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Cleavage of Abasic Sites in DNA by Intercalator-amines

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Abstract—Anthraquinone and naphthalene diimide intercalators with amine-containing side chains cleave plasmid DNA at abasic sites (apurinic or apyrimidinic (AP) sites). The intercalator-amine is substantially more effective than the amine itself; many intercalators with diamine side chains cleave most of the abasic sites at micromolar concentration (30 min at 37°C). Intercalators with two amino moieties in the side chain are more efficient than those with one, arguing for a role for each of two amines in the cleavage mechanism. Side chains ending in tertiary amines are somewhat more effective than those ending in primary amines, indicating that imine formation is not required for cleavage at the abasic site. We also report a systematic study of abasic site cleavage by polyamines, including piperidine, spermine, spermidine and 12 other di-, tri- and tetra-amines. For polyamines as well as intercalator-amines, examples with three carbon atoms between neighboring nitrogens atoms cleave most efficiently. This may reflect a particularly favorable geometry for proton abstraction for these species. The effect of nitrogen–nitrogen spacing on the pK_a values of the nitrogens may contribute as well. Overall, cleavage of plasmid DNA at adventitious abasic sites by intercalator-amines bearing two nitrogens in a single side chain occurs readily. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

The binding of intercalators to DNA continues to be studied in a variety of contexts.[1] Complex structures built out of intercalators linked to other moieties are of interest in terms of, inter alia, molecules which interact with DNA via both intercalation and groove interactions,[1,2] molecules which cleave DNA via either photochemical[3,4] or oxidative[5–9] pathways, and electron transfer in DNA scaffolding.[3,4] These complex intercalators are generally built from modular building blocks. The DNA binding properties of the building blocks themselves are therefore of interest as one designs new species which interact with DNA.

Recent studies from a number of laboratories have focused on both anthraquinones[10–24] and naphthalene diimides[25–33] as having desirable DNA intercalation and photophysical activities. In our studies of anthraquinones and naphthalene diimides bearing amine side chains with plasmids, we observed single strand cleavage of the plasmid which could not be attributed to photochemical or oxidative mechanisms. Further experiments in our laboratory established that this cleavage resulted from nicking of the plasmid at adventitous abasic sites, e.g. apurinic or apyrimidinic (AP) sites in which the *N*-glycosidic bond that connects the nucleic base to the deoxyribose moiety is broken.[34–36] Abasic sites may have significant biological and medical consequences.[36]

The glycoside in an abasic site exists in equilibrium between the ring-closed form and approximately 1% of the ring-opened aldehydic form.[37] Cleavage occurs by abstraction of a proton α to the aldehyde, followed by β-elimination of the 3′-phosphate. A further δ-elimination may occur, resulting in loss of the sugar from the 5′-phosphate as well.[36,38,39] Amines catalyze this reaction. A number of research groups have studied selected amines,[39–46] but there has been no comprehensive study of the features of the amine which result in efficient cleavage. It does appear that diamines are more effective than monoamines.[39,47] This is presumably because a cationic species is necessary for DNA binding, but a species with a free nucleophile is necessary for abstraction of the proton α to the aldehyde. The diamine can combine both of these features in a single molecule.

Because cleavage by intercalator-amines appeared to occur readily, we have performed a systematic survey of the properties of the amines which lead to efficient cleavage. Herein we report that both anthraquinone and naphthalene diimide intercalators bearing diamine side chain ‘linkers’ (Fig. 1) cleave plasmid DNA at abasic

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Key words: Abasic; plasmid; anthraquinone; naphthalene diimide; polyamine.

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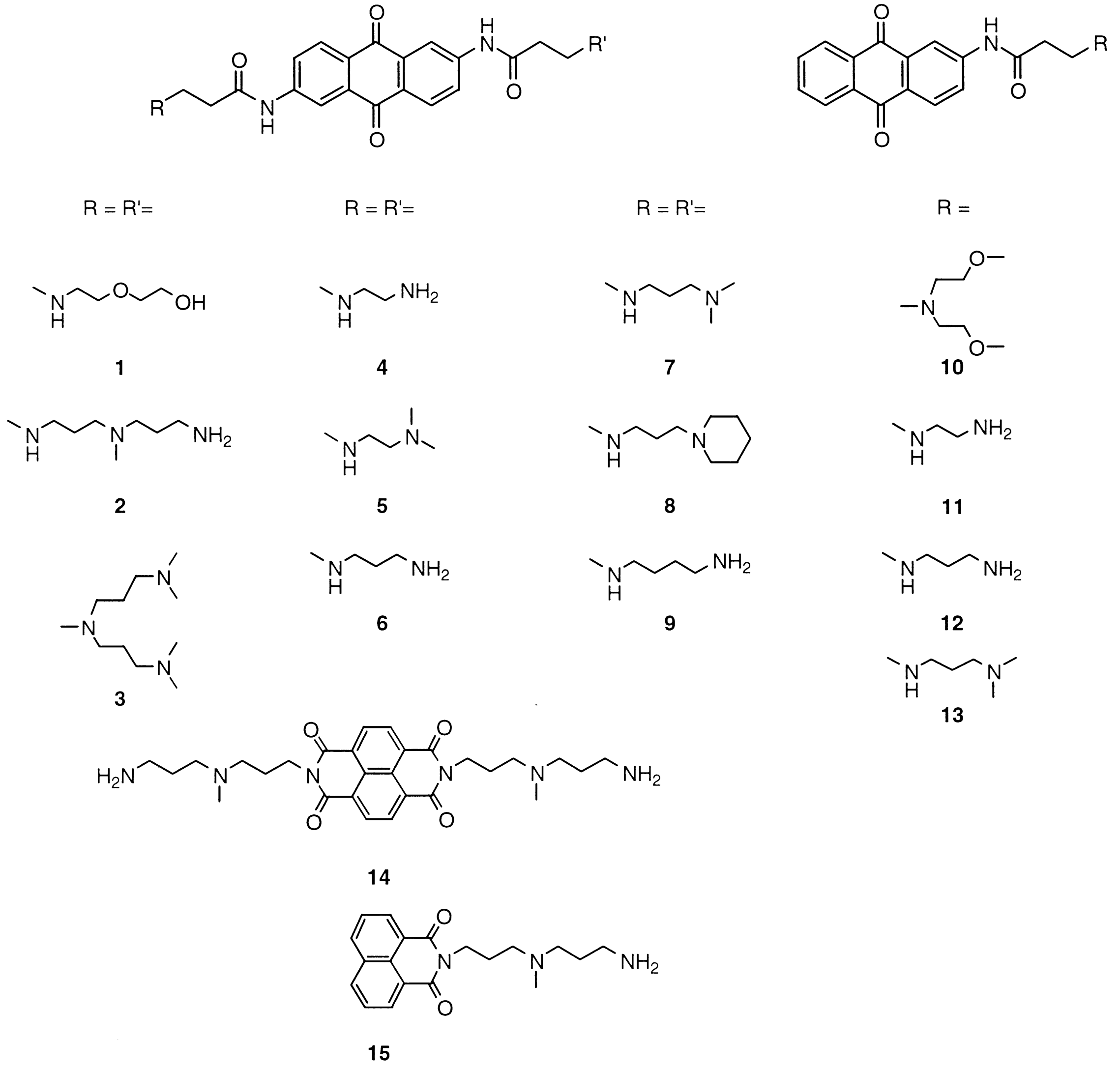

Figure 1. Intercalator-amines used for abasic site cleavage.

sites. The structure of the side chain controls the cleavage efficiency of the molecule. Examples of intercalator-amines containing side chains with one, two and three nitrogens have been synthesized. For the side chains with two nitrogens, the 1,2- 1,3- and 1,4-diamino derivatives have been synthesized. We also report a systematic study of the cleavage of DNA at abasic sites by polyamines themselves. Cleavage of DNA by amine-containing molecules attributed to photochemical or oxidative pathways should be evaluated in light of the fact that plasmid preparations can have adventitious abasic sites.

Results and Discussion

Cleavage occurs at abasic sites in the plasmid

Single-stranded cleavage of plasmids can be due to a variety of mechanisms, including oxidative, photochemical, hydrolytic and reaction at defects in the duplex structure. To establish that cleavage of the plasmids in these studies was at abasic sites, rather than via any other mechanisms, the cleavage activities for selected examples were compared with that of endonuclease IV, an enzyme that recognizes and cleaves AP-sites.[48–51] Five samples with increasing amounts of abasic sites were prepared by heating pUC19 under acidic conditions.[43] The average number of abasic sites per plasmid in each sample was quantified by cleaving the abasic sites with endonuclease IV; the extent of cleavage was calculated using a Poisson distribution.[52] The samples used in most of this work were those heated for 0, 2, 4, 6 and 10 min; these had average numbers of abasic sites of 0.28, 0.44, 0.61, 0.77 and 1.04 per plasmid and are referred to as pUC19 < 0.28 >, pUC19 < 0.44 >, pUC19 < 0.61 >, pUC19 < 0.77 > and pUC19 < 1.04 >, respectively.

Selected pUC19 samples were treated with a variety of intercalator-amines. In no instance was more cleavage observed for the intercalator-amine than for endonuclease IV. In a number of instances (e.g. compounds **6** and **7** in Table 2), cleavage was 90–100%, consistent with nicking of the plasmid DNA only at abasic sites. Figure 2 shows a gel comparing cleavage by **14** and endonuclease IV for plasmid preparations containing increasing amounts of abasic sites. Cleavage to give the nicked plasmid (Form II) clearly increases as the number of abasic sites per plasmid increases. The gel shifts observed on the addition of **14** indicates that this is bound to the DNA, altering its mobility. Figure 3 compares cleavage by 10 μM **14** and 50 μM spermine (conditions designed for partial cleavage of abasic sites) with cleavage by endonuclease IV. The cleavage by both naphthalene diimide **14** and spermine is a function of the number of abasic sites in the plasmid. Finally, naphthalene diimide **14** (10 μM) and spermine (50 μM) were incubated with pBR322 that did not have abasic sites. No cleavage was observed after the plasmid was incubated with either compound for 2 h at 37°C (4 times as long as the customary incubation period).

Reactions were run in a stainless steel incubator to ensure that the cleavage was not photochemical in origin. Control reactions with EDTA indicated that the cleavage was not due to oxidative reactions catalyzed by metals (most reactions were run with EDTA to ensure that oxidative mechanisms were not operative).

Cleavage of abasic sites by amines

Many DNA binding molecules under current study contain amine and polyamine units. In general, DNA binding molecules are studied at micromolar concentrations. To understand the possible role of the amine and polyamine side chains in catalyzing cleavage of abasic sites, we performed a systematic study of the cleavage of plasmids with amines at micromolar concentrations. Our work complements and extends earlier studies,[39–46] which were in general performed at millimolar or greater concentrations of amine. A variety of amines, including mono-, di- tri- and tetraamines, were evaluated with respect to their ability to cleave DNA (Table 1). Amines were tested by incubating with pUC19 < 1.04 > (i.e. pUC19 containing an average of 1.04 abasic sites per plasmid) for 30 min at 37°C at 50 μM [amine]. No evidence of plasmid precipitation was seen for any of the amine cleavage reactions.

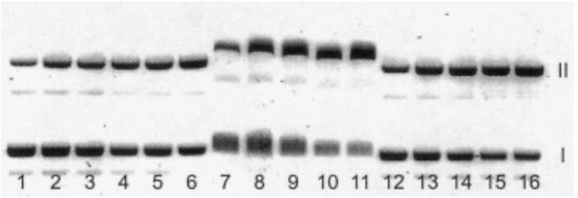

Figure 2. Cleavage of pUC19 by naphthalene diimide **14** and by endonuclease IV as a function of the number of abasic sites per plasmid. Lanes 1–5, 7–11 and 12–16 contained 0.28, 0.44, 0.61, 0.77 and 1.04 abasic sites, respectively. pUC19 was incubated at 37°C for 30 min with the following additions: lanes 1–5, none; lanes 7–11, 10 μM of **14**; lanes 12–16, endonuclease IV (1 μg/mL). In lane 6, pUC16 < 0.44 > was treated with 1 mM $NH_2(CH_2)_3NMe(CH_2)_3NH_2$.

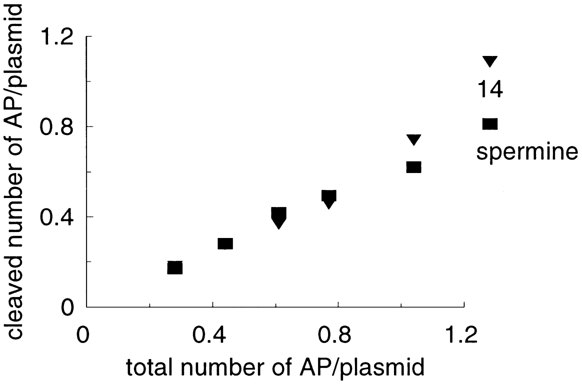

Figure 3. pUC19 Cleavage by naphthalene diimide **14** (10 μM) and spermine (50 μM) as a function of the number of abasic sites per plasmid (determined by endonuclease IV cleavage).

Monoamines, typified by piperidine, showed essentially no cleavage at 50 μM [amine]. In experiments at 20-fold higher (1 mM) concentration, monoamines did cleave abasic sites, consistent with previous literature findings.[39,40]

Most polyamines cleave abasic plasmids at 50 μM [amine]. We observed that the extent of cleavage is a function of the distance between the nitrogen atoms in the polyamine; compounds with three carbon atoms between the nitrogens cleave more readily than those with other spacings between the nitrogens. This was observed for diamines, triamines and tetraamines, each of which is discussed individually below.

Two diamines, $NH_2(CH_2)_2NH_2$ and $Me_2N(CH_2)_3NH_2$, were evaluated. It was found that the diamine with two carbons between adjacent nitrogen atoms ($NH_2(CH_2)_2NH_2$) cleaved only 4% of the available abasic sites, far less than the diamine with three carbons between adjacent nitrogen atoms, $Me_2N(CH_2)_3NH_2$, which cleaved 33% of the available abasic sites (Table 1).

Triamines with two carbon atoms between each of the adjacent nitrogen pairs showed little or no cleavage (0–12%) at 50 μM [amine]. However, triamines with three-carbon atoms between each of the adjacent nitrogen pairs generally cleaved more than half of the available abasic sites (57–69%). The triamine with one two- and one three-carbon atom spacing, $NH_2(CH_2)_2NH(CH_2)_3NH_2$, was midway between the two- and three-carbon atom spacing examples in cleavage ability (27%). Spermidine, $NH_2(CH_2)_3NH(CH_2)_4NH_2$, with one three- and one four-carbon atom linker, cleaved 19% of available AP sites, similar to the two-, three-linker species and less than the three-, three-linker species. Triamines with three carbons between adjacent nitrogens were also tested at different amine concentrations; the relative orders of activity were the same at each concentration (data not shown). One cyclic triamine (1,5,9-triazacyclodecane), with three-carbon linkers between each of the nitrogens, was tested. It cleaved 45% of the available abasic sites, which is lower than linear triamines with three-carbon linkers (57–69%) but higher than linear triamines with other linker patterns (0–27%).

Two tetraamines were evaluated, spermine ($NH_2(CH_2)_3NH(CH_2)_4(CH_2)_3NH_2$) and the branched $N((CH_2)_2$

Table 1. Cleavage of pUC19 < 1.04 > plasmid by amines

Amine (50 μM)	Number of N	Number of C between N	Nicks/plasmid	%[a] cleaved
$N((CH_2)_2NH_2)_3$	4	2, 2, 2	0.12	12
$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	4	3, 4, 3	0.62	60
$Me_2N(CH_2)_2NH(CH_2)_2NH_2$	3	2, 2	0.12	12
$NH_2(CH_2)_2NH(CH_2)_2NH_2$	3	2, 2	0.01	1
$MeNH(CH_2)_2NMe(CH_2)_2NHMe$	3	2, 2	0.06	6
$Me_2N(CH_2)_2NMe(CH_2)_2NMe_2$	3	2, 2	0.00	0
$NH_2(CH_2)_2NH(CH_2)_3NH_2$	3	2, 3	0.28	27
$NH_2(CH_2)_3NMe(CH_2)_3NH_2$	3	3, 3	0.72	69
$NH_2(CH_2)_3NH(CH_2)_3NH_2$	3	3, 3	0.63	61
$Me_2N(CH_2)_3NH(CH_2)_3NMe_2$	3	3, 3	0.59	57
1,5,9-triazacyclodecane	3	3, 3, 3	0.47	45
$NH_2(CH_2)_3NH(CH_2)_4NH_2$	3	3, 4	0.20	19
$NH_2(CH_2)_2NH_2$	2	2	0.04	4
$Me_2N(CH_2)_3NH_2$	2	3	0.34	33
Piperidine	1	—	0.00	0

[a] % of cleavage compared to endonuclease IV.

$NH_2)_3$; these gave 60 and 12% cleavage, respectively. Overall, there is a consistent pattern that polyamines with three carbon atoms between each of the adjacent nitrogen pairs are the most efficient in cleaving abasic sites (discussed in more detail below). Polyamines such as spermine and spermidine are used in DNA packaging. We observe that both cleave abasic sites efficiently; this was also seen at 1 mM concentrations by Male et al.[44] The ability of spermine and spermidine to cleave abasic sites in DNA at low concentrations may have biological significance.[44,45]

Cleavage of abasic sites by intercalator-amines

Anthraquinone and naphthalene diimide intercalators containing diamine and triamine side chains are able to cleave abasic plasmid DNA efficiently at micromolar concentrations. Figure 1 shows the intercalator-amines synthesized for this work. Compounds **1**–**9** and **14** have two side chains extending off opposite sides of the molecule; these are expected to adopt a threading intercalation geometry in DNA with one side chain each in the major and minor groove.[11,26] Compounds **10**–**13** and **15** have only one side chain. Anthraquinones with mono-, di- and triamine side chains were synthesized. For the polyamine side chains, the number of carbon atoms between the nitrogen atoms was varied. Cleavage reactions were done with five samples of pUC19 containing different average numbers of abasic sites per plasmid, < AP/plasmid > : pUC19 < 0.28 > , pUC19 < 0.44 > , pUC19 < 0.61 > , pUC19 < 0.77 > and pUC19 < 1.04 > .

Initial studies were performed to determine the concentrations of intercalator-amines necessary for cleavage using pUC19 < 0.44 > . The cleavage activity of intercalator-amines **6**, **8** and **14** was evaluated at 0.1, 1.0 and 10 μM intercalator (Fig. 4). Cleavage increased as the concentration of the intercalator increased. All tested compounds cleaved less than 2% at 0.1 μM. At 1 μM, these compounds cleaved 16–25% of the abasic sites. At 10 μM, **6**, **8** and **14** cleaved 66, 55 and 64%, respectively, of the abasic sites of pUC19 < 0.44 > . The cleavage efficiency observed herein is approximately equal to that observed for Lys-Trp-Lys,[41–43] 9-amino-ellipticene[53,54] and acridine–amine conjugates.[55]

To evaluate the fundamental aspects of the side chain necessary for cleavage, side chains with one, two and three amines were synthesized. Intercalator-amines with one amino moiety in the side chain (e.g. **1** and **10**) did not cleave pUC19 < 0.44 > at 0.1–10 μM. Intercalator-amines with three amino moieties in the side chain (e.g. **2** and **3**) gave 13 and 8% cleavage, respectively, at 1 μM, but partial precipitation of plasmid was observed. At 10 μM, **3** precipitated the plasmid completely; **2** showed 30% cleavage but with significant precipitation. Most of our studies were concentrated on intercalator-amines containing two amino moieties in each side chain, discussed in detail in the sections which follow. These systems combine maximum cleavage activity with minimum precipitation. Results of cleavage of AP DNA by anthraquinone-amines and by naphthalene diimide-amines containing two amino moieties in each side chain are summarized in Tables 2 and 3, respectively.

Precipitation of the plasmid by the anthraquinone-amines

Tetraamines are well known to aggregate DNA due to electrostatic interactions of the protonated amine with

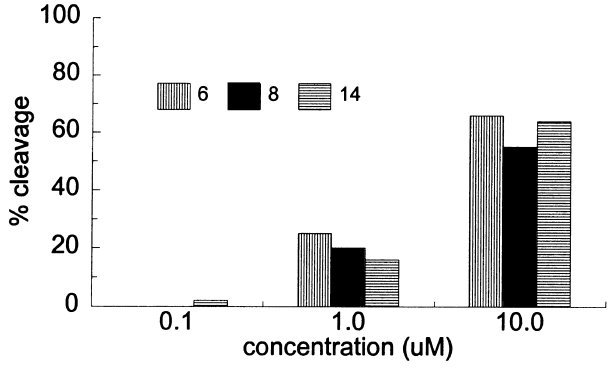

Figure 4. Cleavage of pUC19 < 0.44 > by intercalator-amines as a function of concentration.

Table 2. Anthraquinone-amines (side chain only shown)

Side chains		Nicks/plasmid	% cleaved[a]	AP/plasmid	(intercalator) (μM)
Bis-substituted					
$NHCH_2CH_2NH_2$	**4**	0.16, 0.20, 0.26	21, 26, 34	0.77	4, 6, 8
$NHCH_2CH_2NH_2$	**4**	0.36	35	1.04	10
$NHCH_2CH_2NMe_2$	**5**	0.34	77	0.44	10
$NHCH_2CH_2NMe_2$	**5**	0.29, 0.43, 0.48	38, 56, 62	0.77	4, 6, 8
$NHCH_2CH_2NMe_2$	**5**	0.70	67	1.04	10
$NHCH_2CH_2CH_2NH_2$	**6**	0.11, 0.29[b]	25, 66	0.44	1, 10
$NHCH_2CH_2CH_2NH_2$	**6**	0.47, 0.76[b]	61, 99	0.77	4, 6
$NHCH_2CH_2CH_2NH_2$	**6**	0.95[b]	91	1.04	10
$NHCH_2CH_2CH_2NMe_2$	**7**	0.40[b]	91	0.44	10
$NHCH_2CH_2CH_2NMe_2$	**7**	0.57, 0.67, 0.56	74, 87, 73	0.77	4, 6, 8
$NHCH_2CH_2CH_2NMe_2$	**7**	1.04	100	1.04	10
$NHCH_2CH_2CH_2N(CH_2)_5$	**8**	0.09, 0.24	20, 55	0.44	1, 10
$NHCH_2CH_2CH_2CH_2NH_2$	**9**	0.33[b]	75	0.44	10
$NHCH_2CH_2CH_2CH_2NH_2$	**9**	0.42[b], 0.43[b], 0.44[b]	55, 56, 57	0.77	4, 6, 8
$NHCH_2CH_2CH_2CH_2NH_2$	**9**	0.41[b]	39	1.04	10
Mono-substituted					
$NHCH_2CH_2NH_2$	**11**	0.18	41	0.44	10
$NHCH_2CH_2NH_2$	**11**	0.21	27	0.77	4
$NHCH_2CH_2CH_2NH_2$	**12**	0.30	68	0.44	10
$NHCH_2CH_2CH_2NH_2$	**12**	0.56	73	0.77	4
$NHCH_2CH_2CH_2NMe_2$	**13**	0.36	82	0.44	10
$NHCH_2CH_2CH_2NMe_2$	**13**	0.57	74	0.77	4

[a] % cleavage compared to endonuclease IV, i.e. (nicks/plasmid)/(AP/plasmid).
[b] Partial precipitation of the plasmid observed.

Table 3. Naphthalene diimide-amines (side chain only shown)

Side chains		Nicks/ plasmid	% cleaved[a]	AP/ plasmid	(intercalator) (μM)
Bis-substituted					
$CH_2CH_2CH_2NH_2$	**14**	0.18	64	0.28	10
$CH_2CH_2CH_2NH_2$	**14**	0.07, 0.28	16, 64	0.44	1, 10
$CH_2CH_2CH_2NH_2$	**14**	0.37	61	0.61	10
$CH_2CH_2CH_2NH_2$	**14**	0.46	60	0.77	10
$CH_2CH_2CH_2NH_2$	**14**	0.74	71	1.04	10
Mono-substituted					
$CH_2CH_2CH_2NH_2$	**15**	0.22	50	0.44	10
$CH_2CH_2CH_2NH_2$	**15**	0.68	65	1.04	10

[a] % cleavage compared to endonuclease IV.

the phosphate backbone.[56] Some of the structures in this work are expected to be at least tetraprotonated under the experimental conditions. For example, the two bis-substituted anthraquinones with triamine side chains **2** and **3** (overall hexaamines) should be at least tetraprotonated at pH 7.5 (the pH of the experiments). The parent amine for the side chain, $H_2NCH_2CH_2CH_2NHCH_2CH_2CH_2NH_2$, has p$K_a$ values of 10.7, 9.6 and 7.7 at $\mu = 0.1$ M).[57] In line with the expectation that highly charged derivatives will precipitate the DNA, **2** and **3** precipitated most of the plasmid at 10 μM; significant precipitation was seen even at 1 μM (data not shown).

Precipitation also consistently occurred for **9**, with a $H_2NCH_2CH_2CH_2CH_2NH_2$-based side chain. 1,4-Butanediamine has pK_a values of 10.7 and 9.5 ($\mu = 0.1$ M) and **9** is also expected to be a tetracation under the conditions of the experiment. Compounds with $H_2NCH_2CH_2CH_2CH_2NH_2$-based side chains showed some precipitation above 4 μM anthraquinone ($H_2NCH_2CH_2CH_2NH_2$ has pK_a values of 10.5 and 8.8, $\mu = 0.1$ M) The $H_2NCH_2CH_2CH_2NMe_2$-based structures (side chain pK_a values of 10.2 and 8.2, $\mu = 0.1$ M) precipitate less, although slight precipitation was observed for **7** at 10 μM. The greater aggregation of the structure ending in a primary amine may be simply a pK_a effect or may be due also to hydrogen bonding of the primary amine with the DNA. If the compounds selectively precipitate either the intact supercoiled or nicked form of the plasmid, quantification of the bands does not give an accurate picture of the extent of abasic cleavage. Thus, experiments in which precipitation was observed (indicated in the tables) should be interpreted with caution. Structures with side chains based on ethylenediamine derivatives did not precipitate the plasmid ($CH_3NHCH_2CH_2NH_2$ has pK_a values of 10.1 and 7.2, $\mu = 0.1$ M). Morier-Teissier et al. have also noted DNA condensation on treatment of plasmids with anthraquinones bearing amine side chains.[58]

The intercalator aids abasic site cleavage

To determine the extent to which the intercalator aids the cleavage, sets of experiments were run in which the intercalator with two amino moieties in each side chain and the corresponding diamines were tested for cleavage activity. The anthraquinone itself without a diamine, e.g. **1** and **10** (monoamine side chains), did not give significant cleavage at 10 μM. The intercalator-amines (diamine side chains) were substantially more effective than the corresponding diamines. Experiments were conducted with pUC19 < 0.44 > at 10 μM concentration. The intercalator-amines containing two amino moieties in each side chain cleave 41–91% under these conditions (Tables 2 and 3). The amines evaluated were

$NH_2(CH_2)_2NH_2$ (corresponding to **4** and **11**), $Me_2N(CH_2)_2NH_2$ (corresponding to **5**), $MeNH(CH_2)_3NH_2$ (corresponding to **6**, **12, 14** and **15**) and $Me_2N(CH_2)_3NH_2$ (corresponding to **7** and **13**). In each case, no cleavage of abasic sites was observed for the amine itself at 10 μM, indicating that the anthraquinone plays a significant role in directing the cleavage (10 μM data for amines not shown). Lhomme and colleagues have found similar effects in acridine–amine systems;[59] they also have found that efficiency can be further enhanced 100- to 1000-fold by appending an adenine to the intercalator-amine species to target the abasic site specifically.[55]

Only one side chain is needed for cleavage

The 2,6-bis-substituted anthraquinones are threading intercalators, with one side chain each in the major and minor groove of DNA.[10,11] They were compared to anthraquinones with a single side chain. The mono- and bis-substituted anthraquinones are expected to bind with similar association constants to the DNA.[11] In each case, the 2,6-bis-substituted compound (**4**, **6** and **7**) cleaves with similar efficiency to its 'mono-substituted' analogue (**11**, **12** and **13**, respectively) at equimolar concentrations of intercalator (Table 2). This indicates that only one side chain is required for cleavage.

Cleavage is most efficient with a three carbon linker between the nitrogens

The role of the amines in the sidechain is twofold: to enhance DNA binding by electrostatic attraction between the protonated amine and the phosphate backbone of DNA and to remove the proton α to the carbonyl in the elimination of phosphate (unprotonated amine). To evaluate the role of nitrogen–nitrogen spacing in the same side chain on cleavage, compounds with two, three and four carbons between the nitrogens were compared. In the mono-substituted anthraquinone series, three compounds were synthesized: **11** with two carbon atoms between adjacent nitrogens, and **12** and **13** with three carbon atoms between adjacent nitrogens. For pUC19 < 0.77 > with 4 μM anthraquinone, cleavages of 27, 73 and 74% were observed for **11**, **12** and **13**, respectively. Data for pUC19 < 0.44 > with 10 μM intercalator showed a similar pattern (Table 2), with the compounds with three carbon atoms between adjacent nitrogens cleaving more efficiently than those with two carbon atoms between adjacent nitrogens.

In the bis-substituted anthraquinone series, compounds with two-carbon (**4** and **5**), three-carbon (**6, 7** and **8**) and four-carbon linkers (**9**) were studied. Experiments were run under a number of different conditions. Figure 5 shows the general pattern of cleavage. Under most conditions, examples with three carbon linkers cleave more efficiently than those with two- or four-carbon linkers. Most effective cleavage with a three-carbon linker was also observed for the polyamines themselves (above). The observation that cleavage in the series spermidine > N^8-acetylspermidine > N^1-acetylspermidine occurs in the order shown[45] is also consistent with the three-carbon spacing giving the most efficient cleavage. A previous study on acridine 1,2- and 1,3-amines also observed similar effects as a function of the nitrogen spacing.[55] The 1,3-nitrogen spacing may have the best stereochemical features for abstraction of the proton which leads to cleavage. However, the spacing of the nitrogens also governs their relative pK_a values: the further apart the nitrogens, the higher the pK_a of the second nitrogen. More highly protonated species bind better to the DNA. However, cleavage is presumably catalyzed by a neutral amine, which removes the proton α to the carbonyl group. The 1,3-amine spacing may give pK_a values which best balance these two competing phenomena.[55]

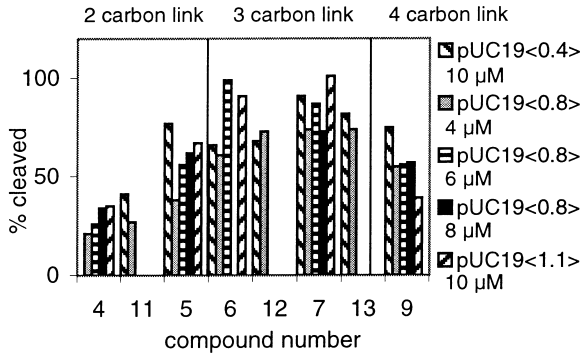

Figure 5. General pattern of abasic site cleavage by intercalator-amines showing that the three carbon link between two nitrogens in general gives more efficient cleavage than the two- or four-carbon link. Legend shows plasmids and intercalator-amine concentrations used in each experiment.

We also compared intercalators with one amino moiety in each of two side chains extending in opposite directions from the central core with intercalators bearing one side chain with two amines. Specifically, pUC19 < 0.44 > was incubated with 10 μM of bis-substituted **1** (one nitrogen in each side chain) and mono-substituted **11**, **12** and **13** (each bearing two amino groups in the same side chain). The mono-substituted diamines **11**, **12** and **13** cleaved 41, 68 and 82%, respectively, of the abasic sites (Table 2). However, bis-substituted diamine **1** with the two nitrogens in opposite side chains did not cleave the abasic sites under our conditions. This indicates that cleavage is substantially favored by placement of the two amino moieties in the same side chain.

Nitrogen substitution

Depending on the system, cleavage may occur either directly from the aldehydic form of the sugar or through an imine formed by condensation of the aldehyde with a primary amine.[36] In this study, anthraquinones with side chains ending in a tertiary amine cleave somewhat more efficiently than those ending in a primary amine: **5** is more efficient than **4** and **13** is more efficient than **12** under all conditions (Fig. 5). This indicates that formation of an imine intermediate with the primary amine is not required for cleavage with these species. It is still possible that an iminium intermediate is formed with the secondary nitrogen in the chain, although this would presumably be a less efficient catalytic pathway than imine formation.[60,61] In the polyamine series (no intercalator) triamines $NH_2(CH_2)_3NH(CH_2)_3NH_2$, $NH_2(CH_2)_3NMe(CH_2)_3NH_2$, and $Me_2N(CH_2)_3NH(CH_2)_3NMe_2$ all cleave to similar extents, 61, 69 and 57%, respectively. This also argues against a mandatory imine intermediate in the cleavage pathway.

Conclusions

This work shows that simple polyamines at low concentrations (50 μM) can cleave plasmid DNA at abasic sites. This concentration is significantly less than that used in previous studies of polyamines (mM or greater concentrations). The nitrogen substitution pattern (e.g. primary, secondary, tertiary) has little effect on the extent of cleavage, arguing against a mandatory imine intermediate in the cleavage pathway.

Anthraquinone and naphthalene diimide intercalators containing diamine and triamine side chains are able to cleave abasic plasmid DNA efficiently at micromolar concentrations. The intercalator-amine is substantially more effective than the amine itself. Cleavage requires two amines in a single side chain; anthraquinones with a single amine in each of two side chains on opposite sides of the molecule do not give abasic site cleavage. Side chains with tertiary amines are somewhat more effective than those with primary amines, indicating that imine formation is not required for cleavage of the abasic site.

For both amines and intercalator-amines, examples with three carbon atoms between neighboring nitrogens atoms cleave more efficiently than those with other spacings. This spacing may give the most favorable stereochemistry for proton abstraction. However, the relative pK_a values of the two nitrogens may also be important. Cleavage efficiency presumably involves a competition between better binding of more highly protonated species (more carbon atoms between adjacent nitrogens) and better cleavage with at least one neutral amine (fewer carbon atoms between adjacent nitrogens).

These observations have general significance for cleavage of plasmids with DNA binding species bearing polyamine side chains: plasmid cleavage with DNA-binders bearing attached polyamine moieties attributed to photochemical or oxidative pathways should be evaluated in light of the fact that plasmid preparations can have adventitious abasic sites. The intercalator-amines studied herein may be used as reagents to test the integrity of plasmid preparations. Taking into consideration the ease of synthesis, tendency to precipitate DNA and cleavage efficiency, compounds **5** and **13** are the reagents of choice.

Experimental

General

The plasmids pUC19 and pBR322 were purchased from Bayou Biolabs (Harahan, LA) and Pharmacia, respectively. Endonuclease IV was a gift from Dr. Yoke Kow.[48,62] NMR spectra were recorded on Varian Unity+ 300 and 500 MHz spectrophotometers. Mass spectra were obtained on a VG 70-SE spectrometer. Elemental analyses were done on a Perkin–Elmer Series II Analyzer.

Preparation of AP DNA

To produce abasic sites, pUC19 (500 μg/mL) in 10 mM sodium citrate buffer containing 0.1 M NaCl (pH 5.0) was heated at 70°C for various lengths of time.[43] The reaction was stopped by chilling rapidly on ice. The samples were dialyzed in sodium phosphate buffer, pH 7.5. The numbers of abasic sites were quantitated by cleaving these sites with endonuclease IV (6.0 μg/mL), 10 mM Tris buffer, pH 7.5, 1 mM EDTA, 50 mM NaCl, incubation for 30 min at 37°C. Reaction mixtures were analyzed by agarose gel electrophoresis as described below. The average numbers of abasic sites per plasmid were calculated from the extent of cleavage using the Poisson distribution.[52] The samples used in most of this work were those heated for 0, 2, 4, 6 and 10 min; these had average numbers of abasic sites of 0.28, 0.44, 0.61, 0.77 and 1.04 per plasmid and are referred to as pUC19 < 0.28 >, pUC19 < 0.44 >, pUC19 < 0.61 >, pUC19 < 0.77 > and pUC19 < 1.04 >, respectively. Note that even pUC19 which had not been heated under acidic conditions had approximately 0.28 abasic sites per plasmid. The pBR322 did not contain significant abasic sites, as shown by the endonuclease IV assay.

Cleavage of AP DNA

For the cleavage reactions, AP plasmid DNA (0.5 μg) was incubated in Eppendorf tubes in a stainless steel incubator with different amines and intercalator-amines for 30 min at 37°C in 10 mM sodium phosphate buffer, pH 7.5. Reaction mixtures were analyzed by 1% agarose gel, containing 0.5 μg/mL ethidium bromide in the gel and in the buffer. The electrophoresis was carried out in 1×TAE buffer, pH 8.0, for 2 h at 5 V/cm. The gel was scanned with a Molecular Dynamics FluorImager SI. Bands corresponding to supercoiled (Form I) and nicked (Form II) plasmid were quantitated with ImageQuaNT gel scanning software. The data were corrected for the decreased stainability of Form I DNA, assuming that the supercoiled plasmid was 1.22 times more fluorescent than the relaxed DNA at the concentrations of ethidium used.[52] The number of breaks was calculated assuming a Poisson distribution. Control reactions with 1 mM EDTA gave the same results as those without EDTA, indicating that the cleavage was not due to the presence of metals.

Syntheses

Starting reagents were from Aldrich or Fluka. The anthraquinone-amines with two side chains were prepared following the procedure of Neidle and coworkers[10] in which 2,6-bis(3-chloropropionamido)-anthracene-9,10-dione is treated with an excess of amine; displacement of chloride gives the desired product.

Anthraquinone examples with one side chain followed a similar procedure, but starting from the 2-(3-chloro-propion–amido)-anthracene-9,10-dione. Examples ending in primary amines were prepared from the mono-BOC-protected diamines. Representative procedures for the two compounds which are overall the most useful cleaving reagents, **5** (two side chains) and **13** (one side chain), are given first below, followed by chemical characterization of the rest of the compounds. Compound **8** has been described previously.[10] The syntheses of **1** and **3**[63] and the imides[64] will be reported elsewhere. Concentrations were measured using extinction coefficients of 26,000 M^{-1} cm^{-1} and 12,400 M^{-1} cm^{-1} for the naphthalene imides[25] and 9400 M^{-1} cm^{-1} for the anthraquinones.

2,6-(*N*,*N*-Dimethyl-1,2-diaminoethano-3-propionamido)-anthracene-9,10-dione (5). 2,6-Bis(3-chloropropionamido)anthracene-9,10-dione (0.33 g, 0.79 mmol) and *N*,*N*-dimethyl-1,2-diaminoethylamine (0.69 g, 7.9 mmol) were refluxed in absolute ethanol for 72 h. The reaction mixture was filtered hot and the residue was discarded. The filtrate was removed under reduced pressure. Ethyl ether and petroleum ether were then added to the crude product and allowed to cool at 4°C. A precipitate developed; this was filtered and the residue washed with ether. The product (0.27 g) was obtained as yellow–orange powder in 65% yield. ^{1}H NMR (300 MHz, D_2O) δ 2.67 (t, $J=6.8$ Hz, 4H, CH_2CO), 2.80 (s, 12H, N(CH_3)$_2$), 3.06 (t, $J=6.8$ Hz, 8H, NH$CH_2$$\mathrm{CH_2}$CO and NH$CH_2$$\mathrm{CH_2CH_2}$), 3.20 (t, $J=6.8$ Hz, 4H, CH_2N ($\mathrm{CH_3}$)$_2$), 7.64 (d, $J=8.4$ Hz, *ArH*, 2H), 7.78 (d, $J=8.4$ Hz, *ArH*, 2H), 7.89 (s, *ArH*, 2H). HRMS $(M+H)^+$ calcd for $C_{28}H_{39}N_6O_4$ 523.3032, found 523.3007. Anal. calcd for $C_{28}H_{38}N_6O_4{\cdot}3H_2O{\cdot}HCl$: C, 51.85; H, 6.99. Found: C, 51.96; H, 6.72.

2-(3-Chloropropionamido)anthracene-9,10-dione. 2-Aminoanthraquinone (2.86 g, 12.8 mmol) and 3-chloropropionyl chloride (24.4 g, 192 mmol) were refluxed neat for 12 h. The mixture was cooled to 5°C, filtered and washed with ether (6×15 mL) and 1,4-dioxane (3×15 mL).

Recrystallization was carried out from DMF:EtOH (4:1, v/v) to give 2.16 g (54% yield) of a yellow-brown powder. ^{1}H NMR (DMSO-d_6) δ 2.93 (t, $J=6.3$ Hz, 2H, COCH_2), 3.93 (t, $J=6.3$ Hz, 2H, CH_2Cl), 7.90–8.20 (m, 6H, *ArH*), 8.48 (br s, 1H, *ArH*), 10.73 (s, 1H, *NH*CO). Anal. calcd for $C_{17}H_{12}NO_3Cl$: C, 65.08; H, 3.86; N, 4.46. Found: C, 65.08; H, 3.74; N, 4.61.

2-(*N*,*N*-Dimethyl-1,3-diaminopropano-3-propionamido)-anthracene-9,10-dione (13). 2-(3-Chloropropionamido)-anthracene-9,10-dione (0.198 g, 0.630 mmol) and *N*,*N*-dimethyl-1,3-diaminopropylamine (0.51 g, 5.0 mmol) were refluxed in absolute ethanol for 72 h. The reaction mixture was filtered hot and the residue was discarded. The filtrate was removed under reduced pressure. Ethyl ether was added to the crude product and allowed to cool at 4°C. A precipitate developed; this was filtered and the residue washed with ether. The product (0.15 g) was obtained as an orange–brown powder in 63% yield. ^{1}H NMR (300 MHz, D_2O) δ 2.17 (m, 2H, $\mathrm{CH_2}$ $CH_2$$\mathrm{CH_2}$), 2.83 (t, $J=6.3$ Hz, 2H, CH_2CO), 2.91 (s, 6H, N(CH_3)$_2$], 3.15 (t, $J=7.8$ Hz, 2H, CH_2N), 3.26 (t, $J=$ 7.8 Hz, 2H, CH_2N), 3.33 (t, $J=6.3$ Hz, 2H, $\mathrm{COCH_2}$ CH_2N), 7.34 (m, 2H, *ArH*), 7.52–7.87 (m, 5 H, *ArH*). HRMS calcd for $C_{22}H_{25}N_3O_3$ 379.1896, found 379.1889. Anal. calcd for $C_{22}H_{25}N_3O_3{\cdot}H_2O{\cdot}2HCl$: C, 56.18; H, 6.21; N, 8.93. Found: C, 55.94; H, 6.60; N, 8.96.

2,6-Bis(3,3-diamino-*N*-methyldipropanylamino-3-propionamido)anthracene-9,10-dione (2). The BOC-protected product was prepared on a 0.23 g scale (72%) by refluxing 2,6-bis(3-chloropropionamido)anthracene-9,10-dione (0.16 g, 0.38 mmol) and *N*-BOC-3,3-diamino-*N*-methyl-dipropyl-amine[65,66] (0.46 g, 1.9 mmol) for 24 h following the procedure described above. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.36 (s, 18H, (CH_3)$_3$ CO), 1.46–1.80 (m, 8H, $\mathrm{CH_2}$$CH_2$$\mathrm{CH_2}$), 2.19 (s, 6H, N$CH_3$), 2.37 (m, 8H, CH_2), 2.48–2.82 (m + DMSO-d_6, CH_2), 2.79 (m, 8H, NHCH_2), 2.94 (dt, $J=6.0$ Hz, 4H, BOCNHCH_2), 6.75 (s, br, 2H, *NH*BOC), 7.64 (d, $J=8.7$ Hz, 2H, *ArH*), 7.69 (d, $J=8.7$ Hz, 2H, *ArH*), 7.81 (s, 2H, *ArH*), 10.56 (2H, NHCO).

Trifluoroacetic acid was added to the protected amine until all the solid dissolved. The trifluoroacetic acid was evaporated under reduced pressure. The deprotected product **2** was put into $CHCl_3$:H_2O (1:1 v/v) and the water layer separated. The water layer was evaporated under reduced pressure to give the product (90%) as an orange powder. ^{1}H NMR (300 MHz, D_2O) δ 2.2 (m, 4H, $\mathrm{CH_2}$$CH_2$$\mathrm{CH_2}$), 2.4 (m, 4H, $\mathrm{CH_2}$$CH_2$$\mathrm{CH_2}$), 3.01 (s, 6H, N$CH_3$), 3.05–3.18 (m, 12H, CH_2CO and CH_2 $\mathrm{NCH_3}$), 3.52 (m, 4H, CH_2NH), 3.72 (br, 8H, $\mathrm{NH_2}$$CH_2$ and CH_2NH), 7.55 (d, $J=8.5$ Hz, 2H, *ArH*), 7.72 (d, $J=8.5$ Hz, 2H, *ArH*), 7.93 (s, 2H, *ArH*). MS (FAB, thioglycerol) $(M+H)^+$ 637.5, 581.1 ($((M+H)^+ + H_2)$-C_3H_8N), 509.3 ($(M+H_2)^+$-$C_7H_{17}N_2$), 492.2 ($(M+H)^+$-$C_7H_{19}N_3$).

2,6-Bis(1,2-diaminoethano-3-propionamido)anthracene-9,10-dione (4). The BOC-protected product was prepared on a 0.47 g scale (80% yield) by refluxing 2,6-bis(3-chloro-propionamido)-anthracene-9,10-dione and *N*-BOC-1,2-diaminoethane for 72 h. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.36 [s, 18H, (CH_3)$_3$CO), 2.56 (t, $J=6.4$ Hz, 4H, NHCH_2), 2.82 (t, $J=6.4$ Hz, 4H, NHCH_2), 3.01 (dt, $J=6.4$ Hz, 4H, BOCNHCH_2), 6.75 (br s, 2H, *NH*BOC), 8.07 (d, $J=8.8$ Hz, 2H, *ArH*), 8.17 (d, $J=8.8$ Hz, 2H, *ArH*), 8.46 (s, 2H, *ArH*), 10.73 (2H, *NH*CO); the COCH_2 resonance is presumably under a solvent peak. Anal. calcd for $C_{34}H_{46}N_6O_8$: C, 61.25; H, 6.95; N, 12.60. Found: C, 61.27; H, 7.08; N, 12.49.

Deprotection with trifluoroacetic acid gave **4** in 90% yield as a yellow powder. ^{1}H NMR (300 MHz, D_2O) δ 2.90 (t, $J=5.4$ Hz, 4H, COCH_2), 3.41 (m, 12H, $\mathrm{NH_2}$$CH_2$), 7.62 (d, $J=8.1$ Hz, 2H, *ArH*), 7.79 (d, $J=8.1$ Hz, 2H, *ArH*), 7.96 (s, 2H, *ArH*). HRMS $(M+H)^+$ calcd for $C_{24}H_{31}N_6O_4$ 467.2407, found 467.2435.

2,6-Bis(1,3-diaminopropano-3-propionamido)anthracene-9,10-dione (6). This was prepared as described for **2**. The initial condensation with *N*-BOC-1,3-diaminopropane was run for 18 h (0.27 g scale, 75% yield). ^{1}H

NMR (500 MHz, DMSO-d_6) δ 1.36 (s, 18H, $(CH_3)_3$CO), 1.51 (quintet, J = 6.8 Hz, 4H, $CH_2CH_2CH_2$), 2.80 (t, J = 6.4 Hz, 4H, $COCH_2CH_2NH$), 2.95 (t, J = 6.8 Hz, 4H, CH_2NHBOC), 6.80 (s, 2H, NHBOC), 8.06 (d, J = 8.8 Hz, 2H, ArH), 8.16 (d, J = 8.8 Hz, 2H, ArH), 8.45 (s, 2H, ArH), 10.73 (2H, NHCO); the remaining $COCH_2$ and $NHCH_2$ resonances are presumably under solvent peaks. Anal. calcd for $C_{36}H_{50}N_6O_8$: C, 62.23; H, 7.25; N, 12.09. Found: C, 61.88; H, 7.20; N, 11.74.

Deprotection with trifluoroacetic acid gave **6** in 92% yield as an orange powder. ^{1}H NMR (500 MHz, D_2O) δ 2.18 (quintet, J = 7.8 Hz, 4H, $CH_2CH_2CH_2$), 2.99 (t, J = 6.4 Hz, 4H, $COCH_2$), 3.17 (t, J = 7.8 Hz, 4H, NH_2CH_2), 3.27 (t, J = 7.8 Hz, 4H, $NHCH_2$), 3.50 (t, J = 6.4 Hz, 4H, $COCH_2CH_2NH$), 7.68 (d, J = 8.8 Hz, 2H, ArH), 7.81 (d, J = 8.8 Hz, 2H, ArH), 7.98 (s, 2H, ArH). HRMS $(M+H)^+$ calcd for $C_{26}H_{35}N_6O_4$ 495.2720, found 495.2639.

2,6-Bis(*N*,*N*-dimethyl-1,3-diaminopropano-3-propionamido)anthracene-9,10-dione (7). This was prepared as described for **5** (0.17 g, 64%). ^{1}H NMR (300 MHz, D_2O) δ 1.81–1.98 (quintet, J = 7.2 Hz, 4H, $CH_2CH_2CH_2$), 2.64 (t, J = 6.3 Hz, 4H, $COCH_2$), 2.73 (s, 12H, $NH(CH_3)_2$), 2.79 (t, J = 7.2 Hz, 4H, $CH_2NH(CH_3)_2$), 2.90–3.10 (m, 8H, $NHCH_2CH_2CO$ and $NHCH_2CH_2CH_2$), 7.54 (d, J = 8.4 Hz, 2H, ArH), 7.62 (d, J = 8.4 Hz, 2H, ArH), 7.70 (s, 2H, ArH). HRMS $(M+H)^+$ calcd for $C_{30}H_{43}N_6O_4$ 551.3346, found 551.3313. Anal. calcd for $C_{30}H_{42}N_6O_4\cdot H_2O\cdot 2HCl$: C, 56.16; H, 7.23; N, 13.09. Found: C, 56.33; H, 7.14; N, 12.83.

2,6-Bis(1,4-diaminobutano-3-propionamido)anthracene-9,10-dione (9). This was prepared following the procedure described for **2** using *N*-BOC-1,4-diaminobutane (76 h reflux). Deprotection of the crude BOC-protected product gave **9** as an orange powder. ^{1}H NMR (300 MHz, D_2O) δ 1.63–1.86 (m, 8H, $CH_2CH_2CH_2CH_2$), 2.99 (t, J = 6.3 Hz, 4H, $COCH_2$), 3.06 (t, J = 6.8 Hz, 4H, $NHCH_2CH_2CH_2$), 3.19 (t, J = 6.8 Hz, 4H, $NHCH_2CH_2CH_2$), 3.35 (t, J = 6.3 Hz, 4H, $NH_2CH_2CH_2CO$), 7.69 (d, J = 8.4 Hz, 2H, ArH), 7.89 (d, J = 8.4 Hz, 2H, ArH), 8.02 (s, 2H, ArH). HRMS $(M+H)^+$ calcd for $C_{28}H_{39}N_6O_4$ 523.3033, found 523.2965.

2-[Bis(2-methoxyethyl)amino-3-propionamido]anthracene-9,10-dione (10). This was prepared following the procedure for **13**. The crude product was purified by column chromatography on silica gel (70–270 mesh, 60 Å, 13×6 cm) with ethyl acetate and hexane (50:50) to give 0.17 g (67%) of an orange powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.50 (t, J = 6.0 Hz, 2H, CH_2CO), 2.75 (t, J = 5.1 Hz, 4H, CH_2NH), 2.82 (t, J = 6.0 Hz, 2H, CH_2NH), 3.23 (s, 6H, OCH_3), 3.47 (t, J = 5.1 Hz, 4H, OCH_2), 7.68 (m, 2 H, ArH), 8.14–8.24 (m, 4H, ArH), 8.34 (dd, J = 8.4, 1.8 Hz, 1H, ArH), 11.28 (s, 1H, NHCO). MS $(M+H)^+$ 411.5, 278.3 ($(M+H)^+$ - $C_6H_{15}NO_2$).

2-(1,2-Diaminoathano-3-propionamido)anthracene-9,10-dione (11). The BOC-protected product was prepared on a 0.14 g (67%) scale using *N*-BOC-1,2-diaminoethane following the procedure described above for **13**. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.36 (s, 9H, $(CH_3)_3$CO], 2.57 (t, J = 6.0 Hz, 2H, CH_2NH), 2.83 (t, J = 6.0 Hz, 4H, CH_2NH), 3.01 (dt, J = 6.0 Hz, 2H, CH_2NHBOC), 6.69 (br s, 1H, NHBOC), 7.92–8.30 (m, 6H, ArH), 8.49 (s, 1H, ArH), 10.70 (1H, NHCO); the $COCH_2$ resonance is presumably under the DMSO-d_6 peak. Anal. calcd for $C_{24}H_{27}N_3O_5$: C, 65.89; H, 6.22; N, 9.61. Found: C, 65.90; H, 6.26; N, 9.35.

The BOC-protected product was deprotected with trifluoroacetic acid to give **11** in 91% yield as a yellow powder. ^{1}H NMR (300 MHz, D_2O) δ 2.87 (t, J = 6.3 Hz, 2H, CH_2CO), 3.44–3.51 (m, 6H, CH_2NH and CH_2NH_2), 7.32 (d, J = 8.4 Hz, 1 H, ArH), 7.55 (d, J = 8.4 Hz, 1 H, ArH), 7.60–7.90 (m, 5 H, ArH). HRMS $(M+H)^+$ calcd for $C_{19}H_{20}N_3O_3$ 338.1505, found 338.1523.

2-(1,3-Diaminopropano-3-propionamido)anthracene-9,10-dione (12). The BOC-protected product was prepared following the procedure above for **2** on a 0.29 g scale (81%) using *N*-BOC-1,3-diaminopropane. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.37 (s, 9H, $(CH_3)_3$CO), 1.54 (quintet, J = 6.8 Hz, 2H, $CH_2CH_2CH_2$), 2.56 (m, 4H, $COCH_2CH_2NH$ and $NHCH_2CH_2CH_2$), 2.84 (t, J = 6.4 Hz, 2H, CH_2CO), 2.98 (dt, J = 6.8 Hz, 2H, CH_2NHBOC), 6.78 (s, br, 1H, NHBOC), 7.80–8.23 (m, 6 H, ArH), 8.47 (s, 1 H, ArH), 10.60 (1H, NHCO). Anal. calcd for $C_{25}H_{29}N_3O_5\cdot 0.5\ H_2O$: C, 65.2; H, 6.57; N, 9.12. Found: C, 65.6; H, 6.37; N, 8.77.

Deprotection of this product with trifluoroacetic acid gave **12** in 89% yield as an orange powder. ^{1}H NMR (300 MHz, D_2O) δ 2.16 (quintet, J = 7.6 Hz, 2H, $CH_2CH_2CH_2$), 2.88 (t, J = 6.0 Hz, 2H, CH_2CO), 3.15 (t, J = 7.6 Hz, 2H, CH_2NH), 3.26 (t, J = 7.6 Hz, 2H, CH_2NH), 3.42 (t, J = 6.0 Hz, 2H, CH_2NH_2), 7.20–7.80 (m, 7 H, ArH). HRMS $[M+H]^+$ calcd for $C_{20}H_{22}N_3O_3$ 352.1661, found 352.1683.

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