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Synthesis and Properties of Oligodeoxyribonucleotides Containing 4-*N*-Acetylcytosine Bases

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Abstract: Oligodeoxyribonucleotides containing 4-*N*-acetyl-2'-deoxycytidines (ac^4dC) were synthesized by the *H*-phosphonate method. Thymidine 3'-*O*-(3,4-dichloro)phthalate bound to a solid support was employed as the starting material for the solid-phase synthesis. The (3,4-dichloro)phthaloyl (DCP) linker was found to be cleaved by treatment with 10% DBU in CH_3CN for 5 min without loss of the 4-*N*-acetyl group of 2'-deoxycytidine. The thermal stability of the duplexes containing ac^4dC was investigated.

Keywords: nucleic acids; solid-phase synthesis; nucleosides; acylation

4-*N*-Acetylcytidine (ac^4C) and 4-*N*-acetyl-2'-*O*-methylcytidine (ac^4Cm) are widely occurring modified nucleosides found in tRNAs [1-6]. It is known that ac^4C existing at the first letter of the anticodons of some tRNAs selectively forms the Watson-Crick type base pair with guanosine [2]. The 4-*N*-acetylation of cytidine was found to stabilize the *C3'-endo* conformation of the ribose moiety [7]. In order to elucidate the structural and functional roles of 4-*N*-acetylcytosine, the chemical synthesis of oligonucleotides containing this modified base is of great importance. However, it is difficult to obtain such nucleic acids by the standard solid-phase method because the 4-*N*-acetyl group is readily cleaved under aqueous basic conditions prescribed for removal of the *N*-protecting groups and liberation of the products from the solid support [8]. In this paper, we report a new strategy for the solid-phase synthesis of oligodeoxyribonucleotides containing 4-*N*-acetylcytosine bases.

In the current solid-phase synthesis of DNA, oligonucleotides bound to solid supports *via* a succinyl linker are liberated by treatment with aqueous ammonia. Letsinger *et al* have reported a more labile oxalyl linker for the synthesis of base-sensitive oligonucleotides [9]. This linker can be cleaved by treatment with *n*-$PrNH_2$ under anhydrous conditions. However, it was found that the 4-*N*-acetyl group of cytosine was simultaneously removed during liberation of the oligomer from the solid support by treatment with *n*-$PrNH_2$ in CH_3CN. In contrast to this fact, a non-nucleophilic strong base DBU does not affect the 4-*N*-acetyl group under anhydrous conditions. Brown *et al* have reported the nucleoside bound to the solid support *via* the succinyl or phthaloyl linker can be liberated with the DBU treatment by the intramolecular nucleophilic attack of the neighboring deprotonated amide group [10]. However, the cleavage of these linkers with DBU was less satisfactory [11]. Therefore, we

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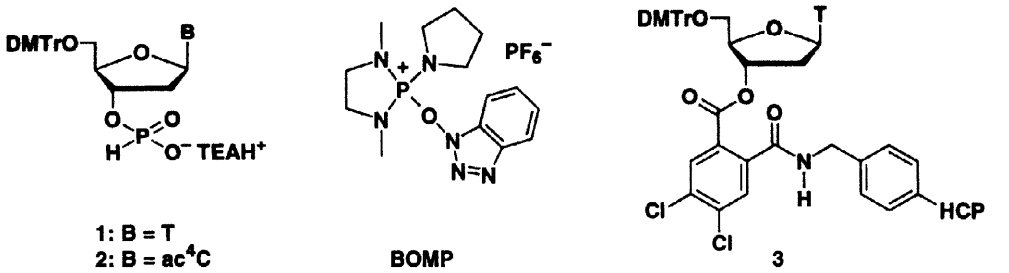
report here a more labile (3,4-dichloro)phthaloyl (DCP) linker which can be cleaved with DBU by a similar mechanism along with the formation of a (3,4-dichloro)phthalimide derivative (Scheme 1).

Scheme 1



5'-*O*-Dimethoxytritylthymidine was treated with (3,4-dichloro)phthalic anhydride in pyridine in the presence of DMAP for 6 h. After the usual workup, 5'-*O*-dimethoxytrityl-thymidine 3'-*O*-(3,4-dichloro)phthalate was obtained in 96% yield as a triethylammonium salt. The resulting compound was condensed with the amino group (35.0 μmol/g) on the highly cross-linked polystyrene (HCP) resin [12] in the presence of DCC in CH_2Cl_2 for 6 h. After capping of the unreacted amino group on the resin with Ac_2O-pyridine (1:9, v/v) for 3 h, 5'-*O*-dimethoxytritylthymidine 3'-*O*-(3,4-dichloro)phthalate bound to the HCP resin **3** was obtained with the loading amount of 13.7 μmol/g. When this resin was treated with 10% DBU in CH_3CN for 5 min, 95% of the nucleoside was released from the solid support as evidenced by the DMTr assay.

Next, the synthesis of 4-*N*-acetyl-2'-deoxycytidine 3'-*H*-phosphonates **2** was examined. In this case, the aqueous treatment after phosphonylation should be carried out under extremely mild conditions to avoid deacetylation. For example, use of aqueous pyridine containing Et_3N resulted in quick deacetylation. In contrast, the *N*-acetyl group of 2'-deoxycytidine found to be stable in aqueous pyridine in the absence of a strong base for several hours. Consequently, 5'-*O*-dimethoxytrityl-4-*N*-acetyl-2'-deoxycytidine [13] was treated with 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-on in dioxane-pyridine (3:1, v/v) for 15 min and the reaction was quenched by simple addition of water [14]. After the usual workup and silica gel column chromatography, the *H*-phosphonate unit of 4-*N*-acetyl-2'-deoxycytidine **2** was obtained in 80% yield (Scheme 1).

Solid-phase synthesis of 13mers having various sequences was started from the anchor nucleoside **3**. Condensation was carried out by using the *H*-phosphonate units **1** and **2** (0.05 M) with BOMP (0.2 M) in pyridine for 1 min as described by us previously [15]. The average coupling yield was generally higher than 98%. After chain elongation and oxidation, the 13mers bearing the 4-*N*-acetylcytosine bases on the HCP resin were treated with 10% DBU in CH_3CN for 5 min under anhydrous conditions. The HCP resin was removed by filtration and the filtrate was quickly acidified by addition of 50% acetic acid in CH_3CN. After evaporation, the crude mixture was diluted with water and passed through a column of cation-exchange resin to remove the DBU salt. The eluate was concentrated to dryness and the product was purified successively by anion-exchange and reversed-phase HPLC. The

HPLC profiles of the crude products indicated that less than 10% (per ac^4dC) of the oligomers containing the deacetylated dC, which could be separated easily by HPLC, were formed. In general, the isolated yields of the 13mers containing one or three ac^4dC residues (see Table 1) were 20-30%. The purified products were treated with nuclease P1 to give T, pT, ac^4dC and p-ac^4dC in identical ratio for all the cases.

Next, the thermal stability of the duplexes containing one or three 4-*N*-acetylcytosine bases was investigated. The Tm values of the duplexes in the phosphate buffer (pH 7.0) containing 1.0 M NaCl are listed in Table 1 along with those of the corresponding 13mer bearing unmodified dC [16]. In general, the 13mers having the 4-*N*-acetyl cytosine bases have slightly higher Tm values compared with those of the unmodified duplexes except for entry 2. These results suggest that the 4-*N*-acetyl group does not interfere with the duplex formation.

Table 1. Thermal stability of duplexes containing dC or ac^4dC[a]

entry	duplex	X = C	X = ac^4C	
		Tm (°C)	Tm (°C)	ΔTm (°C)
1	d(TTTTTTXTTTTTT)•d(AAAAAAGAAAAAA)	43.6	44.0	+0.4
2	d(XTTTTTTTTTTTT)•d(AAAAAAAAAAAAG)	45.7	45.5	–0.2
3	d(TTTTTTTTTTTXT)•d(AGAAAAAAAAAAA)	45.3	46.1	+0.8
4	d(TTTTTXXXTTTTT)•d(AAAAAGGGAAAAA)	34.2	36.3	+2.1
5	d(XTTTTTXTTTTXT)•d(AGAAAAGAAAAAG)	31.1	33.0	+1.9

[a] Conditions: 10 mM sodium phosphate buffer (pH 7.0), 1.0 M NaCl, 0.1 mM EDTA and 2.0 μM of the each oligomer.

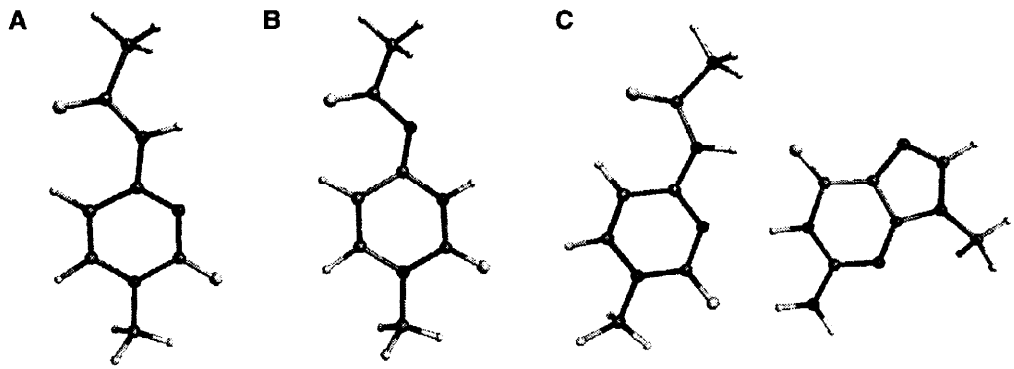


Figure 1. The optimized geometries calculated at the MP2/6-31G*//HF/6-31G* level: (A) m^1ac^4C (amino form), (B) m^1ac^4C (imino form), (C) m^1ac^4C•m^9G base pair.

Preliminary *ab initio* calculations for 1-methyl-4-*N*-acetylcytosine at the MP2/6-31G*//HF/6-31G* level indicated that the most stable orientation of the acetyl group is feasible for the Watson-Crick type base pair (Figure 1A). This result is consistent with the crystal structure of 4-*N*-acetylcytidine [17]. Moreover, the amino form of 1-methyl-4-*N*-acetylcytosine was found to be 1.33 kcal/mol more stable than the tautomeric imino form which can form a base pair with adenine (Figure 1B). In the case of unmodified 1-

methylcytosine, the amino form is 1.19 kcal/mol more stable than the imino form. The hydrogen bonding energies of C•G and ac^{4}C•G base pairs were estimated to be -25.97 and -25.54 kcal/mol, respectively. These results indicated that the 4-*N*-acetylation does not appreciably affect the Watson-Crick type base pair (Figure 1C).

The thermal stability of duplexes with mismatch base pairs ac^{4}C•X (X = A, C and T) was also investigated. In each case, the duplex was apparently destabilized by the one-base mismatch. The ΔTm values of these mismatched duplexes containing ac^{4}dC were similar to those of the corresponding unmodified duplexes as shown in Table 2.

Table 2. Thermal stability of full-matched and mismatched duplexes containing dC or ac^{4}dC[a]

entry	duplex	X = C		X = ac^{4}C	
		Tm (°C)	ΔTm (°C)	Tm (°C)	ΔTm (°C)
1	d(TTTTTTXTTTTTT)•d(AAAAAAGAAAAAA)	43.6	—	44.0	—
2	d(TTTTTTXTTTTTT)•d(AAAAAAAAAAAAA)	38.0	–5.6	38.0	–6.0
3	d(TTTTTTXTTTTTT)•d(AAAAAACAAAAAA)	22.6	–21.0	20.8	–23.2
4	d(TTTTTTXTTTTTT)•d(AAAAAATAAAAAA)	29.3	–14.3	30.7	–13.3

[a] Conditions: 10 mM sodium phosphate buffer (pH 7.0), 1.0 M NaCl, 0.1 mM EDTA and 2.0 μM of the each oligomer.

In summary, the present (3,4-dichloro)phthaloyl (DCP) linker was highly effective for the solid-phase synthesis of oligodeoxyribonucleotides bearing 4-*N*-aceylcytosine bases. It was found that the thermal stability of the duplexes was increased by 4-*N*-acetylcytosine which could selectively form the base pair with guanine. The results are consistent with the accurate codon-anticodon interaction including the ac^{4}C•G base pair found in some tRNAs [2]. Solid-phase synthesis of various oligodeoxyribonucleotides bearing 4-*N*-acetylcytosine and other unmodified nucleobases by the *H*-phosphonate method without *N*-protection [15] are now under study.

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