Chemical and Metagenomic Studies of the Lethal Black Band Disease of Corals Reveal Two Broadly-Distributed, Redox-Sensitive Mixed Polyketide/Peptide Macrocycles

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Looekeyolide B

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X : parts per Million : 1H
Looekeyolide B
S10

Looekeyolid B

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Figure S13. Electron Paramagnetic Resonance spectrum of looekeyolide B with added FeCl.
Figure S14. Maximum quantum yields (Fv/Fm) (mean ± SE) of *M. cavernose* exposed to either treatment (color) or control (grey) Phytagel strips in 2013 (n=6), 2014 (n=10), and 2016 (n=6). Statistical comparisons (t-test) between treatments are displayed for each experiment.
Table S1. Specificity codes for adenylation domains from LklG and LklII.

| A Domain   | 23  | 23  | 23  | 27  | 29  | 30  | 32  | 33  | 33  | 51  | Specificity                  |
|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----------------------------|
| LklG_A     | G   | L   | F   | W   | I   | G   | A   | S   | G   | K   | 2-ketoacid                  |
| CrpDM2_A   | V   | A   | I   | F   | L   | G   | S   | S   | G   | K   | 2-hydroxyisocaproate        |
| CseA_A1    | V   | G   | V   | W   | V   | G   | T   | S   | G   | K   | 2-ketoisocaproate           |
| Vlm1_A1    | A   | A   | L   | W   | I   | A   | V   | S   | G   | K   | 2-ketoisovalerate           |
| PksJ_A1    | V   | G   | W   | T   | T   | A   | A   | I   | C   | K   | 2-ketoisocaproate           |
| LklI_A     | D   | A   | W   | F   | L   | G   | N   | V   | V   | K   | L-Leucine                   |
| BarD_A     | D   | A   | I   | L   | L   | G   | G   | A   | A   | K   | L-Leucine                   |
| AerB_A     | D   | A   | W   | F   | L   | G   | N   | V   | V   | K   | L-Leucine                   |

*: CrpDM2: cryptophycin NRPS CrpD module 2; CseA: cereulide NRPS CseA; Vlm1: valinomycin NRPS Vlm1; PksJ: bacillaene NRPS PksJ; BarD: barbamide NRPS BarD; AerB: aeruginosin NRPS AerB.
**Supplementary Methods**

*Biofilm formation by coral-associated isolates in the presence of looekeyolide B*

Bacterial isolates were grown overnight in marine broth at 30 °C, including five *Halomonas* strains (strains BBD45, BBD48, R1t3, R1t4, FL8), three *Vibrio* strains (strains BBD44, BBD50, BBD93), three *Alteromonas* strains (strains FL4, FL17, FL44), and two *Oceanospirillum* strains (strains BBD11, BBD12) isolated from *Montastraea cavernosa* (with the exception of R1t3 and R1t4, from *Acropora palmata*) surface mucus collected in the Florida Keys. Cells were washed twice with sterile marine broth, diluted 1:100 in fresh media, and 100 µL of diluted culture was added per well of a 96-well polystyrene plate, with or without 1-mg/L looekeyolide B. Plates were incubated overnight inside a sealed bag containing a damp paper towel at 30 °C. Biofilm formation was evaluated by the addition of 0.2% crystal violet solution for 15 mins to the wells with overnight cultures. Cultures with the unbound dye were decanted and the wells were gently washed three times with distilled water. 200 µL of 33% acetic acid was pipetted up and down in each well and 125 µL was distributed to a fresh 96-well plate. Absorbance at 590 nm was measured with a Victor-2 multimode microtiter plate reader (Perkin-Elmer, Waltham, MA, USA).

*Electron Paramagnetic Resonance (EPR) spectroscopy*

EPR spectra were collected on a Bruker ELEXSYS E500 spectrometer using a HighQ perpendicular mode cavity. Temperature was controlled using an Oxford Instruments CF905 helium cryostat with a Lakeshore Cryotronics ITC model 336 temperature controller using a calibrated Cernox temperature sensor. All samples were prepared in a glove bag under Argon atmosphere using degassed solutions. Solutions were degassed by sparging with Argon for 2 hours and prepared as follows. 50 mL of 50 mM HEPES buffer was prepared at pH 7.4 and subsequently sparged with Argon via septum in a round bottom flask. A 63 mM FeCl₃ solution was prepared by measuring 353 mg FeCl₃ into a prepared round bottom flask that was then evacuated and replaced with Argon atmosphere. 20.7 mL of prepared HEPES buffer were transferred from the degassed solution to the flask containing FeCl₃ via gas tight syringe. The solution remained clear indicating the absence of any dissolved oxygen. A solution blank was prepared with 200 µL of buffer and 200 µL of DMSO (as a glassing agent) added to an eppendorf tube in a glove bag under Argon. 200 µL was then transferred to a 5-mm quartz EPR tube and capped with a schlenk adapter. The sample tube was flame sealed upon removal from the glove bag. To the remaining 400 µL of buffer/DMSO, 10µL of 63 mM FeCl₃ was added to the solution and mixed. 200 µL was then transferred to a 5 mm quartz EPR tube and flame sealed as above as a FeCl₃ standard for EPR. A FeCl₃ standard plus EDTA was prepared with 200 µL of FeCl₃ buffer solution and 2.4 mg of EDTA. After mixing, this solution was transferred to a 5mm quartz EPR tube and flame sealed as above as a FeCl₃ standard for EPR. A FeCl₃ standard plus EDTA was prepared with 200 µL of FeCl₃ buffer solution and 2.4 mg of EDTA. After mixing, this solution was transferred to a 5mm quartz EPR tube and flame sealed as above. Approximately 1 mg of looekeyolide B was dissolved in 400 µL of pH 7.4 HEPES buffer and 400 µL of DMSO. Two flame sealed EPR tubes were prepared with the looekeyolide/buffer solution without iron. To the remaining 400 µL of solution 5 µL of the 63 mM FeCl₃ solution was added. The resulting solution was placed into two 5 mm quartz EPR tubes and flame sealed. EPR parameters were as follows: 9.41GHz, 3550 Gauss center field and 7000 Gauss sweep width, 25dB microwave attenuation corresponding to 0.6325 mW, 5 Gauss modulation amplitude, 100 kHz modulation frequency, 40 ms conversion time, 7001 data points per scan.

*Pulse amplitude modulated (PAM) fluorometry to assess application of looekeyolide to corals*

Pulse Amplitude Modulated Fluorometry (Model: Walz Diving-PAM ; LED emission maximum 650 nm) was used in separate experiments to assess the photosynthetic performance of multiple colonies of *Montastraea cavernosa* that were exposed to Phytagel embedded with looekeyolides dissolved in EtOH (treatment) molded onto plastic window screen and cut into strips (2.5 cm x
2.5 cm) following previously described methods. To account for the application of the strips, separate coral colonies were exposed to Phytagel with solvent only and no lookeyolides (control). Lookeyolide B was tested in 2013 at a concentration of 0.04 mg LK B g wet wt⁻¹, and in 2016 LK-B was tested at a higher concentration of 0.18 mg LK-B g wet wt⁻¹, encompassing a range of natural concentrations of the compound (~0.1-0.2 mg g wet wt⁻¹). In 2014, a mixture of LK-A and lyngbic acid (~2:1 based on H NMR) was tested at a concentration of 0.36 mg g wet wt⁻¹. In 2013 and 2014, control and treatment corals were held in separate aquaria, and in 2016 a control and a treatment coral were held in the same tank.

After 48 hours of exposure, maximum quantum yield (Fv/Fm) was measured directly underneath both the treatment and control strips of each coral. All measurements were taken after dark-adapting each colony for a minimum of 90 minutes. Dark yields were obtained by administering a saturating pulse (>8000 μmol photons m⁻² s⁻¹) with the diving PAM to the coral tissue directly underneath each treatment and control strip. The fiber topic tip (5.5mm active diameter) was standardized to a distance of 10mm from the coral surface, and initial fluorescence values (Fo) were between 300-500 units. Internal settings on the diving-PAM were as follows (measuring intensity = 4, saturation intensity = 10; saturating width = 0.8s).

Supplementary Results

In silico analysis of biosynthesis of lookeyolide A

The Lkl PKSs and NRPSs contained eight functional modules. LklD was a partial PKS and contained one ketosynthase (KS) and one acyltransferase (AT) domain. It may form a fully functional PKS module with LklE that contained one methyltransferase (MT), one ketoreductase (KR), and thiolation (T) domain. LklB was a bimodular PKS. In all four Roseofilum genomes, only the first 150 amino acid residues of the AT domain in the second module (module 4) of LklB showed high similarities to other AT domains. It remains unclear whether another AT domain compensates its function or this region is difficult for metagenomic sequencing to produce an accurate reading.

To predict substrate specificity of the AT domains in the Lkl PKSs, we aligned them with malonyl-CoA specific AT domains from PKSs in the biosynthesis of cyanobacterial natural products curacin (CurG, CurH, CurL, CurJ, CurK, CurL),² cryptophycin (CrpA, CrpB),³ microcystin (McyG, McyE),⁴ and methylmalonyl-CoA specific AT domains from niddamycin (NidA),⁵ and erythromycin (DEBS1)⁶ (Fig. 4b, main paper). All AT domains in the Lkl PKSs shared the sequence motif of QGHGANVGRF in that is characteristic to activate malonyl-CoA.⁷ A methylation modification on carbon or oxygen atom at the β position of the growing polyketide intermediate can be performed by the MT domain of PKS. In total, five MT domains were identified in the Lkl PKSs and shared characteristic motif I and II with those from other PKSs (Figure 4c, main paper). Among them, the domains from LklA, LklB, and LklE also contained one motif I-post that is featured only in C-MT domains. This bioinformatics analysis indicates that the final product contains three C-Me and two O-Me modifications. We further bioinformatically analyzed seven KR domains in five PKSs and NRPS LklG. Two out of three catalytic residues in the KR domain from LklB’s second module were mutated, indicating that it is not functional (Figure 4c, main paper). Based on their sequence features,⁹,¹⁰ LklB_KR1 and LklD_KR were A-type KR domains while those from LklA, LklE, and LklF belonged to B-type. The outcome for the KR domain from NRPS LklG is unpredictable with the current understanding.
LklG was a single modular NRPS comprised of one condensation (C), one adenylation (A), one KR, and one T domain. This type of domain organization is similar to the second module of CrpD in the cryptophycin biosynthesis and several other NRPSs, all of which use 2-hydroxy- or 2-ketoacid but not amino acid as the substrates. We predicted the A domain specificity of this enzyme on the basis of binding pocket residue motifs. The conserved Asp235 involved in ionic interaction with the amino group of the substrate amino acid was replaced by Gly235 in the LklG A domain (Table S1), indicating the preferred use of 2-keto acid as its substrate. LklII (C-A-T-E-C-T) is likely another single modular NRPS appended with a C-terminal thioesterase (TE) domain. This enzyme was predicted to activate L-leucine as the substrate, similar to BarD and AerB in the biosynthesis of cyanobacterial natural products barbamide and aeruginosin respectively. Its epimerase (E) domain might convert the substrate into D-form. Interestingly, there were one additional C and one additional T domain in LklI. The function of this didomain is unclear and might be evolutionary remnants of a complete NRPS module. The similar domain organization was found in AerG for the biosynthesis of aeruginosin in the cyanobacterium Planktothrix agardhii CYA126/8.

Based on the functional predictions of all biosynthetic enzymes and the elucidated structure of lookeyolide A, a biosynthetic scheme of this compound was proposed (Figure 4a, main text). The isolated cluster is missing one loading module and one PKS module (module 1) that together synthesize a butyrate intermediate. Another possibility is that the missing loading module directly activates a butyryl-CoA substrate. LklA-F activate six molecules of malonyl-CoA, reduce five keto groups, introduce three C-Me side chains, and promote two O-Me modifications. The stereochemistry of the hydroxyl groups are labeled based on the bioinformatic prediction of the conserved motifs of KR domains. LklG may activate the methionine-derived 2-keto-4-(methylthio) butyrate whose keto group is reduced to a hydroxy group by the embedded KR domain. Next, LklI incorporates D-leucine into the biosynthetic intermediate that is then cleaved off by the catalysis of the TE domain through macrocyclization. The cyclized product might further undergo a nucleophilic attack at the keto group with the C9 hydroxyl group able to generate a tetrahydro-2H-pyran moiety in an automatic manner. LkhI introduces another hydroxyl group at the C8 position. Finally, a sulfoxide group is produced, likely by the oxidation of susceptible sulfur atom with reactive oxygen species. The exact order of these tailoring reactions requires future studies. All of these results indicated that all five isolated lklA gene clusters are highly likely to be responsible for the biosynthesis of lookeyolide A.

**Electron Paramagnetic Resonance (EPR) spectroscopy**

Lookeyolide B was tested for iron binding activity due to structural similarity to several siderophore species. X-band EPR was employed to investigate iron chelation. Lookeyolide B was measured in HEPES buffer in water: DMSO glass at pH 7.4 with and without addition of Fe(III). Spectra were recorded from 0 to 7000 Gauss, encompassing the two major X-band resonance positions for iron signals, being ~1600 Gauss ($g = 4$) and ~3400 Gauss ($g = 2$), respectively. In the lookeyolide B sample no spectral change was observed before and after adding iron, suggesting that iron is agglomerating in the sample and increasing the relaxation rate, resulting in signals that are broadened into the baseline beyond detectability. Control experiments consisting of iron (III) in HEPES buffer with and without EDTA produce a spectrum that conforms to the expectation of a chelated iron signal. Before EDTA addition the magnitude of the iron signal observed is approximately equal in intensity to the trace manganese impurity present in the iron salt and not at all consistent to an iron signal at the concentration used. Upon addition of EDTA to the sample we observed a 1,000-fold increase in intensity of the $g = 4$ absorption peak. As EDTA is a well-known strong chelator of iron in solution and the EDTA was added in excess in order to ensure full chelation this control serves as a baseline for sequestered iron in HEPES buffer. Due to the lack of a coordinated iron signal in the lookeyolide B sample it can be
inferred that looekeyolide B does not effectively sequester iron, indicating that iron is still agglomerating in the solution (Fig. S13).

Pulse amplitude modulated (PAM) fluorometry to assess application of looekeyolides to corals

Across all experiments, application of the Phytagel strips containing looekeyolides had no significant effect on coral Fv/Fm as compared to the application of the control strips. In 2013, dark yields (mean ± SE) were 0.57 ± 0.02 and 0.59 ± 0.03 underneath the treatment and control strips, respectively (t-test, n=6, P=0.73). In 2014, dark yields were 0.51 ± 0.02 and 0.48 ± 0.03 underneath the treatment and control strips, respectively (t-test, n=10, P=0.26). In 2016, dark yields were 0.56 ± 0.04 and 0.59 ± 0.04 underneath the treatment and control strips, respectively (t-test, n=6, P=0.68) (Fig. S14).
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