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Structure–Metabolism Relationships of Ring-A Halogenated Analogues of 17α-Ethynyloestradiol

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The metabolic fates of 2-chloro-, 2-bromo-, 4-bromo- and 2-iodo-17α-ethynyloestradiol (EE_2) in rats were determined. 6,7-^{3}H-labelled analogues (0.1–2.0 μmol/kg) were administered i.v. to anaesthetized animals. The metabolites of all four compounds were rapidly and extensively excreted in bile (79–93% of the dose over 6 h). Unlike EE_2 and 2-fluoro-EE_2 (2-FEE_2), neither 2-chloro(Cl)-(2.0 μmol/kg),2-bromo(Br)-(0.1 μmol/kg), nor 2-iodo(I)-EE_2-(0.1 μmol/kg) underwent C-2 hydroxylation in female rats; 2-$BrEE_2$ was similarly refractory in male rats; 4-$BrEE_2$(0.1 μmol/kg), in females, was subject to approx. 2-fold greater C-2 hydroxylation than 2-FEE_2 but this equalled only approx. 60% of that undergone by EE_2. All three of the C-2 halogenated derivatives were substantially excreted unchanged except for conjugation. 2-$ClEE_2$ alone was C-4 hydroxylated to an appreciable extent. The oxidative metabolism of 2- and 4-$BrEE_2$ in rats was sexually differentiated: 2-$BrEE_2$ yielded an alkyl hydroxylated metabolite and a two-component dihydroxylated fraction in the ratio 1:0.09 and 1:0.76 in males and females, respectively; 4-$BrEE_2$ underwent C-2 and alicyclic (C-15) hydroxylation in the ratio 1:4.8 and 1:0.07 in males and females, respectively. 2-$ClEE_2$ formed much less alkyl monohydroxylated metabolite (C-16 hydroxylated for 2-Cl- and 2-IEE_2) than did either 2-$BrEE_2$ or 2-IEE_2. The observed structure–metabolism relationships are discussed.

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INTRODUCTION

17α-Ethynyloestradiol (EE_2), the standard oestrogen of combined oral contraceptives, is substantially metabolized via C-2 hydroxylation in man [1] and rats [2]. The catecholoestrogen undergoes catechol-*O*-methylation [2, 3] and/or glucuronylation [2, 4].

Ring-A oxygenated oestrogen metabolites—catechol and methoxy derivatives—may display reduced classical oestrogen activity [5] but are either reported or conjectured to possess a variety of physiological [6, 7] and toxic [8, 9] activities *in vivo*. They and their reactive intermediates also have a number of pathophysiological effects on isolated cells [10–12].

The involvement of catechols in mediating biological and toxic actions of steroidal oestrogens has been investigated using analogues which are presumed to be partially or completely resistant to aromatic hydroxylation *in vivo* but retain receptor-mediated activity [7, 13]. Dissociating receptor binding from catecholoestrogen formation could have practical significance since catechol metabolites are postulated to induce the tumours associated with EE_2 [14, 15]. Halogenation, and especially fluorination, of C-2 and C-4 is the favoured chemical modification for blocking aromatic hydroxylation: fluoro (F) [16] and, to a lesser extent, chloro (Cl) [17] derivatives retain receptor binding affinity, and even bromo(Br)-oestrogens may possess appreciable receptor-mediated activity [18]. Unfortunately C-2 and C-4 functionalized 17β-oestradiol(E_2) analogues undergo oxidative dehalogenation by hepatic and renal microsomes, though divergent rates of metabolism have been reported [19–21]. However, recent studies [22] have shown that 2- and 4-FE_2 were not subject to this biotransformation in rats; suggesting artifactual enhancement of defluorination *in vitro*. In contrast, and emphasizing the complexity of substituent effects *in vivo*, 2-FEE_2 yields 2-$OHEE_2$ in rats [23].

The present work completes a systematic investigation of structure–metabolism relationships amongst ring-A halogenated EE_2 analogues.

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A preliminary report on part of this work has been published elsewhere [24].

EXPERIMENTAL

Materials

[6,7-^{3}H]Oestrone ([^{3}H]E_1; 48.1 Ci/mmol; radiochemical purity 99%) and [6,7-^{3}H]EE_2 ([^{3}H]EE_2; 53.7 Ci/mmol; radiochemical purity 99%) were purchased from NEN Research Products (Dreieich, Germany). Unlabelled oestrogens and conjugate hydrolases were products of Sigma Chemical Co. (Poole, England). Reagents for chemical syntheses were obtained from Aldrich Chemical Co. (Gillingham, England). Standards of 6α- and 16α-OHEE_2 were provided by Schering AG (Berlin, Germany).

Chemical syntheses

2-ClEE_2 and [^{3}H]2-ClEE_2. A mixture of 2- and 4-phenylselenyloestrone (PhSeE_1; 85:15) was prepared in 75% yield from E_1 according to the method of Ali and Van Lier [25]. The mixture (2.4 mmol) was dissolved in dry dichloromethane (DCM), iodine monochloride (3.6 mmol) was added, and the whole stirred at room temperature for 12 h. Following addition of saturated aqueous sodium sulphite (40 ml), the organic layer was separated and the aqueous phase extracted with DCM (30 ml × 3). The pooled organic solutions were dried ($MgSO_4$), concentrated, and chromatographed on a silica column. Elution with DCM gave 2-ClE_1 in 76% yield from 2-PhSeE_1. 2-ClE_1 (0.69 mmol) was converted to its 3-0-*t*-butyldimethylsilyl (TBDMS) derivative by a standard method [26]. Ethynylation was carried out as described previously [26]. 2-ClEE_2 (0.54 mmol) recrystallized from petroleum ether–ethyl acetate (3:2, v/v) gave: m.p. 195–200°C; C 72.43%, H 7.01% ($C_{20}H_{23}ClO_2$ requires C 72.6%, H 7.01%); *m/z* 330 (M^+; 45%), 312 (5%), 262 (27%), 247 (100%); δ 0.95 (3H,s), 1.00–2.45 (13H,m), 2.60 (1H,s), 2.8–2.92 (2H,m), 5.60 (1H,s), 6.72 (1H,s), 7.20 (1H,s) and 8.40 (1H, br s).

Efficient chlorination of 2-PhSeE_1 could not be reproduced on the small scale of radiochemical syntheses [27]. However, it was previously observed [28] that treatment of E_1 with *N*-fluoropyridinium triflate (NFPT) in 1,1,2-trichloroethane under reflux conditions yields *o*-chlorinated analogues. [^{3}H]E_1 (500 μCi, 5 μmol) was reacted with NFPT (10 μmol) at reflux in 1,1,2-trichloroethane (3 ml) for 24 h. The reaction mixture was reconstituted in methanol, and chromatographed on a Techopak C_{18} column (10 μm, 30 × 0.39 cm i.d.; HPLC Technology, Macclesfield, England) using a gradient of acetonitrile (20–75% over 40 min) in ammonium dihydrogen orthophosphate buffer (43 mM, pH 3.0) at 1.5 ml/min. E_1 (18% eluted radiolabel), 2/4-FE_1 (5%; isomers unresolved; R_t 24.7 min), 2-ClE_1 (24%; R_t 27.0 min) and 4-ClE_1 (53%; R_t 27.6 min) were monitored at 280 nm and quantified by liquid scintillation counting. They were characterized by electron impact (EI) mass spectrometry. [^{3}H]2-ClE_1 co-eluted with authentic unlabelled material. It was isolated by isocratic elution (acetonitrile–buffer, 1:1 v/v) of the reaction mixture from a Techopak column; material was extracted from the eluate with ether following evaporation of the acetonitrile. To remove contaminating [^{3}H]4-ClE_1 (5% radiolabel), the recovered steroid was chromatographed on a Lichrosorb diol column (10 μm, 25 × 0.46 cm i.d., HPLC Technology); *n*-hexane (1.5 ml/min) resolved the 2- and 4-isomers at R_t 24.5 and 18.7 min, respectively. [^{3}H]2-ClE_1 (25.0 μCi, 100 μCi/μmol), obtained at a radiochemical purity >99%, was diluted with carrier (final sp.act. 5.1 μCi/μmol) and reacted with TBDMS chloride (33 μmol) and imidazole (67 μmol) at room temperature for 12 h to give the TBDMS ether in 85% yield. Addition of carrier 2-ClE_1 was dictated by the very low efficiency of the ethynylation reaction at lower masses (*ca* 1 μmol) of substrate; unavoidably, the [^{3}H]2-ClEE_2 had only a modest specific activity. Treatment with lithium trimethylsilyl acetylide as before [23] and chromatographic purification (C_{18} column; acetonitrile–buffer, 1:1 v/v; R_t 15.5 min) enabled 77% conversion of TBDMS intermediate to radiochemically pure product (R_t 2-ClEE_2, gradient elution from C_{18} column, 22.0 min). The absence of co-chromatographing [^{3}H]2-ClE_1 was confirmed using gradient elution (5–10% propan-1-ol in hexane over 20 min; 1.5 ml/min) from a diol column; authentic 2-ClEE_2 and 2-ClE_1 had R_t of 16.2 and 8.1 min, respectively.

2- and 4-BrEE_2 and [^{3}H]2- and [^{3}H]4-BrEE_2. 2- and 4-BrEE_2 were prepared as described previously [26].

[^{3}H]EE_2 (160 μCi; 235 μCi/μmol) in absolute ethanol (0.5 ml) was mixed with *N*-bromosuccinimide (1.0 μmol) freshly dissolved in ethanol. The solution was left at room temperature for 18 h. The monobrominated products (69% radiolabel by C_{18}-HPLC) were isolated from the reaction mixture, reconstituted in methanol (500 μl), by gradient elution from a C_{18} column as described above. Radiochemically homogeneous [^{3}H]2- and [^{3}H]4-BrEE_2 were obtained in 21.5 and 37.3% yield, respectively. They co-chromatographed with unlabelled standards (R_t 2- and 4-BrEE_2 26.0 and 27.0 min, respectively) and gave identical EI spectra [26].

2-IEE_2 and [^{3}H]2-IEE_2. EE_2 (1.7 mmol) in ethanol (80 ml) was treated with *N*-iodosuccinimide (NIS; 2 mmol) at room temperature for 1 h. Saturated sodium sulphite (20 ml) was added, the organic layer separated, and the aqueous phase extracted with DCM (50 ml × 3). Pooled organic solutions were dried ($MgSO_4$). 2-IEE_2 (0.32 mmol, 19% yield) was isolated by elution from a silica column with DCM, and gave: m.p. 213–214°C; C 56.65%, H 5.45% ($C_{20}H_{23}I\ O_2$ requires C 56.88%, H 5.49%); *m/z* 422 ($M^{+\cdot}$, 74%),

354 (30), 339 (100%); δ 0.95 (3H,s), 1.00–2.50 (13H,m) 2.60 (1H,s), 2.88–2.95 (2H,m), 5.1 (1H,s), 5.3 (1H,s), 6.75 (1H,s) and 7.6 (1H,s).

[^{3}H]EE$_2$ (161 μCi; 100 μCi/μmol) in ethanol (0.5 ml) was mixed with an ethanolic solution of NIS (1.21 μmol) at room temperature; higher equivalents of NIS yielded excessive amounts of disubstituted product. After 2 h, [^{3}H]2-IEE$_2$ (13.4 μCi, 8.3% yield; R_t 27.5 min) and unreacted [^{3}H]EE$_2$ (41.4%; R_t 21.5 min) were isolated by gradient elution from a C_{18} column. The salvaged [^{3}H]EE$_2$ was recycled. The combined yields of [^{3}H]2-IEE$_2$ (19.8 μCi) were radiochemically homogeneous, co-chromatographed with unlabelled standard and gave the correct mass spectrum.

Animal experiments

Male and female Wistar rats were anaesthetized as described previously [29], and cannulated via the jugular vein and common bile duct with 0.4 mm i.d.–0.8 mm o.d. cannulae (Portex Ltd, Hythe, England).

[^{3}H]2-ClEE$_2$ (2 μmol/kg) in saline–polyethylene glycol (PEG) 200 (1:1, v/v) was administered i.v. to females (200 ± 4 g body wt; mean ± SD, $N = 4$). Its low specific activity necessitated administration of a relatively large dose.

[^{3}H]2-BrEE$_2$ (0.1 μmol/kg) in saline–PEG 200 (1:1) was administered i.v. to males (205 ± 5 g; $N = 4$) and females (238 ± 13 g; $N = 4$).

[^{3}H]4-BrEE$_2$ (0.1 μmol/kg) in saline–PEG 200 (1:1) was administered i.v. to males (220 ± 12 g; $N = 4$) and females (209 ± 11 g).

[^{3}H]2-IEE$_2$ (0.1 μmol/kg) in saline–PEG 200 (1:1) was administered i.v. to females (200 ± 7 g; $N = 4$).

Bile was collected hourly for 6 h except as indicated, and assayed for radioactivity [30]. The rats were killed by cervical dislocation and major organs were removed for determination of residual radioactivity [2].

Chromatographic analysis of biliary metabolites

Metabolite conjugates in bile (0–2 h pooled collections) from rats given either 0.1 or 2 μmol/kg of steroid were hydrolysed with arylsulphohydrolase-β-glucuronidase, and aglycones extracted into ether [29]. Ether-extracted metabolites were analysed by reversed-phase HPLC with radiometric quantification [23].

Isolation and identification of metabolites

Larger doses of the analogues were administered to enable isolation of metabolites in amounts sufficient for mass spectrometric characterization.

2-ClEE$_2$. Female Wistar rats (215–230 g) were administered [^{3}H]2-ClEE$_2$ (1.5 μCi; 14 μmol/kg) in saline–PEG 200 (1:2, v/v) via a jugular vein cannula. Bile was collected in a single 3-h fraction. Three aliquots (300 μl; *ca* 180 μg equiv.) were incubated [29] with arylsulphohydrolase-β-glucuronidase (125 U arylsulphohydrolase). Liberated aglycones were extracted with ether (5 ml × 2) and chromatographed in methanol by gradient elution from a Techopak column: 20–75% acetonitrile in ammonium dihydrogen orthophosphate (43 mM, pH 3.0) over 40 min at 1.5 ml/min. Metabolites B and C (Table 1) were located by spectrophotometry (280 nm) and liquid scintillation counting. They were recovered by ether extraction (5 ml × 2) of concentrated eluate fractions. The more polar metabolite (B; R_t 12.0 min) was further purified on a diol column to remove endogenous material; aliquots were eluted (R_t 16.8 min) with propan-1-ol (5–50% over 30 min) in hexane at 1.5 ml/min.

Table 1. Metabolites of C-2 halogenated [^{3}H]EE$_2$ analogues in male and female rats

Analogue & metabolites	% Radioactivity (mean ± SD, $N = 4$) Females	Males
2-ClEE$_2$ (2 μmol/kg)	83.3 ± 11.2	
(A) [diOH-?]	0.5 ± 0.3	
16-OH-{+4-MeO-16OH-}(B)	3.4 ± 1.5	
4-OH-(C)	10.0 ± 2.5	
2-BrEE$_2$ (0.1 μmol/kg)	62.1 ± 9.2	70.1 ± 5.1
4,16-diOH-{+diOH-}(D)	9.6 ± 2.4	2.4 ± 1.0
xOH-(E)	12.7 ± 0.8	25.5 ± 3.6
2-IEE$_2$ (0.1 μmol/kg)	76.7 ± 18.0	
(G)[xOH-?]	4.5 ± 4.1	
16OH-(H)	14.4 ± 3.6	
(I)[4-MeO-?]	0.5 ± 0.8	

Metabolites recovered from enzymic hydrolysates of 0–2 h bile collections by ether extraction were quantified by radiometric C_{18}-HPLC. [?], assignment based upon chromatographic characteristics; { }, co-eluting metabolite; xOH, position of alicyclic hydroxylation uncertain.

2-BrEE$_2$. Female rats (190–200 g) were administered [^{3}H]2-BrEE$_2$ (3.1 μCi; 13 μmol/kg) i.v. in saline–PEG 200 (2:3). Deconjugated biliary metabolites D–F were isolated from 0–3 h bile collections by C_{18}-HPLC as described above. The most polar isolated fraction (D; R_t 12 min) was comprised of two subfractions (D1 and D2; ratio of ^{3}H, 3:2), which could be resolved (R_t 16.0 and 19.2 min) with a modified gradient of acetonitrile (20–45% over 40 min) in phosphate buffer.

4-BrEE$_2$. Female rats (230 g) received [^{3}H]4-BrEE$_2$ (4.2 μCi, 16 μmol/kg) i.v. saline–PEG 200 (2:3). Bile was collected for 3 h. Deconjugated biliary metabolites I–III (Table 2) were isolated by C_{18}-HPLC. The most

Table 2. Metabolites of [^{3}H]4-BrEE$_2$ in male and female rats

Analogue & metabolites	%Radioactivity (mean ± SD, $N = 4$) Females	Males
4-BrEE$_2$ (0.1 μmol/kg)	46.3 ± 2.3	65.9 ± 4.4
15-OH-(I)	2.8 ± 0.1	21.9 ± 2.0
2-OH-(II)	35.1 ± 3.2	5.5 ± 1.8
2-MeO-(III)	7.0 ± 0.2	0.9 ± 0.9

Metabolites recovered from enzymic hydrolysates of 0–2 h bile collections by ether extraction were quantified by radiometric C_{18}-HPLC.

polar aglycone metabolite (I; R_t 13.5 min) was further purified by sequential reversed-phase and diol-phase (R_t 17.7 min) HPLC as described above for 2-BrEE$_2$ and 2-ClEE$_2$, respectively.

2-IEE$_2$. Female rats (200 g) received [^{3}H]2-IEE$_2$ (2 μCi; 12 μmol/kg) i.v. in saline–PEG 200 (1:2). Bile was collected for 3 h. The sole phase I metabolite of 2-IEE$_2$ formed at this higher dose (G) was isolated by C_{18}-HPLC and further purified by diol-phase HPLC to remove endogenous contaminants.

Mass spectrometry

Standards and radiochromatographically homogenous isolated metabolites were analysed by direct-probe EI mass spectrometry [29].

RESULTS

Excretion and tissue residues of radiolabelled material

The radiolabelled metabolites of all four analogues (0.1 or 2 μmol/kg dose) were rapidly and extensively excreted in bile: for 2-Cl-, 2-Br- (males), 2-Br-(females), 4-Br-(males), 4-Br-(females) and 2-IEE$_2$, the 0–1 h recovery of radiolabel was 38.0 ± 11.3% (mean ± SD, $N = 4$), 64.7 ± 6.4, 42.7 ± 9.9, 32.4 ± 2.1, 56.0 ± 9.1 and 60.0 ± 13.8%, respectively; the corresponding 0–6 h recoveries were 79.4 ± 8.6, 92.8 ± 3.2, 82.8 ± 4.3, 88.6 ± 2.5, 85.2 ± 4.9 and 79.4 ± 8.6%.

Six hours after administration of the above doses, only residual levels of radiolabelled material remained in the tissues examined: *ca* 1–7% of the dose in liver and <0.1% in each of the other organs.

Biliary metabolites

The biliary metabolites (0.1 or 2 μmol/kg dose) were in every case largely recovered as ether-extracted aglycones (73–98% of incubated radioactivity) following incubation of pooled 0–2 h bile collections, containing 63–88% of recovered radiolabel (mean values), with arylsulphohydrolase-β-glucuronidase. Bile from females administered larger doses (12–16 μmol/kg) of steroid yielded 75–94% of excreted radiolabel as ether-extracted aglycones following enzymic hydrolysis.

2-ClEE$_2$. Following administration of 2 μmol/kg [^{3}H]2-ClEE$_2$ to female rats, the radiolabelled material extracted from bile hydrolysates was resolved into unchanged 2-ClEE$_2$ and three polar metabolite fractions (A–C) by C_{18}-HPLC [Fig. 1(a); Table 1]. Females given a larger dose of [^{3}H]2-ClEE$_2$ (14 μmol/kg) excreted 73–97% of the radiolabel in bile over 0–3 h. Only two aglycone metabolites—B and C—were observed at the higher dose. Metabolite A (R_t 9.5 min) was tentatively identified, by analogy with the chromatographically cognate dihydroxylated metabolite of 2-BrEE$_2$ [D; R_t 12.0 min; Fig. 1(a and b)], as a dihydroxylated product. Metabolite B (R_t 12.0 min; 8% eluted radiolabel at 14 μmol/kg) gave molecular ions at m/z 348 and 346 (B2; Table 3) indicative of oxygen insertion with retention of chlorine, but additional intense $M^{+\cdot}$ at m/z 378 and 376 (B1) suggested a co-chromatographing C-4 methoxy derivative. Its polarity is typical of oestrogen metabolites with an alkyl hydroxyl group, e.g. 16-OHEE$_2$ [31]. However, the multiple ring-D fragmentations [32] of 16α-OHEE$_2$, i.e. [M-96/98/99/101], were obtained in altered form. The dominant [M-99] peak (m/z 213; 100%), arising from loss of ring D (carbons 15–17, 19 and 20) with proton migration to the major fragment [32], was replaced by [M-97] (m/z 279 and 249), the putative product of ring-D fission with proton migration to the major fragment; [M-99] peaks (m/z 277 and 247) occurred at reduced intensities. The ions at m/z 194 and 224 are, respectively, chloro and chloro-methoxy analogues of the characteristic steroid ion m/z 160, which comprises rings A and B with either C-11 or, less likely, C-14 [32]. B1's base-peak $M^{+\cdot}$ is typical of ring-A methoxy derivatives [23, 33]. B1 and B2 are identified as 2-Cl-4-methoxy-16OHEE$_2$ and 2-Cl-16OHEE$_2$, respectively.

Metabolite C (R_t 18.5 min, 16% radiolabel at 14 μmol/kg) gave the $M^{+\cdot}$ of a monoxygenated product at m/z 348/346 and fragments, e.g. m/z 212/210, diagnostic of an hydroxyl functionalized A ring [23]. It was identified as 2-Cl-4-OHEE$_2$.

2-BrEE$_2$. Male and female rats given 0.1 μmol/kg [^{3}H]2-BrEE$_2$ excreted conjugates of the unchanged steroid and its two metabolites D and E [Fig. 1(b and c); Table 1]. Males formed twice as much of E.

Females excreted 79% of a 13 μmol/kg dose into bile over 3 h. An additional metabolite (F; R_t 21.5 min; 7% eluted radiolabel) was formed at this dose.

Chromatographic analysis of 0–20, 20–40 and 40–60 min and 1–2 h bile fractions from females given 0.1 μmol/kg revealed a progressive development of the metabolic profile: the metabolite ratio D:E was 0.3, 0.4, 0.5 and 0.6, respectively. Fractions D (R_t 12 min; 5% at 13 μmol/kg) and E (R_t 15.0 min; 11% at 13 μmol/kg) were identified as di- and monohydroxylated products (m/z at 408/406 and 392/390), respectively (Table 3). The subfractions of D resolved by diol-HPLC yielded the same $M^{+\cdot}$ but notably different fragments: D2 underwent the extensive dehydration characteristic of 6α-OHEE$_2$ (68% [M-18] at m/z 294); D1, the prominent loss of 97 amu observed in spectra of metabolites B1 and B2. The peaks in D1's spectrum at [M-97/98/99] and m/z 256/254 (bromo-hydroxyl analogues of m/z 160) indicated oxygen insertion into the D ring and at C-4, respectively. The positions of hydroxylation in D2 and E could not be assigned but the metabolites' polarity suggested alicyclic functionalization. Metabolite F yielded $M^{+\cdot}$ and fragments consistent with 2-Br-4-OHEE$_2$.

4-BrEE$_2$. Male and female rats metabolized 4-BrEE$_2$ (0.1 μmol/kg) to three monoxygenated products, I–III (Fig. 2; Table 2). The proportions of unchanged

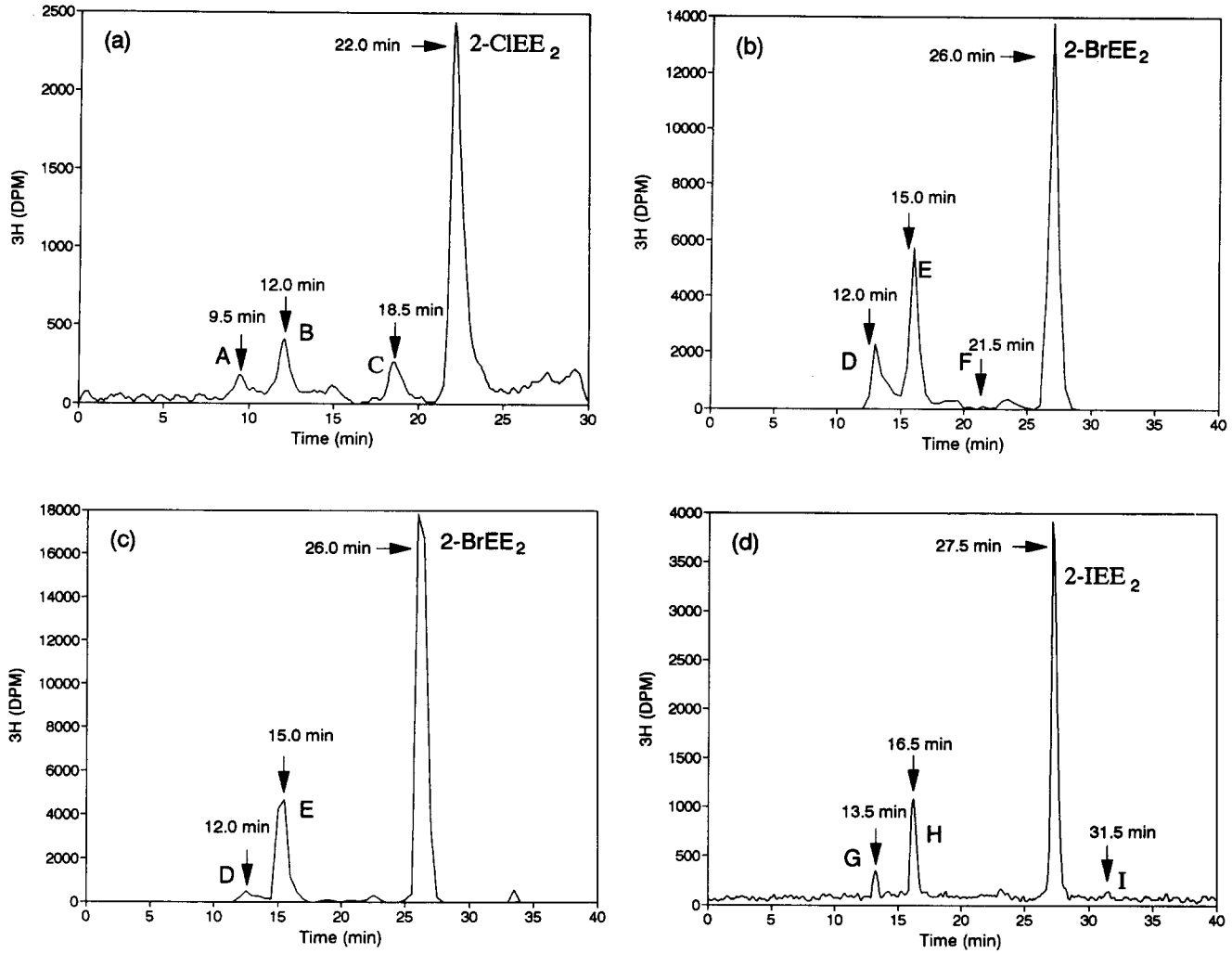

Fig. 1. High-performance liquid radiochromatograms of the phase I metabolites of C-2 halogenated [^{3}H]EE$_2$ analogues in rats: (a) 2-ClEE$_2$ (2 μmol/kg); (b) 2-BrEE$_2$ (females); (c) 2-BrEE$_2$ (males); (d) 2-IEE$_2$. Except as indicated, the steroid was given i.v. to female rats at a dose of 0.1 μmol/kg. Metabolites recovered from bile hydrolysates (pooled 0–2 h collections) and unlabelled standards were resolved on a C$_{18}$ column using a gradient of acetonitrile (20–75% over 40 min) in phosphate buffer. Metabolite F of 2-BrEE$_2$ was only formed at a higher dose (13 μmol/kg).

steroid and of all three metabolites in bile were sexually differentiated.

Females excreted 69% of a 16 μmol/kg dose into bile over 3 h. Metabolites I–III were formed in proportions approximately equal to those observed at the lower dose.

Metabolite I (R_t 13.5 min; 5% eluted radiolabel at 16 μmol/kg) was identified as an alkyl monohydroxylated derivative. The dominant fragmentation [M-68] [loss of $CH_2.C(OH).C{\equiv}CH$], yielding m/z 322 and 324 (Table 3), indicated an unsubstituted C-16 (EE$_2$ and 16α-OHEE$_2$ give [M-68] and [M-84], respectively) but peaks at [M-83] due to complete loss of a 17α-ethynylated D ring were absent. Instead, m/z 304 and 306 ([322/324-H_2O]), and m/z 291 and 293 ([M-99]) arising from fission of an hydroxylated D ring (16α-OHEE$_2$ undergoes [M-99] to give base-peak m/z 213), are formed. The disproportionately strong peak at m/z 291 and the peak at m/z 289 ([M-101]) probably represent a third fragmentation route, viz loss of the secondary hydroxyl group and C-8 hydrogen, via dehydration, followed by fission of ring D with migration of two hydrogens. The hydroxyl group is assigned to C-15.

Metabolites II (R_t 23 min; 29% at 16 μmol/kg) and III (R_t 28.5 min, 7% at 16 μmol/kg) were unambiguously identified from their mass spectra as 2-OH-4-BrEE$_2$ and 2-methoxy-4-BrEE$_2$, respectively.

2-IEE$_2$. Female rats metabolized 2-IEE$_2$ (0.1 μmol/kg) to one major (H), one minor (G) and one trace (I) aglycone metabolite [Fig 1(d); Table 1] but eliminated most of the steroid by conjugation alone.

Only metabolite H (R_t 16.5 min; 18% eluted radiolabel) was formed at the higher dose (12 μmol/kg). G (R_t 13.5 min), solely from its extreme polarity, was

Table 3. Mass spectra (direct-probe EI) of ring-A halogenated [^{3}H]EE$_2$ analogues and their metabolites isolated from bile of female rats

Analogue & metabolite	Molecular and fragment ions
2-ClEE$_2$	332($M^{+\cdot}$, 25) 330(68), 264(10), 262(29), 249(37), 247(100), 233(6), 219(7), 206(12), 196(17), 194(48), 167(19).
B(1)	378($M^{+\cdot}$, 44), 376(100), 281(17), 280(17), 279(40), 278(34), 277(20), 226(15), 224(47), 197(22).
B(2)	348($M^{+\cdot}$, 19) 346(60), 251(11), 250(13), 249(59), 248(26), 247(25), 196(21), 194(52), 167(27).
C	348($M^{+\cdot}$, 30), 346(74), 278(35), 265(38), 263(100), 222(11), 212(16), 210(45).
2-BrEE$_2$	376($M^{+\cdot}$, 41), 374(43), 308(26), 306(28), 293(97), 291(100), 277(6), 263(7), 250(10), 240(41), 238(48).
D(1)	408($M^{+\cdot}$, 92), 406(96), 390(12), 388(12), 311(79), 310(62), 309(100), 308(42), 307(32), 293(22), 291(17), 268(20), 266(20), 256(61), 254(91).
D(2)	408($M^{+\cdot}$, 57), 406(64), 390(60), 388(60), 375(99), 373(100).
E	392($M^{+\cdot}$, 37), 390(41), 374(15), 372(17).
F	392($M^{+\cdot}$, 49), 390(52), 324(36), 322(36), 309(96), 307(100), 256(44), 254(51).
4-BrEE$_2$	376($M^{+\cdot}$, 48), 374(51), 308(28), 306(29), 293(95), 291(100), 277(7), 263(7), 250(10), 240(40), 238(46), 226(10), 224(14), 213(13), 211(10).
I	392($M^{+\cdot}$, 76), 390(69), 374(12), 372(10), 324(100), 322(91), 306(40), 304(57), 293(38), 291(52), 289(21), 240(34), 238(60). 226(38), 224(41).
II	392($M^{+\cdot}$, 98), 390(100), 324(49), 322(49), 309(93), 307(95), 268(12), 266(15), 256(38), 254(48), 242(13), 240(19), 229(20), 227(24).
III	406($M^{+\cdot}$, 100), 404(99), 338(34), 336(40), 323(49), 321(61), 282(11), 280(10), 270(21), 268(31), 256(10), 254(13), 243(16), 241(14).
2-IEE$_2$	422($M^{+\cdot}$, 81), 354(30), 339(100), 325(4), 311(5), 298(10), 286(40), 272(8), 259(12).
H	438($M^{+\cdot}$, 100), 341(47), 340(25), 339(27), 325(7), 311(10), 298(15), 286(52), 272(11), 259(28).

Relative intensities given in parentheses.

deduced to be a product of alicyclic hydroxylation [31]. I (R_t 31.5 min) eluted shortly after the parent compound, a characteristic of A-ring methoxy derivatives (cf. Fig. 2, metabolite III); it was tentatively identified as 2-I-4-methoxy EE$_2$.

Metabolite H gave the $M^{+\cdot}$ of a monohydroxylated product, and underwent the multiple D-ring fragmentations ([M-97/98/99]) noted previously with metabolites of 2-Cl- and 2-BrEE$_2$. The hydroxyl group is assigned to C-16.

Schematic pathways based upon the minimum number of biotransformations required to account for the identified metabolites are given in Figs 3 and 4.

DISCUSSION

The outstanding feature of the structure–metabolism relationships of C-2 haloestrogens, as reported here and previously [22, 23], is the sharply differentiated inhibition of C-2 hydroxylation *in vivo*: whereas chloro, bromo and iodo substituents on EE$_2$ completely block this biotransformation, fluorine is partially displaced to yield substantial amounts of 2-OHEE$_2$ [23] even though 2-FE$_2$ is refractory to defluorination [22].

Nevertheless, irrespective of their various effects on oxidative pathways, none of the halogens significantly

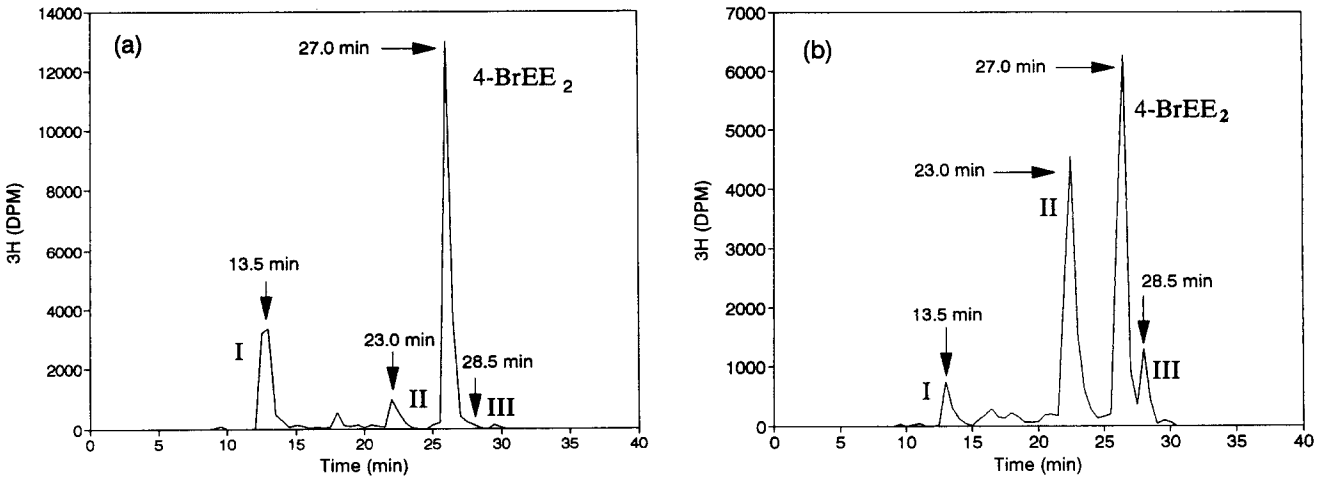

Fig. 2. High-performance liquid radiochromatograms of the phase I metabolites of 4-BrEE$_2$ in (a) male and (b) female rats. The steroid was given i.v. at 0.1 μmol/kg. Metabolites recovered from bile hydrolysates (pooled 0–2 h collections) and unlabelled standards were resolved on a C$_{18}$ column using a gradient of acetonitrile (20–75% over 40 min) in phosphate buffer.

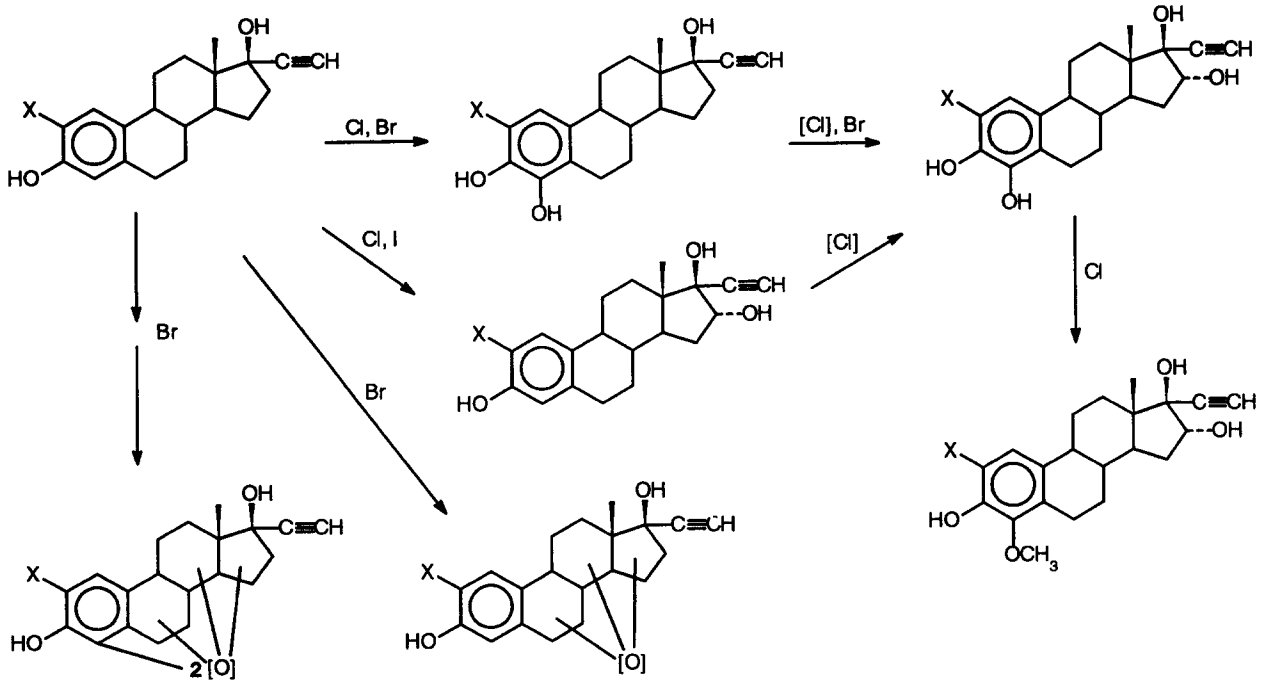

Fig. 3. Metabolic pathways of 2-Cl, 2-Br- and 2-IEE$_2$ in female rats and of 2-BrEE$_2$ in male rats. [X] indicates pathway forming a putative intermediate (non-excreted) metabolite. [O] indicates position of hydroxylation uncertain. - - - represents unknown configuration. Of the excreted metabolites, only those characterized by mass spectrometry are depicted.

influences elimination of parent compound and metabolites via conjugation and biliary excretion [2, 29].

Hypothetical explanations for the ethynyl group's facilitation of oxidative defluorination have been discussed previously [23]. Carbon–halogen bond strength is clearly not the enabling substituent characteristic—indeed the great strength of the C–F bond militates against direct oxygen insertion, the favoured mechanism for oestrogen C-2 hydroxylation [34]. However, the alternative defluorination pathway proposed by Morgan *et al.* [23] suggests a mechanistic rationale for the selectivity of dehalogenation; the steroid is metabolized to a 1,2-epoxide intermediate, rearranges with elimination of HF, and yields an *o*-quinone which undergoes facile reduction to the C-2 catechol. Discrimination between fluorine and the other halogens could occur at two steps in this sequence: 1,2-epoxidation might be subject to steric hindrance by the larger halogens (fluorine is isosteric with hydrogen); rearrangement of the epoxide resulting in explusion of the halogen would be favoured by fluorine's maximal electronegativity. Arene oxide metabolites have been implicated in the fluorine-selective dehalogenation of *p*-substituted phenylpiperazines [35].

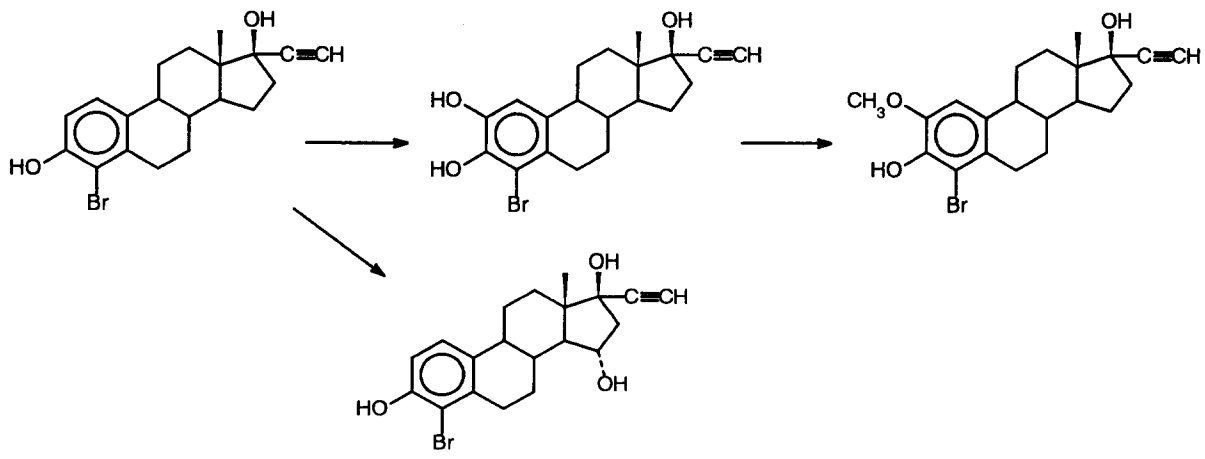

Fig. 4. Metabolic pathways of 4-BrEE$_2$ in male and female rats. Metabolites were characterized by mass spectrometry.

Both the complete and partial blockages of C-2 hydroxylation allowed substituent-dependent redirections along lesser pathways of oxidative metabolism. In the case of 2-FEE_2, enhanced C-4 hydroxylation was accompanied by distal suppression of alicyclic biotransformations [2, 23, 31]. These actions might be partly attributable to short-range electronic influences and long-range effects (conformational-electronic), respectively [36]. At the μg/kg doses, only 2-FEE_2 (0.7 μmol/kg) and 2-$ClEE_2$ (2.0 μmol/kg) yielded identifiable quantities of metabolites functionalized solely at C-4. 2-$BrEE_2$ (0.1 μmol/kg) underwent C-4 hydroxylation though the moiety was apparently confined to a difunctionalized metabolite. Since 2-Br-4-$OHEE_2$ was excreted only at the higher (13 μmol/kg) dose—which suggests saturation of the preliminary or subsequent C-16 pathway—the monohydroxylation at C-4 of the relatively large lower dose of 2-$ClEE_2$ (2 μmol/kg) may have been dose related. C-2 hydroxylation is seemingly much less influenced by the *m*-halogen's identity. Thus C-4 fluorine and bromine reduce equally, by *ca* 40%, C-2 hydroxylation of E_2 [22, 29] and EE_2 [2], respectively, in female rats. The C-2 chloro substituent, in comparison with the fluoro [23], not only restricted *m*-hydroxylation but also catechol methylation. Likewise, the C-4 bromine decreased O-2 methylation (methylated catechol: catechol, 0.7 and 0.2 for EE_2 [2] and 4-$BrEE_2$, respectively) whilst a C-4 fluorine has only a small effect (methylation ratio, 0.6 and 0.8 for E_2 and 4-FE_2 [22, 29].

Unlike C-4 pathways, C-15 and C-16 alicyclic hydroxylations of oestrogens, alone or in combination with A-ring biotransformations, are significant metabolic routes in rats [2, 29]. C-16 functionalization, on its own a minor route for EE_2 [2, 32], demonstrated a direct relationship to halogen size. This may have arisen indirectly from localized influences on substrate–enzyme interactions as well as from long-range effects.

To summarize, 2-FEE_2 was metabolized exclusively by A-ring pathways, 2-$ClEE_2$ and 2-$BrEE_2$ preferentially via, respectively, C-4 and alicyclic hydroxylations, and 2-IEE_2 largely by C-16 hydroxylation. Although this trend from A- to D-ring hydroxylation corresponds to the gradations in the halogens' physico-chemical properties, modest dose-related influences on metabolism, as mentioned above, cannot be excluded.

The oxidative metabolism of 2- and 4-$BrEE_2$ was sexually differentiated at μg/kg doses. 2-$BrEE_2$ yielded the monohydroxylated E and the dihydroxylated fraction D at the ratio of 1:0.09 and 1:0.76 in male and female rats, respectively. 4-$BrEE_2$ underwent C-2 and alicyclic hydroxylation at the ratio 1:4.8 and 1:0.07 in males and females, respectively. Sex-linked differences in phase I metabolism were observed previously with E_2 [29] and EE_2 [2]. They and 4-$BrEE_2$ are metabolized to the greater quantity of C-2 monofunctionalized (hydroxylated and methoxylated) products by female (Wistar) rats: representing 67, 71 and 42% of biliary radioactivity, respectively, against the 4, 50 and 6% produced by males. The latter form greater quantities of D-ring and multifunctionalized [2, 31] metabolites: 64, 24 and 22% vs 5, 10 and 3% for females. In effect, the 17α-ethynyl group narrows sexual differentiation by restricting D-ring hydroxylation in males. The C-4 bromine enhances metabolic dimorphism via an impeding of C-2 hydroxylation; this allows dominant male-selective expression of C-15 monofunctionalization. It also impedes catechol methylation in both sexes, reducing the ratio of methylated catechol: catechol from 1.5 (EE_2) to *ca* 0.2 (4-$BrEE_2$) in males and from 0.7 to 0.2 in females; O-3 methylation [2, 31] appears to be completely inhibited.

The sex-selective formation of 4-Br-15$OHEE_2$ contrasts with the sex-specific metabolism of E_2 to 15α-OHE_1 and 2-methoxy-15α-OHE_1 by male rats [29]. 15α-hydroxylation in rats was attributed previously to male-specific *P*450 IIC13 [29, 37], but apparently female Wistars possess at least low-level activity against certain hindered substrates. Substituents which indirectly restrict EE_2 aromatic hydroxylation can exert regioselective influences on D-ring metabolism. Thus, whereas C-15 hydroxylation is the major route for 4-$BrEE_2$, the C-16α/β-pathways dominate the biotransformation of 11β-methoxyEE_2 in rats and C-15 hydroxylation is insignificant [38].

Substitution of an additional bromine at C-2 renders EE_2 inert to phase I reactions in male rats and nearly so in females [30]. Comparison of the present work with studies on 2,4-dibromoEE_2 [30] and 2,4-dibromoE_2 [39] indicates that 2,4-dibromination is necessary for inhibition of C-15 hydroxylation in male rats, and 17α-ethynylation for complete blocking of D-ring oxygenation. The bromines' influence is potentially explained by the observation that C-2 bromination and 2,4-dibromination alter the configuration of the oestrogen backbone in several regions, including the D-ring [40]. Nevertheless, a fluorine at C-2 of EE_2 has the same effect as the two bromines on D-ring hydroxylation [23]. Such long-range effects epitomize the subtlety of steroids' structure–metabolism relationships, and represent the major challenge to accurate prediction of these relationships.

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