Letter

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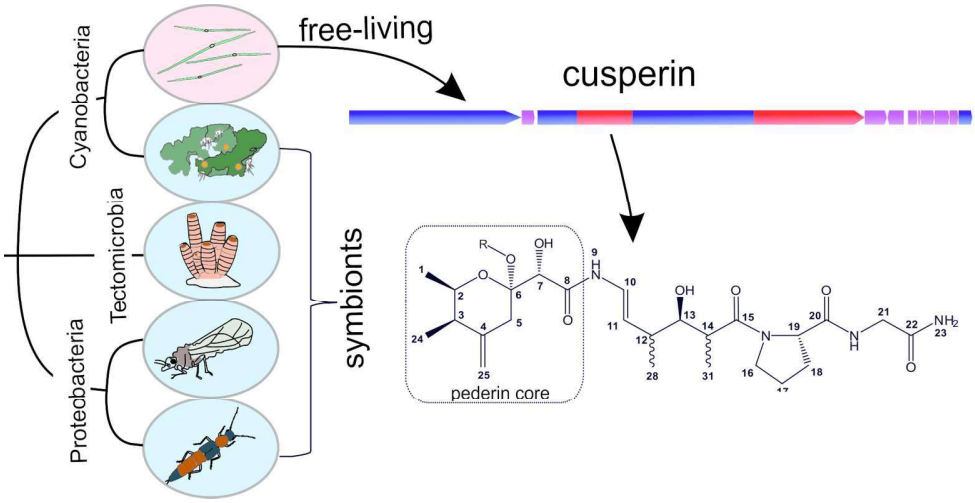

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Discovery of a pederin family compound in a non-symbiotic bloom-forming cyanobacterium

Andreja Kust#[†, ‡, §], Jan Mareš#[†, ‡, §], Jouni Jokela[‖], Petra Urajová[†], Jan Hájek[†, §], Kumar Saurav[†], Kateřina Voráčová[†], David P. Fewer[‖], Esa Haapaniemi[⊥], Perttu Permi[⊥, ¶], Klára Řeháková[‡], Kaarina Sivonen[‖] and Pavel Hrouzek*[†, §]

[†]*Institute of Microbiology CAS, Center Algatech, Třeboň, Czech Republic;*

[‡]*The Czech Academy of Sciences, Biology Centre, Institute of Hydrobiology, České Budějovice, Czech Republic;*

[§]*University of South Bohemia, Faculty of Science, České Budějovice, Czech Republic;*

[‖]*Department of Microbiology, University of Helsinki, FI-00014 Helsinki, Finland;*

[⊥]*Department of Chemistry, University of Jyväskylä, FI-40010 Jyväskylä, Finland*

[¶]*Department of Biology and Environmental Science, Nanoscience Center, University of Jyväskylä, FI-40010 Jyväskylä, Finland*

[#]These authors contributed equally to this work

Correspondence: P Hrouzek, Institute of Microbiology CAS, Center Algatech, Novohradská 237, Opatovický Mlýn, 379 81 Třeboň, Czech Republic, Email: hrouzekp@gmail.com, Tel.: +420-384-340-470, Fax: +420-384-340-415.

Abstract

The pederin family includes a number of bioactive compounds isolated from symbiotic organisms of diverse evolutionary origin. Pederin is linked to beetle-induced dermatitis in humans and pederin family members possess potent antitumor activity caused by selective inhibition of the eukaryotic ribosome. Their biosynthesis is accomplished by a polyketide/non-ribosomal peptide synthetase machinery employing an unusual trans-acyltransferase mechanism. Here we report a novel pederin type compound, cusperin, from the free-living cyanobacterium *Cuspidothrix issatschenkoi* (earlier *Aphanizomenon*). The chemical structure of cusperin is similar to that of nosperin recently isolated from the lichen cyanobiont *Nostoc* sharing the tehrahydropyran moiety and major part of the linear backbone. However, the cusperin molecule is extended by a glycine residue and lacks one hydroxyl substituent. Pederins were previously thought to be exclusive to symbiotic relationships. However, *C. issatschenkoi* is a non-symbiotic planktonic organism and a frequent component of toxic water blooms. Cusperin is devoid of the cytotoxic activity reported for other pederin family members. Hence, our findings raise questions about the role of pederin analogues in cyanobacteria and broaden the knowledge of ecological distribution of this group of polyketides.

Pederin is a toxin containing two tetrahydropyran cores that causes dermatitis[1]. Pederin was firstly reported from the beetle *Paederus fuscipes* and widely believed to be produced by eukaryotes[2,3]. However, the discovery of the pederin biosynthetic gene cluster demonstrated that bacterial symbionts of insects, marine invertebrates, and lichens are the true producers of these compounds[4]. Biosynthesis of pederin and its homologues is accomplished by hybrid polyketide/nonribosomal peptide synthases (PKS/NRPS) employing an unusual trans-acyltransferase (AT) PKS mechanism[5], in which the PKS enzymatic modules utilize a common AT encoded in a separate gene. The biosynthesis of all pederin family compounds

seems to share the first several steps forming the typical tetrahydropyran moiety attached to an acyl chain with a glycine residue[6].

Metagenomic sequencing of symbiotic consortia has recently uncovered a large and highly specific repertoire of natural products, particularly in bacterial symbionts of marine sponges[7,8]. Pederins are currently also believed to be specifically associated with prokaryote-eukaryote symbioses as they have been reported exclusively from symbiotic associations[6,7,9,10,11]. However, a recent discovery of a pederin analogue in a purportedly free-living strain of the proteobacterium *Labrenzia* sp. PHM005 isolated from marine sediment challenged this hypothesis[12].

The potential of free-living organisms to produce pederins is further corroborated with our report of a novel pederin analogue, cusperin, identified for the first time in a non-symbiotic cyanobacterium, *Cuspidothrix issatschenkoi* (Usačev) Rajaniemi et al. *Cuspidothrix* (earlier *Aphanizomenon*) is a frequent bloom-forming species in freshwater bodies[13], which belongs to the lineage of typical freshwater planktonic heterocytous cyanobacteria, clearly distant from all groups of the typical pederin producers (Figure 1).

[Figure 1]

Inspection of a draft genome of *C. issatschenkoi* strain CHARLIE-1 led to the identification of a previously unreported PKS/NRPS gene cluster, exhibiting >70% similarity with the nosperin biosynthetic gene cluster[6]. The core region of this biosynthetic gene cluster is almost identical to that reported for pederin compounds (Figure 2a, Supplementary Table 1, 2). The putative biosynthetic gene cluster (~54 kb) consisted of 11 protein coding ORFs: two genes encoding multidomain PKS/NRPS proteins, eight accessory genes, and a single gene encoding a peculiar AT in *trans* position (Figure 2a). Further evidence suggesting the presence of a *trans*-AT PKS system in the cusperin synthetase is the absence of AT-domains

in the deduced PKS proteins (Figure 2b). Detailed analysis of the individual components of the gene cluster are provided in Supporting Information.

[Figure 2]

Analysis of methanol extracts of *C. issatschenkoi* CHARLIE-1 led to the identification of two putative products of the PKS/NRPS gene cluster. Their chemical structures were elucidated using a combination of high-performance liquid chromatography connected to high resolution mass spectrometer with electrospray ionization (HPLC-ESI-HRMS/MS) and nuclear magnetic resonance (NMR) spectroscopy (Figure 3, 4 Supplementary Figures 1–6, Supplementary Tables 3, 4). A prominent chromatographic peak was obtained by HPLC-MS analysis of *Cuspidothrix* extract containing a compound detected at *m/z*=561.2896 $[M+Na]^+$ with calculated elemental composition of $C_{26}H_{42}N_4O_8+Na^+$ (Δ 0.2 ppm) corresponding to cusperin A (Figure 3). Its MS/MS exhibited attributes analogous to those reported for nosperin, e.g. the loss of methanol (producing ion a) and the loss of a whole substituted tetrahydropyran moiety (producing ion b) identical to those of nosperin[6] (Figure 3b).

[Figure 3]

Data from the analysis of ^1H, ^1H-^1H NOESY, ^{13}C-HSQC, ^{13}C-HMBC and ^{15}N-HSQC spectra (Supplementary Figures 2–6) of cusperin showed high similarity to nosperin NMR data[6] which proved that their general structure was the same (Supplementary Table 4). Compared to nosperin, cusperin lacked C-28 hydroxyl, C-20 hydroxyl was changed to carbonyl and next to it there was an extra amide group (C-21-NH). Due to the lack of the hydroxyl, C-28 showed a methyl signal (δC 19.8, δH 1.00) and this difference affected the nearby shift values (Supplementary Table 4). Four carbonyl signals were found (Supplementary Figure 3), one more than in nosperin as one hydroxyl of nosperin is replaced with carbonyl in cusperin. Cusperin NH-9 signal (d, δH 8.40) matched with nosperin (d, δH 8.57). The extra C-21-NH

signal (dd, δH 7.11) of cusperin was broad but the signals dd structure could be seen which was characteristic for glycine in peptide structures. ^{15}N-HSQC spectrum showed the δH 7.11 proton connection to a nitrogen atom (Supplementary Figure 4). The most important ^{13}C-HMBC and ^{1}H-^{1}H NOESY correlations are shown in Supplementary Figure 3 and 5.

The MS/MS analysis further detected the presence of a second cusperin variant with *m/z* at 547.2740 $[M+Na]^+$ and calculated elemental composition of $C_{25}H_{40}N_4O_8+Na^+$ (Δ –0.3 ppm). While cusperin A provided ions corresponding to $[M-CH_2OH+H]^+$ in both MS and MS/MS (*m/z* 507. 2812, Δ 0.3ppm), cusperin B provided only $[M-H_2O+H]^+$ fragment (Supplementary Figure 6). This corresponds to the losses of a methoxy- and a hydroxy- group from the molecular ion of cusperin A and B, respectively. Based on the combination of MS and NMR data we concluded that cusperin A presents the methylacetal variant (bearing methoxy group on C-6) and cusperin B presents the hemiacetal variant (bearing hydroxy group on C-6). Purification (see Supporting Information and Supplementary Table 5) yielded 0.3 mg of cusperin A and B in molar ratio 70/30 (Supplementary Figure 6).

The origin of the structural differences between cusperin and nosperin as well as the occurrence of cusperin A and B variants were explained by bioinformatic prediction of cusperin biosynthesis (Figure 2b). First, the presence of a terminal glycine in cusperin instead of a reduced biketide was consistent with an additional NRPS module encoded in *cusC* containing an A-domain with a predicted substrate specificity for glycine (Supplementary Table 2). The second difference observed was the lack of the C-12 hydroxyl in cusperin, explained by the lack of cytochrome-450 like protein responsible for the hydroxylation step in nosperin[6]. Four nitrogen atoms were predicted in the cusperin scaffold based on the presence of three NRPS modules each incorporating a single amino acid and an asparagine synthetase responsible for the final transamination step (Figure 2b). The number of nitrogen atoms was

further confirmed by ^{15}N isotopic substitution (*m/z* at 565.2685, Δ –0.9 ppm). The predicted product of *cusF* was an *O*-methyltransferase, likely involved in facultative *O*-methylation of the hydroxy group at C6, which results in formation of the observed cusperin methylacetal/hemiacetal variants (cusperin A and B). The individual predicted biosynthetic steps are explained in detail in Supporting Information.

[Figure 4]

The cusperin gene cluster exhibited high similarity in the arrangement of the first five PKS/NRPS modules to those observed in several pederin members (pederin, theopederins, mycalamides, onnamides) which allowed us to infer the absolute configuration at chiral carbons C2, C3, C6, and C7 (Figure 4b). Besides, the arrangement of the first 10 modules was consistent with the nosperin gene cluster, further suggesting the configuration of C10, C11 and C13 are identical to nosperin. Comparison of the NMR coupling constants and shift values (Supplementary Table 4) further indicates that the stereochemistry is likely identical between these two compounds (Figure 4b). Bioinformatics analysis of the specific sequence motifs of the KR domains have again confirmed D-OH configuration at C2 and C13 based on the presence of a conserved aspartate residue recently demonstrated to play a key role in stereospecificity of the KR domain[14,15,16] (Supplementary Table 6). Finally, the lack of the epimerization domain in the NRPS module 10 supported the incorporation of an L-Pro residue as per bioinformatics analysis of pathway A-domains. Equivalent to nosperin, C12 and C14 remained without stereochemical assignments.

The strong cytotoxic activity of pederin family members was found due to their specific binding to the large ribosomal subunit[17]. Recent structure-activity studies defined the key features of pederin analogues involved in their action[18]. Firstly, the presence of alkoxy- or hydroxy- substitution at C–10 facilitates the molecule rotation and subsequent binding, while

the lack of any substituent at this position leads to diminished activity[18]. Additionally, the presence of methoxy- group at C–13 is favoured over the hydroxyl group. In comparison to other natural pederin analogues, cusperin A and B exhibited only a weak cytotoxic activity (Supplementary Table 7). The cusperin molecule features both traits, which do not favour the interaction with ribosome as C–10 is not functionalized and C–13 bears the less efficient –OH group.

The bioactivity of cusperin demonstrates that not all natural pederin analogues exert high cytotoxic activity and thus they do not act generally and exclusively as ribosome poisons. Interestingly, high production levels of cusperin A and B were recorded in the *Cuspidothrix* culture (up to 2.5 mg/g of dry weight). With regard to the high content and easy isolation protocol, discovery of cusperin opens up an opportunity to address the ecological role of this type of compounds.

Cuspidothrix, a bloom-forming cyanobacterium which has the potential to produce compounds that are a threat to human health[19], has been characterized as the first free-living cyanobacterium producing a pederin-type compound, cusperin. Our findings contradict the recently suggested restricted occurrence of pederin family members in symbiotic bacteria, and dispute their general role directly associated with specific symbioses between eukaryotes and bacteria[6]. Cusperin production may however play a role in other microbe-microbe relationships occuring in freshwater blooms that are currently not well characterized. The selective advantage for the production of a pederin family compound by a non-symbiotic organism remains unknown and further studies are needed to decipher its possible ecological significance; especially as cytotoxic activity is weak compared to other pederins.

METHODS

Cultivation of the strain. *C. issatschenkoi* CHARLIE-1 was isolated from a water bloom sample collected from fishpond Papež near Dobříš, Czech Republic. A monoclonal culture was established from a single filament and maintained in the culture collection of the Biology Centre of CAS, Institute of Hydrobiology in 250 ml Erlenmeyer flasks with WC medium[20] at 21°C and a light intensity of 50 μmol m^{-2} s^{-1} (16:8 L: D cycle). For isolation procedure the batch culture of the strain was grown in 10 l Erlenmeyer flasks using BG-11 medium bubbled with 1.5% CO_2 enriched air at 25°C with an illumination of 100 μmol m^{-2} s^{-1}. The biomass for isolation of ^{15}N isotopically substituted cusperin was cultivated under the same cultivation conditions in BG-11 medium supplemented with $Na^{15}NO_3$ (Sigma Aldrich, Cat. No. 364606) and the culture was re-inoculated twice each time after 20 days of cultivation period. Strain identification was performed based on morphological characters[21] and position in a phylogenetic tree based on the 16S rRNA gene.

Phylogenetic analysis. The 16S rRNA gene sequences of pederin family compound producers available through NCBI and the cusperin producer *C. issatschenkoi* CHARLIE-1 were aligned with a set of closest BLAST hits, representatives of the corresponding bacterial phyla, and a representative set of cyanobacterial sequences. A phylogenetic tree was constructed using the Bayesian Inference. See Supporting Information for details.

Genomic and bioinformatic analysis. Single filaments of *C. issatschenkoi* CHARLIE-1 were isolated using the glass capillary technique and prepared for whole genome sequencing as described previously[22]. The filaments were utilized as a template for whole-genome amplification by multiple-displacement amplification (MDA) using Repli-G Mini Kit (Qiagen). Sixteen MDA products that passed the quality check were pooled and sent for commercial *de novo* genome sequencing (EMBL Genomics Core Facility, Heidelberg, Germany) using Illumina MiSeq Pair-End library with 250 bp reads, 350 bp average insert

length, and 1.4 Gbp data yield. The data from Illumina were assembled using CLC Bio Genomics Workbench v. 10.5 (Qiagen). The genomic assembly is available under NCBI accession number PGEM00000000.1 and the cusperin biosynthetic gene cluster is available under accession number MG518226.

Genomic scaffolds were investigated using BLASTp in Geneious Pro R10 (Biomatters) software package to identify putative PKS/NRPS gene clusters, employing cyanobacterial A-domains and KS-domains as queries. In target genomic regions, open reading frames were predicted (Glimmer 3)[23], the deduced proteins were functionally annotated (BLASTp and CDD searches), and in the putative PKS/NRPS proteins individual enzymatic domains were identified (antiSMASH 4.0)[24]. The substrate specificities of KS domains in the *trans*-AT PKS modules were predicted based on similarity to the closest functionally characterized hits in the BLASTp analysis (Supplementary Table 2), which were unequivocally represented by KS domains from the nosperin pathway (analysed by phylogenetics in a previous study[6]). The predicted specificity of NRPS adenylation domains was inferred using a combination of tools implemented in antiSMASH 4.0 (Stachelhaus code, NRPS Predictor3, pHMM search). A biosynthetic scheme was constructed based on the functional annotation of the deduced PKS/NRPS domains and accessory enzymes, and by comparing to known biosynthesis pathways of pederin-family compounds. Stereochemical assignment of cusperin was assessed based on NMR data, specific KR sequence motifs[14,15,16] and overall arangement of NRPS/PKS modules[14].

HPLC-ESI-HRMS. Crude extract of *C. issatschenkoi* CHARLIE-1 was analyzed using high-performance liquid chromatograph (Dionex UltiMate 3000 UHPLC+ (Thermo Scientific, Sunnyvale, CA, USA) connected to Bruker Impact HD high resolution mass spectrometer (Bruker, Billerica, Massachusetts, USA) with electrospray ionization in positive mode.

Separation was performed on RP-C18 column (Phenomenex Kinetex, 2.6 μm; 4.6 × 150, Torrance, CA, USA) using H_2O (A) and acetonitrile (B) (both containing 0.1% HCOOH) as a mobile phase at a flow rate of 0.5 ml min^{-1}. The linear gradient was as follows: A/B 85/15 (0 min), 85/15 (in 1 min), 0/100 (in 19 min), 0/100 (in 25 min), and 85/15 (in 30 min). The following settings of mass spectrometer were used: drying temperature, 200 °C; drying gas flow, 12 l min^{-1}; nebulizer gas pressure, 3 bar; capillary voltage, 3.8 kV; end plate offset, 500 V. Spectra were collected in the range *m/z* 20–2000 with a spectra rate of 2 Hz. The automated fragmentation was used in the method with the precursor ion selection 20–2000 *m/z* for with the isolation window width of 1Da. The ramping of the collision energy was based on the molecular mass: 20 eV at 400 *m/z* and 70 eV at 1000 *m/z* for analysis. The mass spectrometer was calibrated with sodium formate clusters at the beginning of each analysis. Further the lock mass calibration was applied during the analysis using hexakis (1*H*,1*H*,2*H*-perfluoroethoxy) phosphazene (ES-TOF m/z 622.02896, 97%) purchased from ABCR GmbH & Co., KG, Germany. Quantification of cusperin A and B in the *C. issatschenkoi* CHARLIE-1 extract was performed using method described above with purified compounds as external standard (concentrations: 2.5, 5, 10, 25, 50, 100 μg.ml^{-1} were used to establish the calibration curve). See Supporting Information for details.

Extraction, purification and quantification. Freeze-dried biomass of *C. issatschenkoi* CHARLIE-1 was extracted twice with 400 ml methanol/water (40/60, v/v) using bath sonicator, resulting supernatants were combined and partially evaporated on rotary evaporator and remaining solvent was diluted by distilled water so that the final MeOH concentration did not exceed 5%. Further the sample was loaded on DCS 18 SPE cartridge and retained compounds were eluted with MeOH. Eluent was evaporated till dryness and reconstituted in 10 ml of MeOH prior the high-performance liquid chromatography (HPLC) purification. The

compounds were purified using three consecutive preparative HPLC separation steps (for detailed information on the purification procedure see the Supporting Information).

Nuclear magnetic resonance spectroscopy. All NMR spectra were collected using a Bruker Avance III HD 800 MHz NMR spectrometer, equipped with cryogenically cooled TCI ^{1}H, ^{13}C, ^{15}N triple resonance probehead. Data were collected at 25 °C. For the assignment of cusperin ^{1}H, ^{13}C and ^{15}N resonances, ^{1}H preset experiment along with two-dimensional total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) experiments as well as heteronuclear single quantum coherence (^{13}C HSQC and ^{15}N HSQC) and heteronuclear multiple bond correlation (^{13}C HMBC) experiments were employed. For details of the NMR measurement please see Supporting Information.

Cytotoxicity testing. In order to test the antiproliferative and cytotoxic activity usually reported for pederin family members, cusperin bioactivity was tested on human cervical cancer cells (HeLa). For cell maintenance and plating before the experiment see Supporting Information. Serial dilutions (in the range from 0.5 – 20 μM) of cusperin A and B variants were added as technical triplicates so the concentration of the vehicle (MeOH) did not exceed 1%. The viability was determined after 72 h of exposure. To assess cell viability we used three end-point methods: the ATP content measured using the CellTiter-Glo® Luminescent Viability Assay (Promega, G7570), MTT assay, and determination of cell counts (384-well plates) of treated and control cells at 72 h by optical microscopy. The output data represents results of three independent experiments. For detailed method description see Supporting Information.

Accession Codes. PGEM00000000.1 and MG518226.

Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. The Supporting Information file contains additional details on methods and results concerning the phylogenetic analysis, genomic and bioinformatic analysis, HPLC-MS/MS, and cytotoxic tests, Supplementary Figures 1, 2, 3, 4, 5 and 6; Supplementary Tables 1, 2, 3, 4, 5, 6 and 7, and their captions.

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Figure 1. Phylogenetic position of *Cuspidothrix issatschenkoi* CHARLIE-1 among the producers of pederin family compounds. The unrooted tree was inferred from 16S rRNA gene data using Bayesian Inference (BI). BI posterior probabilities are indicated near nodes. The major bacterial lineages containing producers of pederin analogues are color-shaded. The strains producing pederin family compounds are printed in red, representative structures of the products are shown for each group.

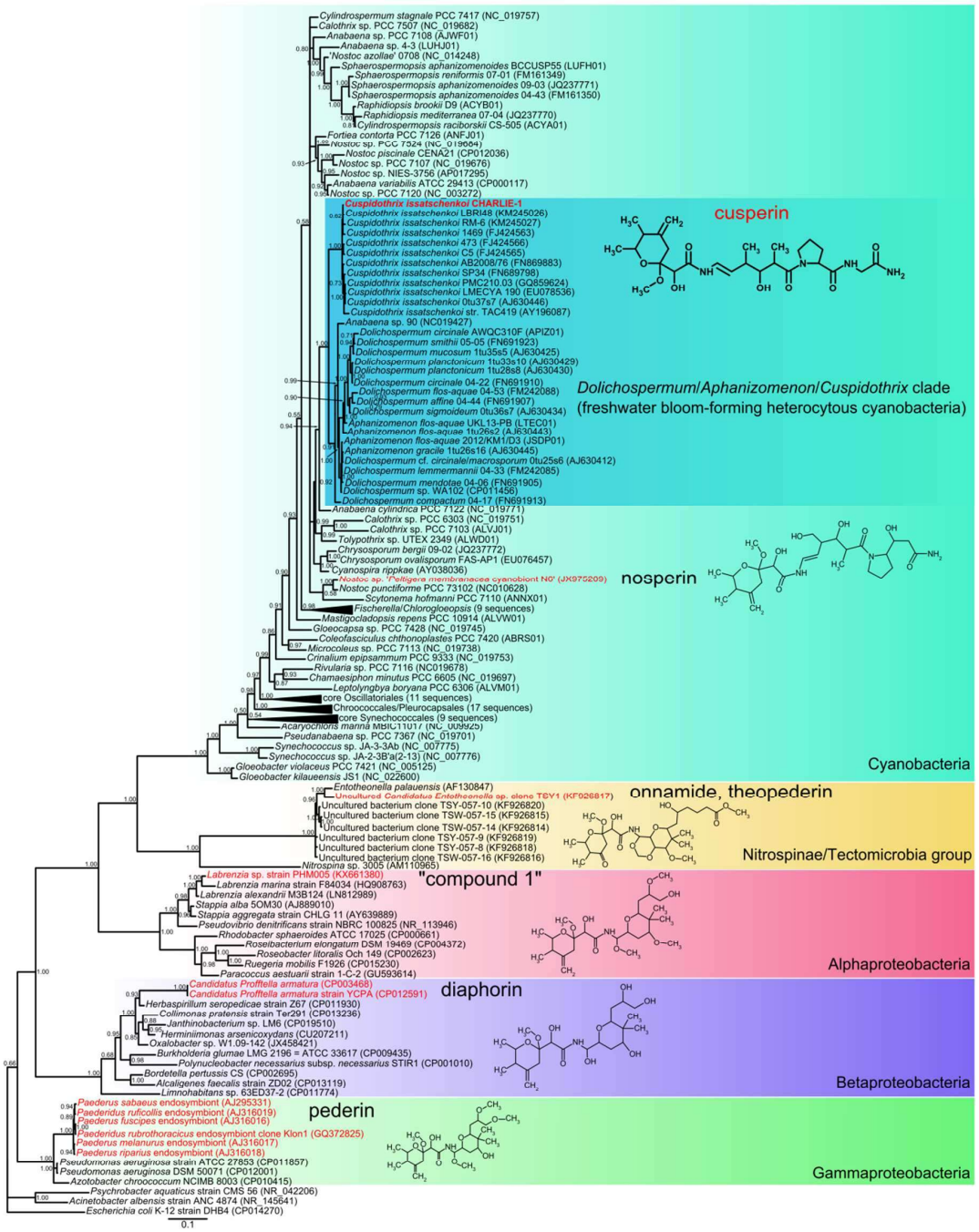


Figure 2. The predicted biosynthetic pathway of cusperin. a) Gene arrangement of the proposed cusperin biosynthetic gene cluster and comparison to nosperin biosynthetic gene cluster. Homologous regions are indicated by grey shading. b) Proposed biosynthesis of

cusperin A and B is co-linear with the arrangement of modules in CusA and CusC. Part of the pathway orthologous to pederin, onnamides, theopederins, and diaphorin is indicated by a dashed box. The structure of nosperin is shown for comparison, differences from cusperin A are highlighted in red colour. A, adenylation domain; ACP, acyl carrier protein; C, condensation domain; CR, crotonase (enoyl-CoA hydratase); DH, dehydratase; GNAT, GCN5-related N-acetyltransferase; MT, methyltransferase; NRPS, non-ribosomal peptide synthetase; KR, ketoreductase; KS, ketosynthase; PCP, peptidyl carrier protein; PKS, polyketide synthase; TE, thioesterase.

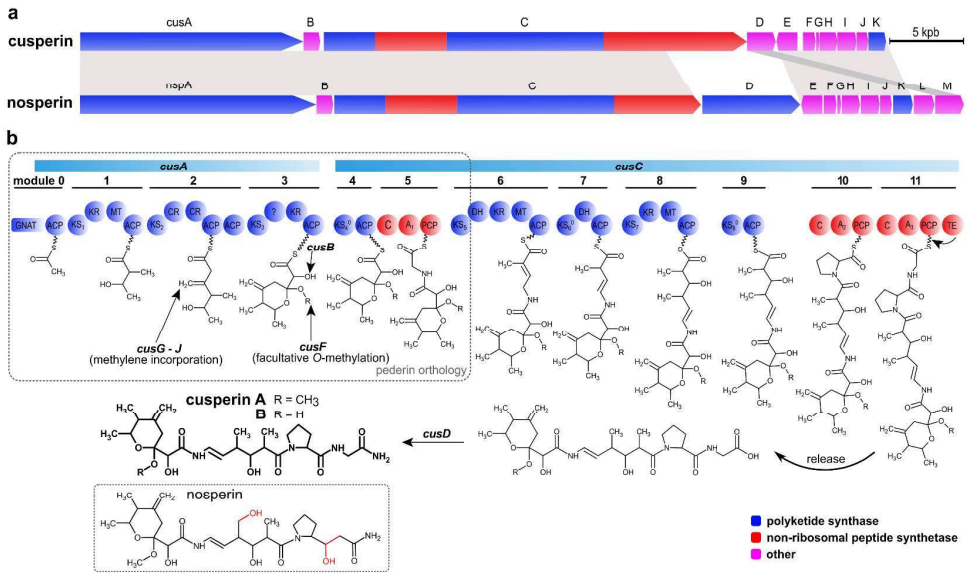


Figure 3. Structural analysis of cusperin A. a) HPLC-HRMS/MS base peak chromatogram of *Cuspidothrix* crude extract. b) Fragmentation pattern and HRMS/MS spectrum of cusperin A.

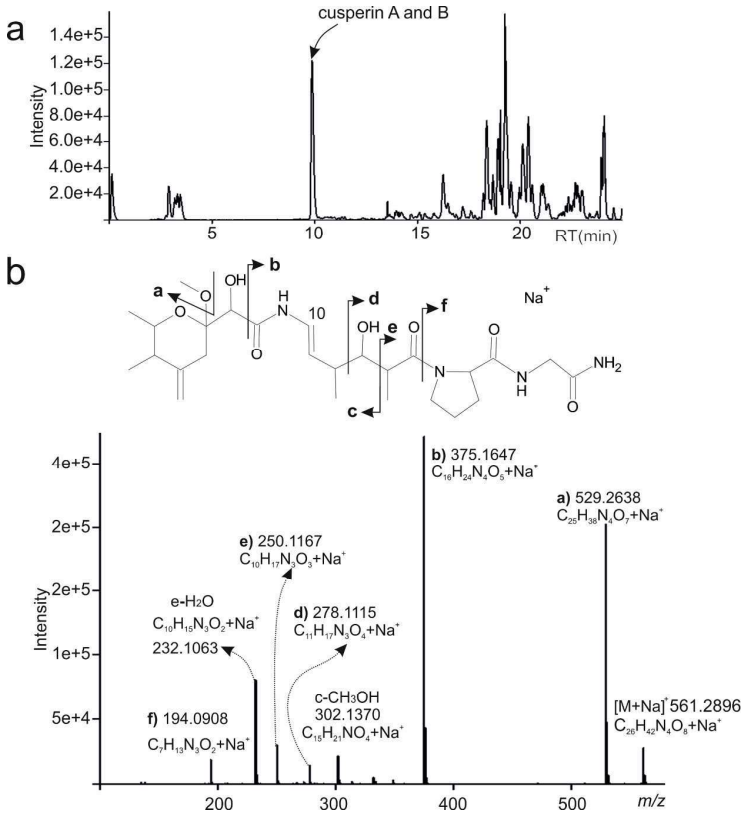
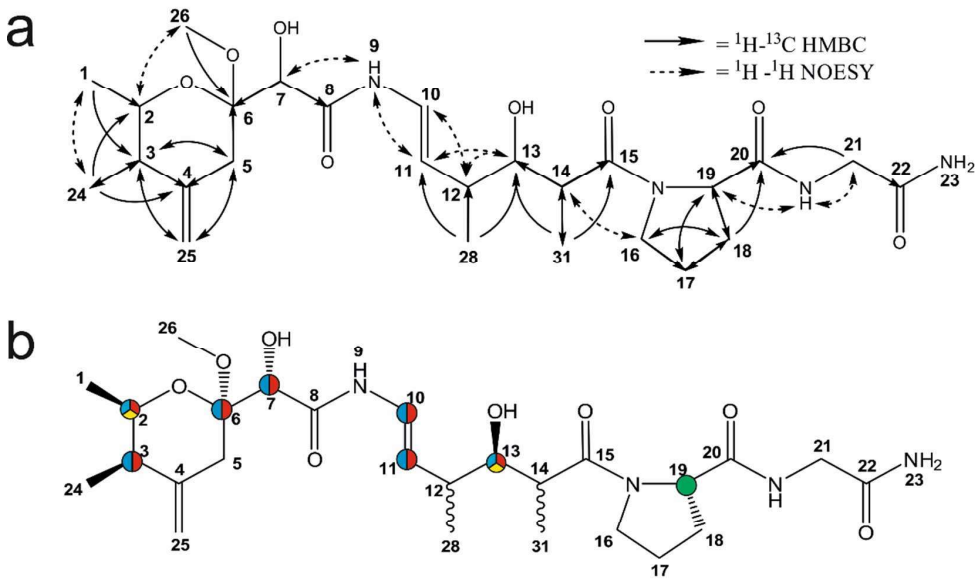


Figure 4. NMR analysis and stereochemical assignment of cusperin A. a) ^{13}C HMBC (solid arrows) and ^{1}H-^{1}H NOESY (dashed arrows) correlations of cusperin. b) Stereochemical arrangement of cusperin chiral centres based on NMR and bioinformatic analysis. Blue, domain organization identical to nosperin, supporting a similar arrangement of chiral centers; red, NMR coupling constants and shifts; yellow, ketoreductases (KRs) predicted specificity based on a specific sequence motif (the presence of a conserved Asp residue); green, nonribosomal peptide synthase (NRPS) domain prediction (absence of an epimerase domain).



Supporting Information Available: This material is available free of charge via the Internet.