COMMUNICATIONS

Byproduct with Altered Fluorescent Properties Is Formed during Standard Deprotection Step of Hexachlorofluorescein Labeled Oligonucleotides

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HEX-labeled oligonucleotides obtained via typical synthetic protocols may contain more than 15% of material with altered spectral characteristics. We discovered hexachlorofluorescein residue transformation unknown earlier for standard DNA ammonolysis step. HEX residue reacts with ammonium hydroxide yielding acridine derivative, which has differed UV−VIS and fluorescent properties compared to HEX. Therefore, for critical bioassays where sensitivity and/or fluorescent signal differentiation (e.g., in quantitative or multiplexed assays) are essential, the careful RP-HPLC purification step is required.

INTRODUCTION

Fluorescently labeled oligonucleotides are now routinely used in molecular biology and medical diagnostics (*1, 2*). Most common methods of such oligomer synthesis assume the introduction of a label during the chain elongation process. In this case, a fluorophore undergoes all deblocking procedures and may be modified. For example, ammonolysis leads to partial conversion of the thiourea fragment of the FITC-label to urea and/or guanidine fragments (*3*).

It is a generally accepted opinion that carefully synthesized RT-PCR probes are not needed in additional purification (*4*). HEX (hexachlorofluorescein), being one of the popular red fluorescent dyes, is widely used in PCR-based assays, including multiplexed RT-PCRs (*5*). We have performed more than 200 HPLC separations of various HEX-labeled oligonucleotides obtained after automated synthesis and purified by PAGE, ion exchange chromatography, or RP cartridges. These oligonucleotides had >95% purity by gel electrophoresis analysis. In all of them, we found the presence of more than 15% full-size oligomers with altered fluorescent and UV−VIS spectral properties. For typical HPLC profiles and separation conditions, please see the Supporting Information. In the case of RT-PCR, the presence of such byproduct may lead to a decline in the sensitivity of RT-PCR assays and may have an impact on quantitation (*6*). Moreover, the fluorescent signal of these byproducts may interfere with signals from other labels, such as fluorescein, leading to a false positive response. Therefore, it is very important to understand the nature of this phenomenon.

RESULTS AND DISCUSSION

As a model object for investigation of the chemical mechanism of HEX-labeled oligonucleotide transmutation, we chose the simplest target oligonucleotide, 5′-HEX-T_{10}. The HPLC profile of the 5′-HEX-T_{10} postammonolysis mixture exhibited two main peaks (Figure 1A), labeled **1** and **2**. Fraction **1** demonstrated spectral properties expected for HEX-derivatives, while **2** had hypochromic shifts both in VIS (42 nm) (Figure 1B) and in fluorescent emission spectra (18 nm) (Figure 1C). Because of a significant shift of the emission maximum, this byproduct interferes with the working range of FITC and FAM labels. This can lead to false positive detection errors of multiplexed fluorescent-based assays.

The ratio of **1/2** peak intensities depends on ammonolysis conditions and, as shown on the Figure 1A, varies from 20% to 60% total oligonucleotide content. The yield of compound **1** is reduced with prolongation of time and/or elevation of ammonolysis temperature (see Figure 1A). The highest yields of oligonucleotide **1** were obtained with 25% ammonia either at room temperature/24 h or at 50 °C/5 h. More gentle deblocking conditions enhanced **1/2** ratio, but decreased overall yield of oligonucleotide **1** possibly because of incomplete removal of pivalic protection groups from 3,6-positions of the fluorophore.

Repeating ammonolysis of HPLC purified HEX-T_{10} (**1**) led to the appearance of the same compound **2** on the HPLC profile (data not shown). It is noteworthy that the alkaline treatment (1 M NaOH) in the same conditions did not cause such transformations (data not shown). It means that the presence of ammonium hydroxide is essential for change of the HEX label.

We were not able to detect the difference in molecular masses of oligonucleotides **1** and **2** within the mass accuracy of the MALDI TOF linear mode (±1 Da). To obtain better isotopic resolution, the synthesis of low-molecular-weight HEX−isopropylphosphate was undertaken.

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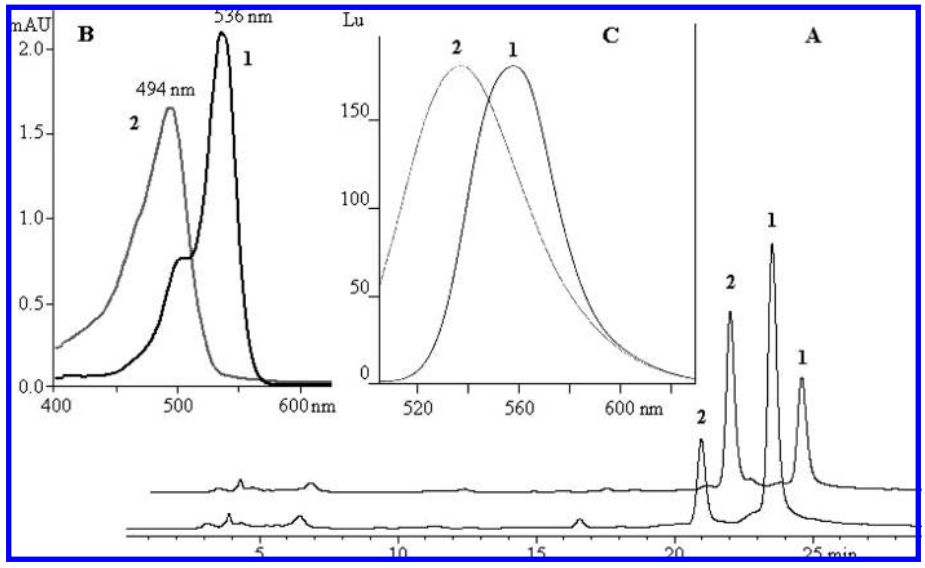

Figure 1. Transformation of 5′-HEX-T_{10} oligonucleotide. A. HPLC profiles of 5′-HEX-T_{10} isolation after ammonolysis at 50 °C/5 h (bottom curve) and at 50 °C/24 h (top curve). Column Diasorb C16T, 0.4 × 25 cm (Elsica, RF). Gradient of acetonitrile (10−20%/35 min) in 0.1 M ammonium acetate, flow 0.8 mL/min, temperature 35 °C. UV detector, λ 260 nm. B. VIS spectra of HEX-T_{10} **1** and of its acridine analogue **2** in 0.1 M NH_4OAc buffer, pH 6.7, normalized to adsorption maximum at 260 nm (UV areas are very similar, not shown). C. Fluorescence emission spectra of HEX-T_{10} **1** and of its acridine analogue **2** in the same buffer, λ_{ex} 480 nm.

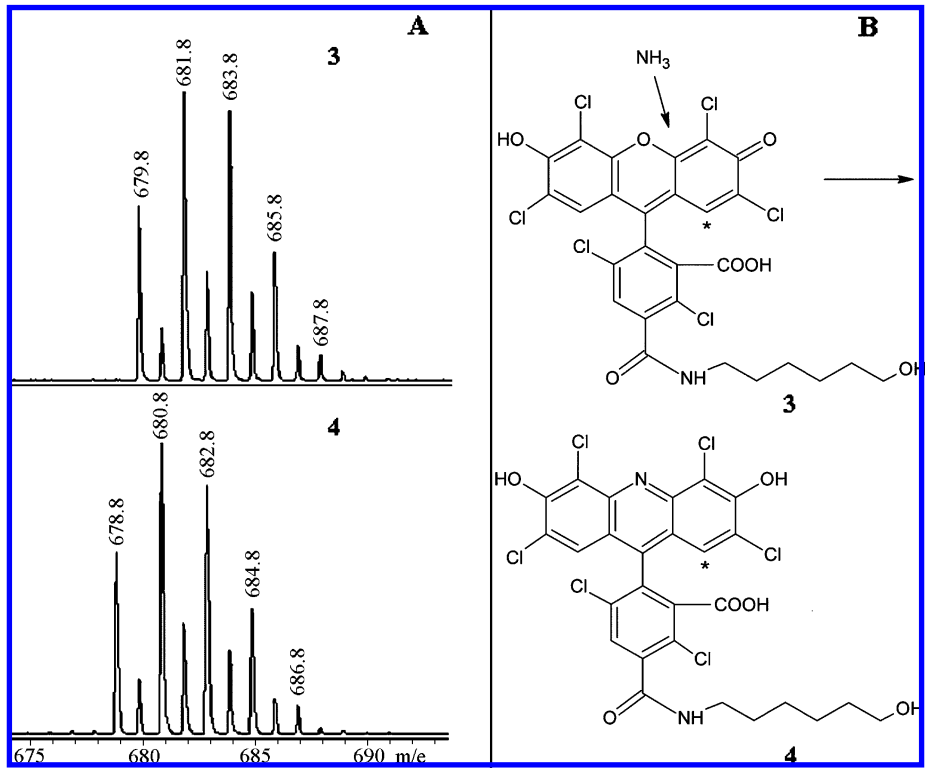

Figure 2. MALDI mass spectra (A) and the structures (B) of model fluorone **3** and acridine **4** derivatives. The arrow marks the interquartile point of ammonia nucleophilic attack. Asterisks mark locations of one of the protons that differed in ^{1}H NMR spectra of **3** and **4**.

Intermediate HEX−cyanoethylisopropylphosphite obtained by condensation of HEX-amidite (Glen Research, USA) with dry isopropyl alcohol in the presence of tetrazole was converted with standard iodine−water−pyridine oxidizer to corresponding phosphotriester, which without isolation was treated with 25% ammonia−tetrahydrofuran (9:1). Since phosphotriester had low solubility in this mixture, ammonolysis time was increased up to 66 h/60 °C. Under these conditions, significant hydrolysis of the phosphoester bonds occurred. HPLC profile of the resulting reaction mixture contained mainly two aromatic nonphosphorylated products, **3** and **4** (Figure 2A) in 7.5:1 integral ratio, according to adsorption at 260 nm. This pair of **3** and **4** had the same differences in fluorescent and VIS spectra as the pair of **1** and **2** (Figure 1B,C).

Mass spectra of compounds containing six chlorine atoms have very specific isotope signal distribution due to the natural abundance of ^{35}Cl and ^{36}Cl. The mass spectrum of **3** exhibits the same shape of the isotopic envelope as theoretically simulated (program *IsoPro* v 3.0 (*7*)) spectrum for 2,4,5,7,2′,5′-hexachloro-4′-(6″-hydroxyhexylaminocarbonyl)-fluorescein (simulation not shown). All signals observed in the mass spectrum (reflectron mode) of **4** were shifted by −1 Da in comparison

with corresponding isotopic signals of compound **3**, while the isotopic distribution remained the same (Figure 2A). These data prove that there are the same number of chlorine atoms in molecules **3** and **4**, and that there is a possibility of single oxygen atom substitution by NH group.

^{1}H NMR spectra of **3** and **4** were practically identical, including the aliphatic proton field (1−3 ppm, 12H) and the peak of the aromatic proton at the phenyl C3′ (7.63 ppm, 1H). The most important similarity is that the signals of both H atoms at C1 and C8 (Figure 2B) in both heterocycles **3** and **4** are singlets (6.96 ppm, 2H, for HEX (**3**), and 6.87 ppm, 2H, for **4**), which suggests preservation of the symmetry of the heterocycle after reaction with ammonia. There exists the sole possibility of symmetric incorporation of ammonia into the fluorone structure, the substitution of heterocyclic O10 with the NH group. Therefore, compound **4** is the acridine derivative—2,4,5,7-tetrachloro-3,6-dihydroxy-9-(2′,5′-dichloro-4′-[6″-hydroxyhexylaminocarbonyl]-6′-carboxyphenyl)acridine (see Figure 2B).

We observed a similar reaction of water ammonia with tetrabromo- (eosine) and tribromofluorescein (data not shown) and did not see it for the dibromo derivatives of fluorescein. In agreement with these data, all attempts at long-term ammonolysis (60 °C/6 days) of oligonucleotides labeled with nonhalogenated fluorescein (FAM-label) or with TET-label (containing dichloroxanthene group) also failed to lead to the noticeable formation of acridine structures (like **2** and **4**).

We speculate that the presence of more than two halogen atoms in the xanthene cycle of fluorones at o- and p-positions to carbon atoms near bridge atom O10 significantly facilitates ammonia nucleophilic attack leading to substitution of O10 by NH and formation of acridine structure (see Figure 2B; the most stable tautomer is shown). This effect makes possible the conversion of hexachlorofluoresceins into acridine derivatives (**2**, **4**) and explains the stability of the twice- or non-halogenated xanthene cycle of fluorone residues at the same mild conditions of ammonolysis.

The reaction of fluorone transformation to acridines under very stringent conditions was described a very long time ago, in 1899. Fluorescein ammonium salt in a dry ammonia stream has been converted to 3,6-diaminoacridine derivative at 180−200 °C in 5 days (*8*). However, at lower temperature (110 °C) dry ammonia did not change tetrachloroeosine (*9*). In turn, we did not detect 3,6-hydroxyl substitution with water ammonia at temperatures up to 70 °C.

Here, for the first time we report partial transformation of hexachlorofluorescein derivatives under mild ammonolysis conditions applied in obtaining oligonucleotides.

CONCLUSIONS

There is a possibility of conversion of halogenated fluorone labels of oligonucleotides into their acridine derivatives in mild ammonolysis conditions, which may be useful for future bioconjugate transformations.

During the standard ammonolysis step of hexachlorofluorescein- (HEX-) labeled oligonucleotides, this label undergoes substantial transformation into corresponding acridine derivative.

Post synthetic HPLC purification of HEX-labeled oligonucleotides is essential for demanding applications, where sensitivity and/or fluorescent signal differentiation (e.g., in quantitative and multiplexed assays) are critical and the possibility of false positive response must be minimized.

Supporting Information Available: Additional information as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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