Supporting Information

Deciphering Chemical Mediators Regulating Specialized Metabolism in a Symbiotic Cyanobacterium

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Experimental Procedures

General experimental procedures

NMR data were recorded on either an AVANCE III 500 MHz or 600 MHz instruments with a CryoProbe (Bruker Biospin, Rheinstetten, Germany). Spectra were referenced to the residual solvent peak, DMSO-d6 (δ=2.49, δc=39.5), pyridine-d5 (δ=7.22, δc=124.0), and CD3OD (δ=3.30, δc=47.6). LC-HRMS measurements were performed using either Exactive or Q Exactive Orbitrap high performance benchtop device with an electrospray ion source and an Accela HPLC system (Thermo Fisher Scientific, Bremen, Germany) consisting of an autosampler equipped with a column oven, a 1250 pump and a PDA detector. The purification of compounds by the preparative and semi-preparative HPLC was carried out using Shimadzu preparative HPLC system. UV spectra were measured by Shimadzu UV-1800 spectrophotometer (Shimadzu Deutschland, Jena, Germany). IR spectra were measured by JASCO FT/IR-4100 spectrometer (JASCO Deutschland, Pfungstadt, Germany).

Conventional cultivation of cyanobacteria

*N. punctiforme* cultures were maintained in liquid BG11[11] media under continuous white light illumination with an intensity of 30 µmol photons m⁻² s⁻¹ at 23 °C. Mutant strains were cultivated in BG11₀ media supplemented with 2 µg mL⁻¹ streptomycin.

To analyze metabolite influence on RIPP4 reporter mutant strain[2] or WT, cells were grown in 12-well plates or 35 mL flasks on a rotary shaker (45 rpm) under conventional cultivation conditions. The desired metabolite was fractioned by HPLC, dried and dissolved in 80 µL 60% methanol and added to the culture. A culture supplemented with 80 µL of 60% methanol and an untreated culture served as controls. Reporter cultures were analyzed by confocal fluorescence microscopy after 24 h. The WT strain was analyzed by RT-qPCR and RNA sequencing.

High light and high CO₂ (HL/HC) cultivation of cyanobacteria

For high light and high CO₂ cultivation of *N. punctiforme* WT and the mutant strain AraC_PK51 100 mL HD cultivators were used together with the HDC 9.100 universal platform (CellDEG GmbH, Germany), connected to a CO₂ compartment. Cells were shaken at 120 rpm. A high-power LED light source (RX400, AP673L, Valoya, Finland) was used for illumination. Light intensity and CO₂ partial pressure were controlled by a growth control unit (CellDEG GmbH, Germany). The cultivation was started with a light intensity of 70 µmol photons m⁻² s⁻¹ and a concentration of 3% CO₂. After one week of cultivation, the light intensity was set to 165 µmol photons m⁻² s⁻¹ and the CO₂ concentration was increased to 6%. Cultures were harvested after 20 d.

For high density cultivation of reporter mutant strains 10 mL HD cultivators were used together with a HDC9.10B platform (CellDEG GmbH, Germany). Into the lower vessel of the cultivator 200 mL bicarbonate buffer was applied. The buffer was obtained by mixing 3 M solutions of KHCO₃ and K₂CO₃ with ratios of 1:1 and 4:1, to provide CO₂ partial pressures of 5 and 32 mbar. Cells were shaken at 250 rpm and illuminated with a full-spectrum LED-panel (HYG05-D100°3W-W, RoHS). The bicarbonate buffer was exchanged every 72–120 h. The cultivation was started with an initial light intensity of 60 µmol photons m⁻² s⁻¹ and 5 mbar bicarbonate buffer. After 10 d the light intensity was set to 120 µmol photons m⁻² s⁻¹ and 32 mbar bicarbonate buffer was used. The strains were analyzed by confocal fluorescence microscopy after 10 d, 20 d, and 30 d.

Generation of the AraC_PK51 overexpression mutant

For the generation of the AraC_PK51 overexpression mutant a plasmid backbone (pJK008) was constructed, containing a streptomycin resistance cassette and a *N. punctiforme* promoter region (5' UTR of RS16340) followed downstream by an EcoRI restriction site. pJK008 was created by integration of the promoter region into the EcoRI site of pRL1049 by using HiFi-Builder (NEB, Frankfurt a.M., Germany) mediated homologous recombination. The promoter region was amplified from genomic DNA of *N. punctiforme* using PCR.

Therefore, the primers J056 and J057, containing the required homology sequence to the target vector and the missing bases for regeneration of EcoRI restriction site, were used (Table S9). The regulatory gene RS10525 was amplified by PCR, using the primer pairs J072/J073 and genomic DNA as template (Table S7). The regulatory gene was introduced into pJK008 by using HiFi-Builder (NEB, Frankfurt a.M., Germany) and transformed into the *E. coli* XL-1 blue (Agilent Technologies, Waldbronn, Germany) by chemical transformation. Before further transformation into *N. punctiforme* the construct was sequenced (GATC Biotech, Cologne, Germany).

For transformation into *N. punctiforme*, a dense culture was harvested, washed 4 times with sterile water and concentrated in a final volume of 400 µL sterile water. The overexpression plasmid (10 µg) was added to the cells and introduced by electroporation, using an electroporator (MicropulseSTM, Biorad, Munich, Germany) with the following conditions: 4 ms, 1.5 kV, 1 pulse. Cell suspension was spread on BG11₀ plates covered with HATF filters and placed under low light (10 µmol photons m⁻² s⁻¹) for 2 days. After another 5–7 days under standard light conditions (30 µmol photons m⁻² s⁻¹) the filters were transferred onto selective BG11₀ plates containing 2 µg mL⁻¹ streptomycin. The non-resistant cellular background died of after 2–3 weeks and potential mutant colonies appeared on the plates.
The colonies were transferred into liquid media and the correct genotype was confirmed by isolation of total DNA with the GenElute™ Plant Genomic DNA Miniprep Kit and subsequent PCR. For PCR the primer pair D021_S1/D022_S2, binding at the flanking region of insertion site of the plasmid backbone, was used.

**Treatment of N. punctiforme with nostovalerolactone (3), N1 (1) and N2 (2)**

In preparation of the second RNA sequencing study *N. punctiforme* cultures (10 mL) were freshly inoculated. After two days of conventional cultivation the cultures were treated with nostovalerolactone, both nostocides (N1, N2) or a combination of both nostocides and nostovalerolactone (final concentration: 15 µM) and further cultivated conventionally. All treatments were performed in duplicates, respectively. After 24 h the cells were harvested, and RNA was extracted. Prior to RNA sequencing, the purified RNA was analyzed by RT-qPCR. An RNA sample from an untreated *N. punctiforme* culture was used as a control.

**Bioactivity-guided fractionation of chemical mediators activating the AraC regulatory gene RS10525**

To identify factors activating the AraC regulatory gene *RS10525* *N. punctiforme* WT cultures were treated with arabinose or individual HPLC fractions of the WT HL/HC supernatant. For this, *N. punctiforme* cultures (4 mL) were freshly inoculated in 12-well-plates. To analyze the effect of arabinose on the transcriptional activity, cultures were treated with different concentrations of arabinose (0.1 mM, 1 mM, 10 mM, 100 mM) after two days of conventional cultivation. Cultures were further cultivated conventionally. After 24 h the cells were harvested, and RNA was extracted and purified. The purified RNA was analyzed by RT-qPCR. An RNA sample from an untreated *N. punctiforme* culture was used as a control.

To analyze the potential influence of secreted metabolites on the activity of the AraC regulatory gene *RS10525* the supernatant of an HL/HC cultivated *N. punctiforme* WT culture was analyzed by HPLC and fractionated. The fractions were dried and dissolved in 100 µl of 60% methanol. Fractions were added separately to a 2 day-old conventionally grown *N. punctiforme* culture. After 24 h of conventional cultivation, the cells were harvested, and RNA was extracted and purified. The purified RNA was analyzed by RT-qPCR. An RNA sample from an *N. punctiforme* culture treated with pure 60% methanol was used as a control.

**RNA Extraction**

For RNA extraction from *N. punctiforme* strains, 2 mL of liquid culture were pelleted and dissolved in 1 mL Trizol™ Reagent (Life Technologies GmbH, Darmstadt, Germany). Samples were frozen in liquid nitrogen. Frozen samples were shaken on a thermo shaker at 1400 rpm and 65 °C for 20 min. The aqueous phase was extracted by chloroform and the therein contained RNA was precipitated with equal volumes of isopropanol and a high salt solution (0.8 M trisodium citrate, 1.2 M NaCl), washed with ethanol and resuspended in sterile, RNase-free water. RNA samples were cleaned up with the RNEasy™ Mini KIT (Qiagen, Hilden, Germany), including on-column DNase digestion to eliminate possible DNA contaminations. The purity of the samples was checked by PCR.

**Semi-quantitative gene expression analysis by RT-qPCR**

RNA samples were transcribed into cDNA, using the Maxima™ H Minus cDNA synthesis kit (Thermo Scientific, USA). For real-time qPCR a Lightcycler 96 (Roche Diagnostics, Germany) was utilized together with a Sybr green based detection system (Blue’ S’ Green qPCR mix, Biozyn Scientific, Germany). For each BGC a specific primer pair was used amplifying an amplicon with a size of 96–164 bp (Table S10). For standardization the gene *rnpB* was used as a housekeeping gene, as already described.[3] For each reaction three technical replicates were carried out. From the raw data Ct values were calculated by the LightCycler 96 SW software (version 1.1, Roche Diagnostics, Germany). Ct values were used to calculate relative expression levels between control (untreated WT) and sample strains by using the ΔΔCt method.

**Transcriptome analysis by RNA sequencing**

Sequencing of extracted *N. punctiforme* mRNA was conducted by Novogene Europe (Cambridge, UK), including RNA quality control and rRNA depletion. The sequencing was performed on an Illumina Novaseq 6000 with a PE 150 platform. The initial RNA sequencing study was conducted with RNA from conventionally grown *N. punctiforme* WT, *N. punctiforme* WT grown under HL/HC cultivation conditions, conventionally grown *N. punctiforme* AraC_PKS1 and *N. punctiforme* AraC_PKS1 grown under HL/HC cultivation conditions. The cells were harvested after 20 d. All samples were analyzed in triplicates. For the second RNA sequencing study *N. punctiforme* WT was treated with the *pks1* derived compounds separately and in combination (final concentration 15 µM). The cells were harvested after 24 h of incubation. As only a limited amount of the *pks1* product was available, only one timepoint was chosen for RNA sequencing and the analysis was performed in duplicates. The incubation time was chosen according to the bioactivity-guided *ripp4* reporter studies. Here the strongest effect on *ripp4* transcription was detected 24 h after addition of fraction V.

Sequencing data were deposited in NCBI under the accession numbers PRJNA666330 and PRJNA718147.

The raw sequencing data was uploaded to the Galaxy web platform and its public server[4] (usegalaxy.eu) was used for data processing. HISAT (version 2.1.0)[5] was used to map paired-end reads to *N. punctiforme* PCC 73102 reference genome (Genbank GCA_000020025.1; RefSeq GCF_000020025.1). Mapped reads were trimmed by BAM filter (version 0.5.9) to remove unmapped reads...
and reads smaller than 20 bp. Trimmed reads were counted, using HTSeq (version 0.9.1).\cite{[6]} Counted reads were further processed and normalized with the R (version 4.0.2, R Foundation for Statistical Computing, Vienna, Austria) package DESeq2 (version 1.30.1)\cite{[7]} to obtain log2 fold changes between control and sample strains. For all samples the transcript levels of a conventionally grown N. punctiforme WT strain was used as reference for the calculation of the log2 fold changes. Log2 fold changes ± 1 and a p-value ≤ 0.01 were used as threshold values to indicate statistically significant transcriptional changes. The data of transcriptome analysis shown in Figure 1a were obtained as described earlier.\cite{[8]}

Confocal fluorescence microscopy
Confocal fluorescence microscopy was performed on a Zeiss LSM 780 (Carl Zeiss Mikroskopie GmbH, Jena, Germany), Axio Observer Z1equipped with a diode laser (405 nm) and an AxioCam digital microscope camera. A PlanApo 1.4/63 × oil immersion objective and filter presets for eCFP and chlorophyll α detection (excitation at 405/633 nm, detection at 450–550 nm / 650–725 nm) were used for image acquisition. For operating the microscope, image acquisition and processing, the Zen Software (version 2.3) was used.

Sample preparation and HPLC analysis
For HPLC analysis cell pellets (~ 0.3 g wet weight) were resuspended in MilliQ water and sonicated (pulse on 2 s, pulse off 2 s, 10 min, Bandelin Sonoplus HD3100) on ice. To remove cell debris, cells were centrifuged. Cell-free supernatant was loaded onto a SepPak Plus C18 column (Waters GmbH, Eschborn, Germany) and washed with 2 mL of 5% methanol, before matrix-bound metabolites were eluted with 2 mL of 100% methanol. The methanolic extracts were vacuum-dried and the resulting pellets dissolved in 60% methanol. Samples were centrifuged and filtered (Acrodisc 4 mm Syringe filters; Pall GmbH, Germany) to remove any remaining debris. Filtered samples were analyzed by HPLC.

HPLC analysis was conducted on a Shimadzu SCL-10AVP HPLC-system. Samples were loaded (20–30 μL) on a SymmetryShield RP18 column (3.5 μm, 4.6 mm × 100 mm) and a SymmetryShield Sentry Guard column (3.5 μm, 3.9 mm × 20 mm). Compound separation was achieved by applying an acetonitrile (0.05% TFA) in MilliQ water gradient from 2% to 60% in 40 min, at a flow-rate of 1 mL min⁻¹. Afterwards the acetonitrile proportion was increased to 100% within 2 min and held for 3 min. Annotation of nostopeptolides, anabaenopeptin and microviridin based on authentic standards (Figure S55).

For relative compound quantification (Table S8) 10 mL of N. punctiforme WT and AraC_PKSI mutant cultures grown under HL/HC conditions and conventionally were analyzed by HPLC. Peak areas of known compound peaks were quantified and compared to the WT HL/HC culture.

For RP-HPLC analysis of Figure 2 and Figure S46, extracts were subjected to reversed-phase HPLC (Symmetry C18 5 μm, 3.9 × 150 mm, Waters GmbH, Eschborn, Germany) with a gradient system; solvent A (MilliQ water), solvent B (acetonitrile), 5% B for 10 min, to 99% B in 30 min, to keep for 1 min, at a flow-rate of 1 mL min⁻¹.

Isolation of nostoclide N1 (1), N2 (2), nostovalerolactone (3), and 9-dehyдоностовалеролактон (4)
The cultured cells (wet weight 9.15 g) from N. punctiforme PCC 73102 AraC_PKSI mutant (500 mL culture) were suspended with 200 mL of 80%aq. methanol (v/v) and then lysed by a sonicator (BANDELIN SONOPULS HD2200, BANDELIN electronic, Berlin, Germany) using a sonotrode MS73 with 2 min sonication at 50% duty cycle at a room temperature. The obtained extract was centrifuged at 9,000 × g for 15 min at 15 °C and the residue was extracted with methanol (200 mL × 2 times) as described above. These extracts were combined and concentrated under reduced pressure. The resulting residue was dissolved in 200 mL of 90%aq. methanol (v/v) and then washed with 200 mL of hexane. Theaq. methanol phase was subjected to flash column chromatography (2.5 × 6.0 cm, LiChroprep C18, 40 – 63 μm, Merck, Darmstadt, Germany) and eluted with 20%aq. (200 mL, v/v), 50%aq. (100 mL, v/v), 100% methanol (100 mL), and dichloromethane (100 mL).

The 100% methanol eluted fraction was further subjected to flash column chromatography (2.5 × 6.0 cm, LiChroprep C18, 40 – 63 μm) and eluted with 50%, 70%, 80%aq. methanol and 100% methanol (each 100 mL). The 80%aq. methanol fraction was subjected to reversed-phase HPLC (Nucleosil 100-7C18, 21.2 × 250 mm, Machery-Nagel, Düren, Germany) using a gradient system; solvent A (MilliQ water containing 0.1% trifluoroacetic acid (v/v, TFA)), solvent B (83%aq. acetonitrile (v/v)), 20% B for 10 min, to 100% B in 40 min, and kept for 10 min, at a flow-rate of 16 mL min⁻¹. The nostoclide N1 and N2 containing fraction was further subjected to reversed-phase HPLC (Nucleosil 100-7 C18, 10 × 250 mm) with an isocratic mobile phase 50:50 (83%aq. acetonitrile (v/v) / MilliQ water containing 0.1% TFA (v/v)) at a flow-rate of 6 mL min⁻¹ to yield nostoclide N1 (1, 530 μg) and N2 (2, 930 μg).

The culture supernatant (500 mL) was subjected to Amberlite XAD4 (3.0 × 7.0 cm, Sigma-Aldrich, Taufkirchen, Germany), washed with 150 mL of MilliQ water and eluted with 300 mL of methanol followed by 150 mL of acetone. The methanol / acetone eluted fractions and nostovalerolactone-containing fractions from cell extracts were subjected to reversed-phase HPLC (LiChroSpher WP300 RP-18, 10 × 250 mm, Merck, Darmstadt, Germany) with a gradient system; solvent A (MilliQ water), solvent B (acetonitrile), 5% B for 10 min, to 45% B in 20 min, to 100% B in 10 min, at a flow-rate of 6 mL min⁻¹, to yield nostovalerolactone (3, 16.8 mg).
Isolation of nostoveralactone (3) from 1-\(^{13}\)C AcONa and 1,2-\(^{13}\)C\(_2\) AcONa fed culture supernatants

\(N.\ punctiforme\) PCC 73102 AraC\(_{-}\)PKS1 mutant cultures, with a volume of 100 mL, were cultivated for 12 d under HL/HC cultivation conditions prior to feeding with labeled acetates (1-\(^{13}\)C AcONa or 1,2-\(^{13}\)C\(_2\) AcONa). The cultures were fed with a mixture of labeled acetate (2 mg) and unlabeled acetate (2 mg) on days 12, 16, 18 and 21 after inoculation. The labeled acetates, 1-\(^{13}\)C AcONa and 1,2-\(^{13}\)C\(_2\) AcONa, were fed separately. Cultures were harvested on day 24. The supernatants of the fed AraC\(_{-}\)PKS1 mutant cultures (100 mL culture supplemented with a total amount of 8 mg 1-\(^{13}\)C AcONa and 1,2-\(^{13}\)C\(_2\) AcONa, respectively) were subjected to Amberlite XAD4 (2.0 × 7.0 cm), washed with 75 mL of MilliQ water and eluted with 150 mL of methanol followed by 150 mL of acetone. The methanol eluted fractions were subjected to reversed-phase HPLC (LiChroSpher WP300 RP-18, 10 × 250 mm) with a gradient system; solvent A (MilliQ water), solvent B (acetonitrile), methanol eluted fractions were subjected to reversed-phase HPLC (LiChroSpher WP300 RP-18, 10 × 250 mm) with a gradient system; solvent A (MilliQ water), solvent B (acetonitrile), 10% B for 10 min, to 100% B in 30 min, at a flow-rate of 6 mL min\(^{-1}\), to yield 1-\(^{13}\)C acetate labeled nostoveralactone (4.0 mg) and 1,2-\(^{13}\)C\(_2\) acetate labeled nostoveralactone (5.9 mg), respectively.

**Nostoclide N1 (1).** Pale yellow powder. UV (MeOH) \(\lambda_{max}\) 236 nm (ε 9.210), 358 nm (ε 16.210), IR (ATR), 1675, 1444, 1196, 1135, 1030, 839, 803, 720 cm\(^{-1}\) (Figure S4). HRESIMS, \(m/z\) 293.1166 [M+H]\(^{+}\) (calcd for C\(_{18}\)H\(_{17}\)O\(_{3}\), 293.1172), \(m/z\) 291.1022 [M–H]\(^{−}\) (calcd for C\(_{17}\)H\(_{16}\)O\(_{2}\), 291.1016).

**Nostoclide N2 (2).** Yellow powder. UV (MeOH) \(\lambda_{max}\) 241 nm (ε 8.780), 370 nm (ε 16.440), IR (ATR), 1670, 1438, 1191, 1130, 1022, 840, 804, 714 cm\(^{-1}\) (Figure S14). HRESIMS, \(m/z\) 323.1278 [M+H]\(^{+}\) (calcd for C\(_{20}\)H\(_{20}\)O\(_{3}\), 323.1273), \(m/z\) 321.1121 [M–H]\(^{−}\) (calcd for C\(_{20}\)H\(_{19}\)O\(_{2}\), 321.1130).

**Nostoveralactone (3).** Pale yellow powder. UV (MeOH) \(\lambda_{max}\) 224 nm (ε 39.330), 230 nm (ε 39.330), 302 nm (ε 9.780). IR (ATR), 1739, 1591, 1427, 1294, 1020, 990, 869, 811 cm\(^{-1}\) (Figure S24). HRESIMS, \(m/z\) 361.1645 [M–H+O+H]\(^{+}\) (calcd for C\(_{20}\)H\(_{20}\)O\(_{5}\), 361.1646), \(m/z\) 377.1605 [M+H]\(^{+}\) (calcd for C\(_{20}\)H\(_{19}\)O\(_{4}\), 377.1595).

9-Dehydronostoveralactone (4). Pale yellow powder. HRESIMS, \(m/z\) 361.1643 [M+H]\(^{+}\) (calcd for C\(_{20}\)H\(_{20}\)O\(_{5}\), 361.1646), \(m/z\) 359.1501 [M–H]\(^{−}\) (calcd for C\(_{20}\)H\(_{19}\)O\(_{4}\), 359.1489).

Allelopathy assay

\(N.\ punctiforme\) PCC 73102 and Anabaena sp. 7120 served as indicator strains to visualize a potential allelopathic effect against the nostoclides. Therefore, both strains were homogenized, using a syringe and plated on a solid BG11\(_{0}\) plate (1.2% bacto agar). The dried paper disks (6 mm) soaked with 25 µg of the two nostoclides, respectively and in combination were placed on the top agar. A paper disc soaked in 60% methanol served as negative control, another disc with 10 µg streptomycin served as antibiotic control. Plates were kept under standard cultivation conditions for two weeks.

Preparation of \(C.\ reinhardtii\) and \(N.\ punctiforme\) for chlorophyll fluorescence measurements

\(C.\ reinhardtii\) strain SAG 73.72 (mt\(^{+}\)) (obtained from the algal culture collection in Göttingen) was cultivated in Tris-acetate-phosphate (TAP) medium\(^{[8]}\) under continuous illumination at 50 µmol photons m\(^{-2}\) s\(^{-1}\) and agitated on an orbital shaker (200 rpm) at 20 °C. Cell concentrations were determined using a Coulter counter (Multisizer 4e, Beckmann Coulter, Brea CA, United States) with a 30-µm capillary and analyzed in the size range 3.5 - 13 µm. A 2-day-old \(C.\ reinhardtii\) pre-culture was used to inoculate a 24-well plate (Greiner CELLSTAR, Kremsmünster, Austria) with an initial concentration of 2 × 10\(^{6}\) cells mL\(^{-1}\). For experiments with \(N.\ punctiforme\) a pre-culture was mixed thoroughly using a pipette to achieve an equal distribution of filaments before inoculation. Due to the filamentous growth of \(N.\ punctiforme\) no cell counts were determined. 5 µL of a 2 mM nostoclide stock solution (dissolved in ethanol) were added to 1 mL of cell suspension per well and the incubation continued for 24 h under the culture conditions described above before the cell density was reassessed.

Absorption spectra

Absorption spectra of \(C.\ reinhardtii\) were measured with a microtiter plate reader (TECAN Spark, Tecan, Männedorf, Switzerland). The spectra were corrected for light scattering by subtracting the absorption value measured at 750 nm.

Chlorophyll fluorescence measurements

Variable chlorophyll fluorescence was measured with a Pulse-amplitude modulated (PAM) fluorometer (PAM101/103, Walz, Effeltrich, Germany) according to the saturating pulse method by Schreiber et al. (1994).\(^{[15]}\) Saturating pulses were applied with a duration of 800
Structure elucidation of nostoclide N1 (1) and N2 (2)

The molecular formula (C_{20}H_{32}O_{7}) of compound 1 (UV_{max} 355 nm) was deduced from its high-resolution mass spectrum (m/z 293.1166 [M+H]^+), Figure S3, S5–S6). 1H NMR data analysis indicated two sets of aromatic protons, two doublet protons, H7/H11 and H8/H10 (δ 7.67 and δ 6.81), one doublet proton, H14/18 (δ 7.24) and two triplet protons H15/17 and H16 (δ 7.28 and δ 7.19) (Figure S7–S8, and Table S1). The correlation between H7/H11 and H8/H10 in the 1H–1H COSY spectrum and the HMBC correlations from H7/H11 to the phenolic carbon C9 (δ 159.8), and H8/H10 to C6 (δ 126.1) pointed to a p-hydroxyphenyl moiety (Figure 2C, Figure S9–S12, and Table S1). The second set of aromatic protons suggested the presence of a phenyl group, owing to the 1H–1H COSY spin system (H14 to H18) and the HMBC correlation between H15/H17 and C13 (δ 139.7). The third unit is a 3,4,5-trisubstituted butenolide, according to the observed HMBC correlations of the singlet methyl protons 3-Me (δ 2.19) to C2 (δ 148.1), C3 (δ 126.3), and C4 (δ 151.6), of the singlet methylene protons H12 (δ 3.72) to C1 (δ 172.6), C2, and C3, and of the singlet methane proton H5 (δ 6.21) to C3 and C4 (Figure 2C, Figure S9–S12, Table S1). These three partial units are connected through C5/C6 and C12/C13 as indicated by the HMBC correlations from H5 to C7/C11 (δ 133.4), Figure S11 from H12 to C14/C18 (δ 129.4) (Figure 2C, Figure S9–S12, Table S1). From HR-ESIMS data (m/z 323.1278 [M+H]^+) the molecular formula (C_{20}H_{32}O_{7}) of compound 2 (UV_{max} 365 nm) was deduced. The 8-OMe (δ 3.89 and δC 56.2) signals observed in the 1H NMR and HSQC spectra suggested that the structure of 2 represents an 8-methoxy derivative of 1 (Figure S13–S22). 1H–1H COSY, HSQC, and HMBC analyses corroborated the proposed structure of 2 (Figure 2C, Figure S13–S22, Table S2).

The configuration of the C4/C5 double bonds of 1 and 2 was determined to be Z on the basis of the observed NOE between 3-CH3 and H5 (Figure 2C, Figure S12, S22). Taken together, 1 and 2 (Figure 2C) were identified as new congeners of photosynthesis-inhibiting nostoclides I and II that were previously isolated from a symbiotic Nostoc sp. strain of the lichen Pettigera canina. The novel nostoclides of N. punctiforme were named N1 and N2. In contrast to nostoclides I and II, both congeners of N. punctiforme lack the characteristic chlorine modification at the p-hydroxyphenyl moiety and differ in the substitution of the butenolide moiety.

Structure elucidation of nostovalerolactone

High-resolution ES-MS data (m/z 377.1605 [M+H]^+) of 3 (UV_{max} 300 nm) indicated its molecular formula (C_{20}H_{32}O_{7}) (Figure S23, S25–S26). The 1H and 13C NMR spectra of 3 in DMSO-d6 showed the presence of isomers (major/minor 3:1). Figure S27–S28, Table S3). Since these isomers could not be separated by HPLC, the structure elucidation was performed using the conformational isomers in equilibrium. Two 1H–1H COSY correlation systems from an oxymethine proton H10 (δ 3.76) to a methylene proton H17 (δ 1.30) (a correlation between H13 (δ 6.21) and H14 (δ 6.01) is observed in pyridine-d5) and from a methylene proton H18 (δ 1.28) to triplet methyl protons H19 (δ 0.85) were further connected on the basis of HMBC correlations between H19/C17 (δ 31.0) (Figure 2C, Figure S29–S31, Table S3). HMBC correlations of the doublet methylene protons H8 (δ 1.77 and 2.56) to C3 (δ 105.2), C7 (δ 190.8), C9 (δ 77.1), and C10 (δ 86.1) connected C3 and C7 to C9. This linkage was supported by additional HMBC correlations of the alkenyl proton 7-CH= (δ 9.66) to C3/C8, of the singlet methyl protons 9-Me (δ 1.17) to C8 (δ 50.4) and C9/C10, and of the tertiary hydroxyl proton 9-CH(OH) (δ 4.48) to C8/C9. Finally, two HMBC correlations from exomethylene protons H6 (δ 4.71 and 4.92) to C4 (δ 179.6) and C5 (δ 152.8) suggested the presence of a tetratic acid moiety. Although no HMBC correlations from any protons to carbonyl carbon C1 (δ 168.6) were detectable, the NMR spectral comparison between 3 and the structurally related agglomeren11 pointed to a 5-methylidenetetronate substructure in 3 (Figure S51). As we noted a discrepancy of 28 Da (C=O) between the proposed structure and the deduced molecular formula (C_{20}H_{32}O_{7}), we further analyzed NMR data extensively. The detailed HMBC analysis revealed a long range 1J-HMBC correlation from H6 to the hemiacetal carbon C2 (δ 93.6), which is adjacent to the oxygen at C10 (Figure S51). This ether bond connection indicated that 3 harbors a fused bicyclic structure containing a δ-valerolactone unit instead of a γ-butyrolactone. This ring formation is also supported by a weak long range 1J-HMBC correlation from H10 to C3. Finally, this structure was confirmed by the labeling pattern of 1-13C and 2-13C sodium acetate feeding experiments (Figure S46–S51, Table S4). The configurations of the two double bonds (C12 to C15) were determined as E by NOE (H12/H14 and H13/H15) in pyridine-d5 (Figure S32–S37, Table S5). Taken together, compound 3, named nostovalerolactone, represents a novel polyketide featuring a unique bicyclic moiety combining a persubstituted δ-valerolactone unit and an oxepane-like unit. The structure of 3 indicated that two sets of NMR signals derive from a conformational isomer in the oxepane ring, owing to the flexibility of the seven-membered ring.12 In the course of the analysis, we also discovered a congener of 3, 9-dehydroxynostovalerolactone (4). Notably, 4 has reduced flexibility in the oxepane ring, which is reflected by a single set of 1H and 13C NMR signals (Figure S38–S45, Table S6).
### Table S1. \(^1\)H and \(^{13}\)C NMR Data for Nostoclide N1 (1) in CD\(_3\)OD.

| Position | \(^1\)H (J=Hz) | \(^{13}\)C | HMBC (\(^1\)H to \(^{13}\)C) |
|----------|----------------|------------|--------------------------|
| 1        | 172.6          |            | H12                      |
| 2        | 148.1          | 3-CH\(_3\), H12 |
| 3        | 126.3          | 3-CH\(_3\), H5, H12 |
| 3-CH\(_3\) | 2.19 (s)  | 10.2            |                          |
| 4        | 151.6          |            | H5, H12                  |
| 5        | 6.21 (s)       | 111.1      | H7, H11                  |
| 6        | 126.1          | H8, H10    |                          |
| 7,11     | 7.67 (d 8.6)  | 133.4      | H7, H11                  |
| 8,10     | 6.81 (d 8.6)  | 116.7      | H8, H10                  |
| 9        | 159.8          | H7, H11    |                          |
| 12       | 3.72 (s)       | 30.2       | H14, H18                 |
| 13       | 139.8          | H15, H17   |                          |
| 14,18    | 7.24 (d 7.4)  | 129.4      | H12, H14, H, 16, H18    |
| 15,17    | 7.28 (t 7.4)  | 129.7      | H15, H17                 |
| 16       | 7.19 (t 7.4)  | 127.5      | H14, H18                 |

### Table S2. \(^1\)H and \(^{13}\)C NMR Data for Nostoclide N2 (2) in CD\(_3\)OD.

| Position | \(^1\)H (J=Hz) | \(^{13}\)C | HMBC (\(^1\)H to \(^{13}\)C) |
|----------|----------------|------------|--------------------------|
| 1        | 172.5          |            | H12                      |
| 2        | 148.2          | 3-CH\(_3\), H5, H12 |
| 3        | 126.0          | 3-CH\(_3\), H5, H12 |
| 3-CH\(_3\) | 2.18 (s)  | 9.60            |                          |
| 4        | 151.6          | H5, H12    |                          |
| 5        | 6.21 (s)       | 111.4      | H7, H11                  |
| 6        | 126.5          | H10        |                          |
| 7        | 7.48 (d 1.8)  | 133.4      | H5, H11                  |
| 8        | 148.9          | H7, H10, 8-OCH\(_3\) |                          |
| 8-OCH\(_3\) | 3.89 (s)  | 56.2       |                          |
| 9        | 149.1          | H11        |                          |
| 10       | 6.80 (d 8.3)  | 116.3      |                          |
| 11       | 7.23 (m)       | 126.1      | H5, H7                   |
| 12       | 3.71 (s)       | 30.0       | H14, H18                 |
| 13       | 139.4          | H12, H15, H17 |                          |
| 14,18    | 7.24 (m)       | 128.7      | H12, H14, H18            |
| 15,17    | 7.27 (m)       | 129.5      | H15, H17                 |
| 16       | 7.18 (t 6.9)  | 127.5      | H18                      |
Table S3. $^1$H and $^{13}$C NMR Data for Nostovalerolactone (3) in DMSO-$d_6$.

| Position | Major $^1$H (J=Hz) | $^{13}$C | $^1$H-$^1$H COSY $^1$H ($^1$H to $^1$H) | HMBC $^1$H to $^{13}$C |
|----------|-------------------|---------|---------------------------------|------------------|
| 1        | 168.6             |         |                                 |                  |
| 2        | 93.6              | H6      |                                 |                  |
| 3        | 105.2             | 7-OH, H8|                                 |                  |
| 4        | 179.6             | H6      |                                 |                  |
| 5        | 152.8             | H6      |                                 |                  |
| 6        | 4.71 (d 1.5)      | 88.5    |                                 |                  |
| 7        | 4.92 (d 1.5)      | 190.8   | H6, H8                         |                  |
| 8        | 9.66 (s)          |         |                                 |                  |
| 9        | 1.77 (d 13.7)     | 50.4    | 7-OH, 9-OH, 9-CH$_3$            |                  |
| 10       | 2.56 (d 13.7)     |         |                                 |                  |
| 11       | 77.1              | H8, 9-OH, 9-CH$_3$, H10, H11     |                  |
| 12       | 4.48 (s)          |         |                                 |                  |
| 13       | 4.17 (s)          |         |                                 |                  |
| 14       | 2.17 (s)          | 23.8    | H8, 9-OH, H10                  |                  |
| 15       | 3.76 (dd 8.4, 4.4)| 86.1    | H11                            | H8, 9-OH, 9-CH$_3$, H11, H12 |
| 16       | 2.09 (m)          | 31.7    | H12, H10                       | H12, H13         |
| 17       | 2.18 (m)          | 129.3   | H12                            | H12, H13         |
| 18       | 5.52 (m)          | 130.4   | H13, H11, H14                  |                  |
| 19       | 6.00 (m)          | 131.56  | H13                            | H11, H15         |
| 20       | 5.95 (m)          | 105.3   |                                 |                  |
| 21       | 5.52 (m)          | 132.1   | H14, H16                       | H13, H17         |
| 22       | 2.01 (m)          | 31.6    | H15, H17                       | H14, H18         |
| 23       | 1.30 (m)          | 31.0    | H16, H19                       | H15, H16, H19    |
| 24       | 1.28 (m)          | 21.6    | H16, H17                       | H16, H17, H19    |
| 25       | 0.85 (t 7.2)      | 13.8    | H18                            | H17              |

| Position | Minor $^1$H (J=Hz) | $^{13}$C | $^1$H-$^1$H COSY $^1$H ($^1$H to $^1$H) | HMBC $^1$H to $^{13}$C |
|----------|-------------------|---------|---------------------------------|------------------|
| 1        | 168.9             |         |                                 |                  |
| 2        | 93.4              |         |                                 |                  |
| 3        | 105.3             | 7-OH, H8, H10 |                                 |                  |
| 4        | 179.2             | H6      |                                 |                  |
| 5        | 153.1             | H6      |                                 |                  |
| 6        | 4.68 (d 1.4)      | 88.2    |                                 |                  |
| 7        | 4.90 (d 1.4)      | 190.5   | H8                              |                  |
| 8        | 10.11 (s)         |         |                                 |                  |
| 9        | 1.67 (d 13.2)     | 46.9    | 7-OH, 9-OH, 9-CH$_3$            |                  |
| 10       | 2.65 (dd 13.1, 1.3)|       |                                 |                  |
| 11       | 76.6              | H8, 9-OH, 9-CH$_3$, H10, H11     |                  |
| 12       | 4.17 (s)          |         |                                 |                  |
| 13       | 4.14 (s)          | 24.0    | H8, 9-OH, H10                  |                  |
| 14       | 3.42 (t 6.4)      | 87.0    | H11                            | H8, 9-OH, 9-CH$_3$, H11, H12 |
| 15       | 2.28 (t 6.4)      | 33.0    | H11                            | H12, H13         |
| 16       | 5.52 (m)          | 129.2   | H12                            | H12, H13         |
| 17       | 6.00 (m)          | 131.58  | H12                            | H12, H13         |
| 18       | 5.95 (m)          | 130.4   | H13                            | H15              |
| 19       | 5.52 (m)          | 132.1   | H14, H16                       | H13, H17         |
| 20       | 1.99 (m)          | 31.6    | H15, H17                       | H14, H18         |
| 21       | 1.30 (m)          | 31.0    | H16, H19                       | H15, H16, H19    |
| 22       | 1.28 (m)          | 21.6    | H19                            | H16, H17, H19    |
| 23       | 0.84 (t 7.2)      | 13.8    | H18                            | H17              |
Table S4. Enriched $^{13}$C NMR Data for Nostovaleralactone (3), obtained by feeding of $1^{-13}$C and $1,2^{-13}$C$_2$ AcONa, in DMSO-$d_6$.

| Position | Major $^{13}$C | Minor $^{13}$C | $1^{-13}$C AcONa | $1,2^{-13}$C$_2$ AcONa, $J_{CC}$ Hz | $^{13}$C enrichment %$^b$ |
|----------|---------------|----------------|------------------|---------------------------------|------------------|
| 1        | 168.6         | 168.9          | 2.7              | $1.3, J_{1,2} = 77$             | $-0.4$           |
| 2        | 93.6          | 93.4           | 0.3              | $2.3, J_{1,2} = 77$             | $0.4$            |
| 3        | 105.2         | 105.3          | 0.2              | $1.9, J_{3,7} = 57$             | $0.3$            |
| 4        | 179.6         | 179.2          | 0.2              | $-0.1$                          | $-0.4$           |
| 5        | 152.8         | 153.1          | 0.0              | 0.1                             | 0.4              |
| 6        | 88.5          | 88.2           | 0.0              | $-0.3$                          | 0.2              |
| 7        | 190.8         | 190.5          | 2.1              | $1.4, J_{3,7} = 57$             | $-0.3$           |
| 8        | 50.4          | 46.9           | 2.1              | $1.5, J_{6,9} = 36$             | 0.0              |
| 9        | 77.1          | 76.6           | $-0.3$           | $1.2, J_{6,9} = 36$             | $-0.2$           |
| 9-CH$_3$ | 23.8          | 24.0           | $-a$             | $-a$                            | $-a$             |
| 10       | 86.1          | 87.0           | 2.0              | $1.4, J_{10,11} = 40$           | $-0.1$           |
| 11       | 31.7          | 33.0           | $-0.1$           | $1.3, J_{10,11} = 40$           | $-0.2$           |
| 12       | 129.3         | 129.2          | 2.2              | $1.2, J_{12,13} = 71$           | $-0.2$           |
| 13       | 131.56        | 131.58         | 0.0              | $1.3, J_{12,13} = 71$           | 0.0              |
| 14       | 130.4         | 130.4          | 2.0              | $1.3, J_{14,15} = 71$           | $-0.2$           |
| 15       | 132.1         | 132.1          | $-0.1$           | $1.3, J_{14,15} = 71$           | $-0.1$           |
| 16       | 31.6          | 31.6           | 2.0              | $1.4, J_{16,17} = 34$           | $-0.1$           |
| 17       | 31.0          | 31.0           | $-0.1$           | $1.4, J_{16,17} = 34$           | 0.1              |
| 18       | 21.6          | 21.6           | 2.0              | $1.4, J_{18,19} = 34$           | $-0.2$           |
| 19       | 13.8          | 13.8           | $-0.1$           | $1.3, J_{18,19} = 34$           | $-0.2$           |

$^a$ 9-CH$_3$ is used to normalize all values in unlabeled and labeled 3. $^b$ Integrations were combined with signals of major and minor compounds. $^{13}$C enrichment % is obtained by $1.1\% \times \left[\text{intergrated intencity (labeled 3)} / \text{intergrated intencity (unlabeled 3)}\right] - 1.1\%$. $^{13}$ Bold values are $^{13}$C enriched.
Table S5. $^1$H and $^{13}$C NMR Data for Nostovalerolactone (3) in Pyridine-$d_5$.

| Position | $^1$H (J=Hz) | $^{13}$C | $^1$H-$^1$H COSY | HMBC |
|----------|--------------|---------|----------------|------|
| Major    |              |         | ($^1$H to $^1$H) | ($^1$H to $^{13}$C) |
| 1        |              | 173.3   | 96.2           | H6   |
| 2        |              | 96.2    | 107.1          | H8   |
| 3        |              | 107.1   | 182.1          | H6   |
| 4        |              | 182.1   | 154.5          | H6   |
| 5        |              | 154.5   | 4.84 (brs)     | H6   |
| 6        | 4.84 (brs)   | 90.6    | 5.37 (brs)     |      |
| 7        | 5.37 (brs)   |         | 2.46 (br)      | H8   |
| 8        | 2.46 (br)    | 48.6    | 3.234 (d 12.8) | 9-CH$_3$ |
|          | 3.234 (d 12.8)|       |                 |      |
| Minor    |              |         | ($^1$H to $^1$H) | ($^1$H to $^{13}$C) |
| 1        |              | 173.6   | 96.1           | H6   |
| 2        |              | 96.1    | 107.1          | H8   |
| 3        |              | 107.1   | 182.4          | H6   |
| 4        |              | 182.4   | 154.6          | H6   |
| 5        |              | 154.6   | 4.84 (brs)     | H6   |
| 6        | 4.84 (brs)   | 90.6    | 5.37 (brs)     |      |
| 7        | 5.37 (brs)   |         | 2.38 (d 12.8)  | H8   |
| 8        | 2.38 (d 12.8)| 52.5    | 3.238 (d 12.8) | 9-CH$_3$ |
|          | 3.238 (d 12.8)|       |                 |      |
| 9        |              | 76.6    | 24.21          | H8   |
| 9-CH$_3$ | 1.44 (s)     | 24.21   | 2.46 (br)      | H8   |
| 10       | 3.94 (m)     | 89.4    | H11            | H8   |
| 11       | 2.73 (m)     | 34.6    | H10, H12       | H12, H13 |
|          | 2.90 (m)     |         |                 |      |
| 12       |              | 130.1   | H11, H13       | H10, H11, H14 |
| 13       | 5.89 (m)     | 130.1   | H12, H14       | H11, H14, H15 |
| 14       | 6.21 (m)     | 130.1   | H13, H15       | H12, H13, H15, H16 |
| 15       | 5.53 (m)     | 130.1   | H14, H16       | H13, H14, H16, H17 |
| 16       | 1.97 (m)     | 32.9    | H15, H17       | H14, H15, H18 |
| 17       | 1.23 (m)     | 32.2    | H16            | H15, H16, H19 |
| 18       | 1.21 (m)     | 22.9    | H19            | H16, H19 |
| 19       | 0.82 (m)     | 14.5    | H18            | H17, H18 |
|          |              |         |                 |      |

nd; not determined.
Table S6. $^1$H and $^{13}$C NMR Data for 9-Dehydronostovalerolactone (4) in DMSO-$d_6$.

| Position | $^1$H ($J$=Hz) | $^{13}$C | $^1$H-$^1$H COSY (H to H) | HMBC (H to $^{13}$C) |
|----------|----------------|--------|---------------------------|----------------------|
| 1        | nd             |        |                          |                      |
| 2        | nd             |        |                          |                      |
| 3        | nd             |        |                          |                      |
| 4        | 4.53 (d 1.4)   | 180.7  | H6                       |                      |
| 5        | 5.73 (d 1.4)   | 86.0   | H6                       |                      |
| 6        | 195.3          |        | H8                       |                      |
| 7        | 6.09 (t 1.2)   | 119.4  | 9-CH$_3$, H10            |                      |
| 8        | 9.73 (d 1.4)   | 160.3  | 9-CH$_3$                 |                      |
| 9-CH$_3$ | 1.95 (s)       | 15.3   | H8                       |                      |
| 10       | 3.94 (dd 7.2, 5.3) | 74.8 |        |                      |
| 11       | 2.14 (m)       | 38.3   | H10, H12                 |                      |
| 12       | 5.51 (m)       | 128.1  | H11, H13                 |                      |
| 13       | 5.98 (m)       | 132.1  | H12, H11                 |                      |
| 14       | 5.96 (m)       | 130.3  | H15, H12; H16            |                      |
| 15       | 5.54 (m)       | 132.4  | H14, H16                 | H13, H17             |
| 16       | 2.01 (m)       | 31.8   | H15, H17                 | H14, H18             |
| 17       | 1.28 (m)       | 30.9   | H16, H18                 |                      |
| 18       | 1.24 (m)       | 21.6   | H19, H16, H17, H19       |                      |
| 19       | 0.85 (t 7.0)   | 13.8   | H18, H17                 |                      |

nd; not determined.
## Table S7

| Gene name (NPUN_RS...) | Annotation | Size (aa) | Characterized homolog | Identities/Positives (%) | Proposed/confirmed function |
|------------------------|------------|----------|-----------------------|--------------------------|-----------------------------|
| 10535                  | nitrogenase| 88       | -                     | -                        | unknown                     |
| 10530                  | hypothetical protein| 105 | -                     | -                        | unknown                     |
| 10525 (mvIА)          | response regulator transcription factor | 256 | BacR                  | 32/66                    | response regulator[19]       |
| 37470 (mvIB)          | phosphopantetheine-binding protein | 153 | -                     | -                        | unknown                     |
| 10515 (mvIC)          | 4'-phosphopantetheinyl transferase superfamily protein | 251 | PPT_IN                 | 43/60                    | sfp-type phosphopantetheinylase[15] |
| 10510 (mvID)          | 3-oxoacyl-ACP synthase III family protein | 344 | AbmA1                  | 46/56                    | ketoacyl-S-ACP synthase[19]  |
| 10505 (mvIE)          | HAD-IIIC family phosphatase | 638 | AbmA2                  | 41/62                    | glyceryl-S-ACP synthase[19]  |
| 10500 (mvIF)          | acyl carrier protein | 73  | AbmA3                  | 38/66                    | acyl carrier protein (ACP)[10] |
| 10495 (mvIG)          | dehydrogenase catalytic domain-containing protein | 253 | AbmA4                  | 42/64                    | Acetyltransferase[16]        |
| 10490 (mvIH)          | FAD-dependent oxoreductase | 406 | OxyE                   | 30/52                    | FAD-dependent hydroxylase[17] |
| 10485 (mvII)          | AMP-binding protein | 1284| FAAL domain: PuwC, ACAD domain: Bcd | 63/73 29/47 | fatty acyl-AMP ligase[18] butyryl-CoA dehydrogenase[19] |
| 10475 (mvIJ)          | type I polyketide synthase | 1850 | Mgi                    | 53/70                    | type I polyketide synthase[20] |
| 10470 (mvIK)          | type I polyketide synthase | 2016 | HctD                   | 51/67                    | type I polyketide synthase[21] |
| 10465 (mvIL)          | SDR family oxoreductase | 1872 | Mgi                    | 53/71                    | type I polyketide synthase[22] |
| 10460 (mvIM)          | type I polyketide synthase | 1080 | Curl                   | 52/67                    | type I polyketide synthase[22] |
| 10455 (mvIN)          | alpha/beta hydrolase | 366 | AbmA5                  | 40/59                    | lyase[16]                   |
| 10450 (mvIO)          | thioesterase | 264 | AbmT                   | 37/52                    | type II thioesterase (editing role)[16] |
| 10445 (mvIP)          | SRPBCC domain-containing protein | 149 | -                     | -                        | unknown                     |
| 10440 (mvIQ)          | NAD(P)/FAD-dependent oxoreductase | 514 | CHMO                   | 25/40                    | cyclohexanone monooxygenase[23] |
| 10435 (mvIR)          | hypothetical protein | 253 | -                     | -                        | unknown                     |
| 10430 (mvIS)          | hypothetical protein | 144 | -                     | -                        | unknown                     |
| 10425                  | IS630 family transposase | 143 | -                     | -                        | unknown                     |
| 10420 (ncIА)          | acyl carrier protein | 81  | ACP_BORBUS             | 35/65                    | acyl carrier protein[24]    |
| 10415 (ncIВ)          | beta-ketoacyl-ACP synthase II | 422 | RedR                  | 44/61                    | 3-oxoacyl-ACP-synthase II[25] |
| 10410 (ncIС)          | ketoacyl-ACP synthase III | 333 | RedP                  | 41/59                    | 3-oxoacyl-ACP synthase III[26] |
| 10405 (ncIД)          | aromatic amino acid lyase | 569 | FevV                   | 34/50                    | tyrosine ammonia lyase[26]   |
| 10400 (ncIЕ)          | long-chain fatty acid-CoA ligase | 512 | MoeA4                   | 39/55                    | acyl-CoA ligase[27]          |
| 10395 (ncIФ)          | thiamine pyrophosphate-binding protein | 586 | ScyA                   | 61/75                    | acylcoylin synthase[28]      |
| 10390 (ncIГ)          | 3-oxoacyl-ACP synthase | 386 | RedP                   | 26/44                    | 3-oxoacyl-ACP synthase III[22] |
Table S8. Relative quantification of nostoclide N1 and N2 and nostovalerolactone in WT *N. punctiforme* PCC 73102 and the AraC_PKS1 mutant. Shown are mean production levels from three independent biological replicates. The production level in the WT strain under HL/HC conditions was set to 1 (Reference).

|        | WT HL/HC (Ref) | WT CONV | AraC_PKS1 conv | AraC_PKS1 HL/HC |
|--------|----------------|---------|----------------|-----------------|
| N1     | 1              | n.d.    | 0.57 ±0.24     | 4.80 ±1.80      |
| N2     | 1              | n.d.    | 70.84 ±47.44   | 91.68 ±72.77    |
| Nvl    | 1              | n.d.    | 3.47 ±0.99     | 20.62 ±13.10    |

Table S9. PCR primer for creation of overexpression mutants

| Name       | Sequence                                                                 | Description                                                                 |
|------------|--------------------------------------------------------------------------|-----------------------------------------------------------------------------|
| J056_FW    | tcgatgataagctgtaacatgAAGTACCTTATAGATTATATTGCAGTCAA TAAA                  | 5' UTR of RS16340                                                          |
| J057_RV    | cgcaagggcccttgcgttcaagaattcCTTTTTCCTATGGATTGCTATATGAC                    | 5' UTR of RS16340                                                          |
| J072_FW    | cgcaagggcccttgcgttcaagaattcTGGTTTAAACTAGCACAAAATCCT                     | PKS1 regulator (RS10525)                                                   |
| J073_RV    | tagccaatccatatgagaaaaaggtGAGAGATGCTAGATGTATTCCG                         | PKS1 regulator (RS10525)                                                   |
| D021_S1    | AGGATGACGATGAGCGCATT                                                    | Sequencing pRL1049                                                         |
| D022_S2    | CGGCACCTCGGACAGAATTGCC                                                   | Sequencing pRL1049                                                         |

* lower case letters indicate homology regions mandatory for HiFi Builder homologues recombination
** upper case letters indicate target specific sequence
## Table S10. RT qPCR Primer

| Name  | Sequence                     | Description                  | Amplicon size [bp] |
|-------|------------------------------|------------------------------|--------------------|
| C3_FW | CGTGGTTGACTGGAGATGCT         | Npun_RS10475, PKS1           | 108                |
| C3_RV | AAGCCTCTCGTGCGGTTTTA         | Npun_RS10475, PKS1           | 108                |
| D132_FW | GCGGACTAGCTCATCAGACC     | Npun_RS15970, PKS2           | 118                |
| D133_RV | GTACCATCCCACAACCCAT      | Npun_RS15970, PKS2           | 118                |
| D136_FW | AGAACGGGCCTACTCTTTTT    | Npun_RS17005, PKS3           | 105                |
| D137_RV | TTTCTTGTTGATGAGCGG       | Npun_RS17005, PKS3           | 105                |
| D138_FW | ACTCTAGGGAATGTTCCA       | Npun_RS17455, PKS4           | 144                |
| D139_RV | CCTGAAGATGACGCAGAT        | Npun_RS17455, PKS4           | 144                |
| C21_FW | GGGGAATGGAAAGCATGGGAGA   | Npun_RS33540, PKS5           | 125                |
| C21_RV | ATTAACGGCCCTTTCTCTGTG     | Npun_RS33540, PKS5           | 125                |
| C8_FW | AGCGGCAACATATCCCGCAA     | Npun_RS15385, NRPS1          | 113                |
| C8_RV | ACGCCAACCCTGCTCTATTCC    | Npun_RS15385, NRPS1          | 113                |
| Cp_FW | CCTCAATACCAAGTACGAGCCT    | Npun_CR074, NRPS2            | 136                |
| Cp_RV | CTCAAGTGCAGATCTGAGTCTA   | Npun_CR074, NRPS2            | 136                |
| C10L_FW | GCCGGAATTGGGGAGGAGCACGA | Npun_RS16255, Ripp1a         | 111                |
| C10L_RV | TCCCAAACCACATCTGAGCA     | Npun_RS16255, Ripp1a         | 111                |
| D134_FW | CGAAAGAAGCAGTCATCAA      | Npun_RS16310, Ripp1b         | 164                |
| D135_RV | TTGCTTTTCTTGATCAC        | Npun_RS16310, Ripp1b         | 164                |
| C11a_FW | AGCAGACATCATAGCTCACCCT   | Npun_RS16795, Ripp3          | 130                |
| C11a_RV | GGGTGCAAGAAAGGGGCTACA    | Npun_RS16795, Ripp3          | 130                |
| D140_FW | GCCGATCCTCAATGCTGCCGC   | Npun_RS25510, Ripp4          | 103                |
| D141_RV | CGTGTGATCTAGACGCTGCT    | Npun_RS25510, Ripp4          | 103                |
| D150_FW | GCTAGGAGCTACTGAGAACA     | Npun_AF077, Ripp5            | 97                 |
| D151_RV | AGCAGACATCATACCTGAATCG   | Npun_AF077, Ripp5            | 97                 |
| apt_RT_FW | GAAATTGAGCGGTTTTTGA   | Npun_RS12350, Apt            | 136                |
| apt_RT_RV | GGCTAGTGAGCCTGATCTA   | Npun_RS12350, Apt            | 136                |
| NosA_RT_FW | GTTTGCCTCTTCTGCTGAAC | Npun_RS1097, NosA            | 109                |
| NosA_RT_RV | GCCGATGACAGGCGTTATCAA | Npun_RS1097, NosA            | 109                |
| J093_FW | ATGCCTACAAAACAGCTCAA     | Npun_RS38845, Mvd            | 96                 |
| J094_RV | GAACCTCTGGTCTCTCTCTAG    | Npun_RS38845, Mvd            | 96                 |
| mpB_RT3_FW | GCGGTTGAGATCGATCGCTA | Npun_RS018, RnpB            | 110                |
| mpB_RT3_RV | TCTGTGATCGATATCTGACG | Npun_RS018, RnpB            | 110                |
| J097_FW | GCATGAATTAGGTGAGATG      | Npun_RS10525, PKS1 Reg       | 130                |
| J098_RV | GTGATGATCGCTGATTA       | Npun_RS10525, PKS1 Reg       | 130                |
| J099_FW | CAGAAGAAAGGCAGTCTAGA    | Npun_RS16090, PKS2 Reg       | 117                |
| J0100_RV | CGCAGATCGCTCTGAAA      | Npun_RS16090, PKS2 Reg       | 117                |
Figure S1. Cyanobacterial BGCs containing AraC_PKS1 (RS10525) homologous genes. AraC regulator homologs are highlighted in red.
Figure S2. Influence of AraC_PKS1 overexpression on the transcription of NRPS, PKS and RiPP BGCs in N. punctiforme. Transcript levels of respective BGCs were determined by RT-qPCR. The relative changes in transcript levels in the AraC_PKS1 mutant are shown as log₂-fold changes compared to the WT transcript level. The range of non-significant changes between log₂ fold -1 and 1 is highlighted in gray. The pks1 BGC is highlighted in blue.
Figure S3. The UV/Vis spectrum of nostoclide N1 (1) in RP-HPLC (A) and in methanol (B).
Figure S4. The IR spectrum of nostoclide N1 (1).
Figure S5. The positive HR-ESI-MS spectrum of nostoclide N1 (1).

Figure S6. The negative HR-ESI-MS spectrum of nostoclide N1 (1).
Figure S7. $^1$H NMR spectrum of nostoclide N1 (1) in CD$_3$OD.

Figure S8. $^{13}$C NMR spectrum of nostoclide N1 (1) in CD$_3$OD.
Figure S9. $^1$H-$^1$H COSY spectrum of nostoclide N1 (1) in CD$_3$OD.

Figure S10. HSQC spectrum of nostoclide N1 (1) in CD$_3$OD.
**Figure S11.** HMBC spectrum of nostoclide N1 (1) in CD$_3$OD.

**Figure S12.** NOESY spectrum of nostoclide N1 (1) in CD$_3$OD.
Fig S13. The UV/Vis spectrum of nostoclide N2 (2) in RP-HPLC (A) and in methanol (B).
Figure S14. The IR spectrum of nostoclide N2 (2).
Figure S15. The positive HR-ESI-MS spectrum of nostoclide N2 (2).

Figure S16. The negative HR-ESI-MS spectrum of nostoclide N2 (2).
Figure S17. $^1$H NMR spectrum of nostoclide N2 in CD$_3$OD (2).

Figure S18. $^{13}$C NMR spectrum of nostoclide N2 in CD$_3$OD (2).
Figure S19. $^1$H-$^1$H COSY spectrum of nostoclide N2 in CD$_3$OD (2).

Figure S20. HSQC spectrum of nostoclide N2 in CD$_3$OD (2).
Figure S21. HMBC spectrum of nostoclide N2 in CD$_3$OD (2).

Figure S22. NOESY spectrum of nostoclide N2 in CD$_3$OD (2).
Figure S23. The UV/Vis spectrum of nostovalerolactone (3) in RP-HPLC (A) and in methanol (B).
**Figure S24.** The IR spectrum of nostovalerolactone (3).
**Figure S25.** The positive HR-ESI-MS spectrum of nostovalerolactone (3).

**Figure S26.** The negative HR-ESI-MS spectrum of nostovalerolactone (3).
Figure S27. $^1$H NMR spectrum of nostovalerolactone (3) in DMSO-$d_6$.

Figure S28. $^{13}$C NMR spectrum of nostovalerolactone (3) in DMSO-$d_6$. 
Figure S29. $^1$H-$^1$H COSY spectrum of nostovalerolactone (3) in DMSO-d$_6$.

Figure S30. HSQC spectrum of nostovalerolactone (3) in DMSO-d$_6$. 
Figure S31. HMBC spectrum of nostovalerolactone (3) in DMSO-d6.
Figure S32. $^1$H NMR spectrum of nostovalerolactone (3) in pyridine-d$_5$.

Figure S33. $^{13}$C NMR spectrum of nostovalerolactone (3) in pyridine-d$_5$. 
Figure S34. $^1$H–$^1$H COSY spectrum of nostovalerolactone (3) in pyridine-$d_5$.

Figure S35. HSQC spectrum of nostovalerolactone (3) in pyridine-$d_5$.  
Figure S36. HMBC spectrum of nostovalerolactone (3) in pyridine-d$_5$.

Figure S37. NOESY spectrum of nostovalerolactone (3) in pyridine-d$_5$. 
Figure S38. The positive HR-ESI-MS spectrum of 9-dehydronostovalerolactone (4).

Figure S39. The negative HR-ESI-MS spectrum of 9-dehydronostovalerolactone (4).
Figure S40. $^1$H NMR spectrum of 9-dehydronostovalerolactone (4) in DMSO-$d_6$.

Figure S41. $^{13}$C NMR spectrum of 9-dehydronostovalerolactone (4) in DMSO-$d_6$. 
Figure S42. $^1$H-1H COSY spectrum of 9-dehydronostovalerolactone (4) in DMSO-d$_6$.

Figure S43. HSQC spectrum of 9-dehydronostovalerolactone (4) in DMSO-d$_6$. 
Figure S44. HMBC spectrum of 9-dehydronostovalerolactone (4) in DMSO-d$_6$.

Figure S45. Selected $^1$H-$^1$H COSY (bold line) and HMBC (arrow) correlations of 9-dehydronostovalerolactone (4).
Figure S46. Reversed-phase HPLC profiles of supernatants from *N. punctiforme* PCC 73102 AraC_PKS1 with feeding $^{13}$C labeling substrates. All methanol eluted fractions from XAD4 were dissolved in 4 ml methanol (per 100 mL culture volume) and analyzed 20 µL. Two independent $^{13}$C$_6$ Glucose feeding experiments showed less nostovalerolactone (3) production clearly.

Figure S47. $^{13}$C NMR spectra of nostovalerolactone (3) obtained by four $^{13}$C labeled substrates feeding experiments. All purified nostovalerolactone (3) were measured in DMSO-d$_6$. 
Figure S48. Expanded $^{13}$C NMR spectra of nostovalerolactone (3) obtained by four $^{13}$C labeled substrates feeding experiments. A) Observed enriched carbons from labeled acetate feeding experiments in $^{13}$C NMR spectra of nostovalerolactone (3). B) and C) expanded $^{13}$C NMR spectra of nostovalerolactone (3), obtained by four $^{13}$C labeled substrates feeding experiments, in DMSO-d$_6$ with carbon numbers (Major isomer; black number, minor isomer; gray number).
Figure S49. Expanded $^{13}$C NMR spectra of nostovalerolactone (3) obtained by four $^{13}$C labeled substrates feeding experiments. A), observed enriched carbons from labeled acetate feeding experiments in $^{13}$C NMR spectra of nostovalerolactone (3). B) and C) expanded $^{13}$C NMR spectra of nostovalerolactone (3), obtained by four $^{13}$C labeled substrates feeding experiments, in DMSO-d$_6$ with carbon numbers (Major isomer; black number, minor isomer; gray number).
Figure S50. Expanded $^{13}$C NMR spectra of nostovalerolactone (3) obtained by four $^{13}$C labeled substrates feeding experiments. A) Observed enriched carbons from labeled acetate feeding experiments in $^{13}$C NMR spectra of nostovalerolactone (3). B) expanded $^{13}$C NMR spectra of nostovalerolactone (3), obtained by four $^{13}$C labeled substrates feeding experiments, in DMSO-$d_6$ with carbon numbers (Major isomer; black number, minor isomer; gray number).
Figure S51. Process of structure elucidation of nostovalerolactone (3). A) As the biosynthetic pathway suggested a possible tetronate structure for 3, 13C NMR chemical shifts in DMSO-d6 (indicated in the structure) were compared between 3 and agglomerin A. However, the molecular formula obtained by HR-MS indicated an additional C=O group in 3. B) The addition of C=O resulted in δ-valerolactone instead of tetronate, but the observed carbon δ 93.6 ppm excludes carbonyl carbon. C) 3 long range HMBC correlations indicated a fusion of the δ-valerolactone and oxepane-like units via a hemiacetal carbon δ 93.6 and did not support 10-OH, which has never been observed as hydroxy proton by NMR. D) Analysis of 13C acetate labeled nostovalerolactone resulted in the structure of nostovalerolactone.

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Figure S52. Comparison of BGC transcription in WT and mutant strains of *N. punctiforme* grown under different cultivation conditions. The heatmaps visualize the log₂ fold transcriptional changes of all genes of the relevant BGCs in comparison to the WT strain.
Figure S53. The AraC-PKS1 gene is activated by an unknown chemical mediator present in the HL/HC supernatant but not by arabinose. A) Transcriptional changes of the AraC_PKS1 gene RS10525 (AraC_Reg) and pks1 BGC (representative gene RS10475) upon arabinose addition. B) Transcriptional changes of AraC_Reg and pks1 BGC upon addition of individual HPLC fractions from the supernatant of a WT culture grown under HLHC conditions. Only fraction F05 is able to activate the AraC-PKS1 gene. C) HPLC profiles of the supernatant of a WT culture grown under HLHC cultivation conditions. Active fraction F05 is highlighted in pink. Known metabolite peaks are labeled. F05 does not contain any previously known metabolite. Structural elucidation of the active compounds will require further upscaling. (Mvd: microviridin, Nos1052: nostopeptolides 1052, NosA: nostopeptolide A, Apt: nostamide A, N1, N2: nostoclides, Nvl, nostovalerolactone).
Figure S54. Influence of nostoclides on photosynthetic organisms. A) Influence of nostoclides on the morphology of *N. punctiforme*. Fluorescence micrographs show conventionally grown *N. punctiforme* WT, AraC_PKS1 and PKS1 reporter strains. Both mutant strains show a higher number of broken cells. Labels: V, vegetative cells; H, heterocysts, B, broken cells. B) Influence of nostoclides on the growth of *N. punctiforme* or *Anabaena sp.* 7120. Allelopathy assays do not show an influence of nostoclides N1 or N2 on the growth of *N. punctiforme* or *Anabaena sp.* 7120. Cultures were grown for 14 d. Streptomycin was used as an antibiotic control and 60% methanol as blank. C) Influence of nostoclides on the growth and in vivo absorption spectra of *C. reinhardtii*. The column chart on the left side shows the cell density of *C. reinhardtii* 24 h after addition of 10 µM nostoclide N1 or N2. The initial cell density of 2 x 10^6 cells ml^-1 is indicated with a dashed line. No significant influence on the growth was detectable. Ethanol equivalent to the volume used for treatments and a sample without any treatment were used as controls. The graph on the right side shows in vivo absorption spectra of the nostoclide-treated cell suspensions. D) Photosystem II quantum yield (YPSII) of *N. punctiforme* and *C. reinhardtii* 24 h after addition of 10 µM nostoclide N1 or N2 measured after adaptation to darkness for 5 min (D) and after subsequent actinic illumination with 700 µmol photons m^-2 s^-1 for 3 min (L1) followed by 2000 µmol photons m^-2 s^-1 for another 3 min (L2). Ethanol equivalent to the volume used for treatments and a sample without any treatment were used as controls. No significant differences were detected between treatments vs. the respective ethanol control groups (i.e. all p values were > 0.05). In B and C, the error bars indicate standard deviations, and the statistical analyses were performed using one-way ANOVA followed by Tukey’s multiple comparisons post hoc test. Compared to an untreated control sample, ethanol did not significantly affect the result (not shown).
Figure S55. Annotation of nostopeptolide 1052 (Nos1052), nostopeptolide A (NosA), nostamide A (Apt) based on comparison to authentic standards. Annotation is shown for supernatant fractions of the WT *N. punctiforme* and the AraC_PKS1 mutant as an example. The entire spectra are shown in Figure 1E.

**Dataset S1 (separate file).**
RNA Sequencing data of *N. punctiforme* WT and AraC_PKS1 mutant.

**Dataset S2 (separate file).**
RNA Sequencing data of *N. punctiforme* WT treated with nostoclides or nostovalerolactone.
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