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GAS CHROMATOGRAPHY OF ECDYSTEROIDS AS THEIR TRIMETHYLSILYL ETHERS

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SUMMARY

Conditions for the preparation of some 60 silyl ethers of ecdysteroids using trimethylsilylimidazole are reported. The thin-layer and gas–liquid chromatographic properties of the resulting derivatives, together with information on mass spectrometry as an aid to structure identification, are described.

INTRODUCTION

The ecdysteroids, a family of polyhydroxylated steroids structurally related to ecdysone (Fig. 1), are important as the moulting hormones of insects and crustaceans as well as other arthropods. These compounds are also encountered in certain species of plants particularly conifers and ferns[1]. To date over 100 different structures have been reported.

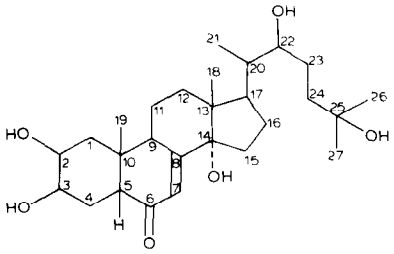

Fig. 1. The structure of ecdysone showing the numbering.

A variety of methods have been developed for the analysis of ecdysteroids including thin-layer chromatography (TLC)[2,3], high-performance liquid chromatography (HPLC)[4,5], gas–liquid chromatography (GLC)[6,7] and radioimmunoassay (RIA)[8] or a combination of more than one of these methods (*e.g.* HPLC–RIA).

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GLC, when combined with electron-capture detection (ECD) or mass spectrometry (MS) (using single ion monitoring, SIM), provides a method of great sensitivity and specificity for the analysis of these compounds. However, the ecdysteroids are involatile and thermally unstable and require conversion to their trimethylsilyl (TMS) ethers for GLC. The derivatisation procedure requires careful control because of the different rates of reaction of the various hydroxyls present.

Though fast atom bombardment MS has established itself as the best method for determining the molecular mass of the ecdysteroids, the electron impact mass spectra (EI) of their silyl ethers remains one of the best sources of structural information.

We present here the conditions required for making some 60 silyl ethers of ecdysteroids, together with their relative retention data in GC.

MATERIALS AND METHODS

The ecdysteroids used in this study were gifts from a number of sources.

Silylation

A sample of ecdysteroid (0.2–1.0 mg) was dissolved in acetone and methanol to give a concentration of 500 μg ml^{-1}. A 50-μl aliquot (approx. 25 μg) of this solution was evaporated to dryness under nitrogen in a 1-cm^3 ReactiVial (Pierce and Warriner, Chester, U.K.). This sample was redissolved in a mixture of pyridine (65 μl) and trimethylsilylimidazole (TMSI, 35 μl) and the vial sealed. The mixture was allowed to react at either room temperature (30 min), 120°C (5 h) or 140°C (60 h) depending on the degree of silylation required. After reaction, an aliquot (usually 10 μl) was diluted with purified "ECD"-grade toluene (see ref. 6) to give a concentration of 1–10 ng ml^{-1}. Samples of this solution (1 μl) were taken for GLC–ECD as described below.

The remainder of the reaction mixture was either stored at 0–4°C, at which temperature the silyl ethers are stable for several weeks, or purified by TLC.

Purification of ecdysteroid TMS ethers by TLC

The TMS ethers were purified using preparative TLC on 0.6 mm thick 20 cm × 20 cm plates coated with methanol-washed silica gel 60 P F_{254} (E. Merck, Darmstadt, F.R.G.). The volume of the reaction mixture was first reduced by evaporation of some of the pyridine under a stream of nitrogen. The concentrated solution was applied to the origin of the plate which was developed in toluene–ethyl acetate (7:3, v/v) for 15 cm. The tetrakis-TMS ether of ecdysone was also spotted onto a portion of the plate for comparison. The derivatives were located on the plate by visualisation at 254 nm when present in sufficient quantity. They were recovered from the plate by removing the appropriate zone of silica gel and eluting with diethyl ether. After evaporation of the ether the samples were redissolved in ECD-grade toluene for analysis by GLC. Recoveries of ecdysteroid TMS ethers were generally 80–90% of the material applied to the plate. The R_F values for these derivatives are given in Table I.

GC of ecdysteroid TMS ethers

GC was performed using a Pye Unicam Series 104 gas chromatograph, fitted with a ^{63}Ni electron capture detector, on silanised glass columns (0.9 m or 1.5 m × 4 mm I.D.) containing 1.5% (w/w) OV 101 silicone phase (Magnus Scientific, Aylesbury, U.K.) on Chromosorb W. The chromatographic conditions were a column temperature of 285°C, detector temperature 300°C, and a carrier gas (oxygen free nitrogen) flow-rate of 50–60 ml min^{-1}. Samples (1–2 μl) were injected directly onto the top of the column using a 5-μl syringe fitted with an 11-cm needle.

RESULTS AND DISCUSSION

The hydroxyl groups present on the ecdysteroid possess a range of reactivities towards TMSI, due to their different steric environments. Previous work with model compounds[6] together with ecdysone and 20-hydroxyecdysone has established the following order of reactivity of the hydroxyl groups. 2β, 3β, 22*R*, 25 > 20*S* ⩾ 14α. The reaction conditions employed in this study exploit these differences to allow the formation of a number of different derivatives. Thus the reaction of 20-hydroxyec-

TABLE I

R_F VALUES OF THE TMS ETHERS OF ECDYSTEROIDS ON SILICA

Parent compound	*R_F value of the derivative formed after silylation for:*		
	30 min at room temperature	*5 h at 120°C*	*60 h at 140°C*
Ecdysone	0.72	0.72	0.82
20-Hydroxyecdysone	0.65	0.69	0.75
Ajugasterone C	0.63	0.8	0.8
Calonysterone	0.7	0.76	0.76
Carpesterol	0.7	0.7	0.7
Cyasterone	–	–	0.72
Dacrysterone	0.54	077	0.81
2-Deoxyecdysone	0.74	0.74	0.78
2-Deoxy-3-epiecdysone	0.74	0.74	0.77
2-Deoxy-20-hydroxyecdysone	0.74	0.68	–
20-Hydroxyecdysone 2-cinnemate	0.64	0.81	0.75
Inokosterone	0.65	0.72	0.8
22-Isoecdysone	–*	0.77	0.79
Kaladasterone	0.7	0.73	0.75
Makisterone A	0.67	0.72	0.78
Muristerone	–*	0.47*	0.59
Polypodine B	0.65	0.74	0.76
Polypodine B 2-cinnamate	0.4	0.76	0.83
Ponasterone A	0.67	0.78	0.71
Ponasterone C	0.34	0.71	–
Ponasterone C 2-cinnamate	0.38	0.75	0.78
Poststerone**	0.6–0.8	0.6–0.8	0.6–0.8
Pterosterone	0.71	0.71*	0.73

* Silylation of these ecdysteroids under these conditions formed mixed derivatives.

** Too small a sample to be viewed under UV illumination.

TABLE II

PROPOSED DERIVATIVES OF SOME ECDYSTEROID TMS ETHERS

Parent compound	*Conditions of silylation*					
	30 min, room temperature		*5 h, 120°C*		*60 h, 140°C*	
	No. of silyl groups	*Position of hydroxyl groups silylated*	*No. of silyl groups*	*Position of hydroxyl groups silylated*	*No. of silyl groups*	*Position of hydroxyl groups silylated*
Ecdysone	4	2,3,22,25	4	2,3,22,25	5	2,3,14,22,25
20-Hydroxyecdysone	4	2,3,22,25	5	2,3,20,22,25	6	2,3,14,20,22,25
2-Deoxyecdysone	3	3,22,25	3	3,22,25	4	3,14,22,25
2-Deoxy-3-epiecdysone	3	3epi,22,25	3	3epi,22,25	4	3epi,14,22,25
2-Deoxy-20-hydroxyecdysone	3	3,22,25	4	3,20,22,25	5	3,14,20,22,25
Inokosterone	4	2,3,22,26	5	2,3,20,22,26	6	2,3,14,20,22,26
Makisterone A	4	2,3,22,25	5	2,3,20,22,25	6	2,3,14,20,22,25
Ponasterone A	3	2,3,22	4	2,3,20,22	5	2,3,14,20,22
Poststerone	2	2,3	2	2,3	3	2,3,14
Pterosterone	4	2,3,22,24	4	2,3,20,22,24	6	2,3,14,20,22,24

dysone with TMSI, at room temperature for 30 min (or longer) produces the 2,3,22,25-tetrakis-TMS ether, 5 h at 120°C the 2,3,20,22,25-pentakis-TMS ether, and 60 h at 140°C the fully derivatised 2,3,14,20,22,25-hexakis-TMS ether.

Thus our knowledge of the rate of silylation of hydroxyl groups located on these carbons allows us to identify tentatively the derivatives formed from ecdysteroids that possess hydroxyl groups on C-2, C-3, C-14, C-20, C-22 and C-25. Ecdysteroids containing only hydroxyl groups on these carbons and their proposed derivatives are shown in Table II. This table also includes inokosterone and pterosterone which have an hydroxyl group on C-26 and C-24 respectively, both of which are in unhindered positions and are very likely to be readily silylated. These proposed derivatives have yet to be confirmed by MS. GC retention time data are given in Table III.

The remaining ecdysteroids either possess an additional chemical moiety or a hydroxyl group in such a position that it would be unwise to predict its rate of silylation without confirmation of the structure of the derivatives by MS. In cyasterone a lactone ring occurs on the side chain on C-24 which increases the molecular weight and the polarity of the silyl ether in comparison with other ecdysteroids. This causes the derivative to have a longer retention time, and poor peak shape at the temperature used for other ecdysteroid derivatives (Table III).

A group of plant ecdysteroids were also studied that have an additional chemical group, a cinnamate ester, present on the C-2 hydroxyl. These ecdysteroids, originally isolated from the bark of *Dacrydium intermedium* by Russell *et al.*[9], are 20-hydroxyecdysone-2-cinnamate, polypodine B-2-cinnamate and ponasterone C-2-cinnamate. These compounds also exhibited poor GC characteristics with broad peak shape and multiple product formation (which may have arisen from partial hydrolysis of the ecdysteroid derivative and formation of a silyl ether of the parent compound and cinnamic acid on silylation).

Four of the ecysteroids studied possess a C-5 β-hydroxyl group. These were dacrysterone (5β-hydroxymakisterone A) kaladasterone (2β,3β,5β,14α,20,22-hexahydroxycholest-7-en-6-one), muristerone (11α-hydroxykaladasterone) and polypodine B (2β,3β,5β,14α,20,22,25-heptahydroxycholest-7-en-6-one). A mass spectrum of the derivative formed after silylation of polypodine B at 120°C for 5 h is shown in Fig. 2. This indicates that six of the seven hydroxyl groups have been silylated to form the hexakis TMS ether. Given that the 14α-hydroxyl group is not silylated in ecdysone and 20-hydroxyecdysone under these conditions we deduced that the 5β-hydroxyl group had been derivatised. As yet we do not know whether this hydroxyl group is silylated readily at room temperature. Extrapolating this result to other ecdysteroids with a 5β-hydroxyl group it is likely that the hexakis TMS ether of dacrysterone and the pentakis TMS ether of kaladasterone are formed at 5 h, whilst 60 h at 140°C, probably results in complete silylation (heptakis and hexakis TMS ethers respectively).

In ajugasterone C (11α-hydroxyponasterone A) and muristerone A an extra hydroxyl group is present on the C ring at C-11α. Only small quantities of these two ecdysteroids were available and mass spectra were not obtained, it is therefore not possible to propose the rate of silylation for this group. Additionally, with muristerone A, multiple peaks were obtained on GC suggesting that, either the compound was impure (also indicated by HPLC) or that some of the hydroxyl groups were only partially silylated under the conditions employed.

TABLE III

GAS CHROMATOGRAPHIC PROPERTIES OF ECDYSTEROID TMS ETHERS

GC conditions: 0.9 m × 4 mm I.D. column, 1.5% OV-101 silicone phase, gas flow-rate 50–60 ml min^{-1}, temperature 285°C, detector temperature 300°C.

Ecdysteroid	*Conditions of silylation*					
	39 min, room temperature		*5 h, 120°C*		*60 h, 140°C*	
	t_R	t_R *relative to ecdysone*	t_R	t_R *relative to ecdysone*	t_R	t_R *relative to ecdysone*
Ecdysone	6.9	1	6.9	1	5.85	1
20-Hydroxyecdysone	7.95	1.15	9.45	1.37	7.55	1.3
Ajugasterone C	6.55	0.95	7.8	1.13	6.15	1.05
Calonysterone	9.25	1.34	10.5	1.52	10.5	1.79
Carpesterol	–		27.5*	4	–	
Cyasterone	–		19.8**	2.78	19.5	3.3
Dacrysterone	12.7*	1.84	9.85	1.43	–	
2-Deoxyecdysone	5.9	0.85	5.9	0.85	5.3	0.9
2-Deoxy-3-epiecdysone	5.5	0.8	5.5	0.8	5.3	0.9
2-Deoxy-20-hydroxyecdysone	7.1	1.03	8.5	1.23	–	
20-Hydroxyecdysone 2-cinnamate	11.6	1.68	>30	–	8.6	
Inokosterone	9.9	1.43	10.8	1.57	8.0	
22-Isoecdysone	7.5 → 6.5***	1.09	6.5	0.94	6.4	
Kaladasterone	6.8	0.98	6.3	0.91	4.1	
Makisterone A	10.0	1.45	11.4	1.65	9.15	
Muristerone	**	–	9.25**	1.34	5.1**	
Polypodine B	9.1	1.32	10.7	1.55	7.5	
Polypodine B 2-cinnamate	11.0*	1.59	≈10.6*	–	**	
Ponasterone A	5.9	0.85	6.2	0.9	4.4	
Ponasterone C	11.0	5.9	9.0	1.3	–	
Ponasterone C 2-cinnamate	**	–	≈10.0	–	–	
Poststerone	1.65	0.24	1.65	0.24	1.3	
Pterosterone	8.7	1.26	9.1	1.32	8.5	

* Very broad peak.
** Mixed derivatives.
*** If left longer than 30 min at room temperature.

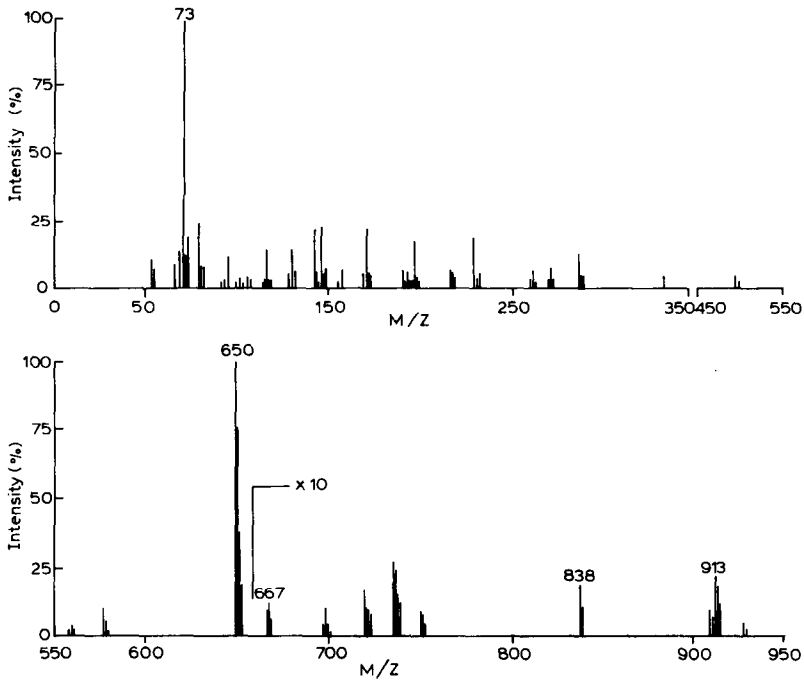

Fig. 2. The EI mass spectrum of the hexakis-TMS ether of polypodine B. The strong ion at *m/z* 650 arises from cleavage between C-20 and C-22 this is characteristic of ecdysteroids with hydroxyl groups at both of these positions. The ion at *m/z* 261 results from the side chain fragment of the same cleavage.

Calonysterone [2β,3β,6,20,22,25-hexahydroxycholesta-5,8(9),14-trien-7-one] is an unusually modified ecdysteroid[10], lacking a 14α-hydroxyl, with a ketone on C-7 (as opposed to C-6), an hydroxyl group on the C-6 position and a higher degree of unsaturation than "normal". This compound yielded two products, one after derivatisation for 30 min at room temperature and the other after 5 h at 120°C. No further derivatisation occurred by increasing the silylation time or temperature and it was concluded that after 5 h the ecdysteroid was fully derivatised as the hexakis TMS ether. The derivative formed by silylation at room temperature was assumed to be the pentakis TMS ether where all but the hydroxyl group on C-20 had been derivatised.

The final ecdysteroid investigated was a synthetic analogue, 22-isoecdysone differing from ecdysone (which has a 22*R* configuration) only in being its optical antipode at C-22. If this difference has no effect on the rate of silylation compared to ecdysone we would expect two derivatives to be formed (tetra and pentakis TMS). In fact three derivatives were observed. After a 30 min silylation at room temperature a single derivative was detected with a retention time of 7.5 min. Further silylation at room temperature resulted in a second peak gradually developing with a shorter retention time (6.5). This derivative was obtained as a single peak by silylation for 5 h at 120°C. Silylation at 140°C for 60 h then resulted in the formation of a third derivative with a retention time of 6.4 min. From these results we speculate that the C-22 hydroxyl group on 22-isoecdysone does not silylate as readily as the C-22 hy-

droxyl of ecdysone. Hence the first derivative was the tris-TMS ether where the C-2, C-3 and C-25 hydroxyl groups were silylated and the derivative formed after 5 h at 120°C was the tetrakis TMS ether where the C-22 group had also been fully derivatised.

CONCLUSION

GC with ECD (or GC with SIM) provides a specific and highly sensitive method for the qualitative and quantitative analysis and identification of most of the ecdysteroids which we have been able to investigate. The ability to form a number of different derivatives for each compound by varying the reaction conditions is useful in the identification of these compounds by GC–MS and as derivatives in the same way that acetates and acetonides are used in TLC and HPLC.

REFERENCES

1 C. Hetru and D. H. S. Horn, in J. A. Hoffmann (Editor), *Progress in Ecdysone Research*, Elsevier/North-Holland Biomedical Press, Amsterdam, 1980, p. 13.
2 E. D. Morgan and C. F. Poole, in J. E. Treherne, M. J. Berridge and V. B. Wigglesworth (Editors), *Advances in Insect Physiology 12*, Academic Press, London, New York, 1976, p. 17.
3 I. D. Wilson, *J. Chromatogr.*, 318 (1985) 373.
4 R. Lafont, G. Somme-Martin, B. Mauchamp, B. F. Maume and J.-P. Delbeque, in J. A. Hoffmann (Editor), *Progress in Ecdysone Research*, Elsevier/North-Holland Biomedical Press, Amsterdam, 1980, p. 45.
5 I. D. Wilson, C. R. Bielby and E. D. Morgan, *J. Chromatogr.*, 238 (1982) 97.
6 C. F. Poole and E. D. Morgan, *J. Chromatogr.*, 115 (1975) 587.
7 C. R. Bielby, A. R. Gande, E. D. Morgan and I. D. Wilson, *J. Chromatogr.*, 194 (1980) 43.
8 J. D. O'Connor and D. W. Borst, *Science*, 178 (1972) 418.
9 G. B. Russell, D. G. Fenemore, D. H. S. Horn and E. J. Middleton, *Aust. J. Chem.*, 25 (1972) 1935.
10 L. Canonica, B. Danieli, G. Ferrari, J. Krepinsky and G. Rainoldi, *Chem. Commun.*, (1973) 737.