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Synthesis and Structure–Activity Relationships of Lapacho Analogues. 1. Suppression of Human Keratinocyte Hyperproliferation by 2-Substituted Naphtho[2,3-*b*]furan-4,9-diones, Activation by Enzymatic One- and Two-Electron Reduction, and Intracellular Generation of Superoxide

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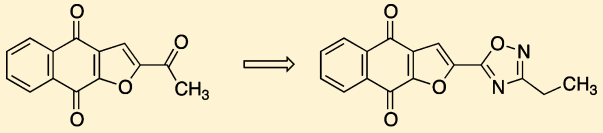
Supporting Information

ABSTRACT: A series of linearly anellated lapacho quinone analogues substituted at the 2-position of the tricyclic naphtho-[2,3-*b*]furan-4,9-dione system were synthesized and evaluated for their ability to suppress keratinocyte hyperproliferation using HaCaT cells as the primary test system. While very good in vitro potency with IC_{50} values in the submicromolar range was attained with electron-withdrawing substituents, some compounds were found to induce plasma membrane damage, as evidenced by the release of LDH activity from cytoplasm of the keratinocytes. The most potent analogue against keratinocyte hyperproliferation was the 1,2,4-oxadiazole **18**, the potency of which was combined with comparably low cytotoxic membrane damaging effects. Structure–activity relationship studies with either metabolically stable or labile analogues revealed that the quinone moiety was required for activity. Selected compounds were studied in detail for their capability to generate superoxide radicals both in isolated enzymatic one- and two-electron reduction assays as well as in a HaCaT cell-based assay.

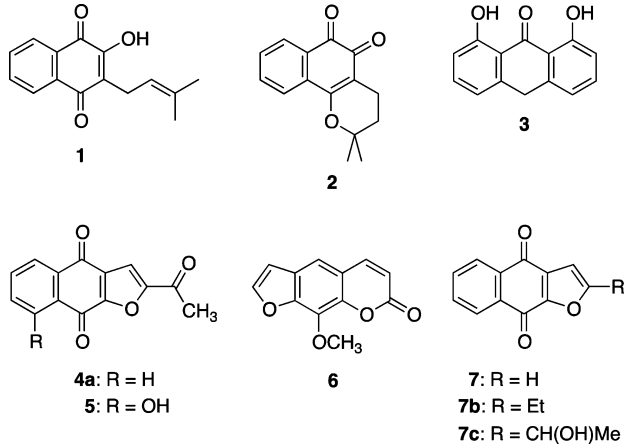


INTRODUCTION

Lapacho is a commercial natural product obtained from the inner bark and heartwood of Tabebuia trees indigenous to the Amazonian rainforest.[1,2] It is commonly known as "pau d'arco", "ipê-roxo" or "taheebo" and has been used as a longtime folk medicine in South America against malignant, inflammatory, infectious, stomach, and skin diseases.[1,2] The stem bark of the tree shows a wide range of biological properties such as antibacterial, antifungal, and antiinflammatory and, in particular, antitumor activity.[3−6] Today, lapacho is acclaimed to be a wonder drug for curing cancer[2] and also used against disorders of the immune system, for example psoriasis.[1]

Lapacho contains a number of naturally occurring quinones, of which lapachol (**1**) and the angularly anellated *β*-lapachone (**2**) are the research molecules of interest, as these naphthoquinones have been related to the anticancer molecular pharmacology of the plant extract.[2] Among the most significant quinones of the lapacho extract, *β*-lapachone also displayed activity comparable to that of the antipsoriatic drug anthralin (**3**) against the growth of human keratinocytes, which we have reported in an earlier paper.[7] While lapachol was inactive, however, the linearly anellated naphtho[2,3-*b*]furan-4,9-diones **4a** and **5** (Chart 1) were among the most potent lapacho constituents. Moreover, activity against tumor cell lines of naphtho[2,3-*b*]furan-4,9-diones[8] has stimulated the attention of medicinal chemists, and as a consequence, several synthetic analogues have been described.[9−14]

Chart 1. Quinones Derived from Lapacho (1, 2, 4a, 5), Related Structures (7, 7a, 7b), and Antipsoriatic Drugs (3, 6)



While most of these studies have focused on antitumor activities, the effects of lapacho analogues, their mechanism of action, and structure–activity relationships (SAR) in human

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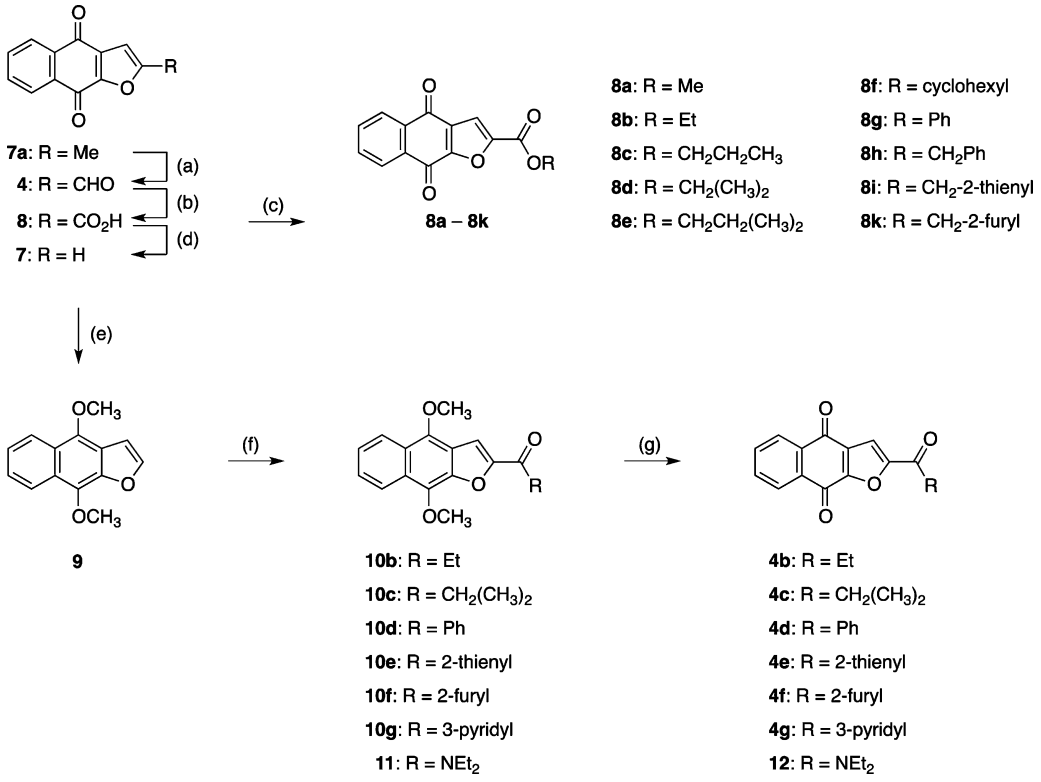
Scheme 1[a]



[a]Reagents: (a) SeO_2, SiO_2, microwave, 120 W; (b) H_2O_2 30%, HOAc, 75 °C; (c) DCC, DMAP, MeOH, appropriate alcohol, 0 °C, RT, 3 h; (d) quinoline, $Cu_2Cr_2O_5$, reflux; (e) $Na_2S_2O_4$, Bu_4NBr, THF, KOH, Me_2SO_4, N_2; (f) *n*-BuLi, THF, −15 °C, appropriate *N*,*N*-dimethylcarboxamide, N_2; (g) $(NH_4)_2[Ce(NO_3)_6]$, MeCN, H_2O, 0 °C.

keratinocytes are still poorly understood. As part of our ongoing interest in tricyclic structures with antiproliferative activity such as anthracenones,[15] naphtho[2,3-*b*]thiophen-4(9*H*)-ones,[16] acridones,[17] phenoxazines, and phenothiazines,[18] we extended our studies to linearly anellated lapacho analogues. Examples for tricyclic antiproliferative agents with proven clinical utility against psoriasis are the anthracenone derivative anthralin (**3**) and the furobenzopyranone derivative 8-methoxypsoralen (**6**).[19,20] Results from our preliminary experiments revealed that from the linearly anellated lapacho analogues, the 2-acetyl substituted **4a** showed enhanced activity in comparison to its unsubstituted analogue **7** and its side chain reduced forms, the corresponding alkyl and alcohol analogues **7b** and **7c**, respectively. It is for this reason that we decided to carry out an extensive study to explore the effect of introducing other acyl and bioisosteric functionalities at the 2-position of the tricyclic furan ring system.

With respect to psoriasis, a common, immune-mediated inflammatory and scaling skin disease, mainly characterized by excessive growth of keratinocytes,[21] we have tested the novel 2-substituted naphtho[2,3-*b*]furan-4,9-diones as inhibitors of keratinocyte hyperproliferation, which is one of the hallmarks of the disease. We also have measured the generation of superoxide radicals after incubation of representative analogues via both one- and two-electron transfer by flavoenzymes and subsequent autoxidation reactions, as well as their contribution to intracellular superoxide generation in keratinocytes in order to determine their ability to redox cycle. The biological implications of these results with respect to oxidative stress[22] in human keratinocytes and to the potential therapeutic action of lapacho quinone analogues for the treatment of psoriasis or other hyperproliferative skin disorders are discussed.

CHEMISTRY

The starting 2-methyl-naphtho[2,3-*b*]furan-4,9-dione (**7a**) was synthesized following a literature method.[23] Selective oxidation of the methyl group with SeO_2 over a solid support under microwave irradiation[24] gave the carbaldehyde **4**, and this in turn was oxidized to the carboxylic acid **8** using H_2O_2 and glacial acetic acid. 2-Carboxylic ester analogues **8a**−**8k** were obtained from **8** and appropriate alcohols by Steglich esterification (Scheme 1).[25]

As also outlined in Scheme 1, the 2-acyl analogues **4b**−**4g** as well as the carboxamide **12** were prepared from the basic structure **7**. The latter was obtained from acid **8**, which was decarboxylated by means of copper chromite in quinoline in analogy to a literature procedure.[23] Unfortunately, quinone **7** is completely resistant to electrophilic substitution because of the deactivation of the furan ring by the quinone carbonyl groups. Accordingly, to obtain an electron-rich nucleophilic system, **7** was subjected to reductive methylation with dimethyl sulfate and $Na_2S_2O_4$ in the presence of tetrabutylammonium bromide following the procedure of Tanaka.[26]

Next, 4,9-dimethoxynaphtho[2,3-*b*]furan (**9**) was treated with *n*-butyllithium and acylated with the appropriate *N*,*N*-dimethylcarboxamides to afford the 2-acylated naphthohydroquinone dimethyl ethers **10b**−**10g**. With *N*,*N*-diethyl-2,2,2-trifluoroacetamide, the desired trifluoroacetyl analogue could not be isolated. However, the reaction proceeded in an unexpected manner. Thus, carboxamide **11** was formally

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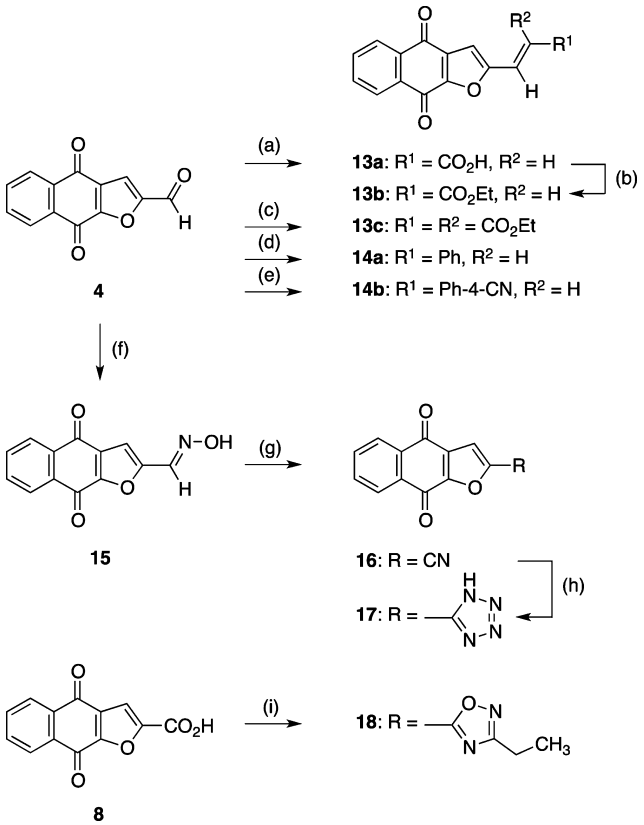
derived from the organolithium intermediate, evidently by elimination of the trifluoromethyl group instead of diethylamine. A similar observation had been described in the literature.[27] Final oxidation of the naphthohydroquinone ethers with diammonium cerium(IV) nitrate proceeded with concomitant ether cleavage to give the desired 2-acylated target compounds **4b**–**4g**. In a similar manner, carboxamide **12** was prepared from **11**.

As a key intermediate for the preparation of 2-substituted naphtho[2,3-*b*]furan-4,9-diones, carbaldehyde **4** was also used for the synthesis of analogues bearing α,β-unsaturated side chains. These were obtained by one of the following synthetic routes shown in Scheme 2. In the first approach, Knoevenagel

Scheme 2[a]



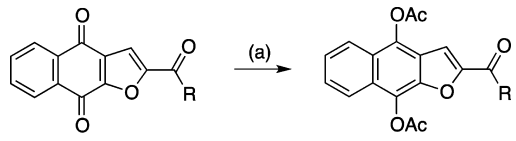
[a]Reagents: (a) malonic acid, pyridine, piperidine 110 °C; (b) DCC, DMAP, EtOH, 0 °C, RT, 3 h; (c) diethyl malonate, benzene, piperidine, HOAc, reflux; (d) $PhCH_2PO(OEt)_2$, NaH, DMSO, N_2, 60 °C; (e) (4-cyanobenzyl)triphenylphosphonium chloride, NaH, DMSO, N_2, 60 °C; (f) $NH_2OH{\cdot}HCl$, K_2CO_3, EtOH 70%; (g) Ac_2O, reflux; (h) NaN_3, NH_4Cl, DMF, 90 °C; (i) DCC, CH_2Cl_2, 0 °C, *N*-hydroxypropionamidine, pyridine, reflux.

condensation[28] with malonic acid in pyridine provided the acrylic acid analogue **13a**. The ^1H NMR showed the characteristic coupling pattern of an ethenyl spacer, and coupling constants of the olefinic protons (J = 16 Hz) indicated compound **13a** was in the *E*-configuration. Esterification gave **13b**, and **13c** was obtained from the reaction of carbaldehyde **4** with diethyl malonate. In the second approach, stilbene **14a** was obtained from the Horner–Emmons reaction[29] of carbaldehyde **4** and diethyl benzylphosphonate. In the third approach, nitrile analogue **14b** was prepared from (4-cyanobenzyl)triphenylphosphonium chloride and carbaldehyde **4** through a Wittig-type[30] olefination reaction. The stereochemical assignment of stilbenes **14a** and **14b** as *E*-isomers was performed through ^1H NMR.

Carbaldehyde **4** was also transformed into the corresponding oxime **15** by treatment with hydroxylamine hydrochloride, which furnished nitrile **16** upon dehydration with acetic anhydride. Introduction of a tetrazole moiety at the 2-position as a bioisostere for the carboxylate group[31] was accomplished according to the procedure described by Lofquist.[32] Thus, nucleophilic attack of NaN_3 on nitrile **16**, followed by ring closure, gave the 5-tetrazole analogue **17** (Scheme 2). Furthermore, 1,2,4-oxadiazole analogue **18** as a bioisosteric replacement of an ester group[33,34] was prepared in a one-pot reaction[35] from the activated carboxylic acid **8** and *N*-hydroxypropionamidine.

Finally, analogues **4b** and **4d** were converted to the corresponding diacetates **19b** and **19d**, respectively, by reductive acetylation[36] using zinc in acetic anhydride (Scheme 3).

Scheme 3[a]



[a]Reagents: (a) Zn, Ac_2O, pyridine.

BIOLOGICAL EVALUATION AND DISCUSSION

Inhibition of Keratinocyte Hyperproliferation. Compounds prepared in this study were evaluated in a keratinocyte culture model. Drugs that suppress keratinocyte hyperproliferation are potentially useful in the treatment of psoriasis because a rebalanced homeostatic control of keratinocyte growth and differentiation is crucial for recovery from psoriatic to normal epidermis.[37] As antihyperproliferative action in cell cultures may be critical in the management of the hyperproliferative component of psoriasis, the sensitivity of HaCaT keratinocytes to each lapacho analogue was determined directly by counting the dispersed cells under a phase-contrast microscope after 48 h of treatment. HaCaT is a rapidly dividing immortalized human keratinocyte line,[38] which mimics the hyperproliferative epidermis found in psoriasis, as it exhibits a similar keratinization pattern to psoriatic skin, and these cells have been widely used as a suitable model for the preclinical evaluation of novel antipsoriatic agents.[39] The biological data for the novel compounds are summarized in Table 1.

In this model, a total of 38 naphtho[2,3-*b*]furan-4,9-diones and related structures were tested, of which 15 showed activity in the low micromolar range (IC_{50} < 1 μmol/L). In an earlier report, we established the importance of having a 2-acetyl functionality at the furan moiety of the tricyclic system, in enhancement of the antihyperproliferative activity.[7] We have now undertaken a study directed toward understanding the SAR at the 2-position of the tricyclic ring system. Introduction of alkyl substituents such as methyl (**7a**) or ethyl (**7b**) was disadvantageous to suppression of keratinocyte hyperproliferation as compared to unsubstituted **7**. However, potency clearly

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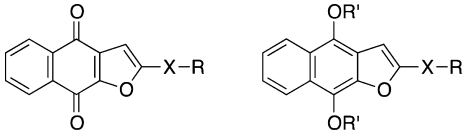
Table 1. Suppression of Human Keratinocyte Hyperproliferation by 2-Substituted Naphtho[2,3-*b*]furan-4,9-dione Analogues of Lapacho and Related Structures



10b,d, 19b,d

compd	X	R	AA[a] IC_{50} (μmol/L)	LDH[b] (mU/mL)
4	CO	H	2.76	186[c]
4a	CO	Me	0.50	255[c]
4b	CO	Et	0.63	270[c]
4c	CO	CH_2Me_2	0.44	*d*
4d	CO	Ph	0.72	318[c]
4e	CO	2-thienyl	0.67	169[c]
4f	CO	2-furyl	0.55	167[c]
4g	CO	3-pyridyl	0.37	202[c]
7	H		3.85	*d*
7a	Me		7.54	*d*
7b	Et		12.18	*d*
7c[e]	CHOH	Me	3.70	*d*
8	CO	OH	>30	*d*
8a	CO	OMe	0.92	190[c]
8b	CO	OEt	0.61	187[c]
8c	CO	*O*-*n*-Pr	1.32	*d*
8d	CO	OCH_2Me_2	0.99	*d*
8e	CO	$OCH_2CH_2Me_2$	2.86	*d*
8f	CO	*O*-cyclohexyl	2.93	*d*
8g	CO	OPh	>30	*d*
8h	CO	OCH_2Ph	>30	*d*
8i	CO	OCH_2-2-thienyl	14.34	*d*
8k	CO	OCH_2-2-furyl	8.23	*d*
10b	CO	Et, R′ = Me	>30	*d*
10d	CO	Ph, R′ = Me	>30	*d*
12	CO	NEt_2	0.24	226[c]
13a	CH=CH	CO_2H	>30	*d*
13b	CH=CH	CO_2Et	2.51	*d*
13c	CH=C	$(CO_2Et)_2$	1.11	186[c]
14a	CH=CH	Ph	>30	*d*
14b	CH=CH	Ph-4-CN	7.73	*d*
15	CH=	N–OH	2.34	*d*
16	CN		0.89	181[c]
17	5-(1*H*-tetrazole)		>30	380[c]
18	5-(1,2,4-oxadiazole)	3-Et	0.17	157[c]
19b	CO	Et, R′ = Ac	0.80	185[c]
19d	CO	Ph, R′ = Ac	0.92	193[c]
20	NO_2		2.00	*d*
anthralin			0.70	239[c]
β-lapachone			0.90	268[c]
menadione			15.80	*d*

[a]Antihyperproliferative activity against keratinocytes. IC_{50}, concentration of test compound required for 50% inhibition of cell growth (HaCaT). Inhibition of cell growth was significantly different with respect to that of the control, $N = 3$, $P < 0.05$. [b]Activity of LDH (mU) release in HaCaT cells after treatment with 2 μmol/L test compound ($N = 3$, SD < 10%); [c]Values are significantly different with respect to vehicle control (0.2% DMSO in the culture medium, 110 mU/mL), $P < 0.05$. Brij 35 (polyoxyethyleneglycol dodecyl ether)/ultrasound was the positive control (409[c] mU/mL). [d]Not determined. [e]Reference 7.

increased in the series **7b** (alkyl, IC_{50} 12.18 μmol/L) < **7c** (alcohol, IC_{50} 3.70 μmol/L) < **4a** (ketone, IC_{50} 0.50 μmol/L), i.e., with the degree of oxidation of the functional group-bearing carbon of the 2-substituent. Once we had determined that a carbonyl group at the 2-position was optimal for substitution, we decided to investigate the breadth of substituents that would not only maintain or enhance the potency but also ideally show low cytotoxicity in the LDH assay as stated below. Thus, compounds **4b–4g** explore the consequences of varying the substituent attached to the 2-carbonyl group. There appears to be no special requirement for the nature of this group, as all compounds were active in the low micromolar range. Small and branched alkyl groups (**4b**, **4c**), and similarly, phenyl (**4d**) or heterocyclic rings (**4e–4g**), were well tolerated. The nicotinyl (3-pyridyl) analogue **4g** (IC_{50} 0.37 μmol/L) was the strongest suppressor of keratinocyte hyperproliferation in the 2-acyl series. However, exchanging the methyl group of ketone **4a** for a hydroxyl group to give the acid **8** resulted in lack of cellular activity. This may be attributed to the inability of the free acid to cross the cellular membrane. Indeed, there was a clear distinction in activity between the free acids and their corresponding esters. Thus, comparing acid **8** with its esters **8a–8k**, activity was restored when the carboxyl group was esterified, with the ethyl ester **8b** (IC_{50} 0.61 μmol/L) being the most potent suppressor of keratinocyte hyperproliferation of all esters studied. Lengthening, branching, or cyclizing the alkyl chain of **8b**, as in **8c**,**d** and **8e** or **8f**, respectively, decreased potency up to 5-fold, while a terminal phenyl ring (**8g** and **8h**) was detrimental to activity. Also, replacement of the phenyl ring of **8h** with a thiophene (**8i**) or a furan ring (**8k**) gave only moderately active compounds.

Compounds **13a–13c** explore the effect of inserting a double bond between the furan moiety of the tricyclic system and the carboxyl group. While the free acrylic acid **13a** had no significant effect on keratinocyte hyperproliferation, its corresponding ethyl ester **13b** as well as ester **13c** demonstrated activity in this assay, supporting the hypothesis that cellular ineffectiveness of the free acids **8** and **13a** was due to insufficient cell penetration. Furthermore, the stilbenes **14a** and **14b** were inactive or only moderately potent in the HaCaT hyperproliferation assay. Moreover, when replacing the carboxylic ester of **8b** by an amide group, potency was enhanced. Thus, diethyl amide **12** (IC_{50} 0.24 μmol/L) was among the most potent compounds of this series.

We next investigated the influence of electron-withdrawing groups other than a carbonyl at the 2-position. While the nitro derivative **20** and the oxime **15** were about 4-fold less potent than the genuine lapacho constituent **4a**, nitrile **16** displayed activity in the low micromolar range. Encouraged by this result, we also looked for bioisosteric replacement for the carboxylic acid moiety in **8**. For example, replacement with a tetrazole is well-known and can improve permeability, as this group is almost 10 times more lipophilic while having similar acidity to that observed for carboxylic acids.[31] However, a tetrazole substituent (**17**) did not result in any appreciable activity. Finally, oxadiazoles have been used as bioisosteric replacements for carbonyl containing compounds such as esters or amides.[33,34] Therefore, we decided to incorporate a 1,2,4-oxadiazole moiety to mimic the ester or amide substituent of **8b** and **12**, respectively. This resulted in oxadiazole **18**, which was the most potent suppressor of keratinocyte hyperproliferation in this series. With an IC_{50} of 0.17 μmol/L, it was slightly more potent than amide **12** and 4- and 5-fold more potent than the

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standard antipsoriatic agent anthralin and the naturally occurring lapacho compound β-lapachone (Table 1), respectively.

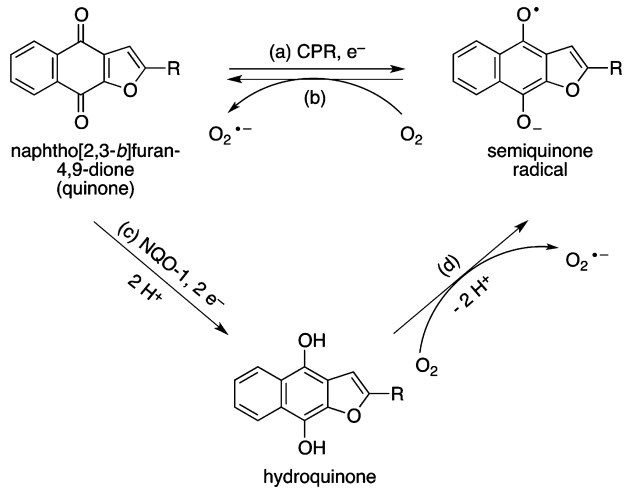
Compounds **10b** and **10d** as well as **19b** and **19d** (Table 1) were designed to elucidate the role of the quinone moiety in the biological action of the lapacho analogues. Both pairs of compounds are closely related congeners of their parent 2-acylnaphtho[2,3-*b*]furan-4,9-diones **4b** and **4d**, the biological activity of which was supposed to be masked in the naphthohydroquinone dimethyl ether (**10b**, **10d**) or diacetate (**19b**, **19d**) form, respectively. In the case of the ethers **10b** and **10d**, the quinone system was masked in such a way to prevent formation of the parent quinones, whereas acetates **19b** and **19d** were designed as prodrugs, being cleaved and oxidized efficiently to parent **4b** and **4d** in the keratinocyte cultures by esterases. As expected, both **10b** and **10d**, which are stable and cannot be cleaved to the hydroquinone forms, were inactive against keratinocyte hyperproliferation, suggesting that the presence of a quinone moiety is a prerequisite for activity in our novel compounds. In contrast, the effect of reductive acetylation of the quinone moiety was quite different, with the metabolically labile esters **19b** and **19d** suppressing keratinocyte hyperproliferation in the low micromolar range. Both precursors were only slightly less active than their corresponding parent molecules, with IC_{50} values of 0.80 for **19b** vs 0.63 μmol/L for **4b**, and 0.92 for **19**d vs 0.72 μmol/L for **4d**, respectively.

Lactate Dehydrogenase Release. HaCaT keratinocytes were also tested for their susceptibility for the action of the compounds on plasma membrane integrity. This was assessed by the activity of LDH (lactate dehydrogenase) released into the culture supernatant, which is commonly used as an indicator of plasma membrane damage.[40,41] As shown in Table 1, the most potent lapacho quinone analogues in the keratinocyte hyperproliferation assay were additionally tested for LDH release, which was apparent for all compounds and exceeded that of the vehicle control. Surprisingly, the inactive tetrazole **17** was the most potent inducer of membrane damage. The antipsoriatic agent anthralin, the clinical efficacy of which is limited by irritation of the nonaffected skin surrounding the psoriatic lesion,[19] and both lapacho constituents, the 2-acetyl analogue **4a** and β-lapachone, released significantly large amounts of LDH. In addition, the 2-acyl analogues **4b**, **4d** and the carboxamide **12** were essentially equivalent or even stronger inducers of cell lysis. In all other cases, cytotoxic membrane damaging effects as compared to anthralin were substantially reduced for the lapacho compound analogues, in particular, when the 2-substituent was terminated with a heterocycle such as thiophene, furan, and oxadiazole as in **4e**, **4f**, and **18**, respectively.

Superoxide Generation and Redox Cycling. Two major mechanisms for the biological action of quinoid compounds have been identified. First, some compounds unsubstituted at one or both positions of the quinone ring are potent electrophiles, capable of reacting with, e.g., sulfur nucleophiles in a 1,4-reductive addition of the Michael type, and thus can arylate tissue components and causing cell death.[42] In the case of the lapacho naphtho[2,3-*b*]furan-4,9-dione analogues, however, both electrophilic positions of the quinone ring are fused to a furan ring, rendering them inappropriate for such a reaction.

Second, there is circumstantial evidence to support the idea that both the desired biological activity and also some other effects such as skin toxicity of quinones are linked to ROS generation.[43−47] A characteristic feature of the quinone moiety of anthraquinones and naphthoquinones in general is its ability to undergo reversible oxidation−reduction processes. Enzymes such as NADPH-cytochrome P-450 oxidoreductase (CPR) catalyze one-electron reduction of the quinone, yielding the corresponding semiquinone radical as outlined in pathway a of Scheme 4, whereas NAD(P)H:quinone oxidoreductase 1

Scheme 4. Redox cycling Process of a 2-Substituted Naphtho[2,3-*b*]furan-4,9-dione[a]



[a](a) one-electron reduction by CPR/NADPH to its semiquinone radical and (b) reoxidation back to the quinone resulting in superoxide generation; (c) two-electron reduction by NQO-1/NADPH to its hydroquinone and (d) subsequent autoxidation via one-electron transfer to molecular oxygen with formation of the semiquinone radical and superoxide.

(NQO-1) catalyzes formally the two-electron reduction, resulting in the formation of a hydroquinone (pathway c, Scheme 4).[43,48] On the one hand, the semiquinone radical in turn can react with molecular oxygen and generate superoxide radical (pathway b, Scheme 4), while on the other, the hydroquinone autoxidizes with transfer of an electron to molecular oxygen resulting in semiquinone radical and concomitant superoxide generation (pathway d, Scheme 4). In such a fashion, both one- and two-electron reduction products of the quinone then participate in redox cycling to generate superoxide as the primary oxygen radical, and the cycle results in an apparent nonending shunting of electrons from cellular reducing equivalents to molecular oxygen generating potentially damaging reactive oxygen species (ROS).[45] Superoxide can react directly with some biomolecules or with H_2O_2, the latter formed by enzymatic or spontaneous dismutation of superoxide, which may generate even more deleterious oxygen species such as hydroxyl radicals (•OH).[22] The formation of various oxygen intermediates and the redox cycling of the quinone may ultimately lead to a cellular condition known as oxidative stress,[22] which can affect cell behavior in many ways.[49]

To provide information on the fundamental capability of naphtho[2,3-*b*]furan-4,9-dione derivatives to redox cycle, we have measured the generation of superoxide after incubation of selected quinones of this series both in isolated enzyme assays and in a HaCaT cell-based assay.

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Superoxide generation is commonly determined by the rate of reduction of ferricytochrome *c* to ferrocytochrome *c*.[50] To enhance the specificity of the assay,[50] cytochrome *c* was succinoylated before use. The specificity was further validated by addition of superoxide dismutase (SOD), as only the SOD-inhibitable extent of cytochrome *c* reduction would indicate a superoxide-dependent reaction. Catalase was added to scavenge H_2O_2, and the high affinity iron chelator DTPA was added to prevent the generation of hydroxyl radicals.[51]

Superoxide Generation Through One-Electron Reduction by CPR/NADPH. To examine one-electron reduction of naphtho[2,3-*b*]furan-4,9-diones, we incubated human recombinant CPR with NADPH and the test compounds and measured the rate of superoxide generation. As shown in Table 2, all of the four lapacho quinone analogues tested were excellent substrates for CPR. The naturally occurring lapacho constituent **4a** produced the highest level of superoxide as determined by the rate of reduction of SOD-inhibitable succinoylated cytochrome *c*. The unsubstituted basic structure **7** showed a reduction rate comparable to that of menadione[48,52] (2-methylnaphthalene-1,4-dione), which is a well-characterized redox cycler through one- and two-electron reduction and was used as a positive control. Also, introduction of a 2-methyl group or an oxadiazole as in **7a** and **18**, respectively, significantly increased the rate of cytochrome *c* reduction as compared to controls, but the generation of superoxide was substantially less than that generated by the 2-acetyl substituted **4a**. These results clearly indicate that one-electron reducing enzymes (e.g., CPR) activate naphtho[2,3-*b*]furan-4,9-diones into metabolic products that are superoxide generators, and furthermore, the rate of superoxide generation depends upon the nature of the 2-substituent.

Table 2. Rates of Superoxide Generation by Naphtho[2,3-*b*]furan-4,9-diones through One-Electron Reduction by Human Recombinant CPR and Two-Electron Reduction by Human Recombinant NQO-1

compd	$O_2^{\bullet-}$ (CPR)[a]	$O_2^{\bullet-}$ (NQO-1)[b]
4a	3.0 ± 0.2[c]	1.4 ± 0.6[c]
7	2.2 ± 0.4[c]	0.4 ± 0.3
7a	1.2 ± 0.2[c]	0.2 ± 0.2
18	1.8 ± 0.2[c]	1.9 ± 0.6[c]
menadione	2.1 ± 0.4[c]	1.0 ± 0.4[c]
control[d]	0.2 ± 0.1	0.2 ± 0.1

[a]Superoxide generation in the presence of CPR is expressed as SOD-inhibitable reduction of succinoylated cytochrome *c* (μmol/L/min/2 mU enzyme) by the test compound (100 μmol/mL). [b]Superoxide generation in the presence of NQO-1 is expressed as SOD-inhibitable reduction of cytochrome *c* (μmol/L/min/unit enzyme) by the test compound (25 μmol/mL). Each value represents the mean ± SD, $N \geq 3$. [c]Values are significantly different from vehicle control, $P < 0.0001$. [d]Rate of reduction with no test compound present (DMSO).

Superoxide Generation through Two-Electron Reduction by NQO-1/NADPH. As a further step toward investigating the role of redox cycling in the biological action of the novel compounds, we evaluated the impact of two-electron reduction by human recombinant NQO-1/NADPH on superoxide generation. As depicted in Table 2, superoxide generation after enzymatic two-electron reduction of the basic structure **7** and its 2-methyl analogue **7a** followed by hydroquinone autoxidation did not significantly exceed the control value. By contrast, incubation with both the 2-acetyl (**4a**) and oxadiazole (**18**) analogues resulted in significantly increased superoxide generation, which was also observed for menadione as a positive control. There is considerable data indicating a role for NQO-1 in the detoxification of redox cycling quinones[53,54] because two-electron reduction bypasses semiquinone radical generation and leads to the formation of a stable hydroquinone that can be readily conjugated and excreted. However, this is true only in certain cases because not all hydroquinones are redox-stable. Originally, the two-electron reduction product of menadione was regarded as a stable hydroquinone, but later it was found that oxygen radicals were produced in the presence of NQO-1.[54] This is also confirmed by our study, which shows a significant increase in superoxide production during the reduction of menadione by human recombinant NQO-1 (Table 2). Likewise, a significant increase in superoxide generation was observed after incubation with lapacho constituent **4a** and our novel analogue **18**, indicating autoxidation of their hydroquinones and redox cycling upon reduction by NQO-1. The susceptibility to autoxidation of the hydroquinone is important for it can determine whether or not activation takes place within the cell.[54] Thus, depending upon the properties of the hydroquinone that is formed and its ability to redox cycle, appreciable amounts of superoxide may be produced (pathway d, Scheme 4). While an electron-releasing group such as the 2-methyl of analogue **7a** may impair redox cycling of the quinone, an electron-withdrawing substituent as in **4a** or **18** rather enables autoxidation of the corresponding hydroquinone generated in the presence of NQO-1.

Taken together, the results obtained from the isolated enzyme assays indicate that superoxide radicals are, indeed, generated following incubation of naphtho[2,3-*b*]furan-4,9-diones in the presence of flavoenzymes that act as electron donors. Under normal conditions, the antioxidant defense systems such as superoxide dismutase present in the cell are able to control or prevent adverse effects of oxygen radicals.[22] Therefore, it was necessary to show that excessive quantities of superoxide are also generated within keratinocytes by redox cycling of lapacho analogues resulting from the normal enzyme activities of the cell.

Superoxide Generation in Keratinocytes. To directly assess the level of superoxide generated in quinone-treated cells, we employed flow cytometry in HaCaT keratinocytes and a fluorescent probe, dihydroethidium (DHE). This is widely used for detecting intracellular superoxide.[55] The red fluorescence arising from DHE is usually equated to intracellular superoxide generation, and recent studies proved that the product of the DHE/superoxide reaction is 2-hydroxyethidium (2-OH-E^+) and that 2-OH-E^+ is a specific marker for superoxide.[56,57] In these cell-based experiments, we have also employed menadione as a positive control, as intracellular generation of superoxide after treatment of cells with this agent by the use of DHE has been described.[56,58]

Acute and Chronic Treatment of HaCaT Keratinocytes with Lapacho Analogues. In the acute and chronic treatment experiments, neither the basic structure **7** nor its 2-methyl analogue **7a** had any significant impact on the 2-OH-E^+ signals generated in HaCaT keratinocytes (Table 3). In contrast, quantitative measurements of the mean fluorescence intensities from the samples after exposure to **4a** and, in particular, **18**, demonstrated markedly enhanced superoxide generation in keratinocytes in both sets of experiments. Table 3 also presents the levels of 2-OH-E^+ fluorescence in keratinocytes treated with

Table 3. Superoxide Generation in Human Keratinocytes Following Acute and Chronic Treatment with Naphtho[2,3-*b*]furan-4,9-diones

compd	acute treatment[a] $O_2^{\bullet-}$ (MFI)[d]	chronic treatment[b] $O_2^{\bullet-}$ (MFI)[d]	dicoumarol pretreatment[b,c] $O_2^{\bullet-}$ (MFI)[d]
4a	20.5 ± 7.2[e]	725.5 ± 97.2[e]	741.4 ± 127.9[e]
7	13.0 ± 0.6	452.5 ± 39.2	601.1 ± 82.1[e]
7a	6.4 ± 0.2	420.2 ± 290.0	830.9 ± 317.3[e]
18	17.0 ± 3.7[e]	1287.0 ± 218.2[e]	1279.0 ± 320.1[e]
menadione	44.0 ± 11.6[e]	431.9 ± 83.5	457.2 ± 103.4
control[f]	7.1 ± 5.2	367.5 ± 80.1	407.59 ± 68.7

[a]HaCaT keratinocytes were incubated with test compound (50 μmol/L) for 30 min. [b]HaCaT keratinocytes were incubated with test compound (5 μmol/L) for 18 h. [c]HaCaT keratinocytes were preincubated with the NQO-1 inhibitor dicoumarol (5 μmol/L) for 25 min before treatment with test compounds. [d]Superoxide generation is expressed in terms of changes in mean fluorescence intensity (MFI) of 2-OH-E$^+$. Each value represents the mean ± SD, $N = 3$. [e]Values are significantly different from vehicle control, $P < 0.05$. [f]Mean fluorescence intensity with no test compound present (DMSO).

the known redox cycler menadione. Clearly, menadione stimulated 2-OH-E$^+$ fluorescence, but a significant increase was observed only after acute treatment at a relatively high concentration of 50 μmol/L. This concentration of menadione has been reported to generate superoxide in human spermatozoa.[58]

Effects of Pretreatment with the NQO-1 Inhibitor Dicoumarol. To determine whether two-electron reduction of the quinones by NQO-1 is involved in intracellular superoxide generation, we measured 2-OH-E$^+$ fluorescence in keratinocytes after preincubation with dicoumarol, a potent inhibitor of NQO-1.[48,59] The concentration of dicoumarol required for specific inhibition of NQO-1, without significant impact on the activity of other quinone reducing enzymes, has been reported to be around 5 μmol/L.[59] The results of the NQO-1 inhibition experiments are summarized in Table 3. After chronic treatment with lapacho quinone analogues, when keratinocytes were pretreated with dicoumarol, 2-OH-E$^+$ fluorescence was significantly increased in each case, whereas it was not appreciably changed in the case of menadione. The latter finding is somewhat unexpected because it has been demonstrated that dicoumarol increases the availability of menadione for one-electron reduction, indicating that NQO-1 plays a protective role in preventing its redox cycling by competing with the one-electron reduction pathway.[48] However, chronic treatment of keratinocytes with menadione did not significantly increase superoxide generation, whether or not dicoumarol was added, suggesting that redox activation and ensuing potential suppression of keratinocyte proliferation of menadione were neglectable at concentration of 5 μmol/L. Indeed, as compared to the lapacho quinone analogues (Table 1), relatively high concentrations of menadione were required for suppression of HaCaT cell hyperproliferation (IC_{50} = 15.8 μmol/L) under identical conditions. Even though menadione has proven to be a useful model compound to study the consequences of oxidative stress both in vitro and in vivo,[48,54] the results from our studies do not lend support to an important role of ROS generated by this quinone, at least in this model of keratinocyte hyperproliferation.

Next, it is clear from these results that although menadione and the lapacho compound analogues both possess a quinone function, they elicit different responses to NQO-1 reduction and superoxide levels in keratinocytes. Surprisingly, inhibition of NQO-1 and consequently of the two-electron reduction pathway by dicoumarol increased the levels of superoxide produced by **7** and **7a**, which were not significantly increased when keratinocytes were treated without the NQO-1 inhibitor dicoumarol. This strongly suggests that under normal conditions these quinones were reduced by the two-electron pathway involving NQO-1 activity, which can be considered relatively nontoxic. The resulting hydroquinones are stable and can be readily inactivated by conjugation. This is in good agreement with the results obtained for **7** and **7a** in the isolated enzyme assay with NQO-1 (Table 2), which did not produce appreciable amounts of superoxide. However, inhibition of NQO-1-mediated metabolism of **7** and **7a** in keratinocytes by dicoumarol increased the availability of these quinones for one-electron reduction and redox cycling, possibly reflecting competition between NQO-1 and CPR, i.e., pathways c and a, respectively, of Scheme 4. As a consequence of the inhibited competition, enhanced 2-OH-E$^+$ fluorescence was observed, indicative of the predominating CPR pathway with the consequent generation of superoxide, which is also consistent with the results of **7** and **7a** in the isolated enzyme assay (CPR, Table 2).

Furthermore, only the 2-acetyl and oxadiazole analogues **4a** and **18**, respectively, were potent superoxide generators in the chronic treatment experiments under conditions comparable to that of the hyperproliferation model. Interestingly, the presence of dicoumarol did not potentiate this increase significantly (Table 3), suggesting that in contrast to **7** and **7a** NQO-1 cannot protect keratinocytes against quinone-induced oxidative stress by these compounds. In support of this, our experiments using recombinant NQO-1 revealed that unlike **7** and **7a**, the autoxidation of both **4a** and **18** resulted in significantly enhanced superoxide generation (Table 2).

How lapacho compound analogues act at the molecular level to suppress keratinocyte hyperproliferation is not clear at present. However, the observation that suppression of keratinocyte hyperproliferation was accompanied by increased superoxide generation in lapacho compound analogues treated cells suggests that ROS mediate, or at least contribute to, the mechanism of their growth-suppressing effects. Of interest in this regard, tricyclic antipsoriatic drugs such as anthralin[60,61] (**3**) or 8-methoxypsoralen[62,63] (**6**, Chart 1) have been reported to exert their action through a ROS-dependent mechanism, and also, keratinocyte growth suppression by a novel antipsoriatic benzodiazepine seems to be ROS-mediated.[64] Of further interest is the finding that keratinocytes have drastically lower mitochondrial superoxide dismutase activity than other skin cells, inducing a superoxide-driven impairment of mitochondrial function, which might be a prerequisite for keratinocyte differentiation.[65] This points to a higher susceptibility of these cells to oxidative stress, which would be enhanced in the presence of redox cycling agents. Moreover, the relative deficiency of the protective enzymes superoxide dismutase, catalase, and glutathione peroxidase in psoriatic tissue[66−68] renders the psoriatic epidermis particularly sensitive to the action of ROS, and this may as well increase the selective cytotoxicity of ROS-generating antipsoriatic drugs to psoriatic involved versus normal epidermis.

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CONCLUSIONS

We have designed and prepared novel compounds to answer a number of questions concerning SAR for suppression of keratinocyte hyperproliferation among a series of lapacho naphtho[2,3-*b*]furan-4,9-dione analogues. In particular, the consequences of introducing a variety of substituents at the 2-position of the tricyclic ring system have been investigated. The need for electron-withdrawing substituents such as acyl, carboxylic ester, carboxamide, or cyano groups is evident. While very good in vitro potency was attained with these substituents, a number of compounds evaluated was found to induce plasma membrane damage, as evidenced by the release of LDH activity from cytoplasm of the keratinocytes. The three most active analogues in this series were the nicotinyl derivative **4g**, carboxamide **12**, and 1,2,4-oxadiazole **18**, which showed potent suppression of keratinocyte hyperproliferation with IC_{50} values in the submicromolar range. It is noteworthy that potency of oxadiazole **18** was combined with comparably low cytotoxic keratinocyte membrane-damaging effects. As an important aspect with respect to SAR, comparison of the metabolically stable **10b** and the labile analogue **19b**, which were employed as masked quinones, indicates that the quinone moiety is a requirement for activity, as only **19b** was active.

In the redox cycling experiments, we studied compounds **4a**, **7**, **7a**, and **18** in detail. Several conclusions can be drawn from the superoxide generation data available (Tables 2 and 3). Generally, this class of compounds is susceptible to one-electron reduction, with all analogues tested generating superoxide to some degree in the presence of CPR. However, cell-based assays reveal that two-electron reduction of our lapacho quinone analogues in keratinocytes may lead either to activation or deactivation of redox cycling, and the balance between these processes will presumably depend upon the relative rates of the one- and two-electron steps and the stability of the particular hydroquinones that are formed according to Scheme 4. If the quinones are preferentially and rapidly reduced to stable hydroquinones (pathway c), and if autoxidation of the latter (pathway d) is comparatively slow under physiological conditions, conjugation may occur before reoxidation to the semiquinone (pathway d). Consequently, both one-electron transfer (pathway a) and autoxidation of the hydroquinone (pathway d) and ensuing superoxide generation will be bypassed in keratinocytes. This was observed for the basic structure **7** and 2-methyl analogue **7a**, which are only moderate inhibitors of keratinocyte hyperproliferation. In contrast, quinones that slowly form reactive hydroquinones will not be prevented from redox cycling in this manner because their persistence in the keratinocyte will permit one-electron transfer to molecular oxygen to continue. Moreover, superoxide will additionally be generated through autoxidation of the hydroquinone. Such a scenario may be envisaged for 2-acetyl analogue **4a** or oxadiazole **18**, which both suppress hyperproliferation of keratinocytes in the low micromolar range. Of interest, both **4a** and **18** are substituted in the 2-position of the naphtho[2,3-*b*]furan-4,9-dione with an electron-withdrawing acetyl or oxadiazole group, respectively. The potential impact of the functional chemistry of the quinone is supported by experimental observation that electron-withdrawing groups make the hydroquinone more susceptible for autoxidation.[45]

In summary, the most favorable benefit/toxicity profile compared with the standard antipsoriatic drug anthralin was determined for oxadiazole analogue **18**. On the basis of our in vitro studies, suppression of keratinocyte hyperproliferation seems to be superoxide-mediated, leading to subsequent regulation of keratinocyte growth. These results support further development of the lapacho analogue class of compounds for psoriasis and other hyperproliferative skin diseases.

EXPERIMENTAL SECTION

Melting points were determined with a Kofler melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian Mercury 400 plus spectrometer (400 MHz), using tetramethylsilane as an internal standard. Fourier transform IR spectra were recorded on a Jasco FT/IR-4100 (ATR) spectrometer by applying ATR correction. Mass spectra (EI, unless otherwise stated) were obtained on a Finnigan MAT GCQ instrument (70 eV). Thin layer chromatography (TLC) was conducted on Merck 60 F_{254} precoated silica gel plates. Chromatography refers to column chromatography, which was performed on Acros Organics silica gel (0.060−0.200 mm, 6 nm) with CH_2Cl_2 as eluant unless otherwise stated. Yields have not been optimized. Elemental analyses were performed by the Microanalysis Laboratory, University of Münster, using a Vario EL III elemental analyzer. Analytical data confirmed the purity of the test compounds was ≥95%.

Compounds **4a**,[69] **7a**,[23] **7b**,[69] **7c**,[7] **9**,[26] and **20**[70] were prepared as described.

4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]furan-2-carbaldehyde (4). To a solution of **7a**[23] (850 mg, 4.00 mmol) in CH_2Cl_2 (20 mL) was added SeO_2 (650 mg, 6.00 mmol). SiO_2 (4 g) was then added, excess solvent was evaporated, and the material was exposed to microwave irradiation (Discover Labmate, CEM) at 120 W (3 × 5 min, TLC-control). The product was purified by chromatography to afford yellow crystals; 50% yield; mp 194 °C (ref 26 190−101 °C).

General Procedure for the Oxidative Demethylation of Compounds 10b−10g. 2-Propionylnaphtho[2,3-*b*]furan-4,9-dione (4b). To a suspension of **10b** (250 mg, 0.88 mmol) in MeCN (8 mL) and H_2O (1.5 mL) at 0 °C was added dropwise within 20 min $(NH_4)_2[Ce(NO_3)_6]$ (1.23 g, 2.25 mmol) in MeCN (2.5 mL) and H_2O (2.5 mL) under vigorous stirring. The mixture was allowed to react for 20 min under the same conditions. Then it was poured onto ice−water and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic phase was washed with a saturated solution of NaCl (2 × 30 mL), dried over Na_2SO_4, and then concentrated and purified by chromatography to afford yellow needles; 75% yield; mp 227 °C; FTIR 1669 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 8.28−8.23 (m, 2H), 7.83−7.80 (m, 2H), 7.61 (s, 1H), 3.07 (q, J = 7.44 Hz, 2H), 1.27 (t, J = 7.44 Hz, 3H). MS m/z = 254 (76, M^+), 225 (100). Anal. $C_{15}H_{10}O_4$ (C, H).

Analogously, compounds **4c−4g** and **12** were prepared from **10c−10g** and **11**, respectively. See Supporting Information for details.

Naphtho[2,3-*b*]furan-4,9-dione (7). A mixture of **8** (1.00 g, 4.13 mmol), quinoline (20 mL), and chromium copper oxide (200 mg) was refluxed for 2 h and then cooled, diluted with CH_2Cl_2 (50 mL), and filtered. The solution was thoroughly washed with HCl (2 mol/L, 5 × 50 mL), and the combined organic phase was dried over Na_2SO_4. Then the solvent was evaporated and the product purified by chromatography to afford light-yellow, felted needles; 56% yield; mp 225 °C (ref 71 220−221 °C).

4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]furan-2-carboxylic Acid (8). To a suspension of **4** (400 mg, 1.76 mmol) in glacial HOAc (40 mL) was added H_2O_2 (30%, 15 mL) at 75 °C, and the mixture was stirred for 1 h. Then it was concentrated in vacuo, and the product was filtered by suction, washed with H_2O (30 mL), and dried in a drying apparatus to provide a bright-yellow powder; 91% yield; mp 236 °C. FTIR 3105, 1709, 1667 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 8.21−8.16 (m, 2H), 7.76−7.74 (m, 2H), 7.65 (s, 1H). MS m/z = 242 (100, M^+). Anal. $C_{13}H_6O_5$ (C, H).

General Procedure for the Preparation of Esters 8a−8k. Methyl 4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]furan-2-carboxylate (8a). To the carboxylic acid **8** (200 mg, 0.83 mmol) in dry MeOH (80 mg, 2.48 mmol) was added DMAP (10 mg, 0.08 mmol) under stirring. Then DCC (200 mg, 0.91 mmol) was added at 0 °C,

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and the mixture was stirred for 5 min and then for an additional 3 h at 20 °C. The mixture was filtered, the residue washed with CH_2Cl_2 (20 mL), and the filtrate evaporated and dissolved in CH_2Cl_2 (100 mL). The solution was washed with HCl (0.5 mol/L, 2 × 50 mL), then with a saturated solution of $NaHCO_3$ (2 × 50 mL), and the organic phase was dried over Na_2SO_4. Then the solvent was evaporated and the product purified by chromatography (CH_2Cl_2/EtOAc, 97/3) to give light-yellow crystals; 52% yield; mp 197 °C. FTIR 1738, 1681 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.27−8.22 (m, 2H), 7.81−7.79 (m, 2H), 7.64 (s, 1H), 4.00 (s, 3H). MS m/z = 256 (3, M^+), 241 (100). Anal. $C_{14}H_8O_5$ (C, H).

Analogously, compounds **8b−8k** were prepared from **8**. See Supporting Information for details.

General Procedure for Acylation of 4,9-Dimethoxynaphtho[2,3-*b*]furan (9). 1-(4,9-Dimethoxynaphtho[2,3-*b*]furan-2-yl)-propan-1-one (10b). To a mixture of *n*-butyllithium (2.5 M, 1.33 mL, 3.32 mmol) in THF (3 mL) at −15 °C under N_2 was added dropwise a solution of **9**[26] (380 mg, 1.69 mmol) in THF (15 mL). The mixture was stirred for 4 h at −15 °C, and then *N*,*N*-dimethylpropionamide (360 mg, 3.54 mmol) in THF (2.5 mL) was added. The mixture was stirred at rt for 3 h, then poured onto ice−water, acidified with 10% HCl, and extracted with ether (3 × 50 mL). The combined organic phase was washed with a saturated solution of NaCl (50 mL), dried over Na_2SO_4, and then concentrated and purified by chromatography to afford a yellow solid; 46% yield; mp 125 °C. FTIR 1552, 1076 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.27−8.22 (m, 2H), 7.82−7.79 (m, 2H), 7.60 (s, 1H), 4.31 (s, 3H), 4.24 (s, 3H), 3.06 (q, J = 7.45 Hz, 2H), 1.26 (t, J = 7.45 Hz, 3H). MS m/z = 284 (60, $M^{\bullet+}$), 269 (100).

Analogously, compounds **10c−10g** were prepared from **9** and the appropriate *N*,*N*-dimethylcarboxamides, and **11** was prepared from **9** and *N*,*N*-diethyl-2,2,2-trifluoroacetamide. See Supporting Information for details.

(*E*)-3-(4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]furan-2-yl)acrylic Acid (13a). To a solution of malonic acid (130 mg, 1.20 mmol) in pyridine (20 mL) was added carbaldehyde **4** (230 mg, 1.00 mmol) and refluxed at 110 °C until the formation of CO_2 bubbles had ceased. The solution was allowed to cool to rt, then poured onto ice−water (50 mL) and acidified with 36% HCl. The precipitate was filtered off, washed with H_2O (20 mL), and then dried in vacuo and recrystallized from MeOH to give yellow crystals; 41% yield; mp 288 °C. FTIR 1667 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.12−8.04 (m, 2H), 7.91−7.83 (m, 2H), 7.56 (s, 1H), 7.54 (d, J = 16.04 Hz, 1H), 6.59 (d, J = 16.04 Hz, 1H). MS m/z = 268 (100, M^+). Anal. $C_{15}H_8O_5$ (C, H).

(*E*)-Ethyl 3-(4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]furan-2-yl)-acrylate (13b). was prepared from **13a** as described for **8a**. See Supporting Information for details.

Diethyl 2-[(4,9-Dioxo-4,9-dihydronaphtho[2,3-b]furan-2-yl)-methylene]malonate (13c). Carbaldehyde **4** (200 mg, 0.88 mmol), diethyl malonate (0.14 g, 0.88 mmol), piperidine (0.15 mL), and glacial HOAc (0.5 mL) in benzene (15 mL) were added to a molecular sieve type 4A (200 mg). The mixture was refluxed for 2 h, then cooled to rt, and the benzene phase was washed with a solution of 17% NaCl (2 × 10 mL). The organic phase was dried over Na_2SO_4 and then concentrated and purified by chromatography to afford yellow crystals; 43% yield; mp 178 °C. FTIR 1739, 1719, 1667 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.15−8.13 (m, 2H), 7.73−7.70 (m, 2H), 7.48 (s, 1H), 7.15 (s, 1H), 4.48 (q, J = 7.04 Hz, 2H), 4.28 (q, J = 7.04 Hz, 2H), 1.37 (t, J = 7.04 Hz, 3H), 1.29 (t, J = 7.04 Hz, 3H). MS m/z = 368 (18, $M^{\bullet+}$), 284 (100). Anal. $C_{20}H_{16}O_7$ (C, H).

(*E*)-2-Styrylnaphtho[2,3-*b*]furan-4,9-dione (14a). To a suspension of NaH (60% in mineral oil, 0.15 g, 3.87 mmol) in DMSO (7.75 mL) in a dry, 50 mL three-necked flask, purged with N_2 at 45 °C, was added diethyl benzylphosphonate (0.35 g, 1.55 mmol). The mixture was stirred for 10 min, then carbaldehyde **4** (0.35 g, 1.55 mmol) in DMSO (7.0 mL) was added, and the mixture was heated at 60 °C and stirred for 30 min (TLC-control). Then it was poured into H_2O (30 mL), acidified with HCl (2 mol/L), and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic phase was washed with H_2O (3 × 30 mL), dried over Na_2SO_4, and then concentrated and purified by chromatography to afford red, felted needles; 12% yield; mp 239 °C. FTIR 1656 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.25−8.17 (m, 2H), 7.78−7.74 (m, 2H), 7.57−7.54 (m, 2H), 7.49 (d, J = 16.04 Hz, 1H), 7.42−7.35 (m, 3H), 6.98 (d, J = 16.04 Hz, 1H), 6.95 (s, 1H). MS m/z = 300 (100, M^+). Anal. $C_{20}H_{12}O_3$ (C, H).

(*E*)-4-(2-(4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]furan-2-yl)-vinyl)benzonitrile (14b). **14b** was prepared from **4** and (4-cyanobenzyl)triphenylphosphonium chloride in a similar manner as described for **14a**. See Supporting Information for details.

4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]furan-2-carbaldehyde Oxime (15). A mixture of carbaldehyde **4** (300 mg, 1.33 mmol), hydroxylamine hydrochloride (300 mg, 4.32 mmol), and K_2CO_3 (300 mg, 2.16 mmol) in 70% EtOH (15 mL) was stirred at rt for 2 h. Then H_2O (30 mL) was added and the mixture was cooled overnight at 6 °C. The precipitate was sucked off by filtration, washed with H_2O, and dried in a drying apparatus to provide an orange−yellow powder; 94% yield; mp 275 °C. FTIR 1669 cm^{-1}. ^1H NMR ($CDCl_3$) δ 9.91 (s, 1H), 8.21−8.18 (m, 2H), 7.78−7.74 (m, 2H), 7.62 (s, 1H). MS m/z = 241 (100, M^+). Anal. $C_{13}H_7NO_4$ (C, H, N).

4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]furan-2-carbonitrile (16). A solution of oxime **15** (200 mg, 0.83 mmol) in acetic anhydride (20 mL) was refluxed for 2 h. It was allowed to cool to rt, then poured onto ice−water (100 mL), and the precipitate was filtered and washed with H_2O (30 mL). The product was dried in vacuo at 50 °C and purified by chromatography to afford yellow needles; 65% yield; mp 207 °C. FTIR 2241, 1680 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.28−8.23 (m, 2H), 7.85−7.83 (m, 2H), 7.60 (s, 1H). MS m/z = 223 (100, M^+). Anal. $C_{13}H_5NO_3$ (C, H, N).

2-(1*H*-Tetrazol-5-yl)naphtho[2,3-*b*]furan-4,9-dione (17). To a solution of **16** (300 mg, 1.35 mmol) in DMF (20 mL) was added NaN_3 (100 mg, 1.50 mmol) and NH_4Cl (10 mg, 0.14 mmol), and the mixture was stirred at 90 °C for 2 h. Then it was cooled to rt, treated with a saturated solution of $NaHCO_3$ (20 mL), extracted with EtOAc (2 × 20 mL), and acidified with 36% HCl. It was then extracted with CH_2Cl_2 and dried over Na_2SO_4. The solution was evaporated, and the product was treated with a few drops of H_2O to induce precipitation of a yellow powder; 56% yield; mp 287 °C dec. FTIR 1672 cm^{-1}. ^1H NMR (DMSO-d_6) δ 8.16−8.11 (m, 2H), 7.93−7.89 (m, 2H), 7.69 (s, 1H). MS m/z = 266 (9, M^+), 238 (100). Anal. $C_{13}H_6N_4O_3$ (C, H, N).

2-(3-Ethyl-1,2,4-oxadiazol-5-yl)naphtho[2,3-*b*]furan-4,9-dione (18). To a solution of **8** (650 mg, 2.70 mmol) in dry CH_2Cl_2 (15 mL) was added DCC (0.28 g, 1.35 mmol) under N_2, and the mixture was stirred at 0 °C for 1 h. Then it was filtered, and the solvent was evaporated. The residue was treated with pyridine (15 mL), and a solution of *N*-hydroxypropionamidine[72] (110 mg, 1.30 mmol) in pyridine (2.5 mL) was added dropwise at rt. Then the mixture was refluxed for 3 h, cooled to rt, poured onto ice−water (100 mL), acidified with HCl (2 mol/L), and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic phase was washed with H_2O (2 × 50 mL) and dried over Na_2SO_4. The solution was evaporated, and the product was purified by chromatography (CH_2Cl_2/EtOAc, 95/5) to afford yellow crystals; 18% yield; mp 202 °C. FTIR 1669, 1634 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.27−8.22 (m, 2H), 7.83−7.81 (m, 2H), 7.76 (s, 1H), 2.88 (q, J = 7.44 Hz, 2H), 1.41 (t, J = 7.44 Hz, 3H). MS m/z = 294 (100, M^+). Anal. $C_{16}H_{10}N_2O_4$ (C, H, N).

2-Propionylnaphtho[2,3-*b*]furan-4,9-diyl Diacetate (19b). To a suspension of **4b** (150 mg, 0.59 mmol) and zinc dust (77 mg, 1.18 mmol) in acetic anhydride (5 mL) with stirring was added slowly pyridine (1.2 mL). An exothermic reaction ensued, and the mixture was stirred at rt for 1 h. Then it was filtered and treated with H_2O (10 mL). The product was dried in vacuo at 50 °C and purified by chromatography (CH_2Cl_2/EtOAc, 97/3) to afford white−yellow crystals; 20% yield; mp 145 °C. FTIR 1756, 1719, 1688 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.00−7.98 (m, 2H), 7.58−7.51 (m, 2H), 7.47 (s, 1H), 3.04 (q, J = 7.44 Hz, 2H), 2.59 (s, 3H), 2.57 (s, 3H), 1.26 (t, J = 7.44 Hz, 3H). MS m/z = 340 (3, $M^{\bullet+}$), 256 (100). Anal. $C_{19}H_{16}O_6$ (C, H).

Analogously, compound **19d** was prepared from **4d**. See Supporting Information for details.

Keratinocyte Culture and Determination of Cell Proliferation. HaCaT keratinocytes[38] were cultured, and the cell proliferation assay was performed as described previously.[73] After 48 h of incubation, cell growth was determined by enumerating the dispersed cells by phase contrast microscopy. Inhibition was calculated by the comparison of the mean values of the test compound ($N = 3$) with the control ($N = 6-8$) activity: (1 − test compound/control) × 100. Inhibition was statistically significant compared to that of the control (Student's t test; $P < 0.05$). IC_{50} values were obtained by nonlinear regression.

Lactate Dehydrogenase Release. The assay was performed as described.[40,41] HaCaT cells were incubated with the test compounds (2 μM) for 4 h at 37 °C. Extracellular LDH activity was measured using the UV method with pyruvate, and NADH and is expressed in mU/mL. Appropriate controls with the vehicle were performed ($P < 0.01$; $N = 3$, SD < 10%). Brij 35 (polyoxyethyleneglycol dodecyl ether)/ultrasound was the positive control.

Superoxide Generation Assays. Enzymatic One-Electron Reduction. Cytochrome c was succinoylated according to the procedure of Kuthan et al.[74] The extent of succinoylation was determined by the trinitrobenzenesulfonic acid method according to the method Finkelstein et al.[75] The assay was performed as described with modification.[76] The reaction mixtures, totaling 1 mL in volume, contained CPR (EC 1.6.2.4; human recombinant, Calbiochem, 2 mU), catalase (EC 1.11.1.6, bovine liver, Sigma, 70 U), NADPH (0.2 mmol/L), succinoylated cytochrome c (30 μmol/L), and sufficient buffer (K_2HPO_4/KH_2PO_4, 150 mmol/L; DTPA, 1 mmol/L, bidistilled H_2O, pH 7.4). After 2 min of incubation at 37 °C, succinoylated cytochrome c (30 μmol/L) was added and superoxide generation was initiated by addition of NADPH (0.2 mmol/L) and test compound (100 μmol/mL, DMSO).

Enzymatic Two-Electron Reduction. The same reaction mixtures were used as described above, except that NQO-1 (DT-diaphorase, EC 1.6.5.5, human recombinant, Sigma, 0.5 U) was used for enzymatic reduction of the test compound (25 μmol/mL, DMSO), native cytochrome c (30 μmol/L) was used for the detection of superoxide, and the assays were run at 20 °C.

For both assays, a parallel set of experiments was run to determine the SOD-inhibitable portion of the total rate of superoxide generation. Addition of SOD (EC 1.15.1.1, bovine erythrocytes, Sigma, 75 U) enabled the background reduction rate to be subtracted. The rate of superoxide generation was measured by the rate of increase in absorbance at 550 nm on a Merck Spektroquant Pharo 100 spectrophotometer. Rates of reaction were calculated from the initial 2 min of the reaction, and results were expressed in terms of μmol of SOD-inhibitable (succinoylated) cytochrome c reduced per liter per minute per unit enzyme using a molar extinction coefficient of 21.1 mM^{-1} cm^{-1} for reduced cytochrome c.[50] Controls were performed with no test compound present (DMSO, final concentration of 0.1%). All reactions were carried out in triplicate.

Intracellular Superoxide Generation. To detect intracellular superoxide levels,[77] HaCaT keratinocytes were cultivated as described.[73] Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, no. E15−810, PAA) in 6-well plates (2.5×10^5 cells/mL) supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) in a CO_2 incubator for 24 h at 37 °C. Then the medium was replaced, and cells were treated for 18 h (chronic treatment) with the test compound (5 μM, DMSO, final concentration of DMSO in the culture medium was 0.1%) in CO_2 incubator for 24 h at 37 °C. Also, acute treatment experiments (30 min, 50 μM test compound) were carried out. In a separate set of experiments, keratinocytes were preincubated with the NQO-1 inhibitor dicoumarol[48,59] (5 μmol/L) for 25 min before treatment test compounds were added. After incubation with test compound, DHE (10 μmol/mL) was added, cell culture plates were treated on a shaker (200 rpm) for 5 min, and cells were allowed to load DHE for an additional 25 min under incubation conditions. Then the medium was removed, cells were washed with PBS (0.5 mL/well) and then trypsinized (0.3 mL/well) for 7 min, treated with FACS buffer (FACSFlow, BD Biosciences, No. 342003, 0,7 mL), centrifuged (1000g), and resuspended in FACS buffer (0.5 mL). Experiments with DHE were performed in the dark. Controls were performed with no DHE and with no test compound (DMSO alone). DHE-treated keratinocytes were immediately evaluated by flow cytometric measurements, which were performed at different time points (0, 10, 20, 30, 60, 90 min). Keratinocytes were kept in CO_2 incubator at 37 °C between measurements. All experiments were run in triplicate.

Flow Cytometry. The fluorescence of the oxidized product 2-OH-E^+ was monitored on a FACSCalibur (Becton Dickinson) flow cytometer equipped with an argon laser ($\lambda = 488$ nm) as a light source, and the data was collected in the FL2 channel ($\lambda = 550-600$ nm, 42 nm bandpass). For each sample, 10000 live cells were examined, and dead cells were gated out for analysis. The recorded histograms were analyzed using the software CellQuest Pro and were compared with histograms of untreated control cells. Data were expressed as mean fluorescence intensity (MFI).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary chemical data of compounds **4c−4g**, **8c−8k**, **10c−10g**, **11**, **12**, **13b**, **14b**, and **19d** and table of analytical data of all test compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ DEDICATION

Dedicated to Prof. Dr. Dr. Wolfgang Wiegrebe, Regensburg, on the occasion of his 80th birthday.

■ ABBREVIATIONS USED

CPR, NADPH-cytochrome P450 oxidoreductase; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DHE, dihydroethidium; DMAP, 4-dimethylaminopyridine; DTPA, diethylene triamine pentaacetic acid; NQO-1, NAD(P)H:quinone oxidoreductase 1; ROS, reactive oxygen species; rt, room temperature; SAR, structure−activity relationships; SOD, superoxide dismutase

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