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Methidium Intercalator Inserted into Synthetic Oligonucleotides

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Abstract: A new methidium intercalator phosphoramidite has been synthesized. Methidium incorporation into an oligonucleotide during the synthesis was confirmed by UV and MALDI TOF MS data. UV melting experiments showed enhanced stability of a duplex, containing internal methidium.

Conjugates of intercalating dyes with oligonucleotides have recently gained attention as sequence-specific agents having an increased ability to bind single- and double-stranded DNA. Their properties have been investigated in a number of studies.[1-4] Intercalators covalently attached to an oligonucleotide have also proved to be successful models for fluorescent energy transfer experiments.[5] We report here the synthesis of methidium intercalator phosphoramidite, which allows multiple introductions of the dye into synthetic oligonucleotides in any position.

The phosphoramidite with an intercalator insert was prepared as shown in Figure 1. 2,7-Diamino-9-(*p*-carboxyphenyl)-10-methylphenanthridinium chloride (**1**) was synthesized starting from 4,4′-dinitro-2-aminobiphenyl as previously described.[6] Before coupling with the linker **3,** the amino groups of the dye were protected by acylation with trifluoroacetic anhydride. A flexible hydrophilic linker for the intercalator insert was designed to make possible the introduction of methidium at any desired position. Selective protection of the 1,2-diol fragment in 1,2,3-butanetriol was followed by mesylation and substitution with a triethyleneglycol residue. Conversion of hydroxyl to azide, reaction with DMTCl after removal of the isopropylidene protection group, and reduction of the azide derivative with Ph_3P formed aminoalcohol **3** with an overall yield of 41%. Finally, **3** was coupled with an activated methidium derivative and phosphytilated to give the phosphoramidite **4**.

The synthesis of an oligonucleotide with an internal methidium insert, 5′-CCATG-MET-GCTAT, was performed on a DNA synthesizer ASM-2U (Russia) at the 0.2 μmole scale. Since monomer **4** has limited solubility in acetonitrile, a 0.05M solution in dichloromethane-acetonitrile mixture (8:2 v/v) was used. No modifications in the reaction cycle were made for intercalator phosphoramidite. After a standard deprotection procedure (concentrated ammonia, 55°C, 6 hs), the oligonucleotide was purified by reversed phase HPLC (Hypersil® ODS column, 250 cm x 4.6 mm, A=0.1M TEAA, B=50% MeCN in 0.1M TEAA. DMT on: 20-100% B in 30 min.; DMT off: 0-50% B in 30 min). Increased retention times were observed for both DMT-

protected and fully deblocked oligonucleotide relative to unmodified oligomers. The UV spectrum of the oligonucleotide showed an additional absorbance maximum at 520 nm, which concurs with spectral data previously reported for methidium covalently linked to TpT dinucleotide via a phosphoramidate derivative.[7]

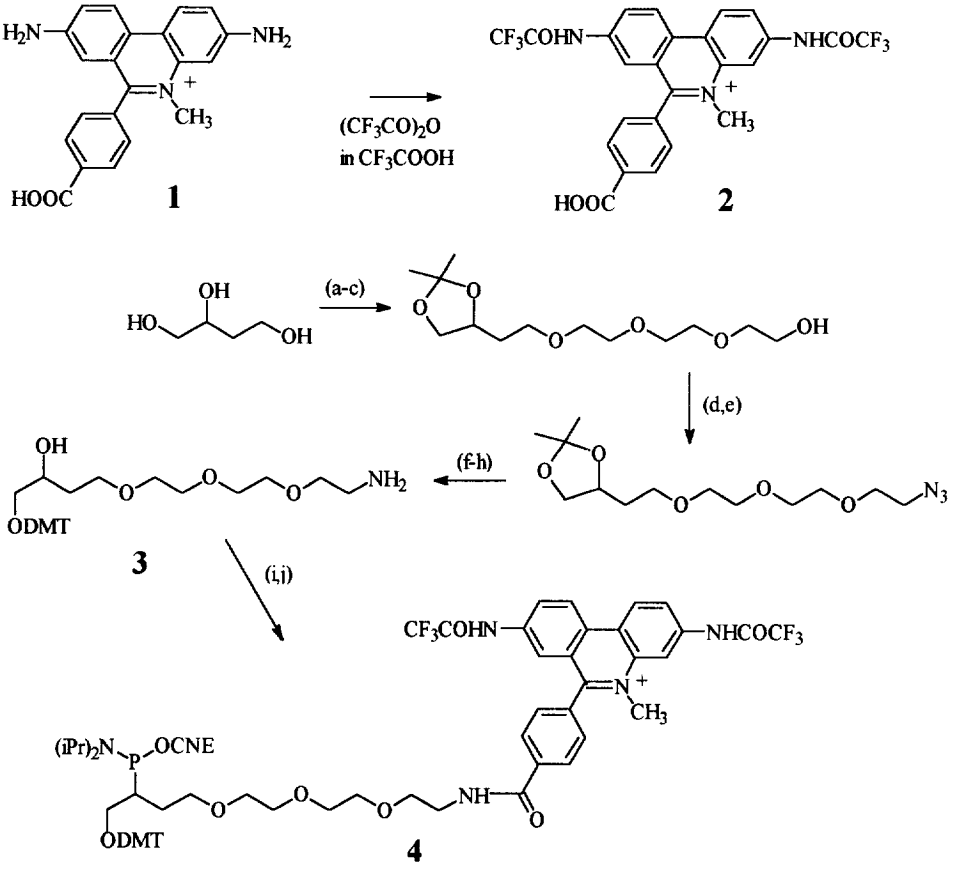

Fig. 1. The synthetic scheme for preparation of methidium phosphoramidite (**4**). (a) Me_2CO, $CHCl_3$, TsOH, reflux with aseotropic removal of water; (b) MsCl in Py; (c) triethyleneglycol, Na salt; (d) MsCl in Py; (e) LiN_3 in DMF, 130°C; (f) 80% AcOH; (g) DMTCl in Py; (h) Ph_3P in Py; (i) imidazolide derivative of **2** (from **2** and Im_2CO) in Py; (j) $(iPr_2N)_2POCH_2CH_2CN$, tetrazole in MeCN/DCM.

The structure of the conjugate was confirmed by analysis of the failure sequences that are always present in the crude oligonucleotide synthetic mixtures and are usually observed by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI TOF MS)[8,9] (Figure 2).

The sequence was reconstructed based on the mass difference between pairs of signals from

neighboring fragments, which was in good agreement with their theoretical values (for pdC, 289.2 Da; pdT, 304.2 Da; pdA, 313.2 Da; pdG, 329.2 Da; and for linker with dye, 624.6 Da). The mass of the smallest oligomer chain that could be observed on the spectrum corresponded within 0.8 Da to trimer 3′-TAT. The range below 800 Da was not interpretable due to high chemical noise background. Minor signals in the area of 3300 Da are actually a set of different N-1 sequences containing random deletions of the four possible bases across the chain. A plausible reason is either insufficient deblocking or capping along the synthetic cycle, which also explains the peak at 3004.0 Da, corresponding to the full-length product but without the linker with dye insert. Signals marked as +p are oligomers containing a 5′-phosphate group; the origin of the phosphate remains unclear.[9]

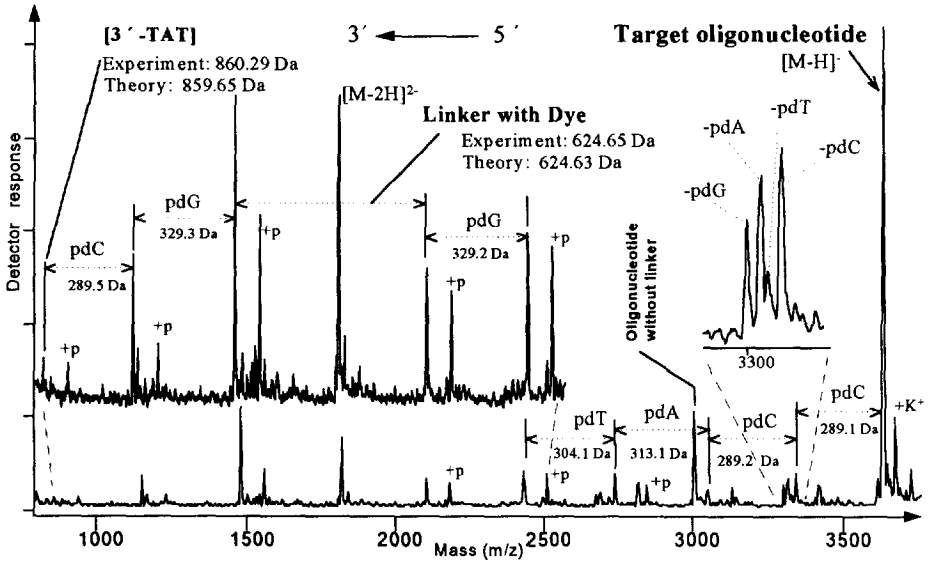

Fig. 2 Delayed Extraction MALDI TOF mass spectrum of the crude modified oligonucleotide (DMT-OFF) containing methidium dye. Matrix: 3-hydroxypicolinic acid (50g/l)/diammonium citrate (100 mM) 10/1(v/v)[10]

The effect of the internal methidum insert on the duplex stability was evaluated by determining the melting temperatures (T_m).[11] A complementary target 10-mer sequence, 5′-ATAGCCATGG-3′, was used in melting experiments for hybridization with an oligonucleotide either containing a methidium insert or without modification (Figure 3). A considerable increase in T_m value, by 8.1°C, was observed for a duplex containing one modified strand (T_m=46.7°C), relative to an unmodified duplex (T_m=38.6°C).

In summary, we have synthesized a new intercalator insert, allowing the introduction of a methidium dye into the oligonucleotides at any position. As shown by UV melting studies, modification of the oligonucleotide strand results in considerable increase in duplex stability.

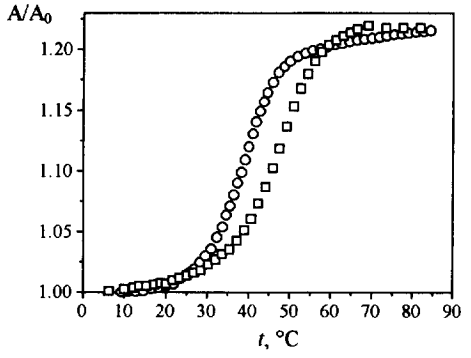

Fig. 3. Plots of A^{260}/A_0^{260} vs. *t* for duplexes with two unmodified strands (○) and with one strand containing internal methidium (□).

Acknowledgments

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References and Notes

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10. The spectrum was obtained with DE Voyager BioSpectrometry Workstation (PerSeptive BioSystems, Framingham, MA), linear mode, +25 kV accelerating voltage, 100 ns extraction delay, 337 nm N_2 laser.
11. T_m values were determined as the midpoint of transitions from melting experiments on a Shimadzu UV160U spectrophotometer at 260 nm, in 0.01 M sodium phosphate, 0.1 M NaCl, pH 7, at an oligonucleotide concentration of approximately 2 μM.

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