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Dudawalamides A–D, Antiparasitic Cyclic Depsipeptides from the Marine Cyanobacterium *Moorea producens*

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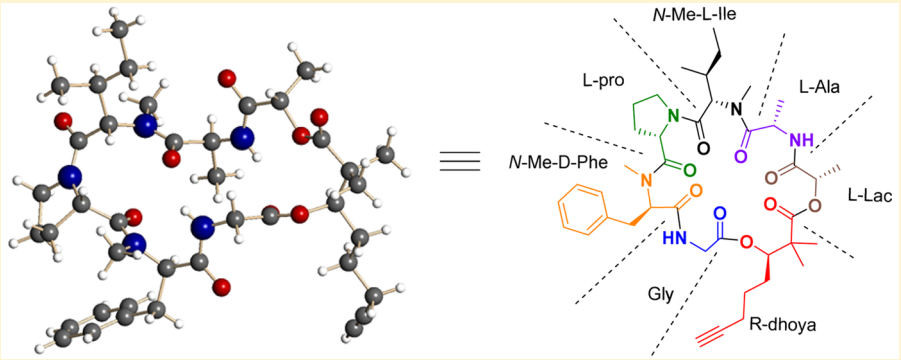
Supporting Information



ABSTRACT: A family of 2,2-dimethyl-3-hydroxy-7-octynoic acid (Dhoya)-containing cyclic depsipeptides, named dudawalamides A–D (**1**–**4**), was isolated from a Papua New Guinean field collection of the cyanobacterium *Moorea producens* using bioassay-guided and spectroscopic approaches. The planar structures of dudawalamides A–D were determined by a combination of 1D and 2D NMR experiments and MS analysis, whereas the absolute configurations were determined by X-ray crystallography, modified Marfey's analysis, chiral-phase GCMS, and chiral-phase HPLC. Dudawalamides A–D possess a broad spectrum of antiparasitic activity with minimal mammalian cell cytotoxicity. Comparative analysis of the Dhoya-containing class of lipopeptides reveals intriguing structure–activity relationship features of these NRPS–PKS-derived metabolites and their derivatives.

Marine cyanobacteria are one of the most prolific sources of biologically active secondary metabolites, often of mixed peptide and polyketide biosynthetic origin.[1,2] Investigation of these marine bacteria has resulted in the discovery of a wealth of biologically active compounds, and the majority of these are peptides and peptide-derived compounds. Of special note, a number of cyanobacterial peptides and related hybrid metabolites have been pursued as therapeutic lead compounds.[2−4] Recent examples include dolastatin 10, curacin A, largazole, and the carmaphycins.[5−7] Expanding on the known assembly of cyanobacterial peptides, we have reported a number of cyclic depsipeptides including viequeamides A and B, yanucamides A and B, mantillamide, antanapeptins A–D, apratoxins D, F, and G, and wewakpeptins A–D.[8−12]

Kulolide, isolated from the cephalaspidean mollusk *Philinopsis speciosa* by Scheuer and co-workers, was the first cyclic depsipeptide reported to contain the Dhoya (2,2-dimethy-3-hydroxy-7-octynoic acid) moiety.[13] Since the isolation of kulolide in 1993, a number of structurally similar cyclic depsipeptides have been isolated from marine cyanobacteria that contain either the Dhoya or the 3-hydroxy-2-methyl-7-octynoic acid (Hmoya) moiety.[8,14−19] These molecules are likely produced by hybrid biosynthetic pathways that are composed of nonribosomal peptide synthetases (NRPS) and polyketide synthetases (PKS).[20,21] Depsipeptides containing Dhoya and Hmoya possess an array of biological activities including cytotoxicity to P388 murine leukemia cells, LoVo human colon cancer cells, H-460 human lung cancer cells, and brine shrimp, as well as in various antiparasitic assays.[8,14−19]

In our continuing search for biologically active compounds from marine organisms, the extract of the marine cyanobacterium *Moorea producens* collected from Dudawali Bay, Papua New Guinea, was found to contain several related molecules

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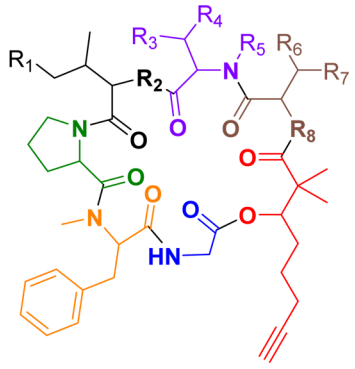
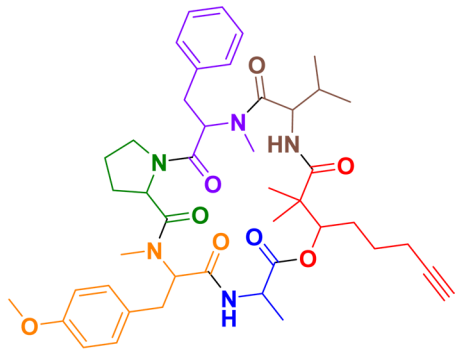


Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
Dudawalamide A (1)	CH_3	CH_3N	H	H	H	H	H	O
Dudawalamide C (3)	H	O	CH_3	CH_3	CH_3	CH_3	CH_3	NH
Dudawalamide D (4)	CH_3	O	CH_3	CH_3	CH_3	CH_3	CH_3	NH



Dudawalamide B (2)

Figure 1. Structures of dudawalamides A–D (**1**–**4**).

that shared similar fragmentation patterns by MS/MS analysis.[22] In this study, we present the isolation, structure elucidation, and biological evaluation of four structurally similar cyclic Dhoya-containing depsipepeptides, the dudawalamides A–D, from this Papua New Guinea collection. The planar structures of these compounds were determined by 2D NMR and mass spectrometric methods whereas the absolute configurations were determined by X-ray crystallography, chiral-phase GCMS, chiral-phase HPLC, or modified Marfey's analysis. Biological evaluation of these four new compounds revealed selective antiparasitic activity with low host cell cytotoxicity as well as some preliminary structure–activity relationships (SAR).

RESULTS AND DISCUSSION

A sample of the cyanobacterium *M. producens* was collected in 2006 from shallow marine habitats in Dudawali Bay, Papua New Guinea. The sample was preserved in 1:1 2-propanol–seawater and then subjected to CH_2Cl_2–MeOH (2:1) extraction, yielding an extract mass of 1.99 g. The extract was fractionated on normal-phase silica vacuum liquid chromatography in a gradient from 100% hexanes to 100% EtOAc to 100% MeOH, providing nine subfractions (A–I). Two relatively polar fractions (F, G), eluting with 80% to 100% EtOAc, were further purified by reversed-phase HPLC and analyzed by LCMS to afford a new family of metabolites called dudawalamides A–D (**1**–**4**). This collection also yielded the known cyanobacterial natural products apratoxin A and majusculamides A and B,[23,24] identified by comparison with authentic standards and published data, respectively. The structures of the new dudawalamides A–D (**1**–**4**) were determined by 1D and 2D NMR, mass spectroscopy (MS), and X-ray crystallography (Figures 1 and 2).

Positive ion LC-LRESI-MS analysis of the extract revealed four new compounds with protonated molecules at *m*/*z* 752.40, 786.47, 794.87, and 808.93 (LC-HRMS gave *m*/*z* 752.4220, 786.4429, 794.4694, and 808.4849 for molecular formulas of $C_{40}H_{57}N_5O_9$, $C_{44}H_{59}N_5O_8$, $C_{43}H_{63}N_5O_9$, and $C_{44}H_{65}N_5O_9$, respectively). Their closely aligned molecular formulas suggested that they were related to one another and, hence, were given the names dudawalamides A–D (**1**–**4**).

Dudawalamide A (**1**, $C_{40}H_{57}N_5O_9$ for 15 degrees of unsaturation) was found as the major component in the mixture (0.7% of extract). ^{13}C NMR data showed the presence

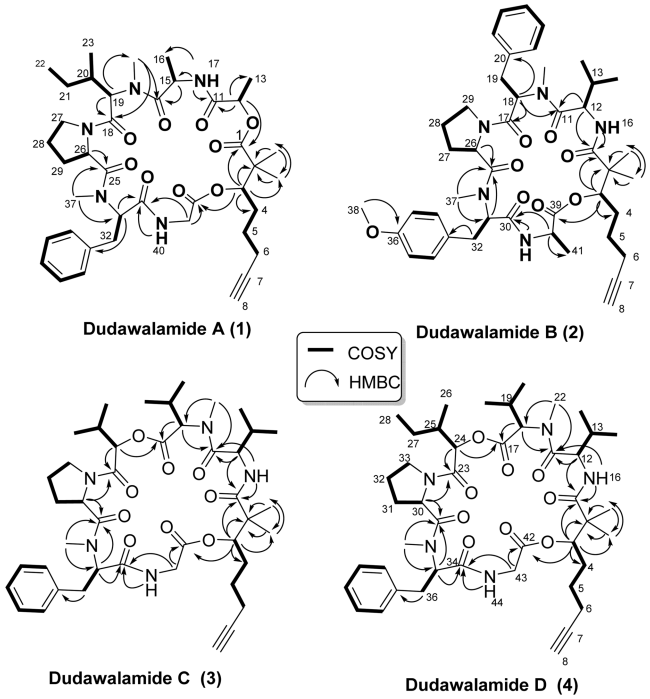


Figure 2. Key 2D NMR correlations of dudawalamide A–D (**1**–**4**).

of seven carbonyls at δ_C 168–175 and a phenyl ring, accounting for 11 degrees of unsaturation and leaving four additional double bonds or rings unassigned. The ^1H NMR spectrum was well dispersed and showed several proton resonances indicative of amide bonds (e.g., NH and NCH_3 groups) and an aromatic ring structure. The presence of four shielded methyl groups composed of three doublets and one triplet was suggestive of isoleucine- and alanine-type moieties. Integrated ^1H NMR, COSY, HSQC, and HMBC data were used to assign six partial structures consisting of the amino acids glycine, *N*-methylphenylalanine, *N*-methylisoleucine, proline (accounting for a 12th degree of unsaturation), alanine, and lactic acid.

A final partial structure was shown by these same NMR methods to be 2,2-dimethyl-3-hydroxy-7-octynoic acid. An HSQC experiment revealed that a doublet methine proton at δ_H 5.58 (J = 10.8 Hz) was connected to a carbon at δ_C 77.1, indicating that it was also attached to an oxygen atom. COSY

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Table 1. NMR Data ($CDCl_3$) for Dudawalamide A (1) (^1H 500 MHz, ^{13}C 125 MHz)

residue	position	δ_C, type	δ_H (J in Hz)	HMBC	COSY
Dhoya	1	174.6, C			
	2	47.6, C			
	3	77.1, CH	5.58, d (10.8)	1, 2, 4, 9, 10, 38	4
	4a	28.0, CH_2	1.87, m	5	3, 4b, 5
	4b		1.64, m	3, 6, 7	3, 4a
	5	24.4, CH_2	1.63, m	6, 7	4, 6
	6a	17.9, CH_2	2.26, m	4, 5, 7	5, 6b
	6b		2.39, m	4, 5, 7	5, 6a
	7	83.7, C			
	8	69.3, CH	1.99, t (2.7)		
	9	23.9, CH_3	1.25, s	3, 7, 10	
	10	17.6, CH_3	1.33, s	3, 7, 9	
Lac	11	169.8, C			
	12	70.9, CH	5.40, q (6.7)	1, 11, 13	13
	13	18.4, CH_3	1.40, d (6.8)	11, 12	12
Ala	14	174.9, C			
	15	46.2, CH	4.64, m	14, 16	16
	16	15.3, CH_3	1.51, d (7.2)	15	15
	17	NH	6.78, d (5.6)	11, 15, 16	
N-Me-Ile	18	168.5, C			
	19	56.6, CH	4.85, d (11.0)	18, 24	20
	20	33.9, CH	2.03, m	19	19, 23
	21	23.9, CH_2	1.43, m	19, 20, 22, 23	20, 22
	22	10.9, CH_3	0.83, t (7.0)	20, 21	21
	23	15.0, CH_3, CH_3	0.77, d (6.5)	19, 20, 21	20
	24	30.3, CH_3	3.26, s	14, 18, 19	
Pro	25	175.0, C			
	26	58.4, CH, CH_2	4.64, m	25, 27, 28, 29	29b
	27	46.3, CH_2	3.33−3.46, m	28, 29	28a, 28b
	28a	21.3, CH_2	0.89 m	27, 29	27
	28b		1.43 m		27
	29a	30.8, CH_2	0.88 m	28	26, 28
	29b		1.84 m	28	26, 28
N-Me-Phe	30	171.4, C			
	31	57.1, CH	5.81, dd (12.2, 4.8)	30, 33	32a, 32b
	32a	34.3, CH_2	2.86, m	31, 33, 34	32b
	32b		3.58, m	31, 33, 34	32a
	33	137.1, C			
	34	129.1, CH	7.18−7.22, m	32, 33, 35, 36	35
	35	128.3, CH	7.24, d (7.4)	33, 34	34, 36
	36	126.4, CH	7.15−7.19, m	35	35
	37	30.3, CH_3	3.26, s	25, 31	
Gly	38	172.6, C			
	39a	40.9, CH_2	4.50, dd (18.0, 8.1)	30, 38	39b
	39b		3.57, m	30, 38	39a
	40	NH	9.00, dd (8.2, 4.3)	30	39a, 39b

and HMBC correlations were used to consecutively assign the adjacent C-4, C-5, and C-6 methylenes (δ_C 28.0, 24.4, and 17.9). The distal methylene protons (δ_H 2.26−2.39; δ_C 17.9) showed HMBC correlations to a terminal alkyne group with a quaternary carbon at δ_C 83.7 and a methine carbon at δ_C 69.3 (δ_H 1.99), thereby accounting for two more degrees of unsaturation. Additionally, two- and three-bond HMBC correlations were observed between two singlet methyl groups to a quaternary carbon (δ_C 47.6), an ester carbonyl carbon (δ_C 174.6), and the above-described C-3 oxygen-bearing carbon atom (δ_C 77.1), thus completing this partial structure as 2,2-dimethyl-3-hydroxy-7-octynoic acid. This final residue accounted for all of the remaining atoms in dudawalamide A (**1**), and thus the final degree of unsaturation was an overall macro-ring structure.

HMBC correlations were used to elucidate the sequence of residues in dudawalamide A (**1**). Both the α-proton and *N*-methyl protons of the *N*-Me-isoleucine residue showed HMBC correlations to the alanyl carbonyl carbon at δ_C 174.9, revealing that an amide bond bridged these two residues. An HMBC correlation was also observed between the NH proton of the alanyl residue to the carbonyl carbon of the lactic acid (δ_C 169.8), indicating a second amide linkage. The α-methine proton at δ_H 5.40 of the lactic acid showed an HMBC correlation to the carbonyl carbon of the Dhoya moiety (δ_C 174.6), revealing this ester linkage in dudawalamide A (**1**). In

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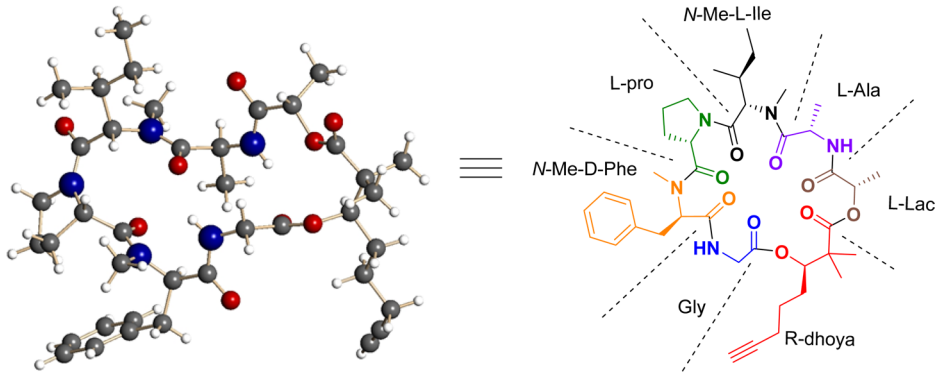

Figure 3. Isotropic X-ray crystallographic structure and line drawing of dudawalamide A (**1**) with solvent water molecules deleted.

turn, the deshielded C-3 proton of the Dhoya group showed an HMBC correlation to the glycyl carbonyl at δ_C 172.6, indicating the presence of a second ester bond. HMBC correlations were also observed from the glycyl α-methine proton to the *N*-methylphenylalanine carbonyl and from the latter residue's *N*-methyl group to the carbonyl of the proline, establishing the two additional amide linkages between these three amino acids. However, the α-methine proton of proline (δ_H 4.64) showed only a very weak HMBC correlation through its N atom to the next residue in the sequence (*N*-Me-isoleucine); we have previously observed a similar small or absent heteronuclear coupling in other proline-containing metabolites.[8,14] However, to account for the last degree of unsaturation based on HRMS data, the dudawalamide A (**1**) structure must have an overall macrocyclic structure. Summarizing, HMBC and MS data identified the residue sequence of dudawalamide A (**1**) as cyclo-[Pro-*N*-Me-Phe-Gly-Dhoya-Lac-Ala-*N*-Me-Ile] (Figure 2, Table 1).

Confirmation of the structure of dudawalamide A (**1**) and determination of its absolute configuration were achieved by X-ray crystallography of crystals obtained from a mixture of CH_2Cl_2 and hexanes containing residual acetone. This provided unambiguous confirmation of the structure of dudawalamide A (**1**) and revealed that the Lac, Ala, *N*-Me-Ile, and Pro residues all have an L-configuration, while the Dhoya residue was *R* (Figure 3). Remarkably, the *N*-Me-Phe was shown to be of D-configuration. This is the first occurrence of *N*-Me-D-Phe in a metabolite of the "kulolide superfamily".[8]

Three analogues of dudawalamide A (**1**), designated as dudawalamides B–D (**2**–**4**), were also isolated from the same *M. producens* extract. Their planar structures were determined by 2D NMR, and the absolute configurations of dudawalamides B (**2**) and D (**4**) were elucidated by a combination of chiral-phase HPLC, chiral-phase GCMS, and modified Marfey's analysis. However, due to the limited quantities of isolated dudawalamide C (**3**), its configurations were only partially determined.

Comparison of the ^{1}H and ^{13}C NMR spectra for dudawalamide B (**2**) with those of dudawalamide A (**1**) indicated that these metabolites were closely related; both possessed two *N*-methyl amide peaks, doublet methyl groups indicative of alanine, aryl protons indicative of phenylalanine, and the alkyl methylene protons and two singlet methyl signals indicative of a Dhoya residue. The most noticeable differences were the presence in dudawalamide B (**2**) of doublet methyl groups indicative of a valine residue, and the aryl protons and deshielded singlet methyl group suggestive of *O*-methyltyrosine. With interplay of TOCSY, COSY, HSQC, and HMBC experiments, the residues of dudawalamide B (**2**) were elucidated as Dhoya, proline, alanine, phenylalanine, and two residues not observed in dudalwalmide A, valine and *N*,*O*-dimethyltyrosine. The sequence of the residues of dudawalamide B (**2**) was deduced from the HMBC correlations as cyclo-[Dhoya–Val–*N*-Me-Phe–Pro–*N*,*O*-diMe-Tyr–Ala] (Figure 2, Table 2).

The ^{1}H NMR spectra of two additional analogues, dudawalamide C (**3**) and dudawalamide D (**4**), were well dispersed and contained several resonances indicative of two *N*-methyl groups, alkyl methylene and two singlet methyl signals indicative of a Dhoya residue, and aromatic protons suggesting a phenylalanine residue, similar to the structure of dudawalamide A (**1**). Two shielded methyl doublets were suggestive of valine residues in dudawalamides C (**3**) and D (**4**). A combination of ^{1}H and ^{13}C NMR as well as COSY and HSQC spectra were used to deduce partial structures for these two additional dudawalamides; both possessed Dhoya, valine, proline, *N*-methylphenylalanine, and glycine residues. The difference between them was the presence of a hydroxyisovaleric acid (Hiva) in dudawalamide C versus a 2-hydroxy-3-methylpentanoic acid (Hmpa) unit in dudawalamide D (**4**). HMBC correlations delineated the sequence of residues in these analogues, establishing the sequence for dudawalamide C (**3**) as cyclo-[Dhoya–Val–*N*-Me-Val–Hiva–Pro–*N*-Me-Phe–Gly] and for dudawalamide D (**4**) as cyclo-[Dhoya–Val–*N*-Me-Val–Hmpa–Pro–*N*-Me-Phe–Gly] (Table 3 and Supporting Information, Figure 2).

To determine the absolute configurations of the residues for dudawalamides B–D, a combination of chiral-phase GCMS, chiral-phase HPLC, and modified Marfey's analysis was used (Figure 4).[8] The configuration of the Dhoya residue was determined for each compound by hydrogenation (H_2/Pd) followed by acid hydrolysis and then reacting the hydrolysate with CH_2N_2 to give the methyl ester of the 2,2-dimethyl-3-hydroxyoctanoic acid (Dhoaa) unit. The retention time of this dudawalamide-derived fragment was compared by chiral-phase GCMS to previously prepared synthetic standards.[25] Interestingly, while the Dhoya moiety configuration was determined to be *R* in dudawalamide A (**1**) by X-ray diffraction analysis and confirmed via this chiral-phase GCMS investigation, the opposite configuration (*S*) was observed for all of the other analogues, dudawalamides B–D (**2**–**4**). Furthermore, the configurations of all of the amino acids in dudawalamides B–D were determined to be L by hydrolysis and either direct analysis by chiral-phase HPLC or modified Marfey's analysis

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Table 2. NMR Data (in $CDCl_3$) for Dudawalamide B (2) (^1H 500 MHz, ^{13}C 125 MHz)

residue	position	δ_C, type	δ_H (J in Hz)	HMBC	COSY
Dhoya	1	176.6, C			
	2	46.1, C			
	3	77.5, CH	5.23, dd (11.2, 2.3)	1, 2, 4, 5, 6, 39	4a, 4b
	4a	27.8, CH_2	1.84−1.73, m	6	4b, 5
	4b		1.62, m	6	4a, 5
	5	24.7, CH_2	1.45−1.53, m	3, 6, 7	4a, 4b, 6
	6	18.3, CH_2	2.24, td (6.8, 2.6)	4, 5, 7, 8	5
	7	83.7, C			
	8	69.3, CH	1.97, t (2.6)	6	
	9	17.6, CH_3	1.30, s	2, 10	
	10	23.5, CH_3	1.22, s	9	
Val	11	172.3, C			
	12	55.7, CH	4.31, dd (9.5, 7.2)	1, 11, 13, 15	13, 16
	13	31, CH	1.95−1.85, m	14, 15	12, 14, 15
	14	19.5, CH_3	1.01, d (6.6)	12, 13, 15	13
	15	18.8, CH_3	0.94, d (6.6)	12, 13, 14	13
	16	NH	5.73, d (7.2)	1, 12, 13	12
N-Me-Phe	17	169.1, C			
	18	54.3, CH	5.11, dd (12.2, 3.4)	11, 17, 19, 20, 24	17a, 17b
	19a	38, CH_2	3.00, dd (11.7, 3.4)	18, 20, 21	18, 19b
	19b		3.20, m	18, 20, 21	18, 19a
	20	137.8, C			
	21	130.1, CH	7.50−7.41, m	19, 20	22
	22	128.7, CH	7.26, m	20, 21, 23	21, 23
	23	127.1, CH	7.24, m	21, 22	22
	24	32.4, CH_3	3.62, s	11, 18	
Pro	25	171.7, C			
	26	56.5, CH	3.19, m	25, 27, 28	27a, 27b
	27a	30.2, CH_2	0.13, ddt (12.9, 6.5)		26, 27b, 28a
	27b		0.63, tt (10.2, 7.8)	26	26, 27a, 28b
	28a	22.3, CH_2	1.27, m	27	28b, 29a, 29b
	28b		1.38, m		28a, 29a, 29b
	29a	46.4, CH_2	3.25, m	26, 27, 28	28a, 28b, 29b
	29b		3.41, ddd (12.0, 8.3, 3.8)	26, 27, 28	28a, 28b, 29a
N,O-diMe-Tyr	30	169, C			
	31	63.4, CH	3.95, dd (8.9, 4.5)	25, 30, 32, 37	32a, 32b
	32a	34.4, CH_2	3.60, m	30, 31, 33	31, 32b
	32b		2.69, dd (14.5, 8.9)	30, 31, 33	31, 32a
	33	130.2, C			
	34	130.7, CH	7.00, d (8.2)	36	35
	35	114.4, CH	6.80, d (8.3)	36	34
	36	158.9, C			
	37	31.1, CH_3	2.80, s	25, 31	
	38	55.6, CH_3	3.76, s	36	
Ala	39	169.9, C			
	40	48.7, CH	5.01, dd (8.7, 6.6)	30, 41	42, 41
	41	18.4, CH_3	1.57, d (6.9)	39, 40	40
	42	NH	9.05, d (9.0)	30	40

along with authentic standards. By chiral-phase HPLC, the configuration of the Hiva residue in dudawalamide C (**3**) was established to be L, whereas the Hmpa residue in dudawalamide D (**4**) was determined to be D-*allo*. Unfortunately, due to limited quantity of isolated dudawalamide C (**3**), the configurations of only three residues were determined (*S*-Dhoya, L-Hiva, and L-Val).

The dudawalamides were evaluated for their cytotoxic properties using the H-460 human lung cancer cell line and for antiparasitic properties using malaria, leishmaniasis, and Chagas disease assays.[26−30] Intriguingly, while the dudawalamides are structurally quite similar to one other, they show quite different biological activities. In general, they exhibit broad antiparasitic activity but little to no cytotoxicity against H-460 cells relative to the control at the maximum concentration of 30 μM. Dudawalamides A (**1**) and D (**4**) showed the most potent activities against *P. falciparum* with IC_{50}'s of 3.6 and 3.5 μM, respectively. However, these two were distinguished in their activity to the other two parasites, with **1** possessing weaker activity against both *Trypanasoma cruzi* and *Leishmania donovani*, whereas **4** was relatively potent against *L. donovani* (2.6 μM) (Table 4). Dudawalamides B (**2**) and C

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Table 3. NMR Data (in $CDCl_3$) for Dudawalamides C (3) and D (4) (^1H 500 MHz, ^{13}C 125 MHz except Where Noted)

dudawalamide C (3)				dudawalamide D (4)			
residue	position	δ_C, type	δ_H, mult (J in Hz)	residue	position	δ_C, type	δ_H, mult (J in Hz)[a]
Dhoya	1	175, C		Dhoya	1	175, C	
	2	46.8, C			2	46.8, C	
	3	77.6, CH	5.00, dd (10.0, 2.4)		3	77.8, CH	4.97, m
	4a	28.5, CH_2	1.59, m		4a	28.6, CH_2	1.58, m
	4b		1.80, m		4b		1.80, m
	5	25.1, CH_2	1.52, m		5	25.1, CH_2	1.52, m
	6	18.4, CH_2	2.22, m		6	18.4, CH_2	2.23, m
	7	84.1, C			7	84, C	
	8	69.2, CH	1.96, t (2.5)		8	69, CH	1.97, s
	9	18.7, CH_3	1.38, s		9	18.7, CH_3	1.37, s
	10	24.2, CH_3	1.22, s		10	24.1, CH_3	1.21, s
Val	11	172, C		Val	11	172, C	
	12	53.2, CH	5.18, dd (8.5, 2.6)		12	53.3, CH	5.17, d (8.6)
	13	32, CH	2.08, m		13	32, CH	2.08, m
	14	15.8, CH_3	0.77, d (6.9)		14	15.8, CH_3	0.78, d (6.5)
	15	20.2, CH_3	0.95, d (6.8)		15	20.3, CH_3	0.95, m
	16	NH	6.62, d (8.6)		16	NH	6.65, d (8.6)
N-Me-Val	17	168.8, C		*N*-Me-Val	17	168.7, C	
	18	63.9, CH	3.87, d (11.0)		18	64, CH	3.87, d (11.0)
	19	26.8, CH	2.41, m		19	26.9, CH	2.41, dq (13.6, 6.7)
	20	17.9. CH_3	0.89, d (6.8)		20	17.9, CH_3	0.90, d (6.8)
	21	20, CH_3	0.97, d (6.8)		21	19.9, CH_3	0.95, m
	22	28.8, CH_3	2.65, s		22	28.7, CH_3	2.66, s
Hiva	23	167.5, C		Hmpa	23	167.7, C	
	24	77.2, CH	4.78, d (5.2)		24	75, CH	4.97, m
	25	29.7, CH	2.03, m		25	36, CH	1.71, m
	26	17.3, CH_3	0.99, d (6.9)		26	14.1, CH_3	0.95, m
	27	19.2, CH_3	1.00, d (6.9)		27	26.5, CH_2	1.40, m
Pro	28	172.1, C			28	12, CH_3	0.92, m
	29	55.4, CH	4.31, dd (8.1, 5.2)	Pro	29	171.9, C	
	30a	28.1, CH_2	0.88, m		30	55.6, CH	4.34, dd (8.1, 5.2)
	30b		0.94, m		31a	28, CH_2	0.88, m
	31a	25.5, CH_2	2.03, m		31b		0.96, m
	31b		1.65, m		32a	25.5, CH_2	1.61, m
	32a	47.5, CH_2	3.37, m		32b		2.03, dt (13.4, 6.9)
	32b		3.80, dt (9.7, 7.1)		33a	47.4, CH_2	3.36, q (8.1)
N-Me-Phe	33	169.7, C			33b		3.76, q (7.5)
	34	63.8, CH	4.77, m	*N*-Me-Phe	34	169.8, C	
	35a	34, CH_2	2.95, dd (14.5, 11.5)		35	63.9, CH	4.74, dd (11.6, 2.9)
	35b		3.38, m		36a	33.9, CH_2	2.94, dd (14.6, 11.5)
	36	138.6, C			36b		3.42, dd (14.7, 2.8)
	37	127, CH	7.22, m		37	138.7, C	
	38	129, CH	7.28, d (1.5)		38	127, CH	7.22, t (7.4)
	39	129.4, CH	7.12, m		39	129, CH	7.29, m
	40	29.3, CH_3	2.79, s		40	129.4, CH	7.12, d (7.6)
Gly	41	169.7, C			41	29.4, CH_3	2.80, s
	42a	40.2, CH_2	4.02, dd (18.3, 1.9)	Gly	42	169.7, C	
	42b		4.67, ddd (18.2, 9.8, 1.9)		43a	40.2, CH_2	4.03, d (17.9)
	43	NH	8.93, d (9.6)		43b		4.67, d (18.0, 9.7)
					44	NH	8.91, d (9.7)

[a]Data collected at 800 MHz.

(3) were significantly less potent than dudawalamides A and D against *P. falciparum*, and 2 also showed decreased potency to the other two parasites.

Intriguing SAR features emerge from comparison of the Dhoya class of depsipeptides in several measures of bioactivity. The constellation of changes between residues 1−3 of dudawalamide A (1) and dudawalamide D (4), L-Lac to L-Val, L-Ala to L-*N*-Me-Val, and L-*N*-Me-Ile to D-*allo*-Hmpa, results in at least a 5-fold difference for *Leishmania* inhibition (Table 4). Because residues 1 and 2 in dudawalamide D (4) are rather similar in dudawalamide B (2), and the latter has weak activity against *Leishmania*, it is possible that stereochemical inversion at residue 3 is responsible for this change in activity (Tables 4 and 5). In the case of wewakpeptins A and C,

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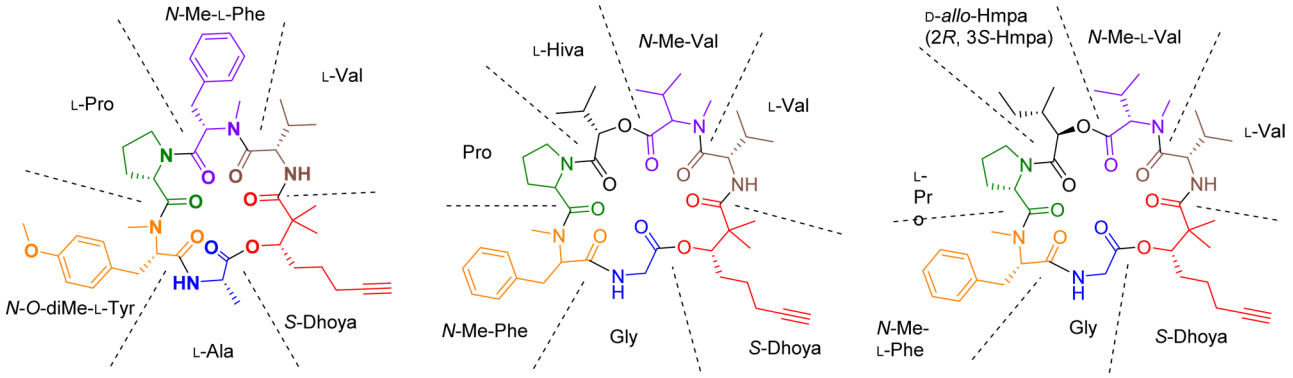

Dudawalamide B (2) **Dudawalamide C (3)** **Dudawalamide D (4)**

Figure 4. Stereostructures of dudawalamides B–D (**2**–**4**).

Table 4. Biological Activities of Dudawalamides A–D (1–4)

compound	*Plasmodium falciparum* (IC_{50}, μM)	*Trypanosoma cruzi* (% GI at 10 μg/mL)[a]	*Leishmania donovani* (IC_{50}, μM)
dudawalamide A (**1**)	3.6	12	>10
dudawalamide B (**2**)	8.0	7	>10
dudawalamide C (**3**)	10	not tested	not tested
dudawalamide D (**4**)	3.5	60	2.6

[a]Percentage growth inhibition.

modification of residue 6 from L-Hiva to D-Pla results in a 20-fold decrease in neuro-2a cytotoxic activity (Table 5).[12] Although the configuration of dudawalamide C is only partially defined, it is noteworthy that dudawalamides C (**3**) and D (**4**) are only different by one methyl group at residue 3, L-Hiva to D-*allo*-Hmpa, but this single methyl group and stereochemical inversion result in a more than 3-fold difference in their activity for *P. falciparum*. Thus, it appears that minor changes in configuration and sequence of residues strongly impact the bioactivity of these Dhoya-containing natural products. Nevertheless, it will be important to more fully explore these SAR relationships in the future using a synthetic medicinal chemistry approach.

The dudawalamides A–D (**1**–**4**) join several other Dhoya-containing cyclic lipopeptides such as the wewakpeptins (isolated from *Lyngbya semiplena*),[12,13] kulolide (isolated from a mollusk),[13,31] pitipeptolides A and B (isolated from *Moorea* sp.),[31−33] antanapeptin A (isolated from *Moorea* sp.),[19] mantillamide A,[10] cocosamides A and B (isolated from *Moorea* sp.),[34] and the recent viequeamides (isolated from *Rivularia* sp.).[8] These natural products are members of the "kulolide superfamily", which is characterized by a β-hydroxyoctanoic acid derivative in addition to a sequence of amino acids or hydroxy acid residues in an overall cyclic structure (Table 5). The dudawalamides A–D (**1**–**4**), like other members of the "kulolide superfamily", are believed to derive from a mixed NRPS–PKS biosynthetic pathway. Specifically, the biosynthesis of the distinctive Dhoya moiety likely arises via PKS acetate extensions (possibly to a hexanoic or hexenoic acid starter unit) and terminal desaturation by oxidative dehydrogenation, and *S*-adenosylmethionine is likely responsible for methylation at C-2 of this residue.[35,36]

In summary, we report the isolation of four new Dhoya-containing natural products, dudawalamides A–D (**1**–**4**), from the marine cyanobacterium *M. producens*. This newly identified group of natural products further demonstrates that the Dhoya residue can be considered a molecular fingerprint of cyanobacterial secondary metabolism. The dudawalamides exhibit variable antiparasitic activity against malaria-, leishmaniasis-, and Chagas disease-causing organisms, with many of the IC_{50} values in the low micromolar range. This set of natural products further expands on SAR features for Dhoya-containing cyanobacterial compounds, generally known as the "kulolide superfamily".

EXPERIMENTAL SECTION

General Experimental Procedures. A Jasco P-2000 polarimeter was used to measure optical rotations. IR spectra were measured on a

Table 5. Selected Dhoya-Containing Secondary Metabolites from the "Kulolide Superfamily"[8,35]

	residue								
compound	1	2	3	4	5	6	7	8	9
dudawalamide A	*R*-Dhoya	L-Lac	L-Ala	*N*-Me-L-Ile	L-Pro	*N*-Me- L-Phe	Gly		
dudawalamide B	*S*-Dhoya	L-Val	*N*-Me-L-Phe	L-Pro	*N*-Me-O-Me-L-Tyr	L-Ala			
dudawalamide C	*S*-Dhoya	L-Val	*N*-Me-Val	Hiva	Pro	*N*-Me-Phe	Gly		
dudawalamide D	*S*-Dhoya	L-Val	*N*-Me-L-Val	D-*allo*-Hmpa	L-Pro	*N*-Me- L-Phe	Gly		
viequeamide A	*S*-Dhoya	L-Val	*N*-Me-L-Val	D-*allo*-Hmpa	L-Pro	*N*-Me- L-Val	L-Thr		
viequeamide B	*S*-Dhoya	L-Val	*N*-Me-L-Val	L-Pla[a]	L-Pro	*N*-Me- L-Ala	L-Val		
kulolide	*R*-Dhoya	L-Val	*N*-Me-D-Val	L-Pla[a]	L-Pro	L-Val	L-Ala		
pitipeptolide A	*S*-Dhoya	L-Val	*N*-Me-L-Phe	L-Hmpa	L-Pro	L-Ile	Gly		
wewakpeptin A	*R*-Dhoya	L-Ile	*N*-Me-L-Ala	*N*-Me- L-Val	*N*-Me- L-Val	L-Hiva	L-Pro	L-Pro	L-Val
wewakpeptin C	*R*-Dhoya	L-Ile	*N*-Me-L-Ala	*N*-Me- L-Ala	*N*-Me- L-Val	D-Pla	L-Pro	L-Pro	L-Val

[a]Pla = Phenyl Lactic Acid.

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Thermo Electron Corporation Nicolet IR 100 FT-IR. NMR spectra were recorded on a Varian 500 MHz spectrometer (500 and 125 MHz for the ^{1}H and ^{13}C nuclei, respectively) and Bruker 800 MHz spectrometer (800 and 200 MHz for the ^{1}H and ^{13}C nuclei, respectively) using $CDCl_3$ as solvent from Cambridge Isotope Laboratories, Inc. (99.8% D). Spectra were referenced to residual $CDCl_3$ solvent as internal standard (δ_H 7.26 and δ_C 77.1). LCMS data for stereochemical analysis of the hydrolysates of **1**–**4** were obtained with a Thermo Finnigan Surveyor Autosampler-Plus/LC-Pump-Plus/PDA-Plus system and a Thermo Finnigan LCQ Advantage Max mass spectrometer (monitoring 200–600 nm and *m/z* 250–2000 in positive ion mode). LC-HRMS data for analysis of compounds **1**–**4** were obtained on an Agilent 6239 HR-ESI-TOFMS equipped with a Phenomenex Luna 5 μm C18 100 Å column (4.6 × 250 mm). GCMS was conducted with a Thermo Electron Corp. DSQ/TRACE-GC-Ultra GCMS system with a Cyclosil-B column (Agilent Technologies J&W Scientific, 30 m × 0.25 mm). Semipreparative HPLC purification was carried out using a Waters 515 with a Waters 996 photodiode array detector using Empower Pro software. All solvents were HPLC grade.

Organism Collection and Identification. The *Moorea producens* sample (PNG-04/22/06-2) yielding compounds **1**–**4** was collected in April 2006 at a depth of 3–9 m by snorkel near Dudawali Bay in Papua New Guinea (10°17.274′ S and 151°00.390′ E). The specimen, measuring 8 L in total biomass, was stored in 70% EtOH at −20 °C until extraction. The 16S rRNA gene sequences of algal biomass for this sample are available in the DDBJ/EMBL/GenBank databases under acc. no. EU492878.1.

Extraction and Isolation. Approximately 1 L of the preserved *M. producens* biomass was filtered using cheesecloth and extracted six times with 500 mL of CH_2Cl_2–MeOH (2:1) with heating at gentle reflux. The combined organic layers were dried under vacuum to afford 1.99 g of dark green solids. A small portion was preserved for future analysis, and the rest was purified on silica gel (type H, 10–40 μm, Sigma-Aldrich) using vacuum liquid chromatography with a hexanes–EtOAC–MeOH gradient to generate nine subfractions, A–I (100% hexanes, 10% EtOAc–90% hexanes, 20% EtOAc–80% hexanes, then 20% increments to 100% EtOAc, 25% MeOH–75% EtOAc, and 100% MeOH, named A–I, respectively). The most cytotoxic fractions in the H-460 lung carcinoma cell line were F (90% EtOAc–hexanes; 17.6 mg) and G (100% EtOAc, 29.2 mg). Fraction F exhibited 33% and 99% inhibition at 1 and 10 μg/mL, respectively, while fraction G exhibited 7% and 89% inhibition at 1 and 10 μg/mL, respectively, against the H-460 cell lines. These fractions (combined weight 46.8 mg) were filtered using a Waters RP C18 SPE cartridge with 100% MeCN followed by 100% MeOH. The MeCN filtrate from both fractions was further purified by RP-HPLC using a Phenomenex Kinetex C-18, 100 Å, 150 × 10 mm column using linear gradient elution with MeCN–H_2O (50:50) for 5 min to MeCN–H_2O (90:10) in 30 min at 3 mL/min. The purification of both fractions yielded a combined yield of dudawalamide A (**1**, 1.8 mg), dudawalamide B (**2**, 1.1 mg), dudawalamide C (**3**, 0.3 mg), dudawalamide D (**4**, 0.8 mg), and the known compounds apratoxin A (1.0 mg) and majusculamides A and B (2.0 mg combined).

*Dudawalamide A (**1**):* white, crystalline solid; mp 96 °C, $[\alpha]^{25}_D$ −62.5 (*c* 0.17, $CHCl_3$); IR (neat) ν_{max} 3385, 3270, 3050, 2987, 2945, 2876, 1733, 1512, 1488, 1376, 1285, 1195, 1094 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data, Table 1; (+)-HRESIMS *m/z* 752.4220 $[M + H]^+$ (calcd for $C_{40}H_{57}N_5O_9$, 752.4235).

*Dudawalamide B (**2**):* colorless oil; $[\alpha]^{25}_D$ −79.4 (*c* 0.17, $CHCl_3$); IR (neat) ν_{max} 3286, 2920, 2852, 1742, 1660, 1633, 1512, 1458, 1305, 1249, 1142 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data, Table 2; (+)-HRESIMS *m/z* 786.4429 $[M + H]^+$ (calcd for $C_{44}H_{59}N_5O_8$, 786.4442).

*Dudawalamide C (**3**):* colorless oil; $[\alpha]^{25}_D$ −10.8 (*c* 0.11, $CHCl_3$); IR (neat) ν_{max} 3287, 2963, 2923, 2853, 1744, 1645, 1499, 1407, 1308, 1258, 1194, 1124, 1090, 1021, 801 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data, Table 3 and Supporting Information; (+)-HRESIMS *m/z* 794.4694 $[M + H]^+$ (calcd for $C_{43}H_{63}N_5O_9$, 794.4704).

*Dudawalamide D (**4**):* colorless oil; $[\alpha]^{25}_D$ −50.1 (*c* 0.16, $CHCl_3$); IR (neat) ν_{max} 3281, 2964, 2927, 2361, 1747, 1660, 1500, 1447, 1406, 1307, 1193, 1123 cm^{-1}; ^{1}H NMR ($CDCl_3$, 800 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data, Table 3 and Supporting Information; (+)-HRESIMS *m/z* 808.4849 $[M + H]^+$ (calcd for $C_{44}H_{65}N_5O_9$, 808.4861).

X-ray Crystallographic Data for Compound 1. The single-crystal X-ray diffraction studies were carried out on a Bruker Kappa APEX-II CCD diffractometer equipped with Cu Ka radiation (λ = 1.5478). A 0.33 × 0.30 × 0.08 mm colorless plate was mounted on a Cryoloop with Paratone oil. Data were collected in a nitrogen gas stream at 100(2) K using ϕ and ϖ scans. The crystal-to-detector distance was 50 mm, and the exposure time was 1 s per frame using a scan width of 1.0°. Data collection was 99.7% complete to 68.00° in θ. A total of 42 468 reflections were collected covering the indices $-17 \le h \le 17$, $-12 \le k \le 11$, and $-18 \le l \le 18$. A total of 8830 reflections were found to be symmetry independent, with an R_{int} of 0.0395. Indexing and unit cell refinement indicated a primitive, monoclinic lattice. The space group was found to be $P2_1$. The data were integrated using the Bruker SAINT software program and scaled using the SADABS software program. Solution by direct methods (SHELXT) produced a complete phasing model consistent with the proposed structure.

All non-hydrogen atoms were refined anisotropically by full-matrix least-squares (SHELXL-2014). All hydrogen atoms were placed using a riding model. Their positions were constrained relative to their parent atom using the appropriate HFIX command in SHELXL-2014. The absolute configurations of the stereocenters in dudawalamide A were established by anomalous dispersion using the Parson's method with a Flack parameter of 0.023(47). Crystallographic data are summarized in Table S1 in the Supporting Information; CCDC 1549804.

Hydrogenations. A 0.2–0.4 mg aliquot of compounds **2**–**4** was dissolved in 300 μL of EtOH, and a catalytic amount of 10% Pd/C was added under argon. The argon was replaced with hydrogen gas followed by stirring for 3–4 h at room temperature. The crude reaction product was filtered with a Nalgene syringe filter (2 μm, 13 mm) and dried under reduced pressure. The crude product was used without purification for acid hydrolysis reactions.

Acid Hydrolysis and Chiral HPLC. Hydrogenated compounds **2**–**4** were dissolved in 500 μL of 6 N HCl in sealed tubes, flushed with N_2, and heated at 105 °C for 16 h. The reaction mixture was dried using a stream of N_2 and resuspended in MeOH, and an aliquot was taken for further analysis. The configuration of the proline residue in **2**–**4** was determined by chiral-phase HPLC (Chirex 3126, 4.6 × 50 mm, D-penicillamine) using isocratic conditions [2 mM $CuSO_4$–MeCN (95:5), 0.8 mL/min] and matched the retention time (t_R, min) of L-Pro (8.1 min; D-Pro, 11.8 min). The Hiva residue in **3** and Hmpa residue in **4** were analyzed using the same chiral-phase HPLC system with slightly varying conditions. The Hiva residue in **3** was analyzed using 2 mM $CuSO_4$–MeCN (85:15), 0.8 mL/min, while the Hmpa residue in **4** was analyzed using 2 mM $CuSO_4$–MeCN (87.5:12.5), 0.8 mL/min. Dudawalamide C (**3**) was thus shown to possess L-Hiva (t_R for L-Hiva, 16.6 min; D-Hiva, 24.2 min). The retention times for the authentic Hmpa standards were as follows: L-Hmpa (20.4 min), D-Hmpa (31.0 min), L-*allo*-Hmpa (18.5 min), D-*allo*-Hmpa (26.6 min). For dudawalamide D (**4**), the results were consistent with D-*allo*-Hmpa (26.7 min).

Modified Marfey's Analysis. A second portion of the acid hydrolysis product of compounds **2**–**4** was reacted with D-FDVA (0.1% in acetone and 0.1 M $NaHCO_3$) and heated at 90 °C in a sealed tube for 5 min. The reaction was quenched with 50 μL of 1 N $HCl_{(aq)}$ and diluted with 100 μL of MeCN, and 10 μL of this mixture was analyzed by HPLC-ESIMS in comparison to authentic amino acid standards that had been derivatized with D-FDVA. The *N*-Me-Phe, Pro, Val, *N*-Me-Val, and *N*,*O*-diMe-Tyr residues in **2**–**4** were analyzed by RP-HPLC [HP LiChrospher 100 RP-18 (5 μm, 4 × 125 mm); 0.8 mL/min, UV detection at 340 nm] using a linear gradient (30/70 to

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70/30 MeCN−H_2O in 0.1% HCOOH for 60 min). The retention times for the authentic amino acid standards were as follows: *N*-Me-L-Phe (18.5 min), *N*-Me-D-Phe (20.1 min), L-Pro (8.1 min), D-Pro (10.7 min), L-Val (34.4 min), D-Val (51.7 min), *N*-Me-L-Val (15.7 min), *N*-Me-D-Val (21.0 min), L-Pro (8.1 min), D-Pro (10.7 min), *N*,*O*-diMe-L-Tyr (17.2 min), *N*,*O*-diMe-D-Tyr (18.9 min), L-Ala (13.9 min), and D-Ala (13.0 min). The hydrolysate peaks with the expected masses for dudawalamide B (**2**) were found at 34.4, 18.5, 8.1, 17.2, and 13.9 min, which correspond to L-Val, N-Me-L-Phe, L-Pro, *N*,O-diMe-L-Tyr, and L-Ala. For dudawalamide D (**4**), the retention time matched those for L-Val (34.4 min), *N*-Me-L-Val (15.7 min), L-Pro (8.1 min), and *N*-Me-L-Phe (18.5 min).

Dhoya Configuration Analysis. The hydrolysates of compounds **1**−**4** were used for the Dhoya configuration analysis. A small portion of each was dissolved in MeOH, and CH_2N_2 (in ether) was added. The reaction mixture was stirred for 10 min, dried under N_2, and extracted several times with CH_2Cl_2−H_2O and a few drops of 1 N HCl. The combined organic layer was dried under N_2, redissolved in CH_2Cl_2, and analyzed by chiral-phase GCMS (J &W Scientific Cyclosil C, 0.25 μm, 30 mm × 0.250 mm) using a stepwise temperature gradient [40 to 100 °C (20 °C/min), hold for 5 min, then 100 to 110 °C (5 °C/min)]. The Dhoaa synthetic standards (3.0 mg) were subjected to the same reaction conditions as above to give the methyl-ester derivatives.[25] The Dhoaa residue deriving from compound **1** matched the t_R of *R*-Dhoaa while those from compounds **2**−**4** matched the t_R of *S*-Dhoaa (t_R for *S*-Dhoaa, 51.5 min; *R*-Dhoaa, 52.8 min, Supporting Information).

Cytotoxicity Assay. H-460 human lung cancer cells were added to 96-well plates at 3.33 × 10^4 cells/mL in Roswell Park Memorial Institute (RPMI) 1640 media with fetal bovine serum (FBS) and 1% penicillin−streptomycin. The cells were incubated overnight (37 °C, 5% CO_2) in a volume of 180 μL per well to permit recovery before treatment with test compounds. The test substances were dissolved in DMSO to a stock concentration of 1 mg/mL. Working solutions were prepared through serial dilution in RPMI 1640 media without FBS, with 20 μL added to each well to give final compound concentrations of 10, 3, 1, 0.3, 0.1, 0.03, 0.01, 0.003, and 0.001 μg/mL. Negative control wells were prepared by adding an equal volume of RPMI 1640 media lacking FBS for each plate. Plates were incubated for approximately 48 h prior to MTT staining. Plates were read with a ThermoElectron Multiskan Ascent plate reader at 570 and 630 nm to define cell viability.[26,27]

Malaria, Leishmaniasis, and Chagas Disease Antiparasite Assays. Dudawalamides A−D (**1**−**4**) were screened through established assays for inhibitory activity to the parasites causing malaria, leishmaniasis, and Chagas disease (Table 4). The malaria assay was conducted on the chloroquine-resistant strain W2 of *Plasmodium falciparum*,[37,38] using chloroquine as the positive control. The causal agent of visceral leishmaniasis, *L. donovani* (WR2801), was tested in the axenic amastigote stage,[28] using amphotericin B as positive control. A transgenic β-galactosidase-expressing strain of *Trypanosoma cruzi* (Tulahuen, clone C4) (ATCC PRA33) was used in the Chagas disease assay, using benznidazole as the positive control.[39] Activity was expressed as percent growth inhibition relative to controls. All initial screenings were done at 10 μg/mL.[28−30] The IC_{50} determination was calculated with the LSW Excel add-on using the following concentrations: 10, 2, 0.4, and 0.08 μg/mL.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00034.

^{1}H NMR, ^{13}C NMR, COSY, HSQC, and HMBC spectra for compounds **1**−**4**, low- and high-resolution LC-MS/MS spectra of compounds **1**−**4**, chiral-phase GCMS analysis, and X-ray crystallographic data for compound **1** (PDF)

(CIF)

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Notes

The authors declare no competing financial interest.

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