Synthesis of 5-[(Methylthio)methyl]-2′-deoxyuridine, the Corresponding Sulfoxide and Sulfone, and Their 5′-Phosphates: Antiviral Effects and Thymidylate Synthetase Inhibition

Charles L. Schmidt, Charles T.-C. Chang, Erik De Clercq, Johan Descamps, and Mathias P. Mertes*

Department of Medicinal Chemistry, University of Kansas, Lawrence, Kansas 66045, and the Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium. *Received August 27, 1979*

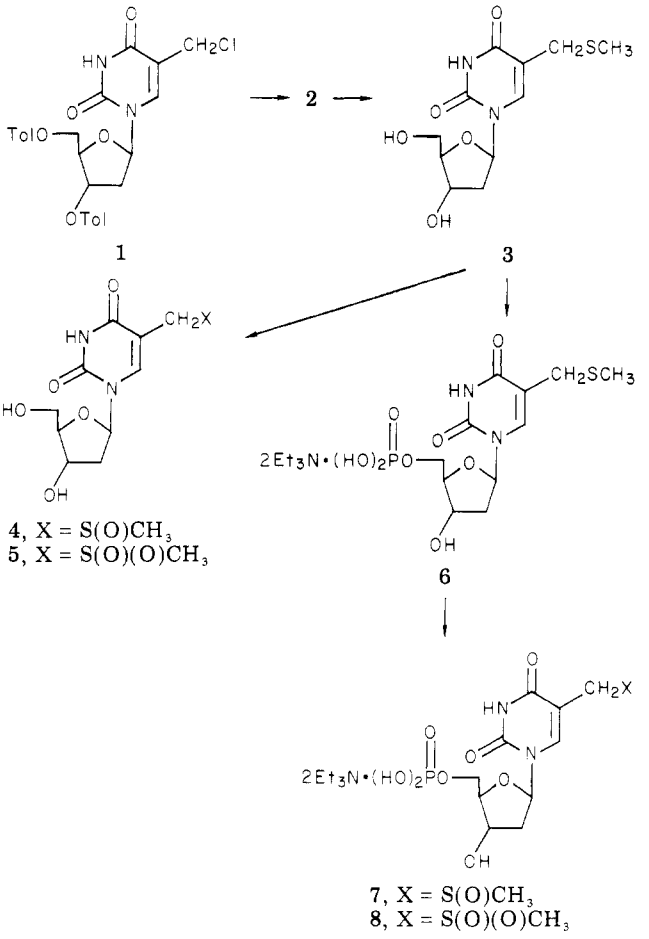
Substitution on the α position of thymidine with methylthio (**3**) and methylsulfonyl (**5**) groups gave antiviral agents that were specific and relatively nontoxic inhibitors of herpes simplex virus replication in cell culture. The thioether (**3**) was effective against both types 1 and 2 of herpes simplex virus, whereas the activity of the sulfone derivative (**5**) was restricted to herpes simplex virus type 1. The sulfoxide derivative 1-(2-deoxy-β-D-ribofuranosyl)-α-(methylsulfinyl)thymine (**4**) was inactive as an antiviral agent. The 5′-phosphates of these three thymidine derivatives were relatively potent inhibitors of thymidylate synthetase (K_i values range from 7.8 to 1.9 μM). It is improbable that the inhibition of this enzyme accounts for the anti-herpes activity of compounds **3** and **5**.

In addition to the clinically effective iodo and trifluoromethyl derivatives, other 5-substituted 2′-deoxyuridine nucleosides have been identified as potent antiviral agents.[1] The activity of these nucleosides is restricted to DNA viruses and certain oncornaviruses. Emphasis on selective activity has revealed differential inhibitory actions against herpes simplex (HSV 1 and 2) and vaccinia virus. 5-Nitro- and 5-cyano-2′-deoxyuridine had greater inhibitory potency against vaccinia replication, while the 5-vinyl, 5-ethynyl, 5-(propynyloxy), 5-ethyl, 5-propyl, 5-allyl, and (*E*)-5-(2-halovinyl) derivatives exhibited potent anti-herpes actions.[1,2] The most potent and least toxic agents in the latter series were the (*E*)-5-(2-iodovinyl) and (*E*)-5-(2-bromovinyl) derivatives. However, significant selectivity, as measured by the ratio of in vitro toxic and effective doses ($TD_{50}/ED_{50} > 100$), also was observed for the 5-propyl and 5-vinyl nucleosides. On this basis the title compound, 5-[(methylthio)methyl]-2′-deoxyuridine (**3**) was synthesized as an isoelectronic analogue of the active alkyl, vinyl, and allyl derivatives in the quest for greater efficacy and safety as anti-herpes agents. In addition, the corresponding sulfoxide (**4**) and sulfone (**5**) nucleosides were prepared.

Previous studies have questioned a relationship between antiviral potency of 5-substituted 2′-deoxyuridines against DNA viruses and thymidylate synthetase inhibition. 5-(Trifluoromethyl)-2′-deoxyuridine, an effective inhibitor of herpes simplex replication, is also a potent inhibitor of thymidylate synthetase. The 5′-phosphates (**6–8**) of the title compounds were prepared for evaluation as in vitro inhibitors of thymidylate synthetase.

The most convenient preparation of these compounds commenced with the 5-(chloromethyl) derivative (**1**) of the diesterified β anomer of 2′-deoxyuridine.[3] Treatment of (**1**) with an excess of methanethiol and 1 equiv of sodium methoxide in methanol gave the diprotected 5-[(methylthio)methyl] nucleoside **2** (Scheme I). Use of an excess quantity of sodium methoxide and methanethiol (10 times the equivalent amount) afforded the deprotected nucleoside **3** directly. However, it was our experience that the two-step reaction involving transesterification of **2** with potassium carbonate in methanol as the second reaction gave a higher yield of pure nucleoside **3**.

Scheme I



The oxidation of 5-[(methylthio)methyl]-2′-deoxyuridine (**3**) with 1 equiv of sodium metaperiodate at 0 °C for 4 h afforded the sulfoxide **4** as the major product, with only a trace of the sulfone **5** evident. Alternatively, oxidation of **3** at 25 °C for several days with an excess of sodium metaperiodate gave predominantly the sulfone **5**. Proton magnetic resonance showed a singlet for the methyl in thioether **3** at δ 1.97, whereas a downfield chemical shift to δ 2.46 for the sulfoxide **4** and 3.17 for the methyl in the sulfone **5** was observed, which is consistent with the electronic effects of the substituents. Unlike the thioether

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(3) (a) Brossmer, R.; Rohm, E. *Hoppe Seyler's Z. Physiol. Chem.* **1967**, *348*, 1431. (b) Wilson, R. S.; Mertes, M. P. *Biochemistry* **1973**, *12*, 2879–2886.

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Table I. Antiviral and Antimetabolic Activities of 1-(2-Deoxy-β-D-ribofuranosyl)-α-(methylthio)thymine (3), 1-(2-Deoxy-β-D-ribofuranosyl)-α-(methylsulfinyl)thymine (4), and 1-(2-Deoxy-β-D-ribofuranyl)-α-(methylsulfonyl)thymine (5) in PRK Cell Cultures

	ID_{50},[a] μg/mL				
compd	HSV 1	HSV 2	vaccinia	[*Me*-^{3}H]dThd incorp	[2-^{14}C]Urd incorp
3	6 (4-10)	4	200	100	>400
4	>400	>400	>400	400	>400
5	4	≥400	200	300	>400
5-I-dUrd	0.15	0.3 (0.2-0.4)	0.2	2.5	1.2

[a] Concentration required to reduce virus-induced cytopathogenicity or [*Me*-^{3}H]dThd or [2-^{14}C]dUrd incorporation into host cell DNA by 50%. For herpes simplex virus, the data represent the mean values obtained with three different strains of HSV type 1 (KOS, F, and Mc Intyre) and three different strains of HSV type 2 (LYONS, 196, and G); the range of values is indicated in parentheses.

and the sulfone, the sulfoxide **5** gave a doublet for the signal assigned to the methyl group; these were of equal intensity and presumably arise from different chemical shifts in the diastereoisomeric pair.

Phosphorylation of the methylthio derivative **3** using a modification of the method of Sowa and Ouchi[4] gave the phosphate **6**. Oxidation of the thioether **6** with sodium metaperiodate at 0 °C gave the sulfoxide **7** and, under more strenuous conditions, afforded the sulfone **8**. Verification of the assigned structures was made by the corresponding proton NMR shifts for the methyl groups which agreed with those observed for the nucleosides.

Results

Compounds **3–5** were evaluated for their antiviral activity in primary rabbit kidney (PRK) cell cultures inoculated with herpes simplex virus (type 1 or 2) or vaccinia virus. Using the minimal inhibitor concentration for 50% reduction in virus-induced cytopathogenicity (ID_{50}) as a measure of potency, the standard compound, 5-iodo-2′-deoxyuridine, was effective against both herpes simplex and vaccinia virus at a concentration of about 0.2 μg/mL (Table I). The 5-[(methylthio)methyl] nucleoside **3**, in the same test, was effective against herpes simplex types 1 and 2 at 6 and 4 μg/mL, respectively[5] (Table I). While the iodo compound was equally inhibitory to herpes and vaccinia virus, the ID_{50} of the methylthiomethyl derivative (**3**) for vaccinia virus was about 50-fold higher than its ID_{50} for HSV 1 or 2 (Table I). That **3** is relatively nontoxic was established by the absence of a microscopic alteration of cell morphology at a dose 100 times greater than the ID_{50} for herpes control. Additionally, **3** was found to be equally effective against several clinical and laboratory strains of both types 1 and 2 of herpes simplex virus.[5] In parallel tests, the sulfoxide **4** and the sulfone **5** have also been examined for antiviral activity in PRK cell cultures. The ID_{50} of the sulfoxide **4** for both herpes simplex and vaccinia virus was >400 μg/mL (Table I), which is in marked contrast to the anti-herpes activity of **3**. The sulfone **5**, however, had an ID_{50} of 4 μg/mL for inhibition of herpes simplex virus type 1, but its ID_{50} for HSV 2 and vaccinia was markedly higher (>400 and 200 μg/mL, respectively; Table I). Neither compound **5** nor compound **4** caused a microscopically detectable alteration of cell morphology at 400 μg/mL, the highest concentration tested.

In an attempt to establish the mechanism of action of 5-[(methylthio)methyl]-2′-deoxyuridine and its congeners **4** and **5**, the compounds were examined for inhibition of [^{3}H]thymidine and 2′-deoxy[^{14}C]uridine incorporation into the DNA of PRK cells (Table I; see also ref 5 and 6).

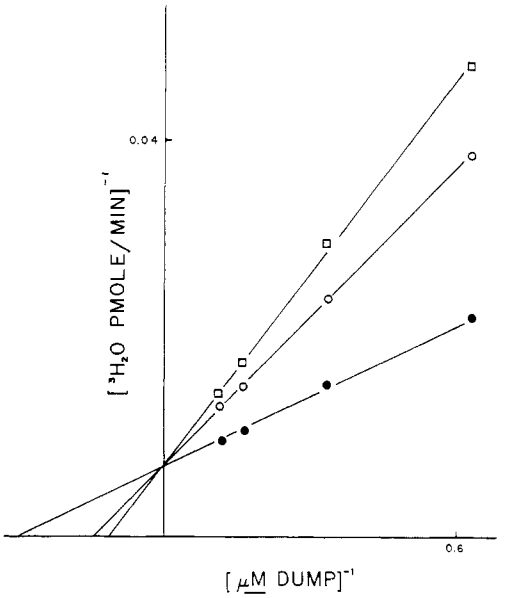

Figure 1. Double-reciprocal plot of the velocity of formation of ^3H_2O vs. concentration of substrate, 2′-deoxyuridine 5′-phosphate (dUMP), with and without added inhibitor, 1-(2-deoxy-β-D-ribofuranosyl)-α-(methylsulfinyl)thymine 5′-phosphate (**7**): no inhibitor (●); 2.0 μM inhibitor (○); 4.0 μM inhibitor (□).

While **3**, **4** and **5** caused a 50% reduction in thymidine incorporation at a concentration of 100, 400, and 300 μg/mL, respectively, a similar reduction in 2′-deoxyuridine could not be achieved at a concentration of 400 μg/mL (Table I). Another observation[5] bearing on the mechanism of action is that compound **3** was ineffective in controlling replication of a thymidine kinase deficient (TK^-) mutant of HSV 1 at 200 μg/mL.

All three nucleotides (**6–8**) were evaluated for in vitro inhibition of thymidylate synthetase purified from amethopterin-resistant *Lactobacillus casei*. The assay method used in this study is a measure of ^3H_2O released from 2′-deoxy[5-^{3}H]uridine 5′-phosphate, the substrate for the enzymatic transformation. The observed substrate K_m in these studies was 3.2 μM in analysis of inhibitors **6** and **7** and 3.9 μM for the experiments using compound **8**. Previous values[7] for the substrate K_m using this enzyme ranged from 0.7 to 5.2 μM. For all three nucleotides (**6–8**) the double-reciprocal plots of velocity vs. substrate con-

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(5) De Clercq, E.; Descamps, J.; Schmidt, C. L.; Mertes, M. P. *Biochem. Pharmacol.* **1979**, *28*, 3249–3254.
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(7) (a) Daron, H. H.; Aull, J. L. *J. Biol. Chem.* **1978**, *253*, 940–945. (b) Dunlap, R. B.; Harding, N. G. L.; Huennekens, F. M. *Biochemistry* **1971**, *10*, 88–97. (c) Crusberg, T. C.; Leary, R.; Kisliuk, R. L. *J. Biol. Chem.* **1970**, *245*, 5292–5296.

centrations show competitive inhibition. The thioether **6** is the least effective inhibitor, with a K_i of 7.8 μM. Using the K_i/K_m ratio as a measure of affinity compared to substrate, a value of 2.4 for compound **6** suggests that it is a reasonably potent inhibitor. The sulfoxide **7** and the sulfone **8** are equally effective and about four times more potent than the thioether **6**. For **7** (Figure 1) the K_i is 1.9 μM, which gives a K_i/K_m ratio of 0.61; similarly, nucleotide **8** has a calculated K_i of 2.2 μM. Using the observed substrate K_m of 3.8 μM for the latter study, a K_i/K_m of 0.58 for **8** suggests there is little difference in enzyme affinity between the sulfoxide and the sulfone.

Irreversible inhibition of this enzyme recently has been observed for two affinity labels, 5-nitro-[8] and 5-(α-bromoacetyl)-2′-deoxyuridine 5′-phosphate.[9] Compounds **6–8**, when incubated with the enzyme at 37 °C in the absence of the cofactor, N^5,N^{10}-methylenetetrahydrofolic acid, failed to show any significant enzyme inactivation. After 1 h, the control experiment without inhibitor showed 80% of the original enzyme activity, while the assays containing inhibitors **6–8** showed 85 to 95% remaining activity. The higher values observed using the inhibitor are not unexpected; we have observed that incubation of this enzyme with a reagent that binds competitively to the active site stabilizes the enzyme.

Discussion

Among the 5-substituted 2′-deoxyuridines, the ethyl, vinyl, (2-halovinyl), and propyl derivatives all show anti-herpes activity at concentrations that are significantly lower than those showing cytotoxicity. The methylthiomethyl nucleoside **3** is an effective anti-herpes agent, has little activity against vaccinia, and is not cytotoxic at doses 100 times the ID_{50} for inhibition of herpes simplex replication. Although the corresponding standard compound 5-iodo-2′-deoxyuridine is about 20–40 times more potent as an antiviral agent, it shows toxicity, as measured by 2′-deoxy[^{14}C]uridine incorporation, at doses 4–10 times the anti-herpes concentration. In contrast with the thioether **3**, the sulfoxide **4** does not exhibit anti-herpes activity; **4** is not cytotoxic either. The sulfone **5**, however, exhibits a selective inhibitory activity toward herpes simplex type 1; it has little activity against HSV 2 or vaccina, and, akin to **3**, **5** is not cytotoxic at doses 100 times the ID_{50} for inhibition of HSV 1 replication. It is remarkable that transition of the thioether **3** to the sulfone **5** virtually abolished the anti-HSV (type 2) activity of the compound, while retaining its anti-HSV (type 1) activity.

The differences in the selectivity noted for the antiviral activity of the compounds prepared in this study contrast sharply with 5-iodo-, 5-(trifluoromethyl)-, and 5-formyl-2′-deoxyuridine. These compounds inhibit both vaccinia and herpes simplex replication at relatively low concentrations (within the 0.1–1 μg/mL range). However, all three agents are relatively toxic; i.e., the antiviral index[2a,5] for the 5-(trifluoromethyl) and 5-formyl derivatives is 0.25.

A possible clue to the mechanism causing antiviral activity was the observation that the antiviral effects of 5-nitro-, 5-fluoro-, and 5-cyano-2′-deoxyuridine could be readily reversed by addition of thymidine to the cell culture medium.[6] All of these derivatives, as the 5′-phosphates, are potent in vitro inhibitors of thymidylate synthetase, with 5 to 100 times greater affinity for the enzyme than the substrate.

In addressing the mechanism of the antiviral action of compound **3**, the following observations are pertinent. Compound **3** is uniquely selective against herpes simplex virus, it is not cytotoxic, it is ineffective against TK$^-$ mutants of herpes simplex virus, it does not inhibit 2′-deoxy[^{14}C]uridine incorporation into cellular DNA, and, finally, its antiviral activity is reversed upon the addition of high concentrations of thymidine.[5] All of these would support the proposal that **3** must be converted to the nucleotide form prior to exerting its antiviral effect. Regardless of the fact that its nucleotide (**6**) is an inhibitor of thymidylate synthetase, this mechanism is probably not responsible for the anti-herpes action, since antiviral concentrations have no effect on incorporation of 2′-deoxy[^{14}C]uridine into DNA (Table I). The only route for this incorporation into DNA is via thymidylate synthetase. Further evidence that inhibition of thymidylate synthetase is not the mechanism for antiviral activity stems from the observation that both the sulfoxide **7** and the sulfone **8** are potent inhibitors of the enzyme; yet, the corresponding nucleoside of **7** (**4**) has no antiviral activity, while the sulfone **5** is a potent inhibitor of HSV 1 replication.

Further work is necessary to establish the mechanism of action and the potential clinical usefulness of compounds **3** and **5** as antiviral agents.

Experimental Section

Melting points were determined with a Thomas-Hoover melting point apparatus and are uncorrected. Proton magnetic resonance spectra were obtained with a Varian EM 360, and chemical shifts are reported relative to tetramethylsilane as an internal standard for the nucleosides or as an external standard for the nucleotides; unreported chemical-shift assignments were as expected. Ultraviolet spectra were measured with a Cary 219 spectrophotometer. Thin-layer chromatography was run on precoated silica gel 60 F-254, 0.25-mm thick plates supplied by E. M. Laboratories, Inc. Column chromatography was run on silica gel 60 (70–230 mesh) supplied by E. M. Laboratories, Inc., and on DE-32 DEAE-cellulose supplied by Whatman, Ltd. All solvent proportions are given by volume.

Thymidylate synthetase purified from methotrexate-resistant *Lactobacillus casei* was purchased from the New England Enzyme Center, Tufts University, at a specific activity of 1.1 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 50 mM mercaptoethanol. The substrate 2′-deoxy[5-^{3}H]uridine 5′-phosphate at a specific activity above 15 Ci/mmol was purchased from Moravek Biochemicals, Industry, 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-(2-Deoxy-β-D-ribofuranosyl)-α-(methylthio)thymine 3,5-Di-*p*-toluate (2). Sodium metal (0.2 g, 8.7 mmol) was dissolved in anhydrous methanol (150 mL), the solution was cooled in an ice bath, and methanethiol was bubbled into the solution with stirring until the weight gain was 3.9 g; this represents a 10% excess of sodium and a tenfold excess of methanethiol. 5-(Chloromethyl)-1-(3,5-di-*O*-toluoyl-2-deoxy-β-D-ribofuranosyl)-uracil[3] (1; 4.0 g, 8.0 mmol) was added and the mixture was stirred at room temperature for 48 h. The precipitate which formed was collected by filtration and dried. The filtrate was evaporated to dryness and the residue was triturated with a small volume of methanol to yield additional white solid. The combined solids (ca. 4 g) were recrystallized from ethanol (200 mL) and then dried in vacuo to yield 2.6 g (62%) of product: mp 174–175.5 °C; NMR ($CDCl_3$) δ 7.65 (s, 1,6-H), 7.65 (q, 8, toluoyl H's), 6.37 (t, 1, $J_{1',2'}$ = 7 Hz, C-1′), 1.9 (s, 3, SCH_3). Anal. ($C_{27}H_{28}N_2O_7S$), 524.6, C, H, N.

1-(2-Deoxy-β-D-ribofuranosyl)-α-(methylthio)thymine (3).

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Method A. Sodium metal (0.3 g, 13 mmol) was dissolved in methanol (100 mL, dried by distillation from magnesium) and then methanethiol was bubbled into the solution until the weight gain was 2.0 g (42 mmol). 5-(Chloromethyl)-1-(3,5-di-*O*-toluoyl-2-deoxy-β-D-ribofuranosyl)uracil (1; 2.0 g, 4 mmol) was added and the mixture was stirred at room temperature for 24 h. The solution was neutralized with Amberlite IR 120 resin (H^+ form), then the resin was removed by filtration and washed with hot methanol, and the filtrate and wash were evaporated to dryness in vacuo. The residue was extracted with several portions of anhydrous ether to leave a white solid (0.75 g, 65%). Recrystallization of a portion of the solid from water gave crystals of mp 177–178.5 °C; NMR (Me_2SO-d_6) δ 7.70 (s, 1, 6-H), 6.05 (t, 1, $J_{1',2'}$ = 7 Hz, 1'-H), 1.97 (s, 3, SCH_3); UV λ_{max} ($\epsilon \times 10^{-3}$), pH 1, 267 nm (9.8); pH 11, 265.5 (7.2). Anal. ($C_{11}H_{16}N_2O_5S$), 288.3, C, H, N.

Method B. Compound **2** (2.3 g, 4.4 mmol) and potassium carbonate (0.5 g, 3.6 mmol) were added to anhydrous methanol (200 mL), and the solution was stirred at room temperature for 48 h. The resulting solution was neutralized with Amberlite IR-120 (H^+ form), the resin was removed by filtration and washed with hot methanol, and the combined filtrate and wash were evaporated to dryness in vacuo. The residue was extracted with several portions of anhydrous ether to yield a white solid (1.13 g, 89%), which was shown by TLC (ethyl acetate–ethanol, 4:1) to be identical with the product from method A.

1-(2-Deoxy-β-D-ribofuranosyl)-α-(methylsulfinyl)thymine (4). The thioether **3** (0.3 g, 1.04 mmol) was suspended in water (2.0 mL), and the solution was stirred and cooled in an ice bath. To this solution was added dropwise an aqueous solution of sodium metaperiodate (1.04 mmol, 2.1 mL of a solution of 0.53 g in 5.0 mL of water). The reaction was stirred at 0 °C for 4 h and then ethanol (10 mL) was added. The inorganic precipitate was removed by filtration, the filtrate was washed with ethanol, and the combined filtrate and wash were evaporated to dryness. The residue was adsorbed onto silica gel by dissolving in methanol, adding silica gel (2 g), and evaporating the solvent. The silica gel with compound adsorbed was applied to a column of silica gel (1.7 × 15 cm) packed in ethyl acetate–acetone (1:1), and the column was eluted with 400 mL of the same solvent, followed by 10% methanol in ethyl acetate–acetone (1:1) to remove the product: TLC R_f 0.06 (ethyl acetate–ethanol, 4:1). The solvent was evaporated and the residue recrystallized by dissolving in ethyl acetate (30 mL) containing a minimum of methanol and then letting the solution stand in a sealed tank containing a large volume of ethyl acetate. After several days, the resulting crystals were collected by filtration and dried in vacuo to yield 0.22 g (67%) of product: mp 177–180 °C; NMR (Me_2SO-d_6) δ 7.75 (s, 1, 6-H), 6.01 (t, 1, 1'-H), 2.46 [d, 3, $S(O)CH_3$], UV λ_{max} ($\epsilon \times 10^{-3}$), pH 1, 270 nm (15.0); pH 11, 268 (10.5). Anal. ($C_{11}H_{16}N_2O_6S \cdot 0.5H_2O$), 313.4, C, H, N.

1-(2-Deoxy-β-D-ribofuranosyl)-α-(methylsulfonyl)thymine (5). To 144 mg of 1-(2-deoxy-β-D-ribofuranosyl)-α-(methylthio)thymine (**3**; 0.5 mmol) was added a solution of sodium metaperiodate (2.0 mmol, 2.0 mL of a solution of 1.07 g in 10.0 mL of water). The mixture was stirred at room temperature for 56 h and then ethanol (4 mL) was added. The precipitate which formed was removed by filtration and washed with ethanol, and the filtrate and wash were combined and evaporated to dryness. The residue was adsorbed onto silica gel (1 g), applied to a column of silica gel (1.7 × 15 cm), and eluted with ethyl acetate–acetone (1:1). The material with R_f 0.37 (ethyl acetate–ethanol, 4:1) was evaporated to dryness to yield 30 mg of product (19%), which could be crystallized from ethanol to yield needles: mp 213–215 °C; NMR (Me_2SO-d_6) δ 7.73 (s, 1, 6-H), 6.00 (t, 1, 1'-H), 3.17 (s, 3, SO_2CH_3); UV λ_{max} ($\epsilon \times 10^{-3}$), pH 1, 268.5 nm (10.3); pH 11, 267 (7.3). Anal. ($C_{11}H_{16}N_2O_7S$), 320.3, C, H, N.

1-(2-Deoxy-β-D-ribofuranosyl)-α-(methylthio)thymine 5'-[Bis(triethylammonium) Phosphate] (6). Water (0.22 mL) was added dropwise over a 30-min period to a cold (0 °C) solution of phosphorous oxychloride (2.0 mL, 22 mmol) in anhydrous acetonitrile (5 mL). The mixture was stirred at 0 °C for 1 h and then pyridine (1.9 mL, 24 mmol) was added dropwise to the cold solution. The mixture was diluted to a total volume of 10 mL with acetonitrile and then stirred at 0 °C for 30 min . To the thiomethyl nucleoside **3** (0.3 g, 1 mmol) was added 2.1 mL of cold reagent, and the mixture was stirred at 0 °C for 4 h. After this time, water (1.0 mL) was added dropwise, and the mixture was stirred for 30 min at 0 °C. This solution was applied to a column (2.6 × 40 cm) of DEAE-cellulose (HCO_3^- form) and eluted with 700 mL of a gradient of 0.01 to 0.3 M ammonium formate buffer (pH 4.5). Fractions 35–44 (10 mL/fraction) were pooled and lyophilyzed, and the resulting material was applied to a second column (2.6 × 70 cm) of DEAE-cellulose (HCO_3^- form) and eluted with 1000 mL of 0.01 to 0.3 M triethylammonium bicarbonate buffer gradient. Fractions 85–99 (10-mL fraction) were pooled, lyophilyzed, and again chromatographed under the same conditions. Fractions 85–100 were pooled, lyophilyzed, and dried at 15 μmHg at room temperature for 24 h to yield 0.2 g (31%) of white solid: NMR (D_2O) δ 7.68 (s, 1, 6-H), 6.11 (1, t, 1-H), 1.95 (s, 3, SCH_3); UV λ_{max} ($\epsilon \times 10^{-3}$), pH 1, 267 nm (8.6), λ_{min} 238 (3.4); pH 11, 266 (6.3). Anal. [$C_{11}H_{15}N_2O_8PS \cdot 2(C_2H_5)_3NH \cdot 3H_2O$], 624.7, C, H, N.

1-(2-Deoxy-β-D-ribofuranosyl)-α-(methylsulfinyl)thymine 5'-[Bis(triethylammonium) Phosphate] (7). To a solution of the methylthio nucleotide **6** (62 mg, 0.1 mmol) in water (0.5 mL) cooled to 0 °C in an ice bath was added dropwise, with stirring over a 15-min period, 0.5 mL of a solution of sodium metaperiodiate (424 mg in 10 mL of water). The mixture was stirred at 0 °C for 5 h, then applied to a column (0.9 × 20 cm) of DEAE-cellulose (HCO_3^- form), and eluted with 150 mL of a gradient of 0.01 to 0.3 M triethylammonium bicarbonate buffer. Fractions 18–24 (3.0 mL/fraction) were pooled and lyophilyzed, then applied to a second column (2.4 × 40 cm) of DEAE-cellulose, and eluted with 1000 mL of a 0.01–0.3 M triethylammonium bicarbonate buffer gradient. Fractions 75–80 (10.0 mL/fraction) were pooled, lyophilyzed, and applied to a column (0.9 × 15 cm) of Sephadex G-10 and eluted with 0.05 M triethylammonium bicarbonate. Fractions 6 and 7 (2.0 mL/fraction) were lyophilyzed and dried at 15 μmHg for 36 h to yield the product **7**: NMR (D_2O) δ 7.77 (s, 1, 6-H), 6.20 (t, 1, 1'-H), 2.67 (d, 3, $SOCH_3$); UV λ_{max}, pH 7, 270 nm; pH 11, 268. Anal. ($C_{11}H_{15}N_2O_8PS \cdot 2(C_2H_5)_3NH \cdot 2.5H_2O$), 631.7, C, H, N.

1-(2-Deoxy-β-D-ribofuranosyl)-α-(methylsulfonyl)thymine 5'-[Bis(triethylammonium) Phosphate] (8). Compound **6** (37 mg, 0.06 mmol) and sodium metaperiodate (29 mg, 0.14 mmol) were combined in water (0.5 mL) and the solution was stirred at room temperature for 5 days. The mixture was applied to a column (1.5 × 30 cm) of DEAE-cellulose (HCO_3^- form) and eluted with 400 mL of a gradient from 0.01 to 0.3 M triethylammonium bicarbonate buffer. Fractions 16–21 (10.0 mL/fraction) were pooled, lyophilyzed, and rechromatographed in the same fashion. Fractions 17–20 (10.0 mL/fraction) were pooled, lyophilyzed, and applied to a column (0.9 × 30 cm) of Sephadex G-10 and eluted with 0.05 M triethylammonium bicarbonate buffer. Fractions 5 and 6 (2.0 mL/fraction) were pooled and lyophilyzed, then dried at room temperature at 15 μmHg for 2 days to yield the desired product **8**: NMR (D_2O) δ 8.01 (s, 1, 6-H), 3 (obscured by quartet for Et_3NH, SO_2CH_3); UV λ_{max}, pH 7, 269 nm; pH 11, 267.5. Anal. ($C_{11}H_{15}N_2O_{10}PS \cdot 2(C_2H_5)_3NH \cdot H_2O$), 620.8, C, H, N.

Thymidylate Synthetase Assays. The enzyme was assayed by modification of the radioisotope assays described by Roberts[10] and Lomax and Greenberg.[11] The solution, 0.1 mL, contained 25 mM mercaptoethanol; 0.22 mM *dl*-tetrahydrofolic acid; 6.75 mM formaldehyde; 5 mM sodium bicarbonate; 3 mM magnesium chloride; 0.12 mM EDTA, 6 mM Tris–acetate buffer, pH 6.8; 5 μL of the diluted enzyme solution; substrate; and, when indicated, inhibitor. Control reactions lacked the cofactor, tetrahydrofolic acid. The substrate 2'-deoxy[5-^{3}H]uridine 5'-phosphate was used at a specific activity of 500 μCi/μmol. The assays were started by the addition of the enzyme to the complete mixture then incubated at 30 °C. Incubation was stopped at 30 s by the addition of 50 mL of 20% trichloroacetic acid. A 20% aqueous suspension of charcoal (0.25 mL) was added, and the solution vortexed and allowed to stand for 15 min. The suspension was filtered through a glass-wool plugged Pasteur pipet, and 0.1 mL of the filtrate was counted in a scintillation fluid containing 0.5% 2,5-diphenyl-

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oxazole and 10% Beckman BBS-3 solubilizer in toluene. Counting efficiency was 33%; control samples lacking the cofactor were found to have less than 5% of the respective sample counts. On the basis of specific activity, velocity is reported in the adjusted value of picomoles of ^3H_3O formed per minute in the assay.

Preincubation 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. After incubation for the indicated time period, the assay for the 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 enzyme assay. A high substrate concentration (40 μM) was used in these assays to afford reasonably high velocity and to competitively reduce any enzyme inactivation by the inhibitor during the assay. The assay was run for 30 s and treated as described in the enzyme assay section. Inactivation of the enzyme was measured by comparing the velocity at time zero to that at the indicated incubation times. Under the conditions of the assay, the uninhibited enzyme retained 95% of the initial activity after 20 min of incubation.

Antiviral and Antimetabolic Assays. The methodology for measuring the inhibition of virus-induced cytopathogenicity in primary rabbit kidney (PRK) cell cultures and the incorporation of 2'-deoxy[*methyl*-^{3}H]thymidine or 2'-deoxy[2-^{14}C]uridine into DNA of these cells has been described previously.[5,6]

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Quantitative Structure-Activity Correlations of Rifamycins as Inhibitors of Viral RNA-Directed DNA Polymerase and Mammalian α and β DNA Polymerases

Roy S. Wu,[1]

Biotech Research Laboratories, Inc., Rockville, Maryland 20852

Mary K. Wolpert-DeFilippes,

Drug Evaluation Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Silver Spring, Maryland 20910

and Frank R. Quinn*

Drug Design and Chemistry Section, Laboratory of Medicinal Chemistry and Biology, Developmental Therapeutics Program, Division of Cancer Treatment National Cancer Institute, Bethesda, Maryland 20205. *Received July 18, 1979*

Twenty-two 3-substituted rifamycins were tested for inhibition of mammalian α and β DNA polymerase and viral RNA-dependent DNA polymerase ("reverse transcriptase"). Quantitative structure-activity relationships (QSAR) were formulated for the three systems. Inhibition is linearly dependent on the partition coefficient and is highly favored by the presence of bulky hydrazones or oximes. None of these agents proved to be a selective or specific inhibitor of reverse transcriptase. A correlation in terms of log P and $(\log P)^2$ was obtained from data on a more closely related set of analogues from a published study. For murine reverse transcriptase, $\log P_0 = 5.1$.

The rifamycins (Figure 1) represent a class of compounds obtained from chemical modification of some metabolic products produced by *Streptomyces mediterranei*.[2,3] Early studies showed that the rifamycins had good antimicrobial activity, which led to their clinical use as antibiotics for the treatment of tuberculosis and bacterial infections.[4] Their chief mechanism of action appears to be as inhibitors of bacterial RNA polymerase.[5] In addition, there have been reports that rifamycins are inhibitors of mammalian DNA polymerases and viral RNA-dependent DNA polymerase ("reverse transcriptase" or RT).[6] The finding of inhibitory effects on viral DNA polymerases suggested that systematic screening of several rifamycin analogues might produce a potent and specific inhibitor of the viral enzyme. Such an inhibitor would be of interest because it could potentially block the transformation of a cell by an RNA tumor virus.

In this paper, we present the results of our efforts to find a specific inhibitor of RNA-directed DNA polymerase by comparing the activities of several 3-substituted rifamycins (Table I) in inhibiting the viral enzyme as well as mammalian α and β DNA polymerases. In addition, we have attempted to test the hypothesis of whether increasing the size and the lipophilicity of the "tail" bound to rifamycin

(1) Address: Laboratory of Molecular Pharmacology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20205.

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