Identification of ecdysone 25-*O*- β -D-glucopyranoside as a new metabolite of ecdysone in the nematode *Parascaris equorum*

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A major metabolite of [³H]ecdysone injected into adults of the nematode *Parascaris equorum* maintained *in vitro* for 48 h was secreted into the culture medium. The compound could be hydrolysed with a crude hydrolase preparation from *Helix pomatia*, yielding ecdysone. Sufficient quantity of this material for identification was produced by administration of ecdysone to the parasites. The resulting compound was purified by h.p.l.c. and identified as ecdysone 25-*O*- β -D-glucopyranoside by n.m.r. spectroscopy and by fast atom bombardment mass spectrometry of the conjugate and of the sugar released by enzymic hydrolysis. The significance of formation of the glucoside is uncertain.

INTRODUCTION

The occurrence of free and polar conjugated ecdysteroids (insect moulting hormones) has been reported not only in arthropods, but also in several non-arthropod phyla (for references see reviews in Hoffmann & Porchet, 1984; Mercer, 1985; Rees & Mercer, 1986). Amongst the nematodes, the swine parasite, *Ascaris suum*, contains ecdysone and 20-hydroxyecdysone as major ecdysteroid components (Fleming, 1985; Cleator *et al.*, 1987). In studies to examine the fate of ecdysteroids in adult females of this species, [³H]ecdysone was converted into a compound, 'U', chromatographing just after 20-hydroxyecdysone on reversed-phase h.p.l.c. as well as into a series of apolar compounds. Compound U was hydrolysed completely by *Helix pomatia* hydrolases, yielding ecdysone, was not hydrolysed by KHCO₃, and apparently formed two separate acetone compounds (M. Cleator & H. H. Rees, unpublished work).

The present paper reports the purification and identification of compound U. Since large quantities of *A. suum* are difficult to obtain locally, we examined the related equine parasite, *Parascaris equorum*. It was shown that both male and female adults of this species metabolized [³H]ecdysone to compound U, substantial amounts of which were excreted into the culture medium (G. M. O'Hanlon, J. G. Mercer & H. H. Rees, unpublished work). This compound U was hydrolysed by *Helix* enzymes, releasing ecdysone, but not by treatment with KHCO₃, as for the sample from *Ascaris*.

To obtain sufficient compound U for identification, unlabelled ecdysone was injected into adult *P. equorum* of mixed sex which were maintained in simple medium. Since in the foregoing experiments with [³H]ecdysone far more compound U was recovered in the culture medium than in the nematodes themselves, and as isolation of compound U from the former source should be easier than from the nematodes, the medium was used as a source of this compound for purification. Part of this work was presented at the VIIIth Ecdysone Workshop, Marburg, Germany (March 1987).

MATERIALS AND METHODS

H.p.l.c.

Reversed-phase h.p.l.c. was carried out on a radial compression module Radial-PAK C₁₈ column (10 cm × 8 mm; particle size 5 μ m; Millipore–Waters) eluted at a flow rate of 1 ml/min with either a linear gradient (30 min) of methanol in water changing from 1:1 (v/v) to 1:0 (v/v) (system 1) or a concave gradient (30 min; no. 10 gradient on a Waters 660 gradient programmer) of methanol in water changing from 9:11 (v/v) to 1:1 (v/v) (system 2). Adsorption h.p.l.c. was carried out on a radial compression module NH₂ Radial-PAK column (10 cm × 8 mm; particle size 10 μ m; Millipore–Waters) eluted isocratically at a flow rate of 1 ml/min with methanol/dichloroethane (1:4, v/v) (system 3).

Enzymic hydrolysis of ecdysone metabolite

Purified compound U (25 μ g) was incubated with a crude aryl sulphatase preparation from *Helix pomatia* (8100 units; 1 unit will hydrolyse 1.0 μ mol of *p*-nitrocatechol sulphate/h at pH 5.0 at 37 °C; Sigma) in 0.1 M-Mes buffer, pH 5.5 (1 ml), at 37 °C for 24 h. The sample was then partitioned four times with butanol to extract the released ecdysteroid. The aqueous phase was desalted by passage down a Dowex 1-X8 –400 anion exchange resin column (4 ml bed volume), which had been pretreated by washing with 0.1 M-NaOH followed by de-ionized water until the washings were neutral. The sugar was eluted with 3 bed vol. of water, evaporated to dryness under nitrogen using ethanol as an azeotrope and vacuum-desiccated before derivatization for g.c.–m.s. analysis.

Mass spectrometry

Negative ion f.a.b. mass spectra were obtained on a VG 7070H mass spectrometer linked to a Finnigan Incos 2300 data system using a primary atom beam of xenon, and glycerol as the probe solvent.

Trimethylsilyl (TMS) ethers of carbohydrate samples were analysed by g.c.–m.s. using a Pye–Unicam 204 gas

Abbreviation used: f.a.b., fast atom bombardment.

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chromatograph linked to the aforementioned mass spectrometer. Sugars were derivatized by reaction with *NO*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in dry pyridine (1:1, v/v; room temperature; 30 min). the reaction mixture (1 μ l) was injected, without dilution, on-column via an SGE OCI-III injector onto a fused-silica BP-1 coated (0.25 μ m film thickness) capillary column (25 m $\times$ 0.22 mm i.d.). Helium carrier gas was at a linear flow rate of approx. 100 cm/s and the g.c. oven temperature was programmed from 50 to 280 °C at 8 °C/min. Other conditions were: g.c.-m.s. interface temperature, 280 °C; source temperature, 210 °C; emission current, 200 μ A; ionization potential, 70 eV; scan range, 40–700; accelerating voltage, 4 kV.

N.m.r. spectroscopy

¹H-n.m.r. spectra were obtained at 400.13 MHz on a Bruker WH400 spectrometer. Samples were dissolved in ²H₂O and spectra were referenced to 3-trimethylsilyl-2,2,3,3-tetradeuteriopropionic acid. Two-dimensional COSY spectra (Bax & Freeman, 1981) were accumulated overnight with a 2K $\times$ 2K data set, and nuclear Overhauser enhancement difference spectra (Sanders & Meresh, 1982) over 43 h with a 1.5 s pre-irradiation time.

Production and isolation of ecdysone metabolite

To obtain sufficient of the ecdysone metabolite U for identification, unlabelled ecdysone was administered to adult *Parascaris equorum* of mixed sex, which were cultured for 48 h and the culture medium extracted. For this, 540 nematodes were each injected with 2 μ l of ecdysone (50 μ g; Simes, Milan, Italy) in ethanol/water (1:1, v/v). The nematodes were then incubated at 37 °C in groups of ten in 800 ml tissue culture flasks (Nunc) containing 250 ml of culture medium [25 ml of concentrated Earle's balanced salt solution (Gibco) and 7.5 ml of sterile 7.5% (w/v) aqueous NaHCO₃ solution were made up to 250 ml with double-distilled water; the solution also contained 2.5 ml of penicillin/streptomycin (12 500 units of penicillin and 12 500 μ g of streptomycin; Gibco) and 0.625 ml of nystatin (6250 units; Gibco)]. The medium was changed after 24 h and the incubation terminated after 48 h by removal of the nematodes. [³H]U (2.5 μ Ci) prepared by metabolism of [23,24-³H]ecdysone (18 μ Ci total; specific radioactivity 60 Ci/mmol) by adult *P. equorum* (20 worms; 24 h) was used as marker compound throughout the following purification procedure. The bulked medium was acidified to pH 4 with 2 M-acetic acid and chromatographed in 4-litre portions on an Amberlite XAD-2 column (170 g; BDH) to remove the bulk of the salts, followed by elution of the ecdysteroid with ethanol (2 litres) (Thompson *et al.*, 1987). The combined ethanolic eluates were evaporated to dryness under vacuum (13.5 g), partitioned between methanol/water (7:3, v/v) and hexane, and the latter phase back-extracted with methanol/water. The bulked methanolic fractions were dried onto Celite (Koch-Light) which was applied to two silicic acid columns (50 g). The columns were each eluted successively with 500 ml volumes of chloroform, methanol/chloroform (1:4, v/v), methanol/chloroform (1:1, v/v) and methanol. The methanol/chloroform (1:1, v/v) fraction containing the metabolite U was evaporated to dryness (4.34 g) and aliquots loaded in 2 ml of methanol/water (1:9, v/v) onto 17 SEP-PAK cartridges (Millipore-Waters). The

cartridges were each eluted successively with methanol/water (1:9, v/v; 4 ml), methanol/water (3:2, v/v; 6 ml) and methanol (6 ml). The methanol/water (3:2, v/v) fraction, which contained compound U, was purified by successive chromatography on the following h.p.l.c. systems, yielding 0.98 mg ecdysone equivalents of material: (i) system 1, U 10.5 min (cf. 20-hydroxyecdysone 9 min and ecdysone 15 min), (ii) system 3, U 13.5 min (cf. ecdysone and 20-hydroxyecdysone 5–6 min), and (iii) system 2, U 14.5 min (cf. 20-hydroxyecdysone 12.5 min and ecdysone 25 min).

RESULTS

N.m.r. spectroscopy

The ¹H-n.m.r. spectrum of compound U showed all the resonances expected for ecdysone, each without significant change in shift, plus seven multiplets (two overlapping) lying in the region expected for sugar resonances. Analysis of the shifts and couplings, aided by a two-dimensional COSY experiment, identified the sugar moiety unambiguously as β -D-glucopyranoside (1-H, δ 4.65 p.p.m., d, 7.9 Hz; 2-H, δ 3.21 p.p.m., dd, 7.9, 9.4 Hz; 3-H, δ 3.48 p.p.m., dd, 9.4, 9.0 Hz; 4-H, δ 3.34 p.p.m., dd, 9.0, 9.8 Hz; 5-H, δ 3.46 p.p.m., complex; 6a-H, δ 3.90 p.p.m., dd, 2.1, 12.3 Hz; 6b-H, δ 3.66 p.p.m., dd, 6.4, 12.3 Hz) No other new resonances were present.

The observation that no ecdysone C–H resonance was significantly shifted on derivatization pointed to glucosidation at the quaternary C-25. This was confirmed by nuclear Overhauser enhancement difference spectroscopy, with irradiation at the coincident methyl protons 26/27 and at H-1 of the sugar ring. The methyl irradiation brought a 5% enhancement to sugar H-1, and irradiation of H-1 0.5% to the same methyls, together with the expected enhancements of H-3 and H-5 (5.5% together). In contrast, irradiation at methyl-21 had no effect on the sugar ring. These data unambiguously confirm the presence of a 25-O–1 link.

F.a.b.-m.s.

A major peak at high mass in the negative ion f.a.b. mass spectrum of compound U corresponding to the $[M-H]^-$ ion occurred at m/z 625, indicating a molecular mass of 626. This is consistent with the structure of compound U being an ecdysone mono-hexoside.

G.c.-m.s. of sugar released on enzymic hydrolysis

The sugar released on enzymic hydrolysis of conjugate U was characterized further by g.c.-m.s. As expected, under the derivatization conditions employed, authentic D-glucose yielded two anomers eluting at 12.5 and 13.3 min, respectively (Sweeley *et al.*, 1963). These peaks had similar retention times and mass spectra to the derivatized sugar from the hydrolysate of compound U. G.c.-m.s. co-chromatography of derivatized authentic D-glucose and nematode-derived sugar confirmed that the latter is glucose.

DISCUSSION

The results demonstrate that the ecdysone metabolite U isolated from *Parascaris equorum* is ecdysone 25-O- β -D-glucopyranoside (Scheme 1). Such a structure can be reconciled with the properties observed for [³H]U. Although the metabolite exhibits polar properties, as

Scheme 1. Structure of ecdysone 25-*O*- β -D-glucopyranoside

expected, on an adsorption h.p.l.c. column, it chromatographs near 20-hydroxyecdysone on a reversed-phase system, behaviour expected of a compound only slightly more polar than ecdysone. It may be that under the conditions of the reversed phase h.p.l.c., the compound adopts a folded conformation which does not reflect its true polarity. The apparent formation of a diacetone derivative by compound U can be explained if an acetone derivative is formed on the 2,3-positions of ecdysone as well as on the glucose moiety.

Ecdysone 25-*O*- β -D-glucopyranoside constitutes a new ecdysteroid conjugate. The only other identification of an ecdysteroid glucoside reported from invertebrates is that of 26-hydroxyecdysone 22-glucoside from tobacco hornworm (*Manduca sexta*) eggs during embryogenesis and newly hatched larvae (Thompson *et al.*, 1987). However, several ecdysteroid glycosides have been characterized from plants (Horn & Bergamasco, 1985; Saatov *et al.*, 1984, 1986), including 2-deoxyecdysone 25- β -D-glucopyranoside (blechnoside B) from the fern *Blechnum minus* (Suksamrarn *et al.*, 1986).

The significance of the formation of ecdysone 25-*O*- β -D-glucopyranoside from ecdysone in *Parascaris equorum* awaits elucidation. The observed elevated ecdysteroid concentration in the body fluids of mammalian hosts harbouring helminth infections is suggestive of ecdysteroid excretion by the parasites and may be of value in detecting such infections (Koolman & Moeller, 1986). Indeed, ecdysteroid excretion has been demonstrated in the rat cestode *Hymenolepis diminuta* cultures *in vitro*

(Mercer *et al.*, 1987). It is possible that metabolites such as the glucoside could be of value in increasing the specificity of detection of helminth infections and aid in distinguishing the type of helminthiasis.

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