Viennamycins: Lipopeptides Produced by a *Streptomyces* sp.

Paulina Bekiesch, Martin Zehl, Elizabeth Domingo-Contreras, Jesús Martín, Ignacio Pérez-Victoria, Fernando Reyes, Arthur Kaplan, Christian Rückert, Tobias Busche, Jörn Kalinowski, and Sergey B. Zotchev*

Cite This: *J. Nat. Prod.* 2020, 83, 2381–2389

**ABSTRACT:** Extracts from *Streptomyces* sp. S4.7 isolated from the rhizosphere of edelweiss, an alpine medicinal plant, exhibited activity against Gram-positive bacteria. LC-HRMS analyses of the extracts resulted in the detection of two unknown, structurally related lipopeptides that were assumed to be responsible for the antibacterial activity. LC-MS guided isolation and structure elucidation of viennamycins A and B (1 and 2) by HR-MS/MS, 1D and 2D NMR, and Marfey’s analyses revealed them to be novel compounds, with viennamycin A containing cysteic acid, a unique feature for lipopeptides. Tests for antibacterial, antifungal, and cytotoxic activities of purified viennamycins, both with and without divalent cations, did not reveal any bioactivity, suggesting that their biological function, which could not be determined in the tests used, is atypical for lipopeptides. The genome of *Streptomyces* sp. S4.7 was sequenced and analyzed, revealing the viennamycin biosynthetic gene cluster. Detailed bioinformatics-based analysis of the viennamycin gene cluster allowed elucidation of the biosynthetic pathway for these lipopeptides.

Many bacterial species produce structurally diverse secondary metabolites that are not required for normal proliferation of the producer but supposedly provide an advantage in competition for nutrients or and defense against predators. Cyclic lipopeptides are produced by representatives of several bacterial genera, including *Bacillus*, *Paenibacillus*, *Pseudomonas*, and *Streptomyces*. These natural products feature a hydrophobic cyclic peptide moiety linked to a hydrophobic acyl chain and thus have amphiphilic properties that have a decisive role in their modes of action. Most of the acidic cyclic lipopeptides, such as daptomycin and friulimycin, have antibacterial activity due to their ability to target the membrane lipid bilayer and disrupt its normal function by causing depolarization. The acyl chain of cyclic lipopeptides plays an important role in the interaction of these compounds with the membrane, while the peptide part appears to be important for stabilization of the molecular 3D structure with the help of a metal ion. In particular, the cyclic peptide moieties of the antibiotics daptomycin, taromycin, calcium-dependent antibiotic, and friulimycin contain an Asp-X-Asp-Gly motif that is involved in binding Ca$^{2+}$ ions, which is essential for their antibacterial activity. Recently, a family of novel lipopeptides named cadasides has been discovered via heterologous expression of a biosynthetic gene cluster identified in a soil metagenome library. The cyclic peptide core of cadasides lacks the canonical Ca$^{2+}$-binding motif, and the complete peptide moiety features five aspartate residues, making these compounds more acidic compared to other reported lipopeptide antibiotics.

Maximum antibacterial activity of cadasides required a significantly higher concentration of Ca$^{2+}$ ions (100 mM), which is unlikely to be relevant for antibacterial therapy, but might reflect the environmental conditions at the spot where the metagenomics DNA was isolated from (high calcium carbonate concentration).

Biosynthesis of acidic lipopeptides starts with priming of the nonribosomal peptide synthetase (NRPS) complex with an acyl chain that is sometimes unsaturated due to the action of specific dehydrogenases and reductases. The acyl starter is then condensed with amino acid residues by NRPS extension modules that may also feature epimerase and methyl transferase domains. The synthesis is completed by the release of the acylated peptide chain from the NRPS and its cyclization by the thioesterase domain linked to the last NRPS module. The modularity of NRPSs and availability of a vast variety of adenylation domains responsible for the choice and activation of amino acid extension blocks offer an opportunity for engineering these biosynthetic systems to afford novel analogues of lipopeptide antibiotics.

Received: February 10, 2020  
Published: July 31, 2020
In this work, we describe the isolation and characterization of two novel acidic lipopeptides, viennamycins A and B (1 and 2), one of which features cysteic acid (Cya), a building block unique to lipopeptides. The cyclic peptide core of viennamycins lacks the Ca^{2+}-binding motif, and they are not active against bacteria, fungi, or the human cancer cells tested. We also report the biosynthetic gene cluster (BGC) for viennamycins, which harbors, besides the genes for lipopeptide scaffold assembly enzymes, a gene for cysteic acid synthase that may be exploited in combinatorial biosynthesis. Analysis of this BGC was instrumental in establishing the correct amino acid sequence of both viennamycins and confirms once more the complementarity between the information obtained from this analysis and that obtained by spectroscopic approaches to establish the correct structure of complex natural products.

RESULTS AND DISCUSSION

Streptomyces sp. S4.7 was isolated from the rhizosphere of edelweiss [Leontopodium nivale subsp. alpinum (Cass.) Greuter], a traditional medicinal and decorative plant collected in the Austrian Alps.6 The isolate was identified as a member of the Streptomyces genus using 16S rRNA gene-based taxonomy, showing the closest similarity to various endophytic streptomycetes isolated from Camellia sp. (e.g., GenBank: MN337312, MN337313). The isolate exhibited optimal growth at 28 °C on solid ISP2 medium, while displaying strong agarase activity. The isolate was identified as a member of the Streptomyces genus using 16S rRNA gene-based taxonomy, showing the closest similarity to various endophytic streptomycetes isolated from Camellia sp. (e.g., GenBank: MN337312, MN337313). The isolate exhibited optimal growth at 28 °C on solid ISP2 medium, while displaying strong agarase activity. The latter made collection of spores problematic, and agar was later replaced with gellan gum, which to a large extent alleviated the problem. Strain S4.7 was cultivated in liquid fermentation media S254, S288, and S294,7 followed by extraction of the cultures with n-butanol. The dried extracts were dissolved in methanol and tested for bioactivity (see Experimental Section). The extract prepared from the culture grown in medium S288 adjusted to pH 10 (and acidified prior to extraction) proved to be bioactive against Bacillus subtilis. The bioactive extract was fractionated by flash chromatography (see Experimental Section), and the bioactive fraction was subjected to LC-HRMS analysis. The latter revealed two major components, whose fragmentation patterns clearly suggested them being lipopeptide congeners with the peptide moiety composed of 12 amino acids.

The less abundant component yielded an [M + 2H]^{2+} ion at m/z 690.8382, which corresponds to a calculated molecular formula of C_{63}H_{93}N_{13}O_{22}S (calcd m/z 690.8377, Δ = 0.7 ppm). De novo sequencing yielded parts of the primary structure, including a linear N-terminal peptide chain consisting of Asn-Ile-Leu-Phe-Asp-Asp with a C10 acyl moiety featuring two double bonds attached to the N-terminus. For the remaining part of the peptide, neither the directionality of obtained sequence tags nor the linkage between different parts could be safely determined from the MS/MS spectra. This is because fragmentation of the side-chain-to-tail cyclized peptide part starts with a ring cleavage on one of several possible positions that typically results in branched peptide fragments without an intact C-terminus.

The major component gave HR-ESIMS signals from the [M + 2H]^{2+} ion at m/z 708.8213 and the [M + H]^+ ion at m/z 1416.6354. The MS/MS data indicated a structure largely identical to the less abundant congener, with the only detectable difference being the exchange of Asp in position 4 by a nonstandard amino acid with a residue mass of 150.9934 Da.

A large-scale culture yielded the two pure viennamycins, whose structures were elucidated by means of NMR, HR-MS/MS, and Marfey’s analysis.

ESI-TOF-MS analysis of viennamycin A (1) indicated a molecular formula of C_{65}H_{95}N_{15}O_{24}S. The peptideptic nature of the molecule anticipated by HR-MS/MS analysis was confirmed by the presence in its 1H NMR spectrum of signals in the amino acid NH and α-CH region and signals of numerous carbonyl groups in its 13C NMR spectrum (Table 1). Analysis of HSQC, COSY, and HMBC spectra revealed the presence of Pro, Ala, Glu, Asp, Asn, Thr, N-Me-Val, lle, and 2 × Gly residues. Apart from the signals of these common amino acids, a methine (δ_3 4.38/δ_2 51.7) and a methylene (δ_3 2.78 and 2.81/δ_2 52.2) mutually coupled in the COSY spectrum were observed. These chemical shift values are in agreement with the presence of a cysteic acid (Cya) unit in the molecule,8 which was further supported by the presence of a sulfur atom in the molecular formula of the compound, as deduced from HRMS measurements, and the excellent fit to the mass difference measured for the nonstandard amino acid in position 4 (observed 150.9934 Da, calculated 150.9939 Da). Finally, the lipophilic moiety of the molecule was identified as (2Z,4E)-8-methylnona-2,4-dienoic acid (MNDA) using COSY and HMBC correlations and comparison of the NMR data to those of cadaside B and ramoplanin A3.9 The Z and E configurations of the Δ^2 and Δ^4 double bonds present in this fragment were assigned based on the measured J_{2,3} and J_{4,5} coupling constant values of 11.3 and 15.0 Hz, respectively.3,9 The attachment of this residue to the NH of the peptide was confirmed by HMBC correlations from the NH (δ_4 8.19 ppm) and Hα (δ_4 4.63 ppm) protons of the Asn residue to carbon C-1 of MNDA (δ_1 166.1 ppm). De novo sequencing of the compound using HRMS/MS led to results that were not in full agreement with the amino acid sequence deduced from BGC analysis (see below). Initial sequencing of the peptide using amide NH and Cα proton HMBC correlations also led to inconclusive results due to the overlapping of some key signals. Therefore, the ester bond of the peptide was hydrolyzed in basic conditions (LiOH), and LC-HRMS/MS analysis of the resulting hydrolyzed molecule was performed. Although not very intense, key fragments obtained during this analysis (Figure 1) led to the confirmation of the sequence obtained from BGC analysis. Additionally, key NOESY correlations observed between inter-residue protons, especially those between the Ala and Pro protons, confirmed the sequence MNDA-Asn-Ile-Phe-Cya-Asp-Thr-N-Me-Val-Gly-Gly-Glu-Ala-Pro with the C-terminus forming an ester with the Thr side chain (Figure 2). Hydrolysis of the peptide under acidic conditions followed by derivatization with i- and d-FDVA and Marfey’s...
analysis confirmed the presence in the molecule of L-Pro, L-Ala, D-Glu, D-N-Me-Val, L-Asp, D-Cya, D-Phe, L-Ile, and L-Asn. A molecular formula of C_{64}H_{93}N_{13}O_{21} was assigned to viennamycin B (2) based on ESI-TOF measurements. Compared to 1, this formula accounted for one additional carbon atom with respect to 1 and the absence of the sulfur and one of the oxygen atoms present in the latter. These observations were in agreement with the replacement of the Cya residue in 1 by Asp in the structure of 2. The major differences in the NMR data of both compounds (Tables 1 and 2) were observed in the region around the Cya residue of viennamycin A, where the key signals for this residue were

| unit | position | δ_{C} | δ_{H} (mult., J in Hz) | unit | position | δ_{C} | δ_{H} (mult., J in Hz) |
|------|----------|------|------------------------|------|----------|------|------------------------|
| MND A | 1 | 166.1 | 5.67, d (11.4) | (6) Thr | NH | 8.36, d (7.5) |
|      | 2 | 119.5 | 6.36, dd (11.3, 11.3) | α | 52.5 | 4.82, t (7.8) |
|      | 3 | 141.1 | 7.43, dd (14.9, 11.2) | β | 71.7 | 4.99, m |
|      | 4 | 127.6 | 5.95, ddd (15.0, 6.9, 6.9) | γ | 16.5 | 1.03, m |
|      | 5 | 142.9 | 2.11, m | | | |
|      | 6 | 30.6 | 1.26, m | (7) N-Me Val | N-Me | 30.0 | 2.85, s |
|      | 7 | 38.0 | 0.86, d (6.5) | α | 65.0 | 4.26, d (9.1) |
|      | 8 | 27.5 | 1.53, septet (6.6) | β | 28.3 | 2.15, m |
|      | 9, 10 | 22.3 | 6.00, br s | γ | 18.9 | 0.70, d (6.3) |

A molecular formula of C_{64}H_{93}N_{13}O_{21} was assigned to viennamycin B (2) based on ESI-TOF measurements. Compared to 1, this formula accounted for one additional carbon atom with respect to 1 and the absence of the sulfur and one of the oxygen atoms present in the latter. These observations were in agreement with the replacement of the Cya residue in 1 by Asp in the structure of 2. The major differences in the NMR data of both compounds (Tables 1 and 2) were observed in the region around the Cya residue of viennamycin A, where the key signals for this residue were

| unit | position | δ_{C} | δ_{H} (mult., J in Hz) | unit | position | δ_{C} | δ_{H} (mult., J in Hz) |
|------|----------|------|------------------------|------|----------|------|------------------------|
|      | 1 | 8.19, d (8.0) | | (8) Gly | NH | 6.90, m |
|      | 2 | 7.60, m | | | | |
|      | 3 | 8.25, m | | | | |
|      | 4 | 8.28, m | | | | |
|      | 5 | 8.25, m | | | | |

A molecular formula of C_{64}H_{93}N_{13}O_{21} was assigned to viennamycin B (2) based on ESI-TOF measurements. Compared to 1, this formula accounted for one additional carbon atom with respect to 1 and the absence of the sulfur and one of the oxygen atoms present in the latter. These observations were in agreement with the replacement of the Cya residue in 1 by Asp in the structure of 2. The major differences in the NMR data of both compounds (Tables 1 and 2) were observed in the region around the Cya residue of viennamycin A, where the key signals for this residue were

| unit | position | δ_{C} | δ_{H} (mult., J in Hz) | unit | position | δ_{C} | δ_{H} (mult., J in Hz) |
|------|----------|------|------------------------|------|----------|------|------------------------|
|      | 1 | 8.19, d (8.0) | | (8) Gly | NH | 6.90, m |
|      | 2 | 7.60, m | | | | |
|      | 3 | 8.25, m | | | | |
|      | 4 | 8.28, m | | | | |
|      | 5 | 8.25, m | | | | |

A molecular formula of C_{64}H_{93}N_{13}O_{21} was assigned to viennamycin B (2) based on ESI-TOF measurements. Compared to 1, this formula accounted for one additional carbon atom with respect to 1 and the absence of the sulfur and one of the oxygen atoms present in the latter. These observations were in agreement with the replacement of the Cya residue in 1 by Asp in the structure of 2. The major differences in the NMR data of both compounds (Tables 1 and 2) were observed in the region around the Cya residue of viennamycin A, where the key signals for this residue were

| unit | position | δ_{C} | δ_{H} (mult., J in Hz) | unit | position | δ_{C} | δ_{H} (mult., J in Hz) |
|------|----------|------|------------------------|------|----------|------|------------------------|
|      | 1 | 8.19, d (8.0) | | (8) Gly | NH | 6.90, m |
|      | 2 | 7.60, m | | | | |
|      | 3 | 8.25, m | | | | |
|      | 4 | 8.28, m | | | | |
|      | 5 | 8.25, m | | | | |

A molecular formula of C_{64}H_{93}N_{13}O_{21} was assigned to viennamycin B (2) based on ESI-TOF measurements. Compared to 1, this formula accounted for one additional carbon atom with respect to 1 and the absence of the sulfur and one of the oxygen atoms present in the latter. These observations were in agreement with the replacement of the Cya residue in 1 by Asp in the structure of 2. The major differences in the NMR data of both compounds (Tables 1 and 2) were observed in the region around the Cya residue of viennamycin A, where the key signals for this residue were

| unit | position | δ_{C} | δ_{H} (mult., J in Hz) | unit | position | δ_{C} | δ_{H} (mult., J in Hz) |
|------|----------|------|------------------------|------|----------|------|------------------------|
|      | 1 | 8.19, d (8.0) | | (8) Gly | NH | 6.90, m |
|      | 2 | 7.60, m | | | | |
|      | 3 | 8.25, m | | | | |
|      | 4 | 8.28, m | | | | |
|      | 5 | 8.25, m | | | | |

A molecular formula of C_{64}H_{93}N_{13}O_{21} was assigned to viennamycin B (2) based on ESI-TOF measurements. Compared to 1, this formula accounted for one additional carbon atom with respect to 1 and the absence of the sulfur and one of the oxygen atoms present in the latter. These observations were in agreement with the replacement of the Cya residue in 1 by Asp in the structure of 2. The major differences in the NMR data of both compounds (Tables 1 and 2) were observed in the region around the Cya residue of viennamycin A, where the key signals for this residue were

| unit | position | δ_{C} | δ_{H} (mult., J in Hz) | unit | position | δ_{C} | δ_{H} (mult., J in Hz) |
|------|----------|------|------------------------|------|----------|------|------------------------|
|      | 1 | 8.19, d (8.0) | | (8) Gly | NH | 6.90, m |
|      | 2 | 7.60, m | | | | |
|      | 3 | 8.25, m | | | | |
|      | 4 | 8.28, m | | | | |
|      | 5 | 8.25, m | | | | |
replaced by those of a methine (δH 4.63/δC 49.8) and a methylene (δH 2.45 and 2.57/δC 37.2) compatible with a second Asp residue in the molecule. The presence of this Asp was confirmed, and its absolute configuration established to be D by Marfey’s analysis, where the presence of both L- and D-Asp derivatives could be observed in contrast with the analysis of viennamycin A, where only derivatized L-Asp was detected.

While the cysteate residue has not been reported as part of lipopeptide structures, it was identified in the JBIR-95 heptapeptide isolated from Kibdelosporangium sp. AK-AAS6. Its congener, JBIR-78, contained D-aspartate at the same position, which resembled the situation with viennamycins. Analysis of the gene cluster governing the biosynthesis of JBIR-78 and -95 revealed an NRPS with an adenylation domain with apparently relaxed substrate specificity, capable of activating either aspartate or cysteate. The gene for cysteate synthase was also identified in the JBIR-78 and -95 biosynthesis gene cluster. The latter enzyme apparently catalyzes conversion of L-phosphoserine and sulfite to L-cysteate and inorganic phosphate. It appeared therefore interesting to identify and analyze the viennamycin biosynthesis gene cluster of Streptomyces sp. S4.

Figure 1. MS/MS fragmentation of hydrolyzed viennamycin A.

Figure 2. Key NOESY correlations observed in the structure of viennamycin A (1).
The genome of *Streptomyces* sp. S4.7 was sequenced (see Experimental Section for details), and its size was determined to be 7.92 Mb, with a composition highly similar to that of *Streptomyces niveus* SCSIO, a marine-derived producer of sesquiterpenoid naphthoquinones, marfuraquinocins, and phenaziterpenes.\(^\text{11}\) antiSMASH 5.0-assisted analysis of the S4.7 genome, followed by manual curation, identified 38 secondary metabolite BGCs (Table S1, Supporting Information).

### Table 2. NMR Data (500 MHz, DMSO-\(d_6\) at 30 °C) of Viennamycin B (2)

| unit position | \(\delta_C\) | \(\delta_H\) (mult., \(J\) in Hz) | unit position | \(\delta_C\) | \(\delta_H\) (mult., \(J\) in Hz) |
|---------------|--------------|---------------------------------|---------------|--------------|---------------------------------|
| MNDA          | 166.2        |                                 | (6) Thr       | CO           | 170.1                           |
|               | 119.3        | 5.63, d (11.2)                  |               | CO           | 170.1                           |
|               | 141.3        | 6.37, dd (11.2, 11.2)           |               | \(\alpha\)   | 52.1                            |
|               | 127.5        | 7.42, dd (15.0, 11.2)           |               | \(\beta\)    | 71.7                            |
|               | 143.1        | 5.96, ddd (15.1, 6.9, 6.9)      |               | \(\gamma\)   | 16.4                            |
|               | 30.6         | 2.11, m                         |               | 1.02, br s   |                                 |
| 7             | 38.0         | 1.26, m                         | (7) N-Me Val  | N-Me         | 30.0                            |
| 8             | 27.5         | 1.53, septet (6.6)              |               | CO           | n. d.                           |
| 9, 10         | 22.8         | 0.86, d (6.5)                   |               | \(\alpha\)   | 65.2                            |
|               |              |                                 |               | \(\beta\)    | 28.3                            |
|               |              |                                 |               | 2.15, m      |                                 |
| (1) Asn       | NH           | 8.19, d (8.0)                   | (8) Gly       | NH           | 6.90, br t                      |
|               | CO           | 171.2                           |               | \(\gamma\)   | 19.5                            |
|               | \(\alpha\)  | 49.8                            |               | \(\gamma\)   | 19.5                            |
|               | \(\beta\)   | 37.0                            | a. 2.56, m    | \(\gamma\)   | 19.5                            |
|               |              | b. 2.37, dd (15.4, 7.8)         |               | 6.90, br t   |                                 |
| \(\gamma\)NH\(_2\) | a. 7.28, br s |                                 |               | \(\alpha\)   | 41.4                            |
|               |              | b. 6.85, br s                   |               | a. 3.97, m   |                                 |
| (2) Ile       | NH           | 7.60, d (8.5)                   | (9) Gly       | NH           | 8.63, m                         |
|               | CO           | 170.9                           |               | CO           | 170.0                           |
|               | \(\alpha\)  | 57.3                            | 4.16, m       | \(\alpha\)   | 43.8                            |
|               | \(\beta\)   | 37.5                            | 1.49, m       | a. 3.84, m   |                                 |
|               | \(\gamma\)  | 23.8                            | 0.73, m; 1.00, m 0.0 | \(\beta\)   | 26.3                            |
|               | \(\delta\)  | 11.6                            | 0.58, t (7.2) | a. 4.07, m   |                                 |
| \(\beta\)-CH\(_3\) | 15.5         | 0.50, d (6.5)                   |               | b. 2.02, m   |                                 |
|               |              |                                 |               | b. 1.71, m   |                                 |
| (3) Phe       | NH           | 8.19, d (8.0)                   | \(\gamma\)   | 30.7                      | 2.28, m                         |
|               | CO           | 170.9                           | \(\delta\)   | 174.3                    |                                 |
|               | \(\alpha\)  | 54.2                            | 4.59, m       | \(\delta\)   | 174.3                          |
|               | \(\beta\)   | 38.2                            | a. 3.14, m    | \(\delta\)   | 174.3                          |
|               |              | b. 2.69, m                      | \(\delta\)   | 174.3                    |                                 |
| \(\gamma\)   | 138.6        |                                 | \(\delta\)   | 174.3                    |                                 |
| \(\delta, \delta\) | 129.7       | 7.25, m                         | \(\alpha\)   | 46.4                      | 4.57, m                         |
| \(\epsilon, \epsilon\) | 128.4       | 7.23, m                         | \(\beta\)    | 17.4                      | 1.30, d (6.4)                   |
| \(\zeta\)    | 126.5        | 7.14, m                         | \(\beta\)    | 17.4                      | 1.30, d (6.4)                   |
| (4) Asp       | NH           | 8.08, d (8.0)                   | \(\gamma\)   | 47.0                      | 3.53, m                         |
|               | CO           | 171.7                           | \(\delta\)   | 47.0                      | 3.53, m                         |
|               | \(\alpha\)  | 49.8                            | 4.63, m       | \(\delta\)   | 47.0                      | 3.53, m                         |
|               | \(\beta\)   | 37.2                            | a. 2.57, m    | \(\delta\)   | 47.0                      | 3.53, m                         |
|               |              | b. 2.45, m                      | \(\delta\)   | 47.0                      | 3.53, m                         |
| \(\gamma\)   | n. d.        |                                 | \(\delta\)   | 47.0                      | 3.53, m                         |
| (5) Asp       | NH           | 8.43, d (6.3)                   | \(\gamma\)   | 172.0                    |                                 |
|               | CO           | 170.9                           | \(\gamma\)   | 172.0                    |                                 |
|               | \(\alpha\)  | 50.2                            | 4.60, m       | \(\gamma\)   | 172.0                    |                                 |
|               | \(\beta\)   | 36.7                            | a. 2.66, m    | \(\gamma\)   | 172.0                    |                                 |
|               |              | b. 2.54, m                      | \(\gamma\)   | 172.0                    |                                 |

The genome of *Streptomyces* sp. S4.7 was sequenced (see Experimental Section for details), and its size was determined to be 7.92 Mb, with a composition highly similar to that of *Streptomyces niveus* SCSIO, a marine-derived producer of sesquiterpenoid naphthoquinones, marfuraquinocins, and phenaziterpenes.\(^\text{11}\) antiSMASH 5.0-assisted analysis of the S4.7 genome, followed by manual curation, identified 38 secondary metabolite BGCs (Table S1, Supporting Information).

Detailed analysis of the NRPS-encoding gene clusters supported by the data on viennamycins' structure allowed precise identification of the BGC responsible for their biosynthesis (Figure 3), for which no close homologue could
be found in the publicly accessible databases.\textsuperscript{12} The viennamycins' BGC (cluster 37, Table S1) spans 57 kb and contains 13 genes (Table 3). Analyses of gene products in the viennamycin gene cluster supports the proposed biosynthetic pathway, which could start with the activation of 8-methylnonanoic acid by the acyl-CoA ligase VieF and its tethering on the acyl carrier protein VieI, where dehydrogenation and reduction by VieG and VieH, respectively, would afford the lipid part of viennamycins, the \((2Z,4E)-8\text{-methylnona-2,4-dienoyl}\) moiety. The first module of the NRPS, VieJ, then condenses the latter moiety with Asn, Ile, Phe, and Cya (viennamycin A) or Asp (viennamycin B), with Phe and Cya/Asp being epimerized in-synthesis via action of cognate epimerase domains. The Cya building block is synthesized by the cysteate synthase VieA. The acylated peptides are then extended via the action of NRPS proteins VieK, -L, and -M, and the final linear product is released and cyclized by the thioesterase domain of VieM. The VieC and VieD proteins constitute the ABC transporter that is responsible for the efflux of viennamycins. The presence in the cluster of the gene vieB encoding a DedA family membrane protein is intriguing, since these proteins were shown to play various important roles in bacteria. In \textit{Borrelia burgdorferi} and \textit{Escherichia coli} the DedA family proteins were shown to be essential,\textsuperscript{13} and other DedA-deficient bacteria exhibit mutant phenotypes such as defective cell division, temperature sensitivity, and loss of proton motive force.\textsuperscript{14} Since lipopeptide antibiotics' main target is the bacterial membrane, it is thus tempting to speculate that viennamycins target DedA family proteins, and VieB represents a modified target that these lipopeptides cannot bind. The viennamycins were tested against a panel of 14 Gram-positive bacteria (including \textit{Streptomyces coelicolor} A(3)2, 11 Gram-negative bacteria, yeast, two filamentous fungi, and four cancer cell lines (see Experimental Section). No antibacterial, antifungal or cytotoxic activity of viennamycins was revealed in these tests both with and without the presence of Ca\textsuperscript{2+} ions at concentrations up to 5 mM (test conditions did not allow higher calcium concentrations). A disk diffusion assay using purified viennamycins with \textit{B. subtilis} as test organism, which was used to detect the bioactive fraction where viennamycins were originally identified, also failed to show antibacterial activity, even in the presence of up to 5 mM Ca\textsuperscript{2+}, Mn\textsuperscript{2+}, Mg\textsuperscript{2+}, or Zn\textsuperscript{2+} ions. Obviously, if a more rigorous bioactivity-guided fractionation was used from the start, a different result would have been obtained. It appears likely that viennamycins have a very specific biological activity/function in the producing microorganism that could not be revealed in the tests applied.

![Figure 3. Organization of the viennamycin biosynthetic gene cluster in the genome of \textit{Streptomyces} sp. 4.7.](https://dx.doi.org/10.1021/acs.jnatprod.0c00152)

### Table 3. Genes in the Viennamycin Biosynthetic Gene Cluster and Their Deduced Products

| gene  | putative product                      | proposed function                                      |
|-------|--------------------------------------|--------------------------------------------------------|
| vieA  | cysteate synthase                    | biosynthesis of cysteic acid                           |
| vieB  | DedA family membrane protein         | transport and/or resistance                            |
| vieC  | ABC transporter, permease component  | viennamycin efflux                                     |
| vieD  | ABC transporter ATP-binding protein  | viennamycin efflux                                     |
| vieE  | MbtH-like protein                    | NRPS accessory protein                                 |
| vieF  | Acyl-CoA ligase                      | activation of 8-methylnonanoic acid                    |
| vieG  | Acyl-CoA dehydrogenase               | generation of the \((2Z,4E)-8\text{-methylnona-2,4-dienoyl}\) moiety |
| vieH  | Acyl-CoA reductase                    | generation of the \((2Z,4E)-8\text{-methylnona-2,4-dienoyl}\) moiety |
| vieI  | acyl carrier protein                 | attachment of the 8-methylnonanoyl moiety             |
| vieJ  | NRPS                                | activation and condensation of Asn, Ile, Phe (with epimerization) and Asp/Cya (with epimerization) with acyl moiety |
| vieK  | NRPS                                | activation and condensation of Asp, Thr, Val (with N-methylation and epimerization) with the product of VieJ |
| vieL  | NRPS                                | activation and condensation of 2 × Gly and Glu (with epimerization) with the product of VieL |
| vieM  | NRPS                                | activation and condensation of Ala and Pro with the product of VieL, peptide chain release, and cyclization |

\[\text{https://dx.doi.org/10.1021/acs.jnatprod.0c00152}\]
signals of the residual solvent as internal reference (δH 2.51 ppm and δC 39.55 ppm). ESI-TOF mass spectra were acquired using a maxis HD ESI-Qq-TOF mass spectrometer. Medium-pressure fractionation of crude extracts was performed using a CombiFlash RF system. Semipreparative and preparative HPLC separations were performed on a Gilson FX-281 HPLC system with UV detection at 210 and 280 nm and automatic fraction collection.

**Cultivation of Streptomyces sp. S4.7 for Viennamycin Production.** A seed culture of Streptomyces sp. 4.7 was prepared by inoculating 10 mL of TSB seed medium (tryptone soy broth 30 g/L) in a 40 mL tube (150 mm × 25 mm) with 100 μL of glycerol stock of spores and incubating for 2 days at 28 °C and 200 rpm. A 2.5 mL amount of this culture was used to inoculate a second seed culture (50 mL of TSB medium in baffled 250 mL Erlenmeyer flasks). A 5% aliquot of the second seed culture (6.25 mL) was transferred to each of forty-eight 500 mL flasks containing 125 mL of the production medium (5288 pH 10 medium) consisting of glycerol 15.0 g/L, soyl meal 10.0 g/L, NaCl 5.0 g/L, CaCO3 1.0 g/L, and Co2Cl4 (0.1% TFA) in 35 min, then cultivated at 28 °C and 200 rpm for 10 days.

**Extraction of Culture Broth and Fractionation.** Cultures were acidified to pH 4 with HCl and extracted with an equal volume of n-butanol for 60 min. The organic phase was evaporated to dryness under reduced pressure at 40 °C. The residue was dissolved in a 1/50 volume of methanol, and this extract fractionated by flash chromatography using a linear gradient from 0 to 100% methanol in 45 min on a 25 g RP18 column and size exclusion chromatography on Sephacryl LH-20 with methanol as mobile phase. Upscaled production was followed by n-butanol extraction and purification using C18 reversed-phase column chromatography and repeated preparative reversed-phase HPLC.

**Mass Spectrometric Analysis.** The crude extract and fractions were analyzed by LC-MS on an UltimaMate 3000 RSLC-series system (Dionex) coupled to an HCT 3D quadrupole ion trap mass spectrometer equipped with an orthogonal ESI source. Fractions containing viennamycins A and B were analyzed by direct infusion HR-MS/MS on an ESI-Qq-TOF mass spectrometer in positive mode. The fragmentation patterns were used for de novo sequencing. The isolated viennamycins A and B were also analyzed by LC-MS on a Vanquish Horizon UHPLC system coupled to the ESI source of an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) in positive and negative ion mode. The molecular formula for viennamycin A was supported by the presence of the protonated molecule at m/z 1416.6341 (calcd for C63H94N13O22S, 1416.6341, m/z 1416.6352, Δ = 0.7 ppm), and that for viennamycin B by the presence of a protonated molecule observed at m/z 1380.6679 (calcd for C63H94N13O22S, m/z 1380.6682, Δ = 0.2 ppm). Isolation and Purification. The culture broth of strain S4.7 was acidified to pH 4 and extracted with an equal volume of n-butanol added to the fermentation flasks. The resulting mixture was stirred for 1 h and centrifuged at 8000 rpm (1 h), and the pellet was discarded. The mixture was decanted in a separatory funnel, and the organic phase collected and evaporated to dryness using a rotary evaporator. A 5.66 g amount of extract was obtained using this procedure. LC-MS analysis identified the presence of both viennamycins A (1) and B (2) in this extract. The crude extract was subjected to reversed-phase chromatography (C18 silica gel 40–60 μm, h = 11.2 cm, eluent 0–3.5 cm) using mixtures of H2O–CH3CN (0–100%) TFA (0.1% TFA). The chromatographic separation started with a 5% CH3CN (0.1% TFA) hold for 5 min followed by a linear gradient to 100% CH3CN (0.1% TFA) in 35 min and a final isocratic step with 100% CH3CN (0.1% TFA) for 10 min. A flow of 18 mL/min was used, and 50 18 mL fractions were collected. Fractions containing the two compounds of interest were pooled to produce two subfractions, A (413 mg), containing essentially compound 1, and B (200.9 mg), containing compounds 1 and 2. Subfraction A was dissolved in methanol and subjected to preparative reversed-phase HPLC (Zorbax SB-C8, 21.2 × 250 mm, 7 μm) using H2O–CH3CN (0.1% TFA) mixtures in the following conditions: 30% CH3CN (0.1% TFA) for 1 min followed by a gradient from 30% to 50% CH3CN (0.1% TFA) in 35 min, flow: 20 mL/min. Fractions containing viennamycin A (69.3 mg) were further purified by semipreparative HPLC (XBridge Phenyl 10 × 150 mm, 5 μm) using the following conditions: 25% CH3CN (0.1% TFA) for 1 min followed by a gradient from 25% to 50% CH3CN (0.1% TFA) in 30 min, flow: 4.7 mL/min, to afford 6.1 mg of viennamycin A (1). Subfraction B was dissolved in methanol and subjected to preparative reversed-phase HPLC (Zorbax SB-C8, 21.2 × 250 mm, 7 μm) using H2O–CH3CN (0.1% TFA) mixtures in the following conditions: 25% CH3CN (0.1% TFA) for 1 min followed by a gradient from 25% to 55% CH3CN (0.1% TFA) in 35 min, flow: 20 mL/min. Fractions enriched in viennamycin B (5.5 mg) were subjected to semipreparative HPLC (XBridge Phenyl 10 × 150 mm, 5 μm) using the following conditions: 30% CH3CN (0.1% TFA) for 1 min followed by a gradient from 30% to 55% CH3CN (0.1% TFA) in 35 min, flow: 4.7 mL/min, to afford 1.9 mg of viennamycin B (2).

**Viennamycin A (1):** white amorphous solid; δH 3.60 (Hα, d, J = 7.6 Hz, 1H), δC 163.1 (C27), 151.9 (C11), 151.8 (C10), 145.0 (C17), 138.4 (C22), 136.8 (C26), 133.0 (C23), 126.0 (C24), 122.6 (C4), 121.8 (C5), 117.9 (C25), 113.8 (C1), 113.1 (C9), 110.1 (C6), 103.4 (C21), 102.1 (C14), 46.7 (C18), 33.7 (C15), 33.4 (C16), 21.3 (C30), 20.7 (C29), 20.5 (C31), 15.8 (C28), 14.2 (C19), 13.7 (C2), 13.1 (C13), 12.9 (C7), 12.2 (C8), 12.1 (C12), 4.7 (C8a, C9a, 1H). EI-HRMS (m/z) calcd for C63H94N13O22S: 1416.6352, [M + H]+ 1416.6341 (Δ = 0.7 ppm); 1433.6600 [M+NH4]+ (Δ = 0.7 ppm); 1433.6600 [M+Na+] (Δ = 1.2 ppm).

**Viennamycin B (2):** white amorphous solid; δH 3.76 (Hα, d, J = 7.6 Hz, 1H), δC 162.7 (C27), 151.9 (C11), 151.8 (C10), 145.0 (C17), 138.4 (C22), 136.8 (C26), 133.0 (C23), 126.0 (C24), 122.6 (C4), 121.8 (C5), 117.9 (C25), 113.8 (C1), 113.1 (C9), 110.1 (C6), 103.4 (C21), 102.1 (C14), 46.7 (C18), 33.7 (C15), 33.4 (C16), 21.3 (C30), 20.7 (C29), 20.5 (C31), 15.8 (C28), 14.2 (C19), 13.7 (C2), 13.1 (C13), 12.9 (C7), 12.2 (C8), 12.1 (C12), 4.7 (C8a, C9a, 1H). EI-HRMS (m/z) calcd for C63H94N13O22S: 1380.6679, [M + H]+ 1380.6682 (Δ = 0.2 ppm); 1397.6946 [M + NH4]+ (Δ = 1.0 ppm). 2387
0.1% TFA for 2 min followed by a gradient from 10% to 15% CH₃CN + 0.1% TFA in 45 min. Retention times of both isomers under these conditions were as follows: l-Cya: 12.24; d-Cya: 13.02 (Figure S34). Although some racemization occurred during the acid hydrolysis of the peptide, the major isomer obtained in this process displayed a retention time of 12.93 min after derivatization, confirming the presence of n-Cya in the molecule (Figure S35).

**Genome Sequencing and Analysis.** Genomic DNA was isolated as described previously. From the genomic DNA, three sequencing libraries were prepared, two for sequencing on the MiSeq platform (Illumina Inc., NL) and one for sequencing on the MinION platform (Oxford Nanopore Technologies, UK). The two former consisted of a TruSeq DNA PCR-free library and a Nextera Mate Pair library with an insert size of ~9 kbp. Both were run in a 2 x 300 nt run using a 600 cycle MiSeq reagent kit v3 (Illumina Inc., NL). For ONT sequencing, the rapid sequencing kit SQK-RAD004 was used to prepare the library, which was in turn run on a R9.4 flow cell. Base-calling of the raw data was performed with Albacore v2.3.1. The Canu assembler v1.6 was used to assemble the ONT data into 3 contigs, which were subsequently polished using the Illumina data and the Pilon polisher v1.2217 for a total of 10 rounds. For the first 5, Bwa Mem v2.8 was used as a mapper; for the final 5 Bowtie2 v2.3.0 was applied. In addition, the Illumina data were assembled into 5 scaffolds containing 105 contigs using Newbler v2.8. Both assemblies were combined and manually curated using Conseal, resulting in the complete genome of *Streptomyces* sp. S.4.7, consisting of one linear chromosome of 7,920,066 bp (70.72% G+C) and two circular plasmids of 10,119 bp (70.92% G+C) and 10,110 bp (70.37% G+C), respectively. Annotation was performed using Prokka v1.11,22 resulting in the prediction of 6931 coding sequences CDS, 6 tRNA operons, 79 tRNAs, and 32 noncoding RNA elements. The annotated genome was deposited at DDBJ/ENA/GenBank under the accession CP048397–CP048399.

**Antimicrobial and Cytotoxic Activities.** Compounds were tested in antimicrobial assays with and without the addition of CaCl₂ against the growth of Gram-positive bacteria (methylcillin-resistant *S. aureus* (MRSA) MB5393 and 1415-10, methylcillin-sensitive *S. aureus* (MSSA) ATCC29213 and MB2865, *S. epidemidis*, *S. pneumoniae* ATCC46619, *S. pyogenes*, *Streptomyces coelicolor A3(2)*, and Gram-negative bacteria (*A. baumannii* MB5973, 1799, and 1710) and in a cytotoxicity assay against the human cell lines Hep G2 (liver carcinoma), S. pyogenes, MB2865, and MB5973, 17999, and 1710) and in a cytotoxicity assay against the human cell lines Hep G2 (liver carcinoma), S. pyogenes, ASpergillus niger CBS 110271. Concentrations of compounds used ranged from 64 to 0.125 µg/mL in the antibacterial and antifungal assays and from 10 to 0.02 µM in the cytotoxicity tests.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c00152.

Secondary metabolite biosynthesis gene clusters in the genome of *Streptomyces* sp. S4.7, UV-vis and HRMS spectra for 1 and 2, NMR (COSY, TOCSY, NOESY) spectra for 1 and 2, HSQC and HMBE spectra for 2, HPLC traces of the l- and d-FDVA derivatives of single amino acids and hydrolysates of 1 and 2, LC-MS data of 1 and 2 (PDF)

#### AUTHOR INFORMATION

**Corresponding Author**

Sergey B. Zotchev — Department of Pharmacognosy, University of Vienna, 1090 Vienna, Austria; orcid.org/0000-0002-9324-245X; Phone: +43-1-4277-55296; Email: sergey.zotchev@univie.ac.at; Fax: (+43)-1-4277-855296

**Authors**

Paulina Bekiesch — Department of Pharmacognosy, University of Vienna, 1090 Vienna, Austria

Martin Zehl — Department of Analytical Chemistry, Faculty of Chemistry, University of Vienna, 1090 Vienna, Austria; orcid.org/0000-0001-9685-0373

Elizabeth Domingo-Contreras — Fundación Medina, Centro de Excelencia en Investigación de Medicamentos Innovadores en Andalucía, 18016 Granada, Spain

Jesús Martín — Fundación Medina, Centro de Excelencia en Investigación de Medicamentos Innovadores en Andalucía, 18016 Granada, Spain

Ignacio Pérez-Victoria — Fundación Medina, Centro de Excelencia en Investigación de Medicamentos Innovadores en Andalucía, 18016 Granada, Spain; orcid.org/0000-0002-4556-688X

Fernando Reyes — Fundación Medina, Centro de Excelencia en Investigación de Medicamentos Innovadores en Andalucía, 18016 Granada, Spain; orcid.org/0000-0003-1607-5106

Arthur Kaplan — Department of Pharmacognosy, University of Vienna, 1090 Vienna, Austria

Christian Rückett — Center for Biotechnology, Bielefeld University, 33615 Bielefeld, Germany

Tobias Busche — Center for Biotechnology, Bielefeld University, 33615 Bielefeld, Germany

Jörn Kalinowski — Center for Biotechnology, Bielefeld University, 33615 Bielefeld, Germany

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jnatprod.0c00152

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This study was supported by the Austria Wirtschaftsdienst, grant P1715748, the University of Vienna, and the Bielefeld University. We are thankful to Olha Schneider and Jaime Felipe Guerrero-Garzón for help with some bioassays, to Mercedes de la Cruz and Bastien Cautain for performing the antibacterial and cytotoxicity tests, respectively, and to Anna Fabisikova for help with the LC-MS analyses.

**REFERENCES**

1. Hamley, I. W. Chem. Commun. 2015, 51, 8574–8583.
2. Balleza, D.; Mescola, A.; Marin-Medina, N.; Ragazzini, G.; Pieruccini, M.; Facci, P.; Alessandrini, A. Biophys. J. 2019, 116, 503–517.
3. Wu, C.; Zhang, Z.; Lemetre, C.; Ternei, M. A.; Brady, S. F. J. Am. Chem. Soc. 2019, 141, 3910–3919.
4. Schwarzer, D.; Finking, R.; Marahiel, M. A. Nat. Prod. Rep. 2003, 20, 275–287.
5. Brown, A. S.; Calcott, M. J.; Owen, J. G.; Ackerley, D. F. Proc. Natl. Acad. Sci. U. S. A. 2004, 101, 11977–11979.
6. Oberhofer, M.; Hess, J.; Leutgeb, M.; Gossnitzer, F.; Rattei, T.; Wassersch, C.; Zotchev, S. B. Front. Microbiol. 2019, 10, 2531.
7. Wink, M. J. 2012. Compendium of Actinobacteria from Dr. Joachim M. Wink, University of Braunschweig, an Electronic Manual A v a i l a b l e o n l i n e at: https://www.dsmz.de/bacterial-diversity/including-the-important-bacterial-group-of-the-actinomycetes.html.
8. Schwarzer, D.; Finking, R.; Marahiel, M. A. Nat. Prod. Rep. 2018, 15, 1201–1228.
9. Oberhofer, M.; Hess, J.; Leutgeb, M.; Gossnitzer, F.; Rattei, T.; Wassersch, C.; Zotchev, S. B. Front. Microbiol. 2019, 10, 2531.
10. Wink, M. J. 2012. Compendium of Actinobacteria from Dr. Joachim M. Wink, University of Braunschweig, an Electronic Manual Including the Important Bacterial Group of the Actinomycetes. Available online at: https://www.dsmz.de/bacterial-diversity/compendium-of-actinobacteria.html.
11. Schwärzer, D.; Finking, R.; Marahiel, M. A. Nat. Prod. Re 2003, 35, 1201–1228.
12. Schwarzer, D.; Finking, R.; Marahiel, M. A. Nat. Prod. Rep. 2013, 20, 275–287.
13. Brown, A. S.; Calcott, M. J.; Owen, J. G.; Ackerley, D. F. Nat. Prod. Rep. 2019, 10, 2531.
14. Wink, M. J. 2012. Compendium of Actinobacteria from Dr. Joachim M. Wink, University of Braunschweig, an Electronic Manual Including the Important Bacterial Group of the Actinomycetes. Available online at: https://www.dsmz.de/bacterial-diversity/compendium-of-actinobacteria.html.
(10) Takeda, K.; Kemmoku, K.; Satoh, Y.; Ogasawara, Y.; Shin-Ya, K.; Dairi, T. ACS Chem. Biol. 2017, 12, 1813–1819.

(11) Song, Y.; Huang, H.; Chen, Y.; Ding, J.; Zhang, Y.; Sun, A.; Zhang, W.; Ju, J. J. Nat. Prod. 2013, 76, 2263–2268.

(12) Kautsar, S. A.; Blin, K.; Shaw, S.; Navarro-Muñoz, J. C.; Terlouw, B. R.; van der Hooft, J. J. J.; van Santen, J. A.; Tracanna, V.; Suarez Durán, H. G.; Pascal Andreu, V.; Selem-Mojica, N.; Alanjary, M.; Robinson, S. L.; Lund, G.; Epstein, S. C.; Sisto, A. C.; Charkoudian, L. K.; Collemare, J.; Linnington, R. G.; Weber, T.; Medema, M. H. Nucleic Acids Res. 2019, 48, D454–D458.

(13) Doerrler, W. T.; Sidkar, R.; Kumar, S.; Boughner, L. A. J. Bacteriol. 2013, 195, 3–11.

(14) Panta, P. R.; Kumar, S.; Stafford, C. F.; Billiot, C. E.; Douglass, M. V.; Herrera, C. M.; Trent, M. S.; Doerrler, W. T. Front. Microbiol. 2019, 10, 2532.

(15) Schneider, O.; Simic, N.; Aachmann, F. L.; Rückert, C.; Kristiansen, K. A.; Kalinowski, J.; Jiang, Y.; Wang, L.; Jiang, C. L.; Lale, R.; Zotchev, S. B. Front. Microbiol. 2018, 9, 3139.

(16) Koren, S.; Walenz, B. P.; Berlin, K.; Miller, J. R.; Bergman, N. H.; Phillippy, A. M. Genome Res. 2017, 27, 722–736.

(17) Walker, B. J.; Abeel, T.; Shea, T.; Priest, M.; Abouelliel, A.; Sathkumar, S.; Cuomo, C. A.; Zeng, Q.; Wortman, J.; Young, S. K.; Earl, A. M. PLoS One 2014, 9, e112963.

(18) Li, H. arXiv 2013, 1303.3997v1 [q-bio.GN].

(19) Langmead, B.; Salzberg, S. L. Nat. Methods 2012, 9, 357–359.

(20) Margulies, M.; Egholm, M.; Altman, W. E.; Attiya, S.; Bader, J. S.; Bemben, L. A.; Berka, J.; Braverman, M. S.; Chen, Y. J.; Chen, Z.; Dewell, S. B.; Du, L.; Fierro, J. M.; Gomes, X. V.; Godwin, B. C.; He, W.; Helgesen, S.; Ho, C. H.;Irzyk, G. P.; Jando, S. C.; Alenquer, M. L.; Jarvie, T. P.; Pirro, C. B.; Kim, J. B.; Knight, J. R.; Lanza, J. R.; Leamon, J. H.; Lefkowitz, S. M.; Lei, M.; Li, J.; Lohman, K. L.; Lu, H.; Makhijani, V. B.; McDade, K. E.; McKenna, M. P.; Myers, E. W.; Nickerson, E.; Noble, I. R.; Plant, R.; Puc, B. P.; Ronan, M. T.; Roth, G. T.; Sarkis, G. J.; Simons, J. F.; Simpson, J. W.; Srinivasan, M.; Tartaro, K. R.; Tomasz, A.; Vogt, K. A.; Volkmer, G. A.; Wang, S. H.; Wang, Y.; Weiner, M. P.; Wu, P.; Begley, R. F.; Rothberg, J. M. Nature 2005, 437, 376–380.

(21) Gordon, D.; Green, P. Bioinformatics 2013, 29, 2936–2937.

(22) Seemann, T. Bioinformatics 2014, 30, 2068–2069.

(23) Martinez, J.; Sousa, T.; da S.; Crespo, G.; Palomo, S.; Gonzalez, I.; Tormo, J. R.; de la Cruz, M.; Anderson, M.; Hill, R. T.; Vicente, F.; Genilloud, O.; Reyes, F. Mar. Drugs 2013, 11, 387–398.

(24) Zhang, L.; Ravipati, A. S.; Koyyalamudi, S. R.; Jeong, S. C.; Reddy, N.; Bartlett, J.; Smith, P. T.; de la Cruz, M.; Monteiro, M. C.; Melguizo, A.; Jiménez, E.; Vicente, F. Asian Pac. J. Trop. Med. 2013, 6, 673–681.