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Cytotoxic bromoditerpenes from the red alga *Sphaerococcus coronopifolius*

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ABSTRACT

Three new brominated diterpenes (**1**, **2**, **8**), along with six previously reported metabolites (**3**–**7**, **9**), were isolated from the organic extract of *Sphaerococcus coronopifolius*, collected in Palaiokastritsa bay at the west coasts of Corfu Island. The structures of the new natural products, as well as their relative stereochemistry, were established by means of spectral data analyses, including 2D experiments. The absolute stereochemistry of **2** and **4** as well as the structure revision of the previously reported metabolite **3** were established by X-ray crystallographic analyses. The cytotoxicity of the isolated metabolites was evaluated against the NSCLC-N6-L16 and A549 human lung cancer cell lines.
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1. Introduction

Diterpenes are widespread metabolites of 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 is yielding an extended variety of interesting diterpenes having di-, tri- or tetra-cyclic and often rearranged carbon skeletons, most of which contain one or more bromine atoms.[3] These halogenated metabolites have been suggested to function as chemical defence against marine herbivores.[4–6] Moreover some of these halogenated metabolites have been proven to possess insecticidal,[7] antibacterial,[8] antifungal[9] and antiviral activities.[10]

In the course of our ongoing investigations towards 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 west coasts of Corfu Island. In this report we describe the isolation and structure elucidation of three new metabolites (**1**, **2** and **8**) along with the already described metabolites **3**,[14] **4** (bromosphaerodiol),[15] **5** (12*R*-hydroxy-bromosphaerol),[14] **6** (12*S*-hydroxy-bromosphaerol),[16] **7** (bromosphaerol)[17–19] and **9** (sphaerococcenol-A),[18,20] all of which were obtained from the organic extract of *S. coronopifolius*. The structures of **2** and **4**, the determination of their absolute stereochemistry, and the structure of **3** and its relative stereochemistry were confirmed by X-ray analyses. Moreover, detailed analyses of the 2D spectra of metabolites **4**–**6** allowed full assignment of ^{13}C and ^{1}H signals, which have not been reported before.

Cytotoxicity evaluation performed on the NSCLC-N6-L16 and A549 human lung cancer cell lines showed metabolites **2**, **3** and **8** to possess significant activity on both cell lines.

2. 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 as well as normal and reversed phase high pressure liquid chromatography (HPLC) separations, using mixtures of cyclohexane/EtOAc or CH_3CN, respectively, as mobile phase, to yield compounds **1**–**9** in pure form.

Compound **1** was isolated after purification by HPLC, as a colourless oil. The molecular formula $C_{20}H_{32}Br_2O$ was deduced from HRFAB-MS data in combination with the NMR data (Tables 1 and 2). The LRCI-MS ions at m/z 429:431:433 $[MH–H_2O]^+$, with relative intensities 1.0:2.0:0.7, and at m/z 349:351 $[MH–H_2O–HBr]^+$ with relative intensities 1.0:1.0 indicated the presence of two bromine atoms. In the IR spectrum, the intense wide band at ν_{max} 3478 cm^{-1} indicated the presence of hydroxyl group in the molecule. The ^{13}C NMR exhibited 20 signals corresponding to three quaternary carbons, seven methines, six methylenes and four methyls. The ^{1}H and ^{13}C NMR spectra displayed resonances for two secondary methyls ($\delta_{H/C}$ 0.89/19.1; 1.00/23.9) of an isopropyl group linked to a methine ($\delta_{H/C}$ 2.04/27.5) attached on another methine ($\delta_{H/C}$ 2.21/45.8), two

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Table 1
^{1}H NMR data[a] and NOESY correlations of compounds **1**, **2** and **8**

No.	**1**			**2**			**8**		
	^{1}H (δ)	m (*J*)	NOESY	^{1}H (δ)	m (*J*)	NOESY	^{1}H (δ)	m (*J*)	NOESY
1	α2.39	m	16	4.76	dd 9.3, 2.5	9, 16, 17b	5.97	br d 10.4	9, 10, 16
	β1.84	dddd 17.6, 5.4, 4.9, 2.4	16, 17b						
2	5.73	dddd 10.2, 4.9, 2.4, 2.4		6.04	dd 10.3, 2.5	17b	5.70	ddt 10.4, 5.0, 2.5	
3	5.61	dddd 10.2, 4.9, 2.4, 2.4		5.95	dd 10.3, 4.9	20	α1.99	m	10
							β2.11	m	17b
4	2.21	m	17b	2.41	dd 4.9, 3.9	6β, 17b, 20	1.73	m	17b
5	—	—	—	—	—	—	—	—	—
6	a1.72	m		α1.79	br ddd 14.2, 13.7, 3.9	15	α1.77	tdd 13.7, 4.2, 1.7	15
	b1.72	m		β1.63	ddd 14.2, 3.4, 3.4	4, 18	β1.55	m	
7	α1.87	m		α1.88	ddd 13.7, 3.9, 3.4		α1.01	dt 13.3, 3.3	14
	β1.13	ddd 13.7, 13.7, 3.9	14, 17a	β1.10	ddd 13.7, 13.7, 3.4	14, 17a	β1.94	m	17a
8	—	—	—	—	—	—	—	—	—
9	1.36	d 10.8	14, 17a	1.51	d 9.8	1, 14, 17a	2.51	d 12.8	1, 16, 17a, 17b
10	2.52	m	15, 20	2.64	dd 9.8, 9.3	15, 19	2.77	ddq 12.8, 4.1, 2.1	1, 3α, 15, 18, 19, 20
11	—	—	—	—	—	—	—	—	—
12	a1.64	m		3.18	ddd 10.8, 9.3, 7.3	13β, 14, 16	—	—	—
	b1.64	m							
13	α2.43	m	15	α2.32	m	15	α2.63	dd 18.2, 1.2	14
	β1.99	dq 13.7, 3.9	14	β2.33	m	12, 14	β2.99	dd 18.2, 7.9	14
14	3.97	dd 12.2, 3.9	7β, 9, 13β	3.90	dd 10.3, 6.3	7β, 9, 12, 13β	3.74	dd 7.9, 1.2	7α, 13α, 13β, 15
15	1.26	s	10, 13α	1.23	s	10, 13α	0.77	s	6α, 10, 14
16	1.36	s	1α, 1β	1.50	s	1, 12	1.34	s	1, 9
17	a3.79	d 10.2	7β, 9	a3.85	d 10.3	7β, 9	a3.89	d 10.8	7β, 9
	b3.58	dd 10.2, 1.0	1β, 4	b3.50	br d 10.3	1, 2, 4	b3.72	dd 10.8, 1.7	3β, 4, 9
18	2.04	dhept 3.4, 6.8		2.12	dhept 3.9, 6.8	6β, 19, 20	1.96	m	10
19[b]	0.89	d 6.8	10	0.93	d 6.8	10, 18	0.88	d 6.6	10
20[b]	1.00	d 6.8		1.02	d 6.8	3, 4, 18	0.93	d 6.6	10
11-OH							3.47	br s	

[a] ^{1}H (400 MHz) recorded in $CDCl_3$ (δ_H 7.24, δ_C 77.0), chemical shifts are expressed in parts per million and *J* values in Hz.
[b] Positions can be interchanged.

quaternary methyls ($\delta_{H/C}$ 1.26/15.5 and 1.36/35.6), one halomethine ($\delta_{H/C}$ 3.97/68.5), two sp^2 methines ($\delta_{H/C}$ 5.61/124.8 and 5.73/126.4), two aliphatic methines ($\delta_{H/C}$ 1.36/52.9 and 2.52/34.7), one halomethylene ($\delta_{H/C}$ 3.79, 3.58/40.9) and five aliphatic methylenes ($\delta_{H/C}$ 2.39, 1.84/30.0; 1.72/25.5; 1.87, 1.13/36.6; 1.64/46.1 and $\delta_{H/C}$ 2.43, 1.99/30.4). With an unsaturation degree of 4, the structure was suggested to contain, besides the double bond, three rings. All protonated carbons and their protons were assigned by the COSY and HMQC experiments. The structure elucidation was assisted by analyses of the HMBC experiments. The correlation in the HMBC experiments, between H_3-19 and H_3-20 (δ_H 0.89 and 1.00) with C-4 (δ_C 45.8), confirmed the position of the isopropyl group. The correlation of H-17a (δ_H 3.79) with C-4 (δ_C 45.8) and of H-17b (δ_H 3.58) with C-4 (δ_C 45.8), C-5 (δ_C 40.1) and C-6 (δ_C 25.5), secured the position of the bromomethyl group on C-5. The other bromine atom was positioned on C-14, as concluded by the correlations of C-14 (δ_C 68.5) with H-9 (δ_H 1.36), H_2-12 (δ_H 1.64), H-13α (δ_H 2.43) and H_3-15 (δ_H 1.26). The position of the double bond between C-2 and C-3 was

Table 2
^{13}C NMR data[a] and $^{13}C \rightarrow {}^{1}H$ HMBC correlations of compounds **1**, **2** and **8**

No.	**1**		**2**		**8**	
	^{13}C (δ)	HMBC	^{13}C (δ)[b]	HMBC	^{13}C (δ)	HMBC
1	30.0	2, 3, 10	79.5	3, 10	128.8	
2	126.4	1α, 1β	127.5		127.7	4
3	124.8	1α, 1β	129.1	1	22.6	1, 4
4	45.8	2, 3, 17a, 17b, 19, 20	46.9	2, 17a, 19, 20	41.9	17a, 19, 20
5	40.1	1α, 3, 7α, 17b	42.3	3, 10	40.0	1, 4, 17a
6	25.5	7α, 17b	26.9	17b	24.7	7β, 17a, 17b
7	36.6	6, 15	36.2	15	29.3	6α, 15
8	41.1	6, 9, 10, 14, 15	43.2	9, 15	39.7	7α, 9, 15
9	52.9	7α, 10, 15, 16	50.5	7α, 10, 15, 16	42.2	14, 15, 16, 11OH
10	34.7	1β, 2, 9	38.8	1, 2, 9	35.3	1, 4, 9
11	73.2	9, 16	75.3	9, 12, 16	76.3	13β, 16, 11OH
12	46.1	13α, 16	76.0	13α, 13β, 16	216.6	13α, 13β, 14, 16, 11OH
13	30.4		39.9		42.4	
14	68.5	9, 12, 13α, 15	65.7	13α, 15	74.5	13α, 15
15	15.5	9, 14	14.4	9, 14	17.2	9
16	35.6		27.3		31.2	9
17	40.9		41.4	6α, 6β, 10	40.2	
18	27.5	4, 19, 20	28.3	19, 20	25.8	4, 19, 20
19[c]	19.1	20	19.2	20	19.2	4, 20
20[c]	23.9	19	24.2	19	25.8	4, 19
11-OH	—	—	—	—	—	—

[a] ^{13}C NMR (50.3 MHz) recorded in $CDCl_3$ (δ_H 7.24, δ_C 77.0), chemical shifts are expressed in parts per million.
[b] ^{13}C NMR (50.3 MHz) recorded in acetone-d_6 ($\delta_C[CD_3]$ 29.8).
[c] Positions can be interchanged.

established from correlations of H-2 (δ_H 5.73) with C-1 (δ_C 30.0), C-4 (δ_C 45.8) and C-10 (δ_C 34.7) and of H-3 (δ_H 5.61) with C-1 (δ_C 30.0), C-4 (δ_C 45.8) and C-5 (δ_C 40.1). Moreover the correlations between H_3-15 (δ_H 1.26) with C-8 (δ_C 41.1), C-7 (δ_C 36.6), C-9 (δ_C 52.9) and C-14 (δ_C 68.5) and of H_3-16 (δ_H 1.36) with C-11 (δ_C 73.2), C-9 (δ_C 52.9) and C-12 (δ_C 46.1), confirmed the positions of the quaternary methyl groups. Comparison of the NMR data of **1** with reported values for bromosphaerol,[17–19] led to the assignment of the structure as the $\Delta^{2,3}$ isomer of bromosphaerol. The relative stereochemistry of **1** was assigned on the basis of NOESY experiments. The strong NOE correlations between H-14/H-9, H-14/H-13β, H-14/H-7β, H-7β/H-17a, H-17a/H-9, H-17b/H-4 and H-17b/H-1β, established the stereochemistry at C-4, C-5, C-9 and C-14. The strong NOE correlations between H_3-15/H-13α, H-10/H_3-15 and H-10/H_3-19 established the stereochemistry at C-8 and C-10. The absence of any correlation between H_3-15/H_3-16 or H-10/H_3-16 and the NOE correlations between H_3-16/H-1α and H_3-16/H-1β confirmed the equatorial configuration of H_3-16. The large coupling constant J values of H-7β, H-9, H-10 and H-14 supported the axial configuration of these protons. In view of the above-mentioned data metabolite **1** was named bromosphaerol-B.

Compound **2** was purified by means of HPLC, and was isolated as colourless crystals. Combination of its ^{13}C NMR data and HRFAB-MS measurements suggested a molecular formula of $C_{20}H_{32}Br_2O_4$. The LRCI-MS peaks at m/z 477:479:481 $[MH-H_2O]^+$, with relative intensities 1.4:2.2:1.0, and at m/z 459:461:463 $[MH-2H_2O]^+$, with relative intensities 0.9:1.9:1.0, 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: three singlets, nine doublets, four triplets and four quartets. Strong IR absorptions at ν_{max} 3448 cm^{-1} and ^{13}C NMR signals at δ_C 75.3 (C-11) and 76.0 (C-12) indicated the presence of hydroxyl groups. One more oxygenated carbon resonated in lower fields at δ_C 79.5 (C-1), characteristic for the presence of a hydroperoxy group. Among the other carbons two were olefinic resonating at δ_C 127.5 (C-2) and 129.1 (C-3), and two were brominated resonating at δ_C 65.7 (C-14) and 41.4 (C-17). Furthermore, the ^1H NMR spectra revealed signals due to a halomethine proton at δ_H 3.90 (H-14), two halomethylene protons at δ_H 3.85 and 3.50 (H-17a and H-17b), two olefinic protons at δ_H 6.04 (H-2) and 5.95 (H-3), two oxygenated methine protons at δ_H 4.76 (H-1) and 3.18 (H-12), two secondary methyls of an isopropyl group at δ_H 0.93 (H_3-19) and 1.02 (H_3-20) attached to a methine at δ_H 2.12 (H-18), and two quaternary methyls at δ_H 1.23 (H_3-15) and 1.50 (H_3-16). All protonated carbons and their protons were assigned on the basis of their COSY and HMQC correlations. The structure elucidation was assisted by analyses of the HMBC experiments. Based on the correlations of H-1 methine (δ_H 4.76) with C-3 (δ_H 129.1) and C-10 (δ_H 38.8), and of H_2-13 methylene protons (δ_H 2.33 and 2.32) and H_3-16 (δ_H 1.50) with C-12 (δ_C 76.0), observed in the HMBC spectrum, the hydroperoxy and the secondary hydroxyl group were placed on C-1 and C-12, respectively. The position of the olefinic bond between C-2 and C-3 was established from correlations of H-2 (δ_H 6.04) with C-4 (δ_C 46.9) and C-10 (δ_C 38.8) and of H-3 (δ_H 5.95) with C-1 (δ_C 79.5) and C-5 (δ_C 42.3). The correlation in the HMBC experiments, between H_3-19 (δ_H 0.93) and H_3-20 (δ_H 1.02) with C-4 (δ_C 46.9), confirmed the position of the isopropyl group on C-4. The correlation of H-17a (δ_H 3.85) with C-4 (δ_C 46.9) and of C-17 (δ_C 41.4) with H_2-6 (δ_H 1.79 and 1.63) and H-10 (δ_H 2.64), secured the position of the bromomethyl group on C-5. The position of the second bromine atom at C-14 was suggested by the correlation of C-14 (δ_C 65.7) with H_3-15 (δ_H 1.23) and H-13α (δ_H 2.32). Moreover the correlations between H_3-15 (δ_H 1.23) with C-8 (δ_C 43.2), C-7 (δ_C 36.2), C-9 (δ_C 50.5) and C-14 (δ_C 65.7) and of H_3-16 (δ_H 1.50) with C-11 (δ_C 75.3), C-9 (δ_C 50.5) and C-12 (δ_C 76.0), confirmed the positions of the quaternary methyl groups. Comparison of the NMR data of **2** with the NMR data of **3** and reported values for 12*S*-hydroxy-bromosphaerodiol,[14] showed close similarity even though the structure described in the literature was presented with hydroxy instead of hydroperoxy group. This led us to assign **2** as 1*S*-hydroperoxy-12*R*-hydroxy-bromosphaerol-B. This was confirmed by all NOESY correlations. The NOE correlations between H-14/H-9, H-14/H-13β, H-14/H-7β, H-14/H-12, H-7β/H-17a, H-9/H-17a, H-9/H-1, H-17b/H-1, H-17b/H-4 and H-12/H-13β, established the stereochemistry at C-4, C-5, C-9, C-14 and C-12. The NOE correlations between H_3-15/H-13α, H-10/H_3-15 and H-10/H_3-19 established the stereochemistry at C-8 and C-10. The absence of any correlation between H_3-15/H_3-16 or H-10/H_3-16 and the NOE correlations between H_3-16/H-1 and H_3-16/H-12 confirmed the equatorial configuration of H_3-16. The large coupling constants of H-7β, H-9, H-10 and H-14 supported the axial configuration of these protons. Definite proof of the proposed structure (Fig. 1) and the determination of the absolute stereochemistry of the metabolite were provided by extensive X-ray crystallographic analysis[21] that was performed on a single crystal of **2** (Fig. 2).

Compound **3** after purification by HPLC was isolated as colourless crystals. Comparison of its ^1H NMR and MS spectra with literature data showed them to be identical to those of 12*S*-hydroxy-bromosphaerodiol.[14] However, the fact that C-1 was resonating at unusually low fields (δ_C 79.2) compared with bromosphaerodiol (**4**)[15] (C-1 signal at δ_C 66.2) was indicative of the presence of a hydroperoxy group on C-1. We therefore assigned **3** as 1*S*-hydroperoxy-12*S*-hydroxy-bromosphaerol-B (Table 3). The definite proof of the structure and the determination of the relative stereochemistry of the metabolite were provided by extensive X-ray crystallographic analysis[22] that was performed on a single crystal of **3** (Fig. 3).

Compound **4** after purification by HPLC was isolated as colourless crystals and identified by comparison of its ^1H NMR and MS spectra with previously reported data as being bromosphaerodiol (**4**).[15] Extensive analyses of the ^{13}C NMR, HMQC and HMBC spectra allowed ^{13}C and ^1H NMR assignments for bromosphaerodiol (**4**). The absolute stereochemistry of the metabolite was also confirmed by X-ray crystallography[23] performed on a single crystal of **4** (Fig. 4).

Compound **5** after purification by HPLC was isolated as colourless oil and identified by comparison of its ^1H NMR and MS spectra

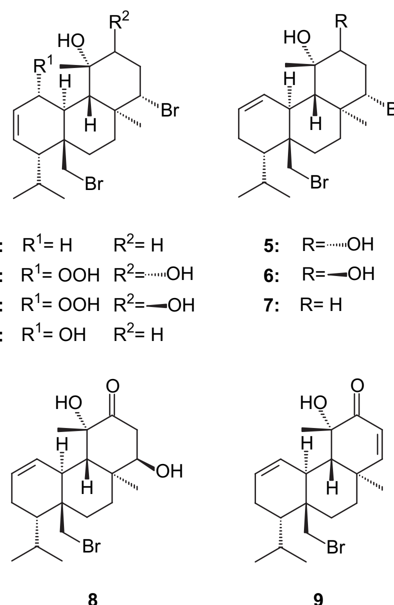

Figure 1. Metabolites isolated from *S. coronopifolius*.

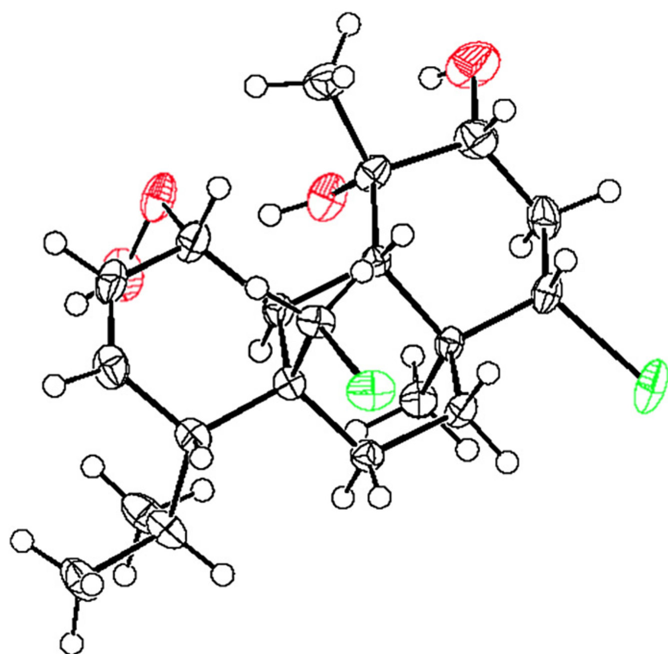

Figure 2. Molecular model of **2** provided by X-ray crystallography.

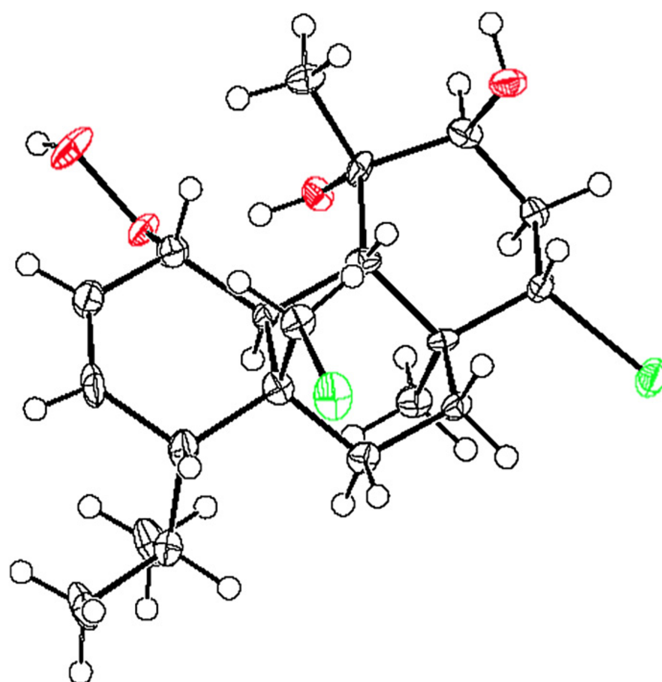

Figure 3. Molecular model of **3** provided by X-ray crystallography.

with previously reported data as being 12*R*-hydroxy-bromosphaerol (**5**).[14] Extensive analyses of the ^{13}C NMR, HMQC and HMBC spectra allowed the ^{13}C and ^{1}H NMR assignment for 12*R*-hydroxy-bromosphaerol (**5**).

Compound **6** after purification by HPLC was isolated as colourless oil and identified by comparison of its ^{1}H NMR and MS spectra with previously reported data as being 12*S*-hydroxy-bromosphaerol (**6**).[16] Extensive analyses of the ^{13}C NMR, HMQC and HMBC spectra allowed the ^{13}C and ^{1}H NMR assignments for 12*S*-hydroxy-bromosphaerol (**6**).

Compound **8** was purified by means of HPLC separations and was isolated as colourless oil. Both ^{13}C NMR data and HRFAB-MS measurements supported the molecular formula $C_{20}H_{31}BrO_3$. The LRCI-MS showed $[MH]^+$ peaks at m/z 399:401 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 1703 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 **8** (Table 2) exhibited signals for 20 carbons, with the

Table 3
NMR data[a] of compounds **3**–**6**

No.	3			4			5			6		
	^{1}H (δ)	m (*J*)	^{13}C (δ)	^{1}H (δ)	m (*J*)	^{13}C (δ)	^{1}H (δ)	m (*J*)	^{13}C (δ)	^{1}H (δ)	m (*J*)	^{13}C (δ)
1	4.63	dd 9.6, 2.8	79.2	4.29	br d 8.3	66.2	5.99	br d 10.4	128.8	5.97	br d 10.4	128.5
2	6.07	dd 10.2, 2.8	124.6	5.73	dd 10.4, 2.9	128.7	5.67	ddt 10.4, 5.8, 2.9	126.4	5.69	ddt 10.4, 5.0, 2.5	127.5
3	5.95	dd 10.2, 5.4	131.0	5.80	br dd 10.4, 5.4	128.2	α1.91	m	21.8	α1.93	m	21.9
							β2.12	m		β2.10	m	
4	2.42	dd 5.4, 4.1	46.1	2.39	dd 5.4, 3.3	45.8	1.73	m	42.5	1.72	br s	42.6
5	—	—	42.6	—	—	42.3	—	—	40.5	—	—	41.1
6	α1.76	ddd 14.3, 13.7, 3.4	26.1	α1.77	m	25.9	α1.77	m	24.9	α1.75	m	24.9
	β1.61	ddd 14.3, 3.8, 3.4		β1.59	ddd 14.1, 3.7, 3.7		β1.50	m		β1.51	m	
7	α1.85	ddd 13.7, 3.8, 3.4	36.0	α1.89	ddd 13.7, 3.7, 3.7	36.2	α1.78	m	36.0	α1.81	m	36.4
	β1.22	td 13.7, 3.4		β1.14	td 13.7, 3.7		β1.18	m		β1.29	m	
8	—	—	41.5	—	—	41.3	—	—	42.0	—	—	41.8
9	1.93	d 10.9	47.3	1.57	d 10.8	52.6	1.38	d 10.8	48.7	1.78	d 11.2	45.9
10	2.56	dd 10.9, 9.6	37.3	2.49	dd 10.8, 8.3	44.0	3.02	dm 10.8	37.5	2.97	dm 11.2	36.8
11	—	—	74.1	—	—	72.0	—	—	73.5	—	—	74.8
12	3.50	dd 3.4, 2.4	79.6	α1.80	ddd 14.9, 3.3, 3.3	45.2	3.34	dt 11.6, 5.4	76.9	3.45	br s	79.4
				β1.63	ddd 14.9, 13.3, 4.6							
13	α2.72	ddd 13.6, 13.3, 2.4	37.0	α2.45	tdd 13.3, 12.9, 3.3	30.2	α2.33	ddd 12.8, 12.4, 11.6	37.9	α2.70	ddd 13.7, 12.8, 2.9	37.4
	β2.13	dt 13.6, 3.4		β1.94	dddd 13.3, 4.6, 3.8, 3.3		β2.21	ddd 12.4, 5.4, 3.3		β2.14	dt 13.7, 3.7	
14	4.51	dd 13.3, 3.4	63.8	3.99	dd 12.9, 3.8	69.5	3.88	dd 12.8, 3.3	63.0	4.46	dd 12.8, 3.7	63.4
15	1.27	s	14.6	1.30	s	14.1	1.25	s	13.6	1.27	s	14.9
16	1.46	s	29.6	1.41	s	33.0	1.41	s	30.9	1.44	s	31.8
17	a3.95	d 10.6	39.7	a3.89	d 10.4	39.9	a3.89	d 10.4	40.3	a3.93	d 10.8	40.5
	b3.47	br d 10.6		b3.50	br d 10.4		b3.59	dd 10.4, 1.7		b3.60	dd 10.8, 2.1	
18	2.12	m	27.5	2.13	dhept 3.3, 6.6	27.2	1.93	m	25.8	1.93	m	25.9
19[b]	0.94	d 6.8	18.9	0.95	d 6.6	19.0	0.89	d 7.1	19.9	0.89	d 7.0	19.7
20[b]	1.02	d 6.8	23.9	1.01	d 6.6	23.8	0.95	d 7.1	26.1	0.95	d 7.0	25.9

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

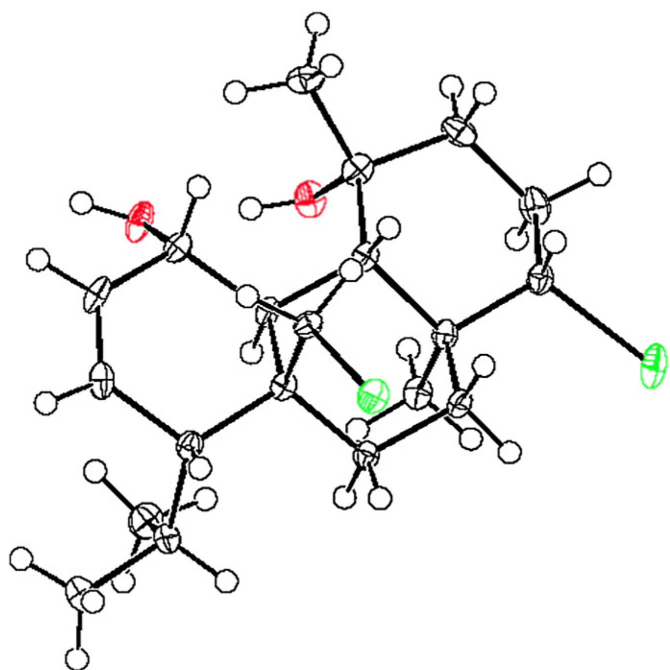

Figure 4. Molecular model of **4** provided by X-ray crystallography.

multiplicities of the carbons determined from the DEPT spectra as: four quaternary carbons, seven methines, five methylenes and four methyls. Among the carbons, one was a carbonyl, resonating at δ_C 216.6, two were olefinic resonating at δ_C 128.8 (C-1) and δ_C 127.7 (C-2), one was brominated resonating at δ_C 41.4 (C-17), and two were oxygenated resonating at δ_C 76.3 (C-11) and 74.5 (C-14). The ^{1}H NMR spectra displayed signals for two olefinic protons at δ_H 5.97 (H-1) and 5.70 (H-2), one oxygenated methine proton at δ_H 3.74 (H-14), two halomethylene protons at δ_H 3.89 and 3.72 (H-17a and H-17b), two secondary methyls of an isopropyl group at δ_H 0.88 (H_3-19) and 0.93 (H_3-20) attached to a methine at δ_H 1.96 (H-18), two quaternary methyls at δ_H 0.77 (H_3-15) and 1.34 (H_3-16), and one exchangeable proton at δ_H 3.47 (C11-OH). With an unsaturation degree of 5, the structure was suggested to contain besides the carbonyl group, one double bond and three rings. All protonated carbons and their protons were assigned by the COSY and HMQC experiments. The NMR data comparison of **8** with those of sphaerococcenol-A (**9**)[18,20] suggested that metabolite **8** was its 14-hydroxy-13,14-dihydro derivative. Based on the correlations of carbonyl C-12 (δ_C 216.6) with H_2-13 (δ_H 2.99 and 2.63), H-14 (δ_H 3.74), H_3-16 (δ_H 1.34) and C11-OH (δ_H 3.47) observed in the HMBC spectrum, the carbonyl group was placed on C-12. The position of the olefinic bond between C-1 and C-2 was established from correlations of H-1 (δ_H 5.97) with C-3 (δ_C 22.6), C-5 (δ_C 40.0) and C-10 (δ_C 35.3) and of C-2 (δ_C 127.7) with H-4 (δ_H 1.73). The correlation in the HMBC experiments, between H_3-19 and H_3-20 (δ_H 0.88 and 0.93) with C-4 (δ_C 41.9), confirmed the position of the isopropyl group on C-4. The correlation of H-17a (δ_H 3.89) with C-4 (δ_C 41.9), C-5 (δ_C 40.0) and C-6 (δ_C 24.7), and of H-17b (δ_H 3.72) with C-4 (δ_C 41.9) secured the position of the bromomethyl group on C-5. The oxygenated methine was positioned on C-14, as concluded by the correlations of C-14 (δ_C 74.5) with H-13α (δ_H 2.63) and H_3-15 (δ_H 0.77), and of H-14 (δ_H 3.74) with C-9 (δ_C 42.2) and C-12 (δ_C 216.6). Moreover the correlations between H_3-15 (δ_H 0.77) with C-8 (δ_C 39.7), C-7 (δ_C 29.3), C-9 (δ_C 42.2) and C-14 (δ_C 74.5) and of H_3-16 (δ_H 1.34) with C-11 (δ_C 76.3), C-9 (δ_C 42.2) and C-12 (δ_C 216.6), confirmed the positions of the quaternary methyl groups. The relative stereochemistry of **3** was assigned by NOE experiments. The NOE correlations between H-1/H-9, H-1/H_3-16, H-9/H_3-16, H-17a/H-9 and H-17b/H-9, H-17a/H-7β and H-17b/H-4, established the stereochemistry at C-4, C-5, C-9 and C-11. The correlations between H-10/H_3-15, H-10/H_3-20 and H-10/H-18 established the stereochemistry at C-8 and C-10. The absence of any correlation between H-14/H-9 and the NOE correlations between H_3-15/H-14, H-14/H-7β and H-14 with both H-13α and H-13β, established the stereochemistry at C-14. The large coupling constant of H-9 and H-10 supported the trans diaxial configuration of these protons. According to the above observations, the structure of metabolite **8** was established as 14*R*-hydroxy-13,14-dihydro-sphaerococcenol-A.

Table 4
In vitro cytotoxicity of metabolites **1**–**9**

Compound	IC50 (μg/mL)	
	Cell line	
	NSCLC-N6-L16	A549
1	Inactive	Inactive
2	9.5	12
3	6	5
4	Inactive	Inactive
5	>30	>30
6	Inactive	>30
7	Inactive	Inactive
8	5	4
9	Inactive	Inactive

Metabolites **1**–**9** were evaluated for their cytotoxicity against the NSCLC-N6-L16 and A549 human lung cancer cell lines. The results of the cytotoxic activity of the tested compounds after 48 h of incubation are given in Table 4. Compounds **2**, **3** and **8** were more potent as cytotoxic agents against the tested cell lines, whereas **5** and **6** exhibited lower activity. It seems that the presence of the hydroperoxy group or an additional hydroxy group on C-14 of sphaerococcenol-A increases the cytotoxicity of the molecules. These preliminary results might help a structure–activity study in the future for the synthesis of related compounds possessing selectivity on the NSCLC-N6-L16 and A549 cancer cell lines.

3. Experimental

3.1. 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 experiments (^{1}H–^{1}H COSY, HMQC, HMBC, NOESY) were performed using standard Bruker microprograms. High-resolution mass spectra data were provided by the University of Notre Dame, Department of Chemistry and Biochemistry, Indiana, USA. Low-resolution chemical ionisation (positive) MS data were recorded on a Thermo DSQ Mass Detector using direct exposure probe (DEP) and methane as the CI gas. Vacuum column chromatography (VCC) 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} aluminium 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 Spherisorb S10 W HPLC normal phase column, 25 cm×10 mm or a Kromasil 100 C18, 5 μm 25 cm×8 mm.

3.2. Plant material

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

3.3. 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 VCC on silica gel, using cyclohexane with increasing amounts (10%) of EtOAc and finally MeOH as mobile phase. The CH_3CN soluble part (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 to yield pure compound **8** (5.1 mg). Peak X_b (retention time 11.97 min) (21.0 mg) was subjected again to reversed phase HPLC chromatography, using CH_3CN as mobile phase to yield pure compound **3** (4.9 mg), while peak XI_b (retention time 12.52 min) (86.3 mg) with similar re-purification yielded pure compounds **2** (4.6 mg), **4** (8.5 mg) and **5** (54.4 mg). The CH_3CN soluble part (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 to yield pure compound **6** (42.1 mg). Fraction II_a (20% EtOAc in cyclohexane) (4.01 g) was subjected to GCC, using cyclohexane with increasing amounts (2%) of EtOAc as mobile phase. Fraction XI_c (10% EtOAc in cyclohexane) (6.2 mg) was subjected to normal phase HPLC chromatography, using 2% EtOAc in cyclohexane as mobile phase to yield pure compound **1** (3.0 mg). Part (50 mg) of the VII_c fraction (8% EtOAc in cyclohexane) (2.85 g) was subjected to normal phase HPLC chromatography, using 5% EtOAc in cyclohexane as mobile phase to yield pure compounds **7** (21.0 mg) and **9** (18.7 mg).

3.3.1. Compound 1

Colourless oil; $[\alpha]_D^{20}$ +63.3 (*c* 2.8, $CHCl_3$); UV $\lambda_{max}^{CHCl_3}$ (log ε): 245 (2.47) nm; IR ($CHCl_3$) ν_{max} 3478, 2951 cm^{-1}; HRFAB-MS (*m*/*z*): 445.0739 $[M-H]^+$ (calcd for $C_{20}H_{31}^{79}Br_2O$: 445.0742); NMR data ($CDCl_3$), see Tables 1 and 2; CIMS, *m*/*z* (rel int. %): 429:431:433 $[MH-H_2O]^+$ (4:8:3), 367:369 $[MH-HBr]^+$ (10:9), 349:351 $[MH-HBr-H_2O]^+$ (100:97), 335:337 (18:17), 305:307 (20:19), 269 $[MH-H_2O-2HBr]^+$ (77), 255 (39), 225 (17), 173 (17), 147 (23), 109 (36), 95 (35), 79 (12).

3.3.2. Compound 2

Crystalline solid; $[\alpha]_D^{20}$ +82.2 (*c* 1.5, $CHCl_3$); UV $\lambda_{max}^{CHCl_3}$ (log ε): 251 (2.88), 321 (1.80) nm; IR ($CHCl_3$) ν_{max} 3448, 3235, 2952, 1683 cm^{-1}; HRFAB-MS (*m*/*z*): 477.0621 $[M-OH]^+$ (calcd for $C_{20}H_{31}^{79}Br_2O_3$: 477.0640); NMR data ($CDCl_3$ and acetone-d_6), see Tables 1 and 2; CIMS, *m*/*z* (rel int. %): 477:479:481 $[MH-H_2O]^+$ (14:22:10), 459:461:463 $[MH-2H_2O]^+$ (15:33:17), 441:443:445 $[MH-3H_2O]^+$ (30:61:31), 415:417 $[MH-HBr]^+$ (3:4), 397:399 $[M-H_2O-HBr]^+$ (63:60), 379:381 $[MH-HBr-2H_2O]^+$ (98:100), 363:365 $[MH-H_2O_2-H_2O-HBr]^+$ (35:34), 347:349 (10:12), 283 $[MH-H_2O_2-H_2O-2HBr]^+$ (44), 265 (16), 241 (29), 173 (20), 133 (22), 83 (39).

3.3.3. Compound 3

Crystalline solid; $[\alpha]_D^{20}$ +29.8 (*c* 0.8, $CHCl_3$); NMR data ($CDCl_3$), see Table 3; CIMS, *m*/*z* (rel int. %): 477:479:481 $[MH-H_2O]^+$ (4:6:3), 459:461:463 $[MH-2H_2O]^+$ (27:50:25), 441:443:445 $[M-3H_2O]^+$ (23:45:22), 415:417 $[MH-HBr]^+$ (3:4), 397:399 $[M-H_2O-HBr]^+$ (36:34), 379:381 $[MH-HBr-2H_2O]^+$ (99:100), 363:365 $[MH-H_2O_2-H_2O-HBr]^+$ (32:30), 283 $[MH-H_2O_2-H_2O-2HBr]^+$ (62), 265 (31), 159 (15), 133 (12), 83 (32).

3.3.4. Compound 4

Crystalline solid; $[\alpha]_D^{20}$ −35.6 (*c* 0.8, $CHCl_3$); NMR data ($CDCl_3$), see Table 3; CIMS, *m*/*z* (rel int. %): 445:447:449 $[MH-H_2O]^+$ (1:2:1), 427:429:431 $[MH-2H_2O]^+$ (2:4:2), 365:367 $[MH-HBr-H_2O]^+$ (47:45), 351:353 $[MH-CH_3Br-H_2O]^+$ (14:12), 347:349 (34:35), 321:323 $[MH-HBr-H_2O-CHMe_2]^+$ (26:26), 303 $[MH-2HBr]^+$ (9), 285 $[MH-2HBr-H_2O]^+$ (92), 271 $[MH-HBr-CH_3Br-H_2O]^+$ (100), 267 $[MH-2HBr-2H_2O]^+$ (86), 241 (26), 173 (17), 159 (35), 121 (15), 83 (53).

3.3.5. Compound 5

Colourless oil; $[\alpha]_D^{20}$ −43.8 (*c* 0.9, $CHCl_3$); NMR data ($CDCl_3$), see Table 3; CIMS, *m*/*z* (rel int. %): 463:465:467 $[MH]^+$ (3:4:2), 445:447:449 $[MH-H_2O]^+$ (14:21:12), 427:429:431 $[MH-2H_2O]^+$ (7:12:6), 383:385 $[MH-HBr]^+$ (34:35), 369:371 $[MH-CH_3Br]^+$ (100:98), 365:367 $[MH-HBr-H_2O]^+$ (81:80), 351:353 (62:59), 347:349 (34:35), 303 $[MH-2HBr]^+$ (39), 285 $[303-H_2O]^+$ (54), 267 $[285-2H_2O]^+$ (48), 243 (21), 229 (20), 201 (13), 161 (18), 109 (21), 95 (17), 83 (16).

3.3.6. Compound 6

Colourless oil; $[\alpha]_D^{20}$ −0.8 (*c* 1.0, $CHCl_3$); NMR data ($CDCl_3$), see Table 3; CIMS, *m*/*z* (rel int. %): 463:465:467 $[MH]^+$ (2:3:1), 445:447:449 $[MH-H_2O]^+$ (5:8:4), 427:429:431 $[MH-2H_2O]^+$ (4:7:4), 383:385 $[MH-HBr]^+$ (18:15), 365:367 $[MH-HBr-H_2O]^+$ (100:96), 351:353 $[MH-CH_3Br-H_2O]^+$ (48:46), 347:349 $[365-H_2O]^+$ (24:24), 303 $[MH-2HBr]^+$ (20), 285 (81), 267 (38), 229 (13), 201 (12), 189 (12), 135 (16), 109 (25), 95 (21), 83 (28).

3.3.7. Compound 8

Colourless oil; $[\alpha]_D^{20}$ −51.5 (*c* 1.5, $CHCl_3$); UV $\lambda_{max}^{CHCl_3}$ (log ε): 250 (2.79), 414 (2.27), 672 (1.80), 505 (1.43), 536 (1.35) nm; IR ($CHCl_3$) ν_{max} 3468, 2932, 1703 cm^{-1}; HRFAB-MS (*m*/*z*): 399.1533 $[M+H]^+$ (calcd for $C_{20}H_{32}^{79}BrO_3$: 399.1535); NMR data ($CDCl_3$), see Tables 1 and 2; CIMS, *m*/*z* (rel int. %): 399:401 $[MH]^+$ (2:2), 381:383 $[MH-H_2O]^+$ (23:19), 363:365 $[381-H_2O]^+$ (23:22), 337 $[MH-H_2O-CHMe_2]^+$ (19), 319 $[MH-HBr]^+$ (21), 301 $[MH-HBr-H_2O]^+$ (59), 283 $[MH-HBr-2H_2O]^+$ (100), 269 (20), 239 (27), 229 (26), 161 (23), 121 (19), 91 (24), 81 (18).

3.4. X-ray crystallographic analysis

Single crystal X-ray diffraction data of compounds **2**–**4** were collected at 120 K on a Nonius Kappa CCD diffractometer with a graphite-monochromated Mo Kα radiation (λ=0.71073 Å) using the Nonius Collect Software. After the initial corrections and data reduction, intensities of reflections were used to solve (by direct methods) and refine the structures (on F^2) using the WINGX program.[24] A weighting scheme based upon $P=[F^2_o+2F^2_o]/3$ was employed. All the hydrogen atoms were located from difference maps and included in the refinements as riding. The absolute structures of compounds **2** and **4** were estimated by applying the Flack parameter.[25]

3.4.1. Single-crystal X-ray crystallography of 2

Number of measured, independent and observed parameter: 46250, 4590 and 4143. R_{int}=0.034. Suitable colourless blocks of **2** were obtained by slow evaporation of a saturated solution of MeOH. The crystals belong to the orthorhombic system, space group $P2_12_12_1$ (#14) with unit-cell dimensions a=8.3510(6) Å, b=10.2940(8) Å, c=23.368(2) Å, $\alpha=\beta=\gamma=90°$; V=2008.3 Å^3, Z=4, d_{calcd}=1.641 g/cm^3. The refined structural model converged to

a final R_1=0.037, wR_2=0.075 and S=1.11 for 4590 observed reflections [$I/2\sigma(I)$] and 263 parameters. Flack parameter 0.003 (11).

*3.4.2. Single-crystal X-ray crystallography of **3***

Number of measured, independent and observed parameter: 26389, 9390 and 7075. R_{int}=0.058. Suitable colourless blocks of **3** were obtained by slow evaporation of a saturated solution of MeOH. The crystals belong to the triclinic system, space group $P1$ (#1) with unit-cell dimensions a=7.399(2) Å, b=7.734(5) Å, c=18.953(9) Å, α=97.397(5)°, β=99.209(3)°,γ=98.512(4)°, V=1045.8 Å^3, Z=1, d_{calcd}=1.605 g/cm^3. The refined structural model converged to a final R_1=0.079, wR_2=0.093 and S=1.02 for 9390 observed reflections [$I/2\sigma(I)$] and 491 parameters. Flack parameter 0.042 (8).

*3.4.3. Single-crystal X-ray crystallography of **4***

Number of measured, independent and observed parameter: 46993, 4447 and 4000. R_{int}=0.039. Suitable colourless blocks of **4** were obtained by slow evaporation of a CH_2Cl_2/EtOAc (1:1) solution. The crystals belong to the orthorhombic system, space group $P2_12_12_1$ (#14) with unit-cell dimensions a=8.6872(6) Å, b=9.7897(8) Å, c=23.0472(18) Å, α=β=γ=90°, V=1960 Å^3, Z=4, d_{calcd}=1.573 g/cm^3. The refined structural model converged to a final R_1=0.037, wR_2=0.048 and S=1.11 for 4447 observed reflections [$I/2\sigma(I)$] and 223 parameters. Flack parameter 0.003 (8).

3.5. Determination of cytotoxicity

The NSCLC-N6-L16 cell line,[26] derived from a human non-small-cell broncho-pulmonary carcinoma (moderately differentiated, rarely keratinizing, classified as T2N0M0), along with A549 lung cancer cell line type II (reference number CCL6185) that was provided from the American National Cancer Institute, was used for the experiments. The cells were cultured in RPMI 1640 medium with 5% foetal calf serum, to which were added 100 IU penicillin ml^{-1}, 100 μg streptomycin ml^{-1} and 2 mM glutamine, at 37 °C in an air/carbon dioxide (95:5, v/v) atmosphere. In these conditions, cell doubling time was 48 h. Cells used in the experiment never exceeded 35 passages. Experiments were performed in 96 wells microtiter plates (2×10^5 cells ml^{-1}). Cell growth was estimated by colourimetric assay based on the conversion of tetrazolium dye (MTT) to a blue formazan product by live mitochondria.[27] Eight repeats were performed for each concentration. Control growth was estimated from 16 determinations. Optical density at 570 nm corresponding to solubilised formazan was read for each well on Titertek Multiskan MKII.

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22. Crystallographic data (excluding structure factors) for the structure of **3** has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication 671358 CCDC. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 (0) 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).
23. Crystallographic data (excluding structure factors) for the structure of **4** has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication 670886 CCDC. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 (0) 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).
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