5-Iminodaunorubicin. Reduced Cardiotoxic Properties in an Antitumor Anthracycline

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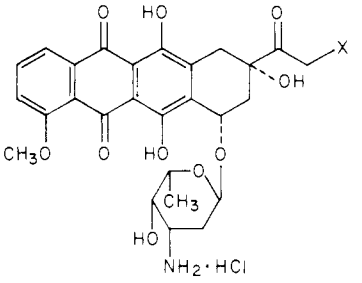
Treatment of daunorubicin with methanolic ammonia affords 5-iminodaunorubicin, the first quinone-modified analogue of either daunorubicin or adriamycin. This product retains antileukemic activity in mice, is less cardiotoxic by electrocardiographic measurements in rats, and is nonmutagenic in *Salmonella typhimurium* (Ames test).

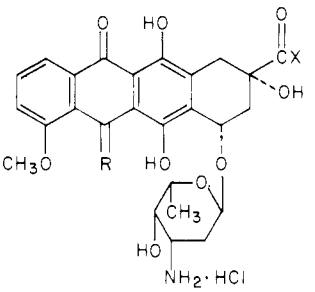
Development of the important anticancer drugs adriamycin (**1**) and daunorubicin (**2**) has brought about



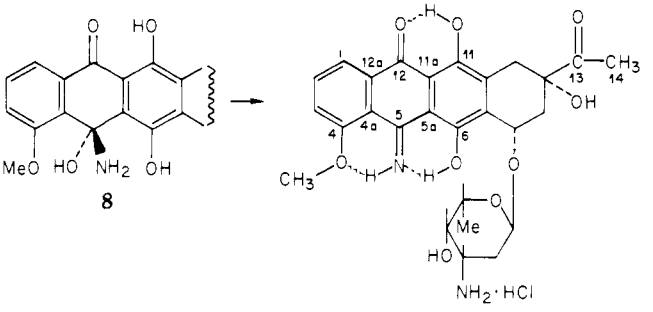
1, X = OH
2, X = H

major advances in the treatment of a variety of solid tumors and leukemias in humans.[1-3] However, these agents produce cardiotoxic side effects that are dose dependent and cumulative, so that dose levels must be limited, and sometimes successful courses of treatment must even be halted to avoid the risk of fatal heart failure.[2,4,5] Perhaps the best solution to this problem would be development of structural analogues in which antitumor efficacy is retained while cardiotoxic effects are minimized, but a rational approach to such analogues requires much more than is presently known about the mechanisms of biological action of **1** and **2**. The interaction of these drugs with DNA is important and has been studied widely,[2,3] particularly the intercalation of **1** and **2** into the helical form of DNA, but various biochemical mechanisms are accessible to these molecules. Several current studies point to the quinone unit of **1** and **2** as a key site of biological action that might involve the redox properties of the quinone function[6-9] or possibly its alkylating potential.[10] Analogues modified at the quinone would be important for further mechanistic studies that might lead to a separation of the antitumor and cardiotoxic effects. Several hundred analogues of **1** and **2** have been synthesized for evaluation of antitumor properties,[3,11] but surprisingly it appears that none has provided a structural change at the quinone function. So far as we are aware, this report is the first of such an analogue.

Chemistry. Derivatization of the quinone was first observed during ammonolysis of the ester **3** (obtained[12] by side-chain degradation of **1**) to form the amide **4**. Thin-layer chromatographic analysis showed that the product **4**, reddish orange like **1** and **2** and other anthracyclines, was accompanied by one or two violet byproducts. The byproducts appeared to arise from ring amination, according to mass spectral and ^1H NMR analyses and a shift in the UV–visible absorption spectrum to longer wavelength (Table I). When the reaction was conducted in cold methanol solution saturated with ammonia, ring amination appeared to be favored, whereas conversion to the amide appeared to be favored in liquid ammonia. The amide **4** was best obtained if ester **3**, as a thin film on the walls of a flask, was exposed to an atmosphere of ammonia at −10 to −15 °C for several days, followed by chromatographic purification. The violet byproducts were not fully purified and characterized, and they were assumed to be the 5-imino ester **5** and the 5-imino amide **6** by analogy with the product (**7**) from



3, R = O; X = OCH_3
4, R = O; X = NH_2
5, R = NH; X = OCH_3
6, R = NH; X = NH_2



8

7

daunorubicin. There was no evidence that the violet byproducts could be hydrolyzed back to the parent quinones nor were they converted with excess 30% hydrogen peroxide at room temperature.

It was of interest to extend the amination to daunorubicin (**2**) because the reaction appeared to give negligible cleavage of the glycosidic bond or other types of degradation. Daunorubicin hydrochloride was best treated with methanolic ammonia at 0–5 °C for 39 h to give a single blue-violet product **7** that was chromatographically purified as the hydrochloride in 57% yield. A minor blue-violet byproduct was observed (≤2%) but not isolated and identified. The selectivity of the amination to produce a single major product seemed surprising at first. Attack by NH_3 was expected to occur at either of the quinone carbonyls to produce two isomeric products. However, there are reasons why amination at C-5 to give the 5-imino derivative **7** might be preferred. First, quaternization of the quinone carbon at the intermediate stage of the amination, as in **8**, should be favored at C-5 since it permits relief of the steric strain from the three peri substituents at C-4, C-5, and C-6. Furthermore, in **7** the imino group at the 5 position can be stabilized by H bonding to the 4-OCH_3 as shown in the structure, as well as by H bonding

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Table I. Comparison of UV-Visible Spectra[a]

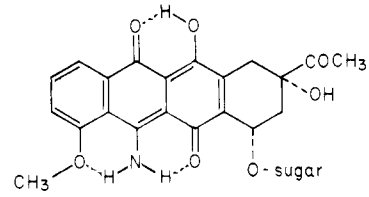
compd	λ_{max}, nm ($\epsilon \times 10^{-3}$), in MeOH
4	233 (38.4), 252 (26.0), 288 (8.87), 478 (12.4), 494 (12.3), 530 (6.64)
crude 5 + 6	220 (26.2), 233 (23.0), 252 (27.7), 303 (6.15), 310 sh, 518 sh, 550 (13.9), 591 (16.1)
2[b]	234 (37.5), 252 (26.1), 290 (8.60), 480 (12.1), 495 (12.3), 532 (6.30)
7	220 (29.8), 233 sh, 252 (32.0), 307 (6.93), 335 sh, 357 sh, 520 sh, 551 (16.6), 592 (19.8)

[a] Obtained on Cary 11 or Cary 14 recording spectrometers. [b] Data from G. Cassinelli and P. Orezzi, *G. Microbiol.*, 11, 167 (1963).

of the 6-OH to N. A precedent for this type of stabilization is found in the observation[13] that treating 1-methoxyanthraquinone with methanolic ammonia produced in high yield a single quinone imine which was weakly H bonded to the methoxyl, whereas 2-methoxyanthraquinone did not form an imine even at 100 °C.

The elemental analysis of product **7** confirmed that a second N had been introduced into the molecule. A shift in UV–visible absorption to longer wavelengths in going from **2** to **7** is shown in Table I and corresponds to the shift from **4** to **5** and **6**. Data in support of structure **7** as 5-iminodaunorubicin were observed in the NMR spectra, which are reported in Table II. The ^{1}H NMR spectrum of **7** in dimethyl-d_6 sulfoxide (Me_2SO) solution showed two H-bonded protons at δ 13.47 and 9.54. Both signals were broad singlets, unlike the sharp singlets typical of the H-bonded phenolic OH's in anthracyclines such as **1** and **2**. Clearly the proton at δ 9.54 was only weakly H bonded, but as is characteristic of H bonding both protons kept the same chemical shift and signal broadening in all samples and Me_2SO solutions of **7**. Addition of a small amount (0.2 molar equiv) of trifluoroacetic acid[14] to the ^{1}H NMR solution did not produce sharpening of these signals as we expected, but it did cause the appearance of a third proton as a very sharp singlet, strongly H bonded at δ 15.80. Sharpening of the other two protons was achieved only by irradiation, first at δ 13.47 and then at δ 9.54, showing that these protons were coupled and thus indicating that both were H bonded about the N. The only arrangement of atoms in this product that can accommodate three H bonds is structure **7**, having the imino group at C-5. If alternatively the imino group had been introduced at C-12, only two of the three protons could be H bonded. Therefore, we have assigned the 5-NH to the signal at δ 9.54 (weak H bond to 4-OCH_3), the 6-OH to δ 13.47 (normal H bond to N), and the 11-OH to δ 15.80 (strong H bond to C=O). The stronger nature of the H bond to the C-12 carbonyl in **7** is attributed to the effect at C-12 of the more electron-releasing imine at C-5 (relative to the carbonyl at C-5 in **2**). By similar reasoning we assign the stronger H bond in **2** to the 6-OH, based on the electron-releasing effect of the 4-methoxyl on the carbonyl at C-5. It appears that the phenolic OH's in **1** and **2** have not been individually assigned in previous literature, and this no doubt reflects the lack of study of quinone modifications.

The ^{13}C NMR data supplement the above observations. In particular, placement of the imino function on one of the quinone carbons was clearly confirmed. In **2** the quinone carbons were indistinguishable at δ 185.9 and 186.1, but on going to **7** they were shifted strongly upfield and became well separated at δ 172.7 and 179.3. The shift to δ 172.7 is the largest for any carbon and clearly involves the site of amination, although it does not permit a choice between C-5 and C-12 that would confirm the assignment based on ^{1}H NMR data. The site of amination might independently be defined from the changes at C-4a and C-12a, since these sites were already distinguished in the starting material **2**. Indeed, the larger upfield shift at C-4a (3.3 ppm) than at C-12a (1.9 ppm) in going from **2** to **7** does support the assignment of **7** as the 5-imino compound, but the effects were not large enough for this to be used as an independent criterion. Other changes in the ^{13}C NMR spectra were also small, except at C-6 and C-6a where appreciable downfield shifts occurred. Perhaps this can be attributed to the existence in some proportion of the tautomer **7a** with a carbonyl at C-6 and an amino group



7a

at C-5. Also, the observed coupling of the protons about the N might be better explained in the tautomer **7a** than in structure **7**. All of the data are consistently explained only by the 5-imino structure **7** or the tautomer **7a**. More detailed NMR studies of **7** that confirm these assignments and explore the substituent effects and tautomerism of the aglycon will be the subject of a separate report.

It is possible that the presence of the adjacent methoxyl group and the resultant arrangement of hydrogen bonds in structure **7** are essential for the formation of **7** and for

Table II. Comparison of NMR Data

compd	^{1}H NMR[a] chemical shift, δ (Me_2SO-d_6 soln, Me_4Si internal ref)[e]										
	H-1	H-2	H-3	4-OCH_3	$COCH_3$	H-1′	H-5′	5′-CH_3	6-OH	11-OH	=NH
2	7.83, m	7.59, m	7.83, m	3.96, s	2.30, s	5.29, br s	4.22,[b] q	1.17,[b] d	14.05, s	13.26, s	
7	7.95, m	7.79, m	7.58, m	4.10, s	2.31, s	5.47, br s	4.20,[c] m	1.19,[b] d	13.47, br s	15.80,[d] s	9.54, br s

compd	^{13}C NMR[a] chemical shift, δ (D_2O soln, dioxane internal ref)																
	C-1	C-2	C-3	C-4	4-OCH_3	C-4a	C-12a	C-5	C-12	C-5a	C-11a	C-6	C-11	C-6a	C-10a	C-13	C-14	5′-CH_3
2	120.0 or 120.1	137.2	120.1 or 120.0	161.0	57.1	119.1	134.0	185.9 or 186.1	186.1 or 185.9	110.9	110.9	156.4	154.7	134.9 or 135.0	135.0 or 134.9	215.8	25.1	16.6
7	119.7	134.3	117.1	159.8	57.2	115.8	132.1	172.7	179.3	103.9	109.4	160.9	155.4	141.2	135.4	216.0	25.2	16.6
diff						3.3	1.9	13.2	6.8			3.5		6.1				

[a] Varian XL-100 spectrometer. Shift assignments are based on proton noise decoupling and on previous assignments by A. Arnone et al.[14] and L. H. Wright, J. A. Chan, J. A. Schroer, and A. A. Aszalos, *J. Org. Chem.*, 42, 2344 (1977). [b] $J_{5',6}$ = 6-7 Hz. [c] Quartet obscured. [d] Sharp singlet appeared after adding trifluoroacetic acid to the solution. [e] m = multiplet, br = broad, s = singlet, d = doublet, q = quartet.

Table III. Comparison of Biological Test Data

compd	act. vs. leukemia P388 in mice:[a] % T/C (dose, mg/kg) qd 1-9	act. vs. leukemia P388 in mice:[a] % T/C (dose, mg/kg) q4d 5, 9, 13	cardiotoxicity in rats:[b] min cumulative cardiotoxic dose, mg/kg	mutagenicity in *S.t.*:[c] TA 98, revertants/nmol	isolated helical DNA in soln:[d] ΔT_m, C	inhibn of synth[e] in leukemia L1210 cells: ED_{50}, μM DNA	ED_{50}, μM RNA
1	176 ± 72 (1.56)	120 ± 12 (16.0)	11		13.4	1.5	0.7
	197 ± 26 (0.78)	159 ± 20 (8.0)					
	174 ± 14 (0.39)	146 ± 15 (4.0)					
	160 ± 7 (0.20)	130 ± 13 (2.0)					
2	148 ± 35 (1.56)	126 ± 12 (16.0)	14	100 ± 30	11.2	1.0	0.3
	160 ± 27 (0.78)	132 ± 4 (8.0)					
	153 ± 9 (0.39)	128 ± 12 (4.0)					
	144 ± 9 (0.20)	126 ± 15 (2.0)					
7	153 ± 3 (4.0)	126 ± 3 (24.0)	64	1	6.25	1.6	1.3
	170 ± 15 (2.0)	116 ± 14 (12.0)					
	164 ± 11 (1.0)	124 ± 1 (6.0)					
	145 ± 8 (0.5)	130 ± 6 (3.0)					
		126 ± 8 (1.5)					

[a] Reference 15. Mice injected ip with leukemia cells on day 0 were treated ip on days 1-9 or on days 5, 9, and 13 with the specified dose. Activity is defined as an increase in survival time of (treated mice)/(control mice) (% T/C) ≥ 125. The T/C data for 1 and 2 are averages of eight tests (qd 1-9) or 27 tests (q4d 5, 9, and 13). Data for 7 are from duplicate tests. [b] Reference 16. The cumulative dose is the minimum required to produce significant electrocardiographic changes; note that the present results are in mg/kg rather than mg/m² as in ref 16, Table 4. [c] Reference 17. *S.t.* = *S. typhimurium*. Reported mutagenicities of 1 and 2 were 108 and 356 revertants/nmol, respectively, in ref 17a. The observed values from four determinations on 7 were from 0 to 2 revertants/nmol, with and without microsomal activation. Doses up to a maximum dose of 32 μg/plate showed no toxicity to the cells, whereas 2 was toxic above 8 μg/plate. In tester strain TA100, 2 was but weakly mutagenic (0.5-2 revertants/nmol) and 7 showed no effect. [d] The increase in thermal denaturation temperature was measured as in ref 12 except that the 0.010 M phosphate buffer was at pH 7.0 and contained EDTA at 10^{-3} M plus 5% Me_2SO to solubilize the compounds. [e] Procedure the same as that given in ref 12, except for addition of 1% Me_2SO. We are indebted to D. L. Taylor and staff at SRI for these and the ΔT_m data.

the stability of the imino group. There has been no evidence that the imino group is readily hydrolyzed, so that it is highly doubtful that any of the properties of 7 can be explained by its hydrolysis to 2. For example, even when 7 was treated with 0.2 N hydrochloric acid at room temperature for 1 month to hydrolyze the glycosidic link and give the 5-imino aglycon (≥80% yield), little daunomycinone (≤10%) was formed by hydrolysis of the imine. Further synthesis of imino compounds is planned, including examples with and without the adjacent methoxyl.

Biology. Amide 4, despite degradation of the side chain of 1 and 2, showed good activity against transplanted tumors in mice,[15] as measured by the increase in survival time of treated animals compared to controls (% T/C). Against P388 lymphocytic leukemia, T/C was 151%, and against B16 melanoma, it was 161% (single tests). In both screens the optimum dose was 6.25 mg/kg in a schedule of nine daily doses (qd 1-9). This was an increase in both potency and efficacy relative to 3.[12]

In Table III the test data for 5-iminodaunorubicin (7) are compared with data for 1 and 2. Leukemia P388 in mice is considered the in vivo screen[15] most predictive of anticancer activity for human therapy. The test is made more stringent if the leukemia is allowed to progress for 4 days after implantation and administration of the test compound is delayed until the 5th day (q4d 5, 9, and 13 instead of qd 1-9). The data show that in both dose schedules 7 retains the antitumor activity of the parent 2, with minor variation in efficacy (% T/C) and potency (dose required for best % T/C), and approaches the activity of 1. The imino compound 7 was also active against B16 melanoma in mice when administered in nine daily doses (T/C = 222 ± 17% at an optimum dose of 2 mg/kg) or in a single dose on day 1 (T/C = 173 ± 8% at 6.25 mg/kg), but not if the single dose was delayed to day 5 (T/C = 122 ± 2% at 12.5 mg/kg).

Data from the Zbinden cardiotoxicity test[16] in rats show that the dose of 7 must be increased 4.5-fold relative to 2 or sixfold relative to 1 in order to produce a comparable effect in the heart. Cardiotoxicity is thus correspondingly reduced, and this is a highly encouraging indication toward a solution of this serious toxicity problem. Comparison of data from two species is difficult, but the reduction in rat cardiotoxicity is especially encouraging relative to the retention of antileukemic potency in the mouse. How well these data predict for human treatment is not established as yet, but the Zbinden test is the best short-term test for cardiotoxicity currently in routine operation, and further tests of 7 are in progress at the National Cancer Institute.

Data from several in vitro tests indicate an alteration in the DNA interactive properties of 7 that may be significant to its mechanism of action. In particular, it was unexpected that 5-iminodaunorubicin (7) would show almost complete loss of mutagenicity to *Salmonella typhimurium* in the Ames test,[17] which is a useful predictive test for carcinogenicity in humans. The parent anthracyclines (1 and 2) are potent mutagens in several test systems and are carcinogenic in animals.[15a,18-20] The carcinogenicity common to most anticancer drugs is a subject of current interest.[21,22] Though not a concern like cardiotoxicity or other short-term toxic effects in human therapy, carcinogenicity does become a consideration as cancer treatment is improved to provide long-term survivals. How well the nonmutagenicity of 7 in salmonella strains will predict for human therapy is, again, not established. This compound was found to be about as mutagenic as 1, though less mutagenic than 2, when tested in V79 Chinese hamster cells,[23] which suggests that some process for transformation to a mutagen may be present in mammalian cells that is absent in bacteria. In any case, nonmutagenicity of 7 in the Ames test at least indicates the loss of some form of interaction with DNA that is brought about by the minor modification in structure at

the quinone. Whether this is related to the reduced cardiotoxicity in the Zbinden test may be an interesting subject for further study.

Table III presents data from other in vitro tests of interaction with DNA. The increase (ΔT_m) in thermal denaturation temperature[12] of helical DNA in the presence of the test compound is a measure of its stabilization of the helical form of DNA, presumably by intercalative binding. This effect is significantly weaker for **7**. On the other hand, **7** retains the potency of **1** and **2** as an inhibitor of DNA and RNA synthesis[12] in L1210 lymphoid leukemia.

Thus, apart from its potential as an anthracycline anticancer agent with reduced cardiotoxicity, **7** is a useful subject for mechanism of action studies, and its properties suggest to us that hitherto overlooked modifications of the quinone should continue to be explored.

Experimental Section

Solutions were evaporated in vacuo using a rotary evaporator. Melting points are uncorrected. IR spectra were taken on a Perkin-Elmer 137 spectrometer. Mass spectra were recorded on an LKB Model 9000 spectrometer at 12 eV. The R_f values are from thin-layer chromatography on silica gel GF 250-μm plates (Analtech).

5-Iminodaunorubicin (7). To a stirred solution of 1700 mL of methanolic ammonia (saturated at 0 °C) in an ice bath was added a solution of 28.8 g (51.0 mmol) of daunorubicin hydrochloride in 500 mL of methanol. The cold solution was stirred briefly (1 h), stored at 0–5 °C for 39 h, and evaporated. To remove traces of ammonia, the violet residue was thrice dissolved in 400 mL of $CHCl_3$–CH_3OH (4:1) and the solution evaporated, yielding 33.9 g: R_f 0.12 (violet, trace impurity), 0.14 (violet, **7**), 0.21 (orange, trace **2**), and 0.71, 0.75 (magenta, trace aglycons) in $CHCl_3$–CH_3OH–H_2O (40:10:1). The product was isolated by chromatography of the residue in two equal batches. A solution of 16.9 g in 200 mL of $CHCl_3$–CH_3OH (6:1) was added to a column (6.7 × 86 cm) of silica gel (200–325 mesh, Mallinckrodt SilicAR CC-7), which was eluted with the same solvent. After collection (100-mL fractions, monitored by TLC) of 13.7 L of initial eluate, a 5.4-L fraction was evaporated to yield 9.67 g, which was precipitated by dissolving in 100 mL of $CHCl_3$–CH_3OH (4:1) and adding 400 mL of ether dropwise. The precipitate was washed with ether and dried in vacuo at 25 °C to yield 8.79 g of **7** as a homogeneous blue-violet powder: mp 175–178 °C dec (preheated to 160 °C); IR (Nujol) 2.92 (OH), 5.82 (C=O), 6.31 μm (C=O of chelated quinone, C=N). The combined yield was 16.8 g (57%). Anal. ($C_{27}H_{30}N_2O_9$·HCl·H_2O) C, H, Cl, N.

(7*S*,9*S*)-7-[(3-Amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,9,11-trihydroxy-4-methoxy-5,12-dioxonaphthacene-9-carboxamide Hydrochloride (4). A solution of 0.478 g (0.88 mmol) of the ester **3** as free base (isolated before conversion to the HCl salt[12]) and 0.054 g (1.0 mmol) of NH_4Cl was evaporated to leave a film on the walls of a 1-L flask. (The NH_4Cl is required for disproportionation to give the evaporated product as the HCl salt; alternatively, NH_4Cl may be omitted if the HCl salt of **3** is used.) The flask was cooled to −60 °C and 150 mL of ammonia was condensed into it with stirring. The solution was allowed to evaporate while the flask was rotated at room temperature to leave a violet residue on the wall under an atmosphere of ammonia. The flask was stoppered immediately, stored at −10 to −15 °C for 6 days, and flushed with N_2. The residue was dissolved several times in $CHCl_3$–CH_3OH (1:1), and the solution was evaporated to afford an amber residue, 0.55 g [R_f 0.17 (violet) and 0.25 (4, reddish orange) in $CHCl_3$–CH_3OH–H_2O (20:10:1)]. A solution in 40 mL of $CHCl_3$–CH_3OH (1:1) was added to a column (2 × 42 cm) of 200–325 mesh silica gel (50 g, Bio-Sil A), which was eluted with $CHCl_3$–CH_3OH–H_2O (40:10:1). An initial 100 mL of eluate was collected. The reddish orange product was then collected in a 200-mL fraction, which was evaporated. The residue was dissolved in 15 mL of $CHCl_3$–CH_3OH (1:1) and 2 mL of 0.1 M HCl in CH_3OH (some dissociation to the free base appeared to occur during chromatography) and reprecipitated with 20 mL of ether to yield 0.307 g (59%) of **4**: mp 189–192 °C dec; IR (Nujol) 2.90 (OH), 6.00 (amide C=O), 6.20, 6.31 μm (C=O, chelated quinone); MS [as the $(Me_3Si)_6$ derivative] m/e 945 (M − CH_3), 930 (M − $2CH_3$). Anal. ($C_{28}H_{28}N_2O_{10}$·HCl·$1.5H_2O$) C, H, Cl, N.

When **4** was treated with methanolic ammonia, as described for **7**, the product was a mixture of **5** and **6** containing <5% of **4**, according to MS analysis of the $(Me_3Si)_6$ derivatives: m/e 944 [$(Me_3Si)_6$ of **6** − CH_3], 887 [$(Me_3Si)_5$ of **5** − CH_3]; R_f 0.15 (**6**, violet), 0.25 (**4**), 0.33 (**5**, violet) in $CHCl_3$–CH_3OH–H_2O (20:10:1).

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