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Depsipeptides from a Guamanian marine cyanobacterium, *Lyngbya bouillonii*, with selective inhibition of serine proteases

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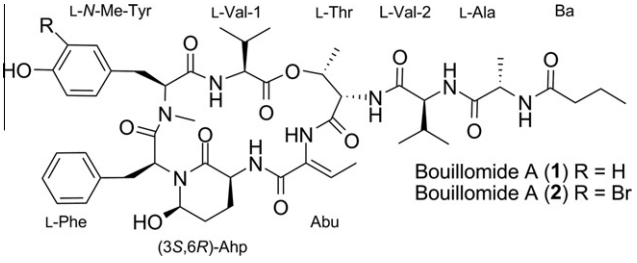
ABSTRACT

Bouillomides A (**1**) and B (**2**) are two depsipeptide analogues of dolastatin 13. Isolated from a Guamanian sample of *Lyngbya bouillonii*, the planar structures were elucidated on the basis of HR-ESI-MS and NMR data, while the absolute configurations were determined by employing functional group conversions, modified Marfey's analysis, and detailed analyses of ROESY correlations. Compounds **1** and **2** selectively inhibited serine proteases elastase (IC_{50} = 1.9 μM for both) and chymotrypsin (IC_{50} = 0.17 and 9.3 μM, respectively) while showing no inhibition of trypsin (IC_{50} >100 μM).

A number of cyanobacterial compounds inhibit serine proteases, which are enzymes central to the regulation of digestion in most animals.[1,2] The family of inhibitory depsipeptides that are exemplified by dolastatin 13[3] is one of the most widely distributed in Nature. While the patriarchal compound was originally isolated from the sea hare *Dolabella auricularia*, subsequent studies showed that the metabolite was actually produced by cyanobacteria upon which the nudibranch fed.[4,5] Dolastatin 13 analogues have now been isolated from marine,[6–11] freshwater,[12–14] and terrestrial[15] cyanobacterial strains collected all over the world. The distinguishing features of these depsipeptides are a 3-amino-6-hydroxy-2-piperidone (Ahp) residue contained within a 20-membered macrocycle, which is formed via an ester linkage between a threonine hydroxyl group and the hydrophobic C-terminus of the precursor peptide.[4] The remaining units are highly variable though, particularly the residues that cap the threonine N-terminus. The responsible biosynthetic gene clusters, therefore, have a high degree of similarity in their non-ribosomal peptide synthetase (NRPS) domains. Since stringent substrate specificity is not always observed with NRPS domains, even analogues from the same strain will display remarkable structural diversity. While the actual ecological role of these compounds remain uncertain,[16] the active sites of several proteases (namely chymotrypsin, trypsin, and elastase) are fairly well conserved.[2] Production of these suites of structurally diverse serine protease inhibitors is therefore proposed to be primarily for their anti-predation effect.[1]

As part of a larger program examining the unique chemical diversity inherent to marine cyanobacteria, two new members of the dolastatin 13 family, bouillomides A (**1**) and B (**2**) were discovered. Discussed herein are the isolation, structure elucidation, and protease screening of these two compounds, which were isolated from a Guamanian sample of *Lyngbya bouillonii* (L. Hoffman and V. Demoulin).

A mass guided approach was used to purify bouillomides A (**1**) and B (**2**) from the crude extract of the *L. bouillonii* sample. First detected by LC–MS analyses, the compounds exhibited pseudomolecular ions with isotopic patterns that indicated that the two compounds differed by the substitution of a bromine. Accordingly, the total crude extract was subjected to a modified Kupchan protocol, in which the extract was partitioned successively[17] between hexanes, CH_2Cl_2, CH_3OH, and H_2O. Compounds **1** and **2** were isolated from the CH_2Cl_2 partition. Three rounds of fractionation using RP-HPLC afforded the amorphous white powders, bouillomides A (**1**) and B (**2**).



The planar structure of bouillomide A (**1**) was elucidated using the HR-ESI-MS, and NMR data. The HR-ESI-MS spectrum for **1** displayed a pseudomolecular ion at *m/z* = 983.4843 $[M+Na]^+$ consistent with a molecular formula of $C_{49}H_{68}N_8O_{12}$. The ^{13}C NMR data

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showed signals for eight amide carbons (δ_C = 165.8–172.6 ppm) and another carbon resonance attributed to an ester carbonyl, based on the carbon chemical shift of δ_C 171.9 and the low-field resonance observed for an acyloxy proton at δ_H = 5.52 ppm in the ^{1}H NMR spectrum. Taken together these data suggested a depsipeptide structure for **1**. All peaks in the ^{1}H and ^{13}C spectra were quickly assigned from analyses of the TOCSY, COSY, HSQC, HMBC, and ROESY data (Table 1).

The 1D and 2D NMR data showed that **1** was an assemblage of eight amino acid subunits (alanine, 3-amino-6-hydroxy-2-piperidone (Ahp), threonine, 2-amino-2-butenoic acid (Abu), *N*-Me-tyrosine, phenylalanine, and two valines) and one butanoic acid (Ba) subunit (Fig. 1). The Ala, Val-1, Val-2, and Ba units were elucidated through analyses of the spin systems observed in TOCSY experiments, after excitation of their respective alpha protons, and identification of their respective amide carbonyl resonances on the basis of HMBC correlations observed. Due to the small mag-

Table 1
NMR data of bouillomide A (**1**) in DMSO-d_6[a]

Unit	Position	δ_C, type[b]	δ_H, mult. (*J* in Hz)	COSY	HMBC (H to C)[c]	ROESY[c]
Val-1	1	171.9, qC		—	—	—
	2	56.1, CH	4.63, m	3, NH	1	3, 4, 5, NH
	3	30.7, CH	2.06, m	2, 4, 5	1	2, 4, 5
	4	19.2, CH_3	0.86, d (7.0)	3	2, 3, 5	2, 3, 5, 4(Thr)
	5	17.4, CH_3	0.74, d (7.0)	3	2, 3, 4	2, 3, 4, 4(Thr)
	NH	—	7.52, d (8.0)	2		2, 2(*N*-Me-Tyr)
N-Me-Tyr	1	169.4, qC	—	—	—	—
	2	60.8, CH	4.87, dd (11.5, 2.0)	3a, 3b	1	3a, NH(Val-1), 2(Phe)
	3a	32.8, CH_2	3.08, dd (14.0, 12.0)	2	4, 5/9	2, 3b, 5/9
	3b		2.70, dd (12.0, 2.0)	2	4, 5/9	3a, 5/9
	4	127.3, qC	—	—	—	—
	5/9	130.4, CH	6.97, d (8.5)	6/8	3a, 3b, 6/8, 7	6/8, 2(Phe)
	6/8	115.3, CH	6.76, d (8.5)	5/9	4, 7	5/9
	7	156.4, qC	—	—	—	—
	N-Me	30.4, CH_3	2.74, s		2, 1(Phe)	
Phe	1	170.5, qC	—	—	—	—
	2	50.2, CH	4.72, dd (11.5, 4.5)	3a, 3b	1, 2(Ahp), 6(Ahp)	3b, 2(*N*-Me-Tyr), 5/9(*N*-Me-Tyr)
	3a	35.3, CH_2	2.86, dd (14.0, 12.0)	2, 3b	4, 5/9	3b, 5/9, 6(Ahp)
	3b		1.81, dd (14.0, 3.0)	2, 3a	4, 5/9	2, 3a, 5/9
	4	136.7, qC	—	—	—	—
	5/9	129.4, CH	6.82, d (7.0)	6/8	3a, 3b, 7	3a, 3b, 6/8, 5b (Ahp), 6(Ahp)
	6/8	127.8, CH	7.14, t (7.5)	5/9, 7	4, 7	5/9
	7	126.2, CH	7.14, t (7.5)	6/8	4, 5/9	
Ahp	2	168.7, qC	—	—	—	—
	3	48.2, CH	3.77, m	4a, 4b, NH	2	5b, NH
	4a	21.9, CH_2	2.40, m	3, 4b, 5a		4b, NH
	4b		1.56, m	3, 4a,		4a, 5a, NH
	5a	29.4, CH_2	1.71, m	4a, 5b, 6		4b, 5b, 6
	5b		1.52, m	5a, 6		3, 5a, 6, 5/9(Phe)
	6	73.7, CH	5.06, s	5a, 5b, OH	2, OH	5a, 5b, OH, 3a(Phe), 5/9(Phe)
	NH	—	7.19, m	3		3, 4a, 4b, NH(Abu)
	OH	—	6.20, br s	6		6
Abu	1	165.8, qC	—	—	—	—
	2	130.1, qC	—	—	—	—
	3	131.6, CH	6.49, q (7.0)	4	1, 4	4
	4	13.1, CH_3	1.47, q (7.0)	3	2, 3, 1(Thr)	3, NH
	NH		9.25, br s			4, 2(Thr), 3(Thr), NH(Ahp)
Thr	1	172.6, qC	—	—	—	—
	2	55.3, CH	4.62, br s	NH		3, 4, NH, NH(Abu)
	3	71.7, CH	5.52, br s	4		2, 4, NH, NH(Abu)
	4	18.0, CH_3	1.21, d (6.5)	3	2, 3	2, 3, 4(Val-1), 5(Val-1)
	NH		7.93, d (6.5)	2		2, 2(Val-2), 3(Val-2)
Val-2	1	171.8, qC	—	—	—	—
	2	57.1, CH	4.36, t (7.0)	3, NH	1	3, 4, 5, NH(Thr)
	3	30.7, CH	2.04, m	2, 4, 5	1	2, 4, 5, NH(Thr)
	4	19.2, CH_3	0.85, d (7.0)	3	1, 2, 3	2, 3, 5
	5	17.7, CH_3	0.80, d (7.0)	3	1, 2, 3, 4	2, 3, 4, NH
	NH		7.71, br s	2		3, 5, 2(Ala)
Ala	1	172.5, qC	—	—	—	—
	2	48.0, CH	4.34, dd (7.5, 7.0)	3, NH	1, 3	3, NH (Val-2)
	3	18.0, CH_3	1.18, d (7.0)	2	1, 2, 1(Ba)	2, NH
	NH		8.03, d (7.5)	2	2, 3, 1(Ba)	2, 3, 2(Ba)
Ba	1	171.9, qC	—	—	—	—
	2	37.0, CH_2	2.07, t (7.0)	3	1, 3, 4	3, NH(Ala)
	3	18.7, CH_2	1.48, sxt (7.0)	2, 4	1, 2, 4	2, 4
	4	13.1, CH_3	0.83, t (7.0)	3	2, 3	3

[a] Measured at 500 MHz (^{1}H) and 125 MHz (^{13}C).
[b] Determined from HSQC and/or HMBC spectra.
[c] Refers to correlations on the same unit unless otherwise indicated.

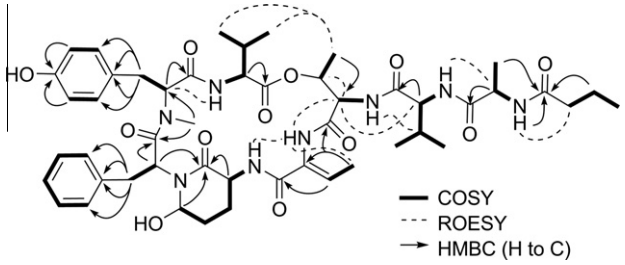

Figure 1. Key 2D NMR correlations for **1**.

nitude of the $^{3}J_{H,H}$ between the stereogenic centers, the entire proton spin system corresponding to the Thr units was not observed after irradiation of the corresponding amide proton in the 1D TOCSY experiment. However, this unit could be easily identified based on COSY and HMBC correlations from the terminal methyl group to H-3 and C-2, respectively. *N*-Me-Tyr and Phe units were deduced based on the distinctive chemical shifts of their diastereotopic β-protons and the HMBC correlations observed to the aryl rings. In the former case, the standard *para*-substitution of the Tyr ring was evident based on the coupling pattern of the aromatic protons ($\delta_{H\text{-}5/H\text{-}9}$ 6.97, d (8.5 Hz); $\delta_{H\text{-}6/H\text{-}8}$ 6.76, d (8.5 Hz)). The sole N-methylation was assigned to the Tyr unit due to an HMBC correlation from the *N*-CH_3 to the alpha carbon of Tyr. The two most distinctive spin-systems, which focused the elucidation on the dolastatin 13 family of depsipeptides,[3] were the Ahp and Abu residues. The latter was deduced based on HMBC correlations from the vinyl methyl group to the corresponding sp^2 carbons of that residue. The network of proton signals observed in the TOCSY spectrum when H-3 was irradiated could be ascribed to the former unit, completing the identification of the components of **1**.

The sequence of these nine subunits was determined based on detailed analyses of the HMBC and ROESY correlations (Fig. 1). Starting from the *N*-Me-Tyr residue, a clear HMBC correlation was observed from the proton signal of the *N*-CH_3 group to the amide carbonyl of the adjacent Phe unit. This nascent unit was connected to the Ahp group through incorporation of the amino terminus on the basis of HMBC correlations from the α-proton resonance of Phe across the tertiary nitrogen to C-2 of the Ahp group. This unit was then expanded to include the Abu and Thr units sequentially, based on a suite of ROESY correlations observed between the resonances for the amide protons of Ahp, Abu, and the α-proton of Thr. That these units formed a macrocycle was finally deduced based on ROESY correlations observed between the methyl groups of Thr and Val-1. The nitrogen of this Thr unit served as an anchor point for the remaining acyclic subunits of **1**. Specifically, a ROESY correlation was observed between the amide proton resonance of Thr and the alpha proton of Val-2, thus establishing their order. The remaining two residues, Ala and Butanoic acid (Ba), were appended sequentially to the amino terminus of Val-2 to complete the structure determination of **1**. Evidence in support of this order included a ROESY correlation between the amino proton resonance of Val-2 and the alpha proton of Ala and an HMBC correlation from the latter proton to the amide carbonyl of Ba. Additional support for our proposed structure also comes from comparison of the NMR data (^{13}C, ROESY, and HMBC) with those observed for several recent dolastatin 13 analogues.[7,10,18] In particular molassamide,[18] isolated from *Dichothrix utahensis*, bears the closest structural resemblance to **1**, as they possess identical macrocycles, and the reported ^{13}C NMR signals from these compounds are in good agreement.

The planar structure of bouillomide B (**2**) was deduced in a similar manner to that described above. The HR-ESI-MS data showed a pseudomolecular ion cluster at m/z = 1061.3960 $[M+Na]^+$ with an isotopic pattern suggestive of a brominated analogue ($C_{49}H_{67}N_8O_{12}Br$). The observed bathochromic shift in the UV spectrum of **2** (λ_{max} 283 and 290 nm), compared to **1** (λ_{max} 279 and 286 nm), indicated that one of the aromatic chromophores was halogenated. Analyses of the 1D and 2D NMR data (Supplementary data, Table S1) verified this hypothesis as **2** was clearly comprised of the same nine basic amino acid residues as **1**, with the exception of a brominated *N*-Me-Tyr unit. The observed ^{1}H NMR proton–proton coupling patterns for this aryl unit indicated a 1,2,4-trisubstituted ring with bromination at the 2-position ($\delta_{H\text{-}5}$ = 7.25, d (1.5 Hz); $\delta_{H\text{-}8}$ = 6.99, d (8.0 Hz); $\delta_{H\text{-}9}$ = 6.95, dd (8.0, 1.5 Hz)). Further analyses of the spectral data in a similar manner to that described for **1** defined the planar sequence depicted.[7,10,18]

The absolute configuration of the individual amino acid subunits was determined using a combination of functional group interconversions, ROESY correlations, and the modified Marfey method. Bouillomides A (**1**) and B (**2**) were first oxidized under classic Jones' conditions with CrO_3.[11] This transformation converted the Ahp unit into a 5-carboxy-δ-lactam, which after acid hydrolysis, was liberated as glutamic acid.[19] The hydrolysate was then derivatized with L-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) and the resulting mixture analyzed by HRESI-LC–MS. Standards of the respective L- and D-amino acids were coupled with L-FDLA and analyzed under the same conditions to confirm the identity of the components in the hydroylzate. For both **1** and **2**, these analyses established the L-configurations of the Phe, Ala, Thr, and Val residues after comparison with the appropriate standards. Peaks with the correct retention times and pseudomolecular ions consistent with L-glutamic acid, which must have originated from the oxidation of the L-Ahp units, were also present in the LC–MS chromatograms of **1** and **2**. With the absolute configuration of the amino-bearing center of the Ahp units defined, ROESY correlations within these units were used to define the relative stereochemistry with respect to the C-6 hydroxyl groups. Specifically, the resonance for H-3 displayed a ROESY crosspeak with H-5, suggestive of a 1,3-diaxial relationship. This proton resonance in turn displayed a small $^{3}J_{H,H}$ with H-6 that was equatorial. Thus a 3*S*,6*R* absolute configuration was assigned to the stereocenters in the Ahp unit. Furthermore, ROESY correlations in both **1** and **2** between the CH_3 and NH of the Abu unit assigned a (*Z*)-configuration to the double bond.

Under this oxidation and hydrolysis sequence, no signals were observed for the tyrosine units. Therefore, a portion of **1** was hydrolyzed, in the presence of 0.1% w/v of phenol, without prior oxidation with Jones' reagent. These conditions have been shown to preserve easily oxidizable aromatic units.[20] Under these modified conditions, the L-FDLA coupling successfully yielded di-L-FDLA-L-*N*-Me-Tyr, which could be identified by HR-ESI-LC–MS after comparison with the standards. These data established the presence of adjacent L-*N*-Me-Tyr and L-Phe residues; a configuration, that is, conserved in nearly all the members of the dolastatin 13 family.[4] In solution, a ROESY correlation is typically observed between the alpha protons of these residues, which was also the case for **1**. This observation proved useful, as while no standards for the Br-*N*-Me-Tyr unit in **2** were available, a ROESY correlation between the alpha protons of the L-Phe and Br-*N*-Me-Tyr residues suggested that this latter residue had an L-configuration in **2**. Careful comparison of the carbon chemical shifts between the backbone carbons in **1** and **2**, along with other related members of this structural family, verified this stereochemical assignment. As mentioned earlier, the closest structural relative was molassamide,[18] which has an L-Thr unit in place of the L-Val-2 unit in **1**. No other brominated molassamide cogeners are known though.

Given the SAR trends previously noted for this series of Ahp-containing molecules, bouillomides A (**1**) and B (**2**) were screened against common serine proteases. Dolastatin 13 analogues have consistently been reported as inhibitors of serine proteases. The

specificity of this inhibition, for chymotrypsin or trypsin, depends strongly on the hydrophobicity or hydrophilicity, respectively, of the subunits neighboring the Ahp moiety.[11,21] Regardless of the identity of the neighboring subunits, these Ahp-containing compounds should inhibit elastase.[7] Compounds **1** and **2** were no exception to these trends, inhibiting chymotrypsin (IC_{50} = 0.17 and 9.3 μM, respectively) while displaying no inhibition of trypsin at 100 μM, the highest concentration tested. Furthermore, both these compounds demonstrated the same elastase inhibition with IC_{50} values of 1.9 μM. A similar, though more potent, serine protease activity profile was reported for molassamide.[18] The observed inhibition appears to be specific to serine proteases though as **1** does not inhibit the aspartic protease BACE1 at concentrations up to 30 μM.

Acknowledgments

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Supplementary data

Supplementary data (NMR data table for **2** and spectra/data for NMR, MS, UV, IR, IC_{50} curves and general experimental) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.10.062.

References and notes

1. Lesk, A. M.; Fordham, W. D. *J. Mol. Biol.* **1996**, *258*, 501–537.
2. Baptista, A. M.; Jonson, P. H.; Hough, E.; Petersen, S. B. *J. Mol. Evol.* **1998**, *47*, 353–362.
3. Pettit, G. R.; Kamano, Y.; Herald, C. L.; Dufresne, C.; Cerny, R. L.; Herald, D. L.; Schmidt, J. M.; Kizu, H. *J. Am. Chem. Soc.* **1989**, *111*, 5015–5017.
4. Weckesser, J.; Martin, C.; Jakobi, C. *Syst. Appl. Microbiol.* **1996**, *19*, 133–138.
5. Harrigan, G. G.; Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Nagle, D. G.; Paul, V. J. *J. Nat. Prod.* **1999**, *62*, 655–658.
6. Harrigan, G. G.; Yoshida, W. Y.; Moore, R. E.; Nagle, D. G.; Park, P. U.; Biggs, J.; Paul, V. J.; Mooberry, S. L.; Corbett, T. H.; Valeriote, F. A. *J. Nat. Prod.* **1998**, *61*, 1221–1225.
7. Taori, K.; Paul, V. J.; Luesch, H. *J. Nat. Prod.* **2008**, *71*, 1625–1629.
8. Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J. *J. Nat. Prod.* **1999**, *62*, 1702–1706.
9. Williams, P. G.; Moore, R. E.; Paul, V. J. *J. Nat. Prod.* **2003**, *66*, 1356–1363.
10. Matthew, S.; Ross, C.; Rocca, J. R.; Paul, V. J.; Luesch, H. *J. Nat. Prod.* **2007**, *70*, 124–127.
11. Taori, K.; Matthew, S.; Rocca, J. R.; Paul, V. J.; Luesch, H. *J. Nat. Prod.* **2007**, *70*, 1593–1600.
12. Zafrir, E.; Carmeli, S. *J. Nat. Prod.* **2010**, *73*, 352–358.
13. Okino, T.; Qi, S.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *J. Nat. Prod.* **1997**, *60*, 158–161.
14. von Elert, E.; Oberer, L.; Merkel, P.; Huhn, T.; Blom, J. F. *J. Nat. Prod.* **2005**, *68*, 1324–1327.
15. Matern, U.; Oberer, L.; Falchetto, R. A.; Erhard, M.; Konig, W. A.; Herdman, M.; Weckesser, J. *Phytochemistry* **2001**, *58*, 1087–1095.
16. Rounge, T. B.; Rohrlack, T.; Kristensen, T.; Jakobsen, K. S. *BMC Microbiol.* **2008**, *8*, 141–151.
17. Kupchan, S. M.; Streelman, D. R.; Sneden, A. T. *J. Nat. Prod.* **1980**, *43*, 296–301.
18. Gunasekera, S. P.; Miller, M. W.; Kwan, J. C.; Luesch, H.; Paul, V. J. *J. Nat. Prod.* **2010**, *73*, 459–462.
19. Harada, K.; Fujii, K.; Mayumi, T.; Hibino, Y.; Suzuki, M.; Ikai, Y.; Oka, H. *Tetrahedron Lett.* **1995**, *36*, 1515–1518.
20. Muramoto, K.; Kamiya, H. *Anal. Biochem.* **1990**, *189*, 223–230.
21. Perona, J. J.; Craik, C. S. *Protein Sci.* **1995**, *4*, 337–360.