70.0 to 70.5 °C in five different runs.

Acknowledgment. We are indebted to Drs. Robin Hood and Ole Mols and Maya Kemff of the Wayne State University Department of Chemistry for their expert assistance in obtaining NMR and MS data. This work was supported by National Cancer Institute Grant CA 32038 and Wayne State University Grant-in-Aid 167-1006.

Registry No. **10a**, 102830-98-8; **10b**, 102830-99-9; **10b**·HCl, 102831-00-5; **10c**, 102831-01-6; **10d**, 102831-02-7; **10e**, 102831-03-8; **11a**, 17241-59-7; **11b**, 24094-45-9; **11c**, 22296-59-9; **11d**, 56670-70-3; **11e**, 481-72-1; **12**, 6736-63-6; **13a**, 102830-93-3; **13b**, 102830-94-4; **13c**, 102830-95-5; **13d**, 102830-96-6; **13e**, 102830-97-7.

Thiol Addition to Quinones: Model Reactions for the Inactivation of Thymidylate Synthase by 5-*p*-Benzoquinonyl-2′-deoxyuridine 5′-Phosphate

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The reaction of methyl mercaptoacetate (**5**) with phenyl-*p*-benzoquinone (**6**) or 5-*p*-benzoquinonyl-3′,5′-di-*O*-acetyl-2′-deoxyuridine (**10**) resulted in the formation of the three possible adducts to the quinone rings of **6** and **10**; an additional product in the reaction with **10** was the unsubstituted hydroquinone (**14**). Both reactions were found to be solvent dependent; in buffered aqueous acetonitrile the meta and para adducts of **10** were formed in the ratio of 2:1. In ethyl acetate the ortho adduct and the reduction product of **10** were isolated in a ratio of 2:3. The second-order rate constant for the reaction of **5** with **10** in acetonitrile was 0.53 M^{-1} s^{-1}; the reaction was accelerated by the addition of water. Although the initially proposed mechanism-based enzyme inactivation cannot be excluded, the results of the model reactions support the alternative mechanism, active-site thiol addition to the quinone ring. If this is true the title compound would be classed as an affinity label, not a mechanism-based inhibitor.

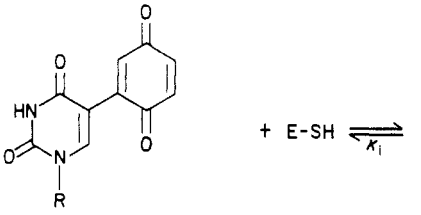
The design and synthesis of affinity labels as enzyme inhibitors has been a useful approach for probing the mechanism of enzyme catalysis and for the development of agents for disease control. Three criteria normally are employed in the verification that a compound is an affinity label:[1] (1) A reversible enzyme–inhibitor complex is formed. (2) Time-dependent loss of enzyme activity is observed in agreement with the standard equation (eq 1) describing the reaction,[2] where K_i is the inhibitory constant, K_m the Michaelis constant, and [I] and [S] are the concentrations of inhibitor and substrate. (3) Nonspecific vs. active-site directed inactivation is distinguished by the decreased rate of enzyme inactivation in the presence of substrate; this "substrate protection" analysis also must satisfy the kinetic equation (eq 1).

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

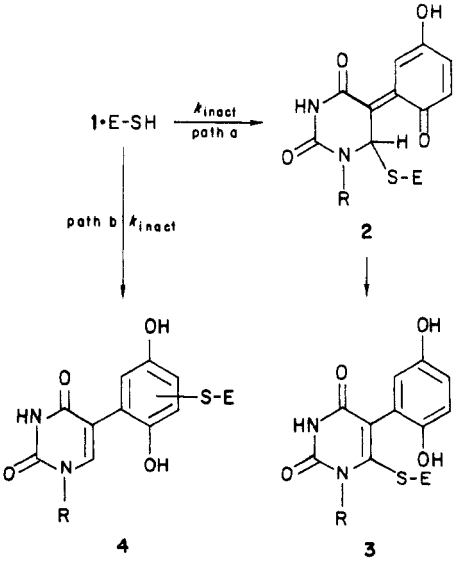
Contemporary research in the design of a class of affinity labels termed mechanism-based inhibitors center on the utilization of the chemical mechanism of the catalytic event promoted by the enzyme for the activation of the inhibitor in the noncovalent complex of enzyme and inhibitor.[3] The activated inhibitor subsequently reacts with the enzyme resulting in covalent bond formation and enzyme inactivation, which may or may not be irreversible.

5-*p*-Benzoquinonyl-2′-deoxyuridine 5′-phosphate (**1a**) has been reported to be a possible mechanism-based inhibitor of thymidylate synthase.[4] The reductive alkylation reaction catalyzed by this enzyme is initiated by active-site thiol addition to carbon-6 of the α,β-unsaturated carbonyl moiety in the pyrimidine ring.[5] On this basis, compound **1** was designed as a potential mechanism-based inhibitor affording the reactive intermediate [2], which would be ex-

Scheme I. Possible Mechanisms for the Inactivation of Thymidylate Synthase by 5-*p*-Benzoquinonyl-2′-deoxyuridine 5′-Phosphate (**1a**)



1a, R = 2′-deoxyribose 5′-phosphate
b, R = 2′-deoxyribose



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pected to rearrange to **3** resulting in inactivation of the enzyme by pathway a in the sequence proposed in Scheme I.

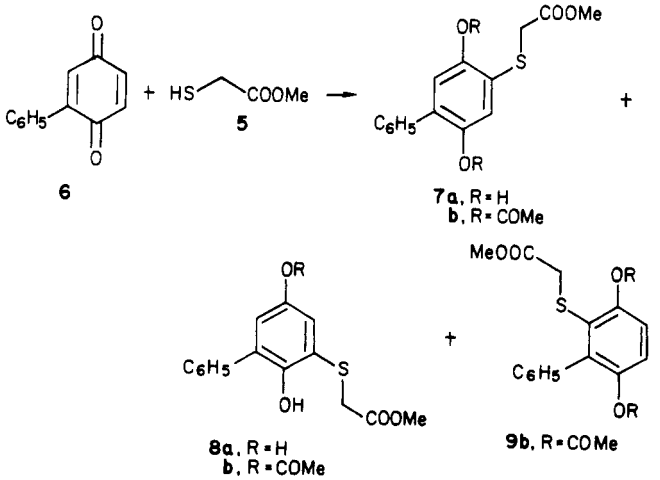
Compound **1a** displayed high affinity for thymidylate synthase (K_i = 2 μM), rapid time-dependent inactivation with an observed rate constant (k_{inact}) of 0.065 s^{-1}, and substrate protection in agreement with eq 1, and the inactivation was irreversible when the enzyme–inhibitor complex was dialyzed for 48 h in the presence of 2-mercaptoethanol. These findings verify that compound **1a** is an affinity label; however, there is no distinguishing characteristic in kinetic analysis that differentiates a mechanism-based inhibitor from an alkylating-type affinity label. It was noted that an alternative pathway giving similar results would involve the addition of the active-site thiol directly to the quinone ring to give **4** (pathway b, Scheme I); in this case compound **1** would be classified as an alkylating-type affinity label.

Model reactions were examined for the reaction of mercaptans with the nucleoside **1b**, its diacetyl ester compound **10**, and related *p*-benzoquinones. The results of these studies demonstrate that the bimolecular reaction involving addition of thiol directly to the quinone ring of the nucleoside **10** clearly is a favored reaction. The reaction is affected by solvent and displays regioselectivity, and an alternative redox reaction was observed under certain conditions. Reaction at carbon-6 of the pyrimidine ring was not observed in these studies.

Results

The thiol methyl mercaptoacetate (**5**) was chosen for the model studies principally because the pK_a (7.91) was similar to that of the active-site thiol in thymidylate synthase (pK_a = 8).[6,7] Other advantages gained in this choice included no interferring ultraviolet absorption or NMR peaks in the region under study. Phenyl-*p*-benzoquinone (**6**) was selected as a model thiol acceptor, since it was found to rapidly inactivate thymidylate synthase.[4]

Treatment of phenyl-*p*-benzoquinone (**6**) with an excess of the thiol **5** in acetonitrile afforded a 1:1 mixture of the para and meta adducts **7** and **8**. On the other hand, the

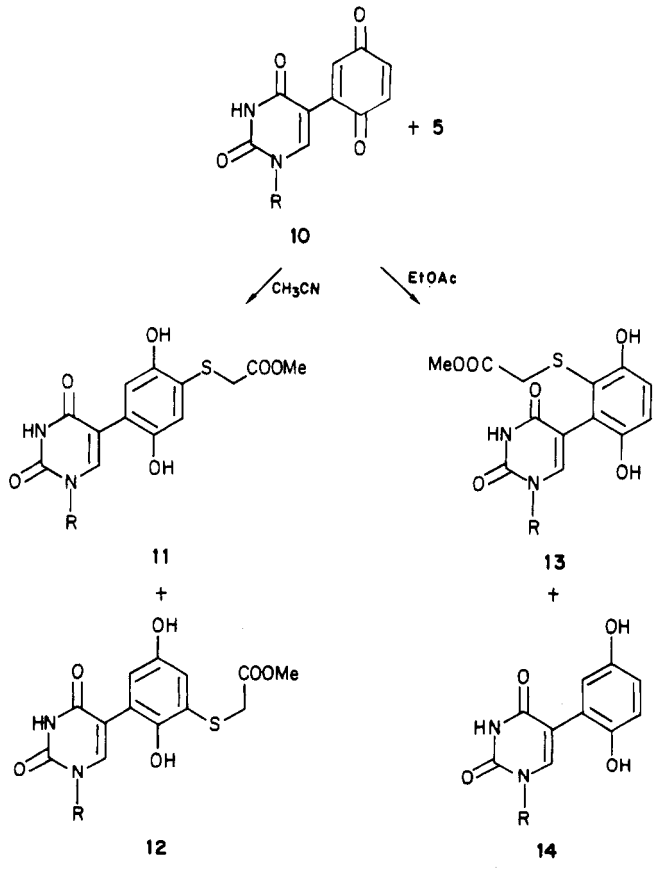


predominant product in ethyl acetate was the ortho isomer **9**. No evidence for reduction of **6** to phenyl-*p*-hydroquinone was observed. The ^1H and ^{13}C NMR spectra of the diacetate derivatives **7b**–**9b** were used in structural characterization. The key features in this analysis were (1) the hydroquinone ring protons in the para isomer **7b** appeared as two singlets (7.28, 7.14); (2) the meta coupling pattern observed for compound **8b** (J_{meta} = 2.66 Hz); and (3) although the ortho protons of the diacetate of **9b** were magnetically equivalent, by use of the attached proton test (APT), the ^{13}C NMR spectra of this compound clearly showed 6 and 4 ppm downfield shifts for the quaternary carbons of the hydroquinone ring bearing the thiol and phenyl substituents.

Studies on the reaction of methyl mercaptoacetate (**5**) with the quinone nucleoside in aprotic solvents employed the 3′,5′-di-*O*-acetyl derivative **10** because of the enhanced stability and solubility of this compound. The synthesis of **10** followed the reported procedure[4] with one modification. Ceric(IV) ammonium nitrate rather than silver(II) oxide was used in the last step of the synthesis, oxidative demethylation.

Treatment of an acetonitrile solution containing 0.015 M phosphate buffer (pH 7.4) and 5-*p*-benzoquinonyl-3′,5′-di-*O*-acetyl-2′-deoxyuridine (**10**) with a excess of the thiol **5** gave the para and meta adducts **11** and **12** in a ratio of 1:2. The latter structure (**12**) was confirmed by the ^1H NMR spectrum, which showed the characteristic meta proton coupling pattern (J_{meta} = 2.85 Hz) also observed in the meta hydroquinone **8**. The para isomer **11** showed the corresponding uncoupled hydroquinone protons as singlets at 7.06 and 6.73.

In contrast to the results obtained from the reaction of **5** with phenyl-*p*-benzoquinone (**6**) in ethyl acetate, the major product isolated from the reaction of the nucleoside **10** with a 20 M excess of **5** was the reduction product **14**.



Oxidation of **14** to the starting quinone **10** using ferric chloride confirmed the structure. The second product, the ortho isomer **13**, and the reduction product **14** were formed in a 2:3 ratio. The former structure was verified by the

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Table I. Observed Rate of the Reaction of 5-*p*-Benzoquinonyl-3′,5′-di-*O*-acetyl-2′-deoxyuridine (**10**) and 5-*p*-Benzoquinonyl-2′-deoxyuridine (**1b**) with Methyl Mercaptoacetate (**5**) in Acetonitrile and Buffered Aqueous Acetonitrile Solutions at 37 °C

quinone	% H_2O, 10^2 M	[5] M	k_{obsd}, min^{-1}	k_2, M^{-1} s^{-1}
10	none	0.0096[a]	0.30	
		0.0048	0.15	
		0.0029	0.092	
		0.00096	0.026	0.53
10	0.015 (0.78)	0.0015[b]	0.11	
		0.0026	0.20	
		0.0036	0.31	
		0.0061	0.57	
		0.012	1.18	1.7
10	0.022 (1.1)	0.0020[c]	0.26	
		0.0039	0.58	
		0.0077	1.0	2.3
10	0.050 (2.8)	0.00098[d]	0.23	
		0.0019	0.55	
		0.0039	1.1	4.7
1b	75	0.01[e]	>100	$>1 \times 10^3$

[a] The concentration of quinone **10** was 9.7×10^{-5} M. From the equation, $\log k_{obsd} = \log k_2 + n \log [5]$; the value of n is 1.06. [b] The concentration of the quinone **10** was 1.6×10^{-4} M. [c] The concentration of the quinone **10** was 1.4×10^{-4} M. [d] The concentration of the quinone **10** was 7.4×10^{-5} M. [e] The concentration of the quinone **1b** was 8.4×10^{-4} M in a solution containing 0.1 M phosphate buffer (pH 6.8) at an ionic strength of 0.5 (KCl) with the addition of 25% (v/v) of acetonitrile. This reaction was examined at 25 °C.

^1H and ^{13}C NMR of the mixture of rotomers. The ortho protons of the hydroquinone ring of **13** appeared as a singlet at 6.97 and a doublet centered at 6.39 ppm; the latter doublet is assigned to one of these protons in the two rotational isomers. The pyrimidine ring proton resonance of the mixture of rotamers **13** also was a doublet centered at 7.68 ppm.

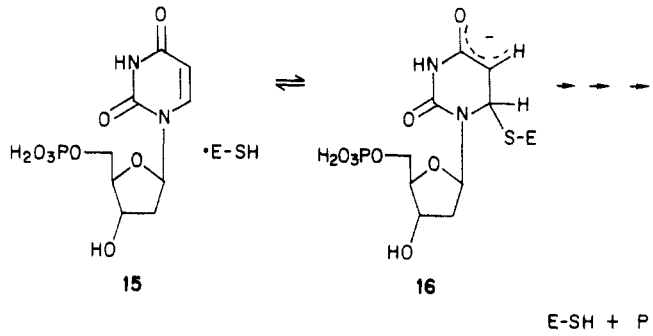
The observed rates of the reaction of the nucleoside diacetate **10** with **5** were determined in acetonitrile by following the time-dependent decrease in the ultraviolet absorption band of **10** at 390 nm. The observed rates of the reaction are listed in Table I for the pseudo-first-order reaction using an excess of the thiol **5**.

From the results in Table I a notable increase in the reaction rate was observed in protic solvents; a 9-fold rate enhancement resulted when 0.05% water was present. At higher levels of water the reaction was much faster. Treatment of the unprotected nucleoside quinone **1b** with a 100-fold excess of methyl mecaptoacetate (**5**) in aqueous buffer (pH 6.8) containing 25% acetonitrile was examined by use of a stopped-flow spectrophotometer. The reaction was completed within 15 ms, the minimum mixing time for the reaction cell. Estimating that these reactions have a $t_{1/2}$ less than 5 ms, under the conditions of the reaction the second-order rate constant exceeds 1×10^3 M^{-1} s^{-1}. From this estimate it appears that thiol addition to quinones in neutral aqueous solution is much faster ($\sim 10^3$) than the corresponding Michael addition to simple α,β-unsaturated carbonyls or to quinones in nonaqueous solvents.

Discussion

The principal question addressed in these model studies is the mechanism for the inactivation of thymidylate synthase by compound **1a** (Scheme I). Kinetic results for the inactivation suggest a high-affinity enzyme–inhibitor complex as the initial product.[4] Presuming that the structure of this complex allows for addition of the active-site thiol to any of the three available reaction sites on the quinone or carbon-6 of the pyrimidine ring, the chemical reactivity of the various α,β-unsaturated carbonyl groups could be the primary factor in the enzyme inactivation. An alternate mode of inactivation of thymidylate synthase by **1a** would be by a simple redox reaction wherein the quinone is reduced with concurrent oxidation of the thiol groups in thymidylate synthase. The latter is unlikely, since activity was not restored when the inactivated enzyme complex was dialyzed in the presence of mercaptoethanol. Such treatment is used routinely to reactivate thymidylate synthase after standing in the presence of oxygen.

Scheme II. First Catalytic Step in Thymidylate Synthase Catalysis[5]



The first question posed in this study is the regioselectivity of the reaction of thiols with a simple quinone (**6**) as compared to the quinone nucleoside **10**. The reaction of methyl mercaptoacetate (**5**) with either quinone could result in addition to the ortho, meta, or para carbons of the quinone ring. An additional reaction site in the quinone **10** is adduct formation at carbon-6 of the uracil ring.

Wilgus and co-workers[8] reported the formation of the meta and para adducts in a ratio of 5:1 from the reaction of a heterocyclic thiol with phenyl-*p*-benzoquinone (**6**) in a protic solvent. We found that the reaction of **6** with methyl mercaptoacetate (**5**) in a polar aprotic solvent gave the meta and para adducts (**8**, **7**) however, in a 1:1 ratio.

Nucleophilic addition to conjugatively substituted benzoquinones has been examined by Rozeboom and coworkers[9] who concluded that consideration of the energy levels for the lowest unoccupied molecular orbital (LUMO) of substituted quinones leads to more accurate prediction of regioselectivity than do resonance arguments. On this basis they suggested that donor substituents and conjugating substituents on *p*-benzoquinones give regioselective nucleophilic addition in the order of meta > para > ortho. Although no preference for meta addition was noted in the reaction of the thiol **5** with phenyl-*p*-benzoquinone (**6**) in acetonitrile, the addition of **5** to the nucleoside quinone **10** confirmed the prediction based on the LUMO, wherein a 2:1 ratio of the meta/para adducts (**12**, **11**) was observed. In contrast, the principal addition products formed by using ethyl acetate were the ortho adducts of both **6** (**9**) and **10** (**13**). Wilgus and co-workers[8] noted formation of ortho adducts only when the quinone was substituted with an electron-accepting group such as acetyl or a carboxylate ester. No obvious explanation is apparent to account for the solvent-dependent reversal in regioselectivity in the addition of the thiol **5** to the quinones **6** and **10**. Another unusual finding in this study was the formation of the reduction product **14** from the reaction of **10** and **5** in ethyl acetate.

The second point raised in these studies is the relative

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Table II. Rate Constants For the Reaction of Thiols and Thymidylate Synthase with α,β-Unsaturated Carbonyls

substrate	reagent	conditions	k_2, M^{-1} s^{-1}	k_{cat}, s^{-1}
γ-methyl-Δ-$^{\alpha\beta}$-butenolide[a]	cysteine	pH 7.4, 25 °C	0.037	
methyl acrylate[b]	3-mercaptopropionic acid	pH 8.1, 30 °C	0.11	
quinone **10**	methyl mercaptoacetate	acetonitrile, 37 °C	0.53	
		0.05% aq acetonitrile, 37 °C	4.7	
2-methylnaphthoquinone[c]	glutathione	70% ethanol	0.14	
quinone **1b**	methyl mercaptoacetate	75% aq acetonitrile, 25 °C, pH 6.8	$>1 \times 10^3$ [d]	
	thymidylate synthase[e]	pH 6.8, 30 °C	5×10^2	
phenyl-*p*-benzoquinone (**6**)	thymidylate synthase[e]	pH 6.8, 30 °C	2.6×10^3	
quinone **1a**	thymidylate synthase[e]	pH 6.8, 30 °C	3.2×10^4	0.065
2′-deoxyuridine 5′-phosphate	thymidylate synthase[f]	pH 7.4, 30 °C	$>7.6 \times 10^5$	3.7

[a] Reference 16. [b] Reference 17. [c] Reference 18. The rate was calculated from the data and presumes a bimolecular reaction. [d] Calculated from the reaction of the quinone and thiol in a ratio of 1:100 where the half-life was less than 0.005 s. [e] Reference 4. [f] The value given is for product formation in the enzyme-catalyzed reaction from the ratio k_{cat}/K_m determined from the specific activity (3.2 μM min^{-1} mg^{-1}) and a K_m of 5 μM. This is the value if thiol addition is the rate-limiting step in the reaction.

reaction rates for the addition of thiols to quinones as compared to the addition to α,β-unsaturated carbonyls. The proposal that thymidylate synthase is inactivated by thiol addition to carbon-6 of the pyrimidine ring of compound **1a** (pathway a, Scheme I) is based on the chemical mechanism for the first catalytic step in the reaction (Scheme II). In this mechanism the protein thiol group at the active site of the complex **15** adds to the α,β-unsaturated carbonyl moiety represented by carbons 4, 5, and 6 of the uracil ring (Scheme II).[5] There is ample evidence of this Michael-type nucleophilic addition to carbon-6 of the nucleotide **15**. By use of models, it was observed that exchange of the proton on carbon-5 or dehalogenation of 5-halouracil derivatives is catalyzed by base[10] or thymidylate synthase[11] and proceeds through covalent addition at carbon-6. 5′-Thio-5′-deoxyuridine in neutral aqueous solution exists in equilibrium with the cyclized product resulting from thiol addition to carbon-6.[12] Other nucleophilic addition reactions to carbon-6 in uracil and 5-substituted uracil derivatives involve bisulfite,[13] hydroxylamine,[14] and thiol.[15]

Kupchan and co-workers[16] examined the bimolecular reaction of cysteine with an α,β-unsaturated lactone at pH 7.4 and found a second-order rate of 0.037 M^{-1} s^{-1}. Similarly, Friedman and co-workers[17] found a rate constant of 0.11 M^{-1} s^{-1} for the addition of 3-mercaptopropionic acid to methyl acrylate at pH 8.1 (Table II).

In attempts to determine the rate of thiol addition to phenyl-*p*-benzoquinone (**6**) we found the reaction in aprotic solvents to be sensitive to both light and oxygen. However, when an aprotic solvent was used, the reaction of methyl mercaptoacetate (**5**) with the nucleoside **10** was neither light nor air sensitive. A calculated second-order rate constant of 0.53 M^{-1} s^{-1} was determined by using dry acetonitrile. This is slightly faster than the bimolecular rate calculated from the data reported by Nickerson and co-workers[18] for the reaction of glutathione with 2-methylnaphthoquinone in 70% ethanol (Table II).

The kinetic results for the inactivation of thymidylate synthase by the phosphate **1a** support the mechanism for the enzyme inactivation by **1a** as proceeding in a unimolecular reaction via the reversible enzyme–inhibitor complex (Scheme I). The second-order rate constant for the reaction calculated from k_{inact}/K_i is 32×10^3 M^{-1} s^{-1}. Neither the nucleoside **1b** nor phenyl-*p*-benzoquinone (**6**) has any detectable affinity for the active site of thymidylate synthase; however, they both inactivated the enzyme in a bimolecular reaction with second-order rate constants of 0.5×10^3 and 2.6×10^3 M^{-1} s^{-1}, respectively, at pH 6.8 (Table II). These rates are similar to that estimated for the reaction of **1b** with **5** in aqueous media.

The apparent bimolecular rate constant for the enzymatic reaction catalyzed by thymidylate synthase is 7.6×10^5 M^{-1} s^{-1} (Table II). Given the generally accepted mechanism proceeding via enzyme thiol addition at carbon-6 of the substrate (Scheme II), this value is a minimum rate for the addition reaction and applies only if this is the rate-limiting step in the reaction. The corresponding value for the inactivation of the enzyme by **1a** is 20 times slower than the catalyzed reaction.[4] However, the kinetic results do not distinguish nucleophilic addition to carbon-6 (pathway a, Scheme I) or to the quinone ring (pathway b, Scheme I).

The initial conclusion from these comparative rates is that thiol addition to carbon-6 in **1a** is not precluded if the catalytic features employed in the enzyme reaction also

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play a role in the reaction of the complex of enzyme·**1a**. However, the rates of enzyme inactivation by **1a**, **1b**, and phenyl-*p*-benzoquinone (**6**) also are in the range estimated for thiol addition to quinones in aqueous media. On this basis it is reasonable to suggest that compound **1a** inactivates thymidylate synthase by a general mechanism consistent with pathway b in Scheme I. That is, **1a** acts as an affinity label rather than a mechanism-based inhibitor. This conclusion is based on the regioselectivity of the reaction and a comparison of the reaction rates. In the former, no evidence was obtained for the Michael addition of the model thiol **5** to carbon-6 of the pyrimidine ring in **10**. The primary reaction in polar media was addition to the meta and para positions of the quinone ring in agreement with literature precedent for substituted quinones. The second argument for this proposal is that the second-order rates for the reaction of the enzyme thiol or the model thiol **5** to either phenyl-*p*-benzoquinone (**6**), the nucleotide **1a**, or its nucleoside **1b** in polar media are comparable and 1000 times faster than thiol addition to simple α,β-unsaturated carbonyls as represented by carbons 4, 5, and 6 of the pyrimidine ring.

Experimental Section

Materials and Methods. Fisher Scientific HPLC grade acetonitrile, Aldrich dimethoxybenzene and acetic anhydride, and 5-iodo-2′-deoxyuridine from U.S. Biochemical Corp. were used as received. All other materials from commercial suppliers were purified prior to use. Solvents were distilled; ethyl acetate and acetonitrile were stored over 3-Å molecular sieves after distillation. For the experiments in which "wet" acetonitrile was required, doubly deionized water was added to the dried acetonitrile. Dry methanol and ethanol were distilled over magnesium metal. Pyridine was distilled and sequentially dried over 3-Å molecular sieves. Phenyl-*p*-benzoquinone (Pfalz and Bauer) was recrystallized from petroleum ether (bp 35–60 °C) yielding yellow platelike crystals, mp 111–112 °C (uncorrected). Methyl mercaptoacetate was distilled under reduced pressure through a 12-in. Vigreux column and stored under argon. All glassware for synthetic reactions was dried in an oven at 130 °C for 24 h and cooled with a stream of nitrogen prior to use. Silica gel 60 was used in column chromatography. Buffers were prepared in doubly deionized water with AR grade chemicals. ^{1}H NMR spectra were determined on the following spectrometers: Varian T-60A equipped with a T7 Fourier transform accessory, Varian FT-80A, and Nicolet 1280. ^{13}C NMR spectra were obtained from a Nicolet 1280 spectrometer. ^{1}H and ^{13}C NMR spectra were taken in either deuterated acetonitrile (CD_3CN) or deuterochoroform ($CDCl_3$), as noted. Chemical shifts are expressed in parts per million downfield from the internal standard, tetramethylsilane (Me_4Si). Both broad-band-decoupled ^{13}C and attached proton test (APT) ^{13}C NMR spectra were recorded. The computer program used to obtain the APT spectra was written by Ashraf N. Abdel-Sayed at the University of Illinois Medicinal Chemistry Department. Mass spectra were recorded on a Reibermag GC-MS instrument. High-resolution mass spectra were recorded on a Varian MAT 112SA mass spectrometer. UV spectral data were collected on a Durram, Carey 118, Carey 219, and Hewlett-Packard 8451A diode array spectrophotometers. The pH of the buffer solutions was measured with an Altex (Beckman) 71 pH meter. IR spectra were taken on a Nicolet MXI FTIR instrument. The HPLC system used was a Waters Associates unit with an Autolab System I computing integrator using a Whatman Partisil PXS 10/25 ODS-2 reverse-phase column. The mobile phase was 50:50 acetonitrile/water adjusted to pH 4.5 with acetic acid. TLC analyses were performed with Whatman K6F silica gel plates. Microanalyses were obtained from a Hewlett-Packard 185B at the Department of Medicinal Chemistry, University of Kansas.

2-Phenyl-5-[(carbomethoxymethyl)thio]hydroquinone (7a) and 2-Phenyl-6-[(carbomethoxymethyl)thio]hydroquinone (8a). Phenyl-*p*-benzoquinone (**6**, 92.1 mg, 0.5 mmol) was dissolved in 20 mL of acetonitrile (dried over activated 3-Å molecular sieves) under nitrogen. Methyl mercaptoacetate (**5**, 0.447 mL, 5 mmol) was added in one portion at 25 °C. The yellow solution was stirred under nitrogen at 25 °C until the yellow color was gone. The acetonitrile and excess methyl mercaptoacetate were removed by blowing a stream of nitrogen into the reaction mixture. The residue was chromatographed on Florasil with 10% hexane in ethyl acetate. The mixture of products **7a** and **8a** was isolated as an oil: UV λ_{max} (CH_3CN) 318 nm; ^{1}H NMR ($CDCl_3$) 7.23–7.52 (m, 10 H), 7.11 (s, 1 H), 6.99 (d, 1 H, J = 3.1 Hz), 6.90 (s, 1 H), 6.84 (d, 1 H, J = 3.1), 3.72 (s, 3 H), 3.69 (s, 3 H), 3.52 (s, 2 H), 3.51 ppm (s, 2 H); ^{13}C (CD_3CN) 171.90 and 171.81 (carbonyl carbon of thiol group), 151.16, 150.79, 148.30 and 147.71 (C1, C4), 138.92 and 138.48 (C7-Ph), 131.28 and 130.45 (C2), 130.08 (C9-Ph), 130.02 (C6 meta isomer), 129.04 (C8- and C10-Ph), 128.15 (C5 para isomer), 121.55 (C6 para isomer), 121.08 (C3 para isomer), 119.78 (C5 meta isomer), 117.78 (C3 meta isomer), 53.29 and 53.20 (OCH_3), 38.59 and 37.57 ppm (CH_2 thiol group).

2-Phenyl-5-[(carbomethoxymethyl)thio]-1,4-diacetoxybenzene (7b) and 2-Phenyl-6-[(carbomethoxymethyl)thio]-1,4-diacetoxybenzene (8b). A mixture of **7a** and **8a** was prepared as described on a 1 mmol scale. The reaction mixture was concentrated under reduced (aspirator) pressure. Acetic anhydride (0.5 mL, 5.3 mmol) and 1 drop of sulfuric acid were added to the residue under nitrogen. The reaction was stopped after 1.5 h, and 20 mL of ice water was added to the reaction mixture. The product separated as an oil and was extracted with ethyl acetate (3 × 25 mL). Solvent was removed in vacuo and the crude mixture of **7b** and **8b** chromatographed on silica gel with 30% ethyl acetate in hexane eluant. The products were isolated as an unresolvable mixture (217 mg, 58% yield from phenyl-*p*-benzoquinone): UV λ_{max} (CH_3CN) 246 nm; mass spectrum, m/e (relative intensity) 376 (M + 2, 2), 375 (M + 1, 4.7), 374 (M, 24), 333 (11), 332 (48), 291 (18), 290 (100), 258 (50), 230 (40), 229 (15), 184 (10), 43 (3); ^{1}H NMR ($CDCl_3$) 7.33–7.39 (m, 10 H), 7.28 (s, 1 H), 7.24 (d, 1 H, J = 2.64 Hz), 7.14 (s, 1 H), 7.04 (d, 1 H, J = 2.68 Hz), 3.73 (s, 3 H), 3.72 (s, 3 H), 3.64 (s, 2 H), 3.63 (s, 2 H), 2.34 (s, 3 H), 2.28 (s, 3 H), 2.08 (s, 3 H), 2.07 ppm (s, 3 H); ^{13}C ($CDCl_3$) 169.5 (thiol carbonyl), 168.82, 168.76, 168.68, and 168.31 (acetyl carbonyls), 148.43, 147.62, 145.54, and 144.30 (C1-, C4-hydroquinone), 136.90 and 136.60 (C7-Ph), 136.23 and 135.88 (C2), 130.41 (C5 para isomer and C6 meta isomer), 128.78 (C9-Ph), 128.39 (C8-Ph), 127.98 (C10-Ph), 125.97 (C6 para isomer), 124.60 (C3 para isomer), 123.11 (C5 meta isomer), 122.72 (C3 meta isomer), 52.61 (OCH_3), 35.73 and 35.64 (CH_2S thiol group), 20.99, 20.71, and 20.44 ppm (acetyl CH_3's). Anal. Calcd for $C_{19}H_{18}O_6S$ (M_r 374.1) C, 60.95; H, 4.85. Found: C, 60.58; H, 4.99.

2-Phenyl-3-[(carbomethoxymethyl)thio]-1,4-diacetoxybenzene (9b). Phenyl-*p*-benzoquinone (**6**, 184 mg, 1 mmol) was dissolved in 20 mL of dry ethyl acetate under a nitrogen atmosphere. Methyl mercaptoacetate (**5**, 0.224 mL, 265 mg, 2.5 mmol) was added in one portion. The reaction flask was wrapped in aluminum foil, and the solution was stirred under nitrogen at ambient temperature. Within 6 h all the yellow color was gone indicating that the quinone starting material had been consumed. The reaction mixture was left to stir an additional 60 h. To this solution was added acetic anhydride (0.566 mL, 613 mg, 6 mmol) and 1 drop of concentrated sulfuric acid. The reaction mixture was stirred for approximately 2 h. At this time TLC analysis indicated that the reaction was complete. It was then poured over ice (approximately 100 mL) and extracted with ethyl acetate (2 × 50 mL). The organic layers from the extraction were combined and dried over sodium sulfate. After filtration the solvent was removed under reduced pressure. According to TLC a mixture of three products was obtained, and one product was predominant. The mixture of products was chromatographed on silica gel with 30% ethyl acetate in hexane eluant. The fractions containing the major product were combined and concentrated. A ^{1}H NMR spectrum revealed that the desired product **9b** had been isolated, but that it was contaminated with a small amount of the other isomers. From the NMR spectrum it was clear that the minor products were the two previously characterized products, 2-phenyl-5-[(carbomethoxymethyl)thio]-1,4-diacetoxybenzene (**7b**) and 2-phenyl-6-[(carbomethoxymethyl)thio]-1,4-diacetoxybenzene (**8b**). No further attempt was made to isolate and quantitate the minor products. The sample of (**9b**) was further purified by chromatography on silica gel with 25% ethyl acetate in hexane; ^{1}H NMR ($CDCl_3$) 7.38 (m, 5 H), 7.15 (s, 2 H), 3.56 (s, 3 H), 3.11

(s, 2 H), 2.35 (s, 3 H), 1.87 ppm (s, 3 H); ^{13}C NMR ($CDCl_3$) 169.4, 169.2 (carbonyl carbons of the thiol and acetate groups), 150.3 and 146.1 (C1 and C4), 140.15 (C2), 135.6 (C7-Ph), 136.9 and 136.6 (C3), 129.68 (C10-Ph), 128.62 (C12-Ph), 128.33 (C8-Ph), 127.81 (C9- and C11-Ph), 124.37 and 122.57 (C5 and C6). Anal. Calcd for $C_{19}H_{18}O_6S$ (M_r 374.1): C, 60.95; H, 4.85. Found: C, 60.59; H. 4.95.

5-*p*-Benzoquinonyl-2′-deoxyuridine (1b).[4] This was prepared by a modified procedure[19] using ceric ammonium nitrate (904 mg, 1.64 mmol) dissolved in 10 mL of deionized water. A solution of 5-(2,5-dimethoxyphenyl)-2′-deoxyuridine[4] (0.1 g, 0.3 mmol) in 30 mL of acetonitrile was added dropwise to this orange solution. After the reaction was complete according to TLC analysis, the acetonitrile was removed in vacuo, ethyl acetate was added to the orange residue, the solution was passed through Florasil, and **1b** was purified as described in 33% yield (30 mg). The purity of the product was confirmed by elemental analysis and HPLC analysis.[4]

5-*p*-Benzoquinonyl-3′,5′-di-*O*-acetyl-2′-deoxyuridine (10). By use of the same method as described for the synthesis of 5-(*p*-benzoquinonyl)-2′-deoxyuridine, 200 mg (0.45 mmol) of 5-(2,5-dimethoxyphenyl)-3′,5′-di-*O*-acetyl-2′-deoxyuridine was converted to the quinone[4] in 37% yield: ^1H NMR (CD_3CN) 9.25 (s, 1 H, NH), 8.02 (s, 1 H, C6-H), 7.24 (d, 1 H, C6-quinone H, $J_{4,6}$ = 2.42 Hz), 6.82 (d, 1 H, C3-quinone H, $J_{3,4}$ = 10.10 Hz), 6.78 ppm (dd, 1 H, C4-quinone H, $J_{3,4}$ = 10.18 Hz, $J_{4,6}$ = 2.43 Hz).

5-[3-[(Carbomethoxymethyl)thio]-2,5-dihydroxyphenyl]-3′,5′-di-*O*-acetyl-2′-deoxyuridine (12) and 5-[4-[(Carbomethoxymethyl)thio]-2,5-dihydroxyphenyl]-3′,5′-di-*O*-acetyl-2′-deoxyuridine (11). 5-(*p*-Benzoquinonyl)-3′,5′-di-*O*-acetyl-2′-deoxyuridine (**10**, 94 mg, 0.23 mmol) was dissolved in 75 mL of acetonitrile under a nitrogen atmosphere. Methyl mercaptoacetate (**5**, 0.425 mL, 0.504 mg, 4.75 mmol) was added in one portion; the solution remained bright yellow. When the buffer (25 mL of pH 7.4, 55 mM phosphate buffer) was added, a clear colorless solution was formed instantly. This solution was extracted with 75 mL of ethyl acetate, the organic layer was washed twice with 50 mL of water, and the aqueous layer was extracted again with 50 mL of ethyl acetate. The combined ethyl acetate layers were dried over magnesium sulfate and concentrated under reduced pressure. The residue was purified on silica gel with 20% hexane in ethyl acetate. The mixture of products **11** and **12** was obtained in 57% yield: UV (CH_3CN) λ_{max} 265, 316 nm, λ_{min} 250, 306 nm; mass spectrum, *m/e* (relative intensity) 524 (M^+, 3.1), 324 (15), 292 (11), 264 (4.3) 220 (9.2), 81 (74), 43 (100); ^1H NMR ($CDCl_3$) 7.83 (s, 1 H, C6-H), 7.78 (s, 1 H, C6-H), 7.06 (s, 1 H, para isomer C6-hydroquinone), 6.90 (d, 1 H, J = 2.88 Hz, meta isomer C6-hydroquinone), 6.73 (s, 1 H, para isomer C3-hydroquinone), 6.80 (d, 1 H, J = 2.83 Hz, meta isomer C4-hydroquinone), 6.34 (m, 2 H, C1′-H both isomers), 5.24 (d, 2 H, J = 6 Hz, C3′-H both isomers), 4.36 (m, 2 H, C4′-H both isomers), 4.31 (s, 4 H, C5′-H both isomers), 3.72 (s, 3 H, OCH_3 para isomer), 3.70 (s, 3 H, OCH_3 meta isomer), 3.56 (s, 2 H, CH_2S meta isomer), 3.54 (s, 2 H, CH_2S para isomer), 2.12 (s, 6 H, acetyl CH_3's both isomers), 1.96 (s, 3 H, acetyl CH_3 para isomer), 1.93 ppm (s, 3 H, acetyl CH_3 meta isomer); ^{13}C NMR ($CDCl_3$) 171.45 (C=O thio ester), 170.88, 170.56 (C=O acetyl), 164.13, 163.43 (C4), 151.31, 149.71, 149.42, and 147.95 (C2-, C5-hydroquinone), 147.51 (C2), 140.42 (C6), 125.23, 123.68, 121.93, 120.62, 120.11, 120.05, 118.49, and 115.86 (C3, C4, C1 and C6 of hydroquinone), 113.27, 111.73 (C5), 85.63, 85.45 (C1′), 82.63, 82.48 (C4′), 74.40, 74.20 (C3′), 64.06, 64.88 (C5′), 53.05, 53.00 (OCH_3-thiol ester), 38.33, 37.92 (CH_2S), 37.66, 37.46 (C2′), 20.93, 20.56 ppm (acetyl CH_3). Anal. Calcd for $C_{22}H_{24}N_2O_{11}S$ (M_r 524.1): C, 50.38; H, 4.61; N, 5.34. Found: C, 50.70; H, 4.90; N, 5.30.

5-[6-[(Carbomethoxymethyl)thio]-2,5-dihydroxyphenyl]-3′,5′-di-*O*-acetyl-2′-deoxyuridine (13) and 5-(2,5-Dihydroxyphenyl)-3′,5′-di-*O*-acetyl-2′-deoxyuridine (14). 5-(*p*-Benzoquinonyl)-3′,5′-di-*O*-acetyl-2′-deoxyuridine (**10**, 0.1 g, 0.27 mmol) was dissolved in 90 mL of dry ethyl acetate, which was under a nitrogen atmosphere. Methyl mercaptoacetate (**5**) was added in one portion (**5**, 0.24 mL, 285 mg, 2.68 mmol). The clear bright yellow solution was stirred at 25 °C under nitrogen for 6 days. At this time the reaction was not complete according to UV analysis, so another aliquot (0.24 mL, 2.68 mmol) of **5** was added. After a total of 11 days the solvent and excess thiol were removed under vacuum and the pale yellow residue was chromatographed on silica gel with 20% ethyl acetate in hexane as eluant. Two products were obtained in a ratio of 3:2, **14**/**13**. Compound **14** eluted from the column first: UV λ_{max} (EtOAc) 270 nm, sh 320 nm; mass spectrum, *m/e* (relative intensity) 420 (M^+, 20), 220 (60), 81 (100); ^1H NMR ((CD_3COCD_3) 7.85 (s, 1 H, C6-H) 6.69 (m, 2 H, hydroquinone H), 6.31 ppm (t, 1 H, C1′, J = 7 Hz). Compound **13**: UV λ_{max} (CH_3CN) 264.5, 315 nm, λ_{min} 246, 296 nm.; mass spectrum, *m/e* (relative intensity) 524 (M^+, 0.5), 492 (6.6), 324 (3.9), 292 (62.1), 264 (11.7), 250 (6.2), 220 (4.6), 219 (3.7), 218 (2.0), 201 (1.0), 81 (100), 43 (93.8); ^1H NMR ($CDCl_3$) 8.88 (s, 1 H, NH), 7.71 (s, 0.5 H, C6-H), 7.64 (s, 0.5 H, C6-H) 6.97 (s, 1 H, hydroquinone H), 6.94 (s, 0.5 H, hydroquinone H), 6.92 (s, 0.5 H, hydroquinone H), 6.35 (m, 1 H, C1′-H), 3.66 (s, 3 H, OCH_3), 3.35 ppm (m, 1 H, CH_2S); ^{13}C NMR ($CDCl_3$) 171.70, 171.42 (C=O of thiol ester), 170.44, 170.42 (C=O 3′,5′-acetyl) 163.61, 163.46 (C4), 152.47, 152.36, 150.28, and 150.18 (C2-, C5-hydroquinone), 148.43, 148.39 (C2), 141.19, 140.73 (C6), 124.14, 124.11, 121.26, 120.86, 118.88, 118.72, 117.26, and 117.25 (C1-, C3-, C4-, C6-hydroquinone), 110.87, 110.76 (C5), 85.26, 84.93 (C1′), 82.39, 82.16 (C4′), 74.28, 73.88 (C3′), 63.90, 63.80 (C5′), 53.03, 53.01, (OCH_3 of thiol ester), 38.43, 38.25 (SCH_2), 37.86, 37.64 (C2′), 20.91, 20.21, and 20.09 (acetyl CH_3). Anal. Calcd for $C_{22}H_{24}N_2O_{11}S$ (M_r 524.1): C, 50.38; H, 4.61; N, 5.34. Found: C, 50.68; H, 4.89; N, 4.99.

The structure of the hydroquinone **14** was confirmed by oxidation to the corresponding quinone. Compound **14** (62 mg, 0.15 mmol) was dissolved in 20 mL of acetonitrile, and approximately 50 mg of ferric chloride (0.3 mmol) and 2.5 mL of deionized water were added. The reaction was complete within 5 min according to TLC analysis. Ethyl acetate was added to the reaction mixture. This solution was washed with water (3 × 10 mL) and dried over magnesium sulfate. After filtration the solvent was removed in vacuo to yield a orange–yellow solid. This crude product was purified on silica gel using 20% hexane in ethyl acetate eluant to give 5-*p*-benzoquinonyl-3′,5′-di-*O*-acetyl-2′-deoxyuridine (**10**) in 66% yield. The structure was confirmed by use of ultraviolet and mass spectroscopy, ^1H and ^{13}C NMR, and chromatographic comparison to the quinone **10**.

Kinetic Experiments. Absorbance changes (at 390 and 315 nm) for a freshly prepared acetonitrile solution of 5-*p*-benzoquinonyl-3′,5′-di-*O*-acetyl-2′-deoxyuridine (**10**) after addition of an aliquot of freshly prepared solution of methyl mercaptoactate (**5**) were followed at 37 °C on a Carey 118 spectrophotometer. The solvent was deoxygenated before preparation of the solutions. Initial concentrations of quinone were 3.2×10^{-5}, 7.5×10^{-5}, 1.0×10^{-4}, and 1.6×10^{-4} *M*. The amount of water in the acetonitrile was varied by adding known amounts of water to acetonitrile dried over 3-Å seives. The pseudo-first-order rate constants, k_{obsd}, and the calculated second-order rate constant, k_2, are listed in Table I. The absorbance changes (at 390 nm) for freshly prepared solutions of either phenyl-*p*-benzoquinone (**6**) or 5-*p*-benzoquinonyl-2′-deoxyuridine in 0.1 M phospahte buffer (pH 7) after addition of an aliquot of a freshly prepared solution of methyl mercaptoacetate (in acetonitrile) were monitored with a Durrum stopped-flow spectrophotometer at 25 °C.

Acknowledgment. This research was supported by a research grant from the National Cancer Institute (CA 7522) and by a training grant (GM 1341) from the Institute of General Medical Sciences of the National Institutes of Health and administered by the Center for Biomedical Research at this University. We appreciate the cooperation afforded by Professors James Gearian and William Dunn of the Department of Medicinal Chemistry and Pharmacognosy in providing facilities, supplies, and helpful discussion for the completion of the latter part of this work, Dr. Robert Kleps and Mike Matau for use of the Nicolet 1280 spectrometer and for their generous assistance in the Nuclear Magnetic Resonance Laboratory of the Research Resources Center at the University of Illinois, and Pro-

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fessor Earl Huyser of the University of Kansas for his assistance with the stopped-flow experiments.

Registry No. **1b**, 87414-25-3; **5**, 2365-48-2; **6**, 363-03-1; **7a**, 102830-51-3; **7b**, 102830-53-5; **8a**, 102830-52-4; **8b**, 102830-54-6; **9b**, 102851-29-6; **10**, 87414-26-4; **11**, 102830-55-7; **12**, 102851-30-9; **13**, 102830-57-9; **14**, 102830-56-8; 5-(2,5-dimethoxyphenyl)-2′-deoxyuridine, 76756-32-6; 5-(2,5-dimethoxyphenyl)-3′,5′-di-*O*-acetyl-2′-deoxyuridine, 87414-24-2; thymidylate synthase, 9031-61-2; 5-*p*-benzoquinonyl-2′-deoxyuridine-5′-phosphate, 87414-22-0.

Carbocyclic Analogues of 5-Halocytosine Nucleosides[1]

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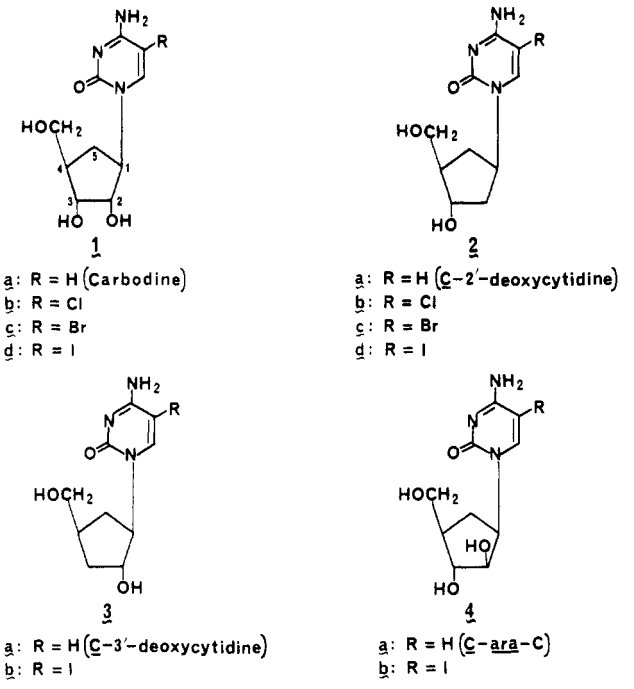
Carbocyclic analogues of 5-halocytosine nucleosides were prepared by direct halogenation of the carbocyclic analogues of cytidine, 2′-deoxycytidine, 3′-deoxycytidine, or ara-C. The 5-chloro and 5-bromo derivatives of the cytidine (carbodine) and of the 2′-deoxycytidine analogues and the 5-iodo derivatives of all four of the cytosine nucleoside analogues were prepared. All of the *C*-5-halocytosine nucleosides, as well as the parent *C*-cytosine nucleosides, were tested against a strain of herpes simplex virus type 1 (HSV-1) that induces thymidine kinase in host cells. Carbodine, 5-bromocarbodine, *C*-2′-deoxycytidine, *C*-5-bromo-2′-deoxycytidine, the four *C*-5-iodocytosine nucleosides, and *C*-ara-C inhibited replication of this strain of HSV-1 in cultured cells. Most of these compounds were tested also against the type 2 virus (HSV-2) in vitro and were active. The greatest activity observed was exerted by *C*-5-iodo-2′-deoxycytidine in inhibiting replication of HSV-1 in L929 cells. In tests against these DNA viruses, carbodine, a ribofuranoside analogue that had been shown previously to be highly active against human influenza A virus in vitro, was the most active compound against HSV-2 and one of the most active compounds against HSV-1 in Vero cells. 5-Bromocarbodine was active against influenza virus, but it was less active than carbodine.

The carbocyclic analogue (**1a**, carbodine) of cytidine was synthesized from the carbocyclic analogue of uridine.[3] Carbodine is active against lymphoid leukemia L1210 in mice,[3,4] is metabolized to the triphosphate in mammalian cells,[5,6] and selectively inhibits the replication of influenza virus (strains A_0/PR/8/34 and A_2/Aichi/2/68) in cultures of kidney cells.[6] However, carbodine was not effective against influenza A virus infections in mice when it was administered intraperitoneally or intranasally.[6] *C*-2′-Deoxycytidine (**2a**) and *C*-3′-deoxycytidine (**3a**), synthesized by the methods used for carbodine,[2,4] were not active (at 200 mg/kg per day, qd 1–9) against L1210 leukemia in mice[2] nor were these compounds active against influenza virus in cultured cells.[6] The carbocyclic analogue (**4a**, *C*-ara-C) of arabinofuranosylcytosine (ara-C) is quite active against L1210 leukemia in mice (T/C, 204% at 150 mg/kg per day, qd 1–9);[7] it also showed modest activity in tests against influenza virus in vitro.[6] Some 5-halo derivatives of these fundamental carbocyclic analogues of the cytosine nucleosides have been prepared and evaluated for antiviral activity. The results of these investigations are described in this report. Earlier, we had synthesized 5-methylcarbodine and reported its modest activity in vitro against influenza virus;[6] and, recently, Herdewijin et al.[8] described the synthesis and antiherpetic activity in vitro of *C*-5-(2-bromovinyl)-2′-deoxycytidine.

Chart I



Chemistry. 5-Chlorocarbodine (**1b**), *C*-5-chloro-2′-deoxycytidine (**2b**), 5-bromocarbodine (**1c**), and *C*-5-bromo-2′-deoxycytidine (**2c**) were prepared by direct halogenation of the parent analogues (**1a** and **2a**). Acetylation of the hydroxyl groups of **1a** or **2a** was allowed to

(1) In this report, as in earlier publications of this series, carbocyclic analogues of nucleosides are named by prefixing *C*- to the names of the corresponding true nucleosides; e.g., *C*-5-halocytosine nucleosides should be read as "carbocyclic analogues of 5-halocytosine nucleosides". However, the carbocyclic analogue (*C*-cytidine) of cytidine was also named *carbodine*;[2] therefore, the *C*-cytidines described in this report are named as derivatives of carbodine. In the Experimental Section, carbocyclic analogues of nucleosides are named, in accordance with the systematic nomenclature employed by *Chemical Abstracts*, as cyclopentyl derivatives of 4-amino-2(1*H*)-pyrimidinone.

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