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Lagunamide C, a cytotoxic cyclodepsipeptide from the marine cyanobacterium *Lyngbya majuscula*

Ashootosh Tripathi [a], Jonathan Puddick [b], Michele R. Prinsep [b], Matthias Rottmann [c], Kok Ping Chan [d], David Yu-Kai Chen [e], Lik Tong Tan [a,*]

[a] Natural Sciences and Science Education, National Institute of Education, Nanyang Technological University, 1 Nanyang Walk, Singapore 637616, Singapore
[b] Department of Chemistry, University of Waikato, Private Bag 3105, Hamilton 3205, New Zealand
[c] Swiss Tropical and Public Health Institute, Parasite Chemotherapy, Socinstrasse 57, CH-4002 Basel, Switzerland and University of Basel, CH-4003 Basel, Switzerland
[d] Chemical Synthesis Laboratory, Institute of Chemical and Engineering Sciences, 11 Biopolis Way, Helios #03-08, Singapore 138667, Singapore
[e] Department of Chemistry, Seoul National University, Gwanak-1 Gwanak-ro, Gwanak-gu, Seoul 151-742, South Korea

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ABSTRACT

Lagunamide C (**1**) is a cytotoxic cyclodepsipeptide isolated from the marine cyanobacterium, *Lyngbya majuscula*, from the western lagoon of Pulau Hantu Besar, Singapore. The complete structural characterization of the molecule was achieved by extensive NMR spectroscopic analysis as well as chemical manipulations. Several methods, including the advanced Marfey's method, a modified method based on derivatization with Mosher's reagents and analysis using LC–MS, and the use of $^{3}J_{H-H}$ coupling constant values, were utilized for the determination of its absolute configuration. Compound **1** is related to the aurilide-class of molecules and it differs mainly in the macrocyclic structure by having a 27 membered ring system due to additional methylene carbon in the polyketide moiety. Lagunamide C displayed potent cytotoxic activity against a panel of cancer cell lines, such as P388, A549, PC3, HCT8, and SK-OV3 cell lines, with IC_{50} values ranging from 2.1 nM to 24.4 nM. Compound **1** also displayed significant anti-malarial activity with IC_{50} value of 0.29 μM when tested against *Plasmodium falciparum*. In addition, lagunamide C exhibited weak anti-swarming activity when tested at 100 ppm against the Gram-negative bacterial strain, *Pseudomonas aeruginosa* PA01.

1. Introduction

Marine cyanobacteria have emerged over the past 40 years as one of the richest groups of marine organisms in producing structurally diverse bioactive secondary metabolites (Gerwick et al., 2001; Tan, 2007). Cyanobacterial strains belonging to *Lyngbya*, *Symploca*, and *Oscillatoria* genera, in particular, are prolific producers of a number of structurally unique compounds with several bioactivities, such as antimicrobial, cytotoxic, and neurotoxic properties (Tan, 2010; Nunnery et al., 2010). This makes marine cyanobacterial compounds an attractive source of therapeutic agents for the treatment of cancer diseases. In recent years, a growing number of cyanobacterial natural products, including viridamides A and B (Simmons et al., 2008) and gallinamide A (Linington et al., 2009), have been shown to exhibit significant antiprotozoal activities, particularly as antimalarial agents (Nunnery et al., 2010).

In our quest for novel bioactive secondary metabolites from local strains of marine cyanobacteria, we chanced upon a persistent strain of filamentous marine cyanobacterium, *Lyngbya majuscula* Agardh ex Gomont, from the western lagoon of Pulau Hantu Besar, Singapore. Chemical investigation of its organic extract, using vacuum flash chromatography (VFC), RP-HPLC, and NMR, yielded a number of known and new bioactive secondary metabolites, including the recently reported lagunamides A and B. The present study represents a continued chemical investigation on the same cyanobacterial samples. Further examination of one of the VFC-derived fractions eluted with 100% EtOAc and subsequent RP-HPLC, resulted in the isolation of a new cytotoxic cyclic depsipeptide, lagunamide C (**1**). In this study, we wish to report on the complete structural elucidation as well as the biological property of lagunamide C (**1**). Compound **1** shares structural similarities with the aurilide class of molecules *viz*., lagunamides, kulokekahilide-2, palau'amide, and it differs primarily in the polyketide-derived moiety as well as having a 27-membered macrocyclic ring system (Williams et al., 2003; Suenaga et al., 2004; Nakao et al., 2004; Han et al., 2006; Tripathi et al., 2010) (see Fig. 1).

2. Results and discussion

Lagunamide C (**1**) possesses a molecular formula of $C_{46}H_{73}N_5O_{10}$ as suggested by HRESIMS based on the $[M+Na]^+$ ion peak at *m/z*

* Corresponding author. Tel.: +65 6790 3820; fax: +65 6896 9432.
E-mail address: liktong.tan@nie.edu.sg (L.T. Tan).

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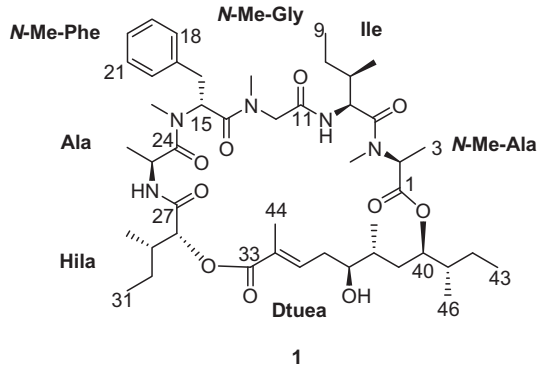

Fig. 1. Chemical structure of lagunamide C (**1**).

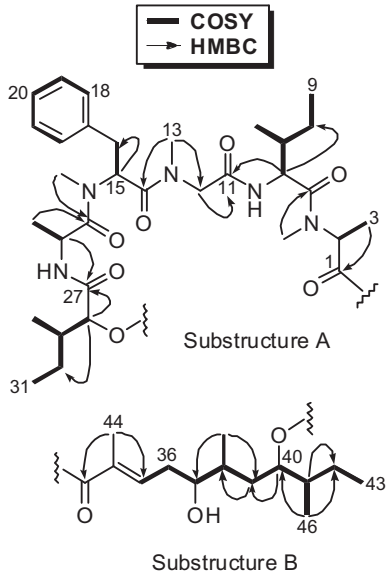

Fig. 2. Planar structure of lagunamide C (**1**) showing key COSY and HMBC correlations.

878.5250. The ^{1}H NMR data, recorded in CD_3OD, indicated the peptidic nature of **1** and suggested the presence of at least three *N*-methyl amide groups at δ 2.90, 3.06, and 3.32. Furthermore, the ^{13}C NMR spectrum (in CD_3OD) of **1** exhibited the presence of seven carbonyl carbons attributable to ester/amide functionalities and a mono-substituted phenyl ring (δ 137.3, 129.6, 128.0, and 126.4) system. Analyses of the 1D and 2D NMR spectra led to the establishment of a hybrid structural framework consisting of polypeptide and polyketide sections (substructures A and B in Fig. 2). Detailed 2D NMR analysis of **1** allowed the assignment of five proteinogenic amino acids, namely *N*-methyl-alanine (*N*-Me-Ala; C1–C4), isoleucine (Ile; C5–C10), *N*-methyl-glycine (*N*-Me-Gly; C11–C13), *N*-methyl-phenylalanine (*N*-Me-Phe; C14–C23), and alanine (Ala; C24–C26) in substructure A (Fig. 2). The sixth and final residue in substructure A was deduced to be a hydroxy acid, 2-hydroxyisoleucic acid (Hila; C27–C32), owing to the attachment of H-28 to an oxymethine carbon (δ 76.6). The sequence of *N*-Me-Ala-Ile-*N*-Me-Gly-*N*-Me-Phe-Ala-Hila was subsequently deduced from key HMBC correlations (Fig. 2), including H-2 and H-3/C-1, H-4 and H-6/C-5, H-6/C-11, H-12/C-11, H_3-13/C-12 and C-14, H-15/C-14, H-23/C-24, H-25 and H_3-26/C-24, H-25/C-27, as well as H-28/C-27 (Table 1).

The polyketide-derived portion (substructure B in Fig. 2) in lagunamide C (**1**) was elucidated by COSY spectral data with correlations starting from the olefinic proton at H-35, via the allylic methylene protons, H_2-36ab, the oxymethine proton, H-37, and the methine proton at H-38. The continued COSY correlations can be traced forward between methyl protons at H_3-45 to the methine proton at H-38, extending to the methylene protons at H_2-39ab, and oxymethine proton at H-40. Additional COSY correlations observed between H-40/H-41, H-41/H_2-42ab and H_3-46, and H_2-42ab/H_3-43 completed substructure B. In addition, these correlations were further supported by various spin systems observed in the TOCSY spectral data. Furthermore, long range HMBC correlations observed between H_3-44/C-33 and C-35, H_3-45/C-37 and C-39, H-39a/C-38, H-40/C-39, H_3-46/ C-40 and C-41, H-41/C-42 strongly supported the structural assignment of the polyketide segment as 5,8-dihydroxy-2,6,9-trimethyl-undec-2-enoic acid (Dtuea) moiety (Fig. 2). The position of the hydroxy group at C-37 was consistent with the aurilide class of compounds and was suggested on the basis of the characteristic chemical shifts

Table 1
1D NMR spectroscopic data for lagunamide C (**1**) in CD_3OD.

Unit	C/H No.	δH (*J* in Hz)[a]	δC[b]	HMBC
N-Me-Ala	1		171.7	
	2	3.96, q (7.0)	59.2	1, 3, 4
	3	1.43, d (7.0)	12.8	1, 2
	4	3.32, s	36.5	2, 5
Ile	5		172.0	
	6	5.06, d (6.2)	53.7	5, 7, 8, 11
	7	1.83, m	38.3	
	8a	1.64, m	23.5	
	8b	1.30, m		
	9	0.93, m	9.0	7, 8
	10	1.04, d (6.8)	14.9	6, 7, 8
	*N*H			
N-Me-Gly	11		170.3	
	12	4.20, d (18.3)	51.6	11, 13
		3.57, d (18.3)		11, 13
	13	2.90, s	35.5	12, 14
N-Me-Phe	14		171.5	
	15	5.47, dd (10.3, 5.2)	54.0	14, 16, 17, 23
	16a	3.04, m	34.8	15, 17, 18, 19
	16b	2.94, dd (14.3, 5.2)		15, 17, 18, 19
	17		137.3	
	18/22	7.20, m	128.0	16, 19, 21
	19/21	7.18, m	129.6	20, 18, 22
	20	7.28, m	126.4	19, 21
	23	3.06, s	29.5	15, 24
Ala	24		173.8	
	25	4.52, q (6.9)	45.3	24, 26, 27
	26	0.87, d (6.9)	14.7	24, 25
	*N*H			
Hila	27		171.8	
	28	4.82, m	76.6	27, 29, 30, 32, 33
	29	1.86, m	37.5	30, 31
	30a	1.49, m	26.4	29, 31
	30b	1.34, m		
	31	0.97, m	10.6	28, 29, 30
	32	1.14, m	13.5	28, 29
Dtuea	33		169.4	
	34		127.6	
	35	7.30, brd	145.8	34, 44
	36a	2.24, m	29.6	34, 35, 37
	36b	2.04, m		44
	37	3.76, brd (3.2)	70.4	
	38	2.14, m (3.2, 3.6, 8.4)	40.4	37
	39a39b	1.24, m (8.4, 11.2)	36.8	37, 38,40, 41
		1.10, m (3.6, 4.1)		40, 45
	40	4.92, m (4.1, 10.4, 11.2)	78.2	1, 38, 39, 41, 42, 46
	41	1.80, m (10.4)	34.9	42, 43, 46
	42	1.37, m	20.5	40, 41, 43
	43	0.91, m	10.6	40, 42
	44	1.93, s	11.4	33, 34, 35
	45	0.90, m	11.9	37, 39
	46	0.92, m	10.9	40, 41

[a] Recorded at 400 MHz. Coupling constants (Hz) are in parentheses.
[b] Recorded at 100 MHz.

of H-37 (δ 3.76) and C-37 (δ 70.4) (Williams et al., 2003; Nakao et al., 2004; Tripathi et al., 2010). The *E*-geometry of the double bond $\Delta^{34,35}$ was assigned on the basis of the ^{13}C NMR signals observed for the methyl group CH_3-44 at δ 11.4 (Couperus et al., 1976). Substructures A and B were connected on the basis of HMBC correlations observed for the α-proton (H-28) of Hila to C-33, the carbonyl carbon in substructure B, and between H-40 of substructure B with the C-1 carbonyl carbon of substructure A through an ester bond. The cyclic nature of the depsipeptide satisfies the final degree of unsaturation in providing a 27-membered ring in lagunamide C (**1**).

Stereochemical assignments of the amino acids in **1** were based on the advanced Marfey's method coupled with LC–MS analysis of the acid hydrolysate, revealing the absolute configurations of Ala, *N*-Me-Phe, *N*-Me-Ala and Ile to be L, D, L, and L-*allo*, respectively (Marfey, 1984; Fuji et al., 1997a,b). Isomers of 2-hydroxyisoleucic (Hila) acids were synthesized by diazotization of the corresponding amino acids in dilute perchloric acid (Mamer, 2000). The replacement of the amino group by a hydroxyl group is known to occur with retention of configuration at the α-carbon owing to anchiomeric participation of the neighboring carboxyl function (Mamer and Reimer, 1992). The synthesized Hila isomers were derivatized with Mosher's acids (*S*- and *R*-MTPACl) and subsequently analyzed on LC–MS to provide the absolute configuration of Hila in **1** as D-*allo*. In addition, the $\Delta\delta_{(S-R)}$ values obtained by ^{1}H NMR analysis of *S*- and *R*-MTPA esters of the hydroxy group at C-37 in **1**, revealed unambiguously that C-37 possesses the *S*-configuration.

The relative configurations of C-38, C-40, and C-41 were determined using $^3J_{H,H}$ values as well as 2D NOESY correlations. Having the absolute configuration at C-37 determined as *S* allowed the construction of four (out of six) possible conformations for C-37 and C-38 (Fig. 3a). The protons at H-37 and H-38 displayed a small coupling constant ($^3J_{H\text{-}37,H\text{-}38}$ = 3.2 Hz, obtained through HOM2DJ experiment in CD_3OD at 400 MHz), indicating these protons to be in a *syn* conformation. A NOESY correlation observed between H_3-45 and H-36 would be satisfied by three possible relative conformations. However only one conformation (37*S* and 38*S**), can satisfy the NOESY correlation observed between H-37 and H_3–45 (highlighted in box in Fig. 3a). Likewise, the protons H-38 and H-39a displayed large coupling constant for H-38 and H-39a

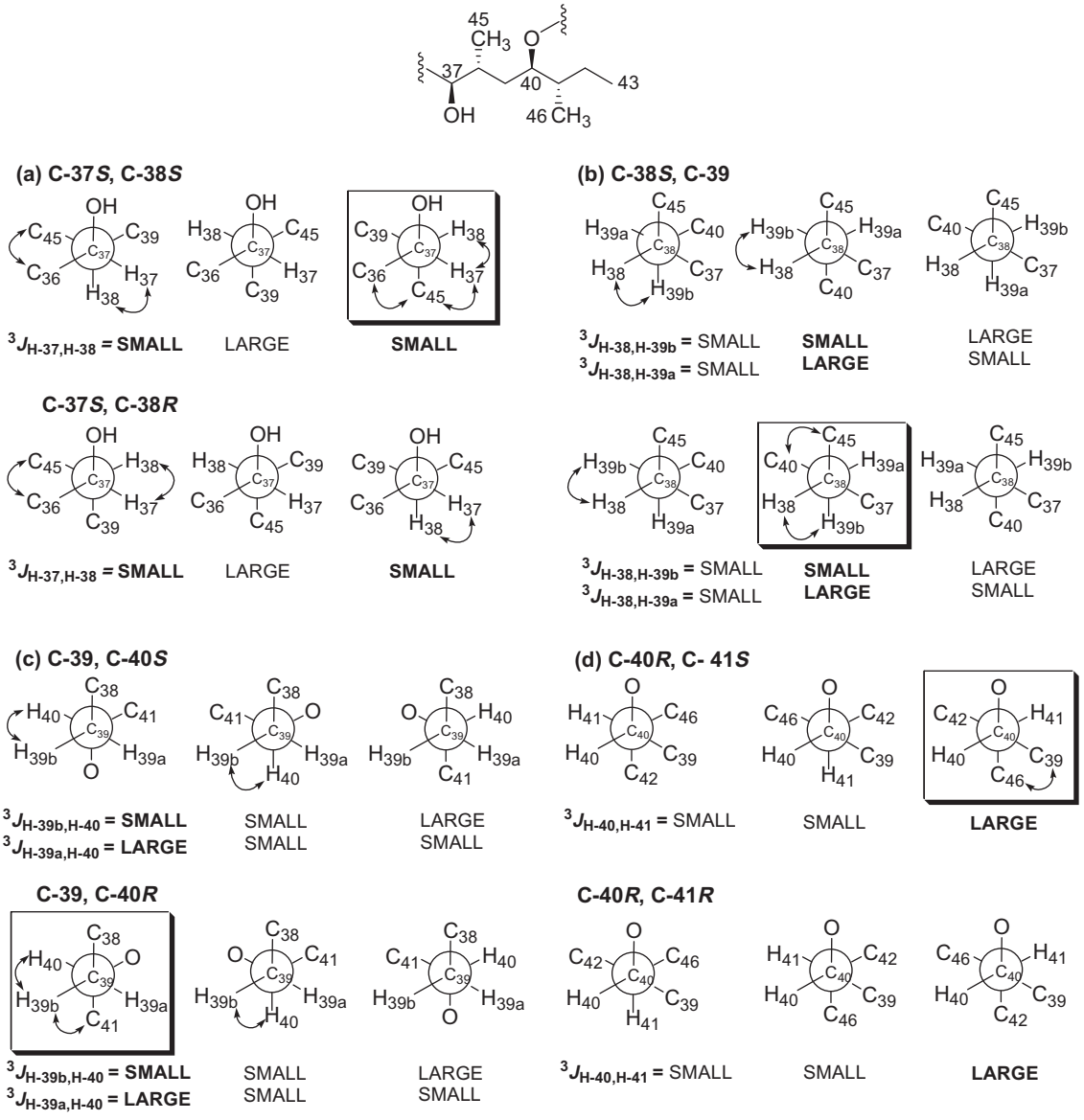

Fig. 3. Newman projections for (a) C-37/C-38; (b) C-38/C-39; (c) C-39/C-40; and (d) C-40/C-41. Labels below projections denote predicted size of the $^3J_{H-H}$ coupling constant values. Predicted sizes (in bold) are consistent with observed $^3J_{H-H}$ coupling constant values. Observed NOESY correlations are presented as double-arrow arch.

($^3J_{H\text{-}38,H\text{-}39a}$ = 8.4 Hz, obtained through HOM2DJ experiment), which produced two possible conformations. However, only one conformation (highlighted in box in Fig. 3b) displayed a NOESY correlation between H_3-45 and H-40, facilitating us to fix the relative conformation of H-39a and H-39b at C-39 (Fig. 3b).

The continued stereochemical analysis of the spin system from C-39 to C-40 revealed small coupling constant between H-39b and H-40 ($^3J_{H\text{-}39b,H\text{-}40}$ = 4.1 Hz), indicating the protons to be in *syn* orientation but large coupling constant between H-39a and H-40 ($^3J_{H\text{-}39a,H\text{-}40}$ = 11.2 Hz), indicating these to be in *anti* conformation. This allowed only two (out of six) possible conformations for C-39 and C-40 (Fig. 3c). However, a strong NOESY correlation observed between H-39b and H-41 suggested the relative configuration for C-40 to be 40*R** (highlighted in box in Fig. 3c). Subsequent analysis of the spin system from C-40 to C-41 indicated large coupling constant between H-40 and H-41 ($^3J_{H\text{-}40,H\text{-}41}$ = 10.4 Hz), suggesting these protons to be in *anti* orientation. However, a strong NOESY correlation observed between H_3-46 and H-39b allowed the 40*R**, 41*S** configurations at C-40 and C-41 (highlighted in box in Fig. 3d). Having confirmed the absolute configuration of C-37 as *S*, the absolute configurations of 37*S*, 38*S*, 40*R*, and 41*S* were therefore inferred in the Dtuea moiety in **1**.

A separate study performed on a related compound, kulokekahilide-2, showed the phenomenon of intramolecular ester exchange between the adjacent hydroxyl groups within the polyketide moiety (Umehara et al., 2009). On the same line, an experiment was performed for compound **1** using different two solvents, DMSO-d_6 and CD_3OD, to investigate any probable intramolecular ester exchanges. Interestingly, the occurrence of the ester exchange was not observed in the ^{1}H NMR spectra of lagunamide C (**1**) taken over the span of 98 h, indicating its structural stability in different deuterated solvents (refer to Supplementary Information). Compound **1** also showed well-dispersed proton NMR signals in CD_3OD rather than $CDCl_3$, as shown by kulokekahilide-2.

The report of lagunamide C (**1**) in this study represents a new sub-class of aurilide-related compound isolated from a marine cyanobacterial sample. Biosynthetically, the polyketide portion in **1** could be derived from the starter unit, 2-methylbutyryl-CoA (via isoleucine), and further chain extension by combination of methylmalonyl-CoA and acetyl-CoA.

Structural perspective of natural products belonging to the aurilide class of compounds, including lagunamides (Tripathi et al., 2010), palau'amide (Williams et al., 2003), and kulokekahilide-2 (Nakao et al., 2004), provided some interesting insights into the construction of these metabolites. Firstly, within the polypeptide chain, the only conserved residue is *N*-Me-Gly (Table 2). Secondly, the structural comparison suggests that the *N*-methyltransferase domain, which is usually integrated in the adenylation domains, may either be expressed or suppressed for the tailoring of Ala unit at the sixth position (Table 2). In addition, substrate specificity for Ala appeared to be conserved at this position. Thirdly, the position of the α-hydroxy residue is conserved in the different compounds

Table 2
Polypeptide-derived sequences in aurilides, kulokekahilide-2, pulau'amide, lagunamide A, and lagunamide C. Conserved amino acid units are indicated in bold.

Compounds	Positions					
	1	**2**	**3**	**4**	**5**	**6**
Lagunamides A or C	Hila	Ala	*N*-Me-Phe	***N*-Me-Gly**	Ile	*N*-Me-**Ala**
Kulokekahilide-2	Hica	Ala	*N*-Me-Phe	***N*-Me-Gly**	Ile	**Ala**
Palau'amide	Hica	Ala	*N*-Me-Phe	***N*-Me-Gly**	Ile	*N*-Me-**Ala**
Aurilide	Hila	Val	*N*-Me-Leu	***N*-Me-Gly**	Val	*N*-Me-**Ala**
Aurilide B	Hila	Val	*N*-Me-Ile	***N*-Me-Gly**	Val	*N*-Me-**Ala**
Aurilide C	Hiva	Val	*N*-Me-Ile	***N*-Me-Gly**	Val	*N*-Me-**Ala**

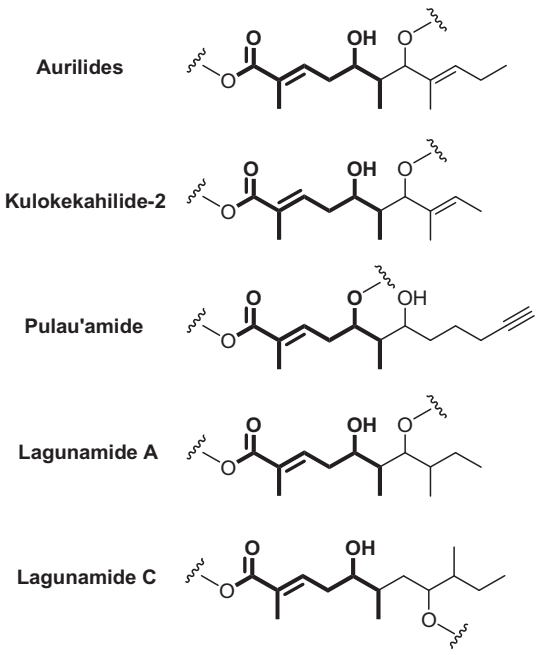

Fig. 4. Polyketide-derived moieties in aurilides, kulokekahilide-2, pulau'amide, lagunamides A and C. Conserved sequences are highlighted in bold.

but the adenylation substrate specificity is relaxed for the precursor Val, Leu, or Ile (Table 2). Lastly, although kulokekahilide-2 (Nakao et al., 2004), palau'amide (Williams et al., 2003), and lagunamides A and B (Tripathi et al., 2010) differ largely in the polyketide-derived moiety, they share certain sequences as indicated in Fig. 4.

A series of biological assays were conducted and revealed that **1** exhibited potent cytotoxic properties against a range of cancer cell lines, including P388 (murine leukemia), A549 (lung carcinoma), PC3 (prostate cancer), HCT8 (ileocecal colorectal adenocarcinoma), and SK-OV (ovarian) cancer cell lines with IC_{50} values of 24.4 nM, 2.4 nM, 2.6 nM, 2.1 nM, and 4.5 nM, respectively. Lagunamide C (**1**) was also tested for its anti-malarial activity against the NF54 strain of malaria parasite and showed significant *in vitro* activity against *Plasmodium falciparum* with IC_{50} value at 0.29 μM. Furthermore, compound **1** exhibited weak anti-swarming activity (49% inhibition compared to control) when tested at 100 ppm against the Gram-negative bacterial strain, *Pseudomonas aeruginosa* PA01. It is of interest to note that a related natural product, aurilide, was recently found to activate mitochondria-induced apoptosis by selectively binding to the protein prohibitin (Sato et al., 2011). Research is currently being conducted to determine if the mode of action of lagunamide C operates in a similar fashion to aurilide.

3. Conclusions

A new cyclic depsipeptide, lagunamide C (**1**), was isolated from the marine cyanobacterium, *L. majuscula*, collected from Pulau Hantu Besar, Singapore. Lagunamide C represents a new subclass of aurilide-related compounds by having a ring expansion due to the additional methylene carbon in the polyketide-derived moiety. Compound **1** was tested against a panel of five cancer cell lines, including P388, A549, PC3, HCT8, and SK-OV cell, with IC_{50} values ranging from 2.1 nM to 24.4 nM. It also possesses significant anti-malarial property. The isolation of compound **1** in this study, along with other reports of highly cytotoxic depsipeptides, such as apratoxin A and largazole, underscores the importance of filamentous marine cyanobacteria as a prolific source of potential therapeutic

drugs (Luesch et al., 2001; Taori et al., 2008; Lemmens-Grubert et al., 2009).

4. Experimental

4.1. General experimental procedures

Optical rotation was measured on a Bellingham Stanley ADP 440 polarimeter. UV and IR spectra were measured on a Varian Cary 50 UV visible spectrophotometer and a Perkin–Elmer spectrum 100 FT-IR spectrophotometer, respectively. ^{1}H, ^{13}C, and 2D NMR spectra were recorded in CD_3OD on a 400 MHz Bruker NMR spectrometer using the residual solvent signal (δH at 3.31 ppm and δC at 49.1 ppm) as internal standards. HRESIMS and LC–MS/MS data were obtained using a Bruker Daltonics MicrOTOF™ mass spectrometer. HPLC isolation of lagunamide C (**1**) was conducted on a Shimadzu LC-8A Preparative LC equipped with Shimadzu SPD-M10A VP diode array detector, while an Agilent 1100 series coupled with an Agilent LC/MSD trap XCT mass spectrometer equipped with an ESI interface system was used for the detection of the Marfey-derivatized alanine, *N*-methyl-phenylalanine, *N*-methyl-alanine, and isoleucine as well as for Mosher's derivatized α-hydroxy acids in lagunamide C (**1**). Cell viability in 96-well plates was measured using a Bio Rad Benchmark plus microplate reader.

4.2. Marine cyanobacterial samples

Samples (about 1.5 L) of the filamentous marine cyanobacterium, *L. majuscula*, were collected by hand from shallow waters during low tides at Pulua Hantu Besar on June 25, 2007 and stored in 70% aq. EtOH at −20 °C before workup. A voucher specimen of this microalga is maintained at NIE under the code TLT/PHB/002.

4.3. Extraction and isolation

The thawed cyanobacterial samples (∼169 g dry wt.) were exhaustively extracted using $CHCl_3$/MeOH (1:1) to produce an organic extract (∼2 g). The extract was then fractionated on normal phase silica VFC using a stepwise gradient solvent system from hexane, to EtOAc, and MeOH. Fractions eluted with EtOAc (100%) and EtOAc:MeOH (9:1) were found to possess 100% toxicity (tested at 10 ppm) in the brine shrimp toxicity assay. Each fraction was passed through a SEP-PAK RP-18 cartridge by eluting with 100% MeOH. The cleaned fraction, which initially eluted with EtOAc:MeOH (9:1), was subjected to C18 RP-HPLC [Phenomenex Sphereclone 5 μm ODS, 250 × 10.00 mm, MeOH/H_2O (81:19) at 3.0 mL/min, UV detection at 230 nm] to obtain lagunamide C (**1**, 9.2 mg, 0.5 % of extract, t_R = 33.1 min). Additional source of compound **1** (6.9 mg, 0.35 % of extract, t_R = 43.1 min) was also obtained after the C18 RP-HPLC [Phenomenex Sphereclone 5 μm ODS, 250 × 10.00 mm, MeOH/H_2O (78:22) at 3.0 mL/min, UV detection at 230 nm] of the fraction initially eluted with 100% EtOAc. Both sources of lagunamide C were pooled together (on the basis of ^{1}H NMR spectra) and due to its impurity were subjected to further C18 RP-HPLC [Phenomenex Sphereclone 5 μm ODS, 250 × 10.00 mm, MeOH/H_2O (87:13) at 3.2 mL/min, UV detection at 230 nm] to yield pure lagunamide C (**1**, 4.7 mg, 0.3 % of extract, t_R = 19.2 min, refer to Supplementary Information).

4.4. Lagunamide C (*1*)

White, amorphous solid; $[\alpha]_D^{25}$ −36 (*c* 0.5, MeOH); UV(MeOH) λ_{max} 220 nm (log ε 2.89); IR (neat) 3316, 2914, 2486, 2251, 2054, 1694, 1439, 1276 cm^{-1}; ^{1}H NMR (400.13 MHz, CD_3OD) and ^{13}C NMR (100.62 MHz, CD_3OD) data, see Table 1; HRESIMS *m/z* $[M+Na]^+$ 878.5225 (calcd for $C_{46}H_{73}N_5O_{10}Na$, 878.5250).

4.5. Advanced Marfey's analysis of amino acid residues in *1*

A sample of compound **1** (1.0 mg) was treated with 6 N HCl (1 mL) at 110 °C for 18 h. The resulting hydrolysate was concentrated to dryness, re-dissolved in H_2O (0.6 mL) and divided into two equal portions. Each portion was combined with either a 1% solution of 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (L-FDAA, Marfey's reagent, 50 μL) in acetone or a racemic mixture of a 1% solution of 1-fluoro-2,4-dinitrophenyl-5-LD-alaninamide (LD-FDAA, 50 μL) in acetone and 1 M $NaHCO_3$ (25 μL), and the two mixtures were heated at 40 °C for 45 min. Both reaction mixtures were cooled to r.t. and quenched by the addition of 2 N HCl (25 μL), dried, and redissolved in MeCN (500 μL). The aliquots were subjected to reversed-phase LC–MS (Agilent 1100 series) according to the advanced Marfey's method (Phenomenex, Luna, 150 × 2.0 mm, 5 μm, 100 Å; MeCN in 0.1% (v/v) aqueous HCOOH; at 0.20 mL/min) using a linear gradient (10–50% MeCN over 60 min) (Marfey, 1984; Fuji et al., 1997a,b). An Agilent 1100 series MSD spectrometer was used for detection in ESI (negative mode). The retention times and ESIMS product ions (t_R in min, *m/z*$[M-H]^-$) of the L-FDAA-derivatized amino acids in the hydrolysate of the first portion were observed to be Ile (45.1, 382.0), *N*-Me-Phe (48.6, 430.1), Ala (28.3.0, 340.0), and *N*-Me-Ala (35.2, 354.0). The reaction with racemic LD-FDAA in the second portion gave rise to two peaks for each corresponding amino acid moiety and the retention times and ESIMS product ions (t_{R1}/t_{R2}, min, *m/z* $[M-H]^-$) were observed to be Ile (45.1/52.4, 382.0), *N*-Me-Phe (47.9/48.6, 430.1), Ala (28.3/49.5, 340.0), and *N*-Me-Ala (35.2/36.1, 354.0). Consequently, the absolute configurations of the moieties in the hydrolysate of **1** were confirmed as D-*N*-Me-Phe, L-Ala, and L-*N*-Me-Ala.

For the analysis of the Ile unit in **1**, additional lagunamide C (0.5 mg) was hydrolyzed in 6 N HCl at 110 °C for 20 h and was derivatized with Marfey's reagent (L-FDAA) as described above. Two portions each of 0.5 mg standard L-Ile and L-*allo*-Ile were dissolved in 100 μL of H_2O. A 1.0% solution of L-FDAA (100 μL) and 1 N $NaHCO_3$ (20 μL) were added to one portion each of L-Ile and L-*allo*-Ile and to the other portions were added 1.0% solution of D-FDAA (100 μL) and 1 N $NaHCO_3$ (20 μL). All four mixtures were then heated at 40 °C for 45 min. The solution were cooled to r.t., neutralized with 2 N HCl (10 μL) and evaporated to dryness. The residues were then resuspended in MeCN (500 μL). The aliquots were subjected to reversed-phase LC–MS (Agilent 1100 series) according to the Marfey's method [column: Phenomenex, Luna, 150 × 2.0 mm, 5 μm, 100 Å; mobile phase, MeCN in 0.1% (v/v) aqueous HCOOH; flow rate, 0.20 mL/min] using a linear gradient (30–70% MeCN over 40 min) (Marfey, 1984; Fuji et al., 1997a,b). An Agilent 1100 series MSD spectrometer was used for detection in API-ES (negative mode). The derivatized Ile residue in the hydrolysate of **1** eluted at the same retention time as the derivatized standard L-*allo*-Ile (14.2 min) but not that of L-Ile (13.0 min), D-Ile (=D-FDAA derivatized L-Ile , 17.3 min) and D-*allo*-Ile (=D-FDAA derivatized L-*allo*-Ile, 17.8 min).

4.6. Preparation of isomers of 2-hydroxyisoleucic acid (Hila)

L-Isoleucine (100 mg, 0.75 mmol) was dissolved in 50 mL of 0.2 N perchloric acid (0 °C). To this was added a cold (0 °C) solution of $NaNO_2$ (1.4 g, 20 mmol) in 20 mL of H_2O with rapid stirring. With continued stirring the reaction mixture was allowed to reach r.t. until evolution of N_2 subsided (about 30 min). The solution was then brought to boil for 3 min, cooled to r.t., and saturated with NaCl. The mixture was then extracted with Et_2O and dried under vacuum to give 2*S*, 3*S*-Hila (L-Hila). The three other stereoisomers 2*R*,

3*R*-Hila (D-Hila), 2*R*, 3*S*-Hila (D-*allo*-Hila), and 2*S*, 3*R*-Hila (L-*allo*-Hila) were synthesized in a similar manner from D-Ile, D-*allo*-Ile, and L-*allo*-Ile, respectively (Mamer, 2000; Mamer and Reimer, 1992).

4.7. Absolute configuration of Hila unit in **1**

Determination of the absolute configuration for the 2-hydroxy-isoleucic acid (Hila) residue in **1** was accomplished by a modified method based on Mosher's reagents and analysis using LC–MS. Acid hydrolysis of lagunamide C (**1**, 1.0 mg) was achieved in 1 mL of 6 N HCl placed in a sealed reaction vial at 110 °C for 18 h. Trace HCl was removed under a stream of N_2 gas and the resulting hydrolysate was divided into two equal portions (0.5 mg each), and to each sample was added 0.5 mL of pyridine. To one portion was added 2.5 mg of *R*-MTPACl (α-methoxy-α-trifluoromethylphenylacetic acid) and to the other 2.5 mg of *S*-MTPACl. The reaction was carried out for 10 h at r.t. and the solvent was evaporated under N_2. In a similar manner, each isomer of Hila (0.5 mg each) was derivatized with either *R*- or *S*-MTPACl (1.0 mg each in 0.5 mL pyridine). All derivatized samples were subjected to reversed-phase LC–MS (Agilent 1100 series) [column: Phenomenex, Luna, 150 × 2.0 mm, 5 μm, 100 Å; mobile phase, MeCN in 0.1% (v/v) aqueous HCOOH; flow rate, 0.20 mL/min] using a linear gradient (30–70% MeCN over 60 min). An Agilent 1100 series MSD spectrometer was used for detection in API-ES (negative mode). The retention times and ESIMS product ions (t_R in min) of the *S*-MTPACl monoderivatized standard hydroxy amino acids were observed to be L-Hila (30.1 min), L-*allo*-Hila (30.7 min), D-Hila (=*R*-MTPACl derivatized L-Hila, 28.5 min), and D-*allo*-Hila (=*R*-MTPACl derivatized L-*allo*-Hila, 29.4 min). Consequently, the absolute configuration of the Hila moiety in the hydrolysate of **1** derivatized with *S*-MTPACl was confirmed as D-*allo*-Hila since it eluted at 29.4 min.

4.8. Preparation of MTPA (α-methoxy-α-trifluoro-methyl-phenylacetate) esters of **1**

Two portions of lagunamide C (**1**, 0.5 mg each) were reacted with *R*- or *S*-MTPACl (α-methoxy-α-trifluoro-methyl-phenylacetylchloride) (5.0 mg) in 0.5 mL of pyridine for 10 h at r.t., and the solvent was then evaporated under N_2. The corresponding esters were subjected to NMR analysis.

S-MTPA Ester: ^{1}H NMR (400 MHz, CD_3OD) δ 7.210 (H-35), 2.204 (H-36a), 2.041 (H-36b), 3.512 (H-37), 2.141 (H-38), 1.102 (H-39a), 1.012 (H-39b), 4.839 (H-40), 2.248 (H-41), 1.207 (H-42a), 1.413 (H-42b), 0.932 (H-43).

R-MTPA Ester: ^{1}H NMR (400 MHz, CD_3OD) δ 7.227 (H-35), 2.234 (H-36a), 2.097 (H-36b), 3.542 (H-37), 2.032 (H-38), 1.013 (H-39a), 1.001 (H-39b), 4.810 (H-40), 2.236 (H-41), 1.181 (H-42a), 1.401 (H-42b), 0.915 (H-43).

4.9. Anti-swarming assay based on P. aeruginosa PA01

Anti-swarming assay based on the bacterial *P. aeruginosa* PA01 were carried out using 0.5 mg of lagunamide C added into 5 mL of molten STA (soft top agar) [constituted with 200 mL deionised H_2O, 1.3 g agar (Technical agar # 3 Oxoid), 2 g tryptone (Difco), 1 g NaCl (Sigma)] and poured immediately over the surface of a solidified luria bertini agar (LBA, 40 g/L from MoBio) plate as an overlay. The plate was point inoculated with 5 μL of the bacterial strain once the overlaid agar had solidified and incubated at 37 °C for 3 days. The experiment was performed in triplicate along with MeOH treated plate as control and the extent of swarming was determined by measuring the area of the colony using a leaf surface meter (Area Meter AM200, ADC Bioscientific Ltd.).

4.10. Cytotoxicity assay

Test solution was added to A549 lung carcinoma, PC3 prostate cancer cells, HCT8 ileocecal colorectal adenocarcinoma, and SK-OV ovarian cancer cell lines and P388 murine leukemia cells (ATCC CCL46) in a series of eight twofold dilutions and cells were then incubated (35 °C, 72 h). Media, solvent, cells, and positive controls were included in each assay run. 3(4,5-Dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to wells, then cells were incubated for a further four hours. Cell viability was determined by measurement of formazan production via a spectrophotometer at 540 nm. The percentage inhibition of cell growth was determined by comparison of test well absorbance to that of a control and a plot of logarithm of sample concentration versus absorbance was used to determine the IC_{50} (ng/mL) value (concentration of the test sample required to reduce the cancer cell line growth by 50 %, respectively).

4.11. In vitro antimalarial assay

P. falciparum drug-sensitive NF54 and chloroquine-resistant K1 strains were cultivated in a variation of the medium previously described (Dorn et al., 1995; Trager and Jensen, 1976) consisting of RPMI 1640 supplemented with 0.5% ALBUMAX® II, 25 mM Hepes, 25 mM $NaHCO_3$ (pH 7.3), 0.36 mM hypoxanthine, and 100 μg/ml neomycin. Human erythrocytes served as host cells. Cultures were maintained in an atmosphere of 3% O_2, 4% CO_2, and 93% N_2 in humidified modular chambers at 37 °C. Compounds were dissolved in DMSO (10 mM), diluted in hypoxanthine-free culture medium and titrated in duplicates over a 64-fold range in 96 well plates. Infected erythrocytes (1.25% final hematocrit and 0.3% final parasitemia) were added into the wells. After 48 h incubation, 0.5 μCi of [^{3}H] hypoxanthine was added per well and plates were incubated for an additional 24 h. Parasites were harvested onto glass-fiber filters and radioactivity was counted using a Betaplate liquid scintillation counter (Wallac, Zurich). The results were recorded and expressed as a percentage of the untreated controls. Fifty percent inhibitory concentrations (IC_{50}) were estimated by linear interpolation (Huber and Koella, 1993).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2011.08.019.

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