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Steroidal and Triterpenoidal Fungal Metabolites as Ligands of Liver X Receptors

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Abstract Cholesterol homeostasis is tightly controlled process that involves a variety of regulators including liver X receptors (LXR). Agonists of LXR are expected to increase cholesterol efflux, lower LDL, and raise HDL levels. Screening of a natural product library of microbial extracts using a LXR-scintillation proximity assay (SPA) binding assay and bioassay-guided fractionation of a number of fungal extracts led to the isolation of five ergostane and a cycloartane derivative. These compounds exhibited IC_{50} value ranging 0.5~9 μM in the binding assay for α-receptor and a number of these showed *in vitro* agonist activity in the coactivator association assays but lacked the cell based LXR activation. The isolation and LXR activity of these compounds are described.

Keywords steroids, triterpenoids, LXR receptor agonists, microbial products, cholesterol lowering agents

Introduction

Liver X receptors (LXR) are members of a superfamily of nuclear hormone receptors. There are two subtypes of these receptors, LXRα and LXRβ [1~3]. They play a role in cholesterol homeostasis [4]. The α-subtype is predominantly found in the liver whereas the β-subtype is ubiquitously expressed. Oxysterols have been identified as endogenous ligands for both LXR subtypes [5~8]. LXR receptors form heterodimers with the retinoid X receptor (RXR) and regulate the expression of a number of genes involved in cholesterol and fatty acid metabolism including adenosine friphosphate binding cassette frasporter A1 (ABCA1). ABCA1 mediates the efflux of cholesterol from the cell and onto the apolipo protein (Apo) A1 protein of HDL particles. Nonsteroidal agonists of LXR that increased the expression levels of ABCA1 raised the HDL levels in mice [9]. Therefore, LXR agonists are expected to provide an opportunity for the development of drugs to increase reverse cholesterol transport and thus decrease the burden of atherosclerosis [4].

Recently we reported a number of structurally diverse natural products as LXR ligands derived from plants [10~12], and a *Streptomyces* sp. [13] Continued screening of a microbial extract library employing the ligand binding domain of LXRα and β receptor using radioactive ligand and scintillation proximity binding assays (LXR-SPA) [14] allowed identification of a number of extracts that exhibited binding inhibitory activities. Standardized bioassay-guided fractionation of these extracts led to the isolation of ergostane derivatives: ergosterol, **2** and **3** from *Tolypocladium niveum*, **4** from *Colletotrichum dematium* and **5** from *Acremonium sordidulum* and a cycloartane derivative **7**, from an unidentified fungus. These compounds inhibited LXR activities to different degrees with LXRα binding IC_{50} ranges of 0.5~6.5 μM. The isolation, structure, LXR binding and agonist activities are detailed herein.

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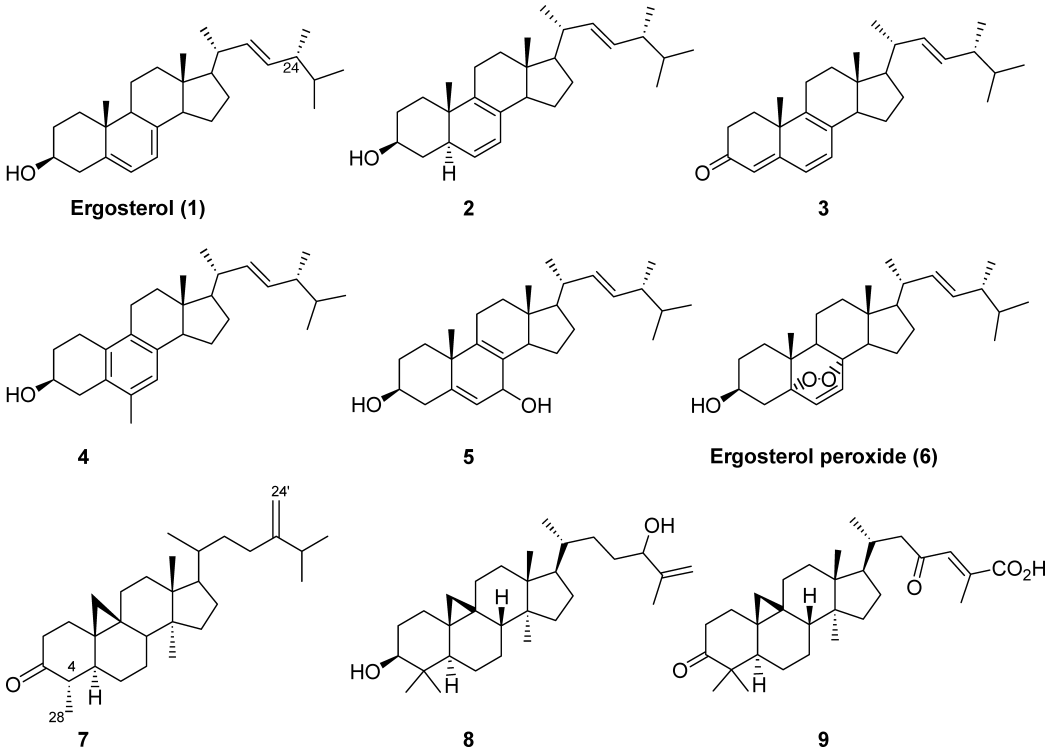

Fig. 1 Chemical structures of compounds **1**–**9**.

Results and Discussion

Compounds 2 and 3

These two ergostane derivatives along with ergosterol (**1**) were isolated from the fermentation broth of *Tolypocladium niveum* collected from Puerto Rico. Sephadex LH 20 followed by two steps of reversed phase HPLC and silica gel chromatographies afforded the three compounds. The production of ergosterol (**1**) was substantially higher than **2** and the enone **3**. The structure of ergosterol was confirmed by direct comparison with an authentic sample. The structure of **3** (MW 392) was elucidated as ergostan-4,6,8,22-tetraen-3-one by the comparison of published spectroscopic data of the compound originally reported from the oyster fungus, *Pleurotus ostreatus* [15]. Mass spectral analysis of **2** produced a molecular ion at *m*/*z* 396 suggesting the presence of 4 extra hydrogen atoms. ^{1}H NMR spectrum of **2** was similar to the spectrum of **3** except for the absence of the enone proton and the presence of methine at C-3 (δ_H 3.71, m) indicating that this compound was ergostan-6,8,22-trien-3-ol which has been reported from soil amoebae, *Acanthamoeba polyphaga*, *Neaegleria lovaniensis* and *N. gruberi* and was characterized as a mono acetate [16∼18]. The ^{1}H NMR data of this compound is reported here for the first time and is consistent with the ^{1}H NMR spectral data of the acetate [16,18] except for the acetate induced changes.

Compound 4

Colletotrichum dematium, the fungus that produced **4**, was isolated from *Monotropa uniflora* collected in New Jersey. The strain was cultured in a liquid media and extracted with MEK. Sephadex LH 20 chromatography followed by two successive steps of reversed phase HPLC afforded 12.7 mg of **4** (MW 394). The structure of **4** was elucidated by 2D NMR spectroscopy as ring B aromatic steroid with the migration of C-10 methyl group to C-6. The structure was confirmed by comparison of the published data of the compound isolated from soil amoeba *Acanthamoeba polyphaga* [19].

Compound 5

This compound was produced by a fungal strain identified as *Acremonium sordidulum* isolated from sand collected at a beach in Puerto Rico and was grown on FFL medium. It was isolated from a MEK extract of the fermentation broth after Sephadex LH 20 and silica gel TLC affording **5** (1 mg, 33 mg/liter). **5** was characterized by mass and 2D NMR spectral analysis. While the 7*R* analog was prepared in 6∼9% yield from ergosterol (**1**) by *in vitro* singlet oxygen reaction [20] this is first report of this compound from nature. This broth also produced small amounts of

Table 1 LXR activities of steroids and triterpenoids (**1**~**7**) and comparator compounds

Compound	LXR SPA binding IC_{50} (μM)		Coactivator association HTRF assay, EC_{50} (μM)		Transactivation max fold induction	
	LXRα	LXRβ	LXRα	LXRβ	LXRα	LXRβ
Ergosterol (**1**)	6.5	—	—	—	—	—
2	2.8	—	—	—	—	—
3	2.3	>15	1.0	>50	—	—
4	9.0	1.3	1.96*	>50	NA	NA
5	2.3	—	—	—	—	—
Ergosterol peroxide (**6**)	1.6	>50	1.7	—	NA	—
7	0.5	1.0	>10	>50	NA	NA
8	1.2	NT	NT	NT	NT	NT
9	1.7	NT	NT	NT	NT	NT
[$^3H_2$]-F_3-methyl AA	0.035	0.025	0.035	0.016	20	35
22-(*R*)-OH-cholesterol	70% @ 100 μM	60% @ 100 μM	>15	>15	4	8

Blank space (Not Tested), NA (not active at 10 μM), * Partial agonist max 36%

compound **4**.

Cycloeucalenone (7)

It was isolated from an unidentified fungus collected from New Jersey. Extraction with MEK and Sephadex LH 20 and two successive steps of reversed phased chromatographies afforded **7** (14 mg, 35 mg/liter). The structure of **7** was elucidated as cycloeucalenone (24-methyl-29-norcycloart-24(24′)-en-3-one) by ESIMS (MW 424) and comparison of NMR spectral data with the published data. Both α and β isomers of C-4 have been reported [21, 22]. The ^{1}H NMR spectrum of **7** exhibited a doublet of doublets at δ_H 2.41 with $J_{4,5}$=14 Hz and $J_{4,28}$=6 Hz suggesting an *anti*-relationship of H-4 and H-5 and establishing H_β at C-4. Both cycloeucalenone (**7**) and 4-epicycloeucalenone were originally isolated from a banana peel, *Musa sapientum* and this is first report of its production by fungal species.

LXR Activity

Compounds were first evaluated for their ability to displace the tritiated ligand {[$^3H_2$]-F_3-methyl AA {3-chloro-4-(3-(7-(2,3-ditritio-propyl)-3-trifluoromethyl-6-(4,5)-isoxazolyl)-propylthio)-phenyl acetic acid} in the LXR-SPA receptor binding assays using ligand binding domains (LBD) of α and β LXR and the results are expressed as IC_{50} (concentration of compound required to exhibit 50% displacement of the radio ligand) [14]. Following the measurements of the binding activity, many of these compounds were further evaluated for their functional activity in a coactivator association and transactivation assays. The coactivator association assay determines the association of recombinant steroid receptor coactivator 1 (SRC1) protein with recombinant LXRα and β LBD using homogeneous time resolved fluorescence (HTRF) detection method and results are expressed as EC_{50} (effective concentration of compound requiring 50% stimulation of coactivator association). The transactivation assay determines the activation of receptors expressed in HEK-293 cells and data is expressed as fold induction compared to DMSO control [14].

Of the ergosterol (**1**) series, the B-ring $\Delta^{6,8}$-diene **2** exhibited LXR α binding affinity with an IC_{50} of 2.8 μM whereas the isomer, ergosterol (**1**), with $\Delta^{5,7}$-diene was 2 fold less active and exhibited binding affinity with IC_{50} of 6.5 μM. The trienone **3** showed slightly better affinity for the α-receptor (IC_{50} 2.3 μM). Of the three non-aromatic mono-oxygenated ergosterol derivatives the better of the three compounds, **3** was further evaluated. It did not show any binding to LXRβ (IC_{50}>15 μM). In coactivator association assays, it was an agonist of LXRα and stimulated the α-receptor with EC_{50} value of 1 μM and was not effective for the β-receptor. The B-ring aromatic compound **4** exhibited better binding inhibition for LXRβ (IC_{50} 1.3 μM) compared to LXRα (IC_{50} 9 μM). It was a partial agonist (36% maximal stimulation) of the α-receptor (EC_{50} 1.96 μM) as measured by coactivator association assays and was not effective for LXRβ (EC_{50}>50 μM). Of the mono-oxygenated compounds, **4** was tested in the transactivation assay. This compound did

not show any activity in the transactivation assay at 10 μM. Of the two di- and tri-oxygenated ergosterol derivatives, **5** and ergosterol peroxide, showed binding affinities to LXRα with IC_{50} of 2.3 and 1.6 μM, respectively. Ergosterol peroxide did not have any effect on the binding affinity for LXRβ. It exhibited LXRα agonist activity (EC_{50} 1.7 μM) in the coactivator association assay. However it had no effect on the transactivation activity at 10 μM.

The 29-nor-cycloartane derivative, cycloeucalenone (**7**) displayed the best binding affinity of all with IC_{50} values of 0.5 and 1.0 μM, respectively, for LXRα and β exhibiting 2~3 fold better affinity for LXRα than plant derived cycloartanes (**8**, **9**) [12] suggesting a positive role played by either addition of the methyl group at C-24 or subtraction of the C-29 methyl group. However, this compound was not an agonist at >10 μM against either of the two receptors and was not effective in the transactivation assays.

It has been reported that cholesterol and a number of hydroxy cholesterol derivatives such as 7α, and 22*S*-, have no effect on the LXR activation and others such as 20*S*-, 22*R*-, 24*R*-, 24*S*-, 25-, 27-hydroxy, 24*R*,25 and 24*S*,25-epoxy, and 24-keto cholesterol have varying degree of receptor activation [6~8, 23].

In summary, we have described the isolation and LXR activities of a number of unsaturated steroids of ergosterol and 29-nor-cycloartane families. While a number of oxycholesterols are natural agonists of LXR receptors, the activity of these compounds adds to the growing reservoir of understanding of LXR receptor binding.

Experimental

General Experimental Procedures

All ACS grade solvents were obtained from Fisher Scientific. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. The UV spectra were recorded in MeOH on a Beckman DU-70 Spectrophotometer. IR spectra were recorded on Perkin-Elmer Spectrum One spectrometer. HRESIMS were obtained on a Thermo Quest FTMS spectrometer using electrospray ionization. The NMR spectra were recorded on a Varian INOVA 400 or 500 FTNMR spectrometers at 400 or 500 MHz for ^{1}H and 100 or 125 MHz for ^{13}C in $CDCl_3$. Agilent HP1100 was used for analytical HPLC.

Seed Media

KFA in g/liter of distilled water: corn steep liquor (5), tomato paste (40), oat flour (10), glucose (10), trace elements, agar (4) in distilled water at pH 6.8. YMEJ in g/liter of distilled water: yeast extract (4), malt extract (8), glucose (4), junlon (1.5), pH adjusted to 7.0.

Production Media

DEF2: This liquid medium consists of three solutions that are prepared and mixed at different proportions. Solution A consists of in g/900 ml distilled water: glycerol (60), mono sodium glutamate (10), L-tryptophan (0.7), NH_4Cl (3), K_2HPO_4 (1), $CaCO_3$ (1), $MgSO_4 \cdot 7H_2O$ (0.58), MES (20), salts (20 ml), pH adjusted to 6.0 by addition of NaOH. Solution B in g/100 ml distilled water: glucose (60) and autoclave. Solution 3 salts in mg/liter: $FeSO_4 \cdot 7H_2O$ (500), $ZnSO_4 \cdot 7H_2O$ (500), $MnSO_4 \cdot H_2O$ (100), $CuSO_4 \cdot 5H_2O$ (50), $CoCl_2 \cdot 6H_2O$ (40), conc. HCl (50 ml/liter). A 45 ml aliquot of solution 1 was added to a 250 ml flask followed by 5 ml of solution 2 and 20 ml of salts. NPF2 medium, components in g/liter of distilled water: glucose (150), urea (4), N. Z. amine type A (4), K_2HPO_4 (0.5), $MgSO_4 \cdot 7H_2O$ (0.25), KCl (0.25), $ZnSO_4 \cdot 7H_2O$ (0.9), $CaCO_3$ (16.5) without any pH adjustment. FFL medium, components in g/liter in distilled water: glycerol (100), glucose (70), L-tryptophan (0.7), NH_4Cl (3), mono sodium glutamate (10), amicase (8), MES (20), K_2HPO_4 (1), $MgSO_4 \cdot 7H_2O$ (0.5), 85% lactic acid (5 ml), trace elements (20 ml), adjust pH to 6.0 before addition of $CaCO_3$ (1 g).

Fermentation and Isolation of 2 and 3

The producing organism a fungus, *Tolypocladium niveum* (JP3248) was isolated from leaf litter of *Guare guidonia* collected from Puerto Rico and grown on a YMEJ seed medium for 3 days at 25°C followed by a transfer and growth on a production medium NPF2 for 18 days at 22°C in a 250 ml Erlenmeyer flask containing 50 ml of the medium. 2 liters fermentation broth was extracted with 2 liters MEK by shaking for 1 hour. The extract was concentrated to dryness affording 2.1 g residue which was chromatographed on a 1.5 liters Sephadex LH 20 in methanol. The active fractions were pooled which contained a large amounts of ergosterol which was collected by filtration and a 50 mg portion was chromatographed on a Zorbax RX C_8 (21×250 mm) column eluting with a 50 minutes gradient of 40~90% aqueous CH_3CN+0.1% TFA followed by holding at 90% for 25 minutes then at 99% at a flow rate of 8 ml/minute. The fractions eluting at 88 minutes was concentrated to give 22 mg of mixture and purified by preparative TLC on silica gel (3% MeOH-CH_2Cl_2). Compounds eluting at Rf values of ~0.5~7 (8 mg) was rechromatographed on a semi prep Zorbax RX C_8 (9.4×250 mm) eluting with 85% aqueous CH_3CN +0.1% TFA at a flow rate of 4 ml/minute. The major compound **2** (3 mg) eluted at 29 minutes and the minor compound **3** (1 mg) eluted at 40 minutes which were lyophilized to give

colorless powder. **2**: ^{1}H NMR ($CDCl_3$) δ 0.68 (3H, s, H_3-19), 0.86 and 0.87 (3H each, d, J=6.5 Hz, H_3-26, 27), 0.94 (3H, s, H_3-18), 0.95 (3H, d, J=6.5 Hz, H_3-21), 1.07 (3H, d, J=6.5 Hz, H_3-28), 1.17～1.35 (4H, m), 1.37～1.54 (6H, m), 1.70～2.0 (5H, m), 2.0～2.15 (3H, m), 2.25～2.42 (2H, m), 3.72 (1H, m, H-3), 5.23 and 5.26 (1H each, dd, J=15, 7 Hz, H-22 and H-23), 5.29 (1H, br d, J=9.5 Hz, H-7), 6.15 (1H, dd, J=10, 3 Hz, H-6); ESIMS (m/z) 397 (M+H).

Fermentation and Isolation of 4

The producing organism a fungus, *Colletotrichum dematium* (GB4417) was a single spore isolated from sporodochia formed on stems of *Monotropa uniflora*, collected in Califon, New Jersey. Inoculum was grown on YMEJ medium and then the cells were used to inoculate NPF2 media at the condition described for **3** and **2** except for harvesting after 21 days. A 2 liters fermentation broth was extracted with 2 liters MEK and concentrated to give 2 g which was dissolved in 30 ml MeOH and chromatographed on 1 liter of Sephadex LH 20 in MeOH to give an active fraction weighing 300 mg. A 2/3rd portion of the fraction was chromatographed on a reversed phase Zorbax RX C_8 (21×250 mm) column with a 30 minutes gradient of 20 to 80% aqueous CH_3CN with a hold for 20 minutes at a flow rate of 8 ml/minute. The active fraction (30 mg) eluted in 80% of CH_3CN at 46～50 minutes was rechromatographed on same HPLC column using a 30 minutes gradient of 80～90% aqueous CH_3CN at a flow rate of 8 ml/minute. Fractions eluting at 21～25 minutes were pooled and lyophilized to give 12.7 mg of **4** as a gum.

Fermentation and Isolation of 5

The producing fungus, *Acremonium sordidulum* (F-094151), was recovered from sand collected at a beach in Puerto Rico. For the production of the compound, seed flasks of KF seed medium were prepared from a fresh slant culture on potato dextrose agar (Difco), as described [24]. Two milliliter aliquots of the seed cultures were used to inoculate 250 ml unbaffled Erlenmeyer flasks containing 50 ml of FFL production medium. The production flasks were incubated at 22°C in a rotatory shaker at 220 rpm for 20 days. A 30 ml MEK extract was chromatographed on 70 ml Sephadex LH 20 in MeOH and the active fraction (10 mg) was chromatographed on silica gel TLC (5% MeOH - CH_2Cl_2). The fraction eluting at Rf ～0.2 was concentrated to afford 1 mg of **5** as gum.

Ergosterol Peroxide (6)

Ergosterol and its peroxide are components of the fungal cell wall. The peroxide was isolated from a series of cultures and identified by direct comparison with an authentic sample obtained from our collection. The biological data reported here is from a sample from our collection.

Fermentation and Isolation of 7

The producing organism, an unidentified Coelomycete (JP4567) was isolated from live leaves of Sweet gum collected in New Jersey and was grown on FFL media. A 2-liter broth was extracted with 2 liters MEK and the extract (1.6 g) was chromatographed on 1 liter of Sephadex LH 20 in MeOH. A 2/5th portion of an 800 mg active fraction was chromatographed on a similar Zorbax RX C_8 column eluting with a 30 minutes gradient of 20～80% aqueous CH_3CN +0.1% TFA with a hold for 20 minutes at a flow rate of 8 ml/minute. The active fraction (96 mg) eluted with 80% CH_3CN and was further chromatographed on the same column with a 30 minutes gradient of 80～90% aqueous CH_3CN at a flow rate of 8 ml/minute eluting active compound at 36～40 minutes which upon lyophilization afforded 14 mg of **7** as a gum.

LXR-SPA Binding Assays

LXR scintillation proximity assay (LXR-SPA) was performed using the GST-LXR ligand binding domain (LBD) of the α or β receptor and the ligand [$^3H_2$]F_3-methyl AA (**12**) as detailed by Menke *et al* [14].

Coactivator-association Assays

The agonist activity of compounds were measured in an *in vitro* coactivator association assays. In this assay, the association of recombinant steroid receptor coactivator 1 (SRC1) coactivator protein with recombinant LXR α and β ligand binding domains were measured using a HTRF assay as described earlier [14].

LXRα and β Cell Based Transactivation Assay

Cell-based transactivation assay using a chimeric LXR constructs were used to measure the LXRα and/or β agonist or antagonist functional activity in HEK-293 cells. This assay uses fusion proteins with the yeast Gal4 DNA binding domain connected to the hinge region and the LBD domain of either LXR receptors and has been previously described [14, 25].

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