Acta Biochimica Polonica

Vol. 43 No. 1/1996

133–142

QUARTERLY

This paper is dedicated to Professor David Shugar who has been a frequent source of inspiration, on the occasion of his 80th birthday

Molecular mechanism of thymidylate synthase-catalyzed reaction and interaction of the enzyme with 2- and/or 4-substituted analogues of dUMP and 5-fluoro-dUMP*

Wojciech Rode[a] and Andrzej Leś[b]

[a]*M. Nencki Institute of Experimental Biology, Polish Academy of Sciences, L. Pasteur 3, 02–093 Warsaw, Poland*
[b]*Department of Chemistry, University of Warsaw, Pasteur 1, 02 093 Warsaw, Poland*

Key words: thymidylate synthase, inhibition specificity, dUMP analogues

Thymidylate synthase is a target enzyme in anticancer, antiviral, antifungal and antiprotozoan chemotherapy. With two dUMP analogues, 5-fluoro-dUMP (FdUMP) and 5-(trifluoromethyl)-dUMP (CF_3dUMP), strong thymidylate synthase inhibitors and active forms of drugs, the inhibition mechanism is based on the reaction mechanism. Recent comparative studies of new dUMP analogues, containing more than one substituent in the pyrimidine ring, showed that substitution of the pyrimidine ring $C_{(4)}$=O group in FdUMP by either $C_{(4)}$=N-OH group (in N^4-hydroxy-FdCMP) or $C_{(4)}$=S group (in 4-thio-FdUMP) preserves high inhibitory potency of the drug but may alter its specificity for thymidylate synthases from various sources, which differ in sensitivity to slow-binding inhibition by FdUMP. Informations suggesting mechanisms responsible for the foregoing have been reviewed, including results of molecular modeling studies suggesting interaction of the pyrimidine $C_{(4)}$=O group, or its modification, with the $N^{5,10}$-methylene-

Thymidylate synthase (EC 2.1.1.45) catalyzes the C(5) methylation of 2'-deoxyuridylate (dUMP) in a concerted transfer and reduction of the one-carbon group (at the aldehyde oxidation level) of $N^{5,10}$-methylenetetrahydrofolate, with concomitant production of dihydrofolate and thymidylate. *In vivo* folate cofactor and product of the reaction are usually in their γ-oligoglutamate forms, preventing transport through the cell membrane [1, 2]. As the sole *de novo* source of thymidylate synthesis in cells, the enzyme is a target in anticancer, antiviral, antifungal and antiprotozoan chemotherapy [3–7]. Two dUMP analogues, 5-fluoro-dUMP (FdUMP) and 5-(trifluoromethyl)-dUMP (CF_3dUMP), strong thymidylate synthase inhibitors, are active forms of drugs used in chemotherapy, such as 5-fluorouracil, 5-fluoro-2'-deoxyuridine and 5-fluorocytosine, and 5-(trifluoromethyl)-2'-deoxyuridine. With both inhibitors the inhibition mechanism is based on the reaction mechanism, and in order to understand the former, it is essential to elucidate the latter.

The essential step of thymidylate synthase catalysis involves formation of a ternary sub-

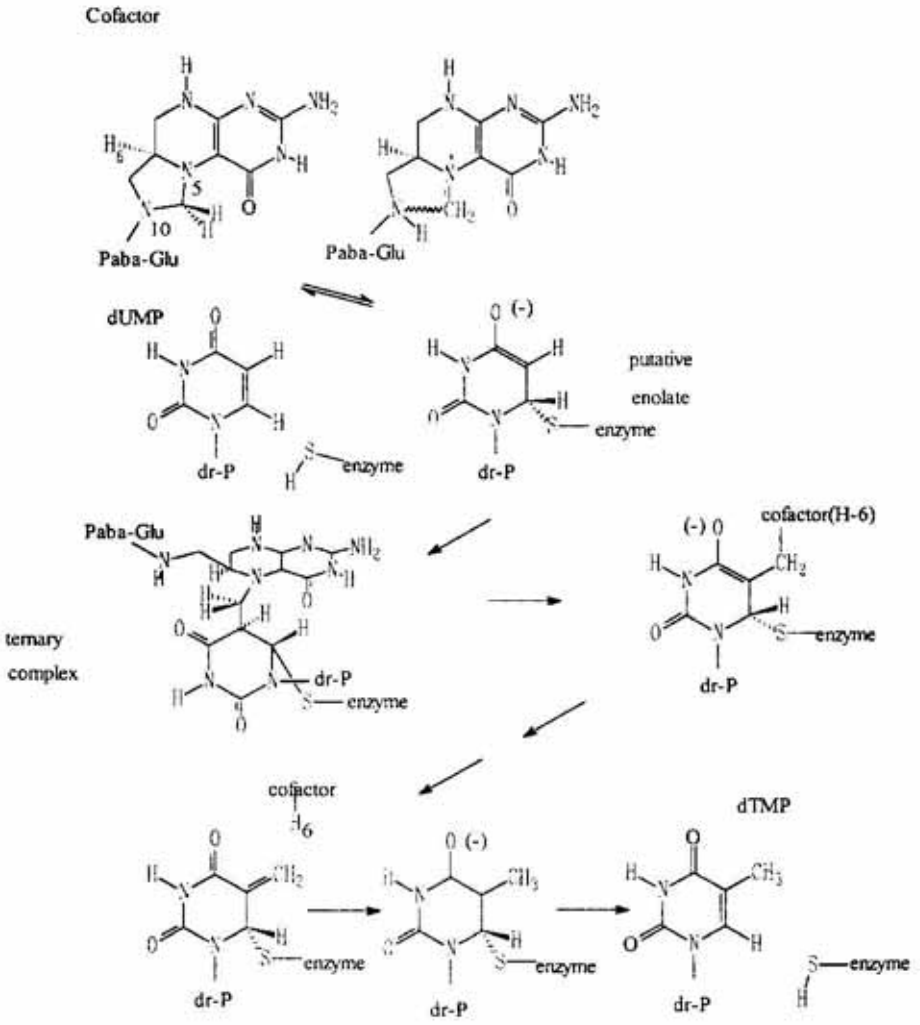
*Supported by the State Committee for Scientific Research (Grants No. 6 6253 92 03p/02 and 0914/P3/94/06).

Abbreviations: MEP, molecular electrostatic potential; RHF, restricted Hartree-Fock calculations.

strate-enzyme-cofactor molecular complex which splits, after some bond rearrangement, into the product, thymidylate (dTMP), dihydrofolate and enzyme (Scheme 1). Thymidylate synthase activates dUMP by nucleophilic attack, at $C_{(6)}$ of the pyrimidine, by the cysteine residue present in the enzyme active center. At this step the negative charge of cysteine residue is delocalized, probably towards the $C_{(4)}$=O of dUMP, forming the corresponding enolate anion, the $C_{(5)}$ position of which is thought to become strongly nucleophilic. The latter facilitates a consecutive attack of the methylene residue, in the form of the iminium ion, resulting from opening of the imidazolidine ring of the cofactor. This leads to formation of the covalent intermediate, ternary complex of the enzyme with substrate and cofactor. Next, the $C_{(5)}$ hydrogen dissociates, as proton, from dUMP pyrimidine ring, with concomitant β-elimination of tetrahydrofolate, still remaining bound in the active center. The latter enables hydride transfer from the pteridine $C_{(6)}$, resulting in reduction of the pyrimidine $C_{(5)}$ methylene group to a methyl one. The reaction is completed by regeneration of the pyrimidine 5,6 double bond, with concomitant elimination of the cofactor to form dTMP and enzyme (reviewed in [8–10]).

Most of the dUMP analogues that are good inhibitors of thymidylate synthase, involve an electron-withdrawing substituent at the pyrimidine $C_{(5)}$ (reviewed in [11–13]). The most prominent examples are the already mentioned FdUMP and CF_3dUMP (Scheme 2).

Inhibition of thymidylate synthase by FdUMP involves time-dependent formation of a ternary covalently bound complex of the enzyme with FdUMP and $N^{5,10}$-methylenetetrahydrofolate, in a reaction similar to that involving dUMP. However, at this step the reaction stops, as the $C_{(5)}$ fluorine atom fails to dissociate (due



Scheme 1. Mechanism of the reaction catalyzed by thymidylate synthase.

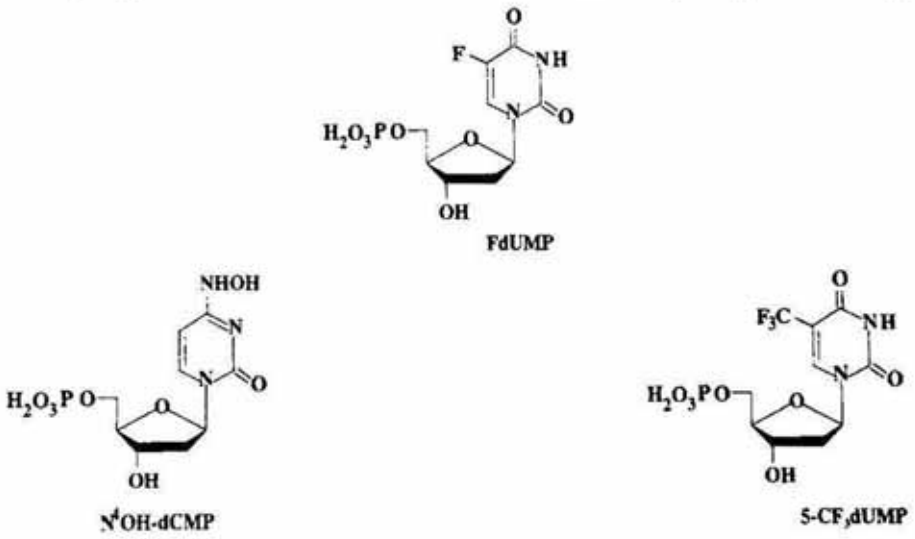
to the strength of the C–F bond). This results in a slowly reversible enzyme inactivation. Thymidylate synthase inactivation is caused also by CF_3dUMP but the inhibitor binds to the enzyme in the absence of $N^{5,10}$-methylenetetrahydrofolate. The nucleophile cysteine attack, at $C_{(6)}$ of the pyrimidine, by cysteine residue leads to activation of the trifluoromethyl group and release of fluoride ion, followed by reaction of the resulting $C_{(5)}$=CF_2 group with a nucleophile (tyrosine residue) of the enzyme, forming a covalent complex [14].

A rare example of a dUMP analogue that is $C_{(4)}$-substituted and, nevertheless, a strong thymidylate synthase inhibitor, is N^4-hydroxy-2'-deoxycytidine-5'-monophosphate (N^4-OH-dCMP; Scheme 2). Like FdUMP, it inactivates the enzyme *via* time-dependent formation of a ternary covalently bound complex with thymidylate synthase and $N^{5,10}$-methylenetetrahydrofolate [15, 16]. However, unlike with FdUMP, this mechanism-based process is arrested at some step prior to abstraction of the $C_{(5)}$ hydrogen [17].

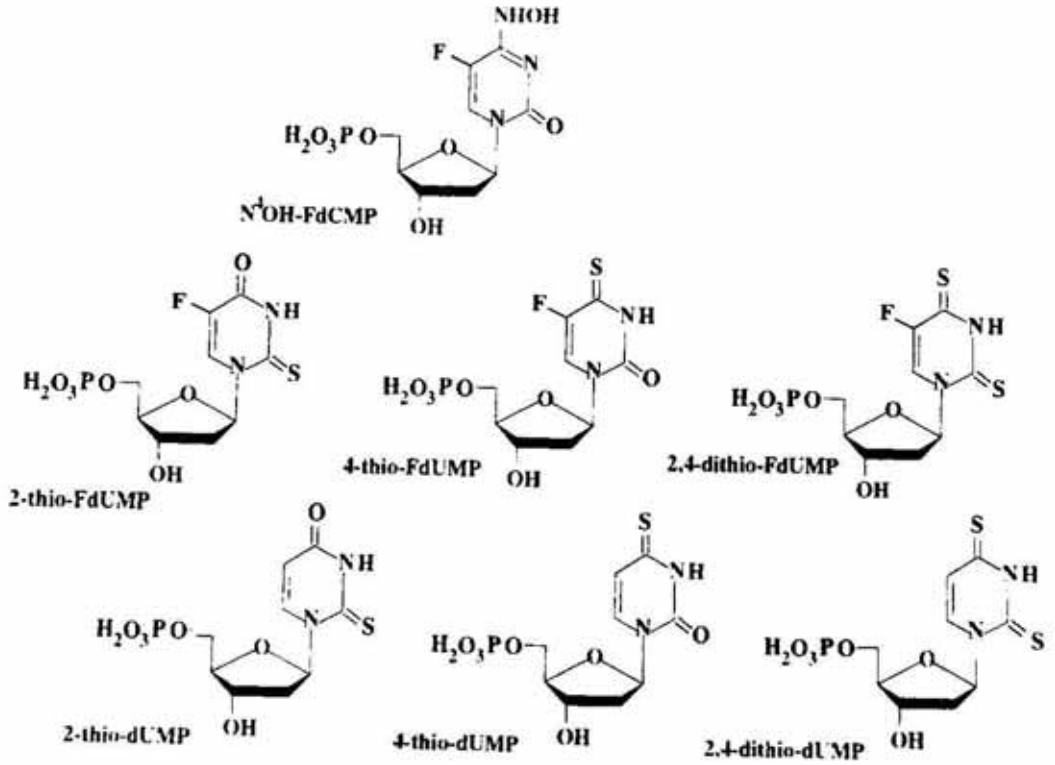
Recently, in search of potent and selective thymidylate synthase inhibitors, several new dUMP analogues, containing more than one substituent in the pyrimidine ring, including N^4-hydroxy-5-fluoro-2'-deoxycytidine-5'-monophosphate (N^4-OH-FdCMP), 2-thio-FdUMP, 4-thio-FdUMP, 2,4-dithio-FdUMP and 2,4-dithio-dUMP were tested (Scheme 3). Comparative studies showed that substitution of the pyrimidine ring $C_{(4)}$=O group in FdUMP by either $C_{(4)}$=N-OH group (in N^4-OH-FdCMP) or $C_{(4)}$=S group (in 4-thio-FdUMP) preserves high inhibitory potency of the drug but may alter its specificity for thymidylate synthases from various sources, which differ in sensitivity to slow-binding inhibition by FdUMP (Table 1). This phenomenon seems to be due to some interplay between the substituents at $C_{(4)}$ and $C_{(5)}$ in their interaction with the enzyme [17, 18]. An additional support for this interpretation comes from the finding that specificity of 2-thio-FdUMP for inactivation of thymidylate synthases (Table 1) paralleled that of FdUMP [18, 19].

All three FdUMP analogues inactivate the enzyme in time- and $N^{5,10}$-methylenetetrahydrofolate-dependent manner. However, although in all three cases the inactivation mechanisms are apparently based on the reaction mechanism, they are not necessarily identical. Enzyme inactivation by either 2-thio-FdUMP or 4-thio-FdUMP seems to be due to the presence of the 5-fluoro substituent, since both 2-thio-dUMP and 4-thio-dUMP behave as thymidylate synthase substrates [19, 20]. Thus, although neither 2-thio nor 4-thio substituent in FdUMP molecule appears to influence the inactivation mechanism, only the latter influences its selectivity.

In N^4-OH-FdCMP molecule, the $C_{(4)}$=N-OH substituent is probably the cause of inactivation, and the 5-fluoro substituent potentiates this process [17]. To explain the latter phenomenon, pointing clearly to an interplay between the $C_{(4)}$=N-OH and $C_{(5)}$–F substituents, an intramolecular hydrogen bonding N^4-O-H ··· F-



Scheme 2. Structures of FdUMP, 5-trifluoromethyl-dUMP and N^4-hydroxy-dCMP.

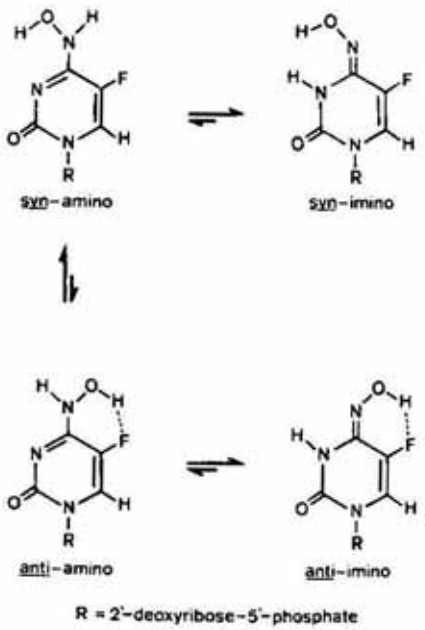


Scheme 3. Structures of dUMP, FdUMP and their 2- and/or 4-substituted analogues.

$C_{(5)}$ was hypothesized, influencing an assumed *syn-anti*, relative to $N_{(3)}$, equilibrium of rotamers around the $C_{(4)}$–N^4 bond (Scheme 4), and resulting in stabilization of the rare species *anti*, found to be the only inhibitory form [17]. However, results of *ab initio* quantum mechanical calculations brought such a mechanism into question [21]. Thus, an interplay between the

Table 1
Parameters of interaction of thymidylate synthases from different sources with dUMP, FdUMPand FdUMP analogues [17–19, 24, 32].

Enzyme source	K_m (μM) for dUMP	K_i (slow binding) (nM) for				
		FdUMP	2-Thio-FdUMP	4-Thio-FdUMP	N^4-OH-dCMP	N^4-OH-FdCMP
Mouse						
Ehrlich carcinoma	1.3	5.5	79	60	50	20
L1210	2.6	1.8	41	102	63	73
L1210 FdUrd-resistant	2.5	12.0	297	14	184	93
Thymus	4.2	2.6				
Rat						
Colon tumour K-12	3.2	120		400		
Regenerating liver	3.4	10	64	850	890	50
Man						
Colon tumour HCT-8 cells	2.8	130		140		
Leukemia CCRF-CEM cells	2.3	4		180		
Tapeworm						
Hymenolepis diminuta	5.4	114	632	910	920	50

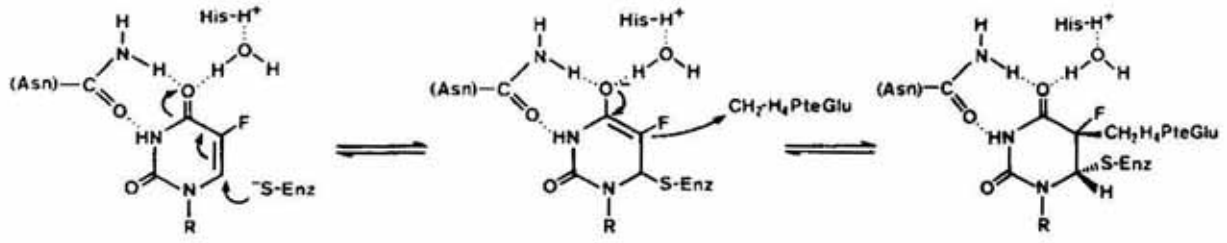


Scheme 4. Amino-imino and syn-anti equilibria for N^4-OH-FdCMP and hypothetical stabilization of the syn rotamers by intramolecular hydrogen bonding [15].

$C_{(4)}$=N-OH and $C_{(5)}$–F, as well as between the $C_{(4)}$=S and $C_{(5)}$–F groups, seems to be due to alteration in the pyrimidine $C_{(4)}$ region rather than to some intramolecular bonding.

It should be noted that a crucial role has been ascribed to dUMP $C_{(4)}$=O and a non-dissociated $N_{(3)}$-H groups of the pyrimidine moiety in the specificity of enzyme binding, *via* an active center asparagine residue and an ordered water molecule [22, 23]. This phenomenon, resulting in the discrimination by thymidylate synthase active center between dUMP and dCMP, with the asparagine residue proposed to stabilize, by hydrogen bonding, the partial negative charge developed on O^4 of covalently bound dUMP [8], has been suggested to be mechanism-based [22]. Since strong mechanism-based inactivation by FdUMP or 4-thio-FdUMP of thymidylate synthase has been shown to depend on a non-dissociated $N_{(3)}$-H group [24], the active center asparagine appears to be involved in the interaction (cf. Scheme 5). The same is probably true for N^4-OH-FdCMP and N^4-OH-dCMP, as (i) the most stable appear to be their imino forms ([21] and references therein), with a non-dissociated $N_{(3)}$-H and $C_{(4)}$=N-OH (imitating the $C_{(4)}$=O) groups (Scheme 4); (ii) comparison of thymidylate synthase inactivation by N^4-OH-dCMP and dCMP showed a lack of activity of the latter [17], indicating again a strong demand for the structure involving $N_{(3)}$-H and $C_{(4)}$=N- groups; and (iii) thymidylate synthase slow-binding inhibition by FdCMP [25] and 2-thio-FdCMP (unpublished), both involving $-N_{(3)}=C_{(4)}(-NH_2)-$ structure, was about 10^3-fold weaker than by N^4-OH-FdCMP [17], involving $-N_{(3)}(-H)-C_{(4)}(=N-OH)-$ structure. Hence, substitution in FdUMP (or dUMP) of the pyrimidine $C_{(4)}$=O with a substituent preserving the non-dissociated $N_{(3)}$-H group, and capable of serving as hydrogen acceptor in a hydrogen bond, might hinder pyrimidine recognition by thymidylate synthase, sensitizing it to variations of nonconservative amino-acid residue(s) in the enzyme active center.

The latter explanation is supported, at least in case of the $C_{(4)}$=S substitution, by the results presenting the Molecular Electrostatic Potential (MEP) for the optimized molecular structures, calculated with the *ab initio* RHF/6-31G** method for a rectangular mesh of 51 × 51 grid points separated by 0.2 Å. One mesh belonged to the molecular plane and the other one was located 1.6 Å above the plane. In Table 2 selected MEP values were gathered for the grid points located 1.6 Å strictly above the $C_{(5)}$ and below (or above) the $C_{(6)}$ carbon atoms of the pyrimidine ring. The minimal MEP values in the vicinity (within the sphere of 3.0 Å) of the Y atom (Y = O, S, N) at the $C_{(4)}$ and the position of the bottom of the MEP well were added (Leś,



Scheme 5. Possible involvement of the active center asparagine in thymidylate synthase inactivation by FdUMP (R = 2'-deoxyribose-5'-phosphate).

A., unpublished). Positive MEP values below the $C_{(6)}$ carbon facilitate the nucleophilic attack of the negatively charged cysteine residue. Negative MEP values in the vicinity of the Y atom suggest an increased tendency to stabilize the incipient enolate anion that is thought to be formed just after cysteine addition to the $C_{(6)}$ atom. Weakly positive or negative MEP values above the $C_{(5)}$ carbon facilitate the electrophilic attack of the incipient iminium ion resulting from opening of the imidazolidine ring of $N^{5,10}$-methylenetetrahydrofolate (Scheme 1). The 4-thio-substituted (but not 2-thio-substituted) derivatives of either 1-methyl-uracil or its 5-fluoro congener, models of 4-thio-substituted derivatives of either dUMP or FdUMP, appear to show impaired fitness into the network of hydrogen bonds in the enzyme active center cleft not only due to its excessive molecular volume but also due to reduction of electron density in interaction regions (distant shallow MEP well). However, the same does not seem to apply to the 4-hydroxyimino substituent, affecting MEP values around the $C_{(5)}$ and $C_{(6)}$ rather than around $C_{(4)}$ (Table 2).

An additional suggestion, concerning participation of the pyrimidine $C_{(4)}$=O group, or its modification, in thymidylate synthase reaction, is provided by initial results of molecular modeling studies on a possible influence of the $N^{5,10}$-methylenetetrahydrofolate $N_{(10)}$ on this reaction (Leś, A., unpublished). The quantum mechanical *ab initio* Restricted Hartree Fock (RHF/3-21G) calculations have been performed for a series of model compounds that mimic the essential building blocks of the ternary complex. The molecular geometry parameters (bond lengths, bond angles and tetrahedral angles) were varied until the minimal total energy was obtained. The Gaussian 92 code was used in the geometry optimization.

Preliminary results were obtained for neutral systems modeling three possible reaction paths that can occur when the imidazolidine ring of the cofactor is opened and the incipient methyl group is attached to the $C_{(5)}$ carbon atom of the dUMP pyrimidine ring (Scheme 6). The theoretically predicted energy difference between the A and C products is estimated to be some 10 kcal · mol^{-1}, in favour of A. The product of reaction B is highly energetic and should not compete with the two others. An interesting conclusion that emerges from the above theoretical studies is that the OH-group can block the $N_{(10)}$ nitrogen atom of the cofactor making impossible the $C_{(5)}$ hydrogen abstraction.

A more detailed study of this situation was performed for another model system simulating the involvement of the N^4-hydroxy-dCMP (Scheme 7). The ionized (deprotonated) cysteine residue of thymidylate synthase active center was modeled by the thiomethyl anion

Table 2

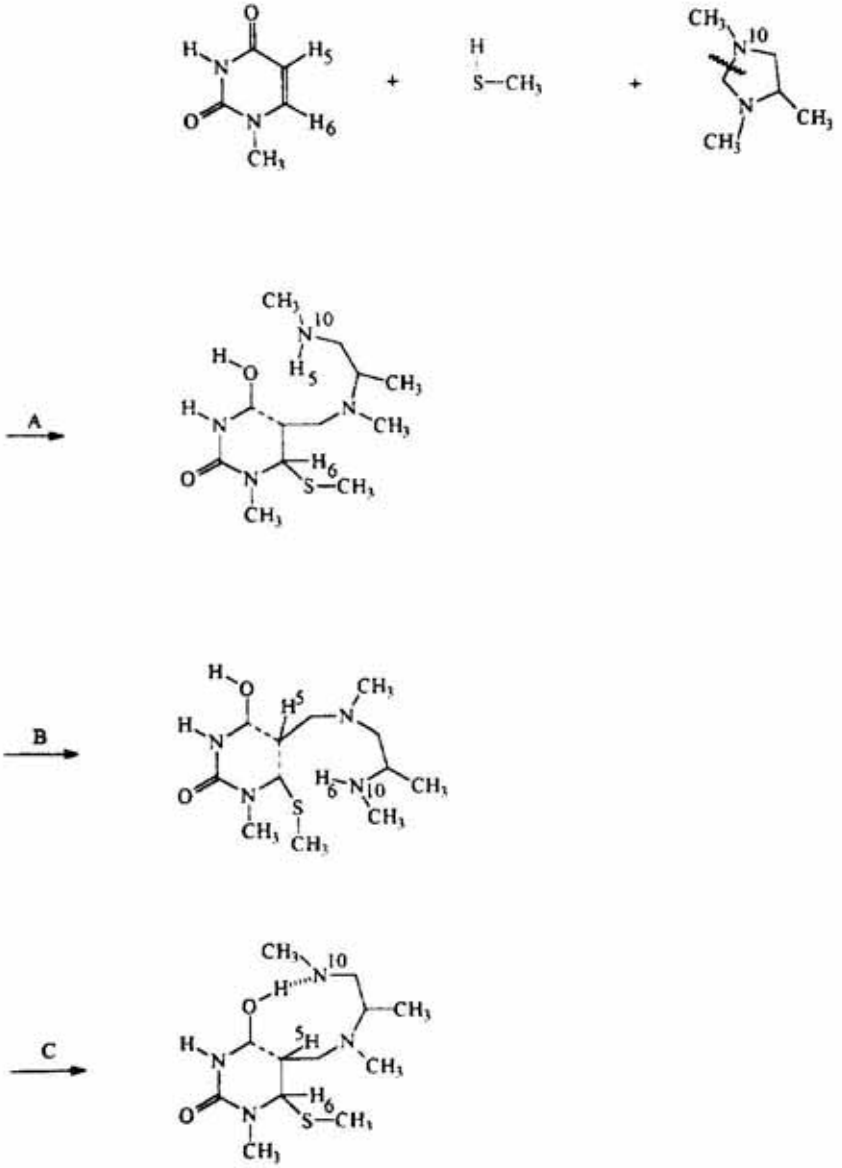
Molecular electrostatic potentials (in 10^{-3} atomic units[a]) of several 1-methyl-uracil analogues, substituted at $C_{(2)}$, $C_{(4)}$ and $C_{(5)}$ with R_2, R_4 and R_5, respectively

R_2	R_4	R_5	MEP in-plane[a] (10^{-3} a.u.[b])	Distance from Y[c] (Å)	MEP above $C_{(5)}$ (10^{-3} a.u.[b])	MEP below $C_{(6)}$ (10^{-3} a.u.[b])
O	O	H	–94	1.19	3	30
O	S	H	–66	1.74	8	36
S	O	H	–85	1.25	18	39
S	S	H	–58	1.70	19	45
O	O	F	–88	1.18	27	35
O	S	F	–67	1.70	31	43
S	O	F	–79	1.28	39	45
S	S	F	–58	1.84	41	52
O	N-OH	H	–86	1.34	–6	18
O	N-OH	F	–75	1.22	17	22

[a]MEP in-plane denotes the bottom of the MEP well found in the vicinity (sphere of 3.0 Å radius) of Y, at the distance presented in the next column.

[b]10^{-3} atomic unit = 0.6275 kcal · mol^{-1} = 0.027211 eV.

[c]Y denotes O, S, or N atoms in $C_{(4)}$=O, $C_{(4)}$=S and $C_{(4)}$=N-OH residues, respectively.

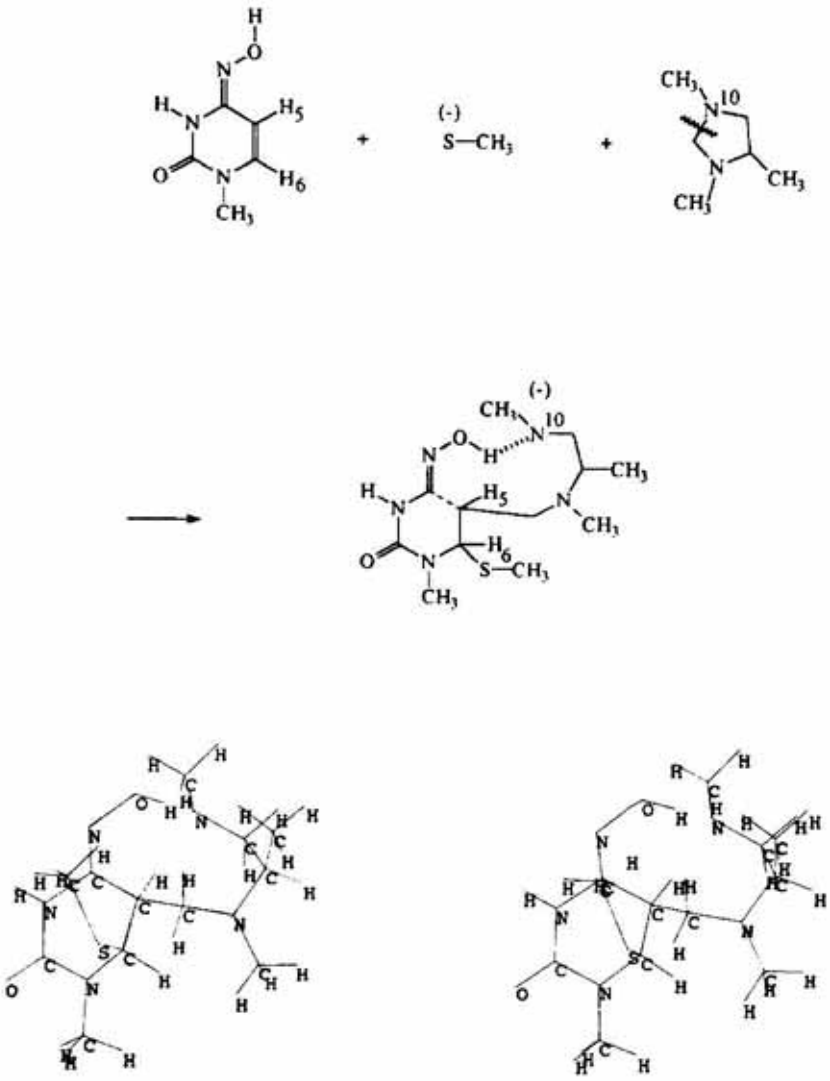


Scheme 6. Models of possible (A, B, and C) reaction channels in the course of ternary complex formation.

$(S\text{-}CH_3)^{(-)}$. The resulting molecular anion is stable during the optimization of the geometry. The most interesting feature of the system is the strong intramolecular N-OH ····· $N_{(10)}$ hydrogen bond. The theoretical calculations suggest that the hydrogen atom can reside almost in the middle of the N(oxime)-$N_{(10)}$ distance. The estimated energy output of some +15 kcal · mol^{-1} suggests that the formation of such a ternary complex should be a weakly endothermic reaction. However, such an energy loss can easily be recovered by possible formation of two–three hydrogen bonds with the environment.

The foregoing result suggests an explanation of the mechanism of thymidylate synthase inactivation by N^4-hydroxy-dCMP, in particular the arrest of liberation of the $C_{(5)}$ hydrogen in the enzyme-inhibitor-cofactor complex. Although formation of the N^4-OH ····· $N_{(10)}$ hydrogen bond has been hypothesized [17], its preventing the $C_{(5)}$ hydrogen abstraction was not suspected. Besides, the possibility of interaction between the pyrimidine $C_{(4)}$=O and $N^{5,10}$-methylenetetrahydrofolate $N_{(10)}$, reflected by the abortive reaction channel C (Scheme 6) that under conditions of the uninhibited reaction is probably prevented by the structure of enzyme active center, indicates why inactivation by $C_{(4)}$-substituted dUMP analogues might be sensitive to variations of nonconservative amino-acid residue(s) in active centers of different thymidylate synthases.

In striking contrast to the 2-thio and 4-thio analogues of dUMP, which are good substrates, and to the 2-thio and 4-thio analogues of



Scheme 7. Model of N^4-hydroxy-dCMP complex with the enzyme and cofactor. The lower panel shows the stereo view of the resulting molecular anion. The intramolecular N-OH ···· N(10) bond is characterized by O ···· N distance of 2.4 Å, H ···· $N_{(10)}$ distance of 1.2 Å and the $OHN_{(10)}$ angle of 172°.

FdUMP, which are good slow-binding inhibitors of thymidylate synthase, 2,4-dithio-dUMP is not a substrate and 2,4-dithio-FdUMP is not a good inhibitor. Instead, 2,4-dithio-dUMP is a weak inhibitor, competitive *vs* dUMP and although 2,4-dithio-FdUMP behaves as a typical slow-binding inhibitor of the enzyme, it is 10^3–10^4-fold less potent than the corresponding 2-thio and 4-thio congeners (Table 3).

Theoretical comparison, based on calculations of the HOSE indices [26], of pyrimidine ring approximate electron distributions, reflected by participations of hypothetical limiting structures (not shown) with the same global charge, but different localization of electron pairs forming π bonds, and thus different local polarity, for available nucleoside structures of Urd, 2-thio-Urd, 4-thio-Urd and 2,4-dithio-Urd [27–30] was made. This pointed to higher participation of two structures forming aromatic hybrids, analogous to Kekulé structures, with negative charges localized on $C_{(2)}$ and $C_{(4)}$, and positive charges on $N_{(1)}$ and $N_{(3)}$, in 2,4-dithiouridine (16.8%) than in monothio-substituted (about 12%) or nonsubstituted uridine (6.1%). The result, indicating enhanced aromaticity [31] of the 2,4-dithio-substituted pyrimidine ring, was further supported by comparison of the aromaticity indices [33]. It suggests that lack of substrate activity of 2,4-dithio-dUMP may result from increased pyrimidine ring aromaticity of the latter, leading to

Table 3
Interactions of 2 and/or 4 thio-substituted analogues of dUMP and FdUMP with L1210 thymidylate synthase

Compound	Activity	K_m or K_i	Reference
dUMP	Substrate	10^{-5}	19
2-Thio-dUMP	Substrate	2×10^{-5}	19
4-Thio-dUMP	Substrate	10^{-5}	20
2,4-Dithio-dUMP	Inhibitor (C[a])	3×10^{-5}	33
FdUMP	Inhibitor, S-B[b]	2×10^{-9}	24
2-Thio-FdUMP	Inhibitor, S-B	4×10^{-8}	18
4-Thio-FdUMP	Inhibitor, S-B	10^{-7}	24
2,4-Dithio-FdUM	Inhibitor, S-B	10^{-5}	33

[a]Classic; [b]Slow-binding.

resistance of $C_{(6)}$ to nucleophilic attack by the enzyme active center cysteine.

REFERENCES

1. Santi, D.V. & Danenberg, P.V. (1984) Folates in pyrimidine nucleotide biosynthesis; in *Folates and Pterines* (Blakley, R.L. & Benkovic, S.J., eds.) vol. 1, pp. 345–398, Wiley, New York.
2. Rode, W. (1986) Thymidylate biosynthesis: biological role and regulation in animal cells. *Post. Biochem.* **32,** 401–420 (in Polish).
3. Danenberg, P.V. (1977) Thymidylate synthetase — a target enzyme in cancer chemotherapy. *Biochim. Biophys. Acta* **473,** 73–92.
4. Heidelberger, C., Danenberg, P.V. & Moran, R.G. (1983) Fluorinated pyrimidines and their nucleosides. *Adv. Enzymol.* **54,** 57–119.
5. Jackman, A.L., Jones, T.R. & Calvert, A.H. (1985) Thymidylate synthetase inhibitors: experimental and clinical aspects; in *Experimental and Clinical Progress in Cancer Chemotherapy* (Muggia, F.M., ed.) pp. 155–210, Martinus Nijhoff Publishers, Boston.
6. Douglas, K.T. (1987) The thymidylate synthesis cycle and anticancer drugs. *Med. Res. Rev.* **7,** 441–475.
7. Rathod, P.K., Leffers, N.P. & Young, R.D. (1992) Molecular targets of 5-fluoroorotate in the human malaria parasite, Plasmodium-falciparum. *Antimicrob. Agents Chemother.* **36,** 704–711.
8. Matthews, D.A., Villafranca, J.E., Janson, C.A., Smith, W.W., Welsh, K. & Freer, S. (1990) Stereochemical mechanism of action for thymidylate synthase based on the X-ray structure of the covalent inhibitory ternary complex with 5-fluoro-2′-deoxyuridylate and 5,10-methylenetetrahydrofolate. *J. Mol. Biol.* **214,** 937–948.
9. Stroud, R.M. & Finer-Moore, J.S. (1993) Stereochemistry of a multistep/bipartite methyl transfer reaction: thymidylate synthase. *FASEB J.* **7,** 671–677.
10. Carreras, C.W. & Santi, D.V. (1995) The catalytic mechanism and structure of thymidylate synthase. *Annu. Rev. Biochem.* **64,** 721–762.
11. Lewis, C.A., Jr. & Dunlap, R.B. (1981) Thymidylate synthase and its interaction with 5-fluoro-2′-deoxyuridylate; in *Topics in Molecular Pharmacology* (Burgen, A.S.V. & Roberts, G.C.K., eds.) pp. 170–219, Elsevier/North-Holland Biomedical, New York.
12. Santi, D.V. (1980) Perspectives on the design and biochemical pharmacology of inhibitors of thymidylate synthetase. *J. Med. Chem.* **23,** 103–111.
13. De Clercq, E., Balzarini, J., Torrence, P.F., Mertes, M.P., Schmidt, C.L., Shugar, D., Barr, P.J., Jones, A.S., Verhelst, G. & Walker, R.T. (1981) Thymidylate synthetase as target enzyme for the inhibitory activity of 5-substituted 2′-deoxyuridines on mouse leukemia L1210 cell growth. *Mol. Pharmacol.* **19,** 321–330.
14. Eckstein, J.W., Foster, P.G., Finer-Moore, J., Wataya, Y. & Santi, D.V. (1994) Mechanism-based inhibition of thymidylate synthase by 5-(trifluoromethyl)-2′-deoxyuridine 5′-monophosphate. *Biochemistry* **33,** 15086–15094.
15. Lorenson, M.Y., Maley, G.F. & Maley, F. (1967) The purification and properties of thymidylate synthetase from chick embryo extracts. *J. Biol. Chem.* **242,** 3332–3344.
16. Goldstein, S., Pogolotti, A.L., Jr., Garvey, E.P. & Santi, D.V. (1984) Interaction of N^4-hydroxy-

2'-deoxycytidylic acid with thymidylate synthetase. *J. Med. Chem.* **27,** 1259–1262.

17. Rode, W., Zieliński, Z., Dzik, J.M., Kulikowski, T., Bretner, M., Kierdaszuk, B., Cieśla, J. & Shugar, D. (1990) Mechanism of inhibition of mammalian tumor and other thymidylate synthases by N^4-hydroxy-dCMP, N^4-hydroxy-5-fluoro-dCMP, and related analogues. *Biochemistry* **29,** 10835–10842.
18. Dzik, J.M., Zieliński, Z., Cieśla, J., Bretner, M., Kulikowski, T., Shugar, D., Bertino, J.R. & Rode, W. (1993) Interaction of 2-thio-5-fluoro-dUMP and 4-thio-5-fluoro-dUMP with mammalian normal and tumour, and helminthic, thymidylate synthases: influence of C(4)-substituents on specificity for enzyme inactivation. *Biochem. Biophys. Res. Commun.* **195,** 1301–1308.
19. Bretner, M., Kulikowski, T., Dzik, J.M., Balińska, M., Rode, W. & Shugar, D. (1993) 2-Thio derivatives of dUrd and 5-fluoro-dUrd and their 5'-monophosphates: synthesis, interaction with tumor thymidylate synthase, and *in vitro* antitumor activity. *J. Med. Chem.* **36,** 3611–3617.
20. Kalman, T.I., Bloch, A., Szekeres, G.L. & Bardos, T.J. (1973) Methylation of 4-thio-2'-deoxyuridylate by thymidylate synthase. *Biochem. Biophys. Res. Commun.* **55,** 210–217.
21. Leś, A., Adamowicz, L. & Rode, W. (1993) Structure and conformation of N^4-hydroxycytosine and N^4-hydroxy-5-fluorocytosine. A theoretical ab initio study. *Biochim. Biophys. Acta* **1173,** 39–48.
22. Hardy, L.W. & Nalivaika, E. (1992) Asn (177) in *Escherichia coli* thymidylate synthase is a major determinant of pyrimidine specificity. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 9725–9729.
23. Liu, L. & Santi, D.V. (1992) Mutation of asparagine 229 to aspartate in thymidylate synthase converts the enzyme to a deoxycytidylate methylase. *Biochemistry* **31,** 5100–5104.
24. Dzik, J.M., Kulikowski, T., Zieliński, Z., Cieśla, J., Rode, W. & Shugar, D. (1987) Interaction of 5-fluoro-4-thio-2'-deoxyuridine 5'-phosphate with mammalian tumour thymidylate synthase: role of the pyrimidine N(3)-H dissociation. *Biochem. Biophys. Res. Commun.* **149,** 1200–1207.
25. Liu, L. & Santi, D.V. (1994) 5-Fluoro-2'-deoxycytidine 5'-monophosphate is a mechanism-based inhibitor of thymidylate synthase. *Biochim. Biophys. Acta* **1209,** 89–94.
26. Krygowski, T.M., Anulewicz, R. & Kruszewski, J. (1983) Crystallographic studies and physicochemical properties of π-electron compounds. III. Stabilization energy and the Kekulé structure contributions derived from experimental bond lengths. *Acta Cryst.* **B39,** 732–739.
27. Green, E.A., Rosenstein, R.D., Shiono, R., Abraham, D.J., Trus, B.L. & Marsh, R.E. (1975) The crystal structure of uridine. *Acta Cryst.* **B31,** 102–107.
28. Hawkinson, S.W. (1977) The crystal and molecular structure of 2-thiouridine. *Acta Cryst.* **B33,** 80–85.
29. Lesyng, B. & Saenger, W. (1981) Influence of crystal packing forces on molecular structure in 4-thiouridine. Comparison of *anti* and *syn* forms. *Z. Naturforsch.* **36c,** 956–960.
30. Saenger, W. & Suck, D. (1971) Kristall und Molekül von 2,4-Dithiouridin-monohydrat. *Acta Cryst.* **B27,** 1178–1186.
31. Minkin, W.I., Glukhovtsev, M.N. & Simkin, B.Ya. (1994) *Aromaticity and Antiaromaticity. Electronic and Structural Aspects.* Wiley, New York.
32. Cieśla, J., Gołos, B., Dzik, J.M., Pawełczak, K., Kempny, M., Makowski, M., Bretner, M., Kulikowski, T., Machnicka, B. & Rode, W. (1995) Thymidylate synthases from *Hymenolepis diminuta* and regenerating rat liver: purification, properties, and inhibition by substrate and cofactor analogues. *Biochim. Biophys. Acta* **1249,** 127–136.
33. Dzik, J.M., Bretner, M., Kulikowski, T., Gołos, B., Jarmuła, A., Poznański, J., Rode, W. & Shugar, D. (1996) Synthesis and interactions with thymidylate synthase of 2,4-dithio-analogues of dUMP and 5-fluoro-dUMP. *Biochim. Biophys. Acta* **1293,** in print.