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_3isoG_d) and 2′-deoxy-5-methylisocytidine (= $p_3me^5isoC_d$), 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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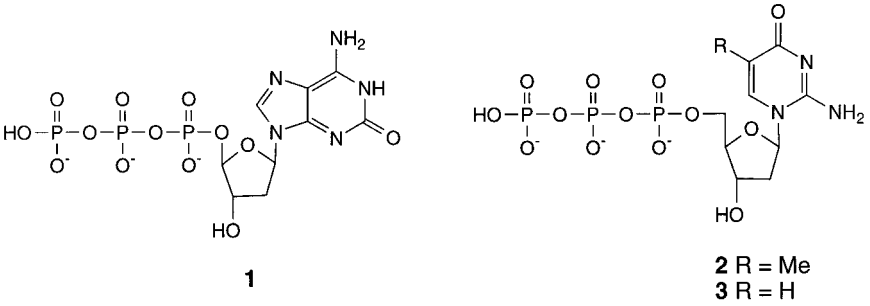
activities of the resulting oligonucleotides. Indeed, increasing the diversity of oligonucleotide functionality has already proved effective in the *in vitro* selection experiments of *Tarasow et al.* [9] using a 5-substituted deoxyuridine. Key to such selection experiments is the ready availability 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_3isoG_d; **1**) and the first detailed synthetic procedure for 2′-deoxy-5-methylisocytidine ($p_3me^5isoC_d$, **2**), a more useful analog of p_3isoC_d (**3**).



1

2 R = Me
3 R = H

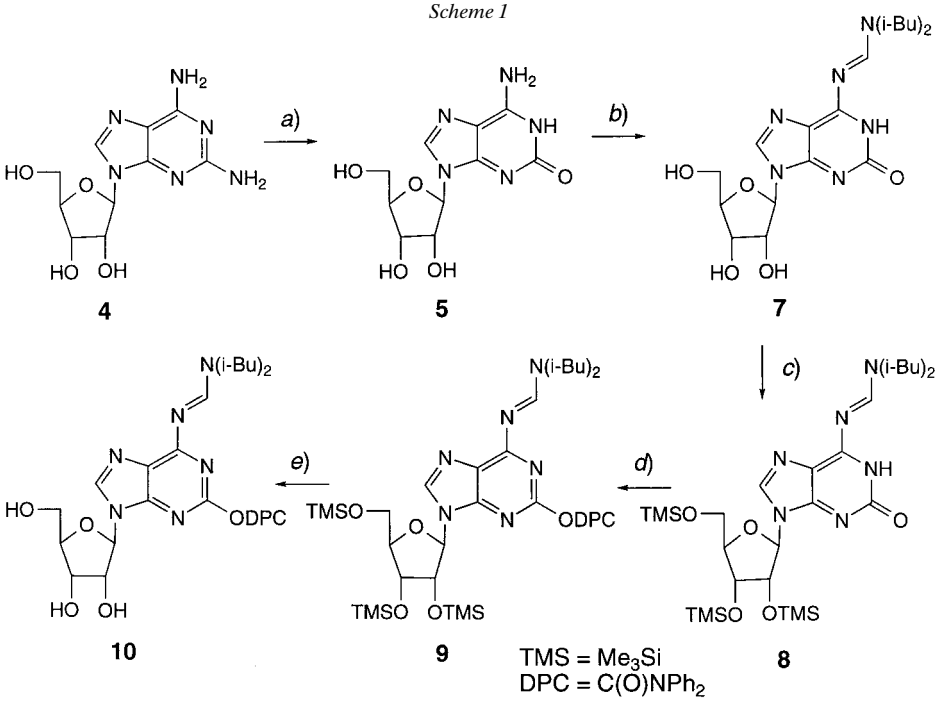
Results and Discussion. – *Deoxyisoguanosine Triphosphate.* The triphosphate **1** of 2′-deoxyisoguanosine has previously been synthesized by a photochemical procedure using 2′-deoxyadenosine triphosphate as the starting compound. However, the method gave p_3isoG_d only in low yield along with inseparable impurities [8]. An alternative conversion of 2′-deoxyadenosine triphosphate to p_3isoG_d using $[Fe^{II}H_e(edta)]$ 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′-deoxyriboside, 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.

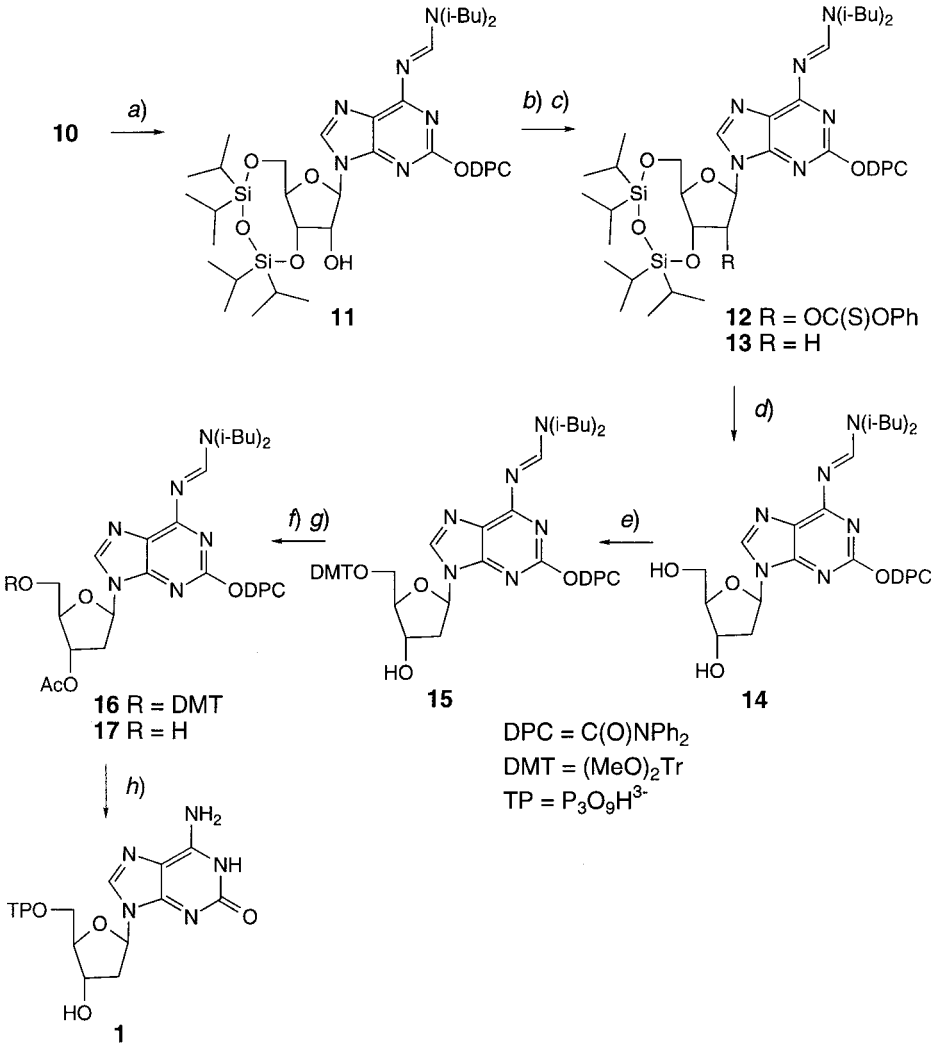
Scheme 1



a) AcOH, $NaNO_2$, H_2O; 90%. *b*) $(i\text{-Bu})_2NCH(OMe)_2$ (**6**), DMF; 98%. *c*) Me_3SiCl, Et_3N, THF; 96%. *d*) $Ph_2NC(O)Cl$, pyridine, $(i\text{-Pr})_2NEt$. *e*) TsOH, CH_2Cl_2/THF; 95% (yield for 2 steps).

Protected 2′-deoxyisoguanosine **17** was converted in a one-pot reaction to p_3isoG_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 I_2/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 p_3isoG_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 p_3isoG_d selectively (Fig., *Lane 3*), as the major product synthesized from

Scheme 2



a) Dichlorotetraisopropyldisiloxane, pyridine; 93%. *b*) PhOC(S)Cl, pyridine, 4-(dimethylamino)pyridine (DMAP), CH_2Cl_2; 86%. *c*) Bu_3SnH, 2,2′-azobis[isobutyronitrile] (AIBN), toluene; 82%. *d*) KF dihydrate, [18]crown-6, THF; 70%. *e*) $(MeO)_2TrCl$, DMAP, pyridine; 49%. *f*) Ac_2O, DMAP, Et_3N, pyridine. *g*) HCl, MeOH, $CHCl_3$; 45% (yield for 2 steps). *h*) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, tributylammonium pyrophosphate, Bu_3N, I_2, NH_3; 17%.

an $isoC_d$-containing template in the presence of p_3isoG_d and standard triphosphate is full length (primer + 7). Elongation of the primer with the same $isoC_d$-containing template in the absence of p_3isoG_d yielded only aborted product (primer + 2), stopping cleanly opposite me^5isoC_d in the template (Fig., *Lane 5*).

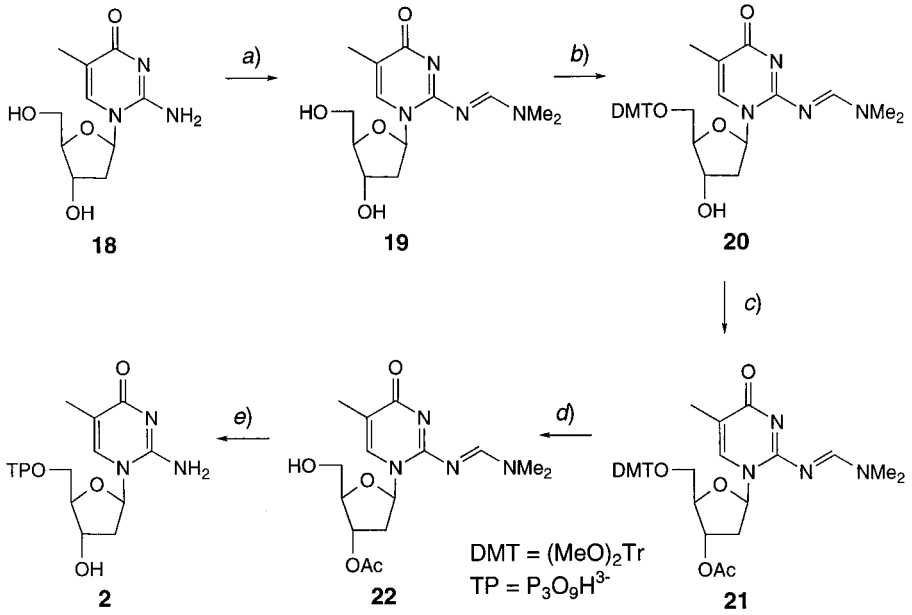
Fig. 1. *Primer-extension assay of p_3isoG_d* (**1**) *with 2.5 U of* Klenow *fragment at 37°*. Fluorescein-labeled primer **I** is extended in the presence of control template **II** and p_3T (*Lane 2*). Primer **I** is elongated to full-length product in the presence of me^5isoC_d-containing template **III**, p_3T, and either p_3isoG_d (*Lane 3*) or a substituted p_3isoG_d from a related work (*Lane 4*) [18], but not in the presence of only template **III** and p_3T (*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 $p_3me^5isoC_d$ (**2**) following the synthetic procedure [8] previously used in the synthesis of p_3isoC_d (**3**); however these authors reported no experimental procedures, yields, or product characterization.

Like p_3isoG_d (**1**), $p_3me^5isoC_d$ (**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 me^5isoC_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 $p_3me^5isoC_d$ (**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 $p_3me^5isoC_d$ (**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, $p_3me^5isoC_d$ (**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-tetraisopropyldisiloxane 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 $p_3me^5isoC_d$ (**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 C_{18}* 6 μm 60 Å, 25 × 100 mm); purification of triphosphates: flow rate 5.1 ml/min; solvent *A:* 25 mM (Et_3NH)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 (^{1}H) 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 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 NH_4OAc and 87 mM AcOH in H_2O/MeCN/i-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}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}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 ($CHCl_3$/MeOH 82.5 : 17.5): R_f 0.38. ^{1}H-NMR ((D$_6$)DMSO): 0.81–0.98 (*m*, 2 *Me*$_2$CHCH$_2$); 1.97–2.19 (*m*, 2 Me$_2$C*H*CH$_2$); 3.28–3.50 (*m*, 2 Me$_2$CHC*H*$_2$); 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}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 anh. 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 ($CHCl_3$/MeOH 9 : 1): R_f 0.48. ^{1}H-NMR ($CDCl_3$): 0.10, 0.14, 0.18 (3*s*, 3 Me_3Si); 0.91–1.10 (*m*, 2 *Me*$_2$CHCH$_2$); 2.03–2.32 (*m*, 2 Me$_2$C*H*CH$_2$); 3.32–3.61 (*m*, 2 Me$_2$CHC*H*$_2$); 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}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 anh. 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 $CHCl_3$/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% MeOH/CH_2Cl_2) to give **9** as a yellow foam. ^{1}H-NMR ($CDCl_3$):-0.10, 0.14, 0.18 (3*s*, 3 Me_3Si); 0.93 (*dd*, 2 *Me*$_2$CHCH$_2$); 1.93–2.20 (*m*, 2 Me$_2$C*H*CH$_2$); 3.18 (*d*, 1 Me$_2$CHC*H*$_2$); 3.47–3.64 (*m*, 1 Me$_2$CHC*H*$_2$); 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}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 $CHCl_3$/dil. aq. $NaHCO_3$ soln. The org. phase was dried (Na_2SO_4) and evaporated, and the residue was purified by CC (10% MeOH/$CHCl_3$; R_f 0.27); **10** (8.08 g, 95% for 2 steps). Light yellow foam. ^{1}H-NMR ($CDCl_3$): 0.88 (*m*, 2 *Me*$_2$CHCH$_2$); 1.83–2.17 (*m*, 2 Me$_2$C*H*CH$_2$); 3.18 (*m*, 1 Me$_2$CHC*H*$_2$); 3.37–3.58 (*m*, 1 Me$_2$CHC*H*$_2$); 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). ^{13}C-NMR ($CDCl_3$): 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.

N^6-*[(Diisobutylamino)methylidene]*-O^2-*(diphenylcarbamoyl)-3′,5′*-O-*(1,1,3,3-tetraisopropyldisiloxane-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). Dichlorotetraisopropyldisiloxane (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. $NaHCO_3$ soln. was added, the solution extracted with $CHCl_3$, the org. layer dried (Na_2SO_4) and evaporated, and the residue purified by CC ($CHCl_3$, then 10% MeOH/$CHCl_3$, R_f 0.77); (**11**, 31.6 g, 93%). Light yellow foam. ^{1}H-NMR ($CDCl_3$): 0.90–1.19 (*m*, 4 *Me*$_2$C*H*, 2 *Me*$_2$CHCH$_2$); 1.93–2.20 (*m*, 2 Me$_2$C*H*CH$_2$); 3.19 (*d*, 1 Me$_2$CHC*H*$_2$); 3.57 (*m*, 1 Me$_2$CHC*H*$_2$); 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). ^{13}C-NMR ($CDCl_3$): 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.

N^6-*[(Diisobutylamino)methylidene]*-O^2-*(diphenylcarbamoyl)-2′*-O-*(phenoxythiocarbonyl)-3′,5′*-O-*(1,1,3,3-tetraisopropyldisiloxane-1,3-diisoguanosine* (**12**). Compound **11** (36.8 mmol, 31.6 g) was co-evaporated with pyridine and then dissolved in CH_2Cl_2 (400 ml). Pyridine (147 mmol, 11.9 ml), DMAP (9.19 mmol, 1.11 g), and *O*-phenyl carbonochloridothioate (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/CH_2Cl_2, then 10% MeOH/CH_2Cl_2): **12** (31.6 g, 86%). Light yellow foam. ^{1}H-NMR ($CDCl_3$): 0.87–0.96 (*m*, 4 *Me*$_2$C*H*, 2 *Me*$_2$CHCH$_2$); 1.92–2.20 (*m*, 2 Me$_2$C*H*CH$_2$); 3.18 (*m*, 1 Me$_2$CHC*H*$_2$); 3.54 (*m*, 1 Me$_2$CHC*H*$_2$), 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). ^{13}C-NMR ($CDCl_3$): 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-N^6-*[(diisobutylamino)methylidene]*-O^2-*(diphenylcarbamoyl)-3′-5′*-O-*(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)isoguanosine* (**13**). Compound **12** (31.8 mmol, 31.6 g) was co-evaporated with toluene, dissolved in anh. 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/Bu_3SnH 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. $NaHCO_3$/$CHCl_3$ soln., the org. layer dried (Na_2SO_4) and evaporated, and the residue purified by CC ($CHCl_3$, then 5% MeOH/$CHCl_3$; R_f 0.79): **13** (22.0 g, 82%). Light yellow foam. ^{1}H-NMR ($CDCl_3$): 0.78–1.11 (*m*, 4 *Me*$_2$C*H*, 2 *Me*$_2$CHCH$_2$); 1.82–2.21, 2.52–2.66 (2*m*, 2 Me$_2$C*H*CH$_2$, 2 H–C(2′)); 3.02–3.58 (*m*, 2 Me$_2$CHC*H*$_2$); 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). ^{13}C-NMR ($CDCl_3$): 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-N^6-*[(diisobutylamino)methylidene]*-O^2-*(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 H_2O (50 ml) and extracted with $CHCl_3$. The org. layer was dried (Na_2SO_4) and evaporated and the residue purified by CC ($CHCl_3$, then 10% MeOH/$CHCl_3$; R_f 0.23): **14** (11.0 g, 70%). Light yellow foam. ^{1}H-NMR ($CDCl_3$): 0.81–1.02 (*m*, 2 *Me*$_2$CHCH$_2$); 1.86–2.18 (*m*, 2 Me$_2$C*H*CH$_2$); 2.20 (*m*, 1 H–C(2′)); 2.62 (*m*, 1 H–C(2′)); 3.12 (*d*, 1 Me$_2$CHC*H*$_2$); 3.43 (*m*, 1 Me$_2$CHC*H*$_2$); 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). ^{13}C-NMR ($CDCl_3$): 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-N^6-*[(diisobutylamino)methylidene]-5′*-O-*(4,4′-dimethoxytrityl)*-O^2-*(diphenylcarbamoyl)isoguanosine* (**15**). Compound **14** (13.7 mmol, 8.26 g) was co-evaporated with pyridine and dissolved in anh. pyridine (200 ml). DMAP (3.44 mmol, 419 mg) and (MeO)$_2$Tr-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. $NaHCO_3$ soln./$CHCl_3$. The combined org. phase was dried (Na_2SO_4) and evaporated and the residue purified by CC (hexanes/AcOEt/$CHCl_3$/Et_3N 20:35:35:1, then AcOEt/MeOH/Et_3N 80:2:1; R_f 0.57): **15** (6.13 g, 49%). Light-yellow foam. ^{1}H-NMR ($CDCl_3$): 0.90 (*dd*, 2 *Me*$_2$CHCH$_2$); 1.91–2.18 (*m*, 2 Me$_2$C*H*CH$_2$); 2.51 (*m*, 2 H–C(2′)); 3.18 (*d*, 1 Me$_2$CHC*H*$_2$); 3.30 (*m*, 1 Me$_2$CHC*H*$_2$); 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). ^{13}C-NMR ($CDCl_3$): 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*-N^6-*[(diisobutylamino)methylidene]-5′*-O-*(4,4′-dimethoxytrityl)*-O^2-*(diphenylcarbamoyl)-isoguanosine* (**16**). Compound **15** (1.01 mmol, 911 mg), DMAP (0.25 mmol, 31 mg), Et_3N (2.52 mmol, 0.351 ml), and Ac_2O (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. $NaHCO_3$ soln.), the org. layer dried (Na_2SO_4) and evaporated, and the residue (R_f 0.78 5% MeOH/$CHCl_3$) used in the next step without further purification.

3′-O-*Acetyl-2′-deoxy*-N^6-*[(diisobutylamino)methylidene]*-O^2-*(diphenylcarbamoyl)isoguanosine* (**17**). Unpurified **16** (directly from the previous reaction) was dissolved in a soln. of MeOH (5 ml) and $CHCl_3$ (1 ml). The soln. was cooled to 0°, and 10% anh. HCl in MeOH (5 ml) was added. Stirring was continued at 0° for 5 min. The mixture was then neutralized with aq. $NaHCO_3$ soln. and extracted ($CHCl_3$). The resulting org. layer was dried (Na_2SO_4) and evaporated and the residue purified by CC (5% MeOH/$CHCl_3$; R_f 0.59): **17** (290 mg, 45% for 2 steps). Pale yellow foam. ^{1}H-NMR ($CDCl_3$): 0.87–0.98 (*m*, 2 Me_2CHCH_2); 2.11 (*s*, Ac); 1.93–2.42 (*m*, 2 Me_2CHCH_2, 2 H–C(2′)); 3.19, 3.54 (2*m*, 2 Me_2CHCH_2); 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). ^{13}C-NMR ($CDCl_3$): 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 anh. pyridine (0.17 ml) and anh. dioxane (0.51 ml). A soln. of 2-chloro-4*H*-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 I_2 in pyridine/H_2O 98:2 was added (3.74 ml). Stirring was continued for 15 min, and the excess I_2 was destroyed by addition of 5% aq. Na_2SO_3 soln. H_2O (6 ml) was added and the mixture stirred for 30 min. Then 25% aq. NH_3 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 $1.1 \cdot 10^4$ м$^{-1}$ cm^{-1} [14]. UV (H_2O): 247, 292. ^{1}H-NMR (D_2O): 1.09 (*t*, Et_3N); 2.33 (*m*, 1 H–C(2′)); 2.49 (*m*, 1 H–C(2′)); 3.00 (*q*, Et_3N); 4.00 (*m*, 2 H–C(5′)); H–C(3′) and H–C(4′) superimposed by D_2O; 6.09 (*t*, H–C(1′)); 8.00 (*s*, H–C(8)). ^{31}P-NMR (D_2O: –07.04 (*d*); –8.5 (*d*); –20.73 (*t*). HPLC/ESI-MS (pos. mode): 508 ($[M+H]^+$), 525 ($[M+NH_4)^+$). HPLC/ESI-MS (neg. mode): 506 ($[M-H]^-$), 525 ($[(M-H+Na)-H]^-$).

2′-Deoxy-N^2-*[(dimethylamino)methylidene]-5-methylisocytidine* (**19**). To a soln. 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 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/$CHCl_3$; R_f 0.35): 677 mg (97%) of **19**. White solid product. ^{1}H-NMR (CD_3OD): 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 (2*s*, Me_2N); 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). ^{13}C-NMR (CD_3OD): 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)*-N^2-*(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 Et_3N (4.57 mmol, 0.636 ml) were added, and the mixture was stirred at r.t. for 8 h (TLC (10% MeOH/$CHCl_3$): R_f 0.29). MeOH (3 ml) was added, the mixture evaporated, aq. $NaHCO_3$ soln. (30 ml) added, and the mixture extracted with AcOEt (3 × 30 ml). The combined org. layer was dried (Na_2SO_4) and evaporated, and the residue was purified by CC (10% MeOH/$CHCl_3$, then 17.5% MeOH/$CHCl_3$): **20** (1.03 g, 75%). White foam. ^{1}H-NMR ($CDCl_3$): 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 (2*s*, Me_2N); 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). ^{13}C-NMR ($CDCl_3$): 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)*-N^2-*[(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), Et_3N

(4.29 mmol, 0.596 ml), and Ac_2O (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. $NaHCO_3$ soln. (30 ml) was added and the mixture extracted with AcOEt (3×30 ml). The combined org. layer was dried (Na_2SO_4) and evaporated, and the residue was purified by CC (17.5% MeOH/$CHCl_3$; R_f 0.68): **21** (0.974 g, 89%). White foam. ^{1}H-NMR ($CDCl_3$): 1.62 (*s*, Me–C(5)); 2.07 (*s*, Ac); 2.40–2.55 (*m*, 2 H–C(2′)); 3.08, 3.12 (2*s*, Me_2N); 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′), $(MeO)_2$*Tr*); 7.18–7.37, 7.43 (*m*, *d*, 9 H, $(MeO)_2$*Tr*); 7.70 (*s*, H–C(6)); 8.82 (*s*, N=CH). ^{13}C-NMR ($CDCl_3$): 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-*N^2*-[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. $NaHCO_3$ soln. was added dropwise to pH 7, the mixture was extracted with AcOEt (3×20 ml), the extract dried (Na_2SO_4) and evaporated, and the residue purified by CC (17.5% MeOH/$CHCl_3$): **22** (241 mg, 64%). Colorless stars. TLC (17.5% MeOH/$CHCl_3$): R_f 0.46.

Method b: To a soln. of **21** (0.2 mmol, 128 mg) in MeCN containing 1% H_2O (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/$CHCl_3$): **22** (28.9 mg, 43%). Light yellow stars. ^{1}H-NMR ($CDCl_3$): 1.93 (*s*, Me–C(5)); 2.09 (*s*, Ac); 2.28–2.45 (*m*, 2 H–C(2′)); 3.09, 3.13 (2*s*, Me_2N); 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). ^{13}C-NMR ($CDCl_3$): 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-4*H*-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 I_2 (0.449 mmol, 114 mg) in pyridine (10 ml) and H_2O (0.2 ml) was added, and the resulting soln. was stirred for 15 min. The reaction was quenched by addition of 5% Na_2SO_3 soln. The solvent was evaporated at r.t. and the residue dissolved in H_2O (7.5 ml) and stirred at r.t. for 30 min. Conc. aq. NH_3 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 (H_2O): 261. ^{1}H-NMR (D_2O): 1.25 (*t*, Et_3N); 1.88 (*s*, Me–C(5)); 2.45 (*m*, 2 H–C(2′)); 3.15 (*q*, Et_3N); 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)). ^{13}C-NMR (D_2O): 9.6 (Et_3N), 14.0, 39.1, 47.6, 65.9, 70.8, 86.3, 89.6, 116.0, 137.4, 155.4, 175.7, 177.6. ^{31}P-NMR (D_2O): –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 ($[M+NH_4]^+$). 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 me^5isoC_d incorporation: 5′-d(AAA AAA AGT CAT AGC TGT TTC CTG)-3′ (**II**).
me^5isoC_d-Containing template: 5′-d(AAA A(me^5isoC)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 $MgCl_2$, 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 p_3isoG_d (**1**), or $p_3me^5isoC_d$ (**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 µl). Loading buffer (5–6 µl, 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 × 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 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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REFERENCES

[1] J. A. Piccirilli, T. Krauch, S. E. Moroney, S. A. Benner, *Nature (London)* **1990**, *343*, 33.
[2] S. A. Benner, R. K. Allemann, A. D. Ellington, L. Ge, A. Glasfeld, G. F. Leanz, T. Krauch, L. J. MacPherson, S. E. Moroney, J. A. Piccirilli, E. G. Weinhold, *Cold Spring Harbor Symp. Quant. Biol*. **1987**, *52*, 53.
[3] A. Rich, in ' Horizons in Biochemistry'. Eds. M. Kasha, and B. Pullman, Academic Press, New York, 1962, pp. 103–126.
[4] Y. Tor, P. B. Dervan, *J. Am. Chem. Soc*. **1993**, *115*, 4461.
[5] T. Horn, C.-A. Chang, M. L. Collins, *Tetrahedron Lett*. **1995**, *36*, 2033.
[6] F. Seela, C. Wei, *Helv. Chim. Acta* **1997**, *80*, 73.
[7] C. Roberts, R. Bandaru, C. Switzer, *J. Am. Chem. Soc*. **1997**, *119*, 4640.
[8] C. Y. Switzer, S. E. Moroney, S. A. Benner, *Biochemistry* **1993**, *32*, 10489.
[9] T. M. Tarasow, S. L. Tarasow, B. E. Eaton, *Nature (London)* **1997**, *389*, 54.
[10] H. Kamiya, H. Kasai, *J. Biol. Chem*. **1995**, *270*, 19446.
[11] J. Hovinen, E. Azhayeva, A. Azhayev, A. Guzaev, H. Lonnberg, *J. Chem. Soc., Perkin Trans. 1* **1994**, 211.
[12] J. G. Moffat, H. G. Khorana, *J. Am. Chem. Soc*. **1961**, *83*, 649.
[13] J. Ludwig, *Acta Biochem. Biophys. Acad. Sci. Hung*. **1981**, *16*, 131.
[14] F. Seela, C. Wei, Z. Kazimierczuk, *Helv. Chim. Acta* **1995**, *78*, 1843.
[15] C. Roberts, R. Bandaru, C. Switzer, *Tetrahedron Lett*. **1995**, *36*, 3601.
[16] S. C. Jurczyk, J. T. Kodra, J. D. Rozzell, S. A. Benner, T. R. Battersby, *Helv. Chim. Acta* **1998**, *81*, 793.
[17] J. Ludwig, F. Eckstein, *J. Org. Chem*. **1989**, *54*, 631.
[18] J. T. Kodra, S. A. Benner, *Synlett* **1997**, *8*, 939.
[19] A. M. Bukowska, J. T. Kusmierek, *Acta Biochim. Pol*. **1996**, *43*, 247.
[20] J. R. Hwu, M. L. Jain, S.-C. Tsay, G. H. Hakimelahi, *J. Chem. Soc., Chem. Commun*. **1996**, 545.

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