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Note

Analysis of ecdysones by gas chromatography using electron capture detection

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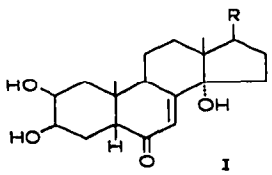
Ecdysone (Ia) and related compounds are generally recognized as being hormones controlling moulting in insects and crustaceans[1]. Their presence in sub-microgram quantities in insects makes their study difficult. Sensitive, but unspecific, bioassays such as the Calliphora test are available but in order to learn more about the sequence of events in the moulting process, the site of production and amount of the hormone, its manner of transport and target organs, an accurate chemical assay, capable of detecting sub-nanogram quantities in biological fluids would be valuable. In addition, over thirty ecdysones have been recognized in plants[2], in amounts as high as 0.1% by weight, so that screening of plants is important, as they provide the best source of the compounds for physiological study.

We have described earlier a method suitable for the detection by gas chromatography (GC) of 10^{-7} g of Ia or Ib as their methoxime-trimethylsilyl (TMS) ethers[3].



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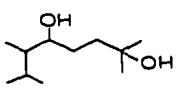
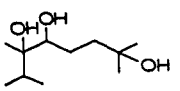
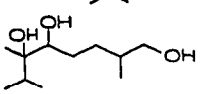
(Ia) Ecdysone, R =



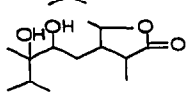
(Ib) 20-Hydroxyecdysone, R =



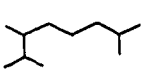
(Ic) Inokosterone, R =



(Id) Cyasterone, R =



(Ie) 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one, R =



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Ikekawa *et al.*[4] have claimed an improved method of detecting heptafluorobutyrate TMS ethers which is more sensitive. We now describe a method of determining the TMS ethers of ecdysones which is both highly sensitive, simple to perform and applicable directly to crude biological samples. The method requires treating the sample with trimethylsilylimidazole and detecting the TMS ethers of ecdysones directly by GC with the electron capture detector (GC–ECD)

EXPERIMENTAL AND RESULTS

The method of Ikekawa *et al.*[4] required first heating the ecdysone at 100° for 1 h with trimethylsilylimidazole. We found that using trimethylsilylimidazole, this always produces several peaks, and for 20-hydroxyecdysone (Ib), the three major peaks could be identified as the hexakis-TMS ether, the pentakis-TMS ether and an epimer of the hexakis-TMS ether (Fig. 1). Exact conditions varied with the batch of reagent. The effect is problably due to traces of trimethylchlorosilane. Trimethylsilylimidazole prepared in the laboratory gave the hexakis ether as described by Ikekawa, but addition of less than 1% of trimethylchlorosilane caused isomerization. Trimethylsilylimidazole rapidly converts ecdysone and 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one (Ie) to their completely protected silyl ethers in less than 30 min at room temperature giving single peaks on GC. Under the same conditions, 20-hydroxyecdysone produces

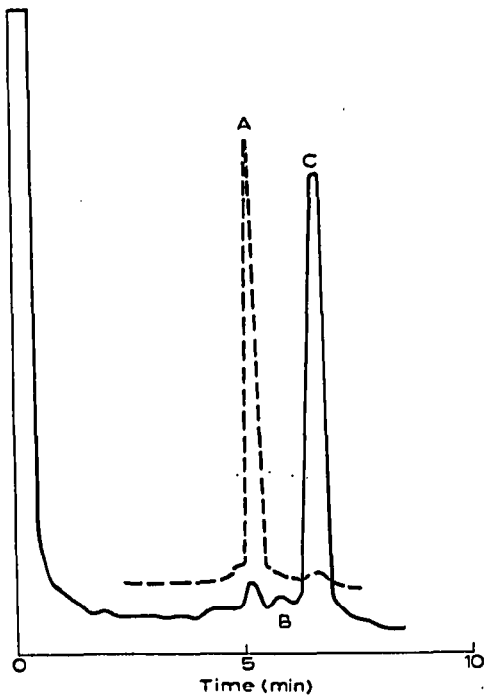

Fig. 1. GC analysis of the silyl ethers of 20-hydroxyecdysone produced by treatment with trimethylsilylimidazole at 40–45° for 30 h (———) and at 140° for 40 h (— — —). Operating conditions: 3-ft. column of 1% OV-101 on CQ, at 268°; nitrogen flow-rate, 35 ml min^{-1}; FID. A = Isomerized hexakis-TMS ether (5.0 min); B = pentakis-TMS ether (5.6 min); C = hexakis-TMS ether (6.4 min).

mainly the pentakis-TMS ether (approx. 95%), the hindered 20-OH group remains unreacted. At 40–45° conversion of the 20-OH group to silyl ether is slow, at 100–110°, reaction is complete 90 min, at 140–150° for 40h or with added trimethylchlorosilane at 100°, the product of shorter retention time is virtually the sole product. The mass spectrum of this product indicates it is a hexakis-TMS ether of the 5β-isomer with epimerization of at least one carbon atom.

The detection limit of 50×10^{-9} g with a flame ionization detector (FID) is too low for insect work. Ikekawa *et al.*[4,5] converted their TMS ethers into a heptafluorobutyrate-TMS ether. The method has the disadvantage of requiring a further reaction step and does not give complete conversion to one product, though it is sensitive to the ECD down to 10^{-12} g. We find that, by good fortune, the TMS ethers of ecdysones are as sensitive to the ECD as the heptafluorobutyrate derivatives. The response of the ECD is linear over the range $5–700 \times 10^{-12}$ g for compounds Ia–Ie. Fig. 2 is a typical calibration curve, and Fig. 3 shows the ECD response to 25×10^{-12} g of inokosterone hexakis-TMS ether.

This sensitivity to ECD was unexpected, since sterols and their TMS ethers generally are not detected at low levels. From our preliminary work, it would appear that the necessary structural element is the 7-en-6-one group. Some 4-en-3-ones were 1000 times less sensitive, though methoximes of the 7-en-6-one are as sensitive as the parent ketone.

We have successfully used flophemesyl derivatives for the GC of human steroid hormones at low concentration[6]. Still greater sensitivity might be achieved with flophemesyl derivatives of ecdysones, however, when Ia or Ib are treated with flophemesylamine only three hydroxy groups are converted to flophemesyl ethers (probably those at the 2, 3, and 22 positions). The resulting derivatives did not give good peak shape on chromatography and there was some loss due to absorption on

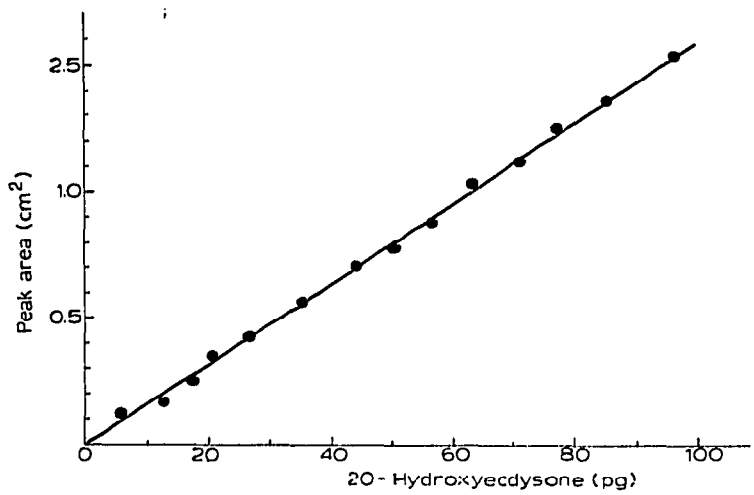

Fig. 2. Response of ECD to the isomerized hexakis-TMS ether of 20-hydroxyecdysone shown as picograms 20-hydroxyecdysone taken against peak area in cm². Pye Model 84 chromatograph; ECD detector; pulse width, 0.75 μsec; pulse period, 50 μsec; pulse height, 47–60 V; detector temperature, 300°; column, as in Fig. 1.

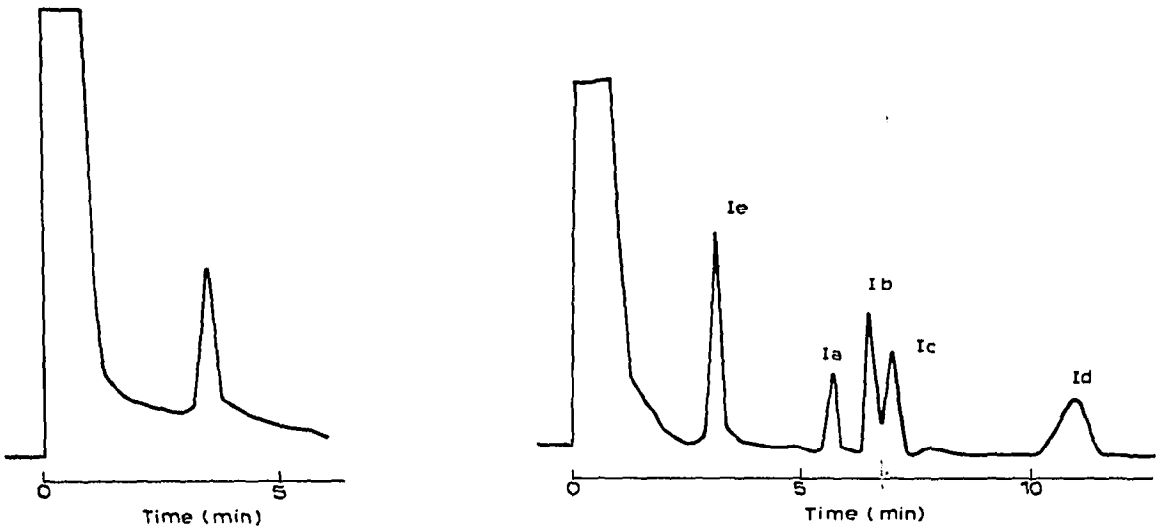

Fig. 3. Electron capture response to 25 pg of the hexakis-TMS ether of inokosterone. Operating conditions, as in Figs. 1 and 2; temperature, 275°.

Fig. 4. Separation of a mixture of TMS ethers of ecdysones. Approximately 30 pg each of the TMS ethers of compounds Ia–Ie were mixed and injected in 1 μl benzene. Column, as in Fig. 1; temperature programmed from 250° to 300° at 4° min^{-1}; flow-rate, 45 ml min^{-1}; ECD conditions, as in Fig. 2.

the column at low concentrations. If the remaining free hydroxyl groups are converted to TMS ethers, the flophemesyl groups are cleaved at the same time.

The TMS ethers of ecdysones are excellent derivatives for their analysis in biological materials as they are sufficiently volatile in GC to produce good peak shape, are well separated (Fig. 4) and detectable at picogram levels by electron capture. For example, 20-hydroxyecdysone (Ib) was readily detected and determined quantitatively in a 300-g sample (fresh weight) of the common barnacle, *Balanus balanoides* after preliminary clean-up, treatment with trimethylsilylimidazole, thin-layer chromatography on silica gel and GC–ECD. Compound Ib was first isolated from a crustacean, *Jasus Lalandei*[7], but this is the first report of an ecdysone from the sub-class Cirripedia.

ACKNOWLEDGEMENTS

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