Side-Chain Modified Ergosterol and Stigmasterol Derivatives as Liver X Receptor Agonists

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Abstract

A series of stigmasterol and ergosterol derivatives, characterized by the presence of oxygenated functions at C-22 and/or C-23 positions, were designed as potential LXR agonists. The absolute configuration of the newly created chiral centers was definitively assigned for all the corresponding compounds. Among the sixteen synthesized compounds, **21**, **27** and **28** were found to be selective LXRα agonists, whereas **20**, **22**, and **25** showed good selectivity for the LXRβ isoform. In particular, **25** showed the same degree of potency as 22*R*-HC (**3**) at LXRβ, while it was virtually inactive at LXRα (EC_{50} = 14.51 μM). Interestingly, **13**, **19**, **20** and **25** showed to be LXR target gene-selective modulators, by strongly inducing the expression of *ABCA1*, while poorly or not activating the lipogenic genes *SREBP1* and *SCD1*, or *FASN*, respectively.

Introduction

Oxysterols are 27-carbon intermediates or end-products of cholesterol metabolism, structurally characterized by the presence of oxygenated functions such as hydroxy, keto, hydroperoxy, epoxy and carboxy moieties. They are produced *in vivo* through both enzymatic- and non-enzymatic (auto-oxidation) processes.[1,2] Specific enzymes of the cytochrome P450 (CYP) family preferentially oxidize the cholesterol side chain (7α-hydroxycholesterol (**1a**), 24(*S*)-hydroxycholesterol (**2**), 22(*R*)-hydroxycholesterol (22*R*-HC, **3**), and 24(*S*),25-epoxycholesterol (**4**) are examples of oxysterols generated by CYPs, see Figure 1), whereas the double bond of the cholesterol B-ring represents a privileged target for free-radical-involving reactions. Thus, 7-ketocholesterol (**5**), 7β-hydroxycholesterol (**1b**), 5α,6α- and 5β,6β-epoxycholesterols (**6a**,**b**) constitute the main non-enzymatically produced oxysterols (Figure 1).[1,2]

A broader definition for the class of oxysterols is not limited to cholesterol oxidation products, but includes also steroidal oxygenated derivatives that humans can assimilate by diet, either as primary constituents (plants and shellfish sterols) or as storage and cooking-derived components.[1]

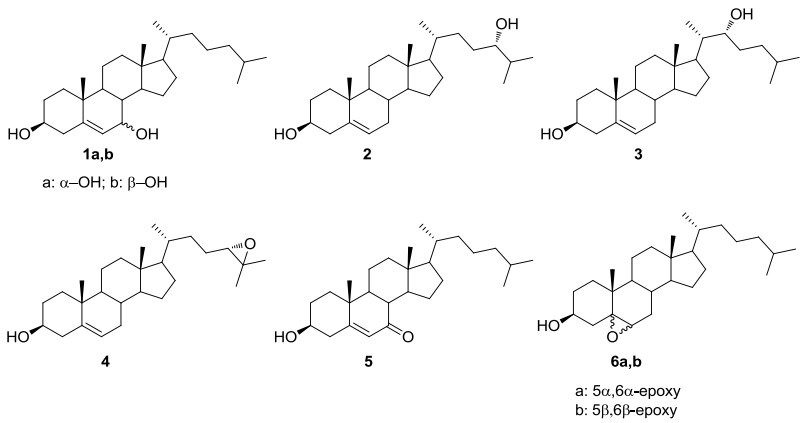

Figure 1. Examples of enzymatically and non-enzymatically produced oxysterols.

The past two decades have evidenced an exponential increase in the number of studies on the physiological roles of mammalian oxysterols, as well as on their contribution to the pathogenesis of different diseases.[3,4,5,6] The major breakthrough was the identification of a specific subset of oxysterols (**2-4**)[7,8] as endogenous ligands of Liver X Receptor α and β (LXRs).[9,10,11,12,13] Thus, given the action of LXRs (α and β isoforms) as whole-body cholesterol sensors and key regulators of lipogenesis, oxysterols have the potential to assume a key role in the modulation of lipid metabolism and glucose homeostasis.

LXRs and their ligands can also suppress inflammatory responses, either by activating the genes that encode anti-inflammatory proteins or by suppressing the genes that are under the control of proinflammatory transcription factors.[4,14]

However, the functions of oxysterols are not limited to their LXR binding,[15] but they significantly interact with other cellular proteins, giving rise to different effects. Examples of proteins affected by oxysterols are: a) insulin-induced gene (INSIG) proteins, regulating the function of sterol response element binding protein (SREBP);[16] b) Niemann-Pick C1 (NPC1) and oxysterol-binding protein family (OSBP/ORP), involved in cholesterol metabolism;[17] and c) Smoothened oncoprotein, interfering with the Hedgehog signalling.[18]

So far the oxysterol medicinal chemistry has been mainly focused on the identification of LXR modulators, although the number of the studied natural and synthetic oxysterol derivatives is only marginal when compared to that of the non-steroidal ligands.[19,20]

The first series of synthetic steroidal ligands allowed the identification of the minimal pharmacophore for LXRα, i.e. a sterol with a hydrogen bond acceptor at C-24.[21] The most potent derivative of this series, namely cholenic acid dimethylamide **7**, was an efficacious LXRα agonist,[21] able to promote a gene–selective modulation (Figure 2).[22] 5α,6α-Epoxycholesterol (**6a**), identified in processed food, was shown to be a LXR modulator with cell and gene-context-dependent activities,[23] whereas the two 5β-cholane derivatives 3α,6α,24-trihydroxy-24,24-di(trifluoromethyl)-5β-cholane (ATI-829, **8**)[24] and 3α,6α,24-trihydroxy-22-en-24,24-di(trifluoromethyl)-5β-cholane

(ATI-111, **9**),[25] whose design was inspired by the structure of the potent non-steroidal agonist *N*-(2,2,2-trifluoroethyl)-*N*-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]benzene sulfonamide (T0901317),[26] demonstrated antiatherosclerotic effects.[24,25] In view of the well-known effect of phytosterols in reducing blood cholesterol,[27] and considering the fact that the treatment of intestinal cells with these compounds was found to increase the expression of LXR target genes,[28] Kaneko *et al.*[29] studied the LXR activity of a series of phytosterols, including natural and semi-synthetic derivatives. They identified (22*E*)-ergost-22-ene-1α,3β-diol (YT-32, **10**)[29] as a potent and non-isoform selective LXR agonist, able to selectively induce the expression of ABC transporter genes in the intestine. Interestingly, the oral administration of **10** resulted in the inhibition of the intestinal cholesterol adsorption without increasing plasma triglyceride levels, in contrast to what observed with non-steroidal ligands.[19,30]

To our knowledge, the study of phytosterols as LXR agonists is limited to the mentioned compound **10**, to the plant hormone 28-homobrassinolide (**11**),[31] and to 24(*S*)-saringosterol (**12**), a minor component isolated from marine seaweeds which showed to act as a selective LXRβ agonist.[32]

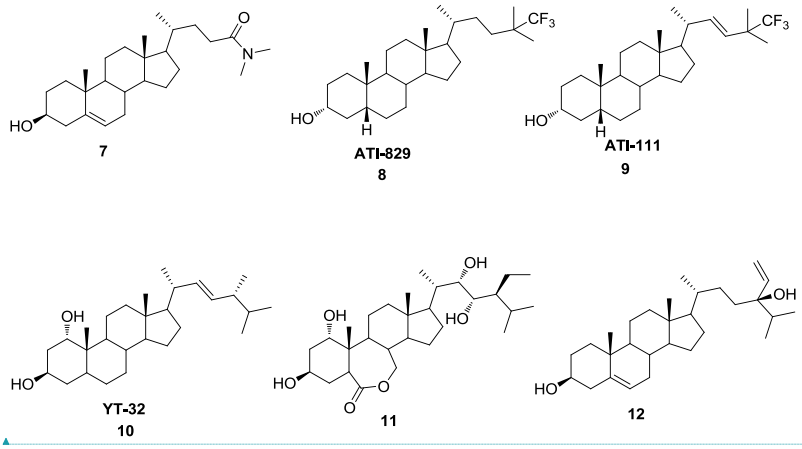

Figure 2. Examples of steroidal LXR agonists

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Therefore, intrigued by the ability of some phytosterols to interfere with cholesterol homeostasis by acting as analogs of endogenous oxysterols,[33] we engaged ourselves in a vast research project aimed at synthesizing stigmasterol and ergosterol derivatives characterized by the presence of oxygenated functions, structural features known to be crucial for LXR activation, at all the possible side-chain positions. Due to the lack of previous structure-activity relationship (SAR) studies for this class of derivatives we considered reasonable to chose the starting point on the basis of the synthetic accessibility. Therefore, herein, we present the synthesis and the biological evaluation of the first 16 derivatives **13-28**, functionalized at C-22 and/or C-23 positions (Figure 3).

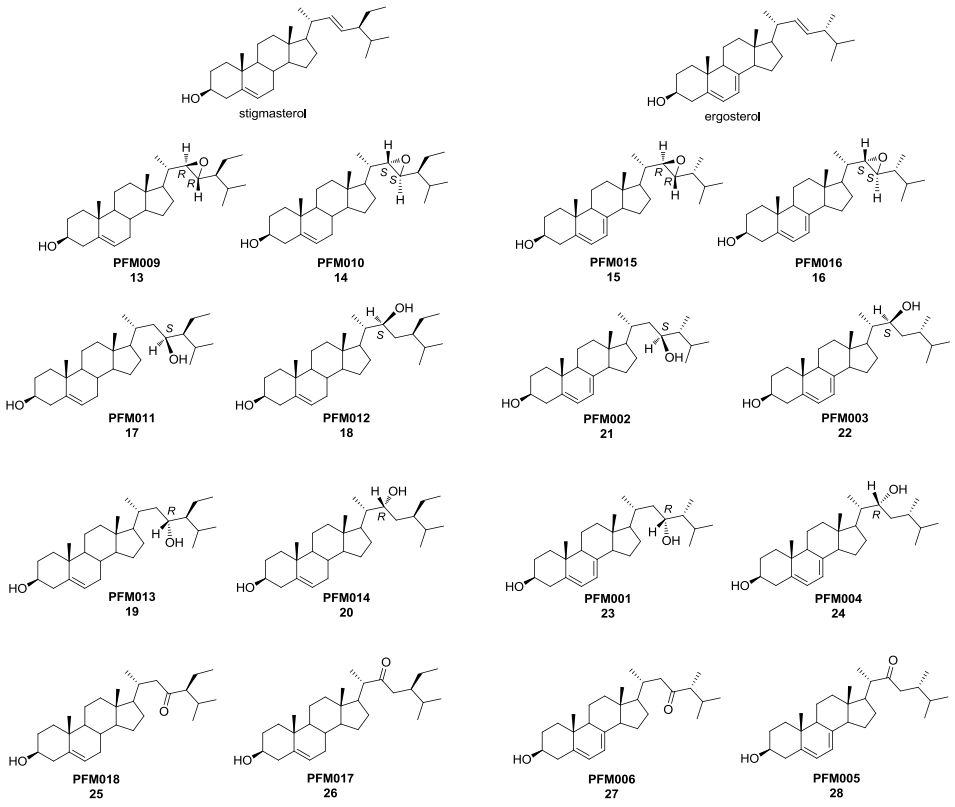


Figure 3. Structures of the compounds reported in the paper

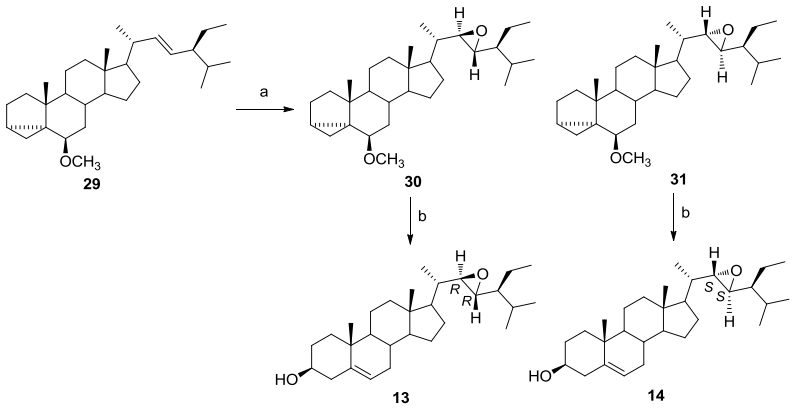
Results and Discussion

Chemistry

The two series of derivatives herein reported include a) four different epoxides **13**-**16**, obtained by oxidizing the double bond between positions C-22 and C-23 of stigmasterol and ergosterol; b) eight isomeric alcohols **17**-**24**, deriving from the reductive opening of each epoxide, and c) four different ketones **25**-**28**, resulting from the oxidation of the corresponding alcohols.

(22*E*)-3α,5α-cyclo-6β-methoxystigmast-22-ene (**29**), obtained in two steps from stigmasterol, as already reported,[34] represented the starting material for the preparation of the stigmastane derivatives (Scheme 1). The epoxidation reaction of **29** resulted in the formation of the two diastereoisomeric epoxides **30** and **31**, which were separated by chromatography in 30 and 18% yield, respectively.[35] The recovery of the 3β-hydroxy-5,6-ene moiety was performed by the known two-step procedure,[35] consisting first in the treatment with glacial acetic acid, followed by the alkaline hydrolysis in the presence of hydroalcoholic potassium carbonate solution. Thus, starting from **30** and **31**, we obtained the desired (22*R*,23*R*)-22,23-epoxystigmast-5-ene-3β-ol (**13**) and its (22*S*,23*S*)-isomer **14**, respectively.

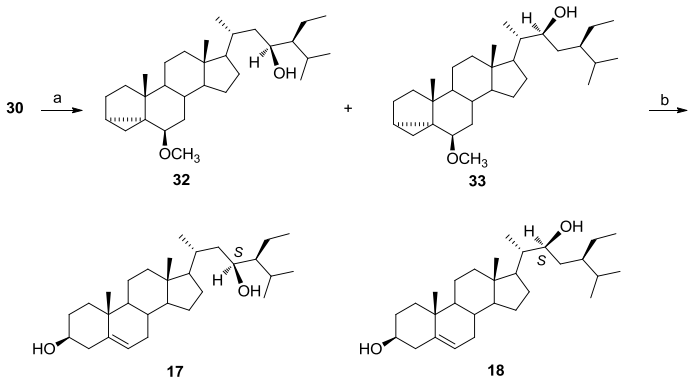
Scheme 1. Synthesis of (22*R*,23*R*)-22,23-Epoxystigmast-5-ene-3β-ol (13) and (22*S*,23*S*)-22,23-Epoxystigmast-5-ene-3β-ol (14)[a]



[a]Reagents and conditions: (a) *i*. *m*-CPBA, $NaHCO_3$, CH_2Cl_2, reflux, 2 h; *ii*. mpc; (b) *i*. glacial AcOH, reflux, 5h; *ii*. K_2CO_3, MeOH/H_2O, reflux**,** 3 h.

The $LiAlH_4$-promoted reductive opening of the oxirane ring of **30** (Scheme 2) gave the inseparable mixture of the corresponding 23*S*- and 22*S*-hydroxy derivatives **32** + **33**, which was first treated with glacial acetic acid and then in basic conditions to afford, after medium pressure chromatography (mpc), pure samples of (23*S*)-3β-stigmast-5-ene-3,23-diol (**17**) and (22*S*)-3β-stigmast-5-ene-3,22-diol (**18**).

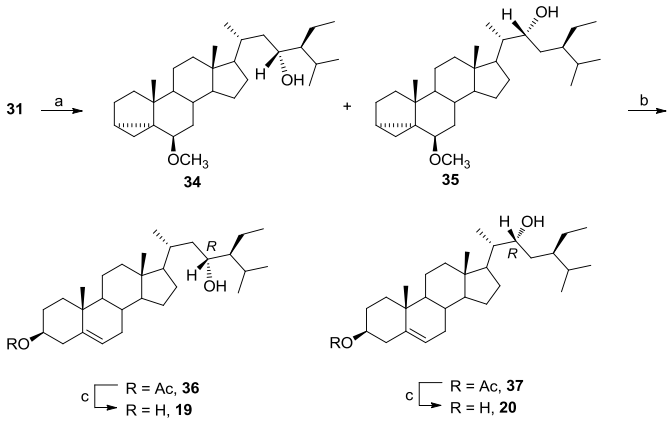
Scheme 2. Synthesis of (23*S*)-3β-Stigmast-5-ene-3,23-diol (17) and (22*S*)-3β-Stigmast-5-ene-3,22-diol (18)[a]



[a]Reagents and conditions: (a) $LiAlH_4$, THF, reflux, 36 h; (b) *i*. glacial AcOH, reflux, 6 h; *ii*. 2M KOH, MeOH, reflux, 3h; *iii*. mpc.

Similarly, the reductive opening of the epoxide **31** gave the inseparable mixture of the corresponding 23*R*- and 22*R*-hydroxy derivatives **34** + **35** (Scheme 3). In this case, the chromatographic separation of the two components of the mixture was only possible as 3β-acetate form. Thus, the mixture **34** + **35** was heated in glacial acetic acid and the crude submitted to mpc to achieve the two pure isomers **36** and **37**. Their final alkaline hydrolysis gave the desired (23*R*)-3β-stigmast-5-ene-3,23-diol (**19**) and (22*R*)-3β-stigmast-5-ene-3,22-diol (**20**), respectively, thus completing the series of stigmastanediols.

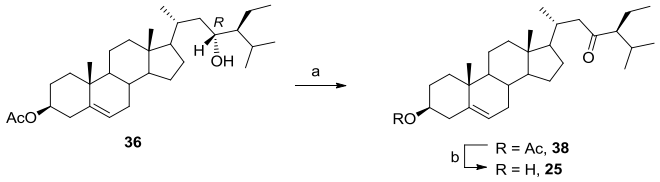
Scheme 3. Synthesis of (23*R*)-3β-Stigmast-5-ene-3,23-diol (19) and (22*R*)-3β-Stigmast-5-ene-3,22-diol (20)[a]



[a]Reagents and conditions: (a) $LiAlH_4$, THF, reflux, 36 h; (b) *i*. glacial AcOH, reflux, 6h; *ii*. mpc; (c) 2M KOH, MeOH, reflux, 3h.

Swern oxidation of (23*R*)-3β-acetoxystigmast-5-ene-23-ol (**36**) afforded the corresponding 23-keto derivative **38** (Scheme 4), which under basic hydrolysis gave the desired 3β-hydroxystigmast-5-ene-23-one (**25**).

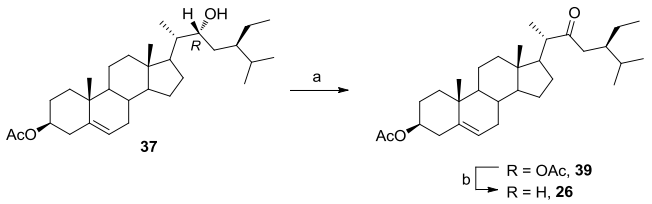
Scheme 4. Synthesis of 3β-Hydroxystigmast-5-ene-23-one (25)[a]



[a]Reagents and conditions: (a) $(COCl)_2$, DMSO, CH_2Cl_2, -78ºC, 2h, then Et_3N, r.t.; (b) 2M KOH, acetone, reflux, 3h.

Analogously, (22*R*)-3β-acetoxystigmast-5-ene-22-ol (**37**) was converted into the desired 3β-hydroxystigmast-5-ene-22-one (**26**) (Scheme 5).

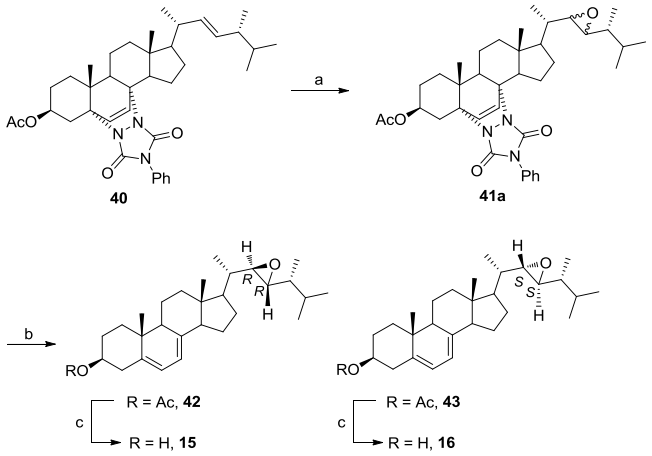
Scheme 5. Synthesis of 3β-Hydroxystigmast-5-ene-22-one (26)[a]



[a]Reagents and conditions: (a) $(COCl)_2$, DMSO, CH_2Cl_2, -78ºC, 2h, then Et_3N, r.t.; (b) 2M KOH, acetone, reflux, 3h.

3β-Acetoxy cycloadduct **40**, obtained by Diels-Alder cycloaddition between ergosterol-3β-acetate and 4-phenyl-1,2,4-triazoline-3,5-dione,[36] constituted the starting material for the synthesis of the ergostane derivatives: Its epoxidation reaction with *m*CPBA gave access to the inseparable mixture of the two diastereoisomeric epoxides **41a**.[36] In an analogous manner the corresponding mixture of 3β-tetrahydropyranyl-protected epoxides **41b** was also prepared starting from 3β-tetrahydropyranyloxy cycloadduct.[37] The treatment of the mixture **41a** with anhydrous potassium carbonate resulted in the retro 1,4-cycloaddition reaction, affording, after mpc, the two single isomers **42** and **43** (Scheme 6). The minor, less polar component **42**, whose absolute configuration was assigned as 22*R*,23*R* (*vedi infra*), was submitted to alkaline hydrolysis to furnish (22*R*,23*R*)-22,23-epoxyergosta-5,7-diene-3β-ol (**15**). The same procedure starting from the major, more polar epoxide **43** gave the corresponding (22*S*,23*S*)-22,23-epoxyergosta-5,7-diene-3β-ol (**16**).

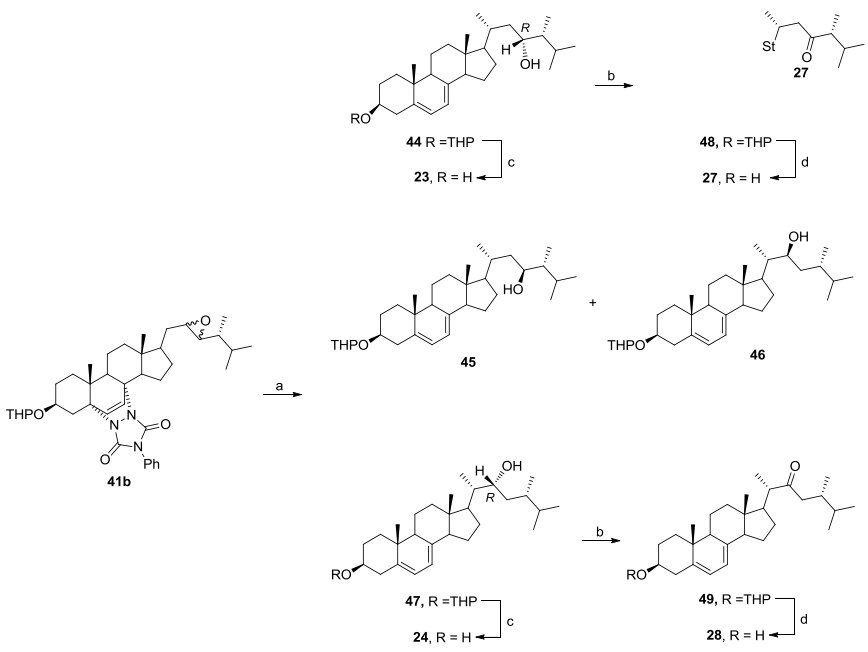
Scheme 6. Synthesis of (22*R*,23*R*)-22,23-Epoxy-3β-ergosta-5,7-diene-3-ol (15) and (22*S*,23*S*)-22,23-Epoxy-3β-ergosta-5,7-diene-3-ol (16).[a]



[a]Reagents and conditions: (a) *m*CPBA, CH_2Cl_2, r.t., 5h; (b) K_2CO_3, DMF, reflux, 6h; (c) 2M KOH, EtOH, reflux, 15 min.

The reductive opening of the epoxide mixture **41b** gave, after separation by mpc, three different fractions, constituted by (23*R*)-3β-tetrahydropyranyloxyergost-5,7-diene-23-ol (**44**), the inseparable mixture of (23*S*)- and (22*S*)-3β-tetrahydropyranyl-protected diols (**45** + **46**), and (22*R*)-3β-tetrahydropyranyloxyergost-5,7-diene-22-ol (**47**) (Scheme 7). The deprotection of the 3β-hydroxy group of **44** by pyridinium *p*-toluenesulfonate (PPTS)[38] provided the desired (23*R*)-3β-ergost-5,7-diene-3,23-diol (**23**).

Scheme 7. Synthesis of (23*R*)-3β-Ergost-5,7-diene-3,23-diol (23), (22*R*)-3β-stigmast-5-ene-3,22-diol (24), 3β-Hydroxyergosta-5,7-diene-23-one (27), and 3β-Hydroxyergosta-5,7-diene-22-one (28)[a]



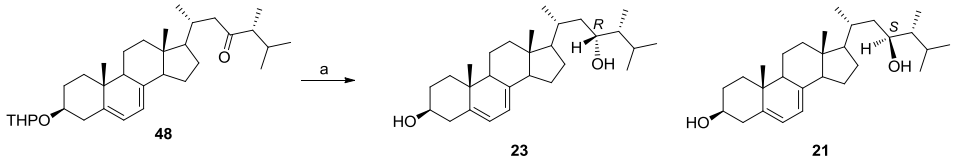
[a]Reagents and conditions: (a) $LiAlH_4$, THF-Et_2O, reflux, 36 h; (b) $(COCl)_2$, DMSO, CH_2Cl_2, -78ºC, 2h, then Et_3N, r.t.; (c) PPTS, EtOH, reflux, 1h; (d) PPTS, acetone, reflux, 5h.

Subsequent Swern oxidation of the single alcohol **44** afforded the 3β-tetrahydropyranyl-23-keto derivative **48**, which was deprotected under analogous mild acidic conditions to finally afford 3β-hydroxyergosta-5,7-diene-23-one (**27**). An analogous sequence, starting from the more polar, pure 22*R*-hydroxy derivative **47** gave access to the desired (22*R*)-3β-ergost-5,7-diene-3,22-diol (**24**) and 3β-hydroxyergosta-5,7-diene-22-one (**28**).

The ergostanediol series was completed by reducing the 3β-tetrahydropyranyl-23-keto derivative **48** with sodium borohydride, achieving almost quantitatively the mixture of the two 23-hydroxy epimers, which, after deprotection at C-3 position, gave the already obtained (23*R*)-3β-ergost-5,7-diene-3,23-diol (**23**), and the missing 23S-epimer **21** (Scheme 8). Analogously, starting from the

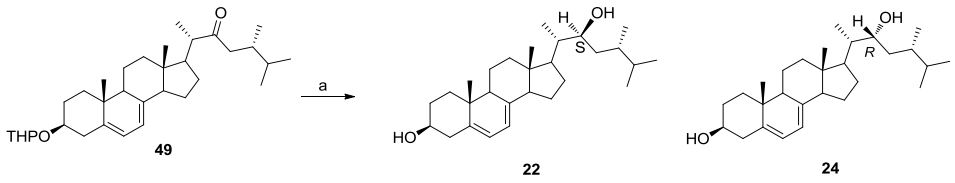
22-keto derivative **49**, (22*S*)-3β-ergost-5,7-diene-3,22-diol (**22**) was achieved along with the already obtained **24** (Scheme 9).

Scheme 8. Synthesis of (23*R*)-3β-Ergost-5,7-diene-3,23-diol (23) and (23*S*)-3β-Ergost-5,7-diene-3,23-diols (21)[a]



[a]Reagents and conditions: (a) *i*. $NaBH_4$, THF, 2-propanol, H_2O, r.t.; *ii*. PPTS, EtOH, reflux, 1h; *iii*. mpc.

Scheme 9. Synthesis of (22*S*)-3β-Ergost-5,7-diene-3,22-diol (22) and (22*R*)-3β-Ergost-5,7-diene-3,22-diol (24)[a]



[a]Reagents and conditions: (a) *i*. $NaBH_4$, THF, 2-propanol, H_2O, r.t.; *ii*. PPTS, EtOH, reflux, 1h; *iii*. mpc.

Absolute Configuration Assignment

The workflows for the stereochemical elucidation of the newly created asymmetric centers are depicted in the figures 4 and 5.

In the case of the members of the stigmastane series we took advantage of the X-ray single crystal diffraction analysis reported for (22*R*)-3β-stigmast-5-ene-3,22-diol, the only known derivative among the stigmastanediols here reported.[39,40] By comparison of its reported spectroscopic data with those of our compounds, we established that the more polar diol **20** corresponded to (22*R*)-3β-

stigmast-5-ene-3,22-diol. Since **20** had been obtained from the reductive opening of the more polar oxirane isomer **14**, as a consequence, the latter had to be endowed with the 22*S*,23*S*-absolute configuration. Thus, the other diol deriving from its reductive opening, namely **19**, was assigned instead with the 23*R*-configuration (Figure 4). By exclusion, the diols **17** and **18** were characterized by the *S*-configuration at the newly formed side-chain chiral center, and the less polar epoxide **13** by 22*R*,23*R*-configuration. The respective position of the hydroxyl group in the two diols **17** and **18** was definitively established by their comparison with the compounds resulting from the reduction of 3β-hydroxystigmast-5-ene-22-one (**26**).

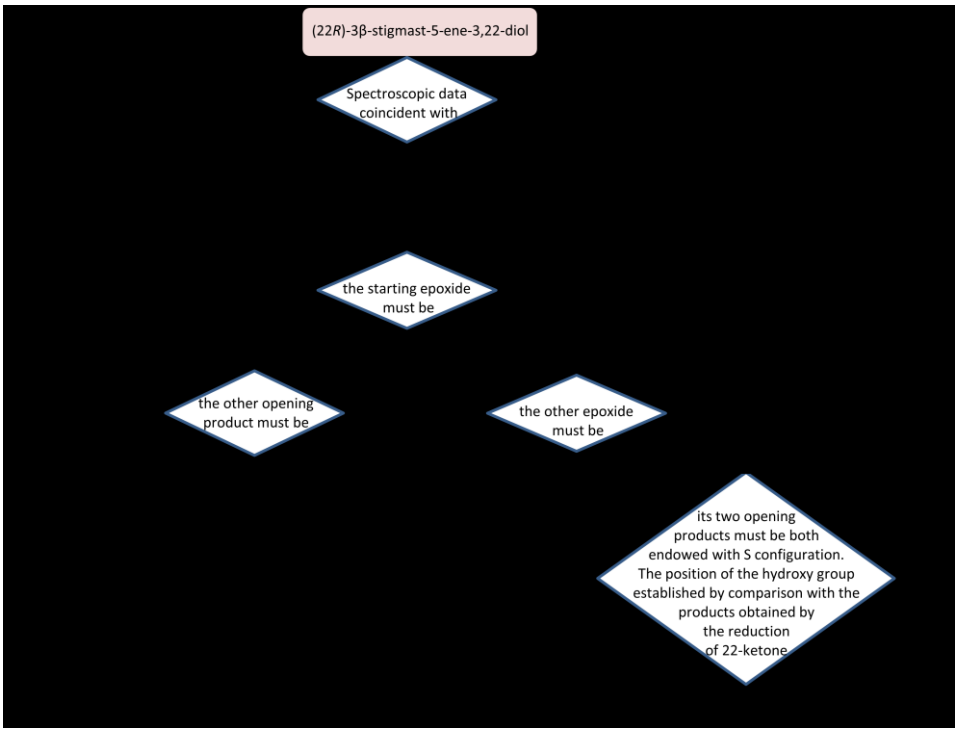


Figure 4. Flowchart for the structural assignment of the stigmastane derivatives

Although the synthesis of some of our ergostane derivatives had been already reported, their structural assignment had been only presumed.[36,41,42] To unambiguously proceed with the structural elucidation, the diol **23**, derived by the hydrolysis of **44**, the less polar, major fraction obtained by the reductive opening of the epoxide mixture **41b** (Scheme 7), was submitted to single crystal X-ray analysis (Figure 4) and thus characterized as the (23*R*)-isomer. Consequently, the diol **24**, since obtained by the hydrolysis of the other more abundant isomer **47** resulting from the same opening reaction (Scheme 7), was assigned as (22*R*)-3β-ergost-5,7-diene-3,22-diol (Figure 5). Since these two major isomers surely derived from the opening of a unique epoxide, the absolute configuration 22*S*,23*S* was assigned to the more abundant epoxide **16**, and consequently, the 22*R*,23*R*-configuration to the less abundant **15**. The diol obtained by the reduction of the 23-keto derivative **27**, different from **23**, had to be the (23*S*)-isomer **21**, as well as the other diol deriving from the 22-keto derivative **28** and distinct from **24**, was the 22S-derivative **22**.

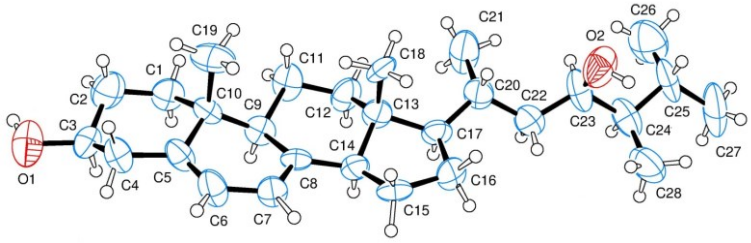


Figure 4. X-ray structure of **23**. A crystallization water molecule is omitted for clarity. Ellipsoids enclose 50% probability

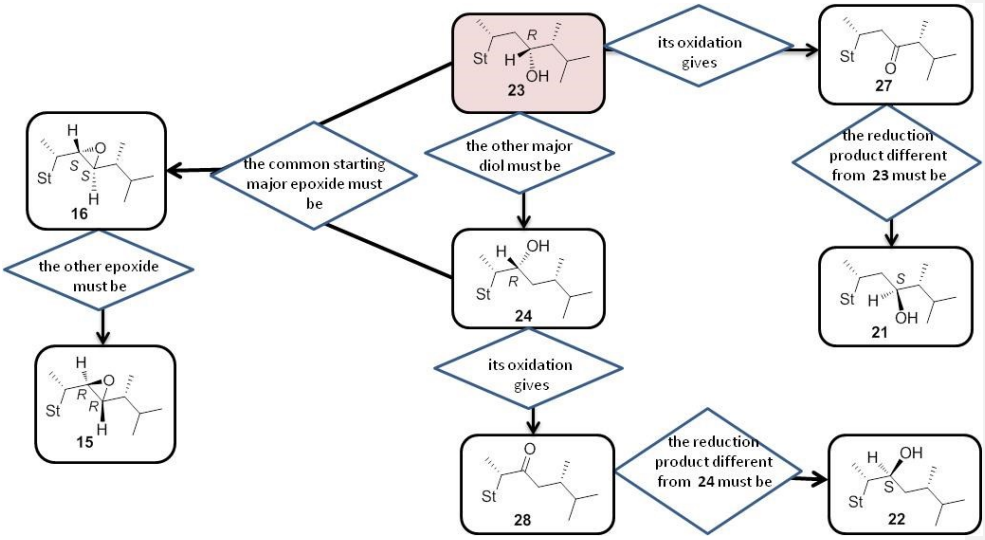


Figure 5. Flowchart for the structural assignment of the ergostane derivatives.

LXRs Activity

All the synthesized compounds were first tested for their ability to activate LXRs by using luciferase assays with GAL-4 chimeric receptors. These were performed by co-transfecting plasmids encoding hLXRα- and β-binding domains fused to GAL-4, with the respective responsive element conjugated with the luciferase reporter gene into the human embryonic kidney 293 cells. Results of the assays are listed in Table 1: most of the compounds exhibited low micromolar LXRs activity retaining or, in some cases, improving the magnitude of activity of the endogenous ligand 22*R*-HC (**3**).

Table 1. LXR Agonist Profile of Compounds 13-28

Compd	LXRα EC_{50} (μM)[a] ± SD (95% C.I.)[b]	Efficacy (%)[b] ± SD	LXRβ EC_{50} (μM)[a] ± SD (95% C.I.)[b]	Efficacy (%)[b] ± SD
22*R*-HC (3)	6.71 ± 0.71 (5.4 - 8.2)	100	4.75 ± 0.12 (3.4 - 6.4)	100

13	2.09 ± 0.57 (1.1 - 3.4)	488.8 ± 89.1	3.79 ± 0.82 (2.5 - 5.6)	142.1 ± 16.8
14	16.43 ± 0.62 (13.1 - 24)	56.8 ± 4.6	12.72 ± 2.7 (11.6 - 14.9)	36.3 ± 3.0
15	4.11 ± 0.31 (3.5 - 4.7)	93.2 ± 5.8	7.2 ± 0.94 (0.1 - 15.7)	30.4 ± 10.6
16	1.5 ± 0.12 (1 - 2.1)	51.7 ± 3.8	1.96 ± 0.05 (1.0 - 2.3)	48.5 ± 13
17	NA[c]	-	NA[c]	-
18	NA[c]	-	NA[c]	-
19	3.2 ± 0.54 (2.0 - 4.7)	489.3 ± 70.1	2.7 ± 1.16 (1.8 - 3.9)	115.1 ± 2.8
20	6.93 ± 1.9 (2.1 - 11.8)	208.3 ± 69.3	2.31 ± 0.36 (0.3 - 14.6)	90.8 ± 13.2
21	8.07 ± 1.60 (7.7 - 8.8)	159.3 ± 41.3	NA[c]	-
22	6.61 ± 1.69 (4 - 8.7)	74.7 ± 11.1	1.96 ± 0.1 (0.6 - 6.5)	41.9 ± 19.2
23	15.75 ± 0.65 (14.5 - 17)	63.9 ± 26.9	NA[c]	-
24	NA[c]	-	NA[c]	-
25	14.51 ± 1.86 (7.4 - 23.2)	12.4 ± 4.2	6.02 ± 1.2 (4.7 - 7.5)	46.4 ± 8.5
26	NA[c]	-	NA[c]	-
27	5.58 ± 0.30 (4.6 - 6.4)	150.6 ± 4.8	NA[c]	-
28	8.51 ± 0.42 (7.5 - 9.7)	70.2 ± 3.9	NA[c]	-

[a]Fifty% maximal activation (EC_{50}) ± standard deviations (SD) was determined by dose-response curve of titrating concentrations of compounds **13**-**28** (32, 16, 8, 4, 2 and 1 μM) tested by luciferase assays. The results were mean of three-five independent experiments; [b]Efficacy: % of compound effect ± SD versus 8 μM of 22R-HC; [c]NA: not active.

Concerning the isoform selectivity profile, besides non-selective and poorly preferential LXRα agonists (**13**, **16** and **19**) (Supplementary Figure 1 A-D), other compounds, such as **21**, **27** and **28**, deserve to be highlighted as selective LXRα agonists. Among them, the derivative **27** showed to be the most promising α-selective agonist thanks to its lower EC_{50} value and higher efficacy respect to the reference compound **3**. Furthermore, **20**, **22** and **25** showed a good selectivity for the LXRβ isoform in terms of EC_{50}. In particular, **25** can be considered a LXRβ-selective agonist, being

virtually inactive (EC_{50} = 14.51 μM) at LXRα (Supplementary Figure 1 B and D). Of note, **25** while showing approximately 50% of efficacy in terms of LXRβ activation, was endowed with the lowest efficacy at LXRα, as compared to the 22*R*-HC (**3**) (Table 1); Thus, confirming its selectivity for the LXRβ isoform. From a structural point of view, all the selective LXRα agonists are ergostane derivatives, whereas the preferential LXRβ ligands belong to the two classes; however the most interesting compound in this sense, namely **25**, is a stigmastane derivative.

Moreover, the skeleton system more than the nature and, where applicable, the stereochemistry of the side-chain modification, appeared to strictly influence both potency and isoform selectivity. Indeed, with *R*,*R*-epoxy derivatives **13** and **15**, as the only exceptions, any equally side-chain modified ergostane and stigmastane derivatives did not show similar biological profile.

We also evaluated the selectivity of our compounds within the nuclear receptor superfamily by luciferase assays using GAL4-RXR, -PPARγ, -PXR and -FXR plasmids. No compound was able to activate RXR or PPARγ, whereas we observed a slight activation of PXR by **21**, **22** and **15**, and a strong FXR activation by **20** (Supplementary Figure 2).

Gene expression profile

LXR agonists induce the expression of target genes, which are involved in cholesterol homeostasis, particularly in the reverse cholesterol transport pathway.[43] Indeed, LXR agonists induce the expression of ABCA1 both in macrophages and in many tissues of the periphery such as the intestine.[44] Moreover, ABCA1 regulates cholesterol efflux to APOAI acceptors.[45] In the liver, LXR activation promotes the biosynthesis of fatty acids, a process also termed as *de novo* lipogenesis by inducing the expression of the master regulator of hepatic lipogenesis sterol-regulatory element-binding protein 1C (SREBP-1c), as well as several downstream genes in the SREBP-1c pathway, including steroyl CoA desaturase 1 (SCD1) and fatty acid synthase (FASN).[43] Therefore, we investigated by quantitative PCR (qPCR) the expression of *ABCA1*, *SREBP1c*, *FASN*, and *SCD1,*

by using RNA from monocytic U937 cells (Figure 6) and from hepatic HepG2 cells (Figure 7) stimulated with our compounds, the non-steroidal agonist T0901317 or the endogenous ligand 22*R*-HC (**3**) as positive controls. As shown in Figure 6A, all the compounds, except **18** and **15**, were able to induce *ABCA1* expression, although to a different extent. With most derivatives, a mild up-regulation of the gene expression (2 fold) was observed, whereas, interestingly, with **13**, **19**, **20** and **25** we detected a strong induction of *ABCA1* expression comparable to that caused by T0901317. Noteworthy, for all our compounds the level of up-regulation of *SREBP-1c* was much lower than that observed for T0901317 and comparable to the level obtained with the natural ligand 22*R*-HC (**3**) (Figure 6B). The effects observed on *FASN* and *SCD1* genes were even more interesting: no compound up-regulated the mRNA levels of *FASN* (Figure 6C); a slight activation (below 2-fold) of *SCD1* was detected only for **16** and **25**, with the latter being statistically significant (Figure 6D). Also the natural ligand 22*R*-HC (**3**) did not induce up-regulation of *FASN* and *SCD1* transcripts (Figures 6C and 6D). These data were confirmed at later time points (*i.e.* 16 hours, *data not shown*). Then, we evaluated the induction of genes involved in the lipogenesis using the hepatic cell line HepG2. [43,22] By qPCR analysis we observed only a significant up-regulation of *SREBP-1c* induced by **13** and **16** compounds, while all the other compounds turned out to be negative (Figure 7A). No compound up-regulated the mRNA levels of *FASN* (Figure 7B) and *SCD1* (Figure 7C). According to all these evidences, the derivatives **13**, **19**, **20** and **25**, being strong inducers of *ABCA1,* poor activators of *SREBP1c* and *SCD1* in the U937 cell line, showed to be very promising derivatives. Over the time, indeed, substantial efforts have been dedicated to the identification of LXR ligands able to turning on ABC transporter genes, without affecting lipogenic genes levels. This task is still one of the major challenge to the discovery of a clinically useful LXR modulator for atherosclerosis. According to the isoform selectivity profile, **13** and **19** were non-selective ligands, **20** and **25** were a preferential and a selective LXRβ agonist, respectively, thus evidencing that in our model the ability to not up-regulate the genes involved in lipogenesis was not a phenomenon exclusive of LXRβ-selective modulators.

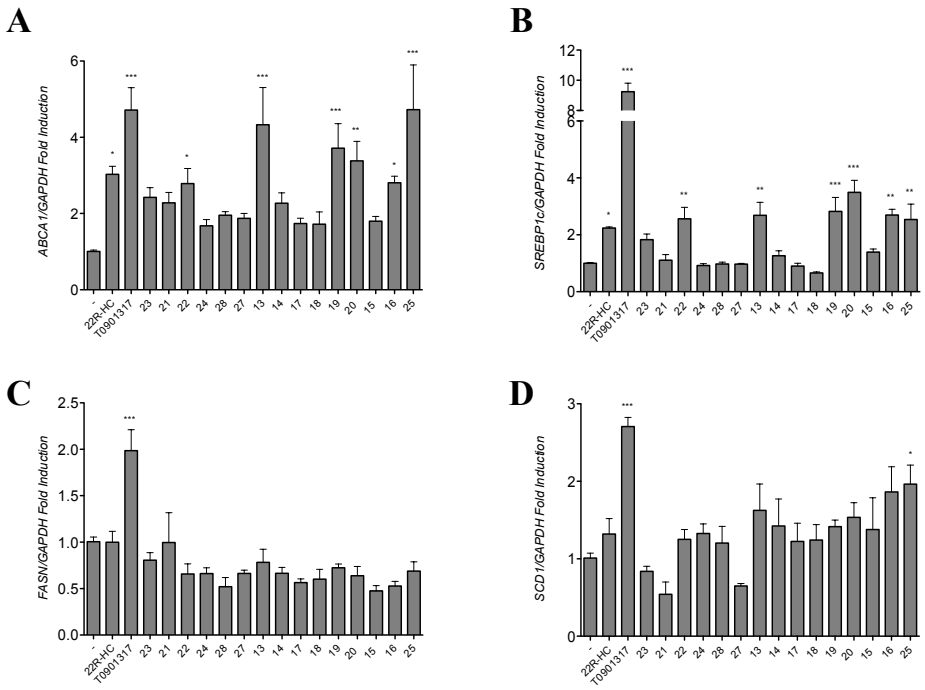


Figure 6. Regulation of *ABCA1* (A), *SREBP1c* (B), *FASN* (C), and *SCD1* (D) genes by the title compounds assessed by qPCR. U937 cells differentiated with PMA for 72 hours were treated with T0901317 (10 μM), 22*R*-HC (**3**) or with the tested compound (10 μM). The results show mean ± SD of three biological samples. (n = 3/group). *p < 0.05, **p < 0.01, ***p < 0.001.

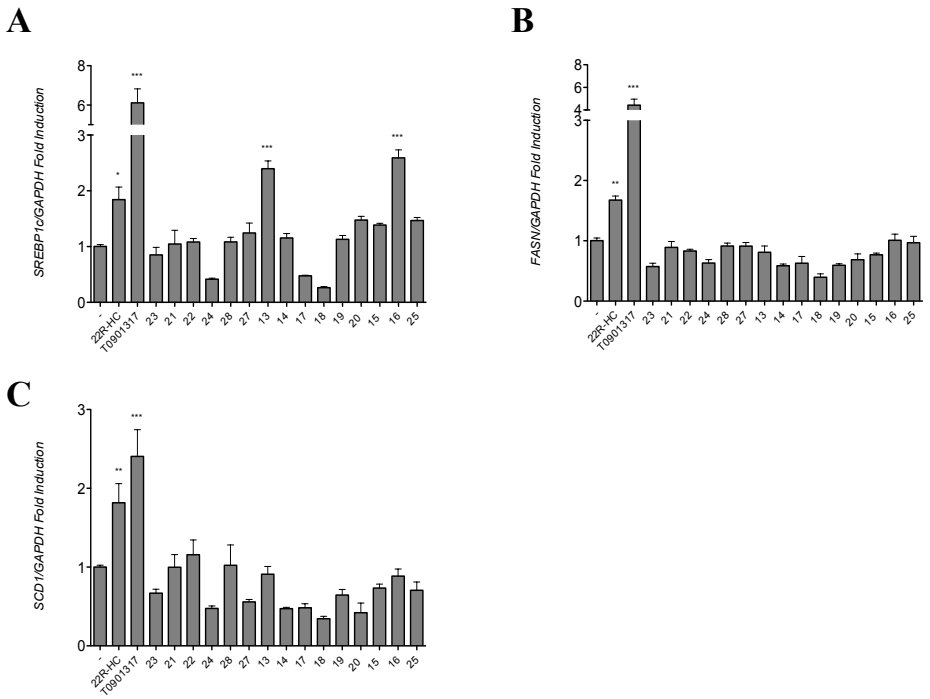


Figure 7. Regulation of *SREBP1c* (A), *FASN* (B), and *SCD1* (C) genes by the title compounds assessed by qPCR. HepG2 cells were treated with T0901317 (10 μM), 22*R*-HC (**3**) (10 μM) or with the tested compound (10 μM). The results show mean ± SD of three biological samples. (n = 3/group). *p < 0.05, **p < 0.01, ***p < 0.001.

Being LXRs not only transcriptional regulators of the cholesterol and lipid homeostasis, but also able to exert potent anti-inflammatory effects through the interference of TLRs 2, 4 and 9 signaling,[46] we decided to verify whether our compounds were also capable of modulating genes involved in the inflammatory pathways, such as the *MCP-1/CCL2* and *TNFα* genes, which have been shown to be inhibited when LXRs are engaged in the presence of LPS.[47] To this purpose, we treated differentiated U937 cells for 6 hours with our compounds in combination with LPS (100 ng/ml) and then we evaluated the treated cells for the expression of *CCL* and *TNFα* by qPCR. Most

of the compounds were able to inhibit *CCL2* expression with **21**, **22**, **19** and **16** showing the same grade of potency of the positive control T0901317 (Figure 8A). Most of the compounds were also able to inhibit *TNFα* with **21**, **13**, **19** and **25** being the most active (Figure 8B). Similar results were obtained by using the endogenous ligand 22*R*-HC (**3**) (data not shown).

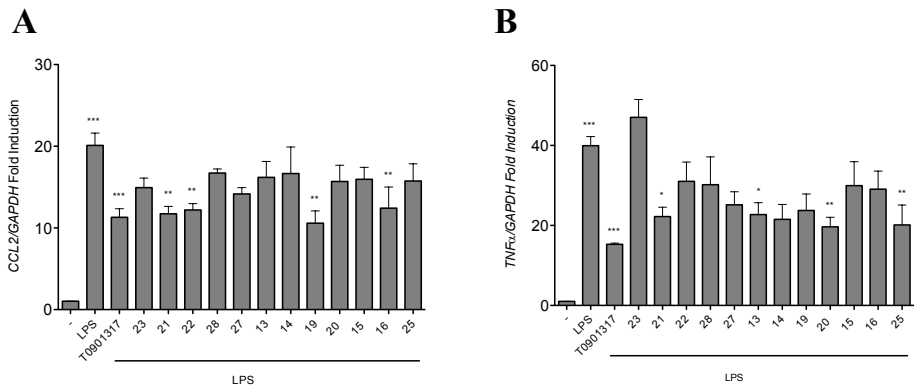


Figure 8. Regulation of *CCL2* (A) and *TNFα* (B) genes by the title compounds assessed by qPCR. U937 cells differentiated with PMA for 72 hours were treated with LPS (100 ng/ml) in combination with T0901317 (10 μM) or with the title compounds (10 μM). The results show mean ± SD of three biological samples. (n = 3/group). *p < 0.05, **p < 0.01, ***p < 0.001.

Conclusions

In summary, out of sixteen side-chain modified stigmasterol and ergosterol derivatives, we identified three selective LXRα agonists, namely **21**, **27** and **28**, and a selective LXRβ agonist, **25**. Additional novelty of our compounds concerns the gene expression profile, very different from that of the non-steroidal modulator T091317. Some of our compounds, indeed, when tested on U937 cells strongly up-regulated *ABCA1* expression without affecting lipogenesis-associated genes, as confirmed by tests on HepG2 cells. Thereby, we can hypothesize for our compounds a more pronounced effect on cholesterol homeostasis, especially on the reverse cholesterol transport pathway, than on lipogenesis. However, we cannot completely rule out the possibility that these results may be also associated to off-target effects, namely independent of LXR activation. This possibility deserves a careful investigation *in vitro* by using *LXRα* and/or *LXRβ* knockout cells and *in vivo* in appropriate models, such as $Lxr\alpha^{-/-}$, $Lxr\beta^{-/-}$ and $Lxr\alpha\beta^{-/-}$ mice. Gene expression data indicate two stigmastane analogues, namely **13** and **25**, as the most promising of the whole series; thus, evidencing the potential of the stigmastane scaffold as a starting point for designing LXR modulators.

Experimental Section

Chemistry. Melting points were determined by the capillary method on a Büchi 535 electrothermal apparatus and are uncorrected. ^{1}H- and ^{13}C NMR spectra were taken on a Bruker AC 400 spectrometer as solutions in $CDCl_3$ unless otherwise indicated. The spin multiplicities are indicated by the symbols s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and bs (broad). Flash chromatography was performed on Merck silica gel (0.040-0.063 mm). Medium pressure chromatography (mpc) was performed on Merck LiChroprep Si 60 Lobar columns. Microanalyses were carried out on a Carlo Erba 1106 elemental analyzer and the results were within ± 0.4% of the theoretical values. All solvents were distilled and dried according to standard procedures. Purity was determined by microanalysis to be >95% for all final compounds.

(22*R*,23*R*)-22,23-Epoxystigmast-5-ene-3β-ol (13). The epoxide **30** (0.067 g, 0.15 mmol) was refluxed in glacial acetic acid (5 mL) for 5 h. The residue obtained by the removal of the solvent *in vacuo* was directly dissolved in methanol/water (2:1, 12 mL) and the resulting solution treated with K_2CO_3 (0.26 g, 1.86 mmol) and refluxed for 3 h. After cooling the reaction mixture was extracted with CH_2Cl_2 (3x10 mL) and the combined organic layers dried over Na_2SO_4. The solvent was removed *in vacuo* and the residue submitted to mpc. Elution by light petroleum–ethyl acetate (80:20) afforded pure sample of **13**: 36% yield; mp 173.2-175.4 °C; ^{1}H NMR (400 MHz) δ 0.69 (s, 3H), 2.28-2.29 (m, 2H), 2.49-2.50 (m, 1H), 2.75 (dd, 1H, *J* = 9.32 and 2.21 Hz), 3.52 (m, 1H), 5.35-5.36 (m, 1H); ^{13}C NMR (100 MHz) δ 11.82, 12.45, 16.17, 19.37, 19.54, 20.17, 20.85, 21.01, 24.53, 27.93, 29.13, 31.61, 31.88 (2C), 36.48, 37.22, 38.66, 39.55, 42.24, 42.62, 48.28, 50.07, 53.42, 56.35, 62.14 (2C), 71.74, 121.54, 140.79; Anal. Calcd for $C_{29}H_{48}O_2$: C, 81.25%; H, 11.29%. Anal. Found: C, 81.17%; H, 11.24%.

(22*S*,23*S*)-22,23-Epoxystigmast-5-ene-3β-ol (14). The epoxide **31** was treated as reported for compound **30** to furnish **14** in 29% yield; mp 127.8-130.2 ºC; ^{1}H NMR (400 MHz) δ 0.68 (s, 3H), 2.24-2.30 (m, 2H), 2.49-2.54 (m, 2H), 3.53 (m, 1H), 5.35-5.37 (m, 1H); ^{13}C NMR (100 MHz) 11.97, 12.36, 16.28, 19.36 (2C), 20.92, 21.06, 24.51, 27.07, 29.31, 29.68, 31.62, 31.87 (2C), 36.48, 37.25, 38.87, 39.67, 42.27, 42.67, 48.77, 50.17, 56.02, 56.32, 58.55, 63.13, 71.77, 121.67, 140.67; Anal. Calcd for $C_{29}H_{48}O_2$: C, 81.25%; H, 11.29%. Anal. Found: C, 81.33%; H, 11.27%.

(22*R*,23*R)*-22,23-Epoxyergosta-5,7-diene-3β-ol (15). 2*M* KOH solution (0.2 mL) was added to a solution of **42** (0.037 g, 0.08 mmol) in EtOH (3.8 mL) and the resulting mixture was refluxed for 15 min. After cooling the reaction mixture was extracted with EtOAc (4x5 mL) and the combined organic layers were washed with brine (8 mL), dried over Na_2SO_4, filtered and the solvent removed *in vacuo* to give a residue which was submitted to flash chromatography. Elution with light petroleum–ethyl acetate (80:20) afforded **15** in 64% yield; mp: 163.8-165.2 ºC; ^{1}H NMR (400 MHz) δ 0.61 (s, 3H), 3.61-3.65 (m, 1H), 5.39-5.41 (m, 1H), 5.57-5.58 (m, 1H); ^{13}C NMR (100 MHz) 11.9, 13.7, 16.2, 16.3, 19.5, 20.4, 21.0, 23.2, 26.8, 31.1, 31.9, 37.0, 38.3, 39.0 (2C), 40.7,

42.3, 43.2, 46.2, 54.0, 55.6, 60.4, 64.3, 70.3, 116.5, 119.5, 139.8, 140.8; Anal. Calcd for $C_{28}H_{44}O_2$: C, 81.50%; H, 10.76%. Anal. Found: C, 81.17%; H, 10.74%.

(**22*S*,23*S*)-22,23-Epoxyergosta-5,7-diene-3β-ol (16)**. The derivative **43** was treated as reported for **42** to furnish **16** in 89% yield: mp: 138.3-139.6 ºC; ^{1}H NMR (400 MHz) δ 0.60 (s, 3H), 3.60-3.66 (m, 1H), 5.39-5.41 (m, 1H), 5.56-5.58 (m, 1H); ^{13}C NMR (100 MHz) 11.8, 12.5, 16.2, 17.1, 18.5, 20.2, 21.0, 23.3, 27.8, 31.0, 31.9, 37.0, 38.3, 39.0, 39.8, 40.7, 42.5, 43.2, 46.1, 53.3, 54.0, 63.1, 63.8, 70.3, 116.5, 119.4, 140.0, 140.7; Anal. Calcd for $C_{28}H_{44}O_2$: C, 81.50%; H, 10.76%. Anal. Found: C, 81.32%; H, 10.77%.

(23*S*)-3β-Stigmast-5-ene-3,23-diol (17) and (22*S*)-3β-Stigmast-5-ene-3,22-diol (18). $LiAlH_4$ (0.25 g, 6.71 mmol) was portion wise added to the solution of the epoxide **30** (0.27 g, 0.61 mmol) in anhydrous THF (15 mL). The resulting mixture was refluxed for 36 h under an argon atmosphere. After cooling, first EtOAc and then water were carefully added. The organic phase was separated and the water phase extracted with EtOAc (3x15 mL). The combined organic phases were washed with brine (20 mL) and then dried over Na_2SO_4. After filtration, the solvent was evaporated *in vacuo* to give a residue, which was dissolved in glacial acetic acid (5 mL) and the resulting solution refluxed for 6 h. After cooling, the mixture of **32** + **33**, obtained by the removal of the solvent *in vacuo,* was directly dissolved in methanol (16 mL) and treated with 2M KOH solution (8 mL). After refluxing for 3 h, the reaction mixture was extracted with EtOAc (3 x 15 ml). The combined organic layers were dried over Na_2SO_4, filtered and the solvent removed *in vacuo*, to give a residue which was submitted to mpc. Elution by light petroleum–ethyl acetate (70:30) afforded pure samples of the desired compounds in 69% total yield; **17**: mp 178.2-181.4 ºC; ^{1}H NMR (400 MHz) δ 0.69 (s, 3H), 2.23-2.31 (m, 2H), 3.53 (m, 1H), 3.91 (m, 1H), 5.36 (m, 1H); ^{13}C NMR (100 MHz) 11.79, 13.82, 18.28, 19.38 (2C), 19.85, 21.03, 21.12, 24.24, 28.45, 28.54, 31.58, 31.82 (2C), 34.16, 36.44, 37.19, 39.73, 42.22, 42.35, 42.46, 49.13, 50.01, 56.66, 56.88, 70.55, 71.73, 121.62, 140.72; Anal. Calcd for $C_{29}H_{50}O_2$: C, 80.87%; H, 11.70%. Anal. Found: C, 80.63%; H, 11.72%; **18**: mp 168.9-172.4 ºC; ^{1}H NMR (400 MHz) δ 0.70 (s, 3H), 2.24-2.31 (m, 2H), 3.53 (m, 1H), 3.75 (t, 1H, *J*

= 6.77 Hz), 5.35 (d, 1H, *J* = 5.21 Hz); ^{13}C NMR (100 MHz) 11.39, 11.73, 11.85, 18.89, 19.10, 19.38, 21.05, 23.27, 24.18, 27.8, 28.84, 31.58, 31.80, 31.88, 35.77, 36.43, 37.20, 39.72, 39.92, 42.04, 42.20 (2C), 50.02, 52.58, 56.61, 71.71, 71.86, 121.60, 140.73; Anal. Calcd for ($C_{29}H_{50}O_2$): C, 80.87%; H, 11.70%. Anal. Found: C, 80.70%; H, 11.65%.

(23*R*)-3β-Stigmast-5-ene-3,23-diol (19). A solution of **36** (0.03 g, 0.06 mmol) in MeOH (3 mL) was treated with 2*M* KOH solution (1 mL) and the resulting mixture was refluxed for 30 min. After cooling the reaction mixture was extracted with EtOAc (3x10 mL) and the combined organic layers were dried over Na_2SO_4, filtered and the solvent removed *in vacuo*. The residue, thus obtained, was purified by flash chromatography: elution with light petroleum–ethyl acetate (80:20) afforded **19** in 55% yield: mp 158.1-158.6 ºC; ^{1}H NMR (400 MHz) δ 0.72 (s, 3H), 2.27-2.28 (m, 2H), 3.51 (m, 1H), 3.69-3.74 (m, 1H), 5.35 (d, 1H, *J* = 5.25 Hz); ^{13}C NMR (100 MHz) 11.94, 14.48, 18.63, 18.96, 19.17, 19.36, 21.09, 21.44, 24.25, 27.80, 28.50, 31.67, 31.90 (2C), 32.78, 36.51, 37.28, 39.87, 41.11, 42.32, 42.51, 50.17, 52.49, 56.92 (2C), 70.25, 71.76, 121.60, 140.81; Anal. Calcd for $C_{29}H_{50}O_2$: C, 80.87%; H, 11.70%. Anal. Found: C, 80.67%; H, 11.66%.

(22*R*)-3β-stigmast-5-ene-3,22-diol (20). The derivative **37** was treated as reported for compound **36** to furnish **20** in 72% yield: mp: 149.2-149.9 ºC; ^{1}H NMR (400 MHz) δ 0.72 (s, 3H), 3.51 (m, 1H), 3.69-3.74 (m, 1H), 5.35 (d, 1H, *J* = 5.25 Hz); ^{13}C NMR (100 MHz) 11.75 (2C), 12.32, 17.74, 19.38, 20.45, 21.11, 23.60, 24.45, 27.50, 28.92, 29.65, 30.11, 31.70, 31.92, 36.54, 37.30, 39.81, 41.53, 42.33, 42.59, 42.70, 50.22, 53.07, 56.39, 71.39, 71.78, 121.59, 140.85; Anal. Calcd for $C_{29}H_{50}O_2$: C, 80.87%; H, 11.70%. Anal. Found: C, 80.91%; H, 11.69%.

(23*R*)-3β-Ergost-5,7-diene-3,23-diol (23) and **(23*S*)-3β-Ergost-5,7-diene-3,23-diol (21).** $NaBH_4$ (0.13 g, 3.44 mmol) was added to a solution of the ketone **48** (0.10 g, 0.2 mmol) in THF-2-propanol (2:1, 6 mL) and the resulting mixture was stirred at room temeprature overnight. The reaction mixture was then diluted with H_2O (5 mL) and extracted with Et_2O (3x5 mL). The combined organic layers were washed with brine (10 mL), dried over Na_2SO_4, filtered and the solvent removed *in vacuo*. The residue, thus obtained, was dissolved in EtOH (10 mL) and treated with

PPTS (0.012 g, 0.047 mmol). After refluxing for 1 h, the reaction mixture was allowed to cool to room temperature, and the solvent was removed *in vacuo* to give a residue, which was submitted to mpc. Elution with light petroleum–ethyl acetate (90:10) afforded pure samples of the desired compounds in 78% total yield; **21**: mp 129.8-130.7 ºC; ^{1}H NMR (400 MHz) δ 0.65 (s, 3H), 3.62-3.69 (m, 2H), 5.40-5.42 (m, 1H), 5.59 (dd, 1H, *J* = 7.89, 2.35 Hz). ^{13}C NMR (100 MHz) 10.54, 11.71, 16.27, 17.92, 20.67, 21.04, 21.75, 23.05, 27.74, 28.40, 31.92, 35.75, 36.97, 38.32, 39.09, 40.70 (2xC), 43.00, 45.32, 46.16, 54.37, 56.76, 70.44, 73.30, 116.33, 119.54, 139.80, 141.24; Anal. Calcd for $C_{28}H_{46}O_2$: C, 81.10%; H, 11.18%. Anal. Found: C, 80.97%; H, 11.19%.

(22*R*)-3β-Ergost-5,7-diene-3,22-diol (24) and **(22*S*)-3β-Ergost-5,7-diene-3,22-diol (22).** The derivative **49** was treated as reported for **48** to furnish pure samples of the desired compounds **24** and **22** in 83% total yield. **22**: mp 117.3-121.0 ºC; ^{1}H NMR (400 MHz) δ 0.65 (s, 3H), 0.79 (d, 3H, *J* = 6.84 Hz), 3.63-3.66 (m, 1H), 3.78-3.81 (m, 1H), 5.40-5.42 (m, 1H), 5.59-5.61 (m, 1H); ^{13}C NMR (100 MHz) 11.79, 12.49, 15.55, 16.02, 16.22, 21.04, 21.12, 23.15, 23.80, 27.39, 29.53, 31.92, 34.60, 35.27, 36.98, 38.30, 39.09, 40.72, 43.00, 46.14, 52.73, 54.01, 70.40, 71.67, 116.43, 119.54, 139.90, 140.96; Anal. Calcd for $C_{28}H_{46}O_2$: C, 81.10%; H, 11.18%. Anal. Found: C, 81.09%; H, 11.17%.

(23*R*)-3β-Ergost-5,7-diene-3,23-diol (23). PPTS (0.010 g, 0.039 mmol) was added to a solution of **44** (0.050 g, 0.1 mmol) in EtOH (5 mL) and the resulting mixture was refluxed for 5 h. After cooling the solvent was removed *in vacuo* and the residue was purified by flash chromatography. Elution with light petroleum–ethyl acetate (80:20) furnished **23** in 70% yield: mp 167.8-169.4 ºC; ^{1}H NMR (400 MHz) δ 0.68 (s, 3H), 3.68 (m, 1H), 3.82 (m, 1H), 5.44 (s, 1H), 5.61 (s, 1H). ^{13}C NMR (100 MHz) 9.83, 11.88, 16.25, 18.45, 18.79, 21.05, 21.50, 22.98, 28.33, 29.55, 31.92, 33.11, 36.97, 38.33, 39.18, 40.73, 42.11, 43.01, 45.37, 46.17, 54.52, 56.47, 70.38, 70.64, 116.34, 119.53, 139.80, 141.19; Anal. Calcd for $C_{28}H_{46}O_2$: C, 81.10%; H, 11.18%. Anal. Found: C, 81.26%; H, 11.16%.

(**22*R*)-3β-Ergost-5,7-diene-3,22-diol (24).** The derivative **47** was treated as reported for **44** to furnish **24** in 73% yield: mp 197.7-201.2 ºC; ^{1}H NMR (400 MHz) δ 0.65 (s, 3H), 0.79 (d, 3H, *J* = 6.84 Hz), 3.63-3.66 (m, 1H), 3.78-3.81 (m, 1H), 5.40-5.42 (m, 1H), 5.59-5.61 (m, 1H); ^{13}C NMR (100 MHz) 11.79, 12.49, 15.55, 16.02, 16.22, 21.04, 21.12, 23.15, 23.80, 27.39, 29.53, 31.92, 34.60, 35.27, 36.98, 38.30, 39.09, 40.72, 43.00, 46.14, 52.73, 54.01, 70.40, 71.67, 116.43, 119.54, 139.90, 140.96; Anal. Calcd for $C_{28}H_{46}O_2$: C, 81.10%; H, 11.18%. Anal. Found: C, 80.86%; H, 11.20%.

3β-Hydroxystigmast-5-ene-23-one (25). A solution of DMSO (0.03 g, 0.38 mmol) in anhydrous CH_2Cl_2 (0.5 mL) was added to a solution of oxalyl chloride (0.025 g, 0.2 mmol) in anhydrous CH_2Cl_2 (1 mL), kept at -60 °C under an argon atmosphere. After the resulting mixture was stirred for 15 min at -60 °C, the solution of alcohol **36** (0.048 g, 0.1 mmol) in anhydrous CH_2Cl_2 (1 mL) was added. The mixture was stirred for 2 h at -55/60 °C before the addition of Et_3N (0.08 g, 0.76 mmol). After the reaction mixture was allowed to reach room temperature, stirring was continued for 15 min, and then water (10 mL) was added. The reaction mixture was extracted with CH_2Cl_2 (3x5 mL), the combined organic layers were washed with brine (10 mL), dried over Na_2SO_4, filtered and the solvent removed *in vacuo*, to give the crude ketone **38**, which was dissolved in acetone (2 mL) and treated with 2*M* KOH solution (0.5 mL). The resulting solution was refluxed for 40 min, cooled and extracted with EtOAc (3x5 mL). The combined organic layers were dried over Na_2SO_4, filtered and the solvent removed *in vacuo* to give a residue then submitted to flash chromatography. Elution with light petroleum–ethyl acetate (80:20) furnished **25** in 51% yield: mp: 174.2-174.8 ºC; ^{1}H NMR (400 MHz) δ 0.62 (s, 3H), 2.40-2.42 (m, 2H), 3.40-3.47 (m, 1H), 5.26 (d, 1H, *J* = 4.9 Hz); ^{13}C NMR (100 MHz) 11.77, 12.02, 16.57, 18.46, 19.37, 19.61, 21.04, 23.90, 24.52, 27.64, 28.93, 31.64, 31.83, 31.90, 36.50, 37.27, 39.66, 39.82, 42.29, 42.47, 43.27, 49.66, 50.12, 51.95, 56.11, 71.72, 121.51, 140.78, 214.50; Anal. Calcd for $C_{29}H_{48}O_2$: C, 81.25%; H, 11.29%. Anal. Found: C, 81.32%; H, 11.31%.

3β-Hydroxystigmast-5-ene-22-one (26). The derivative **37** was treated as reported for the compound **36** to furnish **26** in 59% yield: mp: 151.7-152.8 ºC; ^{1}H NMR (400 MHz) δ 0.72 (s, 3H), 3.48-3.53 (m, 1H), 5.32-5.33 (m, 1H); ^{13}C NMR (100 MHz) 11.85, 19.35, 19.70, 20.06, 21.18, 21.57, 24.22, 28.37, 29.17, 31.62, 31.71, 31.85, 36.47, 37.24, 39.66, 42.26, 42.42, 50.06, 51.04, 55.70, 56.82, 60.82, 71.73, 121.52, 140.81, 214.58; Anal. Calcd for $C_{29}H_{48}O_2$: C, 81.25%; H, 11.29%. Anal. Found: C, 81.49%; H, 11.28%.

3β-Hydroxyergost-5,7-diene-23-one (27). PPTS (0.010 g, 0.039 mmol) was added to a solution of **48** (0.050 g, 0.1 mmol) in acetone (5 mL) and the resulting mixture refluxed for 5 h. After cooling the solvent was removed *in vacuo* and the residue was purified by flash chromatography. Elution with light petroleum–ethyl acetate (80:20) furnished **27** in 78% yield**:** mp 104.4-105.6 ºC; ^{1}H NMR (400 MHz) δ 5.57 (m, 1H), 5.39 (m, 1H), 3.6 (m, 1H), 5.38-5.40 (m, 1H), 5.56-5.58 (m, 1H); ^{13}C NMR (100 MHz) 11.8, 12.6, 16.2, 18.6, 20.0, 21.0, 21.4, 22.9, 28.2, 30.0, 31.8, 32.2, 36.9, 38.3, 39.0, 40.7, 42.9, 46.1, 49.0, 52.7, 54.4, 55.5, 70.3, 116.4, 119.4, 139.91, 140.9, 215.1; Anal. Calcd for $C_{28}H_{44}O_2$: C, 81.50%; H, 10.75%. Anal. Found: C, 81.73%; H, 10.71%.

3β-Hydroxyergost-5,7-diene-22-one (28). The derivative **49** was treated as reported for **48** to furnish **28** in 58% yield: mp 118.2-122.6 ºC. ^{1}H NMR (400 MHz) δ 0.65 (s, 3H), 3.63-3.68 (m, 1H), 5.39-5.40 (m, 1H), 5.57-5.59 (m, 1H); ^{13}C NMR (100 MHz) 11.94, 15.88, 16.23, 16.70, 18.17, 20.95, 23.19, 27.34, 31.82, 31.92, 33.61, 36.93, 38.26, 38.95, 40.65, 43.00, 46.07, 46.66, 49.98, 51.81, 53.66, 70.30, 116.56, 119.42, 139.97, 140.47, 214.79; Anal. Calcd for $C_{28}H_{44}O_2$: C, 81.50%; H, 10.75%. Anal. Found: C, 81.70%; H, 10.78%.

(22*R*,23*R*)-22,23-Epoxy-3α,5α-cyclo-6β-methoxystigmastane (30) and (22*S*,23S*R*)-22,23-Epoxy-3α,5α-cyclo-6β-methoxystigmastane (31). $NaHCO_3$ (7.34 g, 87 mmol,) and 77% *m*-CPBA (3.54 g, 16.8 mmol) were added to the solution of (22*E*)-3α,5α-cyclo-6β-methoxystigmast-22-ene[34] (**29**) (3.0 g, 7.0 mmol) in $CHCl_3$ (60 mL) and the resulting mixture was refluxed for 2 h. After cooling the reaction mixture was washed with 10% $Na_2S_2O_3$ solution (3x50 mL), water (50 mL), and then dried over Na_2SO_4. The solvent was removed *in vacuo* and the residue submitted to mpc.

Elution by light petroleum–ethyl acetate (95:5) afforded pure samples of **30** and **31** in 30% and 18% yields, respectively. Their spectral data were in agreement with those previously reported.[48]

(23*R*)-3β-Acetoxystigmast-5-ene-23-ol (36) and (22*R*)-3β-Acetoxystigmast-5-ene-22-ol (37). $LiAlH_4$ (0.22 g, 5.94 mmol) was portion wise added to the solution of the epoxide **31** (0.24 g, 0.54 mmol) in anhydrous THF (15 mL). The resulting mixture was refluxed for 36 h under an argon atmosphere. After cooling, first EtOAc and then water were carefully added. The organic phase was separated and the water phase extracted with EtOAc (3x15 mL). The combined organic phases were washed with brine (20 mL) and then dried over Na_2SO_4. After filtration, the solvent was evaporated *in vacuo* to give the mixture of **34** + **35**, which was dissolved in glacial acetic acid (10 mL) and the resulting solution refluxed for 3 h. After cooling, the solvent was removed *in vacuo* to give a residue, which was submitted to mpc. Elution by light petroleum–ethyl acetate (80:20) afforded pure sample of (23*R*)-3β-acetoxystigmast-5-ene-23-ol (**36**): 36% yield; mp 132.1-132.6 °C; ^{1}H NMR (400 MHz) δ 0.70 (s, 3H), 2.01 (s, 3H), 2.30 (d, 2H, *J* = 7.56 Hz), 3.67-3.71 (m, 1H), 4.58 (m, 1H), 5.36 (d, 1H, *J* = 4.25 Hz); ^{13}C NMR (100 MHz) 11.86, 14.56, 18.53, 18.82, 19.03, 19.22, 20.93, 21.37 (2C), 24.17, 27.69 (2C), 28.45, 31.72, 31.79, 32.70, 36.47, 36.89, 38.02, 39.68, 40.93, 42.38, 49.88, 52.33, 56.70, 56.76, 70.04, 73.88, 122.53, 139.51, 170.48. Further elution with the same eluent afforded (22*R*)-3β-acetoxystigmast-5-ene-22-ol (**37**): 21% yield; mp 123.9-125.2 °C; ^{1}H NMR (400 MHz) δ 0.70 (s, 3H), 2.03 (s, 3H), 2.31 (d, 2H, *J* = 7.11 Hz), 3.71 (d, 1H, *J* = 10.12 Hz), 4.60 (m, 1H), 5.37 (s, 1H); ^{13}C NMR (100 MHz) 11.77, 11.81, 12.26, 17.53, 19.26, 20.52, 20.96, 21.41, 23.50, 24.36, 27.39, 27.69, 28.62, 29.77, 31.80 (2C), 36.50, 36.93, 38.04, 39.61, 41.30, 42.45, 42.57, 49.97, 52.90, 56.18, 71.19, 73.88, 122.51, 139.59, 170.54.

3β-Acetoxy-5α,8α-(3,5-dioxo-4-phenyl-1,2,4-triazolidino)-22,23-epoxyergost-6-ene (41a). 77% *m*-CPBA (0.42 g, 1.87 mmol) was added to the solution of ergosterol acetate adduct **40**[36] (1.0 g, 1.63 mmol) in CH_2Cl_2 (10 mL) and the resulting mixture was stirred at room temperature for 5 h. Then, the reaction mixture was filtered and the solution washed with 5% $NaHCO_3$ solution (2x10 mL) and brine (10 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent was

removed *in vacuo* to give a residue which was submitted to flash chromatography. Elution by light petroleum–ethyl acetate (90:10) afforded the desired compound **41a** in 80% yield: mp: 138.1-144.1 ºC; ^{1}H NMR (400 MHz) δ 2.25-2.75 (m, 4H), 3.15-3.25 (m, 1H), 5.40 (m, 1H), 6.25 (m, 1H), 6.40 (m, 1H), 7.25-7.50 (m, 5H). ^{13}C NMR (100 MHz) δ: 12.3, 12.8, 13.0, 13.4, 17.1, 17.3, 18.5, 19.2, 20.1, 20.3, 21.1, 22.2, 25.7, 30.7, 30.9, 33.5, 37.8, 39.3, 40.9, 42.3, 44.0, 48.8, 52.6, 54.9, 60.1, 62.8, 63.9, 64.6, 64.7, 65.1, 70.2, 126.0, 127.6, 128.6, 128.9, 131.5, 135.0, 135.3, 146.4, 148.8, 148.9, 169.8.

(22*R*,23*R)*-3β-Acetoxy-22,23-epoxyergosta-5,7-diene (42) and **(22*S*,23*S)*-3β-Acetoxy-22,23-Epoxyergosta-5,7-diene (43)**. Anhydrous K_2CO_3 (0.13 g, 0.93 mmol) was added to a solution of epoxide **41a** (0.59 g, 0.93 mmol) in anhydrous DMF (50 mL). The resulting mixture was refluxed for 6 h, then cooled to room temperature and neutral alumina was added. The resulting mixture was filtered, and treated with water to yield a precipitate, which was then filtered *in vacuo* washing with water. The solid was submitted to mpc. Elution with light petroleum–ethyl acetate (90:10) afforded pure samples of the desired compounds in 65% total yield; **42**: mp 158.8-160.2 °C; ^{1}H NMR (400 MHz) δ 0.62 (s, 3H), 2.05 (s, 3H), 2.46-2.48 (m, 2H), 2.60-2.62 (m, 1H), 4.71 (m, 1H), 5.40-5.41 (m, 1H), 5.57-5.58 (m, 1H). ^{13}C NMR (100 MHz) 11.92, 13.70, 16.09, 16.29, 19.51, 20.43, 20.94, 21.43, 23.21, 26.83, 28.06, 31.10, 36.60, 37.04, 37.87, 39.00, 42.29, 43.20, 46.00, 53.94, 55.63, 60.37, 64.22, 72.74, 116.52, 120.19, 138.59, 141.05, 170.56; **43**: mp 133.5-135.2 °C; ^{1}H NMR (400 MHz) δ 0.61 (s, 3H), 2.05 (s, 3H), 2.37-2.52 (m, 3H), 2.69 (d, 1H, J = 7.70 Hz), 4.71 (m, 1H), 5.40 (bs, 1H), 5.57 (bs, 1H). ^{13}C NMR (100 MHz) 11.88, 12.56, 16.13, 17.14, 18.57, 20.24, 20.92, 21.43, 23.32, 27.80, 28.05, 31.00, 36.61, 37.05, 37.86, 38.92, 39.89, 42.50, 43.22, 45.95, 53.28, 53.97, 63.07, 63.83, 72.72, 116.53, 120.08, 138.78, 140.94, 170.57.

(23*R*)-3β-(Tetrahydro-2*H*-pyran-2-yloxy)ergost-5,7-diene-23-ol (44) and (22*R*)-3β-(Tetrahydro-2*H*-pyran-2-yloxy)ergost-5,7-diene-22-ol (47). $LiAlH_4$ (1.87 g, 49 mmol) was portion wise added to the solution of the epoxide **41b** (2.83 g, 4.2 mmol) in anhydrous THF (110 mL). The resulting mixture was refluxed for 36 h under an argon atmosphere. After cooling, first

EtOAc and then water were carefully added. The organic phase was separated and the water phase extracted with EtOAc (3x25 mL). The combined organic phases were washed with brine (30 mL), dried over Na_2SO_4, filtered and the solvent removed *in vacuo* to give a residue which was submitted to mpc. Elution with light petroleum–ethyl acetate (95:5) gave a pure sample of **44** in 20.5% yield: mp 95.1-96.9 ºC; ^{1}H NMR (400 MHz) δ 0.66 (s, 3H), 3.49-3.51 (m, 1H), 3.62-3.74 (m, 2H), 3.93-3.95 (m, 1H), 4.74-4.77 (m, 1H), 5.39 (s, 1H), 5.57 (s, 1H); ^{13}C NMR (100 MHz) 9.79, 11.82, 16.14, 18.40, 18.76, 19.78, 19.95, 20.97, 21.46, 22.94, 25.42, 28.20, 28.28, 29.50, 29.92, 31.13, 31.22, 33.05, 37.19, 37.34, 38.17, 38.46, 38.68, 39.17, 42.09, 42.95, 45.35, 46.12, 54.46, 56.45, 62.54, 62.77, 70.53, 74.55, 74.69, 96.60, 97.00, 116.31, 116.40, 119.34, 119.47, 139.89, 140.14, 140.78, 141.01. Further elution gave the inseparable mixture of (23*S*)-3β-tetrahydropyranyloxyergost-5,7-diene-23-ol (**45**) and (22*S*)-3β-tetrahydropyranyloxyergost-5,7-diene-22-ol (**46**) in 30% yield. Following elution afforded a pure sample of **47** in 21% yield: mp 180.2-181.5 ºC; ^{1}H NMR (400 MHz) δ 0.63 (s, 3H), 1.07 (d, 3H, *J* = 6.50 Hz), 3.47-3.50 (m, 1H), 3.61-3.65 (m, 2H), 3.76 (d, 1H, *J* = 10.66 Hz), 3.91-3.93 (m, 1H), 4.73-4.75 (m, 1H), 5.37 (s, 1H), 5.55 (s, 1H); ^{13}C NMR (100 MHz) 10.54, 11.68, 11.76, 12.48, 15.57, 16.06, 16.18, 17.91, 19.84, 20.01, 20.67, 21.01, 21.74, 23.05, 23.15, 25.45, 27.40, 27.73, 28.23, 28.38, 29.57, 29.96, 31.17, 31.26, 34.59, 35.31, 35.73, 37.23, 37.37, 38.22, 38.50, 38.73, 39.12, 40.72, 42.97, 43.05, 43.20, 45.35, 46.11, 46.16, 52.75, 53.98, 54.34, 56.78, 62.61, 62.84, 71.61, 74.20, 74.60, 74.68, 96.67, 97.05, 116.33, 116.43, 116.52, 119.36, 119.48, 139.98, 140.07, 140.22, 140.31, 140.57, 140.81, 141.07.

3β-(Tetrahydro-2*H*-pyran-2-yloxy)ergost-5,7-diene-23-one (48). A solution of DMSO (0.20 g, 2.51 mmol) in anhydrous CH_2Cl_2 (0.5 mL) was added to a solution of oxalyl chloride (0.17 g, 1.32 mmol) in anhydrous CH_2Cl_2 (1 mL), kept at -60 °C under an argon atmosphere. After the resulting mixture was stirred for 15 min at -60 °C, the solution of alcohol **44** (0.33 g, 0.66 mmol) in anhydrous CH_2Cl_2 (2 mL) was added. The mixture was stirred for 2 h at -55/60 °C before the addition of Et_3N (0.51 g, 5.0 mmol). After the reaction mixture was allowed to reach room

temperature, stirring was continued for 15 min, and then water (10 mL) was added. The reaction mixture was extracted with CH_2Cl_2 (3x5 mL), the combined organic layers were washed with brine (10 mL), dried over Na_2SO_4, filtered and the solvent removed *in vacuo*, to give a residue which was submitted to flash chromatography. Elution with light petroleum–ethyl acetate (90:10) furnished **48** in 65% yield: mp 135.9-136.3 ºC; ^{1}H NMR (400 MHz) δ 0.66 (s, 3H), 3.49 (m, 1H), 3.63 (m, 1H), 3.93 (m, 2H), 5.38 (s, 1H), 5.56 (s, 1H); ^{13}C NMR (100 MHz) 11.82, 12.58, 16.23, 18.67, 20.07, 21.38, 22.98, 25.48, 28.20, 30.08, 31.30, 32.28, 37.28, 38.00, 39.09, 43.01, 46.16, 49.09, 52.80, 54.47, 55.66, 62.91, 74.70, 74.76, 97.13, 116.53, 119.45, 139.89, 140.91, 214.81.

Biology. T0901317, GW4064 and 9-*cis*-retinoic acid were purchased from Sigma. Rosiglitazone was purchased from Cayman Chemical (Ann Arbor, MI).

Cell Culture and Co-transfection Assays. Human embryonic Kidney 293 cells (American Type Culture Collection) were cultured in Dulbecco's Modified Eagle's medium containing 10% of fetal bovine serum at 37°C in humidified atmosphere of 5% CO_2. We transiently transfected HEK293 cells (4x104 cells per well) in 48 well plate with the reporter plasmids pMH100X4-TK-luc (100 ng/well), Renilla (22 ng/well) together with 100 ng/well of pCMX-Gal4-RXR, pCMX-Gal4-PPAR-γ, pCMX-Gal4-PXR, pFA-CMV-FXR pCMX-Gal4-LXR-α or pCMX-Gal4-LXR-β plasmids using X-tremeGENE 9 DNA Transfection Reagent (Roche). Six hours after transfection, we treated the cells with the appropriate compound for 24 hours. We analyzed luciferase activities by luciferase Dual Reporter Assay Systems (Promega) according to the manufacturer's protocol. GAL4-LXRs, GAL4–PPAR-γ, GAL4-RXR and TK-MHC100-luc plasmids were described in Villablanca *et al*.[49] GAL4–PXR was a kind gift of Dr. Enrique Sainz (The Scripps Research Institute, La Jolla, USA). GAL4-FXR was a kind gift of Dr. Daniel Merk (Goethe-University Frankfurt am Main). The results obtained by luciferase assays and reported in Table 1 are from three to five independent experiments.

Quantitative Real-Time-PCR. U937 cell line was differentiated in foam macrophages with phorbol 12-myristate 13-acetate (PMA) 10 ng/ml (Sigma) for 72 hours at 37°C in 10 mm dish at the concentration of $3x10^6$ cells in 10 ml RPMI 10% FBS. At day 3 nuclear receptor ligands were added for 6 hours. HepG2 cells were treated with the ligands as described by Quinet *et al*.[22] Total RNA was purified by TRIZOL (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed incubating 2 μg of total RNA 1 hour at 42 °C with MLV-reverse transcriptase (Promega). Quantitative PCR was performed using Sybr Green Master Mix (Applied Biosystems) and real-time PCR (Viia 7 Real Time PCR System, Applied Biosystems). All PCR reactions were done in triplicate. The comparative Ct method was used to quantify transcripts that were normalized for human GAPDH. We used the following primer pairs:

GAPDH-F ACA TCA TCC CTG CCT CTA CTG

GAPDH-R ACC ACC TGG TGC TCA GTG TA

ABCA1-F CCA GGC CAG TAC GGA ATT C

ABCA1-R CCT CGC CAA ACC AGT AGG A

SREBP-1c-F GGC GGG CGC AGA TC

SREBP-1c-R TTG TTG ATA AGC TGA AGC ATG TCT

MCP-1-F AGA AGC TGT GAT CTT CAA GAC CAT T

MCP-1-R TGC TTG TCC AGG TGG TCC AT

FAS-F ACA GCG GGG AAT GGG TAC T

FAS-R GAC TGG TAC AAC GAG CGG AT

SCD1-F TTC AGA AAC ACA TGC TGA TCC TCA TAA TTC

SCD1-R ATT AAG CAC CAC AGC ATA TCG CAA GAA AGT

TNFα-F TCT TCT CGA ACC CCG AGT GA

TNFα-R CCT CTG ATG GCA CCA CCA G

Statistical analysis. Data are expressed as mean ± SEM and were analyzed for significance by ANOVA with Dunnet's multiple comparison tests. The analysis was performed with Prism software. Data in Table 1 are expressed as EC_{50} ± SD. In particular, the standard deviations were obtained by calculating the mean of the EC_{50} of each experiment (three to five independent experiments). The efficacy (%) of the compounds was calculated as the percentage of the compound effect, in terms of LXRα or β activation, versus 8 μM of 22*R*-HC ± SD. The analyses were performed with Prism software.

X-ray Analysis. A single crystal of compound **23** was submitted to X-ray data collection on an Oxford-Diffraction Xcalibur Sapphire 3 diffractometer with a graphite monochromated Mo-Kα radiation (λ = 0.71073 Å) at 293 K. The structure was solved by direct methods implemented in SHELXS program (version 2013/1).[50] The refinement was carried out by full-matrix anisotropic least-squares on F^2 for all reflections for non-H atoms by means of the SHELXL program (version 2013/4).[50] Crystallographic data (excluding structure factors) of **23** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC 1526884. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; (fax: + 44 (0) 1223 336 033; or e-mail: deposit@ccdc.cam.ac.uk).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website

Copies of ^{1}H- and ^{13}C-NMR spectra of final compounds and intermediates, SMILES strings, dose-response curves for activation of LXRs by **13**-**28** and activation of RXR, PPARγ, PXR and FXR nuclear receptors by **13**-**28**.

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ACKNOWLEDGMENTS

This work was supported by the Italian Association for Cancer Research (AIRC) to V.R.

ABBREVIATIONS USED

ABCA1, ATP-binding cassette transporter A1; CYP, cytochrome; DMF, dimethyl formamide; DMSO, dimethyl sulfoxide; FASN, fatty acid synthase; FXR, farnesoid X receptor; hLXR, human liver X receptor; INSIG, insulin-induced gene; LPS, lipopolysaccharides; LXR, liver X receptor; MCP-1, monocyte chemoattractant protein-1; *m*CPBA, *m*-chloroperoxybenzoic acid; mpc, medium pressure chromatography; NPC1, Niemann-Pick C1; ORP, OSBP-related protein; OSBP, oxysterol-binding protein; PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; PPARγ, peroxisome proliferator-activated receptor γ; PPTS, pyridinum *p*-toluenesulfonate; PXR, pregnane X receptor; qPCR, quantitative polymerase chain reaction; RNA, ribonucleic acid; RXR, retinoid X receptor; SCD1, stearoyl-CoA desaturase 1; SD, standard deviation; SREBP, sterol response element binding protein; TLR, toll-like receptor; TNF, tumor necrosis factor;

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