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Isolation and Structure Determination of Aplidinones A–C from the Mediterranean Ascidian *Aplidium conicum*: A Successful Regiochemistry Assignment by Quantum Mechanical ^{13}C NMR Chemical Shift Calculations

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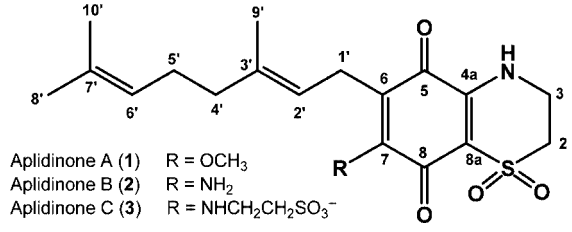
A chemical investigation of a Mediterranean ascidian, *Aplidium conicum*, has resulted in the isolation of the new metabolites aplidinone A (**1**), B (**2**), and C (**3**). The structures of aplidinones A–C were determined by interpretation of spectroscopic data, whereas the regiochemistry was determined by the comparison of experimental ^{13}C NMR chemical shifts with those predicted by GIAO shielding calculations.

Introduction

Many natural products of mixed terpene and quinone/hydroquinone biosynthesis have been reported from both marine and terrestrial sources. Examples based on the farnesyl quinone/hydroquinone skeleton include avarone and avarol from the marine sponge *Dysidea avara*,[1] cyclozonarone from the marine brown alga *Dictyopteris undulata*,[2] and farnesylhydroquinone from the tree *Wigandia kunthii*.[3] Marine ascidians within the family Polyclinidae have proven to be a rich source of several closely related terpene quinones/hydroquinones; remarkable examples are prenylated quinones,[4] longithorones,[5] and chromenols.[6] Of the approximately one dozen prenylated quinones isolated from the genus *Aplidium*, the first examples of terpene quinones containing the unusual 1,1-dioxo-1,4-thiazine ring are conicaquinones A and B from the tunicate *Aplidium conicum*, which we reported recently.[7] Further investigation of its secondary meabolite composition resulted in the isolation of thiaplidiaquinones A and B, inducing apoptosis in Jurkat cells because of a rapid overproduction of intracellular reactive oxygen species (ROS), which mediate the collapse of the mitochondrial transmembrane potential ($\Delta\Psi_m$).[8]

Deeper understanding of the extract of *Aplidium conicum* has now led to the isolation of three further novel geranylated quinones **1**–**3**, which also feature a 1,1-dioxo-1,4-thiazine ring fused with a quinone moiety. We report here on the structural elucidation of these novel compounds, named aplidinone A (**1**), B (**2**), and C (**3**), which are discussed on the basis of their spectroscopic data, specifically those obtained from 2D NMR experiments. The assignment of the regiochemistry of the 1,1-dioxo-1,4-thiazine ring was achieved by a comparison of the experimental ^{13}C NMR chemical shifts with those predicted by DFT (density functional theory) GIAO (gauge including atomic orbitals) shielding calculations.[9–11]



Aplidinone A (**1**) R = OCH_3
Aplidinone B (**2**) R = NH_2
Aplidinone C (**3**) R = $NHCH_2CH_2SO_3^-$

Results and Discussion

Specimens of *Aplidium conicum* Olivi (= *Amoroucium conicum*; order Aplousobranchiata, family Polyclinidae), collected by hand whilst SCUBA diving at Capo Caccia (Alghero, Italy), were homogenised and exhaustively extracted with methanol followed by chloroform. The combined extracts were concentrated, and the resulting aqueous residue was partitioned between water and ethyl acetate. The water portion was re-extracted with *n*BuOH. The ethyl acetate soluble material was chromatographed on a SiO_2 flash column using a gradient elution (*n*-hexane → ethyl acetate → methanol). The fractions eluted with AcOEt/MeOH (from 9:1 to 7:3) were further purified by normal

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phase HPLC and afforded aplidinone A (**1**, 6.0 mg) and B (**2**, 1.5 mg). The *n*BuOH phase was concentrated in vacuo and then subjected to CC (RP-18 MPLC) with a H_2O/MeOH gradient elution; the fraction eluted with H_2O/MeOH (1:1) was separated by C_{18} reverse phase HPLC to give aplidinone C (**3**, 6.0 mg) in a pure form.

Aplidinone A (**1**) was obtained as an amorphous solid with the molecular formula $C_{19}H_{25}NO_5S$ as indicated by HRFABMS (*m*/*z* = 378.1398, $[M - H]^-$). The facile loss of 64 amu observed in its FAB spectrum is attributed to the expulsion of SO_2, which suggests the presence of a sulfone functionality.

Analysis of the NMR spectra of aplidinone A reveals the presence of three moieties (Figure 1): a geranyl chain (**I**), a methoxylated 1,4-benzoquinone (**II**), and a 1,1-dioxothiazine ring (**III**).

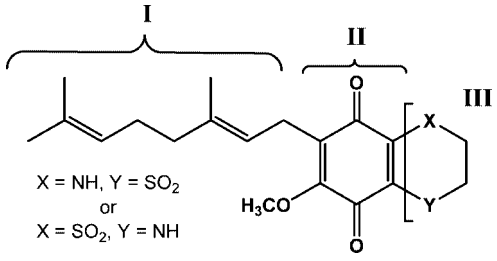


Figure 1. Substructures **I**, **II**, and **III** of compound **1**.

The ^{13}C NMR spectrum (Table 1) contains nineteen signals. Analysis of the ^1H- and HSQC NMR spectra indicates that two methine, five methylene, and four methyl carbon atoms are present in compound **1**. Signals for two olefinic protons [δ = 4.99 ppm (t, J = 7.1 Hz) and δ = 5.05 ppm (t, J = 6.4 Hz)], as well as for three methyl [δ = 1.58 ppm (s, 3 H), 1.66 (s, 3 H), and 1.69 ppm (s, 3 H)] and three methylene groups [δ = 3.09 ppm (d, J = 7.1 Hz), δ = 1.94 ppm (m) and δ = 2.03 ppm (m)], are present in the ^1H NMR spectrum. HMBC and COSY NMR correlations for the above signals allowed us to establish the presence of a geranyl chain in **1** (substructure **I**, Figure 1). The *cis* or (*E*) configuration at the C2′/C3′ double bond was established on the basis of the relatively upfield signal for the C-9′ methyl resonance (δ = 16.1 ppm).

In addition, the ^{13}C NMR spectrum includes two conjugated carbonyl signals (δ = 179.3 and 174.2 ppm) and four signals at δ = 109.2, 126.7, 144.3, and 157.3 ppm, which are attributed to two tetrasubstituted double bonds. These carbon atoms compose the skeleton of a tetrasubstituted 1,4-benzoquinone chromophore (substructure **II**, Figure 1), as indicated by UV data (see Experimental Section).

The remaining resonances of the ^1H NMR spectrum of **1** are a D_2O-exchangeable proton at δ = 6.83ppm (NH) coupled to a multiplet at δ = 4.06 ppm (m, 2 H), which in turn is coupled to a triplet at δ = 3.28 ppm (J = 6.6 Hz, 2 H); this suggests the presence of a $–NHCH_2CH_2SO_2–$ unit (substructure **III**, Figure 1). Finally, the ^1H NMR spectrum also contains a methyl singlet resonating at δ = 4.25 ppm, which is assigned to the methoxy group.

The analysis of the HMBC NMR correlations, some of which are illustrated in Figure 2, allowed us to connect the three substructures **I**–**III**. Unfortunately, the lack of key diagnostic long-range heterocorrelations in the HMBC NMR spectrum of **1**, prevented us from distinguishing between

Table 1. NMR spectroscopic data of compounds **1**–**3**.

Position	**1**[a] δ_C	δ_H, mult., (J in Hz)	HMBC[c]	**2**[a] δ_C	δ_H, mult., (J in Hz)	HMBC[c]	**3**[b] δ_C	δ_H, mult., (J in Hz)	HMBC[c]
2	49.0	3.28, t (6.6)	3	48.6	3.28, t (6.6)	3	49.0	3.34[d]	3
3	40.5	4.06, m	2, 4a	40.1	4.09, m	4a	41.0	4.01[d]	2, 4a
4a	144.3	–	–	149.5	–	–	151.0	–	–
5	179.3	–	–	173.1	–	–	174.5	–	–
6	126.7	–	–	108.9	–	–	110.0	–	–
7	157.3	–	–	147.8	–	–	149.0	–	–
8	174.2	–	–	175.4	–	–	176.6	–	–
8a	109.2	–	–	109.2	–	–	109.0	–	–
1′	22.2	3.09, d (7.1)	5, 6, 7, 2′, 3′	23.4	3.13, d (7.0)	5, 6, 7, 2′, 3′	24.0	3.33[d]	5, 6, 7, 2′, 3′
2′	119.2	4.99, t (7.1)	6, 1′, 4′, 9′	119.5	4.97, t (7.0)	–	124.8	5.04, t (7.0)	4′, 9′
3′	137.6	–	–	138.4	–	–	137.0	–	–
4′	39.7	1.94, m	2′, 3′, 5′, 6′, 9′	39.5	2.03, m	5′	40.5	2.03, m	2′, 3′, 5′, 6′, 9′
5′	26.6	2.03, m	3′, 4′, 6′, 7′	26.3	2.08, m	4′	27.8	2.10, m	3′, 4′, 6′, 7′
6′	124.1	5.05, t (6.4)	8′, 10′	123.8	5.02, t(6.2)	–	125.0	5.10, t (7.0)	8′, 10′
7′	131.5	–	–	131.8	–	–	133.0	–	–
8′	25.6	1.66, s	6′, 7′,10′	25.7	1.67, s	6′, 7′,10′	26.1	1.68, s	6′, 7′,10′
9′	16.1	1.69, s	2′, 3′, 4′	16.2	1.73, s	2′, 3′, 4′	16.5	1.73, s	2′, 3′, 4′
10′	17.7	1.58, s	6′, 7′, 8′	17.7	1.59, s	6′, 7′, 8′	17.8	1.61, s	6′, 7′, 8′
1′′	–	–	–	–	–	–	41.5	4.04[d]	7, 2′′
2′′	–	–	–	–	–	–	51.5	3.08, t (6.5)	1′′
OCH_3	62.3	4.25, s	7	–	–	–	–	–	–
NH-4	–	6.83, bs	–	–	7.35, bs	–	–	6.71[e]	–
NH-1′′	–	–	–	–	–	–	–	8.99[e]	–

[a] ^1H NMR and ^{13}C NMR shifts are referenced to $CDCl_3$ (δ_H = 7.26 ppm and δ_C = 77.0 ppm). [b] ^1H NMR and ^{13}C NMR shifts are referenced to CD_3OD (δ_H = 3.34 ppm and δ_C = 49.0 ppm). [c] Carbon atoms coupled with the given proton(s). [d] Overlapped. [e] The chemical shift value was obtained from an ^1H NMR spectrum performed in C_6D_5N (δ_H = 7.19, 7.55 and 8.71 ppm).

the alternative structures **1a** and **1b**, and did not allow us to assign the regiochemistry of the 1,1-dioxo-1,4-thiazine ring.

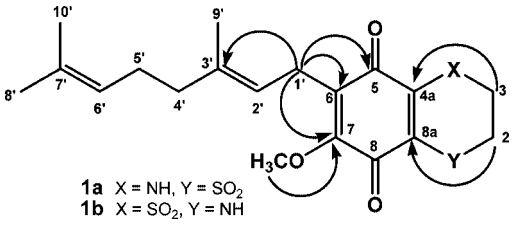


Figure 2. Diagnostic HMBC NMR correlations (H⌒C) of compound **1**.

The molecular formula $C_{18}H_{24}N_2O_4S$ of aplidinone B (**2**) was determined by interpretation of the $[M - H]^-$ pseudomolecular ion at m/z = 363.1369 in the HRFABMS in conjunction with NMR spectroscopic data. Comparison of the ^{13}C NMR spectrum (see Table 1) of **2** with that of **1** shows twelve almost coincident signals (deviation < 2 ppm). Ten of the above signals are assigned to the carbon atoms of a geranyl chain, while the other two are assigned to the two methylene carbon atoms C-2 and C-3. The remaining six ^{13}C NMR signals were assigned to the carbon atoms of a tetrasubstituted 1,4 benzoquinone moiety, whose presence is supported by UV data (see Experimental Section). The ^1H NMR spectrum of aplidinone B displays a close resemblance to that of aplidinone A, and the only observed difference is the absence of the methoxy signal. Since no signals for quinone ring protons are observed, it is evident that **2** is like **1** except for the substituent at C-7, which has an NH_2 function at this position as indicated by comparison of the molecular formulae of **1** and **2**.

Analysis of HMBC NMR correlations (see Table 1) confirms the whole structure of **2**, except for the regiochemistry of the 1,1-dioxo-1,4-thiazine ring.

The molecular formula $C_{20}H_{28}N_2O_7S_2$ of compound **3** was unequivocally established by HRFABMS and corroborated by NMR spectroscopic data. The close similarity of **1**, **2**, and **3** was suggested by the comparison of their ^1H and ^{13}C NMR spectra; these compounds appear to be different only by the substituent at C-7. The key distinguishing feature of the ^1H NMR spectrum (CD_3OD) of **3** is the presence of two mutually coupled methylene signals at δ = 3.08 ppm (t, J = 6.5 Hz) and δ = 4.04 ppm (partially overlapped). Moreover, the ^1H NMR spectrum performed in C_6D_5N shows, in addition to the signals for these methylene protons [δ = 3.60 ppm (m) and δ = 4.30 ppm (m)], two signals that are due to D_2O-exchangeable protons at δ = 6.71 (NH-4) and 8.99 ppm (NH-1′′), the latter being coupled with the protons of the downfield methylene group. Taking into account the molecular formula, these data suggest that the substituent at C-7 is a $-NHCH_2CH_2SO_3^-$ group.

A detailed analysis of the ^1H and ^{13}C NMR spectra of aplidinone C, aided by COSY, HSQC, and HMBC 2D NMR experiments, allowed the assignment of all the proton and the carbon signals (see Table 1). Analogously to compounds **1** and **2**, the NMR spectroscopic data of compound **3** is incomplete and thus the regiochemistry of its 1,1-dioxo-1,4-thiazine ring cannot be assigned. Unfortunately, in addition to the lack of key diagnostic NMR correlations in the 2D NMR spectra, the novelty of the structures **1**–**3** prevented us from defining their regiochemistry by comparison of our experimental data with those of known compounds.

Some papers have recently appeared in the literature regarding structure validation of natural products by GIAO calculation of ^{13}C NMR chemical shifts.[12–14] These papers

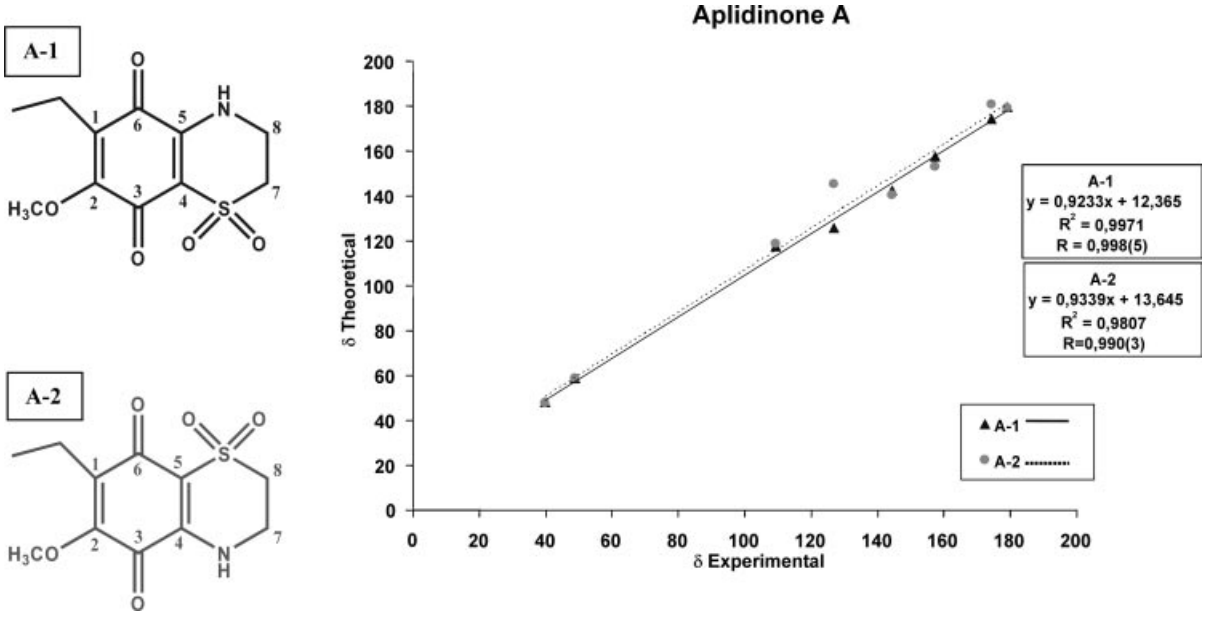


Figure 3. Correlation plot of calculated versus experimental ^{13}C NMR chemical shifts for the rightly (A-1) and the wrongly (A-2) proposed structures of aplidinone A.

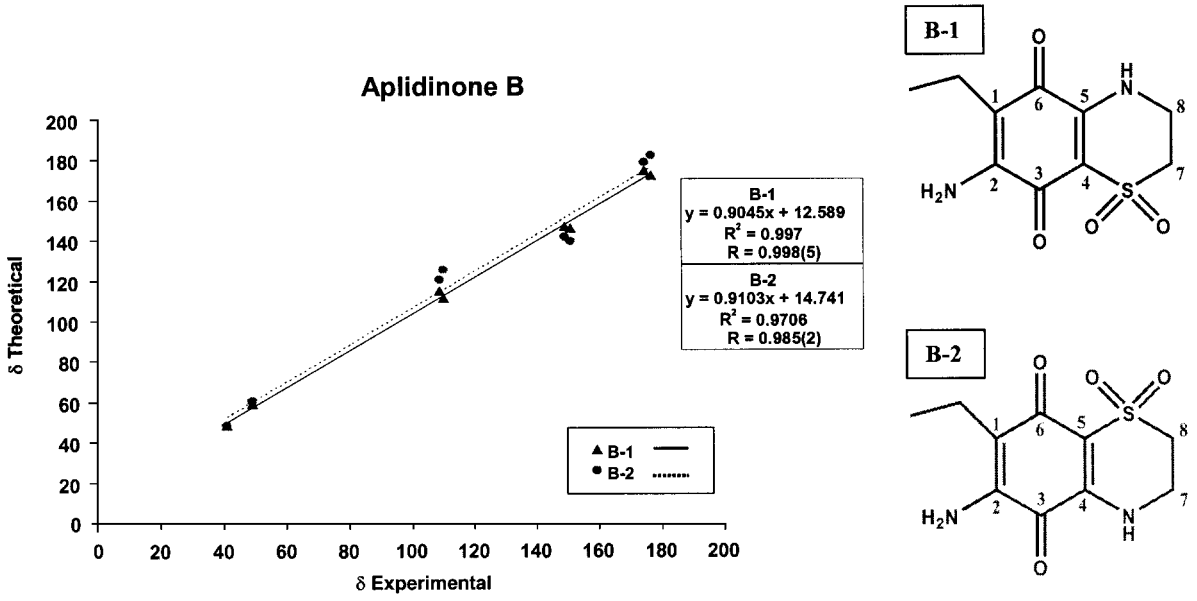
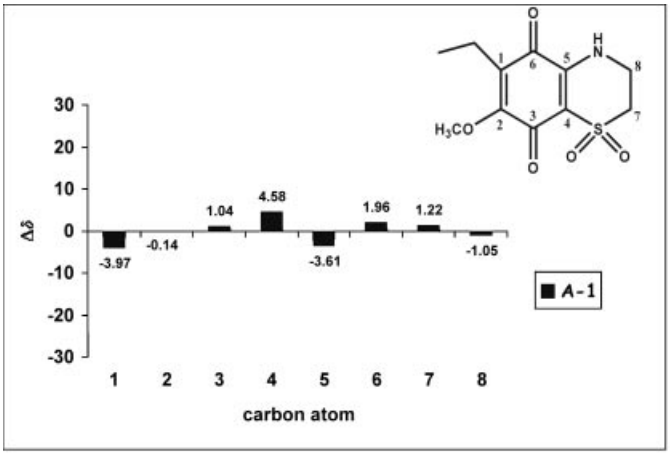


Figure 4. Correlation plot of calculated versus experimental ^{13}C NMR chemical shifts for the rightly (B-1) and the wrongly (B-2) proposed structures of aplidinone B.



A-1

carbons	δ exper.	δ theor.	δ scal. theor.	Δδ
1	126.7	125.68	122.73	-3.97
2	157.3	157.47	157.16	-0.14
3	174.2	174.17	175.24	1.04
4	109.2	117.42	113.78	4.58
5	144.3	142.27	140.69	-3.61
6	179.2	179.63	181.16	1.96
7	49.0	58.74	50.22	1.22
8	39.7	48.05	38.65	-1.05

A-2

carbons	δ exper.	δ theor.	δ scal.theor.	Δδ
1	126.7	145.29	140.96	14.26
2	157.3	152.86	149,06	-8.24
3	174.2	180.78	178.96	4.76
4	144.3	140.46	135.79	-8.51
5	109.2	118.97	112.78	3.58
6	179.2	179.24	177.31	-1.89
7	49	58.69	48.23	-0.77
8	39.7	47.74	36.51	-3.19

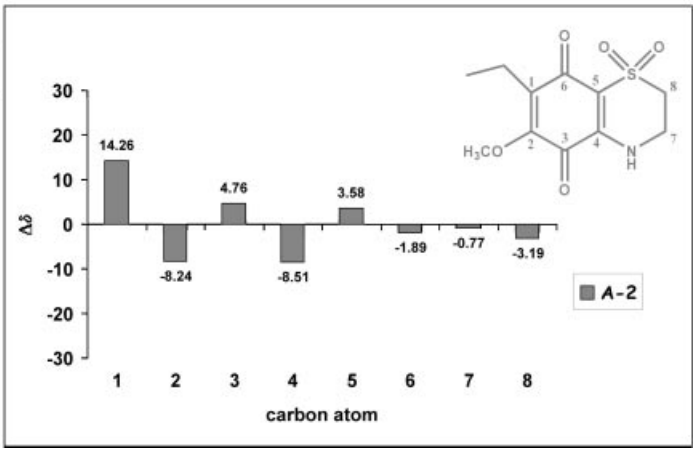


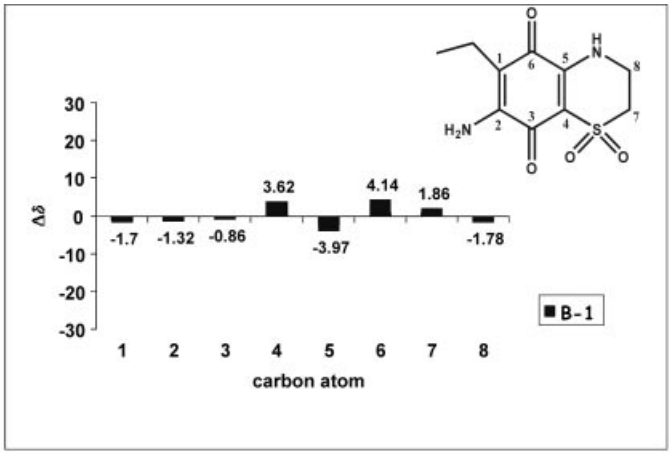
Figure 5. Differences between scaled and experimental ^{13}C NMR chemical shifts for the rightly (A-1) and wrongly (A-2) proposed structures of aplidinone A.

indicate that ^{13}C NMR chemical shifts can be predicted with a degree of accuracy that is adequate for discrimination between trial structures, provided that a careful geometry optimization is performed prior to the calculation.

The rigid bicyclic moiety of aplidinones is particularly suitable for such a calculation, but the flexible geranyl side chain, with many local conformational energy minima, would require an unnecessarily heavy amount of computational work. Therefore, DFT calculations were performed on the four model compounds A-1 and A-2 (related to the two possible regioisomers of aplidinone A, Figure 3) and B-1 and B-2 (related to the regioisomers of aplidinone B, Figure 4), in which the geranyl chain of the natural product was replaced by an ethyl group, which is much shorter, although its influence on the ^{13}C NMR chemical shifts of the bicyclic system is basically the same. The four structures were fully optimized by using the Gaussian03W package[15] at the B3LYP level with the 6-31(+)G-2d basis set.

The theoretical GIAO ^{13}C NMR chemical shifts for the optimized structures A1 and A2 were then calculated and plotted against the corresponding experimental ^{13}C NMR chemical shifts of aplidinone A (Figure 3). Analogously, the theoretical ^{13}C NMR chemical shift values calculated for B1 and B2 were calculated and plotted against the corresponding ^{13}C NMR chemical shifts of aplidinone B (Figure 4). For each structure, the values for the intercept, slope, and correlation coefficient (R) of the least-squares linear fits are reported in the respective figure. The R value for structure A-1 [0.998(5)] is remarkably higher than that observed for structure A-2 [0.990(3)]. Literature data[12] show that for a correct structure, R values are usually greater than or equal to 0.995. Therefore, our results strongly suggest that the regiochemistry of aplidinone A is that of the model compound A-1 rather than that of A-2. Likewise, the results obtained for structures B-1 [R = 0.998(5)] and B-2 [R = 0.985(2)], respectively, suggest that aplidinone B has the same regiochemistry as model compound B-1.

An alternative and probably more intuitive approach has been proposed[12] to analyze the results of the GIAO calculation of ^{13}C NMR chemical shifts. This approach is based on the difference ($\Delta\delta$) between the experimental chemical shift (CS_X) and the "scaled theoretical" chemical shift (CS_{SX}). The scaled theoretical chemical shift of a carbon atom X of a given compound, CS_{SX}, is obtained from: $CS_{SX} = (CS_X - Intercept)/Slope$, where *Intercept* and *Slope* are the least-squares parameters obtained by the linear cor-



B-1				
carbons	δ exper.	δ theor.	δ scal. theor.	Δδ
1	110.0	110.55	108.30	-1.7
2	149.0	146.17	147.68	-1.32
3	176.6	171.55	175.74	-0.86
4	109.0	114.46	112.62	3.62
5	151.0	145.58	147.03	-3.97
6	174.5	174.17	178.64	4.14
7	49.0	58.60	50.86	1.86
8	41.0	48.07	39.22	-1.78

B-2				
carbons	δ exper.	δ theor.	δ scal.theor.	Δδ
1	110,0	124.86	120.97	10.97
2	149.0	140.71	138.38	-10.62
3	176.6	181.55	183.24	6.64
4	151.0	138.64	136.10	-14.90
5	109.0	120.01	115.64	6.64
6	174.5	178.17	179.53	5.03
7	49.0	60.12	49.85	0.85
8	41.0	47.88	36.40	-4.60

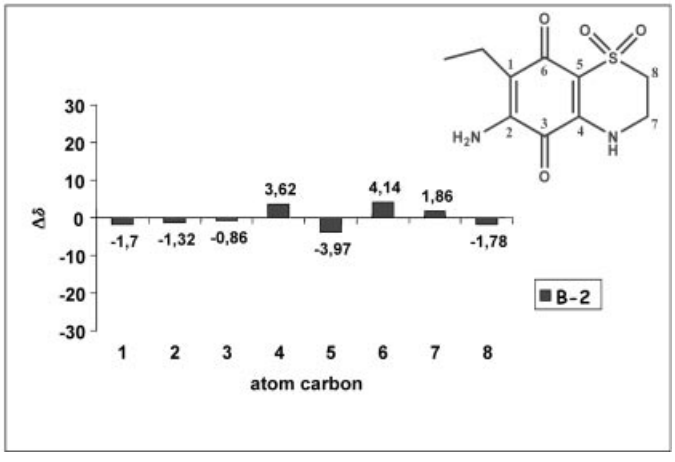


Figure 6. Differences between scaled and experimental ^{13}C NMR chemical shifts for the rightly (B-1) and wrongly (B-2) proposed structures of aplidinone B.

relation plot. In graphical terms, $\Delta\delta$ represents the horizontal distance between the points and the least-square line for the plots in Figure 3 and Figure 4. The rationale of this approach is that any systematic errors involved in the calculation of the chemical shifts is cancelled out, so that emphasis is given to the actual influence on the chemical shifts of the structural feature of the molecule.

The $\Delta\delta$ values for each carbon atom of model structures A-1 and A-2, and B-1 and B-2 are shown in Figure 5 and Figure 6, respectively. The difference in the quality of the results for model compounds A-1 and B-1 compared to A-2 and B-2, respectively, is apparent here. For example, calculations performed by using model compound A-1 resulted in relatively small $\Delta\delta$ values – the largest deviation was + 4.68 ppm for C-4. In contrast, when model compound A-2 was used for the calculations, large deviations were observed for C-1, C-2, C-4, and C-5, and the largest deviation was as high as + 14.26 for C-1 (Figure 5). Thus, the results of the calculations allowed us to determine the regiochemistry of aplidinone A and B (as indicated by structures **1** and **2**) with a high degree of certainty.

As for the regiochemistry of aplidinone C, the ^{13}C NMR chemical shifts observed for the bicyclic system are almost superimposable on those observed for aplidinone B, thus suggesting aplidinone C (**3**) has the same regiochemistry as that proposed for aplidinone B (**2**).

Experimental Section

General Procedures: Low and high resolution resolution FAB mass spectra (glycerol matrix) were obtained with a VG Prospec (FISONS) mass spectrometer. NMR experiments were performed on a Bruker AMX-500 spectrometer; chemical shifts are referenced to the residual solvent signal ($CDCl_3$: δ_H = 7.26 ppm, δ_C = 77.0 ppm; CD_3OD: δ_H = 3.34 ppm, δ_C = 49.0 ppm; C_6D_5N: δ_H = 7.19, 7.55 and 8.71 ppm).

Homonuclear (^1H–^1H) and heteronuclear (^1H–^{13}C) connectivities were determined by magnitude-mode COSY and phase-sensitive GE-HSQC NMR experiments, respectively, multiplicity-edited and optimized for a $^1J_{CH}$ of 150 Hz. Two- and three-bond ^1H–^{13}C connectivities were determined by GE-HMBC NMR experiments and optimised for a $^{2,3}J_{CH}$ of 10 Hz. Medium-pressure liquid chromatography (MPLC) was performed using a Büchi 861 apparatus with SiO_2 (230–400 mesh) packed columns. High performance liquid chromatography (HPLC) separations were achieved using a Waters 501 apparatus equipped with an RI detector. UV spectra (MeOH) were recorded on a Shimadzu UV-1204 instrument.

Collection, Extraction, and Isolation of Aplidinones A–C: Specimens of *Aplidium conicum* Olivi (= *Amoroucium conicum*; order Aplousobranchiata, family Polyclinidae), collected by hand whilst SCUBA diving at Capo Caccia (Alghero, Italy), were homogenised and exhaustively extracted with methanol followed by chloroform. The combined extracts were concentrated, and the resulting aqueous residue was partitioned between water and ethyl acetate. The ethyl acetate soluble material was chromatographed on a SiO_2 flash column using gradient elution (*n*-hexane→ethyl acetate→methanol); the resulting fractions eluted with AcOEt/MeOH (9:1) were further purified by normal-phase HPLC (SiO_2 column, ethyl acetate/*n*-hexane, 75:25) and afforded 1.5 mg of aplidinone B (**2**). The fractions eluted with AcOEt/MeOH (7:3) were re-chromatographed by HPLC (SiO_2 column, ethyl acetate/*n*-hexane, 1:1) to yield pure aplidinone A (**1**, 3 mg). Subsequently, the polar layer was re-extracted with *n*BuOH. The *n*BuOH solution was concentrated in vacuo and then subjected to CC (RP-18 MPLC) with a H_2O/MeOH gradient elution; the fraction eluted with H_2O/MeOH (1:1) was separated by C_{18} reverse- phase HPLC (Luna C_{18}, MeOH/H_2O, 1:1) to give pure aplidinone C (**3**, 5 mg).

Aplidinone A (1): Orange amorphous solid. FABMS negative ion: m/z = 378 $[M - H]^-$, 314 $[(M - SO_2) - H]^-$. HRFABMS ($C_{19}H_{24}NO_5S$): calcd. m/z = 378.1375 $[M - H]^-$; found m/z = 378.1398. UV (CH_3OH): λ_{max} (ε) = 310 nm (5700). ^1H NMR and ^{13}C NMR spectroscopic data and HMBC correlations ($CDCl_3$) are reported in Table 1.

Aplidinone B (2): Violet amorphous solid. FABMS negative ion: m/z = 363 $[M - H]^-$, 299 $[(M - SO_2) - H]^-$. HRFABMS ($C_{18}H_{24}N_2O_4S$): calcd. m/z = 363.1384 $[M - H]^-$; found m/z = 363.1369. UV (CH_3OH): λ_{max} (ε) = 330 nm (2600). ^1H NMR and ^{13}C NMR spectroscopic data and HMBC correlations ($CDCl_3$) are reported in Table 1.

Aplidinone C (3): Red-violet amorphous solid. FABMS negative ion: m/z = 471 $[M - H]^-$, 391 $[(M - HSO_3)]^-$. HRFABMS ($C_{20}H_{27}N_2O_7S_2$): calcd. m/z = 471.12597 $[M - H]^-$; found m/z = 471.13056. UV (MeOH): λ_{max} (ε) = 340 nm (2900). ^1H NMR and ^{13}C NMR spectroscopic data and HMBC correlations (CD_3OD) are reported in Table 1.

Computational Methods: DFT calculations were performed on the model compounds indicated in Figure 3 and Figure 4 at the B3LYP level, with the 6-31(+)G-2d basis set, using the Gaussian03W package (Revision B05).[15] The geometry of the model compounds, together with that of tetramethylsilane (TMS), were fully optimized. The theoretical ^{13}C NMR chemical shift values were obtained by subtracting the GIAO-calculated[12] ^{13}C NMR isotropic magnetic shielding (IMS) for each carbon atom X from the average GIAO ^{13}C NMR IMS of TMS: $CS_{SX} = IMS_{TMS} - IMS_X$. The least-squares linear fitting parameters of correlation plots between computed and experimental chemical shift values and a functional expression of the same parameters (see Results and Discussion) were employed to discriminate between trial structures.

Supporting Information (see footnote on the first page of this article)**:** ^1H NMR spectra of compounds **1**–**3** and COSY, ^{13}C NMR, HMBC, and FABMS spectra of **3**.

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