Stability and Polymerase Recognition of Pyridine Nucleobase Analogues: Role of Minor Groove H-bond Acceptors

Yoonkyung Kim, Aaron M. Leconte, Yoshiyuki Hari and Floyd E. Romesberg

General Methods. All synthetic reactions were carried out in oven-dried glassware under argon atmosphere with commercially available anhydrous solvents (Aldrich) and monitored by thin layer chromatography (0.25 mm E. Merck silica gel plates, 60F-254) using UV light or 5% ethanolic p-anisaldehyde solution as a developing agent. 4-Iodopyridine (Ryscor) and other reagents (Aldrich) were used without further purification. 1H, 13C and 31P NMR spectra were recorded on Bruker DRX-600, Bruker DRX-500 or Bruker AMX-400 spectrometers. Mass spectroscopic data were obtained from The Center for Mass Spectrometry at The Scripps Research Institute.

Procedure for Synthesis and Purification of Oligonucleotides. Oligonucleotides were synthesized using standard β-cyanoethylphosphoramidite chemistry by means of an Applied Biosystems Inc. 392 DNA/RNA synthesizer. Trityl-deprotected oligonucleotides were cleaved from the CPG support and deprotected by a 16 h reaction with concentrated ammonia at 55° C. The oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis (10-20%, 8 M urea) and visualized by UV shadowing. Following excision from the gel, the oligonucleotides were electroeluted and ethanol precipitated. Purified oligonucleotides were dissolved in water, and their concentration was determined by using the calculated extinction coefficient at 260 nm (http://paris.chem.yale.edu/extinct.html); note that for oligonucleotides containing an unnatural residue, dC replaced it in the calculated sequence.

Thermal Denaturation. Oligonucleotide duplex denaturation temperature measurements were made using a Cary 300 Bio UV-visible spectrometer. Buffer contained 10 mM PIPES, pH 7.0, 10 mM MgCl2, and 100 mM NaCl with a ssDNA concentration of 3 μM. Following rapid annealing and cooling, measurements of absorbance at 260 nm were taken at 0.5°C/min intervals over the temperature range 20-80°C. Melting temperatures were obtained from one scan by means of the derivative method using the Cary Win UV software.

Steady State Single Nucleotide Incorporation Assay. DNA polymerase I (large fragment, exonuclease deficient) from E. coli was purchased from New England Biolabs. Primer oligonucleotides were 5'-radiolabeled with T4 polynucleotide kinase (New England Biolabs) and [γ-32P]-ATP (Amersham Biosciences). Primers were annealed to template oligonucleotides (final concentration of duplex DNA: 40nM) in the reaction buffer (final concentration: 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM DTT, and 50 μg/mL acetylated BSA) by heating to 90°C, followed by slow cooling to ambient temperature over approximately 2 h. Assay conditions contained 40 nM primer template, 0.1-1.2 nM enzyme,. A 2X DNA-enzyme mixture was added to an equal volume (5 μL) of 2X dNTP stock solution, incubated at 25°C for 2-12 min, and quenched by the addition of 20 μL of loading dye (95% formamide, 20 mM EDTA, and sufficient amounts of bromophenol blue and xylene cyanole). The reaction mixtures were resolved by 15% polyacrylamide gel electrophoresis, and the radioactivity was quantified by means of a PhosphorImager (Molecular Dynamics) and ImageQuant software. The Michaelis-Menten equation was fit to a plot of kobs versus triphosphate concentration using the program Kaleidagraph (Synergy Software). The data presented are averages of three independent determinations.

Nucleotide Synthesis (General). Previously, we reported on the synthesis of the phosphoramidite 5b with a 3-pyridine nucleobase from aldehyde 1. Using a similar synthetic route, phosphoramidites 5a and 5c were synthesized (Scheme 1). Briefly, treatment of 1 with pyridyllithium followed by ring-closure reaction afforded a mixture of 2 and 3, which can be resolved by column chromatography. 2 was deprotected under acidic conditions to give free nucleoside 4, which was converted to 5 by dimethoxytritylation and phosphitylation. Oligonucleotides containing these pyridine nucleobases were prepared by the phosphoramidite protocol on an automated DNA synthesizer. The 2-pyridine analogue triphosphate, 6a, was synthesized from the corresponding free nucleoside 4a using standard conditions.
Representative Procedure of PMB Protected Nucleoside. A solution of aldehyde 1 (555 mg, 1.12 mmol) in THF (2.3 ml) was added to a 2-pyridyllithium solution, prepared from bromopyridine (a,b) or iodopyridine (c) (0.18 ml, 1.87 mmol) and n-BuLi (1.6 M in hexane, 1.26 ml, 2.02 mmol) in THF (7.5 ml), at –78 °C. After being stirred for 1.5 h at –78 °C and for 3.5 h at rt, the reaction mixture was quenched by addition of aqueous saturated NaHCO₃ and extracted with Et₂O (3 x 20 mL). The combined organic layer was dried over Na₂SO₄ and concentrated in vacuo. Flash chromatography (30-60% EtOAc:hexanes) gave appropriate compounds (Rf = 0.4-0.5, hexane/EtOAc=1:2), which were dissolved in pyridine (40 ml). DMAP (cat.), diisopropylethylamine (0.46 ml, 2.63 mmol) and MsCl (0.13 ml, 1.67 mmol) were added at 0 °C, and after being stirred for 20 h at rt, the mixture was concentrated in vacuo. Flash chromatography (30-50% EtOAc:hexanes) resolved the two stereoisomers (2a and 3a) and yielded 212 mg of 2 (43 % from 1). The correct stereochemistry was confirmed by 1D nOe.

Compound 2a. 1H NMR (500 MHz, CDCl₃) δ 8.52 (d, J = 4.6 Hz, 1H), 7.64 (td, J = 7.7, 1.6 Hz, 1H), 7.50 (d, J = 7.7 Hz, 1H), 7.25 (d, J = 8.6 Hz, 2H), 7.16 (dd, J = 7.6, 4.6 Hz, 1H), 6.87 (d, J = 8.6 Hz, 4H), 5.24 (dd, J = 10.1, 5.8 Hz, 1H), 4.51 (s, 2H), 4.45 (d, J = 11.5 Hz, 1H), 3.56 (dd, J = 10.1, 5.2 Hz, 1H), 2.53 (dd, J = 10.1, 5.1 Hz, 1H), 2.03 (dd, J = 13.2, 10.1, 6.0 Hz, 1H); 13C NMR (125 MHz, CDCl₃) δ 161.54, 159.11, 159.09, 148.77, 136.59, 130.20, 130.07, 129.26, 129.21, 129.13, 122.26, 113.74, 113.65, 84.08, 81.03, 80.60, 72.99, 70.69, 70.61, 55.21, 39.00; MS (MALDI), 436 (MH+).

Compound 2b. 1H NMR (500 MHz, CDCl₃) δ 8.60-8.54 (m, 2H), 7.69 (d, J = 7.7 Hz, 1H), 7.28-7.23 (m, 5H), 6.88 (d, J = 8.6 Hz, 2H), 6.88 (d, J = 8.7 Hz, 2H), 5.14 (dd, J = 10.8, 5.1 Hz, 1H), 4.52 (s, 2H), 4.48 (s, 2H), 4.28 (td, J = 4.8, 2.1 Hz, 1H), 4.18-4.14 (m, 1H), 3.81 (s, 6H), 3.62 (dd, J = 10.1, 4.4 Hz, 1H), 3.55 (dd, J = 10.1, 5.2 Hz, 1H), 2.36 (ddd, J = 13.2, 5.1, 1.1 Hz, 1H), 1.89 (ddd, J = 13.2, 10.8, 5.8 Hz, 1H); 13C NMR (125 MHz, CDCl₃) δ 159.28, 159.22, 133.73, 130.15, 129.98, 129.35, 129.26, 113.86, 113.78, 84.03, 81.12, 78.27, 73.15, 70.78, 70.60, 55.25, 41.18, 40.73; HRMS (ESI) calcd for C₂₆H₂₉NO₅ (MH⁺) 436.2118, found 436.2107.

Compound 2c. 1H NMR (500 MHz, CDCl₃) δ 8.63 (brs, 2H), 7.31 (brs, 2H), 7.25 (d, J = 8.6 Hz, 1H), 7.23 (d, J = 8.8 Hz, 1H), 6.88 (d, J = 8.8 Hz, 2H), 6.88 (d, J = 8.6 Hz, 2H), 5.10 (dd, J = 10.5, 5.4 Hz, 1H), 4.51 (s, 2H), 4.48 (s, 2H), 4.29 (td, J = 4.8, 2.0 Hz, 1H), 4.12-4.11 (m, 1H), 3.81 (s, 3H), 3.61 (dd, J = 10.1, 4.4 Hz, 1H), 3.54 (dd, J = 10.1, 5.1 Hz, 1H), 2.38 (ddd, J = 13.1, 5.4, 1.1 Hz, 1H), 1.83 (ddd, J = 13.1, 10.5, 5.8 Hz, 1H); 13C NMR (125 MHz, CDCl₃) δ 159.28, 159.22, 130.20, 129.98, 129.35, 129.26, 113.86, 113.78, 84.03, 81.12, 78.27, 73.15, 70.78, 70.60, 55.25, 41.18, 40.73; HRMS (ESI) calcd for C₂₆H₂₉NO₅ (MH⁺) 436.2118, found 436.2107.

Representative Procedure for Deprotection of PMB Protected Nucleoside. A solution of 2a (337 mg, 0.77 mmol) in 20 % TFA/CH₂Cl₂ (3.72 ml) was stirred for 40 min at rt and then the solvent was removed in vacuo. Flash chromatography (3-10% MeOH : CH₂Cl₂) yielded 150 mg of 4 (86 %).
Compound 4a. $^1$H NMR (500 MHz, CD$_2$OD) δ 8.49 (d, J = 5.0 Hz, 1H), 7.82 (td, J = 7.8, 1.6 Hz, 1H), 5.26 (dd, J = 10.1, 6.0 Hz, 1H), 4.36-4.35 (m, 1H), 4.07-4.05 (m, 1H), 3.76 (dd, J = 11.8, 4.1 Hz, 1H), 3.66 (dd, J = 11.8, 5.5 Hz, 1H), 2.35 (ddd, J = 13.1, 6.0, 1.9 Hz, 1H), 2.04 (ddd, J = 13.1, 10.1, 5.7 Hz, 1H); $^{13}$C NMR (125 MHz, CD$_2$OD) δ 162.66, 149.64, 139.15, 124.32, 122.41, 89.74, 81.83, 74.11, 64.07, 43.92; HRMS (ESI) calcd for C$_{10}$H$_{13}$NO$_3$ (MH$^+$) 196.0968, found 196.0969.

Compound 4b. $^1$H NMR (500 MHz, CDCl$_3$) δ 8.61 (s, 1H), 8.55 (m, 1H), 7.69 (d, J = 7.8 Hz, 1H), 7.31-7.28 (m, 1H), 5.21 (ddd, J = 11.6, 5.5, 1.9 Hz, 1H), 4.51-4.48 (m, 1H), 4.07-4.04 (m, 1H), 3.85 (dd, J = 11.6, 4.2 Hz, 1H), 3.78 (dd, J = 11.6, 4.6 Hz, 1H), 2.32 (ddd, J = 13.4, 5.5, 2.0 Hz, 1H), 2.05 (ddd, J = 13.4, 10.2, 6.3 Hz, 1H); $^{13}$C NMR (125 MHz, CD$_2$OD) δ 149.14, 148.15, 136.36, 125.33, 101.15, 89.61, 79.25, 74.49, 64.03, 44.99; HRMS (ESI) calcd for C$_{10}$H$_{13}$NO$_3$ (MH$^+$) 196.0968, found 196.0966.

Compound 4c. $^1$H NMR (600 MHz, CD$_2$OD) δ 8.52 (brs, 2H), 7.54 (brs, 2H), 5.17 (dd, J = 10.3, 5.7 Hz, 1H), 4.33-4.32 (m, 1H), 4.00 (td, J = 4.8, 2.4 Hz, 1H), 3.68 (d, J = 5.0 Hz, 2H), 2.31 (ddd, J = 12.8, 5.7, 1.7 Hz, 1H), 1.89 (ddd, J = 12.8, 10.3, 5.8 Hz, 1H); $^{13}$C NMR (150 MHz, CD$_2$OD) δ 163.60, 149.64, 119.09, 89.64, 79.90, 74.29, 63.99, 44.67; HRMS (ESI) calcd for C$_{10}$H$_{13}$NO$_3$ (MH$^+$) 196.0968, found 196.0969.

General Procedure for Phosphoramidite Synthesis. DMTrCl (1.3 eq.) was added to a solution of free nucleoside 4 (1 eq.), Et$_3$N (3.5 eq.) and DMAP (cat.) in pyridine (final concentration ca. 0.6 M) and the mixture was stirred overnight. Flash chromatography (EtOAc:hexanes) gave DMTr-protected nucleoside, which was dissolved in CH$_2$Cl$_2$ (final concentration 0.3-0.4 M). To this solution, DMAP (cat.), diisopropylethylamine (5 eq.) and (i-Pr)$_2$NP(Cl)OCH$_2$CH$_2$CN (>2 eq.) were added at 0 °C. After being stirred for 30 min, warming to rt, the reaction mixture was quenched by addition of aqueous saturated NaHCO$_3$, and extracted with CH$_2$Cl$_2$ (20 ml x 3). The combined organic layer was dried over Na$_2$SO$_4$ and concentrated in vacuo. Flash chromatography (20-50% EtOAc:hexanes with 1% triethylamine) gave phosphoramidite 5.

Compound 5a. $^{31}$P NMR (162 MHz, CDCl$_3$) δ 148.70, 148.17; HRMS (ESI) calcd for C$_{40}$H$_{48}$N$_3$O$_6$P (MNa$^+$) 720.3173, found 720.3171.

Compound 5b. $^{31}$P NMR (162 MHz, CDCl$_3$) δ 148.62, 148.52; HRMS (ESI) calcd for C$_{40}$H$_{48}$N$_3$O$_6$P (MH$^+$) 698.3353, found 698.3352.

Compound 5c. $^{31}$P NMR (162 MHz, CDCl$_3$) δ 148.62, 148.57; HRMS (ESI) calcd for C$_{40}$H$_{48}$N$_3$O$_6$P (MH$^+$) 698.3353, found 698.3351.

Triphosphate Synthesis (6a). POCl$_3$ (8.6 μl, 92.3 μmol) was added to a solution of 4a (12.0 mg, 61.5 μmol), Proton-Sponge® (19.8 mg, 92.4 μmol) in PO(OMe)$_3$ (370 μl) at –20 °C and the reaction mixture was stirred for 2 h at –15°C. After addition of Bu$_3$N (91 μl, 0.38 mmol) and 0.5 M tetrabutylammonium pyrophosphate solution (prepared from tetrabutylammonium pyrophosphate and DMF, 614 μl, 0.31 mmol), the reaction mixture was further stirred for 15 min at –15°C. Then, 0.5 M triethylammonium bicarbonate solution (1 ml) and H$_2$O (10 ml) were added. The mixture was lyophilized and purified by reverse-phase HPLC (C18, 1-32% MeCN in 0.1 M triethylammonium bicarbonate buffer (pH 7.5)) to afford 6a.

Compound 6a. $^{31}$P NMR (162 MHz, D$_2$O) δ -10.11 (d, J = 20 Hz), -10.68 (d, J = 20 Hz), -22.78 (t, J = 20 Hz).

References