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Antifungal isopimaranes from *Hypoestes serpens*

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Dedicated to the memory of Professor Jeffrey B. Harborne

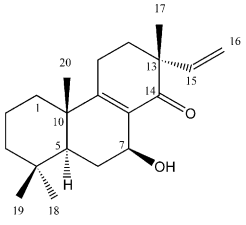
Abstract

Five isopimarane diterpenes (7β-hydroxyisopimara-8,15-dien-14-one, 14α-hydroxyisopimara-7,15-dien-1-one, 1β,14α-dihydroxyisopimara-7,15-diene, 7β-hydroxyisopimara-8(14),15-dien-1-one and 7β-acetoxyisopimara-8(14),15-dien-1-one) have been isolated from the leaves of *Hypoestes serpens* (Acanthaceae). All compounds exhibited antifungal activity against both the plant pathogenic fungus *Cladosporium cucumerinum* and the yeast *Candida albicans*; two of them also displayed an acetylcholinesterase inhibition. The structures of the compounds were determined by means of spectrometric methods, including 1D and 2D NMR experiments and MS analysis.

Keywords: *Hypoestes serpens*; Acanthaceae; Diterpenes; Isopimaranes; Antifungal activity; Acetylcholinesterase inhibition

1. Introduction

Hypoestes serpens (Vahl) R. Br. (Acanthaceae) is a plant used in Malagasy folk medicine to treat high blood pressure and infectious vaginitis. In previous work, a fusicoccane diterpenoid with relaxant activity on isolated rat aorta was identified from a defatted chloroform extract of the leaves (Andriamihaja et al., 2001). As part of an ongoing search for novel bioactive compounds, further investigation of the leaves of *Hypoestes serpens* was undertaken. A dichloromethane extract of the leaves showed interesting antifungal activity against both *Cladosporium cucumerinum* and *Candida albicans* in bioautographic TLC assays and from this extract two antifungal diterpenoids (a fusicoccane and a dolabellane) were isolated (Rasoamiaranjanahary et al., 2003). Using bioactivity-guided fractionation procedures, five antifungal isopimaranes (**1–5**) have now been isolated, two of which exhibited an acetylcholinesterase (AChE) inhibitory activity. A great deal of research is presently being devoted to the discovery of potent AChE inhibitors which are able to maintain high levels of acetylcholine at the muscarinic and nicotinic receptors in the central nervous system and thus find use in the treatment of Alzheimer's disease. In this paper, the isolation and structural elucidation of these diterpenoid compounds, together with their biological activities are described.



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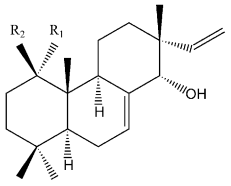
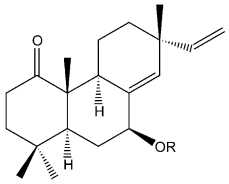


2 $R_1 = R_2 = O$
3 $R_1 = H$; $R_2 = OH$



4 R = H
5 R = $COCH_3$

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2. Results and discussion

Repeated open column chromatography of the dichloromethane extract of the leaves guided by TLC bioautography afforded five isopimarane diterpenoids: 7β-hydroxyisopimara-8,15-dien-14-one (**1**), 14α-hydroxyisopimara-7,15-dien-1-one (**2**), 1β,14α-dihydroxyisopimara-7,15-diene (**3**), 7β-hydroxyisopimara-8(14),15-dien-1-one (**4**) and 7β-acetoxyisopimara-8(14),15-dien-1-one (**5**).

Compound **1** was obtained as an amorphous powder and its molecular formula was assigned as $C_{20}H_{30}O_2$ after HR-ESIMS analysis ($\Delta + 0.2$ mmu). The presence of three rings in the structure of **1** was deduced from the molecular formula and the observation, in the ^{13}C NMR spectrum, of four unsaturated carbons at δ_C 115.0, 131.4, 140.1 and 168.9 ppm, and in addition a ketonic carbon at 204.2 ppm. Analysis of NMR spectroscopic data revealed resonances for two geminal methyl groups [δ_H 0.88 (3H, *s*), 0.96 (3H, *s*)], two non-geminal methyl groups [δ_H 1.05 (3H, *s*), 1.19 (3H, *s*)] and a vinyl function [δ_H 4.98 (1H, *d*, $J = 18.0$ Hz) and 5.10 (1H, *d*, $J = 10.5$ Hz) with δ_C 115.0 and δ_H 5.78 (1H, *dd*, $J = 18.0/10.5$ Hz) with δ_C 140.1]. From these structural elements, compound **1** appeared to be a diterpenoid belonging to the pimarane group (Lago et al., 2000; Prawat et al., 1993; Touché et al., 1997). The structure of the pimarane skeleton could then be deduced by a careful analysis of the NMR spectra data of **1**. The ^{13}C NMR spectrum of **1** presented two quaternary sp^2 hybridized carbons (δ_C 131.4 and 168.9) which suggested the position of a double bond between C-8 and C-9. Long-range ^1H–^{13}C correlations between the protons at δ_H 5.78 (H-15) and 1.19 (Me-17) and the ketonic carbon at 204.2 ppm supported the C-14 position for the ketonic function, which in addition presented a chemical shift value in agreement with its conjugation with the double bond at C-8 and C-9. Furthermore, the UV spectrum of **1** which exhibited an absorption band at 260 nm corroborated the presence of an α,β-unsaturated ketone. A methine group was also deduced from the NMR spectra data of **1** with chemical shifts at δ_H 4.60 and δ_C 63.1. According to the molecular formula of **1** and its characteristic low field values, this methine group bore a hydroxyl moiety. Furthermore, this methine group was located at C-7 because of the observation of HMBC correlations between the proton at δ_H 4.60 and the ^{13}C NMR signals at δ_C 44.8 (C-5), 26.5 (C-6), 131.4 (C-8), 168.9 (C-9) and 204.2 (C-14).

The spatial relationships in the molecule were then deduced from the NOESY spectrum of **1**. In particular, the cross-peaks implicating Me-18, H-7, H-5 indicated that these protons were all on the same face of the molecule (α face) while those observed with Me-17, Me-19 and Me-20 established that these other substituents were on the other side of the molecule (β face). It should be noted that the chemical shifts attributed to C-17 and C-19, δ_C at 24.0 and 21.7 respectively, suggested for them an axial position (Beier, 1978). Finally, the relative configuration, thus obtained, showed that compound **1** belonged in fact to the isopimarane group and was defined as 7β-hydroxyisopimara-8,15-dien-14-one.

Compound **2** was obtained as an amorphous powder and its molecular formula, $C_{20}H_{30}O_2$, deduced from HR-ESIMS analysis ($\Delta + 0.2$ mmu) indicated that it was an isomer of **1**. The NMR spectra of compound **2** were very similar to those recorded for **1**, suggesting the presence of another isopimarane structure. Nevertheless, some differences were observed between the two compounds. The NMR spectral data of **2** indicated the presence of an oxygenated methine (δ_H 3.64 and δ_C 79.7), an ethylenic group (δ_H 5.70 and δ_C 125.5) associated with a non-hydrogenated sp^2 carbon (δ_C 137.5) and a non-conjugated carbonyl function (δ_C 215.5). The ethylenic proton and the oxygenated methine were assigned to the 7- and 14-positions, respectively, according to the HMBC correlations observed between the protons at δ_H 5.88 (H-15), 0.89 (Me-17) and 5.70 (H-7), and the ^{13}C signal at δ_C 79.7 (C-14) and between the proton at δ_H 3.64 (H-14) and the ^{13}C signals at δ_C 125.5 (C-7), 135.7 (C-8), 41.1 (C-13), 146.2 (C-15) and 22.1 (Me-17). The position of the carbonyl group at C-1 was established by HMBC correlations observed between the ^{13}C signal at δ_C 215.5 and the protons at δ_H 1.61 (H-5), 2.77 (H-9) and 1.15 (Me-20). Following a NOESY experiment, the stereochemistry of **2** was determined to be the same as **1**. In addition, the common cross-peaks detected for Me-20, H-11β, H-12β, Me-17 and H-14 suggested that all these protons were on the same face of the molecule (β face). The H-9 showed correlations with H-5α and with the multiplet around δ_H 2.00 (H-11α and H-12α), implicating a *syn*-relationship among these different protons. The structure of **2** was thus established as 14α-hydroxyisopimara-7,15-dien-1-one.

The molecular formula $C_{20}H_{32}O_2$ of **3** was determined by HR-ESIMS ($\Delta + 0.3$ mmu). Spectral data of **3** showed that its structure was very close to that of compound **2** with the presence of a double bond between C-7 (δ_C 126.7) and C-8 (δ_C 137.7) and an oxygenated methine at C-14 (δ_C 79.7). However, compound **3** had an additional hydroxyl group at C-1 instead of a carbonyl function, as indicated by the ^1H and ^{13}C NMR spectra which showed additional deshielded signals at δ_H 3.72 *m* (H-1) and at δ_C 71.2 (C-1). The stereochemistry of **3** was assigned to be the same as **2**. The β-position of the hydroxyl function at C-1 was deduced by the presence of a strong NOE contact between the geminal proton at δ_H 3.72 and the protons at δ_H 1.53 (H-5α) and 0.91 (Me-18) (which was equatorial) on the α face of the molecule. Thus, the structure of **3** was established as 1β,14α-dihydroxyisopimara-7,15-diene.

Compound **4** had the same molecular formula as **2** ($C_{20}H_{30}O_2$) as deduced from HR-ESIMS analysis (Δ

+0.2 mmu). NMR analysis of the two compounds revealed that the only differences concerned the positions 7, 8 and 14. In fact, long-range ^{1}H–^{13}C correlations between the ethylenic proton at δ_H 5.59 (H-14) and the carbon signals at δ_C 147.5 (C-15) and 26.1 (Me-17) indicated an ethylenic function at the C-8 and C-14 positions of the molecule. Furthermore, the position of the oxygenated methine (δ_H 4.20 and δ_C 72.9) at C-7 was deduced by the observation of the HMBC correlations between the latter and the carbon signals at δ_C 138.6 (C-8), 38.7 (C-9) and 136.1 (C-14). By analysis of its NOESY spectrum, the stereochemistry of **4** was assigned as in **1**. Thus, the presence of important NOE contacts between the proton at δ_H 4.20 (H-7) and the signals at δ_H 2.06 (H-5), 2.70 (H-9) and 0.96 (Me-18) indicated that these groups were located on the same face of the molecule (α face). On the basis of these data compound **4** was shown to be 7β-hydroxyisopimara-8(14),15-dien-1-one.

The structure of **5** was determined by comparison of its NMR spectra with those of **4**, which were very similar. However, the ^{1}H and ^{13}C NMR spectra showed that instead of a hydroxyl group at C-7, an acetoxyl group was present in compound **5** (Tables 1 and 2). The stereochemistry of **5** (similar to **1**) and the 7β-location of the acetoxyl group was corroborated by the presence of common NOE contacts between the signals at δ_H 5.31 (H-7), 1.82 (H-6α) and 0.90 (Me-18). Finally, the structure of **5** was assigned as 7β-acetoxyisopimara-8(14),15-dien-1-one.

All compounds displayed antifungal activity against both the plant pathogenic fungus *Cladosporium cucumerinum* and the yeast *Candida albicans* (Rahalison et al., 1991, 1994). The most active compounds were compounds **2** and **4** (0.5 μg was the minimum amount required for inhibition in the TLC bioautographic assay against both *Cladosporium cucumerinum* and *Candida albicans*). Compounds **2** and **4** were more active than the reference compound, miconazole (minimum amount required to inhibit fungal growth on TLC plates: 1 μg). Compound **1** had the same value as miconazole. The other compounds (**3** and **5**) were slightly active with values of 25–50 μg against *Cladosporium cucumerinum* and *Candida albicans*. As *Candida albicans* is one of the causative agents of vaginitis, the inhibitory activity of the pure compounds supports the use of *Hypoestes serpens* in traditional medicine to treat this complication.

Compounds **1** and **2** also exhibited acetylcholinesterase inhibitory activity. The minimum amounts required for inhibition of the enzyme acetylcholinesterase in a TLC bioautographic assay (Marston et al., 2002) were determined as 0.5 μg for **1** and 0.2 μg for **2**. The minimum amount of galanthamine, an Amaryllidaceae alkaloid recently introduced for the treatment of Alzheimer's disease, required to inhibit the enzyme was 0.01 μg.

Table 1
^{1}H NMR spectral data of compounds **1–5** ($CDCl_3$, 500 MHz)

Position	1	2	3	4	5
1α	1.16 *m*		3.72 *m*		
1β	1.78 *m*				
2α	1.58 *m*	2.15 *dt* (12.5/5.0)	1.68 *m*	2.17 *dt* (12.5/5.0)	2.16 *ddd* (12.0/4.5/4.0)
2β	1.66 *m*	2.75 *dd* (12.5/5.0)	1.19 *m*	2.79 *ddd* (12.5/12.0/5.0)	2.83 *ddd* (12.0/5.5/1.0)
3α	1.23 *m*	1.66 *dt* (13.0/5.0)	1.59 *m*	1.75 *m*	1.75 *m*
3β	1.44 *m*	1.77 dd (13.0/5.0)	1.86 *m*	1.83 *t* (5.0)	1.84 *m*
5α	1.50 *m*	1.61 *m*	1.53 *dd* (12.0/5.0)	2.06 *m*	1.89 *m*
6α	1.89 *m*	2.01 *m*	2.03 *m*	1.77 *t* (2.5)	1.82 *m*
6β	1.61 *m*	2.15 *m*	1.96 *m*	1.70 *m*	1.75 *m*
7[a]	4.60 *dd* (5.0/1.5)	5.70 *dd* (4.0/2.0)	5.69 *dd* (3.0/2.0)	4.20 *t* (2.5)	5.31 *t* (3.0)
9α		2.77 *m*	2.80 *dd* (6.0/2.5)	2.70 *td* (7.0/2.0)	2.60 *ddd* (7.0/6.5/2.0)
11α	2.33 *dt* (10.0/4.5)	2.03 *m*	1.60 *m*	1.48 *m*	1.50 *m*
11β	2.33 *dt* (10.0/4.5)	1.30 *m*	1.40 *m*	2.10 *m*	2.05 *m*
12α	1.93 *dd* (10.0/4.0)	2.06 m	2.00 *m*	1.44 *m*	1.40 *m*
12β	1.77 *dd* (10.0/4.0)	1.30 m	1.33 *m*	1.44 *m*	1.40 *m*
14α				5.59 *d* (2.0)	5.73 *d* (2.0)
14β		3.64 *s*	3.64 *s*		
15	5.78 *dd* (18.0/10.5)	5.88 *dd* (18.0/11.0)	5.91 *dd* (17.5/10.5)	5.77 *dd* (17.5/10.0)	5.71 *dd* (17.5/11.5)
16-*trans*	4.98 *d* (18.0)	5.12 *dd* (18.0/1.5)	5.09 *dd* (17.5/1.5)	4.92 dd (17.5/1.5)	4.88 *dd* (17.5/1.5)
16-*cis*	5.10 *d* (10.5)	5.16 *dd* (11.0/1.5)	5.13 *dd* (10.5/1.5)	4.96 *dd* (10.0/1.5)	4.92 *dd* (11.5/1.5)
17 β	1.19 *s*	0.89 s	0.89 *s*	1.04 *s*	1.04 *s*
18 α	0.96 *s*	0.95 s	0.91 *s*	0.96 *s*	0.90 *s*
19 β	0.88 *s*	1.14 s	0.96 *s*	1.08 *s*	1.08 *s*
20 β	1.05 *s*	1.15 s	0.87 *s*	1.13 *s*	1.16 *s*
OCOMe					2.00 *s*

[a] 7α position for compounds **1**, **4** and **5**.

Table 2
^{13}C NMR spectral data of compounds **1–5** ($CDCl_3$, 125 MHz)

Position	1	2	3	4	5
1	35.2	215.5	71.2	216.2	216.2
2	18.7	35.8	33.9	36.3	36.2
3	41.1	41.8	25.7	43.1	43.2
4	33.0	32.6	32.5	33.0	33.0
5	44.8	51.5	43.7	48.4	49.8
6	26.5	23.2	23.6	29.3	27.9
7	63.1	125.5	126.7	72.9	75.2
8	131.4	137.5	137.7	138.6	133.7
9	168.9	39.3	38.2	38.7	39.0
10	40.1	48.7	39.0	52.5	52.6
11	21.9	21.2	18.7	19.9	20.1
12	35.1	27.5	27.4	34.2	34.2
13	47.2	41.1	41.1	37.5	37.6
14	204.2	79.7	79.7	136.1	138.5
15	140.1	146.2	146.6	147.5	146.9
16	115.0	113.6	113.5	111.0	111.7
17	24.0	22.1	22.1	26.1	26.5
18	32.8	32.0	33.3	32.0	32.0
19	21.7	22.6	22.6	22.6	22.3
20	17.9	14.0	15.0	13.5	13.7
OCOMe					170.3
OCO*Me*					21.5

3. Experimental

3.1. General

Silica gel 60 (70–200 μm 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. Low-pressure LC (LPLC) was performed using a Lobar LiChroprep RP-18 column (40–63 μm; 310×25 mm; Merck). 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. NMR spectra were obtained on a Varian Unity Inova-500 spectrometer, at 500 MHz (proton) and 125 MHz (carbon); δ in ppm rel. to Me_4Si (internal standard), J in Hz. HR-ESIMS was recorded on a Brüker 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 No. 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 CC 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 F5 (950 mg) was applied to a silica gel 60 column (35–70 μm) eluted with $CHCl_3$–EtOAc (25:1) to give two fractions (F51, F52). Fraction F51 (250 mg) was separated on a silica gel column (35–70 μm) eluted with toluene–EtOAc (24:1) to afford six fractions (F511 to F516). Gel filtration of F512 (60 mg) on Sephadex LH-20 with hexane–CH_2Cl_2 (2:1) as solvent gave compound **5** (7 mg). Fraction F6 (750 mg) was fractionated by gel filtration on Sephadex LH-20 with $CHCl_3$–MeOH (1:1) as mobile phase to give three fractions (F61 to F63). Fraction F61 (100 mg) was separated on a silica gel column (35–70 μm) successively using CH_2Cl_2–EtOAc (9:1) and $CHCl_3$–EtOAc (25:1) as mobile phase to afford compound **1** (20 mg). Fraction F10 (250 mg) was fractionated by LPLC on a Lobar B LiChroprep RP-18 column eluted with MeOH–H_2O (8:2) to give 5 fractions (F101–F105). Compounds **2** (9 mg) and **4** (7mg) were isolated from fractions F103 (35 mg) and F104 (40 mg) respectively by silica gel CC (35–70 μm) eluted with $CHCl_3$–EtOAc (25:1). Fraction F12 (650 mg) and fraction F13 (500 mg) were applied to silica gel columns (35–70 μm) successively eluted using toluene–EtOAc (5:1) and $CHCl_3$–EtOAc (25:1) as solvents, and then purified by filtration on Sephadex LH-20 with hexane–CH_2Cl_2 (2:1) as mobile phase, yielding compound **3** (7 mg).

3.4. 7β-Hydroxyisopimara-8,15-dien-14-one (*1*)

Amorphous powder. $[\alpha]_D$: +55.5 ($CHCl_3$, *c* 0.8); UV λ_{max} nm (log ε): 243 (2.92); ^1H and ^{13}C NMR are given in Tables 1 and 2, respectively. EI-MS 70 eV m/z (rel.int.): 302 $[M]^+$ (63), 284 (22), 274 (43), 269 (15), 241 (6), 234 (42), 219 (23), 206 (100), 191 (44), 178 (26), 165 (30), 149 (23), 145 (18), 121 (21), 119 (27), 105 (40). D/CI-MS m/z (rel. int.): 320 $[M+H_2O]^+$ (16), 302 (100), 285 (28). HR-ESIMS m/z: 325.2140 $[M+Na]^+$ (calc. for $C_{20}H_{30}O_2$ + Na 325.2138).

3.5. 14α-Hydroxyisopimara-7,15-dien-1-one (*2*)

Amorphous powder. $[\alpha]_D$: +18.0 ($CHCl_3$, *c* 0.9); UV λ_{max} nm (log ε): 201 (2.93); ^1H and ^{13}C NMR are given in Tables 1 and 2, respectively. EI-MS 70 eV m/z (rel. int.): 284 $[M-H_2O]^+$ (18), 269.21 (100), 241 (24), 227 (15), 185 (18), 139 (24), 107 (12), 105 (12). D/CI-MS m/z (rel. int.): 320 $[M+H_2O]^+$ (45), 302 (50), 285 (100). HR-ESIMS m/z: 325.2140 $[M+Na]^+$ (calc. for $C_{20}H_{30}O_2$ + Na 325.2138).

3.6. 1β,14α-Dihydroxyisopimara-7,15-diene (**3**)

Amorphous powder. $[\alpha]_D$: −16.0 ($CHCl_3$, *c* 0.6); UV λ_{max} nm (log ε): 201 (2.77), 244 *sh* (2.47); ^1H and ^{13}C NMR are given in Tables 1 and 2, respectively. EI-MS 70 eV *m*/*z* (rel. int.): 286 $[M-H_2O]^+$ (23), 271 (100), 245 (15), 253 (22), 241 (16), 218 (21), 203 (23), 187 (18), 169 (20), 147 (30), 119 (24), 105 (38). D/CI-MS *m*/*z* (rel. int.): 322 $[M+H_2O]^+$ (30), 304 (90), 287 (100), 269(29). HR-ESIMS *m*/*z*: 327.2298 $[M+Na]^+$ (calc. for $C_{20}H_{32}O_2$ + Na 327.2295).

3.7. 7β-Hydroxyisopimara-8(14),15-dien-1-one (**4**)

Amorphous powder. $[\alpha]_D$: −3.1 ($CHCl_3$, *c* 0.7); UV λ_{max} nm (log ε): 203 (4.06); ^1H and ^{13}C NMR are given in Tables 1 and 2, respectively. EI-MS 70 eV *m*/*z* (rel. int.): 302 $[M]^+$ (93), 287 (34), 269 (65), 251 (21), 241 (100), 223 (21), 213 (18), 195 (21), 171 (40), 152 (38), 139 (42), 131 (51), 113 (100). D/CI-MS *m*/*z* (rel. int.): 320 $[M+H_2O]^+$ (30), 302 (70), 285 (100). HR-ESIMS *m*/*z*: 325.2140 $[M+Na]^+$ (calc. for $C_{20}H_{30}O_2$ + Na 325.2138).

3.8. 7β-Acetoxyisopimara-8(14),15-dien-1-one (**5**)

Amorphous powder. $[\alpha]_D$: +5.0 ($CHCl_3$, *c* 0.5); UV λ_{max} nm (log ε): 203 (4.11); ^1H and ^{13}C NMR are given in Tables 1 and 2, respectively.

3.9. Biological assays

TLC bioautographic assay followed standard protocols (Rahalison et al., 1991 and 1994 for the antifungal test and Marston et al., 2002 for the bioautographic enzyme assay).

3.9.1. 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. These solutions (10 µl) were applied to the TLC plate.

3.9.2. Chromatogram preparation

Al-backed silica gel 60 F_{254} TLC sheets (Merck) were used for TLC bioautographic assay with *Cladosporium cucumerinum* and enzyme. Glass-backed silica gel 60 F_{254} TLC plates (Merck) were used 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.

3.9.3. Bioautographic assay with Cladosporium cucumerinum

A spore suspension of *Cladosporium cucumerinum* (ATCC No. 16402) 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.

3.9.4. Bioautographic assay with Candida albicans

Candida albicans was obtained from clinical isolates of the Service de Dermatologie, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland. 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.

3.9.5. Bioautographic enzyme assay

A solution of acetylcholinesterase in 0.05 M Tris–HCl (pH 7.8) was sprayed over the developed TLC plate. After 20 min incubation at 37 °C in a moist atmosphere, the bioautograms were then sprayed with a 1:4 mixture of solutions of naphthylacetate (250 mg) in ethanol (100 ml) and of Fast Blue B salt (400 mg) in distilled water (160 ml). After 1–2 min, active compounds appeared as clear spots against a purple background.

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