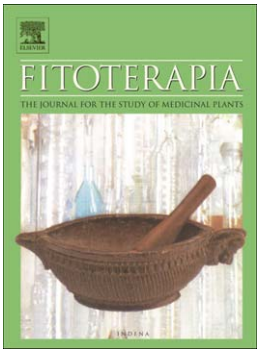

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Structural elucidation and evaluation of multidrug-resistance modulatory capability of Amarissinins A-C, diterpenes derived from *Salvia amarissima*.

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Abstract

Three new diterpenes (amarissinins A-C, **1**-**3**) containing several oxygenated functionalities were isolated from the leaves and flowers of *Salvia amarissima*. The structures of these compounds were established through the analysis of their NMR spectroscopy and mass spectrometry data. The structures of compounds **1** and **2** were confirmed by single crystal X-ray diffraction. Compound **2** was identified as a C-10 epimer of dugesin F (**5**). The cytotoxic activity of these compounds against five human cancer cell lines was determined. Additionally, the capability to modulate the multidrug resistance (MDR) in the MCF-7 cancer cell line resistant to vinblastine was tested.

Keywords: Lamiaceae, *Salvia amarissima*, clerodane diterpenes, cytotoxic, MDR modulation

1. Introduction

Genus *Salvia* grouped 900 species with a sub-cosmopolitan distribution.[1] In Mexico, about 275 species with a high degree of endemism are distributed,[2] this fact makes the country the major center of diversification.[3] In addition, many *Salvia* species are used with ethnomedical purposes to alleviate a wide variety of ailments;[4] and constitute a rich source of terpenoids, mainly diterpenes.[5] This kind of isolates are responsible, in many cases, for the pharmacological effects attributed to these species.[6] Diterpenes isolated from *Salvia* possess a large number of carbon skeletons, for example microphyllane[7] and salvileucalane[8] among others, which originated through rearrangement of clerodane-type intermediates. Biological properties of these compounds are also relevant as anti-inflammatory, anti-viral, antiprotozoal, cytotoxic and phytotoxic agents.[9]

Another remarkable fact of the phytochemical study of the *Salvia* genus is the variant metabolic profile, which could be due to varying external stimuli and growth conditions[10] as well as by the effect induced by their associated endophytic microorganism.[11] This pattern is exemplified by the chemical constituents of *S. microphylla*. The variety of secondary metabolites isolated from this specimen, collected in different geographical regions, is very different.[5,12-14] In this context, the same behavior was observed in *S. amarissima*, an endemic herb in Mexico. A previous phytochemical study of the non-polar extract obtained from its leaves, collected in Oaxaca valley, leads to the isolation of amarisolide, a glycosylated clerodane diterpene.[15] Recently, further investigation of the acetone extract of the leaves and flowers from *S. amarissima*, collected in the mountains surrounding the Teotihuacan Valley, State of Mexico, leads to the identification of teotihuacanin (**4**), a rearranged clerodane diterpene with potent modulatory activity of

MDR in the MCF-7 cancer cell line resistant to vinblastine. In this study, the isolation of amarisolide was not reported.[16]

This investigation describes the structural elucidation of three new diterpenes isolated by chromatographic methods from a Me_2CO-soluble extract of the leaves and flowers of *S. amarissima*. One of these is a 9-10-*seco*-clerodane (**1**) and the other two are clerodanes (**2-3**). These compounds were named amarissinins A-C (**1**-**3**, Fig. 1), and the results of their cytotoxicity against a panel of five human cancer cell lines and the MDR modulatory activity in a breast cancer cell line (MCF-7) resistant to vinblastine are reported.

2. Experimental

2.1 General experimental procedures

Melting points (uncorrected) were determined on a Fisher-Johns apparatus. Optical rotations were measured on a Perkin-Elmer 343 polarimeter. UV spectra were recorded on a Shimadzu UV 160U spectrophotometer. IR spectra were obtained on a Bruker Tensor 27 spectrometer. 1D and 2D NMR experiments were performed on a Varian Unity Plus 500. X-Ray crystallographic data were obtained on a Bruker D8 Venture κ-geometry diffractometer with a Cu Kα radiation (λ = 1.54178 Å). Chemical shifts were referred to TMS. *J* values are given in Hz. HRDARTMS were recorded on a JEOL AccuTOF JMS-T100LC mass spectrometer. Column chromatography (CC) assisted with vacuum was performed on silica gel 60 (Merck G), unless otherwise stated. Silica gel 230-400 mesh (Macherey–Nagel) was used for flash chromatography. TLC was carried out on precoated Macherey–Nagel Sil G/UV254 plates of 0.25 thickness, and spots were visualized by spraying with 3% $CeSO_4$ in H_2SO_4 2 N, followed by heating. Energy minimization was

performed with MM2 force field using ChemBio3D Ultra1.0.1986-2009 Cambridge Soft Corporation. The 3D minimized structure was visualized with WebLab ViewerLite software.

2.2 Plant material

The leaves and flowers of *S. amarissima* were collected in the mountains surrounding the Valley of Teotihuacan, State of Mexico, in June 2014, and identified by M. Sci. María del Rosario García Peña. A voucher specimen was deposited (MEXU-1407290) at the National Herbarium, Instituto de Biología, Universidad Nacional Autónoma de México.

2.3 Extraction and isolation

The dried and ground plant material (700 g) was extracted 3 times by percolation for 3 h with Me_2CO (6 L) to obtain a dried extract (37.5 g), which was dissolved in n-hexane (0.5 L) and partitioned with a mixture of MeOH-H_2O (4:1) (0.3 L x 4). The residue of hexanic fraction was 19.7 g. The hydroalcoholic fraction (17.7 g) was concentrated to one fifth of its original volume and partitioned again with EtOAc (0.3 L x 4). The EtOAc fraction (11.6 g) was subjected to silica gel CC (7.0 x 22.0 cm, 200 mL) using mixtures of n-hexane-EtOAc (frs. 1-55) and EtOAc-Me_2CO (frs. 56-69). The fractions obtained from this column were analyzed by TLC and grouped as follows: fraction A (0.24 g, eluted with n-hexane-EtOAc 7:3), fraction B (1.64 g, eluted with n-hexane-EtOAc 3:2), fraction C (1.36g, eluted with n-hexane-EtOAc 1:1), fraction D (0.8g, eluted with EtOAc), and fraction E (1.81 g, eluted with EtOAc-Me_2CO 4:1). Fraction A was submitted to silica gel CC (1.0 x 15 cm, 20 mL) eluted with n-hexane-EtOAc 3:2 to give compound **2** (135.2 mg) which was crystalized from AcOEt/n-hexane. Fraction B was subjected to successive silica gel CC (3.0 x 18 cm, 50 mL; and 2.0 x 15 cm, 50 mL) eluted with mixtures n-hexane-EtOAc 1:1 and

$CHCl_3$-MeOH 98:2 to give by crystallization from acetone/n-hexane the compound **3** (657 mg). The constituents from fraction C were separated by successive silica gel CC (2.5 x 22.0 cm, 40 mL) eluted with mixtures n-hexane-EtOAc 1:1 (frs. 1-20) and n-hexane-EtOAc 3:7 (frs. 21-35). From frs. 16-23 was isolated by crystallization from acetone/n-hexane the compound **1** (278 mg). Frs. 26-30 yielded by crystallization from acetone/n-hexane the compound **4** (60 mg).

*Amarissinin A (**1**)*: yellowish-white crystals, mp 230-232 °C; $[\alpha]^{25}_{D}$ +93 (*c* 0.15, Me_2CO); UV (MeOH) λ_{max} (log ε) 211 (4.25), 330 (4.52); IR (KBr) ν_{max} 3270, 1780, 1687, 1645, 875 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$- DMSO-d_6) see Table 1; HRMS (DART-TOF+) *m/z* 371.11323 $[M + H]^+$ (calcd for $C_{20}H_{19}O_7$, 371.11308). X-ray crystallographic analysis of amarissinin A (**1**): Moiety formula: $C_{20}H_{18}O_7$, MW = 370.34, trigonal, space group $P3_1$, unit cell dimensions a = 16.8901(3) Å, b = 16.8901(3) Å, c = 5.8211(10) Å, α = 90°, β = 90°, γ = 120°, V = 1438.1(6) $Å^3$, Z = 3, D_c = 1.283 g/cm^3, $F(000)$ = 582. A total of 3,936 reflections were collected, with 1968 independent reflections (R_{int} = 0.0625). The structure was solved by direct methods and refined by full-matrix least-squares on F^2, with anisotropic temperature factors for non-hydrogen atoms at final R indices [$I > 2\sigma(I)$], R_1 = 0.0368, wR_2 = 0.0772; R indices (all data), R_1 = 0.0543, wR_2 = 0.0836. Flack parameter = 0.01(11). Crystallographic data reported in this paper have been deposited in the Cambridge Crystallographic Data Centre (deposition number: CCDC 1456506). The data can be obtained free of charge via http://www.ccdc.cam.ac.uk./data_request/cif.

*Amarissinin B (**2**)*: colorless crystals, mp 132-134 °C; $[\alpha]^{25}_{D}$ +16 (*c* 0.10, $CDCl_3$); UV (MeOH) λ_{max} (log ε) 207 (4.21), 297 (3.96); IR (KBr) ν_{max} 3359, 1733, 1704, 873 cm^{-1}; ^1H

and ^{13}C NMR ($CDCl_3$- DMSO-d_6) see Table 1; MS (DART-TOF+) *m/z* 357 $[M+H]^+$; HRMS (DART-TOF+) *m/z* 357.13338 $[M + H]^+$ (calcd for $C_{20}H_{21}O_6$, 357.13381).

*Acetylamarissinin B (**2a**)*. A 0.8 mL amount of acetic anhydride was added by drops to a solution of compound **2** (61.6 mg, 0.17 mmol) dissolved in pyridine (0.4 mL). The reaction mixture was stirred at room temperature for 72 h. After this, the reaction mixture was worked up as is usual to obtain a residue (73.8 mg), which was purified by CC (n-hexane-EtOAc 3:2 and 2:3) to give 20.8 mg of **2a** as colorless crystals: $[\alpha]^{25}_D$ +27.7 (*c* 0.26, $CDCl_3$); UV (MeOH) λ_{max} (log ε) 209 (3.99), 296 (3.73); ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 7.38 (1H, t, *J* = 1.7 Hz, H-16), 7.33 (1H, dt, *J* = 1.5, 0.8 Hz, H-15), 7.06 (1H, d, *J* = 5.2 Hz, H-3), 6.53 (1H, ddd, *J* = 9.5, 5.2, 3.2 Hz, H-2), 6.33 (1H, dd, *J* = 1.7, 0.8 Hz, H-14), 6.24 (1H, dd, *J* = 9.5, 2.3 Hz, H-1), 5.71 (1H, s, H-20), 5.25 (1H, t, *J* = 8.0 Hz, H-12), 4.15 (1H, d, *J* = 8.6 Hz, H-19 *Pro* R), 3.85 (1H, dd, *J* = 8.7, 1.7 Hz, H-19 *Pro* S), 3.17 (1H, br s, H-10), 2.96 (1H, q, *J* = 6.9 Hz, H-8), 2.72 (1H, dd, *J* = 13.0, 8.3 Hz, H-11a), 2.56 (1H, d, *J* = 13.2 Hz, H-6α), 2.33 (1H, dd, *J* = 13.0, 8.0 Hz, H-11b), 2.31 (1H, dd, *J* = 13.0, 1.7 Hz, H-6β), 1.96 (3H, s, CH_3), 1.22 (3H, d, *J* = 6.9 Hz, CH_3-17); ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 206.4 (C-7), 168.9 (C-18), 167.8 (C-1'), 143.8 (C-15), 139.5 (C-16), 134.6 (C-1), 130.7 (C-4), 128.5 (C-3), 127.7 (C-13), 126.3 (C-2), 108.4 (C-14), 97.9 (C-20), 74.8 (C-19). 74.5 (C-12), 59.0 (C-9), 49.1 (C-8), 45.3 (C-10), 43.5 (C-6), 43.3 (C-5), 42.2 (C-11), 21.5 (C-2'), 9.4 (C-17); ESIMS+ *m/z* 421.2 $[M + Na]^+$; X-ray crystallographic analysis of acetylamarissinin B (**2a**): Moiety formula: $C_{22}H_{22}O_7$, MW = 398.39, monoclinic, space group $P2_1$, unit cell dimensions *a* = 18.8711(11) Å, *b* = 9.2292(5) Å, *c* = 22.5216(13) Å, α = 90°, β = 105.731(2)°, γ = 90°, *V* = 3775.6(4) Å^3, *Z* = 8, D_c = 1.402 g/cm^3, *F*(000) = 1686. A total of 15,458 reflections were collected, with 8321 independent reflections (R_{int} =

0.0401). The structure was solved by direct methods and refined by full-matrix least-squares on F^2, with anisotropic temperature factors for non-hydrogen atoms at final *R* indices [$I > 2\sigma(I)$], R_1 = 0.0319, wR_2 = 0.0814; *R* indices (all data), R_1 = 0.0339, wR_2 = 0.0833. Flack parameter = 0.04(3). Crystallographic data reported in this paper have been deposited in the Cambridge Crystallographic Data Centre (deposition number: CCDC 1482296). The data can be obtained free of charge via http://www.ccdc.cam.ac.uk./data_request/cif.

*Amarissinin C (**3**)*: colorless crystals, mp 268-270 °C; $[\alpha]^{25}_{D}$ +85 (*c* 0.18, $CDCl_3$); UV (MeOH) λ_{max} (log ε) 206 (3.90); IR (KBr) ν_{max} 3424, 3254, 1771, 1723, 876 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$- DMSO-d_6) see Table 1; HRMS (DART-TOF+) *m/z* 375.14503 $[M + H]^+$ (calcd for $C_{20}H_{23}O_7$, 375.14438).

2.4 Cell lines and culture medium

All the isolates were screened *in vitro* against five human cancer cell lines: MCF-7 and MDA-MB-231 (mammary adenocarcinoma), HeLa (cervix carcinoma), HCT-15 and HCT-116 (colon adenocarcinoma) cell lines, supplied by the National Cancer Institute (NCI, USA). Cytotoxicities were determined following protocols established by the NCI.[17] **C**ell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L- glutamine, 10000 units/mL penicillin G sodium, 10000 μg mL^{-1} streptomycin sulfate, 25 μg mL^{-1} amphotericin B (Gibco), and 1% non-essential aminoacids (Gibco). Cells were maintained at 37 ℃ in a humidified atmosphere with 5% CO2.

2.5 Cytotoxicity and multidrug-resistance modulation assays

The cytotoxic activity and modulation of multidrug resistance assays were evaluated by the sulforhodamine B (SRB), method as previously described.[17] The cells were removed

from the tissue culture flasks by treatment with trypsin and diluted with fresh media. Cell suspensions (100 μL, containing 5000 or 7500 cells per well) were placed into 96 well microtiter plates (Costar), and incubated at 37◦ C for 24 h in a 5% CO2 atmosphere. A 100 μL aliquot of the test compounds at concentrations ranging from 1 x 10^{-3} to 50 μM was added to each well. The cultures were exposed for 48 h to the drug. After the incubation period, cells were fixed by addition of 50 μL of cold 50% aqueous trichloroacetic acid. The plates were incubated at 4◦ C for 1 h, washed with tap H_2O, and air-dried. The cells were stained with 0.4% SRB, washed with 1% aqueous acetic acid and air-dried. The plates were placed on a shaker for 5 min, and the absorption was determined at 515 nm using an ELISA plates reader (Bio-Tex Instruments). The values were estimated from a semilog plot of the drug concentration (μg/mL) against the percentage of growth inhibition.[17] The multidrug-resistance modulation assay was carried out as previously described.[18]

3. Results and discussion

Compound **1** (Fig. 1) was isolated as yellowish-white crystals. Its HRMS (DART-TOF+) showed a pseudomolecular ion peak at *m/z* 371.11323, which suggested a molecular formula $C_{20}H_{19}O_7$ (calcd 371.11308), indicating 12 degrees of unsaturation. The IR spectrum showed absorption bands for an hydroxyl group (3270 cm^{-1}) and for carbonyls of a γ- lactone (1780 cm^{-1}), a δ- lactone (1687 cm^{-1}) and a α,β- unsaturated ketone (1645 cm^{-1}), as well as a furan ring (875 cm^{-1}). The ^{13}C NMR spectra confirmed the presence of three carbonyl groups, and showed six signals for non-protonated carbons (Table 1). The DEPT experiment displayed the additional signals for six methines, four methylenes and one vinylic methyl group. The ^{1}H NMR spectrum showed three different spin systems

characteristic of clerodane diterpenes (Table 1);[9] one of these systems is for the 18,19- γ- lactone with signals at δ_H 4.85 (d, J = 8.7 Hz, H-19$_{Pro\text{-}R}$) and 4.45 (d, J = 8.7 Hz, H-19$_{Pro\text{-}S}$). The second spin system is for a monosubstituted furan ring, with signals at δ_H 7.91 (br s, H-16), 7.46 (br s, H-15) and 6.60 (br s, H-14). A third spin system consists of two methylenes at δ_H 2.55 (ddd, J = 13.1, 12.5, 5.2 Hz), 2.34 (ddd, J = 13.1, 12.5, 5.2 Hz); and 1.73 (ddd, J = 13.1, 12.5, 4.9 Hz), 1.67 (ddd, J = 13.1, 12.5, 4.9 Hz) assigned to the protons of H_2-7 and H_2-6 on the basis of their COSY correlations (Fig. 2). This was supported by the HMBC correlations (Fig. 2) of H_2-6 with C-19 (δ_C 69.4) and C-5 (δ_C 57.0). The HMBC heteronuclear correlations of H_2-19, H_2-6 and two additional signals of vinylic protons at δ_H 6.03 (d, J = 10.1 Hz) and 6.77 (dd, 10.1, 3.2 Hz) assigned to H-1 and H-2, with a signal at δ_C 197.1 established the position of the α,β- unsaturated ketone at C-10. In the COSY spectrum, these last two signals displayed cross peaks with the signals of a methylene at 2.81 (dd, J = 19.5, 3.2 Hz) and 2.70 (br d, J = 19.5 Hz), assigned to H_2-3; which in turn correlated in the HMBC spectra with a signal at δ_C 75.6 (C, C-4). The chemical shift of C-4 suggested the presence of a hydroxyl group at this position.[19] The above discussion indicated that **1** is a 9,10-*seco*-clerodane. This assumption was confirmed by the comparison of its spectroscopic data with those described for salvianduline B, which is closely similar.[20] In addition, the HMBC spectrum of compound **1** displayed cross peaks of H-14 and H-16 with a signal at δ_C 151.3(C), assigned to C-12, indicating that **1** differ from salvianduline B by a C-11, C-12 double bond. The above was confirmed by the HMBC cross peaks of H-11 (δ_H 6.16, s) with C-8 (δ_C 120.6, C), C-9 (δ_C 149.8, C), C-13 (δ_C 119.0, C) and C-20 (δ_C 18.3, CH_3). In order to establish the relative stereochemistry, the NOESY spectrum was acquired; nevertheless it did not provide enough information about

its spatial conformation; fortunately, compound **1** crystallized in a good shape and its structure was confirmed by single crystal X-ray diffraction method (Fig. 3). This determination also allowed the establishment of its absolute configuration using Cu Kα radiation (Flack parameter = -0.01 (11); λ = 1.54178 Å).[21]

Compound **2** has the molecular formula $C_{20}H_{20}O_6$, deduced from its HRMS (DARTTOF+, $[M+H]^+$ *m/z* 357.13338, calcd 357.13381). The ^{13}C NMR spectrum displayed 20 signals, and together with the analysis of the ^1H NMR spectrum which showed signals for a 18,19-γ-lactone (δ_C 168.5, C-18; δ_C 75.8, C-19; δ_H 4.46, 4.17, H_2-19) and a furan ring (δ_C 128.2, C-13; δ_H 7.40, H-16; 7.38, H-15; 6.45, H-14). Additional signals in the ^1H NMR spectrum at δ_H 7.03 (d, J = 5.2 Hz), 6.48 (ddd, J = 9.6, 5.2, 3.3 Hz) and 6.20 (dd, J = 9.6, 2.2 Hz) were assigned to H-3, H-2 and H-1 respectively. In the COSY spectrum, the signal for H-1 had a cross peak with a signal at δ_H 3.26 (br s), which belongs to H-10. The above information indicated the presence of a 1,3-diene in ring A of the clerodane framework.[5] The presence of the signal at δ_H 4.85 (s) in the ^1H NMR spectrum suggested the presence of an hemiacetalic function in the structure of **2**. Its position was established at C-20 on the basis of its HMBC cross peaks with C-8 (δ_C 49.1), C-9 (δ_C 59.9), C-11 (δ_C 42.7) and C-12 (δ_C 74.0). Comparison of the NMR data of **2** with those described for dugesin F (**5**, Fig. 1), a clerodane with antiviral activity isolated from *S. dugessi*;[22] indicated that both are epimers at C-10. This assumption was supported by the differences in the values of the specific optical rotations (compound **2**: $[\alpha]^{25}_D$ +16.0, *c* 0.10, $CDCl_3$, versus compound **5**: $[\alpha]^{25}_D$ - 26.1, *c* 0.08, $CDCl_3$, literature). Similar cases of diastereomeric clerodanes have been reported previously; in these cores, the spectroscopic differences were due to an α or β orientation of substituents in the *cis*-decalin framework

(e.g. cardiophyllidin and salvimicrophyllin B, as well as in linearolactone and salvimicrophyllin C).[5] The relative stereochemistry of **2** was determined considering its coexistence with **4** and the same biosynthetic origins in the plant. These led us to establish the disposition of both, the C-17 methyl group and C-19 methylene, as *α*. Thereby, the NOESY correlations (Fig. 4) of H_3-17 with H-12 and H-20 determined that the furan ring and the hemiacetalic proton are also as *α*- oriented. The NOE cross peak of H_2-19 with H-10 established the decalin ring fusion as *α*- *cis*. This disposition of the decalin ring was confirmed by the negative Cotton effect observed at 310 nm of the electronic circular dichroism (ECD) curve for **2** (Fig. S1, Supporting Information).[5] In addition, compound **2** was derivatized with Ac_2O in pyridine under standard conditions to give **2a**, which crystallized from AcOEt/hex. Thereby, the derivatization of **2** allowed the confirmation of its structure and stereochemistry through the analysis of single crystal X-ray diffraction of compound **2a** (Flack parameter = 0.04(3), Fig. 5).

Compound **3** showed a pseudo-molecular ion peak at *m/z* 375.14503 from the HRMS (DART-TOF+), consistent with the molecular formula $C_{20}H_{23}O_7$. The IR spectrum showed absorption bands for hydroxyl groups, *γ*- and *δ*- lactones, and a furan ring. The analysis of ^1H and ^{13}C NMR spectra confirmed the presence of the 18,19- *γ*- lactone (δ_C 175.0, C-18; δ_C 71.4, C-19; δ_H 4.89, H-19$_{Pro\text{-}S}$; δ_H 4.29, H-19$_{Pro\text{-}R}$); a 17,12- *δ*- lactone (δ_C 172.8, C-17; δ_C 71.2, C-12; δ_H 5.66, H-12) and the furan ring (δ_C 144.2, C-16; 138.7, C-15; 127.4, C-13; 108.8, C-14; δ_H 7.44, H-16; δ_H 7.37, H-15; δ_H 6.39, H-14). In the ^1H NMR spectra, two signals of vinylic protons at δ_H 6.07 (d, J = 10.4 Hz) and 5.83 (dd, J = 10.4, 3.7 Hz) appeared, assigned to H-2 and H-3 by the COSY correlations between them. In the ^{13}C NMR spectra, two signals for oxygenated carbons at δ_C 75.9 and 47.9 were assigned to C-4

and C-10 respectively, taking into account their HMBC correlations between H-2 and H-3 with C-4, and from H_2-1, H-8, H_2-19 and H_3-20 with C-10. Comparison of this NMR data with those described for the hydroxyclerodane: infuscatin (**6**, Fig. 1)[19] indicated that **3** differs from **6** by the absence of a hydroxyl group at C-8 and by the double bond at C-2. Concerning to the stereochemistry, the value of the H-12 coupling constant ($J_{11\alpha\text{-}12}$ = 8.5 Hz) indicated an α- oriented furan ring. The NOESY interaction of H_3-20 with H-8 and H_2-19 determined a *cis*-fused δ- lactone and α-disposition of the C-19 methylene. The NOESY correlations (in $CDCl_3$) between both hydroxyl groups suggested the same disposition. In addition, the NOESY cross peaks (in DMSO-d_6) of OH-4 with H-19$_{Pro\text{-}S}$; and of OH-10 with H-11α and H_3-20 established their dispositions as α.

The cytotoxicity of acetone extract, an ethyl acetate fraction rich in terpenoids and compounds **1**-**3,** was assayed against a panel of human cancer cell lines of breast (MCF-7 and MDA-MB-231), cervix (HeLa) and colon (HCT-15 and HCT-116), using the same method previously described.[17] The ethyl acetate fraction showed significant cytotoxic activity against the HeLa cancer cell line (IC_{50} = 1.05 ± 0.21 μg/mL). Compound **1** exhibited moderated cytotoxic activity against the cancer cell lines MCF-7 (IC_{50} = 18.2 ± 2.02 μg/mL), MDA-MB-231 (IC_{50} = 19.3 ± 0.82 μg/mL) and HeLa (IC_{50} = 14.0 ± 1.04 μg/mL). Previously, we described the effect of re-sensitization to vinblastine by teotihuacanin (**4**) which is one of the most potent MDR modulators in mammalian cancer cells ($RF_{MCF\text{-}7/Vin}^{+}$ > 10703).[16] For this reason, in this work also we decided to assay the capability to modulate the MDR by the acetone extract, ethyl acetate fraction and compounds **1**-**3** in the MCF-7 cancer cell line (Table 2). The soluble acetone extract (AE), as well as the ethyl acetate (EAF) fraction, showed at 25 μg/mL a capability 1.5-2 fold

higher than reserpine to modulate the resistance to vinblastine in phenotype MCF-7/Vin$^-$ (AE: $RF_{MCF\text{-}7/Vin^-}$ = 36 and EAF: $RF_{MCF\text{-}7/Vin^-}$ = 54); and in the MCF-7/Vin$^+$ cell line, both extract and fraction showed an equipotent MDR modulation as reserpine. Compound **1** had the same reversal fold as reserpine in MCF-7/Vin$^+$. Compound **2** enhanced twofold the susceptibility to vinblastine in comparison with reserpine. Compounds **1**-**3** were less active as MDR modulators than teotihuacanin (**4**) and the enhancement in the vinblastine susceptibility by acetone extract and ethyl acetate fraction may be due to the presence of teotihuacanin (**4**) in both. Finally, further phytochemical investigation of *S. amarissima* aimed at establishing the mechanism of action of the multidrug resistance by teotihuacanin (**4**) is underway.

Supporting Information

Supplementary data (Experimental procedures, 1D- and 2D-NMR spectra of compounds **1**-**3**) associated with this article can be found in the online version. CCDC 1456506 and 1482296 contains the supplementary crystallographic data of **1** and **2a**.

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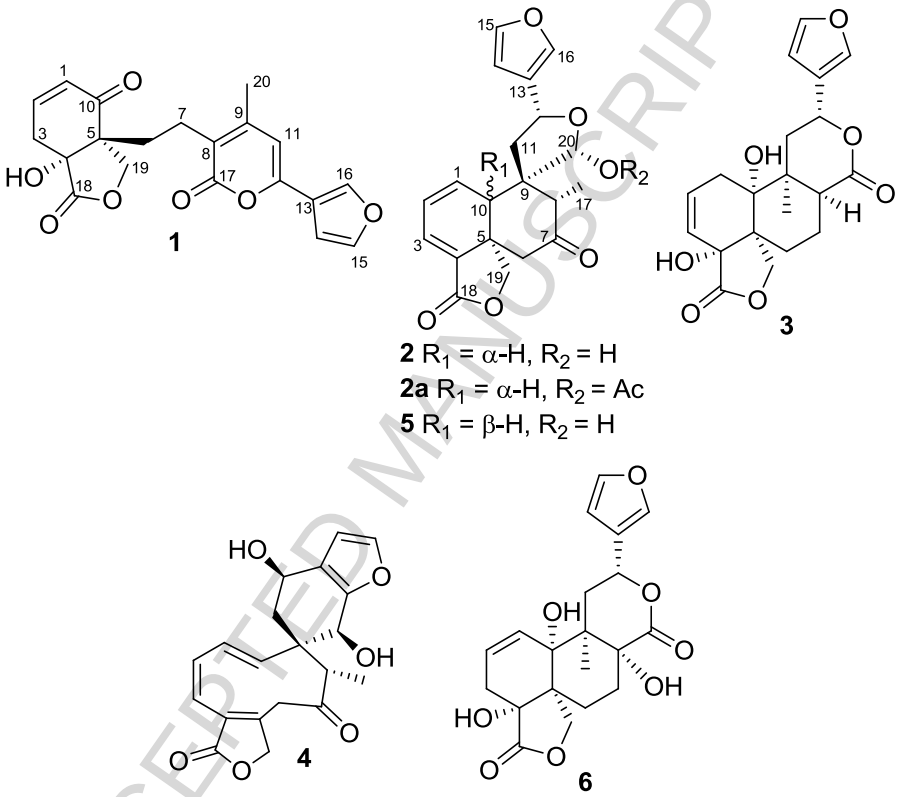

Figure 1. Structures of the new compounds (**1**-**3**), acetylamarissinin B (**2a**), teotihuacanin (**4**), dugesin F (**5**) and infuscatin (**6**).

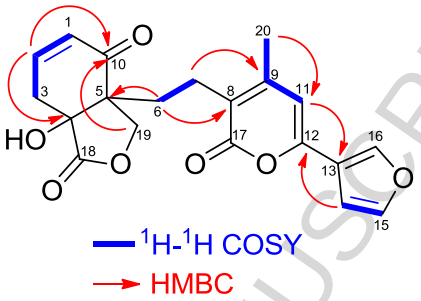

Figure 2. Key ^{1}H-^{1}H COSY and HMBC correlations for **1**.

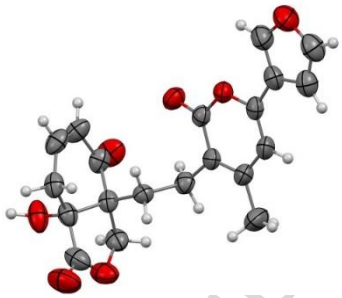

Figure 3. ORTEP drawing for compound **1**.

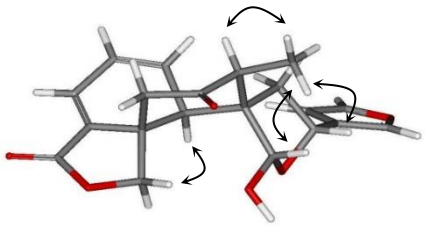

Figure 4. Key NOESY correlations for **2**.

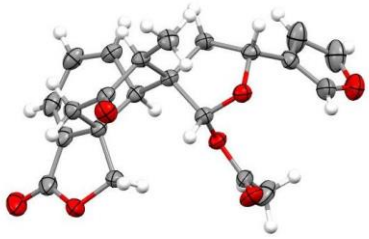

Figure 5. ORTEP drawing for compound **2a**.

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of Compounds 1-3 in $CDCl_3$ (δ in ppm, J in Hz)

	1[a]		**2**		**3**[a, c]	
position	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)	δ_C
1a	6.03 d (10.1)	127.3, CH	6.20 dd (9.6, 2.2)	135.6, CH	2.48 dd (18.9, 4.9)	29.5, CH_2
1b					2.37 br d (18.9)	
2	6.77 dd (10.1, 3.2)	142.5, CH	6.48 ddd (9.6, 5.2, 3.3)[b]	125.9, CH	6.07 d (10.4)	130.2, CH
3a	2.81 dd (19.5, 3.2)	29.7, CH2	7.03 d (5.2)	128.2, CH	5.83 dd (10.4, 3.7)	126.2, CH
3b	2.70 br d (19.5)					
4		75.6, C		130.8, C		75.9, C
5		57.0, C		43.4, C		40.1, C
6a	1.67 ddd (13.1, 12.5, 4.9)	28.6, CH_2	2.28 d (13.3)[b]	43.7, CH_2	1.65 m[b]	24.5, CH_2
6b	1.73 ddd (13.1, 12.5, 4.9)		2.56 br d (13.3)[b]		1.29 dd (13.7, 9.0)	
7a	2.55 ddd (13.1, 12.5, 5.2)	21.8, CH_2		207.9, C	2.07 m	16.8, CH_2
7b	2.34 ddd (13.1, 12.5, 5.2)				1.65 m[b]	
8		120.6, C	2.91 q (6.9)	49.1, CH	2.47 m[b]	43.0, CH
9		149.8, C		59.9, C		41.2, C
10		197.1, C	3.26 br s	45.2, C		47.9, C
11a	6.16 s	104.1, CH	2.60 dd (13.1, 8.1)[b]	42.7, CH_2	3.11 dd (16.1, 8.5)	33.7, CH_2
11b			2.31 dd (13.1, 8.1)[b]		1.90 d (16.1)	
12		151.3, C	5.18 t (8.1)	74.0, CH	5.66 d (8.5)	71.2, CH
13		119.0, C		128.2, C		127.4, C
14	6.60 br s	106.2, CH	6.45 d (1.5)[b]	109.0, CH	6.39 s	108.8, CH
15	7.46 br s	143.6, CH	7.38 t (1.5)	143.8, CH	7.37 s	138.7, CH
16	7.91 br s	141.3, CH	7.40 br s	140.0, CH	7.44 s	144.2, CH
17		161.5, C	1.18 d (6.9)	9.3, CH_3		172.8, C
18		173.7, C		168.5, C		175.0, C
19-*Pro R*	4.85 d (8.7)	69.4, CH_2	4.17 d (9.3)	75.8, CH_2	4.29 d (8.3)	71.4, CH_2
19-*Pro S*	4.45 d (8.7)		4.46 dd (9.3, 1.5)		4.89 d (8.3)	
20	2.10 s	18.3, CH_3	4.85 s	98.3, CH	1.26 s	27.4, CH_3

[a] In $CDCl_3$-DMSO-d_6, [b]Overlapped, [c]OH-4 signal at δ_H 6.71 (br s), and OH-10 signal at δ_H 5.35 (br s); assigned by HMBC correlations.

Table 2. Modulation of Vinblastine Cytotoxicity in Drug-Sensitive MCF-7 and Multidrug-Resistant MCF-7/Vin by acetone extract, EtOAc fraction and compounds **1-3**

	IC_{50} (µg/mL)			reversal fold[e]		
Sample[a]	MCF-7/Vin$^-$	MCF-7/Vin$^+$	MCF-7 sens	$RF_{MCF\text{-}7/Vin^-}$	$RF_{MCF\text{-}7/Vin^+}$	$RF_{MCF\text{-}7\ sens}$
Vinblastine	1.08 ± 0.06	1.56 ± 0.23	0.0032 ± 0.004			
AE[b]	0.03 ± 0.01	0.22 ± 0.04	0.0011 ± 0.0002	36.0	7.1	2.9
EAF[c]	0.02±0.009	0.29 ± 0.18	0.0003 ± 0.00008	54.0	5.4	1.1
1	0.35 ± 0.11	0.27 ± 0.17	0.0002 ± 0.0001	3.1	5.8	16.0
2	0.02 ± 0.015	0.59 ± 0.25	0.0007 ± 0.0003	54.0	2.6	4.6
3	0.45 ± 0.22	1.44 ± 0.30	0.0026 ± 0.001	2.4	1.1	1.2
Reserpine[d]	0.037 ± 0.01	0.31 ± 0.19	<0.000128	29.2	5.0	25.0

[a]Serial dilutions from 0.000128 to 10 µg/mL of vinblastine in the presence or absence of extract, fraction or compound (25 µg/mL). [b]Acetone extract. [c]Ethyl acetate fraction. [d]Reserpine = 5 µg/mL as positive control. [e]RF = IC_{50} Vinblastine/IC_{50} Vinblastine in the presence of compound. Each value represents the mean ± SD from three independent experiments.

Graphical Abstract