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Identification of the 22-phosphate esters of ecdysone, 2-deoxyecdysone, 20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone from newly laid eggs of the desert locust, *Schistocerca gregaria*

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1. The four major ecdysteroid (insect moulting hormone) conjugates present in the newly laid eggs of the desert locust, *Schistocera gregaria*, have been purified by reversed-phase and anion-exchange high-performance liquid chromatography. 2. The steroid moieties were identified as ecdysone, 2-deoxyecdysone, 20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone. 3. Phosphate analysis of acid-hydrolysed samples showed a steroid:phosphate ratio of approx. 1:1 for all four compounds. 4. The intact conjugates were identified as ecdysone 22-phosphate, 2-deoxyecdysone 22-phosphate, 20-hydroxyecdysone 22-phosphate and 2-deoxy-20-hydroxyecdysone 22-phosphate by fast atom bombardment mass spectrometry and ^{1}H, ^{13}C and ^{31}P n.m.r. 5. The significance of ecdysteroid phosphates as a source of free hormone during embryogenesis is discussed.

The occurrence of both free and conjugated ecdysteroids in the mature ovaries and eggs of insects is well documented (for a review see Hoffmann *et al.*, 1980). Although the function of egg ecdysteroids is still not clear, studies carried out on the developing eggs of a number of insect species indicate an apparent correlation between peaks of free ecdysteroids and the synthesis of embryonic membrane/cuticle (Dorn & Romer, 1976; Bulliere *et al.*, 1976; Hoffmann *et al.*, 1980; Matz, 1980; Cavallin & Fournier, 1981; Imboden & Lanzrein, 1982; Scalia & Morgan, 1982). In most of these studies the hormone titres do not include polar ecdysteroid conjugates, which are the major form of ecdysteroids in the eggs of some insect species: *Bombyx mori* (Mizuno & Ohnishi, 1975), *Galleria mellonella* (Hsiao & Hsiao, 1979), *Locusta migratoria* (Hoffmann *et al.*, 1980) and *Schistocerca gregaria* (Gande *et al.*, 1979; Dinan & Rees, 1981*a*). Such ecdysteroid conjugates can be hydrolysed with a crude arylsulphatase preparation from *Helix pomatia*. In *S. gregaria*, 95% of the ovarian ecdysteroids are in the polar conjugate form which are passed into the newly laid eggs (Dinan & Rees, 1981*a*). The steroid moieties of the latter conjugates have been identified as ecdysone, 2-deoxyecdysone, 20-hydroxyecdysone, 2-deoxy-20-hydroxyecdysone, 26-hydroxyecdysone (Dinan & Rees, 1981*a*; Isaac *et al.*, 1981*a*) and 20,26-di-hydroxyecdysone (R. E. Isaac & H. H. Rees, unpublished work).

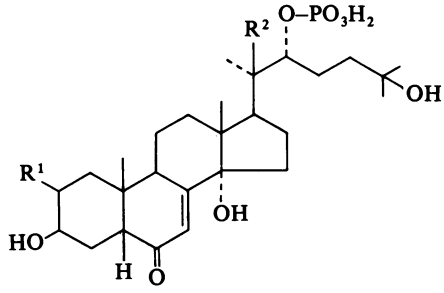
The paucity of information on the nature of ecdysteroid conjugates, which have also been found as metabolites of ecdysone in insect larvae (Koolman, 1982), has restricted studies on ecdysteroid metabolism and its control in insects. In the present paper we report the unequivocal identification of the major ecdysteroid conjugates in newly laid eggs of *S. gregaria* as ecdysone 22-phosphate (1), 2-deoxyecdysone 22-phosphate (2), 20-hydroxyecdysone 22-phosphate (3) and 2-deoxy-20-hydroxyecdysone 22-phosphate (4). A preliminary report of part of this work has appeared (Isaac *et al.*, 1982*b*).

Abbreviations used: FAB, fast atom bombardment; h.p.l.c., high-performance liquid chromatography.

Materials and methods

Chemicals

[1α,2α-^{3}H]Cholesterol (43 Ci/mmol) was obtained from Amersham International, Amersham, U.K., and ecdysone was from Simes, Milan, Italy. The following ecdysteroids were gifts: 20-hydroxyecdysone from Dr. G. B. Russell, D.S.I.R., New Zealand; 2-deoxyecdysone from Professor E. Ohnishi, Nagoya University, Nagoya, Japan; 2-deoxy-20-hydroxyecdysone from Dr. D. H. S. Horn,



(1) $R^1 = OH, R^2 = H$
(2) $R^1 = R^2 = H$
(3) $R^1 = OH, R^2 = OH$
(4) $R^1 = H, R^2 = OH$

C.S.I.R.O., Melbourne, Australia. H.p.l.c. grade solvents were purchased from Rathburn Chemicals, Walkerburn, U.K., *Helix pomatia* arysulphatase preparation was from Sigma, and glycerol and xenon (99.995%) were obtained from BDH Chemicals.

Insects

The rearing of *S. gregaria* and the collection of eggs were carried out as described previously (Dinan & Rees, 1981*a*). Newly laid eggs were stored at −20°C until they were extracted.

Extraction of ecdysteroid conjugates from newly laid eggs

Eggs were macerated and extracted four times in methanol/water (7:3, v/v; 250ml/100g of eggs) and the extract was partitioned between methanol/water (7:3, v/v) and hexane to remove apolar material. A mixture of polar ecdysteroid conjugates was isolated from the aqueous methanol fraction by chromatography on a silicic acid column, as described by Dinan & Rees (1981*a*,*b*).

Formation of [^{3}H]ecdysteroid conjugates

Radioactivity was incorporated into conjugated ecdysteroids from cholesterol substrate by injecting adult female locusts with [1α,2α-^{3}H]cholesterol (5μCi, 0.1nmol) as described by Dinan & Rees (1981*b*). The [^{3}H]ecdysteroid conjugates were isolated from newly laid eggs by the procedure already described, the incorporation of ^{3}H from cholesterol, in a number of experiments, being in the region of 0.5–0.9%.

Radiochemical methods

Radioactivity was assayed on a Beckman model LS 9800 liquid scintillation spectrometer after the addition of Scintran Cocktail T (4ml) (BDH). Counting efficiencies for ^{3}H were approx. 40%.

Hydrolysis of ecdysteroid conjugates

Samples were dissolved in 0.1M-Mes (4-morpholine-ethanesulphonic acid) buffer (1ml), pH5.5, and incubated with a crude arylsulphatase preparation (250units; one unit will hydrolyse 1.0μmol of nitrocatechol sulphate in 1h at pH5.0 and 37°C) from *Helix pomatia* for 17h at 37°C. Reactions were terminated by the addition of ethanol (4ml) and the protein precipitate was extracted twice with methanol (4ml). Free ecdysteroids were isolated from the combined alcoholic extracts by chromatography on a silicic acid column (Dinan & Rees, 1981*a*).

High performance liquid chromatography

A Waters instrument (Water Associates, Northwich, U.K.), incorporating two M6000A pumps in conjunction with a model 660 solvent programme controller, U6K injector and a model 441 detector set at 254nm, was used. Free ecdysteroids were chromatographed either on a reversed-phase column (Ultrasphere-ODS; 15cm × 4·6mm internal diameter; Anachem, Luton, U.K.) as described by Isaac *et al.* (1982*a*) or on an aminopropyl column (APS Hypersil; 25cm × 4·6mm internal diameter; Shandon Southern Products, Runcorn, U.K.) as described by Dinan *et al.*, 1981. Ecdysteroid conjugates were separated on the following h.p.l.c. systems: system 1, ion suppression reversed-phase chromatography on (*a*) a Partisil ODS-3 column (Whatman, Maidstone, U.K.; Magnum 9, 50cm × 9.4mm internal diameter) eluted at a flow rate of 4ml/min with methanol in 20mM-sodium acetate buffer, pH5.5 (7:13, v/v) or (*b*) a Partisil ODS-3 column (25cm × 4.6mm internal diameter) eluted at a flow rate of 2ml/min with a linear gradient (30min) of methanol in 20mM-sodium citrate buffer, pH6.5 changing from 1:9 (v/v) to 7:3 (v/v); system 2, reversed-phase chromatography on a Partisil ODS-3 column (Magnum 9, 50cm × 9.4mm internal diameter) eluted at a flow rate of 4ml/min with a linear gradient (30min) of methanol in water changing from 1:5 (v/v) to 7:3 (v/v); system 3, anion-exchange chromatography on either (*a*) a Partisil SAX column (Whatman, Magnum 9, 50cm × 9.4mm internal diameter) eluted at a flow rate of 6ml/min with 0.1M-ammonium acetate or (*b*) a Partisil SAX column (25cm × 4.6mm internal diameter) eluted at a flow rate of 2ml/min with 0.1M-ammonium acetate. Samples collected from h.p.l.c. were concentrated under reduced pressure and were desalted whenever buffer was employed in the mobile phase. In the case of radioactive samples, fractions (2ml) were collected and scintillation cocktail (4ml) was added directly before radioassay.

Desalting of samples

Ecdysteroid conjugates were loaded onto reversed-phase SEPPAK cartridges (Waters Associates) in water (5ml), salt was eluted with water (5ml) and the ecdysteroid conjugates were recovered by eluting with methanol (5ml).

Assay of inorganic phosphate

Samples of purified ecdysteroid conjugates were hydrolysed with 0.5M-HCl at 110°C in sealed vials. After 6h, the reaction mixtures were evaporated to dryness under N_2 and the residues were redissolved in distilled water. The inorganic phosphate liberated was determined by the method of Chen *et al.* (1956).

Mass spectrometry

Electron impact, chemical ionization and FAB mass spectra were recorded on a VG Micromass 7070F spectrometer. Negative-ion FAB mass spectra were obtained using a primary atom beam of xenon with energy 8keV. Samples, as solutions in methanol, were added to glycerol on the probe tip prior to FAB.

Analysis of phosphoric acid by g.l.c./mass spectrometry

The residue after acid hydrolysis of the conjugates was subjected to the derivatization procedure of Graff *et al.* (1980). Then, the phosphoric acid derivative (tris-trimethylsilyl phosphate) was analysed directly by g.l.c./mass spectrometry on a Pye–Unicam 204 gas chromatograph fitted with a 1.5m × 4mm glass column [3% OV-17 on Gas Chrom Q (100–120mesh); temperature, 130°C] coupled via a single-stage all-glass jet separator to a VG Micromass 7070F mass spectrometer.

N.m.r. spectroscopy

Fourier transform n.m.r. spectra were recorded on either a Bruker 400MHz (S.E.R.C. High Field NMR Service, Chemistry Department, University of Sheffield) or a Bruker 250MHz (Bruker Spectrospin, Coventry, U.K.) instrument. Samples were dissolved in (^{2}H)methanol and the ^{1}H and ^{13}C n.m.r. spectra were referenced, respectively, to tetramethylsilane and methanol ($C^2H_3O^2H$) at δ 49.0p.p.m. downfield from tetramethylsilane, whereas ^{31}P n.m.r. spectra were referenced to external H_3PO_4.

Results

Isolation of the two major ecdysteroid conjugates (1) and (2)

Anion-exchange h.p.l.c. [system 3(*a*); Fig. 1] of the [^{3}H]ecdysteroid conjugate fraction from newly laid eggs (10g) resulted in the separation of the two radioactive and u.v.-absorbing compounds, (1) and (2). The radioactivity recovered in (1) and (2) was 70% of the total applied to the column. For structural studies, non-radioactive samples of (1) and (2) were isolated from newly laid eggs (110g) on the same h.p.l.c. system. Compound (1) was further

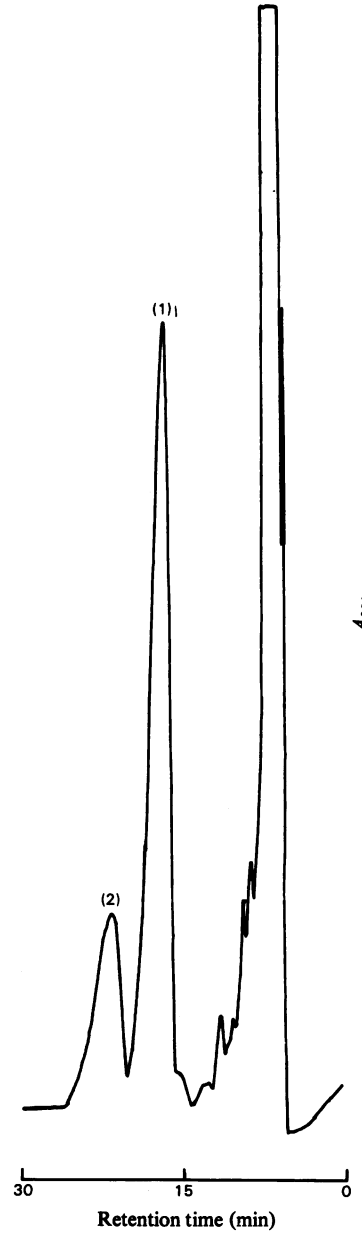


Fig. 1. *Isolation of ecdysteroid conjugates (1) and (2) by anion-exchange h.p.l.c.*
Retention volumes for (1) and (2) on h.p.l.c. system 3(*a*) were 87ml and 115ml, respectively. The elution was monitored by measuring the u.v. absorbance at 254nm.

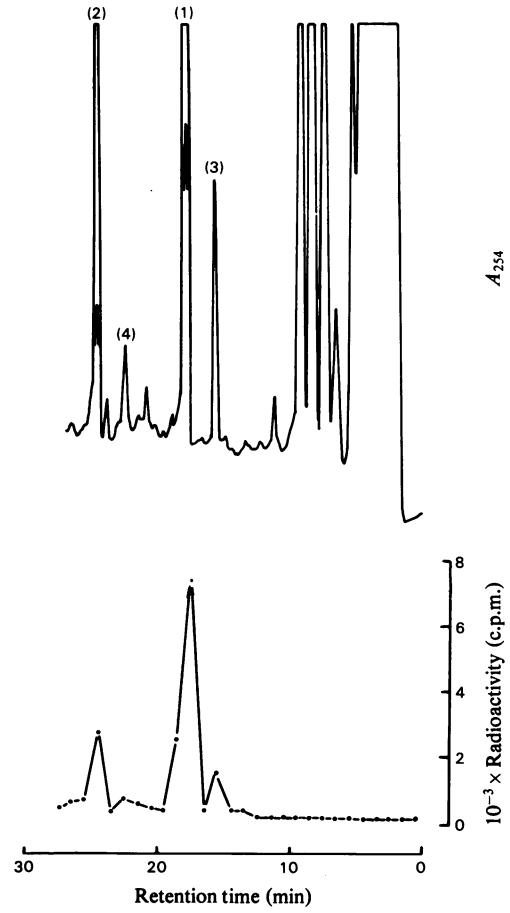


Fig. 2. *Separation of [^3H]ecdysteroid conjugates by ion-suppression reversed-phase h.p.l.c.*
Compounds (3) and (4) were separated from the major ecdysteroid components (1) and (2) by using a methanol/sodium citrate buffer, pH 6.5, gradient (system 1*b*) for elution. The chromatography of (1) (36 ml), (2) (50 ml), (3) (31 ml) and (4) (45 ml) was monitored by measuring the u.v. absorbance at 254 nm and by collecting fractions at 1 min intervals for determination of radioactivity.

purified by ion-suppression reversed-phase h.p.l.c. [system 1(*a*); retention volume, 65 ml]. Final purification of both (1) and (2) was achieved by reversed-phase h.p.l.c. [system 2(*a*); retention volume for (1), 87 ml and for (2), 115 ml]. U.v. spectra of (1) and (2) showed an absorbance maximum at 242 nm (methanol) which is characteristic of the α,β-unsaturated oxo group of ecdysteroids (ε_{242} 12400 litre · mol^{-1} · cm^{-1}) and indicated a yield of 3.4 mg for (1) and 1.7 mg for (2). A third ecdysteroid conjugate, later shown to be identical with compound (3), was isolated in smaller amounts from the anion-exchange h.p.l.c. [system 3(*a*); retention volume, 170 ml].

Isolation of ecdysteroid conjugates (3) and (4)

The less abundant ecdysteroid conjugates (3) and (4) were separated by chromatography of the ecdysteroid conjugate fraction from a second batch of newly laid eggs (340 g), on ion-suppression reversed-phase h.p.l.c. [system 1(*b*); Fig. 2]. Further purification of (3) and (4) was achieved by anion-exchange h.p.l.c. [system 3(*b*); retention volumes for (3), 32 ml and for (4), 42 ml] followed by reversed-phase h.p.l.c. [system 2; retention volume for (3), 26 ml and for (4), 60 ml]. Compound (3) isolated during the separate extraction of (1) and (2) was also purified further by the foregoing three step procedure. Both samples of (3) were combined for structural analysis. U.v. spectra of (3) and (4) showed an absorbance maximum at 242 nm (methanol) and indicated a total yield of 2.3 mg and 0.15 mg of (3) and (4), respectively.

Identification of the ecdysteroid conjugates (1–4)

Hydrolysis of (1), (2), (3) and (4) with *H. pomatia* enzymes released ecdysone, 2-deoxyecdysone, 20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone, respectively, which were identified by co-chromatography with authentic compounds on two h.p.l.c. systems (reversed-phase and aminopropyl columns) and by electron impact mass spectrometry (Dinan & Rees, 1981*a*; Isaac *et al.*, 1981*a*). The recoveries of (1), (2), (3) and (4) were between 70 and 80% of the maximum expected on the basis of the previously observed yields of ecdysone, 2-deoxyecdysone, 20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone from enzymic hydrolysis of the crude conjugate fraction (Dinan & Rees, 1981*a*; Isaac *et al.*, 1981*a*).

Mass spectrometry

Electron impact and chemical ionization mass spectrometry of the conjugates gave ions characteristic of the respective ecdysteroids but not of the intact molecules or of the conjugating moiety. This probably resulted from the thermal elimination of the conjugating moiety on heating the sample prior to ionization. The problem of thermal lability of the ecdysteroid conjugates was overcome by the use of FAB mass spectrometry. This technique is particularly suitable for involatile and thermally labile compounds as the samples are ionized at ambient temperature in the liquid phase (Barber *et al.*, 1981). The major peaks at high mass in the negative ion FAB mass spectra of compounds (1), (2), (3) and (4) occurred at *m/z* 543, 527, 559 and 543, respectively (Fig. 3). Since $[M-H]^-$ ions are generally observed in the negative ion mode (Taylor, 1981), molecular weights of 544, 528, 560

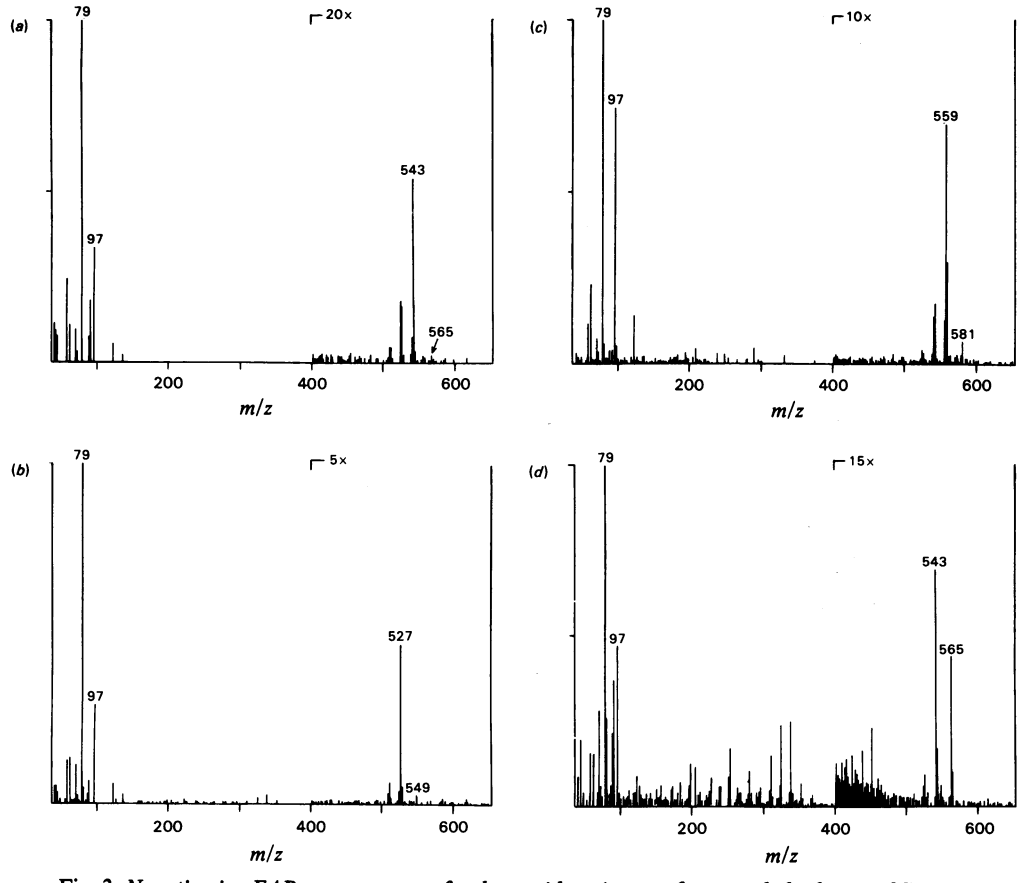


Fig. 3. *Negative-ion FAB mass spectra of ecdysteroid conjugates from newly laid eggs of S. gregaria*
The negative ion FAB mass spectra of ecdysone phosphate (*a*), 2-deoxyecdysone phosphate (*b*), 20-hydroxyecdysone phosphate (*c*) and 2-deoxy-20-hydroxyecdysone phosphate (*d*) show distinct $[M-H]^-$ ions at *m/z* 543, 527, 559 and 543, respectively.

and 544 are indicated for (1), (2), (3) and (4), respectively. The observation also of $[M-H]^-$ peaks for the monosodium salts of (1), (2), (3) and (4) at *m/z* 565, 549, 581 and 565, respectively, confirms the assignment of molecular weights. The detection of sodium salts is common in FAB mass spectrometry, especially for acidic samples (Taylor, 1981). Structures which are consistent with the mass spectra for (1), (2), (3) and (4) are the phosphate and sulphate esters of the parent ecdysteroids. The observation of the $[M-H]^-$ ions does not distinguish between these two possibilities. However, the prominent peaks at *m/z* 79 and 97 in the negative ion spectra of all four compounds correspond to PO_3^- and $H_2PO_4^-$ ions, respectively (Fig. 3). In contrast, sulphate esters of sterols yield large peaks at *m/z* 80 (SO_3^-) and 97 (HSO_4^-) (Rose *et al.*, 1983). The stability of these oxyanions dictates that they give rise to very large peaks in negative ion FAB mass spectra which are thus highly diagnostic of the conjugate moiety.

Definitive corroboration of the presence of phosphate in (1) and (2) was obtained by acid hydrolysis of the conjugates, followed by combined g.l.c./mass spectrometry of the liberated phosphate as its trimethylsilylated derivative. In each case, tris-trimethylsilyl phosphate was observed with a retention time and a mass spectrum identical with that of an authentic sample. In addition, proton-decoupled ^{31}P n.m.r. of (1) and (2) gave signals at δ +2.69 p.p.m. and δ +1.55 p.p.m., respectively, which is consistent with the presence of phosphate. Quantitative determination of inorganic phosphate in acid-hydrolysed samples of (1), (2), (3) and (4) showed an ecdysteroid/phosphate molar ratio of 1:0.91, 1:0.94, 1:0.90 and 1:0.90, respectively. The data presented demonstrate conclusively that the four ecdysteroid conjugates isolated are steroid phosphates.

Table 1. *^1H Chemical shifts (δ) of characteristic protons of ecdysteroid phosphates isolated from S. gregaria eggs and of authentic ecdysteroids*

P.m.r. spectra were recorded with (^2H)methanol as the solvent and either tetramethylsilane or CH_3OH (δ 3.3 p.p.m. downfield from tetramethylsilane) as reference. Chemical shifts are given downfield from tetramethylsilane.

	δ (p.p.m.)							
	3H signals				1H signals			
Compounds	C-18	C-19	C-21	C-26/27	C-2	C-3	C-22	C-7
Ecdysone	0.74 (s)	0.97 (s)	0.94, 0.97 (d,*J* 7 Hz)	1.20, 1.21 (2s)	3.85 (m,$W_{\frac{1}{2}}$ 21 Hz)	3.96 (m,$W_{\frac{1}{2}}$ 7 Hz)	3.61 (m,$W_{\frac{1}{2}}$ 16 Hz)	5.81 (d,*J* 2 Hz)
Ecdysone 22-phosphate (1)	0.75 (s)	0.97 (s)	0.97, 1.00 (d,*J* 7 Hz)	1.18. 1.19 (2s)	3.86 (m,$W_{\frac{1}{2}}$ 21 Hz)	3.97 (m,$W_{\frac{1}{2}}$ 8 Hz)	4.20 (m,$W_{\frac{1}{2}}$ 20 Hz)	5.81 (d,*J* 2 Hz)
2-Deoxyecdysone	0.73 (s)	0.96 (s)	0.93, 0.95 (d,*J* 7 Hz)	1.19/1.20 (2s)	—	*	*	5.80 (d,*J* 2 Hz)
2-Deoxyecdysone 22-phosphate (2)	0.74 (s)	0.96 (s)	0.96, 0.98 (d,*J* 8 Hz)	1.17, 1.19 (2s)	—	3.98 (m,$W_{\frac{1}{2}}$ 18 Hz)	4.20 (m,$W_{\frac{1}{2}}$ 22 Hz)	5.80 (d,*J* 2 Hz)
20-Hydroxyecdysone	0.89 (s)	0.96 (s)	1.19 (s)	1.19, 120 (2s)	3.84 (m,$W_{\frac{1}{2}}$ 21 Hz)	3.95 (m,$W_{\frac{1}{2}}$ 7 Hz)	3.33 (m,$W_{\frac{1}{2}}$ 14 Hz)	5.80 (d,*J* 2 Hz)
20-Hydroxyecdysone 22-phosphate (3)	0.89 (s)	0.96 (s)	1.26 (s)	1.18, 1.19 (2s)	3.84 (m,$W_{\frac{1}{2}}$ 22 Hz)	3.95 (m,$W_{\frac{1}{2}}$ 8 Hz)	4.07 (m, $W_{\frac{1}{2}}$ 22 Hz)	5.80 (d,*J* 2 Hz)
2-Deoxy-20-hydroxy-ecdysone 22-phosphate (4)	0.89 (s)	0.95 (s)	1.26 (s)	1.18, 1.20 (2s)	—	3.98 (m,$W_{\frac{1}{2}}$ 18 Hz)	4.07 (m,$W_{\frac{1}{2}}$ 22 Hz)	5.80 (d,*J* 2 Hz)

* Low sample concentration limited the observation of these proton signals.

Fourier transform p.m.r. spectroscopy

The p.m.r. spectra of the phosphate esters of ecdysone (1), 2-deoxyecdysone (2), 20-hydroxyecdysone (3) and 2-deoxy-20-hydroxyecdysone (4) were compared with the spectra of ecdysone and 20-hydroxyecdysone obtained using the same solvent [(^2H)methanol] (Table 1). The methyl proton signals were readily assigned by comparison with published spectra (Horn, 1971). The C*H*-OH signals of C-2, C-3 and C-22 were assigned by their width at half height (Horn, 1971; Mori, 1973) and by comparison of the spectra of ecdysone before and after acetylation of the hydroxy groups (spectra not shown). The only major differences between the spectra of (1) and (2) and those of the corresponding free ecdysteroids were the downfield shifts of the C-21 methyl [+0.03 p.p.m., (1) and (2)] and C-22 proton signals [+0.59 p.p.m., (1) and (2)]. Smaller changes in the chemical shifts of the C-26/C-27 resonances were also observed (Table 1). Similarly, the spectra of (3) and (4) show a marked downfield shift for the C-21 methyl (+0.07 p.p.m.) and the C-22 proton signals (+0.74 p.p.m.) as compared with the spectrum of 20-hydroxyecdysone. It is apparent that in all four ecdysteroid conjugates (1–4), introduction of the phosphate group only affects the resonances of protons at or close to C-22. The p.m.r. spectra provide conclusive evidence that the phosphate group of the ecdysteroid conjugates (1), (2), (3) and (4) is attached to the steroid through an ester linkage at C-22.

Table 2. *Chemical shifts (δ) of the hydroxy-substituted carbon atoms of ecdysteroid phosphates isolated from S. gregaria eggs and of authentic ecdysteroids in proton-decoupled ^{13}C n.m.r. spectra*

Spectra were recorded with (^2H)methanol as the solvent. Chemical shifts are downfield from tetramethylsilane which was used as a reference. The type of peak observed in the off-resonance decoupled spectra is indicated below the δ value. The δ for the C-2 and C-3 signals are interchangeable, except for compound (2). — indicates a non-substituted carbon atom.

	δ (p.p.m.)					
Compound	C-2	C-3	C-14	C-20	C-22	C-25
Ecdysone	68.7 (d)	68.5 (d)	85.2 (s)	—	75.3 (d)	71.5 (s)
Ecdysone 22-phosphate (1)	68.7 (d)	68.6 (d)	85.4 (s)	—	79.3 (d)	71.8 (s)
2-Deoxyecdysone 22-phosphate (2)	—	65.6 (d)	85.6 (s)	—	79.1 (d)	71.8 (s)
20-Hydroxyecdysone	68.7 (d)	68.6 (d)	85.3 (s)	77.9 (s)	78.4 (d)	71.3 (s)
20-Hydroxyecdysone 22-phosphate (3)	68.7 (d)	68.6 (d)	85.3 (s)	78.5 (s)	82.9 (d)	71.4 (s)

^{13}C Fourier transform n.m.r. spectroscopy

Proton noise-decoupled and off-resonance decoupled spectra of the phosphate conjugates of ecdysone (1), 2-deoxyecdysone (2) and 20-hydroxyecdysone (3) were recorded and compared with the

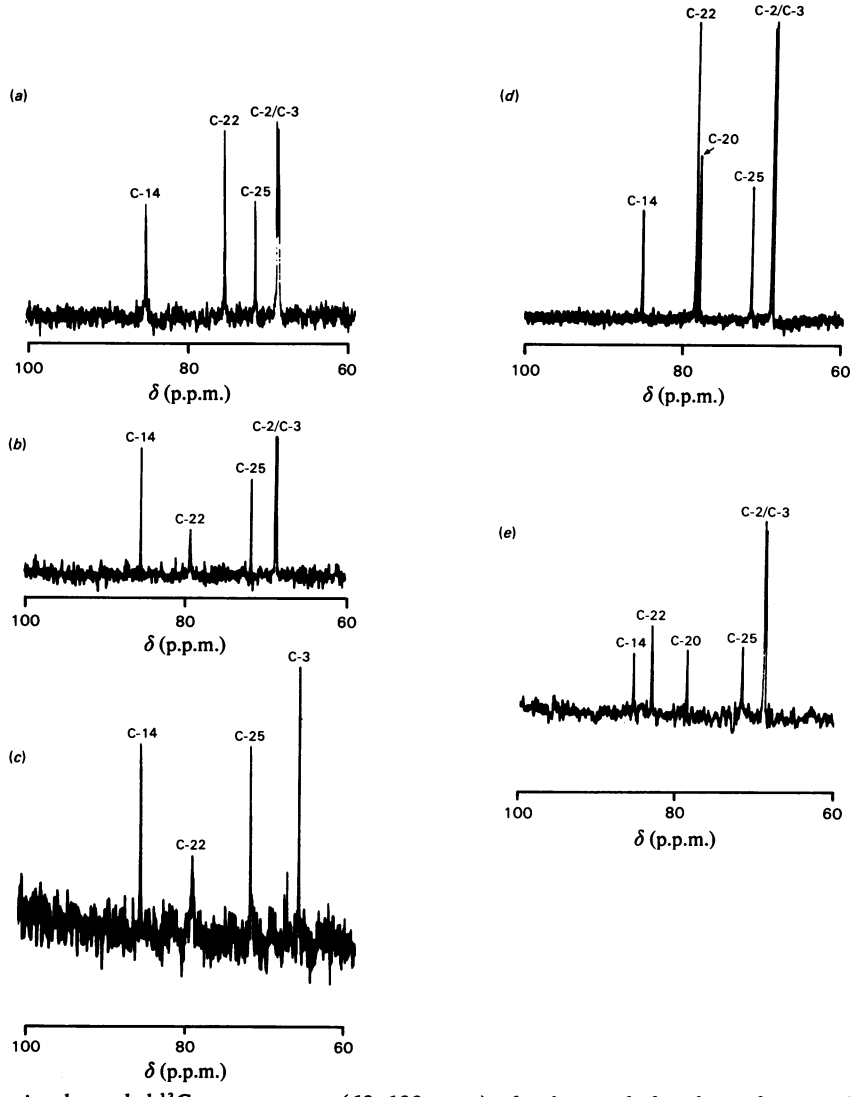


Fig. 4. *Proton noise decoupled ^{13}C n.m.r. spectra (60–100p.p.m.) of ecdysteroid phosphates from newly laid eggs of S. gregaria and of authentic ecdysone and 20-hydroxyecdysone*

The chemical shifts of hydroxy-substituted carbon atoms in the ^{13}C spectra of (*a*) ecdysone, (*b*) ecdysone 22-phosphate, (*c*) 2-deoxyecdysone 22-phosphate, (*d*) 20-hydroxyecdysone and (*e*) 20-hydroxyecdysone 22-phosphate were assigned from the off-resonance decoupled spectra. Chemical shifts are relative to tetramethylsilane at 0p.p.m.

spectra of ecdysone and 20-hydroxyecdysone (Table 2). The small amount of 2-deoxy-20-hydroxyecdysone phosphate conjugate (4) isolated did not afford a useful ^{13}C n.m.r. spectrum. The signals were assigned by off-resonance decoupling and by comparison with the data published by Krepinsky *et al.* (1977). The ^{13}C spectra of all three ecdysteroid phosphates showed no signals in addition to those of the parent ecdysteroids and the solvent (methanol). The major difference between the spectra of the free ecdysteroids and the phosphorylated derivatives was the chemical shift and intensity of the C-22 signals (Fig. 4 and Table 2). Comparison of the spectra of ecdysone phosphate (1) and 2-deoxyecdysone phosphate (2) with that of the ecdysone revealed a downfield shift of +4.0p.p.m. and +3.8p.p.m., respectively, for the C-22 resonance. In the spectrum of (2), the upfield shift (−3.0p.p.m.) of the resonance of C-3, relative to ecdysone, is attributed to the absence of an hydroxy group at the adjacent C-2 [cf. the upfield

shift (−3.0p.p.m.) of the C-22 signal in ecdysone relative to 20-hydroxyecdysone]. The presence of a phosphate group had a similar effect on the C-22 resonance of 20-hydroxyecdysone phosphate (3), which was observed downfield (+4.5p.p.m.) from the C-22 signal of 20-hydroxyecdysone. Peak broadening of the C-22 signals of all three ecdysteroid phosphates was attributed to doublets arising from $^{13}C-^{31}P$ coupling ($^2J_{cop.}$ approx. 4.7Hz). Less significant changes were observed as predicted (cf. Uesugi *et al.*, 1978; Hesbain-Frisque *et al.*, 1981), in the chemical shifts of carbons close to C-22. For example, with respect to ecdysone the corresponding 22-phosphate ester showed shifts at C-20, C-21, C-23, C-24 of −2.2p.p.m., +0.3p.p.m., −0.4p.p.m. and −1.24p.p.m., respectively. These observations, together with the absence of any effect on the C-2, C-3, C-14 and C-25 signals, demonstrates unequivocally that the phosphate ester linkage in compounds (1), (2) and (3) is through the oxygen function at C-22.

Discussion

The combined data demonstrate that compounds (1), (2), (3) and (4) isolated from newly laid eggs of *S. gregaria* are ecdysone 22-phosphate, 2-deoxyecdysone 22-phosphate, 20-hydroxyecdysone 22-phosphate and 2-deoxy-20-hydroxyecdysone 22-phosphate, respectively. The unambiguous demonstration of the covalent steroid–phosphate bond relied upon FAB mass spectrometry. This technique is particularly useful in distinguishing between phosphate and sulphate esters of ecdysteroids (R. E. Isaac, D. R. Greenwood & H. H. Rees, unpublished work). The identification of ecdysteroid conjugates as metabolites of ecdysone has relied heavily in the past upon hydrolysis with commercial enzyme preparations. The fact that the ecdysteroid phosphates isolated from *S. gregaria* eggs were readily hydrolysed by an arylsulphatase preparation from *Helix pomatia* illustrates the danger of making any assumption regarding the structures of ecdysteroid conjugates on this basis.

In *S. gregaria*, the ecdysteroid phosphates, which are synthesized by the adult female insect, accumulate in the terminal öocyte (Gande *et al.*, 1979; Dinan & Rees, 1981*a*). In the closely related species, *Locusta migratoria*, the follicle cells have been shown to synthesize ecdysteroids (Goltzené *et al.*, 1978), but it is not known whether this is also the site of production of ecdysteroid conjugates, which are also the major form of the hormone in the mature ovaries of this species (Hoffmann *et al.*, 1980). The isolation of the phosphate esters of ecdysone, 20-hydroxyecdysone and the corresponding 2-deoxy compounds from eggs of *S. gregaria* may suggest a branched pathway in the biosynthesis of the steroid moiety of 20-hydroxyecdysone phosphate. It is conceivable that either the C-20 or C-2 hydroxylation reaction may be the final oxidation step in the biosynthesis. A branched pathway has also been suggested for the synthesis of 20-hydroxyecdysone in the ovaries of *Bombyx mori* on the basis of the identification of 2,22-dideoxy-20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone (Ohnishi *et al.*, 1981). It is possible that the formation of the phosphate esters of ecdysteroids could promote the overall synthesis of ecdysteroids by facilitating the removal of free steroid from the site of synthesis. The exact stage in the ecdysteroid biosynthetic sequence at which phosphorylation occurs is not clear. At least it must be a late step because the C-22 hydroxylation itself apparantly occurs late in ecdysteroid biosynthesis (Rees, 1983). Whether ecdysteroid 22-phosphates can serve as substrates for later hydroxylation reactions (for example, at C-2) is not clear. In vertebrates, the direct hydroxylation of steroid sulphate esters without prior removal of the sulphate group has been demonstrated (Gustaffson & Ingelman-Sundberg, 1974; Einarson *et al.*, 1976; Tsoutsoulis & Hobkirk, 1980).

The possibility that ecdysteroid conjugates might serve as storage forms of ecdysteroids has been considered (Willig *et al.*, 1971). The C-22 hydroxy of ecdysteroids, which has been shown to be essential for hormonal activity (for review see Bergamasco & Horn, 1980), appears to be exclusively phosphorylated in adult female *S. gregaria*. Although the exact function of the ecdysteroid conjugates in the developing eggs is not known, the ecdysteroid 22-phosphates probably represent inactive storage forms of the hormone. Indeed, we have demonstrated that embryonic tissues can enzymically hydrolyse ecdysteroid 22-phosphates to liberate edcysteroids (Rees *et al.*, 1981; Isaac *et al.*, 1983*a*). Other factors that might contribute to the changes in free ecdysteroid titre during embryogenesis of *S. gregaria* include embryonic synthesis of ecdysteroids from cholesterol or precursors present in the öoplasm as well as metabolism of the hormone to inactive products. Of relevance to the latter process is the isolation of ecdysonoic acid, 20-hydroxyecdysonoic acid and a phosphate conjugate of ecdysone acetate, which are all considered to be hormonally inactive metabolites of ecdysone, from developing eggs of *S. gregaria* (Isaac *et al.*, 1981*b*; Isaac *et al.*, 1983*b*; R. E. Isaac, H. Desmond & H. H. Rees, unpublished work). The occurrence of any ecdysteroid biosynthesis by the embryo from cholesterol or related sterols remains to be determined.

In the related insect, *Locusta migratoria*, the major ecdysteroid conjugate in newly laid eggs was identified as the 22-adenosinemonophosphate ester of 2-deoxyecdysone by n.m.r. spectroscopy of the intact conjugate and by mass spectrometry of the

ecdysteroid and adenosine moieties after either enzymic or acid hydrolysis (Tsoupras *et al.*, 1982). In this species, it has been shown that the ecdysteroid conjugates can bind to the major yolk protein, vitellin (Lagueux *et al.*, 1981*a*). This phenomenon was used in the purification of the 22-adenosinemonophosphate ester of 2-deoxyecdysone. However, it is significant that the amount of this compound isolated represented only approx. 6% of the expected amount of 2-deoxyecdysone conjugate in the newly laid eggs as calculated from radioimmunoassay measurements (Lagueux *et al.*, 1981*b*).

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