Targeting RORs nuclear receptors by novel synthetic steroidal inverse agonists for autoimmune disorders

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Abstract

Designing novel inverse agonists of NR RORγt still represents a challenge for the pharmaceutical community to develop therapeutics for treating immune diseases. By exploring the structure of NRs natural ligands, the representative arotenoid ligands and RORs specific ligands share some chemical homologies which can be exploited to design a novel molecular structure characterized by a polycyclic core bearing a polar head and a hydrophobic tail. Compound MG 2778 (**8**), a cyclopenta[a]phenantrene derivative, was identified as lead compound which was chemically modified at position 2 in order to obtain a small library for preliminary SARs. Cell viability and estrogenic activity of compounds **7**, **8**, **19a**, **30**, **31** and **32** were evaluated to attest selectivity. The selected **7**, **8**, **19a** and **31** compounds were assayed in a Gal4 UAS-Luc co-transfection system in order to determine their ability to modulate RORγt activity in a cellular environment. They were evaluated as inverse agonists taken ursolic acid as reference compound. The potency of compounds was lower than that of ursolic acid, but their efficacy was similar. Compound **19a** was the most active, significantly reducing RORγt activity at low micromolar concentrations.

Key words: NR RORγt, inverse agonists, Gal4 UAS-Luc co-transfection, autoimmune disorders

1. Introduction

Nuclear receptors (NRs) form a family of transcription factors that are composed of modular protein structures with DNA- and ligand-binding domains (DBDs and LBDs). The DBDs confer gene target site specificity, whereas LBDs serve as control switches for NR function. In each case the overall fold of the LBD is conserved and the ligand is bound entirely within the protein, completing the

core as the protein refolds around it.[1] It was shown that despite the chemical diversity of the natural nuclear receptor ligands, their volumes are highly conserved.[2]

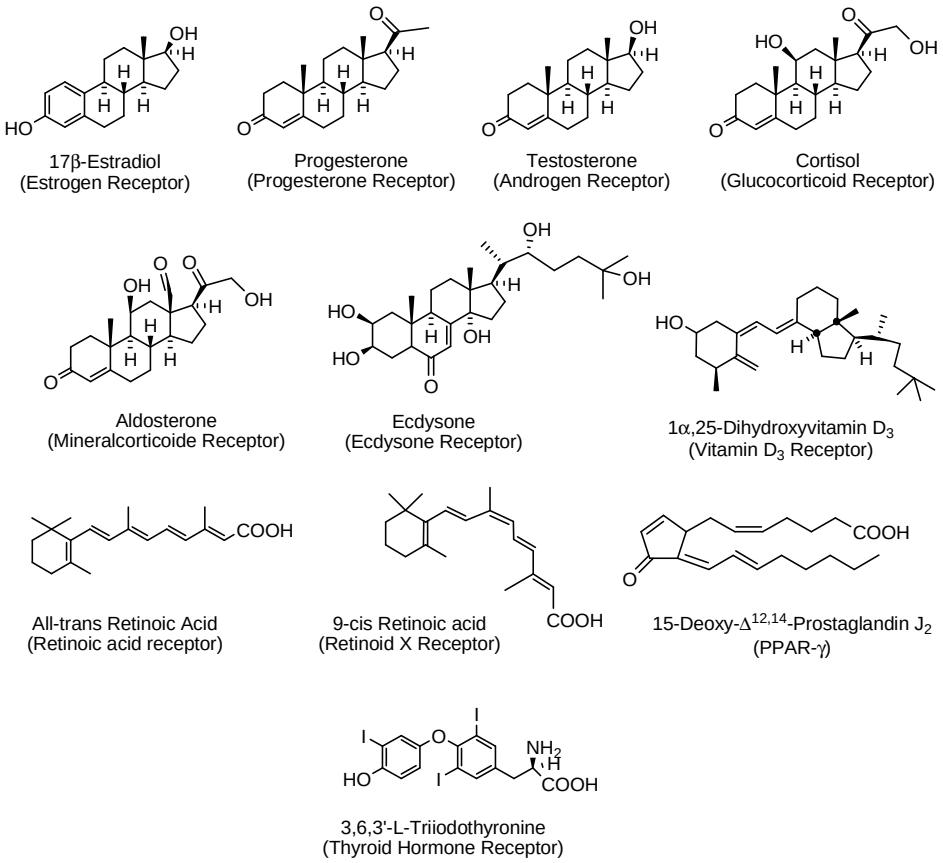


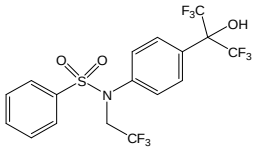
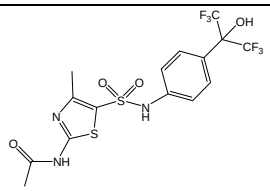
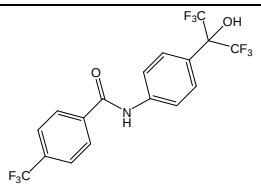
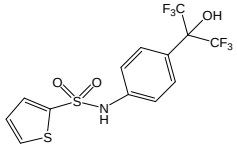
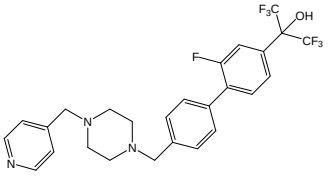
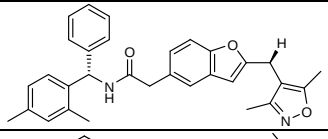
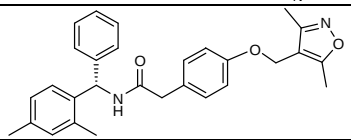
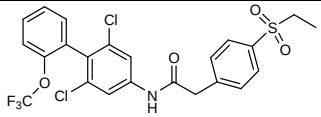
Figure 1. The natural ligands of nuclear receptors[2]

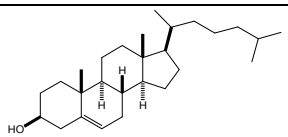
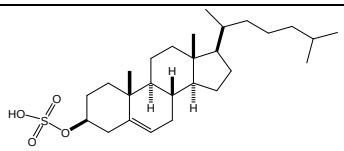
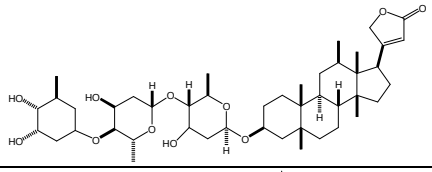
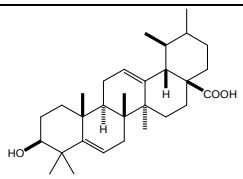
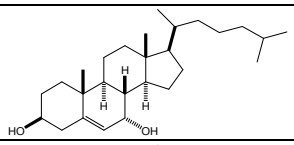
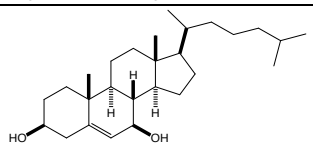
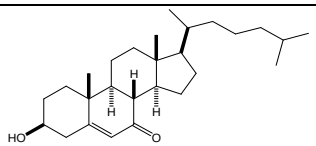
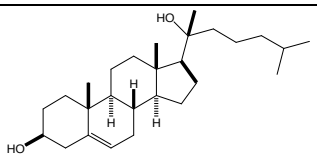
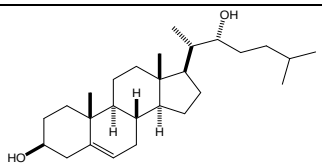
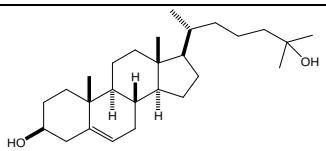
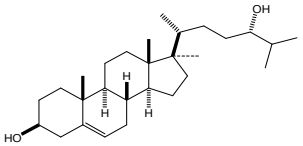
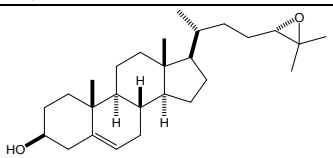
For many NRs, both endogenous and synthetic small molecule ligands bind to small pockets within the LBDs, resulting in conformational changes that regulate transcriptional activity. This property of NRs has proven to be a rich source as targets for developing of therapeutics for a myriad of human diseases, ranging from inflammatory diseases and cancer to endocrine and metabolic diseases.[3]

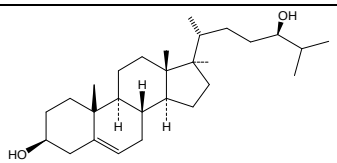
The retinoic acid nuclear receptors subfamily includes RARα, RARβ and RARγ and it is evolutionarily closed to the retinoic acid receptor-related orphan receptors subfamily, which is constituted by RORα, RORβ and RORγ or RORc. RORγt is a splice variant of RORγ and is encoded by a single gene called RORc. RORγt is selectively expressed in thymocytes (T cells) and appears to drive the activation and differentiation of CD4+ and CD8+ cells into IL17-producing T helper cells (T_H17) and cytotoxic T cells (Tc17). T_H17 and Tc17 are effector cells that promote inflammation, adaptive immunity, and autoimmunity by producing IL17 and other inflammatory cytokines such as IL21. Both synthetic and putative endogenous agonists of RORγt have been

shown to increase the basal activity of RORγt enhancing T_H17 cell proliferation. Among the various transcriptional regulators RORγ is a uniquely tractable drug target for manipulating T_H17 cell development and function in the context of autoimmune diseases.[4] The RORγt LBD is an ideal domain to target via small molecules. Small molecules targeting RORs come in at least two types: inverse agonists, which block ROR-dependent transcriptional activity; and agonists, which enhance the transactivation of RORs.[5]

Table 1. Structure of RORs ligands[7]

Name	Structure	Receptor preferences	Ligand type	Ref.
T0901317		RORα, RORγ, LXRα, LXRβ, PXR, FXR, other	RORs: inverse agonist LXRS, PXR, FXR: agonist	8; 9
SR1001		RORα RORγ	Inverse agonist	11
SR1078		RORα RORγ	Agonist	12
SR3335		RORα	Inverse agonist	6
SR2211		RORγ	Inverse agonist	12
TMP778		RORγ	Inverse agonist	13
TMP920		RORγ	Inverse agonist	13
GSK805		RORγ	Inverse agonist	13

Cholesterol		RORα	Agonist	14
Cholesterol sulfate		RORα	Agonist	14
Digoxin		RORγ	Inverse agonist	15
Ursolic acid		RORγ	Inverse agonist	16
7α-Hydroxycholesterol		RORα RORγ	Inverse agonist	17
7β-Hydroxycholesterol		RORα RORγ	Inverse agonist	17
7-Ketocholesterol		RORα RORγ	Inverse agonist	17
20α-hydroxycholesterol		RORγ	Agonist	18
22R-Hdroxycholesterol		RORγ	Agonist	18
25-Hydroxycholesterol		RORγ	Agonist	18
24S-hydroxycholesterol		RORα RORγ	Inverse agonist	19
24,25-Epoxy-cholesterol		RORγ	Inverse agonist	19

24R-Hydroxycholesterol		RORγ	Inverse agonist	19

Since the discovery of the first small molecule T0901317[6,8,9] (Table 1), many RORγt ligands with agonistic and inverse agonistic activity have been disclosed in the literature.[7,10] Using the T0901317 scaffold as a lead compound, a series of synthetic RORγ inverse agonists have been developed, including SR1001, SR1555, and SR2211.[6-11] Some structurally complex natural products, such as digoxin and ursolic acid have also been reported to be RORγ inverse agonists.[15,16] Dan Littman's group, who discovered the crucial role for RORγt in T_H17 cells, identified the cardiac glycoside digoxin as a specific inhibitor for RORγt transcriptional activity using a chemical library screening.[15] They confirmed that digoxin inhibited murine T_H17 cell differentiation without affecting other T cell lineages, and it was efficient in a mouse EAE model. Digoxin was also identified in a random screening campaign, as an inhibitor of mouse and human T_H17 cell differentiation, and the crystal structure of the LBD of RORγt in complex with digoxin at 2.2 A° resolution has been solved. (Fig. 2). [15,17]

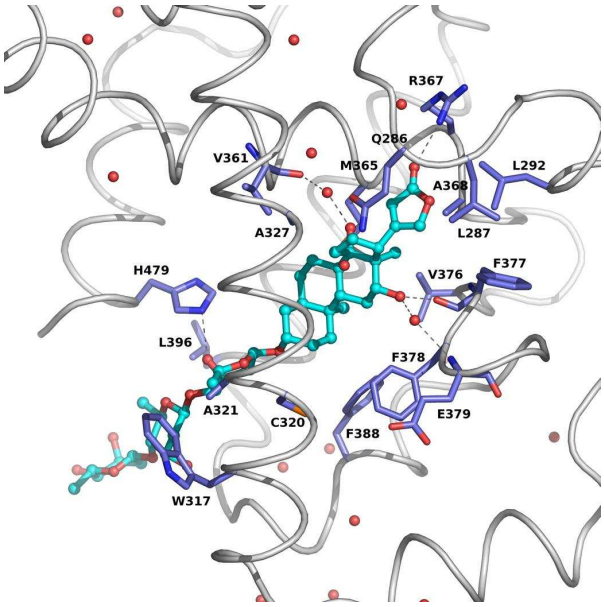


Figure 2. Digoxin binding mode in the RORγt ligand binding domain.[15]

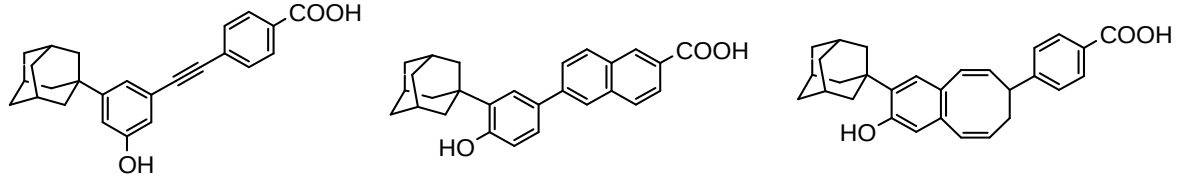
Ursolic acid, another natural product, was also found in a compound library screening as an inhibitor of RORγt.[16] Importantly, both digoxin and ursolic acid have cholesterol-like chemical structures, which might account for their similar action on the NR.

Recently, a team at Genentech identified N-isobutyl- N-((5-(4-(methylsulfonyl)phenyl)thiophen-2-yl)methyl)-1-phenylmethanesulfonamide as a RORγt inverse agonist via a biochemical screening campaign.[20] Although the development of RORγt inverse agonists has shown significant promise, [21,22] the development of new RORγt selective modulators with therapeutic potential still remains an urgent need.

Wang et al.[23] first reported that the natural products 7α-hydroxycholesterol[17] and 24S-hydroxycholesterol[19] were inverse agonists (i.e. functional antagonists) of RORα and RORγ that suppressed transcriptional activities in hepatocytes. Oxysterols are well known natural ligands for the related NR including the liver X receptor (LXR), therefore their interaction with the LBDs of RORs was not surprising.[24] Most small molecule inhibitors and drugs are based on cyclic systems, which leads to a stiffening of the molecule, resulting in enhanced target affinity due to less entropy loss upon binding. The structural homology of NRs suggested to evaluate ligands for other class of receptors as possible cognate compounds that opportunely modified could switch their target classes becoming specific RARs/RORs agonists or inverse agonists.

1.1 Designing a lead compound

Very recently, the authors were involved in expanding their research in the field of inflammatory and auto-immune diseases, by modulating the activity of NRs. Looking through the NRs superfamily and the chemical variety of the ligands scaffolds (polyenes, polycyclic compounds, aromatic or aliphatic rings, eicosanoids, farnesoids, oxysterols, and tryptamine) (Figure 1), it could seem very unlikely that a novel RORγ inverse agonist lead candidate could be designed. However, the authors decide to explore the possibility to target RORγ receptor with a novel lead candidate, characterized by a cyclopenta[a]phenantrene scaffold. The design of a novel RORγ inverse agonist lead candidate was rationalized by means of a structure-based approach founded on hybridization of chemical structures, which mix the features of RORγ natural ligands (cholesterol-like derivatives, digoxin, ursolic acid) with the features of representative arotenoids (Figure 3).[24] This choice was made because RARs and RORs receptors are evolutionarily closed and shared sequence homology.[6]



20.7 CD437 CD153

Figure 3. Arotenoids selective ligands

The envisaged novel RORs inverse agonist may result then conceptually defined by the following attributes:

1) A central polycyclic fused structure is present in other natural ligands of different classes of NRs assuring a suitable molecular volume to fulfill the LBD of the ROR receptors. The conservation of volumes among the natural ligands of nuclear receptors is likely to be a useful criterion in the design of high-affinity analogs.[2] It serves as a linker and supporting structure for the other fundamental chemical functions necessary for delivering the biological activity of the compound.
2) An aromatic ring as usually represented in arotenoids[24]
3) A large lipophilic scaffold (cyclic, polycyclic or poly methylated scaffold) mimicking the cyclic RA function or other bulky substituents connected to the polycyclic linker
4) A polar terminus corresponding to or mimicking the RA and ursolic acid acidic function (COOH or any of the known bioisosters or derivatives)
5) A hydroxylic function, as represented in arotenoids, cholesterol-like ligands and ursolic acid

The molecular structure of a lead compound might be the tetracycle MG 2778 as shown in Figure 4:

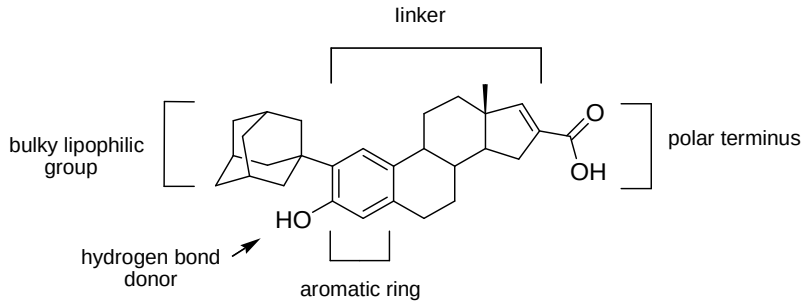


Figure 4. Lead structure of MG 2778. The molecule can be divided into three parts: an acidic head, a cyclopenta[a]phenantrene backbone, and a lipophilic tail. The activity was investigated after structural modification of lipophilic group.

The early objective was to develop an efficient synthetic path for obtaining the proposed compound as described in Figure 4 (MG 2778). MG 2778 is a cyclopenta[a]phenantrene derivative bearing an adamanthyl group at 2 position. This large group in position 2 was placed also because it was found to be effective in reducing hormonal effects of estrone and estradiol analogs in non-feminizing neuroprotective agents and so preventing estrogen receptor binding.[25] It also has an α-β-unsaturated carboxylic group at 16 and a phenolic hydroxyl at position 3. Next, with the aim to obtain preliminary SARs, a small series of analogs modified at position 2 of the polycyclic nucleus with

groups other than adamanthyl but maintaining the lipophilic and bulky features was planned, since a suitable substitution at this position is considered significant for giving selectivity. To synthesize 2-substituted analogs we adopted methods such as Friedel-Crafts alkylation, acylation and Suzuki-Miyaura cross-coupling reactions on aromatic ring. Six compounds (**7**, **8**, **19a**, **30**-**32**) were tested for cytotoxicity and estrogen receptor activity. The selected four non-cytotoxic compounds (**7**, **8**, **19a**, **31**) were assayed in a Gal4 UAS-Luc co-transfection system in order to determine their ability to modulate RORγt activity in a cellular environment. They were evaluated as inverse agonists taken ursolic acid as reference compound.[26] Results from the synthetic work and preliminary biological evaluation are reported.

2. Results and Discussion

2.1 Docking simulations of MG 2778 (8) in RORγt LBD

We further employed molecular modelling analysis to simulate MG 2778 (**8**) binding in the RORγt binding pocket. We selected the crystal structure of RORγt in complex with one of the best-known inverse agonists, digoxin (PDB code 3B0W).[27] Computer docking simulation of compound **8** was performed using Maestro 10.5 Glide software SP precision.

Figure 5 shows the binding mode of the most favoured pose of compound **8** in the presumptive binding site in comparison with digoxin. We found that compound **8** could be readily accommodate in the pocket. Moreover, RORγt shows a binding pocket mostly characterized by hydrophobic residues (Leu-287, Leu-292, Trp-317, Cys-320, Ala-321, Ala-327, Val-361, Met-365, Ala-368, Val-376, Phe-377, Phe-378, Phe-388, Leu-396) which suggests a binding interaction mode mainly characterised by hydrophobic interactions. No direct interaction between compound **8** and the residues responsible for digoxin binding was found.[15] However, even if the molecular volume of compound **8** is smaller than that of digoxin, it is possible that the bulky substituent in position 2 of the cyclopenta[a]phenanthrene core (which occupies the position of the first sugar ring in digoxin) might be sufficient to disturb the polar interactions observed in the agonist-bound RORγt LBD, involving His-479, Tyr-502 and Phe-506 which would be important to stabilize the active conformation of helix H12.[15,17]

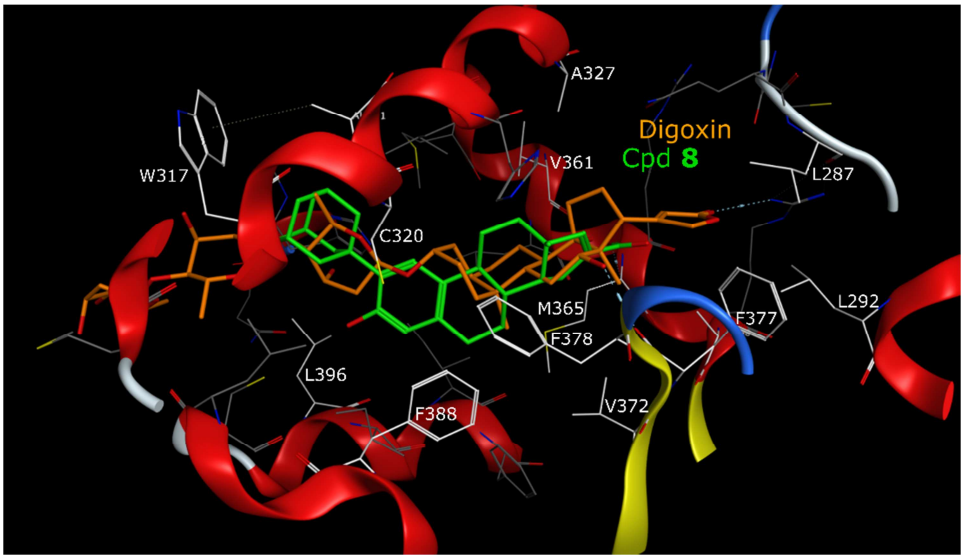


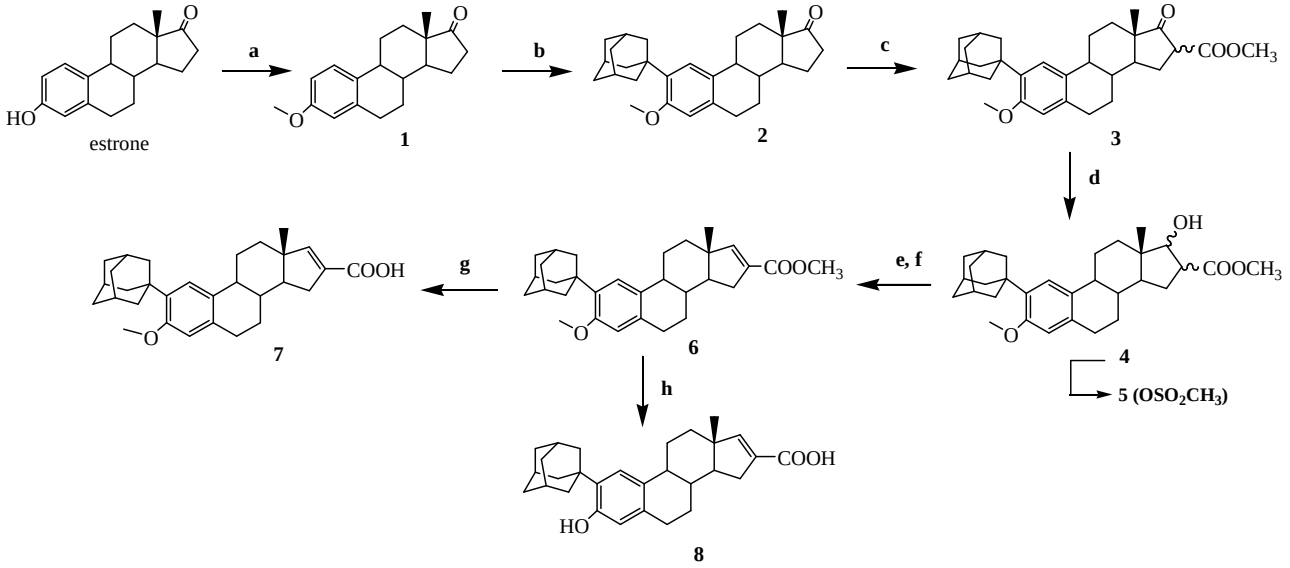
Figure 5. Comparison of the crystallographic structure of digoxin (in orange) in complex with RORγt ligand binding domain (Protein Data Bank code 3B0W) and the energetically most favourable pose of compound **8** (in green) obtained by molecular docking simulation. Hydrophobic residues are shown in white. Hydrogen atoms are omitted.

2.2 Chemistry

The synthetic work has been organized into four schemes that describe the optimized synthetic pathways as a result of trials to improve yields and purity of reaction products. The schemes report the routes carrying to final compounds for the synthesis of which the pre-formed polycyclic scaffold 3-hydroxyestra-1,3,5(10)-triene-17-one (estrone) was selected as starting material. In all cases, the early protection of phenolic OH was necessary to prevent unresolvable mixtures formation along the pathway. Schemes 1 and 2 describe two alternative routes to obtain compound **8** (named MG 2778) by performing the same reactions in a different order. For this purpose, intermediate **1** was obtained from the starting commercial estrone by alkylating with CH_3I in the presence of Bu_4NI and NaOH 10% in CH_2Cl_2 at 70° C (99% yield).[28] As previously reported,[29] compound **1** was submitted to a Friedel-Crafts reaction conducted with adamanthanol, BF_3 Et_2O in hexane for 4 h. The reaction proved to be highly region-specific yielding only the 2-adamanthyl substituted compound **2** (95%yield). The following 16-C methoxycarbonylation reaction[30] was carried out with dimethyl carbonate, NaH at refluxing (85° C) for 3h yielding compound **3** (yield 93%). In order to form the 16-17 double bond, at first the 17-carbonyl group was reduced to secondary alcohol **4** by a chemo-selective reaction with $NaBH_4$[31] in a mixture of THF/CH_3OH 9:1 for 1 h at room temperature (yield 90%). The obtained alcohol **4** was mesylated with MsCl in anhydrous CH_2Cl_2[32] giving the intermediate ester 17-methylsulfonate **5**, which by treatment with DBU in benzene[32] for 6h at 60°C and after Flash Chromatography purification, furnished the precursor intermediate **6** (60% yield).

The last step to produce the designed compound **8** was attempted with various hydrolytic methods and most of them failed. Among all, the treatment with, MeOH, NaOH 2M, in CH_2Cl_2[33] for 96 h gave the acid derivative **7** by 95% yield and only the method involving the use of $NaSCH_3$ in NMP at refluxing for 9h[34] was successful in giving the desired compound **8** with a yield of 56%.

Scheme 1. Synthesis of compound **8**

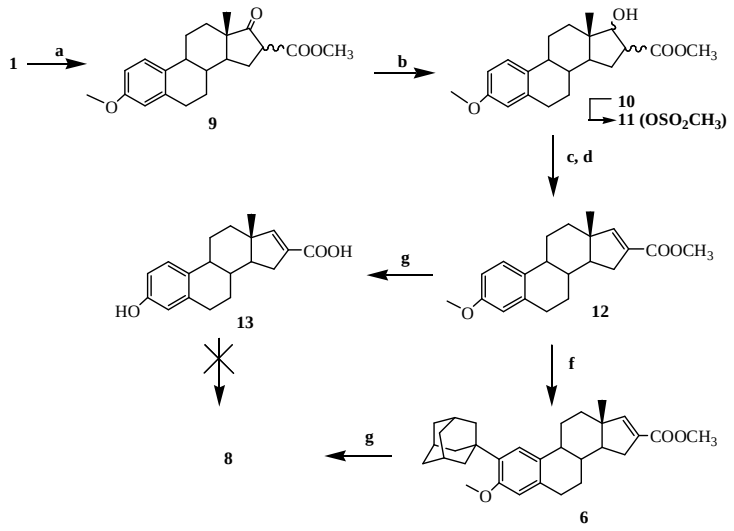


Reagents and conditions: a) CH_3I, Bu_4N+I-, CH_2Cl_2, NaOH 10%, ref., 3h, 99%; b) 1-adamantanol, BF_3Et_2O, hexane, 4h, 95%; c) $C_3H_6O_3$, NaH, ref., 3h, 93%; d) $NaBH_4$, THF/CH_3OH 9:1, 1h, 90%; e) MsCl, Et_3N, anhydrous CH_2Cl_2; f) DBU, C_6H_6, ref., 6h, 60%; g) NaOH, MeOH, CH_2Cl_2, 96 h, 90%; h) $NaSCH_3$, NMP, ref., 9h, 56%.

In scheme 2, the route to compound **8** was set up in an attempt to improve the work up of reaction mixtures. Indeed, through the previous reactions scheme 1, with compounds bearing the 2-adamanthyl substitution the procedure resulted difficult. Thus, the adamanthyl moiety was inserted at the end of the pathway. Henceforward, compound **1** was transformed into the 16-methoxycarbonylated derivative **9**[30] (93%) that was reduced to the 17-hydroxylic derivative **10**[31] (60%)**.** Then, the last was mesylated to compound **11** and this reacting with DBU produced the precursor compound **12**[32] (84%) showing the 16-17 double bond. At this point, the introduction of the adamanthyl group again produced only compound **6** but unfortunately with low yields (12%).[29] Evidently, the presence of the 16-17 double bond provoked the formation of byproducts in the F-C reaction. Following, compound **13** gave the described acid **8** by reacting with $NaSCH_3$ and NMP at reflux.[34] Accordingly, by comparing the two synthetic pathways (scheme 1 and 2), it was concluded that by the pathway in scheme 2 the scope to facilitate the synthetic work was achieved, but despite

the laborious work up, the pathway in scheme 1 was undoubtedly the more advantageous because of the higher yields.

Scheme 2. An alternative pathway for the synthesis of compound **8**

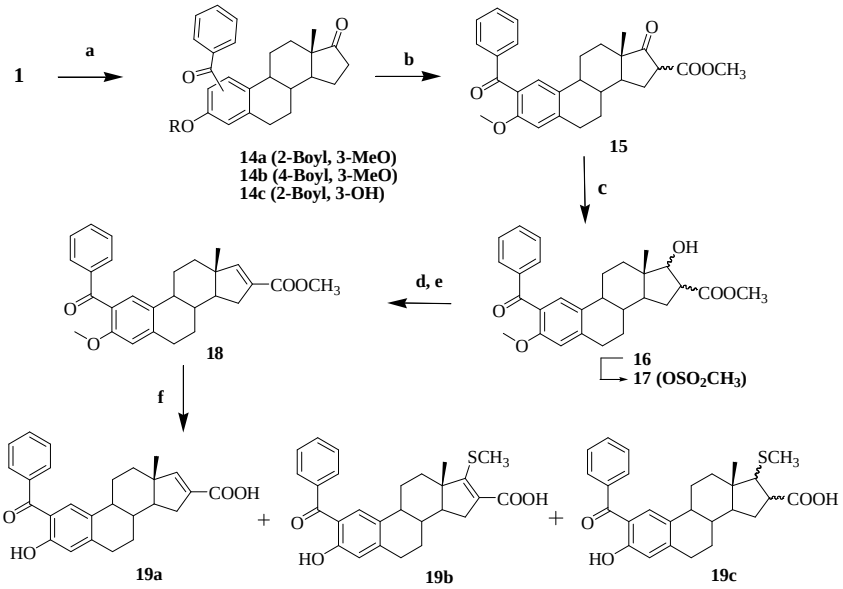


Reagents and conditions: a) $C_3H_6O_3$, NaH, ref., 3h, 95%; b) $NaBH_4$, THF/CH_3OH 9:1, 1h, 60%; c) MsCl, Et_3N, anhydrous CH_2Cl_2, 84%; d) DBU, C_6H_6, ref., 6h, 99%; e) NaOH, MeOH, CH_2Cl_2, 96 h, 90%; f) 1-adamanthanol, BF_3Et_2O, hexane, 4h, 12%; g) $NaSCH_3$, NMP, ref., 5h, 32%.

Next, in view of the synthesis of various 2-substituted analogs of **8**, the synthetic work has proceeded with an assessment of the reactivity of 3-methoxylated estrone **1** towards the Friedel-Crafts (F-C) acylation and the Suzuky-Miyaura (S-M) cross-coupling reaction. For this purpose, following the above useful pathway and carrying out the same kind of reactions as in scheme 1, scheme 3 describes the synthesis of 2-benzoyl-compound **14**. The 3-methoxy-estrone **1** was submitted to the F-C reaction with benzoyl chloride in the presence of $AlCl_3$ in CH_2Cl_2 at 0°C for 3 h.[35] In this case, a mixture of three compounds was obtained that were separated by Flash Chromatography. As expected, due to the more electron-rich position 2, the 2-benzoyl-3-methoxy-derivative **14a** was retrieved in greater amount (58%), the 4-benzoyl-methoxy isomer **14b** (31%) and in lesser amount the 2-benzoyl-3-hydroxy derivative **14c** (2%). The last formed due to the demethylating property of reaction conditions. Compound **14a** was then transformed into the 16-methoxycarbonylated derivative **15** (33%)[30] before being selectively reduced to the 17-hydroxylic compound **16** by $NaBH_4$ (97%).[31] This compound was first mesylated (**17,** 17-OSO_2CH_3)[32] and thereafter by treatment with DBU, compound **18** (17-H)[32] showing the 16-17 double bond, was obtained (21%). Finally, compound **18** was reacted with $NaSCH_3$ in DMF[34] for 1 h when at this time the starting compound disappeared on monitoring the reaction progress by TLC. After work-up of the reaction mixture, the raw material was purified by Flash Chromatography giving three

compounds, identified as **19a, b** and **c.** Unfortunately, the desired compound **19a** was present in lesser amount (25%), **19b** (37,5%) and **19c** (37%). The different reactivity of benzoyl compound **18** in comparison with compound **6** (schemes 1 and 2) towards $NaSCH_3$ has not been understood. In this case, the F-C acylation reaction of 3-methoxy-estrone, as for some reported alkylation[35] other than with adamanthanol, was proved not to be a regio-specific reaction. Therefore, it is possible to conclude that the lack of region specificity of F-C acylation towards position 2 together with the low yields of compound **19a** might represent a drawback for the future synthesis of novel 2-substituted analogs.

Scheme 3. Synthesis of compound **19**



Reagents and conditions: a) benzoyl chloride, $AlCl_3$, anhydrous DCM, 3h, 91%; b) $C_3H_6O_3$, NaH, rif., 3h, 31%; c) $NaBH_4$, THF/CH_3OH 9:1, 0.5 h, 94%; d) MsCl, Et_3N, anhydrous CH_2Cl_2; e) DBU, C_6H_6, rif., 5h, 21%; f) $NaSCH_3$, DMF, 1h., 63%.

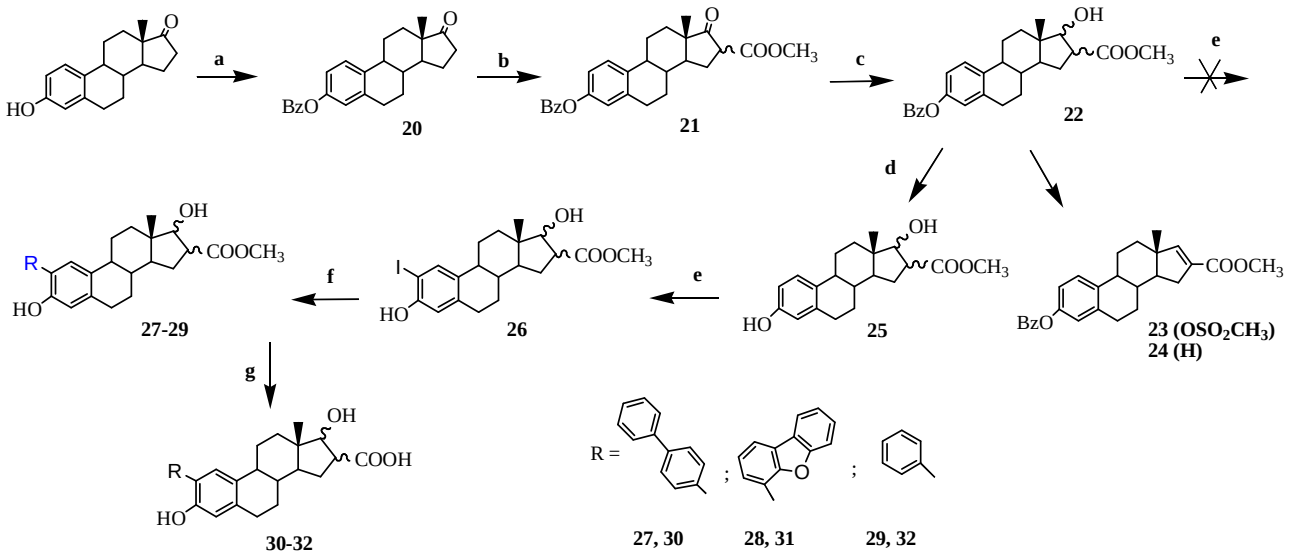
It is known that the Suzuki-Miyaura cross-coupling reaction[36] is a robust method to obtain a variety of aromatic derivatives because of the large amount of commercially available boronic acids, therefore it was planned to study also the possibility to obtain novel 2-substituted analogs by this kind of cross-coupling. In scheme 4, the synthesis of three novel 2-substituted compounds by this method is reported.

Preliminary results suggested an optimal pathway where the starting estrone was protected as benzyl ether, easily removable later in the path, giving compound **20** (BzCl, Bu_4NI) (99%)[28] that was then transformed into the 16-methoxycarbonylated derivative **21**, as before (81%).[30] This was first selectively reduced with $NaBH_4$ to the corresponding alcohol **22** (68%).[31] After mesylation of

17-hydroxy (**23**) and the next treatment with DBU, compound **24** was obtained (54%). In previous experiments it was seen that as for 3-methoxy compound **12**, also the 2-benzyloxy derivative **24** resulted not to be a suitable intermediate for iodination step. Thus, compound **22** was catalytically reduced (Pd/C 10%, H_2)[37] producing the 2,17-dihydroxylic derivative **25** (93%) that was submitted to the successful iodination to compound **26** with NIS, $(CF_3SO_3)_3In$ in CH_3CN for 8h.[38] Bromination had previously been carried out on 3-methoxy-estrone **1** (scheme 1) but it was slightly region-selective (data not shown) and mainly with the 2-Br-derivatives the cross-coupling did not take place later in the synthesis. The iodination of compound **25** with NIS yielded the desired 2-iodinated product **26** (51%) and a little amount of 4-iodinated and 2,4-diiodinated as deduced from ^1H NMR spectrum of the reaction mixture. Therefore, iodinating with NIS and $(CF_3SO_3)_3In$ proved to be more region-selective compared with the other methods carried out (data not shown). It is worth to underline that the chromatographic purification of **26** in presence of other two iodinated compounds was only feasible when the two phenolic and alcoholic hydroxyls were free. Unfortunately, for compound **26** 16-17 double bond formation was no longer possible. Preliminarily, the S-M cross-coupling reaction of compound **26** was accomplished with three boronic acids of different hindrance and following two different methods: conventional synthesis[38] and MW added organic synthesis.[39] The first one provided only complex mixtures, while the second one was found to be successful due to the following advantages: shorter reaction times, higher yields, less by-products and thus easier to process mixtures. After flash chromatography purification, compounds **27-29** were obtained in good yields 26%, 33%, 42%, respectively.

Finally, the three methyl esters **27-29** were transformed into the corresponding acids by treatment with MeOH-NaOH 10% giving the compounds **30** (99%), **31** (98%) and **32** (97%).[40] For all the synthesized compounds, complete characterization was carried out by mono-dimensional ^1H- ^{13}C- and bi-dimensional HSQC, HMBC and COSY NMR experiments.

Scheme 4. Synthesis of compounds **30-32**



Reagents and conditions: a) BzCl, $Bu_4N^+I^-$, CH_2Cl_2, NaOH 10%, rif., 3h, 99%; b) $C_3H_6O_3$, NaH, rif., 3h, 31%; c) $NaBH_4$, THF/CH_3OH 9:1, 1h, 68%; d) Pd/C, H_2, EtOAc, r.t., 8h, 93%; e) NIS, $(CF_3SO_3)_3In$, CH_3CN, 8h, 51%; f) 1. $C_{12}H_{11}BO_2$, $Pd(PPh_3)_4$, K_2CO_3, $C_4H_8O_2$, MW (160° C), 30 min, 33%; 2. $Pd(PPh_3)_4$, K_2CO_3, $C_4H_8O_2$, MW (160° C), 30 min, 42%; 3. $C_6H_7BO_2$, $Pd(PPh_3)_4$, K_2CO_3, $C_4H_8O_2$, MW (160° C), 30 min, 26%; g) MeOH, NaOH 10%, rif, 1h, 99%.

Furthermore, it is noted that the synthesis described in scheme 4, despite the successful S-M cross-coupling on the iodinated **26**, presents a strong restriction due to the impracticality to obtain the designed compounds with 16-17 double bond. Indeed, iodination reaction with NIS didn't work with compounds **12** and **24** and additionally the chromatographic purification of the 2-iodinated derivative was achievable only with the di-hydroxylic compound **26** that however was not suitable for the removal of 17-alcoholic OH by the method reported before.

2.3 Biology

2.3.1 Effect of compounds 7, 8, 19a, 30-32 on cell viability.

In order to verify whether the synthetic RORγt inverse agonists had any effect on cell growth and survival, MTT assay was performed on HepG2 cells. As shown in Figure 6, compound **19a** was found to be toxic at the highest concentrations tested (25 μM, $p<0.01$ *vs* vehicle; 50 μM, $p<0.001$ *vs* vehicle), whereas compounds **30** and **32** caused a significant decrease of cell viability even at lower concentrations. No cytotoxic effects were observed on after incubation of HepG2 cells with compounds **7, 8** and **31**.

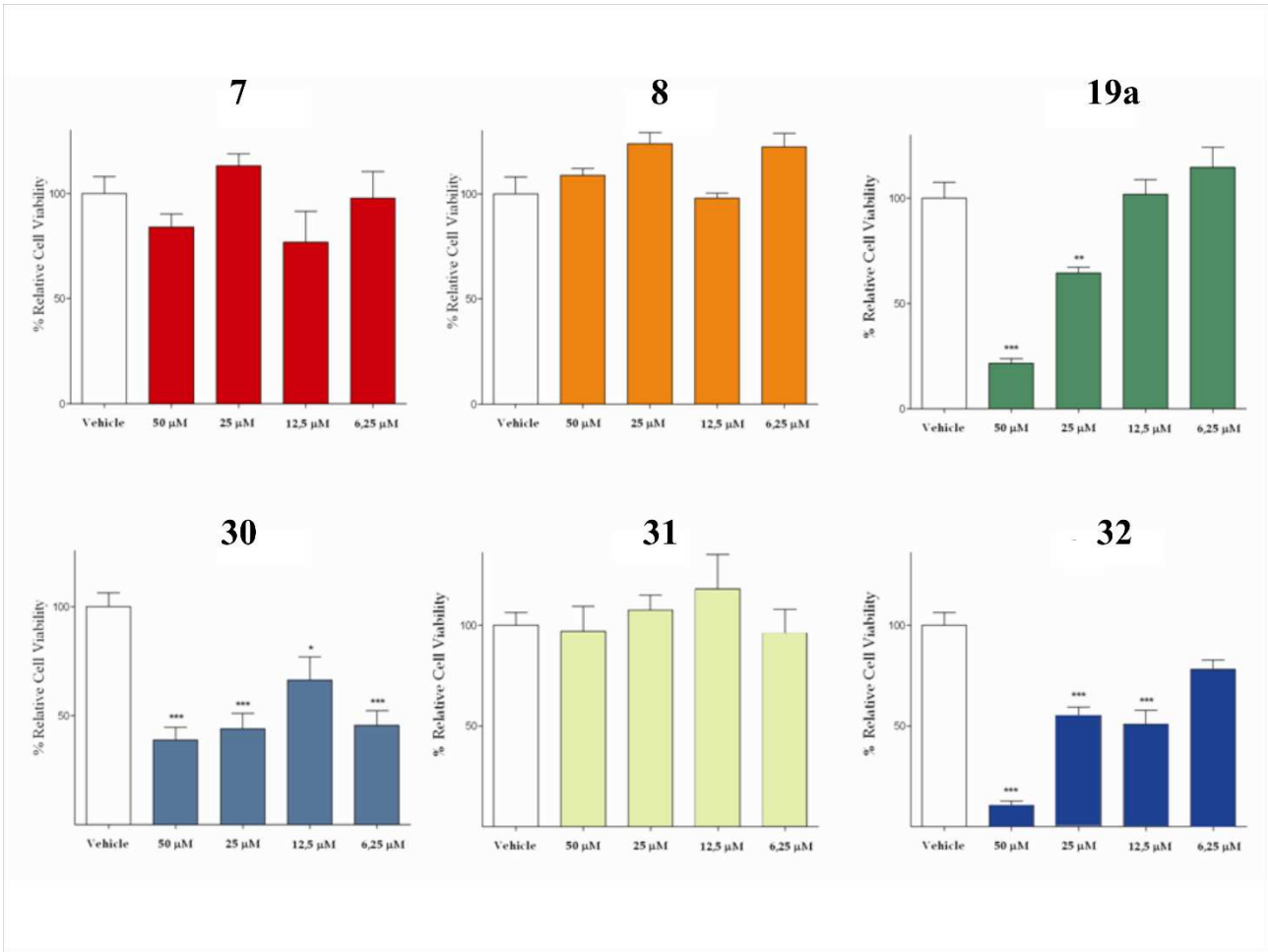
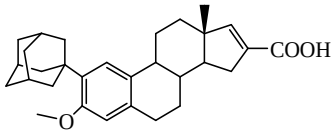
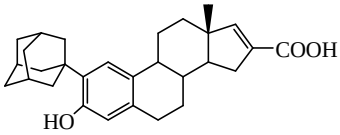
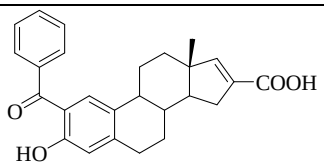
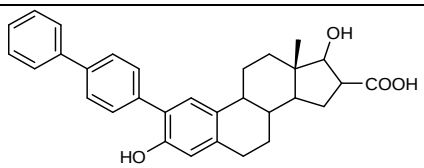
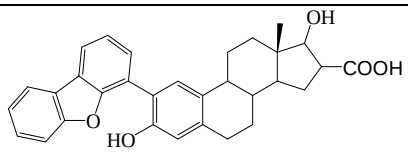
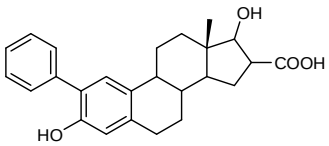


Figure 6. Cell viability assay on HepG2 cells treated with the synthetic compounds **7, 8, 19a** and **30-32**, reported as percentage of viable cells with respect to control treated with medium. Results are mean ± SEM. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs vehicle, one-way ANOVA followed by Dunnett post hoc test. Three independent experiments were performed in quadruplicate.

Table 2. Structure of compounds tested for cytotoxic and estrogenic activity

Compound	Structure	Formula	MW
7		$C_{30}H_{38}O_3$	446.62
8		$C_{29}H_{36}O_3$	432.27
19a		$C_{26}H_{26}O_4$	402.18
30		$C_{31}H_{32}O_4$	468.59
31		$C_{31}H_{30}O_5$	482.57
32		$C_{25}H_{28}O_4$	392.49

2.3.2 Estrogenic activity of the synthetic compounds 7, 8, 19a and 30-32.

Estrogenic activity of the novel steroidal compounds **7, 8, 19a** and **30-32** was evaluated because of the molecular structure being derived from estrone, a known estrogenic agent. Real time PCR analysis was performed on RNA extracts from an estrogen-receptors (ERs) expressing cell line (MCF-7). Cells were treated with the compounds in order to test whether the expression of GREB1 and CXCL12, two target genes for ERs, was altered. Figure 7 shows that the expression of both GREB1 and CXCL12 was increased by compound **8** ($p<0.05$ and $p<0.001$ for GREB and CXCL12 mRNA expression *vs* vehicle, respectively), **30** ($p<0.001$ for GREB and CXCL12 mRNA expression *vs* vehicle), **31** ($p<0.001$ for GREB and CXCL12 mRNA expression *vs* vehicle) and **32**

(p<0.001 and p<0.05 for GREB and CXCL12 mRNA expression *vs* vehicle, respectively), while neither compound **7** nor **19a** display any estrogenic activity.

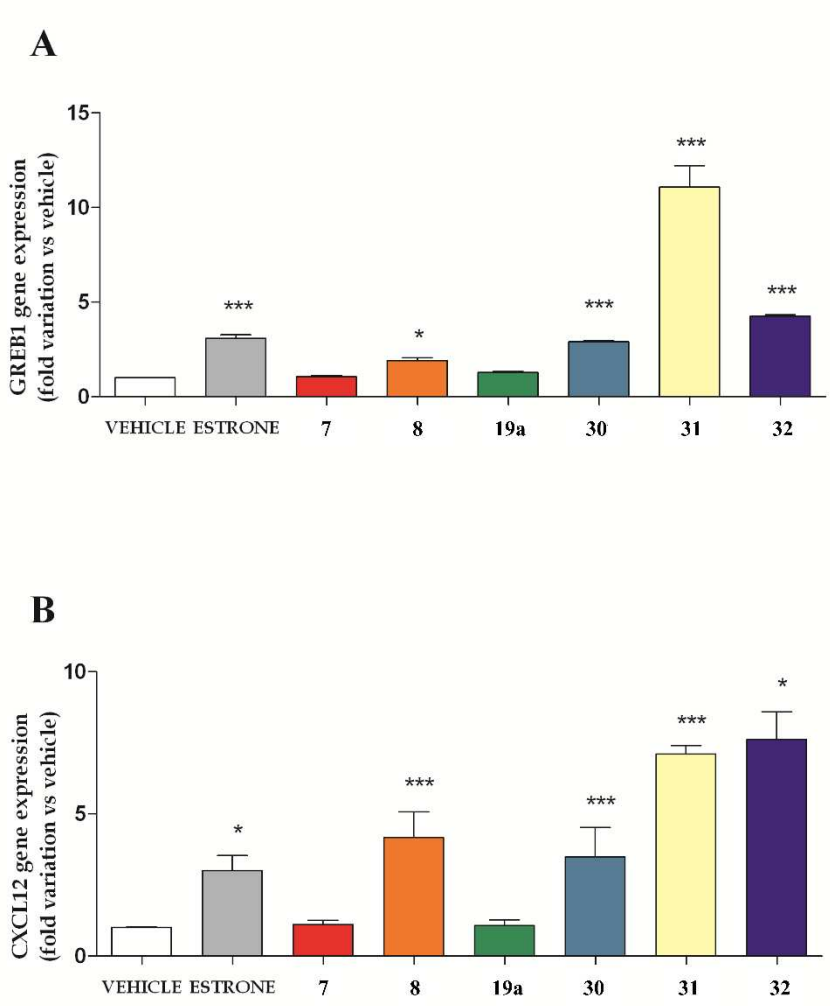


Figure 7. Gene expression of GREB1 (A) and CXCL12 (B) in MCF-7 cells treated with the synthetic compounds **7, 8, 19a** and **30-32**. All the compounds were tested at 2 µM concentration. Results are mean ± SEM. * p<0.05 and *** p<0.001 vs vehicle, one-way ANOVA followed by Dunnett post hoc test. Three independent experiments were performed in triplicate.

2.3.3 Evaluation of inverse-agonist activity of compounds 7, 8, 19a and 31 on RORγt

Based on the MTT assay results, where compounds **7, 8** and **31** did not display any cytotoxic activity, and compound **19a** was cytotoxic only at the highest concentrations (25-50 µM), compounds **7, 8, 19a** and **31** were selected for evaluating their ability to modulate the in vitro RORγt activity in a cellular environment by means of a Gal UAS-Luc cotransfection system taken ursolic acid as reference compound. Since the absence of *in vitro* cytotoxicity at low concentrations is a promising feature for candidate drugs designed for lifetime lasting diseases such as autoimmune

diseases, no further in vitro characterization of **30** and **32** was performed. To ascertain whether HEK-293 cells had been successfully transfected with the plasmids, RORγt protein expression was evaluated by means of Western Blot analysis. As shown in Figure 8, the cells transfected with all three plasmids (RORγ-Gal4, UAS-Luc, NanoGlo) express RORγt, whereas the cells transfected with the plasmids UAS-Luc and NanoGlo do not express the protein containing the RORγt LBD. Densitometric analysis confirmed that RORγt is not expressed in lanes 2 and 3 (Data not shown).

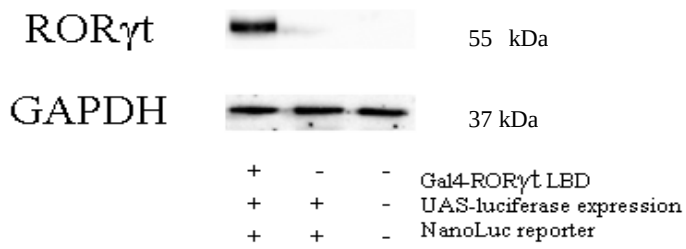


Figura 8. Western blot analysis of RORγt (58 kDa) protein in whole protein extracts of HEK-293 cells transfected with Gal4-RORg LBD plasmid, UAS-luc and NanoLuc reporter plasmid (+) or not-transfected cells (-). GAPDH (43 KDa) was used as loading control.

Figure 9 shows the ability of the tested compounds to decrease activity, as luminescence lessening, at various concentrations. After 2 μM treatment only compound 19a displayed a slight but significant activity, at 5 μM both compounds **8** and **19a** decreased activity in a significant amount, at 10 μM a dramatic decrease in RORγ activity could be observed after addition for all the tested compounds ($p<0.001$), and finally, at 20 μM all compounds showed an inhibitory effect comparable to that of ursolic acid. Fig. 9 shows that compounds **7**, **8**, **19a** and **31** displayed a concentration-dependent activity. Extrapolated IC50 values were similar for compounds **19a** and **31** (4,4 μM and 4,7 μM, respectively), and increased for compounds **7** and **8** (6,8 and 6,5 μM, respectively). The most relevant outcome of the *in vitro* RORγt inhibitory activity by the selected compounds was that compound **19a** significantly reduced RORγt activity at low concentrations (2-5 μM, $p<0.05$ *vs* vehicle).

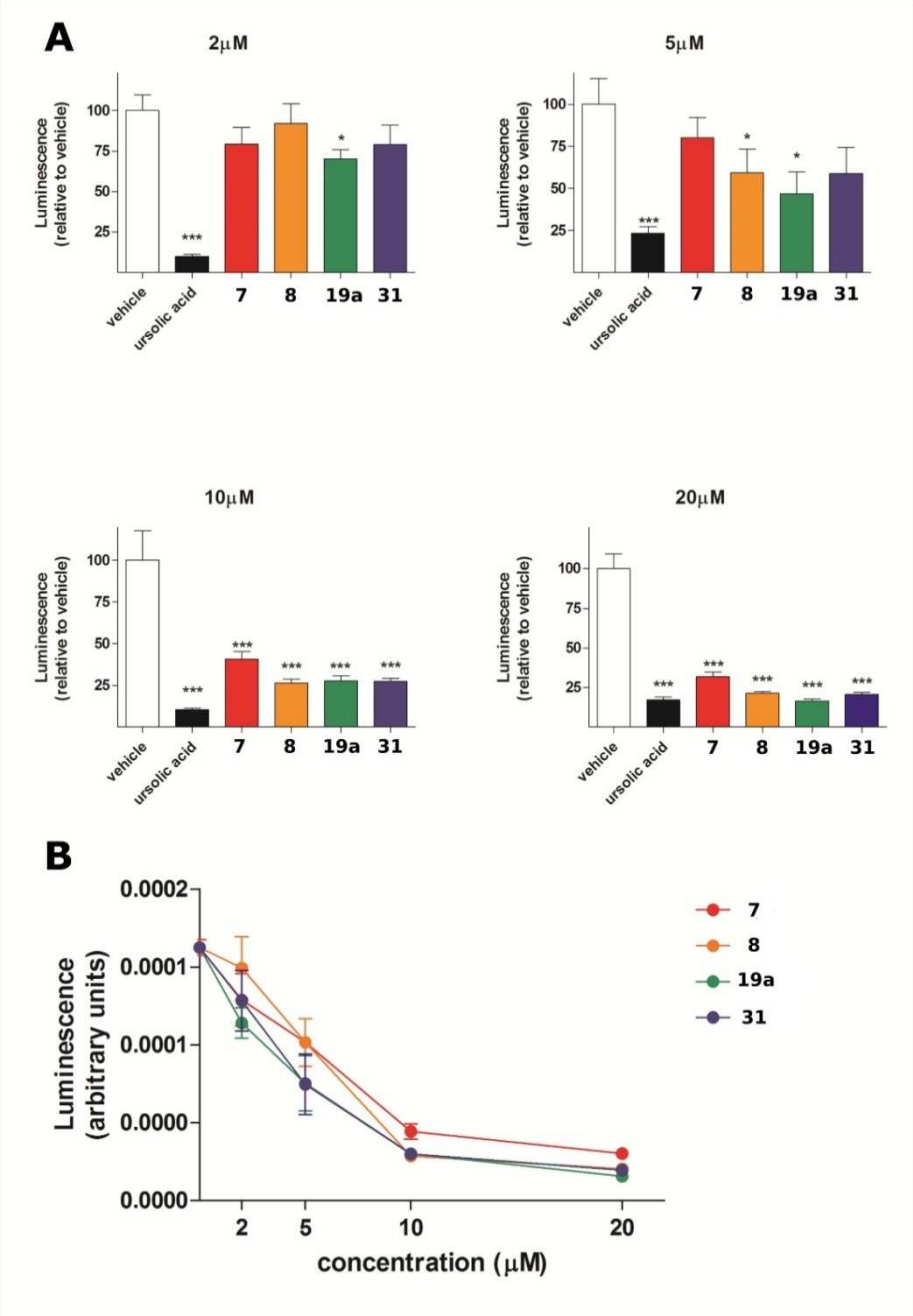


Figure 9. Evaluation of inverse-agonist activity of compounds **7, 8, 19a** and **31** on RORγt (A) and dose-dependent efficacy (B). Results are mean Â± SEM. * $p<0.05$ and *** $p<0.001$ vs vehicle, one-way ANOVA followed by Dunnett post hoc test. Three independent experiments were performed in triplicate.

2.3.4 Effect of compounds 7, 8, 19a and 31 on cell cycle distribution

In order to complete the characterization of the selected synthetic compounds, we analysed their effect on cell cycle distribution both in HepG2 and HEK-293 cells. Fig. 10 shows the effect of compounds **7**, **8**, **19a** and **31** on both cell viability of HepG2 and HEK-293 either transfected or not with RORγt- Gal4 plasmid, and cell cycle distribution. After confirming the absence of cytotoxicity

of the selected synthetic compounds on both cell lines, we also demonstrated that cell cycle distribution was not affected even after incubation with the highest concentrations (10 and 20 μM) tested previously (see Section 2.3.3).

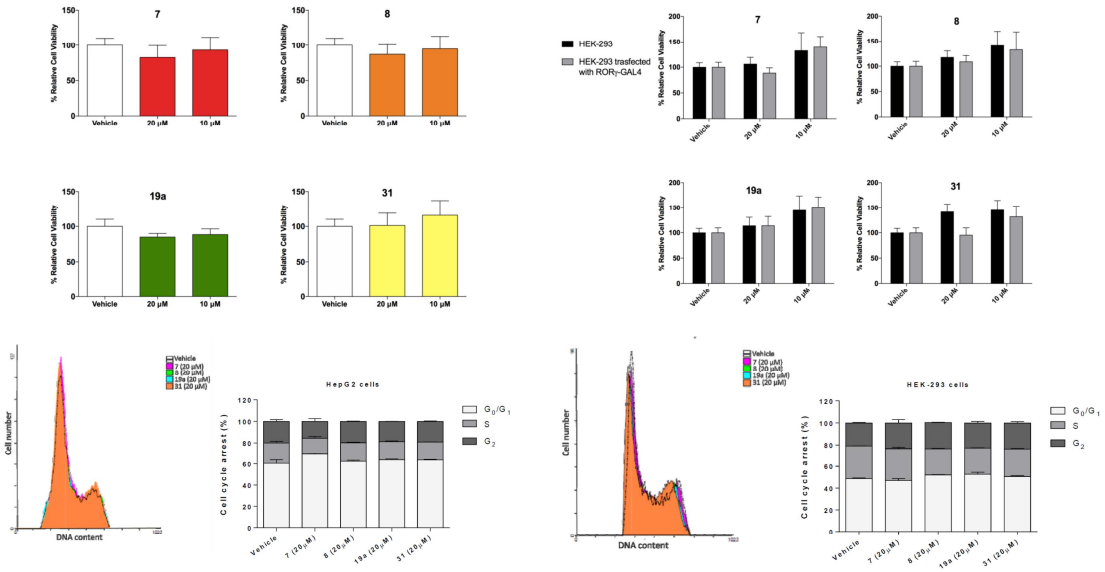


Fig. 10. Cell viability of HepG2 (left) and HEK-293 (right) cells after incubation with compounds **7**, **8**, **19a** and **31** at 10 and 20 μM. Below, cell cycle distribution analysis at 20 μM. The results are expressed as mean ± SEM. Three independent experiments were performed in duplicate.

2.4 Docking study of compound 19a

Compound **19a** was docked using the crystal structure of RORγt in complex with digoxin (PDB code 3B0W).[27] Computer docking simulation of **19a** was performed using Maestro 10.5 Glide software SP precision. The most favoured pose of **19a** (Figure 11) in the presumptive binding site is similar to the one found for **8** (Figure 5). Compound **19a** could be readily accommodated in the pocket, but also in this case, no significant interactions with residues responsible for digoxin binding were found.[15] Again, we can suggest the possibility that the substituent in position 2 (benzoyl group in this case), could perturb the interactions necessary for RORγt activity.[15,17]

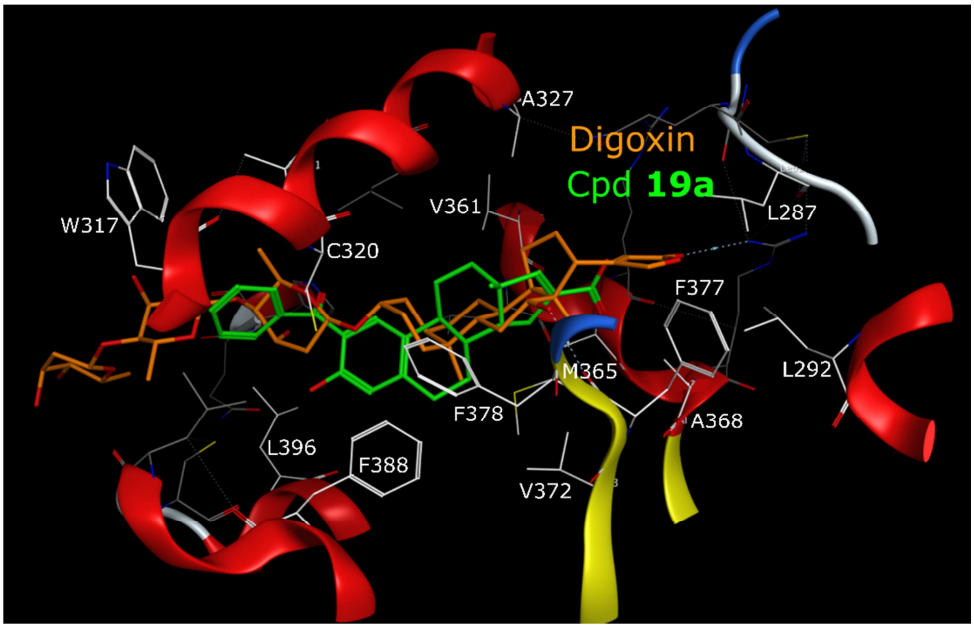


Figure 11. Comparison of the crystallographic structure of digoxin (in orange) in complex with RORγt ligand binding domain (Protein Data Bank code 3B0W) and the energetically most favourable pose of **19a** (in green) obtained by molecular docking simulation. Hydrophobic residues are shown in white. Hydrogen atoms are omitted.

3. Conclusion

Looking the NRs natural ligands structure through, representative arotenoids ligands and RORs ligands, by means of a structure-based approach founded on hybridization of chemical structures, a lead compound **8** (MG 2778) was identified, synthesized and chemically modified in order to obtain a small series of novel steroidal compounds acting as RORγ inverse agonists. Docking simulations of compounds **8** and 1**9a** into RORγt LBD in complex with digoxin showed a potential binding affinity.

The four non-cytotoxic compounds **7**, **8**, **19a** and **31** were tested by means of a Gal UAS-Luc co-transfection system taken ursolic acid as reference compound, resulting to act as RORγt inverse agonists in a dose dependent manner. Considering these preliminary biological results, we can propose that using the tetracycle scaffold is an appropriate approach for the further design of RORγt inverse agonists. Regarding the bound groups at 2, 3 and 16 positions, we can deduce that a bulky alkyl or aryl group in the 2 position is necessary in order to reduce estrogenic activity, although low estrogenic activity is maintained in presence of the free 3-phenolic OH as for compound **8** with respect to compound **7** (3-OCH_3). However, no estrogenic activity was observed for compound **19a** having the free 3-OH. In this case, we suggest the existence of a H-bond, between the carbonyl of the flexible benzoylic group and the phenolic OH. Probably, this event could hamper the interaction

of the OH itself at the ER, however, at the docking simulation of **19a** in RORγt LBD (Figure 10) we didn't see it. The polar terminus (16-COOH) is essential for activity while the 16-17 double bond not as noted for compound **31** that was active as well as compounds showing the double bond at that position. The potency of our compounds is lower than that of ursolic acid, the strongest known RORγt inverse agonist, but their efficacy is similar. In particular, compound **19a** was the most active, causing a significant reduction of RORγt activity at low micromolar concentrations. From the above considerations, we can conclude that **19a** may represent a good candidate for further in vitro and in vivo characterization and may serve as a useful tool for developing RORγt inverse agonists.

4. Experimental section

Melting points were determined on a Buchi M-560 capillary melting point apparatus and are uncorrected. ^{1}H NMR spectra were determined on Bruker 300 and 400 MHz spectrometers, with the solvents indicated; chemical shifts are reported in δ (ppm) downfield from tetramethylsilane as internal reference. Coupling constants are given in hertz. In the case of multiplets, chemical shifts were measured starting from the approximate centre. Integrals were satisfactorily in line with those expected based on compound structure. Mass spectra were obtained on a Mat 112 Varian Mat Bremen (70 eV) mass spectrometer and Applied Biosystems Mariner System 5220 LC/MS (nozzle potential 140 eV). Column flash chromatography was performed on Merck silica gel (250−400 mesh ASTM); chemical reactions were monitored by analytical thin-layer chromatography (TLC) on Merck silica gel 60 F-254 glass plates. Microwave assisted reactions were performed on a CEM Discover® monomode reactor with a built-in infrared sensor assisted-temperature monitoring and automatic power control; all reactions were performed in closed devices under pressure control. Solutions were concentrated on a rotary evaporator under reduced pressure. The purity of new tested compounds was checked by HPLC using the instrument HPLC VARIAN ProStar model 210, with detector DAD VARIAN ProStar 335. The analysis was performed with a flow of 1 mL/min, a C-8 column of dimensions 250 mm X 4.6 mm, a particle size of 5 mm, and a loop of 10 mL. The detector was set at 254 nm. The mobile phase consisted of phase A (Milli-Q H_2O, 18.0 MU, TFA 0.05%) and phase B (95% MeCN, 5% phase A). Gradient elution was performed as reported: 0 min, % B ¼ 10; 0e20 min, % B ¼ 90; 25 min, % B ¼ 90; 26 min, % B ¼ 10; 31 min, % B ¼ 10.

Starting materials were purchased from Sigma-Aldrich and Alfa Aesar, and solvents were from Carlo Erba, Fluka and Lab-Scan. DMSO was obtained anhydrous by distillation under vacuum and stored on molecular sieves.

Dulbecco's modified Eagle's medium (DMEM), was obtained from Sigma-Aldrich Italy (Milan, Italy). Dulbecco's modified Eagle's medium (DMEM), was obtained from Sigma-Aldrich Italy (Milan, Italy). Foetal bovine serum (FBS), glutamine and penicillin-streptomycin (pen-strep) solutions were obtained from Gibco (Life Technologies Italia, Monza, Italy).

4.1 Synthesis

*4.1.1 General procedure for the synthesis of of-protected estrone derivatives **1** and **20**.* As a typical procedure, the synthesis of the-3-methoxy-estrone derivative is described in detail. A mixture of commercial estrone (1.00 g, 3.70 mmol) and tetrabutylammonium iodide (0.068 g, 0.185 mmol) was suspended in CH_2Cl_2 (18 mL). Methyl iodide (0.875 mL, 14.06 mmol) and a 10% NaOH solution (18 mL) were added. The mixture was refluxed at 70°C for 3 h. The reaction was monitored by TLC analysis (eluent chloroform/methanol 95:5). At the end of the reaction, the two phases were clearly transparent and were separated. The aqueous phase was extracted with CH_2Cl_2 (30 mLx3) and the combined organic phases were washed with brine, dried over sodium sulphate, filtered and evaporated under vacuum to give a white solid product (1.045 g).

(8R,9S,13S,14S)-7,8,9,11,12,13,15,16-octahydro-3-methoxy-13-methyl-6*H*-cyclopenta[*a*]phenanthren-17(14*H*)-one (**1**). Yield 99%; R_f = 0.88 (chloroform/methanol, 95:5); mp = 177-178°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.84 (s, 3H), 1.49 (m, 1H), 1.50 (m, 1H), 1.52 (m, 1H), 1.53 (m, 1H), 1.61 (m, 1H), 1.65 (m, 1H), 1.97 (m, 1H), 2.05 (m, 1H), 2.09 (m, 1H), 2.27 (m, 1H), 2.42 (m, 1H), 2.51 (m, 1H), 2.53 (m, 1H), 2.93 (m, 2H), 3.80 (s, 3H), 6.67 (d, *J* = 2.73 Hz, 1H), 6.75 (dd, *J* = 8.61 Hz, *J* = 2.73 Hz, 1H), 7.23 ppm (d, *J* = 8.61 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.86, 21.60, 25.94, 26.57, 29.68, 31.60, 35.88, 38.39, 43.99, 48.03, 50.43, 55.22, 111.59, 113.89, 126.35, 132.03, 137.77, 157.91, 220.94 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{19}H_{25}O_2^+$, 285.1855; found, 285.1865.

(8R,9S,13S,14S)-3-(benzyloxy)-7,8,9,11,12,13,15,16-octahydro-13-methyl-6*H*-cyclopenta[*a*]phenanthren-17(14*H*)-one (**20**). Compound **20** was prepared as for compound **1** by reacting estrone (2.50 g, 9.25 mmol), tetrabutylammonium iodide (0.178 g, 0.462 mmol), benzyl bromide (4.18 mL, 35.14 mmol) in a mixture of CH_2Cl_2 /10% NaOH solution (45 mL each). After the workup, the obtained residue was washed with hexane to remove excess benzyl bromide yielding 3.301 g of yellow solid. Yield 99%; R_f =0.38 (n-hexane/ethyl acetate, 8:2); mp = 128-129°C; ^{1}H NMR (400 MHz, $CDCl_3$): 0.91 ppm (s, 3H), 1.50 (m, 1H), 1.52 (m, 1H), 1.53 (m, 1H), 1.56 (m, 1H), 1.62 (m, 1H), 1.65 (m, 1H), 1.99 (m, 1H), δ 2.04 (m, 1H), 2.09 (m, 1H), 2.17 (m, 1H), 2.29 (m, 1H), 2.43 (m, 1H), 2.51 (m, 1H), 2.88 (m, 2H), 5.04 (s, 2H), 6.73 (d, *J* = 2.7 Hz, 1H), 6.79 (dd, *J* = 8.6, 2.8 Hz, 1H), 7.20 (d, *J* = 8.4 Hz, 1H), 7.34 – 7.29 (m, 1H), 7.40 – 7.35 (m, 2H), 7.45 –

7.41 ppm (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 13.89, 21.62, 25.95, 26.58, 29.69, 31.63, 35.90, 38.40, 44.04, 48.04, 50.47, 70.00, 112.42, 114.94, 126.37, 127.45, 127.88, 128.57, 132.36, 137.29, 137.82, 156.90, 220.94 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{25}H_{29}O_2^+$, 361.2168; found, 361.2149.

4.1.2 (8R,9S,13S,14S)-7,8,9,11,12,13,15,16-octahydro-2-adamantyl-3-methoxy-13-methyl-6*H*-cyclopenta[*a*]phenanthre ne-17(14*H*)-one (**2**). Into a two-necked 100 mL round-bottomed flask, compound **1** (1.08 g, 3.80 mmol) and 1-adamantanol (0.70 g, 4.60 mmol) were placed and stirred for 15 min in hexane at 0°C. Under N_2 atmosphere, BF_3 Et_2O (1.6 mL, 12.74 mmol) was added dropwise with a syringe. The mixture was stirred at room temperature for 4 h. The reaction was monitored by TLC analysis (eluent cyclohexane/ethyl acetate, 8:2). At the end of the reaction, the mixture was transferred to a single-necked round-bottomed flask and the solvent was removed under vacuum. The obtained residue was treated with water to obtain a yellowish solid. The solid was filtrated and dried overnight under vacuum to yield 1.55 g of yellow powder. Yield 95%; R_f = 0.50 (cyclohexane/ethyl acetate, 8:2); mp = 253°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.93 (s, 3H), 1.47 (m, 1H), 1.53 (m, 1H), 1.54 (m, 1H), 1.56 (m, 1H), 1.64 (m, 1H), 1.65 (m, 1H), 1.76 (6H), 1.97 (m, 1H), 2.01 (m, 1H), 2.06 (m, 1H), 2.11 (3H), 2.20-2.08 (6H), 2.30 (m, 1H), 2.47 (m, 1H), 2.53 (m, 2H), 2.92 (m, 2H), 3.83 (s, 3H), 6.63 (s, 1H), 7.18 ppm (s, 1H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 13.90, 21.61, 26.04, 26.63, 29.17, 29.30, 31.60, 35.92, 36.94, 37.16, 38.55, 40.79, 44.36, 48.08, 50.42, 55.03, 112.09, 123.68, 131.03, 134.72, 136.07, 156.87, 221.12 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{29}H_{39}O_2^+$, 419.2950; found, 419.2932.

4.1.3 (8R,9S,13S,14S)-7,8,9,11,12,13,15,16-octahydro-2-benzoyl-3-methoxy-13-methyl-6*H*-cyclopenta[*a*]phenanthre ne-17(14*H*)-one (**14**). In a dried round-bottomed flask, a suspension of anhydrous $AlCl_3$ (1.260 g, 9.453 mmol) in 15 mL of anhydrous CH_2Cl_2 was prepared. The mixture was cooled to 0°C and benzoyl chloride (0.880 mL, 7.574 mmol) was added dropwise. The mixture turned pink and was stirred for 1 h at room temperature. After this period, the mixture was cooled again at 0°C and then a solution of compound **1** (1.077 g, 3.787 mmol) in anhydrous CH_2Cl_2 (10 mL) was added dropwise. The reaction mixture turned yellow immediately and it was kept at 0°C for all the duration. The progression of the reaction was monitored by TLC analysis (hexane/ethyl acetate 6:4). At the end of the reaction, the suspension was poured into an ice/water mixture and it was acidified with concentrated HCl. The two phases were separated: the aqueous phase was extracted with CH_2Cl_2 and the resulting organic phase was washed with saturated sodium bicarbonate solution, brine and dried over sodium sulphate. The mixture was filtered, and the solvent evaporated under vacuum to yield 1.344 g of white solid. Yield 58% **14a**, 31% **14b**, 2%;

14c; R_f = 0.49 (hexane/ethyl acetate, 6:4); mp = 229°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.91 (s, 3H), 1.45 (m, 1H), 1.49 (m, 1H), 1.50 (m, 1H), 1.55 (m, 1H), 1.59 (m, 1H), 1.60 (m, 1H), 1.92 (m, 1H), 2.05 (m, 1H), 2.05 (m, 1H), 2.06 (m, 1H), 2.25 (m, 1H), 2.32 (m, 1H), 2.51 (m, 1H), 2.97 (m, 2H), 3.68 (s, 3H), 6.70 (s,1H), 7.30 (s, 1H), 7.39-7.46 (m, 2H); 7.50-7.57 (m, 1H), 7.81 ppm (dd, *J* = 8.3, 1.3 Hz, 2H); ^{13}C NMR (400 MHz, $CDCl_3$): δ 13.83, 21.56, 25.77, 26.40, 29.94, 31.45, 35.81, 38.26, 43.80, 47.94, 50.35, 55.64, 111.79, 126.47, 127.07, 128.08, 129.77, 131.98, 132.64, 138.20, 140.97, 155.54, 196.47, 220.60 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{26}H_{29}O_3^+$, 389.2117; found, 389.2212.

4.1.4 General procedure for the synthesis of derivatives (**3**,**9**,**15**,**21**). As a typical procedure, the synthesis of the methyl 2-adamantyl-3-methoxy-16-carboxylate estrone derivative **3** is described in detail. Compound **2** (0.640 g, 1.53 mmol) was suspended in dimethyl carbonate (11.2 mL, 132.91 mmol) and NaH (0.320 g, 13.33 mmol) was added. A catalytic amount of CH_3OH was added. The mixture was refluxed for 3h at controlled temperature (85°C). The reaction was monitored by TLC analysis (eluent cyclohexane/ethyl acetate, 8:2). At the end of the reaction, the mixture was cooled at room temperature and quenched with CH_3OH (1 mL). The mixture was acidified with glacial acetic acid and poured into water (150-200 mL). The suspension was stirred and once the precipitate was formed, filtrated to obtain a yellow precipitate that was dried overnight under vacuum to yield 0.511 g of yellow powder.

(8R,9S,13S,14S)-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-2-adamantyl-3-methoxy-13-methyl-17-oxo-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylate (**3**). Yield 93%; R_f = 0.30 (cyclohexane/ethyl acetate, 8:2); mp = 180-181°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.98 (s, 3H), 1.37 (m, 1H), 1.51 (m, 1H), 1.52 (m, 1H), 1.57 (m, 1H), 1.63 (m, 1H), 1.65 (m, 1H), 1.76 (6H), 1.85 (m, 1H), 1.99 (m, 1H), 2.03 (m, 1H), 2.11 (3H), 2.20-2.08 (6H), 2.29 (m, 1H), 2.44 (m, 1H), 2.88 (m, 2H), 3.21 (dd, *J* = 9.9, 8.5 Hz, 1H), 3.76-3.79 (s, 3H), 3.80 (s, 3H), 6.61 (s, 1H), 7.14 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.31, 25.90, 26.40, 29.17, 29.30, 32.50, 36.90, 36.94, 37.16, 38.40, 40.79, 44.80, 47.87, 50.42, 52.50, 54.10, 55.03, 112.09, 124.20, 131.03, 134.72, 136.07, 156.87, 169.87, 212.05 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{31}H_{41}O_4^+$, 477.3005; found, 477.3015.

(8R,9S,13S,14S)-methyl7,8,9,11,12,13,14,15,16,17-decahydro-3-methoxy-13-methyl-17-oxo-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylate (**9**). Compound **9** was prepared as for compound **3** by reacting compound **1** (1.045 g; 3.674 mmol) with dimethyl carbonate (26.9 mL, 319 mmol) and NaH (0.768 g, 32.01 mmol). Yield 93%; R_f = 0.33 (hexane/ethyl acetate, 8:2); ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.98 (s, 3H), 1.37 (m, 1H), 1.51 (m, 1H), 1.52 (m, 1H), 1.57 (m, 1H), 1.63 (m,

1H), 1.65 (m, 1H), 1.85 (m, 1H), 1.99 (m, 1H), 2.03 (m, 1H), 2.29 (m, 1H), 2.44 (m, 1H), 2.88 (m, 2H), 3.21 (dd, *J* = 9.9, 8.5 Hz, 1H), 3.76-3.79 (s, 3H), 3.80 (s, 3H), 6.67 (d, *J* = 2.73 Hz, 1H), 6.75 (dd, *J* = 8.61 Hz, 2.73 Hz, 1H), 7.23 ppm (d, *J* = 8.61 Hz, 1H).

(8R,9S,13S,14S)-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-2-benzoyl-3-methoxy-13-methyl-17-oxo-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylate (**15**). Compound **15** was prepared as for compound **3** by reacting compound **14** (1.259 g; 3.241 mmol) with dimethyl carbonate (23.7 mL, 281.5 mmol) and NaH (0.677 g, 28.23 mmol). An amount of 1.290 g of a crude product was obtained, and this was purified by silica gel flash column chromatography (hexane/ethyl acetate) to give 0.430 g of compound **15**. Yield 33%; R_f = 0.35 (hexane/ethyl acetate, 2:1); mp = 114°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.99-0.96 (m, 3H), 1.40(m, 1H), 1.41 (m, 1H), 1.42 (m, 1H), 1.45 (m, 1H), 1.52 (m, 1H), 1.90 (m, 1H), 2.07 (m, 1H), 2.07 (m, 1H), 2.24 (m, 1H), 2.26 (m, 1H), 2.27 (m, 1H), 2.91 (m, 2H), 3.22 (dd, *J* = 9.9, 8.4 Hz, 1H), 3.68 (s, 3H), 3.77 (s, 3H), 6.70 (s,1H), 7.29 (s, 1H), 7.39-7.46 (m, 2H); 7.50-7.57 (m, 1H), 7.81 ppm (dd, *J* = 8.3, 1.3 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 13.27/14.34, 25.70, 26.31, 26.39, 30.05, 31.57/31.77, 37.82, 43.78, 47.82, 48.89, 52.61, 54.01, 55.62, 111.75, 126.49, 127.05, 128.09, 129.77, 131.75, 132.69, 138.14, 140.91, 155.54/155.56, 169.80/170.32, 196.48, 211.79 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{28}H_{31}O_5^+$, 447.2171; found, 447.2157.

(8R,9S,13S,14S)-methyl 3-(benzyloxy)-7,8,9,11,12,13,14,15,16,17-decahydro-13-methyl-17-oxo-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylate (**21**). Compound **21** was prepared as for compound **3** by reacting compound **20** (3.301 g; 9.157 mmol) with dimethyl carbonate (67.03 mL, 795.46 mmol) and NaH (1.914 g, 79.78 mmol) to yield 3.104 g of yellow powder. Yield 81%; R_f = 0.27 (hexane/ethyl acetate, 8:2); mp = 155°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.98 ppm (s, 3H), 1.45 (m, 1H), 1.46 (m, 1H), 1.48 (m, 1H), 1.51 (m, 1H), 1.62 (m, 1H), 1.63 (m, 1H), 1.98 (m, 1H), 2.03 (m, 1H), 2.07 (m, 1H), 2.25 (m, 1H), 2.40 (m, 1H), 2.89 (m, 2H), 3.21 (dd, *J* = 9.9, 8.5 Hz, 1H), 3.76 (s, 3H), 5.04 (s, 2H), 6.74 (d, *J* = 2.6 Hz, 1H), 6.79 (dd, *J* = 8.6, 2.7 Hz, 1H), 7.20 (d, *J* = 8.5 Hz, 1H), 7.32 (t, *J* = 7.1 Hz, 1H), 7.38 (t, *J* = 7.3 Hz, 2H), 7.43 ppm (d, *J* = 6.9 Hz, 2H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 13.29, 25.78, 26.54, 29.56, 31.94, 36.90, 37.94, 43.99, 47.95, 48.94, 52.57,54.07, 69.97, 112.47, 114.92, 126.32, 127.42, 127.87, 128.55, 132.00,137.22, 137.67, 156.94, 169.85, 212.90 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{27}H_{31}O_4^+$, 419.2222; found, 419.2237.

4.1.5 General procedure for the synthesis of derivatives (**4**,**10**,**16**,**22**). As a typical procedure, the synthesis of methyl 17-hydroxy-2-adamantyl-3-methoxy-13-methyl-16-carboxylate **4** is described in detail. Compound **3** (1,711g, 3.59 mmol) was suspended in a mixture THF/CH_3OH 9:1 (20 mL).

The mixture was cooled and stirred for 15 min at 0°C, then $NaBH_4$ (0.156 g, 4.12 mmol) was added carefully in portions. The temperature was maintained at 0°C and the reaction was monitored by TLC analysis (eluent cyclohexane/ethyl acetate, 2:1). The reaction was completed in 0.5h. The mixture was acidified with HCl 2N solution and extracted with ethyl acetate. The combined organic phases were washed with brine, dried over sodium sulphate, filtered and evaporated to dryness to yield 1.725 g of spongy solid. The crude product was purified by silica gel flash column chromatography (hexane/ethyl acetate) to give 1.53 g of white solid.

(8R,9S,13S,14S)-methyl 7,8,9,11,12,13,14,15,16,17-decahydro-17-hydroxy-2-adamantyl-3-methoxy-13-methyl-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylate (**4**). Yield 90%; R_f = 0.57 (hexane/ethyl acetate, 2:1); mp = 210-211°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.83 (s, 3H), 1.18 (m, 1H), 1.32 (m, 1H), 1.35 (m, 1H), 1.45 (m, 1H), 1.55 (m,1H), 1.68 (m, 1H), 1.76 (6H), 1.91 (m, 1H), 1.80 (m, 1H), 2.02 (m, 1H), 2.02 (3H), 2.10 (6H), 2.32 (m, 1H), 2.35 (m, 1H), 2.83 (m, 2H), 3.14 (dd, *J* = 18.7, 9.1 Hz, 1H), 3.72 (s, 3H), 3.80 (s, 3H), 3.89 (d, *J* = 10.0 Hz, 1H), 6.59 (s, 1H), 7.16 ppm (s, 1H); ^{13}C NMR (300 MHz, $CDCl_3$): δ 11.67, 26.71, 27.25, 27.74, 29.48, 29.71, 37.24, 37.49, 37.62, 38.72, 41.11, 44.44, 44.57, 44.69, 48.96, 52.04, 55.10, 82.16, 112.39, 124.03, 131.70, 135.07, 136.25, 156.87, 175.94 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{31}H_{43}O_4^+$, 479.3161; found, 479.3149.

(8R,9S,13S,14S)-methyl 7,8,9,11,12,13,14,15,16,17-decahydro-17-hydroxy-3-methoxy-13-methyl-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylate (**10**). Compound **10** was prepared as for compound **4** by reacting compound **9** (1.195, 3.49 mmol) with $NaBH_4$ (0.151 g, 4.005 mmol) for 0.5 h, to give 1.166 g of spongy solid. The crude product was purified by silica gel flash column chromatography (hexane/ethyl acetate) to give 0.717 g of white solid. Yield 60%; R_f = 0.53 (hexane/ethyl acetate, 2:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.83 (s, 3H), 1.18 (m, 1H), 1.32 (m, 1H), 1.35 (m, 1H), 1.45 (m, 1H), 1.57 (m, 1H), 1.63 (m, 1H), 1.68 (m, 1H), 1.85 (m, 1H), 1.99 (m, 1H), 2.02 (m, 1H), 2.32 (m, 1H), 2.35 (m, 1H), 2.83 (m, 2H), 3.14 (dd, *J* = 18.7, 9.1 Hz, 1H), 3.72 (s, 3H), 3.80 (s, 3H), 3.88 (d, *J* = 10.0 Hz, 1H), 6.67 (d, *J* = 2.73 Hz, 1H), 6.75 (dd, *J* = 8.61 Hz, 2.73 Hz, 1H), 7.23 ppm (d, *J* = 8.61 Hz, 1H).

(8R,9S,13S,14S)-methyl 2-benzoyl-7,8,9,11,12,13,14,15,16,17-decahydro-17-hydroxy-3-methoxy-13-methyl-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylate (**16**). Compound **16** was prepared as for compound **4** by reacting compound **15** (0.564g, 1.263 mmol) with $NaBH_4$ (0.055 g, 1.449 mmol) for 0.25 h, to give 0.553 g of spongy solid. Yield 97%; R_f = 0.54 (hexane/ethyl acetate, 1:1); mp = 250°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.84 (s, 3H), 1.23 (m, 1H), 1.31(m, 1H), 1.31 (m, 1H), 1.47 (m, 1H), 1.70 (m, 1H), 1.87 (m, 1H), 1.87 (m, 1H), 1.93 (m, 1H), 2.01 (m,1H), 2.17 (m, 1H), 2.98 (m, 2H), 3.14 (dd, *J* = 18.8, 9.1 Hz, 1H), 3.67 (s, 3H), 3.72 (s, 3H), 3.87 (d, *J* =

10.0 Hz, 1H), 6.68 (s,1H), 7.30 (s, 1H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.54 (t, *J* = 7.3 Hz, 1H), 7.83 – 7.77 ppm (m, 2H); ^{13}C-NMR (101 MHz, $CDCl_3$): δ 11.28, 27.19, 27.44, 29.67/29.25, 30.01, 37.16/37.07, 38.14, 43.75, 44.05, 44.38, 48.65, 51.86, 55.64, 81.71, 111.76, 126.41, 127.16, 128.11/128.07, 129.79, 132.33, 132.59, 138.27, 141.02, 155.47, 175.41, 196.53 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{28}H_{33}O_5^+$, 449.2328; found, 449.2548.

(8R,9S,13S,14S)-methyl 3-(benzyloxy)-7,8,9,11,12,13,14,15,16,17-decahydro-17-hydroxy-13-methyl-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylate (**22**). Compound **22** was prepared as for compound **4** by reacting compound **21** (3.104 g, 7.42 mmol) with $NaBH_4$ (0.322 g, 8.52 mmol) for 0.5 h, to give 3.586 g of orange solid. The crude product was purified by silica gel flash column chromatography (cyclohexane/ethyl acetate) to give 2.44 g of white solid. Yield 68%; R_f = 0.45 (cyclohexane/ethyl acetate, 2:1); mp = 186°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.84 ppm (s, 3H), 1.38 (m, 1H), 1.41 (m, 1H), 1.55 (m, 1H), 1.56 (m, 1H), 1.77 (m, 1H), 1.90 (m, 1H), 2.05 (m, 1H), 2.12 (m, 1H), 2.26 (m, 1H), 2.36 (m, 1H), 2.89 (m, 2H), 3.13 (m, 1H), 3.73 (s, 3H), 3.88 (d, 1H), 5.03 (s, 2H), 6.72 (d, *J* = 2.7 Hz, 1H), 6.78 (dd, *J* = 8.4, 2.6 Hz, 1H), 7.20 (d, *J* = 8.5 Hz, 1H), 7.31 (dd, *J* = 8.5, 5.9 Hz, 1H), 7.37 (dd, *J* = 8.1, 6.5 Hz, 2H), 7.43 ppm (d, *J* = 6.9 Hz, 2H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 11.29, 26.24, 27.32, 29.33, 29.69, 37.16, 38.19, 43.90, 44.04, 48.62, 51.88, 69.93, 81.76, 112.29, 114.79, 126.35, 127.43, 127.84, 128.53, 132.64, 137.25, 137.81, 156.74, 175.57 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{27}H_{33}O_4^+$, 421.2379; found, 421.2364.

4.1.6 General procedure for the synthesis of derivatives (**6**,**12**,**18**). As a typical procedure, the synthesis of methyl 2-adamantyl-3-methoxy-16-carboxylate derivative **6** is described in detail. In a double-necked round bottomed flask compound **4** (0.659g, 1.377 mmol) was dissolved in anhydrous CH_2Cl_2. Under a N_2 atmosphere, triethylamine (0.273mL, 1.956 mmol) was added dropwise to the solution and then methanesulfonyl chloride (0.112 mL, 1.456mmol) was poured into the mixture. The obtained solution was stirred overnight. The mixture was then washed with water, saturated $NaHCO_3$ solution, brine, filtered and evaporated under vacuum to yield a yellow solid (**5**). The obtained residue (0.741 g, 1.331 mmol) was then dissolved in benzene (20 mL), and DBU (0.397 mL, 2.662 mmol) was added. Under a N_2 atmosphere, the reaction mixture was refluxed for 5 h. The progress of the reaction was monitored by TLC analysis (hexane/ethyl acetate 2:1). Even though the reaction was not completed, the mixture was cooled and washed with equivalent volumes of 5% HCl solution, brine and saturated $NaHCO_3$ solution. The organic phase was evaporated to dryness under vacuum and the obtained crude product was purified by silica gel

flash column chromatography (hexane/ethyl acetate) to give 0.179 g of white solid correspondent to the desired product (**6**) and 0.287 g of starting material.

(8S,9S,13S,14S)-methyl 7,8,9,11,12,13,14,15-octahydro-2-adamantyl-3-methoxy-13-methyl-6*H*-cyclopenta [*a*] phenanthrene-16-carboxylate (**6**). Yield 60%; R_f = 0.80 (hexane/ethyl acetate, 2:1); mp = 179°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (s, 3H), 1.50 (m, 1H), 1.63 (m, 1H), 1.65 (m, 1H), 1.70 (m, 1H), 1.77 (6H), 1.91 (m, 1H), 1.97 (m, 1H), 2.05 (3H), 2.09-2.06 (6H), 2.10 (m, 1H), 2.27 (m, 1H), 2.37 (m, 1H), 2.42 (m, 1H), 2.58 (m, 1H), 2.86 (m, 2H), 3.76 (s, 3H), 3.81 (s, 3H), 6.62 (s, 1H), 6.92 (d, *J* = 1.7 Hz, 1H), 7.14 (s, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 16.48, 26.68, 28.13, 29.48, 29.55, 31.51, 35.38, 37.24, 37.49, 37.73, 41.11, 44.90, 47.50, 51.73, 55.30, 55.34, 112.44, 123.60, 131.85, 135.14, 135.26, 136.16, 155.12, 157.02, 166.78 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{31}H_{41}O_3^+$, 461.3056; found, 461.3067.

(8S,9S,13S,14S)-methyl 7,8,9,11,12,13,14,15-octahydro-3-methoxy-13-methyl-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylate (**12**). Compound **12** was prepared as for compound **6** by reacting compound **10** (0.660 g, 1.916 mmol) with triethylamine (0.379 mL, 2.722 mmol) and methanesulfonyl chloride (0.119 mL, 2.026 mmol). The obtained crude product **11** (0.659 g, 1.560 mmol) was treated with DBU (0.466 mL, 3.120 mmol) and after the work-up, it was purified by silica gel flash column chromatography (hexane/ethyl acetate) to give 0.523 g of white solid. Yield 84%; R_f = 0.83 (hexane/ethyl acetate, 2:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (s, 3H), 1.50 (m, 1H), 1.63 (m, 1H), 1.65 (m, 1H), 1.70 (m, 1H), 1.91 (m, 1H), 1.97 (m, 1H), 2.10 (m, 1H), 2.27 (m, 1H), 2.37 (m, 1H), 2.42 (m, 1H), 2.58 (m, 1H), 2.86 (m, 2H), 3.76 (s, 3H), 3.81 (s, 3H), 6.67 (d, 1H), 6.75 (d, *J* = 1.7 Hz, 1H), 7.23 ppm (d, 1H).

(8S,9S,13S,14S)-methyl 7,8,9,11,12,13,14,15-octahydro-2-benzoyl-3-methoxy-13-methyl-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylate (**18**). Compound **18** was prepared as for compound **6** by reacting compound **16** (0.656 g, 1.462 mmol) with triethylamine (0.289 mL, 2.077 mmol) and methanesulfonyl chloride (0.119 mL, 1.546 mmol). The obtained crude product **17** (0.495 g, 0.940 mmol) was treated with DBU (0.317 mL, 2.126 mmol) and after the work-up, it was purified by silica gel flash column chromatography (hexane/ethyl acetate) to give 0.104 g of white solid. Yield **21%;** R_f = 0.66 (hexane/ethyl acetate, 2:1); mp = 107°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.91 (s, 3H), 1.54 (m, 1H), 1.61 (m, 1H), 1.68 (m, 1H), 1.72 (m, 1H), 1.74 (m, 1H), 1.90 (m,1H), 2.04 (m, 1H), 2.35 (m, 1H), 2.38 (m,1H), 2.39 (m, 1H), 2.60 (m, 1H), 2.89 (m, 2H), 3.68 (s, 3H), 3.71 (s, 3H), 6.65 (s, 1H), 6.85 (d, *J* = 1.8 Hz, 1H), 7.22 (s, 1H), 7.44 (t, *J* = 7.4 Hz, 2H), 7.58 – 7.50 (m, 1H), 7.84 – 7.77 (m, 2H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 15.95, 26.04, 27.65, 29.99, 31.23, 34.78, 37.10, 43.95, 46.99, 51.54, 54.88, 55.60, 111.57, 126.47, 126.79, 128.09, 129.83, 132.52, 132.59,

132.60, 134.72, 141.27, 154.60, 155.43, 166.51, 196.63 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{28}H_{31}O_4^+$, 431.2222; found, 419.2473.

4.1.7 (8S,9S,13S,14S)-7,8,9,11,12,13,14,15-octahydro-2-adamantyl-3-methoxy-13-methyl-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylic acid (**7**). In a round bottomed flask, compound **6** (0.166g, 0.360 mmol) was dissolved in a mixture of CH_2Cl_2/CH_3OH (9:1), and then 2 mL of 3M methanolic NaOH solution were added. The mixture was stirred at room temperature for 96 h. The progression of the reaction was monitored by TLC analysis (hexane/ethyl acetate 2:1). At the end of the reaction, 1M HCl solution was added and the organic phase was extracted with $CHCl_3$. The combined organic phases were washed with 1M HCl solution, brine and dried over sodium sulphate. After filtration, the organic phase was evaporated to dryness to yield 0.153 g of white solid. Yield 95%; R_f = 0.49 (hexane/ethyl acetate, 2:1); mp = over 300°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (s, 3H), 1.46 (m, 1H), 1.58 (m, 1H), 1.65 (m, 1H), 1.72 (m, 1H), 1.77 (6H), 1.81 (m, 1H), 1.95 (m, 1H), 2.03 (m, 1H), 2.05 (3H), 2.09-2.06 (6H), 2.27 (m, 1H), 2.36 (m, 1H), 2.37 (m, 1H), 2.56 (m, 1H), 2.88 (m, 2H), 3.80 (s, 3H), 6.61 (s, 1H), 7.05 (d, *J* = 1.7 Hz, 1H), 7.14 (s, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 16.38, 26.65, 28.13, 29.46, 29.59, 31.19, 35.20, 37.22, 37.47, 37.72, 41.09, 44.85, 47.79, 55.27, 55.35, 112.43, 123.62, 131.77, 134.74, 135.15, 136.19, 157.03, 157.88, 170.75 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{30}H_{39}O_3^+$, 447.2899; found, 447.2878. RP-C8 HPLC: t_R = 19.80 min, 98.9% (A%).

4.1.8 (8S,9S,13S,14S)-7,8,9,11,12,13,14,15-octahydro-2-adamantyl-3-hydroxy-13-methyl-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylic acid (**8**). Compound **7** (0.103 g, 0.217 mmol) was dissolved in 5 mL of NMP and treated with a suspension of $NaSCH_3$ (0.092 g, 1.32 mmol) in 5 mL of NMP. The mixture was refluxed for 5 h and monitored by TLC analysis. Once the starting material spot disappeared on TLC, a mixture of water and ice was added, and then 1M HCl solution until pH=1. The mixture was extracted with ethyl acetate, washed with water, brine and dried over sodium sulphate. The solvent was evaporated under vacuum and the black residue obtained was dissolved with diluted NH_3 solution. The solution was acidified again with 1M HCl until pH=1 to obtain a subtle precipitate. The suspension was centrifugated and the supernatant discarded. The obtained powder was dried to yield 0,057 g of final product. Yield 56%; R_f = 0.49 (hexane/ethyl acetate, 2:1); mp = over 300°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (s, 3H), 1.46 (m, 1H), 1.72 (m,1H), 1.75 (m, 1H), 1.76 (m, 1H), 1.77 (6H), 1.93 (m, 1H), 1.97 (m, 1H), 2.06 (m, 1H), 2.07 (3H), 2.11 (6H), 2.30 (m, 1H), 2.31 (m, 1H), 2.42 (m, 1H), 2.66 (m, 1H), 2.88 (m, 2H), 6.39 (s, 1H), 7.03 (d, *J* = 1.7 Hz, 1H), 7.12 ppm (s, 1H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 16.98, 26.16, 28.91,

29.46, 29.59, 32.06, 34.85, 36.45, 37.47, 37.65, 40.07, 44.52, 47.39, 55.15, 116.3, 123.74, 131.74, 133.69, 134.19, 134.73, 151.14, 157.60, 168.99 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{29}H_{37}O_3^+$, 433.2743; found, 433.2761. RP-C8 HPLC: t_R = 16.59 min, 99.1% (A%).

4.1.9 (8S,9S,13S,14S)-7,8,9,11,12,13,14,15-octahydro-2-benzoyl-3-hydroxy-13-methyl-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylic acid (**19**). Compound **18** (0.124 g, 0.289 mmol) was dissolved in 5 mL DMF and treated with $NaSCH_3$ (0.123 g, 1.759 mmol). The mixture was refluxed for 1 h and monitored by TLC analysis (hexane/ethyl acetate 1:1). Once the starting material spot disappeared on TLC, DMF was evaporated under vacuum and the residue was acidified with 1M HCl. The mixture was extracted with ethyl acetate, washed with water, brine and dried over sodium sulphate. The solvent was evaporated under vacuum to give 0.074 g of a spongy yellow solid. The crude product was purified by RP-C18 flash column chromatography (tetrahydrofuran/water 8:2) to give a solid correspondent to the products: 25% **19a**, 37,5% **19b** and 37% **19c** as approximately evaluated by ^{1}H-NMR. The mixture was further separated by a flash column chromatography (Ethyl acetate/hexane 8:2) yielding the desired compound 0.0185 g. Overall yield 16%; mp = over 300°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.87 (s, 3H), 1.56 (m, 1H), 1.66 (m, 1H), 1.68 (m, 1H), 1.70 (m, 1H), 1.71 (m, 1H), 1.91 (m,1H), 2.02 (m, 1H), 2.31 (m, 1H), 2.31 (m,1H), 2.36 (m, 1H), 2.58 (m, 1H), 2.91 (m, 2H), 6.69 (s, 1H), 6.90 (d, *J* = 1.7 Hz, 1H), 7.28 (s, 1H), 7.42 (t, *J* = 7.4 Hz, 2H), 7.57 – 7.50 (m, 1H), 7.83 – 7.78 (m, 2H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 16.09, 26.11, 27.56, 29.98, 31.16, 34.81, 37.12, 43.99, 47.10, 55.62, 111.77, 126.27, 126.78, 128.07, 129.79, 132.52, 132.61, 132.61, 134.88, 141.17, 154.67, 155.45, 166.40, 196.66 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{26}H_{27}O_4^+$, 403.1909; found, 403.1889. RP-C8 HPLC: t_R = 17.75 min, 98.7% (A%).

4.1.10 (8R,9S,13S,14S)-methyl 7,8,9,11,12,13,14,15,16,17-decahydro-3,17-dihydroxy-13-methyl-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylate (**25**). Into a double-necked round bottomed flask, previously dried in oven, about 0.300 g of Pd/C 10% and approximately 40 ml of ethyl acetate were placed. After connecting the flask to an elastomer balloon containing hydrogen gas, the mixture was stirred at room temperature for 1h to saturate the suspension of Pd/C with hydrogen. Then, compound 22 (2.121 g, 5.04 mmol) in 20 mL of ethyl acetate was added dropwise to the suspension, and the mixture was stirred under hydrogen at atmospheric pressure and heated by means of an oil bath at 50 °C for 8 h, monitoring the progression of the reaction by TLC analysis (cyclohexane/ethyl acetate 2:1). At the end of the reaction the mixture was filtered, and the solution was concentrated to dryness on a rotavapor to give 1.550 g of white solid. Yield 93%; R_f = 0.20

(cyclohexane/ethyl acetate, 2:1); mp = 125°C ^{1}H NMR (300 MHz, DMSO-d6): δ 0.76 (s, 3H), 1.14 (m, 1H), 1.21 (m, 1H), 1.24 (m, 1H), 1.25 (m, 1H), 1.32 (m, 1H), 1.41 (m, 1H), 1.81 (m, 1H), 1.82 (m, 1H), 1.83 (m, 1H), 2.11 (m, 1H), 2.26 (m, 1H), 2.71 (m, 2H), 3.04 (q, *J* = 8.7 Hz, 1H), 3.60 (s, 3H), 3.77 (s, 1H), 5.00 (dd, *J* = 8.8, 5.4 Hz, 1H), 6.43 (d, *J* = 2.6 Hz, 1H), 6.50 (dd, *J* = 8.4, 2.5 Hz, 1H), 7.03 (d, *J* = 8.3 Hz, 1H), 8.99 ppm (s, 1H); ^{13}C-NMR (75 MHz, DMSO-d6): δ 12.11, 26.62, 27.46, 28.59, 29.63, 37.08, 38.71, 43.88, 44.53, 46.82, 48.94, 51.72, 80.89, 113.32, 115.38, 126.64, 130.85, 137.71, 155.37, 175.70 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{20}H_{27}O_4^+$, 331.1909; found, 331.1901.

4.1.11 (8R,9S,13S,14S)-methyl 7,8,9,11,12,13,14,15,16,17-decahydro-3,17-dihydroxy-2-iodo-13-methyl-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylate (**26**). Compound **25** (1.550 g, 4.69 mmol), N-iodosuccinimide (1.161 g, 5.160 mmol), Indium (III) trifluoromethanesulfonate (0.264 g, 0.47 mmol) were mixed together and dissolved in acetonitrile. The mixture was stirred overnight in the dark (wrapped in foil) at room temperature. The progression of the reaction was monitored by TLC analysis (cyclohexane/ethyl acetate 1:1). At the end of the reaction water was added and the organic phase was extracted with ethyl acetate. The combined organic phases were washed with brine and dried over sodium sulphate. After filtration, the solvent was evaporated under vacuum to yield 2.183 g of yellow product. The product was purified by silica gel column chromatography (*d* = 3 cm, *l* = 35 cm, 230-400 mesh, eluent cyclohexane/ ethyl acetate 1:1) to yield 0.639 g of white product. Yield 30%; R_f = 0.66 (cyclohexane/ethyl acetate, 1:1); mp = 179°C ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.82 (s, 3H), 1.16 (m, 1H), 1.31 (m, 1H), 1.32 (m, 1H), 1.48 (m, 1H), 1.51 (m, 1H), 1.76 (m, 1H), 1.88 (m, 1H), 2.05 (m, 1H), 2.09 (m, 1H), 2.16 (m, 1H), 2.24 (m, 1H), 2.78 (m, 2H), 3.13 (dd, *J* = 18.7, 9.2 Hz, 1H), 3.72 (s, 3H), 3.88 (d, *J* = 10.1 Hz, 1H), 5.74 (s br, 1H), 6.68 (s, 1H), 7.51 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.33, 26.28, 27.10, 29.23, 29.28, 37.02, 37.94, 43.52, 44.15, 45.99, 48.53, 51.99, 81.68, 82.23, 115.00, 134.75, 135.20, 138.94, 152.83, 175.63 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{20}H_{26}IO_4^+$, 457.0876; found, 457.0853.

*4.1.12General procedure for the synthesis of derivatives (***27**, **28**, **29**). As a typical procedure, the synthesis of (8R,9S,13S,14S)-methyl 7,8,9,11,12,13,14,15,16,17-decahydro-3,17-dihydroxy-13-methyl-2-(4-byphenyl)-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylate **27** is described in detail. Compound **26** (0.200 g, 0.438 mmol) was dissolved in dioxane (2 mL) and then biphenyl boronic acid (0.174 g, 0.880 mmol), potassium carbonate (0.243 g, 1.760 mmol) and tetrakis(triphenylphosphine)palladium (0) (0.050 g, 0.045 mmol) were added. The mixture was microwave irradiated at 160°C (power set point 250 W, ramp time 60 sec, hold time 30 min). The

reaction progression was monitored by TLC analysis (hexane/ethyl acetate 1:1). At the end of the reaction, the mixture was diluted with water (10 mL) and extracted with ethyl acetate. The combined organic phases were dried over sodium sulphate, filtered and the solvent removed under vacuum. The crude product was purified by silica gel flash-column chromatography (hexane/ethyl acetate) to give 0.081 g of compound **27**.

(8R,9S,13S,14S)-methyl 7,8,9,11,12,13,14,15,16,17-decahydro-3,17-dihydroxy-13-methyl-2-(4-byphenyl)-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylate (**27**). Yield 33%; R_f = 0.54 (hexane/ethyl acetate, 1:1); mp = 232°C; ^{1}H NMR (400 MHz, MeOD-d_4): δ 0.94 (s, 3H), 1.46 (m, 1H), 1.65 (m, 1H), 1.69 (m, 1H), 1.79 (m, 1H), 1.81 (m, 1H), 1.85 (m, 1H), 1.90 (m, 1H), 1.91 (m, 1H), 1.93 (m, 1H), 2.03 (m, 1H), 2.65 (m, 1H), 2.74 (m, 1H), 2.76 (m, 1H), 2.95 (m, 1H), 3.70 (s, 3H), 3.82 (d, *J* = 10.5 Hz, 1H), 6.75 (s, 1H), 7.39 (s, 1H), 7.41 (m, AA'BB', 2H), 7.45 (m, AA'BB', 2H), 7.62 (m, 1H), 7.68 (m, 2H), 7.65 (m, 2H) ppm. ^{13}C-NMR (101 MHz, MeOD-d4): δ 11.64, 24.70, 26.41, 29.32, 34.27, 36.48, 39.02, 42.54, 44.97, 47.91, 53.39, 55.64, 84.23, 114.35, 127.01, 127.28, 127.50, 127.61, 127.67, 128.78, 129.03, 137.41, 138.56, 139.34, 140.25, 142.65, 158.70, 174.60 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{32}H_{35}O_4^+$, 483.2535; found, 483.2547.

(8R,9S,13S,14S)-methyl 7,8,9,11,12,13,14,15,16,17-decahydro-3,17-dihydroxy-13-methyl-2-(4-dibenzofuranyl)-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylate (**28**). Compound **28** was prepared as for compound **27** by reacting compound **26** (0.308 g, 0.675 mmol) with 4-(dibenzofuranyl)-boronic acid (0.287 g, 1.356 mmol), potassium carbonate (0.375 g, 2.710 mmol) and tetrakis(triphenylphosphine)palladium (0) (0.078 g, 0.068 mmol). The obtained crude product was purified by silica gel flash column chromatography (hexane/ethyl acetate) to give 0.129 g of white solid. Yield 42%; R_f = 0.53 (hexane/ethyl acetate, 1:1); mp = 215°C; ^{1}H NMR (400 MHz, MeOD-d_4): δ 0.80 (s, 3H), 1.14 (m, 1H), 1.15 (m, 1H), 1.24 (m, 1H), 1.31 (m, 1H), 1.42 (m, 1H), 1.43 (m, 1H), 1.52 (m, 1H), 1.72 (m, 1H), 1.94 (m, 1H), 2.07 (m, 1H), 2.24 (m, 1H), 2.82 (m, 2H), 3.16 (q, *J* = 6.82 Hz, 1H), 3.70 (s, 3H), 3.91 (d, *J* = 5.07 Hz, 1H), 7.13 (d, *J* = 9.04 Hz, 1H), 7.59 (d, *J* = 2.24 Hz, 1H), 7.65 (dd, *J* = 9.12, 1.94 Hz, 1H), 7.88 (m, *J* = 7.54, 1.12 Hz, 1H), 7.98 (m, *J* = 7.45, 0.98 Hz, 1H), 8.08 (d, *J* = 8.94 Hz, 1H), 8.15 (d, *J* = 9.14 Hz, 1H), 8.22 (s, 1H), 8.55 ppm (s, 1H). ^{13}C-NMR (101 MHz, MeOD-d4): δ 10.45, 27.02, 27.76, 29.53, 30.01, 32.31, 38.45, 44.12, 45.02, 47.54, 47.35, 55.72, 82.01, 113.06, 124.23, 125.08, 125.10, 126.11, 126.40, 127.21, 128.05, 128.86, 130.09, 131.20, 134.89, 138.55, 139.71, 142.89, 149.81, 154.89, 159.11, 169.81 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{32}H_{33}O_5^+$, 497.2328; found, 497.2341.

(8R,9S,13S,14S)-methyl 7,8,9,11,12,13,14,15,16,17-decahydro-3,17-dihydroxy-13-methyl-2-phenyl-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylate (29). Compound **29** was prepared as for

compound **27** by reacting compound **26** (0.131 g, 0.287 mmol) with phenyl boronic acid (0.070 g, 0.577 mmol), potassium carbonate (0.158 g, 1.150 mmol) and tetrakis(triphenylphosphine)palladium (0) (0.033 g, 0.029 mmol). The obtained crude product was purified by silica gel flash column chromatography (hexane/ethyl acetate) to give 0.081 g of white solid. Yield 26%; R_f = 0.47 (hexane/ethyl acetate, 1:1); mp = 240°C; ^{1}H NMR (400 MHz, MeOD-d_4): δ 0.89 (s, 3H), 1.22 (m, 1H), 1.34 (m, 1H), 1.37 (m, 1H), 1.54 (m, 1H), 1.55 (m, 1H), 1.91 (m, 1H), 1.94 (m, 1H), 1.97 (m, 1H), 2.23 (m, 1H), 2.36 (m, 1H), 2.41 (m, 1H), 2.85 (m, 2H), 3.18 (q, *J* = 8.8 Hz, 1H), 3.71 (s, 3H), 3.96 (d, *J* = 10.5 Hz, 1H), 6.62 (s, 1H), 7.14 (s, 1H), 7.29 – 7.27 (m, 1H), 7.41 – 7.38 (m, 2H), 7.54 – 7.51 ppm (m, 2H); ^{13}C NMR (101 MHz, MeOD-d_4): δ 10.74, 26.17, 27.23, 28.30, 28.94, 37.36, 38.51, 43.91, 46.43, 48.30, 48.85, 50.78, 82.90, 115.51, 125.89, 126.08, 127.17, 127.65, 129.12, 131.38, 136.76, 139.31, 151.47, 176.20 ppm. ^{13}C-NMR (101 MHz, MeOD-d4): δ 10.74, 26.17, 27.23, 28.30, 28.94, 37.36, 38.51, 43.91, 46.43, 48.30, 48.85, 50.78, 82.90, 115.51, 125.89, 126.08, 127.17, 127.65, 129.12, 131.38, 136.76, 139.31, 151.47, 176.20 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{26}H_{31}O_4^+$, 407.2222; found, 407.2234.

4.1.13 General procedure for the synthesis of derivatives (**30**, **31**, **32**). As a typical procedure, the synthesis of (8R,9S,13S,14S)-7,8,9,11,12,13,14,15,16,17-decahydro-3,17-dihydroxy-13-methyl-2-(4-byphenyl)-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylic acid **30** is described in detail. Compound **27** was dissolved in 8 mL of methanol and then 4 mL of 10% NaOH solution were added. The mixture was heated to reflux for 1 h and monitored by TLC analysis (hexane/ ethyl acetate, 2:1). As the starting reagent spot disappeared, the solvent was reduced with rotavapor and the mixture acidified with concentrated HCl until pH=1. The suspension was centrifugated and the supernatant discarded. The obtained powder was dried to yield 0,069 g of final product.

(8R,9S,13S,14S)-7,8,9,11,12,13,14,15,16,17-decahydro-3,17-dihydroxy-13-methyl-2-(4-byphenyl)-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylic acid (**30**). Yield 98%; mp = over 300°C; ^{1}H NMR (400 MHz, MeOD-d_4): δ 0.94 ppm (s, 3H), 1.46 (m, 1H), 1.65 (m, 1H), 1.69 (m, 1H), 1.79 (m, 1H), 1.81 (m, 1H), 1.85 (m, 1H), 1.90 (m, 1H), 1.91 (m, 1H), 1.93 (m, 1H), 2.03 (m, 1H), 2.65 (m, 1H), 2.74 (m, 1H), 2.76 (m, 1H), 2.95 (m, 1H), 3.82 (d, 1H), 6.77 (s, 1H), 7.39 (s, 1H), 7.41 (m, AA'BB', 2H), 7.45 (m, AA'BB', 2H), 7.64 (m, 1H), 7.68 (m, 2H), 7.69 ppm (m, 2H); ^{13}C-NMR (101 MHz, MeOD-d4): δ 11.12, 25.94, 26.17, 29.23, 34.16, 36.72, 38.98, 42.57, 44.89, 47.95, 53.47, 79.66, 114.12, 127.07, 127.34, 127.54, 127.69, 127.82, 128.87, 128.92, 137.42, 138.43, 139.24, 140.32, 142.68, 158.76, 177.55 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{31}H_{33}O_4^+$, 469.2379; found, 469.2354. RP-C8 HPLC: t_R = 18.89 min, 99.23% (A%).

(8R,9S,13S,14S)-7,8,9,11,12,13,14,15,16,17-decahydro-3,17-dihydroxy-13-methyl-2-(4-dibenzofuranyl)-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylic acid (**31**). Compound **31** was prepared as for compound **30.** Yield 97%; mp = over 300°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.81 (s, 3H), 1.14 (m, 1H), 1.15 (m, 1H), 1.24 (m, 1H), 1.31 (m, 1H), 1.42 (m, 1H), 1.43 (m, 1H), 1.52 (m, 1H), 1.72 (m, 1H), 1.94 (m, 1H), 2.07 (m, 1H), 2.24 (m, 1H), 2.82 (m, 2H), 3.16 (q, *J* = 6.82 Hz, 1H), 3.91 (d, *J* = 5.07 Hz, 1H), 7.13 (d, *J* = 9.04 Hz, 1H), 7.59 (d, *J* = 2.24 Hz, 1H), 7.65 (dd, *J* = 9.12, 1.94 Hz, 1H), 7.89 (m, *J* = 7.54, 1.12 Hz, 1H), 7.98 (m, *J* = 7.45, 0.98 Hz, 1H), 8.08 (d, *J* = 8.94 Hz, 1H), 8.15 (d, *J* = 9.14 Hz, 1H), 8.22 (s, 1H), 8.59 ppm (s, 1H); ^{13}C-NMR (101 MHz, DMSO-d_6): δ 10.94, 26.98, 27.88, 29.72, 30.04, 32.24, 38.74, 44.17, 45.09, 47.53, 47.88, 82.04, 113.12, 124.12, 125.06, 125.07, 126.15, 126.34, 127.93, 128.01, 128.92, 130.14, 131.24, 134.93, 138.56, 139.72, 142.82, 149.82, 154.83, 159.14, 169.87 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{31}H_{31}O_5^+$, 483.2171; found, 483.2161. RP-C8 HPLC: t_R = 14.32 min, 98.8% (A%).

(8R,9S,13S,14S)-7,8,9,11,12,13,14,15,16,17-decahydro-3,17-dihydroxy-13-methyl-2-phenyl-6*H*-cyclopenta[*a*]phenanthrene-16-carboxylic acid (**32**). Compound **32** was prepared as for compound **30.** Yield 90%; mp = over 300°C; ^{1}H NMR (400 MHz, MeOD-d_4): δ 0.94 (s, 3H), 1.46 (m, 1H), 1.65 (m, 1H), 1.69 (m, 1H), 1.79 (m, 1H), 1.81 (m, 1H), 1.85 (m, 1H), 1.90 (m, 1H), 1.91 (m, 1H), 1.93 (m, 1H), 2.03 (m, 1H), 2.65 (m, 1H), 2.74 (m, 1H), 2.76 (m, 1H), 2.95 (m, 1H), 3.82 (d, 1H), 6.88 (s, 1H), 7.38 (s, 1H), 7.40 (m, 1H), 7.42 (m, 2H), 7.45 ppm (m, 2H); ^{13}C-NMR (101 MHz, MeOD-d4):δ 11.48, 26.12, 26.17, 29.42, 34.16, 36.72, 39.65, 42.57, 44.12, 47.93, 53.47, 79.66, 113.01, 127.69, 127.81, 128.87, 128.92, 129.04, 137.42, 138.76, 139.24, 158.76, 177.52 ppm. HRMS (ESI-MS, 140 eV): m/z [M + H^+] calculated for $C_{25}H_{29}O_4^+$, 393.2066; found, 393.2973. RP-C8 HPLC: t_R = 11.81 min, 99.1% (A%).

4.2 Biology

4.2.1 Cell viability assay

Cell viability was determined by the 3-(4,5-dimethyl- thiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St Louis, MO, USA) assay, as previously described.[41] Briefly, HepG2 cells were cultured in DMEM supplemented with 1% glutamine, pen-strep and 10% FBS; cells were seeded in 96-multiwells culture plates at a concentration of (5000 cells/well) and treated with compounds **7**, **8**, **19a**, **30**, **31** and **32** (6.25, 12.5, 25, 50 μM) for 24 h. The formazan absorbance was measured at 570 nm, using a Multilabel Plate Reader VICTOR™ X3 (Wallac Instruments, Turku, Finland). Three independent experiments were performed in quadruplicate.

4.2.2 Cell cycle distribution analysis

Cell cycle distribution analysis was evaluated by flow cytometry (Epics XL, Beckmann Coulter) with CXP software, according to an already described method.[42] Cells (200000 per well), 24 hours after seeding into 6 well-plates, were treated with compounds 7, 8, 19a, 31 at 20 µM for 24 h. The cells were washed with PBS and fixed with ethanol 70%. After 15 min of incubation, cells were resuspended with RNase A (0,1 mg/mL) and 25 µL of propidium iodide (1 mg/mL) for 15 min at room temperature. The results of the different experiments were analyzed with CXP software. Three independent experiments were performed in duplicate.

4.2.2 Evaluation of the estrogenic activity by qRT-PCR

The estrogenic activity of the compounds **7**, **8**, **19a**, **30**, **31** and **32** was evaluated in the human breast adenocarcinoma cell line MCF-7 which highly expresses estrogen receptor (ER).[43] MCF-7 cells were cultured in high glucose DMEM without phenol red supplemented with 1% glutamine, pen-strep and 10% FBS, and seeded in 6-well culture plates at a concentration of (250000 cells/well). Samples were treated with compounds **7**, **8**, **19a**, **30**, **31** and **32** (2 µM) for 24 h. Estrone (2 µM) was used as positive control. At the end of the incubation period, MCF-7 were scraped away from cell culture dishes and total RNA was extracted and purified by means of the SV Total RNA Isolation System (Promega Corporation, Madison, WI), as already described.[44] Integrity and quantity of RNA were evaluated by an RNA 6000 Nano assay in an Agilent BioAnalyser (Agilent Technologies Inc., Palo Alto, CA, USA). The relative expression of GREB-1 and CXCL12, two genes which increase their transcription after the activation of ER[45] was determined by real-time PCR (Eco™ Illumina, Real-Time PCR system, San Diego, CA, USA) using One Step SYBR PrimeScript RT-PCR Kit (Takara Bio, Inc., Otsu, Shiga, Japan). PCR amplifications were tested for linearity and efficiency using standard curves obtained with serial dilution of cDNA; the specificity of amplification and absence of dimers were confirmed by melt-curve analysis. All genes were normalized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used in this study are listed in Table 3. Expression levels of GREB-1 and CXCL12 genes were calculated by the ΔΔCt method using the Eco™ Software v4.0.7.0. Modifications of mRNA levels were expressed as fold variation compared with that of untreated cells. Three independent experiment were performed in triplicate.

Gene	Forward primer	Reverse primer	RefSeq	Size(bp)
GREB-1	**5'-gtt-ctg-aag-cta-gac-acg-ga-3'**	**5'-ttg-agc-aatcgg-tcc-acc-aa-3'**	**NM_014668.3**	**185**
CXCL12	**5'-tac-aga-tgc-cca-tgc-cga-tt-3'**	**5'-gaa-tcc-act-tta-gct-tcg-gg-3'**	**NM_000609.6**	**157**
GAPDH	**5'-aca-tca-aga-agg-tgg-tga-agc-a-3'**	**5'-gtc-aaa-ggt-gga-gga-gtg-ggt-3'**	**NM_001289746.1**	**119**

Table 3. Primer sequences used in this study, NCBI reference sequences and amplicon sizes (base pairs).

4.2.3 Cell transient transfection assays

HEK293 cells were maintained in DMEM supplemented with 10% FBS, 1% penicillin and streptomycin. Cells were plated at a concentration of 30000/well in 96-well plates, according to an already described method.[26] HEK293 cells were transiently transfected in OPTIMEM (Gibco) medium using lipofectamine 2000 (Invitrogen) following manufacturer instructions. Each plate was cotransfected with 0,2 µg of Gal4-RORg LBD plasmid (Gal4-driven reporter assays), 0.1 µg UAS-luciferase expression plasmid (both plasmids were kindly furnished by prof. Griffin) and 0.01 µg of NanoLuc reporter plasmid (Promega, Italy). 6 h after transfection medium was replaced with DMEM supplemented with 1% FBS. The following day compounds **7**, **8, 19a** and **32** were added at different concentrations (2, 5, 10, 20 µM). Ursolic acid was used as a positive control. 48 h after transfection luminescence emission was measured using Nano-glo dual-luciferase reporter assay system (Promega) following manufacturer instructions with a Perkin Elmer en-vision system. All the assays were performed in triplicate, and the standard errors were calculated accordingly.

4.2.4 Western blot analysis.

Protein expression levels of RORγt in transient transfected HEK293 cells was evaluated by Western blot analysis, as already described.[46] Briefly, 20 µg per lane of proteins were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels (100 mV for 15′ and 150 mV for 90′) and transferred to a 0.45µm nitrocellulose membrane (Bio-Rad Laboratories S.r.l., Segrate, Milan, Italy) at 250 mA for 90 min in the presence of 25 mM Tris - 192 mM glycine. Mouse monoclonal anti-RORγ (Millipore, Billerica, MA, USA) and rabbit polyclonal anti-GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA) primary antibodies (both diluted 1:500) were used to detect RORγt and GAPDH (used as a loading control). Signal intensity of immunoreactive bands was analyzed by the Quantity One software (Bio-Rad Laboratories S.r.l.).

4.2.5 Statistical analysis

Comparison of the experimental data obtained from control cell cultures and those treated with the synthetic compounds was made by one-way analysis of variance (ANOVA). In the case of

significant differences ($\alpha = 0.05$), the analysis of variance was followed by the Dunnett *post-hoc* test. $P < 0.05$ was considered statistically significant. If not otherwise stated, data are presented as mean ± standard deviation.

4.3 Molecular docking simulations

The 3D structure of orphan nuclear receptor RORγt in complex with the inverse agonists, digoxin, was retrieved from the Protein Data Bank (www.rcsb.org, PDB code 3B0W).

Prior to docking simulation, protein structure was processed with Maestro 10.5 Protein Preparation tool using OPLS-2005 force field. Maestro 10.5[47] Receptor Grid tool was used for the docking site identification, indicating bound digoxin as the grid centre and a length of 10 Å. Molecular structures of compounds **8** and **19a** used for virtual docking were designed using the Builder tool of MOE 2015.10[48] and prepared for the docking simulation with Maestro 10.5[47] Ligand Preparation tool using OPLS-2005 force field. The docking simulations were performed with Maestro 10.5[47] Glide software SP precision, using flexible ligand sampling and performing post-docking minimization.

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