Reaction of Anthracycline Antitumor Drugs with Reduced Glutathione. Formation of Aglycon Conjugates†

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Abstract: Anaerobic reduction of adriamycin (**1**) with bi(3,5-dimethyl-6-(hydroxymethyl)-2-oxomorpholin-3-yl) (DHM-3 dimer), bi(3,5,5-trimethyl-2-oxomorpholin-3-yl) (TM-3 dimer), or dithionite in the presence of excess reduced glutathione (GSH, **9**) yielded the aglycon conjugate, 7-deoxy-7-*S*-glutathionyladriamycinone (**11a,b**), as a mixture of diastereomers together with 7-deoxyadriamycinone (**12**) via 7-deoxyadriamycinone quinone methide. Anaerobic reaction of adriamycin with reduced glutathione also yielded, in a slower reaction, the conjugates with the glutathione serving both as the reducing agent and as the nucleophile reacting with the quinone methide, as proposed in Scheme 2. A separate reductive cleavage of **11a,b** to **12** established reversibility of the nucleophilic addition. The proposed mechanism was further supported by the reaction composition at termination as a function of conditions (Tables 1 and 3). At higher concentrations of **1** and **9**, a 99% yield of salt **13**, consisting of the anion of **11b** and adriamycin cation, precipitated and was characterized spectroscopically. The conjugate **11b** was subsequently separated from adriamycin by extraction. The anthracyclines daunomycin (**2**) and menogaril (**10**) also reacted with glutathione with and without an additional reducing agent to yield the respective aglycon conjugates, 7-deoxy-7-*S*-glutathionyldaunomycinone (**14a,b**) and 7-deoxy-7-*S*-glutathionylnogarol (**15a,b**), characterized predominantly by HPLC electrospray mass spectrometry. Enzyme-catalyzed formation of **11a,b** may be relevant to tumor cell resistance to adriamycin.

Introduction

The anthracycline adriamycin (**1**) is a broad spectrum antitumor drug of significant clinical utility.[1] Of current interest are the mechanisms of its cytotoxicity and multidrug resistance often induced upon its administration. Probable mechanisms for adriamycin cytotoxicity include DNA cleavage through interaction with DNA–topoisomerase complexes[2] and catalysis of superoxide formation amongst other reactive oxygen species through redox cycling.[3] The former process appears to be augmented through adriamycin interaction with the cell membrane.[4] The latter process is thought to result in oxidative stress, which in the myocardium is a possibler source of cardiotoxicity.[5] Relevant to the results presented here was the observation that intravenous administration of reduced glutathione (γ-glutamylcysteinylglycine, GSH) before and after adriamycin in a rat model eliminated delayed cardiotoxicity.[6] Multidrug resistance is characterized by resistance to several drugs developed by a variety of tumor cells upon treatment with **1** or other single drugs. Mechanisms proposed for tumor cell multidrug resistance include overexpression of P-170-glycoprotein or other proteins, resulting in enhanced efflux of the drug, and increased glutathione concentration and overexpression of glutathione transferase.[7] Glutathione transferase catalyzes the formation of drug–glutathione conjugates, which commonly, but not always,[8] have lower cytotoxicity and/or may be expelled from the cell.[9,10] Of particular relevance here is a significant increase in glutathione concentration and glutathione transferase activity in adriamycin insensitive cells relative to sensitive cells.[11,12]

Adriamycin also has the potential for bioreductive activation to a quinone methide species with possible alkylating ability.[13-15] *In vitro* anaerobic two-electron reduction of the quinone functionality of daunomycin (**2**), the 14-deoxy analog of adriamycin, to hydroquinone **3** results in glycosidic cleavage to quinone methide **4**.[16] The quinone methide reacts reversibly with *N*-acetylcysteine (**5**) to form the diastereomeric aglycon conjugates **6a** and **6b** in their hydroquinone redox states.[17] Oxidation of **6a** and **6b** by molecular oxygen or by daunomycin yields the isolable conjugates in their quinone redox states, *N*-acetyl-7-*S*-cysteinyl-7- deoxydaunomycinones (**7a** and **7b**). With daunomycin as the oxidizing agent the process is a chain reaction. In competition with formation of **6a** and **6b** is tautomerization of **4** to 7-deoxydaunomycinone (**8**). These reactions are summarized in Scheme 1. With 0.02 M *N*-acetylcysteine and 0.4 molar equiv of reducing agent in water at pH 7, **7a** and **7b** were formed in 65% yield with 35% of **8**.[17] Other thiols which reacted with **4** to yield conjugates were cysteine and 1-thio-β-D-glucose; however, the yield with 2

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Scheme 1

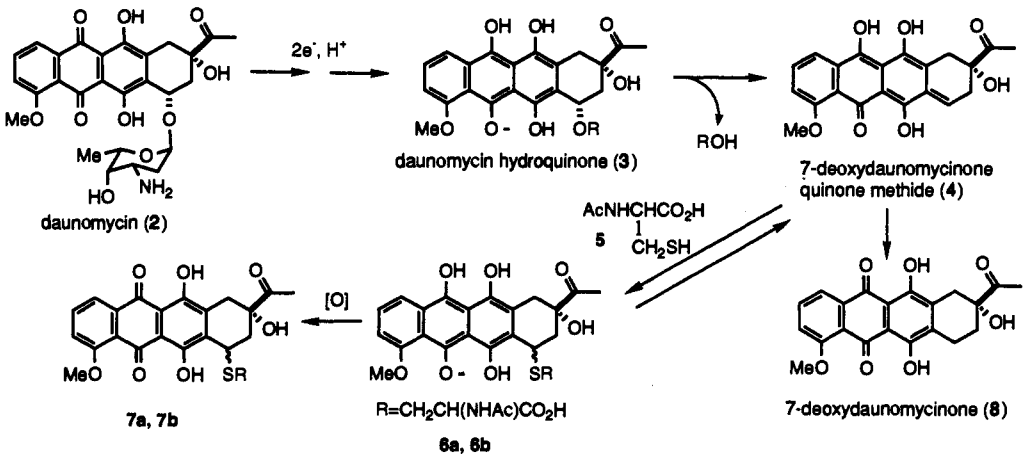


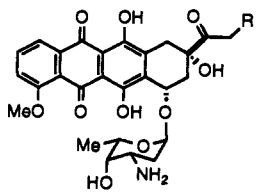
Table 1. Mixture Composition from Reaction of Reduced Glutathione (9) with Adriamycin (1) as a Function of Conditions

entry number	reducing agent	medium	[1], M	[Tris],[a] M	[9], M	reaction time	reaction mixture 1	12	11a + 11b	11a/11b
1	DHM-3 dimer, 1 mol equiv	H_2O, degassed	0.0010	0.015	0.010	15 min	3%	81%	13%	0.5
2	DHM-3 dimer, 1 mol equiv	D_2O, degassed	0.0010	0.060	0.050	15 min	0%	48%	49%	
3	NaS_2O_4, 0.8 mol equiv	D_2O, degassed	0.0010	0.060	0.050	15 min	0%	77%	21%	
4	TM-3 dimer, 1 mol equiv	MeOD, degassed	0.0010	0.015	0.010	30 min	5%	64%	21%	0.5
			0.0010	0	0.010	30 min		100%		
5	none	H_2O, degassed	0.0010	0.015	0.010	24 h	68%	16%	7%	
6	none	D_2O	0.0020	0.15	0.10	20 h	100%	0%	0%	
7	none	D_2O, degassed	0.0040	0.12	0.080	5 h	48%[b]	trace	51%[b]	0.04
8	none	D_2O, degassed	0.0014	0.042	0.028	26 h	58%	0%	42%	0.2
			0.0014	0.042	0.028	70 h	42%	0%	58%	0.2
			0.0014	0.042	0.028	6 days	28%	0%	72%	0.3

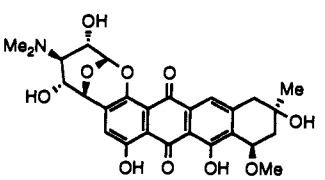
[a] Concentration before reaction with adriamycin hydrochloride and reduced glutathione. [b] Predominantly in the form of the adriglu salt, 13.

$\times 10^{-3}$ M cysteine was only 5%, and conjugate formation with thioglucose required a pH of 8.1.[17] Conjugates **7a** and **7b** were also formed from anaerobic reaction of leucodaunomycin, an isolable tautomer of daunomycin hydroquinone, with *N*-acetylcysteine.[18]

Since reduced glutathione (GSH, **9**) is the most abundant thiol in cells and since conjugation of drugs with glutathione modifies activity, reaction of the quinone methides from reduction of antitumor anthracyclines with GSH seems probable and might be of biological significance, especially with regard to cell resistance. However, reductive activation of daunomycin with carbon dioxide radical anion generated by pulse radiolysis in the presence of 4.5×10^{-4} M GSH in pH 7 water was reported to yield no detectable aglycon conjugate and formation of more than 90% 7-deoxydaunomycinone (**8**).[19] We now report that under conditions of higher GSH concentration aglycon conjugates are formed. Further, adriamycin is a little more reactive than daunomycin, and reduced glutathione can serve both as the reducing agent and as the nucleophile. We also report that glutathione reacts with menogaril (**10**), another anthracycline antitumor drug in clinical trials, to yield nogarol conjugates.



R=OH, adriamycin (1)
R=H, daunomycin (2)



menogaril (10)

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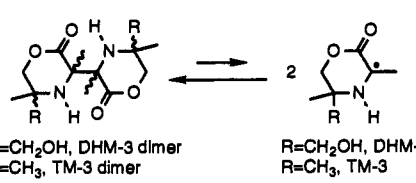
Results and Discussion

Adriamycinone–Glutathione Conjugate Formation upon Reduction of Adriamycin with Various Reducing Agents. The initial reactions were performed with reductive activation of adriamycin using the water soluble reducing agent bi(3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl) (DHM-3 dimer). DHM-3 dimer reacts through homolysis of the 3—3′ bond to yield the one-electron reducing agent 3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl (DHM-3). In the absence of reduced glutathione, DHM-3 dimer is known to reduce anthracycline antitumor drugs to their 7-deoxyaglycons in anaerobic medium.[20] Anaerobic reaction of adriamycin with a buffered aqueous solution of DHM-3 dimer in the presence of 0.010 M GSH gave a low yield of two new products ultimately determined to be the diastereomers of 7-*S*-glutathionyl-7-deoxyadriamycinone (**11a** and **11b**) (see entry 1 of Table 1). The major product was 7-deoxyadriamycinone (**12**). Because of the low to modest yields of **11a,b** and their instability, isolation and spectral characterization awaited a fortuitous result described below. Formation of **11a,b** most likely followed the path for formation of the cysteinyl aglycon conjugates **7a,b** shown in Scheme 1 via reaction with the analogous quinone methide.



R=CH_2OH, DHM-3 dimer
R=CH_3, TM-3 dimer

R=CH_2OH, DHM-3
R=CH_3, TM-3

In an attempt to improve the yield of **11a,b** deuterium oxide was employed as the solvent, and the concentration of GSH was increased by a factor of 5. Deuterium oxide was selected to take advantage of the primary deuterium kinetic isotope effect for

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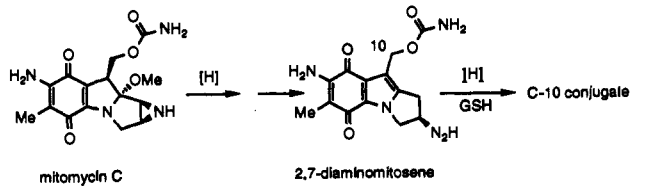
tautomerization of anthracycline-derived quinone methides to their respective 7-deoxyaglycons.[21] Provided the rate-controlling step for formation of **11a,b** did not involve a proton transfer, nucleophilic addition would then be more competitive with the tautomerization. Further, higher GSH concentration should increase the rate of nucleophilic addition. These adjustments in the reaction conditions increased the yield of **11a,b** by a factor of 4 (Table 1, entry 2).

The reaction was also run with dithionite as the reducing agent. In this case, substoichiometric amounts of dithionite were employed relative to adriamycin. Since all the adriamycin was completely destroyed (Table 1, entry 3), the chain mechanism for nucleophilic addition to anthracycline-derived quinone methides must be operating.[16,22] In the chain-carrying step the hydroquinone from nucleophilic addition of GSH is oxidized by adriamycin to yield the adduct in its stable quinone state and adriamycin hydroquinone. In the absence of this oxidation, the hydroquinone can eliminate GSH to reform the quinone methide, which will then irreversibly tautomerize to 7-deoxyadriamycinone. Reversibility of nucleophilic addition was established by anaerobic reduction of **11a,b** with DHM-3 dimer to form 7-deoxyadriamycinone (**12**) in 80% yield. The factor of 2 reduction in the yield of **11a,b** using dithionite relative to the yield for the experiment in entry 2 of Table 1 remains unexplained.

The adriamycin aglycon glutathione conjugates were also produced by reduction with bi(3,5,5-trimethyl-2-oxomorpholin-3-yl) (TM-3 dimer) in methanol-*d* solvent (Table 1, entry 4). TM-3 dimer reduces anthracyclines analogously to DHM-3 dimer.[14,16] This table entry also reports no conjugate formation in the absence of Tris, tris(hydroxymethyl)aminomethane. In all of these experiments adriamycin was used as its hydrochloride salt, and reduced glutathione in the acidic form. Consequently, both species rapidly reacted with Tris to form Tris·H^+ and in so doing created Trizma buffer. The measured pH, pD, or apparent pD was in the range 6–8 except in the absence of Tris. Low pH most likely favors tautomerization of quinone methide to **12**, which is probably acid catalyzed, and disfavors conjugate formation, which is probably base catalyzed via deprotonation of the thiol group of **9**.

Mitomycin C is another clinically important quinone antitumor drug for which glutathione conjugate formation has been reported upon reductive activation.[23] Enzymatic reduction of mitomycin C with the two-electron reducing agent DT-diaphorase or chemical reduction with sodium borohydride, both in the presence of reduced glutathione, yielded a glutathione conjugate. The conjugate structure was not explicitly proposed but was most likely the product of substitution of the carbamate group at the 10-position of 2,7-diaminomitosene for a glutathionyl group. 2,7-Diaminomitosene is the initial product from reduction of mitomycin C with DT-diaphorase; glutathione conjugate formation then required a subsequent reduction of the diaminomitosene. Interestingly, conjugate formation was not significantly inhibited by an aerobic medium.



Conjugate Formation in the Absence of an Additional Reducing Agent. Of particular significance was the subsequent observation that adriamycin reacted with reduced glutathione in the absence of a reducing agent such as DHM-3 dimer. The reaction was slow; only a low yield of **11a,b** was observed even after 24 h (Table 1, entry 5). Apparently, GSH was serving both as a reducing agent and as a nucleophile since **12** was also a product and its formation requires a reduction. Formation of the aglycon conjugates was not simply a nucleophilic displacement reaction because even with higher concentrations of GSH no reaction was observed in the presence of molecular oxygen (Table 1, entry 6). Molecular oxygen oxidizes reduced states of anthracyclines back to their quinone state very rapidly. Further, lack of reaction in the presence of molecular oxygen could not simply be explained through oxidation of GSH to the disulfide, oxidized glutathione, since GSH was still present even after 20 h.

A fortuitous result which made characterization of **11a,b** possible was the fact that at higher concentrations of all reagents **11b** crystallized in high yield from the reaction medium as salt with adriamycin, which we have named adriglu salt (**13**) (Table 1, entry 7). HPLC analysis of redissolved salt showed two peaks of equal area, with one at the retention time of adriamycin and the other at the retention time of **11b**. Treatment of **13** with aqueous ammonium hydroxide followed by extraction of the adriamycin into chloroform yielded a solution of pure **11b** in the form of its ammonium salt. The ^{1}H NMR spectrum of adriglu salt is reported in Table 2 in comparison with that of adriamycin and reduced glutathione. The spectrum showed some peak broadening most likely because of the size of the salt; however, the appropriate signals were observed. Some of the assignments were made from homonuclear COSY data. Of particular significance was an upfield shift of approximately 1 ppm for the resonance of the proton at position 7 of the conjugate anion relative to the resonance for the analogous proton in adriamycin. The conjugate anion also showed approximately a 0.5 ppm downfield shift for the resonance of one of the protons at position 10 and 0.5 ppm upfield shift for the resonance of the protons at position 14, again relative to the resonances for the analogous protons in adriamycin. These shifts are reasonable for the conjugate anion. Further structural information was obtained by electrospray mass spectrometry, and the spectrum of **13** is shown in Figure 1. Ions were observed for protonated adriglu salt semiquinone at m/z 1249.5 (calcd, 1249.4) as well as for protonated adriamycin semiquinone dimer at m/z 1090.3 (calcd, 1090.4) and for protonated semiquinone–hydroquinone dimer of **11b** at m/z 1410.3 (calcd, 1410.4). The peaks are broad, and expansion shows that they also represent ions for the other quinone redox states. Formation of anthracycline semiquinone and hydroquinone states in the mass spectrometer has been reported in fast atom bombardment mass spectrometry.[21,24] The ions at m/z 705.3 and 545.3 are assigned to protonated semiquinone of **11b** and protonated semiquinone of adriamycin (calcd, 705.2 and 545.2, respectively). The remaining ions are fragment ions of those described above as established by MS/MS except m/z 935.6 and 416.2, which appear to be from impurities. Fragment ions at m/z 398.2 and 308.2 have the mass to charge ratio for 7-deoxyadriamycinone cation and GSH cation, respectively. Fragmentation spectra for m/z 1410, 1249, and 1090 are provided as supplementary material. The use of mass spectrometry, including the electrospray technique, for the characterization of glutathione conjugates has recently been reveiwed.[25] Complete elemental analysis including analysis for sulfur was performed; however, a good fit to the adriglu salt formula was apparently plagued by varying states of hydration, as indicated by reproducibility with samples which appeared to be chromatographically pure. Because of instability, further efforts at purification proved futile.

The reaction of adriamycin with 0.028 M GSH in anaerobic D_2O was then monitored as a function of time. No 7-deoxyadriamycinone (**12**) was observed at any of the time points, and the yield of **11a,b** increased from 42% after 1 day to 72% after

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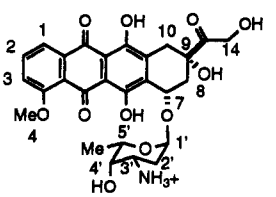
Table 2. ^{1}H NMR Data

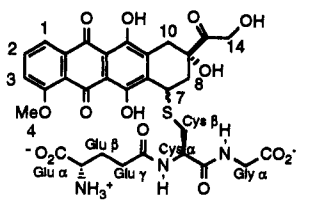
Compound	Position													
	1	2	3	4	7	8	9	10	14	1'	2'	3'	4'	5'
adriamycin (1) 400 MHz DMSO-d_6	7.9-8.0 m	7.9-8.0 m	7.7 m	4.02 s	5.32 d J=3	2.19 d J=12 2.14 dd J=4,12	5.50 s OH	3.02 d J=18 2.90 d	4.93 t J=6 OH 4.62 d	4.96 t J=4	1.91 dt J=3,12 1.71 dd J=4,12	3.38 m	5.5 d J=6 OH 3.62 m	4.22 q J=6 1.18 d J=6
adriglu salt (13), 400 MHz, DMSO-d_6 + D_2O														
adriamycin cation	7.8-8.0 m	7.8-8.0 m	7.55-7.75 m	3.98 s	5.33 bs	2.05-2.35 m	-	2.97 d J=18 2.89 d J=18	4.71 d J=21 4.57 d J=21	3.94 bs	1.85-2.05 m 1.65-1.70 m	3.3-3.5 m	3.55-3.7 m	4.19 q J=7
conjugate anion	7.8-8.0 m	7.8-8.0 m	7.55-7.75 m	3.98 s	4.26 bs	2.05-2.35 m	-	3.45 d J=18 ? 2.80 d J=18 ?	3.92 bs					

	Cys α	Gly α	Glu α	Cys β	Glu γ	Glu β
glutathione, 300 MHz, DMSO-d_6 + D_2O						
9	4.3-4.45 X of ABX	3.75 s	3.31 t J=8	2.6-2.85 AB of ABX	2.2-2.4 m	1.8-2.0 m
adriglu salt (13), 400 MHz, DMSO-d_6 + D_2O						
conjugate anion	4.55-4.65 m	3.60 s	3.45 m	2.7-2.95 m	2.35-2.5 m	1.85-2.05 m



adriamycin cation

13



conjugate anion

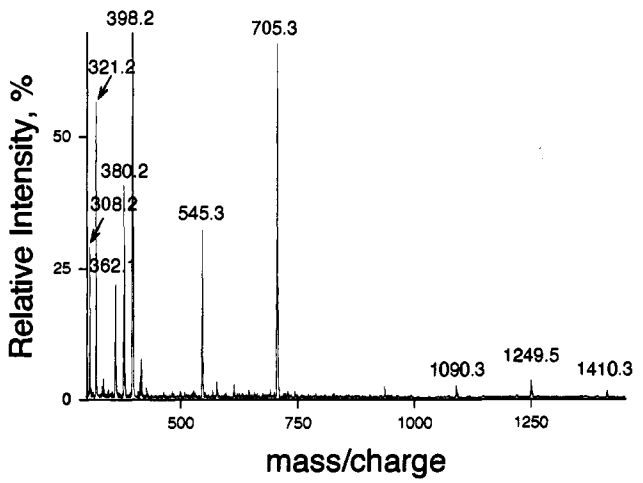


Figure 1. Positive ion electrospray mass spectrum of the adriglu salt, **13**, showing molecular ions at m/z 1090.3, 1249.5, and 1410.3, which represent a cluster dimer of **1**, the adriglu salt, **13**, and a cluster dimer of **11b**, respectively (see Results and Discussion section for further discussion). Ions at m/z 545.3 and 705.3 represent **1** and **11b**, respectively. The ions appearing below m/z 400 are fragment ions generated at the orifice (308.2, 321.2, 362.1, 380.2, and 398.2) and can be observed in the MS/MS of 545.3 and 705.3 MS/MS of ions m/z 1090.3, 1249.5, and 1410.3 are provided as supplementary material.

6 days (Table 1, entry 8). During this same time period the ratio of **11a** to **11b** approximately doubled. This experiment when compared with that in entry 5 of Table 1 further points to the favorable effects of D_2O vs H_2O and a higher concentration of GSH. Comparison of entries 7 and 8 of Table 1 indicates that GSH addition to quinone methide is reversible at least at modest to low reagent concentrations. At the high concentrations of the experiment described in entry 7, **11b** greatly predominates because of precipitation of the adriglu salt (**13**). This suggests that **11b** is the kinetically favored diastereomer. However, in the absence of crystallization of **13** and with a longer reaction time, diastereomer **11a** becomes a significant product. The structure of **11a** was inferred from HPLC electrospray MS data for a mixture of **11a** and **11b**. Both compounds gave mass spectra with base peaks at m/z 705 for the protonated semiquinone; small peaks were also observed at m/z 727 for the semiquinone plus a sodium ion and at m/z 1409 for the monoprotonated dimer of the semiquinone. Further evidence for reversibility of glutathione addition to quinone methide comes from reduction of **11a,b** with DHM-3 dimer to 7-deoxyadriamycinone (**12**), as mentioned earlier. The proposed steps in the formation of **11a,b** are summarized in Scheme 2. Precedent for the reduction of adriamycin to the semiquinone state comes from the observed reduction of the quinone menadione to its semiquinone by reduced glutathione, which required a pH of at least 7.5.[26,27] Although menadione glutathione conjugates were also observed, the proposed mechanism is somewhat different and involves nucleophilic addition to the quinone followed by oxidation.[26] Adriamycin semiquinone is shown to disproportionate to adriamycin and adriamycin hydroquinone; alternatively, semiquinone could be reduced by a second molecule of GSH. Rapid disproportionation of anthracycline semiquinones to quinone and hydroquinone redox states in protic medium has been observed in pulsed radiolysis experiments;[28] however, in the less protic medium, 5%/95% H_2O/DMSO (dimethyl sulfoxide), disproportionation appears to be significantly slower.[29]

Reactions of Daunomycin and Menogaril with Reduced Glutathione. Daunomycin (**2**) was reacted anaerobically with GSH in Tris-buffered deuterium oxide, and the reaction mixture was analyzed as a function of time. Over a period of 10 days,

(26) Takahashi, N.; Shreiber, J.; Fischer, V.; Mason, R. P. *Arch. Biochem. Biophys.* **1987**, *252*, 41.
(27) Miura, T.; Muraoka, S.; Ogiso, T. *Chem. Pharm. Bull.* **1992**, *40*, 709.
(28) Svingen, B. A.; Powis, G. *Arch. Biochim. Biophys.* **1981**, *209*, 119. Land, E.; E. J.; Mukherjee, T.; Swallow, A. J.; Bruce, J. M. *Br. J. Cancer* **1985**, *51*, 515.
(29) Gaudiano, G.; Frigerio, M.; Bravo, P.; Koch, T. H. *J. Am. Chem. Soc.* **1992**, *114*, 3107.

Scheme 2

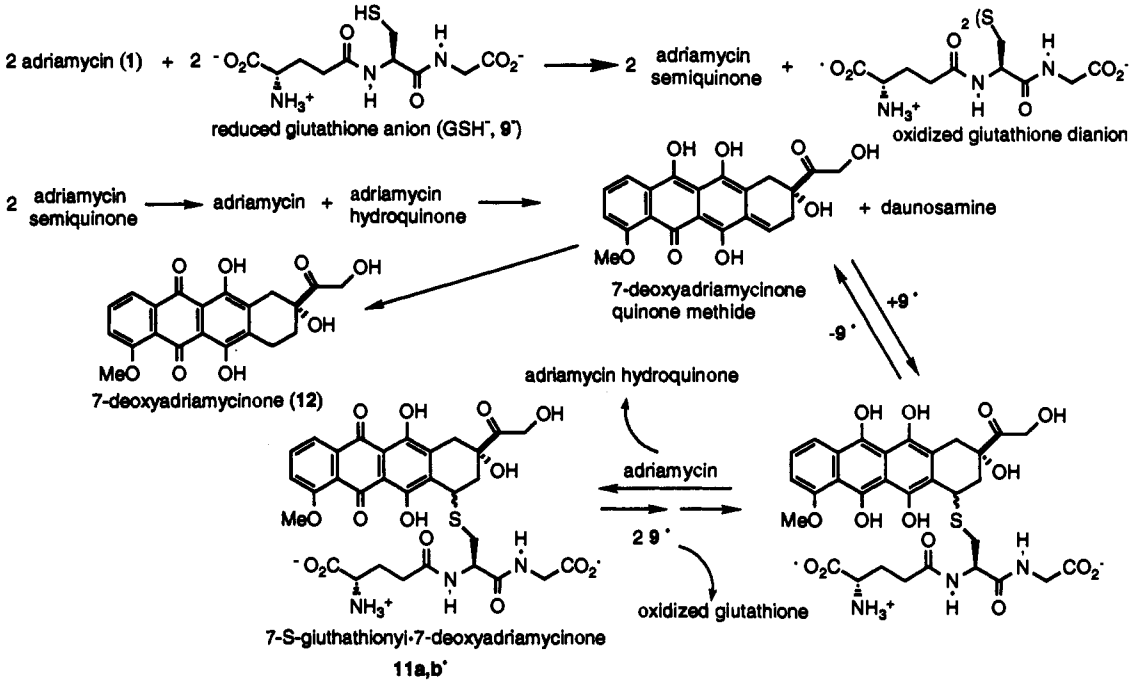
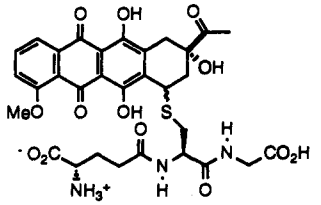


Table 3. Mixture Composition from Reaction of Reduced Glutathione (**9**) with Daunomycin (**2**) or Menogaril (**10**) as a Function of Conditions

entry number	reducing agent	medium	[**2**] or [**10**], M	[Tris]/[Tris·H⁺],[a] M	[**9**], M	reaction time	reaction mixture: **2** or **10**	**8** or **16**	**14a + 14b** or **15a + 15b**	**14a/14b** or **15a/15b**
			Daunomycin (**2**)							
1	none	D_2O, degassed	0.0014	0.042	0.028	16h	93%	0%	5%	0.3
			0.0014	0.042	0.028	65 h	77%	0%	23%	0.3
			0.0014	0.042	0.028	5 days	59%	0%	40%	0.5
			0.0014	0.042	0.028	10 days	40%	0%	60%	0.5
2	NaS_2O_4, 1 molar equiv	MeOD, degassed	0.00010	0.0020/0.00010	0.0010	10 min	0%	55%[b]	0%	
			Menogaril (**10**)							
3	none	D_2O, degassed	0.00010	0.0030	0.0020	4 days	60%	0%	40%	1
4	TM-3 dimer, 0.5 molar equiv	MeOH, degassed	0.00040	0.044/0.024	0.012	15 min[c]	0%	0%	95%	0.3

[a] Concentration before reaction with anthracycline and/or reduced glutathione. [b] The balance of the product mixture consisted of the products of air oxidation of 7-deoxydaunomycinone quinone methide.[30] [c] The same result was observed after 30 min, 60 min, and 20 h.

daunomycin was slowly destroyed with formation of two products (entry 1, Table 3). The products were assigned to the structures **14a** and **14b**, the diastereomers of 7-*S*-glutathionyl-7-deoxydanumycinone, on the basis of HPLC/electrospray mass spectrometric M + 1 ions at m/z 689 for both products and in analogy with the products of the reaction of adriamycin. Comparison of entry 1 of Table 3 with entry 8 of Table 1 indicates that the reaction of GSH with daunomycin occurs a little more slowly than the reaction with adriamycin. This may result from a small difference in the redox potential of the two anthracyclines. Again reversibility was apparent from a change in the ratio of **14a** to **14b** with increasing reaction time.



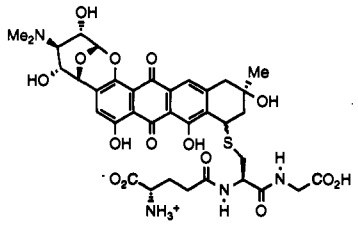
7-S-glutathionyl-7-deoxydaunomycinone (**14a,b**)

Dauomycin was also reduced anaerobically with 1 molar equiv of dithionite in the presence of a modest concentration of GSH in methanol-*d* solvent. Quinone methide formation was observed spectroscopically by the appearance of an absorption band at 610 nm.[16] After 10 min when the quinone methide absorption had decreased to one-third of its maximum concentration, the mixture was exposed to air to oxidize any quinone methide thiol adducts in the hydroquinone state to **14a,b**. HPLC analysis of the reaction mixture, however, showed only 7-deoxydaunomycinone and the products of reaction of quinone methide **4** with molecular oxygen.[30] As stated in the Introduction, efficient trapping of 7-deoxydaunomycinone quinone methide with *N*-acetylcysteine employed a 20 times higher concentration of thiol.

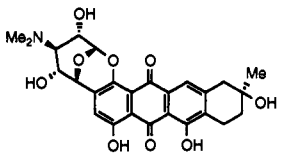
The anthracycline antitumor drug menogaril similarly reacted with reduced glutathione even in modest concentration in the absence of a reducing agent to give a 1:1 mixture of the diastereomers of 7-*S*-gluthathionyl-7-deoxynogarol (**15a,b**), as shown in entry 3 of Table 3. Again the structures were characterized from the appearance of M + 1 ions at m/z 817 for both diastereomers in the electrospray mass spectrum. A high yield of the 7-deoxynogarol glutathione conjugates was obtained upon reduction of menogaril with 0.5 molar equiv of bi(3,5,5-trimethyl-2-oxomorpholin-3-yl) (TM-3 dimer). The quinone methide from reduction of menogaril with substoichiometric amounts of reducing agent has a much longer lifetime than the quinone methides from reduction of adriamycin or daunomycin.[31] In fact 7-deoxynogarol quinone methide in the absence of hydroquinones does not tautomerize to 7-deoxynogarol (**16**) but only dimerizes at the 7-position to form an aglycon dimer.[31]

(30) Gaudiano, G.; Koch, T. H. *J. Am. Chem. Soc.* **1990**, *112*, 9423.
(31) Boldt, M.; Gaudiano, G.; Haddadin, M. J.; Koch, T. H. *J. Am. Chem. Soc.* **1989**, *111*, 2283.

Consequently, facile formation of the glutathione conjugates **15a,b** with reductive activation was not surprising. Earlier, conjugates were characterized from reaction of the quinone methide with *N*-acetylcysteine,[31] imidazole,[32] and guanosine.[33]



7-S-glutathionyl-7-deoxynogarol (15a,b)



7-deoxynogarol (16)

Conclusions. Nonenzymatic *in vitro* formation of glutathione conjugates to aglycons of anthracyclines, which occurs via a quinone methide, requires a high concentration of glutathione, a pH in the range 6–8, and an anaerobic medium. In the absence of an additional reducing agent, substantial time periods are also required. Some of these requirements might not be likely conditions *in vivo*; although, concentrations of glutathione can be as high as 5–10 mM.[9] In any case, the identification and characterization of the conjugates now open the possibility for observing their formation *in vivo*, with HPLC or better with HPLC electrospray mass spectrometry as an analytical tool.

Experimental Section

General Remarks. UV–vis spectra were recorded with a Hewlett-Packard 8452A diode array spectrometer. ^{1}H NMR spectra were obtained with Bruker Model Am-400 and Varian Model VXR-500S spectrometers operating at 400 and 500 MHz, respectively; chemical shifts are reported in parts per million on the δ scale from internal 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt, and coupling constants are in hertz. HPLC analyses were performed with Hewlett-Packard 1090 liquid chromatograph equipped with a diode array UV–vis detector and workstation. Chromatographies were performed with Hewlett-Packard 5-μm C_{18} microbore column, 2.1 mm i.d. × 100 mm, eluting at 0.3 mL/min with mixtures of methanol and 0.3% aqueous ammonium formate adjusted to pH 4 with 98% formic acid. The following eluting conditions (*e.c.*) were used (*e.c.* #, time, min (% methanol)): #*1*, isocratic (60); #*2*, isocratic (55); #*3*, isocratic (30); #*4*, 0 (30), 0.5 (30), 1.5 (42), 8 (52), 10 (90). The retention times were (**compound**, min (*e.c.* #)): **1**, 7.0 (#*1*), 11.4 (#*4*); **2**, 7.5 (#*1*); **8**, 8.0 (#*1*); **10**, 7.0 (#*2*); **11a**, 1.5 (#*1*), 1.9 (#*2*), 6.2 (#*4*); **11b**, 1.8 (#*1*), 2.3, (#*2*), 7.0 (#*4*); **12**, 11.8 (#*4*); **14a**, 1.3 (#*1*); **14b**, 2.4 (#*1*); **15a**, 2.0 (#*2*), 4.0 (#*3*); **15b**, 2.2 (#*2*), 4.8 (#*3*); **16**, 8.0 (#*2*). Yields from UV–vis/HPLC analyses were based on peak areas monitoring at 480 nm and assuming $\epsilon_{480} = 10^4$ L mol^{-1} cm^{-1}. The freeze–pump–thaw degassing procedure was that reported previously.[21] Tris and Tris·HCl were purchased from Boehringer Mannheim Gmbh, Germany. DHM-3 and TM-3 dimers were prepared as described earlier.[20,34] All other chemicals were purchased from Aldrich Chemical Co., Milwaukee, WI. Daunomycin hydrochloride and adriamycin hydrochloride were a gift from Farmitalia-Carlo Erba, Milan, Italy. Menogaril and 7-deoxynogarol were a gift of the Upjohn Co., Kalamazoo, MI. Most of the experiments with adriamycin were performed with pure adriamycin hydrochloride; however, some as noted were performed with clinical samples of adriamycin hydrochloride containing five parts by weight lactose. The presence of lactose did not seem to affect the reactivity. The purity of sodium dithionite was checked by iodine titration in water containing 2% sodium bicarbonate. Elemental analyses were performed by Atlantic Microlab, Norcross, GA, or Galbraith, Knoxville, TN.

Mass Spectrometry. Mass determinations (MS) and collision-induced dissociations (MS/MS) were carried out on an API-III triple quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada) equipped with a nebulization-assisted electrospray ion source[35] and a high-pressure collision cell. Operating parameters were previously described;[36] data collection and analysis were carried out using programs provided with the Sciex instrument. Samples were analyzed by direct infusion of sample prepared in 50% MeOH, 0.1% formic acid, or by HPLC directly coupled to the mass spectrometer (LC/MS) with post-column splitting so that 10% went to the mass spectrometer. HPLC was performed on a Hewlett-Packard C18 column, as described above, utilizing an Applied Biosystems Model 140A syringe driven pump at a flow rate of 200 μL/min. Initial buffer was 0.05% ammonium formate adjusted to pH 4 with formic acid, and elution was accomplished with a gradient into MeOH. In order to demonstrate the presence of GSH in the antibiotic adducts, initial fragmentation of the compound was accomplished by raising the orifice voltage to 95 V, followed by MS/MS of the selected fragment in the second quadrupole with argon (MS/MS/MS).

Reduction of Adriamycin (1) with 1 Molar Equiv of DHM-3 Dimer in Water, in the Presence of 10 Molar Equiv of Glutathione (9). To a nitrogen-bubbled solution of 15.3 mg of **9** and 9.1 mg of Tris in 5.0 mL of water was added 2.9 mg of adriamycin hydrochloride to obtain a 1.0×10^{-3} M concentration of the anthracycline. A 3.5-mL aliquot of this solution was syringed into a serum-stoppered vial flushed with nitrogen. The vial contained 1.1 mg of DHM-3 dimer, which quickly was dissolved by shaking. In a few minutes the solution turned brownish and some precipitate separated. After 15 min the vial was opened and the precipitate collected by suction filtration. The filtrate, containing 17% of the original content of anthracycline as measured by UV, upon HPLC analysis showed **11a** (25%), **11b** (50%), **1** (18%), and **12** (10%). The red precipitate appeared to be 95% pure **12**.

Reduction of Adriamycin (1) with 1 Molar Equiv of DHM-3 Dimer in Deuterium Oxide in the Presence of 50 Molar Equiv of Glutathione (9). An experiment was run as described in the previous paragraph, using a solution 5.0×10^{-2} M in **9**, 6.0×10^{-2} M in Tris, and 1.0×10^{-3} M in **1** in deuterium oxide. When the reaction was over (15 min) and the precipitate (**12**) separated by suction filtration, the filtrate, containing 58% of the original content of anthracycline as measured by UV, upon HPLC analysis showed 85% of **11a** + **11b** and 11% of **12**.

Reduction of Adriamycin (1) with 0.8 Molar Equiv of Sodium Dithionite in Deuterium Oxide in the Presence of 50 Molar Equiv of Glutathione (9). For this experiment the adriamycin employed was half from a pure sample and half from a clinical sample containing lactose (1:5, adriamycin: lactose, w/w). A 3.5-mL aliquot of a nitrogen-bubbled deuterium oxide solution, 1.0×10^{-3} M in **1**, 5.0×10^{-2} M in **9**, and 6.0×10^{-2} M in Tris, was introduced into a serum-stoppered vial containing 0.6 mg of 79% pure sodium dithionite under nitrogen. Almost immediately the orange solution turned green. After 1 min the green color faded and some red precipitate (**12**) separated. After 15 min the precipitate was collected by suction filtration. The filtrate, containing 25% of the original anthracycline content as measured by UV, upon HPLC analysis showed 85% of a 1:2 mixture of **11a** + **11b** and 8% of **12**. No unreacted adriamycin was detected.

Reaction of Adriamycin (1) with Glutathione (9): (a) in the Absence of Air, in Water. An aqueous solution 1.0×10^{-3} M in **1**, 1.0×10^{-2} M in **9**, and 1.5×10^{-2} M in Tris was prepared under a nitrogen flow in a vacuum tube. After freeze–thaw degassing (three cycles) and sealing, the tube was kept in the dark at ambient temperature for 24 h. HPLC analysis of the reaction mixture showed 7% of **11a** + **11b**, 68% of reacted **1**, and 16% of **12**.

(b) In the Presence of Air. When a similar experiment was run by letting a deuterium oxide solution 2.0×10^{-3} M in **1**, 0.10 M in **9**, and 0.15 M in Tris react for 20 h in the presence of air, HPLC analysis only showed unreacted **1**. For this experiment the adriamycin was from a clinical sample containing five parts by weight lactose. Ellman's analysis[37] for the presence of unoxidized **9** was performed in dimethyl sulfoxide (DMSO) and showed a residual 1.0×10^{-3} M concentration. The use of DMSO instead of water as the solvent for the analysis gives better results because the absorption maximum in DMSO occurs at 478 nm (412 nm in water), completely out of the absorption region of the Ellman's reagent.

Reaction of Adriamycin (1) with 20 Molar Equiv of Glutathione (9) in Deuterium Oxide: Synthesis of the Adriglu Salt (13). A solution was made 4.0×10^{-3} M in **1**, 8.0×10^{-2} M in **9**, and 0.12 M in Tris by dissolving 40 mg of adriamycin hydrochloride, 0.42 g of **9**, and 0.25 g of Tris in 17 mL of nitrogen-bubbled deuterium oxide, in a round-bottom flask provided with a tube for vacuum attachment. The adriamycin was from a clinical sample containing five parts by weight lactose. The solution

(32) Gaudiano, G.; Egholm, M.; Haddadin, M. J.; Koch, T. H. *J. Org. Chem.* **1989**, *111*, 8291.
(33) Egholm, M.; Koch, T. H. *J. Am. Chem. Soc.* **1989**, *111*, 5090.
(34) Bennett, R. W.; Wharry, D. L.; Koch, T. H. *J. Am. Chem. Soc.* **1980**, *102*, 2345.
(35) Covey, T. R.; Bonner, R. R.; Shushan, B. I.; Henion, J. *Rapid Commun. Mass Spectrom.* **1988**, *2*, 249.
(36) Resing, K. A.; Johnson, R. S.; Walsh, K. A. *Biochemistry* **1993**, *32*, 10036.
(37) Ellmann, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70.

was freeze–thaw degassed (one cycle) and the flask sealed under vacuum. The flask was then kept in the dark at ambient temperature. After ca. 1 h some red precipitate began to separate. After 5 h the flask was opened and the jelly precipitate collected by centrifugation. The filtrate (accounting for 17% of the original anthracycline content, as measured by UV) upon HPLC analysis showed 12.5% **11a**, 48% **11b**, and 40% **1**. The precipitate (**13**), washed with water and dried, weighted 39 mg (82%, based upon state of hydration, *vide infra*). Chromatographic analysis of it showed only two peaks of equal area, corresponding to **11b** and **1**, and only traces of **12**. The material was quite soluble in dimethyl sulfoxide, somewhat soluble in methanol, not very soluble in water and ethanol, and insoluble in acetonitrile or tetrahydrofuran. An analytically pure sample was obtained by recrystallization from hot methanol (10 mL/mg). The pure material had the following properties: UV (dimethyl sulfoxide) λ_{max}, nm (ϵ, L mol^{-1} cm^{-1}) 290 (sh, 2.0×10^4), 396 (sh, 7.3×10^3), 484 (2.5×10^4), 500 (2.5×10^4), 534 (1.5×10^4); in 9:1 water/dimethyl sulfoxide, 256 (5.2×10^4), 290 (sh, 1.8×10^4), 384 (sh, 6.1×10^3), 478 (2.4×10^4), 496 (2.4×10^4), 534 (1.4×10^4); ^{1}H NMR, see Table 2; mass spectrum, see Figure 1, Results and Discussion section, and the supplementary material. Anal. Calcd for $C_{58}H_{62}N_4O_{25}S$: C, 55.86; H, 5.01; N, 4.49; S, 2.57. Found: C, 49.99 (Galbraith Labs) or 50.8 and *53.8* (Atlantic Microlab); H 5.63 (Galb) or *5.35* (Atl); N, 4.18 (Galb) or 4.2 and *3.85* (Atl); S, *2.26* (Atl) (underlined values were from the same sample). With three water molecules of hydration the calulated values for $C_{58}H_{68}N_4O_{28}S$ are C, 53.5; H, 5.3; N, 4.3; S, 2.5; and with eight water molecules of hydration the calculated values for $C_{58}H_{78}N_4O_{33}S$ are C, 50.0; H, 5.6; N, 4.0; S, 2.3. Adriamycin and **11b** were separated from their salt (**13**) as follows: 10 mg of crude **13** was dissolved in 15 mL of 2×10^{-2} M aqueous ammonia. The solution was extracted three times with equal volumes of chloroform. HPLC analysis showed only **1** in the organic extracts and **11b** (containing ca. 5% of unextracted **1**) in the aqueous layer.

When the reaction between **1** and **9** was attempted with the same conditions, but using a lower concentration of Tris (8.0×10^{-2} M instead of 0.12 M), only unreacted **1** was obtained, even letting the reaction run for 21 h.

Reaction of Adriamycin (1) with 20 Molar Equiv of Glutathione (9) in Deuterium Oxide, Monitoring with Time. A solution was made 1.4×10^{-4} M in **1**, 2.8×10^{-3} M in **9**, and 4.2×10^{-3} M in Tris in nitrogen-bubbled deuterium oxide. Aliquots (1.0 mL) of this solution were introduced into each of three ampules (A, B, C) provided with tubes for vacuum attachment. After freeze–thaw degassing (two cycles) and sealing under vacuum the ampules were kept in the dark at ambient temperature. After 1 day they had all become turbid. No detectable color change was observed. Ampule A was opened after 26 h, ampule B after 70 h, and ampule C after 6 days. By this time the solution in ampule C had become almost limpid. The content of the ampules was examined by HPLC after limpidification by addition of a few drops of dimethyl sulfoxide. The following was the result: **11a** + **11b**, A 42%, B 58%, C 72% (**11a**/**11b**, A 0.18, B 0.21, C 0.33); adriamycin (**1**), A 58%, B 42%, C 28%. The compounds were identified by reverse-phase HPLC electrospray mass spectrometry: **11a**, *m/z* 1409.4 (protonated semiquinone dimer, calcd 1409.4), 727.3 (semiquinone + sodium ion, calcd 727.2), 705.3 (protonated semiquinone, calcd 705.2); **11b**, *m/z* 1409.2, 727.1, 705.3

Reduction of Adriamycin (1) with 1 Molar Equiv of TM-3 Dimer in Methanol-*d* in the Presence of 10 Molar Equiv of Glutathione (9): (a) in the Presence of 15 Molar Equiv of Tris. A solution 1.0×10^{-3} M in **1**, 1.0×10^{-2} M in **9**, and 1.5×10^{-2} M in Tris was made by dissolving 40 mg of adriamycin hydrochloride, 212 mg of **9**, and 125 mg of Tris in 70 mL of nitrogen-bubble methanol-*d*. TM-3 dimer (19 mg) was added, and the dark brown solution was kept under constant flow of nitrogen for 30 min. At this time the reaction mixture was filtered from a small amount of undissolved **9** and analyzed by HPLC to show 21% of **11a** + **11b** (**11a**/**11b** = 0.5), 5% of **1**, and 64% of **12**. The filtrate was rotary-evaporated, and water (25 mL) was added to the residue. The undissolved **12** was removed by suction filtration. After washing with chloroform the filtrate showed (HPLC) the presence of **11a** and **11b** and only minor peaks having the anthracycline-type chromophore. Reverse-phase HPLC electrospray mass spectrometry showed strong M + 1 ions at *m/z* 705 for both **11a** and **11b**. Most of the water was rotary-evaporated and the concentrate submitted to preparative HPLC to separate **11a** from **11b**. The column was a Ranin Dynamax C18, 3 μm, 50 mm × 10 mm. The eluent was a mixture of methanol and water, at 3 mL/min, using the following gradient (time, min (% MeOH)): 0 (25), 1 (28), 4 (30), 6 (60), 8 (90). The combined fractions containing **11b** (retention time 7 min) were washed with chloroform, filtered, and rotary-evaporated. The dark reddish residue (95% pure by HPLC) was very soluble in water, somewhat soluble in methanol, and almost insoluble in dimethyl sulfoxide and gave the following UV spectrum in water, λ_{max}, nm (ϵ, L mol^{-1} cm^{-1}): 256 (1.9×10^4), 290 (sh, 7.2×10^3), 386 (sh, 2.9×10^3), 484 (9.4×10^3), 496 (9.4×10^3), 532 (sh, 5.8×10^3). The less pure **11a** (retention time 3.5 min) gave the following UV spectrum, λ_{max}, nm (rel intensity): 258 (2.3), 290 (sh, 0.75), 386 (sh, 0.30), 488 (0.99), 500 (1.0), 532 (sh, 0.63).

(b) In the Absence of Tris. When a similar reaction was run on a small scale (2.5 mL) without Tris, the only detected reaction product was **12**.

Reaction of 11a and 11b with DHM-3 Dimer. A 0.95-mL aliquot of a 3.2×10^{-3} M freshly made acetonitrile solution of DHM-3 dimer was introduced into a 10-mL flask provided with a tube for vacuum attachment. The solvent was evaporated with a stream of nitrogen. A pH 8 Trizma-buffered aqueous solution of a 1:2 mixture of **11a** and **11b** was prepared at a total 1.0×10^{-3} M anthracycline concentration (A_{490} = 1, measured in a 1-mm cuvette). Nitrogen was bubbled through the solution. A 3-mL aliquot was quickly introduced into the flask and immediately freeze–thaw degassed (three cycles), and the flask was sealed. Soon the solution became turbid. After 26 h the flask was opened and the precipitate collected by suction filtration. The filtered solution was almost colorless. The precipitate was dissolved in dimethyl sulfoxide. HPLC analysis of the solution showed a large peak (80% of the total area) corresponding to **12**, along with several minor peaks of unidentified anthracyclines.

Reaction of Daunomycin (2) with 20 Molar Equiv of Glutathione (9) in Deuterium Oxide, Monitoring with Time. An experiment was set up in the same way as described above for the time-monitored experiment with adriamycin, using four separate ampules (A, B, C, D). No precipitate was obtained in any of the ampules, and no change in the UV spectrum was observed. The amplues were opened after 16 h (A), 65 h (B), 5 days (C), and 10 days (D) and analyzed by HPLC. They all showed three peaks, corresponding to **14a**, **14b**, and unreacted **2**. The following was the **14a** + **14b**, A 5%, B 23%, C 40%, D 60% (**14a**/**14b**, A 0.3, B 0.27, C 0.5, D 0.5); daunomycin, A 93%, B 77%, C 59%, D 40%. Reverse-phase HPLC electrospray mass spectrometry showed strong M + 1 ions at *m/z* 689 for both **14a** and **14b**.

Reduction of Daunomycin (2) with 1 Molar Equiv of Sodium Dithionite in Methanol-*d* in the Presence of 10 Molar Equiv of Glutathione (9). A 3.3-mL aliquot of a nitrogen-bubbled solution 1.0×10^{-4} M in **2**, 1.0×10^{-3} M in **9**, 2.0×10^{-3} M in Tris, and 1.0×10^{-4} M in Tris·HCl was introduced into a serum-stoppered, nitrogen-flushed, 10-mm cuvette. A 50-μL aliquot of a solution made by dissolving 2.0 mg of 79% pure sodium dithionite in nitrogen-bubbled deuterium oxide (1.3 mL) was introduced into the cuvette. Spectroscopic monitoring showed a quick change (5 s) of the quinone chromophore (486 nm) to hydroquinone (430 nm), which in turn was replaced by the quinone methide chromophore (610 nm) in less than 60 s. The intensity of the quinone methide band decreased to ca. one-third of the original intensity in 10 min. At this time air was let into the cuvette and the reaction mixture analyzed by HPLC. The chromatographic pattern matched the one seen when daunomycin quinone methide was exposed to air, in the absence of reactive nucleophils,[30] showing 55% of **8** and ca. 35% of the oxidation products of quinone methide. No **14a** or **14b** could be detected.

Reaction of Menogaril (10) with 20 Molar Equiv of Glutathione (9) in Deuterium Oxide. A 1.0×10^{-4} M solution of **10** was prepared by adding a solution of 0.60 mg of **10** in 0.50 mL of dimethyl sulfoxide to 9.5 mL of nitrogen-bubbled deuterium oxide containing 2.0×10^{-3} M **9** and 3.0×10^{-3} M Tris. A 2.5-mL aliquot of this solution was freeze–thaw degassed (three cycles) in a cell provided with a spectroscopic cuvette and a degassing chamber. The cell was sealed and kept in the dark at ambient temperature. Occasionally UV–vis spectra were taken, showing no change with time (λ_{max} = 474 nm). After 4 days a small amount of precipitate was observed. The cell was opened and the solution filtered from the precipitate. The absorbance of the filtrate at 474 nm was half as intense as the original solution. HPLC analysis showed the presence of **15a** and **15b** (total area 80%) in a 1:1 ratio and unreacted **10** (20%). The precipitate was unreacted **10**.

Reduction of Menogaril (10) with 0.5 Molar Equiv of TM-3 Dimer in Methanol, in the Presence of 30 Molar Equiv of Glutathione (9). A solution 4.0×10^{-4} M in **10**, 1.2×10^{-2} M in **9**, 4.4×10^{-2} M in Tris, and 2.4×10^{-2} M in Tris·HCl was made by dissolving 54 mg of **10**, 0.92 g of **9**, 1.32 g of Tris, and 0.95 g of Tris·HCl in 250 mL of argon-bubbled methanol. TM-3 dimer (14 mg) was added, and the mixture was kept stirring under argon. Samples of the clear, orange-brown solution (whose apparent pH was 7.5) were taken out of the flask after 15 min (A), 30 min (B), and 60 min (C) and analyzed by HPLC. A fourth sample (D) was freeze–thaw degassed, sealed under vacuum, and opened for HPLC analysis after 20 h. The chromatograms all showed ca. 95% of **15a** + **15b** (**15a**/**15b** = 0.3), on unreacted **10**, and no 7-deoxynogarol (**16**). The UV–vis

spectrum in methanol showed λ_{max}, nm (rel intensity): 238 (3.2), 264 (1.4), 296 (sh, 0.7), 476 (1). Attempts to separate and purify **15a** and **15b** by flash chromatography failed. A partial separation of the two epimers (**15a**, retention time 4.0 min; **15b**, retention time 4.8 min) was obtained by reverse-phase HPLC using the eluting conditions #3. Reverse-phase HPLC electrospray mass spectrometry showed strong M + 1 ions at m/z 817 for both **15a** and **15b**.

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Supplementary Material Available: Figure showing the MS/MS of ions 1410, 1249, and 1090 appearing in the mass spectrum (Figure 1) of the adriglu salt (**13**) (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in teh microfilm version of the journal, and can be ordered from the ACS; see any current masthead-page for ordering information.