ORGANIC LETTERS

2013
Vol. 15, No. 22
5882–5885

Jahnellamides, α-Keto-β-Methionine-Containing Peptides from the Terrestrial Myxobacterium *Jahnella* sp.: Structure and Biosynthesis

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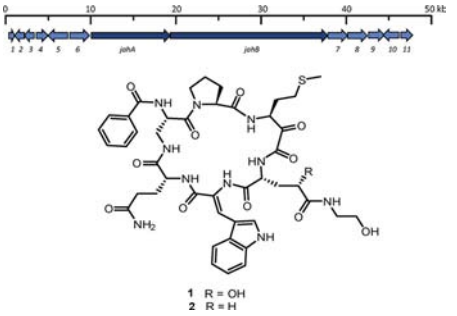
Received October 15, 2013

ABSTRACT



Two new cyclic peptides, termed jahnellamides A and B, were isolated from the myxobacterium *Jahnella* sp. Their structures were solved by NMR, ESIMS, and chemical derivatizations. Jahnellamides are a new class of α-ketoamide-containing peptides comprised of nonproteinogenic amino acids, including α-keto-β-methionine and 4-hydroxyglutamic acid. Moreover, *in silico* analysis of the genome sequence along with feeding experiments allowed us to identify and annotate a candidate nonribosomal peptide synthetase biosynthetic gene cluster containing a polyketide synthase module involved in the formation of the α-ketoamide moiety.

Even though finding new scaffolds from natural products has become increasingly difficult, myxobacteria are still a rich source of biologically active secondary metabolites with distinctive structural features.[1] To increase chemical diversity and reduce rediscovery rates in our screening programs, we have been using NMR based technology to profile crude extracts prepared from new myxobacterial families[2] and underexplored species. Using this strategy, a methanol extract from *Jahnella* sp. (strain SBSr007) exhibiting strong antifungal activity was profiled by LC-SPE-NMR/MS. Analysis of this extract showed the presence of at least three different classes of peptides. The following structural studies led to the isolation of two new cyclic peptides containing an α-keto-β- methionine residue, termed jahnellamide A (**1**) and B (**2**) (Figure 1), together with the known peptides microsclerodermin D[3] and pedein A.[4]

The HRESI-MS spectrum of jahnellamide A (**1**) dissolved in MeOH displayed three ion peaks at m/z 947.3702 $[M+H]^+$, 965.3834 $[M+H_2O]^+$, and 979.3968 $[M+CH_3OH]^+$ supporting a molecular formula of $C_{44}H_{54}N_{10}O_{12}S$ (calcd for $C_{44}H_{55}N_{10}O_{12}S$, 947.3722) and requiring 23 degrees of unsaturation. The HSQC spectrum of **1** in CD_3OD exhibited signals characteristic of a peptide containing aromatic and oxygenated residues, including five α-amino methines (δ_C 52.4, δ_H 4.58; δ_C 61.3, δ_H 4.33; δ_C 53.8, δ_H 4.83; δ_C 53.7, δ_H 4.86; δ_C 57.6, δ_H 4.31), one oxymethine (δ_C 69.5, δ_H 4.16), one oxymethylene (δ_C 61.6, δ_H 3.62),

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10.1021/ol402967y
Published on Web 11/07/2013

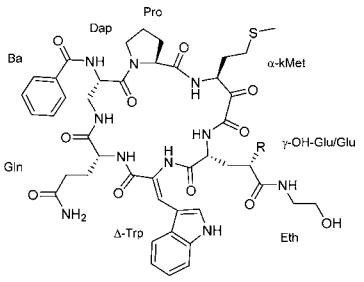

Figure 1. Structures of jahnellamide A (**1**) and B (**2**).

and one olefinic methine (δ_C 127.2, δ_H 8.13). Moreover, the ^{13}C NMR spectrum showed signals corresponding to nine carbonyls at δ 167.7−179.2 and five sp^2 quaternary carbons at δ 134.5 (C-2_{Ba}), 120.4 (C-$2_{\Delta\text{-Trp}}$), 110.7 (C-$3'_{\Delta\text{-Trp}}$), 129.5 (C-$3a'_{\Delta\text{-Trp}}$), and 137.5 (C-$7a'_{\Delta\text{-Trp}}$). A detailed analysis of the 2D NMR data (HSQC, HSQC-TOCSY, HMBC, COSY) of **1** clearly established the presence of 2,3-diaminopropionic acid (Dap), proline, 4-hydroxyglutamic acid (γ-OH-Glu), and glutamine along with benzoic acid (Ba) and an ethanolamine (Eth) moiety (see Table S1). Key HMBC correlations from the olefinic singlet at δ 8.13 to the carbon resonances at δ 167.7 (C-$1_{\Delta\text{-Trp}}$), 130.1 (C-$2'_{\Delta\text{-Trp}}$), 120.4, and 129.5, and from the aromatic proton at δ 7.71 (H-$2'_{\Delta\text{-Trp}}$) to the carbon resonances at 110.7, 137.5, and 120.4, determined the presence of an α,β-dehydrotryptophan (Δ-Trp) residue. The structure of the remaining $C_6H_9NO_2S$ unit was assembled as follows. HSQC-TOCSY, COSY, and HMBC implied the presence of a modified methionine. Indeed, the aminomethine proton at δ 4.83 and the methylene proton at δ 1.81 showed long-range correlations to a quaternary carbon resonance at δ 99.4 that was diagnostic of a hemiketal functional group. An unassigned carbon resonance at δ 174.2 remained, and no HMBC correlation from the proton at δ 4.83 to the latter carbon was observed. Additionally, a ^{13}C NMR spectrum of **1** recorded in DMSO-d_6 clearly showed C-$2_{\alpha\text{-kMet}}$ resonating at δ 192.0 suggesting conversion from a hemiketal to a carbonyl (see Table S2).[5] Taken together, these data indicated the presence of an α-keto-β-methionine (α-kMet) residue. Further evidence supporting the presence of an α-ketoamide functionality was obtained from the ESI-MS spectrum, where an ion peak at m/z 979.3968 $[M+CH_3OH]^+$ was indicative of **1** forming a hemiketal with MeOH at C-$2_{\alpha\text{-kMet}}$.

A semiselective HMBC spectrum in CD_3OD was used to increase the resolution of the overlapped carbonyl region, and long-range correlations between α-protons and carbonyl carbons of adjacent amino acids allowed us to establish the following two partial sequences: γ-OH-Glu-Δ-Trp-Gln and Dap-Pro (see Figure S1). Connectivity between Gln and the Dap β-amine was apparent from HMBC correlations between the β-methylene protons of Dap (δ_H 4.34, 3.25) and the carbonyl resonance at δ 175.0 (C-1_{Gln}). An HMBC cross peak from the α-proton at δ 4.83 (H-$3_{\alpha\text{-kMet}}$) to the carbonyl at δ 176.7 (C-1_{Pro}) attached the α-kMet residue to proline. In addition, an HMBC correlation from the equivalent methylene at δ 3.36 (H-1_{Eth}) to the carbonyl at δ 177.8 (C-$5_{\gamma\text{-OH-Glu}}$) linked the ethanolamide moiety to the δ-carboxylic acid of γ-OH-Glu, while the connectivity of the benzoic acid to the N-terminus of Dap was deduced from a long-range correlation from the proton at δ 4.58 (H-2_{Dap}) to the carbon resonance at δ 170.3 (C-1_{Ba}). Lastly, NMR data acquired in DMSO-d_6 allowed us to complete the planar structure of **1**. In particular, closure of the macrocyclic ring was deduced from an ROE correlation between the amide proton at δ 8.94 ($NH_{\gamma\text{-OH-Glu}}$) and the α-proton at δ 4.50 (H-$3_{\alpha\text{-kMet}}$) along with an HMBC correlation from H-$2_{\gamma\text{-OH-Glu}}$ (δ 4.69) to the carbonyl resonance at δ 161.2.

The Pro residue was assigned as *trans* on the basis of the differential value of ^{13}C chemical shifts of C_β and $C\gamma$ ($\Delta\delta_{\beta\gamma}$) of around 3 ppm along with an observed ROE correlation between H-$5a_{Pro}$ and H-2_{Dap} (see Table S2).[6] Comparison of the chemical shift of H-$3_{\Delta\text{-Trp}}$ (δ 7.85 in DMSO-d_6) with that of keramamide F (δ 7.83)[7] and with those of the geometrical isomers of methyl-α-acetamido-6-methylindole-3-acrylate [δ 7.69 (*Z*) and 7.00 (*E*)][8] indicated the geometry of the α,β double bond to be *Z*. ROE correlations from H-$2'_{\Delta\text{-Trp}}$ (δ 7.48) to $NH_{\Delta\text{-Trp}}$ (δ 8.41) and H-$2_{\gamma\text{-OH-Glu}}$ (δ 4.70) further supported this result. The absolute configurations of L-Dap, L-Pro, D-Gln, and 4*S*-hydroxy-D-Glu residues were assigned by acid hydrolysis and derivatization of **1** with L-FDLA (1-fluoro-2,4-dinitrophenyl-5-L-leucinamide) and D-FDLA followed by MS-detected chromatographic comparison of derivatives of amino acid standards.[9] The L configuration of α-kMet was established by treating **1** with H_2O_2/NaOH followed by acid hydrolysis and application of the advanced Marfey method.

Jahnellamide B (**2**) showed an ion peak at m/z 931.3762 $[M+H]^+$ ($C_{44}H_{54}N_{10}O_{11}S$, calcd for $C_{44}H_{55}N_{10}O_{11}S$, 931.3772), which is 16 mass units lower than that of **1**. The 2D NMR data for **2** closely resembled those of **1** with the exception that resonances belonging to γ-OH-Glu were replaced by resonances belonging to a glutamic acid residue. The *Z* geometry of the α,β double bond in Δ-Trp was deduced from a strong ROE correlation between H-$2'_{\Delta\text{-Trp}}$ and H-2_{Glu} (see Table S3). Marfey's analysis was not performed due to the insufficient quantity of **2**. However, we assumed identical configurations for **1** and **2** at comparable chiral centers because their structures and NMR data are very similar.

With the structures of the jahnellamides in our hands, a tentative biosynthetic scheme was proposed by using a

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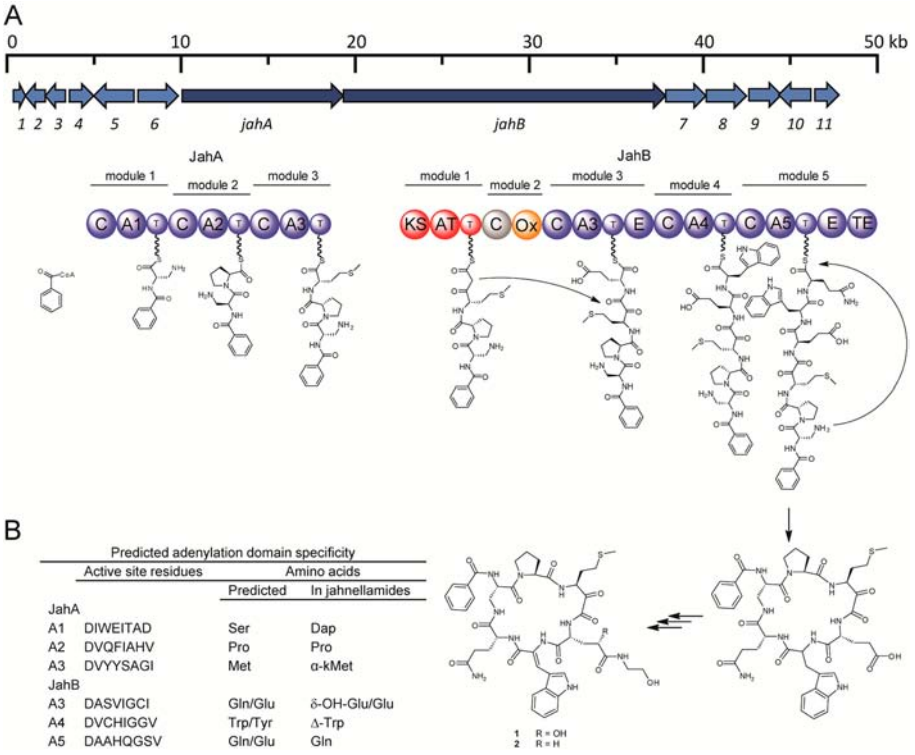

Figure 2. (A) Putative gene cluster of jahnellamides in *Jahnella* sp. and proposed biosynthetic pathway of **1** and **2**. (B) Prediction of A domain substrate specificity.

combination of feeding experiments with labeled precursors and *in silico* analyis of the draft genome sequence of *Jahnella* sp. ^{2}H NMR analysis of **1** obtained from an L-[2,3,3-$^2H_3$]serine feeding experiment showed resonances for H-2_{Eth} (δ 3.62) and H-$3a_{Dap}$ (δ 4.34) indicating that both Eth and Dap originate from serine (see Figure S2). It is worth mentioning that no deuterium was observed at δ 4.58 (H-2_{Dap}) which is consistent with the possible role of dehydroalanine as an intermediate during the conversion from serine to Dap.[10,11] The biosynthetic origin of the α-kMet residue was studied as follows. Intact incorporation of L-[U-^{13}C, ^{15}N]methionine (7%) was measured by HRESI-MS (see Figure S3a) while addition of [2-^{13}C] acetate led to enhancement of the ^{13}C NMR signal of C-$1_{\alpha\text{-kMet}}$ (δ 174.2) (see Figure S4). However, feeding experiments with [1-^{13}C] acetate did not result in the labeling of C-$1_{\alpha\text{-kMet}}$. Taken together, these data suggest that the α-kMet residue is formed by condensation of methionine to the carbon at position 2 of a malonate moiety, and therefore α-kMet has a hybrid NRPS/PKS origin. Additionally, HRESI-MS spectra showed intact incorporation of L-[ring-$^{13}C_6$]phenylalanine (25%), and [$^2H_5$]benzoic acid (29%), while incorporation of [$^2H_7$]cinnamic acid was only observed for the aromatic ring (33%) (see Figure S3b−d).

On the basis of the chemical structure of the jahnellamides along with the results from the feeding experiments, we postulated **1** and **2** to be assembled by a modular mixed NRPS/PKS pathway. Analysis of the *Jahnella* sp. draft genome sequence with anti-SMASH[12] allowed us to identify a gene cluster containing one NRPS gene and one hybrid NRPS/PKS gene with modular organization. The predicted substrate specificity for each module was in good agreement with the structures of **1** and **2**, and with our feeding experiment results. Further analysis of the adenylation domain's substrate specificity was performed by using NRPSpredictor2.[13] The candidate *jah* gene cluster (~50 kb in size) comprises 13 putative open reading frames (ORF) including a trimodular NRPS (*jahA*) and a pentamodular NRPS/PKS (*jahB*) (Figure 2). However, the exact boundaries of the gene cluster have not been determined yet. In fact, gene inactivation in the producing strain has not been successful so far, in particular due to the fact that *Jahnella sp.* does not grow in suspension. Our analysis indicates that assembly starts with the condensation of benzoyl-CoA to Dap which originates from serine (*vide supra*). This is in agreement with the fact that the JahA

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starter module has a C-A-T domain organization characteristic of N-acylated NRPS products.[14] Assembly then proceeds with sequential incorporation of Pro and Met. The growing peptide chain is transferred onto JahB, where malonyl-CoA is incorporated by the PKS part. Our incorporation experiments suggested the α-kMet residue is formed by condensation of the C-2 carbon of an acetate moiety to Met. Consequently, we theorized that the additional condensation and oxidation domains of the PKS module catalyze an oxidation at the methylene carbon of the malonyl residue with subsequent elimination of the malonyl carbon at position 1. Interestingly, this catalytic domain architecture is also present in the hybrid PKS-NRPS encoded by MXAN_3779 of *Myxococcus xanthus*, which is responsible for the biosynthesis of the α-ketoserine-containing peptide named myxoprincomide.[15] Thus, we predict that formation of the α-keto functionality in the jahnellamides occurs in similar fashion to that proposed for myxoprincomide. Next, assembly continues with the consecutive incorporation of Glu, Trp, and Gln. The presence of E domains downstream of the C-A-T motifs in modules 3 and 5 of JahB correlates nicely with the presence of D-Glu and D-Gln. Finally, the C-terminal thioesterase (TE) domain of JahB would catalyze the peptide bond between the C-terminus of Gln and the β-amine of Dap and offload of the prejahnellamides as cyclic peptides. Subsequently, jahnellamides are formed by condensation of ethanolamine to Glu and desaturation of the α- and β-carbon of the tryptophane residue. Jahnellamide B is considered to be a precursor of **1**, which bears a hydroxyl group at position 4 of the glutamate residue. The roles of the remaining ORFs have yet to be established; nonetheless a function such as tailoring enzymes, facilitation of building block biosynthesis, or compound export is expected (see Table S4). Our feeding experiments clearly determined that L-serine is the precursor for L-Dap and ethanolamine, which are both expected to be provided by enzymes encoded within the cluster. Upstream of *jahA* we located *orf2* and *orf3*. Interestingly, their sequences are similar to those of *sbnB* and *sbnA*, respectively, which are genes encoding L-Dap synthetases.[11] Therefore, conversion of L-serine to L-Dap in JahA may be catalyzed by the synergetic action of *orf2* and *orf3*. The *orf8* and *orf9* are putative desaturases and therefore are proposed to be responsible for the formation of Δ-Trp, as demonstrated for *Chromobacterium violaceum*.[16] The decarboxylation of serine to produce ethanolamine is most likely performed by the PLP-dependent decarboxylase *orf10*.[17] The hydroxylation of the Glu residue in **1** is expected to result from the action of a P450 oxidase or nonheme oxidoreductase enzyme encoded elsewhere in the *Jahnella* sp. genome. Unfortunately, we were not able to confirm the proposed functions of the genes in the *jah* gene cluster because mutagenesis is not established for this strain (*vide supra*). Additionally, precursor-directed biosynthesis was applied to generate analogues of **1**. A 4-fluorobenzoic acid derivative and a 2-fluoroethan-1-amine analogue of **1** (**3** and **4**, respectively; see Figures S4 and S5) were detected by LC-HRMS in a methanol extract of *Jahnella* sp. However their low yields prevented their isolation and confirmation of their structure by NMR.

Jahnellamide A showed neither antifungal activity nor cytotoxicity toward HCT-116 cells, and the antifungal activity of the extract was traced to the microsclerodermins. However the intriguing structural features of the jahnellamides warrant further evaluation of its biological function.

In summary, jahnellamides are a new class of cyclic peptides that contain unusual amino acids, including 4-hydroxyglutamic acid, α,β-dehydrotryptophan, 2,3-diaminopropionic acid, and the previously undescribed α-keto-β-methionine. **1** and **2** are characterized by a six-residue lactam ring formed by cyclization between the C-terminus and the β-amine of Dap. Exocyclic to the lactam, a benzoic acid is linked to the α-amine of Dap and an ethanolamine moiety is connected to the δ-carboxylic acid of glutamic acid. Although a few examples of α-ketoamide-containing cyclic peptides have been reported from Lithistid demosponges,[18] the jahnellamides are the first myxobacteria-derived examples of this class of natural products. In fact, the jahnellamides show a slight structural similarity to the strong thrombin inhibitors, cyclotheonamides. These peptides are characterized by the presence of an α-Keto-Arg moiety and a pentaresidue macrocycle formed by condensation of the C-terminus to the β-amine of Dap.[5,19] However, the jahnellamides are unprecedented in their hexapeptide macrocycle and also in the presence of the α-keto-β-methionine and ethanolamine residues. Besides, our feeding experiments and *in silico* analysis demonstrated a hybrid NRPS/PKS biosynthetic origin for **1** and **2**. These data establish an excellent stage for further investigations of jahnellamide biosynthesis, in particular in the formation of the α-keto-amide functionality. In closing, identification of two different structural classes of peptides in strain SBSr007 provides another example of the notable biosynthetic capabilities of myxobacteria.

Acknowledgment. Work in the laboratory of R.M. was supported by the Bundesministerium für Bildung und Forschung (Grant No. 0315798) and the Deutsche Forschungsgemeinschaft.

Supporting Information Available. Experimental details, ^{1}H and ^{13}C NMR assignments, Figures S1−S5, and 1D and 2D NMR spectra for **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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