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Effect of the Terminal Amino Group of a Linker Arm and Its Length at the C5 Position of a Pyrimidine Nucleoside on the Thermal Stability of DNA Duplexes

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2′-Deoxyuridine derivatives bearing a substituent at the C5-position, which has a different chain length and a different functional group (methyl or amino), were synthesized and incorporated into oligodeoxyribonucleotides. The effect of the substituent groups in the major groove on the stability of the duplexes was investigated by UV melting experiments. It was found that the stabilization of these duplexes by a terminal amino group depended on the length of a linker arm.

Key Words: oligodeoxyribonucleotide; C5-substituted 2′-deoxyuridine; thermal stability.

INTRODUCTION

There is great interest in modified oligodeoxyribonucleotides (ODN) as research tools for molecular biology and antisense molecules. An appropriate chemical modification of ODN leads to improved hybridization properties, nuclease resistance, and cell permeability. To date, various chemically modified ODNs have been synthesized and their ability for the antisense effect has been investigated. The modification at the 5′-end and 3′-end, internucleotide phosphodiester of ODNs, the 1′-position and 2′-position of the sugar, C5 of a pyrimidine, or N^4 of cytosine has been reported (*1*). The C5-position of a pyrimidine is a suitable site for the modification of oligonucleotides. The substituent at C5 of a pyrimidine does not inhibit the base pairing with adenine on a complementary strand, but the thermal stability of the duplex depends on the kind of tethers and functional groups. For example, C5-propagyl substitution of 2′-deoxyuridine conferred significant stability to duplexes and triplexes (*2*). This indicates that their modification should bring about perturbation to the stability of a duplex DNA. In a previous paper (*3*), we reported the effect of acridine-modification at the C5-position on duplex stability. The acridine modification causes stabilization

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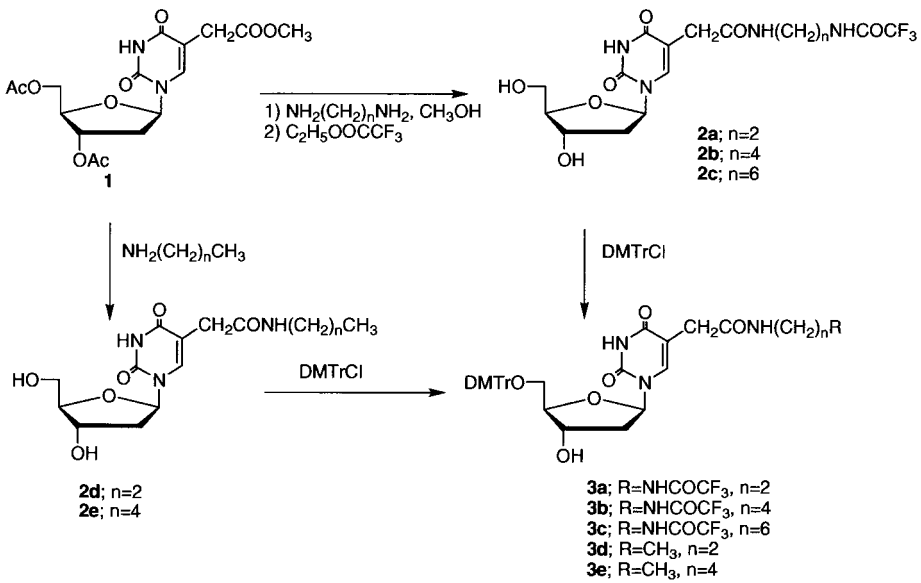
of a duplex-form by interaction between the acridine moiety and DNA, and the length of the spacer arm has a slight effect on the interaction. However, the relationship between the duplex stability and the length of spacer remains to be clarified. The structure of a tether to introduce a functional group on DNA is important for the preparation of hybridization probes or antisense molecules. Here we investigated the effect of the length of a tether between an oligodeoxyribonucleotide and a functional group on the duplex stability. An amino group was attached on the tether as the functional group because the amino group can interact with the DNA backbone and is useful for further functionalization.

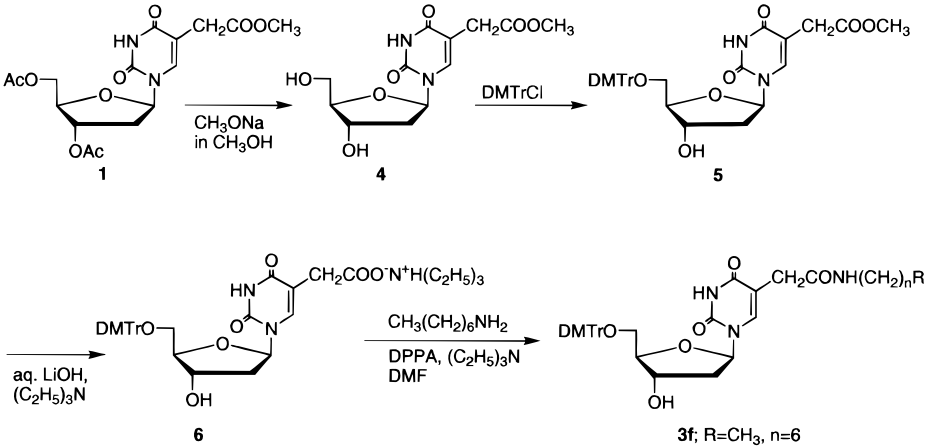
RESULTS AND DISCUSSION

Synthesis of Modified Nucleotides

The C5-Substituted nucleosides bearing an amine linker were prepared from 5-methoxycarbonylmethyl-2′-deoxyuridine (**1**) as described previously (*4*). Most of the amines reacted with **1** in satisfactory yields by a direct aminolysis of a methyl ester group as shown in Scheme 1. In the reactions with diamines, the terminal amino group at the C5-substituent was protected with a trifluoroacetyl group without purification. Heptylamine could not react with **1** by aminolysis because of its low nucleophilicity. Therefore, the corresponding nucleoside was synthesized by another route as shown in Scheme 2. The methoxycarbonyl group in **4** was hydrolyzed after protection of the 5′-hydroxyl group, followed by condensation of the resulting carboxyl group with heptylamine using diphenylphosphoryl azide (DPPA) as a condensing reagent. The 5′-hydroxyl group of the modified nucleosides was protected with a dimethoxytrityl



SCHEME 1.

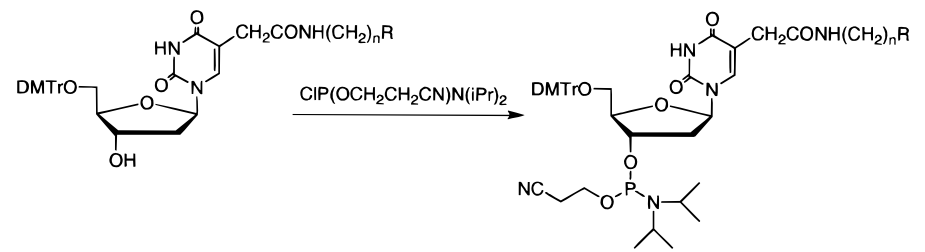


SCHEME 2.

group and all 5′-protected modified nucleosides were converted to nucleoside 3′-phosphoramidites as shown in Scheme 3.

Synthesis of the Modified Oligodeoxyribonucleotides

The modified oligodeoxyribonucleotides (ODNs) were synthesized on a DNA synthesizer. The coupling yields of the modified nucleoside 3′-phosophoramidites on a CPG support are shown in Table 1, with abbreviations and retention times of ODNs on a reversed-phase HPLC. The coupling yields decreased with an increase in the chain length of the C5-substituent, particularly for the nucleosides bearing a terminal methyl group (**7d–f**). The synthesized ODNs were deprotected and purified in the usual manner. The longer retention time on a reversed-phase HPLC column correlates



3a; R=NHCOCF$_3$, n=2:
3b; R=NHCOCF$_3$, n=4:
3c; R=NHCOCF$_3$, n=6:
3d; R=CH$_3$, n=2
3e; R=CH$_3$, n=4
3 f; R=CH$_3$, n=6

7a; R=NHCOCF$_3$, n=2:
7b; R=NHCOCF$_3$, n=4:
7c; R=NHCOCF$_3$, n=6:
7d; R=CH$_3$, n=2
7e; R=CH$_3$, n=4
7 f; R=CH$_3$, n=6

SCHEME 3.

TABLE 1

Abbreviations, Coupling Yields, and Retention Times of Modified ODNs

Abbreviations	X^a	Coupling yield (%)[b]	T_R/min[c]
N-ODN	T	99	16.5[d]
ODN-2N	**2a′**	100	15.8
ODN-4N	**2c′**	97	15.9
ODN-6N	**2e′**	96	16.7
ODN-2C	**2f**	100	18.0
ODN-4C	**2g**	97	21.1
ODN-6C	**2h**	87	26.0

Note. ODN sequence: 5′d(CATAGGAGAXGCCTA)3′.

[a] **2a′**, **2c′**, and **2e′** were the deprotected nucleosides of **2a**, **2c**, and **2d** by an alkaline treatment, respectively.

[b] Coupling yields are for incorporation of the modified nucleosides.

[c] HPLC condition is in the experimental section except the gradient. Gradient: 10% B to 35% B in 35 min.

[d] HPLC condition is in the experimental section except the gradient. Gradient: 3% B to 53% B in 35 min.

with the stronger hydrophobicity of the substituent. The structure of the ODNs was confirmed by a nuclease digestion.

Thermal Stability of the Duplexes Consisted of the Modified ODNs and Its Complementary DNA

Thermal stabilities of the ODN/DNA duplexes were investigated by UV melting experiments. The melting temperatures (T_m) of the duplexes are summarized in Table 2. For the ODN/DNA duplexes, most of the ODNs, except **ODN-2N**, showed lower T_m values than a normal ODN (**N-ODN**). The T_m values decreased with increasing chain length of the side arm, especially for **ODN-2N**, **-4N**, and **-6N**, which has a terminal amino group on the C5-substituent. In comparison of the different terminal groups with the same linker length, the duplexes formed from the ODN bearing a terminal amino group always had higher T_m values than those from the ODN bearing a terminal methyl group. This result suggests that the alkyl linker arm interfered with

TABLE 2

Melting Points and Thermodynamic Parameters of ODN/DNA Duplexes

ODN	T_m(°C)	ΔH^0(kJ mol^{-1})	ΔS^0(J mol^{-1} K^{-1})	ΔG_{37}(kJ mol^{-1})
N-ODN	50.6	−496	−1420	−55.5
ODN-2N	53.7	−516	−1460	−63.1
ODN-4N	49.8	−521	−1500	−55.7
ODN-6N	47.9	−559	−1630	−53.4
ODN-2C	47.6	−526	−1530	−51.4
ODN-4C	46.1	−481	−1390	−49.8
ODN-6C	47.1	−414	−1180	−48.0

Note. Measurement condition under Experimental.

the duplex formation and the terminal amino group stabilized the duplexes. This effect of the amino linker arm on the duplex stability is consistent with our previous results (*5*), which indicated that the stability of the duplexes depends on the number of amino groups in the linker arm and the length of the arm.

To gain detailed information on the stability of duplexes formed from the ODNs, thermodynamic parameters for the duplex formation were estimated from the curve-fitting for each melting curve. The calculation was performed by a nonlinear, least squares calculation program, "TMSPEC," which was developed by Kodama *et al.* (*6*). The results are shown in Table 2. In the ODNs bearing a terminal amino group on the linker arm, ODNs bearing the longer linker arm showed a more negative ΔH, which suggests the stabilization of the duplex, and a more negative ΔS, which suggests the destabilization of the duplexes. Hence, this destabilization is governed by a decrease in ΔS for the ODN bearing the longer linker arm. The ODNs bearing a terminal methyl group and the longer linker arm showed a less negative ΔH and ΔS. Since their duplexes had almost the same T_m values, an unfavorable ΔH of the duplex formation bearing a longer alkyl group was compensated by a favorable ΔS. This suggests that the long alkyl linker destabilizes the duplex in terms of enthalpy, but the long linker with a terminal amino group stabilizes the duplex in terms of enthalpy. In comparison of the different terminal groups bearing the same linker length, the differences between the enthalpy change of a terminal amino group and a methyl group ($\Delta\Delta H^0$), are $+10$ kJ mol^{-1}, for **ODN-2N** and **-2C**, -40 kJ mol^{-1} for **ODN-4N** and **-4C**, and -145 kJ mol^{-1} for **ODN-6N** and **-6C** from Table 2. It is suggested that the duplexes consisting of **ODN-4N** or **-6N**, which have a long linker, were stabilized by the contribution of enthalpy change compared with those consisting of **ODN-4C** or **-6C**, although the alkyl group is disadvantageous for the duplex formation in terms of entropy change. On the other hand, the **ODN-2N**/DNA duplex was stabilized by an increase in ΔS value compared with the **ODN-2C**/DNA. That is to say, the large negative $\Delta\Delta H^0$ in the long linker arms suggests that the terminal amino groups at the side chain interact with moieties of the complementary strand on the DNA duplex. Dande *et al.* reported that the side chain of 5-(aminohexyl)-2′-deoxyuridine was placed at the 3′-side in the major groove could have an electrostatic interaction with an electronegative atom in the major groove (*7*). In their model, the terminal amino group at the linker arm interacted with *O*6 of 5′-side G on the complementary strand. Therefore, in our duplexes it is also estimated that the terminal amino group at the side chain of ODN could interact with electronegative atoms in the major groove such as *O*6 and *N*7 of G-12 and G-13 and *N*7 of A14 in the complementary stand. One of the possible structures of each duplex, which were constructed by modeling of the modified ODN/DNA duplex based on Dande's report, is shown in Fig. 1.

In conclusion, the side chain at the C5-position of a pyrimidine nucleoside could interact with the DNA backbone and affect the duplex stability according to the length of the linker arm. The terminal amino group on the linker arm has a stabilization effect by the entropy for the ODN bearing a short linker and by the enthalpy for the ODN bearing a long linker. This knowledge is useful in the development of DNA probes or antisense molecules.