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WIDE SPECTRUM OF STEROIDS SERVING AS SUBSTRATES FOR THE FORMATION OF LIPOIDAL DERIVATIVES IN ZR-75-1 HUMAN BREAST CANCER CELLS

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Summary—Recently, several natural steroids have been found to be esterified to long-chain fatty acids (FAE) in various mammalian tissues. The purpose of the present study was to determine the ability of a series of ^{3}H-labeled steroids to serve as substrates for the formation and accumulation of such non-polar derivatives in intact cells, using the hormone-responsive ZR-75-1 human breast cancer cell line as model. All 14 steroids tested were found to be converted, directly or following further metabolism, to lipoidal ester derivatives. The percentage of intracellular steroids recovered as FAE derivatives was usually substantial (14–90%), especially in the case of C-19 steroids (75–90%). The composition of the lipoidal steroid fractions recovered from the labeled cell extracts was characterized by chromatographic comparison with synthetic steroid FAEs and by saponification of the steroid FAEs and identification of the released steroidal moieties. Following metabolism, most steroid substrates were converted into multiple lipoidal esters. Furthermore, 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, as well as androst-5-ene-3β, 17β-diol formed lipoidal diesters in addition to the monoester form. The high level of intracellular steroid FAE accumulation reported in this study suggests that these yet poorly known steroid derivatives may play important functions in the regulation of steroid hormone metabolism and action.

INTRODUCTION

Several steroids, including pregnenolone [1–5], 17α-hydroxy-pregnenolone [4], 3β-hydroxy-5α-pregnan-20-one [3], dehydroepiandrosterone [4–8], androst-5-ene-3β,17β-diol [6–8], estradiol-17β [9–10], androsterone [11] and corticosterone [12] have been shown to undergo esterification to long-chain fatty acids in a number of mammalian tissues. The resulting fatty acid esters (FAEs) are much less polar than the parent steroid and can regenerate the free-steroid moiety following mild alkaline hydrolysis [1–12] or through an esterase activity present in the same tissue [8, 10, 13]. Although the role of these lipoidal steroids is at present unknown, it has been postulated that they could mimic the pharmacokinetic properties of synthetic steroid FAEs (e.g. 19-nortestosterone undecanoate, estradiol valerate, etc.) by behaving as a long-acting depot form of the parent steroid, thus delaying its metabolic inactivation [7, 14, 15].

We have recently characterized the properties and the kinetics of formation of the FAEs of dehydroepiandrosterone (DHEA) and androst-5-ene-3β,17β-diol (Δ^5-diol) in the ZR-75-1 human breast cancer cell line incubated with radiolabeled precursors [7]. In order to assess the potential of other natural steroids to serve as substrates for biological fatty acylation, we have measured the formation of lipoidal derivatives in intact ZR-75-1 cell monolayers following incubation with 14 common steroids, using the synthetic steroid palmitoyl esters as references to characterize the derivatives obtained. This report provides the preliminary description of the lipoidal steroid derivatives formed and accumulated in ZR-75-1 cells from the various substrates used, some of them for the first time. Evidence is also presented showing that lipoidal steroids markedly differ in their net rate of accumulation over a 24-h incubation period, as well as in the fraction of total intracellular steroid products that they represent. Part of this work has been presented in a preliminary report [16].

Abbreviations: ADT, androsterone (5α-androstane-3α-ol-17-one); DHEA, dehydroepiandrosterone; DHT, 5α-dihydrotestosterone; Δ^5-diol, androst-5-ene-3β,17β-diol; 3α-diol, 5α-androstane-3α,17β-diol; 3β-diol, 5α-androstane-3β,17β-diol; DOC, 11-deoxycorticosterone (pregn-4-ene-21-ol-3,20-dione); E_2, estradiol; E_3, estriol; EpiADT, epiandrosterone (5α-androstane-3β-ol-17-one); FAE(s), fatty acid ester(s); FAE_1, fatty acid monoester; FAE_2, fatty acid diester; HBSS, Hanks' balanced salts solution; Δ^5-P, pregnenolone; 17-OH-P, 17α-hydroxyprogesterone; 17-OH-Δ^5-P, 17α-hydroxy-pregnenolone; T, testosterone.

EXPERIMENTAL

Chemicals

The following radioactive steroids were purchased from Amersham (Arlington Heights, Ill.): [4,7-^{3}H] pregnenolone (Δ^5-P, 10 Ci/mmol); 17α-hydroxy[7(n)-^{3}H]pregnenolone (17-OH-Δ^5-P, 12 Ci/mmol); 17α-hydroxy-[1,2,6,7-^{3}H]progesterone (17-OH-P, 74 Ci/mmol); 11-deoxy[1α,2α(n)-^{3}H]corticosterone (DOC, 46 Ci/mmol); [1,2,6,7-^{3}H]corticosterone (92 Ci/mmol); dehydro[1,2,6,7-^{3}H]epiandrosterone (DHEA, 100 Ci/mmol); [1,2,6,7-^{3}H]testosterone (T, 91.7 Ci/mmol); 5α-dihydro[1α,2α(n)-^{3}H]testosterone(DHT, 60 Ci/mmol); 5α[1α,2α(n)-^{3}H]androstane-3α,17β-diol (3α-diol, 47 Ci/mmol); 5α-[1α,2α(n)-^{3}H]androstane-3β,17β-diol (3β-diol, 60 Ci/mmol); [2,4,6,7,16,17-^{3}H] estradiol (E_2, 140 Ci/mmol) and [2,4,6,9-^{3}H]estriol (E_3, 70 Ci/mmol). [1,2(n)-^{3}H]Androst-5-ene-3β,17β-diol (Δ^5-diol, 46.9 Ci/mmol) and [1,2(n)-^{3}H] androsterone (ADT, 53.3 Ci/mmol) were obtained from New England Nuclear (Lachine, Qué, Canada). All radiolabeled steroids were repurified prior to use by liquid chromatography on Sephadex LH-20 columns, as described [17]. All radioinert steroids were purchased from Steraloids (Pawling, N.Y.). Palmitoyl chloride, culture media and supplements, and all chemical reagents were from Sigma (St Louis, Mo.).

Synthesis of standard ^{3}H-labeled steroid FAEs

The palmitoyl esters of monohydroxy steroids and the dipalmitoyl esters of dihydroxy steroids were synthesized essentially as described [9]. Briefly, the equivalent of 25 μCi of the ^{3}H-labeled steroid, together with 300 μg of the non-radioactive steroid added as a carrier, were dissolved in 400 μl of anhydrous pyridine. The solution was coolled to 0°C and 100 μl of palmitoyl chloride was then added. The mixture was kept overnight at room temperature, and the crude steroid palmitoyl ester fraction was isolated from the acidified solution exactly as described by Mellon-Nussbaum *et al.*[9]. Palmitic acid was removed from this fraction by chromatography on a neutral alumina column (Fisher A540) using $CHCl_3$ to selectively elute the steroid palmitate(s). The steroid FAE was then purified by TLC using as a solvent system petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v) containing 1% (w/v) butylated hydroxytoluene as antioxidant. The area of the chromatogram containing the ^{3}H-steroid FAE was detected using a Berthold thin-layer scanner, and the product was recovered from the silica gel by elution with diethyl ether.

In addition, aliquots of the radioactive steroid FAE isolated at the end of this procedure were subjected to saponification by dissolving in 90% aqueous MeOH containing 0.6% (w/v) Na_2CO_3 (pH ~ 11) and heating at 60°C for 24 h. These reaction conditions were found to be adequate for the complete hydrolysis of all synthetic steroid FAEs. The mixture was then neutralized with aqueous acetic acid (0.68%, v/v) and extracted 3 times with diethyl ether. The products of the alkaline hydrolysis were then isolated by TLC using different solvent systems and identified with authentic steroids chromatographed in parallel [7]. This saponification procedure confirmed in all cases that the steroidal moiety of the synthetic radiolabeled palmitates described above corresponded to that of the original ^{3}H-labeled precursor steroid used for synthesis, and was unchanged by the acylation procedure.

[^{3}H]Δ^5-diol-3β-palmitate, [^{3}H]5α-androstane-3α,17(α,β)-diol-3α-palmitate and [^{3}H]5α-androstane-3(α,β),17β-diol-17β-palmitate were synthesized by the chemical reduction of the palmitoyl esters of [^{3}H]DHEA, [^{3}H]ADT and [^{3}H]DHT, respectively, prepared as described above. Briefly, the radioactive FAE precursor was dissolved in MeOH, and following the addition of an excess of $NaBH_4$, the solution was kept for 30 min at room temperature. The reduced steroid FAE was then purified by TLC, with petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v, containing 1% butylated hydroxytoluene, w/v) as solvent and identified by its mobility, which was lower than that of the ketosteroid FAE precursor. It was further characterized by alkaline hydrolysis, which yielded the expected reduced form of the steroidal moiety of the precursor FAE.

[^{3}H]E_2-17β-palmitate was obtained after selective hydrolysis of [^{3}H]E_2-3,17β-dipalmitate prepared from [^{3}H]E_2 as described above, by limited saponification with methanolic $NaHCO_3$ [9], followed by purification by TLC using petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v, and containing 1% butylated hydroxytoluene, w/v) as solvent system. This system resolves well E_2-17β-dipalmitate from E_2-3-palmitate and E_2-3,17β-dipalmitate.

After their purification, all synthetic steroid palmitates were dissolved in isooctane and stored under a N_2 atmosphere at −20°C. Radioactive concentrations were determined by liquid scintillation spectrometry with correction for quenching using the external standard method.

Cell culture and incubation with ^{3}H-labeled steroids

Stock ZR-75-1 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, Md) and routinely grown in Phenol Red-free RPMI 1640 supplemented as described [18]. For studies of radiolabeled steroid incorporation, cells were plated in triplicate in 100-ml petri dishes at 5×10^5 cells/dish and grown for 7–8 days in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM Na pyruvate, 100 nM bovine insulin, antibodies and 5% (v/v) dextran-coated charcoal-treated fetal bovine serum (HyClone, Logan, Ut.). Cells were then incubated for 24 h in fresh medium of identical composition containing the indicated concentration of ^{3}H-labeled steroid (added as an ethanolic stock solution to 0.1%, v/v, final concentration). Medium

was then removed and cells were quickly washed with 2 × 10 ml of ice-cold HBSS and harvested with 2 ml 0.05% trypsin/0.02% EDTA (w/v) in Ca^{2+}, Mg^{2+}-free HBSS. The cell suspension was transferred directly to 20 × 150mm glass tubes and frozen in an EtOH/solid CO_2 bath.

Analysis of biosynthetic steroid FAEs

After thawing, the cell suspension was extracted 3 times with 5 ml diethyl ether, and the pooled ether extracts were brought to dryness under N_2 prior to redissolution with 70 or 90% (v/v) aqueous MeOH, as indicated. Free steroids and their FAEs were then separated by 2 cycles of solvent partition using either isooctane/90% aqueous MeOH (1:1, v/v) or hexane/70% aqueous MeOH (1:1,v/v) as 2-phase systems, as detailed in the next section. The isooctane or hexane extracts were then evaporated to dryness and redissolved in isooctane. This fraction, which contained the cell-derived steroid FAEs, was then analyzed by TLC on GF-254 silica gel plates using 1–3 developments, as indicated, with petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v, containing 1% butylated hydroxytoluene, w/v). The radioactive areas were located and the FAEs eluted with diethyl ether prior to liquid scintillation spectrometry, as described above for the ^3H-labeled synthetic FAEs. Aliquots of the isolated FAE fractions were also subjected to mild alkaline hydrolysis, and the free steroids thus released isolated by TLC on silica gel plates using $CHCl_3$/acetone (7:3 or 8:2, v/v) as a mobile phase. The steroids were identified by comparison with the mobility of standard steroids chromatographed in parallel.

In the case of the 5α-androstanes, further confirmation of the identity of the steroidal moiety of the biosynthetic steroid FAE was provided by HPLC analysis. Radiolabeled steroids released by saponification were extracted with diethyl ether, and following evaporation of the extract and redissolution with MeOH/H_2O (1:1, v/v), were injected on a Radial-Pak Novapak C_{18} reversed phase HPLC column (8 mm × 10 cm) and eluted with H_2O/CH_3CN/tetrahydrofuran (65:23:12, v/v/v) at a flow rate of 1.5 ml/min. The HPLC system was a Waters Associates unit including a model 510 pump, a U6K injector and a C_{18} pre-column. The radioactivity of the eluate was monitored using a Berthold LB506B HPLC Radioactivity Monitoring System with Formula 963 (Dupont–New England Nuclear) as a scintillation mixture at a flow rate of 3.0 ml/min. The efficiency obtained for ^3H was about 20%. In this HPLC system, T, 3α-diol, 3β-diol, EpiADT, DHT and ADT were completely resolved, with retention times of 9.6, 20.0, 12.8, 16.6, 18.5 and 26.1 min, respectively.

Efficiency of the extraction and solvent partition procedures for free and lipoidal steroids

The efficiency of the extraction procedure with diethyl ether was estimated for the various steroids as follows. The equivalent of 1×10^6 dpm of free steroid or of its mono- or dipalmitoyl ester was dissolved in 2 ml of HBSS and extracted 3 times with 3 ml of diethyl ether. The combined ether extracts were then evaporated to dryness, redissolved in 2 ml MeOH, and 100 μl aliquots used for radioactivity determination. The radioactivity left in the aqueous phase was also determined, and the extraction efficiency measured as:

$$EE = \frac{(\text{dpm in the ether fraction})}{(\text{dpm in ether fraction} + \text{dpm in aqueous fraction})} \times 100. \quad (1)$$

The efficiency of the solvent partition system for separating free steroids from their FAE derivatives was also determined. Briefly, the equivalent of 5×10^5 dpm of free steroid or 3×10^5 dpm of the corresponding mono- or dipalmitoyl ester was dissolved in 8 ml of 70 or 90% (v/v) aqueous MeOH, and 8 ml of hexane or isooctane, respectively, were then added. The 2 phases were vigorously shaken for 30 s by vortex mixing, the alkane fraction collected, and reextracted with 8 ml of 70 or 90% (v/v) aqueous MeOH. The pooled methanolic fractions as well as the alkane phase were evaporated to dryness, and their radioactivity determined. The partition efficiency of the free steroid (PES) and that of its palmitoyl ester (PEP) were defined as:

$$PES = \frac{M}{M + A} \times 100 \quad (2)$$

and

$$PEP = \frac{A}{M + A} \times 100 \quad (3)$$

where A and M are the total radioactivity recovered into the alkane and methanolic phases, respectively.

Calculation of the degree of conversion of substrate to FAE

The extent of conversion of the various steroid substrates tested into FAEs in intact cells was calculated as:

$$\%\ (\text{conversion to FAE}) = \frac{(RA_{FAE})}{(RA_{pre})} \times 100 \quad (4)$$

where RA_{FAE} and RA_{pre} are the total radioactivity recovered in the FAE fraction and the total precursor radioactivity initially added, respectively.

The importance of FAE accumulation relative to the intracellular pool of free steroids was calculated as:

$$\%\ (\text{FAE accumulation}) = \frac{(RA_{FAE})}{(RA_{FAE} + RA_{free})} \times 100 \quad (5)$$

where RA_{free} is the total intracellular radioactivity recovered in the free steroid fraction.

RESULTS

Comparison of the efficiency of isolation procedures

In order to validate comparisons between different steroids as substrates for transformation into lipoidal derivatives in intact cells, it was first necessary to assess the efficiency of the extraction and solvent partition procedures used for isolation of the various compounds. Three extraction steps with diethyl ether were sufficient to extract $98.7 \pm 0.6\%$ ($n = 24$, EE value equation (1)) of the initial amounts added of 12 different steroids studied. In the case of the 2 most polar steroids studied, namely E_3 and corticosterone, the recovery was somewhat less (90 and 89% respectively). The extraction of all synthetic steroid palmitoyl esters prepared for this study was essentially complete with the standard procedure used (data not shown). The amounts of free steroids recovered at the end of the isolation procedure were thus corrected for the minor losses due to the extraction procedure as calculated from these data.

As shown in Table 1, the isooctane/90% (v/v) aqueous MeOH partition system [3] was excellent in selectively concentrating free steroids in the methanolic phase in all 12 cases studied. However, partition of the steroid FAEs into the isooctane fraction was highly dependent upon their polarity, as judged from the observation that the monopalmitoyl ester form of a given steroid had significantly lower PEP values than the corresponding diester in the case of Δ^5-diol, 3α-diol, 3β-diol and E_2. Moreover, since the mean PEP value was ≤90% for most steroid monopalmitates studied, we attempted to increase the polarity of both solvent phases of the partition system in order to improve the selectivity of extraction of the steroid FAE from the methanolic phase. The use of the 70% (v/v) aqueous MeOH/hexane phase system [19] considerably improved the partition of steroid monopalmitates into the alkane phase (Table 1). Since both solvent partition systems were used in the course of the present investigation, the yields of free and lipoidal steroids were corrected using the data from Table 1 in order to account for differences in extraction efficiencies due to the nature of the steroidal moiety and of the solvent system used.

Conversion of steroid substrates to lipoidal derivatives in intact ZR-75-1 cell monolayers

The ability of 14 different steroids to serve as substrates for the formation of FAE-like derivatives was assessed in intact ZR-75-1 cells grown in monolayer culture (Table 2). Accumulation of lipoidal derivatives which could regenerate the precursor steroid or a free steroid derived from metabolism of the precursor following alkaline hydrolysis (see below) could be demonstrated with all the precursors tested. The most efficient substrate for the net accumulation of lipoidal derivatives was DOC, while 17-OH-Δ^5-P, 17-OH-P and E_3 were the least utilized for that conversion among the precursors studied.

On the other hand, the accumulation of lipoidal derivatives relative to the total amount of radiolabeled intracellular products, was unrelated to the extent of conversion of the precursor steroid. These percentages were very reproducible in successive experiments, and were independent of precursor concentration over a wide range of values (data not shown). When regrouped according to the class of steroid, it is clear that the FAE-like products derived from the C_{19}-steroids represented a larger fraction of the total intracellular metabolites than those formed from either C_{21} steroids or C_{18} phenolic estrogens.

Table 1. Partition efficiencies of free steroids (PES) and steroid mono- or dipalmitates (PEP) with 2 different phase systems

	(A) Isooctane/90% (v/v) aqueous MeOH		
Free steroid	PES	Steroid FAE	PEP
Δ^5-P	98.7 ± 0.6	Δ^5-P-Pal_1	96.3 ± 0.3
17-OH-Δ^5-P	98.6 ± 0.3	17-OH-Δ^5-P-3β,17α-Pal_2	97.9 ± 0.5
17-OH-P	99.2 ± 0.1	17-OH-P-Pal_1	80.6 ± 2.8
DOC	99.3	DOC-Pal_1	90.6 ± 1.2
T	95.0 ± 0.3	T-Pal_1	85.1 ± 2.5
DHT	93.0 ± 1.3	DHT-Pal_1	91.9 ± 0.1
3α-diol	95.6 ± 0.5	3α-diol-3α-Pal_1	80.0 ± 2.7
		3α-diol-3α,17β-Pal_2	97.8 ± 0.3
3β-diol	98.3 ± 0.4	3β-diol-17β-Pal_1	78.0 ± 1.0
		3β-diol-3β,17β-Pal_2	98.1 ± 0.7
ADT	96.2 ± 0.6	ADT-Pal_1	91.6 ± 0.8

(B) Isooctane/90% (v/v) aqueous MeOH (System 1) and hexane/70% (v/v) aqueous MeOH (System 2)

Free steroid	PES System 1	Steroid FAE	PEP System 1	PEP System 2
DHEA	95.1 ± 0.1	DHEA-Pal_1	89.5 ± 2.0	98.6 ± 0.2
Δ^5-diol	95.7 ± 0.2	Δ^5-diol-3β-Pal_1	80.0 ± 3.5	97.7 ± 0.3
		Δ^5-diol-3β,17β-Pal_2	97.5 ± 0.3	99.4 ± 0.4
E_2	94.9 ± 0.8	E_2-17β-Pal_1	58.6 ± 2.6	95.3 ± 0.3
		E_2-3,17β-Pal_2	95.3 ± 0.7	98.4 ± 0.6

Radiolabeled free steroids and the corresponding synthetic steroid mono- (Pal_1) or dipalmitoyl (Pal_2) esters were separated by solvent partition using either isooctane/90% (v/v) aqueous MeOH (A,B) or hexane/70% (v/v) aqueous MeOH (B), and the partition efficiency values determined as described in "Experimental". Data are expressed as mean ± SD of 2–3 determinations.

Table 2. Extent of conversion of various steroid substrates to lipoidal derivatives and fraction of total intracellular radioactivity recovered as lipoidal steroids

Precursor	Concentration (nM)	% Conversion ($\times 10^2$)	% intracellular FAE accumulation
Δ^5-P	370	18.9 ± 0.4	14.2 ± 0.6
17-OH-Δ^5-P	540	6.6 ± 1.0	52.1 ± 1.9
17-OH-P	92	3.9 ± 0.3	45.4 ± 1.4
DOC	61	163 ± 5	56.1 ± 0.8
Corticosterone	62	11 ± 4	29.1 ± 3.1
E_2	303	14.2 ± 1.2	39.3 ± 0.1
E_3	412	0.7 ± 0.2	14.4 ± 2.1
DHEA	90	16 ± 8	75.0 ± 2.6
Δ^5-diol	150	16.6 ± 0.2	77.1 ± 6.4
T	76	22.2 ± 0.8	75.1 ± 0.2
DHT	116	80.7 ± 0.7	79.5 ± 0.8
3α-diol	160	31.8 ± 0.4	89.6 ± 0.4
3β-diol	93	48.3 ± 2.6	78.9 ± 2.5
ADT	44	126 ± 2	82.7 ± 2.8

Steroidal precursors were added to growth medium at the concentration indicated, and following a 24-h incubation, intracellular metabolites were extracted and analyzed as described in "Experimental". Data are expressed as means ± SD of determinations from a representative experiment.

There was no direct relationship between the polarity of the precursor steroid and the relative importance of the corresponding lipoidal derivatives.

A preliminary characterization of the steroidal moiety of the lipoidal derivatives formed from some of the precursors studied was performed using as criterion co-chromatography of the cell-derived FAEs with the synthetic mono- or dipalmitoyl ester forms of the corresponding free steroids. In addition, the isolated biosynthetic FAE-like fractions were saponified, and the free steroids thus released were identified by comparison of their TLC mobility with that of authentic standards. In some cases, lipoidal fractions identified after saponification as mono- or diesters of a given steroid did not exactly comigrate with standard palmitoyl esters. Such differences can probably be accounted by the presence of different fatty acyl moieties in the biosynthetic steroid FAE.

(a) Pregnenolone (Δ^5-P) and 17α-OH-pregnenolone (17-OH-Δ^5-P). Following a 24-h incubation with the C_{21}-5-ene-steroids Δ^5-P and 17-OH-Δ^5-P, no free steroid other than the respective precursor was recovered, suggesting that cytochrome $P450_{17\alpha}$ and 3β-hydroxy-5-ene-steroid dehydrogenase 5-ene:4-ene-isomerase activities are absent in ZR-75-1 cells. Moreover, a single lipoidal derivative was recovered following cell incubation with either [^{3}H]Δ^5-P or 17-OH-[^{3}H]Δ^5-P, which comigrated with the monopalmitoyl esters of the corresponding steroids on TLC silica gel plates (Fig. 1A and B). Moreover, alkaline hydrolysis of the respective lipoidal fraction yielded a more polar compound which had the same chromatographic behavior on TLC or HLPC as the parent steroid. These results are consistent with the presence in ZR-75-1 cells of one or several enzyme activities responsible for the direct fatty acylation of Δ^5-P and 17-OH-Δ^5-P.

(b) Dehydroepiandrosterone (DHEA) and androst-5-ene-3β,17β-diol (Δ^5-diol). As already reported [7], DHEA and Δ^5-diol were interconvertible via 17β-hydroxysteroid dehydrogenase activity in ZR-75-1 cells. Incubation with both DHEA and Δ^5-diol led to the intracellular accumulation of DHEA-FAE (fraction 2), Δ^5-diol-FAE$_1$ (fraction 1) (at both 3β- and 17β-hydroxy positions) as well as Δ^5-diol-3β,17β-FAE$_2$ (fraction 3) (Fig. 1C and D and Ref. [7]). The occurrence and formation of DHEA-FAE and the mono- and diacyl derivatives of Δ^5-diol has already been reported in several human breast cancer cell lines [7, 8].

(c) Estradiol (E_2) and estriol (E_3). In keeping with reports on other human breast cancer cell lines [10] and on mammary tumor biopsies [20, 21], E_2 was used as substrate for the formation of the corresponding E_2-17β-FAE$_1$ (Fig. 1E). No lipoidal derivative of E_2 corresponding to the 3-acyl ester was found in ZR-75-1 cells incubated for various time periods with [^{3}H]E_2. In addition, the accumulation of small amounts of a lipoidal derivative even less polar than E_2-17β-monopalmitate was observed, which yielded free E_2 upon saponification and had chromatographic properties somewhat similar to those of synthetic E_2-3,17β-dipalmitate using various TLC solvent systems.

Although over 60% of total free steroid contents was represented by estrone, no lipoidal derivative of estrone could be detected in cell extracts.

On the other hand, E_3 was the steroid least efficiently converted into lipoidal derivatives among the substrates tested, with a total net rate of conversion of FAE at only about 5% that of E_2 (Table 2). About 85% of total free-intracellular radioactivity was recovered as free E_3. The only lipoidal fraction recovered from cell incubations with [^{3}H]E_3 had the properties of a fatty acyl monoester of E_3 (Fig. 1F). Too little product was formed to allow assignment of the ester group position on the lipoidal E_3-derivative.

(d) Testosterone (T) and 5α-androstane derivatives. The five C_{19} androgens or androgen metabolites tested as substrates for the formation of lipoidal derivatives, namely T, DHT, 3α-diol, 3β-diol, and ADT were converted to 4 radioactive TLC fractions (Fig. 1G–K). In order to increase the absolute yield of each of these metabolic fractions, ZR-75-1 cells

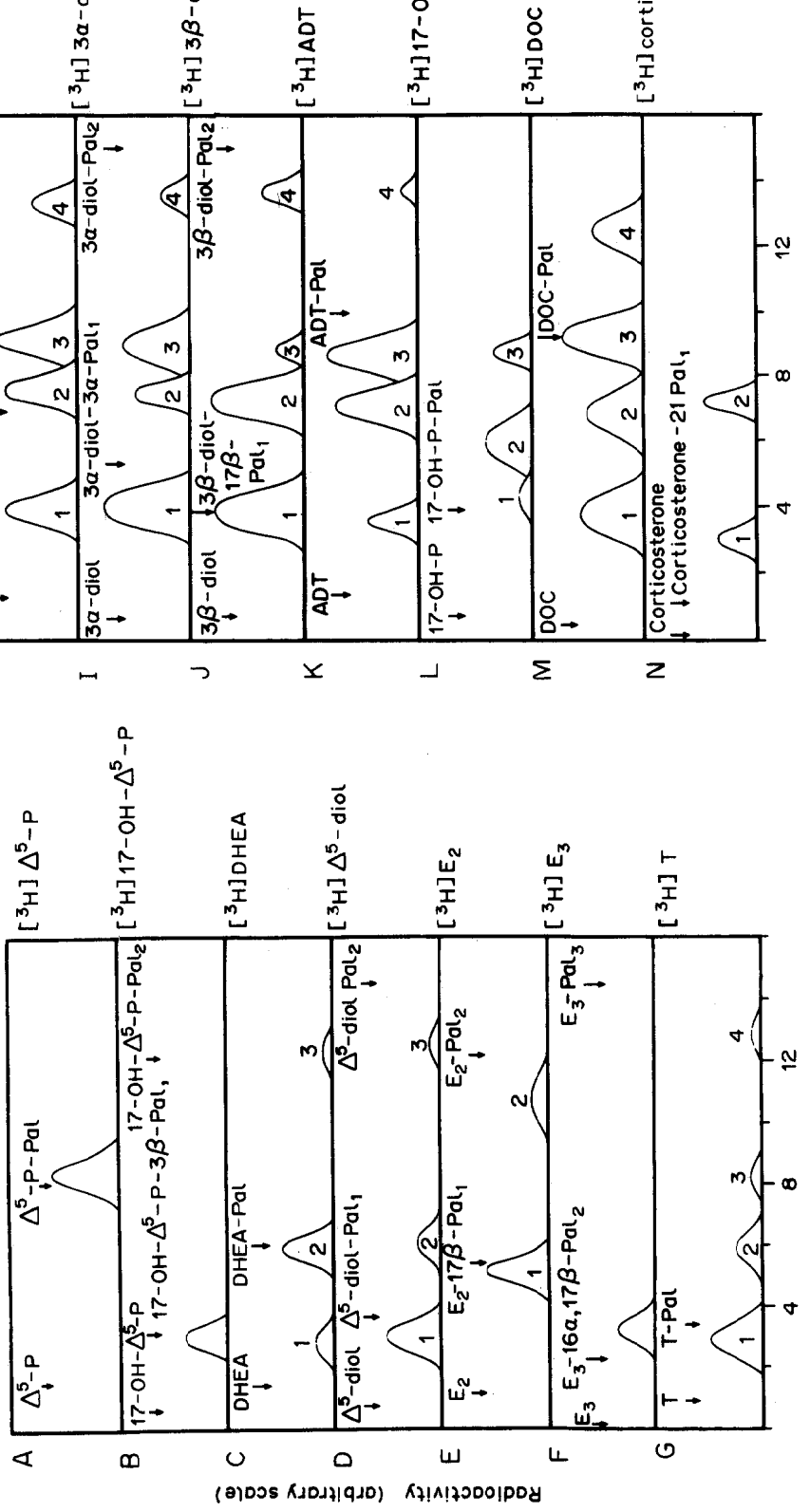

Fig. 1. Radiochromatograms of the lipoidal fractions recovered from ZR-75-1 cells following a 24-h incubation with selected ^{3}H-labeled steroid precursors. Ether extracts were prepared, and the lipoidal fraction separated from the free steroid fractions by solvent partition using hexane 70% (v/v) aqueous MeOH as 2-phase system. The lipoidal fraction thus obtained was resolved on TLC GF-254 silica gel plates with petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v) as mobile phase, using 1 (A–K), 2 (L) or 3 (M, N) developments. Radioactivity was detected using a Berthold thin-layer scanner. Arrows designate the mobility of the indicated precursor, as well as that of standard tritiated palmitoyl (Pal) esters (Pal_1 and Pal_2 refer to mono- or dipalmitate, respectively). Characterization of the various peaks is described in the text.

Table 3. Steroid composition of the lipoidal fractions obtained following a 24-h incubation of ZR-75-1 cells with ^{3}H-labeled 5α-androstanes

Concentration (nM)	Precursor[a] [^{3}H]3α-diol 1695	[^{3}H]3β-diol 940	[^{3}H]ADT 620	[^{3}H]DHT 175
3α-diol FAE_1	34 (5.3)	<1 (<0.15)	2.5 (0.59)	12 (1.1)
3α-diol FAE_2	3.3 (0.51)	<1 (0.15)	<1 (<0.15)	<1 (<0.15)
3β-diol FAE_1	22 (3.4)	54 (12.7)	4.8 (1.1)	48 (4.5)
3β-diol FAE_2	4.7 (0.73)	6 (1.4)	<1 (<0.15)	7 (0.65)
ADT-FAE	21 (3.2)	<1 (<0.15)	59 (13.8)	12 (1.1)
EpiADT-FAE	15 (2.3)	39 (9.1)	29 (6.8)	21 (2.0)

[a]Cells were incubated for 24 h with the indicated concentrations of ^{3}H-labeled precursor, and the 4 lipoidal fractions accumulated at the end of this period were isolated by TLC using petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v + 1%, w/v, butylated hydroxytoluene) (1 development). The individual fractions obtained for each type of incubation were saponified and, following extraction of the released steroid(s), analyzed by HPLC as described in "Experimental". Data are expressed as % of total lipoidal steroids represented by the indicated species (amounts formed given in parentheses are expressed as pmol/24 h).

were incubated for 24 h with about 10-fold higher concentrations of [^{3}H]3α-diol, [^{3}H]3β-diol and [^{3}H]ADT than those used for the first screening experiments. The TLC radioactive zones obtained after extraction of the lipoidal derivatives were individually eluted, saponified, and analyzed by HPLC following isolation of the free steroids thus released. As shown in Table 3, the 4 lipoidal fractions recovered following incubation with the radiolabeled 5α-androstanes represented up to 6 types of FAE. Thus, fraction 1 (cf. Fig. 1) included the monoesters of 3α- and 3β-diol, fraction 2, the monoesters of EpiADT, fraction 3, those of ADT, while fraction 4 comprised the diesters of 3α- and 3β-diol. The free steroid fraction recovered from ZR-75-1 cells following these incubations was complex and, quite predictably, indicated extensive interconversions among the precursors used, with ADT and EpiADT being the dominant free steroids found (>50% of total; data not shown).

Interestingly, DHT did not directly form lipoidal derivatives in ZR-75-1 cells, but was extensively metabolized via 3β-reduction, and to a much lesser degree, by 3α-reduction, prior to fatty acylation of the 3β-diol and 3α-diol thus formed. Furthermore, approximately 33% of all lipoidal derivatives formed following incubation of cells with [^{3}H]DHT were FAE of EpiADT and ADT. Although all substrates formed substantial amounts of 3β-acyl esters (3β-diol-FAE_1, 3β-diol-FAE_2, and EpiADT-FAE), 3β-diol was not itself a precursor of 3α-acyl esters (Table 3). Thus, the pattern of lipoidal derivatives formed from the four 5α-androstanes studied indicates that although 5α-dihydro, 3α-hydroxysteroid and 5α-dihydro, 3β-hydroxysteroid dehydrogenase activities, as well as 17β-hydroxysteroid dehydrogenase activity are present in the ZR-75-1 cell line, the equilibrium conditions greatly favor the 3β- over the 3α-reduction pathways for the metabolism of DHT.

In the case of T, the pattern of metabolites formed was almost indistinguishable from that observed when [^{3}H]DHT was used as precursor, except that 9% of the total lipoidal fraction was represented by esters of T itself included in fraction 1 (Fig. 1G). The findings for T are consistent with the already reported presence of high levels of 5α-reductase activity in the ZR-75-1 cell line [22].

(e) 17α-hydroxyprogesterone (17-OH-P), deoxycorticosterone (DOC) and corticosterone. Metabolism of C_{21}-4-ene-steroids in breast cancer cells is poorly known. Thus, in the limited scope of the present study, the complex pattern of metabolites formed from these substrates precluded the definitive identification of the steroidal moiety of the lipoidal derivatives accumulated following incubation of ZR-75-1 cells with steroids belonging to this category. Nevertheless, some features of these non-polar metabolites deserve mention.

With 17-OH-P as precursor, at least 3 discrete lipoidal fractions could be identified (Fig. 1L). Saponification of these derivatives less polar than 17-OH-P yielded several free steroid species, most of which being more polar than 17-OH-P itself. A minor fraction of the lipoidal derivatives was tentatively identified as acyl esters of 17-OH-P (fraction 3). One of the major non-polar radioactive fractions (fraction 2; cf. Fig. 1L) was represented by FAE-like derivatives of a steroid with a TLC mobility similar to that of 5α-pregnan-3β,17α-diol-20-one. The other important lipoidal fraction (fraction 1) corresponds to lipoidal derivatives of even more polar steroids.

As mentioned earlier, among the precursors studied, DOC was the steroid most extensively used for the formation of lipoidal derivatives. In fact, only 15–20% of the total radioactivity was recovered as free DOC at the end of the 24-h incubation, thus indicating that this steroid was extensively metabolized in ZR-75-1 cells. Four broad radioactive fractions were present as the lipoidal derivatives formed from [^{3}H]DOC (Fig. 1M). No definitive identification was obtained for the free steroids released after alkaline hydrolysis of these non-polar metabolites. No significant amount of the acyl esters of DOC itself was recovered. However, over 90% of all the radioactivity present in lipoidal derivatives was recovered in free steroids more polar than DOC, among which

was a steroid species with TLC mobility identical to that of 5α-pregnan-3β,21-diol-20-one (fraction 3, Fig. 1M).

Corticosterone was a much poorer substrate than DOC for the accumulation of lipoidal steroids, a feature which hampered characterization of its FAE-like derivatives. Nevertheless, the 2 major non-polar radioactive fractions recovered yielded steroids with chromatographic properties similar to those of free corticosterone (~20%, fraction 1) and 5α-pregnan-3β,11β,21-triol-20-one (~50%, fraction 2) (Fig. 1N).

DISCUSSION

The present study was designed as a survey of the potential importance of fatty acylation in steroid metabolism in a human breast cancer cell line. We have been able to establish that 7 steroids known to serve as substrates for fatty acylation in other biological systems, namely Δ^5-P [1–5], 17-OH-Δ^5-P [4], DHEA [4–8], Δ^5-diol [6–8], E_2 [9,10], ADT [11] and corticosterone [12] also form lipoidal derivatives in ZR-75-1 human breast cancer cells. In addition, we provide evidence for the biosynthesis of non-polar esters of steroids as yet unknown to serve as substrates for this reaction in intact tissues, namely T, 3α-diol, 3β-diol, EpiADT and E_3. Furthermore, it is clear from the data obtained with the C_{21}-4-ene-steroids that several unknown metabolites of 17-OH-P, DOC and corticosterone are also extensively converted to lipoidal derivatives in ZR-75-1 breast cancer cells.

Although no direct demonstration that fatty acids were actually linked through ester bonds to steroids in the lipoidal derivatives are described here, the following criteria strongly suggest that these biosynthetic products result from fatty acylation of steroids: (i) their solvent partition properties and TLC mobility indicate that they are much less polar than the parent steroid; (ii) they are completely hydrolyzed under relatively mild conditions [1–12]; and (iii) they cochromatograph with the synthetic mono- or dipalmitoyl esters of the parent steroid. These non-polar steroidal derivatives thus resemble in every respect the steroid FAE previously described [1–13].

It must be indicated that the absolute extent of conversion of steroids to their lipoidal derivatives as measured here is small (≤ 1%, cf. Table 2) as compared to the rates of other metabolic conversions such as redox reactions and sulfoconjugation. For instance, E_2 is converted to estrone and estrogen sulfates at 13- and 18-fold higher overall net rates, respectively, than to the E_2-17β-FAE_1 over a 19-h period in ZR-75-1 cells (C. Thériault, R. Poulin, D. Poirier and F. Labrie, unpublished data). Thus, fatty acylation is likely to represent only a minor fraction of the metabolic flux observed at any time for a given steroid substrate of extracellular origin. However, since once formed, lipoidal steroids are exclusively sequestrated inside the cells [7–10] by virtue of their highly lipophilic character, accumulation of these derivatives constitutes a major fraction of the metabolites present in the intracellular compartment. The latter properties are probably responsible for the fact that they have been overlooked in previous studies designed to identify metabolic products present in the incubation media alone or pooled with the cellular fraction.

Among the most notable features of the present work is the wide spectrum of steroids able to serve as substrates for the formation of lipoidal derivatives in ZR-75-1 cells, as well as the structural characteristics influencing the accumulation of these compounds. This study was intended to measure the extent of accumulation of lipoidal steroids for several substrates, and accordingly, prolonged (24 h) incubation periods were chosen, during which the precursors tested were extensively metabolized, thus providing additional information about preferred metabolic pathways. Notwithstanding the limitations of the influence of metabolism, the data herein presented illustrate the quantitative importance of the distribution of steroid FAE as intracellular steroid depots under a hormonally inactive form. In fact, all steroids tested were directly converted to lipoidal derivatives and/or further metabolized to derivatives ultimately providing the steroidal moiety for fatty acylation. However, although several hydroxy groups were found to presumably accept ester linkages (e.g. at C-3[α,β], C-17[α,β], C-21), certain configurations were more favourable for fatty acylation. For instance, the large extent of esterification (≥ 75%) of the C_{19}-5-ene-steroids (DHEA and Δ^5-diol) as well as the 3-hydroxy-5α-androstanes (3α-diol, 3β-diol, ADT and EpiADT), as compared with the complete absence of esterification of DHT suggests that the presence of a C-3 hydroxy group strongly influences at least a subset of enzymatic reactions catalyzing this transformation. Nevertheless, esterification can occur at both the 3- and 17-hydroxy groups, as shown here for Δ^5-diol, 3α-diol and 3β-diol. We have previously demonstrated that the 3β configuration was preferred over the 17β configuration for the esterification of Δ^5-diol to fatty acids [7]. The position(s) of the ester group in the case of 3α-diol and 3β-diol remain(s) to be elucidated.

Possibly relevant to these findings is our observation that lipoidal monoesters of E_2 were exclusively formed at the 17β-hydroxy group, although minor amounts of the putative E_2-3,17β-FAE_2 were also detected. Our localization of the E_2 ester linkage at the 17β-hydroxy position confirms that previously reported by various groups [9, 10, 20]. The presence of a hydroxyl group at C-16 (i.e. E_3) strongly decreased the net rate of esterification of the phenolic estrogen, indicating the specificity of the reaction for the 17β-hydroxy group of the molecule, the accessibility of which being likely hampered by the vicinity of the bulky 16α-hydroxy group. Interestingly,

DHEA, Δ^5-diol and T are all competitive inhibitors of E_2 fatty acylation in breast tumor microsomal preparations [24] and there is kinetic evidence that E_2 and T are esterified by the same enzymatic activity in bovine placenta cotyledons [19]. It will thus be of interest to determine whether fatty acylation of steroids is carried out by a single or multiple enzyme(s), and whether any of these reactions can be catalyzed by the acyl CoA: cholesterol acyltransferase activity present at high levels in ZR-75-1 breast cancer cells (R. Poulin, D. Poirier and F. Labrie, unpublished results).

The present identification of biosynthetic lipoidal diester derivatives of 3α-diol and 3β-diol, and possibly E_2, together with that already demonstrated for Δ^5-diol [7, 8], is especially intriguing because of the extreme hydrophobicity of these derivatives. Although their abundance as measured in the present study was low, it may become greater following longer periods of accumulation, as shown for the Δ^5-diol3β,17β-FAE_2 recovered in ZR-75-1 cells incubated for up to 72 h with either [^{3}H]DHEA or [^{3}H]Δ^5-diol [7]. Since the FAE_2 derivatives are degraded at a much slower rate than the monoester forms of the same steroid [7], the storage of these compounds is likely to significantly increase the metabolic half-life of the active free steroid. More extended periods of labeling will be required in order to investigate this possibility.

The significance of steroid fatty acylation in endocrine glands and target tissues is far from being understood. In the case of the ZR-75-1 cell line, the formation of lipoidal derivatives from precursors such as Δ^5-P and 17-OH-Δ^5-P is difficult to explain on physiological grounds. Due to the absence of 5-ene-3β-hydroxysteroid dehydrogenase/5-ene:4-ene isomerase and 17α-hydroxylase (C-17,20-lyase activities), neither Δ^5-P nor 17-OH-Δ^5-P are metabolized to active hormones such as Δ^5-diol or progesterone in this model. However, other breast tumor tissues possess the enzymatic activities required to synthetize active hormones from Δ^5-P and 17-OH-Δ^5-P [25, 26].

It is also conceivable that other cell types present in the mammary gland could use Δ^5-P and 17-OH-Δ^5-P as well as their FAE derivatives for the biosynthesis of active hormones [4]. Interestingly, the esterification of Δ^5-P to long-chain fatty acids has been described in mammalian adrenal glands[1–5], with the subsequent conversion of these Δ^5-P-FAE to the corresponding fatty acyl derivatives of 17-OH-Δ^5-P and DHEA [4]. However, unlike in ZR-75-1 cells, free 17-OH-Δ^5-P does not directly serve as substrate for fatty acylation in adrenal homogenates [4, 5]. The significance of these differences remains to be determined.

A novel feature uncovered in the present study is the very high rate of fatty acylation observed with 5α-androstane substrates. Except for the presence of low levels of lipoidal esters of T, all non-polar derivatives formed from T, DHT, 3α-diol, 3β-diol and ADT were hydrolyzed to 3-hydroxy-5α-androstane steroids. Of special interest are the findings that DHT was metabolized more to 3β-hydroxylated derivatives (76%) than to 3α-hydroxylated derivatives (24%), and that no 3α-hydroxylated derivatives were recovered when [^{3}H]3β-diol was used as precursor. This metabolic pattern suggests that 5α-dihydro, 3β-hydroxysteroid dehydrogenase activity exerts a strong "draining" effect on the metabolism of DHT in ZR-75-1 cells, as observed in other tissues [27]. Since the free 3β-diol is a full estrogen [28–30] with an affinity for the estrogen receptor greater than that of DHT [18, 31, 32], the extensive conversion of DHT to free, as well as mono- and diacyl esters of 3β-diol raises the question of the form(s) under which high concentrations of T and DHT exert estrogenic effects in breast cancer cells [18, 31–33] and other estrogen target tissues [32]. As found in MCF-7 breast cancer cells [34], the ZR-75-1 cell line also possesses substantial 3α-hydroxysteroid dehydrogenase activity. However, unlike 5α-dihydro,3β-hydroxysteroid dehydrogenase, there is evidence that the 5α-dihydro,3α-hydroxysteroid dehydrogenase reaction is greatly favored in the oxidative direction (i.e. towards DHT formation), as illustrated by the high rates observed for the accumulation of the lipoidal esters of 3β-diol and EpiADT with [^{3}H]3α-diol and [^{3}H]ADT as precursors (Table 3).

Thus, all 5α-androstanes studied are efficient substrates for the accumulation of lipoidal esters which can regenerate either DHT precursor and/or the estrogenic 3β-diol. There is a wide occurrence of high metabolic activity directed towards the formation and interconversion of 5α-reduced androgen metabolites in breast cancer [22, 34] as well as breast adipose tissue [35]. These compounds can exert either androgen receptor-mediated inhibition [18] or estrogen receptor-mediated stimulation of cell growth [28, 33], as well as strongly antagonize the expression of aromatase activity in human breast carcinoma cells [36, 37]. Therefore, the presence of important fatty acylation activity using these bioactive steroids as subsrates may profoundly affect their kinetics and efficiency of action.

Finally, preliminary data have been presented on the formation of a complex array of lipoidal derivatives following the incubation of ZR-75-1 cells with C_{21}-4-ene-steroids. Quite remarkably, incubation with DOC led to the greatest accumulation of lipoidal derivatives among all steroids tested. A property common to the metabolism of 17-OH-P, DOC and corticosterone is the extensive conversion of these precursors to highly polar metabolites, which could be recovered both in free and esterified forms. Among the major steroidal moieties released by saponification of the various lipoidal fractions extracted from ZR-75-1 cells incubated with these precursors were the putative 5α-reduced, 3β-hydroxylated forms of these steroids. The definitive identification of the

lipoidal esters of these polar metabolites still awaits further characterization. Related to this hypothesis is the demonstration that progesterone is converted to substantial amounts of 5α-pregnane-3β-ol-20-one (allopregnanolone) and its FAE forms in bovine corpora lutea [3, 38]. Furthermore, the likelihood of the occurrence of important amounts of 5α-reduced derivatives of C_{21}-4-ene-steroids in our system is reinforced by the identification of 5α-pregnan-3β,6α-diol-20-one as the terminal product of progesterone metabolism in a human breast cancer cell line [39]. Fatty acylation of 5α-reduced, 3β-hydroxylated metabolites of C_{21}-4-ene-steroids in ZR-75-1 cells is consistent with the high 5α-reductase and 5α-dihydro-3β-hydroxysteroid dehydrogenase activities present in this cell line, and with the already mentioned, efficient transformation of the 3β-hydroxy-5α-androstanes to lipoidal derivatives.

In conclusion, a wide spectrum of steroid substrates can be converted to and accumulated as lipoidal derivatives endowed with the properties of steroid FAE in the ZR-75-1 human breast cancer cell line. The rather unexpected generality of this metabolic pathway, as well as its prevalence as the main intracellular state of several steroids, suggest that they play key physiological roles which remain to be determined. The hydrophobic nature of steroid FAE selectively partitions these compounds in the tissue compartment and is likely to be determinant in the influence of the fatty acylation pathway on steroid metabolism at the cellular level. In view of the unique biochemical character of these metabolites and the ever expanding number of steroid substrates and tissues actively involved in their biosynthesis, we propose the name of "liposteroids" to refer to this novel class of steroidal derivatives.

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