Leucodaunomycins: New Intermediates in the Redox Chemistry of Daunomycin

Donald M. Bird,[†,‡] Giorgio Gaudiano,[‡] and Tad H. Koch*[,‡]

Contribution from the Department of Chemistry, U.S. Air Force Academy, Colorado Springs, Colorado 80840, and the Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215. *Received June 21, 1990*

Abstract: The reduction of daunomycin (**1a**) to the hydroquinone state has been described in the literature as leading either to elimination of the sugar daunosamine with formation of 7-deoxydaunomycinone (**5**) under anaerobic conditions or to subsequent oxidation by molecular oxygen, yielding reactive oxygen species under aerobic conditions. The conditions under which the hydroquinone of **1a** (**7**) tautomerizes, producing an isolable reduced form of **1a**, referred to as leucodaunomycin, (8*S*)-*cis*-8-acetyl-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-6a,7,8,9,10,10a-hexahydro-5,8,12-trihydroxy-1-methoxy-6,11-naphthacenedione (**3**), are described here. The synthesis and characterization of the four diastereomers, A–D, of **3**, along with studies of aerobic and anaerobic chemistry of two isomers, A and C, are reported. Under aerobic conditions, the isomers of **3** react with oxygen to form **1a**, **5**, 7-epidaunomycinone (**8**), 7-deoxy-7-ketodaunomycinone (**9**), 7-deoxy-7,13-epidioxydaunomycinol (**10**) and daunomycinone (**6**), and the rate constant is a function of pH and stereochemistry and varies from 3.0×10^{-4} to 2.6×10^{-2} s^{-1} at pH 7.4. Under anaerobic conditions at pH 7.4 and 25 °C, an aqueous solution of isomer A of **3** forms a 97:3 ratio of **5** to **6**, and the reaction follows consecutive first-order kinetics with a k_1 of 1.0×10^{-2} s^{-1} for the tautomerization of **3** to hydroquinone **7** and a k_2 of 3.3×10^{-2} s^{-1} for the tautomerization of quinone methide **2** to **5**. Under identical conditions, isomer C of **3** forms a 96:4 ratio of **5** to **6**, and the reaction follows first-order kinetics with a k of 1.2×10^{-3} s^{-1} for tautomerization of **3** to **7**. Finally, the ability of **3** to react with *N*-acetyl-L-cysteine and participate in an autocatalytic cycle in the presence of **1a** to form 7-[(*N*-acetyl-L-cysteinyl)thio]-7-deoxydaunomycinone (**11**) is described.

Introduction

The anthracycline antitumor drugs daunomycin (**1a**) and adriamycin (**1b**) are the products of aerobic fermentation of the microorganism *Streptomyces peucetius* and have been used extensively in the treatment of various solid tumors and leukemias.[1-3] Most believe that anthracycline cytotoxicity is expressed by a variety of biological pathways and that any one drug's cytotoxicity is the total of its various modes of action.[4] The favored modes of anthracycline cytotoxicity center on interaction of the drug with DNA, with membranes, and with proteins such as topoisomerase, and on the catalytic redox chemistry of the anthracyclines to produce reactive oxygen species.[5,6]

Several researchers have demonstrated that the anthracyclines have the ability to intercalate in DNA, where they are believed to stabilize the helical structure and prevent replication.[7-9] Another target for drug attack is the cell membrane and the lipids associated therewith. Membranes containing lipids with acidic head groups have been shown to associate strongly with anthracycline drugs.[10] Membrane properties such as fluidity, ion transport, glycoprotein synthesis, phospholipid structure,[11] and protein transport[12] are altered significantly in the presence of adriamycin. While a direct correlation between cytotoxicity and anthracycline–membrane interaction has not been made, researchers have found that drug association can lead to genetic changes in the membrane that increase the drug efflux and effectively make the cells resistant to a broad spectrum of antitumor drugs.[13]

The in vivo and in vitro redox chemistry of the anthracyclines has received widespread attention. The two most popular schemes for redox-mediated cytotoxicity involve the formation of reactive oxygen species and the production of quinone methides, reactive aglycon tautomers presumably capable of covalently binding to a variety of biological molecules.

Formation of the reactive oxygen species in vivo is generally considered to be an aerobic process in which a single electron transfer leads to the formation of the anthracycline semiquinone radical, which subsequently transfers that electron to oxygen, leading to the formation of reactive oxygen species such as the superoxide radical, hydrogen peroxide, and hydroxyl radical.[14-16] The chemistry of anthracycline quinone methides has been studied intensively because quinone methides are proposed as possible reactive forms for covalent binding to biological molecules.[17] Daunomycin and adriamycin have been shown to bind covalently to both DNA and proteins. Crothers and co-workers have shown that the intercalation of **1** in DNA is an exothermic process with a preference for binding to G + C base pairs.[9] Sinha's work has also provided evidence for the covalent binding of anthracyclines to DNA and proteins under anaerobic conditions.[18-20] Koch and Fisher have shown that anthracycline quinone methides can react with electrophiles and nucleophiles.[21-23] Electrophilic attack by

[†] U.S. Air Force Academy.
[‡] University of Colorado.

(1) Arcamone, F. *Doxorubicin Anticancer Antibiotics*; Academic Press: New York, 1981.
(2) Carter, S. K. *J. Natl. Cancer Inst.* **1975**, *55*, 1265.
(3) Henry, D. W. *ACS Symp. Ser.* **1976**, *No. 30*, 15.
(4) Lown, J. W., Ed. *Anthracycline and Anthracenedione-Based Anti-Cancer Agents*; Elsevier: Amsterdam, 1988.
(5) Abdella, B. R. J.; Fisher, J. *Environ. Health Perspect.* **1985**, *64*, 3. Fisher, J. F.; Aristoff, P. A. *Prog. Drug Res.* **1988**, *32*, 411.
(6) Powis, G. *Pharmacol. Ther.* **1987**, *106*, 2380. Powis, G. *Free Radical Biol. Med.* **1989**, *6*, 63.
(7) Berg, H.; Horn, G.; Luthardt, U. *Electrochem. Bioenerg.* **1981**, *8*, 537.
(8) Crooke, S. T.; Duvernay, V. H.; Galvan, L.; Prestayko, A. W. *Mol. Pharmacol.* **1978**, *14*, 290.
(9) Chaires, J. B.; Dattagupta, N.; Crothers, D. M. *Biochemistry* **1982**, *21*, 3933.
(10) Goormaghtigh, E.; Chatelain, P.; Caspers, J.; Ruysschaert, J. M. *Biochem. Pharmacol.* **1980**, *29*, 3003.
(11) Wheeler, C.; Rader, R.; Kessel, D. *Biochem. Pharmacol.* **1982**, *31*, 2691.
(12) Eilers, M.; Endos, T.; Schatz, G. *J. Biol. Chem.* **1989**, *264*, 2945.
(13) Kolata, G. *Science* **1986**, *231*, 220.
(14) Bachur, N. R.; Gordon, S. L.; Gee, M. V. *Cancer Res.* **1978**, *38*, 1745.
(15) Lown, J. W.; Sim, S.-K.; Chen, H.-H. *Can. J. Biochem.* **1978**, *56*, 1042.
(16) Lown, J. W.; Chen, H.-H. *Can. J. Chem.* **1981**, *59*, 390.
(17) Moore, H. W.; Czerniak, R. *Med. Res. Rev.* **1981**, *1*, 249.
(18) Sinha, B. K.; Chignell, C. F. *Chem.-Biol. Interact.* **1979**, *28*, 301.
(19) Sinha, B. K. *Chem.-Biol. Interact.* **1980**, *30*, 67.
(20) Sinha, B. K.; Sik, R. H. *Biochem. Pharmacol.* **1980**, *29*, 1867.
(21) Kleyer, D. L.; Koch, T. H. *J. Am. Chem. Soc.* **1984**, *106*, 2380.
(22) Fisher, J.; Ramakrishnan, K.; Becvar, J. E. *Biochemistry* **1983**, *22*, 1347.

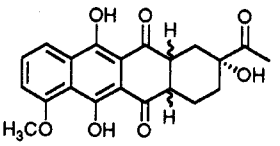
0002-7863/91/1513-308$02.50/0

a proton restores the quinone chromophore while nucleophilic attack yields a hydroquinone adduct, which can eliminate the nucleophile to re-form the quinone methide unless it is oxidized to a quinone adduct.[5] In vivo, adduct formation would seem to favor quinone methide reacting with electrophiles; however, except for the proton, appropriate biological electrophiles are rare. Association with nucleic acids could cause reaction with weakly electrophilic or nucleophilic positions of guanine and cytosine to be favorable.[22,24,25]

Both association with DNA and reductive activation would seem to be necessary for covalent binding of the drug to DNA. However, if the mode of association is intercalation, reductive activation is inhibited.[13] Furthermore, if the drug is reduced prior to intercalation, the half-life of the quinone methide (**2**) of daunomycin, 15 s at 25 °C,[26] is probably insufficient to allow migration from the site of reduction and intercalation to occur. As reported earlier[27] and discussed in more detail here, a possible solution to this problem is the formation of leucodaunomycins (**3**), hydroquinone tautomers of **1a**. These leuco tautomers are the stereoisomers of (8*S*)-*cis*-8-acetyl-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-6a,7,8,9,10,10a-hexahydro-5,8,12-trihydroxy-1-methoxy-5,12-naphthacenedione (**3**). Heretofore, the anaerobic reduction of **1a** was believed to lead only to reductive elimination of the sugar moiety through the quinone methide **2**. Since the sugar moiety seems to be a key factor for intercalation,[2] this reduced form of **1a** with the sugar moiety attached may be able to intercalate and then react with DNA through formatioin of the quinone methide **2**. This would be consistent with the hypothesis that to achieve covalent binding **1a** must be associated with the target molecule prior to activation and that reduction of **1a** does not occur to a significant extent after intercalation.[5,18-20,28]

Results and Discussion

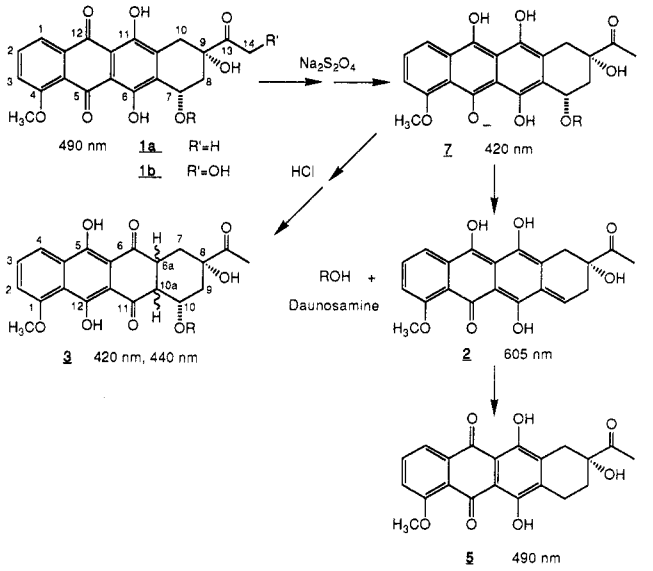
Preparation of Leucodaunomycin. The discovery of leucodaunomycin was the fortuitous result of an experiment by Boldt in here examination of solvent effects on the rate of daunosamine elimination from the hydroquinone state of **1a**.[29] Reduction of **1a** in pure acetonitrile resulted in formation of a species with spectral properties quite similar to those reported by Brand and Fisher for leuco-7-deoxydaunomycinone, 2(*R*)-acetyl-1,2,3,4,4a,12a-hexahydro-2,6,11-trihydroxy-7-methoxy-5,12-naphthacenedione (**4**), from the reduction of 7-deoxydaunomycinone (**5**).[30] Treatment of the acetonitrile solution with a small amount of base in the presence of oxygen resulted in the restoration of the normal chromophore of **1a**, and HPLC analysis indicated that only **1a** was present.



4

The low solubility of **1a** in acetonitrile was a limiting factor in the use of acetonitrile as the solvent for synthesis of **3**. In their study of the tautomeric instability of the hydroquinone of **5**, Brand and Fisher noted that the leuco products were very unstable in the presence of base.[30] At pH 7 and above, the phenolic OH groups of hydroquinone **7** are partially deprotonated,[31] a condition that most likely favors the elimination reaction to form **5**; thus, adidic pH was proposed as a favorable condition for the desired tautomerization of **7**. The solubility of **1a** in water, along with the ability to control accurately the pH of aqueous solutions, made water the preferred solvent for synthesis of **3**. When 1 equiv of sodium dithionite was added to a deoxygenated aqueous solution of **1a** adjusted to pH 3, the four diastereomers of **3** were produced along with **5** and a significant amount of unreduced **1a**. Incomplete reduction is consistent with the fact that the emf of sodium dithionite under acidic conditions is 0.08 V while under basic conditions it is 1.13 V.[32] To maximize the reducing ability of sodium dithionite and the stabilizing effect of acidic conditions, 0.25 equiv of deoxygenated HCl was injected into the reaction solution to reduce the pH to 3 immediately after **1a** was reduced with 1 equiv of sodium dithionite. This procedure resulted in the complete reduction of **1a**, formation of the least amount of **5** and the largest amount of **3**, and is described in more detail in the Experimental Section. Scheme I shows the proposed mechanism for the formation of **3** as well as the competing reaction that leads to the formation of **5**. Two major (A and C) and two minor (B and D) isomeric products were detected in the ratio of 2.9:1.0:4.7:1.2, in order of increasing retention time as determined by photodiode array detection on reverse-phase HPLC. The individual diastereomers were isolated by reverse-phase flash chromatography eluting with a gradient of methanol and aqueous formic acid followed by rotary evaporation to removed the methanol and lyophilization to remove the water. Only isomers A and C were isolated in sufficient quantity and purity for further characterization and study. The total yield of **3**, determined chromatographically, was approximately 40% prior to separation into the individual diastereomers. However, the lengthy process of lyophilization, chromatography, and a second lyophilization reduced the overall isolated yield to approximately 10%.

Scheme I



Characterization of Isomers A and C of Leucodaunomycin. UV–vis spectral properties were determined in water and methanol. Because both isomers A and C were unstable in water near pH 7, the pH was lowered to 3.0 with HCl. HPLC analysis after the accumulation of spectral data showed the presence of only isomer A or C, as appropriate. In methanol, isomer C was relatively stable; however, isomer A was unstable. Even when HCl was

(23) Guadiano, G.; Egholm, M.; Haddadin, M. J.; Koch, T. H. *J. Org. Chem.* **1989**, *54*, 5090. Egholm, M.; Koch, T. H. *J. Am. Chem. Soc.* **1989**, *111*, 8291.
(24) Pullman, B.; Pullman, A. *Adv. Heterocycl. Chem.* **1971**, *13*, 77.
(25) Kwiatkowski, J. S.; Pullman, B. *Adv. Heterocycl. Chem.* **1975**, *18*, 199.
(26) Fisher, J.; Abdella, B. R. J.; McLane, K. *Biochemistry* **1985**, *24*, 3562.
(27) Bird, D. M.; Boldt, M.; Koch, T. H. *J. Am. Chem. Soc.* **1988**, *111*, 1148.
(28) Mimnaugh, E. G.; Trush, M. A.; Ginsburg, E.; Gram, T. E. *Cancer Res.* **1982**, *42*, 3574.
(29) Boldt, M. Substituent Effects on the Redox Chemistry of Anthracycline Anti-Tumor Drugs. M.S. Thesis, University of Colorado, Boulder, CO, 1987.
(30) Brand, D. J.; Fisher, J. *J. Am. Chem. Soc.* **1986**, *108*, 3088. Brand, D. J.; Fisher, J. *J. Org. Chem.* **1990**, *55*, 2518.
(31) Bouma, J.; Beijnen, J. H.; Bult, A.; Underberg, W. J. M. *Pharm. Weekbl. Sci. Ed.* **1986**, *8*, 109.
(32) (a) Sodium Hydrosulfite. Technical Data Sheet No. 76-69-683P, Virginia Chemicals Inc., Portsmouth, VA. (b) Sodium Hydrosulfite. Technical Data Sheet No. 76-70-184V, Virginia Chemicals Inc., Portsmouth, VA.

Table I. UV–vis Spectral Properties of Isomers A and C of Leucodaunomycin (**3**)

water		methanol[a]	
λ_{max}, nm	ϵ, M^{-1} cm^{-1}	λ_{max}, nm	ϵ, M^{-1} cm^{-1}
Isomer A			
245	18 100	245	19 300
272	20 500	267	20 700
397 sh	6 300	397 sh	6 400
422	11 800	420	12 000
444	12 100	446	12 300
Isomer C			
243	21 600	242	24 700
270	21 800	268	21 600
397 sh	7 700	399 sh	9 100
420	13 900	418	16 200
444	14 300	444	17 600

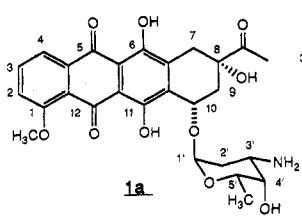
[a] These data were calculated, correcting for the presence of reaction products, by using known extinction coefficients for **1a** and assuming that all components other than isomer A have the same extinction coefficients as **1a**.

added to the methanol to reduce the pH of the solution to an apparent pH of 3.0, isomer A decomposed; however, the rate was slow enough to allow the collection of spectral data. UV–vis spectral data are shown in Table I. The actual spectra of isomers A and C of **3** in water were previously reported.[27] Comparison of the UV–vis spectral data to those collected by Brand and Fisher for the diastereomers of **4** supports the assignment of structure **3** to leucodaunomycin. The loss of daunosamine would not be expected to affect significantly the chromophore and indeed the maxima reported for **4**, 266.5, 376 (shoulder), 397.5, 417.5, and 442 nm,[30] compare quite favorably to those reported in Table I for **3**.

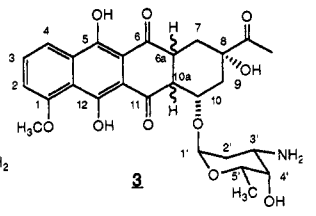
The mass spectral data also support the assignment of structure **3** to leucodaunomycin. The use of positive and negative ion fast atom bombardment mass spectrometry has been useful for obtaining mass spectral data for daunomycin and its derivatives.[21,33] The only peak in the negative ion FAB spectrum of **3** was at m/z 529 and was attributed to **3** plus an electron. The positive ion spectrum gave peaks at m/z 552, 530, and 401. The m/z 552 peak was attributed to **3** plus a sodium ion and the m/z 530 peak to **3** plus a proton. The peak at m/z 401 was attributed to 8-acetyl-6a,7,8,9,10,10a-hexahydro-5,8,10,12-tetrahydroxy-1-methoxy-6,11-naphthacenedione plus a proton. This molecule is the leucodaunomycin equivalent of daunomycinone (**6**), which is likely formed by hydrolytic cleavage of the amino sugar from daunomycin in the mass spectrometer.

NMR data were difficult to acquire because of the instability of **3**. NMR measurements focused on isomer C since it was more stable than isomer A. Methanol-d_4 acidified with a small amount of DCl to stabilize the compound was used for the NMR experiment. Complete ^1H NMR data for isomer C of **3** are presented in the Experimental Section, and the important chemical shifts are summarized in Table II along with those for **1a**, **4**, and **5**. Assignments were based upon the splitting patterns and the integration of the peaks. The chemical shifts of the protons on carbon-7 of **3** are shifted upfield nearly 1 ppm from those observed for the corresponding protons of **1a**, consistent with changing from α to an aromatic ring to β to a carbonyl group. The single proton on carbon-10 is shifted upfield 0.2 ppm in the transition from **1a** to **3**. As the data show, this shift is over 0.8 ppm with the aglycons in the transition from **5** to **4**. The upfield shift for these protons of **3** is consistent with the disappearance of aromaticity in ring B; the less dramatic change relative to that observed in the aglycons is consistent with the presence of an oxygen atom on carbon-10 in compound **3**. The new resonances for protons at carbons-6a and -10a in **3** are 3.61 and 3.78 ppm, respectively. These chemical shifts are consistent with methine protons α to a carbonyl. The resonance at 3.78 ppm is assigned to the proton

Table II. Comparison of ^1H NMR Data for Compounds **1a**, **3**, **4**, and **5**[a]



1a



3

^1H	**1a**[b]	**5**[c]	**3**[b]	**4**[c]
7	2.80	2.91	1.9	1.92
	3.34	3.05		2.31
9	2.10	1.94	2.1	1.72
	2.78	2.01		
10	4.91	2.93	4.73	2.05
		3.15		2.48
10a			3.78	2.81
6a			3.61	3.33
1′	5.40		4.96	

[a] **1a**, daunomycin; **5**, 7-deoxydaunomycinone; **3**, leucodaunomycin; **4**, leuco-7-deoxydaunomycinone. For purposes of comparison, the numbering system used for **3** has been used for all four compounds. [b] Data collected in methanol-d_4. [c] Data collected in $CDCl_3$ and reported by Brand and Fisher.[30]

on carbon-10a because it is further downfield, as would be expected because of the oxygen bonded to carbon-10. As the table shows, the reverse is true for the aglycons. The downfield resonance in **4** is assigned to the proton on carbon-6a because it is α to one carbonyl and γ to another, as well as being β to a hydroxyl.

Unfortunately, the stability and solubility of **3** precluded the use of ^{13}C NMR, and the ^1H NMR was of only sufficiently good quality to justify the structure of **3** without the assignment of stereochemistry at carbons-6a and -10a. The structural assignment is secure from the spectroscopic data, the related work of Brand and Fisher[30] on the structure of leuco-7-deoxydaunomycinone (**4**), and the chemical reactivity (vide infra). The UV–vis spectra for the isomers of **3** are virtually identical with those published by Brand and Fisher for **4**,[30] as would be expected for compounds with the same chromophore. Further, treatment of both isomers A and C with base in the presence of oxygen in acetonitrile solvent resulted in the quantitative regeneration of daunomycin (**1a**), as shown by coinjections on the HPLC. The mass spectral data confirmed that the reduction and subsequent tautomerization resulted in a product molecule with a mass 2 greater than the starting material. Finally, the data presented later for the anaerobic chemistry of **3** show that **3** decomposes almost exclusively to 7-deoxydaunomycinone (**5**), presumably through hydroquinone **7** and quinone methide **2** via the same mechanism by which **1a** reductively eliminates daunosamine (Scheme III).

Aerobic Chemistry of Isomers A and C of Leucodaunomycin. To understand the chemistry of **3** in aqueous medium, its disappearance was first examined as a function of pH under aerobic conditions. The disappearance of isomers A and C was observed spectroscopically at pH 6.4, 7.0, and 7.4. These pH levels were chosen because they gave measurable rates of disappearance of **3** and are physiologically feasible.

Figure 1 shows some of the spectroscopic data collected when isomer A of **3** was adjusted to pH 6.4 under aerobic conditions. Though the rates were different, the spectral changes at pH 7.0 and 7.4 were identical with those at pH 6.4. Each set of spectra showed the disappearance of **3** by the decrease in absorbance at 420 and 444 nm along with the appearance of products with absorbance at 490 nm. A Kezdy–Swinbourne plot[34] of the absorbance versus time data at pH 6.4, to find absorbance at the infinity point, was nonlinear indicating that more than one measurable process was taking place. Kezdy–Swinbourne plots of absorbance versus time data for reaction of isomer A at pH

(33) Dass, C.; Seshadri, R.; Israel, M.; Desiderio, D. M. *Biomed. Environ. Mass Spectrom.* **1988**, *17*, 37.

(34) Kezdy, F. J.; Kaz, J.; Bruylants, A. *Bull. Soc. Chim. Belges* **1958**, *67*, 687. Swinbourne, E. S. *J. Chem. Soc.* **1960**, 2371.

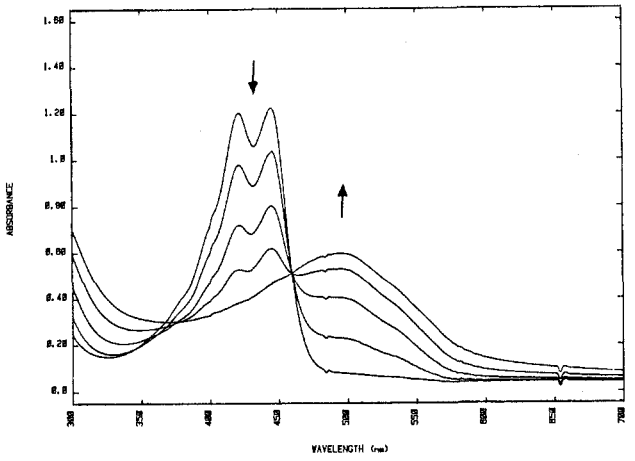


Figure 1. UV–vis absorption spectra of a reaction mixture in which 1.12 × 10^{-4} M aqueous solution of isomer A of **3** was adjusted to pH 6.4 with pHydrion standard buffer under aerobic conditions at 25 °C as a function of time. Scans are 1 s in duration and occurred at the following times after mixing: 5, 120, 360, 900, and 10 800 s.

Table III. Rate Constants and Product Ratios for Reaction of Leucodaunomycin (**3**) as a Function of Isomer, Anaerobicity, and pH

pH	k, s^{-1}	product ratios[a] **8+9**[b]**:10:6:1a:5**
	Aerobic Medium	
isomer A of **3**		
6.4		2.4:3.2:1.5:1:1
7.0	1.1×10^{-2}	2.1:3.1:1.6:1:1
7.4	2.6×10^{-2}	2.6:3.3:1.5:1:1
isomer C of **3**		
6.4	3.0×10^{-4}	3.5:1.6:1:2.8:2.7
7.0	1.0×10^{-3}	3.1:2.2:1:2.6:1.1[c]
7.4	1.5×10^{-3}	2.4:1.9:1:3.7:1.2

pH	k_1, s^{-1}	k_2, s^{-1}	**5:6**
	Anaerobic Medium[d]		
isomer A of **3**			
7.4	1.0×10^{-2}	3.3×10^{-2}	97:3
isomer C of **3**			
7.4	1.2×10^{-3}		96:4

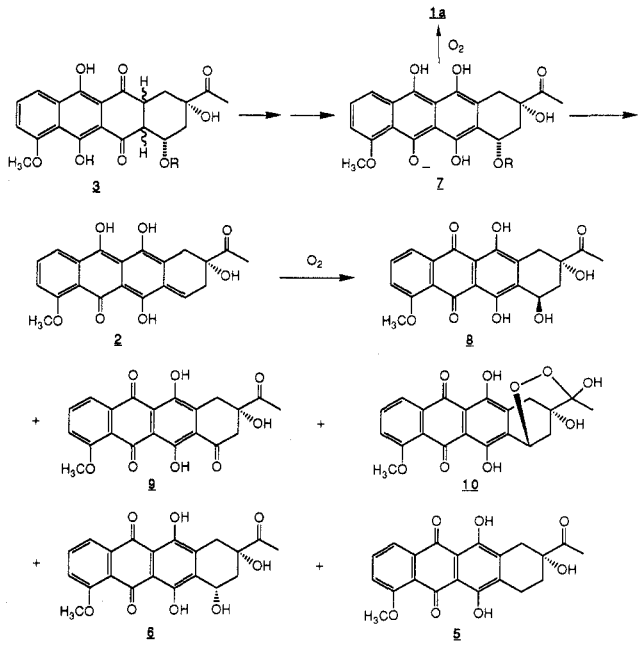
[a] The assumption of equal extinction coefficients for all products at 480 nm was made based upon all products bearing the daunomycin-like quinone chromophore. [b] The peaks for **8** and **9** were not well resolved; ratio of **8** to **9** was approximately 3:2. [c] HPLC peaks for **8+9** and **10** in this case consisted of at least one additional component each. [d] Rate constants are defined in Scheme III.

7.0 and 7.4 were linear. Least-squares analysis of these data gave the pseudo-first-order rate constants reported in Table III. The increase in rate constant with pH is consistent with a base-catalyzed transformation of **3** and confirms the previous observations that the stability of **3** decreases with increasing pH. HPLC analysis of reaction mixtures from experiments at pH 6.4, 7.0, and 7.4 showed six major product peaks in each. These were identified as 7-epidauomycinone (**8**), 7-deoxy-7-ketodaunomycinone (**9**), 7-deoxy-7,13-epidioxydaunomycinol (**10**), daunomycinone (**6**), daunomycin (**1a**), and 7-deoxydaunomycinone (**5**), in order of increasing retention time, by chromatographic and spectroscopic comparison with authentic samples (Scheme II).[35]

The spectroscopic changes resulting from adjusting a solution of isomer C of **3** to pH 6.4 and 7.4 under aerobic conditions were similar to those shown in Figure 1 for isomer A. As with isomer A, each set of spectra showed a sharp isosbestic point at 456 nm along with decreasing absorbances at 420 and 444 nm and increasing absorbance at 490 nm. The linear least-squares kinetic analysis gave the pseudo-first-order rate constants reported in Table III. Again the order is consistent with what would be expected for a base-catalyzed reaction. Additionally, the rates

(35) Gaudiano, G.; Koch, T. H. *J. Am. Chem. Soc.* **1990**, *112*, 9423.

Scheme II



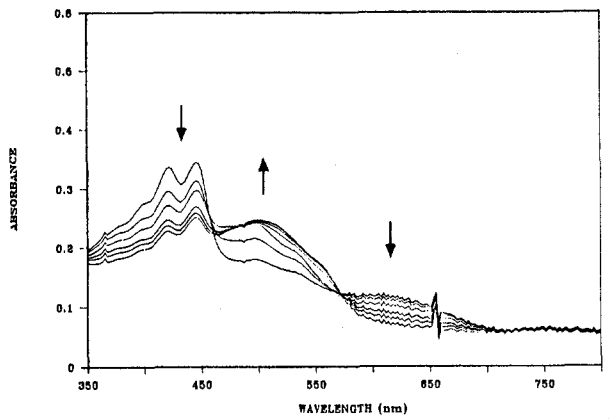


Figure 2. UV–vis absorption spectra of a reaction mixture in which 4.40 × 10^{-5} M aqueous solution of isomer A of **3** was adjusted to pH 7.4 under anaerobic conditions at 25 °C as a function of time. Scans are 1 s in duration and occurred at the following times after mixing: 65, 95, 125, 180, 240, and 360 s.

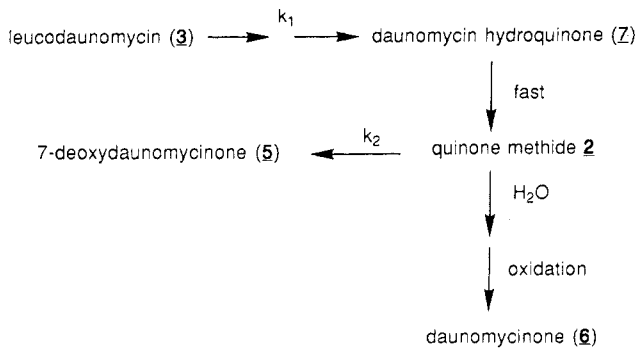
for isomer C are significantly slower than for isomer A, as would be expected from the previously mentioned observation that isomer C is more stable than isomer A. HPLC analysis of the experiments at pH 6.4 and 7.4 showed the same six products as were formed from isomer A of **3**, and product ratios are compared in Table III.

The products of aerobic reaction of **3** are also products of reaction of quinone methide **2**, from reductive glycosidic cleavage of **1a**, with molecular oxygen.[35] Consequently, they are shown in Scheme II as resulting from leucodaunomycin tautomerizing to hydroquinone **7** followed by glycosidic cleavage to quinone methide **2** and reaction of **2** with molecular oxygen. A proposed mechanism for formation of **6** and **8–10** from reaction of **2** with molecular oxygen appears elsewhere.[35]

Anaerobic Chemistry of Isomers A and C of Leucodaunomycin. The complexity of the aerobic reactions of **3** coupled with the fact that the anaerobic chemistry of the anthracyclines is believed to play an important biological role in their activity prompted the investigation of the anaerobic chemistry of isomers A and C of **3**. These studies focused only on pH 7.4 since this is physiological pH and since the predominant effect of pH in the aerobic studies was on the rate.

The spectroscopic changes that occurred during the first 65 s after an aqueous solution of isomer A of **3** was freeze–thaw

Scheme III

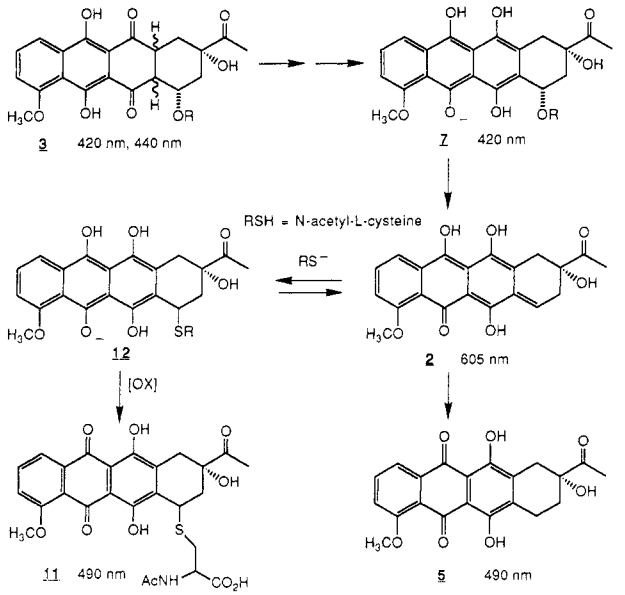


degassed and then adjusted to pH 7.4 are shown in Figure 1 of the earlier communication.[27] The decrease in absorbance at 420 and 444 nm was accompanied by a rise in absorbance at 490 and 605 nm. The spectroscopic changes that occurred after 65 s are shown in Figure 2 and indicate that the absorbance at 605 nm also decreased while the absorbance at 490 nm continued to rise with product formation. HPLC analysis after the reaction showed the presence of 7-deoxydaunomycinone (**5**) and daunomycinone (**6**) in a ratio of 97:3. HPLC analysis verified that no **6** was present in the starting materials. The decrease in absorbance at 420 and 444 nm along with the rise at 605 nm is attributed to the tautomerization to hydroquinone **7** followed by elimination of daunosamine to form quinone methide **2**, which absorbs at 605 nm.[21] Subsequently, **2** tautomerizes to form **5**, which absorbs at 490 nm or is attacked by water to form **6** in its hydroquinone state. This reaction mechanism is summarized in Scheme III. Nonlinear least-squares analysis of the change in the average absorbance at 618–622 nm fit consecutive first-order kinetics. The absorbance by **2** was monitored in this region rather than at 605 nm to minimize interference from absorbance by quinones. The rate constants, k_1 and k_2, defined in Scheme III are reported in Table III. For comparison, an experiment was performed in which daunomycin (**1a**) was anaerobically reduced with 1 equiv of sodium dithionite in pH 7.4 water. HPLC analysis of this reaction showed the presence of **5** and **6** in a ratio of 98:2, confirming that **6** is also a minor product of the anaerobic reduction of **1a**, and in about the same ratio as observed after the reaction of **3** upon adjustment of the pH to 7.4. Further, linear least-squares analysis of the average absorbance at 618–622 nm gave a pseudo-first-order rate constant of 3.2×10^{-2} s^{-1}, the rate constant for tautomerization of quinone methide **2** to **5**. The nearly equal rate constants for tautomerization of the transients generated from isomer A of **3** and daunomycin (**1a**) is kinetic evidence that the transients observed during both reactions are the same quinone methide **2**.

The spectral changes that took place when a solution of isomer C of **3** was anaerobically adjusted to pH 7.4 were similar to those shown for isomer A under aerobic conditions in Figure 1 except isosbestic points were observed at both 365 and 456 nm. Only very minor absorbance changes at 605 nm were apparent. First-order linear least-squares analysis of the decreasing absorbance at 444 nm as a function of time gave the pseudo-first-order rate constant k_1 reported in Table III. Scheme III also shows the proposed mechanism for reaction of isomer C. In this case, the only slow step is the tautomerization of isomer C to hydroquinone **7**. The subsequent steps are identical with those proposed for isomer A, but the quinone methide was not observed spectroscopically because the first tautomerization is so much slower than the following steps, elimination of daunosamine and tautomerization of **2** to **5**. HPLC analysis of the reaction mixture yielded virtually the same result as with isomer A, with the only products being **5** and **6** (see Table III).

The kinetic data are consistent with mechanisms for product formation with quinone methide **2** as an intermediate as shown in Schemes II and III except for reaction of isomer A of **3** at pH 7.4 in aerobic medium. Under these conditions, if the rate-controlling step were formation of **2**, then k should equal k_1 determined for reaction in anaerobic medium. In fact k (aerobic) is

Scheme IV



2.6 times k_1 (anaerobic). A possible explanation is that tautomerization of the carbonyl group at the 11-position (see Table II for numbering system) occurs faster than tautomerization of the carbonyl at the 6-position, that glycosidic cleavage occurs after the first tautomerization to give a tautomer of **2**, and that molecular oxygen reacts rapidly with this tautomer.

Reaction of Isomer A of Leucodaunomycin with *N*-Acetyl-L-cysteine at pH 7.4 under Anaerobic Conditions. Several reports suggest that the reductive activation of the anthracyclines can lead to covalent binding to proteins and nucleic acids.[18-20,36,37] Since **3** is reduced by two electrons when compared to **1a**, investigation of its potential for reacting with a biological-type molecule was undertaken. On-going work in the laboratory with *N*-acetyl-L-cysteine and the existence of authentic samples of 7-[(*N*-acetyl-L-cysteinyl)thio]-7-deoxydaunomycinone (**11**) made *N*-acetyl-L-cysteine the preferred candidate for investigation.

Isomer A of **3** was anaerobically adjusted to pH 7.4 in the presence of a 60-fold excess of *N*-acetyl-L-cysteine, and the reaction mixture was allowed to sit for 20 h prior to exposure to air. HPLC analysis showed three product peaks. The first two peaks, in a ratio of 1:2, accounted for 14% of the total anthracyclines. These peaks were identified as the diastereomers of **11** by coinjection with authentic samples on the HPLC. The third product, accounting for 86% of the total anthracyclines, was **5**. Thus, nucleophilic attack by *N*-acetyl-L-cysteine accounted for 14% of the products while the electrophilic attack by H^+ accounted for 86% of the products as shown in Scheme IV. No daunomycinone (**6**) was detected. A similar experiment was performed in deuterium oxide with a 250-fold excess of *N*-acetyl-L-cysteine with anticipation of a higher yield of adduct formation because of the known kinetic isotope effect on protonation of **2** to give **5**.[21,26] HPLC analysis, however, showed that 95% of the **3** initially present was converted to **5** and only 5% was converted to **11**, with only the more predominant isomer from the previous experiment being detected. Scheme IV shows the mechanism for the formation of **11**. The mechanism for formation of **5** is the same as is shown in Scheme III. In the absence of an oxidant, the predominant reaction is formation of **5**. At pH 7.4, one of the phenolic hydrogens of **12**, such as the one at C-5, is likely deprotonated. This possibly accounts for the observed deuterium isotope effect as follows. In deuterium oxide the phenolic hydrogens will exchange with deuterons. If the C-5 phenolic group of the hydroquinone **12** is anionic, elimination of the group at C-7 to form quinone methide **2** should not show a large isotope effect. However,

(36) Ramakrishnan, J.; Fisher, J. *J. Med. Chem.* **1986**, *29*, 1215.
(37) Boldt, M.; Gaudiano, G.; Haddadin, M. J.; Koch, T. H. *J. Am. Chem. Soc.* **1989**, *111*, 2283. See also: Reference 23.

formatioin of trapped product requires a deuteride transfer, possibly to **5**, which is forming during the reaction. The kinetic isotope effect on deuteride transfer should result in less efficient trapping to form **11** and more favorable nucleofuge departure to re-form **2**. While an isotope effect is also observed in the conversion of **2** to **5**,[21,26] this effect must be less than that observed in the formation of **11**.

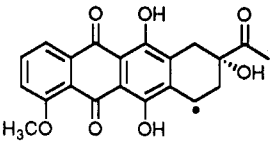
In their investigtion of the reaction of quinone methide **2** with nucleophiles, Ramakrishnan and Fisher observed that when 0.4 equiv of reducing agent was used to reduce **1a** in the presence of a 60-fold excess of *N*-acetyl-L-cysteine the product analysis showed 65% of adduct **11** and 35% of **5**.[36] From these results they proposed an autocatalytic mechanism in which hydroquinone **12** is oxidized by **1a**, perpetuating the cycle. In light of this work, investigation of the trapping of *N*-acetyl-L-cysteine by **3** in the presence of **1a** was pursued.

Isomer A of **3** was adjusted to pH 7.4 in D_2O in the presence of a 250-fold excess of *N*-acetyl-L-cysteine and a 4-fold excess (relative to **3**) of **1a**. HPLC analysis after only 2 h showed that 66% of the total anthracycline content was **1a**, 11% was **5**, and 23% was adduct **11**. Since only 20% of the initial anthracycline content was **3**, a significant amount of **1a** must have participated in redox chemistry resulting in **5** and **11**. The increased yield of trapped product when compared to the previous experiment in deuterium oxide was presumably the result of having a readily available oxidant, daunomycin, on hand to continue the autocatalytic cycle.

Significance of Leucodaunomycin. Herein, we report the first isolation and investigation of a form of daunomycin (**1a**) at the hydroquinone redox state. Prior to this, all reductions of the quinone functionality of **1a** resulted in either elimination of the sugar daunosamine or, when molecular oxygen was present, formation of reactive oxygen species. Of biological importance is the question of whether or not **3** can be formed in vivo. While the precise answer to this question must await direct observation, the potential for in vivo formation certainly exists. A region of reduced pH would be conducive to formation of **3** and these regions are believed to exist especially within tumor cells.[38] Boldt's initial discovery of **3** was the result of an anaerobic reduction of **1a** in acetonitrile, an aprotic solvent.[29] Certainly hydrophobic regions exist within the cell and cell membrane where the tautomerization of the daunomycin hydroquinone might be favored over daunosamine elimination. In vivo, an advantage in terms of cytotoxicity for forming leucodaunomycin as a precursor to quinone methide is that the reduction can take place at a location in the cell more remote from the quinone methide target molecule; the half-life of the C isomer of leucudaunomycin, for example, is 24 times the half-life of quinone methide. Even if in vivo chemistry does not permit the formation of **3**, it may still be important biologically as an antitumor drug. Anthracyclines such as **1** are strong DNA intercalators, yet intercalation has been shown to inhibit biological reductive activation.[39,40] Since **3** is already in a reduced state, the necessity for an adequate reducing agent has been eliminated. The ability of **3** to form covalent bonds, at least with sulfur nucleophiles, has now been demonstrated.

Ferradine and co-workers[41] have recently shown that daunomycin (**1a**) intercalated in DNA can be reduced anaerobically by carbon dioxide radical anion from γ radiolysis. 7-Deoxydaunomycinone (**5**) was formed without covalent binding or daunomycin or its aglycon to DNA. The absence of covalent binding may reflect the absence of a suitable oxidizing agent for the anticipated DNA–7-deoxydaunomycinone hydroquinone transient. Without the oxidizing agent, coupling to DNA would likely reverse. In vivo, molecular oxygen might serve the role of oxidant if a DNA–7-deoxydaunomycinone hydroquinone adduct were formed as a transient species from reaction of leucodaunomycin with DNA.

Also of interest are the products resulting from the aerobic chemistry of **3**. These products themselves may exhibit biological activity, or transients formed during the oxidation of **3** may be important in coupling the aglycon portion of the drug to biological macromolecules. As presented elsewhere,[35] a logical transient in the oxidation of quinone methide **2** is the one-electron oxidation product of **2**, namely, semiquinone methide (**13**). Semiquinone



13

methide might couple with carbon radical sites created in macromolecules via oxidative stress induced by daunomycin through catalytic production of reactive oxygen species. Such a mechanism for coupling the aglycon to macromolecules was proposed much earlier without an established mechanism for formation of semiquinone methide **13**.[18-20,42] An advantage to this mode of coupling is the creation of a carbon–carbon bond, which would not likely cleave upon any subsequent reduction of the quinone functionality.

Regardless of the efficacy of **3** as an antitumor drug, its isolation and characterization has opened a new avenue for investigating the redox chemistry of daunomycin and other anthracycline antitumor drugs.

Experimental Section

General Remarks. HPLC was performed with a Hewlett-Packard Model 1090 liquid chromatograph equipped with a diode array detector and DPU multichannel integrator. Chromatography was performed with a 5-μm C_{18} reverse-phase microbore column, 2.1 mm i.d. and 100 mm long from Hewlett-Packard, eluting with a mixture of methanol (A) and 3% ammonium formate in water adjusted to pH 4 with 98% formic acid (B) using a gradient from 40% A–60% B for the first 2 min to 60% A–40% B at 12 min. UV–vis spectral data were collected with a Hewlett-Packard 8450 or 8452A spectrophotometer. Mass spectral data were collected from a V. G. Instruments 7070 EQ-HF high-resolution mass spectrometer. Proton NMR spectra were determined on a Bruker WM 250 spectrometer. Chemical shifts are reported in parts per million on the δ scale. Elemental analysis of **3** was not performed because the relatively large quantities required for the analysis were not practically obtainable with the current method of synthesis.

All solvents were HPLC or spectroanalyzed grade and were used after filtration through 0.2- or 0.5-μm filters. No other purification was used. Sodium dithionite, approximately 80% pure, was obtained from Sigma Chemical Co. and used as received. Tris(hydroxymethyl)aminomethane (Tris) and tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) were obtained from Sigma. Daunomycin was a gift from Farmitalia Carlo-Erba, Milan, Italy. All other solvents and chemicals were obtained from Aldrich, Sigma, J. T. Baker, Fisher, or E. M. Science Chemical Co.

Deoxygenation Procedures. Methanol and water solutions, placed in individual compartments of a multicompartment cell,[43] were oxygen degassed on a high-vacuum line as previously described.[43] The cell was sealed by tightly closing the high-vacuum stopcocks and was stored in a nitrogen glovebag. When the freeze–thaw degassing procedure was not used, solvents were deoxygenated by boiling for 15–20 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.

Lyophilization Procedure. Prior to lyophilization, the heavy-walled, round-bottom glass receptacles were silanized by soaking in a 5% solution of dichlorodimethylsilane in chloroform for 1 h followed by thorough rinsing with water and baking for 2 h at 180 °C. This procedure was necessary to prevent decomposition of **3** to **5** during lyophilization. The liquid solution of drug was then placed in the receptacles and frozen with liquid nitrogen. The flasks were attached to the lyophilization apparatus, which was then packed in dry ice–isopropyl alcohol. Lyophilization was

(38) Tannock, I. F.; Rotin, D. *Cancer Res.* **1989**, *49*, 4373. Vaupel, P.; Kallinowski, F.; Okunieff, P. *Ibid.* **1989**, *49*, 6449.
(39) Kalyanaraman, B.; Peres-Reyes, E.; Mason, R. P. *Biochim. Biophys. Acta* **1980**, *630*, 119.
(40) Berlin, V.; Haseltine, W. W. *J. Biol. Chem.* **1981**, *29*, 4747.
(41) Rouscilles, A.; Houee-Levin, C.; Gardes-Albert, M.; Ferradine, C. *Free Radical Biol. Med.* **1989**, *6*, 37.
(42) Bachur, N. R. *Cancer Treat. Rep.* **1979**, *63*, 817. Pan, S.-S.; Pederson, L.; Bachur, N. R. *Mol. Pharmacol.* **1981**, *19*, 184.
(43) Bird, D. M.; Boldt, M.; Koch, T. H. *J. Am. Chem. Soc.* **1987**, *109*, 4046.

carried out under a vacuum of 10^{-2} Torr or less until all water was removed. The resulting dry material was a fluffy powder, which was easily removed from the flask.

Synthesis of Leucodaunomycin (3). To 61.42 mg (1.09×10^{-4} mol) of daunomycin hydrochloride in a 250-mL round-bottom flask inside the nitrogen glovebag was added 100 mL of deoxygenated water. Within the nitrogen glovebag, 1.00 mL of deoxygenated water was transferred by syringe to a vial containing 56.38 mg of 80% pure sodium dithionite (2.59×10^{-4} mol). By use of a 1.00-mL syringe, 0.45 mL of the sodium dithionite solution (1.17×10^{-4} mol) was transferred to the solution of daunomycin hydrochloride. Immediately after the reduction, 0.24 mL of pH 0.7 deoxygenated aqueous HCl was injected into the solution. The final pH was 3.1. The solution was stirred and allowed to sit for 30 min. The solution was then removed from the glovebag and extracted twice with dichloromethane. Prior to lyophilization, HPLC analysis showed four isomers of **3** in a ratio of 2.9:1:4.7:1.2, in order of increasing retention time. After lyophilization, the resultant yellow-orange powder was subjected to flash chromatography on a 40-mm column packed with 150 mm of C_{18} reverse-phase flash chromatography packing material purchased from J. T. Baker, Inc. The column was eluted with 200 mL of each of six different solutions in the order shown: (A is methanol; B is 1.5% formic acid in water) (1) 35% A–65% B, (2) 40% A–60% B, (3) 45% A–55% B, (4) 50% A–50% B, (5) 55% A–45% B, (6) 60% A–40% B. Fractions containing approximately 50 mL were collected and subjected to HPLC analysis. Isomerically pure fractions were combined, the methanol was removed by rotary evaporation, and then the water was removed by lyophilization. Only isomers A and C were present in sufficient quantities to isolate in pure form. Prior to separation into the individual diastereomers, the total yield of **3** was approximately 40%, with the balance of the material being 7-deoxydaunomycinone (**5**). However, the lengthy isolation process reduced the overall yield to approximately 10%. ^{1}H NMR spectroscopy was accomplished for isomer C by using CD_3OD solvent acidified with DCl to stabilize the compound: δ 1.22 (d, J = 6.5 Hz, 5′-CH_3), 1.79–2.18 (m, 7-H, 9-H, 2′-H), 2.28 (s, 8-$COCH_3$), 3.60 (m, 3′-H, 6a-H), 3.73 (m, 4′-H, 10a-H), 3.98 (s, 1-OCH_3), 4.13 (q, J = 6.5 Hz, 5′-H), 4.72 (m, 10-H), 4.94 (m, 1′-H), 7.30 (d, J = 7.6 Hz, 4-H), 7.71 (t, J = 7.7 Hz, 3-H), 7.93 (d, J = 7.4 Hz, 2-H). Positive and negative ion fast atom bombardment mass spectral data were acquired by using a glycerol matrix. A mixture of the diastereomers of **3** was dissolved in a minimum amount of methanol that had been acidified with hydrochloric acid for stabilization of the compound. The methanol solution was then applied to the matrix and the methanol allowed to evaporate. Positive ion MS m/z (relative intensity) 552 (100, M + Na^+), 530 (80, M + 1), 401 (27); negative ion MS m/z 529 (100, M). UV–vis spectral data for isomers A and C were obtained in pH 3.0 water and in methanol with an apparent pH of 3.0 and are reported in Table I. Due to the instability of isomer A in methanol, the data presented were corrected for product formation by using known extinction coefficients for **1a** and assuming that all components other than isomer A had the same extinction coefficients as **1a** at the various wavelengths.

Kinetics of the Disappearance of Isomers A and C of 3 at pH 6.4, 7.0, and 7.4 under Aerobic Conditions. To 1.27 mg of isomer A of **3** (2.20×10^{-6} mol) in a 10-mL volumetric flask was added water, which had been adjusted to a pH of 3.1 with concentrated HCl. To each of two 100-mL volumetric flasks were added one capsule of either pH 6.4 or pH 7.0 pHydrion standard buffer and water. Buffer solution of pH 7.4 was prepared by diluting 3.00 g of Tris-HCl (1.9×10^{-2} mol) and 0.48 g of Tris (4.0×10^{-3} mol) to 200 mL with water. A quartz cuvette was filled with 1.0 mL of the solution of **3** and covered with a septum cap. The cuvette and a sample of the appropriate buffer were equilibrated to 25.0 °C. The cuvette was placed in the thermostated cell holder and 1.0 mL of the buffer was injected.

For pH 6.4, spectra were taken from 300 to 700 nm every 60 s from 0 to 1200 s, every 300 s from 1500 to 3600 s, every 600 s from 4200 to 7200 s, and every 3600 s from 10800 to 14400 s. These and spectra described in subsequent experiments showed a clean isosbestic point at 456 nm. Based upon a comparison of HPLC retention times and UV–vis absorption spectra obtained with the HPLC diode array detector with those for authentic samples,[35] products in order of elution were identified as **8**, **9**, **10**, **6**, **1a**, and **5** in this and subsequent experiments and were formed in the ratios reported in Table III.

For pH 7.0, spectra were taken from 300 to 700 nm every 15 s from 15 to 300 s, every 60 s from 360 to 600 s, and every 300 s from 900 to 3600 s. The absorbance at 444 nm was then recorded versus time. A Kezdy–Swinbourne plot[34] using a τ of 120 s was used to determine the absorbance at the infinity point. A linear least-squares treatment of a plot of ln $(A - A_\infty)$ versus time gave an apparent first-order rate constant of 1.1×10^{-2} s^{-1} with a correlation coefficient of 1.00. Similar experiments were performed with isomer A of **3** at pH 7.4 and with isomer C of **3** at pH 6.4, 7.0, and 7.4, and the rate constants and product ratios are again reported in Table III. With isomer C of **3** at pH 7.0, the peaks corresponding to **8** + **9** and **10** were broad and represented at least one additional product eluting at approximately the same time and bearing a leucodaunomycin-like chromophore.

Kinetics of the Disappearance of Isomer A of 3 at pH 7.4 under Anaerobic Conditions. To a 5-mL volumetric flask was added 0.39 mg of isomer A of **3** (6.76×10^{-7} mol) and water, which had been adjusted to a pH of 2.5 with concentrated HCl. To a 10-mL volumetric flask were added 0.30 g of Tris-HCl (1.9×10^{-3} mol), 0.050 g of Tris (4.1×10^{-4} mol), and water. By use of a syringe, 1.0 mL of the drug solution was transferred to the center compartment of a three-compartment cell, and 2.0 mL of the Tris buffer solution was transferred to the large compartment. The cell was subjected to the freeze–thaw degassing procedure and then equilibrated to 25.0 °C. After mixing, spectra were taken from 350 to 800 nm at 5 s intervals from 15 to 435 s. Only data from 15 to 360 s were used for analysis due to precipitation of **5**. The spectra for the first 6 min showed a sharp isosbestic point at 462 nm and a rise and fall in the absorption at 618–622 nm. Nonlinear least-squares fitting of the absorption data for 618–622 nm versus time to the consecutive first-order rate equation (shown below), including the compensation for end absorption by **5** in the 618–622-nm region, gave k_1 and k_2 reported in Table III and defined in Scheme III. Both rate constants are the average of two measurements with an average deviation from the mean of 0.05×10^{-2} s^{-1} for k_1 and 0.02×10^{-2} s^{-1} for k_2. The rate law is

$$A_t = \frac{[3]_0}{k_2 - k_1}[(k_1\epsilon_2 - k_2\epsilon_5)e^{-k_1 t} + (k_1\epsilon_5 - k_1\epsilon_2)e^{-k_2 t} + \epsilon_5(k_2 - k_1)]$$

A_t is the absorbance at 618–622 nm at time t; $[3]_0$ is the initial concentration of isomer A of **3**; k_1 is the pseudo-first-order rate constant for elimination; k_2 is the pseudo-first-order rate constant for tautomerization; and ϵ_2 and ϵ_5 are the molar extinction coefficients of quinone methide **2** and 7-deoxydaunomycinone (**5**) at 618–622 nm. This rate law was reported earlier but contained a typographical error.[27] The extinction coefficients for **2** and **5** at 618–622 nm were determined in the nonlinear least-squares analysis to be 8100 ± 400 and 160 ± 10 M^{-1} cm^{-1}, respectively. HPLC analysis of this and the subsequent reaction showed formation of only **5** and **6** (Table III).

Kinetics of the Disappearance of Isomer C of 3 at pH 7.4 under Anaerobic Conditions. The reaction cell was prepared similarly to that described for the reaction of isomer A of **3** under anaerobic conditions. After mixing the reagents, spectra were taken from 350 to 800 nm at 60 s intervals from 60 to 3900 s. Only the data through 1440 s were used for determination of the rate constant due to light scattering caused by the precipitation of **5** after that time. The absorbance at 444 nm was recorded versus time and a Kezdy–Swinbourne plot[34] using a τ of 600 s was used to determine the absorbance at the infinity point. A linear least-squares treatment of a plot of ln $(A - A_\infty)$ versus time gave the first-order rate constant shown in Table III with a correlation coefficient of 1.00.

Experiment To Determine the Rate of Quinone Methide (2) Tautomerization in pH 7.4 Water by Reducing Daunomycin (1a). To a 10-mL volumetric flask were added 1.39 mg of daunomycin hydrochloride (**1a**; 2.46×10^{-6} mol), 61.5 mg of Tris-HCl (3.89×10^{-4} mol), and 10.7 mg of Tris (8.84×10^{-5} mol). To the center compartment of a multicompartment cell[43] was added 3.0 mL of the pH 7.4 Tris-buffered solution of **1a**. To the right side-arm of the cell was added 0.16 mg of 80% pure sodium dithionite (7.38×10^{-7} mol, 1.0 equiv) and to the middle side-arm was added 1.0 mL of water. The cell was subjected to the freeze–thaw degassing procedure and equilibrated to 25.0 °C. The stopcocks of the cell were then manipulated to mix the water with the sodium dithionite and then this solution with the solution of **1a**. The average absorbance from 618 to 622 nm was determined from 10 s after mixing the dithionite solution with the solution of **1a** until 120 s at 2-s intervals and from 125 to 240 s at 5-s intervals. The absorbance at the infinity point was determined by using a τ of 30 s. Linear least-squares analysis of a plot of ln $(A - A_\infty)$ versus time gave a pseudo-first-order rate constant of 3.2×10^{-2} s^{-1}. HPLC analysis showed only two products, **5** and **6**, in a ratio of 98:2.

Experiment To Trap *N*-Acetyl-L-cysteine with Isomer A of 3 under Anaerobic Conditions at pH 7.4. To a 5-mL volumetric flask was added 0.93 mg of isomer A of **3** (1.6×10^{-6} mol) and water which had been adjusted to a pH of 2.5 with concentrated HCl. To a separate 5-mL volumetric flask were added 21.16 mg of *N*-acetyl-L-cysteine (1.3×10^{-4} mol) and Tris buffer solution that was 0.19 M in Tris-HCl and 0.04 M in Tris, pH 7.4. With a syringe, 1.0 mL of the drug solution (3.25×10^{-7} mol) was transferred to the center compartment of the three-compartment cell and 2.0 mL of the *N*-acetyl-L-cysteine solution (5.19×10^{-5} mol, a 60-fold excess) was transferred to the large compartment. The cell was freeze–thaw degassed and then equilibrated to 25.0 °C. After mixing the solutions, the cell was allowed to sit for 20 h before opening

for HPLC analysis. HPLC analysis showed three product peaks. The largest peak, accounting for 86% of the product, was **5**. The other two peaks, accounting for 14% of the product, were the two diastereomers of 7-[(*N*-acetyl-L-cysteinyl)thio]-7-deoxydaunomycinone (**11**), as proven by coinjection with authentic samples[36] onto the HPLC and purity analyses with a UV-vis diode array detector. The ratio of the two diastereomers was 1:2. When daunomycin was reduced at pH 7 with 0.4 mol equiv of NADPH and ferredoxin reductase at ambient temperature in the presence of a 60-fold excess of *N*-acetyl-L-cysteine, the ratio of the diastereomers of **11** was 1:2.6.[36]

A similar experiment was run for 20 h using the exact same procedure with deuterated solvents and a 250-fold excess of *N*-acetyl-L-cysteine. HPLC analysis using method B showed 95% **5** and 5% **11**. Additionally, only the isomer formed in the highest proportion above was detected.

Experiment To Trap *N*-Acetyl-L-cysteine with Isomer A of 3 in the Presence of 1a at pH 7.4 under Anaerobic Conditions Using Deuterated Solvents. To a 3-mL volumetric flask were added 0.54 mg of isomer A of **3** (9.36×10^{-7} mol) and deuterium oxide, which had been adjusted to an apparent pH of 3 with DCl. To a 10-mL volumetric flask were added 65.5 mg of *N*-acetyl-L-cysteine (4.01×10^{-4} mol), 0.300 g of Tris-HCl (1.90×10^{-3} mol), 0.0489 g of Tris (4.04×10^{-4} mol), and D_2O. To a 5-mL volumetric flask were added 1.76 mg of daunomycin hydrochloride (3.12×10^{-6} mol) and the Tris-buffered *N*-acetyl-L-cysteine solution. With a syringe, 1.0 mL of the solution of **3** (3.12×10^{-7} mol) was transferred to the center compartment of the three-compartment cell, and 2.0 mL of the pH 7.4 buffered daunomycin-*N*-acetyl-L-cysteine solution (1.25×10^{-6} mol of daunomycin hydrochloride, 8.02×10^{-5} mol of *N*-acetyl-L-cysteine) was transferred to a second compartment. The solutions were freeze-thaw degassed, equilibrated to 25.0 °C, and then mixed. HPLC analysis after 2 h showed three product peaks in addition to unreacted daunomycin. Of the total anthracyclines present, 11% was **5**, 23% was the diastereomers of **11** in a ratio of 1:3.9, and 66% was **1a**.

Acknowledgment. This investigation was supported by PHS Grant CA-24665 and the NIH Small Instrumentation and Biomedical Sciences Support Grants to the University of Colorado (DHHS). We thank Professors Federico Arcamone and Sergio Penco of Farmitalia Carlo-Erba, Milan, Italy, for a generous sample of daunomycin. T.H.K. also thanks the University of Colorado Council on Research and Creative Work for a faculty fellowship.

9-(Difluorophosphonoalkyl)guanines as a New Class of Multisubstrate Analogue Inhibitors of Purine Nucleoside Phosphorylase

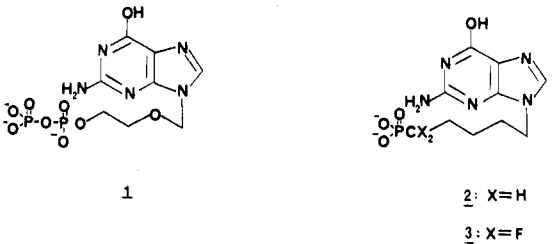
S. Halazy, A. Ehrhard, and C. Danzin*

Contribution from the Merrell Dow Research Institute, 16 Rue D'Ankara, B.P. 447 R/9, F-67009 Strasbourg Cedex, France. *Received June 29, 1990*

Abstract: 9-(5,5-Difluoro-5-phosphonopentyl)guanine (**3**) was synthesized as a potential multisubstrate analogue inhibitor of purine nucleoside phosphorylase (EC 2.4.2.1, PNP). At pH 7.4, **3** has a K_i value 18-, 26-, 25-, and 5.5-fold lower than that of the nonfluorinated analogue 9-(5-phosphonopentyl)guanine (**2**) regarding PNP from human erythrocyte, rat erythrocyte, calf spleen, and *Escherichia coli*, respectively. Further studies with human erythrocytic PNP show that at pH 6.2 the difference in K_i value is more pronounced (K_{i2}/K_{i3} is 96), and at pH 8.8, where **2** and **3** are both essentially present in the unprotonated form, the ratio is 8. The superiority of the difluorophosphonate **3** over the phosphonate **2** is explained by electronic as well as by steric effects.

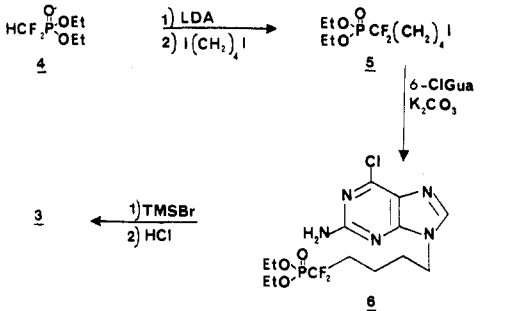
Purine nucleoside phosphorylase (EC 2.4.2.1; PNP), a key enzyme in the purine salvage pathway,[1] is believed to be a target for the design of immunosuppresive agents.[2] PNP inhibitors might also be useful in the treatment of T-cell leukemia, gout,[2] and some parasitic diseases.[3] PNP catalyzes the reversible phosphorolysis of guanosine and inosine nucleosides (or deoxynucleosides) to their respective free base and ribose 1-phosphate (or deoxyribose 1-phosphate). This reaction proceeds via a ternary complex of enzyme, nucleoside, and orthophosphate.[1] Based on the finding that the diphosphate derivative of acyclovir (**1**, Chart I) is a very potent inhibitor of the human enzyme[4] (K_i = 8.7 nM, when determined in the presence of 1 mM orthophosphate), metabolically stable "multisubstrate" acyclic nucleotide analogues containing a purine and a phosphate-like moiety such as 9-phosphonoalkyl derivatives of hypoxanthine and guanine have been designed and synthesized.[5,6] The most potent inhibitor of human erythrocytic PNP in this series was 9-(5-phosphonopentyl)guanine

Chart I



1 2: X=H 3: X=F

Scheme I



(1) Parks, R. E., Jr.; Agarwal, R. P. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1972; Vol. 7, pp 483-514.

(2) (a) Kazmers, I. S.; Mitchell, B. S.; Dadonna, P. E.; Wotring, L. L.; Townsend, L. B.; Kelley, W. N. *Science* **1981**, *214*, 1137-1139. (b) Gilbertsen, R. B.; Sircar, J. C. In *Comprehensive Medicinal Chemistry*; Sammes, P. G., Ed.; Pergamon Press: Oxford, 1990; Vol. 2, pp 443-480. (c) Stoeckler, J. D.; Ealick, S. E.; Bugg, C. E.; Parks, R. E., Jr. *Fed. Proc., Fed. Am Soc. Exp. Biol.* **1986**, *25*, 2773-2778.

(3) Dadonna, P. E.; Wiesmann, W. P.; Milhouse, W.; Chern, J.-W.; Townsend, L. B.; Hershfield, M. S.; Webster, H. K. *J. Biol. Chem.* **1986**, *261*, 11667-11673.

(4) (a) Tuttle, J. V.; Krenitsky, T. A. *J. Biol. Chem.* **1984**, *259*, 4065-4069. (b) Krenitsky, T. A.; Tuttle, J. V.; Miller, W. H.; Moorman, A. R.; Orr, G. F. *J. Biol. Chem.* **1990**, *265*, 3066-3069.

0002-7863/91/1513-315$02.50/0