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Short Report

Biotransformation of a Tetrahydrofuran Lignan by the Endophytic Fungus *Phomopsis* Sp.

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A biotransformação da lignana tetraidrofurânica, (-)-grandisina, pelo fungo endofítico *Phomopsis sp*, obtido de *Viguiera arenaria*, conduziu à formação de um novo metabólito caracterizado como 3,4-dimetil-2-(4'-hidróxi-3',5'-dimetóxifenil)-5-metóxi-tetraidrofurano. O metabólito foi analisado contra o parasita *Trypanosoma cruzi*, o agente causador da doença de Chagas, e mostrou uma atividade tripanocida (IC_{50} 9,8 μmol L^{-1}) similar ao precursor natural (IC_{50} 3,7 μmol L^{-1}).

The biotrasformation of the tetrahydrofuran lignan, (-)-grandisin, by the endophitic fungus *Phomopsis* sp, obtained from *Viguiera arenaria*, led to the formation of a new compound determined as 3,4-dimethyl-2-(4'-hydroxy-3',5'-dimethoxyphenyl)-5-methoxy-tetrahydrofuran. The metabolite was evaluated against the parasite *Trypanosoma cruzi*, the causative agent of Chagas's disease, and showed a trypanocidal activity (IC_{50} 9.8 μmol L^{-1}) similar to the natural precursor (IC_{50} 3.7 μmol L^{-1}).

Keywords: biotransformation, 2,5-diaryltetrahydrofuran, endophytic fungus, (-)-grandisin, trypanocidal activity

Introduction

The lignan family is a large group of natural products with noteworthy therapeutic activity as illustrated by podophyllotoxin derivatives (*e. g.* etoposide) with antitumoral activity.[1] In particular, the optically active tetrahydrofuran lignans display a wide range of pharmacological activities, including anti-parasite,[2-4] antibacterial,[5] antifungal,[6] anti-tumoral,[7] anti-inflammatory[8,9] and platelet-activating factor (PAF) inhibition.[10-12]

Several non-symmetrical 2,5-diaryltetrahydrofuran lignans, shown in Table 1, have been isolated from *Piper* species (Piperaceae) containing either piperonyl, veratryl or 3,4,5-trimethoxyphenyl substituent at 2 and 5 positions and methyl groups at 3 and 4 positions attached to the tetrahydrofuran ring, as illustrated by (+)-calopiptin (**1**, *P. schmidtii*), 5-[*rel*-(2*S*,3*S*,4*S*,5*S*)-5'-(3'',4''-dimethoxyphenyl)-3',4'-dimethyl-2'-tetrahydro-furanyl)]-1,3-benzodioxole (**2**, *P. wightii*), (2*S*,3*R*,4*S*,5*R*)-3,4-dimethyl-2,5-bis(3',4'-dimethoxyphenyl)-tetrahydrofuran (**3**, *P. clarkii*), (±)-galgravin (**4**, *P. futokadsura, P. hancei, P. puberculum, P.wallichii*), (-)-galbelgin (**5**, *P. attenuatum*, *P. futokadsura, P. thomsoni, P. wightii*), (+)-grandisin (**6**, *P. polysyphorum*), (+)-machilin G (**7**, *P. schmidtii, P. wightii*), (+)-veraguensin (**8**, *P. cuneifolium, P. futokadsura, P. puberculum*) and (-)-zuonin A (**9**, *P. schmidtii)*.[13] Other bis-tetrahydrofuran lignans have also been described from *Achillea lingulata* (Asteraceae).[14]

In previous reports, Martins *et al.*[3] described the isolation of (-)-grandisin (**6**) from *Piper solmsianum* and Lopes and co-workers[4,15] from *Virola surinamensis* (Rol.) Warb, which belong to Piperaceae and Myristicaceae, respectively. The tetrahydrofuran lignan (-)-grandisin (**6**) showed a potent trypanocidal activity against the trypomastigote form of *Trypanosoma cruzi,* responsible for Chagas´s disease in Latin America, at 5 μg mL^{-1} leading to a total parasite lysis after 24 h, without hematiae damage.[4] Furthermore, veraguensin (**8**) also isolated from *Virola surinamensis*[4] preserved this anti-parasite pattern along with a potent platelet-activating factor (PAF) inhibitory activity that may be involved in the mechanisms of the parasite growing and differentiation.[16] Nevertheless, attempts to overcome

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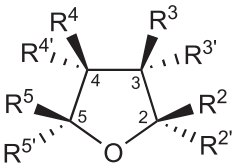
Table 1. Non-symmetrical 2,5-diaryltetrahydrofuran lignans isolated from *Piper* species



Compound	R^2	$R^{2'}$	R^3	$R^{3'}$	R^4	$R^{4'}$	R^5	$R^{5'}$
1	piperonyl	H	H	CH_3	CH_3	H	veratryl	H
2	veratryl	H	H	CH_3	CH_3	H	H	piperonyl
3	veratryl	H	CH_3	H	CH_3	H	veratryl	H
4	veratryl	H	CH_3	H	CH_3	H	H	veratryl
5	H	veratryl	CH_3	H	H	CH_3	veratryl	H
6	3',4',5'-trime thoxyphenyl	H	H	CH_3	CH_3	H	H	3',4',5'-trime thoxyphenyl
7	piperonyl	H	H	CH_3	H	CH_3	veratryl	H
8	veratryl	H	H	CH_3	CH_3	H	veratryl	H
9	piperonyl	H	CH_3	H	CH_3	H	H	Piperonyl

Modified since were included higher number of examples with tetrahydrofuran skeleton in relation of the original.[13]

the poor water solubility of compounds **6** and **8** for *in vivo* evaluation, comprising semi-synthetic modifications such as *O*-demethylation of the methoxy groups attached to the aromatic rings, led to byproducts due to the presence of two benzylic carbons in the tetrahydrofuran ring that were very unstable under acidic or base conditions.[17]

The biotransformation of the naturally occurring 2,5-diaryl-3,4-dimethyltetrahydrofurans with PAF inhibitory activity, such as (+)-veraguensin (**8**), (+)-galbelgin (**3**) and galgravin (**4**) named as 7,7'-epoxylignans, has been investigated in order to obtain novel and bioactive derivatives. In the series, carrying a 3,4-dimethoxyphenyl substituents pattern, both methyl groups at *para*-position of the aromatic rings were metabolized and cleaved by *Aspergillus niger* yielding the corresponding 4,4'-*O*-demethyl derivatives.[18] Similar results were also achieved using furofuran lignans with the 2,6-diaryl-3,7-dioxabicyclo[3.3.0]octane skeleton,[19] such as (+)-magnolin and (+)-eudesmin. Replacing the 3,4-dimethoxyphenyl groups by 3,4,5-trimethoxyphenyl (e. g. (+)-yangabin) and 4,5-dihydroxy-3-methoxyphenyl groups on the lignans lead to a stable *para*-methoxy groups not cleaved by *A. niger*. On the other hand, the use of larvae of *Spodoptera litura*[20] or rats as a source of liver metabolized enzymes[21,22] allowed the *O*-demethylation at the *para*-position of both aromatic rings of furofuran lignans containing either di- or trimethoxyphenyl groups, being the pre-formed hydroxyl group frequently conjugated to glycosides. The grandisin (**6**) has also been di-O-demethylated by Lepidopterae and Coleoptera species.[23]

The biotransformation reactions of lignans, including podophyllotoxin, and neolignans was described in a recent review.[24]

Endophytic fungi are prolific producers of natural products, since they live inside the health plant tissues without causing any apparent disease.[25] These environmental conditions might stimulate the fungi to produce a range of metabolic enzymes as a requirement for their survival in the presence of toxic defense compounds of plants.[26] Endophytes have also been successfully used in the biotransformation of natural products.[27,28]

These findings stimulated us to investigate about 20 endophytic strains from our collection of fungi[29] as biocatalyst systems and effective method for regio- and stereoselective production of novel and bioactive products. This procedure could convert unreactive methoxy groups to hydroxyl functions of a tetrahydrofuran lignan under mild conditions which may increase the solubility of **6**. Thus, this study focuses on the biotransformation of **6** by four different endophytic fungi with the aim to obtain potential trypanocidal derivatives with improved drug-like properties.

Experimental

General procedures

GC-MS were measure at 70 eV on a Shimadzu Model: QP2010, 120 ºC (oven temp.), using a DB-5MS (30 m ×

0.25 mm × 0.25 μm). ESI analysis were performed on a ultrOTOF$_Q$-ESI-TOF MS (End Plate: 4000 Volts, Capillary: 4500 Volts, Capillary Exit: 300 Volts, Skimmer 1: 50 Volts, Skimmer 2: 25 Volts, Transfer: 90 μs, Collision Exit Gate: 80 μs).^{1}H NMR spectrum was measured on a Bruker DPX-400 (400 MHz) while 2D experiments (HSQC and HMBC) were recorded in a Bruker DRX-400 (400 and 100 MHz) using $CDCl_3$ (Aldrich) as solvent and TMS as int. standard. Chemical shifts were reported in units (ppm) and coupling constants (*J*) in Hz. TLC: silica gel 60 F254 pre-coated (layer thickness 0.25 mm, Merck); CC: silica gel with hexane-EtOAc 4:6, v/v. The instrumentations for HPLC analysis consisted of a Shimadzu (SCL-10Avp, Japan) multisolvent delivery system, Shimadzu SCL-M10vp Photodiode Array detector, and an Intel Celeron computer for analytical system control and data collection. Chromatography was carried out using the isocratic method with methanol:water 7/3, v/v within 20 minutes during the screening phase for the evaluation of grandisin biotransformation (condition A). The mobile phase used during the isolation of the metabolites was methanol:water 6:4, v/v within 60 minutes (condition B). A CLC-ODS (M)- 4.6 × 250 mm Shimadzu column was used at a flow of 1.0 mL min^{-1}. The spectral data from the detector were collected over de 200-400 nm range of absorption chromatograms, and the chromatograms were plotted at 225 nm.

Grandisin was obtained from dichloromethane extracts from twigs of *Virola surinamensis,* which yielded two steroids, two lignans (compounds **6** and **8**), five flavonoids and one polyketide.[4]

Microorganisms and culture

The endophytic fungi have been isolated and identified by their rDNA sequences as previously described.[29] The strains are maintained on PDA slants at 4 ºC. Initially the fungi were screened for their ability to transform grandisin. Fungi were cultivated on PDA in Petri dish for 7 days at 30 ºC and agar pieces (1 cm^2) containing fungal mycelia was used as inocullum. Strain SS42 was also identified by "Micoteca URM/ Depto. de Microbiologia/ CCB/ UFPE" (Av. Prof. Nelson Chaves, s/n, 50670-420 Recife-PE, Brazil).

A two-step culture was used for each fungus. Initially, the fungi were inoculated into 20 mL of Czapek medium, supplemented with 3% of sucrose and the cultures were incubated at 30 ºC for 3 days, with shaking at 120 ºC rpm. After cultivation the resulting mycelia were harvested by filtration and transferred to 20 mL of Czapek medium, containing 1% of sucrose and supplemented with a solution of grandisin in DMSO, resulting 0.4 mg mL^{-1} as final concentration in the media. The cultures were reincubated at the same conditions for 8 days.

Extraction of the metabolites

The culture broths were separated from mycelia by vacuum filtration, followed by 3 times partition sequentially with ethyl ether, ethyl acetate and *n*-butanol (10 mL). The solvents were evaporated and the crude extracts were analyzed by TLC and HPLC (condition A).

Isolation of the metabolites

After the preliminary screening, *Phomopsis* sp (VA35) culture was scaled up to 150 mL using the same conditions already described, including the proportion of grandisin (**6**) (60 mg), but changing the fermentation time to 9 days. The ethyl ether and ethyl acetate extracts were joined because they showed similar profiles. The crude extract obtained was purified by Silica-gel column chromatography using hexane/ethyl acetate 1:1, yielding 4 fractions. Fraction 3 was further purified through HPLC (Condition B) to yield compound **10** (1.0 mg).

In vitro trypanocidal assay

The bioassays were carried out using the blood of infected Swiss albino mice, collected by cardiac puncture on the parasitemy peak (7th day) after infection with Y strains of *T. cruzi*. The infected blood was diluted with the blood health mice to achieve a concentration of 10^6 trypomastigote *per* mL. The assays were performed on titration microplates (96 wells) in duplicate. The compound **10** was dissolved in dimethyl sulfoxide (DMSO) and was added into the infected mouse to provide concentration of 0.5, 2.0, 8 and 32 μmol L^{-1}. The plates were incubated at 4 ºC and the numbers of parasites were counted after 24 h. Negative and positive controls containing either DMSO or crystal violet at 250 μg mL^{-1}, respectively were run in parallel.[30]

Results and Discussion

The microbial transformations of (-)-grandisin (**6**) by endophytic fungi were investigated in a small scale screening test (8 mg) monitored by TLC and HPLC analyses (condition A). Among the 20 endophytic fungi, *Curvularia lunata* (SS-42), isolated from *Smallanthus sonchifolius,* and *Phomopsis* sp. (VA-35), obtained from *Viguiera arenaria*, showed better results in the biotransformation of **6**. *Phomopsis* sp. was selected to be cultivated in large

scale experiments because of its great ability to generate metabolites in the preliminary tests.

The metabolization of compound **6** (60 mg), with a retention time of 40.23 min as indicated by HPLC analysis (condition B), led to the generation of a major metabolite (**10**) with a distinct retention time of 10.35 min. Compound **10** was purified by column chromatography, followed by HPLC (condition B). Condition B was established by exploring its different interaction properties with the reverse phase column (C-18), in a manner similar to that previously reported for butyrolactones isolated from *Virola surinamensis*.[31,32]

GC-MS analysis showed that compound **10** has a molecular weight lower than grandisin with a $[M]^{+\cdot}$ at *m/z* 282 and fragments (*m/z* 68, 85, 100, 167 and 182) suggesting the molecular formula $C_{15}H_{22}O_5$. In fact, the High Resolution Mass Spectrometry confirms the proposed molecular formula with a detectable $[M + Na]^+$ 305.1368 and a corresponding theoretic mass of 305.1365.

The structure of compound **10** was established by 1D and 2D NMR data. For instance, the ^{1}H NMR spectrum of compound **10** showed two methoxy proton signals at δ 3.37 and 3.83 with a relative integration of 3:6, which were characteristic of groups attached to aliphatic and aromatic skeleton, respectively. Moreover, the *ortho*-aromatic proton signals were assigned at δ 6.52 (s, 2H), while the methyl groups at C-13 and C-12 were observed at δ 1.10 and δ 0.87 as two doublets (*J* 6.9 Hz).

Nearly all relevant spectral details of the isolated metabolites were closely comparable to the corresponding ^{13}C and ^{1}H features of grandisin,[4,33] but no data for the second aromatic ring were observed by the integration of signals. In addition, a hemiacetal proton was observed at δ 4.71 (d, 1H), attached to a carbon at δ 111.1 as observed in the HMQC experiment. Also, the signal at δ 3.83 was assigned to the methoxy groups at *meta* positions of the remaining aromatic ring linked to the tetrahydrofuran nucleus, indicating the presence of a free hydroxyl group at *para* position. Methoxy aliphatic protons at δ 3.37 and methyl protons at δ 1.10 showed correlation in the HMBC with carbon at δ 111.1, unequivocally establishing the methoxy group at C-5. These data confirmed the presence of a single aromatic ring in compound **10**, and an extra methoxy group at C-5. The main HMBC correlations for compound **10** are shown in Figure 1 and data described in Table 2.

Thus, from the foregoing spectral data the structure of compound **10** was characterized as 3,4-dimethyl-2-(4'-hydroxy-3',5'-dimethoxyphenyl)-5-methoxy-tetrahydrofuran. To our knowledge, this compound has not been previously described in the literature.

We propose that the peculiar aliphatic methoxy groups of compound **10** should be generated firstly by a microbial oxidative metabolism at position C-5 followed by rearrangement to a lactone displacing the aromatic ring. Further reduction to the hemiketal function and *O*-methylation of the nearly formed hydroxyl group might explain how the fungus converted the 3,4,5 trimethoxyphenyl group of compound **6** to methoxy group of the metabolite **10**.

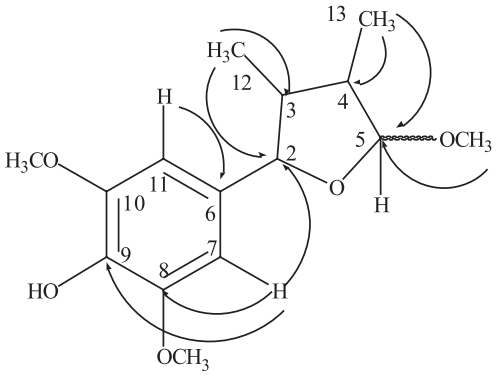


Figure 1. Selected HMBC correlations for metabolite **10** obtained in the biotrasformation process.

The HPLC separation (condition B) provided a second metabolite with a retention time of 12.50 min. GC-MS analysis showed that this compound has the same molecular weight and fragments presented by compound **10**. The High Resolution Mass Spectrometry detected $[M + Na]^+$ 305.1368. ^{1}H NMR spectrum revealed the same integrals, but insufficient data could be obtained from 2D analysis because of the small isolated quantity.

The results from ^{1}H NMR spectrum, GC-MS and high resolution mass spectrometry suggested that this secondary compound might be an epimer of compound **10**.

Table 2. ^{1}HMR (400 MHz) and ^{13}C (100 MHz) data for compound **10** ($CDCl_3$)

Position	Conpound **10**	
	δH	δC
2	4.36 (d, *J* 9.6, 1H)	86.7
3, 4	1.78 (m, 2H)	45.8
5	4.71 (d, *J* 4.7, 1H)	111.1
6	----	130.8
7, 11	6.52 (s, 2H)	103.2
8, 10	----	143.6
9	----	143.2
12	0.87 (d, *J* 6.9, 3H)	14.0
13	1.10 (d, *J* 6.9, 3H)	15.7
OH	5.41 (s, 1H)	----
OMe	3.37 (s, 3H)	56.1
OMe	3.83 (s, 6H)	55.6

The percentage of lyses observed for the trypomastigote form of *T. cruzi* treated with compound **10**, as outlined in Figure 2, was similar to the corresponding natural product **6**.[4] Based on experiments involving the Y strains of *T. cruzi* compounds **6** and **8** were able to cause lyses of parasites with IC_{50} 3.7 and 2.3 μmol L^{-1} respectively,[33,34] while the biotransformation product **10** displayed IC_{50} 9.8 μmol L^{-1}.

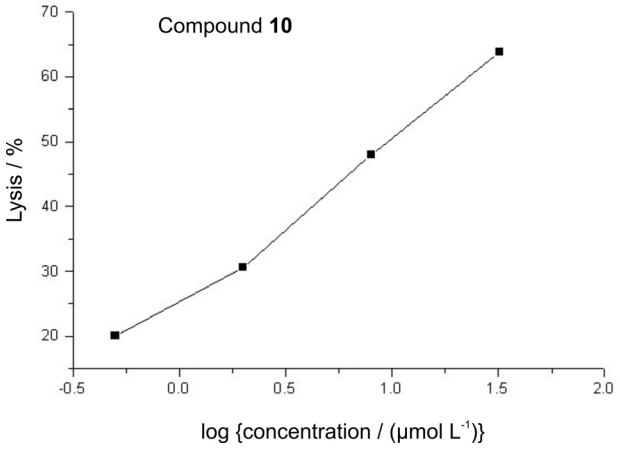


Figure 2. Dose-response curve for lytic activity of compound **10** on trypomastigote forms of *T. cruzi.*

Conclusions

The product 3,4-dimethyl-2-(4'-hydroxy-3',5'-dimethoxyphenyl)-5-methoxy-tetrahydrofuran (**10**) was successfully obtained from the biotransformation of grandisin (**6**) by the endophytic fungus *Phomopsis* sp. The evaluation of the trypanocidal activity of compound **10** and its corresponding natural precursor **6** gave similar results, revealing that a unique methoxylated aromatic ring bound to the central tetrahydrofuran scaffold might preserve the trypanocidal activity, this represents an interesting molecular simplification for synthetic purposes.

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Supplementary Information

^{1}H NMR, HMQC and high resolution spectra of **10** and ^{1}H and ^{13}C NMR spectra of **6** are available free of charge at http://jbcs.sbq.org.br, as PDF file.

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