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Novel Cytotoxic Brominated Diterpenes from the Red Alga *Laurencia obtusa*

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Received February 21, 2003

Five new brominated diterpenes, along with two known, have been isolated from the organic extract of the red alga *Laurencia obtusa*, collected from the coastal rocks of Preveza in the Ionean Sea, Greece. The novel metabolites prevezols B−E possess two new carbon skeletons, to the best of our knowledge, unprecedented in the literature. The structures and the relative stereochemistry of the new natural products were established by means of spectral data analyses. The new metabolites were tested for their cytotoxic activity against five human cell lines. Two metabolites have exhibited significant cytotoxicity.

Introduction

Species of the red alga genus *Laurencia* (Huds.) Lamoroux elaborate an astonishing variety of structurally unusual secondary metabolites and have been the subject of intensive research since the earliest studies on marine organisms.[1] The vast majority of *Laurencia* metabolites are C_{15} acetogenins,[2−4] halogenated diterpenes,[5−7] and sesquiterpenes,[8−12] although many other structural classes have been reported.[13]

In the course of our ongoing research activities toward the isolation of biologically active compounds from marine organisms of the Greek seas,[4,7,11,12] we studied *Laurencia obtusa* collected from the coastal rocks of Preveza in the Ionean Sea. In this paper, we describe the isolation and structure elucidation of the new metabolite prevezol C (**1**) along with the revised structure of metabolite prevezol B (**2**), two novel diterpenes, prevezols D−E (**3** and **4**), and the new metabolite neorogioldiol B (**6**) along with the known metabolites neorogioldiol (**5**) and *O*,[11]15-cyclo-14-bromo-14,15-dihydrorogiol-3,11-diol (**7**) all of which were obtained from the non polar fractions of the organic extract (Chart 1). Following extensive analyses of the 1D and 2D NMR spectra, the structure of previously published prevezol B (**2a**) was revised as **2**, and the new assignments are now reported. Assessment of the new compounds' cytotoxicity was performed on the following human tumor cell lines: MCF7 (derived from a mammary adenocarcinoma), PC3 (derived from a prostate adenocarcinoma), HeLa (derived from cervix adenocarcinoma), A431 (derived from epidermoid carcinoma), K562 (a chronic myelogenous leukemia cell line).

Results and Discussion

The alga was collected during the summer of 2000 from the coast area north of the city of Preveza in central Greece.The CH_2Cl_2/MeOH extract of the freeze-dried alga was subjected repeatedly to vacuum column chromatography (VCC) on silica gel and normal-phase high-pressure liquid chromatography (HPLC), using mixtures of cyclohexane/EtOAc as the mobile phase, to yield compounds **1**−**7** in pure form.

Compound **1** was isolated as a colorless oil whose ^{13}C NMR data and HRFAB-MS measurements supported the

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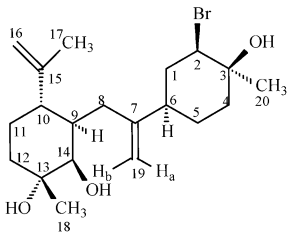
10.1021/jo0342323 CCC: $25.00
Published on Web 08/26/2003

CHART 1



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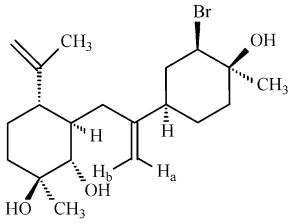

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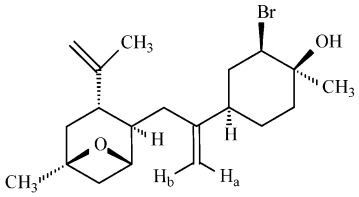
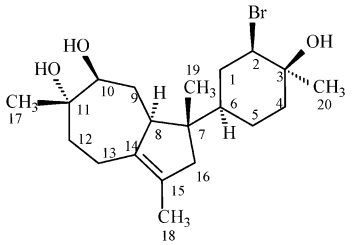


2a



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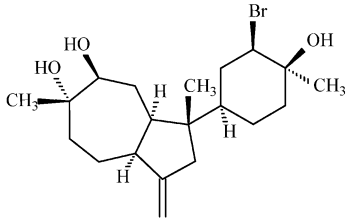

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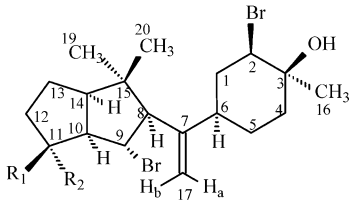
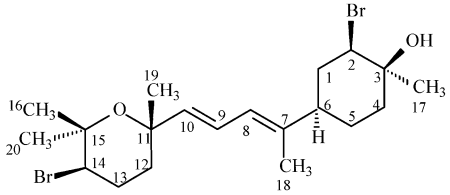


5 R_1: -CH_3 R_2: -OH
6 R_1: -OH R_2: -CH_3



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molecular formula $C_{20}H_{33}O_3Br$. The EIMS showed $[M - H_2O]^+$ peaks at *m*/*z* 382, 384 with intensities of 1/0.95, indicating the presence of one bromine atom. The presence of hydroxyl groups was indicated by the strong IR absorption at 3614 cm^{-1}. The ^{13}C NMR spectrum along with the DEPT experiments showed the presence of 20 carbons corresponding to four quaternary, five methine, eight methylene, and three methyl carbon atoms. Among the carbons, three were bonded to oxygens resonating at δ_C 73.8 (d), 72.3 (s), and 70.3 (s), one was brominated resonating at δ_C 65.7 (d), and four were olefinic. The two double bonds were on olefinic methylenes, and their presense was evident from the methylene peaks at δ_C 112.1 and 110.2 ppm. Furthermore, the ^1H NMR spectrum revealed signals due to a halomethine group at δ_H 4.13 (1H, dd), an α-hydroxy methine at δ_H 3.60 (1H, d), two exocyclic methylene groups at δ_H 4.87 (1H, brs), 4.80 (1H, brs), and 4.75 (2H, brs), two tertiary methyl groups at δ_H 1.60 and 1.31, and one vinylic methyl group at δ_H 1.66. With four degrees of unsaturation and two double bonds, metabolite **1** was suggested to possess a bicyclic structure.

Comparison of spectral data (Table 1) of **1** with literature values suggested that metabolite **1** was related to the unique brominated *Laurencia* diterpene prevezol A.[7]

The NMR data of **1** showed resonances corresponding to the six-membered brominated ring of prevezol A, suggesting that this moiety also exists in metabolite **1**. Strong correlations observed in the HMBC spectrum between C-6 (δ_C 44.2) and C-8 (δ_C 35.2) with the methylene protons H-19a (δ_H 4.87) and H-19b (δ_H 4.80) confirmed the position of the $\Delta_{7,19}$ terminal methylene group. Additionally, the isopropylidene group was positioned at carbon C-10 (δ_C 43.7) on the basis of the long-range correlations of C-10 with H-17 (δ_H 1.66) and H-16 (δ_H 4.75). The low-field resonances of the oxygenated carbons supported the presence of two hydroxyl groups

TABLE 1. NMR Data of Compounds 1 and 2[a]

	prevezol C (**1**)			prevezol B (**2**)		
no.	δ_H ^{1}H	δ_C ^{13}C	NOESY	δ_H ^{1}H	δ_C ^{13}C	NOESY
1	2.18 (m) (-α)	39.9	H-1*β*, H-2, H-6	2.14 (m)	39.4	H-2, H-19a
	2.01 (m) (-*β*)		H-1α, H-5*β*, H-19a			
2	4.13 (dd, 12.29, 4.10)	65.7	H-1α, H-4α, H-6, H-20	4.13 (dd, 9.95, 6.63)	66.0	H-1, H-4α, H-6, H-20
3		70.3			70.4	
4*β*	2.07 (m)	37.4	H-4α, H-5*β*, H-5α, H-20	2.04 (m)	37.6	H-4α, H-20
4α	1.52 (m)		H-2, H-4*β*, H-20	1.47 (m)		H-2, H-4*β*, H-5, H-20
5	1.76 (m) (-*β*)	26.1	H-1*β*, H-4*β*, H-5α, H-19a	1.59 (m)	26.8	H-4α, H-19a
	1.55 (m) (-α)		H-4*β*, H-5*β*, H-14			
6	1.91 (m)	44.2	H-1α, H-2, H-20	1.99 (m)	45.8	H-2
7		150.2			149.6	
8a	2.10 (m)	35.2	H-9, H-16, H-17	2.23 (m)	38.3	H-10, H-14, H-16, H-17, H-19b
8b	1.88 (m)		H-9, H-14, H-19b	2.07 (m)		H-17
9	2.29 (br t, 11.80)	35.6	H-8a, H-8b, H-11α, H-14, H-17, H-19b	2.01 (m)	43.1	H-11α, H-18, H-19b
10	2.09 (m)	43.7	H-11*β*, H-12*β*, H-16	2.02 (m)	52.6	H-8a, H-16, H-17
11α	1.84 (m)	27.6	H-9, H-11*β*	1.46 (m)	28.6	H-9
11*β*	1.46 (m)		H-10, H-11α, H-12*β*, H-12α, H-17	1.58 (m)		H-12α
12*β*	1.96 (m)	34.6	H-10, H-11*β*, H-12α, H-18	1.57 (m)	37.6	H-14
12α	1.75 (m)		H-11*β*, H-12*β*, H-18	1.96 (m)		H-11*β*
13		72.3			72.9	
14	3.60 (d, 4.43)	73.8	H-5α, H-8b, H-9, H-18, H-19b	4.02(d, 11.20)	75.9	H8a, H-12*β*, H-19b
15		147.3			146.7	
16	4.75 (br s)	112.1	H-8a, H-10, H-17	4.75 (br s)	113.1	H-8a, H-10, H-17
17	1.66 (s)	18.6	H-8a, H-9, H-11*β*, H−16	1.60 (s)	19.1	H-8a, H-8b, H-10, H-16
18	1.60 (s)	30.1	H-12*β*, H-12α, H-14, H-19b	1.40 (s)	23.5	H-9, -OH (2.35)
19a	4.87 (br s)	110.2	H-1*β*, H-5*β*, H-19b	4.88 (br s)	110.2	H-1, H-5
19b	4.80 (br s)		H-8b, H-9, H-14, H-18, H-19a	4.86 (br s)		H-8a, H-9, H-14
20	1.31 (s)	30.5	H-2, H-4*β*, H-4α, H-6	1.31 (s)	30.5	H-2, H-4*β*, H-4α
−OH				2.35 (br s)		H-18
−OH				1.88 (br s)		

[a] All spectra were recorded in $CDCl_3$. Chemical shifts are expressed in ppm. *J* values in parentheses are in Hz.

that were placed on carbons C-14 (δ_C 73.8) and C-13 (δ_C 72.3) because of the strong correlations of C-14 with protons H-8 and H-10 (δ_H 2.09) as well as C-13 with protons H-12 and H-18 (δ_H 1.60). Moreover, strong correlations that were observed in the ^{1}H−^{1}H COSY spectrum of **1** between the protons H-14/H-9, H-9/H-10, H-10/H-11, and H-11/H-12 confirmed the positions of the two hydroxyl groups on the adjacent carbons C-13 and C-14.

The stereochemical configuration of the asymmetric centers was resolved by combination of NOESY data and analyses of coupling constants. The strong NOE effect between H-2 (δ_H 4.13)/H-4α (δ_H 1.52) and H-2/H-6 (δ_H 1.91) showed the cis orientation of these protons and suggested chair conformation for the six-membered ring. Moreover, the coupling constant of H-2 (12.29, 4.10 Hz) indicated the axial configuration of H-2 and the NOE interactions between H-2/H-20 and H-4α, H-4*β*/H-20 revealed the equatorial configuration of the methyl group on the brominated six-membered ring.

The strong NOE effects between H-9 (δ_H 2.29) and H-11α (δ_H 1.84) as well as between H-10 and H-12*β* (δ_H 1.96) were indicative of the chair conformation of the second six-membered ring and the axial orientation of these protons. Moreover, the coupling constant (4.43 Hz) between H-14 (δ_H 3.60) and H-9 (δ_H 2.29) confirmed the equatorial position of H-14. Additionally, H-14 exerts NOE enhancement on H-18 (δ_H 1.60), which in combination with the NOE effect between H-18 and both H-12α and H-12*β* suggested equatorial configuration for H-18. Thus, both hydroxyl groups on the second six-membered ring have axial configuration. Additionally, a strong correlation between H-17 and H-9 indicated equatorial configuration for the isopropylidene group. Since there were no NOE enhancements linking the stereocenters C-6 and C-9 in metabolite **1**, named prevezol C, the relative stereochemistry was established as 2*R**,3*S**,6*R**,9*R**,10*S**,13*R**,14*R** or 2*R**,3*S**,6*R**,9*S**,10*R**,13*S**,14*S**.

Compound **2**, following HPLC purifications, was isolated as a colorless oil. The molecular formula $C_{20}H_{33}O_3Br$ for **2** was deduced from the HREI-MS data in combination with the NMR spectra (Table 1). The dominant $[M - H_2O]^+$ peaks in the HREI mass spectrum, at *m*/*z* 382 and 384 with intensities of 1/0.95, indicated the presence of one bromine atom in the molecule.

Comparison of the spectral data of **2** with those of **1** revealed a great similarity in the structures of the two compounds even though compound **2** exhibited identical spectral characteristics with previously published prevezol B (**2a**).[7]

The EI LRMS and IR spectra were almost identical with those recorded for **1.** The most significant differences between metabolite **2** and **1** were observed as follows: in the ^{1}H NMR chemical shifts of α-hydroxy methine H-14 (δ_H 4.02 in **2**; δ_H 3.60 in **1**), the methine H-9 (δ_H 2.01 in **2**; δ_H 2.29 in **1**) and the methyl H-18 (δ_H 1.40 in **2**; δ_H 1.60 in **1**); in the ^{13}C NMR chemical shifts of the methine carbons C-14 (δ_C 75.9 in **2**; δ_C 73.8 in **1**) and C-9 (δ_C 43.1 in **2**; δ_C 35.6 in **1**) as well as the methyl carbon C-18 (δ_C 23.5 in **2**; δ_C 30.1 in **1**). Additionally, a significant difference was observed at the magnitude of the coupling constants for H-14 (J = 11.20 Hz in **2**; J = 4.43 Hz in **1**).

These data led to the assumption that compound **2** was a stereoisomer of **1**.

The strong NOE effect between H-9 and H-11α (δ_H 1.46) as well as H-9 and H-18 confirmed the chair conformation for the second six-membered ring and the axial (α) configuration of these protons. Additionally, a significant correlation was observed between H-14 and H-12*β* (δ_H 1.57) indicating their cis (ax−ax) orientation, while the observed coupling constant (11.20 Hz) between H-14 (δ_H 4.02) and H-9 (δ_H 2.01) revealed their trans (ax−ax) spatial proximity. Thus, both hydroxyl groups on the second six-membered ring have equatorial configuration.

On the basis of the above findings and since there were no NOE enhancements linking the stereocenters C-6 and C-9 the relative stereochemistry of metabolite **2** is proposed as 2*R**,3*S**,6*R**,9*R**,10*S**,13*S**,14*S** or 2*R**,3*S**,6*R**,9*S**,10*R**,13*R**,14*R**.

In the present study, the higher magnetic field (500 MHz) resolved the ^{1}H NMR spectrum and allowed solid assignment of the H-14 in axial orientation, thus excluding the possibility of an ether bridge. Additionally, the presence of three free hydroxyl groups was confirmed by the mass spectrum of the methylation product of metabolite **2** with NaH/MeI. Moreover, the NOE effect between H-18 and H-9 does not support migration of C-18 on C-12, as was earlier proposed.[7]

Thus, we suggest revision of the earlier published structure **2a** for prevezol B (**2**) as shown in this paper.

Compound **3** was purified by means of HPLC separation and was isolated as a colorless oil. Both ^{13}C NMR data and HREI-MS measurements supported the molecular formula $C_{20}H_{33}O_3Br$. The HREI-MS showed $[M - H_2O]^+$ peaks at *m*/*z* 382, 384 with intensities of 1/0.95, indicating the presence of one bromine atom. The presence of hydroxyl groups was indicated by the strong IR absorption at 3437 cm^{-1}. The ^{13}C NMR spectrum along with the DEPT experiments showed the presence of 20 carbons corresponding to five quaternary, four methine, seven methylene, and four methyl carbon atoms. Among the carbons, three were bonded to oxygens resonating at δ_C 77.4 (d), 72.5 (s), and 70.4 (s), one was brominated resonating at δ_C 67.3 (d), and two were olefinic quaternary carbons resonating at δ_C 127.2 and 127.1 ppm. Furthermore, the ^{1}H NMR spectrum revealed signals due to a halomethine proton at δ_H 4.15 (1H, dd), an α-hydroxy methine at δ_H 3.82 (1H, d), three tertiary methyl groups at δ_H 1.40, 1.30, and 0.75, and one vinylic methyl group at δ_H 1.60. With four degrees of unsaturation, the structure was suggested to contain, besides the double bond, three rings. All protonated carbons and their protons were matched precisely by the ^{1}H COSY and HMQC experiments. From comparison of the NMR data (Table 2) of **3** with those of compounds **1** and **2** it was evident that in these three molecules there were significant similarities focused on the brominated six-membered ring.

Moreover, strong long-range correlations between carbon C-6 (50.3 ppm) with H-8 (δ_H 0.91) and H-19 (δ_H 0.75) and carbon C-7 (34.3 ppm) with H-8 and H-19 observed in the HMBC experiments and confirmed the connection between carbons C-6 and C-7 in the molecule. Furthermore, correlations between carbon C-9 (δ_C 40.9) with H-8 and H-10 (δ_H 3.82) (confirmed by COSY), carbon C-11 (δ_C 72.5) with H-10, H-9 (δ_H 2.28), H-12 (δ_H 2.02), H-13 (δ_H 2.67) and H-17 (δ_H 1.40), carbon C-14 (δ_C 127.2) with H-12 (δ_H 2.02) and H-18 (δ_H 1.60), carbon C-15 (δ_C 127.1) with H-16 (δ_H 1.92) and H-18, as well as carbon C-16 (δ_C 42.7) with H-8, H-18 and H-19 confirmed connectivities between carbons C-7 and C-16. Additionally, the characteristic correlation of the methine C-8 with H-13 (δ_H 2.67) suggest a cyclization between C-8 and the quaternary olefinic carbon C-14.

The stereochemical configuration of the chiral centers was resolved by a combination of NOESY data and analysis of coupling constants. The above data indicated that the six membered brominated ring had the same relative stereochemistry as in compounds **1** and **2**. The observed coupling constant (11.61 Hz) between H-10 (δ_H 3.82) and H-9*β* (δ_H 2.28) revealed their trans (ax-ax) spatial proximity. Additionally, the dihedral angle between H-10 and H-9α (δ_H 2.05) was evidently approaching 90° as we did not observe any coupling between these two protons. Moreover, the strong NOE effect between H-10/H-12α (δ_H 1.53) and H-10/H-8 (δ_H 0.91) indicated their cis (α) orientation, while strong correlations of H-9*β* (δ_H 2.28) with the methyl groups H-17 (δ_H 1.40) and H-19 (δ_H 0.75) suggested cis (*β*) orientation for these protons. Thus, both hydroxyl groups of the seven-membered ring have equatorial orientation. The axial orientation of methyl H-17 was further supported by the absence of NOE effect between H-10/H-17. Finally, the spatial proximity of H-10 with H-12α and H-17 with H-13*β* suggest a chairlike conformation for the seven-membered ring.

On the basis of the above observations, and since there are not convincing enhancements connecting the streocenters C-6 and C-7, the relative stereochemistry of the novel brominated diterpene **3**, named prevezol D, was proposed as 2*R**,3*S**,6*R**,7*S**,8*S**,10*S**,11*S** or 2*R**,3*S**,6*R**,7*R**,8*R**,10*R**,11*R**.

Compound **4**, after HPLC purification, was isolated as a colorless oil. The composition of metabolite **4** was deduced to be $C_{20}H_{33}O_3Br$ from the HRFAB mass spectrum, and the NMR data (Table 2) suggested metabolite **4** to be an isomer of prevezol D (**3**). The general appearance of the NMR data showed a close resemblance between metabolites **4** and **3**. Comparison of the NMR spectra of compounds **4** and **3** indicated that the difference between these two structures lay on the position of the double bond (tetrasubstituted double bond $\Delta_{14,15}$ in **3**; exocyclic methylene $\Delta_{15,18}$ in **4**). This assumption was confirmed by HMBC experiments that revealed connectivities of vinylic protons H-18 (δ_H 4.68) with C-14 (δ_C 48.1) and C-16 (δ_C 45.6) signals, as well as protons H-16 with C-15 (δ_C 147.7) and H-14 (δ_H 1.60) with C-13 (δ_C 24.8).

The stereochemical configuration of the asymmetric centers was determined by analysis of the NOESY spectrum along with the coupling constants of the NMR spectrum and was found to be the same as in metabolite **3**. The NOE interactions between H-10 (δ_H 3.92)/H-14 (δ_H 1.60), H-10/H-8 (δ_H 0.95), and H-8/H-14 implied that these protons were cofacial (α). Noteworthy differences in the NOESY spectrum compared to metabolite **3** are the interaction between H-9*β*/H-12*β* and the absence of

TABLE 2. NMR Data of Compounds 3 and 4[a]

	prevezol D (**3**)				prevezol E (**4**)		
no.	δ_H ^{1}H	δ_C ^{13}C	HMBC	NOESY	δ_H ^{1}H	δ_C ^{13}C	NOESY
1α	2.18 (m)	34.6	H-5	H-1*β*, H-2, H-6, H-9α	2.18 (m)	34.7	H-1*β*, H-2, H-6, H-19
1*β*	1.97 (m)			H-1α, H-19	2.01 (m)		H-1α, H-19
2	4.15 (dd, 12.29, 4.10)	67.3	H-1α, H-1*β*, H-4*β*, H-20	H-1α, H-4α, H-6, H-20	4.14 (dd, 12.28, 4.43)	67.2	H-1α, H-6, H-20
3		70.4	H-1α, H-4*β*, H-5, H-20			70.4	
4*β*	2.08 (m)	37.7	H-5, H-20	H-4α, H-20	2.05 (m)	37.6	H-4α, H-20
4α	1.47 (m)			H-2, H-4*β*, H-5, H-6	1.60 (m)		H-4*β*, H-6
5	1.56 (m)	20.8	H-1α, H-4α	H-4α, H-6, H-19	1.55 (m)	21.0	H-6
6	1.10 (m)	50.3	H-1*β*, H-4*β*, H-8, H-19	H-1α, H-2, H-4α, H-5, H-8, H-16α	1.12 (m)	51.0	H-1α, H-2, H-4α, H-5, H-8, H-16α
7		34.3	H-8, H-19			37.3	
8	0.91 (m)	40.9	H-6, H-13α, H-16*β*, H-19	H-6, H-9α, H-10	0.95 (m)	43.7	H-6, H-9α, H-10, H-14, H-16α
9*β*	2.28 (m)	40.9	H-8, H-10	H-9α, H-17, H-19	1.59 (m)	43.0	H-12*β*, H-17, H-19
9α	2.05 (m)			H-1α, H-8, H-9*β*	2.08 (m)		H-8, H-19
10	3.82 (d, 11.61)	77.4	H-8, H-9α, H-17	H-8, H-12α, -OH (2.32)	3.92 (d, 10.58)	75.9	H-8, H-14
11		72.5	H-9*β*, H-10, H-12*β*, H-13α, H-17			72.8	
12*β*	2.02 (m)	38.0	H-9*β*, H-13α, H-17	H-12α, H-13α, H-13*β*, H-17	2.06 (m)	37.7	H-9*β*, H-12α, H-13, H-17
12α	1.53 (m)			H-10, H-12*β*, H-13α	1.47 (m)		H-12*β*, H-13, H-17, H-18
13	2.67 (dt, 15.02, 3.42) (-α) 1.79 (m) (-*β*)	24.9	H-12*β*	H-12*β*, H-12α, H-13*β*, H-18 H-12*β*, H-13α, H-17	1.78 (m)	24.8	H-12*β*, H-12α, H-14, H-18
14		127.2	H-12*β*, H-18		1.60 (m)	48.1	H-8, H-10, H-13, H-18
15		127.1	H-16α, H-18			147.7	
16α	1.92 (d, 12.63)	42.7	H-8, H-18, H-19	H-6, H-16*β*, H-18	1.86 (d, 12.29)	45.6	H-6, H-8, H-16*β*
16*β*	1.59 (m)			H-16α, H-19	2.02 (m)		H-16α, H-18, H-19
17	1.40 (s)	22.6	H-10	H-9*β*, H-12*β*, H-13*β*, -OH (2.32)	1.33 (s)	23.4	H-9*β*, H-12*β*, H-12α
18	1.60 (s)	19.9	H-16α, H-16*β*	H-13α, H-16α	4.68 (br s)	108.0	H-12α, H-13, H-14, H-16*β*, H-19
19	0.75 (s)	18.9	H-8, H-16α	H-1*β*, H-5, H-9*β*, H-16*β*	0.75 (s)	19.3	H-1α, H-1*β*, H-9α, H-9*β*, H-16*β*, H-18
20	1.30 (s)	30.5	H-4α	H-2, H-4*β*	1.30 (s)	30.5	H-2, H-4*β*
-OH	2.32 (s)			H-10, H-17	2.32 (s)		
-OH	1.87 (s)				1.87 (s)		

[a] All spectra were recorded in $CDCl_3$. Chemical shifts are expressed in ppm. *J* values in parentheses are in Hz.

nOe between H-10/H-12α and H-17/H-13*β*. These observations suggest a boat like conformation for the seven-membered ring.

The relative stereochemistry of metabolite **4**, named prevezol E, since there were no convincing NOE correlations between the stereocenters C-6 and C-7, was established as 2*R**,3*S**,6*R**,7*S**,8*R**,10*S**,11*S**,14*S** or 2*R**,3*S**,6*R**,7*R**,8*S**,10*R**,11*R**,14*R**.

Compound **5** was purified along with compound **6**, following chromatographic separations and was isolated as a colorless oil. Compound **5** was identified by comparison of its NMR spectra with previously reported data[5] as being neorogioldiol. Metabolite **6** was found to share the same molecular formula ($C_{20}H_{32}O_2Br_2$) with neorogioldiol and to exhibit similar spectral characteristics. Comparison of the spectral data of **6** (Table 3) with those of neorogioldiol (**5**) revealed a great similarity in the structures of the two compounds. The most significant differences between metabolites **6** and **5** were observed in the ^{1}H NMR chemical shifts of halomethine H-9 (δ_H 4.36 in **6**; δ_H 3.74 in **5**) and the methine H-10 (δ_H 2.59 in **6**; δ_H 2.70 in **5**), as well as in the ^{13}C NMR chemical shifts of the methyl carbon C-18 (δ_C 28.9 in **6**; δ_C 24.3 in **5**), the methine carbon C-9 (δ_C 51.8 in **6**; δ_C 54.3 in **5**), and the quaternary carbon C-11 (δ_C 78.0 in **6**; δ_C 80.5 in **5**). These data led to the assumption that compound **6** must be a stereoisomer of neorogioldiol (**5**).

The strong NOE effect between H-9/H-20 and H-9/H-12*β* (δ_H 1.78) indicated a cis orientation of these protons, while the observed coupling constant between H-9/H-8 (11.95 Hz) and H-9/H-10 (9.05 Hz) confirmed the axial configuration of H-9. Moreover, NOE interactions between H-8/H-19, H-14/H-19, and H-10/H-14 suggested cis (α) orientation for these protons and confirmed the cis fusion of the five-membered rings. The equatorial configuration of the methyl H-18 was confirmed from a strong NOE correlation between H-18 and H-10, as well as by the absence of an NOE effect between H-18 and H-9 (observed in neorogioldiol **5**) suggesting an inversion of configuration at C-11 in comparison to **5**.

The relative stereochemistry of metabolite **6**, named neorogioldiol B, since there are no unambiguous NOE enhancements linking stereocenters C-6 and C-8, was deduced as either 2*R**,3*S**,6*R**,8*S**,9*S**,10*R**,11*S**,14*R** or 2*R**,3*S**,6*R**,8*R**,9*R**,10*S**,11*R**,14*S**.

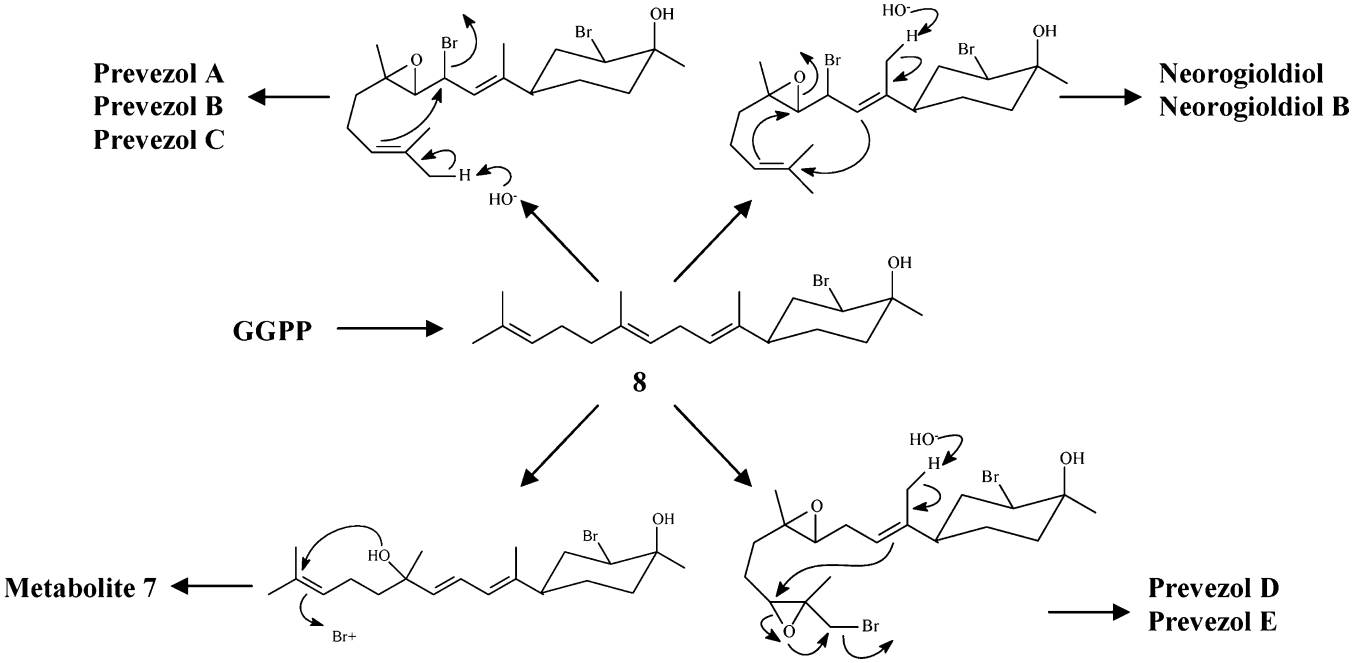
Compound **7** was purified by HPLC as a colorless oil and identified by comparison of its NMR spectra (Table 3) with literature values[5] as being the prenylbisabolane *O*,[11]15-cyclo-14-bromo-14,15-dihydrorogiol-3,11-diol. The

TABLE 3. NMR Data of Compounds 6 and 7[a]

	compound **6**			compound **7**	
no.	δ_H ^{1}H	δ_C ^{13}C	NOESY	δ_H ^{1}H	δ_C ^{13}C
1α	2.34 (m)	40.5	H-1*β*, H-2, H-6, H-8	2.08 (m)	38.7
1*β*	1.98 (m)		H-1α, H-17a	2.15 (m)	
2	4.09 (dd, 12.46, 4.26)	65.8	H-1α, H-4α, H-6, H-16	4.15 (dd, 12.12, 4.61)	65.7
3		70.3			70.2
4*β*	2.08 (m)	37.6	H-4α, H-5*β*, H-5α, H-16	2.04 (m)	37.4
4α	1.49 (m)		H-2, H-4*β*	1.49 (m)	
5*β*	1.85 (m)	26.3	H-4*β*, H-5α, H-17a	1.76 (m)	25.8
5α	1.60 (m)		H-4*β*, H-5*β*, H-6, H-8	1.48 (m)	
6	1.82 (m)	46.1	H-1α, H-2, H-5α, H-8, H-19	2.00 (m)	48.5
7		147.6			140.3
8	2.58 (d, 11.95)	67.4	H-1α, H-5α, H-6, H-19	5.80 (d, 10.59)	123.7
9	4.36 (dd, 11.95, 9.05)	51.8	H-12*β*, H-17b, H-20	6.25 (dd, 15.71, 10.59)	122.4
10	2.59 (dd, 11.95, 9.05)	61.0	H-14, H-18	5.62 (d, 15.71)	140.6
11		78.0			73.6
12*β*	1.78 (m)	43.7	H-9, H-12α, H-20	1.80 (m)	32.4
12α	1.63 (m)		H-12*β*	1.92 (m)	
13*β*	1.65 (m)	23.4	H-13α, H-20	2.23 (m)	27.6
13α	1.54 (m)		H-13*β*, H-14	2.12 (m)	
14	2.41 (m)	54.8	H-10, H-13α, H-19	4.07 (dd, 7.51, 3.41)	59.4
15		41.4			74.7
16	1.31 (s)	30.5	H-2, H-4*β*	1.32 (s)	28.8
17	5.20 (br s) (-a)	112.2	H-1*β*, H-5*β*, H-17b	1.31 (s)	30.5
	4.82 (br s) (-b)		H-9, H-17a, H-20		
18	1.42 (s)	28.9	H-10	1.71 (s)	14.9
19	0.99 (s)	30.8	H-6, H-8, H-14, H-20	1.29 (s)	29.3
20	0.73 (s)	19.6	H-9, H-12*β*, H-13*β*, H-17b, H-19	1.37 (s)	28.8

[a] All spectra were recorded in $CDCl_3$. Chemical shifts are expressed in ppm. *J* values in parentheses are in Hz.

SCHEME 1. Hypothetic Biogenetic Scheme for Compounds 1−7



assignment of the values for compound **7** was based on extensive analysis of the 1D and 2D NMR spectra and is reported here with a solid set of data to supplement literature misassignments due to the scarcity and instability of metabolite **7**.

All metabolites reported here could arise from the same speculative biogenetic sequence (Scheme 1) explaining the structural diversity of isolated *Laurencia* diterpenes sharing the hydroxylated bromocyclohexane ring.[5−7] Prevezols A, B (**2**), and C (**1**) could be biosynthesized from intermediate **8** by allylic bromination, epoxidation, subsequent nucleophilic attack of the isopropylidene double bond to form the second six-membered ring, and epoxide opening. The same intermediate **8** following abstraction of the allylic proton on C-17 by a basic enzyme can give rise to a subsequent cascade intramolecular cyclization to form the tricyclic stereoisomers neorogioldiol (**5**) and neorogioldiol B (**6**). Prevezols D (**3**) and E (**4**) could be formed by allylic bromination, bis epoxidation of the resulting intermediate with concomitant abstraction of the allylic proton, nucleophilic attack on the less substituted side of the epoxide ring that would lend to trans-

TABLE 4. In Vitro Antitumor Activities of the Compounds against Various Cell Lines

		IC_{50} (μM)				
cell line	origin	**1**	**2**	**3**	**4**	**6**
MCF7	breast	140.5	135.6	>200	>200	172.3
PC3	prostate	158.8	80.4	>200	>200	50.8
HeLa	cervix	80.5	78.0	120.6	>200	34.4
A431	epidermis	78.4	65.2	135.5	>200	65.8
K562	myelogenic	123.5	76.4	156.9	>200	76.4

position of the epoxide, and final closure of the third ring. Metabolite **7** can be seen as the product of dehydrogenation, hydroxylation, and nucleophilic attack of the unpaired electrons of the hydroxyl group.

The new metabolites were evaluated for their cytotoxicity against five human cell lines. The results of the antitumor activity of the test compounds are given in Table 4. Compounds **2** and **6** were consistently more potent as cytotoxic agents than metabolites **1**, **3**, and **4** for the majority of the cell lines tested and showed an inhibitory activity at doses lower than 100 μM. Comparing the cell lines, PC3 and HeLa cells were more sensitive to compound **6** as shown by the IC_{50} values (50.8 and 34.4, respectively). Compound **1** showed significant cytotoxity only against HeLa and A431 cell lines. Compound **3** was moderately active, and compound **4** was inactive against all cell lines tested with an IC_{50} value higher than 200 μM.

Cell lines PC3 and K562 were proven sensitive to the relative stereochemistry of the diol structural motif, as metabolite **2** was twice as active compared to the stereoisomer **1**. Comparing metabolites **3** and **4** is clear that the exocyclic methylene abolishes activity, since the cyclopentene ring is relatively active against HeLa, A431 and K562 cell lines. Comparison of the two most active metabolites (**2** and **6**) reveals as common structural feature besides the presence of the brominated cyclohexane ring and the methylene substituent, the *S** configuration of the quaternary hydroxylated carbon C-13 and C-11, respectively.

For clarity, SEM values are not included in the tables, but they were in all cases less than 5% of the mean.

Experimental Section

General Experimental Procedures. General experimental details are the same as those reported in Iliopoulou et al.[11]

Plant Material. The alga was collected by hand at Preveza in the Ionean Sea, Greece, at a depth of 0.5−1 m during the summer of 2000. A voucher specimen is kept at the Herbarium of the Pharmacognosy Laboratory, University of Athens.

Extraction and Isolation. The alga was initially freeze-dried (151.4 g dry weight) and then exhaustively extracted at room temperature with mixtures of CH_2Cl_2/MeOH (2/1). The organic extract after evaporation of the solvents afforded a dark oily residue (5.4 g). The crude extract was subjected to VCC on Si gel using cyclohexane with increasing amounts (10%) of EtOAc and finally MeOH. The IV_a and V_a fractions (30% and 40% EtOAc in cyclohexane) (780 mg) was further purified by VCC on Si gel using cyclohexane with increasing amounts (5%) of EtOAc. Fractions IX_b (40% EtOAc) (80.3 mg) and XI_b (50% EtOAc) (158.2 mg) were subjected to normal-phase HPLC chromatography, using as mobile phase cyclohexane/EtOAc (85/15), to yield pure compounds **1** (9.4 mg), **2** (6.7 mg), **3** (7.5 mg), **4** (2.5 mg), **5** (1.5 mg), **6** (2.5 mg), and **7** (3.2 mg).

Conditions of Cell Culture. Cells were grown as monolayer cell cultures in Dulbecco's Modified Eagle's Medium (containing 4.5 g glucose/lt) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin) at 37 °C in an incubator with humidified atmosphere and 5% CO_2. Cells were passaged by trypsinisation 1−2 times a week to keep them in log phase. Chronic myelogenous leukaemia derived K-562 cells were grown as suspension culture in RPMI 1640 medium under the same conditions.

Determination of Cytotoxicity. Cells were seeded into 96-well plates (100μL/well at a density of 1×10^5cells/mL) and exposed to various concentrations of the compounds for 72 h. The cytotoxicity was determined with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye reduction assay[14] as previously modified.[15] Briefly, after incubation with the test compounds, MTT solution (5 mg/mL in PBS) was added (20 μL/well). Plates were further incubated for 4 h at 37 °C, and the formazan crystals formed were dissolved by adding 100 μL/well of 0.1 N HCl in 2-propanol. Absorption was measured by an enzyme-linked immunosorbant assay (ELISA) reader at 545 nm, with reference filter at 690 nm. For each concentration at least nine wells were used from three separate experiments. One hundred microliters of RPMI 1640 supplemented with the same amount of MTT solution and solvent was used as blank solution. Data obtained were presented as IC_{50} (μM), which is the concentration of the compound where $100(A_0 - A)/A_0 = 50$. In this formula, A is the optical density of the wells after 72 h of exposure to test compound and A_0 is the optical density of the control wells.

Compound 1: colorless oil; $[\alpha]_D = -13.57$ (*c* 0.28, $CHCl_3$); UV λ_{max} (*n*-hexane) (log ϵ) 201.3 (3.85) (nm); IR ($CHCl_3$) ν_{max} 3614, 2973, 1641, 1450, 1377, 1163, 1102, 1022, 907 cm^{-1}; HRFAB-MS (*m/z*) 365.1494 $[M + 1 - 2H_2O]^+$ (calcd for $C_{20}H_{30}O^{79}Br$ 365.1481); NMR data ($CDCl_3$), see Table 1; EIMS 70 eV, *m/z* (rel int) 384, 382 $[M - H_2O]^+$ (16:16), 366, 364 $[M - 2H_2O]^+$ (5:5), 313 (36), 284 (66), 267 (31), 191 (80), 173 (69), 135 (95), 109 (100), 79 (82), 43 (98).

Compound 2: colorless oil; $[\alpha]_D = +33.33$ (*c* 0.09, $CHCl_3$); UV λ_{max} (*n*-hexane) (log ϵ) 208 (3.60) (nm); IR (film) ν_{max} 3437 (broad), 2948, 1643, 1439, 1377, 1166, 1111, 1007, 907 cm^{-1}; HREI-MS (*m/z*) 382.1507 $[M - H_2O]^+$ (calcd for $C_{20}H_{31}O_2{}^{79}Br$ 382.1508); NMR data ($CDCl_3$), see Table 1; EIMS 70 eV, *m/z* (rel int) 384, 382 $[M - H_2O]^+$ (0.5:0.5), 366, 364 $[M - 2H_2O]^+$ (0.8:0.8), 313 (3), 285 (11), 267 (9), 191 (30), 173 (46), 133 (79), 122 (98), 93 (96), 43 (100).

Compound 3: colorless oil; $[\alpha]_D = +17.95$ (*c* 0.39, $CHCl_3$); UV λ_{max} (*n*-hexane) (log ϵ) 211 (3.25) (nm); IR (film) ν_{max} 3437 (broad), 2935, 1647, 1631, 1452, 1379, 1107, 1004, 733 cm^{-1}; HREI-MS (*m/z*) 382.1518 $[M - H_2O]^+$ (calcd for $C_{20}H_{31}O_2{}^{79}Br$ 382.1508); NMR data ($CDCl_3$), see Table 2; EIMS 70 eV, *m/z* (rel int) 384, 382 $[M - H_2O]^+$ (36:37), 366, 364 $[M - 2H_2O]^+$ (2:2), 341, 339 (3:3), 285 (55), 243 (41), 189 (100), 173 (65), 147 (35), 107 (52), 91 (35), 43 (73).

Compound 4: colorless oil; $[\alpha]_D = +8$ (*c* 0.20, $CHCl_3$); UV λ_{max} (*n*-hexane) (log ϵ) 230.2 (3.12) (nm); IR ($CHCl_3$) ν_{max} 3683, 3584, 3564, 2938, 1605, 1457, 1380, 1163, 1104, 924 cm^{-1}; HRFAB-MS (*m/z*) 383.1629 $[M + 1 - H_2O]^+$ (calcd for $C_{20}H_{32}O_2{}^{79}Br$ 383.1586); NMR data ($CDCl_3$), see Table 2; EIMS 70 eV, *m/z* (rel int) 384, 382 $[M - H_2O]^+$ (12:12), 366, 364 $[M - 2H_2O]^+$ (6:6), 341, 339 (20:20), 285 (49), 241 (33), 191 (49), 173 (41), 147 (35), 119 (60), 91 (41), 43 (100).

Compound 6: colorless oil; $[\alpha]_D = -39.28$ (*c* 0.14, $CHCl_3$); UV λ_{max} (*n*-hexane) (log ϵ) 202 (3.84) (nm); IR ($CHCl_3$) ν_{max} 3596, 2929, 1645, 1456, 1377, 1243, 1182, 1102, 898 cm^{-1}; HRFAB-MS (*m/z*) 365.1456 $[M + 1 - H_2O - HBr]^+$ (calcd for $C_{20}H_{30}O^{79}Br$ 365.1481); NMR data ($CDCl_3$), see Table 3; EIMS 70 eV, *m/z* (rel int) 384, 382 $[M - HBr]^+$ (1.2:1.2), 351, 349

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(1.6:1.6), 326, 324 (100:100), 285 (12), 227 (43), 211 (11), 159 (12), 133 (20), 119 (22), 91 (22), 43 (35).

Acknowledgment. Financial support from the Greek General Secretariat for Research and Technology is gratefully acknowledged.

Supporting Information Available: ^{1}H and ^{13}C spectra for compounds **1**−**7**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO0342323