# 5-*p*-Benzoquinonyl-2′-deoxyuridine 5′-Phosphate: A Possible Mechanism-Based Inhibitor of Thymidylate Synthetase

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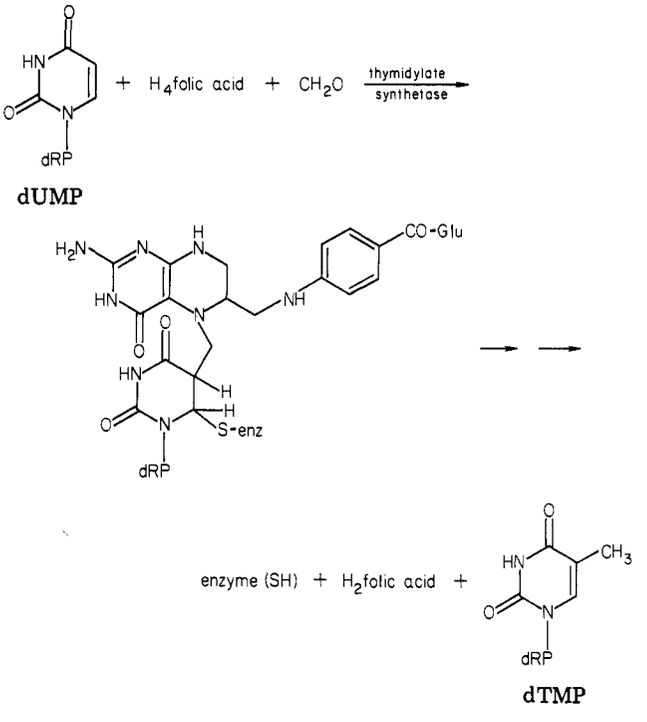
The title compound (1), designed as a suicide inhibitor of thymidylate synthetase, can be prepared by silver(II) oxide oxidative demethylation of the corresponding dimethoxyphenyl derivative. Compound 1 shows time-dependent inactivation of thymidylate synthetase (methotrexate-resistant *Lactobacillus casei*) and saturation kinetics, and the inactivation is responsive to substrate protection. The inactivation is not reversible on prolonged dialysis in attempts to remove the inhibitor. The rate constant for inactivation is 0.065 $s^{-1}$; the dissociation constant ($K_I$) was estimated to be 2 $\mu$M. The kinetics of this inactivation are compared to inactivation caused by model thiol reagents that do not have affinity for the active site of thymidylate synthetase.

Thymidylate synthetase (EC 2.1.1.45) is recognized as a clinically effective target enzyme for the control of neoplastic cell proliferation.[1,2] Specific, but nonselective inhibition of this enzyme by 5-fluoro-2′-deoxyuridine 5′-phosphate is important in the chemotherapeutic control of certain cancers by 5-fluorouracil. Problems invariably arise, however, since the proliferation of normal cells is also inhibited. For this reason, efforts in several laboratories have been directed to the search for an effective inhibitor of this enzyme specifically in tumor cells or viral-infected host cells.

The overall catalytic process promoted by this protein is a two-step alkylation–reduction reaction wherein a carbon–carbon bond is formed between carbon 5 of the substrate (2′-deoxyuridine 5′-phosphate) and a one-carbon synthon generated from formaldehyde. The enzyme catalyzes a reaction whereby the nucleophilic character of carbon 5 of the substrate, 2′-deoxyuridine 5′-phosphate (dUMP), is amplified by reaction with a cysteine residue at the active site of the enzyme.[3,4] Subsequent reaction with the cofactor $N^5$,$N^{10}$-methylenetetrahydrofolic acid results in formation of the covalent complex of enzyme, substrate, and cofactor, which is proposed as a transient intermediate in the reaction (Scheme I). The intermediate in this reaction is subsequently reduced by the enzyme cofactor, tetrahydrofolic acid, to give the 5-methyl derivative of the substrate and the oxidized cofactor, 7,8-dihydrofolic acid.[1,2] Mechanism studies with enzyme purified from amethopterin-resistant *Lactobacillus casei* have supported the view that the initial reaction in this sequence is the addition of the thiol group of cysteine-198 in this protein to carbon 6 of the pyrimidine ring in the substrate.[3-6]

Since the initial description of the concepts of suicide or $k_{cat}$ inhibitors,[7,8] a number of valuable active-site probes useful for mechanistic studies have been described. The predominant activity in this field has centered around the pyridoxal-associated transaminase enzymes, where selective inhibition of GABA transaminase could result in the discovery of alternative therapeutic agents for the control of convulsive disorders.[9]

Scheme I[a]




[a] dRP = 2-deoxyribose 5-phosphate.

The title compound (I) has been designed as a suicide inhibitor of thymidylate synthetase. The chemical analogy of 1 to the substrate, together with the fact that large substituents in the 5-position of the pyrimidine ring do not interfere with the active-site binding, suggests that 1 should selectively bind to thymidylate synthetase.[10] Previous studies have shown that substituents in the 5-position of the pyrimidine ring that afford increased electron delocalization enhance the first step in the catalytic sequence, the addition of a thiol anion to carbon 6, and that such compounds can be considered substrates for the enzyme.[11] The requirement for suicide inactivation

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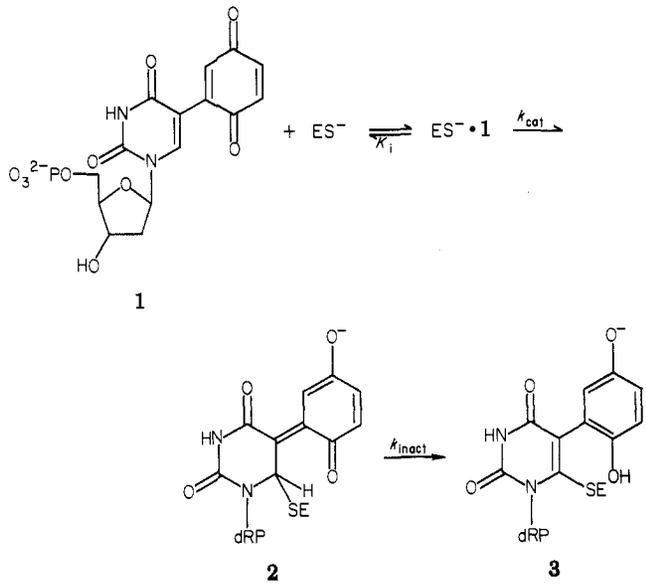
Scheme II. Postulated Mechanism for Suicide Inactivation of Thymidylate Synthetase ($ES^-$) by 5-p-Quinonyl-2'-deoxyuridine 5'-Phosphate (1)[a]




[a] dRP = 2'-deoxyribosyl 5'-phosphate.

is satisfied if the product of the enzyme-catalyzed reaction is a chemically reactive intermediate, such as **2** depicted in Scheme II. Although other reactions can occur, a reasonable pathway from **2** to a low-energy product is a proton shift from carbon 6 to the quinone ring to give **3**, wherein the quinone ring of **1** is now reduced. Inherent in the kinetics of this sequence is the expectation that the limiting rate of the inactivation process will be dependent on the concentration of the association complex. If the reaction follows this sequence, the product, **3**, with enzyme covalently bonded to an $sp^2$ carbon, should be a useful probe for further studies of the active-site chemistry of thymidylate synthetase.

**Chemistry.** Two synthetic approaches were explored for the synthesis of **1**, both of which required the synthesis of a 5-aryl-substituted pyrimidine nucleoside and the corresponding nucleotide. At the time this work was initiated the synthesis of several 5-arylpyrimidines had been described; however, no published data were evident for the synthesis of the corresponding 5-arylpyrimidine nucleosides. Two methods were explored for the synthesis of these unsymmetrical biaryl derivatives, a photochemical approach and a palladium-catalyzed coupling reaction.

An unsuccessful attempt for the direct synthesis of the nucleoside **5a** evolved from a report that enones undergo a Michael reaction with aryl–palladium complexes.[12] The coupling reaction between 5-(chloromercuri)-2'-deoxyuridine and *p*-benzoquinone with lithium tetrachloropalladate[13] failed to give adduct formation. Since unsaturated acetals and ketals have been arylated with phosphine-complexed palladium(II) acetate and triethylamine,[14] the corresponding bis(ethylene ketal) of *p*-benzoquinone was prepared.[15] A series of reactions between 5-(chloromercuri)-2'-deoxyuridine and *p*-benzoquinone bis(ethylene ketal) were run with lithium tetrachloropalladate in slight excess while altering the solvent conditions (methanol or methanol–tetrahydrofuran), the reaction temperature (ambient temperature to reflux), or the workup conditions (hydrogen sulfide, sodium borohydride, or cupric chloride). In all cases, 2'-deoxyuridine was the only nucleoside isolated. Aprotic reaction conditions with bis(benzonitrile)palladium chloride as catalyst in benzonitrile–tetrahydrofuran (1:1) solvent system at reflux for 8 h under argon also gave 2'-deoxyuridine upon workup. No evidence for any adduct formation was found, and under all of these reaction conditions, the bis(ketal) underwent general decomposition and polymerization reactions, which prevent this from being a useful pathway even if the coupling reaction proceeded.

The alternate route utilizing a photochemical coupling reaction was ultimately successful for the synthesis of the nucleoside **4a** and the corresponding nucleotide **6**.[16]

Standard procedures were followed in the initial attempts to demethylate the nucleoside **4a** to give the hydroquinone **8**. Boron tribromide in acetonitrile resulted in monodemethylation and glycoside bond cleavage. Treatment of **4a** with trimethylsilyl iodide in acetonitrile at 25 °C afforded 1-acetyl-5-(2,5-dimethoxyphenyl)uracil as the only characterized product, presumably arising from reaction of the trimethylsilyl derivative of the substituted uracil with the solvent and subsequent hydrolysis.

Snyder and Rapaport[17] reported a reliable reaction for the conversion of *o*- and *p*-dimethoxyphenyl and naphthyl derivatives to the corresponding quinones. The reaction is described as an acid-catalyzed oxidative demethylation utilizing silver(II) oxide.[18] Both the unprotected nucleoside **4a** and the diacetyl derivative **4b** were rapidly converted to the quinones **5a** and **5b** on addition of 4 equiv of silver(II) oxide to a dioxane solution of **4a** or **4b**, followed by acidification with nitric acid (Scheme III). The reaction also worked well in water for conversion of the nucleotide **6** to the title compound **1**.

The purity and stability of compound **1** could not be determined by HPLC with the standard anion-exchange column, since, under the described conditions, the product, starting material, and decomposition products had the same retention time. However, if the described methanol–phosphate buffer combination was used, complete resolution was possible on a reverse-phase (ODS) column.

The quinone products had a ultraviolet maximum in acid at 250 nm, which is at an unusually low wavelength for a pyrimidine nucleoside. However, the extinction coefficient ($\epsilon$ 14 000) suggests that the maximum is a composite of the out of plane *p*-quinone and pyrimidine ring (shoulder 275 nm) contributions.[16] The expected long-wavelength (385 nm), low extinction coefficient band ($\epsilon$ 1800) is characteristic for substituted *p*-benzoquinones.

The nucleotide **1** and, to some extent, the nucleoside **5a** were found to be unstable in aqueous solution in the presence of nucleophiles, particularly as the pH of the mixture approached 7. Both **5a** and **1** when treated with sodium borohydride gave, in each case, a single product by liquid chromatography with the expected ultraviolet absorption spectrum characteristic of hydroquinones wherein loss of the long-wavelength band at 385 nm is common. Further treatment of the hydroquinone nucleotide **7** with hydrogen peroxide gave the quinone **1**.

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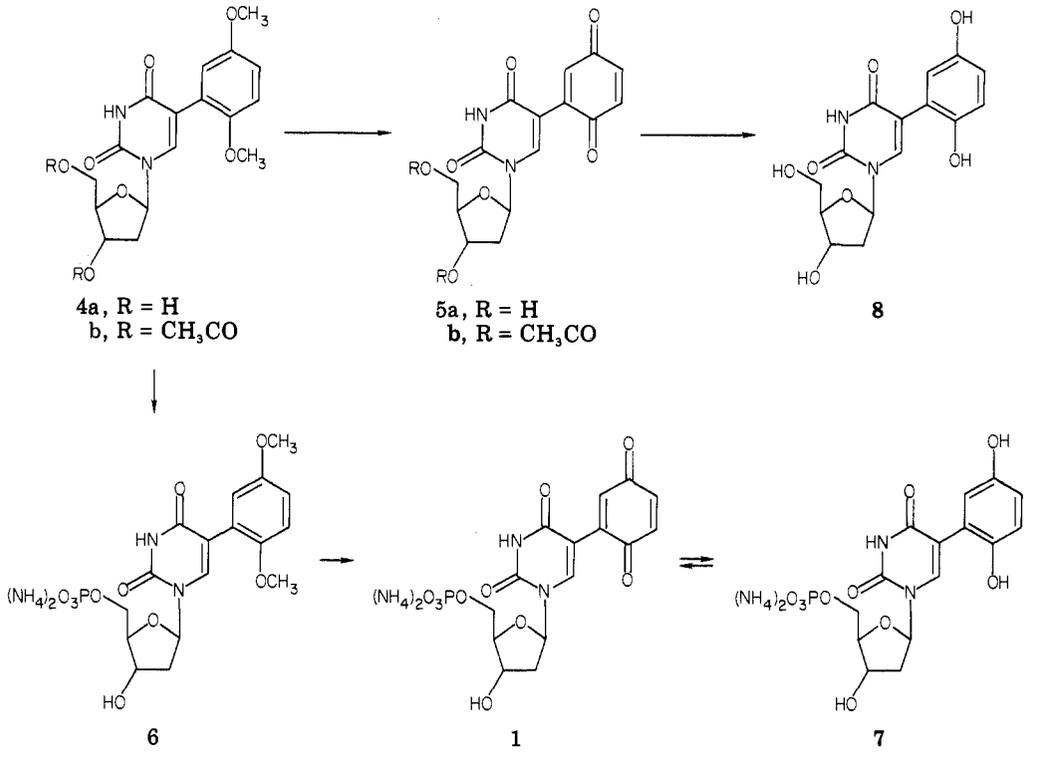
Scheme III




The stability of the quinone nucleotide 1 in the presence of various nucleophiles was examined. Compound 1 is stable in acidic media; treatment with various nucleophiles at higher pH values and acidification of the resulting mixture at given times allowed for liquid chromatographic analysis of the reaction products with a reverse-phase column. Preliminary studies show a half-life of less than 1 h in 1 M Tris–acetate, pH 6.8, or 0.02 M sodium acetate, pH 7.5. Compound 1 was most stable at pH 6.8 in 0.1 M phosphate buffer ($t_{1/2} \approx 10$ h), and in the enzyme incubation buffer mixture of 0.012 M phosphate, pH 6.8, 0.006 M magnesium chloride, and 0.024 M ethylenediaminetetracetic acid, the $t_{1/2}$ exceeded 20 h. Mixing a neutral solution of 1 with 1 equiv of 2-mercaptoethanol or glutathione showed essentially complete reaction of 1 in 1 min. The $t_{1/2}$ in the presence of arginine was less than 5 min.

**Enzyme Inactivation Studies.** Thymidylate synthetase in a crude extract from methotrexate-resistant *Lactobacillus casei* was purified and crystallized according to the method of Maley and co-workers[19] to give, after activation by dialysis in 50 mM 2-mercaptoethanol, a preparation with a specific activity of 3.2 μmol of product $mg^{-1}$ $min^{-1}$ based on the radioisotope assay.[11d] Removal of the 2-mercaptoethanol was accomplished with a sephadex G-25 column and 0.05 M phosphate buffer at pH 6.8.

Incubation of compound 1 with thymidylate synthetase was performed at 30 °C in a mixture containing buffer, magnesium chloride, EDTA, and enzyme. Immediately after the addition of inhibitor, a zero-time aliquot of 50 μL was removed and assayed (30 s) at 50 mM substrate concentration for remaining enzyme activity. Similarly, assays were run by removing aliquots each 10 s to determine time-dependent loss of active enzyme. A plot of the logarithm of the percentage of remaining active enzyme vs. time was linear during the early time course of the assay where inhibitor concentration exceeded that of enzyme.

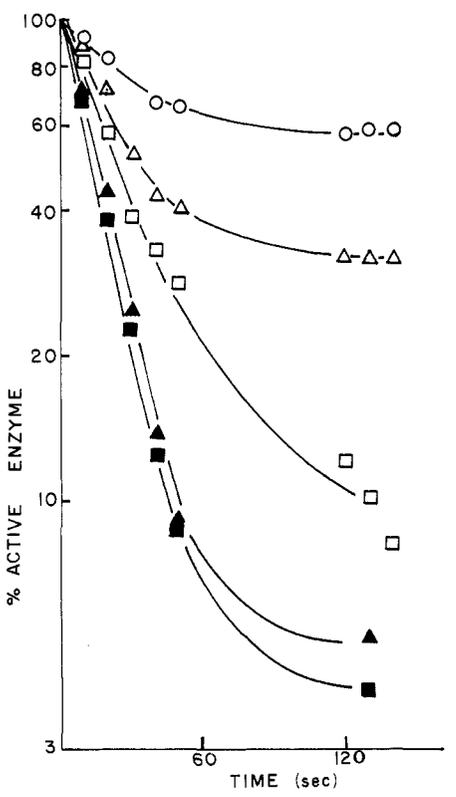



**Figure 1.** Plot of the natural logarithm of the percentage of active mercaptoethanol-free thymidylate synthetase (0.24 μM, sp act. 3.2 μM $min^{-1}$ $mg^{-1}$) vs. time (seconds) in the presence of varying concentrations of inhibitor 5-*p*-benzoquinonyl-2'-deoxyuridine 5'-phosphate (1): 0.55 μM, (○), 0.87 μM (△), 1.33 μM (□), 3.33 μM, (▲), 5.56 μM (■). The observed rates of inactivation for these concentrations of 1 were essentially the same when the enzyme concentration was reduced to 0.08 μM.

The change in the rate of inactivation is ascribed to loss of inhibitor by secondary reactions and a change to second-order kinetics for a brief period until the inhibitor

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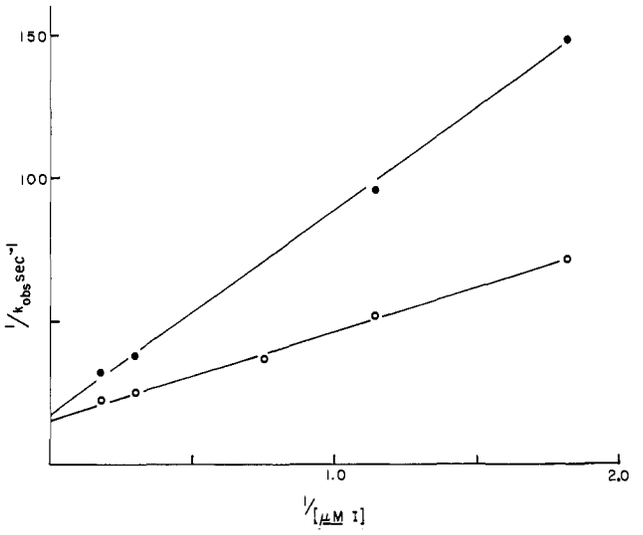


**Figure 2.** Double-reciprocal plot of the observed rate constants for the inactivation of mercaptoethanol-free thymidylate synthetase (sp act. 3.2 $\mu$M mg$^{-1}$ min$^{-1}$) vs. the concentration of the inhibitor 5-*p*-benzoquinonyl-2'-deoxyuridine 5'-phosphate (1) in the absence (○) and the presence (●) of 10 $\mu$M substrate, 2'-deoxyuridine 5'-phosphate.

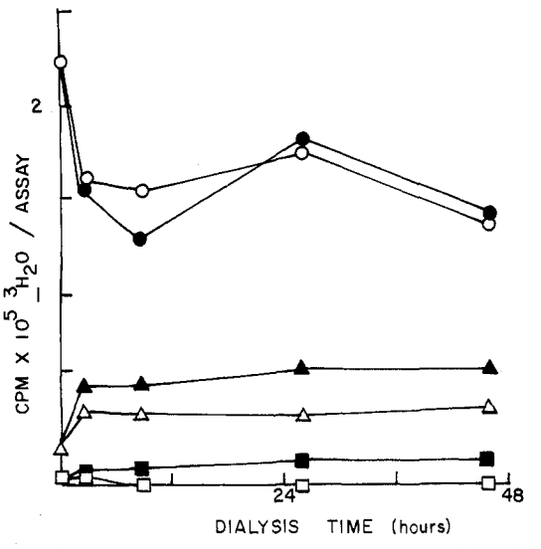


**Figure 3.** Plot of the activity in counts per minute of thymidylate synthetase vs. the time in hours for the dialysis of control (○) and inactivated enzyme in 0.1 M potassium phosphate buffer, pH 6.8. The inactivated enzyme was prepared by the addition of 1 in concentrations of 0.87 (△) and 3.33 $\mu$M (□) of 5-*p*-quinonyl-2'-deoxyuridine 5'-phosphate (1); incubation was continued for an additional 5 min before dialysis. The enzyme concentration was 0.24 $\mu$M; sp act. 3.2 $\mu$M min$^{-1}$ mg$^{-1}$. The dialysis was done in absence (○, △, □) and presence (●, ▲, ■) of 50 mM 2-mercaptoethanol.

concentration falls to zero. If the initial time points (up to 50 s) were used, the observed rate of enzyme inactivation could be determined (Figure 1). The observed rates of inactivation during the initial time course showed a saturation effect (3.33 and 5.56 $\mu$M 1) and essentially complete enzyme inactivation. The expected course for the reaction would be initial formation of a reversible complex of enzyme and inhibitor, followed by reaction of this complex to give the inactivated enzyme. If this is true, the expression relating the velocity of the inactivation process as developed by Kitz and Wilson[20] (eq 1) can be used to establish the dependency on the concentration of the reversible enzyme–inhibitor complex.

$$1/k_{obsd} = \left[\frac{K_i[S]}{K_m k_{cat}} + \frac{K_i}{k_{cat}}\right]\frac{1}{[I]} + \frac{1}{k_{cat}} \qquad (1)$$

A plot of the reciprocals of the observed rate constants for inactivation and the inhibitor concentrations (Figure 2) gave a rate constant ($k_{cat}$) of 0.065 s$^{-1}$ for inactivation of the enzyme at saturating levels of inhibitor and a $K_i$ of 2.0 $\mu$M. Verification that the intermediate ES$^-$–1 complex is formed at the active site of the enzyme was obtained from substrate protection studies. The decreased rates of inactivation in the presence of 10 $\mu$M substrate were plotted according to eq 1. Under these conditions, the $k^s_{cat}$ was found to be 0.057 s$^{-1}$. This equation also affords an independent determination of $K_m$ or $K_i$; using the observed $K_i$ of 2.0 $\mu$M, the $K_m$ value was 4.4 $\mu$M.

The corresponding nucleoside **5a** also was examined for inactivation of thymidylate synthetase. At 100 $\mu$M concentration, complete inactivation was observed. At 10 $\mu$M of **5a** the half-life for inactivation was 143 s; the corresponding half-life for enzyme inactivation by the nucleotide 1 at 1 $\mu$M was 32 s. The calculated second-order rate for inactivation of thymidylate synthetase by the nucleoside **5a** was 500 M$^{-1}$ s$^{-1}$.

The irreversible nature of the reaction of 1 with enzyme was examined by dialysis of the inactivated enzyme in the presence and absence of 0.05 M 2-mercaptoethanol. Enzyme was inactivated by adding 0.87 and 3.3 $\mu$M 1 to the enzyme, and the mixture was incubated for 5 min. The results (Figure 3) compare the activity of the control enzyme to that of the inhibited enzyme. After dialysis for 26 h, the control enzyme had 80% of the initial activity; the presence of 2-mercaptoethanol did not significantly affect the activity. After 5 min incubation, the inhibited enzyme had 7 and 0% of the initial activity at 0.87 and 3.3 $\mu$M of inhibitor. After 3 h dialysis, the solution containing the lower concentration of inhibitor recovered a portion of the original activity: from 7 to 16% and, in 2-mercaptoethanol, 24%. There was little change in activity during the remaining dialysis period. At the higher concentration of inhibitor, dialysis in the presence of 2-mercaptoethanol showed only a slight recovery (6%) of activity. After dialysis for 7 days the activity of the control enzyme fell to 50% and there was no significant change in the activity of the inhibited enzyme.

The reaction of model quinones with thymidylate synthetase was examined by incubation of varying concentrations of *p*-benzoquinone or phenyl-*p*-benzoquinone with the enzyme. The experiment was designed to examine the kinetics of inactivation under pseudo-first-order conditions where the concentration of inhibitor exceeds that of enzyme. The assay for remaining activity was run by removing aliquots at the indicated time points and adding the substrate and cofactor. Presumably, the excess benzoquinone is rapidly reduced by cofactor, since, under normal assay conditions, 1 mM of *p*-benzoquinone does not inhibit the enzyme.

The results in Table I show that *p*-benzoquinone rapidly inactivates the enzyme; the half-life for the reaction is 90 s when a 1 $\mu$M concentration of the inhibitor is used. At the same concentration, phenyl-*p*-benzoquinone inactivates the enzyme with a calculated half-life of 200 s (Figure 4). The kinetics of this inactivation were examined under pseudo-first-order conditions by using three concentrations of *p*-benzoquinone (Table I). A plot of $k_{obsd}$ vs. concentration of *p*-benzoquinone gave an origin intercept with a slope equal to the second-order rate constant ($k_2$) for the

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Table I. Kinetics of Inactivation of Thymidylate Synthetase by 5-*p*-Quinonyl-2'-deoxyuridine (5a), *p*-Benzoquinone, and Phenyl-*p*-benzoquinone[a]

| compd | concn, μM | substrate, μM | $k_{obsd}$,[e] $s^{-1}$ |
|---|---|---|---|
| 5-*p*-quinonyl-2'-deoxyuridine (5a)[b] | 5.56 | 0 | 0.0029 |
| | 10.0 | 0 | 0.0048 |
| *p*-benzoquinone[c] | 0.6 | 0 | 0.0025 |
| | 1.0 | 0 | 0.0077 |
| | 1.0 | 10 | 0.0025 |
| | 2.0 | 0 | 0.0126 |
| phenyl-*p*-benzoquinone[d] | 1.0 | 0 | 0.0034 |
| | 3.0 | 0 | 0.0070 |
| | 10.0 | 0 | 0.025 |
| | 30.0 | 0 | 0.078 |

[a] The compounds were incubated with enzyme in 0.1 M phosphate buffer at pH 6.8 at 30 °C. Aliquots were removed at time 0, 10, 20, 30, 40 and 50 s and assayed for remaining enzyme activity by using saturating amounts of substrate (50 μM) in a 60-s assay. [b] The concentration of enzyme was 0.24 μM based on a dimer molecular weight of 70 000 and a specific activity of 3.2 μM $mg^{-1}$ $min^{-1}$. [c] The concentration of enzyme in this preparation is 0.06 μM based on a molecular weight of 70 000 and a specific activity of 3.2 μM/$mg^{-1}$ $min^{-1}$. The substrate $K_m$ for this enzyme preparations was 5.6 μM. [d] The enzyme concentration was 0.18 μM. [e] The calculated second-order rate constant for enzyme inactivation by 5a is $0.5 \times 10^3$ $M^{-1}$ $s^{-1}$, by *p*-benzoquinone is $6.7 \times 10^3$ $M^{-1}$ $s^{-1}$, and by phenyl-*p*-benzoquinone is $2.6 \times 10^3$ $M^{-1}$ $s^{-1}$.

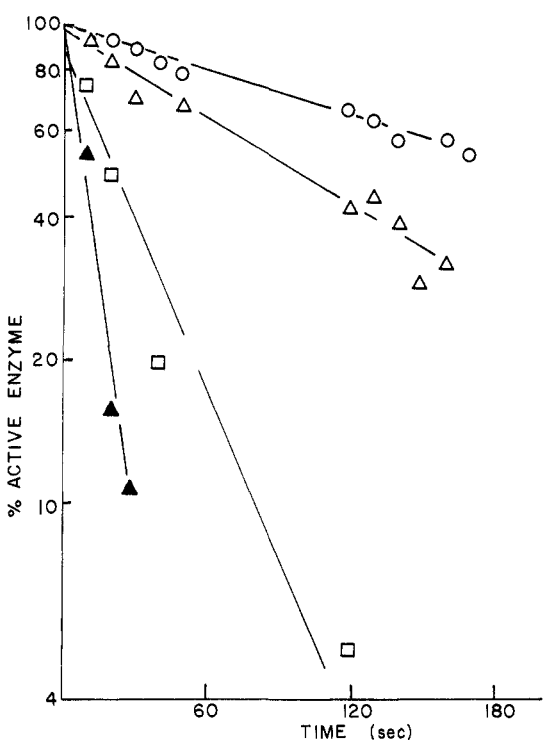


**Figure 4.** Plot of natural logarithm of the percentage of mercaptoethanol-free active thymidylate synthetase vs. time (seconds) in the presence of varying concentrations of phenyl-*p*-benzoquinone: 1 μM (○); 3 μM (△), 10 μM (□), 30 μM (▲).

inactivation of thymidylate synthetase by *p*-benzoquinone ($6.7 \times 10^3$ $M^{-1}$ $s^{-1}$, eq 2 and 3). If the enzyme inactivation

$$E + I \xrightarrow{k_2} EI \quad (2)$$

$$k_{obsd}[E] = k_2[E][I] \quad (3)$$

caused by *p*-benzoquinone takes place by reaction at the active-site thiol residue, the rate constant for the same

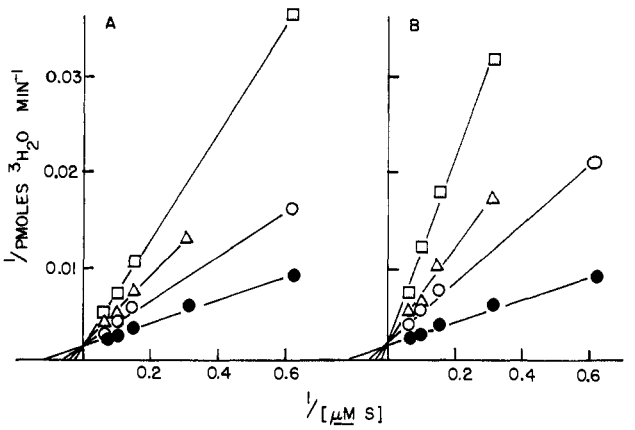


**Figure 5.** Double-reciprocal plot of the velocity of the reaction vs. concentration of 2'-deoxyuridine 5'-phosphate. The product reported is one-fourth of the total amount of $^3H_2O$ formed in each assay as described under Experimental Section. (A) Product of the reaction of 5-*p*-quinonyl-2'-deoxyuridine 5'-phosphate (1) and 2-mercaptoethanol. (B) Product of the reaction of 5-*p*-quinonyl-2'-deoxyuridine 5'-phosphate (1) plus sodium borohydride (7): 0 μM (●), 2.5 μM (○), 5 μM (△), 10 μM (□).

reaction in the presence of substrate ($k^s$) should be reduced in accord with eq 4. The $k_{obsd}$ for 1 μM benzoquinone was

$$k^s = k/\left(1 + \frac{[S]}{K_m}\right) \quad (4)$$

found to be 0.0077 $s^{-1}$; in the presence of 10 μM substrate, the $k_{obsd}$ was found to be 0.0025 $s^{-1}$. The substrate $K_m$ thus calculated from eq 4 is 4.8 μM; the agreement with the observed $K_m$ (7.6 μM) suggests that *p*-benzoquinone inactivates this enzyme in a second-order reaction by reaction at the active site. The calculated second-order rate constant for enzyme inactivation by phenyl-*p*-benzoquinone is $2.6 \times 10^3$ $M^{-1}$ $s^{-1}$.

The product of the reaction of 1 with 10 molar equiv of sodium borohydride is proposed to be the hydroquinone derivative 7, which would not be expected to inactivate the enzyme. However, incubation of 5.56 μM 7 with the enzyme slowly inactivated the enzyme ($k_{obsd} \approx 0.003$ $s^{-1}$). The product of the reaction of the quinone nucleotide 1 with 1.2 equiv of 2-mercaptoethanol failed to inactivate thymidylate synthetase at a concentration of 5.56 μM. The dissociation constants of the enzyme–inhibitor complex of the hydroquinone nucleotide 7 and the product formed on treatment of 1 with 2-mercaptoethanol were determined from the double-reciprocal plots of velocity vs. substrate concentration at three concentrations of inhibitor (Figure 5). The $K_m$ for this enzyme preparation was 7.6 μM. The dissociation constant ($K_i$) for the hydroquinone 7 was 1.6 μM, and that for the mercaptoethanol–1 adduct was 2.6 μM; both inhibitors were competitive with the substrate.

## Discussion

Two modifications in the standard assay method for inactivation and determination of enzyme activity were required for these studies. It was necessary to remove 2-mercaptoethanol from the enzyme preparation, since it is recognized that thiols readily add to quinones to give the substituted hydroquinone.[21] This was evident in the initial studies with standard enzyme preparations containing at least 10 μM 2-mercaptoethanol where no enzyme inactivation was observed by treatment with compound I.

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Table II. Kinetics of Thymidylate Synthetase Inactivation[a]

| compd | $k_{cat}$, $s^{-1}$ | $K_i$, M | $k_2$,[b] $M^{-1}$ $s^{-1}$ | conditions (pH; buffer; temp, °C) |
|---|---|---|---|---|
| iodoacetamide | | | 7[c] | 7; phosphate; 24 |
| *N*-ethylmaleimide | | | 13[c] | 7; phosphate; 4 |
| methyl methanethiolsulfonate | | | $1.6 \times 10^3$[d] | 6.8; phosphate; 0 |
| *p*-benzoquinone | | | $6.7 \times 10^3$ | 6.8; phosphate; 30 |
| phenyl-*p*-benzoquinone | | | $2.6 \times 10^3$ | 6.8; phosphate; 30 |
| 5-*p*-quinonyl-dUMP[e] (1) | 0.065 | $2 \times 10^{-6}$ | $32 \times 10^3$ | 6.8; phosphate; 30 |
| 5-(α-bromoacetyl)-dUMP | 0.0025 | $4 \times 10^{-6}$ | $0.6 \times 10^3$[g] | 6.8; Tris-acetate; 30 |
| 5-nitro-dUMP | 0.146 | $5 \times 10^{-7}$[f] | $300 \times 10^3$[h] | 6.8; Tris-acetate; 30 |
| 5-fluoro-dUMP | | $5 \times 10^{-7}$[i] | $330\text{-}2800 \times 10^3$[j] | |

[a] Unless otherwise indicated, the enzyme source was from amethopterin-resistant *Lactobacillus casei*. [b] In some cases the second-order rate constant was estimated from the ratio $k/K_i$. [c] Calculated from data furnished by F. Maley (Galivan, J.; Noonan, J.; Maley, F. *Arch. Biochem. Biophys.* 1977, *184*, 336-345.) [d] Lewis, C. A., Jr.; Munroe, W. A.; Dunlap, R. B. *Biochemistry* 1978, *17*, 5382-5387. [e] dUMP, 2'-deoxyuridine 5'-phosphate. [f] A value for the $K_i$ of $0.3 \times 10^{-7}$ M also is reported: Wataya, Y.; Matsuda, A.; Santi, D. V. *J. Biol. Chem.* 1980, *255*, 5538-5544. [g] Brouillette, C. B.; Chang, C. T.-C.; Mertes, M. P. *J. Med. Chem.* 1979, *22*, 1541-1544. [h] Maggiora, L. M.; Chang, C. T.-C.; Torrence, P. F.; Mertes, M. P. *J. Am. Chem. Soc.* 1981, *103*, 3192-3198. [i] Danenberg, P. U.; Lockshin, A. *Biochem. Pharmacol.* 1981, *13*, 69-90. [j] $330 \times 10^3$ $M^{-1}$ $s^{-1}$ at pH 7.4, *N*-methylmorphiline, 24 °C, Santi, D. V.; McHenry, C. S.; Sommer, H. *Biochemistry* 1974, *13*, 471-480. $420 \times 10^3$ $M^{-1}$ $s^{-1}$, pH 7.1, Tris-HCl, 25 °C, mouse ascites tumor enzyme, Myers, C. E.; Young, R. C.; Chabner, B. A. *J. Clinical Invest.* 1975, *56*, 1231-1238. $2800 \times 10^3$ $M^{-1}$ $s^{-1}$, pH 7.4, Tris-HCl, 37 °C, human leukemia cell enzyme, Lockshin, A.; Danenberg, P. U. *Biochem. Pharmacol.* 1981, *30*, 247-257.

The second modification in our normal assay procedure was to replace Tris–acetate with phosphate as the buffer. Although phosphate is recognized to affect the active-site binding of nucleotides,[22] this was chosen as the buffer because it appeared to stabilize our mercaptoethanol-free enzyme preparation. Secondly, phosphate is a considerably weaker nucleophile than Tris or other amine buffers at pH 6.8; the latter were found to react with the title compound at pH 6.8.

The inactivation of thymidylate synthetase by 5-*p*-quinonyl-2'-deoxyuridine 5'-phosphate (1) was found to be time dependent and demonstrated saturation kinetics, and the rate of inactivation decreased in the presence of substrate. The derived equation (eq 1) that relates the rate of inactivation as a function of the reversible enzyme–inhibitor complex satisfies the mechanistic requirements for the enzyme inactivation as proceeding according to eq 5.

$$E + 1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}1 \xrightarrow{k_{cat}} E\text{-}1 \qquad (5)$$

When eq 1 in the absence of substrate was used, the dissociation constant ($K_i$) estimated from the slope of the plot in Figure 2 was 2.0 μM; this compares favorably with the $K_i$ of the precursor 5-(2,5-dimethoxyphenyl)-2'-deoxyuridine 5'-phosphate (**6**) and similar 5-aryl-substituted substrate analogues.[23] Furthermore, the dissociation constants of the hydroquinone nucleotide **7** and the adduct of mercaptoethanol and **1** determined from double-reciprocal plots of velocity vs. substrate concentration calculated to be 1.6 and 2.6 μM, respectively. These results agree with earlier studies that have shown that the dissociation constants for inhibitors are generally within a narrow range in a given series of compounds with reasonably similar electronic characteristics in the group substituted on carbon 5 of the substrate.[10]

Additional evidence for the formation of the reversible E·1 complex was obtained from the incubation studies incorporating substrate in addition to the enzyme and inhibitor. If eq 1 is satisfied, the intercept from the plot of Figure 2 at high inhibitor concentration should and does calculate for a rate equal to that observed in the absence of substrate. A further application of eq 1 is made when the $K_m$ was found to be 4.4 μM, again in good agreement with the $K_m$ of 7.6 μM determined by the double-reciprocal plot of velocity vs. substrate in a separate experiment.

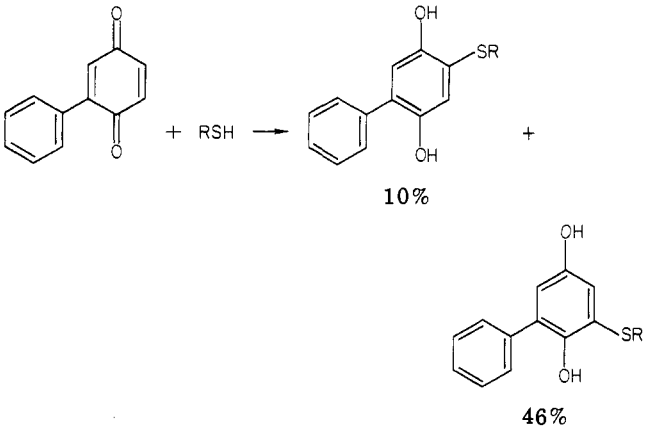
The essential structural requirements for the quinone group for enzyme inactivation by compound **1** were confirmed when the hydroquinone nucleoside **8** and the mercaptoethanol adduct of **1** did not show time-dependent inactivation of the enzyme. An exception was noted with the hydroquinone nucleotide **7**, which unexpectedly inactivated the enzyme at a rate one-fifteenth as fast as the quinone **1**. Possible mechanisms for this inactivation currently are being examined.

The inactivation reproduced in Figure 1 shows a change in rate of the reaction after 1 min of incubation even where the concentration of inhibitor exceeds that of enzyme. We have examined the stability of **1** in the presence of buffer and salts in the incubation mixture without enzyme and have found that **1** decomposes slowly to give a single, as yet unidentified, material ($t_{1/2} > 20$ h). Stronger nucleophiles, such as 2-mercaptoethanol, glutathione, and arginine, rapidly reacted with compound **1** ($t_{1/2} < 5$ min). Considering the multiplicity of nucleophilic residues on the protein, it would not be unusual to find that the inhibitor **1** also interacted to some extent with the enzyme at nucleophilic residues not at the active site. We cannot at this time say whether or not the enzyme also would be inactivated in these nonspecific reactions; however, it is clear that inhibitor would be lost. In any case, the observed results with the initial linear rates of the inactivation where inhibitor concentration is in excess of enzyme does fit the model; within these constraints the observed $k_{cat}$ of 0.065 $s^{-1}$ is considered to be a minimum value.

*p*-Quinones are recognized to be highly reactive reagents that can undergo both redox and electrophilic reactions.[24] While it is commonly accepted that the major reaction with thiols is nucleophilic addition to *p*-quinones to give the α-substituted hydroquinone,[21] alternative modes of reaction via redox reactions cannot be excluded. With this background, it was not unexpected that the nucleoside **5a** inactivated the enzyme. However, the rate of inactivation by **5a** was considerably slower than observed with compound **1**.

(22) Galivan, J. H.; Maley, G. F.; Maley, F. *Biochemistry* 1976, *15*, 356–362.
(23) Unpublished results from these laboratories.
(24) (a) Webb, J. L. "Enzyme and Metabolic Inhibitors"; Academic Press: New York, 1966; Vol. 3, p 421–594. (b) Finley, K. T. "The Chemistry of Quinoid Compounds", Part 2; Patai, S., Ed.; Wiley: New York, 1974.

Scheme IV




The inactivation of thymidylate synthetase by thiol reagents has been a subject of study in several laboratories. Maley and co-workers[25] reported inactivation of this enzyme on treatment with *N*-ethylmaleimide or iodoacetamide; calculations from the published data gave estimated second-order rate constants of 13 and 7 $M^{-1}$ $s^{-1}$, respectively (Table II). It was noted by these authors that a 1:1 equivalent of inhibitor–dimeric enzyme afforded complete inactivation. Dunlap and co-workers[26] also reported the inactivation of thymidylate synthetase by methyl methanethiosulfonate at a rapid rate; under their conditions, 1.5 mol of this reagent reacted per mole of dimeric enzyme. Since protection against inactivation by these three reagents was afforded by the substrate 2′-deoxyuridine 5′-phosphate, the inactivation appears to be a result of reaction at the active-site thiol.

In the present study we have found that both *p*-benzoquinone and phenyl *p*-benzoquinone are highly reactive reagents that inactivate thymidylate synthetase and, at least in the one case examined (*p*-benzoquinone), appear to react with the active-site thiol. Viewing the title compound 1 as a monosubstituted *p*-benzoquinone, a more appropriate model for comparison is phenyl-*p*-benzoquinone. The latter reacts rapidly with thiol reagents to give both the 2,5-disubstituted and the 2,6-disubstituted compounds (Scheme IV).[27] The time-dependent inactivation of the enzyme by phenyl-*p*-benzoquinone (Figure 4) shows first-order kinetics. The order of the reaction can be obtained by analysis of the logarithm of eq 3 as shown in eq 6, where *n* is the number of molecules of the inhibitor

$$\ln k_{obsd} = \ln k_2 + n \ln [I] \qquad (6)$$

that react with one molecule of enzyme.[28] The value for *n* in this experiment is 0.94 or approximately unity; the corresponding value of *n* for *p*-benzoquinone is 1.3 and for **5a** is 0.86.

Two other analogues of the substrate that are thought to react with the enzyme to form a covalent bond are the 5-fluoro and the 5-nitro derivatives, potent inhibitors that are slowly reversible under certain dialysis conditions. In the first case, the inactivation is reversible with an overall dissociation constant of $10^{-11}$ M for the complex with enzyme found in human leukemic cells.[29] In the second example, 5-nitro-2′-deoxyuridine 5′-phosphate is a potent affinity label for thymidylate synthetase[11] that was found to be reversible on prolonged dialysis;[11b] the dissociation constant for breakdown of the covalent complex was $10^{-10}$ M.[11d]

The reversibility of the product of the reaction of 1 with the enzyme was examined by dialysis. Two inhibitor concentrations were chosen to give partial inactivation and complete inactivation. For the latter preparation, the results in Figure 3 show little recovery of activity even after dialysis for 7 days.

The results of this study show that 5-*p*-quinonyl-2′-deoxyuridine 5′-phosphate (1) is a possible mechanism-based inhibitor of thymidylate synthetase. The chemical reactivity of this compound can be compared to that of the thiol reagents from the second-order rate calculated from the ratio $k_{cat}/K_i$, which gives a value of $32 \times 10^3$ $M^{-1}$ $s^{-1}$, a thousand times faster than *N*-ethylmaleimide and clearly faster than the corresponding nucleoside or the model compound that does not have active-site affinity, phenyl-*p*-benzoquinone. On the other hand, compound 1 has one-half the reactivity ($k_{cat}$) as 5-nitro-2′-deoxyuridine 5′-phosphate (Table II). The predominating factor in the rapid second-order rate of the 5-nitro and the 5-fluoro[30] derivatives can be accounted for by the tenfold greater affinity evident in comparing the dissociation constants. Dialysis studies have shown that under certain conditions the enzyme complex resulting from inactivation by these two compounds can be reversed; the overall dissociation constant describing the breakdown of these complexes to give active enzyme is estimated to be $10^{-10}$ M. Based on these studies, compound 1 offers the advantage over the other affinity labels for this enzyme in that prolonged dialysis of the enzyme–1 complex, even in presence of high concentrations of 2-mercaptoethanol, does not restore activity.

The proposed mechanism of this reaction in Scheme II depicts 1 as a suicide inhibitor; that is, the covalent interaction complex **2** is a chemically reactive intermediate formed by the active-site catalytic residue. However, at the present time, while 1 can be classified as a mechanism-based inhibitor, in order to classify 1 as a suicide inhibitor, more studies are required to establish the structure of the inactivated enzyme–inhibitor complex. Given that the reaction proceeds as described in eq 5, the most probable alternative to Scheme II would be the addition of the enzyme thiol in the complex E·1 directly to the quinone ring. Model studies are planned to examine the structural chemistry of Michael addition reactions to the nucleoside **5a** and the nucleotide 1 and more clearly define the action of compound 1.

## Experimental Section

All melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. IR spectra were measured with a Beckman IR-33, UV spectra were obtained with a Cary Model 219 recording spectrophotometer, and $^1H$ NMR spectra were obtained with a Varian FT 80 Model or T-60 or a Brucker WP80. Microanalyses were obtained from a Hewlett-Packard 185B, and mass spectra were obtained from a Varian CH5 spectrometer. Unless indicated otherwise, C, H, and N analyses were ±0.4% of the calculated values. DEAE-cellulose

(25) Galivan, J.; Noonan, J.; Maley, F. *Arch. Biochem. Biophys.* **1977**, *184*, 336–345.
(26) Lewis, C. A., Jr.; Munroe, W. A.; Dunlap, R. B. *Biochemistry* **1978**, *17*, 5382–5387.
(27) (a) Porter, R. F.; Rees, W. W.; Frauenglass, E.; Wilgus, H. S.; Nawn, G. H.; Chiesa, P. P.; Gates, J. W., Jr. *J. Org. Chem.* **1964**, *29*, 588–594. (b) Wilgus, H. S.; Frauenglass, E.; Jones, E. T.; Porter, R. F.; Gates, J. W., Jr. *J. Org. Chem.* **1964**, *29*, 594–600.
(28) Edwards, J. B.; Keech, D. B. *Biochim. Biophys. Acta* **1967**, *146*, 576–583.
(29) See Lockshin, A.; Danenberg, P. V. *Biochem. Pharmacol.* **1981**, *30*, 247–257, and references therein.
(30) Santi, D. V.; McHenry, C. S.; Sommer, H. *Biochemistry* **1974**, *13*, 471–480.

was a product of Whatman Biochemical Ltd.; 2'-deoxyuridine (B grade) and 2'-deoxyuridine 5'-phosphate were purchased from Calbiochem or Sigma Chemical Co.

High-performance LC was performed with a Waters Model M6000A and a Perkin-Elmer LC-15 UV detector with a Partisil PXS 10/25 SAX (strong anion exchange) or Partisil PXS 10/25 ODS-II column. Thymidylate synthetase from methotrexate-resistant *Lactobacillus casei* (purchased from the New England Enzyme Center, Tufts University) was purified and used at a specific activity of 3.2 μmol of TMP formed $min^{-1}$ (mg of protein)$^{-1}$ by using the radioisotope assay. The enzyme was activated by dialysis for 24 h at 4 °C against 0.1 M potassium phosphate (pH 6.8) containing 50 mM mercaptoethanol. The substrate 2'-deoxy[5-$^3$H]uridine 5'-phosphate at a specific activity above 15 Ci/mmol was purchased from Moravek Biochemicals, Industry, CA, and diluted with cold substrate purchased from Sigma Chemical Co., St. Louis, MO, to give a specific activity of 500 μCi/μmol. The cofactor, *dl*-tetrahydrofolic acid, was also purchased from Sigma Chemical Co.

**Treatment of 5-(2,5-Dimethoxyphenyl)-2'-deoxyuridine (4a) with Boron Tribromide or Trimethylsilyl Iodide.** 5-(2,5-Dimethoxyphenyl)-2'-deoxyuridine[16] (**4a**; 55 mg, 0.15 mmol) was dissolved in 5 mL of dry acetonitrile, and over a period of 5 min, 0.8 mL of a 1 M acetonitrile solution of boron tribromide (0.8 mmol) was added to the stirred solution at 25 °C. After 4 h, the yellow solution was neutralized to pH 8 by the addition of 1 M potassium hydroxide. The reaction mixture was resolved in one case on silica (15% methanol in chloroform) and in another similar reaction on DEAE-cellulose and eluted with a linear gradient of 300 mL each of 0.01 and 0.3 M triethylammonium bicarbonate buffer (pH 7.5). In these and subsequent reactions, in addition to starting material, the only characterized product (~10% yield) was the monomethyl ether of 5-(2,5-dihydroxyphenyl)uracil arising from monodemethylation and glycosidic bond cleavage: mass spectrum, *m/e* 234 (100, $M^+$), 219 (20), 191 (20), 176 (20), 147 (30); $^1$H NMR ($CD_3OD$) δ 7.63 (s, 1 H, $C_6$ H), 6.97 (s, 3 H, aromatic), 3.92 (s, 3 H, $OCH_3$).

A solution of 5-(2,5-dimethoxyphenyl)-2'-deoxyuridine (**4a**; 100 mg, 0.27 mmol) in 1 mL of anhydrous acetonitrile under an argon atmosphere was treated with approximately 3 equiv of iodotrimethylsilane (0.12 mL, 0.84 mmol) added in one portion with vigorous stirring. A precipitate formed instantaneously, which slowly went back into solution. After 13 h at room temperature, 0.2 mL of methanol was added to quench the reaction. The reaction mixture was stirred overnight and then neutralized with 0.1 mL of triethylamine, and the resulting precipitate was filtered. After the solvent was removed from the filtrate, the residue was resolved on a silica gel column to give a brown solid (23 mg), which was further purified by washing with methanol to give an off-white solid, which has been tentatively identified as 1-acetyl-5-(2,5-dimethoxyphenyl)uracil (27%): mp 235 °C dec; mass spectrum, *m/e* 277, 254, 249, 248, 233, 219, 217, 205, 190, 174, 163, 162; IR (KBr) no O–H stretch, 1730, 1650, 1635 $cm^{-1}$; $^1$H NMR [$(CD_3)_2SO$] δ 7.33 (s, 1 H, $C_6$ H), 6.87 (m, 3 H, aromatic), 3.78 (s, 3 H, $OCH_3$), 3.75 (s, 3 H, $OCH_3$), 3.37 (s, 3 H, $CH_3CO$), 3.31 (s, 1 H, N-3); UV ($H_2O$) $\lambda_{max}$ 260 nm (ε 9800), sh 292, $\lambda_{min}$ 245 (8900); UV (0.1 N HCl) $\lambda_{max}$ 260 nm (ε 9900), sh 292, $\lambda_{min}$ 245 (9200); UV (0.3 N NaOH) $\lambda_{max}$ 292 (ε 13 200) nm, $\lambda_{min}$ 267 (8800). Anal. ($C_{14}H_{14}N_2O_5 \cdot H_2O$, $M_r$ 308.3) H, N; C: calcd, 54.54; found, 54.90.

**5-(2,5-Dimethoxyphenyl)-3',5'-diacetyl-2'-deoxyuridine (4b).** A pyridine mixture (10 mL) containing 900 mg of 5-(2,5-dimethoxyphenyl)-2'-deoxyuridine (**4a**; 2.5 mmol) and 0.6 mL of acetic anhydride was heated to 45 °C for several minutes to dissolve the nucleoside, and the reaction solution was allowed to stand overnight at 25 °C. After the addition of 0.5 mL of ethanol, the reaction was evaporated, and the residue was redissolved in 50% ethanol–water and evaporated to dryness twice. After trituration of the residue with ether, 910 mg (82%) of **4b** was collected as a solid: $^{13}$C NMR [$(CD_3)CO$] 170.2, 170.1 (acetyl C=O), 161.8 (C4), 153.7, 151.8 (C2, C5 phenyl), 150.4 (C2), 138.9 (C6), 123.1, 117.8, 113.9, 112.8, 112.0 (C1, C3, C4, C6 phenyl; C5), 85.1 (C1'), 82.4 (C4') 74.6 (C3'), 64.1 (C5'), 56.1, 55.3 ($OCH_3$), 37.1 (C2'), 13.4, 12.8 ($CH_3$) ppm; mass spectrum, *m/e* 448 (9, $M^+$) 248 [100, 5-(2,5-dimethoxyphenyl)uracil], 233 (10), 219 (8), 217 (10), 205 (9), 190 (8), 174 (10), 162 (12), 117 (6); UV ($CH_3OH$) $\lambda_{max}$ 265 nm. Anal ($C_{21}H_{24}N_2O_9$, $M_r$ 448.4) C, H, N.

**5-p-Quinonyl-2'-deoxyuridine (5a).** 5-(2,5-Dimethoxyphenyl)-2'-deoxyuridine (**4a**; 12 mg, 0.037 mmol) was dissolved in 1.5 mL of dioxane, and 20 mg of silver(II) oxide[17,18] (0.16 mmol) was added. The reaction was initiated by the addition of 0.05 mL of 6 N nitric acid to the vigorously stirred solution; an intense yellow color developed immediately. After 10 min, TLC on silica showed complete conversion of the starting material to a new yellow spot with a lower $R_f$. Water and ethyl acetate were added, and this mixture was extracted several times with ethyl acetate. The combined organic layers were dried and evaporated; the residue was resolved on silica with 15% ethanol in chloroform. The quinone **5a** was isolated in 60% yield: $^1$H NMR ($D_2O$) δ 8.08 (s, 1 H, $C_6$ H), 7.9–6.9 (m, 3 H, quinone H), 6.20 (t, 1 H, $J$ = 6 Hz, $C_{1'}$ H); the assignments for the remaining sugar protons were as expected; UV (0.04 M ammonium formate, pH 4.4) $\lambda_{max}$ 250 nm (ε 14 000), sh 275 nm, $\lambda_{min}$ 327 nm (750), $\lambda_{max}$ 385 nm (1800). Anal. ($C_{15}H_{14}N_2O_7 \cdot H_2O$, $M_r$ 352.3) C, H, N.

**5-p-Quinonyl-3',5'-diacetyl-2'-deoxyuridine (5b).** By the same method as described for the synthesis of **5a**, 135 mg of the diacetyl derivative **4b** (0.3 mmol) was converted to 5-*p*-quinonyl-3',5'-diacetyl-2'-deoxyuridine (**5b**) in 63% yield: $^{13}$C NMR ($CDCl_3$) 187.1, 186.2 (quinone C=O), 170.4, 170.3 (acetyl C=O), 160.7 (C4), 148.7 (C2), 142.6 (C6), 136.8, 136.5, 135.8, 134.2 (quinone CH, C), 105.5 (C5), 85.9 (C1'), 82.8 (C4'), 74.2 (C3'), 63.8 (C5'), 38.2 (C2'), 20.84, 20.78 ($CH_3$) ppm; mass spectrum, *m/e* 418 (100, $M^+$), 407 (60), 377 (74), 236 (54), 219 (74, 5-*p*-quinonyluracil), 201 (44), 176 (76), 159 (90). Anal ($C_{19}H_{18}N_2O_9 \cdot 0.5H_2O$, $M_r$ 427.4) C, H, N.

**5-(2,5-Dimethoxyphenyl)-2'-deoxyuridine 5'-Phosphate (6) Bis(triethylammonium) Salt (6).** The nucleoside **4a** (150 mg, 0.4 mmol) was mixed at 0 °C with 0.8 mL of a freshly prepared solution containing (v/v) phosphorus oxychloride (2.01), acetonitrile (5.87), water (0.22), and pyridine (1.9).[31] After the mixture was stirred for 10 h at 0 °C, water (0.5 mL) was added to the homogeneous solution; the mixture was neutralized to pH 7.5 by the addition of a 2.0 M solution of triethylammonium bicarbonate buffer (pH 7.5). After diluting to 50 mL with water, the mixture was resolved on DEAE-cellulose with a gradient composed of 500 mL each of 0.01 and 0.3 M triethylammonium bicarbonate (pH 7.5). The product **6** was obtained in 72% yield.[16] A sample of the free acid of **6** for $^{13}$C NMR analysis was obtained by passage of an aqueous solution of the salt through a Dowex 50 ($H^+$ form) column: $^{13}$C NMR ($^2H_2O$) 164.4 (C4), 154.7, 152.9 (C2, C5 phenyl), 151.9 (C2) 141.2 (C6), 125.3, 113.0 (C5, C1 phenyl), 118.4, 115.1, 113.4 (C3, C4, C6 phenyl), 88.7 (C4'), 86.6 (C1'), 72.1 (C3'), 62.7 (C5'), 56.4, 56.2 ($CH_3$), 41.3 (C2').

**5-p-Quinonyl-2'-deoxyuridine 5'-Phosphate (1).** The ammonium salt of 5-(2,5-dimethoxyphenyl)-2'-deoxyuridine 5'-phosphate (**6**; 28 mg, 0.060 mmol) was dissolved in 0.06 mL of water, and 62 mg of freshly prepared silver(II) oxide (0.50 mmol) was added. The reaction was started by the addition of 0.2 mL of 6 N nitric acid to the vigorously stirred solution. Over a 10-min period an orange color developed, and the precipitate dissolved slowly. The solution was neutralized to pH 4 by the addition of 2 M ammonium formate buffer (pH 4.4), and the entire mixture was diluted with sufficient water (50 mL) to give a final concentration of salts of less than 0.1 M. This solution was applied at 2 °C to a 1.5 × 75 cm column of DEAE-cellulose (formate), the column was washed with 200 mL of water, and the reaction products were resolved with a linear gradient of 500 mL of water and 500 mL of 0.4 M ammonium formate buffer (pH 4.4). The product **1** was collected in 54% yield in the first yellow band (~0.1 M buffer) in 98% pure form. Unless an excess of silver(II) oxide was used, unreacted starting material **6** followed the product and contaminated the latter fractions of **1**: UV (0.1 M ammonium formate, pH 4.4) $\lambda_{max}$ 250 nm, 385 nm, $\lambda_{min}$ 327 nm; $^1$H NMR ($^2H_2O$) δ 8.05 (s, 1 H, $C_6$ H), 7.27, 7.07, 6.9 (m, 3 H, quinone H), 6.3 (t, 1 H, $C_{1'}$ H); the assignments for the remaining sugar protons were as expected.

High-performance LC with 30% methanol and 70% 0.1 M potassium dihydrogen phosphate and a reverse-phase Partisil 5 ODS column resolved the product **1** ($V_R$ 0.74), starting material **6** ($V_R$ 2.10), minor side products ($V_R$ 1.37, 3.21), and decomposition

(31) Sowa, T.; Ouchi, S. *Bull. Chem. Soc. Jpn.* **1975**, *48*, 2084–2090.

products arising from 1 ($V_R$ 0.10, 0.53).

The column fractions containing 1 were passed through a Dowex 50 ($H^+$) column, and the resulting acidic solution of 1 was freeze-dried to give the product as a yellow solid without significant decomposition. In contrast, freeze-drying the ammonium formate buffer solutions of 1, any increase in pH, or the addition of nucleophiles (acetate, thiols, or alkylammonium compounds such as Tris) above a pH of 6 decomposed 1 to give products with retention times ($V_R$) of 0.10 and 0.53.

Reduction of an aqueous solution of either 1 or **5a**, with a 4-equiv excess of sodium borohydride gave products presumed from UV and liquid chromatography ($V_R$ 0.53 from 1) to be the hydroquinone derivatives **7** and **8**: UV for the nucleoside **8** (0.1 M ammonium formate, pH 4.4) $\lambda_{max}$ 265 nm ($\epsilon$ 9000), $\lambda_{min}$ 245 nm (8000).

Treatment of an aqueous solution of the hydroquinone nucleotide **7** with a slight excess of 3% aqueous hydrogen peroxide gave a product with the same ultraviolet spectra and liquid chromatography retention time as the quinone 1.

**Enzyme Assay.** The enzyme was assayed by modification of the radioisotope assays described by Roberts[32] and Lomax and Greenberg.[33] The solution (0.1 mL) contained 25 mM mercaptoethanol, 0.22 mM *dl*-tetrahydrofolic acid, 6.75 mM formaldehyde, 5 mM sodium bicarbonate, 3 mM magnesium chloride, 0.12 mM EDTA, 6 mM potassium phosphate buffer, pH 6.8, 10 $\mu$L of the diluted enzyme solution, substrate, and, when indicated, inhibitor. Control reactions lacked the cofactor, tetrahydrofolic acid. The substrate 2′-deoxy[5-$^3$H]uridine 5′-phosphate was used at a specific activity of 500 $\mu$Ci/$\mu$mol. For maximum velocity studies for active enzyme determination the substrate concentration was 50 $\mu$M. The assays were started by the addition of the enzyme to the complete mixture and then incubated at 30 °C. Incubation was stopped at 30 s by the addition of 50 $\mu$L of 20% trichloroacetic acid. A 20% aqueous suspension of charcoal (0.25 mL) was added, and the solution was vortexed and allowed to stand for 15 min. The suspension was filtered through a cotton-plugged Pasteur pipet, and 0.1 mL of the filtrate was counted in Beckman Ready Solve HP scintillation fluid. Counting efficiency was 33%; control samples lacking the cofactor were found to have less than 5% of the respective sample counts. Velocity is reported in the adjusted value of picomoles of $^3H_3O$ formed per minute in the assay.

**Preincubation Studies.** The enzyme concentration was determined by absorbance at 278 nm by using an extinction coefficient of $1.05 \times 10^5$ and a dimer molecular weight of 70 000. Passage of a concentrated solution of the activated enzyme through a 1 × 20 cm column of Sephadex G-25 with 0.05 M phosphate buffer at pH 6.8 gave the pure enzyme free of 2-mercaptoethanol. This enzyme preparation was reasonably stable for several days at 2 °C; the specific activity of the enzyme used in the preincubation studies ranged from 2.6 to 3.2 $\mu$mol of product formed min$^{-1}$ (mg of protein)$^{-1}$. The enzyme in concentrations ranging from 0.1 to 0.4 $\mu$M was preincubated at 30 °C in 50 $\mu$L of solution containing 6 mM potassium phosphate buffer, pH 6.8, 3 mM magnesium chloride, 0.12 mM ethylenediaminetetracetic acid, and varying concentrations of the compounds tested for inactivation of the enzyme. Substrate protection was evaluated by including the indicated concentration of substrate at a specific activity of 500 $\mu$Ci/$\mu$mol. After incubation for the indicated time period, the assay for remaining active enzyme was started by the addition of 50 $\mu$L of a solution containing buffer and other components of the assay to give the same concentrations as noted in the enzyme assay. A high substrate concentration (50 $\mu$M) was used in these assays to afford reasonably high velocity and to competitively reduce the enzyme inactivation by the inhibitor during the assay. The assay was run for 60 s and treated as described in the enzyme assay section. Inactivation of the enzyme was measured by comparing the velocity at time zero to that at the indicated incubation times. Under the conditions of the assay, the uninhibited enzyme retained 96% of the initial activity after 4 min of incubation.

**Dialysis Studies.** The enzyme was preincubated with varying concentrations of inhibitor for 5 min. Control and inhibited enzyme preparations were then dialyzed in two changes of a 4000-fold volume of a solution containing 0.01 M potassium phosphate buffer, pH 6.8. Dialysis was performed with and without 50 mM 2-mercaptoethanol.

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# Synthesis and Monoamine Oxidase Inhibitory Activities of $\alpha$-Allenic Amines in Vivo and in Vitro. Different Activities of Two Enantiomeric Allenes[1]

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A series of 15 $\alpha$-allenic amines, including primary, secondary, and tertiary ones, was synthesized, partly by organocopper chemistry. Their ability to inhibit mouse and rat brain mitochondrial monoamine oxidase (MAO) in vivo and in vitro, respectively, was evaluated. Almost all compounds were quite potent inhibitors of MAO, some as potent as deprenyl. Like deprenyl, most of the compounds were selective inhibitors of the B form of MAO. The two enantiomeric forms of *N*-methyl-*N*-(2,3-pentadienyl)benzylamine (**2**) were prepared and the *R*-(−) form was found to be 2.7 times as active as the (+) form in vivo and 25 times as active in vitro. Most of the compounds were tested for their ability to potentiate the phenylethylamine (PEA) response in mice, and a good correlation between the potency of MAO inhibition and PEA potentiation was found. Compound **5**, as the only compound tested, did not potentiate the blood pressure response to tyramine.

Mitochondrial monoamine oxidase (MAO) is an enzyme that is involved in the oxidative deamination of transmitter amines.[2] The existence of two forms of MAO was first reported by Johnston,[3] who found that one form, which he named MAO-A, was inactivated by the acetylene inhibitor clorgyline, while the other form, which he named

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