The binding modes of a rationally designed photoactivated DNA nuclease determined by NMR

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ABSTRACT

The complex between the rationally designed synthetic DNA cleaving agent netropsin-diazene and the double-stranded DNA oligomer 5'-CGCAAAGGC-3'-5'-GCCTTTGCG-3' was characterized by two-dimensional NMR spectroscopy in solution. Photolysis of netropsin-diazene bound to DNA generates a trimethylenemethane diradical intermediate that induces single-strand breaks in the DNA. The π -diyl trimethylenemethane based compounds are a new class of DNA nucleases. We tested the following design criteria: (i) binding of the diazene and subsequent reactive diyl to the DNA, (ii) sequence selectivity in the ligand binding and (iii) prevention of diyl dimerization. Sixteen NOE derived ligand-DNA distance restraints were used to obtain the energy minimized model of the complex. The ligand is bound to the minor groove of the oligomer with the diazene at the 5' end of the A-tract in the predominant conformation of the complex. This form of the complex exchanges with a minor conformation in which the ligand is in the opposite orientation. The DNA maintains a B-form structure. Netropsin-diazene has fulfilled all of the design criteria, binding to the DNA duplex studied in the minor groove of the central AAAA tract in a 1:1 mode, preventing diyl dimerization and other side reactions from occurring.

INTRODUCTION

NMR spectroscopy has become an integral part of the iterative design and testing of new oligo(pyrrolicarboxamide) ligands based on distamycin A (1) (Fig. 1) for the sequence specific recognition of AT and/or GC containing tracts of DNA (1–10). Oligo(pyrrolicarboxamide)-based ligands have been modified to sequence specifically alkylate and/or cleave DNA (5,11–19). The development of artificial DNA cleaving agents requires the simultaneous solution of the problem of DNA sequence recognition and the introduction of a functionality that effects cleavage in a specific fashion.

A DNA cleaving agent (3) based on the DNA binding ligand netropsin (2) and a trimethylenemethane diradical (TMM) precursor has been designed and synthesized (Fig. 1) (20). Netropsin (2) is a di-peptide that binds in the minor groove of DNA at sites of four or five successive A,T base pairs (3,11,21–23). The design criteria were to: (i) bind the diazene and subsequent reactive diyl to the DNA, (ii) achieve sequence selectivity in the ligand binding and (iii) prevent diyl dimerization side reactions by physical separation from other diyls.

TMM diyls are less reactive towards hydrogen atom abstraction than are the enediyne derived 1,4 σ -diyls (24,25). TMM diyls can be generated by the thermolysis or photolysis of diazenes (20,25,26). Photoactivated DNA cleavage has the advantage of allowing precise control of the initiation and duration of the cleaving reaction (Fig. 1). In principle, the reduced reactivity of the soft π -diyl trimethylenemethane relative to the very reactive, toxic 1,4 σ -diyls generated from the natural products may result in decreased overall toxicity (27).

In this report we describe the use of NMR spectroscopy to evaluate the success of this design strategy by determining the binding modes of netropsin-diazene 3 to d(CGCAAAGGC):d(GCCTTTGCG).

MATERIALS AND METHODS

Synthesis of ligands and oligonucleotides

The netropsin-diazene 3 was synthesized as described previously (20). The oligomers d(CGCAAAGGC) and d(GCCTTTGCG) were synthesized and purified as reported previously (28). The numbering scheme for the oligomer is:

5'- C1 G2 C3 A4 A5 A6 A7 G8 G9 C10 -3'
3'- G20 C19 G18 T17 T16 T15 T14 C13 C12 G11 -5'

Sample preparation

NMR samples were prepared by dissolving the decamer oligonucleotide duplexes in 0.5 ml of 25 mM Na₂PO₄ buffer (pH 7.0) and then lyophilizing to dryness. For experiments carried out in D₂O the solid was redissolved in 0.5 ml 99.96% D₂O (Cambridge Isotope Laboratories), while for experiments in H₂O a 90% H₂O/10% D₂O mixture (0.5 ml) was used. Solid netropsin-

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Figure 1. Structure of distamycin (1), netropsin (2) and netropsin-diazene (3). The numbering scheme for netropsin-diazene is shown as well as diyl formation by photolysis. The diazene chromophore is specifically excited by irradiation with light from a 450 W Hanovia lamp, transmitted through Pyrex and a 3.9% naphthalene-ethanol liquid filter.

diazene **3** was suspended in H₂O and titrated with HCl until a clear yellow solution was obtained, yielding stock solution of 40 mM as determined by UV absorbance. The extinction coefficient of netropsin (**2**) ($\epsilon_{296} = 2.15 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) was used for the netropsin-diazene **3**. The ligand stock solution was stored at -70°C . Extinction coefficients at 260 nm for d(CGCAAAGGC) and d(GCCTTTTGCG) were calculated to be $1.02 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ and $8.72 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ respectively (29). The concentration of the double-stranded DNA sample was 2.3 mM.

NMR experiments

NMR experiments were performed at 600 MHz on a Bruker AMX-600 spectrometer or at 500 MHz on a GE Omega spectrometer. Netropsin-diazene **3** was titrated into the NMR sample containing duplex DNA in ~ 0.2 mole equivalents per

addition. One-dimensional spectra in H₂O were acquired with 8192 complex points averaged over 128 scans with a spectral width of 12 048 Hz using a 1–1 jump and return pulse sequence to suppress the water signal.

NOESY spectra in D₂O were collected at 25°C , with 1024 complex points in t_2 using a spectral width of 6024 Hz and a mixing time of 200 ms. 512 t_1 experiments were recorded and zero-filled to 1024 points. For each t_1 value, 64 scans were signal averaged using a recycle delay of 2 s. A presaturation pulse was applied during the recycle and mixing periods to suppress the residual HDO resonance. NOESY spectra in H₂O were acquired at 25°C , replacing the last 90° pulse by a 1–1 jump and return sequence (30) to suppress the solvent signal as described previously (4). The spectra were collected with 2048 complex points in t_2 using a spectral width of 13 514 Hz and a mixing time of 200 ms. 480 t_1 experiments with 64 scans were recorded and zero-filled to 2048 points. TOCSY spectra in D₂O were acquired at 25°C , with 1024 complex points in t_1 with a spectral width of 4800 Hz and a mixing time of 60 ms. All spectra were acquired in TPPI mode.

The data were processed with FELIX v. 2.30 (Biosym Technologies, San Diego) on a Silicon Graphics IRIS/4D workstation. Skewed sine bell functions were used for apodization of the free induction decays. NOESY spectra of the ligand–DNA complex in D₂O and H₂O enabled the assignment of the DNA resonances using standard methods (31,32). Ligand resonances were assigned as described below (4,6,28,33).

Distance restraints

A model of the netropsin-diazene complex with d(CGCAAAGGC):d(GCCTTTTGCG) was built based on the NMR data. Intermolecular distance restraints were generated from the volume integrals of the cross peaks in the H₂O NOESY spectrum acquired at a mixing time of 200 ms as described previously (4,6). The cross peak volumes were classified semi-quantitatively into three categories: strong (1.8–2.8 Å), medium (1.8–3.5 Å) or weak (1.8–5.0 Å) relative to the volume integrals of the cytosine H5–H6 cross peak volumes. A pseudoatom was generated for each of the following sets of ligand protons: (i) the six *N,N*-dimethyl protons, (ii) the four methylene protons 7a, 7b, 8a and 8b and (iii) the four methylene protons 9a, 9b, 10a and 10b (see Fig. 1 for numbering scheme). Distance restraints from DNA protons to these pseudoatoms were given a range of 1.8–7.0 Å. The 16 intermolecular ligand–DNA restraints used in the modeling are listed in Table 3.

Model refinement

The model of the d(CGCAAAGGC):d(GCCTTTTGCG) duplex was constructed using the Biopolymer module of Insight II (Biosym) from standard B-form DNA. The netropsin-diazene was assembled using the sketcher module of Insight II (Biosym) and assigned AMBER type potentials. The partial charges on the atoms were obtained by a MOPAC calculation on the ligand. The netropsin-diazene model was manually docked into the minor groove with the help of the Builder module of Insight II on the Silicon Graphics workstation. Energy minimizations were performed using the Discover module of Insight II. A force constant of $50 \text{ (kcal/mole)/\AA}^2$ was used for the experimentally derived NOE restraints. The cut-off distance for non-bonded interactions was set at 18 Å with a switching distance of 2 Å. A distance-dependent dielectric of the form $\epsilon = R$ was used to account for solvent effects. The energy of the complexes was

Figure 2. Expansion of the aromatic to sugar H1' region of a NOESY spectrum of the 1:1 complex of netropsin-diazene **3** with d(CGCAAAAGGC):d(GCCTTTTGCG) (in D₂O, 25°C, $\tau_{\text{mix}} = 200$ ms). Sequential aromatic to C1'H connectivities for the 5'-AAAA-3' strand are shown as a solid line; those for the 5'-TTTT-3' strand are shown as a broken line. NOE cross peaks from ligand pyrrole protons to various DNA protons are labeled. The labeling scheme is as according to Figure 1.

initially minimized using 100 steps of a steepest descents algorithm and 3000 steps of conjugate gradient minimization. The final model was created by subjecting the minimized starting model to 13 ps of restrained molecular dynamics (rmd) at 200 K, which was subsequently cooled to 100 K over 10 ps, followed by 4 ps of rmd at 100 K. The model was then energy minimized by conjugate gradient minimization to a final rms derivative of <0.05 (kcal/mole)/Å². The minor form of the complex was not modeled, but is undoubtedly very similar.

RESULTS

NMR resonance assignments

Part of a NOESY spectrum of the 1:1 netropsin-diazene-DNA complex in D₂O is shown in Figure 2. The chemical shifts of the deoxyribose H1' and DNA base H6/H8/H2 resonances and of the ligand resonances are listed in Table 1 and Table 2, respectively. Assignments of the deoxyribose H1', H2', H2'', H3', thymine methyl and base H5, H6, H8 and H2 resonances were made from the D₂O NOESY spectra, using standard methods (31). Assignments were extended to some of the deoxyribose H4' resonances as well. The correct assignment of the adenosine base H2 resonances established the orientation of the ligand in the complex. These assignments were made based on cross peaks from the H2 protons to intrastrand and interstrand deoxyribose H1' protons in the D₂O NOESY. Crosspeaks from the H2 protons to thymidine imino protons in the H₂O NOESY confirm these assignments. Intraresidue NOE cross peak patterns arising from deoxyribose protons as well as interresidue NOE cross peak patterns indicate that the DNA is B-form in the complex and that there is no obvious distortion of the DNA helix as a result of ligand binding.

The ligand methylene 9a, 9b, 10a and 10b (see Fig. 1 for numbering scheme) proton resonances and *N,N*-dimethyl reson-

Figure 3. Schematic of selected intermolecular NOEs between the netropsin-diazene and d(CGCAAAAGGC):d(GCCTTTTGCG) in the major form of the complex. The black diamonds are pseudoatoms.

ances were assigned using the D₂O NOESY and TOCSY spectra. The resonance at 3.06 p.p.m. integrates to six protons in the spectrum and gives strong NOESY cross peaks to four other ligand resonances. These four resonances give rise to strong TOCSY and NOESY cross peaks to each other, indicating that they are J-coupled and are close in space. However, the resonance at 3.06 p.p.m. does not give rise to TOCSY crosspeaks to these four resonances. This information is consistent with our assignments of the resonance at 3.06 p.p.m. to the *N,N*-dimethyl protons and the other four resonances to the adjacent methylene protons 9a, 9b, 10a and 10b (see Table 2). Among these four resonances, it was impossible to determine which resonance corresponded with which of the four methylene protons, so that specific assignments could not be made.

Two of the methylene resonances 9a, 9b, 10a and 10b give NOE crosspeaks to a ligand amide resonance. This amide resonance at 8.23 p.p.m. is assigned to NH-3. Several broad resonances in the region 2.2–2.5 p.p.m. give rise to NOESY cross peaks to another ligand amide resonance at 9.45 p.p.m. This amide must be NH-1 and the broad upfield resonances are assigned to the ligand methylene protons 7a, 7b, 8a and 8b. These methylene protons have additional NOESY cross peaks to DNA A7 and A4 H2 protons and to an unassigned proton of the bicyclic diazene framework. The third amide resonance at 9.23 p.p.m. is assigned to NH-2. The two netropsin-diazene pyrrole H3-1 and H3-2 resonances are degenerate in chemical shift. They were assigned based on their chemical shift and based on NOESY cross peaks to the ligand NH-1, NH-2 and NH-3 resonances and to various DNA resonances.

The bicyclic diazene moiety contains seven protons, labeled 1, 3, 4a, 4b, 5a, 5b and 6 in Figure 1. We observe intramolecular NOEs within the bicyclic diazene moiety and from methylene protons 7a, 7b, 8a and 8b to the bicyclic diazene framework. However, using both NOESY and TOCSY spectra we were unable to unambiguously assign the diazene resonances. No intermolecular NOESY cross peaks from any of the seven bicyclic diazene resonances to the DNA were observed (35).

Table 1. Chemical shift assignments of the d(CGCAAAAGGC):d(GCCTTTTGGC) duplex, free and in the netropsin-diazene:DNA complex^a

	H6/H8			H1'			H2		
	Free	Complex	$\Delta\delta$	Free	Complex	$\Delta\delta$	Free	Complex	$\Delta\delta$
Strand 1									
C1	7.62	7.64	+0.02	5.73	5.76	+0.03			
G2	7.93	7.96	+0.03	5.86	5.87	+0.01			
C3	7.31	7.32	+0.01	5.42	5.44	+0.02			
A4	8.19	8.28	+0.09	5.74	5.60	-0.14	7.17	7.15	-0.02
A5	8.08	8.19	+0.11	5.75	5.88	+0.13	7.00	7.35	+0.35
A6	7.99	7.86	-0.13	5.81	5.51	-0.30	7.03	7.50	+0.47
A7	7.87	7.69	-0.18	5.89	5.42	-0.47	7.43	7.67	+0.24
G8	7.39	7.39	0.00	5.52	5.41	-0.11			
G9	7.56	7.60	+0.04	5.92	5.92	0.00			
C10	7.36	7.44	+0.08	6.15	6.20	+0.05			
Strand 2									
G11	7.95	8.00	+0.05	5.97	6.01	+0.04			
C12	7.51	7.56	+0.05	6.08	6.12	+0.04			
C13	7.59	7.67	+0.08	6.00	6.01	+0.01			
T14	7.49	7.54	+0.05	6.09	6.07	-0.02			
T15	7.49	7.51	+0.02	6.14	6.11	-0.03			
T16	7.48	7.33	-0.15	6.10	5.67	-0.43			
T17	7.30	7.12	-0.18	5.81	5.34	-0.47			
G18	7.90	7.85	-0.05	5.79	5.76	-0.03			
G19	7.33	7.35	+0.02	5.75	5.77	+0.02			
C20	7.92	7.97	+0.05	6.12	6.15	+0.03			

^aChemical shifts are given in p.p.m. (± 0.01 p.p.m.). The residual HDO resonance is referenced to 4.84 p.p.m. (20°C) for the free DNA and to 4.78 p.p.m. (25°C) for the complex.

Intermolecular contacts

NOE cross peaks from DNA protons to ligand pyrrole protons H3-1 and H3-2 and amide protons NH-1 and NH-2 indicate that the ligand pyrrole-pyrrole ring system spans the 5'-A4-A5-A6-A7-3' sequence. This is further supported by cross peaks from DNA protons to the ligand methylene protons at either end of the molecule (Table 3 and Fig. 3).

A set of NOEs connects the dimethylamino protons to the H2 proton of A7, although a weaker set to A4 H2 also occurs which cannot be explained by a single model of the ligand-DNA complex (Fig. 4). The presence of two mutually exclusive sets of NOESY cross peaks clearly indicates that the ligand is in one predominant orientation with a minor alternate conformation and is consistent with the occurrence of flip-flop exchange between the two conformations (Fig. 5) (36,37). Based on relative intensities of ligand-DNA NOE cross peaks in the two ligand conformations, the major form is preferred over the minor form in approximately a 3:1 ratio. The set of NOEs which defines the major form contains cross peaks from the methylene protons 9a, 9b, 10a and 10b to the A7 H2 proton (Fig. 4) and cross peaks from the *N,N*-dimethyl protons to the A7 H2 proton as well as to G8, G9 and T14 deoxyribose H1' protons.

In the major form of the complex, the bicyclic diazene moiety of the ligand points toward the 5'-end of the A-rich DNA strand

and is located near the C3/G18 and A4/T17 base pairs (Fig. 6). The ligand arrangement is determined by specific hydrogen bonds between the amide protons of the ligand and the DNA. Evidence for these hydrogen bonds is provided indirectly by NOE cross peaks between the ligand amide protons and DNA A4, A5, A6 and A7 base H2 protons in the H₂O NOESY spectrum. Additionally, NOESY contacts to the DNA from the ligand H3 pyrrole protons, methylene protons on both ends of the ligand and the *N,N*-dimethyl protons support this model. Although the dimethylammonium proton is likely to be involved in hydrogen bonding interactions with the DNA, specific acceptors cannot be resolved by the current data and model.

The minor form is defined by cross peaks from the 9a, 9b, 10a and 10b ligand methylene protons to the A4 H2 proton (Fig. 4) and from the *N,N*-dimethyl protons to the A4 H2 proton and to the G18 and C19 deoxyribose protons. NOEs are observed between NH-1 of the ligand and A5, A6 and A7 H2 protons. In the minor orientation the diazene is located near the A7/T14 and G8/C13 base pairs.

These results are in agreement with the observed cleavage pattern on a 517 bp fragment of DNA in which the highest frequency of cleavage occurred at the 5' end of apparent netropsin binding sites (20). Although the present study demonstrates a specificity for the AAAA site, a complete characterization of the sequence specificity of netropsin-diazene for A,T containing sites

would require independent determination of the binding affinity of netropsin-diazene for all possible 4 bp DNA sequences (42).

Table 2. Chemical shift assignments of netropsin-diazene 3 complexed with d(CGCAAAGGC):d(GCCTTTTGCG)^{a,b}

Proton	Chemical shift
NH-1	9.45
H3-1	6.59
NH-2	9.23
H3-2	6.59
NH-3	8.23
C(4a,b)H ^c , C(5a,b)H ^c	1.10, 1.14, 1.81, 1.87
C(7a,b)H ^c , C(8a,b)H ^c	2.2–2.5
C(9a,b)H ^c , C(10a,b)H ^c	3.31, 3.40, 3.57, 3.60
C(1)H ^{c,d} , C(3)H ^{c,d} , C(6)H ^{c,d}	5.34, 5.39, 5.73, 5.74
<i>N,N</i> -(CH ₃) ₂	3.06

^aChemical shifts are given in p.p.m. (± 0.01 p.p.m.), with the residual HDO resonance referenced to 4.78 p.p.m. (25°C).

^bRefer to Figure 1 for numbering system used.

^cResonances assigned as a group.

^dMore than one resonance is observed for some of the diazene protons because the ligand binds the DNA diastereotopically (35).

Table 3. Ligand–DNA contacts for the netropsin-diazene complex with d(CGCAAAGGC):d(GCCTTTTGCG)

Netropsin-diazene	DNA	NOE classification ^a
NH-2	A5-H2	w
H3-1	A5-H2	s
NH-1	A5-H2	s
H3-2	A6-H2	s
NH-2	A6-H2	s
H3-1	A6-H1'	m
NH-2	A6-H1'	w
H3-2	A7-H1'	m
H3-2	T16-H1'	m
H3-1	T17-H1'	m
<i>N,N</i> -(CH ₃) ₂	A7-H2	p
H7a, H7b, H8a, H8b	A4-H2	p
H9a, H9b, H10a, H10b	G8-H1'	p
H9a, H9b, H10a, H10b	G9-H1'	p
H9a, H9b, H10a, H10b	T14-H1'	p
H9a, H9b, H10a, H10b	T15-H1'	p

^aNOE classification: s = strong (1.8–2.8 Å), m = medium (1.8–3.5 Å), w = weak (1.8–5.0 Å), p = pseudo atom (1.8–7.0 Å).

Figure 4. Expansion of the aromatic to upfield region of a NOESY spectrum of the 1:1 complex of netropsin-diazene with d(CGCAAAGGC):d(GCCTTTTGCG) (in D₂O, 25°C, $\tau_{\text{mix}} = 200$ ms). Resonances assigned to the ligand *N,N*-dimethylethylene diamino protons and to the A4 H2 and A7 H2 protons are labelled. The presence of these two sets of crosspeaks between the ligand and DNA protons at either end of the A tract clearly indicates that the ligand is exchanging between two conformations.

Figure 5. Schematic representation of the model and exchange dynamics of the characterized netropsin-diazene–DNA complex. The square represents the bicyclic diazene on the netropsin-diazene molecule.

DISCUSSION

DNA cleavage

The most important feature of the netropsin-diazene:DNA complex is the placement of the bicyclic diazene unit in the minor groove near the H1', H4' and H5' protons of bases C3, A4, G18 and C19 in the major form. These protons are the likely targets for radical initiated cleavage reactions (27,38–40). The diazene is localized to a very short segment of the DNA by the binding properties of the netropsin section of the molecule. However, from this study there are insufficient data to characterize the precise orientation of the diazene moiety with respect to the DNA.

Figure 6. Stereo view of a molecular model of the 1:1 netropsin-diazene:DNA complex with d(CGCAAAAGGC):d(GCCTTTTGCG) obtained by energy minimization using semiquantitative distance restraints derived from NOESY data. For clarity, hydrogen atoms are omitted for the DNA but not for the ligand molecule.

The diazene moiety may not have a preferred orientation in the complex with respect to the DNA. This is supported by the complete lack of intermolecular NOESY crosspeaks from the diazene moiety to the DNA in the presence of intramolecular NOESY crosspeaks from the diazene to the netropsin moiety.

Recent studies have shown that photolysis of netropsin-diazene **3** in the presence of DNA leads to the formation of single stranded breaks in the phosphodiester backbone of the DNA (20). Based on the NMR derived model, the soft π -trimethylenemethane diyl (TMM) that is formed upon photolysis of the diazene is localized in the area near the backbone of the DNA. One possible cleavage mechanism involves the abstraction of a hydrogen atom from the sugar-phosphate backbone to give a DNA centered radical that can react further, ultimately leading to strand scission (26). Other diyl-dependent cleavage mechanisms are also accommodated by the minor groove binding and localization of the diazene. Thus far, it is unclear what mechanism the diradical generated from the netropsin-diazene uses to effect DNA strand cleavage, only that the cleavage is diyl-dependent. Work is in progress to characterize the cleavage chemistry (41).

Molecular design

The netropsin-diazene satisfies the first design criterion listed above by binding to the DNA duplex in the minor groove of the central AAAA tract in a 1:1 mode. The netropsin-diazene **3** retains the DNA binding sequence specificity of netropsin (**2**), fulfilling the second design criterion. Intermolecular dimerization of two netropsin-diazene molecules is prevented as a consequence of the 1:1 sequence selective binding mode, fulfilling the third design criteria. Diyl side reactions are prevented by the physical separation of diazenes from each other. Distamycin-like molecules form either 1:1 ligand:DNA or antiparallel side-by-side 2:1

ligand:DNA complexes. The diazene moiety of the bound netropsin-diazene is physically separated from other diazenes which are bound to the DNA or free in solution in these complexes. We observe only the 1:1 netropsin-diazene:DNA complex and there is no evidence to suggest that 2:1 binding exists in this system.

Complex stoichiometry and binding affinity

Netropsin-diazene **3** is complexed to the DNA in a 1:1 ratio at all stoichiometries tested, up to a 2:1 ligand DNA ratio. The DNA resonances broaden and shift upon addition of ligand and only one set of DNA resonances exists throughout the titration, indicating that the ligand is binding to the DNA in fast exchange on the NMR time scale (34,43). In contrast, the parent compounds distamycin A (**1**) and netropsin (**2**) have been shown to bind DNA in slow exchange on the NMR time scale, although reorientation on a symmetric sequence can be detected (34,43,45,46). The higher exchange rate of **3** suggests that its binding affinity is reduced relative to **1** and **2**. Differences in shape, charge distribution and/or hydrogen bonding could be responsible for the lowered DNA binding affinity of netropsin-diazene **3**.

Netropsin (**2**) is a di-cation while netropsin-diazene **3** is a mono-cation at physiological pH. The positively charged tail groups in these molecules contribute to the electrostatic attraction to the polyanionic DNA. The hydrophobic bicyclic diazene moiety has a pK_a of -1.4 and therefore remains uncharged at physiological pH (47). The bulky diazene is positioned in the minor groove in the area of the exocyclic amino group of the C3•G18 base pair, which may prevent the diazene from binding deeply into the groove. The substitution of an AT base pair at that position in the duplex may be more sterically favorable.

Netropsin-diazene **3** differs further from netropsin (**2**) and distamycin (**1**) in that the cationic tail consists of a dimethylammo-

nium group instead of an amidinium group (Fig. 1). P3 is a distamycin derivative in which an acetyl moiety is substituted for the formyl group and a dimethylammonium group is substituted for the amidinium group. P3 binds calf thymus DNA with a 28-fold lower affinity than distamycin (1) (48). It is likely that the lowered affinity of P3 for DNA is at least partially due to the dimethylammonium tail and suggests that part of the difference in the affinity of 3 and netropsin also comes from the nature of the tail.

The netropsin-diazene complex compares well with the complexes of distamycin (1) with AAAA:TTTT as well as with AATT:AATT, in which the major orientation of ligand has been observed with the formyl end of the distamycin lying towards the 5'-end of the A-tract and the minor form in which the orientation of the distamycin is flipped head to tail (33,43). Netropsin (2) shows little or no orientational preference in binding to the AAAA:TTTT site (34,37,44).

Future design directions

Distamycin analogs and lexitropsins have been modified to carry out DNA alkylation and cleavage (5,11–19). The TMM based DNA cleaving moiety adds new versatility to these molecules. The netropsin-diazene molecule demonstrates that TMM can be used to cleave DNA and the binding and placement of the diazene could be optimized by building more sophisticated systems. New sequence selective reagents based on the TMM could be generated by increasing the number of pyrrolicarboxamide and/or introducing imidazolecarboxamide units into the ligand, or by linking molecules with selective binding properties together (8).

CONCLUSION

We have characterized the DNA binding modes of a new rationally designed DNA cleaving agent based on trimethylene-methane (TMM) using the powerful tools of two-dimensional NMR spectroscopy. The molecule appears to have fulfilled all of the criteria used in its design. Netropsin-diazene 3 binds to the DNA duplex studied in the minor groove of the central AAAA tract in a 1:1 mode, preventing diyl dimerization and presumably preventing other side reactions from occurring. The data does not, however, allow us to describe the precise orientation of the TMM diyl with respect to the minor groove. Further studies on the binding properties and cleavage mechanism of netropsin-diazene 3 and related molecules will prove useful in the further development of this new class of DNA nucleases.

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