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Antifungal diterpenes from *Hypoestes serpens* (Acanthaceae)

Lalao Rasoamiaranjanahary[a,c], Andrew Marston[a], David Guilet[a], Kurt Schenk[b], Fanantenanirainy Randimbivololona[c], Kurt Hostettmann[a,*]

[a]*Institut de Pharmacognosie et Phytochimie, Université de Lausanne, CH-1015 Lausanne, Switzerland*
[b]*Institut de Cristallographie, Université de Lausanne, CH-1015 Lausanne, Switzerland*
[c]*Laboratoire de Pharmacologie Générale et de Pharmacocinétique, Université d'Antananarivo, BP 8351, 101 Antananarivo Madagascar*

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Dedicated to Meinhart H. Zenk on the occasion of his 70th birthday

Abstract

Two new diterpenes, fusicoserpenol A and dolabeserpenoic acid A, with antifungal activity, were isolated from leaves of *Hypoestes serpens* (Acanthaceae). Their structures were elucidated by means of spectrometric methods including 1D and 2D NMR experiments and MS analysis. X-ray crystallographic analysis confirmed the structure of fusicoserpenol A and established the relative configuration.

Keywords: *Hypoestes serpens*; Acanthaceae; Fusicoccane; Dolabellane; Antifungal activity

1. Introduction

Hypoestes serpens (Vahl) R. Br. (Acanthaceae) is a herbaceous plant which grows in the central region of Madagascar. According to ethnobotanical investigations, a decoction of the leaves is used in traditional Malagasy medicine for the treatment of high blood pressure. Previous work on the defatted chloroform extract of the leaves reported the isolation of a fusicoccane diterpene which had a relaxant activity on isolated rat aorta (Andriamihaja et al., 2001).

As part of our ongoing search for novel bioactive compounds, the leaves of *Hypoestes serpens* were investigated. The dichloromethane extract of the leaves showed interesting antifungal activity against both the plant pathogenic fungus *Cladosporium cucumerinum* and the yeast *Candida albicans* in bioautographic TLC assays. Therefore, the investigation of this extract was undertaken. In the present paper we describe the isolation and identification of two novel antifungal diterpenes from the dichloromethane extract.

2. Results and discussion

Bioautographic TLC assay-guided fractionation by repeated open column chromatography led to the isolation of fusicoserpenol A (**1**) and dolabeserpenoic acid A (**2**).

The D/CI-MS spectrum of compound **1** showed a molecular ion signal at m/z 306 $[M]^+$ and the ^{13}C NMR spectrum displayed 20 carbon signals, corresponding to a molecular formula of $C_{20}H_{34}O_2$. This was confirmed by HR-ESIMS, which showed an ion peak at m/z 329.2457 $[M+Na]^+$. The ^{13}C NMR spectrum of fusicoserpenol A exhibited only two unsaturated carbons at δ_C 111.3 and 154.9 ppm, thus implicating the presence of three ring structures in the molecule. Analysis of NMR data revealed resonances for an isopropyl moiety [δ_H 0.80 (3H, *d*, $J=6.5$ Hz), 0.93 (3H, *d*, $J=7.0$ Hz) and 1.69 (1H, *m*)], two non-geminal methyl groups [δ_H 1.06 (3H, *s*), 1.10 (3H, *s*)] and an exomethylene function [δ_H 4.73 (1H, *d*, $J=2.0$ Hz), 4.82 (1H, *d*, $J=2.0$ Hz) and δ_C 111.3]. From these structural elements, compound **1** appeared to be a diterpenoid belonging to the fusicoccane (dicyclopenta[*a,d*]cyclooctane) group (Andriamihaja et al., 2001; Tori et al., 1993). Inspection of the gDQF-COSY spectrum indicated a vicinal relationship between the two methine signals at δ_H 1.82 and 2.27, which were attributed to positions 3 and 7 of the

* Corresponding author. Tel.: +41-21-692-45-61; fax: +41-21-692-45-65.
E-mail address: kurt.hostettmann@ipp.unil.ch (K. Hostettmann).

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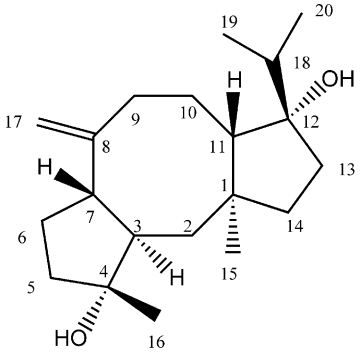

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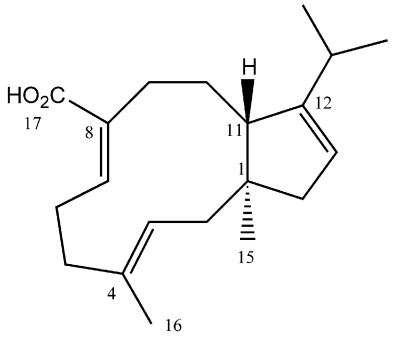

2

methylcyclopentane unit. The last methine group [δ_H 1.61 (1H, *m*, H-11) and δ_C 50.0 (C-11)] was subsequently associated with the isopropylcyclopentane substructure. Long-range ^1H-^{13}C couplings deduced from the gHMBC spectrum of **1** (Table 1) revealed that the methine H-11 correlated with six carbons- among them one of the methyl groups (δ_H 1.10 and δ_C 23.5) attributed to Me-15 and one quaternary carbon substituted by a hydroxyl function (δ_C 89.4). Furthermore, cross-peaks between the isopropyl group and this oxygenated carbon suggested substitution of the latter by the isopropyl moiety and led to attribution of the oxygenated carbon to the C-12 position. Other NMR data deduced from gDQF-COSY, gHSQC and gHMBC corroborated these different structural elements and confirmed the substitution pattern of the isopropylcyclopentane substructure. In addition, careful analysis led to the attribution of the neighbouring methylene groups at the C-2 and C-10 positions, characterized by chemical shifts at δ_H 1.44 (2H, *m*, H-2 and H-10), 1.62 (1H, *m*, H-10) and 1.72 (1H, *m*, H-2) and δ_C 31.8 (C-10) and 32.1 (C-2). The substitution pattern of the methylcyclopentane unit was then deduced by examination of several major long-range ^1H-^{13}C couplings. In particular, the correlation between the methine group at δ_H 1.82 and the carbon C-1 led to the distinction between the hydrogen atom at the 3-position [δ_H 1.82 (1H, *m*, H-3) and δ_C 51.0 (C-3)] and the other methine group at the 7-position [δ_H 2.27 (1H, *m*, H-7) and δ_C 51.5 (C-7)]. The other correlations observed for the methine H-3 with the carbon atom at δ_C 23.6, associated with the remaining methyl group [δ_H 1.06 (3H, *s*, H-16)], and with the carbon atom at δ 81.5, supported the substitution of C-4 by this methyl moiety and by a second hydroxyl function. Finally, the exact location of the exomethylene unit was deduced from heteronuclear long-range correlations between H-17 (δ_H at 4.73 and 4.82) and C-7. It should be noted that the different elements of stereochemistry could not be deduced from the analysis of the NOESY spectrum because of the overlapping of signals at δ_H 1.44, 1.45, 1.69 and 1.70 ppm. X-ray crystallographic analysis was used in order to confirm this structure and establish the relative stereochemistry (Fig. 1). Accordingly, fusicoserpenol A (**1**) was determined to be 4α,12α-dihydroxy-8(17)-fusicoccene.

Table 1
^{13}C and ^1H NMR spectral data of compounds **1** and **2** in $CDCl_3$

	1			**2**	
	δ_C	δ_H, mult, *J* (Hz)	HMBC	δ_C	δ_H, mult, *J* (Hz)
1	43.6			45.9	
2	32.1	1.44 *m*	C_1, C_3, C_4, C_7	41.2	1.63 *dd* (13.0/4.5)
		1.72 *m*	C_1, C_3, C_4, C_{15}		2.24 *m*
3	51.0	1.82 *m*	C_1, C_4, C_7, C_{16}	125.1	5.27 *dd* (12.5/3.5)
4	81.5			135.4	
5	40.1	1.69 *m*	C_3, C_4, C_6, C_7, C_{16}	39.9	2.22 *m*
		1.71 *m*	C_4, C_6, C_{16}		2.27 *m*
6	26.0	1.45 *m*	C_4, C_5, C_7	26.0	2.27 *m*
		1.60 *m*	C_4, C_5, C_{16}		3.35 *m*
7	51.5	2.27 *m*	C_2, C_3, C_6, C_8, C_{17}	150.4	5.90 *dd* (12.0/3.5)
8	154.9			128.7	
9	29.9	2.18 *m*	C_6, C_7, C_8, C_{10}, C_{11}, C_{17}	34.3	2.25 *m*
		2.23 *m*	C_6, C_7, C_8, C_{10}, C_{11}		2.61 *m*
10	31.8	1.44 *m*	C_1, C_{11}, C_{12}	26.4	1.41 *m*
		1.62 *m*	C_9, C_{12}		1.77 *m*
11	50.0	1.61 *m*	C_1, C_{10}, C_{12}, C_{13}, C_{14}, C_{15}	47.1	2.17 *m*
12	89.4			154.0	
13	43.3	1.44 *m*	C_1, C_{10}, C_{11}, C_{12}, C_{14}, C_{15}	118.4	5.18 *d* (1.0)
		1.71 *m*	C_1, C_{10}, C_{11}, C_{12}, C_{14}, C_{15}		
14	46.4	1.06 *m*	C_1, C_{11}, C_{13}, C_{15}	48.5	1.97 *dd* (15.5/3.0)
		1.70 *m*	C_1, C_2, C_{11}, C_{12}, C_{13}		2.20 *m*
15	23.5	1.10 *s*	C_1, C_{11}, C_{14}	22.4	1.15 *s*
16	23.6	1.06 *s*	C_3, C_4, C_5, C_6, C_7	15.9	1.51 *s*
17	111.3	4.73 *d* (2.0)	C_7, C_8, C_9	172.9	
		4.82 *d* (2.0)	C_7, C_8, C_9		
18	23.9	1.69 *m*	C_{12}, C_{13}, C_{14}, C_{19}, C_{20}	26.9	2.16 *m*
19	18.1	0.80 *d* (6.5)	C_{12}, C_{18}, C_{20}	21.8	1.05 *d* (6.5)
20	18.1	0.93 *d* (7.0)	C_{12}, C_{18},C_{19}	22.6	0.90 *d* (7.0)

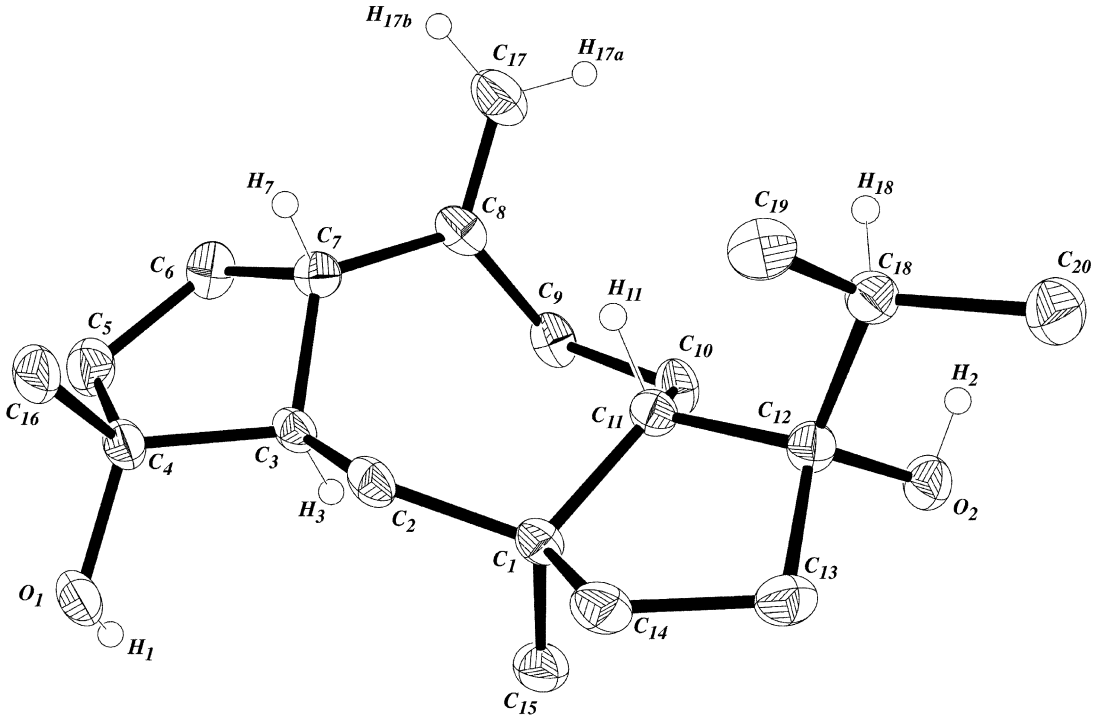

Fig. 1. ORTEP style (20%) representation of **1**.

The molecular formula of **2** was established as $C_{20}H_{30}O_2$ by HR-EIMS. The ^{1}H NMR data of **2** (Table 1) indicated the presence of an isopropyl group [δ_H 0.90 (3H, *d*, $J=7.0$ Hz), 1.05 (3H, *d*, $J=6.5$ Hz) and 2.16 (1H, *m*)], two methyl units [δ_H 1.15 (3H, *s*), 1.51 (3H, *s*)] and three ethylenic protons [δ_H 5.18 (1H, *d*, $J=1.0$ Hz), 5.27 (1H, *dd*, $J=3.5$, 12.5 Hz) and 5.90 (1H, *dd*, $J=3.5$, 12.0 Hz)]. Furthermore, the ^{13}C NMR data showed that compound **2** presented seven sp^2-hybridized carbon atoms (Table 1), three of which were protonated (δ_C 118.4, 125.1 and 150.4), three were non-protonated (δ_C 128.7, 135.4 and 154.0) and one at δ_C 172.9 was associated with a carbonyl moiety. The occurrence of these four unsaturations implied consequently the presence of only two ring structures in the molecule. In addition, these spectral data were quite similar to those of dolabella-3,7,18-trien-17-oic acid isolated from *Trichilia trifolia* (Meliaceae) (Ramirez et al., 2000), suggesting that **2** possessed the same basic dolabellane structure (cyclopentacycloundecane). Careful examinations of the gDQF-COSY, gHSQC and gHMBC spectra indicated that compound **2** differed from the known dolabella-3,7,18-trien-17-oic acid by the substitution pattern of the isopropylcyclopentane substructure. Thus, in compound **2**, the isopropyl moiety substituted the unsaturated C-12 position, as shown by the long-range couplings observed between the two methyl groups H-19 and H-20 with the signal carbon at δ_C 154.0 (C-12). The stereochemistry of the double bonds at C-3 and C-7 were determined to be *E* and *Z*, respectively, by comparison with the NMR data (chemical shifts and coupling constants) reported for the crystallized dolabella-3,7,18-trien-17-oic acid (Ramirez et al., 2000). From biogenetic considerations, the cyclopentane and the undecane rings in compound **2** are most likely joined in a *trans* fashion, as observed in **1**. Therefore, compound **2**, named dolabeserpenoic acid A, was identified as (3*E*,7*Z*)-dolabella-3,7,12-trien-17-oic acid.

From a chemotaxonomic point of view, it is important to mention that fusicoserpenol A (**1**) is the second fusicoccane derivative isolated from *H. serpens* (Andriamihaja et al., 2001) and dolabeserpenoic acid A (**2**) is only the second dolabellane diterpenoid reported from the whole genus *Hypoestes* (Al-Rehaily et al., 2002). In fact, eight fusicoccane-type and one dolabellane-type diterpenes have already been identified in four species of the genus *Hypoestes*: *H. forskalei* (Muhammad et al., 1997, 1998), *H. rosea* (Adesomoju et al., 1983; Adesomoju and Okogun, 1984), *H. serpens* (Andriamihaja et al., 2001) and *H. verticillaris* (Al-Rehaily et al., 2002). It is intriguing to note that fusicoccane and dolabellane derivatives of *Hypoestes* species usually have intracyclic unsaturations and at least one ketonic function—this is quite different from the chemical characteristics observed for fusicoserpenol A (**1**) and dolabeserpenoic acid A (**2**). The presence of **1** and **2** in *H. serpens* seems to consolidate the singularity of the biochemical characteristics of this *Hypoestes* species. Furthermore, fusicoccanes and especially dolabellanes

are not common in higher plants and are generally isolated from liverworts (Asakawa, 2001).

The minimum amounts of compounds **1** and **2** required for inhibition in the TLC bioautographic assay were determined as 2 μg for **1** against both *Cladosporium cucumerunum* and *Candida albicans* and 5 μg for **2** in the two tests. This compares well with the value of 1 μg obtained for the antifungal standard miconazole.

3. Experimental

3.1. General

Silica gel 60 (70–200 and 35–70 μm, SDS, Peypin, France) and Sephadex LH-20 (Pharmacia, Sweden) were used for CC and Al-backed silica gel 60 F_{254} plates (Merck) for TLC. Optical rotations were recorded on a Perkin-Elmer-241 polarimeter at r.t. UV spectra were measured on a Varian DMS 100S UV–vis spectrophotometer. IR spectra were recorded on a Perkin-Elmer 781 spectrometer. NMR spectra were obtained on a Varian Unity Inova-500 spectrometer, at 500 (proton) and 125 MHz (carbon); δ in ppm rel. to Me_4Si (internal standard), J in Hz. HRESIMS was recorded on a Bruker FTMS, Apex 2, 4.7T instrument. MS data were recorded at 70 eV on a Finnigan-MAT/TSQ-700 triple-stage quadrupole instrument; m/z (rel. intensity in%).

3.2. Plant material

Leaves of *Hypoestes serpens* were collected from Fandriana (Central and South Highlands of Madagascar) in September 2000 and identified by the Parc Botanique et Zoologique de Tsimbazaza (P.B.Z.T) Antananarivo, Madagascar. A voucher specimen has been deposited at the Institute of Pharmacognosy and Phytochemistry, University of Lausanne (voucher N° 2000070).

3.3. Extraction and isolation

Air-dried and powdered leaves (750 g) were extracted at room temperature with dichloromethane to give 15 g of extract. This extract was fractionated by column chromatography on silica gel 60 (70–200 μm), eluting with a petrol ether–EtOAc gradient (6:1 to 0:1), giving 16 fractions (F1–F16). Fraction F12 was separated by silica gel 60 (35–70 μm) using toluene–EtOAc (5:1) as mobile phase to give 4 fractions (F121–F124). Finally, F122 was further purified by gel filtration on Sephadex LH-20 with hexane–CH_2Cl_2 (2:1) as eluent to afford compound **1** (25 mg). Fraction F5 was applied to a silica gel 60 column (35–70 μm) eluted with $CHCl_3$–EtOAc (25:1) to give 2 fractions (F5-1, F5-2). Gel filtration of F5-2 on Sephadex LH-20 with hexane–CH_2Cl_2 (2:1) gave compound **2** (12 mg).

3.4. 4α,12α-Dihydroxy-8(17)-fusicoccene (fusicoserpenol A, 1)

Colourless crystals obtained from hexane, mp 87–90 °C. $[\alpha]_D$: +19.4 ($CHCl_3$, c 0.5); UV λ_{max} nm (logϵ): 204 (3.83); ^1H and ^{13}C NMR are given in Table 1. EI-MS 70 eV m/z (rel.int.): 288 $[M–H_2O]^+$ (18), 263 (34), 245 (15), 227 (18), 189 (18), 149 (15), 128 (64), 125 (100), 95 (15), 81 (18), 71 (20), 55 (38). D/CI-MS m/z (rel.int.): 306 $[M]^+$ (42), 289 (30), 271 (100). HR-ESIMS m/z: 329.2457 $[M+Na]^+$ (calc. for $C_{20}H_{34}O_2$ +Na 329.2451).

3.5. Crystallography for compound 1

$C_{20}H_{34}O_2$, $M=306.47$ g mol^{-1}; colourless platelet with dimensions $0.8\times0.6\times0.14$ mm^3 and habitus: {110} prism and {001} pinacoid; monoclinic space group $P2_1$ with $a=9.275(5)$, $b=21.997(5)$, $c=9.275(5)$ Å, $\beta=96.719$ (5), $V=1879$ (2) Å^3, $Z=4$; $\rho_{calc}=1.083$ g cm^{-3}, $\mu_{MoK_\alpha}=0.067$ mm^{-1}; $\lambda=0.71069$ Å, $F(000)=680$. At $T=293$ K, collected 9888 reflections with $h(-13,10)$, $k(-29,26)$, $l(-13,12)$ in the range $2.21\leqslant\theta\leqslant24.99°$ on a Bruker SMART as hemisphere (0.3° and 60 s per frame), integration carried out using SAINT (Bruker, 1996), no absorption correction. Structure solved with the help of SIR97 (Altomare et al., 1997) and refined by means of SHELXL (Sheldrick, 1997). 5591 unique reflections with $R_{int}=0.0846$, 4175 reflections $>4\sigma(F_o)$, 413 parameters, 1 restraint, $R_1^{obs}=0.0558$, $wR_2^{obs}=0.1439$, $R_1^{all}=0.0750$, $wR_2^{all}=0.1539$, $\chi^2=0.972$, weights $=[\sigma(F_0^2+(0.1P)^2]^{-1}$, $P=\frac{1}{3}\left[\mathrm{Max}(F_0^2,0)+2F_c^2\right]$, largest residua: 0.187, −0.198 e. Å^{-3}. *CIF* has been deposited under number *CCDC 184061* at the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK (fax +44 1223 336 033 or e-mail deposit@ccdc.cam.ac.uk).

3.6. (3E,7Z)-Dollabella-3,7,12-trien-17-oic acid (dolabeserpenoic acid A, 2)

Amorphous powder. $[\alpha]_D$: −32.1 ($CHCl_3$, c 1.5), UV λ_{max} nm (logε): 204 (4.01), 245 sh. (3.31); ^1H and ^{13}C NMR are given in Table 1. EI-MS 70 eV m/z (rel.int.): 302 $[M]^+$ (21), 259 (15), 191 (18), 189 (13), 165 (14), 150 (15), 149 (30), 136 (54), 135 (100), 121 (78), 107 (43), 93 (54), 91 (33), 79 (25), 55 (20). HR-ESIMS m/z: 325.2134 $[M+Na]^+$ (calc. for $C_{20}H_{30}O_2$ +Na 325.2138).

3.7. Biological assay

TLC bioautographic assay followed a standard protocol (Rahalison et al., 1991, 1994).

3.7.1. Sample preparation

Geometric dilutions were obtained from stock solutions of isolated and reference compounds at a concentration of 1 mg/ml in an appropriate solvent. Ten microliters of these solutions were applied to the thin layer chromatography plate.

3.7.2. Chromatogram preparation

Al-backed silica gel 60 F_{254} TLC sheets (Merck) were used for TLC bioautographic assay with *Cladosporium cucumerinum*, and glass-backed silica gel 60 F_{254} TLC plates (Merck) for the *Candida albicans* assay. After application of the sample on the plate, it was developed in hexane–EtOAc (1:1) as solvent system and thoroughly dried for complete removal of solvents.

3.7.3. Bioautographic assay with Cladosporium cucumerinum

A spore suspension of *Cladosporium cucumerinum* in a nutritive medium was sprayed over the chromatogram. After 3 days incubation at room temperature in polystyrene boxes with a moist atmosphere, active compounds appeared as clear zones against a dark background.

3.7.4. Bioautographic assay with Candida albicans

Yeast inoculums (approx. 10^7 cells/ml) in molten malt agar MA were distributed over the chromatogram. After solidification of the medium as a thin layer (approx. 1 mm layer thickness), TLC plates were incubated overnight at 30 °C in polystyrene boxes in a moist atmosphere. The bioautograms were then sprayed with an aqueous solution of methylthiazolyltetrazoliumchloride MTT (2.5 mg/ml). Active compounds appeared as clear spots against a purple background. Miconazole was used as reference compound.

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