Symplostatin 3, a New Dolastatin 10 Analogue from the Marine Cyanobacterium *Symploca* sp. VP452

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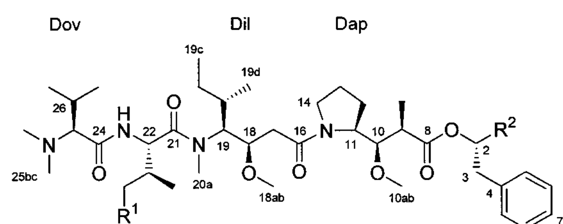
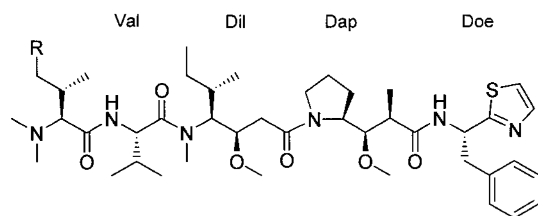
Symplostatin 3 (**1**), a new analogue of dolastatin 10 (**2**), has been isolated from a tumor selective extract of a Hawaiian variety of the marine cyanobacterium *Symploca* sp. VP452. Compound **1** differs from **2** only in the C-terminal unit; the dolaphenine unit is substituted by a 3-phenyllactic acid residue. Symplostatin 3 (**1**) possesses IC_{50} values for in vitro cytotoxicity toward human tumor cell lines ranging from 3.9 to 10.3 nM. It disrupts microtubules, but at a higher concentration than **2**, correlating with the weaker in vitro cytotoxicity.

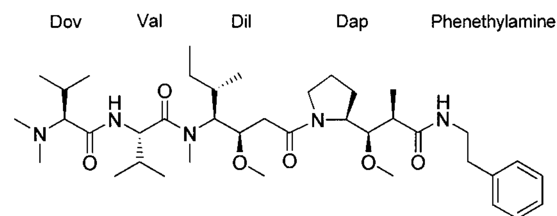
Cyanobacteria are ubiquitous prokaryotic organisms that have been shown to be a rich source of bioactive compounds, mainly peptides or hybrids of peptide-polyketide biosynthesis.[1] These metabolites have been isolated directly from cyanobacteria or from macroorganisms such as sponges or tunicates that harbor cyanobacterial symbionts.[2] Cyanobacterial metabolites have also been found in opisthobranch mollusks that sequester these compounds through diet,[3] but their movement through the food chain has not always been immediately recognized by researchers. The most notable example of initially misidentifying the true source of such metabolites are the dolastatins,[4] cytotoxins found in exceedingly low yields in the sea hare *Dolabella auricularia*, a generalist herbivore known to feed on macroalgae and cyanobacteria.[3] Our research has aimed at finding antitumor compounds from marine cyanobacteria, and we and others have been encountering dolastatins and analogues of these compounds in our collections, providing evidence for the cyanobacterial origin of these cytotoxins.[5] Since dolastatin 10 (**2**)[6] is in human clinical trials for the treatment of cancer,[7] our finding of an analogue, symplostatin 1 (**3**),[8] and more recently dolastatin 10 (**2**) itself[8b] in strains of *Symploca* spp. has been most significant. Herein we report the discovery of another dolastatin 10 analogue, termed symplostatin 3 (**1**), from Hawaiian *Symploca* sp. VP452.[9] Structurally it most closely resembles the dolastatin 10 analogue isodolastatin H (**4**) found in the Japanese sea hare *D. auricularia*.[10]



1 R^1 = H (Val) R^2 = CO_2H (3-Phenyllactic acid)
4 R^1 = CH_3 (Ile) R^2 = CH_2OH (3-Phenylpropane-1,2-diol)



2 R = H (Me_2Val, Dov)
3 R = CH_3 (Me_2Ile)



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Results and Discussion

Symploca sp. VP452, which taxonomically appeared to be *S. hydnoides*, was collected at Kaneohe Bay, Oahu, and was consecutively extracted with CH_2Cl_2 and MeOH to yield VP452L and VP452A, respectively. Both extracts showed tumor selectivity in the Corbett assay (Table 1). After solvent partition (see Experimental Section), the *n*-BuOH layers of the two extracts exhibited similar cytotoxicity, displayed almost identical ^{1}H NMR spectra, and thus were combined. Cytotoxicity-guided normal-phase chromatography of the pooled fractions followed by several reversed-phase chromatographic steps afforded symplostatin 3 (**1**) as a colorless amorphous solid (0.014% dry wt).

The NMR spectra coupled with a $[M + H]^+$ peak at *m*/*z* 747.4898 from HRFABMS analysis indicated a molecular formula for **1** of $C_{40}H_{66}N_4O_9$. The ^{1}H NMR spectrum revealed a 3:1 conformational ratio in various organic solvents (CD_2Cl_2, $CDCl_3$, CD_3CN), displayed broad signals as known for dolastatin 10 (**2**) and symplostatin 1 (**3**), and overall suggested a close relationship to these compounds. By using deuterated MeOH as the NMR solvent, most of

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Table 1. Evaluation of VP452 Extracts in the Differential Cytotoxicity (Corbett) Assay[a]

extract	dilution	leukemia L1210	mouse solid tumor C38	human solid tumor H15	normal cells fibroblast
VP452L	1/4	800	>950	750	0−850[b]
VP452A		400	200−360	150−260	0−420[b]

[a] Zone units of inhibition (200 units = 6.5 mm). Extracts are tumor selective if zone differentials between tumors and normal cells are >250 units and solid tumor selective if zone differentials between solid tumors and leukemia/normal cells are >250 units. [b]The zero value is most important. The fibroblast cells are alive at zero; they are just not replicating. This is usually a good sign, and most good antitumor agents have this property. They inhibit the normal cell replication without killing the cells that thus can recover after the drug is gone.

Table 2. NMR Spectral Data for Both Conformers of Symplostatin 3 (**1**) in MeOH-d_3 (ratio 3:2) at 500 MHz (^{1}H) and 125 MHz (^{13}C)

unit	C/H no.[a]	major conformer δ_H (J in Hz)	major conformer δ_C[b]	minor conformer δ_H (J in Hz)	minor conformer δ_C[b]	HMBC[c]
3-phenyllactic acid	1		not observed		not observed	
	OH	not observed		not observed		
	2	5.13, dd (11.4, 6)	75.8,[d] d	5.21, dd (10.9, 6)	75.5,[d] d	
	3	3.04, dd (−14.8, 11.4), 3.26, dd (−14.8, 6)	38.3, t	3.06, dd (−15, 10.9), 3.26, dd (−15, 6)	38.3, t	H-5/5′
	4		138.3, s		138.8, s	H-3, H-6/6′
	5/5′	7.26, m	130.4, d × 2	7.26, m	130.1, d × 2	H-3
	6/6′	7.27, m	129.5, d × 2	7.27, m	129.6, d × 2	
	7	7.19, m	127.8, d	7.19, m	127.9, d	H-5/5′
Dap	8		175.9, s		175.9, s	H-9, H-9a, H-10
	9	2.50, quint (7.0)	43.5, d	2.43, m	44.1, d	H-9a, H-10
	9a	1.14, d (7.0)	13.0, q	1.24, d (6.8)	14.8, q	H-9, H-10
	10	3.73, dd (7.0, 3.6)	82.8, d	3.45, dd (9.1, 1.6)	86.2, d	H-10ab
	10ab	3.14, s	60.8, q	3.32, s	61.9, q	H-10
	11	3.85, ~dt (7.5, 3.6)	60.6, d	3.65, m	61.1, d	H-10
	12	1.50, m, 1.81, m	26.0, t	1.40, m, 1.82, m	26.8, t	
	13	1.71, m, 1.90, m	25.4, t	1.69, m, 1.88, m	24.4, t	
	14	3.40, m, 3.52, m	48.8, t	3.20, m, 3.63, m	48.0, t	H-12, H-13
Dil	16		172.3, s		172.2, s	H-17
	17	2.45, m, 2.48, m	38.1, t	2.46, m, 2H	37.1, s	
	18	4.02, br	79.9, d	4.07, br	79.9, d	H-17, H-18ab
	18ab	3.266, s	58.5, q	3.272, s	58.6, q	
	19	4.75, br	58.2,[e] d	4.78, br	58.2,[e] d	H-19d, H-20a
	19a	1.78, m	33.8, d	1.81, m	33.8, d	H-19c, H-19d
	19b	0.97, m, 1.42, m	27.2, t	0.97, m, 1.42, m	27.2, t	H-19c, H-19d
	19c	0.85, t (7.4)	11.0, q	0.85, t (7.4)	11.0, q	
	19d	0.99, d (6.5)	16.3, q	0.99, d (6.5)	16.0, q	
	20a	3.13, s	33.0, q	3.19, s	32.9, q	
Val	21		174.6, s		174.5, s	H-20a, H-22
	22	4.68, t (8.4)	57.0, d	4.72, t (8.3)	57.0, d	H-22b
	22a	2.08, m	31.8, d	2.11, m	31.7, d	H-22, H-22b, H-22c
	22b	1.02, d (6.8)	19.2, q	1.048, d (7)	19.2, q	H-22a, H-22c
	22c	1.050, d (7.0)	19.2, q	1.08, d (7)	19.6, q	H-22b
	23	8.61, d (8.4)		8.59, d (8.3)		
Dov	24		167.1, s		167.0, s	H-25, H-26
	25	3.65, d (5.7)	74.2, d	3.67, d (6.2)	74.2, d	H-25bc, H-26, H-27, H-28
	25bc	2.88, s	42.6, q × 2	2.89, s	42.6, q × 2	H-25, H-25bc
	26	2.39, m	28.8, d	2.40, m	28.8, d	H-25, H-27, H-28
	27	0.96, d (6.8)	16.7, q	0.97, d (6.8)	16.7, q	H-25
	28	1.059, d (6.8)	19.9, q	1.064, d (7.0)	19.9, q	H-25, H-27

[a] Numbering system for dolastatin 10 (**2**)[6] and isodolastatin H (**4**)[10] adopted. [b]Multiplicity deduced from the HMQC spectrum. [c]Protons showing long-range correlation with indicated carbon. [d]Signal not observed in the ^{13}C NMR spectrum presumably due to broadening. Chemical shift was obtained from the HMQC spectrum. [e]Signal of very low intensity.

the signals sharpened, but the conformational ratio changed to 3:2. We chose MeOH-d_3 for the NMR analysis since we found that the better HMBC spectra obtained in this solvent (due to sharper signals) outweighed the somewhat complicated analysis caused by the unfavorable conformational ratio. ^{1}H NMR, ^{13}C NMR, and HMBC data (Table 2) were diagnostic for an *N*,*N*-dimethylamino acid residue, which contained an isopropyl unit as in dolastatin 10 (**2**) rather than the sec-butyl group found in symplostatin 1 (**3**). Therefore, the N-terminal unit was dolavaline (Me_2-Val, Dov). A COSY cross-peak between H-22 and H-23 and further HMBC analysis (Table 2) clarified that the only exchangeable proton observed (H-23) belonged to a valine residue located on the C-terminus of Dov. The dolaisoleuine (Dil) moiety was identified as the next part of the sequence. Even though no evidence for the linkage between C-18 and C-19 was found by HMBC or ^{1}H−^{1}H COSY experiments on account of the extreme broadness of the corresponding NMR signals, a ROESY cross-peak between H_3-18ab and H-19 ultimately established the connectivity of the two Dil subunits. The intact dolaproine (Dap) unit could be assembled on the basis of ^{1}H−^{1}H COSY and HMBC data, mainly because H-10 and H-11 gave rise to sharp signals. The connectivity of the N-terminus of the Dap unit was decided on the basis of strong cross-peaks in the ROESY spectrum between resonances of H_2-17 and H_2-14 of the major conformer; this allowed the expansion of the partial structure to Dov-Val-Dil-Dap and indicated trans config-

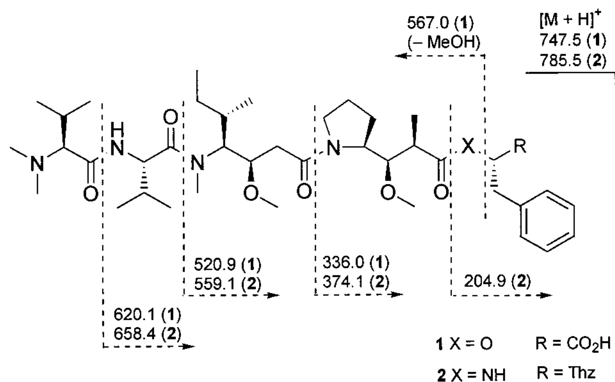


Figure 1. Characteristic fragmentations of symplostatin 3 (**1**) and dolastatin 10 (**2**) by ESI-MS/MS.

uration around the Dil–Dap amide bond. As with dolastatin 10 (**2**),[11] trans–cis isomerization around the Dil–Dap amide bond is responsible for the occurrence of two conformers in slow exchange. For the minor conformer, H_2-17 comes in NOE contact with H-11, therefore indicating a cis configuration. This is in agreement with the carbon chemical shift differences $\Delta(\delta_{C-12} - \delta_{C-13})$ for the proline-derived group of 0.6 ppm for the major conformer and of 2.4 ppm for the minor conformer (Table 2), indicative for trans and cis configuration, respectively,[11b] and similar to those values found for dolastatin 10 (**2**).[11,12] Aromatic signals were characteristic of a monosubstituted phenyl group, but no thiazole signals for the dolaphenine (Doe) unit as in **2** and **3** were present. Benzylic protons (H_2-3) showed COSY cross-peaks to a methine proton that was attached to an oxygenated sp^3 carbon resonating at δ_{C-2} 75.8/75.5 (major/minor). The signal due to the methine proton (H-2) appeared as a broad doublet in the ^{1}H NMR spectrum of **1**, sharpening to a doublet of doublets at lower concentration, and did not give any HMBC correlation. Its multiplicity suggested the lack of additional homonuclear coupling partners, so that C-1 appeared to be a nonprotonated carbon. Since all ^{1}H NMR signals were accounted for and the molecular formula required another CHO_2, a 3-phenyllactic acid residue was postulated as the C-terminal unit. This left the connection of C-2 via an ester linkage to the Dap unit as the only logical choice and was consistent with the low-field chemical shift of the H-2 signal of δ 5.13/5.21 (major/minor, Table 2), leading to the gross structure shown for **1**. The presence of a carboxylic acid functionality is consistent with the chromatographic behavior of **1**. It streaked on Si gel, in contrast to symplostatin 1 (**3**), and eluted with a more polar solvent mixture during C_{18} step-gradient chromatography compared to compound **3** using the same fractionation procedure.[8b]

Further evidence for the correct planar structure was provided by ESI-MS/MS studies on symplostatin 3 (**1**) in comparison with dolastatin 10 (**2**). The fragmentation pattern of the $[M + H]^+$ peak of **1** was as expected similar to the one for the $[M + H]^+$ peak of **2**, but differed for the C-terminus (Figure 1). It confirmed the established sequence from NMR studies and was in agreement with a C-terminal 3-phenyllactic acid residue in **1**. The first-generation fragment ions of the $[M + H]^+$ peak arose from the same fragmentation for compounds **1** and **2** (Scheme 1). Formation of one second-generation and two third-generation ions was accompanied by a loss of 148 amu only for **1** but not for **2** (Scheme 1), which can be explained by a McLafferty-type elimination of 3-phenylpropenoic acid on the C-terminus of **1**.[13]

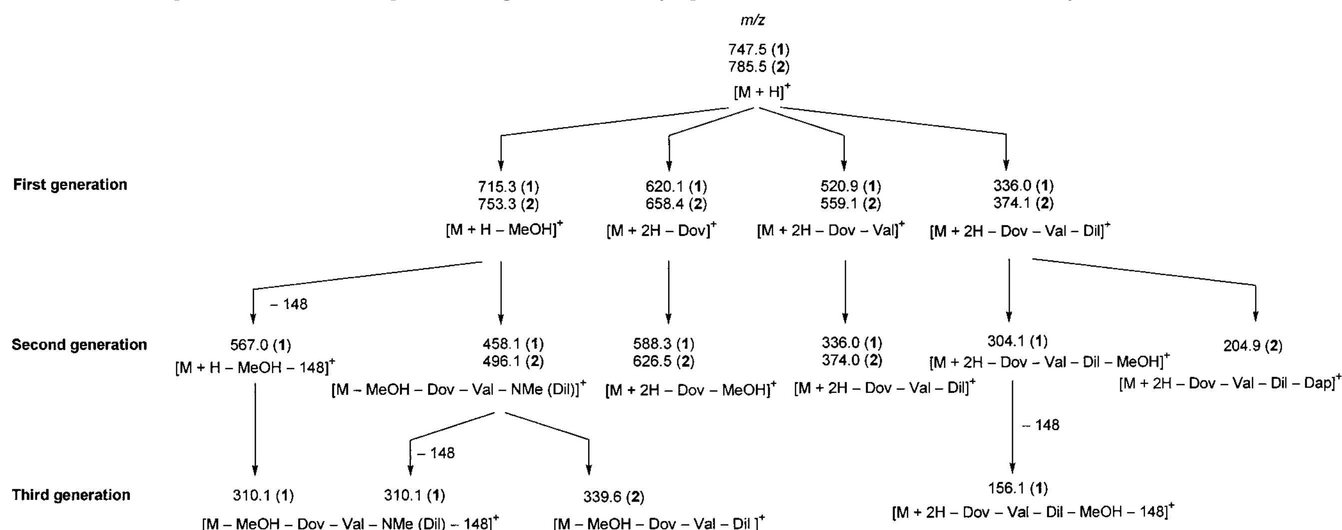
A related C-terminal unit, a 3-phenylpropane-1,2-diol, was found in the dolastatin 10 analogue isodolastatin H (**4**).[10] Compounds **1** and **4** therefore vary in the oxidation state of C-1.[14] Upon acid hydrolysis of **1**, a 3-phenyllactic acid was indeed liberated and shown to be the *S* isomer by chiral HPLC analysis and comparison of its retention time with those of authentic *R* and *S* standards. Further analysis revealed that the configurations of the Dov and Val units were both L like in dolastatin 10 (**2**). Since the ^{13}C NMR chemical shifts for the Dil and Dap units of **1** and **2** in MeOH-d_3 have been found to be similar (see Supporting Information, Table 3),[12] we propose that the same Dil and Dap units exist in **1**. Furthermore, in all of the naturally occurring congeners, i.e., dolastatin 10 (**2**), symplostatin 1 (**3**), dolastatin H, and isodolastatin H (**4**), the common units, including Dil and Dap, have the same configurations, and thus on biogenetic grounds the same Dil and Dap units are most likely present in symplostatin 3 (**1**) as well. It is also noteworthy that the ^{1}H NMR chemical shift data for symplostatin 3 (**1**) support the folding of the peptide chain postulated for dolastatin 10 (**2**). Conformational analysis for **2** suggested that spatial proximity of Dov and at least one aromatic ring of Doe resulted in the shielding of H-25.[11] The H-25 signal for **1** in MeOH-d_3 appears 0.97 ppm more downfield for both trans and cis conformers compared with **2** in MeOH-d_3 (see Supporting Information, Table 3), presumably due to the absence of the thiazole ring.

Symplostatin 3 (**1**) possesses IC_{50} values for in vitro cytotoxicity of 3.9 and 10.3 nM against KB and LoVo cell lines, respectively. In comparison with the values we obtained for dolastatin 10 (**2**),[8b] the substitution of Doe by the 3-phenyllactic acid unit causes approximately a 100-fold reduction of in vitro cytotoxicity. Unfortunately, scarcity of material has prevented us so far from in vivo studies with **1**, but we expect in vivo antitumor activity comparable to that of isodolastatin H (**4**) based on reported structure–activity relationships. Modification of the Doe unit disclosed that the thiazole ring is not important for activity,[15] and this led to the ongoing clinical evaluation of the synthetic dolastatin 10 analogue TZT-1027 (**5**),[16] which is structurally equivalent to auristatin PE.[15c,d] Substitution of Doe by 3-phenyl-1,2-propanediol in **4** yielded an active compound in vivo, and only a higher dose of **4** was required to obtain antitumor activity similar to that of dolastatin 10 (**2**).[10,17] The phenyl group on the C-terminus is critical for activity, and a C_2 unit between the phenyl group and the linkage to the Dap unit is needed for significant activity in vivo.[10,15b] Those structural requirements are all fulfilled by symplostatin 3 (**1**).

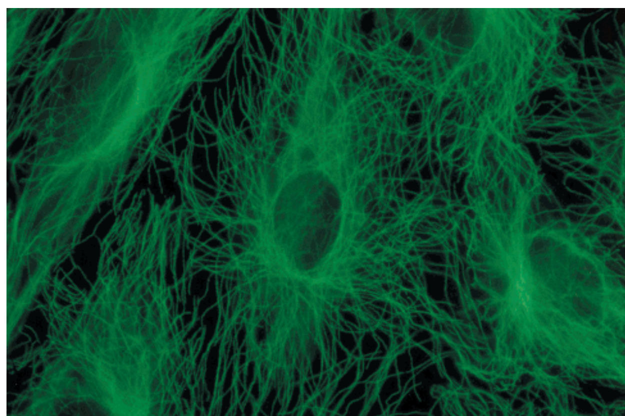
The effects of symplostatin 3 (**1**) on cellular microtubules and actin filaments were evaluated in A-10 cells. Symplostatin 3 (**1**) caused microtubule depolymerization and did not cause any loss of cellular microfilaments (Figure 2). In addition to the loss of cellular microtubules, compound **1** initiated the breakdown of the nucleus into micronuclei. The microtubule effects of symplostatin 3 (**1**) are indistinguishable from the effects of dolastatin 10 (**2**) and symplostatin 1 (**3**), but the least cytotoxic compound **1** was also least potent in this assay. A concentration of 0.1 μg/mL of **1** led to microtubule disruption, and an increase to 1 μg/mL caused total depolymerization of all the cellular microtubules.

Symplostatin 3 (**1**) is another example of a dolastatin 10 analogue isolated from a marine cyanobacterium, following the previous isolation of symplostatin 1 (**3**)[8] and dolastatin

Scheme 1. Comparison of the Mass Spectral Fragmentation of Symplostatin 3 (**1**) and Dolastatin 10 (**2**) by ESI-MS/MS[a]



[a] See also Pettit et al.[6] for fragmentation of dolastatin 10 (**2**) by SP-SIMS coupled with CAD



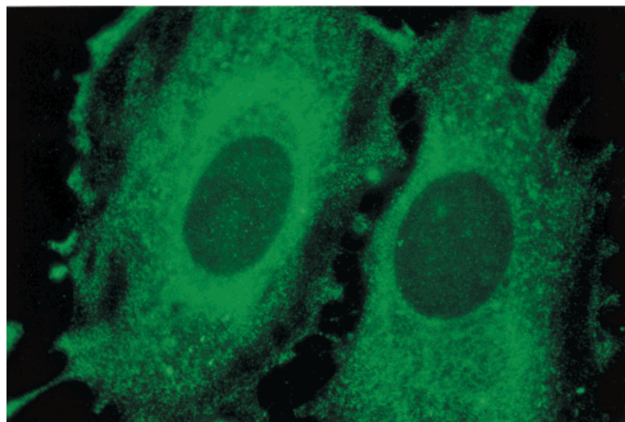


Figure 2. Microtubule-disrupting effects of symplostatin 3 (**1**). A-10 cells were treated for 24 h with either vehicle control (top) or symplostatin 3 (**1**) (bottom). Following incubation the cells were fixed and microtubules visualized by indirect immunofluorescence with an anti-β-tubulin antibody.

10 (**2**)[8b] from cyanobacterial sources. Like dolastatin H and isodolastatin H (**4**), which were found in the sea hare *D. auricularia*,[10] compound **1** does not have the Doe unit, and the new unit is connected via an ester linkage to the residual modified tetrapeptide. Symplostatin 3 (**1**) exhibited strong in vitro cytotoxicity, but was significantly less potent than dolastatin 10 (**2**).

Experimental Section

General Experimental Procedures. All NMR experiments were run on a Varian Unity Inova 500 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded in MeOH-d_3 at 500 and 125 MHz, respectively, using residual solvent signals as internal references. The HMQC experiments were optimized for $^1J_{CH}$ = 140 Hz, and the HMBC experiments for $^nJ_{CH}$ = 7 Hz. HRFABMS was obtained on a VG-ZAB instrument in the positive mode. MS/MS measurements were performed in the positive mode after infusing the analyte dissolved in methanol directly into the electrospray ionization unit of the mass spectrometer (quadrupole ion trap mass spectrometer model LCQ, Thermo Finnigan Corp., San Jose, CA) with mass screening covering the range 80−1000 amu. Capillary temperature was held at 200 °C, and sheath nitrogen flow was set at 60, corresponding to approximately 60 psi. ESI source needle voltage was set at 4.5 kV, leading to an average current of 80 μA, and capillary voltage was set at +10 V. Standards of *N,N*-dimethylvaline for the chiral HPLC analysis were synthesized by an established method.[18]

Biological Material. Cyanobacterium VP452 was collected on August 14, 1997, from patch reefs inside Kaneohe Bay, Oahu (Hawaii), at 3−4 m depth. The organism was morphologically consistent with *S. hydnoides*.[19] Unfortunately a voucher sample was not retained.

Extraction and Isolation. The freeze-dried organism VP452 (13.5 g) was first extracted with CH_2Cl_2 and then with MeOH to yield VP452L (159.7 mg) and VP452A (2.249 g), respectively. VP452L was partitioned between hexanes and 80% aqueous MeOH, and the solvent-evaporated methanolic phase was then partitioned between *n*-BuOH and H_2O. VP452A was partitioned between *n*-BuOH and H_2O as well, and the cytotoxic *n*-BuOH layers from both extracts were concentrated to dryness (92.4 and 116.2 mg, respectively) and combined. This portion was subjected to Si gel chromatography eluting first with CH_2Cl_2 and followed by CH_2Cl_2 solutions containing 2%, 5%, 10%, 20%, and 50% of MeOH and final washing with MeOH. The fractions eluting with 10−50% MeOH (85.3 mg) were pooled since they all exhibited approximately the same cytotoxicity. The pooled fractions were applied on a C_{18} Sep Pak, and elution was initiated with 10% MeCN in H_2O, followed by H_2O solutions containing progressively increasing amounts of MeCN. The portion eluting with 40% MeCN in H_2O (22.4 mg) was subjected to semipreparative reversed-phase HPLC (Econosil C18, 10 μm, 250 × 10 mm, 2.5 mL/min; PDA detection from 210 to 540 nm) using a MeCN−0.02 N TFA/H_2O linear gradient (20−100% MeCN over

50 min and then 100% MeCN for 20 min). The fraction eluting between 30 and 35 min was concentrated and rechromatographed on a different reversed-phase column (YMC-Pack ODS-AQ-323, 5 μm, 250 × 10 mm) using a MeOH−0.02 N TFA/H_2O linear gradient (50−100% MeOH over 50 min and then 100% MeOH for 20 min). Symplostatin 3 (**1**) eluted at t_R 27.6 min (1.9 mg, 0.014% dry wt).

Symplostatin 3 (1): colorless amorphous solid; $[\alpha]^{24}_D$ −46° (*c* 0.35, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.36) nm; IR (film) ν_{max} 3442 (br), 2923, 2851, 1709, 1682, 1639, 1457, 1417, 1206, 1136, 1098 cm^{-1}; ^1H NMR, ^{13}C NMR, and HMBC data, see Table 2; HRFABMS *m/z* $[M + H]^+$ 747.4898 (calcd for $C_{40}H_{67}N_4O_9$, 747.4908).

Acid Hydrolysis of 1 and Chiral HPLC Analysis. A sample of compound **1** (0.3 mg) was treated with 6 N HCl at 110 °C for 18 h. The hydrolyzate was concentrated to dryness and analyzed by chiral HPLC [column, Chirex phase 3126 (D) (4.6 × 250 mm), Phenomenex; solvent, 2 mM $CuSO_4$; flow rate, 1.0 mL/min; detection at 254 nm] for its amino acid content. *N,N*-Dimethylvaline (Me_2Val) and valine (Val) eluted at t_R 10.5 and 25.3 min, respectively. The retention times (t_R, min) of the authentic amino acids were as follows: L-Me_2Val (10.5), D-Me_2Val (12.4), L-Val (25.3), and D-Val (44.5), indicating the presence of L-Me_2Val and L-Val in the hydrolyzate. The stereochemistry of the 3-phenyllactic acid residue was determined by analysis of the acid hydrolyzate of **1** on a different chiral column [column, CHIRALPAK MA(+) (4.6 × 50 mm), Daicel Chemical Industries, Ltd.; solvent, 2 mM $CuSO_4$−MeCN (85:15); flow rate 1.0 mL/min; detection at 254 nm]. 3-Phenyllactic acid eluted after t_R 59.5 min, corresponding to the retention time of an authentic standard of L-3-phenyllactic acid and therefore indicating *S* configuration [t_R of D-3-phenyllactic acid: 41.0 min]. The amino acids eluted within the first few minutes under these conditions.

Cytotoxicity and Cytoskeletal Assays. Extracts VP452L and VP452A were tested in tumor selectivity assays that were performed as described previously.[20] Fractionation of these extracts was guided by monitoring cytotoxicity toward KB and LoVo cell lines. The IC_{50} values for cytotoxicity in vitro were determined using the SRB assay.[21] The effects of symplostatin 3 (**1**) on cellular microtubules and actin filaments were evaluated in A-10 cells as previously described.[8,22]

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Supporting Information Available: Copies of ^1H NMR and ^{13}C NMR spectra of **1** in MeOH-d_3 and NMR spectral data for **2** in MeOH-d_3 (Table 3) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) We assign dolastatin analogues that we isolate from cyanobacteria the trivial names lyngbyastatins (from *Lyngbya* spp.) and symplostatins (from *Symploca* spp.) to express the relation to the dolastatins and their true origin.[5a] The name symplostatin 2 has been given to a dolastatin 13 analogue: Harrigan, G. G.; Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Nagle, D. G.; Paul, V. J. *J. Nat. Prod.* **1999**, *62*, 655−658.

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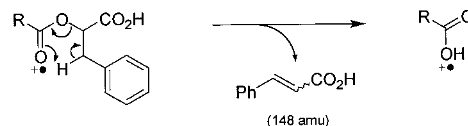
(12) For dolastatin 10 (**2**), the conformational ratio in MeOH-d_3 is also approximately 3:2, but the major conformer has cis configuration around the Dil−Dap amide bond (see Supporting Information).[6]

(13) According to:



(14) Furthermore, in contrast to compounds **1**−**3**, compound **4** has an isoleucine residue in place of the valine unit.

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(17) SAR studies showed that the substitution of Val by Ile, such as in compound **4**, does not reduce the antitumor activity, but affects the optimal dose.[15b]

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