Organic LETTERS

Letter

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Pseudoxylallemycins A–F, Cyclic Tetrapeptides with Rare Allenyl Modifications Isolated from *Pseudoxylaria* sp. X802: A Competitor of Fungus-Growing Termite Cultivars

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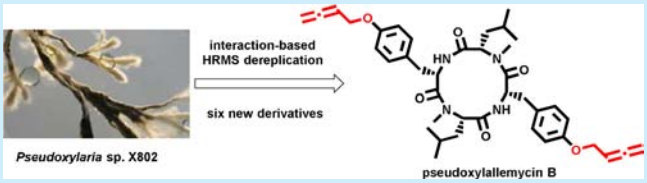
Supporting Information

ABSTRACT: Based on fungus–fungus pairing assays and HRMS-based dereplication strategy, six new cyclic tetrapeptides, pseudoxylallemycins A–F (**1**–**6**), were isolated from the termite-associated fungus *Pseudoxylaria* sp. X802. Structures were characterized using NMR spectroscopy, HRMS, and Marfey's reaction. Pseudoxylallemycins B–D (**2**–**4**) possess a rare and chemically accessible allene moiety amenable for synthetic modifications, and derivatives A–D showed antimicrobial activity against Gram-negative human-pathogenic *Pseudomonas aeruginosa* and antiproliferative activity against human umbilical vein endothelial cells and K-562 cell lines.



Obligate mutualisms, such as fungal agriculture in insects,[1] are widespread in nature and often play dominant roles in ecosystems, as the combined characteristics of different organisms allow adaptation to previously inaccessible niches.[2,3] Termites of the subfamily Macrotermitinae live in an obligate symbiosis with a specialized fungal cultivar *Termitomyces* sp. (Basidiomycotina),[4,5] which is cultivated on fecal deposits of dead predigested plant material (the fungus comb). Under healthy conditions, *Termitomyces* sp. is the only fungus present and visible in the fungus combs. *Pseudoxylaria*, a subgenus within the genus *Xylaria* (Ascomycota: Xylariaceae), is, however, frequently found on deteriorating comb material and is presently considered a stowaway fungus, waiting as a substrate specialist and opportunistic weed until conditions are favorable for outcompeting *Termitomyces*.[6] Members of the Xylariaceae family are ecologically important due to their saprotrophic nature and are known for their outstanding biosynthetic capabilities to produce structurally diverse metabolites with a broad spectrum of biological activities.[7] Based on the competitive and/or antagonistic behavior, we hypothesized that termite-associated *Pseudoxylaria* may produce biologically active small molecules upon exposure to other fungi from the same ecological niche. Using a MS-based imaging and dereplication strategy, we identified six new cyclic peptides, pseudoxylallemycins A–F.

Pseudoxylaria sp. X802 was obtained from fungal comb material of a *Microtermes* sp. colony collected in South Africa (Table S1). Based on 99% ITS sequence identity, *Pseudoxylaria* sp. X802 (KX097055) is most closely related to *Xylariaceae* sp. OTU8 (FJ425677.1, Supporting Information). To investigate its potential to produce bioactive metabolites, we subjected isolate X802 to fungus–fungus pair challenge assays against different *Termitomyces* spp. (fungal cultivar) and other naturally co-occurring fungi (*Cladosporium perangustum*, *Pleosporales* sp. *Alternaria* sp., *Hypocrea virens*, *Fusarium* sp., and *Coriolopsis* sp.).[8] *Pseudoxylaria* sp. X802 showed strong antifungal activity against all four *Termitomyces* species and weak or moderate antifungal activity against other fungi.

Comparative LC-MS analysis of mycelium extracts of unchallenged versus challenged *Pseudoxylaria* sp. X802 revealed a strongly induced and complex mixture of secondary metabolites upon cocultivation. In addition, we observed an enhanced rate of exudation of droplets (guttation) in close proximity to the competitor.[9] To analyze the secondary metabolites produced by *Pseudoxylaria* sp. X802, we first harvested the guttation droplets produced in coculture with *Coriolopsis* sp. Subsequent HPLC followed by ESI-HRMS analysis indicated several abundant, but so far unreported, protonated molecular ions (*m*/*z* 549.3434 (**1**), 685.3956 (**2**), 617.3695 (**3**), 633.3642 (**4**) $[M + H]^+$), which correlated with distinct UV absorption at 195 nm (Supporting Information).[10] To visualize the distribution of the identified ions, we performed MALDI-TOF MS imaging of the zone of inhibition and guttation

Received: May 17, 2016
Published: June 24, 2016

DOI: 10.1021/acs.orglett.6b01437
Org. Lett. 2016, 18, 3338–3341

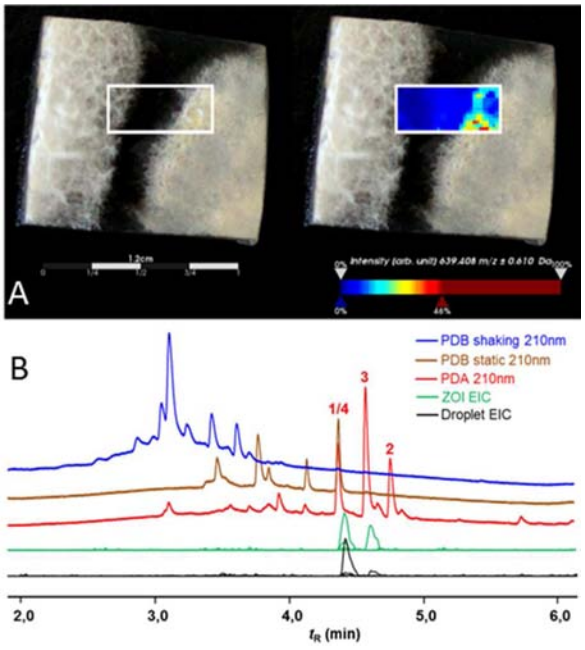


Figure 1. (A) Interaction assay of X802 against co-isolate *Coriolopsis* sp. Visualization of guttation droplets of X802 using MALDI-TOF MS imaging of m/z 639.408 ± 0.610 Da ($[M + Na]^+$ of **3**). (B) HPLC chromatogram (210 nm) of cultivation conditions (PDB shaking, PDB static, PDA plates) and LC-MS chromatogram (EIC mode) of secreted droplet (black) and zone of inhibition (ZOI, green).

droplets from the paired fungus−fungus interaction assays (Supporting Information). In accordance with the LC-HRMS data, differential MS imaging analysis (mycelium vs droplets) revealed several ions (m/z) correlated to compounds **1**−**4** within the guttation droplets. We then investigated different cultivation conditions of *Pseudoxylaria* sp. X802 to optimize production yield in pure culture and to fully characterize the potentially new metabolites. While shaking liquid cultures afforded only trace amounts of the respective compounds, standing liquid culture and agar plate cultivation stimulated the production of all unidentified metabolites in varying ratios depending on the growth conditions (Figure 1B). Generally, maximum ion abundance was detectable by LC-MS after 4−5 weeks of cultivation. For a first preparative scale cultivation, *Pseudoxylaria* sp. X802 was grown on PDA plates for 5 weeks, and plates fully covered with mycelium were extracted using a previously established procedure.[11] Crude extracts were purified using a SPE C18 cartridge followed by Sephadex LH20 column chromatography. For final purification, enriched fractions were subjected to semipreparative reverse-phase HPLC (C18 column), yielding four new compounds (**1**−**4**) with matching HRMS and UV spectra predicted from the droplet analysis. In addition, LC-(HR)MS analysis of fractions highly enriched in compounds **1**−**4** revealed two additional minor m/z congeners, pseudoxylallemycin E (**5**, m/z 619.3851 $[M + H]^+$) and pseudoxylallemycin F (**6**, m/z 687.4117 $[M + H]^+$), that differ from compounds **2** and **3** by two mass units.

Compounds **1**−**6** were named pseudoxylallemycins A−F (Figure 2) after the producer *Pseudoxylaria* sp. X802. Their planar structures were determined by the combination of HRMS, 1D and 2D NMR spectroscopy, and the absolute configuration by using NOESY experiments, Marfey's reaction,[12] and CD spectroscopy (Supporting Information). The molecular formula of pseudoxylallemycin A (**1**) was assigned as $C_{32}H_{44}O_4N_4$ based on ESI-HRMS (m/z 549.3434 $[M + H]^+$, calcd 549.3435, Δ = −0.28 ppm). The ^{1}H NMR spectrum revealed 22 protons, pointing to a symmetric structure. ^{1}H NMR signals at δ_H 4.81 ppm (δ_C 50.9 ppm) and δ_H 3.83 ppm (δ_C 58.3 ppm) indicated the peptidic nature of **1** (α-position of amino acid residue). Subsequent analysis of COSY and HMBC spectra allowed the determination of all bond connectivities, and compound **1** was assigned as *cyclo*-(*N*-methyl-L-leucine-L-phenylalanine)$_2$. ESI-HRMS analysis of pseudoxylallemycin B (**2**) indicated a molecular formula of $C_{40}H_{52}O_6N_4$ (m/z 685.3956 $[M + H]^+$, calcd 685.3960, Δ = −0.57 ppm). Again, the ^{1}H NMR spectrum showed only 26 proton signals, indicating a symmetric molecule. Detailed comparison of ^{1}H and ^{13}C NMR spectra revealed the presence of a modified tyrosine instead of a phenylalanine moiety. Modification of tyrosine was identified as an unusual allenic ether C-4 building block. This structural proposal was supported by the presence of an additional methine group (δ_H 5.44 ppm, δ_C 87.1 ppm), two methylene groups (δ_H 4.50 ppm, δ_C 65.1 ppm; δ_H 4.94 ppm, δ_C 77.0 ppm), and one quaternary carbon (δ_C 208.7 ppm). COSY (H-17 to H-18 and H-20) and HMBC correlations (H-17 to C-18 and C-19, H-18 to C-19 and C-20, H-20 to C-17, C-18, and C-19) as well as an IR absorption band at 1955 cm^{-1} confirmed the presence of an allene moiety. Compound **2** was therefore assigned as *cyclo*-(*N*-methyl-L-leucine-4-(buta-2,3-dienyloxy)-L-phenylalanine)$_2$. In contrast, 48

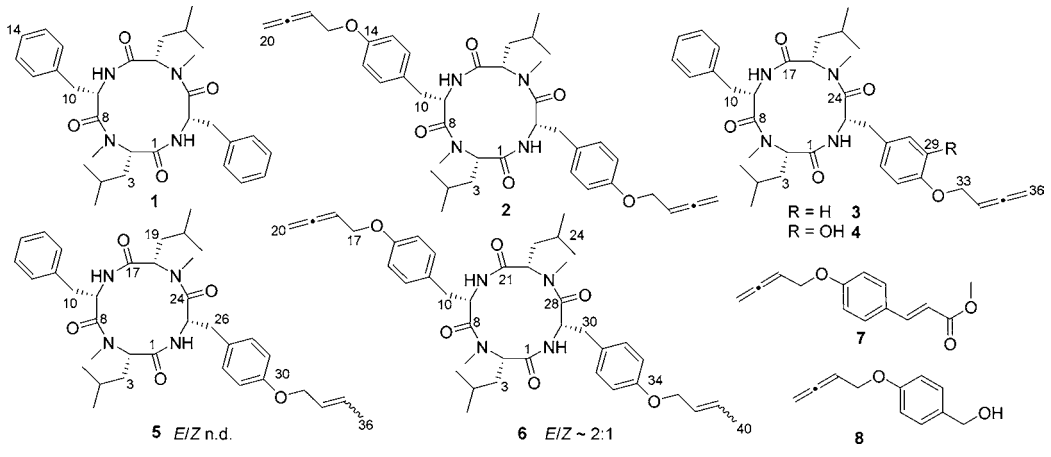


Figure 2. Structures of pseudoxylallemycins A−F (**1**−**6**), eucalyptene A (**7**), and terricollene C (**8**).

DOI: 10.1021/acs.orglett.6b01437
Org. Lett. 2016, 18, 3338−3341

proton signals in the ^{1}H NMR spectrum of pseudoxylallemycin C (**3**, $C_{36}H_{48}O_5N_4$, *m/z* 617.3695 $[M + H]^+$, calcd 617.3697, Δ = −0.38 ppm) indicated an asymmetric architecture. Again, sequence analysis based on COSY and HMBC correlations suggested the presence of a phenylalanine and an allenic ether modified tyrosine. This indication was confirmed by analysis of HRMS/MS fragment patterns. Similarly, pseudoxylallemycin D (**4**, $C_{36}H_{48}O_6N_4$, *m/z* 633.3642 $[M + H]^+$, calcd 633.3647, Δ = −0.73 ppm) revealed 48 proton signals with a different aromatic substitution pattern. The additional 16 mass units indicated a hydroxyl group, which was positioned at position C-29 based on 2D NMR and detailed HRMS/MS fragmentation analysis. Finally, comparative 1D and 2D NMR analysis and characteristic MS fragmentation patterns of compounds **5** and **6** indicated the presence of the same cyclic peptide core structure as for **2** and **3**, but now carrying an alkenyl ether modified tyrosine instead of an allenic ether. In addition, we also isolated the known allenic aromatic ethers eucalyptene A (**7**) and terricollene C (**8**), as well as three known cytochalasin derivatives (19,20-epoxycytochalasin C (**9**), 19,20-epoxycytochalasin D (**10**), and 19,20-epoxycytochalasin Q (**11**); see Supporting Information).

Pseudoxylallemycins A−F (**1**−**6**) are medium-ring-sized cyclic peptides containing hydrophobic and aromatic amino acids and an alternating pattern of N-methylation, with *N*-methyl groups present at the leucine but not the phenylalanine or tyrosine residues. Derivatives **2**−**4** carry a rare allenyl (C-4 fragment) modification at the aromatic moiety, which can be easily modified, using metal-catalyzed cycloaddition,[13] to yield derivatives applicable for target identification or mode of action studies.[14] Medium-ring-sized cyclic peptides have been reported from a diverse range of micro-organisms and often display antimicrobial or cytotoxic activity. Most compounds similar to pseudoxylallemycins are, for example, hirsutide from the spider-derived entomopathogenic fungus *Hirsutella* sp.,[15] the symmetric tetrapeptide *cyclo*-(L-Ile-*N*-methyl-L-Phe)$_2$ from the fungus *Onychocola schlerotica*,[16] and xyloallenolide B, which was isolated from a mangrove endophytic *Xylaria* sp.[17] Allene moieties are rare in natural products.[18] However, they are often attributed to the biological activities of the compounds, such as enzyme inhibition, cytotoxicity, and/or antiviral action.[19] Yet, little is known about the microbial biosynthesis of allenes. Several models for biosynthetic pathways have been proposed, including the formation of an alkenyl unit by enzymatic desaturation, followed by oxidative or reductive rearrangements.[20] So far, we were not able to identify any predicted biosynthetic precursors of **2**−**4** using MS, IR, and comparative NMR analysis. However, it is likely that alkene derivatives **5** and **6**, eucalyptene A (**7**), and terricollene C (**8**) are shunt products of the biosynthetic pathway. Efforts to sequence and assemble the genome of *Pseudoxylaria* sp. X802 and to analyze the biosynthetic origin of pseudoxylallemycins are currently underway.

Since *Pseudoxylaria* sp. X802 exhibited antifungal activity against several fungi in cocultivation, pseudoxylallemycins A−F were evaluated for their antimicrobial properties. So far, none of the isolated compounds were responsible for the observed antifungal activity, implying that *Pseudoxylaria* sp. X802 produces additional as yet unidentified antifungal metabolites. We also evaluated the antibacterial properties (Table 1) and antiproliferative and cytotoxic activities (Table 2) of compounds **1**−**4**. Pseudoxylallemycins A−D (**1**−**4**) showed antimicrobial activity against the Gram-negative human pathogen *Pseudomonas aeruginosa* and *Mycobacterium vaccae* with minimal inhibition concentrations (MICs) of 12.5−25.0 μg/mL. Additionally,

Table 1. Minimal Inhibitory Concentrations (μg/mL) of Pseudoxylallemycins A−D (1−4) and Ciprofloxacin (cip) toward Gram-Positive and Negative Bacteria

	antimicrobial activity MIC (μg/mL)		
compound	*Staphylococcus aureus* SG511	*Mycobacterium vaccae* 10670	*Pseudomonas aeruginosa* K799/61
1	>100	12.5	12.5
2	>100	25	12.5
3	>100	12.5	12.5
4	>100	25	12.5
cip	0.2	0.4	0.2

Table 2. Antiproliferative Effect and Cytotoxicity of Pseudoxylallemycins A−D (1−4)

	antiproliferative effect GI_{50} (μg/mL)		cytotoxicity CC_{50} (μg/mL)
compound	HUVEC[a]	K-562[b]	HeLa[c]
1	14.0	19.4	40.4
2	9.8	25.5	30.3
3	4.3	4.2	10.3
4	33.8	42.8	49.5

[a]Human umbilical vein endothelial cells. [b]K-562 cell line: human immortalized myelogenous leukemia line. [c]HeLa: human cervical cancer cell line.

compounds **1**−**4** exhibited antiproliferative activity against human umbilical vein endothelial cells (HUVEC) and K-562 cell lines, with GI_{50} values ranging from 4.2 μg/mL (**3**, K-562) to 42.8 μg/mL (**4**, K-562), in addition to cytotoxic activity (HeLa cells) with a CC_{50} value as low as 10.3 μg/mL (**3**).

To our knowledge, this is the first study to explore the biosynthetic potential of a *Pseudoxylaria* sp. associated with the fungus-growing termite genus *Microtermes*. Using ecological-based pair challenging assays, MS-based imaging, and HRMS analysis of guttation droplets, we were able to identify six new cyclotetrapeptides, pseudoxylallemycins A−F (**1**−**6**), with derivatives B−D carrying a rare allenyl (C-4 fragment) modification. Pseudoxylallemycins A−D show promising antimicrobial activity and antiproliferative and cytotoxic properties.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b01437.

Isolation and characterization of fungal strains, challenge assays, and MS imaging, HRMS, ^{1}H NMR, ^{13}C NMR, and 2D NMR spectra of isolated compounds (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful for financial support from the German National Academy of Sciences Leopoldina (LPDS 2011-2) and the Daimler Benz foundation to C.B., the Villum Kann Rasmussen Foundation for a Young Investigator Fellowship (VKR10101) to

DOI: 10.1021/acs.orglett.6b01437
Org. Lett. 2016, 18, 3338−3341

M.P., the Deutsche Forschungsgemeinschaft for Grant SFB 1127 (ChemBioSys) to C.H. and M.G.A., and the ERC for a Marie Skłodowska-Curie Individual Fellowship (IF-EF) to M.G.A. We thank Mrs. Heike Heinecke (Hans Knöll Institute) for recording NMR spectra, Mrs. Andrea Perner (Hans Knöll Institute) for HRMS measurements, and Ms. Victoria Challinor (University of Copenhagen) for comments on a draft of the manuscript. We thank Z. Wilhelm de Beer, Michael J. Wingfield, and the staff and students at the Forestry and Agricultural Biotechnology Institute, University of Pretoria, for hosting fieldwork, and the Oerlemans family (Mookgophong) for permission to sample colonies at their farm.

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DOI: 10.1021/acs.orglett.6b01437
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