Biotransformation of ent-pimaradienoic acid by cell cultures of Aspergillus niger


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ABSTRACT

Microbial transformation stands out among the many possible semi-synthetic strategies employed to increase the variety of chemical structures that can be applied in the search for novel bioactive compounds. In this paper we obtained ent-pimaradienionic acid (1, PA, ent-pimara-8(14),15-dien-19-oic acid) derivatives by fungal biotransformation using Aspergillus niger strains. To assess the ability of such compounds to inhibit vascular smooth muscle contraction, we also investigated their spasmylic effect, along with another five PA derivatives previously obtained in our laboratory, on aortic rings isolated from male Wistar rats. The microbial transformation experiments were conducted at 30 °C using submerged shaken liquid culture (120 rpm) for 10 days. One known compound, 7α-hydroxy ent-pimara-8(14),15-dien-19-oic acid (2), and three new derivatives, 1β-hydroxy ent-pimara-6,8(14),15-tetra-19-oic acid (3), 1x,6x,14β-trihydroxy ent-pimara-7,15-dien-19-oic acid (4), and 1x,6x,7α,11x-tetrahydroxy ent-pimara-8(14),15-dien-19-oic acid (5), were isolated and identified on the basis of spectroscopic analyses and computational studies. The compounds obtained through biotransformation (2–5) did not display a significant spasmylic activity (values ranging from 0% to 16.8% of inhibition); however the previously obtained diterpene, methyl 7α-hydroxy ent-pimara-8(14),15-dien-19-oate (8), showed to be very effective (82.5% of inhibition). In addition, our biological results highlight the importance to study the antispasmodic potential of a large number of novel diterpenes, to conduct further structure–activity relationship investigations.

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1. Introduction

The plant kingdom is a rich and promising source of novel biologically active chemical entities. Not only can natural compounds serve as new drugs themselves, but they can also be suitable prototypes to optimize drug structure via a number of semi-synthetic strategies, generating compounds with biological properties distinct from those of the parent structure.

Microbial transformation stands out among the many possible semi-synthetic strategies employed in drug discovery: it increases the variety of chemical structures that can be applied in the search for novel bioactive compounds, leading to new sustainable routes to synthesize fine chemical and drugs. Microbial enzymatic systems can perform highly regio- and stereoselective reactions at non-activated carbons, which is difficult to achieve by classic organic chemistry. Strains of Aspergillus, for example, can introduce hydroxyl groups into different classes of bioactive natural compounds, such as terpenoids, flavonoids, coumarins, and lignans.

Diterpenes are a class of plant-derived compounds with a wide spectrum of biological actions—they possess antinociceptive and anti-inflammatory activities, antiparasitic, antimicrobial, and antioxidant activities. In particular, ent-pimaradienionic acid (PA, ent-pimara-8(14),15-dien-19-oic acid), a diterpene with a well-established antibacterial effect, has also attracted considerable attention from the scientific community because it inhibits phenylephrine-, KCl-, and CaCl2-induced vascular contraction. Therefore, PA might be potentially applicable in the treatment of arterial hypertension.

Considering the semi-synthetic potential of fungal biotransformation and as part of our ongoing efforts to explore the antihypertensive potential of diterpenes, in this paper we obtained PA derivatives by fungal biotransformation using Aspergillus niger.
strains. To assess the ability of such compounds to inhibit vascular smooth muscle contraction, we also investigated their spasmyotic effect, along with another five PA derivatives previously obtained in our laboratory, on aortic rings isolated from male Wistar rats.

2. Materials and methods

2.1. General procedures

The NMR spectra were run on a Bruker DPX 400 spectrometer (400 MHz for $^1$H and 100 MHz for $^{13}$C). Samples were dissolved in CDCl$_3$, and the spectra were calibrated with the solvent signals at $\delta$ 7.26 ($^1$H) and $\delta$ 77.0 ($^{13}$C). The biotransformation procedures were performed on a Cientec CT-713 rotary shaker. The high-resolution ESI-MS spectra were recorded on an Ultro-TOF Bruker instrument using a positive ion mode. Optical rotation of novel compounds were measured in CHCl$_3$ in a JASCO DIP-370 polarimeter.

2.2. Substrate and microorganisms

ent-Pimara-8(14),15-dien-19-oic acid (PA, 1, 600.0 mg) was isolated from the dichloromethane extract of Viguiera arenaria roots, as previously described by Ambrósio et al. The A. niger strain used in the biotransformation processes was provided by Prof. Dr. Suraia Said from the Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Brazil. This microorganism was maintained as a conidial suspension on silica gel (6-12 mesh, grade 40, desiccant-activated), at 4 °C.

2.3. Microbial transformation

The microbial transformation experiments were conducted using submerged shaken liquid culture and two-stage fermentation procedures. An inoculum of 2.1 × 10$^6$ conidia mL$^{-1}$ was aseptically added to seven Erlenmeyer flasks (1000 mL) containing 500 mL of seed medium, as previously described in the literature. Cultures were incubated at 30 °C using a shaker operating at 120 rpm. After 48 h of incubation, the resulting mycelia were harvested, rinsed, and aseptically transferred to seven Erlenmeyer flasks (2000 mL) containing 1000 mL of sterile medium consisting of sucrose (3.0%), NaNO$_3$ (15.0); Glucose, 5.5; and CaCl$_2$ (0.001%). After 24 h, 25 mL PA in dimethylsulfoxide (DMSO) was added to each flask, and cultures were incubated for 4–5 mm long. Two stainless-steel stirrups were placed through the lumen of each ring. One stirrup was connected to an isometric force transducer, to measure tension in the vessels. The rings were placed in a 5 mL organ chamber containing Krebs solution, gassed with 95% O$_2$/5% CO$_2$, and maintained at 37 °C. The Krebs solution consisted of (mmol L$^{-1}$): NaCl, 118.0; KCl, 4.7; KH$_2$PO$_4$, 1.2; MgSO$_4$, 1.2; NaHCO$_3$, 15.0; Glucose, 5.5; and CaCl$_2$, 2.5. After pH checking (pH 7.0), the rings were stretched until they reached a basal tension of 1.0 g, determined by length-tension relationship experiments, and were allowed to equilibrate for 60 min; during this time, the bath fluid was changed every 15–20 min. Endothelial integrity was assessed qualitatively by the degree of relaxation caused by acetylcholine (1 μmol L$^{-1}$) in the presence of contractile tone induced by phenylephrine (0.1 μmol L$^{-1}$). To study endothelium-intact vessels, a ring was discarded if relaxation with acetylcholine was not 80% or greater.

2.4. Isolation of diterpenes

AnE was first chromatographed over silica gel 60H (Merck, art. 7736) using vacuum liquid chromatography; an increasing gradient of EtOAc in n-hexane was used as eluent, which afforded seven fractions of 300 mL each: AnE1 (13.4 mg; n-hexane), AnE2 (30.2 mg; 20% EtOAc), AnE3 (201.0 mg; 40% EtOAc), AnE4 (112.5 mg; 60% EtOAc), AnE5 (189.3 mg; 80% EtOAc), AnE6 (125.0 mg; EtOAc), and AnE7 (975.5 mg; MeOH). Fraction AnE3 was initially partitioned by medium pressure chromatography (flash chromatography) using an isocratic n-hexane/EtOAc 4:1 mixture as the mobile phase; five new fractions were obtained (AnE3.1-AnE3.5). 7α-Hydroxy ent-pimara-8(14),15-dien-19-oic acid (2; 42.0 mg) was isolated from fraction AnE3.2 (85.0 mg) after classic chromatography (n-hexane/EtOAc 7:3 + 1% HOAc). TLC analysis (Whatman, 250.0 μm layer) of AnE4 revealed a main spot that was purified by flash chromatography (n-hexane/CHCl$_3$ 1:1 + 1% HOAc), to furnish diterpene 3 (87.5 mg; 1β-hydroxy ent-pimara-8(14),15-trien-19-oic acid). AnE5 was chromatographed by classic chromatography (n-hexane/EtOAc 1:4), which yielded 60 fractions of 10 mL each; these fractions were grouped into six fractions (AnE5.1–AnE5.6) after TLC analysis, and fraction AnE5.4 corresponded to the diterpene 1x,6δ,14β-trihydroxy ent-pimara-7,15-dien-19-oic acid (4; 40.0 mg). Finally, compound 5 (33.8 mg; 1α,6β,7 α,11α-tetrahydroxy ent-pimara-8(14),15-dien-19-oic acid) was isolated from fraction AnE6 by flash chromatography using n-hexane/EtOAc 2:3 + 1% HOAc as eluent. The $^1$H NMR spectra of fraction AnE7 did not display characteristic signals of ent-pimarane-type diterpenes.

2.5. Preparation of endothelium-intact vessels

Male Wistar rats weighing between 200 and 250 g (50–60 days old) were anesthetized and killed by aortic exsanguinations in accordance with the Ethical Animal Committee from the University of Franca. The thoracic aorta was quickly removed, cleaned of adherent connective tissues, and cut into rings (each one measuring 4–5 mm long). Two stainless-steel stirrups were passed through the lumen of each ring. One stirrup was connected to an isometric force transducer, to measure tension in the vessels. The rings were placed in a 5 mL organ chamber containing Krebs solution, gassed with 95% O$_2$/5% CO$_2$, and maintained at 37 °C. The Krebs solution consisted of (mmol L$^{-1}$): NaCl, 118.0; KCl, 4.7; KH$_2$PO$_4$, 1.2; MgSO$_4$, 1.2; NaHCO$_3$, 15.0; Glucose, 5.5; and CaCl$_2$, 2.5. After pH checking (pH 7.0), the rings were stretched until they reached a basal tension of 1.0 g, determined by length-tension relationship experiments, and were allowed to equilibrate for 60 min; during this time, the bath fluid was changed every 15–20 min. Endothelial integrity was assessed qualitatively by the degree of relaxation caused by acetylcholine (1 μmol L$^{-1}$) in the presence of contractile tone induced by phenylephrine (0.1 μmol L$^{-1}$). To study endothelium-intact vessels, a ring was discarded if relaxation with acetylcholine was not 80% or greater.

2.6. Effect of PA derivatives on phenylephrine-induced rat aorta contractions

After equilibration, cumulative concentration–response curves for phenylephrine (10$^{-10}$ – 10$^{-5}$ M) were constructed. The curves were obtained using endothelium-intact rings and applying a stepwise increase in phenylephrine concentration. Additions were made as soon as a steady response was reached at the preceding concentration. The curves were determined without pre-incubation (control group) or after 60-min incubation with PA derivatives at a concentration of 100 μM, as previously described by our research group for PA. The maximum effect elicited by the tested compound (E$_{max}$) in the absence (control group) or presence of PA derivatives was used as the pharmacological parameter to calculate the percentage of inhibition for each compound. The diterpenes which promoted a percentage of inhibition that was not statistically different from its E$_{max}$ as compared with the control group were considered to promote 0% inhibition.

2.7. Statistical analysis

Statistical differences between the E$_{max}$ values were calculated using one-way analysis of variance (ANOVA). Post-hoc comparisons were performed after ANOVA analysis, using a Newman–Keuls
multiple comparison test. Data are expressed as the mean ± S.E.M.; P values of less than 0.05 were considered significant in all the analyses. Data represent the mean of responses observed in vascular rings of six to seven animals.

2.8. Computational methods

We used the program package Gaussian09, the B3LYP hybrid functional, and Dunning's cc-pVDZ basis set to optimize the full geometry and calculate the vibrational frequency. The nature of the stationary point was determined using the eigenvalues obtained from the harmonic vibration analysis. ¹H and ¹³C NMR chemical shift values were calculated within the Gauge-Independent Atomic Orbital (GIAO) using the ‘mixed’ option, which requires stepwise calculation of spin–spin coupling constants and includes the Fermi Contact contribution, improving the final results. All the calculations were performed at the B3LYP/cc-pVDZ level of theory. In order to compare the GIAO isotropic magnetic shielding (IMS) values with experimental chemical shifts, the NMR parameters of the reference molecule tetramethylsilane (TMS) were calculated at the same level of theory. The hydroxyl group at C-1 and considering the coupling constants of the germinal proton in the ¹H NMR spectrum of compound 1 presented the characteristic PA signals corresponding to the vinylic protons H-14 (J = 11.5, 4.9 Hz), H-16a (J = 10.2 Hz). In compound 2, the ¹H–¹H COSY, HMBC, HMQC, and NOE; computational methods also supported the spectrometric identification of the novel diterpenes. Tables 1 and 2 list the ¹H and ¹³C NMR chemical shifts; Figures 2 and 3 represent the main HMBC and NOE correlations, respectively.

Several authors have investigated the microbial transformation of natural compounds by Aspergillus strains; these species promote the introduction of hydroxyl groups into various plant-derived products. Here, we found that A. niger catalyzed the hydroxylation of non-activated carbons in the chemical structure of PA, mainly at C-1, C-6, C-7, C-11, and C-14 (Fig. 1), which is difficult to achieve by classic synthetic methodologies and represents an important strategy to introduce a reactive center into the poorly functionalized PA skeleton. Besides this reaction, A. niger also promoted dehydrogenation at C-6 and C-7 in compound 3, as well as transmigration of the double bond from C-8/C-14 to C-7/C-8 in compound 4. The whole process yielded about 8, 16.7, 6.9, and 5.6% of compounds 2, 3, 4, and 5, respectively, on the basis of their weights relative to the starting substrate.

We obtained compound 3 (colorless and amorphous solid, [a]D 25 = -410, c 0.0013, CHCl₃); we determined its molecular formula as C₂₀H₂₅O₂ using HR-ESIMS, which evidenced an increment of 14 a.m.u. relative to PA. The ¹H NMR spectrum of compound 3 (Table 1) presented the characteristic PA signals corresponding to the vinylic protons H-14 (δH 5.29, 1H, s(br)), H-15 (δH 5.71, 1H, dd, J = 17.2, 10.2 Hz), H-16a (δH 4.84, 1H, dd, J = 17.2, 1.7 Hz), and H-16b (δH 4.96, 1H, dd, J = 10.2, 1.7 Hz); we also detected the typical methyl protons H-17 (δH 1.05), H-18 (δH 1.31), and H-20 (δH 0.68). The signal relative to one oxymethine proton appeared at δH 3.57 (1H, dd, J = 11.4, 4.9 Hz). The HMBC spectrum revealed long-range correlations, particularly between H-20 (δH 0.68) and the carbinolic carbon at δH 78.2, suggesting the presence of a hydroxyl group at C-1. We assigned the α-orientation to OH-1, on the basis of data previously reported for correlated compounds containing a hydroxyl group at C-1 and considering the coupling constants of the germinal proton in the ¹H NMR spectrum. Computational methods corroborated this assignment: the coupling constant calculated for H-1 (J = 11.6, 5.5 Hz) was quite similar to the experimental value (J = 11.5, 4.9 Hz). Compared with PA, the ¹H NMR spectrum of compound 3 also indicated the presence of two olefinic protons at δH 6.04 (1H, dd, J = 10.2, 1.7 Hz) and δH 5.97 (1H, d, J = 10.2 Hz). In

![Figure 1. Chemical structures of the pimarane-type diterpenes 1-5.](image-url)
the HMBC spectra (Fig. 2), the proton resonance at $\delta_{H} 5.97$ correlated with C-8 ($\delta_{C} 135.9$), while the chemical shift of the hydrogen at $\delta_{H} 6.04$ correlated with C-5 ($\delta_{C} 54.2$), attesting to the presence of a double bond between C-6 and C-7. Therefore, we established the structure of metabolite 3 as $1b$-hydroxy ent-pimara-6,8(14),15-trien-19-oic acid.

Using HR-ESIMS, we determined a molecular formula of $C_{20}H_{30}O_{5}$ for compound 4 ($\delta_{D} = 138.25$, c 0.0014, CHCl$_3$); this represents an increment of 48 a.m.u. compared with PA and suggests that compound 4 contains three hydroxyl functionalities. The $^1H$
NMR (Table 1) spectrum of compound 4 displayed the characteristic signals of PA resonance at $\delta_H 5.66$ (1H, dd, $J = 17.2, 10.4$ Hz), 4.97 (1H, dd, $J = 17.2, 1.7$ Hz), 5.07 (1H, dd, $J = 10.4, 1.7$ Hz), 1.08 (3H, s), 1.29 (3H, s), and 0.89 (3H, s), attributed to H-15, H-16a, H-16b, H-17, H-18, and H-20, respectively. The $^1$H NMR (Table 1) and $^{13}$C NMR (Table 2) data of compound 4 also revealed three oxymethine protons, at $\delta_H 3.70$ (1H, dd, $J = 5.1, 2.2$ Hz); such protons do not exist in the chemical structure of PA. The HMBC spectrum of compound 4 (Fig. 2) evidenced that the proton resonance at $\delta_H 4.37$ correlated with C-2 (58.8), C-3 (73.3), C-4 (82.5), and C-10 (40.5). We attributed the proton at $\delta_H 4.57$ to H-7, on the basis of the NOE enhancement observed for this hydrogen and the protons H-6 (58.8), H-7 (4.57, low intensity), and H-18 (3H, s, $\delta_H 1.28$) indicated that the hydroxyl group at C-6 is $\alpha$-oriented. The coupling constants of the oxymethine protons were close to the values obtained by computational methods (Table 1), reinforcing the relative stereochemistry proposed for the OH functionalities introduced by A. niger in the chemical skeleton of PA. The above findings led us to identify compound 5 as $1\alpha,6\beta,7\alpha,11\beta$-tetrahydroxy ent-pimar-8(14),15-dien-19-oic acid.

### 3.2. Effect of PA derivatives on phenylephrine-induced rat aorta contraction

We evaluated the ability of the PA derivatives (2–10) to inhibit the rat aorta contraction induced by phenylephrine, an $\alpha_1$-selective adrenergic agonist. Table 3 summarizes the % of inhibition of such compounds.

Smooth muscle cells in blood vessel walls contain calcium channels; activation of $\alpha_1$-adrenergic receptors in these channels promotes calcium influx and elicits contractile response through two different molecular mechanisms: regulation of inositol phospholipid hydrolysis and prompt contractions irrespective of extracellular Ca$^{2+}$ by controlling the opening of membrane channels that allow influx of extracellular Ca$^{2+}$.30,31

Analysis of the results depicted in Table 3, allow us to conclude that compounds 2, 3 and 6–10 were able to modify the drug-receptor binding or influence the intracellular transduction signals. These results, in addition to the studies previously reported in the literature,30,32,33 reinforce the importance of this class of natural products in the search for novel effective compounds to inhibit vascular smooth muscle contraction and further application in the treatment of hypertension.

Our research group has demonstrated that minor modification in the chemical structure of diterpenes, mainly in ent-pimaranes and ent-kaurenes, significantly affects their antispasmodic properties.30,33 In these studies we also have pointed out the importance to investigate a great number of chemical correlated diterpenoids, aiming to better understand the structure-activity relationship associated with this biological property.

Scientific reports have demonstrated that the presence of one hydrogen bond donor (HBD) group, such as hydroxyl, carboxylic acid, or aldehyde functions, in the chemical skeleton of ent-kaurene and ent-pimarane-type diterpenes is a very important structural requirement related with their antispasmodic activity.30,33 However, a careful observation of the results obtained in this study (Table 3), allow us to propose additional trends in the structure-activity relationship presented by such compounds. Comparison of the inhibition elicited by PA (1: 73.2%) and 9 (58.8%) with the value displayed by 10 (10.5%), lead us to conclude that the

### Table 3

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<th>Compounds</th>
<th>% of inhibition</th>
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<tr>
<td>PA (1)*</td>
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<td>61.5</td>
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*% of inhibition displayed by PA previously reported (Tirapelli et al.27).
presence of one hydrogen bond acceptor (HBA) group at C-19, instead of an HBD group, drastically diminishes the activity of ent-pimarane diterpenes with antispasmodic properties. In general, this report highlights the importance to study novel ent-pimarane and ent-kaurane diterpenes in a structure-activity context, to promote the total elucidation of the structural features involved in the antispasmodic activity displayed by this class of natural compounds.

4. Conclusions

Incubation of ent-pimara-8(14),15-dien-19-oic acid (PA, 1) with A. niger, afforded three new compounds. The main microbial reactions involved hydroxylation, dehydrogenation between C-6 and C-7, and transmigration of the double bond from C-8/C-14 to C-7/C-8. The two latter reactions are not common in microbial transformation promoted by Aspergillus species and denote that the biotransformation process based on A. niger strains increases the diversity of chemical entities. Among the evaluated compounds, only diterpene methyl 17β-hydroxy-ent-pimara-8(14),15-dien-19-oate (8) showed to be more effective than PA, thus denoting that this metabolite could potentially exert antihypertensive effects in vivo. In a structure-activity relationship context, our results highlight that it is important to study the antispasmodic potential of a large number of novel ent-pimarane and ent-kaurane diterpenes, to promote the total elucidation of the structural features involved in the antispasmodic activity displayed by this class of natural products.

Acknowledgment

The authors wish to thank FAPESP (Process No. 2007/54762-8 and 2009/12458-6), CAPES and CNPq for funds and grants.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.07.009.

References and notes