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Note

Catalysed derivatisation of trimethylsilyl ethers of ecdysterone

A preliminary study

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Ecdysteroids are a class of polyhydroxylated steroids of major importance in controlling the moult cycle of arthropods[1,2]. Over thirty ecdysteroids have also been isolated from higher plants although their role is obscure[3]. Whilst radioimmunoassay[4,5] is preferred for routine measurement of ecdysteroids, cross-reactivity occurs to a greater or lesser extent with all antisera. In contrast, gas chromatographic (GC) analysis of ecdysteroids, although laborious, can be used to separate and quantify individual ecdysones[6,7].

During a preliminary investigation of the ecdysteroids of crustaceans using GLC, inconsistent derivatisation of ecdysterone with *n*-trimethylsilylimidazole was observed, using previously published techniques[4,8,9]. This paper reports on a catalysed derivatisation procedure which has been found to be both rapid and reproducible.

EXPERIMENTAL

Equipment and chromatographic conditions

All analysis were performed using a Pye Unicam GCV gas chromatograph equipped with a 20-mCi ^{63}Ni electron-capture detector. A glass column (6 ft. × 4 mm) packed with 3% OV-101 on Chromosorb W HP (80–100 mesh), preconditioned with "Silyl 8" (Pierce) was used. The column temperature was 270°C, detector and injector temperature 310°C. Nitrogen carrier gas flow was 50–55 ml min^{-1}. Detector attenuation was normally mid-range (64×10^{-11}–32×10^{-11} A).

Reagents

Ecdysterone and *n*-trimethylsilylimidazole (TMSI) were obtained from Sigma. Further TMSI, and electron-capture grade hexane were purchased from Phase-Sep. All derivations were performed in 0.3-ml reactivials (Pierce). Other reagents were laboratory grade unless specified.

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Derivatisations

Methanolic solutions of ecdysterone (1 ng μl^{-1}) were evaporated and 20 μl of TMSI added to the dried extract. Dry potassium acetate (5 mg) was subsequently added. Vials were incubated for periods up to 3 h at 20°C. Reactions were terminated by adding 50 μl of hexane followed by the same volume of ultra pure water. After vigorous shaking, the hexane phase was removed and dried over anhydrous sodium sulphate. Up to 1 μl containing 25–1000 pg of ecdysterone of the hexane phase could then be injected directly on-column.

RESULTS

A typical reaction sequence for the potassium acetate catalysed reaction is shown in Fig. 1. After only 1 min of derivatisation, a prominent peak eluting at 5.1 min was observed. However, after 5 min of derivatisation, a small peak eluting at 4.3 min can be observed. Thirty minutes after initiation of derivatisation, most of the derivative elutes at 4.3 min. An example of these reaction kinetics is shown in Fig. 2. Reaction follows a hyperbolic sequence; after 2 h incubation at room temperature, ecdysterone is quantitatively silylated.

To test whether crude biological material interfered with the catalysed derivatisation reaction, purified haemolymph from *Palaemon elegans* was derivatised in the normal way. The results of such a derivatisation, using purified haemolymph from a prawn in late premoult are shown in Fig. 3. The peak eluting at 4.3 min

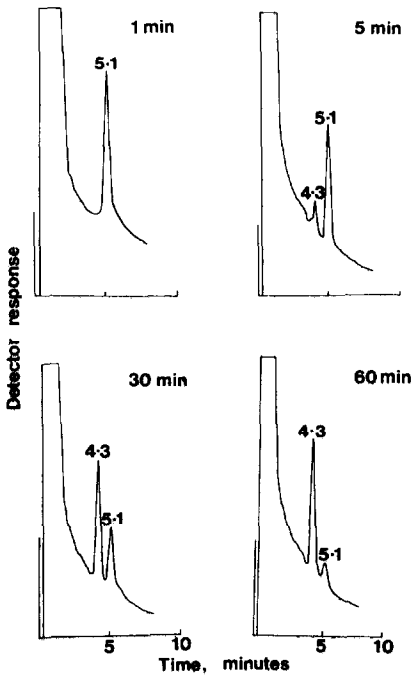

Fig. 1. Typical chromatograms showing the conversion of the peak eluting at 5.1 min, to peak eluting at 4.3 min. Solid potassium acetate catalysed reaction, incubated at 20°C for 1, 5, 30, 60 min. Chromatographic conditions as described in the text.

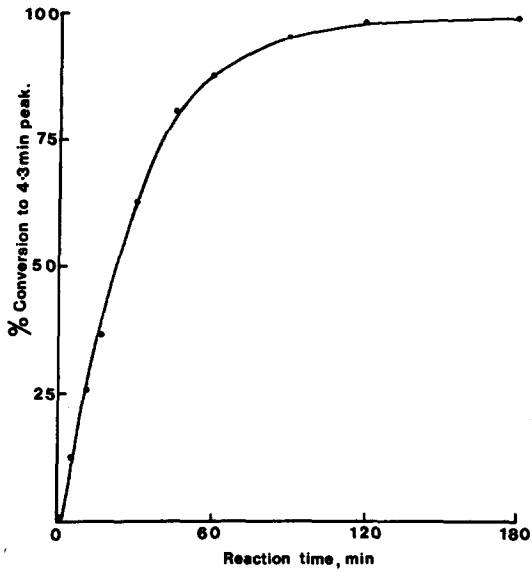

Fig. 2. Reaction curve showing the conversion of the compound eluting at 5.1 min to the compound eluting at 4.3 min during the potassium acetate catalysed reaction. Chromatographic conditions as described in the text.

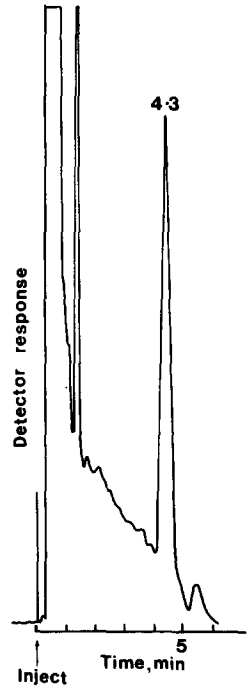

Fig. 3. Gas chromatographic response to a derivatised extract of haemolymph from a prawn sampled during late premoult. This represents about 1 ng of ecdysterone. Chromatographic conditions as described in the text.

represented approximately 1 ng of ecdysterone. The identity of the small peak eluting at approximately 5.5 min is unknown.

DISCUSSION

Several workers have reported derivatisation procedures for ecdysteroids using *n*-trimethylsilylimidazole[4,6,8–10]. However, during preliminary experiments using the methods of[4,9], variable derivatisation was observed, resulting in a mixture of products eluting at 4.3 and 5.1 min depending on the batch or supplier of TMSI. It has been suggested[10] that commercial preparations of TMSI often contain trichloromethylsilane (TCMS). 1% TCMS catalyses the formation of TMS ethers of ecdysterone[10] and thus could be considered as a catalyst. However, addition of more than 1% TCMS causes troublesome isomerisation reactions, and some commercial TMSI has been found to be unusable as a silylating agent due to traces of imidazole which profoundly inhibit the formation if TMS ethers of ecdysterone[8].

In view of the difficulty in producing fully silylated derivatives of ecdysteroids, some workers[8,9] employ mild derivatisation conditions which result in incomplete silylation (*i.e.* without derivatisation of the highly sterically hindered α14-OH group). This method was investigated, but generally this reaction was very slow, and the high temperatures (100°C) used in the process frequently destroyed ecdysterone before the reaction was completed.

In a reappraisal of the conditions used to create TMS ethers of ecdysteroids, it has been noted[11] that the previously published methods of producing ecdysteroid derivatives may yield mixed derivatives. However, it has been noted that acetates in general[9] and potassium acetate (among other compounds)[11] greatly accelerate rates of catalysation, indeed it has been reported that the addition of solid potassium acetate to the silylation medium allows quantitative derivatisation to occur within 3 h at room temperature[11].

The results using solid potassium acetate as a catalyst presented here, indicate that this reaction is indeed rapid, full derivatisation occurring within 3 h at 20°C. Using mild reaction conditions[11] it has been shown that TMS ethers formation proceeds via the tetrakis and pentakis ether, and finally after extensive incubation, to the hexakis ether which displays a somewhat shorter retention time on OV-101 columns than the tetrakis or pentakis ether. It seems possible therefore that the peak eluting at 4.3 min in the present study might also represent the fully silylated (hexakis) derivative, but, without GC–MS analysis it is impossible to determine with certainty the nature of the derivatives formed in this reaction (see ref. 11 for a full discussion of TMS derivatives of ecdysterone analysed by GC–MS).

In the present study, the detection limit for ecdysterone was in the region of 25 pg. It has been reported[10] that the lower limit for detection of TMS ethers of ecdysterone is about 5 pg. The reason for this difference is not clear, although it may suggest that the catalysed derivatisation results in the formation of an enol ether, since the α-β unsaturated ketone is thought to be an important part of the electrophore for detection[11].

It has been observed[12] that the presence of crude biological material may inhibit the formation of TMS ethers of ecdysteroids, but purification of haemolymph from *Palaemon elegans*, using the method of ref. 9, and potassium acetate catalysed TMSI derivatisation demonstrated that this did not occur.

The preliminary results presented here suggest that the potassium acetate derivatisation of ecdysterone with TMSI might be a rapid and reliable method for producing TMS ethers of ecdysterone, using commercially available TMSI. However, further studies are necessary to determine whether this method can be used for other ecdysones, and importantly to determine the exact nature of the derivatives formed during this reaction.

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