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Synthesis and antifungal activity of C-21 steroids with an aromatic D ring

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ABSTRACT

Six analogues of salpichrolides with a simplified side chain (6–11) were synthesized using a new methodology to obtain steroids with an aromatic D-ring. The key step was the elimination of HBr in a vicinal dibromo D-homosteroid by treatment with 1,4-diazabicyclo[2.2.2]octane (DABCO). All new compounds were completely characterized by 2D NMR techniques and tested on two fungal pathogenic species, *Fusarium virguliforme* and *Fusarium solani*.

1. Introduction

Withanolides are a group of naturally occurring C-28 steroids isolated from several genera of the *Solanaceae*, that exhibit a variety of biological effects such as antifeedant, immunosuppressive, antifungal and chemopreventive activities [1,2]. In particular, a small group of withanolides with a six-membered aromatic D ring, the nicandrenoids, were isolated from the Peruvian "shoofly" plant *Nicandra physaloides* [3]. Nicandrenone (**1**) (Fig. 1) is the most prominent example of this group and is known by its potent insect-repellent activity [4,5]. It was thought that withanolides with an aromatic ring D were restricted to *N. physaloides* until a new family of this type of withanolides and related ergostane derivatives (termed salpichrolides), were isolated in the early 90's from *Salpichroa origanifolia* (Lam.) Thell [6,7]. Salpichrolide A (**2**) was the major component and it was the first withanolide having a 5,6-epoxide with α-stereochemistry (Fig. 1) [7]. Some of the salpichrolides exhibit activity as feeding deterrents on *Musca domestica*, *Tribolium castaneum* and *Ceratitis capitata* [8–10]. Significant development delays from larvae to puparia were observed in treatments with the natural salpichrolides A (**2**), G (**3**) and B (**4**) (Fig. 1). *In vitro* antiproliferative activity studies against a panel of human breast cancer cell lines (performed on a series of 22 naturally occurring withanolides) showed that the activity depended on the tumor cell line only in the case of withanolides with an aromatic ring D [11]. Preliminary testing carried out recently in our laboratory has shown that the natural salpichrolides **2–5** have antifungal activity against two fungal pathogenic species, *Fusarium virguliforme* and *Fusarium solani*. These species are responsible of the sudden death syndrome (SDS) of soybean, which may produce yield losses between 20% and 80% depending on the time of infection [12].

The interesting biological properties of withanolides with an aromatic ring D prompted us to evaluate the activity of more simple structures based on this modified steroid nucleus. The first total synthesis of the nicandrenones was described by Corey and coworkers in 2000 [13]. Previously, Blumbach et al. carried out a biogenetically-inspired aromatization of an androstane D-ring, in which the former C/D methyl is incorporated into the new D-ring [14,15]. We have now developed a simple methodology for the preparation of a series of ring D aromatic steroids with a simplified side chain (**6–11**) (Fig. 1) from readily available steroids, and evaluated their antifungal activity compared to the natural salpichrolides **2–5**.

2. Experimental

2.1. General

Mps were taken on a Fisher-Johns apparatus and are uncorrected. IR spectra were recorded in thin films using KBr disks on a Nicolet Magna 550 FT-IR spectrophotometer, values are given in cm^{-1}. NMR spectra were recorded on Bruker AC-200 (^1H at 200.13 MHz, ^{13}C at 50.32 MHz) or Avance II 500 (^1H at 500.13 MHz, ^{13}C at 125.77 MHz) spectrometers. 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 using standard Bruker

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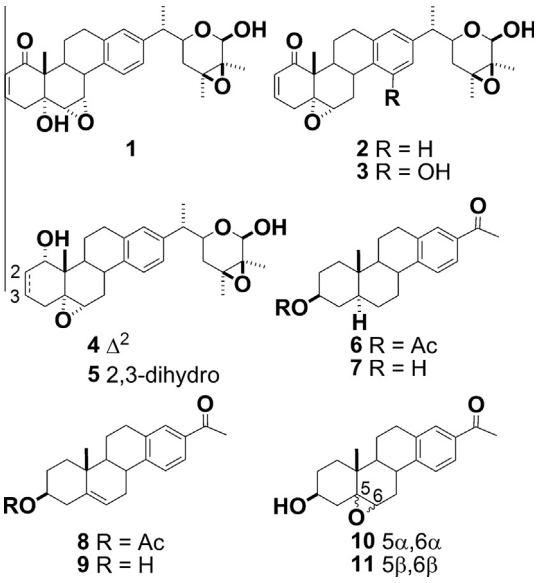


Fig. 1. Structures of compounds **1–11.**

software. Exact mass spectra were obtained using a VG 7070 spectrometer or a Bruker micrOTOF-Q II mass spectrometer, equipped with an ESI source operating in positive mode. Microwave assisted reactions were carried out on a CEM Discover reactor, mode Discover (closed vessel) Power max: on, with air cooling of the reaction vessel during irradiation. Flash column chromatography was carried out on Kieselgel S 0.040–0.063 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. Solvents were evaporated at reduced pressure and *ca.* 40–50 °C. Compounds **2–5** and **16** were obtained following the procedures described previously by us [6,16]. 3β-Acetoxy-17β,18-cyclo-5αH-pregnan-20-one (**12**) was prepared as described in the Supplementary Data. DBU-HBr_3 complex and DABCO-bromine tetrameric complex were obtained following literature procedures [17,18].

2.2. Chemistry

2.2.1. 3β-Acetoxy-17(13 → 18)-abeo-5α,13β-pregn-17-en-20-one (**13**)

To a solution of compound **12** (1.40 g, 3.91 mmol) in 1,2-dichloroethane (120 mL) $BF_3{\cdot}Et_2O$ (349 μL, 1.86 mmol) was added dropwise. After stirring for 1.5 h at 50 °C, the mixture was poured onto 5% aqueous sodium bicarbonate and extracted with dichloromethane. The organic layer was dried with sodium sulphate and the solvent evaporated under vacuum. The resulting solid was purified by flash chromatography (hexane–ethyl acetate 90:10) to give compound **13** (1.22 g, 87%) as a white solid: mp 128–130 °C (from hexane–ethyl acetate); IR (KBr): 2938.2, 2850.1, 1733.5, 1666.2, 1370.6, 1035.4 cm^{-1}; ^1H NMR (500.13 MHz, $CDCl_3$) δ 6.57 (1H, bs, H-18), 4.69 (1H, m, H-3), 2.46 (1H, m, H-16β), 2.28 (3H, s, H-21), 2.08 (1H, m, H-15α), 2.06 (1H, m, H-16α), 2.02 (3H, s, 3-acetate), 2.02 (1H, m, H-7β), 1.87 (1H, m, H-12β), 1.86 (1H, m, H-11α), 1.84 (1H, m, H-13), 1.82 (1H, m, H-2α), 1.78 (1H, dt, 13.0 and 3.6 Hz, H-1β), 1.62 (1H, m, H-4α), 1.49 (1H, qd, *J* = 12.0 and 3.8 Hz,H-2β), 1.36 (1H, m, H-4β), 1.27 (2H, m, H-6), 1.19 (1H, m, H-5), 1.16 (2H, m, H-11b and H-12α), 1.13 (1H, m, H-8), 1.03 (1H, m, H-1α), 0.99 (1H, m, H-15β), 0.88 (1H, m H-7α), 0.79 (3H, s, H-19), 0.79 (1H, m, H-14), 0.78 (1H, m, H-9); ^{13}C NMR (125.77 MHz, $CDCl_3$): 199.64 (C-20), 170.68 (3-acetate), 145.08 (C-17), 138.54 (C-18), 73.60 (C-3), 53.34 (C-9), 45.32 (C-14), 44.12 (C-5), 42.37 (C-13), 40.58 (C-8), 36.63 (C-1), 35.66 (C-10), 33.89 (C-4), 32.45 (C-12), 30.47 (C-7), 28.29 (C-6), 27.40 (C-2), 25.79 (C-11), 25.30 (C-21), 24.97 (C-15), 24.12 (C-16), 21.46 (3-acetate), 12.14 (C-19); HRMS-ESI: calculated for $C_{23}H_{35}O_3$ 359.2581, found 359.2581.

2.2.2. 3β-Acetoxy-17β,18α-dibromo-17(1318)-abeo-5α,13β-pregnan-20-one (**14**)

To a solution of compound **13** (1.20 g, 3.35 mmol) in 1,2-dichloroethane (14 mL) a solution of bromine (4.02 mmol) in 1,2-dichloroethane (2.57 mL) was added in the dark at 0 °C. After stirring for 10 min, the mixture was poured onto a saturated solution of sodium bisulphite, extracted with dichloromethane and the organic layer was dried with sodium sulphate. The solvent was evaporated under vacuum and the resulting solid was purified by flash chromatography (hexane–ethyl acetate 95:5 → 90:10) to give compound **14** (1.61 g, 93%) as a white solid: mp 175–177 °C (from hexane–ethyl acetate); IR (KBr): 2937.3, 2862.1, 1719.5, 1448.2, 1244.8, 1027.1, 736.8 cm^{-1}; ^1H NMR (500.13 MHz, $CDCl_3$) δ: 4.68 (1H, m, H-3), 4.55 (1H, bs, H-18), 2.44 (3H, s, H-21), 2.31 (1H, ddd, *J* = 16.2, 12.7, 3.9, H-16α), 2.14 (1H, m, H-16β), 2.03 (1H, m, H-15α), 2.02 (3H, s, 3-acetate), 2.00 (1H, m, H-7β), 1.95 (1H, m, H-13), 1.84 (1H, m, H-11β), 1.83 (1H, m, H-2α), 1.78 (1H, dt, *J* = 13.0 and 3.3 Hz, H-1β), 1.62 (1H, m, H-4α), 1.55 (2H, m, H-12), 1.49 (1H, m, H-2β), 1.36 (1H, m, H-4β), 1.35 (1H, m, H-15β), 1.33 (1H, m, H-6α), 1.24 (1H, m, H-6β), 1.18 (1H, m, H-11α), 1.17 (3 H, m, H-5, H-8 and H-14), 1.02 (1H, td, *J* = 13.4 and 3.7, H-1α), 0.90 (1H, m, H-7α), 0.79 (3H, s, H-19), 0.77 (1H, m, H-9); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ: 198.80 (C-20), 170.69 (3-acetate), 73.56 (C-3), 71.59 (C-17), 61.47 (C-18), 52.43 (C-9), 43.90 (C-5), 41.00 (C-8), 40.78 (C-13), 40.57 (C-14), 36.58 (C-1), 35.60 (C-10), 33.83 (C-4), 31.83 (C-12), 30.57 (C-7), 29.18 (C-16), 28.25 (C-6), 27.34 (C-2), 25.76 (C-15), 24.66 (C-11), 23.46 (C-21), 21.45 (3-acetate), 12.10 (C-19); HRMS-ESI: calculated for $C_{23}H_{34}Br_2NaO_3$ 539.0767, found 539.0780. $C_{23}H_{34}Br_2O_3$; calculated: C, 53.30; H, 6.61. Found: C, 53.26; H, 7.06.

2.2.3. 3β-Acetoxy-17(13 → 18)-abeo-5α-pregna-13,15,17-trien-20-one (**6**)

To a solution of compound **14** (1.60 g, 3.09 mmol) in acetonitrile (80 mL) was added 1,4-diazabicyclo[2.2.2]octane (DABCO) (1.39 g, 12.4 mmol). After stirring for 1.5 h under reflux, the mixture was poured onto HCl 1 N (40 ml) and concentrated to a third of its volume. Water was added to the residue and then extracted with dichloromethane. The organic layer was washed with a saturated aqueous solution of $NaHCO_3$, dried with sodium sulphate and the solvent evaporated under vacuum. The resulting solid was purified by flash chromatography (dichloromethane) to give compound **6** (964 mg, 88%) as an amorphous solid; IR (KBr): 2940.9, 2860.2, 1731.5, 1681.6, 1604.4, 1361.0, 1308.6, 1247.1, 1028.0, 735.5 cm^{-1}; ^1H NMR (500.13 MHz, $CDCl_3$) δ: 7.71 (1H, dd, *J* = 8.2 and 1.9, H-16), 7.66 (1H, d, *J* = 1.8, H-18), 7.38 (1H, d, *J* = 8.1, H-15), 4.73 (1H, m, H-3), 2.88 (2H, m, H-12), 2.72 (1H, td, *J* = 11.6 and 3.7, H-8), 2.57 (3H, s, H-21), 2.50 (1H, dq, *J* = 12.8 and 3.6, H-7α), 2.04 (3H, s, 3-acetate), 1.98 (1H, dq, *J* = 12.8 and 2.8, H-11α), 1.91 (1H, m, H-1β), 1.89 (1H, m, H-2α), 1.69 (1H, m, H-4α), 1.58 (1H, m, H-2β), 1.50 (2H, m, H-6), 1.41 (2H, m, H-4b and H-11β), 1.30 (1H, m H-7β), 1.27 (1H, m, H-5), 1.12 (1H, m, H-1α), 1.11 (1H, m, H-9), 0.88 (3H, s, H-19); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ: 198.16 (C-20), 170.66 (3-acetate), 147.05 (C-14), 137.26 (C-13), 134.51 (C-17), 128.69 (C-18), 126.33 (C-15), 125.66 (C-16), 73.47 (C-3), 50.62 (C-9), 43.91 (C-5), 38.18 (C-8), 36.36 (C-1), 35.85 (C-10), 33.67 (C-4), 31.75 (C-7), 30.73 (C-12), 28.71 (C-6), 27.25 (C-2), 26.53 (C-21), 22.53 (C-11), 21.42 (3-acetate), 11.52 (C-19); HRMS-ESI: calculated for $C_{23}H_{31}O_3$ 355.2268, found 355.2273.

*2.2.4. 3β-Hydroxy-17(13 → 18)-abeo-5α-pregna-13,15,17-trien-20-one (**7**)*

To a solution of compound **6** (76.2 mg, 0.215 mmol) in tetrahydrofuran (30 mL) and methanol (30 mL) a 5% aqueous solution of KOH (1.25 ml) was added. After stirring for 1 h at room temperature, the reaction mixture was acidified with HCl 1 N (pH 7) and concentrated to a third of its volume. Water was added to the residue and then extracted with dichloromethane. The organic layer was dried with sodium sulphate and the solvent evaporated under vacuum. The resulting solid was purified by flash chromatography (hexane–ethyl acetate 70:30) to give compound **7** (57.8 mg, 86%) as a white solid: mp 153–154 °C (from hexane–ethyl acetate); 3409.3, 2928.6, 2858.4, 1678.7, 1603.9, 1447.4, 1360.3, 1309.4, 1290.4, 1268.9, 1076.0, 1046.9, 735.6 cm^{-1}; ^1H NMR (500.13 MHz, $CDCl_3$) δ: 7.71 (1H, dd, J = 8.2 and 1.7, H-16), 7.66 (1H, d, J = 1.7, H-18), 7.39 (1H, d, J = 8.2, H-15), 3.65 (1H, m, H-3), 2.86 (2H, m, H-12), 2.73 (1H, td, J = 11.6 and 3.6, H-8), 2.57 (3H, s, H-21), 2.51 (1H, dq, J = 12.8 and 3.4, H-7α), 1.99 (1H, m, H-11α), 1.89 (1H, m, H-1β), 1.88 (1H, m, H-2β), 1.65 (1H, m, H-4α), 1.51 (2H, m, H-6), 1.50 (1H, m, H-2α), 1.42 (1H, m, H-11β), 1.35 (1H, m, H-4β), 1.29 (1H, m H-7β), 1.21 (1 H, m, H-5), 1.09 (1H, m, H-9), 1.07 (1H, m, H-1α), 0.87 (3H, s, H-19); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ: 198.21 (C-20), 147.22 (C-14), 137.31 (C-13), 134.46 (C-17), 128.69 (C-18), 126.36 (C-15), 125.64 (C-16), 71.16 (C-3), 50.75 (C-9), 44.10 (C-5), 38.23 (C-8), 37.80 (C-4), 36.60 (C-1), 35.87 (C-10), 31.85 (C-7), 31.28 (C-2), 30.78 (C-12), 28.85 (C-6), 26.54 (C-21), 22.57 (C-11), 11.63 (C-19).

HRMS-ESI: calculated for $C_{21}H_{29}O_2$ 313.2162, found 313.2168.

*2.2.5. 3β-Acetoxy-17(13 → 18)-abeo-13β-pregna-5,17-dien-20-one (**17**)*

Compound **17** was obtained from compound **16** (1.10 g, 3.09 mmol) following the procedure described for compound **13**. The resulting solid was purified by flash chromatography (hexane–ethyl acetate 90:10 → 80:20) to give compound **17** (671 mg, 61%) as a white solid: mp 129–130 °C (from hexane–ethyl acetate); 2940.2, 2858.7, 1732.3, 1667.4, 1367.6, 1244.8, 1031.8 cm^{-1}; ^1H NMR (500.13 MHz, $CDCl_3$) δ: 6.61 (1H, s, H-18), 5.40 (1H, t, J = 2.4 Hz, H-6), 4.61 (1H, m, H-3), 2.48 (1H, m, H-16β), 2.34 (2H, m, H-4), 2.31 (1H, m, H-7β), 2.29 (3H, s, CH_3-21), 2.09 (1H, m, H-16α), 2.05 (1H, m, H-15α), 2.04 (3H, m, 3-acetate), 1.91 (2H, m, H-1b y H-12β), 1.89 (3H, m, H-2α, H-11α y H-13), 1.60 (1H, m, H-2β), 1.59 (1H, m, H-7α), 1.33 (1H, m, H-11β), 1.28 (1H, m, H-8), 1.19 (1H, m, H-12α), 1.15 (1H, m, H-1α), 1.11 (1H, m, H-9), 1.08 (1H, m, H-15β), 0.99 (1H, m, H-19), 0.83 (1H, m, H-14); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ: 199.65 (C-20), 170.52 (3-acetate), 144.88 (C-17), 139.18 (C-5), 138.64 (C-18), 122.19 (C-6), 73.78 (C-3), 49.51 (C-9), 45.86 (C-14), 41.89 (C-13), 37.89 (C-4), 36.74 (C-10), 36.68 (C-1), 36.42 (C-8), 32.11 (C-12), 31.00 (C-7), 27.69 (C-2), 26.06 (C-11), 25.30 (C-21), 25.19 (C-15), 24.11 (C-16), 21.41 (3-acetate), 19.24 (C-19); HRMS-ESI: calculated for $C_{23}H_{32}NaO_3$ 379.2244, found 379.2243.

*2.2.6. 3β-Acetoxy-5α,6β,17β,18α-tetrabromo-17(13 → 18)-abeo-13β-pregnan-20-one (**18**)*

Compound **18** was obtained from compound **17** (630 mg, 1.77 mmol) following the procedure described for compound **14**. Evaporation of the solvent gave a solid that was used without further purification in the next step. An analytical sample (20 mg) purified by flash chromatography (hexane–ethyl acetate 100:0 → 90:10) had mp 169–170 °C (from hexane–ethyl acetate); IR (KBr): 2957.8, 2930.1, 2888.5, 2869.2, 1735.5, 1715.4, 1428.1, 1378.7, 1365.3, 1237.0, 1078.5, 1059.4, 555.1 cm^{-1}; ^1H NMR (500.13 MHz, $CDCl_3$) δ: 5.47 (1H, m, H-3), 4.86 (1H, dd, J = 4.4 and 2.0, H-6), 4.57 (1H, t, J = 2.2, H-18), 2.71 (1H, m H-7α), 2.58 (1H, m, H-4β), 2.45 (3H, s, H-21), 2.40 (1H, m, H-7β), 2.34 (1H, m, H-16α), 2.30 (1H, m, H-4α), 2.18 (1H, m, H-16β), 2.05 (1H, s, 3-acetate), 2.05 (1H, m, H-13), 2.00 (2H, m, H-15α and H-2α), 1.81 (1H, m, H-11α), 1.73 (1H, m, H-1β), 1.69 (2H, m, H-8 and H-9), 1.68 (1H, m, H-1α), 1.64 (1H, m, H-2β), 1.62 (2H, m, H-12), 1.43 (1H, s, H-19), 1.43 (1H, m, H-15β), 1.23 (1H, m, H-11β); ^{13}C NMR (125.77 MHz, $CDCl_3$): 198.60 (C-20), 170.37 (3-acetate), 86.69 (C-5), 71.81 (C-3), 71.15 (C-17), 60.87 (C-18), 55.30 (C-6), 45.82 (C-9), 41.81 (C-4), 41.78 (C-10), 40.65 (C-13), 39.56 (C-14), 36.35 (C-8), 36.30 (C-7), 36.25 (C-1), 31.44 (C-12), 29.02 (C-16), 26.09 (C-2), 25.66 (C-15), 24.80 (C-11), 23.42 (C-21), 21.30 (3-acetate), 20.16 (C-19); HRMS-ESI: calculated for $C_{23}H_{32}Br_4NaO_3$ 694.8977, found 694.8977. $C_{23}H_{32}Br_4O_3$; calculated: C, 40.85; H, 4.77, found: C, 40.65; H, 4.93.

*2.2.7. 3β-Acetoxy-17(13 → 18)-abeo-pregna-5,13,15,17-tetraen-20-one (**8**)*

A solution of compound **18** (590 mg, 0.873 mmol) obtained above in acetonitrile (23 mL) was treated with DABCO following the procedure described for compound **6**. The resulting solid was purified by flash chromatography (dichloromethane) to give compound **8** (178 mg, 58% from **17**) as a white solid mp: 179–180 °C (from hexane); IR (KBr): 2938.6, 1731.9, 1681.2, 1606.2, 1362.9, 1244.6, 1034.6, 813.9, 735.3 cm^{-1}; ^1H NMR (500.13 MHz, $CDCl_3$) δ: 7.73 (1H, dd, J = 8.1 and 1.5 Hz, H-16), 7.68 (1H, bs, H-18), 7.35 (1H, d, J = 8.2 Hz, H-15), 5.53 (1H, d, J = 5.4 Hz, H-6), 4.65 (1H, m, H-3), 2.94 (1H, m, H-12β), 2.85 (1H, m, H-12α), 2.71 (1H, m, H-7β), 2.58 (3H, s, CH_3-21), 2.42 (1H, ddd, J = 13.0, 5.1 and 2.1 Hz, H-4α), 2.34 (1H, m, H-4β), 2.05 (3H, m, 3-acetate), 2.03 (1H, dt, J = 13.5 and 3.4 Hz, H-1β), 2.00 (1H, m, H-11α), 1.96 (1H, m, H-7α), 1.94 (1H, m, H-2α), 1.68 (1H, m, H-2β), 1.46 (1H, m, H-11β), 1.42 (1H, m, H-9), 1.26 (1H, td, J = 13.6 and 3.6 Hz, H-1α), 1.07 (1H, m, CH_3-19); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ: 198.15 (C-20), 170.54 (3-acetate), 146.42 (C-14), 140.13 (C-5), 137.26 (C-13), 134.54 (C-17), 128.86 (C-18), 127.51 (C-15), 125.86 (C-16), 122.41 (C-6), 73.82 (C-3), 48.48 (C-9), 37.77 (C-4), 37.47 (C-10), 36.63 (C-1), 34.94 (C-8), 33.14 (C-7), 30.69 (C-12), 27.51 (C-2), 26.57 (C-21), 22.64 (C-11), 21.42 (3-acetate), 18.46 (C-19); HRMS-ESI: calculated for $C_{23}H_{28}NaO_3$ 375.1931, found 375.1936. Further elution with dichloromethane gave compound **17** (65 mg, 20%).

*2.2.8. 3β-Hydroxy-17(13 → 18)-abeo-pregna-5,13,15,17-tetraen-20-one (**9**)*

Compound **9** was obtained from compound **8** (115 mg, 0.326 mmol) following the procedure described for compound **7**. The resulting solid was purified by flash chromatography (hexane–ethyl acetate 70:30) to give compound **9** (86 mg, 85%) as an amorphous solid; IR (KBr): 3411.0, 2027.3, 1675.2, 1601.0, 1288.4, 1070.0, 736.2 cm^{-1}; ^1H NMR (500.13 MHz, $CDCl_3$) δ: 7.73 (1H, dd, J = 8.1 and 1.4 Hz, H-16), 7.68 (1H, bs, H-18), 7.36 (1H, d, J = 8.2 Hz, H-15), 5.50 (1H, bs, H-6), 3.59 (1H, m, H-3), 2.92 (2H, m, H-12β and H-8), 2.83 (1H, m, H-12α), 2.72 (1H, m, H-7β), 2.58 (3H, s, CH_3-21), 2.39 (1H, ddd, J = 13.0, 4.9 and 2.4 Hz, H-4α), 2.27 (1H, m, H-4β), 2.02 (1H, dt, J = 13.5 and 3.3 Hz, H-1β), 1.99 (1H, m, H-11α), 1.93 (1H, m, H-7α), 1.92 (1H, m, H-2α), 1.60 (1H, m, H-2β), 1.46 (1H, m, H-11β), 1.39 (1H, m, H-9), 1.19 (1H, td, J = 13.6 and 3.7 Hz, H-1α), 1.06 (1H, m, CH_3-19); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ: 198.19 (C-20), 146.56 (C-14), 141.20 (C-5), 137.29 (C-13), 134.51 (C-17), 128.85 (C-18), 127.53 (C-15), 125.84 (C-16), 121.47 (C-6), 71.79 (C-3), 48.58 (C-9), 41.91 (C-4), 37.39 (C-10), 36.87 (C-1), 35.00 (C-8), 33.16 (C-7), 31.38 (C-2), 30.73 (C-12), 26.56 (C-21), 22.69 (C-11), 18.55 (C-19); HRMS-ESI: calculated for $C_{21}H_{27}O_2$ 311.2006, found 311.2011.

*2.2.9. 3β-Hydroxy-5α,6α-epoxy-17(13 → 18)-abeo-pregna-13,15,17-trien-20-one (**10**) and 3β-hydroxy-5β,6β-epoxy-17(1318)-abeo-pregna-13,15,17-trien-20-one (**11**)*

To a solution of compound **9** (80 mg, 0.257 mmol) in chloroform (5.3 mL) were added *m*-chloroperbenzoic acid (67.3 mg, 0.390 mmol) and a 2.5% aqueous solution of Na_2CO_3 (2.7 mL). After stirring for 2 h at room temperature the reaction mixture was diluted with dichloromethane and washed with a saturated aqueous solution of $NaHCO_3$. The organic layer was dried with sodium sulphate and the solvent evaporated under vacuum. The resulting solid was purified by flash chromatography (toluene-ethyl acetate 2:1) to give compounds **10** (53.7 mg, 64%) and **11** (10.1 mg, 12%). Compound **10**: white solid mp 226–228 °C (from hexane); IR (KBr): 3522.1, 2989.3, 2929.0, 1666.9, 1604.9, 1567.4, 1364.3, 1291.0, 1066.5, 979.7, 971.3, 906.1, 804.1, 733.1 cm^{-1}; ^1H NMR (500 13 MHz, $CDCl_3$) δ: 7.71 (1H, dd, J = 8.2 y 1.6 Hz, H-16), 7.63 (1H, bs, H-18), 7.26 (1H, d, J = 8.2 Hz, H-15), 3.98 (1H, m, H-3), 3.11 (1H, d, J = 4.6 Hz, H-6), 2.85 (1H, m H-8), 2.83 (1H, m, H-12β), 2.72 (1H, ddd, J = 15.2, 8.0 and 4.7 Hz, H-12α), 2.72 (1H, m, H-7β), 2.56 (3H, s, CH_3-21), 2.12 (1H, dd, J = 12.6 and 11.4, H-4β), 2.00 (1H, m, H-2α), 1.91 (1H, m, H-7α), 1.89 (1H, m, H-11α), 1.88 (1H, m, H-1β), 1.70 (1H, m, H-9), 1.69 (1H, m, H-2β), 1.50 (1H, td, J = 13.5 and 4.0, H-1α), 1.39 (1H, ddd, J = 12.3, 4.9 and 2.1, H-4α), 1.29 (1H, qd, J = 12.4 and 4.9, H-11β), 1.11 (1H, m, CH_3-19); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ: 198.12 (C-20), 146.67 (C-14), 136.91 (C-13), 134.52 (C-17), 128.71 (C-18), 127.06 (C-15), 125.86 (C-16), 68.66 (C-3), 65.40 (C-5), 59.21 (C-6), 40.68 (C-9), 39.25 (C-4), 35.35 (C-10), 32.91 (C-8), 32.08 (C-1), 30.86 (C-2), 30.56 (C-12), 30.32 (C-7), 26.53 (C-21), 22.28 (C-11), 15.34 (C-19); HRMS-ESI: calculated for $C_{21}H_{27}O_3$ 327.1955, found 327.1966.

Compound **11**: white solid mp 168–170 °C (from hexane); IR (KBr): 3436.9, 2032.6, 2855.9, 1678.8, 1604.8, 1360.3, 1270.2, 1055.0, 734.3 cm^{-1}; ^1H NMR (500 13 MHz, $CDCl_3$) δ: 7.71 (1H, dd, J = 8.2 y 1.6 Hz, H-16), 7.65 (1H, s, H-18), 7.35 (1H, d, J = 8.2 Hz, H-15), 3.76 (1H, m, H-3), 3.25 (1H, d, J = 2.5 Hz, H-6), 2.96 (1H, td, J = 11.4 and 3.0 Hz, H-8), 2.87 (1H, dt, J = 15.6 and 3.3 Hz, H-12β), 2.77 (1H, dt, J = 14.5 and 3.2 Hz, H-7β), 2.75 (1H, m, H-12α), 2.56 (3H, s, CH_3-21), 2.11 (1H, ddd, J = 13.8, 4.6 and 3.0 Hz, H-1β), 2.07 (1H, t, J = 12.4, H-4β), 1.89 (1H, m, H-2α), 1.86 (1H, m, H-11α), 1.65 (1H, ddd, J = 14.0, 11.8 and 0.6 Hz, H-7α), 1.53 (1H, ddd, J = 13.1, 4.9 and 2.3, H-4α), 1.50 (1H, m, H-2β), 1.38 (2H, m, H11β and H-1α), 1.11 (1H, m, H-9), 1.04 (1H, m, CH_3-19); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ: 198.09 (C-20), 145.55 (C-14), 137.35 (C-13), 134.51 (C-17), 128.93 (C-18), 127.43 (C-15), 125.80 (C-16), 69.19 (C-3), 63.99 (C-6), 63.10 (C-5), 49.79 (C-9), 41.82 (C-4), 36.51 (C-1), 35.30 (C-10), 34.62 (C-7), 32.14 (C-8), 30.86 (C-2), 30.84 (C-12), 26.55 (C-21), 23.04 (C-11), 15.89 (C-19); HRMS-ESI: calculated for $C_{21}H_{27}O_3$ 327.1955, found 327.1951.

*2.2.10. Reaction of **13** with DBU-HBr_3*

To a solution of **13** (15 mg, 0.0419 mmol) in acetonitrile (1.0 mL) DBU-HBr_3 was added (59 mg, 0.126 mmol) and the resulting mixture was heated for 30 min at reflux. The residue obtained by conventional work-up was purified by flash chromatography (hexane–ethyl acetate 95:5 → 90:10) to give compound **14** (19.8 mg, 88%).

*2.2.11. Reaction of **13** with DABCO-Bromine complex*

To a solution of **13** (6.4 mg, 0.0179 mmol) in acetonitrile (0.5 mL) DABCO-Bromine complex was added (56 mg, 0.0355 mmol) and the resulting mixture was heated for 20 min under reflux. The residue obtained by conventional work-up was purified by flash chromatography (hexane–ethyl acetate 95:5 → 90:10) to give compound **14** (8.68 mg, 90%).

2.3. Antifungal activity

2.3.1. Fungal inocula

F. virguliforme (Centro de Referencia de Micología, Facultad de Ciencias Bioquímicas y Farmacéuticas. Universidad Nacional de Rosario N CCC220.05) and *F. solani* (Instituto Spegazzini, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata N LPSC868) were cultured in 3% (w/v) malt extract-agar medium (Oxoid Ltd, Basingstoke Hants, England) in 9 cm Petri dishes at 25 °C and in darkness. In order to obtain the spores, fungi was cultured for 7–10 days. Harvesting was carried out by suspending spores in sterilized water (with oligoelements) [19].

2.3.2. Direct bioautography on TLC

A concentration level of 50 μg/spot of each assayed compound was deposited in a thin layer normal phase silica plate separating each spot from the other by a 3 cm distance, in order to establish the diameter of inhibition.

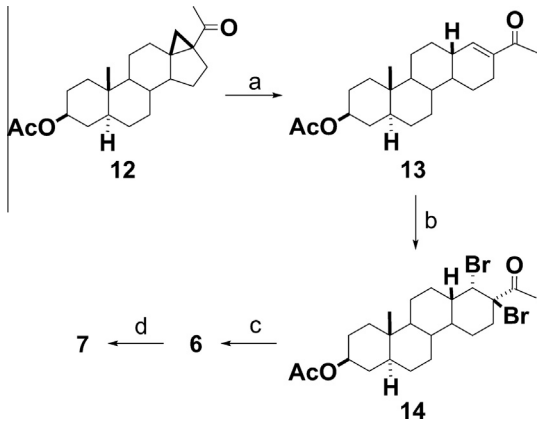
In direct bioautography the plate is dipped in the suspension of spores of fungi. The plate is incubated at 25 °C, in darkness and control humidity and microorganisms grow directly on it. Hence, separation, preconditioning, incubation and visualization are performed directly on the plate. The observation of the inhibition is based on the inhibition diameter caused by the assay compound and visualized after 48 hrs [20].

2.3.3. Broth microdilution test

Test compound were dissolved in methanol to a concentration of 1 μg/μl. Microdilution was performed in sterile disposable microtitre plates (96 U-bottomed wells). Every well was filled with 0.2 ml of 3% (w/v) sterile malt extract broth. Aliquots from 1 to 10 μl of the each compound were dispensed in every well of the row except the first. Compounds were tested by duplicate. Stock inoculum suspension were adjusted to 10^5 CFU/ml. 10 μl were dispensed into each well except the first row. The last row of the plate contains sterile malt extract as positive control. The first row of the plate contains only sterile malt extract broth as negative control of contamination. Microtitre plates were incubated 72 hs at 25 °C in darkness and humidity control. Germ-tube inhibition was determined by direct observation. Minimal inhibition concentration (MIC) was determined as the lowest test compound concentration completely inhibiting spore germination [21].

3. Results and discussion

The strategy followed to obtain compound **6** is shown in Scheme 1. The cyclopropylketone **12** was prepared from pregnenolone acetate (see supplementary data). The D-homosteroid **13** was obtained by acid catalyzed rearrangement of the cyclopropylketone **12**, using BF_3-Et_2O as catalyst [22]. Treatment of **13** with bromine in 1,2-dichloroethane gave the dibromosteroid **14** in 93% yield. The configuration at position 18 was inferred from the ^1H NMR spectra of **14**, given that the H-18 appeared as a narrow triplet (J = 2.2 Hz) at δ 4.55 indicating an equatorial orientation. Several reaction conditions summarized in Table 1 were tried for the aromatization of ring D. Treatment with 1,8-diazabicyclo [5.4.0] undec-7-ene (DBU) (4 eq) at room temperature (entry 1) afforded a mixture of the starting material **14** and compounds **6** and **13** (0.4:1.0). When the temperature was increased, the starting material **14** disappeared in 1 h, and an improvement in the reaction yield was observed (**6**/**13** 1.0:1.0) (entry 2). Further attempts to improve the yield using toluene or DMF as solvents were unsuccessful (entries 3 and 4). Decreasing the amount of DBU (2 eq, entry 5) gave a mixture of **6**, **13** and **15** (0.5:1.0:0.2). Compound **15** could not be isolated, its presence was inferred from the ^1H NMR spec-



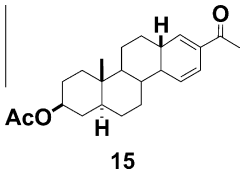
Scheme 1. Reagents and conditions: (a) BF_3-Et_2O, 1,2-dichloroethane, 50 °C, 1.5 h; (b) Br_2, 1,2-dichoroethane, 0 °C, 10 min; (c) DABCO, CH_3CN, reflux, 1 h; (d) KOH, H_2O-MeOH–THF, room temperature, 1 h.

Table 1
Conditions for the D-ring aromatization of compound **14**.

Entry	Conditions (time min)	6 (% yield)	Ratio 6:13:15
1	DBU (4 eq, 0.15 M) CH_3CN (120)[a]	21	0.4:1.0:0
2	DBU (4 eq, 0.15 M) CH_3CN (60)[b]	40	1.0:1.0:0
3	DBU (4 eq, 0.15 M) toluene (60)[b]	28	1.0:1.0:0
4	DBU (4 eq, 0.91 M) DMF (60)[b]	36	1.2:1.0:0
5	DBU (2 eq, 0.076 M) CH_3CN (120)[b]	27	0.5:1.0:0.2
6	DBU (4 eq, 0.15 M) CH_3CN (180)[b]	44	1.3:1.0:0
7	DBU (4 eq, 0.15 M) CH_3CN, O_2 (60)[b]	40	1.0:1.0:0
8	DBU (4 eq, 0.15 M) CH_3CN, N_2 (60)[b]	29	0.6:1.0:0
9	DBU, neat (10)[c]	0	Complex mixture
10	DBU (4 eq, 0.23 M) DMF (10)[c]	30	2.0:1.0:0
11	t-BuOK (4 eq, 0.15 M) t-BuOH (60)[b]	0	Complex mixture
12	2,4,6-collidine (60)[b]	0	Complex mixture
13	Et_3N (4 eq, 0.10 M) CH_3CN (240)[b]	0	0:1.0:0
14	DABCO (4 eq, 0.15 M) CH_3CN (60)[b]	88	1.0:0:0

[a] Room temperature.
[b] Reflux.
[c] Microwave (300 W, 46 psi).

trum of the mixture, that showed a broad singlet at δ 6.59 assigned to H-18 and two doublets at δ 6.49 (J = 10.0 Hz) and δ 6.07 (J = 10.0 Hz) assigned to H-16 and H-15 respectively. According to these results we proposed the mechanism shown in Fig. 2 for the aromatization process. Briefly, DBU may react with H-16 and H-13 leading to the desired HBr abstractions to give an intermediate 13(18),16-diene (path a). This compound would be readily oxidized by air to give **6**. On the other hand, DBU may also react with one of the bromine atoms, regenerating compound **13** by dehalogenation of the vicinal dibromide (path b). Considering that DBU reacts with molecular bromine and HBr to give DBU-hydrobromide-perbromide ($DBUH^+Br_3^-$) [17] that may act as a brominating agent, we propose that path "b" is reversible. When the amount of DBU is decreased (Table 1, entry 5) the increased acidity may promote rearrangement of the intermediate 13(18),16-diene to diene **15**.



When the reaction was carried out in the same conditions as those used in entry 2, but for a longer reaction time (entry 6), an improvement in the product ratio was observed (**6**/**13** 1.3:1). Furthermore when compound **12** was treated with the complex $DBUH^+Br_3^-$, prepared as described in the literature [17], the dibromo steroid **14** was obtained after 30 min. To test if oxygen was involved in the oxidation of **14** to give **6**, the reaction was carried out by bubbling oxygen (entry 7) or nitrogen (entry 8). Although the bubbling of O_2 did not change the **6**/**13** ratio (entry 7), in the absence of oxygen a decrease in yield was observed (entry 8), indicating that O_2 is involved in the oxidation process. Considering that microwave irradiation has proved to be a convenient alternative to many thermal reactions, we tried the reaction using this type of irradiation (entries 9 and 10). In the absence of solvent formation of **6** was not observed, however using DMF as solvent resulted in an increased **6**/**13** ratio (2.0:1.0) although the global yield of **13** decreased.

We then wondered whether this transformation would also occur with other bases. Thus we treated **14** with *t*BuOK, 2,4,6-collidine, Et_3N and 1,4-diazabicyclo[2.2.2]octane (DABCO) (entries 11–14). The first two bases gave a complex mixture in which compound **6** was not observed while Et_3N gave compound **13** and no aromatic product (entry 13). On the other hand we were pleased to find that treatment with DABCO gave the desired compound **6** in 88% yield (entry 14). Considering the proposed mechanism previously described (Fig. 2), the equilibrium in path "b" would be shifted to the left due to the higher brominating efficiency of the DABCO-bromine complex [18] in comparison with DBU-hydrobro-

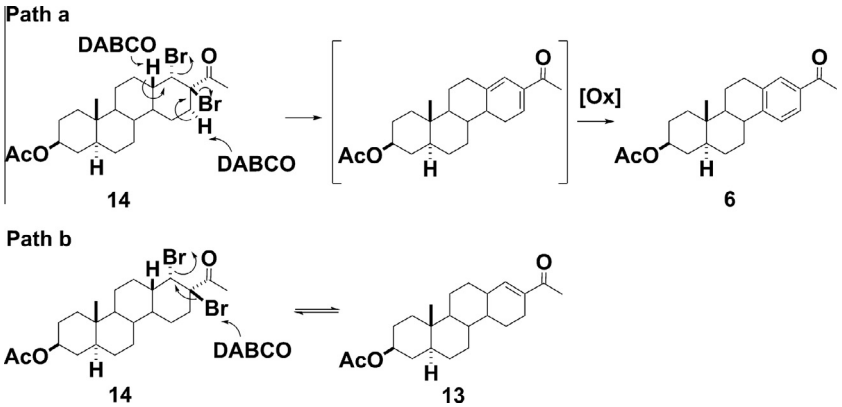
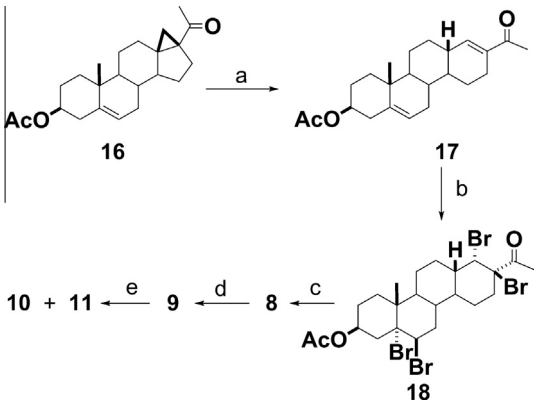


Fig. 2. Proposed mechanism for the aromatization of the D ring.



Scheme 2. Reagents and conditions: (a) BF_3-Et_2O, 1,2-dichloroethane, 50 °C, 1.5 h; (b) Br_2, 1,2-dichoroethane, 0 °C, 10 min; (c) DABCO, CH_3CN, reflux, 1 h; (d) KOH, H_2O-MeOH–THF, room temperature, 1 h; (e) *m*-CPBA, $CHCl_3$, Na_2CO_3-H_2O, room temperature, 2 h.

Table 2
Antifungal activity of compounds **2**–**11** on *F. virguliforme* and *F. solani*.

Compound	F. virguliforme[a]	F. virguliforme[b]	F. solani[a]	F. solani[b]
2	1.5 ± 0.2	33	1.5 ± 0.2	33
3	1.3 ± 0.2	32	1.2 ± 0.2	32
4	1.7 ± 0.2	33	1.5 ± 0.2	33
5	1.5 ± 0.2	33	1.5 ± 0.2	33
6	0.7 ± 0.1	141	0.6 ± 0.1	141
7	1.0 ± 0.1	80	0.9 ± 0.1	80
8	0.5 ± 0.1	142	0.4 ± 0.1	142
9	1.2 ± 0.1	48	1.1 ± 0.1	48
10	1.5 ± 0.1	46	1.2 ± 0.1	46
11	2.0 ± 0.2	31	1.8 ± 0.2	31
Benomyl	3.0 ± 0.2[c]	2	2.0 ± 0.2 [c]	2

[a] Diameters of inhibition zone in cm. Mean values ± standard error from three independent experiments testing 50 μg of compound/spot.
[b] MIC (μM).
[c] 6 μg/spot.

mide-perbromide, finally leading to compound **6**. This possibility was confirmed by treating compound **13** with the tetrameric complex DABCO-bromine prepared as described in the literature [18], that gave dibromo steroid **14** in 90% yield. Finally, treatment of compound **6** with aqueous KOH in MeOH–THF gave compound **7** in 61 % yield from **12**.

Following the above procedure, compounds **8** and **9** were obtained using the cyclopropylketone **16** as starting material. The latter compound was prepared as previously described (Scheme 2) [16]. In this case, treatment of **17** with bromine in 1,2-dichloroethane gave the tetrabromosteroid **18**. Reaction of compound **18** with DBU (4 eq) in CH_3CN (reflux), gave a mixture of **8** and **17** in a 1:1 ratio. As in the case of compound **6**, when DBU was replaced by DABCO an improvement in the reaction yield was observed, giving a 3:1 mixture of **8** and **17**. However in this case, attempts to avoid the formation of the unsaturated D-homosteroid **17** were unsuccessful. Compound **17** was recovered during the purification of **8** by flash chromatography and recycled. In this way **8** was obtained in 74% yield from **17**. Hydrolysis of the acetate at position 3 with KOH (aq) afforded compound **9** in 38 % yield from **16**, this compound had been previously obtained by Blumbach et al. in 16 % yield [15].

Epoxidation of the 5,6-double bond in **9** was achieved by the general protocol described by Ma et al. [23] to give a 5:1 mixture of the 5α,6α (**10**) and 5β,6β (**11**) epoxysteroids.

Compounds **2**–**11** were tested *in vitro* using direct bioautography on TLC [19], for their inhibitory properties towards *F. virguliforme* and *F. solani* (casual agents of sudden death syndrome in soy bean) [15]. A concentration level of 50 μg/spot of each assayed compound was used. Benomyl, a systemic benzimidazole fungicide selectively toxic to microorganisms and to invertebrates, was used as test compound. It showed an inhibition zone of 30 mm at a conc. level of 6 μg/spot (0.02 μmol/spot) and 12 mm at a conc. level of 0.6 μg/spot (0.002 μmol/spot). Most of the new compounds showed a measurable antifungal activity (Table 2). Considering the activity of the synthetic compounds **7**, **9**, **10** and **11**, we can conclude that the antifungal properties of the salpichrolides (**2**–**6**) depend largely on the presence of the aromatic D ring. The results obtained with the synthetic compounds **6**–**11** showed that the presence of a 5,6-epoxy group increases the activity, the β-epoxide being more active, while esterification of the hydroxyl at position three results in a decrease of the antifungal properties.

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.2013.02.003.

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