Isolation and identification of 3-acetylcidysonone 2-phosphate, a metabolite of ecdysone, from developing eggs of *Schistocerca gregaria*

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1. A major ecdysteroid conjugate, which accumulates in the eggs of the desert locust, *Schistocerca gregaria*, during the later stages of embryogenesis, has been isolated by reversed-phase and anion-exchange high-performance liquid chromatography. 2. Hydrolysis of the conjugate with a crude arylsulphatase preparation from *Helix pomatia* liberates mainly ecdysone 3-acetate. 3. The compound was identified as 3-acetylcidysonone 2-phosphate by phosphate analysis of an acid-hydrolysed sample, fast atom bombardment, electron impact and chemical ionization mass spectrometry and ^1H and ^{13}C n.m.r. spectroscopy. 4. The instability of 3-acetylcidysonone 2-phosphate on storage results in the formation of ecdysone 2-phosphate, which was identified by physicochemical techniques. 5. 3-Acetylcidysonone 2-phosphate and ecdysone 2-phosphate are less susceptible than ecdysone 22-phosphate to hydrolysis *in vitro* by an enzyme preparation from *S. gregaria* embryos. 6. The possible role of 3-acetylcidysonone 2-phosphate as an inactive end product of ecdysteroid metabolism is discussed.

Eggs of the desert locust, *Schistocerca gregaria*, contain high concentrations of the 22-phosphate esters of the ecdysteroids (insect moulting hormones), ecdysone, 2-deoxyecdysone, 20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone (Isaac *et al.*, 1982a, 1983a) as well as small amounts of free ecdysteroids (5% of total). The most abundant of these steroid phosphates is ecdysone 22-phosphate, which can undergo enzymic hydrolysis in the developing embryo to release ecdysone (Isaac *et al.*, 1983b); the possibility exists that ecdysone could also arise by synthesis in the embryos. The correlation between peaks of free ecdysteroids in the developing eggs of a number of insect species and the synthesis of embryonic membrane/cuticle (Dorn & Romer, 1976; Bulliere *et al.*, 1976; Lagueux *et al.*, 1979; Matz, 1980; Cavallin & Fournier, 1981; Imboden & Lanzrein, 1982; Scalia & Morgan, 1982) suggests an important function for free ecdysteroids in insect embryogenesis.

In *S. gregaria* embryos, free ecdysone is metabolized, at least in the later stages of development, to ecdysoneic acid (Isaac *et al.*, 1983c) and a polar conjugate of ecdysone 3-acetate (Isaac *et al.*, 1981; R. E. Isaac & H. H. Rees, unpublished work).

These polar compounds are also important metabolites and excretory products of ecdysone in the fifth instar larvae of *S. gregaria* and *Locusta migratoria* (Gibson, 1982).

In the present paper we report the isolation of one of these metabolites, a polar conjugate of ecdysone 3-acetate, from the developing eggs of *S. gregaria* and its identification as 3-acetylcidysonone 2-phosphate (1).

Materials and methods

Generally, the materials and methods were as described previously (Isaac *et al.*, 1983a).

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

Chemicals

Ecdysone was purchased from Simes, Milan, Italy and ecdysone 3-acetate was synthesized by the method of Galbraith & Horn (1969).

High-performance liquid chromatography

Free ecdysteroids were chromatographed on a reversed-phase column (Ultrasphere-ODS, Ana-chem, Luton, U.K.; Isaac *et al.*, 1982b) or on an amino-propyl column (APS-Hypersil, Shandon Southern Products, Runcorn, U.K.; Dinan *et al.*, 1981). Ecdysteroid conjugates were separated on the following h.p.l.c. systems. System 1, reversed-phase chromatography on a Partisil ODS-3 column (Whatman, Maidstone, U.K.; 25 cm $\times$ 4.6 mm internal diameter; particle size, 10 μ m) eluted at a flow rate of 2 ml/min with (a) a linear gradient (30 min) of methanol in 20 mM-sodium citrate buffer, pH 6.5, changing from 1:9 (v/v) to 7:3 (v/v); or (b) a linear gradient (25 min) of methanol in water changing from 1:19 (v/v) to 1:1 (v/v). System 2, anion-exchange chromatography on a Partisil SAX column (25 cm $\times$ 4.6 mm internal diameter; particle size 10 μ m) eluted at a flow rate of 2 ml/min with 0.1 M-ammonium acetate. Samples collected from h.p.l.c. were concentrated under reduced pressure and were desalted as described by Isaac *et al.* (1982a) whenever buffer was employed in the mobile phase.

Analytical methods

Quantitative phosphate and ecdysteroid analysis, hydrolysis of conjugates with *H. pomatia* enzymes, mass spectrometry and Fourier transform n.m.r. spectroscopy were carried out as described by Isaac *et al.* (1983a).

Enzyme preparation from embryos

Embryos were dissected from 15–17-day-old *S. gregaria* eggs and separated from the yolk before being homogenized in 0.154 M-KCl (20%, w/v). A soluble enzyme preparation was obtained from the mitochondrial-lysosomal subcellular fraction (Isaac *et al.*, 1982c) by sonication (6 $\times$ 10 s bursts of a MSE 150W sonic disintegrator) and sedimentation of particulate material at 100 000 g for 60 min in a 40.2 rotor of an L2-65B ultracentrifuge (Beckman Instruments). The supernatant was applied to a Sephadex G-100 column (47 cm $\times$ 2.5 cm), which was equilibrated and eluted (21 ml/h) with 0.1 M-NaCl buffered with 50 mM-sodium citrate (pH 6.5), at 5°C. Fractions (4 ml) were collected and assayed for phosphatase activity towards ecdysone 22-phosphate. The fractions (15–25) corresponding to the enzymic activity were combined and concentrated in a Minicon-B con-

centrator (Amicon, Woking, Surrey, U.K.) to give an enzyme preparation with a protein concentration of 1.33 mg/ml.

Protein assay

Protein concentrations were estimated by the method of Mejbaum-Katzenellenbogen & Dobryzcka (1959), using bovine serum albumin as a standard.

Estimation of the relative hydrolysis of ecdysteroid phosphates by an enzyme preparation from *S. gregaria* embryos

Ecdysteroid phosphates (8 nmol) were incubated with enzyme (110 μ g of protein) from *S. gregaria* embryos in 0.1 M-sodium citrate buffer, pH 4.0, (final volume 150 μ l) for 45 min at 36°C. The reaction was terminated by the addition of methanol (150 μ l). After being stored overnight at –20°C, the samples were centrifuged (5 min, MSE Microcentaur centrifuge) and an aliquot (200 μ l) was analysed directly on reversed-phase h.p.l.c. (Isaac *et al.*, 1982b). The liberated ecdysteroids that co-chromatographed with authentic compounds were quantified by using a u.v. detector (254 nm) linked to an integrator which was calibrated with a standard solution of ecdysone. Enzyme reactions were linear with time for at least 1 h.

Results

Isolation of ecdysteroid conjugate (I) from developing eggs of *S. gregaria*

S. gregaria eggs (280 g) were incubated at 30°C for approx. 15 days (embryogenesis lasts for 16–17 days) and then stored at –20°C until they were extracted with aqueous methanol (Isaac *et al.*, 1983a). The polar ecdysteroid conjugates were isolated from this extract by silicic acid column chromatography (Dinan & Rees, 1981).

Compound (1) [λ_{max} (MeOH) 242 nm] was isolated from the polar ecdysteroid conjugate fraction by ion-suppression reversed-phase h.p.l.c. [system 1(a); retention volume, 50 ml] and was further purified by anion-exchange (system 2; retention volume, 30 ml) and reversed-phase h.p.l.c. [system 1(b); retention volume, 35 ml] to yield 0.84 mg of material (ϵ_{242} 12 400 litre $\cdot$ mol^{–1} $\cdot$ cm^{–1}).

Identification of (I) as 3-acetylcaldysone 2-phosphate

Incubation of a sample of (1), which had been stored at –20°C for approx. 1 month, with a crude arylsulphatase preparation from *H. pomatia* released primarily ecdysone 3-acetate with a lesser

Fig. 1. Negative ion fast atom bombardment mass spectra of 3-acetylcaldysone 2-phosphate (a) and ecdysone 2-phosphate (b).

The mass spectra of 3-acetylcaldysone 2-phosphate (a) and ecdysone 2-phosphate (b) show distinct $[M-H]^-$ ions at m/z 585 and 543, respectively. Large peaks at m/z 79 (PO_3^-) and 97 ($H_2PO_4^-$) are observed in both spectra.

amount (approx. 35%) of ecdysone, both of which were identified by co-chromatography with authentic samples on h.p.l.c. (Isaac *et al.*, 1982b) and by mass spectrometry. All subsequent analyses were carried out on freshly purified samples.

The negative ion FAB mass spectrum of (1) (Fig. 1a) gave as the major ions m/z 585 $[M-H]^-$, 97 $[H_2PO_4]^-$ and 79 $[PO_3]^-$. This spectrum indicates that (1) is a phosphate ester (Isaac *et al.*, 1983a) of molecular weight 586. Both electron impact and chemical ionization mass spectrometry resulted in the thermal elimination of the phosphate moiety. The fragmentation patterns observed were consistent with the identification of the steroid moiety as ecdysone-3-acetate: electron impact, m/z 470

$[M-H_3PO_4-H_2O]^+$, 452 $[M-H_3PO_4-2H_2O]^+$, 410 $[470-CH_3COOH]^+$, 390 $[M-(C_{22}-C_{27})]^+$, 342 $[M-(C_{20}-C_{27})]^+$; chemical ionization (isobutane) m/z 489 $[M+1-H_3PO_4]^+$, 471 $[489-H_2O]^+$, 429 $[489-CH_3COOH]^+$, 411 $[471-CH_3COOH]^+$.

Quantitative estimation of the ecdysteroid (ϵ_{242} 12400) and phosphate moieties present in (1) gave an ecdysteroid/phosphate ratio of 1:0.8.

The Fourier transform p.m.r. spectrum (400 MHz, $C^2H_5O^2H$) of (1) gave signals at δ 0.73 (3H, s, 18- H_3), 0.93 and 0.95 (3H, d, J 6 Hz, 21- H_3), 1.00 (3H, s, 19- H_3), 1.19 and 1.20 (6H, 2s, 26/27- H_3), 2.09 (3H, s, CH_3CO), 3.58 (1H, m, W_1 16 Hz, 22-H) and 5.82 (1H, 7-H) p.p.m. The major differences between this spectrum and that of ecdysone (Isaac *et al.*, 1983a) is the downfield shift of the C-19 methyl (+0.03 p.p.m.) and the shift in the C-2 and C-3 proton signals which are probably hidden by the broad solvent peak at lower field. These observations, together with the lack of any significant changes (compared with ecdysone) in the chemical shifts of the C-21, C-18, C-26/27 methyl signals and the C-22 proton signal, indicate that both the phosphate and acetate substituents are linked through oxygen functions to the A ring of the steroid nucleus.

In the Fourier transform ^{13}C n.m.r. spectrum (100 MHz, $C^2H_5O^2H$) of (1), five of the steroid signals could not be observed above the background noise and the broad solvent peak. However, it was clear that there was little difference in the chemical shifts of the side-chain carbons, including the hydroxy-substituted C-22, between the ^{13}C spectrum of (1) and that of ecdysone (Krepinsky *et al.*, 1977; Isaac *et al.*, 1983a). Signals at 21.2 and 70.8 p.p.m. could be assigned to the methyl carbon of the acetate group (Blunt & Stothers, 1977) and the phosphate-substituted carbon (C-3 or C-2), respectively. The isolation of ecdysone 3-acetate rather than ecdysone 2-acetate after enzymic hydrolysis of (1) with *H. pomatia* enzymes indicates that in the intact compound the phosphate ester is at the C-2 position.

The combined data are consistent with the identification of compound (1) as 3-acetylcaldysone 2-phosphate.

Isolation and identification of a breakdown product of 3-acetylcaldysone 2-phosphate (1)

Compound (1) was found to be unstable when stored over a period of 1 month at $-20^\circ C$. The breakdown product (2) was isolated by ion-suppression h.p.l.c. (system 1(a); retention volume, 44 ml) and was shown to be a phosphate ester of ecdysone by negative ion FAB mass spectrometry (Fig. 1(b); Isaac *et al.*, 1983a) and by the release of ecdysone on hydrolysis of (2) with *H. pomatia*

enzymes. In addition, the ecdysone/phosphate ratio was shown to be 1:0.85.

The Fourier transform p.m.r. spectrum (400 MHz, $C^2H_3O^2H$) of (2) gave signals at δ 0.72 (3H, s, 18- H_3), 0.93 and 0.95 (3H, d, J 6 Hz, 21- H_3), 0.97 (3H, s, 19- H_3), 1.19 and 1.20 (6H, 2s, 26/27- H_3), 4.18 (1H, m, W , 8 Hz, 3-H), 4.37 (1H, m, W , 24 Hz, 2-H) and 5.80 (1H, 7-H). Comparison of this spectrum with that of ecdysone (Isaac *et al.*, 1983a) shows that the C-2 and C-3 protons, which were assigned by their peak widths at half height, have shifted downfield by +0.52 and +0.22 p.p.m., respectively. These observations are consistent with the presence of the electron withdrawing phosphate group at C-2, which deshields the C-2 proton and to a lesser extent the C-3 proton, resulting in the observed downfield shifts. Compound (2) was therefore identified as ecdysone 2-phosphate.

Hydrolysis of 3-acetylcaldysone 2-phosphate (1) and ecdysone 2-phosphate (2) by an enzyme preparation from developing embryos of S. gregaria

Preliminary studies on a phosphatase enzyme from *S. gregaria* embryos, which is active towards ecdysteroid 22-phosphates, have shown that the enzymic activity is localized in the mitochondrial-lysosomal fraction of the cell and is most active at acidic pH (Isaac *et al.*, 1983b).

To compare the relative activity of such an enzyme towards ecdysteroid 22-phosphates and ecdysteroid 2-phosphates, a phosphatase preparation from locust embryos was incubated separately with equimolar amounts of 3-acetylcaldysone 2-phosphate (1), ecdysone 2-phosphate (2) and ecdysone 22-phosphate. The respective reaction products, namely ecdysone 3-acetate and ecdysone, were quantified by reversed-phase h.p.l.c. The greatest activity was towards ecdysone 22-phosphate (11.3 nmol/h per mg of protein) whilst 3-acetylcaldysone 2-phosphate (1) and ecdysone 2-phosphate (2) were significantly less susceptible to hydrolysis (18% and 50%, respectively, of the activity towards ecdysone 22-phosphate). Similar results were obtained for the relative hydrolysis of ecdysone 22-phosphate and compound (1) by a crude mitochondrial-lysosomal preparation.

Discussion

From the evidence presented, we have identified a major ecdysteroid component of the developing eggs of *S. gregaria* as 3-acetylcaldysone 2-phosphate. This compound, which cannot be detected in newly laid eggs, appears during embryonic development as a metabolite of ecdysone (Rees *et al.*, 1981; R. E. Isaac & H. H. Rees, unpublished results). 3-Acetylcaldysone 2-phosphate is consid-

ered to be a hormonally inactive metabolite, since modification of the A ring of the ecdysteroid nucleus reduces hormonal activity (Bergamasco & Horn, 1980).

The observation that 3-acetylcaldysone 2-phosphate is a poor substrate, compared with ecdysone 22-phosphate, for an embryonic phosphatase is consistent with an end-product role for this metabolite. The addition of two distinct substituents on the ecdysteroid molecule is likely to limit any possible recycling of the ecdysteroid moiety to an active free hormone in the essentially closed system of the insect egg. This would be in contrast to the ecdysteroid 22-phosphates, which can serve as inactive storage forms of ecdysteroids (Isaac *et al.*, 1983a). The isolation from the faeces of fifth instar larvae of *S. gregaria* of an ecdysone metabolite, which co-chromatographs with 3-acetylcaldysone 2-phosphate and yields ecdysone 3-acetate on hydrolysis with *H. pomatia* enzymes (Gibson, 1982; R. E. Isaac & H. H. Rees, unpublished work), again suggests that 3-acetylcaldysone 2-phosphate is an end-product of metabolism.

Recently, the 3- (or 2-) acetate derivative of 20-hydroxyecdysone 22-phosphate and the 3- (or 2-) phosphate ester of 20-hydroxyecdysone were isolated from incubations *in vitro* of 20-hydroxyecdysone with Malpighian tubules from *L. migratoria* (Tsoupras *et al.*, 1983). In the present study we have shown that ecdysone 2-phosphate can result from deacylation of 3-acetylcaldysone 2-phosphate on storage. At least, in *S. gregaria* embryos it is not certain whether ecdysteroids can be phosphorylated on the A ring of the steroid nucleus without prior acetylation.

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