JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Article

pubs.acs.org/JACS

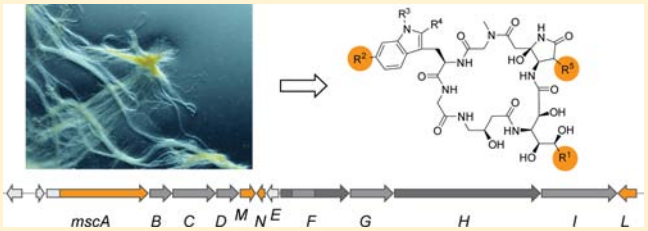
Microsclerodermins from Terrestrial Myxobacteria: An Intriguing Biosynthesis Likely Connected to a Sponge Symbiont

Thomas Hoffmann, Stefan Müller, Suvd Nadmid, Ronald Garcia, and Rolf Müller*

Helmholtz Institute for Pharmaceutical Research Saarland, Helmholtz Centre for Infection Research and Department of Pharmaceutical Biotechnology, Saarland University, Building C 2.3, D-66123 Saarbrücken, Germany

Supporting Information

ABSTRACT: The microsclerodermins are unusual peptide natural products exhibiting potent antifungal activity reported from marine sponges of the genera *Microscleroderma* and *Theonella*. We here describe a variety of microbial producers of microsclerodermins and pedeins among myxobacteria along with the isolation of several new derivatives. A retrobiosynthetic approach led to the identification of microsclerodermin biosynthetic gene clusters in genomes of *Sorangium* and *Jahnella* species, allowing for the first time insights into the intriguing hybrid PKS/NRPS machinery required for microsclerodermin formation. This study reveals the biosynthesis of a "marine natural product" in a terrestrial myxobacterium where even the identical structure is available from both sources. Thus, the newly identified terrestrial producers provide access to additional chemical diversity; moreover, they are clearly more amenable to production optimization and genetic modification than the original source from the marine habitat. As sponge metagenome data strongly suggest the presence of associated myxobacteria, our findings underpin the recent notion that many previously described "sponge metabolites" might in fact originate from such microbial symbionts.



INTRODUCTION

Natural products have a longstanding tradition as leads for the development of new medicines.[1] In addition to well-established and extensively investigated plant, fungal, and bacterial producers of secondary metabolites, newer screening campaigns increasingly include organisms from less studied taxa and previously underexploited habitats such as terrestrial myxobacteria and marine sponges.[2−5] Their potential as sources of novel chemical scaffolds has been clearly demonstrated, and despite the impressive structural diversity originating from these organisms, the overall picture has emerged that structural types obtained from phylogenetically distant producers usually show little overlap.[6] However, as an exception to this general notion, the production of several strikingly similar compounds by unrelated species has also been reported. Some of these findings are parallel discoveries of initially sponge-derived metabolite classes from microbial sources, leading to the assumption that the respective natural products might in fact be produced by bacterial sponge symbionts.[7−9] Support for this theory comes from the identification of filamentous bacteria growing within intercellular space inside the sponge.[8,10] However, studies which unambiguously prove the production of a "sponge metabolite" by a symbiotic bacterium are exceedingly rare.[10,11] The same holds true for marine natural products of other host organisms.[12−14] This shortcoming may be attributed to difficulties with isolation and cultivation of symbiotic microbes under laboratory conditions. Notably, the ability to independently cultivate the "real" producer of a specific secondary metabolite holds great promise, not only for sustained production but also for improving yields using both biotechnological and genetic engineering approaches. These opportunities present an invaluable advantage when further investigating a compound of interest, as the marine organism itself usually faces critical supply limitations and is poorly amenable to genetic manipulation. Moreover, access to a microbial producer facilitates the identification of biosynthetic genes underlying the formation of the metabolite of interest—a crucial prerequisite for understanding the biosynthetic machinery and a promising step toward transferring these genes into a suitable heterologous expression host.[15]

Looking at those natural products from marine sources having apparent microbial counterparts, several cases exist where structures of myxobacterial secondary metabolites are indeed strikingly similar to previously discovered sponge-derived compounds. For example, the cyclodepsipeptide jaspamide (jasplakinolide) isolated from the marine sponge *Jaspis*[16,17] is closely related to the structure of chondramides produced by the myxobacterium *Chondromyces crocatus* Cm c5, suggesting that the biosynthetic pathways responsible for production of these molecules should be largely similar.[18] The same holds true for renieramycin and saframycin MX1, isolated from a *Reniera* sponge and a myxobacterium of the genus *Myxococcus*.[19,20] Moreover, the macrolides salicylihalamide and apicularen were isolated from a *Halicona* sp. sponge and a *Chondromyces* species, respectively.[21,22] Very recently, bengamides were described from a *Jaspis* sponge and a cultured

Received: June 7, 2013
Published: October 14, 2013

   dx.doi.org/10.1021/ja4054509 | J. Am. Chem. Soc. 2013, 135, 16904−16911

myxobacterium.[23,24] Adding to the list of "biosynthetic look-alikes", the structure of pedein from the terrestrial myxobacterium *Chondromyces pediculatus* Cm p3 closely resembles that of microsclerodermin,[25] which was isolated in 1994 from *Microscleroderma* sp., a lithistid sponge harvested in New Caledonia.[26] Upon their finding of pedeins in myxobacteria, Kunze et al. suggested that the origin of microsclerodermins could be a bacterial sponge symbiont closely related to myxobacteria.[25] Indeed, pedein and microsclerodermin are highly similar, and both exhibit potent antifungal activity. To date several new derivatives belonging to the microsclerodermin class of peptides have been identified from various *Microscleroderma* species as well as from a *Theonella* sponge.[27−30] Nevertheless, the biosynthetic machinery behind this natural product remains so far elusive.

In this study we present several terrestrial myxobacteria as alternative producers of microsclerodermins and pedeins. Our data show that *Jahnella* and *Chondromyces* species can produce the identical derivate also known from a *Microscleroderma* species. In addition, they produce new derivatives not previously reported from other sources. Access to genomic sequences for two myxobacterial producers allowed us to establish for the first time a biosynthetic model for microsclerodermin formation and also provided us with an opportunity to probe the molecular basis responsible for the structural diversity observed from microsclerodermins. Moreover, it was shown that the myxobacterial pedeins[25] originate from the same biosynthetic machinery as the microsclerodermins; hence, they belong to the same compound family. Taken together with recent metagenomic studies providing evidence that myxobacterial taxa may even exist as sponge symbionts,[31] our results underpin the assumption that a myxobacterium is the real biosynthetic source of the "marine" natural product microsclerodermin.

EXPERIMENTAL SECTION

Bacterial Strains and Culture Conditions. *Sorangium cellulosum* So ce38 was cultivated in H-medium (2 g/L soybean flour, 2 g/L glucose, 8 g/L starch, 2 g/L yeast extract, 1 g/L $CaCl_2{\cdot}2H_2O$, 1 g/L $MgSO_4{\cdot}7H_2O$, 8 mg/L Fe-EDTA, 50 mM HEPES, adjusted to pH 7.4 with 10 N KOH). Mutants of *S. cellulosum* So ce38 were cultivated in H-medium supplemented with hygromycin B (100 μg/mL) and 1% (w/v) adsorber resin (XAD-16, Rohm & Haas) at 180 rpm and 30 °C. *Jahnella* sp. MSr9139 was cultivated in buffered yeast broth medium VY/2 (5 g/L baker's yeast, 1 g/L $CaCl_2{\cdot}2H_2O$, 5 mM HEPES pH 7.0 with 10 N KOH) at 180 rpm and 30 °C.[32] The *Escherichia coli* strains DH10B and ET12567 harboring the plasmids pUB307 and pSUP*mscH*_KO for conjugation purposes were cultivated in Luria−Bertani (LB) medium at 37 °C. Transformation of strains was performed according to the standard methods described elsewhere.[33] Antibiotics were added with the following final concentrations: chloramphenicol (25 μg/mL), kanamycin sulfate (25 μg/mL), and hygromycin B (100 μg/mL).

Gene Disruption of the *msc*H Locus in So ce38. Gene disruption in So ce38 using biparental mating was carried out according to a previously established protocol.[34] For construction of the plasmid pSUP*mscH*_KO a homologous fragment with the size of 2472 bp was amplified from genomic DNA using the oligonucleotides *mscH*_KO_for (GAT CCA GCG CTG GTT CCT CG) and *mscH*_KO_rev (ACG AGG CTG TCG AAG AGC G) and cloned into pCR TOPO II-vector, resulting in the plasmid pTOPO_*mscH*_KO. The genomic segment was subsequently recovered from this plasmid using the restriction enzymes *Hind*III and *Eco*RV and further integrated into the prepared vector pSUPHyg.

Isolation of Microsclerodermin M from So ce38. The production medium for So ce38 was P38X medium (2 g/L peptone, 2 g/L glucose, 8 g/L starch, 4 g/L probion, 1 g/L $CaCl_2{\cdot}2H_2O$, 1 g/L $MgSO_4{\cdot}7H_2O$, 8 mg/L Fe-EDTA, 50 mM HEPES, adjusted to pH 7.5 with 10 N KOH). A 100 L fermenter with 2% (w/v) XAD-16 adsorber resin (Rohm & Haas) was harvested after 14 days of fermentation. The cells were removed from the XAD before extraction with 3 × 3 L of methanol followed by 1 × 3 L of acetone. The combined fractions yielded 47.2 g dry weight of crude extract. Five grams of this extract was suspended in cold water, the suspension was centrifuged immediately, and the remaining was pellet dissolved in DMSO/MeOH (1/1, v/v) to give a product-enriched solution which was subjected to preparative HPLC using a Waters Autopurifier System equipped with a Waters XBridge C18, 150 × 19 mm, 5 μm d_p column operated at room temperature. The gradient started at 30% B, increased to 50% B in 2 min and to 51% B in another 2 min before increasing to 95% B in 4 min for column flushing. The combined fractions of interest were lyophilized, dissolved in DMSO/MeOH (1/1, v/v), and forwarded to a semipreparative Dionex HPLC system (P680 pump, TCC100 thermostat, and PDA100 detector) equipped with a Phenomenex Fusion C18, 250 × 4.6 mm, 4 μm d_p column. Separation was achieved by a linear gradient using (A) H_2O and (B) ACN at a flow rate of 5 mL/min and 30 °C. The gradient started at 10% B and increased to 30% B in 3 min, followed by an increase to 38% B in 15 min (0.9% B/column volume). UV data were acquired at 316 nm. A maximum of 100 μL of the sample was manually injected before fraction collection, yielding 8.1 mg of microsclerodermin M. Microsclerodermin M: white amorphous solid, $[\alpha]_D^{20}$ = 55.7° (*c* 0.10, DMSO/MeOH 8/2).

Isolation of Microsclerodermins from MSr9139. The strain MSr9139 was cultivated in 3 × 1 L shaking flasks containing 500 mL of buffered VY/2 medium for 30 days. The medium was changed every 24 h by pipetting out the liquid broth. The cell pellet was harvested by centrifugation and lyophilized overnight, followed by extraction with 3 × 300 mL of methanol. The combined fractions yielded an orange-brown crude extract which was further partitioned between hexane and MeOH/H_2O 7/3 (v/v) to yield 170 mg of crude extract out of the aqueous phase. Subsequently, the extract was purified by semipreparative HPLC using an Agilent 1260 Infinity system (G1311C quaternary pump, G1330B thermostat, G1315D DAD detector, and G1328C manual injector) equipped with a Phenomenex Jupiter Proteo, 250 × 10 mm, 4 μm d_p column. Separation was achieved by a linear gradient using (A) H_2O and (B) ACN at a flow rate of 2.5 mL/min and 22 °C. The gradient started at 20% B and increased to 50% B in 35 min (5.7% B/column volume). UV data were acquired at 280 nm. A maximum of 100 μL of the sample was manually injected before fraction collection, yielding 0.7 mg of microsclerodermin D, 0.45 mg of microsclerodermin L, and 0.85 mg of pedein A. Microsclerodermin L: white amorphous solid, $[\alpha]_D^{20}$ = 77.7° (*c* 0.12, MeOH).

LC-MS Data Acquisition. All measurements were performed on a Dionex Ultimate 3000 RSLC system using a BEH C18, 100 × 2.1 mm, 1.7 μm d_p column (Waters, Germany). Separation of a 1 μL sample was achieved by a linear gradient from (A) H2O + 0.1% FA to (B) ACN + 0.1% FA at a flow rate of 600 μL/min and 45 °C. The gradient was initiated by a 0.5 min isocratic step at 5% B, followed by an increase to 95% B in 18 min to end up with a 2 min step at 95% B before reequilibration under the initial conditions. UV spectra were recorded by a DAD in the range from 200 to 600 nm. The LC flow was split to 75 μL/min before entering the maXis 4G hr-ToF mass spectrometer (Bruker Daltonics, Germany) using the Apollo II ESI source. Mass spectra were acquired in centroid mode ranging from 150 to 2500 *m*/*z* at a 2 Hz scan rate.

16S rRNA Gene and Phylogenetic Analysis. Extraction of the 16S rRNA gene was performed in representative microsclerodermin producing strains of *Sorangium*, *Jahnella* and *Chondromyces*. Sequences of other myxobacterial strains used in the analysis were obtained from GenBank. The 16S rRNA gene was amplified using a set of universal primers, and phylogenetic analysis was performed as described in a previous study, but using the MUSCLE alignment algorithm and Neighbor-Joining tree method (JC69) as implemented in the Geneious Pro program version 5.6.5.[35]

Genome Data. The *msc* gene cluster sequence was deposited in GenBank with the accession no. KF657738 for *S. cellulosum* So ce38

Table 1. Overview of Different Microsclerodermins and Pedeins and Their Origin

derivative	R^1	R^2	R^3	R^4	R^5	pyrrolidone confign	sum formula	$(M + H)^+$ (m/z)	*a*	*b*	*c*	*d*	*e*	ref
A	H	H	COOH	OH	i	*S,S*	$C_{47}H_{62}N_8O_{16}$	995.4357	•					26,29
B	H	H	COOH	H	i	*S,S*	$C_{47}H_{62}N_8O_{15}$	979.4407	•					26,29
C	Cl	$CONH_2$	H	H	vii	*R,R*	$C_{41}H_{50}N_9O_{13}Cl$	912.3289	•	•				27
D	Cl	H	H	H	vii	*R,R*	$C_{40}H_{49}N_8O_{12}Cl$	869.3231	•	•	•	•		27, this study
E	H	H	COOH	H	iii	*R,R*	$C_{45}H_{54}N_8O_{14}$	931.3832	•					27
F + G[f]	H	H	H	H	iv	*R,R*	$C_{45}H_{56}N_8O_{12}$	901.4090	•					28
H + I[f]	H	H	H	H	ii	*R,R*	$C_{46}H_{58}N_8O_{12}$	915.4247	•					28
J	H	H	H	H	i	*S,S*	$C_{46}H_{60}N_8O_{12}$	917.4403	•					29
K	H	H	H	OH	i	*S,S*	$C_{46}H_{60}N_8O_{13}$	933.4353	•					29
L	Cl	H	H	OMe	vii	*R,R*	$C_{41}H_{51}N_8O_{13}Cl$	899.3337			•	•		this study
M	H	H	H	H	v	*R,R*	$C_{44}H_{54}N_8O_{12}$	887.3934					•	this study
pedein A[g]	Cl	H	H	OMe	vi	*R,R*	$C_{43}H_{53}N_8O_{13}Cl$	925.3493			•	•		25, this study
pedein B[g]	H	H	H	OMe	vi	*R,R*	$C_{43}H_{54}N_8O_{13}$	891.3883			•	•		25, this study

[a]*Microscleroderma* sp. (3 species). [b]*Theonella* sp. (1 species). [c]*Chondromyces* sp. (2 species). [d]*Jahnella* sp. (2 species). [e]*Sorangium* sp. (11 species). [f]The tryptophan side chain is reduced to an *α,β*-unsaturated amino acid. [g]On the basis of their same biosynthetic origin, we implicitly include pedeins when referring to the microsclerodermin family in this study.

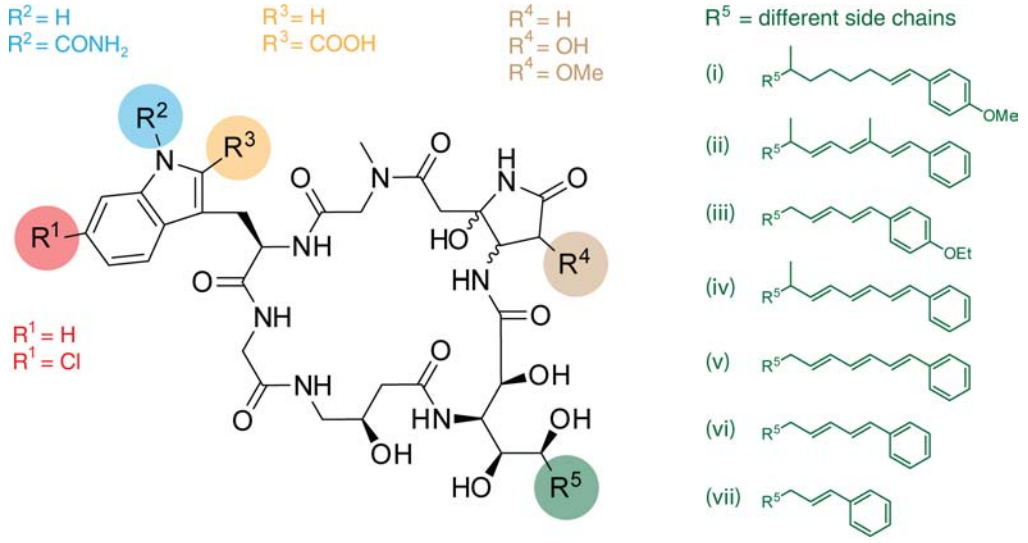


Figure 1. The microsclerodermin scaffold with an overview of the different residues identified so far. Groups R1−R4 are related to the presence of tailoring enzymes during biosynthesis, whereas the side chain R5 is derived by the PKS part of the biosynthetic machinery. The pyrrolidone is reported to have an *R,R* or *S,S* configuration, respectively.

and accession no. KF657739 for *Jahnella* sp. MSr9139 (will be added upon acceptance of the paper).

RESULTS AND DISCUSSION

Production of Microsclerodermins by Terrestrial Myxobacteria. In the course of our screening for bioactive natural products from myxobacteria, we observed antifungal activity in extracts from strain MSr9139, a newly isolated *Jahnella* species. Subsequent HPLC-based purification led to several fractions showing antifungal activity which contained compounds featuring an isotopic pattern typical for chlorination in MS analysis. Two compounds from these fractions could be assigned by their exact mass, fragmentation pattern, and retention time as pedein A (m/z 925.3493, $[M + H]^+$) and pedein B (891.3883 m/z, $[M + H]^+$), antifungal metabolites known from the myxobacterium *Chondromyces pediculatus* Cm p3.[25] Full structure elucidation was carried out for a compound with m/z 869.3231 obtained from another bioactive fraction, and the data unambiguously revealed this candidate as the known marine natural product microsclerodermin D (Table 1, Figure 1, and Figure S1 (Supporting Information)). In addition to this, analysis of the MSr9139 extract led to the isolation and structure elucidation of the new derivative microsclerodermin L, differing from microsclerodermin D by an additional methoxy group, which is also reported for the pedein structure (Table 1 and Figure S3 (Supporting Information)). Notably, the microsclerodermins are the first family of compounds found in the unexplored genus *Jahnella*, a member of the notable secondary metabolite producer myxobacterial family *Polyangiaceae*.[36]

Almost simultaneously, extracts from the myxobacterial strain *Sorangium cellulosum* So ce38 underwent biological profiling and HPLC fractionation, highlighting antifungal activity in the same chromatographic region as previously found from the MSr9139 extract. HPLC purification could narrow down the putatively active compounds to a candidate with m/z 887.3934, and subsequent NMR analysis identified a peptide featuring the pyrrolidone moiety also known from microsclerodermins. NMR data revealed the presence of a new nonchlorinated derivative, microsclerodermin M (Table 1, Figure 2, and Figure S4 (Supporting Information)). It shares the typical cyclic core

dx.doi.org/10.1021/ja4054509 | J. Am. Chem. Soc. 2013, 135, 16904−16911

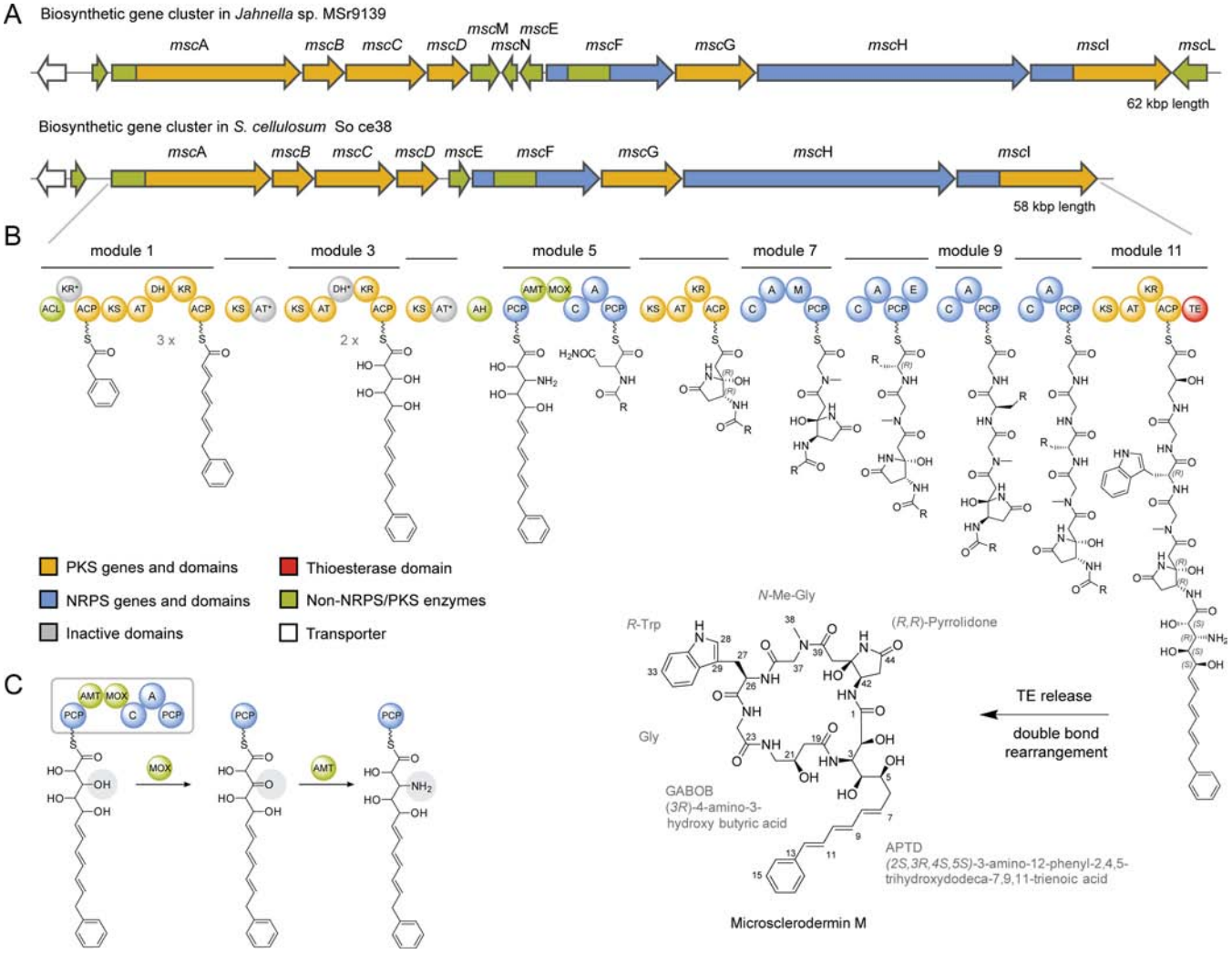


Figure 2. (A) Organization of the *msc* biosynthetic gene cluster in *Jahnella* sp. MSr9139 compared to *Sorangium cellulosum* So ce38. (B) Proposed biosynthetic route to microsclerodermin formation in So ce38. (C) Postulated biosynthetic steps leading to the amino group that is involved in macrolactam formation. Legend: A, adenylation domain; AMT, aminotransferase; ACP, acyl-carrier-protein domain; AT, acyltransferase; C, condensation domain; CoA-Lig, coenzyme A ligase; DH, dehydratase; E, epimerase; KR, ketoreductase; KS, ketosynthase; MT, methyltransferase; MOX, monooxygenase; PCP, peptidyl-carrier-protein domain.

structure with other microsclerodermins but features an unbranched side chain with three double bonds in conjugation to a phenyl moiety. Like the known microsclerodermins, the newly identified derivatives show potent activity against *Candida albicans* (microsclerodermin M, MIC 0.16 μg/mL; microsclerodermin L, MIC 18 μg/mL; microsclerodermin D, MIC 6.8 μg/mL). The stereochemistry of the isolated microsclerodermins was identified by acetonide formation and chemical degradation experiments followed by advanced Marfey analysis (see the Supporting Information). It is identical with that reported for the microsclerodermins C–I and pedeins.[25,27,28]

Having discovered that myxobacteria from three different genera—*Jahnella*, *Sorangium*, and *Chondromyces*—are able to produce microsclerodermin congeners including even the exact same structure as previously described from two species of lithistid sponges (microsclerodermin D) was surprising for two reasons: examples of myxobacteria producing an identical scaffold also known from a phylogenetically distant organism are to date exceedingly rare (even when counting among the bacterial kingdom), and according to previous studies the secondary metabolite profiles from strains belonging to different myxobacterial genera usually exhibit little overlap.[6] In order to shed light on the occurrence of microsclerodermins within the myxobacteria, we conducted a search across high-resolution LC-MS data sets measured from almost 800 extracts, thus covering a sufficiently representative sample including most known myxobacterial taxa (Figure S20 (Supporting Information)). On the basis of the combined evaluation of exact masses, isotope patterns, and retention times we could identify a panel of 15 strains from the suborder *Sorangineae* (no single producer was found within the *Cystobacterineae*) as producers of microsclerodermins. Interestingly, our comprehensive LC-MS survey of myxobacterial secondary metabolomes revealed that producers of microsclerodermins form two mutually exclusive groups: one group comprises 11 *Sorangium* species producing solely the new microsclerodermin M, while the second group includes two strains of *Jahnella* sp. and two *Chondromyces* sp. that produce a variety of different derivatives: i.e., the "marine" microsclerodermin D in addition to the new microsclerodermin L and pedeins A/B (Figures S21 and S22 (Supporting Information)). The various microsclerodermins differ in side chain, tryptophan modification,

dx.doi.org/10.1021/ja4054509 | J. Am. Chem. Soc. 2013, 135, 16904–16911

and oxidation state at the pyrrolidone ring, whereas the peptidic core structure is always identical (Figure 1).

The fact that all microsclerodermins, irrespective of their origin, exhibit an identical macrocycle and in most cases even the same stereochemistry supports the idea of a shared biosynthetic origin or even a shared evolutionary ancestor. Moreover, the finding of a group of terrestrial myxobacteria producing exactly the same compound as found in lithistid sponges[27] (microsclerodermin D) fuels speculation about the actual biosynthetic origin of marine microsclerodermins. We herein propose that the marine microsclerodermins actually originate from a myxobacterium phylogenetically related to the *Sorangineae* suborder—possibly a yet uncultured species of the *Chondromyces*, *Jahnella*, or *Sorangium* taxa living in symbiosis with the sponges from which microsclerodermins were previously isolated (Figure 3). In coincidence with this

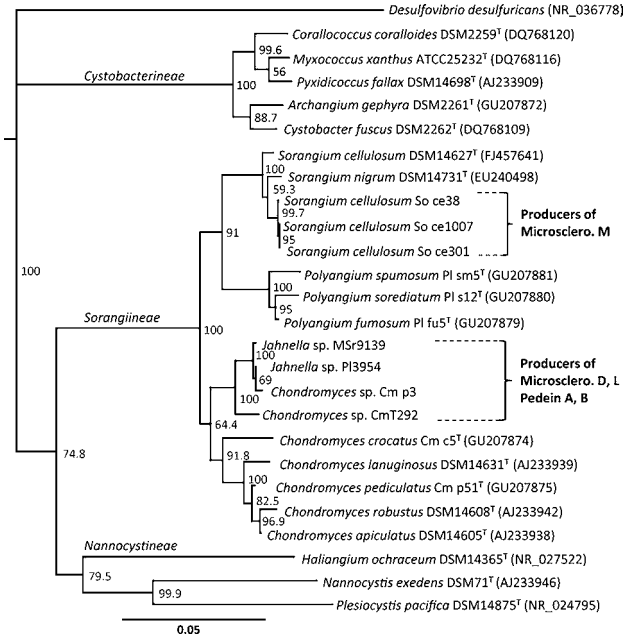


Figure 3. Neighbor-joining tree of myxobacteria inferred from 16S rRNA gene sequences showing the clades of microsclerodermin producing strains in suborder *Sorangiineae*. The numbers at branch points indicate the percentage bootstrap support based on 1000 resamplings. GenBank accession numbers are indicated in parentheses. Bar = 0.05 substitutions per nucleotide position.

hypothesis, phylogenetic studies of sponge metagenomes recently identified δ-proteobacteria in the sponge holobiont.[31] Indeed, the phylogenetic tree presented in the work of Simister et al. lists a clade containing nine myxobacterial species of terrestrial origin, including *Sorangium cellulosum* and *Chondromyces pediculatus*.[31] These data underpin our assumption that an evolutionary link exists between microsclerodermin biosynthesis in terrestrial and marine producers. Notably, 13 out of 15 producers identified by our LC-MS metabolome survey belong to the genera *Sorangium* or *Chondromyces*. In addition, the new *Jahnella* sp. MSr9139 was isolated from a soil sample collected from the same Philippine island where the sponge *Microscleroderma* was initially found.[27] This intriguing finding suggests that myxobacteria are possibly flushed to the ocean and adapted to an association with a sponge. The diversity and density of microbial flora present in sponges appears to be a good niche for a predator and proteo-bacteriolytic myxobacterium;[7] thus, it is expected that in the future myxobacteria will be isolated from this underexplored source.

Access to myxobacterial producers holds a remarkable benefit, as these bacteria may be cultivated in large-scale fermentations, thereby allowing efficient production of the compounds of interest. Microsclerodermin M is produced at 12 mg/L in *S. cellulosum* So ce38 without optimization of growth conditions or genetic modification of the strain. Moreover, it allows us to investigate their biosynthesis, which has not been elucidated from any marine source to date. Thus, we set out to mine genome sequences of the new terrestrial producers for the presence of putative microsclerodermin biosynthetic pathways, using a retrobiosynthetic analysis as the starting point. The genome sequence of the strain *Sorangium cellulosum* So ce38, producer of the new microsclerodermin M, was already available from a previous study.[37] The newly isolated *Jahnella* sp. MSr9139 was selected for additional genome sequencing, as it produces the new microsclerodermin L in addition to known pedeins A and B and the "marine" microsclerodermin D.

Microsclerodermin Biosynthetic Machinery. All microsclerodermins share the same cyclic peptide core but feature different lipophilic side chains and modifications of amino acid residues. On the basis of retrobiosynthetic considerations, the biosynthetic machinery for microsclerodermin formation was expected to consist of a multimodular PKS/NRPS system accompanied by a set of enzymes involved in side chain biosynthesis and postassembly line modification. The core PKS/NRPS modules should be conserved between producers, while enzymes involved in side chain biosynthesis and additional tailoring enzymes—responsible for modifications such as halogenation or oxidation of the pyrrolidone ring—should occur differentially between the two producer groups, as a consequence of evolutionary diversification of the microsclerodermin pathway. Using *S. cellulosum* So ce38 and *Jahnella* sp. MSr9139 as representative strains from both microsclerodermin producer groups, we sought to identify microsclerodermin biosynthetic pathways in their genomes and subsequently elaborate on the molecular basis for the observed structural variations. The genome sequences of both strains were searched in silico for secondary metabolite gene clusters using the antiSMASH analysis pipeline.[38] The assignment of a matching candidate cluster to microsclerodermin biosynthesis was verified in So ce38 via targeted gene disruption by single crossover integration using biparental conjugation (Figure S24 (Supporting Information)). Sequence comparison on the protein and nucleotide level revealed high similarity between gene clusters from both strains (Table 2), and comparison of operons permitted the tentative assignment of cluster boundaries. The microsclerodermin cluster spans a region of 58 kbp (74.7% GC) in So ce38 and 62 kbp (72.4% GC) in MSr9139, respectively. In both strains, genes encoding a major facilitator superfamily transporter (*mscK*) followed by a type II thioesterase (*mscJ*) are located upstream to *mscA*. The core biosynthetic assembly line covers five NRPS modules and three PKS modules encoded on genes *mscA* to *mscI*. An additional halogenase is encoded by *mscL* near the downstream boundary of the cluster in MSr9139 (Figure 2A).

Microsclerodermin biosynthesis is initiated at the side chain to build up a phenyl group in conjugation to a double bond. An activated starter unit such as benzoyl-CoA or *trans*-cinnamoyl-CoA is usually recruited by the enzyme in such a case. However, a retrobiosynthetic proposal tells us that the observed double-bond order of the side chain is likely different during

Table 2. Proteins Involved in Microsclerodermin Biosynthesis As Identified in Two Myxobacterial Strains[a]

	Sorangium cellulosum So ce38		*Jahnella* sp. MSr9139		
protein	length (aa)	domain and position in sequence	length (aa)	domain and position in sequence	identity (%)
MscA	3275	CoA-Lig (264−701), KR[b](1034−1197), ACP (1348−1411), KS (1441−1828), AT (1976−2286), DH (2342−2505), KR′ (2870−3047), ACP′ (3149−3214)	3535	CoA-Lig (215−649), KR[b](1032−1126), MT (1229−1509), ACP (1613−1676), KS (1712−2147), AT (2244−2555), DH (2610−2774), KR′[b] (3132−3309), ACP′ (3411−3476)	65.4
MscB	870	KS (27−451), AT[b] (548−772)	878	KS (39−464), AT[b] (561−792)	73.4
MscC	1551	KS (36−460), AT (557−859), DH[b] (956−1076), KR (1158−1336), ACP (1436−1505)	1549	KS (39−464), AT (561−865), DH[b] (956−1076), KR (1157−1335), ACP (1436−1499)	75.0
MscD	900	KS (36−461), AT[b] (565−678)	848	KS (36−461), AT[b] (564−675)	72.0
MscE	446	putative amidohydrolase	386	putative amidohydrolase	82.9
MscF	2273	PCP (27−98), AMT (329−660), MOX (828−1127), C (1185−1529), A (1673−2082), PCP′ (2169−2237)	2189	PCP (4−75), AMT (280−614), MOX (758−1057), C (1105−1397), A (1594−2000), PCP′ (2088−2149)	76.5
MscG	1548	KS (14−439), AT (534−828), KR (1156−1330), ACP (1441−1509)	1511	KS (14−439), AT (531−850), KR (1136−1312), ACP (1404−1472)	72.1
MscH	4141	C (76−377), A (564−966), MT (1037−1256), PCP (1469−1531), C′ (1554−1850), A′ (2037−2426), PCP′ (2515−2574), E (2591−2905), C″ (3074−3374), A″ (3558−3967), PCP″ (4054−4121)	4106	C (48−346), A (534−936), MT (1007−1225), PCP (1442−1505), C′ (1526−1827), A′ (2013−2405), PCP′ (2492−2551), E (2568−2872), C″ (3043−3343), A″ (3527−3932), PCP″ (4019−4083)	78.8
MscI	2904	C (48−346), A (533−936), PCP (1023−1087), KS (1111−1535), AT (1638−1936), KR (2266−2465), ACP (2549−2612), TE (2634−2888)	2945	C (77−375), A (563−965), PCP (1053−1116), KS (1150−1573), AT (1676−1970), KR (2309−2509), ACP (2597−2660), TE (2683−2945)	77.8
MscJ	257	thioesterase type II	263	thioesterase type II	43.5
MscK	415	major facilitator superfamily (MFS) transporter	450	major facilitator superfamily (MFS) transporter	24.5
MscL			535	tryptophan halogenase	
MscM			438	Fe(II)/α-ketoglutarate dependent oxygenase	
MscN			277	methyltransferase	

[a]Legend: A, adenylation domain; AMT, aminotransferase; ACP, acyl-carrier-protein domain; AT, acyltransferase; C, condensation domain; CoA-Lig, coenzyme A ligase; DH, dehydratase; E, epimerase; KR, ketoreductase; KS, ketosynthase; MT, methyltransferase; MOX, monooxygenase; PCP, peptidyl-carrier-protein domain; TE, thioesterase. [b]Inactive domain.

biosynthesis (Figure 2B). The biosynthetic logic requests the incorporation of C2 units, which is only possible if the double bonds are rearranged (Figure S23 (Supporting Information)). A rearrangement of double bonds has already been reported for other natural products such as bacillaen, rhizoxin, corallopyronin, and ansamitocin where isomerization is likely catalyzed by a dehydratase domain.[39−42] The reason for isomerization of the microsclerodermin side chain remains elusive; however, it is supported by an energetic benefit of a conjugated π system. On the basis of this hypothesis, the only suitable starter unit is phenylacetyl-CoA, which has already been reported for other natural product biosynthesis.[43] The incorporation of a phenylacetate starter unit was indeed verified by feeding experiments using isotope-labeled precursors. Feeding ring-labeled $^{13}C_6$-L-phenylalanine resulted in a mass increase of 6 Da, whereas the fully labeled $^{15}N,^{13}C_9$-L-phenylalanine led to a mass shift of 8 Da, indicating the incorporation of two side-chain carbons (Figure S27 (Supporting Information)). Feeding d_5-benzoic acid or d_7-*trans*-cinnamic acid resulted in no mass increase (Figure S25 (Supporting Information)). We can conclude that the α- and β-carbon atoms of phenylalanine—but not the carboxyl carbon—is incorporated into microsclerodermin. Elongation of the phenylacetate unit is catalyzed by modules MscA and MscC using three times malonate and two times 3-hydroxymalonate as extender units in an iterative manner. Modules 2 (MscB) and 4 (MscD) do only exhibit a combination of a functional KS domain attached to an inactive AT domain as identified by consensus sequence analysis, likely a relic of a former PKS complex.

The PKS-derived unit is forwarded to the first PCP domain of module MscF. This module harbors two additional domains of rather uncommon type showing high homology to the amino transferase family (AMT) and to the monooxygenase family, both located downstream to the PCP domain. A biosynthetic proposal to account for this domain order is based on oxidation of the β-hydroxyl group of the bound intermediate to the respective β-keto functionality followed by conversion to a β-amino moiety that undergoes macrocyclization (Figure 2C). The use of an aminotransferase is known from other natural product biosynthetic pathways, however, not in combination with the initial oxidation step.[44]

Thereafter, biosynthesis continues with a set of NRPS- and PKS-based reaction cycles. Analysis of the A domain specificities in silico is consistent with the amino acids incorporated.[45] We propose the uncommon pyrrolidone moiety is built up by asparagine cyclization. To the best of our knowledge, such an asparagine-derived pyrrolidone system is only found in microsclerodermins and koshikamides, a natural product that was isolated from a *Theonella* species.[46] Indeed, the A domain of MscF is specific for asparagine activation and we did a feeding experiment with fully labeled $^{15}N_2$-$^{13}C_4$-L-asparagine to prove this biosynthetic step. We observed a mass increase of 6 Da according to the incorporation of all carbon and nitrogen atoms of asparagine into the compound (Figures S26 and S28 (Supporting Information)). On the basis of this result, a plausible biosynthetic hypothesis requires the nucleophilic attack of the asparagine side chain to the backbone carbonyl atom. A suitable mechanism is known from the intein-mediated peptide cleavage, where intein initiates an intramolecular asparagine cyclization, notwithstanding the poor reactivity of the side chain's amide.[47] In microsclerodermin biosynthesis, this reaction likely is accompanied by an inversion of the stereochemistry at the α-carbon of the former (*S*)-asparagine. The relative configuration of the pyrrolidone ring was identified

by NOE correlations, whereas the absolute *R,R* configuration is derived from degradation experiments (see the Supporting Information). The stereochemistry at this position is thereby identical with that of microsclerodermins C−I. The protein MscE is most likely responsible for the cyclization step, as it is found in both microsclerodermin clusters and shows similarity to the amidohydrolase class, a fairly promiscuous enzyme family able to act on a variety of substrates. However, the exact mechanism involved in this biosynthetic step remains elusive at present.

The forthcoming NRPS modules correspond to the observed structure of microsclerodermin in terms of domain order and predicted substrate specificity (Figure 2B). For both new microsclerodermins an *R*-configured tryptophan was identified by means of the advanced Marfey method, which is in agreement with the epimerization domain found in module 8. The 3*R* configuration of the γ-aminobutyric acid (GABA) subunit was identified by the same technique (see Figures S13 and S14 (Supporting Information)). Both stereogenic centers have the same configuration as identified in all microsclerodermins so far.

Genetic Basis for the Structural Diversity of Microsclerodermins. The derivatives found in *Jahnella* sp. MSr9139 feature side chains with either one or two double bonds while the side chain in So ce38 comprises strictly three double bonds. As the number of PKS modules encoded in the gene cluster does not match the number of required elongation cycles, an iterative function of the type I PKS subunits MscA and MscC as described for the stigmatellin megasynthase may explain this finding.[48] The KS domains of each module are highly identical for both strains and do not comprise any of the postulated sequence-based identifiers of iterative KS domains.[49] Nevertheless, MscB is grouping with iterative KS domains in a phylogenetic analysis (Figure S11 (Supporting Information)). Comparing the entire module MscA of both clusters revealed the insertion of an additional methyl transferase-like domain into the first part of the protein in MSr9139. This domain is likely inactive on the basis of in silico analysis, as judged by the presence of a corrupted SAM-binding motif (Table S13 (Supporting Information)).[50] Currently, we cannot rule out the possibility that the presence of this additional methyl transferase may influence the iteration process within MscA. In addition to this difference, there is no obvious reason why biosynthesis in So ce38 results in a triene whereas MSr9139 is less strict in iteration. As another hint for a shared origin of the biosynthetic cluster, some of the sponge-derived derivatives exhibit a methyl-branched side chain which could be attributed to this methyl transferase being active in some of the marine producers. Eventually, the variable side chains of the microsclerodermin family are in agreement with the alternating PKS functionality. Halogenation of tryptophan as well as oxidation of the pyrrolidone ring is catalyzed by tailoring enzymes. The halogenase MscL is located downstream to the cluster in MSr9139 and is responsible for chlorination of the tryptophan. It shows 32% identity on a protein level to a tryptophan halogenase from a *Streptomyces* species (PDB entry 2WET_A). There is no analogue of MscL found in So ce38, which is in agreement with the absence of chlorinated products in this strain. Supplementing KBr or NaBr to the MSr9139 cultivation broth led to the production of brominated microsclerodermins on the basis of LC-MS analysis. Another difference is the interregion between the main PKS and NRPS parts. In MSr9139 two additional proteins are found in this region. MscN is a member of the SAM-dependent methyl transferase family, and MscM shows homology to Fe(II)/α-ketoglutarate-dependent dioxygenases. On the basis of the structures produced by MSr9139, we conclude that MscM and MscN are responsible for oxidation and methylation of the pyrrolidone ring, respectively. Modifications at the tryptophan as known from some marine-derived microsclerodermins were not observed in this study. Such modifications are attributed to promiscuous acting enzymes that could be related to the producer strain or even to enzymes related to some sponge symbiont.

■ CONCLUSION

The discovery of microsclerodermins/pedeins from several myxobacteria represents one of the few findings of identical compounds from marine organisms and terrestrial bacteria reported to date. This study thus strengthens the notion that certain natural products, which have been isolated from marine sources such as sponges or other invertebrates, actually originate from associated microbes. Notably, the identification of microsclerodermin-producing myxobacteria provides meaningful hints for future attempts to isolate the symbiotic microbe. This knowledge is considered particularly helpful, because isolation success in many cases critically depends on methods well adapted to the requirements of the genus targeted for isolation, especially when aimed at the rather challenging isolation of slow-growing myxobacteria. Availability of an alternative microbial producer as a sustainable source is an advantage for realizing the potential of a marine natural product for therapeutic applications. Moreover, the myxobacterial producers come along with additional chemical diversity and are amenable to genetic manipulation, as demonstrated in this study. Finally, the identification of two slightly different microsclerodermin biosynthetic gene clusters from two myxobacteria allowed us to establish a conclusive model for microsclerodermin biosynthesis and provided insights into the molecular basis for structural diversity within this compound family. A detailed understanding of microsclerodermin biosynthesis is an important prerequisite for any future efforts toward engineering the pathway for yield improvement or for the production of new derivatives: whether in the native producer, by heterologous expression, or by using synthetic biology approaches.

■ ASSOCIATED CONTENT

Supporting Information

Figures, tables, and text giving NMR spectra, MS data, biosynthesis cluster information, feeding studies, phylogenetic analysis, and inactivation of the *msc* cluster in So ce38. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

R.M.: fax, +49 (0)681 302-70202; e-mail, rom@helmholtz-hzi.de.

Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Research in R.M.'s laboratory was funded by the Bundesministerium für Bildung and Forschung and the Deutsche

Forschungsgemeinschaft. We thank Daniel Krug for advice and discussion, as well as for editing the manuscript. We also thank Jennifer Herrmann for performing the bioactivity assays, Lena Etzbach and Alberto Plaza for help with the NMR data, and Manuel Klos for ozonolysis reactions.

REFERENCES

(1) Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2012**, *75*, 311−35.
(2) Wenzel, S. C.; Müller, R. *Mol. Biosyst.* **2009**, *5*, 567−74.
(3) Weissman, K. J.; Müller, R. *Bioorg. Med. Chem.* **2009**, *17*, 2121−36.
(4) Lane, A. L.; Moore, B. S. *Nat. Prod. Rep.* **2011**, *28*, 411−28.
(5) Winder, P. L.; Pomponi, S. A.; Wright, A. E. *Mar. Drugs* **2011**, *9*, 2643−82.
(6) Wink, J.; Müller, R. *Int. J. Med. Microbiol.* **2013**, DOI: http://dx.doi.org/10.1016/j.ijmm.2013.09.004.
(7) Hentschel, U.; Piel, J.; Degnan, S. M.; Taylor, M. W. *Nat. Rev. Microbiol.* **2012**, *10*, 641−54.
(8) Taylor, M. W.; Radax, R.; Steger, D.; Wagner, M. *Microbiol. Mol. Biol. Rev.* **2007**, *71*, 295−347.
(9) Bewley, C. A.; Faulkner, D. J. *Angew. Chem., Int. Ed.* **1998**, *37*, 2162−2178.
(10) Schmidt, E. W.; Obraztsova, A. Y.; Davidson, S. K.; Faulkner, D. J.; Haygood, M. G. *Mar. Biol.* **2000**, *136*, 969−977.
(11) Unson, M. D.; Faulkner, D. J. *Experientia* **1993**, *49*, 349−353.
(12) Elshahawi, S. I.; Trindade-Silva, A. E.; Hanora, A.; Han, A. W.; Flores, M. S.; Vizzoni, V.; Schrago, C. G.; Soares, C. A.; Concepcion, G. P.; Distel, D. L.; Schmidt, E. W.; Haygood, M. G. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, E295−304.
(13) Lin, Z.; Torres, J. P.; Ammon, M. A.; Marett, L.; Teichert, R. W.; Reilly, C. A.; Kwan, J. C.; Hughen, R. W.; Flores, M.; Tianero, M. D.; Peraud, O.; Cox, J. E.; Light, A. R.; Villaraza, A. J. L.; Haygood, M. G.; Concepcion, G. P.; Olivera, B. M.; Schmidt, E. W. *Chem. Biol.* **2013**, *20*, 73−81.
(14) Kwan, J. C.; Donia, M. S.; Han, A. W.; Hirose, E.; Haygood, M. G.; Schmidt, E. W. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 20655−60.
(15) Wenzel, S. C.; Müller, R. In *Comprehensive Natural Products II, Vol. 2: Natural Products Structural Diversity-II Secondary Metabolites: Sources, Structures and Chemical Biology*; Moore, B. S., Crews, P., Eds.; Elsevier: Amsterdam, 2010; pp 189−222.
(16) Crews, P.; Manes, L. V.; Boehler, M. *Tetrahedron Lett.* **1986**, *27*, 2797−2800.
(17) Zabriskie, T. M.; Klocke, J. A.; Ireland, C. M.; Marcus, A. H.; Molinski, T. F.; Faulkner, D. J.; Xu, C.; Clardy, J. *J. Am. Chem. Soc.* **1986**, *108*, 3123−3124.
(18) Kunze, B.; Jansen, R.; Sasse, F.; Höfle, G.; Reichenbach, H. *J. Antibiot.* **1995**, *48*, 1262−6.
(19) Irschik, H.; Trowitzsch-Kienast, W.; Gerth, K.; Höfle, G.; Reichenbach, H. *J. Antibiot.* **1988**, *41*, 993−8.
(20) Frincke, J. M.; Faulkner, D. J. *J. Am. Chem. Soc.* **1982**, *104*, 265−269.
(21) Erickson, K. L.; Beutler, J. A.; Cardellina, J. H.; Boyd, M. R. *J. Org. Chem.* **1997**, *62*, 8188−8192.
(22) Kunze, B.; Jansen, R.; Sasse, F.; Höfle, G.; Reichenbach, H. *J. Antibiot.* **1998**, *51*, 1075−80.
(23) Hoffmann, H.; Haag-Richter, S.; Kurz, M.; Tietgen, H. Bengamide derivatives, method for the production thereof and use thereof for the treatment of cancer. WO2005044803 A1, 2005.
(24) Johnson, T. A.; Sohn, J.; Vaske, Y. M.; White, K. N.; Cohen, T. L.; Vervoort, H. C.; Tenney, K.; Valeriote, F. A.; Bjeldanes, L. F.; Crews, P. *Bioorg. Med. Chem.* **2012**, *20*, 4348−55.
(25) Kunze, B.; Böhlendorf, B.; Reichenbach, H.; Höfle, G. *J. Antibiot.* **2008**, *61*, 18−26.
(26) Bewley, C. A.; Debitus, C.; Faulkner, D. J. *J. Am. Chem. Soc.* **1994**, *116*, 7631−7636.
(27) Schmidt, E. W.; John Faulkner, D. *Tetrahedron* **1998**, *54*, 3043−3056.
(28) Qureshi, A.; Colin, P. L.; Faulkner, D. J. *Tetrahedron* **2000**, *56*, 3679−3685.
(29) Zhang, X.; Jacob, M. R.; Ranga Rao, R.; Wang, Y. H.; Agarwal, A. K.; Newman, D. J.; Khan, I. A.; Clark, A. M.; Li, X. C. *Res. Rep. Med. Chem.* **2012**, *2012*, 7−14.
(30) Zhang, X.; Jacob, M. R.; Ranga Rao, R.; Wang, Y. H.; Agarwal, A. K.; Newman, D. J.; Khan, I. A.; Clark, A. M.; Li, X. C. *Res. Rep. Med. Chem.* **2013**, 9.
(31) Simister, R. L.; Deines, P.; Botté, E. S.; Webster, N. S.; Taylor, M. W. *Environ. Microbiol.* **2012**, *14*, 517−24.
(32) Garcia, R. O.; Krug, D.; Müller, R. *Methods Enzymol.* **2009**, *458*, 59−91.
(33) Sambrook, J.; Russell, D. W. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001.
(34) Kopp, M.; Irschik, H.; Gross, F.; Perlova, O.; Sandmann, A.; Gerth, K.; Müller, R. *J. Biotechnol.* **2004**, *107*, 29−40.
(35) Garcia, R.; Gerth, K.; Stadler, M.; Dogma, I. J.; Müller, R. *Mol. Phylogenet. Evol.* **2010**, *57*, 878−87.
(36) Garcia, R.; Müller, R. In *The Prokaryotes: Deltaproteobacteria and Epsilonproteobacteria*; Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E., Thompson, F., Eds.; Springer: Heidelberg, Germany, in press.
(37) Jahns, C.; Hoffmann, T.; Müller, S.; Gerth, K.; Washausen, P.; Höfle, G.; Reichenbach, H.; Kalesse, M.; Müller, R. *Angew. Chem., Int. Ed.* **2012**, *51*, 5239−43.
(38) Medema, M. H.; Blin, K.; Cimermancic, P.; de Jager, V.; Zakrzewski, P.; Fischbach, M. A.; Weber, T.; Takano, E.; Breitling, R. *Nucleic Acids Res.* **2011**, *39*, W339−46.
(39) Butcher, R. A.; Schroeder, F. C.; Fischbach, M. A.; Straight, P. D.; Kolter, R.; Walsh, C. T.; Clardy, J. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 1506−9.
(40) Kusebauch, B.; Busch, B.; Scherlach, K.; Roth, M.; Hertweck, C. *Angew. Chem., Int. Ed.* **2010**, *49*, 1460−4.
(41) Erol, O.; Schäberle, T. F.; Schmitz, A.; Rachid, S.; Gurgui, C.; El Omari, M.; Lohr, F.; Kehraus, S.; Piel, J.; Müller, R.; König, G. M. *ChemBioChem* **2010**, *11*, 1253−65.
(42) Taft, F.; Brünjes, M.; Knobloch, T.; Floss, H. G.; Kirschning, A. *J. Am. Chem. Soc.* **2009**, *131*, 3812−3.
(43) Magarvey, N. A.; Beck, Z. Q.; Golakoti, T.; Ding, Y.; Huber, U.; Hemscheidt, T. K.; Abelson, D.; Moore, R. E.; Sherman, D. H. *ACS Chem. Biol.* **2006**, *1*, 766−79.
(44) Tillett, D.; Dittmann, E.; Erhard, M.; von Döhren, H.; Börner, T.; Neilan, B. A. *Chem. Biol.* **2000**, *7*, 753−64.
(45) Röttig, M.; Medema, M. H.; Blin, K.; Weber, T.; Rausch, C.; Kohlbacher, O. *Nucleic Acids Res.* **2011**, *39*, W362−7.
(46) Plaza, A.; Bifulco, G.; Masullo, M.; Lloyd, J. R.; Keffer, J. L.; Colin, P. L.; Hooper, J. N. A.; Bell, L. J.; Bewley, C. A. *J. Org. Chem.* **2010**, *75*, 4344−55.
(47) Mujika, J. I.; Lopez, X.; Mulholland, A. J. *Org. Biomol. Chem.* **2012**, *10*, 1207−18.
(48) Gaitatzis, N.; Silakowski, B.; Kunze, B.; Nordsiek, G.; Blöcker, H.; Höfle, G.; Müller, R. *J. Biol. Chem.* **2002**, *277*, 13082−90.
(49) Yadav, G.; Gokhale, R. S.; Mohanty, D. *PLoS Comput. Biol.* **2009**, *5*, e1000351.
(50) Kozbial, P. Z.; Mushegian, A. R. *BMC Struct. Biol.* **2005**, *5*, 19.