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Note

Analysis of sponge sterols as the trimethylsilyl ethers and as the corresponding 5α- and Δ⁴-3-ketosteroids using open-tubular gas chromatography–mass spectrometry

Application of selective enzymic oxidation

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Marine invertebrates usually possess complex mixtures of C_{25}, C_{26}, C_{27}, C_{28} and C_{29} 4-demethyl sterols and it has been known for many years that sterols from sponges often contain mixtures of Δ^5 and 5α analogues[1]. In more recent studies of sponges, individual sterols were isolated and characterised following preliminary examination by gas chromatography (GC)[2–7]. Combined GC–mass spectrometry (GC–MS) using conventional packed GC columns, of these complex mixtures is very rewarding but is not fully effective. For example, certain sterols (notably the pairs of the Δ^5 and 5α analogues) may not be satisfactorily separated by this type of system. A further problem arises from the occurrence of modified side chains. We have reported two methods of overcoming the first of these difficulties.

Incubation with cholesterol oxidase leads to conversion of the Δ^5- and 5α-3β-hydroxysteroids to Δ^4- and 5α-3-ketosteroids, respectively, which are easily separated on non-polar packed GC columns[8]. More recently, we have demonstrated examples of the resolution of Δ^5- and 5α-steroid pairs by GC on Silanox-type glass open-tubular columns[9]. Both of these methods have been applied to the analysis of sterols from various species of sponges, and some representative results are presented here.

EXPERIMENTAL

Sponges were collected off the north-west coast of Scotland and supplied by the Marine Biological Station (Millport, Cumbrae, Scotland).

Hymeniacidon perleve and *Grantia compressa* (7.2 and 0.95 g dry weight, respectively) were saponified to give non-saponifiable lipid (40.4 and 2.8 mg, respectively)[10]. Thin-layer chromatography (TLC) of these extracts yielded the 4-demethyl sterols (*H. perleve*, 25.8 mg; *G. compressa*, 0.5 mg). The trimethylsilyl (TMS) ethers of the sterols were formed by reaction with bis(trimethylsilyl)trifluoroacetamide (Pierce and Warriner, Chester, Great Britain) at 60° for 10 min.

Preliminary GC was performed on a Pye 104 gas chromatograph fitted with a glass column (4 m × 4 mm I.D.) packed with 1% OV-1 on Gas-Chrom Q (100–120

mesh). Samples were then analysed by GC–MS using open-tubular glass columns (50 m × 0.5 mm I.D.) coated with 6–10 μm Silanox (Cabot, Billericia, Mass., U.S.A.) and OV-1 methyl silicone stationary phase (Applied Science Labs., State College, Pa., U.S.A.)[9] by the method of German and Horning[11]. The columns were installed in an LKB Model 9000 GC–MS instrument with a dry injection device as described previously[9]. Identical chromatograms, obtained on a Pye 104 instrument, were used to calculate retention indices. A sample of the 4-demethyl sterols from *G. compressa* was converted to a mixture of 3-ketosteroids by incubating with cholesterol oxidase (Boehringer, London, Great Britain) in isopropanol–sodium phosphate buffer (pH 7.0) (1:10, v/v)[8].

RESULTS

Erdman and Thomson[2] identified a total of eight sterols in the mixture from *H. perleve* after preliminary GC followed by preparative separation of these sterols using other chromatographic methods. We have examined the sterols (as their TMS ethers) from another specimen of this organism, firstly by GC using a packed OV-1 column, and then by open-tubular GC–MS. The higher resolving power of the open-tubular system enabled many more compounds to be detected (Fig. 1). Most of the constituents of the peaks could be identified by open-tubular GC–MS (Table I). It must be stressed that consideration of the GC retention indices of the sterol TMS ethers played an important part in the identifications. Comparison was made either

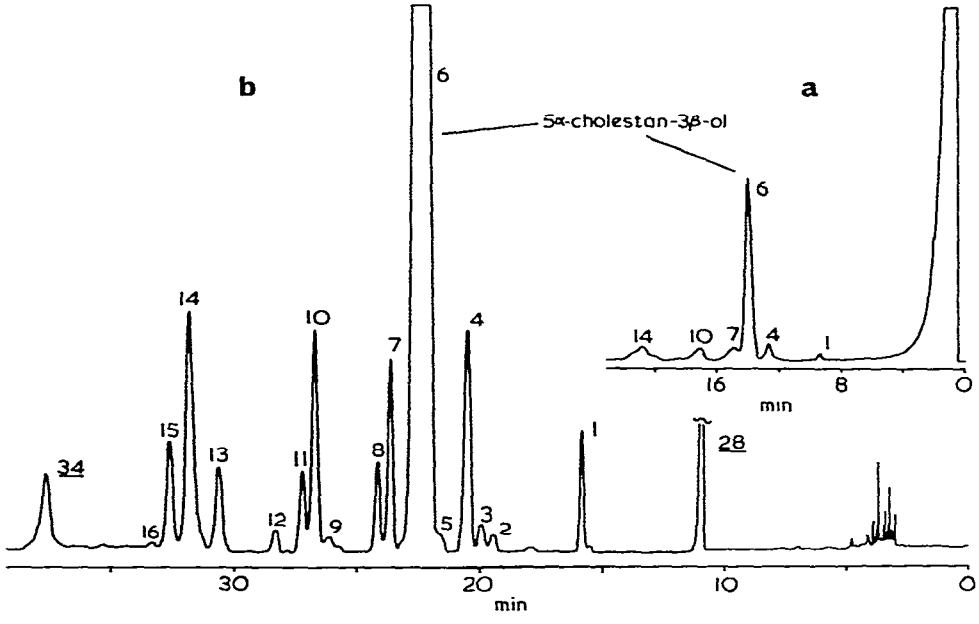

Fig. 1. GC separations of the TMS ethers of the 4-demethyl sterols from *Hymeniacidon perleve* (a) on a packed column (3.6 m × 3 mm I.D.) containing 1% OV-1 on Gas-Chrom Q (100–120 mesh) at 265° with a helium flow-rate of 30 ml/min; and (b) on an open-tubular column (50 m) coated with OV-1 (see Experimental) at 275° with a helium flow-rate of 6 ml/min. The total ion-current chromatograms shown were obtained using an LKB 9000 GC–MS instrument.

TABLE I

GC–MS DATA FOR THE TMS ETHERS OF THE STEROLS FROM *HYMENIACIDON PERLEVE*

GC peak (Fig. 1)	$I^{275°}_{OV\text{-}1}$	$M^{+\cdot}$ *(m/e)*	*Abundance (%)* *	*Base peak* * *(m/e > 100)*	*Some major ions (m/e > 100)*					*Assignment* **
1	3007	444	16	257	429	374	359	354		24-Nor-5α-cholest-22-en-3β-ol
2	3108	456	45	111	366	327	256	129		*cis*-5,22-Cholestadien-3β-ol
3	3120	458	35	257	443	374	366	345	111	*cis*-5α-Cholest-22-en-3β-ol
4	3133	458	26	257	443	374	359	345	111	*trans*-5α-Cholest-22-en-3β-ol
5	3159	458			368	129				5-Cholesten-3β-ol
6	3167	460	56	215	445	370	355	305	257	5α-Cholestan-3β-ol
7	3199	458			443	215				5α-Cholest-24-en-3β-ol
		470	22	129	455	380	365	255	125	5,22-Ergostadien-3β-ol
8	3210	458			443	255				5α-Cholest-7-en-3β-ol
		472	25	257	374	359	125			5α-Ergost-22-en-3β-ol
9	3247	470	25	129	386					5,24(28)-Ergostadien-3β-ol
10	3257	472	6	388	457	374	305	255	215	5α-Ergost-24(28)-en-3β-ol
11	3266	474	39	215	459	384	305			5α-Ergostan-3β-ol
12	3284	484	20	129	469	394	351	255		5,22-Stigmastadien-3β-ol
13	3321	484	21	129	469	394	379	355	343	5,x-(C_{29}-Sterol)
14	3338	486	12	129	471	396	386	371	357	5-Stigmasten-3β-ol
15	3349	488			473	305	257	215		5α-Stigmastan-3β-ol
		484	4	129	386	296	281			5,24(28)-Stigmastadien-3β-ol
16	3358	486	23	388	215					5α-Stigmast-24(28)-en-3β-ol

* Base peaks and molecular ion abundances are not assigned for minor components of unresolved peaks.

** "Classical" side-chains are assumed for components of peaks 2–16.

with authentic compounds or with values calculated from results obtained with analogues. Rationalization of the mass spectra was based on previously published data[10,12–16]. Fig. 1 shows that 5α-cholestan-3β-ol was the major sterol. Seventeen other 4-demethyl sterols were characterised: others were present at too low a level for mass spectra to be obtained.

Many of the sterols of *G. compressa* (Fig. 2a, Table II) were identified using procedures similar to that described for *H. perleve*. In addition, the sterol mixture was incubated with cholesterol oxidase to convert Δ^5- and 5α-sterols to Δ^4- and 5α-3-ketosteroids, respectively. GC of the products revealed a change in the sequence of elution (*cf.* Fig. 2a and b), and comparison of the retention indices of the TMS ethers and the derived 3-ketosteroids provided further evidence for the assignment of structure to the parent sterols. An increase of approximately 48 index units was observed for Δ^5-sterols, whilst 5α-sterols showed a decrease of 23–44 index units, depending on the precise nature of the compound (Table II). High loading of some major constituents, necessitated by the low concentrations of minor constituents, also influenced retention behaviour. Identification of the 3-ketosteroids from their mass spectra was based on data cited in our previous report[17], and in references cited therein. As with *H. perleve*, 5α-cholestan-3β-ol was the major constituent of *G. compressa*; substantial proportions of cholesterol and 5α-cholest-7-en-3β-ol were also present.

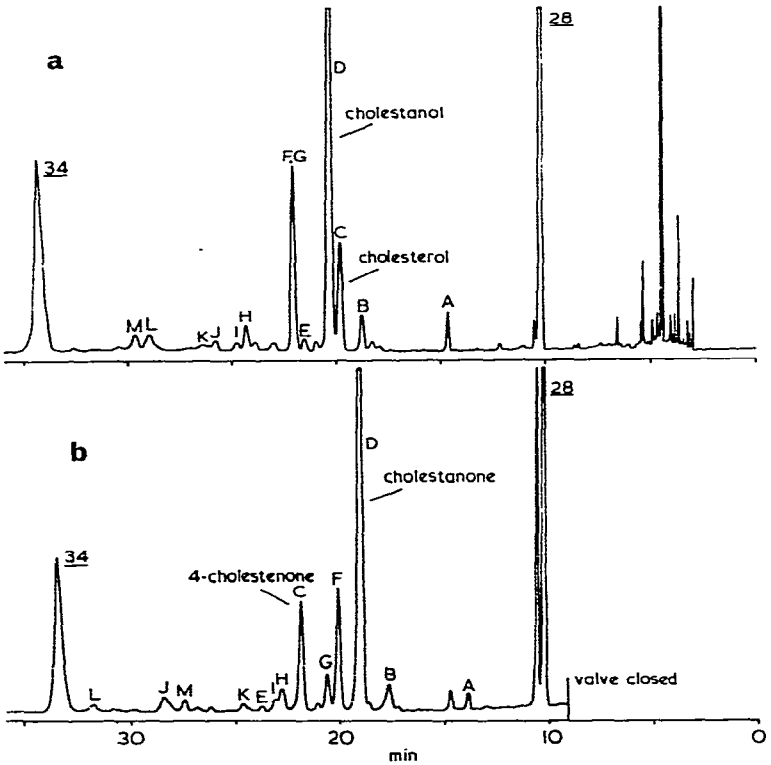

Fig. 2. Total ion-current chromatograms showing open-tubular GC separation of derivatives of the 4-demethyl sterols of *Grantia compressa*. (a) TMS ethers; (b) 3-ketones formed after oxidation with cholesterol oxidase.

TABLE II

COMPARISON OF GC–MS DATA FOR THE 4-DEMETHYL STEROLS OF *GRANTIA COMPRESSA* AS THE TMS ETHERS AND AS THE CORRESPONDING 5α- AND Δ^4-3-KETOSTEROIDS

GC peak (Fig. 2)	$I^{275°}_{OV\text{-}1}$	*ΔI*	$M^{+\cdot}$ *(m/e)*	*Abundance (%)*	*Base peak (m/e > 100)*	*Assignment***
A	3006		444	20	257	24-Nor-5α-cholest-22-en-3β-ol
	2973	−33	370	36	109	24-Nor-5α-cholest-22-en-3-one
B	3130		458	23	257	*trans*-5α-Cholest-22-en-3β-ol
	3107	−23	384	38	123	*trans*-5α-Cholest-22-en-3-one
C	3156		458	24	129	5-Cholesten-3β-ol
	3205	+49	384	28	124	4-Cholesten-3-one
D	3168		460	55	215	5α-Cholestan-3β-ol
	3145	−23	386	43	231	5α-Cholestan-3-one
E	3196		470	17	129	5,22-Ergostadien-3β-ol
	3245	+49	396	46	271	4,22-Ergostadien-3β-ol
F	3207		458	90	255	5α-Cholest-7-en-3β-ol
	3163	−44	384	90	271	5α-Cholest-7-en-3-one
G	3207		472*			5α-Ergost-22-en-3β-ol
	3178	−29	398	21	109	5α-Ergost-22-en-3-one
H	3256		472	4	388	5α-Ergost-24(28)-en-3β-ol
	3223	−33	398	4	314	5α-Ergost-24(28)-en-3-one
I	3264		474	50	459	5α-Ergostan-3β-ol
	3230	−34	400	25	231	5α-Ergostan-3-one
J	3283		484	23	255	5,22-Stigmastadien-3β-ol
	3331	+48	410	34	137	4,22-Stigmastadien-3-one
K	3295		486	15	109	5α-Stigmast-22-en-3β-ol
	3263	−32	412	17	123	5α-Stigmast-22-en-3-one
L	3337		486	18	129	5-Stigmasten-3β-ol
	3385	+48	412	32	124	4-Stigmasten-3-one
M	3348		488	64	215	5α-Stigmastan-3β-ol
	3316	−32	414	33	231	5α-Stigmastan-3-one

* The base peak and molecular ion abundance could not be assigned for the minor component in peak G.

** The first steroid in each pair is that observed in the GC–MS of the trimethylsilyl ethers (Fig. 2a); the second is the corresponding ketone formed after incubation with cholesterol oxidase (Fig. 2b). "Classical" side-chains are assumed for components of peaks B–M. The stereochemistries of C-24 alkylated sterols have not been determined. Besides the sterols shown, mass spectra were obtained of sterols with *I* 3117 (probably *cis*-5α-cholest-22-en-3β-ol), 3183 (di-unsaturated, C_{27}) and 3247 (di-unsaturated, C_{28}). Mass spectra of the corresponding ketones were not obtained because of masking by other steroids, or inadequate amount. Conversely during GC–MS of the 3-ketosteroids some unidentified C_{28} and C_{29} steroids were observed.

CONCLUSIONS

Open-tubular GC–MS has found many applications in the analyses of complicated mixtures. We have briefly illustrated its potential for the analysis of complex mixtures of sterols in marine organisms. In addition, the use of cholesterol oxidase for selective oxidation of Δ^5- and 5α-sterols, in affording new and distinctive GC elution patterns and mass spectra, provides a further aid to elucidating sterol structures. Despite the higher resolving power of the open-tubular GC system employed, mixed peaks still occurred and 24*R* and 24*S* isomers could not be distinguished. Improvements in the resolving power of open-tubular columns, in conjunction with the use of polar-type phases, may overcome these difficulties[16,18]. We are using the techniques outlined here in the analysis of steroids from a variety of natural sources.

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