**Calcaripeptides A-C, Cyclodepsipeptides from a *Calcarisporium* Strain**

Johanna Silber,† Birgit Ohlendorf,‡ Antje Labes,† Christian Näther,§ and Johannes F. Imhoff\*,†

†Kieler Wirkstoff-Zentrum KiWiZ at the GEOMAR Helmholtz Centre for Ocean Research Kiel, Am Kiel-Kanal 44, 24106 Kiel, Germany

‡Formerly Kieler Wirkstoff-Zentrum KiWiZ at the GEOMAR Helmholtz Centre for Ocean Research Kiel, Am Kiel-Kanal 44, 24106 Kiel, Germany

§Institut für Anorganische Chemie, Christian-Albrechts-Universität zu Kiel, Max-Eyth-Strasse 2, 24118 Kiel, Germany

**ABSTRACT**

The isolation and structure elucidation of the novel calcaripeptides A (**1**), B (**2**) and C (**3**) and studies on their biosynthetic origin are described. The calcaripeptides were identified from a *Calcarisporium* sp. strain KF525, which was isolated from the German Wadden Sea. Compounds **1**-**3** are macrocyclic structures composed of a proline and a phenylalanine residue as well as a non-peptidic substructure. Structure elucidation was achieved by applying one- and two-dimensional NMR spectroscopy supported by high resolution mass spectrometry. X-ray crystallography was performed to determine the relative configuration of **1**. The absolute configuration of **1** was assigned by HPLC of the amino acids after hydrolysis of the molecule and derivatization with chiral agents. Studies on the biosynthesis by feeding 13C-labeled substrates revealed that the non-peptidic part of **1** originates from acetate and l-methionine. The involvement of a hybrid between a polyketide synthase and a non-ribosomal peptide synthetase in the biosynthesis of the calcaripeptides is discussed.

With the aim to discover new natural products, fungal strains from the German Wadden Sea were analyzed regarding their metabolite profiles. In Wadden Sea habitats often both true marine organisms and organisms from terrestrial and freshwater habitats frequently occur together. The *Calcarisporium* sp. strain KF525 attracted attention because it produced a set of metabolites that could not be identified by extensive searches of the literature and databases. Fungi of the genus *Calcarisporium* show a widespread occurrence on wood1,2 and leaf litter,3,4 in plants as endophytic fungi5,6 or in coal spoil tips.7 Commonly, *Calcarisporium* spp. are found as mycoparasites or symbionts of higher basidiomycetes and ascomycetes.1,8-12 For some mycoparasitic strains of *Calcarisporium* sp., the ability to reduce feed spoiling molds or fungal plant pathogens, like those responsible for mildews, was observed. Hence, the use of *Calcarisporium* culture filtrates as feed protecting preservatives and as biocontrol agents in crop protection has been discussed.6,11,13,14 It is well known that predatory interactions of microbes are often mediated by enzymes or small molecule compounds and toxins. Even though this has also been assumed to be the case for the antifungal interplay of *Calcarisporium* species,13 few natural products have been described for the genus.15 Among them are antifungal compounds like 15-azahomosterols,16 aurovertins inhibiting mitochondrial ATP synthesis and ATPases17-19 and calcarisporins B1–B4 with calcarisporin B1 showing cytotoxic activity.20,21

The marine-derived isolate KF525 of *Calcarisporium* sp. was shown to produce a metabolite spectrum different from those of other known *Calcarisporium* strains. The isolation of these metabolites from the fungal mycelium yielded the novel, structurally related calcaripeptides A (**1**), B (**2**) and C (**3**) described here. Analysis of the NMR spectroscopic data showed the compounds to be macrocyclic structures consisting of two amino acids (proline and phenylalanine) and a non-peptidic substructure. The non-peptidic chain varies in structure between the three compounds. The absolute configuration of **1** was determined by X-ray crystallography in combination with the configuration data of the amino acids obtained by HPLC analysis after hydrolysis of the molecule and chemical derivatization. The biosynthesis of **1** was investigated by feeding 13C-labeled precursors.

4

**1**

**2**

**3**

1

2

3

5

6

7

8

9

1'

2'

3'

4'

5'

1"

2"

3"

4"

5"

9"

6"

8"

7"



**RESULTS AND DISCUSSION**

Strain KF525 was identified as a *Calcarisporium* sp. based on morphological characteristics, in particular microscopic examination of the conidia and conidiophores, and on sequence analysis of the internal transcribed spacer (ITS) region. As the variation of culture conditions can often be reflected in altered metabolite patterns, the influence of four different culture media as well as static and shaking cultivation conditions on the metabolite production of KF525 was tested. As a response to these varied culture conditions the strain produced diverse metabolite profiles. While known compounds of the genus *Calcarisporium* were not found, new metabolite spectra were identified. An extract obtained from the mycelia of KF525 grown in modified Casamino Acids Glucose medium22 under shaking conditions was fractionated by preparative HPLC yielding compounds **1**-**3**.

High-resolution ESIMS measurements along with the spectroscopic data gave a molecular formula of C27H36N2O5 for **1**, requiring 11 degrees of unsaturation. The structure of **1** was established on the basis of one- and two-dimensional NMR spectra (1H, 13C (1H decoupled and DEPT), COSY, HSQC, and HMBC, see Table 1). The 13C NMR spectrum showed particularly intense signals at δC 129.8 and 130.7 accounting for two magnetically equivalent carbons each (C-6’’ + C-8’’ and C-5’’ + C-9’’). These aromatic carbons together with the further aromatic methine signal of C-7’’ (δC 128.3) and the quaternary carbon C-4’’ (δC 137.4) gave evidence of a monosubstituted benzene. Four carbonyl functions, C-3 (δC 198.6), C-1 (δC 174.2), C-1’ (δC 171.2), and C-1’’ (δC 171.8), were observed, of which the latter three had chemical shifts characteristic of amide and ester carbonyl groups. Additionally, the 13C NMR spectrum revealed the presence of two olefinic carbons, C-4 (δC 136.6) and C-5 (δC 149.6), four methyl groups, six methylene carbons, and five methine carbons.

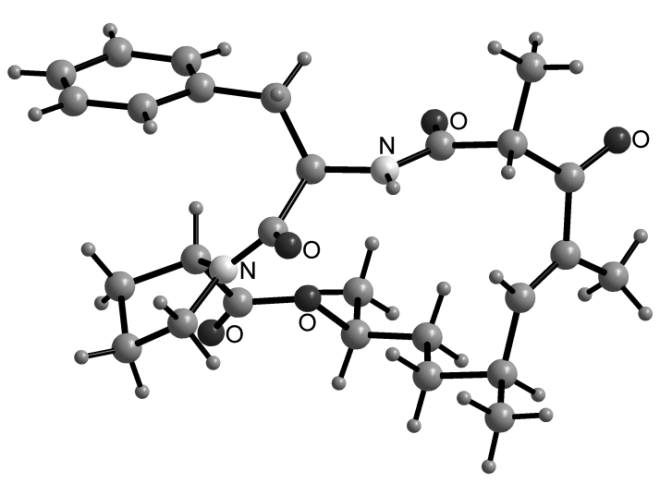
The 1H NMR spectrum showed five aromatic protons (Table 1), corroborating the monosubstituted benzene already deduced from the 13C NMR spectrum. HMBC correlations of H2-3’’ (δH 2.86 and 3.04) to the aromatic carbons (C-4’’, C-5’’+ C-9’’) as well as to C-2’’ and C-1’’ established a phenylalanine residue as a partial structure of **1**. The COSY spectrum displayed couplings between H2-3’ (δH 1.13 and 2.06), H2-4’ (δH 1.79) and H2-5’ (δH 3.36 and 3.43) indicating three consecutive methylene groups characteristic of a proline residue. In addition, correlations of H-2’ (δH 3.92) to H-3b’ in the COSY spectrum and to C-1’ in the HMBC spectrum evidenced that C-2’ was the proline α-carbon. The amide linkage between the phenylalanine and proline residues was proven by an HMBC correlation of H2-5’ to C-1’’ and is consistent with the chemical shift of C-1’’ (171.8). A coupling of H-9 to C-1’ indicated a connection between CH-9 and C-1’, which was further characterized as an ester bond by the respective chemical shifts (δC 75.0, δH 4.65 and δC 171.2). The COSY spectrum revealed that H-9 was part of a proton spin system which reached from 9-CH3 (δH 1.20) to H-5 (δH 6.42) including the methyl group 6-CH3. According to the chemical shifts, CH-5 (δC 149.6, δH 6.42) was olefinic. The methyl-substituted, olefinic carbon C-4 had to be located adjacent to CH-5 due to long-range H,C-couplings of H-5 to C-4 and 4-CH3, and of 4-CH3 (δH 1.73) to C-4 and C-5. The *E*-configuration of the double bond was determined on the basis of the NOESY spectrum in which 4-CH3 showed a more intense cross-peak with H-6 than with H-5. In addition, the configuration was supported by a NOESY cross-peak between 6-CH3 and H-5. HMBC correlations of H-2 (δH 4.23) to 2-CH3, C-1 and C-3 as well as correlations of H-5 and 4-CH3 to C-3 completed the consecutive chain from C-1 to C-9. Finally, correlations of H-2 to C-2’’ and H-2’’ to C-1 linked C-1 to the phenylalanine residue via an amide bond.

The aromatic ring, the proline ring, the macrocycle, four carbonyl groups, and the double bond ∆4,5 accounted for 11 degrees of unsaturation as was required by the molecular formula of **1**. Thus, a cyclodepsipeptide structure was established for **1**.

**Table 1.** 1H (600 MHz) and 13C (150 MHz) NMR Spectroscopic Data of Calcaripeptide A (**1**) in Methanol-*d*4.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| position | δC, type | δH, mult. (*J* in Hz) | COSY | | HMBC | NOESY*a* |
| 1 | 174.2, C |  |  | |  |  |
| 2 | 49.7, CH | 4.23, q (6.9) | 2-CH3 | | 1, 3, 4, 5, 2-CH3, 2'' | 5, 2-CH3 |
| 3 | 198.6, C |  |  | |  |  |
| 4 | 136.6, C |  |  | |  |  |
| 5 | 149.6, CH | 6.42, br d (10.4) | 6, 4-CH3 | | 3, 4, 6, 7, 4-CH3, 6-CH3 | 2, 6, 7a/8a, 7b, 4-CH3, 6-CH3, 2'' |
| 6 | 35.2, CH | 2.54, m | 5, 7b, 6-CH3 | | 4, 5, 7, 6-CH3 | 5, 7a/8a, 8b, 4-CH3, 6-CH3 |
| 7a | 35.7, CH2 | 1.40, m*b* |  | | 5, 6, 8, 6-CH3 | 5, 6, 7b, 9, 6-CH3, 2'' |
| 7b |  | 1.06, m | 6, 8a, 8b | | 5, 8, 9 | 5, 7a/8a, 9, 2'' |
| 8a | 35.9, CH2 | 1.40, m*b* | 7b, 8b, 9 | | 6, 7, 9 | 5, 6, 7b, 9, 6-CH3, 2'' |
| 8b |  | 1.32, m | 7b, 8a, 9 | | 5, 7, 9, 9-CH3 | 6, 9 |
| 9 | 75.0, CH | 4.65, m | 8a, 8b, 9-CH3 | | 7, 9-CH3, 1' | 7a/8a, 7b, 8b, 9-CH3 |
| 2-CH3 | 14.3, CH3 | 1.28, d (6.9) | 2 | | 1, 2, 3 | 2, 5'' + 9'', 6'' + 8'' |
| 4-CH3 | 12.2, CH3 | 1.73, d (1.2) | 5 | | 3, 4, 5, 6, 7, 6-CH3 | 5, 6 |
| 6-CH3 | 20.7, CH3 | 1.01, d (6.6) | 6 | | 5, 6, 7, 8 | 5, 6, 7a/8a |
| 9-CH3 | 21.0, CH3 | 1.20, d (6.2) | 9 | | 8, 9 | 9, 2'' |
| 1' | 171.2, C |  |  | |  |  |
| 2' | 60.5, CH | 3.92, br d (7.0) | 3b' | | 1', 3', 4', 5' | 3a', 3b', 5a', 2'', 3a'', 3b'', 5'' + 9'' |
| 3a' | 28.8, CH2 | 2.06, br dd (12.3, 7.0) | 3b', 4' | | 1', 2', 4', 5' | 2', 3b', 4' |
| 3b' |  | 1.13, m | 2', 3a', 4' | | 1', 2', 4', 5' | 2', 3a', 4', 5a', 5'' + 9'', 6'' + 8'', 7'' |
| 4' | 22.7, CH2 | 1.79, m | 3a', 3b', 5a', 5b' | | 2', 3', 5' | 3a', 3b', 5a', 5b' |
| 5a' | 46.8, CH2 | 3.43, dt (11.8, 8.8) | 4', 5b' | | 2', 3', 4', 1'' | 2', 3b', 4', 5'' + 9'' |
| 5b' |  | 3.36, ddd (12.2, 9.2, 3.2) | 4', 5a' | | 2', 3', 4', 1'' | 4' |
| 1'' | 171.8, C |  |  | |  |  |
| 2'' | 53.8, CH | 5.27, dd (9.1, 5.6) | 3a'', 3b'' | | 1, 1'', 3'', 4'' | 5, 7a/8a, 7b, 9-CH3, 2', 3a'', 3b'', 5'' + 9'' |
| 3a'' | 41.4, CH2 | 3.04, dd (12.9, 5.6) | 2'', 3b'' | | 1'', 2'', 4'', 5'' + 9'' | 2', 2'', 3b'', 5'' + 9'' |
| 3b'' |  | 2.86, dd (12.9, 9.1) | 2'', 3a'' | | 1'', 2'', 4'', 5'' + 9'' | 2', 2'', 3a'', 5'' + 9'' |
| 4'' | 137.4, C |  |  | |  |  |
| 5'' + 9'' | 130.7, CH | 7.22, br d (8.4) | 6'' + 8'' | | 2'', 3'', 7'', 5'' + 9'' | 2-CH3, 2', 3b', 5a', 2'', 3a'', 3b'' |
| 6'' + 8'' | 129.8, CH | 7.31, br dd (8.4, 7.3) | 5'' + 9'', 7'' | | 4'', 6'' + 8'' | 2-CH3, 3b' |
| 7'' | 128.3, CH | 7.25, br t (7.3) | 6'' + 8'' | | 5'' + 9'' | 3b' |
| *a*The NOESY NMR spectrum was recorded at 500 MHz  *b*Proton signals of 7a and 8a overlap. | | | |  |  |  |

A single-crystal X-ray diffraction analysis of a sample recrystallized from MeOH confirmed the structure of **1** and established its relative configuration. It was observed that the asymmetric unit of **1** consists of five different crystallographically distinct molecules with identical relative configuration. Small differences are found only in the conformation of each molecule. Figure 1 shows one of these molecules as a representative. The absolute configuration of **1** was then deduced from the configurations of the phenylalanine and proline residues. Their configurations were assigned by HPLC analyses of their d-FDVA (*N*α-(2,4-dinitro-5-fluorophenyl)-d-valinamide) derivatives (advanced Marfey’s method) after hydrolysis of the molecule. As comparative standards, commercially available l-amino acids were derivatized with d-FDVA and l-FDVA. By comparison of the sample and standards, both the phenylalanine and the proline residue were proven to be l-configured. Combining this information with that of the X-ray analysis, the absolute configurations at all stereogenic centers of **1** were determined as follows: 2*S*, 6*R*, 9*R*, 2’*S*, and 2’’*S*, the conformation of the proline amide bond was *cis*.



**Figure 1.** Molecular structure of one of the five crystallographically distinct molecules in the crystal structure of **1**.

The NMR spectra of **2** were very similar to those of **1** (Table 2). Because the spectra of **2** were recorded in the aprotic solvent acetone-*d*6 instead of methanol-*d*4, an additional signal at δH 7.51 appeared in the 1H NMR spectrum of **2** corresponding to the amide proton NH-2’’. Couplings between NH-2’’ and CH-2’’ in the COSY spectrum confirmed their vicinity as was already deduced from the spectroscopic data of **1**. Furthermore, the NMR spectra of **2** lacked the signal of the methyl group at C-2 which was fully consistent with a mass difference of 14 between structures **1** and **2**. In accordance to the loss of the methyl group, CH2-2 was a methylene group. The signals of H-2a and H-2b (δH 4.32 and 3.15) showed low intensities, and the carbon signal of C-2 (δC 48.2) was weak. Therefore, its chemical shift had to be deduced from the HSQC spectrum. The low signal intensities can be ascribed to the acidic nature of CH2-2 being in the α-position of a β-ketoamide function. The absolute configuration of **2** was postulated in analogy to **1**, yet it was not empirically confirmed.

The NMR spectra of **3** proved that its structurewas almost identical to **1**, except for the lack of the methyl-substituted double bond (Table 2). This was in agreement with a mass decrease of 40 compared to **1**, accounting for C3H4. The structure of **3** was confirmed by the analysis of the two-dimensional NMR spectra. The absolute configuration of **3** was also postulated in analogy to **1**.

Structurally, the calcaripeptides are related to acremolides A and B.23 The compounds share the feature of being cyclodepsipeptides containing an l-proline-l-phenylalanine moiety that together with a non-peptidic partial structure forms the macrocycle. However, the non-peptidic part of the acremolides and calcaripeptides differs and the acremolides possess an additional 7-membered alkyl side chain connected to the ring. The 16- and 14-membered rings of **1**, **2** and **3** are unusual for natural products.

**Table 2.** 1H and 13C NMR Spectroscopic Data of Calcaripeptide B (**2**) (500 MHz and 125 MHz) and Calcaripeptide C (**3**) (600 MHz and 150 MHz) in Acetone-*d*6.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | calcaripeptide B (**2**) | |  | calcaripeptide C (**3**) | |
| position |  | δC, type | δH, mult. (*J* in Hz) |  | δC, type | δH, mult. (*J* in Hz) |
| 1 |  | 167.5, C |  |  | 171.0, C |  |
| 2a |  | 48.2, CH2*a* | 4.32, br s |  | 52.3, CH | 3.66, q (6.9) |
| 2b |  |  | 3.15, br s |  |  |  |
| 3 |  | 196.3, C |  |  | 209.3, C |  |
| 4 |  | 136.0, C |  |  | 44.1, CH | 2.85, m |
| 5a |  | 149.4, CH | 6.40, br d (10.1) |  | 31.8, CH2 | 1.53, m*b* |
| 5b |  |  |  |  |  | 1.06, m |
| 6a |  | 34.6, CH | 2.53, m |  | 35.3, CH2 | 1.67, m |
| 6b |  |  |  |  |  | 1.53, m*b* |
| 7a |  | 35.4, CH2 | 1.44, m*b* |  | 74.0, CH | 4.84, m |
| 7b |  |  | 1.15, m |  |  |  |
| 8a |  | 35.6, CH2 | 1.48, m*b* |  |  |  |
| 8b |  |  | 1.32, m |  |  |  |
| 9 |  | 74.1, CH | 4.63, m |  |  |  |
| 2-CH3 |  |  |  |  | 14.5, CH3 | 1.22, d (6.9) |
| 4-CH3 |  | 11.6, CH3 | 1.70, d (1.3) |  | 15.6, CH3 | 1.02, d (6.7) |
| 6-CH3 |  | 20.6, CH3 | 0.98, d (6.6) |  |  |  |
| 7-CH3 |  |  |  |  | 21.0, CH3 | 1.19, d (6.3) |
| 9-CH3 |  | 20.9, CH3 | 1.18, d (6.2) |  |  |  |
| 1' |  | 171.2, C |  |  | 172.2, C |  |
| 2' |  | 59.7, CH | 3.85, br d (7.5) |  | 59.8, CH | 3.90, m |
| 3a' |  | 29.0, CH2 | 2.01*c* |  | 30.3, CH2 | 1.87, m |
| 3b' |  |  | 1.29, m |  |  | 1.46, m |
| 4a' |  | 22.5, CH2 | 1.77, m |  | 22.4, CH2 | 1.88, m |
| 4b' |  |  |  |  |  | 1.71, m |
| 5a' |  | 46.2, CH2 | 3.39, m |  | 46.3, CH2 | 3.38, m |
| 5b' |  |  | 3.34, m |  |  |  |
| 1'' |  | 170.6, C |  |  | 169.6, C |  |
| 2'' |  | 53.4, CH | 5.18, m |  | 54.2, CH | 4.97, m |
| 3a'' |  | 41.2, CH2 | 3.08, dd (12.8, 5.0) |  | 40.6, CH2 | 3.06, ddt (12.5, 4.7, 2.5) |
| 3b'' |  |  | 2.80, dd (12.8, 9.0) |  |  | 2.93, dddd (12.5, 10.0, 3.2, 2.1) |
| 4'' |  | 137.7, C |  |  | 138.2, C |  |
| 5'' + 9'' |  | 130.3, CH | 7.20, br d (8.4) |  | 130.2, CH | 7.25, br d (7.5) |
| 6'' + 8'' |  | 129.3, CH | 7.31, br dd (8.4, 7.4) |  | 129.2, CH | 7.30, br t (7.5) |
| 7'' |  | 127.7, CH | 7.24, br t (7.4) |  | 127.5, CH | 7.23, br t (7.5) |
| NH-2'' |  |  | 7.51, br d (6.7) |  |  | 7.61, br d (7.5) |
| *a*Signal deduced from the HSQC spectrum. | | | | |  |
| *b*Proton signals of overlap. | | | | |  |
| *c*Signal partially obscured. | | | | |  |

The calcaripeptides were tested for activities against five bacterial test strains, three fungal test strains, one oomycete and two cell lines as well as for inhibition of selected enzyme targets (glycogen synthase kinase-3*β*, acetylcholinesterase, phosphodiesterase 4B2 and protein tyrosine phosphatase 1B). In addition, **1** was tested in further assays including 24 cell lines. Despite the broad panel of 43 assays, neither antibacterial, antifungal and cytotoxic properties, nor inhibition of the enzyme targets could be detected for the calcaripeptides (data not shown, for information on test strains, cell lines and enzymes see SI).

Judging from the structure, a polyketidic biosynthetic origin of the non-peptide substructure of the calcaripeptides was assumed. Polyketides are a structurally diverse class of natural products synthesized by polyketide synthases (PKS). The substrates of PKS enzymes are CoA thioesters of small carboxylic acids, such as acetate and malonate. In order to prove the suggested biosynthetic pathway of **1**, feeding experiments using 13C-labeled precursors were carried out. The feeding experiment with 1-13C-acetate led to an enrichment of the 13C signals in the positions 1, 3, 5, 7, and 9 (Figure 2). Therefore, the chain that connects the carboxy group of the l-proline residue with the amino group of the l-phenylalanine residue is built up by five acetate units with the biosynthesis beginning at 9-CH3 and progressing towards C-1. In addition, a slight enhancement of the 13C NMR signal intensities for C-1’ and C-5’ was observed revealing two acetate building blocks for the l-proline moiety. The incorporation of 13C-labeled acetate into the l-proline substructure shows that a portion of the amino acid was synthesized *de novo,* the labeling pattern is consistent with its formation from -ketoglutarate. The methyl groups of **1**, 2-CH3, 4-CH3 and 6-CH3, originate from S-adenosylmethionine (SAM) as was confirmed by the enhancement of their 13C NMR signals after feeding l-methionine-methyl-13C (Figure 2).



1-13C-acetate

SAM derived methyl group



**Figure 2.** Biosynthetic origin of calcaripeptide A (**1**) as determined by 13C-labeling. For detailed information on the calculation of the 13C enrichment see SI.

According to the feeding studies, a polyketidic origin of the non-peptidic part of the calcaripeptides was confirmed. The overall structure and the biosynthetic origin of the building blocks support the involvement of a hybrid between a polyketide synthase and non-ribosomal peptide synthetase for the peptidic backbone (PKS-NRPS hybrid) in the formation of the calcaripeptides. An increasing number of fungal compounds have recently been shown to be synthesized by this type of enzyme, e.g. fusarin C, aspyridone or pseurotin.24 The genome of *Calcarisporium* sp. strain KF525 is currently under investigation with the aim of identifying genes encoding the respective PKS-NRPS hybrids responsible for the biosynthesis of the calcaripeptides. Reference sequences of the known hybrid PKS-NRPS genes from other fungi are available for comparison.

Promiscuity with respect to the amino acid substrates has been shown for fungal NRPS *in vitro* and *in vivo*.25-27 As promiscuity might thus be a possibility for the postulated NRPS component of the biosynthesis of **1**-**3**, it was tested whether a supply of structurally related, alternative amino acids as substrates for the NRPS would lead to derivatives of **1**. Supplementation of l-tryptophan, l-tyrosine or l-histidine to the culture broth of KF525 did not result in their incorporation into **1** in place of l-proline or l-phenylalanine. Therefore, the enzymes involved in the biosynthesis of the calcaripeptides seem to be specific for their amino acid substrates. Additional supply of l-proline and l-phenylalanine as the naturally incorporated amino acids led to an increased production of **1** displaying potential for an optimization in the fermentation process.

**Experimental Section**

**General Experimental Procedures.** Melting points were determined on an Electrothermal melting point apparatus. Optical rotation measurements were performed on a Perkin Elmer model 241 polarimeter. UV spectra were obtained on a Perkin Elmer Lambda 2 spectrophotometer. NMR spectra were recorded on Bruker DRX 500 (500 and 125 MHz for 1H and 13C NMR, respectively) and Bruker AV 600 spectrometers (600 and 150 MHz for 1H and 13C NMR, respectively), using the residual solvent signals as internal references (δH 3.31 and δC 49.0 for methanol-*d*4; δH 2.05 and δC 29.8 for acetone-*d*6). Measurements of high-resolution mass spectra were conducted on a benchtop time-of-flight spectrometer (micrOTOF II, Bruker) with positive ESI. Analytical reversed-phase HPLC-UV/MS experiments were carried out on a VWR-Hitachi LaChrom Elite system consisting of an L-2130 pump, an L-2450 diode array detector, an L-2200 autosampler, an L-2300 column oven and a Phenomenex Onyx Monolithic column (C18, 100 × 3.00 mm) applying an H2O (A)/MeCN (B) gradient with 0.1% HCOOH added to both solvents (gradient: 0 min 5% B, 4 min 60% B, 6 min 100% B; flow 2 mL min-1). For mass detections the HPLC system was coupled to an ESI-ion trap detector (esquire4000, Bruker Daltonics). Preparative HPLC was performed on a VWR LaPrep system equipped with a P110 pump, a P311 UV detector, a Labocol Vario-2000 fraction collector (LABOMATIC), a Smartline 3900 autosampler (Knauer) and a Phenomenex Gemini-NX column (10µ C18, 100A, Axia, 100 × 50.00 mm). Further compound purifications were conducted on a Merck-Hitachi LaChrom Elite HPLC system consisting of an L-7150 pump, an L-2450 diode array detector, an L-2200 autosampler and Phenomenex Gemini-NX column (5µ C18, 110A, Axia, 100 × 21.20 mm). The eluents for all preparative HPLC separations were H2O (A) and MeCN (B) with 0.1% HCOOH added to both solvents.

**Isolation and Identification of the Fungal Strain.** The strain KF525 was isolated from a water sample collected in the German Wadden Sea. The DNA extraction, the amplification of the internal transcribed spacer region (ITS) and the sequencing were performed using standard protocols28 modified in that the centrifugation was carried out at 8000 × *g* for DNA extraction, DreamTaq Green PCR Master Mix (2x) (Fermentas) was employed for amplification, 35 cycles instead of 30 were conducted for amplification and the primer ITS4 was used for sequencing. The DNA sequence was deposited in GenBank under the accession number KC800713. A sequence analysis in GenBank using the Basic Local Alignment Search Tool (BLAST) gave 91% similarity to *Calcarisporium arbuscula*. A microscopic analysis of the strain showed structures typical of the genus *Calcarisporium*. Taken together, the sequence and morphological data allowed the identification of strain KF525 as a *Calcarisporium* sp., as was additionally confirmed by the Centraalbureau voor Schimmelcultures (CBS, Utrecht, the Netherlands).

**Fermentation.** Cultivation experiments were performed in sixteen 2-L Erlenmeyer flasks, each containing 750 mL of modified Casamino Acids Glucose medium (2.5 g casein hydrolysate, 40 g glucose × H2O, 0.1 g MgSO4 × 7H2O, 1.8 g KH2PO4 per liter of distilled H2O, pH 6.8).22 Cultures were inoculated with a circular agar slant (1.8 cm in diameter) of a preculture grown on solid modified Wickerham medium (3 g malt extract, 3 g yeast extract, 5 g peptone from soymeal, 10 g glucose × H2O, 30 g NaCl, 15 g agar per liter of distilled H2O, pH 6.25).29 The preculture was incubated at room temperature in the dark for 11 days. The main cultures were incubated at 22 °C under shaking conditions (120 rpm) in the dark for 24 days.

**Purification of Calcaripeptides A**-**C.** The culture broth of KF525 was separated into the culture supernatant and the mycelium. The culture filtrate was extracted with EtOAc. The organic solvent was evaporated to dryness *in vacuo* to give 0.92 g of extract. The extract was fractionated by preparative HPLC on a VWR LaPrep system (gradient: 0 min 10% B, 17 min 60% B, 22 min 100% B; flow 100 mL min-1; UV detection at 217 nm) yielding three fractions that contained the calcaripeptides (*t*R 15.7 min, 16.6 min and 17.6 min). These fractions were further purified on a Merck-Hitachi LaChrom Elite system to give 117.9 mg of **1** (isocratic: 42% B; flow 18 mL min-1; UV detection at 220 nm; *t*R 10.7 min), 6.2 mg of **2** (isocratic: 29% B; UV detection at 205 nm; *t*R 27.5 min) and 8.5 mg of a compound mixture containing **3** (gradient: 0 min 40% B, 13 min 65% B; flow 18 mL min-1; UV detection at 205 nm; *t*R 6.2 min). Compound **3** was subjected to a third preparative HPLC purification on the same system (gradient: 0 min 40% B, 13 min 80% B; flow 18 mL min-1; UV detection at 205 nm; *t*R 5.8 min) resulting in a yield of 5.7 mg.

**Calcaripeptide A (1):** white needles or amorphous solid (MeOH); mp 199–201 °C; [α]20D -133 (*c* 1.4 , MeOH); UV (MeOH) λmax (log ε) 201 (4.43), 230 (sh) (4.14) nm; 1D and 2D NMR data, see Table 1; HRESIMS *m/z* 469.2707 [M + H]+ (calcd for C27H37N2O5, 469.2697).

**Calcaripeptide B (2):** colorless oil; [α]20D -113 (*c* 0.31 , MeOH); UV (MeOH) λmax (log ε) 201 (4.35) nm, 230 (sh) (4.09); 1D and 2D NMR data, see Table 2 and SI; HRESIMS *m/z* 455.2538 [M + H]+ (calcd for C26H35N2O5, 455.2541).

**Calcaripeptide C (3):** colorless oil; [α]20D -79 (*c* 0.2 , MeOH); UV (MeOH) λmax (log ε) 201 (4.24) nm; 1D and 2D NMR data, see Table 2 and SI; HRESIMS *m/z* 429.2379 [M + H]+ (calcd for C24H33N2O5, 429.2384).

**X-ray Crystal Structure Determination.** Data collection was performed using an Imaging Plate Diffraction System (IPDS-2) from STOE & CIE at 293 K using Mo-Kα-radiation (=0.71073 Å). Formula: C27H36N2O5, Molecular weight: 468.58 g mol-1, monoclinic, space group C2; unit cell dimensions: a = 44.5917(12) Å, b = 9.6204(2) Å, c = 37.0616(10) Å, = 122.453(2)°, V = 13416.1(6) Å3, Z = 20, Dcalcd = 20, 1.160 Mg/m3 , µ = 0.080 mm. The structure was solved with methods using *SHELXS-97* and refinement was performed against F2 using *SHELXH*. All non-hydrogen atoms were refined anisotropic. The H atoms were positioned with idealized geometry and refined isotropic with Uiso(H) = 1.2 Ueq(C,N) using a riding model (1.5 for methyl H atoms). 27668 measured reflections in the range of 2θ 1.2-24.6° of which 11536 are independent (Rint = 0.0347), 1531 parameters, Gof = 1.080, R1 for 8490 reflections with I>2(I) = 0.0521, wR2 for all reflections = 0.1205. Residual electron density = 0.326/0.164 e/Å3. In one of the five independent molecules slightly enlarged anisotropic displacement parameters are observed for some C atoms indicating for disorder that cannot be resolved successfully. Because no strong anomalous scattering atoms are present the absolute structure and absolute configuration cannot be determined and therefore, Friedel equivalents were merged in the refinement.

Crystallographic data for **1** have been deposited with the Cambridge Crystallographic Data Centre (CCDC-943694). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

**Preparation and Analysis of d- and l-FDVA Derivatives.** The hydrolysis of **1** was achieved by dissolving 2.7 mg of the compound in 1.5 mL of 6 N HCl and subsequent heating at 110 °C overnight. The reaction mixture was then concentrated to dryness and redissolved in 250 µL H2O. For the derivatization, 50 µL of the hydrolysate solution were mixed with 100 µL of a 1% (w/v) solution of d-FDVA (*N*α-(2,4-dinitro-5-fluorophenyl)-d-valinamide) in acetone. After addition of 40 µL of 1 M NaHCO3 and 70 µL DMSO, the mixture was incubated at 60 °C for 2 h. The reaction was stopped by addition of 30 µL of 2 M HCl. The amino acid standards (50 mM l-proline and l-phenylalanine) were derivatized with d-FDVA and l-FDVA in the above described manner. Prior to analytical HPLC-UV/MS analyses the reaction mixtures were diluted 100-fold with MeOH/H2O (1:1). The retention times (min) of the amino acid standard derivatives were as follows: d-FDVA-l-Pro (3.69), l-FDVA-l-Pro (3.48), d-FDVA-l-Phe (4.50) and l-FDVA-l-Phe (4.07). The HPLC analysis of the hydrolysate d-FDVA derivatives showed peaks at 3.69 min and 4.50 min. An additional confirmation of the amino acid configuration was accomplished by spiking the hydrolysate derivatives with the amino acid standard derivatives.

**Biosynthetic Studies.** For the feeding experiments, *Calcarisporium* sp. strain KF525 was cultivated as described in the fermentation section above. Precultures were 7 to 15 days old. Each feeding experiment was performed in one 2-L Erlenmeyer flask, containing 750 mL medium. After five days of cultivation 13C-labeled compounds were added as sterile filtered, aqueous solutions (500 mg sodium acetate-1-13C, Isotec or 250 mg l-methionine-methyl-13C, Cambridge Isotope Laboratories). For the extraction, the supernatant and the mycelium of the cultures were separated after 21 days of incubation. The culture filtrate was extracted with EtOAc as described above, while the mycelium was extracted with EtOH.

The culture filtrate fed with 13C-labeled acetate was purified on a Merck-Hitachi LaChrom Elite system (gradient: 0 min 28% B, 30 min 61% B; flow 18 mL min-1; UV detection at 230 nm; *t*R 17.9 min) to give 8.2 mg of **1**.

In the feeding experiment with 13C-labeled methionine, compound **1** was isolated from the extracts of both the culture filtrate and the mycelium. Afterwards, purified **1** was combined for NMR spectroscopy studies. The extract of the culture filtrate was separated on a Merck-Hitachi LaChrom Elite system (gradient: 0 min 28% B, 20 min 50% B; flow 18 mL min-1; UV detection at 230 nm; *t*R 14.7 min) yielding 1.6 mg of **1**. The purification of compound **1** from the extract of the mycelium was conducted on the same system (gradient: 0 min 35% B, 20 min 50% B, 20.5 min 70% B, flow 18 mL min-1; UV detection at 230 nm; *t*R 12.7 min) with a yield of 0.6 mg.

**Feeding Alternative Amino Acids.** Each amino acid experiment was performed in one 2-L Erlenmeyer flask, containing 750 mL medium. Culture conditions and medium were the same as in the fermentation section described above. After five days of cultivation the respective amino acid was added as a sterile filtered, 50 mL aqueous solution (end concentration in culture medium: 1 g L-1). The added amino acids were l-tryptophan, l-tyrosine, l-histidine, l-proline, l-phenylalanine and l-proline plus l-phenylalanine. l-tyrosine did not dissolve completely and only the dissolved portion was added to the culture. Two cultures without additionally supplied amino acids served as controls in the experiment. The culture filtrate was extracted with EtOAc as described above, while the mycelium was extracted with EtOH. The extracts were analyzed by analytical HPLC-UV/MS.

**Supporting Information.** 1D and 2D NMR spectra of **1**, 1H NMR spectra and 1D and 2D NMR data tables of **2** and **3**, 13C NMR spectra of labeling experiments and calculations for 13C enrichment, information on test strains, cell lines and enzymes of activity assays. This material is available free of charge via the Internet at http://pubs.acs.org.

**Corresponding Author**

\* To whom correspondence should be addressed. Tel: +49-431-6004450. Fax: +49-431-6004452. E-mail: jimhoff@geomar.de.

**ACKNOWLEDGEMENT**

We gratefully thank Dr. K. Schaumann for providing the strain KF525, A. Erhard and the European ScreeningPort for bioactivity and enzyme assays and G. Kohlmeyer-Yilmaz, M. Höftmann as well as Dr. F. Sönnichsen for running and processing NMR experiments. We also thank the Institute of Clinical Molecular Biology in Kiel for providing Sanger sequencing as supported in part by the DFG Cluster of Excellence “Inflammation at Interfaces” and "Future Ocean". We thank the technicians S. Greve and S. Arndt for technical support. This study was performed in the framework of MARINE FUNGI, EU FP7 KBBE program, project no. 265926 at the Kieler Wirkstoff-Zentrum (KiWiZ) at the GEOMAR Helmholtz Centre for Ocean Research Kiel.

**REFERENCES**

(1) Sutton, B. C. *Hyphomycetes from Manitoba and Saskatchewan, Canada*; Commonwealth Mycological Institute: Kew, Surrey, England, 1973; p 143.

(2) Cooper, J. A. *New Zeal. J. Bot.* **2005**, 43, 323-349.

(3) Rambelli, A.; Mulas, B.; Pasqualetti, M. *Mycol. Res.* **2004**, 108, 325-336.

(4) Somrithipol, S.; Jones, E. B. G. *Sydowia* **2006**, 58, 133-140.

(5) Gong, L.-J.; Guo, S.-X. *Afr. J. Biotech.* **2010**, 8, 731-736.

(6) Ji, L. L.; Song, Y. C.; Tan, R. X. *J. Appl. Microbiol.* **2004**, 96, 352-358.

(7) Evans, H. C. *Trans. Br. Mycol. Soc.* **1971**, 57, 255-266.

(8) Barnett, H. L. *Mycologia* **1958**, 50, 497-500.

(9) Barnett, H. L.; Lilly, V. G. *Parasitism of Calcarisporium parasiticum on species of Physalospora and related fungi*; West Virginia University Agricultural Experiment Station, 1958; p 37.

(10) Watson, P. *Trans. Br. Mycol. Soc.* **1955**, 38, 409-414.

(11) Carrión, G.; Rico-Gray, V. *Fungal Divers.* **2002**, 11, 49-60.

(12) Rombach, M. C.; Roberts, D. W. *Mycologia* **1987**, 79, 153-155.

(13) Hijwegen, T. *Neth. J. Plant Pathol.* **1989**, 95, 95-98.

(14) Hijwegen, T.; Verhaar, M. A. *Neth. J. Plant Pathol.* **1993**, 99, 103-107.

(15) *Dictionary of Natural Products*; Chapman & Hall/CRC Press/Hampden Data Services, Ltd., **2012**.

(16) Chrisp, P.; Dewick, P. M.; Boyle, F. T. *Z. Naturforsch. C Biosci.* **1990**, 45, 179-186.

(17) Baldwin, C. L.; Weaver, L. C.; Brooker, R. M.; Jacobsen, T. N.; Osborne Jr, C. E.; Nash, H. A. *Lloydia* **1964**, 27, 88-95.

(18) Mulheirn, L. J.; Beechey, R. B.; Leworthy, D. P.; Osselton, M. D. J. *Chem. Soc., Chem. Commun.* **1974**, 874-876.

(19) Osselton, M. D.; Baum, H.; Beechey, R. B. *Biochem. Soc. Trans.* **1974**, 2, 200-202.

(20) Yu, N.-J.; Guo, S.-X.; Lu, H.-Y. *J. Asian Nat. Prod. Res.* **2002**, 4, 179-183.

(21) Yu, N.-J.; Guo, S.-X.; Xiao, P.-G. *Acta Bot. Sin.* **2002**, 44, 878-882.

(22) Stevens, R. B. *Mycology Guidebook*; University of Washington Press: Seattle and London, 1974; p 703.

(23) Ratnayake, R.; Fremlin, L. J.; Lacey, E.; Gill, J. H.; Capon, R. J. *J. Nat. Prod.* **2008**, 71, 403-408.

(24) Collemare, J.; Billard, A.; Böhnert, H. U.; Lebrun, M.-H. *Mycol. Res.* **2008**, 112, 207-215.

(25) Qiao, K.; Zhou, H.; Xu, W.; Zhang, W.; Garg, N.; Tang, Y. *Org. Lett.* **2011**, 13, 1758-1761.

(26) Krause, M.; Lindemann, A.; Glinski, M.; Hornbogen, T.; Bonse, G.; Jeschke, P.; Thielking, G.; Gau, W.; Kleinkauf, H.; Zocher, R. *J. Antibiot.* **2001**, 54, 797-804.

(27) Xu, Y.; Zhan, J.; Wijeratne, E. M. K.; Burns, A. M.; Gunatilaka, A. A. L.; Molnár, I*. J. Nat. Prod.* **2007**, 70, 1467-1471.

(28) Wiese, J.; Ohlendorf, B.; Blümel, M.; Schmaljohann, R.; Imhoff, J. F*. Mar. Drugs* **2011**, 9, 561-585.

(29) Wickerham, L. J. *Taxonomy of yeasts*; US Dept. of Agriculture: Washington, D. C., 1951; p 56.

**Table of Contents/Abstract Graphic**

