PERGAMON

Phytochemistry 52 (1999) 265–269

PHYTOCHEMISTRY

Two 6-substituted 5,6-dihydro-α-pyrones from *Ravensara anisata*

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Received 8 February 1999; accepted 24 February 1999

Abstract

The leaves and bark dichloromethane extracts of *Ravensara anisata* showed antifungal activity against the yeast *Candida albicans* and the phytopathogenic fungus *Cladosporium cucumerinum* in bioautographic TLC assays. Activity-guided fractionation afforded two new α-pyrones: 6*R**-(4*R**-acetoxy-2*S**-hydroxy-8-phenyloctyl)-5,6-dihydro-2-H-pyran-2-one and 6*R**-(2*S**-acetoxy-4*R**-hydroxy-8-phenyloctyl)-5,6-dihydro-2-H-pyran-2-one. Their structures have been established by NMR spectroscopy, chemical methods and X-ray crystallographic analysis. The antifungal activity against *C. albicans* and *C. cucumerinum* was determined for both compounds.

Keywords: *Ravensara anisata*; Lauraceae; Antifungal activity; *Cladosporium cucumerinum*; *Candida albicans*; α-Pyrones; X-ray analysis

1. Introduction

Ravensara is considered as an endemic genus in Madagascar. Kostermans (1950) identified 27 *Ravensara* species in this genus including *R. anisata* Danguy. He also described *R. anisata* and *R. aromatica* Sonnerat as synonyms. However, studies on their essential oil composition discriminate between the two species: while methylchavicol is the major component of the bark essential oil of *R. anisata*, 1,8-cineol, sabinene and α-terpineol constitute more than 50% of the essential oil of *R. aromatica* (O'Tucker & Maciarello, 1995).

Both species are used in traditional medicine. The essential oil has shown spasmolytic and neurosedative properties (Kostermans, 1950) and antimicrobial activity (de Medeci, Pieretti, Salvatore, Nicoletti & Rasoanaivo, 1992; Raharivelomanana, 1989); spices are made from their fruits and leaves, and the bark is used to perfume local rum (Kostermans, 1950).

In a series of preliminary screenings, both leaves and bark CH_2Cl_2 extracts of *R. anisata* displayed interesting activities against the yeast *C. albicans* (Rahalison, Hamburger, Hostettmann, Monod, & Frenk, 1991) and the phytopathogenic fungus *C. cucumerinum* (Homans & Fuchs, 1970) in bioautographic TLC assays. Activity-guided isolation yielded two new α-pyrones (**1**, **2**). Their structure determination was achieved by 1D- and 2D-NMR spectroscopy including COSY, HSQC, HMBC and DEPT experiments, and chemical methods. The structure of compound **1** was also confirmed by X-ray analysis.

2. Results and discussion

Dried and powdered leaves and bark of *R. anisata* Danguy were separately and successively extracted at room temperature with CH_2Cl_2 and MeOH. The extracts together with the bark essential oil were sub-

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0031-9422/99/$ - see front matter
PII: S0031-9422(99)00158-2

Table 1
^{13}C-NMR shifts (ppm)[a] of compounds **1**, **2** and **2a**

C-atom	1	2	2a	C-atom	1	2	2a
C(2)	165.11	163.78	163.84	C(7′)	31.72	31.42	34.56
C(3)	121.92	121.42	121.37	C(8′)	36.38	35.85	35.67
C(4)	146.11	144.59	144.75	C(1″)	142.94	142.49	142.29
C(5)	29.21	29.15	29.15	C(2″)	129.06[b]	128.34[b]	128.33[b]
C(6)	76.47	75.13	74.89	C(3″)	128.96[b]	128.26[b]	128.26[b]
C(1′)	41.86	39.99	40.02	C(4″)	126.42	125.63	125.68
C(2′)	64.22	68.22	66.59	C(5″)	128.96[b]	128.26	128.26[b]
C(3′)	43.47	42.98	38.93	C(6″)	129.06[b]	128.34	128.33[b]
C(4′)	72.18	67.09	69.64	C=O	173.46	172.02	170.89
C(5′)	35.36	37.07	31.18	–	–	–	170.64
C(6′)	25.72	25.39	24.69	Me	21.02	21.02	21.02
							21.06

[a] In $CDCl_3$.
[b] Assignments may be reversed in each column.

mitted to bioautographic TLC assays against *C. cucumerinum* and *C. albicans*. The lipophilic extracts displayed antifungal activity in both test systems, while the MeOH extracts were inactive. Moreover, the active principles of the extracts were not volatile compounds present in the essential oil.

Compound **1** was obtained by fractionation of the CH_2Cl_2 bark extract on a silica gel column, and further purification by gel filtration on Sephadex LH-20. The EIMS spectrum of **1** exhibited a $[M+H]^+$ peak at m/z 361 in agreement with the molecular formula $C_{21}H_{28}O_5$. This was confirmed by the presence of a peak $[M+NH_4]^+$ at m/z 379 in the D/CIMS spectrum. Nineteen resolved signals were observed in the ^{13}C-NMR spectrum (Table 1) with evidence for the presence of an acetyl group (carbonyl group at

Table 2
^{1}H-NMR shifts (ppm, *m, J* in Hz)[a] of compounds **1**, **2** and **2a**

Position	1	2	2a
3	6.0 *dd* (1.95–9.76)	6.0 *dd* (1.95–9.76)	6.0 *dd* (1.95–9.76)
4	6.86 *m*	6.86 *m*	6.86 *m*
5	2.38 *m*	2.27–2.42 *m*	2.30–2.52 *m*
6	4.62 *m*	4.49 *m*	4.45 *m*
1′	1.70–1.95 *m*	1.84–2.21 *m*	1.87–2.13 *m*
2′	3.60 *m*	5.27 *m*	5.08 *m*
3′	1.54 *m*	1.54–1.72 *m*	1.78–1.87 *m*
4′	5.02 *m*	3.48 *m*	4.93 *m*
5′	1.50–1.58 *m*	1.46–1.50 *m*	1.54 *m*
6′	1.29 *m*	1.36 *m*	1.33 *m*
7′	1.58 *m*	1.62 *m*	1.63 *m*
8′	2.58 *t* (7.82)	2.61 *t* (7.82)	2.60 *t* (7.82)
2″,6″	7.26	7.26	7.27
4″	7.15[b]	7.15[b]	7.15[b]
3″,5″	7.17[b]	7.17[b]	7.17[b]
Me	2.1 *s*	2.1 *s*	2.01 *s*
			2.05 *s*

[a] In $CDCl_3$.
[b] Assignments may be reversed in each column.

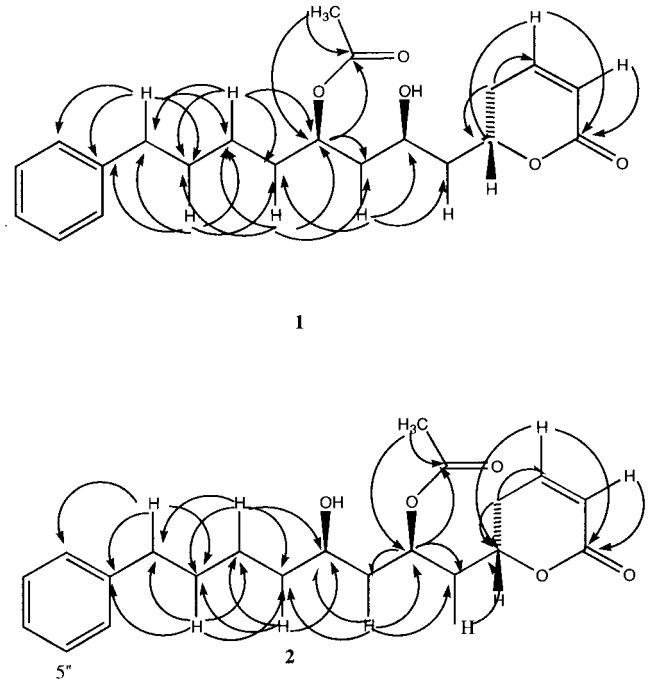

Fig. 1. HMBC correlations observed in compounds **1** and **2**.

$\delta = 174.46$ ppm with a methyl at $\delta = 21.09$ ppm). The presence of seven methylene groups, eight tertiary and three quaternary carbons was deduced from the DEPT spectrum. The presence of an α,β-unsaturated lactone ring was shown by the absorption band at $\nu = 1710$ cm^{-1} in the IR spectrum. Absorptions at $\nu = 3440$ cm^{-1} and $\nu = 2940$ cm^{-1} were attributed to a hydroxyl and a non-substituted phenyl group, respectively. The presence of the latter was confirmed by the typical spin system in the ^{1}H-NMR spectrum (multiplet at 7.15–7.26 ppm) (Table 2), and by the superposition of two pairs of aromatic carbons in the ^{13}C-NMR spectrum.

The positions of the acetyl and methylene groups were established by 2D-NMR experiments including HSQC, COSY and HMBC experiments (Fig. 1.). The relative configurations at C(6), C(2′) and C(4′) were determined by X-ray analysis after crystallisation of **1** from *n*-hexane-EtOAc (Fig. 2). From these data, the structure 6*R**-(4*R**-acetoxy,2*S**-hydroxy,8-phenyloctyl)-5,6-dihydro-2H-pyran-2-one was assigned to compound **1.**

Compound **2** (3 mg) was also obtained from the same fraction but with a residual amount of compound **1,** so that it was difficult to elucidate the structure of **2** from the NMR spectrum of the mixture.

Comparison of the HPLC-UV chromatograms (254 nm) of the CH_2Cl_2 leaves and bark extracts indicated the presence of **1** and **2** in both extracts. Thus, compound **2** was reisolated from the leaf extract.

Similar UV, IR and MS spectra were recorded for

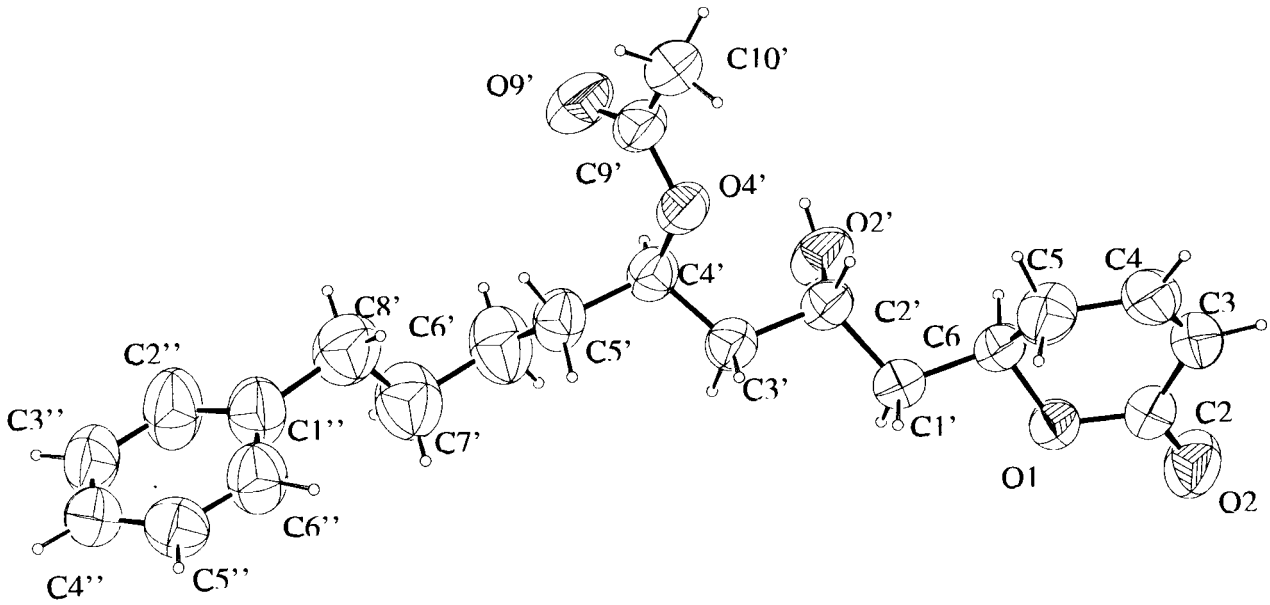

Fig. 2. PLATON (Spek, 1990) drawing of compound **1** showing the molecular structure and crystallographic numbering scheme (thermal ellipsoids at 50% probability level).

compound **2**. Distinctions between **1** and **2** were observed in the NMR spectra: in compound **1**, acetylation of the C(4′) hydroxyl group had a deshielding effect, inducing a lower field chemical shift of the H(4′) (5.02 ppm), while H(2′) gave a signal at 3.60 ppm. Inversely, chemical shifts of 5.27 ppm and 3.48 ppm were recorded for H(2′) and H(4′), respectively, in compound **2** (Table 2). These values were indicative of an acetylated 2′-OH group and a free 4′-OH group. The HMBC experiments showed a clear correlation between the acetyl protons and C(2′) in **2**, while these protons correlated with C(4′) in compound **1** (Fig. 1).

Compounds **1** and **2** and a mixture of **1** and **2** were acetylated and yielded the same product 6-(2,4-diacetoxy,8-phenyloctyl)-5,6-dihydro-2-H-pyran-2-one.

The data obtained, together with an identical optical rotation ($[\alpha]_D = +35°$) for both **1** and **2**, allowed the identification of the α-pyrone **2** as 6*R**-(2*S**-acetoxy,4*R**-hydroxy,8-phenylloctyl)-5,6-dihydro-2H-pyran-2-one.

In addition to **1** and **2**, methylchavicol (**3**), sitosterol (**4**) and 3-(4-methoxyphenyl)-2-propen-1-ol (**5**) have been isolated from the bark of *R. anisata*. They were identified by comparison with literature data (Groebel, Lenoir, & Pernet, 1969; Iribarren & Pomilio, 1984; Naidoo, Drewes, van Staden, & Hutchings, 1992).

The minimum amount of compounds **1** and **2** required to inhibit *C. cucumerinum* fungal growth on TLC plates was 2 μg. This amount was comparable to the minimum quantities in the same assays of miconazole (1 μg) and propiconazole (0.1 μg), two commercially-available reference antifungal compounds. These properties were confirmed in a dilution assay by measurement of their minimum inhibitory concentrations (*MIC*). The *MIC* values of compounds **1** and **2** were 100 μg/ml. The values obtained with the reference standards were 1 μg/ml (propiconazole) and 10 μg/ml (miconazole).

The minimum amount required to inhibit the growth of the yeast *C. albicans* on TLC plates was 10 μg for compound **1** and **2**, while in the dilution assay their *MIC* value was >100 μg/ml (miconazole 0.1 μg/ml).

Over the past 10 years, an increasing number of α-pyrones have been isolated from various plants and notably from the genus *Cryptocarya* (Lauraceae). The compound (2′-acetoxy-6-hept-4-enyl)-5,6-dihydro-2-H-pyran-2-one and cryptofolione were isolated from the bark of *C. latifolia* (Drewes, Horn, & Wijewardene, 1994; Sehlapelo, Drewes, & Scott-Shaw, 1993) and kurzilactone has been isolated from leaves of *C. kurzii* (Fu, Sévenet, Hadi, Remy, & Païs, 1993). Most of these α-pyrone derivatives showed striking features: presence of one or more acylated hydroxyl groups on the aliphatic chain, and cytotoxicity to human tumour cells (Fu et al., 1993; Fang, Anderson, Chang, Fanwick, & McLaughlin, 1990).

Activity-guided fractionation of the dichloromethane extract of *R. anisata* afforded two new α-pyrones with one acetate group on the side chain. Although the *MIC* value of these compounds (100 μg/ml) against *C. cucumerinum*, and *C. albicans* was high compared to standards, it is the first time that an antifungual ac-

tivity is reported for this chemical class of substances. From a chemotaxonomic point of view, these results confirm the similarity of the genera *Cryptocarya* and *Ravensara* which are distinguished only by their fruits (Van der Werf, 1992) On the other hand, this type of compound was described for the first time in the genus *Ravensara* which includes 27 species endemic to Madagascar. Except for some studies on the essential oil of *R. anisata* and *R. aromatica* (O'Tucker & Maciarello, 1995; Raharivelomanana, 1989), and the isolation of methylchavicol and *N*-methylisochorydine from *R. aromatica* (Groebel et al., 1969), the genus *Ravensara* has been poorly investigated.

3. Experimental

3.1. General

M.p: Mettler-FP-80/82 Hot stage-apparatus, uncorrected. α_D: Perkin-Elmer 241 polarimeter $[\alpha]_D$ (solvent used, c in g sample in 100 ml solvent). UV: Perkin-Elmer Lambda 20 spectrophotometer, in MeOH; λ_{max} (nm) (log ϵ). TLC: silica gel 60 F 254 Al sheets (Merck) detection at 254 nm and with Godin reagent (Godin, 1954). Solvent system for TLC: *n*-hexane–EtOAc 1:1. Column chromatography: Sephadex LH-20 (Pharmacia), Silica gel 60 (40–63 μm and 70–200 μm) (Merck). Anal. HPLC: Hewlett-Packard 1090 equipped with a photodiode array detector (DAD). Extracts and fractions were analyzed on a Symmetry C18 column (4.6 × 250 mm i.d.; Waters) with a gradient of MeOH 60–70% in 10 min, 70–75% in 20 min and 75–100% in 20 min at a flow rate of 1 ml min^{-1}. IR spectra: Perkin-Elmer 1600 FTIR spectrophotometer, ν in cm^{-1}. ^1H and ^{13}C-NMR: Varian-Inova-Spectrometer 500 (500 MHz and 125 MHz, respectively); in $CDCl_3$; chemical shifts in ppm as δ rel. to Me_4Si as internal standard, *J* in Hz. Complete attribution was performed on the basis of 2D experiments (COSY, HMBC, HSQC and selective INEPT). M.S: Finnigan-MAT/TSQ-700 triple stage quadripole instrument; *m/z* (rel. intensity in %); EIMS: ionization energy 70 eV; D/CIMS: NH_3, positive ion mode.

3.2. Plant material

Bark and leaves of *R. anisata* Danguy were collected in the forest of Mandraka (Madagascar) in September 1996. A voucher specimen has been deposited at the Institute of Pharmacognosy and Phytochemistry (University of Lausanne) under the number 96201.

3.3. Extraction

Dry and powdered leaves 349 g were successively extracted at room temperature with CH_2Cl_2 (3 × 2500 ml) and MeOH (3 × 2500 ml) to afford 32.8 and 42.7 g of extract. Dried and powdered bark 459 g were successively extracted with CH_2Cl_2 (3 × 2500 ml) and MeOH (3 × 2500 ml) to afford 15.8 and 27.6 g of extract.

3.4. Isolation

Bark CH_2Cl_2 extract 10 g were fractionated on silica gel (70–200 μm) with step gradient (petroleum ether–EtOAc 1:0 to 0:1, then MeOH) into 21 fractions (I-XXI). Gel filtration on Sephadex LH-20 ($CHCl_3$–MeOH 1:1) of fraction XIX and further purification on silica gel (40–60 μm) (*n*-hexane–EtOAc 1:1) yielded 42 mg of compound **1**. Compound **3** was obtained from fraction II after gel filtration and silica gel column chromatography (*n*-hexane–AcOEt 93:7). Gel filtration on Sephadex ($CHCl_3$–MeOH 1:1) of fraction VI, then reprecipitation in $CHCl_3$–MeOH gave compound **4** (50 mg). Fractions X–XVI were combined. After filtration on Sephadex ($CHCl_3$–MeOH 1:1) and silica gel column (*n*-hexane–EtOAc 2:1), compound **5** (10 mg) was obtained.

Leaf CH_2Cl_2 extract 22 g were fractionated in the same way as the bark extract to give 18 fractions I–XVIII. Purification of fraction XVI gave first a mixture of compounds **1** and **2** (3.1 g). Separation of the mixture on silica gel open column chromatography (cyclohexane–EtOAc 1:1) monitored by HPLC, afforded compound **1** (300 mg), a mixture of compounds **1** and **2** (2.5 g) and compound **2** (110 mg).

3.5. Acetylation

Compound **2** (10 mg) was dissolved in Ac_2O–pyridine (2 ml) and stirred at room temperature (24 h). The reaction mixture was diluted with ice water, partioned with EtOAc, and the organic phase yielded 10 mg of compound **2a** (yellow oil). See Tables 1 and 2 for ^{13}C NMR and ^1H NMR. The purity of **2a** was checked by HPLC.

Acetylation of compound **1** (10 mg) and of the mixture of **1** and **2** (10 mg) in the same way, afforded the same compound **2a**.

3.6. 6R*-(4R*-acetoxy,2 S*-hydroxy,8-phenyloctyl)-5,6-dihydro-2H-pyran-2-one (1)

White crystals. Mp 68–70°C. $[\alpha]_D = +35°$ (MeOH, *c* 0.05). UV λ_{max} MeOH nm (log ϵ): 210 (3.8). IR ν cm^{-1}: 3440 (-OH), 2940 (mono-substituted phenyl group), 1710 (α,β-unsaturated δ-lactone), 1390, 1250, 1085. D/CIMS (NH_3) *m/z*: 379 $[M+H+NH_4]^+$, 361 $[M+H]^+$: EIMS 70 eV *m/z* (rel. int): 361 (11), 343, 300 (40), 282 (20), 197 (44), 171 (100), 170 (95),

157 (61). ^{13}C NMR — see Table 1. ^{1}H NMR — see Table 2.

*3.6.1. Crystallographic data for compound **1***

Orthorhombic, space group P $2_12_12_1$, a = 7.0800(4), b = 11.2037(7), c = 25.446(2) Å, Z = 4, D_c = 1.186 g cm^{-3}, 13294 reflections collected, 3901 independent reflections (R_{int} = 0.0319), 2962 were considered observed [$I > 2$ s(I)], final R = 0.0538 (obsd. data), $wR2$ = 0.1588 (all data), goodness of fit 1.029, residual density max/min 0.391/-0.159 e Å^{-3}. Absorption coefficient μ = 0.084 mm^{-1}; no correction for absorption was applied.

Suitable crystals of **1** were grown from EtOAc–*n*–hexane as colourless blocks. Intensity data were collected at 273 K on a Stoe Image Plate Diffraction system using MoKα graphite monochromated radiation. Image plate distance 70 mm, ϕ rotation 0–200°, step $\Delta\phi$ = 1°, 2θ range 3.27–52.1°, $d_{max}-d_{min}$ = 12.45−0.81 Å. The structure was solved by direct methods using the programme SHELXS-97 (Sheldrick, 1990). The refinement and all further calculations were carried out using SHELXL-97 (Sheldrick, 1997). The H-atoms were included in calculated positions and treated as riding atoms using SHELXL-97 default parameters. The non-H atoms were refined anisotropically, using weighted full-matrix least-squares on F^2. No attempt was made to determine the absolute configuration of the molecule. The molecular structure and crystallographic numbering scheme are illustrated in Fig. 2 (Spek, 1990). The bond lengths and angles are normal within experimental error. The crystallographic CIF file has been deposited with the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK, deposition number: 113398.

3.7. 6R-(2 S*-acetoxy, 4R*-hydroxy,8-phenyloctyl)-5,6-dihydro-2H-pyran-2-one (**2**)*

Yellow oil. $[\alpha]_D$ = +35° (MeOH, c 0.05). UV λ_{max} MeOH nm (log ϵ): 210 (3.8). IR ν cm^{-1}: 3440 (-OH), 2940 (mono-substituted phenyl group), 1710 (α,β-unsaturated δ-lactone), 1390, 1250, 1085. D/CIMS (NH_3) m/z: 379 $[M+NH_4]^+$, 361 $[M+H]^+$; EIMS 70 eV m/z (rel. int): 360, 343 (7) 300 (40), 282 (29), 197 (37), 171 (53), 170 (100), 157 (21). ^{13}C NMR — see Table 1. ^{1}H NMR — see Table 2.

Acknowledgements

We thank the Swiss Confederation for a scholarship to the first author (J.O.A.), and the Swiss National Science Foundation for supporting this work.

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