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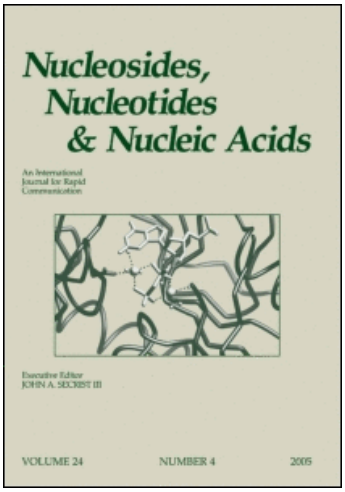

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Introduction of the α-*P*-Borano-Group into Deoxynucleoside Triphosphates Increases Their Selectivity to HIV-1 Reverse Transcriptase Relative to DNA Polymerases

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Introduction of the α-*P*-Borano-Group into Deoxynucleoside Triphosphates Increases Their Selectivity to HIV-1 Reverse Transcriptase Relative to DNA Polymerases

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ABSTRACT

A series of 2′-deoxynucleoside 5′-triphosphates (dNTPs) and their α-*P*-thio or α-*P*-borano analogues, i.e., (*Sp*-dNTPαS), (*Rp*-dNTPαB) and (*Sp*-dNTPαB) were studied as substrates for DNA dependent DNA polymerases and HIV-1 reverse transcriptase (RT). For HIV-1 RT the *Rp*-dNTPαB isomers are 1.2-fold better substrates than natural dNTPs. For DNA polymerases their efficiencies of incorporation are 3-fold (Klenow, Sequenase) and 5-fold (*Taq*) lower than for dNTPs. Thus, introduction of the α-boranophosphate group into dNTPs increases their selectivity to HIV-1 RT relative to bacterial DNA polymerases.

Key Words: Boranophosphates; HIV-1 reverse transcriptase; DNA polymerase; Steady-state kinetics.

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INTRODUCTION

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 borano-group (BH_3). Our previous studies have shown that one stereoisomer (*Rp*-) of the 2′-deoxynucleoside 5′-(α-*P*-borano)triphosphates (*Rp*-dNTPαBs)[4] and the 5-methyl-, 5-ethyl-, 5-bromo- and 5-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. However the kinetic constants for incorporation of dNTPαBs have not yet been published. To better understand the effects of α-boranophosphate substitution on the substrate properties of the nucleoside triphosphates, we performed a comparative steady-state kinetic analysis of the incorporation of dNTPs, *Sp*-dNTPαS, and *Rp*- and *Sp*-dNTPαB stereoisomers by several DNA dependent DNA polymerases and the RNA dependent DNA polymerase, HIV-1 RT.

EXPERIMENTAL

Materials

The enzymes and chemicals were purchased as follows: T7 Sequenase 2.0 and Klenow DNA polymerase from USB, HIV-1 RT from Worthington Biochemicals, [γ-^{32}P]ATP and T4 polynucleotide kinase from New England Biolab, and *Taq* DNA polymerase from Fisher. dNTPαSs were purchased from Amersham and unlabeled ultrapure dNTPs from Pharmacia Biotech. The *Rp*- and *Sp*- stereoisomers of dNTPαSs were separated by HPLC. Synthesis and separation of the *Rp*- and *Sp*- stereoisomers of dNTPαBs have been described previously.[3]

The following template/primer systems were used for dNTPs incorporation:

```
                                  5         10        15        20        25
27-mer template (T1) :      3'-GTC-CTT-GTC-GAT-ACC-GGA-GTC-GAT-CTG
22-mer coding for TMP  : 5'-*CAG-GAA-CAG-CTA-TGG-CCT-CAG-C-3'
21-mer coding for dCMP : 5'-*CAG-GAA-CAG-CTA-TGG-CCT-CAG-3'
20-mer coding for dGMP : 5'-*CAG-GAA-CAG-CTA-TGG-CCT-CA-3'
19-mer coding for dAMP : 5'-*CAG-GAA-CAG-CTA-TGG-CCT-C-3'
```

```
                                  5         10        15        20
27-mer template (T2)        3'-GAG-AGT-GCT-TAC-TGA-CAT-GAT-CGA-ATG
19-mer coding for TMP: HEX-CTC-TCA-CGA-ATG-ACT-GTA-C-3',
```

where * is ^{32}P-radioactive or ***HEX***-label; ***HEX*** is hexachlorofluorescein.

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Steady State Kinetics of Nucleotide Incorporation into DNA

The reaction mixtures for kinetic studies contained 50 mM Tris-HCl at pH 7.8, 5 mM $MgCl_2$, (0.4 mM spermidine – only for *Taq* DNA polymerase), 5 mM DTT, 250 nM DNA primer/template, 0.1–1 nM of the enzyme and a range of six nucleoside triphosphate concentrations. Reaction mixtures (10 μL) equilibrated at 37 °C were initiated with enzyme. Enzyme concentration and reaction times were chosen so that maximal product formation would be ~25% of the template/primer concentration. Reactions were terminated by addition of 10 μL of a stop-solution, containing 20 mM EDTA and 0.1 mM bromophenol blue in 95% (v/v) formamide. Samples (5 μL) were loaded onto 12% polyacrylamide gel containing 8 M urea. Gels were exposed to a phosphorimager screen or quantitated on a Hitachi Laser Fluorescent Scanner FMBIO-100. The steady-state kinetic constants were determined by extrapolation from a Lineweaver-Burk double reciprocal plot.

RESULTS AND DISCUSSION

To examine the kinetics of the analogue incorporation into DNA, a single-nucleotide primer elongation/gel electrophoretic assay was used. Linear transformation of incorporation of TTP and *Rp*-TTPαB stereoisomer into **T1/22** DNA by *Taq* DNA polymerase is presented in Fig. 1. Calculated kinetic constants for the two templates and several DNA polymerases are presented in Tables 1 and 2.

The *Sp*-TTPαB stereoisomer was not a substrate for DNA polymerases. It was shown to be a weak reversible inhibitor and could be classified as fully competitive with the *Rp*-TTPαB isomer (Fig. 2) because, in accordance with the Michaelis-Menten equation, the presence of a constant concentration of competitive inhibitor increases the slope of the double reciprocal plot but has no effect on the $\mathbf{V_{max}}$ value. The affinity of the *Sp*-TTPαB stereoisomer to T7 Sequenase is 21- and 38-fold lower than TTP and the *Rp*-TTPαB stereoisomer, respectively (Table 2). The effect of α-boranophosphate substitution on the stereochemical course of the DNA synthesis ($\mathbf{K_m} = 2.9 \pm 0.9$ μM (TTP), 1.6 ± 1.0 μM (*Rp*-TTPαB) and $\mathbf{K_I} = 61 \pm 9$ μM (*Sp*-TTPαB) for T7 Sequenase is similar to available data for α-thiophosphate substitution: $\mathbf{K_m} = 3.8 \pm 1.2$ μM (dATP), 4.5 ± 1.0 μM (*Sp*-dATPαS) and $\mathbf{K_I} = 30 \pm 15$ μM (*Rp*-dATPαS) for DNA polymerase I.[9] It should be noted that the *Rp*-configuration of dNTPαB corresponds to the *Sp*-configuration of dNTPαS.

Comparison of the data presented in Tables 1 and 2 for TTP and the *Rp*-TTPαB isomer allows us to conclude that the kinetic constants for the incorporation of a natural nucleotide or its analogue by HIV-1 RT and Klenow DNA polymerase are dependent on the nucleotide sequence of template-primer used.[10,11] Kinetic parameters determined here for incorporation of the natural dNTPs are in good accordance with literature data: (HIV-1 RT: $\mathbf{K_m} = 0.03 \pm 0.01$ μM, $\boldsymbol{k_{cat}} = 0.12\ s^{-1}$ for dCTP;[12] *Taq* polymerase: $\mathbf{K_m} = 8.98 \pm 0.24$ for TTP;[13] Klenow: $\mathbf{K_m} = 1.3 \pm 0.1$, $\boldsymbol{k_{cat}} = 3.0 \pm 0.3$ for TTP;[14] and T7 Sequenase: $\mathbf{K_m} = 2.5 \pm 0.6$, $\boldsymbol{k_{cat}} = 0.12 \pm 0.01$ for dCTP[15]).

Data presented in Table 1 indicate that α-*P*-thio- and α-*P*-borano-substitutions of the oxygen atom show opposite effects on their incorporation efficiency by HIV-1

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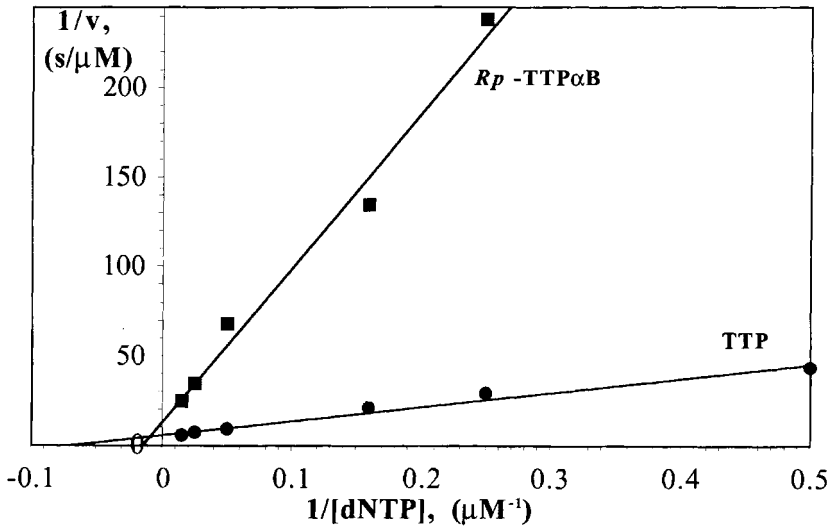

Figure 1. Lineweaver-Burk double reciprocal plot for determination of the steady-state kinetic constants of incorporation of TTP and *Rp*-TTPαB isomer by *Taq* DNA polymerase. Solutions of T1/22-template/primer were preincubated at 60°C with increasing concentrations of TTP(●) or *Rp*-TTPαB isomer (■) and mixed with *Taq* polymerase to start the reactions. After 1 min incubation, the reactions were quenched. The products were separated by 12%-PAGE and quantitated by laser fluorescent imaging. To determine $\mathbf{K_m}$ and $\boldsymbol{k_{cat}}$ values, the inverse values of velocity were plotted against inverse substrate concentration. The straight lines intercept the vertical axis at $\mathbf{1/V_{max}}$ and the horizontal axis at $-\mathbf{1/K_m}$. The determined kinetic constants are presented in Table 1.

RT and the Klenow fragment of DNA polymerase I. The efficiencies of incorporation of *Rp*-dNTPαB isomers by HIV-1 RT in all cases are higher than *Sp*-dNTPαS S isomers, whereas for Klenow fragment the inverse relationship is observed. On average, for HIV-1 RT the *Rp*-dNTPαB isomers are even slightly better substrates than natural dNTPs, whereas their average efficiencies of incorporation are 3-fold (Klenow fragment, T7 Sequenase) and 5-fold (*Taq* DNA polymerase) lower than those for dNTPs. The presence of the α-boranophosphate substituent reduced the steady-state rate constant $\boldsymbol{k_{cat}}$ by 3- to 10-fold for DNA dependent DNA polymerases investigated. Thus, a slower incorporation of the *Sp*-dNTPαS and especially *Rp*-dNTPαB isomers by bacterial DNA polymerases would indicate that phosphodiester bond formation is at least partially rate limiting. As the polymerase reaction occurs through an associative nucleophilic substitution mechanism,[16] the lower electronegativity of the sulfur (2.58) and especially boron (2.04) compared with oxygen (3.44) causes a decrease in the α-phosphorus charge, e.g., from +2.78 to +2.36 upon replacement of an oxygen by a borane.[17] This would make the nucleophilic attack by the 3′-OH group of the DNA primer slower for *Sp*-dNTPαS and especially for *Rp*-dNTPαB isomers than for natural dNTP.

Introduction of the α-boranophosphate group into dNTPs increases their selectivity to HIV-1 RT relative to DNA dependent DNA polymerases. But to

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Table 1. Steady-state kinetic constants of dNTP analogue incorporation in **T1**-template/DNA primers.

Enzyme	dNTP	$\mathbf{K_m}$ (μM)[a]	k_{cat} (s^{-1})[a]	$k_{cat}/\mathbf{K_m}$	**D**[b]
HIV-1 RT	dATP	0.05 ± 0.012	0.69 ± 0.07	13.8	1
	Rp-dATPαB	0.03 ± 0.006	0.57 ± 0.14	19	1.38
	Sp-dATPαS	0.05 ± 0.020	0.63 ± 0.17	12.6	0.91
	TTP	0.05 ± 0.014	0.73 ± 0.14	14.6	1
	Rp-TTPαB	0.04 ± 0.01	0.67 ± 0.17	16.8	1.15
	Sp-TTPαS	0.1 ± 0.037	0.82 ± 0.17	8.2	0.56
	dCTP	0.05 ± 0.014	0.62 ± 0.07	12.4	1
	Rp-dCTPαB	0.023 ± 0.006	0.38 ± 0.13	16.5	1.33
	Sp-dCTPαS	0.06 ± 0.022	0.66 ± 0.14	11	0.89
	dGTP	0.03 ± 0.01	1.54 ± 0.27	51.3	1
	Rp-dGTPαB	0.03 ± 0.009	1.2 ± 0.2	40	0.78
	Sp-dGTPαS	0.04 ± 0.018	1.35 ± 0.2	33.7	0.66
Klenow	dATP	0.04 ± 0.006	13.5 ± 1.1	338	1
	Rp-dATPαB	0.04 ± 0.012	5.0 ± 0.9	125	0.37
	Sp-dATPαS	0.06 ± 0.01	7.9 ± 1.2	131	0.39
	TTP	0.027 ± 0.007	7.8 ± 2.5	289	1
	Rp-TTPαB	0.03 ± 0.014	3.4 ± 0.9	113	0.39
	Sp-TTPαS	0.02 ± 0.008	3.2 ± 0.3	160	0.55
	dCTP	0.02 ± 0.002	29.1 ± 5.8	1455	1
	Rp-dCTPαB	0.02 ± 0.011	3.8 ± 1	190	0.13
	Sp-dCTPαS	0.03 ± 0.013	8.1 ± 2.7	270	0.19
	dGTP	0.03 ± 0.009	25.1 ± 5.4	837	1
	Rp-dGTPαB	0.03 ± 0.002	9.8 ± 3.4	327	0.39
	Sp-dGTPαS	0.05 ± 0.017	17.4 ± 4.8	348	0.42
Taq	dATP	3.5 ± 0.3	0.067 ± 0.009	0.019	1
	Rp-dATPαB	13.5 ± 1	0.029 ± 0.009	0.0021	0.11
	TTP	11 ± 2	0.27 ± 0.06	0.025	1
	Rp-TTPαB	53 ± 10	0.12 ± 0.02	0.0023	0.092
	dCTP	1.5 ± 0.2	0.025 ± 0.005	0.017	1
	Rp-dCTPαB	4.2 ± 0.6	0.001 ± 0.0001	0.0024	0.14
	dGTP	3.0 ± 0.6	0.06 ± 0.005	0.02	1
	Rp-dGTPαB	3.9 ± 0.2	0.035 ± 0.004	0.009	0.45

[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 the mean ± SD.
[b]The discrimination factor of the analogue incorporation compared with unmodified dNTP is given by the ratio of the efficiencies ($k_{cat}/\mathbf{K_m}$) for the analogue divided by that for natural dNTP.

explain fully the accelerating effect of α-*P*-borano-substitution on the efficiency of incorporation of *Rp*-dNTPαB isomers by HIV-1 RT, pre-steady state kinetic analyses and physical studies of the interactions of HIV-1 RT with *Rp*-dNTPαB isomers are necessary.

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Table 2. Steady-state kinetic constants of TTP analogue incorporation into **T2/19** template/primer.

Enzyme	dNTP	$\mathbf{K_m}$ (μM)[a]	k_{cat} (s^{-1})[a]	$k_{cat}/\mathbf{K_m}$	**D**[b]
T7 Sequenase	TTP	2.9 ± 0.9	0.34 ± 0.06	0.12	1
	Rp-TTPαB	1.6 ± 0.2	0.07 ± 0.02	0.044	0.37
	Sp-TTPαB	($\mathbf{K_I}$ = 61 ± 9)	–	–	–
Klenow	TTP	43.2 ± 17	3.4 ± 1	0.079	1
	Rp-TTPαB	6.2 ± 1.1	0.39 ± 0.02	0.063	0.79
HIV-1 RT	TTP	1.2 ± 0.2	1.2 ± 0.1	1	1
	Rp-TTPαB	2.0 ± 0.4	1.8 ± 0.1	0.9	0.9

[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 discrimination factor of the analogue incorporation compared with unmodified dNTP is given by the ratio of the efficiencies ($k_{cat}/\mathbf{K_m}$) for the analogue divided by that for natural dNTP.

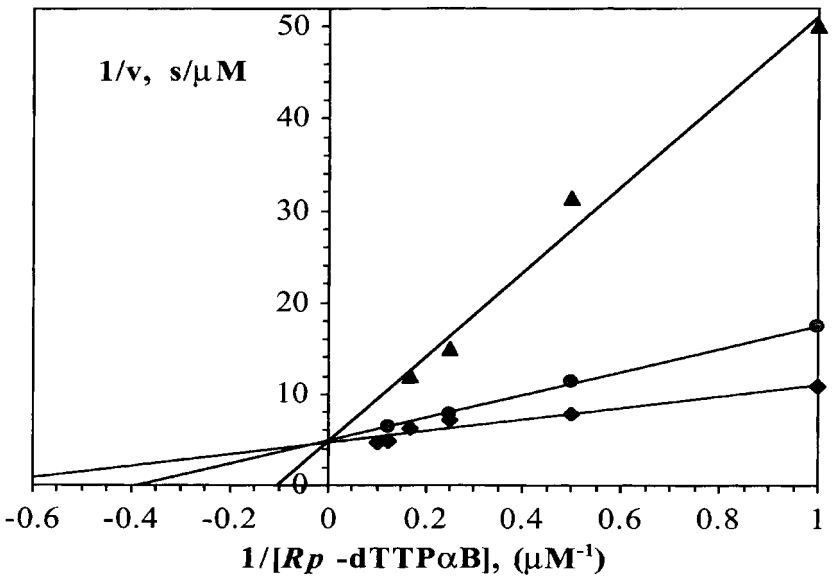

Figure 2. Lineweaver-Burk double reciprocal plot for determination of steady-state kinetic constants of incorporation of *Rp*-TTPαB isomer, alone and in the presence of *Sp*-TTPαB isomer by T7 Sequenase 2.0. Solutions of **T2/19**-template/primer were preincubated at 37°C with increasing concentrations of *Rp*-TTPαB isomer, alone (◆) or in the presence of 80 μM (●) or 270 μM (▲) *Sp*-TTPαB isomer and mixed with T7 Sequenase 2.0 to start the reactions. After 1 min incubation the reactions were quenched. The products were separated by 12%-PAGE and quantitated by laser fluorescent imaging. To determine $\mathbf{K_m}$, $\mathbf{K_I}$ 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 $-\mathbf{1/K_m}$ for incorporation *Rp*-TTPαB isomer alone and at $-\mathbf{1/(K_m(1 + [I]/K_I)}$ for its incorporation in the presence of *Sp*-TTPαB isomer, where **[I]** is the concentration of *Sp*-TTPαB isomer and $\mathbf{K_I}$ is its inhibitory constant. The determined kinetic constants are presented in Table 2.

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CONCLUSIONS

Replacement of the oxygen in α-phosphate of dNTP by a borano-group results in two stereoisomers. The *Sp*-isomer is not a substrate for bacterial DNA polymerases and is only a poor competitive inhibitor of natural dNTP, whereas the *Rp*-isomer is a good substrate for all enzymes investigated.

The α-*P*-borano- and α-*P*-thio- substitutions of the oxygen atom in dNTPs show opposite effects on the incorporation efficiency of the stereochemically equivalent *Rp*-dNTPαB and *Sp*-dNTPαS isomers by HIV-1 RT and Klenow fragment of DNA polymerase I. The *Rp*-dNTPαB isomers are better substrates than the *Sp*-dNTPαS isomers for HIV-1 RT and poorer substrates for Klenow fragment. This distinct behavior makes the *Rp*-dNTPαB isomers promising tools for investigating the mechanism of phosphoryl transfer reactions catalyzed by viral reverse transcriptases and DNA dependent DNA polymerases.

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