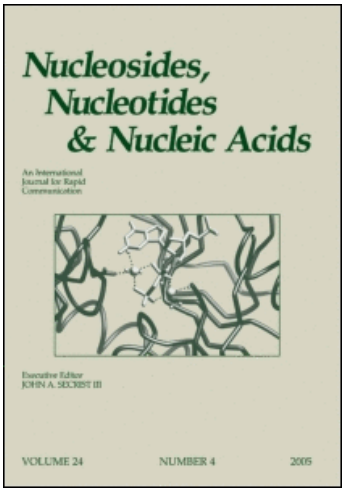

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Incorporation of (α-*P*-Borano)-2′,3′-dideoxycytidine 5′-Triphosphate into DNA by Drug-Resistant MMLV Reverse Transcriptase and *Taq* DNA Polymerase

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ABSTRACT

The *Rp*-stereoisomer of 5′-(α-*P*-borano)triphosphates of 2′-deoxycytidine (*Rp*-dCTPαB) and 2′,3′-dideoxycytidine (*Rp*-ddCTPαB) were synthesized. Their steady-state kinetics of incorporation by ddNTP-resistant enzymes, e.g., MMLV reverse transcriptase (RT) and *Taq* DNA polymerase, were investigated and compared with incorporation of dCTP and ddCTP. The α-boranophosphate substitution in ddCTP results in a 28-fold increase in efficiency of incorporation of the *Rp*-ddCTPαB isomer by MMLV RT, yet has minimal effect on the efficiency of incorporation by *Taq* DNA polymerase.

Nucleoside boranophosphates[1–5] comprise a new class of modified nucleotides in which one non-bridging oxygen atom in the α-phosphate of the nucleoside 5′-triphosphate is replaced by a borane group (BH_3). Our previous studies have shown that one stereo isomer (*Rp*-) of the 2′-deoxynucleoside 5′-(α-*P*-borano)tripho-

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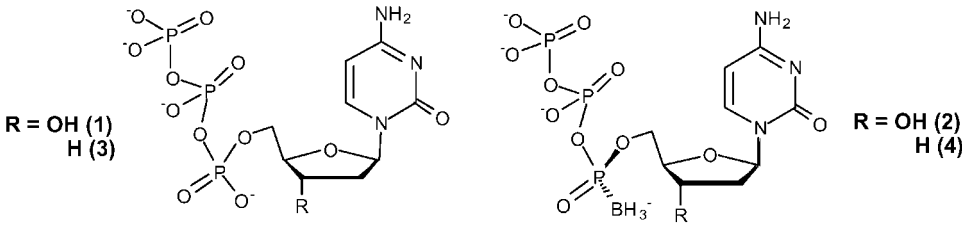
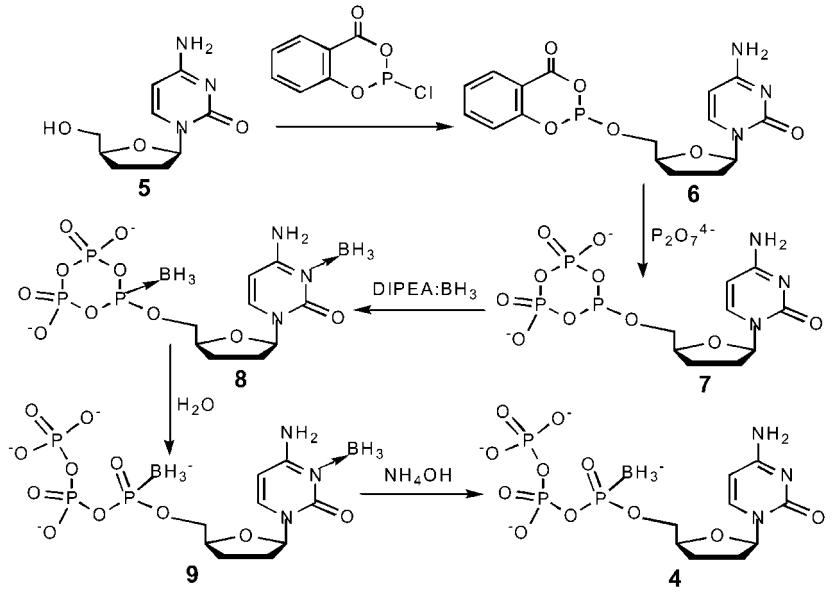
sphates (*Rp*-dNTPαBs)[4] and the 5-methyl-, ethyl-, bromo-, and iodo-*Rp*-dCTPαBs[5] can be successfully incorporated into DNA by DNA polymerases. Moreover, *Rp*-(α-*P*-borano)triphosphates of the clinically relevant antiviral drugs AZT, d4T[6,7] and ddA[8] were shown to be better substrates for wild-type and mutant drug-resistant forms of HIV-1 reverse transcriptase (RT) than nonboronated chain terminators. To obtain insight into the structural basis for discrimination of ddNTPs over dNTPs by viral and bacterial DNA polymerases, we synthesized the *Rp*-stereoisomer of 5′-(α-*P*-borano)triphosphates of 2′-deoxycytidine (*Rp*–dCTPαB, **2**) and 2′,3′-dideoxycytidine (*Rp*-ddCTPαB, **4**). Steady-state kinetics of incorporation of dCTP (**1**), dCTP (**3**), *Rp*-dCTPαB (**2**) and *Rp*-ddCTPαB (**4**) by the ddNTP-resistant enzymes, MMLV RT and *Taq* DNA polymerase, were investigated.



Synthesis of *Rp*-(α-*P*-borano)-2′,3′-dideoxycytidine 5′-triphosphate (4). Dideoxycytidine (ddC, **5**) (0.5 mmol) dried over P_2O_5 under vacuum and suspended in 0.8 mL of anhydrous DMF and 0.2 mL pyridine was phosphitylated with 2-chloro-4*H*-1,3,2-benzodioxaphosporin-4-one (0.55 mmol in 0.8 anhydrous DMF)



Scheme 1.

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at 0°C for 15 min to yield two diastereomers of 2′,3-dideoxycytidine 5′-(4*H*-1,3,2-benzodioxaphosporin-4-one) **6** (Sch. **1**). They were identified by the appearance of a doublet around 127 ppm in the ^{31}P NMR spectra. Compound **6** was treated with tributylammonium pyrophosphate (240 mg in 1 mL anhydrous DMF and 0.15 mL triethylamine) at rt for 1 h to form a 2′,3′-dideoxycytidine 5′-P^2,P^3-dioxo-P^1-cyclo-triphosphate **7**. The upfield shift from ~127 to 107 ppm for trivalent α-phosphorus P^{III}, together with a doublet at −18 ppm for pentavalent phosphorus P^V in ^{31}P NMR, confirmed the complete formation of compound **7**. The borano group was

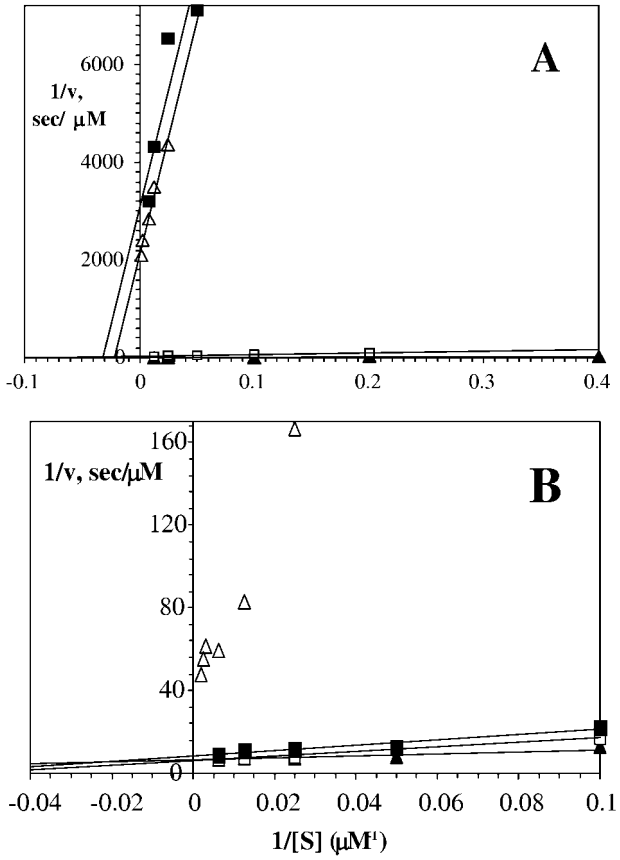

Figure 1. Double reciprocal plot for determination of the steady-state kinetic constants of incorporation of dCTP analogues by *Taq* DNA polymerase (A) and MMLV RT (B). Solutions of 22/27 **T1**-template/primer (350 μM) with increasing concentrations of dCTP (▲), *Rp*-dCTPαB (□′), ddCTP (Δ) or *Rp*-ddCTPαB (■) were preincubated at 60°C (**A**) or 37°C (**B**) and mixed with 0.5 nM *Taq* polymerase (**A**) or 1 nM MMLV RT (**B**) to start the reactions. After 1 min incubation the reactions were quenched. The products were separated by 12%-PAGE and quantified by laser fluorescent imaging. To determine K_m and k_{cat} values, the inverse values of velocity were plotted against inverse substrate concentration. The straight lines intercept the vertical axis at $\mathbf{1/V}_{max}$ value and the horizontal axis at $-\mathbf{1/K}_m$. The determined kinetic constants are presented in Table **1**.

Table 1. Steady-state kinetic constants of incorporation of dCTP analogues.

Enzyme	Substrate	$\mathbf{K}_m$ (μM)[a]	k_{cat} (sec^{-1})[a]	$k_{cat}/\mathbf{K}_m$ ($s^{-1}nM^{-1}$)	S[b]
MMLV RT	dCTP	7.2 ± 1.3	0.086 ± 0.012	12	1
	Rp-dCTPαB	14.8 ± 4.1	0.080 ± 0.025	5.4	2.2
	ddCTP	93 ± 23	0.014 ± 0.005	0.15	80
	Rp-ddCTPαB	14.6 ± 2.8	0.060 ± 0.018	4.1	2.9
Taq DNA polymerase	dCTP	7.1 ± 1.3	0.16 ± 0.06	23	1
	Rp-dCTPαB	11.5 ± 1.7	0.031 ± 0.011	2.7	8.5
	ddCTP	42.6 ± 7.2	0.0007 ± 0.0001	0.017	1360
	Rp-ddCTPαB	36.2 ± 6.7	0.0005 ± 0.0001	0.015	1530

[a]The kinetic constants were determined from double-reciprocal plots of **1**/**v** vs. **1**/**[dNTP]**. Each value is the average of at least six separate experiments and is reported as mean ± SD.
[b]The selectivity for dCTP over its modified analogues is given by the ratio of the efficiencies ($k_{cat}/\mathbf{K}_m$) of incorporation of dCTP to its analogues. The value greater than 1 means that the enzyme discriminates the analog over the dCTP.

introduced by the reaction of derivative **7** with excess borane-diisopropylethylamine complex (4.7 mmol) at rt for 16 h to produce N^3-borano-2′,3′-dideoxycytidine 5′-(α-*P*-borano)cyclotriphosphate **8**. Cyclotriphosphate **8** was converted to N^3-borano-2′,3′-dideoxycytidine 5′-(α-*P*-borano)triphosphate **9** by treatment with H_2O at rt overnight and then to triphosphate **4** by treatment with $NH_3/H_2O:CH_3OH = 1:1$, (v/v) at rt for 8 h. After extraction with Et_2O the water layer was evaporated. For ion-exchange chromatography, the resulting crude mixture was applied to a Poly-sil-CA column and eluted with a linear gradient of 0–0.3 M KH_2PO_4 (pH = 6.5) in 30% CH_3CN. Appropriate fractions were collected and evaporated to give triphosphate **4** as the ammonium salt. The desired fractions were lyophilized, and the excess salt was removed by repeated lyophilization with deionized water for 46% yield of the triethylammonium salt of triphosphate **4**. The stereoisomers were separated on a Delta-Pak C18 cartridge (25 × 40 mm) in isocratic conditions: 2.5% CH_3CN in 0.02 M TEAB at pH 8.0. NMR spectra were recorded on a Varian Inova-400 spectrometer operating at 121.4 Hz (^{31}P) or 400 Hz (^1H). Chemical shift values (δ) are reported relative to H_3PO_4 (85%) for ^{31}P NMR (external standard), *Rp*-isomer of triphosphate **4:** ^1H NMR (D_2O), δ (ppm): 7.96 (d, 1H, H-6), 5.92 (m, 2H, H-5 + H-1′), 4.21 (m, 1H, H-4′), 4.1 (m, 1H, H-5′), 4.02 (m, 1H, H-5″), 2.86 (m, 1H, H-3′), 2.26 (m, 1H, H-3″), 1.91 (m, 2H, H-2′ + 2″), 0.4 − 0.1 (br, 3H, BH_3); ^{31}P NMR (D_2O), δ (ppm): 82 (br, 1P, α-P), −8.9 (m, 1P, γ-P), −21.89 (m, 1P, β-P). UV (H_2O), λmax, nm, (ε, $M^{-1}sm^{-1}$): 272 (9.1).

Steady-State Kinetic Analysis. Steady-state kinetics of incorporation of dCTP, ddCTP, *Rp*-dCTPαB, and *Rp*-ddCTPαB by the ddNTP-resistant enzymes, MMLV RT and *Taq* DNA polymerase, were investigated. Steady-state assays (Fig. **1**) showed that relative to dCTP, the efficiencies of incorporation of ddCTP, *Rp*-dCTPαB, and *Rp*-ddCTPαB isomers were 80-, 2.2-, and 2.9-fold less for MMLV RT, respectively, but 1360-, 8.5-, and 1530-fold less for *Taq* polymerase, respectively (Table 1).

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The α-boranophosphate substitution in ddCTP results in a 28-fold increase in efficiency of incorporation of the *Rp*-ddCTPαB isomer by MMLV RT, whereas it slightly decreases the efficiency of incorporation by *Taq* DNA polymerase.

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