N-(5,5-Diacetoxypent-1-yl)doxorubicin: A New Intensely Potent Doxorubicin Analogue[1]

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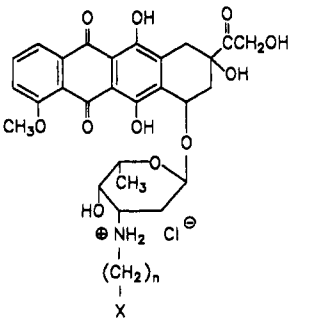
N-(5,5-Diacetoxypent-1-yl)doxorubicin (DAPDOX) (**3**), a new, water-soluble analogue of doxorubicin, has been synthesized by coupling doxorubicin with 5-oxopentane-1,1-diacetate in the presence of $NaBH_3CN$. This analogue was designed to be converted to the corresponding aldehyde, *N*-(5-oxopent-1-yl)doxorubicin, in the presence of carboxylate hydrolases, enzymes that are ubiquitous in tissue. DAPDOX had a half-life of several days in 0.05 M phosphate or 0.05 M acetate buffer solution at pH 4.0. However, in 0.05 M phosphate buffer at pH 7.4 in the presence of 20 unit equiv of porcine liver carboxylate esterase, the half-life of DAPDOX was less than 1 min. *N*-(5-acetoxypent-1-yl)doxorubicin (**4**), which should give rise to *N*-(5-hydroxypent-1-yl)doxorubicin on esterase-mediated hydrolysis, and *N*-(pent-1-yl)doxorubicin (**5**), were also prepared for comparative biological studies. DAPDOX was 150 times more potent than doxorubicin at inhibiting the growth of Chinese hamster ovary (CHO) cells in culture. The compound retained the same degree of potency against a CHO subline 100-fold resistant to doxorubicin (CHO/DOX) that expressed elevated levels of P-glycoprotein. Compounds **4** and **5**, on the other hand, were no more effective than doxorubicin at inhibiting the growth of CHO cells and were 4–7-fold less potent against the CHO/DOX subline. DAPDOX is representative of a new structural class of doxorubicin analogues with unique chemical and biological properties.

The anthracycline antibiotic doxorubicin is effective in the palliative management of a wide variety of human malignancies.[2] However, its clinical utility is limited by dose-dependent cardiomyopathy and by innate and acquired drug resistance. Numerous analogues have been synthesized to overcome these problems, but, so far, none have gained an established clinical role.[3-5] In an effort to develop analogues effective against multidrug-resistant tumor cells that express increased levels of P-glycoprotein (the mdr1 phenotype), we recently reported several doxorubicin analogues, **1**, bearing an alkylating or latent alkylating group on the 3′-amino position of the daunosamine moiety.[6] These analogues were designed on the premise that the alkylating group might bind covalently to nucleophilic groups at or near the anthracycline intercalation (or binding) site, thus preventing drug egress from the cell. Although these alkylating anthracyclines were nearly equally effective at inhibiting the growth of matched pairs of doxorubicin-sensitive and doxorubicin-resistant tumor cells, they were 10–50-fold less potent than the parent compound. Moreover, they were poorly soluble in aqueous media, a property undesirable for clinical development. The reduced potency and limited water solubility of these compounds appeared due to the loss of the basic character of the 3′-amino group of doxorubicin consequent upon its conversion to 3′-amido and 3′-ureido groups in the alkylating derivatives. Thus, it has been reported that doxorubicin analogues bearing *N*-acyl substituents are considerably less potent than those bearing a free amino group[7-15] and show reduced DNA binding affinity.[14,15] These changes have been attributed to the loss of electrostatic binding energy between the protonated amino group and the negatively charged polynucleotide phosphate groups;[7,11,14,16] however, this interpretation must be viewed with caution because recent X-ray crystallographic studies[17] of anthracycline–oligodeoxynucleotide complexes indicate that the amino group does not interact with internucleoside phosphate groups.

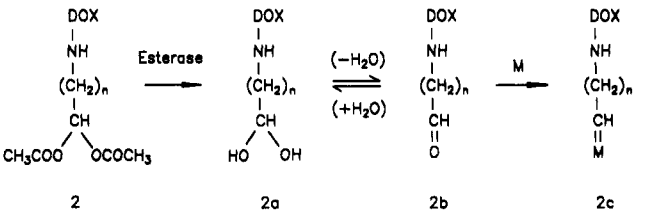
To obtain alkylating doxorubicin analogues with increased potency and enhanced water solubility, we decided to prepare compounds in which the basicity of the 3′-amino group was preserved. Our strategy was to link the amino group through a polymethylene bridge to a chemically reactive functional group, X, to form compounds of the general structure **1** (Chart I). A review of functional groups known to react with nucleophilic centers in bio-

Chart I



1, X = CHO, n = variable
2, X = $CH(OCOCH_3)_2$, n = variable
3, X = $CH(OCOCH_3)_2$, n = 4
4, X = CH_2OCOCH_3, n = 4
5, X = CH_3, n = 4

Scheme I



Where DOX is the 3′-deaminodoxorubicin residue, and M represents a cellular macromolecule such as DNA

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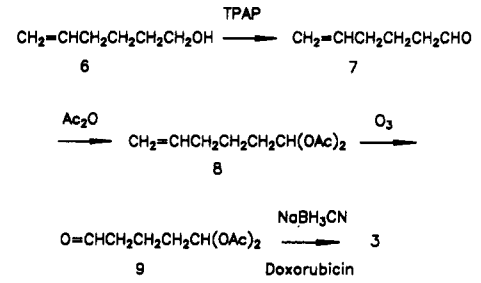
Scheme II



logical macromolecules suggested the use of the aldehyde group. Aldehydes are inherently chemically reactive and readily form addition or condensation products with amino or sulfhydryl groups. The preparation of structures such as **1**, however, pose a formidable synthetic challenge because of the multiple functional groups present in the doxorubicin molecule. To overcome this problem, we decided to introduce the aldehyde group into the side chain in latentiated form. For this purpose we selected the bis(acetoxy) acetal group, a biologically-reversible aldehyde protective group we recently developed to prepare stable precursors of aldophosphamide.[18] Our target compounds, therefore, were bis(acetoxy) acetals of the general structure **2**.

Table I. The Half-lives of Compound **3**[a] at 25 °C in Aqueous Buffers in the Absence or Presence of Pig Liver Carboxylate Esterase[b]

	aqueous conditions	half-life
A	0.05 M acetate buffer, pH 4.0	>10 d
B	0.05 M phosphate buffer, pH 4.0	97 h
C	0.05 M phosphate buffer, pH 7.4	24 h
D	C[c] + esterase (2-fold unit equiv)	27 min
E	C + esterase (5-fold unit equiv)	4 min
F	C + esterase (10-fold unit equiv)	1 min
G	C + esterase (20-fold unit equiv)	20 s

[a] The initial concentration of **3** was 2×10^{-5} M. [b] Two unit equiv of the enzyme per μmole of compound were used, where 1 unit equiv is defined as the amount of enzyme that will hydrolyze 1.0 μmol of ethyl butyrate to butyric acid and ethanol per minute at pH 8.0 at 25 °C. The disappearance of **3** from solution was monitored by HPLC (See Experimental Section). [c] Aqueous condition C.

Table II. Growth Inhibition of Chinese Hamster Ovary (CHO) Cells and a Subline (CHO/DOX) with Acquired Resistance to Doxorubicin[22] in Vitro by Doxorubicin Analogues[a]

	IC_{50}[b,c] (10^{-10} M)	
compd	CHO	CHO/DOX
doxorubicin	600	60000
3	4	7
4	830	3500
5	570	4200

[a] Exponentially growing cells were exposed to varying drug concentrations for 6 days at 37 °C. The cells were then fixed with formaldehyde, stained with crystal violet, and counted under an inverted phase-contrast microscope. [b] The drug concentration that inhibited cell growth by 50% compared to untreated control cultures. [c] At least 7 drug concentrations ranging from 10^{-12} M to 10^{-5} M were used to determine the IC_{50} values. Each value is the average of duplicate determinations.

The mechanism of reversion of **2** to the corresponding free aldehydes is shown in Scheme I. In the presence of carboxylate esterases (EC 3.1.1.1, carboxylate ester hydrolases), enzymes which are ubiquitous in tissue and show low substrate specificity,[19,20] **2** can be hydrolyzed to the corresponding aldehyde hydrate, **2a**. Elimination of water from **2a** generates the free aldehyde, **2b**, which can theoretically react with a nucleophile proximate to, or within, the DNA-binding site to form a covalent drug–DNA adduct, **2c**.

N-(5,5-diacetoxypent-1-yl)doxorubicin (DAPDOX) (**3**) was selected to investigate this approach. For comparative biological studies, we also prepared the *N*-(5-acetoxypent-1-yl) derivative, **4**, which gives rise to an alcohol upon ester group hydrolysis, and the *N*-(pent-1-yl) derivative, **5**.

Chemistry

The synthetic route to **3** is shown in Scheme II. 6-Hexen-1-ol (**6**) was oxidized to the corresponding aldehyde, **7**, in excellent yield with tetrapropylammonium perruthenate (TPAP) in CH_2Cl_2. The aldehyde could also be prepared, although less conveniently, by oxidation of **6** with the Jones reagent (CrO_3 in H_2SO_4) or with pyridinium chlorochromate. Reaction of **6** with acetic anhydride in ether afforded the bis(acetoxy) acetal, **8**, which was oxi-

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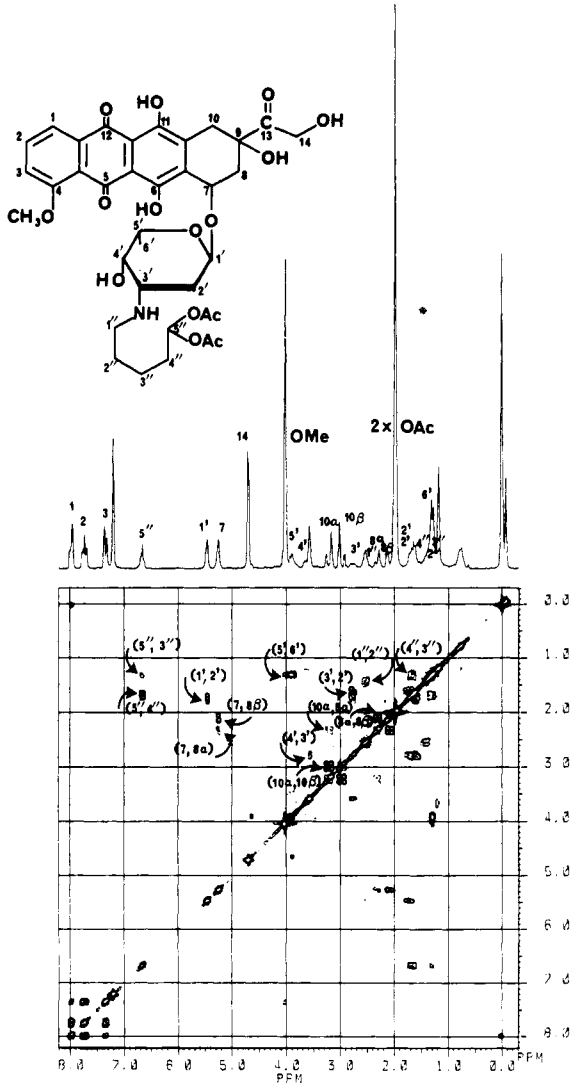

Figure 1. Contour plot showing the COSY spectrum of *N*-(5,5-diacetoxypent-1-yl)doxorubicin (**3**). The spectrum was taken as 256 × 1K complex points and was processed using sinusoidal multiplication prior to both Fourier transforms. The final data matrices, consisting of 512 × 512 real points, were symmetrized prior to plotting. The proton identities are labeled on the high resolution reference spectrum plotted above the contour plot.

dized to the dialdehyde monoacetal, **9**, by treatment with ozone. Condensation of **9** with doxorubicin in the presence of $NaBH_3CN$ afforded *N*-(5,5-diacetoxypent-1-yl)doxorubicin in 64% yield. The HCl salt, **3**, was formed by careful neutralization of a suspension of the free base in water with hydrochloric acid, and lyophilization of the resultant clear solution to dryness. The *N*-(5-acetoxypent-1-yl) and *N*-pent-1-yl analogues, **4** and **5**, were prepared similarly by condensation of doxorubicin with 5-acetoxypentanal and pentanal, respectively.

The structure of **3** was apparent from its ^1H and ^{13}C NMR spectra. The aliphatic component of the ^1H NMR spectrum at 200 MHz was subdivisible into three essentially isolated spin systems. Prominent constituents of these were the H-5″ proton at 6.73 ppm, the H-1′ proton of the daunosamine sugar at 5.62 ppm, and the H-7 proton of the anthracycline at 5.3 ppm. From the COSY spectrum (Figure 1), it was evident that the H-5″ methine proton was correlated to the H-4″ methylene protons at 1.76 ppm. In turn, the H-4″ protons were correlated with a pair of methylene resonances at 1.3–1.4 ppm, which were assigned to the H-2″ and H-3″ protons. Finally, the H-2″ protons were correlated with the H-1″ methylene protons at 2.65 ppm. The assignment of the daunosamine sugar resonances were also evident from the COSY spectrum. Thus, the H-1′ proton was correlated with a resonance signal at 1.82 ppm, clearly attributable to the two H-2′ methylene protons. The latter were further correlated to the H-3′ methine proton at 2.85 ppm. Since the C-6′ methyl group gave rise to a characteristic doublet at 1.35 ppm, the assignment of the resonances at 3.82 and 3.62 ppm to the H-5′ and H-4′ protons follow. Further evidence for the assigned structure derives from the ^{13}C NMR spectrum (see Experimental Section) and the high resolution mass spectrum which indicated an empirical formula of $C_{36}H_{44}NO_{15}$.

Results

Stability Studies. Compounds **3**, **4**, and **5** were readily soluble in water up to 10 mg/mL, the highest concentration tested. Stability studies on **3** are summarized in Table I. The compound was fairly stable in aqueous media, particularly in acetate buffer, pH 4.0, where the half-life exceeded 10 days. In the presence of carboxylate esterase, however, it was rapidly degraded;[21] the half-life with 20 units of esterase per μmole of **3** was only 20 s.

Growth Inhibition Studies. The effects of compounds **3**, **4**, and **5** at inhibiting the growth of Chinese hamster ovary (CHO) cells and a subline with 100-fold resistance to doxorubicin (CHO/DOX) are shown in Table II. The CHO/DOX cells express elevated levels of gp 170 and are cross-resistant with several other antitumor agents including vincristine and vinblastine.[22] Compound **3** was 150-fold more potent than doxorubicin at inhibiting the growth of the CHO cells. Compounds **4** and **5**, on the other hand, did not differ significantly from doxorubicin in their growth-inhibitory potency. Of particular interest, compound **3** was nearly as effective as inhibiting the growth of CHO/DOX cells as the parental cell line. Compounds **4** and **5**, by comparison, were 5–7-fold less potent against CHO/DOX than CHO. However, these compounds were more effective against the resistant cell line than doxorubicin.

Discussion

DAPDOX, one of the new doxorubicin analogues reported here, possesses a number of unique chemical and biological characteristics: It is highly water soluble, it is fairly stable in aqueous media under slightly acidic conditions, it is intensely potent, and it is not cross resistant with doxorubicin against a cell line that expresses elevated levels of P-glycoprotein. No other doxorubicin analogue reported to date combines all of these favorable properties. DAPDOX was designed to be hydrolyzed to the corresponding aldehyde **13** (Scheme I) in the presence of carboxylate esterase. Although we have no evidence, at present, that such an aldehyde intermediate is formed, the stability of DAPDOX in aqueous media and the facility with which it is hydrolyzed in the presence of esterase strongly supports this metabolic pathway. Using ^{31}P NMR spectrometry, we have previously shown that aldo-

(21) A referee has pointed out that the half-life of **3** was not strictly proportional to the enzyme concentration. The most likely reason for this is that the enzyme is partially deactivated by interaction with **3** at low enzyme/drug ratio, an effect that should be progressively less evident at increasing enzyme/drug ratio.

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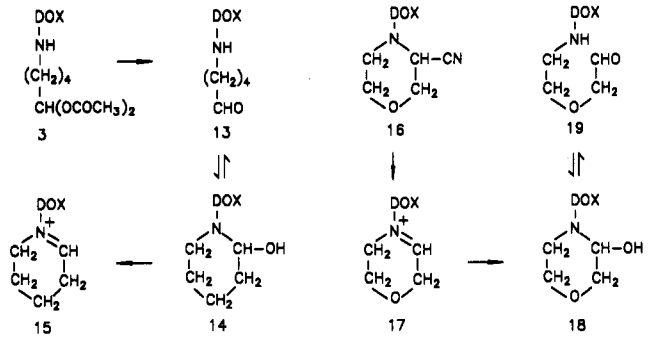
Scheme III



phosphamide acetal diacetate, another geminal bis(acetoxy) compound, is rapidly converted to aldophosphamide in the presence of carboxylate esterase.[18,23] Further circumstantial evidence in favor of the presumed activation pathway is that the structurally related monoacetoxypentyl derivative, **4**, which would give rise to an alcohol upon cleavage of the acetoxy group, was no more effective than doxorubicin at inhibiting the growth of CHO cells and was considerably less active against CHO/DOX cells. If, as seems likely, aldehyde **13** is formed as an intermediate in the activation of DAPDOX (Scheme III), it probably exists in tautomeric equilibrium with the cyclic carbinolamine, **14**. Elimination of hydroxyl anion from **14** would then yield the corresponding iminium analogue, **15**.

The only doxorubicin analogues similarly potent to DAPDOX are a series of α-cyanomorpholino compounds synthesized by Acton et al.,[24-26] as typified by MRA-CN (**16**) (Scheme III), and the barminomycins,[27-29] a family of structurally related daunomycin antibiotics isolated from culture broths of *Streptomyces peucetius*. Consideration of the structures of DAPDOX, the cyanomorpholino derivatives, and the barminomycins suggests that all three classes of compound might give rise to similar reaction intermediates. Thus, Acton and colleagues have hypothesized[25,26] that MRA-CN (**16**) dissociates in solution to give the iminium intermediate, **17**, which can react with biologic nucleophiles. These workers also suggested that the iminium ion could undergo hydration to form the carbinolamine, **18**; the latter presumably exists in tautomeric equilibrium with the acyclic aldehyde, **19**. Barminomycin I exists in solution as a cyclic carbinolamine, and can theoretically ring-open to give an aldehyde tautomer. It also facilely loses the elements of water in solution to form the corresponding imine.[27-29] The presence in all three classes of compound of a substituent group in the sugar moiety capable of existing as an acyclic aldehyde/cyclic carbinolamine tautomeric pair suggests that this structural feature is an important determinant of cytocidal potency. The underlying biochemical mechanisms responsible for this potency have not been clearly established. However, several investigators have shown[30-34] that DNA–DNA crosslinks are formed in cells treated with MRA-CN, and this mechanism appears to be contribute significantly to the marked potency of the compound. We have previously reported[35] that DAPDOX produces DNA–DNA crosslinks in HL-60 cells and a subline resistant to doxorubicin because of altered topoisomerase II (HL-60/AMSA). The precise nature of these crosslinks, however, remains to be established. From mechanistic considerations, it is conceivable that primarily nucleophilic addition occurs at the iminium carbon atom to yield an α-substituted cyclic amine. In this context, Gao et al. have recently reported[36] that, in the presence of formaldehyde, a daunorubicin analogue forms an adduct with a DNA duplex hexamer by covalent binding of the sugar 3′-amino group through a methylene bridge to the 2-amino group of a guanine residue. Similar binding is theoretically possible for the putative cyclic iminium intermediates derived from DAPDOX, MRA-CN, and the barminomycins. However, it is also conceivable that the open-chain N-alkanal group reacts directly with nucleophiles more distal from the DNA binding site.

One advantage of DAPDOX over MRA-CN is that it is highly water soluble, a property which stems from its ionic (hydrochloride salt) nature. Moreover, it is stable in aqueous solution at slightly acidic pH, and thus possesses excellent pharmaceutical characteristics. DAPDOX can also be synthesized from doxorubicin in good yield.

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MRA-CN, by comparison, is formed as a byproduct of reaction in comparatively low yield.

The intense potency of DAPDOX suggests that structurally related compounds might possess comparable biological properties. Indeed, if DAPDOX, the cyanomorpholino anthracyclines, and the barminomycins all have a similar mechanism of action, this new class of doxorubicin analogues should be extremely versatile tools for structure–activity relationship and mechanistic studies. For example, it should be possible to prepare a series of structurally related bis(acyloxy) acetals to answer questions such as the following: How does the chain length and linker composition of the 3′-amino substituent influence drug potency? Are substituents bearing a terminal aldehyde group necessary for intense potency? Is there a marked difference in potency between analogues which can and cannot form cyclic carbinolamines (and, therefore, cyclic iminium intermediates)? Does loss of the basicity of the 3′-nitrogen atom reduce activity?

Finally, since the new analogues are designed to be activated by carboxylate esterases, it should be possible to change the rate of enzymic hydrolysis by altering the composition of the acyloxy groups, and, therefore, the rate at which the putative aldehyde intermediates are formed. The effect of such structural changes on drug potency and antitumor efficacy is of keen interest. It should also be possible, by judicious selection of the acyloxy groups, to modify the lipophilicity and aqueous solubility of the analogues to provide compounds with preselected physicochemical characteristics. Studies addressing these considerations are underway.

Experimental Section

Nuclear magnetic resonance (NMR) spectra (^1H and ^{13}C) were recorded at ambient temperature on an IBM-Bruker Model NR/200 AF spectrometer in the Fourier transform mode in $CDCl_3$ with tetramethylsilane as an internal reference. Chemical shifts (δ) are reported in parts per million (ppm) and coupling constants (J) in hertz. Special NMR techniques used for structural assignment included off-resonance decoupling, selective heteronuclear decoupling, homonuclear shift-correlated 2D-NMR (COSY), homonuclear shift-correlated 2D-NMR with a delay period to emphasize long-range or small coupling (COSYLR), and heteronuclear shift-correlated 2D-NMR with polarization transfer from ^1H to ^{13}C via J_{CH} (XH-CORR). The COSY data were acquired with the normal pulse sequence and a 16-step phase cycle to afford quadrature detection in both frequency domains. The data were collected as 256 × 1K complex points and were processed with sinusoidal multiplication prior to both Fourier transformations with zero filling sufficient to afford a final magnitude-calculated spectrum consisting of 512 × 512 real points. An amount of 320 acquisitions were taken for each experiment, giving an accumulation time of 16 h. These data were plotted as shown in Figure 1. Mass spectral analyses were conducted at TexMS, 15701 W. Hardy Road, Houston, TX by using an atmospheric pressure desorption technique. High-resolution mass spectra were conducted at the Midwest Center for Mass Spectrometry, Lincoln, NE. Samples were formulated in a 3-nitrobenzyl alcohol matrix and analyzed by fast-atom bombardment on a Kratos MS 50 TA instrument. All chemical reactions were conducted in dry glassware and were protected from atmospheric moisture. Solvents were dried over freshly activated (300 °C, 1 h) molecular sieves (type 4A). Evaporations were carried out on a rotary evaporator under aspirator vacuum at a bath temperature of <25 °C. The homogeneity of the products was determined by ascending TLC on silica-coated glass plates (silica gel 60 F 254, Merck) with mixtures of $CHCl_3$–MeOH as the eluting solvent. Preparative separations were performed on thick layers (20 cm × 20 cm × 2 mm) of the same adsorbent or by column chromatography on silica gel (Merck, 230–400 mesh) with mixtures of $CHCl_3$–MeOH as eluent.

5-Hexen-1-al (7). Powdered molecular sieves (type 4A, 500 mg/mmol, 8.5 g) were added to a solution of 5-hexen-1-ol (6) (2 mL, 1.67 g, 17 mmol) and *N*-methylmorpholine *N*-oxide (1.5 equiv, 25.6 mmol, 3.0 g) in CH_2Cl_2 (35 mL). The mixture was stirred for 10 min at room temperature under a nitrogen atmosphere, and then tetrapropylammonium perruthenate (TPAP)[37] (0.30 g, 0.85 mmol, 5 mol%) was added in one portion. The initially green mixture progressively darkened. The reaction was complete after 2 h (as evidenced by TLC). CH_2Cl_2 (35 mL) was added, and the mixture was passed first through a short pad of Celite. The filtrate was evaporated, and the residual crude product was purified by column chromatography on silica gel ($CHCl_3$–MeOH, 98:2) to afford **7** as an oil (1.47 g, 15 mmol, yield 88%): bp 118–118.5 °C/760 mm (lit.[37] 117.5–118 °C/760 mm); ^1H NMR [chemical shift (δ), multiplicity, coupling constant (Hz), number of protons, atom] 9.71 (t, J = 1 Hz, 1 H, H-1), 5.75 (m, 1 H, H-5), 5.04 (m, 2 H, H-6), 2.45 (m, 2 H, H-2), 2.08 (m, 2 H, H-4), 1.72 (m, 2 H, H-3); ^{13}C NMR (ppm, atom) 201.90 (C-1), 137.18 (C-5), 115.07 (C-6), 42.67 (C-2), 32.55 (C-4), 20.75 (C-3).

5-Hexene-1,1-diacetate (8). 5-Hexen-1-al (7) (5 g, 5.9 mL, 50.9 mmol) was added dropwise, with stirring over 5 min at ambient temperature, to a solution of acetic anhydride (6 mL, 62 mmol) and BF_3·Et_2O (0.5 mL) in anhydrous Et_2O (10 mL). The reaction mixture was stirred for 10 min, washed successively with 25% NaOAc solution (20 mL) and H_2O (25 mL × 2), and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was distilled under reduced pressure to give the diacetoxy acetal (8) (9.6 g, 48 mmol, 94%): ^1H NMR 6.83 (t, 1 H, J = 5 Hz, H-1), 5.58 (m, 1 H, H-5), 5.02 (m, 2 H, H-6), 2.12 (s, 6 H, 2 × $OCOCH_3$), 2.05 (m, 2 H, H-2), 1.85 (m, 2 H, H-4), 1.50 (m, 2 H, H-3); ^{13}C NMR 168.42 ($OCOCH_3$), 137.43 (C-5), 114.81 (C-6), 89.92 (C-1), 32.82 (C-2), 32.14 (C-4), 22.23 (C-3), 20.32 ($OCOCH_3$). Anal. ($C_{10}H_{16}O_4$) C, H.

5-Oxopentane-1,1-diacetate (9). A solution of 5-hexene-1,1-diacetate (8) (5 g, 3.5 mL, 25 mmol) in CH_2Cl_2 (5 mL) was placed in a long cylindrical gas absorption vessel with an inlet dispersion tube extending to the base. The vessel was cooled to −70 °C in a dry ice/acetone mixture, and ozone was introduced. Ozonization was continued until all of the compound had reacted (until the mixture turned blue as a result of formation of the ozonide; approximately 20 min). Me_2S (7.25 mL, 0.1 mol, 4 equiv) was added, and the mixture was stirred overnight to reduce the ozonide to the corresponding aldehyde. The excess Me_2S was evaporated, and the residue was chromatographed on a column of silica gel (CH_2Cl_2) to give **9** (4.29 g, 21.25 mmol, 85%) as a syrup: ^1H NMR 9.83 (t, 1 H, J = 1 Hz, H-5), 6.75 (t, 1 H, J = 5 Hz, H-1), 2.66 (dt, 2 H, J = 5.5, 1 Hz, H-4), 2.14 (s, 6 H, 2 × $OCOCH_3$), 2.05 (m, 2 H, H-2), 1.85 (m, 2 H, H-3); ^{13}C NMR 201.43 (C-5), 168.72 ($OCOCH_3$), 89.61 (C-1), 42.86 (C-4), 32.10 (C-2), 20.54 ($OCOCH_3$), 15.62 (C-3). Anal. ($C_9H_{14}O_5$) C, H.

***N*-(5,5-Diacetoxypent-1-yl)doxorubicin Hydrochloride (3).** A stirred solution of doxorubicin hydrochloride (20 mg, 0.035 mmol) and 5-oxopentane-1,1-diacetate (**9**) (14 mg, 2 equiv, 0.07 mmol) in CH_3CN–H_2O (2:1) (5 mL) was treated with a solution of $NaBH_3CN$ (1 M in THF) (24 μL, 0.67 equiv, 0.024 mmol). The mixture was stirred under a nitrogen atmosphere at room temperature in the dark for 1 h. When reaction was complete (as evidenced by TLC), the solution was diluted with H_2O (8 mL) and extracted repeatedly (10 × 10 mL) with $CHCl_3$–MeOH (5:1). The combined extracts were dried and evaporated to give a red amorphous solid (16 mg) [TLC, $CHCl_3$–MeOH, 10:1; R_f = 0.60). Column chromatography of this product ($CHCl_3$–MeOH, 10:1) afforded *N*-(5,5-diacetoxypent-1-yl)doxorubicin (16.3 mg, 0.0224 mmol), yield 64%. The product was suspended in H_2O (1 mL) and carefully acidified to pH 5 by dropwise addition of 0.05 N HCl (ca. 0.5 mL). The clear red solution which formed as lyophilized to afford the title compound as an amorphous powder. It was stored under a nitrogen atmosphere in a tightly stoppered vessel at −78 °C in the dark: ^1H NMR (free base) 14.1 (s, 1 H, OH), 13.2 (s, 1 H, OH), 8.01 (dd, J = 8.2, 0.9 Hz, 1 H, H-1), 7.82 (t, J = 8.2 Hz, 1 H, H-2), 7.39 (dd, J = 8.2, 0.9 Hz, 1 H, H-3), 6.73 (t, J = 5.46, 1 H, H-5″), 5.52 (t, J = 1 Hz, 1 H, H-1′), 5.3 (bs, 1

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H, H-7), 4.75 (s, 2 H, H-14), 4.12 (s, 3 H, 4-CH_3), 3.82 (bs, 1 H, H-5'), 3.62 (m, 1 H, H-4'), 3.25 (d, J = 16 Hz, 1 H, H-10a), 2.95 (d, J = 16 Hz, 1 H, H-10b), 2.85 (m, 1 H, H-3'), 2.65 (m, 2 H, H-1''), 2.35 (m, 1 H, H-8a), 2.25 (m, 1 H, H-8b), 2.01 (s, 6 H, 2 × $OCOCH_3$), 1.82 (m, 2 H, H-2'a), 1.76 (m, 2 H, H-4''), 1.75 (m, 1 H, H-2'b), 1.40 (m, 4 H, H-2'' and H-3''), 1.35 (d, J = 6 Hz, 3 H, H-6'); ^{13}C-NMR (ppm): 213.69 (C-13), 186.92 (C-5), 186.61 (C-12), 169.02 ($OCOCH_3$), 161.07 (C-4), 156.23 (C-6), 155.58 (C-11), 135.73 (C-6a), 135.42 (C-2), 133.72 (C-12a), 133.63 (C-10a), 120.84 (C-4a), 119.78 (C-1), 118.53 (C-3), 111.52 (C-5a), 111.37 (C-11a), 100.42 (C-1'), 90.03 (C-5''), 76.61 (C-9), 69.64 (C-7), 66.92 (C-4'), 66.41 (C-14), 65.46 (C-5'), 56.63 (4-OCH_3), 53.54 (C-1''), 45.43 (C-3'), 35.56 (C-8), 33.86 (C-10), 32.67 (C-2''), 28.46 (C-2'), 27.65 (C-4''), 20.87 (C-3''), 20.74 ($OCOCH_3$), 16.95 (C-6'); MS (electrospray) m/z 730 $(M + H)^+$. HRMS calcd for $C_{36}H_{44}NO_{15}$ 730.2711, found 730.2714.

5-Hexen-1-yl Acetate (10). 5-Hexen-1-ol (3 mL, 2.5 g, 25 mmol) was added over 1 min to a solution of acetyl chloride (4 mL, 4.7 mg, 60 mmol) in $CHCl_3$ (10 mL) contained in a 50-mL round-bottomed flask. The mixture was stirred for 3 h at room temperature and then added, with vigorous stirring, to an ice-cold saturated $NaHCO_3$ solution (20 mL). The organic layer was decanted, dried over Na_2SO_4, and evaporated to dryness. The residual liquid (3.27 g, 23 mmol, yield 92%) was distilled under reduced pressure (bp 56 °C, 10 mm; lit.[38] bp 69 °C, 16 mm): ^{1}H NMR 5.7 (m, 1 H, H-5), 5.2 (m, 2 H, H-6), 3.95 (t, 2 H, J = 5 Hz, H-1), 2.2 (m, 2 H, H-2), 2.10 (s, 3 H, CH_3), 1.75 (m, 2 H, H-3), 1.6 (m, 2 H, H-4); ^{13}C NMR 170.7 ($OCOCH_3$), 137.91 (C-5), 114.43 (C-6), 63.93 (C-1), 32.91 (C-4), 27.65 (C-2), 24.8 (C-3), 20.55 ($OCOCH_3$).

5-Oxopent-1-yl Acetate (11). The acetate **10** (2.56 g, 14.7 mmol) in CH_2Cl_2 (5 mL) was ozonized as described for compound **9**. Me_2S (4.3 mL, 59 mmol, 4 equiv) was added to the blue ozonide solution, and the mixture was stirred overnight. The solvent and unreacted Me_2S was removed by evaporation, and the residue was chromatographed on silica gel (with CH_2Cl_2 as eluent) to afford **11** as an oil (1.82 g, 12.64 mmol, 86%): ^{1}H NMR 9.69 (t, 1 H, J = 1 Hz, H-5), 3.95 (t, 2 H, J = 5 Hz, H-1), 2.45 (dt, 2 H, J = 5.5, 1 Hz, H-4), 2.10 (s, 3 H, CH_3), 1.85 (m, 4 H, H-3, H-2); ^{13}C NMR 201.30 (C-5), 170.30 ($OCOCH_3$), 63.33 (C-1), 42.73 (C-4), 27.54 (C-2), 20.27 ($OCOCH_3$), 18.07 (C-3). Anal. ($C_7H_{12}O_3$) C, H.

***N*-(5-Acetoxypent-1-yl)doxorubicin Hydrochloride (4).** This compound was prepared from doxorubicin hydrochloride (20 mg, 0.035 mmol), 5-oxopent-1-yl acetate (**11**) (10.1 mg, 2 equiv, 0.07 mmol), and $NaBH_3CN$ (1 M in THF) (24 μL, 0.67 equiv, 0.024 mmol) in CH_3CN–H_2O (2:1) (5 mL), as described for compound **3**. When reaction was complete (as evidenced by TLC), the solution was diluted with H_2O (8 mL) and then extracted repeatedly (10 × 10 mL) with $CHCl_3$–MeOH (5:1). The combined extracts were dried and evaporated to give a red amorphous solid (17 mg). Column chromatography of this product [TLC, $CHCl_3$–MeOH, 10:1, R_f = 0.5] afforded *N*-(5-acetoxypentyl)-doxorubicin (14.79, 0.022 mmol), yield 63%. The product was suspended in H_2O (1 mL) and acidified to pH 5 by dropwise addition of 0.05 N HCl (ca. 0.5 mL). The solution was lyophilized to afford the title compound. It was stored under a nitrogen atmosphere in a tightly stoppered vessel at −78 °C in the dark: ^{1}H NMR (free base) 14.05 (s, 1 H, OH), 13.1 (s, 1 H, OH), 8.05 (dd, J = 8.2, 0.9 Hz, 1 H, H-1), 7.89 (t, J = 8.2 Hz, 1 H, H-2), 7.45 (dd, J = 8.2, 0.9 Hz, 1 H, H-3), 5.54 (t, J = 1 Hz, 1 H, H-1'), 5.3 (bs, 1 H, H-7), 4.78 (s, 2 H, H-14), 4.10 (s, 3 H, 4-CH_3), 3.96 (t, J = 5 Hz, 2 H, H-5''), 3.63 (bs, 1 H, H-5'), 3.60 (m, 1 H, H-4'), 3.20 (d, J = 17 Hz, 1 H, H-10a), 2.98 (d, J = 17 Hz, 1 H, H-10b), 2.86 (m, 1 H, H-3'), 2.65 (m, 2 H, H-1''), 2.4 (m, 1 H, H-8a), 2.25 (m, 1 H, H-8b), 2.01 (s, 3 H, $OCOCH_3$), 1.85 (m, 2 H, H-2'a), 1.80 (m, 2 H, H-4''), 1.78 (m, 1 H, H-2'b), 1.75 (m, 2 H, H-3''), 1.40 (d, J = 6 Hz, 3 H, H-6'); ^{13}C-NMR (ppm) 213.66 (C-13), 186.74 (C-5), 186.73 (C-12), 171.10 ($OCOCH_3$), 161.12 (C-4), 156.22 (C-6), 155.68 (C-11), 135.52 (C-6a), 135.52 (C-2), 133.61 (C-12a), 133.78 (C-10a), 120.80 (C-4a), 119.83 (C-1), 118.51 (C-3), 111.61 (C-5a), 111.45 (C-11a), 100.71 (C-1'), 76.73 (C-9), 69.56 (C-7), 66.89 (C-4'), 65.51 (C-5'), 65.46 (C-14), 64.17 (C-5''), 56.69 (4-OCH_3), 54.20 (C-1''), 45.56 (C-3'), 35.56 (C-8), 33.80 (C-10), 32.60 (C-2''), 28.40 (C-2'), 27.38 (C-4''), 23.30 (C-3''), 20.91 ($OCOCH_3$), 17.05 (C-6'); MS (electrospray) m/z 672 $(M + H)^+$; (FAB) 672 $(M + H)^+$, 613 $(M + H^+ - CH_3COO)$, 612 $(M + H^+ - HCOCH_2OH)$; HRMS calcd for $C_{34}H_{42}NO_{13}$ 672.2656, found 672.2657.

***N*-pentyldoxorubicin Hydrochloride (5).** The compound was prepared from doxorobicin hydrochloride (20 mg, 0.035 mmol), pentanal (**12**) (6.0 mg, 2 equiv, 0.07 mmol), $NaBH_3CN$ (1 M in THF) (24 μL, 0.67 equiv, 0.024 mmol) in CH_3CN–H_2O (2:1) (5 mL), as described for **3**. Column chromatography of this product (TLC, $CHCl_3$–MeOH, 10:1; R_f = 0.55) afforded *N*-pentyldoxorubicin (13.3 mg, 0.022 mmol), yield 62%. The product was suspended in H_2O (1 mL) and acidified to pH 5 by dropwise addition of 0.05 N HCl (ca. 0.5 mL). The solution was lyophilized to afford the title compound as an amorphous powder. It was stored under a nitrogen atmosphere in a tightly stoppered vessel at −78 °C in the dark: ^{1}H NMR (free base) 14.0 (s, 1 H, OH), 13.15 (s, 1 H, OH), 8.15 (dd, J = 8.1, 1 Hz, 1 H, H-1), 7.85 (t, J = 8.1 Hz, 1 H, H-2), 7.45 (dd, J = 8.1, 1 H, 1 H, H-3), 5.52 (t, J = 1 Hz, 1 H, H-1'), 5.35 (bs, 1 H, H-7), 4.75 (s, 2 H, H-14), 4.15 (s, 3 H, 4-CH_3), 3.65 (bs, 1 H, H-5'), 3.62 (m, 1 H, H-4'), 3.25 (d, J = 17 Hz, 1 H, H-10a), 3.0 (d, J = 17 Hz, 1 H, H-10b), 2.84 (m, 1 H, H-3'), 2.60 (m, 2 H, H-1''), 2.39 (m, 1 H, H-8a), 2.28 (m, 1 H, H-8b), 1.85 (m, 2 H, H-2'a), 1.79 (m, 1 H, H-2'b), 1.72 (m, 2 H, H-4''), 1.70 (m, 2 H, H-3''), 1.40 (d, J = 6 Hz, 3 H, H-6'), 1.35 (d, J = 5 Hz, 3 H, H-5''); ^{13}C-NMR (ppm) 213.69 (C-13), 186.99 (C-5), 186.68 (C-12), 161.11 (C-4), 156.23 (C-6), 155.64 (C-11), 135.49 (C-6a), 135.73 (C-2), 133.77 (C-12a), 133.63 (C-10a), 120.92 (C-4a), 119.81 (C-1), 118.51 (C-3), 111.57 (C-5a), 111.41 (C-11a), 100.61 (C-1'), 76.71 (C-9), 69.57 (C-7), 66.96 (C-4'), 65.47 (C-14), 65.47 (C-5'), 56.67 (4-OCH_3), 53.26 (C-1''), 45.83 (C-3'), 35.54 (C-8), 33.94 (C-10), 31.67 (C-2''), 27.96 (C-2'), 24.90 (C-3''), 22.30 (C-4''), 17.02 (C-6'), 13.87 (C-5''); MS [FAB, $(C_{32}H_{40}NO_{11})^+$] m/z 614 $(M + H^+)$ 554, $(M + H^+ - HCOCH_2OH)$, 414 (aglycon-7-OH), 398 (aglycon-7-H), 396 (aglycon-7-OH − H_2O), 378 (aglycon-7-OH − $2H_2O$).

Stability Studies. Porcine liver carboxylate esterase was purchased from Sigma Chemical Co., St. Louis, MO, and was used as received. The specific activity of the preparation was 200 units/mg of protein, where 1 unit is defined as the amount that will hydrolyze 1.0 μmol of ethyl butyrate to butyric acid and ethanol per minute at pH 8.0 and 25 °C. Compound **3** was dissolved in the appropriate buffer at a concentration of 20 μM. One-milliliter aliquots of this solution contained in a 5.0-mL screw-capped glass vial, was incubated at 37 °C in either the absence or the presence of the enzyme. At selected time intervals (typically 2, 4, 8, 12, 24, 30, 50, 100, and 250 h in the absence of enzyme), aliquots (20 μL) were removed and analyzed immediately for parent drug by HPLC on a C-18 reverse-phase column (Waters Associates, Milford, MA; μ-Bondapak C-18; 200 × 4.6 mm, i.d.). A solution of CH_3CN–0.05 N acetate buffer, pH 4.0 (2:3), at a flow rate of 1.0 mL/min was used as mobile phase. The retention time of the parent drug **3** was 3.45 min. For the enzyme studies, 2, 5, 10, and 20 units of esterase per μmol of substrate were used. The enzyme was added to 0.2 mL of the drug solution contained in 1.5-mL microcentrifuge tubes that was preequilibrated at 37 °C. At intervals of 0.25, 0.50, 1, 2, 5, 10, 20, 40, and 60 min, the vials were agitated on a Vortex shaker for 20 s and then centrifuged for 4 min at 1000 rpm. Aliquots (40 μL) of the clear supernatant were analyzed by HPLC as described above. In addition to the parent compound, a progressively increasing peak due to the reaction product was present in all of the chromatograms at a retention time of 3.1 min. The half-lives were determined by linear least-squares regression analysis of the pseudo first-order reactions.

Growth Inhibition Studies. The CHO/DOX cells used in this study were originally developed by Sen et al.[21] The cells express elevated levels of P-glycoprotein and are cross-resistant with vincristine and vinblastine. To test for drug growth inhibitory effects, CHO or CHO/DOX cells in exponential growth at an initial density of 1.5 × 10^4 cells/mL were incubated in McCoy's 5A media containing 10% fetal calf serum (FCS) (total final volume, 2 mL), with various drug concentrations over the range 2 × 10^{-5} M to 2 × 10^{-12} M for 6 days at 37 °C in 12-well plates. The cells were fixed with 10% formaldehyde solution (500 μL/

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well) for 10 min, then stained with a solution of 0.1% crystal violet (250 μL/well) for 20 min. The medium was decanted, and the plates were gently rinsed with water and air-dried. The cells were counted under an inverted phase-contrast microscope. The surviving fractions were calculated, dose-response curves constructed, and the IC_{50} values (the drug concentrations that inhibited colony formation by 50%) determined. Each value represents the average of duplicate determinations.

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Hypoxia-Selective Antitumor Agents. 5. Synthesis of Water-Soluble Nitroaniline Mustards with Selective Cytotoxicity for Hypoxic Mammalian Cells

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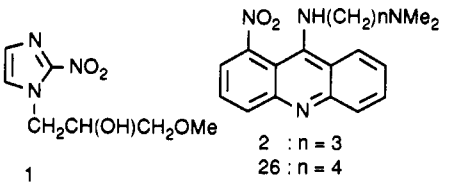
Nitroaniline mustards have potential as hypoxia-selective cytotoxic agents, with reductive metabolism activating the nitrogen mustard by converting the electron-withdrawing nitro group to an electron-donating hydroxylamine or amine. However, the parent compounds have poor aqueous solubility, and their potencies are limited by low reduction potentials ($E_{1/2}$ ca. −600 mV versus the normal hydrogen electrode) and corresponding slow rates of nitro reduction. To address these limitations, a series of 4-nitroaniline mustards bearing hydrophilic side chains attached via an electron-withdrawing carboxamide group was prepared and evaluated for hypoxia-selective cytotoxicity against Chinese hamster cell lines. The *N*-[(*N*,*N*-dimethylamino)ethyl]carboxamide derivatives proved to have excellent aqueous solubility and improved cytotoxic potency, but their reduction potentials, while higher than the non-carboxamide compounds, were still low and little selectivity for hypoxic cells were observed. A series of carboxamides of 2,4-dinitroaniline mustard was also prepared. These compounds had reduction potentials in the desired range ($E_{1/2}$ ca. −450 mV by cyclic voltammetry) and were more toxic to hypoxic than aerobic UV4 cells. The most selective compounds were 5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzamide (**20**, SN 23862) and its water-soluble *N*-[(*N*,*N*-dimethylamino)ethyl]carboxamide analogue. These showed selectivities of 60- to 70-fold for hypoxic UV4 cells. The selectivity of **20** was much superior to that of its aziridine analogue (**23**, CB 1954), which was only 3.6-fold more toxic to hypoxic than oxic cells in the same system. Compound **20** is a much less efficient substrate than CB 1954 for the major aerobic nitroreductase from rat Walker tumor cells, NAD(P)H:quinone oxidoreductase (DT diaphorase). Lack of aerobic bioactivation of **20** by DT diaphorases may be responsible for its higher hypoxic selectivity than that of **23**.

Solid tumors contain a proportion of cells which are either transiently or chronically hypoxic.[1,2] Because of their low proliferative activity[3] and inaccessibility to blood-borne drugs,[4,5] these cells represent a potential clinical problem in the chemotherapy of solid tumors. At the same time, the hypoxic microenvironment offers an attractive target, since nearly all normal tissues are well-perfused, and drugs activated only in hypoxic regions (hypoxia-selective cytotoxins, HSCs) may be truly specific for solid tumors.

The nitro aromatics most thoroughly-studied as HSCs are 2-nitroimidazoles, such as misonidazole (**1**), which undergo metabolic one-electron reduction of the nitro group to the radical anion. In well-oxygenated cells this transformation is efficiently reversed by oxygen, but in hypoxic cells the radical anion is reduced further to form reactive, cytotoxic products.[6] While showing significant hypoxic selectivity, these compounds are not very potent, and it has proven difficult to achieve sufficiently high concentrations in tumors to show therapeutic benefit in vivo, except with 2-nitroimidazoles carrying additional DNA-reactive alkylating functionality.[7,8] While we[9,10] and others[11] have shown that DNA-affinic nitro aromatics (e.g., nitracrine, **2**) show greatly-increased potency as HSCs, the high DNA binding affinity of such compounds appears to hinder their extravascular diffusion.[4,12]



1 **2** : n = 3; **26** : n = 4

As an alternative approach to the design of nitro aromatics as potent HSCs, we have discussed in detail[13] the

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