Synthesis and Hybridization Ability of Oligodeoxyribonucleotides Incorporating *N*-Acyldeoxycytidine Derivatives

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Oligodeoxynucleotides [**11a–i**: $d(T_6XT_6)$] incorporating various *N*-acyldeoxycytidine derivatives (X) have been synthesized through use of a new DBU-labile 4,5-dichlorophthaloyl linker on polymer supports. The hybridization capabilities of these oligonucleotides with the complementary $d(A_6GA_6)$ were examined with the aid of *T*m experiments. It turned out that the *T*m values of DNA duplexes decreased significantly with an increase in the number of the methylene groups in the aliphatic acyl group introduced into one strand of the DNA duplex. Ab initio MO calculations and ^1H NMR analysis suggested that the acyl groups in the tested derivatives were oriented in a manner that made the formation of conventional Watson–Crick-type (W–C-type) base pairs with the guanine residue possible, with the help of an intramolecular hydrogen bond between the amide carbonyl oxygen atom and the 5-vinyl proton of the *N*-acylcytosine residue. Moreover, all the aromatic *N*-acyl groups were found markedly to decrease the thermal stability of the DNA duplexes. Ab initio calculations suggested that the base pairs formed between 4-*N*-acyl-1-methylcytosine derivatives and 9-methylguanine have wholly planar structures as their most stable geometries. Detailed studies of the hydrogen-bond energy of the modified base pairs also suggested that the electronic repulsion between the heteroatom of the acyl group of X and the guanine residue resulted in significant destabilization of the X-G base pair and that the hydrogen-bond network structure of water molecules around the major groove of the DNA duplex is a key factor in the stabilization of DNA duplexes.

Introduction

4-*N*-Acetylcytidine and its 2′-*O*-methylated derivatives are naturally occurring modified nucleosides, found in the first letter of the anticodon loop and in the twelfth position of the D-stem of *E. coli* $tRNA^{Met}$,[1] in thermophilic Archaebacteria tRNA,[2] and in various other tRNAs and rRNAs.[3−10] From the well-known codon−anticodon interaction between mRNA and tRNA, this minor nucleoside base (4-*N*-acetylcytosine) was expected to recognize only the guanine base on mRNA.[11] With this background, we studied whether oligodeoxyribonucleotides containing 4-*N*-acetylcytosine bases were able, like their counterparts containing *N*-unmodified cytosine bases, to hybridize with their complementary DNA strands possessing guanine bases at the opposite sites.[12] In our previous paper,[12] we reported that the presence of 4-*N*-acetyl groups on cytosine bases in oligodeoxynucleotides did not actually impair the duplex stability relative to that of the natural type duplexes, whilst in some cases the thermal stability of duplexes even increased, to a degree of 0.7 °C per modification.

Here we report the synthesis and hybridization capabilities of oligodeoxyribonucleotides containing various *N*-acylated cytosine residues.

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Results and Discussion

Synthesis of Oligodeoxynucleotides Incorporating *N*-Acylated Deoxycytidine Derivatives

The key intermediates − 4-*N*-acyl-5′-*O*-(4,4′-dimethoxytrityl)deoxycytidine derivatives **3a−c** − required for incorporation of these *N*-acyldeoxycytidine derivatives into $d(T_6XT_6)$ were obtained in high yields by treatment of deoxycytidine hydrochloride (**1**) with the corresponding acid anhydride in the presence of triethylamine in DMF at 60 °C for 6 h,[13] followed by in situ treatment of the resulting *N*-acylated products **2a−c** with 4,4′-dimethoxytrityl chloride in pyridine (Method A of Scheme 1).

Six 4-*N*-aroyldeoxycytidine derivatives **2d−i**, on the other hand, were synthesized by trimethylsilylation followed by *N*-acylation with acyl chlorides, as shown in Method B in Scheme 1. These products could easily be extracted and purified by silica gel column chromatography. Compounds **2d−i** were further converted into the 5′-*O*-DMTr derivatives **3d−i** in the usual way. The *H*-phosphonate units **4a−i** were synthesized by phosphitylation of **3a−i** with 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one[14] followed by hydrolysis (Scheme 1).

Oligodeoxynucleotides incorporating *N*-benzoyldeoxycytosine units had been synthesized by Fraser et al.,[15] who used a diisopropylsilanediyl linker for their solid-supported synthesis. However, this method was not applicable to the synthesis of $d(T_6XT_6)$ **11a−i** in which X is an *N*-acyldeoxycytidine **2a−i**; the isolated yield of the modified oligomer **11a**, for example, was extremely poor. Our results appar-

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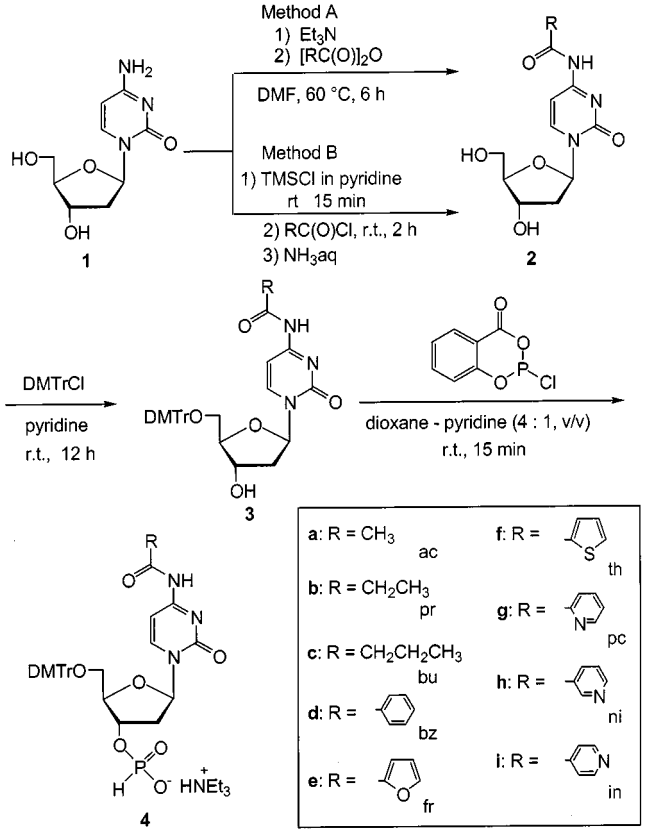
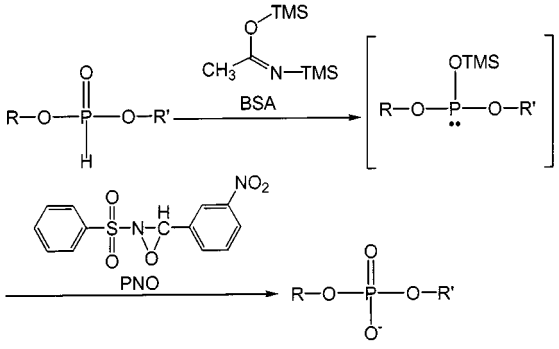


Scheme 1

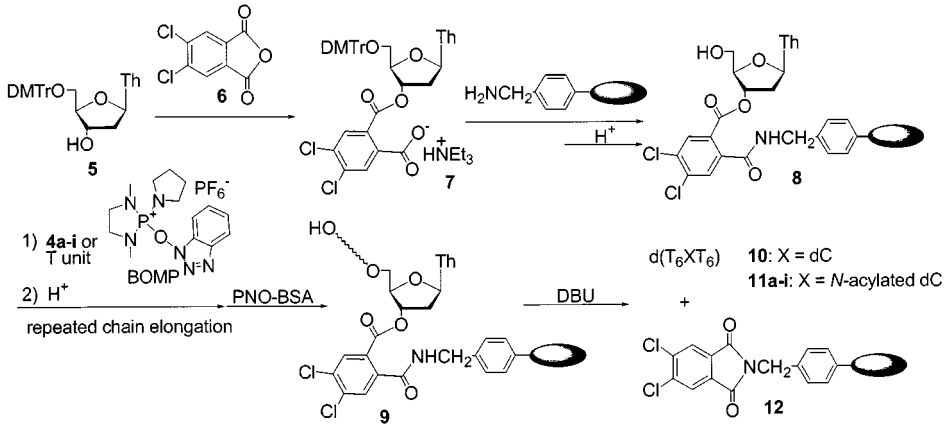
ently indicated that serious side reactions occurred during the synthesis,[16] and the original paper[15] did not actually give any details of the yields of the oligodeoxynucleotides incorporating 4-*N*-benzoyldeoxycytidines. To overcome this problem, we therefore developed a new linker: 4,5-dichlorophthaloyl.[12] This linker was stable during the chain elongation and, when a highly cross-linked polystyrene (HCP) resin[17] was employed for the solid-phase synthesis, it could be removed within 5 min by treatment of the polymer support **9** with 10% DBU in CH_3CN at room temperature, through an intramolecular cyclization with formation of a phthalimide derivative **12** to release the oligodeoxynucleotides (Scheme 2). When an unsubstituted phthaloyl linker was used, 30 min were required for complete cleavage. The thymidine-loaded HCP resin **8** was synthesized by a two-step procedure. Firstly, treatment of 5′-*O*-(4,4′-dimethoxytrityl)thymidine (**5**) with 4,5-dichlorophthalic anhydride (**6**) in pyridine in the presence of 4-(dimethylamino)pyridine (DMAP) gave triethylammonium 5′-*O*-(4,4′-dimethoxytrityl)thymidine-3′-yl (4,5-dichloro)phthalate (**7**) in 96% yield. This compound was then condensed with the amino group (35 μmol/g) of the HCP resin in CH_2Cl_2 in the presence of DCC to give the desired resin after detritylation (**8**: 13.7 μmol/g).

In the solid-phase synthesis of d(T_6XT_6) **11a−i** with X = **2a−i**, the chain elongation was carried out by the modified *H*-phosphonate approach, with the use of 2-(benzotriazol-1-yloxy)-1,1-dimethyl-2-(pyrrolidin-1-yl)-1,3,2-diazaphospholidinium hexafluorophosphate (BOMP) as a powerful condensing agent.[18] For oxidation of the P−H bond of the resulting *H*-phosphonate esters, 2-(phenylsulfonyl)-3-(3-nitrophenyl)oxaziridine (PNO)[19] was used as an effective oxidizing agent in the presence of *N*,*O*-bis(trimethylsilyl)-acetamide (BSA), as shown in Scheme 3. At the final stage in the solid-phase synthesis, *N*-acylated oligodeoxynucleotides were released by treatment with DBU and purified by anion-exchange HPLC to give sufficient amounts of oligodeoxyribonucleotides **11a−i**, as summarized in Table 1.

The structures of these modified oligodeoxynucleotides were confirmed by MALDI TOF mass spectrometry. A typical example of the HPLC profile of the crude material



Scheme 3



Scheme 2

Table 1. Synthesis and hybridization capability of DNA 13mers containing 4-*N*-acyl-2′-deoxycytidine derivatives

Sequence	Compd.	Isolated yield (%)	Hybridization *T*m	Δ*T*m
d(TTTTTTCTTTTTT)	**10**	–	43.6	–
d(TTTTTTC^{ac}TTTTTT)	**11a**	23	44.0	0.4
d(TTTTTTC^{pr}TTTTTT)	**11b**	30	40.9	−2.7
d(TTTTTTC^{bu}TTTTTT)	**11c**	32	37.8	−5.8
d(TTTTTTC^{bz}TTTTTT)	**11d**	27	37.0	−6.6
d(TTTTTTC^{fr}TTTTTT)	**11e**	30	36.8	−6.8
d(TTTTTTCthTTTTTT)	**11f**	20	34.8	−8.8
d(TTTTTTC^{pc}TTTTTT)	**11g**	39	35.5	−8.1
d(TTTTTTC^{nc}TTTTTT)	**11h**	27	36.3	−7.3
d(TTTTTTC^{in}TTTTTT)	**11i**	19	37.8	−5.8

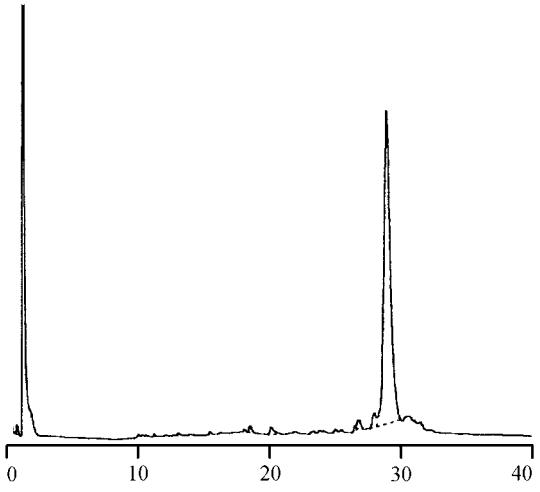


Figure 1. Anion-exchange HPLC profile of crude **11f**

(dT_6CthT_6) (**11f**) obtained after deprotection is shown in Figure 1.

Hybridization Ability of Modified Oligodeoxynucleotides

The thermal stabilities of the duplexes formed between the *N*-acylated oligodeoxynucleotides [**11a−i**: $d(T_6XT_6)$, X = *N*-acylated dC **2a−i**] and the DNA 13mer $d(A_6GA_6)$ were examined. These experiments were carried out at pH = 7.0 in 10 mM phosphate buffer in the presence of 1 M NaCl.

To our surprise, the duplexes incorporating 4-*N*-propionyldeoxycytidine (**2b**) or 4-*N*-butyryldeoxycytidine (**2c**) had stabilities considerably lower than that of the duplex containing 4-*N*-acetyldeoxycytidine (**2a**) (Figure 2, **A**, and Table 1). On addition of a methylene group to the acetyl group, the *T*m value dropped by approximately 3 °C. Thus, it turned out that introduction of a butyryl group into the 4-*N* position in dC resulted in sharp decrease of up to 5.8 °C in the *T*m value of the duplex.

It was found that introduction of a benzoyl group into the cytosine residue of $d(A_6GA_6)/d(T_6CT_6)$ destabilized the DNA duplex considerably, with a substantial decrease in *T*m, of 6.6 °C, being observed. The duplex between $d(A_6GA_6)$ and $d(T_6C^{fr}T_6)$ (**11e**), containing a 4-*N*-furoyldeoxycytidine unit (**2e**: dC^{fr}), was also destabilized to the same degree (by 6.8 °C), as shown in Figure 2 (**B**) and Table 1. The use of dCth **2f**, with a thenoyl group, resulted in a more significant decrease (8.8 °C) in the *T*m value, down to 34.8 °C.

The thermal stabilities of the duplexes between $d(A_6GA_6)$ and oligodeoxynucleotides **11g−i**, possessing 4-*N*-(2-picolyl)deoxycytidine (dC^{pc}) (**2g**), 4-*N*-nicotinoyldeoxycytidine (dC^{nc}) (**2h**), and 4-*N*-isonicotinoyldeoxycytidine (dC^{in}) (**2i**) substituents, were also examined. The presence of the 2-picolyl group resulted in a sharp drop of 8.1 °C in the *T*m value, while the nicotinoyl (nc) group produced a more moderate drop (5.8 °C), as shown in Figure 2 (**C**) and Table 1. The isonicotinoyl (in) group affected the duplex stability to a degree (7.3 °C) intermediate between those of the 2-picolyl and the nicotinoyl (ni) groups. The position of the nitrogen atom in the pyridine ring thus had a pronounced effect on the duplex stability.

Structural Requirements for Preservation of Hybridization Ability in *N*-Acylated Cytosine Residues

Parthasarathy reported that the X-ray crystal structure of 4-*N*-acetylcytidine showed intramolecular hydrogen bonding between the carbonyl oxygen atom and the 5-vinyl proton.[20,21] Actually, the signal of the 5-proton of this nucleoside exhibited a downfield shift of $\delta \approx 1.21$ in the ^{1}H NMR spectrum.[20] In our hands, the ^{1}H NMR spectrum of its deoxy counterpart **2a** showed a lower magnetic field shift ($\Delta\delta \approx 1.5$) of the 5-vinylic hydrogen atom to $\delta \approx 7.2$ from the chemical shift $\delta = 5.70$ of deoxycytidine, as summarized in Table 2. It is reasonable to postulate that this inherent orientation of the acetyl group on the cytosine residue enabled oligodeoxynucleotides containing **2a** to form the usual W−C-type base pairs between the 4-*N*-acetylcytosine and guanine bases, so that they exhibited better hybridization properties than the unmodified oligodeoxynucleotides, as reported previously.[12] It is likely that the 4-*N*-propionyldeoxycytidine (**2b**) and 4-*N*-butyryldeoxycytidine (**2c**) moieties in **11b** and **11c**, respectively, may give rise to similar W−C-type hydrogen bonds at the modified position, as evidenced by the clear-cut low magnetic field shifts of the 5-vinyl protons of **2b** and **2c**, as shown in Table 2. It is also to be expected that the same would be true for other aromatic *N*-acyldeoxynucleosides, since these nucleosides exhibit similar low-field shifts of the resonance signals of their 5-vinyl protons.

The capability of the *N*-acetylcytosine residue to form the W−C-type base pair is also supported by the theoretical ab initio MO calculation reported in the previous paper, which showed that the most stable geometry-optimized structure of 4-*N*-acetyl-1-methylcytosine is that with an intramolecular hydrogen bond between the amide carbonyl oxygen atom and the 5-vinylic proton.[12] This conformer was 3.27

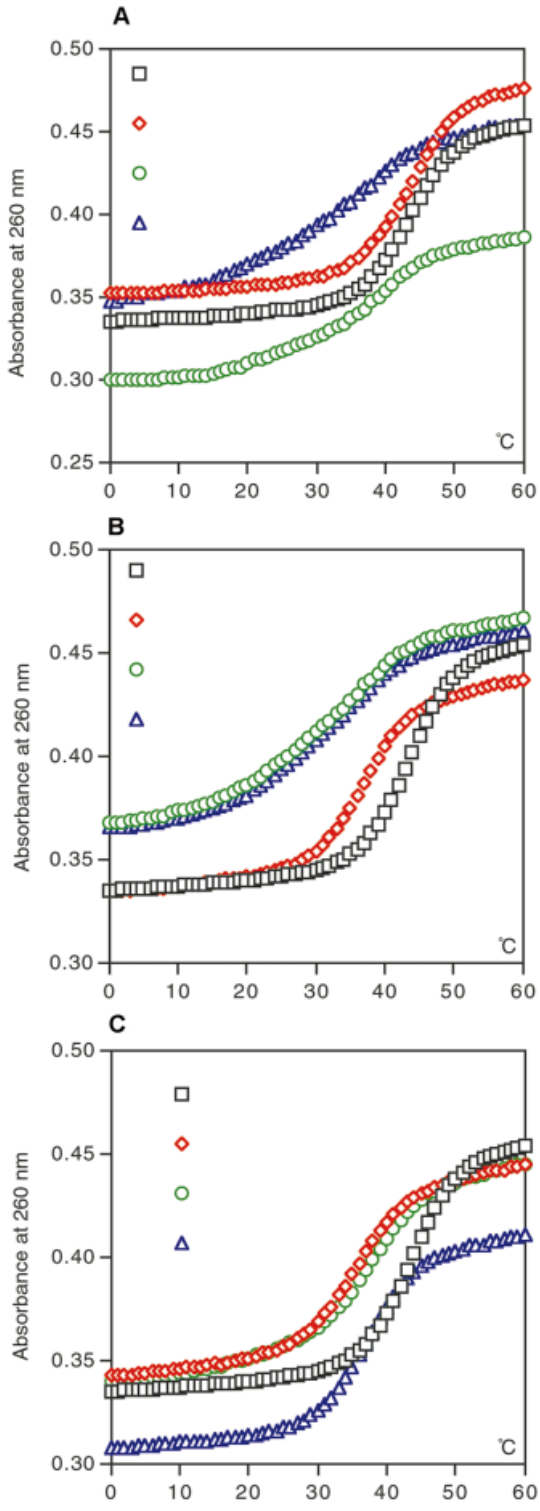


Figure 2. Melting temperature curves of DNA duplexes incorporating 4-*N*-acyldeoxycytidine: panel A: square: normal duplex between d(T_6CT_6) **10** and d(A_6GA_6); diamond: duplex between d($T_6C^{ac}T_6$) **11a** and d(A_6GA_6); circle: duplex between d($T_6C^{pr}T_6$) **11b** and d(A_6GA_6); triangle, duplex between d($T_6C^{bu}T_6$) **11c** and d(A_6GA_6); panel B: square: normal duplex between d(T_6CT_6) **10** and d(A_6GA_6); diamond: duplex between d($T_6C^{bz}T_6$) **11d** and d(A_6GA_6); circle: duplex between d($T_6C^{fr}T_6$) **11e** and d(A_6GA_6); triangle, duplex between d(T_6CthT_6) **11f** and d(A_6GA_6); panel C: square: normal duplex between d(T_6CT_6) **10** and d(A_6GA_6); diamond: duplex between d($T_6C^{pc}T_6$) **11g** and d(A_6GA_6); circle: duplex between d($T_6C^{nc}T_6$) **11h** and d(A_6GA_6); triangle: duplex between d($T_6C^{in}T_6$) **11i** and d(A_6GA_6)

Table 2. The ^{31}P NMR chemical shifts of the 5-vinylic protons in 4-*N*-acylated deoxycytidine derivatives ([D_5]DMSO)

Compd.	δ value	Compd.	δ value
dC	5.70	**2e**	7.27
2a	7.18	**2f**	7.27
2b	7.21	**2g**	7.38
2c	7.21	**2h**	7.31
2d	7.36	**2i**	7.33

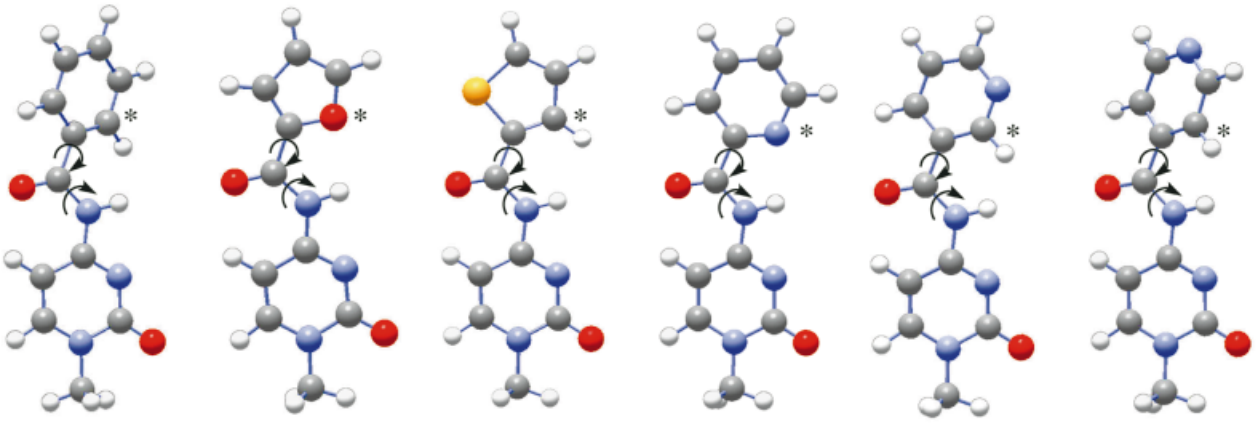
kcal/mol more stable than the second most stable conformer. This great gap between the stabilities of these conformers strongly suggests that the *N*-acetyl-1-methylcytosine exists virtually entirely in this form, as attested by the NMR analysis of **2a**.

Among the oligodeoxynucleotides **11a−i** incorporating *N*-acyldeoxycytidines **2a−i**, only DNA oligomer **11a**, containing *N*-acetyldeoxycytidine (**2a**), showed a slight stabilization effect on DNA duplex formation. Any lengthening of the alkyl chain of the acetyl group resulted in a decrease in the thermal stability of the corresponding DNA duplex. A similar tendency towards decreasing *T*m values has been reported in a paper by Thuong et al., concerning the thermal stabilities of DNA duplexes containing 4-*N*-alkyldeoxycytidine units.[22] These worker observed that when the *N*-alkyl chain on the *exo* amino group of the cytosine base became longer, the thermal stability of DNA duplexes decreased.

Most Stable Geometry-Optimized Structures of 4-*N*-Acyl-1-methylcytosine Derivatives and Base Pairs with 9-Methylguanine Evaluated by Ab Initio MO Calculation

To interpret the results described above, we calculated the most stable geometry-optimized structures of 4-*N*-acyl-1-methylcytosine derivatives **13a−i** by ab initio MO calculation at the HF/6-31G* level. These results are shown in Figure 3 (for the results for **13a−c**, see Figure 11 in the Supporting Information). The most stable geometry-optimized structures of the modified base pairs formed between **13a−i** (meC^R where R = acyl groups) and 9-methylguanine (**14**: meG) were also calculated at the same level base set. These results are shown in Figure 4. In the case of the aliphatic acyl groups, the hydrogen-bond energies are similar (−26.12 to −26.90 kcal/mol) to each other, and so these aliphatic acyl groups did not affect the W−C-type hydrogen bonds essentially. However, the thermal stabilities of the duplexes formed between d(T_6XT_6) **11b** and **11c** (X = **2b**, **2c**) and d(A_6GA_6) decreased with increasing alkyl chain length in the acyl group. From these results, it seems likely that the aliphatic alkyl group disturbs the hydrogen-bond network structure around the major groove of the DNA duplexes. Williams reported that such a water bridge structure is known to be essential for stabilization of DNA duplexes.[23]

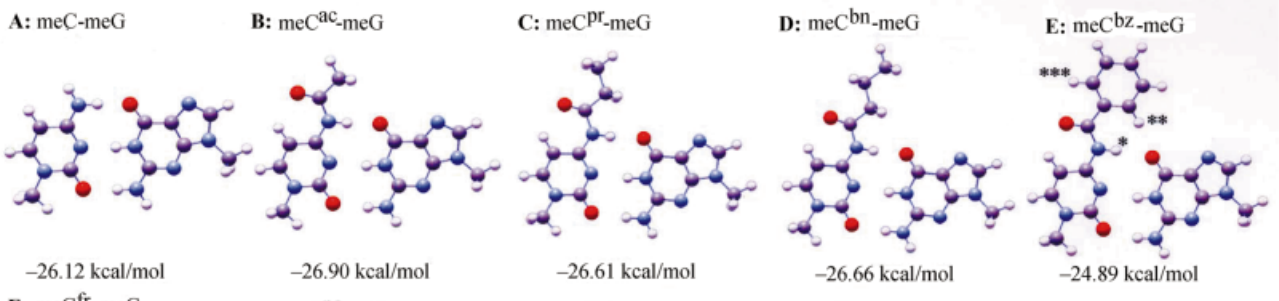
Among the other six 4-*N*-aroyl-1-methylcytosine derivatives, only those with furoyl, thenoyl, and picolyl groups can maintain flat structures suitable for base paring, as shown in Figure 3. Benzoyl, nicotinoyl, and isonicotinoyl groups



meC^{bz} **13d** meC^{fr} **13e** meCth **13f** meC^{pc} **13g** meC^{nc} **13h** meC^{in} **13i**

dihedral	13d	13e	13f	13g	13h	13i
X*-C-C-O (X = C, O or N)	155.0°	180.0 °	180.0 °	180.0 °	157.4 °	155.0 °
O-C-N_4-C_4	6.1°	0.0 °	0.0 °	0.0 °	3.5 °	6.1 °

Figure 3. Most stable geometry-optimized structures of 4-*N*-aroyl-1-methylcytosines determined by ab initio MO calculation at the HF/6-31G* level of theory



distance	A	B	C	D	E	F	G	H	I
NH---O (Å)	1.91	1.89	1.90	1.90	2.05	1.99	2.25	2.02	2.01
CH---O (Å)	-	-	-	-	2.08	2.12	-	2.05	2.07
Mulliken charge									
H*	-	-	-	-	0.52	0.52	-	0.52	0.52
H**	-	-	-	-	0.32	0.34	-	0.34	0.33
H***	-	-	-	-	0.25	-	-	0.25	0.25

Figure 4. Most stable geometry-optimized structures of modified base pairs formed between 4-*N*-acyl-1-methylcytosines and 9-methylguanine determined by ab initio MO calculation at the HF/6-31G* level of theory, the distances between the guanine 6-oxygen atom and one of the aromatic protons of the acyl group capable of hydrogen bonding, and the Mulliken charges of the protons (H*, H**, and H***) marked by asterisks; the values given are the hydrogen-bond energies ΔE, defined as the difference between the energy of the most stable energy-optimized base pair of the 4-*N*-acyl-1-methylcytosine derivatives **13a**–**i** and 9-methylguanine (**14**) and the total energies of the most stable energy-optimized structures of the two components

induced twisted torsion angles around X*−C−C−O and O−C−N_4−C_4. It is interesting that the most stable geometry-optimized structures of the 4-*N*-aroyl-1-methylcytosine derivatives are not always those used in the base-pairing with 9-methylguanine (**14**). As demonstrated in the case of the base pairs meC^{fr}-meG and meC^{nc}-meG, the orientations of the aromatic rings of the furoyl and nicotinoyl groups were different, with complete rotation around X*−C−C−O (Figure 3). In any event, the calculated structures of the base pairs were wholly planar, as shown in Figure 4. Except for the meC^{nc}-meG base pair, the other base pairs showed relatively low hydrogen-bond energies compared with the C-G base pair. This tendency is basically in agreement with the low *T*m values experimentally observed in several DNA duplexes containing 4-*N*-aroyldeoxycytidine derivatives. In particular, the meC^{pc}-meG base pair, computed to have the lowest hydrogen-bond energy among the calculated base pairs, did actually exhibit the lowest *T*m value, as shown in Table 1. A serious factor in this case appears to be electronic repulsion between the nitrogen atom of the pyridine ring and the 6-carbonyl oxygen atom of the guanine residue. This interaction gives rise to strained base pairs. In the meC^{fr}-meG base pair, the orientation of the oxygen atom of the furoyl ring was the reverse of that seen in the monomeric meC^{fr} (**13e** in Figure 3), but in the base pair a new electronic repulsion had arisen between the oxygen and the carbonyl oxygen atom, so that the hydrogen-bond energy was lowered to the second worst value. In meC^{bz}, meC^{nc}, and meC^{in}, on the other hand, one of the aromatic protons can approach the guanine carbonyl oxygen atom within hydrogen-bonding distances, so that this weak interaction can compensate for the loss of energy due to the conformational change from the twist form of these bases to the flat form upon base-paring. The distances between the aromatic proton and the carbonyl oxygen atom are calculated as 2.05 Å, 2.02 Å, and 2.01 Å for meC^{bz}-meG, meC^{nc}-meG, meC^{in}-meG, respectively, as shown in Figure 4 (**E**, **H**, **I**). Actually, electron-density analysis of the base pairs indicated that these protons marked as H** in Figure 4 are positively charged, with Mulliken charges of +0.32, +0.34, and +0.33 for meC^{bz}-meG, meC^{nc}-meG, and meC^{in}-meG, respectively. It is apparent that these protons are more positively charged than the reverse side protons marked as H***, which uniformly have Mulliken charges of 0.25. On the other hand, the protons (H*) of the amide groups each have a Mulliken charge of 0.52 each in these three base pairs. These results strongly suggest that the decrease in the *T*m values in the case of the aromatic acyl groups might be attributable to poor levels of stabilization through hydrogen-bonding energies.

In the *T*m experiments, it was also observed that the location of the nitrogen atom in the pyridinecarbonyl group is important for stabilization of X-G base pairs. Among the duplexes made up of d(T_6XT_6) **11g−i** and d(A_6GA_6), the one containing the isonicotinoyl group is more stable than the others, more stable even (as far as the *T*m values are concerned) than a duplex incorporating the benzoyl group, as shown in Table 1. This result implies that the 4-nitrogen atom contributes to stabilization of the hydrogen-bond network around the major groove, relative to that seen with a benzoyl substituent. In contrast, the worst result was obtained in the case of the picolyl group. This may be explained by the fact that the 2-nitrogen atom of the picolyl group cannot contribute to the outer hydrogen-bonding network in the major groove.

To understand these results, we carried out molecular mechanics computer simulations of these DNA duplexes, using AMBER* with the GB/SA water solvation model.[28] These simulations suggested that the substituents extending from the *exo*-amino group of the cytosine ring are positioned in the direction of the major groove in such a manner that the hydration structure of water molecules[22] is destroyed (see Figure 10 in Supporting Information). In this regard, the (smallest) acetyl group is superior to the other acyl groups. This simulation model also implies that a wholly flat structure in the 4-*N*-aroylcytosine residues does not contribute to stabilization of the duplex, since the acyl residues only extend into the major groove so that the base stacking interaction is impossible.

Conclusion

From these results, it appears likely that the preservation of the hydration structure around the major groove is crucial for the design of *N*-modified deoxycytidines that will maintain the thermal stability. It may also be concluded that planarity of the *N*-aroylcytosine moiety does not essentially contribute to stabilization of DNA duplexes.

Since the major groove provides adequate space for modification of the cytosine amino group, some functional group such as fluorescence and biotin groups can be introduced through the amide linkage, but a certain destabilization effect should be taken into account when such strategies are designed in future.

This *N*-acyl mode for modification of cytosine may provide an alternative to the conventional use of the 5-position of the cytosine base for substitution or introduction of functional groups for gene diagnosis or a variety of physicochemical studies. In applications of work of this kind, it should be taken into account that substituents of the acyl type should be designed not to interfere with this hydration structure and to avoid electronic repulsion between the guanine residue and the substituents. Further studies in this direction are underway.

Experimental Section

General Remarks: ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded at 270, 68, and 109 MHz, respectively. The chemical shifts were measured from tetramethylsilane or DSS for ^{1}H NMR spectra, $CDCl_3$ ($\delta = 77$) or DSS ($\delta = 0$) for ^{13}C NMR spectra, and 85% phosphoric acid ($\delta = 0$) for ^{31}P NMR spectra. UV spectra were recorded with a U-2000 spectrometer. MALDI TOF mass spectra were taken with a Mariner™ spectrometer (PerSeptive Biosystems). Column chromatography was performed with C-200 silica gel purchased from

Wako Co. Ltd., and an aquarium minipump could conveniently be used to attain sufficient pressure for rapid chromatographic separation. Anion-exchange HPLC was done on a Gen-Pak™ FAX column (Waters, 4.6 × 100 mm) with an LC module 1 with a Waters M-741 data module and a Waters column heater, with a 10−77% linear gradient of 25 mM phosphate, 1 M sodium chloride buffer (pH = 6.0) in 25 mM phosphate buffer (pH = 6.0) at 50 °C, at a flow rate of 1.0 mL/min for 30 min. Pyridine was distilled twice from *p*-toluenesulfonyl chloride and from calcium hydride and then stored over 4-Å molecular sieves. Elemental analyses were performed by the Microanalytical Laboratory, Tokyo Institute of Technology at Nagatsuta.

Computational Methods: All ab initio molecular orbital calculations were carried out by using the Gaussian 98 program with an Origin2000/256 supercomputer. The results were analyzed using Gauss View (version 2.0, Gaussian, Inc.) software with a Silicon Graphics Inc. IMPACT O2 workstation. Geometry optimizations and energy calculations were carried out at the HF/6-31G* level. The molecular mechanics simulations of $d(T_4C^*T_4)/d(A_4GA_4)$ were carried out under the following conditions. The initial structures were constructed by use of the structure implemented in Macro Model (version 6.0). The acyl moieties on cytidine derivatives were fixed in the appropriate conformation by application of the geometry obtained by ab initio calculations. The force field used was the AMBER* one, with modification of the distance-dependent dielectric constant to the constant dielectric. The effect of the solvent was included by use of the GB/SA solvent model implemented in the software.

Triethylammonium 5′-*O*-(4,4′-Dimethoxytrityl)thymidine-3′-yl-(4,5-dichloro)phthalate (7): 5′-*O*-(4,4′-Dimethoxytrityl)thymidine (**5**: 1.09 g, 2 mmol) was dried by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (20 mL). 4,5-Dichlorophthalic anhydride (**6**: 1.30 g, 6 mmol) and 4-(dimethylamino)pyridine (12.2 mg, 0.1 mmol) were added to this solution, and the mixture was stirred at room temperature for 6 h. The mixture was concentrated to half its original volume, diluted with $CHCl_3$, and washed three times with 5% $NaHCO_3$ (aq), and the aqueous layer was back-extracted with $CHCl_3$. The organic layer and washings were combined, dried with Na_2SO_4, filtered, and concentrated to dryness under reduced pressure. The residue was subjected on a silica gel column. Chromatography was performed with $CHCl_3$ containing 1% triethylamine, applying a gradient of methanol (1−2%), to give **7** (1.66 g, 96%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 1.28 (t, 9 H, ^3J = 7.3 Hz), 1.34 (s, 3 H), 2.47 (m, 1 H), 2.74 (m, 1 H), 3.08 (q, 6 H, ^3J = 7.2 Hz), 3.52 (m, 2 H), 3.79 (s, 6 H), 4.44 (m, 1 H), 5.66 (m, 1 H), 6.43 (dd, 1 H, $^3J_{1',2'}$ = 8.9, $^3J_{1',2''}$ = 5.6 Hz), 6.85 (d, ^3J = 8.9 Hz, 4 H), 7.12−7.41 (m, 9 H), 7.57 (s, 1 H, 6-H), 7.66 (s, 1 H), 7.93 (s, 1 H). ^{13}C NMR ($CDCl_3$): δ = 8.5, 11.5, 37.5, 45.0, 55.2 63.8, 77.2, 83.3, 84.4, 87.0, 111.5, 113.2, 127.2, 128.0, 128.1, 129.2, 130.1, 130.1, 131.2, 132.5, 134.4, 135.2, 135.6, 138.7, 144.2, 150.3, 158.7, 163.7, 167.6, 170.2. $C_{45}H_{49}Cl_2N_3O_{10}\cdot H_2O$ (880.8): C 61.36, H 5.84, Cl 8.05, N 4.77; found C 61.02, H 5.98, Cl 8.53, N 5.09.

Preparation of Thymidine-Loaded Highly Cross-Linked Polystyrene Support 8: Triethylammonium 5′-*O*-(4,4′-dimethoxytrityl)thymidine-3′-yl-(4,5-dichloro)phthalate (**7**: 70 mg, 87.4 mmol) was dried by repeated coevaporation with dry pyridine and finally dissolved in dry CH_2Cl_2 (2 mL). Dicyclohexylcarbodiimide (90 mg, 437.5 mmol) and HCP (500 mg 17.5 μmol) were added to the solution. The mixture was kept at room temperature for 3 h. The solid support was filtered and washed with pyridine. Unreacted amino groups were blocked by treatment with 10% $(CH_3CO)_2O$ in pyridine. The resulting support **8** was filtered, washed with pyridine, and dried. The degree of thymidine loading was estimated by DMTr cation assay as 13.7 μmol/g.

Evaluation of the Stability of the DMT-T-3′-*O*-(4,5-Dichloro)-phthaloyl Support Towards DBU: The DMT-T-3′-*O*-(4,5-dichloro)-phthaloyl support (9 mg) was suspended in 10% DBU in CH_3CN. At intervals, the support was filtered and washed with pyridine and CH_3CN. The support was treated with 1% TFA in CH_2Cl_2, and the TFA solution was filtered. The filtrate was concentrated to dryness, and the residue was dissolved in perchloric acid/ethanol (3:2, v/v) to estimate the amount of released DMTrT by measurement of the UV absorbance at 498 nm (ε = 71700).

Typical Procedure for Solid-Phase Synthesis: Each chain-elongation cycle consisted of detritylation [1% TFA in CH_2Cl_2 (1 mL), 15 s], washing (CH_2Cl_2, 5 mL), coupling [0.05 M *H*-phosphonate units, 0.2 M BOMP in pyridine (200 mL); 1.5 min], and washing (pyridine, 5 mL). Generally, the average yield per cycle was estimated as 97−99% by the DMTr cation assay. After chain elongation, the DMTr group was removed by treatment with 1% TFA in CH_2Cl_2 (1 mL) for 15 s, and the resin was washed with CH_2Cl_2. The *H*-phosphonate oligomer on the HCP resin was oxidized by treatment with a mixture of PNO (0.2 M) and BSA (0.5 M) in CH_3CN (400 μL) for 10 min. After having been washed with CH_2Cl_2, the oligomer was released from the HCP resin by treatment with 10% DBU in CH_3CN (500 μL) for 5 min. The HCP resin was removed by filtration and washed with CH_3CN. The filtrate was neutralized with 50% CH_3COOH in CH_3CN (1 mL), desalted on Dowex 50 W (NH_3 form), and purified by anion-exchange HPLC. Oligomers were analyzed by nuclease P1 digestion and MALDI TOF mass spectrometry.

***T*m Measurement:** An appropriate oligonucleotide (2.0 μmol) and its complementary DNA 13mer $d(A_6GA_6)$ (2.0 μmol) were dissolved in a buffer consisting of NaCl (1.0 M), sodium phosphate (10 mM), and EDTA (0.1 mM), adjusted to pH = 7.0. The solution containing the oligonucleotides was kept at 60 °C for 10 min for complete dissociation of the duplex into single strands, cooled at the rate of −1.0 °C/min, and kept at 0 °C for 10 min. After that, the melting temperatures (*T*m) were determined at 260 nm, using a Hitachi U-2000 at the rate of 1.0 °C/min.

5′-*O*-(4,4′-Dimethoxytrityl)-4-*N*-propionyl-2′-deoxycytidine (3b): Deoxycytidine hydrochloride (2.68 g, 10 mmol) was dried by repeated coevaporation with dry *N*,*N*-dimethylformamide and was finally dissolved in dry *N*,*N*-dimethylformamide (100 mL). Triethylamine (1.39 mL, 10 mmol) was added to the solution, and the mixture was stirred at room temperature for 30 min. Propionic anhydride (1.42 mL, 11 mmol) was added to the mixture. After the mixture had been stirred at 60 °C for 6 h, the solvent was removed under reduced pressure. The residue was dried by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (100 mL). 4,4′-Dimethoxytrityl chloride (4.07 g, 12 mmol) was added to the solution, and the mixture was stirred at room temperature for 12 h. The mixture was concentrated to half its original volume, diluted with $CHCl_3$, and washed three times with 5% $NaHCO_3$ (aq), and the aqueous layer was back-extracted with $CHCl_3$. The organic layer and the washings were combined and dried with Na_2SO_4, filtered, and concentrated to dryness under reduced pressure. The residue was subject on a silica gel column. Chromatography was performed with $CHCl_3$ containing 1% pyridine, applying a gradient of methanol (1−2%) to give **3b** (5.19 g, 89%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 1.18 (t, 3 H, ^3J = 7.6), 2.24 (m, 1 H, 2′-H), 2.24 (q, ^3J = 7.6 Hz, 2 H), 2.76 (m,

1 H), 3.45 (m, 2 H), 3.81 (s, 6 H), 4.14 (m, 1 H), 4.50 (m, 1 H), 6.29 (dd, 1 H, $^3J_{1',2'}$ = 5.9, $^3J_{1',2''}$ = 5.9 Hz), 6.85 (d, ^3J = 7.9 Hz, 4 H), 7.20−7.42 (m, 10 H, 5-H), 8.23 (d, ^3J = 7.6 Hz, 1 H). ^{13}C NMR ($CDCl_3$): δ = 8.5, 30.2, 41.9, 55.0, 62.7, 70.8, 77.2, 86.5, 86.6, 87.3, 96.6, 113.1, 126.9, 127.8, 127.9, 129.8, 129.9, 135.1, 135.3, 144.0, 144.4, 155.5, 158.4, 162.5, 174.2. MS: *m*/*z* calcd. for ($C_{33}H_{35}N_3O_7$ + H) [M + H^+] 586.2553, found 586.2553.

4-*N*-Butyryl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine (3c): A procedure similar to that described above was performed using deoxycytidine hydrochloride (2.68 g, 10.0 mmol) and butyric anhydride (1.80 mL, 11 mmol), to give **3c** (5.02 g, 84%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 0.87 (t, ^3J = 7.3 Hz, 3 H), 1.58 (m, 2 H, *N*-butyryl), 2.14 (m, 1 H), 2.28 (t, 2 H, ^3J = 7.3), 2.76 (m, 1 H), 3.35 (m, 2 H), 3.71 (s, 6 H), 4.10 (m, 1 H), 4.43 (m, 1 H), 6.23 (dd, 1 H, $^3J_{1',2'}$ = 5.6, $^3J_{1',2''}$ = 5.6 Hz), 6.77 (d, ^3J = 8.6 Hz, 4 H), 7.14−7.34 (m, 10 H, 5-H), 8.15 (d, ^3J = 7.3 Hz, 1 H). ^{13}C NMR ($CDCl_3$): δ = 13.4, 18.1, 39.1, 42.0, 55.1, 62.8, 70.8, 77.2, 86.5, 86.7, 87.4, 96.6, 113.2, 127.0, 127.9, 128.0, 129.9, 129.9, 135.3, 135.4, 144.1, 144.5, 155.5, 158.5, 162.4, 173.4. $C_{34}H_{37}N_3O_7$ (599.7): calcd. C 68.10, H 6.22, N 7.01; found C 68.10, H 6.38, N 6.81.

4-*N*-Furoyl-2′-deoxycytidine (2e): Deoxycytidine hydrochloride (804 mg, 3 mmol) was dried by repeated coevaporation with dry pyridine and finally suspended in dry pyridine (25 mL). Chlorotrimethylsilane (1.90 mL, 15.0 mmol) was added to the solution. After the solution had been stirred for 15 min, 2-furoyl chloride (1.48 mL, 15 mmol) was added, and the reaction was maintained at room temperature for 2 h. The mixture was then cooled in an ice bath, and water (3 mL) was added. After 5 min, aqueous ammonia (29%, 10 mL) was added, and the mixture was stirred at room temperature for 15 min. The reaction mixture was then concentrated to dryness, and the residue was dissolved in water (45 mL). The solution was washed with a 15-mL portion of ethyl acetate. Crystallization began immediately after separation of the layers. Cooling of the solution gave 4-*N*-furoyl-2′-deoxycytidine (684 mg, 71%). M.p. 153−155 °C. ^{1}H NMR ([D_6]DMSO): δ = 2.03 (m, 1 H), 2.29 (m, 1 H), 3.59 (m, 2 H), 3.85 (m, 1 H), 4.14 (m, 1 H), 5.05 (m, 1 H), 5.26 (m, 1 H), 6.11 (dd, 1 H, $^3J_{1',2'}$ = 6.3, $^3J_{1',2''}$ = 6.3 Hz), 6.70 (m, 1 H), 7.27 (d, ^3J = 7.3 Hz, 1 H), 7.71 (m, 1 H), 7.98 (m, 1 H), 8.36 (d, ^3J = 7.3 Hz, 1 H). ^{13}C NMR ([D_6]DMSO): δ = 41.1, 61.1, 70.1, 86.4, 88.1, 96.1, 112.5, 117.4, 145.2, 146.3, 147.8, 154.4, 157.7, 162.7. $C_{14}H_{15}N_3O_6$·1/2H_2O (330.3): C 50.91, H 4.88, N 12.72; found C 50.62, H 4.66, N 12.70.

4-*N*-Thenoyl-2′-deoxycytidine (2f): A procedure similar to that described above was performed with deoxycytidine hydrochloride (804 mg, 3 mmol) and 2-thenoyl chloride (1.59 mL, 15 mmol), to give **2e** (556 mg, 55%). M.p. 205−208 °C. ^{1}H NMR ([D_6]DMSO): δ = 2.04 (m, 1 H), 2.30 (m, 1 H), 3.59 (m, 2 H), 3.86 (m, 1 H), 4.22 (m, 1 H), 5.07 (m, 1 H), 5.27 (m, 1 H), 6.12 (dd, 1 H, $J_{1',2'}$ = 6.3, $^3J_{1',2''}$ = 6.3 Hz), 7.21 (m, 1 H), 7.27 (d, ^3J = 7.3 Hz, 1 H), 7.96 (m, 1 H), 8.25 (m, 1 H), 8.36 (d, ^3J = 7.3 Hz, 1 H). ^{13}C NMR ([D_6]DMSO): δ = 41.1, 61.1, 70.1, 86.4, 88.1, 96.2, 128.8, 131.8, 134.4, 138.8, 145.1, 154.3, 162.1, 162.8. $C_{14}H_{15}N_3O_5S$·1/2H_2O (346.4): C 48.55, H 4.66, N 12.13, S 9.26; found C 48.81, H 4.36, N 12.53, S 9.09.

4-*N*-Picolyl-2′-deoxycytidine (2g): Deoxycytidine hydrochloride (**1**: 804 mg, 3 mmol) was dried by repeated coevaporation with dry pyridine and finally suspended in dry pyridine (25 mL). Chlorotrimethylsilane (1.90 mL, 15.0 mmol) was added to the solution. After the solution had been stirred for 15 min, 2-picolyl chloride hydrochloride (2.67 g, 15 mmol) was added and the reaction mixture was maintained at room temperature for 2 h. The mixture was then cooled in an ice bath and water (3 mL) was added. After 5 min, aqueous ammonia (29%, 10 mL) was added and the mixture was stirred at room temperature for 15 min. The reaction mixture was then concentrated to near dryness and the residue was dissolved in aqueous $NaHCO_3$ (5%, 45 mL) and extracted five times with $CHCl_3$/pyridine (1:1, v/v). The organic layers were combined and dried with Na_2SO_4, filtered, and concentrated to dryness under reduced pressure. The residue was loaded onto a silica gel column. Chromatography was performed with $CHCl_3$ containing pyridine, applying a gradient of methanol (10−15%) to give **2g** (552 mg, 81%) as foam. ^{1}H NMR ([D_6]DMSO): δ = 2.05 (m, 1 H), 2.33 (m, 1 H), 3.60 (m, 2 H), 3.88 (m, 1 H), 4.23 (m, 1 H), 5.08 (t, ^3J = 5.0 Hz, 1 H), 5.28 (d, ^3J = 4.0 Hz, 1 H), 6.11 (dd, 1 H, $^3J_{1',2'}$ = 6.3, $^3J_{1',2''}$ = 6.3 Hz), 7.38 (d, ^3J = 7.6 Hz, 1 H), 7.74 (m, 1 H), 8.07−8.19 (m, 2 H), 8.47 (d, ^3J = 7.3 Hz, 1 H) 8.74 (d, ^3J = 0.5 Hz, 1 H). ^{13}C NMR ([D_6]DMSO): δ = 41.2, 61.1, 70.1, 86.7, 88.2, 95.0, 122.9, 128.4, 138.8, 146.1, 147.9, 149.1, 154.5, 161.2, 163.4. $C_{15}H_{16}N_4O_5$ (332.3): C 54.21, H 4.85, N 16.86; found C 53.89, H 4.68, N 16.81.

4-*N*-Nicotinoyl-2′-deoxycytidine (2h): Deoxycytidine hydrochloride (**1**: 804 mg, 3 mmol) was dried by repeated coevaporation with dry pyridine and finally suspended in dry pyridine (25 mL). Chlorotrimethylsilane (1.90 mL, 15.0 mmol) was added to the solution. After the solution had been stirred for 15 min, nicotinoyl chloride hydrochloride (2.67 g, 15 mmol) was added, and the reaction mixture was maintained at room temperature for 2 h. The mixture was then cooled in an ice bath, and water (3 mL) was added. After 5 min, aqueous ammonia (29%, 10 mL) was added and the mixture was stirred at room temperature for 15 min. The reaction mixture was then concentrated to dryness, and the residue was dissolved in water (45 mL). The solution was washed with a 15-mL portion of ethyl acetate. Cooling of the solution and filtration gave 4-*N*-nicotinoyl-2′-deoxycytidine **2h** (684 mg, 71%). M.p. 161−163 °C. ^{1}H NMR ([D_6]DMSO): δ = 2.05 (m, 1 H), 2.31 (m, 1 H), 3.61 (m, 2 H), 3.87 (m, 1 H), 4.23 (m, 1 H), 5.07 (t, ^3J = 5.3 Hz, 1 H), 5.28 (d, ^3J = 4.0 Hz, 1 H), 6.13 (dd, 1 H, $^3J_{1',2'}$ = 6.3, $^3J_{1',2''}$ = 6.3 Hz), 7.31 (d, 1 H, ^3J = 7.3), 7.54 (m, 1 H), 8.31 (d, ^3J = 7.9 Hz, 1 H), 8.41(d, ^3J = 7.6 Hz, 1 H), 8.76 (d, ^3J = 4.6 Hz, 1 H), 9.09(t, ^3J = 1.0 Hz, 1 H). ^{13}C NMR ([D_6]DMSO): δ = 41.1, 61.1, 70.1, 86.5, 88.2, 96.3, 123.6, 129.4, 136.4, 145.3, 149.6, 153.1, 154.4, 162.9, 166.7. $C_{15}H_{16}N_4O_5$·H_2O (350.3): C 51.43, H 5.18, N 15.99; found C 51.18, H 5.09, N 16.38.

4-*N*-Isonicotinoyl-2′-deoxycytidine (2i): Deoxycytidine hydrochloride (**1**: 804 mg, 3 mmol) was dried by repeated coevaporation with dry pyridine and finally suspended in dry pyridine (25 mL). Chlorotrimethylsilane (1.90 mL, 15.0 mmol) was added to the solution. After the solution had been stirred for 15 min, isonicotinoyl chloride hydrochloride (2.67 g, 15 mmol) was added, and the reaction mixture was maintained at room temperature for 2 h. The mixture was then cooled in an ice bath, and water (3 mL) was added. After 5 min, aqueous ammonia (29%, 10 mL) was added, and the mixture was stirred at room temperature for 15 min. The reaction mixture was then concentrated to dryness, and the residue was dissolved in water (45 mL). The solution was washed with a 15-mL portion of ethyl acetate. Crystallization began immediately after separation of the layers. Cooling of the solution gave 4-*N*-nicotinoyl-2′-deoxycytidine **2i** (684 mg, 71%). M.p. 198−200 °C. ^{1}H NMR ([D_6]DMSO): δ = 2.06 (m, 1 H), 2.28 (m, 1 H), 3.60 (m, 2 H), 3.87 (m, 1 H), 4.22 (m, 1 H), 5.08 (t, ^3J = 5.3 Hz, 1 H), 5.28 (d, ^3J = 4.3 Hz, 1 H), 6.12 (dd, 1 H, $^3J_{1',2'}$ = 6.3, $^3J_{1',2''}$ = 6.3 Hz), 7.33 (d, ^3J = 7.3 Hz, 1 H), 7.87 (d, ^3J = 5.3 Hz, 2 H), 8.42 (d, ^3J =

7.3 Hz, 1 H) 8.76 (d, ^3J = 5.6 Hz, 2 H). ^{13}C NMR ([D$_6$]DMSO): δ = 41.1, 61.1, 70.1, 86.5, 88.2, 96.3, 122.2, 140.6, 145.5, 150.5, 154.4, 162.8, 166.5. $C_{15}H_{16}N_4O_5$ (332.3): C 54.21, H 4.85, N 16.86; found C 54.22, H 4.80, N 17.03.

5′-*O*-(4,4′-Dimethoxytrityl)-4-*N*-furoyl-2′-deoxycytidine (3e): 4-*N*-Furoyl-2′-deoxycytidine (**2e**: 481 mg, 1.5 mmol) was dried by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (15 mL). 4,4′-Dimethoxytrityl chloride (610 mg, 1.8 mmol) was added to the solution, and the mixture was stirred at room temperature for 12 h. The mixture was concentrated to half its volume, diluted with $CHCl_3$, and washed three times with 5% aqueous $NaHCO_3$, and the aqueous layer was back-extracted with $CHCl_3$. The organic layer and washings were combined, dried with Na_2SO_4, filtered, and concentrated to dryness under reduced pressure. The residue was loaded onto a silica gel column. Chromatography was performed with $CHCl_3$ containing 1% pyridine, applying a gradient of methanol (0.5−1%), to give **3e** (804 mg, 86%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 2.29 (m, 1 H), 2.76 (m, 1 H), 3.47 (m, 2 H), 3.79 (s, 6 H), 4.17 (m, 1 H), 4.54 (m, 1 H), 6.30 (dd, 1 H, $^3J_{1',2'}$ = 5.9, $^3J_{1',2''}$ = 5.9 Hz), 6.58 (d, ^3J = 7.9 Hz, 4 H), 6.58 (m, 1 H), 7.13−7.42 (m, 11 H, 5-H), 7.54 (m, 1 H, *N*-furoyl), 8.27 (d, ^3J = 7.6 Hz, 1 H). ^{13}C NMR ($CDCl_3$): δ = 42.1, 55.2, 62.8, 70.9, 77.3, 86.7, 86.9, 87.5, 96.5, 113.0, 113.3, 127.1, 127.8, 128.1, 129.2, 130.0, 130.1, 135.3, 135.5, 144.3, 144.9, 145.9, 146.0, 155.3, 158.6, 161.7. $C_{35}H_{33}N_3O_8 \cdot 1/2H_2O$ (632.7): C 66.45, H 5.42, N 6.64; found C 66.59, H 5.55, N 6.62.

5′-*O*-(4,4′-Dimethoxytrityl)-4-*N*-thenoyl-2′-deoxycytidine (3f): A procedure similar to that described above was performed with 4-*N*-thenoyl-2′-deoxycytidine (**2f**: 505 mg, 1.5 mmol) to give **3f** (880 mg, 92%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 2.30 (m, 1 H), 2.76 (m, 1 H), 3.46 (m, 2 H), 3.79 (s, 6 H), 4.18 (m, 1 H), 4.56 (m, 1 H), 6.31 (dd, 1 H, $^3J_{1',2'}$ = 5.6, $^3J_{1',2''}$ = 5.6 Hz), 6.85 (d, ^3J = 7.9 Hz, 4 H), 7.10−7.68 (m, 13 H), 8.27 (d, ^3J = 7.6 Hz, 1 H). ^{13}C NMR ($CDCl_3$): δ = 41.9, 55.1, 62.7, 70.7, 77.2, 86.5, 86.8, 87.2, 97.2, 113.2, 127.0, 128.0, 129.9, 130.0, 130.5, 133.0, 135.2, 135.4, 138.2, 144.1, 144.4, 158.5, 161.9. MS: *m*/*z* calcd. for ($C_{35}H_{33}N_3O_7S$ + H) [M + H$^+$] 640.2117, found 640.2120.

5′-*O*-(4,4′-Dimethoxytrityl)-4-*N*-picolyl-2′-deoxycytidine (3g): 4-*N*-Picolyl-2′-deoxycytidine (**2g**: 505 mg, 1.5 mmol) was dried by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (15 mL). 4,4′-Dimethoxytrityl chloride (610 mg, 1.8 mmol) was added to the solution, and the mixture was stirred at room temperature for 12 h. The mixture was concentrated to half its volume, diluted with $CHCl_3$, and washed three times with 5% aqueous $NaHCO_3$, and the aqueous layer was back-extracted with $CHCl_3$. The organic layer and washings were combined, dried with Na_2SO_4, filtered, and concentrated to dryness under reduced pressure. The residue was subject on a silica gel column. Chromatography was performed with $CHCl_3$ containing 1% pyridine, applying a gradient of methanol (0.5−1%) to give **3g** (880 mg, 92%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 2.30 (m, 1 H), 2.76 (m, 1 H), 3.46 (m, 2 H), 3.79 (s, 6 H), 4.18 (m, 1 H), 4.56 (m, 1 H), 6.31 (dd, 1 H, $^3J_{1',2'}$ = 5.6, $^3J_{1',2''}$ = 5.6 Hz), 6.85 (d, ^3J = 7.9 Hz, 4 H), 7.10−7.68 (m, 13 H), 8.27 (d, ^3J = 7.6 Hz, 1 H). ^{13}C NMR ($CDCl_3$): δ = 41.9, 55.1, 62.7, 70.7, 77.2, 86.5, 86.8, 87.2, 97.2, 113.2, 127.0, 128.0, 129.9, 130.0, 130.5, 133.0, 135.2, 135.4, 138.2, 144.1, 144.4, 158.5, 161.9. $C_{36}H_{34}N_4O_7$ (634.7): C 68.13, H 5.40, N 8.83; found C 68.10, H 5.19, N 8.49.

5′-*O*-(4,4′-Dimethoxytrityl)-4-*N*-nicotinoyl-2′-deoxycytidine (3h): A procedure similar to that described above was performed with 4-*N*-nicotinoyl-2′-deoxycytidine (**2h**: 505 mg, 1.5 mmol), to give **3h** (880 mg, 92%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 2.30 (m, 1 H), 2.76 (m, 1 H), 3.46 (m, 2 H), 3.79 (s, 6 H), 4.18 (m, 1 H), 4.56 (m, 1 H), 6.31 (dd, 1 H, $^3J_{1',2'}$ = 5.6, $^3J_{1',2''}$ = 5.6 Hz), 6.85 (d, ^3J = 7.9 Hz, 4 H), 7.10−7.68 (m, 13 H, 5-H), 8.27 (d, ^3J = 7.6 Hz, 1 H). ^{13}C NMR ($CDCl_3$): δ = 41.9, 55.1, 62.7, 70.7, 77.2, 86.5, 86.8, 87.2, 97.2, 113.2, 127.0, 128.0, 129.9, 130.0, 130.5, 133.0, 135.2, 135.4, 138.2, 144.1, 144.4, 158.5, 161.9. $C_{36}H_{34}N_4O_7$ (634.7): calcd. C 68.13, H 5.40, N 8.83; found C 67.84, H 5.66, N 8.59.

5′-*O*-(4,4′-Dimethoxytrityl)-4-*N*-isonicotinoyl-2′-deoxycytidine (3i): A procedure similar to that described above was performed with 4-*N*-isonicotinoyl-2′-deoxycytidine (**2i**: 505 mg, 1.5 mmol), to give **3i** (880 mg, 92%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 2.31 (m, 1 H), 2.78 (m, 1 H), 3.48 (m, 2 H), 3.77 (s, 6 H), 4.21 (m, 1 H), 4.62 (m, 1 H), 6.27 (dd, 1 H, $^3J_{1',2'}$ = 5.3, $^3J_{1',2''}$ = 5.3 Hz), 6.84 (d, ^3J = 6.3 Hz, 4 H), 7.12−7.44 (m, 10 H, 5-H), 7.69 (d, ^3J = 5.6 Hz, 2 H), 8.38 (d, ^3J = 7.3 Hz, 1 H), 8.69 (d, ^3J = 5.3 Hz, 2 H). ^{13}C NMR ($CDCl_3$): δ = 41.9, 55.1, 62.4, 70.2, 77.2, 86.5, 86.8, 87.3, 97.4, 113.2, 121.4, 127.0, 128.0, 128.0, 129.9, 130.0, 135.2, 135.5, 140.6, 144.2, 144.9, 150.5, 154.4, 158.5, 162.1, 166.3. $C_{36}H_{34}N_4O_7$ (634.7): C 68.13, H 5.40, N 8.83; found C 67.98, H 5.70, N 8.59.

Typical Procedure for the Synthesis of Triethylammonium 4-*N*-Acyl-5′-*O*-(4,4′-*O*-dimethoxytrityl)-2′-deoxycytidin-3′-yl Phosphonate (4a−i): 4-*N*-Acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine[24] (**3a**: 572 mg, 1 mmol) was dried by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (1 mL) and dry dioxane (3 mL). 2-Chloro-5,6-benzo-1,3,2-dioxaphosphorin-4-one[18] (1.25 M stock solution in dioxane) was added to the solution. After having been stirred at room temperature for 15 min, the mixture was treated with H_2O/pyridine (1:1, v/v) and stirred at room temperature for 30 min. The mixture was concentrated to dryness under reduced pressure. The residue was dissolved in $CHCl_3$ and washed three times with 5% aqueous $NaHCO_3$, and the aqueous layer was back-extracted with $CHCl_3$. The organic layer and the washings were combined and dried with Na_2SO_4, filtered, and concentrated to dryness under reduced pressure. The residue was loaded onto a silica gel column. Chromatography was performed with $CHCl_3$ containing 1% triethylamine, applying a gradient of methanol (4−5%) to give **4a** (588 mg, 80%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 1.29 (t, 9 H, ^3J = 7.6 Hz), 2.20 (s, 3 H), 2.51 (m, 1 H), 2.75 (m, 1 H), 3.06 (q, 6 H, ^3J = 7.2 Hz), 3.45 (m, 2 H), 3.79 (s, 6 H), 4.25 (m, 1 H), 4.98 (m, 1 H), 6.25 (dd, 1 H, $^3J_{1',2'}$ = 5.6, $^3J_{1',2''}$ = 5.6 Hz), 6.84 (d, ^3J = 8.9 Hz, 4 H), 6.89 (d, 1 H, ^1J = 618.9 Hz), 7.12−7.41 (m, 10 H), 8.22 (d, ^3J = 7.6 Hz, 1 H). ^{13}C NMR ($CDCl_3$): δ = 8.38, 24.7, 40.6, 45.3, 55.1, 62.0, 71.6, 84.9, 86.7, 96.3, 113.1, 126.8, 127.8, 128.8, 129.9, 135.1, 135.3, 137.7, 144.1, 154.9, 158.5, 162.7, 171.1. ^{31}P NMR ($CDCl_3$): δ =3.56 (dd, ^1J = 618.9, ^4J = 8.5 Hz). MS: *m*/*z* calcd. for $C_{38}H_{49}N_4O_9P$ (M + H$^+$ − Et_3N) 636.2110, found 636.2200.

Triethylammonium 5′-*O*-(4,4′-Dimethoxytrityl)-4-*N*-propionyl-2′-deoxycytidin-3′-yl Phosphonate (4b): A procedure similar to that described above was performed with 5′-*O*-(4,4′-dimethoxytrityl)-4-*N*-propionyl-2′-deoxycytidine (**3b**: 586 mg, 1 mmol), to give **4b** (518 mg, 69%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 1.18 (t, ^3J = 7.6 Hz, 3 H), 1.33 (t, 9 H, ^3J = 7.3 Hz), 2.33−2.47 (m, 3 H, 2′-H), 2.82 (m, 1 H, 2′′-H), 3.06 (q, 6 H, ^3J = 7.6 Hz), 3.43 (m, 2 H), 3.80 (s, 6 H), 4.30 (m, 1 H), 4.95 (m, 1 H), 6.26 (dd, 1 H, $^3J_{1',2'}$ = 6.3, $^3J_{1',2''}$ = 6.3 Hz), 6.84 (d, ^3J = 7.3 Hz, 4 H), 6.89 (d, 1 H, ^1J = 619.9 Hz), 7.09 (d, ^3J = 9.7 Hz, 1 H), 7.19−7.40 (m, 9 H), 8.21 (d, ^3J = 7.6 Hz, 1 H, 6-H). ^{13}C NMR ($CDCl_3$): δ = 41.9, 55.0, 62.7, 70.7, 77.2, 86.5, 86.8, 87.2, 97.2, 113.2, 127.0, 128.0, 129.9, 130.0, 130.5, 133.0, 135.2, 135.4, 138.2, 144.1, 144.4, 158.5,

161.9. ^{31}P NMR ($CDCl_3$): δ = 3.7. $C_{39}H_{51}N_4O_9P{\cdot}H_2O$ (768.8): C 60.93, H 6.95, N 7.24; found C 60.97, H 6.90, N 6.97.

Triethylammonium 4-*N*-Butyryl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidin-3′-yl Phosphonate (4c): A procedure similar to that described in the case of **4a** was performed with 4-*N*-butyryl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine (**3c**: 600 mg, 1 mmol), to give **4c** (566 mg, 74%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 0.93 (t, ^3J = 7.3 Hz, 3 H), 1.29 (t, 9 H, ^3J = 6.9 Hz), 1.79 (m, 2 H), 2.34−2.48 (m, 3 H), 2.80 (m, 1 H), 3.06 (q, 6 H, ^3J = 6.6 Hz), 3.48 (m, 2 H), 3.79 (s, 3 H), 3.79 (s, 3 H), 4.30 (m, 1 H), 4.98 (m, 1 H), 6.26 (dd, 1 H, $^3J_{1',2'}$ = 5.6, $^3J_{1',2''}$ = 5.6 Hz), 6.84 (d, ^3J = 7.9 Hz, 4 H), 6.89 (d, 1 H, ^1J = 619.9 Hz), 7.15−7.42 (m, 9 H), 8.20 (d, ^3J = 7.6 Hz, 1 H). ^{13}C NMR ($CDCl_3$): δ = 8.5, 13.5, 18.3, 39.5, 41.0, 45.5, 55.2, 62.4, 72.2, 77.2, 86.5, 86.9, 86.9, 90.1, 113.1, 127.7, 129.0, 129.1, 135.3, 135.5, 155.1, 158.6, 162.0, 172.9. ^{31}P NMR ($CDCl_3$): δ = 3.9. $C_{40}H_{53}N_4O_9P{\cdot}H_2O$ (782.9): C 61.37, H 7.08, N 7.16; found C 61.22, H 5.38, N 6.98.

Triethylammonium 5′-*O*-(4,4′-Dimethoxytrityl)-4-*N*-furoyl-2′-deoxycytidin-3′-yl Phosphonate (4e): A procedure similar to that described in the case of **4a** was performed with 5′-*O*-(4,4′-dimethoxytrityl)-4-*N*-furoyl-2′-deoxycytidine (**3e**: 624 mg, 1 mmol) to give **4e** (584 mg, 74%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 1.32 (t, 9 H, ^3J = 7.3 Hz), 2.39 (m, 1 H), 2.86 (m, 1 H), 3.05 (q, 6 H, ^3J = 7.3 Hz), 3.79 (s, 6 H), 4.32 (m, 1 H), 4.96 (m, 1 H), 6.28 (dd, 1 H, $^3J_{1',2'}$ = 5.9, $^3J_{1',2''}$ = 5.9 Hz), 6.84 (d, ^3J = 8.9 Hz, 4 H), 6.89 (d, 1 H, $^1J_{P-H}$ = 620.2 Hz), 7.12−7.42 (m, 13 H), 7.55 (d, ^3J = 1.1 Hz, 1 H), 8.23 (d, ^3J = 7.6 Hz, 1 H). ^{13}C NMR ($CDCl_3$): δ = 8.6, 41.0, 45.5, 55.1, 62.4, 72.0, 72.3, 77.2, 85.4, 85.5, 86.8, 87.0, 96.0, 112.8, 113.1, 115.4, 117.3, 118.7, 122.5, 126.8, 127.7, 128.0, 128.8, 128.0, 128.9, 129.9, 129.9, 135.0, 135.2, 144.0, 144.6, 146.4, 155.0, 158.2, 158.3, 161.3. ^{31}P NMR ($CDCl_3$): δ = 3.8. $C_{41}H_{49}N_4O_{10}P{\cdot}2H_2O$ (824.9): C 59.70, H 6.48, N 6.79; found C 59.55, H 6.50, N 6.68.

Triethylammonium 5′-*O*-(4,4′-Dimethoxytrityl)-4-*N*-thenoyl-2′-deoxycytidin-3′-yl Phosphonate (4f): A procedure similar to that described in the case of **4a** was performed with 5′-*O*-(4,4′-dimethoxytrityl)-4-*N*-thenoyl-2′-deoxycytidine (**3f**: 640 mg, 1 mmol), to give **4f** (552 mg, 81%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 1.31 (t, 9 H, ^3J = 7.3 Hz), 2.38 (m, 1 H), 2.83 (m, 1 H), 3.05 (q, 6 H, ^3J = 7.3 Hz), 3.45 (m, 2 H), 3.79 (s, 6 H), 4.31 (m, 1 H), 4.99 (m, 1 H), 6.27 (dd, 1 H, $^3J_{1',2'}$ = 5.9, $^3J_{1',2''}$ = 5.9 Hz), 6.84 (d, ^3J = 8.9 Hz, 4 H), 6.88 (d, 1 H, $^1J_{P-H}$ = 628.1 Hz), 7.11−7.41 (m, 12 H), 7.89 (m, 1 H), 8.20 (d, ^3J = 7.6 Hz, 1 H). ^{31}P NMR ($CDCl_3$): δ = 3.7. MS: *m*/*z* calcd. for $C_{35}H_{35}N_3O_9PS$ [M ($C_{41}H_{49}N_4O_9PS$) + H^+ − Et_3N] 704.1832, found 704.1835.

Triethylammonium 5′-*O*-(4,4′-Dimethoxytrityl)-4-*N*-picolyl-2′-deoxycytidin-3′-yl Phosphonate (4g): ^{1}H NMR ($CDCl_3$): δ = 1.30 (t, 9 H, ^3J = 7.3 Hz), 2.39 (m, 1 H, 2′-H), 2.88 (m, 1 H), 3.01 (q, 6 H, ^3J = 7.3 Hz), 3.47 (m, 2 H), 3.80 (s, 6 H), 4.33 (m, 1 H), 4.97 (m, 1 H), 6.31 (dd, 1 H, $^3J_{1',2'}$ = 5.9, $^3J_{1',2''}$ = 5.9 Hz), 6.86 (d, ^3J = 7.6 Hz, 4 H), 6.90 (d, 1 H, ^1J = 618.6 Hz, P−H), 7.21−7.55 (m, 11 H), 7.90 (d, ^3J = 5.6 Hz, 1 H), 8.21−8.31 (m, 2 H, 6-H), 8.61(d, ^3J = 4.3 Hz, 1 H), 10.6 (1 H, br, NH). ^{13}C NMR ($CDCl_3$): δ = 8.8, 41.0, 45.5, 55.1, 62.3, 72.0,72.1, 77.2, 85.2, 85.3, 86.7, 86.9, 95.6, 113.0, 122.5, 126.8, 127.7, 127.9, 129.8, 129.8, 129.8, 135.0, 135.2, 137.4, 143.9, 144.5, 147.9, 148.2, 155.0, 158.2, 158.3, 161.3, 163.1. ^{31}P NMR ($CDCl_3$): δ = 3.8. $C_{42}H_{50}N_5O_9P{\cdot}H_2O$ (817.9): C 61.68, H 6.41, N 8.56; found C 61.70, H 5.99, N 8.62.

Triethylammonium 5′-*O*-(4,4′-Dimethoxytrityl)-4-*N*-nicotinoyl-2′-deoxycytidin-3′-yl Phosphonate (4h): ^{1}H NMR ($CDCl_3$): δ = 1.23 (t, 9 H, ^3J = 7.3 Hz), 2.33 (m, 1 H), 2.70 (m, 1 H), 2.96 (q, 6 H, ^3J = 7.3 Hz), 3.39 (m, 2 H), 3.71 (s, 6 H), 4.21 (m, 1 H), 4.91 (m, 1 H), 6.19 (dd, 1 H, $^3J_{1',2'}$ = 5.6, $^3J_{1',2''}$ = 5.6 Hz), 6.77 (d, ^3J = 15.4 Hz, 4 H), 6.79 (d, 1 H, $^1J_{P-H}$ = 619.6 Hz), 7.13−7.34 (m, 11 H), 8.15−8.22 (m, 2 H), 8.67 (d, ^3J = 5.0 Hz, 1 H), 9.13 (d, ^3J = 2.0 Hz, 1 H). ^{13}C NMR ($CDCl_3$): δ = 40.7, 45.4, 55.0, 62.2, 72.0, 77.2, 85.2, 85.3, 86.6, 86.7, 97.7, 113.1, 123.2, 126.9, 127.8, 128.0, 129.7, 129.9, 129.9, 135.1, 135.3, 135.7, 144.0, 144.1, 149.5, 152.9, 158.4, 161.9. ^{31}P NMR ($CDCl_3$): δ = 3.7. $C_{42}H_{50}N_5O_9P{\cdot}2H_2O$ (835.9): C 60.35, H 6.51, N 8.38; found C 59.99, H 6.55, N 8.62.

Triethylammonium 5′-*O*-(4,4′-Dimethoxytrityl)-4-*N*-isonicotinoyl-2′-deoxycytidin-3′-yl phosphonate (4i): ^{1}H NMR ($CDCl_3$): δ = 1.33 (t, 9 H, ^3J = 7.3 Hz), 2.43 (m, 1 H), 2.82 (m, 1 H), 3.06 (q, 6 H, ^3J = 7.3 Hz), 3.48 (m, 2 H), 3.80 (s, 6 H), 4.31 (m, 1 H), 4.99 (m, 1 H), 6.29 (dd, 1 H, $^3J_{1',2'}$ = 5.6, $^3J_{1',2''}$ = 5.6 Hz), 6.85 (4 H, d), 6.90 (d, 1 H, $^1J_{P-H}$ = 619.6 Hz), 7.21−7.42 (m, 11 H), 7.79 (d, ^3J = 5.6 Hz, 1 H), 8.29 (d, ^3J = 7.6 Hz, 1 H, 6-H), 8.81(d, *J* = 6.3 Hz, 2 H). ^{13}C NMR ($CDCl_3$): δ = 8.3, 40.6, 45.2, 54.9, 62.0, 71.7, 77.2, 85.1, 85.2, 86.5, 86.6, 97.4, 113.0, 121.5, 126.8, 127.7, 127.9, 129.6, 129.8, 135.0, 135.1, 140.8, 143.9, 144.2, 150.3, 153.4; ^{31}P NMR ($CDCl_3$): δ = 4.0. $C_{42}H_{50}N_5O_9P{\cdot}2H_2O$ (835.9): C 60.35, H 6.51, N 8.38; found C 60.31, H 6.60, N 7.99.

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