BIOORGANIC CHEMISTRY 22, 405–420 (1994)

Insight into the Chemical Mechanism of Thymidylate Synthase-Catalyzed Reaction through the Evaluation of Chemical Models: The Role of C6 Sulfhydryl Addition During the Reductive Elimination Step of the Reaction[1]

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Received September 2, 1994

Thymidylate synthase catalyzes the last step of the *de novo* synthesis of thymidine-5′-monophosphate (TMP), which has long been a target for the development of effective anticancer agents. Model compounds **(15, 16, 17)** were used to study the effect of C6 nucleophilic addition on the reductive elimination step of the TS-catalyzed reaction. Results suggest that C6 addition facilitates the reductive elimination of the H_2folate moiety of the ternary intermediate **(3)**. Therefore, the reaction pathway (pathway (b)) with the participation of C6 sulfhydryl addition during the reductive elimination process is the energetically favored process. Consequently, the elimination of the cysteine sulfhydryl group from the C6 position is the last step of the reaction before the dissociation of the products from the enzyme.

INTRODUCTION

Thymidylate synthase (TS, EC 2.1.1.45) catalyzes the last step of the *de novo* synthesis of thymidine-5′-monophosphate (TMP), which is essential for DNA synthesis. Selective inhibition of tumor thymidylate synthase has long been a target for developing effective, low-toxicity cancer chemotherapeutic agents (*1*). The elucidation of the detailed TS reaction mechanism might help the design of effective TS inhibitors.

The TS-catalyzed reaction (Scheme 1) involves the 5-methylation of deoxyuridine-5′-monophosphate (dUMP) to give TMP using tetrahydrofolate (H_4folate) as the cofactor, which serves as both the carrier of the one carbon unit and the reducing agent. It has been established that enzymatic reaction is initiated by a

[1] Dedicated to the memory of Professor Mathias P. Mertes. This work is taken in part from the PhD dissertation of B.W.

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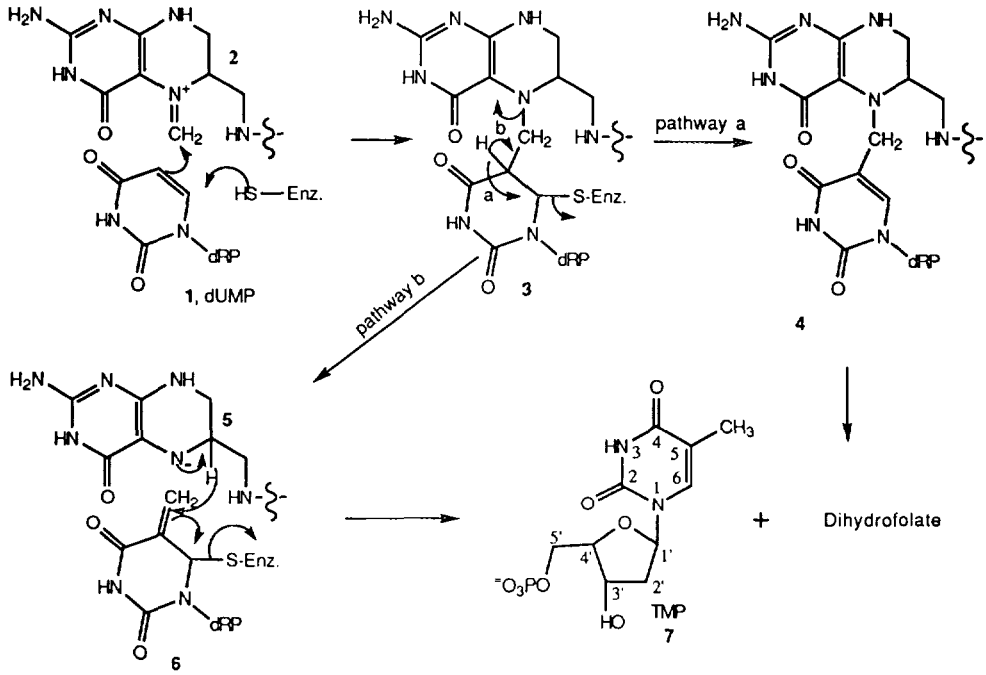
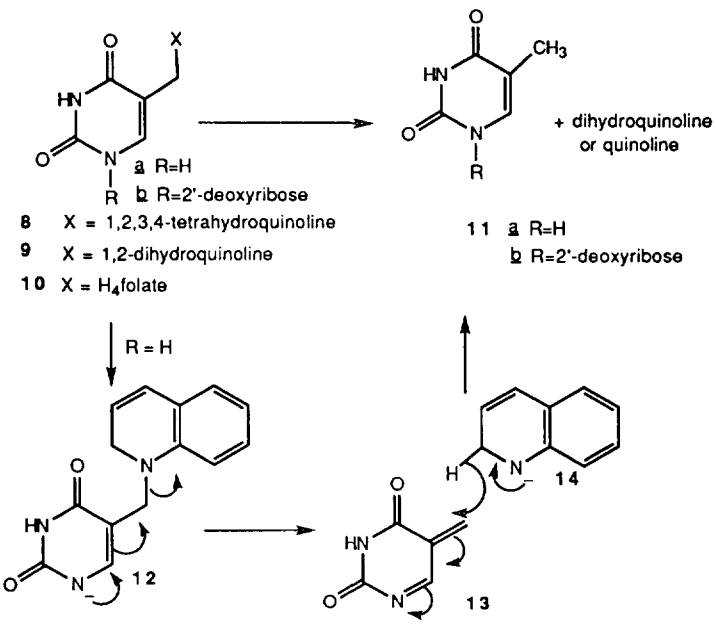


SCHEME 1. Possible mechanisms of the TS-catalyzed reaction.

C6 addition of an active site cysteine sulfhydryl group (*2*, *3*). However, the ternary intermediate **(3)** formed can undergo the subsequent reductive elimination to give dihydrofolate (H_2folate) and TMP **(7)** through two different pathways, which differ in the order of the cleavage of the cofactor moiety. One possible pathway (a) is via the initial elimination of the sulfhydryl group to give the binary intermediate **(4)** as proposed by Friedkin (*4*), which is followed by the reductive elimination of the H_2folate moiety. The second pathway (b) goes through an initial β-elimination of the cofactor to give an exomethylene intermediate **(6)**, which is followed by the reduction of the exomethylene intermediate **(6)** by H_4folate and elimination of the sulfhydryl group to give TMP **(7)**.

In 1972, Wilson and Mertes showed that compounds **(8, 9)**, structurally analogous to the intermediate **(4)** proposed by Friedkin, were converted to thymine analogs **(11)** through reductive elimination upon pyrolysis (Scheme 2) (*5*, *6*). Subsequent studies using H_4folate-substituted model **10** produced similar results (*7*), although the model reactions in Scheme 2 seem to support pathway (a), through intermediate **4**, as the preferred pathway. It is of concern that only N1-unsubstituted models **(8a, 9a, 10a)** underwent the reductive elimination reaction readily under the conditions studied, whereas the model with N1 substituted with a deoxyribose moiety **(8b, 9b)** did not. Because the reductive elimination mechanism of the models can be affected dramatically by the substitution at the N1 position due to the possibility for the N1 unsubstituted ones **(8a, 9a, 10a)** to form N1 anion



SCHEME 2. Possible mechanism for the pyrolysis of proposed TS-catalyzed reaction intermediate models.

upon deprotonation **(12)** (Scheme 2), these model studies might be incomplete when applied to TS catalysis, in which the substrate has N1 substituted with a deoxyribose moiety **(1)**.

An important question remaining in TS catalysis is whether the elimination of the cofactor occurs while the cysteine sulfhydryl group is attached at the C6 position. The aim of this study is to use chemical models to probe the effect of C6 nucleophilic addition on the reductive elimination reaction in intermediate **3** and to establish whether pathway (a) or (b) is the preferred pathway.

THE DESIGN OF THE MODEL SYSTEMS

Models having an N1 glycoside linkage and an intramolecular nucleophile that can readily attack the C6 position were needed for this study. The N1 glycoside linkage was necessary is to eliminate the possibility of N1 ionization and closely mimic the structural features of the intermediates in the TS-catalyzed reaction. Because models with N1 glycoside linkage **(8b, 9b)** have been shown not to undergo the desired reductive elimination reaction in the absence of nucleophilic addition to the C6 position (*5, 6*), the effect of C6 nucleophilic addition can be studied with similar systems through the introduction of an intramolecular nucleophile. In this way, comparison can be drawn to give meaningful information concerning the reductive elimination step of the TS-catalyzed process.

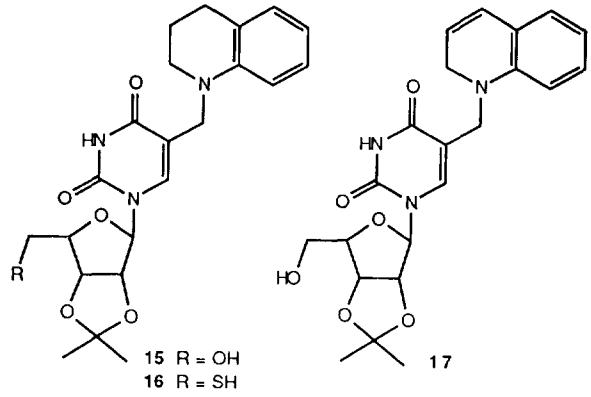


FIGURE 1

It has been shown that the tendency for the 5′-hydroxyl group of uridine to undergo nucleophilic addition to the C6 carbon can be significantly enhanced through the protection of the 2′,3′-hydroxyl groups of uridine with an acetonide (*8*). Similar results were obtained when the 5′-hydroxyl group was replaced by a thiol group (*9, 10*). Such enhanced tendencies of C6 nucleophilic addition by the 5′-substituents are probably due to the conformational changes induced by the acetonide protection.

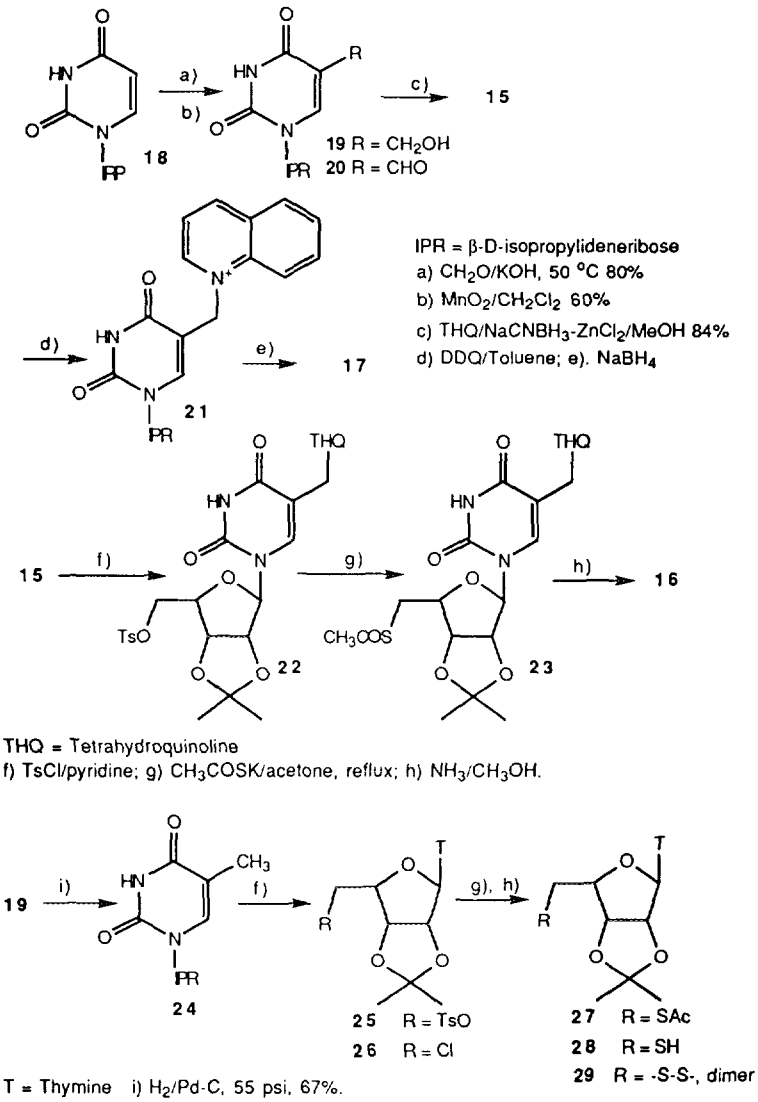
Due to the enhanced capability for the 5′-nucleophile to undergo nucleophilic addition to the C6 position with the protection of the 2′,3′-hydroxyl groups by an acetonide, uridine analogs **(15, 16, 17)** were designed to study the effect of C6 addition on the reductive elimination step of the reaction. Compounds **15** and **17** have a hydroxyl group at the 5′ position, whereas **16** has a 5′-thiol functional group, so that the effect of nucleophiles with different tendencies to undergo C6 addition could also be investigated. As in the models used by Wilson and Mertes, tetrahydroquinoline or dihydroquinoline was used to mimic the H_4folate moiety of the enzymatic system (Fig. 1).

RESULTS AND DISCUSSION

Synthesis

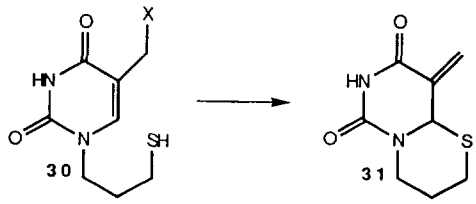
Although reactions of uracil with formaldehyde and amines can afford the corresponding C5-methylamine uracil derivatives (*11, 12*), refluxing isopropylideneuridine **(18)** (*13*) with tetrahydroquinoline and formaldehyde in aqueous solution gave only a low yield of the desired product **(15)** due to the heterogeneous nature of the reaction mixture. Despite the fact that this is a shorter route, experimentally the complexity of the reaction mixture made the purification of the desired product **(15)** difficult. Therefore an alternative method was used for the synthesis of **15.**

The C5 methylhydroxyl analog **(19)** (*14*) was oxidized to its aldehyde derivative



SCHEME 3. Synthesis of models **15, 16, 17** and standards **24, 28.**

(20) with MnO_2 in an 48% overall yield (Scheme 3) (*15*). The tetrahydroquinoline moiety was introduced by reductive amination with sodium cyanoborohydride in the presence of zinc chloride to give **15** (*16*). Oxidation of the tetrahydroquinoline model **(15)** with dichlorodicyanoquinone (DDQ) afforded the quinoline derivative **(21).** Reduction of **21** using sodium borohydride gave the dihydroquinoline model **(17)** (*5, 6*). Due to the instability of the dihydroquinoline model **(17),** it was subjected to pyrolysis without purification.



SCHEME 4. Proposed mechanism for the decomposition of **30.**

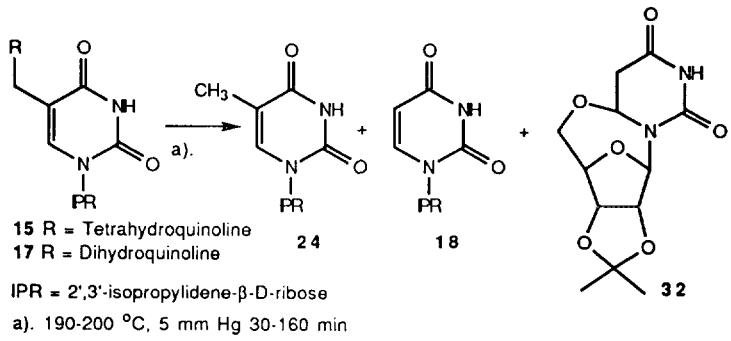
For the synthesis of the corresponding 5′-thiol model **(16), 15** was reacted with toluenesulfonyl chloride (tosyl chloride) followed by refluxing with potassium thioacetate in acetone to give the thioacetyl-isopropylideneuridine derivative **(23)** (Scheme 3). The acetyl protecting group was removed by ammonia to give the thiol model compound **16.**

Compounds **24** and **28** were also synthesized as standards for the expected pyrolysis products of the models **(15, 16, 17).** The synthesis of 5-methylisopropylideneuridine **(24)** was achieved through catalytic hydrogenation of the C5 methylhydroxyl derivative **(19)** (Scheme 3). The thiol analog **(28)** was prepared starting with **24,** following procedures similar to those described for the preparation of model **16.** During the tosylation of **24** to give **25,** the 5′-chloro compound **(26)** was also formed, but not isolated, and the yield of **26** increased with prolonged reaction time. The mixture of the 5′-tosyl analog **(25)** and 5′-chloro analog **(26)** was converted to 5′-thioacetyl derivative **(27)** which underwent aminolysis to afford the final compound **(28),** which is easily air-oxidized to form the disulfide **(29).** The thiol compound **(28),** however, can be obtained by reduction with $NaBH_4$.

The 5′-thiol model **(16)** was not stable at room temperature; it decomposed into a mixture of several components, which may explain the low yield (15%) of this comound **(16)** from the aminolysis of its precursor **(23)** (Scheme 3). In contrast, while similar reaction gave almost quantitative yield of **29** (Scheme 3), the preparation of this model **(16)** only gave 15% of the desired product.

It has been reported that mass spectral analysis of the decomposition of some analogous models (Scheme 4, **30**) gave a mixture containing an intermediate with the same molecular weight as that of the exomethylene intermediate **(31)** (*17*). Because compounds without the intramolecular nucleophile were stable under the same conditions, a mechanism involving the exomethylene intermediate **(31)** was proposed for the decomposition reaction (Scheme 4) (*17*).

The situation for model **16** should be similar to that of **30** and no attempt was made to isolate each component of the decomposition mixture. However, all of these demonstrate the significance of intramolecular C6 addition in facilitating the elimination of the C5-methyl substituent(s) (*8*). It also demonstrates that the nucleophilicity of the intramolecular nucleophile is very important for the model studies. It has to be strong enough so that under pyrolysis conditions, C6 nucleophilic addition can occur. However, it should not be too strong, so that the models are stable enough at room temperature.



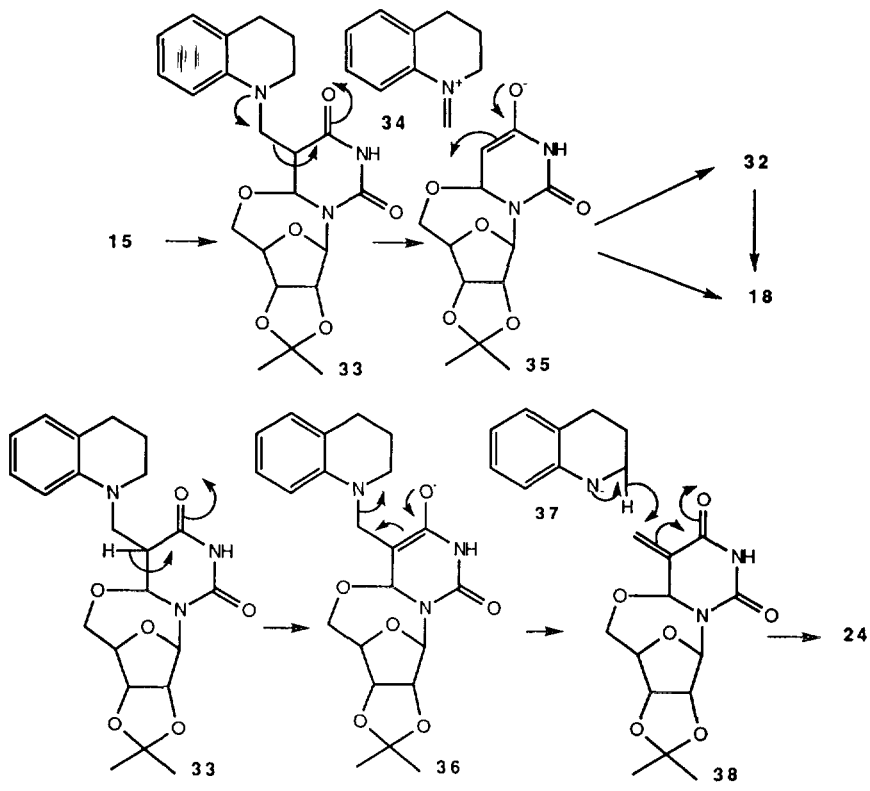
SCHEME 5. Pyrolysis of the model systems **(15, 17)**.

Model Studies

When the 5'-hydroxyl dihydroquinoline model **(17)** was pyrolyzed at 190–200°C under vacuum (5 mm Hg) for 1.5 h, 12% of the reductive elimination product **(24)** was obtained (Scheme 5). However, the yield of the reductive elimination product **(24)** was lower (2%) with the tetrahydroquinoline model **(15).** The formation of the reductive elimination product **(24)** from the pyrolysis of such model systems **(15, 17)** demonstrates for the first time that model systems with N1 glycoside linkage can undergo reductive elimination reactions similar to what was proposed for the TS-catalyzed process. The major chemical difference between the current models **(15, 17)** and those **(8b, 9b)** studied by Wilson and Mertes was the incorporation of an intramolecular nucleophile into the current systems **(15, 17).** The pyrolysis results shown above suggest that the presence of such intramolecular nucleophiles in **15, 17** facilitated the reductive elimination reaction.

Also isolated from the pyrolysis reaction was 2',3'-isopropylideneuridine **(18,** 9%) (Scheme 5). The yield of **18** was much higher (48%) when the pyrolysis was carried out for a shorter time (30 min). When the pyrolysis was carried out for only 30 min, O^6, 5'-cyclo-2',3'-isopropylideneuridine **(32,** 2%) was also isolated (*18*). The isolation of **32** further substantiates the participation of C6 nucleophilic addition during the pyrolytic process of models **15** and **17.**

A detailed analysis of the mechanism for the formation of **18** illustrates further the participation of C6 nucleophilic addition. The formation of **18** is apparently the result of cleavage of the C5–CH2 single bond. Simple cleavage of such a carbon–carbon single bond should be an energetically very unfavorable process. However, if the 5'-hydroxyl group first adds to the C6 carbon of the model compound to give a C6 addition intermediate (**33,** Scheme 6), the cleavage of the C–C single bond is a simple reversal of the C5 alkylation reaction with the imine **(34).** Such a reversal of a condensation reaction is a relatively easy process. The intermediate **(35)** formed then can be readily converted to either O^6, 5'-cyclo-isopropylideneuridine **(32)** or 2',3'-isopropylideneuridine **(18).** The reactive imine intermediate **(34)** can further react with the products formed to give a mixture of many components. This may be the reason that longer reaction time decreases the yield of 2',3'-isopropylideneuridine **(18).**



SCHEME 6. Proposed mechanism for pyrolysis of model **15.**

The isolation of **18** and O^6, 5′-cyclo-2′,3′-isopropylideneuridine **(32)** strongly supports the participation of the 5′-hydroxyl group through C6 addition. Similarly, the mechanism for the formation of the reductive elimination product (**24,** Scheme 6) probably also proceeds through a common intermediate with C6 addition **(33).** The formation of the C6 addition product **(33)** actually changes the chemistry of the C–N bond cleavage from a simple single bond cleavage to generate high-energy intermediates such as a carbocation or a radical to an energetically favorable β-elimination process. Such a change can account for the contribution of C6 addition on the reductive elimination reaction in the enzymatic process. Similarly, the reason that the pyrolysis of the deoxyuridine model **(9b)** (*5, 6*) did not give deoxythymidine is that the 5′-hydroxyl group of deoxythymidine has a much lower tendency for C6 addition (*8*) and the N1 substitution prevents the formation of the exomethylene intermediate (**13,** Scheme 2) through an energetically favorable β-elimination process.

When N1 is not substituted by an alkyl group, the participation of the N1 nitrogen through an anion formation (Scheme 2) is essentially the same as a C6 addition, which facilitates the formation of the exomethylene intermediate **(13).** However, when N1 is substituted with an alkyl group, such ionization by a base

becomes impossible and thus the amide nitrogen cannot participate in facilitating the reductive elimination through a β-elimination process.

Although the current model studies were carried out under more forcing conditions than in the biological system, several factors in the enzyme could be contributing to the lowering of the activation energy in the enzymatic system and force the reaction to go forward. First, as has been proposed by Wilson and Mertes (*5, 6*), enzyme conformational change could provide some driving force for the reaction. Secondly, the reversal of the alkylation, which seems to be the major reason that the pyrolysis of models **15** and **17** gave a low yield of the reductive elimination product **(24)**, can be controlled by the enzyme through conformational changes to favor the forward reaction.

Implications of the Model Thermolysis on the Enzymatic Reaction Mechanism

The chemical feasibilities for the proposed ternary intermediate **(3)** to undergo reductive elimination was demonstrated for the first time with model systems in which there is an N1 glycoside bond. These model studies demonstrated the importance of C6 nucleophilic addition (or its mechanistic equivalent such as N1 ionization) in facilitating the reductive elimination reaction and therefore indicated the significance of C6 nucleophilic addition in the enzymatic reaction (*8*). It showed that increased reducing power does facilitate the reductive elimination reaction. It also indicated that the major differences between the N1-substituted model and the N1-unsubstituted model are due to the participation of the N1 anion of the N1-unsubstituted models **(8a, 9a, 10a)** in the elimination reaction.

Similarly, in the enzyme the reductive elimination reaction can also be facilitated by the C6 sulfhydryl nucleophilic addition. Therefore, the function of C6 nucleophilic addition by an active site thiol group is important not only for the initiation of the overall TS-catalyzed reaction, but also for the facilitation of the reductive elimination step. The function of C6 addition is to change the reaction pathway from a simple C–N bond cleavage in a homolytical or hetereolytical fashion (*16, 19*) to an energetically favorable β-elimination reaction to give an α,β-unsaturated system which then undergoes reduction reaction. Therefore, pathway (b) (Scheme 1) is probably the favored process in the TS-catalyzed enzymatic reaction.

CONCLUSION

In conclusion, three model compounds **(15, 16, 17)** have been prepared through multistep synthesis. The chemical feasibility for the ternary intermediate **(9)** to undergo reductive elimination has been demonstrated using model systems in which there is an N1 glycoside bond. It also shows that efficient catalysis can be afforded by C6 addition for this step of the reaction, therefore supporting pathway (b) as the favored process.

EXPERIMENTAL PROCEDURES

Material and Methods

Proton and carbon nuclear magnetic resonance (^1H, ^{13}C NMR) spectra were obtained on either a Brucker AM-500 or a Varian XL-300 spectrometer. For ^1H NMR and ^{13}C NMR samples, TMS (0.00 ppm) and $CDCl_3$ (77.00 ppm) were utilized, respectively, as an internal standard. Data were reported as chemical shifts in ppm. The numbering of each atom in uridine analogs is similar to that of TMP (Scheme 1, **7**).

Electron-impact (EI) or chemical ionization (CI) mass spectra (MS) were recorded on a Nermag R10-10 (low resolution) mass spectrometer or a V6-ZAB double-focusing high-resolution mass spectrometer. Fast-atom bombardment MS experiments were performed using a Xenon gun operated at 8 keV energy and 0.8 mA emission. Only the major or diagnostically important peaks are reported as *m/e* (relative intensity). Ultraviolet spectra were recorded on either a Hewlett-Packard Diode Array Ultraviolet or a Carey 219 spectrometer. Flash chromatography was performed using Merck silica gel 60 (230–400 mesh). Gravity column chromatography was performed using Merck silica gel (70–260 mesh). Thick-layer chromatography was performed on Analtech Uniplate preparative plates (1 mm) with silica gel GF.

Tetrahydroquinoline (Aldrich) was purified by column chromatography (silica gel, methylene chloride) before use. Dry methanol was distilled from magnesium turnings and stored under argon over activated 3 Å moelcular sieves. All other reagents and solvents were used as obtained commercially without purification.

1,2,3,4-Tetrahydro-1-(2',3'-O-isopropylidenethymidyl)-quinoline (**15**)

To a 50-ml round-bottom flask was added 9.2 g of $ZnCl_2$. After the $ZnCl_2$ was fused by flaming and allowed to cool, 348 mg (1.1 mmol) of 5-formyl-2',3'-*O*-isopropylideneuridine (**20**) dissolved in 40 ml of methanol and 2.6 ml of tetrahydroquinoline (20.3 mmol) were added in sequence. Then the solution was stirred for 20 min at room temperature followed by the addition of 120 mg (1.9 mmol) of $NaCNBH_3$. After reaction at room temperature for 36 h under region with stirring, TLC showed the disappearance of the starting material. To the residue after solvent evaporation was added 20 ml of water. The product was extracted from the aqueous layer with CH_2Cl_2 (30 ml × 4 + 20 ml). After drying the organic layer over anhydrous Na_2SO_4 overnight and solvent evaporation, the residue was purified by column chromatography (15 g of silica gel) using a gradient of 1 to 3% of methanol in methylene chloride to give 370 mg of yellowish foam product (84% yield). uv (CH_3OH): λ_{max} = 259, 303 nm; ^1H NMR ($CDCl_3$): 9.12 (1 H, b, NH), 7.15 (1 H, s, C_6H), 6.95 (2 H, m, aromatic), 6.56 (1 H, t, 7.3 Hz, aromatic), 6.33 (1 H, d, 8.2 Hz, aromatic), 5.65 (1 H, d, 2.9 Hz, $C_{1'}H$), 4.71 (2 H, m, $C_{2'}H$, $C_{3'}H$), 4.15 (3 H, m, $C_{4'}H$, $C_{5'}H_2$), 3.45 (2 H, m, C_5CH_2), 3.29 (2 H, t, 5.8 Hz, NCH_2), 2.74 (2 H, t, 6.4 Hz, $PhCH_2$), 1.93 (2 H, m, $NCH_2C\underline{H}_2$), 1.48 (3 H, s, CH_3), 1.26 ppm (3 H, s, CH_3); ^{13}C NMR ($CDCl_3$): 163.21 (C_4), 150.27 (C_2), 144.58 (N$\underline{C}$CH), 137.52 (C_6), 129.21 (NCC$\underline{C}$H), 127.34 (NCCH$\underline{C}$H), 122.22 (NC$\underline{C}$CH), 116.43

(NCCHCHCH), 113.96 (C_5), 110.58 (NCCH), 109.75 ($(O)_2C(CH_3)_2$), 94.34 ($C_{1'}$), 86.24 ($C_{4'}$), 84.27 ($C_{2'}$), 80.56 ($C_{3'}$), 62.89 ($HOCH_2$), 50.13 (C_5CH_2N), 48.18 (NCH_2), 27.91 (CH_2Ph), 27.20 (CH_3), 25.21 (CH_3), 22.38 ppm (NCH_2CH_2); EIMS m/e (relative intensity): 429 (M^+, 47), 414 (M-CH_3, 3), 297 (M-132, 3), 258 (M-sugar, 9), 132 (96); HRMS: Calcd for $C_{22}H_{27}N_3O_6$: 429.190; found: 429.1914.

1,2,3,4-Tetrahydro-1-(5'-thiol-2',3'-O-isopropylidenethymidyl)-quinoline (**16**)

To 114 mg (0.23 mmol) of 1,2,3,4-tetrahydro-1-(5'-thioacetyl-2',3'-*O*-isopropylidenethymidyl)-quinoline (**23**) in a round-bottom flash was added 5 ml of methanol. After bubbling NH_3 gas at room temperature into the reaction solution for 30 min, TLC showed complete disappearance of the starting material. The solvent was evaporated, and the solid residue was purified by column chromatography (15 g of silica gel) using a gradient solvent of methylene chloride to 3% methanol in methylene chloride to give 16 mg of a white solid product (15% yield). uv (CH_3OH): λ_{max} = 259, 306 nm; ^1H NMR ($CDCl_3$): 9.35 (1 H, NH), 6.99 (3 H, m, aromatic, C_6H), 6.62 (1 H, t, 7.3 Hz, aromatic), 6.39 (1 H, d, 8.2 Hz, aromatic), 5.55 (1 H, b, $C_{1'}H$), 4.5961 (1 H, d, 6.5 Hz, $C_{2'}H$), 4.63 (1 H, m, $C_{3'}H$), 4.26 (1 H, m, $C_{4'}H$), 4.17 (2 H, m, C_5H_2), 3.33 (2 H, t, 5.3 Hz, NCH_2), 2.91–2.79 (4 H, m, C_5CH_2, $PhCH_2$), 2.00 (2 H, t, 5.4 Hz, NCH_2CH_2), 1.52 (3 H, s, CH_3), 1.31 ppm (3 H, s, CH_3); ^{13}C NMR ($CDCl_3$): 163.31 (C_4), 149.87 (C_2), 144.74 (NCCH), 138.64 (C_6), 129.33 (NCCCH), 127.38 (NCCHCH), 122.51 (NCCCH), 116.83 (NCCCHCH), 114.40 (C_5), 110.97 (NCCHCH), 110.66 ($(O)_2C(CH_3)_2$), 95.03 ($C_{1'}$), 86.13 ($C_{4'}$), 84.64 ($C_{2'}$), 83.26 ($C_{3'}$), 50.37 (C_5CH_2N), 48.56 (NCH_2), 41.42 ($HSCH_2$), 27.92 ($PhCH_2$), 27.03 (CH_3), 25.19 (CH_3), 22.53 ppm (NCH_2CH_2); EIMS m/e (relative intensity): 445 (M^+, 2), 256 (M-sugar, 3), 132 (100); HRMS: Calcd for $C_{22}H_{27}N_3O_5S$: 445.1670; found: 445.1678.

1,2,3,4-Tetrahydro-1-(5'-toluenesulfonyl-2',3'-O-isopropylidene-thymidyl)-quinoline (**22**)

To the solution of 323 mg (0.75 mmol) of 1,2,3,4-tetrahydro-1-(2',3'-*O*-isopropylidenethymidyl)-quinoline (**15**) in 40 ml of pyridine was added 194 mg (1.02 mmol) of *p*-toluenesulfonyl chloride. After stirring of the reaction at room temperature under argon atmosphere for 20 h, the reaction was quenched by the addition of 0.5 ml of H_2O. After stirring of the quenched reaction mixture for 30 min, the solvent was evaporated and product was purified by column chromatography (20 g silica gel) using a gradient of 0–3% of methanol in methylene chloride to give 139 mg of an oil product (32% yield). uv (CH_3OH): λ_{max} = 259, 305 nm; ^1H NMR ($CDCl_3$): 9.99 (1 H, b, NH), 7.68 (2 H, d, 8.3 Hz, $SC(CH)_2$, aromatic), 7.23 (2 H, d, 8.2 Hz, $CH_3C(CH)_2$, aromatic), 6.91 (3 H, m, aromatic, C_6H), 6.55 (1 H, t, 7.4 Hz, aromatic), 6.36 (1 H, d, 8.3 Hz, aromatic), 5.38 (1 H, b, $C_{1'}H$), 4.89 (1 H, d, 6.3 Hz, $C_{2'}H$), 4.63 (1 H, dd, 6.2 Hz, 3.4 Hz, $C_{3'}H$), 4.13 (5 H, m, $C_{4'}H$, $C_{5'}H_2$, C_5CH_2), 3.26 (2 H, t, 5.5 Hz, NCH_2), 2.73 (2 H, t, 5.9 Hz, $PhCH_2$), 2.35 (3 H, s, $PhCH_3$), 1.93 (3 H, t, 5.8 Hz, NCH_2CH_2), 1.41 (3 H, s, CH_3), 1.22 ppm (3 H, s, CH_3); ^{13}C NMR ($CDCl_3$): 163.62 (C_4), 149.99 (C_2), 144.93 (NC), 144.72 (SC), 139.19 (C_6), 132.58 (SCCHCHC), 129.69 ($SC(CH)_2$), 129.18 (NCCCH), 127.86

($CH_3C(\underline{C}H)_2$), 127.24 (NCCH$\underline{C}$H), 122.56 (NC$\underline{C}$), 116.70 (NCCCH$\underline{C}$H), 114.29 (C_5), 110.94 (NC$\underline{C}$H), 110.78 ($(O)_2\underline{C}(CH_3)_2$), 96.07 ($C_{1'}$), 85.59 ($C_{4'}$), 84.28 ($C_{2'}$), 81.08 ($C_{3'}$), 69.46 ($C_{5'}$), 50.23 ($C_5\underline{C}H_2N$), 48.55 ($NCH_2$), 27.29 ($PhCH_2$), 26.83 ($CH_3$), 25.03 ($CH_3$), 22.34 ($NCH_2\underline{C}H_2$), 21.54 ppm ($PhCH_3$); EIMS m/e (relative intensity): 583 (M^+, 6), 568 (M–CH_3, 1), 256 (M-sugar, 6), 132 (100); HRMS: Calcd $C_{29}H_{33}N_3O_8S$: 583.1988; found: 583.1994.

1,2,3,4-Tetrahydro-1-(5'-thioacetyl-2',3'-O-isopropylidenethymidyl)-quinoline (**23**)

To 139 mg (0.24 mmol) of 1,2,3,4-tetrahydro-1-(5'-toluenesulfonyl-2',3'-*O*-isopropylidenethymidyl)-quinoline (**22**) in a 25-ml round-bottom flask was added 281 mg (2.47 mmol) of potassium thioacetate and 10 ml of acetone. After refluxing under argon for 5 h, TLC showed the disappearance of the starting material. The reaction solution was filtered with gravity filtration and the residue washed with methylene chloride. After evaporation of solvent, the crude product was purified by column chromatography (10 g of silica gel) using a gradient solvent system of methylene chloride to 2% methanol in methylene chloride to give 114 mg of yellow foam product (98% yield). uv (CH_3OH): λ_{max} = 259, 334 nm; ^1H NMR ($CDCl_3$): 10.01 (1 H, b, NH), 7.00 (3 H, m, aromatic, C_6H), 6.61 (1 H, t, 7.3 Hz, aromatic), 6.29 (1 H, d, 8.3 Hz, aromatic), 5.55 (1 H, b, $C_{1'}H$), 4.91 (1 H, dd, 6.5 Hz, 1.9 Hz, $C_{2'}H$), 4.58 (1 H, dd, 6.4 Hz, 4.0 Hz, $C_{3'}H$), 4.19–4.10 (3 H, m, $C_{4'}H$, $C_{5'}H_2$), 3.34 (2 H, t, 5.4 Hz, NCH_2), 3.11 (2 H, dd, 6.8 Hz, 1.8 Hz, C_5CH_2N), 2.81 (2 H, t, 5.9 Hz, $PhCH_2$), 2.31 (3 H, s, CH_3CO), 2.01 (3 H, t, 5.4 Hz, $NCH_2C\underline{H}_2$), 1.51 (3 H, s, CH_3), 1.31 ppm (3 H, s, CH_3); ^{13}C NMR ($CDCl_3$): 194.46 ($CH_3\underline{C}O$), 163.62 (C_4), 150.01 (C_2), 144.73 (C$\underline{C}$CH), 138.36 (C_6), 129.18 (NCC$\underline{C}$H), 127.28 (NCCH$\underline{C}$H), 122.38 (NC$\underline{C}$CH), 116.71 (NCCCH$\underline{C}$H), 114.33 (C_5), 110.91 (NC$\underline{C}$HCH), 110.61 ($(O)_2\underline{C}(CH_3)_2$), 94.68 ($C_{1'}$), 86.25 ($C_{4'}$), 84.59 ($C_{2'}$), 83.17 ($C_{3'}$), 50.25 ($NCH_2$), 48.57 ($C_5\underline{C}H_2N$), 31.10 ($COS\underline{C}H_2$), 30.42 ($\underline{C}H_3CO$), 27.85 ($Ph\underline{C}H_2CH_2$), 26.94 ($CH_3$), 25.15 ($CH_3$), 22.44 ppm ($NCH_2\underline{C}H_2$); EIMS m/e (relative intensity): 487 (M^+, 7), 256 (M-sugar, 1), 132 (40); HRMS: Calcd for $C_{24}H_{29}N_3O_6S$: 487.1775; found: 487.1776.

2',3'-O-Isopropylidenethymidine (**24**)

Method A. To the solution of 85 mg (0.27 mmol) of 5-methylhydroxyl-2',3'-*O*-isopropylideneuridine (**19**) in 13.5 ml of acetone in a Parr apparatus was added 40 mg of 10% pd on charcoal and 1.5 ml of concentrated HCl (36%). After reaction at room temperature under 50 psi of hydrogen gas for 4 h and 20 min, the reaction solution was filtered and neutralized with saturated Na_2CO_3 solution. Then acetone was evaporated and the product extracted with chloroform (50 ml × 3). The combined organic layers were then dried over anhydrous Na_2SO_4. Evaporation of the solvent gave 54 mg of a colorless viscous oil product (67% yield). uv (CH_3OH): λ_{max} = 264 nm; ^1H NMR ($CDCl_3$): 8.94 (1 H, b, NH), 7.14 (1 H, s, C_6H), 5.50 (1 H, d, 3.1 Hz, $C_{1'}H$), 5.10 (1 H, dd, 6.3 Hz, 2.9 Hz, $C_{2'}H$), 4.98 (1 H, dd, 6.5 Hz, 3.5 Hz, $C_{3'}H$), 4.27 (1 H, m, $C_{4'}H$), 3.82 (2 H, m, $C_{5'}H_2$), 1.92 (3 H, s, C_5CH_3), 1.58 (3 H, s, $(O)C(CH_3)$), 1.36 ppm (3 H, s, $(O)C(CH_3)$; ^{13}C NMR

($CDCl_3$): 163.55 (C_4), 150.39 (C_2), 139.07 (C_6), 114.44 ($(O)_2\underline{C}(CH_3)_2$), 111.26 ($C_5$), 96.47 ($C_{1'}$), 86.76 ($C_{4'}$), 83.19 ($C_{2'}$), 80.27 ($C_{3'}$), 62.71 ($HOCH_2$), 27.27 ($C(\underline{C}H_3)_2$), 25.24 ($C(\underline{C}H_3)_2$), 12.29 ppm ($C_5\underline{C}H_3$); EIMS m/e (relative intensity.): 299 (M + 1,9), 283 (M–CH_3, 15), 126 (M–sugar, 21); HRMS: Calcd $C_{13}H_{18}N_2O_6$: 298.1165; found: 298.1169.

Method B. To a 10-ml round-bottom flask connected to another round-bottom flask, which was immersed into a dry ice–acetone bath and connected to a vacuum pump, was added 175 mg (0.41 mmol) of 1,2,3,4-tetrahydro-1-(2′,3′-*O*-isopropylidenethymidyl)-quinoline (**15**). The reaction was carried out at 195–200°C in an oil bath for 2.67 h under vacuum (5 mm Hg). Fractions collected from the top and the neck of the round-bottom flask were purified by preparative TLC (CH_2Cl_2 : CH_3OH 9 : 1) to give 2 mg (2% yield) of an oily product. The product was identical to an authentic sample prepared by method A as described above (NMR, uv, HPLC, and MS). The fraction at the bottom of the flask was further purified by column chromatography to give 12 mg (9% yield) of isopropylideneuridine (**18**), which was identical to an authentic sample obtained by following method A (NMR, uv, MS).

Method C. To 48 mg (0.11 mmol) of 1,2,3,4-tetrahydro-1-(2′,3′-*O*-isopropylidene-thymidyl)-quinoline (**15**) dissolved in 15 ml of toluene was added 66 mg (0.29 mmol) of dichlorodicyanoquinone (DDQ). After stirring the reaction at room temperature under argon for 4 h, a precipitate was observed, and TLC showed no more starting material. Instead a new spot near the origin of the TLC was observed (solvent system: methylene chloride : methanol, 9 : 1) with no higher R_f material evident. After evaporation of solvent, the residue was dissolved in 10 ml of methanol. To this solution, 37 mg of $NaBH_4$ (1 mmol) was added. The color of the reaction solution changed from purple to light yellow. After stirring the reaction at room temperature under argon for 1 h, two new spots with higher R_fs than the 2′,3′-*O*-isopropylidene-thymidine (**24**) were observed. The residue from solvent evaporation was subjected to the same condition as described in method B for 1.5 h. The residue in the flask was then dissolved in 10 ml of H_2O and extracted with methylene chloride (20 ml × 4). After drying the combined organic layers over anhydrous Na_2SO_4, solvent was evaporated to give 35 mg of product, which was purified by column chromatography (20 g silica gel) using a gradient of 0–2% methanol in methylene chloride. The fraction from column chromatography containing the desired product was further purified with preparative TLC (silica gel CH_2Cl_2 : CH_3OH 9 : 1) to give 4 mg (12% yield) of an oily product, which was shown to be identical with the authentic sample prepared as described in method A (uv, ^1H NMR, MS, HPLC).

5′-Toluenesulfonyl-2′,3′-O-isopropylidenethymidine (**25**)

Method A. To a solution of 150 mg (0.50 mmol) of 2′,3′-*O*-isopropylidenethymidine (**24**) in 10 ml of pyridine was added 154 mg (0.81 mmol) of toluenesulfonyl chloride. The reaction was stirred at room temperature for 20 h and then 3 ml of water was added to quench unreacted toluenesulfonyl chloride. Another 10 ml of water was added after stirring the quenched reaction mixture for 2 h. The product

was extracted with methylene chloride (10 ml × 5). The combined methylene chloride extracts were dried over anhydrous Na_2SO_4 for 1 h. Solvent evaporation gave 212 mg of a white solid product which was purified with preparative TLC plate (CH_2Cl_2 : CH_3OH, 9 : 1) to give 147 mg of a white solid product **(25)**, which was contaminated with a small amount (8%) of 5′-chloro-2′,3′-*O*-isopropylidenethymidine **(26)**. **25:** uv (CH_3OH): λ_{max} = 265 nm; ^1H NMR ($CDCl_3$): 9.77 (1 H, b, NH), 7.76 (2 H, d, 8.3 Hz, $SC(CH)_2$), 7.32 (2 H, d, 8.3 Hz, $CH_3C(C\underline{H})_2$), 7.13 (1 H, s, C_6H), 5.69 (1 H, d, 2.1 Hz, $C_{1'}H$), 4.95 (1 H, dd, 6.5 Hz, 2.1 Hz, $C_{2'}H$), 4.80 (1 H, dd, 6.40 Hz, 3.6 Hz, $C_{2'}H$), 4.28 (3 H, m, $C_{4'}H$, $C_{5'}H_2$), 2.43 (3 H, s, $PhCH_3$), 1.94 (3 H, s, C_5CH_3), 1.54 (3 H, s, CH_3), 1.33 ppm (3 H, s, CH_3); ^{13}C NMR ($CDCl_3$): 164.03 (C_4), 150.20 (C_2), 145.13 ($\underline{C}S$), 137.97 (C_6), 132.43 ($CH_3\underline{C}(CH)_2$), 129.79 (aromatic CH), 127.90 (aromatic CH), 114.48 (C_5), 111.28 ($(O)_2\underline{C}(CH_3)_2$), 94.20 ($C_{1'}$), 84.65 ($C_{4'}$), 84.15 ($C_{2'}$), 80.69 ($C_{3'}$), 69.45 ($C_{5'}$), 26.78 ($CH_3$), 24.96 ($CH_3$), 21.41 ($PhCH_3$), 12.09 ppm ($C_5\underline{C}H_3$); CIMS m/e (relative intensity): 453 (M + 1,16), 437 (M-CH_3, 12), 127 (M-sugar, 55); HRMS: Calcd $C_{20}H_{25}N_2O_8S$: 453.13299; found: 453.1329.

Method B. To a solution of 395 mg (1.32 mmol) of 2′,3′-*O*-isopropylidenethymidine **(24)** in 7 ml of pyridine was added 447 mg (2.35 mmol) of toluenesulfonyl chloride. The reaction was stirred at room temperature for 62 h followed by the addition of 2 ml of water to quench the unreacted toluenesulfonyl chloride. After stirring the quenched reaction mixture for 15 min, product was extracted with 70 ml of chloroform. The combined chloroform layer was then washed with water (2 ml × 2) and dried over anhydrous Na_2SO_4. Solvent evaporation gave 456 mg of a crude product mixture as a white solid which was purified by column chromatography (20 g silica gel) using a gradient solvent system 0–3% methanol in methylene chloride to give 321 mg of a white solid product, which NMR showed to be mixture of 5′-tosyl-2′,3′-isopropylidenethymidine **(25)** and 5′-chloro-2′,3′-*O*-isopropylidenethymidine **(26)** (3 : 2, yield 61%). 5′-Chloro-2′,3′-*O*-isopropylidenethymidine **(26)**: ^1H NMR ($CDCl_3$): 10.28 (1 H, b, NH), 7.21 (1 H, s, C_6H), 5.69 (1 H, b, $C_{1'}H$), 5.06 (1 H, dd, 6.6 Hz, 2.0 Hz, $C_{2'}H$), 4.92 (1 H, dd, 6.6 Hz, 3.6 Hz, $C_{3'}H$), 3.79 (3 H, m, $C_{4'}H$, $C_{5'}H_2$), 1.92 (3 H, s, C_5CH_3), 1.57 (CH_3), 1.36 ppm (CH_3); ^{13}C NMR ($CDCl_3$): 164.32 (C_4), 150.20 (C_2), 138.48 (C_6), 114.29 (C_5), 110.94 ($(O)_2\underline{C}(CH_3)_2$), 94.52 ($C_{1'}$), 86.48 ($C_{4'}$), 84.06 ($C_{2'}$), 80.61 ($C_{3'}$), 44.12 ($ClCH_2$), 26.78 ($CH_3$), 24.96 ($CH_3$), 12.02 ppm ($C_5\underline{C}H_3$).

5′-Thioacetyl-2′,3′-O-isopropylidenethymidine **(27)**

To 320 mg (0.71 mmol) of 5′-toluenesulfonyl-2′,3′-*O*-isopropylideneuridine **(25)** dissolved in 8 ml of acetone was added 223 mg (1.95 mmol) of potassium thioacetate. After refluxing under argon atmosphere for 3 h, the reaction solution was cooled down and the solvent evaporated. The product was purified by column chromatography (20 g silica gel) using a gradient of 0–2% methanol in methylene chloride to give 287 mg of a foamy white product (100% yield). uv (CH_3OH): λ_{max} = 263 nm; 1H NMR ($CDCl_3$): 10.27 (1 H, s, NH), 7.13 (1 H, s, C_6H), 5.59 (1 H, b, $C_{1'}H$), 5.07 (1 H, dd, 6.6 Hz, 1.6 Hz, $C_{2'}H$), 4.76 (1 H, dd, 6.3 Hz, 4.0 Hz, $C_{3'}H$), 4.19 (1 H, m, $C_{4'}H$), 3.28 (2 H, m, $C_{5'}H_2$), 2.37 (3 H, s, $COCH_3$), 1.93

(3 H, s, C_5CH_3), 1.54 (3 H, s, CH_3), 1.34 ppm (3 H, s, CH_3); ^{13}C NMR ($CDCl_3$): 194.53 ($CH_3\underline{C}O$), 164.36 (C_4), 150.15 (C_2), 138.67 (C_6), 114.22 (C_5), 110.86 ($\underline{C}(CH_3)_2$), 94.74 ($C_{1'}$), 86.09 (C_4), 84.23 ($C_{2'}$), 83.17 ($C_{3'}$), 31.06 (SCH_2), 30.36 ($\underline{C}H_3CO$), 26.81 (CH_3), 25.01 (CH_3), 12.10 ppm ($C_5\underline{C}H_3$); CIMS m/e (relative intensity): 357 (M + 1, 100), 341 (M-CH_3, 6), 299 (M-57, 44), 127 (M-sugar, 35); HRMS: Calcd $C_{15}H_{20}N_2O_6S$: 356.1042; found: 356.1049.

5'-Thiol-2',3'-O-isopropylidenethymidine disulfide (**29**)

To 124 mg of 5'-thioacetyl-2',3'-*O*-isopropylidenethymidine (**27**) in a pear-shaped flask was added 5 ml of methanol. After bubbling NH_3 into the reaction mixture at room temperature for 2 h followed by stirring at room temperature overnight, the TLC of the reaction mixture still showed some starting material. Additional NH_3 gas was bubbled into the reaction mixture for another 4 h before the solvent was evaporated. The solid residue was purified by silica gel column chromatography using a gradient of 0–3% methanol in methylene chloride to give 109 mg of white solid product (100% yield). uv (CH_3OH): λ_{max} = 264 nm; ^1H NMR ($CDCl_3$): 10.32 (1 H, s, NH), 7.11 (1 H, s, C_6H), 5.55 (1 H, b, $C_{5'}H$), 5.10 (1 H, m, $C_{2'}H$), 4.84 (1 H, dd, 6.4 Hz, 4.0 Hz, $C_{3'}H$), 4.36 (1 H, m, $C_{4'}H$), 3.10 (2 H, m, $C_{5'}H_2$), 1.91 (3 H, s, C_5CH_3), 1.56 (3 H, s, CH_3), 1.34 ppm (3 H, s, CH_3); ^{13}C NMR ($CDCl_3$): 164.54 (C_4), 150.22 (C_2), 139.08 (C_6), 114.25 (C_5), 110.93 ($\underline{C}(CH_3)_2$), 95.43 ($C_{1'}$), 86.16 (C_4), 84.30 ($C_{2'}$), 83.30 ($C_{3'}$), 41.48 (SCH_2), 26.94 (CH_3), 25.11 (CH_3), 12.21 ppm ($C_5\underline{C}H_3$); EIMS m/e (relative intensity): 626 (M^+, 1), 611 (M-CH_3, 1), 313 (M-313, 3), 127 (35); HRMS: Calcd $C_{24}H_{34}N_4O_{10}S_2$: 626.1714; found: 626.1727.

O^6, 5'-Cyclo-5,6-dihydro-2',3'-O-isopropylideneuridine (**32**)

To a 25-ml round-bottom flask connected to another round-bottom flask immersed into a dry ice–acetone bath and connected to a vacuum pump was added 361 mg of 5-methyl-N_1-tetrahydroquinoline-isopropylideneuridine (**15**). The reaction was carried out at 190–210°C oil bath temperature for 30 min under vacuum (5 mm Hg). After purification of the reaction mixture by column chromatography (25 g of silica gel) with 2% methanol in methylene chloride, 5 mg of an oil product was obtained (**32**). ^1H NMR ($CDCl_3$): 8.32 (1 H, s, NH), 6.22 (1 H, s, $C_{1'}H$), 5.07 (1 H, dd, 3 Hz, 8.4 Hz, $C_{2'}H$), 4.73 (2 H, s, C_5H), 4.52 (1 H, d, 1.8 Hz, C_6H), 4.04 (1 H, d, 6.6 Hz, $C_{3'}H$), 3.73 (1 H, dd, 3 Hz, 12.6 Hz, $C_{4'}H$), 2.97 (1 H, dd, 5.4 Hz, 17.1 Hz, $C_{5'}H$), 2.76 (1 H, dd, 8.4 Hz, 17.1 Hz, $C_{5'}H$), 1.53 (3 H, s, CH_3), 1.36 ppm (3 H, s, CH_3). ^{13}C NMR ($CDCl_3$): 166.80 (C_4), 150.40 (C_2), 112.72 ($(CH_3)_2\underline{C}(O)_2$), 92.06 ($C_6$), 87.48 ($C_{1'}$), 86.90 ($C_{4'}$), 84.05 ($C_{2'}$), 82.31 ($C_{3'}$), 74.38 ($C_{5'}$), 37.95 ($C_5$), 26.23 ($CH_3$), 24.63 ppm (–$CH_3$); EIMS m/e (relative intensity): 285 (M + 1, 1), 269 (M-15, 15); HRMS: Calcd for $C_{12}H_{16}N_2O_6$: 284.1007; found: 284.1014. Further elution gave 120 mg of a white solid product (48% yield), identical with isopropylideneuridine by ^1H and ^{13}C NMR and uv spectra, and MS.

ACKNOWLEDGMENTS

The financial support from NIH (RR5606) and the Wesley Foundation is gratefully acknowledged.

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