Stereochemical Course of Cerium(IV)-Catalyzed Hydrolysis of Cyclic Nucleotides

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Abstract: Ce^{IV} has been shown to catalyze the hydrolysis of thymidine 3′,5′-cyclic thiophosphate (cTMPS) to give a mixture of 3′-TMPS and 5′-TMPS with rates comparable to those previously reported for cAMP and cTMP. The ratio of the 3′-TMPS and 5′-TMPS products was dependent on the absolute configuration of the cTMPS; starting from R_p cTMPS, with the sulfur equatorial, the 3′-TMPS:5′-TMPS ratio was ca. 7:1 whereas for the corresponding S_p cTMPS the ratio was 3:2. Both diastereoisomers of thymidine 3′,5′-cyclic [^{18}O]-thiophosphate have been synthesized, and the stereochemical course of the hydrolysis reaction catalyzed by Ce^{IV} has been determined. In each case, the reaction proceeds with clean *inversion* of configuration, consistent with coordination of the metal ion to the exocyclic oxygen substituent and delivery of the hydroxide nucleophile from one of the remaining metal coordination sites with in-line geometry.

Introduction

Phosphate esters play a vast number of critical roles in biological systems [e.g., information storage and utilization (DNA/RNA); energy transduction (ATP); cellular signaling/communication (cAMP, protein phosphorylation, inositol phosphates etc.)]. The mechanisms by which phosphorylation/dephosphorylation reactions are catalyzed both enzymically and chemically has been an area of considerable interest and controversy.[1,2] Of particular relevance to this present study has been the recent surge of interest in model metal ion catalysis of phosphate hydrolysis that has led to the development of a number of highly efficient artificial nucleases.[3,4] Despite the many different metals that have been studied, the precise details of the origins of the catalysis and its relevance to the biological processes are still far from clear. Determination of the stereochemical course of both chemical- and enzyme-catalyzed phosphoryl and thiophosphoryl transfer reactions has for many years been used as a powerful mechanistic imperative,[1,5] and it is surprising that hitherto this has not been applied to any metal-catalyzed reactions. We report here the first determination of the stereochemical course of a model metal-catalyzed displacement reaction at a phosphoryl center.

Arguably the most impressive rate acceleration reported to date is the catalysis of the hydrolysis of nucleoside 3′,5′-cyclic monophosphates at pH 7 by Ce^{IV} reported Komiyama and colleagues.[4] The half-life for hydrolysis of cAMP at 30 °C was shown to be 7 s, a rate acceleration of over 10^{11}! The origin of this phenomenal rate acceleration is far from clear, particularly because the system is not homogeneous and the number of metal ions and aggregate state of the catalytic species is still undefined. In principle, the metal may function in a number of catalytic roles: as a Lewis acid complexing to the phosphoryl center, making it more electrophilic; as a Lewis acid coordinating to the leaving group, assisting bond cleavage; as a coordination site for the nucleophile and the phosphoryl center, effecting "intramolecular" delivery; as a means to stabilize the negatively charged trigonal bipyramidal transition state (or intermediate). In relation to stabilizing a trigonal bipyramidal intermediate, there is the intriguing possibility that the metal ion or ions could be involved in bidentate coordination to the geminal dianion intermediate, leading to the generation of "small-ring" metal−phosphate chelates that may exploit the high reaction rates widely documented for other small-ring phosphate esters.[6] Such reactions have the potential to proceed via a pseudorotation pathway which has distinct stereochemical implications. It is interesting to note that non-in-line mechanisms for phosphate ester hydrolysis, including the reaction catalyzed by inositol monophosphatase, have recently been claimed to be energetically accessible.[2] The rich array of potential reaction mechanisms encouraged this present study to try to delineate the stereochemical course of a metal-catalyzed phosphate ester hydrolysis.

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Experimental Section

General. Nucleotides were isolated and purified by ion-exchange column chromatography on DEAE Sephadex A25-120, eluting with a linear gradient of triethylammonium bicarbonate buffer (pH 8). HPLC monitoring of hydrolysis reactions was performed on a C8 reversed-phase column with a KH_2PO_4 buffer and methanol gradient. Routine ^1H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker ARX 250 (respective frequencies 250, 101, and 62.9 MHz); the ^{31}P NMR configurational analysis spectra were recorded on a Bruker DRX 400 (162 MHz) spectrometer.

[^{18}O]Phosphorus Oxychloride. Water (ca. 60% enrichment with ^{18}O, 0.75 mL, 39.2 mmol) was added dropwise under a constant stream of nitrogen to phosphorus pentachloride (8.16 g, 39.2 mmol) in an ice−water-cooled two-necked flask. The mixture was stirred for 1 h at room temperature and was then distilled at atmospheric pressure to give the title compound as a colorless liquid (4.58 g, 76%). δ_P ($CDCl_3$, 101 MHz): 4.51.

(2-Chlorophenyl)-[^{18}O]phosphorodichloridate. A mixture of [^{18}O]-phosphorus oxychloride (4.58 g, 29.6 mmol), sodium chloride (0.076 g, 1.30 mmol), and 2-chlorophenol (1.27 g, 9.87 mmol) was heated under reflux for 3 days. The unreacted [^{18}O]phosphorus oxychloride was distilled off at atmospheric pressure, and subsequently the product was distilled under vacuum to give the title compound as a colorless liquid (1.68 g, 69%), bp_8 118 °C. δ_H ($CDCl_3$, 250 MHz): 7.47−7.25 (4 H, m, aryl-H). δ_C ($CDCl_3$, 62.9 MHz): 146.3 (d, $J_{C,P}$ = 10.4 Hz), 131.7 (d), 128.7 (dd, $J_{C,P}$ = 2.3 Hz), 128.4 (dd, $J_{C,P}$ = 2.5), 126.4 (s), 122.4 (d). δ_P ($CDCl_3$, 101 MHz): 4.17.

(2-Chlorophenyl)anilido-[^{18}O]phosphorochloridate. Aniline (1.24 mL, 13.63 mmol) was added dropwise to a ice−water-cooled solution of (2-chlorophenyl)-[^{18}O]phosphorodichloridate (1.68 g, 6.82 mmol) in dry benzene (30 mL). After 30 min, the mixture was allowed to reach room temperature and was left overnight. The precipitate was filtered off, and the filtrate was washed with water, dried over magnesium sulfate, and evaporated in vacuo. The residue was recrystallized from benzene, yielding the title compound as a white solid (1.530 g, 74%), mp 96−98 °C. δ_H ($CDCl_3$, 250 MHz): 7.62−7.08 (10 H, m, aryl-*H* and N*H*). δ_C ($CDCl_3$, 62.9 MHz): 145.2 (d, $J_{C,P}$ = 7.3 Hz), 137.1 (d, $J_{C,P}$ = 1.2 Hz), 131.0 (d), 129.4 (d), 128.1 (dd, $J_{C,P}$ = 1.8 Hz), 126.9 (dd, $J_{C,P}$ = 1.2 Hz), 125.5 (d, $J_{C,P}$ = 7.9 Hz), 124.1 (d), 122.1 (dd, $J_{C,P}$ = 3.7 Hz), 120.3 (dd, $J_{C,P}$ 7.9 Hz). δ_P ($CDCl_3$, 101 MHz): 4.73, 4.69. IR: 3150 (N−H), 1605 and 1595 (aryl), 1500 and 1475 (aryl), 1290 (phenyl-N), 1255 (P═O), 1210 (aryl-O), 920 (P−O), 760 and 695 (C−Cl) cm^{-1}. MS (+FAB): 304 (M^+, ^{18}O-labeled), 302 (M^+).

(2-Chlorophenyl) 5′-Monomethoxytritylthymidine 3′-[^{18}O]Phosphoroanilidate. 2′-Deoxy-5′-monomethoxytritylthymidine (1.839 g, 3.574 mmol) was dried by evaporation from pyridine (3 × 6 mL). (2-Chlorophenyl)anilido-[^{18}O]phosphorochloridate (1.626 g, 5.361 mmol) was dried by evaporation from THF (2 × 6 mL) and was then dissolved in dry pyridine (10 mL) and added dropwise to a solution of the nucleoside in pyridine (10 mL) at 10 °C. After 30 min, the reaction mixture was allowed to reach room temperature and was kept at 24 °C for 42 h. The mixture was cooled with an ice bath, and a solution of sodium acetate (0.61 g) in water (54 mL) was added, followed by extraction with $CHCl_3$ (3 × 70 mL). The combined organic layers were washed with water (2 × 150 mL) and dried over magnesium sulfate. The solvent was removed in vacuo and the residue was evaporated three times from toluene to remove the remaining pyridine. The two diastereoisomers were separated using spinning plate preparative layer chromatography (Chromatotron), eluting with acetone:$CHCl_3$ 3:7, yielding the two diastereoisomers as white solids: least polar diastereoisomer 1.202 g; most polar diastereoisomer 0.908 g (total yield, 77%).

Less Polar Diastereoisomer. δ_H ($CDCl_3$, 250 MHz): 10.03 (1 H, s, N*H*), 7.45 (1 H, d, J = 1.0 Hz, H_6), 7.31−6.71 (23 H, m, aromatic-*H*), 6.37 (1 H, dd, J = 8.8 and 5.7 Hz, $H_{1'}$), 5.32 (1 H, t, J = 6.5 and 6.0 Hz, $H_{3'}$), 4.19 (1 H, d, J = 1.6 Hz, $H_{4'}$), 3.67 (3 H, s, −OCH_3), 3.25 (2 H, dAB-system, $J_{H,P}$ = 2.5 Hz, J = 35.6 and 10.7 Hz, $H_{5'}$), 2.69−2.33 (2 H, m, $H_{2'}$), 1.28 (3 H, s, −CH_3). δ_C ($CDCl_3$, 62.9 MHz): 163.3 (s), 157.8 (s), 149.7 (s), 145.4 (d, $J_{C,P}$ = 6.1 Hz), 142.6 (d, $J_{C,P}$ = 4.9 Hz), 137.4 (d, $J_{C,P}$ = 4.9 Hz), 134.5 (d), 133.6 (s), 129.7 (d), 129.4 (d), 128.4 (d), 127.4 (dd, $J_{C,P}$ = 2.4), 127.1 (d), 126.4 (d), 125.1 (d), 124.5 (d, $J_{C,P}$ = 7.3 Hz), 121.7 (d), 120.4 (dd, $J_{C,P}$ = 2.4 Hz), 117.4 (d), 117.3 (d), 112.4 (d), 110.6 (s), 86.5 (s), 83.4 (dd, $J_{C,P}$ = 9.8 Hz), 77.9 (dd, $J_{C,P}$ = 4.9 Hz), 76.4 (d), 62.4 (t), 54.2 (q), 38.2 (t), 10.6 (q). δ_P ($CDCl_3$, 101 MHz): −2.91 and −2.95. IR: 3200 (N−H), 1690 (C═O), 1500 and 1480 (aryl), 1275 (P═O), 1225 (aryl-O), 1180 (P−O), 1115 (C−O), 1065 (P−O), 1085 (C−O), 945 (P−O), 750 and 700 (C−Cl) cm^{-1}. MS (+FAB): 804 ($M^+ + Na^+$, ^{18}O-labeled), 802 ($M^+ + Na^+$), 782 (M^+, ^{18}O-labeled), 780 (M^+).

More Polar Diastereoisomer. δ_H ($CDCl_3$, 250 MHz): 9.38 (1 H, s, N*H*), 7.45 (1 H, d, J = 1.0 Hz, H_6), 7.32−6.71 (23 H, m, Ar-*H*), 6.36 (1 H, dd, J = 8.5 and 5.7 Hz, $H_{1'}$), 5.38 (1 H, t, J = 6.9 and 5.7 Hz, $H_{3'}$), 4.29 (1 H, d, J = 1.9 Hz, $H_{4'}$), 3.67 (3 H, s, OCH_3), 3.40 (2 H, dAB-system, $J_{H,P}$ = 2.5 Hz, J = 18.3 and 10.7 Hz, $H_{5'}$), 2.63−2.29 (2 H, m, $H_{2'}$), 1.30 (3 H, s, −CH_3). δ_C ($CDCl_3$, 62.9 MHz): 164.3 (s), 159.3 (s), 150.9 (s), 146.7 (d, $J_{C,P}$ = 6.1 Hz), 144.0 (d, $J_{C,P}$ = 2.4 Hz), 138.8 (s), 135.8 (d), 135.0 (s), 131.1 (d), 130.8 (d), 129.8 (d), 128.8 (dd, $J_{C,P}$ = 3.1 Hz), 128.4 ($J_{C,P}$ = 5.5 Hz), 127.8 (d), 126.4 (d), 125.9 (d, $J_{C,P}$ = 7.3 Hz), 123.1 (d), 121.7 (dd, $J_{C,P}$ = 2.4 Hz), 118.8 (d), 118.7 (d), 113.8 (d), 112.0 (s), 87.9 (s), 79.2 (dd $J_{C,P}$ = 5.5 Hz), 77.7 (d), 63.8 (t), 55.6 (q), 39.5 (t), 12.1 (q). δ_P ($CDCl_3$, 101 MHz): −2.77. IR: 3180 (N−H), 1690 (C═O), 1500 and 1480 (aryl), 1275 (P═O), 1225 (C−O), 1105 (C−O), 1070 (P−O), 1035 (C−O), 960 (P−O), 750 and 700 (C−Cl) cm^{-1}. MS (+FAB): 804 ($M^+ + Na^+$, ^{18}O-labeled), 802 ($M^+ + Na^+$), 782 (M^+, ^{18}O-labeled), 780 (M^+).

The experimental procedures for the subsequent reactions of the more polar diastereoisomer were similar to those described for the less polar diastereoisomer, and only spectral characterization is given.

***S$_p$*-(2-Chlorophenyl) Thymidine 3′-[^{18}O]Phosphoroanilidate.** Acetic acid (30 mL, 80%) was added to the less polar diastereoisomer from above (1.2 g, 1.536 mmol) and stirred at room temperature for 2 h. The solvent was evaporated, and the residue was dissolved in $CHCl_3$, subsequently washed with $NaHCO_3$ (saturated) and water, dried over magnesium sulfate, and evaporated. The residue was purified by flash column chromatography (silica, $CHCl_3$:MeOH 9:1), yielding the title compound (0.522 g, 62%) as a white solid.

δ_H (DMSO-d_6, 250 MHz): 11.13 (1 H, s, N*H*), 8.55 (1 H, d, J = 10.1 Hz, N*H*Ph), 7.50 (1 H, d, J = 1.0 Hz, H_6), 7.36−6.72 (9 H, m, Ar-*H*), 6.04 (1 H, dd, J = 8.2 and 6.0 Hz, $H_{1'}$), 5.00 (2 H, m, $H_{3'}$), 3.90 (1 H, m, $H_{4'}$), 3.37 (2 H, m, $H_{5'}$), 2.24 (2 H, m, $H_{2'}$), 1.57 (3 H, d, J = 0.6 Hz, −CH_3). δ_C (DMSO-d_6, 62.9 MHz): 164.0 (s), 150.8 (s), 146.6 (d, $J_{C,P}$ = 5.5 Hz), 140.0 (s), 136.2 (d), 131.0 (d), 129.5 (d), 128.8 (d), 126.6 (d), 124.8 (d, $J_{C,P}$ = 6.7 Hz), 122.1 (d), 121.5 (dd, $J_{C,P}$ = 2.4 Hz), 118.3 (dd, $J_{C,P}$ = 7.9 Hz), 110.1 (s), 85.5 (dd, $J_{C,P}$ = 5.5 Hz), 84.0 (d), 78.5 (dd, $J_{C,P}$ = 5.5 Hz), 61.3 (t), 38.0 (t), 12.6 (q). δ_P (DMSO-d_6, 101 MHz): −0.93 and −0.96. IR: 3420 (O−H), 3200 (N−H), 1690 (C═O), 1500 and 1480 (aryl), 1275 (P═O), 1220 (C−O), 1105 (C−O), 1060 (P−O), 1015 (C−O), 960 (P−O), 750 and 695 (C−Cl) cm^{-1}. MS (+FAB): 532 ($M^+ + Na^+$, ^{18}O-labeled), 530 ($M^+ + Na^+$), 510 (MH^+, ^{18}O-labeled), 508 (MH^+).

***R$_p$*-(2-Chlorophenyl) Thymidine 3′-[^{18}O]Phosphoroanilidate** (410 mg, 73%). δ_H (DMSO-d_6, 250 MHz): 11.15 (1 H, s, N*H*), 8.58 (1 H, bs, N*H*), 7.53 (1 H, s, $H_{6'}$), 7.39−6.74 (9 H, m, Ar-*H*), 6.05 (1 H, dd, J = 8.0 and 6.1 Hz), 5.03 (2 H, m, $H_{3'}$ and −O*H*), 3.98 (1 H, d, J = 1.9 Hz, $H_{4'}$), 3.47 (2 H, bs, $H_{5'}$), 2.21 (2 H, m, $H_{2'}$), 1.60 (3 H, s, −CH_3). δ_C (DMSO-d_6, 62.9 MHz): 164.0 (s), 150.8 (s), 146.6 (d, $J_{C,P}$ = 5.5 Hz), 140.0 (s), 136.2 (d), 131.0 (d), 129.5 (d), 128.8 (d), 126.6 (d), 124.8 (d, $J_{C,P}$ = 7.3 Hz), 122.1 (d), 121.6 (dd, $J_{C,P}$ = 1.6 Hz), 118.3 (dd, $J_{C,P}$ = 7.9 Hz), 110.1 (s), 85.7 (dd, $J_{C,P}$ = 5.5 Hz), 84.1 (d), 78.5 (dd, $J_{C,P}$ = 5.5 Hz), 61.4 (t), 37.9 (t), 12.6 (q). δ_P (DMSO-d_6, 101 MHz): −1.00 and −1.05. IR: 3500 (O−H), 3160 (N−H), 1700 (C═O), 1500 and 1480 (aryl), 1260 (P═O), 1225 (C−O), 1190 (P−O), 1130 (C−O), 1070 (P−O), 1015 (C−O), 960 (P−O), 755 and 690 (C−Cl) cm^{-1}. MS (+FAB): 532 ($M^+ + Na^+$, ^{18}O-labeled), 530 ($M^+ + Na^+$), 510 (MH^+, ^{18}O-labeled), 508 (MH^+).]

***S$_p$*-Thymidine 3′,5′-Cyclic [^{18}O]Phosphoranilidate.** *S$_p$*-(2-Chlorophenyl) thymidine 3′-[^{18}O]phosphoroanilidate (200 mg, 0.393 mmol) was dried by evaporation from THF (3 × 8 mL) and then dissolved in DMSO (8 mL). Resublimed potassium *tert*-butoxide (1102 mg, 9.822 mmol) dissolved in *tert*-butyl alcohol (10 mL) was added, and the

mixture was stirred at room temperature for 43 h. Dowex 50 cation-exchange resin (pyridinium form) was added, and subsequently the mixture was filtered. The filtrate was evaporated, and the residue was purified by flash column chromatography (silica, $CHCl_3$:MeOH 9:1), yielding the title compound (116 mg, 78%).

δ_H (DMSO-d_6, 250 MHz): 11.29 (1 H, s, N*H*), 8.32 (1 H, d, J = 8.8 Hz, N*H*Ph), 7.55 (1 H, s, $H_{6'}$), 7.01 (5 H, m, Ar-*H*), 6.29 (1 H, dd, J = 8.5 and 2.5 Hz, $H_{1'}$), 5.04 (1 H, m, $H_{3'}$), 4.52 (2 H, m, $H_{5'}$), 3.98 (1 H, m, $H_{4'}$), 2.43 (2 H, m, $H_{2'}$), 1.75 (3 H, s, $-CH_3$). δ_C (DMSO-d_6, 62.9 MHz): 164.1 (s), 150.6 (s), 140.0 (s), 137.3 (d), 129.4 (d), 122.0 (d), 118.6 (dd, $J_{C,P}$ = 7.6 Hz), 110.8 (s), 84.0 (d), 76.3 (dd, $J_{C,P}$ = 4.1 Hz), 73.2 (dd, $J_{C,P}$ = 5.6 Hz), 68.8 (dd, $J_{C,P}$ = 8.1 Hz), 34.5 (t), 12.2 (q). δ_P (DMSO-d_6, 101 MHz): 1.74 and 1.70. MS (+FAB): 404 (M^+ + Na^+, ^{18}O-labeled), 402 (M^+ + Na^+), 382 (MH^+, ^{18}O-labeled), 380 (MH^+).

***R*$_p$-Thymidine 3′,5′-Cyclic [^{18}O]Phosphoranilidate** (129 mg, 87%). δ_H (DMSO-d_6, 250 MHz): 8.32 (2 H, bs, N*H*Ph + N*H*), 7.62 (1 H, d, J = 1.2 Hz, $H_{6'}$), 7.43−7.04 (5 H, m, Ar-*H*), 6.41 (1 H, dd, J = 9.0 and 3.0 Hz, $H_{1'}$), 4.97 (1 H, q, J = 9.0 and 9.2 Hz, $H_{3'}$), 4.70 (2 H, m, $H_{5'}$), 4.11 (1 H, m, $H_{4'}$), 2.59 (2 H, m, $H_{2'}$), 1.93 (3 H, d, J = 0.7 Hz, $-CH_3$). δ_C (DMSO-d_6, 62.9 MHz): 164.1 (s), 150.5 (s), 140.6 (s), 137.6 (d), 129.5 (d), 121.6 (d), 117.9 (dd, $J_{C,P}$ = 7.6 Hz), 110.5 (s), 85.0 (d), 77.5 (dd, $J_{C,P}$ = 5.1 Hz) 73.5 (dd, $J_{C,P}$ = 7.1 Hz), 69.7 (dt, $J_{C,P}$ = 9.2 Hz), 34.1 (dt, $J_{C,P}$ = 8.1 Hz), 12.3 (q). δ_P (DMSO-d_6, 101 MHz): −3.12 and −3.16. MS (+ES): 420 (M^+ + K^+, ^{18}O-labeled), 418 (M^+ + K^+), 404 (M^+ + Na^+, ^{18}O-labeled), 402 (M^+ + Na^+).]

***R*$_p$-Thymidine 3′,5′-Cyclic [^{18}O]thiophosphate.** *S*$_p$-Thymidine 3′,5′-cyclic [^{18}O]phosphoranilidate (152 mg, 0.400 mmol) was dried by evaporation from DMF (3 × 5 mL) and dissolved in DMF (3 mL). Sodium hydride (38 mg, 1.60 mmol) was dispersed in DMF (1 mL) and added. After 15 min, carbon disulfide (1.5 mL) was added, and the mixture was stirred for 45 min. Then it was cooled to −78 °C, and Dowex 50 cation-exchange resin (pyridinium form) was added slowly. The mixture was allowed to reach room temperature, and then it was filtered. The filtrate was evaporated and applied to an ion-exchange column. A gradient of triethylammonium bicarbonate buffer (50−600 mM) was applied at 30 mL h^{-1} over 24 h. The title compound (0.293 mmol, 73%) was eluted as the triethylammonium salt. δ_H (D_2O, 250 MHz): 7.24 (1 H, d, J = 0.9 Hz, $H_{6'}$), 6.10 (1 H, dd, J = 8.3 and 3.0 Hz, $H_{1'}$), 4.32−4.12 (2 H, m, $H_{5'}$), 3.75 (1 H, m, $H_{4'}$), 3.00 (6 H, q, NCH_2CH_3), 2.45−2.25 (2 H, m, $H_{2'}$), 1.69 (3 H, s, $-CH_3$), 1.08 (9H, t, NCH_2CH_3). δ_C (D_2O, 62.9 MHz): 167.0 (s), 152.0 (s), 138.1 (d), 112.3 (s), 85.3 (d), 75.5 (dd, $J_{C,P}$ = 6.1 Hz), 74.8 (dd, $J_{C,P}$ = 6.1 Hz), 68.0 (dt, $J_{C,P}$ = 9.8 Hz), 49.3 (t), 34.9 (dt, $J_{C,P}$ = 7.3 Hz), 11.8 (q), 8.6 (q). δ_P (D_2O, 101 MHz): 55.59 and 55.55. MS (−ES): 323 ($M(2D)^-$, ^{18}O-labeled), 322 ($M(D)^-$, ^{18}O-labeled), 321 (M^-, ^{18}O-labeled and $M(2D)^-$), 320 ($M(D)^-$), 319 (M^-).

***S*$_p$-Thymidine 3′,5′-Cyclic [^{18}O]Thiophosphate as the Triethylammonium Salt.** δ_H (D_2O, 250 MHz): 7.54 (1 H, d, J = 1.2 Hz, H_6), 6.46 (1 H, dd, J = 8.8 and 2.4 Hz, $H_{2'}$), 4.52 (2 H, m, $H_{5'}$), 3.98 (1 H, m, $H_{4'}$), 2.63 (2 H, m, $H_{2'}$), 2.00 (3 H, d, J = 0.9 Hz, $-CH_3$). δ_C (D_2O, 62.9 MHz): 162.1 (s), 155.7 (s), 137.4 (d), 112.5 (s), 85.5 (d), 76.6 (dd, $J_{C,P}$ = 4.1 Hz), 75.0 (dd, $J_{C,P}$ = 5.1 Hz), 67.8 (dt, $J_{C,P}$ = 7.6 Hz), 34.6 (dt, $J_{C,P}$ = 8.7 Hz), 12.5 (q). δ_P (D_2O, 101 MHz): 53.70 and 53.66. MS (−ES): 323 ($M(2D)^-$, ^{18}O-labeled), 322 ($M(D)^-$, ^{18}O-labeled), 321 (M^-, ^{18}O-labeled) and ($M(2D)^-$), 320 ($M(D)^-$), 319 (M^-).]

Hydrolysis of Thymidine 3′,5′-Cyclic Phosphate and of *R*$_p$-Thymidine 3′,5′-Cyclic Thiophosphate. The triethylammonium salt of cTMP or cTMPS (13 μmol) was dissolved in Hepes buffer (5 mL; 100 mM solution in water), and cerium diammonium hexanitrate (27 mg, 49 μmol) was added. The suspension was kept at room temperature and was sonicated at regular intervals. Samples were taken 2, 5, 10, 15, 20, 35, and 50 min after addition of the cerium salt, quenched with an equal volume of KH_2PO_4 (0.2 M solution in water), and diluted to 10 times its volume. Part of this solution was taken, filtered, and analyzed by HPLC.

Hydrolysis of *R*$_p$-Thymidine 3′,5′-Cyclic [^{18}O]Thiophosphate. The triethylammonium salt of thymidine 3′,5′-cyclic [^{18}O]thiophosphate (106 μmol) was dissolved in Hepes buffer (106 mL; 100 mM solution in water), and cerium diammonium hexanitrate (581 mg, 1.06 mmol) was added. The suspension was stirred at room temperature for 1.75 h, after which it was quenched by adding potassium dihydrogen phosphate (106 mL, 0.2 M solution in water). The suspension was centrifuged, after which the supernatant liquid was collected. Charcoal (10.6 g) was added to the supernatant, and the resultant suspension was filtered. The charcoal was washed with water (250 mL) to remove inorganic phosphate, and the nucleoside thiophosphate products were eluted from the charcoal by washing with a mixture of methanol (250 mL) and ammonia (250 mL, 40% solution in water). The solvents were evaporated, the residue was dissolved in water, and dithiothreitol (0.15 mL, 1 M solution in water) was added. This was applied to an ion-exchange column. A gradient of triethylammonium bicarbonate buffer (50−600 mM) was applied at 45 mL h^{-1} over 8 h. The 5′- and 3′-thymidine [^{18}O]thiophosphates were isolated as the triethylammonium salts (44 μmol, 42%).

Methylation of the Hydrolysis Products. The bispyridinium salts of 5′- and 3′-thymidine [^{18}O]thiophosphates (7.3 μmol) were dissolved in methanol (0.1 mL), and a freshly prepared ethereal solution of diazomethane (1.5 mL) was added. After the solution was stirred for 4.5 h, the solvents were evaporated, and the residue was dissolved in methanol-d_4. δ_P (CD_3OD, 162 MHz): 32.86, 32.84, 32.83, 32.79, 32.18, 32.16, 32.11, 32.08, 32.06. MS (−ES): 368 ($M(^{18}O)^-$), 367 ($M(^{18}O)^-$ − 1), 366 (M^-), 365 (M^- − 1). MS (+ES): 406 ($M(^{18}O)^+$ + Na^+ + CH_3), 405 ($M(^{18}O)^+$ − 1 + Na^+ + CH_3), 404 (M^+ + Na^+ + CH_3), 403 (M^+ − 1 + Na^+ + CH_3).

Product from Hydrolysis and Methylation of *S*$_p$-Thymidine 3′,5′-Cyclic [^{18}O]Thiophosphate. The residue was dissolved in methanol-d_4. δ_P (CD_3OD, 162 MHz): 32.86, 32.82, 32.81, 32.80, 32.17, 32.13, 32.10, 32.08. MS (−ES): 367 ($M(^{18}O)^-$ − 1), 365 (M^- − 1). MS (+ES): 406 ($M(^{18}O)^+$ + Na^+ + CH_3), 405 ($M(^{18}O)^+$ − 1 + Na^+ + CH_3), 404 (M^+ + Na^+ + CH_3), 403 (M^+ − 1 + Na^+ + CH_3).

Results

The determination of the stereochemical course of displacements at phosphate diesters and monoesters is not straightforward since these are achiral and the stereochemical information is therefore "cryptic". Two different approaches were developed to allow the determination of the stereochemical course of enzyme-catalyzed phosphoryl transfer. The most rigorous approach has employed the stable isotopes of oxygen to generate isotopically chiral materials.[1a,b] Alternatively, for a number of enzymatic and chemical reactions, sulfur has been used as a "surrogate" for one of the oxygen isotopes.[1c,d] In this study of the stereochemical course of a metal-catalyzed phosphoryl ester hydrolysis reaction, we have used thymidine 3′,5′-cyclic [^{18}O]-thiophosphate since the replacement of oxygen by sulfur offers an additional probe of the catalytic mechanism and the site of metal coordination, and furthermore, the stereochemical analysis is easier than for the all oxygen isotope systems. It is certainly possible that the mechanisms for the hydrolysis of phosphate and thiophosphate esters may be different. However, there have been a large number of cases, including the hydrolysis of cyclic nucleotides,[7] where the stereochemical course of a particular enzyme-catalyzed displacement at phosphorus has been studied using both isotopically chiral phosphate *and* thiophosphate esters as substrates, and in all cases the stereochemical course (and by inference the mechanisms) have been the same. It could be argued that such enzyme-catalyzed reactions are likely to be highly constrained by the role and structure of the enzyme; however, in view of the phenomenal rate acceleration achieved by Ce^{IV}, the same argument could be made in this case also. If Ce^{IV} is able to catalyze the hydrolysis of thiophosphates, it is highly likely that the metal-catalyzed hydrolysis of cTMP and cTMPS will follow similar mechanisms. Obviously, for this approach to succeed, it was necessary first to establish that Ce^{IV}

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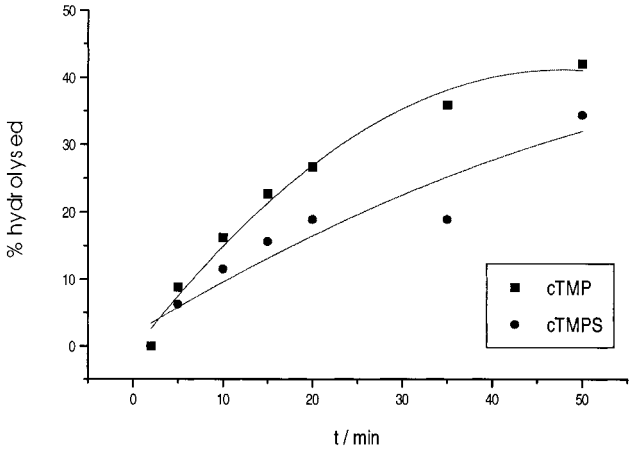


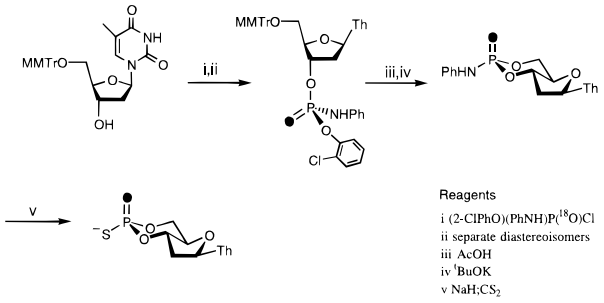
Figure 1. Comparison of the rates of Ce^{IV}-catalyzed hydrolysis of R_p cTMPS and cTMP under similar conditions. The reaction was monitored by quenching samples followed by analysis by HPLC.

could, indeed, catalyze the hydrolysis of cTMPS with similar efficiencies to that previously reported for cTMP.[4]

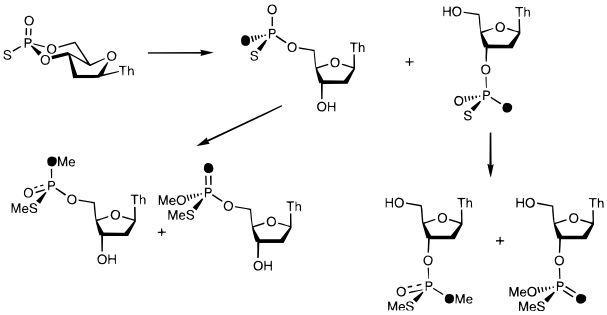
Kinetics of cTMPS Hydrolysis. Treatment of R_p cTMPS with $Ce(NH_4)_2(NO_3)_6$ at pH 7.5 and room temperature led to a rapid hydrolysis of the cyclic thiophosphate, similar to that previously reported for the corresponding cyclic phosphate. The products from this hydrolysis were shown by high-field ^{31}P NMR spectroscopy to be the corresponding thymidine 3′- and 5′-thiophosphates in a ratio of 7:1, which is closely similar to the ratio reported by Komiyama for the products of hydrolysis of cAMP.[4] The hydrolysis rates for cTMP and R_p cTMPS are also closely similar (k_{cTMP}/k_{cTMPS} ca. 1.5 based on initial rates measured by HPLC analysis of products), but in our hands the hydrolyses are not first order (Figure 1) and are complicated by the precipitation of an undefined metal complex. The observation that cTMPS is efficiently cleaved by Ce^{IV} to 3′- and 5′-TMPS is significant on two counts: first, it may suggest that the equatorial P−X position is not the coordination site for the metal, since Ce^{IV} would be expected to have a marked preference for oxygen ligands over sulfur,[8] and second, it provides further supporting evidence to rule out any oxidation processes, since thiophosphates are extremely susceptible to oxidative loss of sulfur. It is also interesting to note the relatively small rate difference between cTMP and cTMPS in the context of the fact that thiophosphate esters usually react significantly slower (typically by factors of ca. >100) than the corresponding phosphate esters via an associative mechanism.[9]

Stereochemistry of R_p cTMPS Hydrolysis. The demonstration that cTMPS is efficiently cleaved by Ce^{IV} allowed us to determine the stereochemical course of this very efficient reaction. Our initial plan was to synthesis the two diastereoisomers of cTMPS by the method of Stec et al.,[10] to hydrolyze these separately in $H_2{}^{18}O$, and to analyze the resulting thymidine 3′-[^{18}O]thiophosphate and thymidine 5′-[^{18}O]thiophosphate to determine the configurations at phosphorus. However, the very dilute solutions used to carry out the Ce^{IV}-catalyzed reaction would have required prohibitive volumes of ^{18}O-labeled water to be used. This problem was solved by incorporation of the

Scheme 1



Scheme 2



oxygen isotope during the synthesis of the diastereoisomers of thymidine 3′,5′-cyclic [^{18}O]thiophosphate (cTMPS) using P[^{18}O]-Cl_3[11] (Scheme 1). The availability of these isotopically labeled chiral molecules ensured that the Ce^{IV}-catalyzed hydrolysis could be carried out in normal water. The hydrolysis of R_p thymidine 3′,5′-cyclic [^{18}O]thiophosphate (cTMPS) led to a mixture of thymidine 3′-[^{18}O]thiophosphate and thymidine 5′-[^{18}O]thiophosphate (ratio ca. 7:1) of unknown configurations at phosphorus. Although the latter is the minor product, a configurational analysis for this isotopically chiral material has already been reported.[12] Methylation of the mixture of thymidine 3′-[^{18}O]thiophosphate and thymidine 5′-[^{18}O]thiophosphate led to the corresponding *O*,*S*-dimethyl triesters as two pairs of diastereoisomers (Scheme 2). High-field ^{31}P NMR spectroscopy not only resolved all of these species but also resolved the isotopomers arising from the bond-order-dependent upfield shift due to the ^{18}O directly bonded to phosphorus.[13] From the isotope shifts for each of the diastereoisomers, it is clear that the thymidine 5′-[^{18}O]thiophosphate has the S_p configuration and is ≥90% ee (Figure 2). The Ce^{IV}-catalyzed hydrolysis proceeds with clean *inversion* of configuration. From the isotope pattern in the ^{31}P NMR spectrum (Figure 2), it is also clear that the related hydrolysis reaction to give the thymidine 3′-[^{18}O]-thiophosphate proceeds stereospecifically. Although hitherto the absolute configurations of the *O*,*S*-dimethyl thymidine 3′-thiophosphate diastereoisomers have not been assigned, it is highly likely that the hydrolysis leading to 3′-TMPS has also occurred with inversion of configuration. On the basis of this reasonable assumption, the assignments of the absolute configurations of the *O*,*S*-dimethyl thymidine 3′-thiophosphate diastereoisomers can be made, and, as shown in Figure 2, the R_p isomer is downfield from the S_p isomer.

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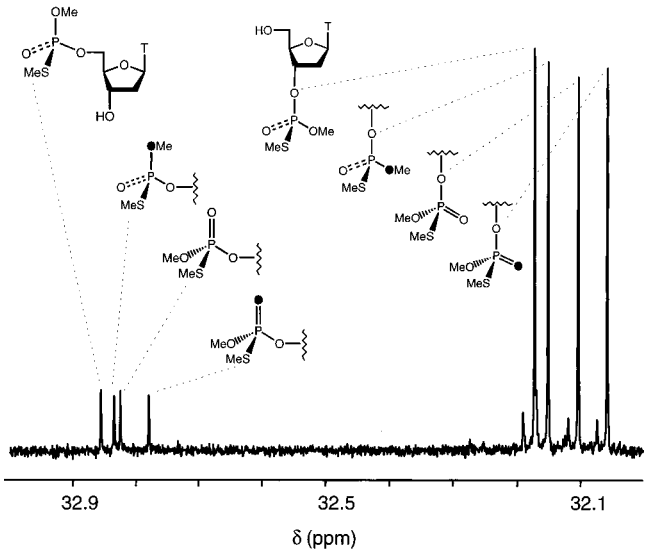

Figure 2. High-field ^{31}P NMR spectrum of the products from Ce^{IV}-catalyzed hydrolysis of R_p thymidine 3′,5′-cyclic [^{18}O]thiophosphate following methylation with diazomethane. The major resonances have been assigned by comparison with authentic materials, and the minor resonances that are unassigned are traces of nucleoside thiophosphates that have also undergone some base methylation.

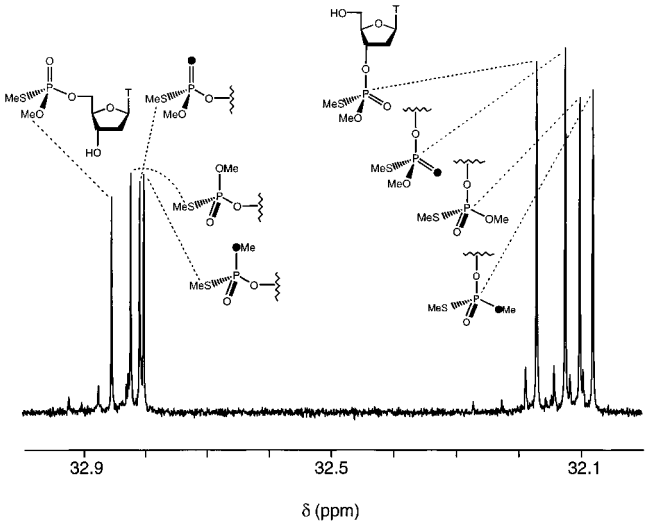

Figure 3. High-field ^{31}P NMR spectrum of the products from Ce^{IV}-catalyzed hydrolysis of S_p thymidine 3′,5′-cyclic [^{18}O]thiophosphate following methylation with diazomethane. The major resonances have been assigned by comparison with authentic materials, and the minor resonances that are unassigned are traces of nucleoside thiophosphates that have also undergone some base methylation.

Stereochemistry of S_p cTMPS Hydrolysis. Interestingly, when S_p thymidine 3′,5′-cyclic [^{18}O]thiophosphate was treated with Ce^{IV} under comparable conditions, efficient hydrolysis was also observed, but the ratio of thymidine 3′-[^{18}O]thiophosphate and thymidine 5′-[^{18}O]thiophosphate products was significantly altered compared with the products of hydrolysis of the R_p cTMPS. The ratio of ca. 3:2 still marginally favored the 3′-[^{18}O]-TMPS. Configurational analysis of these products showed that the hydrolysis of S_p thymidine 3′,5′-cyclic [^{18}O]thiophosphate also proceeded with clean inversion of configuration (Figure 3). The isotopic patterns seen in the ^{31}P NMR spectrum are somewhat more complicated because of the overlap of the isotopomers for the two *O*,*S*-dimethyl thymidine 5′-[^{18}O]-thiophosphate diastereoisomers. As expected, the isotopomeric patterns are the reverse of those seen in Figure 2 from the hydrolysis of the R_p [^{18}O]-cTMPS. Although no systematic study of the relative rates of the Ce^{IV}-catalyzed hydrolyses of R_p and S_p cTMPS diastereoisomers was conducted, the large-scale hydrolyses of the labeled materials was carried out under comparable conditions with similar yields of products, suggesting that the rates may be rather similar.

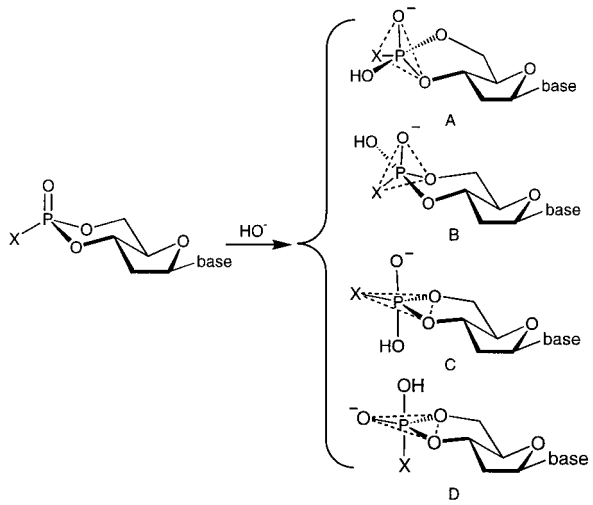

Figure 4. Possible isomeric trigonal bipyramidal intermediates formed in the hydrolysis of a cyclic deoxynucleotide derivative.

Discussion

Most nucleophilic displacement reactions involving phosphate esters are assumed to proceed via five-coordinate, trigonal bipyramidal (tbp) intermediates,[14] although it is difficult to distinguish this from a concerted $S_N2(P)$ reaction involving a trigonal bipyramidal transition state. In principle, four trigonal bipyramidal intermediates are possible for nucleophilic attack on a nucleoside 3′,5′-cyclic phosphate derivative. Figure 4 shows these four isomeric trigonal bipyramids arising from the hypothetical attack of hydroxide. Tbp's A and B have one of the two alternative ring substituents in an apical position, in-line with the nucleophile, such that direct collapse of A and B would lead to the two ring cleavage products with *inversion* of configuration. For tbp's C and D, the hydroxide ion has approached adjacent to the ring substituent, placing the six-membered ring in a diequatorial position and either the $-O^-$ or $-X$ in the apical position. To effect ring cleavage, the leaving group must depart from the preferred apical position, hence tbp's C and D must each undergo a pseudorotation to give an second isomeric tbp before the collapse of this second intermediate to yield the same ring cleavage products. The stereochemical consequence of nucleophilic attack adjacent to the leaving group followed by a single pseudorotation step is that reactions via tbp's C and D would proceed with overall *retention* of configuration.

The stability of trigonal bipyramidal intermediates is dependent on a number of factors. It has long been known, particularly through the early work of Westheimer,[6] that small rings (four- and five-membered) significantly stabilize trigonal bipyramidal intermediates as a result of the marked preference for the ring to span apical−equatorial positions. One consequence of this is that exocyclic displacement reactions in such systems occur with retention of configuration, consistent with a pseudorotation mechanism.[14] The case for six-membered rings is less clear-cut. Calculations suggest that there is a preference for the ring

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to be placed apical−equatorially in the trigonal bipyramidal intermediate,[15] but exocyclic displacement reactions are known to occur with both retention and inversion of configuration suggesting that a six-membered ring can be located either apical−equatorially or diequatorially in the tbp.[14] The relative stability of the alternative trigonal bipyramidal intermediates is also influenced by the apicophilicity of the various substituents. One can apply these simple considerations to the tbp's in Figure 4: intermediates A and B would be favored on the grounds of having the ring spanning apical−equatorial positions. However, if X were strongly apicophilic, tbp D could become of lower energy than A and B. Tbp C would be of higher energy because of having the weakly apicophilic $-O^-$ in the apical position but could be stabilized by neutralization of the charge.

On the basis of simple considerations such as these, the clear expectation would be that base hydrolysis of nucleoside cyclic 3′,5′-phosphates should occur with inversion of configuration via trigonal bipyramidal intermediates A and B ($X = -O^-$). The alternative tbp's are less favorable on the grounds of not only the diequatorial placement of the ring but, more significantly, the placement of a poorly apicophilic $-O^-$ group in the apical position. This has been verified by Mehdi et al.,[16] who have shown that the base hydrolysis of 2′deoxyadenosine cyclic 3′,5′-[^{17}O,^{18}O]phosphate does, indeed, occur with inversion of configuration, consistent with an in-line displacement reaction via A and B. This result is in keeping with the vast majority of reactions of simple phosphate esters which react via either an S_N2(P) mechanism or an addition−elimination reaction with in-line geometry.[14] It is worth considering the base hydrolysis of cyclic nucleotides a little further. For reactions of phosphate ester anions (diesters and monoesters), attack by a nucleophile must overcome the unfavorable electrostatic interaction with the negative charge on the phosphoryl group. Hence, although hydrolysis reactions of phosphate mono- and diester anions are thermodynamically favorable, these reactions have a very high kinetic barrier. In catalyzing such reactions, phosphoryl transferases and phosphatases appear universally to use metal ions. Although these enzymes widely exploit Mg^{2+}, in solution this metal ion shows little catalytic activity, suggesting that some interaction/constraint induced by the enzyme is required in order to achieve catalysis. In terms of hydrolysis of phosphate esters in free solution, the most effective metal catalysts to date are the lanthanides, with Ce^{IV} being the most efficient.[4] One of the roles of the metal ion must be to neutralize the charge on the $-O^-$, hence to overcome the unfavorable electrostatic interaction and to increase the electrophilicity of the phosphoryl center. The metal may also coordinate to any of the other functionality in the trigonal bipyramidal intermediates shown in Figure 4. This could, in principle, dramatically alter the relative stabilities of these intermediates, opening up possibilities for processes involving pseudorotation. The previous observation of the hydrolysis of 3′,5′-UpU catalyzed by imidazole/imidazolium ion being accompanied by isomerization to 2′,5′-UpU is a relevant example of the catalysis of a pseudorotatory process, which in this case is also favored by the involvement of a trigonal bipyramidal intermediate containing a five-membered ring.[17]

The observation of clean inversion of configuration strongly supports an in-line displacement reaction, consistent with the acceleration of the rate of the "normal" uncatalyzed mechanism. Pathways involving adjacent attack accompanied by a pseudorotation step can be rigorously excluded. Importantly, the stereochemical course of this reaction is the same as both the enzyme-catalyzed hydrolysis of cyclic AMPS and dAMP and the base-catalyzed hydrolysis of cyclic dAMP.[7,16] Although it is not possible to conclude precise details of the catalytic mechanism on the basis of these results, they are consistent with the mechanism shown in Figure 5a, in which the metal both acts as a Lewis acid catalyst by coordinating to the $P-O^-$ and also delivers the nucleophile from another coordination site, in line with the appropriate leaving groups. The conclusion that the preferred metal coordination site is the axial $P-O^-$ is supported by the similarities in rate of reaction of both cTMP and R_p cTMPS and by the fact that similar product ratios are seen with both cases. In contrast, when the axial ligand is sulfur, i.e., in S_p cTMPS, the ratio of the respective products is significantly different, possibly due to a switch in coordination to the equatorial $P-O^-$ altering the energetics of the approach, in line with the two alternative leaving groups. The fact that the rate of hydrolysis of S_p cTMPS is not significantly different from that of the R_p isomer suggests that there is considerable flexibility in the metal chelate. More elaborate metal coordination, such as that depicted in Figure 5b, in which more than one metal ion is involved, with coordination to the nucleophile, the leaving group, and the trigonal bipyramidal intermediate can be envisaged. However, the metal chelate shown with the nucleophilic attack adjacent to the coordinated leaving group is ruled out on the basis of the stereochemical observations. The metal chelate with the nucleophile and leaving groups in-line would fit the stereochemical data, but the lack of a significant effect of the replacement of oxygen by sulfur on the reaction rate would, perhaps, be surprising if this were, indeed, the coordinated state.

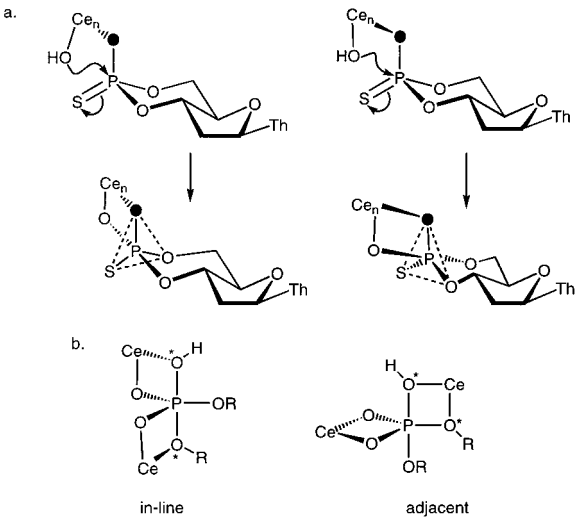

Figure 5. (a) Mechanism of catalysis involving in-line nucleophilic displacement consistent with the observed stereochemistry. The catalysis may involve more that one metal ion, as implied by the Ce_n, and some role of the metal cluster in coordinating to the leaving group cannot be ruled out. (b) Alternative fully coordinated metal−phosphate complexes with the nucleophile and the leaving groups (marked with an asterisk) either in line or adjacent.

Acknowledgment. The authors acknowledge financial support from Albright & Wilson plc and Dr. G. Griffith for assistance with the NMR spectroscopy.

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