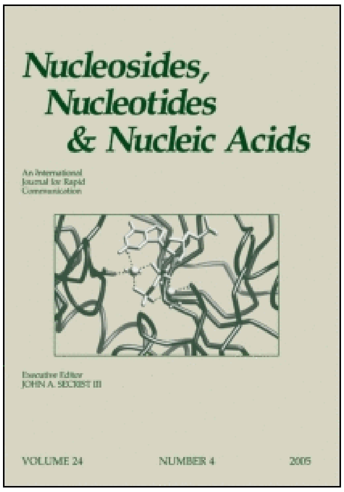

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ULTRAFAST CLEAVAGE AND DEPROTECTION OF OLIGONUCLEOTIDES SYNTHESIS AND USE OF C^{Ac} DERIVATIVES

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ABSTRACT : We have investigated the use of alkylamines as fast cleavage and deprotection reagents for the solid phase synthesis of oligonucleotides and found methylamine/ammonium hydroxide (or methylamine) as an efficient reagent. The transamination side product formed with the commonly used dC^{bz} has been eliminated by the use of dC^{Ac} phosphoramidite. This system has successfully been used in the synthesis of oligonucleotides and oligonucleoside phosphorothioates. DMT dC^{Ac} hydrogen phosphonate and DMT ribo C^{Ac}-2'-OMe phosphoramidite also have been prepared and used in the synthesis of oligonucleotides.

INTRODUCTION

Efforts to reduce the post-synthesis processing of oligonucleotides have primarily focussed on the development of nucleotide reagents carrying more labile amino protecting groups. However, their use has been limited due to their instability during DNA synthesis, reduced shelf life and increased cost. Also, their use did not address the rate of cleavage of oligonucleotides from the solid support, which in our opinion, is as much desirable as reducing the deprotection time.

We reported previously[1] the development of methylamine/ammonium hydroxide as a fast cleavage and deprotection reagent. In order to avoid the transamination side product formation, noticed with the commonly used dC^{bz} phosphoramidite, dC^{Ac} phosphoramidite[2] was developed. We have subsequently reported that the use of methylamine used in conjunction with dC^{Ac} phosphoramidite has dramatically improved the yield of oligoribonucleotides.[3]

In this paper, we publish more details of the fast cleavage and deprotection chemistry including its application to the synthesis of oligonucleotides by the H-phosphonate approach, synthesis of 2'-OMe oligoribonucleotides and synthesis of oligonucleoside phosphorothioates.

RESULTS AND DISCUSSION

We reasoned that alkylamines, which are generally more nucleophilic than ammonium hydroxide, should provide faster cleavage and deprotection kinetics. We have investigated a series of alkylamines: methylamine, ethylamine, n-propylamine, n-butylamine, n-pentylamine, n-hexylamine, dimethylamine, diethylamine, isopropylamine, diisopropylamine, sec-butylamine and their mixtures with ammonium hydroxide[4] and found that methylamine (40% solution in water) or its 1:1 (v/v) mixture with concentrated aqueous ammonium hydroxide[5] gave the fastest kinetics.

Cleavage and Deprotection: The extent of cleavage was determined by measuring 260nm absorbance of the oligonucleotide released from the solid support at different time intervals. Methylamine/ammonium hydroxide (1:1, v/v) or methylamine cleaved oligonucleotides in 5 min where as the commonly used ammonium hydroxide required 60 min for the cleavage.

As the data in TABLE 1 show, methylamine/ammonium hydroxide is much faster than ammonium hydroxide in deprotecting oligonucleotides. The reactivity of the other alkylamines fall in between these two reagents. In this study, dG^{ibu}, dA^{bz}, dC^{ac} and T phosphoramidites were employed. TABLE 2 shows the times required for the complete deprotection at different temperatures.

Side product formation: No side products were formed when methylamine/ammonium hydroxide was used to deprotect dA^{bz}, dG^{ibu} and T as monitored by reverse phase HPLC. However, about 10% transamination product was detected when the commonly used dC^{bz} was used. This was consistent with the observations made by Weber and Khorana[6] in 1972 and subsequently by Miller et al[7], MacMillan et al[8], Hogrefe et al[9] and Polushin et al[10,11] when they used alkylamines for deprotection. The side product is formed in a competitive reaction in which alkylamine attacks the pyrimidine ring of deoxycytidine instead of the carbonyl carbon of the deoxycytidine protecting group. We have rationalized that an increase in the electropositivity of the carbonyl carbon will result in the acceleration of the rate of deprotection, thereby reducing or eliminating the chances of an attack on the pyrimidine ring. In order to check this hypothesis, we have treated the commercially available dC^{ibu} with methylamine/ammonium hydroxide and realized more than 10 fold reduction in the formation of transamination side product. Encouraged by this finding, we have synthesized $dC^{n\text{-}butyryl}$, $dC^{propionyl}$ and dC^{acetyl} nucleosides. As TABLE 3 shows, the expected trend based on the electron donating ability continued, and no side product was detected in the case of dC^{Ac}.

TABLE 1. Deprotection of Oligonucleotides at room temperature

Reagent	Deprotection Time	Reagent	Deprotection Time
NH_4OH	72 h	$C_6H_{13}NH_2/NH_4OH$	12 h
CH_3NH_2/NH_4OH	75 min	$(CH_3)_2NH/NH_4OH$	36 h
$C_2H_5NH_2/NH_4OH$	7 h	$(C_2H_5)_2NH/NH_4OH$	55 h
$C_3H_7NH_2/NH_4OH$	10 h	$(CH_3)_2CHNH_2/NH_4OH$	45 h
$C_4H_9NH_2/NH_4OH$	10 h	$[(CH_3)_2CH]_2NH/NH_4OH$	35 h
$C_5H_{11}NH_2/NH_4OH$	12 h	$C_2H_5CH(NH_2)CH_3/NH_4OH$	50 h

TABLE 2. Time required for complete deprotection of oligonucleotides

Temperature	Methylamine/ Ammonium hydroxide	Ammonium hydroxide
25^0C	75 min	72 h
37^0C	30 min	20 h
55^0C	10 min	4 h
65^0C	5 min	3 h
80^0C	2 min	1 h

TABLE 3. Side product formation by methylamine/ammonium hydroxide

Protected deoxycytidine	Deprotection time by CH_3NH_2/NH_4OH	Side product formed* (N^4-methyldeoxycytidine)
$dC^{benzoyl}$	1 hour	10.0%
$dC^{isobutyryl}$	2 min	0.70%
$dC^{n\text{-}butyryl}$	1 min	0.21%
$dC^{propionyl}$	1 min	0.05%
dC^{acetyl}	< 1 min	0.00%

* The percentage of N^4-methyldeoxycytidine was determined from reverse phase HPLC. Its identity was confirmed by synthesizing[12] it and coinjecting in HPLC.

Synthesis of nucleotide derivatives:

DMT dC^{Ac} phosphoramidite: N^4-Acyl-2'-deoxycytidines 2a-d (FIG. 1) were synthesized in 90-99% yields by selective acylation[13] of 2'-deoxycytidine (1a). dC^{Ac} (2a) was tritylated[14] with 4,4'-dimethoxytrityl chloride to furnish DMT dC^{Ac} (3a) in 85% yield. Its conversion to the phosphoramidite 4a was effected in 85% yield by following the standard procedure[15,16]. It is noteworthy that dC^{Ac} phosphoramidite is obtained with a high purity of 99.66%.

DMT dC^{Ac}---CPG solid support: The 3'-succinate derivative 6a was synthesized from 3a in 83% yield and was loaded on to the CPG support[17] to obtain a DMT dC^{Ac} loading of 31 μmole per gram of the support 6b.

DMT dC^{Ac} H-phosphonate: The H-phosphonate derivative of DMT dC^{Ac} (5) was synthesized by a modification of the literature procedure[18] and was successfully used in the synthesis of oligonucleotides.

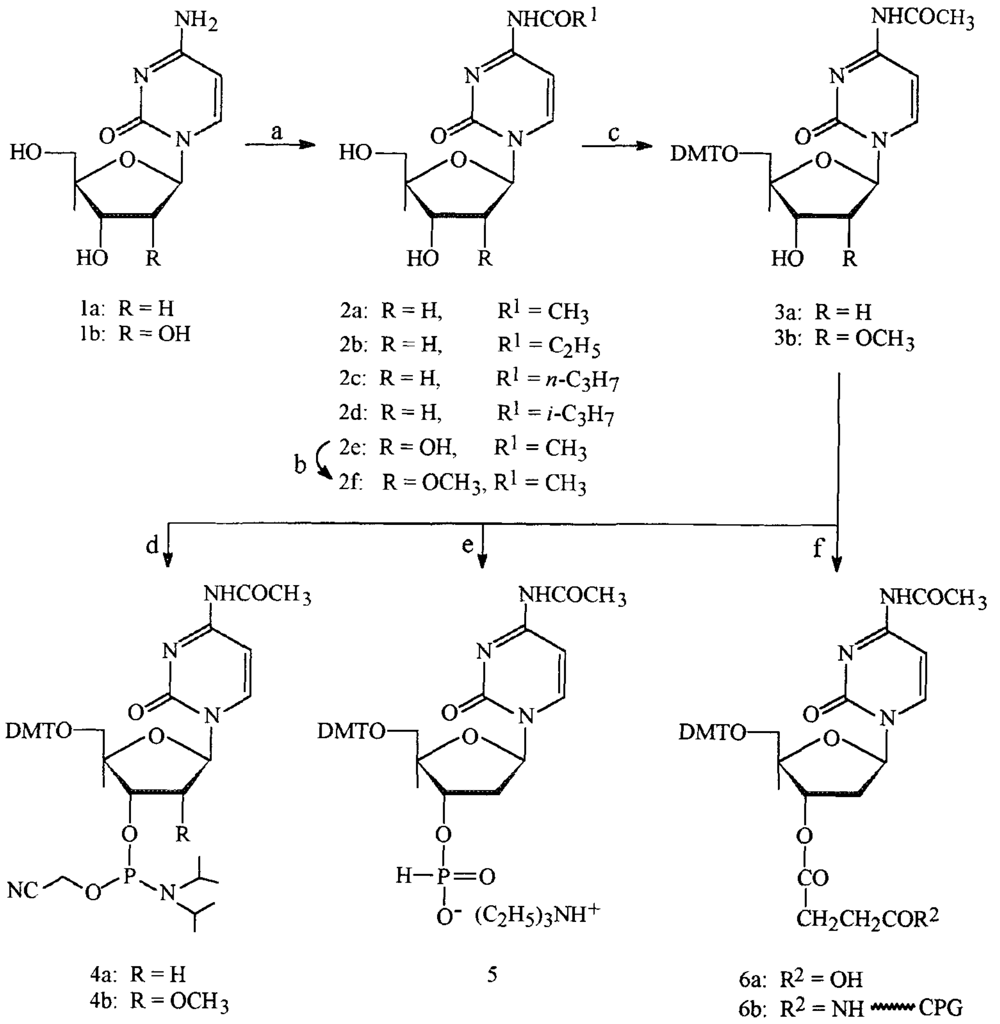
DMT ribo C^{Ac}-2'-OMe phosphoramidite: N^4-Acetylcytidine (2e) was reacted with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane[19] to give N^4-acetyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-cytidine in 90% yield. Treatment of this compound with excess of MeI/Ag_2O[20] in dry benzene at room temperature afforded 70% of the 2'-O-methylderivative. Desilylation[21] using Bu_4NF gave N^4-acetyl-2'-O-methylcytidine (2f) in 60% yield. The compound 2f was tritylated with 4,4'-dimethoxytrityl chloride to yield DMT-2'-O-methylcytidine derivative 3b in 70% yield. Phosphorylation[15,16] of 3b afforded the phosphoramidite 4b in 65% yield.

Synthesis of oligonucleotides:

C^{Ac} phosphoramidite was successfully used in the synthesis of oligonucleotides ranging from 10 to 101 bases. The quality of the oligonucleotides, as monitored by reverse phase HPLC and capillary electrophoresis, was at least as good as the same oligonucleotides synthesized using the C^{bz} phosphoramidite. C^{Ac} phosphoramidite was also successfully used in the synthesis of oligonucleoside phosphorothioates using Beaucage reagent[22] for sulfurization. In the case of oligonucleotide synthesis using 2'-OMe C^{Ac} phosphoramidite, a 15 minute coupling time was used. dC^{Ac} H-phosphonate monomer was successfully employed in the synthesis of normal oligonucleotides as well as oligonucleoside phosphorothioates.

Stability Study: In order to study the stability of DMT dC^{Ac} phosphoramidite, we have performed a quick comparative evaluation[24] by storing dC^{Ac}, dC^{bz} and dC^{ibu} phosphoramidites at 37^{0}C for 3 months and analyzing them by reverse phase HPLC. DMT dC^{Ac} phosphoramidite was found to be at least as stable as the other two phosphoramidites.

In order to test the stability of dC^{Ac} phosphoramidite on a DNA synthesizer, a 35 base oligonucleotide was synthesized on the Beckman Oligo 1000 synthesizer using a fresh dC^{Ac} phosphoramidite solution and a 4 week old solution. Capillary



Reagents and Conditions:

a) $(R^1CO)_2O$/DMF

b) 1. 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane/pyridine; 2. MeI/Ag_2O/benzene; 3. Bu_4NF/THF

c) DMTCl/pyridine

d) 2-Cyanoethyl-N,N-diisopropylmonochlorophosphoramidite/diisopropylethylamine/THF

e) PCl_3/imidazole/CH_3CN

f) 1. Succinic anhydride/DMAP/pyridine; 2. CPG-NH_2/DCC/pyridine

FIGURE 1

electrophoretic analysis showed no significant difference in the quality of the 35 base oligonucleotide.

CONCLUSIONS:

Methylamine/ammonium hydroxide (or methylamine) cleaved and deprotected oligonucleotides significantly faster than the conventional reagent ammonium hydroxide. DMT dC^{Ac} phosphoramidite was synthesized in high purity and it was found to be very stable during storage on the shelf as well as during use in DNA synthesizers. DMT dC^{Ac} H-phosphonate and DMT ribo C^{Ac} 2'-OMe phosphoramidite have successfully been synthesized and used to synthesize oligonucleotides.

EXPERIMENTAL:

General: Methylamine (40% in water), ethylamine (70% in water), other alkylamines and conc. ammonium hydroxide were purchased from Aldrich chemical company. Ultraviolet spectra (UV) were recorded on the Beckman DU-70 spectrophotometer. Infrared spectra (IR) were recorded on the Nicolet 205 FT-IR spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on the Bruker WM 300 spectrometer in DMSO-d_6 or in $CDCl_3$, at room temperature. Chemical shifts are expressed in ppm. For ^{31}P NMR, chemical shifts are expressed downfield from external 85% phosphoric Acid.Capillary electrophoresis (CE) was run on Beckman P/ACE 2000.

Deprotection of oligonucleotides: The oligonucleotides cleaved from the solid support and were incubated at the desired temperature for the appropriate length of time. After evaporating the reagent on a speed-vac concentrator, the residue was digested with phosphodiesterase and alkaline phosphatase, and the nucleosides were analyzed by reverse phase HPLC.

N^4-Acylation of 2'-deoxycytidine (2a-d):2'-Deoxycytidine(1a) was acylated by following the procedure developed by Bhat et al.[13]

N^4-Acetyl-2'-deoxycytidine, dC^{Ac} (2 a): Yield: 98% m.p.: 150 - 170°C (dec.); R_f = 0.6 in methylene chloride-methanol (9:1, v/v) UV($H_2$0): λ max 298 nm and 246 nm; IR (KBr): ν 1651 (vs, C=0 amide ring), 1707 (C=0 of amide), 2800-3650 (NH, OH) cm^{-1}. ^{1}H-NMR (DMSO-$\underline{d}_6$): δ 2.08 and 2.31 (m, 2H, $C_{2'}$ $C\underline{H}_2$), 2.10 (s, 3H, $COC\underline{H}_3$), 3.61 (m, 2H, $C_{5'}$ $C\underline{H}_2$), 3.88 (q, 1H, $C_{4'}\underline{H}$), 4.22 (d, 1H, $C_{3'}\underline{H}$), 5.05 (t, 1H, $C_{5'}$ $\underline{OH}$), 5.27 (d, 1H, $C_{3'}$ $O\underline{H}$), 6.11 (t, 1H, $J_{1',2'}$=6.48 Hz, $C_{1'}\underline{H}$), 7.20 (d, 1H, $J_{5,6}$=7.20 Hz, $C_5\underline{H}$), 8.32 (d, 1H, $J_{5,6}$=7.56 Hz, $C_6\underline{H}$), and 10.85 (s, 1H, $CON\underline{H}$). Analysis: Calcd for C_{11} H_{15} N_3 $0_5.H_20$ (287.75):C, 45.99; H, 5.97; N, 14.63. Found: C, 45.71; H, 6.10; N, 14.38.

N^4-Propionyl-2'-deoxycytidine, dC^{Pr}(2 b): Yield: 96% m.p.: 160-170°C (dec.); UV($H_2$0): λ max 299 nm and 247 nm; IR (KBr): ν 1630 (vs, br, C=0 of ring amide), 1722 (s, C=0 of amide), and 3000-3650 (NH, OH) cm^{-1}.^{1}H-NMR (DMSO-$\underline{d}_6$): δ 1.03 (t, 3H, $CO\text{-}CH_2\text{-}C\underline{H}_3$), 2.02 and 2.26 (2m, 2H, $C_{2'}$ $C\underline{H}_2$), 2.40 (q, 2H, $CO\text{-}C\underline{H}_2CH_3$), 3.60 (m, 2H, $C_{5'}$ $C\underline{H}_2$), 3.81 (q, 1H, C_4,$\underline{H}$), 4.22 (d, 1H, $C_{3'}$ $\underline{H}$), 5.04 (t, 1H, $C_{5'}$ $0\underline{H}$), 5.26 (d, 1H, $C_{3'}$ $0\underline{H}$), 6.11 (t, 1H, $J_{1',2'}$=6.48 Hz, $C_{1'}$ $\underline{H}$), 7.22 (d, 1H, $J_{5,6}$=7.56 Hz, $C_5\underline{H}$), 8.32 (d,1H, $J_{5,6}$=7.56

Hz, $C_6\underline{H}$), and 10.81 (s, 1H, -CON$\underline{H}$). Analysis: Calcd for C_{12} H_{17} N_3 0_5 • 1/4 H_20 (287.78):C, 50.08; H, 6.09; N, 14.60. Found: C, 50.28; H, 5.76; N, 14.15.

N^4-n-Butyryl-2'-deoxycytidine, dC$^{n\text{-}but}$ (2 c): Yield: 90% yield. m.p.: 190-195°C (dec.); UV(H_20): λ max 297 nm and 246 nm; IR (KBr): ν 1650-1690 (vs, br, C=0 of amides), and 2900-3600 (NH, OH) cm^{-1}. ^{1}H-NMR (DMSO-$\underline{d}_6$): δ 0.89 (t, 3H, CH_2 CH_2 $C\underline{H}_3$), 1.56 (q, 2H, $CH_2C\underline{H}_2CH_3$), 2.02 and 2.30 (2m, 2H, $C_{2'}$ $C\underline{H}_2$), 2.41 (t, 2H, -C0-$C\underline{H}_2$), 3.63 (m, 2H, C_5,$C\underline{H}_2$), 3.89 (m, 1H, C_4, $\underline{H}$), 4.23 (t, 1H, $C_{3'}$ $\underline{H}$), 5.03 (t, 1H, $C_{5'}$ 0$\underline{H}$), 5.26 (d, 1H, C_3, 0$\underline{H}$), 6.12 (t, 1H, $J_{1',2'}$=6.27 Hz, C_1, $\underline{H}$), 7.24 (d, 1H, $J_{5,6}$=7.35 Hz, C_5 $\underline{H}$), 8.33 (d, 1H, $J_{5,6}$=7.59 Hz, C_6 $\underline{H}$), and 10.83 (s, 1H, N$\underline{H}$CO). Analysis: Calcd. for C_{13} H_{19} N_3 0_5 • 1/3 H_20 (303.3):C, 51.48; H, 6.51; N, 13.85. Found: C, 51.75; H, 6.33; N, 13.40.

N^4-Isobutyryl-2'-deoxycytidine, dC^{ibu} (2 d): Yield: 95%. This procedure gave higher yield compared to the reported procedure.[24] m.p.: 110-112°C (dec.), mixed m.p. 110-112°C (dec) with an authentic sample. UV(H_20): λ max 297 nm and 246 nm; IR (KBr): ν 1652 (vs, C=0 of ring amide), 1716 (s, C=O of amide), and 2900-3600 (NH,OH) cm^{-1}. ^{1}H-NMR (DMSO-$\underline{d}_6$): δ 1.06 (2s, 6H, 2 X $C\underline{H}_3$), 2.02 and 2.27 (2m, 2H, $C_{2'}$ $C\underline{H}_2$), 2.70 (m, 1H, $C\underline{H}$), 3.59 (m, 2H, C_5, $C\underline{H}_2$), 3.86 (d, 1H, $C_4\underline{H}$), 4.22 (t, 1H, C_3, $\underline{H}$), 5.04 (t, 1H, $C_{5'}$ 0$\underline{H}$), 5.26 (d, 1H, C_3, 0$\underline{H}$), 6.10 (t, 1H, $J_{1',2}$=6.24 Hz, C_1, $\underline{H}$), 7.23 (d, 1H, $J_{5,6}$=7.59 Hz, C_5 $\underline{H}$), 8.32 (d, 1H, $J_{5,6}$=7.62 Hz, C_6 $\underline{H}$), and 10.82 (s, 1H, N$\underline{H}$CO).

N^4-Acetyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxycytidine (3a): N^4-Acetyl-2'-deoxycytidine (2a) was converted to the title compound by following the standard procedure.[14] Yield: 85% m.p.: 120-140^{0}C; R_f = 0.5 in methylene chloride-methanol (95:5). UV(EtOH): λ max 300, 283 and 246 nm; IR (KBr): ν 1660 (vs, C=O of ring amide), 1730 (s, C=O of amide), and 2800-3600 (NH, OH) cm^{-1}; ^{aAc1}H-NMR ($CDCl_3$): δ 2.18 (s, 3H, COC$\underline{H}_3$), 2.22 and 2.78 (2m, 2H, C_2, $C\underline{H}_2$), 3.46 (m, 2H, C_5, $C\underline{H}_2$), 3.57 (q, 1H, $C_4'\underline{H}$), 3.79 (s, 6H, 2x OC$\underline{H}_3$), 4.16 (d, 1H, $C_3'\underline{H}$), 4.51(d, 1H, C_3, O$\underline{H}$), 6.30 (t, 1H, $J_{1',2'}$ = 5.76 Hz, C_1, $\underline{H}$), 6.82-7.40 (m, 14H, $C_5\underline{H}$, and aromatic protons of DMTr), 8.22 (d, 1H, $J_{5,6}$= 7.20 Hz, $C_6\underline{H}$) and 9.30 (br, s IH, CON$\underline{H}$). Analysis: Cacld. for $C_{32}H_{33}N_3O_7$ (571.60): C, 67.24; H, 5.82; N, 7.35. Found: C, 66.92; H, 6.05: N, 6.91.

N^4-Acetyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxycytidine-3'-O-(β-cyanoethyl-N,N-diisopropylphosphoramidite) (4a):

Compound 3a was converted to the phosphoramidite 4a, 85% yield by following the standard proceduresе.[15,16] R_f = 0.6 in CH_2Cl_2-MeOH (95:5); UV (EtOH): λ max: 299 nm, 283 and 236 nm. IR (KBr): ν 1675 (vs, C=O of ring amide), 1720 (s, C=O amide), 2250 (-CN group) and 2966 (vs, NH) cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 1.16 (m, 12H, 2 $CH(C\underline{H}_3)_2$), 2.23 and 2.67 (2m, 2H, C_2, $C\underline{H}_2$),2.25 (s, 3H,-COC$\underline{H}_3$), 2.44 and 2.61 (2t, 2H, -$CH_2C\underline{H}_2$CN), 3.38-3.83 (m, 12 H, C_5, $C\underline{H}_2$, 2$C\underline{H}(CH_3)_2$, 2x-OC$\underline{H}_3$,POC$\underline{H}_2CH_2$), 4.21 (m, 1H, C_4,$\underline{H}$), 4.61 (m, 1H, C_3,$\underline{H}$), 6.25 (dt, 1H, J = 7.2 Hz, $C_1'\underline{H}$), 6.83-7.41 (m, 14H, C_5 $\underline{H}$, and aromatic protons of DMT), 8.23 (dd, 1H, J = 7.2 Hz and J = 7.56 Hz, $C_6\underline{H}$), and 9.70 (br, d, 1H, -CON$\underline{H}$). ^{31}P-NMR ($CDCl_3$): δ 149.43 and 149.99 ppm. Analysis: Calcd. for $C_{41}H_{50}N_5O_8P.H_2O$ (789.82): C, 62.34; H, 6.64; N, 8.87; P, 3.92. Found: C, 62.51; H, 6.84; N, 8.68; P, 3.61. HPLC: Retention times of 7.87 min and 9.4 min corresponding to two diastereomers (99.6% purity). Conditions: C_{18} Microsorb column (Rainin), 5 μ particles, 4.6 mm x 25 cm. Bottle A: 0.1 M Ammonium Acetate pH 6.9, Bottle B: Acetonotrile, Program: Flow rate 1ml/min, 0-20 min at 80%B.

5'-O-(4,4'-Dimethoxytrityl)-N^4-acetyl-2'-deoxycytidine-3'-succinyl-HN-CPG (6b):
The compound 6a was synthesized in 83% yield by succinylation[17] of DMT dC^{Ac} (3a). m.p. 130 - 135°C (dec); UV (EtOH): λ max 292 nm, 283 nm, 275 nm and 236 nm; IR (KBr): ν 1680 (s, C=0 of ring amide), 1760 (vs, C=0 of acid), and 2800 - 3600 (NH, OH) cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 2.20 (s, 3H, $-COC\underline{H}_3$), 2.59 - 2.75 (m, 6H, $C_{2'}C\underline{H}_2$ and $-C\underline{H}_2C\underline{H}_2$ of succinate), 3.43 (m, 2H, $C_{5'}C\underline{H}_2$), 3.79 (s, 6H, 2 x $OC\underline{H}_3$), 4.25 (d, 1H, $C_{4'}\underline{H}$), 5.43 (t, 1H, $C_{3'}\underline{H}$), 6.24 (t, 1H, $J_{1',2'}$ = 6.34 Hz, $C_1'\underline{H}$), 6.82 - 7.36 (m, 14H, $C_5\underline{H}$, and aromatic protons of DMTr group), and 8.13 (d, 1H, $C_6\underline{H}$, $J_{5,6}$ = 7.44 Hz). Analysis: Calcd for $C_{36}H_{37}N_3 0_{10}$ (671.68): C, 64.37; H, 5.55; N, 6.26. Found: C, 64.76; H, 5.91; N, 5.85

The amino-CPG-support was loaded with 6a using standard procedure[17]. The DMT assay showed a nucleoside loading of 31 µmole per gram of the support (6b).

5'-0-(4,4'-Dimethoxytrityl)-N^4-acetyl-2'-deoxycytidine-3'-hydrogen phosphonate triethylammonium salt (5):
The compound (5) was synthesized in 66% yield by following the literature procedure.[18] m.p. 130-145°C (dec.); UV (EtOH): λ max 300, 282 and 234 nm; 1H-NMR ($CDCl_3$):δ1.29 (t, 3H, $NCH_2C\underline{H}_3$), 2.18 (s, 3H, CO $C\underline{H}_3$), 2.57 and 2.72 (2m, 2H, C_2' $C\underline{H}_2$), 3.05 (q, 2H, N $C\underline{H}_2$ CH_3), 3.48 (m, 2H, C_5' $C\underline{H}_2$), 3.78 (s, 6H, 2 X O $C\underline{H}_3$), 4.22 (m, 1H, $C_4'\underline{H}$), 5.03 (m, 1H, $C_3'\underline{H}$), 6.21 (t, 1H, $C_1'\underline{H}$), 6.90 (d, 1H, JpH = 600 Hz, $\underline{H}$-P), 6.83-7.40 (m, 14H, $C_5\underline{H}$, and DMT aromatic protons), 8.29 (d, 1H, $C_6\underline{H}$) and 10.34 (br, s, 1H, $N\underline{H}CO$); ^{31}P-NMR ($CDCl_3$): δ 4.1638 ppm and -0.99 ppm, JH-P = 625.95 Hz HPLC:~100% purity.

3',5'-O-(Tetraisopropyldisiloxane-1,3-diyl)-N^4-acetylcytidine: It was prepared as described by Markiewics et al[19] in 90% yield. UV(EtOH): λ max 298 nm and 248 nm; ^{1}H-NMR ($CDCl_3$): δ 0.97-1.10 (m, 28H, $C\underline{H}_3$ of iPr), 2.30 (s, 3H, $COC\underline{H}_3$), 3.30 (br, s, 1H, $C_{2'}O\underline{H}$), 4.19-4.30 (m, 5H,$C_{2',3',4',5'}\underline{H}$), 5.82 (s, 1H, $C_{1'}\underline{H}$), 7.44 (d, 1H, $C_5\underline{H}$), 8.20 (d, 1H, $C_6\underline{H}$), and 9.97 (br, s, 1H, $N\underline{H}CO$). HPLC: 98.5% purity.

3',5'-O-(Tetraisopropyldisiloxane-1,3-diyl)-2'-O-methyl-N^4-acetylcytidine: It was prepared in 70% yield by following the procedure of Inoue et al.[20] UV(EtOH): λ max 298 nm and 248 nm; ^{1}H-NMR ($CDCl_3$): δ 0.96-1.11 (m, 28H, $C\underline{H}_3$ of iPr), 2.51 (s, 3H, $COC\underline{H}_3$), 3.65 (s, 3H, 2'-$OC\underline{H}_3$), 3.95-4.45 (m, 5H,$C_{2',3',4',5'}\underline{H}$), 5.77 (s, 1H, $C_{1'}\underline{H}$), 7.22 (d, 1H, $C_5\underline{H}$), and 7.93 (d, 1H, $C_6\underline{H}$).

N^4-Acetyl-2'-O-methylcytidine (2f): It was prepared in 60% yield according to the procedure of Chattopadhyaya.[21] m.p. 220-223^{0}C (dec.); UV(H_2O): λ max 298 nm and 248 nm.^{1}H-NMR (DMSO-$\underline{d}_6$): δ 2.10 (s, 3H, $COC\underline{H}_3$), 3.46 (s, 3H, 2'-$OC\underline{H}_3$), 3.71-4.05 (m, 5H, $C_{2',3',4',5'}\underline{H}$), 5.11 (d, 1H, $C_{5'}O\underline{H}$, exchangeable with D_2O), 5.22 (t, 1H, $C_{3'}O\underline{H}$, exchangeable with D_2O), 5.84 (s, 1H, $C_{1'}\underline{H}$), 7.19 (d, 1H, $C_5\underline{H}$); 8.47 (d, 1H, $C_6\underline{H}$), and 10.97 (br, s, 1H, $N\underline{H}CO$, exchangeable with D_2O). HPLC: 98% purity.

5'-O-(4,4'-Dimethoxytrityl)-N^4-acetyl-2'-O-methylcytidine (3b): It was prepared in 70% yield according to the standard procedure.[14] UV(EtOH): λ max 300 nm 282 nm and 234 nm; ^{1}H-NMR ($CDCl_3$): δ 2.22 (s, 3H, $COC\underline{H}_3$), 3.60 (m, 2H, $C_{5'}C\underline{H}_2$), 3.77 (s, 3H, 2'-$OC\underline{H}_3$), 3.82 (s, 6H, 2 x $OC\underline{H}_3$), 3.74 (m,1H, $C_{4'}\underline{H}$), 4.02 (m, 1H, $C_{3'}\underline{H}$), 4.41 (m,1H, $C_{2'}\underline{H}$), 6.00 (s, 1H, $C_{1'}\underline{H}$), 6.85-7.40 (m, 13H, DMT group), 7.06 (d, 1H, $C_5\underline{H}$), 8.53 (d, 1H, $C_6\underline{H}$), and 8.98 (br, s, 1H, $N\underline{H}CO$). HPLC: 98% purity.

5'-O-(4,4'-Dimethoxytrityl)-N^4-acetyl-2'-O-methylcytidine -3'-O-(N,N-diisopropyl)-β-cyanoethyl-phosphoramidite(4b): It was synthesized in 65% yield following the method of Sinha et al.[15] UV(EtOH): λ max 300 nm 284 nm and 236 nm; IR(KBr): ν1660 (vs, CO of ring amide), 1720 (s, CO of Acetamide),2256 (CN groups), and 3000 (br, s, NH) cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 0.86-1.26 (m, 12H, $C\underline{H}_3$iPr), 2.22 (s, 3H, $COC\underline{H}_3$),2.41 and 2.62 (2t, 4H,-$C\underline{H}_2C\underline{H}_2CN$) 3.45-4.29 (m, 16H, $C_{5'}C\underline{H}_2$, 2 x $C\underline{H}$ iPr, 2'-$OC\underline{H}_3$, 2 x $OC\underline{H}_3$, $C_{2',3',4'}\underline{H}$) 6.02 (2s,1H, $C_{1'}\underline{H}$), 6.83-7.43 (m, 14H, DMT aromatic protons and $C_5\underline{H}$), 8.62 (d, 1H, $C_6\underline{H}$), and 9.31 (br, s, 1H, $N\underline{H}CO$). ^{31}P-NMR ($CDCl_3$): δ148.783 ppm and 149.428 ppm. HPLC: Retention times of 7.81 and 8.73 corresponding to the two diastereisomers (98% purity). conditions: C_{18} Microsorb (Rainin column) S_4 particles 4.6 mm x 25 cm . Bottle A: 0.1 M ammonium Acetate (pH 6.9), Bottle B: Acetonitrile, Program Flow rate 1 ml/min, 0-20 min at 80% B.

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