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Belamide A, a new antimitotic tetrapeptide from a Panamanian marine cyanobacterium

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Abstract—The isolation and structure elucidation of belamide A from the marine cyanobacterium *Symploca* sp. is described. Belamide A is a highly methylated linear tetrapeptide with structural analogy to the important linear peptides dolastatins 10 and 15. Disruption of the microtubule network in A-10 cells was observed at 20 μM and displayed classic tubulin destabilizing antimitotic characteristics. The moderate cytotoxicity of belamide A (IC_{50} 0.74 μM vs HCT-116 colon cancer line) provides new insights into structure–activity relationships for this drug class.

Marine cyanobacteria synthesize a myriad of structurally complex and biologically active secondary metabolites.[1] Of specific importance to human health is a diverse family of peptides and depsipeptides known as the dolastatins. Originally isolated from the sea hare *Dolabella auricularia*, dolastatins 10 and 15 display extraordinary cytotoxicity to cancer cells (IC_{50} values 0.059 and 2.9 nM, respectively).[2] Although neither of these two compounds is still undergoing clinical evaluation, they have stimulated extensive synthetic efforts resulting in several drug leads currently in clinical trials.[3] These synthetic efforts have generated considerable structure-activity knowledge in this compound family.[3] Here we report a novel structural analog of dolastatins 10 and 15, belamide A (**1**), as a major metabolite of the marine cyanobacterium *Symploca* sp. Belamide A, a highly methylated tetrapeptide, contains two characteristic residues of dolastatin 15 (Fig. 1), the N-terminal *N*,*N*-dimethylvaline and C-terminal benzyl-methoxypyrrolinone moieties. This letter expands on the repertoire of linear peptides from marine cyanobacteria and contributes to our knowledge of SAR features for this important class of lead anticancer compounds.

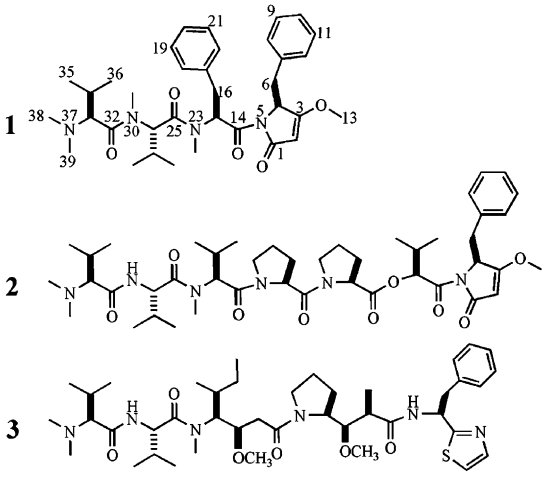

Figure 1. Belamide A (**1**), dolastatin 15 (**2**), and dolastatin 10 (**3**).

The organic oil (CH_2Cl_2–MeOH, 360 mg) of a Panamanian cyanobacterium *Symploca* sp.[4] was initially found

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to be highly cytotoxic to the MCF-7 breast cancer cell line (IC_{50} 8 ng/mL), and was thus fractionated using a silica gel solid phase extraction column and elution solvents of increasing polarity. The resultant four fractions (A–D)[5] were profiled by ^1H NMR, and fraction D exhibited interesting signals typical for a modified peptide. Thus, it was subjected to exhaustive RPHPLC[6] to yield 3.6 mg of pure belamide A (**1**) (Fig. 1).[7]

HRTOF-MS (ES+) measurement indicated a molecular formula of $C_{35}H_{48}N_4O_5$ (M+H obs. *m/z* 605.3643; calcd 605.3624), and was in agreement with the observed physical data.[7] Although **1** readily dissolved in $CDCl_3$, some isochronous proton chemical shifts were observed; these were resolved in C_6D_6 which was used in the remainder of this study (Table 1).

1D NMR spectra were well dispersed in C_6D_6, and indicated the presence of *N*- and *O*-methyl functionalities. Three N-methylated amino acid substructures were deduced from 2D NMR data (^1H–^1H COSY, multiplicity edited ^1H–^{13}C HSQC, and ^1H–^{13}C HMBC) as well as tandem MS data (Fig. 3), including *N*-Me-Phe (C-14 to C-23); *N*-Me-Val (C-25 to C-32) and *N*,*N*-diMe-Val (C-33 to C-39; see Table 1).

The interesting chemical shifts recorded for a methine carbon (C-2, δ_C 95.0; δ_H 4.28) adjacent to the quaternary carbon at δ_C 177.7 (C-3) suggested oxygenation at the latter position. Additionally, HMBC correlations between H-2 (δ 4.28) and C-1 (δ 169) and C-4 (δ 59.9); H-4 (δ 4.57) and C-2 (δ 95.0); H-5 (δ 2.91) and C-3 (δ 177.7) were observed, and defined a substituted pyrrolinone ring (see Fig. 2). By additional HMBC correlations from the C-6 methylene protons to C-7 and C-8/12, one substituent was revealed as a benzyl moiety. A second substituent was a methoxy group (3H at δ 3.38) located at C-3 by a long range ^1H–^{13}C correlation between the methoxy proton resonance (H_3-12) and C-3. These data were reinforced by MS/MS fragments (see Fig. 3), which further supported a benzyl-methoxypyrrolinone moiety present at the C-terminus of **1**.

It was readily apparent that the *N*,*N*-diMe-Val residue formed the N-terminus and the benzyl-methoxypyrrolinone moiety formed the C-terminus of belamide A.

Table 1. NMR data for belamide A (600 MHz for ^1H; 75 MHz for ^{13}C)[a]

Unit	C/H No.	δ_H, mult. (*J* in hertz)	δ_C (75 MHz, mult.)	^1H–^1H COSY	^1H–^{13}C HMBC[b]
Bn-*O*-Me-pyrrolinone	1		169.0 (C)		
	2	4.28 (s)	95.0 (CH)		C1, C4
	3		177.7 (C)		
	4	4.57 (dd, 3.3, 3.6)	59.9 (CH)	H-6	C2
	N-5				
	6	2.91 (dd, 3.0, 2.8)	35.10 (CH_2)	H-4	C3, C7, C8/12
		3.68 (dd, 4.4, 4.5)			
	7		138.1 (C)		
	8/12	7.27 (m)	130.1 (CH)	H-9/11	
	9/11	7.28 (m)	128.6 (CH)	H-8/12	
	10	7.00 (m)	126.8 (CH)		
	13	3.38 (s, 3H)	58.9 (CH_3)		C3
N-Me-Phe	14		172.6 (C)		
	15	2.77 (m)	57.3 (CH)	H-16	C14, C17, C24, C25
	16	2.82 (dd, 3.7, 2.6)	34.1 (CH_2)	H-15	C18/22
		3.52 (dd, 3.4, 3.2)			
	17		134.9 (C)		
	18/22	7.43 (m)	129.2 (CH)	H-19/21	
	19/21	7.09 (m)	128.7 (CH)	H-18/22	
	20	7.08 (m)	127.4 (CH)		
	N-23				
	24	2.75 (s)	32.8 (CH_3)		
N-Me-Val	25		173.0 (C)		
	26	5.65 (d, 10.4)	58.3 (CH)	H-27	C25, C31
	27	2.53 (m)	27.7 (CH)	H-26,28,29	
	28	0.90 (d, 6.7)	19.2 (CH_3)	H-27	C26, C27, C29
	29	1.11 (d, 6.4)	20.1 (CH_3)	H-27	C26, C27, C28
	N-30				
	31	2.57 (s)	30.3 (CH_3)		C32
N,*N*-diMe-Val	32		171.7 (C)		
	33	2.85 (d, 10.3)	68.9 (CH)	H-34	C32, C38, C39
	34	2.33 (m)	28.8 (CH)	H-33,35,36	
	35	1.03 (d, 6.6)	20.3 (CH_3)	H-34	C33
	36	0.57 (d, 6.7)	20.5 (CH_3)	H-34	C33
	N-37				
	38	2.36 (s)	41.4 (CH_3)		C33
	39	2.36 (s)	41.4 (CH_3)		C33

[a] NMR chemical shifts were assigned by ^1H–^1H COSY, ^1H–^{13}C HSQC, and modified 1D ^1H–^{13}C HMBC experiments (C_6D_6; 128.02/7.16 ppm).
[b] Includes data from 1D ^1H–^{13}C HMBC with a delay time for the evolution of long-range heteronuclear coupling optimized for 4 Hz (125 ms).

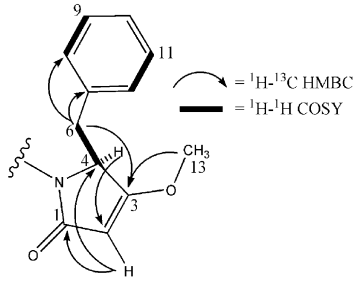

Figure 2. HMBC and COSY correlations for the benzyl-methoxypyrrolinone ('dolapyrrolidone') residue in belamide A (**1**).

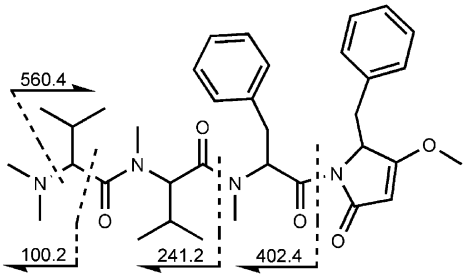

Figure 3. Tandem MS fragments of belamide A (**1**).

Sequencing of the remaining residues was accomplished by HMBC (Table 1) and Tandem MS (Fig. 3). Correlations between H-26 (δ 5.65) and C-31 (δ 30.3) as well as H_3-31 (δ 2.57) and C-32 (δ 171.7) linked the *N*-Me-Val and *N*,*N*-diMe-Val residues. Finally, correlations between H-15 (δ 2.77) and C-25 (δ 173.0) and H-26 (δ 5.65) and C-25 linked the *N*-Me-Val and *N*-Me-Phe residues. Tandem electrospray ionization (ESI) mass spectral fragmentation patterns were straightforward and allowed facile residue sequencing for the planar structure of belamide A (**1**).

The absolute configuration of **1** was based on chiral HPLC analysis.[8] Retention times of the 6 N HCl hydrolysate components of **1** were compared to commercially available amino acid standards, and indicated L-configurations for the *N*-Me-Phe and *N*-Me-Val units. In the case of *N*,*N*-diMe-Val, analytical standards were synthesized from *S*- and *R*-valine using literature methods,[9] and compared with the hydrolysate of **1** by chiral HPLC; this residue was also of L-configuration. Determination of C-4 required ozonolysis of **1** followed by acid hydrolysis which yielded free phenylalanine; this was also determined to be L by chiral HPLC.[8]

Pure belamide A (**1**) assayed against the MCF7 breast cancer cell line displayed an $IC_{50} = 1.6\ \mu M$, while to the HCT-116 colon cancer cell line it was somewhat more potent giving an $IC_{50} = 0.74\ \mu M$.

Bai et al. have shown that β-tubulin binding for this class of linear peptides (there now exist many synthetic analogs) is mainly influenced by the N-terminal residue sequence and configuration at key stereogenic centers.[10] Mitra and Sept have developed an alternative model based on the in silico binding behavior of known tubulin binding compounds (viz. dolastatin 10, cryptophycin 52, and others), in conjunction with multiple X-ray crystal structures of bovine β-tubulin.[11] These models support a single high affinity binding pocket at the 'plus' end of the β-tubulin monomer adjacent to the exchangeable GTP site; however, there is some discussion regarding which residues of dolastatins 10 and 15 are critical for this interaction.[10–13] Interestingly, structural features of both of these two drugs are found in belamide A, and appear to be consistent with the SAR developed from extensive analog work evaluated both in vitro[12] and in vivo.[13]

The N-terminus of belamide A is formed by an *N*,*N*-dimethylvaline residue, a feature found in both dolastatins 10 and 15, and which is reported to be one of the more critical residues for potent biological activity. However, the naturally occurring analog symplostatin 1 possesses an *N*,*N*-dimethylisoleucine residue and is nearly as potent as dolastatin 10,[14] and synthetic analogs possessing a *N*,*N*-dimethylglycine terminus were only modestly diminished in potency.[15] The second residue is also a valyl derivative (as in both **2** and **3**); however, in belamide A this residue is N-methylated. No reported analogs in the dolastatin 10 or 15 series possess N-methylation at this position, and this may explain the lower potency of compound **1**. In the dolastatin 15 series, the third residue is *N*-methylvaline; synthetic studies have shown that slight variations in side chain structure result in analogs with only modest variations in potency. In fact, in the dolastatin 10 series, residues with a bulkier side chain at residue-3 (i.e. isoleucyl derivative) have greater in vivo potency versus the smaller valine analog.[13] Therefore, the larger *N*-methyl Phe residue of belamide A should not necessarily diminish the activity of **1**. Residue-3 of both dolastatins 10 and 15 is N-methylated, as it is in belamide A; this is a required feature for high potency as demonstrated by synthetic analogs.[15]

The final residue of belamide A is precisely as that found for the C-terminal residue of dolastatin 15, benzyl-methoxypyrrolinone. Numerous analogs of the carboxy terminus in both the dolastatins 10 and 15 series have been examined, and in general, this residue is highly tolerant of change, although the required structural feature appears to be a benzyl group. In the case of a terminating benzyl-methoxypyrrolinone residue, the *S*-stereoconfiguration as that found in belamide A has been shown to be critical.[16] Truncated tri- and tetra-peptides have been synthesized and examined for their properties as well. Analogs composed of the N-terminal first three residues or C-terminal four residues of dolastatin 10 were active in inhibiting tubulin assembly, but only weakly cytotoxic.[17]

Given the structural similarity of belamide A to the dolastatins 10 and 15, we were interested to further understand the reduced bioactivity of belamide A. From molecular mechanics calculations of the energy minimized structures of belamide A (**1**) and dolastatin 15 (**2**) and aligning of their C-termini, only a weak overlap correlation was observed.[18] However, alignment of the N-terminal regions of these two linear peptides resulted in a reasonably good fit for the first three residues of both compounds. However, the two adjacent proline residues in **2** result in back-to-back β-turns, thereby

'swinging' the C-terminal pyrrolinone moiety out of conformational overlap with belamide A. Similar analysis was performed with dolastatin 10 (**3**); in this case, alignment of the N-termini of **1** and **3** resulted in a very poor conformational overlap. The dolaproine unit of dolastatin 10 causes the peptide to adopt an overall 'sickle' shape, whereas belamide A retains a more linear conformation.

In the absence of definitive crystal structures of **2** or **3** bound to tubulin, there remains a debate regarding the critical pharmacophore for this family of anticancer agents. Our results for belamide A are intermediate between the pharmacophore hypotheses put forth by Bai et al.[10] (dolavaline-valine-dolaisoleuine) and that of Mitra and Sept[11] (valine-dolaisoleuine-dolaproine). Belamide A displays classic microtubule depolymerizing effects in A-10 cells, including concentration dependent interphase microtubule loss, micronucleation and abnormal mitotic spindle formation at 20 μM, and is a moderately potent cytotoxin to HCT-116 cells (IC_{50} 0.74 μM). These biological effects may be attributed to the N-terminal residue sequence and its hydrophobic binding interaction within the peptide-groove of β-tubulin. However, if belamide A does bind to this peptide-groove, it does so with relatively low affinity, possibly due to one or a combination of missing structural features or steric hindrance, as discussed above. Further biological evaluation of belamide A is needed to fully appreciate the biomedicinal potential of this new modified peptide natural product.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2006.03.082.

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4. Live algal sample collected on 11/3/03 at Salmedina Reef, Portobelo, Panama; N 09°33.799′ × W 79°41.642′; in shallow water by Drs. Kerry L. McPhail and William H. Gerwick.
5. Phenomenex Strata (Si-1; 55 μm particle) NP solid phase extraction column eluted with 100 mL of each the following solvent compositions, fraction: (A) [8:2] hexanes–EtOAc; (B) [1:1] hexanes–EtOAc; (C) 100% EtOAc; (D) 100% MeOH.
6. Phenomenex Synergi 4 μm Fusion-RP 250 × 10 mm column; isocratic [8:2] MeOH–H_2O, then [7:3] MeOH–H_2O.
7. Belamide A (**1**) was isolated as a yellow glassy oil, $[\alpha]_D^{25}$ +16 (*c* 0.002, $CDCl_3$); UV ($CHCl_3$) λ_{max} 240 nm; IR ν_{max} (KBr) 2960, 2926, 2853, 2790, 1997, 1722, 1697, 1630 cm^{-1}.
8. All chiral HPLC analysis was performed using a Phenomenex Chirex 3126 D column with a 2 mM $CuSO_4$/CH_3CN mobile phase except the *N*,*N*-diMe-Val which required 100% 2 mM $CuSO_4$ buffer.
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