A Novel Cytotoxic Alkaloid of Lamellarin Class from a Marine Ascidian *Didemnum* sp.

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Many structurally and pharmacologically novel natural products have been isolated to date from a variety of species of marine ascidians, invertebrate chordates (phylum Chordata, subphylum Urochordata, class Ascidiacea). Major secondary metabolites of ascidians are amino acid-derived compounds.[1] In the chemistry of the genus *Didemnum*, tyrosine and tryptophan are the major amino acid components.[2] Lamellarins, isolated originally from prosobranch mollusc *Lamellaria* sp.[3] and later from the ascidian *D. chartaceum*,[4,5] the sponge *Dendrilla cactos*,[6,7] unidentified *Didemnum* sp.[8,9] and a species of unidentified ascidian,[10,11] are presumably condensation products of 3-(3,4-dihydroxyphenyl) alanines (or 3-hydroxytyrosines, *viz* DOPA's)[12] and showed various bioactivities, such as cytotoxicity,[3,13,14] immunomodulating activity,[8] HIV integrase-inhibitory activity.[11]

We studied a purple unidentified *Didemnum* sp. (other than *D. chartaceum*) to search for novel cytotoxic compounds. In this paper, we describe the structure elucidation of lamellarin β (**1**) using spectroscopic methods including the extensive use of 2-dimensional NMR correlation experiments. Molecular modeling study of the alkaloid was also conducted. The issue of chirality and the shielding effect of phenyl rings are discussed.

The specimens were lyophilized and extracted twice each with 70% methanolic chloroform and methanol. The combined extract was concentrated under vacuum and fractionated between hexane and methanol. The methanol-soluble material was further partitioned between ethyl acetate and water. The ethyl acetate fraction showed cytotoxicity against human acute promyelocytic leukemia cells (HL-60). Bioactivity-guided separation of the ethyl acetate-soluble material through Sephadex LH-20 (MeOH), followed by chromatography on reversed-phase HPLC (ODS-silica), gave a cytototoxic compound, lamellarin β (**1**), along with known compounds, lamellarins G (**2**)[4] and L (**3**).[8]

Experimental Section

Instruments and Data Collection. Low and high resolution mass measurements of DEI and FAB were supplied by the Mass Spec. Facility at the Scripps Research Institute, La Jolla, California, USA. Optical rotations were measured on a Roudolf polarimeter with a 10-cm cell. Infrared spectra were recorded on a Perkin Elmer FT-IR spectrophotometer Model 1600 and ultraviolet spectra on a Perkin Elmer Model Lambda 3B.

NMR spectra were recorded at Varian 400 and 500 MHz and Bruker 200, 300, and 500 MHz instruments. Double Quantum Filtered Correlation Spectroscopy (DQFCOSY),[15] Nuclear Overhauser Exchange Correlation Spectroscopy (NOESY),[16] ^{1}H-detected Heteronuclear Multiple-Quantum ^{1}H-^{13}C Coherence (HMQC),[17] and ^{1}H-detected Heteronuclear Multiple-Bond ^{1}H-^{13}C Correlation (HMBC)[18] experiments were performed using a Varian UN-500 spectrometer. A Brucker WP-200SY spectrometer was used for ^{13}C experiment. All chemical shifts were reported with respect to TMS (δ 0). Typical d1 delay of 0.7 sec was used for the DQFCOSY experiment. For the NOESY experiment, 3 sec d1 delay and 800 msec mixing time were utilized. The homonuclear 2-dimensional NMR spectra were acquired and processed in phase-sensitive modes, such as phase = 1, 2 or 3. Both Gaussian and sine bell functions were used for weighting. HMBC experiment was optimized at 160 Hz for the compounds to suppress $^1J_{CH}$ couplings. Typical d1 delay of 1.0 sec was utilized for the proton-detected heteronuclear 2-dimensional NMR experiments. For processing of those spectra, sine bell and Gaussian weighting functions were used for HMBC and HMQC experiments, respectively.

Collection, Extraction, and Isolation. The purple encrusting ascidian, *Didemnum* sp., collected in the Indian Ocean, was kindly provided by Professor Fenical at the Scripps Institution of Oceanography, La Jolla, USA. The specimens were kept frozen at -20 °C until use. The lyophilized animal (74 g, dry weight) was extracted twice each with 70% MeOH/$CHCl_3$ and methanol. The combined extract was concentrated and partitioned between hexane and methanol. The methanol-soluble material was further partitioned between ethyl acetate and water. Gel-filtration of the ethyl acetate-solubles through Sephadex LH-20 and Spectra Gel HW-40 with methanol gave several fractions which contained lamellarins β (**1**), G (**2**) and L (**3**). Further purification of alkaloids **1-3** was accomplished by reversed-phase HPLC (ODS-silica) in combination with bioassay. To purify compound **1**, 30% CH_3CN in 0.1% trifluoroacetic acid was used as the eluting solvent. Yields of compounds **1-3** were 10 mg, 5 mg, and 8 mg, respectively.

Lamellarin β (1): an amorphous solid; $[\alpha]_D = 0°$; HRDEIMS: M^+ obsd. m/z 473.1094, $C_{26}H_{19}NO_8$, dev. -3.5 ppm; UV (MeOH) λ_{max}: 336 nm (ε 17000), 315 (17000), 278 (23000), 268 (sh), 205 (43000); UV (MeOH + NaOH) λ_{max}: 362 nm (ε 15800), 322 (17600), 287 (23000), 205 (60000); IR (NaCl) ν_{max}: 3700-3000, 1690, 1595, 1420,

1285, 1250, 1187 cm^{-1}.

Molecular Modeling Study. Energy minimization of **1** was performed using a Serena software, pcmodel, which adopts a MMX force field. The actual presentation of **1** (Figure 2) was obtained using a combination of a Chem3D program and a ChemDraw software released from the Cambridge Scientific Computing, Inc.

Cytotoxictiy Assay. Human acute promyelocytic leukemia cells (HL-60) were grown in RPMI 1640 with 10% fetal bovine serum in 5% CO_2 atmosphere. Cytotoxicity of crude extract and subsequently purified compounds were tested against this cell line according to Mosmann's method, colorimetric MTT assay.[19]

Preparation of Acetate Derivative 1a. Acetic anhydride (0.1 mL) was added to a solution of alkaloid (1 mg) in pyridine (0.2 mL) and stirred for 3 hours at room temperature. The reaction was monitored by TLC. Reagents were evaporated under high vacuum and partitioned sequentially between ethyl acetate and ice water, between ethyl acetate and 0.1 $NaHCO_3$ solution, and between ethyl acetate and brine. Finally, the product was dried with $MgSO_4$ and filtered. The solvent was evaporated under vacuum to give the acetate derivative (**1a**) quantitatively.

Pentaacetate 1a: an amorphous solid; $[\alpha]_D = 0°$; LRFABMS, $(M + H)^+$ obsd. *m/z* 684, $(M + Cs)^+$ m/z 816; HRFABMS, $(M + Cs)^+$ obsd. *m/z* 816.0693, $C_{36}H_{29}NO_{13}Cs$, dev. 4.0 ppm (3.3 mmu); ^1H NMR (500 MHz, $CDCl_3$): δ 2.20 (s, 3H), 2.23 (s, 3H), 2.28 (s, 3H), 2.29 (s, 3H), 2.31 (s, 3H), 3.12 (m, 1H), 3.20 (m, 1H), 4.53 (m, 1H), 5.12 (m, 1H), 6.85 (s, 1H), 7.11 (s, 1H), 7.12 (s, 1H), 7.13 (s, 1H), 7.17 (d, 1H, J = 2 Hz), 7.21 (dd, 1H, J = 8, 2), 7.24 (d, 1H, J = 8).

Results and Discussion

Structure Elucidation of the Lamellarin. Lamellarin β (**1**, Table 1) was obtained as an amorphous solid. The high resolution desorption electron ionization mass spectrometry (HRDEIMS) established its molecular formula as $C_{26}H_{19}NO_8$ [obsd. *m/z* 473.1094 M^+, dev. -3.5 ppm]. The high degree of unsaturation (18 degrees) was evident from the molecular formula, and also supported by its UV spectrum [(MeOH) λ_{max}: 336 (ε 17000), 315 (17000), 278 (22000), 265 (sh), and 205 (43000) nm]. A bathochromic shift (1N NaOH), a broad and strong IR absorption band at 3700-3000 cm^{-1}, and ^{13}C NMR data (8 signals downfield of δ 140) suggested the phenolic nature of the compound. A conjugated ester functional group was present, which was indicated by a strong IR band at 1690 cm^{-1} in combination with a carbon signal at δ 154.4.

The proton NMR spectrum of compound **1** showed seven aromatic signals and two mutually coupled methylene resonances [δ 2.93 (t, 2H, J = 7 Hz), 4.54 (dt, 1H, J = 13.5, 7 Hz), and 4.57 (dt, 1H, J = 13.5, 7 Hz)]. Among the aromatic signals, a 1,2,4-trisubstituted benzene ring was very obvious from analysis of coupling constants [δ 6.74 (dd, 1H, J = 8, 2 Hz), 6.73 (d, 1H, J = 2 Hz), and 7.07 (d, 1H, J = 8 Hz)]. Four aromatic singlets either came from four different spin systems or were para-positioned since there was no coupling among them.

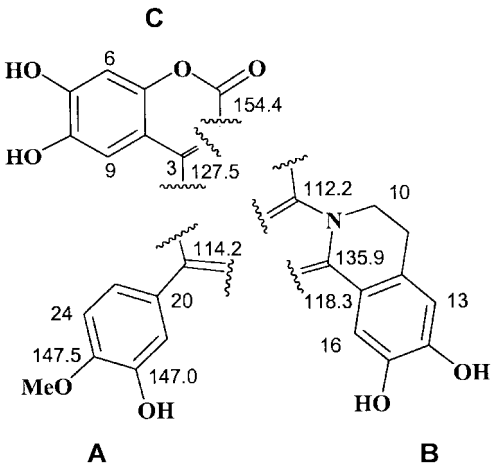
Table 1. NMR Assignments for Lamellarin β (**1**)

C no	$^{13}C^a$	$^1H^b$	HMBC (8 Hz)^b	NOESY^b
1	154.4			
2	112.2			
3	127.5			
4	108.9			
5	146.2			
6	103.2	6.72 (s, 1H)	C4, C5, C7, C8	
7	144.7			
8	142.1			
9	108.5	6.46 (s, 1H)	C3, C5, C7, C8	H21, H25
10	42.0	4.54 (dt, 1H, J = 13.5, 7 Hz) 4.57 (dt, 1H, J = 13.5, 7)	C2, C11, C12, C18	H11
11	27.8	2.93 (t, 2H, J = 7)	C10, C12, C13, C17	H10, H13
12	125.7			
13	115.1	6.67 (s, 1H)	C14, C15, C17	H11
14	146.2			
15	143.2			
16	113.2	6.41 (s, 1H)	C12, C14, C15, C18	H21, H25
17	118.3			
18	135.9			
19	114.2			
20	127.0			
21	117.4	6.73 (d, 1H, J = 2)	C22, C23, C25	H9, H16
22	147.0			
23	147.5			
24	112.9	7.07 (d, 1H, J = 8)	C20, C22, C23	H25, H26
25	121.2	6.74 (dd, 1H, J = 8, 2)	C19, C21, C23	H9, H16, H24
26	55.4	3.88 (s, 3H)	C23	H24

All experiments were performed in DMSO-d_6. Chemical shifts are reported in δ units (downfield of TMS). All $^1J_{CH}$ correlations were determined by HMQC experiments at 500 MHz. [a] ^{13}C NMR was obtained at 50 MHz. [b] Spectra were acquired at 500 MHz.

In the HMBC spectrum of **1**, the proton signals at δ 7.07 (H24) and 6.73 (H21) were coupled to both quaternary carbon signals at δ 147.0 and 147.5. However, the carbon signal at δ 147.5 only correlated to the proton signal H25 at δ 6.74. Therefore, the carbon signal was assigned to C23, since $^4J_{CH}$ couplings were rarely observed in the HMBC experiments (8 Hz). The methoxy signal at δ 3.88 also coupled to the carbon signal at δ 147.5. Furthermore, a correlation between the methoxy signal and the proton signal H24 was observed in NOESY spectra. This allowed the position of the methoxy group to be assigned as C23. The carbon signal C19 at δ 114.2 was long-range coupled to the proton H25 at δ 6.74, which resulted in assignment of subunit **A** (Scheme 1).

Two protons H13 at δ 6.67 and H16 at δ 6.41 were para-positioned since there was no coupling in the proton spectrum of **1**. The assignment of two hydroxyl groups at the



Scheme 1

most deshielded carbons at δ 143.6 and 146.2 was supported by the up-field shifts of two adjacent carbon signals C13 (δ 115.1) and C16 (δ 113.2). Initially, the carbon signal at δ 125.7 was assigned as any carbon within a three-bond range such as C2, C12, or C18. The carbon signal gave two additional correlations to protons H11's (δ 2.93) and to proton H16 (δ 6.41). Hence, the possibility of the carbon being C18 was eliminated again with the restriction of HMBC couplings within three-bond ranges. With a correlation of H13 (δ 6.67) to C17 (δ 118.3), a dihydroisoquinoline moiety, subunit **B**, was constructed.

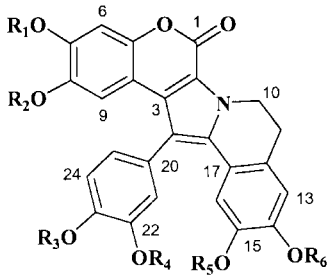
From HMBC correlations by two para-positioned proton singlets at δ 6.72 and 6.46 (H6 and H9, respectively) and the ester functional group, suggested by IR (1690 cm^{-1}) and the carbon signal at δ 154.4, the subunit **C** was very obvious.

The last two connectivities, C3-C19 and C18-C19, were established based on NOESY correlations: H9 to H21, H9 to H25, H16 to H21, and H16 to H25. The large difference in chemical shift between C2 (δ 112.2) and C3 (δ 127.5) could be rationalized if C2 and C3 were α and β to the ester carbonyl C1 (δ 154.4). The overall data explained the 18 unsaturations inherent in the molecular formula. Therefore, the structure was proposed as **1** shown in Figure 1.

Chirality of Molecule. As implied in the NOESY experiment of **1**, the catechol ring at C19 was predicted to be almost orthogonal to the rest of the molecule from molecular modeling study to avoid severe steric interactions. Due to the proximity of the coumarin and the isoquinoline moieties, free rotation of the catechol ring at C19 is seriously restricted. The rotational barrier of the bond between C19 and C20 was in excess of 80 Kcal/mole. Due to the highly restricted rotation of the catechol ring, the molecule could be chiral. However, all lamellarins isolated in this study were racemic as observed before in previous studies,[3-8,10,11] which suggested thermal racemization to be a facile process.[5] One of the lowest energy forms, as illustrated in space-filling model, is presented in Figure 2.

Shielding Effect of Phenyl Ring at C19. The pseudo-orthogonal conformation of the catechol ring at C19 generated significant proton NMR shielding effects of two protons H9 (δ 6.46) and H16 (δ 6.41). Their up-field shifts, compared to other proton signals H6 (δ 6.72) and H13 (δ 6.67), were induced by diamagnetic anistropy of the catechol ring at C19.[20]

In conclusion, we identified a novel cytotoxic compound, lamellarin β, along with previously reported lamellarins G and L, from an unidentified species of marine ascidian *Didemnum*. Due to the steric crowding the alkaloid was predicted to have a catechol ring pseudo-orthogonal to the rest of the molecule. Yet the compound did not show chirality which indicated thermal racemization to be a facile process. The new compound was tested for cytotoxicity



lamellarins

1.	β	$R_1 = R_2 = R_4 = R_5 = R_6 = H$, $R_3 = Me$
2.	G	$R_2 = R_4 = R_6 = H$, $R_1 = R_3 = R_5 = Me$
3.	L	$R_1 = R_4 = R_6 = H$, $R_2 = R_3 = R_5 = Me$
1a.	β acetate	$R_1 = R_2 = R_4 = R_5 = R_6 = Ac$, $R_3 = Me$

Figure 1. Lamellarins and a derivative. Biosynthetically, the compounds appear to be derived from the condensation of three DOPA molecules.[12] The numbering system for the lamellarins used in this study is different from that in previous studies because it is designed to illustrate their relatedness to other DOPA-alkaloids isolated from *Didemnum* species.

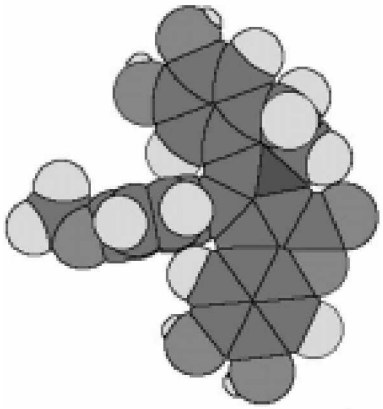


Figure 2. Computer-generated perspective drawing of one of the lowest energy forms for the lamellarin β. The pseudo-orthogonal conformation of the catechol at C19 gave significant shielding effects to the two protons H9 and H16 in the proton NMR spectrum of lamellarin β. The catechol ring was 74 degrees out of the plane of the pyrrole ring since the isoquinoline ring was twisted about 20 degrees. The coumarin moiety and the pyrrole ring were perfectly co-planar.

against various human tumor cell lines in culture. Lamellarin β showed cytotoxicity against human promyelocytic leukemia HL-60 with an IC_{50} of 4.8 μg/mL. The cytotoxicity of lamellarin β, compared to that of the ethyl acetate-solubles (IC_{50} = 4.8 μg/mL), suggested more active compounds yet to be identified. We are currently investigating more potent compounds from this ascidian.

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