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Production of Oosporein and Related Bibenzoquinone Analogues in *Arcopilus amazonicus* Revealed by Genome Mining and Mass Spectrometry Analysis

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Oosporein (OOS) is the major specialized metabolite produced by the fungus *Arcopilus amazonicus*, an endophyte from *Paullinia cupana*. Genes related to the biosynthesis of this molecule in *A. amazonicus* diverge from those found in *Beauveria bassiana*. Furthermore, high-resolution liquid chromatography coupled with mass spectrometry combined with molecular networking, revealed the production of analogous compounds with varying degrees of oxygenation. Based on these analytical data, the analogues were annotated as phoenicin diquinol (**2**), phoenicin quinol (**4**) and phoenicin (**5**). By combining mass spectrometry and genome mining, a comprehensive biosynthetic pathway is proposed for these compounds, including the feasibility of a novel putative biphenol oxidation pathway mediated by the monooxygenase gene *AAm15*, which is exclusive to *A. amazonicus*. Additionally, the production of OOS, **2**, **4** and **5** by a single fungal species is reported for the first time.

Keywords: *Arcopilus*, oosporein, phoenicin, mass spectrometry, biosynthesis

Introduction

Recently, a revision on the Chaetomiaceae, described five new genera of the *Chaetomium globosum* species complex, among them, the new genus *Arcopilus*.[1] Since then, few species have been chemically investigated, with most studies focusing on the endophytic fungus *Arcopilus aureus*.[2-9] More recently, we described a new species as an endophyte from *Paullinia cupana* within this genus, named *Arcopilus amazonicus.*[10]

It was noticed that *A. amazonicus* overexpresses the polyketide pigment oosporein (OOS) in very early stages of development.[10] This molecule was previously described

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mainly in *Beauveria*,[11] and *Chaetomium.*[12] Although it showed toxicity to avians[13] and mice,[14] several biological activities, such as antiviral,[15] antifungal,[16] antibacterial,[17] cytotoxic[18] and insecticidal have been reported.[19,20]

The biosynthesis of OOS within cultures of the entomopathogenic fungus *Beauveria bassiana* was previously investigated.[19] It was stated that a type 1 polyketide synthase (PKS, oosporein synthase 1) was produced via *OpS1* gene to give orsellinic acid. Posteriorly, *OpS4* (salicylate hydroxylase) enables the replacement of the carboxylic acid to give a phenol, 6-methyl-1,2,4-benzenetriol, which undergoes through another hydroxylation via a cupin dioxygenase (*OpS7*), giving 6-methyl-1,2,4,5-benzenetetrol. The latter is dimerized under the action of glutathione S-transferase (GST, *OpS6*) and laccase (*OpS5*) to give OOS.

During the genome mining investigation of *A. amazonicus*, we observed divergence in its OOS biosynthetic gene cluster (BGC) relative to those characterized in *B. bassiana*. Specifically, we found an extra oxygenase gene in *A. amazonicus* that could lead to branching in the OOS biosynthetic pathway. Furthermore, metabolomic studies are lacking for this species, despite its potential to offer a rich variety of metabolites. In this context, mass spectrometry, combined with molecular networking serves as a suitable tool for identifying analogous compounds.[21] Additionally, genomics-driven workflows have the potential to overcome the high rate of compound rediscovery in microbial natural product research.[22] Therefore, the aim of this work was to correlate genome sequencing data with the metabolomic profile of this newly discovered fungal species to elucidate a more comprehensive putative biosynthetic pathway proposal for OOS and to annotate analogous, potentially novel compounds.

Experimental

Fungal strain

A. amazonicus was isolated from the roots of the guarana plant *(Paullinia cupana* var. *sorbilis*). The plant material was collected in Manaus, capital of Amazonas state, Brazil, from the Brazilian Agricultural Research Corporation (Embrapa) campus. Specifically, the collection site was located at 2°53'29.14''S latitude and 59°58'39.90''W longitude, with an elevation of 99 meters above sea level. After isolation of fungal strains, they were identified through morphological characterization and phylogenetic analysis based on the ITS, 28S LSU, partial second largest subunit of ribonucleic acid (RNA) polymerase II (RPB2), and β-tubulin (TUB2) regions.[10] *A. amazonicus* access was registered at the Brazilian National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen, A6FB8EF).

Culture media and chemicals

Potato dextrose broth (PDB), Czapek's medium broth and potato dextrose agar were purchased from Kasvi and prepared according to the instructions of the manufacturer. Hexanes, ethyl acetate (EtOAc), ethanol (EtOH), formic acid (HCOOH), and methanol (MeOH) high-performance liquid chromatography (HPLC) grade were purchased from Tedia and ultrapure water was obtained by purification with a Milli-Q gradient system (Millipore). Mass spectrometry grade solvents water, MeOH and acetonitrile (ACN) were purchased from Merck. Deuterated solvents used: methanol (CD_3OD, 99.8%, Cambridge Isotope Laboratories) and dimethyl sulfoxide ($(CD_3)_2SO$, 99.9%, Sigma-Aldrich). The reagents used for genomic deoxyribonucleic acid (DNA) extraction were: cetyltrimethylammonium bromide (CTAB, 2%, USB™), isopropanol (Nuclear), potassium acetate (5 mol L^{-1}, Sigma-Aldrich), Tris-HCl buffer (pH 8.0, Sigma-Aldrich), ethylenedinitrilotetraacetic acid (EDTA) (0.5 mol L^{-1}, pH 8.0, Invitrogen), sodium chloride (Sigma-Aldrich), chloroform:isoamyl alcohol (24:1, Synth:Cromoline), proteinase K (Invitrogen), RNase A (Sigma-Aldrich), and ethanol (70% and absolute, BIOTEC).

General experimental procedures

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis was performed using an Agilent iFunnel Q-ToF 6550 LC-MS system with chromatographic separation achieved on an analytical C_{18} column (Poroshell 120 SB-Aq 2.7 µm, 2.1 × 100 mm, Agilent). Elution was conducted with a mobile phase consisting of 0.1% HCOOH in deionized water (v/v; solvent A) and 0.1% HCOOH in MeOH (v/v; solvent B) following the gradient: 15% B held for 2 min, 15% to 100% B for 12 min, then maintaining 100% B for 5 min at a flow rate of 350 µL min^{-1}. The MS mass range was *m/z* 150-1000 and MS/MS spectra acquisition was data-dependent, based on the three most intense precursor ions, using a fixed collision energy of 20 eV, and argon was used as collision gas. The operating parameters of the equipment were: sheath gas temperature, 300 ºC; dry gas temperature, 290 ºC; sheath gas flow, 12 L min^{-1}; dry gas flow, 13 L min^{-1}; capillary voltage, 3000 V in negative ion mode; nozzle voltage, 0 V; and nebulizer pressure, 45 psi.

The semi-preparative high-performance liquid chromatography was carried out on a Shimadzu UFLC system (LC-6 AD pump, DGU-20A5 degasser, SPD-20AV UV detector, rheodyne injector, CBM-20A communication module) with a Phenomenex Luna C_{18} column (5 µm, 250 × 10 mm). For column chromatography, the packing material was silica gel (SiliCycle Inc; 70-230 mesh). Thin layer chromatography analysis (analytical and preparative) was performed using precoated silica gel 60 F254 plates (0.25 mm, Merck), and spots were visualized by exposure to ultraviolet light at 254 and 365 nm, by spraying with sulfuric vanillin and heating on a plate. Nuclear magnetic resonance (NMR) spectra were acquired using a Bruker AVANCE III HD NMR spectrometer operating at 11.75 T (^{1}H and ^{13}C at 500 and 125 MHz, respectively).

Molecular networking

Product ion spectra arising from the LC-MS/MS analysis of the ethyl acetate extract from *A. amazonicus* were analyzed and organized in molecular networks by using the Global Natural Product Social Molecular Networking (GNPS2) platform.[23,24] The MS/MS data were converted to the .mzXML format with MS-Convert (ProteoWizard),[25] processed using mzmine 4.5 (mzio GmbH)[26] and then uploaded on the GNPS2 Web platform for creation of feature-based molecular networks.[27] Parameters for molecular network (SERVER:0.1.2;Workflow:2024.062.4) generation were set as follows: precursor ion mass tolerance of 0.02 Da, product ion tolerance of 0.05 Da, and fragment ions below 10 counts were removed from the MS/MS spectra. Molecular networks were generated using six minimum matched peaks and a cosine score of 0.75. Data were visualized using Cytoscape v. 3.10.3 software (Cytoscape Consortium). The MS/MS molecular network is accessible on the GNPS2 website via the link provided in the Supplementary Information (SI) section.

Genome sequencing

Genomic DNA sequencing was performed using the Illumina platform with paired-end reads of 150 base pairs. Quality control and adapter and quality trimming of raw reads were conducted prior to assembly using FastQC[28] (Babraham Bioinformatics) and Ktrim (Kun Sun),[29] respectively. Genome assembly was performed using SPAdes[30] v3.15.5 (St. Petersburg Academic University's Laboratory of Algorithmic Biology). Paired-end reads were provided in forward-reverse (FR) orientation, using the default paired-end configuration (--pe). The careful mode was enabled (--careful) to minimize mismatches and short indels, which is recommended for small to medium-sized genomes such as those of fungi. The coverage cutoff was set to auto (--cov-cutoff auto) to allow SPAdes to automatically estimate and filter low-coverage contigs. K-mer sizes were selected automatically based on read length. The final output included contigs and scaffolds, which were evaluated using QUAST[31] (Gurevich Lab), providing summary statistics such as total assembly size, number of contigs, N50, and GC content.

Genome mining

The genome assembly was analyzed using the Antibiotics & Secondary Metabolite Analysis Shell (AntiSMASH) fungal version 7.0 (FungiSMASH)[32] to predict metabolite biosynthetic gene clusters. The genome was uploaded to the FungiSMASH web platform[33] and analysis was performed using relaxed detection strictness with extra features, such as: knownclusterblast, MIBIG cluster comparison, cluster Pfam analysis, NCBI genomic context links, clusterBlast, activesitefinder, Pfam-based GO term annotation, subclusterblast, RREFinder and TIGRFam analysis. The OOS biosynthetic cluster was identified based on its similarity to the characterized cluster from *Beauveria bassiana* ARSEF 2860 available in the MIBiG[34] repository[35] (BGC0001720). To further annotate genes and validate predicted functions, BLASTp[36] searches were conducted against the National Center for Biotechnology Information (NCBI) non-redundant protein database.[37] In parallel, conserved domain scans were performed using Pfam[38] and InterProScan[39] databases (European Bioinformatics Institute), enabling detailed characterization of functional domains within proteins associated with biosynthetic pathways.

Fermentation, extraction and compounds isolation

The isolate of *A. amazonicus* was cultured in three Erlenmeyer flasks (250 mL), each containing 100 mL of potato dextrose broth for the analytical step. The culture was incubated for 14 days at 28 °C and under constant aeration in a rotary shaker (150 rpm), based on previous cultivation experiments. The fermented broth was then separated from the cells by filtration. Then, the liquid phase was extracted with EtOAc (300 mL, 3×, 1:1 v/v) that was evaporated under reduced pressure to give 135 mg of crude extract for the LC-MS/MS analyses. A twenty-fold scaled-up fermentation was performed following the same microbiological procedures, to afford 2 L of fermented broth, which was extracted with EtOAc (1000 mL, 1×, 1:1 v/v) to give

4.3 g of crude extract used in the isolation attempt. The majority (4.0 g) of this extract was subjected to column chromatography over silica gel (400 g) made up in hexanes and eluted with hexanes followed by hexanes-EtOAc (1:1, v/v), EtOAc and EtOAc-MeOH (1:1, v/v) (700 mL each) to give four main fractions (F1-F4). Fraction F3 (1.2 g) had a portion (100 mg) purified by semi-preparative HPLC (10 injections of 10 mg) eluted with a gradient consisting of H_2O/MeOH (90:10 to 0:100 during 20 min at 7.3 mL min^{-1}) to afford compounds OOS (17.4 mg, t_R = 5.5 min) and orcinol (7.4 mg, t_R = 6.3 min).

Grammarly's artificial intelligence (AI) was utilized for the translation and grammatical review of the manuscript, followed by a final revision by the authors to ensure accuracy and clarity.

Results and Discussion

Genome mining of *Arcopilus amazonicus*

The draft genome of *A. amazonicus* was obtained using the Illumina sequencing platform. The resulting assembly consisted of 225 contigs with a total length of 33.85 Mbp. Contig sizes ranged from 405 bp to 1.13 Mbp, with an average length of 150,464 bp. The assembly demonstrated high contiguity, with an N50 value of 401,751 bp and L50 of 28 contigs, indicating the presence of long, continuous stretches of assembled sequence. The GC content of the assembled genome was 57.85%, which falls within the expected range for filamentous fungi. Additionally, the N90 and L90 values were 131,416 bp and 84 contigs, respectively, reinforcing the quality of the assembly across smaller scaffolds and its usability for genomics studies. Furthermore, this genome represents the first assembly reported for the genus *Arcopilus*, providing a valuable resource for future taxonomic, functional, and comparative studies within the Chaetomiaceae family and related taxa.

Subsequently, the genomic investigation using fungiSMASH resulted in an output predicting the presence of 52 BGCs codifying metabolites biosynthesis. Biosynthetic systems, such as type I polyketide synthase (T1PKS, 29%), fungal-RIPP-like (15%), non-ribosomal peptide synthase (NRPS, 10%), interactive NRPS-T1PKS (10%), terpene (8%) and others (28%) were predicted (Figure S15, SI section). This represents a vast repertoire for the biosynthesis of specialized metabolites with diverse scaffolds and, consequently, varied properties. Additionally, *A. amazonicus* shows great potential for polyketide production, especially those synthesized by T1PKS. It is important to mention that these enzymes are the core of the biosynthesis of many fungal metabolites, which exhibit a wide variety of biological activities, including important drugs and toxins.[40] These characteristics make these genes valuable targets for mining.

Some of these BGCs showed high similarities to previous ones encoding known fungal metabolites. For instance, two T1PKS BGCs, one in region 103.1, showing 100% similarity to *Metarhizium anisopliae* polyketides named BAB and BAA,[41] and another in region 29.1, exhibiting 85% similarity to *Fusarium fujikuroi* genes involved in bikaverin biosynthesis.[42] On the other hand, the BGC that matched with the previously annotated[10] as OOS (region 22.1) displayed only 35% of similarity. (Table S2, SI section). At first, we thought it was a false-positive, but the architecture of this BGC (discussed below) confirmed its identity despite the low score. Additionally, we observed that only 42% of the BGCs had any match with the databases. Moreover, the remaining BGCs (58%) highlight that *A. amazonicus* possesses an intriguing unknown metabolism repertory, indicative of a capability to produce new metabolites yet to be studied further.

LC-MS/MS analysis and OOS identification

The metabolites corresponding to the genome mining matches were directly manually-searched for their corresponding exact *m/z* of protonated ($[M + H]^+$) and sodiated ($[M + Na]^+$) adducts (in positive mode) and deprotonated ($[M - H]^-$) (negative mode), both with tolerances of 5 ppm of error, however, only OOS was detected. Moreover, we searched the full list of recorded *m/z* in databases, such as the libraries available at GNPS2,[23] Atlas of Natural Products,[43] Metlin[44,45] and COCONUT.[46] In addition, all possibilities were checked by manually curating the ion product (MS/MS) spectra, but again, only OOS was annotated.

The analysis of the molecular networks for *A. amazonicus* revealed 7 network families, of which a cluster consisting of 34 connected nodes was the main one (Figures 1a and 1c), due to its size, but also for housing the node corresponding to OOS (cos = 0.72) according to the GPNS2 libraries (Figure 1d). Moreover, the total ion chromatogram showed that OOS (*m/z* 305.0303, t_R = 5.3 min) was the peak with the largest area, reinforcing our observation of direct crystallization of OOS in the crude extract, due to its amount (Figure 1b). Furthermore, we inspected the *m/z* of the main cluster, and found *m/z* 307.0459 (**1**, t_R = 1.6 min), *m/z* 277.0718 (**2**, t_R = 1.7 min), *m/z* 291.0510 (**3**, t_R = 1.9 min), *m/z* 275.0567 (**4**, t_R = 3.0 min), and *m/z* 273.0405 (**5**, t_R = 6.9 min) (Table 1).

To address the identity of compounds **1**-**5**, and aiming to isolate OOS, a large-scale fermentation was

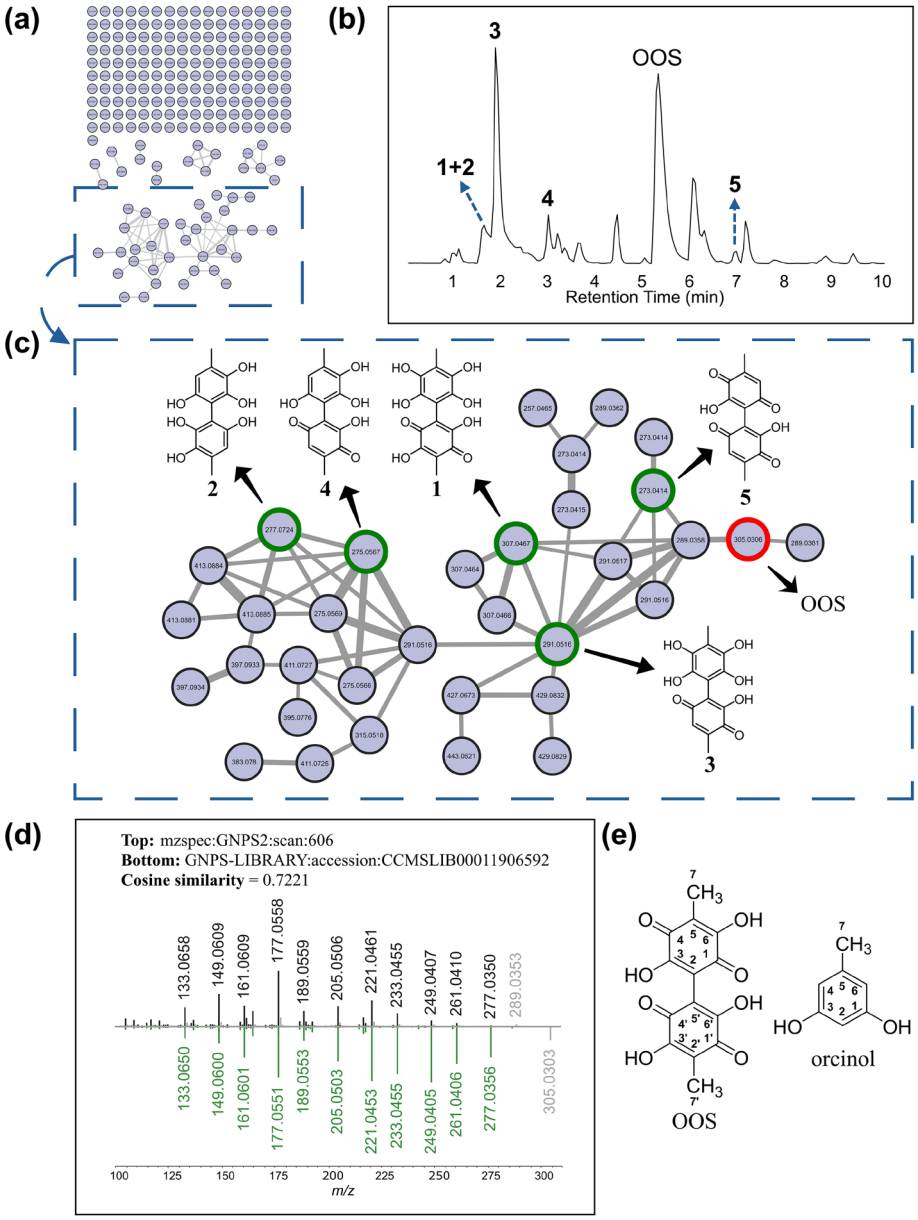

Figure 1. Molecular networks from LC-MS/MS data of *A. amazonicus*. (a) Full feature-based molecular network with singletons. (b) Total ion chromatogram showing elution peaks of OOS and analogous compounds, numbered **1** to **5**. (c) A 35-node molecular family containing the isolated OOS (red circle) and analogous molecules (green circles) with their respective putative structures, while other nodes represent ion source fragments or unannotated compounds. (d) Mirror image comparison between the experimentally obtained mass spectrum of OOS and a reference spectrum from the GNPS library. (e) Structures of the isolated compounds, OOS and orcinol.

Table 1. Retention times and observed masses of OOS and analogous compounds **1**-**5** obtained from LC-MS/MS, along with their molecular formulas, calculated masses and mass errors

Compound	Retention time / min	Molecular formula	Observed mass	Calculated mass	Error / ppm
1	1.6	$C_{14}H_{11}O_8^-$	307.0448	307.0459	3.6
2	1.7	$C_{14}H_{13}O_6^-$	277.0707	277.0718	4.0
3	1.9	$C_{14}H_{11}O_7^-$	291.0500	291.0510	3.4
4	3.0	$C_{14}H_{11}O_6^-$	275.0561	275.0567	2.2
5	6.9	$C_{14}H_9O_6^-$	273.0411	273.0405	3.3
OOS	5.3	$C_{14}H_9O_8^-$	305.0296	305.0303	2.3

OOS: oosporein.

performed to attempt the isolation of these molecules. Furthermore, we isolated OOS and orcinol[9] (not detected by MS, Figures S4-S9, SI section), but unfortunately the other isolated peaks were obtained in derisory amounts, unsuitable for NMR and/or were prone to degradation under the laboratory conditions. Therefore, the inspection of the ^{1}H NMR spectrum (Figure S2, SI section) of OOS, a sole signal corresponding to a singlet at δ_H 1.81 corresponding to the two symmetrical methyl groups was observed, in accordance with previous isolations.[12,18,47] OOS was first isolated from *Oospora colorans*,[48] and it has been reported in species of the genus *Chaetomium* and *Beauveria*.[11,12,19,49]

Fragmentation analysis of bibenzoquinones related to OOS

The absence of chemical information about some of the main produced metabolites, reinforced the importance of manual curation of MS/MS spectra to enhance chemical reliability. Therefore, we believe that mass spectrometry may shed light over the questions about the chemistry of this rare strain, particularly OOS and its plausible analogues observed by molecular networking and genome mining (next section).

To accomplish this, the fragmentation patterns of the MS/MS spectra of OOS and **1**-**5** obtained by high-resolution mass spectrometry with collision-induced dissociation (CID, at 20 eV, using argon) were manually investigated. Furthermore, it was revealed that several ions were common to them, confirming their proximity in the molecular family, thus suggesting a structural relationship among them (Table S1, SI section). Additionally, signals arising from neutral losses of 28 Da and 44 Da, corresponding to eliminations of carbon monoxide (CO) and carbon dioxide (CO_2), respectively, were frequently observed for OOS, **1**, **3** and **5**.

Taking OOS as the seed molecule for our interpretation, the MS/MS spectrum of this compound displayed a base peak (*m/z* 221) is formed through a pathway of ring contractions on both sides of this molecule (due to its symmetrical shape), initiating by three consecutive eliminations of CO (– 28 Da), giving the respective product ions *m/z* 277 (A), *m/z* 249 (B) and *m/z* 221 (C) (*m/z* 305 → *m/z* 277 → *m/z* 249 → *m/z* 221), in which oxygenated methylcyclopentadiene (A, B, C and D ions, Figure 2b) and methylcyclobutene (C and E ions) structures. This set of cascade CO eliminations to form the base peak is followed by a CO_2 neutral loss (– 44 Da) to give *m/z* 177 (*m/z* 221 → *m/z* 177, D ion, Figure 2b). Then, originates two distinct product ions, the *m/z* 149 (*m/z* 177 → *m/z* 149, E ion) via another CO loss and *m/z* 133 (*m/z* 177 → *m/z* 133, F ion).

On the other hand, a competitive pathway is observed originating from the $[M - H]^-$ ion beginning with an initial CO_2 loss, followed by two CO losses and ended with another CO_2 loss (*m/z* 305 → *m/z* 261 → *m/z* 233 → *m/z* 205 → *m/z* 161; forming the ions G, H, I and J). The initial loss of CO_2 is anomalous, as it is common event for flavonoid carbonyl groups[50] but not for *para*-benzoquinones. Nevertheless, after a first ring contraction, 2-hydroxy-1,4-naphtoquinone undergoes a CO_2 elimination by the loss of one carbyl group and an oxygen atom from an hydroxyl,[51] proving the feasibility of this type of loss, at least after the first ring contraction. We believe that different approaches, such as isotope labeling and computational methods, can address this question in the future. Then, the subsequent CO eliminations proceed all with the logic of ring contraction. Furthermore, the ion *m/z* 261 may also yield the ions *m/z* 217 (N ion), *m/z* 189 (O ion) and *m/z* 161 (J ion) (*m/z* 261 → *m/z* 217 → *m/z* 189 → *m/z* 161), comprising a CO_2 loss followed by two consecutive CO losses (Figure 2b).

With hands-on the fragmentation pattern of a validated seed molecule, we expanded the logic of ring contraction for compounds **1**-**5** observed in the molecular network (Figure 1c). Compound **1** shared as product ions K (*m/z* 307 → *m/z* 289, H_2O neutral loss), G (*m/z* 289 → *m/z* 261, CO neutral loss), competing with the formation of M (*m/z* 289 → *m/z* 245, CO_2 neutral loss), N (*m/z* 245 → *m/z* 217, CO neutral loss) and O (*m/z* 217 → *m/z* 189, CO neutral loss) (Figure 2b). This logic of the fragmentation of **1**, let us to propose this compound as a heterodimmer with the same degree of oxygenation of OOS, but consisted of a quinone linked to a quinol biphenyl system.

The mass spectrum of compound **3**, with an $[M - H]^-$ ion at *m/z* 291, displayed that this compound exhibits one oxidation level lower than compound **1** ($\Delta_{m/z}$ = –16 Da). The fragmentation mechanism is also similar, showing eliminations of H_2O, CO and CO_2 in the formation of ions L (*m/z* 273), M (*m/z* 245), P (*m/z* 263), Q (*m/z* 219), S (*m/z* 201), T (*m/z* 173), U (*m/z* 247) and V (*m/z* 137). However, it is not possible to predict which carbon is not hydroxylated by mass spectrometry, and for illustration purposes, only one structure is presented for compound **3** among the possible structures (Figures 1c and 2b).

Moreover, following the same idea of fragmentation, we observed that compound **4** (*m/z* 275.0567, $C_{14}H_{11}O_6$) showed the fragments R (*m/z* 275 → *m/z* 229, CO_2 neutral loss) and V, the later corresponding to the quinone moiety after the loss of the quinol moiety ion, also observed for compound **3**, that shares the same pattern of heterodimmerization. Therefore, compound **4** was

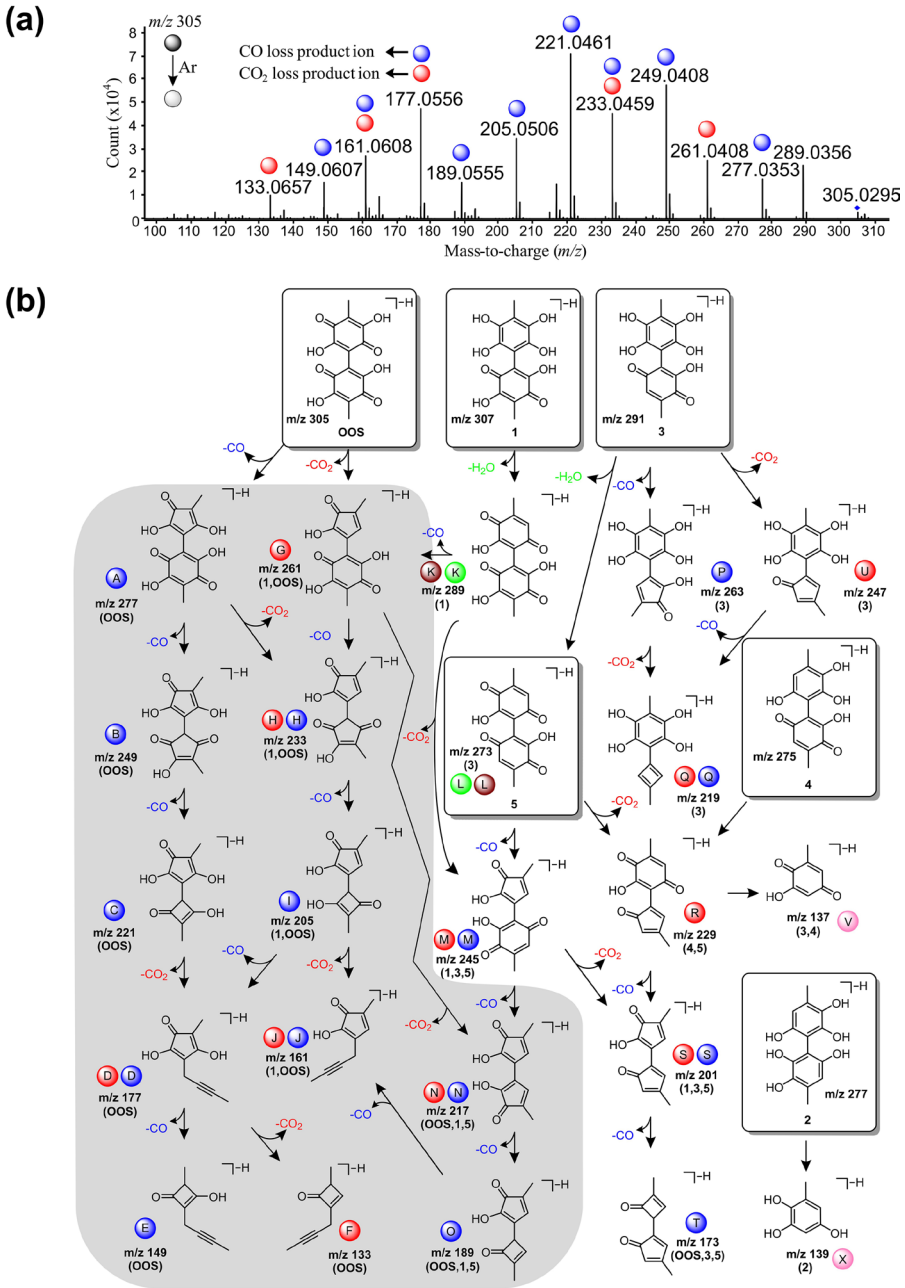

Figure 2. (a) Mass spectrum of OOS. (b) Proposed fragmentation of OOS and **1**-**5**, via successive ring contractions. Ions formed from the loss of CO, CO_2 and H_2O are shown by blue, red and green spheres, respectively. Pink spheres indicate any other losses. It is important to note that CO and CO_2 losses can occur from any of the rings, and for each ion, only one of the many possible structures is displayed. Gray area highlights the ions of OOS fragmentation pathway. Also, originating precursor ions are indicated in parentheses below the respective *m/z*.

annotated at level 3 of confidence as phoenicin quinol.[52] Compound **5** (*m/z* 273.0414, $C_{14}H_9O_6$) displayed similar fragmentation as OOS, being annotated as phoenicin. It is worth noticing that this metabolite, wrongly termed as the new "5,5'-dideoxy-oosporein", was obtained as a shunt metabolite from the OOS biosynthesis in *B. bassiana* mutants lacking the laccase, GST and cupin genes, as well as through heterologous expression in yeast genetically modified by the insertion of the sacilylate hydroxylase gene and the addition of orsellinic acid to the culture medium.[19] Phoenicin (phenicin), was described in 1938 from *Penicillium phoeniceum* extracts by E. Friedheim,[53] and later correlated with the biosynthesis of OOS,[54,55] despite the latter never been observed in *Penicillium* species.[56] Compounds **4** and **5** are recognized as main metabolites from *P. phoeniceum*, but also detected by mass spectrometry in *P. manginii*, *P. chermesinum*, and *P. atrosanguineum*.[57] Finally, compound **2** was annotated as phoenicin diquinol, according to its fragmentation behavior, in which the loss of the quinol moiety yields the *m/z* 139 product ion (loss of

138 Da) and previous observations by mass spectrometry analysis of *P. phoeniceum*.[58] Interestingly, *A. amazonicus* is capable of producing OOS, **2**, **4** and **5** simultaneously, which so far was not observed. Moreover, compounds **2**, **4** and **5** were employed as negolyte in a redox flow battery with potassium ferri/ferrocyanide as a posolyte in the battery, showing an initial capacity of 1.58 Ah L^{-1}.[58]

OOS biosynthetic gene cluster

The analysis of the BGC exhibiting similarity to the OOS cluster revealed 15 genes, including a 8.5 kb gene responsible for codifying a NR-PKS (*AAm11*) with the domains necessary to produce orsellinic acid (OA). This was evidenced by Pfam domain analysis, which indicated the presence of β-ketoacyl synthetase (KS), acyl transferase (AT), thioesterase (TE), starter-unit acyltransferase (SAT) and phosphopantetheine atthachment site (PP-binding) (Figure 3b). Additionally, a BLASTp-based homology analysis showed high amino acid similarity between AAm11 predicted protein with the PKS OpS1 from *Beauveria bassiana* ARSEF 2860 (Table S3, SI section).[19] Considering this similarity, we propose that *A. amazonicus* initiates the biosynthesis of OOS with the

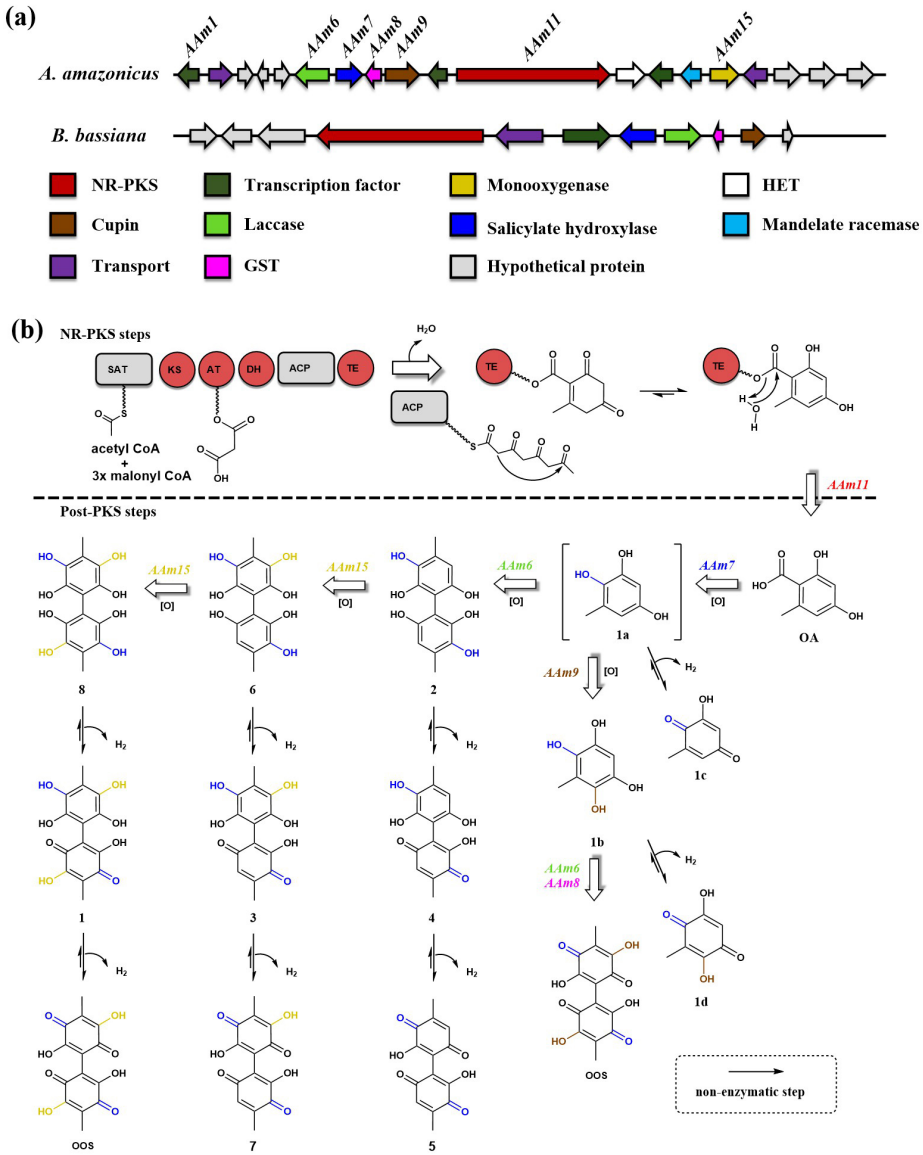

Figure 3. Biosynthesis of OOS and analogous compounds. (a) Schematic representation of biosynthetic gene cluster of *A. amazonicus* and *B. bassiana*. Gene *AAm8* is a glutathione S-transferase (GST) and gene *AAm11* is a non-reducing polyketide synthase (NR-PKS). (b) Proposed biosynthetic pathway with enzyme-catalyzed steps indicated. NR-PKS AAm11 domains include: SAT, starter-unit acyltransferase; KS, β-ketoacyl synthase; AT, acyltransferase; DH, dehydrogenase; ACP, acyl carrier protein; TE, thioesterase. AAm7 is a salicylate hydrolase enzyme, AAm6 is a laccase enzyme, AAm9 is an enzyme of the cupin superfamily, *AAm15* is a monooxygenase and AAm8 is a glutathione S-transferase enzyme. Colors are used to correlate genes and enzymes with the oxygen atoms added to AO.

production of OA by the NR-PKS, as observed previously[19] and based on the observation of this compound in the LC-MS/MS analysis.

Moreover, post-PKS enzymes encoding genes were encountered with hits for salicylate hydroxylase, laccase, glutathione S-transferase (GST), and cupin-family dioxygenase as previous observed for *B. bassiana* (Table S3, SI section).[19] On the other hand, additional genes that may be related with the biosynthesis were observed, especially one responsible for the production of a monooxigenase (*AAm15*); observations that pave the way for an alternative route to produce OOS and related compounds. This hypothesis is corroborated by the production of the biphenyl **2**, which is putatively produced by *P. phoeniceum*,[58] but not by *Beauveria*. Additionally, the BGC associated with compound **5** biosynthesis in *P. atrosanguineum* exhibits high similarity to PKS, salicylate hydroxylase and laccase from *B. bassiana*. However, it lacks genes related to cupin and GST enzymes, which are necessary for OOS biosynthesis (Table S3, SI section).[55]

Through the observance of **2**, in addition to the genes *AAm6* and *AAm7* encoding laccase and salicylate hydroxylase enzymes, respectively, it is plausible that OA is converted in **1a** by the monooxygenase and readily dimerized by laccase to give the key intermediate **2**. This reaction is expected to occur, since phenols are promiscuous substrates for laccases.[59] This intermediate may undergo incomplete spontaneous oxidation to give **4**, and subsequently **5** after the complete process. Therefore, the non-enzymatic conversion of **2** in its semi-oxidized analogue **4**, and subsequently in **5** is proposed herein (Figure 3b).

The presence of genes encoding cupin oxygenase (*AAm9*) and GST (*AAm8*) enzymes corroborates a previously proposed oxidation and dimerization pathway for producing OOS from **1a** in *B. bassiana*.[19] In this pathway, intermediate **1a** is oxidized by cupin to form benzenetetrol **1b**, followed by dimerization promoted by laccase, assisted by GST. The last is a versatile enzyme involved in the detoxification of electrophilic species,[60] and in this context, it scavenges radicals potentially produced by laccase. However, the presence of a monooxygenase gene (*AAm15*) and the detection of intermediates with varying degrees of oxidation, as observed in LC-MS/MS analyses suggest an alternative pathway in *A. amazonicus* (Figure 3b). The gene *AAm15* exhibits similarity to a monooxygenase from *Gnomonia* sp., which is known to promote the hydroxylation of phenols (Table S3, SI section).[61] Based on this information, it is plausible to infer that **2** undergoes two oxidation steps to yield intermediates **6** and **8**, both of which were undetected. While **6** spontaneously oxidizes to generate **3** and **7** (not detected), intermediate **8** is converted into **1** and OOS (Figure 3b).

Therefore, the OOS pathway in *A. amazonicus* diverges in some aspects from previous studies. This suggests that further investigations involving other fungal species are necessary to either confirm distinct pathways among different species or to propose a unified pathway that integrates previous findings.

Conclusions

Oosporein is the major specialized metabolite produced by the endophytic fungus *A. amazonicus.* Genes related to its biosynthesis diverge significantly from their counterparts in *B. bassiana*. Additionally, analogous compounds known to be produced by *Penicillium* species, such as phoenicin, phoenicin quinol, and phoenicin diquinol, were annotated in *A. amazonicus* extracts. The latter appears to be a crucial precursor in the biosynthesis of these compounds via a putative alternative route mediated by the enzyme *AAm15*, which is exclusively present in *A. amazonicus*. Notably, this is the first report of a fungus capable of simultaneously biosynthesizing all these compounds.

Supplementary Information

Supplementary information, including NMR spectra, mass spectra, genome mining results table and molecular network link, is available free of charge at http://jbcs.sbq.org.br as PDF file.

Data Availability Statement

All data generated or analyzed during this study are included in this published article. Additionally, the generated molecular networks can be consulted on the GNPS2 platform through the link present in the SI section.

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Author Contributions

MBAJ was responsible for data curation, formal analysis, investigation, and writing the original draft of the manuscript; AOS for data curation, formal analysis, and investigation; AMR, FEC and MEBY for investigations; TFS for data curation and formal analysis; FMAS, CFFA, RSG, and LSM for essential resources; RSG for formal analysis; GFS forf unding acquisition, methodology development, and the provision of resources; HHFK for conceptualized the study, secured funding, developed the methodology, administered the project, provided resources and supervision, and contributed to the review and editing of the manuscript.

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