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NUCLEOSIDES AND NUCLEOTIDES. 161. INCORPORATION OF 5-(*N*-AMINOALKYL)CARBAMOYL-2'-DEOXYCYTIDINES INTO OLIGODEOXYRIBONUCLEOTIDES BY A CONVENIENT POST-SYNTHETIC MODIFICATION METHOD[1]

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Key Words: nucleoside; 5-trifluoroethoxycarbonyl-2'-deoxycytidine; oligodeoxyribonucleotide; amino-linker; post-synthetic modification

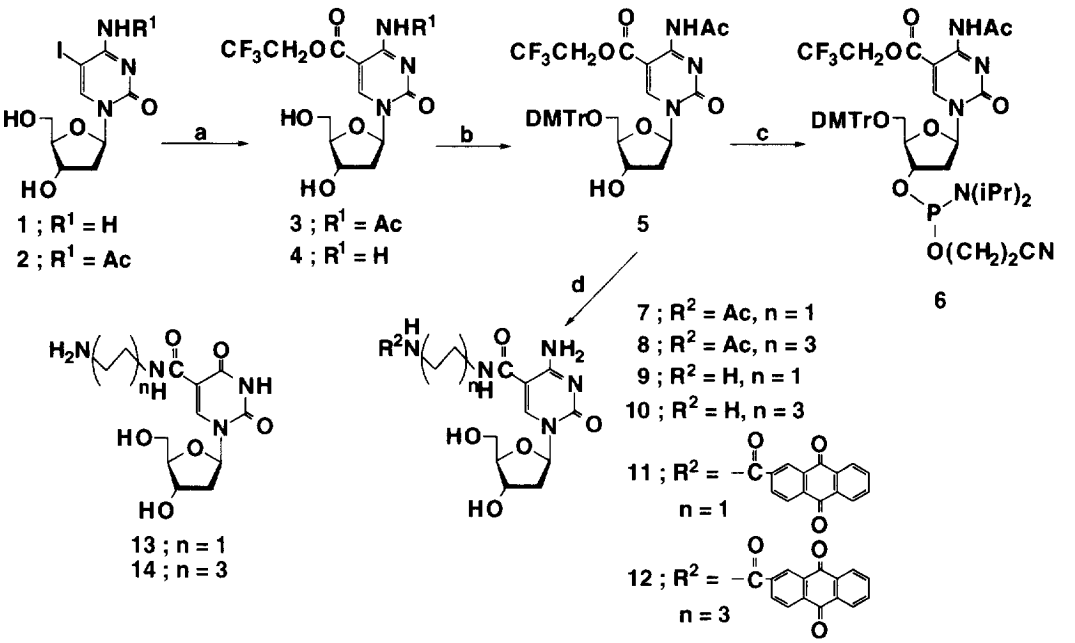
Abstract: 5-Trifluoroethoxycarbonyl-2'-deoxycytidine derivatives were synthesized and incorporated into oligonucleotides. The fully protected oligonucleotides were treated with diaminoethane or 1,6-diaminohexane to give oligonucleotides carrying amino-linkers, which were further derivatized with an intercalator. The properties of these oligonucleotides are described.

We recently reported a novel and convenient method for the post-synthetic modification of oligodeoxyribonucleotides (ODNs), in which 5-methoxycarbonyl-2'-deoxyuridine (**MCdU**) was used as a convertible unit.[2,3] ODNs containing various numbers of **MCdU** at various positions, which were linked to a solid support, were treated with a large excess of a diaminoalkane in MeOH to give the desired ODNs containing 5-(*N*-aminoalkyl)carbamoyl-2'-deoxyuridines (**ACdUs**). This treatment resulted in the simultaneous conversion of the methoxycarbonyl group into an (*N*-aminoalkyl)carbamoyl group, deprotection, and detachment from the solid support. We can easily determine the optimum length of the linker for a desired function using this method without separately synthesizing several mononucleotide units with various linker lengths. We found that an aminohexyl linker was suitable for stabilizing duplex formation with either a DNA or RNA strand. Furthermore, an ODN containing 5-(*N*-aminohexyl)carbamoyl-2'-deoxyuridines is highly resistant to hydrolysis by nucleases, which is a necessary characteristic of antisense molecules.[4]

To extend our method to other complementary bases such as guanine, the development of a 2'-deoxycytidine analogue, instead of **MCdU**, as a convertible unit to be incorporated into ODNs is necessary. However, 5-methoxycarbonyl-2'-deoxycytidine, which was synthesized from the corresponding 5-iodo-2'-deoxycytidine (**1**) via palladium-catalyzed carbonylation with carbon monoxide in MeOH, was found to be much less reactive to appropriate amines than **MCdU** (data not shown). Therefore, we designed N^4-acetyl-5-trifluoroethoxycarbonyl-2'-deoxycytidine (**3**) to be a more reactive convertible unit. In this communication, we describe the synthesis and chemical properties of **3** along with the synthesis of several ODNs with amino-linkers using our post-synthetic modification method with ODNs containing **3**. The thermal stability of these ODNs with their complementary DNA and RNA strands, and the stability of these ODNs to nucleolytic digestion were also studied.

Palladium-catalyzed carbonylation of N^4-acetyl-5-iodo-2'-deoxycytidine (**2**), which was readily prepared from **1** with Ac_2O in DMF in 83% yield, with carbon monoxide in the presence of $(PhCN)_2PdCl_2$, CF_3CH_2OH and Et_3N in DMF gave **3** in 72% yield and **4** in 26% yield (Scheme 1).[4] Before **3** was introduced into ODNs, the chemical reactivity of the trifluoroethoxycarbonyl group with diaminoalkanes was examined. To simplify detection of the reaction, **3** was protected with a dimethoxytrityl group to give **5**. When **5** was treated with a large excess of diaminoethane or 1,6-diaminohexane in MeOH for 16 h at room temperature, followed by acetylation of the terminal amino group and detritylation, 5-(*N*-acetamidoethyl)carbamoyl- (**7**) and 5-(*N*-acetamidohexyl)-2'-deoxycytidines (**8**)[5] were obtained in yields of 98% and 80%, respectively. Under these conditions, no side products, such as 2'-deoxycytidine 5-carboxylate, were detected by HPLC in either reaction. Therefore, these mild conditions for the substitution reaction can be used to introduce amino-linkers at an ODN level.

Scheme 1[a]



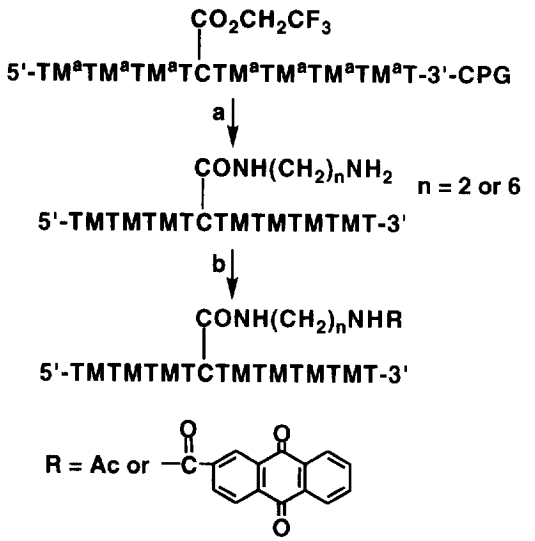
[a](a) CO, $(PhCN)_2PdCl_2$, CF_3CH_2OH, Et_3N, DMF; (b) DMTrCl, pyridine; (c) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, *N*,*N*-diisopropylethylamine, CH_2Cl_2; (d) i) $H_2N(CH_2)nNH_2$, MeOH; ii) Ac_2O, DMF; iii) NH_3/MeOH; iv) 80% AcOH.

To incorporate **3** into an ODN, **5** was then converted into phosphoramidite unit **6**[6] under standard conditions. Compound **6** was incorporated into a 17-mer ODN [5'-$(TM^a)_3$T**X**$(TM^a)_4$T-3'-CPG, where T is thymidine, M^a is N^4-acetyl-5-methyl-2'-deoxycytidine, **X** is **3**, and CPG is a controlled pore glass] using the phosphoramidite method[7] on a DNA synthesizer (Scheme 2). The coupling yield of **6** was 99% using a 0.12 M MeCN solution of **6** and a coupling time of 6 min. The fully protected ODN linked to the solid support was divided into two portions, each of which was treated with a large excess of diaminoethane or 1,6-diaminohexane in MeOH for 16 h at 50 °C, followed by C-18 column chromatography and detritylation to give 5'-$(TM)_3$T**9**$(TM)_4$T-3' (**15**) and 5'-$(TM)_3$T**10**$(TM)_4$T-3' (**16**) after further purification using ion-exchange column

chromatography followed by a C-18 HPLC. Starting from 1 μmol of thymidine residue linked to CPG, 9.5 and 3.3 OD units (at 260 nm) of each ODN were obtained.

Reaction of these ODNs with Ac_2O in Hepes buffer (0.2 M, pH 7.2) for 1 h at room temperature, followed by treatment of the mixture with concentrated NH_4OH overnight gave the desired ODNs [5'-$(TM)_3$T**7**$(TM)_4$T-3' (**17**) and 5'-$(TM)_3$T**8**$(TM)_4$T-3' (**18**)] in good yields after purification with C-18 HPLC. Introduction of an anthraquinone-2-carbonyl group, an intercalator, into the amino terminal of **15** and **16** along with reaction with *N*-(anthraquinone-2-carboxy)-succimide[8] at room temperature overnight gave the desired functionalized ODNs 5'-$(TM)_3$T**11**$(TM)_4$T-3' (**19**) and 5'-$(TM)_3$T**12**$(TM)_4$T-3' (**20**) (Scheme 2). To confirm the presence of these modified nucleosides, these ODNs were hydrolyzed by a mixture of snake venom phosphodiesterase and alkaline phosphatase to the corresponding nucleosides, and the nucleoside composition was analyzed by C-18 HPLC. As examples, the results of ODNs **17** and **18** are shown in Figures 1A, B (for **17**, T:M:**7** = 7.3:9.4:1; for **18**, T:M:**8** = 6.5:9:0.8). These values are consistent with the theoretical values. These results showed that 2'-deoxycytidine with amino-linkers at the 5-position can be easily introduced into ODNs using **3** as a convertible unit.

Scheme 2[a]



[a](a) $H_2N(CH_2)_2NH_2$ or $H_2N(CH_2)_6NH_2$, MeOH, 50 ℃; (b) Ac_2O or ref. 8. T, M, and M[a] correspond to thymidine, 5-methyl-2'-deoxycytidine, and N^4-acetyl-5-methyl-2'-deoxycytidine, respectively.

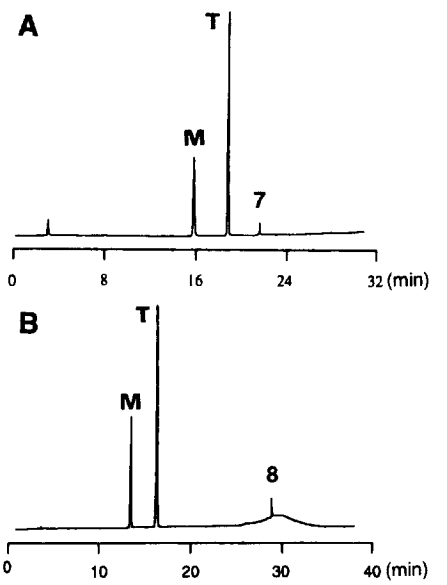


Figure 1. C-18 HPLC profiles of the nucleoside mixtures obtained by enzymatic hydrolysis of **17** (A) and **18** (B). A linear gradient of CH_3CN from 5 to 13% (20 min) and then from 13 to 30 % (5 min) in 0.1 M triethylammonium acetate (pH 6.8) was used.

The stability of the duplexes formed by these ODNs and their complementary DNA, 5'-TG$(GA)_9$G-GT-3' (**21**) or RNA, 5'-UG$(GA)_9$GGU-3' (**22**) was studied by thermal denaturation. 5'-T$(MT)_8$-3' (**23**) was used as a control. Melting temperatures (Tms) are listed in Table 1. With **21** as the complementary strand, the Tm value of the duplex formed by **15** and **21** was slightly lower (ΔTm = -1 ℃) than that of the control duplex formed by **23** and **21** (Tm = 49 ℃), while that of the duplex formed by **16** and **21** was increased to 54 ℃ (ΔTm = +5 ℃). On

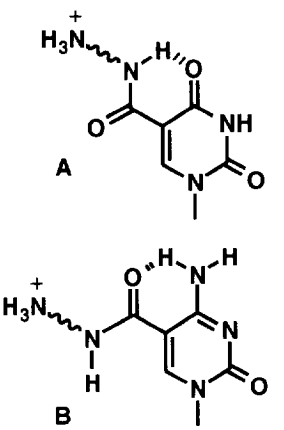
the other hand, when the terminal amino groups in **15** and **16** were acetylated, Tm values of the ODNs **17** and **18** with **21** decreased significantly (47 and 50 ℃, respectively). The anthraquinone-attached ODNs **19** and **20** had higher Tms (54 and 55 ℃, respectively) than the corresponding ODNs with free amino groups. On the other hand, when **22** was used as the complementary strand, the Tms of these ODNs were very similar to those of the DNA-DNA duplexes. These data, together with our previous findings,[2,3] indicate that the terminal ammonium ion plays an important role in stabilizing duplex formation. Although the introduction of the intercalator counteracts the stabilizing effect of the ammonium ion, the resulting intercalation appears to eventually overcome this disadvantage. In our previous report,[2] the length of the amino-linkers in 5'-$(TM)_3$**Y**$(MT)_4$-3', where **Y** is **13** or **14** (Scheme 1), did not correlate with their Tms (53 ℃), which is quite different from the results with ODNs containing **9** and **10** described here. X-ray crystallographic analysis of 5-carbamoyl-2'-deoxyuridine revealed an intramolecular hydrogen-bond between the O^4 atom and one of the carbamoyl protons.[2] If the 5-(*N*-aminoalkyl)carbamoyl groups in **13** and **14** have similar hydrogen-bonds, the aminoalkyl-linkers would be conformationally fixed in one direction in the duplexes (Figure 2A). However, since an intramolecular hydrogen bond in 5-(*N*-aminoalkyl)carbamoylcytosine can be seen in Figure 2B, the positions of the terminal amino groups in **9** and **10** would be different from those in **13** and **14**. This might be reflected by the difference in the Tm values. Therefore, for antisense molecules, ODNs with **10** would be superior to those with **9**, if they have nuclease-resistant properties.

Table 1. Thermal Denaturation of ODNs.[9]

ODNs		Tm (°C)	
		ODN/DNA[a]	ODN/RNA[b]
$T(MT)_8$	(**23**)	49	69
$(TM)_3$**T9**$(TM)_4$T	(**15**)	48	67
$(TM)_3$**T7**$(TM)_4$T	(**17**)	47	65
$(TM)_3$**T11**$(TM)_4$T	(**19**)	54	69
$(TM)_3$**T10**$(TM)_4$T	(**16**)	54	72
$(TM)_3$**T8**$(TM)_4$T	(**18**)	50	69
$(TM)_3$**T12**$(TM)_4$T	(**20**)	55	72

a) Complementary DNA sequence: 5'-$TG(GA)_9GGT$-3'
b) Complementary RNA sequence: 5'-$UG(GA)_9GGU$-3'

Figure 2. Possible Conformations of the Carbamoyl Groups in Uracil and Cytosine Derivatives.



We next examined the stability of ODNs **15** and **16** against nucleolytic digestion using snake venom phosphodiesterase, a 3'-exonuclease. Each ODN was labeled at the 5'-end with ^{32}P,[10] which was incubated with the enzyme, and analyzed by 20% polyacrylamide gel electrophoresis (PAGE) under denaturation conditions. As shown in Figure 3, the phosphodiester linkages of the unmodified ODN **23** was randomly hydrolyzed within 60 min (Figure 3, lane 5). On the other hand, both ODNs **15** and **16** were hydrolyzed at the 3'-side from the modified nucleosides (Figure 3, lanes 10 and 15). The 5'-side of the modified nucleosides were completely resistant to the nuclease for 60 min. Several studies have demonstrated that 3'-exonuclease activities are the major cause of degradation of unmodified ODNs in serum.[11] Previously, we found that ODNs containing **13** and **14** were also stable in a medium containing 10% fetal calf serum, as well as against snake venom phosphodiesterase.

Therefore, based on both the thermal stability and the nuclease-resistance caused by the 5-(*N*-aminohexyl)carbamoyl substituent in either cytosine or uracil, ODNs containing **10** and/or **14** would have properties suitable for use as antisense ODNs.

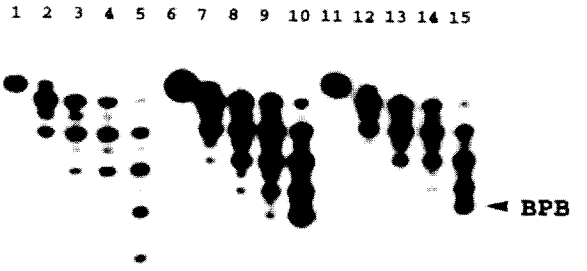


Figure. 3 Polyacrylamide gel electrophoresis of ODNs hydrolyzed by snake venom phosphodiesterase: **23** (lanes 1-5), **15** (lanes 6-10) and **16** (lanes 11-15) were incubated with snake venom phosphodiesterase at 37 ℃ for 0 min (lanes 1, 6, and 11), 10 min (lanes 2, 7, and 12), 20 min (lanes 3, 8, and 13), 30 min (lanes 4, 9, and 14), 60 min (lanes 5, 10, and 15).

In conclusion, we have described a new and convenient post-synthetic modification method for the synthesis of modified ODNs using N^4-acetyl-5-trifluoroethoxycarbonyl-2'-deoxycytidine (**3**) as a convertible unit Amino-linkers were readily introduced into ODNs containing **3**, in which the anthraquinone derivative and acetyl group were also incorporated. Furthermore, we found that ODNs containing **16** stabilized the duplex formation with either the complementary DNA or RNA, and were resistant to hydrolysis by snake venom phosphodiesterase. Applications of these ODNs as antisense ODNs are currently being studied.

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References and Notes

1. For part 160 in the series, see: Haginoya, N.; Ono, A.; Nomura, Y.; Matsuda, A. Submitted for publication.
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4. Synthesis of **3**: A mixture of **2** (1.00 g, 2.53 mmol), $(PhCN)_2PdCl_2$ (10 mg, 0.026 mmol), CF_3CH_2OH (19.7 mL, 0.253 mmol), and Et_3N (0.39 mL) in DMF (100 mL) was heated at 50 ℃ under a CO atmosphere. After 2 h, precipitates were removed by filtration and the filtrate was concentrated *in vacuo*. The residue was purified on a silica gel column with 0-10% EtOH in $CHCl_3$ to give **3** (720 mg, 72%, as a

foam) and **4** (230 mg, 26%, as a powder). Physical data for **3**: ^{1}H NMR (DMSO-d_6) δ 10.57 (1 H, br s, N^3*H*), 9.09 (1 H, s, H-6), 6.05 (1 H, t, H-1', $J_{1',2'ab}$ = 5.9 Hz), 5.31 (1 H, d, 3'-OH), 5.05 (1 H, t, 5'-OH), 4.87 (2 H, q, CF_3CH_2), 4.26-4.23 (1 H, m, H-3'), 3.96 (1 H, dt, H-4'), 3.63 (2 H, ddd, H-5'ab), 2.41-2.17 (2 H, m, H-2'ab), (3H, s, NAc). Physical data for **4**: mp 230-240 ℃ (dec); UV λmax (H_2O) 283 nm (ε 8900), λmax (0.1 N HCl) 287 nm (ε 13700); ^{1}H NMR (DMSO-d_6) δ 8.93 (1 H, s, H-6), 8.07 (1 H, br s, NH_2), 7.50 (1 H, br s, NH_2), 6.05 (1 H, t, H-1', *J* = 6.2 Hz), 5.26 (1 H, d, 3'-OH), 4.95 (1 H, t, 5'-OH), 4.87 (2 H, CF_3CH_2), 4.26-4.23 (1 H, m, H-3'), 3.91 (1 H, dt, H-4'), 3.63 (2 H, m, H-5'ab), 2.32-2.05 (2 H, m, H-2'ab). Anal. Calcd for $C_{12}H_{14}F_3N_3O_6$: C, 40.80; H, 3.99; F, 16.13; N, 11.90. Found: C, 40.61; H, 4.13; F, 16.21; N, 11.92.

5. Synthesis of **8**: 1,6-Diaminohexane (0.5 mL, 7.5 mmol) was added to a solution of **3** (40 mg, 0.057 mmol) in MeOH (5 mL). The mixture was stirred for 17 h at room temperature and the volatile was removed *in vacuo*. Ac_2O (170 μL, 1.14 mmol) was added to a solution of the residue in DMF (5 mL). After 10 min, H_2O (1 mL) was added to the mixture, which was allowed to stand for 2 h. The mixture was concentrated to dryness and the residue was treated with NH_3/MeOH (5 mL) for 2 h at room temperature. The mixture was concentrated to dryness and the residue was purified on a silica gel column with 0-15% MeOH in $CHCl_3$ to give **8** (19 mg, 80%, as a powder); mp 158-161 ℃; FD-MS *m/z* 412 (M^++1); UV λmax (H_2O) 267 nm (ε 8300), λmax (0.1 N HCl) 268 nm (ε 8500); ^{1}H NMR (DMSO-d_6) δ 8.34 (1 H, s, H-6), 8.08 (1 H, t, N*H*CO-5), 7.77 (1 H, t, AcN*H*), 8.03, 7.74 (each 1 H, br s, 4-NH_2), 6.11 (1 H, t, H-1', *J* = 6.4 Hz), 5.23 (1 H, d, 3'-OH), 5.05 (1 H, t, 5'-OH), 4.30 (1 H, m, H-3'), 3.82 (1 H, m, H-4'), 3.61 (2 H, m, H-5'ab), 3.15 (2 H, dt, -CH_2NHCO-5), 3.01 (2 H, dt, -AcNHCH_2-), 2.16 (2 H, m, 2'-ab), 1.78 (3 H, s, Ac). Anal. Calcd for $C_{18}H_{29}N_5O_6 \cdot 1/3\ H_2O$: C, 51.79; H, 7.16; N, 16.78. Found: C, 51.91; H, 6.95; N, 16.56.

6. Synthesis of **6**: 2-Cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.23 mL, 1.28 mmol) was added to a solution of **5** (480 mg, 0.68 mmol) in CH_2Cl_2 (10 mL) containing *N*,*N*-diisopropylethylamine (0.18 mL, 1.0 mmol). The mixture was stirred for 20 min at room temperature and was diluted with $CHCl_3$. The entire mixture was washed with aqueous saturated $NaHCO_3$, dried (Na_2SO_4), and concentrated. The residue was chromatographed over a neutralized silica gel column with hexane:EtOAc 1:2 to give **6** (460 mg, 75%, as a foam): FAB-MS *m/z* 898 (M^+); ^{31}P NMR ($CDCl_3$) δ 151.83 and 148.44 (85% H_3PO_4 as an internal standard).

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9. A solution containing each ODN was heated at 80 ℃ for 10 min, cooled gradually to an appropriate temperature, and used for the thermal denaturation study. Thermal-induced transitions of each mixture of ODNs were monitored at 254 nm by a Gilford Response II. Sample temperature was increased one degree per min. Each Tm represents the average of three measurements. Each sample contained appropriate ODNs (3 μM) and **21** (3 μM) in a buffer of 0.01 M sodium cacodylate (pH 7.0) containing 0.01 M NaCl, or ODNs (3 μM) and **22** (3 μM) in a buffer of 0.01 M sodium cacodylate (pH 7.0) containing 0.1 M NaCl.

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