

## Nocardichelins A and B, Siderophores from *Nocardia* Strain Acta 3026<sup>#</sup>

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An actinomycete, strain Acta 3026, isolated from mangrove soil was characterized and found to belong to the genus *Nocardia*. The strain produces two new cytotoxic metabolites, nocardichelins A (**1**) and B (**2**). Each of the compounds strongly inhibited human cell lines from gastric adenocarcinoma, breast carcinoma, and hepatocellular carcinoma with GI<sub>50</sub> values in a low micromolar to nanomolar range. The structural characterization of the compounds was performed by mass spectrometry and NMR spectroscopy. The nocardichelins represent a new group of siderophores that combine the structural elements of mycobactin-type siderophores from mycobacteria and hydroxamate-type siderophores (desferrioxamine B) produced by streptomycetes. The chromazurol S assay, characteristic for iron(III) complexation, was positive, confirming the role as a siderophore.

Many taxonomically diverse actinomycetes were isolated from a range of European and Malaysian habitats using innovative selective isolation procedures in a project designed to discover novel metabolites. Special attention was directed toward strains isolated from mangrove habitats, as they contain organisms adapted to the marine environment.<sup>1</sup> Representatives of freshly isolated strains were grown in submerged culture in different media, and extracts prepared from mycelia and culture filtrates at various fermentation times were screened by HPLC diode array analysis in combination with an in-house-developed HPLC–UV–vis database<sup>2</sup> to detect novel secondary metabolites for pharmaceutical applications. Strain Acta 3026 was found to be of special interest, as it gave a mycelium extract that contained two related and dominant metabolites. The characteristic spectrum of these metabolites differed from that of 867 reference compounds stored in the HPLC–UV–vis database. This report deals with the taxonomy of the producing strain, fermentation, isolation, structural elucidation, and biological evaluation of the isolated metabolites.

### Results and Discussion

Strain Acta 3026 was isolated from rhizosphere soil samples of a species of *Sonneratia* that had been collected from the mangrove forests at Morib, Selangor, on the west coast of Malaysia. The strain grew well on modified *Micromonospora* agar (MMA),<sup>3</sup> producing an orange substrate mycelium but neither aerial hyphae nor diffusible pigments. The isolate gave whole-organism hydrolysates rich in *meso*-diaminopimelic acid, arabinose, and galactose and contained mycolic acids that comigrated with those of marker *Nocardia* strains, chemotaxonomic properties typical of members of the genus *Nocardia*.<sup>4</sup> This assignment was confirmed by 16S rRNA gene sequence data, which showed that the isolate formed a distinct phyletic line in the *Nocardia* gene tree together with the type strain of *Nocardia carnea* (Figure 1). The 16S rRNA gene sequence is deposited in Genbank under the accession no. EF502100.

Strain Acta 3026 was cultivated in 500 mL Erlenmeyer shake flasks using a complex medium. The production of nocardichelins started after about 3 days, increasing over 7 days of fermentation to reach a maximal amount of 54 and 7.5 mg/L nocardichelin A (**1**) and B (**2**), respectively. The cultures were harvested at maximal nocardichelin production, and metabolites **1** and **2** were isolated from the mycelium by extraction with methanol/acetone. The nocardichelins were re-extracted with *n*-butanol following concentration to an aqueous residue. Pure compounds were obtained by subsequent chromatography using Sephadex LH-20, Toyopearl HW-40 F, and preparative reversed-phase HPLC.

The HPLC-ESIMS spectra revealed molecular masses for **1** [(M + H)<sup>+</sup> = 744.2] and **2** [(M + H)<sup>+</sup> = 716.3]. The exact molecular masses were determined by high-resolution ESI-FT-ICR-MS (Fourier-transform ion cyclotron resonance mass spectrometry), as 744.490588 Da [(M + H)<sup>+</sup>] (**1**) and 716.459100 Da [(M + H)<sup>+</sup>] (**2**), corresponding to the molecular formulas C<sub>40</sub>H<sub>65</sub>N<sub>5</sub>O<sub>8</sub> (**1**) [(M + H)<sup>+</sup><sub>theor</sub> = 744.490591; Δ*m* = 0.003 ppm] and C<sub>38</sub>H<sub>61</sub>N<sub>5</sub>O<sub>8</sub> (**2**) [(M + H)<sup>+</sup><sub>theor</sub> = 716.459290; Δ*m* = 0.266 ppm]. The ESI-FT-ICR-MS measurement of a sample from an intermediate purification step of **1** showed a molecular mass of 797.402054 Da [(M – 3H + Fe + H)<sup>+</sup>]. This corresponds well with the molecular formula C<sub>40</sub>H<sub>62</sub>N<sub>5</sub>O<sub>8</sub>Fe [(M – 3H + Fe + H)<sup>+</sup><sub>theor</sub> = 797.402058; Δ*m* = 0.005 ppm], suggesting that the compounds act as siderophores. Additional experiments described below confirmed this presumption, and as a consequence, the compounds were named nocardichelins A (**1**) and B (**2**).

Amino acid and fatty acid analyses by GC-MS revealed almost all of the constituents needed for the assembly of **1** and **2**, namely, cadaverine, salicylic acid, L-serine, and 2-*cis*-hexadecanoic acid for compound **1** and 2-*cis*-tetradecanoic acid for compound **2**. With the help of tandem MS and 2D NMR experiments the structures of **1** and **2** could be fully elucidated (Figure 2).

The <sup>1</sup>H NMR spectrum of nocardichelin (**1**) showed four signals in the aromatic region around 7 ppm (salicylic acid) and two peaks around 6 ppm assigned to the double bond of the fatty acid. The coupling constant of *J* = 10.24 Hz (**1**) supported the assignment of a *cis*-double bond, as was found from GC-MS experiments. A major peak at 1.22 ppm among a number of signals in the aliphatic region corresponded to 12 CH<sub>2</sub> groups. The correlation of <sup>1</sup>H NMR signals to the corresponding C atoms was carried out by a heteronuclear multiple quantum coherence (HMQC) NMR experi-

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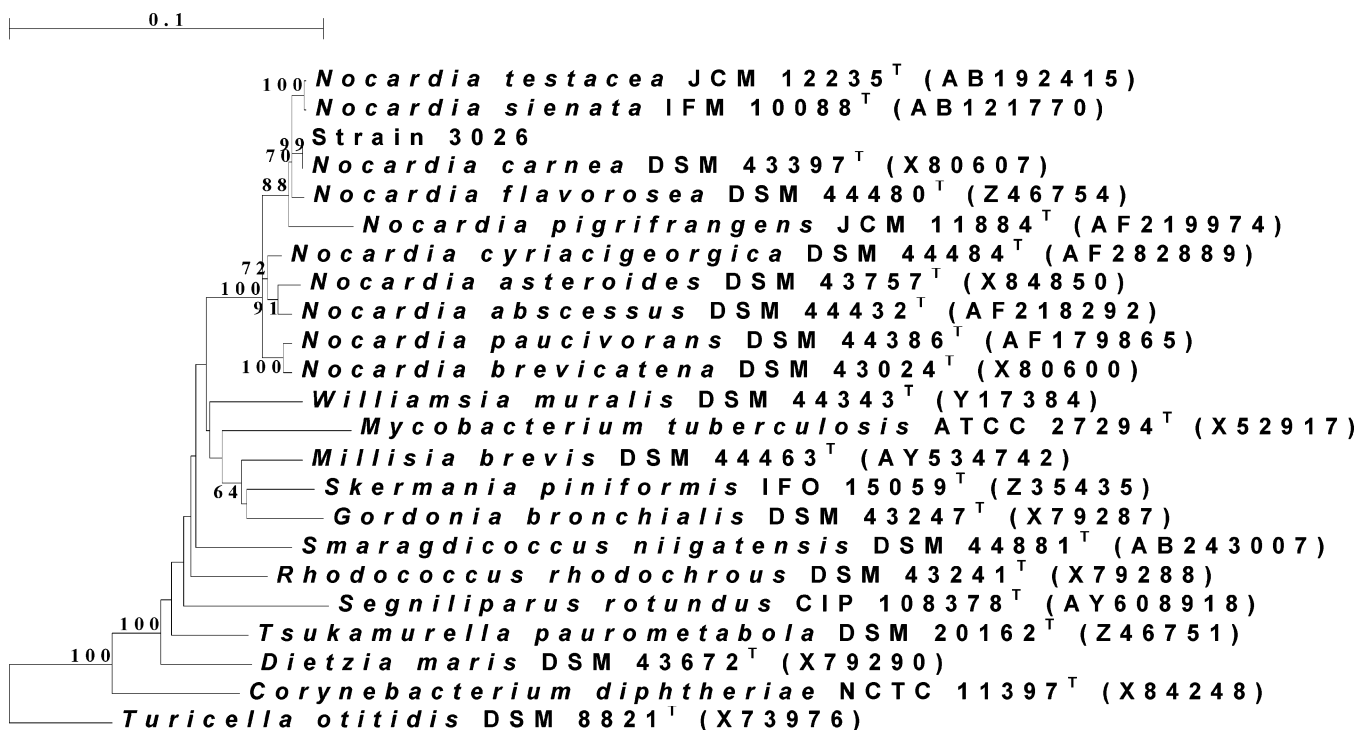
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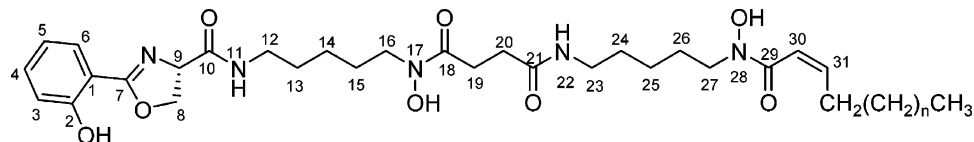
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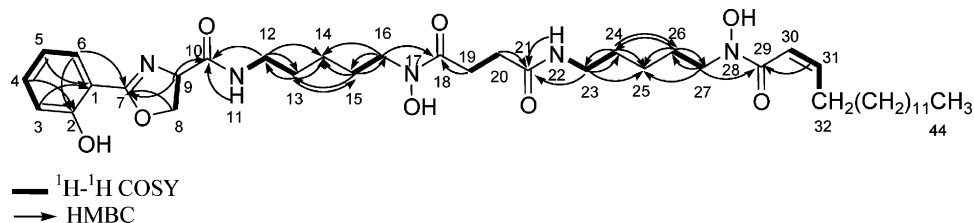
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**Figure 1.** Neighbor-joining tree according to Saitou and Nei<sup>5</sup> based on a nearly complete 16S rRNA gene sequence of strain 3026 showing its position in the *Nocardia* clade. The numbers at the nodes indicate the levels of bootstrap support based on a neighbor-joining analysis of 1000 resampled data sets; only values above 50% are given. The scale bar indicates 0.1 substitution per nucleotide position.



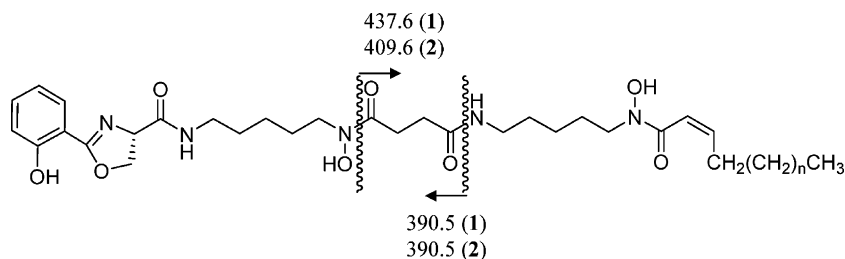
**Figure 2.** Structures of nocardichelins A (1) and B (2). Nocardichelin A (1):  $n = 11$ . Nocardichelin B (2):  $n = 9$ .



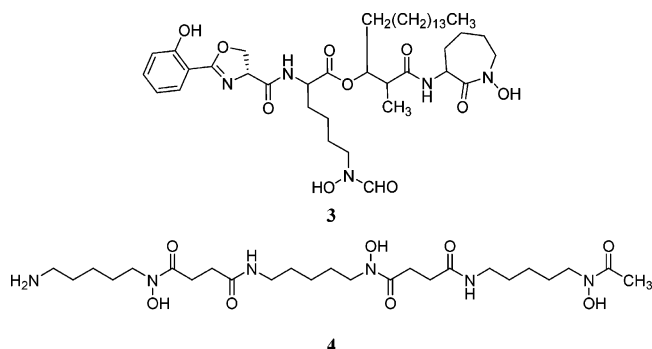
**Figure 3.** Selected 2D NMR correlations for nocardichelin A (1).

ment. Five signals in the <sup>1</sup>H spectrum could not be assigned to any C atom, suggesting the presence of five heteroatom-bound protons (OH or NH). The assignments of NMR spin systems to the constituents mentioned above were derived from the COSY and TOCSY experiments. The <sup>1</sup>H-<sup>1</sup>H-COSY experiment revealed the following spin systems: H-3 to H-6, H<sub>2</sub>-8 to H-9, H<sub>2</sub>-19 to H<sub>2</sub>-20, H-30 to H<sub>2</sub>-33, and H<sub>2</sub>-43 to H<sub>3</sub>-44 (Figure 3). The spin systems H-11 to H<sub>2</sub>-16 and H-22 to H<sub>2</sub>-27 could not be established unambiguously since H<sub>2</sub>-14 and H<sub>2</sub>-25 have the same <sup>1</sup>H chemical shift as the fatty acid methylene signals. This problem was solved with the observed connectivities in the HMBC experiment (Figure 3). The correlations from H<sub>2</sub>-12 to C-13 and C-14, from H<sub>2</sub>-13 to C-12, C-14, and C-15, from H<sub>2</sub>-15 to C-13, C-14, and C-16, from H<sub>2</sub>-16 to C-14 and C-15, from H<sub>2</sub>-23 to C-24 and C-25, from H<sub>2</sub>-24 to C-23, C-25, and C-26, from H<sub>2</sub>-26 to C-24, C-25, and C-27, and from H<sub>2</sub>-27 to C-25 and C-26 gave proof for the assignments of the two cadaverine constituents (Figure 3). Only one NH proton could be assigned for each of the cadaverines. This suggested two OH groups, presumably those with the chemical shift of 9.60–9.64 ppm, directly bound to the N-17 and N-28 of the cadaverines to constitute two hydroxamates. The <sup>13</sup>C chemical shifts of CH<sub>2</sub>-

12 ( $\delta$  38.5 ppm) and CH<sub>2</sub>-23 ( $\delta$  38.2 ppm) compared to the chemical shifts of CH<sub>2</sub>-16 ( $\delta$  46.8 ppm) and CH<sub>2</sub>-27 ( $\delta$  46.6 ppm) strongly support this assumption. All interconnectivities of the nocardichelin constituents were deduced from HMBC experiments (Figure 3). The HMBC correlation from H<sub>2</sub>-8, H-9, and H-6 to C-7 suggested a ring closure between the serine and the 2-hydroxybenzoic acid to form 7-(*O*-hydroxyphenyl)-8,9-dihydrooxazolecarboxylic acid. This was in accordance with the absence of a signal for the OH group of serine. The 7-(*O*-hydroxyphenyl)-8,9-dihydrooxazolecarboxylic acid is connected with a cadaverine via an amide group, which is supported by the observed HMBC correlations from H-9, H-11, and H<sub>2</sub>-12 to C-10. This cadaverine forms a hydroxamate group with a succinic acid, confirmed by the correlations from H<sub>2</sub>-16 and H<sub>2</sub>-19 to C-18. The correlations from H<sub>2</sub>-20, H-22, and H<sub>2</sub>-23 to C-21 showed that the succinic acid is connected to the second cadaverine via an amide group, while the correlations from H<sub>2</sub>-27 and H-31 to C-29 reveal that the second cadaverine also forms a hydroxamate group with the fatty acid. Combining the results of the COSY, TOCSY, and HMBC experiments the constituent connecting the two cadaverine units of the compound was succinic acid. This assignment was confirmed independently



**Figure 4.** Fragment ions of nocardichelins A (1) and B (2). Nocardichelin A (1):  $n = 11$ . Nocardichelin B (2):  $n = 9$ .



**Figure 5.** Structures of brasilibactin A (3) and desferrioxamine B (4).

from the MS/MS fragmentation. Two of the most dominant fragments,  $m/z$  437.5 and 390.5, provided further proof for the presence of succinic acid (Figure 4).

By comparing the molecular formulas of **1** and **2** and considering the results of the fatty acid analysis from GC-MS the difference in nocardichelin A (**1**) and nocardichelin B (**2**) lies in the fatty acid chain length. This was confirmed by 1D and 2D NMR and MS/MS experiments. Figure 4 shows the corresponding fragments at  $m/z$  409.6 and 390.5 of nocardichelin B (**2**).

Nocardichelins A (**1**) and B (**2**) represent a new group of siderophores isolated from a *Nocardia* strain. Members of this genus are a rich source of siderophores, including nocobactin NA,<sup>6</sup> formobactin,<sup>7</sup> BE 32060,<sup>8</sup> amamistatin,<sup>9</sup> asterobactin,<sup>10</sup> brasilibactin A,<sup>11</sup> and nocardimicins.<sup>12</sup> The chemical structures of these siderophores are closely related to mycobactin siderophores isolated from the taxonomically related genus *Mycobacterium*.<sup>12,13</sup> In contrast, comparison of nocardichelins **1** and **2** to brasilibactin A<sup>11</sup> (Figure 5) reveals that only the 4,5-dihydro-2-(2-hydroxyphenyl)-4-oxazolecarboxylic acid moiety is identical. The other part of the nocardichelin structure, particularly the cadaverine–succinic acid–cadaverine motif, shows a greater resemblance to desferrioxamine B (Figure 5).<sup>15</sup> However, instead of an acetyl group attached to the hydroxamate as in desferrioxamine, the nocardichelins contain an unsaturated fatty acid (Figure 2); the latter putatively serves as a membrane anchor and confers a relatively hydrophobic character to the molecules.

With regard to the chemical structure nocardichelins may be considered as hybrids of desferrioxamins and mycobactins, since structural elements of both siderophores are unified. In addition, we suggest the hydroxy group of the phenol at C-2 and the two hydroxamate groups to be putative iron-chelating functional groups of the nocardichelins. In conclusion, the nocardichelins represent a new group of siderophores that combine the structural elements of mycobactin-type siderophores isolated from mycobacteria and nocardiae with the elements of a typical representative hydroxamate siderophore, desferrioxamine B, produced by streptomycetes.

The iron-chelating properties of nocardichelins A and B were confirmed by a positive reaction using the chromazurol S assay.<sup>16</sup> Neither **1** nor **2** showed growth inhibition against Gram-positive and Gram-negative bacteria or against yeasts and filamentous fungi. The cytostatic effects of **1** and **2** were tested against various human

**Table 1.** Cytostatic Activities of Nocardichelins A (1) and B (2) against Selected Human Tumor Cell Lines

	GI <sub>50</sub> <sup>a</sup>			TGI <sup>b</sup>		
	AGS	Hep G2	MCF 7	AGS	Hep G2	MCF 7
<b>1</b>	28.2 nM	282.4 nM	201.7 nM	201.7 nM	470.7 nM	538.0 nM
<b>2</b>	44.7 nM	69.9 nM	1.13 μM	1.5 μM	335.4 nM	3.49 μM

<sup>a</sup> GI<sub>50</sub>: 50% growth inhibition. <sup>b</sup>TGI: 100% growth inhibition.

tumor cell lines. Both compounds showed a strong growth inhibitory activity toward AGS, Hep G2, and MCF 7 tumor cells (see Table 1).

### Experimental Section

**General Experimental Procedures.** Optical rotations were recorded on a Perkin-Elmer 341 polarimeter, IR spectra were acquired in the ATR (attenuated total reflectance) mode on a Perkin-Elmer 881 infrared spectrometer, and UV spectra were acquired on an Ultrospec 2100 pro spectrophotometer. NMR experiments were performed on a DRX 500 NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a BBI probehead with z gradients. LC-MS experiments were performed on an Applied Biosystems QTrap 2000 (Applied Biosystems, Darmstadt, Germany) coupled to an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany). ESI-FT-ICR mass spectra were recorded on an APEX II FTICR mass spectrometer (4.7 T, Bruker-Daltonics, Bremen, Germany).

**Producing Strain.** The *Nocardia* strain was isolated from a soil sample (pH 7.96 ± 0.02) taken from the rhizosphere of a species of *Sonneratia*. Randomly selected seedlings of plants and associated soil were carefully dug out at low tide in the intertidal mangrove forest of Morib, Selangor, on the west coast of Malaysia and air-dried for 5 days. A suspension of 1 g of air-dried rhizosphere soil was prepared in 9 mL of sterile solution of 6% yeast extract and 0.05% sodium dodecyl sulfate. After sonication for 30 min at 25 ± 2 °C, the 10<sup>-1</sup> dilution of the rhizosphere soil was heat-treated at 40 °C for 20 min in a shaking water bath,<sup>17</sup> and 0.1 mL of the 10<sup>-3</sup> dilution was plated onto starch–casein–nitrate agar (SCA)<sup>18</sup> supplemented with filter-sterilized 20 μg/mL nalidixic acid, 50 μg/mL cycloheximide, and 50 μg/mL nystatin. The inoculated plates were incubated at 28 ± 2 °C for five weeks, when an axenic culture of the producing strain was detected and then subcultured onto modified MMA medium.<sup>3</sup> Standard procedures were used for the extraction and analysis of mycolic acids, whole-organism sugars, and the isomeric form of diaminopimelic acid,<sup>19</sup> using appropriate controls. 16S rRNA gene sequencing was carried out after Kim et al.<sup>20</sup> and the resultant sequence compared with corresponding sequences of *Nocardia*-type strains using the neighbor-joining algorithm.<sup>21</sup>

**Screening, Fermentation, and Isolation.** Sample preparation of the culture broth and HPLC diode array analysis of mycelium and culture filtrate extracts were performed according to the protocol described by Fiedler.<sup>2</sup> Strain Acta 3026 was cultivated at 27 °C on a rotary shaker at 120 rpm for 7 days in 500 mL Erlenmeyer flasks with one baffle in a medium consisting of glucose (1%), soluble starch (2%), yeast extract (0.5%), casein peptone (0.5%), NaCl (0.01%), and CaCO<sub>3</sub> (0.1%) in tap H<sub>2</sub>O; the pH was adjusted to 7.6 (1 N NaOH) prior to sterilization. The cultured broth from the 50 Erlenmeyer flasks (4.6 L) was filtered, and the mycelium cake extracted with MeOH/acetone (150 mL × 3). After concentration *in vacuo* the aqueous residue was extracted with *n*-BuOH (50 mL). The raw extract (457 mg) was dissolved in a small volume of MeOH and added to a Sephadex LH-20 column (2.5 × 90 cm; eluent MeOH, flow rate 30 mL/h, UV detection at 310 nm). Fractions containing **1** and **2** were combined (235 mg) and then

separated on a Toyopearl HW-40 F column (same conditions as Sephadex column), resulting in a pure fraction of **1** (14.4 mg) and a mixed fraction of **1** and **2** (83.4 mg). To obtain pure **2**, a part of the mixed fraction was subjected to preparative HPLC using a C18 column (Grom-Sil 300 ODS-5 St, 10  $\mu$ m, 2  $\times$  25 cm) with CH<sub>3</sub>CN/0.1% HCOOH (a linear gradient from 65% to 80% CH<sub>3</sub>CN over 35 min) at a flow rate of 15 mL/min. A total of 7.0 mg of pure **2** was obtained.

**Biological Assays.** An agar plate diffusion assay was used to determine antibacterial and antifungal activities. Briefly, 10  $\mu$ L aliquots of the samples were applied to filter disks (6 mm diameter), which were placed onto inoculated test plates that were incubated for either 24 h (bacteria) or 48 h (fungi) at temperatures that permitted optimal growth of the test organisms.

The inhibitory activities of **1** and **2** on the growth of tumor cells was tested according to NCI guidelines<sup>22</sup> using human cell lines from gastric adenocarcinoma (AGS), breast carcinoma (MCF 7), and hepatocellular carcinoma (Hep G2). Cells were grown in 96-well microtiter plates in RPMI 1640 with 10% fetal calf serum in a humidified atmosphere of 5% CO<sub>2</sub> in air. After 24 h **1** and **2** (*c* = 0.1–10  $\mu$ g/mL) were added as DMSO solutions to the cells and the cells were cultivated for an additional 48 h. Stock solutions were prepared in DMSO; the final DMSO concentration of the cultures was 0.1%. The cell count was surveyed by protein determination with sulforhodamine B.

**Amino Acid and Fatty Acid Analysis.** The composition and configuration of the amino acid and diamines were determined after hydrolysis in 6 M HCl at 110 °C for 21 h. The dry hydrolysate was derivatized to the *N*-(*O*-TFA/ethyl esters and analyzed by chiral GC-MS on a 20 m  $\times$  0.25 mm Lipodex E/PS255 (30:70) capillary column. L-Serine was identified by its EI-mass spectrum, and its configuration was determined by comparison of retention times (nocardichelin A, 21.65 min; nocardichelin B, 21.69 min; D-ser, 20.99 min; L-ser, 21.66 min).

Fatty acids were cleaved from **1** and **2** by methanolysis (0.8 M methanolic HCl at 70 °C for 1.75 h), and the methyl esters were extracted with *n*-hexane and analyzed by GC-MS on a DB-5 capillary. Longer times of hydrolysis (as for amino acid analysis) or methanolysis led to increased isomerization to the more stable *trans*-isomers of the fatty acids.

**Nocardichelin A (1):** colorless solid;  $[\alpha]_D^{20} +11.8$  (*c* 0.187, MeOH); UV (MeOH)  $\lambda_{max}$  (log  $\epsilon$ ) 217 (1.24), 242 (1.00), 248.5 (0.98), 303.5 (0.44); IR  $\nu_{max}$  3304, 3155, 2955, 2914, 2848, 1728, 1638, 1539, 1465, 1260, 1072, 745 cm<sup>-1</sup>; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 500 MHz)  $\delta$  11.7 (1H, br s, -OH-2), 9.64–9.60 (2H, br s, -OH-17, -OH-28), 8.29 (1H, t, *J* = 5.34 Hz, H-11), 7.76 (1H, t, *J* = 5.23 Hz, H-22), 7.62 (1H, dd, *J* = 7.90, 1.5 Hz, H-6), 7.45 (1H, br t, *J* = 7.90 Hz, H-4), 6.99 (1H, d, *J* = 8.30 Hz, H-3), 6.93 (1H, br t, *J* = 7.60 Hz, H-5), 6.29 (1H, d, *J* = 10.24 Hz, H-30), 5.97 (1H, m, H-31), 4.89 (1H, dd, *J* = 7.92, 7.75 Hz, H-9), 4.61 (1H, dd, *J* = 8.53 Hz, H-8a), 4.51 (1H, dd, *J* = 8.14, 7.92 Hz, H-8b), 3.49–3.43 (4H, m, H-27, H-16), 3.08 (2H, m, H-12), 2.99 (2H, m, H-23), 2.56 (2H, t, *J* = 7.26 Hz, H-19), 2.49 (not detectable in the <sup>1</sup>H NMR spectrum, but visible in HMQC, H-32), 2.25 (2H, t, *J* = 7.26 Hz, H-20), 1.51–1.48 (4H, m, H-26, H-15), 1.46 (2H, m, H-13), 1.38–1.36 (4H, m, H-24, H-33), 1.21–1.22 (24H, m, H-14, H-25, H-34 to H-43), 0.84 (3H, t, *J* = 6.84 Hz, H-44); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 500 MHz)  $\delta$  171.9 (C, C-18), 171.2 (C, C-21), 169.3 (C, C-10), 166.0 (C, C-29), 165.6 (C, C-7), 159.0 (C, C-2), 144.2 (CH, C-31), 133.7 (CH, C-4), 127.7 (CH, C-6), 119.4 (CH, C-30), 118.8 (CH, C-5), 116.3 (CH, C-3), 109.9 (C, C-1), 69.0 (CH<sub>2</sub>, C-8), 67.2 (CH, C-9), 46.8 (CH<sub>2</sub>, C-16), 46.6 (CH<sub>2</sub>, C-27), 38.5 (CH<sub>2</sub>, C-12), 38.2 (CH<sub>2</sub>, C-23), 31.0 (CH<sub>2</sub>, C-42), 29.7 (CH<sub>2</sub>, C-20), 28.4 (2 CH<sub>2</sub>, C-13, C-24), 28.2 (2 CH<sub>2</sub>, C-32, C-33), 28.x (8CH<sub>2</sub>, C-34 to C-41), 27.3 (CH<sub>2</sub>, C-19), 25.9 (CH<sub>2</sub>, C-15), 25.6 (CH<sub>2</sub>, C-26), 23.5 (CH<sub>2</sub>, C-25), 23.4 (CH<sub>2</sub>, C-14), 22.1 (CH<sub>2</sub>, C-43), 13.7 (CH<sub>3</sub>, C-44).

**Nocardichelin B (2):** colorless solid;  $[\alpha]_D^{20} +6$  (*c* 0.3, MeOH); UV (MeOH)  $\lambda_{max}$  (log  $\epsilon$ ) 217 (1.38), 242.5 (1.15), 248 (1.13), 304 (0.57); IR  $\nu_{max}$  3304, 3124, 2955, 2914, 2848, 1729, 1638, 1539, 1466, 1260,

1072, 745 cm<sup>-1</sup>; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 500 MHz)  $\delta$  11.7 (1H, br s, -OH), 9.68 (1H, br s, -OH-2), 9.62 (1H, br s, -OH-17, -OH-28), 8.31 (1H, t, *J* = 5.49 Hz, H-11), 7.78 (1H, t, *J* = 5.33 Hz, H-22), 7.62 (1H, dd, *J* = 7.80, 1.53 Hz, H-6), 7.45 (1H, br t, *J* = 7.81 Hz, H-4), 6.99 (1H, d, *J* = 8.31 Hz, H-3), 6.93 (1H, br t, *J* = 7.71 Hz, H-5), 6.29 (1H, d, *J* = 11.14 Hz, H-30), 5.97 (1H, m, H-31), 4.89 (1H, dd, *J* = 7.80, 7.70 Hz, H-9), 4.60 (1H, dd, *J* = 8.42 Hz, H-8a), 4.51 (1H, dd, *J* = 8.14, 7.80 Hz, H-8b), 3.49–3.44 (4H, m, H-27, H-16), 3.08 (2H, m, H-12), 2.99 (2H, m, H-23), 2.56 (2H, t, *J* = 7.25 Hz, H-19), 2.49 (not detectable in the <sup>1</sup>H NMR spectrum, but visible in HMQC, H-32), 2.25 (2H, t, *J* = 7.25 Hz, H-20), 1.50–1.48 (4H, m, H-26, H-15), 1.46 (2H, m, H-13), 1.37–1.34 (4H, m, H-24, H-33), 1.21–1.22 (20H, m, H-14, H-25, H-34 to H-41), 0.84 (3H, t, *J* = 6.73 Hz, H-42); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 500 MHz)  $\delta$  172.0 (C, C-18), 171.3 (C, C-21), 169.3 (C, C-10), 165.6 (2C, C-7, C-29), 159.0 (C, C-2), 144.3 (CH, C-31), 133.7 (CH, C-4), 127.8 (CH, C-6), 119.5 (CH, C-30), 118.8 (CH, C-5), 116.4 (CH, C-3), 109.9 (C, C-1), 69.0 (CH<sub>2</sub>, C-8), 67.3 (CH, C-9), 46.7 (CH<sub>2</sub>, C-16), 46.6 (CH<sub>2</sub>, C-27), 38.5 (CH<sub>2</sub>, C-12), 38.2 (CH<sub>2</sub>, C-23), 31.3 (CH<sub>2</sub>, C-40), 29.7 (CH<sub>2</sub>, C-20), 28.5 (CH<sub>2</sub>, C-24), 28.3 (CH<sub>2</sub>, C-13), 28.2 (2 CH<sub>2</sub>, C-32, C-33), 28.x (6CH<sub>2</sub>, C-34 to C-39), 27.3 (CH<sub>2</sub>, C-19), 25.8 (CH<sub>2</sub>, C-15), 25.6 (CH<sub>2</sub>, C-26), 23.6 (CH<sub>2</sub>, C-25), 23.4 (CH<sub>2</sub>, C-14), 22.1 (CH<sub>2</sub>, C-41), 13.7 (CH<sub>3</sub>, C-42).

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