STRUCTURE-ACTIVITY RELATIONSHIPS OF ESTROGENS: EFFECTS OF ESTERIFICATION OF THE 11β-HYDROXYL GROUP

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ABSTRACT

Fourteen esters (formate, acetate, propionate, butyrate, hexanoate, heptanoate, and benzoate) located at C-11 of 11β-hydroxyestrone and 11β-hydroxyestradiol-17β were synthesized and evaluated for uterotropic and gonadotropin release inhibition in rats, as well as their ability to displace (^{3}H) estradiol-17β from the rat uterine cytosolic estrogen receptor.

The most potent uterotropic agent was 11β-formoxyestrone which was 1,625 or 2,500 times as active as 11β-hydroxyestrone in the uterotropic or gonadotropin release inhibition assay, respectively. 11β-Formoxyestrone was 7.5 times as uterotropic as estradiol-17β and equal to estradiol-17β in inhibiting gonadotropin release. However, the most potent inhibitor of gonadotropin release was 11β-acetoxyestradiol-17β which had 133% of the activity of estradiol-17β, although it had only 38% of the activity of estradiol-17β in the uterotropic assay. Esters larger than the acetoxy group showed sharply decreased activities in either assay. Despite the high estrogenic potency of the 11-formates or 11-acetates, they were rather weak (6% to 35% as active as estradiol-17β) in displacing (^{3}H) estradiol-17β from the rat uterine cytosolic estrogen receptor.

INTRODUCTION

Reports on the use of esterification to increase hormonal activities are legion. Among the examples are the conversion of the progestationally inactive 17-hydroxyprogesterone to the highly active 17-acetoxyprogesterone (1), and the use of androgenic esters, such as testosterone β-cyclopentylpropionate (2), in clinical practice. The classic studies of Miescher, Scholz, and Tschopp 46 years ago on the esters of estrone and estradiol-17β led to interesting discoveries. They found that estrone acetate or propionate approximated estrone in their threshold doses for both the production of vaginal cornification ("estrus") and increase of uterine weight in mice. However, the calculation of absolute potencies was complicated by an increase in the duration of estrus by esters of

estrone or estradiol-17β when the length of the carbon chain of the esters was increased. Thus, from the point of view of total estrogenic activity, they concluded that esterification represented true "activation" of estrogenic compounds (3). Using water-glycerol as the vehicle, Emmens found that the 3-esters of estrone and estradiol-17β were more potent than their parent compounds in producing estrus in rats when administered intravaginally ("topical application") than when injected subcutaneously. By his definition, the esters were thus "true estrogens," although he thought (but did not prove) that the esters were converted to their parent compounds by the vaginal tissues through "local hydrolysis" (4). Segaloff and Nelson (5) and Segaloff (6) showed unequivocally that estrogenic esters are absorbed as such from oily vehicles after subcutaneous or intrasplenic injections in rats, and speculated that the prolonged action of an ester was the result of slow adsorption and protection from metabolism by liver or spleen. The possibility of hydrolysis of an ester at target sites, however, could not be discounted. After nearly 50 years of research on esters of steroid hormones, the question of whether an ester is active *per se* or must be hydrolyzed to its parent compound before it can become biologically active has not been answered. To regard esters as "pro-drugs" can be a dangerous assumption to make, since the hydrolysis of 17-acetoxyprogesterone would result in an inactive compound!

We have reported that hydroxyl groups at C-11 do not favor estrogenic activity (7). Yet, the 11β position has been established as one of the most favorable places (the other being the 7α position) on the steroid structure for modifications (8). Therefore, we investi-

gated the effect of esterifying 11β-hydroxyl groups on estrogenic activity as measured by the uterotropic or gonadotropin release inhibition assay in rats, as well as the ability of the 11β-esters to displace (^{3}H) estradiol-17β from the rat uterine cytosolic estrogen receptor.

EXPERIMENTAL

Infrared spectra were from KBr pellets and obtained from a Perkin Elmer infrared spectrophotometer model 710B. Melting points were determined on a Kofler micro-hotstage, and are uncorrected.

The acyl anhydrides used in the work--acetic, propionic, butyric, hexanoic, and heptanoic--were from Aldrich Chemicals, Milwaukee, WI.

General method of preparation of 11-acyloxyestrogens by acyl anhydride-pyridine:

11β-Hydroxyestrone (9)(500 mg) was dissolved in 5 mL pyridine, 5 mL acyl anhydride added, heated on the steam bath for 15 min, and then allowed to stand at room temperature for 1 h. The mixture was diluted with water to precipitate the 3,11-diester which was collected by filtration and recrystallized from methanol. The yields were usually quantitative.

The diester was selectively hydrolyzed by 5 mL saturated solution of KOH in 50 mL ethanol containing 25% water v/v for 15 min at room temperature. The mixture was diluted with an equal volume of water and then treated with conc HCl to precipitate the 11-ester. The precipitate was collected by filtration and recrystallized from methanol or aqueous methanol. The yields of the 11-esters were approximately 40%.

The 11β-hydroxyestrone 11-esters were reduced by sodium borohydride to the respective 11β-hydroxyestradiol-17β 11-esters by the following method which took into account the difficulty of dissolving 11-esters in ethanol: the 11β-hydroxyestrone 11-ester (100 mg) was dissolved in 5 mL tetrahydrofuran (freshly redistilled over KOH), 5 mL ethanol and 50 mg sodium borohydride added, and allowed to stand at room temperature for 30 min. During the reaction, whenever turbidity occurred, water was added until the solution became clear. After acidification with aqueous 10% HCl, extraction with dichloromethane, evaporation of the extract, and recrystallization of the residue from aqueous methanol (or petroleum ether, bp 60-110°, with some dichloromethane), the 11-esters of 11β-hydroxyestradiol-17β were obtained in yields of 65% to 85%.

11β-Formoxyestrone.--11β-Hydroxyestrone (143 mg) was mixed with 3 mL 88% formic acid and heated on the steam bath for 30 min. After cooling, small colorless prisms of 11β-formoxyestrone crystallized out, which were collected by filtration and recrystallized from methanol to afford 119 mg (84% yield) of the product.

11β-Formoxyestradiol-17β.--11β-Formoxyestrone was reduced by sodium borohydride by the above-mentioned procedure, except that the temperature was maintained at 4° to avoid the possibility of

hydrolyzing the formate. 11β-Formoxyestradiol-17β was obtained in 65% yield after recrystallization from aqueous methanol.

11β-Benzoyloxyestrone.--11β-Hydroxyestrone 3-acetate (mp 185-188°, prepared by pyridine-acetic anhydride acetylation of 11β-hydroxyestrone at room temperature for 30 min) (300 mg) was mixed with 1.5 mL benzoyl chloride, 5 mL pyridine, and 25 mL dichloromethane. (NOTE: Dichloromethane is absolutely necessary for the benzoylation to occur. It apparently dissociates (solubilizes) the pyridine-benzoyl chloride complex which is otherwise insoluble in pyridine.) The mixture was refluxed on the steam bath for 10 min, and then allowed to stand at room temperature for 2 hr. Dilution with water gave a solid which was collected by filtration and recrystallized from methanol to give 225 mg 11β-benzoyloxyestrone 3-acetate. This was subjected to hydrolysis by KOH-aqueous ethanol as described above. After the usual workup and recrystallization from methanol, 175 mg (81% overall yield) of colorless prisms of 11β-benzoyloxyestrone was obtained.

11β-Benzoyloxyestradiol-17β.--11β-Benzoyloxyestrone (160 mg) was reduced by sodium borohydride by the above-mentioned procedure. After the usual workup and recrystallization from aqueous methanol, 153 mg (86% yield) of 11β-benzoyloxyestradiol-17β was obtained.

Physical characteristics of the compounds.--The mp of the compounds are given in Table 1, and their significant ir bands are given in Table 2.

Reference compounds.--Estradiol-17β and estrone were purchased from Searle Chemicals, Inc., Chicago, Ill. 11β-Hydroxyestrone (3,11β-dihydroxy-1,3,5(10)-estratrien-17-one) and 11β-hydroxyestradiol-17β (1,3,5(10)-estratriene-3,11β,17β-triol) were prepared in this laboratory (9).

Bioassays.--The methodologies of uterotropic, inhibition of gonadotropin release, and estrogen receptor assays have been previously published (8). Bioassay data are given in Table 3.

RESULTS AND DISCUSSION

Chemistry

3,11-Diesters of 11β-hydroxyestrone were easily prepared by the use of acyl anhydrides in pyridine at steam bath temperature. As expected, the 3-esters were more easily hydrolyzed by KOH than the 11-esters which are 1,3-diaxial to the sterically hindering 13β-methyl group, thus affording the 11-esters in fair yields. Because valeric anhydride was not commercially available when our work was undertaken, the preparations of 11β-valeroxyestrone and 11β-valeroxyestradiol-17β were not attempted.

Acyl halides, such as benzoyl chloride, can be used to esterify the 11β-hydroxyl group, but it required the use of dichloromethane

to dissolve the benzoyl chloride-pyridine complex in order for the reaction to succeed.

Table 1. Names of the compounds and their mp

Name of Compound	Mp (°C)
1. 11β-Formoxyestrone (11β-formoxy-3-hydroxyl-1,3,5(10)-estratrien-17-one)	260-264
2. 11β-Formoxyestradiol-17β (11β-formoxy-1,3,5(10)-estratriene-3,17β-diol)	225-231
3. 11β-Hydroxyestrone 3,11-diacetate (3,11β-diacetoxy-1,3,5(10)-estratrien-17-one)	185-187
4. 11β-Acetoxyestrone (11β-acetoxy-3-hydroxyl-1,3,5(10)-estratrien-17-one)	< 300
5. 11β-Acetoxyestradiol-17β (11β-acetoxy-1,3,5(10)-estratriene-3,17β-diol)	222-226
6. 11β-Hydroxyestrone 3,11-dipropionate (3,11β-dipropionoxy-1,3,5(10)-estratrien-17-one)	147-150
7. 11β-Propionoxyestrone (3-hydroxy-11β-propionoxy-1,3,5(10)-estratrien-17-one)	247-249
8. 11β-Propionoxyestradiol-17β (11β-propionoxy-1,3,5(10)-estratriene-3,17β-diol)	209-211
9. 11β-Hydroxyestrone 3,11-dibutyrate (3,11β-dibutyroxy-1,3,5(10)-estratrien-17-one)	112-114
10. 11β-Butyroxyestrone (11β-butyroxy-3-hydroxy-1,3,5(10)-estratrien-17-one)	196-198
11. 11β-Butyroxyestradiol-17β (11β-butyroxy-1,3,5(10)-estratriene-3,17β-diol)	166-170
12. 11β-Hydroxyestrone 3,11-dihexanoate (3,11β-dihexanoyloxy-1,3,5(10)-estratrien-17-one)	99-100
13. 11β-Hexanoyloxyestrone (11β-hexanoyloxy-3-hydroxy-1,3,5(10)-estratrien-17-one)	163-167
14. 11β-Hexanoyloxyestradiol-17β (11β-hexanoyloxy-1,3,5(10)-estratriene-3,17β-diol)	183-184
15. 11β-Hydroxyestrone 3,11-diheptanoate (3,11β-diheptanoyloxy-1,3,5(10)-estratrien-17-one)	79-81
16. 11β-Heptanoyloxyestrone (11β-heptanoyloxy-3-hydroxy-1,3,5(10)-estratrien-17-one)	150-153
17. 11β-Heptanoyloxyestradiol-17β (11β-heptanoyloxy-1,3,5(10)-estratriene-3,17β-diol)	170-173
18. 11β-Hydroxyestrone 3-acetate 11-benzoate (3-acetoxy-11β-benzoyloxy-1,3,5(10)-estratrien-17-one)	254-257
19. 11β-Benzoyloxyestrone (11β-benzoyloxy-3-hydroxy-1,3,5(10)-estratrien-17-one)	269-271
20. 11β-Benzoyloxyestradiol-17β (11β-benzoyloxy-1,3,5(10)-estratriene-3,17β-diol)	252-256

Table 2. Ir data (in cm^{-1}) for the compounds. The numbers that are underlined are the most prominent bands. For explanations of symbols, see the footnotes below.

Cpd	17OH	3OH	3K	17K	11K	C=C	C=C	C=C	COC	COC	COC
1.	....	3360	....	1725	1678	1605	....	1500	1230	1195	....
2.	3550	3350	....	....	1685	1605	....	1500	1220	1185	....
3.	....	....	1768	1738	1722	1618	1590	1500	1242	1205	....
4.	....	3440	....	1740	1695	1618	....	1508	1272	1220	....
5.	3410	3300	....	....	1700	1608	....	1508	1260	1242	....
6.	....	....	1752	1738	1720	1605	1585	1500	1205	1138	....
7.	....	3380	....	1721	1679	1595	....	1492	1190	....	....
8.	3450	3350	....	....	1700	1615	1580	1495	1288	1240	....
9.	....	....	1760	1740	1720	1610	1585	1495	1230	1182	1150
10.	....	3450	....	1738	1695	1609	....	1500	1200	....	....
11.	3450	3350	....	....	1700	1620	1588	1500	1290	1250	1200
12.	....	....	1750	1730	1710	1600	1580	1490	1210	1170	1140
13.	....	3375	....	1720(a)		1608	....	1500	1215	1170	....
14.	3550	3375	....	....	1690	1608	....	1505	1290	1265	1223
15.	....	....	1760	1740	1728	1610	1590	1500	1210	1145	....
16.	....	3475	....	1722(a)		1615	....	1508	1220	1160	....
17.	3550	3375	....	....	1688	1608	....	1500	1270	1220	....
18.	....	....	1750	1730	1700	1600	1580	1490	1262	1208(b)	....
19.	....	3375	....	1730	1690	1603	1580	1500	1272	....	....
20.	3375	3250	....	....	1675	1600	1580	1495	1275	....	....

Compound numbers correspond to those in Table 1.

17OH: O-H stretching of 17β-hydroxyl; 3OH: O-H stretching of 3-hydroxyl; 3K: C=O stretching of 3-ester; 17K: C=O stretching of 17-ketone; 11K: C=O stretching of 11-ester; C=C: C-C stretching of ring A double bonds; COC: C-O-C stretching of ester.

a) Combined 17-ketone and 11-ester carbonyls presenting a single band.

b) Assigned to the 3-acetate.

Table 3. In Vitro and In Vivo Data

Compounds[a]		UT[b] (dose)	OV[c] (dose)	OV/UT[d]	RDA[e]
	Estrone	100 (0.5)	100 (4.0)	8.0	46
	11β-Hydroxy-	7.7 (6.5)	0.4 (1000)	154	0
1.	11β-Formoxy-	12500 (0.004)	1000 (0.4)	100	15
4.	11β-Acetoxy-	625 (0.08)	67 (6.0)	75	6
7.	11β-Propionoxy-	50 (1.0)	5 (80)	80	1.2
10.	11β-Butyroxy-	1.3 (40)	0.6 (625)	16	0.5
13.	11β-Hexanoyloxy-	0.1 (500)	inact[f]	...	0.4
16.	11β-Heptanoyloxy-	0.1 (500)	0.04 (10000)	20	0.3
19.	11β-Benzoyloxy-	2 (25)	6 (63)	2.5	0
	Estradiol-17β	100 (0.03)	100 (0.4)	13	100
	11β-Hydroxy-	4.3 (0.7)	0.4 (100)	143	0
2.	11β-Formoxy-	300 (0.01)	57 (0.7)	70	35
5.	11β-Acetoxy-	38 (0.08)	133 (0.3)	3.8	27
8.	11β-Propionoxy-	3 (1.0)	4 (9.0)	9	17
11.	11β-Butyroxy-	0.07 (42)	0.04 (1000)	24	13
14.	11β-Hexanoyloxy-	NC[g]	0.004 (10000)	...	20
17.	11β-Heptanoyloxy-	0.03 (100)	0.005 (7500)	75	16
20.	11β-Benzoyloxy-	0.06 (51)	0.04 (1000)	20	2

a) Underlined numbers are compound numbers given in Table 1.

b) UT: relative uterotropic activity; the ability of a compound to double the weight of rat uterus with the reference compound (which appears first in each of the two divisions) given the value of 100. (Total dose in μg enclosed by parentheses.)

c) OV: relative gonadotropin release inhibition activity; the ability of a compound to halve the weight of parabiotic rat ovary with the reference compound (which appears first in each of the two divisions) given the value of 100. (Total dose in μg enclosed by parentheses.)

d) OV/UT: the total gonadotropin release inhibition dose divided by the total uterotropic dose.

e) RDA: relative displacing activity; the ability of a compound to displace (^{3}H) estradiol-17β from rat uterus cytoplasmic estrogen receptor as compared to estradiol-17β which is given the value of 100.

f) inact: practically inactive; does not halve the weight of parabiotic rat ovary at a total dose of 10,000 μg.

g) NC: not calculable by quantal means, albeit being definitely uterotropic. A total dose of 100 μg increased the uterine weight by 83%, but this was not further increased by a total dose of 500 μg.

The remarkable ease and regiospecificity of preparing 11β-formoxyestrone by merely mixing 11β-hydroxyestrone with 88% formic acid at steam bath temperature deserves further mention. In unpublished results from this laboratory, we have found that the method will also work with 17β-hydroxyl groups: estradiol-17β can be smoothly esterified to estradiol-17β 17-formate by our method. The phenolic 3-hydroxyl group is not attacked by formic acid. On the other hand, formic-acetic anhydride prepared according to Huffman (10) will formylate both 3- and 17β-hydroxyls of estradiol-17β at room temperature when a few drops of pyridine is added as a catalyst. Tserng and Klein have formylated bile acids with formic acid-acetic anhydride-perchloric acid (11). They stated that it was essential to remove all water from the formylation reaction by the use of acetic anhydride-perchloric acid if all hydroxyls (3α, 7α, and 12α) of bile acids were to be completely formylated. It is quite possible, therefore, that the water in 88% formic acid somehow blocked the formylation of phenolic 3-hydroxyls in our experiments, albeit formylation of 11β-hydroxyls (and 17β-hydroxyls) serendipitously occurred in good yields.

We noted that ir spectra (Table 2) supported the structures of our compounds, after comparing our assignments with those made by Neudert and Röpke for various steroidal esters (12). It is noteworthy, however, that 11β-esters exhibit C=O stretching bands that are of wave numbers 20 to 41 cm^{-1} lower than normal when the 3-hydroxyl group is "free." This indicates that, in the KBr pellets, the carbonyl oxygen of an 11β-ester is strongly hydrogen-bonded to the phenolic 3-hydroxyl. Also noteworthy is the shift of the C-O-C band at 1190 cm^{-1}

for 11β-propionoxyestrone to 1288 cm^{-1} for 11β-propionoxyestradiol-17β. We have no explanation for the singular anomaly that is apparently due to the presence of a 17β-hydroxyl group.

Bioassays.--

11β-Formoxyestrone is 125 times as uterotropic as estrone and surpasses estradiol-17β in the same bioassay by a factor of 7.5. It is, to the best of our knowledge, the most potent uterotropic agent for rats. What is even more remarkable is that its rather poor RDA of 15 would never have predicted its high uterotropic potency. The compound is also a good inhibitor of gonadotropin release, being 10 times as active as estrone or equal to estradiol-17β in this regard.

11β-Formoxyestradiol-17β is also a potent estrogen, being 3 times as uterotropic as estradiol-17β but having only 57% of the ability of estradiol-17β to inhibit gonadotropin release. Paradoxically, the 17β-hydroxysteroid was less active than its 17-ketosteroid counterpart in either the uterotropic or gonadotropin release inhibition assay, although its RDA was twice that of its 17-ketosteroid counterpart. Examination of the data in Table 3 reveals the interesting lack of difference between the uterotropic potencies of a 17-ketosteroid and its 17β-hydroxysteroid counterpart when they are 11β-esters (excepting the formate, hexanoate, and heptanoate).

The results (Table 3) of the 11β-esters of estrogens clearly show the futility of any attempt to quantitatively correlate RDA with biological activity. The 11β-esters do not enhance the binding of an estrogenic structure to the putative "estrogen receptor" of the rat uterine cytosol. Therefore, there exists the possibility

that the uterine cytosol estrogen receptor has nothing to do quantitatively with the elicitation of estrogenic activity.

We demonstrated that esterification of the 11β-hydroxyl group can convert a very weak estrogen to a very potent one. 11β-Formoxyestrone is 1,625 or 2,500 times as potent as 11β-hydroxyestrone in the uterotropic or gonadotropin release inhibition assay, respectively. This is reminiscent of the conversion of the progestationally inactive 17-hydroxyprogesterone to the highly active 17-acetoxyprogesterone. Apparently, estrogenic activity is not favored when there is a proton donor or nucleophilic group, such as an hydroxy group, at C-11. The conversion of a proton donor or nucleophile to a proton acceptor (such as the oxygen atoms of an ester) or electrophile (such as the carbonyl carbon of an ester) results in a marked enhancement of estrogenic activity.

In sharp contrast to the results obtained with esters at C-3 or C-17 (3), the data in Table 3 show that uterotropic potency decreases as the size of an 11β-ester group increases. In the estrone series, an 11β-ester is, on the average, 21 times as potent as the next higher homolog as one goes from formate to hexanoate. 11β-Formoxyestrone is 125,000 times as uterotropic as 11β-hexanoyloxyestrone. In the estradiol-17β series, there are also similar decreases in uterotropic potencies as one goes from formate to butyrate. However, in the case of 11β-hexanoyloxyestradiol-17β, its uterotropic potency could not be calculated by quantal means because the uterine weight did not double at the dose of 500 μg after reaching a plateau of 183% of the control uterine weight at the dose of 100 μg. On the other hand, the next higher homolog, 11β-heptanoyloxyestradiol-17β, doubled

the control uterine weight at the dose of 100 μg, giving the compound a potency of only 1/10,000 of that of 11β-formoxyestradiol-17β. The benzoyloxy group is nearly equivalent to a butyroxy group; apparently, it is mainly the size of the 11β-substituent that determines estrogenic potency. From the data of Table 3, it can be readily perceived that the acetoxy group represents a "cut off" limit in the size of the 11β-substituent that can be tolerated by the speculated "physiological estrogen receptor"; any substituent larger than an acetoxy group results in a sharp drop in estrogenic potency as measured by either the uterotropic or gonadotropic secretion inhibition assay.

As we have previously observed (8), uterotropic activities and inhibition of gonadotropin release do not parallel. The most potent inhibitor of gonadotropin release was 11β-acetoxyestradiol-17β which was 133% as active as estradiol-17β. It was, however, only 38% as active as estradiol-17β in the uterotropic assay, thus presenting the best "separation" between uterotropic and gonadtropin release inhibition activities of any highly potent estrogen we have assayed so far. It required only 3.8 times the total dose to double uterine weight to halve the ovarian weight in parabiotic rats.

The results may help one to speculate upon the physicochemical characteristics of whatever it is that is required to interact with an estrogen before typical biological activities may be elicited. There apparently is a proton donor or a nucleophilic group within the speculated "physiological estrogen receptor" in the vicinity of C-11 on the β side of the ligand. Examples of potential proton acceptors other than the 11β-esters that have resulted in potent estrogens are the 11β-methoxyl (13), 11β-nitro (14), and 11β-chloro-

methyl (15) groups. An 11-keto group may be considered as a proton acceptor also, but this modification enhances estrogenic activity only if a 9β configuration and a 17-keto group are also present (15). Finally, the size of the 11β-substituent cannot exceed that of an acetoxy group if considerable estrogenic potency is desired.

The results of esterifying 11β-hydroxyl groups of estrogens are in very sharp contrast to those of similar modifications of androgens and progestogens. The 11β-acyloxy (formoxy or acetoxy) group causes a marked decrease in activity for progesterone derivatives (11β,17-diacetoxyprogesterone had 1/5, and 11β,17-diformoxyprogesterone, 1/13, of the activity of progesterone as measured by the McPhail test in rabbits)(17). 11β-Hydroxytestosterone 11,17-diacetate was found to be essentially inactive in the levator ani and seminal vesicle assays in rats (18).

The effect of the esterification of 11β-hydroxylestrogens strangely resembles that of the esterification of the 16β-hydroxyl group of the cardiac-active aglycone gitoxigenin (3β,14,16β-trihydroxy-5β,14β-card-20(22)-enolide). In their investigations of 31 digitalis glycosides and aglycones, Henderson and Chen found that gitoxigenin was the least potent cardiotoxic agent of the series when tested on cats. Esterification of the 16β-hydroxyl group, however, resulted in a very remarkable increase in potency; indeed, gitoxigenin 16-formate surpassed all of the other 30 compounds in cardiotoxic activity and was 2½ times as active as digoxin. The order of activity of three esters at the C-16 position of gitoxigenin was found to be formate > acetate > propionate (19).

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