Inhibitors of sterol synthesis. Characterization of trimethylsilyl dienol ethers of 3β-hydroxy-5α-cholest-8(14)-en-15-one. Applications in the analysis of mitochondrial metabolites of the 15-ketosterol by gas chromatography–mass spectrometry

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Abstract Derivatization of $\Delta^{8(14)}$-15-ketosterols as bis-TMS dienol ethers facilitates their analysis by gas chromatography–mass spectrometry (GC–MS). Conditions are presented for the preparation of each of the three possible bis-TMS dienol ethers of 3β-hydroxy-5α-cholest-8(14)-en-15-one (**1**), a potent regulator of cholesterol metabolism. Treatment of **1** with N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and pyridine for 20 h at 100°C produced primarily 3β,15-bis(trimethylsilyloxy)-5α-cholesta-7,14-diene. Treatment of **1** with BSTFA-pyridine in the presence of 0.1% perchloric acid for 20 h at 22°C gave mainly the $\Delta^{8(14),15}$ dienol ether. Heating this reaction mixture at 100°C for 4 days gave mainly the $\Delta^{8,14}$ ether. The structures of the three dienol ethers were established by GC–MS and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. The three TMS dienol ethers of **1** were resolved by capillary GC and gave very simple mass spectra upon electron impact with fragmentation limited almost exclusively to the elimination of trimethylsilanol, methyl, and the side chain. The TMS dienol ethers showed reduced artefact formation, improved chromatographic resolution, and increased sensitivity relative to the $\Delta^{8(14)}$-15-ketosterols, properties that improve the detection and identification of minor components in analyses of complex biological mixtures. The utility of this derivatization is illustrated for the $\Delta^{7,14}$ TMS dienol ethers of the 3-deoxy, 3-keto, 3α-hydroxy, and 3β-methoxy analogs of **1** and for $\Delta^{8(14)}$-15-ketosterols in mixtures obtained from incubations of **1** with rat liver mitochondria in the presence of NADPH. —**St. Pyrek, J., W. K. Wilson, S. Numazawa, and G. J. Schroepfer, Jr.** Inhibitors of sterol synthesis. Characterization of trimethylsilyl dienol ethers of 3β-hydroxy-5α-cholest-8(14)-en-15-one. Applications in the analysis of mitochondrial metabolites of the 15-ketosterol by gas chromatography–mass spectrometry. *J. Lipid Res.* 1991. **32:** 1371–1380.

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3β-Hydroxy-5α-cholest-8(14)-en-15-one (**1**) is a potent regulator of cholesterol metabolism (1–6). An understanding of the actions of **1** is critically dependent upon a thorough elucidation of its metabolism. The importance of liver in the metabolism of **1** (7, 8) prompted investigations of the side-chain oxidation of **1** by rat liver mitochondria in the presence of NADPH (9,10). Four major products (**Fig. 1**) were isolated and characterized by nuclear magnetic resonance (NMR) spectroscopy and gas chromatography–mass spectrometry (GC–MS): (25*R*)- and (25*S*)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one (4:1 ratio), 3β-hydroxy-15-oxo-5α-cholest-8(14)-en-26-oic acid, and 3β,25-dihydroxy-5α-cholest-8(14)-en-15-one (10). In addition, 3α,26-dihydroxy-5α-cholest-8(14)-en-15-one and 3β,24-dihydroxy-5α-cholest-8(14)-en-15-one were detected as minor products by capillary GC–MS (10).

In the course of the latter metabolic studies and other analytical investigations of **1** we noted that preparations of the 3β-trimethylsilyl (TMS) ether derivative of **1** frequently showed minor GC–MS components corresponding to TMS dienol ethers of **1**. Similar TMS enol ethers have been observed previously in silylations of hydroxy-keto-steroids (11, 12). After the development of conditions for the conversion of **1** to derivatives enriched in each of the three possible TMS dienol ethers (i.e.,

Abbreviations: amu, atomic mass unit; BSA, N,O-bis(trimethylsilyl)acetamide; BSTFA, bis(trimethylsilyl)trifluoroacetamide; DEPT, distortionless enhancement by polarization transfer; GC, gas chromatography; HETCOR, ^{1}H-^{13}C heteronuclear shift-correlated (spectroscopy); HPLC, high performance liquid chromatography; MS, mass spectrometry or mass spectrum; NMR, nuclear magnetic resonance; SC, side chain; TLC, thin-layer chromatography; TMS, trimethylsilyl; TMSOH, trimethylsilanol; t_R, retention time.

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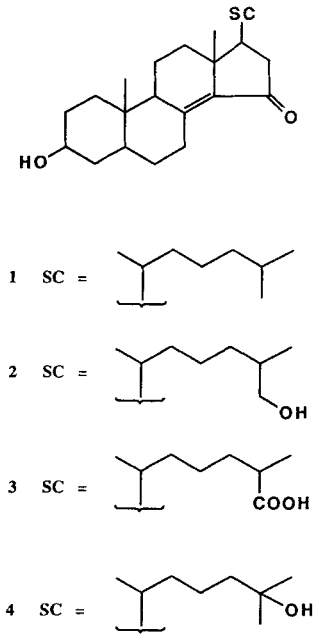

Fig. 1. 3β-Hydroxy-5α-cholest-8(14)-en-15-one (**1**) and its major metabolites upon incubation with rat liver mitochondria. Both the 25*R* and 25*S* epimers of the 26-hydroxy derivative (**2**) are metabolites.

$\Delta^{7,14}$, $\Delta^{8,14}$, and $\Delta^{8(14),15}$), the individual TMS dienol ethers were characterized by GC–MS and ^1H and ^{13}C NMR. Presented here are the synthesis, characterization, and structure determination of the TMS dienol ethers of **1**. We also illustrate the value of such derivatives for GC–MS analysis of biological samples containing $\Delta^{8(14)}$-15-ketosterols.

MATERIALS AND METHODS

Authentic samples of 3β-hydroxy-5α-cholest-8(14)-en-15-one (**1**) (1), 3α-hydroxy-5α-cholest-8(14)-en-15-one (13), 5α-cholest-8(14)-en-15-one (14), 5α-cholest-8(14)-ene-3,15-dione (15), 3β-hydroxy-5α-cholesta-6,8(14)-dien-15-one (16), 5α-cholesta-6,8(14)-diene-3,15-dione (16), and [2,2,3α,4,4-^2H]-**1** (15) were prepared as described previously. [4-^{14}C]-**1**, a gift from Lederle Laboratories (Pearl River, NY), was purified as described previously (10). N,O-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from Petrarch Systems (Bristol, PA). N,O-bis(trimethylsilyl)acetamide-d_{18} (BSA-d_{18}) was obtained from Merck, Sharp, and Dohme (Montreal). Silylating reagents with $HClO_4$ were prepared by adding BSTFA to pyridine solutions containing $HClO_4$.

Unless otherwise specified, electron-impact mass spectra were obtained at 20 eV on an EXTREL ELQ-400 quadrupole GC-MS system interfaced with a Varian chromatograph (model 3400). The fused silica capillary column was introduced directly to the ion source. Mass spectra were obtained with a 1-sec repetition time usually in a range of 100 to 700 amu. Injections were made using either an all-glass falling needle injector (Applied Science, State College, PA) or, in some cases, a split/splitless injector (splitless mode). The following GC capillary columns were used: Rt_x-20 (Restek Corp., Bellefonte, PA), 15 m, 0.25 mm ID, 0.1 μm film thickness, bonded stationary phase of 20% diphenyl–80% dimethyl polysiloxane; DB-5 (J & W Scientific, Folsom, CA), 30 m, 0.25 mm ID, 0.1 μm film thickness, bonded stationary phase of 5% diphenyl- 95% dimethyl polysiloxane; or DB-1, 30 m, 0.25 mm ID, 0.1 μm film thickness, bonded stationary phase of dimethyl polysiloxane. The following GC conditions were used for analysis: condition A: Rt_x-20 column with falling-needle injection and temperature programming from 240 to 280°C at 10°C per min; condition B: as in A but with temperature programming from 220 to 280°C at 5°C per min; condition C: DB-5 column with temperature programming held at 150°C for 1 min then to 280°C at 10°C per min, splitless injection in decane; condition D: as in C but with temperature programming at 40°C per min; condition E, DB-1, falling needle injection, 200°C for 2 min, then to 290°C at 10° per min; condition F: 70 eV, Kratos Concept 1H magnetic GC–MS system (30 m × 0.25 mm ID Rt_x-1 column, 0.1 μm film thickness, split injection, 280°C isothermal). All mass spectra are reported as *m/z* (suggested assignment, relative intensity as % of base peak).

^1H and ^{13}C NMR spectra were recorded on an IBM AF300 spectrometer using $CDCl_3$ as the solvent. ^1H NMR spectra (300.1 MHz) were referenced to tetramethylsilane, and ^{13}C NMR spectra (75.5 MHz) were referenced to $CDCl_3$ at 77.0 ppm. DEPT, HETCOR (~50 increments, δ 0.6–2.6 window in the ^1H dimension), and long-range HETCOR (optimized for J_{CH} = 10 Hz, ~16 increments, δ 0.6–2.6 window in the ^1H dimension) spectra were acquired as described previously (17). Coupling constants were based on first-order analysis of resolution-enhanced signals. NMR samples of **5a** and **5b** had no discernable impurities, the sample of **5d** was ~90% pure, and the sample of **5c** contained 10–20% each of **5a** and **5b**.

Preparation of TMS derivatives

In procedure A, the sterol sample was first dissolved in benzene and evaporated to dryness in a nitrogen stream in order to remove any trace of solvents. The sample was then dissolved in an excess of a 1:1 mixture of BSTFA and pyridine (dried over solid KOH) in a 5-ml conical screw-cap test tube with Teflon liner. For microgram or nanogram amounts of material isolated from biological samples, the sample was evaporated with benzene in the tip of the conical tube, and 10–20 μl of BSTFA-pyridine reagent was added. After 20 h at room temperature under

a nitrogen atmosphere, a portion of the reaction mixture was placed directly on the tip of the falling needle injector. Alternatively, the reagent was removed in a nitrogen stream, and the residue was dissolved in heptane or decane for splitless injection. Conditions in procedure B were the same as in procedure A except that the reaction was conducted for 20 h at 100–110°C in a heating block. BSA-d_{18} was used in place of BSTFA for the preparation of deuterated derivatives. In procedure C, the silylation reagent was BSTFA–pyridine 1:1 containing $HClO_4$ (1 μl per ml), and the reaction was carried out at room temperature for 20 h. The dienol ethers were relatively stable and were only slowly hydrolyzed to mono-TMS ethers.

RESULTS

TMS derivatives of 3β-hydroxy-5α-cholest-8(14)-en-15-one and related sterols

GC retention times are given in **Table 1**, and chemical structures are shown in **Fig. 2**. 3β-Trimethylsilyloxy-5α-cholest-8(14)-en-15-one (**5a**) was obtained by silylation of **1** (106 mg, procedure A, 1 ml of the reagent). Evaporation of reagents in a nitrogen stream followed by further drying in vacuo gave a crystalline residue, 116 mg: mp 168.0–168.5°C; $[\alpha]_D^{25}$ + 111.6° (*c*, 0.9, chloroform); ^1H and ^{13}C NMR, Table 2. Its mass spectrum was virtually identical with that reported previously (15). 3β,15-Bis(trimethylsilyloxy)-5α-cholesta-7,14-diene (**5b**) was prepared by silylation of **1** (1.66 g) by procedure B (10 ml of the reagent) followed by evaporation of the reagent. The crude sample, a mixture containing **5a** (trace), **5b** (95%), **5c** (~4%), and **5d** (trace), was analyzed by ^1H and ^{13}C NMR (**Table 2**) and GC–MS (MS of **5b**, **Table 3**). This crude reaction mixture was, after addition of $HClO_4$ (~1%), heated for 6 days at 100°C. After removal of reagents, this mixture was directly characterized by GC–MS (MS of **5d**, Table 3) and ^1H and ^{13}C NMR (Table 2). GC–MS analysis showed the presence of **5b** (~7%) and **5d** (93%). Heating **1** for 4 days at 100°C in a solution of BSTFA–pyridine containing 0.1% $HClO_4$ also gave a product consisting mainly of **5d** (**Table 4**). 3β,15-Bis(trimethylsilyloxy)-5α-cholesta-8(14),15-diene (**5c**) was made by derivatizing **1** using procedure C followed by evaporation of reagents. The crude product, a mixture containing **5a** (0–3%), **5b** (3–8%), **5c** (77–86%), and **5d** (10–16%), was analyzed directly by GC–MS (MS of **5c**, Table 3) and ^1H and ^{13}C NMR (Table 2). Similar proportions of products were obtained from reaction at 20°C for 4 h or at 80°C for 20 h. The product distributions (Table 4) remained fairly constant during prolonged sample storage at 4°C.

Conditions identical with or similar to those of procedure B were applied for the silylation of 3α-hydroxy-5α-cholest-8(14)-en-15-one,3β-methoxy-5α-cholest-8(14)-en-15-one (10), 5α-cholest-8(14)-en-15-one, and 5α-cholest-8(14)-ene-3,15-dione to give the corresponding 15-trimethylsilyl-$\Delta^{7,14}$ dienol ethers (**6b, 9b, 7b, 8b**). These derivatives gave MS fully consistent with that of the $\Delta^{7,14}$ dienol ether **5b** prepared from **1**. For example, a comparison of the MS of **5b** and the 3α-hydroxy derivative **6b** is given in Table 3.[3]

^1H and ^{13}C NMR assignments of TMS enol ethers 5b, 5c, and 5d

The ^1H and ^{13}C NMR assignments of the dienol ethers (Table 2) were made primarily from DEPT spectra and by comparison with spectra of similar sterols. The chemical shifts of **5b** and **5d** closely matched those (18) of 5α-cholesta-7,14-dien-3β-ol and 5α-cholesta-8,14-dien-3β-ol except for expected differences for C-7, the olefinic carbons, and carbons β and γ to the enol oxygen. The chemical shifts of **5c** also corresponded reasonably closely to those of $\Delta^{8(14)}$ model compounds.

HETCOR (one-bond C–H correlations) and long-range HETCOR spectra (one-, two-, and three-bond C–H correlations) were used to differentiate the remaining unassigned carbons by standard procedures (17). Assigning the olefinic carbons of $\Delta^{7,14}$ dienol ether **5b** required a long-range HETCOR experiment. Of the three quaternary olefinic signals, only δ 123.7 (C-14) showed a long-range correlation to a methyl proton. The remaining carbons, C-15 (δ 144.9) and C-8 (δ 131.0), were distinguished by chemical shift arguments and by differences in their long-range correlations to allylic protons. For $\Delta^{8,14}$ dienol ether **5d**, C-16 (δ 38.74), C-4 (δ 38.68), and C-12 (δ 37.38) were distinguished by their ^1H chemical shifts. Of the four quaternary olefinic signals of **5d**, only δ 122.7 (C-14) showed a long-range correlation to H-18. The remaining carbons were assigned by comparison with spectra of 5α-cholesta-8,14-dien-3β-ol (C-8 and C-9) and **5b** (C-15). For both the $\Delta^{7,14}$ and $\Delta^{8,14}$ dienol ethers, relative to the $\Delta^{7,14}$ and $\Delta^{8,14}$ dienes, C-15 was shifted ~26 ppm downfield and C-14 ~28 ppm upfield, while the remote olefinic carbons were essentially unchanged (± 4 ppm). These trends were used to assign C-8 and C-14 of $\Delta^{8(14),15}$ dienol ether **5c** by comparison with the assignments of 5α-cholest-8(14)-en-3β-ol (18). The ^1H NMR chemical shifts were obtained from a combination of the proton

[3]The MS of **7b**, **8b**, and **9b** are as follows: 3-deoxy analog (**7b**): 456 M^+, 42), 441 (100), 384 (3), 367 (4), 366 (4), 351 (3), 343 (13), 253 (85); 3-keto analog (**8b**); 470 (M^+, 38), 455 (100), 398 (4), 385 (4), 381 (4), 380 (2), 365 (5), 357 (11), 267 (49); 3β-methoxy derivative (**9b**): 486 (M^+, 56), 471 (100), 454 (2), 439 (7), 414 (2), 396 (2), 381 (4), 373 (10), 283 (24), 251 (19). Most ions in the mass spectra can be assigned as losses of CH_3, TMSOH, and SC, analogously to the assignments in Tables 3, 5, and 6; assignments of other ions have been presented in ref. 10.

TABLE 1. Products observed by GC–MS after silylation of $\Delta^{8(14)}$-15-ketosterols with BSTFA-pyridine[a]

Product	Functional Groups: OTMS	Functional Groups: C=C	t_R	Relative Amount[b]	Molecular Ion
			min	%	*m/z*
Silylation of 15-ketosterol **1** by procedure C					
5b	3β,15	$\Delta^{7,14}$	3.47	3–8	544
5d	3β,15	$\Delta^{8,14}$	3.65	10–13	544
5c	3β,15	$\Delta^{8(14),15}$	3.90	77–86	544
5a	3β	$\Delta^{8(14)}$	5.10	0–2	472
Silylation of purified 3β,26-diol **2** (derived from mitochondrial incubation of **1**) by procedure A					
(unidentified)			7.17	5	646
10a	3β,26	$\Delta^{8(14)}$	8.20	90	560
(artefact)[c]			~8.8	5	558
Silylation of purified 3β,26-diol **2** (derived from mitochondrial incubation of **1**) by procedure B					
10b	3β,15,26	$\Delta^{7,14}$	5.18	88	632
10c	3β,15,26	$\Delta^{8,14}$	5.48	<1	632[d]
10d	3β,15,26	$\Delta^{8(14),15}$	5.80	10	632
Silylation of chromatographic zone 2B (derived from mitochondrial incubation of **1**) by procedure B					
(tentatively identified)[e]			3.97	3.1	632
12b	3β,15,25	$\Delta^{7,14}$	4.83	58.8	632
12c	3β,15,25	$\Delta^{8,14}$	5.05	3.0	632
12d	3β,15,25	$\Delta^{8(14),15}$	5.33	14.8	632
10b	3β,15,26	$\Delta^{7,14}$	5.45	2.9	632[d]
(unidentified)[f]			6.31	11.6	630
12a	3β,25	$\Delta^{8(14)}$	7.37	1.9	560
(artefact)[g]			8.88	3.9	558
Silylation of chromatographic zone 3 (derived from mitochondrial incubation of **1**) by procedure B[h]					
13b	3α,15,25	$\Delta^{7,14}$	4.14	3.8	632
11b	3α,15,26	$\Delta^{7,14}$	4.49	67.8	632
15b[i]	3β,15,24	$\Delta^{7,14}$	4.66	9.1	632
11c	3α,15,26	$\Delta^{8(14),15}$	5.11	15.6	632
15c[j]	3β,15,24	$\Delta^{8(14),15}$	5.22	2.4	632[d]
11a[k]	3α,26	$\Delta^{8(14)}$	7.08	trace	560
Silylation of chromatographic zone 4 (derived from mitochondrial incubation of **1**) by procedure B					
14b[l]	3β,15	$\Delta^{7,14}$	5.79		588
14c[l]	3β,15	$\Delta^{8(14),15}$	6.45		588
14a[l]	3β	$\Delta^{8(14)}$	9.43		516

[a]GC condition A with a Rt_x-20 capillary column. Under these conditions, the TMS derivative of cholesterol had a t_R of 3.51 min.

[b]Based on total ion current of the mass chromatogram ($m/z > 100$ or $m/z > 200$).

[c]MS given in Table 6.

[d]These chromatographic peaks included an unidentified component with molecular ion *m/z* 648.

[e]MS: 632 (M^+, 8), 617 (4), 560 (0.4), 546 (1), 542 (1), 527 (1), 460 (2), 459 (1.5), 431 (100), 427 (4), 341 (4), 315 (4), 251 (6), 234 (2), 225 (8), 143 (7), 133 (12), 129 (6). The prominent ion at *m/z* 431 was interpreted as loss of the side chain containing an OTMS group. The intensity of this ion would have pointed to a $\Delta^{8(14),15}$ dienol ether structure except that no corresponding $\Delta^{7,14}$ dienol ether of a shorter t_R was detected and fragment ions diagnostic for the $\Delta^{8(14),15}$ dienol ether were either completely absent (*m/z* 418, 417) or barely detectable (*m/z* 233). Instead, low intensity ions at *m/z* 546, 460, 459, 427, 315, and 225 were present. Based on the short t_R expected for tertiary alcohols (Table 1) and the high abundance of ions corresponding to loss of the side-chain fragment, this component might be the tris-TMS ether of a 20-hydroxylated component. Further studies will be required to establish the nature and origin of this minor component.

[f]A tris-TMS ether of a trihydroxy sterol; MS: 630 (M^+, 25), 615 (6), 525 (6), 468 (2), 453 (1), 429 (100), 339 (4), no other ions of >1% abundance above 100 amu.

[g]Mass spectrum compatible with a bis-TMS ether of a $\Delta^{6,8(14)}$-15-ketosterol with one TMS group in the side chain: 558 (30), 543 (26), 468 (100), 453 (52), 427 (16), 405 (6), 363 (6), 357 (6), 330 (16), 267 (32), 251 (11), 240 (58), 225 (17), 212 (82), 197 (23).

[h]Two additional components both having t_R 3.89 min (combined relative amount, 1.3%; molecular ions 542 and 632) were shown not to contain any radioactivity by subsequent HPLC purification of zone 3.

[i]MS: 632 (M^+, 100), 617 (67), 589 (16), 542 (4), 527 (14), 499 (10), 452 (3), 437 (5), 431 (13), 409 (4), 341 (8), 251 (24), 145 ($CH(=OTMS)CH(CH_3)_2^+$, 28), 143 (8), 107 (13).

[j]MS: 632 (M^+, 16), 617 (10), 589 (7), 542 (7), 527 (2), 499 (4), 431 (100), 341 (6), 251 (7), 233 (5), 107 (11), weak ions at 648, 630, and 531 were attributable to other component(s).

[k]Characterized by derivatization using procedure A.

[l]TMS ether derivatives of methyl 3β-hydroxy-15-keto-5α-cholest-8(14)-en-26-oate; MS of **14b**: 588 (M^+, 82), 573 (100), 557 (6), 516 (10), 499 (5), 498 (5), 483 (14), 431 (13), 393 (10), 341 (16), 251 (28).

and HETCOR spectra. Stereochemistry of CH_2 protons was assigned whenever possible by comparison with reported ^1H assignments of similar sterols (19).

GC–MS analyses of TMS dienol ethers of polar sterols and acids recovered after incubation of 15-ketosterol 1 with rat liver mitochondria

As described previously (9, 10), 3β-hydroxy-5α-cholest-8(14)-en-15-one (**1**) containing [4-^{14}C]-**1** was incubated with rat liver mitochondria in the presence of NADPH, and the radioactive products were separated by preparative thin-layer chromatography (TLC) into four zones (1–4) of decreasing polarity.[4] Zone 2 was further fractionated by reversed-phase high performance liquid chromatography (HPLC) into zones 2A (shorter t_R) and 2B (longer t_R), and pure 3β,26-diol **2** was isolated from zone 2A (containing 8.9–15.3% of incubated ^{14}C) by repeated preparative TLC (10). Material from each of these zones was silylated and analyzed by GC–MS. The product distributions are summarized in Table 1, and MS data are given for the TMS dienol ethers of the 25-hydroxy and 26-hydroxy metabolites in **Table 5.**

GC–MS analysis of **1**, its TMS derivative **5a**, the 3-desoxy analog **7a**, the 3-keto analog **8a**, and certain metabolic samples (see Table 1) showed, in addition to the main peak, small but variable amounts of a minor component 2 amu lower in molecular weight. Rigorous assessment of the purity of **1** by NMR and chromatography ruled out the possibility that the minor components were attributable to impurities in the 15-ketosterols. Mass spectra of these minor components, along with those of authentic $\Delta^{6,8(14)}$-15-ketosterols, are presented in **Table 6**.

DISCUSSION

In our previous work, **1** and its metabolites were derivatized for GC–MS as the 3-TMS ethers. Despite the presence of the underivatized ketone function, this approach has been successfully used to detect low levels of $\Delta^{8(14)}$-15-ketosterols in biological samples (9, 10, 20).

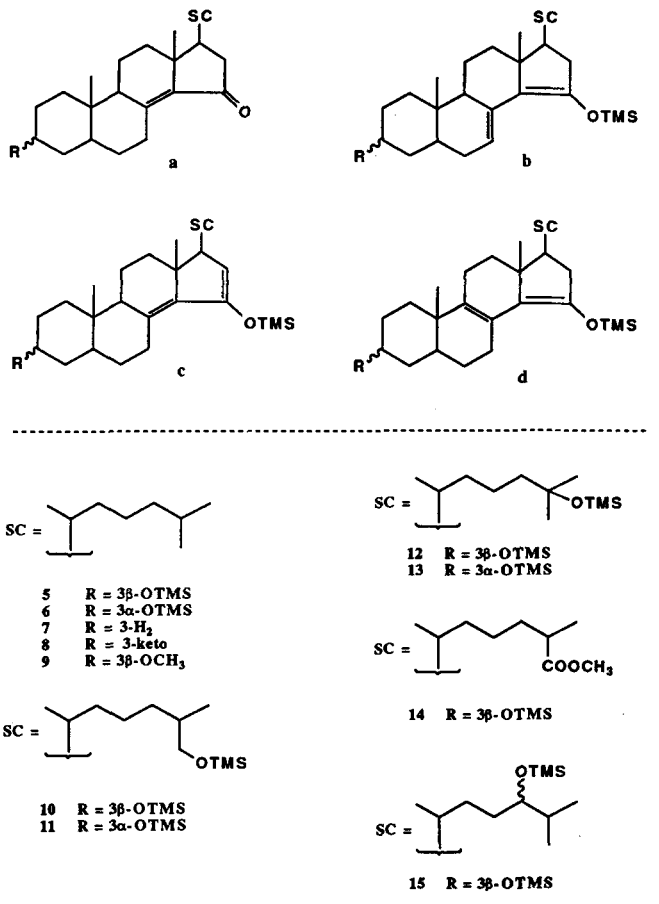

Fig. 2. Trimethylsilyl derivatives of 3β-hydroxy-5α-cholest-8(14)-en-15-one, synthetic analogs having modified C-3 functionality, and major metabolites upon incubation with rat liver mitochondria.

However, the unprotected ketone function presents several potential complications. For example, $\Delta^{8(14)}$-15-ketosterols containing tertiary hydroxyl groups cannot be fully silylated by BSTFA without forcing conditions that also lead to silyl dienol ether byproducts. These and other byproducts are also sometimes observed under mild silylating conditions (11, 12, 21, 22). BSTFA, a convenient silylating reagent because of its potency and volatility, promotes formation of silyl enol ethers more than most other reagents (22). In the preparation of the mono-TMS derivative **5a** from **1**, silyl dienol ether byproducts, a dehydrogenation artefact (vide infra), and other unidentified byproducts were often formed in small and variable amounts. In studies of the metabolism of **1**, such byproducts could be confused with 15-ketosterols of biological origin. Furthermore, the GC analyses of the mono-TMS derivative **5a** and its side-chain hydroxylated analogs sometimes showed peak broadening and tailing that interfered with the resolution of minor components in biological mixtures.

In order to eliminate these problems, we initially pursued a standard approach of protecting the keto group as an oxime, methoxime, or hydrazone derivative prior to

[4]In an additional incubation, a mixture of [2,2,3,4,4-^{2}H]-**1** (~4.8 D/molecule) (15) and [4-^{14}C]-**1** with a final specific radioactivity of 0.114 μCi/μmol (1.96 mg) was incubated with rat liver mitochondria as described previously. The crude product (97% recovery of the incubated radioactivity) was subjected to preparative TLC (zone 2, 0.086 μCi; zone 3, 0.010 μCi; zone 4, 0.032 μCi) followed by silylation and GC–MS analysis (condition C), which showed the following deuterium content; recovered substrate **1**, ~4.6 D/molecule (24% d_4, 69% d_5); the 3β,26-diol metabolite (analyzed as its bis-TMS ether **10a**), ~4.7 D/molecule (27% d_4, 55% d_5); methyl 3β-trimethylsilyloxy-15-keto-5α-cholest-8(14)-en-26-oate (**14a**), ~4.8 D/molecule (24% d_4, 59% d_5); and the 3α, 26-diol (analyzed as its bis-TMS ether **11a**) ~3.8 D/molecule (20% d_3, 56% d_4, 19% d_5). The existence of the d_5 species is possibly due to reincorporation of label in the reduction of the 3-ketone function. The maximum error estimated for the determination of deuterium content by GC–MS, assuming determination of the ion intensity to 2% accuracy, was ±0.3 deuterium atoms for the d_5 molecule.

TABLE 2. ^1H and ^{13}C NMR chemical shifts for TMS dienol ethers of 3β-hydroxy-5α-cholest-8(14)-en-15-one[a,b,c]

	5a $\Delta^{8(14)}$-15-one		**5b** $\Delta^{7,14}$-15-TMS		**5c** $\Delta^{8(14),15}$-15-TMS		**5d** $\Delta^{8,14}$-15-TMS	
Atom	δC	δH (α,β)	δC	δH (α,β)	δC	δH (α,β)	δC	δH (α,β)
1	36.69	1.17,1.66	36.99	1.01,1.78	36.56	1.11,1.65	35.84	*1.2*,1.79
2	31.48	1.71,1.40	31.86	1.66,1.44	31.80	1.71,1.41	32.12	1.73, *~1.5*
3	71.41	3.60	71.50	3.54	71.76	3.57	71.68	3.56
4	38.10	1.56,1.35	38.36	1.58,1.30	38.47	1.53,1.33	38.68	1.54,*1.4*
5	44.28	1.39	39.76	1.41	44.81	1.29	40.76	1.44
6	29.17	*1.32,1.45	30.49	*1.77,1.93	29.07	**1.30,1.37*	25.73	**1.33,1.42*
7	27.60	1.57,4.13[d]	123.32	5.84[e]	27.73	*1.6*,3.52[d]	28.77	*2.60,2.31
8	150.78		130.99		128.12		123.44	
9	50.89	1.84[f]	49.55	1.64	50.12	1.75	137.40	
10	38.76		33.89		37.60		36.47	
11	19.54	*1.53,1.65	20.86	**1.36*,1.56	19.88	*1.37,1.57	22.06	**2.08,2.16*
12	36.96	1.24,2.09	40.68	*1.26,1.98*	35.48	1.40,1.89	37.38	1.33,1.95
13	42.52		44.82		45.76		43.47	
14	140.17		123.70		140.53		122.67	
15	208.05		144.91		154.37		143.93	
16	42.46	*2.33[g],2.03[g]	38.50	*2.18[h],2.12[h]	112.40	4.95[i]	38.74	**2.22*[h],*2.18*[h]
17	50.77	1.46	54.69	1.51	56.90	1.95[j]	53.61	1.49
18	18.74	0.966	17.42	0.842	20.56	1.010	16.03	0.809
19	12.90	0.708	12.38	0.765	12.98	0.747	18.32	0.959
20	34.46	1.57	34.01	1.54	32.35	1.54	34.11	1.57
21	19.21	0.996[k]	19.08	0.911[k]	19.46	0.917[k]	19.11	0.922[k]
22	35.80	*1.06,1.36	36.12	*1.00,1.35	36.03	*1.12,1.42	36.10	**1.0,1.35*
23	23.47	*1.16,1.32	23.82	*1.09,1.37	23.85	**1.20,1.39*	23.77	**1.2,1.4*
24	39.34	1.13,1.13	39.46	1.14,1.14	39.45	1.15,1.15	39.47	1.14,1.14
25	27.94	1.52	27.98	1.53	27.99	1.52	28.00	1.53
26	22.52	0.862[l]	22.52	0.870[l]	22.53	0.865[l]	22.52	0.871[l]
27	22.72	0.862[l]	22.80	0.875[l]	22.76	0.865[l]	22.80	0.874[l]
3-OTMS	0.19	0.118	0.28	0.120	0.24[c]	0.119[c]	0.27	0.118
15-OTMS			0.58	0.166	−0.02[c]	0.211	0.81	0.188

[a]Spectra obtained in 0.05–0.10 M $CDCl_3$ solutions (in some cases containing pyridine and BSTFA byproducts in concentrations up to 0.02 M). Samples of **5b**, **5c**, and **5d** contained other dienol ethers and **5a** in minor (1–15%) amounts that did not interfere with spectral assignments.

[b]^1H NMR chemical shifts (±0.02 ppm for methylene protons) mainly from HETCOR spectra. Values in italics have a greater uncertainty (either weak HETCOR peaks or geminal protons of nearly identical chemical shift). Assignments of α and β protons marked with an asterisk may be interchanged.

[c]$Si(CH_3)_3$ assignments of **5c** uncertain because other peaks were present in the region near 0 ppm.

[d]H-7β, ddd, J = −14.5, 3.5, 2.1 Hz.

[e]ddd, J = 5.2, 2.5, 2.5 Hz.

[f]dd, J = 10.4, 7.2 Hz.

[g]ABX system: J_{AB} = −18.5 Hz, J_{AX} = 7.7 Hz, J_{BX} = 12.2 Hz.

[h]ABX system: J_{AB} = −15.3 Hz, J_{AX} = 7.5 Hz, J_{BX} = 9.5 Hz.

[i]d, J = 1.8 Hz.

[j]dd, J = 9.8, 2 Hz.

[k]d, J = 6.3 ± 0.3 Hz.

[l]d, J = 6.6 Hz.

silylation and GC–MS analysis (21, 22). In each case, complex chromatograms of little analytical value were obtained.[5] However, derivatization of 15-ketosterols as silyl dienol ethers addressed the above problems. The dehydrogenation artefact and unidentified byproducts were reduced to <1%. In addition, the GC retention times of the TMS dienol ether derivatives were considerably shorter than those of the 3-TMS ethers of $\Delta^{8(14)}$-15-ketosterols (Table 1), and the dienol ether peaks consequently appeared sharper and more symmetrical.

From investigating many sets of reaction conditions for preparing silyl dienol ethers, we established procedures (B, C, and D) that gave each of the bis-TMS ethers as the predominant product (Table 4). Thus, heating 15-ketosterol **1** overnight with BSTFA–pyridine (procedure B) yielded **5b** (subsequently identified as 3β,15-bis(trimethylsilyloxy)-5α-cholesta-7,14-diene) in >95% purity, whereas reaction at 22°C afforded **5a** (the 15-keto-3-TMS ether) in ≥99% purity. If the silylation reagent contained $HClO_4$, the room temperature reaction gave **5c** (the $\Delta^{8(14),15}$ dienol ether). Prolonged heating of either **5b** or **5c** at 100°C in BSTFA–pyridine–$HClO_4$ gave mainly the $\Delta^{8,14}$ dienol ether **5d**.

[5]In each case, the chromatograms consisted of multiple peaks, some of which were extremely broad and unresolved from other peaks. Mass spectra of both the narrow and broad peaks generally showed the molecular ions and fragmentation patterns expected for the dimethylhydrazone, oxime, and methoxime derivatives.

TABLE 3. Ion abundances in the mass spectra of bis-TMS dienol ethers of 3β-hydroxy-5α-cholest-8(14)-en-15-one (1) and its 3α isomer

Suggested Assignment[b]	m/z	5b $\Delta^{7,14}$	5c[a] $\Delta^{8(14),15}$	5d[a] $\Delta^{8,14}$	6b $\Delta^{7,14}$
M^+	544	71	14	100	45
$M-CH_3$	529	100	5	56	100
M-TMSO	455	4		2	
M-TMSOH	454	5			5
$M-TMSOH-CH_3$	439	10		18	9
M-SC	431	18	100	2	13
$M-Si(CH_3)_2=CH_2-TMSOH-CH_3$	367	5			4
M-TMSOH-SC	341	16	2	1	11
M-2TMSOH-SC	251	79	2	5	87

[a]Additional ions: **5c**, 418 (8), 417 (6), 345 (3), 233 (3) (corresponding ions in **5c**-d_{18} were at *m/z* 436, 435, 354, and 242); **5d**, 453 (2), 415 (2), 413 (2), 256 (2).

[b]Mass spectra of [2,2,3,4,4-^{2}H]-**5b**, and of **5b**-d_{18} and **5c**-d_{18} (prepared with BSA-d_{18}) were compatible with these assignments.

Using these procedures, we prepared the three bis-TMS ethers **5b, 5c**, and **5d** in sufficient quantity and purity for acquisition of NMR spectra, including ^{1}H, ^{13}C, DEPT, HETCOR, and long-range HETCOR spectra. The $\Delta^{8,14}$ ether **5d** was identified by its lack of ^{1}H olefinic signals, and dienol ethers **5b** ($\Delta^{7,14}$) and **5c** ($\Delta^{8(14),15}$) were differentiated by their C-10 chemical shifts, which are typically $\delta \sim 34$ for Δ^7 species and $\delta \sim 37$–38 for $\Delta^{8(14)}$ species (15, 18). The structure assignments for the dienol ethers were compatible with all of the NMR data.

The $\Delta^{7,14}$ and $\Delta^{8(14),15}$ dienol ethers may be useful in organic synthesis as a complement to the $\Delta^{8,14}$ dienol ethers, whose synthetic utility has been demonstrated previously (23–27). While our work was in progress, preparation of the trifluoromethanesulfonate ester of the $\Delta^{8,14}$-15-ol system was reported in the ergosterol series (25, 26). In that work, MNDO calculations of ΔH_f for three isomeric androstadienols showed the $\Delta^{8,14}$-15-ol species to be 3.3 kcal lower in enthalpy than the $\Delta^{7,14}$-15-ol and 13.0 kcal lower than the $\Delta^{8(14),15}$-15-ol species. These values are consistent with the observed conversion of both **5b** and **5c** to **5d**.

The mass spectra of TMS dienol ethers **5b, 5c** and **5d** are compared in Table 3. The suggested assignments were supported by the analogous fragmentation of the $\Delta^{7,14}$ dienol ethers **6b, 7b, 8b,** and **9b** and by the results of deuterium labeling experiments. The mass spectrum of $\Delta^{8(14),15}$ dienol ether **5c** was readily distinguished from those of the other two dienol ethers (**5b** and **5d**) by the high abundance of its M-SC ion (*m/z* 431, base peak), the lower intensities of its M^+ and $M-CH_3$ ions, and ions at *m/z* 418, 417, 345, and 233 not found in the other two

TABLE 4. Preparation of 15-TMS dienol ethers by treatment of 3β-hydroxy-5α-cholest-8(14)-en-15-one with BSTFA-pyridine under various conditions

Silylation Conditions				Composition of Products[a]			
Time	Temperature	$HClO_4$ Added[b]		$\Delta^{8(14)}$ 15-one **5a**	$\Delta^{7,14}$ 15-OTMS **5b**	$\Delta^{8(14),15}$ 15-OTMS **5c**	$\Delta^{8,14}$ 15-OTMS **5d**
h	°C						
20	22	none	(procedure A)	100[c]	0	0	0
20	100–110	none	(procedure B)	<1	95	4	<1
4	20	0.1%		2	4	85	9
20	22	0.1%	(procedure C)[d]	0–3	3–8	77–86	10–16
20	100	0.1%		0	18	39	44
96	100	0.1%		0	26	1	73

[a]Estimated from the total ion chromatogram; not corrected for the low GC-MS response of **5a**. Samples were either directly injected onto GC-MS or evaporated for 3 min at 30°C followed by dissolution in hexane and GC-MS analysis; $HClO_4$-containing samples that were evaporated under nitrogen at 40°C for 2 h showed increased amounts of **5a** and **5d** and lesser amounts of **5c**.

[b]Concentration (v/v) of $HClO_4$ in BSTFA-pyridine.

[c]Excluding variable amounts of the dehydrogenation artefact.

[d]Essentially similar results after 20 h at 80°C.

[e]Identical results after 144 h.

TABLE 5. Ion abundances in the mass spectra of tris-TMS dienol ethers of 3β,26-dihydroxy-5α-cholest-8(14)-en-15-one (**2**) 3β,25-dihydroxy-5α-cholest-8(14)-en-15-one (**4**), and their 3α epimers[a]

Suggested Assignment	*m/z*	**10b**[b] 3β,26 $\Delta^{7,14}$	**10c**[b] 3β,26 $\Delta^{8(14),15}$	**11b**[c] 3α,26 $\Delta^{7,14}$	**11c**[c] 3α,26 $\Delta^{8(14),15}$	**12b**[d] 3β,25 $\Delta^{7,14}$	**12c**[d] 3β,25 $\Delta^{8(14),15}$	**12d**[d] 3β,25 $\Delta^{8,14}$	**13b**[c] 3α,25 $\Delta^{7,14}$
M^+	632	100	16	100	18	83	25	69	95
$M-CH_3$	617	80	7	92	10	58	13	39	58
M-TMSO	543	3	2	3	1	3	3	5	
M-TMSOH	542	3	2	4	1	5	4	7	8
$M-TMSOH-CH_3$	527	3	0.4	6	1	10	2	19	17
$M-2TMSOH-CH_3$	437	2	1	4	0	3	1	0	7
M-SC	431	13	100	13	100	11	100	1	15
M-SC-13	418	1	5	1	9	1	8	1	
M-SC-14	417	1	4	1	6	1	5	0	
$M-3TMSOH-CH_3$	347	1	0	1	0	2	1	1	6
M-TMSOH-SC	341	8	2	13	3	10	3	0	5
M-2TMSOH-SC	251	20	2	58	3	23	2	4	28
M-ring A-C6-SC	233	2	3	2	5	2	5	2	
	143	8	3	10	2	5	2	15	
$C(CH_3)_2=OTMS^+$	131	5	1	3	1	100	50	100	100

[a]Most species also showed very minor ions at *m/z* 560 ($M-Si(CH_3)_2=CH_2$), 545 ($M-CH_3-Si(CH_3)_2=CH_2$), 470, and 452 (M-2TMSOH).
[b]Material derived from zone 2A, silylated by procedure B.
[c]Material derived from zone 3, silylated by procedure B.
[d]Material derived from zone 2B, silylated by procedure B.

TABLE 6. Mass spectral data for artefacts observed in the GC-MS analysis of $\Delta^{8(14)}$-15-ketosterols and for $\Delta^{6,8(14)}$-15-ketosterols of synthetic origin

Source of MS Data Suggested Assignment[d]	Artefact[a] 3β-OH	Artefact/Synthetic[b] 3β-OTMS	Artefact 3-H_2	Synthetic[c] 3-Keto	Artefact 3β,26-Diol
M^+	398 (75)	470 (16,10)	382 (67)	396 (26)	558 (35)
$M-CH_3$	383 (38)	455 (12,8)	367 (46)	381 (12)	543 (18)
M-ROH	380 (75)	380 (100,77)			468 (100)
$M-ROH-CH_3$	365 (67)	365 (83,100)	349 (4)	363 (5)	453 (49)
M-42	356 (12)		340 (8)	354 (12)	
M-C1...3-H	339 (10)	339 (31,27)		339 (5)	
M-C1...3-2H	338 (7)	338 (17,9)			426 (11)
M-SC	285 (35)	357 (7,12)	269 (35)	283 (23)	
M-SC-14	271 (10)			269 (17)	
M-ROH-SC	267 (32)	267 (70,36)		265 (7)	267 (32)
M-SC-C16...17	258 (100)	330 (25,19)	242 (100)	256 (100)	330 (15)
m/z 251	251 (17)	251 (3,2)			251 (10)
$M-ROH-H_2O-SC$	249 (10)	249 (5,3)			
M-ROH-SC-C16...17	240 (57)	240 (26,50)			240 (65)
M-SC-C15...17	230 (23)		214 (79)	228 (35)	
$M-ROH-SC-C16...17-CH_3$	225 (16)	225 (6,7)		225 (6)	225 (22)
$M-SC-C15...17-CH_3$	215 (15)		199 (59)	213 (14)	
M-ROH-SC-C15...17	212 (69)	212 (61,36)			212 (100)
$M-ROH-SC-C15...17-CH_3$	197 (33)	197 (17,12)			197 (22)
Other ions	253 (12)	352 (6,4)		323 (7)	195 (15)
	239 (16)	253 (8,6)		202 (14)	169 (14)
		239 (10,6)			
R =	H	TMS	H[e]	H[e]	TMS

[a]Similar MS data given in ref. 16 for 3β-hydroxy-5α-cholesta-6,8(14)-dien-15-one.
[b]Relative intensities of the artefact (t_R 12.60 min) associated with **5a** (t_R 12.28 min) and a sample of 3β-trimethylsilyloxy-5α-cholesta-6,8(14)-dien-15-one (t_R 12.63 min), respectively (GC-MS condition E).
[c]MS data for 5α-cholesta-6,8(14)-diene-3,15-dione (t_R 6.41 min); the artefact (t_R 6.40 min) associated with **8a** (t_R 6.29 min) had a similar mass spectrum (GC-MS condition F).
[d]Suggested assignments (unconfirmed by high resolution MS or labeling experiments): *C1...3-H*, radical fragment containing C-1, C-2, C-3, and any C-3 substituent; *SC-C16...17* and *SC-C15...17* are defined analogously.
[e]Suggested losses of water from keto groups.

dienol ethers. Although the mass spectra of $\Delta^{8,14}$ and $\Delta^{7,14}$ TMS dienol ethers were quite similar, they could be distinguished by their GC retention times and by the much higher abundance for the $\Delta^{7,14}$ ethers of ions due to losses of side chain (M-SC, M-TMSOH-SC, and M-TMSOH-TMSOH-SC).

All three dienol ethers had excellent GC and MS properties. Their mass spectra obtained under electron-impact conditions produced an abundant molecular ion, yet retained simple but informative fragmentation patterns. The GC peaks were narrow and symmetrical, and the sensitivity of detection by MS was improved relative to the $\Delta^{8(14)}$-15-ketosterol TMS derivatives. The three TMS dienol ethers were consistently eluted in the order $\Delta^{7,14}$, $\Delta^{8,14}$, and $\Delta^{8(14),15}$ (Table 1).[6] This elution order was very helpful in the identification of minor GC-MS components in complex chromatograms of biological samples.

The utility of this derivatization method is illustrated for the analysis of metabolites obtained after incubation of **1** with rat liver mitochondria in the presence of NADPH. In contrast to previous work (10), the 15-ketosterols were usually derivatized here by procedure B, under which conditions tertiary alcohols were also silylated. This convenient procedure typically gave the $\Delta^{7,14}$ dienol ethers in ~95% purity and only a small amount of the $\Delta^{8(14),15}$ dienol ethers.

A significant concern in assessing the purity of **1** and its metabolites was the differentiation of minor metabolites from artefacts of derivatization and chromatography. Derivatization of **1** as its mono-TMS ether **5a** followed by GC-MS analysis sometimes showed small but variable amounts of an apparent artefact with a slightly longer retention time and a molecular ion 2 amu lower than that of **5a**. This artefact had the same GC-MS as the mono-TMS ether of authentic 3β-hydroxy-5α-cholesta-6,8(14)-dien-15-one (16) and may be formed by dehydrogenation of **1** in the injection port of the gas chromatograph. Other $\Delta^{8(14)}$-15-ketosterols also showed such dehydrogenation artefacts having analogous mass spectra. Various $\Delta^{6,8(14)}$-15-ketosterol standards that were available had retention times and mass spectra similar to those of the artefacts as illustrated in Table 6. Nevertheless, alternative double bond positions could not be ruled out by the observed fragmentation patterns.

When silyl dienol ether derivatives of **1** and its analogs were prepared under stronger silylation conditions, this type of artefact was not observed. Whereas GC-MS analysis of the bis-TMS ether **10a** (prepared by procedure A) showed two minor components of 5% each, silylation at 110°C transformed bis-TMS ether **10a** into two tris-TMS ethers **10b** and **10c** accounting for 98% of the material (Table 1). Under these conditions, only one unidentified trace component was present, a result suggesting that the two 5% components from room temperature silylation may represent artefacts.

The improved GC resolution obtained with the TMS dienol ethers permitted detection of several minor components in samples of rat liver mitochondrial metabolites. For example, the $\Delta^{7,14}$ and $\Delta^{8(14),15}$ tris-TMS ether derivatives of the 3α,26- and 3β,24-diols (zone 3) were easily resolved on both Rt_x-20 and DB-5 capillary columns, whereas we were unable to resolve their bis-TMS ether derivatives (10). Also, the previously undetected 3α,25-diol was resolved as its tris-TMS ether and identified on the basis of its short retention time (and lower polarity on TLC) and by the similarity of its mass spectrum with that of the corresponding 3β,25-diol derivative.

The present investigation illustrates the importance of a thorough understanding of the derivatization of the 15-ketosterol and its metabolites for use in studies involving their quantitation and characterization. Silylation under mild conditions leads to derivatization of unhindered primary and secondary alcohol functions, but tertiary hydroxyl groups (such as that of C-25) are at most partially silylated. Since complete silylation of all hydroxyl groups in the side chain of metabolites of **1** is critical to their characterization by GC-MS, forcing conditions must be employed for complete derivatization. However, silylation of **1** (and of its metabolites containing the $\Delta^{8(14)}$-15-keto system) under forcing conditions can lead to the formation of three isomeric dienol ethers that are separable from each other and from the TMS ether derivative formed under mild conditions. Failure to recognize the formation of the dienol ethers could lead to a misinterpretation of the nature and number of compounds under study. Similar considerations may apply to Δ^5-7-ketosterols and other α,β-unsaturated ketosterols.

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[6]A different retention order was observed for the ethyl dienol ethers of **1** (prepared by treating **1** with $HC(OCH_2CH_3)_3$ in presence of an acid catalyst (23) followed by silylation with BSTFA). Their mass spectra showed numerous intense fragment ions, including ions analogous to those of TMS dienol ethers.

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