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Isolation, Structure, and Synthesis of Dolastatin C, a New Depsipeptide from the Sea Hare *Dolabella auricularia*

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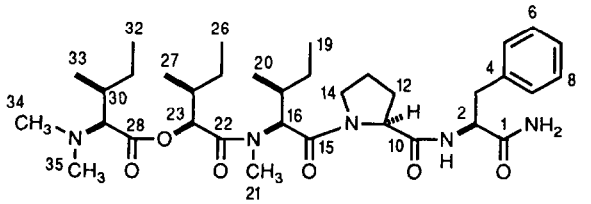
Abstract: Dolastatin C (**1**), a new depsipeptide exhibiting weak cytotoxicity, has been isolated from the Japanese sea hare *Dolabella auricularia*. The structure of **1** was elucidated by spectroscopic analysis and chemical degradation. The synthesis of **1** was carried out to confirm the structure unambiguously.

The Indian Ocean sea hare *Dolabella auricularia* (Aplysiidae) is known to be a rich source of promising antineoplastic peptides,[1] e.g., dolastatins 10[2] and 15.[3] In order to examine the difference in the constituents of *D. auricularia* collected at different locations and further to discover new bioactive compounds, we have investigated the Japanese sea hare *D. auricularia*,[4] resulting in the isolation of a new depsipeptide, dolastatin C (**1**).[5] In this communication we report the isolation, structure elucidation, and synthesis of this compound.

Specimens of *D. auricularia* (33 kg, wet wt) were collected on the Pacific coast of the Shima Peninsula, Mie Prefecture, Japan. Internal organs (20 kg) of the animal were extracted with MeOH, and the extract was partitioned between EtOAc and water. The material obtained from the EtOAc layer (91.4 g), which exhibited moderate cytotoxicity in vitro against HeLa-S_3 cells (IC_{50} 13 μg/mL), was further partitioned between 90% MeOH and hexane. The material from the 90% MeOH portion (30.8 g) was subjected to bioassay-guided chromatographic separation on silica gel, alumina, and ODS silica gel to afford an active fraction (2.5 mg, IC_{50} 0.24 μg/mL), which was finally separated by reversed-phase HPLC to give dolastatin C (**1**)[6] (1.7 mg, 5.1 x 10^{-6}% yield based on wet weight) as an amorphous powder. Dolastatin C (**1**) showed weak cytotoxicity (IC_{50} 17 μg/mL).[7]



1

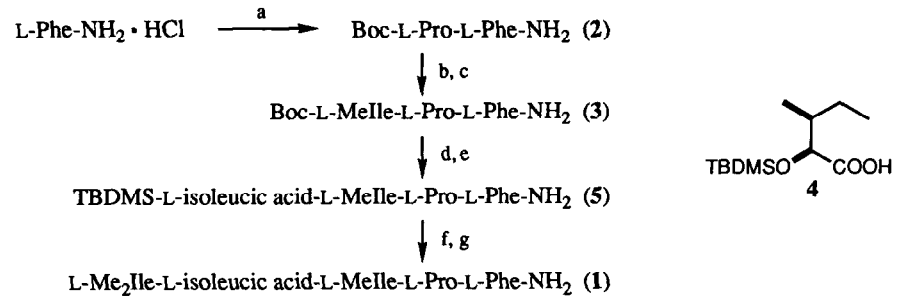
Table 1. NMR Data for **1**.[a]

Position	^1H[b]	^{13}C	HMBC[c]	Position	^1H[b]	^{13}C	HMBC[c]
NH_2	5.33, 6.41 s	–		18a	0.99 ddq (13.6, 2.9, 7.4)	23.8 t	H-20
(Phe)				18b	1.40 ddq (13.6, 8.8, 7.4)		
1	–	172.8 s	H-2, 3	19	0.83 t (7.4)	10.3 q	
2	4.66 ddd (8.2, 6.9 6.6)	53.6 d	H-3	20	0.62 d (6.4)	15.0 q	H-16, 18a
3a	3.08 dd (13.9, 6.9)	37.0 t	H-2, 5, 9	21	3.11 s	30.4 q	H-16
3b	3.22 dd (13.9, 6.6)			(Isoleucic acid)			
4	–	136.5 s	H-2, 3, 6, 8	22	–	170.96 s[e]	
5,9	7.18 m	129.3 d	H-3, 7	23	5.00 d (7.9)	73.8 d	H-27
6,8	7.29 m	128.8 d	H-6, 8	24	1.94 m	36.4 d	H-23, 25a, 26, 27
7	7.24 m	127.2 s	H-5, 9	25a	1.24 m	24.8 t[d]	
NH	6.31 d (8.2)	–		25b	1.66 m		
(Pro)				26	0.88 t (7.5)	10.3 q[f]	
10	–	170.8 s	NH, H-11, 12	27	0.85 d (6.7)	14.3 q	H-23, 25a
11	4.30 dd (7.0, 6.1)	61.3 d	H-12, 13	(Me_2Ile)			
12	2.01 m	28.9 t	H-11	28	–	171.7 s	H-23, 29
13	1.81 m	24.7 t[d]		29	2.91 d (10.4)	72.2 d	H-33, 34, 35
14a	3.50 dt (10.7, 7.3)	48.0 t	H-12	30	1.82 m	33.4 d	H-29, 31a, 32
14b	3.85 dt (10.7, 6.5)			31a	1.15 ddq (13.5, 8.1, 7.5)	25.0 t[d]	
(MeIle)				31b	1.66 m		
15	–	171.01 s[e]		32	0.88 t (7.5)	10.8 q[f]	
16	5.15 d (11.3)	57.6 d	H-20	33	0.82 d (6.7)	15.5 q	
17	2.03 m	32.5 d	H-16, 19, 20	34,35	2.30 s	41.4 q	H-29

[a] Spectra were recorded at 500 MHz for ^1H and at 67.8 MHz for ^{13}C using $CDCl_3$ as solvent and TMS as internal standard. Chemical shifts are in δ values. [b] Coupling constants in Hz are in parenthesis. [c] Parameters were optimized for J_{CH} = 8 Hz. [d,e,f] Signals are interchangeable within the same superscripts.

Dolastatin C (**1**),[8] $[\alpha]^{25}_D$ −136° (*c* 0.066, MeOH), has a molecular formula of $C_{35}H_{57}N_5O_6$ [high-resolution EIMS: *m/z* 643.4299 (M^+), Δ −0.9 mmu]. ^1H and ^{13}C NMR data (Table 1) indicated the peptidic nature of **1**. A detailed analysis of ^1H-^1H and ^{13}C-^1H COSY spectra showed that **1** contained four α-amino acid and one α-hydroxy acid units: phenylalanine (Phe), proline (Pro), *N*-methylisoleucine (MeIle), *N*,*N*-dimethylisoleucine (Me_2Ile), and isoleucic acid. These findings and the presence of a terminal amide group ($CONH_2$) suggested **1** to be a linear depsipeptide. HMBC and difference NOE data for **1** provided information on the sequence of the α-amino and α-hydroxy acid components. The HMBC data summarized in Table 1 allowed us to assign three carbonyl carbons Phe-CO (C1: δ 172.8), Pro-CO (C10: δ 170.8), and Me_2Ile (C28: δ 171.7). The two remaining carbonyl carbons MeIle-CO (C15) and isoleucic acid-CO (C22), however, could not be assigned owing to the close chemical shift values (δ 171.01 and 170.96). The HMBC spectrum showed two cross peaks Pro-CO (C10)/Phe-NH and Me_2Ile-CO (C28)/isoleucic acid-H23 that disclosed the sequences Pro-Phe and Me_2Ile-isoleucic acid. The difference NOEs were observed between isoleucic acid-H23 and MeIle-H21 (18% enhancement on irradiation of H23) and between MeIle-H16 and Pro-H14 (6 and 4% enhancements on irradiation of H16), establishing the two connectivities isoleucic acid-MeIle and MeIle-Pro, respectively. Further the presence of phenylalaninamide terminus (Phe-NH_2) was revealed from NOEs observed between Phe-H2 and NH_2 (6 and 3% enhancements on irradiation of H2). Thus the gross structure of **1** was established to be Me_2Ile-isoleucic acid-MeIle-Pro-Phe-NH_2. Acid hydrolysis of dolastatin C (**1**) with 9 N HCl (110 °C, 72 h) followed by a reversed-phase HPLC separation[9] and chiral HPLC analysis[10] of each component, confirmed the presence of five components described above and showed

Scheme 1



(a) Boc-L-Pro, DEPC, Et_3N, DMF, 0 °C then rt, 87%. (b) 4N HCl, dioxane, rt, 78%. (c) Boc-L-MeIle, DEPC, Et_3N, DMF, 0 °C then rt, 67%. (d) 4N HCl, dioxane, rt, 85%. (e) **4**, Bop-Cl, Et_3N, CH_2Cl_2, rt, 40%. (f) nBu_4NF, THF, rt, 98%. (g) L-Me_2Ile, DCC, DMAP, CH_2Cl_2, rt, 52%.

that all of them had L configuration. Authentic samples of Me_2Ile,[11] MeIle,[12] and isoleucic acid[13] were prepared for comparison purposes in the HPLC analyses.

The structure of dolastatin C (**1**) was further confirmed by synthesis using procedure outlined in Scheme 1. Starting with L-phenylalaninamide,[14] L-Pro and L-MeIle units were attached successively to afford tripeptide Boc-L-MeIle-L-Pro-L-Phe-NH_2 (**3**) (mp 73–74 °C (MeOH), $[\alpha]^{25}_D$ –133° (*c* 0.630, MeOH)) via dipeptide Boc-L-Pro-L-Phe-NH_2 (**2**) (mp 105–106 °C (MeOH), $[\alpha]^{25}_D$ –51.1° (*c* 0.555, MeOH)) using diethyl phosphorocyanidate (DEPC)[15] and hydrochloric acid for the coupling and deprotection steps, respectively. After deprotection of the Boc group of **3**, the resulting tripeptide was coupled with protected L-isoleucic acid **4** (oil, $[\alpha]^{23}_D$ –28.4° (*c* 1.09, MeOH)), prepared from L-isoleucic acid according to the method of Shioiri *et al.*,[16] using bis(2-oxo-3-oxazolidinyl)phosphinic chloride (Bop-Cl)[17] as a coupling reagent to afford tetrapeptide **5** (oil, $[\alpha]^{25}_D$ –143° (*c* 0.574, MeOH)). After deprotection of the silyl ether group of **5**, the resulting tetrapeptide was esterified with L-Me_2Ile using dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP). Dolastatin C (**1**) ($[\alpha]^{25}_D$ –134° (*c* 0.072, MeOH)) thus obtained was identical with the natural compound in all respects ($[\alpha]_D$, IR, 500 MHz ^{1}H NMR, MS, TLC, HPLC), establishing the absolute stereostructure of **1** unambiguously.

Dolastatin C (**1**) is structurally related to dolastatins 10 and 15: these are hydrophobic linear depsipeptides or a peptide with an *N,N*-dimethylamino acid at the N-terminus. Comparison of the cytotoxicities of these peptides, however, suggests that the presence of the terminal *N,N*-dimethylamino acid is not responsible for the significant bioactivity of dolastatins 10 and 15. It should be noted that the known dolastatins have not been isolated thus far from the Japanese sea hare *D. auricularia* which we examined and that it has contained several new peptidic components.

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5. The names, dolastatins A and B have previously been given to the peptides that were isolated from the sea hare *D. auricularia*: Pettit, G. R. Eur. Patent EP 124 984, 1984; *Chem. Abstr.* **1985**, *102*, 72870f. We thank Professor Pettit for informing us of this literature. Professor Pettit and one of the authors (K. Y.) agreed about the name of our depsipeptide to be termed dolastatin C (7th International Symposium on Marine Natural Products, July, 1992, Capri, Italy).
6. Conditions for the chromatographic separation: 1) silica gel, EtOAc/MeOH, step gradient; 2) silica gel, hexane/acetone, step gradient; 3) alumina, EtOAc/MeOH, step gradient; 4) ODS, 70→100% MeOH, linear gradient; 5) silica gel PLC, $CHCl_3$/MeOH 10:1, R_f 0.5; 6) reversed-phase HPLC [column, Develosil ODS-HG-5 (10 x 250 mm) (Nomura Chemical Co., Ltd.); solvent, 60% MeCN; flow rate, 2.0 mL/min; detection at 215 nm], retention time 47.4 min.
7. Since **1** was the major component of the active fraction, the component(s) with the stronger activity than that of **1** must be too minute to be isolated.
8. **1**: UV (MeOH) λmax 207 nm (ε 17500); IR ($CHCl_3$) 3490, 3410, 3350, 1715, 1685, 1635, 1590, 1505 cm^{-1}; EIMS *m/z* 643 (M^+), 586, 485, 447, 382, 256, 114.
9. The amino and hydroxy acids were isolated under the following conditions: column, Develosil ODS-HG-5 (4.6 x 250 mm) (Nomura Chemical Co., Ltd.); solvent, H_2O/MeCN/CF_3COOH 99:1:0.05 (20 min), 99:1:0.05→90:10:0.05 (20 min, linear gradient), and then 90:10:0.05 (20 min); flow rate, 1.0 mL/min; detection at 205 nm. The retention times (min) of components: Phe (37.0), Pro (3.3), MeIle (16.6), isoleucic acid (50.6), and Me_2Ile (19.7).
10. Conditions for the chiral HPLC analysis: column, CHIRALPAK MA(+) (4.6 x 50 mm) (Daicel Chemical Ind., Ltd.); solvent, 2 mM $CuSO_4$ for Pro and Me_2Ile and 2mM $CuSO_4$/MeCN 90:10 for others; flow rate, 0.5 mL/min except for isoleucic acid (1.0 mL/min); detection at 254 nm. The retention times (min) of the authentic amino and hydroxy acids: L-Phe (19.5), D-Phe (13.6), L-Pro (16.2), D-Pro (8.4), L-MeIle (6.9), D-MeIle (5.0), allo-L-MeIle (9.7), allo-D-MeIle (6.0), L-isoleucic acid (63.0), D-isoleucic acid (39.1), allo-L-isoleucic acid (55.3), allo-D-isoleucic acid (32.4), L-Me_2Ile (9.5), D-Me_2Ile (6.9), allo-L-Me_2Ile (12.0), and allo-D-Me_2Ile (8.1).
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