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# Insights into the Biosynthetic Origin of 3-(3-Furyl)alanine in *Stachylidium* sp. 293 K04 Tetrapeptides

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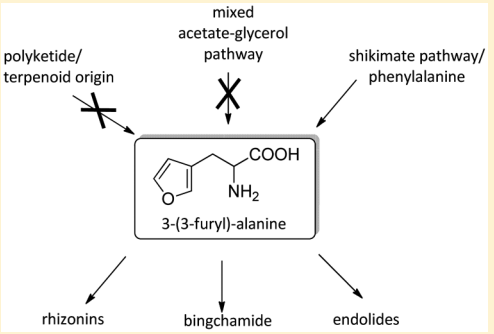
Supporting Information

**ABSTRACT:** The marine-sponge-derived fungus *Stachylidium* sp. 293 K04 produces the *N*-methylated peptides endolide A (**1**) and endolide B (**2**), showing affinity for the vasopressin receptor 1A and serotonin receptor $5HT_{2B}$, respectively. Both peptides feature the rare amino acid 3-(3-furyl)alanine. Isotope labeling experiments, employing several $^{13}C$-enriched precursors, revealed that this unprecedented heterocyclic amino acid moiety in endolide A (**1**) is synthesized from a cyclic intermediate of the shikimate pathway, but not from phenylalanine. Two new tetrapeptide analogues, endolides C and D (**3** and **4**), were characterized, as well as the previously described hirsutide (**5**).

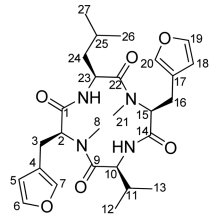


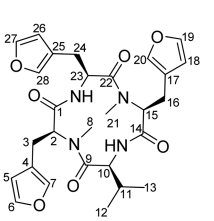

The marine sponge-derived fungus *Stachylidium* sp. 293 K04 produces a plethora of secondary metabolite classes including peptides.[1−3] In a former study we characterized the novel cyclic *N*-methylated peptides endolides A and B (**1** and **2**), which showed affinity for the vasopressin receptor 1A and serotonin receptor $5HT_{2B}$, respectively.[4]

The most intriguing feature of these supposedly non-ribosomal peptides is the incorporation of the nonproteinogenic amino acid *N*-methyl-3-(3-furyl)alanine, to date only reported in the heptapeptides rhizonins A and B and the pentapeptide bingchamide B.[5,6] Rhizonin A was initially considered as a mycotoxin metabolite isolated from the fungus *Rhizopus microsporus*, but was later found to be produced by the bacterial endosymbiont *Burkholderia endofungorum*.[7] Bingchamide B was isolated from a soil-dwelling strain of *Streptomyces bingchenggensis*.

Due to the novel nature of *N*-methyl-3-(3-furyl)alanine, the current study set out to investigate its metabolic origin, employing classical isotope tracer experiments. During the course of this study, we obtained, apart from **1**, three further cyclic *N*-methylated peptide analogues, two of which (**3**, **4**) were structurally unprecedented, whereas hirsutide (**5**) was already described.[8] A further observation during our studies was the production of two thiodiketopiperazines, upon adding phenylalanine to a liquid culture of *Stachylidium* sp. 293 K04. These are bisdethiobis(methylthio)acetylaranotin (BDA) and bisdethiobis(methylthio)acetylapoaranotin (BDAA), belonging to the aranotin group of metabolites.[9]

Here we report the investigation of the biosynthetic origin of the *N*-methyl-3-(3-furyl)alanine moiety of endolide A (**1**) and the description of the new structural analogues **3** and **4**.


endolide A (**1**)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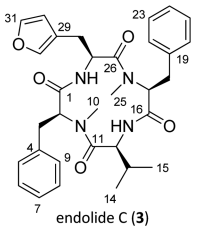
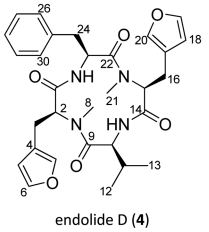
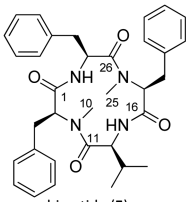

endolide C (**3**)


endolide D (**4**)


hirsutide (**5**)

## RESULTS AND DISCUSSION

For studying the biosynthesis of the unusual *N*-methyl-3-(3-furyl)alanine moiety in *Stachylidium* 293 K04 peptides using isotope-labeled precursors, the major metabolite endolide A (**1**) was targeted. First experiments were initiated to establish an isotope incorporation protocol. Because the peptides are not produced in liquid media, solid biomalt sea-salt-containing agar media were essential for the investigation. Applying LC-MS, production of endolide A (**1**) was detectable around 10 days

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after inoculation onward and up to day 55 (Table S1, Supporting Information). From this time scale experiment, we concluded that the labeled precursors should be added twice, i.e., on day 5 and 10 with a total cultivation time of 30 days (see Experimental Section and Table S1).

The first hypothesis on the biosynthesis of 3-(3-furyl)alanine was based on its structural relationship to the phenylalanine skeleton. Indeed, the oxidative cleavage of a benzene ring as required in this case would not be unusual for fungi. Prominent examples of this are seen in the mycotoxin patulin,[10] the betalain pigments,[11] and the pyrrolobenzodiazepine antitumor antibiotics.[12] Also, the metabolites BDA and BDAA (Figure S1, Supporting Information), isolated from *Stachylidium* sp. 293 K04 in this study, both have an oxidatively cleaved aromatic ring, pointing toward such enzymatic capabilities in the fungus.[13]

Consequently, a labeling experiment with L-[1-$^{13}$C]-phenylalanine was initiated. Incorporation of L-[1-$^{13}$C]-phenylalanine was monitored using LC-MS and further verified after isolation of endolide A (**1**) and other peptides and their $^{13}$C NMR spectroscopic analysis. No shift in the molecular mass for endolide A (**1**) was observed in the LC-MS spectra as compared to the control without labeled phenylalanine (Figure S2). Additionally, no $^{13}$C-enriched signals were observed in the $^{13}$C NMR spectrum of isolated endolide A (**1**) from the same labeling experiment (Figure S3). Validity of this experiment was confirmed by the simultaneous isolation of hirsutide (**5**), a cyclic peptide previously reported from the entomopathogenic fungus *Hirsutella* sp.[8] Hirsutide (**5**) was found to incorporate three L-[1-$^{13}$C]phenylalanine units, as evident from three enhanced $^{13}$C NMR signals for the carbonyl carbons C-1 ($\delta_C$ 170.1), C-16 ($\delta_C$ 171.0), and C-26 ($\delta_C$ 172.1) of the phenylalanine residues (Figure S4). Along with that, a 3 Da shift of the molecular mass of hirsutide (**5**) was observed in the LC-MS spectrum of the extract of the *Stachylidium* sp. 293 K04 culture supplemented with L-[1-$^{13}$C]phenylalanine; that is, $^{13}$C-labeled hirsutide (**5**) showed a signal at $m/z$ 572 $[M + H + 3]^+$ as compared to the unlabeled control with $m/z$ 569 $[M + H]^+$ (Figure S2). Thus, in this experiment it was clearly demonstrated that phenylalanine is not the precursor for 3-(3-furyl)alanine.

In a further set of experiments, general carbon source precursors such as $^{13}$C-labeled glycerol or glucose, which are involved in several biosynthetic pathways, were employed. These building blocks or metabolites derived thereof give specific labeling patterns upon incorporation and help in delineating the involved biosynthetic pathway. Incubation with fully labeled [U-$^{13}$C]glycerol gave a complex $^{13}$C NMR spectrum for the isolated endolide A (**1**) (Figure S5). Detailed analysis of the signal pattern and $^{13}$C−$^{13}$C coupling constants in the inverse gated proton decoupled $^{13}$C NMR spectrum of endolide A (**1**) showed the presence of a coupled assembly of three carbon atoms, i.e., C-1/C-2/C-3, evidenced by a 52.5 Hz coupling between C-1 and C-2 and a 38.4 Hz coupling between C-2 and C-3. Another coupled three-carbon assembly was observed for C-5/C-4/C-7, as evidenced by a 49.5 Hz coupling between C-4 and C-5 and a 72.8 Hz coupling between C-4 and C-7. A single enriched noncoupled $^{13}$C signal was detected for C-6 of the first 3-(3-furyl)alanine moiety (FurAla A, Table 1). This labeling pattern was mirrored in the second 3-(3-furyl)alanine moiety (FurAla B) as shown in Table 1 (for detailed chemical shifts and coupling constants of [U-$^{13}$C] glycerol-derived endolide A (**1**) see also Table S4).

**Table 1. Results of $^{13}$C-Labeling Experiments for Endolide A (1)**

| | | enriched carbon atoms in endolide A | | | |
|---|---|---|---|---|---|
| no. | fed precursor | *N-Me*-FurAla (FurAla A) | Val | *N-Me*-FurAla (FurAla B) | Leu |
| 1 | L-[1-$^{13}$C] phenylalanine | n.e.[a] | n.e. | n.e. | n.e. |
| 2 | [U-$^{13}$C] glycerol | C-1/C-2/C-3, | C-9/C-10, | C-14/C-15/C-16, | C-22/C-23, |
| | | C-5/C-4/C-7, | C-11/C-12, | C-18/C-17/C-20, | C-25/C-27, |
| | | C-6 | C-13 | C-19 | C-24 |
| 3 | D-[1-$^{13}$C] glucose | C-3, C-5 | C-12, C-13 | C-16, C-18 | C-23, C-26, C-27 |
| 4 | [1-$^{13}$C]sodium acetate | C-1, C-6, C-7 | n.e. | C-14, C-19, C-20 | C-22 |
| 5 | L-[*Me*-$^{13}$C] methionine | C-8 | n.e. | C-21 | n.e |

[a]n.e.: not enriched.

It is well established that during glycolysis [U-$^{13}$C]glycerol gives rise to an intact, $^{13}$C-coupled three-carbon unit in the form of phosphoenolpyruvate.[14] Concurrently, [U-$^{13}$C]glycerol is converted in the reductive pentose phosphate cycle, via dihydroxyacetone phosphate and phosphoglyceraldehyde, into fructose-6-phosphate, containing two $^{13}$C-coupled three-carbon units, which with the help of a transketolase gives erythrose-4-phosphate with one intact $^{13}$C-coupled three-carbon unit and a single noncoupled carbon (Figure S6).[15] In the shikimate pathway, phosphoenolpyruvate and erythrose-4-phosphate are the two principle building blocks, the $^{13}$C labeling of which can also be found in any further metabolites derived thereof (Figure 1). Thus, one observed three carbon atom coupled assembly, i.e., C-5/C-4/C-7 (FurAla A), forming one part of the furan ring, could arise from phosphoenolpyruvate, while the second three carbon atom coupled assembly, i.e., C-1/C-2/C-3 (FurAla A), forming the amino acid backbone, along with the single enriched noncoupled C-6 (FurAla A), incorporated also in the furan ring, could arise from erythrose-4-phosphate (Figure S6).

Interestingly, the labeling observed for the 3-(3-furyl)alanine moieties in endolide A (**1**) after adding [U-$^{13}$C]glycerol resembles one of two patterns observed for the dihydrofuranacrylic acid moiety of reductiomycin produced by *Streptomyces xanthochromogenus*, following similar biosynthetic studies using [U-$^{13}$C]glycerol.[16,17] In this context, it is important to note that dihydrofuranacrylic acid shares the basic carbon skeleton with 3-(3-furyl)alanine. Analogous to reductiomycin, we thus conclude that this labeling pattern could be explained only through cleavage of a cyclic structure formed in the shikimate pathway. However, because we observe only one distinct labeling pattern, the intermediate in the case of 3-(3-furyl)alanine must not be symmetrical (Figure S7).

In the labeling study using D-[1-$^{13}$C]glucose, we observed enriched $^{13}$C signals for C-3 ($\delta_C$ 24.5) and C-5 ($\delta_C$ 111.0) of FurAla A and C-16 ($\delta_C$ 24.5) and C-18 ($\delta_C$ 110.9) of FurAla B of endolide A (**1**) (Figure S8). In this way, the involvement of the shikimate pathway was further supported, because it was indeed expected that two carbons should be $^{13}$C enriched. These originate from [3-$^{13}$C]phosphoenolpyruvate via glycolysis and [4-$^{13}$C]erythrose-4-phosphate via the pentose phosphate pathway, following conversion of [1-$^{13}$C]glucose to [6-$^{13}$C]glucose-6-phosphate or [1,6-$^{13}$C]glucose-6-phosphate via glycolysis and gluconeogenesis (Figure S9).[18−20]



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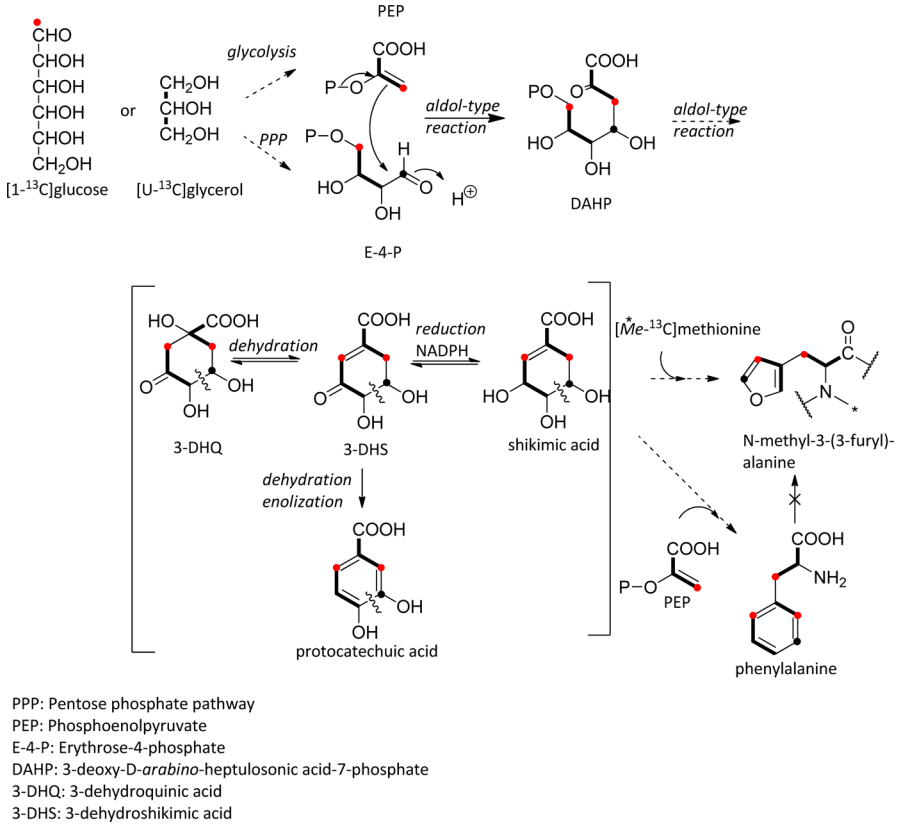


PPP: Pentose phosphate pathway
PEP: Phosphoenolpyruvate
E-4-P: Erythrose-4-phosphate
DAHP: 3-deoxy-D-*arabino*-heptulosonic acid-7-phosphate
3-DHQ: 3-dehydroquinic acid
3-DHS: 3-dehydroshikimic acid

**Figure 1.** Summary of $^{13}C$ enrichment patterns in *N*-methyl-3-(3-furyl)alanine from the biomalt salt agar culture of *Stachylidium* sp. 293 K04 labeled with [U-$^{13}C$]glycerol, D-[1-$^{13}C$]glucose, and L-[*Me*-$^{13}C$]methionine in separate experiments. Bold lines indicate $^{13}C$-labeled isotopomers with directly adjacent $^{13}C$ atoms from [U-$^{13}C$]glycerol. Black dots indicate $^{13}C$-enriched atoms from [U-$^{13}C$]glycerol. Red dots indicate $^{13}C$-enriched atoms from D-[1-$^{13}C$]glucose. Asterisk indicates $^{13}C$-enriched atoms from L-[*Me*-$^{13}C$]methionine. The involvement of phenylalanine as a precursor is excluded.

$^{13}C$-Enrichment after incorporating [1-$^{13}C$]sodium acetate can also be explained by the involvement of phosphoenolpyruvate and erythrose-4-phosphate in the biosynthesis of 3-(3-furyl)alanine (Figure S10). After metabolism in the tricarboxylic acid cycle, [1-$^{13}C$]sodium acetate enters gluconeogenesis as [1,4-$^{13}C$]oxaloacetate.[21] A carboxykinase catalyzes the transformation of oxaloacetate into [1-$^{13}C$]phosphoenolpyruvate,[22] which labels C-7 ($\delta_C$ 141.0) of FurAla A and C-20 ($\delta_C$ 141.1) of FurAla B, respectively. [1-$^{13}C$]Phosphoenolpyruvate is further metabolized to [1,2-$^{13}C$]erythrose-4-phosphate as already described above (Figure S11).[15] This leads to the labeling of C-1 ($\delta_C$ 170.3) and C-6 ($\delta_C$ 144.5) for FurAla A and C-14 ($\delta_C$ 170.7) and C-19 ($\delta_C$ 144.4) of FurAla B.

Finally, for elucidating the biosynthetic origin of the *N*-methyl substituent in 3-(3-furyl)alanine, we added L-[*Me*-$^{13}C$]-methionine, a universal donor of methyl groups frequently used in biosynthetic studies.[23] This resulted in high $^{13}C$-enrichments for C-8 ($\delta_C$ 30.3) and C-21 ($\delta_C$ 30.5) of endolide A (**1**) (Figure S12).

Based on these results (Table 1), a scheme for the biosynthesis of *N*-methyl-3-(3-furyl)alanine is proposed for the first time (Figure 1). This involves both phosphoenolpyruvate and erythrose-4-phosphate as precursor molecules and evidences that the shikimate pathway is the biosynthetic route for the 3-(3-furyl)alanine moiety in endolide A (**1**), with the *N*-methyl group being provided from methionine. The labeling pattern also requires that a cyclic intermediate of the shikimate pathway is involved in the biosynthesis.

In the LC-MS analyses performed during our labeling experiments we noticed that further new cyclic peptide analogues, endolides C and D (**3** and **4**), as well as the known tetrapeptide **5** were produced by the fungus on solid biomalt salt medium supplemented with phenylalanine.

The $^1H$ and $^{13}C$ NMR spectra of the cyclic *N*-methylated peptide analogues **3** and **4** were quite similar to those of the previously reported endolides A and B (**1** and **2**).[4] They showed four $^1H$ NMR resonances at $\delta_H$ 4−5 characteristic for $\alpha$-protons of amino acid residues, along with two singlets at around $\delta_H$ 2.8 for *N*-methyl protons and two characteristic broad doublets at around $\delta_H$ 7.8 indicative of amide protons. In addition $^{13}C$ NMR spectra included four signals at $\delta_C$ 170−173 for four carbonyls, with $\alpha$ carbon signals at $\delta_C$ 50−70. All of these data resulted in the assignment of peptide-like molecules, composed of four amino acid residues, two of which are *N*-methylated (Table 2).

Endolide C (**3**) was assigned a molecular formula of $C_{32}H_{38}N_4O_5$ using HRESIMS, indicating 16 degrees of unsaturation. The $^{13}C$ NMR and DEPT135 spectra revealed 32 resonances, resulting from four methyl groups, three methylenes, five $sp^3$ methines, 13 $sp^2$ methines, and seven nonprotonated carbons. The COSY spectrum revealed correlations of two closely related spin systems, each consisting of five aromatic methine protons, one spanning from H-5 through to H-9 and a second from H-20 through to H-24. HMBC correlations of $H_2$-3 ($\delta_H$ 3.61 and 2.92) to the aromatic carbon C-4 ($\delta_C$ 138.9) as well as to C-2 ($\delta_C$ 63.5) established the first aromatic residue as phenylalanine (Phe A). The second


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Table 2. $^{1}H$ and $^{13}C$ NMR Spectroscopic Data for Endolides C and D (3 and 4)

| 3 | | | | 4 | | | |
|---|---|---|---|---|---|---|---|
| amino acid | pos | $\delta_C$, mult.[a] | $\delta_H$[a] (mult., $J$ in Hz) | amino acid | pos | $\delta_C$, mult.[a] | $\delta_H$[a] (mult., $J$ in Hz) |
| *N*-Me-L-Phe | 1 | 170.4, C | | N-Me- | 1 | 169.8, C | |
| (A) | 2 | 63.5, CH | 4.50, dd (3.3, 11.7) | L-FurAla (A) | 2 | 62.5, CH | 4.25, dd (3.3, 11.7) |
| | 3 | 34.7, $CH_2$ | a 3.61, dd (3.3, 15.0) | | 3 | 24.3, $CH_2$ | a 3.22, dd (3.3, 15.6) |
| | | | b 2.92, dd (11.7,15.0) | | | | b 2.73, m |
| | 4 | 138.9, C | | | 4 | 121.9, C | |
| | 5 | 129.2, CH | 7.27, m | | 5 | 110.9, CH | 6.31, dd (0.9, 1.7) |
| | 6 | 129.5, CH | 7.30, m | | 6 | 144.4, CH | 7.50, t (1.7) |
| | 7 | 127.5, CH | 7.23, m | | 7 | 140.9, CH | 7.38, br s |
| | 8 | 129.5, CH | 7.30, m | | 8 | 30.5, $CH_3$ | 2.71, s |
| | 9 | 129.2, CH | 7.27, m | | | | |
| | 10 | 30.5, $CH_3$ | 2.72, s | | | | |
| L-Val | 11 | 171.8, C | | L-Val | 9 | 172.1, C | |
| | 12 | 56.2, CH | 4.26, dd (7.7, 9.2) | | 10 | 56.2, CH | 4.36, m |
| | 13 | 30.4, CH | 2.04, m | | 11 | 30.4, CH | 2.11, m |
| | 14 | 20.7, $CH_3$ | 0.68, d (6.6) | | 12 | 20.8, $CH_3$ | 0.82, d (6.6) |
| | 15 | 18.4, $CH_3$ | 0.82, d (7.0) | | 13 | 18.4, $CH_3$ | 0.84, d (6.6) |
| | NH-12 | | 7.79, d (9.2) | | NH-10 | | 7.77, d (9.5) |
| *N*-Me-L-Phe | 16 | 171.0, C | | N-Me- | 14 | 170.7, C | |
| (B) | 17 | 63.6, CH | 4.55, dd (3.3, 11.7) | L-FurAla (B) | 15 | 62.7, CH | 4.34, m |
| | 18 | 35.0, $CH_2$ | a 3.67, dd (3.3, 15.0) | | 16 | 24.7, $CH_2$ | a 3.36, dd (3.3, 15.6) |
| | | | b 3.02, dd (11.7, 15.0) | | | | b 2.95, dd (11.3, 15.6) |
| | 19 | 138.9, C | | | 17 | 122.0, C | |
| | 20 | 129.1, CH | 7.27, m | | 18 | 111.0, CH | 6.30, dd (0.9, 1.7) |
| | 21 | 129.5, CH | 7.30, m | | 19 | 144.4, CH | 7.44, t (1.7) |
| | 22 | 127.5, CH | 7.23, m | | 20 | 141.0, CH | 7.37, br s |
| | 23 | 129.5, CH | 7.30, m | | 21 | 30.6, $CH_3$ | 2.78, s |
| | 24 | 129.1, CH | 7.27, m | | | | |
| | 25 | 30.9, $CH_3$ | 2.79, s | | | | |
| L-FurAla | 26 | 172.2, C | | L-Phe | 22 | 172.5, C | |
| | 27 | 51.4, CH | 4.78, dt (9.5, 6.6) | | 23 | 52.2, CH | 4.96, m |
| | 28 | 28.0, $CH_2$ | a 2.90, dd (6.6, 15.0) | | 24 | 38.5, $CH_2$ | a 3.20, dd (6.8, 13.9) |
| | | | b 2.52, dd (6.6, 15.0) | | | | b 2.72, m |
| | 29 | 121.8, C | | | 25 | 139.0, C | |
| | 30 | 112.7, CH | 6.20, br s | | 26 | 130.4, CH | 7.18, m |
| | 31 | 143.2, CH | 7.38, t (1.8) | | 27 | 128.7, CH | 7.24, m |
| | 32 | 141.1, CH | 7.14, br s | | 28 | 126.9, CH | 7.17, m |
| | NH-27 | | 7.93, d (9.5) | | 29 | 128.7, CH | 7.24, m |
| | | | | | 30 | 130.4, CH | 7.18, m |
| | | | | | NH-23 | | 7.93, d (9.5) |

[a]Acetone-$d_6$, 300/75.5 MHz for peptide **3** and 600/150 MHz for peptide **4**

aromatic ring showed similar correlations and hence led to the conclusion that the second aromatic amino acid is also phenylalanine (Phe B). A further $^{1}H-^{1}H$ spin system was assigned to a valine, i.e., using correlations from the methyl protons $H_3$-14 and $H_3$-15 to NH-12 ($\delta_H$ 7.79), through H-13 and H-12, supported by HMBC correlations from H-13 and H-12 to C-11. The last residue displayed carbon resonances characteristic for a 3-substituted furyl moiety, i.e., $\delta_C$ 143.2, 141.1, 121.8 and 112.7. This is confirmed with HMBC correlations from H-30 ($\delta_H$ 6.20) and H-32 ($\delta_H$ 7.14) to the furyl carbon C-29 ($\delta_C$ 121.8). COSY correlations evidenced a spin system from H-27 ($\delta_H$ 4.78) to NH-27 ($\delta$ 7.93). HMBC correlations of H-27 and H-28 with the C-26 ($\delta_C$ 172.2) finalized the assignment of the last residue as 3-(3-furyl)alanine. The sequential relationship of the four amino acid residues was deduced from HMBC correlations of the $\alpha$-protons of the amino acid residues and the carbonyl carbon of the adjacent amino acid, i.e., H-2 (Phe A) to C-11 (Val), H-12 (Val) to C-16 (Phe B), H-17 (PheB) to C-26 (FurAla), and H-27 (FurAla) to C-1 (Phe A). The *N*-methyl substituents were positioned on the relevant amino acid residues Phe A and Phe B, based on HMBC correlations with their adjacent carbonyl carbons and $\alpha$-protons, that is, $H_3$-10 to C-11 and C-2 and $H_3$-25 to C-26 and C-16. This led to the peptide sequence, as cyclo-(*N*-methyl-phenylalanyl, valyl, *N*-methyl-phenylalanyl, furylalanyl).

The $^{13}C$ NMR and DEPT135 spectra of endolide D (**4**) revealed 30 resonances, resulting from four methyls, three methylenes, five $sp^3$ methines, 11 $sp^2$ methines, and seven nonprotonated carbons. The $^{1}H$ and $^{13}C$ NMR spectra displayed characteristic proton and carbon resonances for two magnetically equivalent 3-(3-furyl)alanine moieties, which were *N*-methylated. The remaining proton and carbon resonances in the aromatic region were indicative of a phenylalanine residue. COSY correlations between NH-23 ($\delta_H$ 7.93) and H-23 ($\delta_H$ 4.96) proved that the phenylalanine residue was not *N*-methylated. The last residue was ascribed as valine based on



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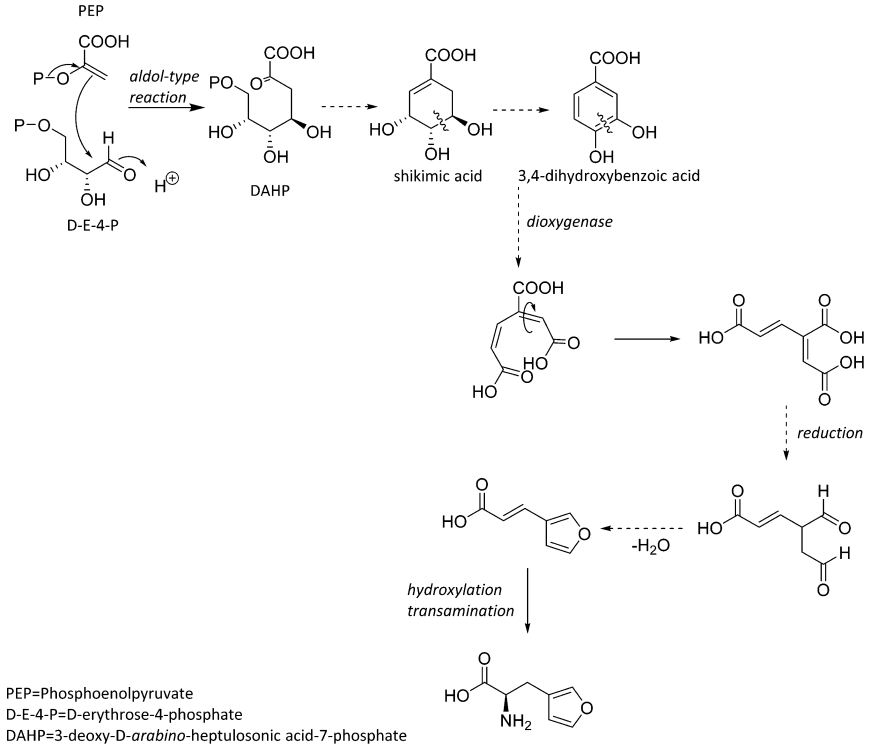


**Figure 2.** Proposed formation of 3-(3-furyl)alanine from a cyclic intermediate of the shikimate pathway. The last step is analogous to the bioconversion of cinnamic acid to phenylalanine.[34] The exact intermediate and sequence of steps are currently not known.

COSY and HMBC correlations. COSY correlations between NH-10 ($\delta_H$ 7.77) and H-10 ($\delta_H$ 4.36) showed that the valine residue was also not *N*-methylated. The sequential relationship of the four amino acid residues was deduced from HMBC correlations of the $\alpha$-protons of the amino acid residues and the carbonyl carbon of the adjacent amino acid, i.e., H-2 (FurAla A) to C-9 (Val), H-10 (Val) to C-14 (FurAla B), H-15 (FurAla B) to C-22 (Phe), and H-23 (Phe) to C-1 (FurAla A), and was supplemented with HMBC correlations of the *N*-methyl substituents. Thus, endolide D (**4**) was assigned as cyclo-[*N*-methyl-3-(3-furyl)-alanyl, valyl, *N*-methyl-3-(3-furyl)-alanyl, phenylalanyl].

High-resolution ESIMS measurements along with further spectroscopic data and literature searches, assigned peptide **5** as hirsutide (Table S5 and Figures S19, S20). The configuration of **5** is identical to that reported, as the specific rotations are comparable (reported specific rotation $[\alpha]^{20}_D$ −192 (*c* 0.2, $CH_2Cl_2$), isolated compound: $[\alpha]^{23}_D$ −122 (*c* 0.125, $CH_2Cl_2$)).

The absolute configurations of the amino acids in the isolated peptides **3** and **4** (as well as **5** for comparison) were assigned after acidic hydrolysis followed by derivatization of the amino acids with Marfey's reagent and HPLC analyses (Advanced Marfey's method).[24] On the basis of comparisons of retention times to standard amino acids we could assign the L-configuration to the amino acids valine and phenylalanine (Figures S21, S22) for all three compounds. For the 3-(3-furyl)alanine moiety, which decomposes upon acid treatment and for which no standard is available, an L-configuration is implied, based on biogenetic considerations. The recently determined X-ray crystal structure of endolide A (**1**) clearly established the L-configuration of 3-(3-furyl)alanine.[4]

Additionally, from a liquid biomalt salt medium supplemented with phenylalanine, the thiodiketopiperazines BDA and BDAA (Figures S23–S26, Table S6), hitherto not encountered in this fungus but previously reported from the fungi *Arachniotus aureus* and *Aspergillus terreus*, were isolated.[9,13] It has been previously established that the biosynthesis of BDA and BDAA involves a phenylalanine intermediate.[25] Through addition of phenylalanine to the liquid cultivation media, biosynthesis of these metabolites was stimulated.[26,27]

The *Stachylidium* sp. 293 K04 is a biochemically versatile fungus, as evident from the secondary metabolite classes (polyketides, phenylpropanoids, peptides) obtained from it so far.[1−4] The exogenous supply of phenylalanine led to the isolation of three additional peptide analogues (**3**−**5**) differing in terms of the incorporated amino acids (phenylalanine, 3-(3-furyl)alanine, and valine), their sequence, and the position of *N*-methylation. This is probably attributed to an increased supply of the precursor amino acid phenylalanine, as seen in precursor-directed biosynthetic studies.[27,28] Nevertheless these peptide analogues were also detected in the LC-MS analyses of the time scale studies. Furthermore, production of endolide A (**1**) was negatively affected in incorporation experiments employing phenylalanine (Figure S2, Table S2), presumably as a result of feedback inhibition where both the primary and secondary metabolites are derived from the same biosynthetic pathway[28] or as a result of an increased pool size of phenylalanine leading to the abundant production of peptides **3**, **4**, and **5**. Overall, this indicates a biosynthetic relationship between the here isolated peptides.

The peptides isolated so far from the *Stachylidium* sp. 293 K04 are proposed to be synthesized by the nonribosomal peptide biosynthetic machinery. This is supported by their structural architecture incorporating unusual amino acid motifs with additional *N*-methylation and cyclization, as frequently encountered in other nonribosomal peptides, *e.g.*, cyclosporins.[29] Contradictory to the number of isolated peptides (**1**−**5**), a single nonribosomal peptide synthetase (NRPS) is


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believed to be involved in their biosynthesis. Promiscuity of the NRPS to alternative amino acid residues is generally encountered, rationalized by the microheterogeneity of the involved adenylation domains accepting different building blocks during peptide synthesis.[30] This was previously observed for structurally related peptides from fungal species, such as 25 naturally occurring cyclosporins[14] and as many as 29 isolated enniatins.[31] Four of these *Stachylidium* sp. 293 K04 peptides (**1**−**4**) contain the rare, nonproteinogenic amino acid *N*-methyl-3-(3-furyl)alanine. From a series of isotope labeling experiments, we outlined the biosynthesis of this rare building block in secondary metabolite peptides, by analyzing the $^{13}C$ labeling pattern in endolide A (**1**). Incubation with $^{13}C$-labeled glycerol was most decisive and resulted in a labeling signature of two assemblies of three coupled $^{13}C$ atoms, with distinct coupling constants, in addition to a single enriched but not coupled carbon atom, in the 3-(3-furyl)alanine moiety. This is clearly implicative of a biosynthesis via the shikimate pathway from phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E-4-P).

It should be noted that cyclization of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP), the condensation product of the precursor molecules PEP and E-4-P, could not be envisioned as a direct source for the 3-(3-furyl)alanine moiety, as deduced from the observed labeling pattern for 3-(3-furyl)alanine. Instead, it is proposed that the ring cleavage of a later stage cyclic intermediate such as shikimic acid or a derivative thereof, e.g., 3-dehydroshikimic acid, 3-hydroxybenzoic acid, or 3,4-dihydroxybenzoic acid, and subsequent cyclization would lead to the formation of the furan moiety, as disclosed from biosynthetic studies for the dihydrofuranacrylic moiety of reductiomycin.[17] In the latter case two labeling patterns were observed indicative of a symmetric intermediate. In our study, however, the involvement of such a symmetrical intermediate is ruled out, as we observe only one distinct pattern. In the case of reductiomycin, 4-hydroxybenzoic acid undergoes two plausible ring cleavage reactions leading to the two labeling patterns observed for the dihydrofuranacrylic acid moiety.[17] Finally, for 3-(3-furyl)alanine the introduction of an amino group, probably from glutamic acid catalyzed by a transaminase, would lead to the basic skeleton of 3-(3-furyl)alanine.[14] The methyl group is provided from *S*-adenosyl-L-methionine, with the help of a methyltransferase,[23] presumably during peptide assembly as seen in cyclosporine biosynthesis.[32]

Moreover, our results exclude other metabolic pathways, such as a polyketide origin, a mixed acetate-glycerol pathway, or terpenoid biosynthesis being involved in the formation of 3-(3-furyl)alanine (Figures S27, S28). Therefore, the shikimate pathway accounts for all the carbon atoms of the *N*-methyl-3-(3-furyl)alanine, apart from the *N*-methyl group, which is provided by the methyl group of methionine (Figure 1). The exact nature of the cyclic intermediate that serves as a precursor for 3-(3-furyl)alanine is currently still elusive (Figure 2).

Concerning the only other known peptides with a 3-(3-furyl)alanine substructure, i.e., rhizonins A and B and bingchamide B, hardly any information is available on their biosynthesis. Genome sequencing of the bingchamide B producing *S. bingchenggensis* revealed a biosynthetic gene cluster containing four genes encoding for nonribosomal peptide synthetases with adenylation domain substrate specificities supposedly correlating with the structure of bingchamide. No experimental proof is, however, provided for this.[33]

As to date no bacteria could be detected in *Stachylidium* sp. 293 K04, the endolides are thus considered the first fungal peptides harboring a 3-(3-furyl)alanine moiety. Hence, the here proposed biosynthetic mechanism for the 3-(3-furyl)alanine supports only fungal biosynthesis, and yet a different pathway could be operative for the synthesis of this moiety in those peptides of bacterial origin, i.e., rhizonins A and B and bingchamide B.

Thus, the information gained from the here applied labeling studies provide us with important data necessary for further biosynthetic investigations, i.e., to determine the exact shikimate intermediate for 3-(3-furyl)alanine biosynthesis. Genomic approaches, such as the currently ongoing sequencing of the *Stachylidium* sp. 293 K04 genome, will result in a detailed view of the assembly of the amino acid building blocks for the respective nonribosomal peptides and may also explain the structural diversity observed for the latter.

## EXPERIMENTAL SECTION

**General Experimental Procedures.** Optical rotations were measured with a Jasco DIP 140 polarimeter. UV and IR spectra were obtained employing PerkinElmer Lambda 40 and PerkinElmer Spectrum BX instruments, respectively. All NMR spectra were recorded in acetone-$d_6$ or methanol-$d_4$ referenced to residual solvent signals with resonances at $\delta_{H/C}$ 2.04/29.8 and $\delta_{H/C}$ 3.35/49.0, respectively, using either a Bruker Avance 300 DPX spectrometer operating at 300 MHz ($^1H$) and 75 MHz ($^{13}C$) or a Bruker Ascend 600 spectrometer operating at 600 MHz ($^1H$) and 150 MHz ($^{13}C$). LCESIMS measurements were conducted employing an Agilent 1100 Series HPLC including DAD, which was coupled with an API 2000, Triple Quadrupole LC/MS/MS, Applied Biosystems/MDS Sciex, and an ESI source. HRESIMS measurements were conducted using a Bruker Daltonics micrOTOF-Q mass spectrometer with an ESI source. HPLC was performed on (a) an HPLC system composed of a Waters 515 pump with a Knauer K-2300 differential refractometer, using a Knauer column (250 × 8 mm, 5 μm, Eurospher II-100 Si), and (b) a Merck-Hitachi system equipped with an L-6200A pump, an L-4500A photodiode array detector, and a D-6000 interface, using Macherey-Nagel columns (Nucleodur $C_{18}$ EC Isis with 250 × 4.6 mm, 5 μm).

**Fungal Material.** The marine-derived fungus *Stachylidium* sp. 293 K04 was isolated from the sponge *Callyspongia* sp. cf. *C. flammea*, collected from the coral reef in Bare Island, New South Wales, Australia. The fungal strain was identified through the Belgian coordinated collections of microorganisms of the Catholic University of Louvain (BCCM/MUCL) by Dr. P. Massart and Dr. C. Decock. A fungal specimen is deposited at the Institute for Pharmaceutical Biology, University of Bonn, isolation number 293 K04.

**Cultivation.** Solid and liquid biomalt salt (BMS) media were used for preparation of precultures and cultures. Biomalt salt medium: 20 g $L^{-1}$ biomalt extract, 15 g $L^{-1}$ agar (for solid medium), and 1 L of artificial seawater: 0.1 g $L^{-1}$ KBr, 23.48 g $L^{-1}$ NaCl, 10.61 g $L^{-1}$ $MgCl\cdot 6H_2O$, 1.47 g $L^{-1}$ $CaCl_2\cdot 2H_2O$, 0.66 g $L^{-1}$ KCl, 0.04 g $L^{-1}$ $SrCl_2\cdot 6H_2O$, 3.92 g $L^{-1}$ $Na_2SO_4$, 0.19 g $L^{-1}$ $NaHCO_3$, and 0.03 g $L^{-1}$ $H_3BO_3$. For the first preculture, the fungal strain was inoculated on BMS agar Petri dishes and incubated at 25 °C for 4 weeks. A seed inoculum from this solid fungal preculture was then used to inoculate 1 L Erlenmeyer flasks (3 flasks), each containing 300 mL of liquid BMS media, which were then shaken at 121 rpm at 25 °C for 10 days (liquid fungal preculture), which was later used to inoculate solid and liquid BMS media.

For the time scale experiment, 1 L of BMS agar medium was prepared and transferred to 20 Petri dishes (30 mL each). From the liquid fungal preculture, 0.5 mL of a liquid seed inoculum was used to inoculate the Petri dishes. Petri dishes were incubated at room


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temperature for 60 days and at defined time intervals (days 5, 10, 15, 20, 30, and 55), and representative Petri dishes were extracted (with EtOAc) and analyzed using LC-MS. LC-MS measurements were performed with the following conditions: RP $C_{18}$ column (Macherey-Nagel Nucleodur 100, 125 × 2 mm, 5 μm) and a 2 mmol $NH_4Ac$ buffered MeOH/$H_2O$ gradient elution system (flow rate 0.25 mL $min^{-1}$), starting from 10% to 100% MeOH over a 20 min period, then isocratic for 10 min. Endolide A (**1**) (*m/z* 515.5 $[M + H]^+$) production was monitored at retention time 19−20 min. Results are summarized in Table S1.

For labeling studies, the fungus was cultivated on solid BMS medium in Fernbach flasks (250 mL each) using a liquid seed inoculum from the liquid fungal preculture (5 mL for each Fernbach flask). Sterile aqueous solutions of labeled precursors (5 mL per feeding per flask) were added twice on day 5 and 10 during a 30-day cultivation period and incubated at room temperature. See Table S2 for concentrations and conditions of labeled precursors. Stable isotope labeled compounds L-[1-$^{13}$C, 99%]phenylalanine, [U-$^{13}$C, 99%]-glycerol, D-[1-$^{13}$C, 99%]glucose, [1-$^{13}$C, 99%]sodium acetate, and L-[Me-$^{13}$C, 99%]methionine were obtained from Cambridge Isotope Laboratories.

For preliminary studies on solid media, 1.5 L of BMS medium was prepared and transferred to six Fernbach flasks (250 mL each). From the liquid fungal preculture, a liquid seed inoculum (5 mL) was used to inoculate each flask. Sterile aqueous solution of D/L-phenylalanine (1 mg $mL^{-1}$) was added to each flask after 3 days. Cultures were incubated at room temperature for 30 days. For preliminary studies in liquid media, 3 L of BMS medium was prepared and transferred to three 5 L Erlenmeyer flasks (1 L medium each). From the liquid fungal preculture a liquid seed inoculum (10 mL) was used to inoculate each flask. A sterile aqueous solution of D/L-phenylalanine (1 mg $mL^{-1}$) was added to each flask after 3 days. Liquid cultures were shaken at 121 rpm at room temperature for 15 days.

**Extraction and Isolation.** *a. Endolide A (1) for Labeling Studies.* At the end of the 30-day cultivation period, the homogenized fungal biomass and cultivation media were exhaustively extracted with EtOAc and concentrated under vacuum (using a vacuum rotatory evaporator, 40 °C) to yield an organic extract. Vacuum liquid chromatography (VLC) was then used for fractionation of the extract using Merck silica gel 60 M (0.040−0.063 mm, 230−400 mesh size) as sorbent. A gradient solvent system of increasing polarity was used for sample elution, starting with 100% petroleum ether to 100% $CH_2Cl_2$ to 100% EtOAc to 100% acetone to 100% MeOH, yielding nine fractions. VLC fraction 4 (50% $CH_2Cl_2$/50% EtOAc) was then subjected to NP-HPLC fractionation (Knauer column; 250 × 8 mm, 5 μm, Eurospher II-100 Si; mobile phase: petroleum ether/acetone (3:1); flow rate 2 mL $min^{-1}$) to yield 10 subfractions. Subfraction 4.6 was further purified using RP-HPLC (Macherey-Nagel column; Nucleodur $C_{18}$ EC Isis, 250 × 4.6 mm, 5 μm; mobile phase: 65% MeOH/$H_2O$; flow rate 1 mL $min^{-1}$). Endolide A (**1**) was collected at retention time ($t_R$) 15 min. For yields see Table S2.

*b. Endolides C and D (3 and 4) and Hirsutide (5).* A 1.5 L amount of BMS agar medium, supplemented with phenylalanine, was exhaustively extracted with EtOAc and concentrated under vacuum (using a vacuum rotatory evaporator, 40 °C) to yield a 315 mg of extract. VLC was then used for fractionation of the extract using Merck silica gel 60 M (0.040−0.063 mm, 230−400 mesh size) as sorbent. A gradient solvent system of increasing polarity was used for sample elution, starting with 100% petroleum ether to 100% $CH_2Cl_2$ to 100% EtOAc to 100% acetone to 100% MeOH, yielding nine fractions. VLC fraction 4 (50% $CH_2Cl_2$/50% EtOAc) was then subjected to NP-HPLC fractionation (Knauer column; 250 × 8 mm, 5 μm, Eurospher II-100 Si; mobile phase: petroleum ether/acetone (3:1); flow rate 2 mL $min^{-1}$) to yield 10 subfractions. Endolide C (**3**) was isolated from subfraction 4.8 using RP-HPLC (Macherey-Nagel column; Nucleodur $C_{18}$ EC Isis, 250 × 4.6 mm, 5 μm; mobile phase: 60% MeOH/$H_2O$; flow rate 1 mL $min^{-1}$) and collected at $t_R$ 20 min, yield 1.5 mg $L^{-1}$. Endolide D (**4**) was isolated from subfraction 4.9 using RP-HPLC (Macherey-Nagel column; Nucleodur $C_{18}$ EC Isis, 250 × 4.6 mm, 5 μm; mobile phase: 60% MeOH/$H_2O$; flow rate 1 mL $min^{-1}$) and collected at $t_R$ 20 min, yield 1.4 mg $L^{-1}$. Hirsutide (**5**) was isolated from subfraction 4.7 using RP-HPLC (Macherey-Nagel column; Nucleodur $C_{18}$ EC Isis, 250 × 4.6 mm, 5 μm; mobile phase: 63% MeOH/$H_2O$; flow rate 1 mL $min^{-1}$) and collected at $t_R$ 20 min, yield 1.0 mg $L^{-1}$.

*Endolide C (3):* white solid, $[\alpha]^{23}_D$ −130 (*c* 0.2, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 204 (3.45), 266 (2.05) nm; IR (ATR) $\nu_{max}$ 3330 (br), 2961, 1704, 1660, 1514, 1362, 1091 $cm^{-1}$; $^1$H NMR and $^{13}$C NMR (Table 2); LRESIMS *m/z* 559.4 $[M + H]^+$, *m/z* 557.6 $[M - H]^-$; HRESIMS *m/z* 581.2731 $[M + Na]^+$ (calcd for $C_{32}H_{38}N_4NaO_5$, 581.2740).

*Endolide D (4):* white solid, $[\alpha]^{23}_D$ −76 (*c* 0.058, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 204 (3.62), 268 (3.16) nm; IR (ATR) $\nu_{max}$ 3331 (br), 2926, 1660, 1505, 1386, 1090 $cm^{-1}$; $^1$H NMR and $^{13}$C NMR (Table 2); LRESIMS *m/z* 549.4 $[M + H]^+$, *m/z* 547.5 $[M - H]^-$; HRESIMS *m/z* 571.2532 $[M + Na]^+$ (calcd for $C_{30}H_{36}N_4NaO_6$, 571.2533).

*Hirsutide (5):* white solid, $[\alpha]^{23}_D$ −153 (*c* 0.125, MeOH), $[\alpha]^{23}_D$ −122 (*c* 0.125, $CH_2Cl_2$); IR (ATR) $\nu_{max}$ 3329 (br), 2961, 1703, 1659, 1512, 1362, 1090 $cm^{-1}$; $^1$H NMR and $^{13}$C NMR (Table S5); HRESIMS *m/z* 569.3119 $[M + H]^+$ (calcd for $C_{34}H_{41}N_4O_4$, 569.3128). Data are in accordance with those in the literature.[8]

**Advanced Marfey's Method.** Peptides **3**, **4**, and **5** (0.5 mg each) were separately dissolved in 6 N HCl (0.5 mL) and heated at 110 °C for 16 h in closed glass vials. After cooling, the solvent was removed using a nitrogen stream and redissolved in 50 μL of $H_2O$. The peptide hydrolysate and 1 mg of each respective standard L- and D-amino acid were resuspended in 50 μL of $H_2O$, and 100 μL of 1% (w/v) L-FDAA (in acetone) and 40 μL of 1 M $NaHCO_3$ were added. The mixture was heated at 70 °C for 40 min. After cooling to room temperature, the reaction was quenched by adding 20 μL of 2 N HCl, and solvents were evaporated to dryness. Samples were resuspended in MeOH (1 mg $mL^{-1}$) for HPLC-MS analyses. The retention times for FDAA derivatives of standard L-valine, D-valine, L-phenylalanine, and D-phenylalanine were 14.4, 18.2, 16.8, and 19.0 min, respectively. Accordingly, the amino acids were assigned in peptide **3** as L-Val (15.0 min) and L-Phe (17.0 min), in peptide **4** as L-Val (14.5 min) and L-Phe (17.0 min), and in peptide **5** as L-Val (14.6 min) and L-Phe (17.1 min).

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00601.

$^1$H and $^{13}$C NMR spectra for compounds **3**−**5**, BDA, and BDAA as well as for labeling experiments; results of advanced Marfey's method; biosynthetic schemes; extraction and isolation of BDA and BDAA (PDF)

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### Notes

The authors declare no competing financial interest.

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