A New Metabolite from the Marine Bacterium *Vibrio angustum* S14

Rocky de Nys,*[,†,‡] Naresh Kumar,[§] Khalil A. Sharara,[§] Sujatha Srinivasan,[⊥] Graham Ball,[§] and Staffan Kjelleberg[‡,⊥]

School of Biological Science, Centre for Marine Biofouling & Bio-Innovation, School of Chemistry, and School of Microbiology & Immunology, University of New South Wales, Sydney, 2052, Australia

Received October 27, 2000

The new metabolite [1-(2′-methylpropoxy)-2-hydroxy-2-methylpropoxy]butane was isolated from the cell-free culture supernatant of the marine bacterium *Vibrio angustum* S14 as part of studies investigating the role of chemical signals in prokaryote−prokaryote and prokaryote−eukaryote interactions. The structure was elucidated by interpretation of its high-field NMR and mass spectrometric data. [1-(2′-Methylpropoxy)-2-hydroxy-2-methylpropoxy]butane induced the acylated homoserine lactone (AHL) reporter system in *Agrobacterium tumefaciens* and bioluminescence in *Vibrio harveyi*.

Extracellular products produced by bacteria play important roles in regulating gene expression of stationary phase phenotypes in bacterial populations.[1,2] The most well-defined of these regulatory systems is the acylated homoserine lactone, or AHL, system.[1,2] Acylated homoserine lactones regulate the expression of a variety of density-dependent phenotypes in Gram negative bacteria including the expression of bioluminescence in *Vibrio harveyi* and *Vibrio fischeri*,[3] the expression of virulence factors (e.g., exoproteases) in *Pseudomonas aeruginosa*,[4] and the production of violaceum in *Chromobacterium violacium*.[5] Recently it has been demonstrated that secondary metabolites from prokaryotes and eukaryotes can specifically interfere with AHL regulatory systems,[6−8] and it has been suggested that this interference has important ecological implications for competition between bacteria and regulating interactions between bacteria and higher organisms.[8] As part of our ongoing research into the biology and chemistry of signaling in bacteria, we investigated the natural products chemistry of *Vibrio angustum* S14. Cell-free cultures of *V. angustum* induce bioluminescence in *V. harveyi*, and extracts of stationary phase supernatant upregulate carbon starvation proteins.[9] We report the isolation of a new compound from the stationary phase cell-free culture of *V. angustum* which induces the AHL reporter system in *Agrobacterium tumefaciens* and bioluminescence in *V. harveyi*.

The isobutanol extract of *V. angustum* S14 afforded one pure metabolite (**1**). The ^{13}C NMR and DEPT-90 and DEPT-135 spectra of **1** showed 12 carbon signals (Table 1) with signals for five methyl, four methylene, and two methine carbons and for one quaternary carbon. These data also contained resonances characteristic of a carbon bearing two oxygens [108.8 (d) ppm] and three carbons bearing a single oxygen [70.4 (t), 72.9 (s), 77.2 (t) ppm]. The ^{1}H NMR spectrum contained three overlapping downfield methyl signals [δ 0.92 (3H), δ 0.93 (3H), δ 0.94 (3H)], two overlapping methyl singlets [δ 1.18 (6H)], two methylene proton signals [δ 1.40 (2H), δ 1.58 (2H)], and six other proton signals [δ 1.89 (1H), δ 3.25 (1H), δ 3.51 (1H), δ 3.61 (1H), δ 3.80 (1H), δ 4.11 (1H)]. Compound **1** had a high-mass ion of 241.1913 by HRESIMS corresponding to $[M + Na]^+$. This information together with the ^{1}H and ^{13}C NMR data showed **1** to have a molecular formula of $C_{12}H_{26}O_3$. Further, from the ^{1}H NMR data and the results of $^{1}H-^{13}C$ and $^{1}H-^{1}H$ 2D NMR shift correlated (HMQC, J = 135 Hz) and DQF-COSY NMR correlation experiments it was possible to establish two $^{1}H-^{1}H$ spin systems. The methylene protons at C-1 [δ 3.51 (dt, J = 6.7, 6.7, 9.2 Hz), 3.80 (dt, J = 6.7, 6.7, 9.2 Hz)] showed a geminal coupling and further coupled to H_2-2 [δ 1.58 (m)], which in turn coupled to the methylene protons H_2-3 [δ 1.40 (m)]. These in turn coupled to the methyl protons H_3-4 [δ 0.93 (t, J = 7.4 Hz)], establishing the first spin system of the molecule. The methylene protons at C-1′ [δ 3.25 (dd, J = 6.3, 8.8 Hz), 3.61 (dd, J = 6.3, 8.8 Hz)] showed a geminal coupling and coupled to the H_1-2′ methine proton [δ 1.89 (m)], which in turn coupled to the methyl protons at H_3-3a′ and H_3-3b′ [δ 0.92 (d, J = 3.6 Hz), 0.94 (d, J = 3.6 Hz)], establishing the second spin system of the molecule. Following the $^{1}H-^{13}C$ data and $^{1}H-^{1}H$ spin analysis, a doubly oxygenated methine carbon, an oxygenated quaternary carbon, and two tertiary methyl groups required positioning within the molecule. These groups and the two spin systems were assembled from the results of a $^{1}H-^{13}C$ correlation experiment (HMBC, J = 8 Hz, Table 1, Figure 1). HMBC correlations between the methyl protons H_3-3″a and H_3-3″b [δ 1.18 (s)] and the carbons δ 72.9 (C-2″) and δ 108.8 (C-1″) established a third fragment of the molecule. Further

Table 1. ^{1}H, ^{13}C, and HMBC Data (**1**)

carbon no.	^{13}C (δ) $CDCl_3$[a]	^{1}H (δ) $CDCl_3$[b]	HMBC correlations H−C
1	70.4 t	3.51 (dt, 6.7, 6.7, 9.2 Hz)	2, 3, 1″
		3.80 (dt, 6.7, 6.7, 9.2 Hz)	2, 3, 1″
2	32.0 t	1.58 (m)	1, 3, 4
3	19.2 t	1.40 (m)	1, 2, 4
4	13.7 q	0.93 (t, 7.4 Hz)	2, 3
1′	77.2 t	3.25 (dd, 6.3, 8.8 Hz)	2′, 3′a, 3′b, 1″
		3.61 (dd, 6.3, 8.8 Hz)	2′, 3′a, 3′b, 1″
2′	28.7 d	1.89 (m)	1′, 3′a, 3′b
3′a	19.1 q[c]	0.92 (d, 3.6 Hz)	1′, 2′, 3′b
3′b	19.1 q[c]	0.94 (d, 3.6 Hz)	1′, 2′, 3′a
1″	108.8 d	4.11 (s)	1, 1′, 3″a, 3″b
2″	72.9 s		
3″a	23.9 t[c]	1.18 (s)	1″, 2″
3″b	23.9 t[c]	1.18 (s)	1″, 2″

[a] Assignments based on DEPT 135 experiment. [b] J values are given in Hz. [c] Interchangeable.

* To whom correspondence should be addressed. Tel: 61-2-93851584. Fax: 61-2-93851558. E-mail: r.denys@unsw.edu.au.
† School of Biological Science, University of New South Wales.
‡ Centre for Marine Biofouling & Bio-Innovation, University of New South Wales.
§ School of Chemistry, University of New South Wales.
⊥ School of Microbiology & Immunology, University of New South Wales.

10.1021/np000512r CCC: $20.00 © 2001 American Chemical Society and American Society of Pharmacognosy
Published on Web 03/24/2001

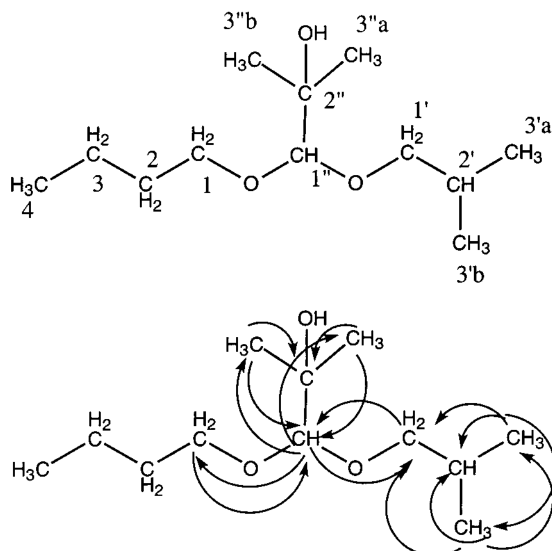

Figure 1. Diagnostic HMBC correlations for **1**.

HMBC correlations between the protons at H-1 and H1′ and the carbon at δ 108.8 (C-1″), and the H-1″ proton and the carbons δ 70.4 (C-1) and δ 77.2 (C-1′), allowed the three groups to be unambiguously assembled to give the structure of **1**. The stereochemistry at the chiral center C1″ remains unresolved. Compound **1** is therefore [1-(2′-methylpropoxy)-2-hydroxy-2-methylpropoxy]butane or 2-methylacetaldehyde *n*-butyl isobutyl acetal.

To investigate the ability of **1** to mediate the expression of AHL-regulated systems, it was tested in the *A. tumefaciens* bioassay. This assay provides a sensitive detection method for AHLs through the induction of the *traI-lacZ* reporter fusion.[10] Compound **1** induced the expression of the *traI-lacZ* reporter fusion at a level of 2.5 μg. This activity was similar to that of the positive control *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) (2.5 μg). However, induction was delayed by 5 h compared to OHHL. Compound **1** also induced bioluminescence in *V. harveyi* at a concentration of 10 μM. Bioluminescence was induced 32-fold by **1** compared to the positive control *N*-(3-hydroxyhexanoyl)-L-homoserine lactone (HBHL) (10 μM), which induced a 128-fold increase. Bioluminescence in *V. harveyi* is regulated by HBHL and an unidentified signal (AI-2) via two separate pathways. Therefore while **1** upregulates bioluminescence, it remains to be determined if this is through mediation of the AHL or AI-2 pathway.

Experimental Section

General Experimental Procedures. All solvents were distilled prior to use. MilliQ water was used in all experiments. Infrared spectra were recorded on a Perkin-Elmer 1600 FTIR using KBr disks. Optical rotation $[\alpha]^{25}_D$ was measured in chloroform using a Jasco DIP-1000 digital polarimeter with a sodium lamp (Na/10) at 589 nm. All NMR spectral analyses were performed on a Bruker DMX 500 MHz NMR instrument. One-bond heteronuclear $^1H-^{13}C$ connectivities were determined by HSQC (135 Hz); two- and three-bond connectivities were determined by HMBC optimized for 8 Hz couplings. High-resolution mass spectra were recorded on a Bruker Bioapex 70e FT/ICR mass spectrometer using an electrospray ionization source.

Bacterial Culture Conditions and Isolation of 1. *V. angustum* S14 was cultured in marine minimal medium with glucose (1 g·L^{-1}) to stationary phase (5 h). Cells were removed by centrifuging and filtration (Millipore GS 0.2 μm). The resulting supernatant (1 L) was extracted three times with isobutanol. The solvent was removed in vacuo and washed with dichloromethane. The dichloromethane-soluble material was separated and the solvent removed in vacuo to yield a single pure metabolite (40 mg).

[1-(2′-Methylpropoxy)-2-hydroxy-2-methylpropoxy]butane (1): clear oil (40 mg, 0.004%); $[\alpha]^{25}_D$ +0.11° (*c* 0.35 $CHCl_3$); IR ν_{max} 3417, 2928, 2932, 2873, 1773, 1451, 1352, 1178, 1114, 1067 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$), see Table 1; ^{13}C NMR (125.65 MHz, $CDCl_3$) see Table 1; LRESIMS *m/z* 241.2 [M + Na]$^+$; HRESIMS *m/z* 241.1913 (calcd for $[C_{12}H_{26}O_3 + Na]^+$ 241.1877).

Agrobacterium Bioassay. The AHL reporter strain *A. tumefaciens* A136 (pCF218) (pCF372) was used to measure the effect of **1** on AHL-regulated *traI-lacZ* expression.[10] *A. tumefaciens* was cultured in AT media seeded with glucose (2 g·L^{-1}), tetracycline (4.5 μg·mL^{-1}), kanamycin (100 μg·mL^{-1}), and spectinomycin (50 μg·mL^{-1}). To view *lac*+ activity 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (40 μg·mL^{-1}) was added. For the assay **1** was dissolved in ethanol and added to a TLC plate (Merck Si gel 60 F_{254}) to give total amounts of 0.1, 0.25, 0.5, 1.0, and 2.5 μg. OHHL was used as a positive control at the same concentrations. The plate was overlaid by agar (1.5% in MilliQ water) and the medium seeded with *A. tumefaciens* and allowed to set prior to incubation overnight at 30 °C. The plates were assessed for the blue pigment due to induction of the *traI-lacZ* fusion.

Luminescence Bioassay. *V. harveyi* UNSW wild-type strain was grown in Luria broth with NaCl (20 g·L^{-1}) (LB20). Cultures were grown overnight and diluted 1:5000 in LB20 media. **1** was dissolved in ethanol and added to 3 mL of diluted culture to give concentrations of 0.01, 0.1, 1, and 10 μM. HBHL was used as a positive control at the same concentrations. Ethanol controls were also included. All cultures were incubated with shaking at 25 °C for 10 h. Samples were taken every hour (100 μL) and bioluminescence (counts per second) measured using a Microbeta Plus liquid scintillation counter (Wallac Inc.).

Acknowledgment. We thank Tim Charlton and the Biomedical Mass Spectrometry Unit at UNSW for providing LRMS. The research was supported by an ARC Research Fellowship to R.d.N. and an ARC Large Grant (A19530672) to S.K.

References and Notes

(1) Fuqua, C.; Winans, C.; Greenberg, E. P. *Annu. Rev. Microbiol.* **1996**, *50*, 727–751.
(2) Swift, S.; Throup, J. P.; Williams, P.; George, P. C.; Salmond, P. C.; Stewart, G. S. A. B. *Trends Biochem. Sci.* **1996**, *21*, 214–219.
(3) Eberhard, A.; Widrig, C. A.; McBath, P., Schineller, J. B. *Arch. Microbiol.* **1986**, *146*, 35–40.
(4) Passador, L. J.; Cook, J. M.; Gambello, M. J.; Rust, L.; Iglewski, B. H. *Science* **1993**, *260*, 1127–1129.
(5) McClean, K. H.; Winson, M. K.; Fish, L.; Taylor, A.; Chhabra, S. R.; Camara, M.; Daykin, M.; Lamb, J. H.; Swift, S.; Bycroft, B. W.; Stewart, G. S. A. B.; Williams, P. *Microbiology* **1997**, *143*, 3703–3711.
(6) Givskov, M.; de Nys, R.; Manefield, M.; Gram, L.; Maximilien, R.; Eberl, L.; Molin, S.; Steinberg, P. D.; Kjelleberg, S. *J. Bacteriol.* **1996**, *178*, 6618–6622.
(7) Schaefer, A. L.; Hanzelka, B. L.; Eberhard, A.; Greenberg, E. P. *J. Bacteriol.* **1996**, *178*, 2897–2901.
(8) Kjelleberg, S.; Steinberg, P. D.; Givskov, M.; Gram, L.; Manefield, M.; de Nys, R. *Aq. Microbiol. Ecol.* **1997**, *113*, 85–93.
(9) Srinivasan, S.; Östling, J.; Charlton, T.; de Nys, R.; Takayama, K.; Kjelleberg, S. *J. Bacteriol.* **1998**, *180*, 201–209.
(10) Fuqua, C.; Winans, S. C. *J. Bacteriol.* **1996**, *178*, 435–440.

NP000512R