The Effect on DNA Cleavage Potency of Tethering a Simple Cyclic Enediyne to a Netropsin Analog

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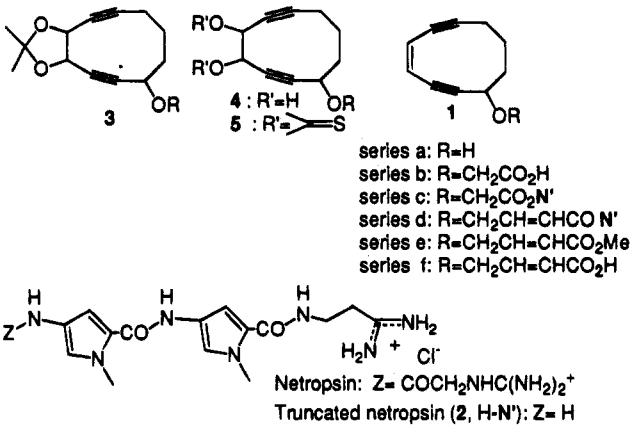
Summary: The attachment of a simple 10-membered monocyclic 3-ene-1,5-diyne to a derivative of netropsin via two-carbon (acetate) and four-carbon (crotonate) tethers enhances the DNA cleavage potency, by a factor of almost 2000 in the best case.

The enediyne antitumor antibiotics are toxic and in certain cases show selective double-strand cleavage of DNA.[1,2] Calicheamicin γ_1^I, for example, bears an "aryl-pentasaccharide" side chain and an "aglycon" which are responsible for (a) chemical activation, (b) formation of an arene 1,4-diradical, (c) association with DNA, and (d) near-synchronous double-strand cleavage. Studies of the natural product[3] and of models[2] have established the mechanism of chemical activation, and labeling studies[1a,4] have provided details regarding the hydrogen atom abstraction responsible for DNA cleavage. The timing of activation and association is not clear,[3a,5,6] and the roles of the oligosaccharide and the enediyne aglycon in DNA recognition and association are the subject of conflicting suggestions.[6,7] It is likely that considerable precision in DNA interaction is required to account for the high potency and the sequence selectivity. Synthesized examples of the cyclic 3-ene-1,5-diyne "warhead" are less potent than the natural products and cause nonselective, single-stranded scission of DNA, presumably because they lack a structural component which can assist in association with DNA and in positioning the enediyne core for maximum effectiveness.[2,6,8] Following the avenue first developed by Nicolaou, model enediynes have been tethered to typical DNA delivery agents, but "the increase in potency of these agents as DNA cleavers is not as dramatic as expected."[2b,9] We report here DNA cleavage studies with a simple enediyne, **1a**,[10] conjugated with netropsin, a minor groove DNA binding agent,[11] *via* acetate and crotonate tethers. This provides the first example in the enediyne series in which cleavage efficiency is strongly enhanced by attachment of a DNA association unit, and the potency is shown to be strongly dependent on the tether and consistent with molecular modeling studies and the first model enediyne to approach the natural enediynes in DNA scission efficiency *in vitro*.

Scheme 1. A Monocyclic Enediyne Tethered to a Netropsin Analog



Cyclodeca-4-ene-2,6-diyn-1-ol (**1a**) cleaves DNA *in vitro* in a single-stranded fashion, as determined by standard procedures. Incubation of **1a** with supercoiled pBR322 plasmid DNA in pH 7.5 buffer at 37 °C followed by electrophoresis of the cleavage products on an agarose

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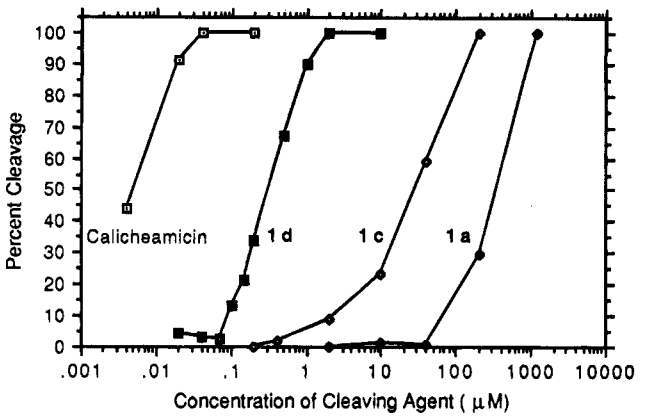

Figure 1. Cleavage of supercoiled pBR322 DNA (30.3 μM/base pair, 50 mM Tris, pH 7.5, 100 mM NaCl; 18 h, 37 °C) by calicheamicin γ1, **1a**, **1c**, and **1d**.

gel allowed for separation of unreacted DNA (form I) from relaxed closed-circular DNA (form II), produced by cleavage of a single strand, and linear DNA (form III), produced by two closely spaced single-strand nicks (<15 base pair separation on complimentary strands) or by double-stranded cleavage. A comparison of the concentrations at which calicheamicin (ca. 0.005 μM) and **1a** (300–400 μM) cause 50% cleavage indicates **1a** is ca. 7 $\times$ 10^4 less potent (Figure 1). There is no evidence for double-strand cleavage by **1a**.

In an effort to increase potency, introduce base pair selectivity, and/or induce double-strand cuts, we tethered **1a** to the truncated netropsin unit **2**[12] *via* an acetate linker. Treatment of **1a** with NaH in THF at −78 °C followed by iodoacetic acid gave acid **1b**.[13] Then reaction of **1b** with amine **2** (DMAP, DCC) gave **1c**[13] in 28% yield (15% recovered **1b**). Comparing the results at 50% cleavage (Figure 1), **1c** (25–35 μM) is about 10–12-fold more potent than **1a**. The reason for the only modest increase in the ability of **1c** to cleave DNA was probed through binding studies and molecular modeling.

CD spectroscopy is useful for evaluating the interaction of netropsin with B–form DNA.[14] Interaction of the enediyne–netropsin conjugates with poly[d(AT)•d(AT)] is associated with a change in ellipticity in the region 300–330 nm. We have used the CD spectral change to compare the DNA binding affinities of the netropsin conjugates and truncated netropsin (**2**). A solution of netropsin, truncated netropsin (**2**), or the conjugate **1c** was titrated into a buffered solution of poly[d(AT)•d(AT)] in increments. The ellipicity at the λ_{max} for each derivative at increasing concentration relative to nucleotide is displayed in Figure 2. The results indicate that netropsin[11b] and truncated netropsin **2** bind strongly to DNA; the conjugate **1c**, however, binds weakly.

Apparent equilibrium binding constants were determined based upon inhibition of ethidium bromide binding to poly[d(AT)•d(AT)][16] by netropsin, truncated netropsin (**2**), and conjugate **1c**. The results of these competition experiments (Figure 3) are consistent with the CD results.

Molecular modeling studies[17] suggested that it is difficult for conjugate **1c** simultaneously to make favorable hydrogen bonding and van der Waals contacts in the manner of netropsin *and* to orient the enediyne portion within the minor groove. This is due to repulsive steric interactions between the enediyne subunit and the "walls" of the minor groove. The modeling also suggested that increasing the length of the tether would allow for the simultaneous placement of both the enediyne terminus and the netropsin unit within the minor groove. A crotonate tether appeared particularly attractive based on the modeling and ease of synthesis.

Enediyne–netropsin conjugate **1d** was synthesized from a precursor to **1a**, the masked enediyne **3a**.[10a] Treatment of alcohol **3a** with the triflate of methyl 4-hydroxycrotonate produced **3d** in 73% yield. Cleavage of the acetonide and treatment of the resulting diol (**4d**) with 1,1′-(thiocarbonyl)diimidazole provided thionocarbonate **5d** in 72% yield. Elimination induced by 1,2,3-trimethyl-1,3-diaza-2-phospholidine[18] at 0 °C provided enediyne **1e** in 40% yield. Hydrolysis of the methyl ester gave carboxylic acid **1f** (81%), which was coupled with amine **2** (DCC/DMAP at 0–3 °C) to give the enediyne–netropsin conjugate **1d**[13] (51%; 11% recovered **1f**). The CD and ethidium competition studies (Figures 2 and 3) indicate that this new conjugate (**1d**) binds strongly to poly[d(AT)•d(AT)].

DNA cleavage analysis (Figure 1 and Table 1) shows strongly increased potency (at 50% cleavage [**1d**] $\cong$ 0.2 μM); conjugate **1d** is almost 2000-fold more potent than **1a** and only ca. 40-fold less than calicheamicin γ1[I]. The crotonate tether compared to the acetate tether increases cleavage effectiveness by ca 150-fold. No significant double-stranded cleavage is observed with either **1c** or **1d**.

Polyacrylamide gel electrophoresis was used to investigate the sequence selectivity of cleavage mediated by **1d** in a singly end labeled HindIII-NciI pBR322 DNA fragment.[19] Cleavage enhancements occurred specifically at the ends of AT sequence segments, consistent with minor groove interaction of the netropsin portion of **1d**. However, unlike netropsin which recognizes four-base-pair-long AT sequence segments,[11] **1d** cuts at the ends of three-base-pair-long AT sequence segments. This result may be due to differences in the hydrogen bonding ability of the amide at the guanidinium end in netropsin which in the case of **1d** is replaced with the enediyne–crotonate tether.

In summary, the combination of a simple enediyne and a conventional minor groove binder can produce an

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(15) The ligand solution was titrated into a solution of 1.3 μM ethidium bromide and 4.0 μM DNA in 10 mM Tris/1 mM EDTA pH 7.1 buffer, and the fluorescence was measured (excitation: 546 nm, emmision: 600 nm, 25 °C). The apparent binding constant was estimated using the equation K_{EthBr}[EthBr] = K_{app}[ligand], where [ligand] is the concentration of the ligand at a 50% reduction of fluorescence and K_{EthBr} is 9.5 $\times$ 10^6 M^{-1}.[15]

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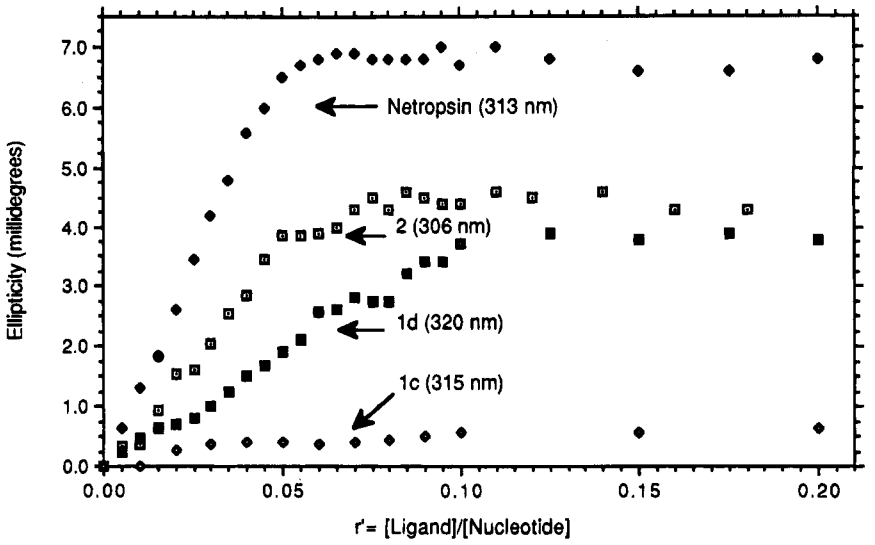

Figure 2. Circular dichroism titrations of poly [d(AT)·d(AT)] with netropsin and compounds **2**, **1c**, and **1d** in pH 7.1 buffer at 25 °C. For netropsin, **2**, and **1d** the DNA was 8.1×10^{-5} M/nucleotide and the buffer was 10 mM Tris/1 mM EDTA. For **1c** the DNA was 7.6×10^{-5} M/nucleotide and the buffer was 20 mM Tris.

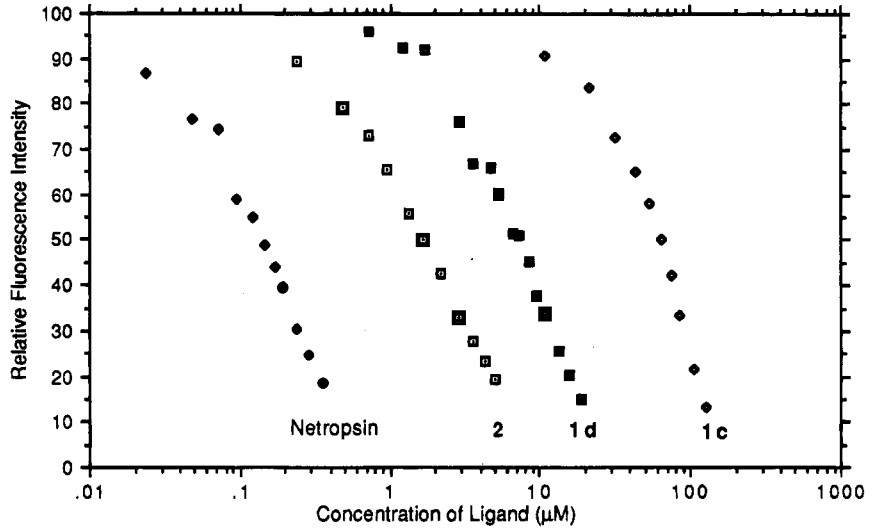

Figure 3. Inhibition of ethidium bromide binding to poly[d(AT)·d(AT)] by the addition of the ligands netropsin, **2**, **1c**, and **1d**. The calculated values of K_{app} are (87, 7.4, 0.19, and 1.7) $\times 10^6$ M^{-1}, respectively.[14]

Table 1. Summary and Comparison of DNA Cleavage

compd	concn at 50% cleavage[a] (mM)	ratio of efficiency rel to calicheamicin $g1^I$
calicheamicin $g1^I$	0.005	1
1d	0.2	40
1c	30	6000
1a	350	70000

[a] Uncertainty is ca. 20%.

effective agent for DNA cleavage. The four-carbon crotonate tether induces a $>10^3$-fold increase in cleavage performance in **1d** compared to the enediyne alone (**1a**) and $>10^2$-fold compared to the conjugate with the acetate tether (**1c**), using a truncated netropsin as a DNA association vehicle. This points the way for design of more efficient DNA cleaving agents by modification of the tether between the enediyne core and the DNA delivery system.

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Supplementary Material Available: The procedures and characterization data for new compounds reported here, as well as NMR spectra for compounds **1b**, **1c**, **1f**, and **1d**, and CD and fluorescence spectra (22 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.