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Transformations of Some Sesquiterpene Lactones by Filamentous Fungi. Cytotoxic Evaluations.

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Biotransformation is an economically and ecologically viable technology which has been used to modify the structures of many classes of biologically active products. Some fungi may be useful for the biotransformation of sesquiterpene lactones (SLs), leading to unusual structural changes that modify their biological activities, and other transformations mimic their biosynthetic pathways, generating evidences for the proposed biogenesis. Eight filamentous fungi were screened for their ability to transform different SLs (**1**-**9**), and microbial reactions yielded compounds **11** -– **18**, which in turn have been isolated as natural products, thus mimicking their biosynthesis. Their structures were identified based on NMR and MS spectroscopic analyses. The cytotoxicities of SLs **1**, **4**, **6**, **7** and **9**, and their biotransformed produts (**10**, **14**, **15** and **17**) against human cancer cell lines U251 (glia), PC3 (prostate), K562 (leukemia), HCT-15 (colon), MCF7 (breast), and SKLU-1 (lung), were determined, confirming that the presence of Michael acceptor is an important feature for the bioactivity.

Keywords: biotransformations, sesquiterpene lactones, germacrolide, filamentous fungi, cytotoxicities

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Introduction

There is an increasing body of information about the use of biocatalysis for selective transformations of synthetic and natural products. The principal advantages of the biocatalytic processes are their mild reaction conditions, environmental safety, and high selectivity [1]. Special attention has been paid to filamentous fungi because they catalyze *situ*-, *regio*- and *stereo*-selective reactions [2]. Sesquiterpene lactones (SLs) constitute a group of compounds mainly isolated from species of the family Asteraceae. SLs possess a wide spectrum of bioactivity [3] such as anti-inflammatory properties [4-7] and cytotoxicity against tumor cell lines [7-10]. It is widely accepted that thiol groups such as cysteine residues in proteins, as well as the free intracellular glutathione (GSH), serve as the major targets for SLs [11,12]. The interaction between SLs and protein thiol groups or GSH leads to reduction of enzymatic activity, or causes the disruption of GSH metabolism and the intracellular cell redox balance [13,14]

The main types of reported biotransformations of SLs with various filamentous fungi include hydrogenations, hydroxylations, acylations, methoxylations, and epoxidations, among others [15-22]. Particularly, the biotransformations of the antimalarial sesquiterpene lactone artemisinin or related compounds have been studied [23-27].

Thus, with the aim of exploring the bioreactivity and bioactivity of several SLs, we carried out the transformations of arglanin (**1**) [28-30], 11,13- dehydroeriolin (**2**) [31, 32], budlein B (**3**) [33,34], ludovicin A (**4**) [28], santamarin (**5**) [28], epoxysantamarin (**6**) [28], parthenolide (**7**) [35], sphaerocephalin (**8**) [36] and deacetylconfertiflorin (**9**) [37] by eight filamentous fungi previously used by our group [38]. The biotransformations afforded the products **10-17**, which were previously characterized as natural products. Therefore, these derivatizations imitate their natural biosynthesis. The cytotoxicities of all the available compounds against human cancer cell lines U251 (glia), PC-3 (prostate), K562 (leukemia), HCT-15 (colon), MCF7 (breast), and SKLU-1 (lung) were also evaluated.

Results and Discussion

The biotransformation of arglanin (**1**) [28] with *Rhizopus nigricans, Beauveria bassiana*, *Curvularia lunata*, *Aspergillus niger, and Cunnighamella blakesleeana* afforded vulgarin (**10**) [39] as the major product (62, 72, 75, 78, 77% yied, respectively), while with *Mucor miehei*, *Rhizopus oligosporus* and *Fusarium moliniforme,* the biotransformation proceeded in relative low yield (< 10% by NMR analysis of the mixture).

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The ^{1}H NMR spectrum of vulgarin **10** did not show the two downfield doublets at δ_H 6.15 (d) and 5.48 (d) derived to the exocyclic vinylic hydrogens in **1**. Instead, a new methyl signal at δ_H 1.27 (d, J = 7 Hz) corresponding to CH_3-13 (δ_H 1.27) and a signal at δ_H 2.35 (dq, J = 13.0, 7.0 Hz) for H-11 appeared. The orientation of the methyl group at C-11 was established as α- by the observed NOESY correlations between H-6β (δ_H 4.17), H_3-15β (δ_H 1.57), H_3-14β (δ_H 1.22) with H-11β (δ_H 2.35). The HMBC and HSQC correlations confirmed the structure of vulgarin (**10**) [39] for this compound.

The stereoselective hydrogenation of the olefin was the transformation observed for 11,13-dehydroeriolin (**2**) with *A. niger, C. blakesleeana, B. bassiana* and *F. moliniforme*, affording eriolin (**11**) [31, 40]. The configuration at C-11 was deduced from the observed NOESY correlation of the β-oriented H-8 (δ_H 4.17) and H-6 (δ_H 2.16) with H-11 (δ_H 2.57), as well as the correlation between H-7α (δ_H 1.93) with CH_3-13 (δ_H 1.27), establishing the 11*R*- configuration [40].

Incubations of budlein B (**3**) [33, 34] with *C. lunata* and *A. niger* in medium B (see Experimental), afforded *allo*-schkuhriolide (**12**) [34]. The formation of **13** can be rationalized via an oxidation of the allylic alcohol of C-14, affording the aldehyde, followed by isomerization of the C1-C10 double bond.

The biotransformation of ludovicin A (**4**) [28] with *C. lunata* in medium B produced tuberiferin (**13,** 31%) [41] and ludovicin B (**14,** 52%) [30]. Compound **13** had the molecular formula $C_{15}H_{18}O_3$ (M^+, *m/z* 248), as determined by EIMS. Its IR spectrum showed bands for a γ-lactone (1768 cm^{-1}) and for an α,β-unsaturated ketone (1675 cm^{-1}). The ^{1}H NMR spectrum revealed the presence of hydrogens representative of an exocyclic methylene conjugated with the γ-lactone at δ_H 6.12 and 5.43 (d, J = 3 Hz for H-13a y H-13b), and the vinylic hydrogens of an α,β-unsaturated ketone at δ_H 6.71 and δ_H 5.91 (d, J = 9 Hz each) corresponding to H-1 and H-2, respectively. The signal at δ_H 2.62 (dq, J = 7 Hz) was assigned to H-4, based on the COSY correlation of H-4 with H-5 (δ_H 2.1, dd, J = 7, 10 Hz) and H-15 (δ_H 1.4, d, J= 7 Hz, 3H). The β-orientation of H-4 was determined by its NOESY correlation with H-6 and H_3-14. The ^{1}H and ^{13}C NMR data and physical properties were in agreement for those of tuberiferin (**13**), previously isolated from *Sonchus tuberifer* [41]. Compound **14** was previously characterized from *A. ludoviciana* ssp *mexicana* [30]. The formation of **13** and **14** from **4** could be explained by the regioselective opening of the oxirane**,** followed by removal of a proton to generate the allylic alcohol **14**; semipinacolic-type rearrangement (or a 1,3-isomerization of the allylic proton from C-3 to C-15, followed by keto-enol tautomerization) would produce the ketone, and a dehydration reaction would afford the α,β-unsaturated ketone **13**.

Santamarin (**6)** [28] was transformed by the different fungi tested to 3α-hydroxyreynosin (**15**) in relatively low yield, but epoxysantamarin (**6**) was relatively efficiently transformed by *C. lunata* and *A. niger* (see Experimental).

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Screening-scale experiments showed that eight microorganisms used in this study were capable of converting parthenolide (**7**) into compound **16 [**35]. However, with *B. bassiana* (medium A) and *C. lunata* (medium B), 1,10-epoxy-11βH,13-dihydroparthenolide (**17**) [42] was obtained. The HRFABMS established a molecular formula of $C_{15}H_{23}O_4$ $[M+H]^+$, 267.1595, calcd. 267.1597, and the ^1H NMR spectrum revealed a signal at δ_H 1.28 (d, J = 7.0 Hz, 3H), which corresponded to CH_3-13, indicative of the hydrogenation of the exocyclic olefin. The presence of an epoxide at C-1 and C-10 in **17** was confirmed on the basis of ^{13}C NMR, HMQC and HMBC experiments. Particularly, H-5 (δ_H 2.82, d, J = 10 Hz) and H-1 (δ_H 2.82, dd; J = 10, 2 Hz) appeared at the same chemical shift, and the corresponding carbons appeared at δ_C 84.3 and δ_C 63.8, respectively. Furthermore, the HMBC correlation of C-5 with H-3β (δ_H 2.16), H-7α (δ_H 2.28) and H_3-15 (δ_H 1.47), C-1 (δ_C 63.8) with H-9β (δ_H 2.25) H-2α, H-3α and H_3-14, confirmed structure **17**.

Sphaerocephalin (**8**) [36] and deacetylconfertiflorin (**9**) [37] were not biotransformed by any of the eight fungi in the tested conditions (using medium A).

The cytotoxic activities of the natural compounds used as starting material (**1**, **4**, **6**, **7** and **9**), and the biotransformation products (**10**, **14**, **15**, and **17**) were evaluated against selected human cancer cell lines: U251 (glia), PC-3 (prostate), K562 (leukemia), HCT-15 (colon), MCF7 (breast), SKLU-1 (lung), following standard procedures, and the results are summarized in Table 1 (some bioactivities were not determined due to the paucity of available material). All the compounds displayed activity (as determined by IC_{50} values below 100 μM), with lactones **10** and **17** being less active. It was confirmed that the cytotoxicities of sesquiterpene lactones reside chiefly on the presence of Michael acceptor groups, although the presence of additional polar groups and stereoelectronic considerations are also important. Parthenolide (**7**) was the most active compound, but displayed limited selectivity.

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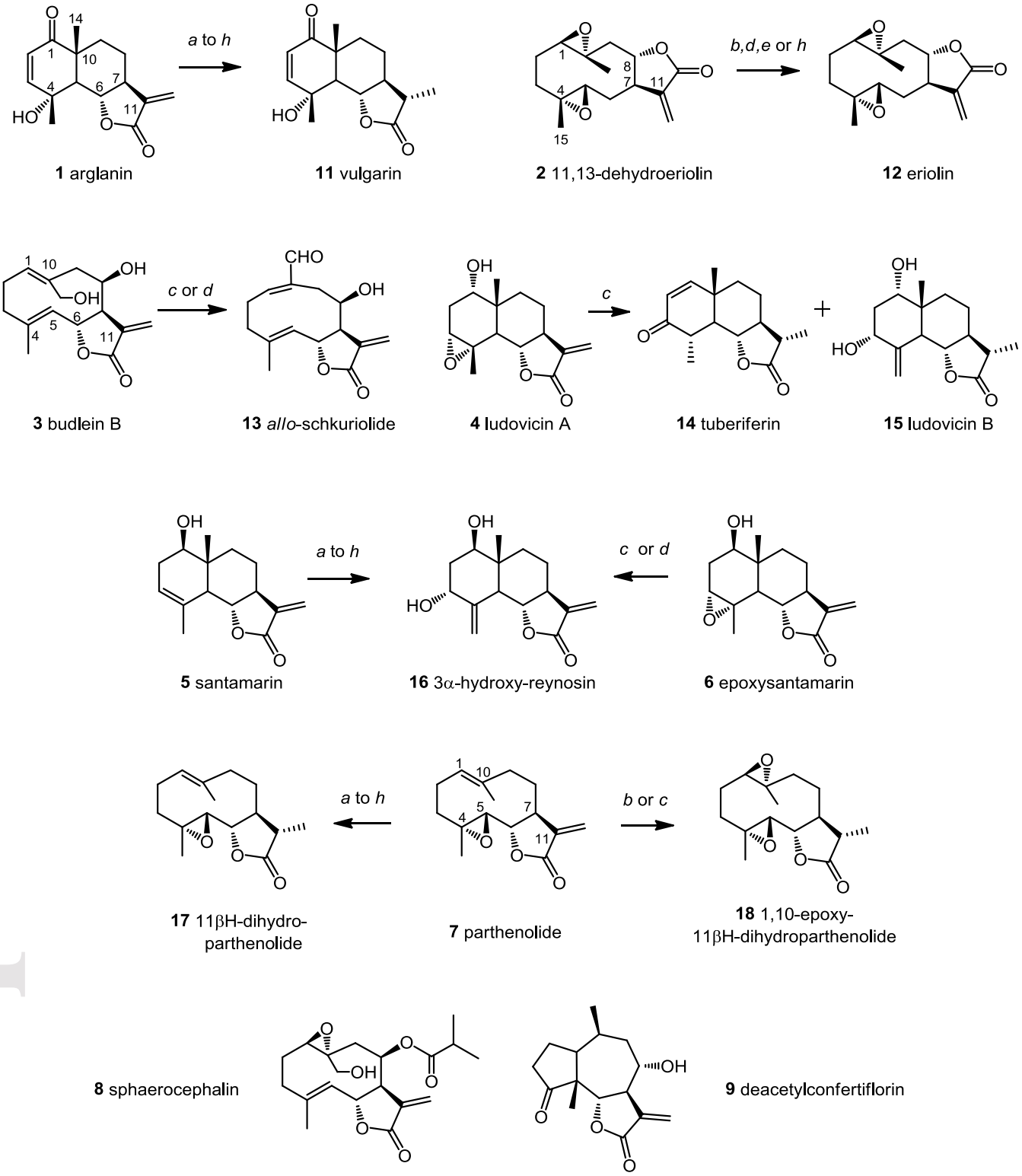

Scheme 1. Biotransformations of sesquiterpene lactones **1** – **6** with filamentous fungi. *a*: *R. nigricans*, *b*: *B. bassiana*, *c*: *C. lunata*, *d*: *A. niger*, *e*: *C. blakesleeana*, *f*: *M. miehei*, *g*: *R. oligosporus*, *h*: *F. moliniforme*.

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Table. Table. Cytotoxicity IC_{50} values (μM) of sesquiterpene lactones against selected cancer cell lines.

Compound	U251 (glía)	PC-3 (prostate)	K562 (leukemia)	HCT-15 (colon)	MCF7 (breast)	SKLU-1 (lung)
1	10.25±0.15	10.98±0.8	12.18±0.64	10.98±0.54	10.05±0.37	nd
4	35.73±0.35	39.76±0.6	36.43±0.4	nd	38.66±0.83	nd
6	26.47±0.5	32.8±0.5	nd	nd	nd	nd
7	5.34±0.12	6.4±0.2	nd	nd	1.32±0.45	7.09±0.9
9	26.57±0.28	24.78±0.9	22.27±0.87	22.14±0.76	20.1±1.9	19.89±0.9
10	65.70±0.52	75.64±0.37	73.29±0.12	84.41±0.27	nd	nd
14	nd	50.09±0.6	36.58±0.3	nd	nd	nd
15	23.12±0.4	27.98±1.49	21.28±0.12	nd	nd	nd
17	72.77±0.81	78.14±1.1	76.97±3.7	nd	nd	nd
Helenalin	0.32±0.02	nd	nd	nd	0.19±0.03	0.21±0.02

The sesquiterpene lactone helenalin was taken as reference [43]. Nd: not determined, due to the paucity of available material.

Conclusions

The hydrogenation of the $\Delta^{11(13)}$ double bond was the common process in microbial transformation of sesquiterpene lactones **1**, **2, 4** and **7** by the filamentous fungi used. The reduction of the $\Delta^{11(13)}$ olefin appears to be dependent on the compound involved, the concentration used, and the fungi assayed. The fungal transformation of the tested lactones are in accordance to the commonly accepted biogenetic routes, since the obtained products have been found also as natural products. The eudesmanolides (**1**, **4**, **5**, **6**) appear to be more reactive toward biotransformations in comparison with germacrolides (**2, 3** and **7**). This is the first report on the biotransformations of compounds **1** – **6**, and it is interesting to note that two of the nine assayed compounds (sphaerocephalin **8** and deacetylconfertiflorin **9**) were not transformed by the tested fungi. The presence of the α-methylene-γ-lactone is responsible in most cases for the cytotoxicities of these substances, and they displayed limited bioselectivities.

Experimental Section

General

Melting points were determined on a Fischer-Jones apparatus. Optical Rotation were measured on a Perkin-Elmer 341 polarimeter. IR: Nicolet Magna FT-IR 750 spectrometer. ^{1}H and ^{13}C NMR spectra were taken on a Varian Unity-plus 500 (at 500/125 MHz) instrument. EI-MS: Jeol JMS-AX505HA mass spectrometer and Jeol JMS-SX 102 A) for HREIMS. TLC spots were revealed by spraying with ceric ammonium sulfate, followed by heating. Vaccum Column Chromatographies

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(VCC) were done following the reported procedures [44]. Column Chromatography (CC) were performed using silica gel 70-230 and TLC using silica gel $60F_{254}$ (Merck) plates.

Fungi, Media and Culture Conditions

Rhizopus nigricans (ATCC 6227b), *Beauveria bassiana* (ATCC 13144), *Curvularia lunata* (ATCC 13432), *Aspergillus niger* (ATCC 16404), *Cunninghamella blakesleeana* (ATCC 8688a), *Mucor miehei* (ATCC 16457), *Rhizopus oligosporus* (ATCC 22959), and *Fusarium moliniforme* (ATCC 102091) were obtained from Instituto de Biotecnología, UNAM, Mexico, maintained on potato dextrose agar (PDA), and stored at 4 °C. The medium A for *R. nigricans, B. bassiana, A. niger, C. blakesleeana, M. miehei, R. oligosporus and F. moliniforme* were prepared by mixing the following ingredients in distilled H_2O (1 L): peptone (1 g), yeast extract (1 g), beef extract (1 g) and glucose (5 g). The pH was adjusted to pH 7 before autoclaving. The medium B for *A. niger, C. blakesleeana* and *C. lunata* was prepared by mixing glucose (10 g), glycerol (10 g), peptone (5 g), yeast extract (5 g), KH_2PO_4 (5 g) and NaCl (5 g). For, the pH was adjusted to 7 before autoclaving.

Incubation Experiments, Recovery and Purification of Products

Eight fungi, *R. nigricans, B. bassiana, C. lunata*, *A. niger, C. blakesleeana, M. miehei, R. oligosporus, F. moliniforme*, were screened for their capabilities to metabolize compounds **1** – **9** at analytical scale (10-15 mg), and according with these results, experiments were carried out on a preparative scale (150-220 mg), following the same experimental processes [38]. Erlenmeyer cell culture flasks (250 mL) containing 125 mL of medium A or medium B were inoculated with a dense suspension (2 mL) of the corresponding fungi. Incubation was maintained at 25°C with gyratory shaking (125 rpm) for 14 days after which the substrates (2-4 mL) in acetone were added. These biotransformation experiments were monitored by TLC, including two controls, "culture control" and "substrate control", for determining that the isolated products are not secondary metabolites from the fungi, and that the culture media have not done any transformation on the substrate.

Cultures were filtered and pooled, and cells were washed thoroughly with water, the liquid was saturated with NaCl, and extracted with CH_2Cl_2. The organic extracts were dried with Na_2SO_4 and concentrated under reduced pressure. The obtained residue was chromatographed by VCC or CC using gradient elution system of *n*-hexane-EtOAc. Representative experimental procedures are described below.

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Biotransformation of Arglanin (**1**) with *C. blakesleeana*

To 72 h of growth of *C. blakesleeana* in medium A, arglanine (**1**) (210.9 mg) was dissolved in acetone (10 mL) and distributed in 13 Erlenmeyer cell culture flasks. After 14 days of incubation and monitoring the reaction by TLC, the crude brownish residue (193.4 mg) was purified by VCC and preparative TLC. This procedure afforded 162.2 mg (77%) of vulgarin (**10**): mp. 175-177 ºC. $[\alpha]_D^{25}$ = +31.58 (c = 9.5 x 10^{-3}, $CHCl_3$). IR ν_{max} ($CHCl_3$) 3571, 2977, 2937, 1789, 1683, cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): 6.61 (1H, d, J = 11.0 Hz, H-3), 5.90 (1H, d, J = 11.0 Hz, H-2), 4.17 (1H, dd, J = 11.5, 10.5 Hz, H-6), 2.44 (1H, d, J = 11.5 Hz, H-5), 2.35 (1H, dq, J = 12.0, 7.0 Hz, H-11), 2.05 (1H, ddd, J = 12.0, 3.0, 3.0 Hz, H-9β), 2.01(1H, dddd, J = 13.0, 12.0, 3.0, 3.0 Hz, H-8), 1.73 (1H, m, H-7), 1.58 (1H, m, H-7), 1.56 (3H, s, CH_3-15), 1.51 (1H, m, H-9α), 1.27 (3H, d, J = 7 Hz, CH_3-13), 1.22 (3H, s, CH_3-14). ^{13}C NMR (125 MHz, $CDCl_3$): 205.6 (C-1), 151.7 (C-2), 170.1 (C-3), 70.1 (C-4), 54.6 (C-5), 79.6 (C-6), 52.4 (C-7), 22-7 (C-8), 34.2 (C-9), 46.2 (C-10), 40.5 (C-11), 179.9 (C-12), 12.5 (C-13), 23.8 (C-14), 19.8 (C-15). EM (IE) *m/z*: 264 [M^+] (15.8), 231 (12.3), 203 (19.2), 132 (36.3), 133 (100), 121 (10.9), 98 (31.5), 93 (28.1), 55 (15.0).

Biotransformation of 11,13-Dehydroeriolin (**2**) *with A. niger*

11,13-Dehydroeriolin (**2**, 200 mg), dissolved in 10 mL de Me_2CO, was distributed among 20 flask containing 125 mL of medium A. The crude product obtained in each case was purified by VCC using *n*-hexane and increasing quantities of EtOAc (5-100%), this afforded 122.2 mg (61.12%) of eriolin (**11**) [30], mp 234-236°C [CH_2Cl_2/*i*-Pr_2O]. Similar conditions and procedures were followed for the biotransformation of **2** (200 mg) with *F. moliniforme*, to obtain **11** (137.2 mg, 68.6%), while with *B. bassiana* (70%) and *C. blakesleeana* (64%), **11** was obtained in *ca.* 5% yield.

Biotransformation of Budlein B (**3**) *with C. lunata*

To 72 h of growth of *C. lunata* in medium B, budlein B (**3**, 189.7 mg) was dissolved in acetone (10 mL) and distributed in 15 Erlenmeyer cell culture flasks. After of 14 days of incubation, the obtained organic residue (150.4 mg) was purified by VCC and preparative TLC, obtaining **3** (142.3 mg, 75%) and **12** (41.7 mg, 22%). Similar conditions and procedures were followed for the biotransformation of **3** (150 mg) with *A. niger*, to obtain **12** (87.2 mg, 58.1%) [34].

Biotransformation of Ludovicin A (**4**) *with C. lunata*

Ludovicin A (**4**, 202.4 mg), dissolved en 10 mL of Me_2CO, was distributed among 20 Erlenmeyer cell culture flasks containing 125 mL of medium B (pH 7). The biotransformation was stopped after 14 days. The crude residue (188.4 mg) was purified by VCC using *n*-hexane and increasing quantities of EtOAc, yielding recovered **4** (24.3 mg, 12%), **13** (62.7 mg, 31%) and **14** (105.3 mg,

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52%) [30]. Tuberiferin (**13**): white solid (62.7 mg, 31%), mp. 145-147 ºC. IR ν_{max} ($CHCl_3$): 1768, 1675, 1457 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$), δ 6.71 (1H, d, J = 9 Hz, H-1), 6.12 (1H, d, J = 3 Hz, H-13a), 5.91 (1H, d, J = 9 Hz, H-2), 5.43 (1H, d, J = 3 Hz, H-13b), 2.62 (1H, dc, J = 7, 4 Hz, H-4), 2.1 (1H, dd, J = 10, 7 Hz, H-5), 3.98 (1H, t, J = 10 Hz, H-6), 2.58 (1H, m, H-7), 2.13 (1H, m, H-8α), 1.79 (1H, ddd, J = 10, 3 Hz, H-8β), 1.75 (1H, m, H-9β), 1.68 (1H, dd, J = 10, 3 Hz, H-9α), 1.40 (3H, d, J = 4 Hz, H-15), 1.17 (3H, s, H-14). ^{13}C NMR (75 MHz, $CDCl_3$), δ 157.8 (C-1), 126.8 (C-2), 200.4 (C-3), 42.1 (C-4), 52.3 (C-5), 82,0 (C-6), 50.2 (C-7), 21.2 (C-8), 37.2 (C-9), 38.5 (C-10), 138.3 (C-11), 170.1 (C-12), 117.5 (C-13), 19.3 8 (C-14), 14.8 (C-15). EM (IE) *m/z*: 248 [M^+, 1.2], 232 (2.1), 218 (2.1), 189 (48.0), 148 (32.5), 108 (18.0) 93 (31.0), 76 (76.0), 51 (26.0), 40 (15.0).

Biotransformation of Epoxysantamarin (**6**) *with C. lunata*

Compound **6** (100.2 mg) was dissolved in acetone (10 mL) and distributed in 15 Erlenmeyer cell culture flasks containing 2 mL of *A. niger* (48 h of growth) in medium A. After 6 days of incubation, the starting material was consumed and the crude residue was purified by CCV and preparative TLC, recovering **6** (17.8 mg, 17.8 %) and **15** (53.4 mg, 53.3%) [28].

Biotransformation of parthenolide (**7**) *with B. bassiana*

Substrate **7** (200 mg) was dissolved in Me_2CO (10 mL) and distributed among 20 Erlenmeyer cell culture flasks and incubated for 14 days. After this time, the cultures were filtered and pooled; the cells were washed thoroughly with water and the liquid was saturated with NaCl and extracted twice with CH_2Cl_2. Extracts were pooled, dried with anhydrous Na_2SO_4, the organic solvent was removed, and the residue was chromatographed on a silica gel column, affording **7** (24.1 mg, 12.1%), **16** (83.9 mg, 42%) and **17** (19.8, mg, 9.9%). 11βH,13-Dihydroparthenolide (**16**): mp. 134-36 °C. IR ν_{max} ($CHCl_3$): 2980, 2932, 1774 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): 5.15 (1H, dd, J = 12.0, 2.3, H-1), 3.80 (1H, t, J = 10.0, 8.4 Hz, H-6), 2.69 (1H, d, J = 10.0, H-5), 2.37 (1H, dddd, J = 13.0, 13.0, 12.0, 5.0 Hz, H-2β), 2.28 (1H, m, H-7), 2.28 (1H, m, H-8β), 2.27 (1H, dq, J = 10.3, 6.8, H-11β), 2.25 (1H, m, H-9β), 2.11 (1H, dddd, J = 13.0, 13.0, 6.0, 2.3 Hz, H-2α), 2.16 (1H, m, H-3β), 1.80 (1H, m, H-8α), 1.80 (1H, m, H-9α), 1.68 (3H, s, CH_3-14), 1.27 (3H, s, CH_3-15), 1.25 (1H, d, J = 6.8, CH_3-13), 1.21 (1H, dt, J = 13.0, 5.6 Hz, H-3α). ^{13}C NMR (75 MHz, $CDCl_3$): 125.2 (C-1), 24.7 (C-2), 36.7 (C-3), 61.4 (C-4), 66.4 (C-5), 82.2 (C-6), 51.9 (C-7), 29.8 (C-8), 41.2 (C-9), 134.4 (C-10), 42.5 (C-11), 177.3 (C-12), 13.3 (C-13), 16.9 (C-14), 17.1 (C-15).

1,10-Epoxy-11βH,13-dihydroparthenolide (**17**): mp. 146-148°C. IR ν 3686, 2972, 2932, 1780, 1602 cm^{-1}. The HRFABMS established a molecular formula of $C_{15}H_{23}O_4$ [M+H]$^+$, 267.1595, Calcd. 267.1597]. ^1H NMR (500 MHz, δ $CDCl_3$): 3.80 (1H, t, J = 10.0, 8.4 Hz, H-6), 2.83 (1H, d, J = 10.0, H-5), 2.37 (1H, dd, J = 11.9 y 2.3, H-1), 2.37 (1H, dddd, J= 13.0, 13.0, 12.0, 5.0 Hz, H-2β), 2.28 (1H, m, H-7), 2.28 (1H, m, H-8β), 2.27 (1H, dq, J = 10.3, 6.8, H-11β), 2.25 (1H, m, H-9β), 2.16 (1H, m, H-3β), 2.11 (1H, dddd, J = 13.0, 13.0, 6.0, 2.3 Hz, H-2α), 1.80 (1H, m, H-8α), 1.80 (1H, m, H-9α),

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1.68 (3H, s, CH_3-14), 1.27 (3H, s, CH_3-15)1.25 (1H, d, J = 6.8, CH_3-13), 1.21 (1H, dt, J= 13.0, 5.6 Hz,H-3α). ^{13}C NMR ($CDCl_3$, 125 MHz): 63.8 (C-1), 23.8 (C-2), 35.2 (C-3), 60.5 (C-4), 64.5 (C-5), 81.4 (C-6), 51.8 (C-7), 25.5 (C-8), 40.1 (C-9), 60.4 (C-10), 42.7 (C-11), 176.8 (C-12), 12.9 (C-13), 16.9 (C-14), 17.4 (C-15).

Cytotoxicity bioassays

Human tumor cell lines were supplied by National Cancer Institute (NCI), USA. The cytotoxicity of the tumors cells with the test compounds was determined using the protein-binding dye sulforhodamine B (SRB) in microculture assay to measure cell growth. The cell lines were cultured in RPM-1640 (Sigma Chemical Co Ltd. St. Louis, MO, USA), supplemented with 10% fetal bovine serum, 2 μM L-glutamine, 100 IU/mL penicillin G, 100 μg/mL streptomycin sulfate and 0.25 μg/mL amphotericin B (Gibco). They were maintained at 37 °C in a 5% CO_2 atmosphere with 95% humidity. For the assay, 5 x 10^4 cell/mL and 100 μL/ well of these cells suspensions were seeded in 96-well microtiter plates, and incubated to allow the cell attachment. After 24 h, 100 μL the test compounds and positive substances were added to each well, and after 48 h adherent cell cultures were fixed *in situ* by adding 50 μL of cold 50% (wt/vol) trichloroacetic acid (TCA) and incubated for 60 min at 4°C. The supernatant was discarded and the plates were washed three times with water and air-dried. Cultured fixed with TCA were stained for 30 min with 100 μL of 4% SRB solution. Protein bound dye was extracted with 10 μM un buffer tris base and the optical densities were read on an Ultra Microplate Reader (Elx 808, BIO-TBK Instruments, Inc.) with a test wavelength of 515 nm. Results were expressed as inhibitory concentration 50 (CI_{50}) values, they were calculated according to the protocol of Monks [45], where a dose-response curve was plotted for each compound, and the concentration giving 50% inhibition (CI_{50}) was estimated from linear regression equations. Helenanin (IC_{50}= 0.32 ± 0.02 μM against U251) was used as positive control.

Supplementary Material

Supporting information for this article is available on the www under http://dx.doi.org/10.1002/MS-number.

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