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Synthesis of structurally simplified analogues of aplidinone A, a pro-apoptotic marine thiazinoquinone

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Dedicated to the memory of Professor Rodolfo A. Nicolaus

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ABSTRACT

The synthesis of analogues of aplidinone A (**7**), a prenylated quinone isolated from the Mediterranean ascidian *Aplidium conicum*, has been performed. This work not only allowed confirming the structural assignment of aplidinone A, previously made with the support of GIAO shielding calculations, but, above all, made a series of structurally related quinone derivatives (compounds **8–13** and the natural metabolite) available for a screening in vitro for cytotoxic and pro-apoptotic activity and for SAR studies. The study evidenced one of the synthetic analogues (**11**) as a potent cytotoxic and pro-apoptotic agent against several tumor cell lines which also inhibits the TNFα-induced NF-κB activation in a human leukemia T cell line. This exemplifies the potential of a natural product to qualify as lead structure for medicinal chemistry campaigns, affording simplified analogues with better bioactivity and easier to synthesize.

1. Introduction

Naturally occurring prenylated 1,4-benzoquinones and hydroquinones are commonly found in a variety of organisms and play important roles in several metabolic processes, such as photosynthesis and electron transport.[1,2] Examples of these compounds have been isolated from marine sources, mostly from tunicates belonging to the order of Aplousobranchiata (family Polyclinidae), such as ascidians of the genus *Aplidium*. They show a wide array of different structures, originated by intra- and inter-molecular cyclizations and/or rearrangements of the original terpene hydroquinone/quinone skeleton, thus giving macrocyclic or polycyclic structures; they are often linked to amino acids or taurine residues.[3–14]

Recently, in the frame of our search for bioactive new molecules from Mediterranean tunicates, we have investigated the ascidian *Aplidium conicum* and this study yielded a large group of new prenylated quinones we named conicaquinones (**1** and **2**),[15] thiaplidiaquinones (**3** and **4**),[16] and aplidinones (**5–7**).[17] These compounds are structurally quite different, but they share the presence of an unusual 1,1-dioxo-1,4-thiazine ring fused to a quinone moiety. As for the aplidinones, the whole of the NMR data were not enough to unambiguously define the structure of the heterocyclic ring; an uncertainty still remained about the relative position of the sulfur and the nitrogen position in the ring. The reported regiochemistry was assigned as suggested for aplidinone A (**7**) by comparison of its experimental ^{13}C NMR chemical shifts with those predicted by GIAO[18] shielding calculations for regioisomers models A1 (**8**) and A2 (**9**). In the present paper we report about a synthetic study performed in order to validate the structural assignment of aplidinone A made by theoretical means. Synthesis of model compounds A1 and A2 has been carried out and allowed us to confirm the proposed regiochemistry. In addition, the designed synthetic procedure was adapted to prepare further quinone analogues, **10–13**. Both aplidinone A and its synthetic analogues were shown to possess interesting cytotoxic effects; SAR studies revealed that **11** is the most potent cytotoxic and pro-apoptotic agent against several tumor cell lines and also inhibits TNFα-induced NF-κB activation in a human leukemia T cell line.

This exemplifies the potential of a natural product to qualify as lead structure for medicinal chemistry campaigns, affording simplified analogues with better bioactivity and easier to synthesize.

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2. Results and discussion

2.1. Synthesis of model compounds A1 (8) and A2 (9): validation of the structure assigned to aplidinone A (7)

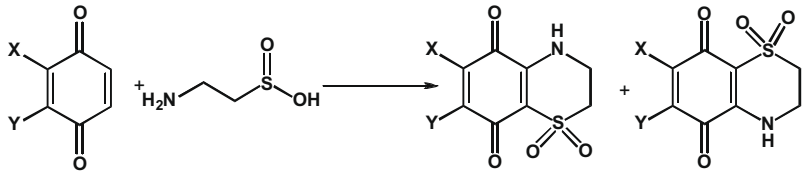
Heterocyclic systems like aplidinone A, having a 1,1-dioxo-1,4-thiazinic ring condensed with a benzoquinone ring, are not common in literature. This kind of compounds has been synthesized for the first time in 1988[20] by condensation of hypotaurine with naphthoquinone using the known conjugate addition reaction of amines and sulfinic acids with naphthoquinones[21] to give substituted naphthoquinones. Later, Harada et al. worked out the synthesis of adociaquinones A and B using the same protocol.[22]

Although this route has the disadvantage of being not regioselective (see Scheme 1), we recognized it as a simple and versatile way to synthesize in relatively few steps aplidinone A derivatives, starting from an appropriate benzoquinone derivative. Thus, compounds **8** and **9** have been prepared according to the synthetic path reported in Scheme 2. The alkylation of methoxy-hydroquinone (**14**) was tested with diethyl sulfate in the presence of potassium carbonate, but the reaction yielded a mixture of mono and bis ether. Satisfactory results, instead, were obtained by using cesium carbonate as base under argon atmosphere. It has been shown that the introduction of an alkyl residue on 1,2,4-trimethoxy benzene may be selective in the 3-position.[23] So, ethylation of compound **15** in the correct position was obtained allowing to react the Li derivative with diethyl sulfate for 24 h at room temperature under argon. The product (**16**) showed the expected ^{1}H NMR spectrum which exhibits the *ortho* coupling between the two aromatic protons (see Section 4). Oxidation of **16** to quinone was carried out with a large excess (molar ratio 8:1) of cerium ammonium nitrate (CAN).

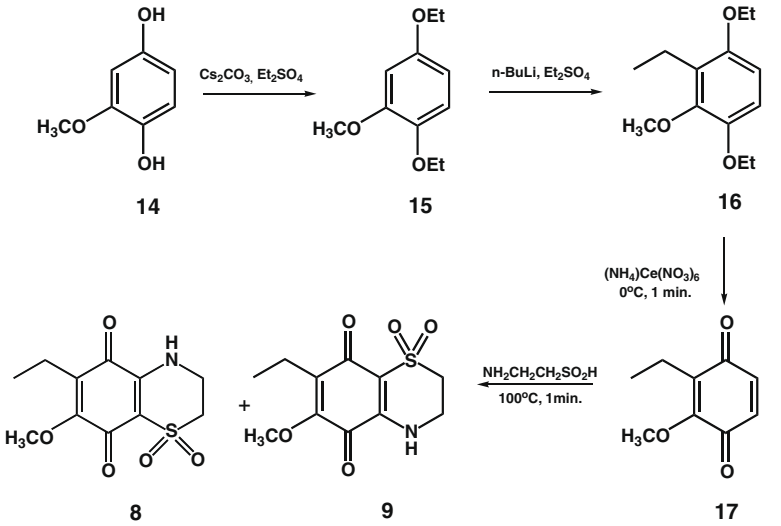
Quinone **17** was obtained with 62% of yield; its condensation with hypotaurine[22] afforded, as expected, a mixture of compounds **8** and **9**. The isolation of the two isomers **8** and **9** was achieved by HPLC separation of this mixture; their structures were univocally assigned by spectral studies as described below.

ESI-MS data (see Section 4) of **8** and **9** indicated that the two compounds were isomers with the molecular formula $C_{11}H_{13}NO_5S$. Analysis of 1D and 2D NMR spectral data of **8** ($CDCl_3$) allowed the assignment of all ^{1}H and ^{13}C NMR signals, which are reported in Table 1. A first set of signals, namely those relevant to the bicyclic nucleus [δ_H: 3.28 (m, 2H-2), 4.07 (m, 2H-3), 4.24 (s, OC*H*$_3$), 6.81 (br s, NH); δ_C: 48.7 (C-2), 39.9 (C-3), 62.2 (OCH_3), 144.2 (C-4a), 179.0 (C-5), 128.6 (C-6), 157.3 (C-7), 173.9 (C-8), 108.7 (C-8a)], appeared almost identical to those of aplidinone A.[17] The remaining signals [δ_H: 2.40 (q, J = 7.5 Hz, 2H-1′), 0.99 (t, J = 7.5 Hz, 3H-2′); δ_C: 16.2 (C-1′), 12.4 (C-2′)] were obviously due to the ethyl group which replaces in structure **8** the geranyl moiety linked to the benzoquinone ring of the natural compound **7**. The position of the ethyl group, with respect to nitrogen and sulfur atoms of the heterocyclic ring, was assigned through assessment of proton–carbon long-range couplings, reported in Table 1. Particularly, diagnostic cross peaks were observed in the HMBC spectrum of **8** between the methylene protons at δ 2.40 (2H-1′) and the carbonyl resonance at δ 179 (C-5), as well as between the sulfur-linked methylene protons at δ 3.28 (2H-2) and the other carbonyl signal at δ 173.9 (C-8). These key correlations indicated that the ethyl chain was linked at C-6, thus defining the structure of **8** as 6-ethyl-7-methoxy-3,4-dihydro-1,1-dioxo-2*H*-1,4-benzothiazine-5,8-dione. It is to be noted that the $^4J_{C8-H2}$ coupling gives rise to a weak correlation peak, which could not be detected in the HMBC spectrum of aplidinone A due to the very small amounts of natural compound isolated.

^{1}H and ^{13}C NMR features of compound **9** were nearly identical to those of **8**, with regard to the number and shape of the signals. However, differences were observed in the chemical shift values of a few NMR signals (see Table 1); significantly different were the



Scheme 1.



Scheme 2.

Table 1
NMR data ($CDCl_3$) of compounds **8** and **9**

Pos.	**8**			**9**		
	δ_C	δ_H, mult. (J in Hz)	HMBC	δ_C	δ_H, mult. (J in Hz)	HMBC
1	–	–	–	–	–	–
2	48.7	3.28 m	3, 8, 8a	48.7	3.28 m	3, 8, 8a
3	39.9	4.07 m	2, 4a	39.7	4.04 m	2, 4a
4	–	–	–	–	–	–
4a	144.2	–	–	142.7	–	–
5	179.0	–	–	176.4	–	–
6	128.6	–	–	152.8	–	–
7	157.3	–	–	139.7	–	–
8	173.9	–	–	178.0	–	–
8a	108.7	–	–	110.1	–	–
1′	16.2	2.40 q (7.5)	5, 6, 7, 2′	17.0	2.50 q (7.5)	6, 7, 8, 2′
2′	12.4	0.99 t (7.5)	6, 1′	13.4	1.08 t (7.5)	7, 1′
OCH_3	62.2	4.24 s	7	60.8	3.91 s	6
NH	–	6.81 br s	–	–	6.50 br s	–

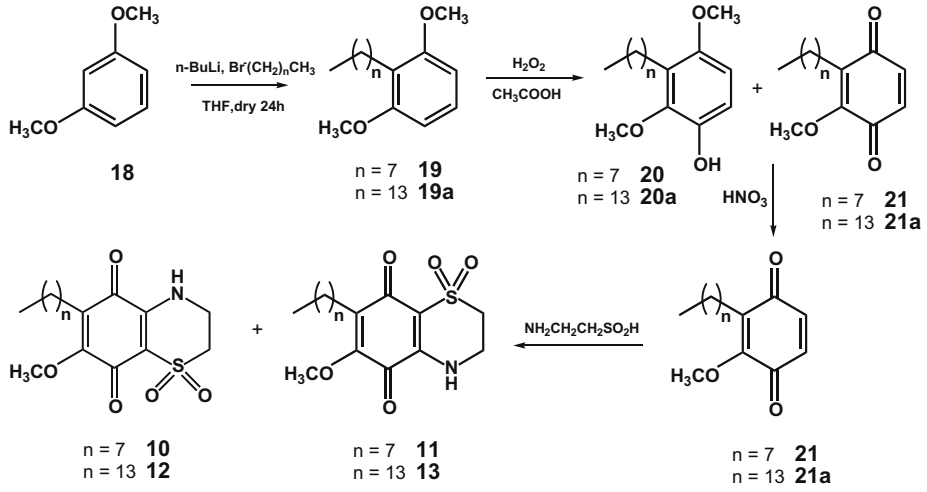
chemical shift values of the carbonyl carbons [δ 176.4 vs δ 179.0 (C-5) and δ 178.0 vs δ 173.9 (C-8)] and of the methoxyl protons (δ 3.91 vs δ 4.24). Thus, it was evident that the two compounds were isomerically differing in the location of the substituents on the quinone ring. The alternative 7-ethyl-6-methoxy-3,4-dihydro-1,1-dioxo-2*H*-1,4-benzothiazine-5,8-dione structure was univocally assigned to **9** by analysis of HMBC spectrum, as well. Both 2- and 1′-methylene protons were indeed correlated to the same carbonyl carbon resonating at δ 178.0 (C-8), thus indicating that in **9** the ethyl chain was linked at C-7 rather than at C-6. The whole series of HMBC correlations were consistent with the proposed assignment (see Table 1).

Compounds **8** and **9** were obtained in a ratio of 8:2 in favor of **9**. The regioselectivity observed in our case merits a comment. Data in the literature are related to reactions of hypotaurine with quinones lacking strong directing groups (i.e., electron releasing groups). In these examples mixtures roughly 1:1 were formed and, therefore, we suggest that the effect of the methoxyl group strongly orients the attack of nucleophilic sulfur on the quinone ring.

The close agreement between the signals of bicyclic skeleton of compound **8** and aplidinone A strongly sustained the structure assignment previously made for the natural compound by theoretical means.[17] In addition, we compared the experimental ^{13}C chemical shifts of **8** and **9** to the corresponding theoretical ones obtained from GIAO calculation[24] (see Section 4). For both structures, the correlation coefficient (R) value was around 0.998, indicative of a very good fit between the two set of parameters and, thus, represented a further validation of the theoretical applied method.

2.2. Synthesis of compounds 10–13

Quinones **10**–**13** were synthesized through a route slightly different from that used for the preparation of **8** and **9**, since trial experiments have shown a drop in the yields of alkylation with long chain alkyl bromides. Therefore, the following route, starting from 1,3-dimethoxybenzene (**18**), in analogy with a similar path reported,[25] was followed (Scheme 3). The lithium derivative of resorcinol dimethyl ether was reacted with two different alkyl bromides; yields were 58% with octyl bromide (1,3-dimethoxy-2-octylbenzene, **19**) and 35% with tetradecyl bromide (1,3-dimethoxy-2-tetradecylbenzene, **19a**). The oxidation of these substrates with peracetic acid (prepared in situ) afforded in both cases mixtures (**20** and **21**; **20a** and **21a**, respectively), which were further oxidized with nitric acid, giving quinones **21** (32% yield) and **21a** (30% yield), respectively, as sole products. Both **21** and **21a** were reacted with hypotaurine and afforded mixtures **10**/**11** and **12**/**13**, respectively. Products **10**–**13** were isolated as individual compounds by HPLC separations; as expected, for each couple of regioisomers, one was formed in large excess in respect to the other. Their chemical characterization was easily performed through spectral analysis (Table 2), referring to the NMR spectral assignments made for **8** and **9**. Actually, carbon and proton resonances relative to the bicyclic nucleus



Scheme 3.

Table 2
NMR data of compounds **10**–**13**

Pos.	10		11		12		13	
	δ_C	δ_H, mult. (*J* in Hz)	δ_C	δ_H, mult. (*J* in Hz)	δ_C	δ_H, mult. (*J* in Hz)	δ_C	δ_H, mult. (*J* in Hz)
1	–	–	–	–	–	–	–	–
2	48.7	3.29 m	48.7	3.28 m	48.8	3.29 m	48.7	3.28 m
3	39.7	4.06 m	39.5	4.06 m	39.7	4.06 m	39.5	4.06 m
4	–	–	–	–	–	–	–	–
4a	144.3	–	142.8	–	144.3	–	142.8	–
6	127.7	–	152.9	–	127.7	–	152.9	–
7	157.6	–	138.8	–	157.6	–	138.8	–
8	174.1	–	178.1	–	174.1	–	178.1	–
1′	22.9	2.38 t (7.5)	23.5	2.47 t (7.5)	22.9	2.38 t (7.5)	23.5	2.47 t (7.5)
2′	28.1	1.36	28.8	1.43	28.1	1.36	28.8	1.43
3′	29.6	1.25	29.5	1.26	29.6	1.25	29.5	1.26
4′–5′	29.1	1.27	29.1	1.26	29.1	1.27	29.1	1.26
6′	31.8	1.25	31.7	1.24	29.1	1.27	29.1	1.26
7′	22.6	1.28	22.3	1.27	29.1	1.27	29.1	1.26
8′	13.9	0.88 t (7.5)	13.6	0.87 t (7.5)	29.1	1.27	29.1	1.26
9′–11′	–	–	–	–	29.1	1.27	29.1	1.26
12′	–	–	–	–	31.8	1.25	31.7	1.24
13′	–	–	–	–	22.6	1.28	22.3	1.27
14′	–	–	–	–	13.9	0.88 t (7.5)	13.6	0.87 t (7.5)
OCH_3	62.2	4.25 s	60.6	3.91 s	62.1	4.26 s	60.7	3.92 s
NH	–	6.81 br s	–	6.50 br s	–	6.81 br s	–	6.50 br s

of the couples of compounds **10**/**12** and **11**/**13** were virtually identical to those of compounds **8** and **9**, respectively, thus defining the structures **10**–**13** as those reported in Figure 1.

2.3. Pro-apoptotic properties of compounds 7–13

A large number of quinones, both synthetic and naturally occurring, have been screened for their antitumor activity; particularly, it has been shown that they lead to oxidative stress by means of an increase of oxygen reactive species (ROS) and the depletion of reduced glutathione (GSH) amount[26] and this action has been related to the beginning of apoptosis.[27,28]

We have previously shown that thiaplidiaquinones **3** and **4** induce generation of ROS and apoptosis in the Jurkat cell line.[16] On this cell line we have now investigated the cytotoxic activity of aplidinone A (**7**), as well as that of its synthetic analogues **8**–**13**. Aplidinone A induced cytotoxicity with an IC_{50} about 45 μM; the cytotoxic activity was enhanced with the modifications introduced in **11** ($IC_{50} \approx 20$ μM) and decreased in **8**–**10**, **12**, and **13** (Table 3).

To further discriminate whether aplidinone A analogues induced cell death by either necrosis or apoptosis we treated the cells with compounds **8**–**11** for 24 h and cell cycle analyses were carried out. Treated cells showed a significant loss of nuclear DNA (chromatinolysis) measured by an increase in the frequency of subdiploid (apoptotic) cells (Fig. 2). As expected, **11** was the most potent apoptotic inducer in this group of analogues.

Comparison of the biological activity of aplidinone A with that of **8**, **10**, and **12**, all having the 6-alkyl-7-methoxy-3,4-dihydro-1,1-dioxo-2*H*-1,4-benzothiazine-5,8-dione structure and differing each other only in the nature of the side chain, indicates a critical role of this part structure for the induction of apoptosis. Further interesting conclusions can be drawn by comparing the cytotoxic effects of compound **10** to those of **11**, having the same side chain and the alternative 7-alkyl-6-methoxy-3,4-dihydro-1,1-dioxo-2*H*-1,4-benzothiazine-5,8-dione structure. This 'unnatural' structure appears to positively affect the cytotoxic activity, since compound **11** is substantially more cytotoxic than the relevant isomer **10** with the 6-alkyl-7-methoxy-3,4-dihydro-1,1-dioxo-2*H*-1,4-benzothiazine-5,8-dione structure.

The cytotoxic effect of compounds **9**, **11**, and **13** was then studied against a series of tumor cell lines by the calcein-AM method. Calcein-AM is a fluorogenic, highly lipid-soluble dye that rapidly penetrates the plasma membrane. Inside the cell, endogenous esterases cleave the ester bonds, producing the hydrophilic and fluorescent dye calcein, which cannot leave the cell via the plasma membrane unless the cells are damaged by cytotoxic compounds. The test compounds were dissolved in DMSO and a dilution series in the medium was prepared. Cells were incubated with the prepared solutions, the final content of DMSO in the cell cultures being never higher than 1%; the IC_{50} values for the test compounds are shown in Table 4. Compound **11** showed potent cytotoxic activities toward all the cell lines tested, indicating that it targets a common pathway in cancer cells. Compounds **9** and **13** were both less active than **11**, demonstrating a definite structure activity relationship for the size of the alkyl chain.

2.4. Inhibition of TNFα-induced NF-κB activation

Another important factor in cancer development and progression is the regulation of many anti-apoptotic genes by the NF-κB family of transcription factors.[29–31] Also in this process, ROS play an important role; in fact, ROS are continuously produced during normal aerobic metabolism by the mitochondria electron chain and their level is related to many different physiological stimuli.[32,33] Low-medium levels of ROS can protect cell from apoptosis by activating antioxidant mechanisms, including activation of the NF-κB pathway.[34] Conversely, high levels of intracellular ROS result in the inhibition of the NF-κB pathway and in the induction of cell death by either apoptosis or necrosis.

Thus, we investigated the effects of synthetic compounds **8**–**13** on the NF-kB pathway (aplidinone A could not be tested due to the very limited amounts of this compound in our hands) through the use of the NF-κB-dependent luciferase assay. As depicted in Figure 3, compound **11** was the only compound that showed a strong anti-NF-κB activity. It is to be noted that compound **8**, lacking cytotoxic activity, at high concentrations also showed some inhibitory activity on the NF-κB pathway indicating that ROS inducing activity is not always coupled with NF-κB inhibition.

3. Conclusions

Our results revealed compound **11** as a potent pro-apoptotic agent, active against several tumor cell lines, and also capable to inhibit the TNFα-induced NF-κB activation in a human leukemia T cell line. Actually, using aplidinone A as a lead structure, we have

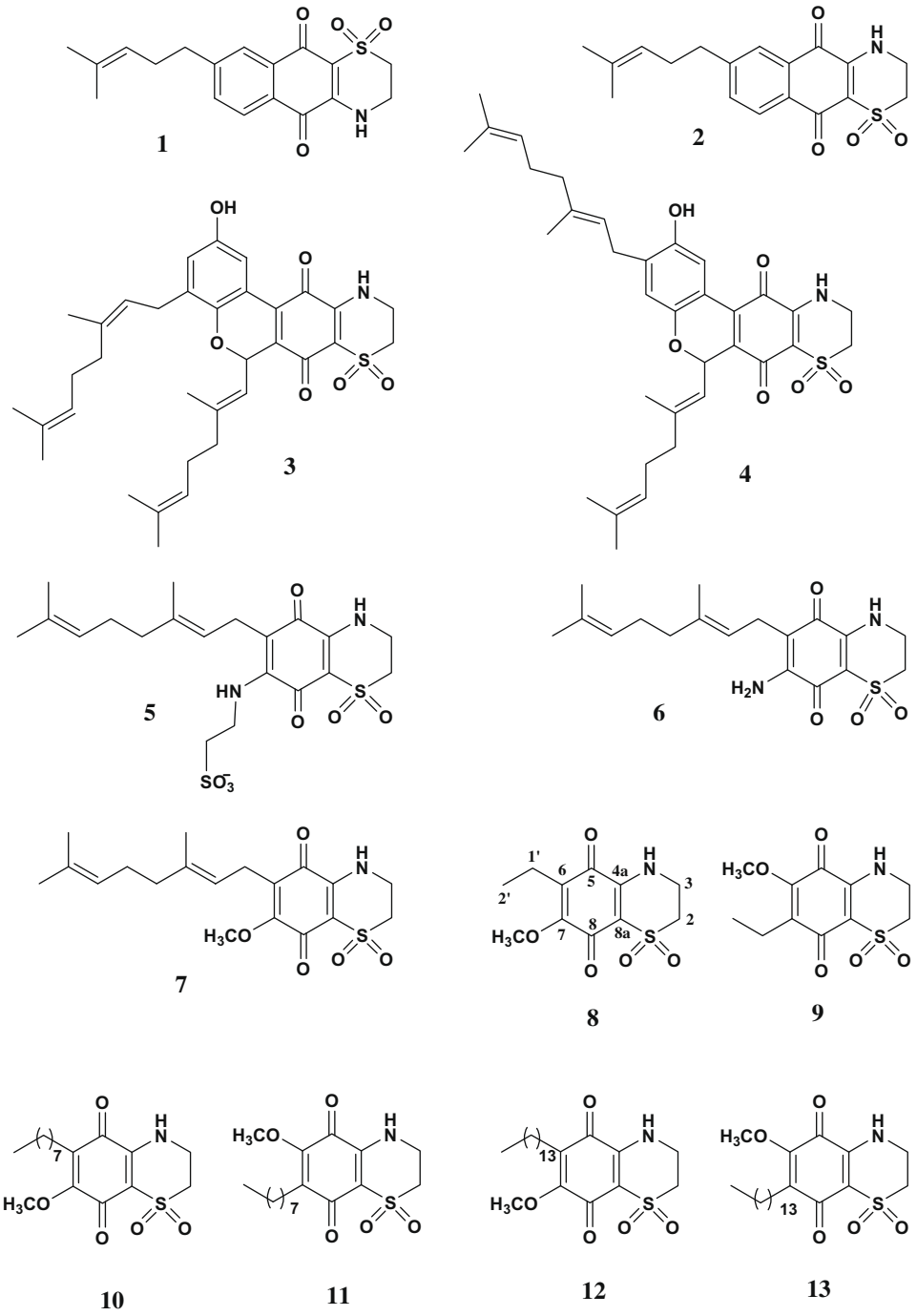


Figure 1. Aplidinone A (**7**) and its synthetic analogues.

Table 3
Cytotoxity of aplidinone A (**7**) and its synthetic derivatives **8**–**13** on Jurkat leukemia cell line

Compound	7	8	9	10	11	12	13
IC_{50}[a]	44.5 ± 6	>50	>50	>50	20.7 ± 2	>50	>50

[a] IC_{50} values of test compound (μM) using PI staining as a cell viability test.

obtained a simplified analogue with better bioactivity and easier to synthesize and this is an illustrative case of the potential of a natural product to act as lead structure for drug discovery. At the moment, there is no evidence about the mechanism by which **11** causes cytotoxic effects. However, a plausible hypothesis based on the structural analogy between quinone derivatives and coenzyme Q is that **11** may interfere with the CoQ binding site of the CoQ-dependent cellular redox systems at the mitochondria and at the plasma membrane. This could lead to a redirection of the normal electron flow, generating an excess of ROS, inhibiting NF-kB activation and inducing apoptosis in tumor cells.

4. Experimental section

4.1. General experimental procedures

ESI mass spectra were performed on a hybrid quadrupole-TOF mass spectrometer, dissolving the sample in MeOH. The spectra were

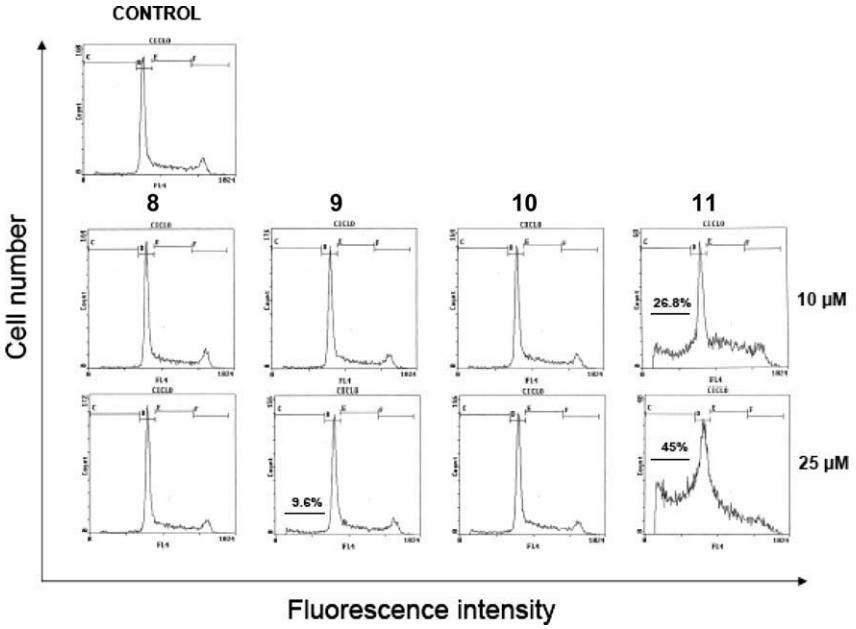


Figure 2. Apoptotic effects of compounds **8**–**9** and **10**–**11** on the human T leukemia Jurkat cell line.

Table 4
Cytotoxity of quinone analogues toward five tumor cells[a]

Compound	Cell line				
	293T	A549	AGS	HCT116	HeLa
9	32.6	>50	>50	>50	>50
11	8.7	13.0	7.2	14.1	24.8
13	47.9	50.8	45.0	44.8	47.9

[a] IC_{50} values of test compound (μM) using calcein-AM as cell viability test.

recorded by infusion into the ESI source using MeOH as the solvent. EI mass spectra were obtained on GC–MS HP 5890, HP 5971A Mass selective detector. ^1H (700 MHz) and ^{13}C (175 MHz) NMR spectra were recorded on a Varian INOVA spectrometer, respectively, chemical shifts were referenced to the residual solvent signal ($CDCl_3$: $\delta_H = 7.26$, $\delta_C = 77.0$). For an accurate measurement of the coupling constants, the one-dimensional ^1H NMR spectra were transformed at 64 K points (digital resolution: 0.09 Hz). Homonuclear (^1H–^1H) and heteronuclear (^1H–^{13}C) connectivities were determined by COSY and HSQC experiments, respectively. Two and three bond ^1H–^{13}C connectivities were determined by gradient 2D HMBC experiments optimized for a $^{2,3}J$ of 8 Hz. Routine ^1H and ^{13}C NMR spectra were recorded with a Varian Mercury 300 MHz or a Varian Gemini 200 MHz. Medium-pressure liquid chromatographies (MPLC) were performed on a Buchi 861 apparatus with SiO_2 (230–400 mesh) packed columns. High performance liquid chromatography (HPLC) separations were achieved on a Knauer 501 apparatus equipped with an RI detector.

4.2. Reagents

Commercial reagents: Sigma–Aldrich. Solvents: Carlo Erba. TLC: Silica Gel 60 F254 (plates 5 × 20, 0.25 mm) Merck. Preparative TLC: Silica Gel 60 F254 plates (20 × 20, 2 mm). Spots revealed by UV lamp then by spraying with 2 N sulfuric acid and heating at

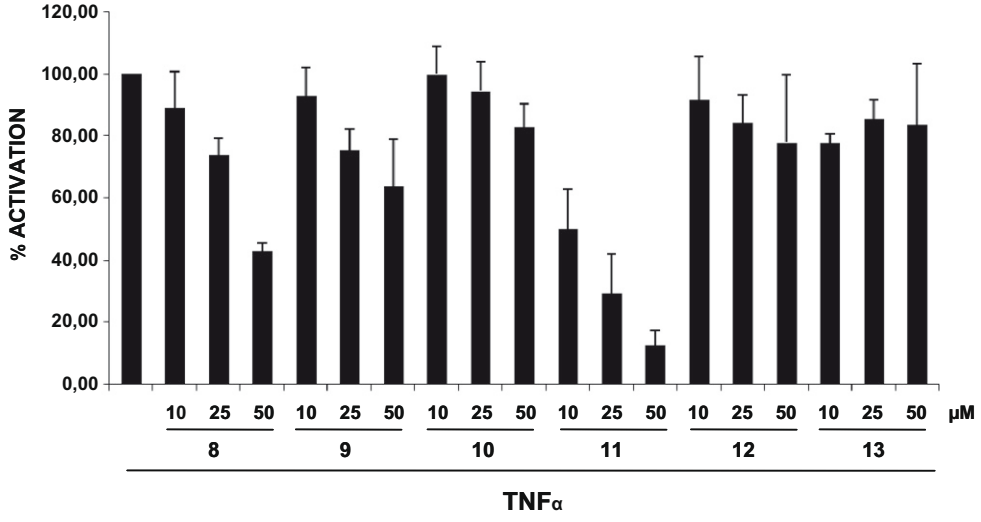


Figure 3. Effect of compounds **8**–**13** in TNFα-mediated NF-κB activation. Results show the percentage of NF-κB-dependent luciferase activity inhibition in 5.1 cells pretreated with the compounds. The luciferase activity induced by TNFα in the absence of the compounds was considered 100% of activation.

120 °C. 'Acidic' silica gel was prepared by treating Silica Gel 60 Merck with 1 N HCl for 24 h, washing with water until the chlorine test was negative, activating for 48 h at 120 °C, then equilibrating with 10% of water. Anhydrous solvents: Sigma–Aldrich (DMF) or prepared by distillation according to standard procedures.

4.3. Cell cultures

The adherent tumoral cell lines 293T (kidney cancer), AGS (gastric cancer), A549 (lung cancer), HeLa (cervix cancer) and HCT116 (colon cancer) were maintained in DMEM medium (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, penicillin (50 U/mL) and streptomycin (50 μg/mL) (complete medium). Jurkat cells (Leukemia) were maintained in exponential growth in RPMI 1640 (Lonza), containing 10% FBS, 2 mM glutamine, and antibiotics. The cells were grown at 37 °C in a 5% CO_2 humidified atmosphere. The 5.1 cell line is a Jurkat derived clone stably transfected with a plasmid containing the luciferase gene driven by the HIV-1-LTR promoter and was maintained in exponential growth in complete RPMI 1640 medium.

4.4. 1,4-Diethoxy-2-methoxybenzene (15)

To 2 g (14.2 mmol) of methoxy-hydroquinone **14**, dissolved in 100 mL of dry DMF, 9.26 g (28.2 mmol) of Cs_2CO_3 were added under argon atmosphere. 3.72 mL (28.2 mmol) of diethyl sulfate were then added drop wise; the mixture was stirred for 30 min and heated for 22 h at 120 °C (bath temp). The dark liquid was cooled and 30 mL of aqueous concd NH_3/methanol 1:1 were added and the mixture was left for 60 min under stirring. Methanol was removed under vacuum; the solution was then poured into 200 mL of cold water and extracted three times with ethyl acetate (50 mL each). The collected organic extracts were washed with brine, dried with sodium sulfate and filtered; the solvent removal afforded **15** (2.70 g, 97%) sufficiently pure for the following reaction. EIMS: $M^{+\cdot} = m/z$ 196. ^{1}H NMR ($CDCl_3$): δ 6.78 (1H, d, J = 8.8 Hz, H-6); 6.51 (1H, d, J = 2.8 Hz, H-3); 6.35 (1H, dd, J = 8.8, 2.8 Hz, H-5); 4.03 (2H, q, J = 14.0, 7.0 Hz, –O*CH*$_2$CH$_3$) 3.98 (2H, q, J = 14.0, 7.0 Hz, –O*CH*$_2$CH$_3$); 3.84 (3H, s, –OCH$_3$); 1.42 (3H, t, J = 7.0 Hz, –OCH$_2$*CH*$_3$); 1.39 (3H, t, J = 7.0 Hz, –OCH$_2$*CH*$_3$).

4.5. 1,4-Diethoxy-2-ethyl-3-methoxybenzene (16)

1.97 g (10 mmol) of 1,4 diethoxy-2-methoxybenzene (**15)** were dissolved in 25 mL of anhydrous ether and 13.5 mL of a *n*-BuLi 1.6 M solution (20 mmol) were added, under argon atmosphere at rt; the mixture was stirred for 1 h. Then, 5 mL of diethyl sulfate (20 mmol) were added and the mixture was left under stirring for 24 h (the end of the reaction was controlled with TLC, eluent: chloroform/hexane 7:3). After this time, 25 mL of aqueous concd NH_3/methanol 1:1 were added and the mixture was left for 20 min under stirring. The mixture was poured into cold water (150 mL) and extracted three times with ether. The ethereal solution was washed with brine several times, dried over sodium sulfate, and filtered. Solvent removal gave 2.20 g of **16** (98%), sufficiently pure for the following reaction. EIMS: $M^{+\cdot} = m/z$ 224. ^{1}H NMR ($CDCl_3$): δ 6.60 (1H, d, J = 8.8 Hz, H-6); 6.43 (1H, d, J = 8.8 Hz, H-5); 4.01 (2H, q, J = 14.0, 7.0 Hz, –O*CH*$_2$CH$_3$); 3.69 (2H, q, J = 14.0, 7.0 Hz, –O*CH*$_2$CH$_3$); 3.77 (3H, s, –OCH$_3$); 2.60 (2H, q, J = 7.6 Hz, –*CH*$_2$CH$_3$); 1.42 (3H, t, J = 7.0 Hz, –OCH$_2$*CH*$_3$); 1.39 (3H, t, J = 7.0 Hz, –OCH$_2$*CH*$_3$); 1.13 (3H, t, J = 7.6 Hz, –CH$_2$*CH*$_3$).

4.6. 2-Ethyl-3-methoxycyclohexa-2,5-diene-1,4-dione (17)

600 mg (3.5 mmol) of **16** dissolved in 90 mL of acetonitrile were added to a solution of CAN (14.4 g, 26.4 mmol) in water (110 mL) at rt. The mixture was diluted with water and extracted with ethyl acetate four times. The organic phase was washed with brine, dried over sodium sulfate, and filtered. Solvent removal gave 360 mg of **17** (62%), subsequently purified by filtration on 'acidic' silica gel (eluent: $CHCl_3$). ESI-MS: MH^+ = 167 m/z. ^{1}H NMR ($CDCl_3$): δ 6.62 (1H, d, J = 10.0 Hz, H-6 or H-5); 6.53 (1H, d, J = 10.0 Hz, H-5 or H-6); 3.96 (3H, s, –OCH$_3$); 2.36 (2H, q, J = 7.4 Hz, –*CH*$_2$CH$_3$); 0.98 (3H, t, J = 7.4 Hz, –CH$_2$*CH*$_3$).

4.7. Condensation of quinone 17 with hypotaurine

325 mg (1.96 mmol) of quinone **17** were dissolved in 26 mL of a mixture of EtOH/CH_3CN 1:1 and heated in a water bath. Then, a solution of hypotaurine (235 mg, 2.15 mmol) in 13 mL of water was added in portions. The yellow solution became orange. Most of the ethanol was removed in vacuo and the residue was poured into water. The mixture was extracted with ethyl acetate (three times) and the organic phase was washed with brine, dried over sodium sulfate, and filtered. Solvent removal afforded a mixture of the isomers **8** and **9** (148 mg, 28%) which was separated as reported below.

4.8. 1,3-Dimethoxy-2-octylbenzene (19) and 1,3-dimethoxy-2-tetradecylbenzene (19a)

Resorcinol dimethyl ether (**18**, 1 g, 7.25 mmol) was dissolved in anhydrous THF (15 mL). Then, 7 mL of a *n*-BuLi 1.6 M solution (11 mmol) were added, under argon atmosphere at 0 °C. After 1 h, the temperature was allowed to rise to 25 °C and the mixture was left under stirring for 2 h. Temperature was again lowered at 0 °C, and the bromoalkane (13 mmol; 2.2 mL of Br-octane or 3.9 mL of Br-tetradecane) was added in portions. Stirring was continued at rt for 18 h. The reaction mixture was poured into water and the organic products extracted with ethyl acetate. The extract was washed with 2 N HCl and water, dried over anhydrous sodium sulfate, and filtered; then, the solvent was evaporated.

Chromatography on silica gel of crude **19** (SiO_2/crude **19** 50:1; eluent: hexane/Et_2O 9:1) gave pure **19** (1.05 g, 58%). EIMS: $M^{+\cdot} = m/z$ 250. ^{1}H NMR ($CDCl_3$): δ 7.12 (1H, t, J = 8.4 Hz, H-5); 6.54 (2H, d, J = 8.4 Hz, H-4 and H-6); 3.81 (6H, s, –OCH$_3$); 2.63 (2H, t, J = 7.4 Hz, –*CH*$_2$(CH$_2$)$_6$CH$_3$); 1.1–1.3 (12H, 6CH$_2$); 0.89 (3H, t, J = 6.4 Hz, –CH$_2$(CH$_2$)$_6$*CH*$_3$).

Chromatography on silica gel of crude **19a** (SiO_2/crude **19a** 50:1; eluent: hexane/ethyl acetate 95:5) gave pure **19a** (850 mg, 35%). EIMS: $M^{+\cdot} = m/z$ 334. ^{1}H NMR ($CDCl_3$): δ 7.12 (1H, t, J = 8.4 Hz, H-5); 6.54 (2H, d, J = 8.4 Hz, H-4 and H-6); 3.81 (6H, s, –OCH$_3$); 2.63 (2H, t, J = 7.4 Hz, –*CH*$_2$(CH$_2$)$_{12}$CH$_3$); 1.1–1.3 (24H, 12CH$_2$); 0.89 (3H, t, J = 6.4 Hz, –CH$_2$(CH$_2$)$_{12}$*CH*$_3$).

4.9. 2-Octyl-3-methoxycyclohexa-2,5-diene-1,4-dione (21) and 2-tetradecyl-3-methoxycyclohexa-2,5-diene-1,4-dione (21a)

The 1,3-dimethoxy-2-alkyl-benzene (2.51 mmol; 630 mg of **19** and 840 mg of **19a**, respectively) was dissolved in a minimum volume of glacial acetic acid. *p*-Toluene sulfonic acid (50 mg) and 0.4 mL of 34% H_2O_2 solution were added and the mixture was heated at 50 °C for 1 h. The mixture was poured into water and extracted with AcOEt (three times). The extract was washed with water and dried over anhydrous sodium sulfate; after filtration, the solvent removed in vacuo. Purification on 'acidic' silica gel, (SiO_2/reaction mixture 15:1; eluent: hexane/ethyl acetate 9:1) afforded the mixtures of compounds **20**/**21** and **20a**/**21a**, respectively, which were submitted to the further oxidation. The mixture of compounds **20** and **21**, as well as that of compounds **20a** and **21a**, was dissolved in glacial acetic acid (3 mL) and 0.23 mL of concd HNO_3 were added in portions at 0 °C under stirring. After 15 h, the reaction was complete (TLC hexane/ethyl acetate 9:1). The

mixture was poured into water and extracted with AcOEt (three times). The extract was washed with water, dried over anhydrous sodium sulfate, and filtered. Removal of the solvent gave crude products **21** (490 mg) and **21a** (620 mg), respectively, which were purified by chromatography on 'acidic' silica gel (SiO_2/compound 20:1; eluent: hexane/ethyl acetate 8:2). Compounds **21** (201 mg, 32%) and **21a** (252 mg, 30%) were thus obtained sufficiently pure for the following reaction.

^{1}H NMR of compound **21** ($CDCl_3$): 6.67 (1H, d, J = 9.9 Hz, H-6 or H-5); 6.58 (1H, d, J = 9.9 Hz, H-5 or H-6); 4.01 (3H, s, $-OCH_3$); 2.42 (2H, t, J = 7.2 Hz, $-CH_2(CH_2)_6CH_3$); 1.1–1.5 (12H, $6CH_2$); 0.87 (3H, t, J = 7.2 Hz, $-CH_2(CH_2)_6CH_3$). ESI-MS: $MH^+ = m/z$ 251.

^{1}H NMR of compound **21a** ($CDCl_3$): δ 6.67 (1H, d, J = 9.9 Hz, H-6 or H-5); 6.58 (1H, d, J = 9.9 Hz, H-5 or H-6); 4.01 (3H, s, OCH_3); 2.42 (2H, t, J = 7.2 Hz, $-CH_2(CH_2)_{12}CH_3$); 1.1–1.6 (24H, $12CH_2$); 0.87 (3H, t, J = 7.2 Hz, $-CH_2(CH_2)_{12}CH_3$). ESI-MS: $MH^+ = m/z$ 335.

4.10. Condensation of quinones 21 and 21a with hypotaurine

290 mg of **21** or 390 mg of **21a** (1.16 mmol) were dissolved in EtOH/CH_3CN 1:1. The solutions were heated at 70 °C and hypotaurine (185 mg, 1.69 mmol), dissolved in the minimum amount of water, was added in portions. The mixtures turned from yellow to red orange. The end of reactions was controlled by TLC (eluent: ethyl acetate/hexane 10:3). After solvent removing in vacuo, each mixture was poured into water and extracted with EtOAc (three times). The organic layer was washed with water, dried over anhydrous sodium sulfate, and filtered. Solvent removal gave residues containing mixtures of compounds **10** and **11** (from **21**, 163 mg, 39% yield) as well as of compounds **12** and **13** (from **21a**, 158 mg, 31% yield); they were separated as reported below.

4.11. Separation of crude mixtures of 8–9, 10–11, and 12–13 isomers

Separation of isomers **8** and **9** mixture (100 mg) was achieved by HPLC on a SiO_2 column (Luna 5 μm, 250 × 4.60 mm) eluting with EtOAc/hexane 6:4 (v/v) and afforded pure compounds **8** (17 mg) and **9** (62 mg). Mixture of isomers **10** and **11** (70 mg), as well as that of **12** and **13** (50 mg), were separated in the same conditions and yielded pure compounds **10** (5 mg), **11** (42 mg), **12** (3 mg), and **13** (30 mg).

6-Ethyl-7-methoxy-3,4-dihydro-1,1-dioxo-2*H*-1,4-benzothiazine-5,8-dione (8)

ESI-MS (positive ion mode): m/z 294 $[M+Na]^+$; HPLC: EtOAc/hexane 6:4 (v/v), (t_R): 13.5 min (single peak). ^{1}H and ^{13}C NMR data are reported in Table 1.

7-Ethyl-6-methoxy-3,4-dihydro-1,1-dioxo-2*H*-1,4-benzothiazine-5,8-dione (9)

ESI-MS (positive ion mode): m/z 294 $[M+Na]^+$; HPLC: EtOAc/hexane 6:4 (v/v), (t_R): 23.0 min (single peak). ^{1}H and ^{13}C NMR data are reported in Table 1.

6-Octyl-7-methoxy-3,4-dihydro-1,1-dioxo-2*H*-1,4-benzothiazine-5,8-dione (10)

ESI-MS (positive ion mode): m/z 378 $[M+Na]^+$; HPLC: EtOAc/hexane 6:4 (v/v), (t_R): 12.0 min (single peak). ^{1}H and ^{13}C NMR data are reported in Table 2.

7-Octyl-6-methoxy-3,4-dihydro-1,1-dioxo-2*H*-1,4-benzothiazine-5,8-dione (11)

ESI-MS (positive ion mode): m/z 378 $[M+Na]^+$; HPLC: EtOAc/hexane 6:4 (v/v), (t_R): 21.0 min (single peak). ^{1}H and ^{13}C NMR data are reported in Table 2.

6-Tetradecyl-7-methoxy-3,4-dihydro-1,1-dioxo-2*H*-1,4-benzothiazine-5,8-dione (12)

ESI-MS (positive ion mode): m/z 462 $[M+Na]^+$; HPLC: EtOAc/hexane 6:4 (v/v), (t_R): 11.0 min (single peak). ^{1}H and ^{13}C NMR data are reported in Table 2.

7-Tetradecyl-6-methoxy-3,4-dihydro-1,1-dioxo-2*H*-1,4-benzothiazine-5,8-dione (13)

ESI-MS (positive ion mode): m/z 462 $[M+Na]^+$; HPLC: EtOAc/hexane 6:4 (v/v), (t_R): 22.0 min. ^{1}H and ^{13}C NMR data are reported in Table 2.

4.12. Computational details

DFT calculations were performed on a Pentium-4 processor at 3.0 GHz using the GAUSSIAN03 package (Revision B.05). The hypothesized rotamers for A1 and A2 were optimized at the hybrid DFT mPW1PW91 level using the 6-31G(d) basis set. GIAO ^{13}C calculations were performed using the mPW1PW91 functional and 6-31G(d,p) basis set, using as input the geometry previously optimized at the mPW1PW91/6-31G(d) level. For these calculations, the IEF-PCM solvent continuum model, as implemented in GAUSSIAN (methanol solvent), was used.

4.13. Cytotoxicity assays in Jurkat cells

The cells (10^6) were treated with increasing concentrations of the compounds for 24 h and cytotoxicity assays were performed by propidium iodide staining. Treated cells were incubated at room temperature for 1 min in the presence of 10 μg/mL of propidium iodide (PI) (in a total volume of 500 μL of PBS). PI is a charged hydrophilic compound, so living cells are impermeable to it. When cell membranes are disrupted and the osmotic equilibrium is impaired (i.e., necrosis or late apoptosis) this compound can rapidly penetrate into the cells and bind nucleic acids by charge affinity. PI bound to nucleic acids exhibits red fluorescence upon excitation with the 488 nm spectral line of the argon-ion laser of the flow cytometer. After incubation, cells were immediately analyzed by flow cytometry using an EPIC XL analyzer (Beckman-Coulter, Hialeah, MI).

4.14. Determination of nuclear DNA loss and cell cycle analysis

The percentage of cells undergoing chromatinolysis (subdiploid cells) was determined as previously described.[19] Briefly, the control and treated cells were fixed in ethanol (70%) for 24 h at 4 °C. Then, the cells were washed twice with PBS containing 4% glucose and subjected to RNA digestion (using RNAse-A, 50 U/mL) and PI (20 mg/mL) staining in PBS for 1 h at RT. After incubation, cells were immediately analyzed by flow cytometry using an EPIC XL analyzer. With this method, low molecular weight DNA leaks from the ethanol-fixed apoptotic cells and the subsequent staining allows determination of the percentage of subdiploid cells (the sub-G_0/G_1 fraction).

4.15. Calcein uptake assay

The tumor adherent cell lines (10^4/well) were cultured in complete medium in 96-well plates and incubated with increasing concentrations of the compounds for another 48 h. After treatment, calcein-AM was added (final concentration 1 μM) and the cells were incubated for 60 min. The uptake was then stopped by transferring the plates on ice and washing the cells twice with HBSS precooled to 4 °C. The fluorescence of the calcein generated within the cells was analyzed in a Tecan Pro plate reader with 485-nm excitation and 535-nm emission filters.

4.16. NF-κB-dependent luciferase assays

To determine NF-κB-dependent transcription of the HIV-1-LTR-luc, 5.1 cells were preincubated for 30 min with the compounds tested as indicated, followed by stimulation with TNFα (2 ng/mL) for six hours. Then, the cells were lysed in 25 mM Tris–phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol. Luciferase activity was measured using an Autolumat LB 9510 (EG&G Berthold, USA) following the instructions of the luciferase assay kit (Promega, Madison, WI, USA) and protein concentration was measured by the Bradford method. The background obtained with the lysis buffer was subtracted from each experimental value, the relative light units (RLU)/μg of protein was calculated and the specific transactivation expressed as the percentage of transcriptional activity compared to TNFα alone (100%) or by showing the absolute RLU numbers. All the experiments were repeated at least four times.

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References and notes

1. Thomson, R. H. *Naturally Occurring Quinones*; Academic Press: London, 1971.
2. Pennock, J. F. In *Terpenoids in Plants*; Pridham, J. B., Ed.; Academic Press: London, 1967; pp 129–146.
3. Fenical, W. *Food-Drugs Sea, Proc. Conf. 4th* **1976**, *4*, 388.
4. Howard, B. M.; Clarkson, K.; Berstein, R. L. *Tetrahedron Lett.* **1979**, 4449.
5. Targett, N. M.; Keeran, W. S. *J. Nat. Prod.* **1984**, *47*, 556.
6. Guella, G.; Mancini, I.; Pietra, F. *Helv. Chim. Acta* **1987**, *70*, 621.
7. Benslimane, A. F.; Pouchus, Y. F.; Le Boterff, J.; Verbist, J. F.; Roussakis, C.; Monniot, F. *J. Nat. Prod.* **1988**, *51*, 582.
8. Sato, A.; Shindo, T.; Kasanuki, N.; Hasegawa, K. *J. Nat. Prod.* **1989**, *52*, 975.
9. Fu, X.; Houssain, M. B.; van der Helm, D.; Schmitz, F. J. *J. Am. Chem. Soc.* **1994**, *116*, 12125. this reference has been corrected in *J. Am. Chem. Soc.* **1995**, *117*, 9381.
10. Rochfort, S. J.; Metzger, R.; Hobbs, L.; Capon, R. *Aust. J. Chem.* **1996**, *49*, 1217.
11. Fu, X.; Houssain, B. M.; Schmitz, F. J.; van der Helm, D. *J. Org. Chem.* **1997**, *62*, 3810.
12. Davis, R. A.; Carroll, A. R.; Quinn, R. J. *J. Nat. Prod.* **1999**, *62*, 158.
13. Garrido, L.; Zubia, E.; Ortega, M. J.; Salva, J. *J. Nat. Prod.* **2002**, *65*, 1328.
14. Shubina, L. K.; Fedorov, S. N.; Radchenko, O. S.; Balaneva, N. N.; Kolexnikova, S. A.; Dmitrenok, P. S.; Bode, A. M.; Dong, Z.; Stonik, V. A. *Tetrahedron Lett.* **2005**, *46*, 559.
15. Aiello, A.; Fattorusso, E.; Luciano, P.; Menna, M.; Esposito, G.; Iuvone, T.; Pala, D. *Eur. J. Org. Chem.* **2003**, 898.
16. Aiello, A.; Fattorusso, E.; Luciano, P.; Macho, A.; Menna, M.; Munoz, E. *J. Med. Chem.* **2005**, *48*, 3410.
17. Aiello, A.; Fattorusso, E.; Luciano, P.; Mangoni, A.; Menna, M. *Eur. J. Org. Chem.* **2005**, 5024.
18. (a) Barone, G.; Gomez-Paloma, L.; Duca, D.; Silvestri, A.; Riccio, R.; Bifulco, G. *Chem. Eur. J.* **2002**, *8*, 3233; (b) Barone, G.; Duca, D.; Silvestri, A.; Gomez-Paloma, L.; Riccio, R.; Bifulco, G. *Chem. Eur. J.* **2002**, *8*, 3240.
19. Macho, A.; Lucena, C.; Calzado, M. A.; Blanco, M.; Donnay, I.; Appendino, G.; Munoz, E. *Chem. Biol.* **2000**, *7*, 483.
20. Schmitz, F. J.; Bloor, S. J. *J. Org. Chem.* **1988**, *53*, 3922.
21. Scribner, R. M. *J. Org. Chem.* **1966**, *31*, 3671.
22. Harada, N.; Sugioka, T.; Soutome, T.; Hiyoshi, N.; Uda, H.; Kuriki, T. *Tetrahedron: Asymmetry* **1995**, *6*, 375.
23. Locksley, H. D.; Murray, I. G. *J. Chem. Soc., C* **1970**, 392.
24. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J. A., Jr.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A. GAUSSIAN03, Revision B.05; Gaussian, Inc., Wallingford CT, 2004.
25. Gonzales, R. R.; Gambarotti, C.; Liguori, L.; Bjorsvik, H. R. *J. Org. Chem.* **2006**, *71*, 1703.
26. Inbaraj, J. J.; Gandhidasan, R.; Murugesan, R. *Free Radical Biol. Med.* **1999**, *26*, 1072.
27. Obberhammer, F.; Fritsch, G.; Schmied, M.; Pavelka, M.; Printz, D.; Purchio, T.; Lassman, H.; Schulte-Hermann, R. *J. Cell Sci.* **1993**, *104*, 317.
28. Ott, M.; Robertson, J. D.; Gogvadze, V.; Zhivotovsky, B.; Orrenius, S. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 1259.
29. Gilmore, T. D. *Oncogene* **2006**, *25*, 6680.
30. Karin, M. *Nature* **2006**, *441*, 431.
31. Hayden, M. S.; Ghosh, S. *Cell* **2008**, *132*, 344.
32. Jezek, P.; Hlavata, L. *Int. J. Biochem. Cell Biol.* **2005**, *37*, 2478.
33. Andreyev, A. Y.; Kushnareva, Y. E.; Starkov, A. A. *Biochemistry (Mosc)* **2005**, *70*, 200.
34. Gloire, G.; Legrand-Poels, S.; Piette, J. *Biochem. Pharmacol.* **2006**, *72*, 1493.