

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 (= p₃isoG_d) and 2'-deoxy-5-methylisocytidine (= p₃me⁵isoC_d), 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.

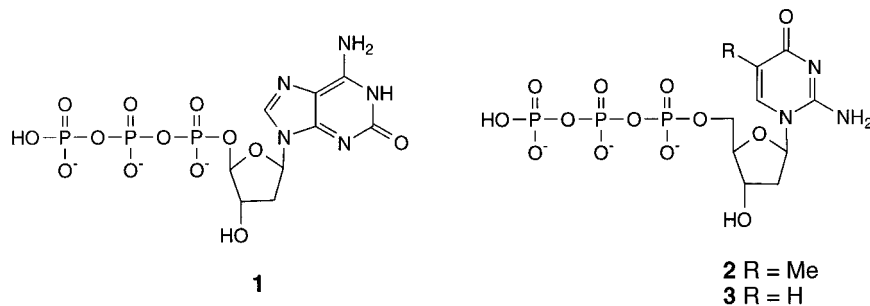
Introduction. – The *Watson-Crick* base pairing between two complementary oligonucleotide strands remains one of the most remarkable examples of molecular recognition. It follows two rules of complementarity: *i*) a large purine from one strand pairs with a small pyrimidine from the other; *ii*) H-bond 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

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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 ($p_3\text{isoG}_d$; **1**) and the first detailed synthetic procedure for 2'-deoxy-5-methylisocytidine ($p_3\text{me}^5\text{isoC}_d$, **2**), a more useful analog of $p_3\text{isoC}_d$ (**3**).

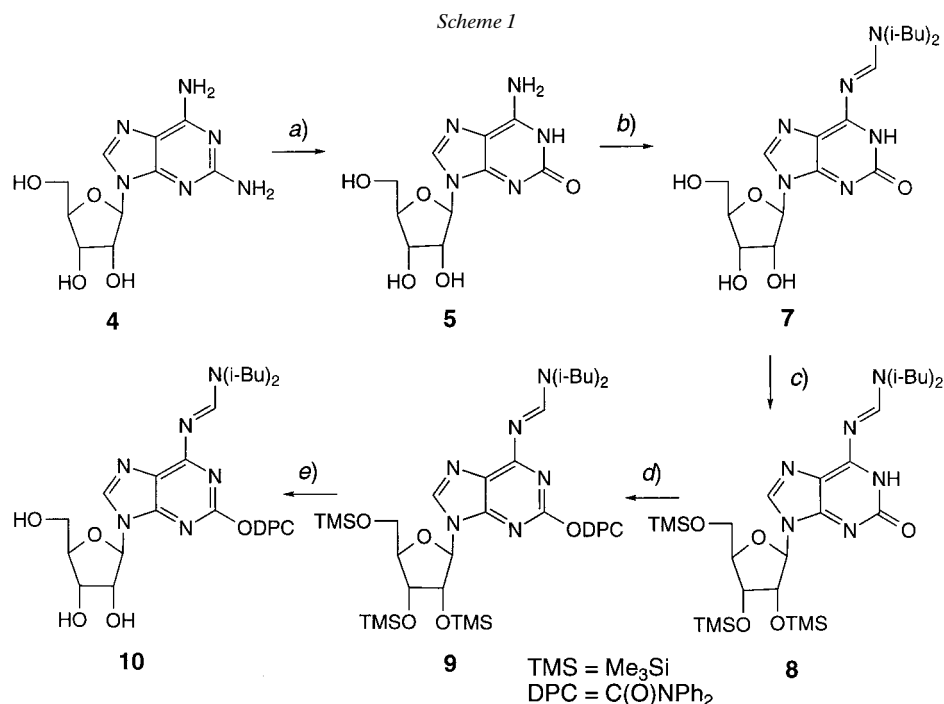


Results and Discussion. – *Deoxyisoguanosine Triphosphate.* The triphosphate **1** of 2'-deoxyisoguanosine has previously been synthesized by a photochemical procedure using 2'-deoxyadenosine triphosphate as the starting compound. However, the method gave $p_3\text{isoG}_d$ only in low yield along with inseparable impurities [8]. An alternative conversion of 2'-deoxyadenosine triphosphate to $p_3\text{isoG}_d$ using $[\text{Fe}^{\text{II}}\text{H}_6(\text{edta})]$ 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 isoG_d 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 POCl_3 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. Diphenylcarbamoyl 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

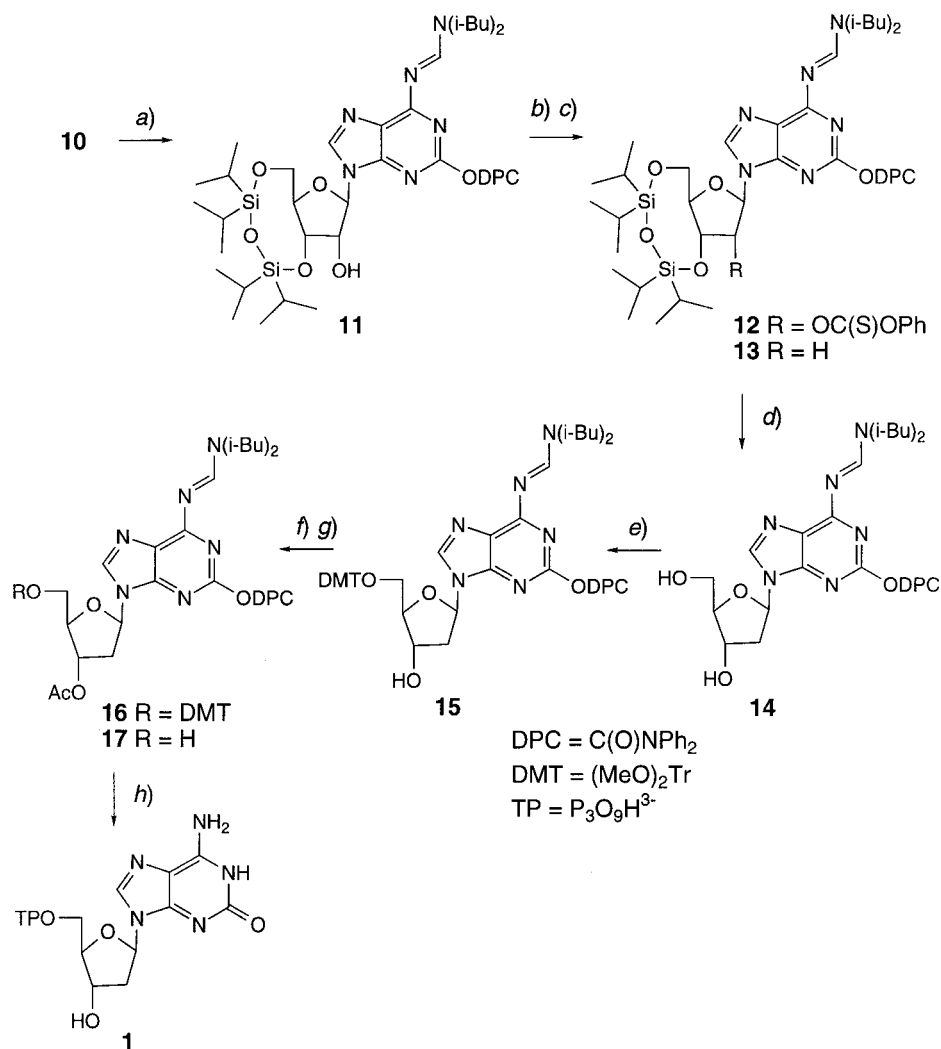
protected 2'-deoxyribose, 3'-*O*-acetyl-2'-deoxy-*O*²-(diphenylcarbamoyl)-*N*⁶-(diisobutylaminomethylidene)isoguanosine (**17**) was then obtained from **10** via **11–16** (Scheme 2). Synthesis of **17** from **5** comprises 11 steps with a reasonable overall yield (11%) and allows a cost-effective large-scale preparation of **1**. Moreover, intermediate **5** is easily purified by recrystallization, and not all intermediates require isolation.



a) AcOH, NaNO₂, H₂O; 90%. b) (i-Bu)₂NCH(OMe)₂ (**6**), DMF; 98%. c) Me₃SiCl, Et₃N, THF; 96%. d) Ph₂NC(O)Cl, pyridine, (i-Pr)₂NEt. e) TsOH, CH₂Cl₂/THF; 95% (yield for 2 steps).

Protected 2'-deoxyisoguanosine **17** was converted in a one-pot reaction to p₃isoG_d (**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 p₃isoG_d (**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 p₃isoG_d selectively (Fig., Lane 3), as the major product synthesized from

Scheme 2



a) Dichlorotetraisopropylsiloxane, pyridine; 93%. b) PhOC(S)Cl, pyridine, 4-(dimethylamino)pyridine (DMAP), CH₂Cl₂; 86%. c) Bu₃SnH, 2,2'-azobis[isobutyronitrile] (AIBN), toluene; 82%. d) KF dihydrate, [18]crown-6, THF; 70%. e) (MeO)₂TrCl, DMAP, pyridine; 49%. f) Ac₂O, DMAP, Et₃N, pyridine. g) HCl, MeOH, CHCl₃; 45% (yield for 2 steps). h) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, tributylammonium pyrophosphate, Bu₃N, I₂, NH₃; 17%.

an isoC_d-containing template in the presence of p₃isoG_d and standard triphosphate is full length (primer + 7). Elongation of the primer with the same isoC_d-containing template in the absence of p₃isoG_d yielded only aborted product (primer + 2), stopping cleanly opposite me⁵isoC_d in the template (Fig., Lane 5).



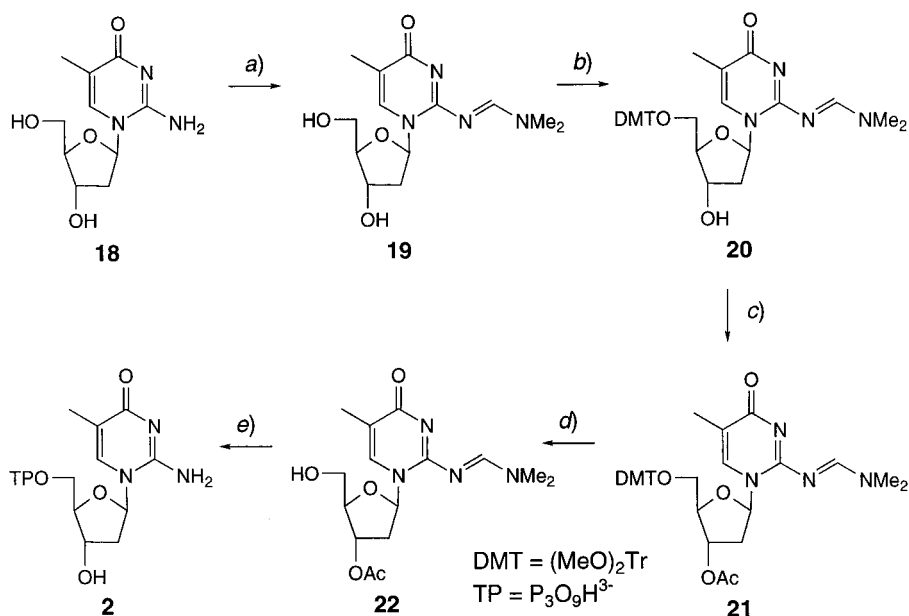
Fig. 1. *Primer-extension assay of p₃isoG_d (1) with 2.5 U of Klenow fragment at 37°*. Fluorescein-labeled primer **I** is extended in the presence of control template **II** and p₃T (Lane 2). Primer **I** is elongated to full-length product in the presence of me⁵isoC_d-containing template **III**, p₃T, and either p₃isoG_d (Lane 3) or a substituted p₃isoG_d from a related work (Lane 4) [18], but not in the presence of only template **III** and p₃T (Lane 5). Primer **I** alone is included for comparison (Lane 1).

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° , 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 p₃me⁵isoC_d (**2**) following the synthetic procedure [8] previously used in the synthesis of p₃isoC_d (**3**); however these authors reported no experimental procedures, yields, or product characterization.

Like p₃isoG_d (**1**), p₃me⁵isoC_d (**2**) was synthesized from the suitably protected nucleoside by the Ludwig-Eckstein method (Scheme 3). Formamidinium protection was chosen for the exocyclic N-atom of me⁵isoC_d (**18**) because 2'-deoxy-*N*²-[(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*²-[(dime-

Scheme 3



a) $Me_2NCH(OMe)_2$, DMF; 97%. *b*) $(MeO)_2TrCl$, DMAP, pyridine; 75%. *c*) Ac_2O , DMAP, Et_3N , pyridine; 89%. *d*) HCl , MeOH; 64%; or $(NH_4)_2Ce(NO_3)_6$, wet MeCN; 43%. *e*) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, tributylammonium pyrophosphate, Bu_3N , I_2 , NH_3 ; 42%.

thylamino)methylidene]-5-methylisocytidine (**22**) was synthesized in an additional 3 steps *via* **21**. Protected me^5isoC_d **22** was then converted to p_3,me^5isoC_d (**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 p_3,me^5isoC_d (**2**) was verified in a primer-extension experiment. Finally, unlike p_3isoC_d (**3**), **2** was stored at -20° for several months without detectable degradation. Therefore, p_3,me^5isoC_d (**2**) appears to be the triphosphate of choice for *in vitro* studies of the $isoC_d \cdot isoG_d$ base pair.

Experimental Part

General. Purine-2,6-diamine riboside and purine-2,6-diamine 2'-deoxyriboside were purchased from *RI Chemical, Inc.* (Orange, CA), 1,3-dichloro-1,1,3,3-tetraisopropylidisiloxane from *Farchan Laboratories, Inc.* (Gainesville, FL), DEAE *Sephadex* and tributylammonium pyrophosphate from *Sigma*, and all other chemicals from *Aldrich Chemical* or *Fisher Scientific*; all were used without further purification. *p*-Toluenesulfonyl chloride was freshly recrystallized from petroleum ether. Acetonitrile and pyridine were dried over 4-Å 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; *Fisher*). TLC: silica gel plates from *Whatman*; visualization by staining with a *Ce/Mo* reagent (2.5% phosphormolybdic acid, 1% $Ce^{IV}(SO_4)_2 \cdot 4H_2O$, 6% H_2SO_4 in H_2O) and heating. Ion-exchange chromatography: DEAE *Sephadex* equilibrated in 0.2M $(Et_3NH)HCO_3$ (pH 7.0); p_3isoG_d (**1**) eluted during a linear gradient to 0.5M $(Et_3NH)HCO_3$, and p_3,me^5isoC_d (**2**) during a linear gradient to 0.3M $(Et_3NH)HCO_3$. HPLC: *Waters-PrepLC-4000* system with a 486 tunable absorbance detector. Reversed-phase HPLC: *Waters-PrepLC-*

25-mm module containing a single *PrepPak* cartridge (*Prep Nova-Pak HR C₁₈* 6 μm 60 \AA , 25 \times 100 mm); purification of triphosphates: flow rate 5.1 ml/min; solvent A: 25 mM (Et_3N)OAc (pH 7.0); solvent B: 20% (v/v) MeCN in solvent A; linear binary gradient for **1** (t_{R} 37 min), 100% A (1 min) and 100% A to 90% A (39 min); for **2** (t_{R} 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 SiMe_4 , at 75.4 MHz (^{13}C) referenced to solvent, and at 121.4 MHz (^{31}P) with H_3PO_4 as standard in the solvents as given. MS: recorded by the Spectroscopy Services of the University of Florida Chemistry Department; *Finnigan MAT LCO* (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 \times 150 mm + guard) (Bellefonte, PA) and isocratically eluting (0.2 ml/min with 10 mM NH_4OAc and 87 mM AcOH in $\text{H}_2\text{O}/\text{MeCN}/i\text{-PrOH}$ 98 : 1.4 : 0.6) the triphosphates.

Isoguanosine (**5**). Purine-2,6-diamine riboside **4** (35.5 mmol, 10.0 g) was suspended in H_2O (250 ml) at 50°; and NaNO_2 (137 mmol, 9.44 g) in H_2O (60 ml) was added. Then AcOH (245 mmol, 14.1 ml) was added at 50° over 1.5 min. The resulting clear soln. was stirred for 3.5 min and then diluted with H_2O (150 ml), and conc. aq. NH_3 soln. was added to pH 8. The soln. was evaporated and the remaining solid washed with H_2O : **5** (9.03 g, 90%). Light yellow powder. $^1\text{H-NMR}$ ((D_6) DMSO): 3.50–3.74 (*m*, 2 H–C(5')); 3.98 (*m*, H–C(4')); 4.12 (*m*, H–C(3')); 4.54 (*m*, H–C(2')); 5.69 (*m*, H–C(1')); 7.97 (*s*, H–C(8)). $^{13}\text{C-NMR}$ ((D_6) DMSO): 61.9, 71.0, 73.2, 86.3, 87.9, 109.8, 138.4, 152.8, 153.6, 156.8.

N^6 -[(*Diisobutylamino*)methylidene]isoguanosine (**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 solvent evaporated. The residue was dried under vacuum at 50° with an oil pump: **7** (13.1 g, 98%). Light yellow foam. TLC ($\text{CHCl}_3/\text{MeOH}$ 82.5:17.5): R_f 0.38. $^1\text{H-NMR}$ ((D_6) DMSO): 0.81–0.98 (*m*, 2 Me_2CHCH_2); 1.97–2.19 (*m*, 2 Me_2CHCH_2); 3.28–3.50 (*m*, 2 Me_2CHCH_2); 3.60–3.78 (*m*, 2 H–C(5')); 4.07 (*m*, H–C(4')); 4.25 (*m*, H–C(3')); 4.59 (*m*, H–C(2')); 5.79 (*m*, H–C(1')); 8.18 (*s*, H–C(8)); 9.21 (*s*, N=CH). $^{13}\text{C-NMR}$ ((D_6) DMSO): 20.2, 20.6, 26.8, 27.5, 53.2, 60.6, 62.3, 71.3, 74.1, 86.6, 88.5, 114.7, 141.9, 155.6, 157.4, 157.7, 163.1.

N^6 -[(*Diisobutylamino*)methylidene]-2',3',5'-tris-*O*-(trimethylsilyl)isoguanosine (**8**). Compound **7** (31.1 mmol, 13.1 g) was co-evaporated with pyridine and dissolved in anhyd. CH_2Cl_2 (130 ml). Under ice-bath cooling, Et_3N (374 mmol, 51.9 ml) and Me_3SiCl (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. NaHCO_3 soln., the aq. phase further extracted with CHCl_3 and the combined org. phase dried (Na_2SO_4) and evaporated. The resulting light yellow foam (19.1 g, 96%) was used in the next step without further purification. TLC ($\text{CHCl}_3/\text{MeOH}$ 9 : 1): R_f 0.48. $^1\text{H-NMR}$ (CDCl_3): 0.10, 0.14, 0.18 (3*s*, 3 Me_3Si); 0.91–1.10 (*m*, 2 Me_2CHCH_2); 2.03–2.32 (*m*, 2 Me_2CHCH_2); 3.32–3.61 (*m*, 2 Me_2CHCH_2); 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')); 6.07 (*m*, H–C(1')); 8.04 (*s*, H–C(8)); 9.41 (*s*, N=CH). $^{13}\text{C-NMR}$ (CDCl_3): –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.

N^6 -[(*Diisobutylamino*)methylidene]-*O*²-(diphenylcarbamoyl)-2',3',5'-tris-*O*-(trimethylsilyl)isoguanosine (**9**). Compound **8** (13.8 mmol, 8.80 g) was co-evaporated with pyridine and dissolved in anhyd. pyridine (70 ml). *N,N*-Diisopropylethylamine (55.2 mmol, 9.6 ml), DMAP (3.45 mmol, 421 mg), and diphenylcarbamoyl chloride (27.6 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 $\text{CHCl}_3/\text{dil. aq. NaHCO}_3$ soln. The org. layer was dried (Na_2SO_4) and evaporated, and the residue was used in the next step without further purification. A small sample was purified by CC (1.5% $\text{MeOH}/\text{CH}_2\text{Cl}_2$) to give **9** as a yellow foam. $^1\text{H-NMR}$ (CDCl_3): 0.10, 0.14, 0.18 (3*s*, 3 Me_3Si); 0.93 (*dd*, 2 Me_2CHCH_2); 1.93–2.20 (*m*, 2 Me_2CHCH_2); 3.18 (*d*, 1 Me_2CHCH_2); 3.47–3.64 (*m*, 1 Me_2CHCH_2); 3.70–4.05 (2*m*, 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). $^{13}\text{C-NMR}$ (CDCl_3): –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.

N^6 -[(*Diisobutylamino*)methylidene]-*O*²-(diphenylcarbamoyl)isoguanosine (**10**). Unpurified **9** (directly from the previous reaction) was dissolved in CH_2Cl_2 (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 Et_3N (82.8 mmol, 11.5 ml) and extracted with $\text{CHCl}_3/\text{dil. aq. NaHCO}_3$ soln. The org. phase was dried (Na_2SO_4) and evaporated, and the residue was purified by CC (10% $\text{MeOH}/\text{CHCl}_3$; R_f 0.27); **10** (8.08 g, 95% for 2 steps). Light yellow foam. $^1\text{H-NMR}$ (CDCl_3): 0.88 (*m*, 2 Me_2CHCH_2); 1.83–2.17 (*m*, 2 Me_2CHCH_2); 3.18 (*m*, 1 Me_2CHCH_2); 3.37–3.58 (*m*, 1 Me_2CHCH_2); 3.63–3.80 (2*m*, 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.

N⁶-[(*Diisobutylamino*)methylidene]-O²-(*diphenylcarbamoyl*)-3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl)isoguanosine (**11**). Compound **10** (39.5 mmol, 24.4 g) was co-evaporated with pyridine and then dissolved in anhyd. pyridine (250 ml). Dichlorotetraisopropylidisiloxane (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 (CHCl₃, then 10% MeOH/CHCl₃, *R*_f 0.77); (**11**, 31.6 g, 93%). Light yellow foam. ¹H-NMR (CDCl₃): 0.90–1.19 (*m*, 4 Me₂CH, 2 Me₂CHCH₂); 1.93–2.20 (*m*, 2 Me₂CHCH₂); 3.19 (*d*, 1 Me₂CHCH₂); 3.57 (*m*, 1 Me₂CHCH₂); 4.03–4.12 (*m*, 2 H–C(5'), H–C(4')); 4.49 (*m*, H–C(3')); 4.78 (*m*, H–C(2')); 6.00 (*m*, H–C(1')); 7.18–7.40 (*m*, 2 Ph); 7.99 (*s*, H–C(8)); 8.98 (*s*, N=CH). ¹³C-NMR (CDCl₃): 12.5, 17.3, 19.8, 20.1, 26.2, 27.1, 52.7, 60.0, 61.9, 71.0, 75.1, 82.0, 88.9, 89.0, 123.7, 125.0, 126.2, 128.9, 140.0, 140.1, 142.3, 149.8, 152.0, 156.2, 159.6, 161.4.

N⁶-[(*Diisobutylamino*)methylidene]-O²-(*diphenylcarbamoyl*)-2'-O-(*phenoxythiocarbonyl*)-3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diisoguanosine (**12**). Compound **11** (36.8 mmol, 31.6 g) was co-evaporated with pyridine and then dissolved in CH₂Cl₂ (400 ml). Pyridine (147 mmol, 11.9 ml), DMAP (9.19 mmol, 1.11 g), and *O*-phenyl carbonochloridithioate (55.1 mmol, 7.62 ml) were added. The mixture turned brown-red and was stirred at r.t. for 19 h. MeOH (5 ml) was added, the mixture was evaporated, and the residue purified by CC (one-step gradient: 1.5% MeOH/CH₂Cl₂, then 10% MeOH/CH₂Cl₂); **12** (31.6 g, 86%). Light yellow foam. ¹H-NMR (CDCl₃): 0.87–0.96 (*m*, 4 Me₂CH, 2 Me₂CHCH₂); 1.92–2.20 (*m*, 2 Me₂CHCH₂); 3.18 (*m*, 1 Me₂CHCH₂); 3.54 (*m*, 1 Me₂CHCH₂); 4.00–4.28 (*m*, 2 H–C(5'), H–C(4')); 4.95 (*m*, H–C(3')); 6.26 (*m*, H–C(2'), H–C(1')); 7.06–7.45 (*m*, 3 Ph); 8.06 (*s*, H–C(8)), 8.96 (*s*, N=CH). ¹³C-NMR (CDCl₃): 12.7, 12.8, 12.9, 13.2, 17.0, 17.2, 19.8, 20.0, 26.2, 27.1, 52.7, 60.0, 60.7, 69.7, 82.1, 83.9, 86.4, 115.5, 121.4, 121.7, 124.8, 126.5, 126.9, 128.9, 129.4, 139.5, 142.2, 152.0, 153.4, 156.4, 159.5, 161.4, 193.6.

2'-Deoxy-N⁶-[(*diisobutylamino*)methylidene]-O²-(*diphenylcarbamoyl*)-3'-5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl)isoguanosine (**13**). Compound **12** (31.8 mmol, 31.6 g) was co-evaporated with toluene, dissolved in anhyd. toluene (200 ml), and degassed with Ar for 30 min. In a second flask, AIBN (2,2'-azobis[isobutyronitrile]; 15.9 mmol, 2.61 g) and tributylstannane (95.4 mmol, 25.7 ml) in toluene (50 ml) were degassed with Ar for 30 min. The first mixture was then heated to 110°, and the AIBN/Bu₃SnH soln. was added dropwise *via* syringe. The mixture was stirred at 100° for 3.5 h. Gas evolution ceased after 20 min. After cooling to r.t., the mixture was concentrated in vacuum and extracted with dil. aq. NaHCO₃/CHCl₃ soln., the org. layer dried (Na₂SO₄) and evaporated, and the residue purified by CC (CHCl₃, then 5% MeOH/CHCl₃; *R*_f 0.79); **13** (22.0 g, 82%). Light yellow foam. ¹H-NMR (CDCl₃): 0.78–1.11 (*m*, 4 Me₂CH, 2 Me₂CHCH₂); 1.82–2.21, 2.52–2.66 (*m*, 2 Me₂CHCH₂, 2 H–C(2')); 3.02–3.58 (*m*, 2 Me₂CHCH₂); 3.71–4.08 (*m*, 2 H–C(5'), H–C(4')); 4.72 (*m*, H–C(3')); 6.29 (*m*, H–C(1')); 7.04–7.56 (*m*, 2 Ph); 8.16 (*s*, H–C(8)); 8.90 (*s*, N=CH). ¹³C-NMR (CDCl₃): 12.4, 12.7, 12.9, 13.2, 16.9, 17.0, 17.2, 17.4, 19.6, 19.7, 27.0, 40.1, 52.7, 59.8, 61.5, 69.5, 82.9, 85.0, 119.4, 125.8, 126.5, 127.1, 128.8, 139.7, 142.0, 151.4, 152.2, 156.1, 159.3, 161.1.

2'-Deoxy-N⁶-[(*diisobutylamino*)methylidene]-O²-(*diphenylcarbamoyl*)isoguanosine (**14**). Compound **13** (26.1 mmol, 22.0 g) was dissolved in THF (200 ml), and KF dihydrate (156 mmol, 14.7 g) and [18]crown-6 (7.82 mmol, 2.07 g) were added. The mixture was vigorously stirred for 2 h and then filtered over *Celite*, and the filtrate was evaporated. The residue was diluted with H₂O (50 ml) and extracted with CHCl₃. The org. layer was dried (Na₂SO₄) and evaporated and the residue purified by CC (CHCl₃, then 10% MeOH/CHCl₃; *R*_f 0.23); **14** (11.0 g, 70%). Light yellow foam. ¹H-NMR (CDCl₃): 0.81–1.02 (*m*, 2 Me₂CHCH₂); 1.86–2.18 (*m*, 2 Me₂CHCH₂); 2.20 (*m*, 1 H–C(2')); 2.62 (*m*, 1 H–C(2')); 3.12 (*d*, 1 Me₂CHCH₂); 3.43 (*m*, 1 Me₂CHCH₂); 3.75–3.98 (*m*, 2 H–C(5')); 4.09 (*m*, H–C(4')); 4.59 (*m*, H–C(3')); 5.92 (*m*, H–C(1')); 7.08–7.50 (*m*, 2 Ph); 7.74 (*s*, H–C(8)); 8.92 (*s*, N=CH). ¹³C-NMR (CDCl₃): 19.6, 20.1, 26.0, 27.0, 40.7, 52.4, 59.8, 63.1, 72.6, 72.7, 87.3, 88.5, 125.4, 126.6, 126.6, 128.8, 141.8, 141.9, 150.9, 152.3, 154.8, 159.6, 161.3.

2'-Deoxy-N⁶-[(*diisobutylamino*)methylidene]-5'-O-(4,4'-dimethoxytrityl)-O²-(*diphenylcarbamoyl*)isoguanosine (**15**). Compound **14** (13.7 mmol, 8.26 g) was co-evaporated with pyridine and dissolved in anhyd. pyridine (200 ml). DMAP (3.44 mmol, 419 mg) and (MeO)₂Tr-Cl (17.2 mmol, 6.12 g) were added at 0°, and the mixture was stirred at this temp. for 30 min and then at r.t. for 15 h. The reaction was quenched by addition of MeOH (5 ml), the mixture was concentrated *in vacuo* and extracted with dil. aq. NaHCO₃ soln./CHCl₃. The combined org. phase was dried (Na₂SO₄) and evaporated and the residue purified by CC (hexanes/AcOEt/CHCl₃/Et₃N 20:35:35:1, then AcOEt/MeOH/Et₃N 80:2:1; *R*_f 0.57); **15** (6.13 g, 49%). Light-yellow foam. ¹H-NMR (CDCl₃): 0.90 (*dd*, 2 Me₂CHCH₂); 1.91–2.18 (*m*, 2 Me₂CHCH₂); 2.51 (*m*, 2 H–C(2')); 3.18 (*d*, 1 Me₂CHCH₂); 3.30 (*m*, 1 Me₂CHCH₂); 3.54 (*m*, 2 H–C(5')); 3.72 (*s*, 2 MeO); 4.09 (*m*, H–C(4')); 4.54 (*m*, H–C(3')); 6.47

(*dd*, H–C(1')); 6.71–6.84 (*m*, 4 H, (MeO)₂Tr); 7.11–7.40 (*m*, 19 H, Ph, (MeO)₂Tr); 7.97 (*s*, H–C(8)); 8.97 (*s*, N=CH). ¹³C-NMR (CDCl₃): 19.7, 20.0, 26.1, 27.0, 40.9, 52.6, 55.0, 55.0, 59.9, 63.8, 71.7, 71.8, 83.5, 83.6, 85.9, 86.3, 113.0, 124.4, 126.3, 126.7, 126.8, 127.6, 127.7, 127.7, 128.0, 128.8, 129.0, 129.8, 129.9, 135.5, 135.6, 139.6, 142.1, 144.5, 152.2, 152.3, 156.0, 158.3, 158.4, 159.5, 161.2.

3'-O-Acetyl-2'-deoxy-N⁶[(diisobutylamino)methylidene]-5'-O-(4,4'-dimethoxytrityl)-O²-(diphenylcarbamoyl)-isoguanosine (**16**). Compound **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 soln. 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 (*R*_f 0.78 5% MeOH/CHCl₃) used in the next step without further purification.

3'-O-Acetyl-2'-deoxy-N⁶[(diisobutylamino)methylidene]-O²-(diphenylcarbamoyl)isoguanosine (**17**). Unpurified **16** (directly from the previous reaction) was dissolved in a soln. of MeOH (5 ml) and CHCl₃ (1 ml). The soln. was cooled to 0°, and 10% anhyd. HCl in MeOH (5 ml) was added. Stirring was continued at 0° 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; *R*_f 0.59): **17** (290 mg, 45% for 2 steps). Pale yellow foam. ¹H-NMR (CDCl₃): 0.87–0.98 (*m*, 2 Me₂CHCH₂); 2.11 (*s*, Ac); 1.93–2.42 (*m*, 2 Me₂CHCH₂, 2 H–C(2')); 3.19, 3.54 (*2m*, 2 Me₂CHCH₂); 3.90 (*m*, 2 H–C(5')); 4.11 (*m*, H–C(4')); 5.53 (*m*, H–C(3')); 6.24 (*dd*, H–C(1')); 7.16–7.50 (*m*, 2 Ph); 7.94 (*s*, H–C(8)); 8.97 (*s*, N=CH). ¹³C-NMR (CDCl₃): 19.6, 19.9, 20.8, 26.0, 26.9, 37.4, 52.6, 59.9, 62.8, 76.3, 86.8, 86.9, 125.6, 126.3 (*br*), 128.8, 141.1, 142.0, 151.3, 151.8, 155.3, 159.5, 161.5, 170.1.

2'-Deoxyisoguanosine 5'-Triphosphate (**1**). Compound **17** (0.170 mmol, 109 mg) was co-evaporated with pyridine and dissolved in anhyd. pyridine (0.17 ml) and anhyd. dioxane (0.51 ml). A soln. 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 (0.261 mmol, 119 mg) in DMF (0.6 ml) and tributylamine (0.71 mmol, 0.17 ml) were added, immediately forming a clear soln. The soln. was stirred for 10 min, then a 1% soln. of I₂ in pyridine/H₂O 98:2 was added (3.74 ml). Stirring was continued for 15 min, and the excess I₂ was destroyed by addition of 5% aq. Na₂SO₃ soln. H₂O (6 ml) was added and the mixture stirred for 30 min. Then 25% aq. NH₃ 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 **1**, calculated with an extinction coefficient at 292 nm of 1.1 · 10⁴ m⁻¹ cm⁻¹ [14]. UV (H₂O): 247, 292. ¹H-NMR (D₂O): 1.09 (*t*, Et₃N); 2.33 (*m*, 1 H–C(2')); 2.49 (*m*, 1 H–C(2')); 3.00 (*q*, Et₃N); 4.00 (*m*, 2 H–C(5')); H–C(3') and H–C(4') superimposed by D₂O; 6.09 (*t*, H–C(1')); 8.00 (*s*, H–C(8)). ³¹P-NMR (D₂O): –07.04 (*d*); –8.5 (*d*); –20.73 (*t*). HPLC/ESI-MS (*pos. mode*): 508 ([*M* + H]⁺), 525 ([*M* + NH₄]⁺). HPLC/ESI-MS (*neg. mode*): 506 ([*M* – H][–]), 525 ([(*M* – H + Na) – H][–]).

2'-Deoxy-N²[(dimethylamino)methylidene]-5-methylisocytidine (**19**). To a soln. of **18** (2.37 mmol, 571 mg) in anhyd. DMF (14 ml), dimethylformamide dimethyl acetal (30.2 mmol, 3.59 g, 4.00 ml) was added. The soln. 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; *R*_f 0.35): 677 mg (97%) of **19**. White solid product. ¹H-NMR (CD₃OD): 2.16 (*s*, Me–C(5)); 2.08–2.19 (*m*, 1 H–C(2')); 2.33–2.42 (*m*, 1 H–C(2')); 3.13, 3.21 (*2s*, Me₂N); 3.73–3.87 (*m*, 2 H–C(5')); 3.96 (*m*, H–C(4')); 4.39 (*m*, H–C(3')); 6.71 (*t*, H–C(1')); 8.03 (*s*, H–C(6)); 8.62 (*s*, N=CH). ¹³C-NMR (CD₃OD): 14.0, 35.5, 41.5, 42.3, 62.7, 71.9, 88.1, 89.2, 117.5, 137.0, 159.1, 160.0, 175.0

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-N²-(dimethylaminomethylidene)-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); *R*_f 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): **20** (1.03 g, 75%). White foam. ¹H-NMR (CDCl₃): 1.58 (*s*, Me–C(5)); 2.20–2.31 (*m*, 1 H–C(2')); 2.48–2.58 (*m*, 1 H–C(2')); 2.98, 3.02 (*2s*, Me₂N); 3.33–3.48 (*m*, 2 H–C(5')); 3.73 (*s*, 2 MeO); 4.19 (*m*, H–C(4')); 4.62 (*m*, H–C(3')); 6.76–6.86 (*m*, 5 H–C(1'), (MeO)₂Tr); 7.14–7.35, 7.42 (*m, d*, 9 H, (MeO)₂Tr); 7.73 (*s*, H–C(6)); 8.69 (*s*, N=CH). ¹³C-NMR (CDCl₃): 13.4, 13.4, 34.9, 35.0, 40.8, 40.9, 41.7, 55.0, 63.6, 71.4, 71.4, 86.2, 86.3, 86.4, 112.9, 117.3, 126.7, 127.7, 127.9, 129.9, 134.0, 135.4, 135.5, 144.3, 157.2, 158.3, 172.6.

3'-O-Acetyl-2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-N²-(dimethylamino)methylidene]-5-methylisocytidine (**21**). Compound **20** (1.71 mmol, 1.03 g) was dissolved in pyridine (20 ml). DMAP (0.43 mmol, 52 mg), Et₃N

(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; R_f 0.68): **21** (0.974 g, 89%). White foam. ¹H-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 (2s, 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)₂Tr); 7.18–7.37, 7.43 (m, d, 9 H, (MeO)₂Tr); 7.70 (s, H–C(6)); 8.82 (s, N=CH). ¹³C-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°, 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): R_f 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. ¹H-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 (2s, 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(6)); 8.76 (s, N=CH). ¹³C-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% Na₂SO₃ 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. NH₃ 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° and further purified by reversed-phase HPLC to give pure **2** (42%), as calculated using an extinction coefficient at 260 nm of 6300 M⁻¹ cm⁻¹ [4]. UV (H₂O): 261. ¹H-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')), H–C(4')); 4.60 (m, H–C(3')); 5.95 (t, H–C(1')); 7.62 (s, H–C(6)). ¹³C-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. ³¹P-NMR (D₂O): –6.53, –6.69 (d, J = 19.3); –9.24, –9.40 (d, J = 19.2), –20.05 (dd). HPLC/ESI-MS (pos. mode): 482 ([M + H]⁺, 499 ([M + NH₄]⁺). HPLC/ESI-MS (neg. mode): 480 ([M – H]⁻), 502 [(M – H + Na) – H]⁻.

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 me⁵isoC_d incorporation: 5'-d(AAA AAA AGT CAT AGC TGT TTC CTG)-3' (**II**).

me⁵isoC_d-Containing template: 5'-d(AAA A(me⁵isoC)A AGT CAT AGC TGT TTC CTG)-3' (**III**).

Control template for isoG_d incorporation: 5'-d(CCC CCC CCC CCC CGT CAT AGC TGT TTC CTG)-3' (**IV**).

isoG_d-Containing template: 5'-d(CCC CCC (isoG)CC CCC CGT CAT AGC TGT TTC CTG)-3' (**V**).

Assays were carried out after heating (95°, 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°), followed by subsequent cooling of the solution to r.t. Solns. were incubated (37°, 20 min) with enzyme (2.5 U *Klenow* fragment), template (18 pmol), primer (15 pmol), and nucleoside triphosphate(s) (20 μM standard nucleoside triphosphate, 200 μM p₃isoG_d (**I**), or p₃me⁵isoC_d (**2**)). All assays were quenched by adding NaOAc soln. (2 μl, 3M, 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 H_2O (5 μ l). Loading buffer (5–6 μ l, 97.5% formamide, 0.3% bromophenol blue, 0.3% xylene cyanol, 10 mM $H_4(edta)$, pH 8.0) was added, and the soln. was heated (2 min, 95°). Aliquots were loaded onto a polyacrylamide gel (20%, 8.3M urea, 16 cm \times 42 cm \times 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 p_3isoG_d or $p_3me^5isoC_d$: 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 p_3isoG_d or $p_3me^5isoC_d$; 4) primer **I**, template **III** or **V**, and appropriate standard triphosphate in the absence of p_3isoG_d or $p_3me^5isoC_d$.

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