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Synthesis of a Chemiluminescent Acridinium Hydroxylamine (AHA) for the Direct Detection of Abasic Sites in DNA

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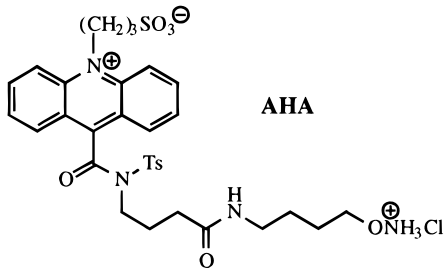
ABSTRACT



The synthesis of a chemiluminescent acridinium hydroxylamine (AHA) for the direct detection of abasic sites in damaged nucleic acids is described. The reagent reacts readily with abasic sites of damaged calf thymus DNA generated in a time-dependent manner under acid/heat depurination conditions. Preliminary results indicate the sensitivity of the direct chemiluminescent detection format is ~0.1 abasic sites detected per 10^6 nucleotides using as little as 200 ng of DNA.

Nucleic acids are known to be particularly susceptible to depurination/depyrimidination reactions, resulting in formation of abasic sites.[1−3] In vivo, abasic site formation occurs spontaneously even under physiological conditions. Various chemical or therapeutic agents can further enhance the rate of abasic site formation in vivo by alkylation of bases that results in destabilization of the glycosidic bond. Alternatively, modified bases can be removed enzymatically to generate abasic sites as intermediates of the base-excision repair process. If unrepaired, abasic sites can lead to cell damage and/or mutagenic induction in vivo.[4] In vitro, abasic site formation can be problematic, as it will occur spontaneously in DNA-based reagents with no mechanism for repair. Thus, methods to monitor, detect, and quantitate abasic site formation are essential for an understanding of the biological significance of abasic sites formed both in vivo and in vitro.

The most recently described methods for detection of abasic sites are based on the reaction of hydroxylamine derivatives with the deoxyribosyl moiety remaining at a damaged DNA site.[5−12] Although useful, these methods generally lack the sensitivity required for the most demanding studies of spontaneous abasic site formation occurring under physiological conditions. A chemiluminescent hydroxylamine

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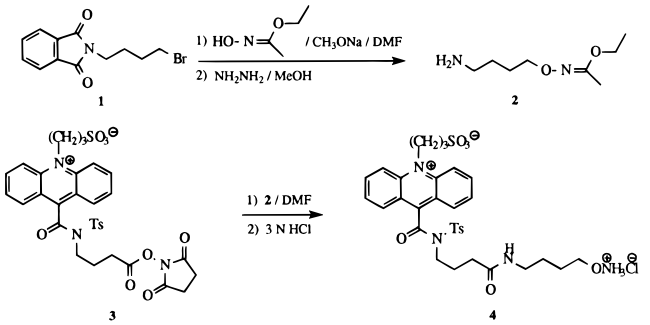
derivative would be able to overcome these sensitivity issues. In fact, the most sensitive method reported to date (0.24 abasic sites detected per 10^6 nucleotides), which has been demonstrated to detect spontaneous abasic site formation under physiological conditions, utilizes a chemiluminescent substrate for the indirect detection of biotin-tagged abasic sites coupled to streptaviden-conjugated horseradish peroxidase in an ELISA-type slot-blot format.[13] Chemiluminescent detection of abasic sites in a direct manner would be less cumbersome and would provide a sensitivity superior to that obtained in an indirect format. We describe here the synthesis and preliminary evaluation of the first chemiluminescent acridinium hydroxylamine (AHA) for the direct detection of abasic sites in damaged DNA at levels previously unobtainable with nonradioactive labels.

The synthesis of AHA (**4**) is depicted in Scheme 1. Briefly, O-alkylation of commercially available ethyl *N*-hydroxyacetimidate with *N*-(4-bromobutyl)phthalimide (**1**) followed by hydrazinolysis of the phthalimide group provided the

Scheme 1



protected hydroxylamine **2** in 20% overall yield for the two steps.[14−16] *N*-Hydroxysuccinimidyl acridinium active ester **3** was prepared in 95% yield by activation of 10-(3-sulfopropyl)-*N*-tosyl-*N*-(3-carboxypropyl)acridinium-9-carboxamide using *N*-succinimidyl trifluoroacetate.[17,18] Subsequent coupling of protected hydroxylamine **2** with acridinium−NHS ester **3** and acidic hydrolysis provided **4** in 94% yield.[16]

The utility of AHA for the direct detection of abasic sites in damaged DNA was demonstrated by labeling calf thymus DNA containing varying concentrations of abasic sites generated in a time-dependent manner. Thus, calf thymus DNA (50 μg/mL) in 10 mM sodium citrate, 100 mM NaCl, pH 5 was heated at 70 °C for 0−60 min.[8] Aliquots were withdrawn at 15 min intervals, and the DNA in each sample was immediately ethanol-precipitated. The abasic site containing DNA samples were redissolved at 25 μg/mL in 20 mM sodium phosphate, 317 μM AHA (∼0.1 mg AHA/assay), pH 6.8 and heated at 37 °C for 2 h. The resulting labeled DNA samples were again isolated by ethanol precipitation and dialyzed against 20 mM sodium phosphate buffer (pH 6.8). Total chemiluminescent emission from 10 ng of each labeled sample was subsequently determined in a direct format using an EG&G Berthold MicroLumat Plus luminometer. Chemiluminescence due to background was eliminated by subtraction of total emission intensity obtained from a DNA sample treated with AHA that had not been subjected to the depurination/depyrimidination conditions. The plot of total chemiluminescent emission intensity versus time of the depurination/depyrimidination reaction is linear with a correlation coefficient of 0.999 (Figure 1). The right-

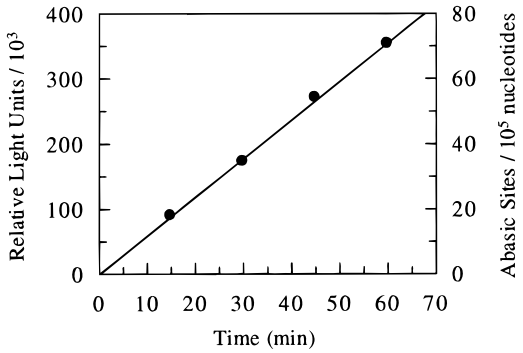


Figure 1. Chemiluminescent intensity of abasic site-labeled DNA versus time of depurination/depyrimidination reaction.

hand axis corresponds to literature values for the ratio of expected abasic sites per 100 000 nucleotides under the depurination/depyrimidination conditions utilized.[8] The absolute sensitivity of a direct chemiluminescent detection format is dependent upon both the quantity of DNA available for analysis and the number of abasic sites present in a given sample. Serial dilution of as little as 200 ng of a DNA sample containing 160 chemiluminescent abasic site labels per 10^6 nucleotides with unmodified calf thymus DNA provides a preliminary sensitivity determination of ∼0.1 abasic sites detected per 10^6 nucleotides.

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(16) Analytical data are as follows. Compound **2**: ^{1}H NMR ($CDCl_3$) δ 3.96 (2H, q, J = 7.1 Hz), 3.89 (2H, t, J = 4.0 Hz), 2.70 (2H, t, J = 5.0 Hz), 1.91 (3H, s), 1.65 (2H, m), 1.53 (2H, m), 1.26 (3H, t, J = 7.7 Hz); ESI/MS m/z 175 $(M + H)^+$. Compound **3**: ^{1}H NMR (DMSO-d_6) δ 9.00 (2H, m), 8.44 (2H, m), 8.22−7.63 (6H, m), 7.17 (2H, m), 5.63 (2H, m), 4.30 (1.3H, m), 3.60−1.60 (15.7H, m); ESI/MS m/z 683 $(M + H)^+$; anal. HPLC (Waters μBondapak C_{18}; 30/70 AcCN/0.1% aqueous formic acid) retention time 4.34 min, 97%. Compound **4**: ^{1}H NMR (CD_3OD) δ 8.97−8.90 (2H, m), 8.48−8.40 (2H, m), 8.19 (0.5H, d, J = 8.4 Hz), 8.00−7.93 (2H, m), 7.88−7.76 (2H, m), 7.63 (0.5H, d, J = 8.1 Hz), 7.14 (3H, s), 5.74 (2H, m), 4.25 (1.5H, m), 4.06 (1.5H, t, J = 5.9 Hz), 3.94 (0.5H, t, J = 6.7 Hz), 3.48−1.66 (4H, m), 1.54−1.42 (3.5H, m), 1.22 (0.5H, m); ESI/MS m/z 671 $(M + H)^+$, 693 $(M + Na)^+$; anal. HPLC (Waters μBondapak C_{18}; 30/70 AcCN/0.05% aqueous TFA) retention time 3.79 min, 98%.
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In summary, the first synthesis of a chemiluminescent acridinium hydroxylamine label for the detection of abasic sites in damaged nucleic acids has been described. Preliminary results indicate AHA reacts readily with abasic sites and provides a highly sensitive method for the direct detection and quantification of such sites in damaged DNA. Direct chemiluminescent detection of labeled DNA in this manner provides a method readily applicable to studies of abasic site formation under physiological conditions. Studies to further evaluate the absolute sensitivity and utility of direct chemiluminescent AHA labeling of abasic sites and an evaluation of AHA-based chemiluminescent conjugates are currently in progress.

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