude product (Compounds Va and Vb). The resulting bile alcohols were resolved by analytical and preparative TLC (silica gel G, solvent system: chloroform–acetone–methanol 70:50:10, v/v/v) yielding 6.5 mg of (24R)-24-methyl-5β-cholestane-3α,7α,12α,25-tetrol, Compound Va, R_f = 0.26, RRT 3.19 min, and 7.5 mg of (24S)-24-methyl-5β-cholestane-3α,7α,12α,25-tetrol, Compound Vb, R_f = 0.33, RRT = 3.14 min (retention time of 5α-cholestane = 6.49 min). For Compound Va, significant peaks in the mass spectrum (EI–MS) were observed at m/z 432 (14%, $M^+ - H_2O$), 414 (14%, M^+ $- 2H_2O$), 399 (0.8%, $M^+ - 2H_2O - CH_3$), 396 (21%, $M^+ - 3H_2O$), 381 (4%, $M^+ - 3H_2O - CH_3$), 367 (43%, $M^+ - C_6H_{11}$), 312 (43%, $M^+ - 2H_2O - CH_3$ − 87), 271 (29%, $M^+ - 2H_2O$ − S.C.), 253 (31%, $M^+ - 3H_2O$ − S.C.), 87 (100%, $C_5H_{11}O$, base peak) (**Fig. 2**). For Compound Vb (EI–MS), 432 (2%, $M^+ - H_2O$), 414 (6%, M^+ $- 2H_2O$), 399 (3%, $M^+ - 2H_2O - CH_3$), 396 (17%, M^+ $- 3H_2O$), 381 (6%, $M^+ - 3H_2O - CH_3$), 356 (9%, M^+

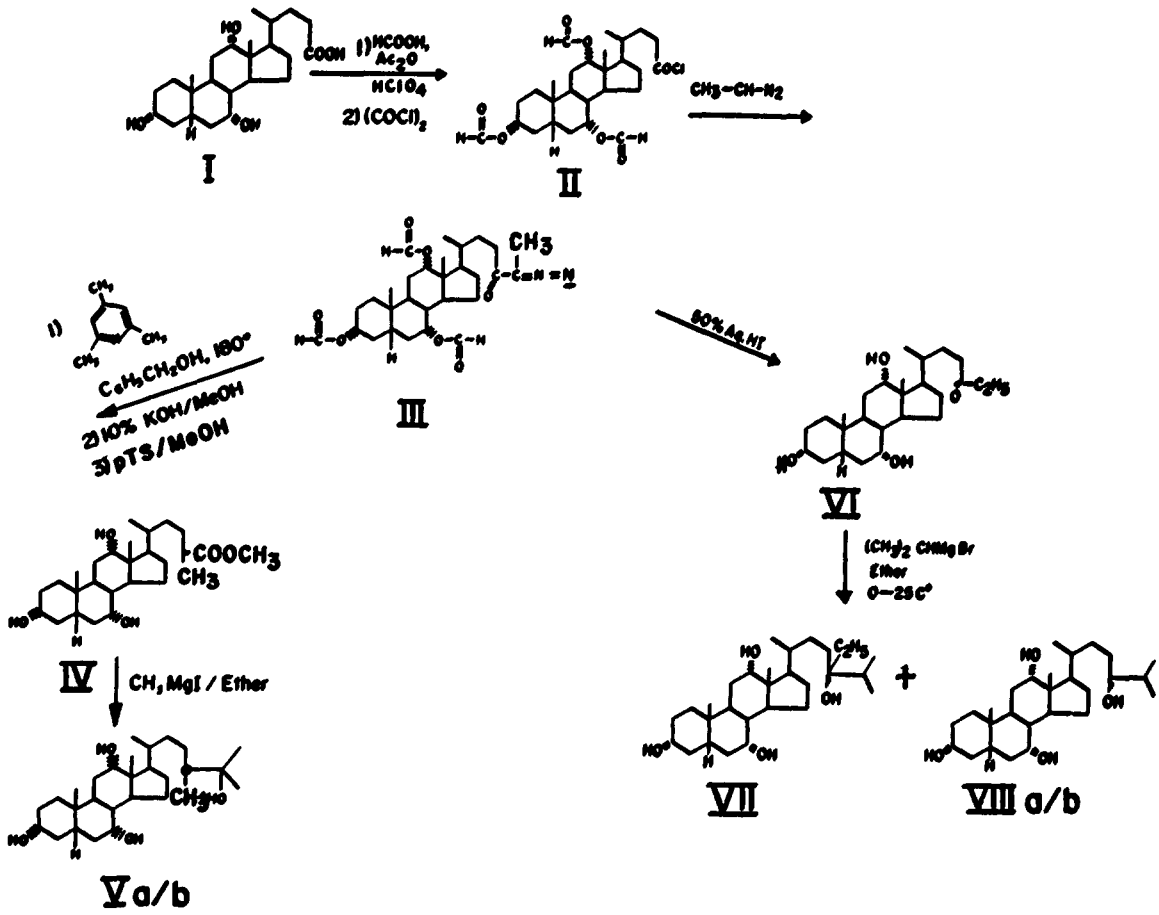


Fig. 1. Synthesis of (24R)- and (24S)-24-methyl-5β-cholestane-3α,7α,12α,25-tetrols and 24-ethyl-5β-cholestane-3α,7α,12α,24ξ-tetrol: I, 3α,7α,12α-triformyloxy-5β-cholan-24-oic acid; II, 3α,7α,12α-triformyloxy-5β-cholan-24-yl-chloride; III, 3α,7α,12α-triformyloxy-25-diazo-24-oxo-27-nor-5β-cholestane; IV, methyl-24-nor-3α,7α,12α,-trihydroxy-5β-cholestanoate; V, (24R)- and (24S)-24-methyl-5β-cholestane-3α,7α,12α,25-tetrols; VI, 3α,7α,12α-trihydroxy-24-oxo-27-nor-5β-cholestane; VII, 24-ethyl-5β-cholestane-3α,7α,12α,24ξ-tetrol; VIII, (24α)- and (24β)-5β-cholestane-3α,7α,12α,24-tetrols.

$- 2H_2O - 59 + H$), 338 (12%, $M^+ - 3H_2O - 59 + H$), 271 (28%, $M^+ - 2H_2O$ – S.C.), 253 (26%, $M^+ - 3H_2O$ – S.C.). The mass spectrum (EI–MS) of the trimethylsilyl ether of epimeric (24R)- and (24S)-24-methyl-5β-cholestane-3α,7α,12α,25-tetrol exhibited prominent peaks at 468 (1%, $M^+ - 3 \times 90$), 378 (1%, $M^+ - 4 \times 90$), 363 (0.8%, $M^+ - 4 \times 90 - 15$), 343 (1%, $M^+ - 2 \times 90$ – S.C.), 253 (6%, $M^+ - 3 \times 90$ – S.C.), 131 (100%, C-25,26,27 scission).

Preparation of 24-ethyl-5β-cholestane-3α,7α,12α,24ξ-tetrol (Fig. 1, VII)

To 150 mg of the diazoketone (III) was added 1.2 ml of H_2O followed by approximately 1–1.5 ml of 50% aqueous hydrogen iodide, which was added dropwise over a period of 3 min until the evolution of nitrogen ceased (14). The mixture was rapidly diluted with 50 ml of ethyl acetate and 10 ml of ether and the resulting organic solution was washed with two 15-ml portions of 1% sodium sulfite solution to remove the iodine color and then three 25-ml portions of water. The organic phase was evaporated and the residue was dried by azeotropic distillation with benzene followed by removal of the solvent under reduced pressure to give 56 mg of Compound VI, 3α,7α,12α-trihydroxy-24-oxo-27-nor-5β-cholestane as a yellow semi-solid. Significant peaks in the mass spectrum were observed at m/z 402 (2%, $M^+ - H_2O$), 384 (26%, $M^+ - 2H_2O$), 369 (5%, $M^+ - 2H_2O - CH_3$), 366 (12%, $M^+ - 3H_2O$), 313 (27%, $M^+ - C_3H_5O - 2H_2O - CH_3$), 295 (36%, $M^+ - C_3H_5O - 3H_2O - CH_3$), 271, (71%, M^+ – S.C. $- 2H_2O$), 253 (47%, $M^+ - 3H_2O$ – S.C.), 57 (100%, base peak, $CH_3CH_2C{\equiv}O$) (**Fig. 3**). To this intermediate Compound VI dissolved in benzene (5 ml) was added isopropylmagnesium bromide in ether. The mixture was left stirring at 0°C for 0.5 hr, and then at room temperature overnight. After the usual workup (10, 11), the reaction mixture was run through a silica gel column using ethyl acetate as eluent. Fractions with the same mobility as bile alcohols were gathered, the solvent was evaporated, and the residue (23 mg) was further resolved by analytical and preparative TLC on silica

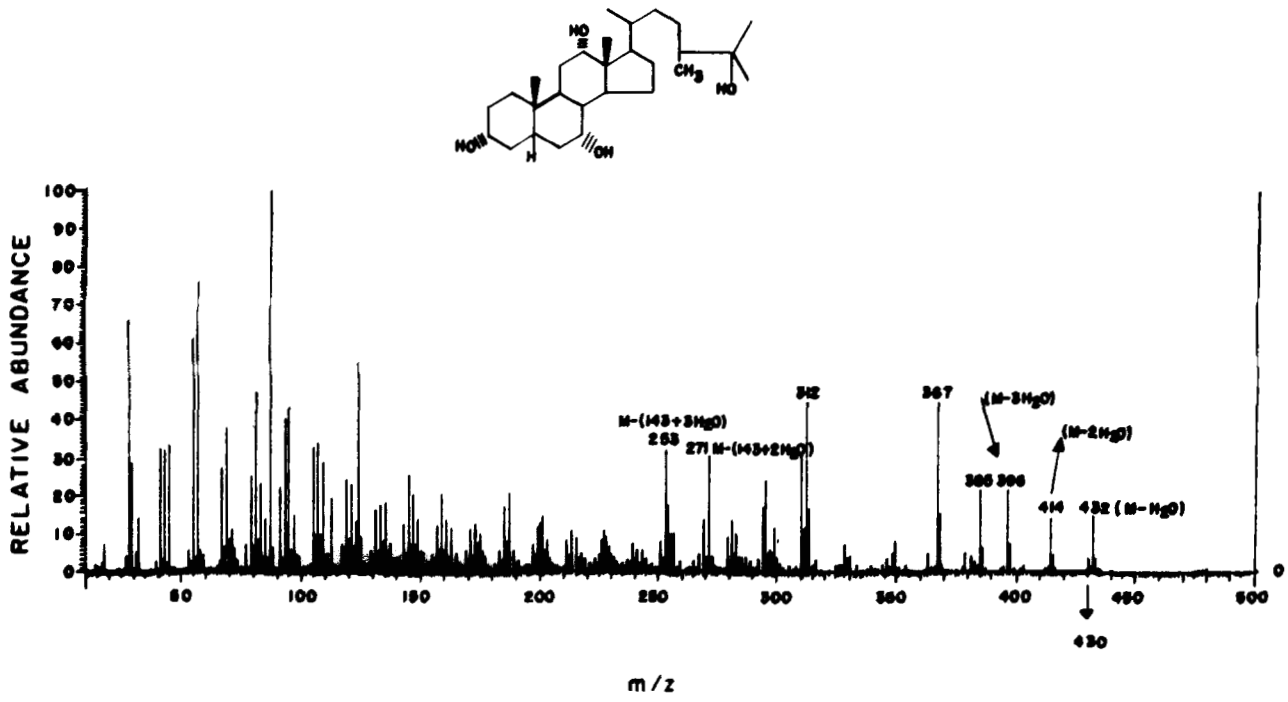
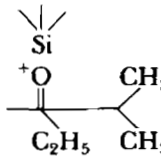


Fig. 2. Mass spectrum of (24R)-24-methyl-5β-cholestane-3α,7α,12α,25-tetrol.

gel G as described above. The following R_f values were observed: R_f 0.45, 24-ethyl-5β-cholestane-3α,7α,12α,24ξ-tetrol, VII [7.6 mg (6%) yield]; GLC-RRT, 3.58 (RT of 5β-cholestane, 6.49 min); EI–MS (TMS derivative) m/z 529, [2%, $M^+ - (2 \times 90 + 43)$]; 482, (1%, $M^+ - 3 \times 90$); 439 [3%, $M^+ - (3 \times 90 + 43)$]; 377 [0.5%, $M^+ - (4 \times 90 + 15)$]; 349 [7%, $M^+ - (4 \times 90 + 43)$]; 253 [8%, $M^+ - (3 \times 90 + SC)$]; 173 (100%,  , C-25,26,27 scission); R_f 0.35, 5β-cholestane-3α,7α,-12α,24β-tetrol, VIIIb, (4.0 mg); GLC-RRT 2.32 (RT of 5β-cholestane, 6.49 min); R_f 0.30, 5β-cholestane-3α,7α,12α,24α-tetrol, VIIIa, (4.0 mg); GLC-RRT 2.32 (RT of 5α-cholestane, 6.49 min). Both VIIIa and VIIIb had the same mass spectra (EI–MS) and proved to be identical with specimens prepared in our laboratory (15).

DISCUSSION

This report describes the synthesis of the following steroidal alcohols: (24R) and (24S) isomers of 24-methyl-5β-cholestane-3α,7α,12α,25-tetrol and 24-ethyl-5β-cholestane-3α,7α,12α,24ξ-tetrol (Fig. 1). These compounds are analogs of certain C_{28} and C_{29} bile alcohols that may be potential intermediates on the pathway leading from β-sitosterol to cholic acid. These compounds will be used as model substances in studies concerning the side chain oxidation of plant sterols.

In the synthesis of these compounds, an apparently more direct route involving alkyl-lithium phenylthiocuprate reagents to diastereoisomerically pure methyl or ethyl substituted sterol side chains failed to yield the desired results (16). On the other hand, syntheses of the (24R) and (24S) isomers of 24-methyl-5β-cholestane-3α,7α,12α,25-tetrol (Fig. 1) were achieved via the classical Arndt-Eistert reaction on the appropriate diazoketone, 3α,7α,12α-triformyloxy-25-diazo-24-oxo-27-nor-5β-cholestane. The resulting 27-nor-5β-cholestanoic acid was transesterified in methanol to which a catalytic amount of *p*-toluenesulfonic acid was dissolved. The subsequent product underwent a Grignard reaction, resulting in a mixture of (24R) and (24S) 24-methyl-5β-cholestane-3α,7α,12α,25-tetrols Va and Vb in a ratio of 51.4:48.6 as determined by GLC and TLC. These diastereoisomers were separated by TLC (11, 17, 18).

The two-step synthesis of 24-ethyl-5β-cholestane-3α,7α,12α,24ξ-tetrol from the intermediary diazoketone involved the reaction of aqueous hydrogen iodide followed by Grignard treatment of the resulting intermediate (14). The 24-ethyl-substituted bile alcohol was separated by preparative TLC using silica gel G plates or precoated plastic sheets. The desired 24-ethyl compound was ac-

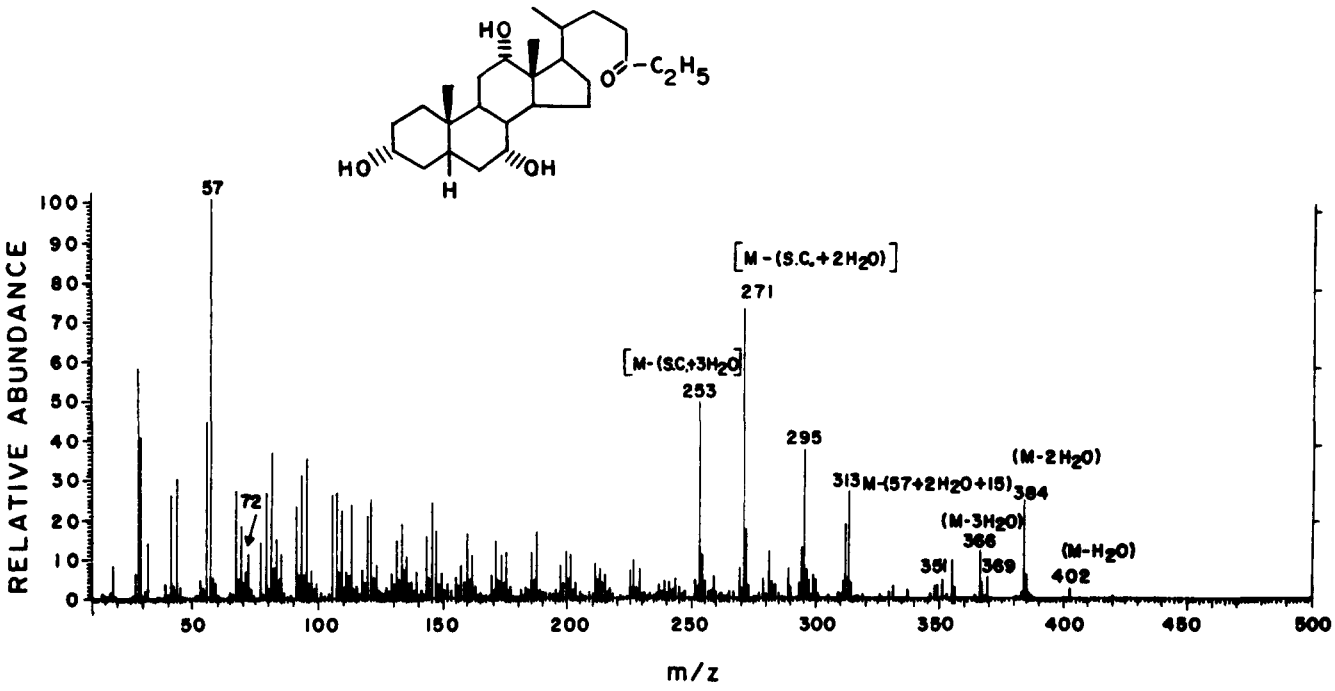


Fig. 3. Mass spectrum of 3α,7α,12α-trihydroxy-24-oxo-27-nor-5β-cholestane.

companied by small quantities of epimeric VIIIa and VIIIb which were easily separated by preparative TLC. The presence of 24α/24β-tetrols was attributed to the coupling reaction between the unreacted triformyloxycholylchloride and isopropylmagnesium bromide.

The TMS derivatives of the bile alcohols were quantitated by GLC on 3% OV-17. The retention times of the TMS ethers of the epimeric 24-methyl-5β-cholestane-3α,7α,12α,25-tetrols were different. The 24R configuration was assigned to the more polar component Va, and consequently the 24S to the less polar component Vb, on the basis of observed polarities in epimeric pairs of 24-alcohols (11, 15, 17, 18).

The identification of these bile alcohols was confirmed by mass spectrometry. The mass spectra of the (24R)- and (24S)-24-methyl-5β-cholestane-3α,7α,12α,25-tetrols (underivatized) were almost identical except for some significant intensity differences. In both epimers, the molecular ion peak was not observed, but an M−18 peak of 14% in Va and 2% in Vb was seen at m/z 432. A peak at m/z 271 was formed by the loss of the side chain and two water molecules. The peak at m/z 253 was due to the loss of the side chain from the fragment at m/z 271. The fragment ions 173 and 131 appeared as base peaks in spectra of the TMS ethers of 24-ethyl-5β-cholestane 3α,7α,12α,24ξ-tetrol and the isomeric (24R) and (24S) 24-methyl-5β-cholestane-3α,7α,12α,25-tetrol, respectively.

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