1,2-Benzisoxazole Phosphorodiamidates as Novel Anticancer Prodrugs Requiring Bioreductive Activation

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Several 1,2-benzisoxazole phosphorodiamidates have been designed as prodrugs of phosphoramide mustard requiring bioreductive activation. Enzymatic reduction of 1,2-benziosoxazole moiety is expected to result in the formation of imine intermediate due to the cleavage of the N−O bond. The imine should then be spontaneously hydrolyzed to a ketone metabolite, thereby facilitating base-catalyzed β-elimination of cytotoxic phosphoramide mustard. As expected, the proposed prodrugs **4**, **9**, and **12** were at least 3−5-fold more potent cytotoxins than control compounds **5** and **15**, which lack in the phosphoramide mustard group. Upon incubation with phenobarb-induced rat liver S-9 fraction, compounds **4**, **9**, and **12** underwent extensive NADPH-dependent metabolism with concomitant generation of alkylating activity under both hypoxic and oxic conditions. Corresponding ketone metabolites were detected for **9** and **15**. NADPH-dependent bioreduction of **15** to its ketone metabolite **16** was located in the microsomal fraction and inhibited by SKF-525A and pCMBA. Compared with phenobarb-induced rat liver microsomal fraction, incubation of **15** with rat or human P450 reductase microsomes showed moderate generation of **16**. Microsomal cytochrome P450 and/or P450 reductase appear to be involved in the reductive metabolism of 1,2-benzisoxazole moiety under hypoxic as well as oxic conditions.

Introduction

In contrast to normal tissues that are well-perfused, solid tumors possess abnormal vasculature and as a result contain subpopulation of hypoxic tumor cells. Hypoxic tumor cells are resistant to radiation and chemotherapy and may lead to increased metastasis and accelerated malignant progression. However, the hypoxic environment of the tumor offers an attractive difference between normal and tumor cells that may be exploited with the use of bioreducible cytotoxins. These compounds are prodrugs that undergo reductive activation to yield cytotoxic metabolites. The reduction is facilitated by the appropriate reductases under the environment of low oxygen tension.[1,2] Many compounds of diverse chemical structures including quinones,[3,4] nitroimidazoles,[5] nitrobenzyl derivatives,[6] benzotriazine di-*N*-oxides,[7,8] and sulfoxide aniline mustards[9] have been designed as hypoxia-selective bioreducible cytotoxins. More recently, nitrobenzyl derivatives,[10] quinones,[11] and nitroheterocyclic[12] prodrugs that release appended phosphoramidate mustard derivatives as DNA-alkylating species after bioreduction have also been explored. The reductases involved include DT diaphorase, cytochrome P450, NADPH-dependent cytochrome P450 reductase, xanthine oxidase, and aldehyde oxidase, among others.[13,14]

Zonisamide, an anti-epileptic agent, is a 1,2-benzisoxazole derivative that undergoes reductive activation, leading to cleavage of the heterocyclic N−O bond. The resulting imine intermediate readily hydrolyzes to the ketone metabolite 2-(sulfamoylacetyl)phenol (SMAP) (Scheme 1). Human and rat liver microsomes have been shown to metabolize zonisamide to SMAP, preferentially under hypoxic conditions, with the suggested involvement of cytochrome P450.[15−20] Additional studies have also reported the ability of liver cytosolic aldehyde oxidase from various strains of rats and other animals to catalyze reduction of zonisamide under hypoxic conditions.[21] On the basis of reductive metabolism of zonisamide to SMAP, 1,2-benzisoxazole phosphorodiamidate prodrugs were designed as bioreducible hypoxia-selective antitumor alkylating agents with the ability to deliver cytotoxic species following reductive activation. The designed prodrugs are expected to undergo bioreduction in a manner similar to zonisamide that sequentially leads to formation of the corresponding imine intermediate and ketone metabolite, thereby facilitating β-elimination of cytotoxic phosphoramide mustard (PDA) (Scheme 2).

Phosphoramide mustard is also derived as the ultimate cytotoxic species following activation of cyclophosphamide (CP), a widely used alkylating anticancer prodrug. Following initial hepatic mixed function oxidase mediated activation of CP, the resulting 4-hydroxy cyclophosphamide (4-OH CP) undergoes ring-opening to aldophosphamide (Aldo), followed by base-catalyzed β-elimination of cytotoxic PDA. Intramolecular cyclization of PDA results in formation of reactive aziridinium ion that cross-links interstrand DNA.[22−23]

In case of 1,2-benzisoxazole phosphorodiamidates, the ketone metabolite releases PDA via β-elimination reaction in a fashion similar to Aldo, except that the electron-withdrawing activating moiety is a substituted phenyl ketone instead of an aldehyde. The utility of phenyl ketone driven β-elimination of PDA derivatives

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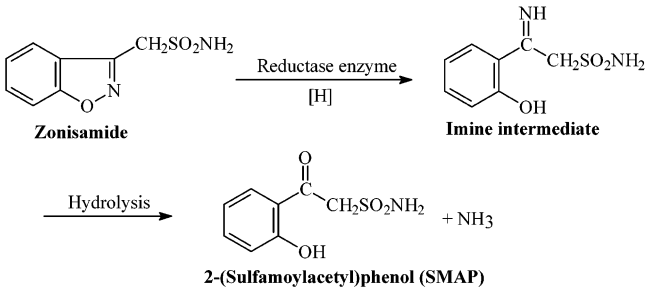
Scheme 1



has been exploited earlier in the design of phenylketophosphamide, which was shown to be a promising antitumor agent.[24]

Chemistry

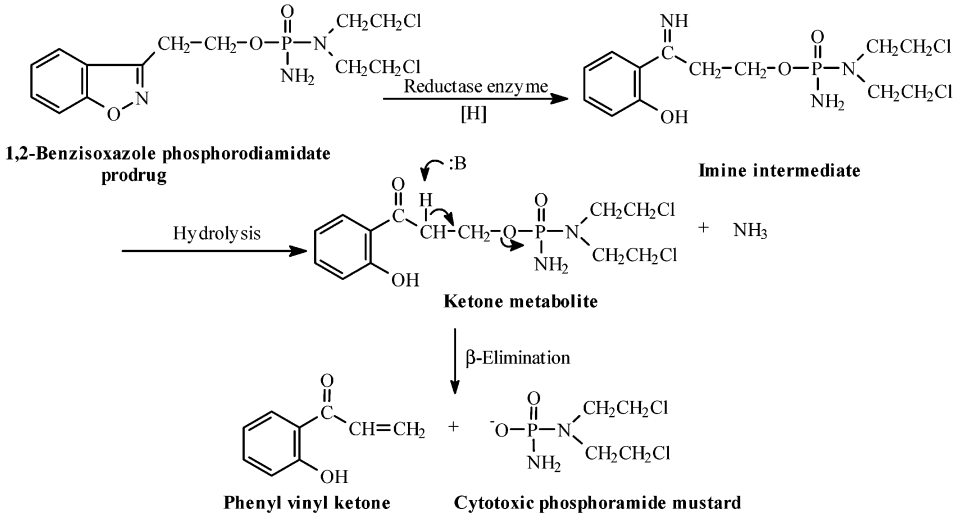
Several 1,2-benzisoxazole phosphorodiamidates (**4**, **9**, and **12**) with the potential to release PDA upon metabolic reduction were synthesized (Scheme 4). Electron-donating 6-methoxy and electron-withdrawing 5-nitro substituents were placed on the heterocycle to explore their influence on bioreduction as well as release of PDA. Additionally, *N*,*N*,*N*′,*N*′-tetramethylphosphorodiamidate (**5**) was prepared as a control compound lacking in alkylating phosphorodiamidate for in vitro cytotoxicity studies. Analogue **15** was chosen as a model test compound for in vitro metabolism studies.

1,2-Benzisoxazole phosphorodiamidates (**4**, **5**, **9**, and **12**) were prepared as outlined in Schemes 3 and 4. Initially, either unsubstituted or methoxy substituted 4-hydroxycoumarin was reacted with hydroxylamine to yield corresponding 1,2-benzisoxazole-3-acetic acid intermediates **1** and **6**, respectively, according to published procedures.[25,26] The 3-acetic acid intermediates were sequentially esterified and reduced to afford corresponding alcohols **3**[27] and **8**. For nitro-substituted analogue, ester **2** was nitrated[28] on the 5-position and then reduced to alcohol **11**. The obtained alcohols (**3**, **8**, and **11**) were sequentially reacted with *n*-butyllithium, *N*,*N*-bis(2-chloroethyl)phosphoramidic dichloride, and ammonia to yield target phosphorodiamidates **4**, **9**, and **12**, respectively. Alcohol **3** was sequentially reacted with *n*-butyllithium and *N*,*N*,*N*′,*N*′-tetramethyldiamidophosphorochloridate to give **5**. Synthesis of **15** was carried out by reaction of 2-hydroxypropiophenone with hydroxylamine that yielded oxime, which was subsequently acetylated and then refluxed with pyridine to obtained cyclized product (Scheme 5).

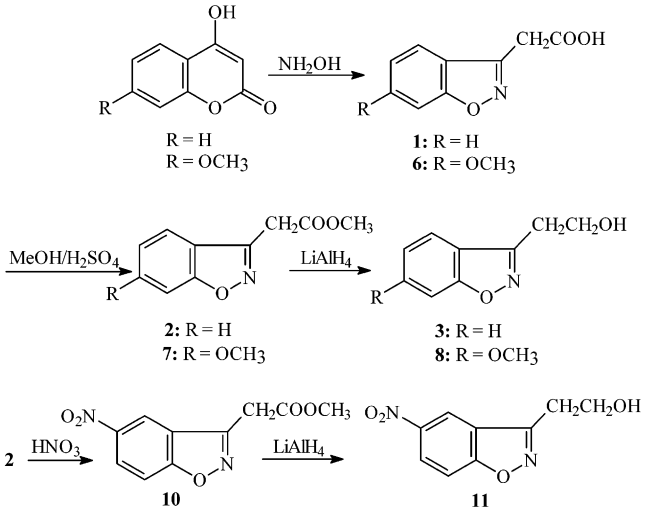
Results and Discussion

In Vitro Cytotoxicity Evaluation. The in vitro cytotoxicity potential of 1,2-benzisoxazole phosphorodiamidates was evaluated against Chinese hamster lung (V-79) fibroblasts in a 96-well microtiter plate assay using the sulforhodamine B (SRB) assay procedure of Skehan et al.[30] with some modifications. The drug exposure period was 48 h. The drug concentration that resulted in 50% reduction in cell-growth (indicated by the absorbance value of bound SRB dye) in drug-treated cells as compared to untreated control cells following incubation was calculated as growth inhibition of 50% (IC_{50}). Results are reported in Table 1. 2-(Methylsulfonyl)ethyl phosphorodiamidate, an Aldo analogue known to deliver PDA under model physiological conditions without enzyme activation requirement,[31] was chosen as a positive control cytotoxin. Among phosphorodiamidate prodrugs, **9** was the most potent cytotoxin, with an IC_{50} value of 241 μM, while **4** and **12** showed slightly weaker cytotoxicity with IC_{50} values of 411 and 311 μM, respectively. A correlation between electron-donating/withdrawing effects of substituents on 1,2-benzisoxazole and their cytotoxic potential could not be drawn, since both methoxy- and nitro-substituent-bearing analogues were more toxic than unsubstituted compound. Analogue **5**, lacking in alkylating phosphorodiamidate mustard, was found to be approximately 3−5-fold less potent than **4**, **9**, and **12**. This suggested that activation of prodrugs leading to expulsion of PDA, an alkylating species, seems to be the mechanism of cytotoxic action for 1,2-benzisoxazole phosphorodiamidates. The weak cytotoxic activity observed for **5** could be attributed to the formation of phenyl vinyl ketone, a potentially reactive Michael-acceptor type byproduct of β-elimination reaction in the reductive activation pathway. This conclusion was also supported by the lack of appreciable cytotoxic activity of **15**, possibly due to the absence of an alkylating PDA moiety as well as the inability of

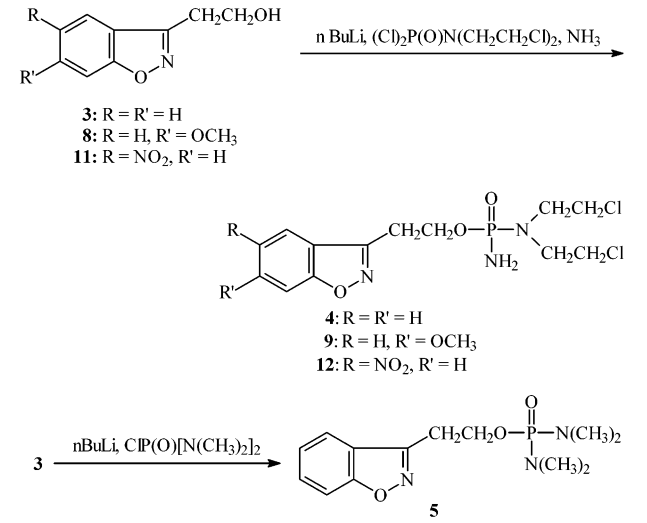
Scheme 2



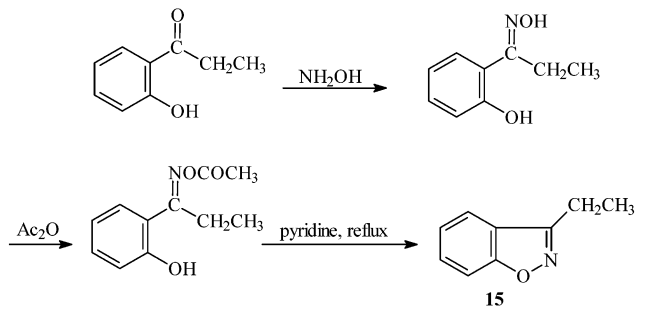
Scheme 3



Scheme 4



Scheme 5



2-hydroxypropiophenone (**16**), a metabolite of **15**, to yield a reactive phenyl vinyl ketone byproduct as in **5**.

In Vitro Metabolism of 1,2-Benzisoxazole Phosphorodiamidates. To determine whether 1,2-benzisoxazole phosphorodiamidate prodrugs undergo reductive metabolism and consequently liberate alkylating species, metabolic stability studies of these prodrugs were performed with rat liver S-9 fraction under hypoxic and oxic conditions. Incubation mixtures were monitored for disappearance of the parent compound and tested for alkylating activity as determined by NBP assay.

Table 1. In Vitro Cytotoxicity of 1,2-Benzisoxazole Phosphorodiamidates against V-79 Cells

compd	IC_{50}[a] (μM)	compd	IC_{50}[a] (μM)
control[b]	45 ± 2	**12**	311 ± 4
4	411 ± 39	**5**	1238 ± 129
9	241 ± 10	**15**	≫2000

[a] IC_{50}: concentration (μM) of drug required to reduce cell growth to 50% of the controls in a SRB assay obtained from the mean ± SD of three experiments. Each experiment comprised of triplicate determinations of cell growth at eight concentrations of test compound. Drug exposure period was 48 h. [b] 2-(Methylsulfonyl)ethyl phosphorodiamidate.

Table 2. Metabolic Stability Profile of 1,2-Bemzisoxazole Phosphorodiamidates upon Incubation with Phenobarb-Induced Rat Liver S-9 Fraction[a]

	% compound remaining[b,c]		
incubation conditions	**4**	**9**	**12**
Hypoxic			
NADPH	34 ± 9	45 ± 4	1 ± 2
no cofactor	98 ± 7	79 ± 4	76 ± 5
Oxic			
NADPH	33 ± 4	46 ± 2	1 ± 1
no cofactor	97 ± 5	78 ± 5	79 ± 5
0.025 M phosphate buffer pH 7.4	97 ± 11	97 ± 6	102 ± 10
boiled S-9, NADPH[d]	96		

[a] Incubation time was 30 min. [b] Mean ± SD from three experiments. [c] The incubation mixtures of phosphorodiamidate prodrugs were quantitatively analyzed for the unchanged starting compounds by TLC densitometry. [d] Mean from two experiments.

Table 3. Generation of Alkylating Activity upon Incubation of 1,2-Bemzisoxazole Phosphorodiamidates with Phenobarb-Induced Rat Liver S-9 Fraction[a]

	absorbance[b,c]		
incubation conditions	**4**	**9**	**12**
control[d]	0.04	0.05	0.04
Hypoxic			
NADPH	2.02	2.35	2.23
no Cofactor	0.38	0.42	0.85
Oxic			
NADPH	1.97	2.31	2.36
no cofactor	0.26	0.32	0.48
0.025 M phosphate buffer pH 7.4	0.49	0.26	0.48
boiled S-9, NADPH	0.44		

[a] Incubation time was 30 min. Incubation samples from metabolic stability experiments were utilized for alkylating activity measurements. [b] Mean of absorbance value at 542 nm from two experiments. [c] Absorbance value is a measurement of NBP alkylation product, which is indicative of alkylating activity. [d] Control sample consisted of the S-9 fraction and NADPH without test compound.

Preliminary experiments of **4** with uninduced rat liver S-9 showed less than 10% metabolism. Upon incubation with phenobarb-induced rat liver S-9 fraction, **4**, **9**, and **12** underwent extensive NADPH-dependent metabolism (Table 2) under both hypoxic and oxic conditions and concomitantly generated substantial alkylating activity (Table 3). Under hypoxic as well as oxic conditions, **12** was almost quantitatively metabolized. In comparison to **12**, analogues **4** and **9** were metabolized to a lesser extent, and 33−46% of the original amount was left unchanged. Control incubations in the absence of NADPH with aqueous buffer (0.025 M phosphate buffer, pH 7.4) or boiled S-9 supplemented with NADPH did not show significant metabolism or alkylating activity. These data provided initial indirect evidence in support

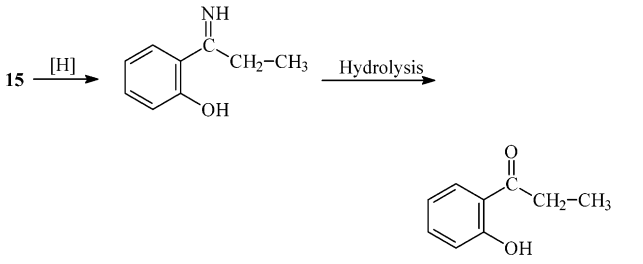
of the proposed bioreductive pathway that **4**, **9**, and **12** undergo metabolism and, thereafter, liberate alkylating species, most likely PDA. Analogue **12** was the most susceptible compound toward metabolism by induced rat S-9. If **12** is quantitatively metabolized to PDA, it may be expected to have the greatest cytotoxicity among tested compounds. However, cytotoxicity data showed that **12** was less potent than **9**. This suggested that, besides reductive activation, some of **12** might be vulnerable to other competing metabolic pathways, e.g., nitro group reduction. In contrast to the enhanced bioreduction of zonisamide to SMAP under a hypoxic environment,[16−21] no significant differences were discernible between the extent of metabolism or alkylating activities under hypoxic and oxic incubation conditions for any of the tested 1,2-benzisoxazole phosphorodiamidate prodrugs. Presumably, the replacement of the sulfamoylmethyl side chain in zonisamide with ethylphosphorodiamidate contributes toward loss of hypoxia-selective bioreduction in the tested prodrugs.

Since the NBP assay does not discriminate between alkylating species, it is conceivable that the alkylating activity observed in the incubation mixtures may arise from chloroacetaldehyde, the possible oxidative *N*-dealkylation product of the chloroethylamine side chain. To investigate if prodrugs generated alkylating species as outlined in the proposed activation pathway (Scheme 2), incubation mixtures of **9** were subjected to LC/MS analysis to detect the presence of the corresponding ketone metabolite. Due to the electron-donating methoxy substituent, it was postulated that the ketone metabolite of **9** might be relatively stable to β-elimination in the incubation mixtures and thereby possible to detect. Under reverse-phase chromatography, **9** eluted at a retention time (t_R) of 13.5 min. The parent compound showed the expected $(M + H)^+$ pseudomolecular ion at *m*/*z* 396 and other characteristic ion-source fragment ions at *m*/*z* 255 and 176 (Figure 1

of the Supporting Information). A closely eluting metabolite peak with $t_R = 13.8$ min showed a $(M + H)^+$ ion with *m*/*z* 399. An increase of 3 amu for the later-eluting peak indicated the presence of a ketone metabolite. A similar shift of 3 amu was observed for ion-source fragment ions with *m*/*z* values of 258 and 179. The metabolite peak showed another distinct fragment ion at *m*/*z* 151 that was not observed for **9**. The metabolite peak was detected in aerobic and hypoxic phenobarb-induced rat liver S-9 incubation mixtures of **9** containing NADPH, but not in the absence of NADPH or when incubations were carried out in the presence of benzaldehyde or 2-hydroxypyrimidine, known cofactors of enzyme aldehyde oxidase. The LC/MS data supported the existence of the ketone metabolite of **9** and, in conjunction with previous metabolism and alkylating activity data, provided persuasive evidence in support of the proposed reductive activation pathway.

In Vitro Metabolism of 15. Compound **15** was selected as a model test compound to measure the rate and extent of bioreduction of 1,2-benzisoxazoles and to identify potential reductase enzyme(s). The ability of **15** to convert to 2-hydroxypropiophenone (**16**) (Scheme 6) could provide additional supportive evidence for the activation pathway of 1,2-benzisoxazole-based prodrugs. Moreover, bioreduction of **4**, **9**, and **12** yields inherently

Scheme 6



2-Hydroxypropiophenone **(16)**

Table 4. Formation of **16** upon Incubation of **15** with Rat Liver Subcellular Fractions in the Presence of Various Cofactors[a]

	% **16** formed[b,c]	
	hypoxic	oxic
Phenobarb-Induced Rat Liver		
S-9 fraction		
NADPH	88.5 ± 3.8	80.9 ± 3.0
NADH	28.5 ± 1.5	14.8 ± 1.6
benzaldehyde	2.0 ± 0.3	0.7 ± 0.3
2-hydroxypyrimidine	0.5 ± 0.1	0.6 ± 0.1
no cofactor	2.2 ± 0.5	0.5 ± 0.1
0.025 M phosphate buffer pH 7.4		nd
boiled S-9, NADPH		0.1 ± 0.0
microsomal fraction		
NADPH	93.0 ± 9.1	73.0 ± 2.9
no cofactor[c]	0.4	0.6
cytosolic fraction		
NADPHc	3.6	0.7
no cofactorc	1.4	0.4
Uninduced Rat Liver		
S-9 fraction		
NADPH	9.4 ± 0.1	9.1 ± 0.1
no cofactor[d]	0.9	1.6

[a] Incubation time was 30 min. [b] Mean ± SD of three experiments. [c] Metabolite **16** was analyzed by HPLC. The amount of **16** was calculated and is reported as percent of total, where the total was based on an equimolar amount of substrate (**15**) used. [d] Mean from two experiments. nd, not detected.

unstable ketone metabolites that are difficult to measure, whereas stable **16** generated from **15** can be easily quantitated against commercially available standard.

Upon incubation of **15** with phenobarb-induced rat liver S-9 fraction, **16** was formed under oxic (80.9%) as well as hypoxic (88.5%) conditions with NADPH cofactor (Table 4). Although formation of **16** was also observed with NADH cofactor, the extent of NADH-dependent formation of **16** was approximately 4−5-fold lower than NADPH-dependent activity. Some degree of hypoxia-selective metabolism was observed with NADH, but not with NADPH cofactor. Incubation of **15** in the presence of benzaldehyde or 2-hydroxypyrimidine, electron donors for enzyme aldehyde oxidase, did not yield significant amounts of **16**. These data suggested the lack of involvement of aldehyde oxidase in activation of **15**. The data were in disagreement with earlier report that demonstrated aldehyde oxidase-mediated metabolism of zonisamide to SMAP in some strains of rat liver cytosol.[21] These conflicting results may be explained due to differences between strains of rats utilized in current experiments and in the literature or most likely due to **15** being a poor substrate for enzyme aldehyde oxidase. Uninduced rat liver S-9 fraction showed approximately 10-fold lower activity in generation of **16** as compared to induced rat liver S-9.

Table 5. Effect of Inhibitors SKF-525A and PCMBA on Formation of **16** upon Incubation of **15** with Phenobarb-Induced Rat Liver Microsomal Fraction[a]

concn of inhibitor (mM)	hypoxic		oxic	
	% **16** formed[b,c]	% inhibn	% **16** formed[b,c]	% inhibn
	SKF-525A			
0	70.8	0	44.9	0
1	54.6	23	36.9	18
2	50.3	29	38.5	14
4	45.0	36	26.9	40
	pCMBA			
0	93.2	0	68.8	0
1	0.7	99	0.5	99
2	1.2	99	0.5	99
4	0.5	99	0.7	99

[a] Incubation time was 30 min. [b] Mean of values obtained from two experiments. [c] See footnote *c* in Table 4.

Investigations with other phenobarb-induced rat liver subcellular fractions showed that the NADPH-dependent metabolic activity of S-9 was primarily located in the microsomal fraction with limited contribution from the cytosolic fraction. These data suggested the possible involvement of NADPH-dependent enzyme(s) cytochrome P450 and/or cytochrome P450 reductase, in the bioreduction of **15**, both of which have been shown to catalyze reduction of a variety of compounds. To test the involvement of these enzymes in the microsomal formation of **16** from **15**, inhibition studies were carried out in phenobarb-induced rat liver microsomes and NADPH with varying concentrations of SKF-525A or *p*-chloromercuribenzoic acid (pCMBA), known inhibitors of cytochrome P450 and cytochrome P450 reductase, respectively (Table 5). SKF-525A inhibited microsomal formation of **16** from **15** under oxic as well as hypoxic conditions in a concentration-dependent manner with 40% (oxic) and 36% (hypoxic) inhibition at 4 mM concentration. This implied possible involvement of cytochrome P450 in the reductive activation of **15**. On the other hand, pCMBA almost quantitatively inhibited formation of **16** from **15** at all tested concentrations under both oxic as well as hypoxic conditions. Results from pCMBA inhibition studies suggested that P450 reductase might also be involved in reduction of **15** to **16**. Alternatively, since P450 reductase activity is essential for cytochrome P450-mediated metabolism, the data might suggest indirect inhibition of cytochrome P450 activity due to inhibition of the P450 reductase.

To investigate the role of P450 reductase, **15** was incubated with rat or human P450 reductase microsomes. In the presence of NADPH, **16** was formed ≤1.7% under hypoxic or oxic conditions and found inhibited due to pCMBA (Table 6). Reduction of **15** due to P450 reductase alone was markedly weaker in comparison with microsomal activity. It has been reported that V-79 fibroblasts contain DT-diaphorase, cytochrome P450 reductase, and xanthine oxidase[33] enzymes, whereas no detectable cytochrome P450 are observed.[34,35] Therefore, in the absence of cytochrome P450, endogenous cytochrome P450 reductase activity in V-79 cells assumes relevance due to its potential ability of reductive activation of phosphorodiamidate prodrugs leading to cytotoxicity.

Table 6. Formation of **16** upon Incubation of **15** with Human or Rat P450 Reductase Microsomes[a]

	% **16** formed[b,c]	
	hypoxic	oxic
Human P450 Reductase Microsomes		
NADPH	1.7	nd
no cofactor	nd	nd
NADPH, 2 mM pCMBA	0.6	nd
Rat P450 Reductase Microsomes		
NADPH	1.7	1.5
no cofactor	nd	nd
NADPH, 2 mM pCMBA	nd	nd

[a] Incubation time was 60 min. [b] Mean of values obtained from two experiments. nd, not detected. [c] See footnote *c* in Table 4.

Conclusions

A series of 1,2-benzisoxazole phosphorodiamidate prodrugs were designed and successfully synthesized. The prodrugs demonstrated in vitro cytotoxicity against V-79 cells. Consistent with the original hypothesis, prodrugs underwent reductive metabolism and generated alkylating activity, however, with equal degree under oxic and hypoxic conditions. Detection of ketone metabolites of **9** and **15** provided additional convincing evidence in support of the proposed metabolic pathway. Model test compound **15** underwent NADPH-dependent metabolic reduction in phenobarb-induced rat liver microsomes under oxic and hypoxic conditions. On the basis of inhibition of microsomal activity due to SKF-525A and pCMBA as well as some metabolic activity observed in human and rat P450 reductase microsomes, cytochrome P450 and/or cytochrome P450 reductase enzymes may be involved in bioreduction of 1,2-benzisoxazoles.

Experimental Section

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and were uncorrected. ^{1}H NMR spectra were recorded on a Bruker Avance 400 (400 MHz) instrument, and chemical shifts are reported as δ values (ppm) downfield from tetramethylsilane as an internal standard. NMR abbreviations used are as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). MS analyses were carried out on an LCQ (Finnigan) ion trap LC/MS instrument. Elemental analyses were performed by Atlantic Microlab, Inc. Norcross, GA. Silica gel GF plates (Analtech) were used for TLC (250 μm, 2.5 × 10 cm) and preparative TLC (1000 μm, 20 × 20 cm). Silica gel (40 μm, Baker) was used for flash column chromatography. NBP spray was used for the detection of potential alkylating compounds as follows: the plates were sprayed with 5% 4-(*p*-nitrobenzyl)pyridine (NBP) in acetone, heated at 100 °C for 5 min, and sprayed with 5% methanolic KOH. Alkylating agents are indicated by the appearance of a blue chromophore. Spectrophotometric analysis was performed on a Milton Roy Spectronic 301 spectrophotometer. Quantitative TLC analyses were done on an Analtech Uniscan Video Densitometer. All organic reagents and solvents were reagent grade and purchased from commercial vendors.

1,2-Benzisoxazole-3-acetic acid (1) was prepared according to the published procedure[25] in 46% yield: mp 120−125 °C (lit.[25] mp 125 °C); TLC R_f = 0.50 in EtOAc/hexane (1:1) with 1% acetic acid; ^{1}H NMR ($CDCl_3$/DMSO-d_6, 8:1) δ 3.93 (s, 2H), 7.22−7.26 (m, 1H), 7.44−7.49 (m, 2H), 7.67 (d, J = 7.9 Hz, 1H).

Methyl 1,2-benzisoxazol-3-yl acetate (2) was prepared according to published procedure[27] with some modifications. To a solution of **1** (1.0 g, 60.0 mmol) in 20 mL of methanol were added 8−10 drops of concentrated sulfuric acid. The mixture was heated under reflux with continuous stirring for a period of 1.5 h. Following reflux, the reaction mixture was

cooled to room temperature and evaporated in vacuo to yield a pale yellow, oily residue. The residue was added with a saturated solution of $NaHCO_3$ (50 mL) to neutralize acid, and the resulting alkaline aqueous mixture was extracted with EtOAc (3 × 50 mL). Organic extracts were pooled, dried over anhydrous sodium sulfate, and filtered, and filtrate was evaporated under reduced pressure to yield a pale yellow, oily product (1.0 g) in 93% yield: TLC $R_f = 0.45$ in EtOAc/hexane (1:5); ^{1}H NMR ($CDCl_3$) δ 3.77 (s, 3H), 4.08 (s, 2H), 7.33−7.37 (m, 1H), 7.56−7.62 (m, 2H), 7.73 (d, $J = 8.0$ Hz, 1H). The obtained product was chromatographically pure and used without additional purification.

2-(1,2-Benzisoxazol-3-yl)ethanol (3) was synthesized according to published procedure[27] with some modifications. A solution of **2** (2.5 g, 13 mmol) in 50 mL of anhydrous THF was added dropwise from a dropping funnel into a stirring suspension of $LiAlH_4$ (0.98 g, 26 mmol) in anhydrous THF (30 mL) in a three-neck flask over a period of 15 min at ice-bath temperature under nitrogen atmosphere. The reaction was allowed to proceed for 15 min and then terminated with dropwise addition of EtOAc (50 mL) at ice-bath temperature with vigorous stirring. The mixture was evaporated under reduced pressure and the residue suspended in 100 mL of EtOAc/hexane (1:1). The suspension was filtered through Celite and filtrate evaporated in vacuo to yield a pale yellow, oily residue. The crude product was dissolved in 10 mL of EtOAc/hexane (1:1) and purified using flash column with eluent EtOAc/hexane (1:1) to give pure **3** as a yellow, oily product (1.0 g, 53% yield): TLC $R_f = 0.49$ in EtOAc/hexane (1:1); ^{1}H NMR ($CDCl_3$) δ 3.26 (t, $J = 6.0$ Hz, 2H), 4.15 (q, $J = 3.9$ Hz, 2H), 7.30−7.37 (m, 1H), 7.55−7.60 (m, 2H), 7.71 (d, $J = 7.9$ Hz, 1H).

2-(1,2-Benzisoxazol-3-yl)ethyl *N*,*N*-bis(2-chloroethyl)-phosphorodiamidate (4) was synthesized according to published procedure[24] with some modifications. A stirring solution of **3** (0.25 g, 1.5 mmol) in anhydrous THF (10 mL) was added to a *n*-butyllithium (0.6 mL, 1.5 mmol, 2.5 M in hexane) solution in anhydrous THF (5 mL) from a dropping funnel at ice-bath temperature in a two-neck round-bottom flask. After complete addition of *n*-BuLi, the mixture was stirred at ice-bath temperature for 15 min, then the ice bath was removed, and stirring was continued at room temperature for additional 90 min. The resulting pale yellow suspension was aspirated into a syringe, transferred into a dropping funnel, and added dropwise to a stirring solution of *N*,*N*-bis(2-chloroethyl)-phosphoramidic dichloride (0.4 g, 1.6 mmol) in anhydrous THF (15 mL) at ice-bath temperature. The reaction mixture was stirred at ice-bath temperature for 1 h and then at room temperature for an additional 2.5 h. Ammonia was bubbled into the reaction mixture with stirring at ice-bath temperature for 45 min. Liquids in the reaction mixture were evaporated in vacuo, and ether (50 mL) was added. Resulting solids were filtered using Celite, and filtrate was evaporated under vacuum to give viscous, pale yellow, oily residue. The crude product was dissolved in EtOAc (10 mL) and purified using flash column with eluent EtOAc/EtOH (10:1) to give pale yellow, oily product that gave white crystals upon overnight storage in freezer. The product was recrystallized using EtOAc/hexane to give light, white-colored solids (0.12 g, 21% yield): mp 80−85 °C; TLC $R_f = 0.56$ in EtOAc/EtOH (10:1); APCI-MS $(M + H)^+$ *m*/*z* calcd 366.05, found 365.9; ^{1}H NMR ($CDCl_3$) δ 3.23−3.61 (m, 10H), 4.39−4.60 (m, 2H), 7.33−7.38 (m, 1H), 7.53−7.70 (m, 2H), 7.72 (d, $J = 7.2$ Hz, 1H). Anal. ($C_{13}H_{18}Cl_2N_3O_3P$) H, Cl, N; C: calcd, 42.62; found, 43.29.

2-(1,2-Benzisoxazol-3-yl)ethyl *N*,*N*,*N*′,*N*′-Tetramethylphosphorodiamidate (5). To a stirring solution of **3** (0.25 g, 1.5 mmol) in anhydrous THF (10 mL) in a two-neck round-bottom flask was added a solution of *n*-butyllithium (0.6 mL, 1.5 mmol, 2.5 M in hexane) in anhydrous THF (10 mL) from a dropping funnel at ice-bath temperature. The reaction mixture was stirred at ice-bath temperature for 15 min followed by removal of the ice bath and continued stirring at room temperature for 60 min. The mixture was aspirated into a syringe, transferred quickly into a dropping funnel, and added dropwise to a stirring solution of *N*,*N*,*N*′,*N*′-tetramethyldiamidophosphorochloridate (0.34 g, 1.65 mmol) in anhydrous THF (12 mL) at ice-bath temperature. The mixture was stirred at ice-bath temperature and then gradually allowed to warm to room temperature and stirred overnight. Reaction mixture was evaporated under vacuum to obtain a yellow-colored oily residue as the crude product. This was dissolved in EtOAc (10 mL) and purification by column chromatography of the resulting solution eluting with EtOAc/EtOH (6:1) afforded pure **5** as a pale yellow oil (0.26 g, 58% yield): TLC $R_f = 0.60$ in EtOAc/EtOH (10:1.5); ^{1}H NMR ($CDCl_3$) δ 2.57 (d, $J = 9.8$ Hz, 12H), 3.38 (q, $J = 6.6$ Hz, 2H), 4.39 (q, $J = 7.4$ Hz, 2H), 7.32−7.35 (m, 1H), 7.54−7.59 (m, 2H), 7.75 (d, $J = 7.9$ Hz, 1H). Anal. ($C_{12}H_{20}N_3O_3P$) H, N; C: calcd, 52.52; found, 52.04.

6-Methoxy 1,2-benzisoxazole-3-acetic acid (6) was prepared according to published procedure[26] and obtained as light-tan-colored solids (0.4 g, 59% yield): mp 175−179 °C (lit.[26] mp 174−175 °C); TLC $R_f = 0.22$ in CH_2Cl_2/EtOH/acetic acid (100:1.25:0.5); ^{1}H NMR ($CDCl_3$/DMSO-d_6, 10:1) δ 3.73 (s, 3H), 3.78 (s, 2H), 6.76 (d, $J = 8.72$ Hz, 1H), 6.83 (d, $J = 2.0$ Hz, 1H), 7.42 (d, $J = 8.7$ Hz, 1H).

Methyl (6-methoxy-1,2-benzisoxazol-3-yl)acetate (7) was prepared from **6** (1.9 g, 9.0 mmol) as in the synthesis of **2** to give product **7** as a pale yellow, crusty solid (2.0 g, quantitative yield): mp 70−74 °C (lit.[26] mp 68−69 °C); TLC $R_f = 0.63$ in CH_2Cl_2/EtOH/acetic acid (100:1.25:0.5); ^{1}H NMR ($CDCl_3$) δ 3.76 (s, 3H), 3.90 (s, 3H), 4.01 (s, 2H),), 6.95 (d, $J = 8.7$ Hz, 1H), 7.01 (d, $J = 2.0$ Hz, 1H), 7.55 (d, $J = 8.0$ Hz, 1H).

2-(6-Methoxy-1,2-benzisoxazol-3-yl)ethanol (8) was prepared by reduction of **7** (1.72 g, 8 mmol) with $LiAlH_4$ as in the procedure for preparation of **3**. Crude product was obtained as a dark yellow, oily residue. The residue was dissolved in 15 mL of EtOAc/hexane (1:1) solvent mixture and purified using column chromatography with the use of EtOAc/hexane (1:1) as the eluting solvent. Pure **8** was obtained as a pale yellow, oily material (1.20 g, 81% yield): TLC $R_f = 0.43$ in EtOAc/hexane (1:1); ^{1}H NMR ($CDCl_3$) δ 3.20 (t, 5.9 Hz, 2H), 3.90 (s, 3H), 4.10 (q, $J = 4.6$, 2H), 6.92−6.95 (d, $J = 8.7$ Hz, 1H), 7.00 (d, $J = 2.0$ Hz, 1H), 7.53 (d, $J = 8.8$ Hz, 1H).

2-(6-Methoxy-1,2-benzisoxazol-3-yl)ethyl *N*,*N*-bis(2-chloroethyl)phosphorodiamidate (9) was prepared from **8** (0.50 g, 2.6 mmol) as in the synthesis of **4** to give a pale yellow, oily substance as a crude product. This was purified using column chromatography with EtOAc/EtOH (10:1.5) as the eluting solvent. Pure **9** was obtained as white-colored solids (0.28 g, 27% yield): mp 86−90 °C; TLC $R_f = 0.69$ in EtOAc/EtOH (10:1); APCI-MS $(M + H)^+$ *m*/*z* calcd 396.05, found 395.9; ^{1}H NMR ($CDCl_3$) δ 3.31−3.59 (m, 10H), 3.90 (s, 3H), 4.46 (d, 2H), 6.94 (d, $J = 8.6$ Hz, 1H), 7.00 (d, $J = 2.0$ Hz, 1H), 7.54 (d, $J = 8.6$, 1H). Anal. ($C_{14}H_{20}Cl_2N_3O_4P$) C, H, Cl, N.

Methyl (5-nitro-1,2-benzisoxazol-3-yl)acetate (10) was prepared from **2** (3.0 g, 15.7 mmol) according to published procedure[28] to obtain the crude product as a yellow oil. The oil was dissolved in CH_2Cl_2 (15 mL) and purified using column chromatography with the use of hexane/EtOAc (4:1) as the eluting solvent. Appropriate fractions containing the product were pooled and evaporated under reduced pressure to give a pale yellow, oily residue that solidified upon addition of a few drops of ether. Resulting solids were recrystallized with methanol to give **10** as white solids (2.7 g, 74% yield): mp 65−67 °C (lit.[28] mp 66−67 °C); TLC $R_f = 0.26$ in hexane/EtOAc (4:1); ^{1}H NMR ($CDCl_3$) δ 3.82 (s, 3H); 4.14 (s, 2H), 7.54 (d, $J = 9.2$ Hz, 1H), 8.51 (d, $J = 6.9$ Hz, 1H), 8.71 (d, $J = 2.2$ Hz, 1H).

2-(5-Nitro-1,2-benzisoxazole-3-yl)ethanol (11) was synthesized from **10** (0.50 g, 2 mmol) according to the procedure utilized for preparation of **3**. After quenching the reaction with EtOAc, the liquids were evaporated under vacuum and the dark residue partitioned between CH_2Cl_2 (100 mL) and 0.5 N HCl (100 mL). The aqueous layer was further extracted with EtOAc (2 × 75 mL). The CH_2Cl_2 and EtOAc extracts were combined and dried under vacuum to obtain the crude product as a dark-colored residue. The residue was subjected to purification with column chromatography with the use of

hexane/EtOAc (2:1) eluting solvent. Fractions containing the desired compound were pooled and the solvent evaporated under vacuum to yield pure product as shiny, yellow crystals (0.14 g, 32% yield): TLC $R_f = 0.69$ in EtOAc/hexane (1:1); ^{1}H NMR ($CDCl_3$) δ 3.31 (t, $J = 5.9$ Hz, 2H), 4.11−4.18 (m, 2H), 7.69 (d, $J = 5.0$ Hz, 1H), 8.49 (d, $J = 5.8$ Hz, 1H), 8.73 (d, $J = 2.2$ Hz, 1H).

2-(5-Nitro-1,2-benzisoxazol-3-yl)ethyl *N*,*N*-bis(2-chloroethyl)phopshorodiamidate (12) was synthesized with the use of **11** (0.21 g) according to the procedure in preparation of **4** on a 1.0 mmol scale. Crude product was obtained as an orange-colored oily residue. The residue was dissolved in EtOAc (15 mL) and purified using column chromatography with EtOAc used as the eluting solvent. Appropriate fractions containing the desired product were pooled and dried under vacuum to give pure **12** as viscous, sticky, yellow oil (0.1 g, 24% yield): TLC $R_f = 0.34$ in EtOAc; ESI-MS $(M + H)^+$ *m*/*z* calcd. 411.03. Found 410.9; ^{1}H NMR ($CDCl_3$) δ 3.34−3.65 (m, 10H), 4.40−4.57 (m, 2H), 7.72 (d, $J = 9.1$ Hz, 1H), 8.49−8.51 (d, $J = 9.1$ Hz, 1H), 8.74 (d, $J = 2.1$ Hz, 1H). Anal. ($C_{13}H_{17}Cl_2N_4O_5P$) H, Cl, N; C: calcd, 37.97; found, 38.68.

1-(2-Hydroxyphenyl)propan-1-one oxime (13) was synthesized according to a published procedure[29] with some modifications. A solution of hydroxylamine hydrochloride (1.5 g, 22 mmol) and sodium acetate trihydrate (3.13 g, 23 mmol) dissolved in 50 mL of EtOH/H_2O (7:3) was added to a solution of 2-hydroxypropiophenone (2.89 g, 19 mmol) in 25 mL of EtOH/H_2O (7:3) solvent. The resulting mixture was heated to reflux. After 2 h, additional hydroxylamine hydrochloride (0.675 g, 9.5 mmol) and sodium acetate trihydrate (9.5 mmol) were dissolved in water (15 mL) and added, and the reflux was continued for additional 30 min. The reaction mixture was cooled to room temperature, concentrated by evaporation under vacuum, and white solids obtained were filtered, washed with water, and dried to give sufficiently pure **13** (2.7 g, 85% yield): mp 90−94 °C; TLC $R_f = 0.46$ in hexane/EtOAc (5:1); ^{1}H NMR ($CDCl_3$) δ 1.25 (t, $J = 7.7$ Hz, 3H), 2.92 (q, $J = 7.6$ Hz, 2H), 6.92−6.96 (m, 1H), 7.01 (d, $J = 8.2$ Hz, 1H), 7.27−7.32 (m, 1H), 7.47 (d, $J = 8.0$ Hz, 1H).

1-(2-Hydroxyphenyl)propan-1-one *O*-acetyloxime (14) was synthesized according to a published procedure[29] with some modifications. Oxime intermediate **13** (3.0 g, 18 mmol) was dissolved in acetic anhydride (6 mL) in a round-bottom flask at room temperature with gentle swirling. The flask became warm to the touch. Swirling was continued for 10 min, following which white precipitates separated out. Water (50 mL) was added to the flask, and the mixture was filtered to obtain white-colored solid residues. The solids were washed with water and dried to give product **14** in 3.35 g yield (89%): mp 87−92 °C; TLC $R_f = 0.66$ in CH_2Cl_2; ^{1}H NMR ($CDCl_3$) δ 1.27 (t, $J = 9.5$ Hz, 3H), 2.28 (s, 3H), 2.94 (q, $J = 9.5$ Hz, 2H), 6.93−6.97 (m, 1H,), 7.12 (d, $J = 8.0$ Hz, 1H), 7.34−7.39 (m, 2H), 7.50 (d, $J = 8.0$ Hz, 1H).

3-Ethyl-1,2-benzisoxazole (15) was prepared according to the published procedure[29] with some modifications. Oxime acetate (**14**) (2.5 g, 12.0 mmol) was heated under reflux with anhydrous pyridine (25 mL) for 3.5 h. Following reflux, the dark yellow reaction mixture was allowed to cool to room temperature and acidified with 5 N HCl (approximately 100 mL). The mixture was extracted with ether (3 × 75 mL). The ether layers were pooled, washed with 1 N HCl (75 mL), dried over anhydrous sodium sulfate, and evaporated under vacuum to give yellow-colored, oily residue as crude product. The crude product was purified using column chromatography with hexane/EtOAc (100:7) as the eluting solvent. Fractions containing the desired compound were combined and dried under vacuum to give **15** as colorless oil (1.5 g, 85% yield): TLC $R_f = 0.74$ in hexane/EtOAc (5:1); ESI-MS $(M + H)^+$ *m*/*z* calcd 148.07, found 147.9; ^{1}H NMR ($CDCl_3$) δ 1.48 (t, $J = 7.5$ Hz, 3H), 3.04 (q, $J = 7.5$ Hz, 2H), 7.31 (m, 1H), 7.53−7.58 (m, 2H), 7.68 (d, $J = 7.1$ Hz, 1H). Anal. (C_9H_9NO) H, N; C: calcd, 73.45; found, 72.49.

In Vitro Cytotoxicity. In vitro cytotoxicity assays were carried out according to a procedure by Skehan et al.[30] in 96-well microtiter plates with some modifications. One hundred microliters of cell suspension (approximately 15×10^3 cells per 100 μL) was transferred to a well and preincubated for 24 h. Fifty microliter of media containing variable concentrations of drugs as well as 50 μL of fresh media was added to each well. Plates were incubated under aerobic conditions for a 48-h period. Cultures were fixed with 50 μL of 50% cold trichloroacetic acid and incubated at 4 °C for 1 h. Plates were then washed five times with tap water to remove trichloroacetic acid, growth medium, and serum protein and then air-dried. The fixed cells were stained for 30 min with 100 μL of 0.4% sulforhodamine B (SRB) solution in 1% acetic acid. At the end of the staining period, plates were rinsed five times with 1% acetic acid to remove unbound dye. The bound dye was solublized with 200 μL of 10 mM Tris buffer for 5 min. A 100-μL aliquot was diluted with 2 mL of 10 mM Tris buffer and absorbance of the resulting solution was measured at 564 nm.

Absorbance is directly proportional to cell-growth after incubation. Percent cell growth following drug treatment was calculated as (absorbance from drug-treated well/absorbance from untreated control well) × 100. Drug concentrations were plotted semilogarithmically versus percent cell-growth values, data were curve-fitted to a first-order decay equation, and IC_{50} values were calculated from the slope (k) of the equation. Each experiment consisted of controls and varying concentrations of drug treatments in triplicates, and three experiments were carried out to obtain the mean IC_{50} value for a compound.

In Vitro Metabolism Studies. Preparation of Rat Liver Fractions. Rat liver S-9 cytosolic and microsomal fractions were prepared as described previously.[32] Protein concentrations were determined by the method of Bradford (Protein Assay Kit, Bio-Rad Laboratories, Inc.) using bovine serum albumin as standard. Protein concentrations for various rat liver fractions were obtained as follows: Uninduced rat liver S-9 fraction, 22.5 mg/mL; phenobarb-induced rat liver S-9 fraction, 17.9 mg/mL (preparation I) and 18.2 mg/mL (preparation II); phenobarb-induced rat liver cytosolic fraction, 12.7 mg/mL; phenobarb-induced rat liver microsomal fraction, 6.3 mg/mL. Cytochrome *c* reductase activity of P450 reductase microsomes was carried out in the following manner: A 2.5 mL reaction mixture containing 0.04 mg/mL protein, 1 mg/mL cytochrome *c*, and 1.6 mM NADPH in 0.025 M phosphate buffer, pH 7.4 was incubated. Incubations were initiated with addition of NADPH, and absorbance was monitored at 550 nm as a function of time. Activity was based on the extinction coefficient of cytochrome *c* (21 cm^2/mmol), the rate of increase in absorbance per minute of incubation, and the amount of protein added to the incubation. Cytochrome *c* reductase activity was obtained as follows: human P450 reductase microsomes, 436.8 nmol/(min × mg protein); rat P450 reductase microsomes, 401.2 nmol/(min × mg protein); phenobarb-induced rat liver microsomal fraction, 202.4 nmol/(min × mg protein).

Incubations. A typical incubation mixture consisted of 2 mL of rat liver fraction containing 5 mM cofactors and 2.5 mM substrates. The test compound was dissolved in methanol/water (50:50) and a 100-μL volume added to the incubation mixture to obtain a final concentration of 2.5 mM. In case of anaerobic incubations, rat liver fraction was transferred to a vial, and the vial was capped with rubber septa with a provision for inlet and outlet needles for nitrogen gas. The fraction was purged with nitrogen for 15−20 min, and septa were sealed. Incubations were initiated with addition of test compound, carried out for 30 min at 37 °C, and terminated with addition of 2 mL of ice-cold methanol. The contents were transferred into a glass test tube and then centrifuged (3000 rpm, 15 min) to allow separation of precipitated proteins. The supernatant was used for either TLC or HPLC analysis. For TLC analysis, 64−80 μL was spotted on a TLC plate and the plates eluted with ethyl acetate/ethanol (10:1) (for compounds **4** and **12**) or with ethyl acetate/ethanol 10:2 (for compound **9**).

For LC/MS analysis of incubation mixtures of **9**, a HP1050 liquid chromatographic system was used for chromatographic separation of ketone metabolite from **9**. A 50 μM aliquot of

supernatant obtained after protein precipitation, as described above, was injected onto a reverse phase C-18 column (Zorbax XDB-C18; 75 × 4.6 mm; 3.5 μm particle size) and eluted with mobile phase consisting of two solvents, (A) 90:10 H_2O/methanol with 10 mM ammonium formate, (B) 10:90 H_2O/methanol with 10 mM ammonium formate. For the first 3 min, isocratic elution at A/B 80:20 was used; thereafter, a linear gradient for 15 min from A/B 80:20 to A/B 20:80 was used. Mobile phase composition was returned to its original value over the next 5 min and allowed to equilibrate for the next analysis. The flow rate throughout the gradient was 0.8 mL/min. For MS analysis, the HPLC effluent was introduced into a Finnigan ion trap (LCQ) mass spectrometer. Electrospray positive ionization in the full scan mode from 100 to 600 *m*/*z* was used for detection. The heated capillary temperature was maintained at 150 °C with a spray voltage at 4.5 kV.

For incubation mixtures of **15**, analyses were carried out by HPLC with UV detection at 254 nm. Chromatography was performed by injecting a 25 μL sample volume on a reverse phase C-18 column (Zorbax XDB-C18; 75 × 4.6 mm; 3.5 μm particle size). Compounds **15** (t_R = 6.2 min) and **16** (t_R = 8.2 min) were separated at a flow rate of 1.0 mL/min with isocratic elution of with a mobile phase composition of methanol/water (50:50). The HPLC system consisted of a 501 HPLC pump (Waters), a Lambda-Max Model 481 LC spectrophotometer (Waters) detector, D-2500 (Hitachi) integrator, and Model U6K (Waters) injector.

For inhibition experiments, 50 μL solutions of SKF-525A in methanol or pCMBA in DMF were added to the microsomal fraction to give the desired inhibitor concentration in 2 mL of incubation mixture. The microsomal fractions were preincubated for 30 min with pCMBA and then added with NADPH. SKF-525A was incubated with microsomes and NADPH for 15 min. Thereafter, oxic samples were added with test compound, and hypoxic samples were purged with nitrogen prior to addition of test compound.

Incubations of **15** with P450 reductase microsomes were carried out at a protein concentration of 1 mg/mL and substrate and cofactor concentrations of 0.25 and 0.5 mM, respectively. Incubations were performed in an identical fashion as above with rat liver fractions, except that the duration of incubation at 37 °C was 60 min.

Determination of Alkylating Activity in the In Vitro Incubation Mixtures by NBP Assay. Following incubation with test compound, the rat liver fraction (2 mL) was added to 2 mL of ice-cold methanol to precipitate proteins. The mixture was centrifuged and 1 mL of supernatant was used for alkylating activity test as described earlier.[32]

Supporting Information Available: LC/MS spectra of **9** and its ketone metabolite.This material is available free of charge via the Internet at http://pubs.acs.org.

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