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Synthesis of Nucleoside 3′-(*S*-Alkyl phosphorothioates) and Their Use as Substrates for Nucleases†

Don Saba and Charles A. Dekker*

ABSTRACT: The synthesis of cytidine, uridine, guanosine, and adenosine 3′-(*S*-methyl phosphorothioates) by treatment of the 2′,5′-di-*O*-(4-methoxytetrahydropyran-4-yl)ribonucleosides with 2-(methylthio)-4*H*-1,3,2-benzodioxaphosphorin 2-oxide is described. These nucleotide analogues are stable compounds both in the solid state and in neutral aqueous solution. All four of these compounds are degraded by RNase T_2 to the parent nucleotides and methanethiol. In addition, cytidine and uridine 3′-(*S*-methyl phosphorothioates) are substrates for bovine pancreatic ribonuclease and guanosine 3′-(*S*-methyl phosphorothioate) is a substrate for RNase T_1 and RNase U_1. When used in conjunction with a chromophore-producing reagent, nucleoside 3′-(*S*-methyl phosphorothioates) provide a means for direct kinetic measurement of ribonuclease activity over a wide pH range (pH 2–9). The reactivities of these substrates with ribonucleases are compared to the reactivities of other synthetic substrates as well as a number of natural substrates. The utility of ribonucleoside 3′-(*S*-methyl phosphorothioates) as substrates for the assay of ribonucleases is discussed.

Nucleoside phosphorothioates and their esters are substrates for a number of enzymes of nucleic acid metabolism (Goody & Eckstein, 1971; Eckstein & Gindl, 1970; Schlimme et al., 1970; Cook, 1970; Cook et al., 1969; Eckstein, 1970). Cook (1970) and Cook et al. (1969) prepared thymidine 5′-(*S*-ethyl phosphorothioate) by condensation of dilithium *S*-ethyl phosphorothioate and 3′-(*O*-acetyl)thymidine using dicyclohexylcarbodiimide. The dinucleoside phosphorothioate Tp(s)T was prepared by the same authors by the reaction of 5′-deoxy-5′-iodo-3′-(*O*-acetyl)thymidine with thymidine 3′-phosphorothioate. Eto et al. (1974) treated the 2′,3′-borate complex of the ribonucleoside 1 with 2-(methylthio)-4*H*-1,3,2-benzodioxaphosphorin 2-oxide (MBTO)[1] (**2**) (Iio et al., 1973) in the presence of cyclohexylamine to form the ribonucleoside 5′-(*S*-methyl phosphorothioate) **3**.

Using the method of Eto et al. (1974), we have prepared the ribonucleoside 3′-(*S*-methyl phosphorothioate) analogues of the four major naturally occurring ribonucleotides in order

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[1] Abbreviations used: MBTO, 2-(methylthio)-4*H*-1,3,2-benzodioxaphosphorin 2-oxide; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; TLC, thin-layer chromatography; DMF, dimethylformamide.

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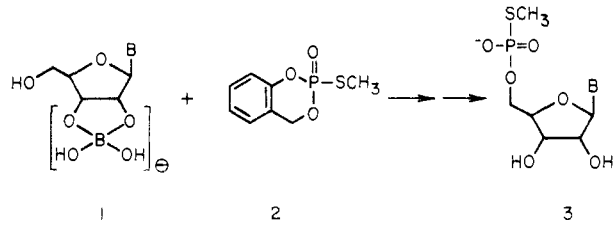

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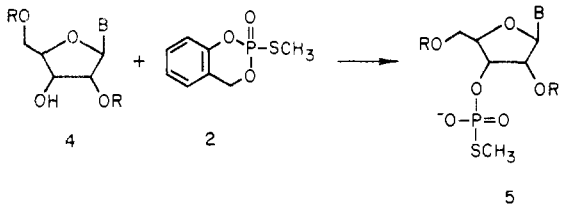
to examine the reactivity of these compounds as substrates for ribonucleases and to gather information regarding the stability of esters and thioesters of ribonucleoside 3′-phosphates toward intramolecular attack by the neighboring *cis*-2′ hydroxyl group.

The preparation of adenosine, guanosine, cytidine, and uridine 3′-(*S*-methyl phosphorothioates) was accomplished in the following manner. To facilitate placement of blocking groups on the 2′ and 5′ positions of adenosine and uridine, we initially acetylated these nucleosides at the 3′ position by treatment with trimethyl orthoacetate and *p*-toluenesulfonic acid followed by silicic acid chromatography, mild acid hydrolysis, and crystallization of the 3′-*O*-acetyl nucleoside from ethanol (Fromageot et al., 1967). The 3′-*O*-acetyl nucleoside was then treated with 5,6-dihydro-4-methoxy-2*H*-pyran in the presence of a small amount of mesitylenesulfonic acid to yield the 2′,5′-di-*O*-(4-methoxytetrahydropyran-4-yl)-3′-*O*-acetyl nucleoside (Reese et al., 1970). The 3′-*O*-acetyl group was then removed by treatment with methanolic ammonia. The resulting 2′,5′-di-*O*-(4-methoxytetrahydropyran-4-yl) nucleoside **4** was treated with MTBO (**2**) to yield the 2′,5′-di-*O*-



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(4-methoxytetrahydropyran-4-yl) nucleoside 3′-(*S*-methyl phosphorothioate) **5**. Mild acid treatment (40% HOAc) is sufficient for removal of the protecting groups. The preparation of the analogous guanosine and cytidine compounds was complicated by the necessity to benzoylate the heterocyclic bases to facilitate crystallization of intermediate products (Reese et al., 1970). Thus guanosine and cytidine were tetrabenzoylated with benzoyl chloride, followed by selective debenzoylation with methanolic sodium methoxide to yield the respective *N*-benzoyl nucleosides (Rammler & Khorana, 1962; Chladek & Smrt, 1964). The *N*-benzoyl nucleosides were then acetylated and protected as in the case of uridine and adenosine. The *O*-acetyl and *N*-benzoyl protecting groups were removed simultaneously by treatment with either 0.1 M NaOH or 33% methylamine in ethanol for cytidine and guanosine derivatives, respectively. The products were then allowed to react with MTBO to form the protected nucleoside phosphorothioates as before.

Experimental Procedures

Organic solvents were purified as follows. Acetonitrile was distilled from phosphorus pentoxide (P_2O_5). Dioxane was refluxed over sodium and distilled. Water was removed from dimethylformamide as the benzene azeotrope; the dimethylformamide was then distilled under reduced pressure. Trimethyl orthoacetate was distilled from calcium hydride, and cyclohexylamine was distilled from KOH. Benzoyl chloride was distilled just before use. All regents except benzoyl chloride were stored over Linde 4A molecular sieves that had been heated to 150 °C for 24 h. All nucleosides and derivatives were dried in vacuo over P_2O_5 for at least 24 h. Mesitylenesulfonic acid was stored at −15 °C over P_2O_5. *p*-Toluenesulfonic acid was dried over P_2O_5 at 100 °C for 15 h and stored at 25 °C over P_2O_5. 4-Methoxy-5,6-dihydro-2*H*-pyran was obtained from Aldrich Chemical Co. 2-Methoxy-4*H*-1,3,2-benzodioxaphosphorin 2-sulfide (Salithion), technical grade, was obtained from Sumitomo Chemical Co. and stored over P_2O_5 in vacuo at 25 °C.

NMR spectra were recorded on a Varian EM-360 NMR spectrometer. Ultraviolet and visible measurements were carried out on a Cary 118 recording spectrophotometer. All melting points were taken on a Thomas-Hoover melting point apparatus and are uncorrected.

Thin-layer chromatography was performed on silica gel 60 F_{254} and cellulose F_{254} plates (EM Laboratories, Inc.). Solvent systems used were as follows: solvent A, 2-propanol/concentrated ammonia/H_2O (7:1:2); solvent B, chloroform/methanol (9:1); solvent C, ethyl ether. Nucleotides and derivatives were detected by viewing under ultraviolet light. Sulfur-containing compounds were detected by a palladium chloride spray reagent (Baumler & Rippstein, 1961).

Ion-exchange chromatography was carried out on Whatman DEAE-cellulose (Whatman DE-52) columns by using a 0–0.1 M linear gradient of triethylammonium bicarbonate. Product elution was followed by UV spectrometry. Eluates were repeatedly concentrated in vacuo from water to remove triethylammonium bicarbonate.

Alumina chromatography was performed on Matheson Coleman and Bell activated alumina (80–200 mesh) containing 6% water by weight. Silicic acid chromatography was performed on Mallinckrodt silicic acid (100 mesh) containing 10% water by weight. Elutions were followed by UV spectrometry.

Concentration of acetic acid solutions of sensitive compounds was carried out at <25 °C. Concentration of solutions of all other sensitive compounds was performed at <35 °C.

Ribonucleases T_1 and T_2 were products of Sankyo Ltd. Ribonuclease A was a product of Worthington Biochemicals. Ribonuclease U_1 was prepared in this laboratory (Kenney & Dekker, 1971).

For Tables I–IV the following buffers were used for determination of the values K_m and V_{max}: buffer 1, 0.1 M imidazole hydrochloride plus NaCl to give μ = 0.2, pH 7.03, 27 °C; buffer 2, same as buffer 1 except pH 7.0 and 26 °C; buffer 3, 0.1 M Tris–acetate plus 0.1 M NaCl, pH 7.0, 25 °C; buffer 4, 0.0001 M sodium citrate plus 0.025 M NaCl (pH-stat assay), pH 6.0, 25 °C; buffer 5, 0.05 M sodium acetate, pH 5.5, 25 °C; buffer 6, 0.01 M Tris-HCl plus 0.10 M KCl, pH 7.4, 25 °C; buffer 7, 0.1 M Tris-HCl, pH 7.5, 23 °C; buffer 8, 0.10 M Tris-HCl, pH 8.0, 25 °C. Substrate concentrations for the determination of K_m and V_{max} ranged from $K_m/5$ to $3K_m$. Concentrations of Ellman's reagent employed ranged from 0.02 to 0.1 mM.

2-(Methylthio)-4H-1,3,2-benzodioxaphosphorin 2-Oxide (MTBO). Salithion (20 g, 92.3 mmol), potassium iodide (1.0 g, 6.0 mmol), and methyl iodide (8.0 mL, 125 mmol) were dissolved in dry acetonitrile (50 mL) and heated to 50 °C. The course of the reaction was followed by thin-layer chromatography on silica gel plates (solvent C). The spots were visualized by UV light and by spraying with a solution of 0.5 g of $PdCl_2$ and 2.0 mL of concentrated HCl in 100 mL of H_2O. With this spray, compounds having a P–S–CH_3 linkage appear as a yellow spot and those having a P=S linkage appear as a dark brown spot on TLC (sensitivity = 10–20 μg of compound) (Baumler & Rippstein, 1961). When very little of the

starting material (R_f = 0.8, brown, solvent C) remained (10–12 h), the mixture was cooled and concentrated to a syrup in vacuo. The thick syrup was extracted with anhydrous ether (2 × 500 mL) with stirring overnight. It was necessary to use a strong magnetic stirrer. Eventually the syrup hardened and then broke up into a yellow powder. The ether extracts were combined, concentrated to a small volume (but not to the viscous stage), and allowed to crystallize at −30 °C. The slightly yellow product (9 g) was recrystallized from ether: yield, 7.5 g (38%); mp 41–42 °C; R_f = 0.6 (silica gel, solvent C); NMR ($CDCl_3$, Me_4Si) δ 6.7–7.2 [m, 4 H, H(5), H(6), H(7), H(8)], 5.22 [s, 1 H, H(4)], 4.96 [s, 1 H, H(4)], 2.02 (d, J = 16 Hz, 3 H, P–S–CH_3). This method was modified from that of Iio et al. (1973).

Uridine 3′-(S-Methyl phosphorothioate). In a 12-mL centrifuge tube 2′,5′-(methoxytetrahydropyranyl)uridine (Green et al., 1970) (200 mg, 0.424 mmol) was dissolved in 3 mL of dry dimethylformamide, MTBO (458 mg, 2.12 mmol) was added, and the solution was cooled with liquid nitrogen until just about the freezing point. Cyclohexylamine (0.79 mL, 7.07 mmol) was then added, and the tube was stoppered and heated to 70 °C for 30 min. The reaction mixture was then evaporated to dryness in vacuo, and the residue was treated with a mixture of $CHCl_3$ (10 mL) and water (10 mL) and shaken well. The $CHCl_3$ was drawn off, and the aqueous layer was briefly evacuated to remove traces of $CHCl_3$, and applied to a column of DEAE-cellulose (2.5 × 35 cm). The column was eluted with a linear gradient consisting of water (1000 mL) in the mixing vessel and 0.10 M triethylammonium bicarbonate (1000 mL) in the reservior at a rate of 250 mL/h. When 8-mL fractions were collected, the product eluted between fractions 80 and 110. The fractions containing product were identified by UV absorption spectra and were pooled and concentrated in vacuo. The solid material was repeatedly evaporated from water until triethylamine could not be detected by smell: yield, 156 mg (54%); R_f = 0.42 (silica gel, solvent A). The resulting glass was dissolved in a minimum of water and transferred to a 50-mL flask. An equal volume of 80% acetic acid was added, and the solution was left at room temperature for 60 min to remove the protecting groups. The acetic acid was removed by concentration to dryness in vacuo followed by addition of a small portion of water and further concentration in vacuo. The acetic acid free residue was then taken up in 1 mL of water. The 1-mL solution was then applied to a Sephadex G-10 column (2.5 × 100 cm) that had been previously equilibrated with 0.025 M ammonium acetate. The column was then eluted with 0.025 M ammonium acetate, and 2-mL fractions were collected. The fractions containing product were determined by UV absorption, pooled, and evaporated to dryness in vacuo. Multiple evaporations from water were necessary to remove ammonium acetate completely. Finally, the product was dissolved in water and applied to a Bio-Rad AG 50 X-2 column (1.5 × 22 cm, sodium form) to convert the product to its sodium salt. The eluant was collected, lyophilized, and dried in vacuo over P_2O_5: yield, 70.4 mg (82%); R_f = 0.24 (cellulose, solvent A); UV λ_{max} 261 nm (ε 9600) at pH 2, 261 (9600) at pH 7, 257 (7670) at pH 11; UV λ_{min} 231 nm (ε 2700) at pH 2, 231 (2640) at pH 7, 245 (5240) at pH 11; NMR (D_2O, Me_4Si) δ 2.25 (d, J = 14 Hz, 3 H, P–S–CH_3), 5.95 [d, J = 8 Hz, 1 H, H(1′)], 8.02 [d, J = 8 Hz, 1 H, H(4)]. Anal. Calcd for $C_{10}H_{14}N_2O_8PSNa·H_2O$: C, 30.46; H, 4.09; N, 7.11; P, 7.86; S, 8.13. Found: C, 30.23; H, 4.13; N, 6.78; P, 7.70; S, 7.70.

Adenosine 3′-(S-Methyl phosphorothioate). In a 12-mL centrifuge tube 2′,5′-di-*O*-(methoxytetrahydropyranyl)-adenosine (Green et al., 1970) (0.200 g, 0.405 mmol) was dissolved in 4.0 mL of dry DMF. MTBO (0.437 g, 2.03 mmol) was added to the solution, the solution was cooled to just above the freezing point in liquid nitrogen, and cyclohexylamine was added (0.33 mL, 3.36 mmol). The solution was then stoppered and heated to 70 °C for 20 min. The product was worked up in the same manner as the uridine derivative: yield, 77.5 mg (87%); R_f = 0.34 (cellulose, solvent A); UV λ_{max} 257 nm (ε 14300) at pH 2, 260 (14400) at pH 7, 260 (14000) at pH 11; UV λ_{min} 232 nm (ε 5230) at pH 2, 230 (4650) at pH 7, 230 (4650) at pH 11; NMR (D_2O, Me_4Si) δ 2.23 (d, J = 18 Hz, 3 H, P–S–CH_3), 8.27 [s, 1 H, H(8)], 8.42 [s, 1 H, H(2)]. Anal. Calcd for $C_{11}H_{15}N_5O_6PSNa·H_2O$: C, 31.66; H, 4.11; N, 16.78; P, 7.42; S, 7.68. Found: C, 31.35; H, 3.98; N, 16.96; P, 7.10; S, 7.43.

2′,5′-Di-O-(methoxytetrahydropyranyl)guanosine. In a 50-mL round-bottom flask 4-methoxy-5,6-dihydro-2*H*-pyran (6.6 g, 58 mmol) was added with stirring to a solution of 3′-*O*-acetyl-N^2-benzoylguanosine (Green et al., 1970) (1.0 g, 2.33 mmol) and anhydrous mesitylenesulfonic acid (0.14 g, 0.70 mmol) in dioxane (15 mL) at 0–2 °C. After 24 h the reaction mixture was neutralized with 0.70 mL of 1.00 M $NaOCH_3$, and the mixture was concentrated to a gum in vacuo and redissolved in CH_2Cl_2. The CH_2Cl_2 solution was applied to a column of neutral alumina (1.4 × 14 cm, 80 g). The column was first washed with CH_2Cl_2 (2–3 L), followed by elution with 3–4 L of $CHCl_3$/MeOH (99:1). The latter eluate was concentrated to dryness, and the residue was taken up in 65 mL of 33% methylamine in ethanol and allowed to stand overnight for ~15 h. The reaction mixture was then carefully concentrated in vacuo to a gum. The gum was taken up in $CHCl_3$ and applied to a column of silicic acid (2.2 × 10 cm, 20 g), and after a wash with $CHCl_3$ the column was eluted with $CHCl_3$/MeOH (99:1). The eluate was concentrated to dryness in vacuo and dried by repeated evaporation from absolute ethanol: yield, 600 mg (50%); UV (95% ethanol) λ_{max} 253 nm (ε 13000); UV λ_{min} 222 nm (ε 3260); R_f = 0.08 (silica gel, solvent B), 0.67 (cellulose, solvent A).

Guanosine 3′-(S-Methyl phosphorothioate). In a 13-mL centrifuge tube 2′,5′-di-*O*-(methoxytetrahydropyranyl)-guanosine (0.250 g, 0.490 mmol) was dissolved in 4.0 mL of DMF. MTBO (0.530 g, 2.46 mmol) was added and the solution was cooled to just above freezing in liquid nitrogen. Cyclohexylamine was added (0.64 mL, 6.52 mmol) and the mixture was stoppered and heated to 70 °C for 20 min. The reaction mixture was then worked up just as for the uridine derivative: yield, 88.5 mg (77%); R_f = 0.23 (cellulose, solvent A); UV λ_{max} 256 nm (ε 11600) at pH 2, 254 (11900) at pH 7, 260 (10700) at pH 11; λ_{min} 229 nm (ε 3500) at pH 2, 228 (4240) at pH 7, 232 (5670) at pH 11; NMR (D_2O, Me_4Si) δ 2.255 (d, J = 13.5 Hz, 3 H, P–S–CH_3), 5.98 [d, J = 5.5 Hz, 1 H, H(1′)], 8.12 [d, J = 6 Hz, 1 H, H(8)]. Anal. Calcd for $C_{11}H_{15}N_5O_7PSNa·H_2O$: C, 30.49; H, 3.95; N, 16.16; P, 7.15; S, 7.40. Found: C, 30.68; H, 3.68; N, 16.05; P, 6.91; S, 7.19.

2′,5′-Di-O-(methoxytetrahydropyranyl)cytidine. In a 50-mL round bottom flask 4-methoxy-5,6-dihydro-2*H*-pyran (8.3 g, 0.073 mol) was added with stirring to a solution of 3′-*O*-acetyl-N^4-benzoylcytidine (Green et al., 1970) (1.0 g, 2.57 mmol) and anhydrous mesitylenesulfonic acid (0.54 g, 2.7 mmol) in 16 mL dioxane at 25 °C. After 20 min, the reaction mixture was neutralized with 2.7 mL of 1.00 M $NaOCH_3$ in MeOH and concentrated in vacuo. The gum was taken up in a minimum of CH_2Cl_2 and applied to a column of alumina (1.4 × 9 cm, 50 g). Initially, the column was washed with

CH_2Cl_2 followed by elution with $CHCl_3$ until no more UV-absorbing material eluted. The eluate was concentrated to a glass which was then dissolved in a solution of 0.10 M NaOH (500 mL) and allowed to stand for 15–20 h at room temperature. The solution was then neutralized with an excess of the pyridinium form of AG-50 resin with stirring for 10 min. The resin was filtered off and washed with a little water. The filtrate was concentrated in vacuo and dried by repeated evaporation from absolute ethanol. The residue was taken up in a minimum of $CHCl_3$ and applied to a column of silicic acid (2.2 × 10 cm, 20 g). After a wash with $CHCl_3$ the column was eluted with $CHCl_3$/MeOH (98:2). The eluate was then concentrated in vacuo and dried over P_2O_5: yield, 400 mg (33%); UV (95% ethanol) λ_{max} 272 nm (ϵ 8000); UV λ_{min} 253 nm (ϵ 6320); R_f = 0.10 (silica gel, solvent B), 0.78 (cellulose, solvent A).

Cytidine 3′-(S-Methyl phosphorothioate). In a 12-mL centrifuge tube 2′,5′-di-*O*-(methoxytetrahydropyranyl)cytidine (0.180 g, 0.382 mmol) was dissolved in 2.7 mL of DMF. MTBO (0.413 g, 1.91 mmol) was added and the mixture was cooled to just above freezing with liquid nitrogen. Cyclohexylamine (0.70 mL, 7.04 mmol) was added, the tube was stoppered, and the mixture was heated at 70 °C for 20 min. The reaction mixture was then worked up in the same manner as the uridine derivative: yield, 58.5 mg (84%); R_f = 0.03 (cellulose, solvent A); UV λ_{max} 275 nm (ϵ 12 700) at pH 2, 269 (8500) at pH 7, 269 (8500) at pH 11; UV λ_{min} 241 nm (ϵ 2350) at pH 2, 249 (6560) at pH 7, 249 (6560) at pH 11; NMR (D_2O, Me_4Si) δ 2.05 (d, J = 14 Hz, 3 H, P–S–CH_3), 6.20 [d, J = 8 Hz, 1 H, H(5)], 7.98 [d, J = 7 Hz, 1 H, H(4)]. Anal. Calcd for $C_{10}H_{15}N_3O_7PSNa\cdot H_2O$: C, 30.54; H, 4.36; N, 10.68; P, 7.88; S, 8.15. Found: C, 30.94; H, 4.32; N, 10.48; P, 7.71; S, 7.81.

Uridine 3′-Methyl Phosphate. The title compound was prepared by methanolysis of the corresponding cyclic 2′,3′-phosphate (Dekker & Khorana, 1954).

Determination of Rate Constants for Hydrolysis of Uridine 3′-(S-Methyl phosphorothioate) and Uridine 3′-Methyl Phosphate. One microliter aliquots of 1 M HCl solutions containing either 2 mg/mL oxygen ester or 2 mg/mL sulfur ester were spotted onto silica gel HF_{254} plates at successive time intervals from 0 to 24 h. The plates were developed in *i*-PrOH/NH_3/H_2O (7:1:2). The developed plates were then scanned on a Kratos/Schoeffel Model SD 300 spectrodensitometer at 260 nm, and the results were integrated on a Hewlett-Packard Model 3380A integrator. Three scans were recorded per lane. The hydrolysis rate constants were calculated by averaging the values obtained from

$$k = \frac{\ln \dfrac{\text{initial absorption of ester}}{\text{absorption of ester at } t_n}}{t_n}$$

for all time intervals. The following results were obtained: for uridine 3′-methyl phosphate, $k = (4.4 \pm 0.6) \times 10^{-1}$ h^{-1}; for uridine 3′-(*S*-methyl phosphorothioate), $k = (1.3 \pm 0.3) \times 10^{-2}$ h^{-1}.

Results

Both 2′,5′-protected and totally unprotected forms of the nucleoside 3′-(*S*-methyl phosphorothioates) were found to be completely stable to decomposition in the solid state and in neutral aqueous solution at room temperature for a period of 9 months. Slight decomposition was noticed, however, on treatment of the 2′,5′-protected substrate with 40% acetic acid at room temperature for periods exceeding 90 min.

Table I: K_m and V_{max} for RNase A

substrate	K_m (M)	V_{max} [μmol/(min·mg of protein)]	solvent	ref
Cp-SCH_3	2.5×10^{-3}	6.04×10^{-1}	3	
C>p	3.3×10^{-3}	2.4×10	1	*a*
Cp-benzyl	3.0×10^{-3}	8.8	2	*b*
Cp-methyl	3.0×10^{-3} [c]	2.2	2	*b*
CpA	1.0×10^{-2}	1.32×10^{4}	2	*b*
CpU	3.7×10^{-3}	1.18×10^{2}	2	*b*
Up-SCH_3	2.5×10^{-3}	5.94×10^{-1}	3	
U>p	5.0×10^{-3}	9.6	1	*a*
UpA	1.9×10^{-3}	5.26×10^{3}	2	*b*
UpU	3.7×10^{-3}	4.8×10	2	*b*

[a] Witzel & Barnard (1962a). [b] Witzel & Barnard (1962b). [c] Assumed.

The protected nucleoside 3′-(*S*-methyl phosphorothioates) produced in the reaction of MTBO with the protected nucleosides are assumed to be the pure 3′ isomer. Evidence in support of this assumption is the release of a stoichiometric amount of thiol on hydrolysis of unprotected product with RNase T_2. Since ribonucleases are believed to be specific for the 3′,5′-phosphodiester linkage, rather than the 2′,5′-phosphodiester linkage, the quantitative release of thiol is strong evidence for the presence of only the 3′ isomer.

It is well established that thiols can be accurately quantitated by the use of a chromophore-producing reagent such as 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) (Ellman, 1959). The thiol undergoes disulfide interchange with Ellman's reagent, releasing the chromophore 5-thio-2-nitrobenzoic acid which has an intense absorption maximum at 412 nm (E_{412} = 13 600) and a pK_a of ~5.

This assay procedure is valid only from pH 5 to pH 10 when Ellman's reagent is used as the chromophore-producing reagent. For assay at more acidic pH values, 4,4′-dithiodipyridine, which produces a neutral chromophore, may be substituted for Ellman's reagent (Grassetti & Murray, 1967). Thus, nucleoside 3′-(*S*-methyl phosphorothioates), used in conjunction with a chromophore-producing reagent, can be employed for direct kinetic measurements of phosphotransferase enzymes having a wide range of pH optima. While enzymatic inhibition has been observed at high concentrations of Ellman's reagent, the rates of enzymatically catalyzed thiol release are unaffected by concentrations of reagent <0.1 mM, the maximum necessary for use under the assay conditions described in this work. In many cases concentrations as low as 0.02 mM can be routinely used.

The nucleoside 3′-(*S*-methyl phosphorothioates) described here are fair substrates for a variety of ribonucleases. The values obtained for K_m for these substrates are comparable to those obtained for similar natural substrates lacking sulfur (Tables I–IV). While the V_{max} values are from 1 to as much as 2 orders of magnitude lower than those values for similar natural substrates, the reaction rates obtained are sufficiently high to provide for sensitive assay of nucleolytic activity.

Discussion

The K_m values obtained for the RNase A catalyzed degradation of uridine and cytidine 3′-(*S*-methyl phosphorothioates) are well within the range of those values obtained for a number of natural and synthetic substrates (Table I). The V_{max} values obtained, however, are far below those values for natural dinucleoside substrates and slightly lower than the V_{max} of nucleoside cyclic phosphates and nucleoside 3′-benzyl and 3′-methyl phosphates. The former result is consistent with the available data in Table I, the substrates bearing a single

Table II: K_m and V_{max} for RNase T_2

substrate	K_m (M)	V_{max} [μmol/(min·mg of protein)]	solvent	ref
Ap-SCH_3	1.00×10^{-4}	9.01	3	
A>p	2.3×10^{-4}	3.60×10^2	4	*a*
ApA	0.39×10^{-4}	9.9×10	5	*b*
ApU	0.82×10^{-4}	2.85×10^2	5	*b*
Cp-SCH_3	9.09×10^{-5}	7.20	3	
C>p	4.0×10^{-4}	3.5×10^2	4	*a*
CpA	0.41×10^{-4}	3.12×10^2	5	*b*
CpU	0.72×10^{-4}	5.50×10^2	5	*b*
Gp-SCH_3	1.43×10^{-4}	3.60×10	3	
G>p	6.5×10^{-4}	2.5×10^2	4	*a*
GpA	0.63×10^{-4}	3.51×10^2	5	*b*
GpU	1.1×10^{-4}	3.31×10^2	5	*b*
Up-SCH_3	2.50×10^{-4}	2.88×10	3	
U>p	6.1×10^{-4}	4.4×10^2	4	*a*
UpA	0.47×10^{-4}	6.35×10^2	5	*b*
UpU	1.2×10^{-4}	7.18×10^2	5	*b*

[a] Sato et al. (1966). [b] Imazawa et al. (1968).

Table III: K_m and V_{max} for RNase T_1

substrate	K_m (M)	V_{max} [μmol/(min·mg of protein)]	solvent	ref
Gp-SCH_3	1.80×10^{-4}	1.23×10^{-2}	3	
G>p	2.50×10^{-3}	9.87	6	*a*
GpC	4.45×10^{-4}	3.94×10^7	7	*b*

[a] Yoshida & Otsuka (1971). [b] Irie (1968).

Table IV: K_m and V_{max} for RNase U_1

substrate	K_m (M)	V_{max} [μmol/(min·mg of protein)]	solvent	ref
Gp-SCH_3	3.85×10^{-4}	3.78×10^{-2}	3	
GpC	2.38×10^{-4}	2.27×10^6	8	*a*

[a] Hashimoto et al. (1971).

nucleoside residue having much lower V_{max} values than the dinucleoside phosphate substrates. The latter result is consistent with our nonenzymatic hydrolysis rate study in which we found that uridine 3′-methyl phosphate hydrolyzes ~34 times faster than uridine 3′-(*S*-methyl phosphorothioate) in 1 M HCl. This reaction rate difference may be due to the poor hydrogen bonding characteristics of sulfur relative to oxygen (Pauling, 1960) which could result in less efficient protonation of the mercaptide leaving group, thus impeding the rate of transphosphorylation relative to the corresponding oxygen ester.

In the case of RNase T_2 the K_m values obtained for the four ribonucleoside 3′-(*S*-methyl phosphorothioate) substrates are lower than the recorded values for nucleoside cyclic phosphates and are only slightly higher than the reported K_m values for dinucleoside phosphate substrates. However, the V_{max} values of the latter are larger than those obtained for the nucleoside 3′-(*S*-methyl phosphorothioate) substrates by a factor ranging from 10 to 75. It can be concluded that the nucleoside 3′-(*S*-methyl phosphorothioates) are fair substrates for RNase T_2.

The K_m value for the interaction of guanosine 3′-(*S*-methyl phosphorothioate) with RNase T_1 is slightly lower than those values for natural substrates, while the V_{max} value for the thioester is 3 orders of magnitude lower than the lowest value of V_{max} for a natural substrate. While the interaction of guanosine 3′-(*S*-methyl phosphorothioate) with RNase U_1 gave K_m and V_{max} values very close to those obtained with RNase T_1, there is insufficient data available for natural substrates to appraise the effect of sulfur on catalysis by RNase U_1.

The advantages of nucleoside 3′-(*S*-methyl phosphorothioates) for the assay of ribonucleases are as follows: (1) accuracy, the quantity measured by the assay is directly related to phosphotransferase activity and is as good as 2–5%; (2) simplicity, the procedure involves only pipetting and the use of a recording spectrophotometer; (3) specificity, the assay is capable of determining the base specificity of a ribonuclease; (4) sensitivity, the assay is sensitive to as little as 20 ng of RNase A. [This level of sensitivity is comparable to that of the assay of Kenney & Dekker (1971) and is $\sim^1/_{50}$ as sensitive as the assay of Blank & Dekker (1981).]

The disadvantages of an assay using these substrates are the following. (1) Since the substrates are not commercially available, they must be prepared in the laboratory. (2) Some RNases may be sensitive to the higher levels of Ellman's reagent used in the assay mixture with the result that inhibition may occur. However, we have had no problems with the RNases described in this work. If necessary, one can use very low levels of Ellman's reagent. (3) Impurities in a crude enzyme solution such as oxidizing agents or thiols can seriously interfere with accurate measurement of released thiol unless the RNase concentration is sufficiently high so as to permit substantial dilution of the crude sample into the assay solution. The usual preliminary dialysis or column chromatography would obviate this problem, however.

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Purification and Some Properties of a Deoxyribonucleic Acid Endonuclease Endogenous to Rat Liver Chromatin†

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ABSTRACT: A deoxyribonucleic acid (DNA) endonucleolytic activity has been purified from a 0.3 M KCl extract of rat liver chromatin by a combination of selective precipitation and ion-exchange and gel filtration chromatography. The purified protein has a molecular weight of 35 000 as determined by Sephadex G-200 gel filtration and sodium dodecyl sulfate–acrylamide gel electrophoresis. The nuclease activity is stimulated by the addition of Mg^{2+} and thus may represent the Mg^{2+}-activated DNase endogenous to chromatin. The purified enzyme has the ability to make both single-strand nicks and double-strand cuts in DNA.

Nuclease digestion studies have proven invaluable in the elucidation of the nucleosome as the basic structural subunit of eukaryotic chromatin. Indeed, one of the first indications of such a chromatin organization was a periodic deoxyribonucleic acid (DNA)[1] digestion pattern observed during chromatin autodigestion mediated by endogenous DNases (Hewish & Burgoyne, 1973a,b). Since then nucleases exogenous to chromatin such as micrococcal nuclease and DNases I and II have been widely used to investigate both the structure of the nucleosome itself and the organization of nucleosomes into domains of higher order structure varying in their sensitivity to digestion [for a recent review see McGhee & Felsenfeld (1980)]. In addition, many workers have utilized the autodigestion of chromatin to examine these topics; this process has been shown to result in the liberation of nucleosome multimers and monomers (Keichline et al., 1976; Krueger, 1978; Suciu, 1979; Chikhirzhina, 1979) and may be able to differentiate between transcriptionally active and inactive chromatin (Paul & Duerksen, 1976a,b). The nature of the DNases responsible for such phenomena is less clear. However, nucleolytic activity has been ascribed to the nonhistone protein component of chromatin and nuclei (O'Connor, 1969; Urbanczyk & Studzinski, 1974; Vinter et al., 1974; Gainuillina et al., 1976; Lambert & Studzinski, 1979), and the purifications to varying degrees of several DNase activities endogenous to chromatin and nuclei have been described (Ishida et al., 1974; Cordis et al., 1975; McGuire et al., 1976; Fischman et al., 1979).

Here we report the purification of a DNA nucleolytic enzyme extracted from rat liver chromatin. The enzyme activity is stimulated by the addition of divalent magnesium cations but differs from those DNases previously described in its mechanism of action on DNA.

Materials and Methods

Frozen rat livers were obtained from Pel-Freeze, standards for $NaDodSO_4$–acrylamide gel electrophoresis was from Pharmacia Fine Chemicals, and standards for gel filtration were from Sigma Chemical Co. Agarose was purchased from SeaKem. All other chemicals were of reagent grade.

Isolation of Nuclei. In a standard preparation nuclei were purified from 20 rat livers as described previously (Wallace et al., 1977).

Isolation of pBR322 DNA. Plasmid DNA was prepared from *Escherichia coli* HB101 essentially by the method of Clewell & Helinski (1969).

Nuclease Assay. The conversion of superhelical pBR322 DNA to relaxed circular and linear forms was used to monitor the nuclease activity throughout the purification. An aliquot (10 μL) of the column fractions to be assayed was incubated in a reaction mixture of 20-μL total volume containing 0.16 μg pBR322 DNA at a final concentration of 0.1 M KCl, 30 mM Tris-HCl (pH 7.0), 2 mM $MgCl_2$, and 40 μM EDTA (derived from the plasmid storage buffer). The reaction was stopped after 15 min at 37 °C by the addition of $NaDodSO_4$ to 0.3%, and the products of digestion were analyzed by agarose gel electrophoresis as described below. One unit of enzyme activity was defined as that amount of enzyme required to convert 0.1 μg of superhelical DNA to other forms in 15 min at 37 °C.

DNA Gel Electrophoresis. Supercoiled, circular relaxed, and linear DNA molecules were resolved in 1% agarose gels containing 36 mM Tris base, 30 mM NaH_2PO_4, and 1 mM EDTA (pH 7.7; also the reservoir buffer). Samples were brought to 0.3% $NaDodSO_4$, 0.05% bromophenol blue, and 10% sucrose and heated to 55 °C for 15 min prior to elec-

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; QAE-Sephadex, quaternary aminoethyl-Sephadex; DNA, deoxyribonucleic acid; Sh, superhelical form of pBR322 DNA; Cr, circular relaxed form of pBR322 DNA; l, linear form (strand) of pBR322 DNA; c, circular strand of pBR322 DNA; DNase, deoxyribonuclease; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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