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

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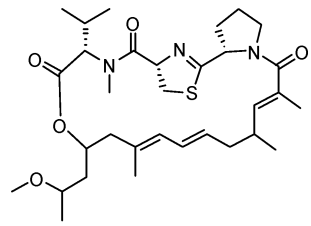
## ABSTRACT



**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 $EC_{50}$ of 4.18 $\mu$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

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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 $Ca^{2+}$ 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 $Ca^{2+}$ 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/nonribosomal 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 ($CH_2Cl_2$/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 $^1$H NMR-guided fractionation, comprised of silica gel column chromatographies and reversed-phased HPLC to afford pure alotamide A (2.8 mg, 0.9%) (**1**) [$[\alpha]^{25}_D$ −1.9 (*c* 0.0158, $CH_2Cl_2$)] (Figure 1), accompanied by the previously reported metabolite lyngbouilloside (3.2 mg, 1.0%) (**2**).[3e]

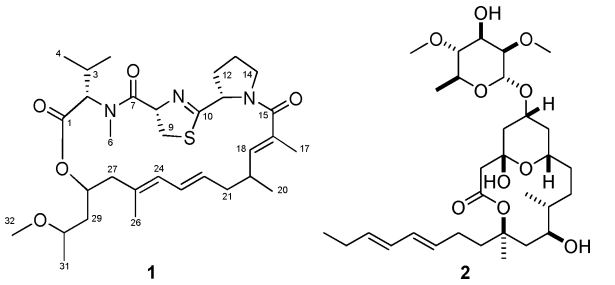

**Figure 1.** Metabolites isolated from a collection of *L. bouillonii* collected from near Alotau, Papua New Guinea, in 2003.

HRESIMS of **1** yielded an $[M + H]^+$ peak at *m*/*z* 588.3477 (calcd for $C_{32}H_{50}O_5N_3S$, 588.3471), consistent with the molecular formula $C_{32}H_{49}O_5N_3S$ 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

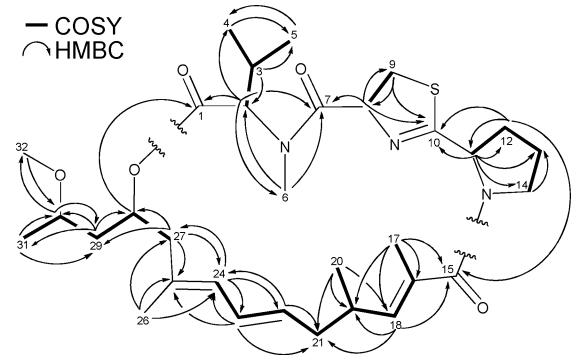


**Figure 2.** Partial structures of alotamide A (**1**) derived from analysis of 2D NMR data and their assembly by HMBC correlations.

deshielded doublet α-amino proton at $\delta$ 4.95 (H2, $\delta_C$ 61.7) was coupled to a multiplet at $\delta$ 2.19 which in turn showed correlations to two methyl groups at $\delta$ 1.03 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 $\delta$ 31.4 (C6) suggested this to be *N*-methylvaline. The H2 resonance was coupled with two carbonyls at $\delta$ 169.7 (C1) and $\delta$ 168.9 (C7); HMBC between the *N*-methyl at $\delta$ 2.84 ($H_3$6) and carbonyl C7 indicated this resonance was associated with an adjacent residue. HMBC connection also was observed between C7 and a methine at $\delta$ 4.80 (H8) which in turn was coupled to a deshielded methylene at $\delta$ 3.41/3.98 (H9a/b; C9 $\delta$35.0). HMBC correlations between H9a and carbon resonances at $\delta$ 76.5 (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 $\delta$ 4.36 (H11, $\delta_C$ 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 of this connectivity and chemical shifts, defined the amino acid proline. Thus, the tripeptide component was found to be composed of *N*-methylvaline (C1−C6), a cysteine-derived thiazolene ring (C7−C9), and a proline residue (C10−C14) and accounted for 5 of the 10 unsaturations present in alotamide (**1**).

A weak HMBC correlation between the H12b proton and carbonyl resonance at $\delta$ 172.1 (C15), together with strong NOE cross peaks between the methine at $\delta$ 4.36 (H11) and methyl at $\delta$ 1.83 (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 ($H_3$17) both showed HMBC correlations to the C15 carbonyl peak and, on the

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**Table 1.** NMR Data of Alotamide A (**1**) in $CDCl_3$

| carbon | $\delta_C$[a] | $\delta_H$, multiplicity ($J$ Hz)[b] | type | COSY | HMBC ($^1H$ 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 | | | | | |
| 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 | |

[a] Recorded at 150 MHz. [b] Recorded at 600 MHz.

basis of a 9.0 Hz coupling of H18 to a high-field methine proton at $\delta$ 2.67, defined an $\alpha,\beta$-unsaturated amide. Despite H18 apparently having an unusually high-field shift for the $\beta$-proton of an $\alpha,\beta$-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 $\delta$ 1.09 and to both protons of a diasterotopic methylene ($H_2$21). HMBC from the doublet methyl to C18, C19, and C21 confirmed these positional assignments. The $H_2$21 protons were in turn coupled to an olefinic proton at $\delta$ 5.63 which was part of a conjugated diene (sequential couplings to $\delta$ 6.23 and 5.68). By HMBC a second olefinic methyl group ($H_3$26) was placed at the distal end of this diene. Moreover, HMBC from $H_3$26 as well as H24 to a methylene at higher field ($\delta$ 2.25) placed it as the other terminating substituent of the diene. The remainder of the polyketide section was deduced from sequential COSY correlations between $\delta$ 2.25, a methine on an oxygen bearing carbon, a high-field methylene, a second oxygen-bearing carbon, and a terminating doublet methyl group ($\delta$ 1.11). HMBC located a methoxy group at the latter oxygenated site, whereas the deshielded nature of H28 was consistent with an ester substituent ($\delta_H$ 5.57, $\delta_C$ 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-trimethyltetradeca-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

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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

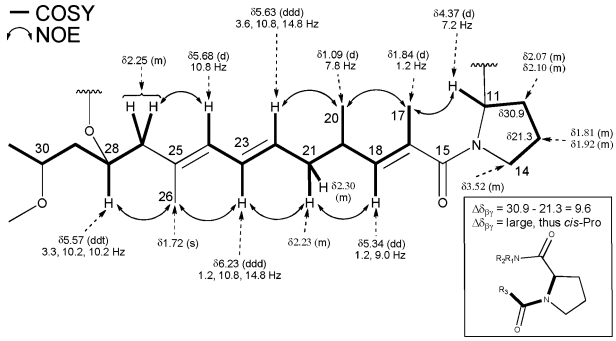


**Figure 3.** Proline amide bond and double-bond geometry assignments for the C11−C32 section of alotamide A (**1**).

determined as *E* from NOE correlations between the $H_3$17 and $H_3$20 as well as between H18 and one of the methylene protons at H21 (Table 1). A *trans*-disubstituted olefin coupling constant of 14.8 Hz was observed between H22 and H23. An NOE between the H26 methyl singlet and H23 vinylic proton was complemented by an NOE between H24 and H27, thus defining the C24−C25 olefin as *E*. This assignment was supported by the upfield chemical shift in C26 ($\delta$ 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 $\beta$ and $\gamma$ 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 six PKS modules) and NRPS pathways (e.g., three 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 $\beta$-branch mechanism using an HMGCoA synthase casette 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 $\mu$M) (Figure 4 and Supporting Information). Although the

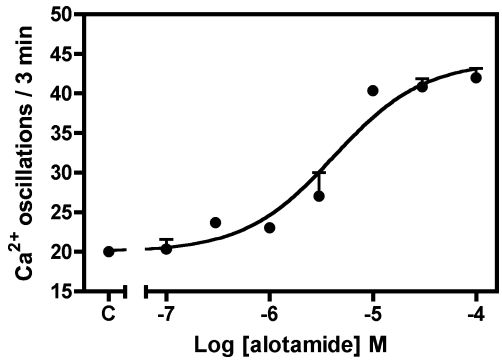


**Figure 4.** Concentration−response profile for alotamide A-induced elevation in the frequency of spontaneous $Ca^{2+}$ oscillations in mouse cerebrocortical neurons. Depicted is a three-parameter logistic fit to the frequency data that yielded an alotamide A $EC_{50}$ value of 4.18 $\mu$M (95% confidence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

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.

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

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