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THE FORMATION OF TRIMETHYLSILYL ETHERS OF ECDYSONES

A REAPPRAISAL

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SUMMARY

The hydroxyl groups of 20-hydroxyecdysone react with trimethylsilylimidazole with varying ease, in the positional order 2,3,22,25 > 20 ≫ 14. The 14α-hydroxyl group can only be silylated under forcing conditions. Confusion in silylation procedures has been caused by failure to recognize incomplete reaction. The conclusions are supported by mass spectra. In the presence of a catalyst, and absence of a 14α-oxy substituent, enol ethers are readily formed, but the rate is considerably reduced with a C-14 substituent present.

INTRODUCTION

The importance of the ecdysones as hormones controlling growth and metamorphosis in arthropods, and their presence in these animals at very low concentrations has meant that considerable attention has been devoted to finding sensitive and accurate methods for their assay. Eight ecdysones have been found in insects and crustaceans; and over forty in higher plants, where their role is obscure. The methods currently used are bioassay[1], radioimmunoassay[2], and gas chromatography (GC). The bioassay and radioimmunoassay are non-specific and technically difficult. GC can distinguish between ecdysones, can be carried out in many laboratories, but originally lacked sufficient sensitivity[3]. Recently, two groups of investigators have found that ecdysones, somewhat unexpectedly are sensitive to the electron capture detector, so that after conversion to their trimethylsilyl ethers they can be directly determined down to the picogram level[2,4].

We have been troubled by the variety of conditions recorded for preparing the trimethylsilyl (TMS) ethers of ecdysones, and by our difficulty in reproducing other results, and so have investigated the matter and found that earlier reports are mis-

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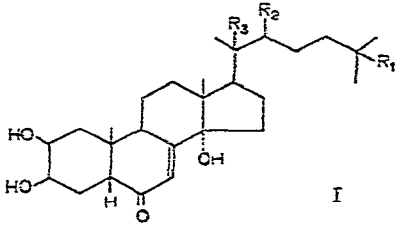
leading and believe that we can now provide reliable information for the preparation of ecdysone TMS ethers.



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Ia: $R_1,R_2 = OH$; $R_3 = H$
Ib: $R_1,R_2,R_3 = OH$
Ic: $R_1,R_2,R_3 = H$

The first report of a TMS ether of ecdysone (Ia) was by heating ecdysone to 80° for 1 min with N,O-bis(trimethylsilyl)acetamide (BSA) in pyridine[5]. A single GC peak was produced, but no evidence was offered on the number of hydroxyl groups silylated. In our earlier work we found complete protection of all hydroxyl groups difficult, particularly for 20-hydroxyecdysone (Ib, β-ecdysone, ecdysterone, crustecdysone), and settled upon a routine method by which the ecdysone was first converted to its methoxime and then silylated with BSA at room temperature for 70 h, this converted the 2,3,22- and 25-hydroxyl groups to silyl ethers[3,6,7]. Two other groups of investigators found the 14α-hydroxyl in some ecdysone analogues was not completely silylated with BSA at 80° in dimethylformamide over 18 h[8,9]. Ikekawa *et al.*[10] claimed that compound Ib could be completely silylated with trimethylsilylimidazole (TMSI) in 1 h at 100°. They claimed the 20-hydroxyl group reacted most slowly, and confirmed the structure of the product by gas chromatography–mass spectrometry (GC–MS). Similar details are given in a further paper by Miyazaki *et al.*[11]. King *et al.*[12] suggested that 30 min at 96° was sufficient for complete reaction but did not state how this was recognized, while Borst and O'Connor[2] used TMSI at 100° for 15 min and stated that the mass spectra were in agreement with those given by Ikekawa *et al.*[10].

We have found that heating compound Ib with TMSI at 100° for 1 h always produced more than one GC peak[4]. Extending the reaction time or increasing the temperature increased the peak of shorter retention time. Both components had mass spectra close to those of 20-hydroxyecdysone hexakis(trimethylsilyl) ether. We examined the matter carefully to see if epimerization was taking place. We found that complete silylation requires longer reaction times than claimed and the published mass spectra can be explained as being due to mixtures.

EXPERIMENTAL

Trimethylchlorosilane (TMCS), N,O-bis(trimethylsilyl)acetamide (BSA) and N-trimethylsilylimidazole (TMSI) were purchased from Koch-Light (Colnbrook, Great Britain) or from Pierce and Warrener (Chester, Great Britain); TMSI was also prepared in the laboratory by the method of Kuhn *et al.*[13]. Standard silylation procedures were followed (*cf.* ref. 14).

The preparation of the model compounds 5α-cholest-7-en-6-one and 14α-hydroxy-5α-cholest-7-en-6-one are described elsewhere[15]. 2β,3β,14α-Trihydroxy-5β-cholest-7-en-6-one (Ic) was prepared as by Thompson *et al.*[9]. 20-Hydroxyecdysone

(Ib) was purchased from Rhoto Pharmaceuticals (Osaka, Japan) and ecdysone (Ia) was a gift from Dr. H. H. Rees, University of Liverpool.

Mass spectra were obtained with a Hitachi–Perkin-Elmer RMU-6E, single-focusing mass spectrometer; ionizing energy 80 or 20 eV, trap current 60 μA, accelerating potential 0.9 kV, solid inlet temperature range 250–280°. Spectra were recorded on the 0–1,200 range with an accuracy of 2 a.m.u. at 1000 a.m.u. For GC–MS a Pye Series 104 gas chromatograph with a 1.5 ft. × 3 mm I.D. column of 1% OV-101 on Gas-Chrom Q, was coupled through a Watson–Biemann separator at 300° with a helium flow-rate of 18 ml/min.

RESULTS AND DISCUSSION

It was found that prolonged heating of the simple model, 14α-hydroxy-5α-cholest-7-en-6-one with TMSI at 100° produced little reaction. Heating overnight at 140° gave almost quantitative yield (by GC) of the trimethylsilyl ether, which has been made on a preparative scale and fully characterized[15]. The rate of reaction of the 14α-hydroxyl group with TMSI is shown in Fig. 1. When the trihydroxy compound Ic was treated with TMSI or BSA at room temperature, a single GC peak was produced, identified as 14α-hydroxy-2β,3β-bis(trimethylsiloxy)-5β-cholest-7-en-6-one, from its mass spectrum (Fig. 2), and its infrared spectrum (ν(O–H) = 3460 cm^{-1}; ν(C=O) = 1660 cm^{-1}), as would have been expected from our earlier work[3,16].

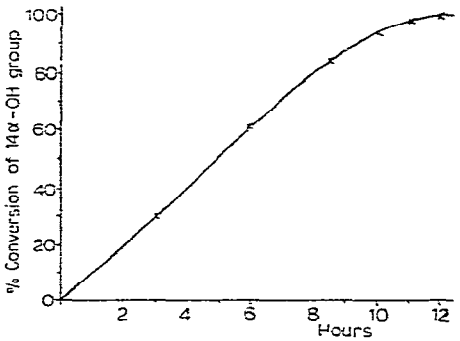

Fig. 1. Rate of reaction of 14α-hydroxyl group in 14α-hydroxy-5α-cholest-7-en-6-one with TMSI at 140°.

Heating compound Ic overnight at 140° in TMSI gave the completely silylated ether in nearly quantitative yield. A small amount of the di-TMS ether was removed by preparative thin-layer chromatography (TLC) and the major product isolated and identified as 2β,3β,14α-tris(trimethylsiloxy)-5β-cholest-7-en-6-one from its mass spectrum (Fig. 3), and its infrared spectrum, which showed the absence of free hydroxyl groups and the presence of the unsaturated ketone group.

A comparison of the mass spectrum of 2β,3β,14α-tris(trimethylsiloxy)-5β-cholest-7-en-6-one with that published by Ikekawa *et al.*[10] showed certain qualitative and quantitative differences. In the light of the slow rate of reaction of the C-14α-hydroxyl group and the conditions for the trimethylsilyl reaction given by Ikekawa *et al.*, the mass spectrum they obtained is better interpreted as that of the di-TMS

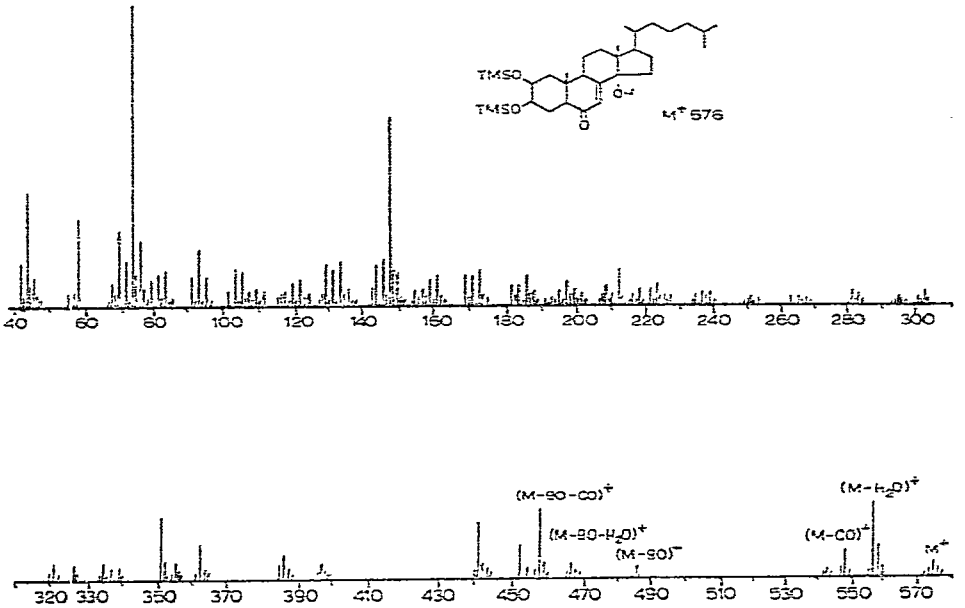

Fig. 2. Mass spectrum of 14α-hydroxy-2β,3β-bis(trimethylsilyloxy)-5β-cholest-7-en-6-one at 80 eV.

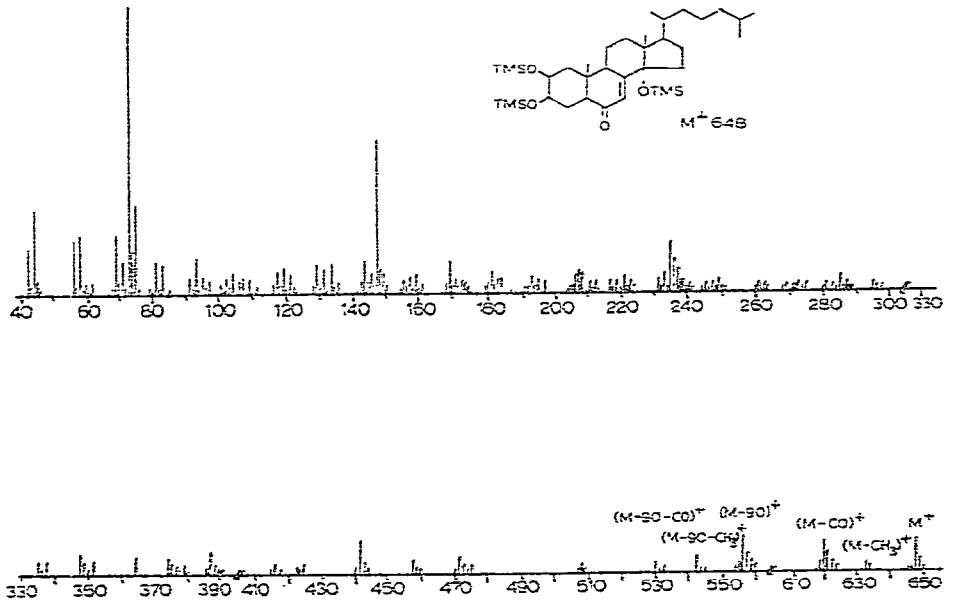

Fig. 3. Mass spectrum of 2β,3β,14α-tris(trimethylsilyloxy)-5β-cholest-7-en-6-one at 80 eV.

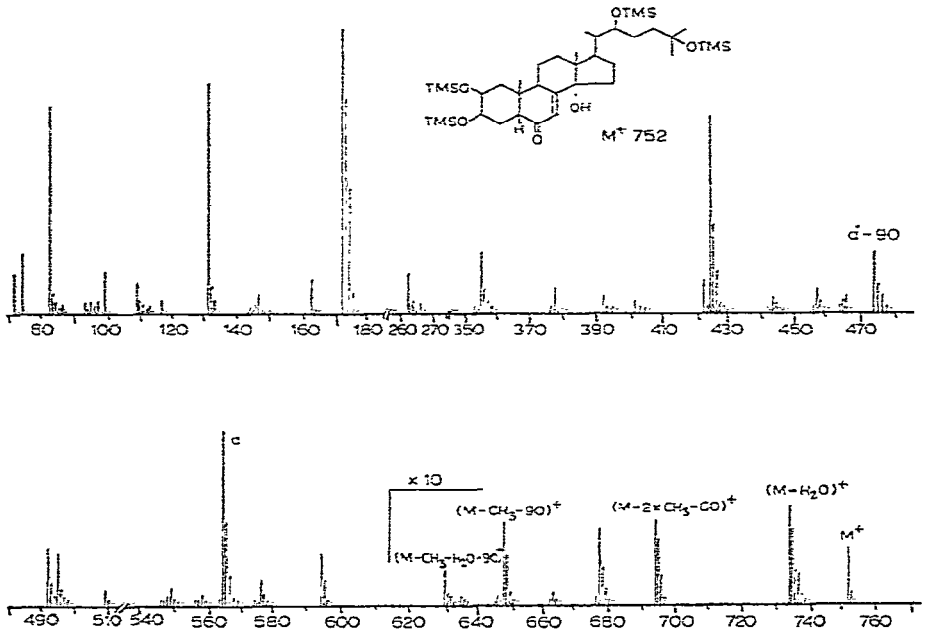

Fig. 4. Mass spectrum of ecdysone 2β,3β,22,25-tetrakis(trimethylsilyl) ether at 20 eV.

ether with a small contribution from the fully trimethylsilylated product. Typical losses in the mass spectrum from the molecular ion are CH_3, CO, and $(CH_3)_3SiOH$, as indicated.

Ecdysone (Ia) at room temperature with TMSI produced one peak on GC. The mass spectrum of this compound (Fig. 4) indicates that the four unhindered hydroxyl groups at C-2, C-3, C-22 and C-25 were all rapidly silylated under these conditions. The 14α-hydroxyl group was only silylated by heating at 140° overnight; the mass spectrum of the pentakis(trimethylsiloxy)ecdysone is given in Fig. 5.

20-Hydroxyecdysone (Ib), when heated at 100° for 1 h in TMSI, as recommended by Ikekawa *et al.*[10], produces two products separated by GC on a 3 ft. × 3 mm I.D. column of 1 % OV-101 on Gas-Chrom Q at 278°, with a carrier-gas flow-rate of 85 ml/min (retention times 3.8 and 4.5 min). The two components were separated by TLC on silica gel, developing in toluene–ethyl acetate (7:3), eluted with diethyl ether and identified as the tetrakis(trimethylsiloxy) ether (Fig. 6), $R_F = 0.54$, and the pentakis(trimethylsiloxy) ether (Fig. 7), $R_F = 0.67$, on the basis of their mass spectra. The pentakis(trimethylsiloxy) ether was obtained virtually quantitatively by heating 20-hydroxyecdysone at 100° for 4 h. Access to the C-20 hydroxyl group is hindered by more rapid formation of the silyl ether at C-22. The hexakis(trimethylsilyl) ether of 20-hydroxyecdysone was virtually the sole product when 20-hydroxyecdysone was heated in TMSI at 140° for approximately 20 h. The mass spectrum of the hexakis ether is given in Fig. 8.

The difficulty of silylating the 14α-hydroxyl group can be compared with some other hindered sterol hydroxyl groups. Maune *et al.*[17] have found the 14β-hydroxyl in cardiac aglycones (cardenolides) was converted to the silyl ether quantitatively

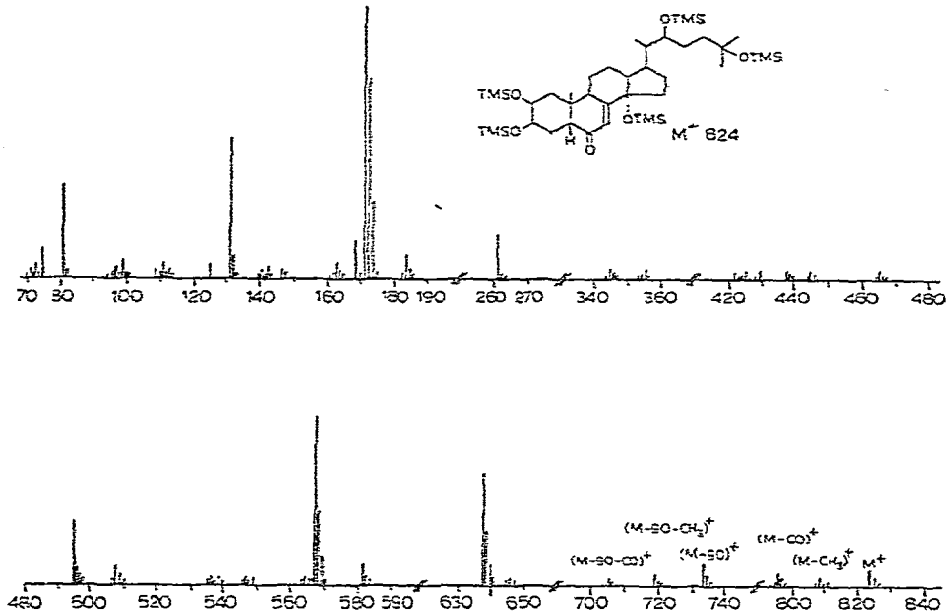

Fig. 5. Mass spectrum of ecdysone pentakis(trimethylsilyl) ether at 20 eV.

with the powerful silylating mixture TMSI–BSA–TMCS at 60° for three days. The hindered 17α-hydroxyl of 3α,17α,20-trihydroxy-5β-pregnane required heating for 15 h for complete reaction under comparable conditions for the 14α-hydroxyl group of ecdysones. The addition of TMCS had a marked effect on the reaction rate of the

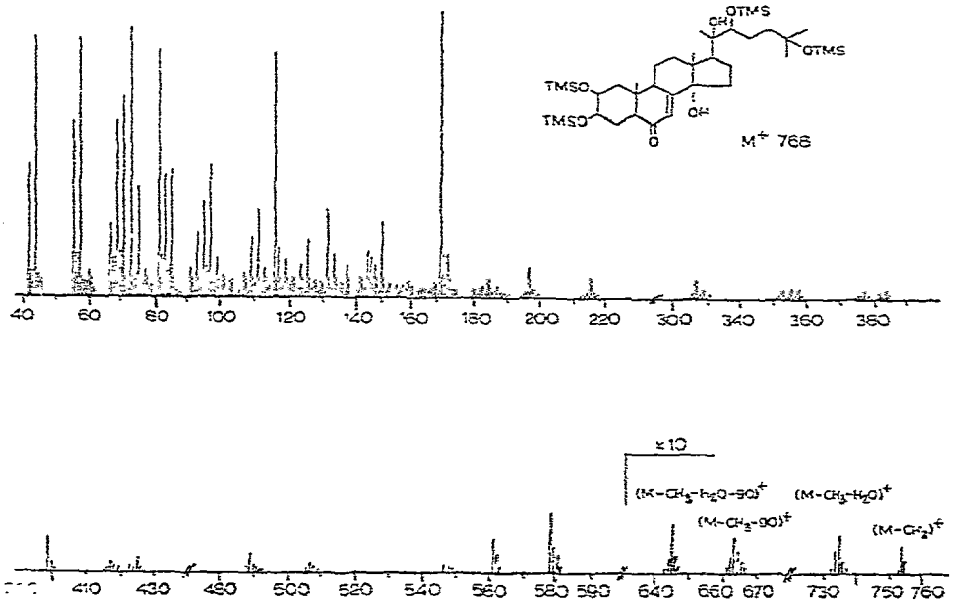

Fig. 6. Mass spectrum of 20-hydroxyecdysone 2β,3β,22,25-tetrakis(trimethylsilyl) ether at 80 eV.

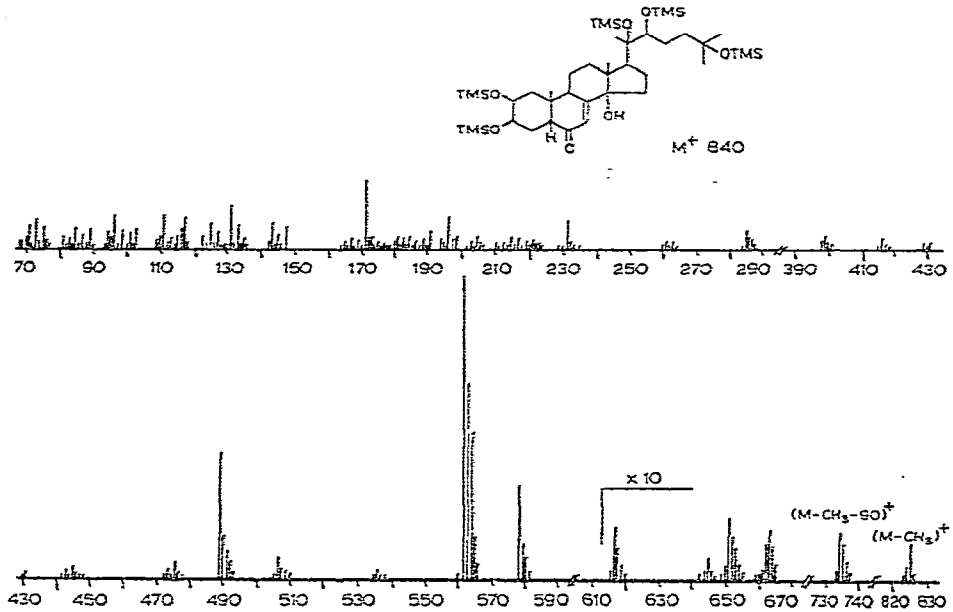

Fig. 7. Mass spectrum of 20-hydroxyecdysone 2β,3β,20,22,25-pentakis(trimethylsilyl) ether at 80 eV.

14α-hydroxyl group, but it also catalysed the formation of the enol ether of the 6-keto group. Addition of 1% TMCS to TMSI enabled the 14α-hydroxyl group to be silylated in 4 h at 100°, but larger quantities caused formation of the enol-TMS ether as well. The addition of traces of TMCS was effective for the pure hormone, but with

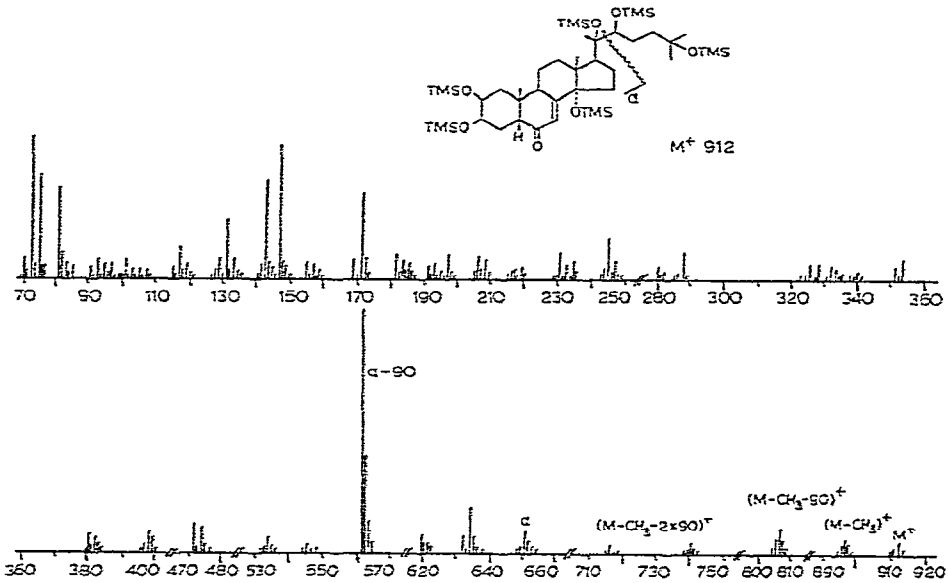

Fig. 8. Mass spectrum of 20-hydroxyecdysone hexakis(trimethylsilyl) ether at 80 eV.

crude biological material its effect was lost, probably because it reacted with other materials. A variety of substances, *e.g.*, piperidine, benzoic acid, catalyse the silylation of hydroxyl groups. Solid potassium acetate increases the rate of reaction of TMSI, enabling the 14α-hydroxyl group to be quantitatively trimethylsilylated in 3 h at room temperature. Our observations on catalysis are only preliminary but do indicate that a rapid method for the formation of the fully silylated derivative without the production of the enol-TMS ether is a possibility.

The ease of formation of enol-TMS ethers in ecdysones is also influenced by the nature of the substituent at C-14. A sample of 5α-cholest-7-en-6-one is easily converted into the enol-TMS ether when heated with TMSI–TMCS (1:1) or with TMSI at room temperature in the presence of potassium acetate. Measurement of UV spectra in hexane gave $\lambda_{max.} = 255$ nm and $\varepsilon_{max.} = 15{,}500$ which is in agreement with the hetero-annular diene structure of 6-(trimethylsiloxy)-5α-cholest-6,8(14)-diene. The mass spectrum had a prominent molecular ion (M^+, *m/e* 456).

In the presence of a C-14 oxy substituent, enol ethers do not form easily and reactions are rarely quantitative. From 14α-hydroxy-5α-cholest-7-en-6-one in TMSI–TMCS (9:1) at 50° was obtained 14α-hydroxy-6-(trimethylsiloxy)-5α-cholest-6,8(9)-diene with $\lambda_{max.} = 283$ nm, $\varepsilon_{max.} = 24{,}000$ and mass spectra (M^+, *m/e* 472). Further heating increases the amount of the 14α-TMS compound and gave rise to other peaks, perhaps due to dehydration products.

The spectra published for 2β,3β,14α-trihydroxycholest-7-en-6-one tris-TMS ether[10], ecdysone pentakis-TMS ether[11], 20-hydroxyecdysone pentakis-TMS ether[10], and hexakis-TMS ether[10] we believe can be interpreted as being mixtures of these compounds with the corresponding bis-, tris-, tetrakis-, and pentakis-TMS ethers respectively. We therefore include spectra of each of these pure derivatives together with that of authentic 20-hydroxyecdysone hexakis-TMS ether, as a guide to those wishing to identify the derivatives. The mass spectra of mixed derivatives can easily arise in the GC–MS of high-molecular-weight substances when a singly pumped mass spectrometer is used. Choosing high temperatures and short columns to overcome the low volatility of the sample and the vacuum restrictions of the mass spectrometer, may produce good peak shape at the expense of resolution.

For the analysis of ecdysones in insect and crustacean materials, we find it convenient to employ mild silylation conditions which leave the 14α-hydroxyl groups unchanged. After preliminary extraction of material, the trimethylsilyl ethers are prepared by heating the dry material at 100° for 6 h in a mixture of pyridine (100 μl) and TMSI (35 μl). After removal of reagents in vacuum, the residue is chromatographed on thin-layer plates, and the appropriate band eluted for GC with the electron capture detector.

CONCLUSIONS

Complete protection of all the hydroxyl groups of ecdysones as trimethylsilyl ethers is more difficult than hitherto realised. The 14α-hydroxyl group is the most hindered and reacts very slowly. Complete silylation can be catalysed by a variety of substances; for convenience in routine analysis of insect material, the 14-hydroxyl group is not protected. The isomeric peaks of shorter GC retention time we reported earlier[4] during silylation of ecdysones, were in fact due to completely silylated products.

ACKNOWLEDGEMENTS

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