The newly discovered N-dealkylation activity of DBM is a potentially valuable tool for the understanding of the chemistry of DBM catalysis at the molecular level. Moreover, we note that this N-dealkylation activity is directly analogous to the peptidylglycine amidating monooxygenase reaction which has been recently shown to be catalyzed by an oxygenase very similar to DBM in its subcellular location and requirement for copper and ascorbate.[58] Furthermore, we note that in view of the central role of DBM in adrenergic neuronal function, these substrate analogues and inhibitors are valuable in designing pharmacologically interesting compounds that might be important for the modulation of dopamine/norepinephrine levels in vivo.[59-62]

Acknowledgment. We gratefully acknowledge support of this research by the National Institutes of Health (GM33950 and HL28167). We are indebted to Dr. R. Thomas Solsten and Dr. Stephen R. Padgette of Monsanto Company, Chesterfield, MO for providing the HPLC-MS spectral data.

(58) (a) Eipper, B. A.; Mains, R. E.; Glembotski, C. C. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5144-5148. (b) Glembotski, C. C.; Eipper, B. A.; Mains, R. E. *J. Biol. Chem.* **1984**, *259*, 6385-6392. (c) Eipper, B. A.; Park, L.; Keutmann, H. T.; Mains, R. E. *J. Biol. Chem.* **1986**, *261*, 8686-8694.

(59) Padgette, S. R.; Herman, H. H.; Han, J. H.; Pollock, S. H.; May, S. W. *J. Med. Chem.* **1984**, *23*, 1331-1357.

(60) Herman, H. H.; Pollock, S. H.; Padgette, S. R.; Lange, J. R.; Han, J. H.; May, S. W. *J. Cardiovasc. Pharmacol.* **1983**, *5*, 725-730.

(61) Pollock, S. H.; Herman, H. H.; Han, J. H.; May, S. W. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1984**, *43*, 1101.

(62) Pollock, S. H.; Herman, H. H.; May, S. W. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1985**, *44*, 879.

(63) BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; DBM, dopamine β-monooxygenase; MAO, monoamine oxidase; α-MePAES, 1-methyl-2-(phenylthio)-1-aminoethane; β-MePAES, 2-methyl-2-(phenylthio)-1-aminoethane; *N*-MePEDA, *N*-methyl-*N*-phenylethylenediamine; β-MePEDA, 2-methyl-2-anilino-1-aminoethane; MES, 2-(*N*-morpholino)-ethanesulfonic acid; 4-OHPAEE, 4-hydroxyphenyl 2-aminoethyl ether; PAES, phenyl 2-aminoethyl sulfide; PAEE, phenyl 2-aminoethyl ether; PEDA, *N*-phenylethylenediamine; SNPA, *N*-succinimidyl *p*-nitrophenylacetate.

A Kinetic Rationale for the Inefficiency of 5-Iminodaunomycin as a Redox Catalyst

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Abstract: The redox chemistry of 5-iminodaunomycin (**1**) is compared with the redox chemistry of daunomycin (**2**) to establish a possible rationale for the inefficiency of 5-iminodaunomycin as an in vivo redox catalyst for the production of reactive oxygen species. Anaerobic reduction of **1** with sodium dithionite in methanol solvent gives 5-imino-7-deoxydaunomycinone (**3**) most likely via the intermediate quinone methide **6**. The slow step is tautomerization of **6** to **3**, a pseudo-first-order process with a rate constant of 2.2×10^{-2} s^{-1} at 25 °C. Anaerobic reduction of **3** with sodium dithionite in methanol solvent gives 2-acetyl-2,11-dihydroxy-7-methoxy-1,2,3,4-tetrahydro-5,12-naphthacenedione (**4**) most likely via the intermediate naphthacenone **8**. The slow step, proposed to be loss of ammonia, occurs with a rate constant of 4.0×10^{-2} s^{-1} at 25 °C. Even traces of molecular oxygen interfere with these two reductions, hence **1** and **3** should be efficient catalysts for the production of reactive oxygen species. Reduction of **4** with sodium dithionite in anaerobic methanol or methanol purged with 2% oxygen rapidly gives 8-acetyl-1-methoxy-7,9,10,12-tetrahydro-6,8,11-trihydroxy-5(8*H*)-naphthacenone (**9**). Naphthacenone **9** is slowly oxidized back to **4** by molecular oxygen with a pseudo-first-order rate constant of 3.3×10^{-4} s^{-1}. Although reduction of daunomycin by sodium dithionite in parallel fashion gives 7-deoxydaunomycinone (**5**), reduction of 7-deoxydaunomycinone does not rapidly give **4** but slowly gives 2-acetyl-1,2,3,4,4a,12a-hexahydro-2,6,11-trihydroxy-7-methoxy-5,12-naphthacenedione (**11**) with a rate constant of 1.5×10^{-4} s^{-1}. Naphthacenedione **11** is reasonably insensitive to molecular oxygen. Upon standing in the presence of oxygen very slow elimination to give **4** and oxidation to give **5** occur. Consequently, a possible explanation for the inefficiency of 5-iminodaunomycin for catalyzing in vivo the reduction of molecular oxygen is the facile formation of naphthacenedione **4** which is an inefficient catalyst. The inefficiency arises because the hydroquinone of **4** tautomerizes to naphthacenone **9** in preference to reduction of molecular oxygen.

5-Iminodaunomycin (**1**) is a semisynthetic derivative of the pharmacologically important anti-tumor drug daunomycin (**2**) in which the C-ring quinone has been transformed regiospecifically into an iminoquinone.[1] It has attracted significant attention because it shows less cardiotoxicity than daunomycin while retaining significant anti-tumor activity.[2] The lower cardiotoxicity of **1** has been rationalized in terms of a diminished potential for catalytic production of reactive oxygen species which presumably attack heart cell membrane lipids.[3-5] Daunomycin and adriamycin efficiently catalyze the production of reactive oxygen species in the presence of either a one- or two-electron reducing agent and molecular oxygen;[6-10] this process is sometimes referred to as radical recycling. 5-Iminodaunomycin has been recently described in an excellent review of the anthracyclines as a redox-incapacitated anthracycline, one which is both more difficult to reduce and/or reoxidize and, hence, cannot catalyze efficiently the reduction of molecular oxygen.[11]

(1) Tong, G. L.; Henry, D. W.; Acton, E. M. *J. Med. Chem.* **1979**, *22*, 36. Acton, E. M.; Tong, G. L. *Ibid.* **1981**, *24*, 669.

(2) "Factors in the Selection of New Anthracyclines": Acton, E. M.; Jensen, R. A.; Peters, J. H. In *Anthracycline Antibiotics in Cancer Therapy*; Muggia, F. M., Young, C. W., Carter, S. K., Eds.; Martinus Nijhoff: Boston, 1982; pp 205-219. Glazer, R. I.; Hartman, K. D.; Richardson, C. L. *Cancer Res.* **1982**, *42*, 117.

(3) Lown, J. W.; Chen, H.-H.; Plambeck, J. A.; Acton, E. M. *Biochem. Pharm.* **1979**, *28*, 2563; **1982**, *31*, 575.

(4) Mimnaugh, E. G.; Trush, M. A.; Ginsburg, E.; Gram, T. *Cancer Res.* **1982**, *42*, 3574.

(5) Davies, K. J. A.; Doroshow, J. H.; Hochstein, P. *FEBS Lett.* **1983**, *153*, 227.

(6) Bachur, N. R.; Gordon, S. L.; Gee, M. V. *Cancer Res.* **1978**, *38*, 1745. Pan, S.-S.; Pedersen, L.; Bachur, N. R. *Mol. Pharmacol.* **1981**, *19*, 184.

(7) Fisher, J.; Ramakrishnan, K.; Becvar, J. E. *Biochemistry* **1983**, *22*, 1347. Fisher, J.; Abdella, B. R. J.; McLane, K. E. *Ibid.* **1985**, *24*, 3562.

(8) Lown, J. W.; Chen, H.-H. *Can. J. Chem.* **1981**, *59*, 390.

(9) Pollakis, G.; Goormaghtigh, E.; Ruysschaert, J.-M. *FEBS Lett.* **1983**, *155*, 267.

(10) Kalyanaraman, B.; Perez-Reyes, E.; Mason, R. P. *Biochim. Biophys. Acta* **1980**, *630*, 119.

(11) Abdella, B. R. J.; Fisher, J. *Environ. Health Perspect.* **1985**, *64*, 3.

0002-7863/87/1509-4046$01.50/0

5-Iminodaunomycin is not a prodrug of daunomycin in that neither daunomycin nor its metabolites are observed as metabolites of **1**. The major compounds found in plasma, liver, heart, lung, and brain of rats receiving high doses of **1** were **1** and 5-imino-13-dihydrodaunomycin. Minor amounts of 5-iminodaunomycinone and 5-imino-13-dihydrodaunomycinone were observed, and no deoxyaglycons of **1** were detected in any tissue.[12] The absence of 7-deoxyaglycon metabolites is consistent with 5-iminodaunomycin being more difficult to reduce because metabolites of this type are diagnostic of anthracycline reduction in a hypoxic environment. The 7-deoxyaglycons are proposed to result from glycosidic cleavage at anthracycline semiquinone or hydroquinone redox states.[6,7,13] Cleavage at the hydroquinone redox state gives a quinone methide intermediate which tautomerizes to the 7-deoxyaglycon.[13]

Recently, we have examined the skin toxicity of a series of anthracycline anti-tumor drugs in a swine model and demonstrated that 5-iminodaunomycin was the most toxic.[14] This observation together with the proposal that 5-iminodaunomycin is redox-incapacitated prompted us to investigate, in vitro, the redox chemistry of **1** in direct comparison with that of **2**.

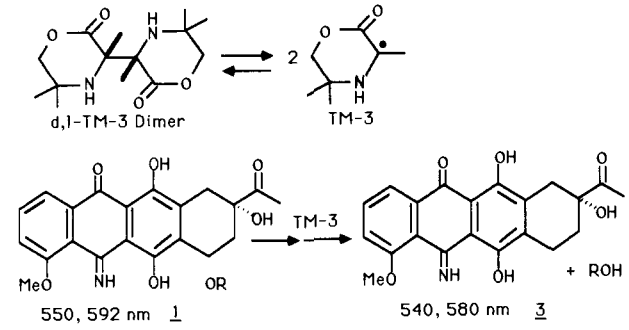
Electrochemical reduction of 5-iminodaunomycin under anaerobic conditions at −0.7 V (SCE) has been shown to yield 5-imino-7-deoxydaunomycinone (**3**) and 2-acetyl-2,11-dihydroxy-7-methoxy-1,2,3,4-tetrahydro-5,12-naphthacenedione (**4**) in two sequential, two-electron processes.[15] The half-wave potentials for the two processes were −0.700 and −0.730 V, respectively. The formation of **3** and **4** was interpreted in terms of initial reduction of **1** to its hydroquinone state followed by glycosidic cleavage to give **3** and subsequent reduction of **3** followed by loss of ammonia to give **4**. Similar electrochemical reduction of daunomycin gave exclusively 7-deoxydaunomycinone (**5**) with an E_0' of −0.66 V (SCE).[16] Dithionite reduction of **5** gives **4** among other products.[17] Half-wave potentials for the first reduction of daunomycin and 5-iminodaunomycin, measured in the same laboratory, are −0.64 and −0.67 V, respectively.[3] Electrochemical measurements in dimethylformamide solvent also demonstrate that imino quinones are more difficult to reduce than corresponding quinones.[18] However, 5-iminodaunomycin has been reduced in vitro by NADPH cytochrome P-450 prepared from rat liver microsomes, and under these reducing conditions **1** stimulated oxygen consumption, although less rapidly than daunomycin.[19]

We establish here that although daunomycin and 5-iminodaunomycin both give 7-deoxyaglycons and naphthacenedione **4** upon reduction, the kinetics are quite different and could be the basis for the inefficiency of 5-iminodaunomycin as a redox catalyst for the production of reactive oxygen species.

Results and Discussion

Reduction of 5-Iminodaunomycin. We established earlier that bi(3,5,5-trimethyl-2-oxomorpholin-3-yl) (TM-3 dimer) is an excellent reagent for elucidating the redox chemistry of the anthracyclines;[13] however, it did not prove to be as useful with 5-iminodaunomycin. In reductions with TM-3 dimer, the actual reducing species is the one electron reducing agent, 3,5,5-trimethyl-2-oxomorpholin-3-yl (TM-3 radical), from homolysis of the 3–3′ bond of TM-3 dimer.[20] Spectroscopic monitoring of a reaction of **1** with TM-3 dimer in apparent pH 8.2 buffered methanol at 25 °C showed only slow conversion of **1** predominantly to **3** with no apparent intermediates. A similar result occurred in methanol-*d* solvent where quinone methide states characteristically have substantially longer lifetimes due to the deuterium kinetic isotope effect.[13] The absence of spectral bands characteristic of hydroquinone and quinone methide intermediates and the time necessary to complete the reduction suggested that the only slow steps in reduction of **1** were bond homolysis of TM-3 dimer and reduction of **1** by TM-3 radical. The rate of reduction, which was slower than the rate of bond homolysis of TM-3 dimer, was consistent with recombination of two TM-3 radicals in competition with reduction of **1** (Scheme I).

Scheme I



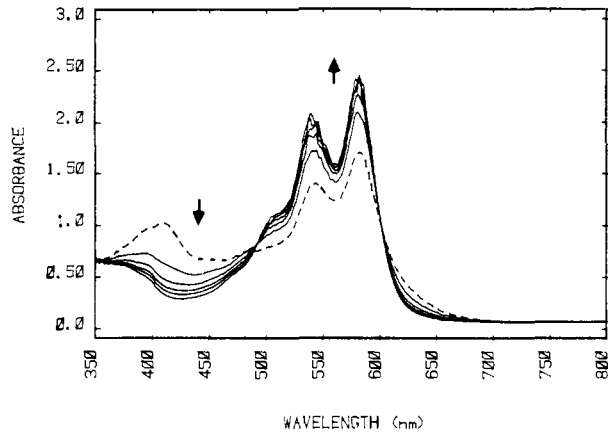
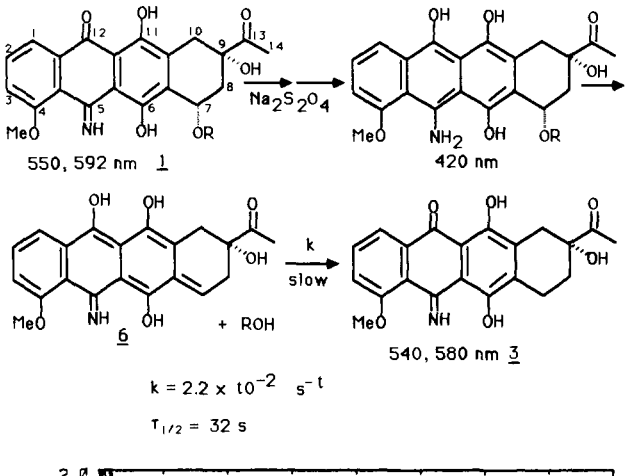


Figure 1. UV–vis absorption spectra of an oxygen-degassed (1:1) Tris–Tris·HCl buffered methanol solution, 8.0×10^{-5} M in 5-iminodaunomycin (**1**) and 7.9×10^{-5} M in sodium dithionite, as a function of time at 25 °C. Scans were 1 s in duration and occurred at the following times after mixing: 30 s (---) and 50, 70, 90, 110, and 150 s (—).

Reaction of 5-iminodaunomycin (**1**) with 1 mol equiv of sodium dithionite, an apparently stronger reducing agent than TM-3, in pH 8.2 buffered, oxygen degassed methanol at 25 °C showed the UV–vis spectral changes displayed in part in Figure 1. Mixing of the reagents resulted in rapid formation of the hydroquinone of **1**, absorbing in the region of 420 nm, and the quinone methide **6** showing end absorption at 630 nm. The quinone methide most likely also absorbs in the region 400–450 nm. In the 5–50 s time period, absorbance in the region of 425 and 630 nm decreased and absorbance in regions of 500, 540, and 580 nm increased due to formation of 5-imino-7-deoxydaunomycinone (**3**). The absorption bands for **3** were assigned from the spectrum of **3** shown in Figure 3. Assignment of the long wavelength absorption at 630 nm and absorption in the region of 400–450 nm to the quinone methide is based upon observation of analogous bands in spectra of quinone methides from reductive glycosidic cleavage of other anthracycline anti-tumor drugs.[13] At 50 s the hydroquinone appears to have disappeared as indicated by the sharp isosbestic

(12) Peters, J. H.; Gordon, G. R.; Kashiwase, D.; Acton, E. M. *Cancer Res.* **1984**, *44*, 1453.
(13) Kleyer, D. L.; Gaudiano, G.; Koch, T. H. *J. Am. Chem. Soc.* **1984**, *106*, 1105. Kleyer, D. L.; Koch, T. H. *Ibid.* **1984**, *106*, 2380. Boldt, M.; Gaudiano, G.; Koch, T. H. *J. Org. Chem.*, submitted for publication.
(14) Averbuch, S. D.; Gaudiano, G.; Koch, T. H.; Bachur, N. R. *J. Clin. Oncol.* **1986**, *4*, 88. Averbuch, S. D.; Boldt, M.; Gaudiano, G.; Stern, J. B.; Koch, T. H.; Bachur, N. R. *J. Clin. Invest.*, submitted for publication.
(15) Tresselt, D.; Ihn, W.; Horn, G.; Berg, H. *Pharmazie*, **1984**, *39*, 417. Ihn, W.; Tresselt, D.; Horn, G.; Berg, H. *Stud. Biophys.* **1984**, *104*, 101. Berg, H. *Ibid.* **1984**, *104*, 13.
(16) Rao, G. M.; Lown, J. W.; Plambeck, J. A. *J. Electrochem. Soc.* **1978**, *125*, 534.
(17) Brand, D. J.; Fisher, J *J. Am. Chem. Soc.* **1986**, *108*, 3088.
(18) Amatore, C.; Anne, A.; Florent, J. C.; Moiroux, J. *J. Electroanal. Chem.* **1986**, *207*, 151.
(19) Bachur, N. R.; Gordon, S. L.; Gee, M. V.; Kon, H. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 954.
(20) Burns, J. M.; Wharry, D. L.; Koch, T. H. *J. Am. Chem. Soc.* **1981**, *103*, 849.

Scheme II



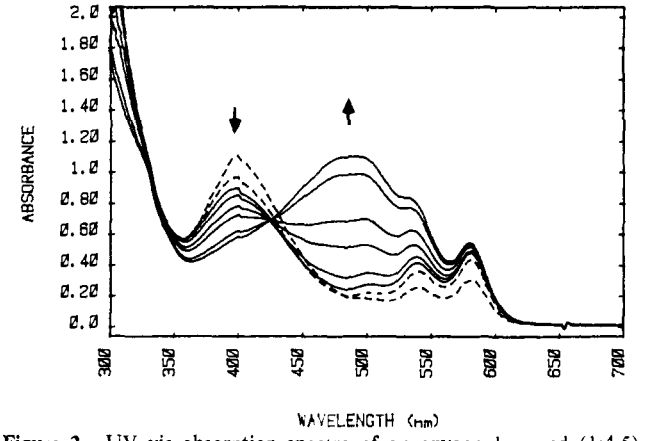
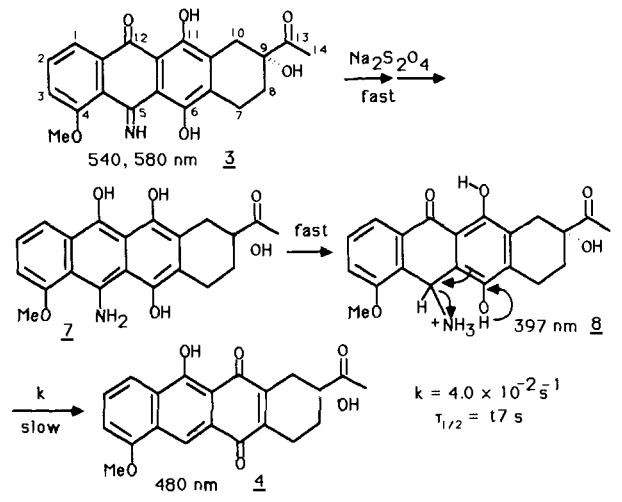


Figure 2. UV–vis absorption spectra of an oxygen-degassed (1:4.5) Tris–Tris·HCl buffered methanol solution, 2.1×10^{-4} M in 5-imino-7-deoxydaunomycinone (**3**) and 1.7×10^{-4} M in sodium dithionite, as a function of time at 25 °C. Scans were 1 s in duration and occurred at the following times after mixing: 6 and 8 s (---) and 10, 12, 18, 24, 30, 42, and 54 s (—). During the first 10 s residual oxygen was scavenged by reduced **3**.

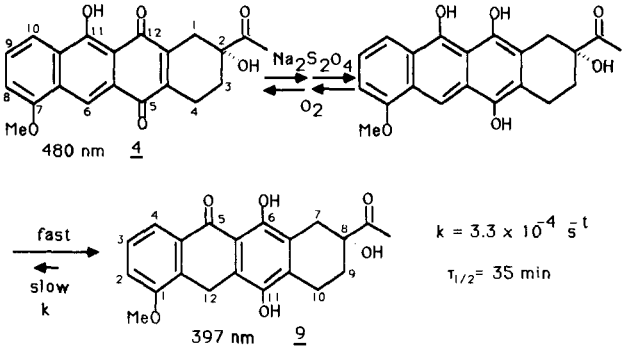
point at 490 nm formed by spectra taken in the time period from 50 to 175 s. In this second time period an absorption rise at 500, 540, and 580 nm was coupled with a fall at 425 and 630 nm characteristic of simple tautomerization of **6** to **3**. The absorbance at 422–424 nm vs. time was used to determine the pseudo-first-order rate constant, $2.2 \times 10^{-2}\ s^{-1}$, for this tautomerization. HPLC analysis of the reaction mixture after no further spectral changes occurred showed 5-imino-7-deoxydaunomycinone (**3**) and a trace of starting material. The reductive glycosidic cleavage process is shown in Scheme II.

Reduction of 5-Imino-7-deoxydaunomycinone. The redox chemistry of 5-imino-7-deoxydaunomycinone (**3**) was similarly investigated by using **3** as the starting material. Pure **3** was prepared by reaction of 7-deoxydaunomycinone (**5**) with methanolic ammonia and characterized by comparison of ^1H NMR and UV–vis spectral data with those in the literature.[15] An anaerobic reaction mixture of **3** and 0.8 mol equiv of sodium dithionite in pH 7.4 buffered methanol at 25 °C gave the spectral changes shown in part in Figure 2. Mixing resulted most likely in rapid formation of the hydroquinone **7** of 5-imino-7-deoxydaunomycinone followed by formation of a tautomer (**8**) of the hydroquinone **7** with absorption at 397 nm in the first 10 s. Assignment of structure **8** (Scheme III) to the species giving the 397-nm band results from absorption by the hydroquinone tautomer of naphthacenedione **4**, 8-acetyl-7,9,10,12-tetrahydro-6,8,11-trihydroxy-1-methoxy-5(8*H*)-naphthacenone (**9**) (Scheme IV, vide infra), at 397 nm.[17] In the time period of 6 to 10 s some 5-imino-7-deoxydaunomycinone reformed most likely from scavenging of residual oxygen by hydroquinone **7**. Rapid formation of some **3** is consistent with the initial rise in the band at 580 nm. Residual

Scheme III



Scheme IV



oxygen is a common problem unless freeze–thaw degassing and sealing with a torch are employed; in our laboratory this degassing procedure has been unsuccessful with dithionite as a reducing agent because of the instability of aqueous dithionite solutions and the time required. In the time period 10–54 s, absorption at 397 nm decreased with increased absorption at 480 nm, characteristic of formation of naphthacenedione **4** via elimination of ammonia from **8**. This transformation occurred with a sharp isosbestic point at 424 nm and accounted for the absorbance increase at 480 nm and the remainder of the absorbance increase at 580 nm shown in Figure 2. During the period 10–54 s the rise in the absorbance at 480 nm accounted for 75% of the rise over the entire reaction. A Kezdy–Swinbourne[21] plot was used to determine the absorbance at 478–480 nm at the infinity point for the elimination reaction. Linear least-squares fit of $\ln (A_\infty - A)$ vs. time gave a rate constant of $4.0 \times 10^{-2}\ s^{-1}$. A second, slower process continued for several hours accompanied by a decrease in the absorbance at 397 nm and the remaining 25% increase at 480 nm. As described below, these changes were most likely a result of the air oxidation of a small amount of naphthacenone **9** to naphthacenedione **4** by oxygen leaking into the cell. Formation of **9** probably resulted from reduction of some **4** by dithionite during the intial 10 s of reaction (vide infra). HPLC analysis of the reaction solution at the end of both reactions showed only the presence of **3** and **4**. The presence of predominantly **3** and **4** after 54 s of reaction was also established by comparison of the sum of the absorption spectra for pure **3** and **4** (Figure 3) with the absorption spectrum of the reaction mixture at 54 s (Figure 2). The processes resulting in reductive elimination of ammonia from 5-imino-7-deoxydaunomycinone (**3**) are summarized in Scheme III.

A second reduction of **3** with sodium dithionite was performed with use of the same conditions except that the progress of the

(21) Kezdy, F. J.; Kaz, J.; Bruylants, A. *Bull. Soc. Chim. Belg.* **1958**, *67*, 687. Swinbourne, E. S. *J. Chem. Soc.* **1960**, 2371. Manglesdorf, J. *J. Appl. Phys.* **1958**, *30*, 443.

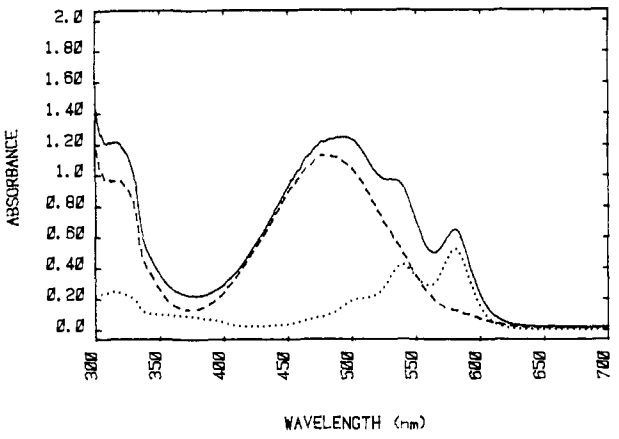


Figure 3. UV–vis absorption spectrum of 2.5×10^{-5} M 5-imino-7-deoxydaunomycinone (**3**) in 1:4.5 Tris–Tris·HCl buffered methanol (···) and of 1.6×10^{-4} M naphthacenedione **4** in Tris buffered methanol (- - -). The sum of the two spectra is shown as the solid line which compares favorably with the spectrum at 54 s in Figure 2.

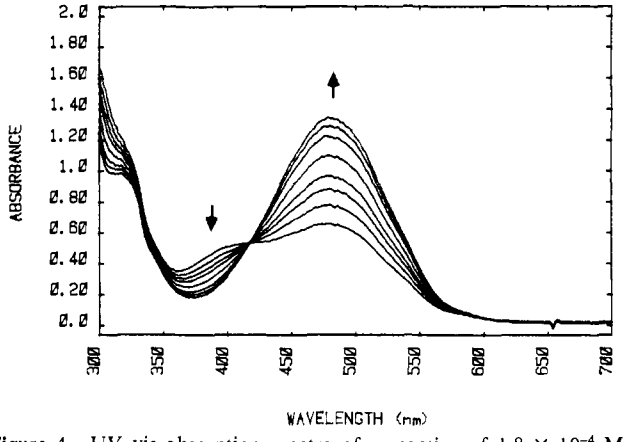


Figure 4. UV–vis absorption spectra of a reaction of 1.8×10^{-4} M naphthacenedione 4 with 1.9×10^{-4} M sodium dithionite in 1:4.5 Tris–Tris·HCl buffered methanol after 600 s and the addition of molecular oxygen. Spectral scans show the conversion of naphthacenone **9** back to naphthacenedione 4. Scans are 1 s in duration and occurred at the following times after mixing: 600, 1200, 1800, 2400, 3600, 5400, 7200, and 10 800 s.

reaction after the first 2 min was monitored by HPLC. A small peak corresponding to naphthacenone **9** appeared in addition to peaks corresponding to **3** and **4**. The identity of **9** was established by spectral/chromatographic analysis of the reaction solution and the reaction solution co-injected with an authentic sample of **9** prepared by dithionite reduction of **4**.[17] The area of the peak corresponding to **9** remained relatively constant until exposure of the reaction solution to oxygen.

The kinetics of oxidation of naphthacenone **9** (Scheme IV) were determined with a solution of **4** anaerobically reduced to **9** with sodium dithionite as described above. Oxygen was bubbled into the cuvette at 25 °C and the spectral changes shown in Figure 4 were observed at 10-min intervals. The pseudo-first-order rate constant for formation of **4** was determined to be 3.3×10^{-4} s^{-1} from the absorbance change at 478–480 nm with use of the Kezdy–Swinbourne method. In Scheme IV the slow step for oxidation of 9 is proposed to be tautomerization to the hydroquinone of **4** because the rate is insensitive to the oxygen concentration. Reduction of **4** but not **1** or **3** could also be performed when the reaction solution was saturated with 2% oxygen in nitrogen.

Reduction of 7-Deoxydaunomycinone. The rates for the redox processes of 7-deoxydaunomycinone (**5**) were also investigated for comparison. Reaction of **5** in degassed pH 7.4 buffered methanol with sodium dithionite gave the spectral changes over the time period 2 min to 4 h shown in Figure 5. During the initial 2 min the hydroquinone (**10**) of **5** with maximum absorption at

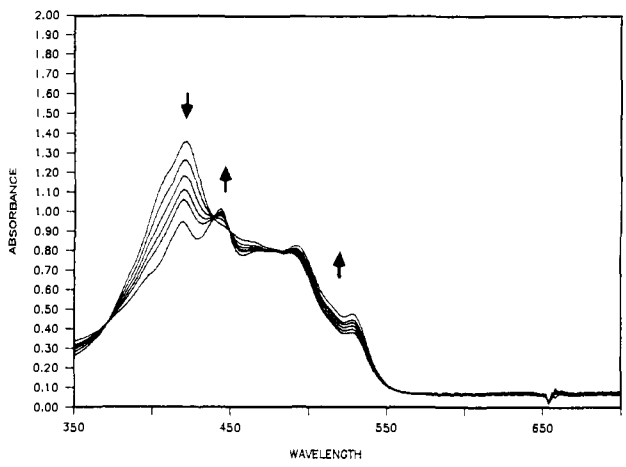
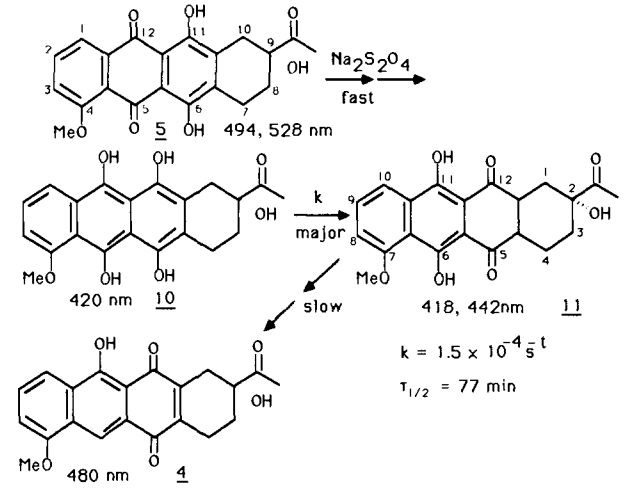


Figure 5. UV–vis absorption spectra of a reaction of 1.1×10^{-4} M 7-deoxydaunomycinone (**5**) and 1.1×10^{-4} M sodium dithionite in Tris–Tris·HCl buffered 7% aqueous methanol as a function of time. Scans are 1 s in duration and occurred at the following times after mixing: 720, 2520, 4320, 6120, 7920, and 14 520 s.

Scheme V



420 nm was rapidly formed. Hydroquinone **10** then slowly disappeared with formation of a mixture of stereoisomers of 2-acetyl-1,2,3,4,4a,12a-hexahydro-2,6,11-trihydroxy-7-methoxy-5,12-naphthacenedione (**11**) first characterized by Brand and Fisher.[17] Because of their yellow color these compounds have been named leuco products. They show absorption at 418 and 442 nm. The increase in bands at 494 and 528 nm is consistent with some air oxidation of the hydroquinone **10** back to **5** by slow leakage of air into the cell over the 4 h time period. The absorbance at 528–530 nm over the first 2 h of reaction as a function of time was zero order and appeared to be dependent only on the rate of leakage of the cell. Non-linear least-squares treatment of the absorbance at 418–420 nm as a function of time, correcting for the small amount of air oxidation using the zero-order rate constant for leakage, gave pseudo-first-order rate constant for tautomerization of hydroquinone **10** to **11** of 1.5×10^{-4} s^{-1}. This rate constant compares favorably with that measured by Brand and Fisher under slightly different conditions.[17] HPLC analysis of the reaction solution showed 46% **5** and 54% **11**. Some of the 7-deoxydaunomycinone (40 out of the 46%) resulted from incomplete initial reduction by dithionite. After exposure for 24 h to molecular oxygen at ambient temperature, the reaction solution showed 53% **5**, 27% **4**, and 20% **11**. Hence, some leuco product (**11**) was slowly converted to **4**, and only a small amount was air oxidized back to 7-deoxydaunomycinone. These reactions are summarized in Scheme V.

Comparison of the Redox Chemistry of Daunomycin and 5-Iminodaunomycin. The observation that reduction of 5-iminodaunomycin (**1**) with TM-3 radical occurs slower than recom-

bination of two TM-3 radicals and similar reduction of daunomycin occurs faster is consistent with the conclusion of others that 5-iminodaunomycin is more difficult to reduce than daunomycin. The difficulty in reducing **1** might have been the explanation for **1** being an inefficient catalyst for production of reactive oxygen species in vivo except for the observation that the enzyme system, cytochrome P-450, is an effective reducing agent for 5-iminodaunomycin.

Assuming now that **1** is reduced in vivo, a possible explanation for inefficiency in radical recycling appears evident in the kinetic measurements presented here. Anaerobic reduction of both daunomycin and 5-iminodaunomycin with dithionite gives rapid formation of the respective 7-deoxyaglycons. In fact 5-iminodaunomycin is reduced to its 7-deoxyaglycon more rapidly than daunomycin, because the rate constant for the slow step, tautomerization of the quinone methide, is 1.7 times larger.[13] Neither 5-iminodaunomycin nor daunomycin can be reduced in vitro in the presence of even traces of molecular oxygen. In both cases if traces of molecular oxygen are present after the degassing process, the initial reduction of the quinones results in subsequent scavenging of the oxygen with reformation of the quinones. Hence, at the intitial oxidation state both **1** and **2** behave similarly. The first difference in redox chemistry appears at the 7-deoxyaglycon state. Both 5-imino-7-deoxydaunomycinone (**3**) and 7-deoxydaunomycinone (**5**) are efficient catalysts for the reduction of molecular oxygen. However, under anaerobic conditions the hydroquinone of **3** has a half-life of only 17 s at 25 °C with respect to formation of naphthacenedione **4**, whereas the hydroquinone of **5** has a half-life of 77 min at 25 °C with respect to formation of naphthacenedione **11**. Naphthacenedione **4** upon reduction even in the presence of molecular oxygen rearranges to naphthacenone **9**, and **9** has a long half-life, 35 min, with respect to reoxidation to **4** by molecular oxygen. Consequently, in vivo if the hydroquinone of 5-imino-7-deoxydaunomycinone experiences a hypoxic environment for only a brief period, it eliminates ammonia to form **4** which is an inefficient catalyst for the formation of reactive oxygen species. The analogous process for the hydroquinone of 7-deoxydaunomycinone is loss of water. It is not observed directly upon reduction of **5** possibly because an intermediate analogous to **8** is not readily formed and/or because at pH 7.4 the leaving group would have to be hydroxide. At pH 7.4 the leaving group in the tautomer of the hydroquinone of 5-imino-7-deoxydaunomycinone (**8**) can be ammonia. Reduction of **5** does eventually form **4**; however, the process is very slow, not well understood, and appears to involve the intermediate formation of **11**. In any event a brief period of hypoxia will not result in the formation of a product from the hydroquinone of 7-deoxydaunomycinone which is a poor catalyst for the production of reactive oxygen species.

The in vitro rates established here predict that 5-imindaunomycin should give 7-deoxyaglycon metabolites. Possibly the lack of observation of 7-deoxyaglycon metabolites[12] resides in the efficient formation of naphthacenedione **4** and naphthacenone **9** and the fluorescence intensity and fluorescence wavelength of **4** and **9**. The analytical method used for the determination of in vivo metabolites was HPLC with fluorescence detection. Fluorescence detection utilized an activation wavelength of 555 nm and an emission wavelength of 610 nm; extracts of liver and stomach contents were also analyzed for daunomycin metabolites with an activation wavelength of 475 nm and an emission wavelength of 580 nm. Fluorescence intensities of **1**, **3**, **4**, and **9** with activation at 475 and 555 nm are compared in Table I. With activation at 555 nm and monitoring at 610 nm, detection sensitivity for **4** is approximately an order of magnitude lower than that for **1** or **3** and for **9** it is zero. With activation at 475 nm and detection at 580 nm, the sensitivity for detection of **4** should be about the same as that for **3** under these conditions but still less than that for **3** with activation at 555 nm and detection at 610 nm. Sensitivity for **9** with the shorter wavelengths is approximately four times less than that for **3** at the shorter wavelengths. Consequently, the analysis of the extracts of plasma, heart, lung, and brain of mice receiving acute doses of 5-iminodaunomycin might have shown reduction metabolites **4** and **9** if the fluorescence detector of the HPLC were set for shorter activation and detection wavelengths especially knowing the chromatographic behavior of the predicted metabolites of reduction.

Table I. Fluorescence Properties of 5-Iminodaunomycin and Its Reduction Products

	excitation wavelengths					
	475 nm		555 nm			
compd (nmol/g)[a]	emission λ (nm)	rel int	emission λ (nm)	rel int	emission λ (nm)	rel int
1 (6)	575[b]	5	605[b]	35	610	30
3 (6)	595[b]	21	595[b]	85	610	40
4 (18)	580[b]	80	590[b]	18	610	10
9 (35)	550[b]	32	none			
9 (35)	580	29				

[a] Concentrations are given in nmol/g of solution. [b] These are wavelengths of maximum fluorescence.

Doroshow has recently implicated the formation of reactive oxygen compounds in at least some aspect of the cytotoxicity of adriamycin but not 5-iminodaunomycin to tumor cells.[22] This observation together with the reported lower cardiotoxicity of 5-iminodaunomycin,[2] reported inability of 5-iminodaunomycin to catalyze the production of reactive oxygen species,[3-5] and the kinetic study described here suggests that the cardiotoxicity and part of the tumor cell toxicity of adriamycin and daunomycin residue in the ability of their 7-deoxyaglycons to serve as catalysts for the intracellular production of reactive oxygen species. Furthermore, the toxicity of 5-iminodaunomycin to skin cells and tumor cells must reside elsewhere, possibly in the formation of a reactive intermediate such as the quinone methide and/or in the formation of naphthacenedione **4**.

Experimental Section

General Remarks. HPLC was performed with a Hewlett-Packard Model 1090 liquid chromatograph with a diode array detector and DPU multichannel integrator with a 10 μm C-18 reverse phase column, eluting with 80% methanol, 19.9% water, and 0.1% trifluoroacetic acid (Method A), or a 5 μm C_{18} column, eluting with 0.02 M phosphoric acid in 65% methanol, 34% water, and 1% tetrahydrofuran (Method B).[12] Ultraviolet and visible spectral data were obtained with either a Hewlett-Packard Model 8450A or Model 8452A rapid-scan spectrophotometer. Fluorescence emission data were obtained on a Perkin-Elmer Model MPF-2A fluorescence spectrometer.

All solvents were HPLC or spectroanalyzed grade. Daunomycin was a generous gift from Drs. Federico Arcamone and Sergio Penco of Farmitalia Carlo Erba, Milan, Italy, and 5-iminodaunomycin from Dr. Steven Averbuch of the National Cancer Institute (NIH). Sodium dithionite, approximately 80% pure, was obtained from the Sigma Chemical Co. and used as received. *d,l*-Bi(3,5,5-trimethyl-2-oxomorpholin-3-yl) (*d,l*-TM-3 dimer) was prepared by photoreductive dimerization of 5,6-dihydro-3,5,5-trimethyl-1,4-oxazin-2-one and separated from its meso isomer by low-temperature flash chromatography.[23] Tris(hydroxymethyl)aminomethane (Tris) and tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl) were obtained from the Sigma Chemical Co.

The values reported for the pH in methanol solvent were those determined with a Chemtrix pH Controller Type 45AR calibrated with standard aqueous buffer solutions and used as a simple pH meter. The measured values were equal to the calculated values from pK_a data in aqueous solution.

Description of the Multicompartment Cell. The cell was constructed of medium-walled glass and consisted of 4 compartments. The large center compartment could hold a volume of 25 mL and had a Pyrex cuvette attached perpendicular to its longitudinal axis. Also attached parallel to the center compartment were two side arm compartments, 10 mL in volume, mounted on opposite sides of the center compartment to form a 120° angle. The spectral cell then bisected and was exterior to this 120° angle. Finally, a third side arm compartment, referred to as the middle side arm, was attached in series to and parallel with the right side arm in such a way that it was interior to the 120° angle mentioned

(22) Doroshow, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4514. Doroshow, J. H. *Biochem. Biophys. Res. Commun.* **1986**, *135*, 330.
(23) Bennett, R. W.; Wharry, D. L.; Koch, T. H. *J. Am. Chem. Soc.* **1980**, *102*, 2345.

above. Each side arm was sealed from the compartments to which it was attached by a high vacuum stopcock to retain the vacuum and allow transfer of solutions from one compartment to another. The large center compartment was also sealed with a high vacuum stopcock which was attached to a tube thal allowed connection to a high vacuum line for the simultaneous freeze–thaw degassing of all cell compartments.

Freeze–Thaw Degassing Procedure. The cells and freeze–thaw degassing procedure for reductions with two-compartment cells were performed on a high vacuum line as described earlier.[13] Methanol and water solutions, placed in individual compartments of the multicompartment cell, were also oxygen degassed on the high-vacuum line. Each cell was frozen in liquid nitrogen and evacuated to 3×10^{-6} Torr or less. The liquid nitrogen was then removed and the solutions were thawed gently with a heat gun. This was repeated four times. On cycles 4 and 5, the solutions were sonicated for 5 to 10 s before refreezing. After sonication on the fifth cycle, the solutions were frozen, evacuated for the final time, and sealed by tightly closing the high-vacuum stopcocks and storing in a nitrogen glovebag.

Deoxygenation of Solutions. When the freeze–thaw degassing procedure was not used, solvents were deoxygenated by boiling for 15 to 20 min under nitrogen which had been passed through a gas purification cylinder capable of reducing the oxygen content to less than 1 ppm. Solvents were then cooled under a stream of nitrogen, covered with a septum, and stored in a glovebag filled with nitrogen.

Reaction of 5-Iminodaunomycin with *d,l*-TM-3 Dimer. To a 50-mL volumetric flask was added 2.23 mg (3.96×10^{-6} mol) of 5-iminodaunomycin hydrochloride. The flask was brought to volume with 2.0×10^{-3} M 1:1 Tris–Tris·HCl buffered methanol and stirred for 30 min. To a 25-mL volumetric flask was added 2.79 mg (9.82×10^{-6} mol) of *d,l*-TM-3 dimer and the solution was brought to volume with dichloromethane. A 1-mL syringe was used to deliver 0.44 mL (1.73×10^{-7} mol) of the TM-3 solution to the chilled (0 °C) cuvette side arm of a two-compartment reaction vessel.[13] The solvent was evaporated under a steady stream of nitrogen. To the second compartment was added 2.0 mL (1.58×10^{-7} mol) of the drug solution via a 1-mL syringe. The solution was freeze–thaw degassed through 5 cycles and sealed under vacuum. The cell was placed in a thermostated cell holder at 25.0 ± 0.1 °C for 25 min. The reagents were mixed and spectra from 300 to 800 nm were periodically taken for 16 h. The cell was opened at 16 h and another spectrum was taken. No reaction intermediates were observed, only a shift in the maxima at 550 and 592 nm for **1** to 540 and 580 nm for **3** at 6 h. No spectral changes occurred between 6 and 16 h nor with admission of molecular oxygen. The color of the solution changed from deep purple at time 0 to a pinkish purple at 16 h. HPLC analysis with Method A eluting at 1.5 mL/min showed predominantly 5-imino-7-deoxydaunomycinone (**3**) at a retention time of 5.4 min and a minor product of 6.3 min, identified as 2-acetyl-2,11-dihydroxy-7-methoxy-1,2,3,4-tetrahydro-5,12-naphthacenedione (**4**). The structures were assigned initially by comparison of UV–vis and mass spectral data for material prepared in a larger scale reaction with those in the literature[15] and subsequently by comparison with material synthesized independently as described below. Similar reduction in methanol-*d* solvent with 9.86×10^{-8} mol of *d,l*-TM-3 dimer and 1.96×10^{-7} mol of 5-iminodaunomycin showed the same spectral changes.

Kinetics of Reduction of 5-Iminodaunomycin (1). To a 25-mL volumetric flask were added 4.1 mg (3.4×10^{-5} mol) of Tris, 5.5 mg (3.5×10^{-5} mol) of Tris·HCl, and 1.13 mg (2.01×10^{-6} mol) of 5-iminodaunomycin hydrochloride. To a 10-mL volumetric flask was added 7.01 mg (3.22×10^{-5} mol) of sodium dithionite. These flasks along with needles, syringes, pipets, cuvettes, and degassed solvents were stored in a nitrogen glovebag for 3 h before bringing solutions to volume. At 3 h, the bag was purged 3 times to eliminate any oxygen introduced with the flasks. The flask containing the drug was brought to volume with degassed spectral grade methanol and serum stoppered and the contents was stirred for 20 min. A 1-mL syringe with a 1.3 cm × 26 gauge needle was used to transfer 1.0 mL of the drug solution to a UV–vis cuvette adapted for a septum screw cap top. The cell was then capped tightly and a second 1.0 mL added through the septum thus creating a positive pressure inside the cuvette. The cell containing 1.61×10^{-7} mol of the drug was placed in a thermostated cell holder at 25.0 ± 0.1 °C for 25 min and purged with a steady flow of nitrogen. The flask containing the sodium dithionate was brought to volume with degassed HPLC grade water, serum stoppered, and shaken. A 100-μL syringe was used to inject 49 μL (1.58×10^{-7} mol) of the sodium dithionate solution into the cuvette containing the drug. The cell was shaken and spectra were taken from 350 to 800 nm every 5 s from 5 s after injection to 175 s. The spectral changes are shown in Figure 1. A clean isosbestic point for formation of 5-imino-7-deoxydaunomycinone (**3**) appeared after 50 s. The first-order rate constant for formation of **3** was determined from the average absorbance at 422 and 424 nm, plotting $\ln (A_t - A_\infty)/(A_0 - A_\infty)$ with the A_∞ determined at 20 min. Thirteen datum points gave a linear plot with slope of 2.2×10^{-2} s^{-1} and a correlation coefficient of 1.00. HPLC analysis was performed with Method A at a flow rate of 1.5 mL/min with monitoring at 550, 580, and 592 nm. The single product formed was characterized as 5-imino-7-deoxydaunomycinone (**3**) by comparison with material prepared independently as described below.

Synthesis of 7-Deoxydaunomycinone (5). A modification of the procedure provided by Farmitalia Carlo Erba, Milan, Italy, was used.[24] Daunomycin hydrochloride (43.7 mg, 7.75×10^{-5} mol) was dissolved in 25 mL of water. This solution was added to a hydrogenation flask containing 0.1 g of 5% palladium on barium sulfate catalyst. The contents of the flask were subjected to hydrogenation for 30 min at ambient temperature and 1 atm of hydrogen. The mixture was filtered, washed first with 25 mL of cold water follwed by 25 mL of cold methanol, and then subjected to soxhlet extraction with dichloromethane to separate **5** from the catalyst. The dichloromethane solution was then concentrated and added dropwise to 50 mL of petroleum ether. The precipitate was collected and dried at ambient temperature in vacuo to yield 24.5 mg of **5** (6.41×10^{-5} mol, 83% yield). The product was identified by spectral comparison with material prepared by dithionite reduction.[25]

Synthesis of 5-Imino-7-deoxydaunomycinone (3). A modification of Acton's procedure for the preparation of 5-iminodaunomycin was used.[1] 7-Deoxydaunomycinone (16.3 mg, 4.26×10^{-5} mol) was dissolved in a minimum amount of dichloromethane. This solution was added to 50 mL of saturated methanolic ammonia in an ice bath and stirred for 1 h. The flask was stoppered and stored in a freezer at 0 °C for 40 h and then removed and placed on a rotary evaporator to remove the solvent. The residue was washed 3 times with chloroform/methanol (6:1, v/v) to remove traces of ammonia. The product was then isolated by flash chromatography on a 10-mm column filled with 230–400 mesh Silica Gel 60 from EM Reagents, eluting with 5% methanol in chloroform and collecting 15-mL fractions. Fractions were monitored by TLC on silica gel plates eluting with the same solvent used for the flash chromatography: R_f 0.33, violet, **3**; 0.63, orange, 7-deoxydaunomycinone. Fractions containing only **3** were combined, rotary evaporated, dissolved in a minimum of dichloromethane, and added dropwise to 50 mL of petroleum ether. The precipitate was collected and dried in vacuo at ambient temperature to yield 8.83 mg of a violet powder, **3** (2.3×10^{-5} mol, 54% yield). ^{1}H NMR and UV–vis spectral analysis of the violet powder confirm its assignment as 5-imino-7-deoxydaunomycinone.[15]

Synthesis of 2-Acetyl-2,11-dihydroxy-7-methoxy-1,2,3,4-tetrahydro-5,12-naphthacenedione (4). Solutions were prepared with the nitrogen glovebag technique. To 8.00 mg of **3** (2.10×10^{-5} mol), 16.8 mg of Tris (1.39×10^{-4} mol), and 106 mg of Tris·HCl (6.71×10^{-4} mol) was added 50 mL of methanol. After all solid material had dissolved, 1.2 equiv of sodium dithionite were added and the flask was removed from the glovebag. Oxygen was bubbled into the flask for 5 min and the reaction solution allowed to sit overnight. The solvent was removed on the rotary evaporator and the residue was then washed 5 times in a separtory funnel with dichloromethane and water. The dichloromethane fraction was placed on the rotary evaporator to remove solvent and the residual solid material dried in vacuo at ambient temperature to yield 6.73 mg (1.8×10^{-5} mol, 86% yield) of a red-orange powder with a melting point of 200–202 °C. The UV–vis spectrum and ^{1}H NMR data matched those reported by Berg[15] and Fisher.[17]

Kinetics of the Reduction of 5-Imino-7-deoxydaunomycinone (3). To 1.98 mg of **3** (5.19×10^{-6} mol), 6.8 mg of Tris (5.62×10^{-5} mol), and 39.7 mg of Tris·HCl (2.51×10^{-4} mol) in a 25-mL volumetric flask were added deoxygenated methanol and a stirbar within a nitrogen glovebag. After 30 min of stirring, all solid material had dissolved. To a 10-mL volumetric flask containing 29.3 mg of 81% pure sodium dithionite (1.36×10^{-4} mol) was added deoxygenated water within the nitrogen glovebag. A quartz cuvette was filled with 2 mL of the solution of **3** and covered with a septum cap. The cuvette was removed from the nitrogen glovebag and placed in a thermostated cell holder[13] at 25.0 °C under a stream of nitrogen to equilibrate for 15 min. With use of a 100-μL syringe, 25 μL of the sodium dithionite solution was withdrawn from the 10-mL volumetric flask, removed from the glovebag, and injected into the cuvette. The cell was vigorously shaken and then spectra were taken from 6 to 54 s at 2-s intervals and then from 10 min to 3 h at 10-min intervals. The spectral changes during the first time interval are shown in Figure 2. The average absorbance at 478 and 480 nm, the λ_{max} of the product naphthacenedione **4**, was then recorded vs. time. Analysis of these kinetic data showed that both a fast and a slow process were taking place as described in the Results and Discussion section. The fast process was the

(24) Penco, S., Farmitalia Carlo Erba, Milan Italy, personal communication, 1986.

(25) Smith, T. H.; Fujiwara, A. N.; Lee, W. W.; Wu, H. Y.; Henry, D. W. *J. Org. Chem.* **1977**, *42*, 3653.

elimination of ammonia to give naphthacenedione **4**, accounting for 75% of the rise in the absorbance at 480 nm, and the slow process was air oxidation of naphthacenone **9** to **4** by oxygen leaking into the cell, accounting for 25% of the rise at 480 nm. A Kezdy–Swinbourne plot using a τ of 20 s was used to determine the absorbance at the infinity point for the fast process. A linear least-squares treatment of a plot of ln ($A_\infty - A$) vs. time gave a first-order rate constant of 4.0×10^{-2} s^{-1} with a correlation coefficient of 1.00. HPLC analysis of the reaction mixture, using Method B and eluting at 2.0 mL/min, showed only two components, which were identified as **3** and **4** from the chromatographic and spectral analysis with the HP 1090 liquid chromatograph, including co-injection of authentic samples.

HPLC Experiment To Isolate the Source of the Slow Rate Constant in the Reduction of 3. To 1.79 mg of **3** (4.69×10^{-6} mol), 8.3 mg of Tris (6.85×10^{-5} mol), and 53.7 mg of Tris·HCl (3.40×10^{-4} mol) in a 25-mL volumetric flask were added deoxygenated methanol and a stirbar inside a nitrogen glovebag. To a 10-mL volumetric flask containing 28.7 mg of 81% pure sodium dithionite (1.34×10^{-4} mol) was added deoxygenated water. Within the glovebag, 2 mL of the solution of **3** was transferred to a small vial and injected with 25 μL of the reducing agent. Starting at 2 min after injection of the reducing agent, 10-μL samples were withdrawn from the vial and injected onto the HPLC, eluting at 2.0 mL/min (Method B). In addition to **3** and **4**, an unknown peak with a shorter retention time than either **3** or 4 was observed. The area of the unknown peak remained relatively constant over several hours but began to decrease after exposure to oxygen. The HP 1090 spectral analysis of the unknown peak produced a spectrum very similar to that of the hydroquinone tautomer of **4**, 8-acetyl-1-methoxy-7,9,10,12-tetrahydro-6,8,11-trihydroxy-5(8*H*)-naphthacenone (**9**). An authentic sample of 4 was then reduced with sodium dithionite to form its hydroquinone tautomer **9**, and the reaction solution and **9** were coinjected onto the HPLC. The area of the unknown peak increased appropriately, and the purity evaluation spectra from the HP 1090 HPLC showed that only **9** was present in the peak.

Kinetics of the Oxidation of the Naphthacenone 9. To 1.70 mg of **4** (4.63×10^{-6} mol), 10.5 mg of Tris (8.67×10^{-5} mol), and 559 mg of Tris·HCl (3.54×10^{-4} mol) in a 25-mL volumetric flask were added deoxygenated methanol and a stirbar inside a nitrogen glovebag. After 1 h of stirring, all of **4** had dissolved. To a 10-mL volumetric flask containing 27.2 mg of 80% pure sodium dithionite (1.25×10^{-4} mol) was added deoxygenated water buffered to pH 8.5 with Tris buffer within the nitrogen glovebag. A quartz cuvette was filled with 2 mL of the solution of **4** and covered with a septum cap. The cuvette was then removed from the nitrogen glovebag and placed in a thermostated cell holder at 25.0 °C under a stream of nitrogen to equilibrate for 15 min. With use of a 100-μL syringe, 30 μL of the sodium dithionite solution was withdrawn from the 10-mL volumetric flask, the syringe was removed from the glovebag, and the 30 μL was injected into the cuvette to convert 4 to naphthacenone **9**. The cell was vigorously shaken, a spectrum was taken, and then the cell was removed from the cell holder. Oxygen was bubbled into the cuvette for 5 min. The cuvette was returned to the thermostated cell holder, and spectra were taken for 3 h at 10-min intervals starting at 10 min after injection of the reducing agent as shown in Figure 4. The average absorbance at 478 and 480 nm was then recorded vs. time. The absorbance at the infinity point was determined with use of a Kezdy–Swinbourne plot with a τ equal to 3000 s. A nonlinear least-squares treatment of the data gave a first-order rate constant of 3.3×10^{-4} s^{-1} with a correlation coefficient of 1.00. HPLC analysis showed the presence of only **4** at the end of the experiment.

Reduction and Kinetics of Reoxidation of 4 in the Presence of 2% Oxygen. To 1.70 mg of **4** (4.63×10^{-6} mol), 10.5 mg of Tris (8.67×10^{-5} mol), and 55.9 mg of Tris·HCl (3.54×10^{-4} mol) in a 25-mL volumetric flask were added deoxygenated methanol and a stirbar inside a nitrogen glovebag. After 1 h of stirring, all of the solid material had dissolved. To a 10-mL volumetric flask containing 27.0 mg of 80% pure sodium dithionite (1.24×10^{-4} mol) was added deoxygenated water which had been buffered at pH 8.5 with Tris buffer. Within the glovebag, 2 mL of the solution of **4** was transferred to a quartz cuvette, which was then sealed with a septum cap. The cuvette was removed from the glovebag and allowed to equilibrate to 25.0 °C in a thermostated cell holder. During equilibration, 2% oxygen in nitrogen was bubbled through the solution in the cuvette for 10 min. With use of a 100-μL syringe, 30 μL of the sodium dithionite solution was withdrawn from the 10-mL volumetric flask, removed from the glovebag, and injected into the cuvette. The cell was shaken vigorously and spectra were taken every 10 min from 10 min after injection of the reducing agent until 3 h. During the run, 2% oxygen in nitrogen was bubbled into the cuvette for 2 min out of every 10 min. The spectra showed that approximately 70% of 4 present was initially reduced to naphthacenone **9**. The average absorbance at 478 and 480 nm was then recorded vs. time. The absorbance at the infinity point was determined with use of a Kezdy–Swinbourne plot with a τ equal to 3000 s. Linear least-squares treatment of the data from multiple experiments gave rate constants ranging from 2.6×10^{-4} to 4.0×10^{-4} s^{-1}, with an average of 3.3×10^{-4} s^{-1}. HPLC analysis with Method B and eluting at 2.0 mL/min showed the presence of only **4** at the end of the experiment.

Kinetics of the Tautomerization of the Hydroquinone (10) of 7-Deoxydaunomycinone. To 1.09 mg of **5** (2.85×10^{-6} mol) and 40 mg of Tris·HCl (2.5×10^{-4} mol) in a 25-mL volumetric flask were added methanol and a stirbar. To 0.754 g of Tris (6.23×10^{-3} mol) and 0.539 g of Tris·HCl (3.40×10^{-3} mol) was added 150 mL of water. A 10-mL portion of the solution of **5** was transferred to the large center compartment of the multicompartment cell. To the middle side arm was added 0.75 mL of Tris buffered water, and to the right side arm was added 0.24 mg of 80% pure sodium dithionite (1.10×10^{-6} mol). The cell was subjected to the freeze–thaw degassing procedure described above. After equilibration to 25 °C in a constant temperature bath, the buffered water was mixed with the reducing agent, which was then mixed with the solution of **5**. The reaction mixture was transferred to the side arms, leaving only enough solution in the center cell to fill the cuvette. The cuvette was then placed in the thermostated cell holder supplied by Hewlett-Packard for the HP 8452A rapid-scan spectrophotometer and equilibrated to 25 °C. Spectra were taken at 10-min intervals from 2 min after mixing until 4 h. The spectral changes showed a fall in absorbance at 420 nm and a rise at 442 nm consistent with conversion of the hydroquinone of **5** to a mixture of stereoisomers of 2-acetyl-1,2,3,4,4a,12a-hexahydro-7-methoxy-2,6,11-trihydroxy-5,12-naphthacenedione (**11**). A rise in the absorbance at 494 and 528 nm was consistent with some air oxidation of **10** back to **5** by the slow leakage of oxygen into the cell, a process which could not be prevented. Over the first 2 h of reaction the absorbance change at 528–530 nm was zero order. Linear least-squares analysis gave a rate constant of 1.5×10^{-9} M/s with a correlation coefficient of 1.00 for leakage of oxygen. Nonlinear least-squares fitting of the absorption at 418–420 nm vs. time to the integrated rate law (shown below), including the zero-order process of oxygen leakage, gave the pseudo-first-order rate constant of 1.5×10^{-4} s^{-1} for tautomerization of **10** to **11**. This rate constant is the average of two measurements with an average deviation from the mean of 0.04×10^{-4} s^{-1}. The average standard deviation in the rate constant for the least-squares fit was 8%.

$$A_t = \frac{(\epsilon_{10} - \epsilon_{11})(k[\mathbf{10}]_0 - k_{ox})e^{-kt} + k_{ox}}{k} + \epsilon_{11}[\mathbf{10}]_0 + \epsilon_5([\mathbf{5}]_0 + k_{ox}t) - \epsilon_{11}k_{ox}t$$

A_t is the absorbance at 418–420 nm at time t where t_0 is 2 min after mixing; ϵ_5, ϵ_{10}, and ϵ_{11} are the molar extinction coefficients of **5**, **10**, and **11** at 418–420 nm; k is the pseudo-first-order rate constant for tautomerization of **10** to **11**; and k_{ox} is the zero-order rate constant for leakage of oxygen. The extinction coefficient ϵ_{10} was determined experimentally to be 19 000 M^{-1} cm^{-1} from a solution of **5** and excess sodium dithionite, and the concentrations of **5** and **10** at time zero were calculated from the extinction coefficients for **5** and **10** at 420 nm and the extinction coefficient for **5** at 528 nm and the absorbances at 420 and 528 nm. The extinction coefficient for **11** at 420 nm was calculated in the nonlinear least-squares analysis to be 12 000 ± 500 M^{-1} cm^{-1}. After 4 h, the cell was opened and 60 μL of the reaction solution was injected onto the HPLC, eluting at 1.5 mL/min and Method B. Oxygen was then bubbled into the reaction solution for 10 min, followed by injection onto the HPLC. A final HPLC analysis was performed 24 h after the cell was opened. Chromatographic analysis performed immediately after addition of oxygen showed that the reaction mixture contained 46% **5** and 54% of a mixture of stereoisomers of 2-acetyl-1,2,3,4,4a,12a-hexahydro-7-methoxy-2,6,11-trihydroxy-5,12-naphthacenedione (**11**), as determined from the purity evaluation spectra of the HP 1090 and the areas of the respective peaks at their wavelengths of maximum absorbance (**11** 442 nm; **5** 494 nm; **4** 480 nm). The structural assignment for naphthacenedione **11** was made by comparison of UV–vis spectra from the HP 1090 HPLC diode array detector with those reported by Brand and Fisher.[17] Of the 46% of **5** present upon opening the cell, 40 out of the 46% was due to incomplete initial reduction of **5**. After 24 h, the reaction mixture contained 53% **5**, 27% 4, and 20% **11**. Of the **11** destroyed in the 24 h after addition of oxygen, 20% was converted to **5** and 80% to **4**.

Fluorescence Emission Spectroscopy of 1, 3, 4, and 9. To 1.15 mg of **1** (2.04×10^{-6} mol) in a 25-mL volumetric flask were added methanol and a stirbar. A 0.17-mL aliquot of this solution was diluted to 3.0 mL giving a solution that was 6 nmol of **1** per g of solution. To 1.08 mg of **3** (2.83×10^{-6} mol) in a 25-mL volumetric flask were added methanol and a stirbar. A 0.13-mL aliquot of the solution of **3** was diluted to 3.0 mL giving a concentration of 6 nmol of **3** per g of solution. To 1.19 mg of **4** (3.24×10^{-6} mol) in a 10-mL volumetric flask were added deoxy-

genated methanol and a stirbar inside a nitrogen glovebag. A 0.13-mL aliquot of the solution of **4** was diluted to 3.0 mL and the solution was removed from the glovebag. The diluted solution contained 18 nmol of **4** per g of solution. Inside the nitrogen glovebag a 0.26-mL aliquot of **4** was added to a cuvette and diluted to 3.0 mL. The cuvette was then sealed with a septum. To 27.7 mg of 80% pure sodium dithionite (1.27×10^{-4} mol) in a 10-mL volumetric flask was added deoxygenated water inside the nitrogen glovebag. With use of a 100-μL syringe, 15 μL of the sodium dithionite solution was injected into the cuvette containing **4** producing **9** with a concentration of 35 nmol/g of solution. Fluorescence spectra of the solutions of **1**, **3**, 4, and **9** were then measured with excitation and emission slits set at 8 nm. The results are summarized in Table I.

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Conformational Analysis of Lipophilic Antifolates: Crystal and Molecular Structures of 6-Substituted 5-Adamantyl-2,4-diaminopyrimidines by X-ray Analysis and Molecular Mechanics Calculations

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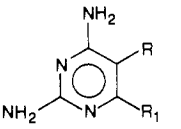
Abstract: The results of crystal structure determinations and molecular mechanics force field calculations on a series of four 6-substituted 5-adamantyl-2,4-diaminopyrimidine antifolates show that the pyrimidine ring and its substituents become more distorted from planarity as the size of the 6-substituent increases. These distortions are caused by the steric interference of the adamantyl hydrogen atoms with those of the 4,6-pyrimidine ring substituents. This series of antifolates shows a 500-fold increase in their cytotoxic activity against mammalian dihydrofolate reductase when the 6-substituent is increased from hydrogen to methyl to ethyl but drops at propyl. Full relaxation MM2P conformational energy profiles for rotation about the pyrimidine–adamantyl bond show that the maximum energy barrier (~6 kcal/mol) is located at 60° within the 0–120° unique conformational space studied. The structurally observed conformations of the 6-H and 6-methyl analogues are located at energy minima, whereas the most active antifolate 6-ethyl and 6-propyl analogues are observed in high energy conformations. The crystal structure of the 6-ethyl antifolate also reveals a (67/33% occupancy) disorder in the methylene carbon of the ethyl side chain which makes several short intramolecular H···H contacts with the adamantyl hydrogen atoms. This conformation may be stabilized in the crystal by intermolecular interactions not considered in the present force field calculations. Such stabilization could also be operative at the enzyme binding site and thus contribute to the 6-ethyl analogue's high potency.

Lipophilic diaminopyrimidines are a class of drugs that act as inhibitors of dihydrofolate reductase (DHFR) and have been developed for use as antimalarial, antibiotic, or anticancer agents. These drugs also show striking differences in their inhibitory activity with only small changes in their chemical structures.[1-3] It has been demonstrated that the principal structural characteristic necessary for binding to dihydrofolate reductase of any species is a 2,4-diaminopyrimidine, *s*-triazine, or pteridine ring structure.[4] Structure–activity studies of 2,4-diaminopyrimidines further indicate that a lipophilic group at position five is also essential for tight binding to DHFR.[5] Large differences in inhibitory potency have been observed concomitant with small changes in the chemical structures of these antifolate drugs.

Comparison of several lipophilic antifolates shows that those with a 5-adamantyl substituent are the most effective inhibitors of mammalian DHFR with potencies greater than the chemotherapeutic agent methotrexate (MTX).[5-8] As illustrated (Table I), these structures have greater binding affinities for DHFR than those antifolates with comparable molar volumes and hydrophobicities.[7,8] Additionally, there is a sharp increase in cytotoxic activity of 5-adamantyl 6-substituted antifolates within the series with 6-H < propyl < methyl < ethyl (Table I). These adamantyl antifolates also have cellular uptake rates about 10000 times more rapid than MTX and show strong cytotoxic activity in culture cells.[9,10]

Table I. Structure–Activity Relationships among Lipophilic Diaminopyrimidine Inhibitors of DHFR



R	R_1	X-ray structure	ID_{50},[a] μM
adamantyl	hydrogen	DAHP	3.3×10^{-7}
adamantyl	methyl	DAMP	6.0×10^{-9}
adamantyl	ethyl	DAEP	2.5×10^{-10}
adamantyl	propyl	DAPP	4.8×10^{-8}
cyclohexyl	methyl	DCXMP	3.9×10^{-7}
hexyl	methyl	DHXMP	1.2×10^{-6}
heptyl	methyl	DHMP	1.6×10^{-6}
tert-butyl	methyl	DTMP	1.9×10^{-5}
1-naphthyl	methyl	DNMP-1	5.6×10^{-4}
2-naphthyl	methyl	DNMP-2	7.0×10^{-8}
methotrexate		MTX	8.0×10^{-9}

[a] 50% growth inhibition of mouse mammary adenocarcinoma cells (TA3) in culture.[7]

In order to investigate the structural, conformational, and electronic properties of this series of 5-adamantyl lipophilic an-

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(1) Hitchings, G. H.; Burchall, J. J. *Adv. Enzymol.* **1965**, *27*, 417–468.
(2) Blakley, R. L.; Benkovic, S. J. *Folates and Pterines*; Wiley: New York, 1984; Vol. 1.
(3) Roth, B.; Cheng, C. C. *Prog. Med. Chem.* **1982**, *19*, 270–331.

0002-7863/87/1509-4053$01.50/0