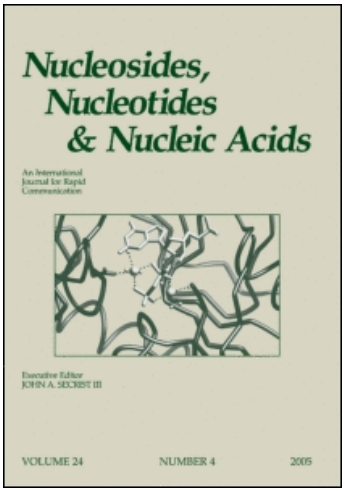

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Synthesis of 5-(1-Propynyl)-2'-Deoxyuridine 5'-(Alpha-P-Borano)Triphosphate and Kinetic Characterization as a Substrate for MMLV Reverse Transcriptase

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SYNTHESIS OF 5-(1-PROPYNYL)-2′-DEOXYURIDINE 5′-(ALPHA-P-BORANO)TRIPHOSPHATE AND KINETIC CHARACTERIZATION AS A SUBSTRATE FOR MMLV REVERSE TRANSCRIPTASE

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▫ *In order to introduce pyrimidine C5-propynyl modification into boranophosphate oligodeoxyribonucleotides (BP–ODNs), 5-(1-propynyl)-2′-deoxyuridine 5′-(α-P-borano) triphosphate (d^{5P}UTPαB) was synthesized. The two diastereomers were separated by reverse-phase HPLC. Kinetic studies showed that the Rp isomer was a slightly better substrate for MMLV reverse transcriptase than thymidine triphosphate or Rp-thymidine 5′-(α-P-borano)triphosphate. Using the Rp isomers of d^{5P}UTPαB and the other three 5′-(α-P-borano) triphosphates, a DNA primer could be extended to the full length of the template.*

Keywords Oligodeoxyribonucleotide Analogs, Boranophosphate, C5-Propynyl Pyrimidines, 5′-(α-*P*-Borano) Triphosphates, MMLV Reverse Transcriptase, Steady-State Kinetics

INTRODUCTION

In a boranophosphate oligodeoxyribonucleotide (BP–ODN), a borano ($-BH_3$) group replaces one non-bridging oxygen of each phosphodiester linkage.[1,2] Besides being remarkably stable against nuclease hydrolysis, BP–ODN is one of only a few oligonucleotide analogues able to induce RNase H-mediated hydrolysis of the complementary RNA strand,[1–4] in a mechanism generally accepted as being most important in efficacy of silencing gene expression using the antisense technique.[5] Previous work in our laboratory showed that a mixed-sequence BP–ODN 15-mer had decreased affinity toward its complementary DNA and RNA strands relative to the natural counterpart, and its RNase H activity, although good, was not as high either.[4,6] On the other hand, Wagner et al.[7] showed that the C5-(1-propynyl) substitution on pyrimidines increased melting temperatures of

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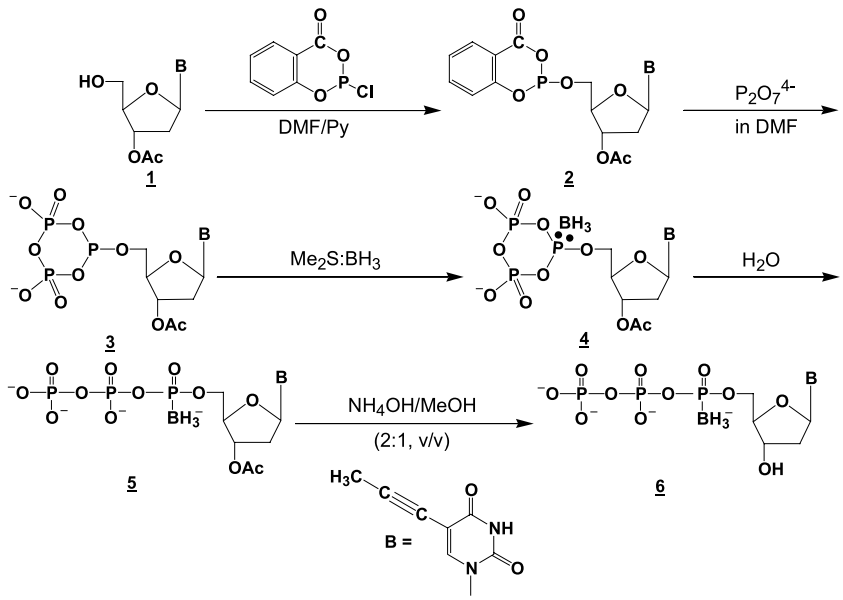
DNA:RNA hybrids, while retaining the ability to induce RNase H activity.[7,8] Moreover, C5-alkyl 2′-deoxycytidine 5′-(α-*P*-borano)triphosphates, when incorporated into DNA, were shown to markedly enhance exonuclease resistance.[9] We propose that introducing C5-(1-propynyl) pyrimidines into boranophosphate ODNs should increase the binding affinity of BP–ODNs with complementary RNA, improve their RNase H activity, and increase their nuclease resistance, thus making them more potent antisense agents. Currently, the most efficient method to prepare mixed-sequence BP–ODNs longer than 10 nucleotides is by template-directed primer extension using polymerase and the *Rp* diastereomers of α-*P*-borano triphosphates. In order to introduce C5-propynyl pyrimidines into BP–ODNs, the corresponding α-*P*-borano triphosphates need to be synthesized. In this work, we demonstrate the synthesis of 5-(1-propynyl)-2′-deoxyuridine 5′-(α-*P*-borano)triphosphate, the separation of the two diastereomers, and the investigation of its substrate properties for MMLV reverse transcriptase, the enzyme used in preparation of BP–ODNs.

RESULTS AND DISCUSSION

5-(1-propynyl)-2′-deoxyuridine 5′-(α-*P*-borano)triphosphate ($d^{5P}UTP\alpha B$) was synthesized through a convenient one-pot salicyl phosphorochloridite approach (Scheme 1).[10] The corresponding 3′-acetylated nucleoside **1** (44 mg), dried over P_2O_5 under vacuum and dissolved in 0.1 mL pyridine/0.4 ml dimethylformamide (DMF), was first phosphitylated by 0.2 mL salicyl phosphorochloridite (freshly



SCHEME 1 Synthesis of 5-(1-propynyl)-2.

prepared 1 M solution in DMF) for 15 min to yield **2**. Tributylammonium pyrophosphate (0.4 mL 0.5 M solution in DMF and 0.1 mL triethylamine) was then added into the reaction mixture. Complete conversion of **2** to **3** was achieved in 15 min. Boronation of **3** was realized using excess borane-dimethyl sulfide complex (1.4 mL 2 M solution in tetrahydrofuran, 4 h). The resulting 5-(1-propynyl)-2′-deoxyuridine 5′-(α-*P*-borano)cyclotriphosphate **4** was then treated with H_2O for 1 h, and NH_4OH:CH_3OH=2:1 (v/v) overnight, to obtain the final product 5-(1-propynyl)-2′-deoxyuridine 5′-(α-*P*-borano)triphosphate **6**. All reactions proceeded at room temperature, and anhydrous conditions were ensured before hydrolysis. The progress of each step before hydrolysis was monitored by ^{31}P NMR: the two diastereomers of **2** gave a doublet around 125 ppm; **3** had a triplet around 104 ppm for the trivalent α-phosphorus P^{III} and a doublet around −19 ppm for pentavalent phosphorus P^{V}; **4** showed a characteristic broad peak around 87 ppm. The crude product was purified by ion-exchange chromatography using a self-packed QA-52 quaternary ammonium cellulose column (1.5 × 30 cm) and a linear gradient of 600 mL each of 5 mM and 350 mM ammonium bicarbonate. The appropriate fractions were collected, evaporated, and repeatedly lyophilized with deionized water to yield the final product d^{5P}UTPαB. **^{1}H NMR** (D_2O), δ (ppm): 7.92 (d, 1H, H-6), 6.13 (m, 1H, H-1′), 4.48 (m, 1H, H-3′), 4.20–3.95 (m, 3H, H-4′, H-5′), 2.35–2.15 (m, 2H, H-2′), 1.87 (s, 3H, CH_3), 0.8 to −0.2 (br, 3H, BH_3). **^{31}P NMR** (D_2O), δ (ppm): 84 (br, 1P, α-P), −7.74 (m, 1P, γ-P), −21.33 (m, 1P, β-P). **MS (ESI)** found: *m/z*: 502.7 (calcd. 503.0 for $BC_{12}H_{19}N_2O_{13}P_3$). **UV** ($H_2O$): λ_{max}=231.1 nm and 292.4 nm. The two diastereomers of d^{5P}UTPαB were well separated by ion-pairing reverse phase HPLC on a Delta-Pak C18 cartridge (25 × 100 mm) with 5% acetonitrile in 0.1 M triethylammonium bicarbonate (TEAB, pH 6.8).

Steady-state kinetics of single nucleotide incorporation showed that the *Rp* diastereomer of the doubly modified d^{5P}UTPαB was as good (or even slightly

TABLE 1 Steady-State Kinetic Constants of Single Nucleotide Incorporation[c]

Substrate	K_M (μM)[a]	k_{cat} (s^{-1})[a,b]	k_{cat}/K_M ($s^{-1}mM^{-1}$)
TTP	14.4 ± 2.6	0.015 ± 0.004	1
d^{5P}UTP	14.1 ± 2.4	0.023 ± 0.005	1.6
Rp-TTPαB	14.6 ± 3.5	0.020 ± 0.003	1.4
Rp-d^{5P}UTPαB	8.9 ± 2.0	0.018 ± 0.008	2

[a]The kinetic constants were derived from fitting Hanes plots of [S]/*v* vs. [S] according to $\frac{S}{v} = \frac{K_M}{V_{max}} + \frac{1}{V_{max}}[S]$, where $V_{max} = k_{cat}$[enzyme]. Each value was the average of six independent experiments and was reported as mean ± SD.

[b]The active site concentration of MMLV reverse transcriptase [enzyme] was determined by active site titration. (From Ref. [11]).

[c]The reaction conditions were as following: pre-annealed primer 5′-HEX-CTC TCA CGA ATG ACT GTA C (19-2, HEX: 4,7,2′,4′,5′,7′-hexachloro-fluorescein) and template 3′-GAG AGT GCT TAC TGA CAT G AT CGA ATG (T-2) was incubated with the enzyme, and then mixed with one of the four thymidine triphosphate analogues to initiate the reaction. The final reaction mixture contained 50 mM Tris-HCl (pH 8.3 at 25°C), 75 mM KCl, 3 mM $MgCl_2$, 15 mM DTT, 350 nM T-2, 300 nM 19-2, 11 nM enzyme, and 0–42 μM triphosphate. All reactions proceeded at 37°C for 8 min.

better) a substrate as the unmodified TTP and the singly modified d^{5P}UTP or *Rp*-TTPαB for MMLV reverse transcriptase (Table 1). When the other three 5′-(α-*P*-borano) triphosphates, dATPαB, dCTPαB, and dGTPαB (*Rp* diastereomers only) were also added, the 19-mer DNA primer could be extended to the full length of the 27-mer template. The resulting oligodeoxyribonucleotide had slower mobility in a polyacrylamide gel than its analogues without C5-propynylation. Work is in progress to optimize conditions and synthesize sufficient amount of BP–ODNs with C5-propynyl substitution for further physicochemical investigation.

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