Targeted Discovery of Tetrapeptides and Cyclic Polyketide-Peptide Hybrids from a Fungal Antagonist of Farming Termites

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1. General Experimental Procedures

**NMR measurements** were performed on a Bruker AVANCE III 500 MHz spectrometer, equipped with a Bruker Cryoplatform. The chemical shifts are reported in parts per million (ppm) relative to the solvent residual peak of DMSO-$d_6$ ($^1$H: 2.50 ppm, quintet; $^{13}$C: 39.52 ppm, heptet) and CD$_3$OH-$d_3$ ($^1$H: 3.31 ppm; $^{13}$C: 49.0 ppm).

**UPLC-ESI-HRMS** measurements were carried out on a Dionex Ultimate3000 system (Thermo Scientific) combined with a Q-Exactive Plus mass spectrometer (Thermo Scientific) equipped with an electrospray ion (ESI) source. Metabolite separation was carried out using a by reverse phase liquid chromatography at 40°C using a Luna Omega C18 column (100 x 2.1 mm, particle size 1.6 µm, 100 Å, Phenomenex) preceded by a SecurityGuard™ ULTRA guard cartridge (2 x 2.1 mm, Phenomenex). Mobile phases were acidified with 0.1% formic acid and consisted of H$_2$O (A) and acetonitrile (B). 5 µl of sample were injected into a gradient as follows: 0–1 min, 5% B; 1–7 min, 99% B; 7–9 min, 99%; 9–10 min, 5% B; 10–13 min, 5% at a constant flow rate of 0.3 mL/min.

Metabolites were detected in positive (MS$^1$) ionization mode within a range of m/z 150 – 1800 with a resolving power of 70,000 at m/z 200. MS$^2$ measurements were performed using combined methods of data-dependent MS$^2$ analysis and Top10 experiments. The resolving power was set to 70,000 at m/z 200 for MS$^1$ and 17500 for MS$^2$, with an isolation window of 1.0 m/z and a stepped normalized collision energy (NCE) of 20/30/40.

**UHPLC-MS measurements** were performed on a Shimadzu LCMS-2020 system equipped with single quadrupole mass spectrometer using a Phenomenex Kinetex C18 column (50 x 2.1 mm, particle size 1.7 µm, pore diameter 100 Å). Column oven was set to 40 °C; scan range of MS was set to m/z 150 to 2,000 with a scan speed of 10,000 u/s and event time of 0.25 s under positive and negative mode. DL temperature was set to 250 °C with an interface temperature of 350 °C and a heat block of 400 °C. The nebulizing gas flow was set to 1.5 L/min and dry gas flow to 15 L/min. **Semi-preparative HPLC** was performed on a Shimadzu HPLC system using a Phenomenex Luna C18(2) 250 x 10 mm column (particle size 5 µm, pore diameter 100 Å) and a Phenomenex Luna Phenyl-Hexyl 250 x 10 mm column (particle size 5 µm, pore diameter 100 Å). **IR spectra** were recorded on an FT/IR-4100 ATR spectrometer (JASCO). **Optical rotations** were recorded in MeOH on a P-1020 polarimeter (JASCO).

**Solid phase extraction** was carried out using Chromabond C$_{18}$ec cartridges filled with 1 g of octadecyl-modified silica gel (Macherey-Nagel, Germany). **Chemicals:** Methanol (VWR, Germany); water for analytical and preparative HPLC (Millipore, Germany), formic acid (Carl Roth, Germany); acetonitrile (VWR as LC-MS grade), media ingredients (Carl Roth, Germany).
2. Strain Isolation and Culturing

Six *Pseudoxylylaria* spp. were isolated from fungus comb material of fungus growing termites. Comb material was incubated in boxes at 30 °C. As soon as fresh fruiting structures appeared on comb material, fungal hyphae were placed on PDA plates and subcultured until pure isolates were obtained. Fungal strains were cultivated on PDA plates for a maximum of four weeks at room temperature and then subcultured by plating mycelium-containing agar pieces (0.5 x 0.5 cm) onto PDA (26.5 g/L potato extract glucose, 20.0 g/L agar).

Table S1: Locations of fungus comb collections for isolation of *Pseudoxylylaria* sp. strains

| Strain | Termite species         | Excavation site      | Isolation date |
|--------|-------------------------|----------------------|----------------|
| X187-2 | *Macrotermes natalensis* | S24 40.434 E28 48.275 | 2018           |
| (X187) |                         |                      |                |
| X3.2   | *Macrotermes natalensis* | S26 50.163 E30 30.490 | 2016           |
| X170 lb| *Odontotermes* spp.     | S25 56.636 E30 35.833 | 2016           |
| X167   | *Odontotermes* spp.     | S25 43.777 E28 14.423 | 2016           |
| MN153  | *Macrotermes natalensis*| S25 44.492 E28 15.663 | 2016           |

3. DNA Extraction and Phylogenetic Classification

DNA extraction was performed from cultures grown on PDA plates for 2-3 weeks at room temperature. After harvesting the mycelium, it was treated with liquid nitrogen and grounded to a fine powder. DNA was obtained by extraction with CTAB-method, purification by chloroform-isoamylalcohol (24:1) and subsequent alcohol precipitation.

Amplification of the partial sequence from the ribosomal DNA (rDNA) and the internal transcribed spacer region (ITS, primer ITS1 5’-TCCGTAGGTGAACCTGCGG-3’ and ITS4 5’-TCCTCCGCTTATTGATATGC-3’) was done by PCR with S7 Phusion Polymerase (Biozym): 98 °C 30 s, 35 cycles of 98 °C 30 s, 55 °C for 30 s and 72 °C for 30 s followed by a final denaturation at 98°C 30 s and extension at 72 °C for 5 min. The positive PCR product was cleaned by gel purification (Zymoclean Gel DNA Recovery Kit, Zymo Research) and sequenced at Eurofins Genomics, Ebersberg.

The sequences were deposited in GenBank (See Figure 1 and 2). In order to characterize the phylogenetic status of strain X187-2 (from now on referred to as X187), we compared parts of the ribosomal DNA and ITS region to a reference data set from Visser et al. Sequences were aligned by the ClustalW algorithm implemented in the MEGA 7 program (standard settings) and trimmed. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-2620.62) is shown in Figure S2.
The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2721)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 29 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 359 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.iii

From ITS phylogenetic analysis as seen in Figure S2, the strain X187 is most closely related to Xylariaceae sp. OTU 10 [FJ425685.1] and therefore belongs to the Pseudoxylaria subclade among other Xylariacea species.
Figure S2. A phylogenetic tree constructed from fungal ITS DNA sequences by maximum likelihood method to show the phylogenetic status of strain X187 among *Pseudoxylaria* strains.
4. Metabolomic Analysis

Fungal plate extracts were analyzed for natural products and compared to extracts from fungal guttation droplets (Figure S6) based on UPLC-HRMS measurements and MS/MS fragmentation based network analysis carried out on the global natural products social molecular networking platform (GNPS).

A molecular network was created using the online workflow at GNPS. The data was filtered by removing all MS/MS peaks within +/- 17 Da of the precursor m/z. MS/MS spectra were window filtered by choosing only the top 6 peaks in the +/- 50 Da window throughout the spectrum. The data was then clustered with MS-Cluster with a parent mass tolerance of 0.02 Da and a MS/MS fragment ion tolerance of 0.02 Da to create consensus spectra. Further, consensus spectra that contained less than 2 spectra were discarded. A network was then created where edges were filtered to have a cosine score above 0.7 for general networks (0.9 for subsequent network creation of isolated compounds) and more than 6 matched peaks. Further edges between two nodes were kept in the network if and only if each of the nodes appeared in each other’s respective top 5 most similar nodes. Finally, the maximum size of a molecular family was set to 25, and the lowest scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS’ spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks.

After filtering for subnetworks not related to instrument blank measurements, two distinct large clusters containing m/z values of unknown metabolites were dominant in networks of both fungal tissue plate extracts (Figure S3), as well as guttation droplets (Figure S4). A set of 4 major and 2 minor compounds were identified by comparison of candidates found in the clusters with their intensity from UPLC-HRMS data (Figure S7). Their great abundance of some compounds in the droplets led to further isolation, purification and structure elucidation, which led to the discovery of pseudoxylaramides A-D and xylacremolides A-B (Figure S15).

The cluster containing isolated molecules xylacremolides A (m/z 403.223) and B (m/z 417.238) (Figure S5) shows signs of several other fragments with a methylation pattern (Δm/z 14.016), as well as 2 fragmentations of double methylation (Δm/z 28.032). Closely related fragments (m/z 448.28 and m/z 462.296) exist for both isolated compounds and are shifted by Δm/z 45.057, potentially corresponding to a C$_3$H$_6$OH moiety but their structure was not elucidated at this moment. A compound with m/z 833.469 could be a potential dimerization product of m/z 417.238. Any masses
corresponding to the structurally closely related acremolides isolated from *Acremonium* sp. could not be detected (Figure S16).v

The cluster containing the identified pseudoxylaramides A-D (m/z 623.381, 609.365, 657.365, 643.349) (Figure S5) contains several related fragments shifted by Δm/z 45.057 (m/z 688.407, 702.422, 668.438), potentially corresponding to a C₂H₄OH moiety. A fragment with m/z 430.24 is strongly linked to pseudoxylaramide A and B by potential loss of the C-terminal N-Met-phenylalanine moiety (Δm/z 179.09), or the C-terminal N-Met-phenylalanine-methylester moiety (Δm/z 193.14).

Figure S3. GNPS network analysis of HR-MS/MS measurement for *Pseudoxylaria* sp. X187-2 (100% MeOH extract, 4 weeks). Precursor ion mass tolerance 0.02 Da, fragment ion mass tolerance 0.02 Da, cosine score 0.7 showing clusters containing (1) pseudoxylaramides A-D, (2) xylacrolemides A-B, (3) phosphocholines, (4) fatty acids. Blue colored nodes: Blank/control data, red colored nodes: Extract data.
Figure S4. GNPS network analysis of HR-MS/MS measurement for *Pseudoxylaria* sp. X187-2 (Droplets 1:10 dilution, 4 weeks). Precursor ion mass tolerance 0.02 Da, fragment ion mass tolerance 0.02 Da, cosine score 0.7 showing MS² clusters containing (1) pseudoxylaramides A-D, (2) xylacrolemides A-B. Blue colored nodes: Blank/control data, red colored nodes: Extract data.

Figure S5. A) GNPS subnetwork cluster showing xylacrolemides A (m/z 403.223), B (m/z 417.238, blue outline), and structurally related fragments isolated from *Pseudoxylaria* sp. X187-2 (Droplets 1:10 dilution, 4 weeks). Precursor ion mass tolerance 0.02 Da, fragment ion mass tolerance 0.02 Da, cosine score 0.9; B) GNPS subnetwork cluster showing pseudoxylaramides A (m/z 623.381), B (m/z 609.365), C (m/z 657.365), D (m/z 643.349, blue outline), and related fragments isolated from *Pseudoxylaria* sp. X187-2 (Droplets 1:10 dilution, 4 weeks). Precursor ion mass tolerance 0.02 Da, fragment ion mass tolerance 0.02 Da, cosine score 0.9.
Figure S6. LC-HRMS analysis of Pseudoxylaria sp. X187 (four weeks, PDA, methanolic extract). ESI-HRMS(+) TIC showing comparison of TIC’s for (A) raw full plate extraction (50 µg/mL) and (B) secreted droplets (20 µL, 1:10 dilution with MeOH).

Figure S7. ESI-HRMS profile of a guttation droplet obtained from Pseudoxylaria sp. X187 (four weeks, PDA, methanolic extract). ESI-HRMS with TIC and XIC’s (5 ppm range) showing: (A) pseudoxylaramide A 1 (HRMS (ESI) m/z [M+H]+ calcld. for C_{35}H_{50}O_{6}N_{4} 623.3803, t_R 9.64 min), (B) pseudoxylaramide C 3 (HRMS (ESI) m/z [M+H]+ calcld. for C_{38}H_{48}O_{6}N_{4} 657.3647, t_R 9.72 min), (C) pseudoxylaramide B 2 (HRMS (ESI) m/z [M+H]+ calcld. for C_{34}H_{48}O_{6}N_{4} 609.3647, t_R 8.76 min), (D) pseudoxylaramide D 4 (HRMS (ESI) m/z [M+H]+ calcld. for C_{37}H_{46}O_{6}N_{4} 643.3490, t_R 8.84 min), (E) xylacremolide A 5 (HRMS (ESI) m/z [M+H]+ calcld. for C_{22}H_{30}O_{5}N_{2} 403.2228, t_R 7.21 min) and (F) xylacremolide B 6 (HRMS (ESI) m/z [M+H]+ calcld. for C_{23}H_{32}O_{5}N_{2} 417.2384, t_R 7.62 min).
5. Isolation of Target Compounds

Large-scale cultivation of *Pseudoxylaria* sp. X187-2 was performed by inoculation of 20 PDA plates (standard 16 mm x 92 mm, 20 mL/plate) using 0.4 L of PDA. Fungal plates were grown for 4 weeks at room temperature.

Mycelium-covered agar plates from a four-week old fungal culture (10 plates) were cut into pieces (0.5 cm x 0.5 cm) and extracted twice with 200 mL MeOH while stirring at 4 °C overnight. MeOH extracts were filtered through filter paper and concentrated using a rotatory evaporator under reduced pressure. The crude extract was dissolved into 20% MeOH (unless stated otherwise: dd H₂O was used as second solvent) and loaded on an activated and equilibrated SPE C18 column (1 g), and fractionated by step-gradient from 20% MeOH to 100% MeOH (10 mL each) resulting in enriched SPE-fractions. Compounds of interest eluted in the 60% MeOH fraction for xylacremolides A,B (5,6) as well as pseudoxylaramides B,D (2,4) and 80% MeOH fraction for pseudoxylaramides A-D (1-4) from the SPE column. The corresponding fractions were concentrated in under reduced pressure to yield a white-brown solid material which was subjected to further purification by semi-preparative HPLC.

The residues obtained from the SPE pre-purification were redissolved in MeOH (5 mg/ml). Separation and fractionation of desired compounds was achieved via semi-preparative HPLC using a Phenyl-Hexyl column (Phenomenex) guided by UV absorption at 190, 210 and 254 nm. Purification of pseudoxylaramides A-D was performed on isocratic conditions (68% ACN, 32% H₂O + 0.1% Formic acid) with a flow of 2.0 mL/min. Purification of xylacremolides A and B was performed using the following gradient (Table S2) at a flow of 2.0 mL/min. Fraction identity and purity was confirmed with LC-MS. Fractions were dried under reduced pressure yielding approx. 1 mg of white solid material and were submitted for NMR analysis and biological activity testing.

Table S2. Gradient used for HPLC purification of xylacremolides A-B.

| t [min] | % A [ACN] | % B [H₂O, 0.1% FA] |
|---------|-----------|-------------------|
| 0       | 45        | 55                |
| 5       | 45        | 55                |
| 25      | 100       | 0                 |
| 26      | 100       | 0                 |
| 29      | 45        | 55                |
| 36      | 45        | 55                |
Figure S8. HPLC purification protocol (λ = 190 nm, 210 nm) of pseudoxylaramides A (fraction 10-12), C (fraction 14-16), B (fraction 1-2) and D (fraction 3-4) of the 80% MeOH SPE fraction.

Figure S9. HPLC purification protocol (λ = 190 nm, 210 nm) of xylacremolides A (fraction 1-2), B (fraction 5-6), and pseudoxylaramides B (fraction 15-16) and D (fraction 17-18) of the 60% MeOH SPE fraction.
6. Characterization of Target Compounds

Pseudoxylaramide A (1) was obtained as a white powder with the molecular formula C$_{35}$H$_{50}$N$_4$O$_6$ with thirteen degrees of unsaturation, as determined by high-resolution ESI-HRMS (HRMS (ESI) $m/z$ [M+H]$^+$ calcd. for C$_{35}$H$_{51}$O$_6$N$_4$ 623.3803, found 623.3776, $\Delta_{ppm}$ -4.49) and NMR spectroscopy in DMSO-$d_6$ and CD$_3$OH-$d_3$ (Table S4). The $^{13}$C NMR spectrum exhibited typical analytical features of a peptide, which includes twenty amide/carboxylic acid carbonyl carbon signals and twenty $\alpha$-carbon resonances. In addition, $^1$H NMR analysis revealed eight exchangeable amide proton signals. Analysis of $^1$H and $^{13}$C NMR data revealed that compound 1 exists as a set of four stable conformers (ratio of approx. 10:7:3:2 in CD$_3$OH-$d_3$ and 10:10:6:3 in DMSO-$d_6$).

![Figure S10. COSY and HMBC correlations for pseudoxylaramide A (1) and B (2)](image-url)

A planar structure of pseudoxylaramide A (1) was assigned based on the distinct signal sets of the major conformer in CD$_3$OH-$d_3$. Interpretation of the COSY, HSQC and HMBC indicated the presence of two N-methyl phenylalanine (N-Me-Phe) and two isoleucine (Ile) residues. The connectivity of the four residues was established by analysis of the HMBC spectrum (Figure S10). The hydroxyl group at C-terminal end of N-Me Phe$^1$ was substituted to O-methyl group [$\delta_{C}$ 51.5, $\delta_{H}$ 3.68] establishing the linkage to carbonyl carbon chemical shift [$\delta_{C}$ 170.8] of the residue. The N-methyl [$\delta_{H}$ 2.97] of N-Me Phe$^1$ displayed clear correlations to C-2 [$\delta_{C}$ 59.0] and C-1' [$\delta_{C}$ 172.4] confirming the connectivity between N-Me Phe$^1$ and Ile$^1$. The NH [$\delta_{H}$ 7.52] of Ile$^1$ showed a heteronuclear correlation to C-1'' [$\delta_{C}$ 170.1] of Phe$^2$. The N-methyl protons [$\delta_{H}$ 2.99] of Phe$^2$ displayed an HMBC correlation with C-1'''' [$\delta_{C}$ 173.2] of Ile$^2$, establishing the sequence from Phe$^2$ to Ile$^2$. The amide proton [$\delta_{H}$ 8.07] of Ile$^2$ showed the long-range coupling to the methyl group [$\delta_{C}$ 21.1] and the methyl protons [$\delta_{H}$ 1.88] showed an HMBC correlation to carbonyl carbon [$\delta_{C}$ 171.4] in Ile$^2$ deducing an OH group of Ile$^2$ was substituted by methyl group. To determine the absolute configurations of $\alpha$-carbon of amino acids in pseudoxylaramide A (1), an acid hydrolysis and Marfey’s method with FDAA (1-fluoro-2,4-dinitrophenyl-L-alanine amide) were performed. After the acid hydrolysis of 1 for an hour with 6 N HCl at 115 °C, the hydrolysate was derivatized with FDAA. The derivatives were analysed by LC-MS.
comparing with FDAA derivatized authentic standard amino acids which corresponded to the L-amino acids.

Pseudoxylaramide C (3) was aquired as a white powder along with 1. Its molecular formula was determined as C_{38}H_{49}O_{6}N_{4} (HRMS (ESI) [M+H]^+ m/z calcd. for C_{38}H_{49}O_{6}N_{4} 657.3647, found 657.3617, Δ_{ppm} -4.41) requiring 13 degrees of unsaturation. After careful analysis of 1D and 2D NMR pseudoxylaramide C (3), one of the isoleucines in 1 was substituted to phenyl alanine in 3.

Pseudoxylamides B and D (2,4) were isolated as white powders (HRMS (ESI) m/z [M+H]^+ calcd. for C_{34}H_{49}O_{6}N_{4} 609.3647, found 609.3627, Δ_{ppm} -3.28); and HRMS (ESI) m/z [M+H]^+ calcd. for C_{37}H_{47}O_{6}N_{4} 643.3490, found 643.3466, Δ_{ppm} -3.90). The difference between 1 and 2 was due to the replacement of the O-methyl in 1 by hydroxyl gourp, as was it between 3 and 4.

**Figure S11. COSY, HMBC and NOESY correlations for xylacremolide A, B (5,6)**

Xylacremolide A (5) was isolated as a white powder with the molecular formula C_{22}H_{30}N_{2}O_{5} and nine degrees of unsaturation which determined by the ESI-HRMS mass spectrum (HRMS (ESI) m/z [M+H]^+ calcd. for C_{22}H_{30}N_{2}O_{5} 403.2228, found 403.2220, Δ_{ppm} -1.73) and 1H and 13C NMR data (Table S6). Interpretation of the 1H and 13C NMR spectra of 5 revealed three amide/ester carbonyl carbon signals [δ_{C} 172.0, 170.6, and 170.2] and two α-carbon/proton signals [δ_{C} 52.6; δ_{H} 4.74 and δ_{C} 48.7; δ_{H} 4.99] and one amide proton [δ_{H} 8.48] in 5 indicating typical features of a peptide-derived compound. The structure of 5 was elucidated by a delicate analysis of the 1D and 2D NMR data. Xylacremolide A (5) consists of three residues including proline (Pro), phenylalanine (Phe), and 3,5-dihydroxy octanoic acid (DHOA). The 1H—13C two-bond coupling between NH [δ_{H} 8.48] of Phe and C-1 [δ_{C} 170.6] of DHOA implied the DHOA → Phe sequence. The β-proton [δ_{H} 3.44] of Pro exhibited heteronuclear correlation to C-1’ [δ_{C} 170.2] of Phe connecting the linkage between Phe and Pro. Unambiguously the NOESY correlations from α- and β-protons [δ_{H} 4.74 and 2.85] of Phe to H-5’’ [δ_{H} 3.16] of proline supported the connectivity of Phe to Pro. It was accounted for 8 of the 9 degrees of unsaturation...
number because of the aromatic ring of phenylalanine, the proline ring, and three carbonyl signals. Finally, the nine degrees of unsaturation number was satisfied by the HMBC correlation between the carbonyl carbon [δC 172.0] of Pro and methane proton [δH 4.66] of DHOA possessing an additional ring.

Xylacremolide B (6) was isolated as a white powder along with xylacremolide A (5). Its molecular weight (HRMS (ESI) m/z [M+H]+ calcd. for C_{23}H_{33}O_{5}N_{2} 417.2384, found 417.2383, Δppm -0.47) was determined by the ESI-HRMS mass spectrum. To determine the structure of 6, a combinational comparison analysis of the 1D and 2D NMR data of 6 with those of 5. The difference between 5 and 6 was due to the replacement of the one methylene proton at C-2 in 5 by a methyl group, 2-CH₃ [δC 8.0; δH 0.81].

**Marfey-Analysis**

**Pseudoxylaramides:**

1 mg of pure compound (pseudoxylaramide A, 1) was dissolved in 1 mL 6 M HCl and hydrolysed for 1 h at 115 °C. The mixture was cooled down quickly on ice and HCl was removed *in vacuo* while washing with H₂O 3 times. The final acid hydrolysis product was frozen and lyophilised prior to the reaction with Marfey’s reagent.

Hydrolysate and standards for L-Ile, D-Ile, L-Me-Phe, D-Me-Phe (1 mg) were resuspended in 200 µL 1 M NaHCO₃. 100 µL of either L-FDAA (Figure S12) or D-FDAA (Figure S13) dissolved in Acetone (10 mg/mL) was added to each sample and the mixtures were incubated for approximately 3 min at 80 °C. The reaction was quenched with addition of 100 µL 2 M HCl. Samples were diluted with 300 µL 50/50 ACN/H₂O and submitted to LC-MS based analysis (Table S3).

| Amino acid          | MW  | RT-L [min] | RT-D [min] | Elution order | Δt [min] |
|---------------------|-----|------------|------------|---------------|----------|
| L-Ile in 1          | 383 | 8.0        | 13.0       | L->D          | 5.0      |
| L-N-Me-Phe in 1     | 431 | 8.3        | 12.5       | L->D          | 4.2      |
| standard L-Ile      | 383 | 8.0        | 13.0       | L->D          | 5.0      |
Figure S12. Marfey analysis of pseudoxylaramide A (1). Comparison of elution profiles for products formed with L-FDAA (A) TIC hydrolysate, (B) m/z [M+H]^+ = 384 Ile-L-FDAA, (C) m/z [M-H]^- = 382 Ile-L-FDAA, (D) m/z [M+H]^+ = 432 N-Me-Phe-L-FDAA, (E) m/z [M-H]^- = 430 N-Me-Phe-L-FDAA.

Figure S13. Advanced Marfey analysis of pseudoxylaramide A (1). Comparison of elution profiles for products formed with D-FDAA (A) TIC hydrolysate, (B) m/z [M+H]^+ = 384 Ile-D-FDAA, (C) m/z [M-H]^- = 382 Ile-D-FDAA, (D) m/z [M+H]^+ = 432 N-Me-Phe-D-FDAA, (E) m/z [M-H]^- = 430 N-Me-Phe-D-FDAA.
Xylacremolides:

1 mg of pure compound (xylacremolide A, 5) was dissolved in 1 mL 6 M HCl and hydrolysed for 1 h at 115 °C. The mixture was cooled down quickly on ice and HCl was removed in vacuo while washing with H₂O three times. The final acid hydrolysis product was frozen and lyophilised prior to the reaction with Marfey’s reagent. Hydrolysate and standards for L-Pro, D-Pro, L-Phe, D-Phe (1 mg) were resuspended in 200 µL 1 M NaHCO₃. 100 µL of L-FDAA (10 mg/mL) dissolved in Acetone was added to each sample and the mixtures were incubated for approximately 3 min at 80 °C. The reaction was quenched with addition of 100 µL 2 M HCl. Samples were diluted with 300 µL 50/50 ACN/H₂O and submitted to LC-MS based analysis (Figure S14).

| Reaction                                | RT m/z 417 (MR + phenylalanine) | RT m/z 367 (MR + proline) |
|-----------------------------------------|---------------------------------|----------------------------|
| L-amino acid standard + L-FDAA          | 10.00                           | 5.9                        |
| D-amino acid standard + L-FDAA          | 11.4                            | 6.45                       |
| Hydrolysis product+ L-FDAA              | 11.4                            | 5.9                        |

Figure S14. Marfey analysis of xylacremolide A (5), comparison with authentic standard compounds: (A) TIC hydrolysate + L-FDAA, (B) m/z [M+H]+ = 417 Phenylalanine-L-FDAA from hydrolysate, (C) m/z [M+H]+ = 367 Proline-L-FDAA from hydrolysate, (D) m/z [M+H]+ = 417 L-Phenylalanine-L-FDAA from authentic standard, (E) m/z [M+H]+ = 367 Proline-L-FDAA from authentic standard, (F) m/z [M+H]+ = 417 D-Phenylalanine-L-FDAA from authentic standard, (G) m/z [M+H]+ = 367 D-Proline-L-FDAA authentic standard.
7. Analytical Data for Pseudoxylaramides A–D and Xylacremolides A–B

**Figure S15.** Structures for pseudoxylaramides A–D (1–4) and xylacremolides A–B (5–6).
**Figure S16.** Structures of closely related acremolides isolated from *Acremonium* sp.\(^5\)
**Pseudoxylaramide A (1):** white solid; $[\alpha]_{25}^{D} -139.80$ (c 0.1 w/v%, MeOH); UV (68% ACN + 32% H$_2$O 0.1% FA), $\lambda_{\max}$ 200, 215 nm; IR (ATR) $\nu_{\max}$ 3310, 2958, 1741, 1629, 1456, 1285 cm$^{-1}$; NMR spectral data, see Table S4; HRMS (ESI) $m/z$ [M+H]$^+$ calcd. for C$_{35}$H$_{51}$O$_6$N$_4$ $+$ 623.3803, found 623.3776, $\Delta$ppm -4.49.

**Pseudoxylaramide B (2):** white solid; $[\alpha]_{25}^{D} -22.96$(c 0.1 w/v%, MeOH); UV (68% ACN + 32% H$_2$O 0.1% FA), $\lambda_{\max}$ 196, 210 nm; IR (ATR) $\nu_{\max}$ 3290, 2957, 1623, 1456, 1352, 1087cm$^{-1}$; NMR spectral data, see Table S4; HRMS (ESI) $m/z$ [M+H]$^+$ calcd. for C$_{34}$H$_{49}$O$_6$N$_4$ $+$ 609.3647, found 609.3627, $\Delta$ppm -3.28.

**Pseudoxylaramide C (3):** white solid; $[\alpha]_{25}^{D} -23.08$ (c 0.1 w/v%, MeOH); UV (68% ACN + 32% H$_2$O 0.1% FA), $\lambda_{\max}$ 192, 210 nm; IR (ATR) $\nu_{\max}$ 3308, 2920, 2359, 1625, 1558, 1030 cm$^{-1}$; NMR spectral data, see Table S5; HRMS (ESI) $m/z$ [M+H]$^+$ calcd. for C$_{38}$H$_{49}$O$_6$N$_4$ $+$ 657.3647, found 657.3617, $\Delta$ppm -4.41.

**Pseudoxylaramide D (4):** white solid; $[\alpha]_{25}^{D} -44.42$ (c 0.1 w/v%, MeOH); UV (68% ACN + 32% H$_2$O 0.1% FA), $\lambda_{\max}$ 198, 210 nm; IR (ATR) $\nu_{\max}$ 3291, 2926, 2359, 1623, 1385, 1090 cm$^{-1}$; NMR spectral data, see Table S5; HRMS (ESI) $m/z$ [M+H]$^+$ calcd. for C$_{37}$H$_{47}$O$_6$N$_4$ $+$ 643.3490, found 643.3466, $\Delta$ppm -3.90.

**Xylacremolide A (5):** white solid; $[\alpha]_{25}^{D} +5.28$ (c 0.1 w/v%, MeOH); UV (68% ACN + 32% H$_2$O 0.1% FA), $\lambda_{\max}$ 201, 218 nm; IR (ATR) $\nu_{\max}$ 3276, 2958, 1771, 1733, 1584, 1455, 1386, 1360, 1195, 953 cm$^{-1}$; NMR spectral data, see Table S6; HRMS (ESI) $m/z$ [M+H]$^+$ calcd. for C$_{22}$H$_{31}$O$_5$N$_2$ $+$ 403.2228, found 403.2220, $\Delta$ppm -1.73.

**Xylacremolide B (6):** white solid; $[\alpha]_{25}^{D} -39.98$ (c 0.1 w/v%, MeOH); UV (75% ACN + 25% H$_2$O 0.1% FA), $\lambda_{\max}$ 201, 219 nm; IR (ATR) $\nu_{\max}$ 3291, 2957, 1731, 1646, 1610, 1558, 1455, 1339, 1201, 1026, cm$^{-1}$; NMR spectral data, see Table S6; HRMS (ESI) $m/z$ [M+H]$^+$ calcd. for C$_{23}$H$_{33}$O$_5$N$_2$ $+$ 417.2384, found 417.2383, $\Delta$ppm -0.47.
Table S4. NMR data for pseudoxyylaramide A (1) and B (2) in CD$_3$OH-d$_3$

| Subunit            | C/H     | δ$^{13} $C, type | δ$^1 $H, mult (J, Hz) | δ$^{13} $C, type$^a$ | δ$^1 $H, mult (J, Hz) |
|--------------------|---------|-----------------|------------------------|----------------------|------------------------|
| N-Me-Phe$^1$       | 1       | 170.8, C        |                        | 173.7, C             |                        |
|                    | 2       | 59.0, CH        | 5.27, dd (11.0, 5.0)   | 58.6, CH             | 5.28, m                |
|                    | 3       | 33.8, CH$_2$    | 3.36, m, 3.07, m       | 29.0, CH$_2$         | 3.36, m, 2.96, m       |
|                    | 4       | 137.0, C        |                        | 138.8, C             |                        |
|                    | 5/9     | 128.7, CH       | 7.20, m                | 128.8, CH            | 7.18-7.30, m           |
|                    | 6/8     | 129.0, CH       | 7.26, m                | 126.2-128.6, CH      | 7.18-7.30, m           |
|                    | 7       | 126.2, CH       | 7.34, m                | 126.2-128.6, C       | 7.18-7.30, m           |
| 1-O-Me             |         |                 |                        |                      |                        |
|                    | 1       | 51.5, CH$_3$    | 3.68, s                | 29.7, CH$_3$         | 2.94, s                |
| 2-N-Me             |         |                 |                        |                      |                        |
|                    | 1'      | 172.4, C        |                        | 172.3, C             |                        |
|                    | 2'      | 53.0, CH        | 4.62, m                | 53.1, CH             | 4.63, m                |
|                    | 3'      | 36.6, CH        | 1.79, m                | 36.8, CH             | 1.76, m                |
|                    | 4'      | 23.9, CH$_2$    | 1.48, m                | 24.3, CH$_2$         | 1.59, m, 1.46 m        |
|                    | 5'      | 9.7, CH$_3$     | 0.90, m                | 9.5, CH$_3$          | 0.86, m                |
|                    | 6'      | 14.1, CH$_3$    | 0.91, m                | 14.4, CH$_3$         | 0.90, m                |
|                    | 2'-NH   |                 |                        |                      |                        |
|                    |         |                 |                        | 7.52, d (9.0)        | 7.50, d (9.0)          |
| N-Me-Phe$^2$       | 1''     | 170.1, C        |                        | 169.5, C             |                        |
|                    | 2''     | 61.9, CH        | 5.35, dd (10.0, 5.5)   | 61.6, CH             | 5.37, m                |
|                    | 3''     | 34.0, CH$_2$    | 3.33, m, 3.10, m       | 33.4, CH$_2$         | 3.12, m, 2.98, m       |
|                    | 4''     | 137.3, C        |                        | 137.0, C             |                        |
|                    | 5''/9'' | 128.7, CH       | 7.18, m                | 128.6, CH            | 7.18-7.30, m           |
|                    | 6''/8'' | 129.2, CH       | 7.24, m                | 126.2-128.6, CH      | 7.18-7.30, m           |
|                    | 7''     | 137.2, C        | 7.32, m                | 126.2-128.6, C       | 7.18-7.30, m           |
| 2''-N-Me           |         |                 |                        |                      |                        |
|                    | 1'''    | 32.4, CH$_3$    | 2.95, s                | 32.1, CH$_3$         | 2.99, s                |
| ile$^3$            |         |                 |                        |                      |                        |
|                    | 1''''   | 173.2, C        |                        | 173.3, C             |                        |
|                    | 2''''   | 54.1, CH        | 4.57, m                | 53.1, CH             | 4.58, m                |
|                    | 3''''   | 35.9, CH        | 1.96, m                | 36.7, CH             | 1.94, m                |
|                    | 4''''   | 24.4, CH$_2$    | 1.64, m                | 24.4, CH$_2$         | 1.52, mm 1.42, m       |
|                    | 5''''   | 9.6, CH$_3$     | 0.89, m                | 9.9, CH$_3$          | 0.87, m                |
|                    | 6''''   | 13.8, CH$_2$    | 0.92, m                | 14.0, CH$_3$         | 0.93, m                |
|                    | 7''''   | 171.4, C        |                        | 171.4, C             |                        |
|                    | 8''''   | 21.1, CH$_3$    | 1.88, s                | 20.9, CH$_3$         | 1.87, s                |
|                    | 2'''''-NH|         | 8.07, d (8.0)         | 8.10, d (8.0)        |                        |

$^a$13$^C$ chemical shift was assigned with HSQC and HMBC.
Table S5. NMR data for pseudoxylaramide C (3) and D (4) in CD$_3$OH-d$_6$.

![Chemical structures of pseudoxylaramide C (3) and D (4)](image)

| Subunit | C/H | $\delta^{13}$C, type | $\delta^1$H, mult (J, Hz) | $\delta^{13}$C, type$^a$ | $\delta^1$H, mult (J, Hz) |
|---------|-----|----------------------|--------------------------|----------------------|--------------------------|
| N-Me-Phe$^1$ | | | | | |
| 1 | 170.9, C | | | 170.4, C | |
| 2 | 58.7, CH | 5.28, dd (10.5, 5.5) | 58.7, CH | 5.24, m | |
| 3 | 32.6, CH$_3$ | 3.37, m, 3.06, m | 31.2, CH$_3$ | 3.07, m, 2.91, m | |
| 4 | 136.5, C | | 137.0, C | | |
| 5/9 | 128.6, CH | 7.18-7.32, m | 128.5, CH | 7.20, m | |
| 6/8 | 126.2-128.6, CH | 7.18-7.32, m | 126.3-128.9, CH | 7.26, m | |
| 7 | 126.2-128.6, CH | 7.18-7.32, m | 126.3-128.9, CH | 7.34, m | |
| 1-O-Me | 51.5, CH$_3$ | 3.70, s | | | |
| 2-N-Me | 29.5, CH$_3$ | 2.94, s | 29.1, CH$_3$ | 2.91, s | |
| Phe | | | | | |
| 1' | 172.8, C | | | 172.7, C | |
| 2' | 51.9, CH | 4.25, ddd (15.0, 9.0, 5.5) | 51.0, CH | 4.28, m | |
| 3' | 37.5, CH$_3$ | 2.96, m, 2.77, m | 35.1, CH$_3$ | 3.01, m, 2.76, dd (14.5, 8.5) | |
| 4' | 136.7, C | | 137.3, C | | |
| 5'/9' | 128.7, CH | 7.18-7.32, m | 128.7, CH | 7.18-7.30, m | |
| 6'/7' | 126.2-128.6, CH | 7.18-7.32, m | 126.3-128.9, CH | 7.18-7.30, m | |
| 8' | 126.2-128.7, CH | 7.18-7.32, m | 126.3-128.9, C | 7.18-7.30, m | |
| 2''-NH | | | 8.07, d (9.0) | | 7.99, d (8.5) |
| N-Me-Phe$^2$ | | | | | |
| 1'' | 170.6, C | | | 169.9, C | |
| 2'' | 59.0, CH | 5.30, dd (10.5, 5.0) | 60.2, CH | 5.34, m | |
| 3'' | 32.0, CH$_3$ | 3.37, m, 3.06, m | 31.5, CH$_3$ | 3.33, m, 2.92, m | |
| 4'' | 137.1, C | | 137.3, C | | |
| 5''/9'' | 128.7, CH | 7.18-7.32, m | 128.9, CH | 7.18-7.30, m | |
| 6''/8'' | 126.2-128.7, CH | 7.18-7.32, m | 126.3-128.9, CH | 7.18-7.30, m | |
| 7'' | 126.2-128.7, C | 7.18-7.32, m | 126.3-128.9, C | 7.18-7.30, m | |
| 2''-N-Me | 32.8, CH$_3$ | 3.01, s | 31.8, CH$_3$ | 3.03, s | |
| Ile | | | | | |
| 1''' | 172.8, C | | | 172.2, C | |
| 2''' | 53.8, CH | 4.56, m | 52.8, CH | 4.62, m | |
| 3''' | 36.2, CH$_3$ | 1.81, m | 36.5, CH$_3$ | 1.61, m | |
| 4''' | 24.2, CH$_2$ | 1.40, m, 1.06 | 24.1 CH$_2$ | 1.03, m, 0.79, m | |
| 5''' | 9.8, CH$_3$ | 0.88, m | 9.7, CH$_3$ | 0.83, m | |
| 6''' | 14.0, CH$_3$ | 0.90, m | 14.5, CH$_3$ | 0.94, m | |
| 7''' | 171.2, C | | 171.3, C | | |
| 8''' | 20.9, CH$_3$ | 1.79, s | 20.9, CH$_3$ | 1.79, s | |
| 2''''-NH | | | 8.03, d (8.0) | | 8.05, d (8.0) |

$^a$C chemical shift was assigned with HSQC and HMBC.
Table S6. NMR data for xylacremolides A (5) and B (6).

![Chemical structure of xylacremolides A and B](image)

xylacremolide A (5, R = H)
xylacremolide B (6, R = CH₃)

| Subunit | C/H            | δ¹³C, type | δ¹H, mult (J, Hz) | δ¹³C, type³ | δ¹H, mult (J, Hz) |
|---------|----------------|------------|-------------------|------------|-------------------|
| 3,5-dihydroxy octanoic acid | 1   | 170.6, C | 173.8, C          |            |                   |
|         | 2   | 44.2, CH₂ | 2.34, dd (14.5, 2.0) | 47.4, CH   | 2.57, qd (6.5, 3.0) |
|         | 3   | 63.0, CH | 4.05, m          | 68.1, CH   | 3.91, m           |
|         | 4   | 25.3, CH₂ | 1.99, m, 1.65, m | 34.0, CH₁ | 1.64, m, 1.34, m |
|         | 5   | 74.3, CH | 4.66, m          | 75.1, CH   | 4.62, m           |
|         | 6   | 21.5, CH₂ | 1.87, m, 1.57, m | 33.0, CH₁ | 1.58, m, 1.54, m |
|         | 7   | 18.9, CH₁ | 1.35, m, 1.25, m | 19.0, CH₁ | 1.30, m, 1.23, m |
|         | 8   | 13.2, CH₃ | 0.87, dd (7.5, 7.5) | 14.3, CH₁ | 0.82, dd (7.0, 7.0) |
|         | 2-Me |          |                   | 8.0, CH₁ | 0.81, d (6.5) |
| Phenylalanine | 1'  | 170.2, C | 170.6              |            |                   |
|         | 2'  | 52.6, CH | 4.74, dddd (17.0, 7.5, 7.5) | 52.5, CH | 4.76, dddd (14.0, 14.0, 8.0) |
|         | 3'  | 38.7, CH₂ | 2.99, dd (13.0, 7.5), | 35.7, CH₁ | 2.96, dd (14.0, 8.0), |
|         | 4'  | 138.5, C | 2.87, dd (17.0, 13.0) | 138.5, C | 2.85, dd (14.0, 8.0) |
|         | 5', 9' | 128.7, CH | 7.21, d (7.0) | 129.6, CH | 7.25, m |
|         | 6', 8' | 130.3, CH | 7.27, m | 128.6, CH | 7.25, m |
|         | 7'  | 126.5, CH | 7.19, dd (7.0, 7.0) | 126.8, CH | 7.16, m |
|         | NH  |          | 8.48, d (7.5) | 8.46, d (8.0) |                   |
| Proline | 1'' | 172.0 | 171.3, C |            |                   |
|         | 2'' | 48.7, CH | 4.99, dd (8.5, 1.0) | 57.8, CH | 4.66, d (8.0) |
|         | 3'' | 30.6, CH₂ | 2.21, m, 1.96, m | 28.0, CH₁ | 2.07, d (12.0, 7.5), 1.71, m |
|         | 4'' | 20.2, CH₁ | 1.79, m, 1.75, m | 24.3, CH₁ | 1.75, m, 1.56, m |
|         | 5'' | 46.0, CH₂ | 3.96, dddd (12.0, 9.5, 2.0), | 46.0, CH₂ | 3.96, dddd (12.0, 9.5, 2.0), |
|         |     |         | 3.16, m | 3.16, m |                   |
8. X-ray Crystallography

The intensity data were collected on a Nonius KappaCCD diffractometer, using graphite-monochromated Mo-K$_\alpha$ radiation. Data were corrected for Lorentz and polarization effects; absorption was taken into account on a semi-empirical basis using multiple-scans.$^vi,vii,viii$

The structure was solved by direct methods (SHELXS)$^ix$ and refined by full-matrix least squares techniques against Fo$^2$ (SHELXL-2018).$^ix$ The hydrogen atoms bonded to the hydroxy-group O3 and the amine group N2 were located by difference Fourier synthesis and refined isotropically. All other hydrogen atoms were included at calculated positions with fixed thermal parameters. All non-hydrogen atoms were refined anisotropically.$^ix$ XP (SIEMENS Analytical X-ray Instruments, Inc.) was used for structure representations.

Crystal Data for 5: C$_{22}$H$_{30}$N$_2$O$_5$, Mr = 402.48 g mol$^{-1}$, colourless prism, size 0.112 x 0.046 x 0.046 mm$^3$, orthorhombic, space group P 2$_1$ 2$_1$ 2$_1$, a = 5.9558(2), b = 15.2186(5), c = 22.7511(7) Å, α = 90$, \beta = 90$, γ = 90°, V = 2062.14(12) Å$^3$, T= -140 °C, Z = 4, $\rho_{\text{calcd.}} = 1.296$ g cm$^{-3}$, μ (Mo-K$_\alpha$) = .92 cm$^{-1}$, multi-scan, transmin: 0.6587, transmax: 0.7456, F(000) = 864, 10710 reflections in h(-7/5), k(-17/18), l(-27/27), measured in the range 2.235° ≤ Θ ≤ 25.682°, completeness Θ$_{\text{max}}$ = 99.9%, 3916 independent reflections, R$_{\text{int}}$ = 0.1034, 2816 reflections with F$_o$ > 4σ(F$_o$), 270 parameters, 0 restraints, R$_1$$_{\text{obs}}$ = 0.0711, wR$_2$$_{\text{obs}}$ = 0.1277, R$_1$$_{\text{all}}$ = 0.1120, wR$_2$$_{\text{all}}$ = 0.1453, GOOF = 1.078, Flack-parameter -0.9(10), largest difference peak and hole: 0.237/-0.315 e Å$^{-3}$.

Supporting Information Available: Crystallographic data deposited at the Cambridge Crystallographic Data Centre under CCDC-1987814 for 5 contain the supplementary crystallographic data excluding structure factors; this data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223-336-033; or deposit@ccdc.cam.ac.uk).
9. Activity Tests

Antimicrobial Activity Assay: Solid phase extracts (SPE extracts given as %MeOH eluent) were concentrated under reduced pressure and submitted for antimicrobial activity tests at a concentration of 1 mg/mL in MeOH. Samples were tested for their ability to inhibit growth of microorganisms (performed by Jena Microbial Resource Collection (Jena, Germany)). The following indicator strains were used: Bacillus subtilis 6633; Staphylococcus aureus SG511, Escherichia coli SG458, Pseudomonas aeruginosa K799/61, Mycobacterium vaccae 10670, Sporobolomyces salmonicolor 549, Candida albicans C.A., Penicillium notatum JP36. Antimicrobial activity was determined by measuring the inhibition zone in mm (Table S7).

Antiproliferative and cytotoxic assays:

To evaluate if the compounds have an antiproliferative and/or a cytotoxic effect on human cells (HUVEC (ATCC CRL-1730), K-562 (DSM ACC 10), HeLa (DSM ACC 57)) the cytotoxicity (cell death) and the antiproliferative activity (retardation of cell proliferation) were measured. The GI50 values were defined as being where the inhibition of proliferation is 50 % compared to untreated control. However, none of the compounds showed any significant inhibitory effects.

Table S7. Antimicrobial activity of Pseudoxylaria sp. extracts. (a) Extract from 1 agar plate, (b) extract combined from 5 agar plates, (c) extract combined from 9 agar plates. (p) Colonies inside inhibition zone, (A) visible hint indicating potential inhibition, (#) calculated average inhibition zone. Extract concentration 1 mg/ml in MeOH.

| Isolate | B. subtilis 6633 | S. aureus SG511 | E. coli SG458 | P. aeruginosa K799/61 | M. vaccae 10670 | S. salmonica 549 | C. albicans C.A. | P. notatum JP36 |
|---------|-----------------|-----------------|---------------|---------------------|-----------------|----------------|-----------------|----------------|
| X187 raw extract | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| X187 (100%) | 12 p | 12 | 0 | 0 | 27p | 0 | 0 | 0 |
| X187-2 (80%) | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| X187-2 (50%) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ciprofloxacin | 29 | 18 | 28.5# | 33.5# | 21p | | | |
| MeOH | 0 | 0 | 10 | 10 | 10 | 0 | 11 |
| Amphotericin B | | | | | | 19p | 21 | 18p |

10. Additional Analytical Data

**Figure S17.** MS/MS spectrum of pseudoxylaramide A (1) (C$_{35}$H$_{50}$O$_6$N$_4$, calculated [M+H]$^+$ = 623.38031).

**Figure S18.** MS/MS spectrum of pseudoxylaramide B (2) (C$_{34}$H$_{48}$O$_6$N$_4$, calculated [M+H]$^+$ = 609.36466).
Figure S19. MS/MS spectrum of pseudoxyllaramide C (3) \(\text{C}_{38}\text{H}_{48}\text{O}_{6}\text{N}_{4}\), calculated \([\text{M+H}]^+ = 657.36466\).

Figure S20. MS/MS spectrum of pseudoxyllaramide D (4) \(\text{C}_{37}\text{H}_{46}\text{O}_{6}\text{N}_{4}\), calculated \([\text{M+H}]^+ = 643.34901\).
Figure S21. MS/MS spectrum of xylacremolide A (5) ([C$_{22}$H$_{30}$N$_2$O$_5$], calculated [M+H]$^+$ = 403.22275).

Figure S22. MS/MS spectrum of xylacremolide B (6) ([C$_{23}$H$_{32}$N$_2$O$_5$], calculated [M+H]$^+$ = 417.23840).
**Figure S23.** $^1$H NMR spectrum of compound 1 in CD$_3$OH.

**Figure S24.** Comparison of $^1$H spectrum in CH$_3$OH and DMSO-$d_6$ for pseudoxyllaramide A and three minor conformers (1 and 1a-c).
Figure S25. Comparison magnified $^1$H spectrum in CH$_3$OH and DMSO-$d_6$ for O-methyl protons, N-methyl protons, and methyl protons in the acetyl group of pseudoxylaramide A and three minor conformers (1 and 1a-c). (Methyl protons of *pseudoxylaramide A (1) and *pseudoxylaramide A (1a)).

Figure S26. Magnified $^1$H spectrum of 1 in CD$_3$OH between 3.85 and 3.65 ppm indicating the ratio (10:7:3:2) of O-methyl protons in pseudoxylaramide A and three minor conformers (1 and 1a-c).
Figure S27. Magnified $^1$H spectrum of 1 in CD$_3$OD between 1.90 and 1.80 ppm indicating the ratio (10:7:3:2) of methyl protons in the acetyl group in pseudoxylaramide A and three minor conformers (1 and 1a-c).

Figure S28. Magnified $^1$H spectrum of 1 in DMSO-$d_6$ between 3.80 and 3.55 ppm indicating the ratio (10:10:5:3) of O-methyl protons of pseudoxylaramide A and three minor conformers (1 and 1a-c).
Figure S29. Magnified $^1$H spectrum of 1 in DMSO-$d_6$ between 1.82 and 1.68 ppm indicating the ratio (10:10:5:3) of methyl protons in the acetyl group of pseudoxylaramide A and three minor conformers (1 and 1a-c).

Figure S30. $^{13}$C NMR spectrum of 1 in CD$_3$OH.
Figure S31. COSY NMR spectrum of 1 in CD$_3$OH.

Figure S32. HSQC NMR spectrum of 1 in CD$_3$OH.
Figure S33. HMBC NMR spectrum of 1 in CD$_3$OH.

Figure S34. $^1$H NMR spectrum of 2 in CD$_3$OH.
Figure S35. COSY NMR spectrum of 2 in CD$_3$OH.

Figure S36. HSQC NMR spectrum of 2 in CD$_3$OH.
Figure S37. HMBC NMR spectrum of 2 in CD$_3$OH.

Figure S38. $^1$H NMR spectrum of 3 in CD$_3$OH.
Figure S39. COSY NMR spectrum of 3 in CD$_3$OH.

Figure S40. HSQC NMR spectrum of 3 in CD$_3$OH.
Figure S41. HMBC NMR spectrum of 3 in CD$_3$OH.

Figure S42. $^1$H NMR spectrum of 4 in CD$_3$OH.
Figure S43. COSY NMR spectrum of 4 in CD$_3$OH.

Figure S44. HSQC NMR spectrum of 4 in CD$_3$OH.
Figure S45. HMBC NMR spectrum of 4 in CD$_3$OH.

Figure S46. $^1$H NMR spectrum of 5 in DMSO-$d_6$. 
Figure S47. $^{13}$C NMR spectrum of 5 in DMSO-$d_6$.

Figure S48. Dept135 NMR spectrum of 5 in DMSO-$d_6$. 
Figure S49. COSY NMR spectrum of 5 in DMSO-$d_6$.

Figure S50. HSQC NMR spectrum of 5 in DMSO-$d_6$. 
Figure S51. HMBC NMR spectrum of S in DMSO-d$_6$.

Figure S52. $^1$H NMR spectrum of 6 in DMSO-d$_6$. 
Figure S53. $^1$H NMR spectrum of 6 in DMSO-$d_6$.

Figure S54. Dept135 NMR spectrum of 6 in DMSO-$d_6$. 
Figure S55. COSY NMR spectrum of 6 in DMSO-\(d_6\).

Figure S56. HSQC NMR spectrum of 6 in DMSO-\(d_6\).
Figure S57. HMBC NMR spectrum of 6 in DMSO-$d_6$.

Figure S58. NOESY NMR spectrum of 6 in DMSO-$d_6$. 
Figure 59. $M^2_S$ of putative xylocremolide congeners C and D varying in PKS chain length and saturation pattern (putative structures are assigned to the $M^2_S$ spectra).
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