Full Papers

Antineoplastic Agents. 510.[1] Isolation and Structure of Dolastatin 19 from the Gulf of California Sea Hare *Dolabella auricularia*[†]

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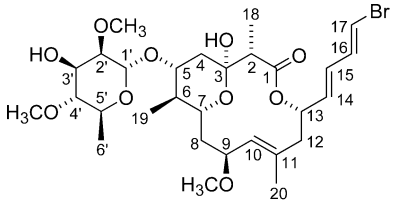
Received April 30, 2003

The Gulf of California shell-less mollusc *Dolabella auricularia* has been found to contain a new 14-membered macrocyclic lactone linked to a 2,4-di-*O*-methyl-L-α-rhamnopyranoside, designated dolastatin 19 (**1**). The new cancer cell growth inhibitor (**1**, 8.33×10^{-8}% yield) was obtained by bioassay (P388 lymphocytic leukemia and human cancer cell lines) directed isolation, accompanied by debromoaplysiatoxin (9.17×10^{-7}% yield) and anhydrodebromoaplysiatoxin (2.0×10^{-7}% yield). The structures were determined on the basis of analyses of high-resolution mass spectra and high-field NMR data. All the relative stereochemistry for the chiral centers was designated by utilizing NMR techniques.

A 1972 collection of the shell-less mollusc *Dolabella auricularia* from the Indian Ocean (Mauritius) followed by subsequent re-collection led to our isolation of a new series of 15 strongly active anticancer peptides designated the dolastatins.[2] Of these, dolastatins 10−15 proved most interesting.[2a] Once their structures were elucidated, dolastatins 10, 11, and 15 were synthesized[3] and subjected to structural modifications.[4] Dolastatin 10 and a synthetic modification, auristatin PE (TZT-1027), as well as two dolastatin 15 derivatives have advanced to clinical trials.[5] Specimens of *D. auricularia* collected from Papua New Guinea provided important additions to these cancer cell growth inhibitory peptides: dolastatins 16−18.[6] Meanwhile Yamada and colleagues[2b,7] have contributed a new series of *D. auricularia* constituents from Japanese collections of this very productive sea hare.

Recent advances in cyanobacterial chemistry have provided increasing evidence of a close relationship of such marine organism components with the majority of *D. auricularia* constituents isolated and characterized in the preceding investigations.[8] The introduction of certain dolastatin peptides such as dolastatins 3, 10, 11−13,[9,10] and 16[11] through consumption of cyanobacteria has recently been further supported by the isolation (∼0.01% yields) of dolastatin 10 from *Symploca* sp. VP642 varieties collected in the Republic of Palau in 1999.[12] Presumably these peptides are ingested during feeding activities and serve as chemical defenses for the sea hare. The variations in antineoplastic constituents of *D. auricularia* led us to sample it in another ocean area quite remote from our prior collections. That research focused on *D. auricularia* from the Gulf of California and afforded the new macrocyclic lactone cancer cell growth inhibitor named dolastatin 19 (**1**), which is related to aurisides isolated from the same species collected in Japan.[13]



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Results and Discusion

Dolabella auricularia (ca. 600 kg wet wt) collected in the Gulf of California in 1996 was extracted with methanol−methylene chloride (1:1), and the methylene chloride fraction was successively partitioned between *n*-hexane and methanol−water (9:1) and methylene chloride and methanol−water (3:2). The methylene chloride fraction (549 g) was separated by gel permeation and partition chromatographic procedures on columns of Sephadex LH-20 with a series of efficient solvent systems (CH_3OH, 3:2 CH_2Cl_2−CH_3OH, 3:1:1 *n*-hexane−toluene−CH_3OH, 8:1:1 *n*-hexane−*i*-PrOH−CH_3OH) as eluents. Each separation procedure was directed by bioassay using the P388 murine lymphocytic leukemia cell line and a selection of human cancer cell lines. Final separation and purification procedures were performed using reversed-phase (C8 and C18) HPLC (6.5:3.5 methanol−water and 4.5:5.5 acetonitrile−water) to yield 0.5 mg of a new cancer cell growth inhibitor (GI_{50} 0.72 μg/mL for breast MCF-7 and 0.76 μg/mL for colon KM20L2) herein named dolastatin 19 (**1**), as well as two known macrolides, debromoaplysiatoxin[14] (5.5 mg) and anhydrodebromoaplysiatoxin[15] (1.2 mg).

Dolastatin 19 (**1**) was obtained as a colorless amorphous powder, and its FABMS spectra showed two quasi-molecular ions in equivalent abundance ratio at *m/z* 641 $[M + Li + 2]^+$ and 639 $[M + Li]^+$ and at *m/z* 657 $[M + Na + 2]^+$ and 655 $[M + Na]^+$, which clearly indicated the presence of a bromine atom and a molecular weight of 634 (for ^{81}Br). The HRFABMS spectroscopic measurement afforded two accurate quasi-molecular ions at *m/z* 641.23617 and

[†] Dedicated to the late Dr. D. John Faulkner (Scripps) and the late Dr. Paul J. Scheuer (Hawaii) for their pioneering work on bioactive marine natural products.

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10.1021/np030198b CCC: $27.50
Published on Web 02/14/2004

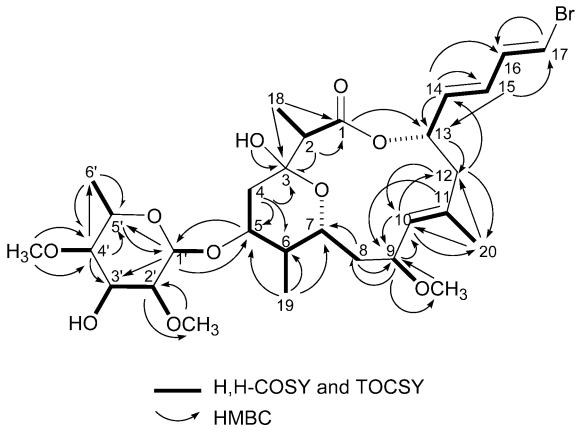

Figure 1.

639.232108 (calc 639.235614), corresponding to molecular formula $C_{29}H_{45}O_{10}Br$ and implying seven degrees of unsaturation. The detailed structural elucidation of dolastatin 19 (**1**) continued with 1D and 2D NMR spectral analyses (^{1}H, APT, ^{1}H−^{1}H COSY, TOCSY, HMQC, and HMBC at 500 MHz). The 29 carbon atoms were confirmed using ^{1}H, APT, and HMQC experiments in CD_3CN and refined to include one carbonyl, five olefinic methines, one olefinic quaternary carbon (δ 119.13 s), one oxygenated quaternary carbon (δ 98.24 s), 11 methines (eight were oxygenated and one was a hemiacetal type at δ 100.02 d), three methylenes, four methyls, and three methoxyl groups, as well as two hydroxy protons at δ 2.97 and 4.47. By employing ^{1}H−^{1}H COSY and TOCSY techniques, three proton-relayed spin−spin systems [A: CH_3−CH−; B: CH_3−CH(O)−CH(O)−CH(O)−CH(O)−CH(O_2)−; C: X−CH=CH−CH=CH−CH(O)−CH_2−C(CH_3)=CH−CH(O)−CH_2−CH(O)−CH(CH_3)−CH(O)−CH_2−] were revealed.

HMBC experiments allowed us to expand the linkages of the three spin−spin systems. In spin−spin system A, both the methine proton at C-2 (δ 2.49 q) and the methyl proton at C-18 (δ 1.08 d) showed HMBC cross-peaks with a carbonyl carbon (C-1, δ 177.43 s) and a quaternary carbon (C-3, δ 98.24 s). Additionally, two *gem*-methylene protons at C-4 (H-4a, δ 2.18 and H-4b, δ 1.23) in spin−spin system C and a hydroxyl proton (δ 4.47 d, 2.0 Hz) also correlated with the oxygenated quaternary carbon (C-3, δ 98.24) in the HMBC spectrum (Figure 1). The hydroxyl proton (δ 4.47) also gave a HMBC cross-peak to C-2. These results identified one linkage between spin−spin systems A and C, which was interlinked with the oxygenated quaternary carbon (C-3). An HMBC correlation was observed from a proton (δ 5.75) at C-13 to the carbonyl carbon (C-1, δ 177.43), creating an ester linkage as another connection between systems A and C to form a 14-membered macrolide.

One methoxyl group (δ 54.74 q and δ 3.12 s) gave double HMBC cross-peaks with a carbon and a proton at C-9 (δ 76.62 d and δ 3.78 dd). The ^{13}C-chemical shift of the quaternary carbon (C-3, δ 98.24 s) strongly suggested that the carbon should be oxygenated to form a hemiketal. However, no other HMBC correlation was observed with C-3. Also, two degrees of unsaturation remained, implying that there were two more rings in this structure. However, 2D-ROESY experiments afforded a valuable NOE correlation from H-4a (δ 2.18) to H-7 (δ 3.57). This NOE cross-peak not only confirmed the presence of a pyran ring by hemiketal linkage between C-3 and C-7 but also confirmed the conformation of the pyran ring (Figure 2), since only in a boat conformation would these protons be close enough in space to exhibit an NOE effect. Also, a *W*-type coupling

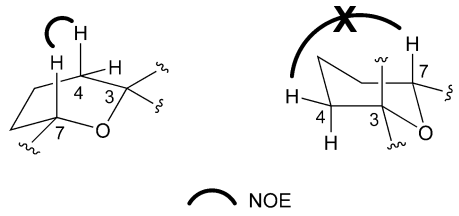

Figure 2.

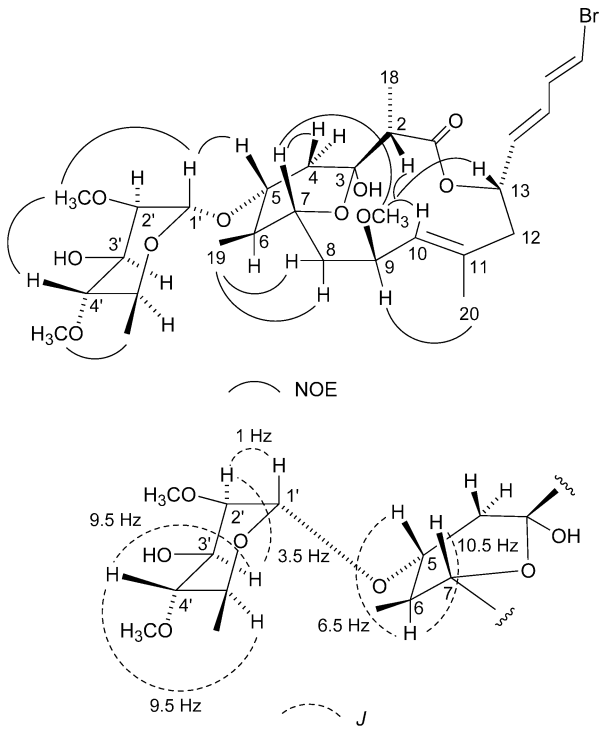

Figure 3.

between a hydroxy proton (δ 4.47 d) and H-4a (δ 2.18) was observed with a coupling constant of 2.0 Hz. According to this interpretation combined with other NOE correlations observed on the macrolide ring, the relative stereochemistry at C-7 was assigned as 7*R**.

Both HMBC and 2D-NOE experiments provided strong evidence regarding the connecting location of spin−spin systems B and C. HMBC correlations from H-5 (δ 3.51) to C-1′ (δ 100.02 d) and from H-1′ (δ 4.86) to C-5 (δ 80.17 d) as well as an NOE correlation between H-5 and H-1′ (Figures 1 and 3) were observed. Furthermore, several HMBC correlations were detected within the spin−spin system B, for example, H-1′/C-2′,H-1′/C-3′,H-1′/C-5′,H-4′/C-3′,H-4′/C-5′,H-4′/C-6′,H-6′/C-4′,H-6′/C-5′, and H-5′/C-1′ (Figure 1). Thus the system B was deduced to be a pyranoside. The two as yet unassigned methoxyl groups (δ 59.11 q/3.39 s and δ 60.92 q/3.46 s) exhibited HMBC correlations with C-2′ (δ 82.00 d/3.35 dd) and C-4′ (δ 84.32 d/2.86 t), respectively. Identification of the mono sugar unit was performed on the basis of its coupling constants (confirmed by its 2D-*J* resolution spectrum) and 2D-ROESY experiments (Figure 3). Both coupling constants between H-3′ and H-4′ and between H-4′ and H-5′ were measured as 9.5 Hz, indicating that the orientation of these protons could be assigned as *ax*/*ax* and the 3.5 Hz coupling constant between H-2′ and H-3′ implied the orientation as *eq*/*ax*. These assignments were reconfirmed by NOE correlations between the methoxy proton (*ax*) at C-2′ and H-4′ (*ax*), as well as between the methoxy proton (*eq*) at C-4′ and the methyl proton (*eq*) at C-5′. Considering the dominant conformer of rhamnose to be $^1C_4$ type, the same

as mannose in carbohydrate chemistry, the 1 Hz coupling constant between H-1′ and H-2′ and NOE correlation between H-1′ (*eq*) and the methoxy proton (*ax*) at C-2′ identified the orientation of H-1′ and H-2′ as *eq*/*eq*. The pyranose ring was thus determined to be 2′,4′-dimethoxy-α-L-rhamnopyranoside.

The overall structure of dolastatin 19 was found to represent a novel 14-membered glycoside macrolide with a hemiketal-bearing ring (**1**). However, the stereochemistry around the 14-membered ring remained to be elucidated. To determine the relative configurations, 2D-ROESY was effectively employed. The NOE correlations from OCH_3 at C-9 to H-7, H-2, and H-13 established that all these protons were located on the same side of the 14-membered ring as H-7. Because the configuration at C-7 was already found to be 7*R**, the chiral centers at C-2, C-3, C-9, and C-13 were deduced to be 2*S**, 3*S**, 9*S**, and 13*S**. The NOE correlation from CH_3 at C-6 to both protons at C-8 indicated that the C-19 methyl must be above the ring and the chirality of C-6 was assigned as 6*S**. The H−H coupling constant at H-6 and H-7 also supported this assignment, because $J_{6,7} = 10.5$ Hz implied that these two protons should be opposite *ax*/*ax* in a boat type conformation. The H−H coupling constant at H-6 and H-5 was measured by 2D-*J* solution experiments to be 6.5 Hz. That value confirmed the dihedral angle (θ) between H-5 and H-6 as about 130°. According to the Karplus calculation, H-5 and H-6 should be vicinal *eq*/*ax* in the boat conformation. Therefore, the configuration at C-5 was deduced to be 5*R**.

Two NOE correlations between OCH_3 at C-9 and H-10 and between CH_3 (C-20) and H-9 pointed to an *E* configuration for the double bond at C-10 and C-11. Chem Draw software was helpful in estimating the proton and carbon NMR data around the conjugated diene in spin−spin system C (C-14−C-17). That inspection suggested that the diene terminated with the bromine atom, as the calculated data most closely tallied with the measurements. The UV spectrum in methanol showed absorptions at λ_{max} 200 and 250 nm, which also supported the result obtained from Chem Draw.[16] On the basis of the H−H coupling constants $J_{14,15}$ (15 Hz) and $J_{16,17}$ (14 Hz), the configuration of the carbon−carbon double bonds in the diene was assigned as *E* geometry. Therefore, all of the relative stereochemistry on the 14-membered macrolide ring was assigned as 2*S**, 3*S**, 5*R**, 6*S**, 7*R**, 9*S**, 13*S**, $E_{10,11}$, $E_{14,15}$, and $E_{16,17}$.

In view of the new and interesting structure of dolastatin 19 (**1**), as well as that of debromoaplysiatoxin and anhydrodebromoaplysiatoxin, which have been isolated from cyanobacteria and red algae, respectively,[14,15] it is very likely that macrolide **1** was obtained from an analogous source by *D. auricularia*. Furthermore, lyngbyaloside, a 16-membered macrolide similar to dolastatin 19, has been isolated from the blue-green alga *Lyngbya bouillonii*.[17] Other 14-membered macrolides similar to dolastatin 19, such as lyngbouilloside[18] and lyngbyaloside B,[19] have also been isolated from cyanobacteria.

Dolastatin 19 (**1**) displayed significant in vitro activity against two of our human cancer cell lines with GI_{50} values of 0.72 μg/mL for breast MCF-7 and 0.76 μg/mL for colon KM20L2. Because of the 8.3×10^{-8}% yield (0.5 mg), further bioevaluation will have to await total synthesis.

Table 1. ^{1}H and ^{13}C NMR Spectral Assignments for Dolastatin 19 (**1**) in Acetonitrile-d_3

carbon no.	^{13}C	^{1}H	HMBC	NOE
1	177.43 s			
2	48.62 d	2.49 q (7.5)	1, 3, 18	OCH_3 at C-9
3	98.24 s			
OH		4.47 d (2.0)	2, 3	
4	40.33 t	2.18 m	3, 5, 6	H-7
		1.23 m	3, 5	
5	80.17 d	3.51 m		
6	43.58 d	1.18 m		
7	73.62 d	3.57 m		H-4a, OCH_3 at C-9
8	40.22 t	2.01 m	7, 9, 10	H-19
		1.46 m		H-19
9	76.62 d	3.78 ddd	8, 10, 11, OCH_3	H-20
		(10.8, 9.0, 2.0)		
OCH_3	54.74 q	3.12 s	9	H-2, H-7, H-10, H-13
10	133.53 s	4.94 d (10.8)	9, 12, 20	OCH_3 at C-9
11	119.13 s			
12	46.89 t	2.30 d (14)	10, 13, 14, 20	
		2.23 d (14)	10, 13, 14, 20	
13	71.71 d	5.75 m (10.5, 6)[a]	1	OCH_3 at C-9
14	133.32 d	5.82 dd (15, 6)	13, 15, 16	
15	129.33 d	6.27 dd (15, 11)	13, 17	
16	137.76 d	6.77 dd (14, 11)		
17	110.77 d	6.50 d (14)	15, 16	
18	12.86 q	1.08 d (7.5)	1, 2,	
19	13.44 q	0.98 d (6.5)	5, 6, 7	H-8a, H-8b
20	16.36 q	1.71 s	10, 12	H-9
1′	100.02 d	4.86 d (1)	2′, 3′, 5′	H-5, H-2′
2′	82.00 d	3.35 dd (3.5, 1)	OCH_3	
OCH_3	59.11 q	3.39 s	2′	H-1′, H-4′
3′	72.19 d	3.55 m (3.5, 9.5)[a]		
OH		2.97 s		
4′	84.32 d	2.86 t (9.5)	3′, 5′, 6′, OCH_3	OCH_3 at C-2′
OCH_3	60.92 q	3.46 s	4′	H-6′
5′	68.38 d	3.53 m (9.5, 6.0)[a]	1′, 4′	
6′	18.07 q	1.15 d(6.0)	4′, 5′	OCH_3 at C-4′

[a] Coupling constant data were obtained from a 2D-*J* resolution spectrum.

Experimental Section

General Experimental Procedures. Solvents used for column chromatography were freshly distilled. Sephadex LH-20, particle size 25−100 μm, used in gel permeation and partition column chromatographic separations was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. The TLC plates were viewed under shortwave UV light and then developed by 20% H_2SO_4 or 3% ceric sulfate−3 N sulfuric acid spray reagent followed by heating at approximately 150 °C. For HPLC separations, a Phenomenex Prepex (particle size 5−20 μm, 10.0 mm × 25 cm) C-8 column and a Phenomenex IB-SIL (partical size 5 μm, 4.6 mm × 25 cm) C-18 were used in reversed-phase mode with a Gilson (model 306) solvent metering pump and a Gilson 118 UV/vis detector at 235 nm.

The optical rotation value for dolastatin 19 was determined with a Perkin-Elmer model 241 polarimeter. The UV spectrum was recorded with a Hewlett-Packard 8450 UV−vis spectrometer. The HRFABMS was measured with a Kratos MS-50 mass spectrometer (Midwest Center for Mass Spectrometry, Lincoln, NE). The ^{1}H and ^{13}C NMR, APT, ^{1}H−^{1}H-COSY, TOCSY (mixing time of 45 and 60 ms), HMQC (optimized for $^1J_{H-C} = 140$ Hz), HMBC (optimized for $^nJ_{H-C} = 8.2$ Hz), ROESY (mixing time of 100 and 150 ms), and 2D-*J* resolution data were recorded (CD_3CN solution) using a Varian VXR-500 instrument.

Marine Animal Collection and Extraction. *Dolabella auricularia* was collected by scuba in April and May 1996 in the Gulf of California at a depth of 5 m off the coast of Danzante Island near Puerto Escondido, Mexico. The fresh sea hare was immediately subdivided and preserved in methanol. The marine animal (600 kg, wet wt) was repeatedly extracted with methylene chloride−methanol (1:1), and water was added to cause phase separation. The methylene chloride fraction was successively partitioned[20] using the system CH_3OH-H_2O (9:1 → 3:2) against *n*-hexane and methylene chloride, respectively, to yield the bioactive methylene chloride fraction (549 g, PS ED_{50} 0.164 μg/mL).

Isolation of Dolastatin 19 (1). Separation of the active methylene chloride fraction was guided by bioassay in the P388 lymphocytic leukemia cell line. A concentrated fraction was obtained by a series of Sephadex LH-20 column chromatographic steps in the sequence methanol, methylene chloride−methanol (3:2), *n*-hexane−2-propanol−methanol (8:1:1), and *n*-hexane−toluene−methanol (3:1:1), respectively, as eluent. Further separation of this fraction was performed by C-8 reversed-phase HPLC with 65% methanol as a mobile phase, then final purification was achieved by ODS-HPLC techniques with 45% or 50% acetonitrile as eluent. By this means three pure compounds were isolated, namely, debromoaplysiatoxin (5.5 mg, 9.17 $\times 10^{-7}$%), anhydrobromoaplysiatoxin (1.2 mg, 2.0×10^{-7}%), and dolastatin 19 (0.5 mg, 8.33×10^{-8}%).

Dolastatin 19 (1): colorless amorphous powder; $[\alpha]_D$ +7.5° (*c* 0.04, CH_3OH); UV (CH_3OH) λ 250, 220 nm; FABMS *m*/*z* 657 $[M + Na + 2]^+$, 655 $[M + Na + 2]^+$, 655 $[M + Na]^+$, 641 $[M + Li + 2]^+$, and 639 $[M + Li]^+$; HRFABMS *m*/*z* 641.23617 and 639.232108 (calc 639.235614). The ^1H, APT, HMQC, HMBC, and ROESY assignments have been summarized in Table 1.

Acknowledgment. We are pleased to acknowledge the very important financial support provided by Outstanding Investigator Grant CA44344-05-12 and Grant RO1 CA 90441-01 with the Division of Cancer Treatment and Diagnosis, NCI, DHHS; the Arizona Disease Control Research Commission; the Fannie E. Rippel Foundation; the Robert B. Dalton Endowment Fund; and G. L. and D. R. Tooker, Dr. J. C. Budzinski, P. Trautman, and S. Schloegel. We also thank the Government of Mexico (especially Dr. D. Lluch Belda, Director, Northeast Biological Research Center; J. Garcia Pamanes, Chief, Department of Marine Biology (UABCS); and C. Sánchez Ortiz, Marine Biologist), and Profs. J. C. Knight and F. Hogan for other helpful assistance.

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NP030198B