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Multi-functionalization of oligodeoxynucleotide: a facile post-synthetic modification technique for the preparation of oligodeoxynucleotides with two different functional molecules

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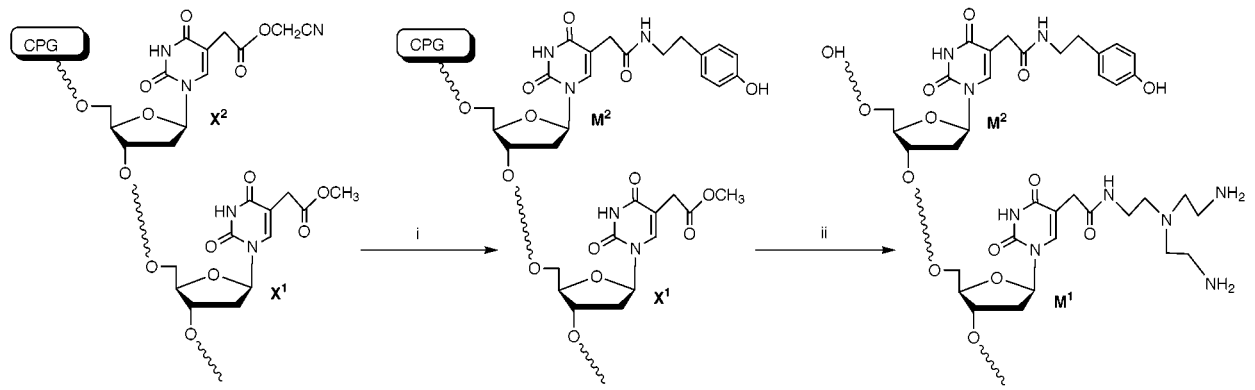
Treatment of the support-bound novel multi-functionalizable oligodeoxyribonucleotides bearing both 5-cyanomethoxycarbonylmethyl- and 5-methoxycarbonylmethyl-uridine with tyramine followed by tris(2-aminoethyl)amine facilitated multi-functionalization of oligodeoxyribonucleotides having different amine molecules.

Modification of oligodeoxyribonucleotides (ODNs) through incorporation of an appropriate molecule such as an intercalating agent, metal chelating agent, fluorescent dye, *etc*. has been widely studied to produce functionalized ODNs which can be used for biological and biophysical studies.[1] Recently, a new and convenient post-synthetic modification method for the functionalization of ODNs has been reported. In this method, ODNs containing a specially designed convertible unit is assembled first by an automated solid-phase synthesizer, then a functional molecule is introduced to the ODNs.[2] Previously, we have reported that the nucleoside analogs 5-methoxycarbonylmethyl- and 5-cyanomethoxycarbonylmethyl-uridine can be utilized as the convertible unit in the post-synthetic modification technique.[3] Thus, the treatment of ODNs substituting the normal thymidine residue with the convertible nucleoside unit with mono-, di- or poly-amine molecules resulted in the corresponding oligomers carrying the amine derivatives at the 5-position of the uridine derivatives *via* nucleophilic displacement reaction. The method is very attractive since one can site-specifically introduce the desired functional molecules into ODNs after the assembly of the oligomer and, therefore, avoid the tedious preparation of each nucleoside precursor and the corresponding phosphoramidite prior to the assembly of the oligomers.

Meanwhile, further investigation of the nucleoside analogs revealed that reactivity towards the amine-involved nucleophilic displacement reaction is significantly different between the analogs. So far, a report dealing with the site-specific modification of ODNs with several different functional molecules *via* a post-synthetic modification technique has not been known. This background prompted us to develop a versatile method which allows the site-specific introduction of several different functional molecules into ODNs by a facile one-pot reaction. Here, we report preliminary results of our study to produce multi-functionalized oligonucleotides *via* a post-synthetic modification technique.

Phosphoramidite derivatives of 5′-*O*-dimethoxytrityl-5-methoxycarbonylmethyl- and 5-cyanomethoxycarbonylmethyl-uridine were prepared according to the previously reported procedure.[3,4] Preparation of CPG-bound ODNs containing 5-methoxycarbonylmethyl- and 5-cyanomethoxycarbonylmethyl-uridine residues was performed by a DNA synthesizer (ABI 381-A) using the corresponding phosphoramidites on a 1 μmol scale. Two different ODNs, namely, ODN-1 (T**X²**TT**X¹**T) and ODN-2 (**X²**TCC**X¹**GCCA**X¹**AG), were prepared (Scheme 1). In the ODNs, **X¹** and **X²** denote the 5-methoxycarbonylmethyl- and 5-cyanomethoxycarbonylmethyl-uridine residues, respectively. An extended coupling period of 360 s for **X¹** and **X²** phosphoramidites was required to obtain reasonable coupling yields (*ca.* 95%) in the synthesis of the ODNs. *N*⁴-Acetyldeoxycytidine (dC^{ac})phosphoramidite[5] was utilized in the synthesis instead of the usual dC^{bz} phosphoramidite to avoid possible transamination[6] during the amine treatment of the oligomers. The normal synthetic protocol was followed otherwise. It should be noted that the sequence of ODN-2 is complementary to the *rev* region of HIV-1 mRNA.[7]

To check the feasibility of the current multi-functionalization concept, we first studied ODN-1. Thus, the CPG support attached to the oligomer (CPG-ODN-1) was removed from the column and placed into a vial with a Teflon-coated screw cap. The support was first treated with a saturated DMA solution of tyramine in the presence of 1,2,4-triazole for 3 days at room temperature.[3b] The support was then washed thoroughly with



CPG-ODN-1 $^{5'}$T**X²**T T**X¹**T $^{3'}$- CPG
CPG-ODN-2 $^{5'}$**X²**TC C**X¹**G CCA **X¹**AG $^{3'}$- CPG

M-ODN-1 $^{5'}$T**M²**T T**M¹**T $^{3'}$
M-ODN-2 $^{5'}$**M²**TC C**M¹**G CCA **M¹**AG $^{3'}$

Scheme 1 *Reagents and conditions*: i, tyramin, 1,2,4-triazole, DMA; ii, 50% tris(2-aminoethyl)amine in dry EtOH.

Table 1 The sequence, yields and nucleoside compositions of the multi-functionalized ODNs (M-ODNs)

Oligodeoxynucleotide sequence	Isolated yield (OD units)	Nucleoside composition A : G : C : T : M_1 : M_2
TM²TTM¹T	13.2	4.0 : 0.9 : 1.1
M²TCOM¹GCCAM¹AG	22.7	1.7 : 1.8 : 4.3 : 1.2 : 1.7 : 1.2

DMA, followed by MeCN.After drying by Savant Speed Vac concentrator, the support was treated with 50% tris(2-aminoethyl)amine in dry ethanol (1 ml) for 48 h.[3a] After collection and concentration with an N_2 evaporator to remove volatile material, the obtained solution was diluted with 1 M tetraethylammonium acetate buffer (pH 7.0) to adjust the volume to approximately 1 ml and applied to gel filtration using a Sephadex G-25 column. The appropriate fractions were collected and purified by reversed-phase (C-18) HPLC. The obtained oligomer was treated with 20% acetic acid to remove the 5′-*O*-DMTr group, followed by EtOH precipitation and gel filtration (Sephadex G-25). The isolated oligomer (M-ODN-1) was subjected to enzymatic digestion to confirm its structure using snake venom phosphodiesterase, alkaline phosphatase and nuclease P1. In reversed-phase HPLC (C-18) analysis, the digested M-ODN-1 gave three peaks corresponding to thymidine and the modified 2′-deoxyuridine residues (**M¹** and **M²**, Scheme 1). The structures of the modified nucleosides were identified by the co-injection of independently prepared authentic samples. As listed in Table 1, the nucleoside composition calculated from the HPLC analysis agrees well with the expected ratio of dT : **M¹** : **M²** (4 : 1 : 1, respectively) for M-ODN-1.

We further examined the applicability of the current method to the ODN consisting of four natural nucleoside units. Thus, CPG-ODN-2 was treated with tyramine and tris(2-aminoethyl)-amine as mentioned above except that the treatment of the CPG-bound oligomer with tris(2-aminoethyl)amine–EtOH mixture was extended to 3 days. After the work up as above, the obtained oligomer (M-ODN-2) was subjected to enzymatic digestion. As shown in Table 1, HPLC analysis of enzyme digested oligomer indicates that the multi-functionalized oligomer was generated *via* the sequential treatment of the amine derivatives (expected ratio of dA : dG : dC : dT : **M¹** : **M²** = 2 : 2 : 4 : 1 : 2 : 1 for M-ODN-2).† Although in Table 1, **M**² residue was slightly over represented in both oligomers, we could not detect any measurable amount of side products which would result from the reaction of the tyramine with the 5-methoxycarbonylmethyl residue. Thus, the introduction of two different functional molecules to DNA having all four natural nucleoside units was also successful. It should be noted that tyramine in M-ODNs can be used as the substrate for ^{125}I labeling *via* the chloramine-T method,[8] and tris(2-aminoethyl)-amine in M-ODNs is effective to enhance the stability of the duplex formed by the ODN and its complement.[9] The T_m study suggested, indeed, that the M-ODN-2 containing duplex has a slightly enhanced stability (higher T_m value) compared to the corresponding duplex having the unmodified oligomer (N-ODN-2).‡ The yields of the multi-functionalized oligomers obtained by the current method were short of excellent but still within the acceptable range (Table 1).

In conclusion, we were able to successfully demonstrate the feasibility of a facile one-pot modification method to produce multi-functionalized oligonucleotides in a post-synthetic manner. The current method would provide an easy access to ODNs which have a combination of a variety of functional molecules useful for chemical and biological studies.

References and notes

† In electron mass spectrometry, *m/z* 4104.2 ($M - 2H^+$, 4104.0 calc. for $C_{139}H_{192}N_{49}O_{76}P_{11}$ in which two tertiary amine functions in the polyamine moieties are presumably protonated) was obtained.

‡ The following T_m values were obtained with complementary DNA (11-mer); M-ODN-2, 44.7 °C, unmodified N-ODN-2, 43.5 °C. Conditions; 2 μM concentration of the each strand, 100 mM NaCl, 50 mM sodium phosphate buffer (pH 7.2).

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