Bi[5,5-bis(hydroxymethyl)-3-methyl-2-oxomorpholin-3-yl] (BHM-3 Dimer). A Low Toxicity, Water-Soluble, One-Electron Reducing Agent

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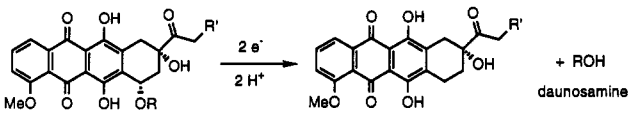
Bi[5,5-bis(hydroxymethyl)-3-methyl-2-oxomorpholin-3-yl] (BHM-3 dimer) was synthesized by condensing tris(hydroxymethyl)aminomethane with ethyl pyruvate to form 5,6-dihydro-5,5-bis-(hydroxymethyl)-3-methyl-1,4-oxazin-2-one (1) followed by photoreduction in 2-propanol. BHM-3 dimer exists in equilibrium with 5,5-bis(hydroxymethyl)-3-methyl-2-oxomorpholin-3-yl (BHM-3) in solution; the rate constant for BHM-3 formation from dimers varies with solvent increasing from 5.3×10^{-6} s^{-1} in acetonitrile to 1.5×10^{-3} s^{-1} in water at 25 °C. BHM-3 reacts as a one-electron reducing agent, and the reduction potential for BHM-3 dimer is estimated at −0.54 V vs NHE from the position of equilibrium for the reduction of the viologen, propyldiquat, to its radical cation. BHM-3 dimer has an octanol/water partition coefficient of 0.054 and shows low mouse toxicity even upon intravenous administration. Intravenously administered BHM-3 dimer projects mice from an immediated subsequent lethal injection of the antitumor drug adriamycin. The properties of BHM-3 dimer are compared with those of other 2-oxomorpholin-3-yl dimers.

Introduction

We have been interested in the synthesis and biological applications of 2-oxomorpholin-3-yl radicals and their associated dimers for some years.[1-4] The parent system is 3,5,5-trimethyl-2-oxomorpholin-3-yl (TM-3) which exists in equilibrium with *meso*- and *dl*-bi(3,5,5-trimethyl-2-oxomorpholin-3-yl)s (*meso*- and *dl*-TM-3 dimers) in solution.[1] Structural factors which lead to the facile homolysis of the 3,3′-bond have recently been discussed on the basis of C–H bond energies at the 3-position of precursor 2-oxomorpholines from photoacoustic calorimetric measurements.[5] Important factors are electronic stabilization and relief of ring and steric strain. At least a portion of the electronic stabilization is from captodative[6] or merostabilization,[7] the synergetic radical stabilization by the electron-donating and -withdrawing functional groups.[8]

TM-3 radical reacts as a one-electron reducing agent with a reduction potential of −0.58 V vs the normal hydrogen electrode (NHE) in methanol at an apparent pH of 7.[9,10] Of particular importance has been the use of TM-3 dimers and a more water-soluble derivative, bi[3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl] (DHM-3 dimers), as *in vitro* reducing agents for the anthracycline and mitomycin antitumor drugs.[11,12] As such they have proven to be useful tools for elucidating the complex redox chemistry of these drugs, which is likely a component of their biological activity. The best known example is the reduction of adriamycin and daunomycin to 7-deoxyadriamycinone and 7-deoxydaunomycinone, respectively. The oxomorpholinyl radical dimers also show low animal toxicity and, consequently, have potential for *in vivo* reduction of antitumor drugs, especially adriamycin, to therapeutic advantage.[13-15]



R'=OH, adriamycin
R'=H, daunomycin

R'=OH, 7-deoxyadriamycinone
R'=H, 7-deoxydaunomycinone

The DHM-3 dimer structure is complicated by four stereocenters which give rise to six possible diastereomers. The diastereomers exist in equilibrium in solution through the facile 3,3′-bond homolysis; as a result, purification has proven to be a challenge.[3] Prior to the synthesis and characterization of DHM-3 dimers, the synthesis of the stereochemically simpler water-soluble system, *meso*- and *dl*-bi[(5,5-bis(hyrodroxymethyl)-3-methyl-2-oxomorpholin-3-yl] (BHM-3 dimers) was attempted but not achieved. We now report a successful synthesis of BHM-3 dimers and comparison with DHM-3 and TM-3 dimers.

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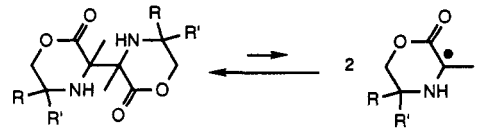


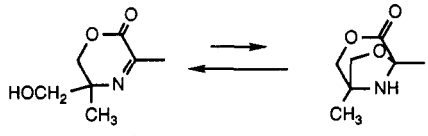
R=R'=CH_3, TM-3 dimers R=R'=CH_3, TM-3

R=CH_3, R'=CH_2OH, DHM-3 dimers R=CH_3, R'=CH_2OH, DHM-3

R=R'=CH_2OH, BHM-3 dimers R=R'=CH_2OH, BHM-3

Results and Discussion

Synthesis of BHM-3 Dimers. Reaction of tris(hydroxymethyl)aminomethane (Tris) with ethyl pyruvate in refluxing butanol gave 5,6-dihydro-5,5-bis(hydroxymethyl)-3-methyl-1,4-oxazin-2-one (1) in 19% yield after silica gel chromatography. Even after high-vacuum rotary evaporation of the chromatography solvent, ethyl acetate, the material was contaminated by about 40% solvent as indicated by ^1H NMR spectroscopy. Spectroscopic and analytical data on a small sample further purified by recrystallization established the structure. In an earlier synthesis via the same reaction, partially purified 1 was characterized as its stable bis-acetate, 5,5-bis(acetoxymethyl)-5,6-dihydro-3-methyl-1,4-oxazin-2-one.[2] The overall yield of 1 was clearly compromised by the multifunctional nature of the starting materials and the product. The isolation of 1 was further complicated by its tautomerization to the valence isomer, 5-(hydroxymethyl)-1-methyl-8-aza-1,7-dioxabicyclo[3.2.1]octan-2-one (2). ^1H NMR spectroscopic observation of a sample of pure oxazinone 1 in deuterioacetonitrile showed that the tautomerization was at its equilibrium point, two parts of 1 to one part of 2, after 18 h at 37 °C. The tautomeric structure was evident from its ^1H NMR spectrum, and its formation paralleled the valence tautomerization of the precursor to DHM-3 dimers, 5,6-dihydro-3,5-dimethyl-5-(hydroxymethyl)-1,4-oxazin-2-one (3) to its valence 4.[3]



oxazinone 3 4

The oxazinone 1, contaminated with ethyl acetate, was photoreductively dimerized in 2-propanol solvent by irradiation at 0 °C with a Pyrex-filtered medium-pressure mercury lamp. Bi(5,5-bis(hydroxymethyl)-3-methyl-2-oxomorpholin-3-yl) was isolated in 25% yield by crystallization. The product was a mixture of a major and a minor diastereomer; some crystallization crops contained more than 95% of the major diastereomer. Both diastereomers were characterized from spectroscopic and analytical data reported in the Experimental Section. The synthesis of BHM-3 dimers is summarized in Scheme I.

Chemical Properties of BHM-3 Dimers. Facile bond homolysis of the 3,3'-bond of BHM-3 dimers was evident from stereochemical equilibration of the major and minor isomers: The major BHM-3 dimer from the synthesis in hexadeuteriodimethyl sulfoxide equilibrated to 2 parts of major isomer to 1 part of minor isomer after several days at ambient temperature. In tetradeuteriomethanol the equilibration was complete at 3 parts of major isomer to 1 part of minor isomer in less than 4 h, and in deuterium oxide the equilibration was complete at 6:1 in 2 h. The proposed intermediate for the equilibration, 5,5-bis(hydroxymethyl)-3-methyl-2-oxomorpholin-3-yl (BHM-3) was observed and characterized by EPR spectroscopic observation in a methanol solution of BHM-3 dimers. The EPR parameters are compared with those for DHM-3 and TM-3 in Table I

Table I. EPR Hyperfine Splitting Constants in Gauss for 2-Oxomorpholin-3-yl Radicals in Methanol at 60 °C

radical	a_{CH_3}	a_N	a_{NH}
BHM-3	10.5	6.2	4.85
DHM-3	10.15	6.4	6.4
TM-3	9.8	6.4	5.2

BHM-3 dimers in deuterium oxide at ambient temperature in a capped NMR tube slowly reacted to form 2-carboxy-4,4-bis(hydroxymethyl)-2-methyloxazolidine (5) based upon the ^1H NMR spectrum of the reaction mixture and a corresponding reaction of DHM-3 dimers. After 5 days the concentration of 5 was twice that of unreacted BHM-3 dimers. At 37 °C and in contact with a slow stream of air, 50% of the BHM-3 dimers was transformed to 5 plus about 5% Tris. Formation of 5 is proposed to occur via molecular oxygen oxidation of BHM-3 to oxazinone 1 followed by its subsequent rearrangement (Scheme II). In a separate NMR experiment, 1 was shown to undergo rearrangement to 5 followed by hydrolysis to Tris and presumably pyruvate. Pyruvate was not observed most likely because of exchange of its methyl protons for deuterons in the deuterium oxide medium.

In a separate experiment BHM-3 dimers in vigorously stirred water were shown to undergo air oxidation with formation of hydrogen peroxide in 93% yield after ca. 1 h. Hydrogen peroxide was detected by spectrophotometric analysis of the product of its reaction with titanium tetrachloride.[16] A 57% yield of hydrogen peroxide was observed after 7 min and 81% after 14 min. Without vigorous stirring, 3 h was required to achieve a yield of 50% hydrogen peroxide. The rate in vigorously stirred water is consistent with the slow step being bond homolysis of BHM-3 dimers (vide infra). Without vigorous stirring at BHM-3 dimer concentrations in excess of the concentration of dissolved oxygen, the slow step is diffusion of molecular oxygen into aqueous medium. Both DHM-3 and TM-3 are known to react with molecular oxygen to form the respective oxazinones and hydrogen peroxide.[3,17]

The first-order constant for bond homolysis of the major diastereomeric BHM-3 dimer was measured at 25 °C in several solvents and is compared with corresponding rate constants for bond homolysis of DHM-3 and TM-3 dimers in Table II. The kinetic measurements were accomplished by using precedented oxidizing agents[2,3,18] to trap BHM-3 radical as it was formed, monitoring the UV-vis absorption change of the trapping agent. The reagents and their products were ferriin, Fe^{3+}(1,10-phenanthroline)$_3$, to ferroin, Fe^{2+}(1,10-phenanthroline)$_3$; $FeCl_3$ to $FeCl_2$; DPPH, diphenylpicrylhydrazyl, to diphenylpicrylhydrazine (6); and isatin (7) to isatide (8).

The reduction potentials for formation of both BHM-3 and DHM-3 dimers from their respective oxazinones, 1 and 3, in apparent pH 7, Tris-buffered methanol were approximated from the equilibrium constants for reduction of paraquat (methylviologen, $PQ^{2+}2Cl^-$) to PQ^+ and the electrochemical reduction potential for PQ^{2+}. The reaction of BHM-3 and DHM-3 dimers with PQ^{2+} was monitored by observing the visible absorption of PQ^+ at 606 nm as

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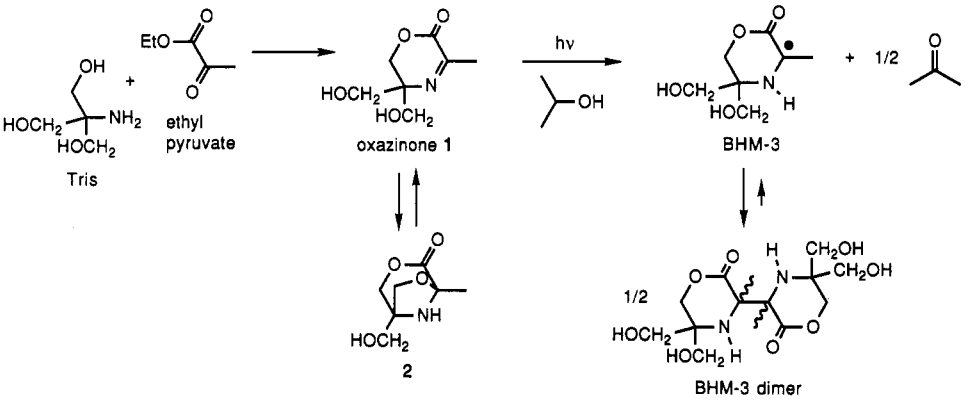
Table II. Rate Constants at 25 °C for Bond Homolysis of Radical Dimers as a Function of Solvent and Structure

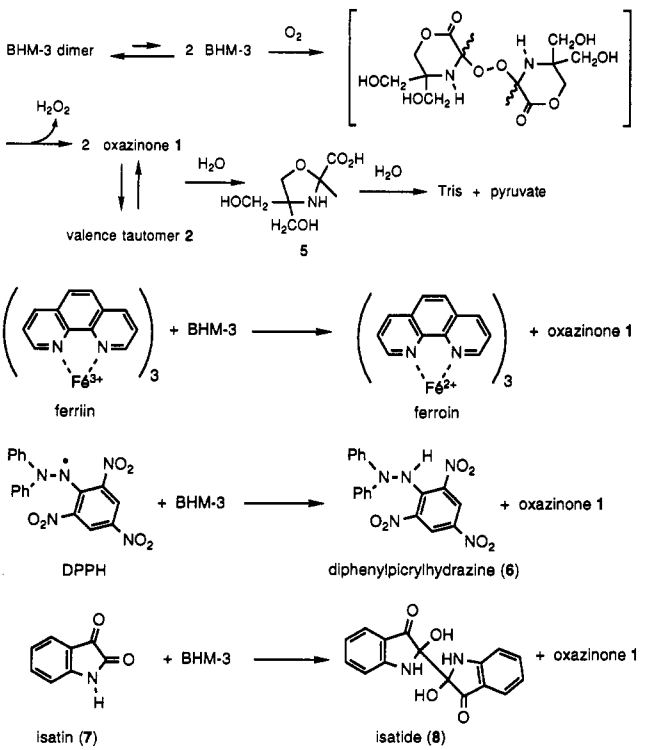
radical dimer	solvent	radical-trapping agent	k, s^{-1}	$t_{1/2}$	ref
BHM-3	CH_3CN	ferriin	$(5.34 \pm 0.02^c) \times 10^{-6}$	36.1 h	-
BHM-3	MeOH	DPPH	$(1.42 \pm 0.04^d) \times 10^{-4}$	81 min	-
BHM-3	MeOH (buffered[a])	$FeCl_3$	$(1.38 \pm 0.14^d) \times 10^{-4}$	84 min	-
BHM-3	H_2O	ferriin	$(1.53 \pm 0.08^d) \times 10^{-3}$	453 s	-
BHM-3	H_2O (buffered[b])	isatin	$(1.60 \pm 0.11^d) \times 10^{-3}$	433 s	-
BHM-3	0.13 M aqueous NaCl	ferriin	$(1.49 \pm 0.01^c) \times 10^{-3}$	465 s	-
DHM-3	CH_3CN	ferriin	1.43×10^{-5}	13.5 h	3
DHM-3	MeOH	DPPH	1.2×10^{-3}	580 s	3
DHM-3	H_2O	ferriin	8.5×10^{-3}	82 s	3
DHM-3	0.13 M aqueous NaCl	ferriin	6.5×10^{-3}	107 s	3
meso-TM-3	CH_3CN	O_2	1.82×10^{-5}	10.6 h	16
dl-TM-3	MeOH	*N*-methylisatin	3.4×10^{-3}	200 s	11
dl-TM-3	90% H_2O/10% MeOH	ferriin		~50 s	12

[a] Buffered to apparent pH 7.2 using imidazole. [b] Buffered to pH 7.0 with Tris/Tris·HCl. [c] Standard deviation obtained from linear least squares fitting of ln $(A - A_\infty)$ vs time for a single measurement. [d] Standard deviation s from the mean of several measurements.

Scheme I



Scheme II



a function of time. The equilibrium absorbance value together with the molar extinction coefficient for PQ^+ equal to 1.57×10^4 M^{-1} cm^{-1} and the initial concentrations of the reactants gave the equilibrium concentrations of the respective radical dimer, PQ^{2+}, PQ^+, and oxazinone. Seven and three measurements, respectively, gave the equilibrium constants, $K_{PQ\text{-}BHM3} = 2.8$ ($s = 2.6$) $M^{1/2}$ and $K_{PQ\text{-}DHM3} = 5.9$ ($s = 6.6$) $M^{1/2}$ using the following equation:

$$K_{\text{radical dimer}} = [PQ^+]_{eq}[\text{oxazinone}]_{eq}[\text{Tris}\cdot H^+]_{eq}/\{[\text{radical dimer}]_{eq}^{1/2}[PQ^{2+}]_{eq}[\text{Tris}]_{eq}\} \quad (1)$$

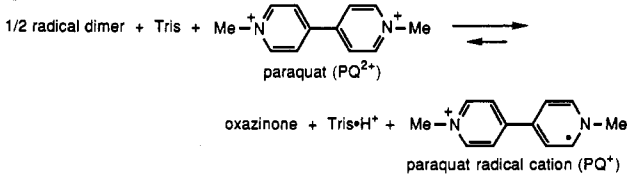
We believe that the significant error in reproducibility resulted from oxidation of PQ^+ by residual oxygen even after careful freeze-thaw degassing. The formal reduction potential ($E^\circ{}'$) for the half-reaction:

$$\text{oxazinone} + \text{Tris}\cdot H^+ + e^- \rightarrow 1/2 \text{ radical dimer} + \text{Tris} \quad (2)$$

was then calculated using the Nernst equation with the average equilibrium constant and the reduction potential of PQ^{2+} in methanol, –0.465 V vs NHE.[10]

$$E^\circ{}'(\text{radical dimer}) = E^\circ{}'(PQ^{2+}) - (RT/nF) \ln K_{\text{radical dimer}} \quad (3)$$

The values are compared with those for TM-3 dimer and bi[5,5-dimethyl-4-ethyl-2-oxomorpholin-3-yl] (DEM-3 dimer) in Table III.



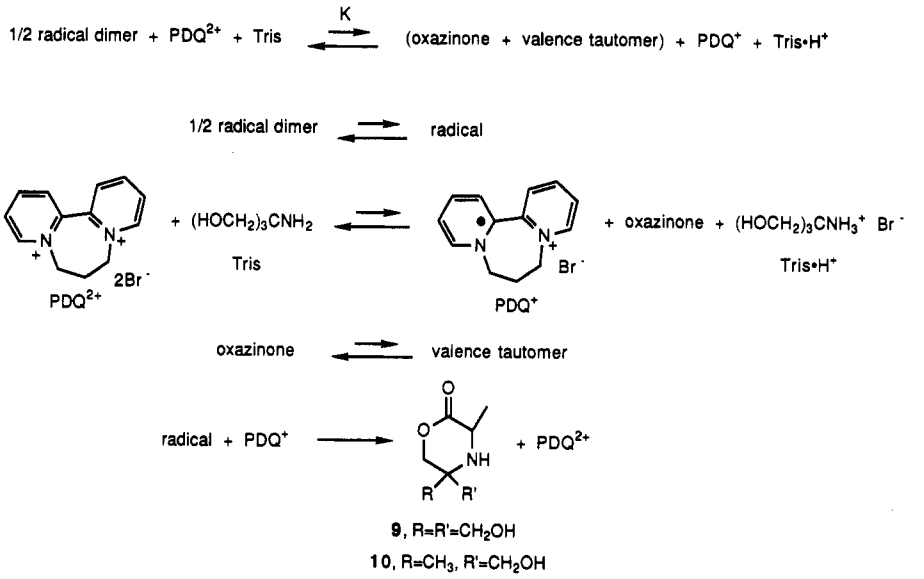
Because of the uncertainty in the equilibrium constants for the reduction of PQ^{2+}, the reduction potentials were also determined from the equilibrium constant for reduction of propyldiquat dibromide ($PDQ^{2+}2Br^-$) to PDQ^+. The effect of residual oxygen was predicted to be much smaller; higher concentrations of reagents could be used because of the smaller equilibrium constant and the lower molar absorptivity of PDQ^+. The reaction of BHM-3 and

Table III. Reduction Potentials for Formation of Oxomorpholinyl Radical Dimers as a Function of Structure in 0.010 M Tris/Tris·HCl-Buffered Methanol Solvent (initial apparent pH 7) Relative to NHE

radical dimer	$E^{\circ\prime}$ (V) (viologen)	radical dimer	$E^{\circ\prime}$ (V) (viologen)
TM-3[a]	−0.58 (PDQ^{2+})	DHM-3	−0.50 (s = 0.03, PQ^{2+}) −0.55 (s = 0.008, PDQ^{2+})
BHM-3	−0.48 (s = 0.03, PQ^{2+}) −0.54 (s = 0.002, PDQ^{2+})	DEM-3[a]	−0.33 (PQ^{2+})

[a] Value from reference corrected for the change in the Tris·H^+/Tris ratio.

Scheme III



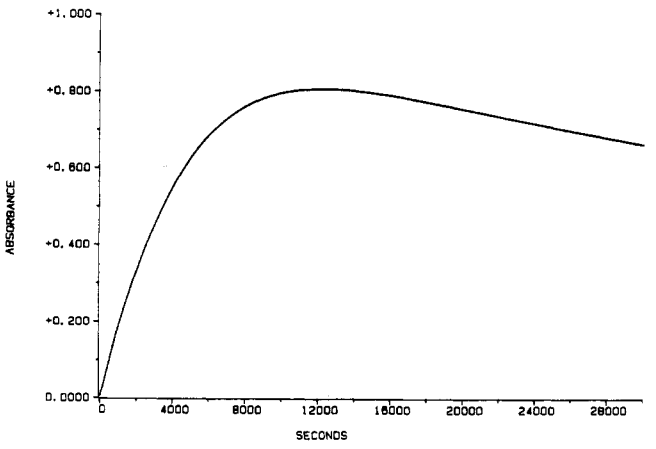


Figure 1. Absorbance at 506 nm versus time for anaerobic reaction of 1.98 × 10^{-4} M BHM-3 dimers with 3.88 × 10^{-4} M propyldiquat (PDQ^{2+}) dibromide in 0.010 M Tris/Tris-HCl buffered methanol (apparent pH 7) at 25 °C.

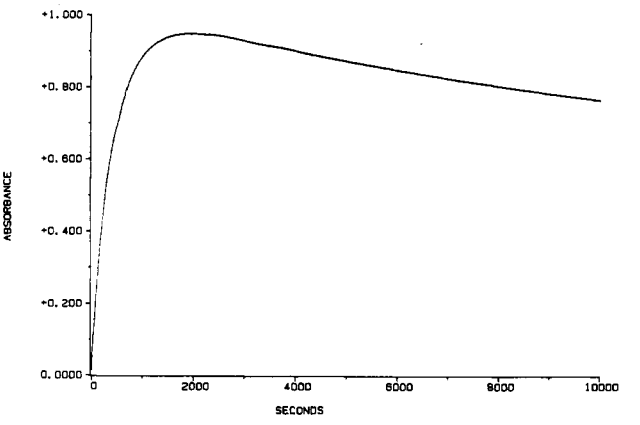


Figure 2. Absorbance at 506 nm versus time for anaerobic reaction of 1.99 × 10^{-4} M DHM-3 dimers with 3.90 × 10^{-4} M propyldiquat (PDQ^{2+}) dibromide in 0.010 M Tris/Tris-HCl buffered methanol (apparent pH 7) at 25 °C.

DHM-3 dimers with PDQ^{2+} was monitored by observing the visible absorption of PDQ^+ at 506 nm as a function of time, shown in Figures 1 and 2. The reactions do not actually come to equilibrium because of a slow subsequent reaction, proposed to be disproportionation of BHM-3 and DHM-3 with PDQ^+ to form 5,5-bis(hydroxymethyl)-3-methyl-2-oxomorpholine (**9**) and 5-(hydroxymethyl)-3,5-dimethyl-2-oxomorpholine (**10**), respectivley, and PDQ^{2+} (see Scheme III) based upon the identical reaction of TM-3 with PDQ^+.[10] The equilibrium absorbance in the absence of disproportionation was estimated using the Kezdy–Swinbourne method[19] for predicting the infinity point of a first-order reaction. The Kezdy–Swinbourne method is applicable because the rate of approach to equilibrium is first-order at absorbances close to equilibrium.[20] The time interval used for the Kezdy-Swinbourne analysis was selected to allow for significant equilibration of oxomorpholinyl radical dimers and yet precede significant disproportionation of oxomorpholinyl radicals with PDQ^+. For the data in Figures 1 and 2, the A_∞ values were calculated to be 0.85 and 0.95, respectively. The A_∞ value together with the molar extinction coefficient for PDQ^+ equal to 4.00 × 10^3 M^{-1} cm^{-1} and the initial concentrations of the reactants gave the predicted equilibrium concentrations of the respective radical dimer, PDQ^{2+}, PDQ^+, and oxazinone. Three measurements established the analogous equilibrium constants, $K_{PDQ\text{-}BHM3}$ = 0.37 (s = 0.03) $M^{1/2}$ and $K_{PDQ\text{-}DHM3}$ = 0.58 (s = 0.17) $M^{1/2}$, correcting for the change in Tris·H^+/Tris ratio with production of HBr. The error is thought to result primarily from the

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error associated with predicting the A_∞ values from the Kedzy–Swinbourne analysis. The formal reduction potential (Table III) was then calculated using the Nernst equation with the average equilibrium constant and the reduction potential of PDQ^{2+} in methanol, –0.562 V vs NHE.[10]

Biological Activity of BHM-3 Dimers. Selection of BHM-3 dimer for testing as an *in vivo* reducing agent for adriamycin was based on its solubility properties and its capacity to reduce adriamycin in human plasma at 37 °C. The partition coefficients of BHM-3 dimers and DHM-3 dimers between 1-octanol and water were determined as a measure of cell membrane permeability. The values are 0.054 and 0.31, respectively. These values are consistent with the observed solubilities in water at ambient temperature, >50 mg/mL and 10 mg/mL, respectively. Both partition coefficients are in the range for biologically permeable compounds.[21] TM-3 and DHM-3 dimers failed to reduce adriamycin appreciably at oxomorpholinyl dimer/adriamycin ratio of 10:1 to 30:1 in nitrogen-purged plasma as indicated by minimal appearance of 7-deoxyadriamycinone on TLC. However, 7-deoxyadriamycinone was observed when TM-3 dimer or DHM-3 dimer was mixed with adriamycin in nitrogen-purged 0.9% saline solution. In contrast, BHM-3 dimer at a BHM-3 dimer/adriamycin ratio of 30:1 reduced adriamycin to its nontoxic 7-deoxy aglycon for up to 60 min in nitrogen-purged plasma at 37 °C. Presumably, a constituent of plasma reacts with TM-3 and DHM-3 dimers in competition with adriamycin.

Previous studies with DHM-3 dimer in mice have demonstrated no apparent acute or chronic toxicity following 0.5 g/kg body weight administered as a single intraperitoneal (ip) or intravenous (iv) dose.[14] Furthermore, DHM-3 dimer, 50 mg/kg ip, rescued mice from high-dose adriamycin.[14] Toxicity studies with BHM-3 dimer in CD2F1 mice showed that a single intravenous injection of over 1.0 g/kg caused no apparent acute or chronic toxicity; whereas, a dose of 1.5 g/kg was acutely lethal to approximately one-third of the animals. The low toxicity is proposed in part to result from oxidative degradation to nontoxic materials. The pathway based upon experiments described above is oxidation to oxazinone 1 followed by hydrolysis to Tris and pyruvate probably via oxazolidine 5. Pyruvate occurs naturally in organisms as part of the citric acid cycle, and Tris is a nontoxic ingredient in many pharmaceutical preparations.

Several schema and doses of BHM dimer as a rescue agent for adriamycin were studied. Generally, effective BHM-3 dimer doses were 10-fold higher than previously shown for DHM-3 dimer.[14] BHM-3 dimer, 500 mg/kg ip, or normal saline (control) was administered 15 min prior to adriamycin, 25 mg/kg ip, and as shown in Figure 3, BHM-3 dimer was effective as a rescue of adriamycin toxicity. Intravenous BHM-3 dimer administered to CD2F1 male mice immediately prior to high-dose adriamycin was effective as a rescue for the acute toxic effcts of high-dose adriamycin. As shown in Figure 4, BHM-3 dimer, 500 mg/kg iv, provided significant protection against the acute toxicity caused by high-dose adriamycin. When BHM-3 dimer was administered after adriamycin at several different doses and time intervals, no rescue from adriamycin toxicity was observed.

Hematology studies performed on animals receiving adriamycin alone and adriamycin plus the BHM-3 dimer rescue are summarized in Table IV. Due to the myelosuppressive effects of adriamycin, a significant lowering of the white blood cell count was observed in the group given adriamycin, 25 mg/kg, iv. BHM-3 dimer administered prior to adriamycin protected against this hematological toxicity. An unexpected finding in the BHM-3 dimer plus adriamycin group was a significant drop in hemoglobin levels compared to the adriamycin and control groups.

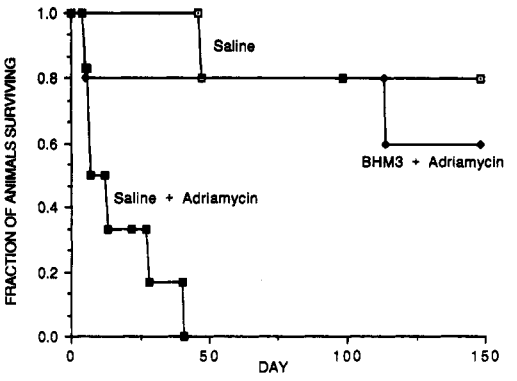


Figure 3. Survival of CD2F1 mice upon intraperitoneal injection of saline, saline followed by adriamycin (25 mg/kg body weight), or BHM-3 dimer (500 mg/kg) followed by adriamycin (25 mg/kg).

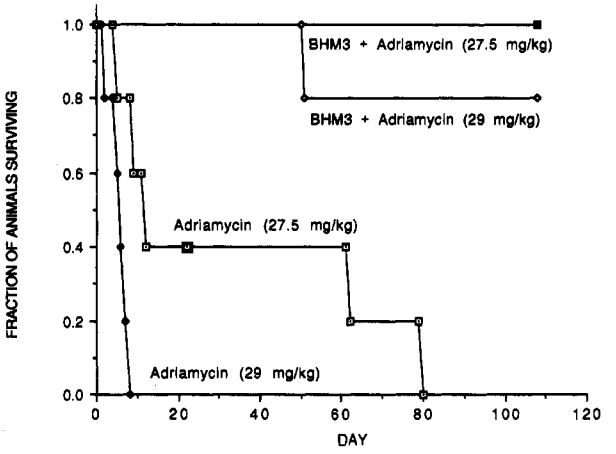


Figure 4. Survival of CD2F1 mice upon intravenous injection of adriamycin (27.5 or 29 mg/kg body weight) or BHM-3 dimer (500 mg/kg) followed by adriamycin (27.5 or 29 mg/kg).

Table IV. White Blood Cell and Hemoglobin Levels in CD2F1 Mice after Adriamycin, 25 mg/kg Body Weight, iv, ±BHM-3 Dimer, 500 mg/kg, iv

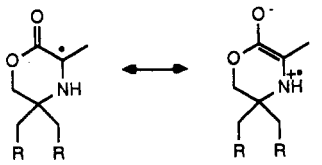
treatment	WBC count (1000/μL)	hemoglobin (g/dL)
control	5.3 ± 1.6	14.7 ± 0.3
adriamycin	1.4 ± 0.9[a]	15.7 ± 0.4
adriamycin + BHM-3 dimer	3.7 ± 1.2	7.9 ± 1.3[b]

[a] $p < 0.01$. [b] $p < 0.003$ compared to control.

Comparison of Oxomorpholinyl Dimers and Radicals. Hydroxyl groups at the 5-methyl substituents of TM-3 affect the chemical, physical, and biological properties of the system. TM-3, DHM-3, and BHM-3 dimers establish a trend. TM-3 dimer undergoes bond homolysis most rapidly, is the strongest reducing agent, and is the least soluble in water. BHM-3 dimer undergoes bond homolysis the slowest, approximately 1 order of magnitude more slowly than TM-3 dimer. BHM-3 dimer is the weakest reducing agent, the most water-soluble reducing agent, and the least toxic reducing agent. DHM-3 dimer

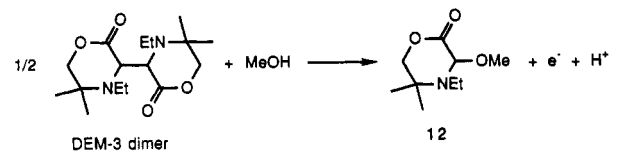
(21) Dennis, M. J. Absorption Processes. In *Comprehensive Medicinal Chemistry*; Hansch, C., Sammes, P. G., Taylor, J. B., Ed.; Pergamon: New York, 1990; Vol. 5, pp 1–43.

shows intermediate properties in all respects. Differences in the rates of bond homolysis probably result in part from differences in radical stability. Recent photoacoustic measurements suggest that oxomorpholinyl radicals receive some stabilization from the captodative effect. Captodative resonance structure 11 places positive charge at the nitrogen. The contribution of this structure is predicted to be less when inductively electron-withdrawing functional groups such as the hydroxyl groups of BHM-3 are present at the 5-methyl substituents. A spectroscopic indicator of the effect is the methyl EPR hyperfine coupling constant compared in Table I; it is inversely related to the electron spin delocalization. The 3-3′-



11

carbon–carbon bond strength is also related to the reduction potential since the 3-3′-bond is broken in the redox process; hence, BHM-3 dimer is predicted to be the weakest reducing agent in the series. The radical dimer, bi[5,5-dimethyl-4-ethyl-2-oxomorpholin-3-yl] (DEM-3 dimer), however, is yet a weaker reducing agent (see Table III).[4,10] Its more positive reduction potential resides predominantly in a change in oxidation product, from a dihydrooxazinone to 5,5-dimethyl-4-ethyl-3-methoxy-2-oxomorpholine (12). All of the radical dimers listed in Table III except DEM-3 dimer will exergonically reduce the anthracyclines, adriamycin and daunomycin, to their respective 7-deoxy aglycons. The reported potentials for these anthracycline vary in the range of −0.31 to −0.46 V vs NHE depending on solvent and method of determination.[22]



DEM-3 dimer 12

Biologically, the oxomorpholinyl radical dimer of intermediate reduction potential and water solubility, DHM-3 dimer, shows the best activity as an antidote for adriamycin with respect to extravasation necrosis[15] and as an adriamycin redox modulator in intraperitoneal chemotherapy.[14] BHM-3 dimer shows some promise as an intravenous modulator of adriamycin activity; it retains reactivity with adriamycin in plasma and is predicted to be more stable to oxidative degradation in the vascular system, primarily because of the slower rate of bond homolysis. BHM-3 dimer appears to be the least toxic of the oxomorpholinyl radical dimers.

Experimental Section

General Remarks. Hewlett-Packard Models 8450A and 8452A spectrometers were used for obtaining UV-vis spectra. ^{1}H NMR spectra were obtained with a ChemMagnetics 200-MHz or a Varian VXR 300-MHz spectrometer. Chemical shifts are reported in ppm downfield from internal tetramethylsilane or if in water, from 3-(trimethylsilyl)propanesulfonic acid sodium salt, and coupling constants are given in hertz. IR spectra were recorded with a Perkin-Elmer (Model 1600) FTIR spectrometer, and EPR measurements were made with a Varian 109E spectrometer equipped with a field/frequency lock. A Fisher Accumet Model 825MP pH meter was used for all pH measurements. Dry column chromatography was performed using the procedure describe by Harwood.[23] Microanalysis were performed by Atlantic Microblab Norcross, GA. Bi[3,5-dimethyl-5-hydroxymethyl)-2-oxomorpholin-3-yl] (DHM-3 dimer) was prepared as described earlier.[3] 1,1′-Propylene-2,2′-bipyridinium dibromide (propyldiquat dibromide, $PdQ^{2+}2BR^-$) was prepared as described by Homer and Tomlinson.[24] Tris(hydroxymethyl)aminomethane (Tris) and tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl) were obtained from Boehringer Mannheim (Indianapolis, IN); ethyl pyruvate and 1,1′-dimethyl-4,4′-bipyridinium dichloride (paraquat) from Aldrich (Milwaukee, WI). The silica gel employed was manufactured by Merck and obtained from EM Science (Philadelphia, PA). All solvents were reagent or spectral grade, from J. T. Baker (Phillipsburg, NJ) or Fisher Scientific (Fair Lawn, NJ).

5,6-Dihydro-5,5-bis(hydroxymethyl)-3-methyl-1,4-oxazin-2-one (1). Tris(hydroxymethyl)aminomethane (99 g, 0.82 mol) and 95 g (0.82 mol) of ethyl pyruvate were dissolved in 1 L of 1-butanol. The mixture in a 2-L three-neck flask equipped with a Dean-Stark trap was refluxed under an atmosphere of nitrogen for 1 h with magnetic stirring. During the reflux period, 0.2 L of distillate was collected in the Dean–Stark trap. The volatile materials from the remaining solution were removed by rotary evaporation at 50 °C first, for 1.5 h with a water aspirator pump, and then with a rotary pump for 1 h at 0.1–0.5 torr. The crude reaction mixture was dry column chromatographed[23] as follows. 2-Propanol (400 mL) was added and the solution warmed until dissolution occurred. The resulting solution was mixed with 480 g of 230–400 mesh silica gel and the solvent was removed by rotary evaporation at 50 °C with a water aspirator pump until volcano-like puffing subsided for at least 15 min (total time ca. 1.5 h). Bumping of the silica gel into the rotary evaporator was prevented by an adaptor bearing a large sintered glass frit located between the evaporator and the flask containing the silica gel. The volume of dry powder was about 1 L which was split into three portions. Each portion was loaded onto a 8 cm × 20 cm high suction filtering funnel with a medium sintered glass frit loaded with ca. 7 cm of clean silica gel. Ethyl acetate was the eluant and 250-mL fractions were collected. The first two fractions which were yellow were discarded. Oxazinone 1 eluted in fractions 3–9 which were slightly yellow or colorless. All three chromatographies were run with the same lower layer of silica gel, decanting away the upper part from the preceding chromatography. To facilitate the removal of the upper layer, it was separated from the lower layer with a disc of filter paper. The fractions containing the oxazinone were combined from the three chromatographies and the solvent was removed by rotary evaporation, first with a water aspirator and second with a rotary pump at 0.1 torr at 40 °C for a total of 6 h. The semisolid residue, 41 g, contained ca 40% solvent as determined by ^{1}H NMR; consequently, the yield was 19%. Further removal of solvent by rotary evaporation was unsuccessful. Further purification was achieved by recrystallization as follows: 1 g was dissolved in 6 mL of warm (70 °C) propiononitrile, and the solution was cooled in the freezer to yield 0.44 g of oxazinone 1, collected by suction filtration. The purified material had the following properties: mp 100–101 °C; silica gel TLC, R_f 0.3 (ethyl acetate); UV (CH_3CN) λ_{max} 322 nm (ϵ 127) with strong end absorption in the region 200–220 nm; IR (Nujol) 3318, 1732, 1651 cm^{-1}; ^{1}H NMR (CD_3CN) δ 2.18 (s, CH_3), 3.05 (t, J = 6 Hz, OH, disappeared with D_2O exchange), 3.47 (dd, J = 6, 12 Hz, CH_2OH, collapsed to d with D_2O exchange), 3.62 (dd, J = 6, 12 Hz, CH_2OH, collapsed to d with D_2O exchange), 4.46 (s, CH_2). Anal. Calcd for $C_7H_{11}NO_4$: C, 48.55; H, 6.36; N, 8.09. Found: C, 48.66; H, 6.41; N, 8.08.

Tautomerization of 5,6-Dihydro-5,5-bis(hydroxymethyl)-3-methyl-1,4-oxazin-2-one (1). The sample of oxazinone used for NMR analysis in CD_3CN as described above was allowed to

(22) Berg, H.; Horn, G.; Jacob, H.-E.; Fiedler, U.; Luthardt, U.; Tresselt, D. *Bioelectrochem. Bioenerg.* **1986**, *16*, 135. Anne, A.; Moiroux, J. *Nouv. J. Chim.* **1985**, *9*, 259. Svingen, B. A.; Powis, G. *Arch. Biochem. Biophys.* **1981**, *209*, 119. Rao, G. M.; Lown, J. W.; Plambeck, J. A. *J. Electrochem. Soc.* **1978**, *125*, 534.

(23) Harwood, L. M. *Aldrichim. Acta* **1985**, *18*, 25.

(24) Homer, R. F.; Tomlinson, T. E. *J. Chem. Soc.* **1960**, 2498.

stand at ambient temperature overnight. The ^{1}H NMR spectrum then showed 95% oxazinone and 5% of its valence tautomer, 5-(hydroxymethyl)-1-methyl-8-aza-1,7-dioxabicyclo[3.2.1]octan-2-one (2). After an additional 18 h at 37 °C the ratio of oxazinone to its valence tautomer was 2/1. The valence tautomer was characterized by the following ^{1}H NMR absorption bands: δ (CD_3CN, 300 MHz) 1.53 (s, CH_3), 3.15 (broad, NH) 3.20 (t, J = 6 Hz, OH, X portion of an ABX pattern), 3.60 (d, J = 8 Hz, CH_2O), 3.6–3.7 (AB portion of an ABX pattern overlapping with signals from the oxazinone, J_{AB} = 12 Hz, CH_2OH), 3.96 (d, J = 8 Hz, CH_2O), 4.26 (unresolved AB pattern, $J_{AB} \sim$ 15 Hz, CH_2-OC=O).

Hydrolysis of 5,6-Dihydro-5,5-bis(hydroxymethyl)-3-methyl-1,4-oxazin-2-one (1). An apparent pH 7.4 deuterium-exchanged buffer was created first by dissolving the appropriate sodium and potassium phosphates in D_2O and evaporating the D_2O. The residue was subsequently dissolved in D_2O to make a 0.5 M solution. Pure 5,6-dihydro-5,5-bis(hydroxymethyl)-3-methyl-1,4-oxazin-2-one (19 mg, mp 100–101 °C) was dissolved in 1.9 mL of the buffer to make a 5.8×10^{-2} M solution. The solution was divided and added to two NMR tubes. The first tube was kept at ambient temperature. The ^{1}H NMR spectrum taken soon after preparation of the sample showed 70% of oxazinone 1, ca. 20% of 2-carboxy-4,4-bis(hydroxymethyl)-2-methyloxazolidine (5), and 10% of what appeared to be a transient (possibly the tautomer 2 of the oxazinone starting material described above) which eventually disappeared. In 1.5 h all of the oxazinone disappeared. The NMR spectrum showed mostly oxazolidine which was characterized by the following resonances: δ (D_2O, 300 MHz) 1.57 (s, CH_3), 3.58 (d, J = 12 Hz, CH_2OH), 3.64 (d, J = 12 Hz, CH_2OH), 3.65 (d, J = 12 Hz, CH_2OH), 3.67 (d, J = 12 Hz, CH_2OH), 3.81 (d, J = 15 Hz, CH_2O), 4.04 (d, J = 15 Hz, CH_2O). The second tube was kept at 37 °C. After a rapid transformation of oxazinone 1 to oxazolidine 5 occurred, a slow hydrolysis to tris(hydroxymethyl)methylamine (Tris) and presumably pyruvate occurred, 25% in 3 days. Tris was characterized by a singlet of δ 3.73. Pyruvate was not visible in the NMR because of exchange of the methyl protons for deuterons. During this extended period, exchange of the methyl protons of the oxazolidine 5 also occurred.

Bi(5,5-bis(hydroxymethyl)-3-methyl-2-oxomorpholin-3-yl) (BHM-3 Dimer). A procedure similar to that described for the synthesis of TM-3 dimers was employed.[25] Oxazinone 1 (40 g of slightly yellow material containing 40% by weight of ethyl acetate) was dissolved in ca. 0.50 L of 2-propanol. The solution at 0 °C was irradiated for 55 h under a nitrogen atmosphere with a 400-W mercury street lamp with the Pyrex housing removed. The progress of the reaction was monitored by UV absorption observing the disappearance of the n–π^* band at 322 nm. Most of the solvent was then removed by rotary evaporation for 1 h at 30 °C; the final weight was ca. 50 g. At this point the material was protected as much as possible atmospheric oxygen. Any precipitate formed was collected by suction filtration. The filtrate was dissolved in ca. 50 mL of ethyl acetate, a seed crystal of BHM-3 dimer added if available, and the solution placed in the freezer. After 2 days, 6 g (25%) of BHM-3 dimer was collected by suction filtration, mp 140–142 °C. Additional material sometimes precipitated from the filtrate with additional time in the freezer. Silica gel TLC eluting with ethyl acetate/2-propanol (9/1, v/v) showed the major diastereomer of BHM-3 dimer at R_f 0.1 and the minor diastereomer at R_f 0.25; oxazinone 1 had an R_f of 0.45. The mother liquors showed larger amounts of the minor, more soluble isomer. The ^{1}H NMR spectrum of the crystalline material also showed a predominant diastereomer as follows: major isomer, δ (DMSO-d_6, 200 MHz) 1.38 (s, CH_3), 2.22 (broad s, NH), 3.1–3.3 (poorly resolved AB portion of an ABX pattern, $J_{AB} \sim$ 12 Hz, CH_2OH, simplified to an AB pattern with D_2O exchange, δ 3.23 and 2.34, J = 12 Hz), 3.3–3.6 (partially resolved AB portion of an ABX pattern overlapping with HOD, $J_{AB} \sim$ 10 Hz, CH_2OH simplified to an AB pattern with D_2O exchange, δ 3.36 and 3.48, J = 10 Hz), 4.10 (d, J = 12 Hz, CH_2O), 4.14 (d, J = 12 Hz, CH_2O), 4.68 (t, J = 6 Hz, OH, disappeared with D_2O exchange), 4.88 (t, J = 6 Hz, OH, disappeared with D_2O exchange); major isomer (D_2O, 300 MHz) 1.52 (s, CH_3), 3.49 (poorly resolved AB pattern, CH_2OH), 3.61 (d, J = 12 Hz, CH_2-OH), 3.73 (d, J = 12 Hz, CH_2OH), 4.33 (d, J = 11 Hz, CH_2O), 4.35 (d, J = 11 Hz, CH_2O); minor isomer δ (D_2O, 300 MHz) 1.60 (s, CH_3), 3.53 (poorly resolved d, CH_2OH), 3.55 (poorly resolved d, CH_2OH), 3.70 (poorly resolved d, CH_2OH), 3.75 (poorly resolved d, CH_2OH), 4.68 (d, CH_2O), 4.73 (d, CH_2O), coupling constants could not be determined because signals were too weak in the presence of the large amount of the major isomer. The IR spectrum of the major isomer showed the following bands: (KBr) 3352, 2922, 2854, 1709, 1460, 1405, 1378, 1298, 1253, 1104, 1028, 890, 842, 770, 726 cm^{-1}. Anal. Cacld for $C_{14}H_{24}N_2O_8$: C, 48.27; H, 6.90; N, 8.05. Found: C, 48.34; H, 6.95; N, 7.99.

Equilibration of Isomeric BHM-3 Dimers. A solution of the major BHM-3 dimer in DMSO-d_6 at ambient temperature in a capped NMR tube was monitored by ^{1}H NMR spectroscopy to observe isomerization to the minor diastereomer and formation of the equilibrium mixture. The diastereomer ratio was determined by measuring the relative areas of the methyl singlets for the major and minor diastereomers at δ 1.38 and 1.49, respectively. The equilibration required days and the equilibrium ratio of the major to the minor diastereomer was 2:1. During the equilibration only traces of the oxazinone 1 were formed.

The equilibration was also performed in freeze-thaw degassed tetradeuteriomethanol at 25 °C. Within 0.5 h, the ratio of diastereomers was 10:1, and within 4 h equilibration was complete at a ratio of 3:1.

Equilibration of Isomeric BHM-3 Dimers in D_2O Followed by Air Oxidation and Hydrolysis. BHM-3 dimer (29 mg) was dissolved in 2.9 mL of pH 7.4 D_2O prepared as described above. The solution was split between two NMR tubes. The 1st tube was kept at ambient temperature. The ^{1}H NMR spectrum taken soon after preparation of the sample showed predominantly the major isomer with very little of the minor isomer of BHM-3 dimer. The tube was left at ambient temperature just capped. The minor isomer reached its maximum concentration (15%) in 2 h. The ^{1}H NMR signals for both isomers then decreased to be slowly replaced by the signals for the oxazolidine 5, presumably arising from rapid hydrolysis of oxazinone 1 formed in a slow oxidation of BHM-3 radical. After 5 days the oxazolidine 5 concentration was about twice the concentration of unreacted BHM-3 dimer. The solution in the second tube was maintained at 37 °C under a slow stream of air. After 1 h 50% of the BHM-3 dimer had been transformed into oxazolidine 5 plus ca. 5% Tris.

Oxidation of Bi[5,5-bis(hydroxymethyl)-3-methyl-2-oxomorpholin-3-yl] (BHM-3 Dimer) by Molecular Oxygen in Water. BHM-3 dimer (5.4 mg, 1.55×10^{-2} mmol) was dissolved in 7.7 mL of distilled water. The solution was vigorously stirred at ambient temperature (ca. 27 °C) in the presence of air. The UV spectrum of the solution showed the rise of an absorption with a maximum at 310 nm, due to the oxazinone, with the intensity of the absorption reaching its maximum (0.36) after 0.5 h. Analyses of hydrogen peroxide were performed as already described using a titanium chloride solution.[3,16] The amount of hydrogen peroxide formed was 57% after 7 min, 81% after 14 min, 93% (maximum value) after ca. 1 h.

The oxidation of 3 mL of the same solution of BHM-3 dimer kept in the presence of air in a 25-mm diameter vial, with no shaking, took 3 h to be 50% complete, based on the hydrogen peroxide analysis.

Rate Constant for Bond Homolysis of BHM-3 Dimer. A radical dimer solution was prepared by dissolving 1.20 mg (3.45×10^{-3} mmol) of BHM-3 dimer (10:1 mixture of diastereomers) in 25 mL of acetonitrile in a volumetric flask. A solution of DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate, 1.67 mg, 4.24×10^{-3} mmol) was prepared in methanol in a 50-mL volumetric flask. A two-compartment cell was charged by placing 0.5 mL (6.89×10^{-5} mmol) of BHM-3 dimer solution in the cuvette and evaporating the solvent with a stream of nitrogen gas. An aliquot of the DPPH solution (2.5 mL, 2.12×10^{-4} mmol) was added to the degassing chamber. The solution was oxygen-degassed on a high vacuum line and then sealed and temperature-equilibrated to 25.0 °C. The cell was shaken to mix the substrate and reducing agent, and the average absorbance at 514–516 nm was monitored every 60 s for 5 h. The initial concentrations of the reactants were 8.47×10^{-5} M DPPH and 2.76×10^{-5} M BHM-3 dimer. The rate constant for the reaction was determined by nonlinear least-squares fitting of absorbance versus time as reported in Table II.

(25) Koch, T. H.; Olesen, J. A.; DeNiro, J. *J. Org. Chem.* **1975**, *40*, 14.

The rate constant for BHM-3 dimer bond homolysis in imidazole-buffered methanol (0.10 M, apparent pH 8) with ferric chloride as a radical-trapping agent was similarly determined except the decay of the absorbance ($FeCl_3$) was monitored at 400 nm. The rate constant in Tris-buffered, distilled, deionized water (8.59×10^{-4} M Tris, 9.45×10^{-3} M Tris·HCl, pH 7.0) was similarly determined with isatin as the radical trapping agent and monitoring the decay of the isatin absorbance at 418–420 nm.

The rate constant was also measured in water, 0.13 M saline, and acetonitrile with ferrin as a radical-trapping agent. Because ferriin trapped radicals very rapidly, it was used successfully in solutions which were degassed with a stream of prepurified nitrogen with no need for the freeze-thaw degassing procedure. A stock solution, 2.62×10^{-2} M in ferrin, was prepared by dissolving 10.6 mg (0.25 mmol) of ferric nitrate nonahydrate and 14.8 mg (0.75 mmol) of 1,10-phenanthroline in 10 mL of distilled, deionized water. A small aliquot (ca. 0.1 mL) of ferriin solution was injected into a nitrogen-degassed, BHM-3 dimer solution in a 1-cm cuvette equipped with a screw cap bearing a septum seal. Absorption by ferroin was monitored at 506–510 nm.

EPR Spectrum of 5,5-Bis(hydroxymethyl)-3-methyl-2-oxomorpholin-3-yl (BHM-3). A sample of BHM-3 dimer (10 mg) was dissolved in 0.5 mL of methanol and the solution added to a quartz EPR tube. The spectrum of BHM-3 was observed at 60 °C without degassing and showed the following parameters: $g = 2.0036$, $a_{NH} = 4.85$ G, $a_N = 6.20$ G, and $a_{CH3} = 10.50$ G.

Reaction of BHM-3 Dimers with Paraquat ($PQ^{2+}2Cl^-$). Paraquat (methylviologen dichloride, 1,1′-dimethyl-4,4′-bipyridinium dichloride) was shown by combustion analysis to have the molecular formula $C_{12}H_{14}N_2Cl_2 \cdot 3H_2O$ and thus the formula weight of 311.21 g/mol. A radical dimer solution was prepared by dissolving 1.63 mg (4.68×10^{-3} mmol) of BHM-3 dimer as a mixture of diastereomers in 50 mL of acetonitrile in a volumetric flask. A stock solution of paraquat was prepared by dissolving 1.64 mg (5.27×10^{-3} mmol) in 10 mL of a 0.10 M solution of Tris and Tris·HCl in methanol in a 100-mL volumetric flask and diluting to the mark with methanol. A two-compartment cell was charged with 1.00 mL (9.36×10^{-8} mol) of the BHM-3 dimer solution in the cuvette and evaporating the solvent with a stream of nitrogen gas. A 3.0-mL aliquot (1.58×10^{-7} mol) of the paraquat stock solution was added to the degassing chamber. The solution was freeze-pump-thaw degassed with sonication for five complete cycles, sealed under vacuum, and equilibrated to 25.0 ± 0.1 °C. The contents of the two compartments were mixed by shaking the cell, and the absorption at 606 nm was monitored as a function of time. The initial concentrations were 3.12×10^{-5} M BHM-3 dimer, 5.27×10^{-5} M PQ^{2+}, and 0.010 M Tris/Tris·HCl. The reaction was repeated seven times, and the results are reported in Results and Discussion and Table III.

Reaction of DHM-3 Dimers with Paraquat ($PQ^{2+}2Cl^-$). Reaction of DHM-3 dimers with paraquat was performed exactly as described for reaction with BHM-3 dimers. The initial concentrations for one example were 3.14×10^{-5} M DHM-3 dimer, 4.95×10^{-5} M PQ^{2+}, and 0.010 M Tris/Tris·HCl. The measurement was repeated three times and the results are reported in Results and Discussion and Table III.

Reaction of BHM-3 Dimers with Propyldiquat ($PDQ^{2+}2Br^-$). Reduction potential measurements were performed in two-compartment cells in which one compartment was a 1-cm cuvette; the two compartments were used to separate the reagents during degassing. The cells were equipped with a 9-mm tube for attachment to a vacuum line with an Ultra-torr union. A radical dimer solution was prepared by dissolving 5.16 mg of BHM-3 dimer as a 10:90 mixture of diastereomers in 25 mL of acetonitrile in a volumetric flask. Combustion analysis of propyldiquat showed it to be hydrated, with the formula $C_{13}H_{14}N_2Br_2 \cdot 1/2H_2O$ and a formula weight of 367.1 g/mol. A stock propyldiquat solution was prepared by dissolving 3.56 mg of propyldiquat dibromide hemihydrate in methanol in a 25-mL volumetric flask. Tris (2.56 mg, 2.11×10^{-2} mmol) and Tris·HCl (37.3 mg, 2.37×10^{-1} mmol) were added to the methanolic solution. The buffered solution had an apparent pH of 7.2 as measured with a saturated KCl glass electrode. A two-compartment cell was charged by placing 1.0 mL (5.93×10^{-7} mol) of the BHM-3 dimer solution in the cuvette and evaporating the solvent with a stream of nitrogen gas. A 3.0-mL aliquot (1.16×10^{-6} mol) of the propyldiquat stock solution was added to the degassing chamber of the same two-compartment cell. The solution was freeze-pump (5×10^{-6} Torr)-thaw-degassed with sonication for five complete cycles and then sealed under vacuum, and temperature was equilibrated to 25.0 ± 0.1 °C. The contents of the two compartments were mixed by shaking the cell, and UV-vis spectral data were collected with the cuvette in a thermostated cell holder at 25.0 ± 0.1 °C. The optical density at 506 nm, measured every 10 s for 30000 s for one measurement, is shown in Figure 1. The experiment was repeated twice. Initial concentrations in the reaction mixture were 1.98×10^{-4} M BHM-3 dimer, 3.88×10^{-4} M PDQ^{2+}, and 0.010 M Tris/Tris·HCl. The measurement was repeated three times and the results are reported in Table III.

Reaction of DHM-3 Dimers with Propyldiquat ($PDQ^{2+}2Br^-$). A radical dimer solution was made by dissolving 1.89 mg of DHM-3 dimer in acetonitrile in a 10-mL volumetric flask. A stock propyldiquat solution was prepared as described above using 3.58 mg of propyldiquat dibromide hemihydrate, 2.43 mg (2.01×10^{-2} mmol) of Tris, and 36.8 mg (2.34×10^{-1} mmol) of Tris·HCl. The experiment was carried out as described for BHM-3 dimers with the exception that spectral data were collected every 10 s for 10000 s as shown in Figure 2. The reaction concentrations initially were 1.99×10^{-4} M DHM-3 dimer, 3.90×10^{-4} M PDQ^{2+}, and 0.010 M Tris/Tris·HCl. The measurement was repeated three times and the results are reported in Table III.

Partition Coefficient of Bi[5,5-bis(hydroxylmethyl)-3-methyl-2-oxomorpholin-3-yl] (BHM-3 Dimer) between 1-Octanol and Water. BHM-3 dimer (33 mg, 9.5×10^{-2} mmol) was dissolved in a nitrogen-bubbled mixture of distilled water (0.50 mL) and distilled 1-octanol (3.0 mL). The mixture was kept under a flow of nitrogen, while sonicating, at ca. 27 °C for 15 min. After quick filtration the two layers were separated and analyzed by measuring the amount of hydrogen peroxide formed upon air oxidation, as follows: (a) 300 μL of the aqueous layer were diluted to 3.0 mL with distilled water and the solution oxidized and analyzed as described above. The concentration of BHM-3 dimer in the original 0.50 mL of the aqueous layer was calculated to be 52 mg/mL; (b) 2.0 mL of the octanol layer were mixed with 2.0 mL of distilled water and vigorously stirred in the presence of air. The hydrogen peroxide analysis[3,16] of the aqueous layer indicated a concentration of 2.8 mg/mL of BHM-3 dimer in the original octanol solution, corresponding to a partition coefficient 0.054.

Partition Coefficient of Bi[3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl] (DHM-3 Dimer) between 1-Octanol and Water. Distilled 1-octanol (2.0 mL) and distilled water (2.0 mL) were combined in a 10-mL vacuum tube and deareated by bubbling nitrogen. DHM-3 dimer (ca. 5 mg), ^{14}C-labeled, showing an activity of 8×10^{-3} μCi/mg,[26] was added, and soon the mixture was freeze-thaw degassed through two cycles. The tube was sealed under vacuum and shaken for several hours at 20 °C. The tube was then opened and the two clear layers soon separated. The radioactivity of 0.50 mL each of the aqueous and organic layers was measured to give 1.81×10^4 (water) and 5.68×10^3 (octanol) cpm, corresponding to a partition coefficient 0.31.

Materials for Biological Experiments. Each vial of clinical grade adriamycin (doxorubicin·HCl, USP, Adria Laboratories, Columbus, OH) containing 10 mg of doxorubicin hydrochloride and 50 mg of lactose was reconstituted with 5.0 mL of sterile 0.9% sodium chloride solution to give a final drug drug concentration of 2.0 mg/mL. TM-3 dimer (10 mg/mL), DHM-3 dimer (10 mg/mL), or BHM-3 dimer (20 mg/mL) solution was prepared by dissolving the oxomorpholinyl dimer in ice-cold 0.9% NaCl solution that had been purged with nitrogen gas for 5 min. The solution was kept cold and protected from air and light. Male CD2F1 mice were obtained from the Frederick Cancer Research Facility, Division of Cancer Treatment, National Cancer Institute and all were between 7 and 9 weeks old. The animals were maintained under standard housing conditions and all experiments were performed after age 12 weeks.

Determination of Reduction of Adriamycin in Human Plasma. The respective oxomorpholinyl dimer was added to 3.0 mL of human plasma containing 1.74 mg of adriamycin at 37

(26) Sankar, I. U.; Gaudiano, G.; Koch, T. H. Unpublished work.

°C under a gentle stream of nitrogen gas. At various time intervals, 0.1 mL of plasma was removed and analyzed for adriamycin and 7-deoxyadriamycinone by TLC as previously described.[14]

***In Vivo* Evaluation of BHM-3 Dimer as a Rescue Agent for Adriamycin.** For intraperitoneal drug experiments, mice were given a single dose of adriamycin, 25 mg/kg body weight. BHM-3 dimer, 500 mg/kg, or saline as a control was administered by the same route at varying intervals before and after the adriamycin injection. For intravenous drug experiments, each animal received either normal saline or BHM-3 dimer, 500 mg/kg, iv bolus, through the same catheter immediately prior to doxorubicin·HCl, 25 mg/kg, iv bolus. The drugs were administered through the same needle and tubing but were separated by a small (0.1 mL) normal saline flush. Lethality was determined by median survival time. For each experiment five mice were used, and all experiments were performed at least twice. Hematology assessment was performed from blood obtained from the retroorbital plexus on day 4 following drug administration. The hematology data were analyzed by two-tailed Student's *t* test.

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