Biotransformation of Jatrophone by *Aspergillus niger* ATCC 16404

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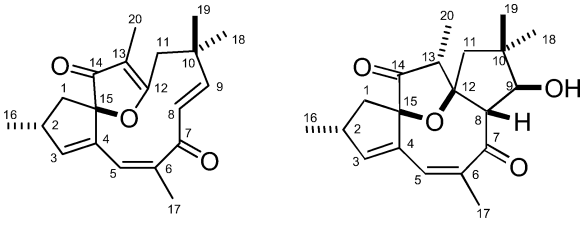
Biotransformation of the diterpene jatrophone (**1**) by *Aspergillus niger* ATCC 16404 afforded the new diterpene 9β-hydroxyisabellione (**2**). The compounds were characterized by spectroscopic analysis. The cytotoxicity of the compounds as IC_{50} values on AGS and lung fibroblasts was 2.4 and 2.8 μM for compound **1** and 53.1 and 260 μM for **2**, respectively. Microbial transformation of **1** into compound **2** strongly reduced the cytotoxicity and enhanced the selectivity against AGS cells.

Key words: Biotransformation, Jatrophone, *Aspergillus niger*

Introduction

The diterpene jatrophone (**1**) (Fig. 1) is the main terpenoid present in the rhizomes of the Euphorbiaceae *Jatropha isabelli* Muell. Arg. Jatrophone was first isolated and described as the antileukemic constituent of *Jatropha gossypiifolia* [1, 2]. Extensive studies on the compound were carried out [2, 3] and several biological activities have been reported for **1**, including the reaction of biological thiols [4], interaction with sRNA from *Escherichia coli* [5], inhibition of insulin release [6], relaxation effect of induced uterine contraction [7], relaxant action in rat portal vein [8], inhibition of lymphocytes activation, probably through inhibition of the protein kinase C pathway [9], antiprotozoal activity [10] and molluscicidal effect [11].

However, no information is available on the possible biotransformation products of this terpenoid. The microbial transformation of jatrophone was planned to obtain new hydroxylated derivatives using *Aspergillus niger*, *Mortierella isabelina* and *Fusarium moniliforme*.



1 **2**

Fig. 1. Structure of the compounds **1** and **2**.

Materials and Methods

General experimental procedures

Melting points were determined on a Kofler hot stage apparatus (Electrothermal 9100) and are uncorrected. Optical rotations were obtained on a Jasco DIP 370 polarimeter, and IR spectra were recorded on a Nicolet Nexus FT-IR instrument. The NMR spectra were recorded on a Bruker Avance 400 NMR spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C, respectively, in $CDCl_3$. MS spectra were measured in a Varian unit at 70 eV. Si gel 60 (Merck, 63 – 200 μm particle size) was used for column chromatography; precoated Si gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) were used for TLC analyses. TLC spots were visualized by spraying the chromatograms with *p*-anisaldehyde-ethanol-acetic acid-H_2SO_4 (2 : 170 : 20 : 10 v/v) and heating at 110 °C for 3 min. Jatrophone (**1**) was isolated from the rhizomes of *Jatropha isabelli* Müll. (Euphorbiaceae) as previously reported [11] in a 0.145 % w/w yield.

Microorganisms

The microorganisms used were either from the American Type Culture Collection (ATCC): *Aspergillus niger* ATCC 16404, *Mortierella isabelina* ATCC 38063 or from the Micoteca Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina: *Fusarium moniliforme* UBA 1061.

Screening scale experiments

Thirty mL of liquid Czapek medium held in 125 mL Erlenmeyer flasks was added with a spore suspension in a first fermentation stage. Stage I cultures were incubated on a rotatory shaker at 250 rpm and 28 °C ± 2 °C for 48 h. As second fermentation stage was initiated by transferring of inoculum from the stage I culture in a final volume of 10 %. After 48 h incubation, 10 mg of substrate (**1**) dissolved in *N*,*N*-dimethylformamide were added to each flask. Samples (5 mL of incubation media) were withdrawn every 12, 24 and 48 h, partitioned with 2 mL of EtOAc and analyzed by TLC. Three experiments with two repetitions each were carried out for every fungal agent. Blank assays without substrate and without fungi were carried out.

Preparative scale experiments and product isolation

Aspergillus niger ATCC 16404 culture. First fermentation stage was performed as it was described for screening experiments. In stage II the substrate (667 mg/L) was added to each of 10 × 1 L Erlenmeyer flasks containing 300 mL of a 72 h old culture. After 24 days, the culture was separated into culture medium and mycelium by filtration. Extraction of the culture filtrates with EtOAc (3 × 500 mL each) after acidification with HCl at pH 5, afforded 400 mg of EtOAc-solubles. TLC analysis of the crude extract (silica gel, PE:EtOAc 7 : 3) showed a main compound, corresponding to jatrophone (R_f = 0.47) and a more polar metabolite (R_f = 0.41). Permeation of the extract on Sephadex LH-20 with MeOH and chromatography of the diterpene mixture on 100 g silica gel (column length 42 cm, internal diameter 1.8 cm) with a PE-PE:EtOAc gradient afforded 30 fractions, pooled together in 5 fraction groups according to the TLC patterns. The first pool did not contain compounds of interest and was discarded. From the fraction pool 2 – 3, some 240 mg of jatrophone was recovered while the pooled fractions 4 – 5 contained a mixture of compounds **1** and **2**. Preparative TLC of the combined pools (silica gel, PE:acetone 8 : 2) yielded 20 mg of jatrophone (**1**, R_f = 0.32) and 13 mg of **2** (R_f = 0.19).

*Jatrophone (**1**):* Colorless crystals, m. p. 154 – 155 °C. – $[\alpha]_D^{20} = +295$ (c = 1.5, $CHCl_3$). – IR (KBr): ν = 2960, 1693, 1660, 1612, 1402 cm^{-1}. – HRMS (ESI): *m/z* = 313.1797 (calcd. 313.1804 for $C_{20}H_{24}O_3H^+$, $[M+H]^+$). – MS (EI): *m/z* (%) = 312.1723 (53) (calcd. 312.1725 for $C_{20}H_{24}O_3$), 297 (11), 284 (29), 269 (21), 242 (48), 227 (22), 213 (25), 199 (17), 189 (100), 188 (31), 175 (43), 173 (61), 160 (62), 147 (37), 145 (31), 125 (25), 91 (33), 83 (36), 81 (89), 77 (25), 69 (25), 53 (91). MS and NMR data in agreement with the structure and with published data.

*9β-Hydroxyisabellione (**2**):* Colorless crystals, m. p. 95 – 97 °C. – $[\alpha]_D^{20} = -76.5$ (c = 0.17, $CHCl_3$). – IR (KBr): ν = 3477, 2927, 1756, 1659, 1462 cm^{-1}. – HRMS (ESI): *m/z* = 353.1736 (calcd. 353.1729 for $C_{20}H_{26}O_4Na^+$, $[M+Na]^+$). – MS (EI): *m/z* (%) = 330.1836 (2) (calcd. 330.1831 for $C_{20}H_{26}O_4$), 313 (12), 312 (49), 297 (22), 269 (18), 203 (12), 189 (11), 164 (18), 163 (100), 149 (11), 137 (11), 135 (17), 131 (12), 107 (14), 91 (29), 79 (12), 77 (13), 69 (11), 55 (15), 53 (11). For the NMR data, see Table 1.

MRC-5 Cell culture

The cytotoxic effect of the assayed compounds, expressed as cell viability, was assessed on a permanent fibroblast cell line derived from human lung (MRC-5) (ATCC CCL-171). MRC-5 fibroblasts were grown as monolayers in minimum essential Eagle medium (MEM), with Earle's salts, 2 mM L-glutamine and 2.2 $g\,L^{-1}$ sodium bicarbonate, supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 100 IU mL^{-1} penicillin and 100 $\mu g\,mL^{-1}$ streptomycin in a humidified incubator with 5 % CO_2 in air at 37 °C. Cell passage was maintained between 10 and 16. The medium was changed every 2nd day.

AGS Cell culture

The cytotoxic effect of the assayed compounds, expressed as cell viability, was assessed on a permanent human epithelial gastric cell line (AGS) (ATCC CRL-1739). The AGS cells were grown as monolayers in Ham F-12 medium containing 1 mM L-glutamine and 1.5 $g\,L^{-1}$ sodium bicarbonate, supplemented with 10 % heat-inactivated FBS, 100 IU/mL penicillin and 100 $\mu g\,mL^{-1}$ streptomycin in a humidified incubator with 5 % CO_2 in air at 37 °C. The

	1		**2**	
	H	C	H	C
1	2.17 dd (13.7; 5.9) 1.88 dd (13.7; 7.8)	42.41 t	2.33 dd (13.2, 6.4) 1.85 dd (13.2, 7.8)	48.26 t
2	3.00 ddq (7.8, 7.0, 5.9)	38.28 d	3.16 ddq br (7.8, 7.1, 6.4)	37.58 d
3	5.83 br s	123.68 d	6.29 br s	154.16 d
4	–	137.05 s	–	139.36 s
5	5.84 br s	147.05 d	6.70 br s	131.86 d
6	–	141.72 s	–	136.71 s
7	–	201.94 s	–	202.08 s
8	6.02 d (16.4)	128.66 d	3.56 d (11.5)	65.97 d
9	6.47 d (16.4)	159.00 d	4.12 d (11.5)	80.08 d
10	–	36.59 s	–	38.05 s
11	2.89 d (14.7) 2.43 d (14.7)	41.17 t	2.07 d (14.7) 1.96 d (14.7)	50.06 t
12	–	183.21 s	–	84.62 s
13	–	112.36 s	2.40 q (7.1)	53.40 d
14	–	203.85 s	–	216.94 s
15	–	99.71 s	–	93.52 s
16	1.10 d (7.0)	18.93 q	1.14 d (7.1)	19.31 q
17	1.90 s	20.69 q	1.87 s	22.04 q
18	1.27 s	30.35 q	1.11 s	21.51 q
19	1.39 s	26.86 q	1.13 s	27.33 q
20	1.77 s	6.00 q	1.01 d (7.1)	7.22 q

Table 1. ^{1}H and ^{13}C NMR data of compounds **1** and **2** (400 and 100 MHz, respectively, $CDCl_3$, δ values in ppm, J values in Hz in parentheses).

cell passage was maintained between 42 and 48. The medium was changed every 2nd day.

Cytotoxicity assay

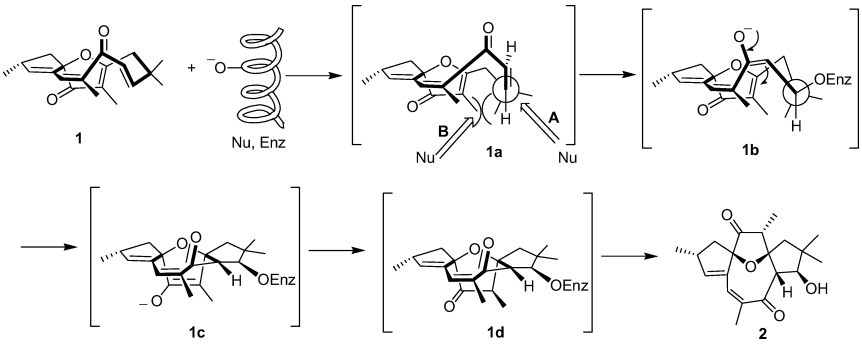
Confluent cultures of MRC-5 as well as AGS cells were treated with medium containing the diterpenes at concentrations ranging from 0 up to 1000 μM. The products were first dissolved in DMSO and then in the corresponding culture medium supplemented with 2 % FBS. The final content of DMSO in the test medium and controls was 1 %. Cells were exposed for 24 h to the test medium with or without the compound (control). Each concentration was tested in quadruplicate together with the control and repeated three times in separate experiments. At the end of the incubation, the neutral red uptake (NRU) assay was carried out [12]. To calculate the IC_{50} values (concentration that produces a 50 % inhibitory effect on the evaluated parameter) the results were transformed to percentage of controls and the IC_{50} values were graphically obtained from the dose-response curves.

Results and Discussion

Microbial transformation of the diterpene jatrophone by *Aspergillus niger* afforded a main compound (**2**) (Fig. 1) with a 0.65 % w/w yield. Under our experimental conditions, neither *Mortierella isabelina* ATCC 38063 nor *Fusarium moniliforme* transformed jatrophone after 21 days culture.

The MS (EI) of compound **2** showed a molecular formula of $C_{20}H_{26}O_4$ indicating 8 unsaturation degrees. The nature of the oxygen functions of **2** followed from the IR spectrum, the main difference to that of jatrophone (**1**) being the presence of a broad absorption band at 3477 cm^{-1}, indicating the presence of OH groups as well as showing two carbonyl absorptions at 1756 and 1659 cm^{-1}, different from that of jatrophone at 1693 and 1660 cm^{-1}. The structure of compound **2** followed from the ^{1}H NMR spectrum which was similar to that of jatrophone. Instead of the pair of doublets at δ = 6.02 and 6.47 (J = 16.4 Hz) for jatrophone, two doublets at δ = 3.56 and 4.12 (J = 11.5 Hz) were observed for compound **2**, suggesting hydroxylation of the double bond. In derivative **2**, a methyl doublet at δ = 1.01 (J = 7.1 Hz) and a CH quartet at δ = 2.40 appeared instead of the olefinic methyl signal at δ = 1.77, indicating the presence of a CH_3–CH sequence.

In the ^{13}C NMR and DEPT spectra of **2** instead of the signals corresponding to the sp^2 CH at δ = 128.66 and δ = 159.00 as well as the sp^2 C at δ = 183.21 and δ = 112.36 of jatrophone, two sp^3 CH resonances at δ = 65.97 and δ = 80.08, one sp^3 C signal at δ = 84.62 and one sp^3 CH signal at δ = 53.40 were observed. Therefore, derivative **2** contains one OH function, and



Scheme 1. Proposed mechanism for the oxidative cyclization of jatrophone mediated by *Aspergillus niger*.

an additional ring is required according to the molecular formula. The placement of the OH group followed from the chemical shifts of the C atoms and HMBC correlations. The coupling constants for the doublets at $\delta = 3.56$ and $\delta = 4.12$ ($J = 11.5$ Hz) suggest a *trans* relationship similar to that observed for the propanethiol adduct of jatrophone [2, 3]. Therefore, the OH group at C-9 and the CH_3 group at C-20 should be β-oriented.

Compound **2** obtained by microbial transformation of jatrophone with *Aspergillus niger* was not reported previously. As the parent compound was obtained from *Jatropha isabelli*, the name 9β-hydroxyisabellione is proposed for compound **2**.

Several studies reported the successful use of microorganisms for bioconversion of diterpenes. They comprise the biotransformation of stemodine by *Rhizopus oryzae* [13], the transformation of isosteviol by *Aspergillus niger*, *Glomerella cingulata* and *Mortierella elongata* [14], biotransformation of baccatin and 1β-hydroxybaccatin I with *Aspergillus niger* [15], microbial transformation of a pimarane derivative with *Gibberella fujikuroi* [16], dehydroabietanol and teideadiol by *Mucor plumbeus* [17], stemodin and several *Stemodia* diterpenes with *Aspergillus niger* [18], *Mucor plumbeus* and *Whetzelinia sclerotiorum* [19].

Recently reported diterpene biotransformations by *A. niger* included the hydroxylation of dehydroabietic acid [20] solidagenone [21] grindelic acid derivatives [22], terpenes from *Stemodia maritima* [18] and isosteviol [23].

The new compound is related to the thiol adducts prepared when studying the reaction of biological thiols with jatrophone [4], which exhibited tumor inhibitory activity.

The stereochemical outcome of the above reaction appears to be an example of stereoselective tandem Michael reactions that led us to rationalize the preferential formation of **2** as the sole product (Scheme 1). Surprisingly, no other regioisomer was formed upon the biocatalysis with *Aspergillus niger* which is explained by conformational restriction of the jatrophone **1** and the sterically hindered oxidase enzyme nucleophile. The first step is rationalized as the oxidase addition of an RO^- group leading regioselectively to the *E*-enolate **1b**. Assuming conformation **1** to be the preferred one for jatrophone, the stereoselectivity depends on the differentiation of the diastereotopic faces of the α,β-unsaturated ketone in **1a** by groups that control the trajectory of the enzyme attack. The Newman projection of **1** (Scheme 1) shows two possible pathways for nucleophilic addition: less hindered **1aA** favored by oxidase attack, and **1aB** disfavored by stereoelectronic effects. Additionally, DFT calculations (B3LYP/6-31G) of the geometries associated with the oxidase addition corroborated that the more stable conformer is **1** (12 kcal mol^{-1}). As depicted in Scheme 1, it affords the *E*-enolate (**1b**) and allows the concomitant generation of the first stereogenic center produced in the Michael-type process through **1aB**. An additional driving force for the cyclization is that the process is exothermic with respect to the products.

The *E*-enolate **1b** also presents stereoelectronic conformer restrictions associated with unfavorable oxygen-oxygen lone pair interactions between butenolidone and enolate moieties. Enolate **1b** attacks selectively the uniquely available *si* face of the butenolide (or the oxonium ion). The enolate attack also shows an antiperiplanar approach of the π systems of the enolate nucleophile and the butenolide double bond. Synclinal arrangement would be preferred for the transition state presented by the oxonium ion intermediate and, therefore, cannot be discarded. The stereogenic centers formed in this manner yield selectively the stereochemistry *trans* as shown in intermediate **1c**.

Finally, hydrolysis of the previously formed enolate **1c** by the abstraction of hydrogen from water or from the acidic medium on its *si* face gives the intermediate **1d** which after enzyme release affords **2** as the sole product of the reaction as depicted in Scheme 1.

The cytotoxicity of the compounds, as IC_{50} values on AGS and human lung fibroblasts, was 2.4 and 2.8 μM for compound **1** and 53.1 and 260 μM for **2**, respectively. Microbial transformation of **1** into **2** strongly reduced the cytotoxicity and enhances the selectivity against AGS cells as can be deduced from the IC_{50} values. Further studies should be undertaken to disclose the cytotoxic effects and the selectivity of the new compound on a panel of tumor cells.

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