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A fluorimetric assay for the spontaneous release of an N7-alkylguanine residue from duplex DNA

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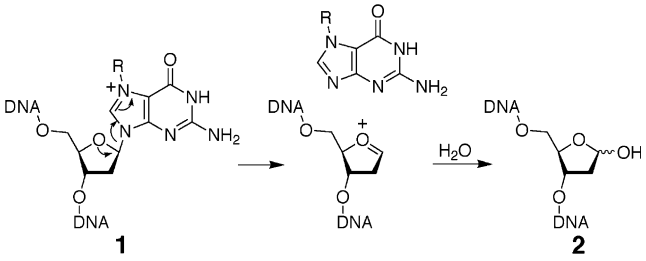
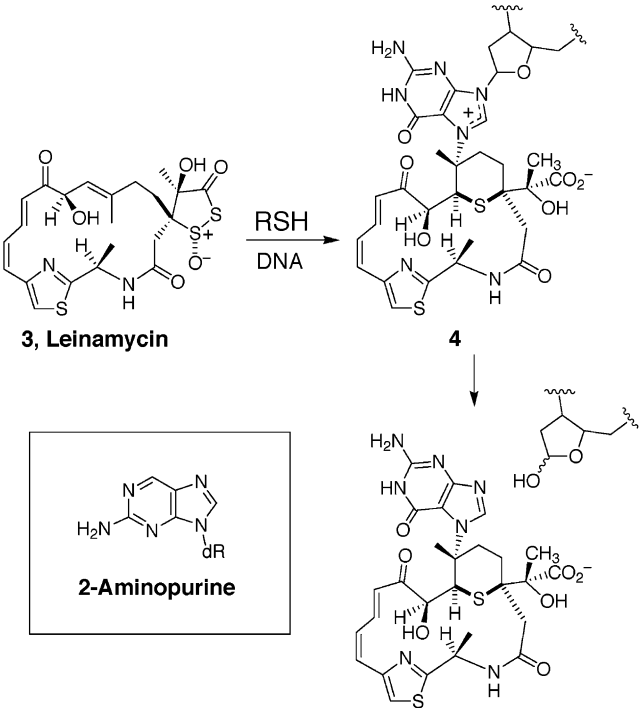
Abstract—A fluorimetric assay for monitoring depurination of the N7-alkylguanine adduct derived from the anticancer natural product leinamycin is described. This general approach could potentially provide the foundation for a high throughput assay that detects DNA-alkylating agents or a convenient continuous fluorimetric assay for base excision repair enzymes.

Many compounds that alkylate DNA are of interest because they possess potent anticancer or mutagenic properties.[1–8] Alkylation at a number of sites on the DNA bases (e.g., N7-guanine and N3-adenine) vastly increases the rate at which the base is released from the DNA backbone by hydrolysis of the glycosidic bond (Scheme 1).[9–14] The rate of this so-called depurination reaction is important because it determines whether cellular DNA-processing proteins are primarily confronted with the alkylated base (**1**) or an abasic site (**2**, Scheme 1). Typically, assays for monitoring depurination rates involve somewhat laborious chromatographic approaches such as gel electrophoretic analysis of oligonucleotides or HPLC and TLC detection of the released base.[15–18] As part of our ongoing studies[17,19–26] of the DNA-alkylating natural product leinamycin (**3**, Scheme 2), we recently set out to develop a fluorimetric assay that could be used to monitor the rate at which the leinamycin–guanine adduct (**4**) undergoes depurination in duplex DNA.



Scheme 1.



Scheme 2.

Keywords: Nucleic acid chemistry; Depurination; DNA alkylation; Antitumor agent; Natural product.
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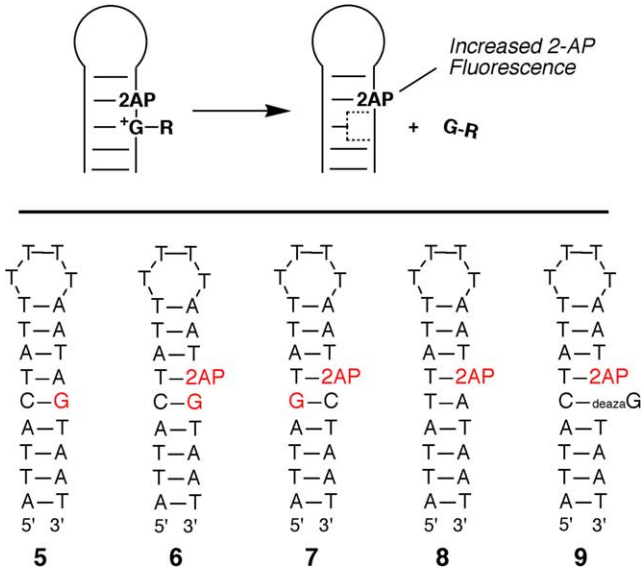

Scheme 3.

Our effort to develop a fluorimetric assay for the rate at which the leinamycin–guanine adduct undergoes depurination began with the design of hairpin duplexes containing 2-aminopurine (2-AP, inset Scheme 2) adjacent to a target guanine residue. The non-natural base, 2-AP, effectively substitutes for adenine in DNA duplexes and displays fluorescence properties that are highly sensitive to its local environment.[27–35] We anticipated that depurination of a 7-alkylguanine residue would significantly alter the surroundings of an adjacent 2-AP, resulting in a readily detectable change in its fluorescence (Scheme 3).

We first confirmed that the oligonucleotides form stable DNA duplexes. The native hairpin **5** has a melting temperature of 57 °C while the 2-AP containing hairpin **6** melts at 52 °C (20 mM MOPS, pH 7, 200 mM NaCl). Hairpin **6** was then alkylated by leinamycin (Scheme 2), followed by removal of unreacted alkylating agent by gel filtration. Leinamycin is known to selectively alkylate guanine residues in duplex DNA.[17,25,26,36] Monitoring the fluorescence of the 2-AP residue in this alkylated duplex (excitation at 317 nm, emission at 362 nm) revealed a time-dependent increase in fluorescence intensity (Fig. 1). The nearly 2-fold increase in fluorescence observed upon loss of the neighboring 7-alkylguanine residue was consistent with expectations, as it is known that the fluorescence of 2-AP increases with increasing exposure to solvent (and decreased stacking interactions with adjacent DNA bases).[27,28,30] Rate constants for the depurination of the leinamycin–guanine adduct were obtained by fitting the fluorescence data to the integrated rate expression for a first order process.[17,37] The rates measured at several different temperatures (Table 1) agree reasonably well with those measured previously for spontaneous release of the leinamycin–guanine adduct from mixed sequence duplex DNA using a traditional HPLC assay[17] When the 2-AP residue is offset from the alkylated guanine residue (hairpin **7**), similar results are obtained (data not shown). When the control

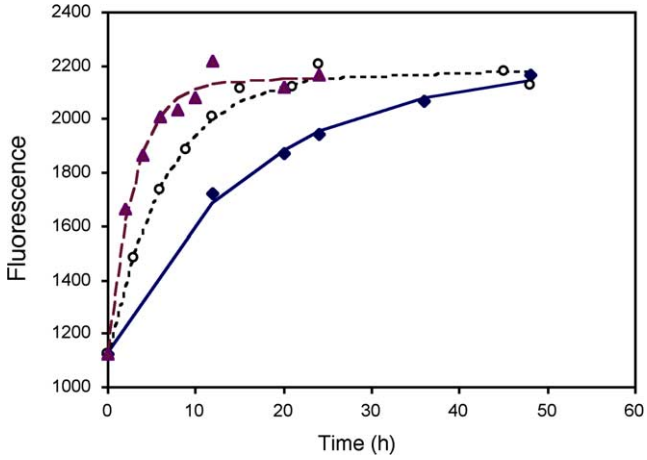

Figure 1. Fluorescence time courses for leinamycin-alkylated hairpin **6** at three different temperatures. The upper curve (▲) 37 °C; middle curve (○) 30 °C; lower curve 25 °C. The lines were obtained by fitting the experimental data to the equation for a first-order chemical process using SigmaPlot 2001 version 7.0. In these assays, the hairpin **6** (6 nmol; 50 μM) was incubated with leinamycin (125 nmol, 1 mM) and 2-mercaptoethanol (1.3 μmol; 10 mM) in 50 mM sodium phosphate buffer, pH 7, containing 500 mM NaCl. The mixture was incubated for 4 h at 4 °C. Excess leinamycin and its byproducts were removed by passing the mixture through a column of G10 Sephadex prepared in the reaction buffer at 4 °C (to prevent depurination of the guanine adduct) while collecting the DNA-containing fractions. For the fluorescence assays, a 1 μM solution of the alkylated hairpin was prepared in 50 mM sodium phosphate buffer, pH 7, containing 500 mM NaCl and maintained at the desired temperature. Aliquots were periodically removed (300 μL) and subjected to fluorimetric analysis (excitation 317 nm, emission 362 nm, 10 nm slit widths, 1 cm pathlength cuvette, photomultiplier voltage 700 V).

Table 1. Rate constants for the depurination of the leinamycin–guanine adduct from hairpin **6** calculated from fluorescence data[a]

Temp (°C)	Fluorescence method		From Ref. 14[b]	
	k (h^{-1})	$t_{1/2}$ (h)	k (h^{-1})	$t_{1/2}$ (h)
37	0.32 ± 0.05	2.2	0.20 ± 0.03	3.5
30	0.15[c]	4.6	N.D.[b]	N.D.
25	0.065 ± 0.003	11	0.039 ± 0.002	18

[a] Rate constants were obtained by fitting the experimental fluorescence data to the equation for a first-order chemical process using SigmaPlot 2001 version 7.0.

[b] Depurination of the leinamycin–guanine adduct from mixed sequence double-stranded DNA measured using HPLC. Rate constant at 30 °C was not reported in Ref. 14.

[c] Result of single measurement.

hairpin **8**, lacking the target guanine residue, was subjected to the standard alkylating conditions (Fig. 1 legend) and then analyzed, no changes from initial fluorescence intensity were observed. Similarly, a hairpin (**9**) containing 7-deazaguanine[38] in place of guanine displayed no change in fluorescence over time. These controls indicate that the fluorescence changes observed for hairpins **6** and **7** are dependent upon initial alkylation at the target guanine residues. Finally, alkylation of the native hairpin **5** (lacking a 2-AP residue), followed by fluorimetric analysis, revealed a fluorescence signal that did not change with respect to time, comparable to that of a blank sample containing only buffer.

In summary, the results reported here show that oligonucleotides containing a 2-AP residue can provide a useful tool for monitoring the spontaneous depurination of a modified base from duplex DNA. This method may provide the foundation for a high-throughput assay that detects DNA-alkylating agents. In addition, this general approach could potentially be applied to the design of convenient continuous fluorimetric assays for monitoring the removal of damaged bases from DNA by base excision repair enzymes.[39,40] Finally, the sensitivity of this assay might be increased through judicious use of fluorescent bases other than 2-AP.[41–44]

Acknowledgements

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