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NUCLEOSIDES AND NUCLEOTIDES. 127. A NOVEL AND CONVENIENT POST-SYNTHETIC MODIFICATION METHOD FOR THE SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES CARRYING AMINO LINKERS AT THE 5-POSITION OF 2'-DEOXYURIDINE[1]

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Key Words: nucleoside; 5-methoxycarbonyl-2'-deoxyuridine; oligodeoxyribonucleotide; amino-linker; post-synthetic modification

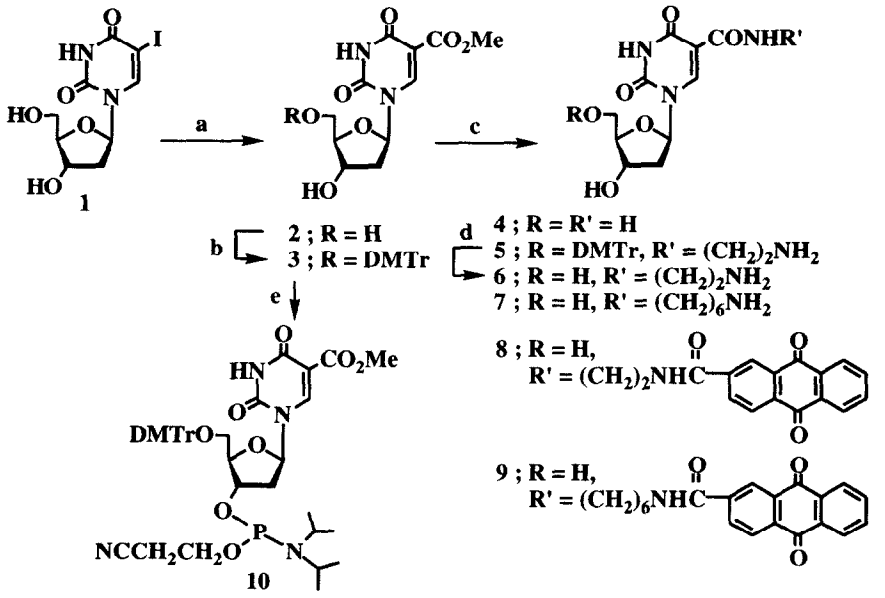
Abstract: 5-Methoxycarbonyl-2'-deoxyuridine has been synthesized and incorporated in oligonucleotides. The fully protected oligonucleotides were treated with diaminoethane or diaminohexane and the oligonucleotides were deprotected and purified to give oligonucleotides carrying amino-linkers, which were further derivatized with an intercalator. Properties of these oligonucleotides are described.

Various oligonucleotide analogues carrying linker groups have been synthesized and further functionalized with intercalating, DNA degrading, alkylating, and fluorescence groups, have been used in biological, biophysical, and chemotherapeutical studies.[2] There are two methods available for introducing linker groups into oligomers. One is to construct a modified mononucleotide unit, which is then incorporated into an oligomer by a usual automated DNA synthesizer. This method is, however, sometimes encumbered with tedious protection-deprotection processes and solubility problems in the mononucleotide units. Especially when the optimum length of the linker group for a desired function is not known, several mononucleotide units having linkers with a variety of lengths have to be constructed and then each oligonucleotide containing them has to be prepared separately. To avoid such time-consuming processes, a post-synthetic modification[3,4] has been developed, from which a nucleotide unit having a leaving group within a molecule, which should be stable under conditions of DNA synthetic cycles, was initially introduced in an oligomer and then appropriately modified linkers and/or further functionalized linkers are introduced into the oligomer.[4] In this way, a variety of linkers can be incorporated at one time.[3] Verdine's group has reported substitutions of O^4-(2,4,6-trimethylphenyl)uracil[3a,b] and O^6-phenylpurine[3c] in preformed oligonucleotides by a variety of amino nucleophiles affording oligonucleotides containing N^4-alkylcytosines and N^6-alkyladenines, respectively, to prove the usefulness of this method. However, introduction of the linkers at the exocyclic amino group reduced the melting temperature (Tm) of duplexes with unmodified complementary oligomers.[3b] Therefore, to make the post-synthetic modification method more useful and reliable, new methods should be developed, in which the linker groups do not destabilize duplex and triplex formations.

During the course of searching for such new methods, we have designed 5-methoxycarbonyl-2'-deoxyuridine (**2**) as a convertible nucleoside. If an oligonucleotide containing **2** reacts smoothly with certain tether groups under mild conditions, this method would solve the previous problems: the stability of duplexes and triplexes would not be reduced by the introduction of **2** due to the electron-withdrawing nature of the 5-

(*N*-alkylamino)carbamoyl group although it has a steric bulkiness around the major groove. The oligonucleotide containing **2** attached to resins can be divided into several portions that can react with tethers having various lengths and functions. In this communication, we describe the synthesis and chemical properties of **2** along with the possibilities of the post-synthetic modification method in oligonucleotides containing **2**. We also deal with introduction of an intercalator into the amino linker and their thermal stabilities with duplex and triplex formations.

Scheme 1[a]



[a]a) CO, $(PhCN)_2PdCl_2$, MeOH, Et_3N, 50 °C; b) DMTrCl, pyridine, room temperature c) NH_3/MeOH, 50 °C from **2** to **4**; $H_2N(CH_2)_2NH_2$, MeOH, room temperature, from **3** to **5**; d) aqueous 80% AcOH, room temperature; e) 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite, *N,N*-diisopropylamine, CH_2Cl_2, room temperature.

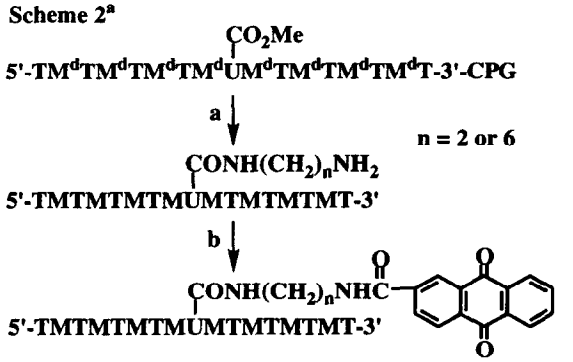
Palladium-catalyzed carbonylation of 5-iodo-2'-deoxyuridine (**1**) with carbon monoxide in MeOH gave **2** in 95% yield (Scheme 1).[5] Before introduction of **2** in oligonucleotides, the chemical reactivity of the methoxycarbonyl group with amines was examined. On treatment of **2** with NH_3/MeOH for 16 h at 50 °C, 5-carbamoyl-2'-deoxyuridine (**4**) was obtained in 95% yield. 5-(*N*-Aminoethyl)carbamoyl-2'-deoxyuridine (**6**)[6] was also readily accessible in 73% yield in 2 steps with the reaction of **3** with diaminoethane in MeOH for 2 days at room temperature followed by deprotection. Under these conditions, no side products such as 2'-deoxyuridine 5-carboxylate were detected on HPLC analyses in either reaction. Therefore, these mild conditions for the substitution reaction can be used for an oligonucleotide level to introduce amino linkers. Conversion of **3** into nucleotide unit **10**[7] was done by standard conditions without any problems.

Compound **10** was then incorporated into two 17-mers [5'-$(TM^d)_4$**2**$(M^dT)_4$-3'-CPG and 5'-**2**$(M^dT)_8$-3'-CPG, where CPG is a controlled-pore glass] using the phosphoramidite method[8] on a DNA synthesizer

(Scheme 2). The coupling yield of **10** was 97% using 0.12 M CH_3CN solution and 6 min for coupling time. Fully protected oligonucleotides linked to the solid support were divided into two portions each, each of which was treated with a large excess of diaminoethane or 1,6-diaminohexane in MeOH at 45 °C overnight, followed by C-18 column chromatography and de-tritylation giving 5'-$(TM)_4$**6**$(MT)_4$-3' (**11**), 5'-$(TM)_4$**7**$(MT)_4$-3' (**12**), 5'-**6**$(MT)_8$-3' (**13**), and 5'-**7**$(MT)_8$-3' (**14**) after purification using ion-exchange column chromatography followed by a C-18 HPLC.[9] Starting from 1 μmol of thymidine residues linked to CPG, 12 to 23 OD units (at 254 nm) of each oligonucleotide were obtained.

Reaction of these oligomers with *N*-[(anthraquinone-2-carbonyl)oxy]succinimide at room temperature overnight furnished the desired functionalized oligonucleotides 5'-$(TM)_4$**8** $(MT)_4$-3'(**15**), 5'-$(TM)_4$**9**$(MT)_4$-3' (**16**), 5'-**8**$(MT)_8$-3' (**17**), and 5'-**9**$(MT)_8$-3' (**18**) (Scheme 2).[10] In Fig. 1a, an HPLC profile of a mixture of **13** and **17** is shown as an example. To confirm the presence of these modified nucleosides, oligomers **13** and **17** were hydrolyzed by a mixture of venom phosphodiesterase and alkaline phosphatase to the corresponding nucleosides, the composition of which was T:M:**6** or **8** = 8:8:1 (Fig. 1b, c). From these experiments, we found that the amino linkers can be easily introduced in the corresponding oligonucleotides at the desired position.

Scheme 2[a]



[a] a) $H_2N(CH_2)_2NH_2$ or $H_2N(CH_2)_6NH_2$ MeOH, 45 °C, then concentrated NH_4OH, room temperature; b) ref. 10b; **T**, **M**, and **M**^{d} correspond to thymidine, 5-methyl-2'-deoxycytidine, and 4-*N*-[(dimethylamino)methylene]-5-methyl-2'-deoxycytidine, respectively.

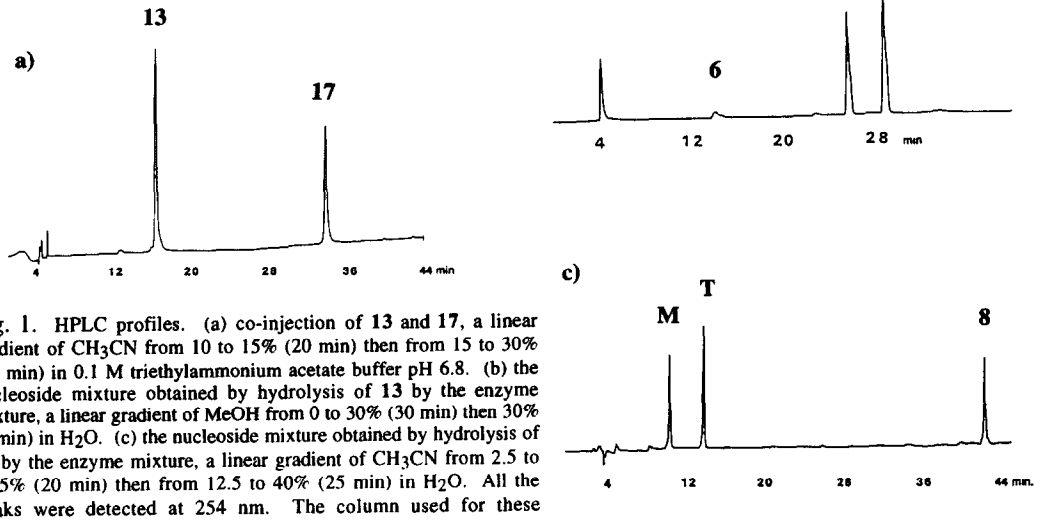


Fig. 1. HPLC profiles. (a) co-injection of **13** and **17**, a linear gradient of CH_3CN from 10 to 15% (20 min) then from 15 to 30% (20 min) in 0.1 M triethylammonium acetate buffer pH 6.8. (b) the nucleoside mixture obtained by hydrolysis of **13** by the enzyme mixture, a linear gradient of MeOH from 0 to 30% (30 min) then 30% (5 min) in H_2O. (c) the nucleoside mixture obtained by hydrolysis of **17** by the enzyme mixture, a linear gradient of CH_3CN from 2.5 to 12.5% (20 min) then from 12.5 to 40% (25 min) in H_2O. All the peaks were detected at 254 nm. The column used for these experiments was Inertsil ODS-2 (GL Science Inc.).

Stability of duplexes and triplexes formed by the oligonucleotides with a target complementary oligomer, 5'-TG(GA)$_9$GGT-3' (**19**), for the duplex formations and a hairpin duplex, 5'-AG(TC)$_9$C(T)$_5$AG(GA)$_9$CT-3' (**20**), for the triplex formations were next studied by thermal denaturation, and the results are summarized in Table 1. 5'-T(MT)$_8$-3' (**21**) was used as a control. The stability of the duplexes did not seem to depend upon the position of the amino linker attached or lengths of the linker, although the Tm values of the duplexes formed by **15** and **16** (the linkers at the 5' position) with **19** are slightly higher than those of **11** and **12** with **19**. However, the Tm values of these duplexes are higher than that of the control duplex between **19** and **21**. The anthraquinone-attached oligomers showed a similar tendency, in which **17** and **18** with **19** are slightly more stable than **15** and **16** with **19** and Tm values of these oligomers are higher than those of the corresponding oligomers having free amino-linkers. The triplex formations using these oligomers with **20** were observed in all the entries, but only with **12**, **17**, and **18**, were the Tm values slightly higher than that of the control triplex. Therefore, lengths of the linkers in each case would be important for triplex formation. For post-synthetic modification it will be useful to find suitable lengths for linker groups.

Table 1. Thermal denaturation of the oligonucleotides.[a]

oligonucleotides	Tm (°C)[b] duplex with **19**	Tm (°C)[c] triplex with **20**	oligonucleotides	Tm (°C)[b] duplex with **19**	Tm (°C)[c] triplex with **20**
(TM)$_4$**6**(MT)$_4$ (**11**)	53	32	**6**(MT)$_8$ (**13**)	54	34
(TM)$_4$**7**(MT)$_4$ (**12**)	53	37	**7**(MT)$_8$ (**14**)	55	34
(TM)$_4$**8**(MT)$_4$ (**15**)	55	33	**8**(MT)$_8$ (**17**)	56	39
(TM)$_4$**9**(MT)$_4$ (**16**)	57	34	**9**(MT)$_8$ (**18**)	58	41
T(MT)$_8$ (**21**)	49	36	T(MT)$_8$ (**21**)	49	36

Duplex Formation

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5'-TMTMTMTMTMTMTMTMT-3'
3'-TGGAGAGAGAGAGAGAGAGGT-5'
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Triplex Formation

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      5'-TMTMTMTMTMTMTMTMT-3'
T T   AGGAGAGAGAGAGAGAGACT-3'
T  T  TCCTCTCTCTCTCTCTCTGA-5'
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a) The solution containing each oligomer was heated at 70 °C for 20 min, then cooled gradually to an appropriate temperature and used for the thermal denaturation study. Thermally induced transitions of each mixture of oligomers were monitored at 254 nm by a Gilford Response II. Sample temperature was increased one degree per one min. Each Tm is given as an average of three measurements. b) Each sample contained appropriate oligonucleotides (3 μM) and **19** (3 μM) in a buffer of 0.01 M sodium cacodylate (pH 7.0) containing 0.01 M NaCl. c) oligonucleotides (3 μM) and **20** (3 μM) in a buffer of 0.01 M sodium cacodylate (pH 7.0) containing 0.5 M NaCl. The second Tm due to dissociation of **20** in all the entries and Tm of **20** itself were 88 °C.

To understand mechanisms to stabilize the duplex and triplex formation, we next studied the physical properties of **4** and **6**. Titration of **6** showed that the pKa was 8.1 which is 1.8 units lower than that of thymidine (pKa = 9.9, a reported value was 9.8[11]). Furthermore, the molecular conformation of **4** was analyzed by X-ray diffraction.[12] An intramolecular hydrogen bond between the O^4 atom and one of the carbamoyl-proton was observed in the crystal; an ORTEP drawing is given as Fig. 2a. This hydrogen bond

together with the electron-withdrawing nature of the 5-(*N*-ethylamino)carbamoyl group would be related to an increase in the acidity of N^3-H in **6** and acceleration of the complex formations, if the 5-(*N*-ethylamino)carbamoyl group has a similar conformation to **4**. It should be noted that the *N*-alkyl group would be conformationally fixed in one direction as shown in Fig. 2b.

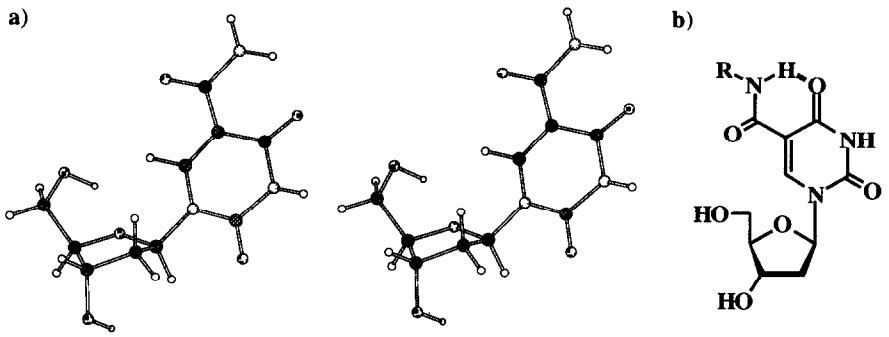

Fig. 2. a) Stereoview of the ORTEP drawing of **4**. b) A possible conformation of the carbamoyl group in **6**.

In conclusion, we have described a new and convenient post-synthetic modification method for the synthesis of modified oligonucleotides using 5-methoxycarbonyl-2'-deoxyuridine (**2**). Amino-linkers were readily introduced in oligonucleotides containing **2**, in which the anthraquinone derivative was also incorporated. This method can be of great value as a post-synthetic modification to find an optimum length of the linker group for a desired function. Further applications will be reported shortly.

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5. Synthesis of **2**: A mixture of **1** (3.56 g, 10 mmol), $(PhCN)_2PdCl_2$ (26 mg, 0.07 mmol), and Et_3N (1.54 mL) in MeOH (300 mL) was heated at 50 °C under a CO atmosphere. After 20 h, precipitates were removed by filtration and the filtrate was concentrated *in vacuo*. The residue was crystallized from EtOH to give **2** (2.73 g, 95%); mp 194-197 °C; ^{1}H NMR (DMSO-d_6) δ 11.55 (1 H, br s, NH), 8.81 (1 H, s,

H-6), 6.10 (1 H, dd, H-1', $J_{1',2'a}$ = 6.1, $J_{1',2'b}$ = 6.3 Hz), 5.27 (1 H, d, 3'-OH), 5.08 (1 H, t, 5'-OH), 4.25 (1 H, m, H-3'), 3.81 (1 H, m, H-4'), 3.70 (3 H, s, OMe), 3.61 (2 H, m, H-5'a,b), 2.21 (2 H, m, H-2'a,b). *Anal.* Calcd for $C_{11}H_{14}N_2O_7$: C, 46.16; H, 4.93; N, 9.79. Found: C, 45.86; H, 4.93; N, 9.72.

6. Synthesis of **6**: Ethylenediamine (2 mL, 23 mmol) was added to a solution of **3** (400 mg, 0.68 mmol, prepared from **2**) in MeOH (4 mL). The mixture was stirred for 2 days at room temperature and the volatile was removed *in vacuo*. The residue in $CHCl_3$ was successively washed with aqueous saturated KH_2PO_4 and brine and the organic phase was dried (Na_2SO_4). The solvent was removed to give crude **5** (690 mg), which was then treated with aqueous 80% AcOH for 5.5 h at room temperature The mixture was concentrated and the residue was crystallized from a mixture of EtOH and Et_2O to give **6** (157 mg, 73%); mp 218 °C (dec.). ^1H NMR (D_2O) δ 8.63 (1 H, s, H-6), 6.29 (1 H, t, H-1', $J_{1',2'}$ = 6.5 Hz), 4.51-4.41 (1 H, m, H-3'), 4.12-4.05 (1 H, m, H-4'), 3.85 (1 H, dd, H-5'a, $J_{a,4'}$ = 4.2, $J_{a,b}$ = 11.0 Hz), 3.77 (1 H, dd, H-5'b, $J_{b,4'}$ = 3.5, $J_{a,b}$ = 11.0 Hz), 3.65 (2 H, dt, -NHCH_2-), 3.16 (2 H, dd, -CH_2NH2), 2.43-2.18 (2 H, m, H-2'a,b). *Anal.* Calcd for $C_{12}H_{18}N_4O_6 \cdot 0.5\ H_2O$: C, 44.58; H, 5.92; N, 17.33. Found: C, 44.52; H, 5.76; N, 17.39.

7. Synthesis of **10**: 2-Cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.28 mL, 1.28 mmol) was added to a solution of **3** (500 mg, 0.85 mmol) in CH_2Cl_2 (10 mL) containing *N*,*N*-diisopropylethylamine (0.29 mL, 1.7 mmol). The mixture was stirred for 50 min at room temperature and was diluted with $CHCl_3$. The whole was washed with aqueous saturated $NaHCO_3$, dried (Na_2SO_4), and concentrated. The residue was chromatographed over a neutralized silica gel column to give **10** (570 mg, 85% as a foam): FAB-MS *m/z* 789 (M^++1); ^{31}P NMR ($CDCl_3$) δ 144.1 and 144.6 (H_3PO_4 as external standard).

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9. In the case using diaminohexane, the oligomers **12** and **14** was purified by gel-filtration column chromatography (Sephadex G-25) prior to using C-18 column chromatography.

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12. Unpublished results from Dr. M. Haratake at the Research Laboratories of Yoshitomi Pharmaceutical Industry Ltd.: Compound **4** was crystallized from aqueous EtOH. The crystal data are as follows: $C_{10}H_{13}N_3O_6$, M = 271.23, monoclinic; space group $P2_1$, a =9.956 (3), b = 20.528 (2), c = 5.539 (1) Å, β = 97.20 (2)°, V = 1123.0 Å^3, Z = 4, D_x = 1.603 Mgm^{-3}, F (000) = 568, μ(Cu$K\alpha$) = 1.542 mm^{-1}. A total of 1732 independent reflections were collected and used for the structure analysis. The final R value was 0.0403.

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