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C(16)-C(22) oxygen-bridged analogues of *ce*DAF-12 and LXR ligands

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ABSTRACT

The DAF-12 receptor in nematodes and the Liver X Receptor (LXR) in mammals are structurally related transcription factors that play key roles in determining the life span of the organism. Both types of receptors are activated by oxysterols, cholesterol metabolites with oxidized side chains. Restricting the movement of the oxysterol side chain to certain orientations may have profound effects in the activity profile, however this has not been explored so far. In a first attempt to obtain analogues of natural ligands of DAF-12 and LXR with restricted side chain mobility we introduced a 16,22-oxygen bridge in 26-hydroxycholesterol, a cholestenoic acid and a dafachronic acid (**5**–**7**). Diosgenin was used as starting material, the key step to obtain the 16,22 epoxy functionality was the one pot formation and reduction of a cyclic hemiketal via the oxocarbenium ion using sodium cyanoborohydride. All new compounds were characterized by NMR and mass spectrometry and assayed as *ce*DAF-12 or LXR ligands in transactivation cell-based assays. The dafachronic acid analogue **7** behaved as a *ce*DAF-12 agonist.

1. Introduction

Nuclear hormone receptors are transcription factors that respond to lipophilic hormones such as steroids, to regulate essential processes in living cells [1]. DAF-12 is a nuclear receptor in *Caenorhabditis elegans*, that controls the choice between reproductive growth and arrest at a long-lived, alternate third larval stage formed under harsh environmental conditions [2,3]. The *Ce*DAF-12 ligands, termed dafachronic acids (DAs) are oxidized cholesterol metabolites. It is known that a C-3 keto group, a double bond at C-4 (Δ^4) or C-7 (Δ^7), and an acidic carboxyl group at the end of the cholesterol side chain are required for efficient *Ce*DAF-12 activation (e.g. Δ^4-DA **1** and the synthetic agonist **2**, Fig. 1) [4,5]. Since many of the molecular and cellular pathways occurring in the nematode show analogies to corresponding pathways on higher animals [2,6], a detailed understanding of DAF-12 function may result central to clarify the molecular mechanism involved in human aging. Using sequence similarity searches, the liver X receptor (LXR) has been identified as one of the human nuclear receptors, the protein sequence of which is most similar to *Ce*DAF-12 [7]. The endogenous LXR ligands are also cholesterol metabolites with an oxidized sterol side chain, some of which are closely related to the dafachronic acids, e.g. 26-hydroxycholesterol **3** and 25*R*-cholestenoic acid **4** (Fig. 1) [8–10]. Once activated, LXR isoforms are involved in many physiological functions being regulators of lipid homeostasis, including reverse cholesterol transport. This has lead to propose LXRs as key factors affecting human life span [7]. Although the ligand binding pockets of DAF-12 and LXR accept structurally similar ligands, molecular modeling and X-ray data indicate marked differences in side chain conformation and binding mode [11,12]. As a first approach to evaluating the effect of restricting side chain flexibility of DAF-12 and LXR ligands we prepared the 16,22-epoxysteroids **5**–**7** that are side chain constrained analogues of natural ligands **3**, **4** and **1** respectively.

2. Experimental

2.1. General

Mps were taken on a Fisher-Johns apparatus and are uncorrected. NMR spectra were recorded on a Bruker Avance II 500 NMR spectrometer (^{1}H at 500.13 MHz, ^{13}C at 125.77 MHz). Chemical shifts are given in ppm downfield from TMS as internal standard, J values are given in Hz. Multiplicity determinations and 2D spectra (COSY, NOESY, HSQC and HMBC) were obtained

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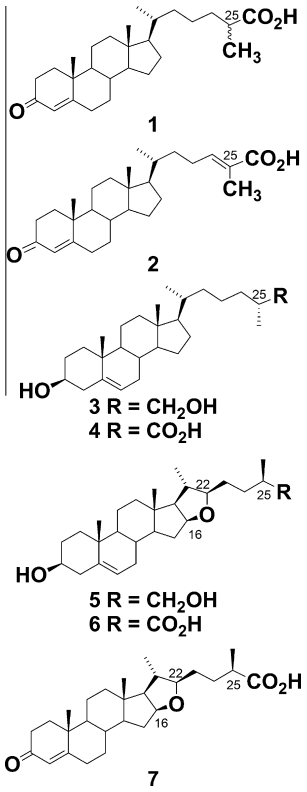


Fig. 1. Structures of DAF-12 and LXR ligands and synthetic analogues.

using standard Bruker software. Exact mass spectra were measured on a Bruker micrOTOF-Q II mass spectrometer, equipped with an ESI source operating in positive mode. Medium Pressure Liquid Chromatography (MPLC) was carried out in a Buchi Sepacore purification system C-615 equipped with two pumps of 10 bar maximum pressure; columns (12 × 75 mm or 12 × 150 mm) were filled with silica gel 60, 0.0040–0.0063 mm. Thin layer chromatography (tlc) analysis was performed on silica gel 60 F254 (0.2 mm thick). The homogeneity of all compounds was confirmed by tlc and high field (500 MHz) ^{1}H NMR. Solvents were evaporated at reduced pressure and ca. 45 °C. 3β,16β-diacetoxy-26-hydroxy-5-cholesten-22-one (**8**) was prepared from diosgenin following the procedure described by Fernández-Herrera et al. [13].

2.2. Chemistry

2.2.1. 3β,16β-Diacetoxy-26-(t-butyldimethylsilyloxy)-cholest-5-en-22-one (*9*)

Imidazole (64 mg, 0.940 mmol) and *t*-butyldimethylsilyl chloride (128 mg, 0.849 mmol) were added successively to a solution of alcohol **8** (160 mg, 0.310 mmol) in anhydrous DMF (1.8 mL) and the solution was stirred for 15 min at 25 °C under a nitrogen atmosphere. The reaction mixture was extracted with ether, the organic layer was washed successively with brine and water and dried with sodium sulphate. Evaporation of the solvent followed by MPLC (Flow rate: 20 mL/min; hexane-ethyl acetate 100:0 → 90:10) gave compound **9** as an amorphous solid (186 mg, 95%); ^{1}H NMR (500.13 MHz, $CDCl_3$) δ_H: 5.36 (1H, d, J = 5.0 Hz, H-6); 4.98 (1H, td, J = 8.0 and 4.6 Hz, H-16); 4.60 (1H, tt, J = 11.0 and 5.5 Hz, H-3); 3.44 (1H, dd, J = 10.0 and 6.0 Hz, H-26a); 3.38 (1H, dd, J = 10.0 and 6.5 Hz, H-26b); 2.96 (1H, m, H-20); 2.60 (1H, m, H-23a); 2.42 (1H, m, H-15β); 2.36 (1H, m, H-23b); 2.31 (2H, m, H-4); 2.03 (3H, s, 3-acetate); 1.96 (3H, s, 16-acetate); 1.95 (1H, m, H-7β); 1.94 (1H, m, H-12β); 1.93 (1H, m, H-17); 1.86 (1H, m, H-2α); 1.85 (1H, m, H-1β); 1.66 (1H, m, H-24a); 1.59 (1H, m, H-2β); 1.55 (3H, m, H-25, H-8 and H-7α); 1.51 (2H, m, H-11); 1.31 (1H, m, H-24b); 1.28 (1H, m, H-12α); 1.14 (1H, m, H-1α); 1.13 (3H, d, J = 7.0 Hz, H-21); 1.04 (1H, m, H-15α); 1.03 (1H, m, H-14); 1.02 (1H, s, H-19); 1.00 (1H, m, H-9); 0.89 (9H, s, $(CH_3)_3$C-Si); 0.87 (3H, d, J = 7.0 Hz, H-27); 0.87 (3H, s, H-18); 0.03 (6H, s, $(CH_3)_2$-Si); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ_C: 213.3 (C-22); 170.5 (3-acetate); 169.8 (16-acetate); 139.6 (C-5); 122.3 (C-6); 75.7 (C-16); 73.8 (C-3); 68.2 (C-26); 55.0 (C-17); 53.9 (C-14); 49.7 (C-9); 43.5 (C-20); 41.8 (C-13); 39.6 (C-12); 38.9 (C-23); 38.0 (C-4); 36.9 (C-1); 36.5 (C-10); 35.4 (C-25); 34.8 (C-15); 31.6 (C-7); 31.2 (C-8); 27.7 (C-2); 27.0 (C-24); 25.9 (($\mathbf{C}H_3)_3$C-Si); 21.4 (3-acetate); 21.1 (16-acetate); 20.7 (C-11); 19.3 (C-19); 18.3 (($CH_3)_3\mathbf{C}$-Si); 16.7 (C-27); 16.6 (C-21); 13.2 (C-18); -5.4 (($CH_3)_2$-Si); HRMS-ESI: calculated for $C_{37}H_{62}NaO_6Si$: 653.4208, found 653.4201.

2.2.2. (22R)-16β,22-Epoxycholest-5-ene-3β,26-diol (*5*)

Method A: A solution of KOH 8% in methanol (0.92 mL, 1.3 mmol) was added to a solution of compound **9** (138 mg, 0.219 mmol) in dichloromethane (0.2 mL) and methanol (4 mL). After stirring for 24 h at 25 °C, water was added to the mixture and a precipitate was formed. The solid was filtered, washed with water and purified by MPLC (Flow rate: 10 mL/min; hexane-ethyl acetate 100:0 → 60:40) to give hemiketal **10** as an amorphous solid (106 mg, 92%): ^{1}H NMR (500.13 MHz, DMSO-d_6) δ_H: 5.27 (1H, d, J = 5.0 Hz, H-6); 4.60 (1H, m, 3-OH); 4.44 (1H, td, J = 7.0 and 6.8 Hz, H-16); 3.26 (1H, m, H-3); 3.39 (2H, dd, J = 5.5.8 and 2.0 Hz, H-26); 2.14 (1H, m, H-4β); 2.09 (1H, m, H-4α); 1.95 (1H, m, H-20); 1.92 (1H, m, H-7β); 1.86 (1H, m, H-15β); 1.77 (1H, m, H-1β); 1.70 (1H, m, H-12β); 1.68 (1H, m, H-2α); 1.65 (1H, m, H-17); 1.61 (1H, m, H-24a);1.53 (1H, m, H-8); 1.52 (1H, m, H-23a); 1.51 (2H, m, H-7α and H-25); 1.49 (2H, m, H-11α and H-24b); 1.40 (1H, m, H-11β); 1.35 (1H, m, H-2β); 1.13 (1H, m, H-12α); 1.12 (2H, m, H-15α and H-23b); 1.09 (1H, m,H-14); 0.98 (1H, m, H-1α); 0.96 (1H, s, H-19); 0.92 (3H, d, J = 7.0 Hz, H-21); 0.90 (1H, m, H-9); 0.88 (9H, s, $(CH_3)_3$C-Si); 0.83 (3H, d, J = 6.5 Hz, H-27); 0.75 (3H, s, H-18); 0.03 (6H, s, $(CH_3)_2$-Si); ^{13}C NMR (125.77 MHz, DMSO-d_6) δ_C: 141.2 (C-5); 120.2 (C-6); 109.5 (C-22); 79.5 (C-16); 69.9 (C-3); 67.3 (C-26); 62.4 (C-17); 55.6 (C-14); 49.5 (C-9); 42.1 (C-4); 40.0 (C-13); 39.1 (C-12); 38.6 (C-20); 36.8 (C-1); 36.1 (C-10); 35.7 (C-24); 35.4 (C-25); 31.5 (C-15); 31.4 (C-7); 31.3 (C-2); 30.9 (C-8); 26.8 (C-23); 25.7 (($\mathbf{C}H_3)_3$C-Si); 20.3 (C-11); 19.0 (C-19); 17.8 (($CH_3)_3\mathbf{C}$-Si); 16.5 (C-27); 15.9 (C-18); 15.7 (C-21); −5.52,−5.54 (($CH_3)_2$-Si); HRMS-ESI: calculated for $C_{33}H_{58}NaO_4Si$: 569.3997, found 569.3981.

Sodium cyanoborohydride (50 mg, 0.796 mmol) was added to a solution of the solid obtained above in dichloromethane (1.6 mL) and MeOH (3 mL) containing a trace of methyl orange. The reaction mixture was acidified with 1 M HCl until the solution turned orange (pH 3) and stirred for 30 min at 25 °C, the orange color was maintained by periodic additions of 1 M HCl (ca. 2 mL) during the reaction. The mixture was diluted with water, concentrated to a third of its volume and extracted with dichloromethane. The organic layer was washed with saturated sodium bicarbonate solution and water and dried with sodium sulphate. Evaporation of the solvent followed by MPLC (Flow rate: 20 mL/min; hexane-ethyl acetate 100:0 → 50:50) gave compound **5** as a white solid (65 mg, 82%), mp 160–162 °C (from hexane-ethyl acetate; lit [14]. 164–166 °C); ^{1}H NMR (500.13 MHz, $CDCl_3$) δ_H: 5.35 (1H, dt, J = 5.3 and 1.8 Hz, H-6); 4.31 (1H, td, J = 7.5 and 5.0 Hz, H-16); 3.51 (1H, m, H-3); 3.50 (1H, dd, J = 10.7 and 6.0 Hz, H-26a); 3.45 (1H, dd, J = 10.5 and 6.0 Hz, H-26b); 3.33 (1H, td, J = 8.0 and 3.5 Hz, H-22); 2.29 (1H, m, H-4β); 2.23 (1H, m, H-4α); 2.01 (1H, m, H-15β); 2.00 (1H, m, H-7β); 1.85 (1H, m, H-1β); 1.84 (1H, m, H-2α); 1.75 (1H, m, H-20); 1.72 (1H, m, H-12β); 1.67 (1H, m, H-25), 1.63 (1H, m, H-8); 1.61 (1H, m, H-17); 1.60 (2H, m, H-23);

1.49 (1H, m, H-2β); 1.47 (1H, m, H-24a); 1.53 (1H, m, H-7α); 1.48 (2H, m, H-11); 1.35 (1H, m, H-24b); 1.31 (1H, m, H-15α); 1.12 (1H, m, H-12α); 1.08 (1H, m, H-14); 1.07 (1H, m, H-1α); 1.02 (1H, s, H-19); 1.00 (3H, d, J = 6.5 Hz, H-21); 0.94 (1H, m, H-9); 0.92 (3H, d, J = 7.0 Hz, H-27); 0.81 (3H, s, H-18); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ_C: 140.8 (C-5); 121.4 (C-6); 90.4 (C-22); 83.2 (C-16); 71.7 (C-3); 68.1 (C-26); 65.1 (C-17); 57.0 (C-14); 50.1 (C-9); 42.2 (C-4); 40.7 (C-13); 39.5 (C-12); 37.9 (C-20); 37.2 (C-1); 36.6 (C-10); 35.7 (C-25); 32.2 (C-15), 32.0 (C-7); 31.6 (C-2 and C-8); 30.4 (C-23); 30.1 (C-24); 20.7 (C-11); 19.4 (C-19); 18.9 (C-21); 16.6 (C-27); 16.4 (C-18); HRMS-ESI: calculated for $C_{27}H_{44}NaO_3$: 439.3183, found 439.3172. Further elution gave enol ether **11** as an amorphous solid (10 mg, 10%), ^{1}H NMR (500.13 MHz, $CDCl_3$) δ_H: 5.35 (1H, d, J = 5.1 Hz, H-6); 4.74 (1H, td, J = 7.3 and 6.8 Hz, H-16); 3.53 (1H, m, H-3); 3.47 (1H, dd, J = 9.5 and 5.0 Hz, H-26a); 3.36 (1H, dd, J = 10.0 and 6.5 Hz, H-26b); 2.46 (1H, d, J = 10.0 Hz, H-17); 2.31 (1H, m, H-4β); 2.23 (1H, m, H-4α); 2.16 (1H, m, H-15β); 2.09 (2H, m, H-23); 2.02 (1H, m, H-7β); 1.85 (1H, m, H-1β); 1.84 (1H, m, H-2α); 1.81 (1H, m, H-12β); 1.63 (1H, m, H-8); 1.60 (1H, m, H-25); 1.58 (3H, d, J = 10.0 Hz, H-21); 1.57 (1H, m, H-24a); 1.56 (1H, m, H-7α); 1.52 (1H, m, H-2β); 1.51 (2H, m, H-11); 1.44 (1H, m, H-15α); 1.24 (1H, m, H-12α); 1.21 (1H, m, H-24b); 1.07 (1H, m, H-1α); 1.03 (1H, s, H-19); 0.98 (1H, m, H-14); 0.95 (1H, m, H-9); 0.89 (9H, s, $(CH_3)_3$C-Si); 0.89 (3H, d, J = 6.5 Hz, H-27); 0.69 (3H, s, H-18); 0.03 (6H, s, $(CH_3)_2$-Si); ^{13}C NMR (125.77 MHz, DMSO-d_6) δ_C: 152.0 (C-20); 140.8 (C-5); 121.4 (C-6); 103.4 (C-22); 84.2 (C-16); 71.7 (C-3); 68.1 (C-26); 64.2 (C-17); 55.0 (C-14); 50.1 (C-9); 43.3 (C-13); 42.3 (C-4); 39.5 (C-12); 37.2 (C-1); 36.6 (C-10); 35.4 (C-25); 34.1 (C-15); 32.2 (C-7); 31.6 (C-2); 31.3 (C-8); 30.7 (C-24); 25.9 (($\mathbf{C}H_3)_3$C-Si); 23.4 (C-23); 21.0 (C-11); 19.4 (C-19); 18.4 ($(CH_3)_3\mathbf{C}$-Si); 16.6 (C-27); 13.9 (C-18); 11.6 (C-21); −5.4 ($(CH_3)_2$-Si.

Method B: A solution of KOH 8% in methanol (1.3 mL, 1.85 mmol) was added to a solution of **9** (190 mg, 0.301 mmol) in dichloromethane (0.27 mL) and methanol (5 mL). After stirring for 24 h at 25 °C, a trace of methyl orange and sodium cyanoborohydride (80 mg, 1.273 mmol) were added, the mixture was cooled to 0 °C, acidified with 1 M HCl until the solution turned orange (pH 3) and stirred for 30 min at 0 °C. The mixture was diluted with water, concentrated to a third of its volume and extracted with dichloromethane. The organic layer was washed with saturated sodium bicarbonate solution and water and dried with sodium sulphate. Evaporation of the solvent followed by MPLC (Flow rate: 20 mL/min; hexane-ethyl acetate 100:0 → 50:50) gave compound **5** (115 mg, 92% from **9**).

*2.2.3. 3β,16β-Diacetoxy-22-oxocholest-5-en-26-oic acid (**12**)*

Compound **8** (120 mg, 0.230 mmol) was dissolved in acetone (15 mL) and Ar was bubbled through the solution for 1 h. The solution was cooled to 0 °C and Jones reagent (0.30 mL) was added dropwise during 5 min. Then isopropyl alcohol (1 mL) was added and the solvent was evaporated. A saturated sodium chloride solution was added and the mixture was extracted with ethyl acetate. The organic layer was washed with water and dried with sodium sulphate. Evaporation of the solvent followed by MPLC (Flow rate: 20 mL/min (hexane-ethyl acetate 100:0 → 90:10) gave compound **12** as an amorphous solid (104 mg, 84%). ^{1}H NMR (500.13 MHz, $CDCl_3$) δ_H: 5.36 (1H, d, J = 5.0 Hz, H-6); 4.97 (1H, td, J = 7.5 and 4.5 Hz, H-16); 4.59 (1H, tt, J = 10.9 and 5.4 Hz, H-3); 2.94 (1H, m, H-20); 2.70 (1H, m, H-4β); 2.46 (1H, m, H-25), 2.40 (1H, m, H-15β); 2.33 (2H, m, H-23); 2.30 (1H, m, H-4α); 2.03 (3H, s, 3-acetate); 1.96 (3H, s, 16-acetate); 1.94 (1H, m, H-7β); 1.92 (1H, m, H-12β); 1.90 (1H, m, H-17); 1.86 (2H, m, H-24a and H-2α); 1.84 (1H, m, H-1β); 1.73 (1H, m, H-24b); 1.57 (1H, m, H-2β); 1.53 (1H, m, H-7α); 1.51 (1H, m, H-8); 1.49 (2H, m, H-11); 1.27 (1H, m, H-12α); 1.19 (3H, d, J = 7.0 Hz, H-27); 1.13 (3H, d, J = 7.0 Hz, H-21); 1.12 (1H, m, H-1α); 1.04 (1H, m, H-15α); 1.02 (1H, s, H-19); 1.01 (1H, m, H-14); 0.98 (1H, m, H-9); 0.86 (3H, s, H-18); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ_C: 212.3 (C-22); 181.2 (C-26); 170.6 (3-acetate); 170.0 (16-acetate); 139.6 (C-5); 122.2 (C-6); 75.7 (C-16); 73.8 (C-3); 55.0 (C-17); 53.9 (C-14); 49.7 (C-9); 43.5 (C-20); 41.9 (C-13); 39.6 (C-12); 38.4 (C-25); 38.1 (C-4); 38.0 (C-23); 36.8 (C-1); 36.5 (C-10); 34.8 (C-15); 31.6 (C-7); 31.2 (C-8); 27.7 (C-2); 26.9 (C-24); 21.4 (3-acetate); 21.1 (16-acetate); 20.7 (C-11); 19.3 (C-19); 16.9 (C-27); 16.8 (C-21); 13.2 (C-18); HRMS-ESI: calculated for $C_{31}H_{46}NaO_7$: 553.3136, found 553.3131.

*2.2.4. (22R)-16β,22:22,26-Diepoxy-3β-hydroxycholest-5-en-26-one (**13**)*

A solution of KOH 8% in methanol (1.8 mL, 2.57 mmol) was added to a solution of compound **12** (92 mg, 0.173 mmol) in dichloromethane (0.30 mL) and methanol (6.0 mL). After stirring for 48 h at 25 °C, the mixture was acidified (pH 3) with 1 M HCl and extracted with diethyl ether. The organic layer was washed with water and dried with sodium sulphate. Evaporation of the solvent followed by MPLC (Flow rate: 20 mL/min; hexane-ethyl acetate 100:0 → 80.20) gave lactone **13** as an amorphous solid (60 mg, 81%). ^{1}H NMR (500.13 MHz, $CDCl_3$) δ_H: 5.35 (1H, dt, J = 5.3 and 1.7 Hz, H-6); 4.73 (1H, m, H-16); 3.53 (1H, tt, J = 11.1 and 4.6 Hz, H-3); 2.39 (1H, m, H-25); 2.28 (1H, m, H-4β); 2.24 (1H, m, H-4α); 2.08 (1H, m, H-20); 2.00 (2H, m, H-15β and H-7β); 1.93 (2H, m, H-23); 1.91 (1H, m, H-17); 1.90 (2H, m, H-24); 1.84 (2H, m, H-2α and H-1β); 1.74 (1H, m, H-12β); 1.64 (1H, m, H-8); 1.57 (1H, m, H-7α); 1.51 (1H, m, H-2β); 1.50 (2H, m, H-11); 1.28 (1H, m, H-15α); 1.28 (3H, d, J = 7.0 Hz, H-27); 1.19 (1H, m, H-12α); 1.12 (1, m, H-14); 1.07 (1H, m, H-1α); 1.07 (3H, d, J = 7.0 Hz, H-21); 1.03 (1H, s, H-19); 0.96 (1H, m, H-9); 0.79 (3H, s, H-18); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ_C: 174.7 (C-26); 140.8 (C-5); 121.3 (C-6); 117.3 (C-22); 83.1 (C-16); 71.6 (C-3); 61.2 (C-17); 56.4 (C-14); 49.9 (C-9); 42.2 (C-4); 42.1 (C-20); 40.4 (C-13); 39.5 (C-12); 37.2 (C-1); 36.6 (C-10); 36.0 (C-25); 31.9 (C-7); 31.7 (C-15); 31.5 (C-2); 31.4 (C-8); 30.3 (C-23); 25.8 (C-24); 20.8 (C-11); 19.4 (C-19); 16.8 (C-27); 16.1 (C-18); 14.7 (C-21); HRMS-ESI: calculated for $C_{27}H_{41}O_4$: 429.2999, found 429.2984.

*2.2.5. (22R)-16β,22-Epoxy-3β-hydroxycholest-5-en-26-oic acid (**6**)*

To a solution of lactone **13** (45 mg, 0.105 mmol) in dichloromethane (4.0 mL) and 2-propanol (1.0 mL) containing a trace of methyl orange, sodium cyanoborohydride (26 mg, 0.414 mmol) was added. The reaction mixture was acidified with 1 M HCl until it turned orange (pH 3) and stirred for 30 min at 0 °C. The mixture was diluted with water, concentrated to a third of its volume and extracted with diethyl ether. The organic layer was washed with a saturated sodium chloride solution and water and dried with sodium sulphate. Evaporation of the solvent followed by MPLC (Flow rate: 20 mL/min; hexane-ethyl acetate 50:50 → 40:60) gave compound **6** as a white solid; mp 188–190 °C from hexane-ethyl acetate (37 mg, 81%); ^{1}H NMR (500.13 MHz, DMSO-d_6) δ_H: 12.00 (1H, br s, $CO_2\boldsymbol{H}$); 5.27 (1H, d, J = 5.0 Hz, H-6); 4.22 (1H, td, J = 7.7 and 5.2 Hz, H-16); 3.27 (1H, m, H-3); 3.23 (1H, m, H-22); 2.32 (1H, m, H-25), 2.13 (2H, m, H-4); 1.93 (1H, m, H-7β); 1.92 (1H, m, H-15β); 1.77 (1H, m, H-1β); 1.69 (1H, m, H-12β); 1.68 (2H, m, H-2α and H-20); 1.57 (1H, m, H-17); 1.54 (1H, m, H-8); 1.52 (1H, m, H-7α); 1.51 (2H, m, H-24); 1.49 (1H, m, H-11α);1.48 (2H, m, H-23); 1.37 (2H, m, H-2β and H-11β); 1.18 (1H, m, H-15α); 1.10 (1H, m, H-12α); 1.07 (1H, m, H-14); 1.04 (3H, d, J = 7.0 Hz, H-27); 0.97 (1H, m, H-1α); 0.96 (3H, d, J = 6.6 Hz, H-21); 0.96 (1H, s, H-19); 0.89 (1H, m, H-9); 0.75 (3H, s, H-18); ^{13}C NMR (125.77 MHz, DMSO-d_6) δ_C: 177.3 (C-26); 141.2 (C-5); 120.2 (C-6); 88.9 (C-22); 82.3 (C-16); 69.9 (C-3); 64.4 (C-17); 56.2

(C-14); 49.5 (C-9); 42.1 (C-4); 40.1 (C-13); 38.7 (C-12); 38.6 (C-25); 37.2 (C-20); 36.8 (C-1); 36.1 (C-10); 31.8 (C-15); 31.3 (C-7); 31.1 (C-8); 30.5 (C-23); 30.3 (C-24); 20.1 (C-11); 19.1 (C-19); 18.7 (C-21); 16.8 (C-27); 16.0 (C-18); HRMS-ESI: calculated for $C_{27}H_{42}NaO_4$: 453.2975, found 453.2984.

2.2.6. (22R)-16β,22-Epoxy-3-oxocholest-4-en-26-oic acid **7**

A suspension of pyridinium chlorochromate (236 mg, 1.092 mmol), barium carbonate (217 mg, 1.099 mmol) and 3 Å molecular sieves (240 mg) in anhydrous dichloromethane (10 mL) was stirred for 5 min under a nitrogen atmosphere. A solution of compound **5** (114 mg, 0.273 mmol) in anhydrous dichloromethane (6.6 mL) was added and stirring continued for 1 h at 25 °C. The reaction mixture was diluted with diethyl ether, percolated through silica gel with ethyl acetate and the solvents evaporated. The residue was dissolved in dichloromethane (2.0 mL), methanol (6.0 mL) and 1 M HCl (0.5 mL) were added and the mixture was vigorously stirred 1 h at 25 °C. After dilution with water, the reaction mixture was extracted with dichloromethane. Evaporation of the solvent followed by MPLC (Flow rate: 20 mL/min; hexane-ethyl acetate 100:0 → 50:50) gave ketoaldehyde **14** as a white crystalline solid (91 mg, 81%); mp:146 °C (from hexane-ethyl acetate); ^{1}H NMR (500.13 MHz, Cl_3CD) δ_H: 9.63 (1H, d, J = 2.0 Hz, H-26); 5.73 (1H, br s, H-4); 4.31 (1H, td, J = 7.8 and 5.3 Hz, H-16); 3.33 (1H, m, H-22); 2.41 (1H, m, H-6β); 2.39 (2H, m, H-2); 2.38 (1H, m, H-25); 2.27 (1H, m, H-6α); 2.04 (1H, m, H-15β); 2.02 (1H, m, H-1β); 1.86 (1H, m, H-7β); 1.77 (2H, m, H-23a); 1.76 (1H, m, H-20); 1.75 (1H, m, H-12β); 1.72 (1H, m, H-8); 1.69 (1H, m, H-1α); 1.61 (1H, m, H-17); 1.60 (1H, m, H-23b); 1.59 (2H, m, H-24); 1.49 (2H, m, H-11); 1.34 (1H, m, H-15α); 1.20 (1H, s, H-19); 1.13 (1H, m, H-12α); 1.11 (3H, d, J = 7.0 Hz, H-27); 1.11 (1H, m, H-14); 1.03 (1H, m, H-7α); 1.00 (3H, d, J = 6.8 Hz, H-21); 0.94 (1H, m, H-9); 0.83 (3H, s, H-18); ^{13}C NMR (125.77 MHz, Cl_3CD) δ_C: 205.2 (C-26); 199.5 (C-3); 171.2 (C-5); 123.9 (C-4); 89.7 (C-22); 83.1 (C-16); 64.9 (C-17); 56.1 (C-14); 53.8 (C-9); 40.8 (C-13); 46.3 (C-25); 39.2 (C-12); 38.6 (C-10); 37.9 (C-20); 35.7 (C-1); 35.3 (C-8); 33.9 (C-2); 32.8 (C-6); 32.0 (C-7 and C-15); 30.7 (C-24); 27.8 (C-23); 20.6 (C-11); 18.8 (C-21); 17.4 (C-19); 16.4 (C-18); 13.4 (C-27). HRMS-ESI: calculated for $C_{27}H_{42}NaO_4$: 453.2975, found 453.2984.

A solution of ketoaldehyde **14** (56 mg, 0.136 mmol) in acetone (15 mL) was treated with Jones reagent (0.176 mL) as previously described for **12**. Evaporation of the solvent followed by MPLC (Flow rate: 20 mL/min; hexane-ethyl acetate 100:0 → 50:50) gave compound **7** as a white solid (49 mg, 84%); mp 146–147 °C (from hexane-ethyl acetate); ^{1}H NMR (500.13 MHz, Cl_3CD) δ_H: 5.73 (1H, br s, H-4); 4.34 (1H, td, J = 7.5 and 5.0 Hz, H-16); 3.36 (1H, td, J = 8.0 and 2.5, H-22); 2.54 (1H, m, H-25); 2.40 (1H, m, H-6β); 2.39 (2H, m, H-2); 2.27 (1H, m, H-6α); 2.05 (1H, m, H-15β); 2.02 (1H, m, H-1β); 1.85 (1H, m, H-7β); 1.78 (1H, m, H-20); 1.75 (1H, m, H-12β); 1.71 (1H, m, H-8); 1.69 (1H, m, H-1α); 1.68 (2H, m, H-24); 1.66 (2H, m, H-23); 1.62 (1H, m, H-17); 1.53 (1H, m, H-11α); 1.43 (1H, m, H-11β); 1.35 (1H, m, H-15α); 1.20 (1H, s, H-19); 1.19 (3H, d, J = 7.00 Hz, H-27); 1.12 (1H, m, H-12α); 1.10 (1H, m, H-14); 1.02 (1H, m, H-7α); 1.00 (3H, d, J = 6.5 Hz, H-21); 0.92 (1H, m, H-9); 0.84 (3H, s, H-18); ^{13}C NMR (125.77 MHz, Cl_3CD) δ_C: 199.6 (C-3); 180.6 (C-26); 171.2 (C-5); 123.9 (C-4); 90.3 (C-22); 83.3 (C-16); 64.8 (C-17); 56.1 (C-14); 53.8 (C-9); 40.8 (C-13); 39.3 (C-25); 39.2 (C-12); 38.6 (C-10); 38.0 (C-20); 35.7 (C-1); 35.3 (C-8); 33.9 (C-2); 32.8 (C-6); 32.03 (C-7); 32.02 (C-15); 31.4 (C-23); 30.9 (C-24); 20.6 (C-11); 18.8 (C-21); 17.4 (C-19); 17.2 (C-27); 16.4 (C-18); HRMS-ESI: calculated for $C_{27}H_{40}NaO_4$: 451.2819, found 451.2815.

2.3. Biological activity

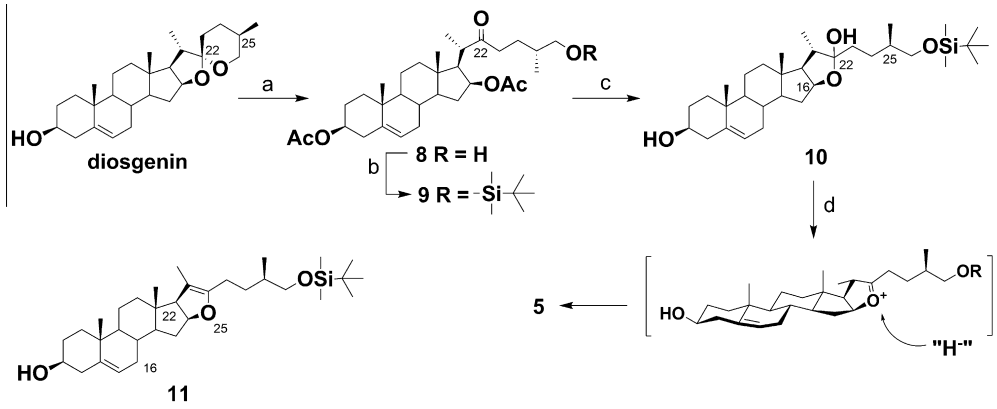
2.3.1. Reporter gene assay

Baby Hamster Kidney cells (BHK) and HEK293T cells were cultured at 37 °C under 5% CO_2 humidified atmosphere in DMEM supplemented with 10% fetal calf serum (FCS) containing penicillin (100 IU/mL), streptomycin (100 mg/mL) and glutamine (2 mM) in p100 plates. For transient transfections, 1×10^5 cells were plated in 24-wells plates and transfected with lipofectamine according to the manufacturer protocol (Lipofectamine 2000, Invitrogen). DAF-12 activity was assessed by transfecting BHK cells with 145 ng CMX-Gal4-DAF-12LBD, which expresses a fusion protein of the Gal4 DNA-binding domain with the LBD of the DAF-12 receptor (referred to as Gal4-DAF-12-LBD) and 350 ng MH100x4-tk-luciferase reporter vector; 100 ng CMX-β-galactosidase was also added as transfection efficiency control. LXRα activity was evaluated in HEK-293T cells by transfecting 0.7 μg of pLRE-LUC, 0.6 μg of pLXRα (gently provided by Dr. Shutsung Liao, University of Chicago), 0.2 μg of pRXR, vectors and 0.6 μg of pRSV-LacZ (Clontech Inc., Palo Alto, CA) as control of transfection. After transfection, the medium was replaced by serum-free medium containing antibiotics. Cells were then incubated during 18 h with steroids at the concentrations indicated. Steroids were applied from 1000-fold stock solutions in dimethylsulfoxide (DMSO). Incubations were stopped by aspirating the medium and washing the cells twice with phosphate buffered saline solution (PBS). Cells were then harvested in lysis buffer and luciferase activity was measured according to the manufacturer protocol (Promega Inc.). Galactosidase activity was measured as previously described [15].

3. Results and discussion

3.1. Chemistry

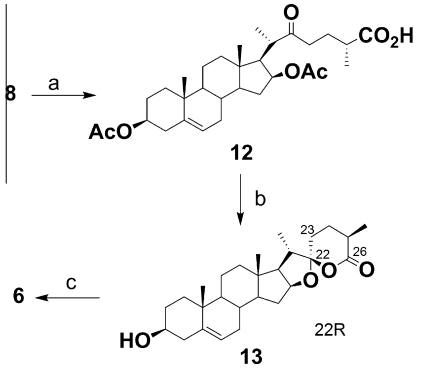
Our approach to obtain the fused tetrahydrofuran ring in the 16,22-epoxysteroids **5**–**7** was based on the stereoselective reduction of an oxocarbenium cation, generated *in situ* in acid media from an appropriate hemiketal. The sequence followed to prepare compound **5** is shown in Scheme 1. Diosgenin was first converted into compound **8** following the procedure of Fernández-Herrera et al. [13] and then the C-26 hydroxyl was protected as the *t*-butyldimethylsilyl ether to give **9**. Removal of the acetates at positions 3 and 16 under basic conditions (8% KOH in methanol) gave hemiketal **10**. The ^{13}C NMR spectrum of **10** showed the absence of a ketone carbonyl group and the presence of a signal at δ 109.5 consistent with the hemiketal function at C-22. The HSQC spectrum showed correlations of the carbon at δ 79.5 (C-16) with H-16 (δ 4.44) and of the carbon at δ 67.3 (C-26) with H-26 (δ 3.38). In the HMBC spectrum correlations of H_3-27 (δ 0.83) with C-26 (δ 67.3) and of H_3-21 (δ 0.92) with C-22 (δ 109.5) were observed. Although only one stereoisomer at C-22 was obtained it was not possible to assign its configuration from the spectroscopic data. Attempts to carry out the oxocarbenium formation/reduction sequence by reaction of the 16,22-hemiketal **10** with triethylsilane and a Lewis acid [16] were unsuccessful. On the other hand treatment of **10** with sodium cyanoborohydride/methanol at pH 3 [17] gave an 8:1 mixture of compound **5** and enol ether **11**. The best yield was obtained by a one-pot procedure for the oxocarbenium formation-reduction-desilylation sequence, that involved alkaline hydrolysis of **9** followed by *in situ* addition of sodium cyanoborohydride and acidification to pH 3 (at 0 °C). In this way compound **9** was converted directly into **5** in 92% yield without formation of enol ether **11**. The ^{1}H NMR



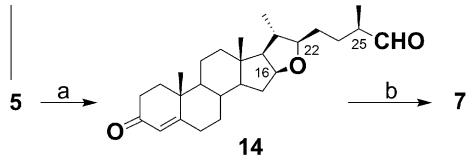
Scheme 1. Reagents and conditions: a) Ref. [11]; b) *t*-Bu(Me_2)SiCl, imidazole, DMF, 15 min, 25 °C (95%); c) 8% KOH in MeOH, CH_2Cl_2/MeOH, 24 hs, 25 °C; d) $NaCNBH_3$, CH_2Cl_2/MeOH, methyl orange, 1 M HCl (pH 3), 30 min, 0 °C (c-d one pot, 92%).

spectrum of **5** showed the resonances of H-16 at δ 4.31 and of H-22 at δ 3.33, both as double triplets (J = 7.4 and 5.8 Hz and J = 8.3 and 3.8 Hz respectively). The HSQC spectrum showed correlations of the carbon at δ 83.2 (C-16) with H-16 and the carbon at δ 90.4 (C-22) with H-22. This was in agreement with the presence of the 16,22-epoxy functionality in compound **5**. The strong correlation between H-16 and H-22 observed in the NOESY spectrum indicated that both hydrogens were on the same side of the 5 membered ring, thus the configuration at C-22 was established as *R*. The high stereoselectivity of the reduction may be explained considering that attack of the reducing agent at C-22 from the β-face of the steroid is hindered, due to the proximity of the angular methyl at C-13.

Compound **8** was readily converted to **6** in three steps (Scheme 2). Jones oxidation of the 26-alcohol to the ketoacid **12**, followed by treatment with 8% KOH in methanol gave lactone **13**. Two non-protonated carbons appeared at δ 174.7 and δ 117.3 in the ^{13}C NMR spectrum which were assigned to C-26 and C-22 respectively. A correlation observed in the HSQC spectrum between signals at δ 4.73 (H-16) and δ 83.1 (C-16) and the $[M+H]^+$ ion at m/z 429.2984 in the HRMS-ESI were in accordance with the proposed structure. The HMBC spectrum showed correlations of the carbon at δ 117.3 (C-22) with H-17 and H-20 and the NOESY spectrum showed a correlation for the pair H_3-21/H-23, indicating a 22*R* configuration. Treatment of **13** with sodium cyanoborohydride in dichloromethane-2-propanol at pH 3, gave the 16,22-epoxyacid **6** (55% yield from **8**) presumably via the oxocarbenium cation (see Scheme 3).



Scheme 2. Reagents and conditions: a) Jones reagent, acetone, 5 min, 0 °C (84%); b) i. 8% KOH in MeOH, CH_2Cl_2/MeOH, 48 h, 25 °C (81%); ii. 1 M HCl (pH 3); c) $NaCNBH_3$, CH_2Cl_2/2-propanol, methyl orange, 1 M HCl (pH 3), 30 min, 0 °C (81%).



Scheme 3. Reagents and conditions: a) i. PCC, $BaCO_3$, MS 4 Å, CH_2Cl_2, 1 h, 25 °C; ii. 1 M HCl, CH_2Cl_2/MeOH, 30 min, 25 °C (81%); b) Jones reagent, acetone, 5 min, 0 °C (84%).

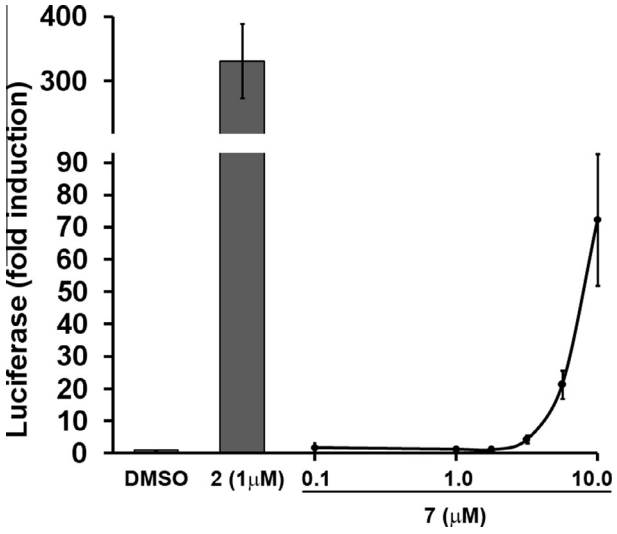


Fig. 2. Activation of *ce*DAF-12 by compound **7** in reporter gene assay. Concentration-response curve of Gal4–DAF-12-dependent reporter activity in the presence compound **7** in BHK-21 cells. Cytomegalovirus-LacZ vector was introduced as control of transfection. Cells were incubated for 18 h with DMSO (vehicle), **2** 1 μM (positive control) and increasing concentrations of **7**. Luciferase activity was measured and corrected for β-galactosidase activity. Values are expressed as means ± S.E.M. (n = 3); fold induction relative to the vehicle.

Attempts to obtain ketoacid **7** from diol **5** in a single step by simultaneous oxidation of the alcohols at C-3 and C-26 with Jones reagent led to varying amounts of the Δ^4-3,6-diketone. Therefore, compound **5** was initially oxidized with PCC followed by acid catalyzed isomerization of the Δ^5 double bond, to give ketoaldehyde **14.** Subsequent oxidation of the 26-aldehyde group with Jones reagent gave ketoacid **7** in 68% yield (from **5**).

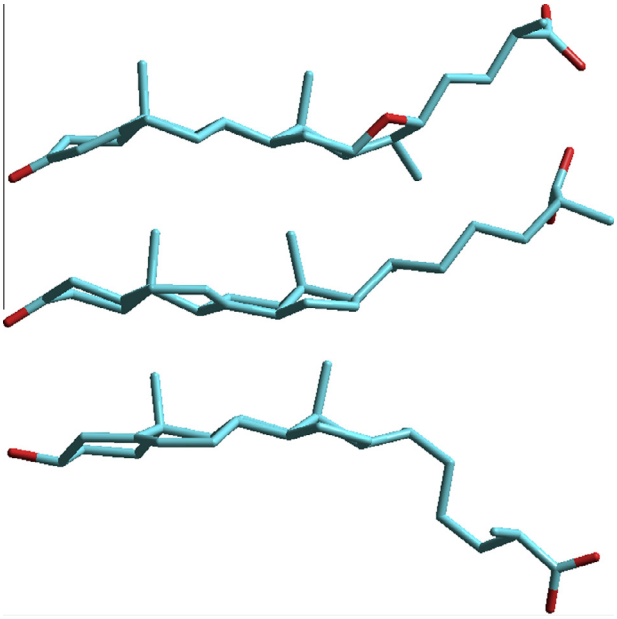


Fig. 3. Side chain orientation of *ce*DAF-12 and LXR ligands. Comparison of the orientation of the side chain in the most stable conformer (HF/6-31G(d,p)) of 16,22-epoxydafachronic acid **7** (top) with that in Δ^7-(25*R*)-dafachronic acid in the LBP of *ce*DAF-12 (middle) [data taken from Ref. [11]] and in (25*R*)-cholestenoic acid **4** in the LBP of LXR (bottom) [data taken from Ref. [12]].

3.2. Biological activity

Transactivation activity of compound **7** on *ce*DAF-12 was evaluated by a reporter gene assay on BHK-21 cells co-transfected with an expression vector codifying the luciferase reporter gene under the control of Gal4 promoter [5], and an expression vector expressing a chimeric protein consisting of the DNA binding domain of Gal4 fused to the *ce*DAF-12 ligand binding domain. Synthetic agonist **2,** previously synthesized by us was used as positive control [5]. Compound **7** behaved as a partial agonist (Fig. 2) with an estimated EC_{50} of >10 μM. On the other hand 3β-hydroxy analogues **5** and **6** were unable to transactivate LXRα in the reporter gene assay (Fig. S1 supplementary data).

4. Conclusions

Overall we have developed a straightforward procedure for the synthesis of 16,22-epoxyoxysterols that exhibit a restricted flexibility of their side chain, using a oxocarbenium formation/reduction sequence. Molecular dynamics (MD) simulations predict that when 25*R*-dafachronic acids bind to the *ce*DAF-12 receptor, their side chain adopts an extended conformation oriented towards the β-face with the carboxyl group above the plane of the steroid nucleus [5,11]. On the other hand, when 25*R*-cholestenoic acid (**4**) binds to the LXR, MD predicts an inverted orientation of the steroid nucleus in the LBP with the side chain towards the α-face of the steroid nucleus [12]. The *R* configuration at C-22 in compounds **5**–**7** fixes the side chain orientation towards the β-face, being similar to that of *ce*DAF-12 bound 25*R*-dafachronic acids but opposite to that of LXR bound cholestenoic acid (Fig. 3). The partial agonist activity of compound **7** on DAF-12 and the lack of activity of compounds **5** and **6** on the LXR found here, give experimental support to these predictions.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2016.05.009.

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