Artigo

ONE STRAIN-MANY COMPOUNDS (OSMAC) METHOD FOR PRODUCTION OF PHENOLIC COMPOUNDS USING *Camarops* sp., AN ENDOPHYTIC FUNGUS FROM *Alibertia macrophylla* (Rubiaceae)[#]

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ONE STRAIN-MANY COMPOUNDS (OSMAC) METHOD FOR PRODUCTION OF PHENOLIC COMPOUNDS USING *Camarops* sp., AN ENDOPHYTIC FUNGUS FROM *Alibertia macrophylla* (RUBIACEAE). Seven phenolic derivatives including *p*-hydroxyphenyllactic acid (**1**), *p*-hydroxybenzoic acid (**2**), *p*-hydroxybenzaldehyde (**3**), phenyllactic acid (**4**), *n*-butyl-3,4-dihydroxybenzoate (**5**), *n*-hexyl-3,4-dihydroxybenzoate (**6**) and *n*-octyl-3,4-dihydroxybenzoate (**7**) were produced using *Camarops* sp. via the one strain-many compounds (OSMAC) approach. Their structures were determined using 2D NMR and ESI-MS spectra and were compared with works reported in the literature. This paper deals with the first report of these compounds in *Camarops* sp.

Keywords: endophytic fungus; *Camarops* sp.; phenolic compounds

INTRODUCTION

Endophytic fungi are a diverse group of organisms ubiquitous in plants and found to maintain an imperceptible association with their hosts for at least a part of their life cycle.[1] These fungi present an enormous ability when it comes to the production of a wide range of new bioactive metabolites from a single strain through the systematic alteration of the cultivation parameters, known as the OSMAC (one strain many compounds) approach. The OSMAC mechanism came into being thanks to the observation that small changes in the cultivation conditions were capable of completely altering the metabolic profile of many microorganisms. Thus, the mechanism is considered a powerful tool which can contribute meaningfully towards elucidating the secondary metabolome of different microbes.[2]

In our recent work, *Camarops* sp. was cultivated in corn where it was found to produce a broad range of eremophilane sesquiterpenes, with antioxidant, anti-inflammatory and cytotoxic activities besides the potent inhibitory activity that was observed against the proteases subtilisin and pepsin.[3,4] To further explore its potential in the production of secondary metabolites, the OSMAC approach was applied aiming at maximizing the chemical diversity of this fungus. Our present work seeks to report the isolation and characterization of the aforementioned seven phenolic derivatives: *p*-hydroxyphenyllactic acid (**1**), *p*-hydroxybenzoic acid (**2**), *p*-hydroxybenzaldehyde (**3**), phenyllactic acid (**4**), *n*-butyl-3,4- dihydroxybenzoate (**5**), *n*-hexyl-3,4-dihydroxybenzoate (**6**) and *n*-octyl-3,4- dihydroxybenzoate (**7**) (Figure 1).

EXPERIMENTAL SECTION

General experimental procedures

^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), HMBC, HSQC and COSY experiments were conducted in a Varian INOVA-500 spectrometer using the residual non-deuterated (DMSO-d_6) signal as an internal standard. Mass spectra were measured using a Thermo Scientific™ LCQ Fleet™ ion trap mass spectrometer set to ESI mode with MeOH:H$_2$O (1:1) being the solvent (cone voltage 25 V). TLC analyses were performed using Merck silica gel 60 (230 mesh) and precoated silica gel 60 PF$_{254}$. Spots on the TLC plates were visualized either under UV light or by spraying with anisaldehyde–H$_2$SO$_4$ reagent followed by heating at 120°C. Analytical and preparative HPLC were performed in a Shimadzu SPD-M20A system using Phenomenex Luna C$_{18}$ column (250 mm x 4.6 mm, 5 μm), Synergi Hydro Phenomenex® C$_{18}$ column (250 mm x 21.2 mm, 4 μm), Supelcosil Supelco® C$_{18}$ column (250 mm x 21.2 mm, 12 μm) and Luna Phenomenex® C$_{18}$ column (150 mm x 21.2 mm, 5 μm). Column chromatography (CC) was carried out over reversed-phase silica gel, 230–400 mesh (Merck).

Plant material

Authenticated *Alibertia macrophylla* K. Schum. (Rubiaceae) was collected from the Mogi-Guaçu Experimental Ecology Station, at Campininha Farm, Mogi-Guaçu, São Paulo, Brazil, in November 2003. Dr. Inês Cordeiro from Institute of Botany, São Paulo, was responsible for the identification of *A. macrophylla* and a voucher specimen was stored at the Herbarium of the Institute of Botany of São Paulo, Brazil (Voucher nº SP 370915).

Fungal isolation and identification

The endophytic fungus *Camarops* sp. was isolated according to the previously described procedure.[5] The pure fungal strain was stored as *Camarops* sp. at the NuBBE fungi collection in Araraquara, Brazil (kept in sterile water at 25 °C).[6] The pure *Camarops* sp. culture was

sent to the Microbial Resources Division, Multidisciplinary Research Center of Agricultural and Biological Chemistry, State University of Campinas (CPQBA/UNICAMP), and was classified by Drs. Lara Durães Sette and André Rodrigues.[4]

Cultivation and isolation of *Camarops* sp. metabolites

The endophytic fungus strain *Camarops* sp. was cultivated in 19 500 mL Erlenmeyer flasks, each containing 300 mL of Czapek's liquid medium of sterilized H_2O. The medium was autoclaved at 121 °C for 40 min. Following sterilization, the medium was in turn inoculated with the endophyte and incubated while stationary at 25 °C for 28 days. The mycelia biomass accumulated in the Erlenmeyers flasks was separated from the aqueous medium by filtration, and the filtrate was subjected to a liquid–liquid partition with EtOAc (3 x 2.5 L). The EtOAc fraction was evaporated deriving in the EtOAc crude extract (0.845 g), which was fractionated by C_{18} RPCC and eluted with H_2O–CH_3OH 90:10 (210 mL), 85:15 (210 mL), 80:20 (210 mL), 75:25 (210 mL), 70:30 (210 mL), 60:40 (210 mL), 50:50 (210 mL), 40:60 (210 mL) and 100% CH_3OH (210 mL), giving rise to eight fractions (Fr1–Fr8). Fraction Fr2 (496 mg) was diluted in H_2O (500 mL) and subjected to a liquid–liquid partition with EtOAc (3 x 250 mL). The EtOAc was evaporated resulting in the EtOAc subfraction Fr2 (96.4 mg), and was further separated by HPLC using C_{18} Synergi Hydro Phenomenex® column (4 μm, 21.2 mm x 250 mm) and H_2O:CH_3OH 85:15 as eluent for 60 min at 10.0 mL/min, leading to 6 subfractions (Fr2-A–Fr2-F). The subfraction Fr2-C was identified as compound **1** (5.7 mg, R_t = 27.0 at 35.0 min). Additionally, the subfraction Fr2-D was identified as compounds **2** and **3** (4.5 mg, R_t = 36.0 at 55.0 min) while Fr2-E as compounds **4** and **5** (11.4 mg, R_t = 56.0 at 60.0 min). Fraction Fr3 (72.7 mg) was further separated by HPLC using C_{18} Supelcosil Supelco® column (12 μm, 21.2 mm x 250 mm) and H_2O:CH_3OH 75:25 as eluent for 60 min at 10.0 mL/min, generating 5 subfractions (Fr3-A–Fr3-E). Subfraction Fr3-C was identified as compounds **4** and **5** (17.1 mg, R_t = 8.5 at 9.5 min) while Fr3-E as compound **4** (3.8 mg, R_t = 10.5 at 12.5 min). The subfraction Fr3-D (34.7 mg) was purified by HPLC using Luna Phenomenex® C_{18} column (5 μm, 21.2 mm x 150 mm) and H_2O:CH_3OH 30:70 as eluted for 25 min at 10.0 mL/min, giving rise to 2 subfractions (Fr3-D-1–Fr3-D-2). The subfraction Fr3-D-1 was identified as compounds **4** and **5** (4.0 mg, R_t = 3.0 at 8.0 min) while Fr3-D-2 as compounds **5**, **6** and **7** (2.0 mg, R_t = 20.0 at 25.0 min).

RESULTS AND DISCUSSION

Through the application of the OSMAC method, the EtOAc crude extract was derived from the cultivation of *Camarops* sp. in Czapek's liquid medium from which seven compounds were obtained. The compounds were identified as *p*-hydroxyphenyllactic acid (**1**)[7, 8], *p*-hydroxybenzoic acid (**2**)[8, 9], *p*-hydroxybenzaldehyde (**3**)[10], phenyllactic acid (**4**)[11], *n*-butyl-3,4-dihydroxybenzoate (**5**)[11, 12], *n*-hexyl-3,4-dihydroxybenzoate (**6**)[11, 12] and *n*-octyl-3,4-dihydroxybenzoate (**7**)[11, 12] (Figure 1).

The NMR ^{1}H spectra of *p*-hydroxyphenyllactic acid (**1**) (Table 1) when analyzed along with HSQC data indicated the presence of two doublets [δ_H 7.00 (2H, *J* = 8.2 Hz, H-3 and H-5, δ_C 129.8) and 6.63 (2H, *J* = 8.2 Hz, H-2 and H-6, δ_C 114.4)], with characteristic coupling constant for *ortho*-positioned hydrogen atoms, showing a disubstituted aromatic moiety. Furthermore, the presence of a methylene group [δ_H 2.85 (*dl*, *J* = 12.8 Hz, 1H, H-7, δ_C 39.3), and 2.62 (*m*, 1H, H-7, δ_C 39.3)], implying the closeness of this hydrogen to a stereogenic center, in addition to the carbon chemical shift led us to conclude that it was a benzylic carbon. An oxygenated methine group [δ_H 3.96 (*sl*,
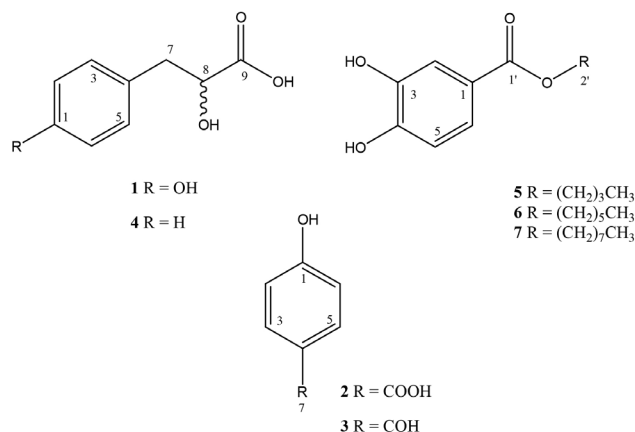

Figure 1. *Compounds isolated from Camarops sp.*

2H, H-8, δ_C 71.4)] characteristic of a carbinolic hydrogen enabled the identification of a hydroxyl group directly bonded to carbon β of an aromatic ring. The HMBC correlations between H-2↔C-1/C-3/C-4, H-3↔C-2/C-5, H-5↔C-2/C-3, H-6↔C-1/C-3/C-4 and H-7↔C-4, confirmed the presence of carbonyl carbon and aromatic unit. The side chain was positioned at C-4 based on the values of chemical shifts and multiplicities of the signals of the aromatic ring signals (Table 1), leading to the identification of compound **1** as *p*-hydroxyphenyllactic acid.[7, 8]

The ^{1}H NMR spectra of *p*-hydroxybenzoic acid (**2**) (Table 1) when analyzed jointly with the HSQC data indicated seven carbons besides the presence of two doublets [δ_H 7.76 (2H, *J* = 8.7 Hz, H-3 and H-5, δ_C 131.1) and 6.81 (2H, *J* = 8.7 Hz, H-2 and H-6, δ_C 114.9)], bearing a characteristic coupling constant of a *para* hydrogen, depicting a disubstituted aromatic ring system. The analysis of HMBC showed correlations of H-2 and H-6↔C-1/C-3 and H-3 and H-5↔C-2/C-3/C-7 confirming structure **2** of *p*-hydroxybenzoic acid.[8, 9]

p-hydroxybenzaldehyde (**3**) was obtained in the mixture with **4**. The ^{1}H spectra of **3** (Table 1) when analyzed together with the HSQC data pointed out a similarity to compound **2**, the difference observed being the presence of one singlet at δ_H 9.79 (H-7), suggesting the existence of an aldehyde function. These data associated with the NMR of compound **2** and the carbon δ_C 190.8 (C-7) in the HMBC data showed the reduction of C-7 carboxylic acid to an aldehyde function, confirming the structure **3** of *p*- hydroxybenzaldehyde.[10]

Phenyllactic acid (**4**) was obtained in a mixture with compound **3**. [1]H spectra of **4** (Table 1) when analyzed along with the HSQC data indicated a similarity with to compound **1**, the difference observed being the aromatic ring which showed a monosubstituted ring. ^{1}H NMR spectra indicated the presence of five sp[2] methine groups at a complex aromatic region in a multiplet form [δ_H 7.23 (*m*, H-1, H-2, H-3, H-5, H-6)]. Compound **4** was thus identified as phenyllactic acid.[11]

The ^{1}H spectra of *n*-butyl-3,4-dihydroxybenzoate (**5**) (Table 1) when analyzed along with the HSQC data indicated the presence of three sp[2] methine groups, showing a trisubstituted aromatic ring system [δ_H 6.82 (*d*, *J* = 8.0 Hz, 1H, δ_C 117.7, H-5), 6.98 (*dd*, *J* = 8.0 and 2.0 Hz, 1H, δ_C 123.4, H-6), and 7.17 (*d*, *J* = 2.0 Hz, 1H, δ_C 113.6, H-2)], three methylene group found to be oxygenated [δ_H 4.30 (*t*, *J* = 6.5 Hz, 2H, δ_C 64.8, H-2'), 1.72 (*m*, δ_C 27.2, H-3') and 1.37 (*m*, δ_C 27.2, H-4')] and one methyl group [δ_H 0.91 (*t*, *J* = 7.0 Hz, 3H, δ_C 13.4, H-5')]. The HSQC correlations together with the HMBC correlations exhibited four quaternary carbons [δ_C 112.6 (C-1); 149.6 (C-3); 153.0 (C-4) and 168.8 (C-1')]. These data associated with the ^{1}H-^{1}H COSY correlations led to a trisubstituted aromatic ring with two hydroxyl groups (C-3 and C-4) and one conjugated carbonyl

Table 1. ¹H- and ¹³C NMR data for compounds **1-5** (500 MHz, CDCl₃)

Position	1 δ ¹H	1 δ ¹³C	Position	2 δ ¹H	2 δ ¹³C	Position	3 δ ¹H	3 δ ¹³C	Position	4 δ ¹H	4 δ ¹³C	Position	5 δ ¹H	5 δ ¹³C
1	-	155.7	1	-	161.6	1	-	163.3	1	7.23 (*m*)	125.9	1	-	112.6
2	6.63 (*d*, 8.2)	114.4	2	6.81 (*d*, 8.7)	114.9	2	7.75 (*d*, 8.5)	132.0	2	7.23 (*m*)	127.8	2	7.17 (*d*, 2.0)	113.6
3	7.00 (*d*, 8.2)	129.8	3	7.76 (*d*, 8.7)	131.1	3	6.92 (*d*, 8.5)	115.8	3	7.23 (*m*)	129.3	3	-	149.6
4	-	130.4	4	-	121.9	4	-	129.3	4	-	138.3	4	-	153.0
5	7.00 (*d*, 8.2)	129.8	5	7.76 (*d*, 8.7)	131.1	5	6.92 (*d*, 8.5)	115.8	5	7.23 (m)	129.3	5	6.82 (*d*, 8.0)	117.7
6	6.63 (*d*, 8.2)	114.4	6	6.81 (*d*, 8.7)	114.9	6	7.75 (*d*, 8.5)	132.0	6	7.23 (m)	127.8	6	6.98 (*dd*, 2.0; 8.0)	123.4
7a	2.62 (*m*)	39.3	7	-	167.7	7	9.79 (*s*)	190.8	7	2.75 (*dd*; 10.0; 5.0) 2.96 (*dd*; 10.0; 5.0)	40.0	1'	-	168.8
7b	2.85 (*dl*, 12.8)	39.3							8	4.11 (*dd*; 5.0; 5.0)	71.1	2'	4.30 (*t*, 6.5)	64.8
8	3.96 (*sl*)	71.4							9	-	175.1	3'	1.72 (*m*)	27.2
9	-	175.5										4'	1.37 (*m*)	27.2
												5'	0.91 (*t*, 7.0)	13.4

ester (C-1'), confirming structure **5** of *n*-butyl-3,4-dihydroxybenzoate. Oddly enough, though all the data as can be seen from our discussion above led us towards the proposed structure, the integration of -CH₂ to the alkyl chain failed to be consistent with it. By so doing, a mass spectrum was obtained and thanks to that it was possible to detect the isomers **5-7**, justifying the congruence of the ¹H NMR data recorded.

The structures of the esters **5-7** were confirmed by mass spectrometry (ESI-MS) which showed the presence of [M-H]⁻ ion peaks at *m/z* 209.23 (*n*-butyl-3,4-dihydroxybenzoate); 237.19 (*n*-hexyl-3,4--dihydroxybenzoate) and 265.27 (*n*-octyl-3,4-dihydroxybenzoate) (Figure 2).[11, 12]

The data obtained from sequencing and BLAST analysis of the 28S rDNA showed that the isolated *AM-02* had 95% sequence identity with several species within the genus *Camarops* (Ascomycota: Boliniales, Boliniaceae). Ecologically, *Camarops* spp. are considered wood-inhabiting fungi and are found in both temperate and tropical regions. Cultures of *AM-02* form abundant white aerial mycelium with the reverse side of the colony becoming orange-brown over the passage of time. *Camarops* spp. are characterized by stromatic ascomata with brown ascospores.[4]

Nonetheless, upon cultivation in malt-extract agar, potato dextrose agar, and oat meal agar, the isolated *AM-02* failed to produce ascomata and ascospores. Moreover, microscopic examination revealed thin-walled hyphae, though without the presence of asexual reproductive structures.[4]

Interestingly, the data derived from molecular and phylogenetic analyses suggest that the *AM-02* strain is related to *Camarops* species and as such there is a plausible likelihood that it represents a new taxon.

Phenolic compounds are known to be of indisputable importance owing to the fact that they are produced by plants or fungi in response to environmental stresses such as pathogens and insects attacks, UV radiation and wounds. They are found to be present in food consumed in human diet as well as in plants used in traditional medicine in many cultures. Some phenols are germicidal and as such are used in formulating disinfectants. Others are found to have anticancer, anti-inflammatory, antiviral, antibacterial, antioxidant and estrogenic activities while some present endocrine disruption.

Among the phenolic compounds, *p*-hydroxybenzoic acid (pHBA) (**2**) is known to play a vital role in three different sectors including
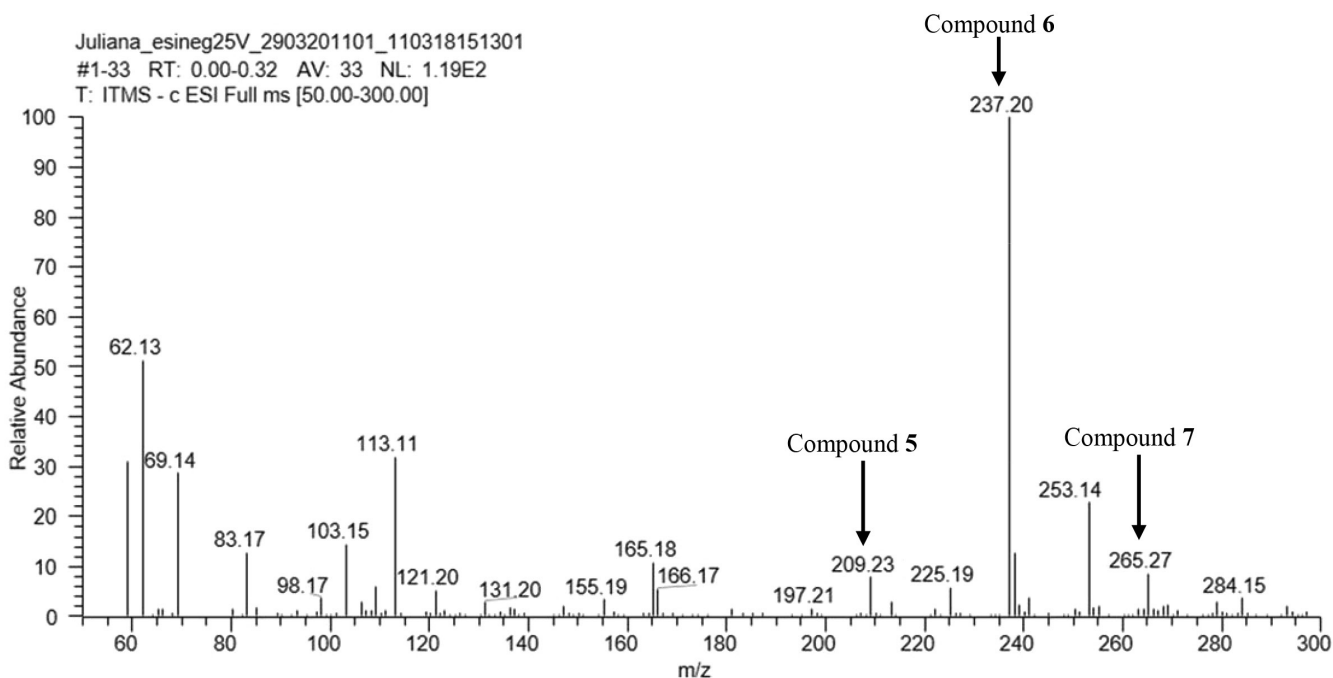
Figure 2. Mass spectrum of compound 5 to 7

the food, electronics and the pharmaceutical industries. In the food industry, it plays the role of an intermediate in the synthesis of red pigment shikonin. In addition, it acts as the main monomer in the production of LCPs (liquid crystal polymers) in the electronic industry. Finally, its role in the pharmaceutical industry is seen to be fundamentally crucial for the fact that it has significant antifungal activity. *P*-hydroxybenzoic acid is obtained by the Kolbe-Schmitt process under severe conditions of temperature and pressure, rendering the process, in effect, extremely costly in industrial scale. In light of that, endophytic fungi are becoming a financially more feasible alternative, considered environmentally renewable and sustainable when it comes to the production of large amounts of *p*HBA.

CONCLUSION

The production of secondary metabolites by fungi is said to be dependent on both the biosynthetic capacity of the microorganisms and the fermentation conditions. In fact, based on our results, the manipulation of the fermentation process parameters is found to be capable of altering the expression of the secondary metabolites produced. Using this strategy, seven known compounds were isolated from *Camarops* sp. following the selection of their growth media composition.

The present study suggests that endophytic fungi are potentially suitable microorganisms for the OSMAC method, and could enhance the chemical diversity of secondary metabolites. This study reinforces the importance of using the OSMAC approach in the biosynthetic exploitation of endophytic fungi as our finding unravels a culture condition which induces this class of fungi towards the production of a wide range of phenolic compounds.

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