$$-(\mathrm{AlaGly})_3\underset{4}{\mathrm{GluGly}}-$$

Finally, we note a recent report in which Takahashi and coworkers challenge the classical "polar" sheet model for *B. mori* silk fibroin and propose instead an apolar antiparallel structure with alanyl methyls extending from both sheet faces. Clearly, the availability of appropriately engineered polypeptides will facilitate the development of an understanding of the factors that control the conformational and crystallization behavior of polymeric solids, both natural and synthetic.

Conclusions

Genes encoding four different chain length variants of polymers of the nonapeptide sequence **1** have been constructed and expressed in *E. coli*, and the corresponding proteins have been isolated and characterized. There was no evidence of genetic instability of the synthetic DNA in the translation system used. Mass spectrometry confirms the size of the largest protein, which was obtained in a yield of approximately 100 mg from a 10-L fermentation. Structural analysis of powders and films prepared from 54 repeat variants of sequence **1** indicates that the polymers form amorphous glasses. These results demonstrate the biological feasibility of generating useful quantities of repetitive artificial proteins of this general class and illuminate further the issues to be addressed in the design of solid-state structure in polymers.

Acknowledgment. We thank Dr. Joseph Cappello of Protein Polymer Technologies, Inc., for the gift of p937.51 and for fruitful discussions regarding the gene construction described herein. Professor Brian Chait and Dr. Ronald Beavis of Rockefeller University provided mass spectra and insight into their interpretation. Dr. Harald Modler recorded the scattering pattern in Figure 6, Mr. Jeffrey Kollodge assisted in thermal analysis, and Professor E. D. T. Atkins of the University of Bristol has made many useful contributions to our thoughts regarding solid-state protein structure. This work was supported in part by the Center for University of Massachusetts-Industry Research on Polymers and in part by a grant from the Polymers and Genetics Programs of the National Science Foundation (NSF DMR 8914359). NMR and infrared spectra were recorded in the spectroscopy facility of the NSF Materials Research Laboratory at the University of Massachusetts.

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Cannizzaro-Based O_2-Dependent Cleavage of DNA by Quinocarcin

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Abstract: A novel mechanism for the reduction of molecular oxygen that results in the O_2-dependent cleavage of both single-stranded and double-stranded DNA by quinocarcin (**1**) is presented. The results are discussed in the context of a redox self-disproportionation (Cannizzaro-type) of the oxazolidine moiety of quinocarcin which produces superoxide.

It is now widely recognized[1] that a variety of clinically significant antitumor antibiotics can mediate oxygen-dependent cleavage of the ribose–phosphate backbone of cellular DNA and RNA. An enormous structural array of interesting natural products and semisynthetic and totally synthetic substances mediate oxidative strand scission of nucleic acids through three main families[2] of reactions: (1) metal-mediated activation of O_2 ultimately producing hydroxyl radical or other reactive oxygen species;[3-14] (2) non-metal-dependent generation of reactive carbon radicals[15] that mediate C–H abstraction from the deoxyribose backbone (the resulting deoxyribosyl radical subsequently reacts with molecular oxygen culminating in strand scission), and (3) photolytic production of hydroxyl radical,[16] which does not require metal participation for the DNA cleavage event. A rich array of chemistry can be found in the metal-dependent family of DNA damaging agents. Many readily oxidizable organic substances are capable of reducing molecular oxygen, resulting in the production of superoxide such as semiquinone radical anions, thiols,[17] and ascorbate, among others. Superoxide is well-documented[6,7,18,19] to be capable of mediating DNA strand breakage via dismutation to hydrogen peroxide and reduction of adventitious metals such as Fe(III) to Fe(II) (Haber–Weiss cycling) culminating in the reduction of hydrogen peroxide by Fe(II), generating the highly reactive hydroxyl radical (Fenton reaction).

The capacity of many antitumor antibiotics to cause oxidative damage to DNA in cancerous tissues is typically inseparable from the nonspecific damage inflicted on healthy cells by these reduced oxygen species and is widely recognized to be associated with the

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undesirable host toxicity of most antitumor drugs. The recognition of new mechanisms for the production of such reactive oxygen species and chemical means to attenuate this reactivity without compromising other modes of action often displayed by such substances, such as nucleic acid alkylation, intercalation, and DNA polymerase inhibition, will be essential to designing more specific and efficacious cancer chemotherapeutic agents. In this paper, we report observations that support an entirely new mechanism for O_2 reduction resulting in cleavage of DNA that is based on an auto-redox disproportionation (Cannizzaro-type) of the oxazolidine hemiacetal of **1** (Chart I).

Quinocarcin (**1**) is a natural secondary metabolite produced by *Streptomyces melanovinaceus* and is the simplest member of the naphthyridinomycin (**4**)/saframycin (**5**) class of antitumor agents.[20,21] Quinocarcin has been shown[20,22] to display weak antimicrobial activity against several Gram-positive microbes but is inactive toward Gram-negative bacteria. As its citrate salt, quinocarcin (named quinocarmycin citrate or KW2152) displays promising antitumor activity[22] against several lines of solid mammalian carcinomas including St-4 gastric carcinoma, Co-3 human colon carcinoma, MX-1 human mammary carcinoma, M5076 sarcoma, B16 melanoma, and P388 leukemia. This substance is currently under evaluation in human clinical trials by the Kyowa Hakko Kogyo Co., Japan. Very recently, the structurally related antitumor antibiotic tetrazomine (**3**) was isolated[23] from *Saccharothrix mutabilis* subsp. *chichijiimaensis* and is reported to display good antimicrobial activity against both Gram-positive and Gram-negative organisms in the 0.78–50.0 μg/mL range and also shows potent activity toward P-388 and L1210 leukemia in vitro.

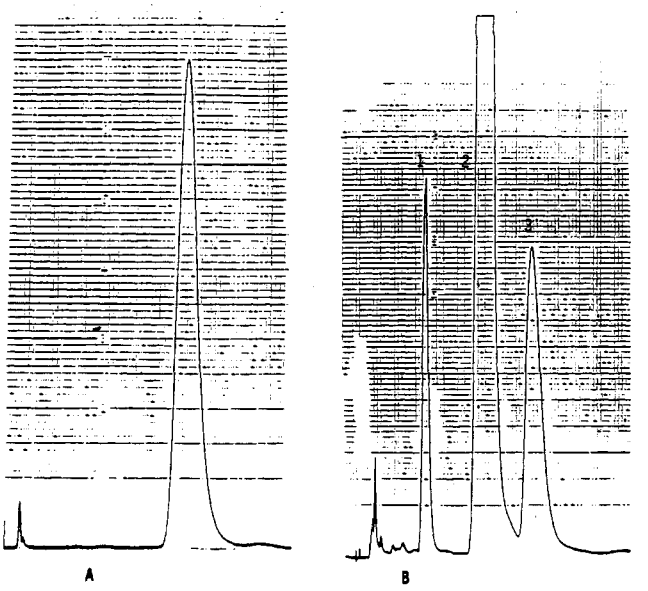

Figure 1. HPLC (C18 Resolve Pack column, 5% MeOH/5% MeCN in 6 mM potassium phosphate (pH 6.8) (isocratic)) of anaerobic quinocarcin (300 mg/mL) at 25 °C: (A) immediately after removal of citrate; (B) after 1 week. Peaks: 1, quinocarcinamide (**10**) 10 min; 2, quinocarcin (**1**) 16 min; 3, quinocarcinol (**2**) 22 min. This corresponds to a rate of disproportionation of $\sim 6 \times 10^{-8}$ M L^{-1} s^{-1}.

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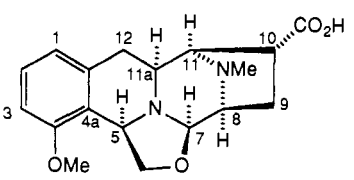
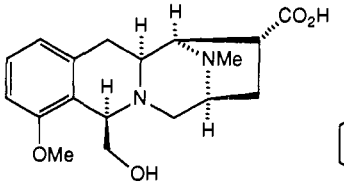
Chart I



1, *QUINOCARCIN, DC-52*



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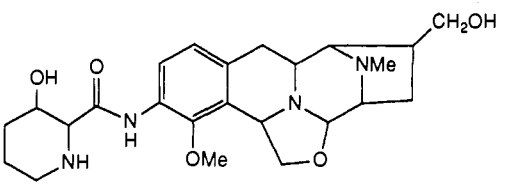
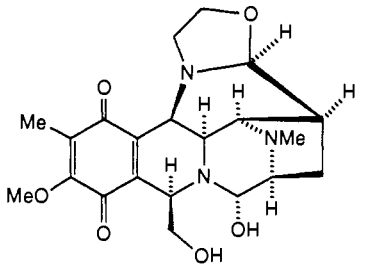
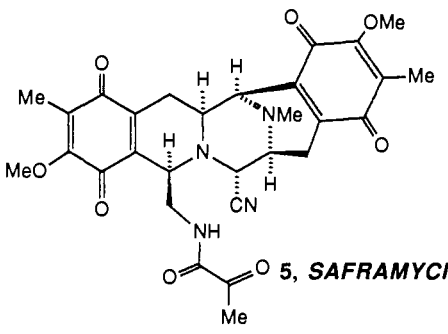


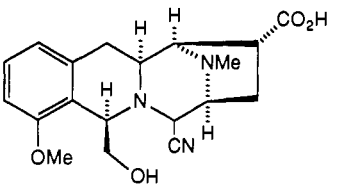
3, *TETRAZOMINE*



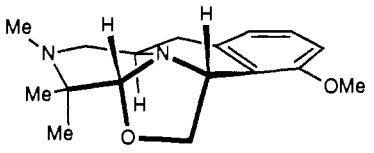
4, *NAPHTHYRIDINOMYCIN*



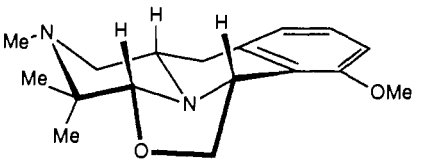
5, *SAFRAMYCIN A*



12, *DX-52-1*



13



14

Our interest in this substance stems from a report by Tomita et al.[24] that recorded the remarkable observation that **1** cleaves plasmid DNA in an O_2-dependent fashion that was reported (1) *not* to be stimulated by the addition of metal ions (Fe^{2+} or Cu^{2+}), (2) to be stimulated by dithiothreitol, (3) to be inihibited by oxygen-free radical scavengers such as methanol, *tert*-butyl alcohol, α-tocopherol, and β-carotene, and (4) to be inhibited by superoxide dismutase (SOD) and catalase. Quinocarcin blocks RNA synthesis in preference to DNA and protein synthesis in P388 leukemia cells.[22] On the other hand, in *Bacillus subtilis*, quinocarcin inhibited [^{3}H]thymidine incorporation, suggesting inhibition of DNA polymerase; therefore, DNA synthesis is thought[24] to be preferentially inhibited in *B. subtilis*. It has been proposed[22,24] that quinocarcin alkylates DNA in the minor groove[25] through the ring-opened form of the oxazolidine (iminium **6**). Similar DNA alkylation has been invoked for **4** and **5**. Indirect support[22,24] for the involvement of the oxazolidine ring in the above context comes from the lack of antitumor and DNA-damaging activity displayed by quinocarcinol (**2**, DC-52d), which is coproduced with **1** by *Streptomyces melanovinaceus*. Quinocarcinol also does not cleave plasmid DNA,[24] which forces the conclusion that the oxazolidine moiety is also responsible for the oxidative degradation of DNA by a previously unrecognized mechanism.

While it is not yet clear whether the antitumor properties of quinocarcin are a manifestation of only one mode of action (i.e., DNA alkylation) or both (DNA alkylation and oxidative DNA cleavage), we were intrigued by the oxidative cleavage observations of the Kyowa Hakko group[24] since **1** does not contain any readily recognizable functionality that would be associated with the capacity for oxidative DNA cleavage, such as metal chelation sites, quinones, and ene–diynes. Most likely, the efficacy of this drug is a delicate and intimate combination of multiple effects that are brought to bear on its macromolecular targets.

We have obtained experimental evidence presented herein that quinocarcin undergoes a redox self-disproportionation reaction that we propose is coupled to the capacity of this substance to effect the production of superoxide in the presence of molecular oxygen and results, at least in part, to Fenton-mediated lesions in DNA; a mechanism for this process is presented in Scheme I. Natural quinocarcin citrate (quinocarmycin citrate), obtained from Kyowa Hakko Kogyo, was separated from citric acid by ion-exchange chromatography (HP-20) and purified to homogeneity by reversed-phase HPLC. When the purified, colorless antibiotic was allowed to stand in carefully deoxygenated water at 25 °C, two new products are produced by HPLC analysis (Figure 1). The slower eluting peak has been isolated and identified as quinocarcinol (**2**); preparation of an authentic sample[20] from **1** rigorously confirms this assignment. The faster eluting peak has been identified as the amide **10** (herein named quinocarcinamide) based on IR, mass spectra, and fully decoupled ^{1}H NMR and ^{13}C NMR spectroscopy. Esterification of **10** with diazomethane in aqueous dioxane produced a substance whose ^{1}H NMR and IR spectra were identical to those of authentic, synthetic material,[26] providing final, rigorous structural confirmation. The identification of the anaerobic redox products **2** and **10** rigorously supports the proposal that **1** undergoes a Cannizzaro-type self-redox disproportionation; i.e., quinocarcin serves as its own reductant. Therefore, as suggested in Scheme I, single electron transfer from **1** with concomitant proton loss from the oxazolidine nitrogen to the ring-opened tautomer (**6**)[27] would furnish radical anion **7** and the oxazolidinyl radical **8**. Radical **8** should be capable of reducing a second equivalent of **6**, ultimately becoming oxazolidinium ion **9** which should hydrolyze to quinocarcinamide (**10**). Evidence for the intermediacy of **9** was secured by running the anaerobic disproportionation in 98% $^{18}OH_2$ and

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(27) It is also conceivable that electron transfer from **1** to molecular oxygen may occur directly or via a metal-mediated process in addition to the transfer shown via **6**.

Table I. Reduction of Nitroblue Tetrazolium by Quinocarcin and DX-52-1[a]

entry	substrate	pH	ΔOD/min at 500 nm ($\times 10^{-4}$)	rate constant, $M^{-1}\ s^{-1}$
1	1.0 mM quinocarcin	6	0	0
2	1.0 mM quinocarcin	7.0	3	4.2×10^{-4}
3	1.0 mM quinocarcin	7.4	5	6.8×10^{-4}
4	1.0 mM quinocarcin	8.0	8	1.1×10^{-3}
5	1.0 mM DX-52-1 (**12**)	7.0	0	0
6	1.0 mM DX-52-1 (**12**)	8.0	0	0
7	1.0 mM **13**	8.0	0	0
8	1.0 mM **14**	8.0	3	4.2×10^{4}
9	control phosphate buffer	8.0	0	0

[a] All reductions were carried out in 20 mM phosphate buffer at the indicated pHs. Formation of the furazan product was monitored using a Varian DMS-80 UV/vis spectrophotometer at 500 nm.

Table II. Cleavage of Plasmid Supercoiled DNA (pBR 322)

entry	conditions	[quinocarcin], mM	S^a
1	pH 5 (20 mM phosphate, 2 h, 37 °C)	1.0	0.3
2	pH 6 (20 mM phosphate, 2 h, 37 °C)	1.0	0.4
3	pH 7 (20 mM phosphate, 2 h, 37 °C)	1.0	1.5
4	pH 8 (20 mM phosphate, 2 h, 37 °C)	1.0	7.8
5	pH 9 (20 mM phosphate, 2 h, 37 °C)	1.0	11.0
6	pH 8 (20 mM phosphate, 4 h, 37 °C)	1.0	19.2
7	H_2O (no buffer, 2 h, 37 °C)	1.0	0.7
8	H_2O (no buffer, 24 h, 37 °C)	1.0	11.4
9	pH 8 (20 mM phosphate, 2 h, 37 °C)	0.1	0.3
10	pH 8 (20 mM Tris, 2 h, 37 °C)	1.0	0.3
11	pH 8 (20 mM phosphate, 2 h, 37 °C)	5.0 (DX-52-1)	0
12	pH 8 (20 mM phosphate, 2 h, 37 °C)	1.0 (quinocarcinamide)	0
13	pH 8 (20 mM phosphate, 2 h, 37 °C)	1.0 (quinocarcinol)	0

[a] The S value for the DNA control represents the amount of nicked open circular DNA present in the starting plasmid and was subtracted from the S values calculated for the individual cleavage reactions. When only forms I (supercoiled) and forms II (nicked open circular) are present, the equation simplifies to $S = -\ln f_I$, where f_I is the fraction of form I molecules. 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 pBR 322 (4362 base pairs).

analyzing the product **10** by mass spectroscopy; greater than 40% ^{18}O was incorporated at the amide carbonyl.[28] Under anaerobic conditions, radical anion **7** subsequently suffers a second electron transfer (presumably from **1** or **8**) with concomitant protonation, resulting in quinocarcinol (**2**). Under aerobic conditions, radical anion **7** can react with molecular oxygen to produce peroxy radical anion **11** which, with nitrogen participation, expels 1 mol equiv of superoxide, regenerating **6**. It is also mechanistically plausible that the putative peroxy radical anion **11** could fragment directly to amide **10** via homolysis of the O–O bond and directly generate hydroxyl radical. A labeling experiment, however, demonstrated that this is not the case. Disproportionation of pure **1** was carried out in water as described above under an atmosphere of 98% $^{18}O_2$. Quinocarcinol (**2**) and quinocarcinamide (**10**) were isolated by reversed-phase HPLC and subjected to mass spectral analysis following diazomethane esterification. The fragmentation pattern of **10** at $m/e = 329$ showed no significant enhancement at the M + 2 peak ($m/e = 331$) that would have been diagnostic for

(28) Mass spectral analysis required esterification of the crude products with diazomethane. The base fragment for the corresponding methyl ester of amide **9** was $m/e = 329$ ($M^+ - CH_2O$); the remainder of the ^{18}O label (~60%) is presumed to reside in the hydroxymethyl moiety due to the ambient electrophilic nature of iminium **8** to hydrolytic capture. Loss of the hydroxymethyl moiety ($m/e = 30$) is the parent ionization process, generating the corresponding stabilized isoquinolinium amide radical cation ($m/e = 329$). It was not possible to quantitate ^{18}O incorporation in the hydroxymethyl moiety ($m/e = 32$) since this appears as a large fragment in both the unlabeled and ^{18}O-labeled samples.

Table III. Effect of Additives on the Cleavage of Plasmid DNA by Quinocarcin[a,b]

entry	reagent	[quinocarcin], mM	% inhibn	% enhancement
1	SOD (10 μg/mL)	1.0	>99	
2	catalase (10 μg/mL)	1.0	33	
3	catalase (100 μg/mL)	1.0	83	
4	deoxygenated mixture[c]	1.0	84	
5	desferal (0.1 mM)	1.0	24	
6	desferal (1.0 mM)	1.0	87	
7	desferal (10 mM)	1.0	>99	
8	Fe(III)/desferal (0.1 mM)	1.0	27	
9	Fe(III)/desferal (1.0 mM)	1.0	91	
10	EDTA (1.0 mM)	1.0	35	
11	DETAPAC (1.0 mM)	1.0	86	
12	picolinic acid (1.0 mM)	1.0	22	
13	picolinic acid (10 mM)	1.0	94	
14	Fe(III) (0.1 mM)	1.0	*d*	
15	H_2O_2 (0.1 mM)	1.0		143
16	H_2O_2 (0.1 mM)	0.1		141
17	DTT (0.1 mM)	1.0	18	
18	DTT (5.0 Mm)	1.0	98	
19	DTT (0.1 mM)	0.1		139
20	DTT (1.0 mM)	0.1		59
21	DTT (5 mM)	0.1	73	

[a] See the Experimental Section. [b] All reactants were mixed at ice bath temperature and brought to a final volume of 10 μL. All reactions were then incubated for 2 h at 37 °C in 20 mM phosphate buffer at pH 8 with 0.15 μg of pBR 322 plasmid DNA. Control reactions for SOD, catalase, iron(III) ammonium sulfate, DTT, desferal, EDTA, DETAPAC (diethylenetriaminepentaacetic acid), H_2O_2, and picolinic acid at the indicated concentrations in the absence of quinocarcin all showed either very marginal or no detectable cleavage of the DNA. [c] The reactants were all mixed at ice bath temperature and then purged with argon gas; the mixture was then brought to 37 °C. [d] No significant inhibition or enhancement of quinocarcin-induced DNA cleavage was observed.

Table IV. Nicking Efficiency as a Function of Superoxide Production (pBR 322 DNA)[a]

entry	reagent	S	ΔΔOD/min[b]	$NE_{superoxide}$
1	ascorbate	5.4	1.653	157
2	DTT	0.28	0.773	19
3	quinocarcin	9.5	0.0013	3.8×10^4
4	**14**	0.38	0.0006	3.3×10^3

[a] All reactions employed 0.15 μg of pBR 322. [b] SOD concentration was 50 μg/mL for ascorbate and 5 μg/mL for all others.

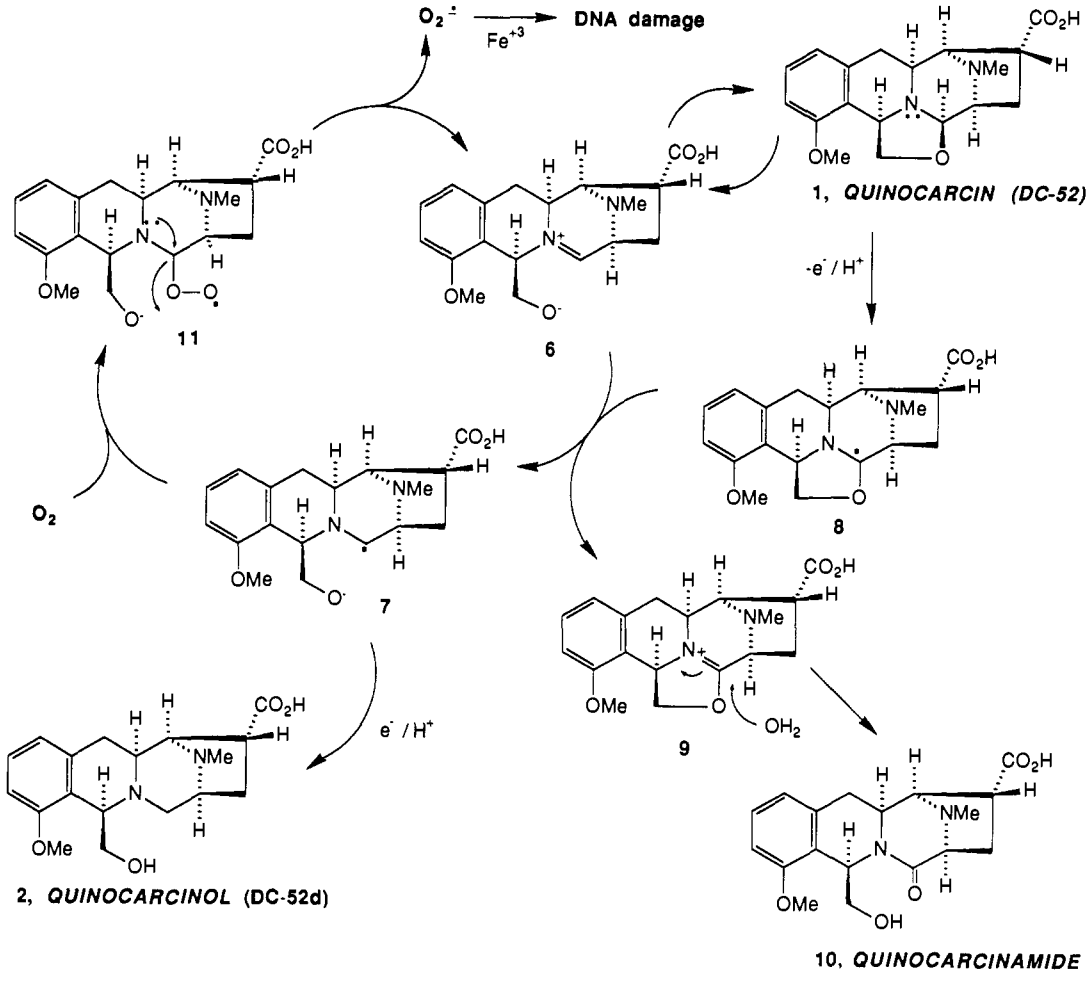
^{18}O incorporation at the amide carbonyl by the peroxide homolysis possibility alluded to above. The authentic ^{18}O-labeled amide **10** obtained as described above from anaerobic disproportionation in $^{18}OH_2$ showed no propensity to exchange with $^{16}OH_2$ after several days at room temperature as evidenced by the same mass spectral analytical protocol. Neither **2**, **10**, nor the semisynthetic cyano derivative of quinocarcin (DX-52-1, **12**) produce superoxide, as evidenced by the complete lack of nitroblue tetrazolium reduction and their corresponding incapacity to mediate oxidative scission of DNA. On the basis of these observations and the chemical redox chemistry of quinocarcin, we propose the Cannizzaro-driven reduction of molecular oxygen as illustrated in Scheme I.

Superoxide production by quinocarcin was previously reported by Tomita,[24] although a mechanistic explanation was not offered. We have carefully examined superoxide production by quinocarcin and two synthetic analogues,[29] **13** and **14**, by following the reduction of nitroblue tetrazolium (NBT)[17,30] under various pH conditions and find (for **1** and **14**) that this reaction is completely

(29) Williams, R. M.; Glinka, T.; Gallegos, R.; Ehrlich, P. P.; Flanagan, M. E.; Coffman, H.; Park, G. *Tetrahedron* **1991**, *47*, 2629.

(30) Tsou, K-C.; Cheng, C.-S.; Nachlas, M. M.; Seligman, A. M. *J. Am. Chem. Soc.* **1956**, *78*, 6139.

Scheme I



inhibited by SOD; the results are collected in Table I. The reduction of NBT by quinocarcin is pH dependent, exhibiting an increased rate of reduction as the pH is raised. This behavior directly parallels the pH dependency for the DNA cleavage reactions described below.

In order to examine the interaction of quinocarcin with DNA, the pure, colorless antibiotic (0.01–50 mM) was allowed to react with pBR 322 supercoiled plasmid DNA between pH 5 and 9 in phosphate buffer (20 mM) at 37 °C for 2 h in the presence of air. Salient experimental results are collected in Tables II and III. Nicking of the DNA was visualized by 0.8% agarose gel electrophoresis; ethidium bromide solution (0.5 μg/mL) was added to the gel after the gel was run. Quinocarcin showed significant nicking of the DNA at 0.1 mM concentration (Table II, entry 9) at pH 8 without the addition of any external reductants. The reaction was found to be pH dependent, optimal cleavage being observed between pH 8 and 9. At lower pH values (pH 5–7) nicking was observed but was significantly less than at higher pH. This is consistent with the obligate participation of the *unprotonated* oxazolidine nitrogen atom in the redox cycle. Consistent with this behavior, quinocarmycin citrate and DX-52-1, neither of which undergoes the Cannizzaro-type self-redox reaction, displayed a markedly inferior relative ability to nick the DNA at the same concentrations as the purified **1**. Exclusion of oxygen significantly inhibited this reaction (84% inhibition) as expected (Table III, entry 4). DTT at low concentrations enhanced the reaction at low concentrations (0.1 mM) of quinocarcin (Table III, entries 19 and 20) but showed *inhibitory* activity at high concentrations (5 mM; Table III, entries 18 and 21). This is presumably due to the capacity of DTT to serve as a competing CH (or SH) substrate with DNA, which is present in much lower relative concentration. Similar observations on the effect of optimum concentrations of reducing agents on the cleavage of DNA by MPE–Fe(II) have been reported.[9] Independent corroboration of this phenomena was obtained by comparing the relative reactivity of quinocarcin in phosphate and Tris buffers. At 20 mM buffer concentrations, Tris effectively inhibited the cleavage of the plasmid DNA (compare Table II, entries 4 and 10) by quinocarcin. Again, this would indicate that potentially any organic substance can compete with DNA for the Fenton-derived oxidant and that the majority of the reactive oxidant is most likely produced in a non-DNA-associated environment. Superoxide dismutase completely inhibited DNA cleavage (Table III, entry 1), consistent with both the capacity of quinocarcin to generate superoxide and the corresponding DNA cleavage event to be exclusively superoxide dependent. Catalase also inhibits the reaction but is not as potent as SOD (Table III, entries 2 and 3). Addition of hydrogen peroxide to quinocarcin/DNA reactions had a potent stimulatory effect on DNA cleavage over control reactions containing hydrogen peroxide at the same concentrations (Table III, entries 15 and 16). Taken together, the above results point strongly to Fenton-type chemistry being responsible for the scission of DNA. Tomita previously reported[24] that the addition of iron or copper salts had no stimulatory effects on the ability of quinocarcin to cleave DNA; we have corroborated this finding (Table III, entry 14). The addition of the potent iron chelator desferal did, however, lead to significant inhibition of DNA cleavage, particularly at high concentrations (Table III, entries 5–9). Other iron chelators (EDTA, DETAPAC; Table III, entries 10 and 11) also exhibit inhibitory activity. Desferal is known[6] to have a high affinity for Fe(III) (log k_f = 30.7), forming a hexacoordinate complex that excludes iron-associated water and uncouples the oxidation of Fe(II) from the formation hydroxyl radical (Fenton reaction). The participation of higher oxidation states of iron and copper in Fenton reactions and related C–H oxidation chemistry is now well recognized.[7] Picolinic acid is known[31] to be a very potent

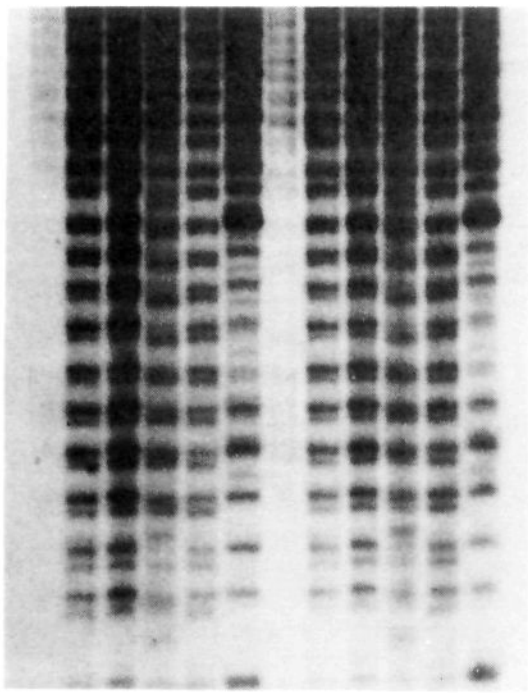


Figure 2. Key: lane 1, single-stranded DNA control; lane 2, single-stranded DNA + quinocarcin (1 mM); lane 3, single-stranded DNA + quinocarcin (5 mM); lane 4, single-stranded DNA + quinocarcin (5 mM) followed by T4 kinase; lane 5, single-stranded DNA + $FeSO_4$ (1 mM); lane 6, Maxim–Gilbert G reaction; lane 7, double-stranded DNA control; lane 8, double-stranded DNA + quinocarcin (1 mM); lane 9, double-stranded DNA + quinocarcin (5 mM); lane 10, double-stranded DNA + quinocarcin (5 mM) followed by T4 kinase; lane 11, double-stranded DNA + $FeSO_4$ (1 mM); lane 12, Maxim–Gilbert G reaction. All reactions with quinocarcin are thiol-free. The photograph of the gel was focused on the clearest region to show the double bands (Tullius bands) characteristic of nonselective hydroxyl radical cleavage. There was no significant evidence for any sequence specificity to the cleavage of this substrate (sequence along the gel is therefore superfluous and has been deleted).

scavenger of hydroxyl radical and inhibitor of the Fe(II)/Fe(III) redox couple. Addition of picolinic acid to reaction mixtures of DNA and quinocarcin at 1 and 10 mM showed 22% and 94% inhibition, respectively. These results also support the notion that adventitious metal in these reaction mixtures in a number of possible oxidation states can be activated by the slow release of superoxide and cause Fenton-related damage to DNA. Thus, these results indicate that, in contrast to the conclusions of Tomita,[24] the DNA cleavage is indeed metal dependent and that the low concentration of adventitious Fe^{3+} is already in excess to that required to effect Fenton-mediated cleavage of the DNA; addition of excess iron would therefore not be expected to have any additive effect. Thus, our data support a hypothesis wherein the limiting reagent in the DNA cleavage mediated by quinocarcin is the slow production of superoxide.

Further evidence for a non-DNA-associated oxidant was obtained from an analysis of the reaction of quinocarcin with a small synthetic oligonucleotide by high-resolution polyacrylamide gel electrophoresis. A synthetic 64-base-pair oligonucleotide with the sequence shown in Figure 2 was 5′-end-labeled with ^{32}P, purified by Sephadex G-50 size exclusion chromatography, and annealed to the complementary strand. Reaction of both the duplex 64-mer and the ^{32}P 5′-end-labeled single-stranded oligonucleotide with quinocarcin (1 and 5 mM, without additional reducing agent added) at 37 °C for 5 h resulted in non-sequence-specific cleavage at every single nucleotide as evidenced by denaturing 20% polyacrylamide gel electrophoresis (Figure 2, lanes 2, 3, 8, and 9). Both the double-stranded and single-stranded substrates were cleaved with roughly equal efficiency, a slight preference being noted for single-stranded DNA.[32] Furthermore, every cleavage band appeared as a doublet, which is characteristic of the 3′-phosphate and 3′-phosphoglycolate ends resulting from nonselective Fenton-mediated cleavage.[8,9] We observed the exact same cleavage pattern when the duplex was incubated with 1 mM $FeSO_4$ under aerobic conditions (Figure 2, lanes 5 and 11). Treatment of the quinocarcin-damaged DNA reaction mixtures with T4 polynucleotide kinase in the absence of ATP or ADP exhibited the expected 3-phosphatase gel band shift of the slower moving band of each doublet (Figure 2, lanes 4 and 10) indicative that the slower moving band of each doublet is the 3′-phosphoryl and the faster moving band is the 3′-phosphoglycolate.

Compared with other antitumor substances that cause oxidative damage to DNA, quinocarcin is rather modest in terms of the rate at which DNA cleavage can be measured at a given concentration of drug. This is a direct manifestation of the very slow production of superoxide by quinocarcin. However, one interesting feature of the cleavage reaction of DNA by quinocarcin is that it is vastly more efficient in effecting DNA cleavage *per equivalent of superoxide* generated than other superoxide generating systems, such as DTT and ascorbate. For a qualitative comparison, we have calculated a nicking efficiency ($NE_{superoxide}$) of DNA cleavage as a function of superoxide production (as measured by NBT reduction) for quinocarcin, DTT, and ascorbate. Total superoxide production was estimated by taking the difference between the $\Delta OD/min$ at 500 nm for NBT reduction for each reagent and subtracting the $\Delta OD/min$ under the same conditions in the presence of SOD. The value obtained ($\Delta\Delta OD/min$) can at least be attributed to specific superoxide-mediated reduction of NBT as opposed to direct reduction of the dye by the organic reductant. The equation used was as follows

$$NE_{superoxide} = \frac{(S)(n_{DNA})/n_{substrate}}{(\Delta\Delta OD/min)_{substrate}}$$

where S is the average number of single hits per DNA molecule calculated as described in the footnotes of Table II and n is the number of molecules present. Quinocarcin is 240 times more efficient in effecting DNA cleavage *per superoxide molecule* than ascorbate and 2000 times more efficient than DTT. Synthetic compound **14** is inferior to quinocarcin but is still relatively efficient per superoxide equivalent (21 times versus ascorbate and 173 times versus DTT). The relative differences between quinocarcin and **14** with respect to DNA cleavage are likely a result of the poor solubility of **14** in water; the HCl or citrate salts of **14** were employed in all these studies resulting in protonation of the amines which attenuates the rate of superoxide release (vida infra). Since quinocarcin and synthetic analogue **14** both produce superoxide very slowly compared to DTT and ascorbate, the observed apparent relative efficiency of DNA cleavage as a function of superoxide production for these oxazolidine-containing substances appears to be a direct manifestation of the relative kinetics of superoxide release, although the mechanism for presentation of the oxidant to DNA may be distinct; a detailed explanation cannot be offered at this time. The rate of formation of superoxide by

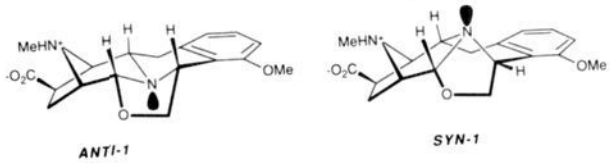
(31) Sheu, C.; Richert, S. A.; Cofre, P.; Ross, B.; Sobkowiak, A.; Sawyer, D. T.; Kanofsky, J. R. *J. Am. Chem. Soc.* **1990**, *112*, 1936 and referenced cited therein.

(32) Celander D. W.; Cech, T. R. *Biochemistry* **1990**, *29*, 1355. Jezewska, M. J.; Bujalowski, W.; Lohman, T. M. *Biochemistry* **1990**, *29*, 5220.

(33) It should be noted that DX-52-1 and various C-8 and C-10 substituted derivatives maintain significant antitumor activity; see ref 22 (Saito et al., 1990). Hirata, T.; Kobayashi, S.; Takahashi, K.; Morimoto, M.; Saito, H.; Sato, A.; Ashizawa, T. European Patent 128,370, 1984.

quinocarcin[34] (see Table I) is extremely slow (10^4–10^5 times slower) relative to the rate-limiting step[18] of the Haber–Weiss/Fenton reaction, which is 76 M^{-1} s^{-1} for the reduction of hydrogen peroxide by Fe(II). In this context, quinocarcin may prove to be a useful mechanistic tool for studying superoxide-mediated reactions where the slow, controlled release of superoxide would be desired.

Remers[25] has conducted molecular mechanics calculations on quinocarcin by docking the drug in the minor groove. From this study, it was concluded that the absolute configuration of quinocarcin is most likely that depicted in Scheme I. The calculations suggested that the lowest energy conformer of **1** orients the piperazine ring in a chairlike conformation, which therefore places the oxazolidine nitrogen lone pair in an antiperiplanar orientation to the oxazolidine methine (*anti*-**1**). Ring opening of the oxa-



ANTI-1 *SYN-1*

zolidine to the iminium species (see **6**, Scheme I) requires nitrogen pyrimidal inversion to a higher energy twist-boat conformer (*syn*-**1**) that was calculated to lie ~10 kcal mol^{-1} above the other conformer. In this situation, the oxazolidine nitrogen lone pair is syn to the methine and antiperiplanar to the C–O bond. It was postulated[25] that the iminium species[33] should be a good alkylator for N-2 of guanine in the minor groove of the sequence d-$(ATGCAT)_2$. Based on the similarity to **4** and **5**,[21] this is a very reasonable expectation. In the present study, we wished to ask a different question regarding the conformational significance of the oxazolidine moiety. As shown in Scheme I, the initial step in the electron transfer between the oxazolidine and the iminimum species involves one-electron loss from the oxazolidine nitrogen with loss of the oxazolidine methine as a proton producing the reduction and oxidation radicals **7** and **8**, respectively. It is reasonable to expect that the trans, antiperiplanar arrangement of the oxazolidine methine and nitrogen lone pair in the lower energy conformer predicted by calculation should also be the most favorable geometry for concomitant one-electron and proton loss in the redox self-disproportionation, since this arrangement provides maximum overlap in the transition state. Synthetic analogues **13** and **14** have the conformations depicted as determined by single-crystal X-ray analyses.[29] Compound **13** mimics the syn conformer of quinocarcin, while **14** mimics the anti conformer of quinocarcin. The relative difference in the capacity of synthetic analogues **13** and **14** to effect both superoxide production (cf. Table I, entries 7 and 8) as well as DNA cleavage (at 5 mM, **13** cleaves plasmid DNA only one-sixth as efficiently as **14**)[29] supports our hypothesis that stereoelectronic control elements of the oxazolidine ring system are intimately related to the biological activities of these substances.

In summary, we have established a previously unrecognized reaction for the reduction of molecular oxygen by a simple heterocyclic ring system. This reduction is driven by the inherent intermolecular redox chemistry of the drug itself, requiring no exogenous reductants; this reaction can be assisted by external reductants, effectively lowering the concentration of drug required to damage DNA. In this context, it is interesting to question the creation of quinocarcinol (**2**) as arising by a genetically encoded biosynthetic pathway or being an artifact of the secondary redox chemistry of quinocarcin discovered herein or a related secondary and endogenous microbial reduction. Chemical means to attenuate the ability of this class of antitumor drugs to produce reactive oxidants (**13** versus **14**, **12**, stabilizers such as citric acid, stimulators such as DTT and hydrogen peroxide) may contribute to possible approaches to designing more selective and less toxic cancer chemotherapeutic agents. Studies aimed at elucidating the details of the expected covalent interactions of this class of compounds with nucleic acid targets are under investigation in these laboratories.

(34) The rate of superoxide release from quinocarcin is of the same order of magnitude as the rate of redox disproportionation (~6×10^{-8} M L^{-1} s^{-1}) of quinocarcin under anaerobic conditions.

Experimental Section

Purification of 1 from Citric Acid. From quinocarcin citrate, which was a gift from Kyowa Hakko Kogyo, was obtained free quinocarcin by dissolving quinocarcin citrate in water and passing it through HP-20 ion-exchange resin (Mitsubishi Corp.) at 4 °C. Citric acid was eluted with water, and subsequently free quinocarcin was eluted with methanol/water (3/1). Free quinocarcin was further purified by HPLC on a C18 Resolve Pack column (Waters) using 5% methanol/5% acetonitrile in 6.0 mM, pH 6.8 potassium phosphate buffer (isocratic). To remove the phosphate buffer from the lyophilized quinocarcin fraction, the residue was dissolved in water and passed through an HP-20 column in the same manner as described above.

Disproportionation of 1 and HPLC Analysis of Products. A 300 μg/mL solution of citrate-free **1** was made up in deionized water which had been deoxygenated under vacuum followed by purging with nitrogen. A sample was then analyzed by HPLC (C18 Resolve Pack column (Waters); 5% methanol/5% acetonitrile in 6 mM potassium phosphate, pH 6.8 (isocratic); and detected by UV at 270 nm) which revealed only the peak at 16 min corresponding to **1**. The solution was then allowed to age at 25 °C under anaerobic conditions, with aliquots being taken periodically and analyzed by HPLC for quinocarcin and formation of **10** and **2** (retention times 10 and 22 min, respectively). Authentic quinocarcinol (**2**) was obtained from quinocarcin by $NaBH_4$ reduction according to Tomita.[20] The authentic sample of quinocarcinol was identical to that obtained by the disproportionation of **1** described above by ^1H NMR, IR, and TLC (silica gel; 10% H_2O in ethanol). Data for quinocarcinamide (**10**). ^1H NMR (300 MHz) (D_2O) δ HOD: 2.34 (1 H, dd, J = 13.6 Hz, J = 9.9 Hz); 2.38 (3 H, s); 2.59 (1 H, ddd, J = 13.6 Hz, J = 7.0 Hz, J = 7.0 Hz); 2.78 (1 H, m); 3.03 (1 H, m); 3.22 (1 H, dd, J = 7.0 Hz, J = 10.3 Hz); 3.58 (1 H, d, J = 6.6 Hz); 3.70 (1 H, dd, J = 11.4 Hz, J = 3.9 Hz); 3.79 (1 H, m); 3.82 (1 H, br s); 3.87 (3 H, s); 3.93 (1 H, dd, J = 11.4 Hz, J = 4.4 Hz); 5.45 (1 H, t, J = 4.0 Hz); 6.94 (1 H, d, J = 7.8 Hz); 7.04 (1 H, d, J = 7.8 Hz); 7.33 (1 H, d, J = 7.8 Hz). IR (KBr): 3601, 3430, 2929, 2340, 2020, 1897, 1792, 1626, 1580, 1388, 1079 cm^{-1}. Additional structural verification was secured through esterification to quinocarcinamide methyl ester as follows. To 2.0 mL of 1 M NaOH was added 5 mg of 1-methyl-3-nitro-1-nitrosoguanidine (Aldrich). The diazomethane formed was carried via nitrogen pressure bubbling through 2.0 mL of a solution of dioxane/H_2O (1/1) containing 1 mg of **10**. Once the dioxane solution turned yellow, the flask was sealed and the mixture was allowed to stand at room temperature for 30 min. The mixture was then concentrated to dryness, affording 1 mg of the corresponding methyl ester whose ^1H NMR matched that of a synthetic sample provided by Prof. S. J. Danishefsky. This procedure was also utilized for aliquoting the ^{18}O experiments for mass spectral determinations (see ref 28).

Reductions of Nitroblue Tetrazolium by 1 and 12–14. Each reaction was performed in triplicate by adding each substrate to an aerated solution of nitroblue tetrazolium (0.12 mM) in 20 mM phosphate buffer at the indicated pH such that the final concentration of substrate was 1.0 mM. The optical absorbance was measured over a 30-min period at 500 nm, and the ΔOD was the average slope for the linear OD change over the reaction time. Addition of SOD (5 μg/mL) completely inhibited the reduction in all relevant cases (entries 2–4 and 8). The rate constants were calculated by assuming that $[O_2]$ does not appreciably change and is in excess (zero order in oxygen); the reaction is second order with respect to [quinocarcin] or [**14**] (i.e., the only substates that effect the reduction of NBT). The second-order rate constants reported in Table I were calculated from the ΔOD measurements and based on an ϵ_0 of 12 200 for the furazan product of NBT at 500 nm.

Cleavage of Supercoiled Plasmid DNA (pBR322). DNA nicking reaction mixtures were made by addition at 0 °C of appropriate amounts of reagent stock solutions to a stock solution of pBR 322 DNA plasmid (Boehringer-Mannheim Biochemical Co.) containing 0.15 μg of DNA/reaction (20 μM base pair concentration). The total volumes of the reaction mixtures were brought up to 10 μL with distilled and deionized water when necessary, and the reaction mixtures were incubated at 37 °C for 2 h in tightly capped plastic Eppendorf tubes. Stock solutions for experiments including DNA were prepared using distilled, deionized water and commercially available reagents: DTT, Sigma; sodium phosphate monobasic, EM Science; sodium phosphate dibasic, 30% hydrogen peroxide, Malinckrodt; superoxide dismutase, beef liver catalase (suspension in water), Boehringer Mannheim Biochemical. Desferal was the generous gift from Ciba-Geigy Co. The cleavage of plasmid DNA was detected by loading the reactions onto 0.8% agarose gels and staining with ethidium bromide after electrophoresis. The

electrophoreses were run for 2 h at 55 V, and the gels were submerged for 15 min in ethidium bromide solution. The electrophoresis gels were immediately visualized on a UV transilluminator and photographed using black and white instant films (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. All reactants were mixed at ice bath temperature and brought to a final volume of 10 μL. All reactions were then incubated for 2 h at 37 °C in the indicated buffer with 0.15 μg of pBR 322 plasmid DNA.

Labeling (5′, ^{32}P) and Reactions of Synthetic 64-mer for High-Resolution Polyacrylamide Gel Electrophoresis. To a solution of the synthetic deoxyoligonucleotide (200 pmol) in 105 μL of deionized distilled H_2O was added 20 μL of polynucleotide kinase buffer, 4 μL (40 units) of T4 polynucleotide kinase (New England Biolabs), and 2 μL (20 μCi) of [γ†^{32}P]ATP (Du Pont). The reaction was incubated for 90 min at 37 °C and 10 min at 65 °C. The solution was loaded onto a 2-mL column of Sephadex G-50 and eluted with TE buffer (10 mM Tris, 1.0 mM EDTA, pH 8). The first radioactive fraction was collected and precipitated with ethanol/3 M NaOAc (pH 5.2) and dried. Annealing was performed by mixing equimolar amounts of the 5′ ^{32}P-end-labeled strand and the complimentary strand in deionized distilled H_2O to a final concentration of 2 pmol/μL; each was heated to 65 °C for 30 min and slowly cooled to 0 °C. The double-stranded and single-stranded DNA substrates (5 pmol in 3 μL of deionized distilled H_2O) were brought to a final reaction volume of 16 μL in phosphate buffer (20 mM, pH 8). Each reaction was incubated at 37 °C for 5 h. None of these reactions contained any additional reducing agents such as DTT. One microliter of 3 M NaOAc (pH 5.2), 5 μL of t-RNA (1 mg/mL), and 100 μL of ethanol were added, and the mixture was cooled to −70 °C for 10 min. Each tube was centrifuged at 14K (rpm, Eppendorf microfuge) for 10 min at 4 °C, the supernatant decanted, and the DNA pellet dried under reduced pressure. To each sample for lanes 3–5 and 10 was added 10 μL of deionized distilled H_2O followed by heating to 90 °C for 10 min and then cooling on ice. To each reaction tube was added 4 μL of polynucleotide kinase buffer and 4 μL (40 units) of T4 polynucleotide kinase (New England Biolabs). After brief vortexing, each reaction was incubated at 37 °C for 1 h. Each sample was ethanol precipitated, centrifuged, and dried for electrophoresis. To each dried pellet of DNA was added 10 μL of loading buffer (formamide, 10 mM EDTA, pH 8, 0.025% xylenecyanol FF and 0.025% bromophenol blue), and the resultant mixture was then heated to 90 °C for 5 min, placed on ice, and immediately loaded onto a 20% denaturing (urea) polyacrylamide gel. After the gel was run (4 h, 1600 V), it was fixed (acetic acid/methanol, 10% each, aqueous), absorbed onto filter blotting paper, and vacuum dried. The bands were visualized by autoradiography.

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