

## Terpene Conjugates of the *Nigella sativa* Seed-Oil Constituent Thymoquinone with Enhanced Efficacy in Cancer Cells

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Thymoquinone (TQ; **1**) is a weak anticancer constituent of black seed oil. Derivatives bearing terpene-terminated 6-alkyl residues were tested in cells of human HL-60 leukemia, 518A2 melanoma, multidrug-resistant KB-V1/Vbl cervix, and MCF-7/Topo breast carcinomas, as well as in non-malignant human foreskin fibroblasts. Derivatives with a short four-atom spacer between quinone and cyclic monoterpene moieties were more antiproliferative than analogues with longer spacers. 6-(Menthoxymethyl)thymoquinone (**3a**) exhibited single-digit micromolar  $IC_{50}$  (72 h) values in all four cell lines. It was seven times more active than TQ (**1**) in 518A2 melanoma cells and four times in KB-V1/Vbl cervix carcinoma cells, while only half as toxic in the fibroblasts. Compound **3a** was also not a substrate for the P-gp and BCRP drug transporters of the resistant cancer cells. The caryophyllyl and germacryl conjugates **3e** and **3f** specifically inhibited the growth of the resistant MCF-7 breast carcinoma cells. Conjugation of TQ with the triterpene betulinic acid *via* the OH group as in **3g** led to a loss in activity, while conjugation *via* the carboxylic acid afforded compound **4** with nanomolar  $IC_{50}$  (72 h) activity against HL-60 cells. All anticancer-active derivatives of TQ (**1**) induced apoptosis associated with DNA laddering, a decrease in mitochondrial membrane potential and a slight increase in reactive oxygen species.

**Introduction.** – Thymoquinone (TQ; **1**) is the active principle of thyme essential oils and the essential oil of black seed (*Nigella sativa*), responsible for many of its antioxidant, anti-inflammatory [1], and antineoplastic effects [2]. TQ (**1**) is of low general toxicity yet showed weak antitumor effects in numerous cancer-cell studies and animal models [3–6]. The molecular mechanism of its action is not fully understood, yet. In certain xenograft models, it was found to delay tumor growth by induction of cell-cycle arrest. In *in vitro* experiments, it induced apoptosis both in p53-dependent and p53-independent ways [7][8]. Lately, the serine/threonine Polo-like kinases (Plk) which are overexpressed in many types of human cancers have been identified as targets for TQ. In HeLa cells, it effected Plk mislocalization, chromosome congression defects, mitotic arrest, and apoptosis [9].

In a preceding article, we have shown that the cell-line specificity and the anticancer activity of TQ can be significantly increased by covalent attachment of fatty acyl residues of defined length and degree of unsaturation [10]. Interestingly, the most active compounds resulted from conjugation with fatty acids that are present in the fixed oil of black seed. In continuation of this study, we now prepared and bio-evaluated conjugates of TQ with various monoterpenes, sesquiterpenes, and the cytotoxic triterpene betulinic acid [11][12] to identify those structural parameters that are associated with anticancer activity. Terpenes were also found in the essential oil of *N. sativa* [13]. We attached esters of various terpene alcohols to C(6) of TQ *via* spacers

of variable length. The resulting derivatives **3** were tested for growth inhibition of cells of the human cancer cell lines HL-60 leukemia, 518A2 melanoma, KB-V1/Vbl cervix carcinoma, and MCF-7/Topo breast adenocarcinoma, and of non-malignant foreskin fibroblasts. Their effects on the mitochondrial membrane potential and the cellular levels of reactive oxygen species (ROS) were also scrutinized.

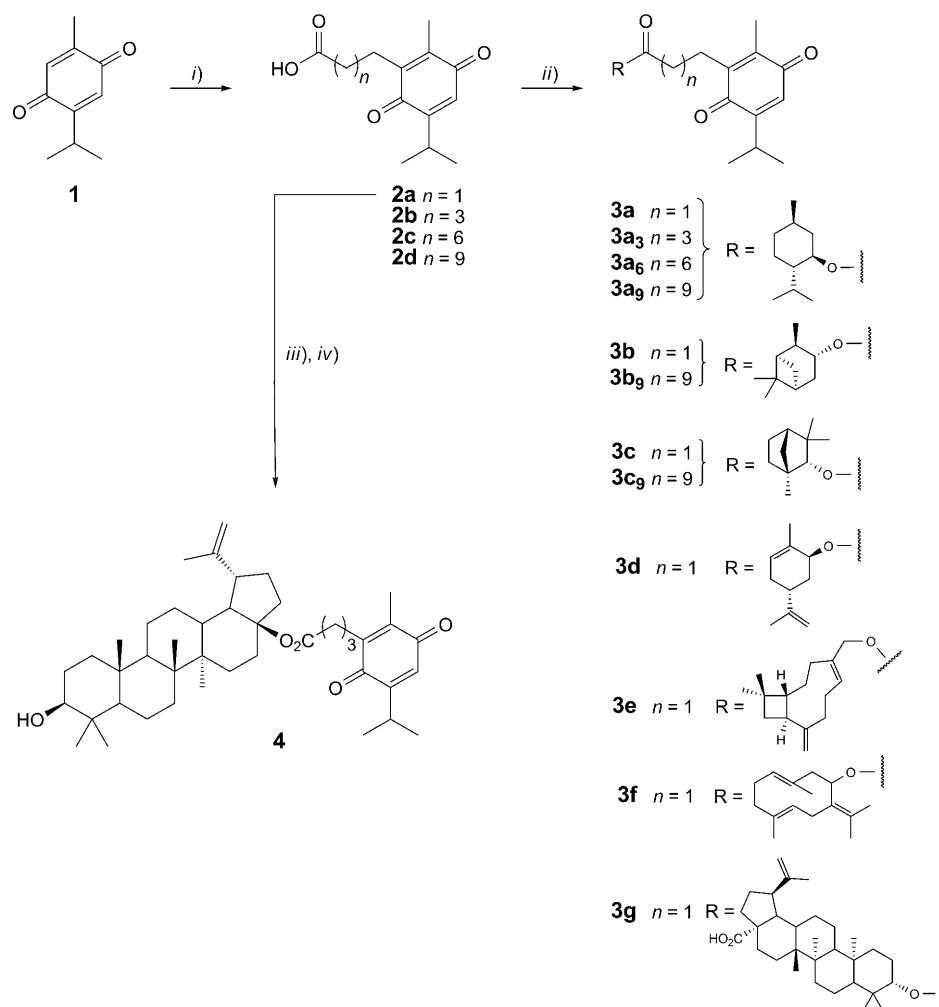
**Results and Discussion.** – *Chemistry.* 3-[2-Methyl-5-(1-methylethyl)-3,6-dioxocyclohexa-1,4-dienyl]alkanoic acids **2** were obtained by treating mixtures of the respective dicarboxylic acid and TQ (**1**) with a solution of  $\text{Ag}_2\text{S}_2\text{O}_8$  in  $\text{H}_2\text{O}/\text{MeCN}$  according to a general procedure [14] (*Scheme*). The acids **2** were then converted to the conjugates **3** by reaction with the respective terpene alcohol, pivalic anhydride, and 4-dimethylaminopyridine (DMAP) according to the general mixed-anhydride protocol [15]. In this way, esters **3** were obtained of 3-(2-methyl-5-(1-methylethyl)-3,6-dioxocyclohexa-1,4-dienyl)propanoic acid (**2a**) with (–)-menthol ( $\rightarrow$  **3a**), (–)-borneol ( $\rightarrow$  **3b**), (+)-fenchol ( $\rightarrow$  **3c**), (–)-carveol ( $\rightarrow$  **3d**), 14-hydroxycaryophyllene ( $\rightarrow$  **3e**), germacrol ( $\rightarrow$  **3f**), and betulinic acid ( $\rightarrow$  **3g**). 14-Hydroxycaryophyllene was obtained by oxidation of (–)-caryophyllene [16] with  $\text{SeO}_2$  by a known procedure [17]. Germacrol was prepared by  $\text{LiAlH}_4$  reduction of germacrone [18] which, in turn, was crystallized from Zdravets oil (*Geranium macrorrhizum*).

Additionally, conjugates of (–)-menthol with a  $\text{C}_6$  spacer ( $\rightarrow$  **3a<sub>3</sub>**) or a  $\text{C}_9$  spacer ( $\rightarrow$  **3a<sub>6</sub>**) were prepared from 5-[2-methyl-5-(1-methylethyl)-3,6-dioxocyclohexa-1,4-dienyl]pentanoic acid **2b** or 8-[2-methyl-5-(1-methylethyl)-3,6-dioxocyclohexa-1,4-dienyl]octanoic acid **2c**, respectively. Three conjugates with  $\text{C}_{12}$  spacers were prepared from 11-(2-methyl-5-(1-methylethyl)-3,6-dioxocyclohexa-1,4-dienyl)undecanoic acid **2d** and either (–)-menthol ( $\rightarrow$  **3a<sub>9</sub>**), or (–)-borneol ( $\rightarrow$  **3b<sub>9</sub>**), or (+)-fenchol ( $\rightarrow$  **3c<sub>9</sub>**).

Since esters and amides of betulinic acid were occasionally found to be more anticancer active than the free acid [19], we also synthesised conjugate **4** by esterification of betulinic acid with 3-(3-hydroxypropyl)-2-methyl-5-(1-methylethyl)-cyclohexa-2,5-diene-1,4-dione, which was available by reduction of **2a** with  $\text{NaBH}_4/\text{I}_2$  [20].

*Growth Inhibition.* The terpene conjugates **3** and **4** were tested for growth inhibition in cells of human HL-60 leukemia, 518A2 melanoma, P-gp-rich KB-V1/Vbl cervix carcinoma, BCRP-rich MCF-7/Topo breast carcinoma by means of the standard MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium hydrobromide) assay. They showed distinctly different activity profiles (*Fig.*). The most active derivatives **3** and **4** were also tested in non-malignant human foreskin fibroblasts (HF). The results were compared with those of the parent compound TQ (**1**). The  $IC_{50}$  values after 72 h exposure to the test compounds are summarized in *Table 1*. We checked that mixtures of conceivable ester hydrolysis products of all included compounds had at least a tenfold greater  $IC_{50}$  value.

In all tested cell lines, the monoterpene derivatives with longer side chains, *i.e.*, **3a<sub>6</sub>**, **3a<sub>9</sub>**, **3b<sub>9</sub>**, **3c<sub>9</sub>**, were much less active than the analogues with the same terpene appendage but shorter spacers, *i.e.*, **3a**, **3a<sub>3</sub>**, **3b**, **3c**, and even less active than TQ (**1**) itself. This is reminiscent of the structure–activity relationship we had found earlier for diamino-platinum(II) complex–terpene conjugates [21]. Derivative **3a** was the most active one on average in this series exhibiting single-digit micromolar  $IC_{50}$  values after 72 h

Scheme. Thymoquinone-Terpene Conjugates **3** and **4**

i) HO<sub>2</sub>C-(CH<sub>2</sub>)<sub>n</sub>-CO<sub>2</sub>H, AgNO<sub>3</sub>, (NH<sub>4</sub>)<sub>2</sub>S<sub>2</sub>O<sub>8</sub>, MeCN/H<sub>2</sub>O, 100°, 12 h. ii) Pivalic anhydride, 4-(dimethylamino)pyridine (DMAP), terpene alcohol, 50°, 12 h. iii) NaBH<sub>4</sub>, I<sub>2</sub>, THF, reflux, 12 h. iv) Pivalic anhydride, DMAP, betulinic acid, 50°, 12 h.

incubation in all four cancer cell lines. It was particularly efficacious in the melanoma cells ( $IC_{50} = 3.9 \pm 0.7 \mu\text{M}$ ) and in the resistant cervix carcinoma cells ( $IC_{50} = 7.0 \pm 1.7 \mu\text{M}$ ), thus surpassing the parent compound TQ (**1**) by factors of seven and four, respectively. Gratifyingly, in the control fibroblasts, compound **3a** exerted only half the toxicity of TQ. Its ratio of  $IC_{50}$  values in the HF vs. in the melanoma cells was 17.6, which is great enough to deliberate further pharmaceutical tests.

In cells of the multidrug-resistant MCF-7/Topo breast carcinoma, most derivatives with shorter spacers, *i.e.*, **3a**, **3a<sub>3</sub>**, **3b**, **3c**, **3e**, and **3f** were active at  $IC_{50}$  values better than

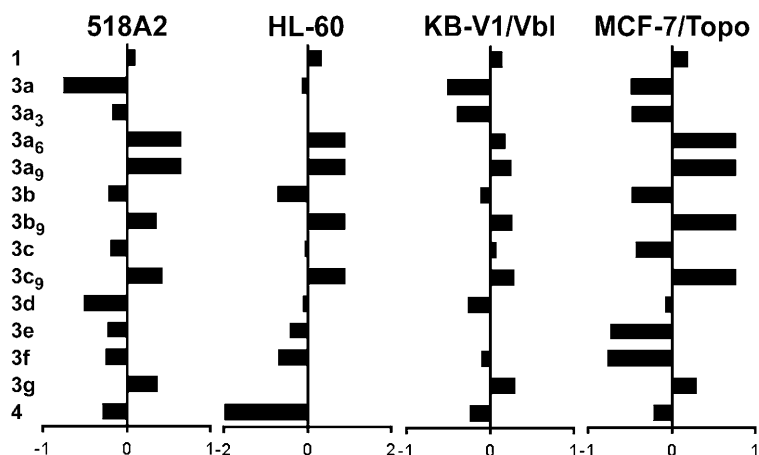


Figure. Cell-line specificities of thymoquinone (**1**), and its terpene conjugates **3** and **4** as deviation of the  $\log (IC_{50}/72 \text{ h})$  of individual derivatives from the mean over all compounds **1**, **3**, and **4**  $\log (IC_{50}/72 \text{ h})$  values for a given cell line. Negative values indicate higher, positive values lower than average activities. Mean  $\log (IC_{50}/72 \text{ h})$ :  $-4.7$  (518A2),  $-4.9$  (HL-60),  $-4.6$  (KB-V1/Vbl), and  $-4.8$  (MCF-7/Topo).

$6 \mu\text{M}$ . The conjugates **3b**, **3e**, and **3f** achieved this effect also in HL-60 leukemia cells. The betulinic acid conjugates differed significantly in their antiproliferative activity. The carboxylic acid **3g** showed much higher  $IC_{50}$  (72 h) values than the alcohol **4** in all cancer cell lines and was also less active than TQ (**1**) in all cell lines except in HL-60 leukemia. In contrast, conjugate **4** was highly efficacious, especially against HL-60 cells ( $IC_{50} = 130 \pm 20 \text{ nM}$ ) where it surpassed TQ (**1**) by a factor of *ca.* 200.

We also tested the most interesting terpene derivatives of TQ for their potential to overcome the multidrug resistance of KB-V1/Vbl and MCF-7/Topo cells. This was achieved by incubating these cells with the test compounds in the presence of selective inhibitors of their respective ABC-transporters, *i.e.*, with  $24 \mu\text{M}$  verapamil hydrochloride added to the P-gp overexpressing KB-V1/Vbl cervix carcinoma cells, and with  $1.2 \mu\text{M}$  fumitremorgin C added to the BCRP-rich MCF-7/Topo breast cancer cells [22]. Ideally, a drug should not be affected by ABC-transporters and thus should be characterized by a ratio  $R$  of its  $IC_{50}$  values with and without addition of specific inhibitors close to 1. This was the case for derivatives **3a** ( $R(\text{KB-V1/Vbl}) = 1.3$ ;  $R(\text{MCF-7/Topo}) = 1.1$ ) and **3b** ( $R(\text{KB-V1/Vbl}) = 0.8$ ;  $R(\text{MCF-7/Topo}) = 0.8$ ). While **3c**, **3e**, and **3f** were good substrates for the P-gp transporter, **3a** and **3b** were not pumped by P-gp. No derivative except **3g** was pumped by the BCRP transporter. This could explain the lower antiproliferative effect of **3g** when compared to **4**. Paradoxically, compound **4** was even less active in the presence of the inhibitors verapamil hydrochloride or fumitremorgin C. This effect could originate from a direct interaction between inhibitors and conjugate. Alternatively, it is known that such inhibitors do not only affect the efflux of anticancer drugs but also their uptake [23].

**Apoptosis Induction.** That compounds **3** and **4** act by inducing apoptosis in cancer cells was first visualized by distinct DNA fragmentation (laddering) in HL-60 cells upon treatment with TQ (**1**), **4**, or selected derivatives **3** at  $5 \mu\text{M}$ . As apoptosis

Table 1. Inhibitory Concentrations<sup>a)</sup> IC<sub>50</sub> [μM] of TQ (**1**), and Conjugates **3** and **4** When Applied for 72 h to 518A2 Melanoma, HL-60 Leukemia, KB-V1/Vbl Cervix Carcinoma, and MCF-7/Topo Breast-Carcinoma Cells, and Human Foreskin Fibroblasts (HF)

Compound/Cell	518A2	HL-60	KB-V1/Vbl	MCF-7/Topo	HF
TQ ( <b>1</b> )	28.3 (± 9.2)	27.8 (± 6.0)	32.3 (± 6.0)	26.7 (± 5.6)	32.6 (± 20.0)
TQ ( <b>1</b> ) <sup>b)</sup>	–	–	24.1 (± 3.5)	23.1 (± 2.5)	–
<b>3a</b>	3.9 (± 0.7)	9.0 (± 2.9)	7.0 (± 1.7)	5.4 (± 1.8)	68.6 (± 4.9)
<b>3a</b> <sup>b)</sup>	–	–	9.3 (± 4.8)	5.8 (± 3.8)	–
<b>3a</b> <sub>3</sub>	14.7 (± 1.8)	11.7 (± 2.3)	9.2 (± 2.5)	5.5 (± 1.7)	–
<b>3a</b> <sub>6</sub>	>100	>100	35.1 (± 10.1)	>100	–
<b>3a</b> <sub>9</sub>	>100	>100	41.3 (± 10.1)	>100	–
<b>3b</b>	13.3 (± 5.6)	2.3 (± 0.5)	17.6 (± 3.9)	5.6 (± 0.1)	41.5 (± 6.8)
<b>3b</b> <sup>b)</sup>	–	–	14.5 (± 3.7)	4.6 (± 2.5)	–
<b>3b</b> <sub>9</sub>	51.7 (± 4.8)	>100	42.4 (± 8.0)	>100	–
<b>3c</b>	14.1 (± 6.8)	10.8 (± 3.1)	27.7 (± 5.9)	6.1 (± 1.0)	59.0 (± 21.8)
<b>3c</b> <sup>b)</sup>	–	–	14.5 (± 3.3)	7.3 (± 2.7)	–
<b>3c</b> <sub>9</sub>	60.0 (± 4.2)	>100	44.7 (± 8.9)	>100	–
<b>3d</b>	6.8 (± 0.6)	9.4 (± 1.3)	12.4 (± 1.4)	14.0 (± 5.1)	–
<b>3e</b>	13.1 (± 2.3)	4.7 (± 1.1)	23.6 (± 1.6)	3.1 (± 0.6)	23.9 (± 0.9)
<b>3e</b> <sup>b)</sup>	–	–	10.0 (± 4.5)	3.5 (± 0.5)	–
<b>3f</b>	12.2 (± 1.5)	2.5 (± 1.7)	17.9 (± 1.1)	2.8 (± 1.8)	48.1 (± 14.1)
<b>3f</b> <sup>b)</sup>	–	–	9.5 (± 5.2)	4.5 (± 2.1)	–
<b>3g</b>	53.3 (± 3.2)	13.7 (± 9.5)	46.2 (± 3.0)	33.6 (± 1.2)	46.2 (± 5.5)
<b>3g</b> <sup>b)</sup>	–	–	79.9 (± 2.0)	13.6 (± 5.9)	–
<b>4</b>	11.4 (± 2.7)	0.13 (± 0.02)	13.1 (± 3.5)	10.0 (± 2.5)	24.5 (± 8.1)
<b>4</b> <sup>b)</sup>	–	–	68.0 (± 6.1)	44.2 (± 7.2)	–

<sup>a)</sup> Values are derived from concentration–response curves obtained by measuring the percentual absorbance of viable cells relative to untreated controls (100%) after 72 h exposure of 518A2 melanoma, HL-60 leukemia, KB-V1/Vbl cervix carcinoma, and MCF-7/Topo breast-carcinoma cells, as well as human foreskin fibroblasts (HF) to the test compounds in the MTT assay. Values represent means of four independent experiments ± standard deviation. <sup>b)</sup> With 24 μM verapamil hydrochloride added in the case of KB-V1/Vbl cells, or with 1.2 μM fumitremogin C added in the case of MCF-7/Topo cells.

proceeding *via* the intrinsic pathway is associated with a damage of the mitochondria, a release of cytochrome c, and an activation of caspase-9, we also analyzed the changes of the mitochondrial membrane potential  $\Delta\Psi_m$  of 518A2 and HL-60 cells upon treatment with the compounds **1**, **3**, and **4** at 5 μM for 24 and 72 h using a kit from *Stratagene*, which is based on the fluorescent cationic dye JC-1 [24]. The ratio of red (JC-1 aggregates in intact mitochondria) to green fluorescence (JC-1 monomers in the cytosol) is decreased in apoptotic cells. In HL-60 cells, we found the most pronounced reductions of cells with intact mitochondrial membranes upon treatment for 72 h with TQ (**1**) (83%), **3a** (78%), and **3b** (80%), relative to untreated vital control cells (100%). In contrast, in 518A2 cells, all tested compounds, *i.e.*, **3a**, **3a**<sub>3</sub>, **3a**<sub>6</sub>, **3a**<sub>9</sub>, **3d**, and **3e**, merely led to a small decrease of cells with intact mitochondria (90 ± 5%).

There is a good deal of evidence for reactive oxygen species (ROS) playing a pivotal role for the anticancer activity as well as for unwanted side effects of established drugs featuring the *p*-quinone motif, *e.g.*, doxorubicin [25]. Hence, we also assessed the ability of the compounds **1**, **3a**, **3b**, **3c**, **3e**, **3f**, **3g**, **3h**, and **4** at 5 μM concentrations to

initiate ROS generation in HL-60 and 518A2 cells by the colorimetric nitroblue-tetrazolium (NBT) assay [26][27]. It is based on the selective reduction of a yellow, water-soluble tetrazolium chloride to an insoluble violet diformazan by superoxide ( $O_2^{\cdot-}$ ). Conjugate **3a**, which had the greatest growth-inhibiting effect in 518A2 cells in the MTT assay, led, like all other test compounds, to similar ROS levels in these cells after 72 h as TQ (**1**). In HL-60 leukemia cells, the pattern of ROS generation was more heterogenous. Two compounds that were quite growth-inhibitory in HL-60 cells in the MTT assays also led to considerable ROS levels. Compound **4** initiated ROS levels six times that caused by TQ, and the caryophyllene derivative **3e** effected three times more ROS than TQ. So, there was some coherence between growth inhibition and ROS initiation in case of the HL-60 cells.

**Conclusions.** – We prepared derivatives of the weak anticancer drug thymoquinone (**1**) with covalently attached terpene residues. Some of these were far more efficacious in certain cancer cell lines than the parent drug, while being considerably less toxic to non-malignant fibroblasts. Generally, their efficacy in cancer cells was dependent both on the nature of the terpene, and the length of the alkyl spacer between *p*-quinone and terpene moieties. Most active were derivatives with short  $C_3$  spacers. The corresponding menthol conjugate **3a** exhibited single-digit micromolar  $IC_{50}$  (72 h) values in all four tested cancer cell lines, and showed the greatest activity of all compounds in cells of 518A2 melanoma and multidrug-resistant KB-V1/Vbl cervix carcinoma, while being virtually nontoxic to non-malignant human fibroblasts. It was also least affected by the resistance-relevant ABC-transporters P-gp and BCRP. The caryophyllene and germacrone conjugates **3e** and **3f**, respectively, achieved the best results of all test compounds against multidrug-resistant MCF-7 breast carcinoma cells. The betulinic acid derivatives **3g** and **4** which differed in the mode of connection also were of quite different antiproliferative activities. Conjugate **4** connected *via* its acid group was *ca.* 200 times more efficacious in HL-60 cells than the parent drug TQ ( $IC_{50} = 130 \pm 20$  nM). This finding is in line with recent reports by other groups on bioactive C(17)-esters of betulinic acid [28][29]. Further investigations such as assessment of caspase kinetics and involvement of pro-/anti-apoptotic proteins are now underway to identify the precise mechanism of apoptosis induced by conjugates of type **3**.

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### Experimental Part

*General.* All reagent-grade chemicals were purchased from *Aldrich*, *Merck*, *Acros*, *Alfa Aesar*, and *ABCR*. All solvents were dried and distilled before use. Germacrone was crystallized from Zdravets oil according to the known procedure [30][31]. Column chromatography (CC):  $SiO_2$  60 (230–400 mesh). Optical rotations: *Perkin-Elmer* polarimeter 241 at 589 nm. IR Spectra: *Perkin-Elmer Spectrum One* FT-IR spectrophotometer equipped with an ATR sampling unit. NMR Spectra: *Bruker Avance 300* spectrometer; chemical shifts ( $\delta$ ) are given in ppm downfield from  $Me_4Si$  as internal standard for  $^1H$  and  $^{13}C$ . EI-MS: *Varian MAT 311A*. Microanalyses: *Perkin-Elmer 2400* CHN elemental analyzer; correct analyses (within  $\pm 0.4\%$  for C, H, N) were obtained for all new compounds **2**, **3**, and **4**.

*Chemistry*<sup>1)</sup>. *Synthesis and Characterization of the Thymoquinone–Terpene Conjugates 3* (see Table 2). *Synthesis of Acids 2: Typical Procedure.* 3-(2-Methyl-5-(1-methylethyl)-3,6-dioxocyclohexa-1,4-dienyl)propanoic acid (**2a**). A mixture of TQ (2-methyl-5-(1-methylethyl)cyclohexa-2,5-diene-1,4-dione; **1**; 500 mg, 3.05 mmol), succinic acid (288 mg, 2.44 mmol), a cat. amount of AgNO<sub>3</sub> and MeCN/H<sub>2</sub>O, 1 : 1 (30 ml) was heated to 100°. A soln. of (NH<sub>4</sub>)<sub>2</sub>S<sub>2</sub>O<sub>8</sub> (695 mg, 3.05 mmol) in H<sub>2</sub>O (3.05 ml) was added dropwise, and the resulting mixture was heated under reflux overnight, then cooled to r.t., diluted with H<sub>2</sub>O, and extracted repeatedly with Et<sub>2</sub>O. The combined Et<sub>2</sub>O extracts were washed with a sat. aq. soln. of NaHCO<sub>3</sub>, acidified with conc. aq. HCl, extracted with CH<sub>2</sub>Cl<sub>2</sub>, and dried (Na<sub>2</sub>SO<sub>4</sub>). The volatiles were evaporated, and the residue was purified by CC (silica gel; AcOEt/cyclohexane 1 : 1) to afford pure **2a** (302 mg, 52%). Yellow oil. *R*<sub>f</sub> (AcOEt/cyclohexane 1 : 1) 0.39. IR (ATR): 2965, 1707, 1643, 1417, 1381, 1248, 1177, 1039, 892, 816, 709. <sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>): 10.8–9.8 (br., 1 H); 6.42 (*d*, *J* = 1.1, 1 H); 2.95 (*dsept.*, *J* = 6.9, *J* = 1.1, 1 H); 2.75 (*t*, *J* = 7.9, 2 H); 2.44 (*t*, *J* = 7.9, 2 H); 1.97 (*s*, 3 H); 1.03 (*d*, *J* = 6.9, 6 H). <sup>13</sup>C-NMR (75.5 MHz, CDCl<sub>3</sub>): 187.9; 186.5; 178.2; 154.6; 142.6; 141.1; 129.9; 32.4; 29.0; 26.6; 21.9; 21.2. EI-MS: 236 (4, *M*<sup>+</sup>), 155 (7), 138 (100), 101 (47), 95 (100), 55 (34), 41 (26).

*Synthesis of Thymoquinone–Terpene Esters 3. Typical Procedure.* (–)-Menthyl 3-(2-Methyl-5-(1-methylethyl)-3,6-dioxocyclohexa-1,4-dienyl)propanoate (**3a**). Acid **2a** (130 mg, 0.55 mmol), (–)-menthol (77 mg, 0.49 mmol), pivalic anhydride (0.11 ml, 102 mg, 0.55 mmol), and a cat. amount of DMAP were dissolved in CHCl<sub>3</sub> (30 ml), and the resulting mixture was heated at 50° overnight. H<sub>2</sub>O was added, and the solvent was evaporated. The residue was dissolved in Et<sub>2</sub>O, the resulting mixture was washed with a sat. aq. NaHCO<sub>3</sub> and brine, dried (Na<sub>2</sub>SO<sub>4</sub>), and concentrated in vacuum. The residue thus obtained was purified by CC (silica gel; AcOEt/cyclohexane 1 : 10) to afford pure **3a** (110 mg, 60%). Yellow oil. *R*<sub>f</sub> (AcOEt/cyclohexane 1 : 1) 0.83. [*α*]<sub>D</sub><sup>24</sup> = –39.9 (*c* = 0.5, CHCl<sub>3</sub>). IR (ATR): 2956, 2928, 2871, 1729, 1702, 1647, 1456, 1369, 1248, 1173, 1149, 982. <sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>): 6.43 (*d*, *J* = 1.2, 1 H); 4.62 (*ddd*, *J* = 4.5, *J* = 10.8, *J* = 11.8, 1 H); 2.99 (*dsept.*, *J* = 6.9, *J* = 1.2, 1 H); 2.38 (*m*, 4 H); 2.00 (*s*, 3 H); 1.9–1.2 (*m*, 9 H); 1.06 (*d*, *J* = 6.9, 6 H); 0.82 (*dd*, *J* = 7.0, *J* = 2.5, 6 H); 0.66 (*d*, *J* = 6.9, 3 H). <sup>13</sup>C-NMR (75.5 MHz, CDCl<sub>3</sub>): 188.0; 186.7; 171.9; 154.6; 143.1; 141.1; 130.0; 74.4; 46.9; 34.2; 33.8; 33.1; 31.3; 26.3; 23.5; 22.6; 21.9; 21.4; 20.7; 16.3. EI-MS: 374 (3, *M*<sup>+</sup>), 238 (100), 236 (46), 220 (59), 175 (19), 138 (100), 83 (100), 57 (44).

9-[3-[2-Methyl-5-(1-methylethyl)-3,6-dioxocyclohexa-1,4-dienyl]propanoyloxy]-5b,8,8,11a,11b-pentamethyl-1-(prop-1-en-2-yl)icosahydro-1H-cyclopenta[*a*]chrysene-3a-carboxylic Acid (**3g**). Acid **2a** (30 mg, 0.12 mmol), pivalic anhydride (0.02 ml, 22 mg, 0.12 mmol), and a cat. amount of DMAP were dissolved in CHCl<sub>3</sub> (25 ml), and the resulting mixture was stirred at 50° for 2 h. Betulinic acid (50 mg, 0.11 mmol) was added, and stirring at 50° was continued overnight. H<sub>2</sub>O was added, and the volatiles were evaporated. The remainder was dissolved in Et<sub>2</sub>O, the resulting mixture was washed with sat. aq. NaHCO<sub>3</sub> and brine, dried (Na<sub>2</sub>SO<sub>4</sub>), and concentrated in vacuum. The residue was purified by CC (silica gel; AcOEt/cyclohexane 1 : 10) to afford pure **3g** (29 mg, 36%). Yellow oil. *R*<sub>f</sub> (AcOEt/cyclohexane 1 : 1) 0.77. [*α*]<sub>D</sub><sup>24</sup> = +8.7 (*c* = 0.5, CHCl<sub>3</sub>). IR (ATR): 3407, 2962, 2929, 2855, 1719, 1649, 1455, 1379, 1285, 1262, 1160, 1107, 1057, 951, 739. <sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>): 9.64 (br., 1 H); 6.46 (*d*, *J* = 1.1, 1 H); 4.71 (*s*, 1 H); 4.49 (*s*, 1 H); 4.43 (*m*, 1 H); 3.01 (*dsept.*, *J* = 6.9, *J* = 1.1, 1 H); 2.81 (br., 1 H); 2.79 (*t*, *J* = 8.5, 2 H); 2.41 (*t*, *J* = 8.5, 2 H); 2.0–1.1 (*m*, 22 H); 1.21 (*s*, 3 H); 1.08 (*d*, *J* = 6.9, 6 H); 0.94 (*m*, 6 H); 0.81 (*m*, 3 H); 0.77 (*m*, 3 H). <sup>13</sup>C-NMR (75.5 MHz, CDCl<sub>3</sub>): 188.1; 186.7; 181.3; 172.3; 154.7; 149.9; 143.2; 141.1; 130.1; 109.9; 81.4; 56.3; 55.4; 50.3; 49.2; 46.5; 42.4; 40.7; 38.4; 37.9; 37.8; 37.0; 34.2; 33.9; 33.2; 31.7; 30.2; 29.7; 26.9; 26.5; 23.7; 22.6; 21.4; 20.9; 19.3; 18.2; 16.5; 16.2; 15.9; 14.6. EI-MS: 674 (2, *M*<sup>+</sup>), 628 (3), 439 (27), 395 (11), 274 (19), 238 (100), 189 (63), 121 (26), 57 (48).

*Synthesis of 3-(3-Hydroxypropyl)-2-methyl-5-(1-methylethyl)-cyclohexa-2,5-diene-1,4-dione.* A soln. of I<sub>2</sub> (107 mg, 0.85 mmol) in THF (3 ml) was added dropwise during 30 min to a soln. of acid **2a** (200 mg, 0.85 mmol) and NaBH<sub>4</sub> (77 mg, 2.04 mmol) in THF (20 ml). After gas evolution had ceased, the mixture was heated under reflux overnight, then cooled to r.t., and the reaction was quenched with MeOH. The volatiles were evaporated, and the remainder was taken up in aq. KOH (20%) and repeatedly extracted with Et<sub>2</sub>O. The combined org. layers were washed with brine, dried (Na<sub>2</sub>SO<sub>4</sub>), and concentrated in

<sup>1)</sup> Synthetic details and characterizations of all new compounds are available, free of charge, from the authors.

Table 2. NMR Data (in CDCl<sub>3</sub>) and Optical Rotations (c=0.5, CHCl<sub>3</sub>) of All New Thymoquinone-Terpene Conjugates **3** and Thymoquinone Mono Acids **2**. Chemical shifts (δ) are given in ppm downfield from Me<sub>4</sub>Si as internal standard for <sup>1</sup>H (300 MHz) and <sup>13</sup>C (75.5 MHz).

	δ(H)	δ(C)	[α] <sub>D</sub> <sup>25</sup>
<b>2b</b>	6.46 ( <i>d</i> , <i>J</i> =1.1, 1 H); 3.01 ( <i>dsept.</i> , <i>J</i> =6.9, <i>J</i> =1.1, 1 H); 2.49 ( <i>t</i> , <i>J</i> =7.9, 2 H); 2.37 ( <i>t</i> , <i>J</i> =7.5, 2 H); 1.99 ( <i>s</i> , 3 H); 1.8–1.4 ( <i>m</i> , 4 H); 1.08 ( <i>d</i> , <i>J</i> =6.9, 6 H)	188.3; 186.9; 179.2; 154.6; 144.6; 134.2; 129.9; 33.6; 26.7; 26.3; 25.6; 24.7; 21.4; 11.7	–
<b>2c</b>	6.45 ( <i>d</i> , <i>J</i> =1.2, 1 H); 3.01 ( <i>dsept.</i> , <sup>3</sup> <i>J</i> =6.9, <i>J</i> =1.2, 1 H); 2.44 ( <i>t</i> , <i>J</i> =7.8, 2 H); 2.32 ( <i>t</i> , <i>J</i> =7.4, 2 H); 1.98 ( <i>s</i> , 3 H); 1.7–1.2 ( <i>m</i> , 10 H); 1.08 ( <i>d</i> , <i>J</i> =6.9, 6 H)	188.2; 186.0; 179.8; 154.5; 145.6; 136.9; 129.9; 33.9; 29.6; 28.9; 28.7; 28.5; 26.6; 26.1; 24.5; 21.1; 13.4	–
<b>2d</b>	6.44 ( <i>d</i> , <i>J</i> =1.1, 1 H); 3.01 ( <i>dsept.</i> , <i>J</i> =6.9, <i>J</i> =1.1, 1 H); 2.30 ( <i>t</i> , <i>J</i> =7.4, 2 H); 2.30 ( <i>t</i> , <i>J</i> =7.6, 2 H); 1.95 ( <i>s</i> , 3 H); 1.3–1.1 ( <i>br.</i> , 16 H); 1.07 ( <i>d</i> , <i>J</i> =6.9, 6 H)	188.2; 187.6; 180.1; 154.7; 145.3; 139.3; 130.0; 34.1; 29.9; 29.8; 29.3; 29.2; 29.0; 28.8; 26.7 24.6; 21.2; 12.0	–
<b>3a<sub>3</sub></b>	6.45 ( <i>d</i> , <i>J</i> =1.1, 1 H); 4.64 ( <i>ddd</i> , <i>J</i> =4.4, <i>J</i> =10.9, <i>J</i> =11.9, 1 H); 3.01 ( <i>dsept.</i> , <i>J</i> =6.9, <i>J</i> =1.1, 1 H); 2.48 ( <i>t</i> , <i>J</i> =7.5, 2 H); 2.29 ( <i>t</i> , <i>J</i> =7.5, 2 H); 1.98 ( <i>s</i> , 3 H); 1.9–1.3 ( <i>m</i> , 13 H); 1.08 ( <i>d</i> , <i>J</i> =6.9, 6 H); 0.86 ( <i>dd</i> , <i>J</i> =7.0, <i>J</i> =2.8, 6 H); 0.72 ( <i>d</i> , <i>J</i> =6.9, 3 H)	188.3; 186.8; 172.9; 154.5; 144.7; 140.1; 129.9; 74.0; 46.9; 34.4; 34.3; 31.3; 29.1; 28.1; 26.4; 26.3; 25.2; 23.4; 21.9; 21.4; 20.7; 16.3	– 35.8
<b>3a<sub>6</sub></b>	6.44 ( <i>d</i> , <i>J</i> =1.2, 1 H); 4.64 ( <i>ddd</i> , <i>J</i> =4.3, <i>J</i> =10.9, <i>J</i> =11.5, 1 H); 2.99 ( <i>dsept.</i> , <i>J</i> =6.9, <i>J</i> =1.2, 1 H); 2.24 ( <i>t</i> , <i>J</i> =7.7, 2 H); 1.95 ( <i>t</i> , <i>J</i> =7.7, 2 H); 1.94 ( <i>s</i> , 3 H); 1.08 ( <i>d</i> , <i>J</i> =6.9, 6 H); 1.9–1.0 ( <i>m</i> , 19 H); 0.87 ( <i>dd</i> , <i>J</i> =6.5, <i>J</i> =2.4, 6 H); 0.72 ( <i>d</i> , <i>J</i> =6.9, 3 H)	187.9; 186.8; 172.8; 154.5; 144.6; 140.0; 129.7; 73.9; 46.9; 40.9; 34.7; 34.3; 31.3; 29.0; 28.9; 28.8; 26.6; 26.5; 26.2; 25.0; 23.4; 21.9; 20.7; 16.3; 13.0	– 41.3
<b>3a<sub>9</sub></b>	6.43 ( <i>d</i> , <i>J</i> =1.2, 1 H); 4.64 ( <i>ddd</i> , <i>J</i> =4.3, <i>J</i> =10.9, <i>J</i> =11.5, 1 H); 3.02 ( <i>dsept.</i> , <i>J</i> =6.9, <i>J</i> =1.2, 1 H); 2.25 ( <i>t</i> , <i>J</i> =7.4, 2 H); 2.24 ( <i>t</i> , <i>J</i> =7.7, 2 H); 1.98 ( <i>s</i> , 3 H); 1.9–1.2 ( <i>m</i> , 25 H); 1.08 ( <i>d</i> , <i>J</i> =6.9, 6 H); 0.86 ( <i>d</i> , <i>J</i> =6.5, 6 H)	188.3; 187.0; 173.4; 152.2; 143.8; 139.2; 129.9; 73.8; 47.0; 40.9; 34.7; 34.3; 31.4; 29.4; 29.3; 29.2; 29.1; 27.1; 26.7; 26.5; 26.2; 23.4; 23.3; 21.9; 20.7; 16.3; 16.1; 14.2	– 23.1
<b>3b</b>	6.47 ( <i>d</i> , <i>J</i> =1.2, 1 H); 4.84 ( <i>m</i> , 1 H); 3.03 ( <i>dsept.</i> , <i>J</i> =6.9, <i>J</i> =1.2, 1 H); 2.81 ( <i>t</i> , <i>J</i> =7.3, 2 H); 2.45 ( <i>t</i> , <i>J</i> =7.3, 2 H); 2.04 ( <i>s</i> , 3 H); 1.4–1.2 ( <i>m</i> , 7 H); 1.09 ( <i>d</i> , <i>J</i> =6.9, 6 H); 0.87 ( <i>s</i> , 3 H); 0.84 ( <i>s</i> , 3 H); 0.78 ( <i>s</i> , 3 H)	188.1; 186.7; 172.9; 154.7; 143.2; 141.1; 130.2; 80.2; 48.7; 47.8; 44.8; 36.8; 33.1; 28.0; 27.1; 26.7; 22.6; 21.4; 19.7; 18.8; 13.5	– 11.2
<b>3b<sub>9</sub></b>	6.44 ( <i>d</i> , <i>J</i> =1.1, 1 H); 4.83 ( <i>tq</i> , <i>J</i> =9.9, <i>J</i> =2.1, 1 H); 3.02 ( <i>dsept.</i> , <i>J</i> =6.9, <i>J</i> =1.1, 1 H); 2.25 ( <i>t</i> , <i>J</i> =7.6, 2 H); 2.5–2.3 ( <i>m</i> , 3 H); 1.98 ( <i>s</i> , 3 H); 2.0–1.2 ( <i>m</i> , 20 H); 1.08 ( <i>d</i> , <i>J</i> =6.9, 6 H); 0.87 ( <i>s</i> , 3 H); 0.79 ( <i>s</i> , 6 H)	188.2; 187.4; 173.9; 154.5; 143.8; 139.5; 130.0; 78.9; 50.5; 48.7; 44.9; 34.4; 30.0; 29.8; 29.7; 29.3; 29.1; 28.7; 28.1; 26.7; 26.6; 26.5; 24.9; 21.4; 21.2; 14.3; 13.5	– 4.4



Table 2 (cont.)

	$\delta(\text{H})$	$\delta(\text{C})$	$[\alpha]_{\text{D}}^{25}$
<b>3c</b>	6.47 ( <i>d</i> , $J=1.2$ , 1 H); 4.34 ( <i>d</i> , $J=1.9$ , 1 H); 3.03 ( <i>dsept.</i> , $J=6.9$ , $J=1.2$ , 1 H); 2.81 ( <i>t</i> , $J=7.3$ , 2 H); 2.46 ( <i>t</i> , $J=7.3$ , 2 H); 2.04 ( <i>s</i> , 3 H); 1.09 ( <i>d</i> , $J=6.9$ , 6 H); 1.7–1.0 ( <i>m</i> , 7 H); 1.07 ( <i>s</i> , 3 H); 0.99 ( <i>s</i> , 3 H); 0.73 ( <i>s</i> , 3 H)	188.1; 186.7; 172.8; 154.7; 143.2; 134.5; 130.1; 86.5; 48.3; 41.3; 39.4; 33.7; 32.8; 29.7; 26.6; 22.6; 20.1; 19.4; 15.6; 15.2; 11.8	13.5
<b>3c<sub>9</sub></b>	6.45 ( <i>d</i> , $J=1.2$ , 1 H); 4.33 ( <i>br</i> , 1 H); 3.02 ( <i>dsept.</i> , $J=6.9$ , $J=1.2$ , 1 H); 2.44 ( <i>m</i> , 2 H); 2.26 ( <i>t</i> , $J=7.7$ , 2 H); 1.99 ( <i>s</i> , 3 H); 1.8–1.2 ( <i>m</i> , 23 H); 1.24 ( <i>s</i> , 3 H); 1.23 ( <i>s</i> , 6 H); 1.08 ( <i>d</i> , $J=6.9$ , 6 H)	188.2; 187.4; 173.9; 154.5; 143.8; 139.5; 130.0; 91.6; 47.9; 43.1; 35.2; 34.4; 30.0; 29.9; 29.8; 29.5; 29.4; 29.3; 29.2; 29.1; 27.1; 25.7; 25.0; 22.9; 17.1; 14.3	1.5
<b>3d</b>	6.43 ( <i>d</i> , $J=1.2$ , 1 H); 5.54 ( <i>m</i> , 1 H); 4.68 ( <i>m</i> , 1 H); 4.67 ( <i>m</i> , 1 H); 4.64 ( <i>m</i> , 1 H); 2.98 ( <i>dsept.</i> , $J=6.8$ , $J=1.2$ , 1 H); 2.78 ( <i>t</i> , $J=7.8$ , 2 H); 2.43 ( <i>t</i> , $J=7.8$ , 2 H); 2.00 ( <i>s</i> , 3 H); 1.66 ( <i>br</i> , 3 H); 1.58 ( <i>m</i> , 3 H); 2.4–1.4 ( <i>m</i> , 5 H); 1.05 ( <i>d</i> , $J=6.8$ , 6 H)	187.9; 186.6; 172.1; 154.5; 143.0; 140.9; 132.6; 130.7; 129.9; 125.9; 109.2; 70.9; 35.7; 33.5; 33.0; 32.9; 26.4; 22.5; 21.3; 20.7; 20.3; 18.7	–46.1
<b>3e</b>	6.47 ( <i>d</i> , $J=1.2$ , 1 H); 5.54 ( <i>t</i> , $J=8.1$ , 1 H); 4.81 ( <i>d</i> , $J=1.2$ , 1 H); 4.73 ( <i>d</i> , $J=1.5$ , 1 H); 4.46 ( <i>br.</i> , 1 H); 3.02 ( <i>dsept.</i> , $J=6.9$ , $J=1.2$ , 1 H); 2.81 ( <i>t</i> , $J=7.9$ , 2 H); 2.45 ( <i>t</i> , $J=7.9$ , 2 H); 2.03 ( <i>s</i> , 3 H); 2.3–1.4 ( <i>m</i> , 10 H); 1.09 ( <i>d</i> , $J=6.9$ , 6 H); 0.98 ( <i>s</i> , 3 H); 0.95 ( <i>s</i> , 3 H)	187.9; 186.7; 172.3; 155.2; 154.7; 143.0; 141.1; 137.9; 130.6; 129.0; 110.8; 68.7; 51.6; 40.3; 40.1; 34.7; 33.2; 32.9; 29.9; 29.2; 27.4; 26.7; 26.2; 25.2; 22.9; 21.4; 11.8	–8.0
<b>3f</b>	6.47 ( <i>d</i> , $J=1.1$ , 1 H); 4.71 ( <i>m</i> , 2 H); 4.47 ( <i>m</i> , 1 H); 3.02 ( <i>dsept.</i> , $^3J=6.9$ , $J=1.1$ , 1 H); 2.79 ( <i>br.</i> , 4 H); 2.61 ( <i>br.</i> , 2 H); 2.40 ( <i>br.</i> , 2 H); 2.02 ( <i>s</i> , 3 H); 2.0–1.4 ( <i>m</i> , 4 H); 1.40 ( <i>s</i> , 6 H); 1.36 ( <i>br.</i> , 3 H); 1.26 ( <i>s</i> , 3 H); 1.09 ( <i>d</i> , $J=6.9$ , 6 H)	188.1; 186.7; 171.6; 154.7; 143.1; 137.8; 134.3; 133.1; 130.6; 130.4; 130.1; 127.0; 68.1; 47.0; 38.7; 33.7; 32.9; 26.7; 22.6; 21.4; 21.3; 21.1; 19.1; 15.6; 11.8	8.7

vacuum. The residue was purified by CC (silica gel; AcOEt/cyclohexane 1:1) to afford pure dione (110 mg, 58%). Yellow oil.  $R_f$  (AcOEt/cyclohexane 1:1) 0.44. IR (ATR): 3384, 2963, 2874, 1644, 1612, 1456, 1384, 1307, 1248, 1191, 1147, 1058, 945, 892, 708.  $^1\text{H-NMR}$  (300 MHz,  $\text{CDCl}_3$ ): 6.43 (*d*,  $J=1.2$ , 1 H); 3.55 (*t*,  $J=6.0$ , 2 H); 2.98 (*dsept.*,  $J=6.9$ ,  $J=1.2$ , 1 H); 2.54 (*t*,  $J=7.3$ , 2 H); 1.97 (*s*, 3 H); 1.63 (*tt*,  $J=6.0$ ,  $J=7.3$ , 2 H); 1.05 (*d*,  $J=6.9$ , 6 H).  $^{13}\text{C-NMR}$  (75.5 MHz,  $\text{CDCl}_3$ ): 188.1; 187.4; 154.5; 144.5; 140.6; 130.1; 61.6; 31.2; 29.2; 26.7; 22.7; 21.3. EI-MS: 222 (100,  $M^+$ ), 204 (33), 189 (74), 179 (80), 161 (84), 151 (65), 135 (42), 121 (18), 105 (36), 91 (39).

**Synthesis of 3-[2-Methyl-5-(1-methylethyl)-3,6-dioxocyclohexa-1,4-dienyl]propyl Betulate (4).** A mixture of betulinic acid (50 mg, 0.11 mmol), pivalic anhydride (0.05 ml, 42 mg, 0.23 mmol), a cat. amount of DMAP and  $\text{CHCl}_3$  (30 ml) was stirred at 50° for 2 h. A soln. of 3-(3-hydroxypropyl)-2-methyl-5-(1-methylethyl)-cyclohexa-2,5-diene-1,4-dione (100 mg, 0.45 mmol) in  $\text{CHCl}_3$  (10 ml) was slowly added, and stirring at 50° was continued overnight.  $\text{H}_2\text{O}$  was added and the solvent was evaporated. The residue was dissolved in  $\text{Et}_2\text{O}$  and washed with sat. aq.  $\text{NaHCO}_3$  and brine, dried ( $\text{Na}_2\text{SO}_4$ ), and concentrated in vacuum. The residue was purified by CC (silica gel; AcOEt/cyclohexane 1:4) to afford pure **4** (27 mg, 39%). Yellow oil.  $R_f$  (AcOEt/cyclohexane 1:1) 0.47.  $[\alpha]_{\text{D}}^{25} = +2.1$  ( $c=0.5$ ,  $\text{CHCl}_3$ ). IR (ATR): 3376, 2960, 2925, 2854, 1710, 1646, 1456, 1378, 1284, 1259, 1161, 1107, 1057, 952, 738.  $^1\text{H-NMR}$  (300 MHz,  $\text{CDCl}_3$ ): 6.44 (*d*,  $J=1.6$ , 1 H); 6.39 (*br.*, 1 H); 4.72 (*br.*, 1 H); 4.59 (*br.*, 1 H); 4.06 (*t*,  $J=6.2$ , 2 H); 3.3–3.1 (*m*, 1 H); 3.00 (*dsept.*,  $J=7.0$ ,  $J=1.6$ , 1 H); 2.9–2.7 (*m*, 1 H); 2.59 (*t*,  $J=6.9$  Hz, 2 H); 1.98

(s, 3 H); 1.67 (br., 5 H); 1.15 (*d*,  $J = 7.0$ , 6 H); 2.3–1.0 (*m*, 24 H); 0.95 (s, 3 H); 0.94 (s, 3 H); 0.91 (s, 3 H); 0.79 (s, 3 H); 0.73 (s, 3 H).  $^{13}\text{C-NMR}$  (75.5 MHz,  $\text{CDCl}_3$ ): 188.0; 187.5; 174.1; 154.5; 144.6; 140.5; 130.0; 109.7; 78.9; 65.9; 56.3; 55.3; 50.5; 49.2; 46.9; 42.4; 40.7; 38.8; 38.4; 37.2; 37.0; 34.3; 32.1; 30.5; 29.7; 27.9; 27.4; 25.5; 24.8; 21.8; 20.8; 19.4; 18.3; 16.1; 16.0; 15.3; 14.7. EI-MS: 660 (2,  $M^+$ ), 410 (13), 395 (5), 233 (17), 189 (28), 135 (18), 69 (31), 57 (100).

**Cell Lines and Culture Conditions.** The human HL-60 leukemia cells were obtained from the German National Resource Center for Biological Material (DSMZ), Braunschweig, and incubated in RPMI (Roswell Park Memorial Institute) Medium 1640 with 10% FCS (fetal calf serum), 100 IU/ml penicillin G, 100  $\mu\text{g/ml}$  streptomycin sulfate, 0.25  $\mu\text{g/ml}$  amphotericin B, and 250  $\mu\text{g/ml}$  gentamycin (all from *Gibco*, D-Eggenstein). The human 518A2 melanoma cells were a gift from the Department of Oncology and Hematology of the Martin-Luther-University Halle-Wittenberg, Germany, the human KB-V1/Vbl cervix carcinoma and the human MCF-7/Topo breast adenocarcinoma cells were obtained from the Institute of Pharmacy of the University Regensburg, Germany, and the human foreskin (HF) fibroblasts from the University Hospital Erlangen, Germany. The 518A2, the KB-V1/Vbl, and the HF cells were cultured in DMEM (*Dulbecco's* modified eagle medium; *Gibco*) containing the same additions as the RPMI medium above. The MCF-7/Topo cells were grown in *Eagle's* minimum essential medium (E-MEM, *Sigma*) supplemented with 2.2 g/l  $\text{NaHCO}_3$ , 110 mg/l sodium pyruvate, and 5% FCS. The cells were maintained in a moisture-saturated atmosphere (5%  $\text{CO}_2$ ) at 37°.

**Inhibition of Cell Growth and Metabolic Activity (MTT Assay).** MTT (= 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium hydrobromide; *ABCR*) was used to identify the metabolic activity of cells, which are capable of reducing it by their mitochondrial dehydrogenases to a violet formazan product. HL-60 Leukemia cells ( $5 \times 10^5/\text{ml}$ ), and cells ( $5 \times 10^4/\text{ml}$ ) of 518A2 melanoma, KB-V1/Vbl cervix carcinoma, MCF-7/Topo breast carcinoma, and foreskin fibroblasts (HF) were seeded, and cultured for 24 h in 96-well microplates [32]. Inhibitors of P-gp or BCRP were optionally added. Incubation (5%  $\text{CO}_2$ , 95% humidity, 37°) of cells following treatment with the test compounds was continued for 72 h. Blank and solvent controls were treated identically. Then, a 5 mg/ml stock soln. of MTT in phosphate-buffered saline (PBS) was added to a final MTT concentration of 0.05% (HL-60, 518A2, and HF) or 0.1% (KB-V1/Vbl, and MCF-7/Topo). After 2 h, the precipitate of formazan crystals was redissolved in a 10% soln. of sodium dodecylsulfate (SDS) in DMSO containing 0.6% AcOH in the case of the HL-60 cells. For the adherent 518A2, KB-V1/Vbl, MCF-7/Topo, and HF cells, the microplates were swiftly turned, flicked, and blotted to discard the medium before adding the solvent mixture. The microplates were gently shaken in the dark for 30 min, and absorbance at 570 and 630 nm (background) was measured with an ELISA plate reader. All experiments were carried out in quadruplicate, and the percentage of viable cells was calculated as the mean  $\pm$  SD relative to the controls (100%).

**Mitochondrial Membrane Potential.** Changes in mitochondrial membrane potentials 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 400g for 5 min. The pellets were resuspended in 500  $\mu\text{l}$  of diluted JC-1 soln. ( $0.1 \times$ ), incubated at 37° for 15 min (HL-60) or 35 min (518A2), and then centrifuged again for 5 min at 400g. After washing, the pellets were resuspended in 100  $\mu\text{l}$  of PBS and transferred into the wells of a black 96-well plate. The red ( $\lambda_{\text{ex}} = 585 \text{ nm}$ ,  $\lambda_{\text{em}} = 590 \text{ nm}$ ) and green ( $\lambda_{\text{ex}} = 510 \text{ nm}$ ,  $\lambda_{\text{em}} = 527 \text{ nm}$ ) fluorescence intensities were measured, and their ratio was calculated [26].

**Generation of ROS (NBT Assay).** HL-60 Cells ( $0.5 \times 10^6/\text{ml}$ ) were plated in 96-well microplates, and test compounds were added after 24 h incubation at 37° to achieve a final concentration of 5  $\mu\text{M}$ . Incubation (5%  $\text{CO}_2$ , 95% humidity, 37°) of cells following treatment with the test compounds was continued for 72 h. After removal of the cell medium by centrifugation, the cells in each well were resuspended in 100  $\mu\text{l}$  0.1% NBT, and the plates were placed in the incubator for 1 h. The reduced NBT was solubilized with 100  $\mu\text{l}$  of 2M KOH and 130  $\mu\text{l}$  of 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 ( $0.5 \times 10^4/\text{ml}$ ) were seeded after trypsinization and incubated in 96-well microplates for 24 h at 37° 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 quadruplicate [27][28].

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