Identification of Ecdysone-22-phosphate and 2-Deoxyecdysone-22-phosphate in Eggs of the Desert Locust, *Schistocerca gregaria*, by Fast Atom Bombardment Mass Spectrometry and N.M.R. Spectroscopy

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Ecdysone-22-phosphate and 2-deoxyecdysone-22-phosphate have been purified by h.p.l.c. from eggs of the desert locust, *Schistocerca gregaria,* and their structures determined primarily by Fast Atom Bombardment mass spectrometry and ^{1}H, ^{13}C, and ^{31}P n.m.r. spectroscopy.

Towards the end of oögenesis in the female adult desert locust, *Schistocerca gregaria*, the ovaries produce relatively large quantities of ecdysteroids (insect moulting hormones) primarily as highly polar conjugates.[1,2] These ecdysteroid conjugates are, in the main, passed into the eggs,[1,2] where the hormones are believed to have an essential role in the development of the embryos.[3] The ecdysteroid moieties of the conjugates in newly laid eggs consist primarily of ecdysone, 2-deoxyecdysone, and 20-hydroxyecdysone.[2,4] These ecdysteroid conjugates of hitherto unknown structures are readily hydrolysed by a crude enzyme preparation from *Helix pomatia*.[1,2] In the present paper we report the isolation of the two most abundant highly polar ecdysteroid conjugates from newly laid eggs of *Schistocerca gregaria* and elucidation of their complete structures as ecdysone-22-phosphate (**1**) and 2-deoxyecdysone-22-phosphate (**2**).

Newly laid eggs (110 g) were extracted with methanol–water (7:3 v/v) and, after partition with hexane, the highly polar ecdysteroids were isolated from the aqueous methanol fraction by chromatography on a silicic acid column as described previously.[2] The highly polar fraction was concentrated and then fractionated by h.p.l.c. on an anion exchange column (Whatman SAX).† The two major u.v. absorbing compounds (**1**) and (**2**) were desalted on reversed phase SEPAK‡ cartridges before being further purified by ion-suppression and reversed phase h.p.l.c.§ On hydrolysis with a crude *Helix pomatia* arylsulphatase preparation, compound (**1**) [3.4 mg ecdysteroid, λ_{max} (MeOH) 244 nm] and compound (**2**) [1.7 mg ecdysteroid, λ_{max} (MeOH) 244 nm] released ecdysone and 2-deoxyecdysone (identified by co-chromatography on

† Retention volumes on a Whatman Magnum 9 Partisil SAX column (50 cm × 9.4 mm i.d.) eluted with 0.1 M ammonium acetate (5 ml/min) were ecdysone-22-phosphate (**1**), 87 ml; 2-deoxyecdysone-22-phosphate (**2**), 115 ml.

‡ Reversed-phase SEPAK cartridges (Waters Associates) were loaded with ecdysteroid phosphates in water (5 ml). Salt was eluted with water (5 ml) and the ecdysteroid phosphates were recovered by eluting with methanol (5 ml).

§ Retention volume for ecdysone-22-phosphate (**1**) on a Whatman Magnum 9 Partisil ODS-3 column (50 cm × 9.4 mm i.d.) eluted with 35% methanol–0.02 M sodium acetate buffer, pH 5.5, was 65 ml. Retention volumes when eluted with a linear gradient (30 min) of 20% → 70% methanol–water were ecdysone phosphate (**1**), 52 ml; 2-deoxyecdysone phosphate (**2**), 76 ml.

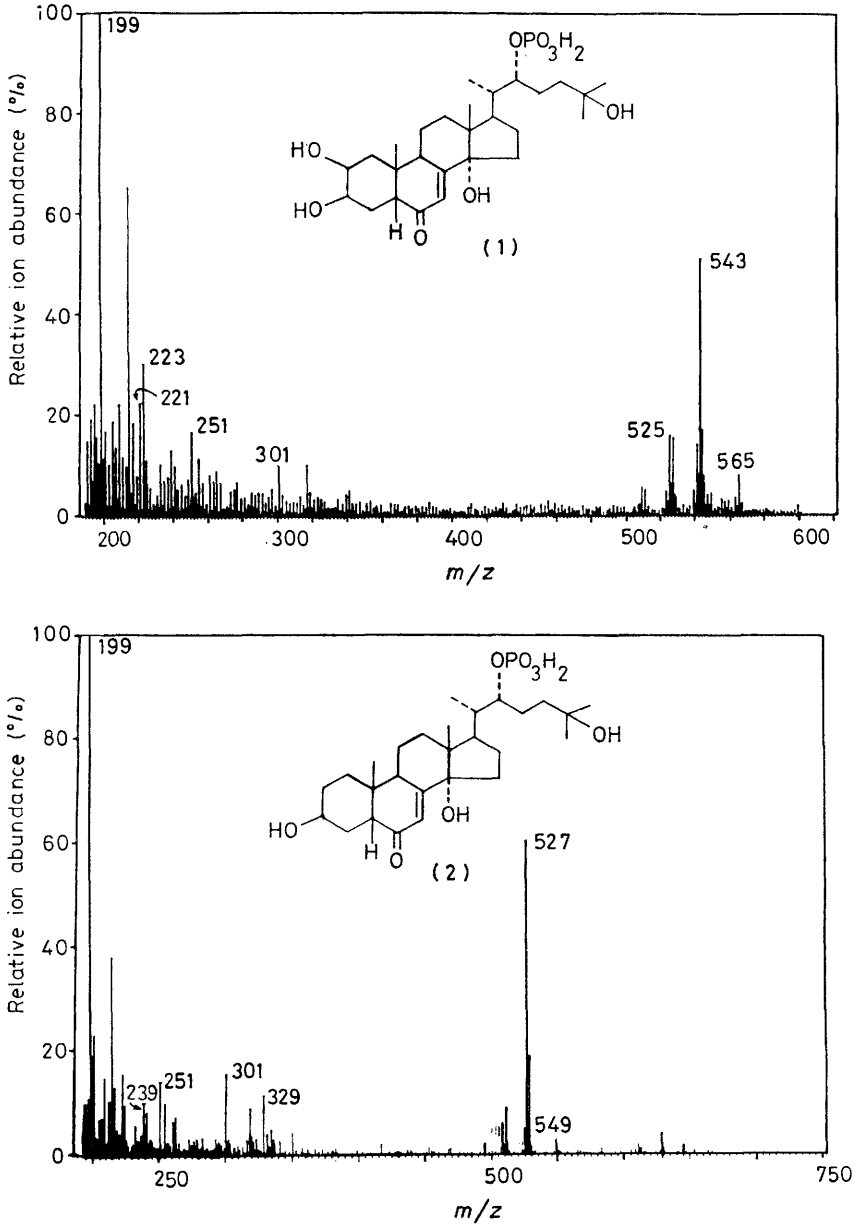

Figure 1. Fast Atom Bombardment mass spectra of ecdysone-22-phosphate (**1**) and 2-deoxyecdysone-22-phosphate (**2**).

h.p.l.c.,[5] with authentic material and by mass spectrometry), respectively. The recoveries of (**1**) and (**2**) correspond to 70 and 80%, respectively, of the maximum expected on the basis of the previously observed[2] yields of ecdysone and 2-deoxyecdysone from enzymic hydrolysis of the crude conjugate fraction.

Chemical ionisation (isobutane) mass spectrometry gave, in the case of (**1**), *m/z* 447 as the ion of highest mass and, in the case of (**2**), *m/z* 431. These ions probably arise through thermal elimination of the conjugate moiety by a process analogous to the commonly observed loss of H_2O from the parent ecdysteroid under the same mass spectrometric conditions. Similarly, other methods of ionisation (field desorption and 'in-beam' chemical ionisation) did not afford helpful spectra for structural elucidation of the intact molecules. This problem was overcome by subjecting the samples to Fast Atom Bombardment (F.A.B.) mass spectrometry.[6] This new technique is particularly suitable for involatile and thermally labile compounds since the samples are ionised at ambient temperature in the solid phase by bombardment with rapidly moving xenon atoms. Negative ion mass spectra (Figure 1) of (**1**) and (**2**) gave as the major ions at high mass, *m/z* 543 and 527, respectively. These are interpreted as $[M-H]^-$ ions and therefore point to molecular weights of 544 for (**1**) and 528 for (**2**). The observation of small $[M-H]^-$ peaks for the monosodium salts of (**1**) and (**2**) at *m/z* 565 and 549, respectively, confirmed the assignment of molecular weights. This phenomenon has been observed commonly in the negative ion F.A.B. mass spectra of many compounds.[7] Possible structures are the phosphate or sulphate esters of ecdysone and 2-deoxyecdysone.

The presence of organic phosphate in samples of (**1**) and (**2**) was demonstrated by assaying[8] for organic phosphate before and after acid hydrolysis (0.5 M HCl at 110 °C for 6 h). This result was confirmed by the proton decoupled ^{31}P n.m.r. spectra (Fourier Transform, 145.8 MHz, CD_3OD), which gave signals (referenced to external H_3PO_4) at δ +2.69 p.p.m. for (**1**) and at δ +1.55 p.p.m. for (**2**). Further corroboration of the presence of phosphate was obtained by acid hydrolysis of conjugates (**1**) and (**2**), followed by g.l.c.–m.s. of the liberated phosphate as its trimethylsilylated derivative.[9] The mass spectrum obtained was identical to that of authentic

tris-trimethylsilyl phosphate, *m/z* 314 (M^+, 19%), 299([$M-CH_3$]$^+$, 100%). Quantitative estimation of the ecdysteroid (ϵ_{244} 12 400) and phosphate[10] moieties present in samples of both (**1**) and (**2**) demonstrated ecdysteroid/phosphate ratios of 1:0.9 (**1**) and 1:0.94 (**2**). In comparison, there were negligible amounts of inorganic sulphate in acid hydrolysed samples of (**1**) and (**2**).[11] Further evidence that the high polarity ecdysteroid conjugates were phosphate esters was obtained by the incorporation of [^{32}P], albeit low, into both (**1**) and (**2**) after administering either [^{32}P]ATP or [^{32}P]PO_4^{3-} to mature adult females.

The ^{1}H n.m.r. spectrum (250 MHz, CD_3OD)¶ of compound (**1**) gave signals at δ 0.75 (3H, s, 18-H_3), 0.97 (3H, s, 19-H_3), 1.00 and 0.97 (3H, d, 21-H_3), 1.18 and 1.19 (6H, 2 s, 26/27-H_3), 3.86 (1H, m, 2-H, $W_{\frac{1}{2}}$ 21 Hz), 3.97 (1H, m, 3-H, $W_{\frac{1}{2}}$ 8 Hz), and 4.20 (1H, m, 22-H, $W_{\frac{1}{2}}$ 20 Hz). This spectrum is similar to that of ecdysone, except for the 22-H and 21-methyl signals which are shifted downfield in the conjugate. For ecdysone the corresponding signals are at δ 3.61 (m, 22-H, $W_{\frac{1}{2}}$ 16 Hz) and δ 0.94, 0.97 (d, 21-H_3). The ^{13}C n.m.r. spectra (62.8 MHz, CD_3OD; δ values with reference to CD_3OD at 49.0 p.p.m.) of (**1**) and ecdysone were compared, the ecdysone ^{13}C signals being assigned by comparison with spectra[12] obtained using C_5D_5N as solvent and by off-resonance decoupling. The ^{13}C spectrum of (**1**) showed that there were no additional ^{13}C signals as compared to ecdysone. The major difference between the spectra was the chemical shift of C-22 [compound (**1**) δ 79.3 p.p.m., ecdysone, δ 75.3 p.p.m.], although other less pronounced differences in chemical shifts for the side-chain carbons were observed. Peak broadening of the C-22 and C-23 signals was attributed to doublets resulting from ^{13}C–^{31}P coupling ($^2J_{COP}$ *ca.* 4.7 Hz; $^3J_{CCOP}$ *ca.* 3.1 Hz). The data from both the ^{1}H and ^{13}C n.m.r. spectra show conclusively that in (**1**) the phosphate moiety is linked to ecdysone through the oxygen function at C-22.

The ^{1}H n.m.r. spectrum (360 MHz, CD_3OD)¶ of compound (**2**) gave signals at δ 0.73 (3H, s, 18-H_3), 0.96 (3H, s, 19-H_3), 0.96 and 0.98 (3H, d, 21-H_3), 1.18 (6H, s, 26/27-H_3), 3.98 (1H, m, 3-H, $W_{\frac{1}{2}}$ 12 Hz), and 4.22 (1H, m, 22-H, $W_{\frac{1}{2}}$ 25 Hz). This spectrum differs significantly from that of 2-deoxyecdysone only in that the 22-H and 21-H_3 signals in the conjugate (**2**) are shifted downfield as in the case of ecdysone conjugate. The combined data indicate that compound (**2**) is 2-deoxyecdysone-22-phosphate.

There have been numerous reports of the occurrence of highly polar ecdysteroid conjugates not only in ovaries and eggs, but also as hormone metabolites in larvae and pupae.[13] This paper is the first report of the complete structural elucidation of highly polar ecdysteroid conjugates in insects. The fact that the ecdysone-22-phosphate (**1**) and 2-deoxyecdysone-22-phosphate (**2**) are hydrolysed by a crude arylsulphatase preparation from *Helix pomatia* illustrates the danger of making any assumptions regarding the structures of ecdysteroid conjugates on the basis of enzymic hydrolysis. That the *Helix pomatia* enzyme preparation contains phosphatase activity is indicated by our observation that it hydrolyses *p*-nitrophenyl phosphate.

Although the exact function of the ecdysteroids present in developing embryos is not known, it is considered that at this stage of development the highly polar conjugates can act as a storage form of the maternal hormone, releasing active ecdysteroids as a result of enzymic hydrolysis.[14] In this context it is interesting that in *Schistocerca gregaria* the phosphate ester is positioned at the 22-hydroxy-group, which has been shown to be essential for hormonal activity.[15]

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¶ Fourier Transform ^{1}H n.m.r., δ values with reference to internal CH_3OH at δ 3.3.

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