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Alotamide A, a Novel Neuropharmacological Agent From the Marine Cyanobacterium *Lyngbya bouillonii*

Irma E. Soria-Mercado^{1,2}, Alban Pereira¹, Zhengyu Cao³, Thomas F. Murray³, and William H. Gerwick^{1,*}

¹ Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography and Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, CA, 92037

² Facultad de Ciencias Marinas, Universidad Autónoma de Baja California, Ensenada, BC, 22800, México

³ Department of Pharmacology, Creighton University School of Medicine, Omaha, NE 68178 USA

Abstract

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wgerwick@ucsd.edu.

Supporting Information Available: Experimental details, full NMR data of alotamide A (**1**), and calcium oscillation data are available free of charge via the Internet at <http://pubs.acs.org>.

1 Alotamide A

Alotamide A (1), a structurally intriguing cyclic depsipeptide, was isolated from the marine mat-forming cyanobacterium *Lyngbya bouillonii* collected in Papua New Guinea. It features three contiguous peptidic residues and an unsaturated heptaketide with oxidations and methylations unlike those found in any other marine cyanobacterial metabolite. Pure alotamide A (1) displays an unusual calcium influx activation profile in murine cerebrocortical neurons with an EC₅₀ of 4.18 μ M.

The secondary metabolites of marine cyanobacteria are among the most structurally intriguing and biologically active in the natural world.¹ The majority of these compounds have been reported from collections of a single species, *Lyngbya majuscula*, accounting for nearly 185 chemical entities reported to date.^{1,2} In turn, the less well explored species *L. bouillonii* has also proven to be a rich source of new natural product chemotypes, including linear tetrapeptides (e.g. lyngbyapeptin)^{3a}, macrolides [e.g. lyngbyaloside and lyngbouilloside (2) among others]^{3b-e}, as well as a group of exceptionally active cyclic depsipeptides (apratoxins A-E).^{3f-h} As part of our assay-based screening program for new neuroactive compounds from cyanobacteria,⁴ we found that the extract of a Papua New Guinea collection of *L. bouillonii* exhibited potent activation of calcium influx in mouse cerebrocortical neurons. A number of critical biological processes such as muscle contraction, neurotransmission, hormone secretion, enzyme regulation and cell membrane permeability, are modulated by calcium ion concentrations in cells.⁵ For example, glutamate mediated intracellular Ca²⁺ overload is known to contribute to neuronal death in several human pathological conditions (hypoxia-ischemia, hypoglycemia trauma, epilepsy)^{6,7} and possibly neurodegenerative disorders such as Alzheimer's, Huntington's, and motor neuron disease.^{8,9} The profile of Ca²⁺ influx induced

by this *L. bouillonii* crude extract was unique, and stimulated an assay-guided isolation of the active constituent and ensuing structure elucidation. The result was the discovery of alotamide A (**1**), a structurally intriguing cyclic depsipeptide of mixed polyketide/non-ribosomal peptide biosynthetic origin.

Samples of *L. bouillonii* were collected by SCUBA in Milne Bay near the town of Alotau, Papua New Guinea. The organic extract (CH₂Cl₂/methanol 2:1, 311.7 mg) was subjected to silica gel vacuum column chromatography (stepwise gradient hexanes/EtOAc/MeOH) to produce nine fractions (A–I). Neurotoxic fraction F was subjected to a combination of further bioassays and ¹H NMR-guided fractionation, comprised of silica gel column chromatographies and reversed-phased HPLC to afford pure alotamide A (2.8 mg, 0.9%) (**1**) {[α]_D²⁵ –1.9 (c 0.0158, CH₂Cl₂)} (Figure 1), accompanied by the previously reported metabolite lyngbouillose (3.2 mg, 1.0%) (**2**).^{3e}

HRESIMS of **1** yielded an [M+H]⁺ peak at *m/z* 588.3477 (calcd for C₃₂H₅₀O₅N₃S, 588.3471), consistent with the molecular formula C₃₂H₄₉O₅N₃S and ten degrees of unsaturation. As described below, extensive analysis of **1** by 2D NMR, including HSQC, HMBC, COSY and NOESY, confirmed the presence of a peptidic C1–C14 fragment as well as a larger polyketide C15–C32 section (Figure 2). A deshielded doublet α-amino proton at δ4.95 (H2, δ_C 61.7) was coupled to a multiplet at δ2.19 which in turn showed correlations to two methyl groups at δ1.03 and 0.74, consistent with the amino acid valine, and confirmed by HMBC (Table 1). An HMBC from H2 to an *N*-methyl carbon resonance at δ31.4 (C6) suggested this to be *N*-methylvaline. The H2 resonance was coupled with two carbonyls at δ169.7 (C1) and δ168.9 (C7); HMBC between the *N*-methyl at δ2.84 (H₃6) and carbonyl C7 indicated this resonance was associated with an adjacent residue. HMBC connection also was observed between C7 and a methine at δ4.80 (H8) which in turn was coupled to a deshielded methylene at δ3.41/3.98 (H9a/b; C9 δ35.0). HMBC correlations between H9a and carbon resonances at δ76.5 (C8) and 173.2 (C10), combined with the unique chemical shifts of C7–C10, identified this to be a cysteine-derived thiazoline ring. A final α-amino methine resonance at δ4.36 (H11, δ_C 60.6) showed HMBC to C10, providing linkage to the third residue. COSY connections were observed from the H11 methine to an adjacent series of methylenes (H12 – H14). H11 also showed HMBC to all three of these methylene carbons, and on the basis this connectivity and chemical shifts, defined the amino acid proline. Thus, the tripeptide component was found to be comprised of *N*-methyl valine (C1–C6), a cysteine-derived thiazolene ring (C7–C9), and a proline residue (C10–C14), and accounted for five of the ten unsaturations present in alotamide (**1**).

A weak HMBC correlation between the H12b proton and carbonyl resonance at δ172.1 (C15), together with strong NOE cross peaks between the methine at δ4.36 (H11) and methyl at δ1.83 (H17) and between H17 and the H14 methylene provided a linkage with the next section of alotamide A. An allylically coupled (*J* = 1.2 Hz) olefinic proton (H18) and olefinic methyl group (H₃17) both showed HMBC correlations to the C15 carbonyl peak, and based on a 9.0 Hz coupling of H18 to a high field methine proton at δ2.67, defined an α,β-unsaturated amide. Despite H18 apparently having an unusually high field shift for the β-proton of an α,β-unsaturated amide, this was supported by comparisons with synthetic compounds which mimic this section of alotamide.¹⁰ H19 was coupled to a doublet methyl group at δ1.09 and to both protons of a diastereotopic methylene (H₂21). HMBC from the doublet methyl to C18, C19 and C21 confirmed these positional assignments. The H₂21 protons were in turn coupled to an olefinic proton at δ5.63 which was part of a conjugated diene (sequential couplings to δ6.23 and 5.68). By HMBC a second olefinic methyl group (H₃26) was placed at the distal end of this diene. Moreover, HMBC from H₃26 as well as H24 to a methylene at higher field (δ2.25) placed it as the other terminating substituent of the diene. The remainder of the polyketide section was deduced from sequential COSY correlations between δ2.25, a methine on an

oxygen bearing carbon, a high field methylene, a second oxygen bearing carbon, and a terminating doublet methyl group ($\delta_{\text{H}}1.11$). HMBC located a methoxy group at the latter oxygenated site, whereas the deshielded nature of H28 was consistent with an ester substituent ($\delta_{\text{H}}5.57$, $\delta_{\text{C}}67.9$). A key HMBC from H28 to C1 located this position as the site of macrolactonization to the tripeptide section of the molecule (Figure 2). Thus, an unusual polyketide fragment, 11-hydroxy-13-methoxy-2,4,9-trimethyltetra-deca-2,6,8-trienoic acid, completed the macrocyclic planar structure of alotamide A (**1**).

Compound (**1**) was ozonolyzed (25 °C, 10 min), followed by oxidative workup (H_2O_2 - HCO_2H) and hydrolysis with 6 M HCl (110 °C, 18 h), and then analyzed by chiral HPLC, revealing the presence of *N*-methyl-*L*(*S*)-valine, *L*(*S*)-proline and *D*(*S*)-cysteic acid. Additional stereochemical analysis of **1** was limited by the amount of available compound at this point in the structure elucidation (< 0.1 mg); thus, only the double bond/proline amide bond geometries were determined (Figure 3). The C16-C17 olefin was determined as *E* from NOE correlations between the H₃17 and H₃20 as well as between H18 and one of the methylene protons at H21 (Table 1). A *trans* disubstituted olefin coupling constant of 14.8 Hz was observed between H22 and H23. An NOE between the H26 methyl singlet and H23 vinylic proton was complemented by an NOE between H24 and H27, thus defining the C24-C25 olefin as *E*. This assignment was supported by the upfield chemical shift in C26 ($\delta_{\text{C}}15.5$) and comparison with related diene-containing model systems.^{9,10} These data established the configuration of these three double bonds as 16*E*, 22*E* and 24*E*. The *cis* conformation for the D-proline amide bond was determined by the diagnostic difference in chemical shifts between the proline β and γ carbons.^{11,12} This assignment was reinforced by NOE data which showed the proximity of the H11 methine and H17 methyl group.

From a biosynthetic perspective, alotamide A (**1**) appears to derive from integration of PKS (e.g. loading plus 6 PKS modules) and NRPS pathways (e.g. 3 NRPS modules). There are four methylations of the polyketide, three of which likely involve SAM as a methyl source (the C30 OCH_3 , C17 and C20 CCH_3 groups). The C26 methyl resides at a predicted carbonyl site in the nascent polyketide, and thus likely involves the β -branch mechanism using an HMGC CoA synthase cassette of enzymes.¹³ In the peptide section, modifications typical of cyanobacterial secondary metabolites are observed (heterocyclization, N-methylation). Cyclization of alotamide A to a 21-membered ring likely occurs concurrent with offloading from the final NRPS module.¹⁴

Alotamide A (**1**) displayed a unique profile when tested in murine cerebrocortical neurons. Specifically, it produced both a concentration-dependent elevation of intracellular calcium concentration as well as an increase in the frequency of spontaneous calcium oscillations in these cells (EC_{50} 4.18 μM) (Figure 4 and Supporting Information). Although the molecular target for this activity is presently unknown, a preliminary pharmacological evaluation has excluded voltage-gated sodium and calcium channels as potential sites of action. Additional possible mechanisms of action include allosteric enhancement of glutamate receptor function or antagonism of voltage-gated-potassium channels. The latter remains an intriguing possibility inasmuch as we have observed similar profiles on spontaneous calcium oscillations in cerebrocortical neurons with potassium channel antagonists. It will therefore be of considerable interest to further characterize the site of action and full pharmacological consequences in mammalian neurons of this new type of cyanobacterial neurotoxin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Tidgewell, K.; Clark, BT.; Gerwick, WH. The Natural Products Chemistry of Cyanobacteria. In: Moore, B.; Crews, P., editors. Comprehensive Natural Products Chemistry. Vol. 2. Elsevier Limited; Oxford, UK: 2009. in press
2. (a) Gerwick GW, Tan LT, Sitachitta N. Alkaloids Chem Biol 2001;57:75–184. [PubMed: 11705123] (b) Tan LT. Phytochemistry 2007;68:954–979. [PubMed: 17336349] (c) Van Wagoner RM, Drummond AK, Wright JL. Adv Appl Microbiol 2007;61:89–217. [PubMed: 17448789]
3. (a) Klein D, Braekman JC, Daloze D, Hoffmann L, Castillo G, Demoulin V. Tetrahedron Lett 1999;40:695–696. (b) Klein D, Braekman JC, Daloze D, Hoffmann L, Demoulin V. J Nat Prod 1997;60:1057–1059. (c) Klein D, Braekman JC, Daloze D, Hoffmann L, Demoulin V. Tetrahedron Lett 1996;37:7519–7520. (d) Klein D, Braekman JC, Daloze D, Hoffmann L, Castillo G, Demoulin V. J Nat Prod 1999;62:934–936. [PubMed: 10395526] (e) Tan LT, Marquez BL, Gerwick WH. J Nat Prod 2002;65:925–928. [PubMed: 12088441] (f) Luesch H, Yoshida WY, Moore RE, Paul VJ. Bioorg Med Chem 2002;10:1973–1978. [PubMed: 11937357] (g) Gutierrez M, Suyama TL, Engene N, Wingerd JS, Matainaho T, Gerwick WH. J Nat Prod 2008;71:1099–1103. [PubMed: 18444683] (h) Matthew S, Schupp PJ, Luesch H. J Nat Prod 2008;71:1113–1116. [PubMed: 18461997]
4. Pereira A, Cao Zhengyu, Murray TF, Gerwick WH. Chem Biol. 2009in press
5. Reuter H. Nature 1983;301:569–574. [PubMed: 6131381]
6. Berman FW, Murray TF. J Neurochem 2000;74:1443–1451. [PubMed: 10737600]
7. Choi DW. Neuron 1998;1:623–634. [PubMed: 2908446]
8. Choi DW. Ann N Y Acad Sci 1994;747:162–171. [PubMed: 7847669]
9. Choi DW. J Neurobiol 1992;23:1261–1276. [PubMed: 1361523]
10. Jew SS, Terashima S, Koga K. Tetrahedron 1979;35:2337–2343.
11. Tan LT, Williamson RT, Gerwick WH, Watts KS, McGough K, Jacobs R. J Org Chem 2000;65:419–425. [PubMed: 10813950]
12. Siemion IZ, Wieland T, Pook KH. Angew Chem Int Ed 1975;14:702–703.
13. Geders TW, Gu L, Mowers JC, Liu H, Gerwick WH, Hakansson K, Sherman DH, Smith JL. J Biol Chem 2007;282:35954–35963. [PubMed: 17928301]
14. Ramaswamy AV, Sorrels CM, Gerwick WH. J Nat Prod 2007;70:1977–1986. [PubMed: 18001088]

Figure 1. Metabolites isolated from a collection of *L. bouillonii* collected from near Alotau, Papua New Guinea in 2003.

Figure 2. Partial structures of alotamide A (**1**) derived from analysis of 2D NMR data and their assembly by HMBC correlations.

Figure 3.
The proline amide bond and double bond geometry assignments for the C11 to C32 section of alotamide A (**1**).

Figure 4. Concentration-response profile for alotamide A-induced elevation in the frequency of spontaneous Ca^{2+} oscillations in mouse cerebrocortical neurons. Depicted is a three parameter logistic fit to the frequency data that yielded an alotamide A EC_{50} value of $4.18 \mu\text{M}$ (95% Confidence Intervals $2.32 - 7.54 \mu\text{M}$).

Table 1

NMR data of alotamide A (**1**) in CDCl₃.

carbon	δ_C^a	δ_H , multiplicity (<i>J</i> Hz) ^b	type	COSY	HMBC (¹ H to ¹³ C)	NOESY
1	169.7					
2	61.7	4.95 d (10.8)	CH	3	1, 3, 4, 5, 6, 7	4, 5
3	25.7	2.19 m	CH	2, 4, 5	1, 2, 4, 5	4, 5
4	17.3	0.74 d (6.6)	CH ₃	3	2, 3, 5	2, 3
5	20.1	1.03 d (6.6)	CH ₃	3	2, 3, 4	2, 3
6	31.4	2.84 s	N CH ₃		2, 7	8, 18, 23, 24
7	168.9					
8	76.5	4.80 ddd (1.8, 8.4, 12.6)	CH	9a, 9b	7, 9, 10	6, 18, 20, 24
9a	35.0	3.41 dd (9.0, 11.4)	CH ₂	8, 9b	8, 10	20
9b		3.98 dd (11.4, 12.6)		8, 9a	7, 8	
10	173.2					
11	60.6	4.36 d (7.2)	CH	12a	10, 12, 13, 14	17, 20
12a	30.9	2.07 m	CH ₂	11, 13a	10, 13, 14	
12b		2.10 m		13a	10, 11, 15	
13a	21.5	1.81 m	CH ₂	12a, 12b, 14	11, 12	
13b		1.92 m		14		
14	45.2	3.52 m	CH ₂	13a, 13b	12, 13	
15	172.1					
16	131.1					
17	14.4	1.83 d (1.2)	CH ₃	18	15, 16, 18, 19	11, 14, 20
18	133.5	5.34 dd (1.2, 9.0)	CH	17, 19	15, 17, 19, 21	6, 8, 21a
19	30.1	2.67 m	CH	18, 20, 21a, 21b		
20	19.4	1.09 d (7.8)	CH ₃	19	18, 19, 21	8, 9a, 11, 17, 22
21a	38.1	2.23 m	CH ₂	19, 22	19	18, 23
21b		2.30 m		19, 22		
22	128.0	5.63 ddd (3.6, 10.8, 14.8)	CH	21a, 21b, 23	21, 24	20
23	129.9	6.23 ddd (1.2, 10.8, 14.8)	CH	22, 24	21, 24, 25	6, 21a, 26
24	126.9	5.68 d (10.8)	CH	23	22, 23, 26, 27	6, 8, 27
25	134.4					

carbon	δ_C^a	δ_H , multiplicity (<i>J</i> Hz) ^b	type	COSY	HMBC (¹ H to ¹³ C)	NOESY
26	15.5	1.72 s	CH ₃		24, 25, 27	23, 28
27	46.5	2.25 m	CH ₂	28	24, 25, 26, 28, 29	24, 29a
28	67.9	5.57 ddt (3.3, 10.2, 10.2)	CH	27, 29b	1	26
29a	43.0	1.58 ddd (3.0, 9.6, 14.2)	CH ₂	30	30	27, 31
29b		1.65 ddd (3.0, 9.3, 14.6)		28	27, 28, 31	31
30	73.2	3.15 m	CH	29a, 31	29, 32	
31	19.0	1.11 d (6.0)	CH ₃	30	29, 30	29a, 29b
32	56.4	3.29 s	OCH ₃		30	

^aRecorded at 150 MHz.

^bRecorded at 600 MHz