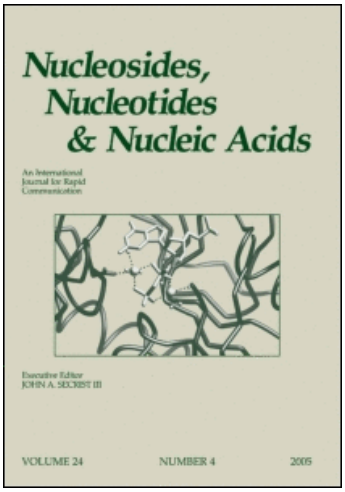

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Semisynthesis of 6-Chloropurine-2'-deoxyriboside 5'-Dimethoxytrityl 3'-(2-Cyanoethyl-*N,N*-diisopropylamino)Phosphoramidite and its Use in the Synthesis of Fluorescently Labeled Oligonucleotides

Md. Jashim Uddin[a]; Michael I. Schulte[a]; Leena Maddukuri[a]; Joel Harp[b]; Lawrence J. Marnett[a]
[a] A. B. Hancock Jr. Memorial Laboratory for Cancer Research, Departments of Biochemistry, Chemistry, and Pharmacology, Vanderbilt Institute of Chemical Biology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA [b] Center for Structural Biology, Vanderbilt University, Nashville, Tennessee, USA

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SEMISYNTHESIS OF 6-CHLOROPURINE-2′-DEOXYRIBOSIDE 5′-DIMETHOXYTRITYL 3′-(2-CYANOETHYL-*N,N*-DIISOPROPYLAMINO) PHOSPHORAMIDITE AND ITS USE IN THE SYNTHESIS OF FLUORESCENTLY LABELED OLIGONUCLEOTIDES

Md. Jashim Uddin,[1] Michael I. Schulte,[1] Leena Maddukuri,[1] Joel Harp,[2] and Lawrence J. Marnett[1]

[1]*A. B. Hancock Jr. Memorial Laboratory for Cancer Research, Departments of Biochemistry, Chemistry, and Pharmacology, Vanderbilt Institute of Chemical Biology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA*
[2]*Center for Structural Biology, Vanderbilt University, Nashville, Tennessee, USA*

□ *An efficient enzymatic synthesis of 6-chloropurine-2′-deoxyriboside from the reaction of 6-chloropurine with 2′-deoxycytidine catalyzed by nucleoside-2′-deoxyribosyltransferase (E.C. 2.4.2.6) followed by chemical conversion into the 5′-dimethoxytrityl 3′-(2-cyanoethyl-*N,N-*diisopropylamino) phosphoramidite derivative is described. The phosphoramidite derivative was incorporated site-specifically into an oligonucleotide and used for the introduction of a tethered tetramethylrhodamine-cadaverine conjugate. The availability of an efficient route to 6-chloropurine-2′-deoxyriboside 5′-dimethoxytrityl 3′-(2-cyanoethyl-N,N-diisopropylamino)phosphoramidite enables the facile synthesis of oligonucleotides containing a range of functional groups tethered to deoxyadenosine residues.*

Keywords Semisynthesis; nucleoside deoxyribosyltransferase; 6-chloropurine-2′-deoxyriboside; fluorescent oligonucleotide

INTRODUCTION

Oligonucleotides containing tethered functional groups (e.g., fluorescent dyes) are useful for polymerase chain reaction (PCR) analysis, probing of whole cells, and characterization of genetic polymorphisms inter alia.[1–3] 6-Chloropurine-2′-deoxyriboside 5′-dimethoxytrityl 3′-(2-cyanoethyl-*N,N*-diisopropylamino) phosphoramidite (**5**) (Scheme 1)

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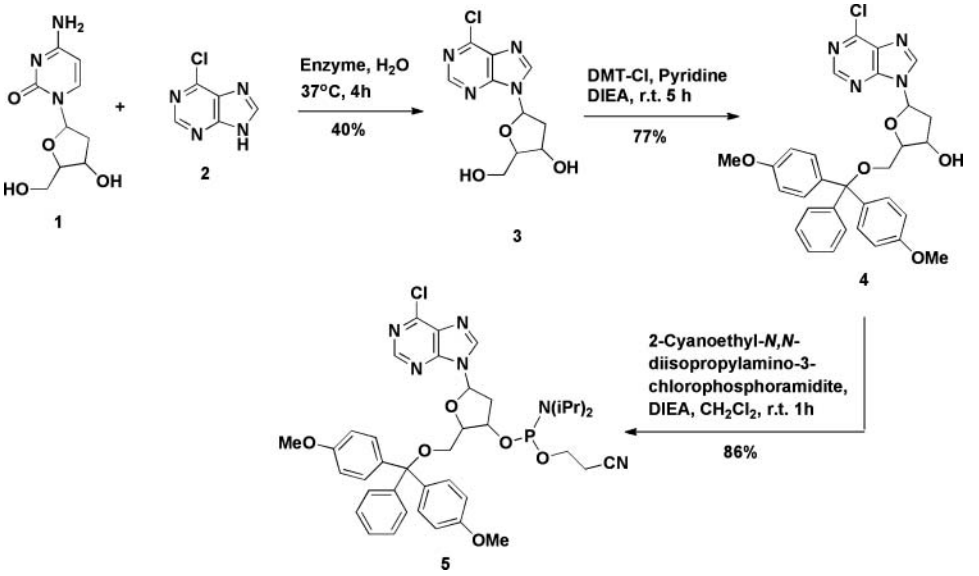
Address correspondence to Lawrence J. Marnett, A. B. Hancock Jr. Memorial Laboratory for Cancer Research, Departments of Biochemistry, Chemistry, and Pharmacology, Vanderbilt Institute of Chemical Biology, Vanderbilt University School of Medicine, Nashville, TN 37232-0146, USA. E-mail: larry.marnett@vanderbilt.edu



SCHEME 1 Semisynthesis of **5**.

is an important precursor that provides access to oligonucleotides adducted through deoxyadenosine residues.[4,5] Synthesis of the precursor, 6-chloropurine-2′-deoxyriboside (**3**) is complicated due to the involvement of unstable intermediates and the requirement for multistep procedures.[6] In addition, chemical synthesis requires removal of protecting groups under conditions that affect the nucleoside scaffold. Some success has been achieved in the synthesis of this type of compound using microbiological[7] or enzymatic[8] methodologies. Both approaches offer distinct advantages over multistep chemical procedures. These methodologies involve the transfer of a sugar residue from a donor to an acceptor base and require the presence of nucleoside phosphorylases or nucleoside deoxyribosyltransferases either as purified enzymes or within the bacterial cells used for synthesis. This report describes a semisynthetic procedure for the preparation of **5** using purified nucleoside 2′-deoxyribosyltransferase (E.C. 2.4.2.6).[9] The phosphoramidite **5** was used in the synthesis of 5′-TATCATGTCTGPu^{Cl}TTCCCGGT-3′ (**6**), which was conjugated with 5-TAMRA cadaverine to give 5′ TATCATGTCTGPu^{TAMRA}ATTCCCGGT-3′ (**7**).

RESULTS AND DISCUSSION

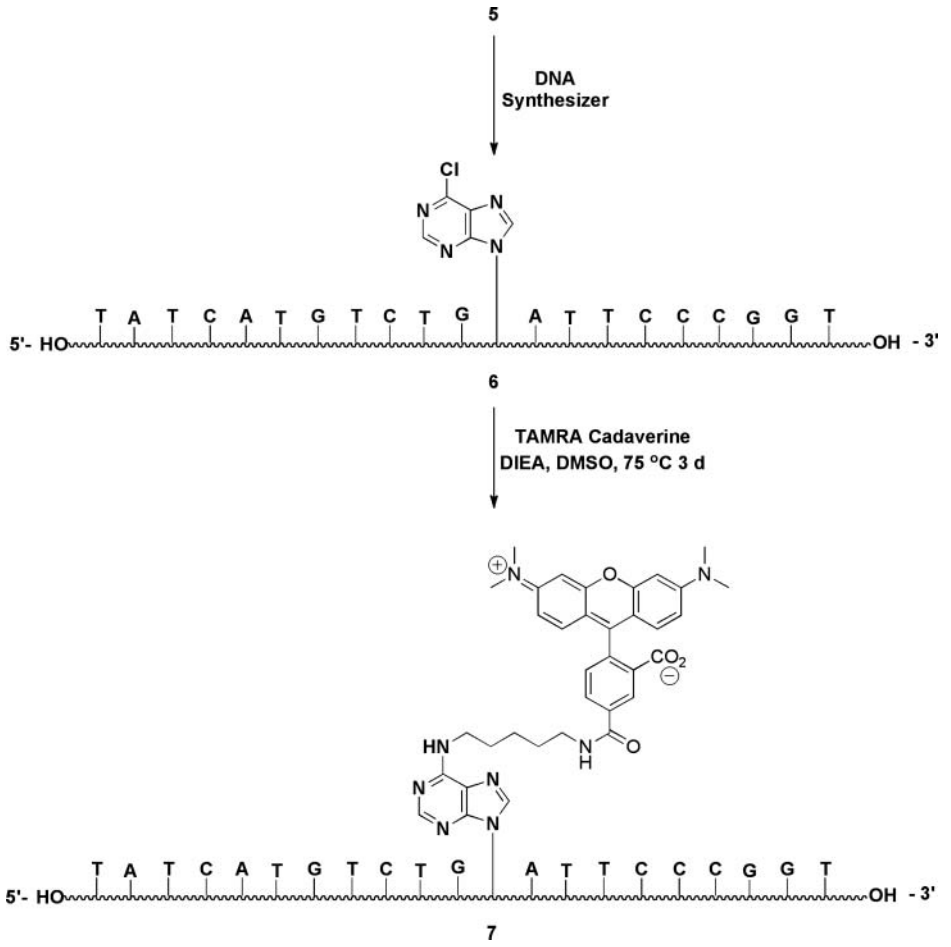
To develop an efficient, alternative route for the synthesis of 6-chloropurine-2′-deoxyriboside, we investigated a number of biosynthetic approaches. Whole cell approaches employing *Bacillus stearothermophilus* as a source of transglycosylase for the reaction of 2′-deoxyinosine and 6-chloropurine in the presence of xanthine oxidase were unsuccessful.[10] We next tried purified nucleoside 2′-deoxyribosyltransferase (E.C. 2.4.2.6)

to catalyze the transglycosylation between 2′-deoxycytidine (**1**) and 6-chloropurine (**2**) in aqueous morpholinoethanesulfonic acid (MES) at pH 6.4.[11] Nuclear magnetic resonance (NMR) analysis and mass spectrometry revealed the presence of some product but purification was difficult. Repetition of this reaction with the omission of MES followed by a silica gel column chromatographic purification afforded **3** in 40% yield. Compound **3** was converted successively into the 5′-dimethoxytrityl derivative (**4**) followed by the 3′-(2-cyanoethyl-*N,N*-diisopropylamino) phosphoramidite derivative (**5**) in good yields (Scheme 1). Phosphoramidite **5** was synthesized as a mixture of two diastereoisomers and one of them was isolated from the diastereomeric mixture by flash chromatography.

All of the compounds were characterized by one-dimensional and two-dimensional NMR and by mass spectrometry. Furthermore, the absolute stereochemistry of compound **3** was unambiguously confirmed by a single crystal x-ray analysis, which showed that compound **3** is a purine—*N9*—glycoside, rather than an *N7*—glycoside. The conformation of the hydroxymethyl group with respect to the sugar ring is *gauche—trans*. The relative configuration of the sugar and base at the glycosidic linkage is β (Figure 1).

The ^{1}H NMR spectrum of each of these compounds contains two characteristic purine protons that can be used for differentiation of other aromatic protons in the molecule. For compound **5**, these characteristic signals, identified as two separate singlets at around 8. 7 and 8.8 ppm, were assigned to the protons at position-2 and position-8 of the purine ring, respectively. The other aromatic protons in the molecule are trityl protons that are assigned as three different multiplets located at 7.28–7.30, 7.15–7.20, and 6.74–6.79 ppm. Assignments were confirmed by a ^{1}H-^{1}H COSY experiment, which revealed the correlations of the neighboring protons in each aromatic system. A single phosphorous atom was detected in the separated diastereomer of **5** at 8.31 ppm in a ^{31}P-NMR experiment.

The oligonucleotide 5′-TATCATGTCTGPu^{Cl}TTCCCGGT-3′ (**6**) was prepared by automated DNA synthesis using **5** for incorporation of the 6-Cl-purine residue. After removal of the protecting groups by hydrolysis with concentrated ammonium hydroxide, the product was desalted by gel filtration chromatography. Nucleophilic displacement of the chloro group of the purine nucleus of **6** with 5-TAMRA cadaverine in the presence of *N,N*-diisopropylethylamine gave the expected fluorescent oligonucleotide 5′ TATCATGTCTGPu^{TAMRA}ATTCCCGGT-3′ (**7**) (Scheme 2). Oligonucleotide **7** was purified by HPLC. The HPLC retention time of compound **7** (16 minutes) was much longer than that of the starting oligonucleotide **6** (8.9 minutes) in the reverse-phase chromatography. Fluorescent oligonucleotide, **7**, was characterized by electrospray mass spectrometry and exhibited a molecular ion at 6381.8 (calcd., 6381.4). The steady-state fluorescence excitation and emission of **7** were determined to be λ_{exc} = 540 nm, λ_{emit} = 572 nm (Figure 2).



SCHEME 2 Synthesis of fluorescent oligonucleotide, **7**.

Semisynthesis of **5** by a combination of enzymatic and chemical synthesis from **2** provides significant advantages over chemical synthesis. Compound **3** is produced in moderate yield in a single step by enzymatic synthesis and can be protected and activated for DNA synthesis by standard chemistry. This enables the site-specific incorporation of 6-chloropurine residues into oligonucleotides for post-synthetic coupling of amine-containing fluorophores or other pharmacophores. This enables introduction of a wide range of functionality into the oligonucleotide scaffold.

EXPERIMENTAL

Materials and Methods

Reagents and Enzymes

Silica gel column chromatography was performed using Sorbent silica gel, standard grade, porosity 60Å, particle size 32–63 μm (230 × 450 mesh),

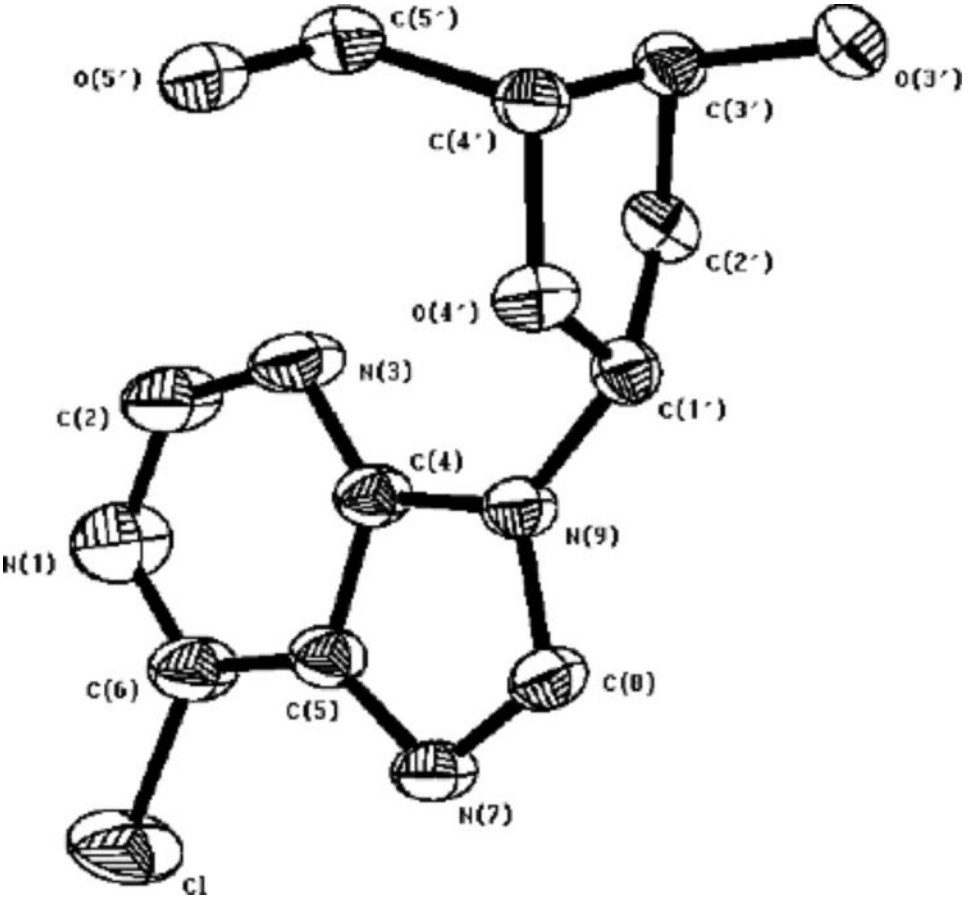


FIGURE 1 Single crystal x-ray structure of **3**.

surface area 500–600 m^2/g, bulk density 0.4 g/mL, pH range 6.5–7.5. High performance liquid chromatography (HPLC) grade solvents were obtained from Fisher (Pittsburgh, PA, USA). Reagent grade chemicals and deuterated solvents were obtained from Aldrich (Milwaukee, WI, USA) and were used as received. Nucleoside 2′-deoxyribosyltransferase (EC 2.4.2.6, transferase) was a generous gift from S. Short (GlaxoSmithKline, Research Triangle Park, NC, USA).

Instrumental Analysis

^{1}H NMR spectra were obtained on a Bruker AV-I console operating at 400.13 MHz. ^{1}H COSY experiments were executed using a 9.4 T Oxford magnet equipped with a Bruker AV-I console operating at 400.13 MHz. Experimental conditions included 2048 × 512 data matrix, 13 ppm sweep width, recycle delay of 1.5 seconds, and 4 scans per increment. The data were processed using a squared sine-bell window function, symmetrized,

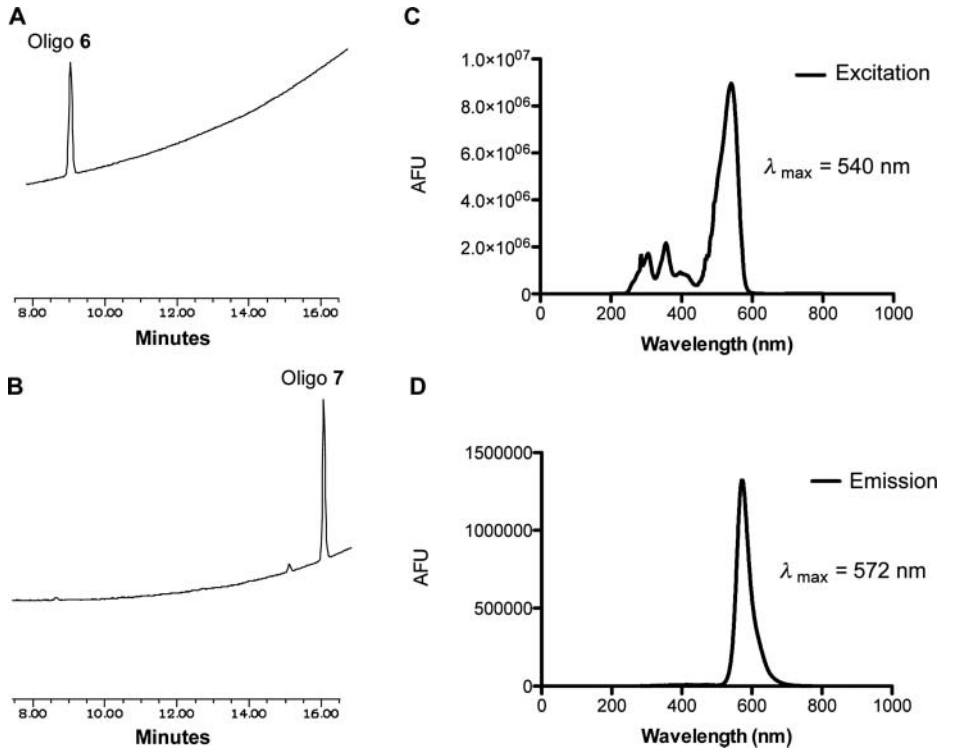


FIGURE 2 (A) HPLC chromatogram of the oligonucleotide, **6** (retention time = 8.9 minutes) with detection at 260 nm; (B) HPLC chromatogram of oligonucleotide, **7** (retention time = 16 minutes) with detection at 540 nm; (C) fluorescence excitation (λ_{max} 540 nm) spectra of compound **7** in Hank's Balanced Salt solution pH 7.4; (D) fluorescence emission (λ_{max} 572 nm) spectra of compound **7** in Hank's Balanced Salt solution pH 7.4.

and displayed in magnitude mode. ^{13}C direct detection, heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation HMBC (HMBC) NMR experiments were conducted using an 11.7 T Oxford magnet equipped with a Bruker DRX console operating at 500.13 MHz. Multiplicity-edited HSQC spectra were acquired using a 2048 × 256 data matrix, a J(C-H) value of 145 Hz resulting in a multiplicity selection delay of 34 ms, a recycle delay of 1.5 seconds, and 16 scans per increment along with GARP decoupling on ^{13}C during the acquisition time (150 ms). The data were processed using a p/2 shifted squared sine window function and displayed with CH/CH_3 signals phased positive and CH_2 signals phased negative. J_1(C-H) filtered HMBC experiments were acquired using a 2048 × 256 data matrix, a J(C-H) value of 9 Hz for detection of long range couplings resulting in an evolution delay of 55ms, a J_1(C-H) filter delay of 145 Hz (34 ms) for the suppression of one-bond couplings, a recycle delay of 1.5 seconds and 128 scans per increment. The HMBC data were processed using a p/2 shifted squared sine window function and displayed

in magnitude mode. Mass spectrometry was performed on a quantum triple quadrupole instrument in ESI positive or negative ion mode operated in-line with a ThermoElectron Surveyor pump and autosampler.

Crystallography

A single crystal of 6-chloropurine-2′-deoxyriboside was used for diffraction data collection at 293 K on an Oxford Diffraction XCalibur PX2 Ultra with an Onyx CCD detector with CuKα radiation. Diffraction data were indexed, integrated, and scaled using Chrysalis software.[12] The structure was solved using SIR2002[13] and refined with SHELXL.[14] The ortep images were prepared using XP.[14]

Fluorescence Spectroscopy

The steady-state fluorescence excitation and emission were determined with a Spex 1681 Fluorolog spectrofluorometer, equipped with a 450 W xenon arc lamp. The excitation and emission monochromator slit widths were 1–2 mm.

HPLC Analysis

The purification of fluorescent oligonucleotide was performed on a Waters 2996 HPLC system with a photodiode array detector using a Phenomenex C18 column (25 × 0.46 cm, 3 mL/min) with 0.1 M aqueous ammonium formate and acetonitrile. HPLC gradients: (A) initially 1% acetonitrile, 15 minutes linear gradient to 10% acetonitrile, 5 minutes linear gradient to 20% acetonitrile, 5 minutes isocratic at 20% acetontrile, 3 minutes linear gradient to 100% acetonitrile, 4 minutes isocratic in 100% acetonitrile followed by 3 minutes linear gradient to initial conditions. (B) Initially 1% acetonitrile, 5 minutes linear gradient to 5% acetonitrile, 25 minutes linear gradient to 8% acetonitrile, 3 minutes linear gradient to 99% acetonitrile, isocratic at 99% acetonitrile for 6 minutes, and 3 minutes linear gradient to the initial conditions, isocratic at 1% acetonitrile for 3 minutes. (C) Initially 1% acetonitrile, 5 minutes linear gradient to 5% acetonitrile, 20 minutes linear gradient to 5.7% acetonitrile, 3 minutes linear gradient to 99% acetonitrile, isocratic at 99% acetonitrile for 5 minutes, and then a 3 minute linear gradient to the initial conditions, isocratic at 1% acetonitrile for 2 minutes.

Chemistry

Synthesis of 6-Chloropurine-2′-deoxyriboside (3)

2′-Deoxycytidine (**1**) (1.25 g, 5.5 mmol), **2** (0.85 g, 5.5 mmol), and deionized water (100 mL) were mixed in a 250 mL bottle and capped. The bottle was agitated in a shaker at 37°C for approximately 1 hour or until all of the **2** was dissolved. Purified *E. coli* nucleoside 2′-deoxyribosyltransferase (E.C.

2.4.2.6) was added (300 μg in 2 μL 100 mM sodium phosphate buffer, pH 6.0) with continued agitation at 37°C for 4 hours. The reaction was cooled to room temperature, and water was removed by rotary evaporation. The resulting residue was purified by flash chromatography using 35:7:1 $CHCl_3$:MeOH:NH_4OH resulting in a white solid. The product was recrystallized using 6:1 EtOAc:MeOH to give colorless crystals of **3** (0.59 g, 40%). ^{1}H NMR (400 MHz, D_2O) 2.62–2.65 (m, 1H), 2.86–2.88 (m, 1H), 3.81–3.84 (m, 2H), 4.17 (q, 1H), 4.67–4.70 (m, 1H), 6.52 (t, 1H), 8.64 (s, 1H), 8.68 (s, 1H); MS (ESI) *m/z* $(M+H)^+$ calcd 271.05; found 271.06

Synthesis of 5′-Dimethoxytrityl 6-chloropurine-2′-deoxyriboside (4)

To a stirred solution of **3** (174 mg, 0.64 mmol) in dry pyridine (2.5 mL) was added diiospropylethylamine (168 mg, 0.96 mmol) followed by dimethoxytrityl chloride (304 mg, 0.9 mmol). The reaction mixture was stirred at room temperature for 5 hours. Then MeOH (1 mL) was added with further stirring for 10 minutes. Water (18 mL) was added and the organic compounds were extracted with CH_2Cl_2 and washed with 10% K_2CO_3. The organic layer was dried with Na_2SO_4 and evaporated in vacuo, and the residue was purified by flash chromatography using 4:95:1 $CHCl_3$:MeOH:diisopropylethylamine to give the product (**4**) as a brown amorphous solid (280 mg, 77%). ^{1}H NMR (400 MHz, DMSO d_6) 2.36–2.40 (m, 1H), 2.85–2.89 (m, 1H), 3.12–3.21 (m, 2H), 4.00–4.04 (m, 1H), 4.49–4.52 (m, 1H), 5.40 (d, 1H, OH), 6.47 (t, 1H), 6.50- 6.78 (m, 4H), 7.13–7.21 (m, 7H), 7.27–7.30 (m, 2H), 8.69 (s, 1H), 8.79 (s, 1H); MS (ESI) *m/z* $(M+H)^+$ calcd 573.18; found 573.20.

Synthesis of 6-Chloropurine-2′-deoxyriboside 5′-dimethoxytrityl 3′-(2-cyanoethyl-N,N-diisopropylamino)phosphoramidite (5)

To a stirred solution of **4** (40 mg, 0.07 mmol) in dry CH_2Cl_2 (0.5 mL) was added diisopropylethylamine (13 mg, 0.1 mmol) followed by β-cyanoethyl-*N,N*-diisopropylamino-3-chlorophosphoramidite (20 mg, 0.084 mmol). The reaction mixture was stirred at room temperature for 1 hour. Then EtOAc (0.5 mL) and water (1 mL) were added, and the organic compounds were extracted and washed with saturated $NaHCO_3$ and water. The organic layer was dried with Na_2SO_4 and evaporated *in vacuo,* and the residue was purified by flash chromatography using 49:50:1 EtOAc:*n*-Hex:triethylamine to give the product **5** as a colorless gummy mass (86% total). A diastereomerically pure single isomer was obtained with a silica column using a gradient elution of EtOAc:*n*-Hex:TEA (10:89:1 to 30:69:1). Single isomer—^{1}H NMR (400 MHz, DMSO d_6) 1.12 (s, 3H), 1.13 (s, 3H), 1.14 (s, 3H), 1.15 (s, 3H), 2.54–2.56 (m, 1H), 2.67 (t, 2H), 3.12–3.14 (m, 1H), 3.22–3.28 (m, 2H), 3.57–3.58 (m, 2H), 3.65–3.68 (m, 2H), 3.71 (s, 3H), 3.72 (s, 3H), 4.18–4.21 (m, 1H), 4.82–4.86 (m, 1H), 6.50–6.52 (m, 1H), 6.74–6.79 (m, 4H), 7.15–7.20

(m, 7H), 7.28–7.30 (m, 2H), 8.67 (s, 1H), 8.82 (s, 1H); MS (ESI) m/z $(M+H)^+$ calcd 773.29; found 773.31.

Synthesis of 5′-TATCATGTCTGPu^{Cl}ATTCCCGGT-3′ (6)

The 21-mer oligonucleotide **6** was synthesized from the 3′ to 5′ end on 0.2 μmol scale using standard automated DNA synthesis using an ABI 395 DNA synthesizer at Midland Certified Reagents. A 0.1 M solution of phosphoramidite **5** in dry acetonitrile was used for standard automated solid-phase synthesis. After synthesis, the oligonucleotide was cleaved from the solid support by incubation at 55°C with concentrated ammonium hydroxide, resulting in complete deprotection of the oligonucleotide. The product was desalted by gel filtration chromatography and the oligonucleotide was purified using reverse-phase HPLC (C18; acetonitrile:water; 10−50% gradient over 50 minutes; monitoring at 254 nm). The purified oligonucleotide exhibited one peak on HPLC. Electrospray mass spectrometry of the oligonucleotide confirmed the formation of 5′-TATCATGTCTGPu^{Cl}ATTCCCGGT-3′ **(6)**, ESMS m/z $[M-H]^-$ calcd 6381.4, found 6381.8.

Synthesis of 5′-TATCATGTCTGPu^{TAMRA}ATTCCCGGT-3′ (7)

Oligonucleotide **6** (1 mg) was mixed with diisopropylethylamine (50 μL), DMSO (100 μL), and TAMRA cadaverine (1.4 mg,) in a plastic test tube. The reaction mixture was stirred at 75°C for 3 days. HPLC analysis showed complete disappearance of **6**. Water was added and the sample was lyophilized to give the crude product, which was purified by sequential chromatography on the three reversed-phase HPLC systems listed above to afford the oligonucleotide **7** (61%). ESMS m/z $[(M-5H)/5z]^-$ calcd 1371.0, found 1371.2.

TABLE 1 Crystallographic parameters

Cell parameters	Values
a	20.658 Å
b	8.156 Å
c	7.047 Å
β	97.637
V (Å^3)	1176.79
Space group	C2
μ (mm^{-3})	2.97
Reflections	2474
Parameters	166
Goodness of Fit (all data)	1.026
R1 (all data)	0.045

Crystal Data

A crystal structure determination of compound **3** was performed, where diffraction data were collected from a single crystal at room temperature (293 K). The crystal was monoclinic with space group C2. Unit cell parameters are shown in Table 1. The structure was phased by direct methods and atom assignments verified as expected. Structure refinement with riding hydrogens and anisotropic temperature factors resulted in a final R1 of 0.045 and a Goodness of Fit (S) of 1.026. The absolute structure was confirmed reasonably well with a Flack-x parameter of 0.084 (esd 0.0227). A displacement thermal ellipsoid plot is shown in Figure 1.

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