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C-26 vs. C-27 Hydroxylation of insect steroid hormones: Regioselectivity of a microsomal cytochrome P450 from a hormone-resistant cell line

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ABSTRACT

Hydroxylation of steroids at one of the side chain terminal methyl groups, commonly linked to C-26, represents an important regulatory step established in many phyla. Discrimination between the two sites, C-26 and C-27, requires knowing the stereochemistry of the products. 26-Hydroxylation of the insect steroid hormone 20-hydroxyecdysone by a microsomal cytochrome P450 was previously found to be responsible for hormonal resistance in a *Chironomus* cell line mainly producing the (25*S*)-epimer of 20,26-dihydroxyecdysone. Here, we studied the 25-desoxy analog of 20-hydroxyecdysone, ponasterone A, to elucidate the stereochemistry of the expected 26-hydroxy product, inokosterone, which occurs as C-25 epimers in nature. We identified the predominant metabolite as the C-25 *R* epimer of inokosterone on comparison by RP-HPLC with the (25*R*)- and (25*S*)-epimers the stereochemistry of which was confirmed by X-ray crystallography. (25*R*)-inokosterone was further oxidized to the 26-aldehyde identified by mass spectroscopy, borohydride reduction and metabolic transformation to 26-carboxylic acid. The (25*S*)-epimers of inokosterone and its aldehyde were minor products. With 20-hydroxyecdysone as substrate, we newly identified the (25*R*)-epimer of 20,26-dihydroxyecdysone as a minor product. In conclusion, the present stereochemical studies revealed high regioselectivity of the *Chironomus* enzyme to hydroxylate both steroids at the same methyl group, denoted C-27.

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Introduction

The ecdysteroid hormones are key players in growth and development of insects and other arthropods. Considerable progress has been achieved in recent years to identify the genes encoding those cytochromes P450, which catalyze the terminal steps of biosynthesis of these highly functionalized steroids from dietary cholesterol and plant sterols, respectively. The biological effects of ecdysteroids are regulated at the levels of biosynthesis and inactivation as well as signaling pathways via nuclear receptors that mediate specific patterns of gene expression [1–3].

20-Hydroxyecdysone (20E)[1] is widely accepted as the most relevant insect steroid hormone as it is essential for molting, postembryonic development and many other processes. Hydroxylation in the side chain of the C-26 (or C-27, depending on the stereochemistry at C-25 [4]) methyl group is considered a major and obviously conserved way of steroid hormone inactivation, as is found in vertebrates, insects and plants [5]. In insects, the oxidation product 20,26-dihydroxyecdysone (2026E) is further modified either by the formation of water-soluble conjugates or by further oxidation to the corresponding 26-carboxylic acid, found in a range of species [2,6]. In larvae of the moth, *Spodoptera littoralis*, 26-hydroxylase activity is induced by hormonally active ecdysteroids and non-steroidal analogs, and evidence has been obtained that the responsible enzyme is a cytochrome P450 located in mitochondria as well as microsomes [7,8]. Very recently, a study of the fly *Drosophila* provided conclusive evidence that hormone inactivation by 26-hydroxylation, performed by CYP18A1, is of vital importance for larval development of this insect [9].

Our previous analysis of the mechanism of hormone resistance in several subclones of an insect cell line from the dipteran *Chironomus tentans* revealed that this insensitivity was due to a high rate of 26-hydroxylation of 20E thus preventing morphological and biochemical differentiation [10]. The responsible 26-hydroxylase was characterized as a cytochrome P450 with strict microsomal localization. Its activity paralleled the level of hormone resistance of the various subclones, and 2026E was identified as the principal metabolite in all resistant cells [10]. Unexpectedly, this product did not give rise to the formation of 26-carboxylic acid; instead, two novel metabolites were isolated as final products, which turned out to be a diastereomeric pair of interconvertable cyclic hemiacetals obviously resulting from an intramolecular addition

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[1] *Abbreviations used:* E, ecdysone; 20E, 20-hydroxyecdysone; 26E, 26-hydroxyecdysone; 2026E, 20,26-dihydroxyecdysone; PonA, ponasterone A; INO, inokosterone.

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reaction of the 26-aldehyde [11]. The stereochemistry of both hemiacetals was (25*S*), as shown by NMR. Since the stereochemistry at C-25 of the steroid alcohol should not change on its further transformation to the aldehyde, the primary and predominant metabolite 2026E has to be assigned (25*S*) configuration.

In the present study, we extended the previous work on 20E [10,11] to its biologically active 25-desoxy analog, ponasterone A (PonA), using cells from the most ecdysteroid-resistant subclone expressing high 26-hydroxylase activity. Like 20E, PonA was expected to become hydroxylated at C-26 thus giving rise to inokosterone (INO). The formation of INO was key to the present study addressing the regio-, respectively, stereochemistry of 26-hydroxylation by the *Chironomus* enzyme. Due to the non-equivalence of the two side chain terminal methyl groups, numbered C-26 and C-27, hydroxylation creates an asymmetric center at C-25 with the possibility of two epimers, designated (25*S*) and (25*R*), respectively. In fact, INO epimers have been found in several plants [12,13]. The 25-hydroxy analog of INO, 2026E, has also been reported to occur as a mixture of C-25 epimers in a plant [14].

Here, we followed two objectives: first, to study the metabolism of PonA by the cytochrome P450 of the ecdysteroid-resistant cell line, and second, to elucidate the stereochemistry of the PonA products in comparison to those of 20E, identified previously [10,11]. To this end, we dissected the oxidation sequence from PonA via INO and its 26-aldehyde derivative to a highly polar product, most likely representing the corresponding 26-carboxylic acid. With reference to stereochemistry, we showed that INO is produced as the (25*R*)-epimer with high preference by the *Chironomus* 26-hydroxylase. Furthermore, we analyzed the stereochemistry at C-25 of the metabolites of 20E revealing the same regioselectivity of the enzyme. Stereochemical aspects of cytochrome P450 catalyzed hydroxylations of ecdysteroids have not yet been studied. Our present studies were made possible by the successful crystallization and definite identification of the natural epimers of INO for use as chromatographic references.

The present study also revealed that the (25*R*)- and (25*S*)-epimers of the 26-hydroxy ecdysteroids under study and also of 26-hydroxycholesterol can be discriminated by conventional RP-HPLC under isocratic conditions.

Experimental

Ecdysteroids and other chemicals

Tritiated PonA ([24,25(n)-^{3}H]ponasterone A) was custom-synthesized from stachysterone A at a specific radioactivity of 7.9 TBq/mmol and of >97% radiochemical purity. Products from [^{3}H]20E were obtained as described [10]. Unlabeled INO, as a mixture of C-25 epimers, was purchased from Rohto Pharmaceutical (Osaka, Japan). Other ecdysteroids were from previous studies. (25*S*)- and (25*R*)-26-hydroxycholesterol were obtained from Research Plus (Barnegat, NJ, USA). (22*R*)-Hydroxycholesterol was from Sigma. Clotrimazol, difenoconazol and standard chemicals were obtained from Merck and Sigma–Aldrich.

Cells and enzyme preparation

All assays were performed with cells from the highly ecdysteroid-resistant subclone ec.r.2 of the epithelial cell line from the dipteran *C. tentans* [10]. Prior to the assays, the cellular membranes were washed by suspension in assay medium (see below) and centrifugation to remove soluble cofactors of enzyme action. Protein concentration was determined using the dye-based reagent from Bio-Rad and bovine serum albumin as a reference.

Enzyme assays

The general assay scheme was the same as in previous work [10]. Varied quantities of cellular membrane protein were incubated in Eppendorf tubes in a total volume of 0.1 mL containing 50 mM potassium phosphate (pH 7.4), 0.1 mM EDTA-Na_2, 0.1 mM DTT, 1 mM NADPH (NADH in some experiments) as cofactor and [^{3}H]-labeled ecdysteroid. The tubes were shaken at 35 °C for varied periods of time. Two kinds of controls were performed: (i) omission of cofactor, and (ii) heat-inactivation of protein (5 min at 95 °C). The fungicidal cytochrome P450 inhibitors clotrimazol and difenoconazol, in methanol solution, were pre-incubated with the protein for 5 min prior to addition of cofactor and labeled substrate. Competitor ecdysteroids, dissolved in methanol/water (1:1; v/v), were mixed with the substrate in buffer prior to the addition of cofactor and protein. The final methanol concentration in the assay never exceeded 1%. IC_{50} values (inhibitor concentrations producing 50% effect) were obtained from single series of experiments each performed on eight concentrations (1 nM–10 μM). Inhibition was calculated from the decrease of [^{3}H]PonA concentration after short-term incubation (5–10 min). Non-standard assay conditions are given in the Section Results.

The assays were terminated by transfer to 95 °C for 5 min followed by centrifugation at 12,000*g* for 5 min. The supernatants were passed through 0.45 μm Ultrafree MC filter units (Millipore) by centrifugation at 5000*g* and then subjected to HPLC analysis (see below). Eventually, as in preparative work, steroid solutions were concentrated on a 100-mg C18 column (Isolute, International Sorbent Technology) and further processed as described [10].

RP-HPLC

A Shimadzu HPLC gradient system was used that consisted of two LC-6A pumps, a SCL-6A control unit and a C-R4A Chromatopak coupled to an UVIS 204 flow-through spectrophotometer (Linear Instruments) set at 245 nm, and to a Ramona-LS system for on-line registration of radioactivity using a solid-phase scintillator flow-through cell (Raytest). Analyses of supernatants from enzyme assays were performed on a 5 μm Nucleosil C18 column (250 × 4.6 mm) equipped with a 1-cm precolumn of the same material. Separation of all metabolites was achieved by a gradient elution program employing increases of methanol concentration in water (v/v) as follows: 0–30 min:33–38% methanol; 30–35 min:38–48% methanol; 35–60 min:isocratic at 48%; 60–70 min:48–80%; 70–80 min:80–33%. The flow rate was 1 mL/min.

Preparative incubations of 20E with cellular preparations of *Chironomus* were performed as described [10]. The main product 2026E was obtained by elution with 33% (v/v) methanol in water. The C-25 epimers of 2026E were separated by elution with 27% (v/v) methanol in water. They were concentrated using 100-mg C18 SepPak columns and eluted with 100% methanol.

The C-25 epimers of commercial INO were separated by preparative RP-HPLC using a 7 μm Eurospher 100-C column (240 × 8 mm) and isocratic elution with 36% (v/v) methanol in water at a flow rate of 2.0 mL/min. The two epimers, assigned INO-1 and INO-2, were well separated eluting after ~42 min and ~49 min, respectively; the difference in elution times was fairly constant at 6.5 min. The pooled eluates of each epimer were diluted with two volumes of water and concentrated by passage over 2-g C18 SepPak columns (Waters) followed by elution with 100% methanol. The solvent was removed using a Speed Vac centrifuge. The first and second eluting epimers, here referred to as INO-1 and INO-2, represented 39% and 61%, respectively, of total INO. Both INO epimers showed ultraviolet absorption spectra, mass spectra and ^{1}H NMR spectra, in accordance with their structure (http://ecdybase.org). In the ^{13}C NMR spectra, the epimers differed in

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