Synthesis of 2'-Deoxyisoguanosine 5'-Triphosphate and 2'-Deoxy-5'-methylisocytidine 5'-Triphosphate

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The syntheses of the 5'-triphosphates of 2'-deoxyisoguanosine (= p,isoGp) and 2'-deoxy-5'-methylisocytidine (= p,me5isoCp), two new bases for the genetic alphabet, are described. The triphosphates were synthesized from the corresponding nucleosides using a transient-protection procedure. The introduction of a methyl group at the 5-position of 2'-deoxyisocytidine remarkably improved the stability of the triphosphate. Characterization of the triphosphates included enzymatic incorporation opposite the complementary base in a template oligonucleotide.

Introduction. – The Watson-Crick base pairing between two complementary oligonucleotide strands remains one of the most remarkable examples of molecular recognition. It follows two rules of complementarity: i) a large purine from one strand pairs with a small pyrimidine from the other; ii) H-bond donors (NH groups) of one base pair with H-bond acceptors (lone pairs of electrons on O- or N-atoms) of the other. Nature only partially exploits the potential of the Watson-Crick formalism, however. Structures for six base pairs can readily be written to conform to Watson-Crick geometry [1][2]. Therefore, it is possible, in principle, to have 12 independently replicating ‘letters’ in the nucleoside ‘alphabet’.

One feasible non-standard base pair is formed between isoguanosine (isoG) and isocytidine (isoC), which forms three H-bonds like a G · C base pair. It was suggested three decades ago by Rich [3] that the (isoG) · (isoC) base pair might have been a component of primitive nucleic acids early in the development of life. In the late 1980’s, the first experimental work was done to explore the oligonucleotide chemistry of this non-standard base pair, including its ability to be incorporated by enzymatic template-directed polymerization. Since then, several other laboratories have made major contributions to developing the chemistry and enzymology of the isoC · isoG base pair, including those of Tor and Dervan [4], Horn and coworkers [5], Seela and Wei [6], and Switzer and coworkers. [7]. A variety of RNA and DNA polymerases have been found that catalyze template-directed incorporation of this base pair into DNA and RNA [8].

An interesting application of the new bases is to expand the genetic alphabet in in vitro selection technology, as increasing the number of replicatable letters would expand the diversity of the oligonucleotide alphabet and likely increase the catalytic
activities of the resulting oligonucleotides. Indeed, increasing the diversity of oligonucleotide functionality has already proved effective in the in vitro selection experiments of Tarasow et al. [9] using a 5-substituted deoxyuridine. Key to such selection experiments is the ready availability of triphosphates of non-standard bases used for PCR amplification of oligonucleotides containing non-standard bases. This has generated substantial interest in synthetic methods for preparing triphosphates of non-standard bases. We report here an improved synthesis of 2'-deoxyisoguanosine 5'-triphosphate (p3isoGd; 1) and the first detailed synthetic procedure for 2'-deoxy-5-methylisocytidine (p3me5isoCd, 2), a more useful analog of p3isoCd (3).

Results and Discussion. – Deoxyisoguanosine Triphosphate. The triphosphate 1 of 2'-deoxyisoguanosine has previously been synthesized by a photochemical procedure using 2'-deoxyadenosine triphosphate as the starting compound. However, the method gave p3isoGd only in low yield along with inseparable impurities [8]. An alternative conversion of 2'-deoxyadenosine triphosphate to p3isoGd using [FeIIHe(edta)] has been reported by Kamiya and Kasai [10]. We were, however, unable to reproduce this procedure.

Our attention, therefore, turned to an alternative conversion of isoGd to its triphosphate. Several procedures have been reported for the conversion of a nucleoside to its triphosphate [4][11][12]. Most attractive is the one-pot procedure of Ludwig, which phosphorylates the sugar of an unprotected nucleoside in situ with POCl3 in trimethyl phosphate followed by the addition of pyrophosphate [13]. This procedure was attempted on 2'-deoxyisoguanosine. However, substantial competing phosphorylation of the base at the 2-oxo position was observed. The high reactivity of the O-atom at C(2) of 2'-deoxyisoguanosine has been previously described [14]. Clearly, base protection at this position prior to the phosphorylation is required. The resulting triphosphate must then tolerate conditions used to remove the protecting group.

Previous work on solid-phase oligonucleotide synthesis with 2'-deoxyisoguanosine in this and other laboratories [5–8][15][16] has shown that formamidine protection is suitable for the exocyclic N-atom. Protection with diisobutylformamide dimethyl acetal (6) [16] confers particular stability against depurination during synthesis. Diphenyl-carbamoyl is an appropriate protecting group for the 2-oxo position of 2'-deoxyisoguanosine [5][6][15][16]. Both protecting groups are readily removed by treatment with aqueous ammonia and consequently are compatible for use with a triphosphate. Thus, protected isoguanosine 10 was prepared from 4 via 5–9 (Scheme 1). The fully
protected 2'-deoxyriboside, 3'-O-acetyl-2'-deoxy-O2-(diphenylcarbamoyl)-N6-(diisobutyraminomethylidene)isoguanosine (17) was then obtained from 10 via 11–16 (Scheme 2). Synthesis of 17 from 5 comprises 11 steps with a reasonable overall yield (11%) and allows a cost-effective large-scale preparation of 1. Moreover, intermediate 5 is easily purified by recrystallization, and not all intermediates require isolation.

Protected 2'-deoxyisoguanosine 17 was converted in a one-pot reaction to p3isoGd (1) by the Ludwig-Eckstein method [17]. In this procedure, salicyl phosphorochloridite, the phosphorylating agent, is reacted in situ with pyrophosphate, followed by oxidation with I2/pyridine, and finally hydrolysis with aqueous ammonia. This treatment applied to 17 also removed the base and sugar protection, and 1 was isolated in 17% yield, after ion-exchange purification followed by reversed-phase HPLC. In addition to spectroscopic methods, the identity of the purified p3isoGd (1) was verified in a simple primer extension experiment (Fig. 1), by incorporating 1 opposite 2'-deoxy-5-methylisocytidine (2) in a template oligonucleotide. The Klenow fragment of DNA polymerase 1, in the presence of standard triphosphate, synthesized full-length product (primer ‡7) from a template containing standard nucleobases with modest amounts of abortion (Fig., Lane 2). As is frequently seen with such runoff experiments, aborted products primarily correspond to n – 1 product (primer ‡6). The Klenow fragment evidently incorporated p3isoGd selectively (Fig., Lane 3), as the major product synthesized from
an isoC₄-containing template in the presence of p₃isoG₄ and standard triphosphate is full length (primer + 7). Elongation of the primer with the same isoC₄-containing template in the absence of p₃isoG₄ yielded only aborted product (primer + 2), stopping cleanly opposite me⁵isoC₄ in the template (Fig., Lane 5).

\[\text{Diagram with reactions and compounds} \]
Deoxyisocytidine Triphosphate. The synthesis of 2'-deoxyisocytidine 5'-triphosphate (3) has been described by Switzer et al. [8]. Unfortunately, the compound was reported to be relatively unstable; after storage for 6 weeks at $-20^\circ$, only 35% of the original triphosphate remained, according to HPLC analysis [8]. This short lifetime may partially explain why isocytidine is excluded from the natural genetic alphabet. In any case, this instability makes in vitro experiments with 3 somewhat troublesome, as one must freshly synthesize the compound before each use.

Tor and Dervan [4] introduced 2'-deoxy-5-methylisocytidine as a substitute for its 5-unsubstituted counterpart in oligonucleotide synthesis. The 5-methyl derivative is more stable towards deamination [16][19], more resistant to acid depyrimidination [19], and cheaper, as the synthesis starts from readily available thymidine instead of the more expensive 2'-deoxyuridine. Exploiting these advantages, Bukowska and Kusmierek [19] synthesized p3me5isoCd (2) following the synthetic procedure [8] previously used in the synthesis of p3isoCd (3); however these authors reported no experimental procedures, yields, or product characterization.

Like p3isoGd (1), p3me5isoCd (2) was synthesized from the suitably protected nucleoside by the Ludwig-Eckstein method (Scheme 3). Formamidine protection was chosen for the exocyclic N-atom of me5isoCd (18) because 2'-deoxy-$N^\gamma$-[(dimethylamino)methylidene]-5-methylisocytidine (19) is considerably more stable to acid conditions than the corresponding N-benzoyl-protected compound, and it is easily detritylated without concomitant depyrimidination [16]. Thus, 2'-deoxy-5-methylisocytidine (18) was prepared as previously described [16] and reacted with dimethylformamide dimethyl acetal to give 19, which was easily purified, in contrast to the corresponding N-benzoyl protected analog [15][16]. 3'-O-Acetyl-2'-deoxy-$N^\gamma$-[(dime-
thylamino)methylidene]-5-methylisocytidine (22) was synthesized in an additional 3 steps via 21. Protected me\textsuperscript{5}isoCd (22) was then converted to p3me\textsuperscript{5}isoCd (2) as described above for 17 → 1. Triphosphate 2 was purified and obtained in 42% yield after reversed-phase HPLC. As with 2'-deoxyisoguanosine 5'-triphosphate (1), the identity of the purified p3me\textsuperscript{5}isoCd (2) was verified in a primer-extension experiment. Finally, unlike p3isoCd (3), 2 was stored at −20°C for several months without detectable degradation. Therefore, p3me\textsuperscript{5}isoCd (2) appears to be the triphosphate of choice for \textit{in vitro} studies of the isoC\textsubscript{d} · isoG\textsubscript{d} base pair.

**Experimental Part**

\textit{General.} Purine-2,6-diamine riboside and purine-2,6-diamine 2'-deoxyriboside were purchased from \textit{RI Chemical, Inc.} (Orange, CA), 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane from \textit{Farchan Laboratories, Inc.} (Gainesville, FL), DEAE Sephadex and tributylammonium pyrophosphate from \textit{Sigma}, and all other chemicals from \textit{Aldrich Chemical or Fisher Scientific}; all were used without further purification. \textit{p}-Toluenesulfonyl chloride was freshly recrystallized from petroleum ether. Acetonitrile and pyridine were dried over 4Å molecular sieves. All reactions were carried out under dry Ar in an oven-dried glass system. 'Evaporation' refers to removal of volatile solvents with a membrane pump. Column chromatography (CC): silica gel (230–425 mesh; \textit{Fisher}). TLC: silica gel plates from \textit{Whatman}; visualization by staining with a Ce/Mo reagent (2.5% phosphormolybdic acid, 1% Ce\textsuperscript{IV}(SO\textsubscript{4})\textsubscript{2} · 4H\textsubscript{2}O, 6% H\textsubscript{2}SO\textsubscript{4} in H\textsubscript{2}O) and heating. Ion-exchange chromatography: \textit{DEAE Sephadex} equilibrated in 0.2m (Et\textsubscript{3}NH)HCO\textsubscript{3} (pH 7.0); p,isoG\textsubscript{d} (1) eluted during a linear gradient to 0.5m (Et\textsubscript{3}NH)HCO\textsubscript{3}, and p,me\textsuperscript{5}isoCd (2) during a linear gradient to 0.3m (Et\textsubscript{3}NH)HCO\textsubscript{3}. HPLC: \textit{Waters-PrepLC-4000} system with a 486 tunable absorbance detector. Reversed-phase HPLC: \textit{Waters-PrepLC-
25-mm module containing a single PrepPak cartridge (Prep Nova-Pak HR C8, 6 µm 60 Å, 25 × 100 mm); purification of triphosphates: flow rate 5.1 ml/min; solvent A: 25 mM (Et3NH)OAc (pH 7.0); solvent B: 20% (v/v) MeCN in solvent A; linear binary gradient for 1 (tq 37 min), 100% A (1 min) and 100% A to 90% A (39 min); for 2 (tq 30 min), 100% A (1 min) and 100% A to 82% A (39 min). UV Spectra: Varian-Cary-1-Bio spectrophotometer. NMR Spectra: Varian-XL-300 spectrometer; at 300 MHz (1H) referenced to SiMe4, at 75.4 MHz (13C) referenced to solvent, and at 121.4 MHz (31P) with H3PO4, as standard in the solvents as given.

MS: recorded with the Spectroscopy Services of the University of Florida Chemistry Department; Finnigan MAT LCQ (San Jose, CA) in electrospray ionization (ESI) mode. HPLC/ESI-MS: Beckman Instruments System Gold 126 pump (Fullerton, CA) with a Keystone Scientific Hypersil ODS column (2 × 150 mm + guard) (Bellefonte, PA) and isocratically eluting (0.2 ml/min with 10 mM NH4OAc and 87 mM AcOH in H2O/MeCN/PrOH 98:1.4:0.6) the triphosphates.

Isoguanosine (5). Purine-2,6-diamine riboside 4 (35.5 mmol, 10.0 g) was suspended in H2O (250 ml) at 50°C, and NaNO2 (137 mmol, 9.44 g) in H2O (60 ml) was added. Then AcOH (245 mmol, 14.1 ml) was added at 50°C over 1.5 min. The resulting clear soln. was stirred for 3.5 min and then diluted with H2O (150 ml), and conc. aq. NH4HCO3 soln. was added to pH 8. The soln. was evaporated and the remaining solid washed with H2O: 5 (9.03 g, 90%). Light yellow powder. 1H-NMR ((D6)DMSO): 3.50–3.74 (m, H–C(3')); 4.54 (m, H–C(2')); 5.69 (m, H–C(1')); 7.97 (s, H–C(8)). 13C-NMR ((D6)DMSO): 61.9, 71.0, 73.2, 86.3, 879, 109.8, 138.4, 152.8, 153.6, 156.8.

N4-(Diisobutylamino)methylideneisoguanosine (7). Isoguanosine (5; 31.8 mmol, 9.00 g) was co-evaporated with pyridine followed by DMF and then suspended in dry DMF (150 ml). Diisobutylformamide dimethyl acetal (6) [16] (44.5 mmol, 9.04 g) was added. The mixture was stirred at r.t. for 23 h, then MeOH (3 ml) was added and the soln. evaporated. The residue was dried under vacuum at 50°C with an oil pump: 7 (13.1 g, 98%). Light yellow foam. TLC (CHCl3/MeOH 82.5 :17.5): 5 (ml) was added, and the mixture was concentrated and extracted with CHCl3/dil. aq. NaHCO3 soln. The org. phase further extracted with CHCl3 and the combined org. phase dried (Na2SO4) and evaporated. The residue was purified by CC (10% MeOH/CHCl3 ; v). Light yellow foam. 1H-NMR (CDCl3): 0.88 (3J, 2 H–C(2')); 4.67 (m, H–C(3')); 4.51 (m, H–C(2')); 5.79 (m, H–C(1')); 7.97 (s, H–C(8)). 13C-NMR ((D4)DMSO): 61.9, 71.0, 73.2, 86.3, 879, 109.8, 138.4, 152.8, 153.6, 156.8.

N4-[(Diisobutylamino)methylidene]-2,3,5-tris-(trimethylsilyl)isoguanosine (8). Compound 7 (31.1 mmol, 13.1 g) was co-evaporated with pyridine and dissolved in anh. CH2Cl2 (130 ml). Under ice-bath cooling, Et3N (374 mmol, 51.9 ml) and Me3SiCl (290 mmol, 35.6 ml) were added (the O-atom at C(2) remained unsilylated under these conditions). The mixture was stirred at r.t. for 1.5 h and then poured into dil. aq. NaHCO3 soln., the aq. phase further extracted with CHCl3, and the combined org. phase dried (Na2SO4) and evaporated. The resulting light yellow foam (191 g, 96%) was used in the next step without further purification. TLC (CHCl3/MeOH 9:1): Rf 0.48. 1H-NMR (CDCl3): 0.10, 0.14, 0.18 (3x, 3 MeSi); 0.91–1.10 (m, 2 Me2CHCH3); 2.03–2.32 (m, 2 Me2CHCH3); 3.32–3.61 (m, 2 Me2CHCH3); 3.76–4.04 (m, 2 H–C(5')); 4.16 (m, H–C(4')); 4.34 (m, H–C(3')); 4.58 (m, H–C(2')); 5.69 (m, H–C(1')); 7.97 (s, H–C(8)). 13C-NMR (CDCl3): −0.8, 0.1, 19.7, 20.0, 26.4, 27.1, 52.8, 60.7, 61.0, 70.9, 75.5, 83.9, 87.6, 114.6, 139.5, 153.7, 157.7, 158.0, 161.9.

N4-[(Diisobutylamino)methylidene]-O2-[(diphenylcarbamoyl)-2,3,5-tris-(trimethylsilyl)isoguanosine (9). Compound 8 (13.8 mmol, 8.80 g) was co-evaporated with pyridine and dissolved in anh. pyridine (70 ml). N,N-Diisopropylethyamine (55.2 mmol, 9.6 ml), DMAP (3.45 mmol, 421 mg), and diphenylcarbamoyl chloride (276 mmol, 6.389 g) were added. The dark orange soln. was stirred at r.t. for 24 h. Then MeOH (5 ml) was added, and the mixture was concentrated and extracted with CHCl3/dil. aq. NaHCO3 soln. The org. layer was dried (Na2SO4) and evaporated, and the residue was used in the next step without further purification. A small sample was purified by CC (1.5% MeOH/CH2Cl2) to give 9 as a yellow foam. 1H-NMR (CDCl3): 0.10, 0.14, 0.18 (3x, 3 MeSi); 0.93 (dd, 2 Me2CHCH3); 1.93–2.20 (m, 2 Me2CHCH3); 3.18 (d, 1 Me2CHCH3); 3.47–3.64 (m, 1 Me2CHCH3); 3.70–4.05 (2m, 2 H–C(5')); 4.12 (m, H–C(4')); 4.31 (m, H–C(3')); 4.51 (m, H–C(2')); 6.00 (m, H–C(1')); 7.22–7.40 (m, 2 Ph); 8.27 (s, H–C(8)); 8.94 (s, N=CH). 13C-NMR (CDCl3): −0.7, 0.1, 19.8, 20.1, 26.1, 27.2, 52.7, 59.9, 60.8, 70.4, 75.8, 83.8, 88.9, 123.7, 126.2, 126.9, 128.8, 140.5, 140.5, 142.3, 152.0, 152.4, 156.0, 159.4, 161.2.

N4-[(Diisobutylamino)methylidene]-O2-[(diphenylcarbamoyl)isoguanosine (10). Unpurified 9 (directly from the previous reaction) was dissolved in CH2Cl2 (200 ml), and TsOH (6 equiv., 82.8 mmol, 15.7 g) in THF (70 ml) was added. The mixture was stirred for 3 min, then the reaction was quenched by addition of Et3N (82.8 mmol, 11.5 ml) and extracted with CHCl3/dil. aq. NaHCO3 soln. The org. phase was dried (Na2SO4) and evaporated, and the residue was purified by CC (10% MeOH/CH2Cl2; Rf 0.27); 10 (8.08 g, 95% for 2 steps). Light yellow foam. 1H-NMR (CDCl3): 0.88 (m, 2 Me2CHCH3); 1.83–2.17 (m, 2 Me2CHCH3); 3.18 (m, 1 Me2CHCH3); 3.37–3.58 (m, 1 Me2CHCH3); 3.63–3.80 (2m, 2 H–C(5')); 4.16 (m, H–C(4')); 4.29
(m, H – C(3’)): 4.82 (m, H – C(2’)); 5.37 (m, H – C(1’)); 7.18 – 7.48 (m, 2 Ph); 7.63 (s, H – C(8’)); 8.99 (s, N = CH).

13C-NMR (CDCl3): 19.6, 20.1, 26.4, 27.0, 52.6, 60.0, 63.1, 72.4, 73.6, 87.1, 90.2, 125.4, 126.7, 127.1, 128.9, 141.8, 142.3, 151.0, 152.8, 154.8, 160.2, 161.4.

N4-[(Diisobutylamino)methylene]-O2-(diphenylcarbamoyl)-3,5’-O-(1,1,3,3-tetraisopropylsiloxane-1,3-diyl)isoguanosine (11). Compound 10 (39.5 mmol, 24.4 g) was co-evaporated with pyridine and then dissolved in anh. pyridine (250 ml). Dichlorotetraethylsiloxane (39.5 mmol, 12.6 ml) was added via syringe, and the mixture was stirred for 16 h at r.t. The mixture was evaporated. Then dil. aq. NaHCO3 soln. was added, the solution extracted with CHCl3, the org. layer dried (Na2SO4) and evaporated, and the residue purified by CC (one-step gradient: 1.5% MeOH/CH2Cl2, then 10% MeOH/CH2Cl2).

Diyl)isoguanosine was stirred for 16 h at r.t. The mixture was evaporated. Then dil. aq. NaHCO3 soln. was added, the mixture was stirred for 16 h at r.t. The mixture was evaporated. Then dil. aq. NaHCO3 soln. was added, the solution extracted with CHCl3, the org. layer dried (Na2SO4) and evaporated, and the residue purified by CC (CHCl3, then 10% MeOH/CHCl3; 1.5% MeOH/CH2Cl2, then 10% MeOH/CH2Cl2): diyl)isoguanosine (10).

The mixture was stirred for 16 h at r.t. The mixture was evaporated. Then dil. aq. NaHCO3 soln. was added, the mixture was stirred for 16 h at r.t. The mixture was evaporated. Then dil. aq. NaHCO3 soln. was added, the solution extracted with CHCl3, the org. layer dried (Na2SO4) and evaporated, and the residue purified by CC (CHCl3, then 10% MeOH/CHCl3; 1.5% MeOH/CH2Cl2, then 10% MeOH/CH2Cl2): diyl)isoguanosine (10).

CC (17.5% MeOH/CHCl₃; stirred at r.t. for 1 h, then EtOH (5 ml) was added, and the mixture was evaporated). The residue was purified by chromatography (DEAE

Compounds 15 (1.01 mmol, 911 mg), DMAP (0.25 mmol, 31 mg), Et₃N (2.52 mmol, 0.351 ml), and Ac₂O (1.2108 mmol, 0.114 ml) were added to a solution of dry pyridine (30 ml). The mixture was stirred at r.t. for 2 h. MeOH (1 ml) was added, the mixture was concentrated in vacuo and extracted (AcOEt/dil. aq. NaHCO₃ soln.), the org. layer dried (Na₂SO₄) and evaporated, and the residue (R₁ 0.78% MeOH/CHCl₃) used in the next step without further purification.

Unpurified 16 (directly from the previous reaction) was dissolved in a solution of MeOH (5 ml) and CHCl₃ (1 ml). The solution was cooled to 0°C, and 10% anh. HCl in MeOH (5 ml) was added. Stirring was continued at 0°C for 5 min. The mixture was then neutralized with aq. NaHCO₃ soln. and extracted (CHCl₃). The resulting org. layer was dried (Na₂SO₄) and evaporated and the residue purified by CC (5% MeOH/CHCl₃; R₂ 0.59): 17 (290 mg, 45% for 2 steps). Pale yellow foam. 

⁴⁺-Deoxy-5'-triphosphate (I). Compounds 17 (0.170 mmol, 109 mg) were co-evaporated with pyridine and dissolved in anh. pyridine (0.17 ml) and anh. dioxane (0.51 ml). A solution of 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (0.19 mmol, 38 mg) [17] in dioxane (0.18 ml) was added, and a white precipitate was formed instantly. The mixture was stirred at r.t. for 10 min, then tributylammonium pyrophosphate (0.616 mmol, 119 mg) in DMF (0.6 ml) and tributylamine (0.71 mmol, 0.17 ml) were added, immediately forming a clear solution. The solution was stirred for 10 min, then 1% soln. of Li₂ in pyridine/H₂O 98:2 was added (3.74 ml). Stirring was continued for 15 min, and the excess Li₂ was destroyed by addition of 5% aq. Na₂SO₄ soln. H₂O (6 ml) was added and the mixture stirred for 30 min. Then 25% aq. NH₄ soln. (30 ml) was added followed by further stirring at r.t. for 5 h. The solvent was then evaporated and the residue purified by ion-exchange chromatography (DEAE Sephadex). Further purification was achieved by reversed-phase HPLC to give 0.029 mmol (17%) of pure I, calculated with an extinction coefficient at 292 nm of 1.1 × 10⁴ M⁻¹ cm⁻¹ [14]. UV (H₂O): 247, 292. 

⁴⁺-Deoxy-5'-[bis(dimethylamino)methylidene]-5-methylisocytidine (19). To a solution of 18 (2.37 mmol, 571 mg) in anh. DMF (14 ml), dimethylformamide dimethyl acetal (30.2 mmol, 3.59 g, 4.00 ml) was added. The solution was stirred at r.t. for 1 h, then EtOH (5 ml) was added, and the mixture was evaporated. The residue was purified by CC (17.5% MeOH/CHCl₃; R₁ 0.35): 677 mg (97%) of 19. White solid product. 

³⁺⁻-Deoxy-5'-[bis(dimethylamino)methylidene]-5-methylisocytidine (20). Compound 19 (2.29 mmol, 677 mg) was co-evaporated with pyridine and then dissolved in dry pyridine (25 ml). DMAP (0.57 mmol, 70 mg), (MeO)₂Tr·Cl (2.74 mmol, 978 mg), and Et₃N (4.57 mmol, 0.636 ml) were added, and the mixture was stirred at r.t. for 8 h (TLC 10% MeOH/CHCl₃; R₁ 0.29). MeOH (3 ml) was added, the mixture evaporated, aq. NaHCO₃ soln. (30 ml) added, and the mixture extracted with AcOEt (3 × 30 ml). The combined org. layer was dried (Na₂SO₄) and evaporated, and the residue was purified by CC (10% MeOH/CHCl₃, then 17.5% MeOH/CHCl₃) of 20 (1.03 g, 75%). White foam.
(4.29 mmol, 0.596 ml), and Ac₂O (2.06 mmol, 0.194 ml) were added. The mixture was stirred at r.t. for 5 h, then MeOH (2 ml) was added and the mixture evaporated. Aq. NaHCO₃ soln. (30 ml) was added and the mixture extracted with AcOEt (3 × 30 ml). The combined org. layer was dried (Na₂SO₄) and evaporated, and the residue was purified by CC (17.5% MeOH/CHCl₃; Rf 0.68); 21 (0.974 g, 89%). White foam. H-NMR (CDCl₃): 1.62 (s, Me–C(5)); 2.07 (s, Ac); 2.40–2.55 (m, 2 H–C(2)); 3.08, 3.12 (2x, Me₂N); 3.42–3.54 (m, 2 H–C(5)); 3.77 (s, 2 MeO); 4.15 (m, H–C(4)); 5.42 (m, H–C(3)); 6.76–6.88 (m, 5 H–C(1), (MeO)₃Tr); 7.18–7.37, 7.43 (m, d, 9 H, (MeO)₃Tr); 7.70 (s, H–C(6)); 8.82 (s, N=CH). ³¹P-NMR (CDCl₃): 13.3, 13.3, 20.7, 34.8, 34.9, 38.4, 40.9, 40.9, 54.9, 63.2, 74.8, 83.7, 85.7, 85.8, 86.7, 112.7, 117.9, 126.8, 127.7, 127.8, 129.7, 132.9, 135.1, 135.1, 144.0, 157.1, 158.4, 158.5, 170.0, 172.1.

³-O-Acetyl-2'-deoxy-N²-[dimethylamino)methylidene]-5-methylisocytidine (22) was prepared a) by the conventional method deprotecting the 5'-position with anh. HCl in MeOH, and b) by a method using ceric ammonium nitrate in wet MeCN [20]. The products obtained in both cases were identical.

Method a: To a soln. of 21 (1.12 mmol, 718 mg) in MeOH (2 ml), a 5% anh. HCl soln. in MeOH (0.2 ml) was added at 0 °C and the mixture was stirred for 3 min. Aq. NaHCO₃ soln. was added dropwise to pH 7, the mixture was extracted with AcOEt (3 × 20 ml), the extract dried (Na₂SO₄) and evaporated, and the residue purified by CC (17.5% MeOH/CHCl₃); 22 (241 mg, 64%). Colorless stars. TLC (17.5% MeOH/CHCl₃): Rf 0.46.

Method b: To a soln. of 21 (0.2 mmol, 128 mg) in MeCN containing 1% H₂O (10 ml), ceric ammonium nitrate (0.02 mmol, 11 mg) was added and the mixture stirred at r.t. for 5 min, then at reflux for 3.5 h (TLC: no starting material left). The solvent was evaporated and the residue purified by CC (17.5% MeOH/CHCl₃); 22 (28.9 mg, 43%). Light yellow stars. H-NMR (CDCl₃): 1.93 (s, Me–C(5)); 2.09 (s, Ac); 2.28–2.45 (m, 2 H–C(2)); 3.09, 3.13 (2x, Me₂N); 3.92–4.02 (m, 2 H–C(5)); 4.13 (m, H–C(4)); 5.37 (m, 1 H–C(3)); 6.75 (dd, H–C(1')); 7.90 (s, H–C(8)); 8.76 (s, N=CH). ³¹P-NMR (CDCl₃); 14.1, 21.0, 35.1, 35.2, 38.6, 41.1, 41.2, 62.1, 75.1, 85.3, 86.2, 86.2, 117.8, 134.2, 157.3, 158.7, 170.5, 172.7.

2'-Deoxy-5-methylisocytidine 5'-Triphosphate (2). Compound 22 (0.408 mmol, 138 mg) was co-evaporated with pyridine and then dissolved in anh. pyridine (0.6 ml) and anh. dioxane (1.75 ml). A soln. of 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (0.45 mmol, 91 mg) in dioxane (0.5 ml) was added (white precipitate). The mixture was stirred at r.t. for 15 min. Tributylammonium pyrophosphate (0.599 mmol, 273 mg) in DMF (1.5 ml) and tributylamine (1.76 mmol, 0.42 ml) were added, and the precipitate was dissolved within a few seconds. The soln. was stirred for another 10 min. Then I₂ (0.449 mmol, 114 mg) in pyridine (10 ml) and H₂O (0.2 ml) was added and the resulting soln. was stirred for 15 min. The reaction was quenched by addition of 5% Na₂SO₄ soln. The solvent was evaporated at r.t. and the residue dissolved in H₂O (7.5 ml) and stirred at r.t. for 30 min. Conc. aq. NH₄ soln. (35 ml) was added, and stirring was continued for 5 h. The solvent was again evaporated at r.t. and the residue purified by ion-exchange chromatography (DEAE Sephadex). The UV-active fractions were evaporated at 20 °C and further purified by reversed-phase HPLC to give pure 2 (42%), as calculated using an extinction coefficient of 260 nm of 6300 M⁻¹ cm⁻¹ [4]. UV (H₂O): 261. H-NMR (D₂O); 1.25 (t, Et₃N); 1.88 (s, Me–C(5)); 2.45 (m, 2 H–C(2)); 3.15 (q, Et₃N); 4.18 (m, 2 H–C(5)); 4.60 (m, H–C(4)); 5.95 (t, H–C(1')); 7.62 (s, H–C(6)). ³¹P-NMR (D₂O); 9.6 (Et₃N), 14.0, 39.1, 47.6, 65.9, 70.8, 86.3, 89.6, 116.0, 137.4, 155.4, 175.7, 177.6. Tris-HCl (pH 7.5) was added and the soln. was adjusted to pH 7.5. The mixture was extracted (30 ml). The combined org. layer was dried (Na₂SO₄) and evaporated, and the residue was purified by CC (17.5% MeOH/CHCl₃); 22 (241 mg, 64%). Colorless stars. TLC (17.5% MeOH/CHCl₃): Rf 0.46.

Enzymatic Incorporation. Triphosphates were tested for site-specific incorporation by primer-extension experiments as previously described [8]. The following deoxyoligonucleotides were synthesized by phosphoramidite chemistry [16].

Primer labeled at the 5' end with fluorescein: 5'-d(CAG GAA ACA GCT ATG AC)-3' (I).
Control template for me²isoC₃g incorporation: 5'-d(AAA AAA AGT CAT AGC TGT TTC CTG)-3' (II).
me²isoC₃g-Containing template: 5'-d(AAA A(me²isoC₃)A AGT CAT AGC TGT TTC CTG)-3' (III).
Control template for isoG₃ incorporation: 5'-d(CCC CCC CCC CCC CGT CAT AGC TGT TTC CTG)-3' (IV).
isoG₃-Containing template: 5'-d(CCC CCC (isoG₃)CC CCC CCC CGT CAT AGC TGT TTC CTG)-3' (V).

Assays were carried out after heating (95 °C, 2 min) primer I with template in buffer soln. (10 mm Tris · HCl, 5 mm MgCl₂, 7.5 mm DTT (dithiothreitol), pH 7.5 at 25 °C), followed by subsequent cooling of the solution to r.t. Solns. were incubated (37 °C, 20 min) with enzyme (2.5 U Klenow fragment), template (18 pmol), primer (15 pmol), and nucleoside triphosphates (20 μm standard nucleoside triphosphate, 200 μm p₂isoG₃ (I), or p₂me²isoC₃g (2)). All assays were quenched by adding NaOAc soln. (2 μl, 3 m, pH 5.2) and EtOH (60 μl). After
quenching, solns. were cooled for 1 h (– 80°). The DNA was collected by centrifugation and redissolved in H2O (5 μl). Loading buffer (5 – 6 μl, 97.5% formamide, 0.3% bromophenol blue, 0.3% xylene cyanol, 10 mM H4(едта), pH 8.0) was added, and the soln. was heated (2 min, 95°). Aliquots were loaded onto a polyacrylamide gel (20%, 8.3 M urea, 16 cm × 42 cm × 0.4 mm). Following electrophoresis (55 W), the gels were visualized with UV light using a Bio-Rad Gel Doc 1000.

Four solns. were incubated to comprise a single experiment for p3isoGd or p3me5isoCd: 1) Primer I in the absence of template; 2) primer I, control template II or IV, and appropriate standard triphosphate; 3) primer I, template III or V, appropriate standard triphosphate, and p3isoGd or p3me5isoCd; 4) primer I, template III or V, and appropriate standard triphosphate in the absence of p3isoGd or p3me5isoCd.

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