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Differential metabolism of diastereoisomeric diterpenes by *Preussia minima*, found as endophytic fungus in *Cupressus lusitanica*

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ABSTRACT

The plant diastereoisomeric diterpenes *ent*-pimara-8(14)-15-dien-19-oic acid, obtained from *Viguiera arenaria*, and isopimara-8(14)-15-dien-18-oic acid, isolated from *Cupressus lusitanica*, were distinctly functionalized by the enzymes produced in whole cell cultures of the fungus *Preussia minima*, isolated from surface sterilized stems of *C. lusitanica*. The *ent*-pimaradienoic acid was transformed into the known 7β-hydroxy-*ent*-pimara-8(14)-15-dien-19-oic acid, and into the novel diterpenes 7-oxo-8 β-hydroxy-*ent*-pimara-8(14)-15-dien-19-oic and 7-oxo-9β-hydroxy-*ent*-pimara-8(14)-15-dien-19-oic acids. Isopimara-8(14)-15-dien-18-oic acid was converted into novel diterpenes 11α-hydroxyisopimara-8(14)-15-dien-18-oic acid, 7β,11α-dihydroxyisopimara-8(14)-15-dien-18-oic acid, and 1β,11α-dihydroxyisopimara-8(14)-15-dien-18-oic acid, along with the known 7β-hydroxyisopimara-8(14)-15-dien-18-oic acid. All compounds were isolated and fully characterized by 1D and 2D NMR, especially ^{13}C NMR. The diterpene bioproduct 7-oxo-9β-hydroxy-*ent*-pimara-8(14)-15-dien-19-oic acid is an isomer of sphaeropsidin C, a phytotoxin that affects cypress trees produced by *Shaeropsis sapinea*, one of the main phytopathogen of *Cupressus*. The differential metabolism of the diterpene isomers used as substrates for biotransformation was interpreted with the help of computational molecular docking calculations, considering as target enzymes those of cytochrome P450 group.

1. Introduction

Cupressus lusitanica is one of the most widely distributed species of Cypress family plants (Cupressaceae family). They are grown in many tropical and subtropical areas for ornamental purposes, mainly used as Christmas trees, and the wood is used for construction purposes [1]. The essential oil of *C. lusitanica* leaves have been evaluated as antimicrobial [2] and antidermatophytic [3]. Only few studies on the chemical composition of its organs and leaves essential oil are found in the literature. Among the compounds accumulated by *C. lusitanica* are terpenoids [4], including monoterpenes, sesquiterpenes and diterpenes, biflavonoids [5], and lignans [6]. The trunk bark and the resin of *C. lusitanica* collected in our region (São Paulo state) was found to contain the diterpenes isopimara-8(14)-15-dien-18-oic acid, sugiol, and huge amounts of E and Z $\Delta^{12(13)}$-communic acids (unpublished results).

C. lusitanica is included in our research program aimed at studying the biochemical aspects of plant colonization by endophytic microorganisms, with emphasis on terpenoidal compounds. We recently described four major sesquiterpenes produced by one *Xylaria* species [7], and found that this fungus can accumulate diterpenes in its essential oil [8] and it is also able to metabolize exogenous carboxylic acids [9]. While some fungal species can be found associated with plants of genus *Cupressus* without causing diseases [7,8,10], others are pathogenic and can cause the development of "Cypress Canker", which reduces its commercial value or result in plant death [11,12]. *Sphaeropsis* and *Seiridium* species have been reported as the most etiological agents identified cause of this disease [12,13], which is spreading worldwide faster than ever [12]. The results of the chemical study of a *Sphaeropsis* species allowed the identification of diterpenes phytotoxic to some Cypress and to Oak [14]. These findings represent an important biochemical aspect, since these phytotoxins show the same isopimarane carbon skeleton of common diterpenes found in the host plant such as the isopimara-8(14)-15-dien-18-oic acid. It seems that terpenoidal compounds, not only diterpenes but also monoterpenes such as β-thujaplicin [15] plays an important role in interactions between plants and pathogens. In view of this evidence, we were encouraged to study the

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metabolism of terpenoids by fungi collected in *C. lusitanica*.

The biotransformation capability of *Preussia minima*, a fungus identified in one of the last collection group of endophytic fungi isolated from *C. lusitanica*, toward the isopimarane diterpene isopimara-8(14)-15-dien-18-oic acid and its isomer *ent*-pimara-8(14)-15-dien-19-oic acid was evaluated in this work.

2. Experimental section

2.1. General experimental procedures

^{1}H, ^{13}C, and 2D-NMR data were recorded on Bruker DRX 400 spectrometers in $CDCl_3$ solution with tetramethylsilane (TMS) as the internal standard. High resolution mass spectrometry analyses were obtained on a Thermo Scientific LTQ Orbitrap Velos Thermo with an ESI ion source. Preparative reversed-phase HPLC separations were achieved using a Luna Phenyl Hexyl column (21.2 × 250 mm, 10 μm) mounted in a Shimadzu SIL-20AP VP coupled with a SPD-20AV ultraviolet and RID-10A refractive index detector systems. Medium pressure column chromatography (MPLC) was performed on a Combiflash RF 200 medium pressure chromatography system from Teledyne-Isco, using RediSep gold silica gel packed columns. TLC was carried out on alumina-foil pre-coated silica gel GF254 plates. Spots were visualized under UV light or by spraying with 10% sulfuric acid in EtOH followed by heating.

The organic solvents dichloromethane, ethyl acetate, methanol, and ethanol were of analytical grade and were obtained from the Synth (Diadema, SP, Brazil) or Quimis (Indaiatuba, SP, Brazil). For HPLC separations, chromatographic grade acetonitrile and methanol (Panreac, Barcelona, Spain) were used. All other chemicals were purchased from the Sigma-Aldrich Chemical, and used without further purification.

2.2. Plant diterpenes isolation

The diterpene *ent*-pimaradienoic acid (**1**, 2.0 g) was isolated from the ethanol extract obtained from 2.5 kg of *Viguiera arenaria* roots, as previously described by Ambrósio et al. [16]. For isolation of isopimara-8(14)-15-dien-18-oic acid (**2**), 950 g of dried and powered trunk bark of *Cupressus lusitanica* were extracted with dichloromethane. The extract (57.7 g) was chromatographed over silica gel under reduced pressure, suing Hexane-ethyl acetate (0.01% formic acid) gradient elution. The fraction eluted with 10% ethyl acetate (2.0 g) was further purified in a medium pressure liquid chromatography system (MPLC, CombiFlash) resulting in 731 mg of diterpene **2**.

2.3. Microorganisms

The fungus *Preussia minima* was isolated from health leaves of *Cupressus lusitanica* collected in São Carlos, São Paulo State, Brazil. A voucher specimen (No. 7281) has been deposited in the Herbarium of the Botanic Department of Universidade Federal de São Carlos, Brazil. It is also deposited in the Laboratório de Bioquímica Micromolecular de Microorganismos (LaBioMMi) - Department of Chemistry, Universidade Federal de São Carlos, Brazil (code LaBioMMi834), and in the Coleção Micológica de Lavras (CML), Department of Phytopathology - Universidade Federal de Lavras, MG (code CML2399). The method of surface sterilization employed in this work was similar to that used by Petrini et al. [17]. After collection, the leaves were washed in abundant water (domestic use grade) and then in distilled water. The leaves were surface sterilized by consecutive immersion in 70% ethanol (2 s), sterile distilled water (2 s), 11% aqueous sodium hypochlorite for 1–5 min and 70% ethanol (2 s), and then in sterile distilled water. The material was placed in Petri dishes containing PDA medium (potato, dextrose and agar) supplemented with $100\,\mu g\,mL^{-1}$ terramycin and incubated at room temperature. Endophytic fungi growing from the plant tissues were picked and re-cultured on PDA to determine culture purity. The fungus were identified based on ITS sequencing (internal transcribed spacer 1 - ITS 1, 5.8S and ITS 2) and deposited under the code LaBioMMi834 at LaBioMMi - Laboratório de Bioquímica Micromolecular de Microorganismos - Department of Chemistry, Universidade Federal de São Carlos, Brazil; and CML2399, at Department of Phytopathology - Universidade Federal de Lavras, MG.

2.4. Biotransformation experiment

The fungus was grown in rotary shaking (128 rpm) at 25 °C in 250 mL Erlenmeyer flasks containing liquid medium (70 mL per flask) composed of glucose ($30\,g\,L^{-1}$), $NaNO_3$ ($3.0\,g\,L^{-1}$), K_2HPO_4 ($1.0\,g\,L^{-1}$), $MgSO_4{\cdot}7H_2O$ ($0.5\,g\,L^{-1}$), KCl ($0.5\,g\,L^{-1}$), $FeSO_4{\cdot}7H_2O$ ($0.01\,g\,L^{-1}$) and yeast extract (20*g*), dissolved in 1.0 L of distilled water and autoclaved at 121 °C. After 24 h of fungal growth, each diterpene [**1** (200 mg) and **2** (110 mg)] were dissolved separately in 20 mL methanol, added to each of the 20 flasks (1.0 mL each), and placed on a rotatory shaker (128 rpm, 25 °C) for fermentation. Parallel control experiments were conducted which included fungus growing without substrate and medium without fungus. After 20 days, the culture medium was filtered and extracted with ethyl acetate (3 × 350 mL). The extract was combined and dried over anhydrous Na_2SO_4, evaporated under reduced pressure, and the crude residues obtained were analyzed by thin layer chromatography.

2.5. Products purification

The extracts obtained (155 mg for substrate **1** and 135 mg for substrate **2**) were chromatographed on MPLC using Hexane 5–65% ethyl acetate with 0.001% formic acid gradient elution to give 26 and 29 fractions. Based on the TLC analysis, the metabolites of diterpenes **1** and **2** were detected in Fr. 14–18 and 13–16, respectively. Fractions containing biotransformed diterpenes were purified by preparative HPLC (mobile phase: 65% acetonitrile in 0.03% aqueous formic acid; flow rate: 8.5 mL/min) to afford compounds **1a** (13.0 mg), **1b** (2.0 mg), and **1c** (5.0 mg) from **1**; and **2a** (1.8 mg), **2b** (2.5 mg), **2c** (2.2 mg), and **2d** (1.7 mg) from **2**. Compound ***1b***: Pale gum; UV (MeOH) λ_{max} (log ε) transparent; ^{1}H and ^{13}C NMR ($CDCl_3$), see Tables 1 and 2; HRESIMS *m/z* 335.2301 $[M+H]^+$ (calcd for $C_{20}H_{31}O_4$, 335.2262). *Compound **1c***: White gum; UV (MeOH) λ_{max} (log ε) 246 (3.83); ^{1}H and ^{13}C NMR

Table 1
^{13}C NMR data for precursors and products (100 MHz, δ in ppm).*

No.	**1***	**1a**#	**1b***	**1c**#	**2***	**2a**#	**2b**#	**2c***	**2d***
1	39.3	40.6	41.3	32.2	38.3	38.9	37.2	38.7	76.5
2	19.3	20.0	19.0	20.6	18.1	18.1	18.2	18.1	24.8
3	38.0	39.5	37.5	39.0	37.0	36.6	36.5	36.7	35.1
4	44.1	44.7	43.9	44.9	47.3	47.0	46.8	47.0	47.3
5	56.2	49.5	54.5	44.7	48.8	48.4	46.0	45.9	48.0
6	24.2	32.6	36.8	39.0	24.9	25.2	34.2	25.0	27.3
7	35.8	74.1	214.5	204.2	35.5	35.5	71.8	71.9	35.7
8	137.9	141.2	78.2	140.4	136.6	135.7	138.2	137.7	135.0
9	50.6	47.2	54.2	75.2	50.5	59.6	48.6	58.1	60.1
10	39.3	40.4	39.7	42.5	37.7	37.8	37.8	38.2	44.1
11	19.6	20.9	17.6	27.3	18.5	66.0	18.3	66.0	65.7
12	36.5	36.7	32.6	31.6	34.4	43.3	35.5	43.1	43.5
13	38.5	39.7	35.2	40.7	37.4	37.2	36.8	37.0	38.2
14	128.0	133.8	42.1	145.4	129.1	127.9	125.8	124.8	130.0
15	147.2	148.1	151.1	145.6	148.9	148.6	148.2	148.7	148.0
16	112.9	113.9	109.2	114.4	110.1	110.5	110.6	111.1	110.5
17	29.4	29.6	25.6	28.5	26.0	26.8	26.3	27.2	25.3
18	184.5	181.7	182.4	181.2	16.7	16.6	16.7	16.8	16.6
19	29.2	29.7	29.2	29.1	185.6	183.1	183.4	182.3	183.1
20	13.9	13.8	16.9	16.3	15.2	15.9	15.3	16.2	9.9

* $CDCl_3$.
\# CD_3OD.

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