Preparation, Resolution, and Biological Evaluation of 5-Aryl-1,2-dihydro-5*H*-chromeno[3,4-*f*]quinolines: Potent, Orally Active, Nonsteroidal Progesterone Receptor Agonists

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Two potent nonsteroidal progestins from the 5-aryl-1,2-dihydro-5*H*-chromeno[3,4-*f*]quinoline class (LG120746 and LG120747) were selected for scale-up, resolution, and biological evaluation of the purified enantiomers. For each quinoline, the levorotatory enantiomer was determined to be the more potent agonist of the human progesterone receptor isoform B (hPR-B) (EC_{50} < 3 nM), but the dextrorotatory enantiomers retained significant PR modulatory activity (EC_{50} < 200 nM). In two in vivo rodent models of progestational activity, a pregnancy maintenance assay and a uterine wet weight assay, the two eutomers displayed potent progesterone-like effects. In a third model for progestational activity, the mammary end bud assay, these compounds were significantly less active. These studies demonstrate that certain members of this class of selective progesterone receptor modulators display encouraging and potentially useful tissue-selective progestational effects.

Introduction

The regulation of gene expression by small molecule modulators of intracellular receptors (IRs)[1] is an attractive opportunity for drug discovery.[2] For example, progesterone plays a central role in regulating female reproductive function via the human progesterone receptor (hPR).[3] Although a number of steroidal progestins are currently marketed (Figure 1), the search for novel agonists of hPR promises to yield important new drugs to address women's health issues, including hormone replacement therapy[4] and cancers of the female reproductive system.[5] In contrast to the human androgen (hAR) and estrogen (hER) receptors, nonsteroidal modulators of hPR have only recently been discovered. Nonsteroidal hPR modulators[6] are particularly interesting due to the possibility of developing drugs with less cross-reactivity than steroids to related IRs such as the human glucocorticoid (hGR), mineralocorticoid (hMR), and androgen (hAR) receptors, as well as molecules which exhibit novel pharmacology.[7]

We have recently described the discovery and preliminary structure−activity relationship studies of a class of potent nonsteroidal hPR modulators based on the 5-aryl-1,2-dihydro-5*H*-chromeno[3,4-*f*]quinoline pharmacophore (**1**, Figure 2).[8] Several members of this class of PR modulators have subnanomolar binding affinities to baculovirus-expressed hPR-A[9] and are as potent and more efficacious in a functional assay using recombinant hPR-B[10] than progesterone, the cognate hormone. These initial studies dealt primarily with racemic compounds,

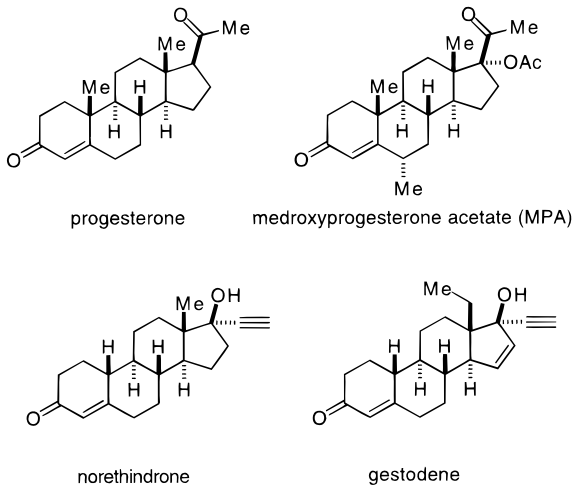

Figure 1. Selected currently marketed steroidal hPR agonists.

although preliminary work indicated that one enantiomer is significantly more active than the other.[8a]

To further study the progestational effects of this series of compounds, two C(9) fluoro derivatives (**2** and **3**, Figure 2) were selected for further biological profiling. Although both C(9) fluoro and C(9) chloro derivatives have potent in vitro and in vivo progestational activity,[8b] certain C(9) chloro compounds also display significant binding affinity for hGR (Table 1), and they were not pursued as hPR modulators. This report describes the scale-up, preparative HPLC separation of enantiomers, and in vitro studies on compounds **2** and **3**. In addition, the in vivo progestational effects of the eutomers (−)-**2** and (−)-**3** were characterized in three rodent models.

Chemistry

The preparation of multigram quantities of **2** and **3** required optimization of several steps of the previously

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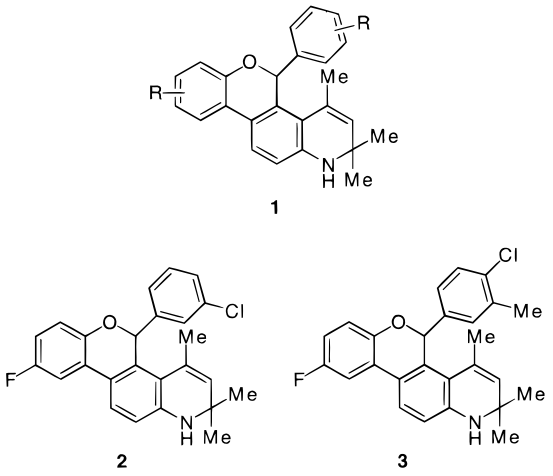
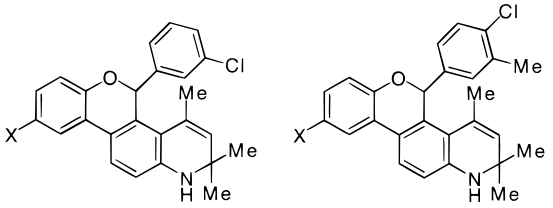


Figure 2. 5-Aryl-1,2-dihydro-5*H*-chromeno[3,4-*f*]quinoline general structure (**1**), LG120746 (**2**), and LG120747 (**3**).

Table 1. In Vitro hPR-B Activity in Cotransfected CV-1 Cells and Binding Affinities to Baculovirus-Expressed hPR-A and hGR[a]



2 (X = F)
4 (X = Cl)

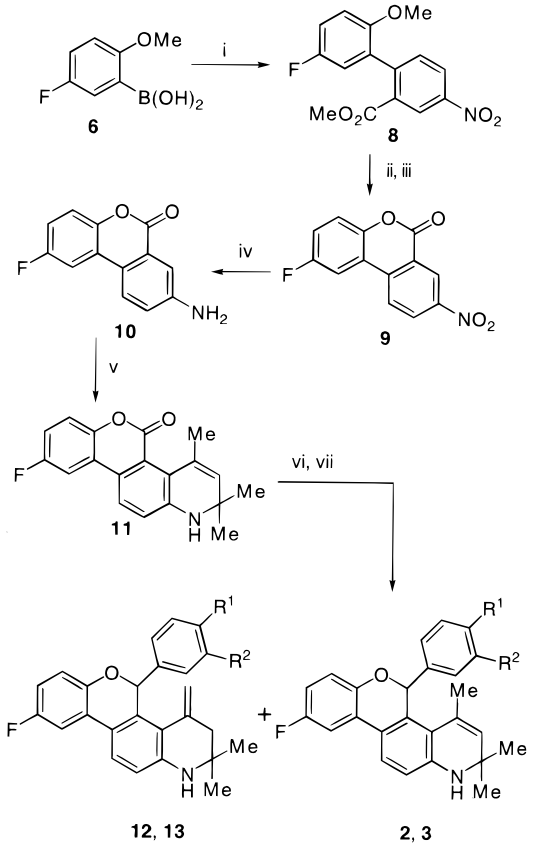
3 (X = F)
5 (X = Cl)

		hPR activity			hGR
ligand	X	EC_{50}[b]	efficacy[c]	K_i[b]	K_i[b]
(±)-**2**	F	2.8 ± 0.7	117 ± 7	0.32 ± 0.11	133
(±)-**4**	Cl	3.6 ± 0.7	95 ± 12	0.48 ± 0.18	6.3
(±)-**3**	F	2.7 ± 0.2	97 ± 6	0.49 ± 0.16	210
(±)-**5**	Cl	9.4 ± 2.6	73 ± 10	0.50 ± 0.15	14

[a] Cotransfection experiment values represent at least triplicate determinations. [b] Values are in nM, mean ± SEM, $N \geq 2$. EC_{50} values represent the concentration required to give half-maximal activation for that ligand. [c] Efficacy expressed as percent relative to progesterone = 100%.

described synthetic route (Scheme 1). Specifically, the palladium-catalyzed coupling reaction of boronic acid **6** with aryl bromide **7** was not scalable (10−15 g scale) due to hydrolysis of the methyl esters during the reaction. This was avoided by utilizing a fluoride-mediated Suzuki cross-coupling reaction,[11] affording the biaryl ester **8** in 75−85% yields on a 20 g scale. Conversion of **8** to the nitrobenzocoumarin **9** proceeded uneventfully using the previously described conditions.[8b] Palladium-catalyzed hydrogenation of the nitro group of **9** also proved problematic upon scale-up, and recourse was made to a zinc-mediated reduction.[12] Subjection of aniline **10** to typical Skraup reaction conditions, which requires high temperatures (120−130 °C) and a high proportion of iodine (30−40 wt %), afforded moderate yields (20−40%) of 1,2-dihydro-2,2,4-trimethylquinoline **11** and gave variable results on a multigram scale. One solution to this problem was simply to run the reaction at high dilution (<0.01 M), affording quinoline **11** in a reproducible 50−60% yield. The next step, generation of the aryllithium reagents, required strict control of reaction temperature (<−65 °C), both during

Scheme 1[a]



[a] (i) Methyl 2-bromo-5-nitrobenzoate (**7**), $(Ph_3P)_4Pd$, CsF, DME, 80 °C; (ii) 20% KOH, EtOH, THF, room temperature; (iii) $SOCl_2$, DCE, 80 °C, then $AlCl_3$, (iv) Zn, $CaCl_2{-}2H_2O$, EtOH, reflux; (v) acetone, I_2, 125−135 °C; (vi) ArLi, THF, −65 °C; (vii) $BF_3{-}OEt_2$, Et_3SiH, CH_2Cl_2, room temperature.

the lithium−halogen exchange process and during the addition of quinoline **11**.[13] Allowing the reaction temperature to rise above approximately −60 °C resulted in significant decomposition of both the aryllithium reagent and the substrate, presumably due to ortholithiation processes. The reduction of the intermediate lactols occurred uneventfully; however, the acidic reaction medium had to be neutralized very carefully to avoid isomerization of the C(3)−C(4) olefin to the exo isomers (**12**, **13**). Using these minor precautions, quinolines **2** and **3** could be prepared reproducibly in 70−80% overall yield from **11** on a 5 g scale.

With a preparative method in hand for multigram quantities of racemic **2** and **3**, the HPLC resolution of the enantiomers was then explored. After a survey of a number of chiral stationary phases, it was found that the "Whelk-O" column developed by Pirkle and coworkers[14] cleanly separated the enantiomers of both **2** and **3**. Using a 10 cm (*R*,*R*)-Whelk-O column, optimization of the mobile phase (heptane/CH_2Cl_2/Et_3N) afforded conditions suitable for separation of up to 400 mg of racemate per 40-min injection, and several grams of each enantiomer could be separated to a purity of greater than 97% ee in a day.

Whereas the free bases of (−)- and (+)-**2** could be recrystallized from hexane (ee >99.5%, mp 133−4 °C), (−)- and (+)-**3** were foams that resisted recrystallization. The low basicity of the quinoline nitrogen of **3** limited the number of potential salts that could be formed, but

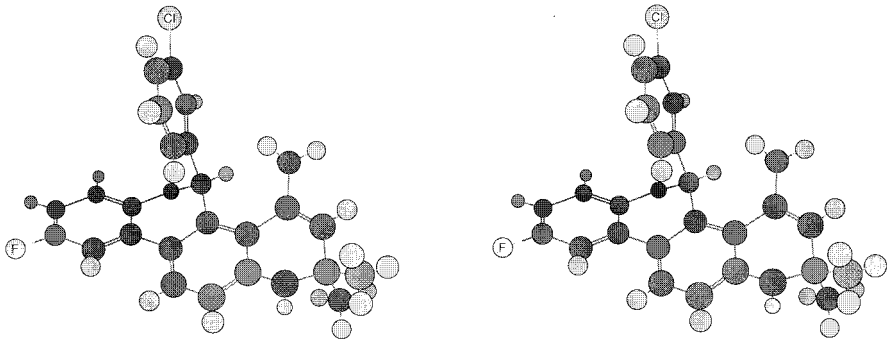


Figure 3. Chem3D representation of (−)-(*S*)-**2** based on X-ray crystallographic coordinates.

Table 2. In Vitro hPR-B Activity in Cotransfected CV-1 Cells and Binding Affinities to Baculovirus-Expressed hPR-A[a]

ligand	hPR activity		
	EC_{50}[b]	efficacy[c]	K_i[b]
progesterone	1.5 ± 0.5	100 ± 0	3.8 ± 0.2
(−)-**2**	1.3 ± 0.3	125 ± 8	0.1 ± 0.1
(+)-**2**	135 ± 28	83 ± 9	30
(−)-**3**-sulfate	2.4 ± 0.6	99 ± 8	0.4 ± 0.3
(+)-**3**-sulfate	147 ± 65	43 ± 5	60

[a] Cotransfection experiment values represent at least triplicate determinations. [b] Values are in nM, mean ± SEM, $N \geq 2$. If no SEM is noted, value is from a single determination. EC_{50} values represent the concentration required to give half-maximal activation for that ligand. [c] Efficacy expressed as percent relative to progesterone = 100%.

suitable crystals of the sulfate hydrate ("**3**-sulfate", >99.5% ee, mp ~230 °C dec) were eventually obtained. The absolute configuration of (−)-**2** was determined to be *S* by X-ray crystallography,[15] and the absolute configuration of (−)-**3** was assigned by analogy. A stereo 3D depiction of (−)-(*S*)-**2** is shown in Figure 3.

In Vitro Biological Activities

As expected from earlier work, the levorotatory enantiomers (−)-(*S*)-**2** and (−)-(*S*)-**3** were the more active enantiomers (Table 2); however, the distomers retained significant (130−150 nM) hPR agonist activity. Indeed, the hPR-A binding affinities of 30 and 60 nM for the "distomers" (+)-(*R*)-**2** and (+)-(*R*)-**3**, respectively, ranked among the most potent reported for nonsteroidal hPR modulators from other compound classes.[6] The binding affinities of 0.11 and 0.36 nM for (−)-(*S*)-**2** and (−)-(*S*)-**3** were among the most potent for any hPR modulator reported to date, exceeding by 1 order of magnitude the K_i of progesterone (3.8 nM) and comparable to that of medroxyprogesterone acetate (0.3 nM) in this assay. In the cotransfection assay, (−)-(*S*)-**2** and (−)-(*S*)-**3** were virtually indistinguishable from progesterone (Figure 4).

In Vivo Biological Activities

To characterize the progestational effects of (−)-(*S*)-**2** and (−)-(*S*)-**3** in vivo, three assays targeting different endpoints were examined. In the uterine wet weight assay, progestins blunt the effects of estrogens on the female uterus.[16] The breast bud assay has been used to characterize the effects of antiprogestins on the differentiation of the mammary gland in estrogen-primed ovariectomized animals[17] and was modified to test for progestational effects in this study.[18] Finally, the ability of these nonsteroidal progestins to maintain

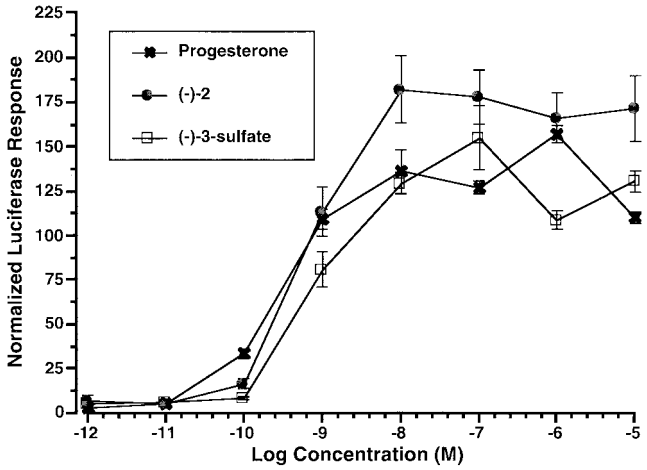


Figure 4. Concentration−response curves for progesterone, (−)-(*S*)-**2**, and (−)-(*S*)-**3**-sulfate. See Experimental Section for details.

pregnancy during simultaneous treatment with the potent steroidal antiprogestin mifepristone (RU486) was also tested.

Inhibition of Uterine Wet Weight. Inhibition of estrogen-induced uterine wet weight in the immature rat was evaluated as a classical endpoint for progestin response. Both enantiomers of **2** were tested in this model (Figure 5, upper panel) in addition to the eutomer of **3** (Figure 5, lower panel). In agreement with the in vitro cotransfection and binding data described above,[19] (+)-(*R*)-**2** was inactive in inhibiting uterine wet weight gains in estradiol-treated animals (Figure 5, upper panel). Conversely, (−)-(*S*)-**2** was as effective as medroxyprogesterone acetate (MPA), a standard marketed progestin, in this assay. Both (−)-(*S*)-**2** and MPA significantly decreased ($p < 0.01$) mean uterine wet weights at doses greater than or equal to 1 mg/kg with a maximum mean efficacy of approximately 35% compared to estrogen-treated animals. (−)-(*S*)-**3**-sulfate was also tested in this assay (Figure 5, lower panel) and was found to be equipotent to MPA throughout the entire dose range evaluated. Both MPA and (−)-(*S*)-**3**-sulfate significantly ($p < 0.01$) decreased estrogen-induced uterine wet weight gain at doses greater than or equal to 0.3 mg/kg with a maximum mean efficacy similar to (−)-(*S*)-**2** and MPA in the previous experiment.

Mammary Lobular-Aveolar Bud Proliferation Assay. Induction of differentiation and proliferation of lobular-aveolar buds in the rat mammary gland has been demonstrated morphologically and proliferatively as a hallmark of progestational action. In ovariecto-

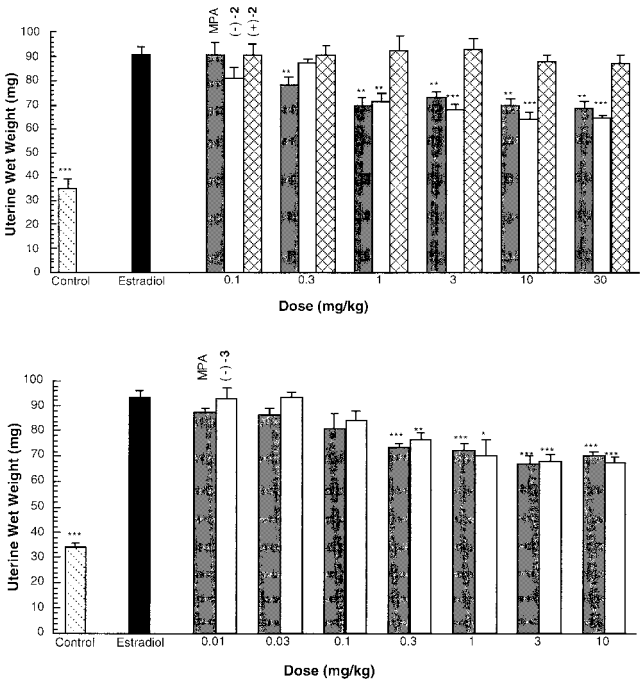


Figure 5. Comparison of inhibition of estradiol-induced uterine wet weight increases by MPA, (+)-(*R*)-**2**, (−)-(*S*)-**2**, and (−)-(*S*)-**3**-sulfate. Bars are designated as follows. Upper panel: control/vehicle (striped bar); estradiol (black bar); MPA + estradiol (gray bar); (−)-(*S*)-**2** + estradiol (dotted bar); (+)-(*R*)-**2** + estradiol (hatched bar). Lower panel: control/vehicle (striped bar); estradiol (black bar); MPA + estradiol (gray bar); (−)-(*S*)-**3**-sulfate + estradiol (dotted bar). Statistically significant (ANOVA) Points are designated: *** $p < 0.001$; ** $P < 0.01$; * $p < 0.05$ vs estradiol-alone treated animals (black bar). See Experimental Section for details.

Table 3. Induction of BrdU Labeling in Lobular-Aveolar Rat Mammary Buds by Progestin Treatment[a]

ligand	fold induction
estrone	1
MPA (3.0 mg/kg)	24
(−)-**2** (3.0 mg/kg)	3.6
(−)-**3**-sulfate (3.0 mg/kg)	2.5

[a] Fold induction by progestin treatment is calculated as the fold increase of the BrdU labeling index over estrone treatment alone. Index is measured from the average of 12 microscopic fields of each inguinal mammary fat pad of treated animals (4 rats/group).

mized rats primed with estrone, treatment with progestins such as MPA can induce a dose dependent increase in mitotic proliferation (as measured by BrdU incorporation) of lobular-aveolar buds.[16] To compare the progestational activity of (−)-(*S*)-**2** and (−)-(*S*)-**3**-sulfate to MPA, these compounds were evaluated in this assay. As described above, (−)-(*S*)-**2**, (−)-(*S*)-**3**-sulfate, and MPA are fully efficacious at a dose of 3 mg/kg in the uterine wet weight assay. As expected, at this dose MPA caused a maximum increase of over 20-fold induction of BrdU labeling in lobular-aveolar buds of the mammary gland over estrone-alone treated animals (Table 3). However, (−)-(*S*)-**2** and (−)-(*S*)-**3**-sulfate induced BrdU labeling only 3.6- and 2.5-fold, respectively, over estrone-alone treated animals. These data suggest a tissue-selective action for the nonsteroidal progestins, which display at least 6-fold less efficacy than MPA at an equivalent high dose.

Antifertility Assay. Implantation in mice occurs around day 4 of pregnancy and is accompanied by a steep increase in circulating progesterone.[20] This extremely sensitive window of progesterone dependency can be manipulated with antiprogestin treatment.[21] When given before the expected time of implantation, a progesterone receptor antagonist, such as mifepristone (RU486),[22] will specifically block implantation and hence the establishment of pregnancy.[23] These well-known antifertility properties of mifepristone can be reversed by high-dose progestin treatment, and the effects of (−)-(*S*)-**2** and (−)-(*S*)-**3**-sulfate were examined using 10−12-week-old pregnant, mature ICR mice. These data are shown in Table 4. Oral administration of RU486 (0.5 mg or ∼15 mg/kg) between days 2 and 4 post coitum reduced the pregnancy rate from a control level of 88.2% to 18.8% (∼80% inhibition; $P < 0.001$), and this dose was chosen for further studies. The observed antifertility effect of RU486 was partially reversed by coadministration of MPA but only with a much higher daily dose (10 mg or ∼300 mg/kg). Of the two test compounds, (−)-(*S*)-**2** completely restored pregnancy in RU486-treated females at doses between 1.0 and 3.0 mg (∼30−100 mg/kg; $P < 0.001$) per day, whereas (−)-(*S*)-**3**-sulfate produced a partial reversal in the same dose range (Table 4), similar to the effects observed with MPA but at lower doses.

Discussion

The relative in vitro activity of the enantiomers of **2** and **3** demonstrated that the levorotatory isomers were 100 times more potent progestins than the dextrorota-

Table 4. Effects of Daily Doses of MPA, (−)-(*S*)-**2**, and (−)-(*S*)-**3**-sulfate on the Outcome of Pregnancy in Mice Simultaneously Treated with RU486 (0.5 mg)[a]

treatment	dose (mg/mouse)	mice	CL	implants	no. pregnant/ no. mated	% pregnant
control	0.1 mL	17	14.2 ± 1.0	13.9 ± 1.0*	15/17	88.2
RU486	0.5 mg	16	13.4 ± 0.9	3.3 ± 1.6	3/16	18.8
MPA + RU486	30	10	10.7 ± 1.7	6.7 ± 2.1**	5/10	50.0
	10	10	12.3 ± 1.2	1.4 ± 1.3***	1/10	10.0
	3	12	13.6 ± 0.8	2.6 ± 1.7***	2/12	16.7
	1	10	11.7 ± 1.2	1.2 ± 1.1***	1/10	10.0
(−)-**2** + RU486	3	9	14.1 ± 0.4	13.7 ± 0.4*	9/9	100
	1	11	14.1 ± 0.6	12.0 ± 1.6*	10/11	91
(−)-**3** + RU486	3	10	13.8 ± 0.4	6.6 ± 2.2**	5/10	50
	1	10	13.0 ± 0.8	2.2 ± 1.3***	3/10	30

[a] Pregnant ICR mice were treated orally with RU486 (0.5 mg/animal) or RU486 (0.5 mg/animal) plus different doses of the test compounds between day 2 and day 4 after mating. Control animals received similar doses of MPA (positive control) or an equivalent volume of corn oil (negative control). Necropsies were performed on day 8 post coitum to determine the outcome of the pregnancy. *$P < 0.001$ compared to RU486-treated group; **$P < 0.005$ and ***$P < 0.001$ compared to oil-treated group (Student *t*-test). CL = corpora lutea.

tory isomers. These in vitro progestational effects were comfirmed in two animal models, while in a third, the mammary bud assay, the nonsteroidal compounds were much less active. Both (−)-(*S*)-**2** and (−)-(*S*)-**3**-sulfate had progestational activity in the uterine wet weight assay comparable to MPA, the most widely prescribed marketed progestin. In the breast, however, both of the nonsteroidal compounds were at least 6-fold less efficacious compared to MPA at high doses, indicating a potentially useful tissue-selective effect.

In the pregnancy maintenance assay, (−)-(*S*)-**2** was able to completely reverse the antifertility effect of the potent steroidal antiprogestin mifepristone (RU486) at a daily oral dose equal to or above 1.0 mg (30 mg/kg); (−)-(*S*)-**3**-sulfate was less efficacious and only produced a partial reversal in this study. Although MPA showed an effect similar to (−)-(*S*)-**3**-sulfate, this steroid required a dose 10 times higher than the nonsteroidal compound. While both test compounds are more potent than MPA, (−)-(*S*)-**2** is both more potent and more efficacious. These results decisively demonstrate that the in vivo activities of the two nonsteroidal progesterone receptor agonists {(−)-(*S*)-**2** and (−)-(*S*)-**3**-sulfate} are progestational in nature and can specifically mimic the action of the natural hormone in regulating pregnancy. These are the first nonsteroidal compounds reported to demonstrate this activity.

The levorotatory *S* enantiomers of the nonsteroidal progestins LG120746 (**2**) and LG120747 (**3**) have potent hPR activity in vitro that translates well to the in vivo situation. As would be anticipated from their nonsteroidal structure, these compounds display novel pharmacology in that the potent progestational effects observed in the rodent uterus are not observed in the breast. Moreover, the biological activity of these nonsteroidal selective progesterone receptor modulators was demonstrated in a mouse pregnancy maintenance assay, a definitive assay for in vivo progestational effects.

Experimental Section[24]

Methyl (5′-Fluoro-2′-methoxy-4-nitro-2-biphenyl)carboxylate (8). In a 500-mL flask, a solution of methyl 2-bromo-5-nitrobenzoate (11.6 g, 44.6 mmol) in DME (60 mL) was treated with tetrakis(triphenylphosphine)palladium (1.54 g, 1.33 mmol, 3.0 mol %). The reaction mixture was stirred at room temperature for 10 min. Cesium fluoride (21.4 g, 141 mmol, 3.10 equiv) was added, followed by a solution of 5-fluoro-2-methoxyphenylboronic acid[8b] (12 g, 71 mmol, 1.5 equiv) in DME (40 mL). The reaction mixture was heated to 80 °C for 6 h, cooled to room temperature, poured into 2.0 M Na_2CO_3 (200 mL), and extracted with EtOAc (3 × 200 mL). The extracts were washed with brine (1 × 100 mL), combined, dried ($MgSO_4$), filtered, and concentrated to an orange oil. Purification by silica gel chromatography (hexane/EtOAc, 9:1) afforded 10.4 g (76%) of **8** as a yellow-orange solid, identical to samples prepared previously.[8b]

8-Amino-2-fluoro-6*H*-dibenzo[*b*,*d*]pyran-6-one (10). In a 500-mL round bottom flask, a slurry of 2-fluoro-8-nitro-6*H*-dibenzo[*b*,*d*]pyran-6-one[8b] (4.5 g, 17.4 mmol) in 95% EtOH/water (250 mL) was treated with zinc dust (5.1 g, 78.3 mmol, 4.5 equiv) and $CaCl_2 \cdot 2H_2O$ (5.1 g, 34.8 mmol, 2 equiv). The reaction mixture was heated to reflux for 15 h, filtered hot through a bed of Celite, and concentrated to half volume. The solution was diluted with EtOAc (400 mL) and washed with 2% HCl (2 × 100 mL) and brine (1 × 100 mL). The aqueous layers were extracted with EtOAc (2 × 250 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated to afford 3.70 g (93%) of the aniline **10** as a yellow solid, identical to samples prepared previously.[8b]

9-Fluoro-1,2-dihydro-2,2,4-trimethyl-5-coumarino[3,4-*f*]quinoline (11). In a 600-mL resealable pressure tube, a suspension of **10** (0.92 g) in acetone (400 mL) was treated with iodine (0.40 g) and heated to 120−130 °C for 36 h. The reaction mixture was cooled to room temperature, concentrated to remove the bulk of the acetone, and dissolved in CH_2Cl_2 (200 mL). The organic layer was washed with 0.5 N $Na_2S_2O_3$ (2 × 200 mL) and saturated $NaHCO_3$ (1 × 100 mL). The aqueous layers were extracted with CH_2Cl_2 (2 × 100 mL). The combined organic layers were dried (K_2CO_3), filtered, and concentrated to afford an orange solid. Purification by silica gel chromatography (hexane/EtOAc, 8:1 to 2:1 gradient) afforded 0.70 g (56%) of **11** as a bright yellow solid, identical to samples prepared previously.[8b]

(±)-5-(3-Chlorophenyl)-9-fluoro-1,2-dihydro-2,2,4-trimethyl-5*H*-chromeno[3,4-*f*]quinoline (2). In a 500-mL three-neck flask, a solution of 3-bromochlorobenzene (7.0 mL, 60 mmol, 7.0 equiv) in THF (75 mL) was cooled to −69 °C (internal temp). A 2.5 M solution of *n*-BuLi in hexanes (24 mL, 60 mmol, 7.0 equiv) was added via syringe pump over a 45-min period (internal temperature ≤−68 °C) and the reaction mixture maintained at ≤−68 °C for 30 min. A solution of **11** (2.64 g, 8.53 mmol) in THF (30 mL) was added via syringe pump over a 45-min period (internal temperature <−64 °C) and the reaction mixture maintained at ≤−65 °C for 30 min. A 1:1 solution of THF/H_2O (40 mL) was added slowly via syringe, and the reaction mixture was allowed to warm to room temperature and poured into saturated NH_4Cl (100 mL). The product was extracted with EtOAc (3 × 100 mL); the extracts were washed with brine (1 × 100 mL), combined, dried ($MgSO_4$), filtered, and concentrated to afford an orange oil. Purification by silica gel chromatography (hexanes/EtOAc, 2:1) afforded 3.42 g (95%) of the hemiacetal as an orange oil, which was not characterized.[8]

In a 200-mL round bottom flask, the hemiacetal was dissolved in CH_2Cl_2 (50 mL) and treated with Et_3SiH (8.1 mL, 51 mmol, 6.0 equiv) and $BF_3{-}OEt_2$ (6.45 mL, 51 mmol, 6.0 equiv). The reaction mixture turned deep green and was stirred at room temperature for 4 h. The reaction mixture was quenched by addition of saturated $NaHCO_3$ (50 mL) added all at once; the resulting biphasic mixture was stirred vigorously for 15 min, diluted with H_2O (50 mL), and extracted with EtOAc (2 × 100 mL). The extracts were washed with brine (1 × 100 mL), combined, dried (Na_2SO_4), filtered, and concentrated. Purification by silica gel chromatography (hexane/EtOAc, 10:1 to 6:1 gradient) afforded 2.78 g (84%, 80% for two steps) of (±)-**2** as a white solid. The analytical data matched that of samples prepared previously.[8b]

(±)-5-(4-Chloro-3-methylphenyl)-9-fluoro-1,2-dihydro-2,2,4-trimethyl-5*H*-chromeno[3,4-*f*]quinoline (3). In a 500-mL three-neck flask, a solution of 5-bromo-2-chlorotoluene (12 g, 58 mmol, 6.0 equiv) in THF (100 mL) was cooled to −69 °C (internal temperature). A 2.5 M solution of *n*-BuLi in hexanes (23 mL, 58 mmol, 6.0 equiv) was added via syringe pump over a 45-min period (internal temperature ≤−65 °C) and the reaction mixture maintained at ≤−65 °C for 30 min. A solution of **11** (3.0 g, 9.7 mmol) in THF (50 mL) was added via syringe pump over a 45-min period (internal temperature <−68 °C) and the reaction mixture maintained at ≤−65 °C for 30 min. A 1:1 solution of THF/H_2O (40 mL) was added slowly via syringe, and the reaction mixture was allowed to warm to room temperature and poured into saturated NH_4Cl (100 mL). The product was extracted with EtOAc (3 × 100 mL); the extracts were washed with brine (1 × 100 mL), combined, dried ($MgSO_4$), filtered, and concentrated to afford an orange solid. The solid was dissolved in EtOAc (50 mL) and triturated with hexanes (300 mL) to afford 3.7 g (88%) of the hemiacetal as an orange solid, which was not characterized.[8]

In a 200-mL round bottom flask, the hemiacetal was dissolved in CH_2Cl_2 (50 mL) and treated with Et_3SiH (8.1 mL, 51 mmol, 6.0 equiv) and $BF_3{-}OEt_2$ (6.45 mL, 51 mmol, 6.0 equiv). The reaction mixture turned deep green and was stirred at room temperature for 12 h. The reaction mixture

was quenched by addition of saturated $NaHCO_3$ (50 mL) added all at once; the resulting biphasic mixture was stirred vigorously for 15 min, diluted with H_2O (50 mL), and extracted with EtOAc (2 × 100 mL). The extracts were washed with brine (1 × 100 mL), combined, dried (Na_2SO_4), filtered, and concentrated. Purification by silica gel chromatography (hexane/EtOAc, 10:1 to 6:1 gradient) afforded 3.1 g (87%, 76% for two steps) of (±)-**3** as a white foam. The analytical data matched that of samples prepared previously.[8b]

HPLC Separation of Enantiomers of 2 and 3. An (*R*,*R*)-Whelk-O1 preparative column (10 μm particle size, 21.1 × 250 mm; Regis Technologies, Inc.) on a Waters Delta Prep 4000 was equilibrated with an eluent of 3% Et_3N in heptane/CH_2Cl_2 (90:10) at a flow rate of 15 mL/min. A 1 g/10 mL solution of **2** in heptane/CH_2Cl_2 (70:30) was prepared, and 4.0 mL of this solution was injected per run. Compound elution was monitored by absorbance detection at 275 nm. The retention time of the first peak was approximately 18 min. The first-eluting enantiomer (levorotatory) was collected until the absorbance began to decline on the LCD at which point the mixed fractions were collected separately. Once the absorbance had returned to a fixed height, the second (dextrorotatory) enantiomer was collected and the flow rate was increased to 40 mL/min until all of the second enantiomer had been collected. The flow rate was lowered to 15 mL/min, and another injection was made. The purity of the combined fractions was 98% ee with 85−90% recovery. Mixed fractions were combined and resubjected to the HPLC conditions. The enantiomers of **3** were resolved in an identical manner to that described above for **2**; 98% ee, 80−85% recovery.

(−)-2: To a 250-mL round bottom flask was added 5.95 g of (−)-**2** prepared above and diethyl ether (30 mL). Mild warming resulted in complete dissolution. To this solution was added hexane (150 mL), and the flask was placed on a rotary evaporator and the ether was removed. During the evaporation crystallization began to occur and the flask was removed and cooled to −4 °C for approximately 3 h. The mother liquors were removed, and the pale yellow crystals were rinsed with cold hexane (3 × 75 mL). The crystals were then collected and dried in vacuo (1 Torr) for 20 h to afford 5.0 g of (−)-**2**. Data for (−)-**2**: mp 134−136 °C; HPLC (*R*,*R*)-Whelk-O1 (5 μm particle size, 4 × 250 mm, EM Separations); 1.0 mL/min (heptane/CH_2Cl_2, 80:20) t_R = 6.3, 99.5% ee; $[\alpha]^{22}_D$ = −372.9°. Anal. ($C_{25}H_{21}ClFNO$) C, H, N.

(+)-2: (+)-**2** was recrystallized in a manner identical to that described above for (−)-**2**: 5.91 g of HPLC-purified (+)-**2** afforded 4.75 g of recrystallized (+)-**2**. Data for (+)-**2**: mp 134−135 °C; HPLC (*R*,*R*)-Whelk-O1; 1.0 mL/min (heptane/CH_2Cl_2, 80:20) t_R = 7.7 min, 99.8% ee; $[\alpha]^{22}_D$ = +373.9°. Anal. ($C_{25}H_{21}ClFNO$) C, H, N.

(−)-3-Sulfate: The free base (−)-**3** (5.4 g, 13 mmol) was dissolved in 150 mL of anhydrous ether and was titrated with concentrated H_2SO_4 ether solution (1/10 volume) until no further precipitation was observed (8 mL of the solution was used, ∼14 mmol). The salt was filtered and washed with ether (2 × 30 mL). The solid salt was dissolved in methanol (30 mL) and was concentrated and dried to give 6.8 g of white foam (> 98% mass recovery, 96% ee). The white foam was dissolved in CH_2Cl_2 (260 mL) and diluted slowly with pentane (230 mL). The solution was left standing at room temperature overnight to afford a white crystalline solid. The solid was filtered, washed with 2:1 mixture of pentane and CH_2Cl_2 (3 × 15 mL), and dried (1 Torr) to afford 6.2 g of (−)-**3**-hydrogen sulfate monohydrate (93% recovery): (*R*,*R*)-Whelk-O1; 1.2 mL/min (heptane/CH_2Cl_2, 80:20) t_R = 4.9 min, 99% ee; $[\alpha]^{22}_D$ = −197°; mp >230 °C dec; ^{1}H NMR (400 MHz, CD_3OD) 7.98 (bs, 1H), 7.62 (bs, 1H), 7.55 (bs, 1H), 7.18 (d, *J* = 8.3, 1H), 7.07 (s, 1H), 6.97 (bs, 1H), 6.95−6.82 (m, 3H), 5.99 (bs, 1H), 2.21 (s, 3H), 2.08 (s, 3H), 1.51 (s, 3H), 1.45 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD) 138.4, 137.4, 136.0, 132.0, 130.1, 128.4, 126.0, 124.5, 120.8, 76.0, 51.7, 24.3, 23.1, 20.1. Anal. ($C_{26}H_{25}ClFNO_5S\cdot H_2O$) C, H, N.

(+)-3-Sulfate. The procedure as described above afforded 1.2 g of (+)-**3**-hydrogen sulfate monohydrate (81% recovery). The NMR data matched that of (−)-**3**-sulfate described above. (*R*,*R*)-Whelk-O1; 1.2 mL/min (heptane/CH_2Cl_2, 80:20) t_R = 6.3 min, 99% ee; $[\alpha]^{22}_D$ = +190°; mp >230 °C dec. Anal. ($C_{26}H_{25}ClFNO_5S-H_2O$) C, H, N.

In Vivo Assays. Uterine Wet Weight Assays. Immature 21-day-old female Harlan Sprague−Dawley rats (Harlan Sprague Dawley Inc., Indianapolis, IN) were housed and compounds were administered under similar conditions as described below for the mammary end bud assay. Briefly, on day 1 of the assay 17β-estradiol (Sigma; St. Louis, MO) dissolved in 100% ethanol and diluted in corn oil was administered sc in an injection volume of 0.1 mL. On the second day of the assay test compounds were dissolved in corn oil (Mazola) in a similar manner and administered by oral gavage in 0.5 mL of vehicle. 24 h after administration of test compounds animals were euthanized, and uteri were excised and blotted dry on filter paper before weighing.

Mammary Lobular-Aveolar Bud Proliferation Assay. Sprague−Dawley (4−5-week-old, ∼100 g) female rats (HSD, Indianapolis, IN.) were ovariectomized by the vendor (bilateral-dorsal surgery) and allowed to acclimate for at least one week. All animals were given food (Harlan Teklab LM485−7012, Indianapolis, IN) and acidified water ad libitum throughout the experiment. Test compounds or progestin standard (MPA, medroxy progesterone acetate; Sigma, St. Louis, MO) were dissolved in 10% EtOH and corn oil (Mazola) and kept at room temperature. Estrone (Sigma, St. Louis, MO) was dissolved in 100% EtOH and corn oil with the EtOH blown off under nitrogen. Animals were randomized into groups of four or five animals and treated for 3 consecutive days with estrone sc (10 μg/animal) and test compounds po in a 0.5 mL volume of vehicle. Estrone-only treated animals were given oral vehicle alone.

After 3 days of consecutive dosing (on the morning of the fourth day) animals were given BrdU (Sigma, St. Louis, MO) 200 mg/kg ip in a volume of 2 mL of phosphate-buffered saline (PBS). Three hours after BrdU administration animals were euthanized with carbon dioxide asphyxiation and necropsied for mammary glands. Right inguinal mammary glands were dissected, taking care to remove the majority of adjacent fat. Mammary gland was embedded in OCT freezing media and frozen sections were cut at 5 μM thickness. Samples were stored at −20 °C until final processing.

Slides were fixed for 10 min at 4 °C in a cold solution of acetone/methanol/formaldehyde (19:9:2), washed with PBS for 10 min at room temperature, and denatured in 0.1 N HCl for 1 h at 37 °C in a water bath under gentle shaking. Slides were neutralized in 0.1 M borate buffer, pH 8.5, at room temperature for 2 × 15 min under gentle agitation and washed in PBS for 10 min at room temperature. Samples were blocked by incubating with 3% bovine serum albumin (BSA) and 3% normal goat serum (NGS) in PBS for 30−60 min at room temperature. Incubation of samples with a primary antibody [anti BrdU mouse monoclonal IgG1; (Becton Dickinson) at a dilution of 1:50 with blocking solution] was completed at 1 h at room temperature. Samples were then washed with PBS for 2 × 5 min at room temperature and incubated with secondary antibody [goat anti-mouse (GAM) IgG conjugated with FITC molecules (Southern Biotechnology Associates, Inc.)], diluted to 1:100 with blocking solution for 1 h at room temperature. Slides were evaluated for FITC staining by capturing digitized images of mammary gland using a Lieca flouresence microscrope (model DMRB) and a Optronix CCD camera system (model VI-470, Goleta, CA). Digitized images were downloaded to a HP P166 computer with a Oculus TCX frame grabber (Coreco, Quebec, Canada). Images were quantified using image analysis software Optimas 6.0 Seattle, WA). Raw data was generated in a blinded fashion and restricted to areas containing lobular buds from each field (3 fields/slide). Labeling index for each field was generated as labeled area divided by lobular areas selected within the field and expressed as a fold increase over estrone alone treated animals.

Statistical Methods. Statistical significance was evaluated in all bioassays using ANOVA followed by one-tailed

Dunnet's *t* test using SuperANOVA (Abacus Concepts Inc, Berkely,CA).

Pregnancy Maintenance Assay. Mature virgin ICR mice (10−12-weeks-old; Harlan Sprague Dawley Inc., Indianapolis, IN) were housed in a light (14 h light:10 h darkness; lights off at 20.00 h) and temperature (22 °C) controlled room, and fed and watered ad libitum (Diet LM-485, Teklad, Madison, WI). Females were caged with fertile males of the same strain overnight, and examined the following morning for vaginal plugs (day 1 of pregnancy). Mating was assumed to have taken place at 02.00 h (time 0).[25] Pregnant mice were treated orally with 0.5 mg of RU486 (∼15 mg/kg) alone, or in combination with different doses of MPA, (−)-**2** or (−)-**3**-sulfate, between days 2 and 4 of pregnancy. Compounds were dissolved in 100% ethanol and diluted with corn oil to appropriate concentrations to give an injection volume of 0.1 mL. Control animals received an equivalent volume of corn oil. Autopsies were carried out at day 8 of pregnancy and the number of implantation sites recorded.

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