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Interaction of Cyanine Dyes with Nucleic Acids. Part 19: New Method for the Covalent Labeling of Oligonucleotides with Pyrylium Cyanine Dyes

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Abstract—New chemistry for the fluorescent labeling of oligonucleotides with cyanine dyes is proposed. It is based on the use of pyrylium salts as amine-specific reagents. Monomethyne pyrylium cyanine dye **1** was covalently linked to 5′-aminoalkyl modified oligonucleotide, with simultaneous conversion of the non-fluorescent dye **1** into fluorescent pyridinium cyanine structure **2**.

There is increasing interest in the use of fluorescently labeled oligonucleotides as hybridization probes for the detection of nucleic acids, primers for DNA sequencing, research tools for the studies on structure and dynamics of nucleic acids and proteins, etc.[1–3] Cyanine dyes are curently the most sensitive nucleic acid fluorescence probes characterized by high extinction coefficients (about 10^5 M^{-1} cm^{-1}) and nucleic acid binding constants.[3] Monomethyne benzothiazole and -oxazole cyanines being virtually non-fluorescent in free state demonstrate >1000-fold fluorescence increase when bound to nucleic acids[4–6] and so are especially suitable for homogeneous detection of nucleic acids.

Fluorescent dyes can be covalently linked to oligonucleotides by a variety of approaches.[1–3,7–10] The most popular methods are based on the reaction of 5′-aminoalkyl modified oligonucleotides with fluorophores bearing amine-specific reactive moieties. The advantage of post-synthetic labeling is that different reporter groups can be conjugated to the same sample of functionalized oligomer. Described synthetic procedures for the labeling of biomolecules with cyanines used dyes derivatized with isothiocyanate[11,12] and *N*-hydroxysuccinimidyl ester[12–14] functional groups. Oligonucleotides were labeled with carbocyanine dyes containing these functions.[15,16] Commercially available phosphoramidites of popular tri- and pentacarbocyanine dyes Cy3™ and Cy5™ can be used for solid-phase synthesis of labeled oligonucleotides.[17] There are, however, only few examples of the use of monomethyne cyanine dyes for oligonucleotide labeling. Oligonucleotide conjugates with thiazole orange (TO) and oxazole yellow (YO) monomethyne cyanines for the detection of specific DNA sequences have been prepared via the coupling of succinimidyl ester of TO dye to 5′-amino modified oligonucleotides[18] and by the reaction of YO iodoalkyl derivative with thiol-modified oligonucleotides,[19] respectively.

We have reported recently a promising new approach to oligonucleotide labeling using pyrylium salts as alternative amine-specific reactive group.[20] This method does not require any preparation of active intermediates. Before we have described new monomethyne benzothiazole cyanine dyes Cyan 39 (**1**) and Cyan 40 (**2a**). Cyan 40 (Abs_{max} 434 nm, Em_{max} 475 nm) with its low intrinsic fluorescence, great fluorescence enhancement upon binding to nucleic acids, and high fluorescence quantum yield for nucleic acid–dye complexes is suitable for the quantification of nucleic acids. At the same time, its close analogue, monomethyne pyrylium cyanine dye Cyan 39 (Abs_{max} 470 nm, Em_{max} 490 nm), has low fluorescence intensity upon binding to nucleic acids.[21] Our approach to oligonucleotide labeling is based on the use of pyrylium cyanine dyes like **1**, as illustrated in Scheme 1.

Pyrylium salts are known to react with primary amines producing pyridinium cations.[22] Thus, in the reaction of aminoalkyl-functionalized oligonucleotide with

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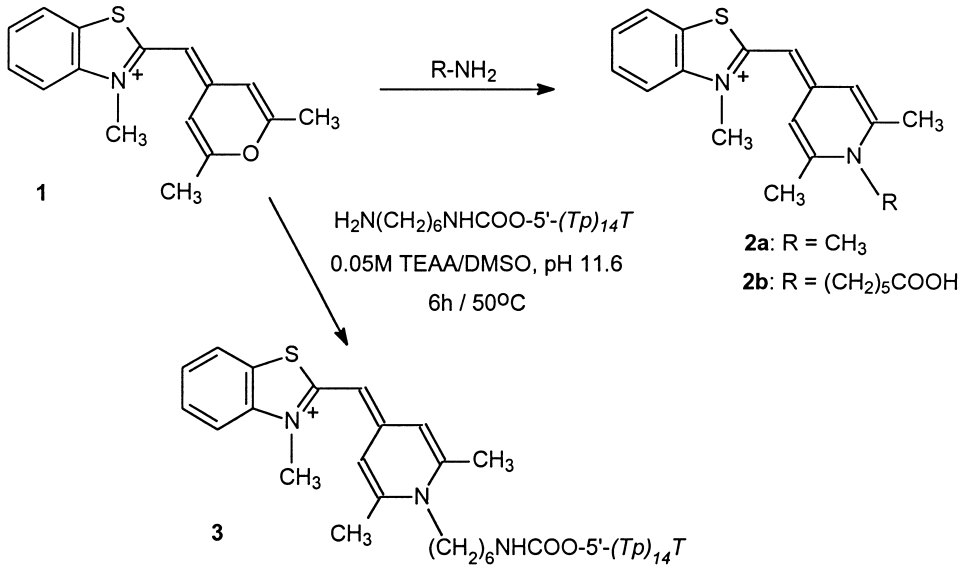

Scheme 1. Reaction of pyrylium cyanine dye **1** with amines and amino-modified oligonucleotide.

amine-specific pyrylium cation, oligonucleotide labeling could be achieved with simultaneous conversion of pyrylium dye into pyridinium derivative. This procedure allowed us to prepare oligonucleotide modified with pyridinium dye **2a** starting from the pyrylium heterocycle of **1**, i.e., fluorescent probe was obtained from almost non-fluorescent predecessor.

Pyridinium cations like **2** are susceptible to nucleophilic displacement of *N*-substituent.[22] Basic pH is essential for the efficient reaction of an aminoalkyl group with pyrylium cation, however at high pH the cleavage of coupling product could be a yield-decreasing side process. So optimum conditions were evaluated by the series of experiments using a model reaction of **1** (prepared by methylation of 2-methylbenzothiazole followed by reaction with 2,6-dimethyl-γ-pyrone[21]) with ω-aminocaproic acid (1:1) as follows: 1 mL of 5 mM ω-aminocaproic acid in buffers of various pH and concentration, and 0.5 mL 10 mM **1** (as perchlorate) in DMSO were mixed and incubated at 50 °C. The course of the model reaction was easily monitored by UV–vis spectroscopy by the shift of the absorption maximum from 470 to 434 nm due to the formation of Cyan 40 chromophore. In sodium carbonate/bicarbonate buffer (0.05 M, pH 9.5) the transformation of **1** to **2b** was achieved in 1 h, with a substantial amount of side products increasing at higher pH. Triethylammonium acetate (TEAA, pH 11.6, final concentration 0.05 M) was found to be the most suitable reaction medium. In this buffer, conversion was completed in 30 min at 50 °C and no side reaction was observed. Structure of product **2b** was confirmed by mass spectrometry.[23] No reaction of exocyclic amino groups of nucleosides (dC, dA and dG) with pyrylium cyanine was observed under reported reaction conditions. These mild conditions were then applied to model oligonucleotide labeling.

5′-Aminohexyl pentadecathymidylate was prepared by the solid-phase carbonyldiimidazole method.[24] Modified oligonucleotide was used without isolation as its purity was found to be sufficient according to its HPLC pattern. Crude 5′-aminoalkyl-T_{15} (4 OD_{260}, 30 nmol) in 80 μL of water was added to 40 μL of 0.2 M TEAA buffer (pH 11.6), and 50 μL of the dye **1** solution in DMSO (10 μmol/mL, 500 nmol) was added with agitation. The progress of labeling reaction was monitored by reverse-phase HPLC. After 6 h of incubation at 50 °C in the dark, oligonucleotide material was precipitated by 2% $LiClO_4$ in acetone and 5′-labeled product was isolated by HPLC. The HPLC profile of the reaction mixture and absorbance spectrum of the dye–T_{15} conjugate are shown in Figure 1. The isolated yield of the labeled oligonucleotide **3** (retention time 24.1 min) was about 70%, based on the crude starting material. In fact, the real coupling efficiency seems to be higher since the starting amino-modified oligonucleotide (rt 20.5 min) completely disappeared whereas peaks at 18–20 min correspond mainly to the impurities present also in crude starting oligonucleotide. The reaction yield in TEAA buffer was higher than that achieved in 'hard' carbonate/bicarbonate buffer (generally 50–60%, with reaction time up to 12 h). The UV–vis spectrum of conjugate clearly confirmed the presence of the cyanine dye residue by its specific absorbance in the visible region. A shift of the absorption maximum of pyridinium cyanine chromophore from 434 to 444 nm was observed in the conjugate due to the dye interaction with oligonucleotide. A similar effect was observed for the interaction of free dye with nucleic acids.[21] The absorbance ratio A_{260}/A_{444} of conjugate **3** indicated a 1:1 ratio of oligonucleotide to dye. The observed value of 0.56 was in good agreement with the calculated ratio of 0.52.[25] The labeled oligonucleotide migrated in the polyacrylamide gel slower than the non-modified oligomer and was easily identified by its greenish fluorescence at 475 nm.

Cyanine-labeled oligonucleotides could be used as fluorescent probes for the detection of nucleic acids. The studies on the interaction of labeled oligonucleotide **3** with complementary nucleic acids were performed by measuring the fluorescence of dye–T_{15} conjugate before

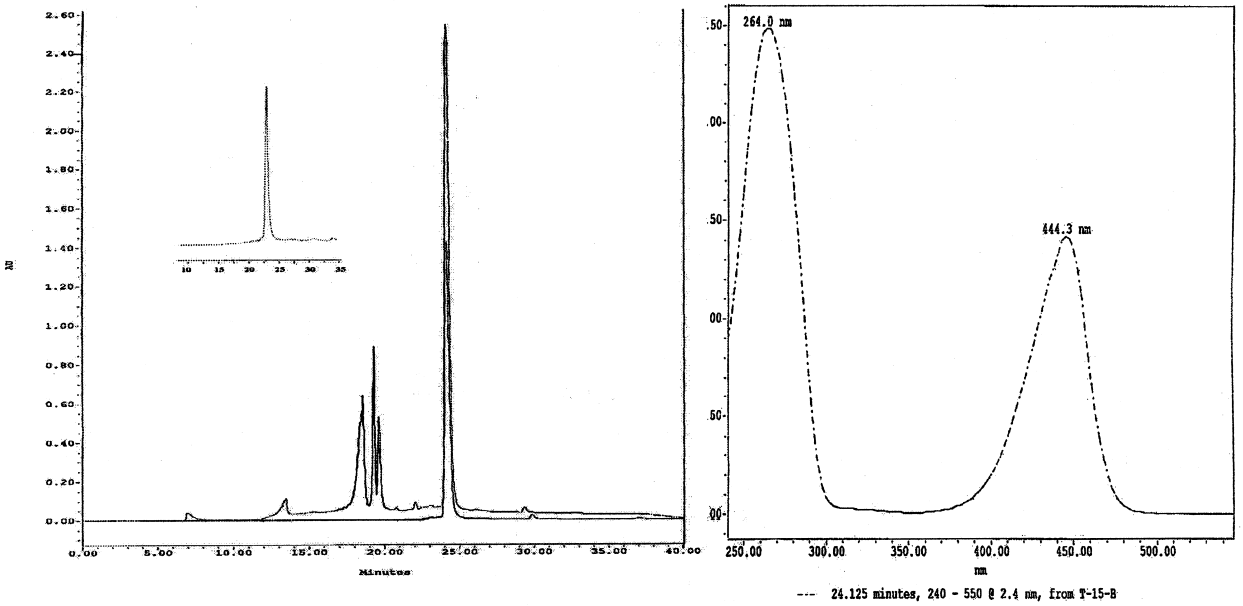

Figure 1. HPLC profile of the reaction mixture of oligonucleotide labeling with **1** recorded at 260 and 444 nm (upper and lower chromatogram, respectively; insert—purified conjugate **3**, 260 nm) and absorbance spectrum of the product.[27]

and after hybridization to the complementary single-stranded poly(rA) and poly(dA). After the formation of duplexes, the fluorescence emission at 475 nm increased 1.9 and 2.1 times, respectively. The fluorescence increase for dye–oligonucleotide hybrid was far lower than that observed for the interaction of free **2a** with double-stranded nucleic acids (ca. 800 times[21]).

TO-labeled oligonucleotides demonstrated a similar effect with a 3.5–5-fold increase of conjugate fluorescence upon hybridization to single-stranded nucleic acid target,[18] whereas emission enhancement upon binding to double-stranded nucleic acids was three orders of magnitude higher for free TO dye.[6,18] There are at least two possible explanations for the relatively low fluorescence increase of cyanine–oligonucleotide conjugates. First, the ratio of one dye residue per 15 base pairs achieved in the formed probe–target duplex could be insufficient to reach a maximum fluorescence emission observed at the ratio 1 dye/2 b.p. for free **2a**.[21] Second, the conjugated fluorophore possibly does not efficiently intercalate into the resulting duplex due to steric factors and interacts just as a cationic molecule. In this case, the choice of appropriate linker would increase the intercalation efficiency.

Thus, we propose a new efficient oligonucleotide labeling method as a simple and convenient alternative for known procedures for nucleic acid labeling. The reaction of pyrylium salts with amino functions can be also used for introducing reporter groups into other biomolecules, including amino acids and peptides (this work is in progress).

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