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5-(α-Bromoacetyl)-2′-deoxyuridine 5′-Phosphate: An Affinity Label for Thymidylate Synthetase

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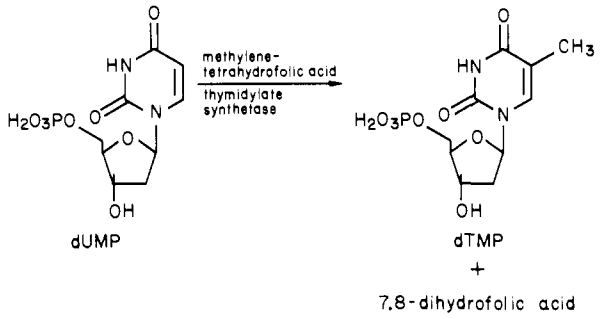
5-(α-Bromoacetyl)-2′-deoxyuridine 5′-phosphate (1) is an active-site-directed irreversible inhibitor of thymidylate synthetase from *Lactobacillus casei*. Analysis of the rate of inactivation of the enzyme in the presence of substrate confirmed the intermediate formation of a reversible enzyme–inhibitor complex.

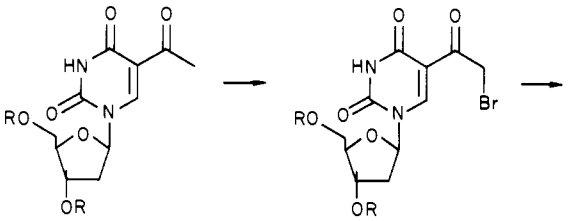
Thymidylate synthetase (EC 2.1.1.45) catalyzes a two-step reductive alkylation of 2′-deoxyuridine 5′-phosphate (dUMP) to give thymidine 5′-phosphate (dTMP).[1] Clinical



dUMP dTMP + 7,8-dihydrofolic acid

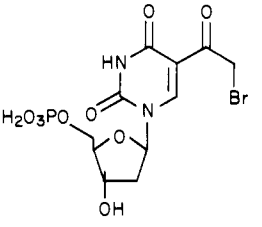
control of cancer growth and viral infection by inhibition of this enzyme has been realized using analogues of either the substrate or product substituted in the 5 position with strong electron-withdrawing groups such as fluoro or trifluoromethyl.[2] In addition to the inherently high enzyme affinity noted for 5-fluoro- and 5-(trifluoromethyl)-dUMP, they irreversibly inactivate thymidylate synthetase.

Recent studies of the mechanism of the first step in thymidylate synthetase catalysis have suggested that the sequence of the reaction is the addition of a cysteine SH group at the active site of the enzyme to carbon-6 of the pyrimidine ring in the substrate dUMP.[3,4] Subsequent steps in the reaction are the addition of the ES covalent complex to the cofactor, followed by rearrangement of the ternary complex to give the product dTMP and the oxidized cofactor 7,8-dihydrofolic acid.[1] A detailed analysis of the reaction requires the interaction of a second nucleophilic group at the active site that functions in abstraction of the carbon-5 proton of the substrate. With this background, the title compound (1) was designed as a mechanism based affinity label for thymidylate synthetase. Three features were embodied in this design: (1) substrate analogy, which should promote enzyme specificity; (2) the carbonyl (acetyl) at carbon-5 of the substrate, because of its inherent electron affinity, should enhance the Michael addition reaction whereby the enzymatic cysteine SH group adds to carbon-6 of the substrate; and (3) a bromo substituted α to a carbonyl group is recognized as a chemically reactive function that interacts with nucleophiles to give covalent bond formation. Thus, 5-(α-bromoacetyl)-2′-deoxyuridine 5′-phosphate (1) could be a



2, R = COC_6H_4-*p*-CH_3
4, R = H

3, R = COC_6H_4-*p*-CH_3
5, R = H



1

substrate competitive inhibitor of thymidylate synthetase by virtue of its analogy to the substrate, the active-site cysteine SH group could reversibly add to carbon-6, and, finally, a second active-site nucleophile could displace the α-bromo group to give a covalent enzyme–inhibitor complex and the resultant enzyme inactivation. Preliminary results have been reported that suggest 1 is an affinity labeling reagent for thymidylate synthetase.[5]

The initial approach to the synthesis of 5-(α-bromoacetyl)-2′-deoxyuridine 5′-phosphate (1) was by bromination of the protected 5-acetyl nucleoside[6] 2 in acetic acid. Under similar conditions, 5-bromouracil is formed in 95% yield from 5-acetyluracil.[7] However, the protected nu-

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Scheme I

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [EI] \xrightarrow{k_2} [EI^*]$$

$$E + S \underset{k_{-SI}}{\overset{k_{SI}}{\rightleftharpoons}} [ES]$$

cleoside **2** gave a 45% yield of the monobrominated product (**3**) that was difficult to separate from α,α-dibromoacetyl nucleoside. A more direct route, bromination of 5-acetyl-2′-deoxyuridine (**4**) in a polar solvent mixture gave 5-(α-bromoacetyl)-2′-deoxyuridine (**5**) in reasonable yield. Phosphorylation of the nucleoside **5** using the procedure of Sowa and Ouchi[8] afforded 5-(α-bromoacetyl)-2′-deoxyuridine 5′-phosphate (**1**), which was resolved using an acidic buffer on DEAE-cellulose.

Compound **5**, the α-bromoacetyl nucleoside, was stable at pH 4.4 in ammonium formate buffer. However, slow decomposition at pH 7.2 in 0.1 M phosphate was observed by high-pressure LC resolution on a reverse-phase column; the half-life at 25 °C was estimated at 25 h. A solution of **5** in dimethyl-d_6 sulfoxide for NMR analysis showed an upfield shift of the α-methylene singlet from δ 4.8 to 4.65, followed after 24 h with loss of the α-methylene resonance and appearance of aldehydic proton resonance at δ 9.1. An analogous dimethyl sulfoxide oxidation of α-bromoacetophenone is reported to give the respective α-ketoaldehyde.[9]

Compound **1** was a competitive inhibitor of thymidylate synthetase with affinity for the enzyme that approximates that of the substrate dUMP; the calculated inhibition constant was 4.1 μM.[5]

Analysis of irreversible inhibition was done by estimating the velocity of enzyme loss over a given time period.[10] When the inhibitor was preincubated with the enzyme in the absence of substrate and the remaining activity estimated at various times, the rate of inactivation of the enzyme followed pseudo-first-order kinetics using concentrations of inhibitor at least 50 times that of enzyme; both time- and concentration-dependent inactivation were noted.[5] The rate constant for inactivation was 0.15 min^{-1}.

Thus, three lines of evidence support the proposal that the title compound (**1**) is an affinity label for thymidylate synthetase:[11] (1) it is a reasonably potent inhibitor that is competitive with substrate dUMP; (2) time-dependent inactivation followed the kinetic expression for conversion of a reversible EI complex to an irreversible EI* complex (Scheme I); (3) finally, the inactivation was dependent on the concentration of the inhibitor.

Further verification of active-site labeling as the mechanism for inactivation of this enzyme by compound **1** utilizes the protection against inactivation afforded by the substrate. Figure 1 shows the results of the incubation of **1** and the enzyme in the presence of substrate over a 10-min period. The observed rate of inactivation of the enzyme by **1** in the presence of the substrate is substantially less than the observed rate of inactivation without substrate.[5] These results would be expected if both **1** and the substrate compete for binding to the active site as shown in Scheme I.

An extension of the analysis of this inhibition as derived by Kitz and Wilson[10b] can be used to quantitatively assess the effect of substrate on the rate of inactivation. The derived expression (eq 1) shows that a plot of the reciprocals of the observed rate vs. inhibitor concentration gives the rate of inactivation as the reciprocal of the intercept.

$$\frac{1}{k_{obsd}} = \left[\frac{K_i[S]}{K_m k_2} + \frac{K_i}{k_2}\right]\frac{1}{[I]} + \frac{1}{k_2} \qquad (1)$$

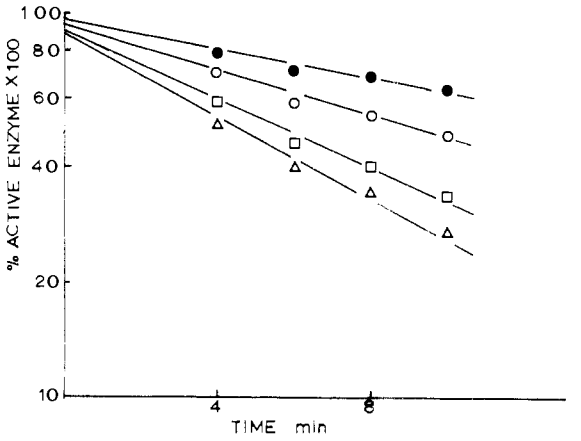


Figure 1. Semilog plot of the percentage of remaining enzyme activity vs. time of incubation for thymidylate synthetase in the presence of 1.2 μM 2′-deoxyuridine 5′-phosphate and varying amounts of affinity label, 5-(α-bromoacetyl)-2′-deoxyuridine 5′-phosphate: 0.4 (●), 1.0 (○), 2.0 (□), 3.0 μM (△).

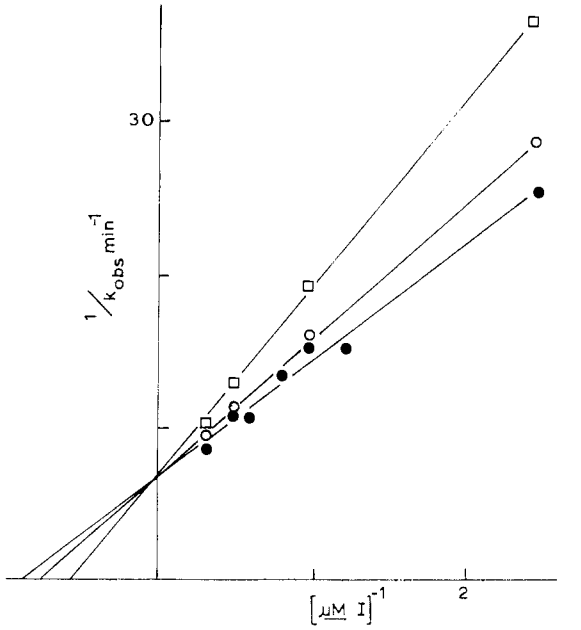


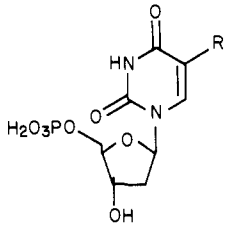
Figure 2. Double-reciprocal plot of the observed rate of inactivation of thymidylate synthetase vs. concentration of 5-(α-bromoacetyl)-2′-deoxyuridine 5′-phosphate in the absence of substrate (●) and in the presence of 1.2 (○) and 2.0 μM (□) 2′-deoxyuridine 5′-phosphate.

Furthermore, the intercept should be the same regardless of substrate concentration. The plot in Figure 2 for the inactivation of the enzyme in the absence and presence of substrate shows the expected pattern of decreasing inactivation rate with increasing substrate concentration (dUMP = 0, 1.2, and 2 μM).

Equation 1 also is valid for the determination of the K_m (enzyme–substrate dissociation constant) and for the K_i, which relates $k_2 + k_{-1}/k_1$. The former value found to be 5.0 μM from a Lineweaver–Burk plot[5] agrees well with the values determined in this study; the respective K_m values are 4.99 and 4.97 μM for substrate concentrations of 1.2 and 2.0 μM.

The inhibition constant for **1** from an earlier study was 4.1 μM;[5] this is an estimate of the enzyme–inhibitor dissociation constant, as derived for a Lineweaver–Burk plot. Using eq 1 relating K_i as the ratio, $k_2 + k_{-1}/k_1$, values of 1.1, 1.0, and 1.3 μM were determined from the respective slopes in Figure 2, where substrate concentrations of 0, 1.2,

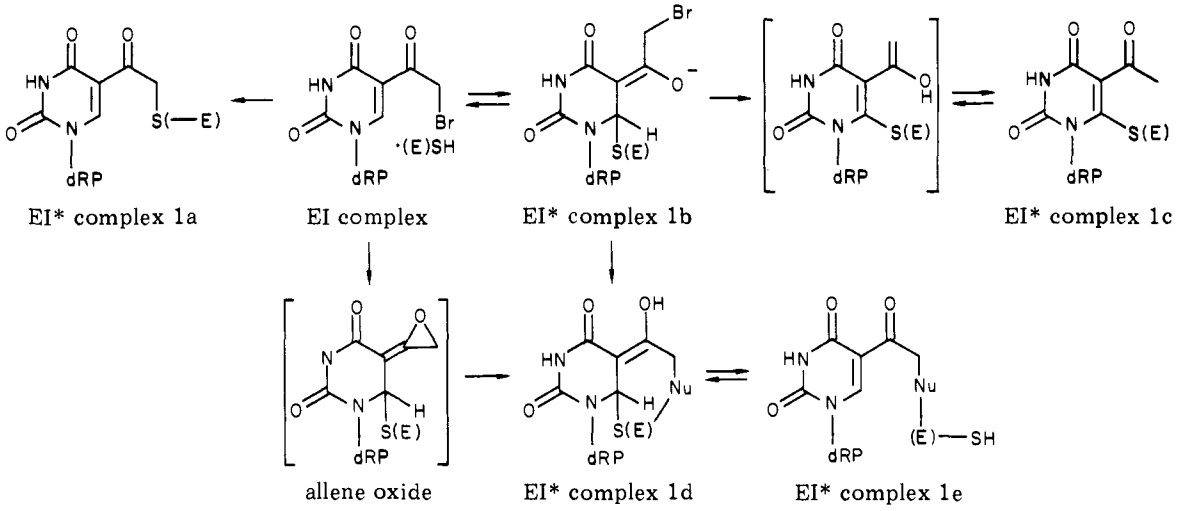
Table I. Irreversible Inhibitors of Thymidylate Synthetase



compd	R	K_I, μM	k_2
5-(α-bromoacetyl)-dUMP (1)	$COCH_2Br$	4.1	0.15 min^{-1}
5-[(iodoacetamido)methyl]-dUMP[a]	CH_2COCH_2I	68	0.057 min^{-1}[b]
5-nitro-dUMP	NO_2	0.03[c]	7 × 10^5 L mol^{-1} s^{-1}[d]
5-fluoro-dUMP	F	0.014[e]	2 × 10^7, 2 × 10^8 L mol^{-1} min^{-1}[f]
5-(trifluoromethyl)-dUMP	CF_3	0.039[e]	
5-formyl-dUMP	CHO	0.017[e] (0.01[g])	

[a] Barfknecht, R. L.; Huet-Rose, R. A.; Kampf, A.; Mertes, M. P. *J. Am. Chem. Soc.* **1976**, *98*, 5041-5043. [b] The enzyme source was Ehrlich ascites tumor. [c] Mertes, M. P.; Chang, C. T.-C.; DeClercq, E.; Huang, G. F.; Torrence, P. F. *Biochem. Biophys. Res. Commun.* **1978**, *84*, 1054-1959; Matsuda, A.; Wataya, Y.; Santi, D. V. *Biochem. Biophys. Res. Commun.* 1978, *84*, 654-659. [d] Chang, C. T.-C.; Torrence, P. F.; Mertes, M. P., unpublished results. [e] Wataya, Y.; Santi, D. V.; Hansch, D. *J. Med. Chem.*, **1977**, *20*, 1469-1473. [f] Santi, D. V.; McHenry, C. S.; Sommer, H. *Biochemistry* **1974**, *13*, 471-480; Myers, C. E.; Young, R. G.; Chabner, B. A. *J. Clin. Invest.* **1975**, *56*, 1231-1238; Danenberg, P. V.; Danenberg, K. D. *Biochemistry* 1978, *17*, 4018-4024. [g] Ehrlich ascites tumor enzyme. Kampf, A.; Barfknecht, R. L.; Shaffer, P. J.; Osaki, S.; Mertes, M. P. *J. Med. Chem.* **1976**, *19*, 903-908.

Scheme II[a]



[a] dRP = 2′-deoxyribose 5′-phosphate

and 2.0 μM were present during incubation.

Mechanism-based irreversible inactivation of thymidylate synthetase has been reported for the 5-fluoro, 5-(trifluoromethyl), 5-nitro, and 5-carboxaldehyde derivatives of the substrate (Table I). One of the most potent irreversible inhibitors, 5-nitro-dUMP, has a K_i of 0.03 μM;[12,13] the second-order rate constant for inactivation of thymidylate synthetase is estimated[14] to be 7 × 10^5 L mol^{-1} s^{-1}.

Another inhibitor that has been examined for the rate of inactivation of thymidylate synthetase from Ehrlich ascites tumor is 5-[(iodoacetamido)methyl]-2′-deoxyuridine 5′-phosphate.[15] It is a reasonably weak competitive inhibitor of the enzyme; however, it irreversibly inactivates the tumor enzyme at one-fourth the rate that 1 inactivates the bacterial enzyme.

Although these results clearly demonstrate that 5-(α-bromoacetyl)-2′-deoxyuridine 5′-phosphate (**1**) is an active-site-directed irreversible inhibitor of thymidylate synthetase, the mechanism of the inhibition is unknown. Several possibilities are noted in Scheme II that are compatible with the results. The first product (**1a**) resulting from bromide displacement by an active-site nucleophile (SH) is reasonable, as is the EI* complex **1c**, which could arise by intramolecular abstraction of the carbon-6 proton by the oxygen anion in **1b** and elimination of bromide. This internal redox reaction would give the irreversible adduct wherein the enzyme is bound to an sp^2 carbon at position 6 of the substrate. Thirdly, as noted in the design of this compound, a second nucleophile at the active site could undergo an intramolecular displacement of bromide to give **1d**, which could exist in equilibrium with **1e**. An alternative route to **1d** requires formation of a highly reactive allene oxide, formed by oxygen anion displacement of bromide. Substituted allene oxides have been synthesized and characterized.[16] Based on model reactions of these allene oxides, the most likely decomposition of the allene oxide if formed as shown in Scheme II would take the form of nucleophilic attack at the terminal carbon of the allene oxide, leading to elimination of the first nucleophile. This would result in a covalently bound species, **1d**.

Experimental Section

Thymidylate synthetase purified from methotrexate-resistant *Lactobacillus casei* was purchased from the New England Enzyme

Center, Tufts University, at a specific activity of 1.03 μmol of TMP formed min^{-1} (mg of protein)$^{-1}$ using the radioisotope assay. The enzyme was activated by dialysis for 4 days at 4 °C against 0.1 M potassium phosphate (pH 6.8) containing 40 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, Calif., and diluted with cold substrate purchased from Sigma Chemical Co., St. Louis, to give a specific activity of 500 μCi/μmol. The cofactor, *dl*-tetrahydrofolic acid, was also purchased from Sigma Chemical Co.

1-(3,5-Di-*O*-*p*-toluoyl-2-deoxy-β-D-ribofuranosyl)-5-(bromoacetyl)uracil (3). The protected nucleoside **2** (1.5 g, 3.0 mmol) was heated to dissolve in 80 mL of glacial acetic acid, and 0.15 mL (0.47 g, 3.0 mmol) of bromine in 60 mL of glacial acetic acid was added dropwise. The solution was stirred for approximately 18 h and then slowly poured onto 600 mL of crushed ice and water. The resulting white precipitate was filtered and chromatographed on a silica gel column (30 × 6 cm; chloroform as eluent). Purity of the fractions were shown by high-pressure LC on an analytical silica gel column (5% methanol–chloroform at 0.8 mL/min). Retention times were as follows: **2**, 4.25 min; **3**, 3.65 min; side product, 3.25 min. Compound **3** was isolated in 46% yield (0.8 g): mp 191–192 °C; NMR ($CDCl_3$) δ 2.4 (d, 6, CH_3-Ph), 4.5 (s, 2, $-CH_2Br$), 6.25 (t, 1 anomeric H), 8.6 (s, 1, H_6), 8.8 (br s, 1 H_3). Anal. ($C_{27}H_{25}BrN_2O_8$; M_r 585.4) C, H, N.

The side product thought to be the dibromoacetyl derivative was isolated before **3** from the preparative column run on the product mixture: mp 148–150 °C; NMR ($CDCl_3$) δ 2.35 (d, 6, CH_3-Ph), 4.7 (s, 3, $H_{3'}$, $H_{5'}$), 6.35 (t, 1, anomeric H), 7.6 (m, 8.7, Ar toluoyl and $-CHBr_2$), 8.8 (s, 1, H_6), 9.75 (s, 1, H_3). Anal. ($C_{27}H_{24}Br_2N_2O_8$; M_r 664.3) H, N; C: calcd, 48.82; found, 49.50.

5-(Bromoacetyl)-2′-deoxyuridine (5). 5-Acetyl-2′-deoxyuridine[6] (**4**; 1.28 g, 4.74 mmol) was suspended in approximately 20 mL of dry chloroform to which had been added 2 mL of dry dimethylformamide and 5 mL of dry methanol to aid dissolution. Bromine (0.28 mL, 4.74 mmol) was added dropwise to the stirred solution under an argon atmosphere. During the course of 5 h, a white solid precipitated from the reaction mixture and was filtered to yield 1 g (60%) of crude **5**. An analytical sample was prepared for elemental analysis by chromatographing 0.1 g on silica gel (7 mm × 21 cm column; 10% methanol–chloroform as eluent): mp 155–168 °C dec; UV (H_2O) λ_{max} 290 nm (ε 13 600), λ_{min} 230 (ε 10 000); UV (0.01 M HCl) λ_{max} 290 nm (ε 13 600), λ_{min} 230 (ε 10 000); UV (0.001 M NaOH, pH 11.5) λ_{max} 290 nm (ε 10 200); NMR (Me_2SO-d_6) δ 2.2 (t, 2, CH_2), 4.78 (s, 2, CH_2Br), 6.18 (t, 1, H_1), 8.88 (s, 1, H_6), 11.72 (s, 1, NH). Anal. ($C_{11}H_{13}BrN_2O_6$; M_r 349.1) C, H, N.

5-(Bromoacetyl)-2′-deoxyuridine 5′-Phosphate (1). The procedure of Sowa and Ouchi[8] was followed for the phosphorylation of **5**. A 10-mL stock solution of the phosphorylating reagent was made at 0 °C by slowly dripping 2.07 mL (0.026 mol) of pyridine into a stirred solution of 2.15 mL (0.024 mol) of $POCl_3$ and 0.27 mL (0.0149 mol) of water, followed by the addition of 5.5 mL of acetonitrile. The solid nucleoside **5** (105 mg, 0.3 mmol) was cooled to 5 °C, and 0.55 mL of the reagent solution was added, followed by stirring at 5 °C for 4.5 h. To the reaction mixture was added 1 mL of ice–water. It was stirred for an additional hour at 5 °C and stored at −25 °C until it was placed on a DE-32 DEAE-cellulose column (formate counterion; 2.6 × 40 cm). A gradient elution of 0.01 to 0.3 M ammonium formate, pH 4.4, was used to elute the phosphate. After lyophilization of the appropriate fractions, determined by UV absorption, the phosphate was analyzed by paper chromatography with uridine and uridine monophosphate (UMP) as internal standards (1-butanol–acetic acid–water, 7:1:2). The R_f values observed were: **5**, 0.65; uridine, 0.30; 1, 0.09; UMP, 0.03. High-pressure LC analysis of the phosphate 1 on a Whatman partisil PXS 10/25 SAX column using 0.007 M KH_2PO_4 at a flow rate of 2 mL/min showed one peak with a retention time of 2.14 min. Anal. ($C_{11}H_7BrN_3O_9P \cdot 1.5H_2O$; M_r 456.1; monoammonium salt) C, N; H: calcd, 4.25; found, 3.65. After further drying at 60 °C and high vacuum for several hours, the sample was analyzed for bromine. Anal. ($C_{11}H_7BrN_3O_9P$; M_r 429.1) Br.

Enzyme Inhibition Studies. The enzyme (5×10^{-8} M) was preincubated at 30 °C in 50 μL of solution containing 5 mM 2-mercaptoethanol; 6 mM magnesium chloride; 0.24 mM EDTA; 12 mM Tris–acetate buffer, pH 6.8; and varying concentrations of inhibitor. Substrate protection was evaluated by including the indicated concentration of substrate at a specific activity of 500 μCi/μmol. After incubation for the indicated time period, the assay for remaining active enzyme was started by the addition of 50 μL of a solution containing buffer and other components of the assay to give the same concentrations as noted in the reported enzyme assay.[5] A high substrate concentration (40 μM) was used in these assays to afford reasonably high velocity and to competitively reduce the enzyme inactivation by the inhibitor during the assay.

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