Conicaquinones A and B, Two Novel Cytotoxic Terpene Quinones from the Mediterranean Ascidian *Aplidium conicum*

Anna Aiello,[a] Ernesto Fattorusso,*[a] Paolo Luciano,[a] Marialuisa Menna,[a] Giuseppe Esposito,[b] Teresa Iuvone,[b] and David Pala[c]

Keywords: Ascidians / Natural products / Quinones / Terpenoids / Thiazinoquinones

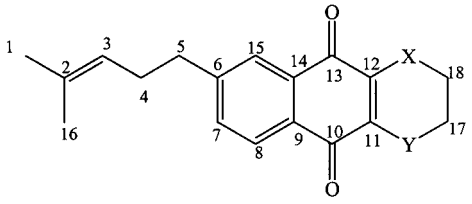
Two new unique cytotoxic metabolites, conicaquinones A (**1**) and B (**2**), each possessing a 1,1-dioxo-1,4-thiazine ring, were isolated from the Mediterranean ascidian *Aplidium conicum*. Their structures were established by an extensive spectroscopic analysis. Compounds **1** and **2** exhibited a strong cytotoxicity in vitro against rat glioma cells.

Introduction

In the last 25 years, ascidians have proven to be a valid potential source of anticancer drug leads because of the number of novel and cytotoxic metabolites they have yielded.[1] It is noteworthy that of the six marine-derived compounds that have reached advanced preclinical and clinical trials as antitumor agents, three — didemnin B, dehydrodidemnin B, and ecteinascidin 743 (ET-743) — are derived from tunicates.[2]

In the course of our continuing search for antitumor agents from marine invertebrates, we have examined the chemistry and the pharmacological properties of the Mediterranean ascidian *Aplidium conicum* (Polyclinidae). Marine ascidians of the order Aplousobranchiata, family Polyclinidae, have been reported to synthesise prenylated quinones and hydroquinones, some of them with interesting biological properties. Indeed, the first biologically active tunicate metabolite, geranylhydroquinone, was isolated from an *Aplidium* sp. and shown to offer protection against leukemia and tumour development in test animals.[3] Further examples include prenylated quinones from *Aplidium* sp.,[4] *A. californicum*,[5] *A. costellatum*[6] and *A. antillense*,[7] dimeric prenylated quinones from *A. longithorax*,[8,9] and chromenols from *A. solidum*[10] and *Amaroucium multiplicatum*.[11]

In this paper, we report the isolation and structural elucidation of two novel cytotoxic terpene quinones, conicaquinones A (**1**) and B (**2**) from *A. conicum* (Figure 1).



1 X = SO_2, Y = NH
2 X = NH, Y = SO_2

Figure 1. Structures of conicaquinones A (**1**) and B (**2**)

Compounds **1** and **2** possess an unusual 1,1-dioxo-1,4-thiazine ring added to the quinone moiety; they exhibited a strong cytotoxicity in vitro against C6 (rat glioma) cells.

Results and Discussion

Specimens of *A. conicum* (170 g dry wt.) were homogenised and exhaustively extracted with MeOH. The extract was concentrated and the residue was partitioned between water and ethyl acetate. The ethyl acetate soluble material was subjected to medium-pressure liquid chromatography (MPLC) on SiO_2 using a gradient elution (*n*-hexane → ethyl acetate → methanol); further purification by normal-phase HPLC (SiO_2 column; ethyl acetate/*n*-hexane, 75:25, v/v) yielded pure conicaquinone A (**1**, 6 mg) and conicaquinone B (**2**, 3 mg).

HRFAB-MS data indicate that the two compounds are isomers with the molecular formula $C_{18}H_{19}NO_4S$; a facile loss of 64 amu observed in their mass spectra, attributable to expulsion of SO_2, suggests the presence in both compounds of a sulfone functionality.

[a] Dipartimento di Chimica delle Sostanze Naturali
Via D. Montesano 49, 80131 Napoli, Italy
Fax: (internat.) + 39-081/678-552
E-mail: fattoru@unina.it
[b] Dipartimento di Farmacologia Sperimentale,
Via D. Montesano 49, 80131 Napoli, Italy
[c] Battelle Memorial Institute
Rue Drize 7, Geneva, Switzerland

Analysis of NMR spectra, including 2D COSY, HSQC, and HMBC, of conicaquinone A and B reveals in both compounds three substructures, consisting of a terpenoid portion, a 1,4-naphthoquinone, and a 1,1-dioxothiazine ring.

The ^{13}C NMR and DEPT spectra of compound **1** reveal 18 carbon atoms, thus confirming the formula suggested by the mass spectral data, and indicating the presence of two methyl groups, four sp^3-hybridised methylene units, four sp^2-hybridised methine units, and eight unprotonated sp^2-hybridised carbon atoms; all the ^1H-^{13}C NMR correlations are evident from the HSQC spectrum. The presence of a 1,4-naphthoquinone chromophore is suggested by IR and UV absorption spectra (see Exp. Sect.); moreover, the ^1H NMR spectrum of **1** contains peaks corresponding to three aromatic protons, two with an *ortho* coupling (J = 7.8 Hz) centred at δ = 8.10 (d) and 7.61 ppm (dd) and one with a *meta* coupling (J = 1.6 Hz) centred at δ = 7.85 ppm. Additional features of the ^1H NMR spectrum of **1** are a signal of an exchangeable proton at δ = 6.78 ppm coupled to a two-proton multiplet at δ = 4.10 ppm, which in turn is coupled to a two-proton multiplet at δ = 3.36 ppm; these signals of an isolated spin system are consistent with an $-NHCH_2CH_2SO_2-$ moiety. Since no signals of quinone ring protons are observed, the $-NHCH_2CH_2SO_2-$ and the 1,4-naphthoquinone units could be fused at positions 11 and 12 to give the monosubstituted naphtoquinone derivative **3** (Figure 2).

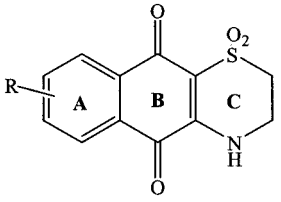


Figure 2. Naphtoquinone derivative **3**

This structure is confirmed by the spectral data reported in the literature for related taurine-containing quinones,[12,13] thus allowing us to identify the tricyclic ring core of conicaquinone A. In particular, the comparison of our ^{13}C NMR spectroscopic data with those reported in the literature provides a way to assign the resonances of the two carbonyl groups present in **1**, and consequently the $-NHCH_2CH_2SO_2-$ annulation regiochemistry, as it is clear that the C=O chemical shifts are not affected by an alkyl substitution on the benzenoid ring.[12]

Analysis of the remaining data in the ^1H NMR spectrum [δ = 5.12 (br. t, J = 7.6 Hz, 3-H), 2.74 (t, J = 7.6 Hz, 5-H), 2.31 (q, J = 7.6 Hz, 4-H), 1.66 (br. s, Me-1), and 1.50 (br. s, Me-16)] and the ^{13}C NMR spectrum [δ = 135.6 (s), 122.9 (d), 34.8 (t), 28.7 (t), 25.3 (q), and 17.5 (q)], obtained by interpretation of one- and two-dimensional spectra, reveals the R group to be a 2-methylpent-2-enyl unit. The chemical shifts and coupling constants of the signals of the aromatic protons (see above) indicate clearly that this fragment must be positioned either at C-6 or at C-7. Its placement at C-6 is confirmed by a whole series of HMBC correlations (see Figure 3), particularly those from the singlet at δ = 7.85 ppm (15-H) to the carbonyl signal at δ = 178.9 ppm (C-13), and from the doublet at δ = 8.10 ppm (8-H) to the carbonyl signal at δ = 174.9 ppm (C-10).

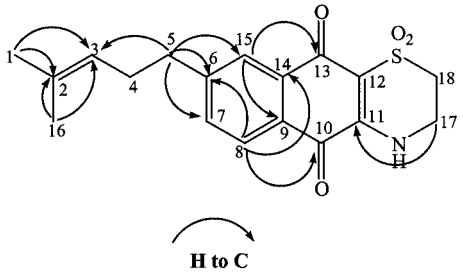


Figure 3. HMBC correlations for conicaquinone A

The spectroscopic data for the more polar metabolite **2** are nearly identical to those of **1**; the mass spectra of both compounds exhibit the same fragment ions, while small differences between the two are visible in their UV (see Exp. Sect.) and NMR (see Table 1) spectra. Thus, it is evident that the two compounds are isomeric thiazinoquinones differing in the location of the homoprenyl group. An important piece of evidence in support of this hypothesis is deduced from an HMBC spectrum; in fact, diagnostic long-range couplings are observed between 15-H (δ = 8.02 ppm) and the carbonyl resonance at δ = 174.8 ppm (C-13), and between 8-H (δ = 7.98 ppm) and the carbonyl resonance at δ = 178.4 ppm (C-10).

Table 1. NMR spectroscopic data for conicaquinones A and B

	Conicaquinone A		Conicaquinone B	
	δ_H (mult., J in Hz)[16]	δ_C[17]	δ_H (mult., J in Hz)[16]	δ_C[17]
1	1.66 (s)	25.3	1.66 (s)	25.7
2		135.6		133.3
3	5.12 (m)	122.9	5.10 (m)	122.2
4	2.31 (q, 7.6)	28.7	2.34 (q, 7.6)	29.3
5	2.74 (t, 7.6)	34.8	2.78 (t, 7.6)	36.5
6		147.3		151.5
7	7.61 (dd, 7.8, 1.6)	135.5	7.48 (br. d, 7.8)	133.2
8	8.10 (d, 7.8)	125.8	7.98 (d, 7.8)	128.2
9		130.6		127.1
10		174.9		178.4
11		146.7		113.4
12		113.4		146.7
13		178.9		174.8
14		129.3		132.6
15	7.85 (d, 1.6)	126.0	8.02 (d, 1.5)	126.5
16	1.50 (s)	17.5	1.52 (s)	17.6
17	4.10 (m)	39.8	3.36 (m)	48.1
18	3.36 (m)	48.2	4.10 (m)	39.8
NH	6.78 (br. s)		6.78 (br. s)	

Conclusion

The study of ascidian secondary metabolites continues to yield unique and pharmacologically active substances.

Based on the reported anticancer and cancer-protective properties of prenylated quinones, conicaquinones A and B were tested in vitro against two different cultured cell lines; cytotoxicity was evaluated on rat glioma (C6) and rat basophilic leukaemia (RBL-2H3) cell lines.

Comparison of the cytotoxic activity of compounds **1** and **2** against C6 and RBL-2H3 cells (see Figure 4) shows a marked and selective effect on rat glioma cells.

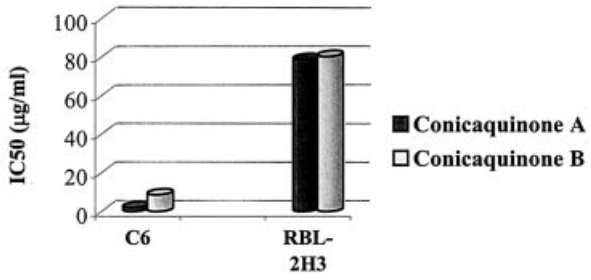


Figure 4. In vitro cytotoxic activity (IC_{50}, µg/mL) of conicaquinones A and B on C6 (rat glioma) and RBL-2H3 (rat basophilic leukaemia) cell lines

Experimental Section

General Remarks: Low- and high-resolution FAB mass spectra (CsI ions, glycerol matrix) were performed with a VG Prospec (FISONS) mass spectrometer. NMR spectroscopic experiments were performed with a Bruker AMX-500 spectrometer; chemical shifts are referred to the residual protonated solvent signal ($CDCl_3$: δ_H = 7.26, δ_C = 77.0 ppm; [D_6]DMSO: δ_H = 2.49, δ_C = 39.5 ppm). Homonuclear (^{1}H-^{1}H) and heteronuclear (^{1}H-^{13}C) connectivities were determined by COSY and HSQC experiments, respectively. Two- and three-bond ^{1}H-^{13}C connectivities were determined by an HMBC experiment optimised for a $^{2,3}J$ coupling constant of 10 Hz. Medium-pressure liquid chromatographies (MPLC) were performed with a Büchi 861 apparatus on SiO_2-packed columns (230−400 mesh). High-performance liquid chromatography (HPLC) separations were achieved with a Waters 501 apparatus equipped with an RI detector. UV spectra (MeOH) were recorded with a Beckman DU 70 instrument. IR (KBr) spectra were recorded with a Bruker model IFS-48 spectrophotometer.

Extraction and Isolation of Conicaquinones A and B: Specimens of *A. conicum* were collected at Capo Caccia (Alghero, Italy) at a depth of 13 m. They were frozen immediately after collection and kept frozen until extraction. A reference specimen has been deposited at the Dipartimento di Chimica delle Sostanze Naturali, University of Naples. The freshly thawed animals (150 g of dry weight after extraction) were homogenised and extracted twice with methanol and then twice with chloroform (4 × 200 mL). The combined extracts were concentrated in vacuo and the resulting aqueous residue was extracted with EtOAc and then with *n*BuOH. Separation of the EtOAc-soluble material (4.8 g) was achieved by gradient silica gel MPLC (hexane → EtOAc → MeOH). Fractions eluted with EtOAc/MeOH (9:1, v/v) were combined and further chromatographed by HPLC on an SiO_2 column (Luna, 5 µm, 250 × 3.00 mm) eluting with EtOAc/hexane (75:25, v/v), affording conicaquinone A (6 mg, 0.004% of dry weight) and B (3 mg, 0.002% of dry weight) as pure compounds.

Conicaquinone A (1): FAB-MS (positive-ion mode): *m/z* = 346 [M + H]$^+$, 282 [(M −SO_2) + H]$^+$. HRFAB-MS: *m/z* = 346.1131; $C_{18}H_{20}NO_4S$ requires 346.1113. UV (MeOH): λ_{max} (ε)= 298 (7400), 261 (16600) nm. IR (KBr): $\tilde{\nu}_{max}$ = 3255, 1685, 1590, 1360, 1310, 1270 cm^{-1}.

Conicaquinone B (2): FAB-MS (positive-ion mode): *m/z* = 346 [M + H]$^+$, 282 [(M −SO_2) + H]$^+$. HRFAB-MS: *m/z* = 346.1128; $C_{18}H_{20}NO_4S$ requires 346.1113. UV (MeOH): λ_{max} (ε) = 300 (8050), 263 (14700) nm. IR (KBr): $\tilde{\nu}_{max}$ = 3255, 1685, 1590, 1360, 1310, 1270 cm^{-1}.

Cytotoxicity Tests: C6 and RBL-2H3 cells (3.5 × 10^6 cells) were plated on 96-well plates in 50-µL drops and allowed to adhere at 37 °C in 5% CO_2/air for 2 h. Thereafter, serial dilutions (1:4, v/v) of the test compounds (50 µL) were added and incubated with the cells for 24 h. Cell viability was assessed through an MTT conversion assay.[14] MTT (5 mg/mL, 25 µL) was added after 24 h, and the cells were incubated for an additional 3 h. After this time, the cells were lysed and the dark blue crystals solubilized with a solution (100 µL) containing 50% (v/v) *N*,*N*-dimethylformamide and 20% (w/v) SDS with an adjusted pH of 4.5.[15] The optical density (OD) of each well was measured with a microplate spectrophotometer (Titerteck Multiskan MCC/340) equipped with a 620-nm filter. The viability of each cell line was calculated as: % dead cells = 100 − (OD treated/OD control) × 100.

Acknowledgments

This work is the result of research supported by MURST PRIN 2001 "Sostanze Naturali ed Analoghi Sintetici con Attività Antitumorale," Rome, Italy. Mass and NMR spectra were recorded at the "Centro Interdipartimentale di Analisi Strumentale", Università di Napoli "Federico II". The assistance of the staff is gratefully acknowledged.

[1] D. J. Faulkner, *J. Nat. Prod. Rep.* **2002**, *19*, 1−49.
[2] K. Rinehart, *Med. Res. Rev.* **2000**, *20*, 1−27.
[3] W. Fenical, *Food-Drugs Sea Proc.* **1976**, *4*, 388−394.
[4] G. Guella, I. Mancini, F. Pietra, *J. Nat. Prod.* **1987**, *50*, 621−626.
[5] B. M. Howard, K. Clarkson, R. L. Berstein, *Tetrahedron Lett.* **1979**, 4449−4452.
[6] N. M. Targett, W. S. Keeran, *J. Nat. Prod.* **1984**, *47*, 556−557.
[7] A. F. Benslimane, Y. F. Pouchus, J. Le Boterff, J. F. Verbist, C. Roussakis, F. Monniot, *J. Nat. Prod.* **1988**, *51*, 582−583.
[8] X. Fu, M. B. Houssain, D. van der Helm, F. J. Schmitz, *J. Am. Chem. Soc.* **1994**, *116*, 12125−12126; this reference has been corrected in: *J. Am. Chem. Soc.* **1995**, *117*, 9381.
[9] R. A. Davis, A. R. Carroll, R. J. Quinn, *J. Nat. Prod.* **1999**, *62*, 158−160.
[10] S. J. Rochfort, R. Metzger, L. Hobbs, R. Capon, *Aust. J. Chem.* **1996**, *49*, 1217−1219.
[11] A. Sato, T. Shindo, N. Kasanuki, K. Hasegawa, *J. Nat. Prod.* **1989**, *52*, 975−981.
[12] F. J. Schmitz, S. J. Bloor, *J. Org. Chem.* **1988**, *53*, 3922−3925.
[13] A. K Alvi, J. Rodriguez, M. C. Diaz, R. Moretti, R. S. Wilhelm, R. H. Lee, D. L. Slate, P. Crews, *J. Org. Chem.* **1993**, *58*, 4871−4880.
[14] T. Mosman, *J. Immunol. Methods* **1983**, *65*, 55−63.
[15] A. W. Opipari Jr., H. M. Hu, R. Yabkowitz, V. M. Dixit, *J. Biol. Chem.* **1992**, *267*, 12424−12427.
[16] Spectrum recorded in $CDCl_3$ at 500 MHz.
[17] Spectrum recorded in [D_6]DMSO at 125 MHz.

Received October 28, 2002
[O02598]