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Novel Locally Active Estrogens Accelerate Cutaneous Wound Healing. A Preliminary Study

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Abstract: New 17β-estradiol (E_2) derivatives **1**−**11** were synthesized from an estrone derivative by addition of organometallic reagents prepared from protected α,ω-alkynols and further elaboration of the addition products. The estrogenic activity of these novel compounds was determined using *in vitro* binding competition assay and transactivation analysis. Among the E_2 derivatives synthesized, compound **2** showed the highest transactivation potency and was therefore tested for its ability to modulate cutaneous wound healing *in vivo.* Compound **2**'s ability to accelerate wound healing in ovariectomized mice and decrease the production of inflammatory molecules was comparable to that of E_2. However, the activity of compound **2** was not superimposable to E_2 with regard to the cells involved in the wound repairing process. When locally administered, compound **2** did not show any systemic activity on ER. This class of compounds with clear beneficial effects on wound healing and suitable for topical administration may lead to the generation of innovative drugs for an area of unmet clinical need.

Keywords: Locally active estrogens; soft drugs; wound healing; estrogen receptor

Introduction

Estrogens regulate diverse aspects of human physiology including fertility control, bone homeostasis, and cardiovascular, brain and skin functions. Reduced estrogen synthesis following the menopause directly contributes to an increased incidence of many age-associated pathologies, such as arthritis, atherosclerosis and osteoporosis. The effects of estrogen are mediated by two cognate receptors, namely, estrogen receptor α (ERα) and estrogen receptor β (ERβ), which are differentially ex-

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pressed in most mammalian tissues.[1−3] ERs regulate target cell activities *via* a direct effect on target gene transcription or by interacting with other nuclear transcription factors or cytoplasmatic signaling molecules.[3] In addition, recent evidence points to the existence of membrane ERs coupled with the G-protein signaling apparatus.[4−6]

Estrogen receptors are expressed by both neutrophils and macrophages, with many reports demonstrating that E_2 can exert anti-inflammatory activities.[7−10] These findings may explain several of the beneficial effects of estrogens in pathologies associated with postmenopause including wound healing, as shown by a series of experiments in ovariectomized rodents or aged humans.[9,10] In wounds E_2 suppresses macrophage production of proinflammatory cytokines, including macrophage migration inhibitory factor (MIF) and tumor necrosis factor α (TNFα), dampens inflammation and enhances dermal matrix deposition.[11] E_2 prevents the neutrophil influx into wounds and the release of neutrophil-derived elastase, which degrades extracellular matrix proteins such as proteoglycans, collagen and fibronectin.[9] E_2 also enhances the production of basic fibroblast growth factor (bFGF) and transforming growth factor β1 (TGF-β1) in fibroblasts.[9,12] Ethynyl estradiol stimulates monocytes/macrophages to produce platelet derived growth factor-A, a potent mitogen for fibroblasts.[13] These E_2 effects may induce fibroblast proliferation, migration and production of extracellular matrix, leading to granulation tissue formation and wound contraction.[12] Moreover, E_2 reduces expression of metalloproteinase P-8 (MMP-8) and 13 (MMP-13) in ovariectomized rats, which inhibits collagenolysis and restores collagen content in the wound bed.[14] Furthermore, E_2 increases tissue inhibitor of metalloproteinase (TIMP) production in fibroblasts,[15,16] which maintains the collagen content as well.

In consideration of their wide range of physiological activities,[2−5] ERs represent a very important drug target. However their well-known proliferative action in reproductive tissues significantly limits their applications in therapy.

In order to exploit the beneficial effects of estrogens on wound healing, while avoiding their undesired side effects, we attempted to obtain locally active estrogens. Therapeutic agents whose biological actions are localized around the area of administration are termed "soft drugs".[17] Limited systemic

(1) Dahlman-Wright, K.; Cavailles, V.; Fuqua, S. A.; Jordan, V. C.; Katzenellenbogen, J. A.; Korach, K. S.; Maggi, A.; Muramatsu, M.; Parker, M. G.; Gustafsson, J. A. International Union of Pharmacology. LXIV. Estrogen Receptors. *Pharmacol. Rev.* **2006**, *58*, 773–781.

(2) Deroo, B. J.; Korach, K. S. Estrogen Receptors and Human Disease. *J. Clin. Invest.* **2006**, *116*, 561–570.

(3) Maggi, A.; Ciana, P.; Belcredito, S.; Vegeto, E. Estrogens in the Nervous System: Mechanisms and Nonreproductive Functions. *Annu. Rev. Physiol.* **2004**, *66*, 291–313.

(4) Moriarty, K.; Kim, K. H.; Bender, J. R. Minireview: Estrogen Receptor-mediated Rapid Signaling. *Endocrinology* **2006**, *147*, 5557–5563.

(5) Heldring, N.; Pike, A.; Andersson, S.; Matthews, J.; Cheng, G.; Hartman, J.; Tujague, M.; Strom, A.; Treuter, E.; Warner, M.; Gustafsson, J. A. Estrogen Receptors: How do They Signal and What Are Their Targets. *Physiol. Rev.* **2007**, *87*, 905–931.

(6) Prossnitz, E. R.; Arterburn, J. B.; Smith, H. O.; Oprea, T. I.; Sklar, L. A.; Hathaway, H. J. Estrogen Signaling through the Transmembrane G Protein-coupled Receptor GPR30. *Annu. Rev. Physiol.* **2008**, *70*, 165–190.

(7) Vegeto, E.; Pollio, G.; Pellicciari, C.; Maggi, A. Estrogen and Progesterone Induction of Survival of Monoblastoid Cells Undergoing TNF-alpha Induced Apoptosis. *FASEB J.* **1999**, *13*, 793–803.

(8) Vegeto, E.; Bonincontro, C.; Pollio, G.; Sala, A.; Viappiani, S.; Nardi, F.; Brusadelli, A.; Viviani, B.; Ciana, P.; Maggi, A. Estrogen Prevents the Lipopolysaccharide-induced Inflammatory Response in Microglia. *J. Neurosci.* **2001**, *21*, 1809–1818.

(9) Ashcroft, G. S.; Dodsworth, J.; van Boxtel, E.; Tarnuzzer, R. W.; Horan, M. A.; Schultz, G. S.; Ferguson, M. W. Estrogen Accelerates Cutaneous Wound Healing Associated with an Increase in TGF-β1 Levels. *Nat. Med.* **1997**, *3*, 1209–1215.

(10) Hardman, M. J.; Emmerson, E.; Campbell, L.; Ashcroft, G. S. Selective Estrogen Receptor Modulators Accelerate Cutaneous Wound Healing in Ovariectomized Female Mice. *Endocrinology* **2008**, *149*, 551–557.

(11) Ashcroft, G. S.; Mills, S. J.; Lei, K.; Gibbons, L.; Jeong, M.-J.; Taniguchi, M.; Burow, M.; Horan, M. A.; Wahl, S. M.; Nakayama, T. Estrogen Modulates Cutaneous Wound Healing by Downregulating Macrophage Migration Inhibitory Factor. *J. Clin. Invest.* **2003**, *111*, 1309–1318.

(12) Fujimoto, J.; Hori, M.; Ichigo, S.; Tamaya, T. Ovarian Steroids Regulate the Expression of Basic Fibroblast Growth Factor and Its mRNA in Fibroblasts Derived from Uterine Endometrium. *Ann. Clin. Biochem.* **1997**, *34*, 91–96.

(13) Shanker, G.; Sorci-Thomas, M.; Adams, M. R. Estrogen Modulates the Inducible Expression of Platelet-derived Growth Factor mRNA by Monocyte/Macrophages. *Life Sci.* **1995**, *56*, 499–507.

(14) Pirila, E.; Ramamurthy, N.; Maisi, P.; McClain, S.; Kucine, A.; Wahlgren, J.; Golub, L. M.; Salo, T.; Sorsa, T. Wound Healing in Ovariectomized Rats: Effects of Chemically Modified Tetracycline (CMT-8) and Estrogen on Matrix Metalloproteinases−8,−13 and Type I Collagen Expression. *Curr. Med. Chem.* **2001**, *8*, 281–294.

(15) Sato, T.; Ito, A.; Mori, Y.; Yamashita, K.; Hayakawa, T.; Nagase, H. Hormonal Regulation of Collagenolysis in Uterine Cervical Fibroblasts. Modulation of Synthesis of Procollagenase, Prostromelysin and Tissue Inhibitor of Metalloproteinases (TIMP) by Progesterone and Oestradiol-17 beta. *Biochem. J.* **1991**, *275*, 645–650.

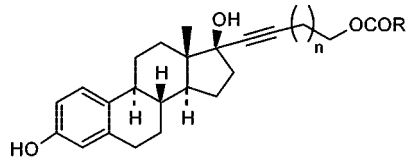
(16) Pirila, E.; Parikka, M.; Ramamurthy, N. S.; Maisi, P.; McClain, S.; Kucine, A.; Tervahartiala, T.; Prikk, K.; Golub, L. M.; Salo, T.; Sorsa, T. Chemically Modified Tetracycline (CMT-8) and Estrogen Promote Wound Healing in Ovariectomized Rats: Effects on Matrix Metalloproteinase-2, Membrane Type 1 Matrix Metalloproteinase, and Laminin-5 gamma2-Chain. *Wound Repair Regen.* **2002**, *10*, 38–51.

(17) Bodor, N. Designing Safer Drugs Based on the Soft Drug Approach. *Trends Pharmacol. Sci.* **1982**, *3*, 53–56.

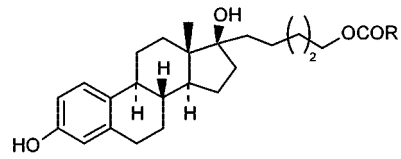
(18) Graffner-Nordberg, M.; Sjodin, K.; Tunek, A.; Hallberg, A. Synthesis and Enzymatic Hydrolysis of Esters, Constituting Simple Models of Soft Drugs. *Chem. Pharm. Bull. (Tokyo)* **1998**, *46*, 591–601.

(19) Laurent, H.; Gerhards, E.; Wiechert, R. New Biologically Active Pregnan-21-oic Acid Esters. *J. Steroid Biochem.* **1975**, *6*, 185–192.

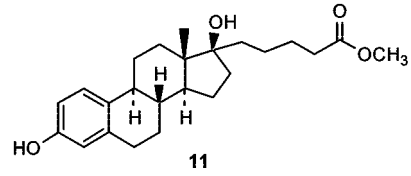
Chart 1. 17α-Estradiol Derivatives **1**−**11** Structure Formulas



1 n = 1 R = CH_3
2 n = 2 R = CH_3
3 n = 2 R = CH_2CH_3
4 n = 2 R = $CH_2CH_2CH_3$
5 n = 2 R = $CH_2CH_2CH_2CH_3$
6 n = 3 R = CH_3
7 n = 5 R = CH_3
8 n = 7 R = CH_3



9 R = CH_3
10 R = CH_2CH_3



11

activity is achieved by rapid catabolism into inactive products in tissues and blood. Most "soft drugs" are characterized by the presence of an ester group that can be hydrolyzed to an inactive or rapidly inactivatable compound.[18−20]

Accordingly it was found that the methyl esters of carboxylic acid analogues of E_2 at C-7 and C-17 have high affinity for the ER whereas the corresponding carboxylic acids are very poor ligands or do not bind at all.[21] From these results it appears that a charged carboxylic acid group in proximity to the steroid system interferes with binding to the ER while masking the charge by esterification nulls this interference. Another study describes the synthesis and biochemical evaluation of estradiol-16α-carboxylic acid esters as locally active estrogens that showed divergence between systemic and local estrogenic action.[22] Besides a comparative analysis of 17α-substituted estradiol derivatives, relative binding affinities showed that any compound with a polar oxygen group in that region has a markedly lower binding affinity.[23]

Finally we have recently reported marked acceleration of healing by the classical SERMS tamoxifen and raloxifene.[10]

On the basis of the above-reported studies, we decided to prepare a series of novel C-17 estradiol derivatives and test their efficacy as locally active estrogens. Thus, starting from estrone, we synthesized 17α-estradiol derivatives **1**−**11** (Chart 1) and tested their ER binding and transactivation activity. In addition, compound **2**, because of its high transactivation activity, was submitted to pharmacological evaluation in our well-characterized murine cutaneous wound healing model[11] where it displayed anti-inflammatory activity and beneficial effects on healing.

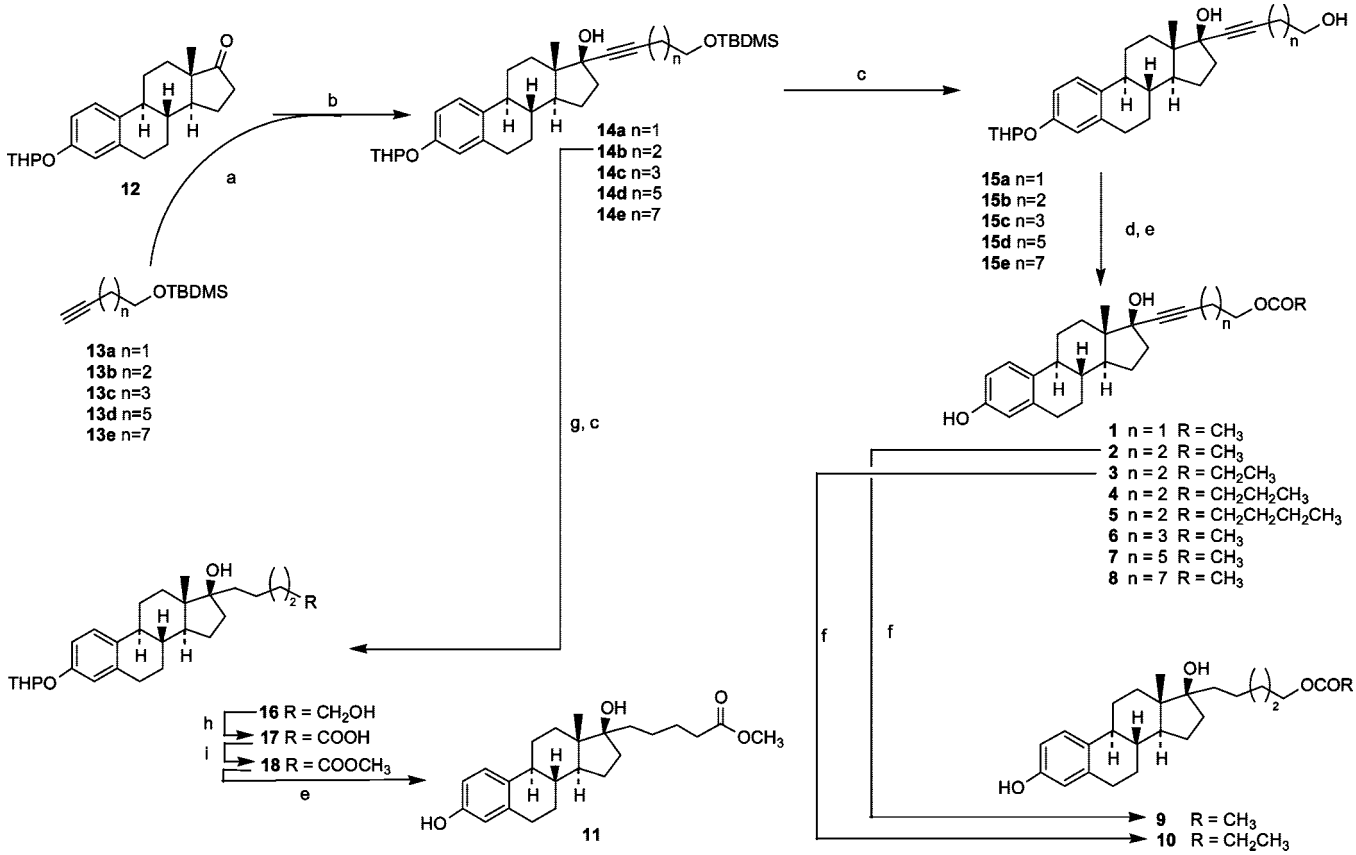
The 17α-position of E_2 was chosen for substitution due to chemical accessibility and on the basis of previous reports demonstrating that substitution at this position does not significantly interfere with the ligand−receptor binding reaction.[23,24]

Results and Discussion

Synthesis of Compounds 1−11. The starting material for the synthesis of **1**−**11** was the known estrone derivative **12**, an epimeric mixture at C(2′), owing to the presence of the THP group on the phenol function. The first step in the synthesis of compounds **1**−**11** was the nucleophilic addition of **12** to the organometallic reagents, prepared from the corresponding known TBDMS protected α,ω-alkynols **13** by the action of *n*-BuLi in THF at −78 °C, to give **14** (Scheme 1). On the basis of previous reports,[25−27] the attack of the organometallic reagents was hypothesized to occur from the α face.[28,29] TBAF deprotection of the side chain hydroxyl group of **14** gave **15**[30] in good yield. Compound **15** was then converted into compounds **1**−**8** by esterification

(20) Lee, H. J.; Soliman, M. R. I. Antiinflammatory Steroids Without Pituitary Adrenal Suppression. *Science* **1982**, *215*, 989–991.
(21) Bucourt, R.; Vignau, Mlli, V.; Richard-Foy, H.; Geynet, C.; Secco-Millet, C.; Redeuilh, G.; Baulieu, E. New Biospecific Adsorbents for the Purification of Estradiol Receptor. *J. Biol. Chem.* **1978**, *253*, 8221–8228.
(22) Labaree, D. C.; Reynolds, T. Y.; Hochberg, R. B. Estradiol-16α-carboxylic Acid Esters as Locally Active Estrogens. *J. Med. Chem.* **2001**, *44*, 1802–1814.
(23) Gao, H.; Katzenellenbogen, J. A.; Garg, R.; Hansch, C. Comparative QSAR Analysis of Estrogen Receptor Ligands. *Chem. Rev.* **1999**, *99*, 723–744.
(24) Kappus, H.; Bolt, H. M.; Remmer, H. Affinity of Ethynyl-estradiol and Mestranol for the Uterine Estrogen Receptor and for the Microsomal Mixed Function Oxidase of the Liver. *J. Steroid Biochem.* **1973**, *4*, 121–128.
(25) Buzby, G. C., Jr.; Smith, H. Ethynylating 17-oxo Steroidal Phenols. U.S. (1972), 5 pp. CODEN: USXXAM US 3646076 19720229. Application: US 70-42880 19700602.
(26) Wust, F.; Spies, H.; Johannsen, B. Synthesis of 17α-Substituted Mercaptoalkynyl Derivatives of 3,17α-Estradiol. *Tetrahedron Lett.* **1997**, *38*, 2931–2932.
(27) Foy, N.; Stéphan, E.; Vessières, A.; Salomon, E.; Heldt, J.-M.; Huché, M.; Jaouen, G. Synthesis, Receptor Binding, Molecular Modeling, and Proliferative Assays of a Series of 17α-Arylestradiols. *ChemBioChem* **2003**, *4*, 494–503.
(28) Miller, T. C.; Christiansen, R. G. Dependence of the Rate, Reversibility, and Stereoselectivity of 17-Ketosteroid Alkynylation on the Alkyne and on the Alkali Metal. *J. Org. Chem.* **1967**, *32*, 2781–2786.

Scheme 1[a]



[a] (a) *n*-BuLi, −78 °C, THF; (b) −78 °C, THF; (c) TBAF, THF, rt; (d) $(RCO)_2O$, py, rt; (e) TsOH, H_2O, MeOH, rt; (f) H_2, 10% Pd(C), EtOH, rt; (g) H_2, 10% Pd(C), dioxane; (h) PDC, DMF, rt; (i) CH_2N_2, $CHCl_3$, rt.

of the primary hydroxylic function with the proper anhydride in pyridine and deprotection of the phenol function with TsOH in aqueous methanol.

We then turned to the preparation of the other estradiol derivatives. Thus, by Pd(C) catalytic hydrogenation **2** and **3** were transformed into the saturated derivatives **9** and **10**.

The preparation of compound **11** was achieved by submitting **14b**[30] to Pd(C) catalytic hydrogenation, removing the side chain protecting group with TBAF (to give **16**[30]), oxidizing with PDC in DMF (to give the carboxylic acid **17**[30]), conversion with CH_2N_2 into the corresponding methyl ester (to give **18**[30]), and removal of the THP protecting group in **18** with TsOH in aqueous methanol (to give **11**).

Assessment of the Activity of the New Chemical Entities on Estrogen Receptors. To evaluate the profile of estrogenicity of the above compounds, we tested their ability to bind the estrogen receptors and to induce transactivation of a reporter gene driven by an estrogen-regulated promoter.

(a) *In Vitro* ER Binding Analysis. Binding affinity was measured experimentally in whole cell binding experiments carried out on the ER positive MCF-7 breast cancer cell line where ERα is preponderant.

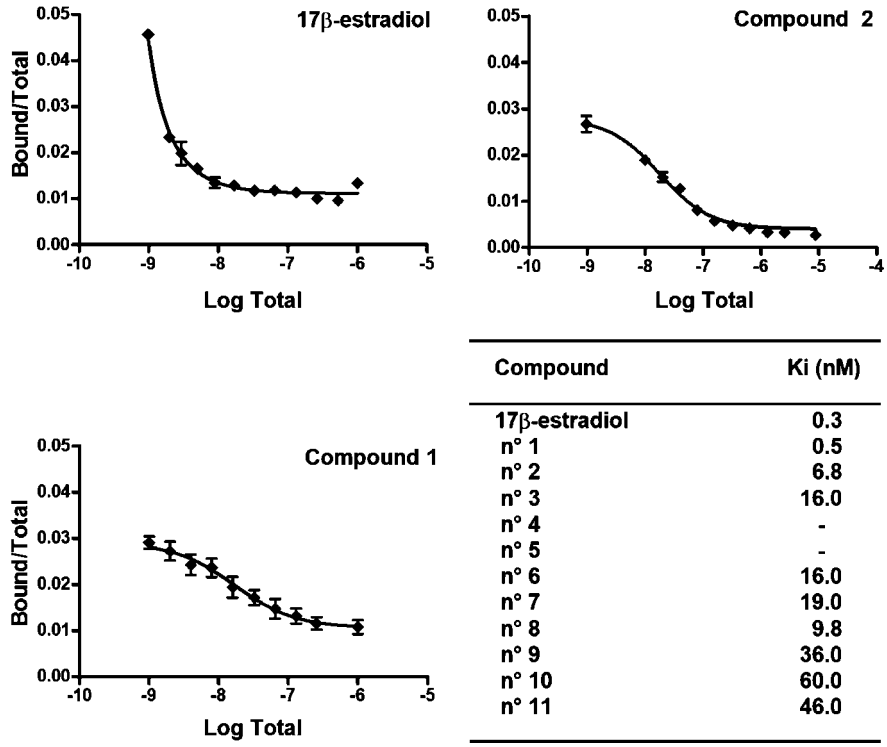
Previous studies[9,10] showed that both ERα and ERβ contribute to the wound healing process. However, we concentrated on ERα due to preliminary studies based on transfection assays. In fact, studying the effect of different ligands in cells (SK-N-BE) transfected with either ERα or ERβ, we found that compounds **1**−**11** have generally higher activity on ERα than on ERβ. We have, therefore, continued our studies using MCF-7 cells which have the advantage of a preponderant expression of ERα.

Competition assays were carried out using tritium labeled E_2 (^{3}H-E_2, 0.5 nM) and increasing concentrations of cold competitor (Figure 1). In these experiments cold E_2 showed a K_i of 0.3 nM, compatible with previously published affinity data from several independent groups.[31−35] Among the compounds synthesized, compounds **1** and **2** displayed relatively high affinity (K_i = 0.5 and 6.8 nM, respectively) for ER in MCF-7 cells. The remaining compounds (K_i

(29) Marini Bettolo, R.; Tsai, C. S. J.; Tsai, Y. R. T.; Wiesner, K. A One Step Synthesis of 17α-Hydroxy Cardenolides and Isocardenolides from C17 Steroidal Ketones. *Heterocycles* **1981**, *15*, 305–308.

(30) Being a diastereomeric mixture no spectroscopic data are presented.

(31) Kwon, J. H.; Katz, L. E.; Liljestrand, H. M. Modeling Binding Equilibrium in a Competitive Estrogen Receptor Binding Assay. *Chemosphere* **2007**, *69*, 1025–1031.



Compound	Ki (nM)
17β-estradiol	0.3
n° 1	0.5
n° 2	6.8
n° 3	16.0
n° 4	-
n° 5	-
n° 6	16.0
n° 7	19.0
n° 8	9.8
n° 9	36.0
n° 10	60.0
n° 11	46.0

Figure 1. Binding affinity of E_2 and of the newly synthesized compounds **1**−**11** as measured by competitive binding assay. Binding affinities of compounds synthesized were measured in a heterologous competitive binding assay in whole MCF7 cells. Binding affinities were obtained by analysis with PRISM5 software, implemented with LIGAND program equations, of at least two independent homologous and heterologous competition curves. Curves were generated by computer fitting as described under Materials and Methods by plotting ^{3}H-E_2 binding versus total ligand (*Y* axis) versus the log of the ligand used in the assay (*X* axis). Values represent the means of a minimum of 2 separate determinations each done in triplicate.

between 10 and 60 nM) also showed a good affinity for MCF-7 ERs.

We decided to avoid binding studies on **4** and **5** since the transactivation studies demonstrated that they were definitely less potent than **2**.

Thus, most of the new compounds showed a good affinity for MCF-7 ERs.

(b) Transactivation Assays. The ability of the novel compounds to transcriptionally activate ERs was tested in an MCF-7 breast cancer cell line stably transfected with a luciferase-reporter plasmid driven by an estrogen-regulated synthetic promoter previously generated and tested in our laboratories.[36] Most compounds were active, with a transactivation potency (EC_{50}) of the same order of magnitude as the natural hormone, E_2 (Figure 2). Among compounds tested, **10** and **11** showed the lowest transactivation potency (EC_{50} = 540 and 1640 nM, respectively), while most compounds synthesized had an EC_{50} between 1 and 10 nM. Interestingly, compound **2**, in spite of its lower potency (EC_{50} = 12 nM), was able to transactivate ERα by around 6-fold, similarly to the natural ligand. Taken together these results led us to conclude that several of the synthesized compounds had a significant affinity for ERs and were able to efficiently induce ER transcriptional activity.

(c) Activity of Compound 2 in Wound Healing. In order to evaluate its biological efficacy in wound healing we tested compound **2**, one of the most estrogenic compounds synthesized. We employed a well-characterized murine model of human age-associated delayed healing, in which we have previously demonstrated the beneficial effects of estrogens on the healing process.[9−11] Administration of

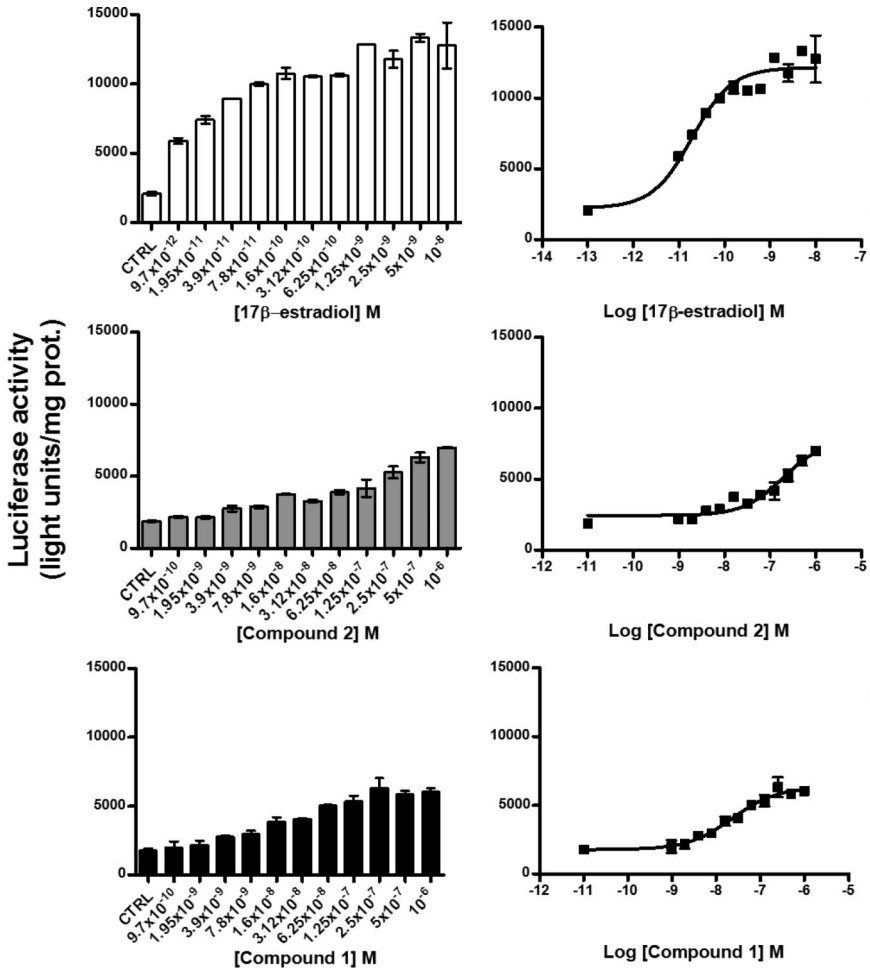
(32) Yoshino, T.; Kato, F.; Takeyama, H.; Nakai, M.; Yakabe, Y.; Matsunaga, T. Development of a Novel Method for Screening of Estrogenic Compounds Using Nano-Sized Bacterial Magnetic Particles Displaying Estrogen Receptor. *Anal. Chim. Acta* **2005**, *532*, 105–111.

(33) Rich, R. L.; Hoth, L. R.; Geoghegan, K. F.; Brown, T. A.; Le Motte, P. K.; Simons, S. P.; Hensley, P.; Myszka, D. G. Kinetic Analysis of Estrogen Receptor/Ligand Interactions. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 8562–8567.

(34) Matthews, J.; Celius, T.; Halgren, R.; Zacharewski, T. Differential Estrogen Receptor Binding of Estrogenic Substances: a Species Comparison. *J. Steroid Biochem. Mol. Biol.* **2000**, *74*, 223–234.

(35) Kuiper, G. G. J. M.; Lemmen, J. G.; Carlsson, B.; Corton, J. C.; Safe, S. H.; Van der Saag, P. T.; Van der Burg, B.; Gustafsson, J. A. Interaction of Estrogenic Chemicals and Phytoestrogens with Estrogen Receptor β. *Endocrinology* **1998**, *139*, 4252–4263.

(36) Ciana, P.; Di Luccio, G.; Belcredito, S.; Pollio, G.; Vegeto, E.; Tatangelo, L.; Tiveron, C.; Maggi, A. Engineering of a Mouse for the In Vivo Profiling of Estrogen Receptor Activity. *Mol. Endocrinol.* **2001**, *15*, 1104–1113.



Compound	EC_{50} (nM)	Fold Induction (10^{-6} M/CTRL)
17β-estradiol	0.02	6.1
1	53.00	2.3
2	12.00	6.2
3	1.30	3.0
4	0.46	2.7
5	2.40	2.8
6	7.40	2.5
7	9.90	2.0
8	31.00	2.6
9	120.00	2.0
10	540.00	2.0
11	1640.00	2.3

Figure 2. Ability of E_2 and compounds **1**−**11** to transcriptionally activate ERs. ER transcriptional activity in the presence of E_2 or heterologous compounds was measured in the clone B17 of MCF-7 cells stably transfected with the reporter plasmid ERE-tk-LUC as described in the methodology section. Left panel: luciferase measured in the presence of increasing concentrations of ligand of selected compounds. Middle panel: examples of plotting of the transactivation data for EC_{50} calculation. Right panel: EC_{50} as calculated by means of sigmoidal dose−response (variable slope) using PRISM software.

either estradiol or compound **2** to ovariectomized (ovx) female mice (as described in the methodology section) substantially accelerated healing (Figure 3).

Accelerated healing correlated with a reduction in wound macrophage numbers in animals treated with either E_2 or compound **2** compared to control ovariectomized mice (Figure 4, A). In contrast, E_2 but not compound **2** treatment reduced wound neutrophil numbers (Figure 4, B). This mirrors our recently reported findings in animals treated with tamoxifen or raloxifene[10] and suggests differential dynamics for the effect of E_2 and these SERM compounds on inflammatory cells, and probably other cell types involved in wound repair.

To investigate this further, we measured wound expression of mediators of the proinflammatory cascade *via* immunohistochemistry (Figure 5) and qPCR (Figure 6). Natural and synthetic estrogen both significantly decreased wound levels of TGFβ1, MIF and TNFα. In contrast immunohistochemical analysis demonstrated that E_2 and compound **2** displayed differential effects on IL-6 protein product, with the synthetic compound treated wounds containing numbers of IL-6 expressing cells not significantly different to wounds from vehicle treated mice (Figure 5). When we measured the mRNA content, however, we found that E_2 and compound **2** were equally potent in decreasing the accumulation of mRNA encoding IL-6, MIF, TGFβ and TNFα proteins. Finally, compound **2** was unable to accelerate re-epithelialization, contrasting with the pronounced effects of E_2 treatment on epidermal repair (as we have previously reported;[10] data not shown).

(d) Systemic Activity of Compound 2. The systemic activity of compound **2** was investigated in male ERE-Luc reporter mice where the expression luciferase gene is ubiquitously regulated by compounds active on ERs.[36] 5 μg/kg of compound **2** or E_2 was administered subcutaneously at groups of a minimum of 5 male mice each.

In order to evaluate the systemic effects of **2** we used males mice because they are the most sensitive system. In fact, the activity of exogenous estrogens can be detected very easily in males because the background is always

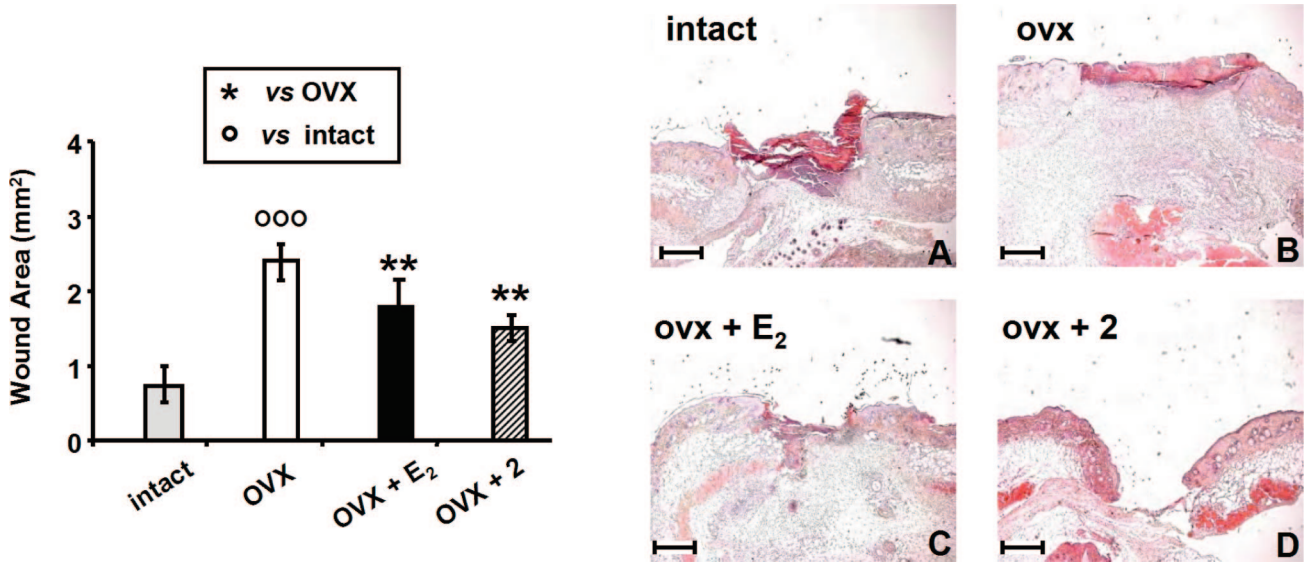


Figure 3. E_2 and compound **2** accelerate wound healing in ovariectomized mice. Left panel: wound area measurement. Results are shown as mean ± SEM, $n = 6$ per group. (**) $p < 0.05$ and (○○○) $p < 0.01$. Right panel: representative hematoxylin and eosin stained sections of full thickness incisional wounds (day 3) from mice: with intact ovaries (A), ovariectomized (B), ovariectomized and treated systemically with E_2 (C) or with compound **2** (D) administered endodermically in the wound area as described in the methodology section. (Bar = 500 μm.)

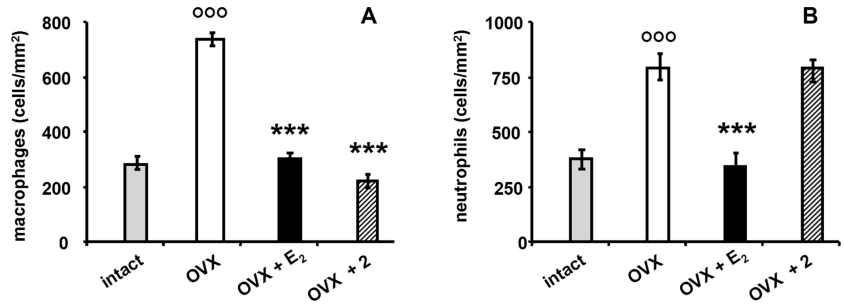


Figure 4. E_2 and compound **2** treatment decreases inflammatory cells in the wound area. Compound **2** was administered topically, as described in the methodological section, and was compared with the effect of systemic E_2. Cells present in the wound area were stained with hematoxylin/eosin and specific antibodies and counted. Results show mean ± SEM with $n = 6$. (***) $p < 0.05$ versus ovx and (○○○) $p < 0.01$ versus intact mice.

very low. In females this is not always the case because of unliganded activation of the receptor which may activate ERs also in ovariectomized animals. On the other hand, repeated experimentation showed that the effect of estrogens in wound healing is the same in males and females (data not shown).

Controls received the vehicle. Animals were then subjected to *in vivo* whole body imaging 6 h after pharmacological treatment. This time was selected on the basis of previous studies showing that the peak of ER activity is generally observed at 6−8 h after administration of estrogenic compounds.[36,37] Figure 7 shows that no ER activity was registered after compound **2** administration. In contrast, E_2 induced a significant increase of luciferase activity in the chest probably due to its action on liver ERs; a trend to increase was observed in all other body areas. Thus this experiment showed the lack of systemic activity of compound **2** when administered topically.

Conclusion

In the present study we have prepared a family of compounds active through the ER characterized by a structure that should ensure their rapid metabolism and limited systemic action when administered *in vivo*. Most of the compounds tested have shown ER binding affinity in the nM range and transactivation potency compatible with a pharmacological application. The biological activity of compound **2** tested in a well-characterized wound healing murine model showed a significant effect on wound healing and inflammatory processes.[9] Compound **2** proved to be as effective as 17β-estradiol in accelerating healing (Figure 3) and affecting the synthesis of molecules of the inflammatory

(37) Ciana, P.; Raviscioni, M.; Mussi, P.; Vegeto, E.; Que, I.; Parker, M. G.; Lowik, C.; Maggi, A. In Vivo Imaging of Transcriptionally Active Estrogen Receptors. *Nat. Med.* **2003**, *9*, 82–86.

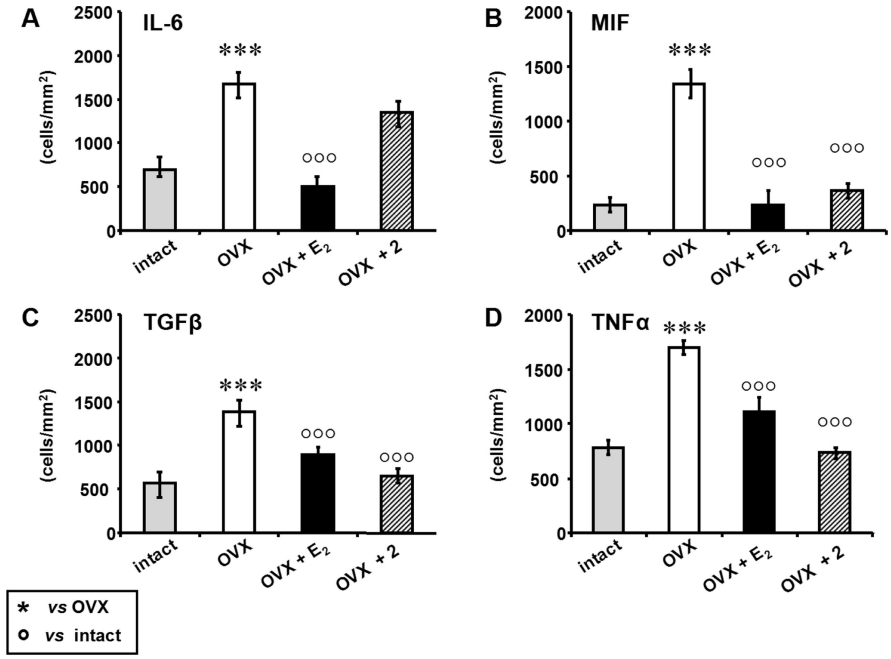


Figure 5. Reduced presence of cytokines in the wound area after treatment with E_2 and compound **2**. Immunocytochemistry was carried out as described in the Experimental Section. Cell counting showed significant changes after treatments with E_2 or compound **2**. Bars show mean ± SEM with $n = 6$. (***) $p < 0.01$ versus ovx and (○○○) $p < 0.01$ versus intact mice.

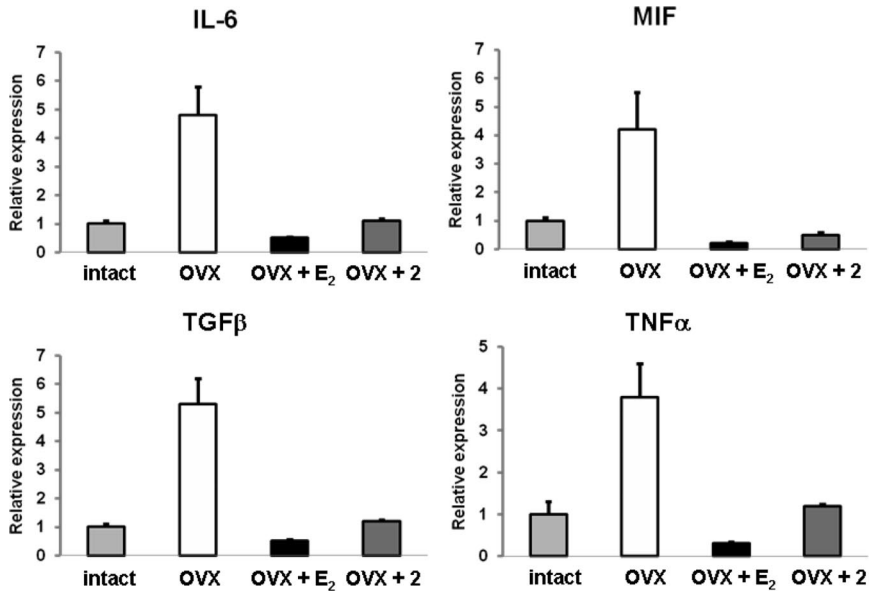


Figure 6. Reduced cytokines mRNA expression in the tissue surrounding the wound area after treatment with E_2 and compound **2**. Cytokine mRNA of the animals treated as in the preceding figure was measured by rtPCR. Data are expressed by considering equal to 1 the quantity of mRNA measured in intact animals. Results shown are the mean of a total of 4 samples measured in duplicates.

cascade. Similarly to the E_2, compound **2** limited the migration of macrophages to the wound granulation tissue (Figure 4) and promoted a rapid resolution of inflammation as indicated by the reduced content and synthesis of wound inflammatory cytokines (Figures 5 and 6).

The present data indicate that the synthetic compound may act with a mechanism that is not entirely superimposable with that of 17β-estradiol. Indeed, the number of neutrophils in the wound area was significantly decreased in animals treated with 17β-estradiol, but not with compound **2** in which case the number of these white cells was equal to that of vehicle treated ovariectomized animals. It is possible that the persistently high IL-6 protein content in wounds from compound **2** treated mice may be a contributing factor in maintenance of neutrophil levels. This interleukin specifically increases the adhesion of neutrophils to dermal fibroblasts,[38] probably limiting the subsequent phagocytosis by macrophages. The role of IL-6 in healing is complex. We have

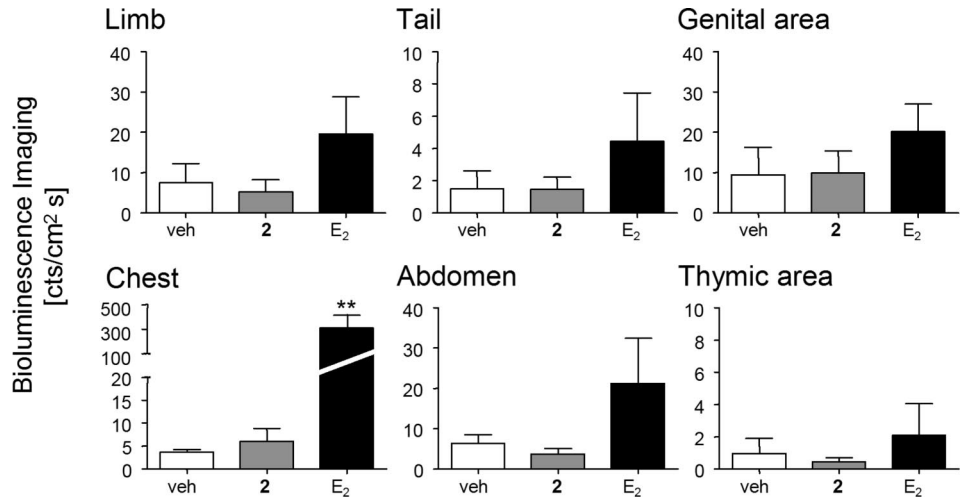


Figure 7. Whole-body bioluminescence imaging fails to detect systemic estrogenic activities of compound **2** in ERE-Luc reporter mice. Groups of 5 ERE-Luc mice were subjected to subcutaneous injection of vehicle, compound **2** (5 μg/kg sc) or E_2 (5 μg/kg sc). Photon emission was measured in selected body regions 6 h after treatment. Bars represent the mean $\pm$ SEM of photon emission measured in a minimum of 5 animals/group. ** $P < 0.01$ versus vehicle. *P* values were calculated with ANOVA followed by Bonferroni *post hoc* test.

previously demonstrated high IL-6 expression in delayed healing wounds[11] yet IL-6 also acts indirectly as a keratinocyte mitogen promoting re-epithelialization.[39] Moreover, the finding that IL-6 mRNA is as low in compound **2** treated wounds as in animals treated with estradiol (Figure 6) suggests that compound **2** possibly delays IL-6 turnover.

The persistently high IL-6 levels in wounds treated with compound **2** may be due to the local activity of the latter. It is, in fact, likely that compound **2** does not interfere with systemic inflammatory processes, as a matter of fact, whole-body bioluminescence imaging fails to detect systemic estrogenic activities of compound **2** in ERE-Luc reporter mice (Figure 7).

Our study, by identifying a new class of molecules locally active through the ERs in the wound healing process opens new avenues in the search of novel drugs for a still unmet medical need.

Experimental Section

Materials and Methods. All solvents were analytical grade. TLC: Merck silica gel 60 F_{254}. CC: silica gel 60, 70−230 mesh ASTM. IR spectra: Shimadzu-FTIR 8400S infrared spectrophotometer, in cm^{-1}. ^1H and ^{13}C NMR: Bruker AC 300P operating at 300.13 and 75 MHz respectively; Varian Gemini-200, at 200 and 50 MHz respectively; δ in ppm relatively to internal standard Me_4Si. Compound purity was evaluated by HPLC analysis: Shimadzu LC-10AD; RID detector; column, Macherey-Nagel 250/4 Nucleosil 100-5; flow, 0.8 mL/min; *T*, 35 °C; all solvents were HPLC grade; t_R in min. Mp: Mettler-FP-61 apparatus (uncorrected). HR mass spectra were recorded with a Micromass Q-TOF micro mass spectrometer (Waters).

Unless otherwise specified, all organic solutions were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure.

Compound **13a** is commercially available, and compounds **12**[40,41] and **13b**−**e**[42−44] were prepared according to the literature. In the case of **13d**,**e** the starting materials for the protection with TBDMS, 7-octyn-1-ol and 9-decyn-1-ol, were

(38) Giuliani, A. L.; Spisani, S.; Cavalletti, T.; Reali, E.; Melchiorri, L.; Ferrari, L.; Lanza, F.; Traniello, S. Fibroblasts Increase Adhesion to Neutrophils after Stimulation with Phorbol Ester and Cytokines. *Cell. Immunol.* **1993**, *149*, 208–222.

(39) Gallucci, R. M.; Simeonova, P. P.; Matheson, J. M.; Kommineni, C.; Guriel, J. L.; Sugawara, T.; Luster, M. I. Impaired Cutaneous Wound Healing in Interleukin-6-deficient and Immunosuppressed Mice. *FASEB J.* **2000**, *14*, 2525–2531.

(40) VanBrocklin, H. F.; Carlson, K. E.; Katzenellenbogen, J. A.; Welch, M. J. 16β-([18F]Fluoro)Estrogens: Systematic Investigation of a New Series of Fluorine-18-labeled Estrogens as Potential Imaging Agents for Estrogen-Receptor-Positive Breast Tumors. *J. Med. Chem.* **1993**, *36*, 1619–1629.

(41) Boucheau, V.; Renaud, M.; Rolland de Ravel, M.; Mappus, E.; Cuilleron, C. Y. Proton and Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Diastereoisomeric 3- and 17β -Tetrahydropyranyl ether Derivatives of Estrone and Estradiol. *Steroids* **1990**, *55*, 209–221.

(42) Guay, B.; Deslongchamps, P. Cascade Polycyclization: Exploration of a Convergent Route to Access Various Tricyclic and Tetracyclic Products Related to Sterols. *J. Org. Chem.* **2003**, *68*, 6140–6148.

(43) Roush, W. R.; Barda, D. A.; Limberakis, C.; Kunz, R. K. Studies on the Synthesis of the Quartromicins: Partial Stereochemical Assignment of Quartromicins A3 and D3 and Diastereoselective Synthesis of the Endo- and Exo-Spirotetronate Subunits. *Tetrahedron* **2002**, *58*, 6433–6454.

(44) Kalivretenos, A.; Stille, J. K.; Hegedus, L. S. Synthesis of β-Resorcylic Macrolides via Organopalladium Chemistry. Application to the Total Synthesis of (S)-Zearalenone. *J. Org. Chem.* **1991**, *56*, 2883–2894.

prepared by isomerization from 3-octyn-1-ol and 3-decyn-1-ol respectively.[45−47]

For biological investigations, unless otherwise specified, chemicals were purchased from Merck (Germany), culture media and additives were purchased from Invitrogen Corporation (Scotland, U.K.), and 17β-estradiol was purchased from Sigma Chemical Co.

Preparation of Compounds 1−8. To an efficiently stirred solution of **13** (10 mmol) in anhydrous THF (15 mL) at −78 °C, a solution of 2.0 M BuLi in cyclohexane (3 mL, 6.0 mmol) was added dropwise in 30 min, under Ar. After three hours a solution of **12** (0.56 g, 1.6 mmol) in anhydrous THF (5 mL) was added in 30 min; the mixture was stirred for 1 h 30 min at −78 °C and then allowed to warm to rt. When TLC showed the end of the reaction (generally after 4 h at rt), the solution was diluted with Et_2O (60 mL) and washed to neutrality with 0.2 M HCl. The organic layer was then washed with brine, dried and filtered; after evaporation of the solvent **14** was obtained as a yellow oil.[48]

To the crude product in THF (3 mL) a solution of 1.0 M TBAF in THF (3.5 mL, 3.5 mmol) was added at 0 °C, and the resulting solution was stirred at rt. When TLC showed the end of the reaction (generally after 3 h), the reaction mixture was diluted with EtOAc (80 mL) and washed with water. The aqueous phase was extracted with EtOAc, and combined organic phases were washed with brine, dried, filtered and evaporated (bath temperature below 40 °C), giving after CC **15** (**15a** eluent, EtOAc/hexane = 50/50, 83% yield; **15b** eluent, EtOAc/hexane = 30/70; 85% yield; **15c** eluent, from EtOAc/hexane = 10/90 to EtOAc/hexane = 30/70, 76% yield; **15d** eluent, from EtOAc/hexane = 20/80 to EtOAc/hexane = 40/60, 80% yield; **15e** eluent, from EtOAc/hexane = 20/80 to EtOAc/hexane = 40/60, 80% yield) as a colorless viscous oil.

To **15** (1.6 mmol) in pyridine (6 mL), the proper anhydride (13 mmol) was added, and the resulting solution was left under stirring at rt until TLC showed the complete disappearance of the starting material (generally 2 h). The solution was then diluted with Et_2O (100 mL) and washed with a 0.7 M HCl (3 × 30 mL). The aqueous phase was washed with Et_2O, and the organic phases were collected, washed with brine and dried. After filtration and evaporation of the solvent, the crude product was dissolved in water (1 mL) and MeOH (7 mL) and *p*-toluensulfonic acid was added to pH 3. The resulting solution was left under stirring at rt until TLC showed the complete disappearance of the starting material (generally 4 h). The solution was then diluted with EtOAc (80 mL) and washed to neutrality with a saturated $NaHCO_3$ aqueous solution, and the aqueous phase was extracted with EtOAc. The combined organic phases were collected, washed with brine and dried. After filtration, evaporation of the solvent gave crude **1−8**, which were purified by CC.

3,17β-Hydroxy-17α-(4′-acetoxy-1′-butyn-1′-yl)estra-1,3,5-(10)-triene (1). CC: EtOAc/hexane = 20/80. Yield: 85%. Viscous oil. IR ($CHCl_3$): 3615 cm^{-1}, 3360 cm^{-1}, 1733 cm^{-1}. ^1H NMR ($CDCl_3$): 7.13 (d, 1H, J_o = 8.4 Hz), 6.64 (dd, 1H, J_m = 2.6 Hz, J_o = 8.4 Hz), 6.57 (d, 1H, ar, J_m = 2.6 Hz), 5.90 (br s,1H, OH), 4.18 (t, 2H, J = 6.8 Hz), 2.86−2.76 (m, 2H), 2.58 (t, 2H, J = 6.8 Hz), 2.40−1.20 (m, 17H), 0.85 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$): 171.2, 153.6, 138.1, 132.2, 126.4, 115.3, 112.7, 85.3, 81.8, 80.0, 62.5, 49.4, 47.2, 43.5, 39.4, 38.9, 32.8, 29.6, 27.2, 26.4, 22.8, 20.9, 19.3, 12.8 ppm. HPLC: EtOAc/hexane = 30/70, t_R = 10.6, area percent = 98. HRMS (ESI): calcd for $C_{24}H_{30}O_4$, 405.2042 $(M + Na)^+$; found 405.2030.

3,17β-Hydroxy-17α-(5′-acetoxy-1′-pentyn-1′-yl)estra-1,3,5-(10)-triene (2). CC: Et_2O/hexane = 70/30. Yield: 94%. Viscous oil. IR (CCl_4): 3613 cm^{-1}, 3412 cm^{-1}, 1744 cm^{-1}. ^1H NMR ($CDCl_3$): 7.14 (d, 1H, J_o = 8.4 Hz), 6.70 (dd, 1H, J_o = 8.4 Hz, J_m = 2.5 Hz), 6.58 (d, 1H, J_m = 2.5 Hz), 6.35 (br s, 1H, OH), 4.19 (t, 2H, J = 6.3 Hz), 2.90−2.70 (m, 2H), 2.65−1.15 (m, 21H), 0.87 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$): 171.6, 153.7, 138.0, 132.1, 126.3, 115.2, 112.7, 84.6, 80.0, 63.2, 49.4, 47.1, 43.5, 39.4, 38.9, 32.8, 29.5, 27.7, 27.1, 26.3, 22.7, 20.9, 15.4, 12.7 ppm. HPLC: EtOAc/hexane = 35/65, t_R = 6.7, area percent = 97. HRMS (ESI): calcd for $C_{25}H_{32}O_4$, 419.2198 $(M + Na)^+$; found 419.2183.

3,17β-Hydroxy-17α-(5′-propanoyloxy-1′-pentyn-1′-yl)estra-1,3,5-(10)-triene (3). CC: Et_2O/hexane = 70/30. Yield: 94%. Viscous oil. IR (CCl_4): 3613 cm^{-1}, 3414 cm^{-1}, 1740 cm^{-1}. ^1H NMR ($CDCl_3$): 7.15 (d, 1H, J_o = 8.4 Hz), 6.63 (dd, 1H, J_o = 8.4 Hz, J_m = 2.7 Hz), 6.57 (d, 1H, J_m = 2.7 Hz), 5.46 (br s, 1H, OH), 4.19 (t, 2H, J = 6.3 Hz), 2.92−2.72 (m, 2H), 2.45−1.22 (m, 20H), 1.13 (t, 3H, J = 7.6 Hz), 0.86 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$): 174.6, 153.4, 138.1, 132.5, 126.4, 115.2, 112.7, 84.7, 84.6, 80.0, 62.9, 49.5, 47.1, 43.6, 39.4, 39.1, 32.9, 29.6, 27.8, 27.5, 27.1, 26.4, 22.7, 15.5, 12.8, 9.1 ppm. HPLC: EtOAc/hexane = 40/60, t_R = 5.0, area percent = 97. HRMS (ESI): calcd for $C_{26}H_{34}O_4$, 433.2355 $(M + Na)^+$; found 433.2348.

3,17β-Hydroxy-17α-(5′-butanoyloxy-1′-pentyn-1′-yl)estra-1,3,5-(10)-triene (4). CC: Et_2O/hexane = 40/60. Yield: 88%. Viscous oil. IR (CCl_4): 3613 cm^{-1}, 3420 cm^{-1}, 1739 cm^{-1}. ^1H NMR ($CDCl_3$): 7.15 (d, 1H, J_o = 8.4 Hz), 6.63 (dd, 1H, J_o = 8.4 Hz, J_m = 2.7 Hz), 6.56 (d, 1H, J_m = 2.7 Hz), 5.14 (br s, 1H, OH), 4.19 (t, 2H, J = 6.3 Hz), 2.95−2.75 (m, 2H), 2.50−1.10 (m, 22H), 1.10−0.80 (m, 6H) ppm. ^{13}C NMR ($CDCl_3$): 173.7, 153.4, 138.2, 132.6, 126.5, 115.2, 112.7, 84.7, 84.6, 80.0, 62.8, 49.5, 47.1, 43.6, 39.4, 39.1, 36.2, 33.0, 29.6, 27.9, 27.2, 26.5, 22.7, 18.5, 15.5, 13.6, 12.8 ppm. HPLC: EtOAc/hexane = 25/75, t_R = 7.5, area percent = 97. HRMS (ESI): calcd for $C_{27}H_{36}O_4$, 447.2511 $(M + Na)^+$; found 447.2529.

(45) Macaulay, S. R. Isomerization of Internal Triple Bonds of Alkyn-1-ols with Sodium Hydride in 1,3-Diaminopropane. *J. Org. Chem.* **1980**, *45*, 734–735.

(46) Denmark, S. E.; Yang, S. M. Intramolecular Silicon-Assisted Cross-Coupling Reactions: General Synthesis of Medium-Sized Rings Containing a 1,3-*cis-cis* Diene Unit. *J. Am. Chem. Soc.* **2002**, *124*, 2102–2103.

(47) Abrams, S. R.; Shaw, A. C. Triple-bond Isomerizations: 2- to 9-Decyn-1-ol. *Org. Synth.* **1988**, *66*, 127–131.

3,17β-Hydroxy-17α-(5′-valeroxy-1′-pentyn-1′-yl)estra-1,3,5-(10)-triene (5). CC: Et_2O/hexane = 40/60. Yield: 85%. Viscous oil. IR (CCl_4): 3613 cm^{-1}, 3420 cm^{-1}, 1739 cm^{-1}. ^1H NMR ($CDCl_3$): 7.15 (d, 1H, J_o = 8.4 Hz), 6.64 (dd, 1H, J_o = 8.4 Hz, J_m = 2.7 Hz), 6.56 (d, 1H, J_m = 2.7 Hz), 5.56 (br s, 1H, OH), 4.19 (t, 2H, J = 6.3 Hz), 2.92−2.70 (m, 2H), 2.45−1.15 (m, 24H), 1.05−0.85 (m, 6H) ppm. ^{13}C NMR ($CDCl_3$): 174.0, 153.5, 138.1, 132.4, 126.4 115.2, 112.7, 84.7, 84.6, 80.0, 62.9, 49.5, 47.2, 43.6, 39.4, 39.1, 34.0, 32.9, 29.6, 27.9, 27.2, 27.0, 26.4, 22.8, 22.2, 15.5, 13.6, 12.8 ppm. HPLC: EtOAc /hexane = 25/75, t_R = 7.2, area percent = 97. HRMS (ESI): calcd for $C_{28}H_{38}O_4$, 461.2668 $(M + Na)^+$; found 461.2663.

3,17β-Hydroxy-17α-(6′-acetoxy-1′-hexyn-1′-yl)estra-1,3,5-(10)-triene (6). CC: EtOAc/hexane = 30/70. Yield: 83%. Viscous oil. IR ($CHCl_3$): 3615 cm^{-1}, 3360 cm^{-1}, 1730 cm^{-1}. ^1H NMR ($CDCl_3$): 7.16 (d, 1H, J_o = 8.5 Hz), 6.64 (dd, 1H, J_m = 2.7 Hz, J_o = 8.5 Hz), 6.57 (d, 1H, J_m = 2.7 Hz), 5.47 (br s, 1H, OH), 4.09 (t, 2H, J = 6.5 Hz), 2.90−2.70 (m, 2H), 2.40−1.25 (m, 23H), 0.87 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$): 171.5, 153.5, 138.2, 132.4, 126.5, 115.3, 112.7, 85.6, 84.2, 80.1, 64.1, 49.5, 47.2, 43.6, 39.4, 39.1, 32.9, 29.6, 27.8, 27.2, 26.4, 25.1, 22.8, 21.0, 18.5, 12.8 ppm. HPLC: EtOAc/hexane = 30/70, t_R = 8.8, area percent = 98. HRMS (ESI): calcd for $C_{26}H_{34}O_4$, 433.2355 $(M + Na)^+$; found, 433.2365.

3,17β-Hydroxy-17α-(8′-acetoxy-1′-octyn-1′-yl)estra-1,3,5-(10)-triene (7). CC: EtOAc/hexane = 30/70. Yield: 94%. Viscous oil. IR ($CHCl_3$): 3615 cm^{-1}, 3375 cm^{-1}, 1722 cm^{-1}. ^1H NMR ($CDCl_3$): 7.14 (d, 1H, J_o = 8.4 Hz), 6.64 (d, 1H, J_o = 8.4 Hz), 6.59 (s, 1H), 5.92 (br s,1H, OH), 4.06 (t, 2H, J = 6.5 Hz), 2.95−2.65 (m, 2H), 2.45−1.12 (m, 27H), 0.87 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$): 171.6, 153.6, 138.1, 132.2, 126.4, 115.3, 112.7, 86.2, 83.8, 80.1, 64.5, 49.4, 47.2, 43.7, 39.4, 39.1, 32.9, 29.6, 28.5, 28.4, 28.3, 27.2, 26.5, 25.3, 22.8, 21.0, 18.7, 12.8 ppm. HPLC: EtOAc/hexane = 30/70, t_R = 6.8, area percent = 98. HRMS (ESI): calcd for $C_{28}H_{38}O_4$, 461.2668 $(M + Na)^+$; found, 461.2676.

3,17β-Hydroxy-17α-(10′-acetoxy-1′-decyn-1′-yl)estra-1,3,5-(10)-triene (8). CC: EtOAc/hexane = 30/70. Yield: 90%. Viscous oil. IR ($CHCl_3$): 3705 cm^{-1}, 3365 cm^{-1}, 1724 cm^{-1}. ^1H NMR ($CDCl_3$): 7.13 (d, 1H, J_o = 8.5 Hz), 6.79 (br s, 1H, OH), 6.67 (dd, 1H, J_o = 8.5 Hz, J_m = 2.4 Hz), 6.58 (d, 1H, J_m = 2.4 Hz), 4.02 (t, 2H, J = 6.7 Hz), 2.90−1.10 (m, 33H), 0.87 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$):171.7, 153.8, 137.8, 131.8, 126.2, 115.3, 112.8, 86.2, 83.6, 80.0, 64.7, 49.3, 47.1, 43.6, 39.4, 38.9, 32.7, 29.5, 29.1, 28.9, 28.7, 28.6, 28.4, 27.2, 26.4, 25.7, 22.7, 20.9, 18.6, 12.8 ppm. HPLC: EtOAc/hexane = 30/70, t_R = 6.1, area percent = 98. HRMS (ESI): calcd for $C_{30}H_{42}O_4$, 489.2981 $(M + Na)^+$; found, 489.2970.

3,17β-Hydroxy-17α-(5′-acetoxypent-1′-yl)estra-1,3,5-(10)-triene (9). To a solution of **2** (0.12 g, 0.30 mmol) in EtOH (5 mL) 10% Pd(C) (25 mg) was added under an inert atmosphere. The resulting suspension was saturated with H_2 and left under stirring at rt for one hour. The whole was then filtered on Celite, and after evaporation of the solvent, the residue was purified by CC (Et_2O/hexane = 60/40) to afford **9** (0.11 g, 92%) as a colorless viscous oil. IR ($CHCl_3$): 3601 cm^{-1}, 3356 cm^{-1}, 1724 cm^{-1}. ^1H NMR ($CDCl_3$): 7.12 (d, 1H, J_o = 8.4 Hz), 6.63 (dd, 1H, J_o = 8.4 Hz, J_m = 2.4 Hz), 6.56 (d, 1H, J_m = 2.4 Hz), 4.08 (t, 2H, J = 6.7 Hz), 2.92−2.68 (m, 2H), 2.40−1.15 (m, 25H), 0.89 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$): 171.6, 153.7, 138.1, 132.2, 126.3, 115.3, 112.7, 83.7, 64.7, 49.4, 46.7, 43.7, 39.6, 36.5, 34.2, 31.5, 29.6, 28.7, 27.4, 26.7, 26.3, 23.3, 23.3, 21.0, 14.4 ppm. HPLC: EtOAc/hexane = 40/60, t_R = 6.0, area percent = 97. HRMS (ESI): calcd for $C_{25}H_{36}O_4$, 423.2511 $(M + Na)^+$; found, 423.2529.

3,17β-Hydroxy-17α-(5′-propanoyloxypent-1′-yl)estra-1,3,5-(10)-triene (10). Compound **10** was prepared from **3**, following the same procedure used for **9** from **2**. CC: Et_2O/hexane = 50/50. Yield: 89%. Viscous oil. IR ($CHCl_3$): 3597 cm^{-1}, 3300 cm^{-1}, 1728 cm^{-1}. ^1H NMR ($CDCl_3$): 7.14 (d, 1H, J_o = 8.4 Hz), 6.63 (dd, 1H, J_o = 8.4 Hz, J_m = 2.7 Hz), 6.56 (d, 1H, J_m = 2.7 Hz), 4.09 (t, 2H, J = 6.6 Hz), 2.95−2.70 (m, 2H), 2.45−1.05 (m, 27H), 0.90 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$): 174.8, 153.6, 138.2, 132.5, 126.4, 115.3, 112.7, 83.5, 64.5, 49.5, 46.7, 43.8, 39.7, 36.6, 34.4, 31.6, 29.7, 28.8, 27.7, 27.5, 26.8, 26.4, 23.4, 23.3, 14.4, 9.2 ppm. HPLC: EtOAc/hexane = 35/65, t_R = 5.8, area percent = 97. HRMS (ESI): calcd for $C_{26}H_{38}O_4$, 437.2668 $(M + Na)^+$; found, 437.2675.

3-[(Tetrahydro-2′-pyran-2′-yl)oxy]-17β-hydroxy-17α-(5′-hydroxy-pent-1′-yl)estra-1,3,5-(10)-triene (16). To a solution of **14b** (0.045 g, 0.081 mmol) in dioxane (5 mL) 10% Pd(C) (10 mg) was added under an inert atmosphere. The resulting mixture was saturated with H_2 and allowed to stir at rt for 4 h (TLC: petroleum ether/Et_2O = 70/30). The mixture was then filtered through Celite and the catalyst washed thoroughly with EtOH. After evaporation of the solvent, the residue was dissolved in THF (3 mL) and 1.0 M TBAF in THF (0.2 mL, 0.2 mmol) was added at 0 °C. The reaction mixture was warmed to rt and left under stirring for 3 h (TLC: EtOAc/petroleum ether = 5/5). The solution was then diluted with EtOAc and extracted with water. The aqueous phase was extracted with EtOAc, and the combined organic phases were washed with brine and dried. After filtration and evaporation of the solvent, CC purification of the residue (Et_2O/petroleum ether = 60/40) gave **16** (0.030 g, 84%) as a white solid.

3-[(Tetrahydro-2′-pyran-2′-yl)oxy]-17β-hydroxy-17α-(4′-carboxy-but-1′-yl)estra-1,3,5-(10)-triene (17). To a solution of **16** (0.075 g, 0.17 mmol) in anhydrous DMF (1.0 mL) PDC (0.38 g, 1.0 mmol) was added. The mixture was left under stirring at rt until complete disappearance of the starting material (∼20 h, TLC: EtOAc/petroleum ether = 80/20). The mixture was diluted with an aqueous solution of acetic acid at pH 5 and extracted with EtOAc. The organic phase was washed with brine and dried over $MgSO_4$. After filtration and evaporation of the solvent, CC purification of the residue (EtOAc/petroleum ether = 80/20) gave **17** (0.038 g, 49%) as a pale yellow solid.

3-[(Tetrahydro-2′-pyran-2′-yl)oxy]-17β-hydroxy-17α-(4′-carboxymethyl-but-1′-yl)estra-1,3,5-(10)-triene (18). To a solution of **17** (0.140 g, 0.31 mmol) in $CHCl_3$ (12 mL) in an ice bath, a solution of CH_2N_2 in Et_2O was added dropwise until complete disappearance of the starting material (TLC: EtOAc /hexane = 3/7). The solvent was then removed under a stream of Ar, and the residue was purified by CC (EtOAc/hexane = 20/80), affording **18** (0.115 g, 79%) as a white foam.

3,17β-Hydroxy-17α-(4′-carboxymethyl-but-1′-yl)estra-1,3,5-(10)-triene (11). To **18** (0.100 g, 0.21 mmol) in MeOH (2.5 mL) an aqueous solution of *p*-toluensulfonic acid (2.0 mL) at pH 3−4 was added, and the mixture was left under stirring at rt until the reaction was completed (∼20 h, TLC: EtOAc/hexane = 4/6). The reaction mixture was diluted with EtOAc and washed with brine. The aqueous phase was extracted with EtOAc and the combined organic phase dried and filtered. After evaporation of the solvent, CC purification of the residue (EtOAc/hexane = 3/7) and crystallization gave **11** (75 mg, 93%) as a white solid. Mp ($CHCl_3$/hexane): 118.2−119.9 °C. IR ($CHCl_3$): 3615 cm^{-1}, 3355 cm^{-1}, 1729 cm^{-1}. ^1H NMR ($CDCl_3$): 7.13 (d, 1H, J_o = 8.4 Hz), 6.63 (dd, 1H, J_o = 8.4 Hz, J_m = 2.7 Hz), 6.56 (d, 1H, J_m = 2.7 Hz), 5.54 (br s, 1H, OH), 3.68 (s, 3H), 2.90−2.70 (m, 2H), 2.50−1.20 (m, 22H), 0.89 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$): 174.5, 153.6, 138.2, 132.4, 126.4, 115.3, 112.7, 83.6, 51.6, 49.5, 46.7, 43.7, 39.6, 36.3, 34.3, 34.2, 31.6, 29.6, 27.5, 26.3, 25.7, 23.4, 23.3, 14.4 ppm. HPLC: EtOAc/hexane = 35/65, t_R = 7.0, area percent = 97. HRMS (ESI): calcd for $C_{24}H_{34}O_4$, 409.2355 $(M + Na)^+$; found, 409.2341.

Cell Culture. All studies were carried out using the B17 clone of MCF-7 obtained in our laboratory by stable transfection of a plasmid containing the luciferase gene under the control of an estrogen responsive promoter.[36]

Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Euroclone, U.K.), 50 U/mL penicillin G, 50 μg/mL streptomicyn sulfate, 2 g/L sodium carbonate and 0.11 g/L sodium pyruvate, at 37 °C at 99% humidity and 5% CO_2. Cells were split twice a week by seeding 2×10^6 cells in 100 mm diameter Petri (Corning) dishes.

Binding Studies. Whole cell binding was carried out as previously described.[49] Briefly, for binding analysis 10^5 MCF-7 cells/well were seeded in twenty-four well plate in phenol red-free RPMI 1640 medium supplemented with 10% charcoal stripped FBS and incubated at 37 °C in a humidified incubator for 24 h. Next, the medium was replaced with fresh medium and 0.5 nM [2,4,6,7-^{3}H]estradiol (Amersham) and increasing concentrations of cold competitors (synthetic compounds or 17β-estradiol at concentrations of 1 nM to 0.25 μM). Nonspecific binding was assessed in the presence of 1 μM 17β-estradiol. Cells were incubated at 37 °C in a humidified incubator for two hours to reach equilibrium of the binding reaction. Cells were then rapidly rinsed with cold PBS three times in order to separate bound from free radioligand and then the radiolabeled 17β-estradiol was extracted by treating the cells with 0.5 M NaOH for 30 min. Radioactivity in 450 μL of cellular extract was quantitated by addition of 4 mL of scintillation fluid (high flash point LSC-cocktail, PerkinElmer) in the liquid scintillation analyzer (Tri-Carb 1600 TR, Packard).

Analysis of binding data was performed by means of nonlinear least squares fitting using PRISM5 software (GraphPad Software Inc.) implemented with LIGAND program equations, to provide the basic molecular and cellular parameters for each ligand studied. Evaluation of the statistical significance of the parameter difference is based on the F-test for the extra sum of squares principle.[50]

Transactivation Studies. For transactivation studies, 10^5 cells/well were seeded in twenty-four well plate in phenol red-free RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% dextran-coated charcoal stripped-FBS, 1% of essential amino acid, 1% of vitamin mixture, 50 U/mL penicillin G, 50 μg/mL streptomicyn sulfate, 2 g/L sodium carbonate and 0.11 g/L sodium pyruvate, and kept at 37 °C in a humidified incubator for 24 h. Next, culture medium was replaced with RPMI 1640 supplemented with 1% stripped FBS and cells were incubated for a minimum of 4 h before adding 17β-estradiol or synthetic compounds (at concentrations between 1 nM and 1 μM). After 24 h cells were rinsed once with PBS before preparing the protein extract for the determination of luciferase content as previously described.[36]

Analysis of transactivation data and calculation of EC_{50} values were performed by means of sigmoidal dose−response (variable slope), using PRISM5 Software (GraphPad Software Inc.).

Wound Healing Experiments. Six-week-old female C57B6 mice were anesthetized with methoxyfluorane and the dorsum shaved and cleaned with ethanol. A subgroup of mice had undergone ovariectomy (or sham procedure) two weeks prior to wounding.

The control of lack of endogenous E_2 production was made by measuring the uterus weight. It is well-known, in fact, that there is a direct relationship between circulating estrogens and uterus weight. This relationship is at the basis of the uterotrofic assay currently in use to assess the estrogenicity degree of any synthetic compound.

Two 1 cm full-thickness incisional wounds equidistant from the midline were made through the skin and panniculus

(48) For the preparation of compound **11** the crude product 3-[(tetrahydro-2′-hydro-pyran-2′-yl)oxy]-17β-hydroxy-17α-[5′-(*tert*-butyldimethylsilyl)oxy-1′-pentyn-1′-yl]estra-1,3,5-(10)-triene **14b** was purified on silica gel (Et_2O/petroleum ether = 20/80, viscous oil, 80% yield) before hydrogenation and TBDMS removal.

(49) Amadasi, A.; Mozzarelli, A.; Meda, C.; Maggi, A.; Cozzini, P., Identification of Xenoestrogens in Food Additives by an Integrated In Silico and In Vitro Approach *Toxicol. Sci.*, in press.

(50) Capra, V.; Veltri, A.; Foglia, C; Crimaldi, L.; Habib, A.; Parenti, M.; Rovati, G. E. Mutational Analysis of the Highly Conserved ERY Motif of the Thromboxane A2 Receptor: Alternative Role in G Protein-coupled Receptor Signaling. *Mol. Pharmacol.* **2004**, *66*, 880–889.

carnosus muscle. Wounds were harvested at day 3 post-wounding ($n = 4-5$ per treatment group) and were processed in formalin for wax embedding.

No differences in the entire wound and in the surrounding area on account of the different animals' treatment with **2** and with E_2 were observed.

(a) Animals' Treatment. Compound **2** was dissolved immediately before treatment in 99% ethanol and diluted in NaCl 9 ‰. For *in vivo* treatment we administered 100 μL of a 100 nM solution in the subcutaneous area surrounding the wound, immediately after generation of the wound and subsequently at 24 and 48 h after wounding. E_2 was administrated by the use of a subcutaneous pellet, releasing 50 μg E_2/kg//day (Innovative Research of America) implanted at the time of wounding.

Systemic administration of E_2 with pellets is most convenient and routinely used in the laboratory. However, as a proof of principle, we also administered E_2 locally sc and proved its efficacy.

Moreover, we know that the differential effects of E_2 and **2** on IL-6 and neutrophils cannot be attributed to the differential route of administration because we tested the effects of sc and systemically administered estradiol and we never observed any difference on all the considered parameters.

(b) Histology, Immunohistochemistry and Image Analysis. Histological sections (5 μm thickness) were prepared from wound tissue fixed in 10% buffered formalin and embedded in paraffin. Sections taken from the center of each wound were stained with hematoxylin and eosin (H and E), or subjected to immunohistochemistry with antibodies raised against IL-6 (goat), TNFα (goat) (both R&D Systems, Oxon, U.K.), TGF-β1 (rabbit; Promega, Southampton, U.K.). As a negative control, sections on each slide were treated with PBS instead of the primary antibody; in all cases these showed no positive staining. Primary antibody was detected using the Vectastain ABC peroxidase kit (Vector Laboratories, Peterborough, U.K.). The images show regions from the centers of the wounds equidistant from the two lateral wound margins. Image analysis and quantification of wound areas and cell numbers per unit area (measured below the clot and above the panniculus muscle) were performed with an Image-Pro Plus program (MediaCybernetics, Finchampstead, U.K.) as previously described.[11]

(c) Real-Time PCR. Total RNA was extracted from frozen wound tissue using Trizol (Invitrogen, Paisley, U.K.) and from macrophages and fibroblasts using RNeasy Mini kit (Qiagen, Crawley, U.K.) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of wound or macrophage RNA using a reverse transcription kit (Promega) and separate AMV-reverse transcriptase (Roche, Lewes, U.K.). Quantitative real-time PCR (qPCR) was performed using the SYBR Green I core kit (Eurogentec, Romsey, U.K.) following the manufacturer's instructions and an Opticon qPCR thermal cycler (Genetic Research Instrumentation, Braintree, U.K.). For each primer set an optimal dilution was determined and melting curves used to determine the specificity of product amplification. Each sample was serially diluted over 3 orders of magnitude, and all samples were run on the same 96 well plate. PCR was carried out using primer pairs designed to murine TGF-β1, TNFα, IL-6, and the housekeeping genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S rRNA (for normalization). Primer sequences were GAPDH (forward, TGCCACTCAGAAGACTGTGG; reverse, GGATGCAGGGATGATGTTCT); 18S rRNA (forward, AGTCCCTGCCCTTTGTACACA; reverse, GATCCGAGGGCCTCACTAAAC); IL-6 (forward, TACCCCAACTTCCAATGCTC; reverse, TGGTCTTGGTCCTTAGCCAC); TNF-α (forward, CTCTTCAAGGGACAAGGCTG; reverse, GGTATGAAGTGGCAAATCGG); TGF-β1 (forward, ATACGCCTGAGTGGCTGTCT; reverse, GTTTGGGACTGATCCCATTG).

Statistical Analysis. If not otherwise stated, statistical differences were determined by using Student's *t*-test, 1-way ANOVA or, for nonparametric data, Mann−Whitney *U* tests. A *P* value of <0.05 was considered significant.

Experimental Animals and Pharmacological Treatments for Systemic Activity Investigation. Animal experimentation was carried out in accordance with European guidelines for animal care and use of experimental animals, approved by the Italian Ministry of Research and University, and controlled by the panel of experts of the Department of Pharmacological Sciences, University of Milan. ERE-Luc mice are transgenic mice engineered to ubiquitously express a transgene, the firefly luciferase, under the control of an estrogen-responsive promoter;[36] these mice allow to spatiotemporally monitor ER transcriptional activity by biochemical or bioluminescence imaging technologies.[37] In the present study, we used heterozygous males of 6 months of age obtained by mating male homozygous ERE-Luc mice with C57BL/6 wild-type female mice. Animals were housed in plastic cages with hardwood chips bedding, fed ad libitum, and provided with filtered water. The animal room was maintained within a temperature range of 22−25 °C and relative humidity of 50% ± 10%. There was a cycle of 12 h light, 12 h dark (lights on at 07:00 a.m.).

(a) Pharmacological Treatments. Stock solutions of 17β-estradiol (E_2, 272 g/mol), and compound **2** (396 g/mol) were made by dissolving the compounds in 99% v/v ethanol to a concentration of 10^{-2} M and kept at −20 °C in the dark. Before the treatments, stock solutions were diluted 1:25 with 99% ethanol. A total of 15 μL of ethanol (containing either 9.5 μL of 2 dilution or 13.8 μL of E_2 solution) were added to 1 mL of maize oil and then mixed until dissolved to a final concentration of 1.5 ug/mL. Treatments were performed in the morning (10:00 a.m.) by administering sc 100 μL of the final oil−hormone solution, corresponding to a dosage of 5 μg/kg to a ∼30 g mouse.

(b) Bioluminescence Imaging. Bioluminescence imaging sessions were carried out 6 h after treatments (4:00 p.m.); mice were anesthetized by sc injection of a solution of ketamine (78 mg/kg) and xilazine (6 mg/kg); the luciferase

substrate luciferin (25 mg/kg) was administered ip 20 minutes before bioluminescence quantification: previous kinetic studies demonstrated that this time is enough to ensure distribution of the substrate at saturating concentration.[51] Bioluminescence was measured by a Night Owl imaging unit (Berthold Technologies, Bad Wildbad, Germany), consisting of a Peltier cooled charge-coupled device slow-scan camera equipped with a 25 mm/f0.95 lens. The camera was operated by WinLight software (Berthold Technologies). For photon emission measurement, mice were placed in a light-tight chamber and luciferase signal was registered for 5 min. For quantification purposes, photon emission was measured in selected body areas by manually superimposing a standardized electronic grid over the regions of interest and integrating the signal from this area (counts per square centimeter per second, cts/cm^2 s). Quantifications were done using WinLight32 imaging software (Berthold Technologies).

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(51) Biserni, A; Giannessi, F; Sciarroni, A. F.; Milazzo, F. M.; Maggi, A; Ciana, P. In Vivo Imaging reveals Selective Peroxisome Proliferator Activated Receptor Modulator Activity of the Synthetic Ligand 3-(1-(4-chlorobenzyl)-3-t-butylthio-5-isopropylindol-2-yl)-2,2-dimethylpropanoic acid (MK-886). *Mol. Pharmacol.* **2008**, *73*, 1434–1443.