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Capillary gas chromatographic behaviour of *tert*.-hydroxylated steroids by trialkylsilylation

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Abstract

Capillary gas chromatographic behaviour was studied for a variety of structurally different bile acids and sterols having one to two *tert*.-hydroxy groups, together with several *sec*.-hydroxy groups, at positions C-3, -5, -7, -12, -14, -17, -20, -24, and/or -25. The *tert*.-hydroxylated steroids were subjected to trimethylsilylation with hexamethyldisilazane/trimethylchlorosilane/pyridine and *N,O*-bis(trimethylsilyl)acetamide/*N*-trimethylsilylimidazole/trimethylchlorosilane, and dimethylethylsilylation with *N,N*,-dimethylethylsilylimidazole. The methylene unit values of the resulting trialkylsilylation products were used for determining their structures of partially and/or fully derivatised ethers. The reactivity of the trialkylsilylation of *tert*.-hydroxy groups was found to be significantly dependent not only on the derivatisation reagents and conditions used, but also on the position and steric factor of the *tert*.-hydroxy groups. The following general order of the decreasing reactivity of *tert*.-hydroxy groups in steroids by trialkylsilyl etherification was observed: 25>20, 24>5β>17α>>14α.

Keywords: Derivatisation, GC; Steroids, *tert*.-hydroxylated; Bile acids; Sterols

1. Introduction

Of the various chromatographic methods available, capillary gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) are now well normalized as one of the most powerful tools for the profile analysis of complicated mixtures of steroids in human biological materials and in naturally occurring products, because of high sensitivity and selectivity as well as excellent resolution efficiency. Hence, a number of separation methods applying GC have been reported for a series of steroids. Prior to capillary GC and GC–MS analyses, hydroxylated steroids are conventionally transformed into suitable derivatives for improvement of peak shape, resolution and sensitivity [1,2]. In particular, trialkylsilyl ether derivatives such as trimethylsilyl (TMS) and dimethylethylsilyl (DMES) ethers have been extensively used as derivatives suitable for the capillary GC and GC–MS determination and separation of closely related bile acids and sterols [3–7]. It is also well normalized that under mild conditions, primary- (*prim*.-) and secondary- (*sec*.-) hydroxy groups present in steroid molecules are readily

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derivatised into their complete persilylated derivatives without difficulty.

Although numerous studies on GC separation and determination of hydroxylated steroids have been reported as their TMS or DMES ether derivatives, they are almost exclusively concerned with compounds having only *prim*.- and *sec*.-hydroxy groups. Analogous GC studies on bioactive steroids having tertiary- (*tert*.-) hydroxy groups, along with *sec*.-hydroxy groups, are limited to some ecdysteroids [8–11], probably owing to the unavailability of authentic reference compounds. To our knowledge, detailed and systematic study on the reactivity of *tert*.-hydroxy groups in steroids by trialkylsilylation has not yet been reported, and, therefore, the reactivity of *tert*.-hydroxylated steroids with trialkylsilylating reagents remains unclear.

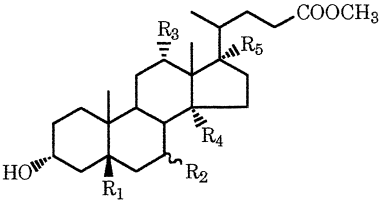
As part of our program on potential steroid metabolites, we have recently synthesized a variety of novel bile acids and sterols, which possess one or two *tert*.-hydroxy groups at positions C-5, -14, -17, -20, -24 and/or -25, together with *sec*.-hydroxy groups at C-3, -7 and/or -12 [12]. In this paper, we examined the capillary GC behaviour of these *tert*.-hydroxylated steroids by derivatisation to TMS and DMES ethers.

2. Experimental

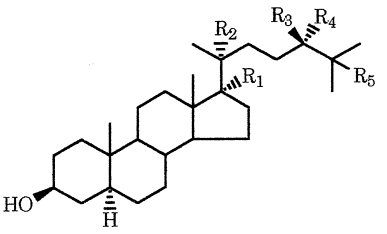
2.1. Materials and reagents

Fig. 1 shows the chemical structures of 22 steroids having one to four hydroxy groups, related to bile acids (5β-cholane series; A/B-ring fusion, *cis*) and sterols (5α-cholestane series; A/B-ring fusion, *trans*) examined in this study, and their abbreviations used are as follows:

- 3α-hydroxy-5β-cholan-24-oic acid (LCA), 3α-OH (**1**);
- 3α,7α-dihydroxy-5β-cholan-24-oic acid (CDCA), 3α,7α-$(OH)_2$ (**2**);



	R_1	R_2	R_3	R_4	R_5
1	H	H	H	H	H
2	H	α-OH	H	H	H
3	H	β-OH	H	H	H
4	H	H	OH	H	H
5	H	α-OH	OH	H	H
6	OH	H	H	H	H
7	OH	α-OH	H	H	H
8	OH	β-OH	H	H	H
9	OH	H	OH	H	H
10	OH	α-OH	OH	H	H
11	H	β-OH	H	OH	H
12	H	α-OH	H	H	OH
13	H	β-OH	H	H	OH
14	OH	α-OH	H	H	OH



	R_1	R_2	R_3	R_4	R_5
15	H	H	H	H	H
16	H	H	C_2H_5	H	H
17	H	H	C_2H_5	OH	H
18	H	H	H	H	OH
19	H	H	C_2H_5	H	OH
20	OH	H	H	H	OH
21	OH	H	C_2H_5	H	OH
22	H	OH	H	H	OH

Fig. 1. Structures of compounds examined.

- 3α,7β-dihydroxy-5β-cholan-24-oic acid (UDCA), 3α,7β-$(OH)_2$ (**3**);
- 3α,12α-dihydroxy-5β-cholan-24-oic acid (DCA), 3α,12α-$(OH)_2$ (**4**);
- 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid (CA), 3α,7α,12α-$(OH)_3$ (**5**);
- 3α,5β-dihydroxy-5β-cholan-24-oic acid, 3α,5β-$(OH)_2$ (**6**);
- 3α,5β,7α-trihydroxy-5β-cholan-24-oic acid, 3α,5β,7α-$(OH)_3$ (**7**);
- 3α,5β,7β-trihydroxy-5β-cholan-24-oic acid, 3α,5β,7β-$(OH)_3$ (**8**);
- 3α,5β,12α-trihydroxy-5β-cholan-24-oic acid, 3α,5β,12α-$(OH)_3$ (**9**);
- 3α,5β,7α,12α - tetrahydroxy - 5β - cholan - 24 - oic acid, 3α,5β,7α,12α-$(OH)_4$ (**10**);
- 3α,7β,14α-trihydroxy-5β-cholan-24-oic acid, 3α,7β,14α-$(OH)_3$ (**11**);
- 3α,7α,17α-trihydroxy-5β-cholan-24-oic acid, 3α,7α,17α-$(OH)_3$ (**12**);
- 3α,7β,17α-trihydroxy-5β-cholan-24-oic acid, 3α,7β,17α-$(OH)_3$ (**13**);
- 3α,5β,7α,17α - tetrahydroxy - 5β - cholan - 24 - oic acid, 3α,5β,7α,17α-$(OH)_4$ (**14**);
- 5α-cholestan-3β-ol (dihydrocholesterol), 3β-OH (**15**);
- (24*R*)-24-ethyl-5α-cholestan-3β-ol (stigmastanol), 24-Et-3β-OH (**16**);
- (24*S*)-24-ethyl-5α-cholestan-3β,24-diol, 24-Et-3β,24-$(OH)_2$ (**17**);
- 5α-cholestan-3β,25-diol, 3β,25-$(OH)_2$ (**18**);
- (24*R*)-24-ethyl-5α-cholestan-3β,25-diol, 24-Et-3β,25-$(OH)_2$ (**19**);
- 5α-cholestan-3β,17α,25-triol, 3β,17α,25-$(OH)_3$ (**20**);
- (24*R*)-24-ethyl-5α-cholestan-3β,17α,25-triol, 24-Et-3β,17α,25-$(OH)_3$ (**21**);
- (20*S*)-5α-cholestan-3β,20,25-triol, 3β,20,25-$(OH)_3$ (**22**).

LCA (**1**), DCA (**4**) and CA (**5**) were purchased from Wako (Osaka, Japan). CDCA (**2**) and UDCA (**3**) were kindly donated by Tokyo Tanabe (Tokyo, Japan). Dihydrocholesterol (**15**) and stigmastanol (**16**) were available from Sigma (St. Louis, MO, USA). The remaining hydroxylated steroids (**6**–**14** and **17**–**22**) having one or two *tert*.-hydroxy groups were synthesized recently in our laboratories [12].

Hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS), *N*-trimethylsilylimidazole (TMSI), *N,N*-dimethylethylsilylimidazole (DMESI), and *N,O*-bis(trimethylsilyl) acetamide (BSA) were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). A mixture of even carbon numbers of C_{20}–C_{38} *n*-alkanes and *n*-dotriacontane (C_{32}) as an internal standard were available from GL Science (Tokyo, Japan). All reagents and solvents used were of analytical reagent grade.

2.2. GC derivatisation of bile acids and sterols

Bile acid samples (**1**–**14**) were first converted into the C-24 methyl esters. Each of the bile acid methyl esters and sterols (**15**–**22**) was then subjected to the following three variants of trialkylsilyl ether derivatisation (two TMS and one DMES) prior to GC analysis.

2.2.1. Methyl esterification of bile acids with diazomethane

For bile acid samples **1**–**14**, a carboxyl group at C-24 was methylated with a solution of diazomethane in ethyl ether, which was generated by a Wheaton generator using *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine and potassium hydroxide as described in a previous paper [13].

2.2.2. Derivatisation to TMS ethers with a mixture of HMDS/TMCS/pyridine

To a steroid sample (~0.2 mg) in 50 μl of dry pyridine was added 100 μl of HMDS and 30 μl of TMCS in pyridine solution, and the mixture was left to stand at room temperature for 1 h. An aliquot of the derivatised sample solution diluted with benzene (200 μl) was injected into the GC together with C_{32}.

2.2.3. Derivatisation to TMS ethers with a mixture of BSA/TMSI/TMCS

To a steroid sample (~0.2 mg) were added 20 μl of TMCS, 20 μl of TMSI and then 20 μl of BSA, and the mixture was heated at 100°C for 1 h. After the reaction, an aliquot of the derivatised solution diluted with benzene (100 μl) was injected into the GC together with C_{32}.

2.2.4. Derivatisation to DMES ethers with DMESI

To a steroid sample (~0.2 mg) was added 50 μl of DMESI, and the mixture was heated at 100°C for 1

h. After the reaction, an aliquot of the derivatised solution diluted with benzene (100 μl) was injected into the GC together with C_{32}.

2.3. Gas chromatography

A Model GC-17A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a data processing system (Chromatopac C-R6A) was employed. It was fitted with a cross-linked 5% phenylpolysiloxane fused-silica capillary column (25 m×0.32 mm I.D.; film thickness, 0.25 μm) (SGE Japan, Yokohama) and operated under the following conditions: linear velocity of carrier gas (helium), 30 cm/s.; make-up gas flow-rate, 60 ml/min; splitting ratio, 1:50; injection port, detector and column oven were maintained at 300, 300 and 280°C, respectively.

2.4. Gas chromatography–mass spectrometry

Electron-impact mass spectra were obtained on a JEOL Model JMC-Automass 150 quadrupole mass spectrometer (Tokyo, Japan) interfaced to a Hewlett-Packard Model 5890 Series III gas chromatograph. A cross-linked dimethylpolysiloxane DB-1 fused-silica capillary column (30 m×0.25 mm I.D.; film thickness, 0.25 μm) (J&W Scientific, Folsom, CA, USA) was inserted into the ion source through the direct inlet and operated under the following conditions: ionization energy, 70 eV; emission current, 300 μA; mass range, 4–1000 a.m.u.; the injection port, column oven and ion source were kept at 280, 300 and 180°C, respectively.

3. Results and discussion

It is generally accepted that the reactivity of hydroxy groups in steroids with trialkylsilylating reagents depends significantly not only on *prim*.-, *sec*.- and *tert*.-hydroxy groups, but also on their position and the steric environment [14]. For instance, the reactivity usually decreases in the order of *prim*.-, *sec*.- and *tert*.-hydroxy groups under the same derivatising conditions. In addition, the following order of the decreasing reactivity of hydroxy groups with TMSI has been reported for the position of ecdysteroids: 2, 3, 22, 25>20>>14 [8].

Of the various GC and GC–MS methods for derivatisation of hydroxylated steroids, trimethyl- and dimethylethylsilylations were chosen, because they react completely with *prim*.- and *sec*.-hydroxy groups of steroids under mild conditions without concomitant formation of by-products and are widely employed in routine analysis [1,2]. A mixed HMDS/TMCS/pyridine system is a typical TMS derivatisation reagent, which is commonly used for steroids having only *prim*.- and *sec*.-hydroxy groups [3,4]. BSA/TMSI/TMCS mixture is known as the most powerful reagent which silylates a highly sterically hindered and/or a less reactive *tert*.-hydroxy group in steroids [14]. Meanwhile, the use of heavier DMES ethers, instead of TMS ethers, provides an excellent resolution of a complex mixture of mono-, di-, and trihydroxylated stereoisomers possessing *sec*.-hydroxy groups [3]. Simultaneous GC analysis of the TMS and DMES ethers of the same compounds, therefore, affords useful information about the number of hydroxy groups present in the molecules [3,4]. Therefore, compounds **1**–**22** were subjected to the three types of trialkylsilylation (two TMS and one DMES) by the derivatisation reagents and procedures described in the Experimental section.

Table 1 compiles the retention data for the GC peaks of all the compounds (**1**–**22**) examined as their trialkylsilylation by HMDS/TMCS/pyridine, BSA/TMSI/TMCS, and DMESI. The retention data were expressed in terms of the relative retention times (RRT; relative to C_{32}) and the methylene unit (MU) values. The MU values were determined by using a mixture of C_{20}–C_{38} *n*-alkanes [3,4]. As expected, reaction of compounds **1**–**5**, **15** and **16**, having one to three *sec*.-hydroxy groups, with HMDS/TMCS/pyridine, BSA/TMSI/TMCS, or DMESI gave, without any difficulty, the completely silylated derivatives cleanly in every case. However, when the procedures were applied to *tert*.-hydroxylated steroids (**6**–**14** and **17**–**22**), the reaction products were significantly influenced by the position and/or steric factor of the *tert*.-hydroxy groups, as well as the silylating reagents employed, to give the corresponding fully or partially silylated derivatives and/

Table 1
Retention data for *tert*.-hydroxylated bile acids and sterols by trialkylsilylation

Steroid		Trimethylsilylation				Dimethylethylsilylation	
		HMDS/TMCS/pyridine		BSA/TMSI/TMCS		DMESI	
		RRT[a]	MU	RRT[a]	MU	RRT[a]	MU
5β-Cholane series							
1	3α-OH	0.98	32.04	0.98	31.98	1.31	33.21
2	3α,7α-$(OH)_2$	1.07	32.40	1.07	32.35	1.80	34.49
3	3α,7α-$(OH)_2$	1.20	32.87	1.21	32.82	2.04	34.98
4	3α,12α-$(OH)_2$	1.00	32.12	1.00	32.07	1.63	34.10
5	3α,7α,12α-$(OH)_3$	1.02	32.19	1.02	32.14	2.19	35.26
6	3α,5β-$(OH)_2$	1.45*	33.58*	1.36	33.28	1.93*	34.67*
7	3α,5β,7α-$(OH)_3$	1.53*	33.80*	1.23	32.89	2.56*	35.82*
8	3α,5β,7β-$(OH)_3$	1.65*	34.06*	1.42	33.47	2.75*	36.10*
9	3α,5β,12α-$(OH)_3$	1.46*	33.58*	1.33	33.22	2.38*	35.51*
10	3α,5β,7α,12α-$(OH)_4$	1.45*	33.57*	1.13	32.56	3.13*	36.63*
11	3α,7β,14α-$(OH)_3$	0.95*	31.88*	0.95*	31.84*	1.59*	33.92*
12	3α,7α,17α-$(OH)_3$	1.59*	33.91*	1.45/1.59*	33.56/33.90*	2.66*	35.98*
13	3α,7β,17α-$(OH)_3$	1.71/1.83*	34.19/34.48*	1.70	34.18	2.97*	36.20*
14	3α,5β,7α,17α-$(OH)_4$	2.24*	35.29*	1.63/1.77*	34.01/34.35*	3.73*	37.33*
5α-Cholestane series							
15	3β-OH	0.91	31.75	0.91	31.69	1.24	32.94
16	24-Et-3β-OH	1.43	33.48	1.43	33.49	1.95	34.73
17	24-Et-3β,24-$(OH)_2$	2.49	35.74	2.50	35.72	3.35*	36.90*
18	3β,25-$(OH)_2$	1.67	34.09	1.67	34.11	3.05	36.52
19	24-Et-3β,25-$(OH)_2$	2.44	35.67	2.44	35.63	4.49	38.05
20	3β,17α,25-$(OH)_3$	2.70*	36.04*	1.82/2.73*	34.46/36.08*	5.01*	38.53*
21	24-Et-3β,17α,25-$(OH)_3$	3.84*	37.43*	2.57/3.87*	35.82/37.47*	7.05*	39.88*
22	3β,20,25-$(OH)_3$	2.10	35.02	2.10	35.03	4.44*	38.04*

[a] Retention times relative to C_{32}; an asterisk indicates the partially silylated product.

or their mixtures, as evidenced by GC–MS analysis (Table 2).

In order to clarify the retention behaviour of *tert*.-hydroxylated steroids, Fig. 2 shows the differences in the MU values (ΔMU) for the two variants of the trimethylsilylation products for the same compounds by HMDS/TMCS/pyridine and BSA/TMSI/TMCS. All of the *sec*.-hydroxylated compounds (**1**–**5**, **15** and **16**) and some of the *tert*.-hydroxylated ones (e.g. **17**–**19** and **22**) had ΔMU values of nearly zero, thus indicating the formation of the same silylated products. However, the other *tert*.-hydroxylated compounds (e.g. **6**–**14**) produced one or two mixed GC peaks with different MU values.

Changes in the MU values caused by different trialkylsilylation procedures were also used for the structural determination of the resulting derivatised products. Our previous study [3] revealed that in a variety of hydroxylated bile acids having only *sec*.-hydroxy groups, a plot of the $\Delta[Um]_{D\text{-}T}$ values (the differences in the MU values between the fully derivatised TMS and DMES ethers for the same compounds) versus the number of hydroxy groups shows a good linearity, indicating that the addition of hydroxy group produces consistent increases (~1 U per hydroxy group) in the $\Delta[Um]_{D\text{-}T}$ values. As the slope of the linear regression line are essentially independent of the other structural characteristics, the deviation of the $\Delta[Um]_{D\text{-}T}$ values of an unknown steroid from the regression line affords useful information not only for estimating the total number of hydroxy groups in the molecules, but also for elucidating the presence of an underivatised *tert*.-hydroxy group.

As expected, plots of the $\Delta[Um]_{D\text{-}T}$ values versus the number of hydroxy groups obtainable from *sec*.-hydroxylated steroids (**1**–**5**, **15** and **16**) by two different trimethylsilylating reagents also gave simi-

Table 2
Gas chromatographic–mass spectrometric data for the TMS and DMES ether derivatives of *tert*.-hydroxylated bile acids and sterols

Steroid	Derivative formed	Major ions above *m*/*z* 200 (relative intensity, %)[a]
5β-Cholane series		
3α,7α-$(OH)_2$	3,7-bisTMS	550 (M, <1%), 460 (M-TMSOH, 11%), 370 (M-2TMSOH, 59%), 355 (M-2TMSOH-CH_3, 96%), 262 (100%)
	3,7-bisDMES	578 (M, <1%), 549 (M-C_2H_5, 4%), 445 (M-DMESOH-C_2H_5, 100%), 370 (M-2DMESOH, 69%), 255 (M-2DMESOH-S.C., 56%)
3α,5β,7α-$(OH)_3$	3,7-bisTMS-5-OH	458 (M-TMSOH-H_2O, 100%), 331 (59%)
	3,5,7-trisTMS	638 (M, <1%), 548 (M-TMSOH, 1%), 458 (M-2TMSOH, 100%), 368 (M-3TMSOH, 11%), 331 (76%)
	3,7-bisDMES-5-OH	472 (M-DMESOH-H_2O, 100%), 368 (M-2DMESOH-H_2O, 7%), 253 (M-2DMESOH-H_2O-S.C., 11%)
3α,7β,14α-$(OH)_3$	3,7-bisTMS-14-OH	533 (M-H_2O-CH_3, 3%), 368 (M-2TMSOH-H_2O, 68%), 353 (M-2TMSOH-H_2O-CH_3, 41%), 253 (M-2TMSOH-H_2O-S.C., 86%), 239 (100%)
	3,7-bisDMES-14-OH	547 (M-H_2O-C_2H_5, 2%), 368 (M-2DMESOH-H_2O, 28%), 353 (M-2DMESOH-H_2O-CH_3, 16%), 253 (M-2DMESOH-H_2O-S.C., 100%)
3α,7α,17α-$(OH)_3$	3,7-bisTMS-17-OH	566 (M, <1%), 429 (M-TMSOH-H_2O-2CH_3+H, 12%), 354 (M-2TMSOH-H_2O-CH_3+H, 34%), 253 (M-2TMSOH-H_2O-S.C., 100%)
	3,7,17-trisTMS	638 (M, <1%), 548 (M-TMSOH, 2%), 393 (M-S.C.-ring D, 7%), 368 (M-3TMSOH, 3%), 343 (M-2TMSOH-S.C., 7%), 211 (100%)
	3,7-bisDMES-17-OH	490 (M-DMESOH, <1%), 421 (M-S.C.-ring D, 4%), 368 (M-2DMESOH-H_2O, 11%), 228 (M-2DMESOH-S.C.-part of ringD, 100%)
5α-Cholestanseries		
3β-OH	3-TMS	460 (M, 13%), 445 (M-CH_3, 16%), 370 (M-TMSOH, 17%), 355 (M-TMSOH-CH_3, 36%), 305 (22%), 215 (M-TMSOH-S.C.-ring D, 100%)
	3-DMES	474 (M, <1%), 445 (M-C_2H_5, 100%), 370 (M-DMESOH, 18%), 215 (M-DMESOH-S.C.-ring D, 10%)
24-Et-3β,24-$(OH)_2$	3,24-bisTMS	576 (M, 1%), 561 (M-CH_3, 15%), 547 (M-C_2H_5, 58%), 533 (100%), 353 (33%)
	3-DMES-24-OH	518 (M, <1%), 500 (M-H_2O, 10%), 489 (M-C_2H_5, 59%), 402 (100%), 257 (M-DMESOH-S.C., 98%), 215 (M-DMESOH-S.C.-ring D, 77%)
3β,25-$(OH)_2$	3,25-bisTMS	548(M,6%),533(M-CH_3,73%),458(M-TMSOH,44%),353(M-2TMSOH-CH_3,51%),345(M-S.C.,78%),215(M-TMSOH-S.C.-ringD,100%)
	3,25-bisDMES	576 (M, 2%), 561 (M-CH_3, 18%), 547 (M-C_2H_5, 100%), 472 (M-DMESOH, 16%), 368 (M-2DMESOH, 21%), 255 (35%), 207 (98%)
3β,17α,25-$(OH)_3$	3,25-bisTMS-17-OH	564 (M, 2%), 549 (M-CH_3, 11%), 363 (M-S.C., 49%), 345 (M-S.C.-CH_3, 46%), 255 (M-TMSOH-H_2O-S.C., 100%)
	3,17,25-trisTMS	636 (M, 2%), 490 (16%), 435 (M-S.C., 94%), 345 (M-TMSOH-S.C., 13%), 255 (M-2TMSOH-S.C., 48%), 239 (100%)
	3,25-bisDMES-17-OH	577 (M-CH_3, <1%), 455 (M-DMESOH-H_2O-CH_3, 28%), 377 (M-S.C., 58%), 359 (M-H_2O-S.C., 83%), 255 (M-DMESOH-H_2O-S.C., 100%)
3β,20,25-$(OH)_3$	3,20,25-trisTMS	636 (M, <1%), 621 (M-CH_3, 5%), 531 (M-TMSOH-CH_3, 8%), 463 (90%), 373 (36%), 345 (M-S.C., 30%), 283 (100%)
	3,25-bisDMES-20-OH	577 (M-CH_3, 3%), 563 (M-C_2H_5, 9%), 470 (M-DMESOH-H_2O, 15%), 359 (36%), 257 (M-DMESOH-S.C., 55%), 203 (100%)

[a] M, molecular ion; TMSOH, trimethylsilanol; DMESOH, dimethylethylsilanol; S.C., side chain.

lar regression lines with nearly identical slopes and intercepts, both of which showed that the addition of one hydroxy group produces ~1-U increase in the $\Delta[Um]_{D\text{-}T}$ values, corresponding to the mass difference of 14 between the DMES and TMS ethers (Fig. 3). Otherwise, if the $\Delta[Um]_{D\text{-}T}$ values of unknown steroids are deviated from the regression lines, they contain less reactive and sterically hindered *tert*.-hydroxy groups to give partially silylated derivatives. The $\Delta[Um]_{D\text{-}T}$ values of a number of *tert*.-hydroxylated steroids examined in this study deviated from the completely derivatised regression lines.

Under the derivatisation procedures used, all of the less hindered *sec*.-hydroxy groups (i.e. 3α, 3β, 7α, 7β, and 12α) in compounds **1**–**22** were rapidly silylated without difficulty and selectivity as has been reported previously [3]. Thus, each derivatisation product to the TMS and DMES ethers of common bile acids (**1**–**5**) and sterols (**13** and **14**) having only *sec*.-hydroxy groups gave a single peak corresponding to the respective completely silylated derivatives. However, the trialkylsilylation of *tert*.-hydroxy groups depends significantly on the silylating reagents and conditions employed to give fully or partially derivatised products.

The trimethylsilylation of 5β-hydroxylated compounds (**6**–**10**) under mild derivatisation conditions (at room temperature for 1 h) with HMDS/TMCS/pyridine gave a single GC peak, but it resulted in incomplete silylation to produce the corresponding 5β-hydroxy-TMS ethers as evidenced by GC–MS. A similar result was also observed for the DMES etherification products with DMESI (at 100°C for 1 h), in which the respective products were identified as the partially derivatised 5β-hydroxy-DMES ethers. On the other hand, when the compounds **6**–**10** were subjected to trimethysilylation with BSA/TMSI/TMCS (at 100°C for 1 h), each of the resulting products had shorter retention times than that observed with HMDS/TMCS/pyridine system, corresponding to the completely derivatised TMS ethers from their GC–MS analyses.

Each of the trimethylsilylation products of compounds (**18** and **19**) having a *tert*.-hydroxy group at the C-25 terminal alkyl side-chain, with two different trimethylsilylating reagents, gave a single GC peak

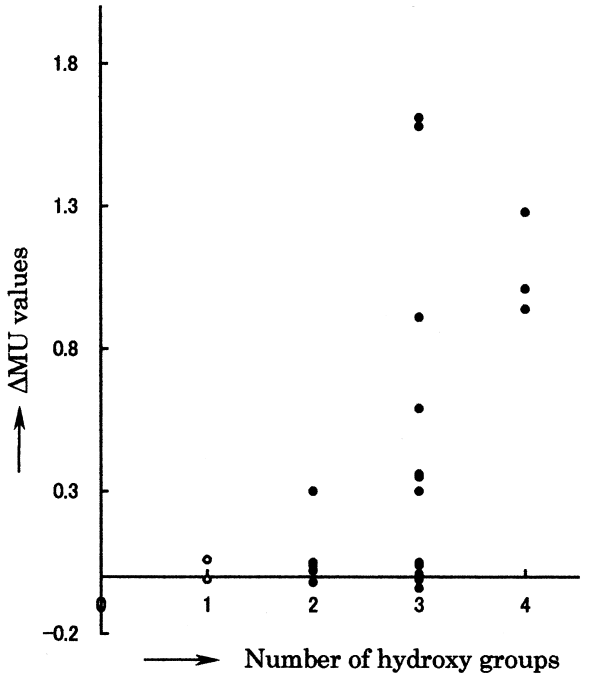


Fig. 2. Relationship between ΔMU values and number of hydroxy groups in *sec*.- and *tert*.-hydroxylated steroids. Open circles show the ΔMU values for steroids having only *sec*.-hydroxy groups.

with an identical retention time, corresponding to their fully derivatised 3β,25-bis(trimethylsiloxy) ethers by GC–MS analysis. The dimethylethylsilylation of these compounds with DMESI also afforded the corresponding persilylated derivatives with longer retention times. Analogously, 20- and 24-*tert*.-hydroxylated steroids (**17** and **22**) were trimethylsilylated completely with HMDS/TMCS/pyridine or with BSA/TMSI/TMCS to give the corresponding persilylated derivatives. On the other hand, the reaction with DMESI failed in producing the corresponding complete 3β,24-bis- and 3β,20,25-tri(dimethylethylsiloxy) ethers and yielded the incomplete 20- and 24-hydroxy-DMES ethers, probably due to the steric hindrance of a bulky ethyl group in DMESI.

We have found that 17α-hydroxylated compounds (**12**–**14**, **20** and **21**) with HMDS/TMCS/pyridine (or DMESI) usually yielded a single GC peak corresponding to the partially derivatised 17α-hydroxy-TMS (or DMES) ethers. However, the silylation of methyl 3α,7β,17α-trihydroxy-5β-cholan-24-oate (**13**) produced two mixed TMS peaks; one of the peaks with a somewhat longer retention time (MU, 34.48) was identified as the incomplete methyl 17α-hydroxy - 3α,7β - bis(trimethylsiloxy) - 5β - cholan - 24-oate by GC–MS analysis and the other (MU, 34.19) with a shorter retention time to the completely derivatised methyl 3α,7β,17α-tris(trimethylsiloxy)-5β-cholan-24-oate. Although the complete derivatisation to the TMS ether of **13** was attained by using BSA/TMSI/TMCS mixture as a silylating reagent, the remaining 17α-hydroxylated compounds resulted in the formation of a mixture of partially and fully silylated steroids under the same conditions. For example, a GC peak (MU, 33.56) eluting first in 3α,7α,17α-triol (**12**) was identified as the fully silylated derivative, while a second peak with MU value of 33.90 was assigned to the methyl 17α-hydroxy - 3α,7α - bis(trimethylsiloxy) - 5β - cholan - 24-oate. Thus, most 17α-hydroxylated steroids were not completely silylated, even though they were treated with the most powerful silylating reagent. According to a previous finding of Morgan and Poole [8], heating 3α,17α,20-trihydroxy-5β-pregnane overnight at 140°C in TMSI afforded the completely silylated ether. However, when **12** was treated with TMSI at 140°C for 12 h, the resulting product still gave a mixture of the two components mentioned above, suggesting that the bulky side chain at C-17 enhances steric hindrance of the 17α-hydroxy group.

Others have pointed out that a highly hindered 14α-hydroxy group in ecdysteroids is silylated with TMSI at 100°C for 15 h [10,11], TMSI/TMCS at 100°C for 4 h [8] or TMSI/TMCS in the presence of solid potassium acetate as a catalyst at room temperature for several hours [8,9]. In methyl 3α,7β,14α-trihydroxy-5β-cholan-24-oate (**11**), however, a 14α-hydroxy group was found to be completely inert against the silylating reagents and derivatising conditions used in this study as well as the reported methods [8–11] to produce the corresponding methyl 14α-hydroxy-bis(trimethylsiloxy)-5β-cholan-24-oate [or bis(dimethylethylsiloxy)]. Again, steric environment of the 14α-hydroxy group in **11** may also contribute to weakening the reactivity.

In conclusion, the reactivity of *tert*.-hydroxy groups in bile acids and sterols with silylating reagents and condition employed was decreased in

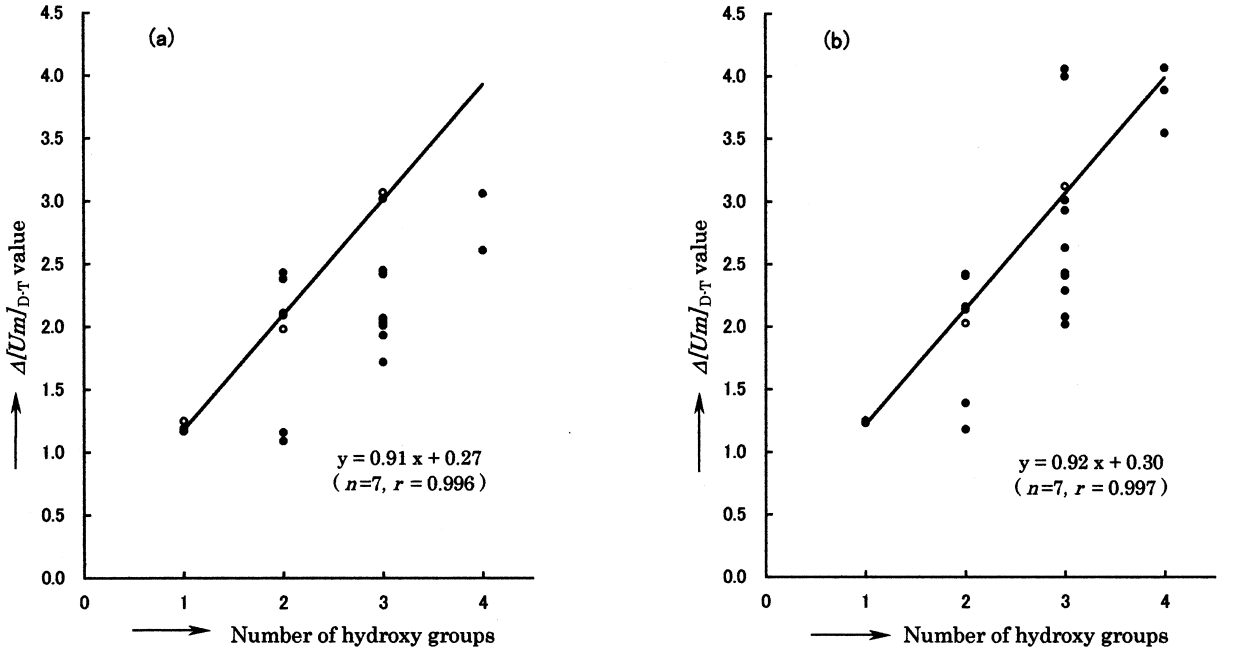


Fig. 3. Relationships between $\Delta[Um]_{D\text{-}T}$ values and number of hydroxy groups in *sec*.- and *tert*.-hydroxylated steroids. Data were obtained from the differences in the MU values between trialkylsilylations by (a) HMDS/TMCS/pyridine and (b) BSA/TMSI/TMCS and DMSI; in each figure, linear regression lines and correlation coefficients (*r*) were calculated from the $\Delta[Um]_{D\text{-}T}$ data (open circle) for *sec*.-hydroxylated steroids (**1**–**5**, **15** and **16**).

the following order: 25>20, 24>5β>17α>>14α. The complete silylation of all of *tert*.-hydroxy groups in steroids was found to be difficult, particularly for the highly hindered 14α- and 17α-hydroxy groups. Attention should, therefore, be paid in the simultaneous GC analysis of a complex mixture of *tert*.-hydroxylated steroids in biological materials. Further studies on the complete or suitable derivatisation procedure of the *tert*.-hydroxylated steroids are being conducted in these laboratories, and the results will be reported elsewhere.

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