Sequence Specific DNA Cleavage by Conjugates of Benzotriazoles and Minor Groove Binders

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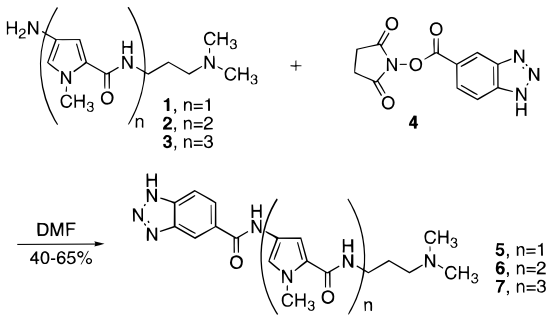
The ability of small molecules to irreversibly modify nucleic acids has generated considerable interest given that many medicinally useful natural products derive their biological activity from their selective interaction with DNA.[1] In addition, compounds which cleave nucleic acids in a sequence specific manner are potentially useful as reagents for accessing structural and genetic information. While DNA cleaving agents range in complexity from hydroxyl radicals to restriction enzymes, much research has focused on the design of novel compounds which can be triggered to generate potent and selective cleavage agents.[2] Recently, we described the development of a new class of DNA cleaving agents termed "benzotriazole photonucleases".[3] These molecules, designed to generate a reactive phenyl radical, were shown to efficiently and selectively cleave DNA upon photochemical activation. While the actual mechanism of cleavage is presently under investigation, it is clear that these novel compounds offer several advantages. Notably, they are readily available, can be easily modified to incorporate a range of functionality, and remain inactive until triggered by light. Herein we describe the synthesis and study of novel conjugates of benzotriazole photonucleases and DNA minor groove binders, agents which have been found to exhibit enhanced cleavage efficiency and unique cleavage selectivity.

The DNA cleavage efficiency and selectivity of our benzotriazole photonucleases were expected to be enhanced by covalent attachment to specific DNA recognition elements. Such a modification would be expected to increase the local concentration of the cleaving subunit in proximity to the DNA, and could also be used to select for specific DNA sequences. While there are several potentially suitable DNA binding compounds, we initially chose to position our triazoles in the minor groove of DNA by attaching them to well-characterized netropsin-like binders.[4] Netropsin and distamycin are di- and tripyrroles which tightly bind to the minor groove of DNA through a combination of electrostatic interactions, hydrogen bonds, and van der Waals contacts.[5] While these natural products preferentially bind four and five base pair (bp) AT tracts, several modifications have recently been reported which allow for the targeting of multiple sites.[6] In addition, new methods for the preparation of such compounds on a solid support provide the basis for further flexible control of sequence specificity by hybrid nucleases.[7]

Scheme 1



Compounds **5**−**7**, which contain a photoactivatable DNA cleaving unit of the benzotriazole type, were prepared to test our initial hypothesis. Since it has been shown that increasing the number of *N*-methylpyrrole units increases the binding affinity of these molecules,[5a] it was decided to systematically explore the effect of one, two, and three pyrrole units on cleavage efficiency and selectivity. The oligo(*N*-methylpyrrolecarboxamide) binding moieties (**1**, **2**, and **3**) were synthesized in four, six, and eight steps, respectively, according to standard literature procedures.[8] The activated ester **4,** prepared from benzotriazole-5-carboxylic acid, was used as a versatile coupling partner in these syntheses, which proceeded smoothly to afford compounds **5**, **6**, and **7** in six to ten steps overall (Scheme 1). Importantly, these molecules all absorb light above 300 nm, allowing for selective photoexcitation in the presence of DNA.[9]

The DNA cleavage ability of **5**, **6**, and **7** was initially tested by monitoring the conversion of circular supercoiled DNA (form I) to circular relaxed (form II) and linear (form III) DNA. Hybrid compounds (3−90 μM) were irradiated with Pyrex-filtered light in the presence of plasmid pBR322 (30 μM bp). Results are shown in Figure 1 for **5** (a), **6** (b), and **7** (c). In all cases, single-strand cleavage (form II) is observed at concentrations as low as 3 μM. Form III DNA (resulting from double-strand cuts or proximal single-strand cuts on opposite strands) is also observed with both **6** and **7**. In the absence of light (lane 7), no cleavage occurs, indicating that these compounds are inactive until triggered photochemically. Two additional points can be gleaned from this experiment. First, this series of benzotriazoles conjugated to DNA minor groove binders is at least 10-fold more effective in cleaving DNA than the previously studied triazole derivatives.[3] Second, the potency of cleavage increases with increasing number of *N*-methylpyrrole units, as would be expected from enhanced association of the agent with the DNA target.

To elucidate the binding mode of these compounds and its effect on cleavage, footprinting experiments with a 167 bp DNA fragment and di- and tripyrrole-containing hybrids **6** and **7** were conducted. The binding of **6** and **7** was determined by identifying those sequences protected from DNase I cleavage.[10]

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(9) UV data for these compounds (H_2O): (**5**) λ_{max} 287 (ϵ 14 206 M^{-1} cm^{-1}), 220 (ϵ 29 178) nm; (**6**) λ_{max} 297 (ϵ 34 165), 230 (ϵ 42 968); (**7**) λ_{max} 309 (ϵ 36 678), 216 (ϵ 42 125) nm.

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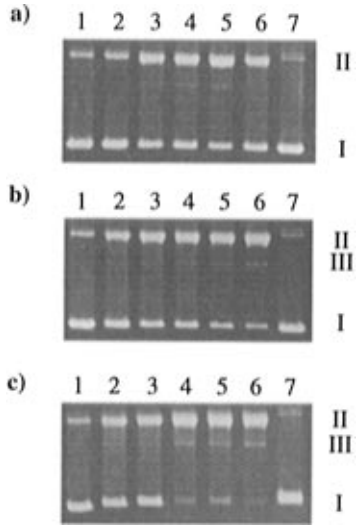

Figure 1. Light-induced cleavage of DNA by hybrid compounds **5** (a), **6** (b), and **7** (c). Supercoiled DNA runs at position I, nicked DNA at position II, and linear DNA at position III. Unless otherwise indicated, all reactions were irradiated for 30 min with Pyrex-filtered light from a 450 W medium-pressure mercury arc lamp. Key: lanes 1−6, DNA + compound at concentrations of 3, 15, 30, 45, 60, and 90 μM; lane 7, DNA + compound (90 μM), no *hν*.

Preincubation of the DNA with 10−50 μM **6** or **7** causes specific inhibition of DNase I cleavage at sites shown in Figure 2a. The three AT rich sequences highlighted were preferentially bound by both **6** and **7** (a fourth is not shown). These locations were also found to be among the primary binding sites for netropsin and distamycin,[11] indicating that, as anticipated, the binding subunits of **6** and **7** are responsible for their DNA recognition properties.

The cleavage selectivity of these benzotriazole hybrids was next investigated by high-resolution denaturing gel electrophoresis. Figure 2b shows the results obtained when these molecules (50 μM) were irradiated in the presence of the same DNA fragment as in Figure 2a. Analysis of the damage products reveals that ***6** cleaves predominantly at one site within the 167 bp fragment*; **7** also cleaves at this site as well as at two additional sites.[12] By comparison with the footprinting data (Figure 2a), we note that the primary cleavage sites occur at the 5′ end of the binding sites. This cleavage is strongly inhibited in the presence of netropsin (data not shown), indicating that damage is primarily initiated by bound nuclease. Interestingly, the nucleotide which is cleaved by **7** but not by **6** lies at the extreme outer edge of the binding site, suggesting that perhaps the additional *N*-methylpyrrole unit of **7** enables it to reach this specific location. Since cleavage occurs only at two of the four binding sites, the specificity of **6** and **7** must therefore arise from a combination of selective binding and intrinsic reactivity.

The cleavage pattern shown in Figure 2b results from photoinduced strand breaks. While additional experiments have shown that this cleavage is enhanced upon basic workup (10% piperidine, 95 °C, 30 min), no new cleavage sites are revealed. These findings suggest that cleavage originates from modification of the nucleotide base. Mechanistic investigations on the related unconjugated benzotriazole photonucleases have supported an electron transfer pathway for DNA cleavage; such a mechanism might also be operating in this case.[13]

The above studies outline the facile preparation of a unique series of triazole photonucleases containing DNA minor groove

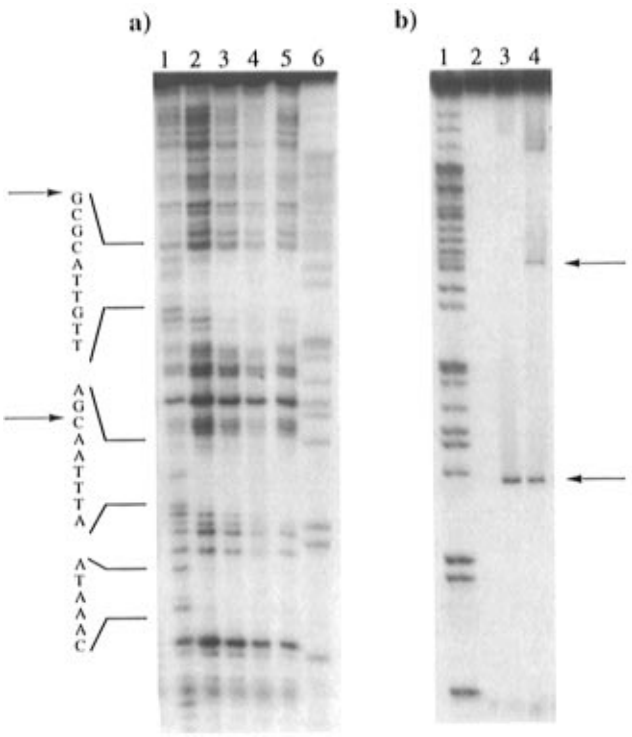

Figure 2. Autoradiograms of 10% denaturing polyacrylamide gels showing binding and cleavage of 3′-^{32}P-end-labeled *Eco*RI/*Rsa*I restriction fragment from plasmid pBR322. Arrows indicate sites of cleavage. (a) DNase I footprinting in the presence of compounds **6** and **7**. Compounds were incubated with the DNA at 37 °C for 30 min and then treated with 0.2 u of DNase I for 2 min. Key: lane 1, DNase I digestion products obtained in the absence of binders; lanes 2−4, digestion products obtained in the presence of 10, 25, and 50 μM **6**; lane 5, digestion products obtained in the presence of 50 μM **7**; lane 6, Maxam−Gilbert G sequencing reaction. (b) Light-induced cleavage by **6** and **7**. Unless otherwise indicated, reactions were irradiated for 30 min with Pyrex-filtered light in 50 mM Tris acetate buffer, pH 7.9 containing 100 μM bp calf thymus DNA.[14] Key: lane 1, Maxam−Gilbert G reaction; lane 2, dark control (50 μM **6**, no *hν*), lanes 3−4, cleavage products obtained with 50 μM **6** and **7**, respectively.

binding units of varying length. These molecules have been assayed for DNA cleavage ability and found to be very effective, surpassing the efficiency of our previously reported benzotriazole derivatives. Binding studies demonstrate that the binder−cleaver conjugates **6** and **7** tightly bind DNA in a manner similar to that of the parent binding subunits (netropsin and distamycin). Upon photoactivation, these compounds cleave DNA in a highly selective fashion, with cleavage occurring at the 5′ end of their binding sites. Additional specificity is undoubtedly provided by the intrinsic chemical reactivity of the benzotriazole subunit, leading to potent, selective, and inducible DNA cleavage by these hybrid compounds.

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Supporting Information Available: Experimental details and characterization data (5 pages). See any current masthead page for ordering and Internet access information.

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(12) Irradiation of netropsin in the presence of DNA shows slight background degradation, but no cleavage in proximity of these sites.
(13) Touami, S. M.; Wender, P. A. Unpublished results. In accordance with this proposal, we have conducted control experiments with **5** and **6**. Under the conditions for DNA cleavage, these compounds remain unchanged as determined by NMR, suggesting that a catalytic pathway is at least partially involved.
(14) Reactions performed in 10 mM sodium phosphate buffer displayed similar efficiencies and selectivities of cleavage.