# Chemoprotective Properties of Phenylpropenoids, Bis(benzylidene)cycloalkanones, and Related Michael Reaction Acceptors: Correlation of Potencies as Phase 2 Enzyme Inducers and Radical Scavengers†

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Induction of phase 2 enzymes (e.g., glutathione transferases, NAD(P)H:quinone reductase, glucuronosyltransferases, epoxide hydrolase) is a major strategy for reducing the susceptibility of animal cells to neoplasia and other forms of electrophile toxicity. In a search for new chemoprotective enzyme inducers, a structure−activity analysis was carried out on two types of naturally occurring and synthetic substituted phenylpropenoids: (a) Ar−CH═CH−CO−R, where R is OH, $OCH_3$, $CH_3$, or Ar, including cinnamic, coumaric, ferulic, and sinapic acid derivatives, their ketone analogues, and chalcones; and (b) bis(benzylidene)cycloalkanones, Ar−CH═C$(CH_2)_n$(CO)C═CH−Ar, where $n = 5$, 6, or 7. The potencies of these compounds in inducing NAD(P)H:quinone reductase activity in murine hepatoma cells paralleled their Michael reaction acceptor activity (Talalay, P.; De Long, M. J.; Prochaska, H. J. *Proc. Natl. Acad. Sci. U.S.A. 85*, **1988**, 8261−8265). Unexpectedly, the bis(benzylidene)cycloalkanones also powerfully quenched the lucigenin-derived chemiluminescence evoked by superoxide radicals. Introduction of *o*-hydroxyl groups on the aromatic rings of these phenylpropenoids dramatically enhanced their potencies not only as inducers for quinone reductase but also as quenchers of superoxide. These potentiating *o*-hydroxyl groups are hydrogen-bonded, as shown by moderate downfield shift of their proton NMR resonances and their sensitivities to the solvent environment. The finding that the potencies of a series of bis(benzylidene)cycloalkanones in inducing quinone reductase appear to be correlated with their ability to quench superoxide radicals suggests that the regulation of phase 2 enzymes may involve both Michael reaction reactivity and radical quenching mechanisms.

## Introduction

Mammalian cells have evolved elaborate biochemical mechanisms for protecting DNA and other macromolecules against damage by electrophiles and reactive oxygen species that arise both from endogenous metabolic processes and from exogenous sources. A particularly important line of cellular defense against these types of toxicities is the coordinate induction of phase 2 enzymes (e.g., glutathione *S*-transferases, EC 2.5.1.18; NAD(P)H:quinone reductase, EC 1.6.99.2) which are principally concerned with the metabolic deactivation of these toxic agents, as well as elevation of glutathione levels. These transcriptionally regulated inductions are easily evoked by administration of a wide variety of natural and synthetic chemical agents.[1] Much persuasive evidence now indicates that elevation of phase 2 enzymes affords protection against the toxic and neoplastic effects of electrophiles and oxidative stress.[2−4] Such protection has been confirmed by demonstrating that cells subjected to chemical induction are resistant to toxicity, by clonal selection for resistance, and by overexpression of genes that code for protective phase 2 enzymes. Recently, transgenic mice lacking quinone reductase or glutathione transferase were shown to be more susceptible to quinone toxicity or carcinogenesis, respectively.[5,6] Our interest in the coordinate induction of phase 2 enzymes in tissues arises from the fact that this strategy reduces susceptibility to carcinogens and the incidence of malignancy.

Extensive efforts to elucidate the molecular mechanism of induction of phase 2 enzymes have focused on identifying the *cis*-acting regulatory sequences, the *trans*-acting transcriptional regulatory factors, and the chemical structures of the inducers.[1,2,4,7−11] A simple assay for determining inducer potency developed in our laboratory involves measurement of quinone reductase (a prototype for phase 2 detoxication enzymes) in murine hepatoma cells grown in 96-well microtiter plates.[12,13] This system reliably predicted the enzyme inducer activity of compounds in animal tissues. At least eight chemically distinct classes of compounds are inducers of quinone reductase in this system.[1,2] Most of these agents are monofunctional since they elevate phase 2 enzymes selectively, without activating the transcription of the *Ah* (*A*ryl *h*ydrocarbon)-dependent cytochromes P450.[14] These chemical classes include (a) *o*- and *p*-diphenols and phenylenediamines that can undergo oxidations to quinones or quinonimines, re-

† Abbreviations and trivial names: quinone reductase, NAD(P)H:(quinone acceptor) oxidoreductase, EC 1.6.99.2, also known as DT diaphorase; CD value, concentration of an inducer required to double the specific activity of quinone reductase in Hepa lclc7 murine hepatoma cells.

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spectively; (b) quinones; (c) various other Michael reaction acceptors; (d) isothiocyanates; (e) hydroperoxides; (f) vicinal dimercaptans; (g) trivalent arsenicals; (h) 1,2-dithiole-3-thiones; and (i) divalent heavy metals. The inducer potencies of these compounds, expressed as the concentrations required to double quinone reductase-specific activities in murine hepatoma cells (CD values), span more than 4 orders of magnitude. There is no obvious common chemical or structural theme that can easily explain the activities and potencies of all of these classes of inducers. Their structural diversity and universal chemical reactivity suggest that passive liganding to a structurally complementary receptor is unlikely to account for their mode of action. Many of the inducers are electrophiles, nearly all can react with sulfhydryl groups by oxidation or electrophilic attack, and inducer potency appears to correlate with their ability to participate as substrates for glutathione transferases.[15] Whereas all inducers are chemically reactive, it is curious that some are powerful oxidants (e.g., peroxides, hydroperoxides) and can cause oxidative damage, others are obviously antioxidants (e.g., BHA, BHT, 2,3-dimercaptopropanol), and a third group is apparently unable to participate directly in oxidoreduction reactions (e.g., isothiocyanates). It is possible that these inducers act by different mechanisms, but recent work from several laboratories[16,17] suggests that oxidants as well as antioxidants and intracellular redox state can regulate gene expression and specifically transcription of phase 2 enzymes.

Michael reaction acceptors (i.e., olefins or acetylenes that are conjugated to electron-withdrawing groups) are a major group of inducers with potencies paralleling the strength of the electron-withdrawing groups and consequently their rate of reaction with nucleophiles.[1] Interestingly, and perhaps not merely by coincidence, Michael reaction acceptor groups are present in many natural products; for example, the plant phenylpropanoids and phenylpropenoids and analogous compounds perform critical physiological functions in the defense strategies of all vascular plants.[18] Moreover, representatives of this class, e.g., cinnamic, ferulic, caffeic, coumaric, and sinapic acids, lignans, and chalcones, as well as coumarins and flavonoids, have been reported to display a wide variety of pharmacological properties, e.g., antioxidant,[19,20] anticancer,[21−23] antimutagenic,[24] and antimalarial[25] activities. One cannot help wondering whether all of these pharmacological properties depend on distinct mechanism(s), or whether, and perhaps more probably, there is a common mechanistic link between these properties that has hitherto eluded identification. Search for this missing link is important, especially since many of these plant constituents eventually become part of the human diet, and some are even ingested in substantial quantities, approaching 1 g/day.[26] In addition, epidemiological and migrational studies have revealed a correlation between high-fiber diet and low incidence of several types of cancer, e.g., stomach, colon, breast, and prostate cancers,[27] which has led to the suggestion that certain plant phenylpropanoids and their derivatives may serve as natural cancer-protective substances.

Bis(benzylidene)cycloalkanones are structurally related to plant phenylpropenoids. They can be viewed as dimeric Michael reaction acceptors and have been shown to exhibit antineoplastic activity.[28] One of the earliest reports on the synthesis of such compounds was published in 1912,[29] and subsequently these compounds have attracted considerable interest in various and sometimes intriguing ways. Some members of this group have found pharmaceutical application as components of choleretic drugs, e.g., 2,6-bis(3-methoxy-4-hydroxybenzylidene)cyclohexanone also known as cyclovalone.[30] Others have been specifically developed as very sensitive pH indicators.[31] Borden[32] at Eastman Kodak synthesized a large number of bis(benzylidene)cycloalkanones as materials for condensation into light-sensitive phenolic polymers suitable for photolithographic purposes. Konrad and Möller[33] at Henkel subsequently developed these substances as sunscreens for the UV-A range (320−405 nm). Most recently, these agents were rediscovered by Markaverich and his colleagues as esterase-insensitive ligands for nuclear type II estrogen receptors and as inhibitors of cell proliferation.[28,34]

In the present study, several bis(benzylidene)cycloalkanone derivatives were synthesized and tested as inducers of quinone reductase with two specific questions in mind: (a) What is the significance of the substituents on the aromatic rings for the inducer activity? and (b) Is the size of the alkanone ring important? In order to answer these questions a detailed structure−activity study was undertaken. First, we examined several "partial molecules", all of which belong to the class of plant phenylpropenoids and their derivatives, i.e., cinnamic acids and methyl cinnamates; coumaric acids and methyl coumarates; coumarins; and chalcones. The lessons that we learned from these "partial molecules" subsequently enabled us to identify bis(benzylidene)cycloalkanones with extraordinary high quinone reductase inducer potency.

A number of phenolic antioxidants, some of which are commonly used as food additives (e.g., 2(3)-*tert*-butyl-4-hydroxyanisole (BHA), 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT)) have been shown to protect against carcinogenesis and mutagenesis.[35−39] The relationship between their antioxidant properties and their ability to serve as chemoprotective agents is probably not a coincidence, since free radical-mediated processes are believed to be associated with the occurrence of many human diseases, including cancer.[40] This prompted us to examine the free radical-scavenging capacity of several quinone reductase inducers obtained in the course of this study. We found that among these compounds some of the most potent inducers display free radical-scavenging activity and that there is good correlation between their potencies as inducers of phase 2 enzymes and their free radical-scavenging capacity.

## Results and Discussion

**Phenylpropenoic Acids and Their Esters as Inducers of Quinone Reductase.** Earlier studies on the phase 2 enzyme inducer potencies of Michael reaction acceptors of the type phenyl−CH=CH−CO−R had shown that these potencies paralleled the electron-withdrawing powers of the R groups: i.e., ketones (R = alkyl or aryl) were more potent than esters (R = *O*-alkyl), and carboxylic acids (R = OH) were barely active.[1] In further studies in this laboratory,[15] a series

of methyl esters of cinnamic acid substituted with electron-withdrawing or electron-donating groups on the aromatic ring were synthesized. Their potencies in inducing quinone reductase activity revealed a general trend: electron-withdrawing substituents at the *meta*-position enhanced the inducer activity, the methyl esters of 3-bromo-, 3-nitro- and 3-chlorocinnamates being the most active. Moreover, the order of potency correlated linearly with the Hammett $\sigma$ and $\sigma^-$ values of these substituents. The chemical approach for methylation that was used,[15] namely, refluxing of methanolic solutions of the corresponding cinnamic acids in the presence of boron trifluoride etherate, did not, however, permit synthesis of the methyl esters of cinnamic acids bearing additional aromatic hydroxyl substituents. Because hydroxylated phenylpropenoids and their derivatives are ubiquitous in nature and there are many reports of their antioxidant, antiinflammatory, antimutagenic, and antitumor activities, it was important to obtain the methyl esters of these hydroxylated cinnamates.

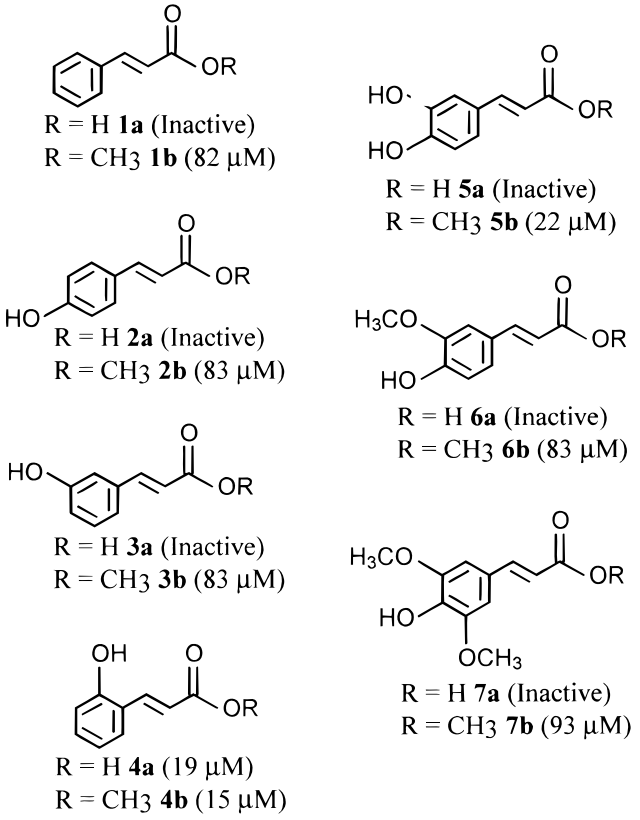
By means of a facile and selective method, involving the refluxing of methanolic solutions of the corresponding cinnamic acids in the presence of small amounts of a cation-exchange resin, we obtained selective methylation of the carboxyl groups of the four most naturally abundant substituted phenylpropenoic acids and tested these compounds for their ability to induce quinone reductase activity in cultured Hepa 1c1c7 murine hepatoma cells.

As expected, the arylpropenoic acids themselves, i.e., cinnamic (**1a**), caffeic (**5a**), ferulic (**6a**), and sinapic (**7a**) acids (Table 1), were inactive as inducers in agreement with their weak Michael acceptor reactivity, in confirmation of previous observations.[1,41] Their methyl esters (**1b**, **6b**, and **7b**) were weak inducers (CD = 82−93 $\mu$M), except for methyl caffeate (**5b**), which was moderately potent (CD = 22 $\mu$M) (Table 1).

Among coumaric (hydroxycinnamic) acids (Table 1), *p*-coumaric (**2a**) and *m*-coumaric (**3a**) acids were also inactive as inducers. Surprisingly, however, *o*-coumaric acid (**4a**) showed moderate inducer potency (CD = 19 $\mu$M). The corresponding methyl esters had inducer potencies similar to those of the methyl esters of the cinnamic acids, again the only exception being methyl *o*-coumarate (**4b**), which was a slightly more potent enzyme inducer (CD = 15 $\mu$M). The last finding suggests that the relatively weak Michael acceptor activity of *o*-coumaric acid (**4a**) was compensated by the potent contribution of the *o*-hydroxyl moiety. These results provided the first demonstration that the presence of a hydroxyl group at the *ortho*-position on the aromatic ring of a Michael reaction acceptor (e.g., cinnamic acid derivative) increases its inducer potency markedly. The differences in inducer potencies between the acids and methyl esters are unlikely to be due to solubility differences alone for two reasons: (i) the *p*- and *m*-hydroxycinnamic acids are inactive, whereas the *ortho*-isomer is active; large differences in solubilities among these isomers are unlikely; and (ii) there is a very small inducer potency difference between *o*-methyl coumarate (CD = 15 $\mu$M) and *o*-coumaric acid (CD = 19 $\mu$M).

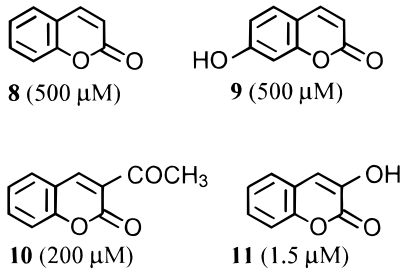
**Hydroxylated Coumarins as Inducers of Quinone Reductase.** Next, several coumarin derivatives were examined (Table 2). These represent another large and diverse group of widely distributed plant metabolites. Coumarins are biosynthesized from cinnamic acid precursors in which the aromatic rings are hydroxylated at the *ortho*-position, the side-chain double bond undergoes light-catalyzed *trans*−*cis* isomerization, and lactonization gives rise to the basic benzopyran-2-one (*cis-o*-coumaric acid lactone) group of the coumarin skeleton.[42] Coumarin (**8**) itself is a very weak inducer of quinone reductase (CD ~ 500 $\mu$M),[1] as is 7-hydroxycoumarin (**9**), the major metabolite of coumarin in humans.[43−45] 3-Acetylcoumarin (**10**) is slightly more potent (CD ~ 200 $\mu$M). When the acetyl group was replaced by a hydroxyl function, as in 3-hydroxycoumarin (**11**), however, a remarkable increase of more than 300-fold in inducer potency was observed (CD = 1.5 $\mu$M). It should be pointed out that this is the only coumarin derivative examined here for which keto−enol

**Table 1.** Potencies of Phenylpropenoic Acids and Their Methyl Esters as Inducers of Quinone Reductase in Murine Hepatoma Cells[a]



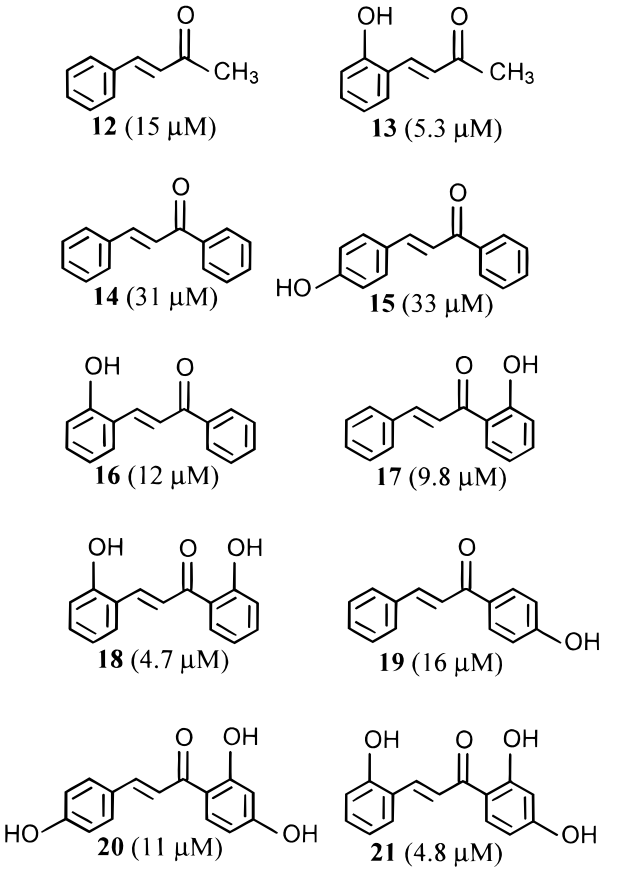

[a] The CD values are shown in parentheses.

**Table 2.** Potencies of Coumarin Derivatives as Inducers of Quinone Reductase in Murine Hepatoma Cells[a]




[a] The CD values are shown in parentheses.

**Table 3.** Potencies of Phenylbutenones and Chalcones as Inducers of Quinone Reductase in Murine Hepatoma Cells[a]




[a] The CD values are shown in parentheses.

tautomerism of the molecule can occur. However, the precise reason for the extraordinary increase in inducer potency of **11** remains unclear.

**Chalcones as Inducers of Quinone Reductase.** Another group of plant products that are structurally and metabolically related to the aforementioned phenylpropenoids are the chalcones, which may be regarded as phenyl ketone analogues of cinnamic acid. The rationale for examining these compounds was twofold: ketones are more potent Michael acceptors than are esters, and numerous reports in the literature describe their antitumor,[12,46,47] antiinflammatory,[19,24] and antimutagenic[48] activities. Notably, structure−activity studies have revealed the absolute requirement for an olefinic function for these biological activities.[19,49] Further, as part of a previous study[1] it had been shown that *trans*-4-phenyl-3-buten-2-one (**12**) (the methyl ketone analogue of cinnamic acid) was a more potent inducer (CD = 15 $\mu$M) than was the corresponding ester, i.e., methyl cinnamate (**1b**) (CD = 82 $\mu$M), in agreement with the order of reactivity of Michael reaction acceptors. In the present study, we found that the *ortho*-hydroxylated derivative of *trans*-4-phenyl-3-buten-2-one (**13**) was 3 times more potent as an inducer (CD = 5.3 $\mu$M) than was the nonhydroxylated compound **12** (Table 3).

The same correlation was found among the conventional chalcones (Table 3). Hydroxyl substitution at position 4 did not affect the inducer potency of chalcones (compare chalcone (**14**), CD = 31 $\mu$M, and 4-hydroxychalcone (**15**), CD = 33 $\mu$M). In contrast, a hydroxyl

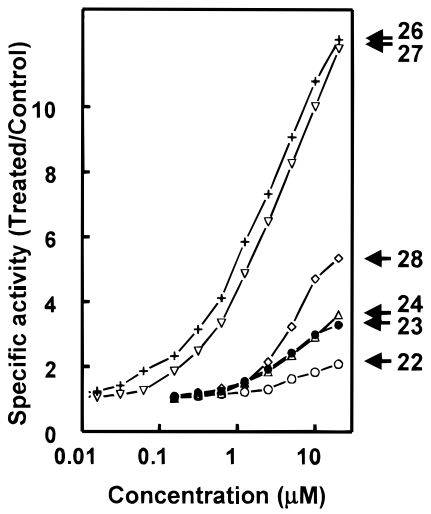
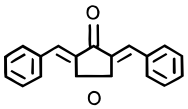
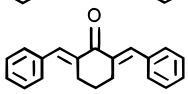
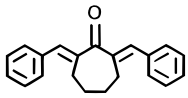
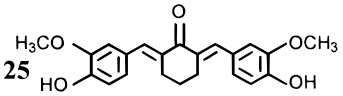
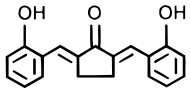
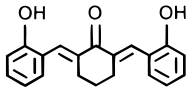
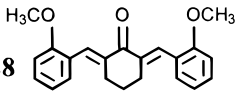



**Figure 1.** Concentration dependence of induction of quinone reductase in murine hepatoma cells grown in 96-well microtiter plate wells by the following bis(benzylidene)cycloalkanones: (○) 2,5-bis(benzylidene)cyclopentanone (**22**); (●) 2,6-bis(benzylidene)cyclohexanone (**23**); (△) 2,7-bis(benzylidene)-cycloheptanone (**24**); (+) 2,5-bis(2-hydroxybenzylidene)cyclopentanone (**26**); (▽) 2,6-bis(2-hydroxybenzylidene)cyclohexanone (**27**); (◇) 2,6-bis(2-methoxy benzylidene)cyclohexanone (**28**). Each data point represents the average of eight replicates, with standard deviation between 5% and 10% of its ordinate value. The graph demonstrates the dramatic increase in potency resulting from *ortho*-hydroxylation (compare **26** and **27** with **22** and **23**).

group at the 2-position increased the inducer potency approximately 3 times (2-hydroxychalcone (**16**), CD = 12 $\mu$M). This was consistent with the findings in the methyl cinnamate series, where a ring hydroxyl group had a strong impact on inducer potency only when located at the *ortho*-position. A 2′-hydroxyl group (i.e., in **17**) had a similar enhancing effect (CD = 9.8 $\mu$M). Moreover, the simultaneous presence of hydroxyl groups at both 2- and 2′-positions (see **18**, CD = 4.7 $\mu$M) led to further enhancement of inducer potency. Introduction of a third hydroxyl group at the 4′-position (as in 2,2′,4′-trihydroxychalcone (**21**) provided no further increase in inducer potency (CD = 4.8 $\mu$M). Interestingly, in the chalcone series, a hydroxyl group at the 4′-position (**19**) led to a 2-fold increase in inducer potency (CD = 16 $\mu$M). Recently 2-hydroxychalcone (**16**) (CD = 12 $\mu$M) and 2′-hydroxychalcone (**17**) (CD = 9.8 $\mu$M) were shown to inhibit proliferation of HeLa cells and were the most potent among many other chalcone and related flavonoid derivatives tested.[50] In addition, it is noteworthy that the related chalcone isoliquiritigenin (4,2′,4′-trihydroxychalcone (**20**), CD = 11 $\mu$M), which occurs naturally in bark and wood of some leguminous trees, was found to be a very strong antitumor agent.[46]

**Bis(benzylidene)cycloalkanones as Inducers of Quinone Reductase.** With these lessons from the naturally existing "partial molecules" and their synthetic analogues, we explored the structurally related bis(benzylidine)cycloalkanones. They contain two Michael reaction acceptor moieties making them good candidates as potential quinone reductase inducers and chemoprotective agents. The unsubstituted compounds were synthesized according to Rumpel,[30] and their inducer potencies were compared (Figure 1, Table 4). We found that although the cyclopentanone derivative **22** was the

**Table 4.** Potencies of Bis(benzylidene)cycloalkanones as Inducers of Quinone Reductase in Murine Hepatoma Cells and as Scavengers of Superoxide Radicals Generated by the Oxidation of Xanthine by Xanthine Oxidase[a]

| | CD (μM) | % Free Radical Scavenging at 50 nM |
|---|---|---|
| **22**  | 16 (7)[a] | 10 (7)[a] |
| **23**  | 2.9 (5) | 11 (5) |
| **24**  | 3.3 (6) | 11 (5) |
| **25**  | 0.9 (3) | 30 (3) |
| **26**  | 0.075 (1) | 70 (1) |
| **27**  | 0.28 (2) | 42 (2) |
| **28**  | 2.2 (4) | 19 (4) |

[a] The potency order ranks for each type of measurement are given in parentheses and are in good agreement. The values for scavenging activity are expressed as percent quenching of luminescence of lucigenin at a 50 nM concentration of each compound.

least potent in this series (CD = 16 $\mu$M), it was comparable in potency to *trans*-4-phenyl-3-buten-2-one (**12**) (CD = 15 $\mu$M). The analogues containing the larger six-membered cycloalkanone ring were considerably more potent, but there was no significant difference in potency between 2,6-bis(benzylidene)cyclohexanone (**23**) (CD = 2.9 $\mu$M) and 2,7-bis(benzylidene)cycloheptanone (**24**) (CD = 3.3 $\mu$M). Substituents at positions 3 and 4 of the aromatic rings were found to contribute further to the inducer potency, which is exemplified by 2,6-bis-(3-methoxy-4-hydroxybenzylidene)cyclohexanone (**25**) (CD = 0.9 $\mu$M).

We next examined whether an aromatic hydroxyl group at position 2 would significantly affect inducer potency, as observed in the plant phenylpropenoids we had tested above. Thus, 2,5-bis(2-hydroxybenzylidene)-cyclopentanone (**26**) and 2,6-bis(2-hydroxybenzylidene)-cyclohexanone (**27**) were synthesized according to the procedures of Borsche and Geyer.[29] When tested as quinone reductase inducers, **26** and **27** were found to be dramatically more potent inducers (leading to at least 12 times increases in enzyme specific activity without any measurable cytotoxicity): 2,6-bis(2-hydroxybenzylidene)cyclohexanone (**27**) (CD = 280 nM) and 2,5-bis(2-hydroxybenzylidene)cyclopentanone (**26**) (CD = 75 nM) (Figure 1, Table 4). We confirmed that this dramatic increase is due specifically to the 2-hydroxyl group by showing that its methylation as in 2,6-bis(2-methoxybenzylidene)cyclohexanone (**28**), led to a large decrease in potency (CD = 2.2 $\mu$M) to a level comparable to that of the unsubstituted cyclohexanone derivative **23** (CD = 2.9 $\mu$M) (Table 4). Although the cytotoxicities of the bis(benzylidene)cycloalkanones have not been extensively investigated, no detectable reduction in cell mass was observed for compounds **22**, **23**, and **26**−**28** under conditions used to determine the CD values (up to a concentration of 20 $\mu$M).

**Free Radical-Scavenging Activity of Quinone Reductase Inducers.** With these extremely potent inducers in hand, we next sought a link between ability to elevate quinone reductase levels and potential free radical-scavenging activity. The rationale for these experiments was based on the recognized role of free radicals in the different stages of carcinogenesis. Furthermore, many of the "partial molecule" inducers are well-established antioxidants, and there is recent interest in the antioxidant function of $\beta$-diketones, especially those related to curcumin (diferuloylmethane).[51]

For this part of the study we utilized the oxidation of xanthine by xanthine oxidase as a system to generate superoxide and the inhibition of the lucigenin-derived chemiluminescence as a measure of the free radical-scavenging activity.[52,53] Again, it was important to start with the "partial molecules", e.g., the chalcones. Although it was not surprising to find that these compounds are good antioxidants, it was quite unexpected that free radical-scavenging activity in the xanthine/xanthine oxidase system correlated with inducer potency in the murine hepatoma system. For example, chalcone (**14**) doubles the specific activity of quinone reductase at a concentration of 31 $\mu$M and inhibits chemiluminescence by 50% at 6.0 $\mu$M. Addition of a hydroxyl group at the *ortho*-position, as in **17**, which increases the inducer potency 3-fold (CD = 9.8 $\mu$M), also leads to an increase in free radical scavenging, inhibiting chemiluminescence by 50% at 2.5 $\mu$M. Simultaneous presence of hydroxyl groups on both aromatic rings at *ortho*-positions, which decreases the CD value to 4.7 $\mu$M (see **18**), leads to a proportional decrease in the concentration needed for 50% inhibition of the chemiluminescence to 1.0 $\mu$M.

The next aim was to determine whether this correlation is also valid for our most potent group of quinone reductase inducers. Figure 2 represents a typical profile of the lucigenin-derived chemiluminescence in the presence of increasing concentrations of 2,5-bis(2-hydroxybenzylidene)cyclopentanone (**26**). Table 4 shows the inhibition of chemiluminescence (based on area under the curve) at 50 nM concentration of the corresponding bis(benzylidene)cycloalkanone. As can be seen, all the bis(benzylidene)cycloalkanones examined in this study are not only extremely potent quinone reductase inducers but also exceptionally strong free radical scavengers. Moreover, inducer potency correlates with free radical-scavenging activity. Among the seven compounds (Table 4) for which measurements of both potency of induction and quenching of luminescence at 50 nM are available, the rank order of potencies in both assays are virtually identical. To confirm that the observed inhibition of chemiluminescence is due to free radical scavenging only and not to inhibition of xanthine oxidase, we compared the rates of enzymatic formation of uric acid

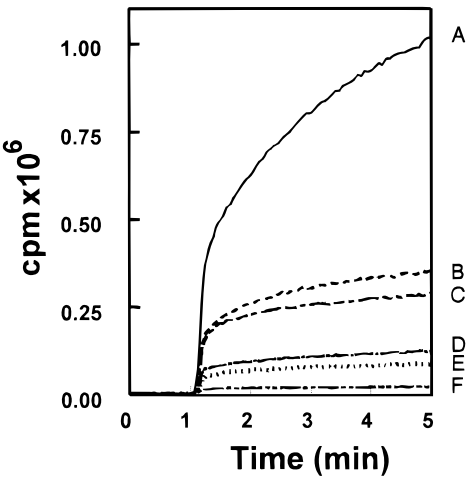



**Figure 2.** Concentration-dependent inhibition by 2,5-bis(2-hydroxybenzylidene)cyclopentanone (**26**) of lucigenin-derived chemiluminescence arising from superoxide generated by the oxidation of xanthine by xanthine oxidase; A, no inhibitor; B, 50 nM; C, 100 nM; D, 500 nM; E, 1 $\mu$M; F, 10 $\mu$M.

from xanthine in the absence and presence of 20 $\mu$M concentrations of the corresponding bis(benzylidene)-cycloalkanones and found that these values were essentially the same.

**Structure and Conformation of Bis(benzylidene)-cycloalkanones. A. Molecular Geometry.** Three geometric isomers of the bis(benzylidene)cycloalkanones can be envisaged, i.e., *E*−*E*, *E*−*Z*, and *Z*−*Z*.[54] On the basis of the symmetrical patterns of their $^1$H NMR spectra, isomer *E*−*Z* was eliminated as a possibility. The stereochemistry (*E*−*E* or *Z*−*Z*) was subsequently established by 2D-NOESY experiments in a mixture of $CDCl_3$/DMSO-$d_6$. Thus, the detection of a cross-peak between the methylene protons of the cyclopentanone

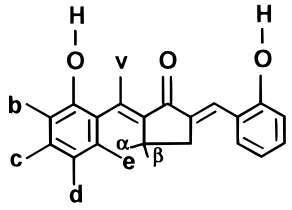



**Figure 3.** Designation of protons in 2,5-bis(2-hydroxybenzylidene)cyclopentanone (**26**). See also Table 5.

ring of 2,5-bis(benzylidene)cyclopentanone (**22**) at $\delta$ 3.14 and the proton at $\delta$ 7.61 (designated e in Table 5 and Figure 3) indicated that these three protons are in spatial proximity, and therefore the stereochemistry is *E*−*E*. Similarly, the cross-peak observed between the methylene protons of the cycloalkanone ring of 2,5-bis-(2-hydroxybenzylidene)cyclopentanone (**26**) at $\delta$ 3.04 and the proton at $\delta$ 7.5 (designated e in Table 5 and Figure 3) established that in this case also the geometry was *E*−*E*. Similar results were obtained when 2,6-bis-(benzylidene)cyclohexanone (**23**) and 2,6-bis(2-hydroxybenzylidene)cyclohexanone (**27**) were examined, thereby establishing that the cyclohexanone derivatives also have the *E*−*E* geometry.

**B. Conformation.** Interestingly, it became clear from the same experiment that the *o*-hydroxyl groups of bis-(benzylidene)cycloalkanones are pointing "up," i.e., away from the methylenic protons ($\alpha$ and $\beta$ in Figure 3) of the cycloalkanone ring. Although this orientation was expected because of steric hindrance, this conformation was established unambiguously for **26** by 2D-NOESY ($CDCl_3$/DMSO-$d_6$) through the observation of cross-peaks between the *o*-hydroxyl proton ($\delta$ 9.49) and the vinyl proton ($\delta$ 7.94), as well as between the *o*-hydroxyl proton and the proton at $\delta$ 7.18 (designated b in Table 5 and Figure 3). In contrast, no cross-peak was observed

**Table 5.** Proton NMR Assignments and Chemical Shifts (ppm) of Bis(benzylidene)cyclopentanone and Bis(benzylidene)cyclohexanone and Their *Ortho*-Hydroxylated Analogues

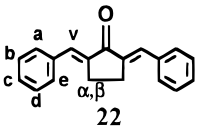
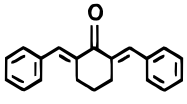
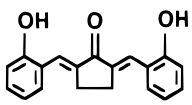
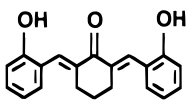
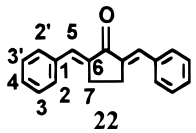
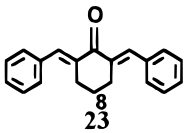
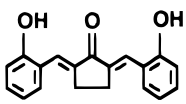
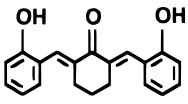
| |  22 |  23 |  26 | | |  27 | |
|---|---|---|---|---|---|---|---|
| **Assignment** | **[$CDCl_3$]** | **[$CDCl_3$]** | **[Acetone]** | **[$CDCl_3$ DMSO]** | **[DMF]** | **[Acetone]** | **[$CDCl_3$ DMSO]** |
| **OH** | - | - | **9.09 (2)** | **9.49** | **10.38** | **8.70 (2)** | **9.28** |
| **vinyl** | **7.53 (2)** | **7.73 (2)** | **7.99 (2)** | **7.94** | **7.99** | **7.97 (2)** | **7.91** |
| **a** | **7.61 (4)** | **7.47 (4)** | - | - | - | - | - |
| **e** | **7.61** | **7.47** | **7.68 (2)** | **7.50** | **7.63** | **7.40 (2)** | **7.27** |
| **b** | **7.45 (4)** | **7.41 (4)** | **7.29 (2)** | **7.18** | **7.28** | **7.21 (2)** | **7.15** |
| **d** | **7.45** | **7.41** | **6.98 (4)** | **6.94** | **6.95** | **6.94 (4)** | **6.91** |
| **c** | **7.40 (2)** | **7.34 (2)** | **6.98** | **6.85** | **6.95** | **6.94** | **6.83** |
| **$CH_2$ (cyclo-** | **3.14 (4)** | **2.90 (4)** | **3.14 (4)** | **3.04** | **3.11** | **2.88 (6)** | **2.86 (4)** |
| **alkanone)** | - | **1.80 (2)** | - | - | - | | **1.75 (2)** |

**Table 6.** $^{13}C$ NMR Chemical Shifts (ppm) of Carbon Atoms of Bis(benzylidene)cyclopentanone and Bis(benzylidene)cyclohexanone and Their *Ortho*-Hydroxylated Analogues

| |  22 |  23 |  26 |  27 |
|---|---|---|---|---|
| **Assignment** | | | | |
| **C=O** | **195.6** | **188.9** | **195.2** | **189.2** |
| **C-6** | **136.9** | **135.6** | **135.7** | **134.9** |
| **C-1** | **135.2** | **135.3** | **122.3** | **122.2** |
| **C-5** | **132.9** | **134.8** | **127.2 (Δ 5.7)** | **131.5 (Δ 3.3)** |
| **C-2** | **130.1** | **129.3** | **128.8** | **129.2** |
| **C-2'** | **130.1** | **129.3** | **156.8** | **155.8** |
| **C-4** | **128.9** | **127.7** | **118.4** | **117.8** |
| **C-3** | **128.2** | **127.5** | **129.8** | **128.95** |
| **C-3'** | **128.2** | **127.5** | **115.2** | **114.9** |
| **C-7** | **26.0** | **27.5** | **25.9** | **27.8** |
| **C-8** | **-** | **22.0** | **-** | **22.6** |

between the *o*-hydroxyl proton and the methylene protons (designated α and *β* in Table 5 and Figure 3) of the cyclopentanone ring (*δ* 3.04) or between the *o*-hydroxyl proton and the proton at *δ* 6.94 (designated d in Table 5 and Figure 3).

Further information on the conformations of these compounds was provided by the $^1H$ NMR resonances of the phenolic protons of 2,5-bis(2-hydroxybenzylidene)-cyclopentanone (**26**) and 2,6-bis(2-hydroxybenzylidene)-cyclohexanone (**27**) (Table 5). These phenolic protons are probably involved in weak hydrogen bonds (most likely with the solvent or between two or more solute molecules), since their chemical shifts are observed near 9−10 ppm. In addition, these are the only protons of **26** and **27** whose chemical shifts are significantly affected by the nature of the solvent; e.g., for 2,5-bis(2-hydroxybenzylidene)cyclopentanone (**26**) the shift was 9.09 ppm in acetone-$d_6$, 9.49 ppm in a mixture of $CDCl_3$/DMSO-$d_6$ (2:8, by volume), and 10.38 ppm in DMF-$d_7$.

**C. Electron Density at Vinyl Carbon Atoms.** Since it had been previously established that the degree of potency of many quinone reductase inducers paralleled their reactivity as Michael reaction acceptors,[1] we next used $^{13}C$ NMR spectroscopy in conjunction with HSQC (heteronuclear single quantum correlation) spectroscopy to determine the effect of the aromatic 2-hydroxyl groups on the electron density of the vinyl carbons, which are the presumed points of attack by intracellular nucleophiles. Significant upfield shifts of the $^{13}C$ resonances of the vinyl carbons of 2,5-bis(2-hydroxybenzylidene)cyclopentanone (**26**) (by 5.7 ppm) and 2,6-bis(2-hydroxybenzylidene)cyclohexanone (**27**) (by 3.3 ppm) were observed in comparison to the chemical shift of the vinyl carbons of the unsubstituted analogues **22** and **23**, respectively (Table 6). Surprisingly, these increases in electron density at the vinyl carbons were associated with very large increases in potency as inducers of QR upon *ortho*-hydroxylation: 200- and 15-fold in **26** and **27**, respectively. This result would not be expected if the inducer potency depended solely on the electrophilicity of the *β*-carbon of the vinyl group. A similar paradoxical finding was the observation that the inducer potencies of various cinnamates did not correlate with the electrophilicity of the *β*-vinyl carbon of substituted cinnamates, as determined by $^{13}C$ NMR.[15] The other differences between the $^{13}C$ resonances associated with hydroxylation were confined to a +25 ppm shift in the carbon atoms carrying the hydroxyl groups, a −13 ppm shift in the carbon atoms flanking the hydroxylated carbons, and a −10 ppm shift of the 4-carbon of the aromatic ring.

Since interaction with intracellular nucleophile(s), e.g., thiols, would be expected to occur via Michael addition on the vinyl carbon atoms, the presence of *o*-hydroxyl groups must make a significant contribution to the rate of addition of $S^-$ anion, although the exact mechanism remains unknown. A parallel can be drawn, however, with the antitumor cytotoxic sesquiterpene lactones from Compositae isolated by Kupchan and colleagues,[55,56] in which a hydroxyl or *O*-acyl group adjacent to the methylene function of these α-methylene-*γ*-lactones enhanced the rate of cysteine addition enormously. This analogy is probably not surprising, because both α-methylenecyclopentanone and α-methylene-*γ*-lactone have the same Michael reaction acceptor, namely, the electrophilic α-methylene carbonyl.

In unrelated studies, Rodriguez et al.[57] have drawn attention to the importance of molecular accessibility factors in the regulation of the reactivity of α,*β*-unsaturated carbonyl systems with sulfhydryl nucleophiles.

## Conclusions

The presence of hydroxyl groups at the *ortho*-position(s) on the aromatic ring(s) of the phenylpropenoids

and bis(benzylidene)cycloalkanones examined leads to significant enhancement of their potencies both as quinone reductase inducers and as free radical scavengers. In the case of the substituted phenylpropenoids, this increase ranges from 3-fold for the ketones (e.g., compare the CD values for *trans*-4-phenyl-3-buten-2-one (**12**) with *2*-hydroxy-*trans*-4-phenyl-3-buten-2-one (**13**) and chalcone (**14**) with 2-hydroxychalcone (**16**)) to 5-fold for the methyl esters of phenylpropenoic acids (e.g., compare methyl cinnamate (**1b**) with methyl *o*-coumarate (**4b**)). Moreover, this effect is additive only when two hydroxyl groups are simultaneously present at *ortho*-positions on both aromatic rings, e.g., as in 2,2′-dihydroxychalcone (**18**). The most pronounced enhancement is within the class of bis(benzylidene)cycloalkanones, where the difference between the potencies of the hydroxylated and the nonhydroxylated derivatives can be dramatic—greater than 200 times (e.g., compare 2,5-bis(benzylidene)cyclopentanone (**22**) with 2,5-bis(2-hydroxybenzylidene)cyclopentanone (**26**)). Taken together, our findings identify two previously unrecognized properties of this class of compounds that are exhibited at very low concentrations and without any detectable cytotoxicity, which make them auspicious candidates as chemoprotective agents: (a) the ability to induce quinone reductase (a prototype of phase 2 detoxication enzyme) and (b) the ability to serve as potent free radical scavengers. That the potencies of these compounds in both processes parallel each other suggests that these properties may be functionally related.

## Experimental Procedures

**Materials.** Xanthine, xanthine oxidase (grade I) from buttermilk, and lucigenin were obtained from Sigma (St. Louis, MO). The structures of all test compounds are shown in Tables 1−4.

Cinnamic acid (**1a**), methyl cinnamate (**1b**), coumarin (**8**), 7-hydroxycoumarin (**9**), *trans*-4-phenyl-3-buten-2-one (**12**), 2-hydroxy-*trans*-4-phenyl-3-buten-2-one (**13**), chalcone (**14**), 4-hydroxychalcone (**15**), 4′-hydroxychalcone (**19**), 2,7-bis(benzylidene)cycloheptanone (**24**), and 2,6-bis(2-methoxybenzylidene)cyclohexanone (**28**) were obtained from Aldrich (Milwaukee, WI); caffeic (**5a**), ferulic (**6a**), and sinapic (**7a**) acids and *p*-(**2a**), *m*-(**3a**), and *o*-(**4a**) coumaric acids were from Lancaster (Windham, NH); 3-acetylcoumarin (**10**), 3-hydroxycoumarin (**11**), 2-hydroxychalcone (**16**), 2′-hydroxychalcone (**17**), 2,2′-dihydroxychalcone (**18**), 4,2′,4′-trihydroxychalcone (**20**), and 2,2′,4′-trihydroxychalcone (**21**) were from Indofine (Somerville, NJ). All other test compounds were synthesized as described below.

**Chemical Syntheses.** Methyl esters of cinnamic and other arylpropenoic acids were prepared by a general procedure involving the refluxing of methanolic solutions (10 mL) of the corresponding acids (usually 1 g) in the presence of a small amount (0.5 mL) of Dowex 50W-8X cation-exchange resin for 2−3 h. The resin was removed by filtration, the solvent was evaporated in a vacuum, and the product was crystallized from suitable mixtures of hexane and acetone. The identities of the compounds were established by $^1$H NMR spectroscopy (300 MHz, $CDCl_3$). Methyl *o*-coumarate (**4b**): $\delta$ (ppm) 8.01 (d, 1H, $J$ = 16 Hz, ph-CH═), 7.48−6.84 (m, 4H, arom), 6.62 (d, 1H, $J$ = 16 Hz, ═CH-CO-), 5.80 (s, 1H, OH), 3.83 (s, 3H, $CH_3$). Methyl *m*-coumarate (**3b**): $\delta$ (ppm) 7.61 (d, 1H, $J$ = 16 Hz, ph-CH═), 7.33−6.79 (m, 4H, arom), 6.40 (d, 1H, $J$ = 16 Hz, ═CH-CO-), 5.57 (s, 1H, OH), 3.80 (s, 3H, $CH_3$). Methyl *p*-coumarate (**2b**): $\delta$ (ppm) 7.65 (d, 1H, $J$ = 16 Hz, ph-CH═), 7.52−6.72 (m, 4H, arom), 6.28 (d, 1H, $J$ = 16 Hz, ═CH-CO-), 5.58 (s, 1H, OH), 3.79 (s, 3H, $CH_3$). Methyl sinapate (**7b**): $\delta$ (ppm) 7.61 (d, 1H, $J$ = 16 Hz, ph-CH═), 6.75 (s, 2H, arom), 6.32 (d, 1H, $J$ = 16 Hz, ═CH-CO-), 5.73 (s, 1H, OH), 3.90 (s, 6H, $CH_3O$), 3.78 (s, 3H, $CH_3$). Methyl caffeate (**5b**): $\delta$ (ppm) 7.56 (d, 1H, $J$ = 16 Hz, ph-CH═), 7.05−6.84 (m, 3H, arom), 6.25 (d, 1H, $J$ = 16 Hz, ═CH-CO-), 5.48 (s, 1H, OH), 5.36 (s, 1H, OH), 3.77 (s, 3H, $CH_3$). Methyl ferulate (**6b**): $\delta$ (ppm) 7.60 (d, 1H, $J$ = 16 Hz, ph-CH═), 7.03−6.90 (m, 3H, arom), 6.28 (d, 1H, $J$ = 16 Hz, ═CH-CO-), 5.89 (s, IH, OH), 3.91 (s, 3H, $CH_3O$), 3.77 (s, 3H, $CH_3$).

2,6-Bis(benzylidene)cyclohexanone (**23**) and 2,5-bis(benzylidene)cyclopentanone (**22**) were synthesized according to the procedure of Rumpel.[30] For the synthesis of **23**, equimolar amounts of cyclohexanone (0.98 g, 0.01 mol) and benzaldehyde (1.08 g, 0.01 mol) were mixed and 3 drops of concentrated HCl was added. The reaction was allowed to proceed for 40 h at room temperature with stirring until solid product was formed. The latter was crystallized from cold $CH_3COOH/H_2O$ (1:1) to yield long bright-yellow needles (1.9 g, 70%). The same procedure, replacing cyclohexanone for cyclopentanone, was used for the synthesis of **22**. $^1$H and $^{13}$C NMR spectroscopic constants of **22** and **23** are given in Tables 5 and 6.

The hydroxylated bis(benzylidene)cycloalkanones were synthesized according to the procedure of Borsche and Geyer.[29] For the synthesis of 2,6-bis(2-hydroxybenzylidene)cyclohexanone (**27**), cyclohexanone (5.0 g, 0.05 mol) and salicylaldehyde (12.1 g, 0.1 mol) were mixed and 37.5 mL of absolute ethanol was added. This was followed by the dropwise addition of 30 mL of 20% NaOH, with constant stirring. The reaction was allowed to proceed for 48 h at room temperature, 200 mL of $H_2O$ was added, and the mixture was neutralized by gently bubbling $CO_2$ through it. The resulting solid material was filtered and crystallized from warm acetone to yield yellow crystals (10 g, 67%). $^1$H and $^{13}$C NMR spectroscopic constants for **26** and **27** are given in Tables 5 and 6.

**NMR Spectroscopy.** Proton NMR spectra were obtained in $CDCl_3$ (unless otherwise specified) at 300 MHz on a Bruker spectrometer at 25 °C. $^{13}$C NMR spectra were obtained in a solvent mixture of $CDCl_3$/DMSO-$d_6$ (2:8) at 400 MHz on a Varian VXR 400 at 30 °C. Two-dimensional NMR was performed in the same solvent mixture on a Varian Unity Plus 600 NMR spectrometer at 30 °C. Chemical shift frequencies were relative to tetramethylsilane.

**Quinone Reductase Activity Assay.** Specific activity of quinone reductase was determined in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates according to minor modifications of the described procedure.[12,13,15,58] Cells (10 000/well) were grown for 24 h in medium supplemented with 10% heat- and charcoal-treated fetal bovine serum and exposed to serial dilutions of inducers for 48 h before specific activities of quinone reductase were measured. All compounds were dissolved in dimethyl sulfoxide and diluted so that the final concentration of solvent was 0.1% by volume in all wells. The specific activities of quinone reductase were normalized to the protein concentrations of the cell extracts. For this purpose, 20-$\mu$L aliquots of the cell lysates were transferred to corresponding wells of a duplicate plate, 300 $\mu$L of bicinchoninic acid reagent was added to each well, and the absorptions at 550 nm were determined according to the procedure of Bradford.[59] The concentration required to double the specific activity of quinone reductase (CD value) was used as a measure of inducer potency.

**Determination of Free Radical-Scavenging Activity.** Assays were carried out at 37 °C in plastic tubes in reaction mixtures gassed with air, which contained in a final volume of 1.00 mL: Dulbecco's phosphate-buffered saline, pH 7.4, 5 $\mu$M lucigenin (1 $\mu$L of a 5 mM solution in $H_2O$), 0.0025 unit of xanthine oxidase (8.5 $\mu$L of a solution containing 0.33 unit/mL of chromatographically purified milk xanthine oxidase; Sigma, grade I, specific activity 0.69 unit/mg protein), and 1 $\mu$L of the test compound in dimethyl sulfoxide, to give final concentrations of 50 nM−10 $\mu$M.[52] After gentle mixing, each assay tube was placed in the holder of Berthold Biolumat model LB9505 luminometer (Bad Wildbad, Germany). Six samples were assayed at one time. After obtaining a stable

baseline (over the period of 1 min), the reaction was initiated by injection of 20 $\mu$L of a xanthine solution to give a final concentration of 56 $\mu$M, and the chemiluminescence was monitored for an additional 4 min. Test compounds were dissolved in dimethyl sulfoxide or acetonitrile to give a final concentration of the organic solvent of 0.1% by volume. The luminescence data were obtained as integrated areas under the curve (during the 4-min interval), and inhibition of chemiluminescence was expressed as a percentage of control area.

**Inhibition of Xanthine Oxidase Activity.** The activity of xanthine oxidase was determined spectrophotometrically by measuring the enzymatic conversion of xanthine to uric acid at 25 °C. Each assay mixture contained in a final volume of 1.00 mL: 0.0025 unit of purified milk xanthine oxidase, 20 $\mu$mol of the test compound in dimethyl sulfoxide (final concentration 0.1% by volume in the reaction), and Dulbecco's phosphate-buffered saline, pH 7.4. Absorbance was monitored at 295 nm, and after the baseline had stabilized, the reaction was started by addition of 2.0 $\mu$L of a 25 mM solution of xanthine to give a final concentration of 50 $\mu$M; then the linear increase in absorbance was recorded.

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