Brominated Diterpenes with Antibacterial Activity from the Red Alga *Sphaerococcus coronopifolius*

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Received March 22, 2008

Four new brominated diterpenes (**1**, **2**, **4**, **5**), along with two previously reported metabolites (**3**, **6**), were isolated from the organic extract of *Sphaerococcus coronopifolius*, collected in Palaiokastritsa Bay at the west coast of Corfu Island. The structures of the new products, as well as their relative configuration, were established by means of spectroscopic data analyses, including 2D NMR experiments. The isolated metabolites were evaluated for their antibacterial activity against a panel of multidrug-resistant (MDR) and methicillin-resistant *Staphylococcus aureus* (MRSA) with MICs in the range 0.5−128 μg/mL.

Diterpenes are widespread metabolites in marine brown algae, but are much less common in Rhodophyta,[1] having been found mainly in species of the genus *Laurencia*[2] and in the unrelated species *Sphaerococcus coronopifolius*. Particularly, the latter yields an extended variety of interesting diterpenes having di-, tri-, or tetracyclic skeletons, often rearranged, most of which contain one or more bromine atoms.[3] These halogenated metabolites have been suggested to function as chemical defense against marine herbivores.[4–6] Moreover some of these halogenated metabolites have been proven to possess antimalarial,[3] insecticidal,[7] antibacterial,[8] antifungal,[9] and antiviral activities.[10]

In the course of our ongoing investigations toward the isolation of bioactive metabolites from marine organisms of the Greek seas,[11–13] we recently studied the chemical composition of the red alga *S. coronopifolius*, collected from the west coast of Corfu Island. In this paper we describe the isolation and structure elucidation of four new metabolites (**1**, **2**, **4**, and **5**), along with the already described metabolites **3**[14] and **6** (1*S*-hydroxy-1,2-dihydrobromosphaerol),[15] all of which were obtained from the organic extract of *S. coronopifolius*. The structures of the new metabolites were elucidated by extensive spectroscopic analyses and their relative configuration was determined by NOESY experiments. Moreover, detailed analyses of the 1D and 2D NMR spectra allowed the revision of the structure for metabolite **3** and the full assignment of the ^{13}C and ^{1}H data, which have not been reported before for **6**.

All compounds were evaluated for antibacterial activity against a panel of multidrug-resistant (MDR) and methicillin-resistant *Staphylococcus aureus* (MRSA) using a microtiter plate based minimum inhibitory concentration (MIC) assay. The metabolites, specifically **4** and **6**, were found to possess highly significant activity in comparison to the standard antibiotic norfloxacin.

Results and Discussion

S. coronopifolius was collected in Palaiokastritsa Bay on the west side of Corfu Island, and the CH_2Cl_2/MeOH extract of the freeze-dried alga was subjected to a series of gravity column chromatography fractionations on silica gel using mixtures of cyclohexane/EtOAc as mobile phase, as well as normal- and reversed-phase high-pressure liquid chromatography (HPLC) separations, using mixtures of *n*-hexane/$CHCl_3$ or CH_3CN, respectively, as eluent, to yield compounds **1**−**6** in pure form.

Compound **1** was isolated after purification by HPLC as a colorless oil. The molecular formula $C_{20}H_{32}Br_2O_2$ was deduced from the HRFABMS in combination with the NMR data (Table 1). The LRCI-MS ions at *m*/*z* 445/447/449 $[MH - H_2O]^+$, with relative intensities 1.4/2.0/1.0, and at *m*/*z* 347/349 $[MH - 2H_2O - HBr]^+$ with relative intensities 1.0/1.0 indicated the presence of two bromine atoms. In the IR spectrum, the intense and broad band at ν_{max} 3377 cm^{-1} indicated the presence of a hydroxyl group in the molecule. The ^{13}C NMR spectrum exhibited 20 signals, which by DEPT spectra corresponded to four quaternary carbons, six methines, six methylenes, and four methyls. The ^{1}H and ^{13}C NMR spectra displayed resonances for two methyls ($\delta_{H/C}$ 0.82/18.7; 0.97/24.8) of an isopropyl group linked to a methine ($\delta_{H/C}$ 1.75/25.9) bonded to another methine ($\delta_{H/C}$ 1.89/41.1), two methyls attached to quaternary carbons ($\delta_{H/C}$ 1.18/15.3 and 1.36/29.6), one oxygenated methine ($\delta_{H/C}$ 4.28/68.9), one halomethine ($\delta_{H/C}$ 3.97/67.7), one trisubstituted double bond ($\delta_{H/C}$ 6.45/133.0 and δ_C 137.2), one aliphatic methine ($\delta_{H/C}$ 1.88/51.2), one halomethylene ($\delta_{H/C}$ 3.81, 3.44/38.7), and five aliphatic methylenes ($\delta_{H/C}$ 1.85, 1.26/28.3; 1.85, 1.35/27.8; 1.87, 1.34/38.0; 1.61, 1.60/44.8; and 2.43, 2.00/29.8). With four degrees of unsaturation, the structure was expected to contain three rings in addition to the double bond. All protonated carbons and the corresponding protons were assigned by COSY and HMQC experiments. The NMR data comparison of **1** with reported values for bromosphaerodiol[16] suggested that metabolite **1** was its 2-hydroxy-$\Delta^{1,10}$ isomer. The correlation in the HMBC experiments, from H_3-19 and H_3-20 (δ_H 0.82 and 0.97) to C-4 (δ_C 41.1), confirmed the position of the isopropyl group. The hydroxyl group was positioned on C-2, as deduced from the correlations of C-2 (δ_C 68.9) with H-3α (δ_H 1.26) and of C-1 (δ_C 133.0) with H-2 (δ_H 4.28). Moreover, the strong correlations between C-1 (δ_C 133.0) and H-9 (δ_H 1.88) as well as the correlations of H-1 (δ_H 6.45) with C-3 (δ_C 28.3), C-9 (δ_C 51.2), and the quaternary carbon C-5 (δ_C 45.5) established the position of the olefinic proton at C-1. The correlation of H-17a (δ_H 3.81) with C-4 (δ_C 41.1), C-5 (δ_C 45.5), and C-6 (δ_C 27.8) and of H-17b (δ_H 3.44) with C-4 (δ_C 41.1), C-5 (δ_C 45.5), and C-10 (δ_C 137.2) secured the position of the bromomethyl group at C-5. The additional bromine atom was positioned on C-14, as concluded by the correlations of C-14 (δ_C 67.7) with H-9 (δ_H 1.88), H_2-12 (δ_H 1.61, 1.60), H-13α (δ_H 2.43), and H_3-15 (δ_H 1.26). Moreover, the correlations of H_3-15 (δ_H 1.18) with C-8 (δ_C 42.6), C-7 (δ_C 38.0), C-9 (δ_C 51.2), and C-14 (δ_C 67.7) and of H_3-16 (δ_H 1.36) with C-11 (δ_C 72.3), C-9 (δ_C 51.2), and C-12 (δ_C 44.8) confirmed the positions of the remaining methyl groups. The relative configuration of **1** was assigned on the basis

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10.1021/np8001817 CCC: $40.75
Published on Web 07/03/2008

Table 1. NMR Data[a] of Compounds **1**−**3**

	1					**2**					**3**		
no.	^{1}H (δ)	m (J)	NOESY	^{13}C (δ)	HMBC (C→H)	^{1}H (δ)	m (J)	NOESY	^{13}C (δ)	HMBC (C→H)	^{1}H (δ)	m (J)	^{13}C (δ)
1	6.45	br s	2, 15, 16	133.0	2, 9	6.57	br s	2, 15, 16	129.0	9	6.46	br s	127.8
2	4.28	dd 10.0, 5.0	1, 3β, 4	68.9	3α, 3β/4	4.60	dd 10.4, 5.0	1, 3β, 4	81.9	3α, 4	4.61	br dd 10.4, 5.4	82.1
3	β 1.85	m	2	28.3	1, 4, 18	β 1.87	m	2	22.4	1, 4, 18	a 1.88	m	22.7
	α 1.26	m	19			α 1.49	m	19			b 1.47	m	
4	1.89	m	2, 17b	41.1	3α, 17a, 17b, 19, 20	1.94	m	2, 17b, 20	40.9	17a, 17b, 18, 19, 20	1.93	m	40.9
5				45.5	1, 3β/4, 17a, 17b, 18				45.7	1, 4, 9, 18, 3β/7α			46.1
6	α 1.85	m	15	27.8	7α, 17a	α 1.88	m	15	27.6		a 1.86	m	27.8
	β 1.35	m	17b, 18, 19			β 1.35	m	18			b 1.35	m	
7	α 1.87	m	14, 17a	38.0	6β, 15	α 1.86	m	9, 14, 17a	37.5	15	a 1.82	m	37.7
	β 1.34	m				β 1.27	m				b 1.39	m	
8				42.6	7β, 9, 15				42.6	9, 15			42.8
9	1.88	m	12β, 14, 16, 17a	51.2	1, 7α, 12, 15, 16	1.80	br s	7α, 12, 14, 17a	49.5	1, 15, 16	2.37	br s	45.8
10				137.2	9, 17b				138.5	9, 17b			139.6
11				72.3	9, 12, 16				74.1	9, 16			74.7
12	β 1.61	m	9, 14, 16 13α	44.8	13α, 16	3.36	dd 12.0, 4.2	9, 13β, 14, 16	75.9	13α, 13β, 16	3.40	dd 3.7, 2.5	78.1
	α 1.60	m											
13	α 2.43	tdd 13.2, 10.0, 7.4	12α, 15	29.8	12	α 2.40	td 12.4, 12.0	15	37.6		α 2.71	ddd 13.3, 13.3, 2.5	36.4
	β 2.00	dtd 13.2, 3.7, 3.3	14			β 2.20	dt 12.4, 4.2	12, 14			β 2.14	ddd 13.3, 4.2, 3.7	
14	3.97	dd 13.2, 3.7	7β, 9, 12β, 13β	67.7	9, 12, 13α, 15	3.91	dd 12.4, 4.2	7β, 9, 12, 13β	62.2	9, 13α, 13β, 15	4.44	dd 13.3, 4.2	62.4
15	1.18	s	1, 6α, 13α	15.3	9, 14	1.13	s	1, 6α, 13α	15.0	9	1.14	s	15.1
16	1.36	s	1, 9, 12β	29.6		1.41	s	1, 12	24.8		1.43	s	25.8
17	a 3.81	d 10.8	7β, 9	38.7	6α	a 3.78	d 11.2	7β, 9	38.2		a 3.91	d 11.2	38.5
	b 3.44	d 10.8	4, 6β, 18			b 3.46	d 11.2	4, 18			b 3.46	d 11.2	
18	1.75	br hept 6.6	6β, 17b, 19, 20	25.9	19, 20	1.78	br hept 6.6	6β, 17b, 19, 20	26.1	19, 20	1.79	m	26.0
19[b]	0.82	d 6.6	3α, 6β, 18	18.7	4, 18, 20	0.83	d 6.6	3α, 18	18.7	4, 18, 20	0.83	d 7.0	18.7
20[b]	0.97	d 6.6	18	24.8	4, 18, 19	0.99	d 6.6	4, 18	24.7	4, 18, 19	0.99	d 7.0	24.7

[a] ^{1}H (400 MHz) and ^{13}C NMR (50.3 MHz) recorded in $CDCl_3$ (δ_H 7.24, δ_C 77.0); chemical shifts are expressed in ppm and J values in Hz. [b] Positions can be interchanged.

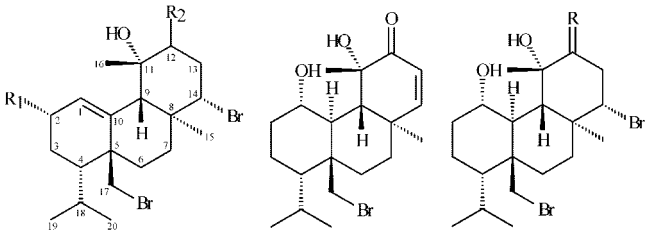

Figure 1. Metabolites isolated from *S. coronopifolius*.

of NOESY data. The strong NOE correlations between H-14/H-9, H-14/H-13β, H-14/H-7β, H-14/H-12β, H-12β/H-9, H-9/H_3-16, H-9/H-17a, H-17a/H-7β, H-17b/H-4, H-17b/H-6β, and H_3-16/H-12β determined the configuration at C-4, C-5, C-9, C-11, and C-14. The strong NOE correlations between H_3-15/H-13α and H-6α/H_3-15 established the configuration at C-8. The NOE correlations between H-2/H-3β and H-2/H-4 determined the configuration at C-4. The large coupling constant values of H-13α and H-14 supported the *trans*-diaxial orientation of these protons. In view of the above-mentioned data metabolite **1** was identified as 2*S*-hydroxyisobromosphaerol (Figure 1).

Compound **2** was purified by means of HPLC and isolated as a white solid. A combination of its NMR data (Table 1) and HRFABMS measurements suggested the molecular formula $C_{20}H_{32}Br_2O_4$. The LRCI-MS peaks at *m/z* 477/479/481 $[MH - H_2O]^+$, with relative intensities 1.1/2.0/1.0, and at *m/z* 459:461:463 $[MH - 2H_2O]^+$, with relative intensities 1.0/1.9/1.3, indicated the presence of two bromine atoms. The ^{13}C NMR spectrum of **2** exhibited signals for 20 carbon atoms with the multiplicities of the carbon signals determined from the DEPT spectra as four singlets, seven doublets, five triplets, and four quartets. Strong IR absorptions at ν_{max} 3422 cm^{-1} and ^{13}C NMR signals at δ_C 74.1 (C-11) and 75.9 (C-12) indicated the presence of hydroxyl groups. An additional oxygenated carbon resonated at lower fields [δ_C 81.9 (C-2)], characteristic for the presence of a hydroperoxy group. Among the other carbons, two were olefinic, resonating at δ_C 129.0 (C-1) and 138.5 (C-10), and two were brominated, resonating at δ_C 62.2 (C-14) and 38.2 (C-17). Furthermore, the ^1H NMR spectra revealed signals due to an olefinic proton at δ_H 6.57 (H-1), one halomethine proton at δ_H 3.91 (H-14), two halomethylene protons at δ_H 3.78 and 3.46 (H-17a and H-17b), two oxygenated methine protons at δ_H 4.60 (H-2) and 3.36 (H-12), two methyls of an isopropyl group at δ_H 0.83 (H_3-19) and 0.99 (H_3-20) attached to a methine at δ_H 1.78 (H-18), and two singlet methyls at δ_H 1.13 (H_3-15) and 1.41 (H_3-16). All protonated carbons and their protons were assigned on the basis their COSY and HMQC correlations. The structure elucidation was assisted by analyses of the HMBC experiments. Based on the correlations of C-2 (δ_C 81.9) with H-3α (δ_H 1.49) and H-4 (δ_H 1.94) and of H_2-13 methylene protons (δ_H 2.40 and 2.20) and H_3-16 (δ_H 1.41) with C-12 (δ_C 75.9), observed in the HMBC spectrum, the hydroperoxy- and hydroxymethines were placed at C-2 and C-12, respectively. The position of the olefinic bond between C-1 and C-10 was established from correlations of H-1 (δ_H 6.57) with C-3 (δ_C 22.4) and C-9 (δ_C 49.5), as well as from the correlations of C-1 (δ_C 129.0) and C-10 (δ_C 138.5) with H-9 (δ_H 1.80). The correlation of H_3-19 (δ_H 0.83) and H_3-20 (δ_H 0.99) with C-4 (δ_C 40.9) confirmed the position of the isopropyl group on C-4. The correlation of H-17a (δ_H 3.78) with C-4 (δ_C 40.9), H-17b (δ_H 3.46) with C-4 (δ_C 40.9) and C-10 (δ_C 138.5), and C-5 (δ_C 45.7) with H-1 (δ_H 6.57), H-4 (δ_H 1.94), H-9 (δ_H 1.80), and H-18 (δ_H 1.78) secured the position of the bromomethyl group on C-5. The position of the second bromine atom at C-14 was indicated by the correlation of C-14 (δ_C 62.2) with H_3-15 (δ_H 1.13) and H_2-13 (δ_H 2.40 and 2.20). Moreover the correlations of H_3-15 (δ_H 1.13) with C-8 (δ_C 42.6), C-7 (δ_C 37.5), C-9 (δ_C 49.5), and C-14 (δ_C 62.2) and of H_3-16 (δ_H 1.41) with C-11 (δ_C 74.1), C-9 (δ_C 49.5), and C-12 (δ_C 75.9) confirmed the positions of these methyl groups. Comparison of the NMR data of **2** with literature data showed a close similarity with those of the previously reported 2*S*,12*S*-dihydroxyisobromosphaerol.[14] The above-mentioned data led us to assign **2** as 2*S*-hydroperoxy-12*R*-hydroxyisobromosphaerol. The proposed structure was confirmed by NOESY correlations (Figure 2). The NOE correlations between H-14/H-9, H-14/H-13β, H-14/H-7β, H-14/H-12, H-13β/H-12, H-9/H-12, H-9/H-7β, H-9/H-17a, H-17a/H-7β, and H-17b/H-4 determined the configuration at C-4, C-5, C-9, C-14, and C-12. The NOE correlations between H_3-15/H-13α and H_3-15/H-6α determined the configuration at C-8. The absence of any correlation between H_3-15/H_3-16 and the NOE correlations between H_3-16/H-1 and H_3-16/H-12 confirmed the equatorial orientation of H_3-16. The NOE correlations between H-2/H-4, H-2/H-3β, and H-3α/H_3-19 and the absence of any correlation between H-2/H-3α established the configuration at the oxygenated carbon C-2. The large coupling constants of H-12, H-13α, and H-14 supported the axial orientation of these protons.

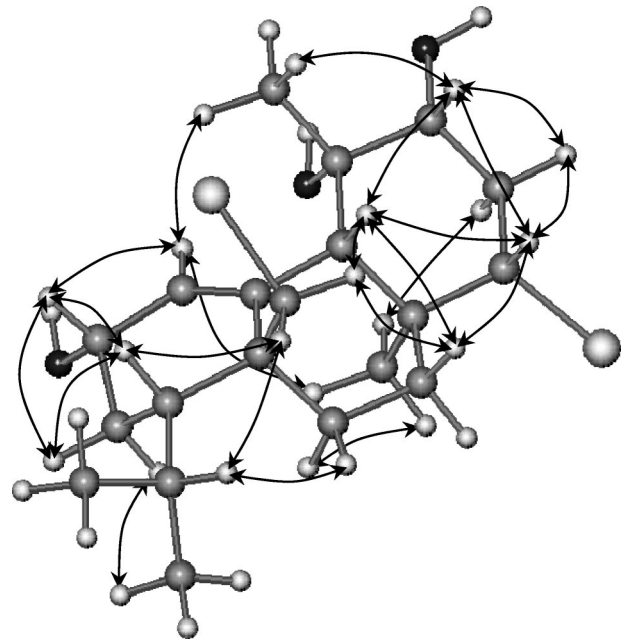

Figure 2. NOE correlations for compound **2**.

Compound **3** after purification by HPLC was isolated as a white solid. Comparison of its NMR (Table 1) and MS spectra with literature data showed them to be almost identical to those for 2*S*,12*S*-dihydroxyisobromosphaerol.[14] However, the fact that C-2 resonated downfield (δ_C 81.9) when compared with 2*S*-hydroxyisobromosphaerol (**1**) (C-2 signal at δ_C 68.9) was again indicative of the presence of a hydroperoxy group at C-2, as seen in **2**. Additionally, several ^{13}C and ^1H NMR chemical shifts were reassigned. On the basis of the above evidence, metabolite **3** was characterized as 2*S*-hydroperoxy-12*S*-hydroxyisobromosphaerol.

Compound **4** was purified by means of HPLC separations and was isolated as a colorless oil. Both ^{13}C NMR data and HRFABMS measurements supported the molecular formula $C_{20}H_{31}BrO_3$. The LREIMS showed $[M - H_2O]^+$ peaks at *m/z* 380/382 with intensities 1.0/1.0, indicating the presence of one bromine atom. The presence of a carbonyl group was evident from the intense IR band at 1698 cm^{-1}, while absorptions at ν_{max} 3468 cm^{-1} indicated the presence of hydroxyl groups in the molecule. The ^{13}C NMR spectrum of **4** (Table 2) exhibited signals for 20 carbons, with the multiplicities of the carbons determined from the DEPT spectra as four quaternary carbons, seven methines, five methylenes, and four methyls. Among

Table 2. NMR Data[a] of Compounds **4**−**6**

	4					**5**					**6**		
no.	^{1}H (δ)	m (J)	NOESY	^{13}C (δ)	HMBC (C→H)	^{1}H (δ)	m (J)	NOESY	^{13}C (δ)	HMBC (C→H)	^{1}H (δ)	m (J)	^{13}C (δ)
1	3.61	ddd 10.4, 10.4, 4.6	2β, 16	75.6	3α/9, 10	3.48	m	2β, 9, 16	74.8	2β, 3α, 3β, 9/10	3.99	m	67.3
2	β 2.00	m	1	29.2		β 1.99	m	1, 3β	29.0		a 1.91	m	34.0
	α 1.46	m	10, 19/20			α 1.46	m	b 1.52		m			
3	α 1.89	m	19/20	23.3		α 1.88	m	20	23.0	2α	a 1.66	m	18.8
	β 1.40	m	17b			β 1.42	m	2β, 4, 17b			b 1.50	m	
4	1.78	m		44.3	17a, 19, 20	1.78	m	3β, 17b	43.7	2β/6β, 17a, 19, 20	1.72	m	43.3
5				43.1	6a, 6b, 7				42.4	3α, 17b			43.9
6	a 1.92	m		27.4	7, 17b	β 1.96	m	7β, 17a, 19	27.6	17a, 17b	α 1.74	m	27.7
	b 1.80	m				α 1.73	m	15, 20			β 1.58	m	
7	a 1.64	m		34.0	6a, 6b, 14, 15	α 1.71	m	6β, 14, 17a	35.6	15	α 1.82	dt 13.4, 4.1	36.7
	b 1.64	m				β 1.31	m				β 1.21	ddd 13.4, 12.9, 4.1	
8				38.0	7, 13, 14, 15				42.4	13β, 14, 15			41.7
9	1.88	m	17a	50.0	7, 10, 14, 15, 16	1.67	m	1, 7β/16, 14, 17a	53.8	7α, 10, 14, 15, 16	1.60	d 10.2	52.3
10	1.86	m	2α, 15, 19/20	48.5	2β, 6a	1.67	m	15, 20	49.6	2α, 4, 6β, 9	2.27	dd 10.2, 9.9	46.1
11				84.2	10, 13, 16				83.9	9/10, 13α, 16			72.1
12				200.6	14,16				205.8	13α, 13β, 16	α 1.78	dt 14.3, 3.2	45.4
											β 1.59	m	
13	5.97	d 9.6		129.9		β 3.19	dd 18.4, 8.2	14, 16	47.0	14	α 2.42	qd 13.1, 3.3	30.0
						α 2.85	dd 18.4, 9.4	15			β 1.90	m	
14	6.88	d 9.6		153.9	15	4.29	dd 9.4, 8.2	7β, 9, 13β	54.8	13α, 13β, 15	3.98	m	69.9
15	0.99	s	10	24.9	7, 9	0.93	s	6α, 10, 13α	13.1	7β, 9, 14	1.31	s	14.5
16	1.35	s	1	27.7	9	1.32	s	1, 13β	25.6	9	1.36	s	32.3
17	a 3.79	d 10.4	9	38.8	6b, 10	a 3.73	d 10.2	6β, 7β, 9	38.0	10	a 3.96	d 10.5	40.9
	b 3.58	dd 10.4, 2.1	3β			b 3.53	dd 10.2, 2.0	3β, 4			b 3.49	br d 10.5	
18	1.70	br hept 6.6		26.1	19, 20	1.65	m		25.9	4, 19, 20	1.97	m	26.5
19[b]	0.98	d 6.6	(2α, 3α, 10)	21.3	20	0.97	d 6.6	6β	21.2	20	1.04	d 6.7	21.2
20[b]	0.95	d 6.6	(2α, 3α, 10)	26.0	19	0.95	d 6.6	3α, 10, 6α	26.0	19	1.01	d 6.7	25.5

[a] ^{1}H (400 MHz) and ^{13}C NMR (50.3 MHz) recorded in $CDCl_3$ (δ_H 7.24, δ_C 77.0); chemical shifts are expressed in ppm and J values in Hz. [b] Positions can be interchanged.

the carbons, one was a carbonyl, resonating at δ_C 200.6 (C-12), two were olefinic, resonating at δ_C 129.9 (C-13) and 153.9 (C-14), one was brominated, resonating at δ_C 38.8 (C-17), and two were oxygenated, resonating at δ_C 84.2 (C-11) and 75.6 (C-1). The proton resonances at δ_H 5.97 (H-13) and 6.88 (H-14) displayed in the ^{1}H NMR spectra defined the isolated AB system of an α,β-unsaturated ketone. Furthermore, the spectra displayed signals corresponding to one oxygenated methine proton at δ_H 3.61 (H-1), two halomethylene protons at δ_H 3.79 and 3.58 (H-17a and H-17b), two methyls of an isopropyl group at δ_H 0.98 (H_3-19) and 0.95 (H_3-20) bonded to a methine at δ_H 1.70 (H-18), and two methyls linked to quaternary carbons at δ_H 0.99 (H_3-15) and 1.35 (H_3-16). With five degrees of unsaturation, the structure was expected to contain three rings in addition to the carbonyl group and the double bond. All protonated carbons and their protons were assigned by COSY and HMQC experiments. The NMR data comparison of **4** with those of sphaerococcenol-A[17,18] suggested that metabolite **4** was its 1-hydroxy-1,2-dihydro derivative. On the basis of the correlations of carbonyl C-12 (δ_C 200.6) with H-14 (δ_H 6.88) and H_3-16 (δ_H 1.35), of H-13 (δ_H 5.97) with the quaternary carbons C-8 (δ_C 38.0) and C-11 (δ_C 84.2), and of H-14 (δ_H 6.88) with C-7 (δ_C 34.0), C-8 (δ_C 38.0), and C-9 (δ_C 50.0), observed in the HMBC spectrum, the carbonyl group was placed at C-12 and the double bond between C-13 and C-14. The correlations between H_3-19 and H_3-20 (δ_H 0.98 and 0.95) with C-4 (δ_C 44.3) confirmed the position of the isopropyl group on C-4. The correlation of H-17a (δ_H 3.79) with C-4 (δ_C 44.3), of H-17b (δ_H 3.58) with C-6 (δ_C 27.4), and of carbon C-17 (δ_C 38.8) with protons H-6a (δ_H 1.92) and H-10 (δ_H 1.86) secured the position of the bromomethyl group at C-5. The oxygenated methine was positioned at C-1, as concluded by the correlations of C-1 (δ_C 75.6) with H-9 (δ_H 1.88) and/or H-3α (δ_H 1.89) and H-10 (δ_H 1.86). Moreover the correlations of H_3-15 (δ_H 0.99) with C-8 (δ_C 38.0), C-7 (δ_C 34.0), C-9 (δ_C 50.0), and C-14 (δ_C 153.9) and of H_3-16 (δ_H 1.35) with C-11 (δ_C 84.2), C-9 (δ_C 50.0), and C-12 (δ_C 200.6) confirmed the positions of the remaining methyl groups. The relative configuration of **4** was assigned on the basis of the NOE experiments. The correlations between H-10/H_3-15 and H-10/(H_3-19 or H_3-20) and H-10/H-18, observed in the NOESY spectra, determined the configuration at C-4, C-8, and C-10. The NOE correlations between H-17a/H-9, H-17b/H-3β, and H-3α/(H_3-19 or H_3-20) determined the configuration at C-5 and C-9. The strong NOE correlations between H-1/H-2β, H-2α/H-10, and H-2α/(H_3-19 or H_3-20) established the configuration at C-1. The absence of any correlation between H_3-15/H_3-16 or H-10/H_3-16 and the strong NOE correlation between H_3-16/H-1 determined the configuration at C-11. According to the above observations, the structure of metabolite **4** was established as 1*S*-hydroxy-1,2-dihydrosphaerococcenol-A.

Compound **5** was isolated after purification by HPLC as a colorless oil. The molecular formula $C_{20}H_{32}Br_2O_3$ was deduced from HRFABMS data in combination with the NMR data (Table 2). The LRCI-MS ions at *m/z* 461/463/465 $[MH - H_2O]^+$, with relative intensities 1.0/2.3/1.3, and at *m/z* 381/383 $[MH - H_2O - HBr]^+$ with relative intensities 1.1/1.0 indicated the presence of two bromine atoms. In the IR spectrum, the broad band at 3414 cm^{-1} indicated the presence of a hydroxyl group, while the intense absorption at ν_{max} 1723 cm^{-1} suggested a carbonyl functionality in the molecule. The ^{13}C NMR exhibited 20 signals corresponding, as determined from the DEPT spectra, to four quaternary carbons, six methines, six methylenes, and four methyls. Among the carbons, one was carbonyl, resonating at δ_C 205.8 (C-12), two were brominated, resonating at δ_C 54.8 (C-14) and 38.0 (C-17), and two were oxygenated, resonating at δ_C 83.9 (C-11) and 74.8 (C-1). The ^{1}H NMR spectra displayed bands corresponding to one halomethine at δ_H 4.29 (H-14) and two halomethylene protons at δ_H 3.73 and 3.53 (H-17a and H-17b), one oxygenated methine proton at δ_H 3.48 (H-1), two methyls of an isopropyl group at δ_H 0.97 (H_3-19) and 0.95 (H_3-20) bonded to a methine at δ_H 1.65 (H-18), and two singlet methyls at δ_H 0.93 (H_3-15) and 1.32 (H_3-16). With four degrees of unsaturation, the structure was suggested to contain three rings besides the carbonyl group. All protonated carbons and their protons were assigned by COSY and HMQC experiments. The NMR data comparison of **5** with those of sphaerococcenol-A[17,18] suggested that metabolite **5** was its 14-bromo-1-hydroxy-1,2,13,14-tetrahydro derivative. On the basis of the correlations of carbonyl C-12 (δ_C 205.8) with H_2-13 (δ_H 3.19 and 2.85) and H_3-16 (δ_H 1.32), observed in the HMBC spectrum, the carbonyl group was placed on C-12. The correlations between H_3-19 and H_3-20 (δ_H 0.97 and 0.95) with C-4 (δ_C 43.7) confirmed the position of the isopropyl group on C-4. The correlation of H-17a (δ_H 3.73) with C-4 (δ_C 43.7) and C-6 (δ_C 27.6), of H-17b (δ_H 3.53) with C-6 (δ_C 27.6) and C-5 (δ_C 43.7), and of carbon C-17 (δ_C 38.0) with proton H-10 (δ_H 1.67) secured the position of the bromomethyl group on C-5. The position of the second bromine atom at C-14 was indicated by the correlation of C-14 (δ_C 54.8) with H_3-15 (δ_H 0.93) and H_2-13 (δ_H 3.19 and 2.85). The oxygenated methine was positioned on C-1, as concluded by the correlations of C-1 (δ_C 74.8) with H-2β (δ_H 1.99), H_2-3 (δ_H 1.88 and 1.42), and H-9 (δ_H 1.67), and/or H-10 (δ_H 1.67). Moreover the correlations of H_3-15 (δ_H 0.93) with C-8 (δ_C 42.4), C-7 (δ_C 35.6), C-9 (δ_C 53.8), and C-14 (δ_C 54.8) and of H_3-16 (δ_H 1.32) with C-11 (δ_C 83.9), C-9 (δ_C 53.8), and C-12 (δ_C 205.8) confirmed the positions of the methyl groups linked to quaternary carbon atoms. The relative configuration of **5** was assigned on the basis of the NOE experiments (Figure 3). The NOE correlations between H-14/H-7β, H-14/H-9, H-14/H-13β, H-9/H-17a, H-7β/H-17a, H-7β/H-6β, H-17a/H-6β, H-17b/H-3β, H-3α/H_3-20, H-3β/H-4, and 17b/H-4 determined the configuration at C-4, C-5, C-9, and C-14. The correlations between H_3-15/H-13α, H_3-15/H-6α, H_3-20/H-6α, H_3-20/H-10, and H_3-15/H-10, observed in the NOESY spectra, established the configuration at C-8 and C-10. Strong NOE correlations between H-1/H-2β, H-2β/H-3β, H-1/H-9, and H-1/H_3-16 determined the configuration at C-1. The strong NOE correlations between H_3-16/H-1 and H_3-16/H-13β determined the configuration at C-11. According to the above observations, metabolite **5** was named 14*S*-bromo-1*S*-hydroxy-1,2,13,14-tetrahydrosphaerococcenol-A.

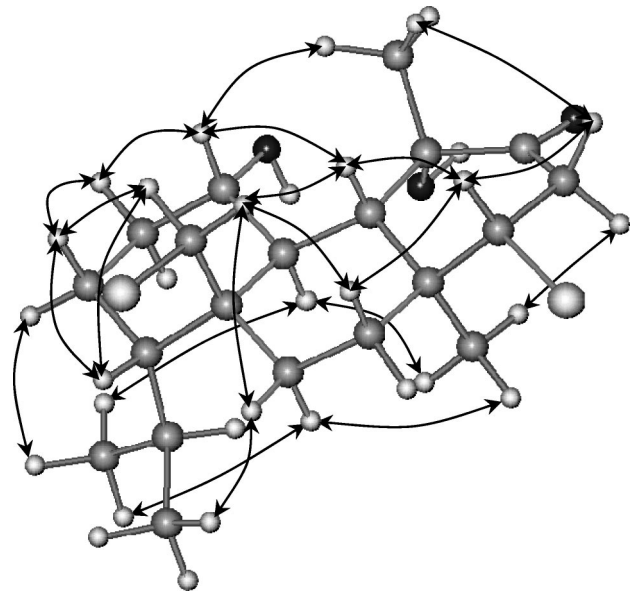

Figure 3. NOE correlations for compound **5**.

Compound **6**, after purification by HPLC, was isolated as a white solid and identified by comparison of its ^{1}H NMR and MS spectra with previously reported data as being 1*S*-hydroxy-1,2-dihydrobromosphaerol.[15] Extensive analyses of the ^{13}C NMR, COSY,

Table 3. MICs of Compounds **1**−**6** and a Standard Antibiotic (Norfloxacin) in μg/mL against MDR and Methicillin Resistant *Staphylococcus aureus*

compound	SA1199B	RN4220	EMRSA-15	ATCC 25943	XU212 (TetK)/	EMRSA-16
1	16	16	16	16	16	16
2	16−32	16	16	32	16	16
3	16	16	16	32	16	16
4	0.5	1	0.5	0.5	1	0.25
5	32	32	128	32	32−64	64
6	1−2	2	2	2	2	1−2
Nor	32	1	0.5	0.5	8	128

HSQC, HMBC, and NOESY spectra allowed full ^{13}C and ^{1}H NMR assignments for **6** (Table 2).

Metabolites **1**−**6** were evaluated for their antibacterial activity against a panel of multidrug-resistant (MDR) and methicillin-resistant *Staphylococcus aureus* strains (Table 3). The minimum inhibitory concentrations (MICs) of **1**−**6** were found to be in the range 0.5−128 μg/mL. Among the metabolites, the highest activity was exhibited by **4**, which was 64 and 512 times more active than the standard antibiotic norfloxacin against *Staphylococcus aureus* SA 1199B (overexpressing the NorA efflux protein) and EMRSA-16 (which expresses mecA and is resistant to methicillin), respectively. The second most potent compound was **6**, having MICs of 1−2 μg/mL. The presence of the α,β-unsaturated ketone moiety at C-12 may well account for the activity of metabolite **4**, with this compound being capable of undergoing Michael-type additions to this moiety via attack at C-14 by biological nucleophiles. In compound **5**, C-13 is conceivably acidic and the elements of HBr could readily eliminate, giving rise to an α,β-unsaturated ketone and generating compound **4** *in situ*. This may account for the antibacterial activity seen for compound **5**. The activity seen for compound **6** is intriguing, and perhaps the bromine atom enhances uptake of this compound. The minor structural alterations in these tricyclic brominated diterpenes that cause such significant variations in their antibacterial activity make these preliminary results a stimulus for further structure−activity investigations.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer model 341 polarimeter with a 10 cm cell. UV spectra were acquired in spectroscopic grade $CHCl_3$ on a Shimadzu UV-160A spectrophotometer. IR spectra were obtained using a Paragon 500 Perkin-Elmer spectrophotometer. NMR spectra were recorded using Bruker AC 200 and Bruker DRX 400 spectrometers. Chemical shifts are given on a δ (ppm) scale using TMS as internal standard. The 2D NMR experiments ($^{1}H-^{1}H$ COSY, HMQC, HMBC, NOESY) were performed using standard Bruker microprograms. The structures in Figures 2 and 3 were generated and optimized (energy: 41.00 and 60.42 Kkcal, respectively) by HyperChem 7.0 molecular modeling and simulation software (force field, MM+; optimization algorithm, Polak-Ribiere). High-resolution mass spectral data were provided by the University of Notre Dame, Department of Chemistry and Biochemistry, IN. Low-resolution electron impact or chemical ionization MS data were recorded on a Thermo DSQ mass detector using a direct exposure probe (DEP) and methane as the CI gas. Vacuum liquid chromatography (VLC) separation was performed with Kieselgel 60 (Merck), gravity column chromatography (GCC) was performed with Kieselgel 60H (Merck), thin-layer chromatography (TLC) was performed with Kieselgel 60 F_{254} aluminum support plates (Merck), and spots were detected after spraying with 15% H_2SO_4 in MeOH reagent and charring. HPLC separations were conducted using an Agilent 1100 model equipped with refractive index detector and a SupelcoSil 5u (250 × 10 mm) HPLC normal-phase column or a Kromasil 100 C18 5u (250 × 8 mm) HPLC reversed-phase column.

Plant Material. *S. coronopifolius* was collected by scuba diving in Palaiokastritsa Bay at the west coast of Corfu Island, Greece, at a depth of 10−15 m in May of 2002. A specimen is kept at the Herbarium of the Laboratory of Pharmacognosy and Chemistry of Natural Products, University of Athens (ATPH/MO/201).

Extraction and Isolation. *S. coronopifolius* was initially freeze-dried (291.4 g dry weight) and then exhaustively extracted with mixtures of CH_2Cl_2/MeOH (3:1) at room temperature. The combined extracts were concentrated to give a dark green residue (8.20 g), which was later subjected to VLC on silica gel, using cyclohexane with increasing amounts (10%) of EtOAc and finally MeOH as mobile phase. The CH_3CN-soluble portion (306.7 mg) of fraction IV_a (60% EtOAc in cyclohexane) (337.8 mg) was subjected to reversed-phase HPLC chromatography, using 100% CH_3CN as mobile phase. Peak V_b (retention time 9.4 min) (3.3 mg) was subjected again to reversed-phase HPLC chromatography, using CH_3CN as mobile phase, to yield pure compound **4** (1.1 mg), while peaks X_b (retention time 12.0 min) (21.0 mg) and XI_b (retention time 12.5 min) (86.3 mg) with similar repurification yielded pure compounds **3** (1.3 mg) and **2** (2.0 mg), respectively. The CH_3CN-soluble portion (323.4 mg) of fraction V_a (70% EtOAc in cyclohexane) (419.8 mg) was subjected to reversed-phase HPLC chromatography, using CH_3CN as mobile phase. Peaks IV_c (retention time 7.8 min) (8.9 mg) and $VIII_c$ (retention time 9.2 min) (11.1 mg) were subjected again to reversed-phase HPLC chromatography, using CH_3CN as mobile phase, to yield pure compounds **1** (1.4 mg) and **5** (1.3 mg), respectively, while peak XII_c (retention time 11.6 min) (48.1 mg) with HPLC normal-phase purification, using 70% $CHCl_3$ in *n*-hexane as mobile phase, yielded pure compound **6** (2.0 mg).

2*S*-Hydroxyisobromosphaerol (1): colorless oil; $[\alpha]^{20}_D$ +8.4 (*c* 2.70, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 245 (2.51) nm; IR ($CHCl_3$) ν_{max} 3377, 2942, 2860 cm^{-1}; NMR data ($CDCl_3$), see Table 1; CIMS *m/z* (rel int %) 445:447:449 $[MH - H_2O]^+$ (1:2:1), 427:429:431 $[MH - 2H_2O]^+$ (1:2:1), 365:367 $[MH - HBr - H_2O]^+$ (12:9), 347:349 $[MH - HBr - 2H_2O]^+$ (14:15), 285 $[MH - 2HBr - H_2O]^+$ (22), 267 $[MH - 2HBr - 2H_2O]^+$ (100), 223 (7), 187 (8), 159 (18), 109 (6), 95 (18), 81 (19); HRFABMS *m/z* 461.0675 $[M - H]^+$ (calcd for $C_{20}H_{31}{}^{79}Br_2O_2$, 461.0691).

2*S*-Hydroperoxy-12*R*-hydroxyisobromosphaerol (2): white solid; $[\alpha]^{20}_D$ +6.6 (*c* 1.70, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 247 (3.00) nm; IR ($CHCl_3$) ν_{max} 3422, 2936 cm^{-1}; NMR data ($CDCl_3$), see Table 1; CIMS *m/z* (rel int %) 477:479:481 $[MH - H_2O]^+$ (43:83:41), 459:461:463 $[MH - 2H_2O]^+$ (12:22:13), 443:445:447 $[MH - H_2O_2 - H_2O]^+$ (6:8:4), 397:399 $[MH - HBr - H_2O]^+$ (21:19), 379:381 $[MH - HBr - 2H_2O]^+$ (42:45), 363:365 $[MH - HBr - H_2O_2 - H_2O]^+$ (31:31), 345:347 (7:9), 337:339 (12:13), 317 (23), 299 $[MH - 2HBr - 2H_2O]^+$ (38), 283 $[MH - 2HBr - H_2O_2 - H_2O]^+$ (100), 265 (39), 239 (16), 201 (17), 159 (29), 95 (23), 83 (79); HRFABMS *m/z* 477.0621 $[M - OH]^+$ (calcd for $C_{20}H_{31}{}^{79}Br_2O_3$, 477.0640).

2*S*-Hydroperoxy-12*S*-hydroxyisobromosphaerol (3): white solid; $[\alpha]^{20}_D$ +3.1 (*c* 2.20, $CHCl_3$); NMR data ($CDCl_3$), see Table 1; EIMS 70 eV, *m/z* (rel int %) 476:478:480 $[M - H_2O]^+$ (2:6:3), 460:462:464 $[M - 2OH]^+$ (4:6:3), 445:447:449 $[M - 2OH - Me]^+$ (3:6:3), 429:431:433 $[M - 2OH - O - Me]^+$ (3:5:3), 415:417 $[M - Br]^+$ (2:2), 383:385 (36:34), 365:367 (26:27), 339:341 $[M - Br - OH - O - Me_2C]^+$ (24:21), 311 (13), 299 (41), 267 $[M - HBr - Br - H_2O_2 - 2OH]^+$ (7), 207 (17), 159 (31), 91 (34), 43 (100).

1*S*-Hydroxy-1,2-dihydrosphaerococcenol-A (4): colorless oil; $[\alpha]^{20}_D$ −32.7 (*c* 0.90, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 247 (3.29) nm; IR ($CHCl_3$) ν_{max} 3468, 1698 cm^{-1}; NMR data ($CDCl_3$), see Table 2; EIMS 70 eV, *m/z* (rel int %) 380:382 $[M - H_2O]^+$ (4:4), 365:367 $[M - OH - O]^+$ (2:2), 301 $[M - Br - H_2O]^+$ (9), 283 $[M - Br - 2H_2O]^+$ (2), 273 $[M - CH_2Br - OH - Me]^+$ (100), 215 (7), 191 (58), 145 (16), 95 (69); HRFABMS (*m/z*): 381.1437 $[M - OH]^+$ (calcd for $C_{20}H_{31}{}^{79}BrO_2$, 381.1429).

14*S*-Bromo-1*S*-hydroxy-1,2,13,14-tetrahydrosphaerococcenol-A (5): colorless oil; $[\alpha]^{20}_D$ −6.6 (*c* 2.70, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 247 (2.67) nm; IR ($CHCl_3$) ν_{max} 3414, 1723 cm^{-1}; NMR data ($CDCl_3$), see Table 2; CIMS *m/z* (rel int %) 461:463:465 [MH −

$H_2O]^+$ (1:2:1), 443:445:447 $[MH - 2H_2O]^+$ (2:4:2), 381:383 $[MH - HBr - H_2O]^+$ (44:40), 363:365 $[MH - HBr - 2H_2O]^+$ (17:20), 352:354 (29:32), 311 (22), 301 $[MH - 2HBr - H_2O]^+$ (100), 283 $[MH - 2HBr - 2H_2O]^+$ (61), 273 (45), 255 (23), 215 (28), 147 (29), 123 (25), 81 (38); HRFABMS (*m/z*) 460.0596 $[M - H_2O]^+$ (calcd for $C_{20}H_{30}{}^{79}Br_2O_2$, 460.0613).

1*S*-Hydroxy-1,2-dihydrobromosphaerol (6): white solid; $[\alpha]^{20}_D$ +5.1 (*c* 2.70, $CHCl_3$); NMR data ($CDCl_3$), see Table 2; CIMS *m/z* (rel int %) 447:449:451 $[MH - H_2O]^+$ (4:6:3), 429:431:433 $[MH - 2H_2O]^+$ (8:18:12), 367:369 $[MH - HBr - H_2O]^+$ (100:98), 349:351 $[MH - HBr - 2H_2O]^+$ (60:63), 335:337 $[MH - CH_3Br - 2H_2O]^+$ (10:8), 287 $[MH - 2HBr - H_2O]^+$ (53), 269 $[MH - 2HBr - 2H_2O]^+$ (46), 255 $[MH - CH_3Br - HBr - 2H_2O]^+$ (11), 203 (9), 175 (10), 147 (7), 105 (11), 83 (47).

Bacterial Strains and Antibiotic. A standard *S. aureus* strain ATCC 25923 and a clinical isolate (XU212), which possesses the TetK efflux pump and was an MRSA strain, were obtained from Dr. E. Udo.[19] Strain RN4220, which has the MsrA macrolide efflux pump, was provided by Dr. J. Cove.[20] EMRSA-15[21] and EMRSA-16[22] were obtained from Dr. Paul Stapleton. Strain SA1199B, which overexpresses the NorA MDR efflux pump, was the gift of Professor G. Kaatz.[23] Norfloxacin was obtained from the Sigma Chemical Co. Mueller-Hinton broth (MHB; Oxoid) was adjusted to contain 20 mg/L Ca^{2+} and 10 mg/L Mg^{2+}.

Antibacterial Assay. Overnight cultures of each strain were made up in 0.9% saline to an inoculum density of 5×10^5 cfu by comparison with a McFarland standard. Norfloxacin was dissolved in DMSO and then diluted in MHB to give a starting concentration of 512 μg/mL. Using Nunc 96-well microtiter plates, 125 μL of MHB was dispensed into wells 1−11. Then 125 μL of the test compound or the appropriate antibiotic was dispensed into well 1 and serially diluted across the plate, leaving well 11 empty for the growth control. The final volume was dispensed into well 12, which being free of MHB or inoculum served as the sterile control. Finally, the bacterial inoculum (125 μL) was added to wells 1−11, and the plate was incubated at 37 °C for 18 h. A DMSO control (3.125%) was also included. All MICs were determined in duplicate. The MIC was determined as the lowest concentration at which no growth was observed. A methanolic solution (5 mg/mL) of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliium bromide (MTT; Lancaster) was used to detect bacterial growth by a color change from yellow to blue.

Acknowledgment. This study was partially supported by the FP-39 EPAN program of the Greek Secretariat for Research and Technology.

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NP8001817