Anthrabenzoxocinones from *Streptomyces* sp. as Liver X Receptor Ligands and Antibacterial Agents

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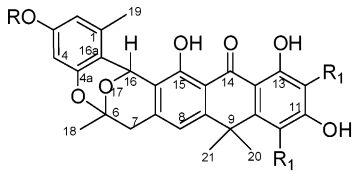
Liver X receptors (LXR) are nuclear hormone receptors that play a critical role in cholesterol homeostasis. They regulate the expression of the ABCA1 gene, which mediates the efflux of cholesterol out of cells. LXR agonists are expected to increase cholesterol efflux, lower LDL, and raise HDL levels. Screening of a natural product library of microbial extracts using a LXR-SPA binding assay and bioassay-guided fractionation of an active extract of a *Streptomyces* sp. (MA6657) led to the discovery of two new hexacyclic aromatic ketones, (−)-anthrabenzoxocinone [(−)-ABX (**1**)], an enantiomer of BE-24566B, and (−)-bischloroanthrabenzoxocinone [(−)-BABX (**2**)]. The IC_{50} values of LXRα-SPA binding are 2 μM for (−)-ABX and 10 μM for (−)-BABX. This extract was also found to inhibit type II fatty acid synthesis, and its active component, (−)-BABX, was responsible for the majority of the inhibition. All three compounds showed good Gram-positive antibacterial activity (MIC 0.5−2 μg/mL). Details of the isolation, structure elucidation, LXR ligand binding, antibacterial activity, and selectivity of inhibition of **1** and **2** are described.

Liver X receptors (LXR) are members of a superfamily of nuclear hormone receptors represented by two subtypes, LXRα and LXRβ.[1−3] These receptors are differentially expressed and have been shown to play a role in cholesterol homeostasis.[4] The α-subtype is predominantly found in the liver, whereas the β-subtype is ubiquitously expressed. High cholesterol loading causes production of oxysterols, which have been identified as endogenous ligands for both LXR subtypes.[5−8] LXR receptors form heterodimers with the retinoid X receptor (RXR) and directly or indirectly regulate the expression of a number of genes involved in cholesterol and fatty acid metabolism including ABCA1. ABCA1 mediates the efflux of cholesterol out of the cells and onto the ApoA1 protein of HDL particles. Nonsteroidal agonists of LXR that increased the expression levels of ABCA1 raised the HDL levels in mice. Therefore, LXR ligands, particularly 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]

Screening of a collection of natural product extract libraries employing the ligand binding domain of LXRα and -β receptors using scintillation proximity binding assays (LXR-SPA)[9] and a radioactive synthetic ligand, [$^3H_2$]-F_3-methyl AA {3-chloro-4-(3-(7-(2,3-ditritiopropyl)-3-trifluoromethyl-6-(4,5)-isoxazolyl)propylthio)phenylacetic acid}, allowed identification of a number of extracts that showed selective binding activity. From these studies the isolation of guttiferone I from *Garcinia humilis* was recently reported as a binding inhibitor of the LXRα receptor.[10] Bioassay-guided fractionation of one of the active extracts of a *Streptomyces* sp. led to the isolation of hexacyclic anthrabenzoxocinone ((−)-ABX, **1**) and bischloroanthrabenzoxocinone [(−)-BABX, **2**)]. (−)-ABX **1** inhibited ligand binding, with IC_{50} values in the range 2−10 μM. The same extract also inhibited type II fatty acid synthesis in a FASII assay, and bischloroanthrabenzoxocinone [(−)-BABX, **2**)] was mainly responsible for FASII inhibitory activity.[11] The isolation, structure elucidation, affinity of LXR ligand binding, fatty acid synthesis inhibition, and antibacterial activities of these compounds are described.



1 (R = R_1 = H), (-)-ABX
2 (R = Me, R_1 = Cl), (-)-BABX

The *Streptomyces* sp. (MA6657) that produced compounds **1** and **2** was obtained from Merck culture collection and was grown on a liquid medium. The broth was extracted with methyl ethyl ketone and chromatographed on Sephadex LH20 followed by reversed-phase HPLC. This procedure afforded compounds **1** (3 mg, 1 mg/L) and **2** (78 mg, 26 mg/L) as amorphous powders.

HRESIMS analysis of anthrabenzoxocinone ((−)-ABX, **1**) produced a molecular formula of $C_{27}H_{24}O_7$. Search of our internal database produced L-755805 as a match for this compound. L-755805 was isolated at Merck as an binding inhibitor of endothelin receptor.[12] The newly isolated compound (**1**) was compared directly with an authentic sample of L-755805 reported by Lam et al.[12] by co-HPLC and ^1H NMR. The two compounds were identical in all aspects except for their specific rotations. L-755805 was reported to have a positive specific rotation $[\alpha]_D$ +690° (*c* 0.5, CH_2Cl_2), and compound **1** exhibited a negative specific rotation $[\alpha]_D$ −371° (*c* 0.08, CH_2Cl_2). These compounds have two chiral centers at bridge heads C-6 and C-16. 1,3-Diaxial ring fusion at C-6 and C-16 dictates the equatorial orientations of CH_3 at C-6 and H at C-16, suggesting the existence of only enantiomeric possibilities and not diastereomeric possibilities. Thus compounds **1** and L-755805 must be enantiomeric. However, the apparent large discrepancies of the specific rotations of the two compounds concerned us and could not be explained by measurement errors. The compound isolated by Lam et al. was available only in microgram quantities to be used as an HPLC reference prohibiting reexamination of the specific rotation. Fortu-

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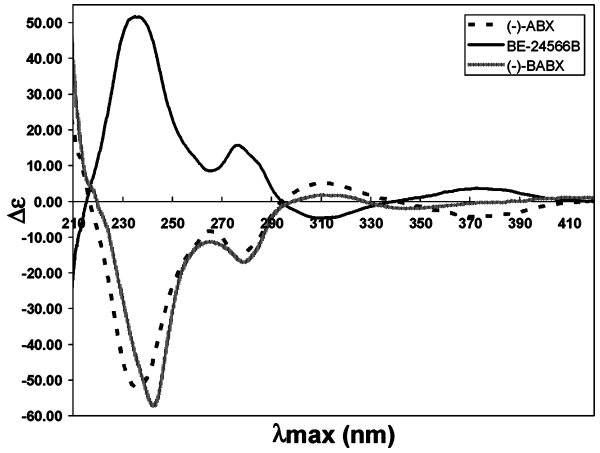
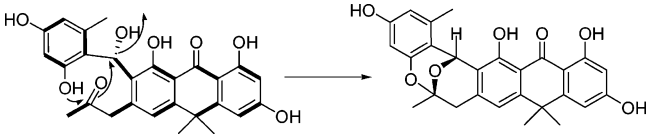


Figure 1. CD spectra of (−)-ABX, (−)-BABX, and BE-24566B.

nately, the same compound was also reported by Banyu Labs as BE-24566B in the patent literature.[13] Unfortunately, the specific rotation of this compound had not been reported. However, we were able to obtain a sample of BE-24566B for comparison. Spectrally and chromatographically all three compounds were indistinguishable. BE-24566B exhibited $[\alpha]_D$ +390° (*c* 0.5, CH_2Cl_2), which was much closer but of opposite sign to compound **1**. To prove that the two compounds were enantiomers, CD spectra were recorded (Figure 1). The CD spectrum of BE-24566B showed positive Cotton effects at λ_{max} 234 ($\Delta\epsilon$ +51.4) and 274 ($\Delta\epsilon$ +15.5) nm, which were opposite in sign to the Cotton effects [λ_{max} 234 ($\Delta\epsilon$ −51.6) and 274 ($\Delta\epsilon$ −15.5)] exhibited by (−)-ABX (**1**) (Figure 1). Therefore, BE-24566B and (−)-ABX (**1**) are enantiomers. The former compound therefore could be designated as (+)-ABX. In all likelihood L-755805 is the same as (+)-ABX, and the higher value of the specific rotation reported is erroneous.

Mass spectral analysis of compound **2** provided a molecular formula of $C_{28}H_{24}O_7Cl_2$, which was supported by ^{13}C NMR. The comparison of ^1H NMR spectra of **1** and **2** in C_5D_5N indicated the presence of a methoxy group and the absence of H-10 and H-12, which were replaced by two chlorine atoms in compound **2**. The ^1H and ^{13}C NMR spectra of **2** displayed four methyl singlets (H_3-18, H_3-19, H_3-20, and H_3-21), one methoxy singlet (δ_H 3.68, δ_C 55.7), *meta*-coupled aromatic protons (H-2 and H-3), an aromatic singlet (H-8), an oxymethine (H-16), and a pair of methylene protons (H_2-7). An HMQC experiment was used to establish one-bond ^1H and ^{13}C connectivity. The methoxy protons afforded an HMBC ($^nJ_{XH}$ = 7 Hz) correlation to C-3 (δ_C 160.7), confirming the location of methoxy at C-3, which was further corroborated by the presence of a major fragment ion at *m/z* 405 due to the loss of 137 amu in the ESIMS arising from the cleavages of C-16/C-16a and C-5/C6. Two- and three-bond HMBC correlations confirmed the assignments of the other part of the structure. As expected, C10−C13, C13a, and C14 did not show HMBC correlations and were assigned on the basis of the chemical shift arguments. Compound **2** displayed a negative specific rotation similar to compound **1** and unlike L-755805 and BE-24566B. The CD spectrum of (−)-BABX (**2**) was similar to the spectrum of (−)-ABX (**1**) except for about 5 nm red shifts of most of the Cotton peaks, suggesting that they possess identical stereochemistries (Figure 1). The bridge head centers C-6 and C-16 dictate the overall stereochemistry placing oxygen O-17 either α or β. To determine absolute stereochemistry, we attempted to crystallize compound **2**. Unfortunately all crystallization efforts failed to provide diffraction quality crystals for X-ray crystallographic analysis. On the basis of these data structure **2** was assigned to (−)-BABX.

Scheme 1. Proposed Biogenesis of Compounds **1**, **2**, and BE-24566B



Biogenetically, the hexacyclic 1,3-dioxane polyketides **1** and **2** most likely originate from a tetracyclic methyl ketone intermediate (Scheme 1). Nucleophilic addition of O-5 to the methyl ketone followed by concomitant addition of the O-17 to C-16 with concomitant elimination of the hydroxy group gives rise to dioxane/pyrene rings (Scheme 1). This would indicate that the stereochemistry of this class of compounds is controlled by the existing chirality of the hydroxy group at C-16. An α-hydroxy group at C-16 would yield compounds with β O-17, and the converse would be the case with a β-hydroxy group at C-16. This would explain the opposite stereochemistries of the compounds **1**, **2**, and BE-24566B (L-755805).

Compounds **1** and **2** were first evaluated in a LXR-SPA binding assay using [$^3H_2$]-F_3-methyl AA, a compound originally identified in the PPAR program as an agonist in a cell-based transactivation assay using a protocol described earlier.[9] (−)-ABX (**1**) exhibited α and β LXR binding affinities each with IC_{50} values of 2 μM. The dichloro compound (−)-BABX (**2**) was generally less active against both receptor bindings and showed IC_{50} values of 10 μM against α and >15 μM against β LXR receptors. These compounds did not exhibit any agonist activities in the HTRF coactivator assay.[9]

In a routine screening of natural product extracts for the discovery of new mode-of-action antibacterial agents using an in vitro fatty acid synthesis inhibition (FASII) assay (obtained as a crude preparation from *Staphylococcus aureus*),[11] the extract producing compounds **1** and **2** was identified as a screening hit. Compound **2** accounted for most of the FASII activity and exhibited an IC_{50} value of 11.4 μg/mL, whereas the des-chloro compound **1** was ~25-fold less active in this assay (IC_{50} = 288 μg/mL). Details of the activity of compound **2** were recently reported.[11] The activities of compounds **1** and **2** are presented in Table 1. In contrast (−)-ABX (**1**) was only 4- to 5-fold less active in a whole cell labeling (macromolecular synthesis inhibition) assay compared to (−)-BABX (**2**), suggesting differences in potency, penetration, or mechanism of action. Both compounds equally inhibited the synthesis of all macromolecules (protein, phospholipids, DNA, RNA, and cell wall), suggesting that the mechanism of antibacterial activity is through multiple or unexplored targets. A selective fatty acid synthesis inhibitor is expected to inhibit the synthesis of phospholipids only. These compounds inhibited the growth of Gram-positive bacteria with MIC values (0.5−2 μg/mL) that are much more potent than expected from FASII inhibitory activity, further indicating that the fatty acid synthesis might not be the primary target to account for their antibacterial activities. BE-24566B was reported as an antibacterial agent.[13] We confirmed the MIC of BE-24566B (Table 1), which showed an IC_{50} value in the FASII assay essentially identical to that of enantiomer **1**, indicating stereochemistry-independent activity and potentially nonselective binding at the active site. These compounds were effective in inhibiting the growth of *E. coli* only when the cell membrane was permeabilized (*E. coli lpx*C strain,

Table 1. Antibacterial Activities of Compounds **1** and **2**

assay	(−)-ABX (**1**)	BE-24566B (+)-ABX (**1**)	(−)-BABX (**2**)	rifampicin	chloro-amphenicol
Staphylococcus aureus FASII (IC_{50}, μg/mL)	288	245	11.4	>250	>250
whole cell labeling (IC_{50}, μg/mL)[a]	1	NT	0.21−0.25	ref 10	ref 10
MIC (μg/mL)					
Staphylococcus aureus MSSA (MB2685)	2	1	0.5	0.002	6.25
S. aureus MRSA mac^R, (COL, MB5393)	2	0.5	1	0.002	6.25
Streptococcus pneumoniae WT (MB212)	1	1	1	0.004	1.56
Enterococcus faecalis Van^S (CL8516)	0.5	1	1	0.008	1.56
Enterococcus faecium Van^R (CL5791)	2	0.5	2	0.04	6.25
Escheria coli (*lpxC*) (MB4902)	2	1	0.5	0.02	1.56

[a] In the *Staphylococcus aureus* strains, these compounds inhibited equally all four macromolecules (phospholipids, protein synthesis, RNA synthesis, and DNA synthesis), exhibiting identical IC_{50} values. MS: methicillin sensitive. MRSA: methicillin resistant *S. aureus*.

MIC = 0.5−1 μg/mL), indicating poor penetration by this class of compounds into Gram-negative bacteria.

In summary, we have described here two new hexacyclic compounds, **1** and **2**, produced by a *Streptomyces* sp. that are inhibitors of the ligand binding activity of LXR receptors. They also inhibited *E. coli* FASII activity and are Gram-positive antibacterial agents.

Experimental Section

General Experimental Procedures. HP1100 was used for analytical HPLC. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. CD spectra were measured on a Jasco 715 machine. The UV spectra were recorded in MeOH on a Beckman DU-70 spectrophotometer. IR spectra were recorded on a 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 500 FT-NMR spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C in C_5D_5N.

Fermentation of *Streptomyces* sp. (MA6657). The producing organism, a *Streptomyces* sp. (MA6657), was obtained from Merck culture collection. Seed culture of the strain was prepared by inoculation from a slant culture in a 50 mL tube containing 10 mL of seed medium consisting of glucose (10 g/L), sucrose (10 g/L), glycerol (10 g/L), bacto-peptone (5 g/L), yeast extract (10 g/L), promine (10 g/L), oat flour (5 g/L), and $CaCO_3$ (1 g/L) in distilled water at pH 7.0. After 1 day at 27 °C, a 5% portion of the seed culture was transferred to 250 mL flasks containing 50 mL of production media consisting of soluble starch (20 g/L), glycerol (10 g/L), glucose (10 g/L), soybean flour (10 g/L), bacto-peptone (5 g/L), yeast extract (5 g/L), and $CaCO_3$ (5 g/L) in distilled water at pH 7.0. The culture was fermented for 12 days at 27 °C at 220 rpm before extraction with methyl ethyl ketone (MEK).

Isolation of Anthrabenzoxocinones 1 and 2. Three liters of MA6657 broth was extracted with 2 L of MEK by shaking for 2 h. The organic layer was separated and concentrated under reduced pressure to give 2.7 g of residue, which was dissolved in 100 mL of MeOH and chromatographed on a 2 L Sephadex LH20 column. The column was eluted with MeOH at a flow rate of 10 mL/min, affording a 240 mg fraction eluting in 0.85−1 column volume that was enriched with compounds **1** and **2**. This fraction was chromatographed in three separate runs on a reversed-phase HPLC column (Zorbax RX C-8, 21 × 250 mm) eluting with a 45 min gradient of 55−90% aqueous CH_3CN containing 0.1% TFA at a flow rate of 8 mL/min. Lyophilization of fractions eluting at 20 and 36−43 min gave compounds **1** (3 mg, 1 mg/L) and **2** (78 mg, 26 mg/L), respectively, as amorphous powders.

Anthrabenzoxocinone (1): $[\alpha]_D^{23}$ −371° (*c* 0.08, CH_2Cl_2); CD (MeOH) $\Delta\epsilon$ (λ_{max}) −51.6 (234), −15.5 (274), +5.0 (309), +3.7 (372). All other spectral properties of **1** were identical to L-755805.[12]

Bischloroanthrabenzoxocinone (2): crystallized from nitromethane as flakes, mp 167−169 °C; $[\alpha]_D^{23}$ −301.6° (*c* 0.6, CH_2Cl_2); UV (MeOH) λ_{max} (ϵ) nm 396 (11 320), 270 (9830); CD (MeOH) $\Delta\epsilon$ (λ_{max}) −56.8 (241), −16.7 (278), +1.7 (311), −2 (345), +1 (403); IR (ZnSe) ν_{max} 3350, 2958, 2872, 1637, 1497, 1466, 1430, 1388, 1364, 1335, 1266, 1249, 1171, 1120, 1090, 840 cm^{-1}, ^{1}H NMR (500 MHz, C_5D_5N) δ 6.59 (1H, d, J = 1.5 Hz, H-2), 6.62 (1H, brs, H-4), 3.30 (1H, d, J = 17.5 Hz, H-7), 3.38 (1H, d, J = 17.5 Hz, H-7), 7.14 (1H, s, H-8), 6.65 (1H, s, H-16), 1.84 (3H, s, H_3-18), 2.67 (3H, s, H_3-19), 2.00 (3H, s, H_3-20), 2.09 (3H, s, H_3-21), 3.68 (3H, s, C-3 OCH_3); ^{13}C NMR (125 MHz, C_5D_5N) δ 137.4 (C-1), 110.1 (C-2), 160.7 (C-3), 100.8 (C-4), 153.7 (C-4a or C-9a), 99.2 (C-6), 41.1 (C-7), 143.3 (C-7a), 118.9 (C-8), 147.1 (C-8a), 40.5 (C-9), 153.8 (C-9a or C-4a), 115.7 (C-10), 161.4 (C-11), 109.8 (C-12), 162.5 (C-13), 107.6 (C-13a), 190.9 (C-14), 111.1 (C-15), 158.1 (C-15a), 123.8 (C-16), 66.3 (C-16), 116.3 (C-16a), 28.4 (C-18), 20.1 (C-19), 29.2 (C-20), 29.4 (C-21), 55.7 (C-3 OCH_3); HMBC ($^nJ_{CH}$ = 7 Hz) C-1 → H-16, 19, C-2 → H-4, 19, C-3 → H-4, OCH_3, C-4 → H-2, C-4a → H-4, C-6 → H-7, 16, 18, C-7 → H-8, 18, C-7a → H-7, 16, C-8 → H-7, C-8a → H-20, 21, C-9 → H-8, 20, 21, C-9a → H-20, 21, C-14a → H-8, C-15 → H-16, C-15a → H-8, C-16a → H-2, 19, C-18 → H-7, C-19 → H-2, C-20 → H-21, C-21 → H-20; HRESIFTMS (*m/z*) 543.0948 (calcd for $C_{28}H_{24}O_7Cl_2$+H, 543.0977).

BE-24566B: $[\alpha]_D^{23}$ +390° (*c* 0.5, CH_2Cl_2); CD (MeOH) $\Delta\epsilon$ (λ_{max}) +51.4 (234), +15.5 (274), −4.7 (309), −4.1 (372).

LXR-SPA Binding Assay. A LXR scintillation proximity assay (LXR-SPA) was performed using GST-LXR ligand binding domain (LBD) receptors for α and β using [$^3H_2$]F_3-methyl AA as detailed by Menke et al.[9]

Cofactor-Association Assays. The agonist activities of these compounds were measured in an in vitro cofactor-association assay. In this assay, the association of recombinant steroid receptor coactivator 1 (SRC1) coactivator protein with recombinant LXRα and -β ligand binding domains was measured using a homogeneous time-resolved fluorescence (HTRF) assay as described earlier.[9]

Antibacterial Assays. The FASII assay was performed using a partially purified preparation of fatty acid synthesis enzymes from *S. aureus* and *n*-octanoyl-CoA and [^{14}C] malonyl CoA as substrates as detailed by Kodali et al.[11] The whole cell labeling and MIC determination methods have also been described. [14]

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