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# Synthesis and Cytotoxic Activity of a Small Naphthoquinone Library: First Synthesis of Juglonbutin

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*Dedicated to Professor Axel Zeeck on the occasion of his 75th birthday*

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A synthetic protocol has been designed to synthesize grecoketidone (**2k**), 5-hydroxylapachol (**2g**), and the recently discovered natural products juglonbutin (**2o**) and its derivatives, leading to a small library of different 1,4-naphthoquinones with the intention of finding new active compounds. Within our collection, 2-*O*-alkylated naphthoquinones with an ester functionality in the side-chain and a free OH group at C-5 showed the best activities. Compounds **2f**, **2m**, and **2n** showed $GI_{50}$ values against 12 tumor cell lines in the lower micromolar range and juglonbutin (**2o**) showed remarkably efficient inhibition of the glycogen synthase kinase 3β with an $IC_{50}$ value of 2.03 μM. Furthermore, studies on the mode of action of the most active cytotoxic compounds have been carried out. To the best of our knowledge, this is the first report on the synthesis of juglonbutin (**2o**) and its biological activity.

## Introduction

Screening for new anticancer drugs is still important and is one of the major goals in medicinal chemistry.[1] Secondary metabolites from the naphthoquinone family and derivatives thereof show different (antibacterial, cytotoxic) biological activities or interact with different enzymes.[2] Examples of antibacterial naphthoquinones are javanicine, which is active against different *Pseudomonas* species, or juglomycin Z, which shows activity against Gram-positive and Gram-negative bacteria.[3,4] Antifungal activity is described for hydroxysesamone.[5]

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Other naphthoquinones show cytotoxic activities, for example, the rhinacanthins show activity against different tumor cell lines.[6] Lapachol is described as a potential anticancer agent and derivatives of lapachol show a wide range of different biological activities.[1,7] 5-Hydroxylapachol, isolated from the root heart wood of *Tectona grandis*, shows cytotoxic activity towards *Artemia salina* (brine shrimp).[8] The synthesis and antiproliferative activity of 5-hydroxylapachol and related naphthoquinones have been reported, and the 5-hydroxy group is described as being very important for the antiproliferative effect.[9,10] Moreover, the highly cytotoxic activity of 5-methoxyjuglone is reported to be caused by the induction of apoptosis due to the activation of different caspases.[11,12]

We became interested in the synthesis of naphthoquinones and their screening for biological activity after finding the new natural products grecoketides A and B produced from *Streptomyces sp.* Acta 1362.[13] Although no biological activity was found for either grecoketide, the aglycon grecoketidone (**2k**) is strongly related to the known active natural products 5-hydroxylapachol (**2g**), goniquinone, and other naphthoquinones.[8,14,15]

Thus, one of our interests was to evaluate the activity profile of grecoketidone (**2k**) in comparison with 5-hydroxylapachol (**2g**). Therefore we planned to synthesize these two naphthoquinones. Both the naphthoquinone products and the side-products arising from the synthesis form a small library of compounds that have been screened for their biological activity in different test systems. As the re-

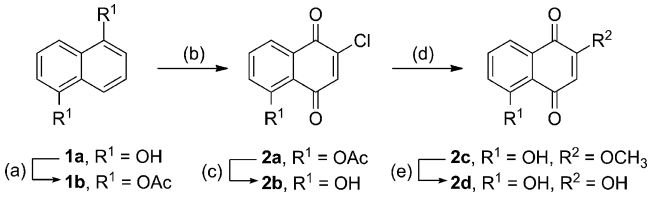
  

cently published juglonbutin (**2o**) should be a realistic target for synthesis by our approach, we became interested in its synthesis for activity screening.[16] The first results of the screening led us to focus on the cytotoxicity of naphthoquinones.

## Results and Discussion

### Chemistry

We synthesized 2-hydroxyjuglone (**2d**) as the key intermediate for the introduction of the desired side-chain at C-3 in five steps (Scheme 1).[17] The synthesis started from 1,5-dihydroxynaphthalene (**1a**). Acetylation of the hydroxy groups in acetic acid anhydride led to 1,5-diacetoxynaphthalene (**1b**), which was oxidized and chlorinated by *N*-chlorosuccinimide to give 5-acetoxy-2-chloro-1,4-naphthoquinone (**2a**). Naphthoquinone **2a** was deprotected with $AlCl_3$ to yield 2-chlorojuglone (**2b**). Treatment of 2-chlorojuglone (**2b**) in alkaline methanol solution provided 2-methoxyjuglone (**2c**), which was converted by hydrolysis into 2-hydroxyjuglone (**2d**).




Scheme 1. Synthesis of the key intermediate 2-hydroxyjuglone (**2d**). Reagents and conditions: (a) $Ac_2O$, pyridine, 35 °C, 1 h, 66%; (b) *N*-chlorosuccinimide, $CH_3COOH/H_2O$, 50 °C, 1 h, 70–75 °C, 1.5 h, 53%; (c) 1. $AlCl_3$, $CH_2Cl_2$, r.t., 3 h, 2. Ice/$H_2O$, $CH_2Cl_2$, r.t., 3 h, 96%; (d) NaOH, $CH_3OH$, r.t., 2 h, 85%; (e) $K_2CO_3$, $CH_3OH/H_2O$, 80 °C, 40 min, 92%.

The alkylation of **2b**–**2d** by different substitution reactions gave naphthoquinone derivatives in one step, with the exception of **2k**, synthesized from **2d**, which required two steps (Scheme 2). For the alkylation at the C-3 position to prepare **2g** and **2k**, we used a synthetic procedure that had been used for lapachol but with a slight modification.[18] Under these conditions we obtained **2g**, together with the two byproducts **2h** and **2i**, similar to the experience of Bonifazi et al. (Scheme 2, b).[8] The precursor molecule of grecoketidone (**2k**), grecoketidone ethyl ester (**2e**), was obtained under these conditions together with **2f** in low yields (Scheme 2, a). More basic reaction conditions led to the formation of **2e** together with **2j** (Scheme 2, c).[19] Similar conditions led to the synthesis of **2l** (Scheme 2, e). The reaction of 2-chlorojuglone (**2b**) with ethyl glycolate led to **2f** as a single product (Scheme 2, i). The related 1,4-naphthoquinones **2m** and **2n** were synthesized starting from 2-hydroxyjuglone (**2d**) by using the corresponding iodo ester as alkylating reagent because hydroxylated ethyl propanoate and butanoate were not accessible (Scheme 2, f,g). The synthesis of **2n** also gave 1,2-naphthoquinone **3a** as a byproduct. Juglonbutin (**2o**) was synthesized together with the chlorinated byproduct **2p** after addition of γ-aminobutyric acid to 2-chlorojuglone (**2b**; Scheme 2, h). The 5-*O*-alkoxylated methoxyjuglone derivative **2q** was synthesized from 2-methoxyjuglone (**2c**) by reaction with ethyl bromoacetate (Scheme 2, j).

We used naphthazarin (**4a**) as the starting material for the synthesis of compound **4e**, with two ester-functionalized chains at C-5-*O* and C-8-*O* (Scheme 3). Methylation of the two hydroxy groups gave 5,8-dimethoxynaphthazarin (**4b**). The methoxy group was introduced at C-2 of **4b** by using a suspension of dioxane/methanol with $H_2SO_4/Fe_2SO_4$.[20] Deprotection of 2,5,8-trimethoxy-1,4-naphthoquinone (**4c**) at C-5-*O* and C-8-*O* with $HNO_3$ and AgO gave compound **4d**, which was treated with ethyl iodoacetate to give the target compound **4e**.

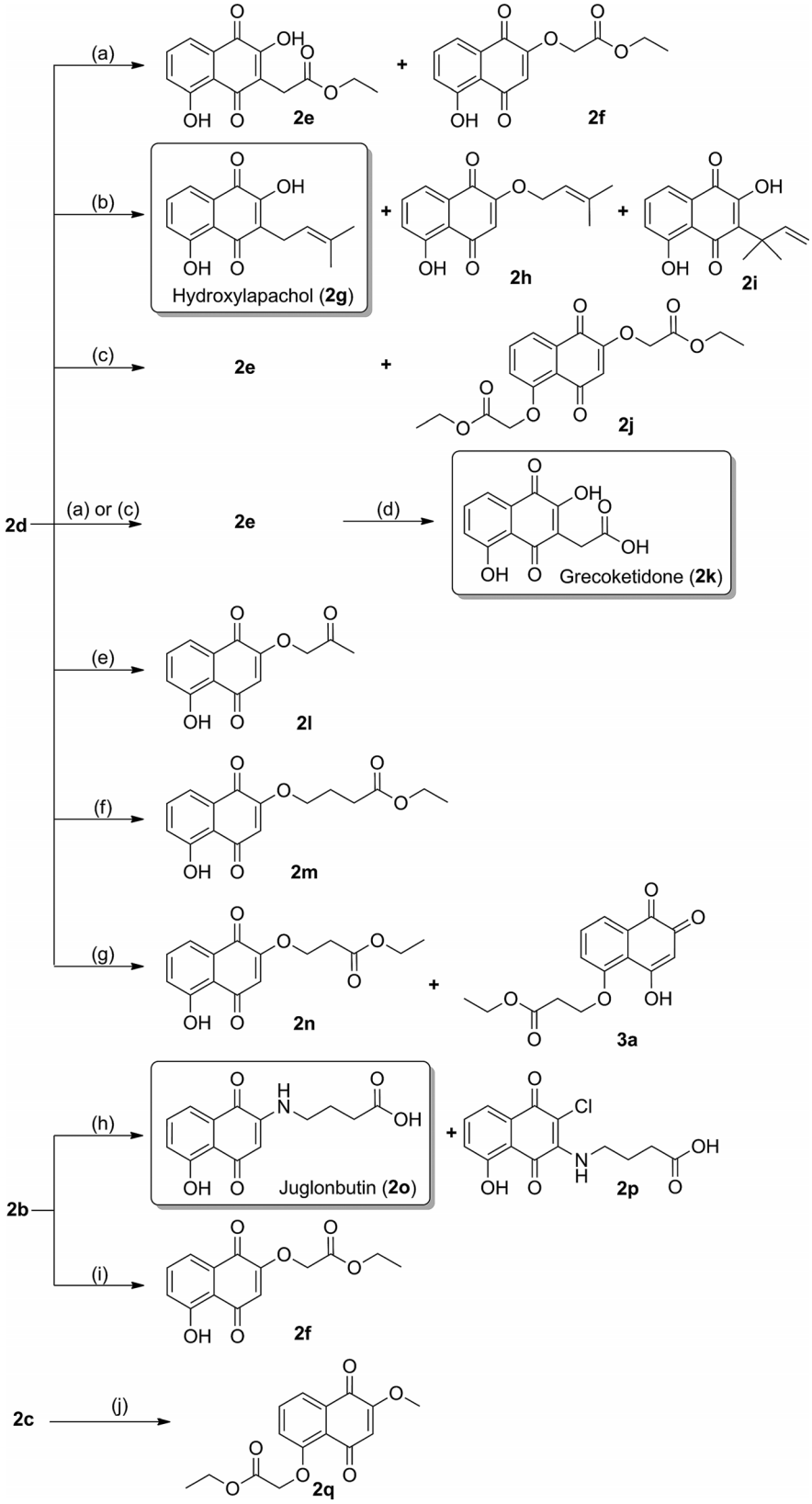
In contrast to the good yields obtained in the synthesis of the key intermediate **2d**, the introduction of side-chains was only achieved in low yields, in agreement with the findings of other groups.[8,19,21,22] One possible reason for this behavior is the mesomeric stabilization of the deprotonated naphthoquinone nucleophile of **2d**, which may inhibit selective substitution.[23] The phenolic hydroxy group at C-5 is presumably chelated, which may protect that position towards substitution. Only a huge excess of bromoalkane prevents the formation of much more complex reaction mixtures, as in the reaction using ethyl bromoacetate leading to **2j**. Compound **2p** is presumably the result of a Michael addition reaction, followed by reduction to the hydroquinone and subsequent oxidation to the quinone.

### Antibacterial Activity and Biochemical Tests

First-order profiling of our library was conducted in different antibacterial agar diffusion assays as well as target-based and cell line assays (detailed results are given in the Supporting Information). The tested naphthoquinones showed good antibacterial activity against the Gram-positive bacteria *B. subtilis* and *S. lentus*, but most showed only moderate activities in all other tests. Higher activities were observed for naphthoquinones with chlorine or *O*-alkylation at the C-2 position. The 2-*O*-ester-functionalized naphthoquinone **2m** showed pronounced activity against *Staphylococcus epidermidis* DSM 3269 with an $IC_{50}$ value of 0.51 μM. It should be highlighted that four compounds, **2c** and **2f**–**2h**, showed similar MIC values against *Staphylococcus aureus* MRS3 in the range of 0.5–2.0 μg/mL, like the reference antibiotics chloramphenicol, kanamycine, and gentamycine. Juglonbutin (**2o**) showed inhibition of glycogen synthase kinase 3β (GSK3β) with an $IC_{50}$ value of 2.03 μM.

GSK3 is a therapeutic target for the treatment of diabetes, inflammation, and multiple neurological diseases.[24] Compounds **2j** and **4e** showed inhibitory effects on the protein tyrosine phosphatase 1B (55% inhibition with $c$ = 10 μM), which is a promising target for the treatment of type 2 diabetes, obesity, and cancer.[25,26]

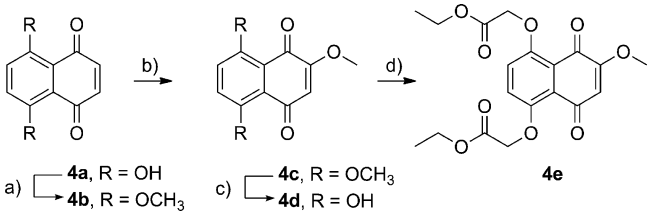



Scheme 2. Synthesis of naphthoquinones **2e**–**2q**. Reagents and conditions: (a) LiH, DMSO, –78 °C, r.t., KI, $BrCH_2COOC_2H_5$, 55 °C, 4 h, 9% (**2e**), 26% (**2f**); (b) 1. LiH, DMSO, –78 °C; 2. KI, $BrCH_2CH{=}C(CH_3)_2$, r.t.; 3. 45 °C, 4 h, 4. 15 h r.t., 28% (**2g**), 2% (**2h**), 8% (**2i**); (c) $K_2CO_3$, ethyl bromoacetate, DMF, r.t., 3 d, 3% (**2e**), 9% (**2j**); (d) 2 N NaOH, EtOH, r.t., 40 min, 89% (**2k**); (e) $Li_2CO_3$, bromo-2-propanone, DMF, r.t., 3 d, 27% (**2l**); (f) $Li_2CO_3$, ethyl 4-iodobutanoate, DMF, r.t., 17 d, 44% (**2m**); (g) $Ag_2O$, ethyl 3-iodopropanoate, $CHCl_3$, reflux, 2 d, 8% (**2n**), 4% (**3a**); (h) $K_2CO_3$, γ-aminobutyric acid, $CH_3CN/H_2O$, reflux, 8 h, 19% (**2o**), 27% (**2p**); (i) 2 N NaOH, ethyl glycolate, $CH_3CN$, 70 °C, 25 h, 30% (**2f**); (j) $K_2CO_3$, ethyl bromoacetate, DMF, r.t., 20 h, 38% (**2q**).

Table 1. Antitumor potency of **2e**–**q**, **3a**, and **4e** against 12 cell lines based on $GI_{50}$ values.

| | $GI_{50}$ [μM] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cell line: | HT29 | 251L | 529L | 629L | 401NL | 462NL | 889L | 1657L | 22RV1 | 1752L | 486L | 1138L |
| **2e** | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 |
| **2f** | 10.1 | 8.11 | 1.02 | 0.96 | 1.70 | 8.14 | 6.87 | 7.54 | 3.58 | 4.19 | 1.09 | 1.13 |
| **2g** | 26.9 | 38.7 | 14.6 | 8.16 | 26.6 | 1.66 | >30 | 67.2 | 9.49 | 16.0 | 23.8 | 3.33 |
| **2h** | >30 | 35.5 | 5.99 | 9.49 | 22.4 | 12.9 | 42.9 | 10.9 | 20.6 | 17.5 | 60.9 | 8.24 |
| **2i** | >30 | 17.9 | 26.4 | 28.6 | 33.0 | 15.2 | 51.2 | 52.6 | 17.8 | 25.3 | 37.8 | 12.6 |
| **2j** | >30 | 9.37 | 7.49 | 9.02 | 8.77 | 8.67 | 10.37 | 9.72 | 6.33 | 9.49 | 9.37 | 1.67 |
| **2k** | >30 | >30 | >30 | 51.7 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 |
| **2m** | 2.13 | 2.87 | 2.24 | 1.62 | 3.53 | 5.26 | 3.45 | 2.59 | 3.25 | 2.15 | 5.05 | 3.08 |
| **2n** | 1.22 | 2.43 | 1.17 | 1.34 | 2.79 | 4.10 | 2.15 | 1.79 | 2.68 | 1.43 | 3.83 | 2.04 |
| **2o** | 28.9 | 26.0 | 25.8 | 17.7 | 22.7 | 26.8 | 29.6 | 29.6 | 29.5 | 27.8 | 30.0 | 29.0 |
| **2p** | 13.2 | 9.89 | 10.1 | 7.55 | 10.7 | 7.71 | 26.4 | 10.5 | 9.10 | 12.0 | 13.3 | 11.0 |
| **2q** | 9.37 | 14.4 | 6.77 | 5.02 | 10.9 | 13.0 | 23.3 | 7.69 | 8.32 | 10.1 | 14.1 | 6.31 |
| **3a** | 10.2 | 12.0 | 3.11 | 7.65 | 7.34 | 11.7 | 8.20 | 3.63 | 8.86 | 6.49 | 11.5 | 4.30 |
| **4e** | 3.42 | 6.85 | 3.62 | 5.14 | 5.25 | 4.93 | 8.32 | 3.58 | 6.70 | 2.99 | 8.16 | 3.33 |




Scheme 3. Synthesis of naphthoquinone **4e**. Reagents and conditions: (a) $CH_3I$, $Ag_2O$, $CHCl_3$, 60–65 °C, 4 d, 69%; (b) $Fe_2(SO_4)_3$, $H_2SO_4$, MeOH, dioxane, reflux, 2 h, 46%; (c) AgO, $HNO_3$, acetone, 0 °C, r.t., 15 min, 27%; (d) ethyl iodoacetate, $K_2CO_3$, DMF, r.t., 3 d, additional $K_2CO_3$, r.t., 2 d, 25%.

### *Growth Inhibitory Activity of 2f–l Against Human Cancer Cell Lines*

Fourteen compounds (**2e**–**k**, **2m**–**q**, **3a**, **4e**) were used for antitumor screening. The primary screening was conducted in a 12 cell line test panel involving different types of cancer cells (colon CXF HT29, stomach GXF 251L, lung LXFA 629L and LXFL 529L, breast MAXF 462NL, melanoma MEXF 462NL, ovary OVXF 899L, pancreas PAXF 1657L, prostate PRXF 22RV1, mesothelioma PXF 1752L, kidney RXF 486L, and uterus UXF 1138L). Their antitumor potency was determined on the basis of their growth inhibition values ($GI_{50}$; Table 1); some of the compounds show growth inhibitory effects with $GI_{50}$ values in the lower micromolar range.

## Mechanism of Action

Within our library of compounds, naphthoquinones substituted at C-3 showed lower activity than C-2-substituted naphthoquinones. Our lead molecule, grecoketidone (**2k**), and its isomer with the ethyl acetate residue at C-3 (**2e**) showed the lowest activity. But the same substituent at the 2-*O*-position (**2f**) led to much higher activity. The influence of the chain length at C-2 on antitumor activity was investigated with compounds **2f**, **2m**, and **2n**. The best activity was observed with two methylene groups (**2n**) between the ether bridge and the ester functionality at C-2. In addition to all the 2-*O*-alkylated naphthoquinones bearing an ester functionality inside the chain, those with a free OH group at C-5 showed higher activities. This supports the proposal of other research groups that a free OH group at C-5 is important for the activity of substituted naphthoquinones.[8,10,27,28] Comparison between **2q** and **4e** seems to suggest that further glycolate groups increase the activity.

In further investigations, the growth inhibitory potentials of all the compounds were evaluated against human osteosarcoma cells U-2 OS, which were then used in further studies on the mechanism of action (Table 2). The $IC_{50}$ value determined for the ester-functionalized 2-*O*-alkylated naphthoquinone **2f** is in the low micromolar range.

Table 2. Ranking of 1,4-naphthoquinones according to their $IC_{50}$ values against human osteosarcoma U-2 OS cells used in mechanism of action studies.

| Grouping [μM] | Naphthoquinone | $IC_{50}$ [μg/mL][a] | $IC_{50}$ [μM][a] |
|---|---|---|---|
| A) 0.1–2.0 | **2f** | 0.14 | 0.5 |
| | **2c** | 0.22 | 1.07 |
| | **2k** | 0.33 | 1.32 |
| | **2m** | 0.60 | 1.97 |
| B) 2.0–20.0 | **2n** | 0.60 | 2.07 |
| | **2q** | 0.69 | 2.39 |
| | **2j** | 1.53 | 4.21 |
| | **4e** | 1.70 | 4.33 |
| | **2h** | 1.20 | 4.64 |
| | **2p** | 5.0 | 16.1 |
| C) 20.0–50.0 | **2i** | 8.08 | 31.3 |
| | **2o** | 12.0 | 43.6 |
| D) >100 | **2g** | 27.8 | 107.8 |
| | **2d** | 31.4 | 165.0 |
| | **2e** | 46.0 | 166.5 |

[a] The $IC_{50}$ values represent the average of two independent measurements and were determined in a MTT assay; 5 d incubation; sigmoidal curve fitting.

To exclude apoptosis induction related to a disruption of the cytoskeletal function, the compound library was tested in routine high-content screening (HCS) on actin filaments (data not shown) and microtubules with the highly active compound **2f** and one representative less-active class B compound, **2j** (Figure 1).

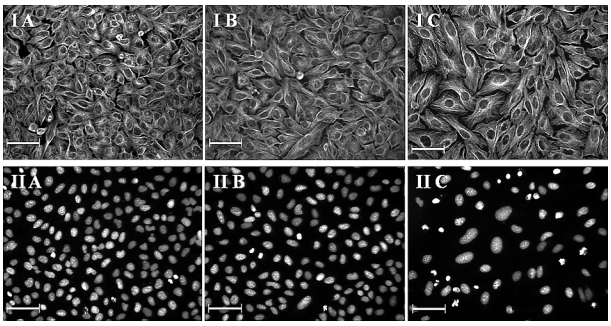



Figure 1. HCS images of microtubules and nuclei of U-2 OS cells treated with **2f** or **2j**. I) Immunofluorescence α-tubulin (secondary antibody coupled to Alexa488). II) Hoechst 33342-stained nuclei. A) Control cells. B) Treatment with 14 μM **2j** for 1 d. C) Treatment with 3.6 μM **2f** for 1 d. Scale bar: 100 μm.

Although **2f** and **2j** do not target tubulin or actin, it was found that hallmarks typical of apoptosis became apparent upon treatment overnight. Incubating human U-2 OS osteosarcoma cells with **2j** (Figure 1, B) caused alterations in cellular morphology compared with the vesicle control (Figure 1, A). In detail, some enlarged nuclei were observed and the number of cells per field of view also decreased. Treatment with **2f** led to an even more pronounced effect (Figure 1, C). Here, nuclei and cells themselves are clearly enlarged and despite this, some late apoptotic fragmented nuclei can be observed.

The phenomenon of enlarged nuclei is often accompanied by a G2/M cell cycle arrest.[29] Therefore human U-2 OS osteosarcoma cells were treated for 3 d with compounds **2f** and **2j** (Figure 2). Indeed, the more active compound **2f** showed an accumulation of cells in the G2/M phase, whereas **2j** has no impact on cell cycle progression in the same concentration range.

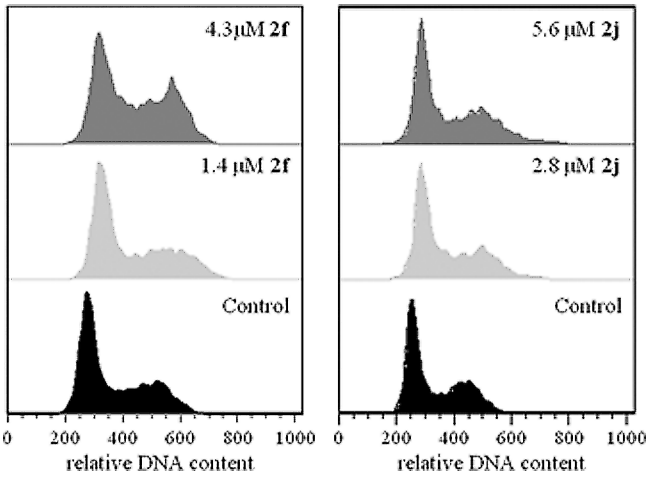



Figure 2. Cell cycle histograms of U-2 OS cells following treatment with naphthoquinones **2f** and **2j.** U-2 OS cells were treated for 3 d with either **2f**, **2j**, or 1 % (v/v) MeOH (control). After DNA staining with propidium iodide, cells were analyzed on a Guava EasyCyte Plus capillary flow cytometer. In total, 5000 viable cells were measured and analysis was conducted with a FlowJo v7.6.1 instrument. In this way cell debris was excluded and histograms display cell count against relative fluorescence in the red channel.

Based on the results from the cell cycle histogram we decided to investigate the impact of the compounds on DNA, especially on DNA double-strand breaks (DSBs), which could be one reason for the apoptosis after the G2/M phase. In further investigations, the compounds were examined after serial dilutions according to their grouping (Table 2). Group A was tested from 0.16–5.00 μM, group B were tested at a 10-fold higher concentration, and groups C and D were tested at concentrations of 50 and 100 μM. The DSBs were analyzed on an automated microscope. The nuclei were segmented on the basis of the Hoechst33342 fluorescence and the intensity of phospho-histone H2A·X (secondary antibody coupled to Alexa488) was determined in these regions of interest (ROIs) with a BD AttoVision v1.6.2 instrument.

During the analysis of the DSBs, it became apparent that some samples led to cell death at high doses, according to their grouping This was observed for the following compounds: Group A: **2c** (5 μM); group B: **2j** (50 μM), **2q** (50 μM), **2n** (50, 25, 12.5, and 6.3 μM) and **4e** (50 μM). All group A compounds except **2k** showed DSBs (Figure 3). Group A member **2c** showed DSBs at concentrations two and four times less than 5 μM (Figure 3). Similar behavior was found for group B members **2j**, **2q**, and **4e**, which also needed to be diluted to see the DSBs instead of cell death (Figure 1). Group B members **2h** and **2p** needed high doses (50μM) to introduce DSBs, and group B member **2n** showed cell death in all cases. Therefore it was decided to test this compound as a group A compound, but then no DSBs were observed. All the compounds from groups C and D were essentially inactive (see the Results in the Supporting Information). Finally, the two compounds **2f** and **2j**, as new compounds, showed in their groups the most pronounced introduction of DSBs observed for phospho-histone H2A·X (see Figures 3 and 4).

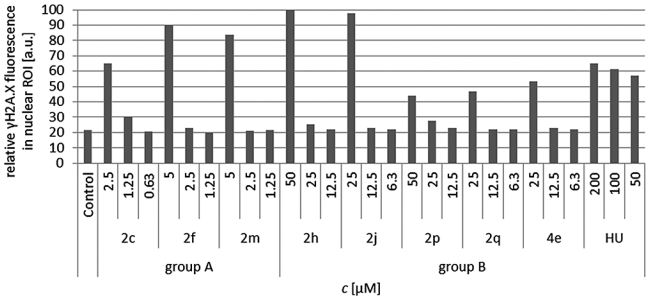



Figure 3. Signal of phosphorylated histone determined as the relative intensity of the secondary Alexa488-coupled antibody in the nuclear area, which is defined by segmentation on Hoechst33342-stained nuclei. Values represent the average of mean values of two independent measurements of selected group A and B compounds. Hydroxyurea was used as a positive control.

To be aware of which mode of action is responsible for the introduction of DSBs, we investigated the influence of our compounds on topoisomerase II and on their ability to create reactive oxygen species (ROS). Only compound **4e** showed the formation of ROS in the cells after treatment with dichlorodihydrofluorescein diacetate (DCFDA) and

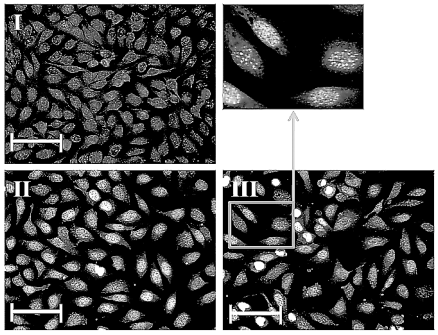



Figure 4. Exemplary images of HCS on histone H2A·X phosphorylation. I) Control. II) Treatment for 1 d at 14 μM **2j**. III) Treatment for 1 d at 7 μM **2f**. The upper right image displays in detail cells treated with **2f** showing H2A·X foci. Scale bar: 100 μm.

comparison with *tert*-butyl hydroperoxide (tBHP) as reference (Figure 5). Naphthoquinones **2c**, **2f**, **2g**, and **2j** showed higher inhibitory activity on topoisomerase II than the reference compound etoposide (see Table S5 in the Supporting Information), which led to the conclusion that topoisomerase II is the major target of these naphthoquinones, which is in good agreement with the inhibition of topoisomerases I and II reported in the literature.[19,30–32]

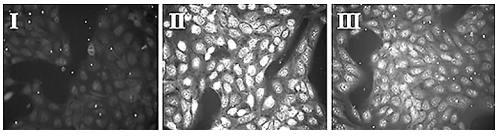



Figure 5. Comparison of levels of ROS in U-2 OS cells induced upon treatment with either tBHP or **4e**. I) Control, II) tBHP, and III) 50μM **4e** after 4 h.

## Conclusions

The library of naphthoquinones synthesized in this work showed good antibacterial activities towards *Bacillus subtilis* and *Staphylococcus lentus*. Naphthoquinones **2c** and **2f**–**h** exhibited similar MIC values against *Staphylococcus aureus* to the tested reference compounds. Juglonbutin (**2o**) showed pronounced activity (2.03 μM) against glycogen synthase kinase 3β, which is a target for the treatment of type 2 diabetes and cancer. Three of our compounds (**2f**, **2m**, and **2n**) showed very good cytotoxic activity against 12 tumor cell lines in the lower micromolar range, but grecoketidone (**2l**), the aglycon of the natural products grecoketides A and B, showed no pronounced cyctotoxic activity, similarly to structural comparable naphthoquinones, for example, 5-hydroxylapachol and gonioquinone.[8,13,14] Within our library of compounds, the cytotoxic activities of the 3-alkylated naphthoquinones were lower than those of their corresponding 2-*O*-alkylated analogues (**2e** in comparison with **2f**). The 2-alkoxylated quinones showed the best activities. The higher cytotoxicities of the 2-monosubstituted naphthoquinones **2f**, **2m**, and **2n** in comparison with the double-substituted naphthoquinones **2j**, **2q**, and **4e** is supported by the results of investigations of other groups. Moreover the 5-hydroxy group is reported to be important for higher cytotoxic activities of naphthoquinones.[8–10,22,27] The ester functionality in the side-chain at C-2 could be the impulse responsible for the higher activity compared with linear chains reported in the literature.[33] Additional investigations concerning the mode of action showed that in most cases DNA double-strand breaks are responsible for the cytotoxicity. The activities of the ester-functionalized 2-*O*-alkylated 1,4-naphthoquinones are much higher than the non-ester-functionalized 2-*O*-alkylated derivatives. Those with a free OH group at C-5 are more potent. Additional ester-functionalized chains on the aromatic ring system increases the activity. Although the quinones are all structural related, a common mode of action could not be found. For example, the main related compounds **2f**, **2m**, and **2j** show the introduction of DSBs, but not **2n**. The two highly active compounds **2f** and **2j** are not involved in the production of ROS, but they show strong inhibitory effects in the topoisomerase II assay, leading to the conclusion that this is the major target of these naphthoquinones.

## Experimental Section

### Chemistry

**General:** Reagents and solvents were commercially available and used without further purification. Reactions were monitored by TLC using plates from Merck (KG 60 $F_{254}$, 0.2 mm layer) and Macherey&Nagel (RP-18W/$UV_{254}$, 0.15 mm layer). TLC plates were colored with vanillin/sulfuric acid (1 g vanillin in 100mL $H_2SO_4$) or anisaldehyde (0.50 mL anisaldehyde in 50 mL $CH_3COOH$ and 1 mL $H_2SO_4$) after heating.[34] Flash chromatography was performed by using a Teledyne Isco CombiFlash Retrieve chromatography separator with commercially available columns (Teledyne Isco). Column chromatography was performed by using silica gel $60^m$ (0.04–0.063 mm/230–400 mesh) from Machery&Nagel. Melting points were determined with a Büchi B-535 or B-545 melting-point instrument. NMR spectra were recorded with a Bruker AV-400 or Varian V NMR-S 600 spectrometer. $^1$H NMR spectra are referenced to TMS (400 MHz spectra) or solvent signals (all 600 MHz spectra and all spectra in [$D_6$]DMSO), and $^{13}$C NMR spectra are referenced to solvent signals. Solvent chemical shifts used for referencing are: $CDCl_3$: $\delta_H$ = 7.26, $\delta_C$ = 77.0 ppm; [$D_6$]-DMSO: $\delta_H$ = 2.50, $\delta_C$ = 39.5 ppm. EI-MS spectra were recorded with a Finnigan MAT-95 spectrometer. The purity of the naphthoquinone library (**2e**–**q**, **3a**, **4a**–**d**) was confirmed by quantitative microanalyses (C, H, and N); the new compounds were in accordance with the theoretical value to within ±0.4%, that is, a purity higher than 95%.

**1,5-Diacetoxynaphthalene (1b):** A solution of 1,5-dihydroxynaphthalene (12.0 g, 74.9 mmol) in acetic acid anhydride (21.0 mL, 223 mmol) and pyridine (134 mL, 1.66 mol) was stirred for 1 h at 35 °C. The reaction mixture was poured onto ice (150 mL) and ethyl acetate (200 mL) was added. The brown precipitate was filtered and the filtrate was washed with water, saturated $CuSO_4$ solution, water, and finally twice with NaCl solution. The organic layer was dried with $Na_2SO_4$ and the solvent was evaporated. Both solids were combined and recrystallized from toluene (12.0 g, 66%). Brown crystals, m.p. 158–159 °C. $R_f$ = 0.65 ($SiO_2$, $CHCl_3$/MeOH,

85:15), 0.05 ($SiO_2$, $CHCl_3$); blue stainable with vanillin/$H_2SO_4$. $^1H$ NMR (400 MHz, $CDCl_3$): $\delta_H$ = 2.46 (s, 6 H, 2 CO-C$H_3$), 7.28 (dd, $^3J$ = 7.9, $^4J$ = 0.5 Hz, 2 H, 2-H, 6-H), 7.50 (t, $^3J$ = 7.9 Hz, 2 H, 3-H, 7-H), 7.78 (dd, $^3J$ = 7.9, $^4J$ = 0.5 Hz, 2 H, 4-H, 8-H) ppm. $^{13}C$ NMR (100 MHz, $CDCl_3$): $\delta_C$ = 21.0 (q, 2 CO-*C*$H_3$), 118.8 (d, C-2, C-6), 119.3 (d, C-4, C-8), 126.0 (d, C-3, C-7), 128.1 (s, C-4a, C-8a), 146.7 (s, C-1, C-5), 169.3 (s, 2 *C*O-$CH_3$) ppm. $C_{14}H_{12}O_4$ (244.24): calcd. C 68.85, H 4.95; found C 68.94, H 4.93.

**5-Acetoxy-2-chloronaphthalene-1,4-dione (2a):** 5-Acetoxy-2-chloronaphthalene-1,4-dione (**2a**) was prepared from 1,5-diacetoxynaphthalene (**1b**; 3.00 g, 12.3 mmol) by dissolving the latter in acetic acid (123 mL) at 50 °C and adding a solution of *N*-chlorosuccinimide (6.58 g, 49.2 mmol) in acetic acid (123 mL) and water (246 mL) at the same temperature within 1 h. The reaction mixture was heated at 70–75 °C for 90 min and then poured onto ice (600 mL). The yellow precipitate was filtered and recrystallized from *n*-butanol (1.63 g, 53%). Yellow needles, m.p. 140–141 °C. $R_f$ = 0.33 ($SiO_2$, $CHCl_3$); blue stainable with vanillin/$H_2SO_4$, yellow stainable with anisaldehyde. $^1H$ NMR (400 MHz, $CDCl_3$): $\delta_H$ = 2.44 (s, 3 H, CO-C$H_3$), 7.10 (s, 1 H, 3-H), 7.42 (dd, $^3J$ = 7.9, $^4J$ = 1.3 Hz, 1 H, 6-H), 7.78 (t, $^3J$ = 7.9 Hz, 1 H, 7-H), 8.13 (dd, $^3J$ = 7.9, $^4J$ = 1.3 Hz, 1 H, 8-H) ppm. $^{13}C$ NMR (100 MHz, $CDCl_3$): $\delta_C$ = 21.4 (q, 2 CO-*C*$H_3$), 123.5 (s, C-4a), 126.5 (d, C-8), 130.8 (d, C-6), 133.3 (s, C-8a), 135.4 (d, C-7), 137.4 (d, C-3), 145.4 (s, C-2), 150.2 (s, C-5), 169.7 (s, *C*O-$CH_3$), 177.9 (s, C-1), 181.6 (s, C-4) ppm. MS (EI): *m*/*z* (%) = 250 (60) $[M]^+$, 208 (100) $[M - C_2H_2O]^+$, 173 (47) $[M - C_2H_2O - Cl]^+$, 145 (10) $[M - CO - Cl]^+$, 43 (28). $C_{12}H_7ClO_4$ (250.63): calcd. C 57.51, H 2.82; found C 57.62, H 2.76.

**2-Chloro-5-hydroxynaphthalene-1,4-dione (Chlorojuglone, 2b):** 2-Chloro-5-hydroxynaphthalene-1,4-dione (**2b**) was synthesized from **2a** (1.00 g, 4.00 mmol) by adding fresh sublimated $AlCl_3$ (3.00 g, 22.5 mmol) portionwise to a stirred solution of **2a** in $CH_2Cl_2$ (33.0 mL) . The reaction mixture was stirred for 3 h before being poured onto ice water (150 mL) and stirring for a further 3 h. The organic layer was extracted with water (3 × 20.0 mL), dried with $Na_2SO_4$, and evaporated under reduced pressure. The product was obtained after column chromatography ($SiO_2$, 1.5 × 20 cm, $CHCl_3$) and recrystallization from 2-propanol (797 mg, 96%). Orange crystals, m.p. 109–110 °C. $R_f$ = 0.71 ($SiO_2$, cyclohexane/ethyl acetate, 60:40), 0.57 ($SiO_2$, $CHCl_3$); blue stainable with vanillin/$H_2SO_4$, orange stainable with anisaldehyde. $^1H$ NMR (400 MHz, $CDCl_3$): $\delta_H$ = 7.19 (s, 1 H, 3-H), 7.31 (dd, $^3J$ = 8.3, $^4J$ = 1.1 Hz, 1 H, 6-H), 7.64 (dd, $^3J$ = 8.3, 7.5 Hz, 1 H, 7-H), 7.72 (dd, $^3J$ = 7.5, $^4J$ = 1.1 Hz, 1 H, 8-H), 11.78 (s, 1 H, 5-OH) ppm. $^{13}C$ NMR (100 MHz, $CDCl_3$): $\delta_C$ = 117.0 (s, C-4a), 121.1 (d, C-8), 125.7 (d, C-6), 131.5 (s, C-8a), 136.3 (d, C-3), 136.9 (d, C-7), 147.5 (s, C-2), 162.1 (s, C-5), 177.7 (s, C-1), 188.3 (s, C-4) ppm. MS (EI): *m*/*z* (%) = 208 (100) $[M]^+$, 173 (97) $[M - Cl]^+$, 145 (35) $[M - Cl - CO]^+$. $C_{10}H_5ClO_3$ (208.60): calcd. C 57.58, H 2.42; found C 57.73, H 2.37.

**5-Hydroxy-2-methoxynaphthalene-1,4-dione (2-Methoxyjuglone, 2c):** To synthesize 2-methoxyjuglone (**2c**), **2b** (1.00 g, 4.80 mmol) was dissolved in methanol (143 mL) and 2 N NaOH (2.40 mL) was added dropwise at 40 °C. The reaction mixture was stirred at room temperature for 2 h. The solvent was evaporated and the solid residue was taken up in $CH_2Cl_2$ and washed with water (3 × 50.0 mL). The organic layer was dried with $Na_2SO_4$ and the solvents evaporated under vacuum. The crude product was purified by column chromatography ($SiO_2$, 5 × 23 cm, cyclohexane/ethyl acetate, 60:40), leading to 2-methoxyjuglone (**2c**) as an orange brown solid (0.83 g, 85%), m.p. 164–165 °C. $R_f$ = 0.41 ($SiO_2$, cyclohexane/ethyl acetate, 60:40), 0.27 ($SiO_2$, $CHCl_3$); orange stainable with vanillin/$H_2SO_4$, yellow stainable with anisaldehyde. $^1H$ NMR (400 MHz, $CDCl_3$): $\delta_H$ = 3.90 (s, 1 H, $OCH_3$), 6.08 (s, 1 H, 3-H), 7.24 (dd, $^3J$ = 8.4, $^4J$ = 1.1 Hz, 1 H, 6-H), 7.56 (dd, $^3J$ = 8.4, 7.6 Hz, 1 H, 7-H), 7.64 (dd, $^3J$ = 7.6, $^4J$ = 1.1 Hz, 1 H, 8-H), 12.20 (s, 1 H, 5-OH) ppm. $^{13}C$ NMR (100 MHz, $CDCl_3$): $\delta_C$ = 57.1 (q, $OCH_3$), 109.9 (d, C-3), 114.6 (s, C-4a), 120.0 (d, C-8), 125.6 (d, C-6), 131.5 (s, C-8a), 135.9 (d, C-7), 161.5 (s, C-2, C-5), 179.8 (s, C-1), 191.1 (s, C-4) ppm. $C_{11}H_8O_4$ (204.18): calcd. C 64.71, H 3.95; found C 64.70, H 3.93.

**2,5-Dihydroxynaphthalene-1,4-dione (2-Hydroxyjuglone, 2d):** 2-Hydroxyjuglone (**2d**) was prepared from **2c** (459 mg, 2.20 mmol) by dissolving **2c** in aqueous $K_2CO_3$ solution (1 N, 6 mL, 56.0 mmol, MeOH/$H_2O$, 1:1) for 40 min at 80 °C under reflux. After cooling to room temperature the reaction mixture was diluted with water (45.0 mL) and adjusted to pH 5 with acetic acid. The precipitate was filtered, washed with water, and recrystallized from 2-propanol (392 mg, 92%). Red crystals, m.p. >300 °C. $R_f$ = 0.09 ($SiO_2$, $CHCl_3$/MeOH, 85:15), 0.34 ($SiO_2$, acetone/$H_2O$, 1:99); red stainable with vanillin/$H_2SO_4$, red stainable with anisaldehyde. $^1H$ NMR (400 MHz, $CDCl_3$): $\delta_H$ = 6.30 (s, 1 H, 3-H), 7.32 (dd, $^3J$ = 8.3, $^4J$ = 0.8 Hz, 1 H, 6-H), 7.58 (dd, $^3J$ = 8.3, 7.8 Hz, 1 H, 7-H), 7.68 (dd, $^3J$ = 7.8, $^4J$ = 0.8 Hz, 1 H, 8-H), 12.32 (s, 1 H, 5-OH) ppm. $^{13}C$ NMR (100 MHz, $CDCl_3$): $\delta_C$ = 110.3 (d, C-3), 114.5 (s, C-4a), 119.7 (d, C-8), 126.7 (d, C-6), 129.3 (s, C-8a), 135.2 (d, C-7), 156.9 (s, C-2), 161.4 (s, C-5), 181.2 (s, C-1), 191.2 (s, C-4) ppm. MS (EI): *m*/*z* (%) = 190 (100) $[M]^+$, 162 (26) $[M - CO]^+$, 134 (25) $[C_8H_6O_2]^+$, 121 (85). $C_{10}H_6O_4$ (190.03): calcd. C 63.16, H 3.18; found C 63.35, H 3.18.

**2,5-Dihydroxy-3-(ethoxycarbonylmethyl)naphthalene-1,4-dione (Grecoketidone Ethyl Ester, 2e):** A solution of 2-hydroxyjuglone (**2d**; 1.50 g, 7.89 mmol) in anhydrous DMSO (9.9 mL) under argon was frozen at –78 °C. The frozen surface was covered with LiH (72.0 mg, 9.06 mmol) and allowed to thaw under inert gas. KI (289 mg, 1.74 mmol) was added to the defrosted solution and after elimination of $H_2$ (gas bubbles) ethyl bromoacetate (1.42 g, 943 µL, 8.53 mmol) was added. The mixture was stirred at 55 °C for 4 h. After cooling to room temperature the mixture was poured onto ice (30 mL). The precipitate was filtered and the filtrate adjusted to pH 2 leading to precipitation. Column chromatography ($SiO_2$, column 5 × 35 cm, cyclohexane/acetone, 6:4, containing 0.1% $CH_3COOH$) of the combined precipitates led to two fractions that were each concentrated in vacuo, diluted in $CHCl_3$, and washed with aqueous NaCl solution and pure water. The organic layers were dried with $Na_2SO_4$ and the solvents evaporated in vacuo.

**2-(Ethoxycarbonylmethoxy)-5-hydroxynaphthalene-1,4-dione (2f):** Fraction 1 gave **2f**, recrystallized from ethanol (562 mg, 26%), m.p. 137–138 °C. $R_f$ = 0.41 ($SiO_2$, cyclohexane/ethyl acetate, 60:40), 0.28 ($SiO_2$, $CHCl_3$), 0.31 ($SiO_2$, $CHCl_3$/methanol, 85:15), 0.53 ($SiO_2$, cyclohexane/acetone, 6:4, with 0.1% $CH_3COOH$); orange stainable with vanillin/$H_2SO_4$, yellow stainable with anisaldehyde.$^1H$ NMR (600 MHz, $CDCl_3$): $\delta_H$ = 1.30 (t, $^3J$ = 7.1 Hz, 3 H, 12-$H_3$), 4.28 (q, $^3J$ = 7.1 Hz, 2 H, 11-$H_2$), 4.71 (s, 2 H, 9-$H_2$), 5.97 (s, 1 H, 3-H), 7.24 (dd, $^3J$ = 8.4, $^4J$ = 1.1 Hz, 1 H, 6-H), 7.56 (dd, $^3J$ = 8.4, 7.6 Hz, 1 H, 7-H), 7.65 (dd, $^3J$ = 7.6, $^4J$ = 1.1 Hz, 1 H, 8-H), 12.06 (s, 1 H, 5-OH) ppm. $^{13}C$ NMR (150 MHz, $CDCl_3$): $\delta_C$ = 14.2 (q, C-12), 62.2 (t, C-11), 65.6 (t, C-9), 110.8 (d, C-3), 114.2 (s, C-4a), 119.7 (d, C-8), 125.3 (d, C-6), 131.1 (s, C-8a), 135.8 (d, C-7), 159.3 (s, C-2), 161.2 (s, C-5), 166.2 (s, C-10), 178.8 (s, C-1), 190.6 (s, C-4) ppm. MS (EI): *m*/*z* (%) = 276 (53) $[M]^+$, 247 (24) $[M - C_2H_5]^+$, 203 (100) $[M - C_3H_5O_2]^+$, 173 (43) $[C_{10}H_5O_4]^+$. $C_{14}H_{12}O_6$ (276.24): calcd. C 60.87, H 4.38; found C 60.74, H 4.28.

**2,5-Dihydroxy-3-(ethoxycarbonylmethyl)naphthalene-1,4-dione (Grecoketidone Ethyl Ester, 2e):** Fraction 2 (**2e**), was diluted in hot

cyclohexane, filtered, and crystallized from cyclohexane (200 mg, 9%). Orange crystals, m.p. 158–160 °C. $^1$H NMR (600 MHz, $CDCl_3$): $\delta_H$ = 1.28 (t, $^3J$ = 6.8 Hz, 3 H, 12-$H_3$), 3.62 (s, 2 H, 9-$H_2$), 4.19 (q, $^3J$ = 6.8 Hz, 2 H, 11-$H_2$), 7.29 (dd, $^3J$ = 8.2, $^4J$ = 1.1 Hz, 1 H, 6-H), 7.55 (dd, $^3J$ = 8.2, $^3J$ = 7.5 Hz, 1 H, 7-H), 7.65 (dd, $^3J$ = 7.5, $^4J$ = 1.1 Hz, 1 H, 8-H), 7.67 (s, 1 H, 2-OH), 12.25 (s, 1 H, 5-OH) ppm. $^{13}$C NMR (150 MHz, $CDCl_3$): $\delta_C$ = 14.1 (q, C-12), 28.4 (t, C-9), 61.3 (t, C-11), 114.2 (s, C-4a), 116.5 (s, C-3), 119.5 (d, C-8), 126.5 (d, C-6), 129.4 (s, C-8a), 135.2 (d, C-7), 154.6 (s, C-5), 161.4 (s, C-2), 169.7 (s, C-10), 180.4 (s, C-1), 189.8 (s, C-4) ppm. MS (EI): *m*/*z* (%) = 276 (19) $[M]^+$, 246 (26) $[M - C_2H_5 - H]^+$, 231 (14) $[M - C_3H_5O]^+$, 203 (19) $[M - C_3H_5O_2]^+$, 174 (38) $[C_{10}H_5O_3]^+$. $C_{14}H_{12}O_6$ (276.24): calcd. C 60.87, H 4.38; found C 60.57, H 4.25. $R_f$ = 0.31 ($SiO_2$, $CHCl_3$/MeOH, 85:15), 0.30 ($SiO_2$, cyclohexane/acetone, 60:40, with 0.1% $CH_3COOH$); red stainable with vanillin/$H_2SO_4$, orange stainable with anisaldehyde.

**2-(Ethoxycarbonylmethoxy)-5-hydroxynaphthalene-1,4-dione (2f) as Single Product:** NaOH (2 N, 0.5 mL, 1 mmol) was added dropwise to a solution of 2-chloro-5-hydroxynaphthalene-1,4-dione (**2b**; 200 mg, 0.96 mmol) and ethyl glycolate (86.0 μL, 0.90 mmol) in $CH_3CN$. The stirred reaction mixture was heated at 70 °C for 11 h followed by the addition of NaOH (2 N, 100 μL) and ethyl glycolate (40.0 μL, 0.42 mmol) and further stirring for 13 h at 70 °C. A final addition of NaOH (2 N, 50 μL) and ethyl glycolate (20.0 μL, 0.21 mmol) and stirring for 1 h at 70 °C gave the final reaction mixture, which was poured onto water. The mixture was extracted with $CHCl_3$. The organic phase was washed with brine and dried with $Na_2SO_4$. Column chromatography ($SiO_2$, column 1 × 30 cm, cyclohexane/acetone, 6:4, containing 0.1% $CH_3COOH$) led after crystallization from ethanol to orange needles of **2f** (79.5 mg, 30%).

**2,5-Dihydroxy-3-(3-methylbut-2-enyl)naphthalene-1,4-dione (Hydroxylapachol, 2g):** A solution of 2-hydroxyjuglone (**2d**; 1.50 g, 7.89 mmol) in anhydrous DMSO (9.9 mL) under argon was frozen at –78 °C. The frozen surface was covered with LiH (72.0 mg, 9.06 mmol) and was then allowed to thaw under an inert gas. KI (289 mg, 1.74 mmol) was added to the thawed solution and after the elimination of $H_2$ (gas bubbles) 1-bromo-3-methyl-2-butene (1.27 g, 933 μL, 8.53 mmol) was added dropwise. The reaction mixture was stirred at 45 °C for 4 h and 15 h at room temperature. The mixture was poured onto ice and the starting material **2d** precipitated and recrystallized from toluene. The filtrate was adjusted to pH 2 and the new precipitate was filtered. Flash chromatography (40 g $SiO_2$ column, toluene/cyclohexane, 7:3, with 0.1% $CH_3COOH$) of the precipitate led to three fractions, which were washed with water, dried with $Na_2SO_4$, filtered, and the solvents evaporated under vacuum.

**2,5-Dihydroxy-3-(2-methylbut-3-en-2-yl)naphthalene-1,4-dione (2i):** Fraction 1, recrystallized from *n*-heptane (167 mg, 8%). Orange crystals, m.p. 151–151 °C (ref.[8] 123–124 °C). $^1$H NMR (600 MHz, $CDCl_3$): $\delta_H$ = 1.57 (s, 6 H, 12-$H_3$, 13-$H_3$), 4.96 (dd, $^3J$ = 10.6, $^2J$ = 0.8 Hz, 1 H, 11-$H_a$), 5.00 (dd, $^3J$ = 17.4, $^2J$ = 0.8 Hz, 1 H, 11-$H_b$), 6.27 (dd, $^3J$ = 17.4, $^3J$ = 10.6 Hz, 1 H, 10-H), 7.28 (dd, $^3J$ = 7.4, $^3J$ = 1.1 Hz, 1 H, 6-H), 7.51 (dd, $^3J$ = 7.5, $^3J$ = 7.4 Hz, 1 H, 7-H), 7.62 (dd, $^3J$ = 7.5, $^3J$ = 1.1 Hz, 1 H, 8-H), 7.97 (s, 1 H, 2-OH), 12.55 (s, 1 H, 5-OH) ppm. $^{13}$C NMR (150 MHz, $CDCl_3$): $\delta_C$ = 28.4 (q, 2 C, C-12, C-13), 41.0 (s, C-9), 109.8 (s, C-11), 115.2 (s, C-4a), 118.9 (d, C-8), 126.7 (d, C-6), 127.5 (d, C-3), 128.5 (s, C-8a), 134.7 (d, C-7), 148.0 (d, C-10), 153.5 (s, C-2), 161.4 (s, C-5), 181.5 (s, C-1), 191.3 (s, C-4) ppm. MS (EI): *m*/*z* (%) = 258 (78) $[M]^+$, 243 (100) $[M - CH_3]^+$, 215 (17) $[M - C_3H_7]^+$. $C_{15}H_{14}O_4$ (258.27): calcd. C 69.76, H 5.46; found C 69.66, H 5.39. $R_f$ = 0.37 ($SiO_2$, toluene/cyclohexane, 70:30, with 0.1% $CH_3COOH$); red stainable with vanillin/$H_2SO_4$, red stainable with anisaldehyde.

**2,5-Dihydroxy-3-(3-methylbut-2-enyl)naphthalene-1,4-dione (Hydroxylapachol, 2g):** Fraction 2, recrystallized from cyclohexane (578 mg, 28%). Orange needles, m.p. 142–143 °C (ref.[13] 144–145 °C). $R_f$ = 0.53 ($SiO_2$, cyclohexane/ethyl acetate, 60:40), 0.21 ($SiO_2$, toluene/cyclohexane, 70:30, with 0.1% $CH_3COOH$); red stainable with vanillin/$H_2SO_4$, red stainable with anisaldehyde. $^1$H NMR (600 MHz, $CDCl_3$, TMS): $\delta_H$ = 1.70 (d, $^4J$ = 1.2 Hz, 3 H, 12-$H_3$), 1.78 (s, 3 H, 13-$H_3$), 3.33 (d, $^3J$ = 7.4 Hz, 2 H, 9-$H_2$), 5.19 (tq, $^3J$ = 7.0, $^4J$ = 1.2 Hz, 1 H, 10-H), 7.28 (dd, $^3J$ = 8.4, $^4J$ = 1.2 Hz, 1 H, 6-H), 7.43 (s, 1 H, 2-OH), 7.53 (dd, $^3J$ = 8.4, $^3J$ = 7.4 Hz, 1 H, 7-H), 7.63 (dd, $^3J$ = 7.4, $^4J$ = 1.2 Hz, 1 H, 8-H), 12.50 (s, 1 H, 5-OH) ppm. $^{13}$C NMR (150 MHz, $CDCl_3$): $\delta_C$ = 17.9 (q, C-13), 22.0 (t, C-9), 25.7 (q, C-12), 114.5 (s, C-4a), 119.2 (d, C-10), 119.3 (d, C-8), 123.1 (s, C-3), 126.2 (d, C-6), 129.4 (s, C-8a), 134.1 (s, C-11), 134.9 (d, C-7), 153.2 (s, C-2), 161.3 (s, C-5), 180.9 (s, C-1), 190.7 (s, C-4) ppm. MS (EI): *m*/*z* (%) = 258 (38) $[M]^+$, 243 (100) $[M - CH_3]^+$, 215 (9) $[M - C_3H_7]^+$. $C_{15}H_{14}O_4$ (258.27): calcd. C 69.76, H 5.46; found C 69.61, H 5.23.

**2-(3-Methylbut-2-enyloxy)-5-hydroxynaphthalene-1,4-dione (2h):** Fraction 3, recrystallized from methanol (33.1 mg, 2%). Orange crystals, m.p. 139–140 °C, (ref.[8] 135–136 °C). $^1$H NMR (600 MHz, $CDCl_3$): $\delta_H$ = 1.76 (s, 3 H, 12-$H_3$), 1.81 (s, 3 H, 13-$H_3$), 4.60 (d, $^3J$ = 6.8 Hz, 2 H, 9-$H_2$), 5.48 (t, $^3J$ = 6.8 Hz, 1 H, 10-H), 6.08 (s, 1 H, 3-H), 7.26 (dd, $^3J$ = 8.3, $^4J$ = 1.1 Hz, 1 H, 6-H), 7.56 (dd, $^3J$ = 8.3, $^3J$ = 7.6 Hz, 1 H, 7-H), 7.66 (dd, $^3J$ = 7.6, $^4J$ = 1.1 Hz, 1 H, 8-H), 12.26 (s, 1 H, 5-OH) ppm. $^{13}$C NMR (150 MHz, $CDCl_3$): $\delta_C$ = 18.4 (q, C-12), 25.8 (q, C-13), 66.7 (t, C-9), 110.0 (d, C-3), 114.2 (s, C-4a), 116.9 (d, C-10), 119.5 (d, C-8), 125.0 (d, C-6), 131.1 (s, C-8a), 135.4 (d, C-7), 140.9 (s, C-11), 160.2 (s, C-2), 161.0 (s, C-5), 179.6 (s, C-1) 191.0 (s, C-4), 166.2 (s, C-10) ppm. MS (EI): *m*/*z* (%) = 258 (38) $[M]^+$, 243 (74) $[M - CH_3]^+$, 215 (13) $[M - C_3H_7]^+$. $C_{15}H_{14}O_4$ (258.27): calcd. C 69.76, H 5.46; found C 69.92, H 5.56. $R_f$ = 0.71 ($SiO_2$, cyclohexane/ethyl acetate, 60:40), 0.52 ($CHCl_3$), 0.09 ($SiO_2$, toluene/cyclohexane, 70:30, with 0.1% $CH_3COOH$); brown stainable with vanillin/$H_2SO_4$, red stainable with anisaldehyde.

**2,5-Bis(ethoxycarbonylmethoxy)naphthalene-1,4-dione (2j):** A solution of 2-hydroxyjuglone (**2d**; 500 mg, 2.63 mmol) in DMF (17.3 mL) was added to a suspension of $K_2CO_3$ (727 mg, 6.26 mmol) in DMF (10 mL) and the mixture was stirred for 15 min before ethyl bromoacetate (1.77 g, 1.14 mL, 10.5 mmol) was added dropwise. The reaction mixture was then stirred for 3 d at room temperature and quenched by pouring onto ice water. The mixture was extracted with ethyl acetate/pentane (1:1) and the organic layer was dried with $Na_2SO_4$, evaporated, and purified by flash chromatography (40 g $SiO_2$ column, hexane/ethyl acetate/$CHCl_3$, 2:1:1). The major fraction led, after recrystallization from ethanol, to 2,5-bis(ethoxycarbonylmethoxy)naphthalene-1,4-dione (**2j**; 89.6 mg, 9%). Yellow needles, m.p. 118–119 °C. $R_f$ = 0.39 ($SiO_2$, cyclohexane/acetone, 60:40, with 0.1% $CH_3COOH$). $^1$H NMR (600 MHz, $CDCl_3$): $\delta_H$ = 1.28 (t, $^3J$ = 7.0 Hz, 3 H, 16-$H_3$), 1.30 (t, $^3J$ = 7.3 Hz, 3 H, 12-$H_3$), 4.26 (q, $^3J$ = 7.0 Hz, 2 H, 15-$H_2$), 4.27 (q, $^3J$ = 7.3 Hz, 2 H, 11-$H_2$), 4.67 (s, 2 H, 9-$H_2$), 4.80 (s, 2 H, 13-$H_2$), 5.97 (s, 1 H, 3-H), 7.21 (dd, $^3J$ = 8.4, $^4J$ = 0.9 Hz, 1 H, 6-H), 7.62 (dd, $^3J$ = 8.3, $^3J$ = 7.8 Hz, 1 H, 7-H), 7.87 (dd, $^3J$ = 7.7, $^4J$ = 1.0 Hz, 1 H, 8-H) ppm. $^{13}$C NMR (150 MHz, $CDCl_3$): $\delta_C$ = 14.1 (q, C-12, C-16), 61.6 (t, C-11), 62.0 (t, C-15), 65.2 (t, C-9), 66.6 (t, C-13), 113.1 (d, C-3), 120.2 (s, C-4a), 121.0 (d, C-6), 121.1 (d, C-8), 133.4 (s, C-8a), 134.2 (d, C-7), 156.7 (s, C-2), 157.6 (s, C-5), 166.3 (s, C-10), 168.1 (s, C-14), 179.5 (s, C-1), 183.6 (s, C-4) ppm. MS (EI): *m*/*z* (%) = 362 (77) $[M]^+$, 316 (29) $[M - C_2H_6O]^+$, 289 (59) $[M - C_3H_5O_2]^+$, 275 (100) $[M - C_4H_7O_2]^+$, 203 (93)

$[C_{10}H_7O_4]^+$, 173 (31) $[C_{10}H_5O_3]^+$. $C_{18}H_{18}O_8$ (362.33): calcd. C 59.67, H 5.01; found C 59.63, H 4.90.

The aqueous solution was acidified with HCl and extracted with ethyl acetate. Column chromatography ($SiO_2$, hexane/ethyl acetate/$CHCl_3$, 2:1:1) gave grecoketidone ethyl ester (**2e**), which was filtered from hot cyclohexane and recrystallized from cyclohexane leading to pure grecoketidone ethyl ester (**2e**, 25 mg, 3%, for characterization data, see above).

**2-(Acetylmethoxy)-5-hydroxynaphthalene-1,4-dione (2l):** A solution of 2-hydroxyjuglone (**2d**; 20.0 mg, 0.11 mmol) in DMF (2.00 mL) was added to a suspension of $Li_2CO_3$ (8.66 mg, 0.12 mmol) in DMF (4.00 mL) and the mixture was stirred for 15 min before bromo-2-propanone (27.3 mg, 16.7 µL, synthesized according to the literature[35]) was added dropwise. The reaction mixture was stirred for 15 h and quenched by pouring onto ice water. The mixture was extracted with ethyl acetate (2 × 15 mL) and the organic layer was dried with $Na_2SO_4$ and removed in vacuo. Recrystallization from methanol led to product **2l** (7.04 mg, 27%). Brown needles, m.p. 176–178 °C (decomp.). $^1$H NMR (600 MHz, $CDCl_3$): $\delta_H$ = 2.34 (s, 3 H, 11-$H_3$), 4.66 (s, 2 H, 9-$H_2$), 5.93 (s, 1 H, 3-H), 7.27 (dd, $^3J$ = 8.3, $^4J$ = 1.1 Hz, 1 H, 6-H), 7.59 (dd, $^3J$ = 8.3, $^3J$ = 7.6 Hz, 1 H, 7-H), 7.67 (dd, $^3J$ = 7.6, $^4J$ = 1.1 Hz, 1 H, 8-H), 12.09 (s, 1 H, 5-OH) ppm. $^{13}$C NMR (150 MHz, $CDCl_3$): $\delta_C$ = 26.5 (q, C-11), 72.9 (t, C-9), 110.7 (d, C-3), 114.1 (s, C-4a), 119.6 (d, C-8), 125.3 (d, C-6), 131.0 (s, C-8a), 135.7 (d, C-7), 159.0 (s, C-2), 161.1 (s, C-5), 178.8 (s, C-1), 190.4 (s, C-4), 201.0 (s, C-10) ppm. MS (EI): *m*/*z* (%) = 246 (30) $[M]^+$, 204 (100) $[C_{10}H_7O_4]^+$, 173 (21) $[C_{10}H_5O_3]^+$. $C_{13}H_{10}O_5$ (246.22): calcd. C 63.42, H 4.09; found C 63.47, H 4.12. $R_f$ = 0.24 ($SiO_2$, cyclohexane/ethyl acetate, 60:40), 0.45 ($SiO_2$, cyclohexane/acetone, 60:40, with 0.1% $CH_3COOH$); orange stainable with vanillin/$H_2SO_4$, yellow stainable with anisaldehyde.

**2,5-Dihydroxy-3-(carboxymethyl)naphthalene-1,4-dione (Grecoketidone, 2k):** Grecoketidone (**2k**) was produced after hydrolyzation of grecoketidone ethyl ester (**2e**; 61.0 mg, 0.22 mmol) by using a mixture of 2 N NaOH (13.5 mL) and EtOH (6.8 mL) at room temperature for 40 min. The red reaction mixture was quenched by acidification with 2 N HCl. The yellow precipitate was filtered and recrystallized from *n*-butanol (48.9 mg, 89%). Red-brown crystals, m.p. 180 °C (decomp.). $R_f$ = 0.23 (RP, 2-propanol/water, 80:20); red stainable with vanillin/$H_2SO_4$, orange stainable with anisaldehyde. $^1$H NMR (400 MHz, [D$_6$]DMSO): $\delta_H$ = 3.41 (s, 2 H, 9-$H_2$), 7.34 (dd, $^3J$ = 8.3, $^4J$ = 1.0 Hz, 1 H, 6-H), 7.57 (dd, $^3J$ = 7.4, $^4J$ = 1.0 Hz, 1 H, 8-H), 7.68 (dd, $^3J$ = 8.3, 7.4 Hz, 1 H, 7-H), 12.38 (s, 1 H, 5-OH) ppm. $^{13}$C NMR (100 MHz, [D$_6$]DMSO): $\delta_C$ = 28.3 (t, C-9), 114.0 (s, C-4a), 117.5 (s, C-3), 119.0 (d, C-8), 125.2 (d, C-6), 130.5 (s, C-8a), 135.6 (d, C-7), 157.6 (s, C-2), 160.2 (s, C-5), 171.4 (s, C-10), 180.5 (s, C-1), 190.9 (s, C-4) ppm. MS (EI): *m*/*z* (%) = 248 (12) $[M]^+$, 204 (100) $[M - CO_2]^+$. $C_{12}H_8O_6$ (248.19): calcd. C 58.07, H 3.25; found C 58.56, H 3.28.

**2-[2-(Ethoxycarbonyl)ethoxy]-5-hydroxynaphthalene-1,4-dione (2n):** $Ag_2O$ (0.92 g, 3.97 mmol) was added in portions and under vigorous stirring to a solution of 2-hydroxyjuglone (**2d**; 200 mg, 1.06 mmol) in $CHCl_3$ (120 mL), followed by ethyl 3-iodopropanoate (280 µL, 479 mg, 2.10 mmol, synthesized according to the literature[35]). The mixture was heated under reflux for 2 d, and then further $Ag_2O$ (243 mg, 1.05 mmol) added and the mixture was heated at reflux for 3 h. The reaction was monitored by TLC ($SiO_2$, cyclohexane/acetone, 6:4, with 0.1% $CH_3COOH$). The reaction mixture was filtered through Celite (ca. 3 g) and concentrated in vacuo. Column chromatography ($SiO_2$, cyclohexane/acetone, 6:4, with 0.1% $CH_3COOH$) gave two fractions, which were concentrated in vacuo and dissolved in diethyl ether. Both fractions were washed with brine and dried with $Na_2SO_4$.

**2-[2-(Ethoxycarbonyl)ethoxy]-5-hydroxynaphthalene-1,4-dione (2n):** Fraction 1, recrystallized from cyclohexane (23.4 mg, 80.6 µmol, 8%). Yellow needles, m.p. 109–111 °C. $R_f$ = 0.53 ($SiO_2$, cyclohexane/acetone, 6:4, with 0.1% $CH_3COOH$); orange stainable with vanillin/$H_2SO_4$, yellow stainable with anisaldehyde. $^1$H NMR (600 MHz, $CDCl_3$): $\delta_H$ = 1.28 (t, $^3J$ = 7.1 Hz, 3 H, 13-$H_3$), 2.92 (t, $^3J$ = 6.3 Hz, 2 H, 10-$H_2$), 4.19 (q, $^3J$ = 7.1 Hz, 2 H, 12-$H_2$), 4.28 (t, $^3J$ = 6.3 Hz, 2 H, 9-$H_2$), 6.13 (s, 1 H, 3-H), 7.25 (d, $^3J$ = 7.3 Hz, 1 H, 6-H), 7.55 (dd, $^3J$ = 7.6, 7.3 Hz, 1 H, 7-H), 7.63 (d, $^3J$ = 7.6 Hz, 1 H, 8-H), 12.18 (s, 1 H, 5-OH) ppm. $^{13}$C NMR (150 MHz, $CDCl_3$): $\delta_C$ = 14.2 (q, C-13), 33.5 (t, C-10), 61.3 (t, C-12), 65.1 (t, C-9), 110.3 (d, C-3), 114.2 (s, C-4a), 119.6 (d, C-8), 125.2 (d, C-6), 131.2 (s, C-4a), 135.6 (d, C-7), 160.2 (s, C-2), 161.2 (s, C-5), 170.1 (s, C-11), 179.1 (s, C-1), 190.9 (s, C-4) ppm. MS (EI): *m*/*z* (%) = 290 (100) $[M]^+$, 202 (53) $[M - C_5H_{10}O_2]^+$, 190 (25) $[C_{10}H_6O_4]^+$, 174 (25) $[C_{10}H_6O_3]^+$. $C_{15}H_{14}O_6$ (290.27): calcd. C 62.07, H 4.86; found C 62.01, H 4.81.

**5-[2-(Ethoxycarbonyl)ethoxy]-4-hydroxynaphthalene-1,2-dione (3a):** Fraction 2, recrystallized from ethanol (12.2 mg, 42.0 µmol, 4%). Red crystals, m.p. 118–120 °C. $R_f$ = 0.13 ($SiO_2$, cyclohexane/acetone, 6:4, with 0.1% $CH_3COOH$), $R_f$ = 0.10 ($SiO_2$, $CHCl_3$/ethyl acetate, 6:4); orange stainable with vanillin/$H_2SO_4$, orange-brown stainable with anisaldehyde. $^1$H NMR (600 MHz, $CDCl_3$): $\delta_H$ = 1.32 (t, $^3J$ = 7.2 Hz, 3 H, 13-$H_3$), 2.95 (t, $^3J$ = 5.7 Hz, 2 H, 10-$H_2$), 4.26 (q, $^3J$ = 7.2 Hz, 2 H, 12-$H_2$), 4.49 (t, $^3J$ = 5.7 Hz, 2 H, 9-$H_2$), 5.94 (s, 1 H, 3-H), 7.23 (d, $^3J$ = 8.1 Hz, 1 H, 6-H), 7.45 (dd, $^3J$ = 8.1, 7.5 Hz, 1 H, 7-H), 7.76 (d, $^3J$ = 7.5 Hz, 1 H, 8-H), 8.82 (s, 1 H, 4-OH) ppm. $^{13}$C NMR (150 MHz, $CDCl_3$): $\delta_C$ = 14.3 (q, C-13), 33.7 (t, C-10), 62.0 (t, C-12), 65.9 (t, C-9), 103.1 (d, C-3), 114.1 (s, C-4a), 123.7 (d, C-8), 126.9 (d, C-6), 131.5 (s, C-8a), 133.2 (d, C-7), 156.4 (s, C-5), 169.9 (s, C-4), 170.5 (s, C-11), 179.2 (s, C-1), 179.4 (s, C-2) ppm. MS ($ESI^+$): *m*/*z* (%) = 313 (35) $[M + Na]^+$, 603 (100) $[2M + Na]^+$. $C_{15}H_{14}O_6$ (290.27): calcd. C 62.07, H 4.86; found C 62.52, H 4.85.

**2-[3-(Ethoxycarbonyl)propyloxy]-5-hydroxynaphthalene-1,4-dione (2m):** Ethyl 4-iodobutanoate (416 mg, 1.72 mmol) was added dropwise to a suspension of 2-hydroxyjuglone (**2d**; 200 mg, 1.06 mmol) and $Li_2CO_3$ (74.0 mg, 1.06 mmol) in DMF (40 mL). The reaction mixture was stirred for 17 d, and during days 10 and 13 further ethyl 4-iodobutanoate (138 mg, 0.57 mmol, synthesized according to the literature[35]) was added. The reaction mixture was finally poured onto water and acidified with HCl (pH 5) and extracted with $CHCl_3$. The organic layer was washed with brine, dried with $Na_2SO_4$, concentrated in vacuo, and subjected to column chromatography ($SiO_2$, cyclohexane/ethyl acetate, 8:2). The product was recrystallized from cyclohexane (142 mg, 0.47 mmol, 44%). Yellow needles, m.p. 93–94 °C. $R_f$ = 0.55 ($SiO_2$, cyclohexane/acetone, 6:4, with 0.1% $CH_3COOH$); orange stainable with vanillin/$H_2SO_4$, yellow stainable with anisaldehyde. $^1$H NMR (600 MHz, $CDCl_3$): $\delta_H$ = 1.26 (t, $^3J$ = 7.1 Hz, 3 H, 14-$H_3$), 2.21 (tt, $^3J$ = 6.8, 6.4 Hz, 2 H, 10-$H_2$), 2.54 (t, $^3J$ = 6.8 Hz, 2 H, 11-$H_2$), 4.08 (t, $^3J$ = 6.4 Hz, 4 H, 2 H, 9-$H_2$), 4.15 (q, $^3J$ = 7.1 Hz, 2 H, 13-$H_2$), 6.09 (s, 1 H, 3-H), 7.25 (d, $^3J$ = 8.1 Hz, 1 H, 6-H), 7.56 (dd, $^3J$ = 8.1, 7.6 Hz, 1 H, 7-H), 7.64 (d, $^3J$ = 7.6 Hz, 1 H, 8-H), 12.2 (s, 1 H, 5-OH) ppm. $^{13}$C NMR (150 MHz, $CDCl_3$): $\delta_C$ = 14.3 (q, C-14), 23.7 (t, C-10), 30.4 (t, C-11), 60.8 (t, C-13), 68.7 (t, C-9), 110.1 (d, C-3), 114.4 (s, C-4a), 119.6 (d, C-8), 125.2 (d, C-6), 131.2 ($s_q$, C-8a), 135.6 (d, C-7), 160.4 (s, C-2), 161.2 (s, C-5), 172.7 (s, C-12), 179.3 (s, C-1), 191.0 (s, C-4) ppm. MS (EI): *m*/*z* (%) = 304 (4) $[M]^+$, 259 (11) $[M - C_2H_5O]^+$, 231 (10) $[C_{10}H_7O_4]^+$, 190 (14) $[C_{10}H_6O_4]^+$. MS

(ESI$^+$): *m*/*z* (%): 327 (100) [M + Na]$^+$; (ESI$^-$): 303 (30) [M – H]$^-$, 363 (10) [M + $CH_3COO$]$^-$. $C_{16}H_{16}O_6$ (304.30): calcd. C 63.15, H 5.30; found C 63.13, H 5.26.

**2-(3-Carboxypropylamino)-5-hydroxynaphthalene-1,4-dione (Juglonbutin, 2o):** A solution of 4-aminobutyric acid (198 mg, 1.92 mmol) in water (1.92 mL) was added dropwise whilst stirring to a solution of 2-chlorojuglone (**2b**; 200 mg, 0.96 mmol) in $CH_3CN$ (20 mL). The reaction mixture was heated at reflux for 8 h. After cooling, the solvents were removed under vacuum and the residue purified by column chromatography ($SiO_2$, ethyl acetate/$CHCl_3$, 1:1, with 0.1% $CH_3COOH$). The two collected fractions were concentrated in vacuo, diluted with ethyl acetate, and washed with brine. The organic layers were dried with anhydrous $Na_2SO_4$ and the solvent removed under vacuo.

**4-[(2-Chloro-5-hydroxy-1,4-dioxo-3-naphthylamino]butanoic Acid (2p):** Fraction 1, crystallized from 2-propanol (81.2 mg, 0.26 mmol). Red needles, m.p. 173–178 °C. $^1$H NMR (600 MHz, [D$_6$]-DMSO): $\delta_H$ = 1.85 (tt, $^3J$ = 7.3, 7.0 Hz, 2 H, 10-H$_2$), 2.29 (t, $^3J$ = 7.3 Hz, 2 H, 11-H$_2$), 3.77 (dt, $^3J$ = 7.0, 6.6 Hz, 2 H, 9-H$_2$), 7.22 (d, $^3J$ = 8.0 Hz, 1 H, 7-H), 7.49 (br. s, 1 H, 3-NH), 7.51 (d, $^3J$ = 7.8 Hz, 1 H, 8-H), 7.70 (dd, $^3J$ = 8.0, 7.8 Hz, 1 H, 7-H), 11.3 (br. s, 1 H, 12-COOH), 12.0 (br. s, 1 H, 5-OH) ppm. $^{13}$C NMR (150 MHz, [D$_6$]-DMSO): $\delta_C$ = 26.0 (t, C-10), 30.7 (t, C-11), 43.2 (t, C-9), 113.6 (s, C-4a), 118.3 (d, C-8), 122.3 (d, C-6), 132.4 (s, C-8a), 137.1 (d, C-7), 144.9 (s, C-3), 145.0 (s, C-2), 160.6 (s, C-5), 174.0 (s, C-12), 175.0 (s, C-1), 183.5 (s, C-4) ppm. MS (ESI$^-$): *m*/*z* (%) = 308 (90) [M – H]$^-$; (ESI$^+$): 310 (36) [M + H]$^+$, 332 (56) [M + Na]$^+$, 366 (12) [M + K + $H_2O$]$^+$. $C_{14}H_{12}ClNO_5$ (309.70): calcd. C 54.29, H 3.91, N 4.52; found C 54.66, H 3.57, N 4.57. $R_f$ = 0.22 ($SiO_2$, ethyl acetate/$CHCl_3$, 1:1, with 0.1% $CH_3COOH$); red stainable with vanillin/$H_2SO_4$, brown stainable with anisaldehyde.

**4-(5-Hydroxy-1,4-dioxo-2-naphthylamino)butanoic Acid (Juglonbutin, 2o):** Fraction 2 crystallized from *n*-butanol (50.8 mg, 185 mmol). Red crystal squares, m.p. 228–230 °C (ref.[16] 228–230 °C). $R_f$ = 0.15 ($SiO_2$, ethyl acetate/$CHCl_3$, 1:1, with 0.1% $CH_3COOH$); pink stainable with vanillin/$H_2SO_4$, red stainable with anisaldehyde. $^1$H NMR (600 MHz, [D$_6$]DMSO): $\delta_H$ = 1.80 (tt, $^3J$ = 7.2, 6.9 Hz, 2 H, 10-H$_2$), 2.31 (t, $^3J$ = 7.2 Hz, 2 H, 11-H$_2$), 3.24 (dt, $^3J$ = 6.9, 6.4 Hz, 2 H, 9-H$_2$), 5.68 (s, 1 H, 3-H), 7.25 (d, $^3J$ = 8.0 Hz, 1 H, 6-H), 7.49 (d, $^3J$ = 7.5 Hz, 1 H, 8-H), 7.56 (dd, $^3J$ = 8.0, 7.5 Hz, 1 H, 7-H), 8.00 (t, $^3J$ = 6.4 Hz, 1 H, 2-NH), 12.08 (br. s, 1 H, 12-OH), 13.41 (s, 1 H, 5-OH) ppm. $^{13}$C NMR (150 MHz, [D$_6$]-DMSO): $\delta_C$ = 22.6 (t, C-10), 30.8 (t, C-11), 41.4 (t, C-9), 98.0 (d, C-3), 114.4 (s, C-4a), 118 (d, C-8), 125.0 (d, C-6), 130 (s, C-8a), 134.0 (d, C-7), 149.7 (s, C-2), 160.1 (s, C-6), 174.0 (s, C-12), 180.6 (s, C-1), 187.8 (s, C-4) ppm. MS (ESI$^-$): *m*/*z* (%) = 274 (100) [M – H]$^-$; (ESI$^+$): *m*/*z* (%) = 276 (60) [M + H]$^+$, 298 (44) [M + Na]$^+$, 314 (12) [M + K]$^+$, 332 (22) [M + K·$H_2O$]$^+$. $C_{14}H_{13}NO_5$ (275.26): calcd. C 61.09, H 4.76, N 5.09; found C 61.53, H 4.77, N 5.06.

**2-Methoxy-5-(ethoxycarbonylmethoxy)naphthalene-1,4-dione (2q):** 2-Methoxyjuglone (**2c**; 300 mg, 1.47 mmol) was dissolved in a suspension of DMF (17.3 mL) and $K_2CO_3$ (483 mg, 1.47 mmol). Whilst stirring, ethyl bromoacetate (315 μL, 2.94 mmol) was added and the reaction mixture was stirred for a further 20 h, poured onto ice (50 mL) and the pH value was adjusted from pH 11 to pH 6 with HCl (2 N, 1 mL). The yellow precipitate was dissolved by addition of ethyl acetate. The organic layer was separated, dried with $Na_2SO_4$, and concentrated in vacuo. Column chromatography ($SiO_2$, 3 × 25 cm, pentane/ethyl acetate, 1:1) led to two fractions. The first fraction contained 2-methoxyjuglone (**2c**; 12.5 mg) and the second fraction was that of the product **2q**, which was obtained, after crystallization from methanol, as yellow crystals (161 mg, 38%), m.p. 134–135 °C. $R_f$ = 0.37 ($SiO_2$, cyclohexane/acetone, 6:4, with 0.1% $CH_3COOH$); orange stainable with vanillin/$H_2SO_4$, yellow stainable with anisaldehyde. $^1$H NMR (600 MHz, $CDCl_3$): $\delta_H$ = 1.29 (t, $^3J$ = 7.2 Hz, 3 H, 13-H$_3$), 3.86 (s, 3 H, 9-OCH$_3$), 4.27 (q, $^3J$ = 7.2 Hz, 2 H, 12-H$_2$), 4.80 (s, 2 H, 10-H$_2$), 6.10 (s, 1 H, 3-H), 7.21 (dd, $^3J$ = 8.5, $^4J$ = 0.8 Hz, 1 H, 6-H), 7.61 (dd, $^3J$ = 8.5, 7.9 Hz, 1 H, 7-H), 7.86 (dd, $^3J$ = 7.9, $^4J$ = 0.8 Hz, 1 H, 8-H) ppm. $^{13}$C NMR (150 MHz, $CDCl_3$): $\delta_C$ = 14.4 (q, C-13), 56.5 (q, C-9), 61.8 (t, C-12), 67.0 (t, C-10), 112.2 (s, C-3), 120.6 (d, C-6), 121.2 (d, C-8), 121.4 (s, C-4a), 133.6 (s, C-8a), 134.2 (d, C-7), 157.7 (s, C-5), 158.7 (s, C-2), 168.4 (s, C-11), 180.3 (s, C-1), 184.2 (s, C-4) ppm. MS (EI): *m*/*z* (%) = 290 (22) [M]$^+$, 261 (27) [M – $C_2H_5$]$^+$, 217 (100) [M – $C_3H_5O_2$]$^+$. $C_{15}H_{14}O_6$ (290.27): calcd. C 62.07, H 4.86; found C 61.79, H 4.65.

**5,8-Dimethoxynaphthalene-1,4-dione (4b):** $Ag_2O$ (500 mg, 2.20 mmol) was added portionwise to a solution of 5,8-dihydroxy-1,4-naphthoquinone (**4a**; 546 mg, 2.90 mmol) in $CHCl_3$ (40 mL), followed by the dropwise addition of methyl iodide (2.0 mL, 32.1 mmol). The reaction mixture was heated to 60–65 °C for 4 d, during which time further $Ag_2O$ (4 × 500 mg, 8.80 mmol) and methyl iodide (3 × 1.5 mL, 72.5 mmol) were added. TLC-analysis ($SiO_2$, cyclohexane/$CHCl_3$/methanol, 7:2:1). After completion of the reaction, the mixture was filtered through Celite (4 g) and washed with $CHCl_3$ The filtrate was concentrated in vacuo (474 mbar, 40 °C) and the orange residue was crystallized from *n*BuOH as orange needles of **4b** (430 mg, 1.97 mmol, 69%), m.p. 157–159 °C (ref.[36] 155 °C). $R_f$ = 0.16 ($SiO_2$, cyclohexane/$CHCl_3$/methanol, 7:2:1); violet stainable with vanillin/$H_2SO_4$, brown stainable with anisaldehyde. $^1$H NMR (400 MHz, $CDCl_3$): $\delta_H$ = 3.96 (s, 6 H, $CH_3$, 5-OCH$_3$, 8-OCH$_3$), 6.78 (s, 2 H, 2-H, 3-H), 7.32 (s, 2 H, 6-H, 7-H) ppm. $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_C$ = 57.0 (q, 5-OCH$_3$, 8-OCH$_3$), 120.5 (s, C-4a, C-8a), 138.5 (d, C-2, C-3, C-6, C-7), 153.8 (s, C-5, C-8), 185.0 (s, C-1, C-4) ppm. MS (EI): *m*/*z* (%) = 218 (100) [M]$^+$, 189 (37) [$C_{10}H_5O_4$]$^+$, 172 (41) [$C_{10}H_4O_3$]$^+$. $C_{12}H_{10}O_4$ (218.21): calcd. C 66.05, H 4.62; found C 66.70, H 4.68.

**2,5,8-Trimethoxynaphthalene-1,4-dione (4c):** A suspension of $H_2SO_4$ (conc., 3.0 mL) and $Fe_2(SO_4)_3$ (800 mg, 0.2 mmol) was added to a solution of 5,8-dimethoxy-1,4-naphthoquinone (**4b**; 218 mg, 100 μmol) in dioxane (30 mL) and methanol (15 mL), and the reaction mixture was heated at reflux for 2 h. The reaction was monitored by TLC ($SiO_2$, $CHCl_3$/ethyl acetate/methanol, 6:3:1). The reaction mixture was poured onto water (100 mL), extracted with $CHCl_3$, and dried with $Na_2SO_4$. The solvent was evaporated (474 mbar, 40 °C) and the residue purified by column chromatography ($SiO_2$, 3.0 × 30 cm, $CHCl_3$/ethyl acetate/methanol, 6:3:1). Two fractions were obtained, the second one containing 2,5,8-trimethoxy-1,4-naphthoquinone (**4c**), which gave orange-colored crystals following crystallization from toluene (248 mg, 1.06 mmol, 46%), m.p. 173–174 °C (ref.[37] 165 °C). $^1$H NMR (600 MHz, $CDCl_3$): $\delta_H$ = 3.82 (s, 3 H, 2-OCH$_3$), 3.94 (s, 6 H, 5-OCH$_3$, 8-OCH$_3$), 6.00 (s, 1 H, 3-H), 7.25 (d, $^3J$ = 9.4 Hz, 1 H, 7-H), 7.32 (d, $^3J$ = 9.4 Hz, 1 H, 6-H) ppm. $^{13}$C NMR (150 MHz, $CDCl_3$): $\delta_C$ = 56.4 (q, 2-OCH$_3$), 57.0 (q, 8-OCH$_3$[a]), 57.1 (q, 5-OCH$_3$[a]), 110.2 (s, C-3), 119.8 (s, C-7), 120.5 (d, C-8a), 121.0 (s, C-4a), 121.4 (d, C-6), 153.5 (s, C-5), 154.5 (s, C-8), 159.4 (s, C-2), 179.5 (s, C-1), 184.9 (s, C-4) ppm. MS (EI): *m*/*z* (%) = 248 (100) [M]$^+$, 233 (60) [M – $CH_3$]$^+$. $C_{13}H_{12}O_5$ (248.23): calcd. C 62.90, H 4.87; found C 63.11, H 4.89. $R_f$ = 0.59 ($SiO_2$, $CHCl_3$/ethyl acetate/methanol, 6:3:1); violet stainable with vanillin/$H_2SO_4$, brown stainable with anisaldehyde. [a] Signals are changeable.

**5,8-Dihydroxy-2-methoxynaphthalene-1,4-dione (4d):** AgO (1.20 g, 9.69 mmol) was added portionwise whilst stirring to a solution of

2,5,8-trimethoxy-1,4-naphthoquinone (**4c**; 217 mg, 0.87 mmol) in acetone (34 mL). After cooling to 0 °C, $HNO_3$ (11.3 mL, 40%) was added dropwise. The reaction mixture was stirred for 15 min at room temp., diluted with water (1:2), and finally extracted with $CHCl_3$ (3×). The organic layer was washed with brine and water (2×), and dried with $Na_2SO_4$. The solvent was evaporated and the residue purified by column chromatography ($SiO_2$, 1 × 20 cm, $CHCl_3$/ethyl acetate, 6:4). The product was obtained after recrystallization from 2-propanol. Black needles (52.0 mg, 236 μmol, 27%), m.p. 192–193 °C (ref.[37] 190–195 °C). $R_f$ = 0.60 ($SiO_2$, cyclohexane/acetone, 6:4, with 0.1% $CH_3COOH$), 0.75 ($SiO_2$, $CHCl_3$/ethyl acetate, 6:4); pink stainable with vanillin/$H_2SO_4$, red stainable with anisaldehyde. $^1$H NMR (600 MHz, $CDCl_3$): $\delta_H$ = 3.92 (s, 3 H, 2-$OCH_3$), 6.16 (s, 1 H, 3-H), 7.19 (d, $^3J$ = 9.4 Hz, 1 H, 7-H), 7.26 (d, $^3J$ = 9.4 Hz, 1 H, 6-H), 12.2 (s, 1 H, 8-OH), 12.6 (s, 1 H, 5-OH) ppm. $^{13}$C NMR (150 MHz, $CDCl_3$): $\delta_C$ = 56.9 (q, 2-$OCH_3$), 110.4 (s, C-3), 110.6 (s, C-4a), 111.5 (s, C-8a), 128.5 (d, C-7), 131.0 (d, C-6), 157.1 (d, C-5), 158.6 (s, C-8), 161.1 (s, C-2), 182.6 (s, C-4), 188.4 (s, C-1) ppm. MS (EI): *m*/*z* (%) = 220 (100) $[M]^+$, 202 (29) $[M - H_2O]^+$. $C_{11}H_8O_5$ (220.18): calcd. C 60.00, H 3.66; found C 59.39, H 3.63.

**5,8-Bis(ethoxycarbonylmethoxy)-2-methoxynaphthalene-1,4-dione (4e):** Ethyl iodoacetate (85.0 μL, 155 mg, 0.72 mmol) was added to a suspension of 5,8-dihydroxy-2-methoxy-1,4-naphthoquinone (**4d**; 28.2 mg, 0.13 mmol) and $K_2CO_3$ (45.3 mg, 0.33 mmol) in DMF (3.0 mL). The reaction mixture was stirred at room temp. for 3 d before further $K_2CO_3$ (11.3 mg, 0.82 mmol) was added and stirring was continued for an additional 2 d at the same temperature. The reaction mixture was then diluted with water (30 mL) and extracted with $CHCl_3$. The organic layer was washed with brine and dried with $Na_2SO_4$. After evaporation of the solvent the product was purified by column chromatography (Combi Flash retrieve, 40 g $SiO_2$ column, $CHCl_3$/ethyl acetate, 6:4) to give, after crystallization, orange crystals (17.5 mg, 44.6 μmol, 25%), m.p. 74–76 °C. $R_f$ = 0.53 ($SiO_2$, cyclohexane/acetone, 6:4, with 0.1% $CH_3COOH$), 0.43 ($SiO_2$, $CHCl_3$/ethyl acetate, 6:4); grey-brown stainable with vanillin/$H_2SO_4$, orange-brown stainable with anisaldehyde. $^1$H NMR (600 MHz, $CDCl_3$): $\delta_H$ = 1.28 (t, $^3J$ = 7.0 Hz, 6 H, 13-$H_3$, 17-$H_3$), 3.83 (s, 3 H, 9-$H_3$), 4.25 (q, $^3J$ = 7.0 Hz, 4 H, 12-$H_2$, 16-$H_2$), 4.71 (s, 4 H, 10-$H_2$, 14-$H_2$), 6.02 (s, 1 H, 3-H), 7.21 (d, $^3J$ = 9.3 Hz, 1 H, 6-H), 7.29 (d, $^3J$ = 9.3 Hz, 1 H, 7-H) ppm. $^{13}$C NMR (150 MHz, $CDCl_3$): $\delta_C$ = 14.3 (q, C-13, C-17), 56.4 (q, 2-$OCH_3$), 61.6 (t, C-12, C-16), 68.0 (t, C-10), 68.3 (t, C-14), 110.2 (d, C-3), 122.1 (s, C-8a), 122.8 (s, C-4a), 123.4 (s, C-7), 125.5 (s, C-6), 152.9 (s, C-8), 153.9 (s, C-5), 159.5 (s, C-2), 168.6 (s, C-11), 168.8 (s, C-15), 179.0 (s, C-1), 184.1 (s, C-4) ppm. MS (EI): *m*/*z* (%) = 392 (68) $[M]^+$, 319 (85) $[M - C_3H_5O_2]^+$, 305 (100) $[M - C_4H_7O_2]^+$, 245 (78) $[C_{12}H_5O_6]^+$, 189 (17) $[C_{10}H_5O_4]^+$. $C_{19}H_{20}O_9$ (392.36): calcd. C 58.16, H 5.14; found C 58.12, H 5.07.

### Biological Assays

**Primary Activity Profiling:** The results and experimental procedures for the antibacterial agar diffusion assays, enzyme assays and three cell line assays are given in the Supporting Information.

**In Vitro Antitumor Activity Assays:** A modified propidium iodide assay was used to determine the cytotoxic activity of the compounds against human tumor cell lines. The test procedure has been described elsewhere.[29] The cell lines tested were derived from patient tumors engrafted as a subcutaneously growing tumor in NMR I nu/nu mice or obtained from the American Type Culture Collection, Rockville, MD, USA, the National Cancer Institute, Bethesda, MD, USA, or the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. In brief, human tumor cell lines were grown at 37 °C in a humidified atmosphere (95% air, 5% $CO_2$) in monolayer cultures in RPMI 1640 medium supplemented with 10% FCS and Phenol Red (PAA, Cölbe, Gemany). Cells were trypsinized and maintained weekly. They were harvested from exponentially growing cultures by trypsination, counted, and plated in 96-well flat-bottomed microplates (140 μL cell suspension, $5 \times 10^3$ to $10 \times 10^3$ cells/well). After a 24 h recovery to allow cells to resume exponential growth, 10 μL of culture medium (6 control wells per plate) or medium containing the test drug were added to the wells. Each drug concentration was plated in triplicate. After 4 d of incubation the culture medium was replaced by a fresh medium containing 6 μg/mL of propidium iodide. Microplates were then kept at –18 °C for 24 h to determine the total cell kill. After thawing of the plates, the fluorescence was measured by using the Cytofluor 4000 microplate reader (Perseptive Biosystems; excitation 530 nm, emission 620 nm). The number of viable cells was proportional to the fluorescence intensity.

### Mechanisms of Action

**Cell Cultures:** The cell lines HCT-116 (human colon carcinoma) and U-2 OS (human osteosarcoma) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and were cultured under the conditions recommended by the depositor. Reagents were purchased from Sigma–Aldrich and plastic ware was obtained from Nunc, BD Falcon, and Sarstedt AG & Co.

**MTT Assay:** Cells were seeded at $6 \times 10^3$ cells per well in 96-well plates in 180 μL complete medium and directly treated with varying concentrations of **2f** or **2j** diluted in MeOH. The compounds were tested in duplicate as well as the internal vesicle control. After treatment for 5 d, 20 μL of 5 mg/mL MTT (Thiazolyl Blue tetrazolium bromide) in PBS was added per well and further incubated for 2 h at 37 °C. The medium was then discarded and cells were washed with 100 μL PBS (phosphate-buffered saline, pH 7.4) before adding 100 μL 2-propanol/10 N HCl (250:1) in order to dissolve formazan granules. The absorbance at 570 nm was measured by using a microplate reader (EL808, BioTek Instruments Inc.) and cell viability was expressed as a percentage relative to the respective methanol control.

**Cell Cycle Analysis:** Cells were seeded at $10^5$ cells per well in 6-well plates in 5 mL complete medium. After a 12 h recovery period, cells were treated for 3 d with varying concentrations of **2f** or **2j** diluted in MeOH. Approximately $10^6$ cells were harvested by centrifugation, washed with ice-cooled PBS, and fixed overnight at –20 °C with 80% MeOH. After fixation, MeOH was removed completely and the cell pellet was washed first with PBS and then with 0.1% saponine in PBS. For DNA staining, cells were incubated for 30 min at 37 °C in a PBS solution containing 20 μg/mL propidium iodide and 100 μg/mL RNase A. The samples were analyzed by a flowcytometric system (EasyCyte Plus, Guava Technologies). In total, 5000 viable cells were acquired per sample and cell cycle histograms were generated after exclusion of cell debris using a Watson algorithm of FlowJo 7.2.6 software (Tree Star Inc.).

**Immunofluorescence:** U-2 OS cells were seeded at $5 \times 10^3$ cells per well in 96-well imaging plates (BD Falcon). At approximately 70% confluency the cells were treated with **2f** or **2j** at the given concentrations and treatment periods indicated for each experiment. Cells were fixed with cold (–20 °C) acetone/MeOH (1:1) for 10 min. After washing with PBS, cells were permeabilized by using 0.01% Triton-X 100 in PBS. The following combinations of primary and secondary antibodies were used: α-tubulin mAb (1:2000, Sigma)/goat-anti-mouse-Alexa488 (1:1000, Molecular Probes); β-actin mAb (1:2000, Cell Signaling)/goat-anti-rabbit-Alexa488 (1:1000, Molecular Probes); phospho-Histone γH2A·X (Ser139) mAb

(1:400, Millipore)/goat-anti-mouse-Alexa488 (1:1000, Molecular Probes). For labelling, cells were incubated with a primary antibody for 45 min at 37 °C, followed by a secondary antibody under the same incubation conditions. The nuclei were stained with Hoechst33342 (5 μg/mL; Molecular Probes) and a whole cell stain (1 μg/mL HCS CellMask™ Red stain, Invitrogen) was used to facilitate later cell segmentation. The samples were imaged on an automated microscope (BD Pathway855) suitable for high-content screening with appropriate filter sets for Alexa488, Hoechst, and rhodamine.

**Level of Reactive Oxygen Species (ROS):** The antioxidative potential of naphthoquinones was evaluated by a counter-screening approach by using a fluorescence-based assay. Reduced, acetylated 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) was deacetylated by cytosolic esterases of living cells to give 2′,7′-dichlorodihydrofluorescein (DCF), which can be oxidized by reactive oxygen species (ROS) to the fluorescent 2′,7′-dichlorofluorescein (DCF). The concentration of DCF generated is directly proportional to the concentration of ROS in a cell.

U-2 OS cells were seeded into 96-well imaging plates (BD Falcon) at a concentration of $5 \times 10^3$ cells per well in McCoy's 5A medium (10% FBS) and incubated for 2 d at 37 °C and 5% $CO_2$. The cells were washed with KRH buffer (119 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM $KH_2PO_4$, 10mM HEPES, 2 mM D-glucose; pH 7.4) and incubated with 10 μM DCFDA in KRH for 1.5 h at 37 °C. Next, the cells were washed twice with KRH buffer to remove extracellular dye. Test compounds in KRH buffer were added at the indicated concentrations (75 μL/well) and the cells were incubated for 1 h at 37 °C. Then 200 μM tBHP in KRH (25 μL/well) was added for 30 min to induce oxidative stress. Resveratrol was used as a positive control. A live-cell assay was carried out by using the Pathway Bioimager 855 (BD, Heidelberg). The fluorescence intensity was quantified for each sample at 535 nm upon excitation with a wavelength of 485 nm.

**Topoisomerase II Assay:** The assay for the determination of the inhibitory activity of **2c**, **2f**, **2g**, and **2j** was performed according to Li et al. with minor modifications.[38] The assay is based on the conversion of supercoiled DNA to relaxed DNA by the topoisomerase II activity. Purified Human DNA Topoisomerase IIa (p170 form) was obtained from TopoGEN and supercoiled pBR322 plasmid DNA was purchased from Fermentas GmbH. The master-mix contained 50 mM Tris HCl (pH 8), 120 mM KCl, 10 mM $MgCl_2 \cdot 6H_2O$, 0.5 mM DL-dithiothreitol, 0.5 mM ATP, and 0.3 μg pBR322 plasmid DNA and placed on ice. Then 10 μg of **2c**, **2f**, **2g**, and **2j**, respectively, dissolved in DMSO were added. The positive control was carried out by using etoposide at concentrations of 10 and 50 μM, respectively. Etoposide is a widely used anticancer agent targeting human DNA topoisomerase type II.[39] For the negative control, DMSO solution (0.1%) was used. The reaction was started by the addition of topoisomerase IIa (2 U) at a final volume of 20 μL. After incubation for 30 min at 37 °C, the reaction was stopped by adding 5 μL buffer [0.77% SDS; 77 mM EDTA/Na (pH 8); 30% sucrose; 0.5% Bromphenol Blue; 0.5% xylene cyanole]. The enzyme activity was visualized by investigating the reaction products (20 μL) on an agarose gel (1%) in TBE (Tris/borate/EDTA) running buffer for 25 h at 15 V and staining with Sybr safe (30 min at 50 rpm). The relaxation activity of the topoisomerase II was quantified by using the Gel-Pro Analyzer (Media Cybernetics) by calculating the area representing the supercoiled pBR322 plasmid DNA.

**Supporting Information** (see footnote on the first page of this article): Additional information on bilogical activity and spectroscopic data.

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