Mechanistic Studies of the Reduction of Daunomycin with Sodium Borohydride. Formation and Reaction of Borate Esters

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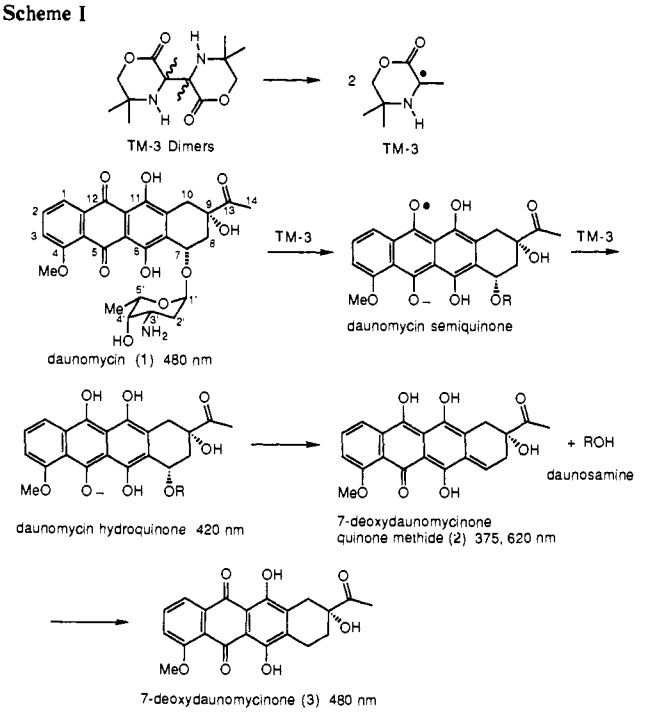
Abstract: Reduction of daunomycin with excess sodium borohydride in methanol degassed with prepurified nitrogen yielded 89% daunomycinol and 11% recovered daunomycin. Monitoring of the reaction by UV-vis spectroscopy revealed the formation of an intermediate with absorptions at 336 and 430 nm, which was assigned the borate ester structure **5** on the basis of the UV-vis absorption bands together with high-field ^{1}H NMR, FTIR, and mass spectral data. Similar results were obtained upon reduction without nitrogen degassing. In contrast, sodium borohydride reduction under strictly anaerobic conditions, achieved with freeze-thaw degassing, predominantly yielded the products of glycosidic cleavage, 7-deoxydaunomycinol (**6**, 58%) along with daunomycinol (**4**, 17%). The sequential formation of two intermediates was observed: first, borate ester **5** and second, a longer lived intermediate with absorptions at 360 and 580 nm. The second intermediate is proposed to be 7-deoxydaunomycinol quinone methide borate ester (**9**) on the basis of the absorption bands, lifetime, and product structures compared with those observed upon reduction with the one-electron reducing agent, bi(3,5,5-trimethyl-2-oxomorpholin-3-yl) (TM-3 dimer). Reduction of 7-deoxydaunomycinone with excess sodium borohydride in nitrogen-degassed methanol yielded 42% 7-deoxydaunomycinol (**6**), 31% 5,7-dideoxydaunomycinol tautomer (1,2,3,4-tetrahydro-2,11-dihydroxy-2-(1-hydroxyethyl)-7-methoxy-5,12-naphthacenedione, **11**), and 27% 7,12-dideoxydaunomycinol tautomer (1,2,3,4-tetrahydro-2,6-dihydroxy-2-(1-hydroxyethyl)-7-methoxy-5,12-naphthacenedione, **12**). Again an intermediate with absorptions at 336 and 430 nm was observed, in this case, assigned to regioisomeric borate esters **13** and **14** on the basis of formation of regioisomeric dideoxydaunomycinol tautomers **11** and **12**. The intermediacy of long-lived borate esters is relevant to the interpretation of studies employing sodium borohydride for the reductive activation of anthracyclines.

Introduction

Daunomycin (**1**) is an anthracycline antitumor drug which is thought to be bioreductively activated.[1] Chemical reagents that have been used to create reduced states of the anthracyclines for investigation of their chemistry are sodium dithionite,[2-4] bi-(3,5,5-trimethyl-2-oxomorpholin-3-yl) (TM-3 dimer),[5-7] hydrogen over palladium,[3,8] and sodium borohydride.[9,10] Dithionite[11] and TM-3 dimer[12] undergo bond homolysis to produce $SO_2^{\bullet-}$ and 3,5,5-trimethyl-2-oxomorpholin-3-yl (TM-3), respectively, which are one-electron donors to the anthracyclines. Carbon dioxide radical anion generated by pulse radiolysis has also been employed as a one-electron donor.[13-15] One-electron reduction of daunomycin produces the semiquinone state, as shown in Scheme I with TM-3. In protic solvents the semiquinone is either reduced to the hydroquinone state by a second molecule of TM-3 or disproportionates to hydroquinone and quinone. The hydroquinone at neutral or basic pH undergoes rapid elimination of the sugar substituent, daunosamine, to form 7-deoxydaunomycinone quinone methide (**2**). The quinone methide has a half-life of 53 s at 25 °C in methanol at an apparent pH of 8, with respect to the pseudo-first-order solvent-catalyzed tautomerization to 7-deoxydaunomycinone (**3**).[5] An alternative pathway to the quinone methide, which continues to be debated, is glycosidic cleavage at the semiquinone state to form a semiquinone methide[16-19] followed by one-electron reduction. Sodium borohydride is commonly

Scheme I



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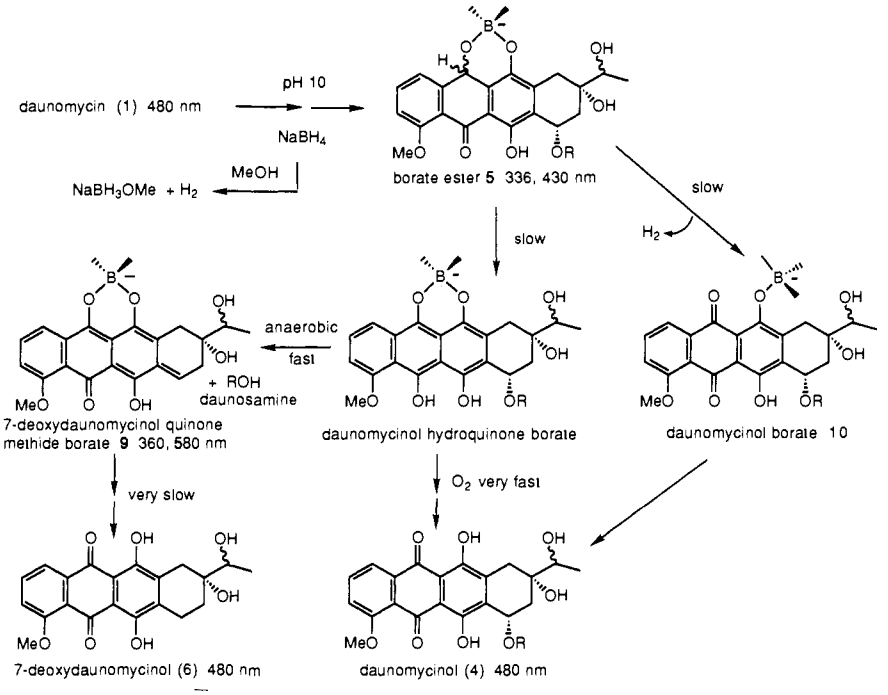
Scheme II



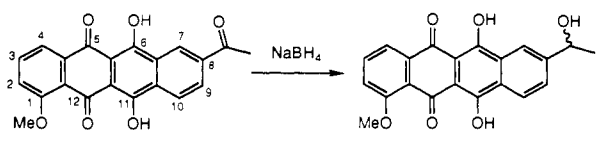
thought to react as a two-electron reducing agent through hydride donation. It reacts with both the carbonyl group at the 13-position[20] as well as the quinone functionality (see Scheme I for the numbering system of daunomycin). With respect to the quinone, Bachur and co-workers have reported that sodium borohydride reduction yields the hydroquinone state, which does not undergo glycosidic cleavage but only "autooxidation" back to the quinone.[16] This result has been interpreted to support the pathway of glycosidic cleavage at the semiquinone state. Yet, both one- and two-electron reducing enzymes cause glycosidic cleavage.[21,22] Interpretations of reactivity as a function of reducing agent are further complicated by the rapid oxidation of anthracycline hydroquinones to semiquinones by mere traces of molecular oxygen.

We report here spectroscopic evidence that the first long-lived intermediate from the reduction of daunomycin with sodium borohydride in methanol is not the hydroquinone but a borate ester with a tetrahedral carbon at the 12-position. The structure of this intermediate is particularly significant because sodium borohydride has been used to generate reactive intermediates in studies of covalent binding of the anthracyclines to DNA through reductive activation.[23]

Results and Discussion

Sodium Borohydride Reduction of Daunomycin. The reduction of daunomycin with 2 mol equiv of sodium borohydride was first examined at an apparent pH of 10 in methanol, oxygen-degassed with prepurified nitrogen. The pH was achieved with a phosphate buffer and is approximately that which also results from addition of sodium borohydride to methanol. The reaction was monitored at 25 °C by UV–vis spectroscopy. During the first 600 s, the quinone band of daunomycin at 480 nm decreased with the formation of new bands at λ_{max} 336 and 430 nm. During the next 5 h, the new bands disappeared with the reappearance of the quinone band at λ_{max} 480 nm. HPLC analysis of the reaction mixture showed 89% diastereomeric daunomycinols (**4**) and 11% recovery of daunomycin, as determined by spectroscopic and chromatographic comparison with authentic samples. A similar experiment without oxygen degassing showed the same spectral changes, with the formation of 82% **4** and 12% recovery of daunomycin. The intermediate with absorptions at 336 and 430 nm was assigned the borate ester structure **5**, as shown in Scheme II, on the basis of evidence to be described.

Rigorous freeze–thaw degassing of the methanol solution of daunomycin prior to mixing with sodium borohydride resulted in some different spectral changes occurring during the reaction and in a different product mixture. The spectral changes are shown in Figures 1 and 2. During the first 1200 s (Figure 1), the formation of the same intermediate, borate ester **5**, appeared as observed during the reaction with less rigorous or no oxygen degassing. However, during the next 20-h period the bands at 336 and 430 nm decreased with the formation of bands at 360 and 580 nm, characteristic of a quinone methide, and bands in the region 460–530 nm, characteristic of a quinone. After 8 days of reaction, hydrochloric acid was added to rapidly protonate any residual quinone methide and solvolyze borate esters. HPLC analysis showed the formation of 17% diastereomeric daunomycinol (**4**) and 58% diastereomeric 7-deoxydaunomycinol (**6**). Six additional products were also observed. One has tentatively been identified as bisanhyrdo-13-dihydrodaunomycinone (**7**, 8%, 6,11-dihydroxy-8-(hydroxyethyl)-1-methoxy-5,12-naphthacenedione). The identification of the other products remains a challenge; none is the product of reduction of 7-deoxydaunomycinone with sodium borohydride (vide infra). The daunomycinol and 7-deoxydaunomycinol were identified by chromatographic and spectral comparison with authentic samples prepared independently. Aerobic sodium borohydride reduction of bisanydrodaunomycinone (**8**, 8-acetyl-6,11-dihydroxy-1-methoxy-5,12-naphthacenedione), obtained from the acid-catalyzed elimination of water and daunosamine from daunomycin,[24] yielded a material which had chromatographic and UV–vis spectroscopic properties identical to the product assigned the structure bisanhydro-13-dihydrodaunomycinone (**7**).



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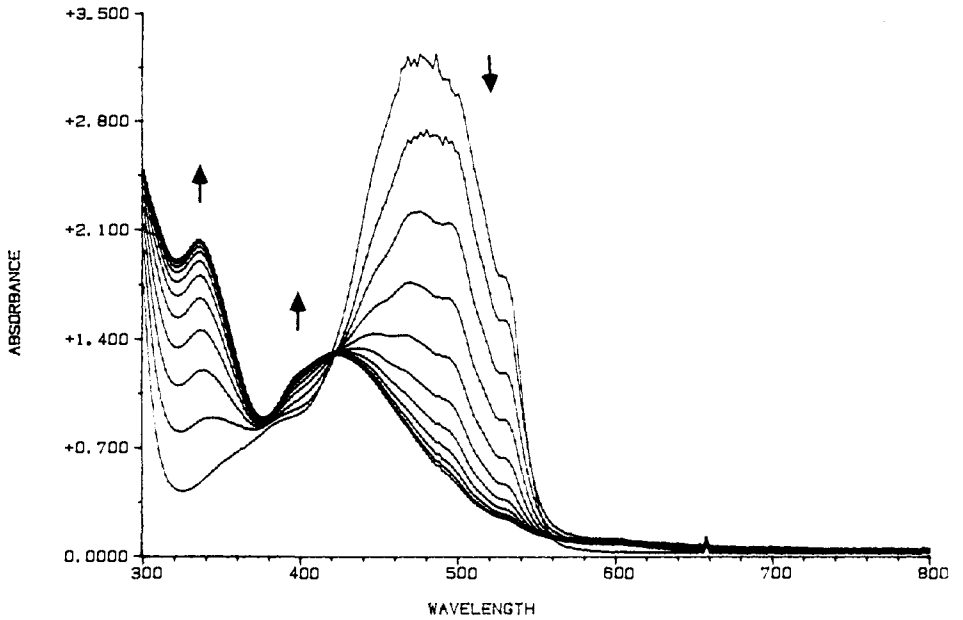

Figure 1. UV–vis spectra of the anaerobic reduction of 2.0×10^{-4} M daunomycin with 2.0×10^{-3} M sodium borohydride in methanol at apparent pH 10 and 25 °C as a function of time. Scans were every 2 min in the time period 0–20 min.

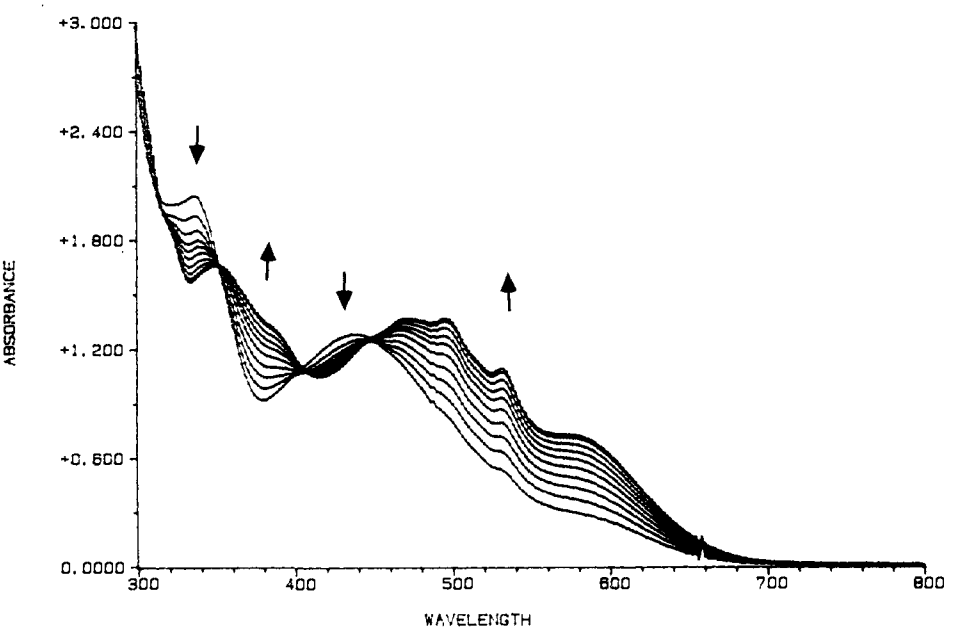

Figure 2. UV–vis spectra of the reaction described in the caption to Figure 1 in the time period 21 min to 20.3 h. Scans were every 2 h.

A mechanism for the formation of daunomycinol and 7-deoxydaunomycinol with the intermediacy of borate ester **5** and the borate ester of 7-deoxydaunomycinol quinone methide **9** is shown in Scheme II. The bisanhydro-13-dihydrodaunomycinone (**7**) is proposed to be a secondary product, resulting from the slow, base-catalyzed elimination of daunosamine and water from daunomycinol during the long reaction period.

The borate ester structure **5** is proposed on the basis of IR, ^{1}H NMR, and mass spectra of the intermediate taken at optimum time during the reduction and the subsequent chemical reactivity. The IR spectrum showed the absence of the carbonyl band at 1723 cm^{-1} for the ketone at the 13-position and a band at 1654 cm^{-1} for a conjugated ketone. The band at 1654 cm^{-1} was twice as intense as that observed for daunomycin at time zero. ^{1}H NMR spectra of daunomycin (**1**), the intermediate **5**, and daunomycinol (**4**) are compared in Table I. Of particular significance was the appearance of two singlets at δ 5.7 ppm in the spectrum of the intermediate, assigned to the proton at the 12-position in the two diastereomers; these were absent when the reduction was conducted with sodium borodeuteride. Also the aromatic protons of **5** resonate at higher field than those of daunomycin and daunomycinol, particulary those at the 3-position, consistent with a chemical change in the electron-withdrawing quinone functionality. The FAB mass spectra of a reaction mixture containing **5** in a glycerol matrix showed positive ions at m/z 532 ($M + H^+$) and 554 ($M + Na^+$) and a negative ion at m/z 530 ($M - H^+$), consistent with the daunomycinol hydroquinone structure. In contrast, a positive ion FAB mass spectrum of the diastereomeric daunomycinols in a glycerol matrix showed an $M + H^+$ peak at m/z 530; consequently, the ions observed at m/z 532 and 554 could not have come from the daunomycinols. Because of the instability of daunomycinol hydroquinone in solution with respect to air oxidation and glycosidic cleavage, we argue that the daunomycinol hydroquinone ions observed in the mass spectra of the reaction mixture resulted from the solvolysis of **5** by the glycerol matrix upon bombardment.

Earlier reductions of daunomycin with the one-electron reducing agent, TM-3 dimer, were conducted in methanol at apparent pH 8.[5] For comparison purposes, this reduction was reinvestigated at pH 10. UV–vis spectral monitoring of the reaction during the

Table I. ^{1}H NMR Data for Daunomycin (**1**), the Mixture of Diastereomeric Transients from Reduction of Daunomycin with Sodium Borohydride (**5**), and Diastereomeric Daunomycinols (**4**) in CD_3OD

species	pattern for indicated position[a]														
(MHz)	1	2	3	4	7	8	10	12	13	14	1′	2′	3′	4′	5′
1	7.99 d	7.85 t	7.59 d	4.04 s	5.11 dd	2.18 dd	3.037 d			2.34 s	5.46 d	1.87 dd	3.57 dt	3.64 bs	1.28 d
(500)	J = 8	J = 8	J = 8		J = 2, 5	J = 5, 15	3.044 d				J = 4	J = 4, 13	J = 12, 4		4.30 q
						2.32 bd	J = 18					2.02 td			J = 7
						J = 15						J = 13, 4			
5	7.68 d	7.64 t	7.09 d	3.94 s	5.19 bs	1.82	2.70	5.70 s[b]	3.60 m[b]	1.26 d[c]	5.42 bs	1.72	3.30[d]	3.45 bs[b]	1.24 d[c]
(500)	J = 8	J = 8	J = 8			2.50 m	3.04 m	5.72 s[b]				1.78 m		3.47 bs[b]	4.19 m
4	7.94 d	7.83 t	7.56 d	4.00 s	5.08 bs	1.91	2.65		3.64 q	1.29 d	5.46 s	1.87 dd	3.50 m	3.63 bs	1.30 d
(300)	J = 8	J = 8	J = 8			2.49 m	3.10 m		J = 6	J = 6		J = 4, 13			4.27 d[b]
												2.02 td			4.29 d[b]
												J = 13, 4			J = 6

[a] Chemical shifts are reported in ppm on the δ scale and coupling constants in hertz. The numbering system is the one used for daunomycin shown in Scheme I. The symbols are defined as follows: b, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. [b] Separate signals were observed for each diastereomer. These signals did not appear when the reduction was performed with $NaBD_4$. [c] Coupling constants could not be determined because of overlapping patterns from the two diastereomers. [d] Pattern was obscured by the signal from CHD_2OD solvent impurity.

20-min reaction period showed spectral changes identical to those reported at pH 8 with the appearance of quinone methide bands at 380 and 610 nm. HPLC analysis of the reaction mixture showed the formation of 7-deoxydaunomycinone (**3**) and recovery of some daunomycin. Kinetic analysis of the 610-nm band as a function of time gave a first-order rate constant for the tautomerization of 7-deoxydaunomycinone quinone methide (**2**) to 7-deoxydaunomycinone equal to 7.1×10^{-3} s^{-1} ($\sigma = 0.3 \times 10^{-3}$ s^{-1}), with a half-life for **2** of 98 s compared with 53 s at pH 8.

The differences in the lifetimes and the UV–vis spectra of the quinone methides generated with TM-3 dimer and sodium borohydride, both at pH 10, indicate that the quinone methides have different structures. Consequently, we have proposed the quinone methide borate ester structure **9** for the second long-lived intermediate formed during anaerobic reduction with sodium borohydride. It is proposed to result from the rearrangement of borate ester **5** to the short-lived transient, daunomycinol hydroquinone borate, followed by glycosidic cleavage. Hydroquinones generated by electrochemical reduction of ortho quinones and 2-hydroxy-1,4-naphthoquinone have been proposed to form cyclic complexes with the borate present in a borate buffer on the basis of spectroscopic and electrochemical measurements.[25] The formation of intermediate **9** must not be competitive with air oxidation of daunomycinol hydroquinone borate as shown in Scheme II because the major products are the daunomycinols (**4**) in the presence of even low concentrations of molecular oxygen. Formation of quinone methide borate ester **9** is consistent with the regiochemistry proposed for the initial reduction of the quinone functionality of daunomycin to form borate ester **5**. The regioselectivity of borohydride for the 12-position of the quinone is also reasonable in terms of steric crowding at the 5-position resulting from the combination of the methoxy group at the 4-position and the daunosaminyl group at the 7-position.

In Scheme II, borate ester **5** is shown to form both 7-deoxydaunomycinol quinone methide borate **9** and daunomycinol borate **10**. As stated earlier, spectroscopic monitoring of the decay of **5** showed the simultaneous formation of both a quinone species and quinone methide species. The quinone species was shown to be predominantly either **10** or daunomycinol (**4**) rather than 7-deoxydaunomycinol (**6**) by its characteristic reaction with TM-3 dimer. Addition of excess TM-3 dimer to the anaerobic reaction mixture after appearance of the quinone bands caused the rapid appearance of an absorption in the region of 610 nm similar to that of quinone methide **2**; the subsequent fall in the absorbance at 610 nm with a rise in absorbance at 420 nm for a hydroquinone species was also characteristic of reduction of daunomycin or daunomycinol with excess TM-3 dimer at pH 10. 7-Deoxydaunomycinol cannot form a quinone methide upon reduction. Quinone species were not subsequently reduced by the excess sodium borohydride added at time zero because by the time the quinone species were formed, the excess sodium borohydride had reacted with the methanol solvent. This was established from spectral changes upon addition of more sodium borohydride to the anaerobic reaction mixture after the appearance of the quinone bands. The addition of sodium borohydride resulted in increased absorption in the region characteristic of borate ester **5** and decreased absorption in the region characteristic of quinones. Further evidence for the deactivation of sodium borohydride by methanol was the lack of reduction of the quinone functionality of daunomycin by a pH 10 methanol solution of sodium borohydride, which had been aged for 3.5 h prior to reaction. The slow reaction of borate ester **5** with solvent to form daunomycinol borate **10** and hydrogen is similar to the reaction of borohydride with methanol to form hydrogen and is reminiscent of the behavior of aluminum alkoxides in the Meerwein–Pondorf–Verley reduction.

Reduction of 7-Deoxydaunomycinone (3) with Sodium Borohydride. Sodium borohydride reduction of the quinone chromophore of daunomycin was subsequently compared with sodium borohydride reduction of the quinone chromophore of 7-deoxydaunomycinone (**3**). UV–vis spectral monitoring of the reduction of **3** degassed with nitrogen again showed the appearance and disappearance of an intermediate with absorptions at λ_{max} 336 and 430 nm, characteristic of a borate ester structure analogous to that of **5**. HPLC analysis of the reaction mixture showed the presence of 42% diastereomeric 7-deoxydaunomycinols (**6**), 31% diastereomeric 5,7-dideoxydaunomycinol tautomers (1,2,3,4-tetrahydro-2,11-dihydroxy-2-(1-hydroxyethyl)-7-methoxy-5,12-naphthacenedione, **11**), and 27% diastereomeric 7,12-dideoxydaunomycinol tautomers (1,2,3,4-tetrahydro-2,6-dihydroxy-2-(1-hydroxyethyl)-7-methoxy-5,12-naphthacenedione, **12**). The products were identified by chromatographic and spectral comparison with samples prepared by aerobic reduction of 7-deoxydaunomycinone with sodium borohydride as described by Brand and Fisher.[26] The mechanism for product formation from the reduction of **3** is shown in Scheme III with the intermediacy of regioisomeric borate esters **13** and **14**. Regioisomeric borate esters are proposed to account for formation of the regioisomeric dideoxydaunomycinol tautomers. The absence of regioselectivity in the reduction of 7-deoxydaunomycinone is consistent with a significant steric effect from the daunosaminyl group at the 7-position during the reduction of daunomycin as suggested above. The formation of substantial amounts of **6** is analogous to the formation of diastereomeric daunomycinols from reduction of daunomycin and is rationalized by reaction of borate esters **13** and **14** with methanol to reform the quinone chromophore.

Significance of Formation of Long-Lived Borate Ester Transients. Sodium borohydride has been employed to reduce daunomycin with the anticipation that the transients produced are the same transients produced in vivo. Of particular significance were studies directed toward establishing covalent bond formation between anthracycline aglycons and DNA with reductive activation.[23] Such experiments were performed in pH 7 buffered water and, consequently, are not directly comparable. Our experiments were performed at pH 10 in methanol because sodium borohydride

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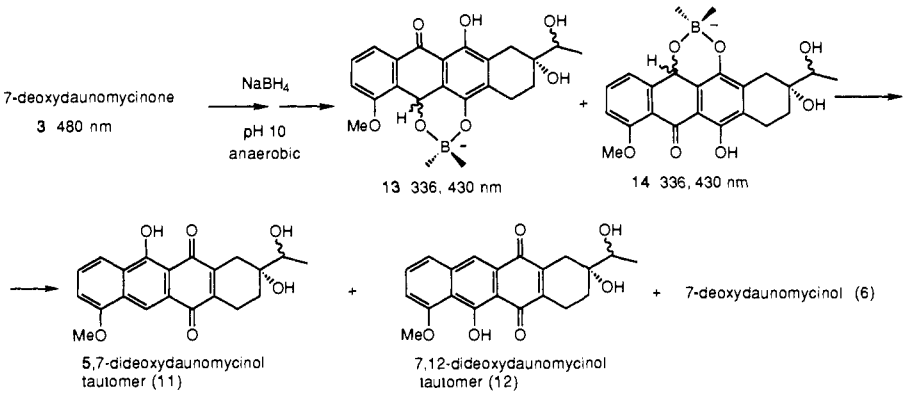
Scheme III



reacts in an acid–base fashion with a protic medium at pH 7 and because no product precipitation occurs in methanol. Product precipitation interferes with spectrophotometric monitoring of the reaction in real time. However, we have also observed the formation of the same long-lived intermediate with absorptions at 336 and 430 nm upon aerobic reduction of both daunomycin and adriamycin in aqueous medium, with the ultimate formation of diastereomeric daunomycinols and adriamycinols. At the very least the results presented here suggest that the species reported to react with DNA in the covalent binding experiments was most likely borate ester **5** or a borate ester intermediate derived from **5**. A semiquinone has also been observed by EPR spectroscopy from the reduction of aqueous daunomycin with sodium borohydride in the presence of molecular oxygen.[27] This semiquinone probably had the side chain reduced at the 13-position and may also have been a borate ester. A structure different from daunomycin semiquinone is consistent with differences in the hyperfine coupling constants with those reported for daunomycin semiquinone generated enzymatically in $DMSO/H_2O$,[28] with TM-3 in methanol,[5] and with alkaline glucose in DMF.[29] We have not observed a significant EPR signal during the aerobic reduction of daunomycin with sodium borohydride in methanol solvent.

Of additional interest is the modulating effect of borate complexation on the reactivity of the quinone methide state. The half-life of quinone methide **2** with respect to solvent protonation to form 7-deoxydaunomycinone (**3**) at 25 °C and an apparent pH of 10 is only 98 s. Quinone methide borate **9** under similar conditions lives for days. Complexation with borate then decreases the basicity of the quinone methide at the 7-position. Does it also change the electrophilicity at the 7-position? The reactivity of **9** has some resemblance to that of the quinone methides from reductive cleavage of the 11-deoxyanthracyclines.[21,30] In particular, the quinone methide from reductive glycosidic cleavage of menogaril, 7-*con*-*O*-methylnogarol, also lives for days under anaerobic conditions;[31] it has been shown to couple with a variety of nucleophiles including 2′-deoxyguanosine.[32] Quinone methide borate **9** and the quinone methides from reductive cleavage of the 11-deoxyanthracyclines are both modified at the 11-position relative to quinone methide **2**. Does an equivalent to the borate ester **9** exist in vivo, such as a phosphate ester, and is it involved in cytotoxicity? These are questions for future thought and investigation.

Experimental Section

General Remarks. HPLC was performed with a Hewlett-Packard 1090 liquid chromatograph equipped with a diode array detector and Hewlett-Packard work station. Chromatography was performed with a 5-μm ODS hypersil reverse-phase microbore column, 2.1 mm i.d. × 100 mm from Hewlett-Packard, eluting with a mixture of methanol (A) and 0.3% ammonium formate buffer adjusted to pH 4 with formic acid (B), using an A:B gradient from 30:70 initially, to 60:40 at 12 min, to 80:20 at 20 min. Materials eluting from the column was detected by absorptions at 440, 480, and 560 nm. HPLC yields were determined by integration of peaks from monitoring at 480 nm, λ_{max} for most of the chromophores, and are uncorrected for small differences in molar absorptivity at this wavelength. UV–vis spectral data were collected using a Hewlett-Packard (HP) 8452 spectrophotometer. ^1H NMR spectra were recorded with a Varian VXR 300- or 500-MHz instrument using TMS or solvent as the internal standard. Chemical shifts are reported in ppm on the δ scale. Infrared spectral data were collected using a Perkin-Elmer 1600 FTIR spectrophotometer, and mass spectral data were obtained with a VG Instruments 7070 EQ-HF high-resolution mass spectrometer equipped with a FAB inlet system.

All solvents used were HPLC or spectroanalyzed grade. Sodium borohydride was obtained from the Aldrich Chemical Co. (Milwaukee, WI). TM-3 was prepared as described earlier.[33] Daunomycin was a gift of Farmitalia Carlo-Erba, Milan, Italy, and (*R*)- and (*S*)-daunomycinols were a gift of Adria Corp. (Columbus, OH). 7-Deoxydaunomycinone was prepared as described earlier.[3] All other solvents and chemicals were obtained from Aldrich, Sigma (St. Louis, MO), J. T. Baker (Phillipsburg, NJ), or Fisher (Fairlawn, NJ). Solvents were deoxygenated by bubbling nitrogen through the solvent for 15–20 min in a vessel equipped with a septum. The nitrogen used was passed through a gas purification cylinder (Labclear, Oakland, CA) capable of reducing the oxygen content to less than 1 ppm.

Three types of multicompartment cells were employed. The first type was constructed of medium-walled glass and consisted of three compartments. The first compartment could hold a volume of 3 mL and had another identical compartment attached at a 45° angle and a Pyrex cuvette attached at a 90° angle. The cell was equipped with a glass tube that allowed connection to a high-vacuum line for the freeze–thaw degassing. The second type of cell consisted of two compartments, one a medium-walled glass tube which could hold a volume of 3 mL, and the other, a Pyrex cuvette attached within the same plane as the first compartment but at a 30° angle. The third type consisted of four compartments. The first compartment could hold a volume of 3 mL; two identical compartments were attached on either side of the 3-mL compartment at 45° angles and a Pyrex cuvette was attached at a 90° angle.

Methanol solutions placed in a multicompartment cell were oxygen-degassed on a high-vacuum line. Each cell was frozen in liquid nitrogen and evacuated to 3×10^{-6} Torr or less. The liquid nitrogen was removed and solutions thawed gently with a heat gun. This was repeated four times. On cycles 4 and 5 the solutions were sonicated 5–10 s before refreezing. After sonication on the fifth cycle the solutions were frozen, evacuated for the final time, and sealed off with a torch.

Reduction of Daunomycin with Sodium Borohydride in Methanol Degassed with Nitrogen. Daunomycin (4.0 mg, 7.1×10^{-6} mol) was dissolved in 25 mL of methanol. A solution of sodium borohydride was prepared by dissolving 10 mg of the solid in 5 mL of cold (0 °C) doubly distilled water buffered with sodium phosphate and brought to pH 10 with 0.1 N NaOH. A 2.5-mL (7.5×10^{-7} mol) aliquot of the daunomycin solution was placed in a quartz cuvette equipped with a septum

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cap and degassed with prepurified nitrogen. The cuvette was then placed into the thermostated cell holder (25 ± 0.1 °C) of an HP 8452 spectrophotometer. The sodium borohydride solution (26 μL, 2 mol equiv) was then injected into the daunomycin solution via syringe. The reaction was monitored with the spectrophotometer from 300 to 800 nm. The cell was stored in the dark at ambient temperature overnight and analyzed by HPLC the following day. Spectroscopic monitoring of the reaction showed a long-lived intermediate which maximized at 600 s; the intermediate showed absorptions at 336 and 430 nm. The quinone band began to reappear starting at 660 s, and the spectrum showed primarily all quinone at 5.5 h. HPLC analysis of the reaction mixture showed 89% diastereomeric daunomycinols (**4**), with t_R = 13.0 and 13.2 min, and 11% diastereomeric 7-deoxydaunomycinols (**6**), with t_R = 14.1 and 14.6 min. An identical experiment was performed in which the solutions were not degassed with nitrogen. Spectroscopic monitoring showed the same spectral change at approximately the same times. HPLC analysis showed primarily daunomycinol (82%) and some recovered daunomycin (12%). Product identifications were made by coinjection with authentic samples and peak purity evaluation with the HPLC diode array detector.

Reduction of 7-Deoxydaunomycinone with Sodium Borohydride in Methanol Degassed with Nitrogen. A solution of 7-deoxydaunomycinone was prepared by dissolving 0.76 mg (2.0×10^{-6} mol) of the solid in 10 mL of unbuffered methanol with stirring overnight. An aliquot (2.0 mL, 2.0×10^{-7} mol) of the 7-deoxydaunomycinone solution was placed in a cuvette equipped with a septum cap. The solution was degassed with prepurified nitrogen. Sodium borohydride was weighted into a microbeaker (0.15 mg, 4.0×10^{-6} mol) which was quickly dropped into the cuvette, and the reaction was monitored with a UV–vis spectrophotometer. The reaction mixture was kept in the dark overnight at ambient temperature and then analyzed by HPLC. The spectral data again showed the formation of an intermediate with maximum absorptions at 336 and 430 nm. The HPLC analysis showed the formation of six products: two 7,12-dideoxydaunomycinol tautomers (**11**) (27%, t_R 11.9 and 12.5 min), and 7-deoxydaunomycinols (**6**) (42%, t_R 13.3 and 14.1 min), and two 5,7-dideoxydaunomycinol tautomers (**10**) (31%, t_R 14.6 and 15.4 min). Product identifications were made by coinjection with authentic samples and comparison of retention times and spectra with those reported by Fisher.[26]

Reduction of Daunomycin with Sodium Borohydride in Freeze–Thaw-Degassed Methanol. A solution of daunomycin was prepared by dissolving 1.7 mg (3.0×10^{-6} mol) in 10 mL of unbuffered methanol. Sodium borohydride (0.10 mg, 3.0×10^{-6} mol) was weighed into a microbeaker, and the microbeaker was placed into one compartment of the three-compartment cell. The daunomycin solution (2.0 mL, 6.1×10^{-7} mol) was loaded into the compartment at the 45° angle via a syringe equipped with a Teflon needle. The daunomycin solution was freeze–thaw-degassed through four cycles as described above. The cell was sealed under vacuum, the contents of the two compartments were mixed by shaking the cell vigorously, and the solution was poured into the third compartment, a Pyrex cuvette. The reaction was monitored with an HP 8452 spectrophotometer, and UV–vis spectra as a function of time are shown in Figures 1 and 2. The cell was opened 8 days later and analyzed by HPLC. Before the cell was opened, a spectrum of the reaction mixture was obtained. Since an intermediate which looked like the quinone methide was still present, hydrochloric acid was added upon opening the cell to destroy any quinone methide quickly so that air-oxidation products would not be formed.[34] The solution was analyzed by HPLC. Eleven products were formed; four of them, the two diastereomeric daunomycinols (**4**, 17%, t_R = 13.0 and 13.2 min) and the two diastereomeric 7-deoxydaunomycinols (**6**, 58%, t_R = 13.3 and 14.1 min), were identified by coinjection with independently prepared samples on an HPLC, with peak purity evaluation by a diode array detector and comparison of HPLC retention times and UV–vis spectra with those for the known materials. A fifth product was tentatively identified as bisanhydro-13-dihydrodaunomycinone (**7**, 8%, t_R = 19.3 min) by chromatographic and UV–vis comparison with a small sample prepared by aerobic sodium borohydride reduction of bisanhydrodaunomycinone (**8**, 8-acetyl-6,11-dihydroxy-1-methoxy-5,12-naphthacenedione), obtained from acid-catalyzed elimination of water and daunosamine from daunomycin.[24]

The effect of adding reducing agents to the freeze–thaw-degassed daunomycin sodium borohydride reaction solution after 18 h of reaction time was explored. The reaction was performed in a four-compartment cell with the fourth compartment used for the additional reducing agent. The reaction was performed as described above, except that after 18 h the reaction solution was mixed with either an additional 10 mol equiv of sodium borohydride or 10 mol equiv of *dl*-bi(3,5,5-trimethyl-2-oxomorpholin-3-yl) (TM-3 dimer). During the 18-h period, the compartment containing the additional reducing agent was warmed a few degrees above the reaction temperature of 25 °C with heating tape to prevent solvent distillation into the compartment. The addition of the extra sodium borohydride resulted in a decrease in the UV–vis absorption at 480 nm and increases at 336 and 430 nm. The addition of TM-3 dimer resulted in a decrease in the absorption at 480 nm and an increase in the region of 610 nm; this was followed by a fall in the band at 610 nm with a rise in the absorbance at 420 nm.

Spectral Characterization of Transient Borate Ester 5. Daunomycin (2.3 mg, 4.1×10^{-6} mol) was dissolved in 1 mL of methanol-d_4. An excess (10 mol equiv) of sodium borohydride or sodium borodeuteride was placed in the solution of daunomycin and mixed. The solution was placed in an NMR tube, and the ^{1}H NMR spectrum was taken when the solution appeared completely yellow. The spectral data are reported in Table I.

Daunomycin (3.4 mg, 6.0×10^{-6} mol) was dissolved in 3 mL of methanol. An excess (10 mol equiv) of solid sodium borohydride was added to the daunomycin solution. An infrared spectrum of the intermediate was acquired by placing a drop of the solution containing the intermediate between two salt plates. The spectrum of daunomycin showed a peak corresponding to the carbonyl of the quinone at 1654 cm^{-1} and a small shoulder corresponding to the side-chain carbonyl at 1723 cm^{-1}. The intermediate showed increased absorption at 1654 cm^{-1} and disappearance of the shoulder 1723 cm^{-1}.

A sample from the reaction of 4.0×10^{-4} M daunomycin with 1.1 mol equiv of sodium borohydride in methanol was analyzed by positive and negative ion FAB mass spectroscopy using a glycerol matrix. Again the analysis was performed when the reaction mixture was completely yellow. The spectra showed positive ions at m/z 532 and 554 and a negative ion at m/z 531.

Anaerobic Reduction of Daunomycin (1) with TM-3 Dimer at pH 10. Daunomycin (2.8 mg, 5.0×10^{-6} mol) was dissolved in 25 mL of methanol. A buffer solution was prepared by dissolving 0.84 mg (0.01 mol) of sodium bicarbonate in doubly distilled water and adjusting the pH to 10 with 2 N sodium hydroxide. A solution of TM-3 dimer was prepared by dissolving 7.1 mg of the solid in methylene chloride. An aliquot (2.5 mL, 5.0×10^{-7} mol) of the daunomycin solution was adjusted to pH 10 by adding 20 μL of the pH 10 buffer. An aliquot of the TM-3 dimer solution (0.5 mL, 5.5×10^{-7} mol) was placed in the Pyrex compartment of a two-compartment cell. The solvent was evaporated with a steady stream of nitrogen. The 2.5-mL aliquot of the pH 10 daunomycin solution was placed in the other compartment of the cell. The solution was freeze–thaw-degassed as described before. The cell was sealed under vacuum, the contents of the cell were mixed, and the cell was placed in a thermostated cell holder (25 ± 0.1 °C) of the HP 8452 spectrophotometer. The reaction was monitored for 20 min scanning every 20 s. Spectral monitoring showed the disappearance of the quinone band at 480 nm with the appearance of bands at 380 and 610 nm for 7-deoxydaunomycinone quinone methide (**2**), maximizing at 100 s, followed by disappearance of the bands of **2** with reappearance of the band at 480 nm. The cell was opened, and the contents were analyzed by HPLC. The analysis showed 76% 7-deoxydaunomycinone (**3**) and 24% recovered daunomycin.

Synthesis[20] and Characterization of Diastereomeric Daunomycinols. Daunomycin (50 mg, 8.9×10^{-5} mol) was mixed with 4 mL of a 1:1 mixture of chloroform and absolute ethyl alcohol. The solution was stirred and kept in a water bath at 20 °C. Potassium borohydride (7.8 mg, 1.4×10^{-4} mol) was added to the daunomycin solution in small portions over a period of 3 min. The solution was stirred for 3.5 h. The solution was the evaporated to dryness and redissolved in 4 mL of a mixture of chloroform and *n*-butanol (8:2). The pH was raised to >12 by adding 0.1 N NaOH, and then the pH was brought to 6.5 by adding 0.1 N HCl. The aqueous phase was separated from the organic phase and washed twice with 2 mL of chloroform and *n*-butanol (8:2). The aqueous phase was evaporated to a minimal volume (<1 mL), and to it was added acetone until precipitation occurred. The solid was isolated by centrifugation and filtration. The solid was washed once with acetone. This product was analyzed by silica gel TLC eluting with 6:1 chloroform/methanol (v/v); five spots were observed at R_f 0.03, 0.08, 0.16, 0.21, and 0.84. The diastereomeric daunomycinols (R_f = 0.08) were isolated by suction chromatography[35] on 230–400 mesh silica gel eluting with a step gradient from 6:1 chloroform/methanol (v/v) to ca. 1:1. Further analysis of the purified product was performed by ^{1}H NMR (Table I) and HPLC which showed the diastereomeric daunomycinols (**4**) at t_R 13.2 and 13.4 min.

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Kinetics and Mechanism of Aliphatic Amine Oxidation by Aqueous $(batho)_2Cu^{II}$ †

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Abstract: The kinetics of oxidation of a large series of aliphatic amines by the "high-potential" oxidant $(batho)_2Cu^{II}$ (batho = 2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonate) was studied under pseudo-first-order conditions (excess amine) in water or in 30% aqueous methanol (v/v) at 25 °C over the pH range 7–11. The oxidations follow bell-shaped pH–rate profiles, with the low-pH leg reflecting the fact that only the free amine base is subject to oxidation and the high-pH leg representing conversion of $(batho)_2Cu^{II}$ to an ineffective oxidant at high pH. The latter is thought to be $(batho)Cu^{II}(OH_2)OH$ on the basis of the observed effect of [batho] on rate at high pH, and curve fitting of the rate data yielded estimates of the unitless K_{eq} values governing this conversion. The variation in rate with degree of N-substitution and other structure–reactivity trends (such as the effect of ring size and the non-rate-retarding effect of 2,4,6-trimethyl substitution on $PhCH_2NR_2$) support a mechanism involving initial outer-sphere one-electron transfer, followed by proton transfer to the solvent, and then a rapid second one-electron oxidation to give imine/iminium product. Inner-sphere coordination of chelating amines shuts down the redox reaction, presumably as a consequence of displacement of the batho ligand(s) needed for high oxidant strength. Deuterium kinetic isotope effect (DKIE) measurements (i) comparing $PhCD_2N(CD_3)_2$ vs $PhCH_2N(CH_3)_2$ (intermolecular DKIE) and (ii) determining N-dealkylation preference in the case of $PhCH_2N(CH_3)CD_2Ph$ (intramolecular DKIE) suggest that the initial electron transfer is mainly rate-limiting. A rate comparison between erythro and threo diastereomers of 1,2-diphenyl-2-piperidinoethanol indicates a stereoelectronic preference for one-electron oxidation at nitrogen when held antiperiplanar to a β-hydroxyl. Stoichiometry studies using an excess of the Cu(II) oxidant reveal regioselective and chemoselective factors governing the overall amine-to-iminium oxidations.

Introduction

Mechanistic diversity in the chemical and enzymatic oxidation of amines is a subject of much current interest. The monooxygenase enzyme cytochrome P-450 achieves oxidative N-dealkylation via hydroxylation at C_α, followed by dissociation of the resulting carbinolamine. A mechanism involving abstraction of a hydrogen atom from C_α by a $(Fe{=}O)^{3+}$ species, followed by $HO^\bullet$ transfer from iron (the "rebound" step) was traditionally considered to rationalize the incorporation of O_2-derived oxygen into the aldehyde product.[1] However, considerations of measured isotope effects and redox potentials,[1-3] and observed suicide inactivation by cyclopropylamines,[4,5] led to a consensus that amine oxidation by cytochrome P-450 (and chemical model systems thereof) involves initial single-electron transfer (SET) to give an aminyl cation radical,[6-8] analogous to electrochemical oxidation of amines.[2] Initial SET is followed by (i) H^+ transfer from C_α and then back-transfer of $HO^\bullet$ from Fe(IV)OH to the resulting C_α radical intermediate or (ii) either H-atom transfer or, more likely, sequential H^+/e^- transfer giving imine/iminium, which is subsequently converted to carbinolamine by addition of the resulting Fe(III)-bound hydroxide.[3] SET oxidation of amines is also described for horseradish peroxidase (HRP),[9,10] which is supposed to be incapable of direct H-atom transfer on account of the inaccessibility of substrates to the "buried" iron center, and thus appears to oxidize "reducing substrates" via their association with the heme edge.[11] Although the C_α primary deuterium kinetic isotope effects for P-450 and HRP oxidation of amines differ considerably,[12,13] these have been rationalized on the basis of recent studies on the acidity of aminyl cation radicals[14] in terms of the presence (P-450) or absence (HRP) of a base to facilitate aminyl deprotonation.[6,8] The flavin-dependent mitochondrial monoamine oxidase (MAO) is also thought to dehydrogenate amines via a stepwise electron/proton/electron transfer mechanism.[15]

Two classes of copper-containing enzymes are known to oxidize amines. The so-called "copper amine oxidases" utilize a covalently bound quinone cofactor to achieve a pyridoxal-like transamination of primary amines to aldehydes, the role of copper being ascribed

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