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Methacrylate Natural Products

Cyaneodimycin, a Bioactive Compound Isolated from the Culture of *Streptomyces cyaneofuscatus* Associated with *Lichina confinis*

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Abstract: Two new compounds, cyaneodimycin (**1**) and cyaneomycin (**2**) bearing a rare methacrylate residue, were isolated from cultures of the bacterium *Streptomyces cyaneofuscatus* associated with the marine lichen *Lichina confinis*. These astonishing structures may be derived from a pyruvate biosynthetic pathway. Cyaneodimycin (**1**) exhibited antiproliferative properties against B16 (IC_{50} = 27 ± 4 μM), HaCaT (IC_{50} = 47 ± 11 μM) and Jurkat cell lines (IC_{50} = 18.5 ± 0.5 μM). Six known compounds **3**–**8** with diversified structures and interesting biological properties (diketopiperazines, actinomycin, dibenzofurane or indole derivatives) were isolated jointly with compounds **1** and **2**. Among them, usnic acid (**8**), a common lichen compound was isolated for the first time from bacteria.

Introduction

Many drugs originate from microorganisms.[1] Additionally, the study of microorganisms from unexplored habitats often results in new chemistries, therapeutic leads and insights into novel bioactive principles, especially if those habitats foster symbiotic relationships among organisms.[2] Among underexplored natural product sources, lichens are unique and are classically described as a symbiotic association between a photobiont (green algae and/or cyanobacteria) and a mycobiont. Long-lived lichen symbioses provide an ecological niche for highly diverse additional microorganisms observed by culture-dependent[3] and -independent approaches.[4–10] Examinations of natural-product profiles of these underexploited bacteria associated with lichens have already started, and have revealed new compounds with cytotoxicity and/or antimicrobial activities,[11] such as angucycline and butenolide,[12] coumabiocins A–F,[13] cladoniamides A–G,[14] uncialamycin[15] and, more recently, unciaphenol.[16] As part of our program focused on the discovery of new natural products from lichen-associated bacteria, we selected an interesting bacterial strain (MOLA 1488) associated with *Lichina confinis* for further study; the EtOAc extract of MOLA 1488 fermentation was found to display significant cytotoxicity against B16 cell lines (IC_{50} = 0.33 ± 0.2 μg/mL). Fermentation of this strain led to the isolation of two new compounds (both methacrylate derivatives) and six known compounds belonging to various structural families (diketopiperazines, indole, phenoxazine or dibenzofurane derivatives). Some of the isolated compounds demonstrated interesting cytotoxic activities. We describe herein the isolation, structural identification and biological properties of cyaneodimycin (**1**), a new methacrylate derivative, jointly with the structural elucidation of cyaneomycin (**2**).

Results and Discussion

The producing bacterial strain MOLA1488 was isolated from a marine lichen, *Lichina confinis* collected on the Brittany coast (Erquy, France). Characterization by 16S rRNA gene sequencing showed the strain to be related at 100 % to *Streptomyces cyaneofuscatus* JCM 4364 after comparisons using the EzTaxon database server.[17] A relatively large-scale (2.7 L) shake fermentation was carried out using modified LB medium over the course of 12 d, to obtain quantities of material sufficient to support full chemical and biological analysis of the metabolites. The excreted metabolites were collected using sterile XAD-7-HP resin, and the solid residue (constituted of resin and bacterial cells) was first extracted three times with acetone/MeOH (50:50, v/v). Concentration of the acetone/MeOH extracts in vacuo

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gave a brownish residue that was partitioned between EtOAc and H_2O. The EtOAc extract, corresponding to the crude material, was used for all experiments (80 mg). A dereplication approach using GNPS (Global Natural Products Social molecular Networking platform at gnps.ucsd.edu, Wang et al.[18]) based on MS^2 fragmentation spectra similarities permitted highlighting of a cluster of diketopiperazines including *cyclo*-(L-Leu,L-Pro), *cyclo*-(L-Pro,L-Val), *cyclo*-(L-Phe,D-Pro), *cyclo*-(Leu,Leu), *cyclo*-(Leu,Phe), *cyclo*-(Val,Leu), *cyclo*-(Val,Phe) (see Supporting Information, Figure S1). Notably, some biological activities had already been reported for this chemical family (antibacterial, antifungal, antifouling, cytotoxic properties among others).[19–21]

Flash chromatography and semipreparative HPLC runs were performed on the EtOAc extract (80 mg, viscous aspect) and led to the isolation of two new compounds **1** (1.5 mg) and **2** (0.2 mg).

Cyaneodimycin (**1**) was isolated as a yellow residue with HRESIMS data of an $[M + Na]^+$ ion at m/z = 379.17327, appropriate for a molecular formula of $C_{18}H_{28}O_7$. The ^1H NMR, JMOD and 2D (COSY, TOCSY, HSQC and HMBC) data were acquired in CD_2Cl_2. These data (^1H NMR and JMOD) revealed the presence of the following groups: carbonyl carbon atoms (δ_C = 167.3 and 176.1 ppm), a methylene group (δ_C = 125.6 ppm and δ_H = 5.54 and 6.04 ppm) and a quaternary carbon atom (δ_C = 136.7 ppm). Signals of three OCH_2 functions (δ_C = 73.4 and 71.5 ppm; δ_H = 3.46–3.56 and 3.36–3.41 ppm; δ_C = 64.9 ppm; δ_H = 4.06 ppm), four methyl groups (δ_C = 18.4, 13.6 and 7.6 ppm; δ_H = 1.90, 1.12 and 0.87 ppm), one CH group (δ_C = 39.9 ppm; δ_H = 2.69–2.73 ppm) and one methylene group (δ_C = 23.6 ppm; δ_H = 1.48 ppm) were also observed in the aliphatic region. On the other hand, ^1H NMR spectroscopic data of **1** revealed the presence of two methylene protons (δ_H = 5.54 and 6.04 ppm) as each of the signals integrated for two protons. Similar observations were shown for signals at δ_H = 1.90 and 4.06 ppm integrating for six and four protons, respectively, and suggesting the presence of two identical methyl groups (C-13 and C-15, δ_C = 18.4 ppm) and two OCH_2 functions (C-3 and C-10, δ_C = 64.9 ppm). ^1H-^1H COSY experiments highlighted correlations between protons H-14 (δ_H = 5.54 and 6.04 ppm) and H-13 (δ_H = 1.90 ppm) and, on the other hand, between protons H-16 (δ_H = 5.54 and 6.04 ppm) and H-15 (δ_H = 1.90 ppm). Additional ^1H-^{13}C HMBC analyses showed connection between H-14 (δ_H = 5.54 and 6.04 ppm) and carbon atoms C-12 (δ_c = 136.7 ppm) and C-11(δ_c = 167.3 ppm), and H-16 (δ_H = 5.54 and 6.04 ppm) and carbon atoms C-2 (δ_c = 136.7 ppm) and C-1 (δ_c = 167.3 ppm). These observations are consistent with the presence of two identical α,β-unsaturated carbonyl groups in the structure of **1**. Moreover, a 3-hydroxy-2-methylpropanoate fragment was identified on the basis of COSY correlations between H-8 (δ_H = 3.46–3.56 ppm), H-7 (δ_H = 2.69–2.73 ppm) and H-9 (δ_H = 1.12 ppm), and HMBC correlations between H-7 (δ_H = 2.69–2.73 ppm) and C-6 (δ_c = 176.1 ppm). At least one ethylene moiety was assigned using COSY correlations between H-17 (δ_H = 1.48 ppm) and H18 (δ_H = 0.87 ppm). Other HMBC correlations from H-3 and H-10 to C-4, C-5 and C-17 allowed the connectivity between the three identified fragments and led to unambiguous assignment of the structure of **1** (Figure 1; Supporting Information, Figures S2–S4).

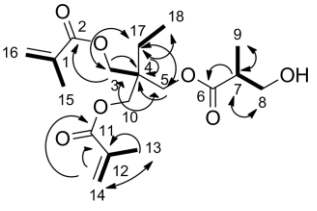

Figure 1. Key COSY (black lines) and HMBC (arrows H to C) correlations for cyaneodimycin (**1**).

Similar ^1H NMR assignments were observed for compound **2** with two major differences: (i) a further CH_2OH group (signal at δ_H = 3.57–3.67 ppm) and (ii) a methylene group (signal at δ_H = 5.62 and 6.14 ppm) integrating only for two protons relative to cyaneodimycin (**1**) (Table 1; Supporting Information, Figures S2 and S7). By analogy with the structure of **1**, we propose the chemical structure for cyaneomycin (**2**) shown in Figure 2.

Interestingly, HRESIMS analysis of compound **2** led to an $[M + H]^+$ ion at m/z = 203.0765, consistent with a formula of $C_{10}H_{19}O_4$. The difference from the molecular formula of **1** corresponds to the loss of two $C_4H_5O_2$ moieties, which could be

Table 1. NMR spectroscopic data for cyaneodimycin **1** and cyaneomycin **2** at 500 MHz.

Cyaneodimycin (**1**) (CD_2Cl_2)						Cyaneomycin (**2**) ($CDCl_3$)		
Position	Carbon type	δ_C	δ_H, mult (J [Hz])	HMBC[a]	COSY	Position	Carbon type	δ_H, mult (J [Hz])
1/12	C (quaternary)	136.7	–	–	–			
2/11	C (quaternary)	167.3	–	–	–			
3/10	CH_2	64.9	4.06, s	C17, C4, C5, C2/11	–	3	CH_2	4.30, s
4	C (quaternary)	42.2	–	–	–	4	C (quaternary)	–
5	CH_2	71.5	3.36–3.41, m	C17, C4, C3/10, C8	–	5	CH_2	3.57–3.67, m
6	C (quaternary)	176.1	–	–	–	6	C (quaternary)	–
7	CH	39.9	2.69–2.73, m	C9, C8, C6	H-9, H-8	7	CH	2.66, m
8	CH_2	73.4	3.46–3.56, m	C9, C7, C6	H-7	8	CH_2	3.57–3.67, m
9	CH_3	13.6	1.12, d (10)	C7, C8, C6	H-7	9	CH_3	n.d.[b]
						10	CH_2	3.57–3.67, m
13/15	CH_3	18.4	1.90, br. s	C1–12, C14/16	H-3/10, H-14/16	15	CH_3	1.96, br. s
14/16	CH_2	125.6	5.54, br. s; 6.04, br. s	C13/15, C1/12, C2/11	H-14/16	16	CH_2	5.62, br. s; 6.14, br. s
17	CH_2	23.6	1.48, q (7.5)	C18, C4, C3, C5	H-18	17	CH_2	1.32, q (7.5)
18	CH_3	7.6	0.87, t (7.5)	C17, C4	H-17	18	CH_3	0.90, t (7.5)

[a] HMBC correlations are from proton(s) to the indicated carbon. [b] n.d.: not determined due to masking by water signal.

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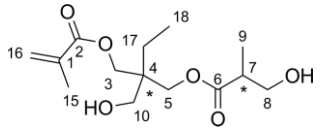

Figure 2. Chemical structure of cyaneomycin **2**.

explained by a hydrolysis process during the deuterium exchange (after the NMR analysis and before or during the MS/MS analysis). All assignments in the ^{1}H NMR data were nevertheless observed for cyaneomycin (**2**) (Table 1 and Supporting Information S7). Fragmentation mass spectrometry analysis (MS/MS) confirmed the presence of these new compounds and inferred the proposed structure. Indeed, several fragments were observed corresponding either to a loss of one, two or three $C_4H_6O_2$, H_2O groups or more complex structures (see Supporting Information S5–S6 and S8–S9). Nonetheless, it is not possible to exclude the possibility that **2** may also be an artefact formed by hydrolysis of **1** during the extraction process of *S. cyaneofuscatus*. The absolute configuration of C7 in compound **1** was determined to be (*R*) based on its optical rotation $[\alpha]_D^{20}$ = –9 (*c* = 0.1, $CHCl_3$) relative to that of commercially acquired (–)-(*R*)-3-hydroxy-2-methylpropionate with $[\alpha]_D^{20}$ = –14 (*c* = 0.1, $CHCl_3$). The absolute configurations of C4 and C7 in compound **2** remain unknown. Attempts at crystallization failed, and the use of electronic circular dichroism methods did not render any exploitable CD signatures. These failures could be explained by the high degree of flexibility of both molecules and by the distant positioning of the chromophore group from the asymmetric carbon atom in **2**. Unfortunately, the small amount of compound **2** that was obtained did not allow derivatization steps, which may be a good alternative to reduce those structural constraints. Cyaneodimycin (**1**) and cyaneomycin (**2**) represent rare examples of acrylate compounds from bacterial fermentation. To the best of our knowledge, **1** and **2** join only three other related natural products: cyrmenins A, B1 and B2, contain *N*-linked β-methoxyacrylate groups and are isolated from two Myxobacteria: *Cystobacter armeniaca* and *Archangium gephyra*.[22,23] Methacrylates **1** and **2** may originate from pyruvate via isobutyraldehyde and isobutyrylCoA leading to methacryloylCoA by a final oxidation step[24] (Figure 3). This methacryloylCoA could be the starter unit for the biosynthesis of **1** and **2**. While several control experiments so far rule out contamination, further experiments will be carried out to determine whether **1** and **2** are synthezised the novo (and by which pathways) or whether they are biotransformation products of synthetic molecules.

In addition to our studies of **1** and **2**, flash chromatography yielded two main fractions from the EtOAc crude extract (80 mg): F1 (68.5 mg oil) and F2 (4.5 mg). These two fractions were partitioned and ultimately enabled the characterization of six known compounds **3**–**8** (Figure 4) already isolated from several sources such as bacteria, sponges, fungi, lichens.[25–30] Semipreparative silica TLC [CH_2Cl_2/EtOAc/MeOH (15:15:3)] of F2 yielded 0.8 mg of compound **3** [3-(hydroxyacetyl)indole], whereas semipreparative HPLC (ACN/H_2O gradient) of F1 afforded usnic acid (**8**) (1 mg). Semipreparative silica TLC (EtOAc, 100 %) of this same fraction (F1) afforded compounds **4**–**7**,

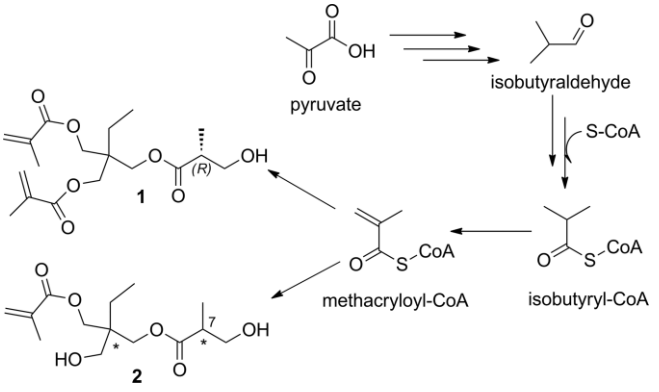

Figure 3. Proposed metabolic synthesis of methacryloylCoA.[24]

whose structural compositions are: *cyclo*-(Phe,Pro) (**4**) (1.2 mg), *cyclo*-(L-Leu,L-Pro) (**5**) (3.6 mg), *N*-acetyl-β-oxotryptamine (**6**) (2.2 mg) and *N*-methyldactinomycin (**7**) (1 mg). The isolation of **4** and **5** confirmed the validity of our dereplication approach. The molecular formula of **7** was determined to be $C_{63}H_{88}N_{12}O_{16}$ (with 26 points of unsaturation) based on HRESIMS and NMR analyses. This compound also showed a maximum absorbance at λ_{max} = 441 nm (in MeOH). All data revealed similarities with actinomycin D with the presence of one additional *N*-methyl group (δ_H = 2.81 ppm; δ_C = 54.80 ppm) (see Supporting Information S14 and S15). Compound **7** corresponds to the *N*-methyldactinomycin on the basis of comparisons with available data.[31] However, the chemical structure given in a patent[31] presents two structural errors but with correct NMR assignments. Consequently, we revised herein the chemical structure of *N*-methyldactinomycin (**7**) (Figure 4, see Supporting Information S14).

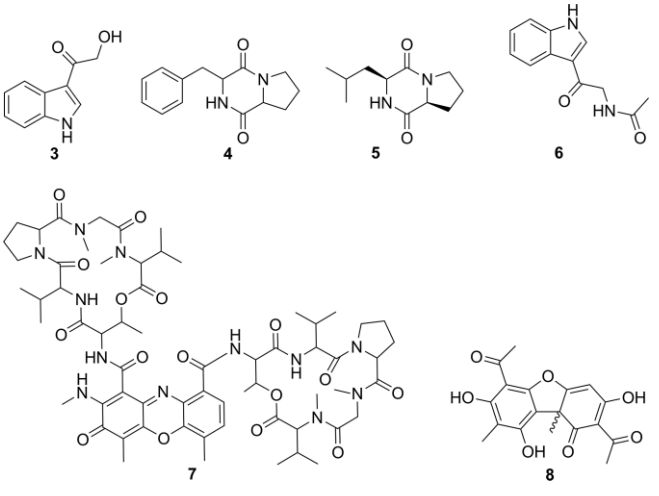

Figure 4. Known compounds **3**–**8** isolated from *Streptomyces cyaneofuscatus* MOLA1488 [**3**: (3-hydroxyacetyl)indole; **4**: *cyclo*-(L-Phe,L-Pro); **5**: *cyclo*-(L-Leu,L-Pro); **6**: *N*-acetyl-β-oxotryptamine; **7**: *N*-methyldactinomycin ; **8**: usnic acid].

Among the known compounds, usnic acid (**8**) (1 mg), a common cytotoxic secondary metabolite in lichens[30,32–35] was isolated and identified for the first time from bacteria.[36] Even though usnic acid is a very widespread lichen compound and is responsible for the green to greenish-yellow color of many

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lichens, this lichen compound has never been reported for the species *L. confinis* from which the present *S. cyaneofuscatus* producer was isolated. The molecular formula of $C_{18}H_{16}O_7$ obtained by HRESIMS and additional MS/MS and NMR spectroscopic data confirmed, on the basis of comparisons with available data,[37] the structural composition of metabolite **8** and its presence in the fermentation of *S. cyaneofuscatus* (see Supporting Information S16–S18). None of the compounds presented herein (**1**–**8**) were reported before. In fact, only four bioactive compounds such as daunomycin, cosmomycin, galtomycin B and maltophilin[38] were putatively detected (exclusively by HPLC-UV/Vis) from *S. cyaneofuscatus* (strain M27) isolated from an unknown lichen.

The MOLA1488 crude extract showed cytotoxic activities in cell viability assays (MTT assay) against murine melanoma cells (B16 cell lines) with an IC_{50} of 0.33 ± 0.2 μg/mL but also against HaCaT cell lines (normal cell lines) with an IC_{50} of 0.25 ± 0.1 μg/mL. Pure cyaneodimycin (**1**) was also tested for cytotoxic properties against the same cell lines and showed an IC_{50} of 27 ± 4 μM and 47 ± 11 μM against B16 and HaCaT cell lines, respectively. By comparison, doxorubicin, used as positive control, exhibited an IC_{50} of 0.008 ± 0.001 μM against B16 cells and an IC_{50} of 0.15 ± 0.08 μM against HaCaT cells. In addition, the activity of **1** was evaluated using leukemic cell lines (Jurkat cell lines) and showed an IC_{50} of 18.5 ± 0.5 μM after 72 h of incubation. The same compound was found to be less active (IC_{50} > 20 μM) after a 16 h incubation with Jurkat cells. The biological properties of cyaneomycin (**2**) were not determined due to its low availability. It was also not surprising that *N*-methyldactinomycin showed potent cytotoxic activities (IC_{50} ≈ 0.05 μM) against various normal or cancer cell lines after 48 h of incubation during preliminary assays (ImPACCell Platform, University of Rennes 1). Indeed, actinomycin compounds are well-known antitumor agents by virtue of their ability to bind DNA and inhibit RNA synthesis.[39] As a result, the presence of compound **7** explains the potent cytotoxic activities of the extract.

Conclusions

We have successfully isolated two new compounds: cyaneodimycin (**1**) and cyaneomycin (**2**) from *Streptomyces cyaneofuscatus* associated with *Lichina confinis*, and compound **1** showed interesting cytotoxic properties. Six previously known compounds were also isolated and reported for the first time from this bacterial species. Moreover, we have unexpectedly isolated a common lichen compound [usnic acid (**8**)] from a bacterial strain. This important observation highlights the possibility of horizontal gene transfer between partners in lichen symbioses. Phylogenetic analysis of polyketide synthase genes have already indicated such horizontal gene transfer events in fungi for the salicylic acid biosynthesis.[40,41] In the present case, *S. cyaneofuscatus* might also be associated with other lichens, in particular those known for usnic acid production, unless *Lichina* is capable of producing usnic acid. However, this capability has not been demonstrated in previously studied individuals. Further experiments and the full genome sequencing of *S. cyaneofuscatus* MOLA1488 are ongoing and will allow us to elucidate the evolutionary background of usnic acid biosynthesis in this bacterium as well as in lichens. From such efforts we will develop a better understanding of the biosynthetic production of methacrylate compounds in this bacterium.

Experimental Section

General Experimental Procedures: All commercial reagents were purchased from Carlo Erba Reactifs and/or from Sigma Aldrich (Val-de-Reuil, France and St Quentin Fallavier, France). For chromatographic analysis, HPLC and LC/MS grade water was obtained from an EasyPure (Barnstead, USA) water purification system. Deuterated solvents were purchased from Euriso-top (Gif-sur-Yvette, France). All spectra were recorded with a Bruker DMX 300 spectrometer [300 MHz (^{1}H) and 75 MHz (^{13}C)] and Bruker 500 cryo-spectrometer [500 MHz (^{1}H) and 125 MHz (^{13}C)] using adequate deuterium solvents. Chemical-shift values were referenced to residual solvent signals for $CDCl_3$ (δ_H/δ_C = 7.21/77.16 ppm) and CD_2Cl_2 (δ_H/δ_C = 5.32/53.84 ppm). HSQC, HMBC, COSY or TOCSY data were recorded using a Bruker DMX 500 cryo-spectrometer instrument. NMR spectroscopic data were processed using the MestReNova version 1.3 software. Optical rotations were measured using a Perkin–Elmer Model 341 polarimeter at 20 °C using a thermostable optical glass cell (1 dm path length and *c* in g/100 mL). HRMS measurements for exact mass determination were performed with a MICROMASS ZabspecTOF spectrometer for electrospray ionization at the CRMPO (Centre Régional de Mesures Physiques de l'Ouest), University of Rennes 1.

Microorganism: *Streptomyces cyaneofuscatus* MOLA1488 (gene bank accession number: KM273905.1) was isolated from the lichen *Lichina confinis* collected in Erquy (Northwest of Rennes, France, 48°37′45″ N, 02°28′30″ W) in April 2012.[3] To identify the strain, its 16S rRNA gene was sequenced using dideoxy termination Sanger sequencing as described previously.[3] Comparisons with sequences in the EzTaxon strain database[17] revealed that the closest phylogenetic neighbor of the strain was *Streptomyces cyaneofuscatus* AY999770, at 100 % sequence identity. The bacteria were stored after growth in Luria-Bertani broth medium (LB) [5 g yeast extract (Sigma–Aldrich, St Louis, MO), 10 g malt extract (Sigma–Aldrich, St Louis, MO) and 5 g NaCl (Sigma–Aldrich, St Louis, MO) for 1 L] with 50 % (v/v) glycerol or 5 % (v/v) DMSO at –80 °C (Banyuls/Mer collection, reference: MOLA1488).

Fermentation of *Streptomyces cyaneofuscatus* MOLA1488: MOLA1488 was cultivated in 50 mL test tubes containing 30 mL of LB medium [5 g yeast extract (Sigma–Aldrich, St Louis, MO), 10 g malt extract (Sigma–Aldrich, St Louis, MO) and 5 g of NaCl (Sigma–Aldrich, St Louis, MO) for 1 L]. The test tube was shaken on an orbital shaker (110 rpm) at 25 °C for 72 h. A 2.7 L (9 × 300 mL) volume of liquid LB medium was then inoculated each with 3 mL (or 1 %) with the test tube cultures.Flasks were incubated at 25 °C with shaking at 110 rpm over the course of 12 d. Sterile resin XAD-7-HP (5 g, Sigma–Aldrich, St Louis, MO), was added in each flask, and then each mixture was shaken under the same conditions (25 °C, 110 rpm) for 4 h. Cultures were centrifuged at 5000 rpm at 4 °C over the course of 15 min. The supernatant was removed, and solid residues (resin and bacteria cells) were lyophilized before further processing.

Extraction and Isolation: The solid residue of 2.7 L of fermentation culture was extracted three successive times with acetone/MeOH (50:50, v/v) (36.23 g). Acetone/MeOH extracts were dried in vacuo and three further successive extractions of the dried extract with

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EtOAc/H_2O (3:1, v/v) were realized. The organic phase (EtOAc extract) was collected and filtered using a Büchner funnel. The residual H_2O in this phase was removed by adding anhydrous sodium sulfate powder (5 g), and the organic phase was dried under vacuum to yield ca. 80 mg of crude extract. The latter was fractionated using various methods [SPOT flash liquid chromatography (Armen Instrument®), semipreparative HPLC and preparative TLC]. For flash liquid chromatography, the stationary phase was a silica column pre-packed normal phase (SiO_2) (FSHP-1207-0025, 25 g, Biotage), and the mobile phase was a gradient: CH_2Cl_2 (A)/EtOAc (B) (100:0 to 0:100 in 75 min). The following gradient was applied at a flow rate of 10 mL/min: initial, 100 % A; 0–10 min, 100 % A linear; 10–40 min, 50 % A linear; 40–45 min, 50 % A linear; 45–75 min, 0 % A linear, followed by washing the column with 100 % MeOH in 30 min. 105 fractions of 10 mL each were collected. Fractions 15–18 (F1) (68.5 mg viscous) and 19–22 (F2) (4.5 mg) were purified by preparative TLC (10–12 µm, Kieselgel 60 F_{254}, Merck 5554) using an automatic TLC sampler III, Camag®. Two solvent systems were applied: CH_2Cl_2/EtOAc/MeOH (15:15:3) for compound **3** and EtOAc (100 %) for compounds **4**–**7**. Consequently, five compounds were obtained in this manner.

3-(Hydroxyacetyl)indole (3): Pale yellow powder. R_f = 0.36 in CH_2Cl_2/EtOAc/MeOH (15:15:3). ^1H NMR and ^{13}C NMR data ($CDCl_3$, 125 MHz) as described in the literature.[28] HRESIMS: m/z = 198.052558 $[M + Na]^+$ (calcd. for $C_{10}H_9NO_2Na$ 198.051453).

***cyclo*-(Phe,Pro) (4):** Yellow powder. R_f = 0.50 in EtOAc (100 %). ^1H NMR and ^{13}C NMR data ($CDCl_3$, 125 MHz) as described in the literature.[26] HRESIMS: m/z = 267.110407 $[M + Na]^+$ (calcd. for $C_{14}H_{16}N_2O_2Na$ 267.109302).

***cyclo*-(L-Leu,L-Pro) (5):** Yellow powder. R_f = 0.38 in EtOAc (100 %). ^1H NMR and ^{13}C NMR data ($CDCl_3$, 125 MHz) as described in the literature.[26] HRESIMS: m/z = 233.126057 $[M + Na]^+$ (calcd. for $C_{11}H_{18}N_2O_2Na$ 233.124952).

***N*-Acetyl-β-oxotryptamine (6):** Yellow powder. R_f = 0.75 in EtOAc (100 %). ^1H NMR and ^{13}C NMR data ($CDCl_3$, 125 MHz) as described in the literature.[25] HRESIMS: m/z = 239.078054 $[M + Na]^+$ (calcd. for $C_{12}H_{12}N_2O_2Na$ 239.078002).

***N*-Methyldactinomycin (7):** Yellow powder. R_f = 0.59 in EtOAc (100 %). λ_{max}(MeOH) = 441 nm. ^1H NMR data ($CDCl_3$, 500 MHz) and ^{13}C NMR data ($CDCl_3$, 125 MHz) available in Table S1 and comparable to literature values.[31] LRESIMS: m/z = 1268.3300 $[M + H]^+$ (calcd. for $C_{63}H_{88}N_{12}O_{16}$ 1268.643576). Separation and purification by semipreparative HPLC was also performed from the EtOAC extract (compounds **1**–**2**) or from F1 (compound **8**) using as first system (S1) a Kromasil C_{18}, 100A column (5 µm, 250 × 10 mm). A gradient system was applied: A (water) and B (acetonitrile). The following gradient was performed at a flow rate of 3 mL/min in the HPLC system: initial, 99 % A; 0–5 min, 99 % A linear; 5–7 min, 90 % A linear; 7–15 min, 75 % A linear; 15–17 min, 75 % A linear; 17–25 min, 50 % A linear; 25–27 min, 50 % A linear; 27–40 min, 0 % A linear; followed by washing and reconditioning of the column. For compound **2** a semipreparative HPLC was performed using as second system (S2) a Prevail Grace C_{18} (5 µm, 250 × 10 mm) column with the following gradient: A (water) and B (acetonitrile) performed at a flow rate of 2.5 mL/min in the HPLC system: initial, 100 % A; 0–5 min, 100 % A linear; 5–35 min, 0 % A linear; 35–45 min, 0 % A linear. Three compounds (**1–2** and **8**) were isolated.

Cyaneodimycin (1): Yellow powder. $[\alpha]_D^{20}$ = –9 (c = 0.1, $CHCl_3$). t_R = 27.50 min in acetonitrile/H_2O gradient (S1). ^1H NMR, ^{13}C NMR and 2D NMR data ($CDCl_3$, 500 MHz) are available in Table 1. HRESIMS: m/z = 379.171598 $[M + Na]^+$ (calcd. for $C_{18}H_{28}O_7Na$ 379.171628).

Cyaneomycin (2): Yellow powder. t_R = 22.96 min in acetonitrile/H_2O gradient (S2). ^1H NMR data ($CDCl_3$, 500 MHz) are available in Table 1. HRESIMS of the cyaneomycin hydrolyzed: m/z = 203.1277 $[M + H]^+$ (calcd. for $C_{10}H_{19}O_4$ 203.12833).

Usnic Acid (8): Pale yellow powder. t_R = 9.55 min in acetonitrile/H_2O gradient (S1). ^1H NMR data comparable to literature values.[42] HRESIMS: m/z = 343.0824 $[M – H]^-$ (calcd. for $C_{18}H_{15}O_7$ 343.082326).

Mass Spectrometry Analysis: Mass spectrometry analysis was carried out using an LC-ESI and ESI-MS^n mass spectrometers as already described.[35,43] A Prevail C_{18} column (5 µm, 250 × 4.6 mm, GRACE®) kept at 30 °C was used. For HPLC a gradient system was applied: A (0.1 % formic acid in water) and B (0.1 % formic acid in acetonitrile). The following gradient was applied at a flow rate of 1 mL/min in the HPLC system: initial, 99 % A; 0.01–5 min, 90 % A linear; 5–7 min, 90 % A linear; 7–15 min, 75 % A linear; 15–17 min, 75 % A linear; 17–25 min, 50 % A linear; 25–27 min, 50 % A linear; 27–40 min, 0 % A linear; followed by washing and reconditioning of the column. A split to 0.2 mL/min was applied before the mass spectrometry system. 20 µL were injected. The MS^n spectra were recorded during the HPLC run using the following conditions: MS/MS analysis with starting collision-induced dissociation energy of 35 eV. The Xcalibur 1.0 software was used for data analyses. Mass spectrometry data were analyzed (molecular networking techniques, dereplication workflow, etc.) with the GNPS Platform (GNPS at http://gnps.ucsd.edu).[18]

Biological Assays: Cytotoxic properties of extracts and pure compounds were determined with a standard tetrazolium-based assay.[44] For the total EtOAc organic extract and cyaneodimycin (**1**), B16 cells were seeded at 15000 cells/mL and HaCaT cells were seeded at 17000 cells/mL in RPMI1640 medium with 5 % fetal calf serum (FCS) at day 0 in the appropriate well plates. Incubation was performed at 37 °C in an atmosphere of 5 % CO_2. After 48 h of incubation, cell growth and viability were measured at 540 nm, using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The cytotoxic activity of cyaneodimycin (**1**) was determined against Jurkat cells seeded at 40000 cells/mL in RMPI media after 16 h (cytotoxic effect) and 5000 cells/mL in RMPI media after 72 h (cytostatic effect) of incubation at 37 °C by the MTT assay above. Each experiment was repeated at least three times.

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