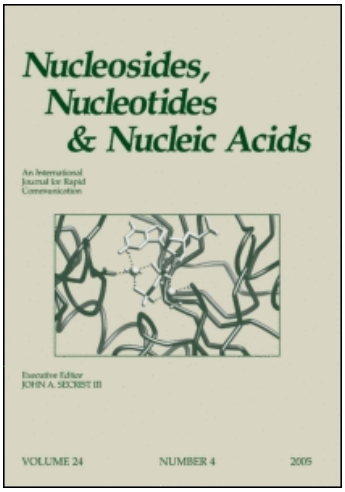

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Quantitative One Step Derivatization of Oligonucleotides by a Fluorescent Label Through Abasic Site Formation

Didier Boturyn[a]; Eric Defrancq[a]; Veacute onique Ducros[b]; Catherine Fontaine[a]; Jean Lhomme[a]
[a] LEDSS, UMR 5616, UniversitC Joseph Fourier, - Grenoble 1, GRENOBLE CEDEX 9, FRANCE [b] Laboratoire de BIOCHIMIE, C-CHU Grenoble, GRENOBLE CEDEX 9

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QUANTITATIVE ONE STEP DERIVATIZATION OF OLIGONUCLEOTIDES BY A FLUORESCENT LABEL THROUGH ABASIC SITE FORMATION

Didier Boturyn[#], Eric Defrancq[#*], Véronique Ducros[+], Catherine Fontaine[#], Jean Lhomme[#*]

[#]LEDSS, UMR 5616, Université Joseph Fourier - Grenoble 1, BP 53, 38041 GRENOBLE CEDEX 9, FRANCE; [+] Laboratoire de BIOCHIMIE C - CHU Grenoble, BP 217, 38043 GRENOBLE CEDEX 9.

ABSTRACT: Reaction of abasic site-containing oligonucleotides with an oxyamino fluorescent label is described. The reaction represents an efficient method to functionalize oligonucleotides at preselected positions.

INTRODUCTION

Radiolabeling and fluorescent labeling of DNA or RNA have found wide applications in DNA hybridization and sequence analysis. Fluorescent dyes conjugated with nucleic acids offer some essential advantages over radioactive labels. These enable detection in real time and avoid the radiation hazards and consequent problems of radioactive waste handling and disposal[1, 2].

Several procedures have been described for fluorescent labeling of DNA. Most of them use labeled primers or labeled nucleoside triphosphates which are then incorporated into DNA by enzymatic reactions[1, 2]. Chemical derivatization of oligonucleotides through abasic site formation has been proposed several years ago by Imbach and Coll.[3] and more recently by Mirzabekov and Coll.[4]. Both methods use amino derivatives which react with the aldehydic function of the abasic site[5] to form an imino coupling product. To stabilize the unstable imino linkage, a further reduction step is required. In this paper, we describe a one step derivatization method in which the abasic site is reacted with a

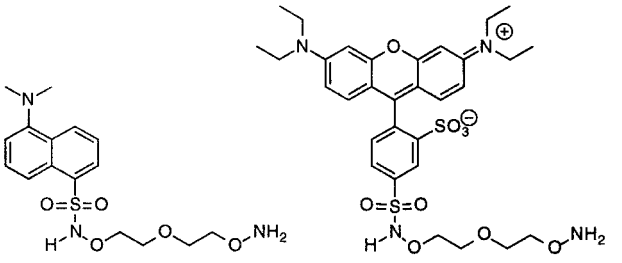
* E-mail : Eric.Defrancq@ujf-grenoble.fr - FAX : +33 (0)4 76 51 43 82

fluorescent label containing an oxyamino group. The oxime ether linkage formed is stable in physiological conditions and thus does not require a subsequent chemical reduction step.

RESULTS AND DISCUSSION

In a general program aimed at designing molecules that specifically recognize abasic sites and that are of interest as cleaving agents for molecular biology experiments[6], or as pharmacological agents to inhibit the repair process in the cell[7], we also engineered fluorescent molecules **1** and **2** that can selectively detect and quantitate abasic sites *in vitro*[8]. We made use of a modular approach in which a highly reactive group towards aldehydes is linked to a detection moiety through a chain possessing the required solubility and DNA interaction properties. For the reactive group we chose the oxyamino function which has already been found to be specific for the abasic site by forming an oxime ether with the aldehydic function of the ring opened deoxyribose residue[9]. Such oxime ethers were found to be stable in physiological conditions[9]. The Dansyl and the Lissamine-Rhodamine B fluorophores were selected as detection moieties. The label and the reactive oxyamino group were tethered by an ethylether linker.

In this paper, we show that such fluorescent probes are efficient tools for the derivatization of oligonucleotides. We report the derivatization of a trinucleotide and an undecanucleotide by the highly fluorescent probe **2** via a one step coupling to an abasic site.



1 **2**

1) Preparation of the abasic site-containing oligonucleotides **5** and **6**:

In a previous paper[10], we reported a new route for the preparation of oligonucleotides containing an abasic site at any preselected position in the sequence. The method involves incorporation of the stable modified nucleoside 8-propylthio-2'-deoxyadenosine into an oligonucleotide by the phosphoramidite approach, subsequent mild oxidative treatment of the resulting oligonucleotide for selective oxidation at sulfur in

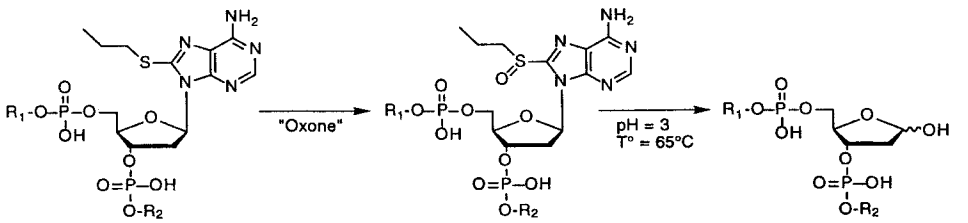


FIGURE 1: Preparation of the abasic site-containing oligonucleotides **5** and **6**.

the modified nucleoside and hydrolytic removal of the oxidized base (figure 1). Using this method we have now prepared two oligonucleotides containing an abasic site: the trimer **5** d(GXA) and the undecamer **6** d(CGCACXCACGC) in which X represents the abasic site.

However, in this previous paper we did not elucidate the oxidation state of sulfur in the two intermediate oligonucleotides obtained after oxidation by oxone (either a mixture of the two diastereoisomeric sulfoxides or a mixture of the sulfone and the sulfoxyde). We thus decided to separate the two isomers **3a** and **3b** of the trimer and purified them by High Performance Liquid Chromatography (HPLC) for subsequent mass spectral investigations. The ElectroSpray Mass Spectrum (ES-MS) of each component **3a** and **3b** (table 1 and figure 5) clearly showed the molecular ion $[M-H]^-$ at 983, which revealed that oxidation of the oligonucleotide gave a mixture of the two diastereoisomeric sulfoxydes. The sulfone derivative that was formed in the same conditions in the case of the nucleoside derivative[11] was not obtained in the oligomer. In the case of the undecamer **6**, we were unable to separate the two isomers **4a** and **4b**.

2) Derivatization of the oligonucleotides **5** and **6** by the fluorescent probe **2**:

Reaction of the highly fluorescent probe **2** (in excess) with the oligonucleotides **5** and **6** was carried out at room temperature in water at pH = 7 for 2 hours. In these conditions, the abasic site oligonucleotides **5** and **6** were stable. The evolution of the reactions was monitored by HPLC. The HPLC profile of the crude mixtures (figure 3) showed that in both cases the reaction was highly selective affording respectively the conjugated oligonucleotides **7** and **8**.

The parasite peaks that appeared at longer retention times correspond to the adducts formed between the probe **2** and the carbonyl contaminants (formaldehyde, acetaldehyde) of the solvent (HPLC pure methanol) used for the HPLC analysis.

TABLE 1: ES-MS of modified oligonucleotides **3a**, **3b**, **7** and **8**.

Oligonucleotides	Calculated Mass	Found Mass
Oxidized Trimer **3a**	983.81	983.84
Oxidized Trimer **3b**	983.81	983.47
Conjugated Trimer **7**	1434.35	1434.29
Conjugated Undecamere **8**	3812.88	3812.54

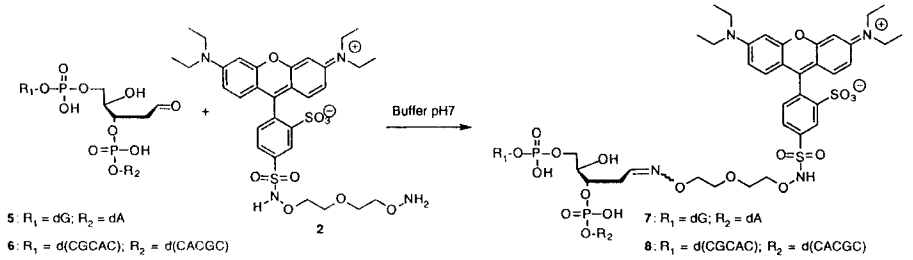


FIGURE 2: preparation of fluorescent oligonucleotides **7** and **8**.

In the case of the conjugated trimer **7** the structure could be precisely established by ^{1}H-NMR spectroscopy and confirmed by ES-MS. In table 2, we report the chemical shifts assigned to the corresponding protons in **7** using DQF-COSY techniques[13]. Two signals were observed for the $H_{1'}$ oximic proton (figure 4B) confirming that a diastereoisomeric mixture of Z/E compounds in a 50/50 ratio was obtained. The same signals had been observed in the case of reaction of oxyamino probe **1** and **2** with 2-deoxyribose[8b]. In the electrospray mass spectrum (table 1 and figure 5) the presence of the peaks at m/e = 1433 and m/e = 716, corresponding respectively to the calculated molecular ions $[M-H]^-$ and $[M-2H]^{2-}$ confirmed the formation of the fluorescent oligonucleotide **7** (the peak at m/e = 1455 corresponds to the $[M-H+Na^+]^-$ adduct).

In the case of the undecamer **8**, the structure was confirmed by the ES-MS spectrum that showed the multicharged ions $[M-5H]^{5-}$ at m/e = 761.6 and $[M-6H]^{6-}$ at m/e = 634.7 corresponding to a mass of 3812.54 (table 1).

The derivatized oligonucleotides **7** and **8** showed excitation and emission maxima respectively at 574 and 592 nm in water solution.

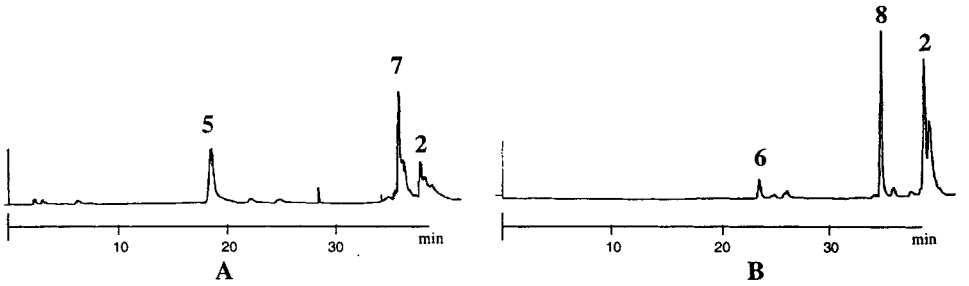


FIGURE 3: HPLC profiles (detection at 260 nm): (**A**) crude reaction mixture of probe **2** with the trimer **5**; (**B**) crude reaction mixture of probe **2** with the undecamer **6**. For the HPLC conditions, see experimental part.

TABLE 2: Chemical shifts of the protons in the conjugated oligonucleotide **7**. **Y** is the abasic site derivatized by probe **2**. * Aromatic protons of Lissamine-Rhodamine B.

R	H_8	H_2	$H_{1'}$	$H_{2',2''}$	$H_{3'}$	$H_{4'}$	$H_{5',5''}$	CH_2O	H-Ar*	CH_3	CH_2CH_3
dG	8.08	/	6.36 6.33	2.77, 2.67	4.97	4.31	3.81				
Y	/	/	7.46 6.98	2.74, 2.60 2.58	4.37	3.97	3.90	4.12- 3.89	8.60-6.60	1.20	3.51
dA	8.18	7.94	6.15 6.12	2.65 2.42	4.66	4.14	4.04				

In conclusion, derivatization of oligonucleotides by reaction of oxyamino labels on the aldehydic form of the abasic site was found to be highly specific. The conjugated oligonucleotides were found to be stable in these conditions. No reduction step of the oxime ether was necessary for the stabilization of the conjugate. In addition, formation of the conjugate was not accompanied by DNA cleavage through β-elimination as was observed on treatment of abasic DNA with the amino reagents such as phenylhydrazine[12a], the tripeptide Lys-Trp-Lys[12b] or 9-aminoellipticine[12c]. In addition this strategy is quite general and could be used to introduce a label either inside the oligonucleotide or at the extremity.

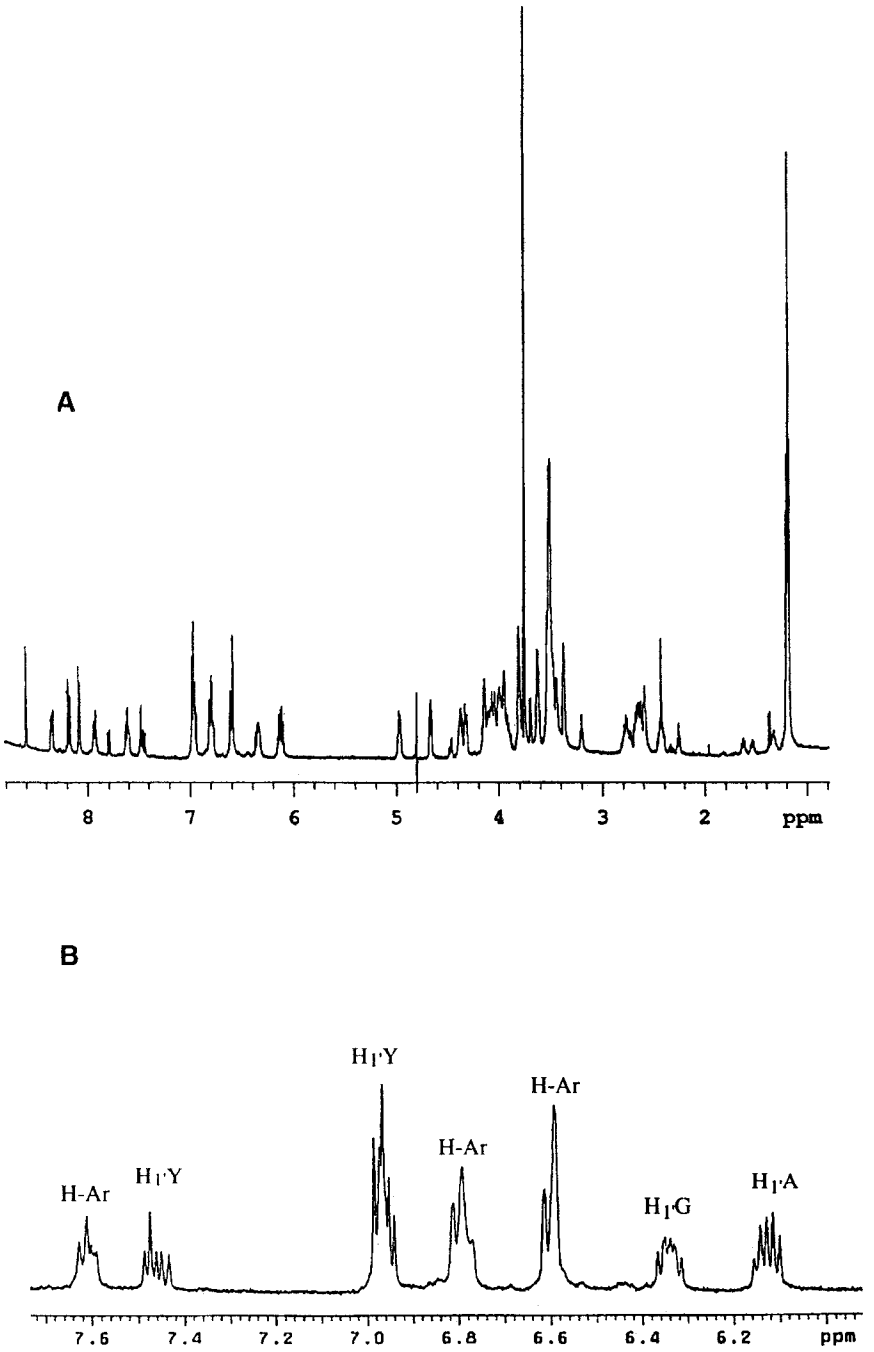


FIGURE 4: ^{1}H-NMR (500 MHz) spectrum of the fluorescent oligonucleotide **7**. **A**: complete spectrum, **B**: expansion of spectrum **A** for the visualisation of the anomeric protons and the oximic proton. **Y** is the abasic site derivatized by probe **2**.

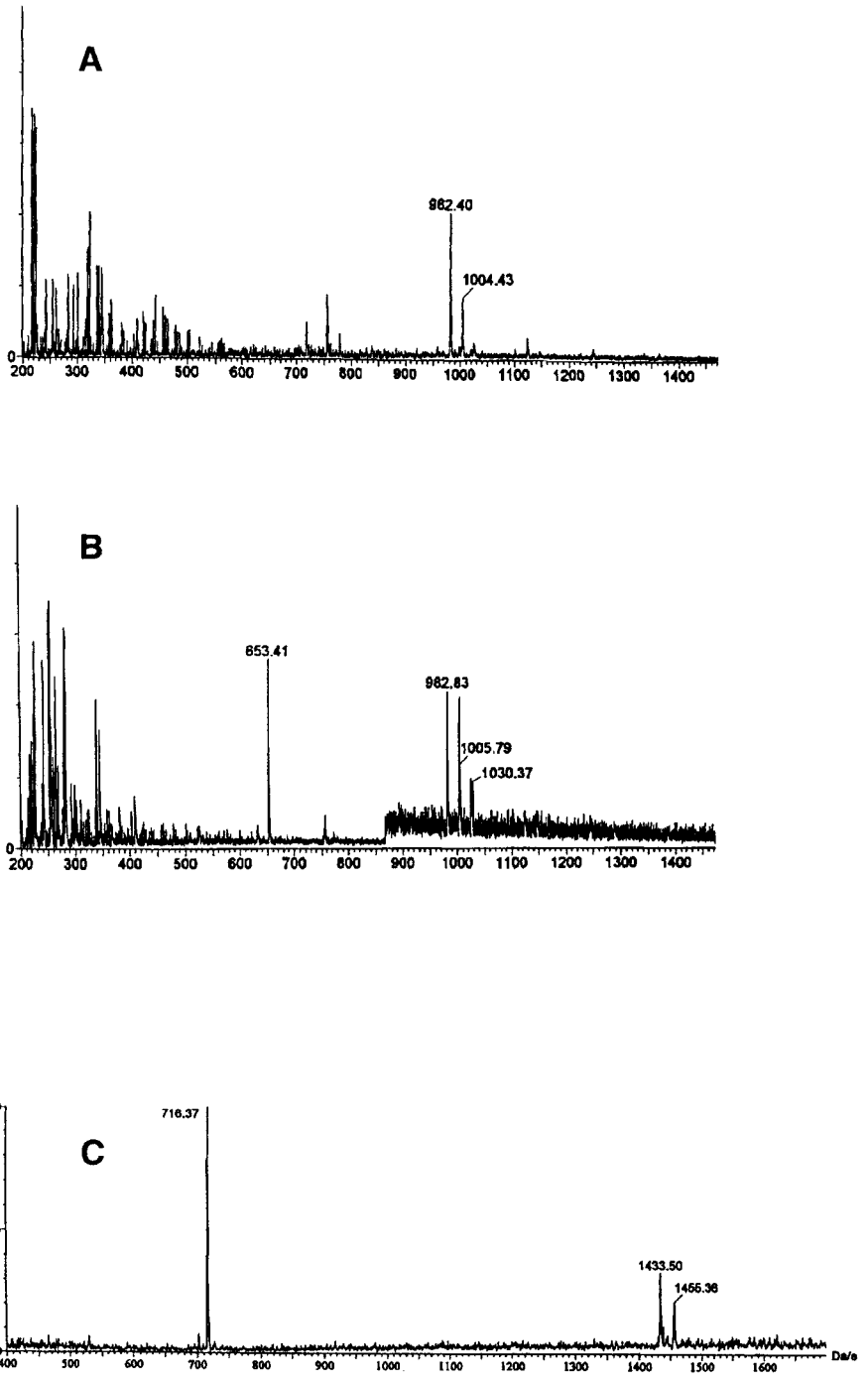


FIGURE 5: ES-MS spectra of the oxidised oligonucleotides **3a** (A) and **3b** (B) and of the derivatized fluorescent oligonucleotide **7** (C).

EXPERIMENTAL PART

Instrument: ^{1}H-NMR spectra were recorded on a Varian Unity + 500 MHz spectrometer using a Nano-NMR Probe[14] with 30 µg of oligonucleotide in 35 µL of D_2O. The probe temperature was 25°C and the spin rate was about 3600 Hz. The 1-D spectrum was obtained with 128 transients and a presaturation delay of 3s. The 2-D correlation map consisted of 512 x 1K data points spectra, each composed of 128 transients. The saturation delay was set at 1.7s. ES-MS spectra were performed on a VG Platform II (Micromass) in the negative ion mode. The eluant was 50% aqueous acetonitrile and the flow rate was 5µL/min. The oligonucleotides were dissolved in 50% aqueous acetonitrile and 1% of NEt_3 was added for the measure. High-Performance Liquid Chromatography (HPLC) analysis was carried out using a Waters chromatograph consisting of two M510 pumps, a M490E detector and a M680 system controler. A µ-Bondapack C18 column (Macherey-Nagel Nucleosil: 10 x 250 mm, 7 µm) was used. Gradient elution was performed by building up a linear gradient starting with solvent A (phosphate buffer pH 7/ MeOH, 95/5 (v/v)) and applying solvent B (MeOH) up to 30% for 20 min with a flow rate of 2 mL/min.

Abasic oligonucleotides **5** *and* **6**: These oligonucleotides were prepared as described[10].

Conjugated oligonucleotides **7** *and* **8**: The oligonucleotides **5** and **6** were dissolved in phosphate buffer (20 mM, pH = 7) and an aqueous/DMSO solution (90/10: v/v) of probe **2** was added. The mixture was stirred for 5h at room temperature then lyophilised. The modified oligonucleotides were purified by HPLC.

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