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Effects of Thymoquinone–Fatty Acid Conjugates on Cancer Cells

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4-Acylhydrazones and 6-alkyl derivatives of thymoquinone (TQ) were tested for growth inhibition of human HL-60 leukemia, 518A2 melanoma, KB-V1/Vbl cervix, and MCF-7/Topo breast carcinoma cells. Unsaturated side chains conferred greater activities than equally long saturated chains. The number of C=C bonds was less decisive than chain length. The 6-hencosahexaenyl conjugate **3e** was most active in all resistant tumor cells, with IC_{50} (72 h) values as low as 30 nM in MCF-7/Topo cells. The conjugates are likely to operate by mechanisms different from that of TQ. For instance, **3e** induced distinct caspase-independent apoptosis in HL-60 and 518A2 cells concomitant with a loss of mitochondrial membrane potential and a subsequent rise in the levels of reactive oxygen species.

Introduction

The *para*-benzoquinone motif in natural products is frequently associated with a high degree of bioactivity.[1] Even the simple thymoquinone (TQ; **1**) displays a variety of pharmacological effects. TQ is a constituent of thyme essential oils and the main bioactive component of the volatile oil of black seed (*Nigella sativa*), responsible for many of its antioxidant, anti-inflammatory,[2] and antineoplastic effects.[3] Although the molecular pathways of TQ action are not fully understood, some details have emerged from studies with xenograft animal models and in vitro tests with tumor cell lines. In animal models TQ exhibited very low toxicity but promising antitumor effects.[4–6] In a xenograft model of HCT-116 colon cancer, TQ significantly delayed tumor growth by induction of cell-cycle arrest.[7] In HCT-116 cells it induced apoptosis, which was associated with a marked increase in p53 and p21WAF1 protein levels and a significant inhibition of the anti-apoptotic Bcl-2 protein. p53-null HCT-116 cells were less sensitive to TQ-induced growth arrest and apoptosis.[8] Alternatively, in cells of myeloblastic leukemia HL-60, TQ can induce apoptosis by p53-independent pathways relying on caspases-8, -9, and -3.[9] In contrast, normal cells and primary mouse keratinocytes were found to be resistant to the apoptotic effects of TQ.[10,11] Very recently, the serine/threonine Polo-like kinases (Plk), which are overexpressed in many types of human cancers, have been identified as targets for TQ and the simple derivative poloxin. TQ can inhibit the kinases Plk1–3 by interfering with the function of their polo-box domains (PBD) and thus with their intracellular localization. In vitro application of TQ and poloxin to HeLa cells led to Plk1 mislocalization, chromosome congression defects, mitotic arrest, and apoptosis.[12] Another major component of black seed oil are unsaturated fatty acids such as linoleic acid (ω-6), α-linolenic acid (ω-3) and docosahexaenoic acid (DHA; ω-3).[13] They are also known to exhibit weak antitumor activity by binding to cognate tumor receptors.[14] Covalent conjugates of DHA with clinically established anticancer drugs such as paclitaxel or doxorubicin were shown to have improved therapeutic indices due in part to a selective accumulation in the tumor tissue.[15,16] Considering the occurrence of antitumor fatty acids in *N. sativa* seed oil, we have investigated covalent conjugates of TQ with a homologous series of fatty acids of varying chain length, branching, and degree of unsaturation. This study is part of a project aimed at improving the efficacy of anticancer drugs in resistant tumor cells by attaching them to acetogenins with beneficial effects on the uptake or on the generation of reactive oxygen species (ROS).[17,18] The acids were attached to C4 of compound **1** by a hydrazide group to give conjugates **2**, or to C6 of **1** as an alkyl residue to give derivatives **3**. Compounds **2** and **3** were tested for antiproliferative activity against the human cancer cell lines HL-60 leukemia, 518A2 melanoma, KB-V1/Vbl cervix carcinoma, and MCF-7/Topo breast adenocarcinoma. Their ability to induce apoptosis in cancer cells was also scrutinized, as was their effect on the pertinent caspases, the mitochondrial membrane potential, and the cellular levels of ROS.

Results and Discussion

Chemistry

The hydrazone conjugates **2** were prepared by a known procedure from TQ and the respective fatty acids **4** (Scheme 1).[16,19–23] The latter were first coupled with mono-Boc-protected hydrazine in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) in DMF to give the corresponding hydrazides RCONHNHBoc. These were deprotected with trifluoroacetic acid (TFA), and the resulting hydrazides **5** were condensed with TQ in the presence of TFA. The 6-alkylthy-

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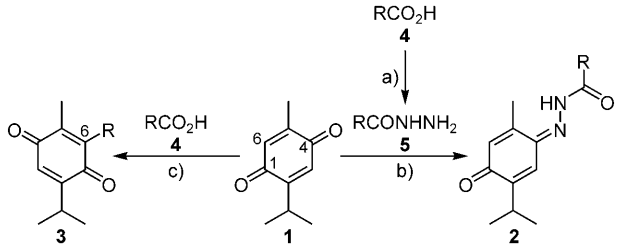

Scheme 1. Reagents and conditions: a) 1. $BocNHNH_2$, EDCI, DMF, RT, 16 h, 2. TFA, CH_2Cl_2, RT, 1 h; b) TFA, MeOH, RT, 16 h; c) $AgNO_3$, $(NH_4)_2S_2O_8$, CH_3CN/H_2O, reflux, 2–12 h.

moquinones **3** were obtained by treatment of the respective carboxylic acids **4** and TQ with a solution of $Ag_2S_2O_8$ in water/acetonitrile.[24,25] In the case of homogeranoic acid,[26] this method failed due to an inexorable *E/Z* isomerization. Thus derivative **3g** was synthesized by a Barbier-type cross-coupling reaction (Figure 1).[27]

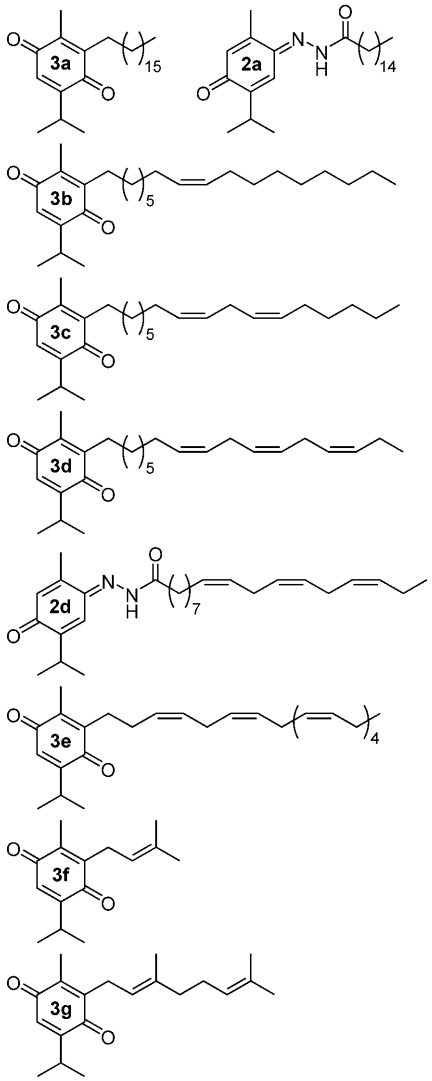

Figure 1. Thymoquinone-4-acylhydrazones **2** and 6-alkylthymoquinones **3**.

Inhibition of tumor cell growth

The conjugates **2** and **3** (Figure 1) were tested for antiproliferative activity in the human cancer cell lines 518A2 melanoma, HL-60 leukemia, P-gp-rich KB-V1/Vbl cervix carcinoma, and breast cancer resistance protein (BCRP)-rich MCF-7/Topo breast adenocarcinoma using the MTT assay. The results were compared with those of the parent compound TQ (**1**). The IC_{50} values after 24, 48, and 72 h exposure to the test compounds are listed in Table 1.

The series of conjugates **2** and **3a–d** feature side chains of similar or identical length, differing only in the number of C=C bonds. In all tested cell lines, the derivatives with saturated appendages (**2a** and **3a**) were less active than all analogues with alkene groups. 6-Heptadecylthymoquinone **3a** was even distinctly less active than the parent TQ in all four cell lines. However, increasing the number of skipped C=C bonds in a residue of constant length such as in the series **3a–d** had little influence on the antiproliferative effect. A dramatic increase in activity resulted only from attaching a longer hendoca-3,6,9,12,15,18-hexaenyl side chain as in **3e**. This conjugate showed IC_{50} (72 h) values below 500 nM for the three tumor cell lines 518A2, KB-V1/Vbl, and MCF-7/Topo. This means a remarkable boost in activity over that of TQ, especially in the MCF-7/Topo breast cancer cells in which **3e** was active at IC_{50} (72 h) = 30 nM. Notably, DHA, the precursor of the C_{21} residue of **3e**, is itself merely weakly antiproliferative in the tested cancer cell lines, with IC_{50} (72 h) > 70 μM. As to cell line specificity, the Bcl-2-overexpressing 518A2 melanoma cells responded well to the unsaturated hydrazide **2d** and also to the "DHA" derivative **3e**. The p53-null HL-60 leukemia cells responded well to the hydrazides **2** but only moderately to the 6-alkyl conjugates **3**, except **3e**. The ABC-transporter-rich KB-V1/Vbl cervix carcinoma and MCF-7/Topo breast adenocarcinoma cells were insensitive to the hydrazones but were effectively inhibited by the 6-alkyl derivatives, especially by conjugate **3e**. This pattern suggests that the fatty acid residues act on specific targets rather than by just enhancing uptake.

Induction of apoptosis

Next, we investigated the ability of compounds **1**, **2a**, **2d**, **3d**, **3e**, and **3g** to induce apoptosis in HL-60 leukemia and 518A2 melanoma cells by means of the TUNEL assay. This allows the detection of late stages of apoptosis by labeling the 3′-OH ends of typical DNA fragments with fluorescein-tagged nucleotides.[28] The assays were conducted with cells that had been exposed to the respective compounds at 5 μM for 24 h and then tested with the in situ Cell Death Detection Kit (Roche). Fluorescence microscopy revealed distinct induction of apoptosis in both cell lines by the conjugates **3d** and **3e** (Table 2).

Induction of caspases

To check the involvement of caspases-3, -8, and -9 in the mechanism of action, we treated cells of HL-60 leukemia and 518A2 melanoma with compounds **1**, **2a**, **2d**, **3d**, **3e**, or **3g** at

Table 1. Inhibition of 518A2, HL-60, KB-V1/Vbl, and MCF-7/Topo cell growth by compounds **1**–**3**.

Compd	IC_{50} [μM][a]		
	24 h	48 h	72 h
518A2 cells:			
1	29.47 ± 6.34	28.53 ± 8.80	28.33 ± 9.17
2a	> 100	> 100	22.16 ± 8.00
2d	94.54 ± 5.46	18.88 ± 2.98	8.36 ± 2.93
3a	> 100	> 100	52.03 ± 6.61
3b	76.31 ± 5.36	46.48 ± 9.12	24.29 ± 7.40
3c	82.92 ± 13.67	23.66 ± 5.42	13.51 ± 3.35
3d	31.39 ± 8.33	14.68 ± 3.70	11.18 ± 4.01
3e	2.69 ± 0.86	1.66 ± 0.56	0.33 ± 0.10
3f	71.24 ± 5.78	52.07 ± 4.83	36.09 ± 4.77
3g	40.35 ± 2.77	31.04 ± 1.27	23.03 ± 7.07
HL-60 cells:			
1	32.18 ± 6.45	30.80 ± 8.77	27.81 ± 5.95
2a	> 100	9.99 ± 0.77	5.77 ± 1.43
2d	18.68 ± 5.92	2.81 ± 1.32	2.75 ± 1.30
3a	> 100	> 100	> 100
3b	24.44 ± 4.20	15.53 ± 2.73	13.54 ± 5.01
3c	44.15 ± 6.88	14.24 ± 2.72	16.77 ± 3.93
3d	29.08 ± 6.90	16.26 ± 3.53	20.78 ± 5.24
3e	6.81 ± 0.56	2.45 ± 0.31	2.12 ± 0.75
3f	56.47 ± 6.98	54.43 ± 3.51	53.97 ± 5.25
3g	> 100	> 100	29.32 ± 11.97
KB-V1/Vbl cells:			
1	46.19 ± 4.79	34.43 ± 9.86	32.31 ± 6.01
2a	> 100	> 100	38.81 ± 12.72
2d	50.85 ± 5.71	15.81 ± 6.63	13.63 ± 1.90
3a	> 100	> 100	61.72 ± 4.05
3b	91.13 ± 8.87	30.77 ± 4.89	16.39 ± 5.58
3c	67.27 ± 3.02	18.59 ± 1.98	13.74 ± 2.90
3d	40.69 ± 11.59	18.83 ± 4.20	10.82 ± 4.12
3e	3.24 ± 0.68	2.48 ± 0.87	0.55 ± 0.12
3f	75.89 ± 6.42	47.46 ± 7.20	39.98 ± 5.63
3g	77.13 ± 8.13	36.81 ± 8.22	16.04 ± 1.47
MCF-7/Topo cells:			
1	34.23 ± 4.25	27.95 ± 7.67	26.68 ± 5.64
2a	> 100	> 100	> 100
2d	> 100	> 100	11.11 ± 1.86
3a	> 100	> 100	> 100
3b	85.12 ± 14.59	21.84 ± 0.79	11.29 ± 5.73
3c	56.46 ± 10.18	9.75 ± 2.14	6.77 ± 3.24
3d	48.03 ± 10.74	8.77 ± 1.90	7.74 ± 2.46
3e	1.08 ± 0.15	1.09 ± 0.19	0.03 ± 0.01
3f	64.80 ± 10.07	41.45 ± 8.63	22.42 ± 7.28
3g	44.66 ± 0.95	34.00 ± 19.93	26.72 ± 2.22

[a] Values are derived from concentration–response curves obtained by measuring the percent absorbance of viable cells relative to untreated controls (100%) after 24, 48, and 72 h exposure of 518A2 melanoma, HL-60 leukemia, KB-V1/Vbl cervix carcinoma, and MCF-7/Topo adenocarcinoma cells to the test compounds in the MTT assay; values represent the mean ± SD of four independent experiments.

Table 2. Percentage of apoptotic 518A2 and HL-60 cells after exposure to selected compounds **1**–**3** for 24 h.

Compd	Apoptotic Cells [%][a]	
	518A2	HL-60
1	3.2 ± 2.1	2.1 ± 1.3
2a	0.0 ± 0.1	2.0 ± 0.9
2d	5.6 ± 3.9	4.7 ± 0.7
3d	9.6 ± 6.3	11.4 ± 6.9
3e	14.2 ± 1.8	8.9 ± 1.1
3g	1.3 ± 1.8	3.8 ± 3.2

[a] Values are derived from the numbers of apoptotic cells as determined by the TUNEL assay using fluorescence microscopy after 24 h exposure of 518A2 melanoma and HL-60 leukemia cells to 5 μM of the test compounds relative to untreated control (0%); values represent the mean ± SD of three independent experiments.

5 μM. The resulting changes in caspase levels were ascertained at regular intervals between 1 and 72 h by a substrate-cleaving fluorescence assay that employs specific dye-tagged oligopeptides (Caspase-Glo Assay, Promega).[29] Generally, in both cell lines, all tested derivatives caused caspase kinetics significantly different from those initiated by TQ (Figure 2). In support of published results,[9] compound **1** initiated an early sharp rise in

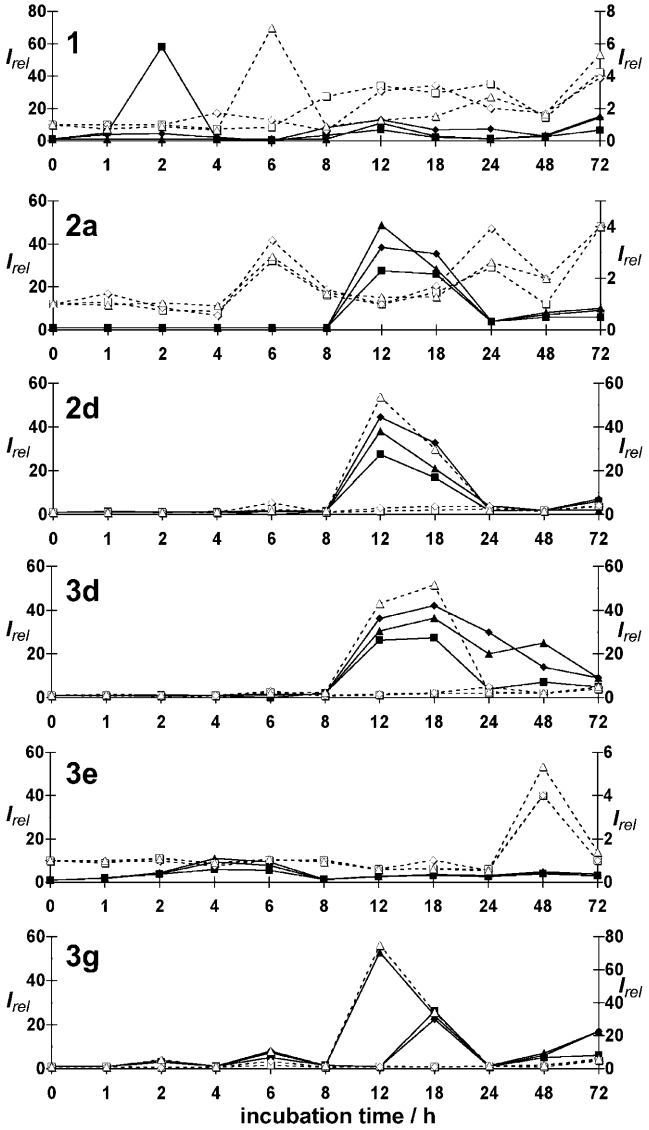

Figure 2. Caspase activation in HL-60 cells (left *y*-axis, continuous lines, full symbols) and 518A2 cells (right *y*-axis, dashed lines, void symbols) treated with 5 μM TQ (**1**) or selected derivatives **2** or **3** for up to 72 h. The activities of caspases-3 (■, □), -8 (◆, ◇), and -9 (▲, △) were quantified by a luminometric assay and are reported as relative luminescence intensities (I_{rel}).

the concentration of effector caspase-3 in HL-60 cells. A series of small maxima for caspases-3, -8, and -9 appeared after 12 h. In contrast, derivatives **2a**, **2d**, and **3d** all gave a simultaneous increase in all three monitored caspases with strong maxima after 12–18 h. Compound **3d** was less active in the MTT test, but more active in the TUNEL assay, than **2a** and **2d**. Conjugate **3e**, while being strongly antiproliferative and pro-apoptotic in HL-60 cells upon 24 h exposure, caused just a small maximum for all caspases after 4–6 h. In the case of geranyl derivative **3g**, which is not more active than TQ in HL-60 cells, there was a delay between the strong maximum of caspase-9 (after 12 h) and the smaller maximum of caspase-3 (after 18 h).

Table 3. ROS generation: percent NBT reduction in 518A2 and HL-60 cells upon exposure to selected compounds for 24 or 72 h.

Compd	NBT Reduction [%][a]			
	518A2, 24 h	518A2, 72 h	HL-60, 24 h	HL-60, 72 h
1	0.82 ± 0.17	1.23 ± 0.12	1.01 ± 0.04	1.04 ± 0.03
2a	1.16 ± 0.03	1.33 ± 0.04	0.98 ± 0.03	2.15 ± 0.02
2d	1.03 ± 0.09	1.70 ± 0.03	1.01 ± 0.02	26.41 ± 1.09
3d	1.00 ± 0.16	1.45 ± 0.03	1.04 ± 0.03	1.07 ± 0.02
3e	4.53 ± 0.50	22.01 ± 0.43	3.63 ± 0.33	4.41 ± 0.26
3g	0.91 ± 0.02	1.38 ± 0.06	1.05 ± 0.06	0.82 ± 0.07

[a] Relative ROS generation (NBT reduction) as determined from percent absorbance of formazan relative to untreated controls (1%) after 24 and 72 h exposure of 518A2 melanoma and HL-60 leukemia cells to test compounds; values represent the mean ± SD of four independent experiments.

In 518A2 cells, TQ treatment led to a small maximum of caspase-9 concentration after 6 h and to an even smaller plateau of caspase-3 between 12 and 24 h. This can be explained with the known Bcl-2-related blockade in the mitochondrial pathway of apoptosis of 518A2 cells.[30,31] Compounds **2d**, **3d**, and **3g**, which performed quite differently in the MTT and TUNEL assays with 518A2 cells, all caused great caspase-9 maxima after 12–18 h. The corresponding caspase-3 maxima were not observed within the 72 h period. The saturated hydrazone **2a** caused only a marginal increase in caspase levels. Conjugate **3e**, the strongest growth inhibitor and apoptosis inducer in 518A2 cells, gave rise to only small maxima of the three caspases after 48 h incubation.

Mitochondrial membrane potential and generation of ROS

Bhalla and colleagues showed that drug-induced apoptosis in HL-60 cells may occur, without the activation of executioner caspases, by an immediate loss of the mitochondrial membrane potential $\Delta\Psi_m$, an increase in ROS, and a release of apoptosis-inducing factor (AIF).[32] Hence, we analyzed the changes in $\Delta\Psi_m$ of 518A2 and HL-60 cells upon treatment with compounds **1**, **2a**, **2d**, **3d**, **3e**, or **3g** at 5 μM for 24 and 72 h using a kit from Stratagene, which is based on the fluorescent cationic dye JC-1.[33] The ratio of red (JC-1 aggregates in intact mitochondria) to green fluorescence (JC-1 monomers in the cytosol) is decreased in apoptotic cells. We found that the decrease in intact mitochondria was small in HL-60 cells, with TQ (24 h: 83%; 72 h: 83%) and **3e** (24 h: 95%; 72 h: 85%) performing best. However, in 518A2 cells, derivative **3e** led to a significant decrease (24 h: 80%; 72 h: 76%), whereas all other compounds left ≥90% of the mitochondria intact.

The ability of compounds **1**–**3** to initiate the generation of ROS in HL-60 and 518A2 cells was finally assessed by the colorimetric nitroblue–tetrazolium (NBT) assay,[34,35] which is based on the selective reduction of a yellow, water-soluble tetrazolium chloride to an insoluble violet diformazan by superoxide ($O_2^{\bullet-}$).

Notable are two conspicuous results listed in Table 3. The α-linolenoyl hydrazone **2d**, which, on par with the 6-hencosahexaenyl conjugate **3e**, exhibited the greatest antiproliferative effect in HL-60 cells upon exposure for 72 h, also caused the most distinct increase in ROS levels in these cells: 26-fold that of the parent TQ. Compound **3e** was the second best inducer of ROS in HL-60 cells and by far the best in 518A2 cells, where it was 22-fold more effective than TQ. Therefore, at least for derivative **3e**, there is reasonable coherence between its antiproliferative activity, apoptosis induction, ROS initiation, and its ability to reduce the mitochondrial membrane potential.

Conclusions

We found that the antiproliferative activity of TQ against resistant cancer cell lines can be significantly improved by attaching fatty acid derived alkenyl groups at position C4 through an acylhydrazone group or directly at C6 by a C–C bond. Although unsaturated residues yield apparently more active conjugates than saturated analogues of equal length, the number of C=C bonds in the side chain is less decisive for the activity than its overall length. This was demonstrated by a comparison of the mono-, bis-, and tris-unsaturated C_{17}-substituted derivatives **3b**–**d** with the hencosahexaenyl analogue **3e**. While the former differed little in their antiproliferative activities, the latter was dramatically more active, especially in the resistant cell lines. The most active derivatives also seem to operate by mechanisms different from that of TQ. For instance, **3e** is a strong inducer of apoptosis in the tested cell lines HL-60 and 518A2. The temporal progress and the magnitude of growth inhibition and apoptosis were largely independent of caspase concentrations but corresponded to changes in the mitochondrial membrane potential and the levels of ROS. These mechanistic peculiarities, together with differences in the rates of cell growth inhibition between hydrazone and alkenyl derivatives with identical residues such as **2d**/**3d**, confute the mere shuttle function of the fatty acid moieties. Further tests are currently underway in order to pinpoint other cancer-relevant targets for TQ–acetogenin conjugates. Preliminary tests for interfer-

ence with the Polo-box domain of Plk by a fluorescence polarization assay based on its binding to a fluorophore-labeled peptide comprising its optimal recognition motif have already been carried out.[12,36] These tests revealed that conjugate **3e** distinctly inhibits the function of the PDB of Plk1, Plk2, and Plk3 (apparent $IC_{50} \leq 10$ μM), whereas compounds **2d** and **3d** were more selective and inhibited only the PDB of Plk2 (apparent $IC_{50} \leq 14$ μM).

Experimental Section

Instrumentation and chemicals

Melting points were recorded on an Electrothermal 9100 apparatus and are uncorrected. IR spectra were measured on a PerkinElmer Spectrum One FTIR spectrophotometer equipped with an ATR sampling unit. NMR spectra were obtained under conditions as indicated on a Bruker Avance 300 spectrometer with tetramethylsilane as an internal standard. MS data were collected with a Varian MAT 311A (EI, 70 eV). HRMS were obtained by peak matching against two bracketing reference mass peaks of perfluorokerosene and iterative scanning/algorithmic averaging. Thymoquinone and its conjugates were kept at 4 °C as 10 mM stock solutions in DMSO. Appropriate test concentrations were obtained by dilution with cell culture medium immediately before use. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from ABCR (Karlsruhe, Germany). DHA was a gift from K.D.-Pharma, Bexbach, Germany.

Methods

1. Cell lines and culture conditions: Human leukemia cells HL-60 were obtained from the German Center of Biological Material (DSMZ), Braunschweig; human melanoma cells 518A2 from the Department of Oncology and Hematology at Martin Luther University, Halle; and the KB-V1/Vbl and MCF-7/Topo cells from the Institute of Pharmacy at the University of Regensburg (Germany). HL-60 cells were grown in RPMI-1640 medium (Gibco) supplemented with 10% fetal calf serum (FCS, Gibco) 100 IU mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin sulfate, 0.25 μg mL^{-1} amphotericin B, and 250 μg mL^{-1} gentamycin. 518A2 and the KB-V1/Vbl cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% FCS, 100 IU mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin sulfate, 0.25 μg mL^{-1} amphtericin B, and 250 μg mL^{-1} gentamycin. MCF-7/Topo cells were grown in Eagle's minimum essential medium (EMEM, Sigma–Aldrich) supplemented with 5% FCS, 2.2 mg mL^{-1} $NaHCO_3$, and 110 μg mL^{-1} sodium pyruvate. Cells were maintained under a moisture-saturated atmosphere (5% CO_2) at 37 °C in 75-mL culture flasks (Nunc, Wiesbaden, Germany). They were serially passaged following trypsinization by using 0.05% trypsin/0.02% EDTA (PAA Laboratories, Cölbe, Germany). Mycoplasma contamination was routinely monitored, and only mycoplasma-free cultures were used.

2. Determination of tumor cell growth (MTT assay): HL-60 cells (0.5×10^6 cells mL^{-1}) were seeded out and cultured for 24 h on 96-well microplates; 518A2, MCF-7/Topo, and KB-V1/Vbl cells (1.7×10^5 cells mL^{-1}) were cultured for 24 h. Incubation (5% CO_2, 95% humidity, 37 °C) of cells following treatment with the test compounds was continued for 24, 48, and 72 h. Blank and solvent controls were incubated under identical conditions. A 5 mg mL^{-1} stock solution of MTT in phosphate-buffered saline (PBS) was then added at a final concentration of 0.05% (HL-60, 518A2) or 0.1% (MCF-7/Topo, KB-V1/Vbl). After 2 h the precipitate of formazan crystals was re-dissolved in a 10% solution of sodium dodecylsulfate (SDS) in DMSO containing 0.6% acetic acid in the case of HL-60 cells. For the adherent 518A2, MCF-7/Topo, and KB-V1/Vbl cells, microplates were swiftly turned to discard the medium prior to adding the solvent mixture. The microplates were gently shaken in the dark for 30 min and left in the incubator overnight to ensure complete dissolution of the formazan. Finally the absorbance at $\lambda = 570$ and 630 nm (background) was measured using an ELISA plate reader. All experiments were carried out in quadruplicate, and the percentage of viable cells quoted was calculated as the mean ± SD with respect to the controls set to 100%.

3. Apoptosis TUNEL assay: Apoptosis was scored by measuring the extent of DNA fragmentation by the terminal deoxytransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay. Following treatment, cells were centrifuged at 400 *g* for 10 min, washed, and fixed with 2% formalin for 7 min, then washed and centrifuged again. Cells were plated on glass slides and permeabilized with a mixture of 0.1% sodium citrate and 0.1% Triton X-100 for 2 min at 4 °C. The cellular DNA was stained with the in situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. The percentage of apoptotic, green-stained cells was counted on a fluorescence microscope (Axiovert 135, Zeiss, Göttingen, Germany), calculated for 300 cells, and expressed as the mean ± SD of four independent experiments.

4. Caspase activity assay: The activity of caspases was determined by a luminometric caspase assay (Promega), according to the manufacturer's protocol. Cellular proteins were extracted from cells following treatment in a lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 1× EDTA-free protease inhibitor mix (Calbiochem). Cell lysates were incubated at 4 °C for 15 min, centrifuged at 800 *g* for 10 min, and the precipitates were discarded. Protein concentrations were measured using the Bradford reagent (Sigma) and bovine serum albumin as a standard. Finally, cell lysates (15 μg cellular protein) were analyzed for caspase activities using the homogeneous luminescent assay. Following the cleavage of the luminogenic substrate containing a caspase-specific peptide sequence, the luminescence was quantified with a Tecan Genios Plus plate reader and assumed to be proportional to the caspase activities in the cell lysates.[29]

5. Mitochondrial membrane potential: Changes in mitochondrial membrane potential were determined by the Mitochondrial Membrane Detection Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's procedure. Following treatment, cell samples were centrifuged at 400 *g* for 5 min. The pellets were resuspended in 500 μL diluted JC-1 solution (0.2×), incubated at 37 °C for 15 min (HL-60) or 35 min (518A2) and then centrifuged again for 5 min at 400 *g*. After washing, the pellets were resuspended in 100 μL PBS and transferred into a well of a black 96-well plate. The red ($\lambda_{ex} =$ 550 nm, $\lambda_{em} = 600$ nm) and green ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 535$ nm) fluorescence intensities were measured and their ratio was calculated.[33]

6. Generation of ROS (NBT assay): HL-60 cells (0.5×10^6 cells mL^{-1}) were plated in 96-well tissue culture plates, and test compounds were added after 24 h incubation at 37 °C to achieve a final concentration of 5 μM. Incubation (5% CO_2, 95% humidity, 37 °C) of cells following treatment with the test compounds was continued for 24 and 72 h. After removal of the cell medium by centrifugation, the cells in each well were resuspended in 100 μL 0.1% NBT, and the plates were placed in the incubator for 1 h. The reduced NBT was solubilized with 100 μL 2 M KOH and 130 μL DMSO for

30 min. The absorbance was measured for each well at 630 and 405 nm (background) using an ELISA plate reader. The adherent 518A2 cells (1.7×10^5 cells mL^{-1}) were seeded out in 96-well tissue culture plates after trypsinization and incubation for 24 h at 37 °C to allow attachment, then treated similarly, only that the medium was removed prior to incubation with NBT for 4 h. All experiments were carried out in double quadruplicate.[34,35]

Syntheses

1. *N*-Boc-protected hydrazides

***N*-Hexadecanoyl-*N'*-*tert*-butoxycarbonylhydrazine**: A mixture of palmitic acid **4a** (1.0 g, 3.9 mmol), dry DMF (20 mL), $H_2NNHBoc$ (0.62 g, 4.7 mmol) and EDCI (2.24 g, 11.7 mmol) was stirred at room temperature overnight. After addition of H_2O the reaction mixture was extracted with EtOAc. The combined organic layers were washed with brine, dried with Na_2SO_4, and concentrated under vacuum. The product was purified by column chromatography (silica gel 60; EtOAc/cyclohexane 4:1). Yield: 1.42 g (98%); colorless solid; mp: 47 °C; R_f = 0.51 (EtOAc/cyclohexane, 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.76 (t, J = 6.9 Hz, 3H), 1.0–1.2 (br m, 24H), 1.34 (s, 9H), 1.52 (t, J = 6.9 Hz, 2H), 2.12 (t, J = 7.5 Hz, 2H), 7.32 (br, 1H), 8.9 ppm (br, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.9, 22.6, 25.3, 26.8 28.0, 29.2, 29.3, 29.4, 29.5, 29.6, 29.7, 31.8, 33.6, 81.1, 156.1, 173.2 ppm; IR (ATR): ν_{max} = 3218, 2923, 2853, 1724, 1670, 1466, 1393, 1368, 1245, 1159, 1057, 1016, 907, 729 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 297 (18) $[C_{17}H_{33}N_2O_2]^+$, 270 (11), 239 (63), 112 (16), 100 (33), 83 (14), 69 (24), 57 (100).

***N*-(α-Linolenoyl)-*N'*-*tert*-butoxycarbonylhydrazine:** Analogously, *N*-(α-linolenoyl)-*N'*-*tert*-butoxycarbonylhydrazine (1.88 g, 89%) was obtained from α-linolenic acid (1.5 g, 5.4 mmol), $H_2NNHBoc$ (0.59 g, 4.5 mmol), EDCI (2.16 g, 11.3 mmol), and dry DMF (20 mL); colorless oil; R_f = 0.55 (EtOAc/cyclohexane 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.81 (t, J = 7.6 Hz, 3H), 1.16 (br m, 8H), 1.32 (s, 9H), 1.49 (m, 2H), 1.8–2.0 (m, 4H), 2.09 (t, J = 7.4 Hz, 2H), 2.64 (t, J = 5.8 Hz, 4H), 5.1–5.3 (m, 6H), 7.32 (br, 1H), 8.85 ppm (br, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.9, 20.3, 25.0, 25.1, 25.2, 26.8, 27.7, 28.7, 28.8, 28.9, 29.2, 33.6, 80.7, 126.8, 127.6, 127.9, 129.7, 129.8, 131.6, 155.9, 172.9 ppm; IR (ATR): ν_{max} = 3266, 3010, 2927, 2855, 1725, 1671, 1456, 1392, 1367, 1246, 1161, 1046, 1016, 872, 720 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 393 (3) $[M+1]^+$, 337 (10), 293 (80), 263 (32), 221 (7), 163 (24), 113 (100).

2. Hydrazides (5)

Hexadecanoylhydrazine (5a): *N*-Hexadecanoyl-*N'*-*tert*-butoxycarbonylhydrazine (1.42 g, 3.83 mmol) was dissolved in CH_2Cl_2 (10 mL) and treated with TFA (6 mL). The resulting mixture was stirred at room temperature for 1 h. The volatiles were evaporated, and the product was purified by column chromatography. Yield: 1.03 g (99%); R_f = 0.14 (EtOAc/cyclohexane, 1:1); colorless solid, mp: 112 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.86 (t, J = 6.9 Hz, 3H), 1.23 (br, 24H), 1.61 (m, 2H), 2.13 ppm (t, J = 7.9 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.1, 22.7, 25.5, 29.3, 29.4, 29.6, 29.7, 31.9, 34.6, 174.1 ppm; IR (ATR): ν_{max} = 3320, 2921, 2849, 1630, 1537, 1462 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 239 (90) $[M-N_2H_3]^+$, 213 (13), 171 (15), 129 (14), 97 (28), 74 (72), 57 (100).

α-Linolenic acid hydrazide (5d): Analogously to the synthesis of **5a**, compound **5d** (812 mg, 58%) was prepared from *N*-(α-linolenoyl)-*N'*-*tert*-butoxycarbonylhydrazine (1.88 g, 4.79 mmol) in CH_2Cl_2 (20 mL) and TFA (5 mL); colorless oil; R_f = 0.23 (EtOAc/cyclohexane, 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.90 (t, J = 7.5 Hz, 3H), 1.24 (br m, 8H), 1.56 (br m, 2H), 1.9–2.1 (m, 4H), 2.20 (t, J = 7.5 Hz, 2H), 2.74 (m, 4H), 4.6–5.2 (br, 2H), 5.29 (m, 6H), 9.07 ppm (br, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.9, 20.4, 24.6, 25.4, 25.5, 27.2, 29.0, 29.1, 29.2, 29.5, 31.4, 126.9, 127.6, 128.1, 130.1, 131.8, 174.2 ppm; IR (ATR): ν_{max} = 3250, 2931, 2858, 1781, 1733, 1664, 1542, 1466, 1374, 1245, 1209, 1164, 1045, 907 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 261 (3) $[M-N_2H_3]^+$, 149 (7), 135 (12), 121 (14), 107 (16), 95 (47), 79 (83), 67 (90), 55 (88), 41 (100).

3. Thymoquinone 4-acylhydrazones (2)

Thymoquinone-4-hexadecanoylhydrazone (2a): Thymoquinone **1** (100 mg, 0.61 mmol) and hydrazide **5a** (165 mg, 0.61 mmol) were dissolved in anhydrous MeOH (20 mL) and treated with TFA (35.3 μL). The mixture was stirred at room temperature overnight and then evaporated. The oily residue was purified by column chromatography. Yield: 71 mg (28%); yellow oil; R_f = 0.55 (toluene); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.85 (t, J = 6.9 Hz, 3H), 1.16 (d, J = 6.9 Hz, 6H), 1.23 (m br, 24H), 1.71 (m, 2H), 2.19 (d, J = 1.2 Hz, 3H), 2.77 (t, J = 7.7 Hz, 2H), 3.14 (sept, J = 6.9 Hz, 1H), 6.37 (q, J = 1.2 Hz, 1H), 7.37 (br, 1H), 10.86 ppm (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.1, 17.5, 21.9, 22.6, 24.8, 26.9, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9, 32.9, 117.1, 129.4, 139.7, 142.1, 152.5, 181.5, 189.7 ppm; IR (ATR): ν_{max} = 3169, 3107, 2914, 2850, 1676, 1644, 1618, 1590, 1539, 1470, 1393, 1314, 1268, 1225, 1205, 1154, 1026, 907, 860 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 416 (32) $[M]^+$, 239 (13), 178 (100); HRMS-EI: *m/z* calcd for $C_{26}H_{44}N_2O$: 416.3403; found: 416.3411.

Thymoquinone-4-α-linolenoylhydrazone (2d): Analogously to **2a**, hydrazone **2d** (163 mg, 47%) was prepared from **1** (130 mg, 0.79 mmol), **5d** (298 mg, 1.02 mmol), TFA (46.2 μL), and anhydrous MeOH (20 mL); yellow oil; R_f = 0.76 (EtOAc/cyclohexane, 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.94 (t, J = 7.5 Hz, 3H), 1.15 (d, J = 6.9 Hz, 6H), 1.24 (m br, 8H), 1.71 (m br, 2H), 1.9–2.1 (m, 4H), 2.05 (t, J = 7.7 Hz, 2H), 2.19 (d, J = 1.1 Hz, 3H), 2.77 (m, 4H), 3.14 (sept, J = 6.9 Hz, 1H), 5.33 (m, 6H), 6.37 (q, J = 1.1 Hz, 1H), 7.36 (s, 1H), 10.85 ppm (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.3, 17.5, 20.5, 21.9, 24.8, 25.5, 25.6, 26.9, 27.2, 29.1, 29.3, 29.4, 29.6, 34.1, 117.2, 127.1, 127.8, 128.3, 129.4, 130.2, 131.9, 141.2, 147.6, 150.2, 178.2, 186.4 ppm; IR (ATR): ν_{max} = 3169, 3108, 3011, 2926, 2855, 1741, 1673, 1639, 1623, 1532, 1464, 1397, 1264, 1156, 1024, 908 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 438 (41) $[M]^+$, 274 (38), 257 (11), 164 (93), 149 (51), 121 (24), 81 (62), 67 (100); HRMS-EI: *m/z* calcd for $C_{28}H_{42}N_2O$: 438.32463; found: 438.3353.

4. 6-Alkyl-thymoquinones (3)

6-Heptadecylthymoquinone (3a): A mixture of **1** (100 mg, 0.61 mmol), stearic acid (139 mg, 038 mmol), $AgNO_3$ (13 mg, 0.08 mmol) and CH_3CN/H_2O 1:1 (15 mL) was stirred and heated at reflux while a solution of $(NH_4)_2S_2O_8$ (139 mg, 0.61 mmol) in H_2O (0.61 mL) was slowly added. The resulting mixture was held at reflux for 4 h, then cooled, diluted with H_2O, and extracted with Et_2O. The organic phases were washed with brine and dried over Na_2SO_4. The volatiles were removed under vacuum, and the residue was purified by column chromatography (silica gel 60; EtOAc/cyclohexane, 1:4). Yield: 106 mg (51%); yellow oil; R_f = 0.79 (EtOAc/cyclohexane, 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.89 (t, J = 6.9 Hz, 3H), 1.13 (d, J = 6.9 Hz, 6H), 1.27 (m, 30H), 2.03 (s, 3H), 2.49 (t, J = 7.9 Hz, 2H), 3.07 (dsept, J = 6.9, 1.2 Hz, 1H), 6.48 ppm (d, J = 1.2 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.1, 21.1, 21.4, 22.7, 26.1, 26.7, 26.8, 28.7, 29.3, 29.4, 29.5, 29.6, 29.7, 29.8, 29.9, 31.9, 129.9, 139.8, 145.4, 154.6, 187.0, 188.5 ppm; IR (ATR): ν_{max} = 2912, 1849, 1647, 1612, 1470, 1379, 1308, 1257, 1078, 1012, 793 cm^{-1}; MS (EI, 70 eV):

m/z (%): 402 (100) $[M]^+$, 360 (12), 177 (27), 137 (9), 43 (6); HRMS-EI: *m/z* calcd for $C_{27}H_{46}O_2$: 402.3498, found: 402.3502.

(8′*Z*)-6-Hepatadeca-8′-enyl)thymoquinone (3 b): Analogously to **3 a**, compound **3 b** (57 mg, 29%) was prepared from **1** (100 mg, 0.61 mmol), oleic acid (138 mg, 0.49 mmol), $AgNO_3$ (13 mg, 0.08 mmol), and $(NH_4)_2S_2O_8$ (139 mg, 0.61 mmol) in CH_3CN/H_2O, 1:1 (15 mL); yellow oil; R_f = 0.82 (EtOAc/cyclohexane, 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.85 (t, *J* 6.9 Hz, 3H), 1.08 (d, *J* = 6.9 Hz, 6H), 1.2–1.4 (m, 22H), 1.96 (m, 4H), 1.98 (s, 3H), 2.45 (t, *J* = 7.8 Hz, 2H), 3.02 (dsept, *J* = 6.9, 1.2 Hz, 1H), 5.31 (t, *J* = 5.6 Hz, 2H), 6.44 ppm (d, ^4J = 1.2 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.1, 21.1, 21.4, 22.7, 24.0, 26.1, 26.6, 26.7, 27.2, 28.7, 29.2, 29.3, 29.5, 29.6, 29.7, 29.9, 31.9, 129.7, 129.9, 130.0, 139.8, 145.4, 154.6, 186.9, 188.5 ppm; IR (ATR): ν_{max} = 2924, 2853, 1647, 1613, 1463, 1306, 1247, 893, 708 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 401 (100) $[M+1]^+$, 277 (8), 227 (25), 191 (5), 179 (57), 137 (27), 81 (23), 41 (37); HRMS-EI: *m/z* calcd for $C_{27}H_{44}O_2$: 400.3341; found: 400.3341.

(8′*Z*,11′*Z*)-6-(Heptadeca-8′,11′-dienyl)thymoquinone (3 c): Analogously to **3 a**, compound **3 c** (80 mg, 40%) was prepared from **1** (100 mg, 0.61 mmol), linoleic acid (137 mg, 0.49 mmol, 0.15 mL), $AgNO_3$ (13 mg, 0.08 mmol), and $(NH_4)_2S_2O_8$ (139 mg, 0.61 mmol) in CH_3CN/H_2O, 1:1 (15 mL); yellow oil; R_f = 0.74 (EtOAc/cyclohexane, 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.86 (t, *J* = 6.9 Hz, 3H), 1.09 (d, *J* = 6.9 Hz, 6H), 1.2–1.4 (m, 1H), 1.9–2.1 (m, 4H), 1.99 (s, 3H), 2.45 (t, *J* = 7.9 Hz, 2H), 2.75 (t, *J* = 5.9 Hz, 2H), 3.02 (dsept, *J* = 6.9, 1.2 Hz, 1H), 5.33 (m, 4H), 6.45 ppm (d, *J* = 1.2 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.0, 21.1, 21.5, 22.6, 25.6, 26.1, 26.7, 27.2, 28.7, 29.2, 29.3, 29.6, 29.8, 29.9, 31.5, 127.9, 128.0, 129.9, 130.0, 130.2, 139.5, 145.4, 154.6, 187.0, 188.5 ppm; IR (ATR): ν_{max} = 3010, 2952, 2925, 2855, 1738, 1649, 1613, 1463, 1378, 1305, 1247, 893, 708 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 398 (100) $[M]^+$, 355 (7), 262 (10), 203 (17), 179 (100), 137 (69), 81 (50), 67 (60); HRMS-EI: *m/z* calcd for $C_{27}H_{42}O_2$: 398.3185; found: 398.3206.

(8′*Z*,11′*Z*,14′*Z*)-6-(Heptadeca-8′,11′,14′-trienyl)thymoquinone (3 d): Analogously to **3 a**, compound **3 d** (57 mg, 30%) was prepared from **1** (100 mg, 0.61 mmol), α-linoleic acid (136 mg, 0.49 mmol, 0.15 mL), $AgNO_3$ (13 mg, 0.08 mmol), and $(NH_4)_2S_2O_8$ (139 mg, 0.61 mmol) in CH_3CN/H_2O, 1:1 (15 mL); yellow oil; R_f = 0.67 (EtOAc/cyclohexane 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.95 (t, *J* = 7.7 Hz, 3H), 1.09 (d, *J* = 6.9 Hz, 6H), 1.2–1.4 (m, 10H), 1.98 (s, 3H), 2.27 (m, 4H), 2.45 (t, *J* = 6.8 Hz, 2H), 2.78 (m, 4H), 3.02 (dsept, *J* = 6.9, 1.2 Hz, 1H), 5.2–5.4 (m, 6H), 6.45 ppm (d, *J* = 1.2 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 11.7, 15.6, 20.5, 21.4, 22.6, 25.5, 25.6, 26.7, 26.7, 27.2, 28.7, 29.2, 29.6, 29.9, 127.1, 127.7, 128.3, 129.9, 130.3, 131.9, 134.2, 139.9, 145.4, 154.6, 187.0, 188.5 ppm; IR (ATR): ν_{max} = 3010, 2961, 2926, 2854, 1741, 1647, 1613, 1463, 1378, 1306, 1247, 1190, 1146, 1106, 894, 707 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 397 (13) $[M+1]^+$, 294 (10), 264 (14), 179 (16), 135 (19), 79 (79), 43 (100); HRMS-EI: *m/z* calcd for $C_{27}H_{40}O_2$: 396.3028; found: 396.3032.

(All-*Z*)-6-(hencosa-3′,6′,9′,12′,15′,18′-hexaenyl)thymoquinone (3 e): Analogously to **3 a**, compound **3 e** (80 mg, 36%) was prepared from **1** (100 mg, 0.61 mmol), docosahexaenoic acid (160 mg, 0.49 mmol, 0.18 mL), $AgNO_3$ (13 mg, 0.08 mmol), and $(NH_4)_2S_2O_8$ (139 mg, 0.61 mmol) in CH_3CN/H_2O, 1:1 (15 mL); yellow oil; R_f = 0.82 (EtOAc/cyclohexane, 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.91 (t, *J* = 7.3 Hz, 3H), 1.09 (d, *J* = 6.9 Hz, 6H), 2.01 (s, 3H), 2.06 (t, *J* = 7.3 Hz, 2H), 2.46 (m, 2H), 2.56 (t, *J* = 7.5 Hz, 2H), 2.7–2.9 (m, 10H), 3.04 (dsept, *J* = 6.9, 1.3 Hz, 1H), 5.2–5.5 (m, 12H), 6.46 ppm (d, *J* = 1.3 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.6, 14.3, 17.6, 21.5, 25.5, 25.6, 26.7, 26.8, 27.8, 127.0, 127.9, 128.1, 128.3, 129.1, 129.9, 132.1, 139.5, 144.4, 154.6, 187.3, 188.5 ppm; IR (ATR): ν_{max} = 3012, 2961, 2928, 2871, 1647, 1613, 1462, 1377, 1305, 1249, 1188, 1147, 1075, 894, 708 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 448 (3) $[M+2]^+$, 400 (9), 346 (33), 312 (22), 298 (15), 231 (31), 203 (46), 179 (85), 119 (47), 79 (100); HRMS-EI: *m/z* calcd for $C_{31}H_{42}O_2$: 446.3185; found: 446.3191.

6-(3′-Methylbut-2′-enyl)thymoquinone (3 f): Analogously to **3 a**, compound **3 f** (54 mg, 47%) was prepared from **1** (100 mg, 0.61 mmol), homoprenoic acid (104 mg, 0.91 mmol), $AgNO_3$ (13 mg, 0.08 mmol), and $(NH_4)_2S_2O_8$ (208 mg, 0.91 mmol) in CH_3CN/H_2O, 1:1 (15 mL); yellow oil; R_f = 0.74 (EtOAc/cyclohexane, 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.08 (d, *J* = 6.9 Hz, 6H), 1.65 (d, *J* = 1.4 Hz, 3H), 1.72 (d, *J* = 1.4 Hz, 3H), 1.99 (s, 3H), 3.02 (dsept, *J* = 6.9, 1.2 Hz, 1H), 3.19 (d, *J* = 7.1 Hz, 2H), 4.92 (tsept, *J* = 7.1, 1.4 Hz, 1H), 6.45 ppm (d, *J* = 1.2 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 11.7, 17.9, 21.5, 25.7, 25.8, 26.7, 119.3, 129.9, 134.2, 140.1, 143.9, 154.5, 186.8, 188.6 ppm; IR (ATR): ν_{max} = 2964, 2928, 2874, 1648, 1612, 1449, 1376, 1304, 1253, 1146, 1044, 945, 912, 893 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 232 (100) $[M]^+$, 189 (100), 175 (46), 147 (22), 105 (35), 91 (38); HRMS-EI: *m/z* calcd for $C_{15}H_{20}O_2$: 232.1463; found: 232.1470.

(*E*)-6-(3′,7′-Dimethylocta-2,6-dienyl)thymoquinone (3 g): $BF_3{\cdot}OEt_2$ (262 mg, 1.85 mmol, 0.23 mL) was added at −78 °C to a stirred solution of **1** (100 mg, 0.61 mmol) in CH_2Cl_2 (20 mL). After 10 min, a solution of tri-*n*-butyl(geranyl)tin (291 mg, 0.68 mmol) in CH_2Cl_2 (5 mL) was added. The cooling bath was removed, and the mixture was allowed to warm to room temperature. After 2.5 h a solution of 10% HCl (6.5 mL) was added, and stirring was continued for 10 min. The mixture was extracted with CH_2Cl_2, the combined extracts were washed with brine, dried with Na_2SO_4, and the volatiles were removed under vacuum. The residue was taken up in CH_3CN/H_2O (1:1, 20 mL) and the resulting solution was treated with ceric ammonium nitrate (835 mg, 1.52 mmol). After stirring at room temperature for 15 min the reaction was quenched with H_2O (50 mL). The mixture thus obtained was extracted with CH_2Cl_2, and the combined extracts were washed with brine, dried over Na_2SO_4, and evaporated. The oily residue was purified by column chromatography (silica gel 60; EtOAc/cyclohexane, 1:4). Yield: 46 mg (25%); yellow oil; R_f = 0.73 (EtOAc/cyclohexane, 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.08 (d, *J* = 6.9 Hz, 6H), 1.61 (br m, 6H), 1.66 (br m, 3H), 1.9–2.1 (m, 4H), 2.00 (s, 3H), 2.54 (m, 2H), 3.02 (dsept, *J* = 6.9, 1.1 Hz, 1H), 4.67 (m, 1H), 5.12 (tsept, *J* = 7.1, 1.3 Hz, 1H), 6.45 ppm (d, *J* = 1.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 11.6, 20.9, 21.4, 25.8, 26.5, 26.8, 35.9, 40.2, 41.5, 124.3, 129.7, 130.1, 132.0, 138.0, 140.1, 145.3, 154.6, 187.2, 188.4 ppm; IR (ATR): ν_{max} = 2962, 2930, 2872, 1762, 1643, 1460, 1377, 1308, 1249, 1148, 1101, 1030, 924, 892 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 300 (31) $[M]^+$, 236 (54), 219 (100), 179 (92), 109 (19), 107 (14); HRMS-EI: *m/z* calcd for $C_{20}H_{28}O_2$: 300.2089; found: 300.2094.

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