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O-(FLUORESCEINYLMETHYL)HYDROXYLAMINE (OFMHA): A FLUORESCENT REAGENT FOR DETECTION OF DAMAGED NUCLEIC ACIDS

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Abstract: 5- and 6-*O*-(Fluoresceinylmethyl)hydroxylamine (OFMHA, **5a**, **b**) were prepared from the corresponding bis-pivaloyl-protected hydroxymethylfluoresceins (**1a**, **b**) in 50–70% yield. The hydroxylamine derivatives reacted smoothly with the abasic sites present in acid/heat stressed calf thymus DNA.

Nucleic acids are susceptible to a variety of damaging physical and chemical forces including UV and ionizing radiation, alkylating and oxidizing reagents, acid, and even some antitumor drugs. The result is most commonly the loss of some purine and pyrimidine bases, which may ultimately lead to errors in the incorporation of nucleotides in vitro or cell damage in vivo.[1] A number of methods have been described for the detection of these abasic sites that are based on the reaction of the remaining deoxyribosyl residue with hydroxylamine derivatives. Talpaert-Borlé, et al.[2] reported the use of [^{14}C]methoxylamine. Dogliotti's group demonstrated that non-isotopic methoxylamine could also be used to quantify abasic sites, based on inhibition of certain endonucleases and polymerases.[3] Other workers have labeled abasic sites with an *O*-biotinylated hydroxylamine,[4,5] then detected the labeled nucleic acid using an avidin-horseradish peroxidase conjugate with a sensitivity of one site per 10 000 bases. Most recently, Defrancq, Lhomme and coworkers[6] have reported the preparation of fluorescent dansyl and Lissamine-Rhodamine B tethered *O*-substituted hydroxylamines to directly detect one abasic site per 100 000 nucleotides in DNA. Those authors recognized that a better fluorophore such as fluorescein would increase the detection limit even more, but they were unsuccessful in preparing such a derivative. We report here the first preparation of two such fluorescein-substituted hydroxylamines, 5- and 6-*O*-(fluoresceinylmethyl)hydroxylamine (OFMHA, **5a**, **b**).[7]

As shown in the Scheme[8] the bis-*O*-pivaloyl protected fluorescein derivatives **1a** and **1b**, obtained from a mixture of the corresponding bis-*O*-pivaloylcarboxyfluorescein isomers by a method previously described,[9] were each reacted under Mitsunobu's conditions with *N,N*-bis-*tert*-butoxycarbonylhydroxylamine (**2**)[10] to give the fully protected, colorless OFMHA isomers **3a** and **3b** in 70–85% yield after flash chromatography on silica. Methanolysis of the pivaloyl groups in the presence of DMAP[9] was problematic. The reaction was slow and the DMAP was subsequently difficult to remove. The pivaloyl protecting groups were removed more conveniently

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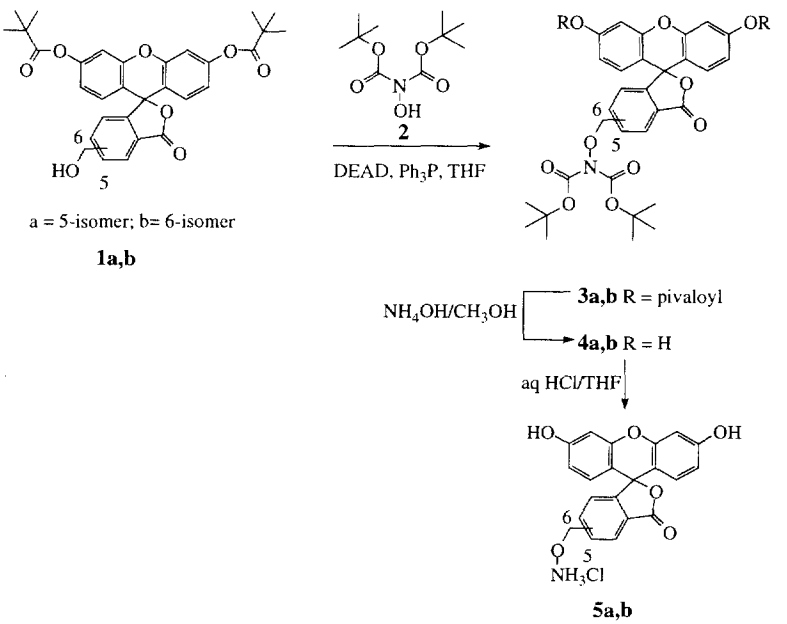
by a brief treatment with an excess of ammonium hydroxide in methanol at ambient temperature. Evaporation of the solvent gave the dark red, bis-*t*-BOC protected hydroxylamines **4a** and **4b**. Without further purification, the *t*-BOC groups were removed using a refluxing mixture of aqueous HCl and THF. After evaporation of the solvent, the crude material was triturated with hot THF to remove the residual pivalamide and afford the desired hydroxylamine hydrochlorides, **5a** and **5b**, in 70–80% yield and greater than 95% purity by analytical HPLC.



Scheme

The fluorescence spectra of 5- and 6-OFMHA at pH 8 are shown in Figure 1. Both isomers showed an emission maximum at 514 nm similar to 5-carboxyfluorescein (5-CF, 520 nm).

The reactivity of 5- and 6-OFMHA with abasic sites of damaged nucleic acids was demonstrated by labeling calf thymus DNA containing varying concentrations of abasic sites prepared according to the method of Ide, et al.[5] Thus, the DNA sample in pH 5 buffer was heated at 70 °C for 0–75 min. Aliquots were withdrawn at 15 min intervals and the DNA from each sample was collected after ethanol precipitation. Each sample was redissolved in sodium phosphate buffer (20 mM, pH 7.1) containing 5-*O*-(fluoresceinylmethyl)hydroxylamine (**5a**, 2.4 mM). After heating at 37 °C for 1 h, the OFMHA-labeled DNA was again isolated by ethanol precipitation, and dialyzed against pH 8 buffer (10 mM Tris, 1 mM EDTA). The concentration of DNA in each sample was determined by UV spectrophotometry, then was adjusted to 1 μg/mL. The total fluorescence intensity was determined using a photon-counting spectrofluorometer with an excitation wavelength of 470 nm (bandwidth 4 nm) and an absorption-type emission filter (503 nm, 50% transmission). Error due to background emission was corrected by subtracting the fluorescence intensity obtained from a DNA sample treated with

OFMHA **5a** that had not been subjected to the depurination/depyrimidination conditions. The results are shown in Figure 2. As expected a linear correlation between fluorescent intensity and time was noted. The right hand axis indicates the expected ratio of abasic sites per 100 000 nucleotides based on the observation that 10 abasic sites per 100 000 nucleotides are generated every 9.3 min under the reaction conditions.[5]

From these preliminary experiments, OFMHA derivatives appear to have great potential in the direct detection of nucleic acid lesions.

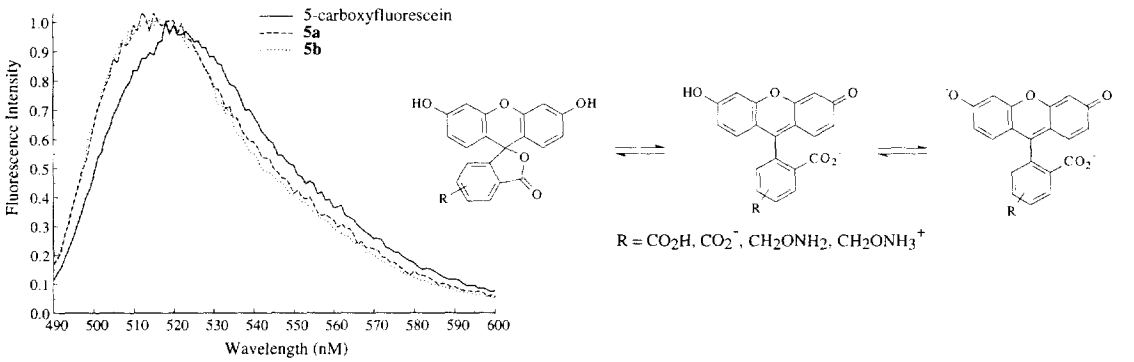


Figure 1. Fluorescence emission comparison between OFMHA and 5-CF.
Conditions: pH 8 buffer (10 mM Tris, 1 mM EDTA), λ_{ex} = 470 nm.

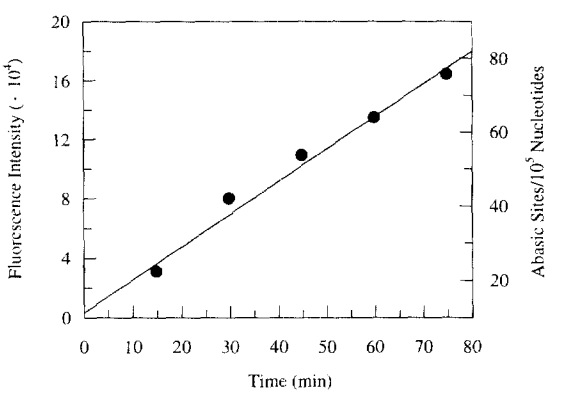


Figure 2. OFMHA-mediated fluorescence detection of abasic sites on acid damaged DNA.

References and Notes

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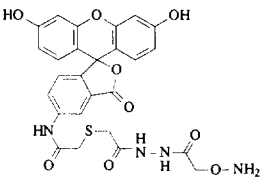

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8. Analytical data: Compound **3a**: ^{1}H NMR ($CDCl_3$) δ 8.11 (1H, s), 7.81 (1H, m), 7.18 (1H, m), 7.06 (2H, m), 6.81 (4H, m), 5.07 (2H, s), 1.58 (18H, s), 1.36 (18H, s); ^{13}C NMR ($CDCl_3$) δ 176.66, 153.43, 152.73, 151.67, 150.10, 137.46, 136.46, 128.95, 126.61, 125.95, 124.31, 117.74, 115.98, 110.38, 84.32, 76.83, 39.10, 28.02, 26.94; ESI/MS *m/z* 746 $(M + H)^+$. Compound **3b**: ^{1}H NMR ($CDCl_3$) δ 8.03 (1H, d, *J* = 7.83), 7.75 (1H, dd, *J* = 1.37, 6.59), 7.27 (1H, bs), 7.05 (2H, d, *J* = 2.20), 6.81 (4H, m), 4.94 (2H, s), 1.48 (18H, s), 1.36 (18H, s); ^{13}C NMR ($CDCl_3$) δ 176.39, 168.80, 153.50, 152.60, 151.44, 149.83, 142.58, 130.49, 128.85, 126.09, 125.31, 123.93, 117.73, 115.92, 110.23, 84.28, 76.86, 39.17, 28.00, 27.04. ESI/MS *m/z* 746 $(M + H)^+$. Anal. HPLC [Waters μBondapak C_{18}; 90:10 AcCN/water] retention time 3.45 min. Compound **4a**: ESI/MS *m/z* 578 $(M + H)^+$. Compound **5a**: ^{1}H NMR (CD_3OD) δ 8.42 (1H, d, *J* = 1.37), 7.95 (1H, dd, *J* = 1.75, 1.65), 7.42 (3H, m), 7.28 (2H, d, *J* = 3.57), 7.12 (2H, dd, *J* = 2.20, 7.00), 5.31 (2H, s); ESI/MS *m/z* 3.78 $(M + H)^+$; Anal. HPLC [Waters μBondapak C_{18}; 30:65:5 AcCN/water/1% aq TFA] retention time 3.47 min, 96%. Compound **5b**: ^{1}H NMR (CD_3OD) δ 8.42 (1H, d, *J* = 7.97), 7.92 (1H, d, *J* = 9.61), 7.50 (1H, s), 7.39 (2H, d, *J* = 9.20), 7.28 (2H, d, *J* = 2.20), 7.12 (2H, dd, *J* = 2.20, 7.01), 5.26 (2H, s); ESI/MS *m/z* 378 $(M + H)^+$. Anal. HPLC [Waters μBondapak C_{18}; 30:65:5 AcCN/water/1% aq TFA] retention time 3.46 min, 96%.
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