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New Synthetic Derivatives from Peroxisomicine A1 and their Biological Activity

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Abstract

Peroxisomicine A1 (PA1) is a dimeric anthracenone obtained from plants of the genus *Karwinskia*. Toxicological studies have demonstrated a selective toxicity of peroxisomicine A1 on various human tumor cell lines *in vitro*. For this reason it has been considered as a possible antineoplastic agent. This article describes the chemical manipulation of this compound with the purpose of finding a possible relationship between the structure and biological activity. Derivatization procedures on the phenolic OH groups, carbonyl group and aromatic ring are presented. Six new compounds were obtained and purified by several chromatographic methods and the pure products were identified and characterized by UV, IR, MS, and ^{1}H and ^{13}C NMR. To evaluate the biological activity of the derivatives, the selective cytoxicity of the compounds on normal and neoplastic human liver cells lines and their effect on catalase activity were analyzed. Cytotoxicity of compounds **2**–**5** suggests that phenolic groups are necessary for the biological action reported for PA1. On the other hand, compound **6**, substituted on the aromatic ring, did not show a significant difference in the selective cytotoxicity assay when compared to peroxisomicine A1.

Keywords: *Karwinskia*, peroxisomicine, hydroxyanthracenone, *parvifolia*, cytotoxicity, catalase, derivatives, diazald.

Introduction

Peroxisomicine A1 (previously named T514) is a toxic substance isolated from plants of the genus *Karwinskia* (Dreyer et al., 1975; Waksman & Ramírez, 1992). Selective toxicity of this substance in several human tumor cell lines has been described, and the potential use of this compound as an antineoplastic agent has been suggested (Piñeyro et al., 1994). The toxicological mechanism of this substance is not completely known. It produces, at sublethal doses, an irreversible and selective damage of yeast peroxisomes *in vivo* (Sepúlveda et al., 1992) and an inhibitory effect *in vitro* on liver catalase activity was also reported (Moreno et al., 1995). Although there is some evidence of the mutagenicity of similar compounds, peroxisomicine A1 did not display a mutagenic activity (Velazco et al., 1996); the structural differences seem to be crucial for the activity of each molecule. Peroxisomicine A1 has been shown to stop proliferating active cells by toxicity, apparently due to induced apoptosis, which reinforces its possible usefulness in therapeutic treatments (Martínez et al., 1999).

Considering the reported effects of peroxisomicine A1 (PA1), the purpose of the present work was to obtain functional derivatives and evaluate their selective cytotoxicity on normal and neoplastic liver cells as well as their effect on liver catalase.

Materials and methods

General experimental procedures

^{1}H and ^{13}C NMR spectra were obtained on a Bruker DPX-400, at 400.13 and 100.62 MHz, respectively, using $CDCl_3$ as solvent. NOE difference experiments were carried out with 100%-deuterated $CDCl_3$ in argon deareated samples. In order to assign all the signals observed, HMBC experiments were optimized for $J = 7$ Hz and 9 Hz. Mass spectra were obtained

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on a HP 5985 mass spectrometer. UV-Vis spectra were recorded on a Beckman DU 7100 and the IR spectra a FT-IR Bruker Vector 22. Mps uncorrected were obtained on an Electrothermal 9100. All evaporations were performed at temperature below 40 °C. Lyophilizations were made in a Virtis Freezemobile 12. Precoated Kieselgel 60 F254 and RP18 F254 (Merck, 0.2 mm thick) were used for TLC, spots being detected in the visible and UV at 366 nm, as well as by spraying with 5% KOH in ethanol. Low-pressure chromatography was accomplished in Lobar Lichroprep Si 60 (40–63 µm) and Lichroprep RP-18 (40–63 µm) from Merck. Analytical HPLC was carried out on a HP 1090 (DAD detector) with a C18 column 100 × 2.1 mm, 5 µm. Elution was accomplished with a mixture of AcCN, MeOH, H_2O, and HOAc according to methodology previously described (Salazar et al., 1996). Preparative HPLC was accomplished on a Waters prep LC 2000, columns 8 × 100 mm and 40 × 100 mm, both 8MBC Radial-Pak C-18, 10 µm. Semi-empirical calculations were done using AM1 method (Macspartan Plus program).

Isolation of peroxisomicine A1

PA1 was obtained from the fruits of *K. parvifolia* as previously described (Waksman & Ramírez 1992); purity and identity were assessed by physico-chemical, chromatographic and spectroscopic methods.

Cytotoxicity assay

The assay procedure was carried out as previously described (Piñeyro et al., 1994), using Chang liver cells and hepatoma cells (Hep-G2) which were obtained from American Type Culture Collection (ATCC, Rockville, MD). Each substance was dissolved in ethanol (1 mg/mL) and further dilutions were done with culture medium. Twelve different concentrations were tested from 0.078 to 160 µg/mL. Aliquots of 5×10^3 cells in 0.1 mL of medium were seeded into each well of 96-well microtitre plates. Culture plates were incubated at 37 °C for 72 h. Cell viability was determined after a 72 h exposure to the substances, using an invertoscope (IM 35 Carl Zeiss). Failure of the cells to attach and spread on the substratum was used as a criterion of cytotoxicity. Trypan blue dye-exclusion test was used to corroborate cytotoxicity (Tolnai, 1975). Cells were incubated for 4 min with 0.167% Trypan blue. Wells where all cells were found detached and non-viable were labeled as total cytotoxic concentration wells (CTmax), while those forming a monolayer, as in controls, were considered to be non-cytotoxic concentration wells (CTmin). Cytotoxic concentration 50% (CT50%) for normal and malignant cells was calculated following the method of Ekwall et al. (1980) as the geometrical mean of total cytotoxic concentration and highest non-toxic concentration tested. Comparison between CT_{50} for normal and malignant cells were made using the Student's *t*-test.

Catalase assay

Catalase test was undertaken as previously reported (Moreno et al., 1995) dissolving the compounds to be tested in EtOH. In order to analyze the effect of the different compounds on catalase activity, the enzyme assay was performed using a fixed concentration of enzyme and substrate and several concentrations of the compounds within a range of 3.9 to 54 µM. In all cases 100% activity was determined without the inhibitor. Absorbance changes at 240 nm not caused by catalase activity were discarded by running spectra for 10 min with samples containing substrate and inhibitor (without enzyme). The extent of inhibition by the addition of samples is expressed as a percentage necessary for 10% inhibition (IC_{10}).

Preparation and purification of PA1 methyl ethers (**2**, **3**, **4**, **5**).

PA1 (250 mg) in ethanol (10 ml) was exposed to diazomethane at room temperature for 45 min. Diazomethane was generated *in situ* by means of the diazald reagent using the Diazald® Kit. The reaction mixture was extracted with EtOAc and precipitated with hexane. The resulting pale yellow residue (265 mg) was purified by means of CC (silica gel 60, EtOAc) to give 10 fractions, which were combined according to TLC monitoring (developed system was also EtOAc) to give 3 fractions. Each fraction was purified by means of low-pressure chromatography (silica gel 60, EtOAc) to obtain four pure compounds: **2** (40 mg), **3** (20 mg), **4** (15 mg), and **5** (15 mg).

Compound 2

UV (MeOH) λ_{max}: 225, 264, 389 nm; FTIR (KBr) ν_{max}: 3426, 2924, 1682, 1626, 1602, 1526, 1382, 1253, 1105, 1069, 706 cm^{-1}; EIMS (70eV) *m/z* (Rel int*)* [M^+] 542 (48), 524 (100), 506 (10), 491 (20); ^{1}H NMR (400.13 MHz): δ 1.32 (s, 3H, CH_3-3′), 1.52 (s, 3H, CH_3-3), 2.83 (d, 1H, *J* = 16.7, H-4′ax), 2.84 (m, 2H, H-2′), 2.89 (m, 2H, H-2), 3.00 (d, 1H, *J*-16.72 Hz, H-4′eq), 3.26 (d, 1H, *J* = 16.1 Hz, H-4eq), 3.29 (d, 1H, *J* = 16.1 Hz, H-4ax), 4.06 (s, 3H, OCH_3-9), 4.12 (s, 3H, OCH_3-9′), 6.85 (d, 1H, *J* = 8.39 Hz, H-5′), 6.89 (d, 1H, *J* = 7.67, H-7′), 7.30 (d, 1H, *J* = 8.1 Hz, H-6), 7.33 (t, 1H, H-6′), 7.39 (d, 1H, *J* = 8.5 Hz, H-5), 7.55 (s, 1H, H-10), 10.17 (s, 1H, OH-8′), 10.25 (s, 1H, OH-8); ^{13}C NMR (100.62 MHz): δ 29.37 (CH_3-3), 29.47 (CH_3-3′), 41.88 (C-4′), 44.31 (C-4), 54.13 (C-2′), 54.58 (C-2), 64.41 (OCH_3-9′), 64.53 (OCH_3-9), 70.82 (C-3′), 71.16 (C-3), 106.10 (C-9a′), 107.53 (C-9a), 111.09 (C-7′), 116.51 (C-8a′), 116.61 (C-8a), 117.06 (C-5′), 118.66 (C-5), 120.35 (C-7), 124.52 (C-10), 130.75 (C-6), 131.33 (C-10′), 133.44 (C-6′), 135.77 (C-4a′), 137.57 (C-4a), 137.96 (C-10a′), 138.33 (C-10a), 153.21 (C-8), 156.67 (C-8′), 159.20 (C-9′), 159.84 (C-9), 195.57 (C-1), 196.24 (C-1′).

*Compound **3***

UV (MeOH) λ_{max}: 225, 266, 393 nm; FTIR (KBr) ν_{max}: 3426, 2924, 1682, 1626, 1602, 1526, 1382, 1253, 1105, 1069, 706 cm^{-1}; EIMS (70eV) *m/z* [M^+] 542 (48), 524 (100), 506 (10), 491 (20); ^{1}H NMR (400.13 MHz): δ 1.30 (s, 3H, CH_3-3′), 1.51 (s, 3H, CH_3-3), 2.68 (d, 1H, *J* = 16.1 Hz, H-4′ax), 2.79 (m, 2H, H-2′), 2.88 (d, 2H, *J* = 16.1 Hz, H-4′eq), 3.25 (d, 1H, *J* = 16.1 Hz, H-4ax), 3.31 (d, 1H, *J* = 16.1 Hz, H-4eq), 4.05 (s, 6H, OCH_3-8′ and OCH_3-9), 6.85 (d, 1H, *J* = 7.9, H-7′), 6.89 (d, 1H, *J* = 8.3 Hz, H-5′), 7.32 (d, 1H, *J* = 8.1 Hz, H-6), 7.37 (d, 1H, *J* = 8.5 Hz, H-5), 7.37 (t, 1H, *J* = 7.9 Hz, H-6′), 7.52 (s, 1H, H-10), 10.21 (s, 1H, OH-8), 15.38 (s, 1H, OH-9′); ^{13}C NMR (100.62 MHz) δ 28.95 (CH_3-3′), 29.34 (CH_3-3), 41.48 (C-4′), 44.28 (C-4), 51.70 (C-2′), 54.51 (C-2), 56.38 (OCH_3-8′), 64.48 (OCH_3-9), 70.62 (C-3′), 71.10 (C-3), 106.05 (C-7′), 110.47 (C-9a′), 115.41 (C-8a′), 116.59 (C-8a), 118.37 (C-5′), 118.50 (C-6′), 119.39 (C-9a), 120.54 (C-7), 124.39 (C-10′), 124.48 (C-10), 131.13 (C-5), 134.16 (C-6), 134.38 (C-4a′), 137.38 (C-4a), 138.22 (C-10a), 139.81 (C-10a′), 153.51 (C-8), 159.79 (C-9), 160.33 (C-8′), 165.76 (C-9′), 195.64 (C-1), 203.30 (C-1′).

*Compound **4***

UV (MeOH) λ_{max}: 224, 256, 378 nm; FTIR (KBr) u $_{max}$: 3426, 2924, 1682, 1626, 1602, 1526, 1382, 1253, 1105, 1069, 706 cm^{-1}; EIMS (70eV) *m/z* [M^+] 556 (20), 538 (100), 520 (60), 458 (40); ^{1}H NMR (400.13 MHz) δ 1.29 (s, 3H, CH_3-3′), 1.49 (s, 3H, CH_3-3), 2.74 (d, 1H, *J* = 16.05Hz, H-4′ax), 2.84 (m, 2H, H-2′), 2.90 (s, 2H, H-2), 3.01 (d, 1H, *J* = 16.72 Hz, H-4′eq), 3.28 (s, 2H, H-4), 3.40 (s, 3H, OCH_3-8), 3.96 (s, 3H, OCH_3-9), 4.12 (s, 3H, OCH_3-9′), 6.83 (d, 1H, *J* = 8.39 Hz, H-5′), 6.89 (d, 1H, *J* = 7.67, H-7′), 7.30 (d, 1H, *J* = 7.81 Hz, H-6), 7.33 (t, 1H, *J* = 8.30 Hz, H-6′), 7.56 (s, 1H, H-10), 7.64 (d, 1H, *J* = 8.4 Hz, H-5), 10.18 (s, 1H, OH-8′); ^{13}C NMR (100.62 MHz) δ 29.31 (CH_3-3), 29.40 (CH_3-3′), 42.11 (C-4′), 44.34 (C-4), 54.01 (C-2′), 54.89 (C-2), 62.30 (OCH_3-8), 63.57 (OCH_3-9), 64.45 (OCH_3-9′), 70.65 (C-3′), 71.13 (C-3), 111.03 (C-7′), 116.41 (C-8a′), 117.33 (C-5′), 119.16 (C-9a), 120.54 (C-7), 122.80 (C-8a), 124.16 (C-5), 124.30 (C-10), 129.73 (C-10a), 130.64 (C-9a′), 131.70 (C-6′), 132.29 (C-6), 135.67 (C-4a′), 137.80 (C-4a), 138.20 (C-10a′), 139.36 (C-10′), 155.61 (C-8), 156.61 (C-8′), 159.24 (C-9′), 159.28 (C-9), 196.13 (C-1), 196.35 (C-1′).

*Compound **5***

UV (MeOH) λ_{max}: 224, 265, 378 nm; FTIR (KBr) ν_{max}: 3426, 2924, 1682, 1626, 1602, 1526, 1382, 1253, 1105, 1069, 706 cm^{-1}; EIMS (70eV) *m/z* [M^+] 556 (20), 538 (100), 520 (60), 458 (40); ^{1}H NMR (400.13 MHz) δ 1.29 (s, 3H, CH_3-3′), 1.52 (s, 3H, CH_3-3), 2.74 (d, 2H, *J* = 16.05 Hz, H-4′ax and H-2′), 2.85 (m, 1H, H-2′), 2.87 (m, 2H, H-2), 2.92 (d, 1H, *J* = 15.88 Hz, H-4′eq), 3.26 (d, 1H, *J* = 16.08 Hz, H-4), 3.32 (d, 1H, *J* = 16.08 Hz, H-4), 4.00 (s, 3H, OCH_3-8′), 4.02 (s, 3H, OCH_3-9′), 4.06 (s, 3H, OCH_3-9), 6.85 (d, 1H, *J* = 7.75 Hz, H-7′), 6.95 (d, 1H, *J* = 8.40 Hz, H-5′), 7.30 (d, 1H, *J* = 7.96 Hz, H-6′), 7.32 (d, 1H, *J* = 7.84 Hz, H-6), 7.39 (d, 1H, *J* = 8.28 Hz, H-5), 7.53 (s, 1H, H-10), 10.22 (s, 1H, OH-8); ^{13}C NMR (100.62 MHz) δ 29.28 (CH_3-3′), 29.33 (CH_3-3), 42.09 (C-4′), 44.34 (C-4), 54.55 (C-2′), 54.55 (C-2), 56.55 (OCH_3-9′), 63.30 (OCH_3-8′), 64.53 (OCH_3-9), 70.83 (C-3′), 71.09 (C-3), 106.49 (C-7′), 116.64 (C-9a), 116.83 (C-9a′), 118.61 (C-5′), 118.61 (C-6′), 120.07 (C-8a′), 120.90 (C-8a), 122.39 (C-7), 123.41 (C-10′), 124.51 (C-10), 129.14 (C-6), 133.77 (C-5), 136.17 (C-4a′), 137.46 (C-4a), 138.29 (C-10a), 138.58 (C-10a′), 153.22 (C-8), 158.72 (C-8′), 159.24 (C-9′), 159.63 (C-9), 195.55 (C-1), 196.39 (C-1′).

Syntheses of 7′chloro-PA1 (**6**)

PA1 (250 mg) was dissolved, with stirring, in 5 ml of dry DMF. A solution of *N*-chlorosuccinimide (143 mg in 10 ml DMF) was added and the reaction vessel was ice-cooled. The reaction was monitored by means of TLC (RP-18, MeOH/H_2O 65:35). The solvent was removed by lyophilization and after it was completed, the residue (230 mg) was redissolved in a mixture of $CHCl_3$: EtOAc (1 : 1) and was precipitated with hexane. The product (200 mg) was chromatographed on a Lobar RP-18 column using AcCN/H_2O/AcOH 50 : 50 : 1.6 as eluent. The combined fractions containing the chlorinated product were purified by means of preparative HPLC at a flow of 70 mL/min, λ 440 nm. The gradient system used consisted of: solvent A (AcCN) and solvent B (1.6% AcOH in H_2O) in the following manner: 0–6.8 min 50% B, and then a linear gradient was applied to reach 100% A in 11.8 min remaining at 100% A for 30 min; this procedure gave 20 mg of pure compound **6**.

*Compound **6***

UV (MeOH) λ_{max}: 268 and 406 nm; ^{1}H NMR (400.13 MHz) δ 1.36 (s, 3H, CH_3-3′), 1.53 (s, 3H, CH_3-3), 2.75 (d, 1H, *J* = 15.7 Hz, H-4′ax), 2.90 (s, 2H, H-2′), 2.92 (s, 2H, H-2), 2.94 (d, 1H, *J* = 16.6 Hz, H-4′eq), 3.15 (d, 1H, *J* = 16.4 Hz, H-4), 3.21 (d, 1H, *J* = 16.4 Hz, H-4), 6.75 (d, 1H, *J* = 8.9 Hz, H-5′), 7.15 (s, 1H, H-10), 7.31 (d, 1H, *J* = 8.4 Hz, H-5), 7.34 (d, 1H, *J* = 8.3 Hz, H-6), 7.40 (d, 1H, *J* = 8.9 Hz, H-6′), 9.95 (s, 1H, OH-8), 10.57 (s, 1H, OH-8′), 16.07 (s, 1H, OH-9), 16.44 (s, 1H, OH-9′); ^{13}C NMR (100.62 MHz) δ 29.70 (CH_3-3), 29.83 (CH_3-3′), 41.43 (C-4′), 43.67 (C-4), 51.33 (C-2′), 51.69 (C-2), 71.34 (C-3′), 71.58 (C-3), 109.15 (C-9a′), 110.24 (C-9a), 110.27 (C-8a′), 113.91 (C-8a), 116.12 (C-5′), 117.93 (C-7′), 119.07 (C-5), 119.07 (C-10), 119.74 (C-7), 125.98 (C-10′), 133.53 (C-6′), 134.24 (C-4a′), 135.73 (C-6), 138.12 (C-10a′), 139.87 (C-10a), 153.80 (C-8), 155.91 (C-8′), 163.68 (C-4a), 165.06 (C-9), 165.92 (C-9′), 203.64 (C-1), 204.42 (C-1′).

Reduction of PA1-9.9′-di-methyl ether (**7**)

Compound **2** (250 mg) dissolved in THF were placed into a 3-liter three-necked flask, fitted with a mechanical stirrer and a short reflux condenser. A solution of lithium borohydride (266.4 mg in 10 ml of THF) was added slowly with occasional cooling to keep the reaction at low temperature. The reaction was monitored by means of TLC (silica gel 60, EtOAc). When the reaction was completed, MeOH was added to the reaction vessel. The solvent was evaporated and redissolved with EtOAc, which was washed several times with H_2O. The two phases were separated and the aqueous phase was successively extracted with EtOAc. The organic phases were evaporated by distillation, redissolved in EtOAc and precipitated with hexane. The pale yellowish residue (180 mg) was purified by means of low-pressure chromatography (silica gel 60) using an elution gradient with EtAcO/Me_2CO 15 : 1 to 1 : 1 and a flow of 1.5 mL/min to obtain 50 mg of pure compound **7**.

Compound 7

UV (MeOH) λ_{max}: 342, 311 nm; FTIR (KBr) ν_{max}: 3404, 2932, 1624, 1574, 1388, 1261, 1105, 1073, 667 cm^{-1}; ^{1}H NMR (400.13 MHz): δ 1.30 (s, 3H, CH_3-3′), 1.50 (s, 3H, CH_3-3), 2.03 (m, 1H, H-2′ax), 2.05 (m, 1H, H-2ax), 2.38 (m, 1H, H-2′eq), 2.43 (m, 1H, H-2eq), 2.76 (s, 2H, H-4′), 3.05 (d, 1H, J = 17 Hz, H-4ax), 3.20 (d, 1H, J = 16.5 Hz, H-4eq), 4.06 (s, 3H, OCH_3-9), 4.18 (s, 3H, OCH_3-9′), 5.39 (s, 1H, H-1), 5.47 (s, 1H, H-1′), 6.87 (d, 1H, J = 7.70 Hz, H-7′), 6.90 (d, 1H, J = 8.90 Hz, H-5′), 7.19 (d, 1H, J = 8.2 Hz, H-6′), 7.22 (d, 1H, J = 8.4 Hz, H-6), 7.42 (d, 1H, J = 8.4 Hz, H-5), 7.52 (s, 1H, H-10), 9.37 (s, 1H, OH-8); 9.42 (s, 1H, OH-8′); ^{13}C NMR (100.62 MHz) δ 30.61 (CH_3-3), 30.82 (CH_3-3′), 40.86 (C-2′), 41.50 (C-2), 41.86 (C-4′), 43.90 (C-4), 63.59 (C-1), 63.87 (C-1′), 64.61 (OCH_3-9′), 64.76 (OCH_3-9), 69.94 (C-3′), 70.04 (C-3), 109.82 (C-7′), 116.19 (C-8a′), 116.29 (C-8a), 117.39 (C-5′), 118.97 (C-5), 119.62 (C-7), 125.19 (C-10), 126.68 (C-10′), 127.41 (C-9a), 127.56 (C-6′), 130.63 (C-6), 132.17 (C-9a′), 132.41 (C-4a′), 133.45 (C-4a), 135.73 (C-10a′), 136.04 (C-10a), 150.09 (C-8), 153.51 (C-8′), 154.24 (C-9′), 154.87 (C-9).

Results and discussion

Hydroxyanthracenones are labile compounds, sensitive to basic and strong acidic media, light and temperature. In all cases, the principal products of decomposition are hydroxyanthraquinones and hydroxyanthrones. This is the reason why all the reactions performed in this study had to be conducted under mild conditions; the presence of hydroxyanthraquinones and hydroxyanthrones was monitored by means of TLC with KOH spray reagent (Wagner et al., 1984). On account of the aforementioned lability of these compounds, few chemical manipulations of these types of substances have been reported in the literature.

In order to block the phenolic OH, an ethereal diazomethane reaction was selected, due to its mildness and selectivity (Gill et al., 1990; Black, 1983); it is well known that tertiary OH does not react under these conditions unless a Lewis acid is used. This reaction produced 265 mg of a yellowish precipitate from which 10 products could be detected by means of TLC. From this mixture, four compounds were isolated, purified and identified (**2**–**5**).

The newly reported ethers could be identified by their spectroscopic properties. UV-Vis spectra showed the characteristic bands reported for these chromophores. In the IR spectra, bands for symmetrical and asymmetrical stretching of the ether at 1253 and 1069 cm^{-1} among others could be observed. Mass spectra obtained from **2** and **3** showed a molecular ion of *m*/*e* 542, which accounts for the addition of two methyl groups to the molecule of peroxisomicine, whereas mass spectra of **4** and **5** showed a molecular ion of *m*/*e* 556, indicating trimethylethers of peroxisomicine. Base peak for compounds **2** and **3** (*m*/*e* 524) and for **4** and **5** (*m*/*e* 538), represents the loss of a molecule of water, characteristic for these compounds; the loss of two molecules of water and subsequent loss of a methyl group were also observed.

Structures for **2** and **3** were proposed on the basis of the foregoing spectroscopic evidence. In the ^{1}HNMR spectrum of **2**, the signals at δ 15.93 and 16.13, corresponding to the deshielded 9 and 9′ enolic protons of peroxisomicine were absent and two new 3H-singlets at 4.12 and 4.06 ppm were present. The ^{13}C NMR spectra showed two additional methoxy signals indicating unambiguously the incorporation of two methyl groups at 9 and 9′ positions of the original molecule. This result was further supported by the relative higher field of the C-1 and C-1′ carbonyl resonance at δ 196.24 and 195.57 ppm, compared to the signals obtained from PA1 at 205.4 and 205.6 ppm, respectively, On the other hand, the ^{1}H NMR spectrum of compound **3** showed two signals at 15.38 ppm and 10.21 ppm for phenolic OH. Furthermore, one signal at 4.05 ppm integrating for 6H was evidence of two methoxy groups in the molecule as suggested by EM results. From these data, it was concluded that this compound was either the 8′, 9 or the 8, 9′ dimethyl derivative. By means of HMQC, HMBC and NOE experiments, the structure for **3** was established as shown; the NOE effects (Fig. 1) were important for assigning some signals, for example those arising from H-5′ and H-7′: upon irradiation of the signal at 6.89 ppm, enhancement of the signals at 7.32 and 7.37 assigned to H-6 and H-6′, respectively, were observed. On the other hand, upon irradiation of the doublet at 6.85 ppm, only enhancement of the signal at 7.37 ppm could be seen, thus showing that the signals from H-5′ and H-7′ are those at 6.89 and 6.85 ppm, respectively. The site of methylation was further proved to be at OH-8′ and OH-9 after examination of the HMBC spectra: the signal at δ 10.21 (phenolic OH) showed correlation with the signals at 120.54 ppm and 153.51 ppm, assigned to C-7 and C-8, respectively, this means that the OH-8 was substituted. Moreover, the signal at 15.38 ppm (enolic OH) correlated with the signals

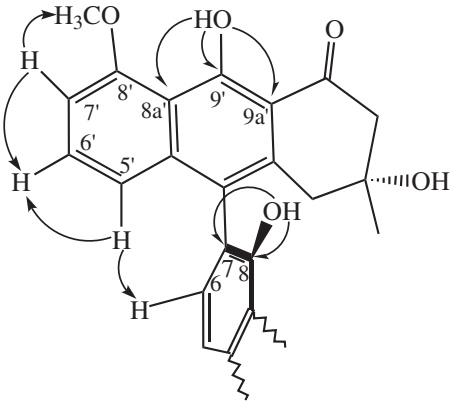


Figure 1. NOEs ↷ and HMBC ↷ for compound **3**.

at 110.47, 115.41 and 165.76 ppm (C-9a′, C-8a′ and C-9′, respectively), confirming that H-9′ is also free. The relative higher field of the C-1 also confirmed the methylation at 9-OH. In all the compounds examined a downfield shift of 0.2–0.4 ppm for the aromatic H *para* to a phenolic group was observed when the phenol was substituted with a methoxy group.

In the ^{1}H NMR spectra obtained from compounds **4** and **5**, only one phenolic signal at *ca*.10 ppm could be observed; three singlets for aromatic methoxy groups were observed in each case both in the ^{1}H and ^{13}C NMR spectra, in agreement with the molecular ion obtained. On account of the disappearance of the two enolic OH, both compounds should have been methylated at the 9 and 9′ position; this is further supported by the relative higher field of the C-1 and C-1′ carbonyl resonance for both compounds, compared with the signals obtained from PA1 as mentioned before. In the ^{1}H NMR spectrum of compound **4**, H-5 appears at lower field than the corresponding H-5 in PA1, suggesting that the 8 position is methoxylated. On the other hand, in the ^{1}H NMR spectrum of compound **5**, the 5′ position is deshielded relative to the H-5′ in peroxisomicine, suggesting a methyl substitution at 8′-OH. In order to confirm these results, assignation of all signals arising from H and C of the molecule were made by means of COSY, HMBC and HMQC experiments. Using semi-empirical calculations, geometry optimization of the two possible isomers, 8, 9, 9′ and 8′, 9, 9′ trimethoxy-peroxisomicine were performed and the internuclear distances thus obtained were used to correlate the data with NOE enhancement experiments. A series of NOE experiments confirmed the structure identity of **4** and **5** (Figs. 2 and 3).

In order to introduce a heavy atom into the molecule, bromination was the first reaction of choice; however, no results could be obtained, even upon milder testing conditions (Br_2/H_2O, Br_2/S_2C, low temperatures) instead brominated anthraquinones were obtained as the main reaction products. For this reason, chlorination was performed using N-chlorosuccinimide, reaction which is described in the literature for obtaining chlorinated hypericine (Cohen et al., 1995). The main product (**6**) was isolated and purified. The ^{1}H NMR spectrum showed the four deshielded phenolic functions and the aliphatic region almost unmodified; in the aromatic region the doublet assigned to H-7′ in peroxisomicine (6.81 ppm) was absent; besides, the signal at 7.37 ppm (assigned to H-6′ in peroxisomicine) was shifted to 7.40 ppm and appeared as a doublet. These experimental data indicated that the compound was substituted at 7′. These data agree with the calculation of HOMO mapped into electronic density for PA1.

Numerous attempts to effect reactions on the carbonyl group were unsuccessful, obtaining the unmodified compound or decomposition products. Gill et al. (1990) described the same behavior when trying to reduce atrochrysone, an hydroxyanthracenone isolated from fungi of the genus *Cortinarius*. They showed that the methylation of the enolic OH enables the reduction of the carbonyl groups

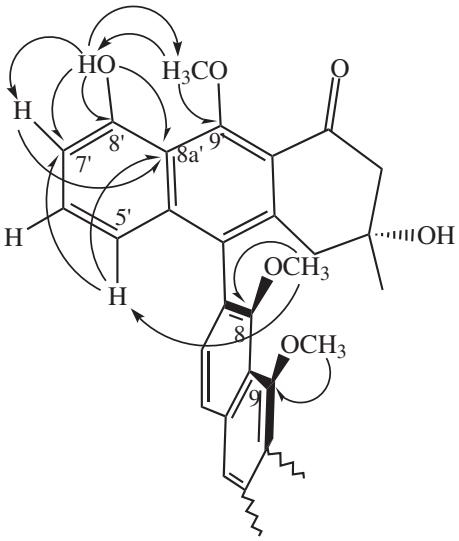


Figure 2. NOEs ↷ and HMBC ↷ for compound **4**.

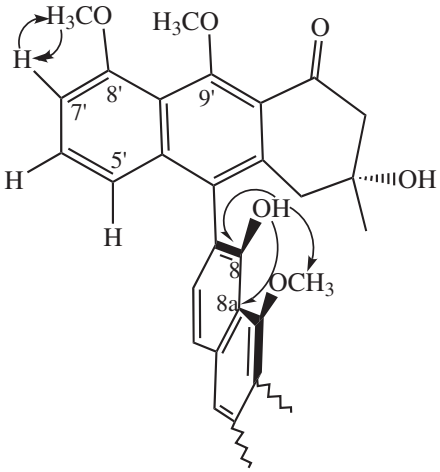


Figure 3. NOEs ↷ and HMBC ↷ for compound **6**.

with $LiBH_4$. Following the same procedure described by them, reduction was made on the 9,9′ dimethyl PA1 obtained previously (compound **2**). The reaction gave 5 products by TLC; the main compound was isolated and purified. IR spectra confirmed the disappearance of the carbonyl band, suggesting total reduction. In the ^{13}C NMR spectrum, the signals assigned to the carbonyl groups in PA1 were also absent, and two signals at 63.87 and 63.59 ppm, arising from the two new methine groups were observed. Reduction of the carbonyl groups leads to the formation of two new chiral centers. It was not possible to know the absolute stereochemistry of this new compound for the same reason that only relative stereochemistry of PA1 is known at the moment (Rodríguez et al., 1998). In order to know the relative stereochemistry of compound **7**, the geometry of all possible stereoisomers (including those conformations coming from axial and equatorial Me groups in C-3 and C-3′) were optimized by means of semi-empirical calculations. Coupling constants (J) found between H-2′ and H-1′ and between H-2 and H-1 were less than 5 Hz. Using Karplus equation, J for the dihedral angle formed by H1′-C1′-C2′-H2′ from each stereoisomer was calculated. Considering these results and the NOE enhancement seen (Fig. 4), the relative stereochemistry of the new chiral center could be established. Compound **7** must be either (M) 1R, 1′R, 3R, 3′R or its enantiomer.

In order to know if chemical modifications altered the biological action of pA1, two *in vitro* assays routinely used in our laboratory were carried out (the selective cytotoxicity and inhibition of catalase activity).

It is well known that PA1 and related compounds inhibit non-competitive catalase activity (Moreno et al., 1995); none of the compounds synthesized could inhibit catalase activity at the same concentration at which peroxisomicine displayed

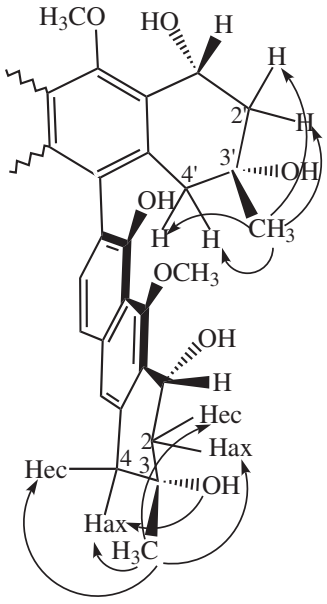
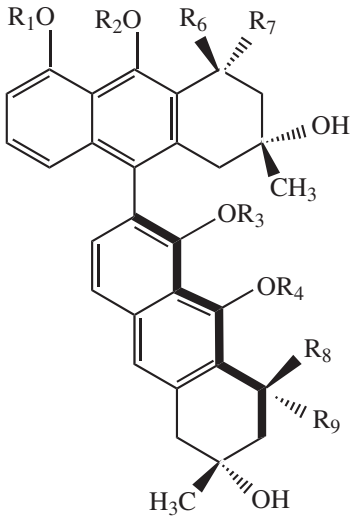


Figure 4. NOEs ↷ for compound **7**.



Compound	R1	R2	R3	R4	R5	R6, R7	R8, R9
1	H	H	H	H	H	C=O	C=O
2	H	Me	H	Me	H	C=O	C=O
3	Me	H	H	Me	H	C=O	C=O
4	H	Me	Me	Me	H	C=O	C=O
5	Me	Me	H	Me	H	C=O	C=O
6	H	H	H	H	Cl	C=O	C=O
7	H	Me	H	Me	H	H,OH	H,OH

Figure 5. Structures of compounds **1**–**7**.

Table 1. Inhibitory effect (IC_{10}) of anthracenonic compounds on catalase activity.

Compound	IC_{10} (μM)
1	0.21
2	24.95
3	23.05
4	49.10
5	40.78
6	7.04
7	8.36

Table 2. CT_{50} (μg/mL) obtained after 72 h exposure to anthracenonic compounds.

Compound	Chang liver cells	Hep G2
1	1.25–2.50	0.05–0.16*
2	3.50–5.00	2.50–3.50
3	5.00	2.50–3.50
4	5.00	3.50
5	2.50–5.00	2.50
6	1.80–2.50	0.11–0.44*
7	3.50–5.00	3.50–7.07

* malignant cells were significantly more sensitive than benign cells $p < 0.05$.

50% inhibition. It was not possible to calculate an IC_{50} with these compounds, because at high concentration interaction between the compounds and H_2O_2 were observed in the UV-Vis spectra; instead the IC_{10} is reported (Table 1).

Cytotoxicity of PA1 and similar compounds is routinely evaluated on benign and neoplastic hepatic human cell lines, because of the selective cytotoxic effect previously reported. Each compound was evaluated at 12 different concentrations, using 3 wells for each concentration. PA1 was the positive control. The procedure was repeated three times in separate experiments. After a 72-h exposure of the cell lines to the compounds, all substances tested displayed cytotoxicity. Nevertheless, the minimum and maximum cytotoxic concentrations were the same for both benign and neoplastic cells, except for compound **6** (Table 2); in this case, the CT_{50} for benign cell was significantly higher than the CT_{50} for cancer cell, as shown previously with PA1.

Conclusions

In the present work, six new compounds were obtained from peroxisomicine A1; they were identified using spectroscopic properties. Results of cytotoxicity of compounds **2**–**5** suggest that phenolic groups are necessary for the biological action reported for PA1. On the other hand compound **6** substituted in the aromatic ring had a selective cytotoxic effect to neoplastic cells as shown previously with PA1. These important results encourage the synthesis of new biologically active compounds from PA1.

Acknowledgments

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