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## The complex metabolism of poststerone in male rats

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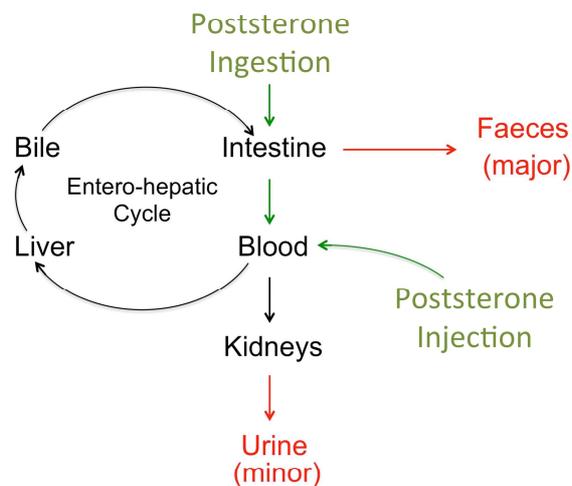
32 **Abstract**

33 Ecdysteroids are not endogenous to mammals, but are normal components of the  
34 food intake of many mammalian species consuming phytoecdysteroid-containing  
35 plants. The most frequently encountered phytoecdysteroid is 20-hydroxyecdysone  
36 (20E). Several pharmaceutical effects have been observed after ecdysteroid injection  
37 or ingestion, but it is not clear to what extent metabolites generated in the  
38 mammalian body contribute to these effects.

39 The C21-ecdysteroid poststerone (Post) is a metabolite of 20E in rodents. Post  
40 analogues are key intermediates in the metabolism of exogenous ecdysteroids  
41 possessing a C20/22-diol. The pharmacokinetics, bioavailability and metabolism of  
42 Post have been assessed in male rats after ingestion and injection. The  
43 bioavailability of Post is significantly greater than that of 20E and the presence of an  
44 efficient entero-hepatic cycle allows Post to be effectively metabolised to a wide  
45 range of metabolites which are excreted mainly in the faeces, but also to some extent  
46 in the urine. Several of the major metabolites in the bile have been identified  
47 unambiguously as 3-*epi*-poststerone, 16 $\alpha$ -hydroxypoststerone, 21-  
48 hydroxypoststerone and 3-*epi*-21-hydroxypoststerone. Conjugates are also present.  
49 Parallels are drawn to the metabolism of endogenous vertebrate steroid hormones,  
50 to which Post bears more similarity than 20E.

51  
52 *Keywords:* anabolic steroid; ecdysteroid; entero-hepatic cycle; pharmacokinetics;  
53 rodent; steroid metabolism

54  
55 **Graphical Abstract**



60  
61

62 **Abbreviations:**

63 20E = 20-hydroxyecdysone, 20,26E = 20,26-dihydroxyecdysone, 14d20E = 14-  
64 deoxy-20-hydroxyecdysone, 6 $\alpha$ OH20E = 6 $\alpha$ -hydroxy-20-hydroxyecdysone,  
65 6 $\alpha$ OH14d20E = 6 $\alpha$ -hydroxy-14-deoxy-20-hydroxyecdysone, Post = poststerone,  
66 14dPost = 14-deoxypoststerone, 3epiPost = 3-*epi*-poststerone, 3-epi21OHPost = 3-  
67 *epi*-21-hydroxypoststerone, HPLC = high-performance liquid chromatography, HPLC-  
68 MS/MS = high-performance liquid chromatography – tandem mass-spectrometry,  
69 NMR = nuclear magnetic resonance spectroscopy, MS = mass spectrometry,  
70

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72  
73

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75 LD and RL are, or were, employees of Biophytis. The support of FUI (Fonds Unique  
76 Interministériel: FUI AAP12\_110426; SARCOB project), and of the Conseil Général  
77 de la Seine is deeply acknowledged. Biophytis had no influence on the study design,  
78 data interpretation or manuscript preparation.  
79

80 **Declaration of Interests:** none

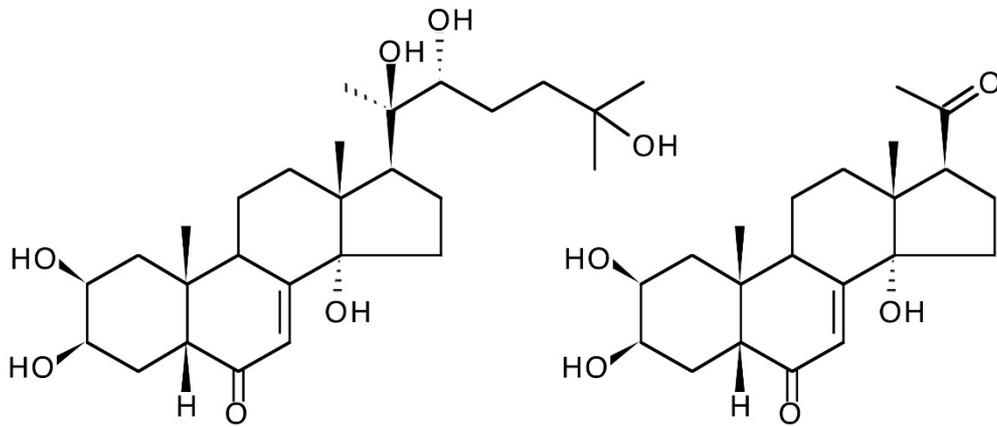
## 81 **1. INTRODUCTION**

82 In a previous paper [Dinan et al., accepted for publication], we have shown that the  
83 fate of ingested 20-hydroxyecdysone (20E) in rats is initially (over the first 2 hours)  
84 uncomplicated, since the bioavailability of 20E is very low and the vast majority of it  
85 remains associated with the upper portions of the intestinal tract, where it undergoes  
86 metabolism only when it reaches the large intestine by 14-dehydroxylation and  
87 reduction of the 6-oxo-group, to produce a clearly defined set of metabolites, based  
88 on permutations of these 2 reactions. Thereafter, the metabolic profile becomes  
89 much more complex and we have proposed that this is a consequence of metabolism  
90 of 20E to poststerone (Post) and of 14d20E to 14deoxy-poststerone (14dPost) and  
91 the establishment of an efficient entero-hepatic cycle, resulting in a multiplicity of  
92 Post metabolites. In this paper we provide strong evidence for this hypothesis in that  
93 we demonstrate that the bioavailability of Post is considerably higher than that of  
94 20E, that Post and its metabolites are found in high concentrations in the bile after  
95 *i.v.* or *per os* administration, and we also identify a number of the many metabolites.

96  
97 Post is a C<sub>21</sub>-ecdysteroid which has been identified in insects and plants. It was first  
98 proposed as a metabolite of 20E in the blowfly *Calliphora stygia*, resulting from  
99 cleavage of the C-20/22 diol [Galbraith et al., 1969]. It was also identified as a  
100 metabolic product of ponasterone A when the latter was injected into larvae of the  
101 silkworm, *Bombyx mori* [Hikino et al., 1975]. These findings, together with the low  
102 biological activity of Post in insect systems [Bergamasco and Horn, 1980; Dinan,  
103 2003], identified Post as a deactivation product of ecdysteroids in insects. Post has  
104 also been detected in a few phytoecdysteroid-containing plants [Hikino et al., 1970;  
105 Vokač et al., 2002], where it is only ever a minor component of the ecdysteroid  
106 'cocktail', so even phytophagous animals would be exposed to very low, or non-  
107 existent, levels of Post in their diet. 20E, the major phytoecdysteroid, found in plants,  
108 has been shown to possess a number of pharmacological effects in mammals,  
109 almost all of which can be considered as beneficial (reviewed in [Bathori and  
110 Pongracz, 2005; Dinan & Lafont, 2006]). Mammals do not synthesise ecdysteroids  
111 themselves and it is not currently established whether the biological activities in  
112 mammals are associated with the administered ecdysteroid alone, or whether  
113 metabolites also contribute to the observed effects.

114 The identification of Post and 14dPost, which have increased structural similarity to  
115 vertebrate steroid hormones (especially to glucocorticosteroids), as significant  
116 metabolites of 20E in mammals enhances the suspicion that these, or similar  
117 metabolites, may possess biological activity in mammals. Recently indeed, evidence  
118 has been presented that Post has an anabolic activity on rat muscle fibres and  
119 activates protein kinase B [Csábi et al., 2019; Issaadi et al., 2019].

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121



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124

**Figure 1:** Structures of 20-hydroxyecdysone (left) and poststerone (right).

## 125 **2. MATERIALS & METHODS**

### 126 **2.1. Reference and radiolabelled ecdysteroids**

127 20E (purity 99.7%) was provided by Dr. Juraj Harmatha (Prague, Czech Republic).  
128 The preparation of 20E tritiated in positions 1 $\alpha$ ,2 $\alpha$  (specific activity = ca. 0.5  
129 TBq/mmol; purity  $\geq$ 90%) and the synthesis of the reference compounds [poststerone  
130 (Post) and 14-deoxy-poststerone (14dPost)] have been previously described  
131 [Kumpun et al., 2011]. Tritiated Post was prepared from [ $^3$ H]20E following the same  
132 protocol as used to obtain non-radiolabelled Post from 20E. 3-*Epi*-poststerone was  
133 prepared from 20E in 3 steps: (1) oxidation to 3-dehydro 20E [Spindler et al., 1977],  
134 (2) reduction to 3-*epi*-20E [Dinan and Rees, 1978] followed by (3) side-chain  
135 cleavage [Petersen et al., 1993], Non-radioactive products were unambiguously  
136 identified by NMR and MS. Radiolabelled products were identified by co-  
137 chromatographed with their unlabelled homologues on RP- and NP-HPLC.  
138

### 139 **2.2. Animals**

140 Male Wistar rats (Charles River, l'Arbresle, France) were used at 12 weeks of age,  
141 weighing ca. 200 g. They had access *ad libitum* to water and a standard diet. All  
142 experiments on animals were carried out in accordance with the European animal  
143 care guidelines (ETS123) concerning the care and the ethical treatment of animals in  
144 research.  
145

### 146 **2.3. Bioavailability of poststerone and an excretion study in rats**

147 Post was administered *per os* to rats (3 animals/group) in water (50 mg/kg body  
148 weight; 10 mL/kg) or intravenously in saline (5 mg/kg body weight; 4 mL/kg). Blood  
149 was collected from the tail at t = 0.08 h, 0.25 h, 0.5 h, 1 h, 3 h, 6 h, 8 h and 24 h.  
150 Blood samples were centrifuged (10,600 x g for 5 min) and plasma samples were  
151 collected, frozen and stored at -80°C until analysis by LC-MS/MS. In a separate  
152 experiment, [ $^3$ H]Post (15  $\mu$ Ci) was administered *per os* to rats. Urine and faeces were  
153 separately collected at 0-8 h, 8-24 h, 24-32 h and 32-48 h after ingestion for  
154 radioactivity measurement and HPLC analysis.  
155

### 156 **2.4. Extraction and purification of rat samples**

157 Faeces were collected and weighed. The volume of urine samples was determined  
158 and 3-times that volume of absolute ethanol was added. Samples were stored at -  
159 80°C until analysis. Faeces were homogenized in ethanol and the volume of the  
160 extract made up to 30 mL with ethanol. The volume of each urine sample was made  
161 up to 25 mL with ethanol. The faecal and urine samples were centrifuged (3,500 rpm  
162 for 12 min at 4°C). The resulting supernatants were dried, and then taken up in 2 mL  
163 methanol. Some lipid-rich faecal samples were also purified by a chloroform-water  
164 (1:1 v/v) partition and the aqueous phase was analysed.

165

## 166 **2.5. Enzymatic hydrolyses**

167 To test for the possible presence of ecdysteroid conjugates, hydrolyses of bile  
168 aliquots were performed by incubating extracts of them overnight with either 1 mg  $\beta$ -  
169 glucuronidase (*Helix pomatia* [G-0751] or *E. coli* [G-5897]; Sigma-Aldrich, St. Louis,  
170 MO) in 500  $\mu$ L sodium acetate buffer (50 mM, pH 5.3). After hydrolysis, the solutions  
171 were dried and then taken up in 100  $\mu$ L water. These solutions were analysed by  
172 HPLC in the presence of reference compounds, with simultaneous monitoring of  
173 radioactivity. In the metabolite preparative experiment the same protocol was used,  
174 except that the amounts of enzyme and buffer volumes were adapted to sample size  
175

## 176 **2.6. Radioactivity measurements**

177 Radioactivity was measured by liquid scintillation counting (Beta-5, Kontron  
178 Instruments, Montigny-le-Bretonneux, France) of 50  $\mu$ L aliquots mixed with 2 mL  
179 scintillation cocktail (Ecoscint, National Diagnostics, Atlanta, GA). In the case of  
180 HPLC analyses, when entering the radioactivity monitor, the effluent was mixed with  
181 scintillation cocktail (3 mL/min). Comparison with the UV traces allowed identification  
182 of the major radioactive metabolites.  
183

## 184 **2.7. Analytical techniques and sample processing**

### 185 **2.7.1. HPLC-MS/MS analyses**

186 Liquid chromatography-mass spectrometry (LC-MS/MS) pharmacokinetic analyses of  
187 plasma samples were performed using an API 3200<sup>®</sup> turbo ion-spray mass-  
188 spectrometer (ABSciex, Framingham, MA) in negative-ion mode (MRM).  
189 Chromatographic separation was achieved on an XBridge<sup>®</sup> Shield RP18 column, (3.5  
190  $\mu$ m, 3.0 x 100 mm; Waters, Milford, MA) at 300  $\mu$ L/min. The mobile phase consisted  
191 of mixtures of solvent A (0.1% trifluoroacetic acid in Milli-Q water) and solvent B  
192 (acetonitrile). The gradient program for HPLC analyses started with 82% solvent A  
193 and 18% solvent B for 1 min, increasing linearly to 30% solvent B at t = 3 min, held  
194 for 2 min, then increasing to 80% solvent B at t = 6 min, held for 1 min, decreased to  
195 18% solvent B at t = 7.5 min and finally held for 6.5 min.  
196

197 Analysis of putative glucuronide conjugates was performed by liquid  
198 chromatography-mass spectrometry (LC-MS) using an API 3200<sup>®</sup> turbo ion-spray  
199 (ABSciex, Framingham, MA) in negative- and positive-ion mode (Q1).  
200 Chromatographic separation was achieved on an Ace C18 column, (5  $\mu$ m, 4.6 x 150  
201 mm; Phenomenex) at 1 mL/min. The mobile phase consisted of mixtures of solvent A  
202 (1% formic acid in Milli-Q water) and solvent B (acetonitrile). The gradient program  
203 for HPLC analyses started with 90% solvent A and 10% solvent B increasing linearly  
204 to 40% solvent B at t = 30 min.  
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209

### 210 2.7.2. Radio-HPLC analyses

211 Radioactive samples were separated on a Spectra series P200 HPLC (Thermo  
212 Separation Products) with a UV detector (254 nm) and a radioactivity monitor ( $\beta$ -  
213 RAM, Lab Logic, Sheffield, UK). An ACE 5C18 column (150 x 4.6 mm i.d.) was eluted  
214 at 1 mL/min with a linear gradient from 90% water+0.1% (v/v) TFA (Solvent A), and  
215 acetonitrile + 0.1% TFA (Solvent B) to 60% Solvent A/40% Solvent B over 30min,  
216 which was then maintained for 5min, before proceeding to 100% Solvent A over 1  
217 min, maintained for 5 min before being returned to starting conditions over 1 min and  
218 re-equilibrated for 3 min.

219

### 220 2.7.3. HPLC Systems used for bile sample analysis and purification

#### 221 *Analytical HPLC/DAD/MS*

222

223

224 Samples were separated on an ACE 5 C18 column (5 $\mu$ m particle size; 150 x 4.6mm)  
225 eluted at 1 mL/min with a linear gradient from 90% H<sub>2</sub>O + 0.1% (v/v) HCOOH  
226 (Solvent A) and ACN + 0.1% (v/v) HCOOH (Solvent B) to 40% Solvent A/60%  
227 Solvent B over 30min, followed by an increase to 90% Solvent B/10% Solvent B over  
228 1min, which was maintained for 9 min, before being returned to starting conditions for  
229 re-equilibration. The Turbo Ion-Spray MS operated in both positive- and negative-ion  
230 modes.

231

#### 232 *Preparative HPLC*

233 *System RP1:* Sunfire C<sub>18</sub> 3.5  $\mu$ m 19 x 100 mm, eluted with a linear gradient from 10-  
234 30% ACN in 0.1% HCOOH/H<sub>2</sub>O over 10 min, 30% ACN for 5 min and then 30-80%  
235 ACN over 5 min; flow-rate = 35 mL/min; detection at 254 nm

236

237 *System NP1:* a preparative Varian Dynamax column (5  $\mu$ m; 250 x 20.4 mm), eluted  
238 isocratically with dichloromethane/isopropanol/water, 125:30:1.5 (v/v/v) at a flow-rate  
239 of 20mL/min and with UV detection at 254 nm.

240

## 241 **2.8. Bile secretion experiments**

242 Three different experiments were performed on male Wistar rats using either  
243 administration of unlabelled or tritiated poststerone. For the first experiment using  
244 non-radiolabelled Post, a catheter was introduced into the bile duct after anaesthesia.  
245 Poststerone was reconstituted in saline and was administered intravenously at a  
246 dose of 10 mg/kg. The bile was collected every 10 min, and each tube was made up  
247 to 500  $\mu$ L with EtOH. Aliquots (50  $\mu$ L) were thereafter analysed by HPLC-MS/MS for  
248 Post content.

249

250 For an experiment using tritiated ecdysteroid, 2 rats were treated with [<sup>3</sup>H]Post  
251 (4.5  $\mu$ Ci) diluted with 10 mg/kg unlabelled ecdysteroid. Ethanol was added to bring  
252 each bile sample to 500  $\mu$ L and aliquots (50  $\mu$ L) were radio-assayed. Aliquots (50  $\mu$ L)

253 of each of the 13 timed samples collected during the 2 h-kinetic were then pooled,  
254 evaporated under a nitrogen gas flow and then analysed by radio-HPLC, before or  
255 after overnight glucuronidase treatment at 37°C.

256  
257 A third preparative experiment was performed with 14 male rats each receiving 2  
258 successive intravenous injections (2.5 mL/kg at 0 h and 1 h) of a poststerone solution  
259 (8 mg/mL in saline), and bile was collected for between 2 and 4 h (depending on  
260 animal survival), which allowed collection of individual bile volumes ranging from 0.7  
261 to 2.3 mL (total bile volume 23.3 mL). Aliquots from each rat bile sample were  
262 assayed by HPLC-MS/MS, both before and after glucuronidase treatment. Then all  
263 the remaining portions of the samples were pooled and treated with glucuronidase;  
264 the pooled bile was added to 23.7 mg glucuronidase in 40 mL 4 mM potassium  
265 phosphate buffer, pH 6.8, incubated at 37°C overnight and then partially purified on a  
266 10 g C18-Sep-Pak Cartridge (Waters), prior to purification by sequential preparative  
267 RP-HPLC and NP-HPLC (as described above in § 2.7.3).

268

## 269 **2.9. NMR analyses**

270 All NMR spectroscopy experiments were recorded on a Bruker AVANCE II 500  
271 Spectrometer in D<sub>2</sub>O (500 MHz for <sup>1</sup>H and 125 MHz for <sup>13</sup>C), at 300K [Girault &  
272 Lafont 1988; Girault,1998]. Presaturation of the solvent was used for all 1D- and  
273 homonuclear 2D-<sup>1</sup>H experiments. 2D-Experiments (<sup>1</sup>H, TOCSY [spin lock times: 15  
274 ms and 35 ms], NOESY [mixing time: 500 ms], <sup>1</sup>H-<sup>13</sup>C, HSQC [correlation one bond  
275 <sup>1</sup>J<sup>1</sup>H-<sup>13</sup>C] and HMBC [correlation <sup>1</sup>H-<sup>13</sup>C via two or three bonds via <sup>2</sup>J<sup>1</sup>H-<sup>13</sup>C or <sup>3</sup>J<sup>1</sup>H-  
276 <sup>13</sup>C, long-range delay: 80 ms]) were performed by Pulse-Field Gradient (PFG)  
277 methods using standard Bruker software. The samples were lyophilised and  
278 dissolved in D<sub>2</sub>O. The errors in the chemical shifts are ≤ 0.01 ppm for <sup>1</sup>H and  
279 ≤ 0.2 ppm for <sup>13</sup>C. TSPD<sub>4</sub>, 3-(trimethylsilyl)[2,2,3,3-d<sub>4</sub>] propionic acid sodium salt, was  
280 used as the internal reference for the proton and carbon shifts

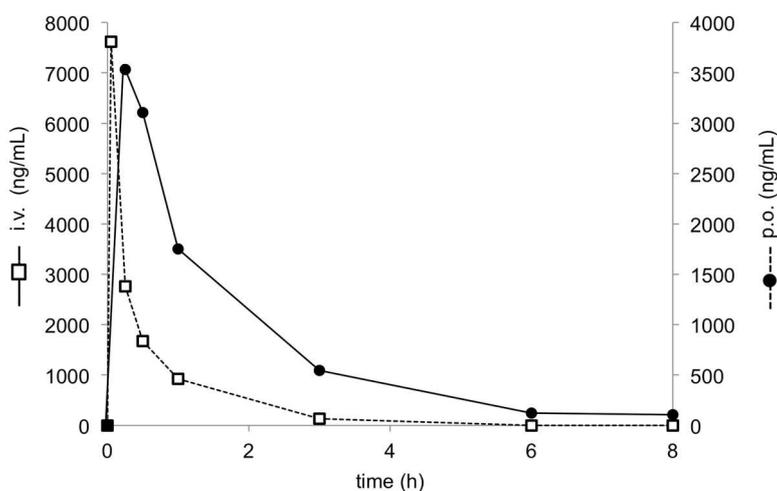
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283 **3. RESULTS**

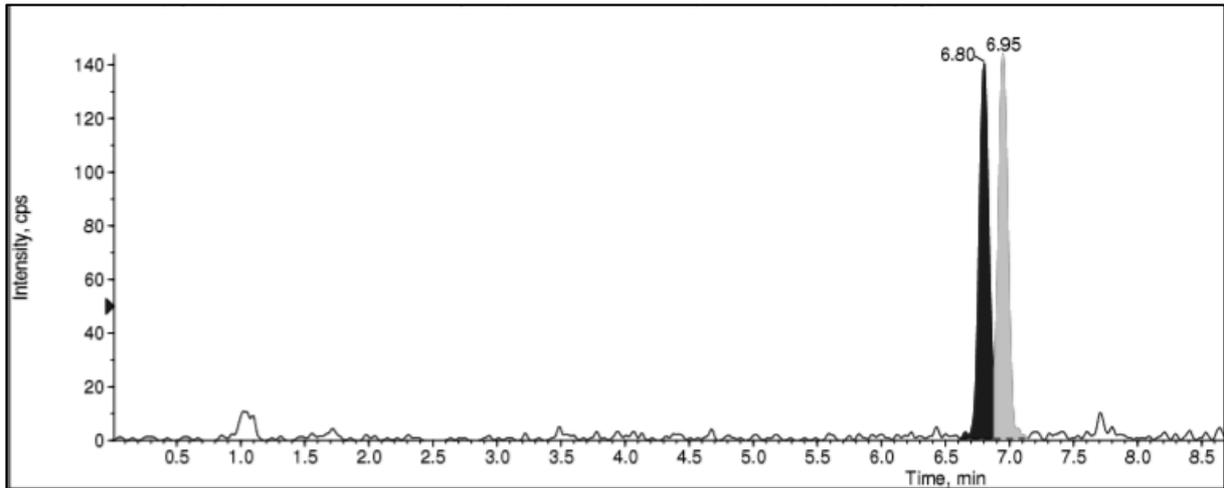
284 **3.1. Bioavailability of poststerone in rats**

285 After oral administration of Post at 50 mg/kg, plasma concentrations are not very  
286 high, reaching a maximum concentration of 3.533  $\mu\text{g/mL}$  after 15 min (AUC = 6885  
287 ng.h/mL). Post plasma concentrations after intravenous administration at 5mg/kg are  
288 initially high ( $C_{\text{max}}$  = 7.617  $\mu\text{g/mL}$  at 3 min) and then decrease rapidly (AUC = 3690  
289 ng.h/mL). The plasma concentrations after oral and intravenous administrations are  
290 compared in Fig. 2. From the AUC, a bioavailability of 18.7% can be calculated, to be  
291 compared with ca. 1% for 20E [Dinan et al., accepted for publication].  
292



293 **Figure 2:** Plasma poststerone kinetics. Levels in rat plasma were measured after  
294 intravenous (5 mg/kg; left axis) or oral (50 mg/kg; right axis) administration. For  
295 clarity, the points at 24 h (< LOQ) are not included.  
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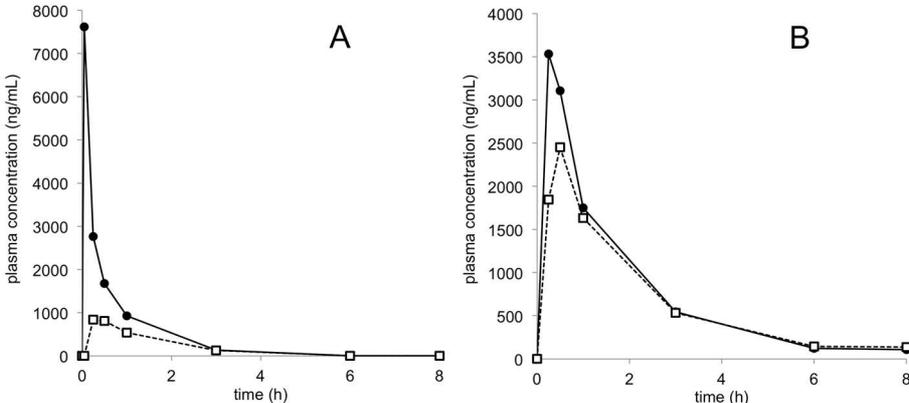
299 HPLC-MS/MS of the plasma samples showed the presence of an additional peak  
300 (6.95 min) eluting very close to Post (6.80 min) and having the same mass (see Fig.  
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**Figure 3:** RP-HPLC separation of a plasma sample from a rat treated *p.o.* with poststerone. The chromatogram shows the presence of 2 closely eluting peaks, corresponding to poststerone (6.80min) and 3-*epi*-poststerone (6.95min) with detection by multiple reaction monitoring in negative-ion mode ( $m/z$ : 361/273).

This peak was identified as 3-*epi*-poststerone by comparison with a synthetic reference. The time-course of its titre in the plasma after oral and intravenous application of Post is shown in Figure 4. It is particularly abundant after oral administration. At this stage, it is difficult to decide whether it is formed in the liver or in the gut wall.



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**Figure 4:** Pharmacokinetics of poststerone. Plasma levels of poststerone (solid line) and 3-*epi*-poststerone (dotted line) were measured by HPC-MS/MS after (A) intravenous (5mg/kg body weight) and (B) oral (50 mg/kg body weight) administration of poststerone to male rats.

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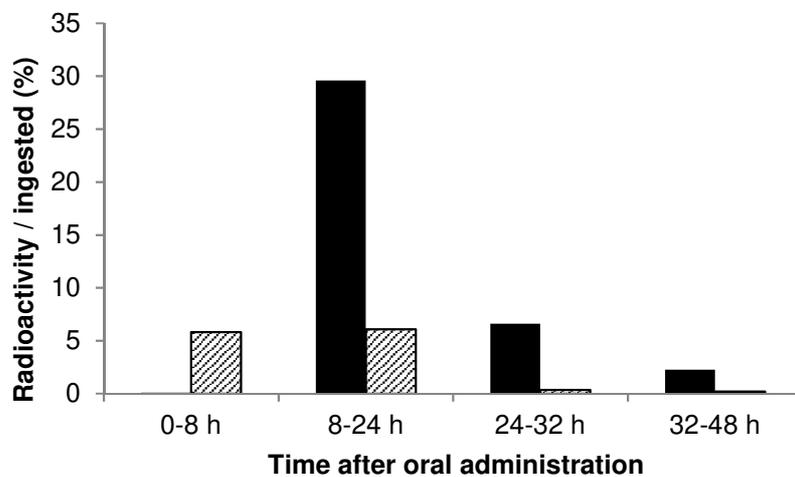
### 322 **3.2. Analyses of faeces and urine samples**

#### 323 *3.2.1. Distribution of radioactivity after oral administration of [<sup>3</sup>H]Post to rats*

324 The amounts of radioactivity measured in the faeces and urine over sequential  
325 periods after the application of [<sup>3</sup>H]Post are shown in Fig. 5. Some radioactivity is still  
326 present in the faeces 32-48 hours after ingestion.

327

328 In the case of poststerone, a quarter of the radioactivity excreted is recovered in the  
329 urine, while for 20E this proportion was only 1.4% [Dinan et al., accepted for  
330 publication]. This result is consistent with the much higher oral bioavailability of  
331 poststerone as compared to 20E. Poststerone, being more bioavailable in the blood  
332 than 20E, is logically found in greater quantities in the urine.  
333



334

335

336 **Figure 5:** Excretion of radioactivity by male rats after oral administration of  
337 [<sup>3</sup>H]poststerone. The histogram shows the distribution of recovered radioactivity in  
338 the faeces (solid bars) and urine (hatched bars) after ingestion of [<sup>3</sup>H]poststerone.

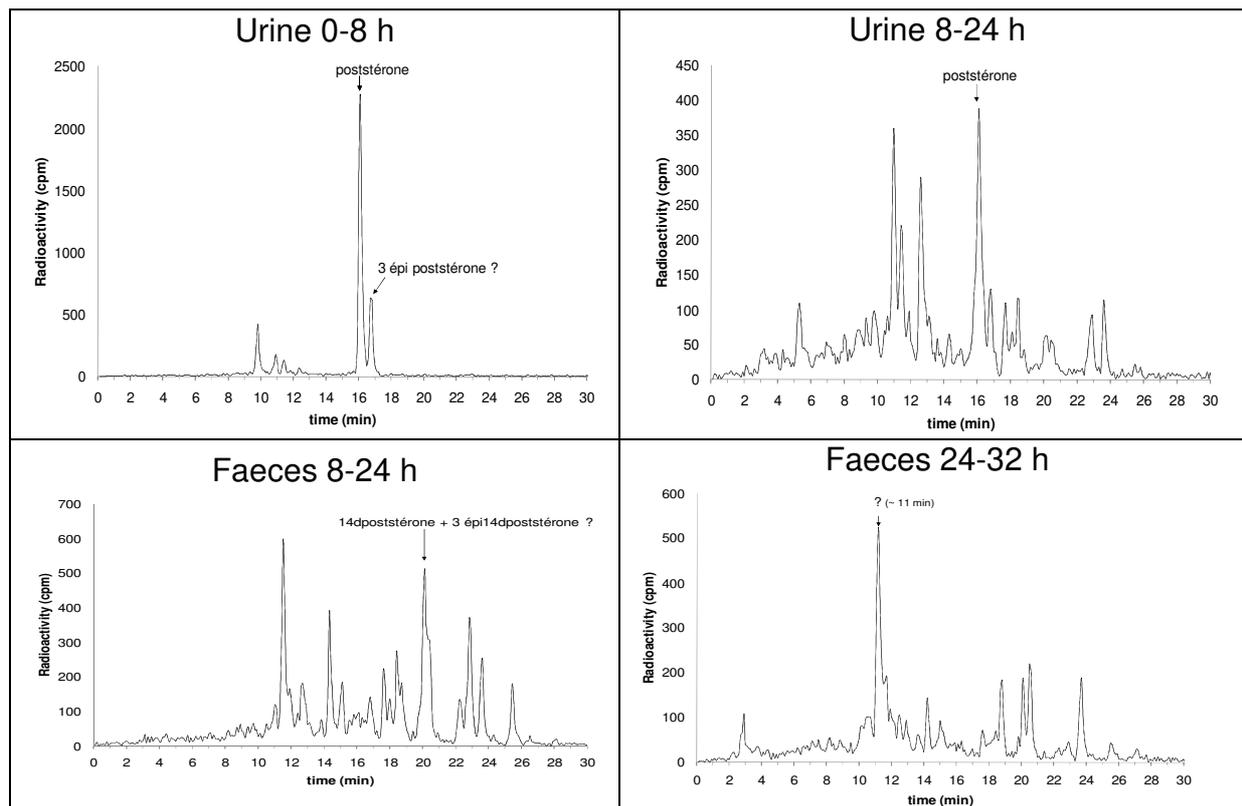
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#### 341 *3.2.2. Qualitative analysis by HPLC of ecdysteroid profiles in faeces and urine of rats*

342 The radiolabelled faecal and urine samples were extracted and separated by RP-  
343 HPLC to assess how metabolite profiles changed with time and excretion route (Fig.  
344 6).

345



346  
 347 **Figure 6:** RP-HPLC of extracts of urine and faeces timed samples after ingestion of  
 348 [<sup>3</sup>H] poststerone.  
 349

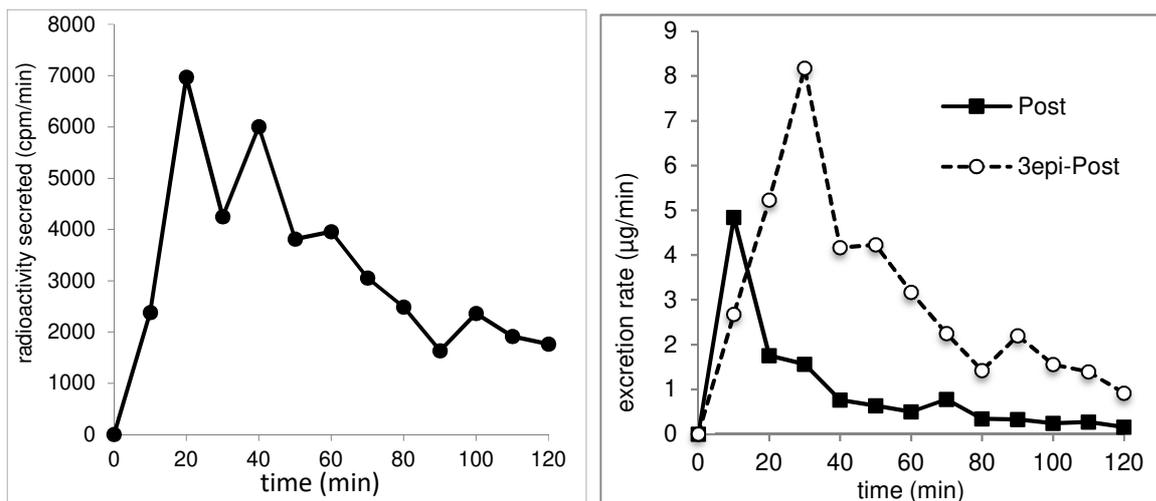
### 350 3.2.3 Metabolism of poststerone by gut microbiota

351 In the previous paper [Dinan et al., accepted for publication], we demonstrated that  
 352 anaerobic incubation of 20E or Post with rat gut contents resulted in their metabolism  
 353 to 14d20E and 14dPost, respectively. No other products were formed under  
 354 anaerobic conditions. In view of the occurrence of the various other metabolites of  
 355 Post in the bile (see below), it seems highly probable that these metabolites are  
 356 formed in the liver, but other possible sites of metabolism (e.g. the gut tissue) cannot  
 357 be excluded at the moment.  
 358

## 359 **3.3. Analysis of bile secretion**

### 360 3.3.1. Kinetics of biliary secretion in rats

361 Bile was collected from the rat bile duct for contiguous 10-min periods over 2h after  
 362 the intravenous application of Post or of 4.5 μCi [<sup>3</sup>H]Post diluted with 10 mg/kg non-  
 363 radioactive Post. The initial analyses of the bile samples for a single rat are  
 364 presented in Fig. 7.  
 365



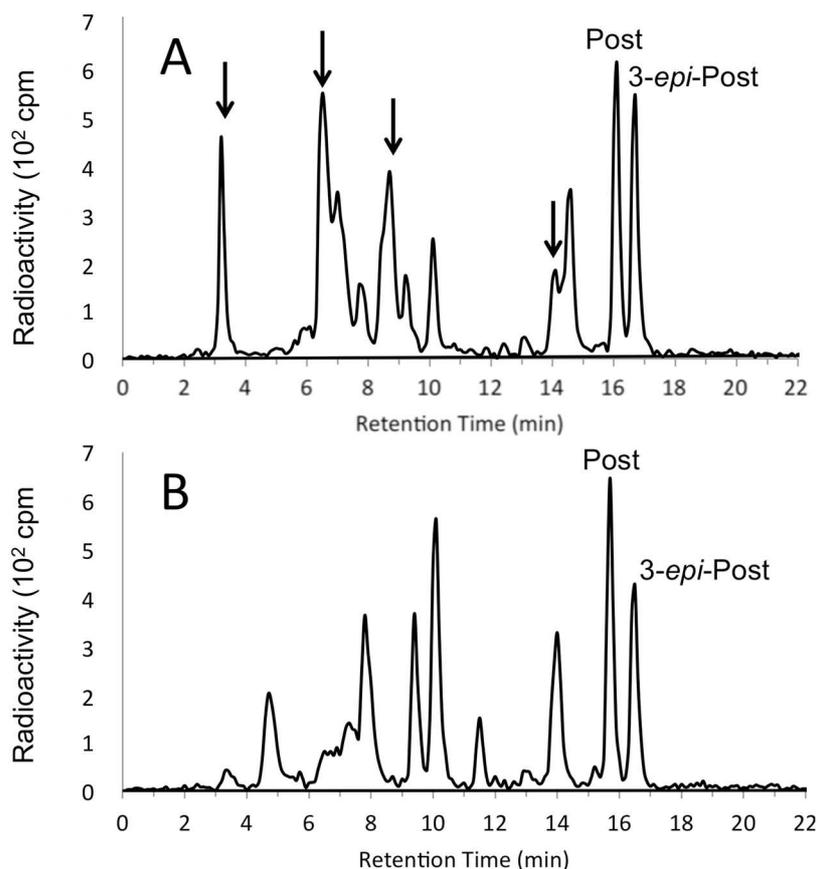
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**Figure 7:** Kinetics of secretion in the bile after *i.v.* injection of poststerone. (A) after injection of  $[^3\text{H}]$ poststerone by radioactivity counting and (B) after injection of unlabelled poststerone by analysis by HPLC-MS/MS where the kinetics of Post and 3epiPost secretion were measured separately.

While the radioactivity eliminated in bile over 2 hours represented 30-40% of the administered dose, the sum of Post+3-*epi*-post accounted for only 5-7% of the administered unlabelled poststerone. This indicated that other poststerone metabolites were present in the bile, as evidenced by radio-HPLC analysis (see below).

### 380 3.3.2. Metabolite profile in bile and the effect of enzymatic hydrolyses

381 Using HPLC analysis of  $[^3\text{H}]$ post-treated rats allowed us to observe the complex  
 382 pattern of labelled metabolites in bile in addition to 3-*epi*-poststerone. (Fig. 8A) The  
 383 presence of conjugates in the bile is apparent from the reduction of certain peaks  
 384 (e.g. at 3.2, 6.6 and 8.7 min), the increase in others (e.g. at 9.5 and 10.1 min) and the  
 385 general shift to later-eluting peaks of lower polarity in the hydrolysed sample (Fig.  
 386 8B).  
 387



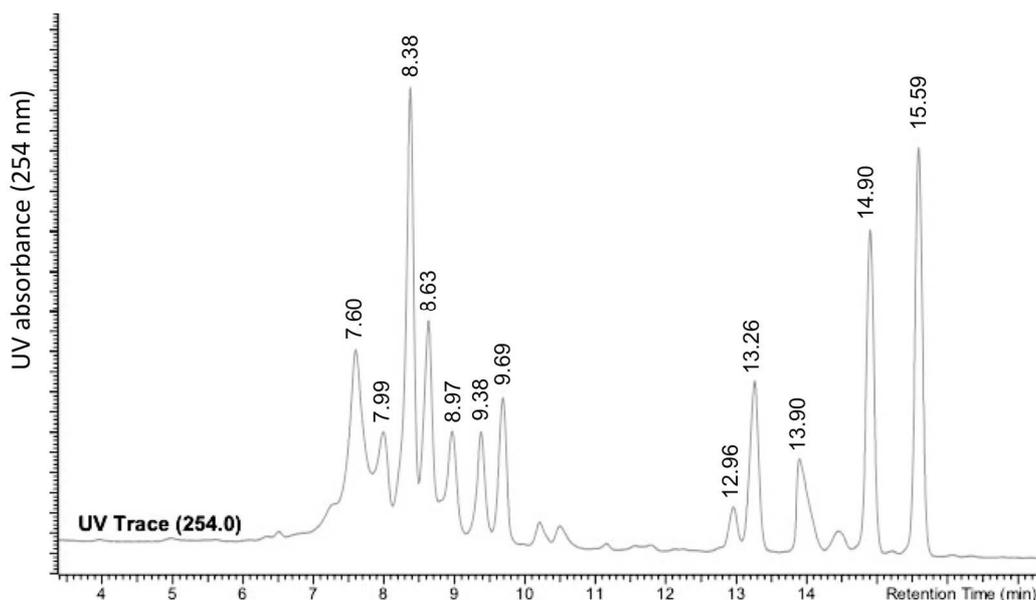
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390 **Figure 8:** The effect of enzymic hydrolysis on the RP-HPLC profile of radiolabelled  
391 rat bile extract. Panel A and Panel B represent the profiles before and after enzymic  
392 hydrolysis with glucuronidase, respectively. The hydrolysis may not be complete  
393 under the conditions used. Arrows indicate the position of major hydrolysable  
394 conjugates (putative glucuronides). HPLC conditions are described in Section 2.7.2.

395

396 HPLC-MS/MS analysis of bile samples (Fig. 9 and Table 1) provided evidence for the  
397 presence of several peaks with a MW of 378 (poststerone+16 amu) that would  
398 correspond to monohydroxylated derivatives of poststerone together with peaks at  
399  $m/z$  537 and 553 (negative-ion mode) that were reduced or disappeared after  
400 glucuronidase treatment and would respectively correspond to mono-glucuronides of  
401 post/3-*epi*-post or mono-hydroxylated derivatives thereof.

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**Figure 9:** HPLC-DAD-MS/MS analysis of bile from rat number 4 injected with poststerone. MS data of the different peaks are reported in Table 1. HPLC conditions are given in Section 2.7.3.

**Table 1:** MS analysis of the different peaks (rat number 4) in positive- and negative-ion modes (molecular mass of poststerone = 362).

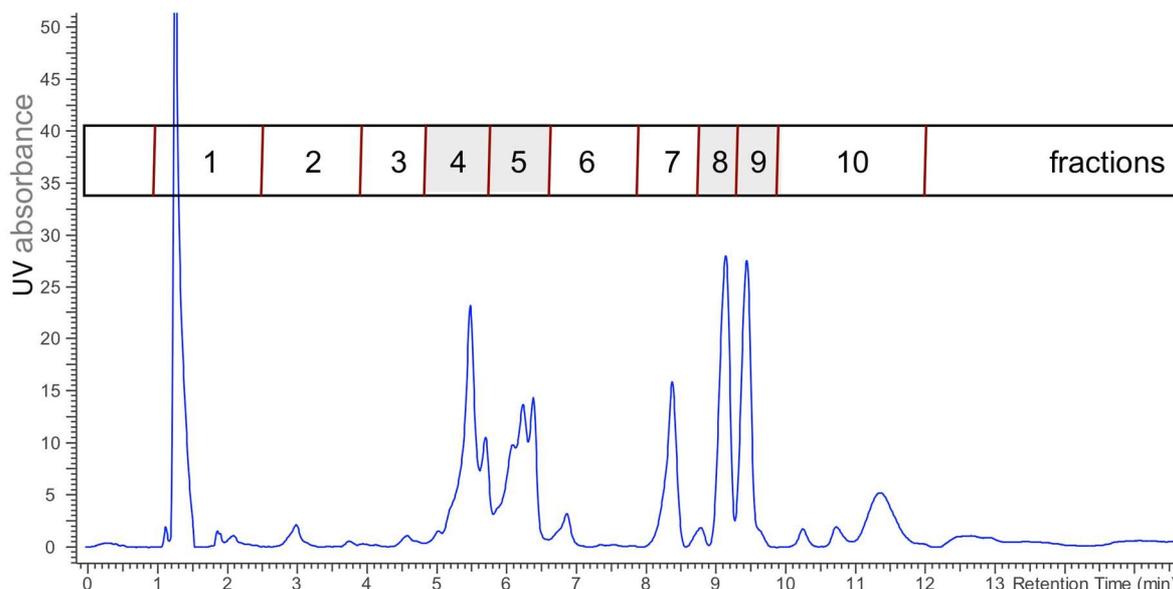
Rt (min.)	Molecular ions		Tentative identification
	Positive-ion mode	Negative-ion mode	
7.60		457 = M+96-1	Post sulfate ?
7.99			?
8.38	379 = M+16+1		hydroxyPost
8.63	379 = M+16+1	553 = M+176+16-1	hydroxyPost + hydroxyPost Glucuronide
8.97		553 = M+176+16-1	hydroxyPost Glucuronide
9.38		553 = M+176+16-1	hydroxyPost Glucuronide
9.69			?
10.21	379 = M+16+1		hydroxyPost
10.43			?
12.96		537 = M+1+176-1	Post Glucuronide
13.26		537 = M+1+176-1	3- <i>epi</i> -Post Glucuronide
13.90			?
14.90	363 = M+1	361 = M-1	Post
15.59	363 = M+1	361 = M-1	3- <i>epi</i> -Post

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In order to reduce the number of metabolites to be isolated, it was decided to hydrolyse the whole set of bile samples before the isolation of the major metabolites.

416 **3.4. Isolation of bile metabolites**

417 The bile extract was first fractionated using HPLC System RP1 to generate 15  
 418 fractions (Fig. 10): 1 (99 mg), 2 (13 mg), 3 (25 mg), 4 (11 mg), 5 (6 mg), 6 (8 mg), 7  
 419 (6 mg), 8 (4 mg), 9 (5 mg), 10 (18 mg), 11, 12, 13 (13 mg), 14 (18 mg) and 15 (5 mg).  
 420 UV and HPLC data led us to focus our efforts on fractions 4,5, 8 and 9. These  
 421 fractions were then further purified by NP HPLC System NP1 (see Supplementary  
 422 Fig. S1). Isolated ecdysteroids were then unambiguously identified by NMR.  
 423 It should be noted that components of potential interest were identified initially on the  
 424 basis of UV-absorbance at 245-248 nm, so metabolites with a modified chromophore  
 425 (e.g. 6 $\alpha$ -hydroxy metabolites), if present, would not be detected.  
 426



427  
 428  
 429 **Figure 10:** Preparative RP-HPLC separation of glucuronidase-treated bile from rats  
 430 treated with poststerone. See text for details of the HPLC separation conditions  
 431 (Section 2.7.3).  
 432

433 **Table 2:** Summary of the chromatographic and physico-chemical data for the  
 434 poststerone metabolites isolated from Fractions 4, 5, 8 and 9 (see Fig. 10).  
 435

N°fraction	RP1	NP1	Compound	HPLC/MS		Amount (mg)	Identification
	Rt (min)	Rt (min)		MW	$\lambda_{max}$		
4	5.10–5.90	10.77	4	378	248	0.40	21-hydroxypoststerone
5	5.95–6.60	10.06	5a	378	248	0.35	3- <i>epi</i> -21-hydroxypoststerone
		15.76	5b	378	245	1.66	16-hydroxypoststerone
8	8.82–9.15	8.1	8	362	?	1.83	poststerone
9	9.20–9.60	7.4	9	362	246	1.01	3- <i>epi</i> -poststerone

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### 438 **3.5. Identification of the metabolites of poststerone**

439 The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra are presented in the Supplementary Information and  
440 the key data are summarised in Tables 3 and 4; They reveal that all the metabolites  
441 isolated in this work, without exception, possess a  $5\beta\text{-H}$  steroid nucleus as present in  
442 Post. This stereochemistry is established unambiguously for each metabolite from  
443 NOESY experiments, where a strong NOE effect between 2-Hax and 9-Hax is  
444 observed and from a typical  $^{13}\text{C}$  chemical shift value of ca. 25 ppm for the C-atom of  
445 the 19-Me. Such NOE and  $^{13}\text{C}$  chemical shifts are also observed with reference  $5\beta\text{-H}$   
446 compounds like Post or 20E.

447

#### 448 3.5.1. 21-Hydroxypoststerone (Compound 4 – 0.46 mg)

449 Turbo Ion-Spray-MS gives positive ions at  $m/z$  **379** ( $\text{M}+\text{H}$ )<sup>+</sup>, **361** ( $\text{MH}-\text{H}_2\text{O}$ )<sup>+</sup>, and **343**  
450 ( $\text{MH}-2\text{H}_2\text{O}$ )<sup>+</sup>. The compound formula is  $\text{C}_{21}\text{H}_{30}\text{O}_6$ , which indicates that it bears one  
451 additional oxygen atom with regard to Post. Examination of the 1D  $^1\text{H}$  NMR spectrum  
452 with respect to that of Post shows disappearance of the C-21 singlet methyl signal  
453 (2.25 ppm, 3H, s) and the presence of a new singlet signal at 4.39 ppm, (2H, s),  
454 corresponding to a new  $\text{CH}_2\text{OH}$  group in this metabolite which could then be readily  
455 assigned as the product of 21-hydroxylation of Post. Thus, the structure of this  
456 metabolite was assigned as 21-hydroxypoststerone.

457

#### 458 3.5.2. 3-Epi-21-Hydroxypoststerone (Compound 5a – 0.35 mg)

459 Turbo Ion-Spray-MS gives positive ions at  $m/z$  **379** ( $\text{M}+\text{H}$ )<sup>+</sup>, **361** ( $\text{MH}-\text{H}_2\text{O}$ )<sup>+</sup>, **343**  
460 ( $\text{MH}-2\text{H}_2\text{O}$ )<sup>+</sup> and **325** ( $\text{MH}-3\text{H}_2\text{O}$ )<sup>+</sup>. In the negative mode, ions were observed at  $m/z$   
461 377 and 359. The compound's formula is  $\text{C}_{21}\text{H}_{30}\text{O}_6$ , which indicates that it bears one  
462 additional oxygen atom in comparison with Post. As for the metabolite Compound 4,  
463 examination of the 1D  $^1\text{H}$  NMR spectrum shows, relative to that of Post, the  
464 disappearance of the C-21 singlet methyl signal (2.25 ppm, 3H, s) and observation of  
465 a new singlet signal at 4.39 ppm, (2H, s), corresponding to a new  $\text{CH}_2\text{OH}$  group in  
466 this metabolite which could be assigned as for Compound 4 (see above) to a C-21  
467 hydroxyl. However, the 1D  $^1\text{H}$  NMR signals of ring-A show significant modifications  
468 (in chemical shift and  $J$ -coupling) with respect to Post. As this compound presents a  
469  $5\beta\text{-H}$  steroid nucleus (see above) and large coupling constants are observed for the  
470 2-H (d,d,d, 11.6, 9, 4.4) and 3-H (d,d,d, 11.4, 9.2, 5) signals, it can be concluded that  
471 these two protons are in axial positions on ring-A. This could only correspond to a 3-  
472 Hax on the  $\beta$ -side of A-ring. This is in agreement with a 3-*epi*-configuration for the 3-  
473 hydroxyl group ( $3\alpha\text{-OH}$ ). NOE are observed between 3-H and 5-H and 19-Me in  
474 agreement with this conclusion. Moreover, comparison of 1D  $^1\text{H}$  NMR signals of ring-  
475 A for 3-*epi*-20E are in perfect agreement with this assignment [Lafont et al., 2002].  
476 Thus, the structure of the metabolite is assigned as 3-*epi*-21-hydroxypoststerone.

477

478 3.5.3. 16 $\alpha$ -Hydroxypoststerone (Compound 5b – 1.66 mg)

479 Turbo Ion-Spray-MS gives positive ions at m/z **379** (M+H)<sup>+</sup>, **361** (MH-H<sub>2</sub>O)<sup>+</sup>, **343**  
480 (MH-2H<sub>2</sub>O)<sup>+</sup> and **325** (MH-3H<sub>2</sub>O)<sup>+</sup>. The compound's formula is C<sub>21</sub>H<sub>30</sub>O<sub>6</sub>, which  
481 indicates that it bears one additional oxygen atom with respect to Post. Examination  
482 of the 1D <sup>1</sup>H NMR spectrum shows a new <sup>1</sup>H broad triplet signal at 4.87 ppm, (1H, tb,  
483 7.5 Hz) corresponding to a new CHOH group in this metabolite with respect to Post.  
484 From TOCSY experiments, *J*-coupling correlations could be observed with 17-H  
485 (3.39 ppm, d, 6.4 Hz), 15 $\beta$ -H (2.64 ppm, d,d, 14.7,9.2 Hz) and 15 $\alpha$ -H (1.72 ppm). ).  
486 The position of the new CHOH group could thus be assigned to C-16. Moreover, one  
487 can observe from NOESY experiments a noe of 16-H with 18-H ( $\beta$ ), and 15-H  $\beta$  (2.64  
488 ppm). These observations allow a  $\beta$ -configuration to be assigned unambiguously to  
489 the 16-H atom. There is also only a very small variation (+0,06ppm) of the <sup>1</sup>H  
490 chemical shift of 18-Me (on the  $\beta$ -side) when compared to Post, providing further  
491 evidence that the 16-OH group is on the  $\alpha$ -side. Thus, the structure of this metabolite  
492 is assigned as 16 $\alpha$ -hydroxypoststerone.

493 3.5.4. 3-Epi-poststerone (Compound 9 – 1.01 mg)

494 Turbo Ion-Spray-MS gives positive ions at m/z **363** (M+H)<sup>+</sup>, **345** (MH-H<sub>2</sub>O)<sup>+</sup>, **327**  
495 (MH-2H<sub>2</sub>O)<sup>+</sup> and **309** (MH-3H<sub>2</sub>O)<sup>+</sup>. The compound formula is C<sub>21</sub>H<sub>30</sub>O<sub>5</sub>, as for Post.  
496 Examination of the 1D <sup>1</sup>H NMR spectrum reveals, as for Post, a C-21 singlet methyl  
497 signal (2.25 ppm, 3H, s) demonstrating no hydroxylation of the acetyl group of Post.  
498 However, the 1D <sup>1</sup>H NMR signals of ring-A shows significant modifications (in  
499 chemical shifts and *J*-coupling) with respect to Post, as seen for metabolite  
500 Compound 5a (see above). As this compound has a 5 $\beta$ -H steroid nucleus (see  
501 above), one can conclude that the 3-hydroxyl group has a 3-*epi*-configuration (3 $\alpha$ -  
502 OH). As for Compound 5a, NOE are observed between 3-H and 5-H and 19-Me, and  
503 the 1D <sup>1</sup>H NMR signals of ring-A for 3-*epi*-20E are in perfect agreement with this  
504 assignment [Lafont et al., 2002]. Thus, the structure of this metabolite is assigned as  
505 3-*epi*-poststerone.

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**Table 3:** <sup>1</sup>H NMR data.

<sup>1</sup> H	Compound 8 Poststerone	Compound 5b 16 $\alpha$ -OHPost	Compound 4 21OHPost	Compound 5a 3epi21OHPost	Compound 9 3epiPost
1-H <sub>ax</sub> $\beta$ 1-H <sub>eq</sub> $\alpha$	1.39 (t, 13) 1.88	1.39 (t, 13.2) 1.88	1.38 (t, 13.2) 1.88 (dd,13.2,4)	1.18 (d,d, 13.6,11.9) 2.15	1.18 (d,d, 13.8,12.1) 2.15
2-H <sub>ax</sub> $\alpha$	4.00 (m, w <sub>1/2</sub> =22) NOE H-9	4.00 (m, w <sub>1/2</sub> =22) NOE H-9	4.00 (m, w <sub>1/2</sub> =22) NOE H-9	3.77 (d,d,d, 11.7, 9.2, 4.4, w <sub>1/2</sub> =27) NOE H-9	3.77 (m, w <sub>1/2</sub> =28) NOE H-9
3-H <sub>eq</sub> $\alpha$	4.08 (m, w <sub>1/2</sub> =8)	4.08 (m, w <sub>1/2</sub> =8)	4.08 (m, w <sub>1/2</sub> =8)	3-H <sub>ax</sub> $\beta$ 3.50 (d,d,d, 11.4, 9.2, 5, w <sub>1/2</sub> =27) NOE H-5 $\beta$ , -1 <sub>ax</sub> $\beta$ , -4 <sub>eq</sub> $\beta$	3-H <sub>ax</sub> $\beta$ 3.50 (m, w <sub>1/2</sub> =27) NOE H-5 $\beta$ , -1 <sub>ax</sub> $\beta$ , -4 <sub>eq</sub> $\beta$
4-H <sub>ax</sub> $\alpha$ 4-H <sub>eq</sub> $\beta$	1.76 1.76	1.76 1.76	1.76 1.76	1.52 (q,13) NOE H-2, -9 1.86	1.53 (t,d,13,12) NOE H-2, -9 1.86
5-H $\beta$	2.36 (t*)	2.36 (t*)	2.36 (t*)	2.25 NOE H-3 <sub>ax</sub> $\beta$ , -19	2.25
7-H	5.98 (d, 2.2) NOE H-15 $\alpha$ , -15 $\beta$	5.94 (s, br)	5.99 (d, 2.2)	5.99 (d, 2.2)	5.99 (d, 2.2)
9-H <sub>ax</sub> $\alpha$	3.14 (m,w <sub>1/2</sub> =22) NOE H-2	3.18 (m,w <sub>1/2</sub> =22) NOE H-2	3.13 (m,w <sub>1/2</sub> =22) NOE H-2	3.14 (m, w <sub>1/2</sub> =22) NOE H-2	3.14 (m, w <sub>1/2</sub> =22) NOE H-2
11-H <sub>ax</sub> $\beta$ 11-H <sub>eq</sub> $\alpha$	1.73 NOE H-19, -18 1.96	1.69 NOE H-19, -18 1.96	1.70 NOE H-19 1.93	1.72 NOE H-19, -18 1.93	1.73 NOE H-19,18 1.96
12-H <sub>ax</sub> $\alpha$ 12-H <sub>eq</sub> $\beta$	2.18 NOE H-9 1.91	2.31 NOE H-9, -17 1.85 NOE H-18	2.12 NOE H-9 1.75	2.14 NOE H-9 1.91	2.18 NOE H-9 1.91
15-H $\beta$ 15-H $\alpha$	2.11 NOE H-18, -7 1.75	2.64 (d,d,14.7, 9.2) NOE H-18, -7, -16 1.72	2.15 NOE H-18 1.77	2.15 NOE H-18, -7 1.77 NOE H-7	2.11 NOE H-18, -7 1.75 NOE H-7
16-H* $\beta$ 16-H* $\alpha$	2.22 1.92	4.87 (t, br, 7.5) NOE H-18, -15 $\beta$ -	2.23 1.96 NOE H-17	2.22 NOE H-18 1.95 NOE H-17	2.22 1.95
17-H $\alpha$	3.33 (t, 9.2)	3.39 (d, 6.4) NOE H-12 $\alpha$	3.25 (t, 8.6)	3.26 (d,d, 9.2, 8.6)	3.33 (t, 9.2)
18-Me $\beta$	0.63 (s)	0.64 (s)	0.64 (s)	0.64(s)	0.63 (s)
19-Me $\beta$	0.99 (s)	0.98 (s)	0.99 (s)	0.97 (s)	0.97 (s)
21-Me	2.25 (s)	2.30 (s)	CH <sub>2</sub> OH 4.39 (s)	CH <sub>2</sub> OH 4.39 (s)	2.25 (s)

Solutions in D<sub>2</sub>O, T= 300K; referenced to TSP-d<sub>4</sub>. Chemical shifts  $\delta$  in ppm,  $\delta$ HDO)= 4.758 ppm,  $\delta$ 19-Me (20E) = 1.00 ppm; Multiplicity of signals : *s* – singlet; *d* – doublet; *t* – triplet; *q* - quadruplet; *m* – multiplet; *br* – broad signal; w<sub>1/2</sub>: width at half-height in Hertz. \*assignments could be reversed. t\* deceptively simple triplet (4-H<sub>ax</sub> and 4-H<sub>eq</sub> isochronous)

**Table 4:**  $^{13}\text{C}$  NMR data

$^{13}\text{C}$	Multiplicity	Compound 8 Poststerone	Compound 5b 16 $\alpha$ OHPost	Compound 4 21OHPost	Compound 5a 3epi21OHPost	Compound 9 3epiPost
C-1	$\text{CH}_2$	37.7	37.7	37.7	43.4	43.5
C-2	CH	69.6	69.3	69.8	73.3	73.4
C-3	CH	69.4	69.2	69.7	76.1	76.2
C-4	$\text{CH}_2$	33.4	33.3	33.8	34.7	34.6
C-5	CH	52.7	52.4	52.6	58.0	58.2
C-6	C	210.9	*	*	*	*
C-7	CH	123.8	123.4	124.1	124.0	124.5
C-8	C	169.3	*	*	*	*
C-9	CH	36.1	35.9	36.3	37.1	37.0
C-10	C	40.6	40.4	40.3	*	41.1
C-11	$\text{CH}_2$	22.5	*	*	*	*
C-12	$\text{CH}_2$	31.8	31.6	31.7	*	32.1
C-13	C	50.4	51.4	50.4	*	50.6
C-14	C	87.4	86.5	87.2	*	87.2
C-15	$\text{CH}_2$	32.9	43.0	*	*	33.5
C-16	$\text{CH}_2$	23.0	72.2 CHOH	*	*	23.0
C-17	CH	61.4	70.7	56.5	56.8	61.6
C-18	$\text{CH}_3$	18.9	19.2	19.3	19.4	18.8
C-19	$\text{CH}_3$	25.4	25.3	25.7	24.9	24.8
C-20	C	219.7	217.1	216.5	216.5	219.6
C-21	$\text{CH}_3$	33.4	33.5	71.2 $\text{CH}_2\text{OH}$	71.2 $\text{CH}_2\text{OH}$	33.4

Solutions in  $\text{D}_2\text{O}$ , T= 300K referenced to TSP-d<sub>4</sub>. Chemical shifts in ppm.

\*signal not detected (too low concentration of the sample)

## 4. DISCUSSION

### 4.1. Comparison of the bioavailabilities of poststerone and 20E

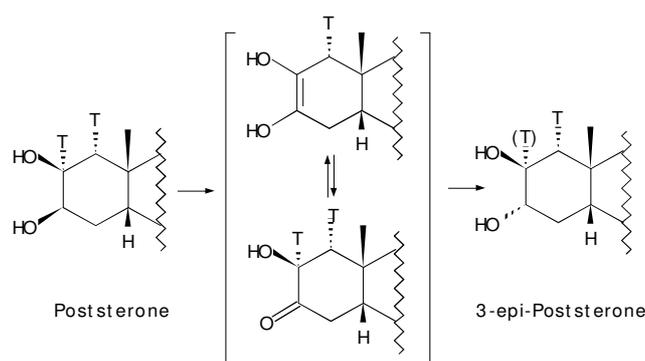
After intravenous injection, Post (5 mg/kg) is rapidly cleared from the blood, being undetectable between 3 and 6 hours after injection. After oral application (50 mg/kg), the maximum concentration found in the blood was 3.53  $\mu\text{g/mL}$  after 15 min, with Post being totally cleared from the plasma between 8 and 24 h after application (AUC = 6890 ng.h/mL). This compares with maximum plasma concentration of 68 ng/mL after 30 min (AUC = 382 ng.h/mL) after oral administration of 20E at 50 mg/kg [Dinan et al., accepted for publication]. Thus, although the oral bioavailability of Post is fairly low (18.7%), it is considerably higher than that of 20E (<1%).

### 4.2. Ecdysteroids in plasma after poststerone application

After oral or intravenous application of poststerone to male rats, not only is Post itself found in the plasma, but also significant amounts of 3-*epi*-poststerone. The epimer is detectable 5 minutes after application in both cases (Fig. 4). Initially, the proportion of 3-*epi*-Post is higher after oral administration, but this would be a consequence of injected Post being rapidly cleared from the plasma before it can be extensively epimerised. When ingested, the Post rapidly equilibrates to give equal concentrations of the 3 $\alpha$ - and 3 $\beta$ -OH epimers. An equivalent epimerisation is not observed for 20E after its intravenous or oral application [Dinan et al., accepted for publication], implying that the presence of a full C8-sterol side-chain with 20/22-diol prevents catalytic interaction with the epimerase or that 20E is preferentially side-chain cleaved before it can react with the epimerase.

### 4.3. Excretion of [<sup>3</sup>H]Post in faeces

By the end of 48 hours, only 51% of the ingested radioactivity has been recovered in the faeces, which is lower than was recovered for 20E (67%; [Dinan et al., accepted for publication]). This difference might be explained by i) the higher proportion of radioactivity found in the urine after ingestion of Post, ii) incomplete extraction of radioactivity from the faeces, and/or potential loss of radioactivity at carbon-2 arising from enolization associated with the epimerization of poststerone (Scheme 1).

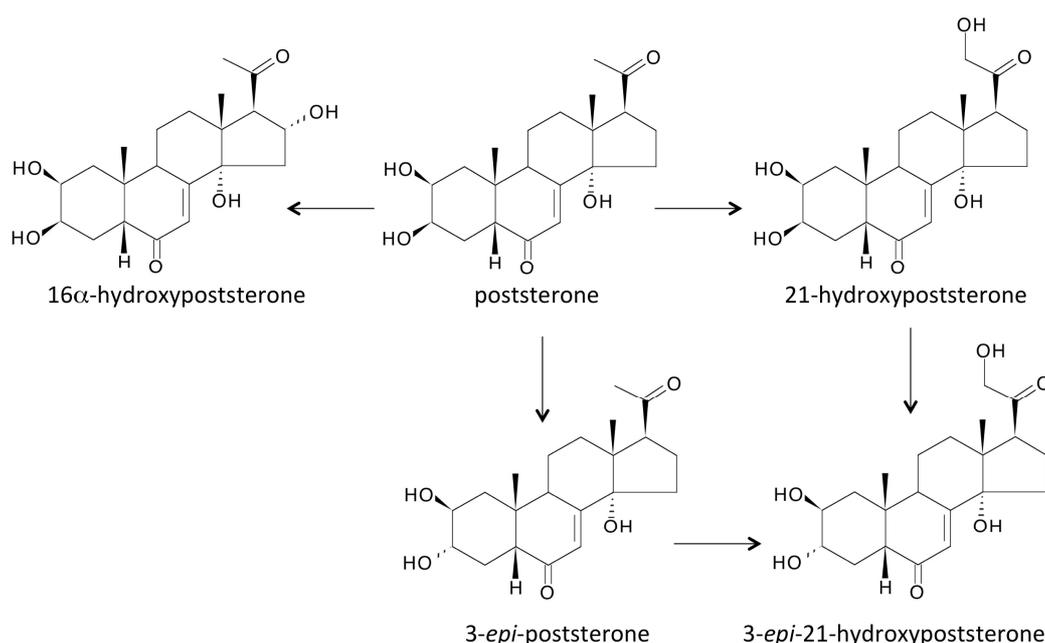


**Scheme 1:** Epimerization of poststerone may lead to a partial loss of tritium in position-2 $\alpha$  owing to enolization of a 3-oxo intermediate.

Studies on steroid excretion in a range of rodent species (summarised in Busso et al., 2020) have shown that, for endogenous steroids, metabolites of less polar molecules are excreted in bile to a greater extent and hence evacuated with the faeces than are the metabolites of more polar steroids. In the case of exogenous 20E/Post this does not appear to be the case, since the more polar 20E is essentially excreted with the faeces, probably reflecting its low bioavailability.

#### 4.4. Bile secretion of injected poststerone and its metabolites

Post can undergo at least 3 metabolic reactions in the liver before being excreted in the bile; 3-epimerization, 21-hydroxylation and 16 $\alpha$ -hydroxylation (Fig. 11). These reactions are very different to those by which 20E is initially metabolised. 20E appears to be predominantly metabolised by the microflora within the gut to 14d20E and 6 $\alpha$ OH20E, with only a small proportion reaching the blood. It can be converted to Post by side-chain cleavage between C-20 and C-22. It is our working hypothesis that this conversion occurs in the (aerobic) tissue of the large intestine, but it still has to be confirmed. Such endogenously-generated Post or exogenous Post is metabolised in an entero-hepatic cycle. In this cycle, Post and its metabolites can also undergo the microbial metabolism which 20E undergoes in the gut lumen (14-dehydroxylation, 6-oxo reduction to 6 $\alpha$ -OH) to increase the complexity of the metabolites even further. The apparent higher excretion rate of 3epiPost over Post in bile (Fig. 7b) appears in contrast to plasma levels of Post and 3epiPost after feeding of Post (Fig. 4a), but this may reflect faster clearance of 3epiPost from the plasma or simply variation between individual rats.



**Figure 11:** Structures of the identified bile metabolites of poststerone.

After intravenous injection of Post, bile contains large amounts of the steroid and its metabolites. Conjugates of these compounds are also present in the bile, since glucuronidase treatment significantly alters the metabolite profile (Fig. 6). To simplify the task, we chose for this study to focus on the free steroids and genins by identifying the metabolites from glucuronidase-treated bile. In addition to Post itself, the major metabolites found in bile were identified as 3-*epi*-poststerone, 21-hydroxypoststerone, 3-*epi*-21-hydroxypoststerone and 16 $\alpha$ -hydroxypoststerone.

#### **4.3. Relationship to vertebrate steroid metabolism**

The absence of even low levels of 3-*epi*-20E, 20,21E or 16 $\alpha$ 20E in the bile after injection of 20E would indicate that the liver enzymes are specific for Post and its C<sub>21</sub> metabolites, while the occurrence of 14dPost and 6 $\alpha$ OHPost show that the gut microflora are capable of metabolising Post as well as 20E. It seems probable that the endogenous substrates for the liver enzymes would be vertebrate steroids, since Post has certain structural features in common with corticosteroids (Supplementary Information Fig. S2; C<sub>21</sub> compounds; cyclopentanophenanthrene ring structure; C-20 ketone, shared connectivity of the B-, C- and D-rings), but also a number of significant differences (A/B-cis ring junction; 14 $\alpha$ -hydroxy-6-oxo-7-ene grouping vs. a 3-oxo-4-ene grouping). A 21-hydroxyl group is characteristic of corticosteroids and the 21-hydroxylase is present in the ER of the adrenal cortex, but the enzyme is not expressed in the liver [Payne & Hales, 2004]. 16 $\alpha$ -Hydroxylation of oestradiol to oestriol or testosterone to 16 $\alpha$ -hydroxy-testosterone is a well-characterized reaction in the liver of mammals, catalysed by CYP3A4, which is credited with being the most important enzyme in drug metabolism in humans because of its high level of expression and broad substrate specificity (Klein & Zanger, 2013). In rats, there are distinct developmental and sex differences in the liver enzymes of steroid metabolism, especially in 16 $\alpha$ -hydroxylase (CYP2C) and 6 $\beta$ -hydroxylase (CYP3A) which are present in adult male liver microsomes, but largely absent from the livers of female and immature animals [Morgan et al., 1985]. This is in addition to the differences in steroid metabolizing enzymes associated with reproductive organs during development in male and female rats [Preslock, 1980]. However, it must be borne in mind that the location and orientation of hydroxylation depends not only on the hydroxylase, but can also depend on the steroid substrate [Wood et al., 1983], so action of such enzymes on Post could result in hydroxylations at other C-atoms.

In rats, the metabolism of 20E appears similar to that in mice. Moreover, we showed that 3-epimerization of Post probably takes place in rodent liver. This reaction seems to operate very efficiently when no side-chain is present, but it is not observed with 20E. On the other hand, previous experiments with ecdysone detected the formation of a 3-epimer of the fully reduced metabolite of 14-deoxyecdysone [(5 $\beta$ -H, 14 $\alpha$ -H)2 $\beta$ ,3 $\alpha$ ,6 $\alpha$ ,22*R*,25-pentahydroxycholestane; Girault et al., 1988].

Type I 3 $\alpha$ -hydroxysteroid dehydrogenase (AKR1C4) is a liver-specific oxidoreductase which is important in bile acid biosynthesis, steroid hormone metabolism and xenobiotic metabolism with broad substrate specificity which has been implicated in the hepatic clearance of 5 $\alpha$ /5 $\beta$ -H steroids [Jin & Penning, 2007; Stayrook et al., 2008], and which can, under certain circumstances, show oxidase activity [Steckelbroeck et al., 2004]. Three other members of the aldo-keto reductase family (AKR1C1, AKR1C2 and AKR1C3) are not only expressed in the liver, but also in the small intestine and these are capable of reducing oxo-groups in mammalian steroids at C-20 (to 20 $\alpha$ -hydroxy), C-3 (to 3 $\alpha$ -hydroxy) and C-17 (to 17 $\beta$ -hydroxy), respectively [Jin & Penning, 2007]. The intestine is capable of synthesizing significant amounts of vertebrate steroid hormones, especially glucocorticoids, and of metabolizing sex steroids [Bouguen et al., 2015], and thus the intestinal epithelial cells may contribute to the metabolism of exogenous ecdysteroids.

Endogenous and exogenous steroids may be subject to enterohepatic cycling and intestinal metabolism in mammals and these processes may contribute significantly to the levels of active compounds in the organism [Adlercreutz & Martin, 1980]. Considerable species differences exist for the extent of biliary and faecal secretion and the nature of the metabolites found, resulting in significant species differences in drug elimination and metabolism. Absorption of steroids from the human small intestine is inversely related to the number of free hydroxyl groups in the molecules [Schedl et al., 1965]. The ability of a specific steroid to enter the enterohepatic cycle is also species-dependent and small changes in structure can affect this. Thus, corticosterone enters the enterohepatic cycle in humans, while cortisol (which differs only by the presence of a 17 $\alpha$ -hydroxyl group) does not [Morris, 2015]. Consequently, steroids entering the enterohepatic cycle have a greater half-life in the body and are metabolised to a greater array of metabolites (free and conjugated). The microbiome of the intestinal tract has the capacity to transform many C19 and C21-steroids [Adlercreutz & Martin, 1980]. Thus, the relative bio-availabilities, half-lives and metabolism of exogenous 20E and Post accord in general terms with those seen for endogenous vertebrate steroids.

Our future studies will focus on the identification of the more minor metabolites of poststerone to be found in the bile and faeces of male rats and to compare the spectrum of metabolites to that formed in females and immature animals and relate this to the location and identity of the metabolising enzymes to verify if they do correspond to some of the enzymes responsible for endogenous steroid metabolism. Further, we wish to elucidate to what extent the many biological activities attributed to exogenous 20E in mammals are a result of its metabolism. If exogenous ecdysteroids are metabolised by the enzymes of endogenous steroid metabolism, as seems likely, it is to be expected that ecdysteroid metabolism in mammals will depend on the sex and stage of development of the animal.

## **5. CONCLUSIONS**

1. Exogenous ecdysteroids possessing a C20/C22-diol can undergo side-chain cleavage in rodents to yield 20-oxo C21-analogues. Thus, 20-hydroxyecdysone (20E), the predominant phytoecdysteroid, is metabolised to poststerone (Post).
2. Post has far greater bioavailability than 20E and is involved in an entero-hepatic cycle, during which it is subjected to metabolism in rodent organs and by the microbiome.
3. Post metabolites have been isolated and identified from rat bile.
4. Post can be subjected to 3-epimerisation, 16 $\alpha$ -hydroxylation and 21-hydroxylation, in addition to the 14-dehydroxylation and 6-oxo reduction reactions already identified for 20E, to produce a complex array of metabolites involving permutations of these reaction.
5. The metabolites are predominantly excreted in the faeces, but in accord with the greater bioavailability of Post over 20E, Post and its metabolites are also present in greater amounts in the urine.

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