Potential Bioreductive Alkylating Agents. 4. Inhibition of Coenzyme Q Enzyme Systems by Lipoidal Benzoquinone and Naphthoquinone Derivatives

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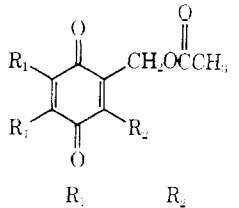
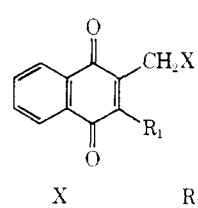
Four quinones with a pentadecyl lipoidal group and a side chain capable of alkylation following reduction of the quinone nucleus were prepared as potential inhibitors of the coenzyme Q mediated enzyme systems, NADH-oxidase and succinoxidase, as well as of neoplastic cells. The results indicated that (a) both enzyme systems are more susceptible to inhibition by benzoquinone derivatives than corresponding naphthoquinones, and (b) compounds containing the pentadecyl side chain are weaker inhibitors of these mitochondrial enzymes than corresponding derivatives without the alkyl chain. All new compounds tested were inactive against the murine ascitic neoplasm, sarcoma 180.

The antineoplastic properties of several chemical agents have been equated with lesions produced at the level of mitochondrial electron transport. Thus, the activity of podophyllotoxin was correlated with its ability to inhibit the respiration of tumor cells.[1] In addition, ethyleneiminoquinones, which have been shown to possess anticancer activity, inhibit tumor respiration,[2,3] and nordihydroguaiaretic acid, a potent inhibitor of mitochondrial electron and energy transfer systems,[4] has been reported to cause the remission of a case of malignant melanoma.[5] This latter agent also has been shown to inhibit cell respiration and glycolysis *in vitro* in the Ehrlich and K-2 carcinomas and leukemia L1210.[6]

Previous papers concerned with bioreductive alkylating agents have reported that a number of benzo-[7] and naphthoquinones,[8] with one or two side chains capable of alkylation following reduction, possessed potent inhibitory activity against adenocarcinoma 755 and sarcoma 180 ascites cells. These compounds have been postulated to form the corresponding dihydroquinones following enzymatic reduction *in vivo;* the formed dihydroquinones would be expected to decompose spontaneously to generate a reactive intermediate, quinone methide, with the capacity to bind covalently to critical biological components. Chemical evidence has been obtained to substantiate the existence of quinone methide *in vitro,* as well as its capability to alkylate amines.[9] Since the naphthoquinones synthesized previously[8] caused moderate inhibition of the activities of the coenzyme Q (CoQ) mediated enzyme systems, NADH-oxidase and succinoxidase of beef heart mitochondria, a lipoidal side chain was incorporated into the structures of benzoquinones **1a,b** or naphthoquinones **2a,b** in an attempt to generate compounds **1c,d** and **2c,d** which more closely mimicked the structure of CoQ.





	R_1	R_2
1a	$-CH_3$	-H
b	$-OCH_3$	-H
c	$-CH_3$	$-(CH_2)_{14}CH_3$
d	$-OCH_3$	$-(CH_2)_{14}CH_3$

	X	R_1
2a	$-OC(=O)CH_3$	-H
b	-Cl	-H
c	$-OC(=O)CH_3$	$-(CH_2)_{14}CH_3$
d	-Cl	$-(CH_2)_{14}CH_3$

It was anticipated that these compounds might act as competitors of CoQ for the electrons of the mitochondrial electron transport chain and that such action would result in the reduction of the inhibitors to the corresponding dihydroquinones with the capacity to spontaneously form quinone methide intermediates which then could act to alkylate the CoQ-requiring enzymes.

Table I

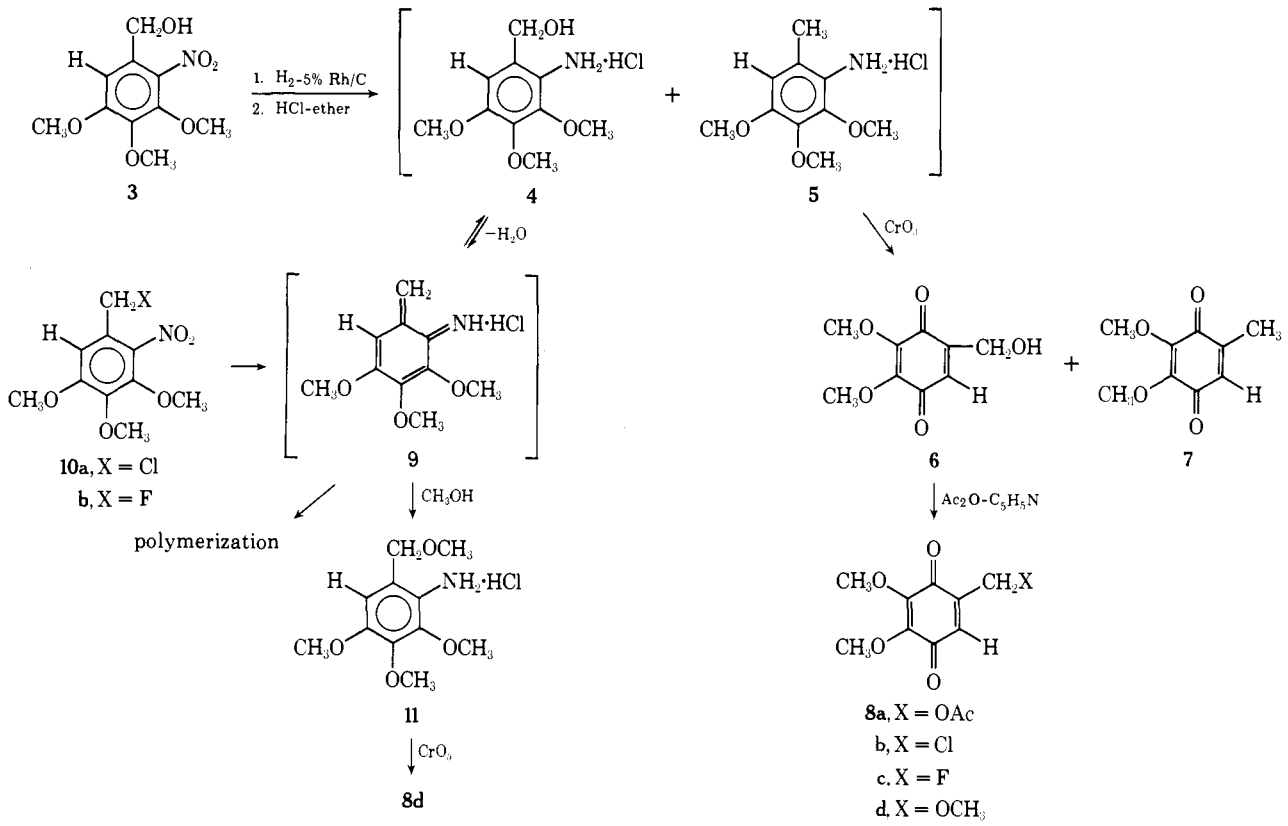
Compd	Mp, °C	Eluent	Recrystn solv	Yield, %
1c	53–56[a]	EtOAc–ligroine (1:40 v/v)	MeOH	29
1d	42–44[b]	EtOAc–ligroine (1:5 v/v)	Ligroine	27
2c	64–66	Pet. ether–EtOAc (3:1 v/v)	Ligroine	23
2d	69–71	EtOAc–ligroine (1:40 v/v)	Ligroine	37

[a]The sample for elemental analysis was sublimed at 230° (0.1 mm). [b]*Anal.* Calcd for $C_{26}H_{42}O_6$: C, 69.33; H, 9.33. Found: C, 70.59; H, 9.38. Nmr ($CDCl_3$) 1.25 (m, alkyl), 2.03 [s, $-OC(=O)CH_3$], 3.94 (s, $-OCH_3$), 4.00 (s, $-OCH_3$), and 4.98 ppm ($-CH_2OAc$).

Chemistry. The alkylquinones **1c,d** and **2c,d** (Table I) were prepared by the thermal decomposition of dipalmitoyl peroxide in the presence of the appropriate quinone.[10] The same procedure was adapted by Catlin, *et al.*,[11] to achieve the alkylation of 2,3-dimethoxy-1,4-benzoquinones. The purification of these pentadecylquinones was achieved by either column chromatography, preparative tlc (silica gel), or a combination of these procedures. While analytical samples of the naphthoquinone derivatives **2c,d** were relatively easy to prepare, those of the benzoquinone analogs **1c,d** were difficult to obtain, particularly compound **1d.** Similar difficulties in the preparation of other benzoquinones with lipoidal side chains have been documented.[11] Thus, although the elemental carbon value of **1d** was out of the range generally considered acceptable (+1.26%), this sample demonstrated only a single spot on tlc (silica gel) employing several solvent systems, and the nmr data (Table I) indicated **1d** to be the desired product. The dipalmitoyl peroxide was synthesized by the procedure of Fieser and Oxford[10] with minor modification. This involved the use of chloroform in place of petroleum ether (bp 20–40°) as the solvent and resulted in a substantial increase in the yield and purity of the peroxide. The syntheses of quinones **1a**[7] and **2a,b**[8] were reported in previous papers.

The route employed for the synthesis of 2-acetoxymethyl-5,6-dimethoxy-1,4-benzoquinone (**8a**) is shown in Scheme I. Compound **3** was prepared according to a reported procedure.[12] Catalytic reduction of compound **3** was accomplished in methanol using 5% Rh/C as the catalyst to yield a crude amine mixture (**4** + **5**). No attempt was made to purify this mixture since compound **4** appeared to be unstable. Attempts to prepare the HCl salts of the amine mixture in ether resulted in a precipitate in

Scheme I



which only part of the material was water soluble; conceivably the hydroxy group of **4** became a more effective leaving group after protonation in the acidic medium. As a result, a reactive *O*-quinomonomethanimine[13] (**9**) was generated, which then polymerized. Oxidation of the crude amine salt with CrO_3 gave the desired 2-hydroxymethyl-5,6-dimethoxy-1,4-benzoquinone (**6**) and the methyl analog **7** in poor yield. Long-range coupling[14] between methylene protons and the olefinic proton of **6** (J = 1.8 Hz) and between the methyl protons and the olefinic proton of **7** (J = 1.5 Hz) was observed. Acetylation of the alcohol **6**, using acetic anhydride in the presence of pyridine, gave a good yield of the acetate **8a**.

Attempts to prepare the chloro analog **8b** by carrying out similar reactions using **10a** as the starting material gave the methoxy analog **8d** instead of the desired chloro derivative. Since the chloro group is a more active chemical leaving group than the hydroxyl group, generation of intermediate **9** was expected after the reduction of the nitro group of **10a** to an amino moiety. It was anticipated that the catalytic reduction in the presence of HCl would yield an amine HCl salt which would serve to decrease the basicity of the amino function and thereby stabilize the molecule. This approach, however, proved unsuccessful. The only quinone isolated from the CrO_3 oxidation step was the methoxymethylbenzoquinone **8d**. The formation of quinone **8d** by CrO_3 oxidation appeared to result from the stability of the methoxy group on compound **11**. Since the fluoro substituent is a relatively poor leaving group, it was anticipated that in the presence of HCl, the fluoro analog **8c** might be obtained by this synthetic approach. However, when **10b** was subjected to these reactions, methoxymethylbenzoquinone **8d** was again obtained.

A different approach to the preparation of compounds **8a–d** was attempted using 2,3,4-trimethoxybenzaldehyde as the starting material (Scheme II). The procedure employed the knowledge that aldehydes are meta directing and the methoxy group is ortho and para directing in electrophilic substitution reactions. Thus, the nitration product of 2,3,4-trimethoxybenzaldehyde (**12**) in concentrated nitric acid would be expected to be the 5-nitro rather than the 6-nitro derivative. No distinctive differences in physical or chemical properties exist between the 5 and 6 isomers of the nitro compound **13** which can be used to unequivocally identify the two isomers. The aldehyde **13** was therefore subjected to $NaBH_4$ reduction in methanol to form the corresponding alcohol **14** which was exhaustively

Scheme II

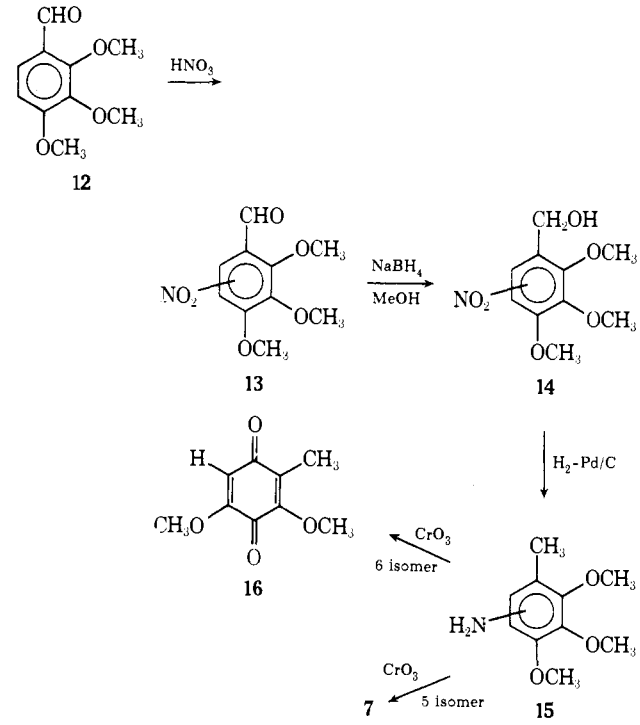
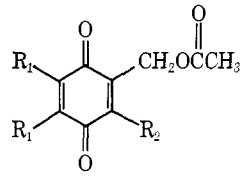
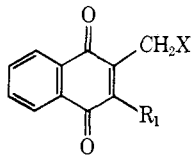


Table II. Inhibition of Beef Heart Mitochondrial NADH-Oxidase and Succinoxidase by Some Benzoquinone and Naphthoquinone Bioreductive Alkylating Agents

		NADH-oxidase (% control act.)[a,b]		Succinoxidase (% control act.)[a,c]	
R_1	R_2	3.3×10^{-4} M[d]	3.3×10^{-5} M[d]	3.3×10^{-4} M[d]	3.3×10^{-5} M[d]
					
$-CH_3$	$-H$	34.7 ± 5.2	39.2 ± 15.6	9.1 ± 0.8	39.3 ± 18.1
$-OCH_3$	$-H$	39.7 ± 17.2	103.9 ± 21.1	13.8 ± 3.6	63.5 ± 26.4
$-CH_3$	$-(CH_2)_{14}CH_3$	22.4 ± 14.4	93.9 ± 24.1	54.9 ± 8.6	89.2 ± 14.9
$-OCH_3$	$-(CH_2)_{14}CH_3$	17.4 ± 0.4	89.9 ± 3.6	50.3 ± 5.6	91.9 ± 8.0
					
X	R_1				
$-O_2CCH_3$	$-H$	31.0 ± 4.4	69.5 ± 15.3	4.3 ± 5.2	95.6 ± 5.4
$-Cl$	$-H$	47.4 ± 7.7	87.6 ± 12.7	24.1 ± 9.8	95.4 ± 5.2
$-O_2CCH_3$	$-(CH_2)_{14}CH_3$	35.0 ± 3.9	80.2 ± 17.6	44.2 ± 8.8	89.9 ± 9.7
$-Cl$	$-(CH_2)_{14}CH_3$	91.9 ± 4.1	113.8 ± 13.3	86.6 ± 2.4	104.4 ± 7.3

[a]Per cent of uninhibited controls ± standard deviation. Mitochondrial protein ranged from 0.5 to 0.7 mg/l. assay flask. [b]Uninhibited NADH-oxidase specific activity ranged from 0.5 to 1.0 $\times 10^{-6}$ atom of oxygen consumed per minute per milligram of protein. [c]Uninhibited succinoxidase specific activity ranged from 0.25 to 0.6 $\times 10^{-6}$ atom of oxygen consumed per minute per milligram of protein. [d]Concentration of inhibitor employed.

hydrogenated to give the amine **15**. Compound **15** was then treated with chromic acid to yield a quinone product which was different from the quinone **7**. No coupling between the methyl protons (1.95 ppm) and the olefinic proton (5.86 ppm) appeared to exist, indicating that the product was quinone **16** and not **7**. Therefore, the nitration product **13** was unambiguously characterized as 2,3,4-trimethoxy-6-nitrobenzaldehyde.

Biological Evaluation. The inhibition of mitochondrial electron transport enzyme systems by a variety of benzoquinones,[15] naphthoquinones,[16] and quinolinequinones[17] has been well documented. To determine the structural specificity of the series of benzoquinones and naphthoquinones described in this report for mitochondrial electron transport systems, particularly with respect to the influence of the lipoidal side chain and the 5,6-dimethoxy groups on inhibitory potency, we have compared the action of these agents on the activities of beef heart mitochondrial succinoxidase and NADH-oxidase. The data presented in Table II demonstrate that all of the compounds tested, at a concentration of 3.3×10^{-4} M, with the exception of the 2-chloromethyl-3-pentadecylnaphthoquinone derivative, inhibited both mitochondrial succinoxidase and NADH-oxidase enzyme systems by 50% or more; however, only the 2-acetoxymethyl-5,6-dimethylbenzoquinone inhibited both enzyme systems by greater than 50% at a concentration of 3.3×10^{-5} M. These findings indicate that (a) both enzyme systems are more susceptible to inhibition by benzoquinone derivatives than to corresponding naphthoquinones, and (b) compounds containing the pentadecyl side chain are weaker inhibitors of these mitochondrial enzymes than the corresponding derivatives without the alkyl chain.

A previous report demonstrated a structural specificity for a 16–17 carbon lipoidal side chain for competition with coenzyme Q requiring systems,[15] whereas a series of hydroxy plastoquinones containing lipoidal side chains was noninhibitory toward the coenzyme Q systems.[18] The inhibition data presented herein suggest a structural specificity for methyl rather than methoxy for the benzoquinone series and the lack of a requirement for the lipoidal side chain employed.

The antineoplastic activity of these compounds was ascertained in mice bearing sarcoma 180 ascites cells. 2-Acetoxymethyl- and 2-chloromethyl-1,4-naphthoquinone[8] were previously reported to be potent inhibitors of the growth of this neoplasm. The incorporation of the pentadecyl side chain into these molecules, however, completely negated their antitumor properties. In the benzoquinone series, all four compounds were inactive against this experimental tumor. The lack of antineoplastic activity of the compounds with lipoidal side chains, coupled with their weaker enzyme inhibitory activity against succinoxidase and NADH-oxidase, suggests that the lipoidal side chain may cause steric interference with the reactive alkylating side chain of the inhibitor molecule or that lipophilicity plays a negative role in the biological action of these compounds.

Experimental Section

Biological Methods. Antineoplastic Activity. Compounds were tested for tumor-inhibitory potency in CD-1 mice bearing sarcoma 180 ascites cells. Complete details of the biological methods have been described earlier.[19]

Determination of NADH-Oxidase and Succinoxidase Activities. Heavy beef heart mitochondria were isolated by differential centrifugation as described by Smith.[20] The activities of the succinoxidase and NADH-oxidase enzyme systems were determined manometrically in the absence and presence of the various inhibitors according to procedures reported earlier.[4a] The various quinone test compounds were added in ethanol which was maintained at a constant level in all of the assay flasks (0.1 ml of ethanol in 3 ml of buffer). Mitochondrial protein was determined by the method of Layne.[21]

Chemical Methods. All melting points were measured on a calibrated Thomas-Hoover capillary melting point apparatus. Analyses were performed by the Baron Consulting Co., Orange, Conn. Spectral data were obtained using a Perkin-Elmer 257 grating ir spectrophotometer, and Varian A-60 and A-60A spectrometers.

The latter instrument used Me_4Si as an internal standard. Where analyses are indicated only by symbols of elements, analytical results obtained for those elements are within ±0.4% of the theoretical values.

2-Nitro-3,4,5-trimethoxybenzyl Fluoride (10b). 2-Nitro-3,4,5-trimethoxybenzyl chloride[12] (**10a**, 1 g, 3.8 mmol) and anhydrous KF (0.45 g, 7.6 mmol) were heated at 120° for 5 hr in 10 ml of ethylene glycol. The solution was cooled to room temperature, and H_2O (30 ml) was added. The mixture was extracted three times with ether; the extracts were combined, washed with H_2O, dried over Na_2SO_4, and evaporated to dryness. The oily substance obtained was chromatographed on silica gel, using as eluent EtOAc–ligroine (bp 65–90°) (1:2 v/v), to yield 0.5 g (54%) of pale yellow crystals, mp 82–83°. *Anal.* ($C_{10}H_{12}NO_5F$) C, H, N.

2-Methoxymethyl-5,6-dimethoxy-1,4-benzoquinone (8d). (a) 2-Nitro-3,4,5-trimethoxybenzyl chloride[12] (0.5 g, 1.9 mmol) in 50 ml of MeOH was added to 2 ml of concentrated HCl and 5% Rh/C (0.25 g), and the mixture was subjected to hydrogenation under 20 psi for 1 hr. The mixture was filtered and the solvent evaporated to dryness under reduced pressure. The crude product was dissolved in 60 ml of H_2O and added dropwise to a cold CrO_3 solution (1.1 g in 25 ml of H_2O). The mixture was stirred at room temperature for 4–5 hr and extracted repeatedly with ether. The ether extracts were combined, washed once with H_2O, dried over Na_2SO_4, and evaporated to dryness. Recrystallization from EtOAc and ligroine gave orange yellow crystals (150 mg, 37%), mp 48–50°. *Anal.* ($C_{10}H_{12}O_5$) C, H.

(b) 2-Nitro-3,4,5-trimethoxybenzyl fluoride (**10b**) was employed as starting material using the procedure described in a.

2-Hydroxymethyl-5,6-dimethoxy-1,4-benzoquinone (6). To 2-nitro-3,4,5-trimethoxybenzyl alcohol[12] (**3**, 18 g, 0.074 mol) in 150 ml of EtOH was added 0.5 g of 5% Rh/C, and the mixture was hydrogenated under 20 psi of pressure for 3 hr. The mixture was filtered to remove the catalyst, and the filtrate was evaporated to dryness. The amorphous residue was dissolved in anhydrous ether, and HCl gas was passed into the solution to form the HCl salt (2.5 g). The amine hydrochloride salt was dissolved in 100 ml of H_2O and added dropwise to a cold solution of CrO_3 (5.5 g in 100 ml of H_2O). The solution was stirred at room temperature overnight and extracted several times with $CHCl_3$. The $CHCl_3$ extracts were combined, dried over Na_2SO_4, and evaporated to dryness. The yellow oil was chromatographed on alumina (neutral, deactivated with 10% H_2O) to give three compounds. The first yellow band gave yellow crystals which were identified as the starting material by ir and nmr. The second yellow band gave 0.5 g (4%) of orange needles (mp 57–59° from ligroine) which were identified as 2-methyl-5,6-dimethoxy-1,4-benzoquinone (**7**) (lit.[22,23] mp 59°): nmr ($CDCl_3$) 2.05 (d, 3, J = 1.5 Hz), 3.98 (s, 3), 4.02 (s, 3), and 6.43 ppm (q, 1, J = 1.5 Hz). The third band yielded 1.5 g (10%) of yellow orange needles, mp 73–75° (from EtOAc + petroleum ether), and was identified as the desired product **6**: nmr ($CDCl_3$) 3.00 (s, 1), 3.95 (s, 3), 4.03 (s, 3), 4.55 (d, 2, J = 1.8 Hz), and 6.68 ppm (t, 1, J = 1.8 Hz). *Anal.* ($C_9H_{10}O_5$) C, H.

2-Acetoxymethyl-5,6-dimethoxy-1,4-benzoquinone (8a). To the alcohol **6** (0.4 g, 2 mmol) in 5 ml of C_6H_6 were added 1 ml of Ac_2O and 1 ml of pyridine. The solution was stirred at room temperature for 1 hr and then heated on a steam bath for 30 min. After cooling the solution was washed with 10% HCl and H_2O, dried over Na_2SO_4, and evaporated to dryness. The residue was recrystallized from ligroine to give 0.3 g (65%) of red needles, mp 54–55°. *Anal.* ($C_{11}H_{12}O_6$) C, H.

2,3,4-Trimethoxy-6-nitrobenzaldehyde (13). 2,3,4-Trimethoxybenzaldehyde (12, 5.5 g, 28 mmol) in 25 ml of glacial AcOH was added dropwise to 50 ml of the acid mixture (AcOH–fuming HNO_3, 1:1 v/v) which was cooled in a salted ice bath to maintain the temperature below 0°. The resulting brownish solution was stirred at 0° for 20 min and poured into 500 ml of ice water. The precipitate was collected and washed with H_2O. Recrystallization from H_2O and EtOH gave pale yellow needles (5 g, 74%), mp 80–82°. *Anal.* ($C_{10}H_{11}NO_6$) C, H, N.

2,3,4-Trimethoxy-6-nitrobenzyl Alcohol (14). The aldehyde **13** (1 g, 4 mmol) was suspended in 20 ml of MeOH and cooled in an ice bath. To the suspension was added 0.2 g (5.4 mmol) of $NaBH_4$ in small portions. A clear solution was obtained which was stirred at ice-cold temperature for 1 hr. The solvent was evaporated to dryness under reduced pressure, and the resulting yellow material was washed with H_2O and recrystallized from ligroine to give yellow needles (0.7 g, 72%), mp 69–71°. An additional recrystallization raised the melting point to 74–75°. *Anal.* ($C_{10}H_{13}NO_6$) C, H, N.

2-Methyl-3,5-dimethoxy-1,4-benzoquinone (16). 2,3,4-Trimethoxy-6-nitrobenzyl alcohol (**14**, 1 g, 4 mmol) in 100 ml of EtOH was hydrogenated under 15 psi of pressure over 0.25 g of 10% Pd/C. The catalyst was removed and the solvent evaporated to dryness under reduced pressure. The residual oil was dissolved in 100 ml of 50% EtOH and added dropwise to a cooled CrO_3 solution (2.2 g in 100 ml of H_2O). The solution was stirred at room temperature overnight and extracted three times with Et_2O (100 ml). Extracts were combined, dried over Na_2SO_4, and evaporated to dryness. The residue was chromatographed on alumina (neutral, 10% H_2O deactivated) and eluted with C_6H_6 to give a yellow powder. Recrystallization from EtOAc and petroleum ether gave yellow needles (250 mg), mp 126–127° (lit.[22] 125°). *Anal.* ($C_9H_{10}O_4$) C, H.

Preparation of Dipalmitoyl Peroxide. Palmitoyl chloride (10 g) in 60 ml of $CHCl_3$ was added with cooling in ice to a solution of 6 g of Na_2O_2 in 40 ml of ice–H_2O. The mixture was shaken for 5 min, and 6 g of Na_2O_2 was added together with more ice and $CHCl_3$. The shaking was continued for 15 min with constant addition of ice to keep the temperature about 5°. Near the end of the reaction, 300 ml of $CHCl_3$ was added, and the $CHCl_3$ layer was separated. The H_2O layer was washed once with 100 ml of $CHCl_3$, and the $CHCl_3$ extracts were combined, dried (Na_2SO_4), and evaporated to dryness. The resulting white crystals were washed with MeOH and dried under vacuum to give 5.9 g of the desired product, mp 70–71° (lit.[10,24] 71.4–71.9°).

General Procedure for Dipalmitoyl Peroxide Alkylation. Quinones (0.6 g) and dipalmitoyl peroxide (3.1 g) were stirred and heated at 90–95° for about 3 hr (until the evolution of CO_2 ceased) in 25 ml of AcOH. The solution was cooled to room temperature and the precipitated white crystals were removed by filtration. The filtrate was evaporated to dryness, and the resulting yellow oily substance was dissolved in 10 ml of ligroine and kept at 5°. White crystals which formed were again removed. The filtrate was evaporated to dryness and chromatographed on a column (3 × 48 cm) of silica gel (150 g). The eluents used in the column are listed in Table I.

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References

(1) V. S. Waravdekar, A. Domingue, and J. Leiter, *J. Nat. Cancer Inst.*, **13**, 393 (1952).
(2) S. Hayashi, H. Ueki, and Y. Ueki, *Gann*, **54**, 381 (1963).
(3) S. Hayashi, H. Ueki, and Y. Ueki, *Gann*, **55**, 1 (1964).
(4) (a) R. S. Pardini, J. C. Heidker, and D. C. Fletcher, *Biochem. Pharmacol.*, **19**, 2695 (1970); (b) C. Bhuvaneswaren and K. Dakshinamurti, *Biochemistry*, **11**, 85 (1972).
(5) C. R. Smart, H. H. Hogle, R. K. Robbins, A. D. Broom, and D. Bartholomew, *Cancer Chemother. Rep., Part I*, **53**, 147 (1969).
(6) D. Burk and M. Woods, *Radiat. Res., Suppl.*, **3**, 212 (1963).
(7) A. J. Lin, L. A. Cosby, C. W. Shansky, and A. C. Sartorelli, *J. Med. Chem.*, **15**, 1247 (1972).
(8) A. J. Lin, R. S. Pardini, L. A. Cosby, B. J. Lillis, C. W. Shansky, and A. C. Sartorelli, *J. Med. Chem.*, **16**, 1268 (1973).
(9) A. J. Lin and A. C. Sartorelli, *J. Org. Chem.*, **38**, 813 (1973).
(10) L. F. Fieser and A. E. Oxford, *J. Amer. Chem. Soc.*, **64**, 2060 (1942).
(11) J. C. Catlin, G. D. Daves, Jr., and K. Folkers, *J. Med. Chem.*, **14**, 45 (1971).
(12) K. I. H. Williams, S. E. Cremer, F. W. Kent, E. J. Sehm, and D. S. Tarbell, *J. Amer. Chem. Soc.*, **82**, 3982 (1960).
(13) F. G. Mann and F. H. C. Stewart, *J. Chem. Soc.*, 4127 (1954).
(14) R. K. Norris and S. Sternhell, *Aust. J. Chem.*, **19**, 617 (1966).
(15) R. S. Pardini, J. C. Catlin, J. C. Heidker, and K. Folkers, *J. Med. Chem.*, **15**, 195 (1972).
(16) (a) J. V. Schnell, W. A. Siddiqui, Q. M. Geiman, F. S. Skelton, K. D. Lunan, and K. Folkers, *J. Med. Chem.*, **14**, 1026 (1971); (b) E. G. Ball, C. B. Anfinsen, and O. Cooper, *J. Biol. Chem.*, **168**, 257 (1947); (c) F. S. Skelton, R. S. Pardini, J. C. Heidker, and K. Folkers, *J. Amer. Chem. Soc.*, **90**, 5334 (1968); (d) J. C. Catlin, R. S. Pardini, G. D. Daves, Jr., J. C. Heidker, and K. Folkers, *ibid.*, **90**, 3572 (1968).
(17) F. S. Skelton, C. M. Bowman, T. H. Porter, K. Folkers, and

R. S. Pardini, *Biochem. Biophys. Res. Commun.*, **43**, 102 (1971).
(18) J. Boler, R. Pardini, H. Mustafa, K. Folkers, R. Dilley, and F. Crane, *Proc. Nat. Acad. Sci. U. S.*, **69**, 3713 (1972).
(19) K. C. Agrawal, B. A. Booth, and A. C. Sartorelli, *J. Med. Chem.*, **11**, 700 (1968).
(20) A. L. Smith, *Methods Enzymol.*, **10**, 81 (1967).
(21) E. Layne, *Methods Enzymol.*, **3**, 447 (1957).
(22) W. K. Anslow, J. N. Ashley, and H. Raistrick, *J. Chem. Soc.*, 439 (1938).
(23) K. Sato, S. Inoue, and H. Sato, *Bull. Chem. Soc. Jap.*, **45**, 3455 (1972).
(24) L. S. Silbert and D. Swern, *J. Amer. Chem. Soc.*, **81**, 2364 (1959).

Cysteine Scavengers. 2. Synthetic α-Methylenebutyrolactones as Potential Tumor Inhibitors†

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Synthetic α-methylenebutyrolactone derivatives related to the naturally occurring antitumor sesquiterpene lactones of plant origin were synthesized as potential experimental antitumor agents on the basis of their possible action as cysteine scavengers. Although these compounds were cytotoxic to cysteine-requiring human lymphoblastic leukemia cells (CCRF-CEM) in continuous culture at doses of 10^{-6}–10^{-7} mol/l., they displayed comparable cytotoxicity against normal human lymphoid cells, and their cytotoxic action did not appear to be a consequence of selective cysteine scavenging.

Human lymphoblastic leukemia cells grown in culture have been shown by Foley and coworkers[1] to possess an absolute nutritional requirement for L-cysteine (L-cystine) which is independent of both their state of ploidy and their population density. In contrast, normal human lymphoid cells in culture are capable of maintaining normal growth in the absence of L-cysteine if they are supplied with L-cysteine precursors such as L-serine and L-homocysteine, or L-cystathionine.[2] That the dependence of various lymphoblastic leukemic cell sublines on exogenous L-cysteine is probably a consequence of one or more enzymatic defects in the methionine–cystathionine–cysteine biosynthetic pathway was indicated by experiments demonstrating a marked deficiency in cystathionase levels in such cells.[2] Accordingly, it was suggested[1,3,4] that rational chemotherapeutic approaches based on the concept of "cysteine scavenging" might be possible, since cultured normal cells can apparently biosynthesize L-cysteine whereas lymphoblastic leukemic cells cannot.[1]

In this context, as part of a larger search for classes of chemical agents capable of blocking exogenous L-cysteine uptake by leukemic cells, we were interested in α-methylenebutyrolactones. Compounds of this type are widely prevalent among terpenoid natural products of plant origin[5,6] and are being discovered with continuing regularity.[7-15] They are noted for their cytotoxic properties, which have been ascribed to a rapid and essentially irreversible 1,4-addition reaction involving the SH group of L-cysteine, either as the free amino acid[16,17] or as part of a peptide chain or protein.[18] Susceptibility to attack by amines has also been demonstrated.[19,20] Some plant lactones containing the α-methylenebutyrolactone moiety have aroused interest in recent years on the basis of encouraging antitumor activity *in vitro*, and efforts to deduce meaningful structure–activity correlations have been reported by two independent groups of investigators.[21,22] Papers describing simple synthetic α-methylenebutyrolactones have likewise appeared intermittently in the literature[23-37] but the biological properties of these small molecules have not been studied to nearly the same extent as those of the more complex terpenoid lactones of natural origin.

In searching for cysteine scavengers that could be used therapeutically, we were especially attracted by the possibility that simple synthetic α-methylenebutyrolactones might be relatively nontoxic because they lack the multifunctional character and complex stereochemistry that Kupchan and coworkers[18,21] have suggested may account for the remarkable toxicity of natural products containing the α-methylenebutyrolactone moiety. According to this view, simple α-methylenebutyrolactones with just the proper degree of chemical reactivity might combine irreversibly with intracellular or extracellular cysteine without interacting with complex biological macromolecules such as respiratory enzymes or membrane proteins.

In the work reported herein, a group of nine synthetic α-methylenebutyrolactones was assayed against human lymphoblastic cells in culture. The compounds were found to be more toxic than expected and, in fact, proved to be no less cytotoxic than several complex α-methylenebutyrolactones of natural origin which were evaluated for comparison.

Chemistry. A modified procedure involving a combination of several published routes[27-29] was employed for the preparation of *trans*-9-methylene-7-oxabicyclo[4.3.0]nonan-8-one (**1**) and *trans*-10-methylene-8-oxabicyclo[5.3.0]decan-9-one (**2**) from cyclohexene oxide and cycloheptene oxide, respectively (Scheme I). The epoxides were condensed with diethyl malonate as described by Newman and VanderWerf[38] and the resultant lactone acids were treated with CH_2O and Et_2NH in the presence of $Et_2NH{\cdot}HCl$,[29] and then with NaOAc in glacial AcOH.[28] Overall yields of **1** and **2** from the epoxides were 15–25%. The identity of each product was established by microanalysis and comparison of ir and nmr spectral data with published values.[27]

3-Methylene-1-oxaspiro[4.5]decan-2-one (**3**) and 3-methylene-1-oxaspiro[4.6]undecan-2-one (**4**) were synthesized conveniently *via* a Reformatsky-type reaction between ethyl α-bromomethacrylate[39] and cyclohexanone or cycloheptanone. This novel α-methylenebutyrolactone synthe-

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