Netropsin and spermine conjugates of a water-soluble quinocarcin analog: analysis of sequence-specific DNA interactions

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Background: Quinocarcin is the simplest of the bioxalmycin/naphthyridinomycin/tetrazomine/saframycin class of anti-tumor antibiotics, which damage DNA in a process that is inhibited by superoxide dismutase (SOD). The oxazolidine moiety of this class of anti-tumor antibiotics undergoes a redox self-disproportionation reaction of the Cannizzaro type. The reaction is proposed to proceed via an intermediate carbon-centered radical, which then reduces molecular oxygen to give superoxide. We set out to determine whether the DNA-cleavage properties of these anti-tumor antibiotics could be retained in less complex analogs of quinocarcin.

Results: A totally synthetic, water-soluble analog of quinocarcin has been prepared. This analog produced superoxide, but had considerably reduced ability to cleave supercoiled circular DNA compared to quinocarcin or tetrazomine. When conjugated to the DNA-binding molecule spermine, however, it cleaved DNA as effectively as quinocarcin at less than 1/10 the concentration. A conjugate with netropsin displayed selective cleavage around the sequence 5'-d(ATTT)-3'. Molecular modeling of the interaction between the conjugate and DNA, together with the pattern of cleavage, indicates that a non-diffusable oxidant is involved in sequence-selective DNA cleavage. The spermine conjugate displayed weak antimicrobial activity.

Conclusions: Knowledge of the stereoelectronic requirements for superoxide production by quinocarcin has allowed us to design a structurally less complex analog which has many of the same physical properties, including water solubility, the ability to produce superoxide and the ability to cleave DNA. Covalently attaching known DNA-binding molecules to this analog gave a compound that produced sequence-specific DNA damage. Our results suggest that a mechanism other than superoxide production can mediate DNA damage by the netropsin conjugate.

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Key words: anti-tumor antibiotics, auto-redox disproportionation, DNA cleavage, netropsin conjugate, quinocarcin

Introduction

It is widely recognized that many clinically significant anti-tumor antibiotics mediate oxygen-dependent cleavage of the ribose-phosphate backbone of cellular DNA and RNA. An enormous array of structurally diverse interesting natural products, semi-synthetic and totally synthetic substances mediate oxidative strand scission of nucleic acids through three main families of reactions: (1) metal-mediated activation of O_2, ultimately producing hydroxyl radical or other reactive oxygen species; (2) non-metal-dependent generation of reactive carbon radicals that mediate C–H abstraction from the deoxyribose backbone, followed by reaction of the resulting deoxyribosyl radical with molecular oxygen, culminating in strand scission; and (3) photolytic production of hydroxyl radical, which does not require metal participation for the DNA-cleavage event. A wide range of chemistry can be found in the metal-dependent family of DNA-damaging agents. For example, many readily oxidizable organic substances, such as semi-quinone radical anions, thiols and ascorbate, can reduce molecular oxygen, resulting in the production of superoxide. Unfortunately, significant non-specific cytotoxicity is observed for many anti-tumor antibiotics that mediate redox cycling of molecular oxygen, since the reactive oxidizing species can be produced both intracellularly as well as extracellularly and are typically freely diffusable. For example, superoxide radical anion is well documented to mediate DNA-strand breakage via dismutation to hydrogen peroxide followed by Fenton-mediated generation of the diffusable and highly reactive hydroxyl radical. It has long been the goal of chemists to devise predictable and, most importantly, selective molecular inhibitors of DNA replication in the rapidly dividing tumor cell. Therefore, methods that improve upon the intrinsic selectivity of drugs that produce reactive oxygen radical species causing non-specific oxidative damage to cellular macromolecules are highly desirable.

Quinocarcin (compound **2**; Fig. 1) is a natural secondary metabolite produced by *Streptomyces melanovinaceus* and is the simplest member of the bioxalomycin/naphthyridinomycin/tetrazomine/saframycin class of anti-tumor/antibiotic compounds [1]. Quinocarcin displays weak antimicrobial activity against Gram-negative organisms and, as the citrate salt, displays promising anti-tumor activity against several cell lines derived from solid mammalian carcinomas, including St-4 (gastric carcinoma), Co-3 (human colon carcinoma), human mammary carcinoma, M5076 (sarcoma) and B16 (melanoma), as well as P388

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(leukemia) [2]. More recently, a natural product structurally related to quinocarcin, tetrazomine (compound **3**; Fig. 1), was isolated from *Saccharothrix mutabilis*. Preliminary anti-tumor/antimicrobial assays of this substance indicate a similar spectrum of biological activity to that displayed by quinocarcin [3]. Additionally, however, tetrazomine shows good antimicrobial activity against both Gram-negative and Gram-positive organisms and activity against the P388 leukemia cell line *in vivo*. Preliminary studies on the modes of action for these compounds disclosed that quinocarcin blocks RNA synthesis in preference to DNA and protein synthesis in P388 leukemia cells [4]. In *Bacillus subtilis*, quinocarcin inhibited [^{3}H]-thymidine incorporation, suggesting that DNA polymerase might be the target of inhibition in that organism [5].

One of several potential ways in which these compounds can damage cells involves an oxygen-dependent mechanism. Superoxide production by quinocarcin was first reported by Tomita *et al.* [5] and a mechanistic explanation has now been offered (Fig. 1) [6,7]. Our interest in these compounds was stimulated by the observation that quinocarcin exhibited the capacity to cleave plasmid DNA in an O_2-dependent reaction that was inhibited by the addition of oxygen-free-radical scavengers such as methanol, *tert*-butanol, α-tocopherol, superoxide dismutase (SOD) and catalase. We have previously reported data that supports the hypothesis that the oxygen-dependent scission of DNA is intimately associated with the propensity of these substances to undergo disproportionation reactions of their oxazolidine ring which, under anaerobic conditions, leads to the redox products **22** and **23** (exemplified for compound **2**, Fig. 1). However, under aerobic conditions, molecular oxygen is proposed to react with an intermediate carbon-centered radical (compounds **20** or **21**) which, with participation of the adjacent non-bonded electron pair on the nitrogen atom, results in the subsequent expulsion of a molar equivalent of superoxide [6] (exemplified in Fig. 2 for the conversion of compound **24** → compound **19** + superoxide radical anion). Superoxide radical anion subsequently suffers Fenton/Haber–Weiss redox cycling ultimately producing hydroxyl radical, which is well documented to mediate sequence-random DNA-strand breaks [8,9].

In an effort to elucidate the stereoelectronic control elements of this reaction, we previously reported the total synthesis and DNA-cleavage abilities for the tetracyclic analogs of quinocarcin **4** and **5** (Fig. 1) [10]. Analog **4**, which possesses the same relative stereochemical configuration as quinocarcin, produces superoxide and damages plasmid DNA. On the other hand, the C-11a-epimer (**5**) is dormant under the same set of conditions (Table 1, entries 7 and 8). The stark difference in reactivity between the closely related compounds **4** and **5** suggested the obligate requirement of a *trans*-antiperiplanar relationship between the non-bonded electron pair of the oxazolidine nitrogen atom and the adjacent methine hydrogen atom at C-4 for the redox disproportionation reaction. Unfortunately, compounds **4** and **5** are virtually insoluble in water resulting in very low aqueous concentrations, low pH ranges (7 or less) and/or mixed alcohol/water solvent systems making further *in vitro* and *in vivo* studies with these systems limited.

The stereochemical insights gained from the analog systems **4** and **5** and the water-soluble nature of quinocarcin, a zwitterionic amino acid, provided the basis for the design and synthesis of compound **1**, reported herein. Based on the mechanistic studies already described on compounds **2–5**, it was anticipated that compound **1** would share the capacity of compounds **2**, **3** and **4**, spontaneously to undergo auto-redox disproportionation at or above pH 7 and mediate oxidative damage to DNA. In addition, the carboxyl moiety of compound **1** was envisioned to provide a site to covalently attach DNA-binding molecules such as spermine and netropsin for the targeted and sequence-selective

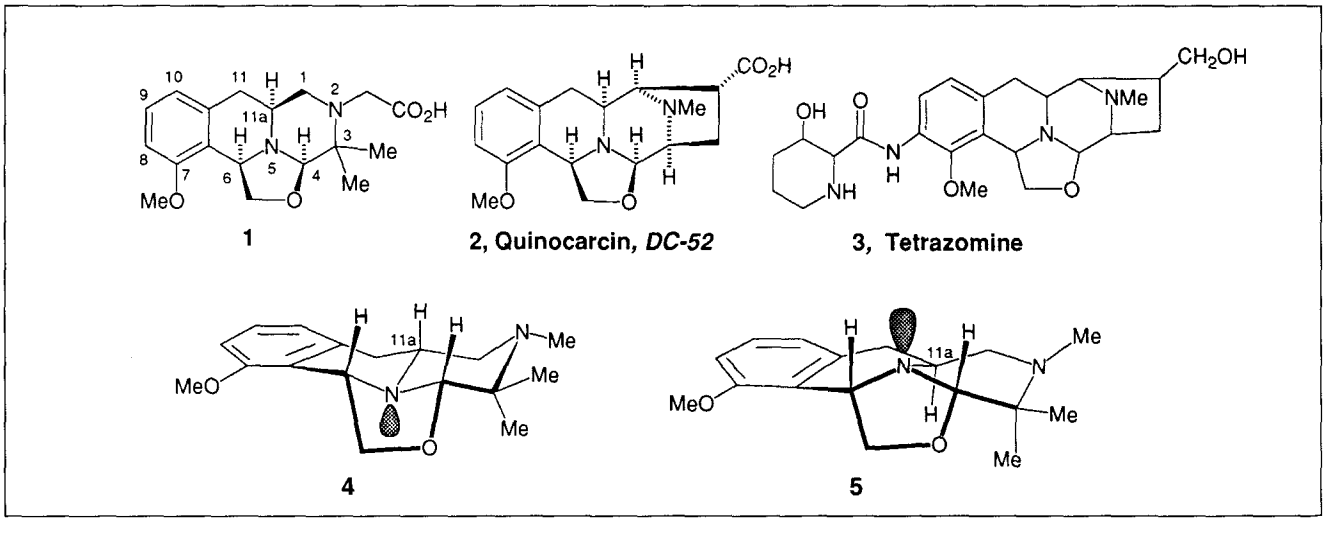

Fig. 1. Structures of quinocarcin, tetrazomine and synthetic structural analogs.

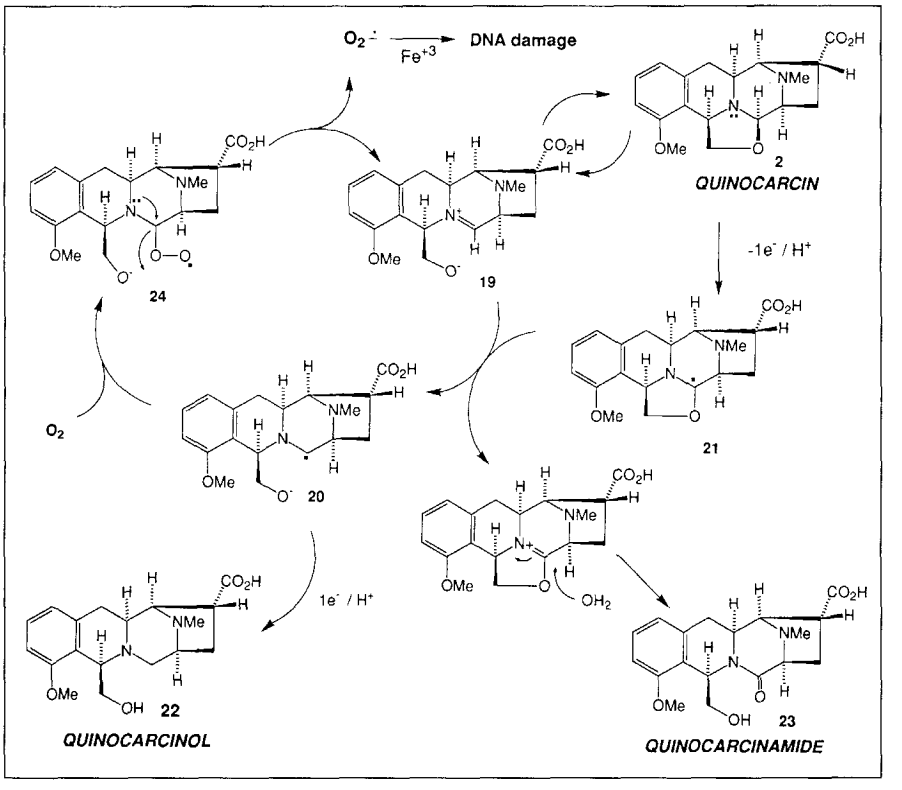

Fig. 2. Proposed mechanism for the autoredox disproportionation of quinocarcin. Under anaerobic conditions, the formation of compounds **22** and **23** is observed. Under aerobic conditions, the production of superoxide is observed, resulting in Fenton/Haber–Weiss cycling and hydroxyl-radical-mediated damage to DNA.

manipulation of DNA cleavage. Here we describe the preparation of conjugates of compound **1** with netropsin **(16)** and spermine **(17)** and the DNA-cleavage chemistry of these molecules.

Results and discussion

Synthesis of a water-soluble quinocarcin analog (1)

Compound **1** was synthesized as illustrated in Fig. 3. The starting material was the β-hydroxyethyl ester **6** obtained as a single diastereomer, as previously described [10]. Saponification of **6** with lithium hydroxide produced the corresponding β-hydroxy acid. This material was carried on without purification by reaction with three equivalents of thionyl chloride in refluxing toluene which resulted in β-elimination and acid-chloride formation.

Table 1. Rates of superoxide production by quinocarcin, tetrazomine and analogs.

Entry	Substrate	pH	Rate ($M\ s^{-1} \times 10^{-9}$)
1	**1** (2 mM)	6	0.21
2	**1** (2 mM)	7	0.82
3	**1** (2 mM)	8	0.99
4	**1** (5 mM)	8	1.56
5	**2** (1 mM)	8	2.18
6	**3** (1 mM)	8	17.5
7[a]	**4** (1 mM)	8	0.82
8[a]	**5** (1 mM)	8	0.0
9	20 mM phosphate buffer (control)	8	0.0

[a]Reactions run in 5 % methanol (aq). All reactions measured the rate of reduction of nitroblue tetrazolium (NBT).

The intermediate α,β-unsaturated acid chloride was then reduced to allylic alcohol **7** with sodium borohydride in 42 % isolated yield from **6**. Reaction of compound **7** with methanesulfonyl chloride and triethylamine followed by addition of 2-amino-2-methyl-1-propanol produced the corresponding allylic amine **8**. The diastereoselective reduction of this material was achieved by reacting compound **8** with one equivalent of palladium chloride under one atmosphere of hydrogen affording compound **9a** as a single diastereomer in 98 % yield. The relative stereochemistry of compound **9a** was determined unambiguously by reacting an aliquot of compound **9a** with trimethyloxonium tetrafluoroborate, producing the N-methylamine **9b**. Spectroscopic data for this material was compared to physical data for the same substance prepared by a different synthetic route [10]. Compound **9b** is the synthetic precursor to compound **4**, for which a single crystal X-ray analysis was obtained [10]. Closure of the C and D rings to form tetracyclic intermediate **11** was achieved by first oxidizing compound **9a** to aldehyde **10**. Subsequent treatment of compound **10** with lithium hydroxide in refluxing ethanol removed the oxazolidinone, furnishing compound **11** in 22 % isolated yield. Formation of compound **1** was accomplished by reacting compound **11** with ethyl bromoacetate in the presence of sodium bicarbonate. The target, compound **1**, was obtained following saponification of ethylester **12** with lithium hydroxide.

Preparation of conjugates of compound 1 with netropsin (16) and with spermine (17)

Netropsin and other distamycin-like peptides are well recognized to bind in the minor groove in AT-rich regions of DNA [11–13]. The flat, slightly curved structures of these

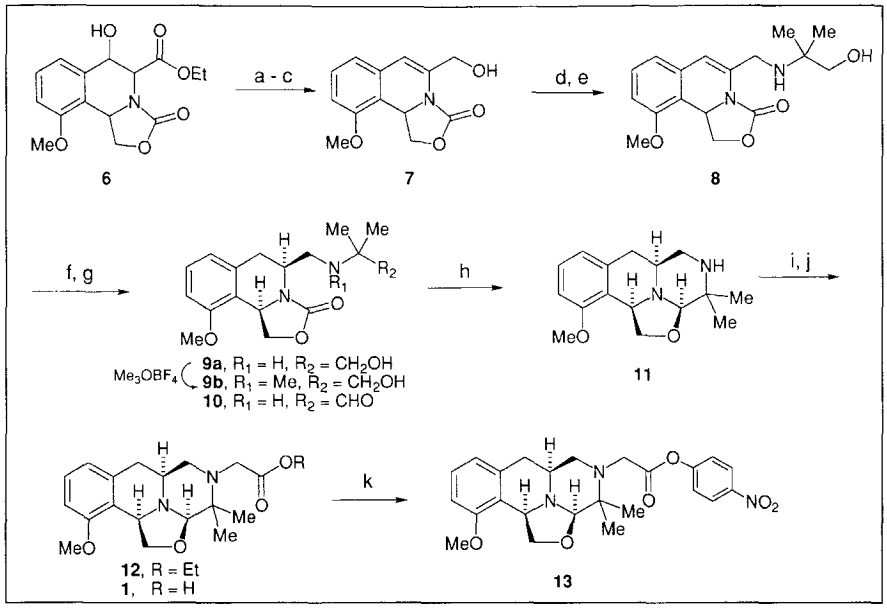

Fig. 3. Preparation of the water-soluble quinocarcin analog **1**. **(a)** LiOH (aq), ethanol (r.t.); **(b)** thionylchloride, toluene (reflux); **(c)** $NaBH_4$, dichloromethane/ethanol (–78 °C to r.t.), 42 % yield from **6**; **(d)** methanesulfonylchloride, triethylamine, dichloromethane (0 °C); **(e)** 2-amino-2-methyl-1-propanol, 46 % yield from **7**; **(f)** $PdCl_2$, H_2 (1 atm) ethanol; **(g)** dimethylsulfoxide, oxalylchloride (–78 °C), 95 % yield from **8**; **(h)** LiOH (aq), ethanol (reflux 24 h), 22 % isolated yield; **(i)** ethylbromoacetate, $NaHCO_3$, DMF; **(j)** LiOH (aq), ethanol (r.t.), 65 % yield from **11**; **(k)** *p*-nitrophenol, DCC, dichloromethane (0 °C to r.t.), 63 % yield.

compounds, coupled with their positive charge, facilitates preferential binding in these regions of the minor groove [14]. A variety of DNA-cleaving compounds have been covalently attached to distamycin peptides in an effort to increase the affinity of these compounds for DNA, to give enhanced DNA cleavage. Such complexes include the highly studied distamycin and penta-*N*-methylpyrrolecarboxamide (P5) Fe/EDTA complexes [15–17]. More recently, other DNA-cleaving compounds, including enediynes and other radical-forming moieties, have been successfully linked to these peptides, altering their capacity to damage DNA [18,19].

It is well known that polyamines such as spermidine and spermine are good DNA-binding molecules. These basic substances are protonated at physiological pH, enabling them to associate strongly with the negatively charged phosphodiester regions of DNA, and to engage in hydrogen-bonding interactions in both the major and minor grooves [20,21]. Thus, in addition to the netropsin conjugate, we also chose to conjugate compound **1** to spermine.

Conjugation of compound **1** to these DNA-binding molecules was carried out by first reacting compound **1** with *para*-nitrophenol and dicyclohexyl carbodiimide (DCC) to afford the activated *para*-nitrophenyl ester **13** (Fig. 3). The dipeptide **14** (Fig. 4) was prepared as described by Bailer *et al.* [22] with minor modifications. Reaction of compound **14** with N,N-dimethylethylenediamine, DCC and N-hydroxybenzotriazole (HOBT) afforded compound **15** in 98 % yield. Reduction of the nitro group by catalytic hydrogenolysis followed by

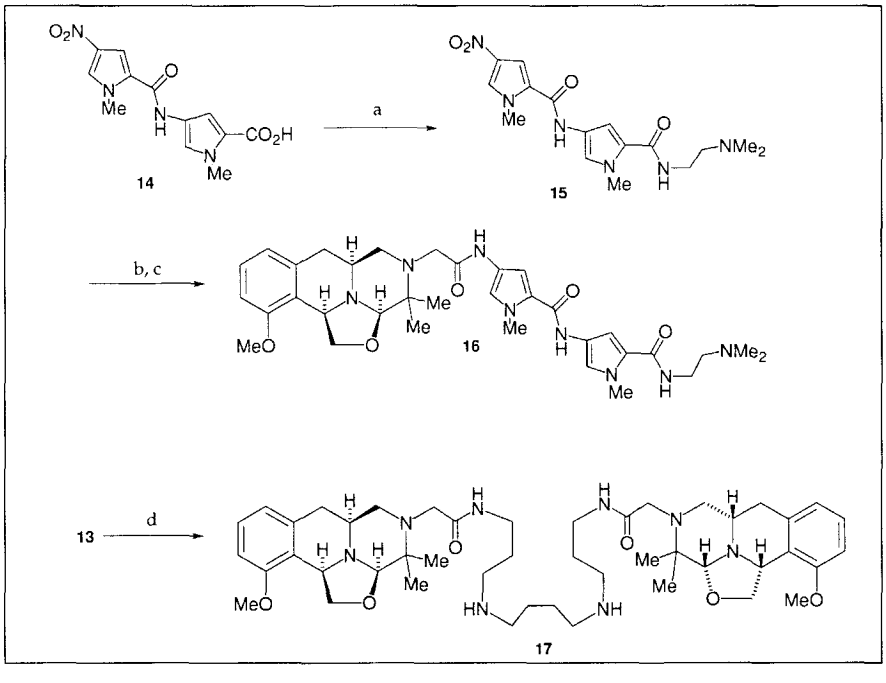

Fig. 4. Preparations of netropsin conjugate **16** and spermine dimer **17**. **(a)** N,N-dimethylethylenediamine, DCC, HOBT, DMF, 98 % yield; **(b)** 5 % palladium on carbon, H_2 (1 atm), DMF; **(c)** **13** (1 eq), Et_3N, DMF, 47 % yield from **15**; **(d)** spermine (0.5 eq), Et_3N, DMF (4 °C), 20 % isolated yield.

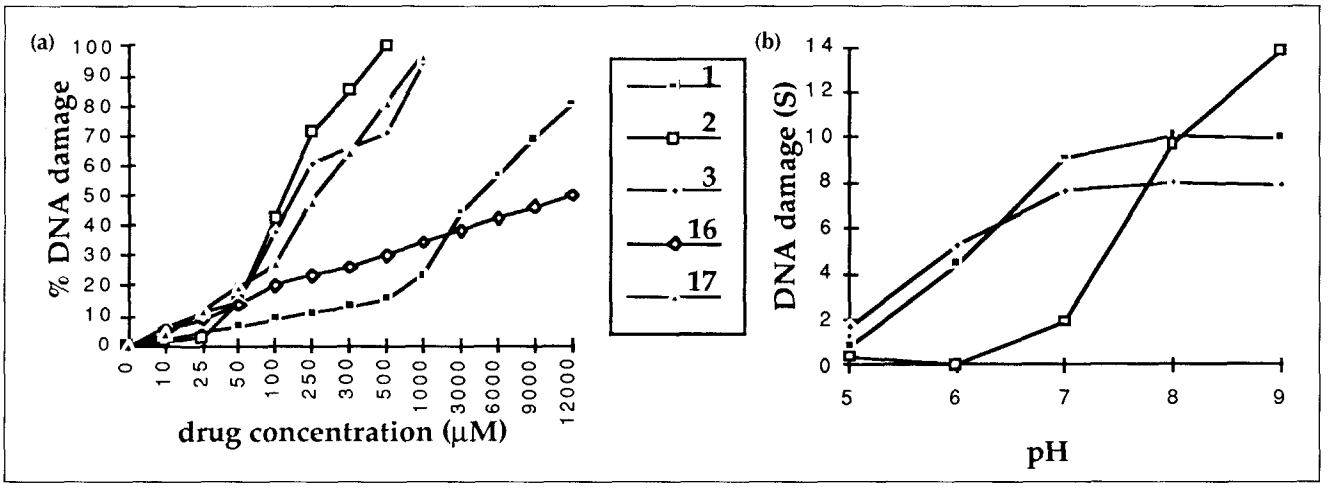

Fig. 5. DNA cleavage by quinocarcin, tetrazomine and designed analogs of quinocarcin. **(a)** Effects of concentration on DNA cleavage for compounds **1**, **2** and **3**, and conjugates **16** and **17**. **(b)** Effects of pH on DNA-cleavage (S) for compound **1** x 10 (◆), quinocarcin (❑) and tetrazomine (■). The cleavage of supercoiled circular DNA was detected by loading the reactions onto 1 % agarose gels containing 0.4 μg ml^{-1} ethidium bromide and running for 2 h at 55 V. The electrophoresis gels were immediately visualized on a UV trans-illuminator and photographed using black and white instant film (Polaroid T667). The measurements of the relative intensities of DNA bands were performed on the photographs using the Dell System 325 computer and Technology Resources Inc. image-processing software. The film used to photograph the gels was confirmed to have a linear response to the range of DNA quantities used. The mean number of single strand scissions (S) per supercoiled DNA substrate was calculated using the Poisson distribution. When only forms I and II are present, the equation simplifies to $S = -\ln f_I$, where f_I is the fraction of form I molecules from the densitometry data. In those cases where form III (linear) DNA was present, S was calculated from $f_I + f_{II} = [1-S(2h + 1)/2L]^{S/2}$ where h is the distance between hits on opposite strands to produce a linear molecule (16 base pairs) and L is the total number of base pairs in ΦX174 (5386 base pairs). The damage to DNA was the percent conversion of form I to form II plasmid DNA from the densitometry data.

immediate reaction with compound **13** produced netropsin conjugate **16** in 52 % yield after chromatography. Similarly, spermine dimer **17** was prepared by reaction of two equivalents of compound **13** with spermine in dimethylformamide (DMF) at 4 °C. Compound **17** probably exists as a mixture of diastereomers (one set of enantiomers plus the *meso*-compound) since compound **1** was prepared as a racemate. The physical data collected for compound **17** (^{1}H NMR and ^{13}C NMR) is consistent with the production of a single diastereomer, but a rigorous stereochemical assignment could not be made.

Reactions with supercoiled circular (ΦX174) DNA

To study the effectiveness of these compounds as DNA-cleaving agents, compounds **1**, **2**, **3** and the conjugates **16** and **17** were reacted under various conditions with

Table 2. Effects of additives on DNA nicking for **1**, **2**, **3**, **16** and **17**.

Entry	Substrate	Conditions	% inhibition	% enhancement
1	**1** (1 mM)	+ 10 μg ml^{-1} SOD	85	
2	**1** (1 mM)	+ 100 μg ml^{-1} catalase	65	
3[a]	**1** (1 mM)	+ 0.1 mM H_2O_2		19
4	**2** (1 mM)	+ 10 μg ml^{-1} SOD	99	
5	**2** (1 mM)	+ 100 μg ml^{-1} catalase	83	
6[a]	**2** (1 mM)	+ 0.1 μg ml^{-1} H_2O_2		143
7	**3** (1 mM)	+ 10 μg ml^{-1} SOD	94	
8	**3** (1 mM)	+ 100 μg ml^{-1} catalase	54	
9[a]	**3** (1 mM)	+ 0.1 mM H_2O_2		68
10	**16** (0.2 mM)	+ 10 μg ml^{-1} SOD	0	0
11	**16** (0.2 mM)	+ 100 μg ml^{-1} catalase	3	
12[a]	**16** (0.2 mM)	+ 0.1 mM H_2O_2		95
13	**17** (0.2 mM)	+ 10 μg ml^{-1} SOD	83	
14	**17** (0.2 mM)	+ 100 μg ml^{-1} catalase	32	
15[a]	**17** (0.2 mM)	+ 0.1 mM H_2O_2		289

All reactions run in 20 mM phosphate buffer (pH 8) at 37 °C. The DNA used was covalently closed circular supercoiled ΦX174.
[a] Values corrected for background cleavage due to H_2O_2 by subtraction of control.

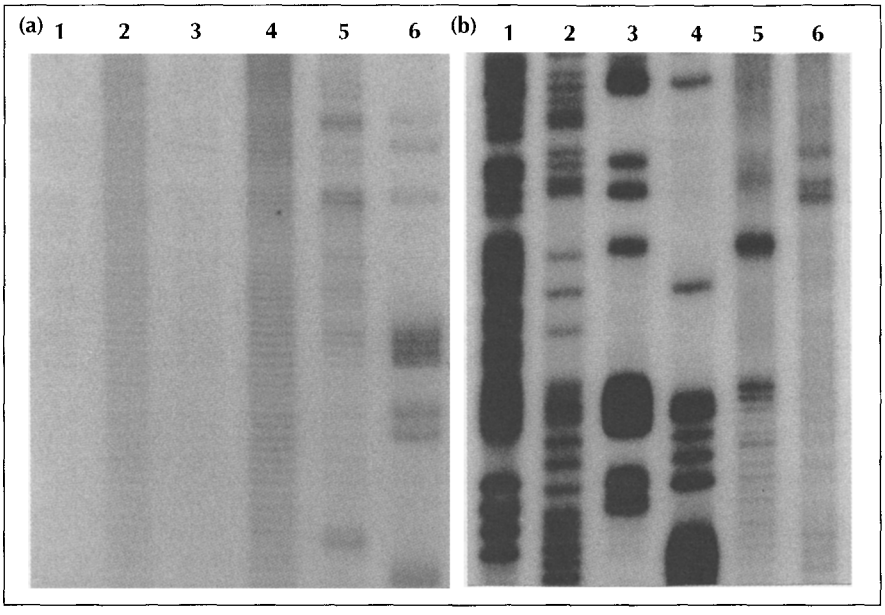

Fig. 6. Cleavage of DNA by compound **1** linked to DNA-binding molecules. **(a)** Compounds **1**, **2** and **17** show random sequence cleavage while compound **16** shows sequence specificity. Reactions of **1**, **2**, **16** and **17** with 3'-^{32}P-labeled, 516-base-pair restriction fragment from pBR322. Lane 1, control; lane 2, 200 μM quinocarcin (**2**); lane 3, 200 μM **1**; lane 4, 200 μM **17**; lane 5, 200 μM **16**; lane 6, Maxam and Gilbert G reaction. **(b)** Cleavage by compound **16** on individual strands of DNA. Reactions of **16** with 3'- or 5'-^{32}P-labeled 516-base-pair restriction fragment. Lane 1, 3'-Maxam and Gilbert G + A reaction; lane 2, 5'-Maxam and Gilbert G + A reaction; lane 3, 3'-Maxam and Gilbert G reaction; lane 4, 5'-Maxam and Gilbert G reaction; lane 5, 3'-labeled DNA + 200 μM **16**; lane 6, 5'-labeled DNA + 200 μM **16**. All reactions run in 10 mM phosphate buffer (pH 8) at 37 °C for 10 h.

supercoiled circular DNA (ΦX174). Compound **1** was observed to cleave this DNA substrate (Fig. 5a), although quinocarcin and tetrazomine were considerably more effective at the same concentrations. From the data shown in Table 2, it appears likely that compound **1** produces superoxide by the same mechanism proposed for quinocarcin and tetrazomine. DNA cleavage mediated by compound **1** is inhibited by the addition of SOD and catalase. As expected, the addition of hydrogen peroxide had a stimulatory effect on DNA cleavage mediated by compound **1**. Compound **1** displays parallel pH trends for DNA damage and superoxide release (as shown by the reduction of nitroblue tetrazolium (NBT)) to both quinocarcin and tetrazomine (Fig. 5b and Table 1)

As shown in Fig. 5a, covalently linking compound **1** to spermine (forming dimer **17**) resulted in more than a ten-fold increase in DNA-cleavage activity as compared to compound **1** alone. These data, and those illustrated in Fig. 6, suggest that this increase in activity renders compound **17** as potent as quinocarcin or tetrazomine. As expected, DNA cleavage by compound **17** was inhibited by the addition of SOD and catalase (Table 2, entries 13 and 14) and was enhanced by the addition of hydrogen peroxide (Table 2, entry 15).

Fig. 5a also shows the different concentration profiles for DNA cleavage by these compounds. At low concentrations (below 25 μM) a slow increase in DNA-cleavage was observed for all compounds. For compounds **2**, **3** and **17**, a marked increase in DNA damage occurs above 25 μM; for compound **1**, this change occurs above 1000 μM. The exception to this trend seems to be compound **16** which maintains a slow increase in DNA-cleavage activity over the entire concentration range. These data suggest that the preferred AT-rich regions of the DNA substrate might be saturated by compound **16** at low drug concentration, and thus increasing the drug concentration only has a moderate effect. Further evidence for sequence-selective cleavage by compound **16** and characterization of the cleavage sites was obtained using ^{32}P-end-labeled restriction fragments, the results of which are described below.

Reactions with ^{32}P-labeled restriction fragments

A 516-base-pair restriction fragment from pBR322 was used for DNA-cleavage studies. The fragment was labeled at the 3' end of one strand by linearizing the plasmid with restriction endonuclease *Eco*RI, then filling in the ends with DNA polymerase in the presence of α-^{32}P-dATP, followed by digestion with *Rsa*I. The desired 3'-^{32}P-end-labeled 516-base-pair fragment was subsequently gel-purified. Fig. 6a depicts the sequence-random nature of cleavage of this substrate mediated by quinocarcin (lane 2). As previously shown using the supercoiled plasmid cleavage assay, quinocarcin is a significantly more efficient DNA-cleaving agent than compound **1** (lane 3). However, as can be seen in lanes 3 and 4 (Fig. 6a), covalent attachment of spermine to compound **1** (producing compound **17**) causes a marked increase in cleavage activity although there is no indication that this damage is sequence specific. The netropsin conjugate (**16**) does, however, exhibit a definite sequence-selective DNA cleavage pattern (Fig. 6a, lane 5).

To fully elucidate the pattern of DNA damage exhibited by compound **16**, the 516-base-pair substrate was labeled at the 5' end of the strand complementary to the one labeled in the 3'-^{32}P substrate. This allowed us to examine the cleavage activity on each individual strand of the double-stranded DNA fragment (Fig. 6b). Lanes 5 and 6 (Fig. 6b) illustrate reactions of compound **16** with the 3'-^{32}P-labeled and 5'-^{32}P-labeled DNA respectively. The data from this experiment is summarized on the histograms in Fig. 7; the highest frequency of cleavage occurred around double-stranded DNA containing the sequence 5'-d(ATTT)-3' (NMR NOESY experiments

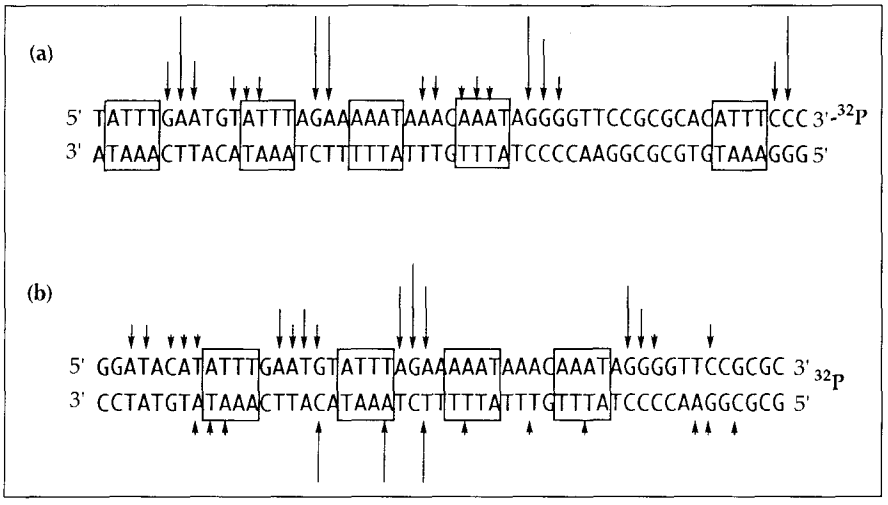

Fig. 7. The selectivity of DNA cleavage by compound **16**. **(a)** Histogram for Fig. 6a, lane 5. **(b)** Histogram for Fig. 6b, lanes 5 and 6. Histograms were prepared by measurements of the relative intensities of DNA bands from the autoradiograms in Fig. 6 using the Dell System 325 computer and Technology Resources Inc. image-processing software. The length of the arrows approximate the relative intensities of the bands by scanning densitometry.

have revealed similar sequence specificity for distamycin A complexed with the double-stranded, self-complementary dodecamer 5'-d(CGCAAATTTGCG)-3' [23]). The actual sites of cleavage however, were two bases to the 3' end of this four-base recognition site. There is also evidence from the histograms (highlighted in boxes, Fig. 7), that in some cases, the drug may be able to bind in two orientations, which would also allow cleavage to occur two bases to the 3' end of the 5'-d(AAAT)-3' recognition sequence. Such an observation is not unexpected since it has been shown that many of the distamycin peptides actually bind in two orientations in the minor groove in a 1:1 or 2:1 ratio of drug to DNA [23,24]. It is also significant to note the apparent directional preference for cleavage exhibited by compound **16**, since little or no cleavage was observed around 3'-d(ATTT)-5' sequences.

Analysis of the data indicates that lesions caused by compound **16** do not necessarily occur on the complementary DNA strands at proximal Watson–Crick base pairs. Proximal double-strand damage is observed in the case of the distamycin–Fe/EDTA affinity-cleaving agents [15–17]. In these systems, the metal that mediates the Fenton-type production of hydroxyl radical is held proximal to the DNA as a consequence of being complexed to the EDTA moiety. If the redox-active oxazolidine portion of DNA-bound compound **16** is generating a (non-diffusable) proximal reactive oxidant, or a highly reactive diffusable oxidant (such as hydroxyl radical) one would expect cleavage to occur on one or both strands of the DNA in close proximity to the site where the drug is bound to the recognition sequence. Alternatively, if superoxide is the only initial species produced by bound drug, the superoxide molecule must diffuse away from the drug to interact with Fe(III) in solution to initiate the Fenton/Haber–Weiss cycling of hydroxyl radical, which is a diffusable sequence-random DNA-damaging agent. In this case, sequence-random cleavage would be expected. The only non-random aspect of DNA cleavage would be a 'footprint' of the bound drug, and this would only be anticipated for a very tightly bound drug.

Examination of the autoradiogram and histogram for compound **16** suggests that an alternative mode of DNA damage may be operating. One plausible explanation for the observed cleavage pattern would be that compound **16** is also operating via a direct hydrogen atom abstraction mechanism. Superoxide production by this class of compounds is proposed to occur as a result of the attack of molecular oxygen on a carbon-centered radical (Fig. 2, compounds **20** or **21**) derived from the auto-redox disproportionation of the oxazolidine ring or by exogenous reduction of compound **19** (Fig. 2) followed by expulsion of superoxide. It is therefore possible that proper orientation of the drug, as dictated by the netropsin moiety, might position a drug-centered radical in the proper geometry to abstract a hydrogen atom from the phosphoribose backbone. In the presence of oxygen, attack of the resulting radical in the phophoribose backbone would ultimately result in scission of the DNA. Experimental support for this hypothesis is presented in Table 2 (entries 10 and 11) which shows that DNA damage caused by compound **16** is not readily inhibited by the addition of SOD and catalase. This situation is in marked contrast to quinocarcin (compound **2**, Table 2, entries 4 and 5), tetrazomine (compound **3**, Table 2, entries 7 and 8), compound **1** (Table 2, entries 1 and 2) and compound **17** (Table 2, entries 13 and 14) whose DNA-cleavage chemistry all display sensitivity to SOD and catalase. These data strongly suggest the intermediacy of a superoxide-independent mechanism in the DNA cleavage exhibited by compound **16**. An issue of further intrigue in the case of compound **16**, involves the exact mechanism for the one electron oxidation or reduction of the bound or unbound drug and the presentation of the radical species to the DNA substrate. Studies are in progress to shed light on these issues.

Based on the observed DNA-cleavage chemistry of compound **16**, we have considered whether it is possible from a structural point of view for compound **16** to bind in the minor groove to a four-base recognition sequence and adopt the requisite geometry to abstract a hydrogen atom two bases to the 3' side. We attempted to answer this question with molecular modeling by docking

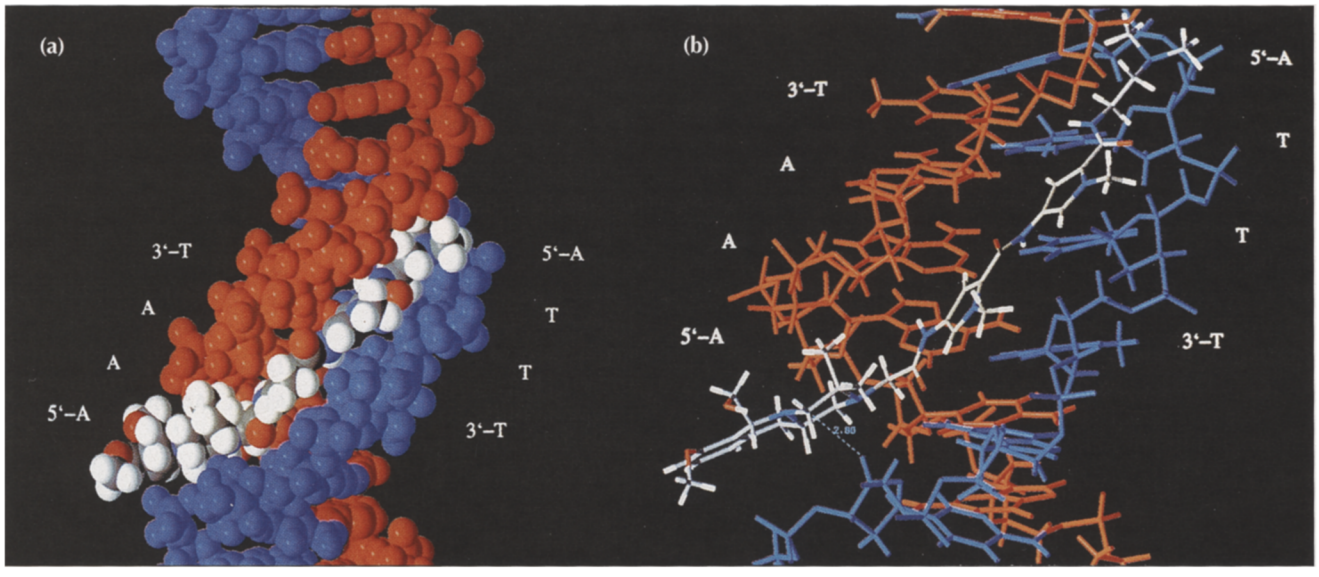

Fig. 8. Netropsin conjugate **16** bound to the double-stranded dodecamer 5'-d(CGCAAATTTGCG)-3'. **(a)** Space-filling model depicting the complex between compound **16** and DNA as manually docked to the 5'-d(ATTT)-3' sequence in the minor groove and minimized. **(b)** Cylindrical bond model showing the 2.8-Å distance between the proposed carbon-centered radical of compound **16** and the C-4' hydrogen two base pairs from the 3' end of the 5'-d(ATTT)-3' sequence. Molecular mechanics were carried out using DREIDING force-field in the molecular modeling package BIOGRAF (Molecular Simulations, Inc.). The double-stranded dodecamer 5'-d(CGCAAATTTGCG)-3' was obtained from the Brookhaven Protein Data Bank.

the 'natural' enantiomer of compound **16** into the dodecamer 5'-d(CGCAAATTTGCG)-3', which contains the 5'-d(ATTT)-3' sequence of interest [25]. By taking advantage of known hydrogen-bonding interactions exhibited by netropsin [25,26], we obtained the minimized structure depicted in Fig. 8. It can be seen from this model, which involves contact with all four bases of the recognition site, that C-7 of the oxazolidine ring of compound **16** is oriented to fall within ~ 3 Å of the C-4' hydrogen atom of the deoxyribose ring two base pairs to the 3' end of this sequence. Hydrogen atom abstraction from C-4' by a radical, followed by reaction of the resulting deoxyribosyl C-4'-radical with molecular oxygen, is well documented [27,28] to result in strand scission producing the corresponding 3'-phosphoglycolate moiety attached to the next base to the 5'-side. Cameron and Uhlenbeck [27] have shown that T4 polynucleotide kinase (T4 PNK) has 3' phosphatase activity, and it is possible to demonstrate whether an oxidatively cleaved DNA product is a 3'-phosphate. Reaction of the DNA-cleavage products with T4 PNK selectively removes the phosphate from the 3'-position, giving a band of slower mobility by polyacrylamide gel electrophoresis (PAGE) due to the removal of one negative charge; the alternative 3'-phosphoglycolate product resulting from C-4' hydrogen atom abstraction and reaction of the resultant radical with dioxygen does not react with T4 PNK. In support of this, we have recently obtained preliminary data which suggests that there may be a strong preference for C-4' hydrogen atom abstraction for this cleavage reaction. Reaction of compound **16** with the 5'-^{32}P-labeled restriction fragment gave DNA products that, upon reaction with T4 PNK, resulted in no apparent band shift (data not shown) suggesting that the 3'-phosphoglycolate product may be formed preferentially (as predicted by the model in Fig. 8) in these reactions.

Quinocarcin and tetrazomine both give a mixture of 3'-phosphate and 3'-phosphoglycolate cleavage products, primarily resulting from non-specific hydrogen atom abstraction on the deoxyribosyl backbone by a diffusable oxidant, such as hydroxyl radical [7]. The 3'-phosphate products from these cleavage reactions gives the expected band shift to a slower-moving band on PAGE. For quinocarcin, the ratio of 3'-phosphate to 3'-phosphoglycolate was determined to be ~6:4; for tetrazomine, the ratio was ~8:2; and for Fe(II)/EDTA, the ratio was ~4:6. These observations, coupled with the unexpected cleavage chemistry exhibited by compound **16**, suggest that there are likely to be multiple modes for the presentation of the DNA-damaging species to DNA. Efforts are under way to clarify these mechanistic questions and to improve upon the selectivity of the drug–nucleic acid interaction.

Antimicrobial activity

The new, synthetic compounds (**1**, **16** and **17**) were assayed for *in vitro* antibacterial activity against the following organisms by the agar disc-diffusion assay: *B. subtilis, Staphylococcus aureus, Micrococcus luteus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Serratia marcescens, Candida albicans, Saccharomyces cerevisiae, Mycobacteria peregrinum, Mycobacteria smegmatis, Mycobacteria fortuitum and Mycobacteria chelonei*. Compound **17** displayed marginal weak anti-microbial activity against *B. subtilis* (at 1 mg ml^{-1}) and *M. smegmatis* (at 10 mg ml^{-1}). Control assays with spermine and compound **1** showed no activity against *M. smegmatis* (>10 mg ml^{-1}). The lack of antimicrobial activity of these compounds, particularly compound **1**,

is somewhat surprising in light of the close structural resemblance of these compounds to quinocarcin and tetrazomine and their demonstrated ability to produce superoxide and damage DNA. Studies are in progress to prepare the C-11a epimers of compounds **1** and **17**, so that their cytotoxic properties can be compared to those of compounds **1** and **17**, and to examine whether these substances can alkylate DNA in the absence of a redox manifold.

Significance

Many anti-tumor antibiotics mediate their effects by generating superoxide, which causes oxidative damage to DNA. This activity also damages healthy cells, resulting in the undesirable host cytotoxicity of many anti-tumor drugs. Although quinocarcin exhibits very promising anti-tumor activity, it suffers from problems of non-specific cytotoxicity. It would thus be highly desirable to find ways to attenuate the production of these reactive intermediates, or to direct the activity of the drugs more specifically.

We have demonstrated that it is possible to prepare a structurally less complex analog of quinocarcin which exhibits most of the physical properties associated with the parent compound. By covalently attaching known DNA-binding molecules to this analog, we have also demonstrated the ability to vary the mechanism and DNA-cleavage specificity of this class of anti-tumor antibiotics. Through the use of synthetic chemistry, we have been able to identify unique stereoelectronic control elements of the oxazolidine ring, which is the biologically-significant functional group responsible for the production of reactive oxidants that ultimately cause damage to nucleic acids. We have been able to prepare, for the first time, a totally synthetic analog of this class of compounds that has markedly different DNA-damaging chemistry from the natural products or the other synthetic analogs. It should now be possible to apply the insights gained from this study to the semi-synthetic alteration of the natural products themselves, with the objective of decreasing their non-specific cytotoxicity and thereby increasing their potential efficacy as chemotherapeutic agents.

Materials and methods

General procedure for reductions of nitroblue tetrazolium (NBT)

Reactions were performed by addition of appropriate amounts of reagent stock solutions to an aerated solution of nitroblue tetrazolium (0.12 mM; Sigma) in 20 mM phosphate buffer (at the indicated pH) containing 1 % Triton X100 detergent (USB) and the final volumes brought to 750 µl with deionized water. The optical absorbance was measured at 25 °C over a 30 min period at 500 nm (Varian DMS 80 UV/vis spectrophotometer) and the ΔOD was the average slope for the linear change in optical density over the reaction time. The rates for superoxide production were calculated by assuming that $[O_2]$ does not appreciably change over this time period and is in excess (zero order in oxygen). The rates (reported in Table 1) were calculated from the ΔOD measurements and based on a molar extinction coefficient (ϵ_0) of 12 200 for the monoformazan product of NBT at 500 nm.

*Reactions of **1,2, 3, 16** and **17** with supercoiled circular DNA (ΦX174)*

All DNA-cleavage reaction mixtures were made up by addition at 0 °C of appropriate amounts of reagent stock solutions (stock solution of **16** was prepared as the hydrochloride salt) to a stock solution of supercoiled circular DNA (ΦX174) containing 0.5 µg DNA per reaction (77 µM base pairs final concentration). The total volumes were brought to 10 µl with deionized water and 80 mM phosphate buffer (pH 8 unless otherwise indicated) to achieve a final concentration of 20 mM phosphate. The reaction mixtures were incubated at 37 °C for 4 h in tightly capped plastic Eppendorf tubes. Stock solutions for experiments including DNA were prepared using sterile, distilled, deionized water and commercially available reagents: sodium phosphate monobasic (EM Science); sodium phosphate dibasic, 30 % hydrogen peroxide (Malinckrodt); superoxide dismutase, beef liver catalase (suspension in water, from Boehringer Mannheim).

*Reactions of **1,2, 16** and **17** with ^{32}P-labeled restriction fragments (pBR322)*

Reactions were made up by additions at 0 °C of appropriate amounts of reagent stock solutions (stock solution of **16** was prepared as the hydrochloride salt) to 3 µl of ^{32}P-labeled DNA (~100 µM base pairs final concentration). The total volume of the reactions was brought to 10 µl with deionized H_2O and enough 80 mM phosphate buffer (pH 8) to achieve a final concentration of 10 mM phosphate. Each reaction was incubated at 37 °C for 10 h. To the reaction mixtures were then added 1 µl of 3M NaOAc (pH 5.2) and the reaction mixture was ethanol-precipitated and dried under reduced pressure. To each pellet was added 10 µl of loading buffer (formamide, 10 mM EDTA, pH 8, 0.025 % xylenecyanol and 0.025 % bromophenol blue) then heated to 90 °C for 5 min and immediately loaded (10 000 cpm/lane as measured by a Packard 1500 liquid scintillation analyzer) on a 20 x 40 cm, 15 % denaturing (urea) polyacrylamide gel containing 20 % formamide and run at 1300 V until the xylenecyanol had reached the bottom of the gel. The gels were then fixed in 5 % acetic acid, 15 % methanol (aq), dried on a Bio-Rad 583 gel dryer and the bands visualized by autoradiography using Kodak X-OMAT AR (35 x 43 cm) film.

Preparation of compounds

Compounds were prepared by standard laboratory procedures as outlined in Figs 3 and 4. Chromatography for compound isolations employed Selectoscientific silica-gel (32–63) or EM Science thin layer chromatography plates (silica gel 60, F254, 20 x 20 cm x 250 µm). ^{1}H NMR and ^{13}C NMR data were collected using a Bruker AC-300 spectrometer and infrared spectral data were collected on a Perkin Elmer 1600 FTIR.

Supplementary material available

Preparation procedures and physicochemical data for compounds **7–17**, preparation and purification procedures for supercoiled plasmid DNA (pBR322) and procedures for 3' and 5'-^{32}P-labeling of restriction fragments.

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