Isolation and Structure of the Human Cancer Cell Growth Inhibitory Cyclodepsipeptide Dolastatin 16[†,‡,1]

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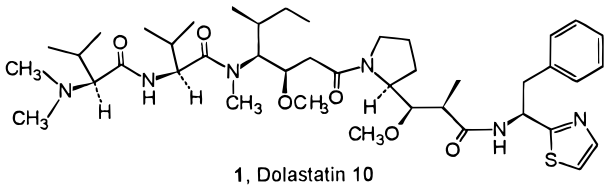
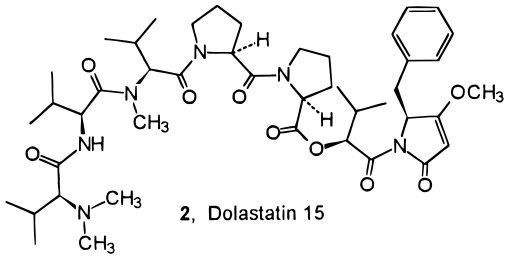
Abstract: An investigation of the sea hare *Dolabella auricularia* from Papua New Guinea has led to discovery of the new cyclodepsipeptide dolastatin 16 (**3**) containing two new amino acid units designated dolamethylleuine (Dml) and dolaphenvaline (Dpv). The structural elucidation was achieved by means of high-field (500 MHz) NMR and tandem MS/MS mass spectral interpretations and allowed the assignment *cyclo*-(Pro-Dpv-Pro-Dml-*O*-Lac-Pro-*O*-Hiv-MeVal). The new depsipeptide exhibited strong inhibition of growth against a variety of human cancer cell lines.

The remarkable sea hare *Dolabella auricularia* (Aplysiidae) enjoys a broad geographical range in tropical to temperate ocean areas. In 1972, we began an intensive investigation of Indian Ocean specimens for antineoplastic constituents that led to discovery of the important anticancer peptides dolastatins 10 (**1**)[2] and 15 (**2**),[3] which are now in cancer phase 1 clinical trials and preclinical development, respectively. Recent studies of *D. auricularia* collected in Japanese ocean areas have led to a different variety of linear and cyclic peptides.[4] We now report that an investigation of antineoplastic components of *D. auricularia* collected (1983) in Papua New Guinea, which was guided by a murine P388 lymphocytic leukemia bioassay, has provided a unique cyclodepsipeptide designated dolastatin 16 (**3**) that strongly inhibits growth of certain human cancer cell lines.

The P388-active dichloromethane-soluble fraction prepared[2c] from 1000 kg (wet weight) of *D. auricularia* was separated by a series of solvent partition, gel permeation (Sephadex LH-20), and partition (LH-20) column chromatography interspersed by high-speed countercurrent distribution procedures.[5] Final separation and purification was accomplished by reversed-phase C8 HPLC with acetonitrile−water (1:1) as mobile



1, Dolastatin 10



2, Dolastatin 15

phase to afford dolastatin 16 (3.1 mg, 3.1×10^{-7}% yield): colorless amorphous powder; $[\alpha]^{20}_{D}$ +15.5° (c = 0.20, CH_3OH). The high-resolution FAB mass spectrum exhibited a protonated molecular ion at m/z 879.525713 $[M + H]^+$, suggesting the molecular formula $C_{47}H_{70}N_6O_{10}$, which was consistent with the carbon and hydrogen totals deduced from the NMR spectra.

The IR spectrum contained typical peptide absorption bands at ν 3300, 1600, and 1540 cm^{-1}. Weak UV absorption at λ 268−250 nm and 240−210 nm indicated the presence of a monosubstituted aromatic unit. Interpretation of the high-field 2D series (500 MHz, Table 1) $^1H-^1H$ COSY, TOCSY, NOESY, ROESY, and HMQC (in $CDCl_3$ and in C_5D_5N) spectra revealed the presence of one *N*-MeVal and three Pro units and one each of lactic acid (Lac) and 2-hydroxyisovaleric acid (Hiv). In addition to these units, two new amino acid components were identified as 2-amino-4-phenylisovaleric acid and 2-methyl-3-aminoisocaproic acid, designated dolaphenvaline (Dpv) and dolamethylleuine (Dml), respectively. Both were also confirmed by HMBC correlations (Table 1).

The bonding sequence of the depsipeptide (**3**) units was first determined by interpretation of the HMBC, NOESY, and ROESY spectra and confirmed by results of tandem mass spectrometry studies. The HMBC correlations from NH [Dpv^2] to CO [Pro^1] indicated the presence of the Pro^1-Dpv^2 segment A. Two HMBC cross-peaks observed between CH_3N[$MeVal^8$]/CO[Hiv^7] and αH[Hiv^7]/CO[Pro^6] confirmed the connections in segment B as Pro^6-*O*-Hiv^7-$MeVal^8$. Another three HMBC correlation sets corresponding to αH[Lac^5]/CO[Dml^4], βH[Dml^4]/CO[Pro^3], and NH[Dml^4]/CO[Pro^3] allowed the structure of segment C to be assigned Pro^3-Dml^4-*O*-Lac^5 (Figure 1). Because the unsaturation number calculated from the molecular formula suggested that dolastatin 16 (**3**) was a cyclic octadepsipeptide, the correct sequence of segments A, B, and C would be one of two possibilities, either *cyclo*-[A-B-C] or *cyclo*-[A-C-B]. Further interpretation of both the NOESY and ROESY spectra afforded the most important evidence used to assign the sequence. The NOE relationships found between αH[$MeVal^8$]/δ2H[Pro^1], αH[Lac^5]/αH[Pro^6], βCH_3[Lac^5]/αH[Pro^6], and δ2H[Pro^3]/αH[Dpv^2], as well as NH[Dml^4]/αH[Pro^3] and the NCH_3 of [$MeVal^8$]/αH [Hiv^7], finally indicated the segment sequence to be *cyclo*-[A-C-B]. The assignment was also in agreement with other HMBC and NOE cross-peak correlations. Those included a strong *hetero*-cross-peak from the α-H

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Table 1. ^{1}H- and ^{13}C-NMR Spectral Data Assignments of Dolastatin 16 (**3**) (in $CDCl_3$)

no.		^{13}C (ppm)	^{1}H (ppm)	*J* (Hz)	HMBC (^{1}H to ^{13}C)	no.		^{13}C (ppm)	^{1}H (ppm)	*J* (Hz)	HMBC (^{1}H to ^{13}C)
Pro[1]	CO	172.24 s					*β*	56.35 d	3.66 m		Pro[3]CO,CO,α,*γ*
	α	61.28 d	4.62 dd	7.2, 2.0	CO,*β*,*γ*,*δ*		*β′*	14.89 q	1.01 d	5.6	CO,α,*β*
	β	30.70 t	2.18 m				*γ*	32.31 d	1.53 m		
			2.26 m		CO,α,*γ*		*δ*	19.73 q	0.87 d	5.6	*β*,*γ*,*δ′*
	γ	24.78 t	1.99 m				*γ′*	20.29 q	0.88 d	5.6	*β*,*γ*,*δ*
			2.08 m		*β*		NH		7.68 d	8.0	Pro[3]CO
	δ	47.55 t	3.45 m		*γ*	Lac[5]	CO	169.20 s			
			3.91 m		α,*β*,*γ*		α	66.64 d	5.18 q	7.0	Dml[4]CO,CO,*β*
Dpv[2]	CO	171.31 s					*β*	17.20 q	1.44 d	7.0	CO,α
	α	50.59 d	4.95 d	7.2	Pro[1]CO,CO,*β*,*γ*,*γ′*	Pro[6]	CO	171.01 s			
	β	40.90 d	1.75 m		*γ*		α	57.82 d	4.45 d	6.4	CO,*β*,*γ*,*δ*
	γ	40.95 t	2.39 m		α,*β*,*γ′*,1,2/6		*β*	30.82 t	2.20 m		CO
			2.52 d	7.6	α,*β*,*γ′*,1,2/6				2.30 m		CO,*δ*
	γ′	15.13 q	0.80 d	5.2	α,*β*,*γ*		*γ*	21.77 t	1.95 m		
	1	140.60 s							2.07 m		*β*
	2/6	129.56 d	7.35 d	6.0	*γ*,4		*δ*	46.43 t	3.42 m		*γ*
	3/4	128.33 d	7.27 d	6.0	1				3.67 m		
	5	126.15 d	7.17 dd	6.0,6.0	2/6	Hiv[7]	CO	169.57 s			
	NH		6.73 d	7.2	Pro[1]CO,CO		α	76.37 d	5.42 m		Pro[6]CO,CO,*β*,*γ*,*γ′*
Pro[3]	CO	171.01 s					*β*	28.29 d	2.18 m		*γ*,*γ′*
	α	58.84 d	4.55 d	6.0	CO,*β*,*γ*,*δ*		*γ*	16.08 q	1.04 d	7.0	α,*β*,*γ′*
	β	25.49 t	1.51 m		CO		*γ′*	19.73 q	1.06 d	7.2	α,*β*,*γ*
			2.40 m		CO,*γ*,*δ*						
	γ	25.01 t	1.73 m		α	MeVal[8]	CO	169.30 s			
			1.84 m				α	59.46 d	5.16 m		CO,*β*,*γ*,*γ′*,CH_3N
	δ	45.89 t	2.52 m		*β*		*β*	25.63 d	2.36 m		α,*γ*,*γ′*
			2.83 m		*γ*		*γ′*	19.73 q	0.91 d	5.6	α,*β*,*γ′*
Dml[4]	CO	174.64 s					*γ′*	17.75 q	0.83 d	5.2	α,*β*,*γ*
	α	38.67 d	2.85 m		CO,*β′*		CH[3]N	29.26 q	3.09 s		Hiv[7]CO

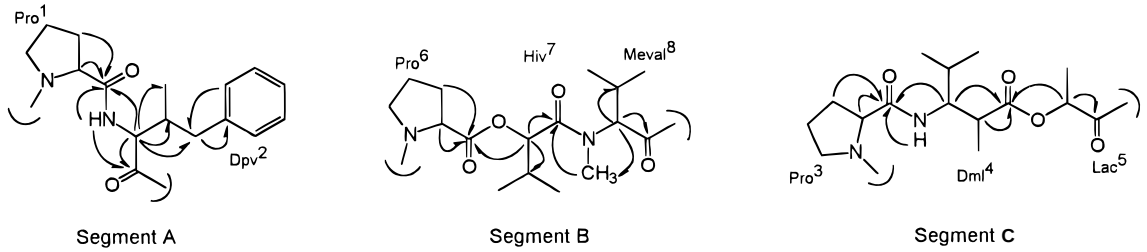

Figure 1. Some HMBC (⟶) correlations.

(*δ* 5.18) of Lac[5] to the CO (*δ* 174.64) of Dml and strong correlations between the α-H of Lac[5] and the α-H (*δ* 4.45) of Pro[6]. Therefore, the overall structure of dolastatin 16 (**3**) was established as *cyclo*-(Pro[1]-Dpv[2]-Pro[3]-Dml[4]-*O*-Lac[5]-Pro[6]-*O*-Hiv[7]-MeVal[8]).

Considerable evidence in support of the overall structure was provided by results of tandem MS/MS analyses. The mass spectrometry data initially suggested a different sequence order involving two of the units; instead of *O*-Lac-Pro-*O*-Hiv, the fragment ions observed appeared to be consistent with Pro-*O*-Lac-*O*-Hiv. However, mass spectrometric analysis of the synthetic unit *O*-Lac-Pro indicated that it underwent an anomalous fragmentation, consistent with that observed in the natural product. The proposed order of the remaining six amino acids was supported by the tandem mass spectrometry results, confirming the structure as *cyclo*-(Pro[1]-Dpv[2]-Pro[3]-Dml[4]-*O*-Lac[5]-Pro[6]-*O*-Hiv[7]-MeVal[8]).

The strong NOE relationship between αH[Lac[5]] and αH[Pro[6]] suggested a *cis* orientation for the Lac[5]-Pro[6] amide bond. That observation was further supported by the difference in chemical shifts of the *β* and *γ* carbons ($\Delta\delta_{\beta\gamma}$ = 9.05 ppm) of the Pro[6] residue.[6,7] The two Pro amide bonds involving MeVal[8]-Pro[1] and Dpv[2]-Pro[3] appeared to be *trans*, as $\Delta\delta_{\beta\gamma}$_ for both Pro units was below 6 ppm (Figure 2).

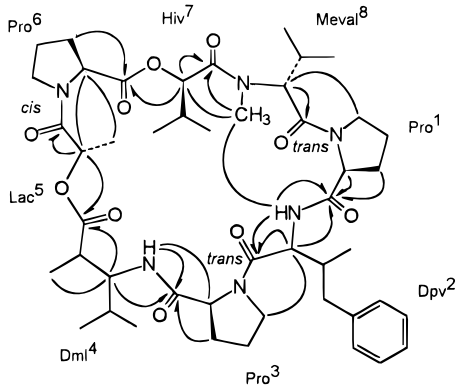

Figure 2. Structure of dolastatin 16 (**3**) and the principal HMBC (⟶) and NOE (—) correlations.

The absolute stereochemistry of the unit components other than Dpv[2] and Dml[4] was determined by chiral HPLC analyses (CHIREX phase 3126) of the dolastatin 16 (**3**) 6 N hydrochloric acid hydrolysate. The configurations of Lac[5] and the three Pro units were established to be all L (*S*), while the MeVal[8] and Hiv[7] units were found to have the D-(*R*)-configuration.[8] Assignment of the remaining chiral centers will require a series of synthetic approaches where the overall objective will be a convenient total synthesis. That research is under

way and will eventually allow a more detailed assessment of the promising antineoplastic activity.

Against a minipanel of the U.S. National Cancer Institute's human cancer cell lines, dolastatin 16 strongly inhibited the growth of the lung (NCI-H460, GI_{50} 0.00096 μg/mL), colon (KM20L2, GI_{50} 0.0012 μg/mL), brain (SF-295, GI_{50} 0.0052 μg/mL), and melanoma (SK-MEL-5, GI_{50} 0.0033 μg/mL) specimens. Dolastatin 16 (**3**) showed mean panel GI_{50} values of 2.5×10^{-7} M against the complete panel[9−12] of 60 human cancer cell lines and relatively low GI_{50}-COMPARE correlations of 0.76 and 0.71 with dolastatins 10 (**1**) and 15 (**2**), respectively. Importantly, against breast cancer lines MCF7, MDA-MB-435, and MDA-N, the GI_{50} values ($\log_{10}$) in that order were found to be −7.32, −7.46, and −7.54 M. Dolastatin 16 gave comparable inhibitory results using five human leukemia cell lines. As indicated above, this potentially valuable new lead will be further pursued.

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(8) Conditions for the chiral HPLC analysis: column, CHIREX phase 3126 (4.6 × 50 mm) (Phenomenex); solvents, 2 mM $CuSO_4$ in H_2O for α-amino acids and lactic acid and 2 mM $CuSO_4$(aq)−CH_3CN (9:1) for 2-hydroxyisovaleric acid; detection at λ 230.4 and 550 nm. By comparison of the retention times (min) with those of the authentic L- and D-α-amino and hydroxy acids [L-MeVal (6.79), D-MeVal (10.91), L-Pro (8.61), D-Pro (18.80), L-Lac (14.14), (*S*)-Hiv (12.04), and (*R*)-Hiv (19.52)], the retention times (min) and absolute configuration of the six components in the acid hydrolysate of dolastatin 16 (**3**) were found to be 8.75 (L-Pro × 3), 10.53 (D-MeVal), 14.50 (L-Lac), and 19.02 (*R*-Hiv).

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