Intensely Cytotoxic Anthracycline Prodrugs: Glucuronides

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We previously reported the synthesis of a series of doxorubicin analogue prodrugs that give rise to intensely cytotoxic metabolites in the presence of carboxylate esterases. We now report studies on structurally related β-glucuronide prodrugs that are converted to similar potent metabolites in the presence of β-glucuronidases. These prodrugs were prepared by reductive condensation of daunomycin or doxorubicin with methyl 1-*O*-[(1′*RS*)-1′-ethoxy-4′-oxobutyl]-2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate in the presence of sodium cyanoborohydride followed by base-mediated cleavage of the glucuronate protective groups. The doxorubicin derivatives were isolated in very low yield, most likely because of the inherent base lability of the parent aglycone. By contrast, fairly good yields of the more base-stable daunomycin analogues were obtained. The target daunomycin glucuronide, *N*-[(4″*RS*)-4″-ethoxy-4″-(sodium 1‴-*O*-β-D-glucopyranuronate)butyl]daunorubicin (**6a**), had a half-life of 30 h when incubated at a concentration of 12 μM in aqueous 0.05 M phosphate buffer, pH 7.4, at 37 °C. Under identical conditions in the presence of 197 units/μmol of *Escherichia coli* β-glucuronidase, **6a** was hydrolyzed with a half-life of 1.7 h. The single metabolite observed was chromatographically identical with that formed from the hydrolysis of *N*-(4,4-diacetoxybut-1-yl)daunomycin by carboxylate esterases. **6a** was approximately 10000-fold more toxic to human A375 melanoma cells in the presence of *E. coli* β-glucuronidase than in the absence of the enzyme. These findings indicate the therapeutic potential of anthracycline glucuronide prodrugs as independent entities or for use in conjunction with enzyme tissue-targeting strategies such as antibody-directed enzyme prodrug therapy (ADEPT) or gene-directed enzyme prodrug therapy (GDEPT).

Introduction

We have reported[1−4] the synthesis of a series of intensely cytotoxic prodrugs derived from the antitumor antibiotics daunomycin and doxorubicin. These compounds contain a ω-bis(acetoxy)alkyl (typified by DAPDOX, **1b**, $n = 4$; Scheme 1) or ω-bis(acetoxy)alkoxyalkyl substituent on the 3′-amino position of the daunosamine sugar. The most active representatives of this class of compounds were 100−1000-fold more potent than doxorubicin against a variety of tumor cell lines in culture and retained full activity against variants of these tumors that were resistant to doxorubicin because of altered topoisomerase II or overexpression of P-glycoprotein (gp 170). The compounds were designed to undergo hydrolysis to the corresponding hemiacetals, **2**, in the presence of carboxylate esterases, enzymes that generally lack substrate specificity and are present in all mammalian tissues including cells in culture.[5,6]

It was anticipated that the hemiacetals, once formed, would spontaneously lose acetic acid to form the corresponding aldehydes, **3**. Structure−activity relationship studies established that the most potent representatives of the series were those in which the bis(acetoxy)acetal groups are linked to the daunosamine sugar by a 3- or 4-carbon (or carbon−oxygen) bridge[3,4] and where the derived aldehydes are capable of forming cyclic carbinolamines, **4**. The ultimate active metabolites of these compounds have not been established but are probably electrophilic iminium ions, **5**, capable of forming covalently bonded adducts with DNA.

Because of the unique biological properties of these compounds, it was of interest to prepare structural analogues that could be activated by hydrolytic enzymes other than carboxylate esterases. Prodrugs of the general structure **6** (Scheme 1) that might be activated by β-glucuronidases were particularly appealing. Levels of β-glucuronidase have been reported[7] to be elevated up to 6-fold in human breast tumors compared to normal peritumor tissue. It is possible, therefore, that such anticancer β-glucuronide prodrugs may be preferentially activated in tumors with intrinsically elevated β-glucuronidase expression. Second, β-glucuronidases have been used in conjunction with the antibody-directed enzyme prodrug therapy (ADEPT) strategy[8,9] for anticancer prodrug design. In this approach, the drug-activating enzyme is first conjugated to monoclonal antibodies (MAbs) directed against tumor-associated antigens. The conjugate is then administered systemically and allowed to localize in the tumor. After the conjugate has cleared from the blood, the prodrug is administered. This leads to selective formation of the cytotoxic metabolite at the target tumor site. β-Glucuronidase-MAb conjugates (or related fusion proteins) have been used to selectively activate various antitumor prodrugs, including nitrogen mustards[10,11] and anthracyclines.[12−14]

To explore the therapeutic potential of β-glucuronide prodrugs related to **1**, we prepared the daunomycin and doxorubicin derivatives **6a**,**b** (Scheme 1). Unlike the *N*-[ω-bis(acetoxy)alkyl] or *N*-[ω-bis(acetoxy)alkoxyalkyl] series, these compounds do not contain ω-ester groups and, therefore, cannot be activated by carboxylate esterases. Instead, they are designed to be activated by β-glucuronidases. Cleavage of the glucuronide moiety leads to the hemiacetals, **7**, which should be inherently unstable and spontaneously lose ethanol to

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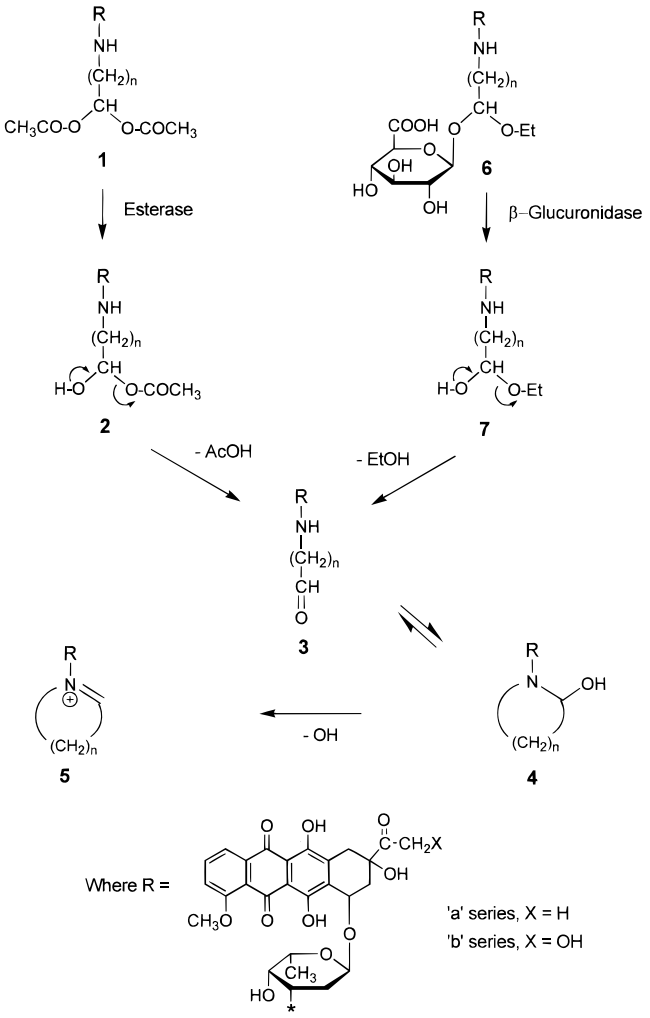
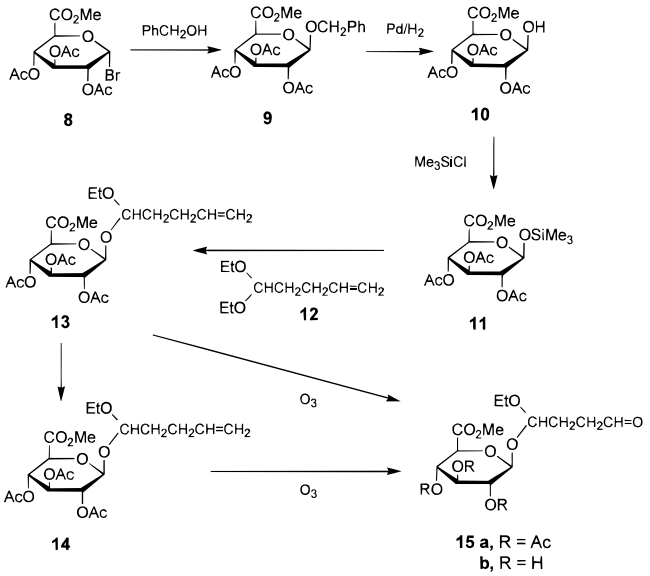
Scheme 1



Scheme 2

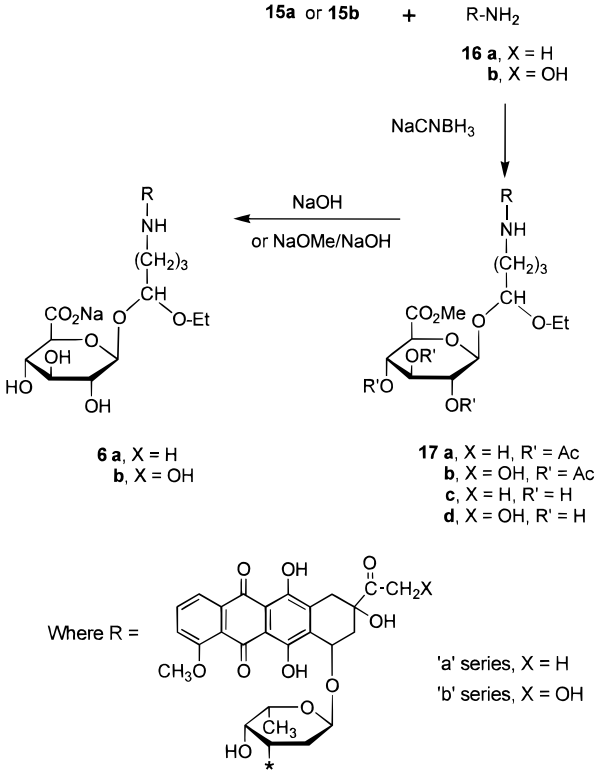


give the aldehyde intermediates, **3**. In this manner, the relatively nontoxic glucuronide prodrugs should be converted to intensely cytotoxic metabolites.

Results and Discussion

The synthetic route to the target glucuronides is shown in Schemes 2 and 3. Reaction of methyl (tri-*O*-acetyl-*β*-D-glucopyranosyl bromide)uronate (**8**) with benzyl alcohol in the presence of $Hg(CN)_2$ afforded methyl 1-*O*-benzyl-2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate, **9**.[15] This compound was debenzylated by catalytic hydrogenation over Pd−C. The product, **10**, was treated with hexamethyldisilazane and chlorotrimethylsilane in pyridine to give the trimethylsilyl ether, **11**.[16] Reaction of **11** with 4-pentenal diethyl acetal, **12**, in the presence of $CF_3SO_3Si(CH_3)_3$ according to the general procedure[17] of Tietze et al. gave the acetal **13**. This compound was deacylated with NaOMe in MeOH to afford, in excellent yield, the methyl glucuronide **14**. Ozonolysis of **13** gave the corresponding aldehyde **15a**. The deacylated analogue **15b** was prepared similarly from **14**.

Scheme 3



The daunomycin derivative **17a** was prepared by reductive condensation of **15a** and daunomycin, **16**, in the presence of sodium cyanoborohydride (Scheme 3). The doxorubicin analogue **17b** was prepared similarly from **15a** and doxorubicin. The deacylated analogues **17c**,**d**, respectively, were synthesized by two methods. In the first, **17a**,**b** were deacylated with NaOMe in MeOH. This led to a moderate yield of the daunomycin derivative **17c** but to an extremely low yield (3.2%) of the doxorubicin analogue **17d**. These same deacylated compounds were also prepared by direct condensation of the parent anthracyclines with **15b**. Again, a good yield of **17c** was obtained but with a poor yield of **17d**. The low yields of the doxorubicin derivatives are probably due to the susceptibility of the 14-hydroxymethyl ketone substituents to degradation by a retro-aldol reaction[18] in alkaline media. Because of this difficulty, no further attempt was made to isolate the free glucuronide **6b**. Daunomycin and analogues, on the other hand, have a methyl ketone substituent in the 14-position of the molecule and are moderately base-stable. The target glucuronide **6a** was isolated as the sodium salt in 31% yield after hydrolysis of **17a** by NaOH and successive passage of the product over ion-exchange

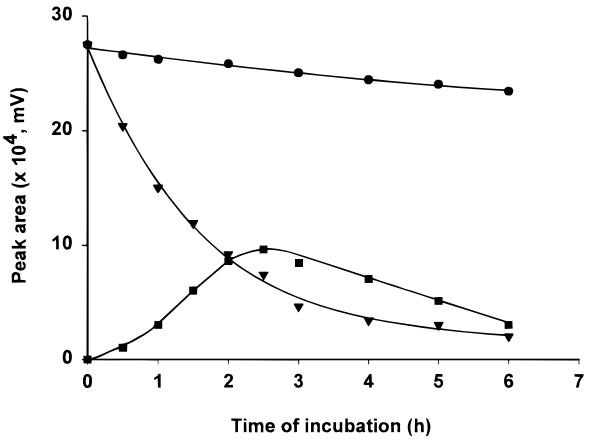


Figure 1. Stability of **6a** (12 μM) in 0.05 M phosphate buffer, pH 7.4, in the absence and presence of *E. coli* β-glucuronidase (197 units/μmol): (●) **6a** in buffer, (▼) **6a** + β-glucuronidase, (■) product.

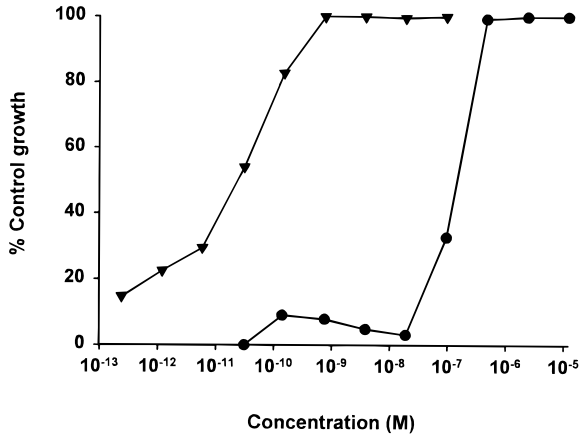


Figure 2. Cytotoxicity of **6a** in the absence and presence of *E. coli* β-glucuronidase (125 units/mL) to A375 human melanoma cells (72-h incubation): (●) **6a**, (▼) **6a** + β-glucuronidase.

resins in the H^+ and Na^+ forms. **6a** was prepared in slightly better yield (44%) by initial deprotection of the acetyl groups of **17a** with NaOMe in MeOH followed by hydrolysis of the methyl glucuronide **17c** by NaOH.

All new compounds had the β-configuration at C-1. This was apparent from the 1-H signals which showed a chemical shift of δ 4.4−4.7 and a relatively large coupling constant of 7.5 Hz.

A new chiral center is formed at C-1′ when acetal **13** is formed from **12**. Therefore, compounds **13**−**15b** consist of a mixture of diastereomers. Unfortunately, it was not possible to crystallize these diastereomers or to separate them by column chromatography. The assignment of the configuration of these compounds is based on their ^{1}H NMR spectra and a comparison of these data with the published data of similarly constituted compounds. Thus, Tietze et al. established NMR rules[17,19−21] for the determination of the configuration of acetal glucosides. According to these rules, the 1′-H signals for (l*R*,l′*R*)- and (l*S*,l′*S*)-glycosides occur at lower fields than those of their l*R*,l′*S* and l*S*,1′*R* counterparts. The spectra of **13**, **14**, and **15b** contained well-defined triplets at δ 4.70−4.77 (l′*S* proton; J = 6.0 Hz) and those of **14** and **15b** (**13** was partially obscured) triplets at δ 4.87−4.81 (1′*R* proton; J = 6.0 Hz). From the relative intensity of these signals, it can be concluded that each product consisted of a mixture of 1′*R* and 1′*S* diastereomers in the ratio 1:1.4. The ^{1}H NMR spectra of the anthracyclines **17a**,**b**,**d** and **6a** were quite complicated, and separate signals for the C-1 and C-1′ protons of the glucuronide moiety were not observed. However, it is likely that these anthracyclines have the same configurations at C-1 and C-1′ as the parent β-glucuronides and in the same ratios.

6a (in the form of its sodium salt) showed no evidence of decomposition when stored over a period of 6 months at 5 °C. When incubated at 37 °C in 0.05 M phosphate buffer, pH 7.4, at a concentration of 12 μM, **6a** was degraded with a half-life of 30 h. Under identical conditions in the presence of *Escherichia coli* β-glucuronidase (197 units/μmol), **6a** was degraded with a half-life of 1.7 h. The disappearance of **6a** from the reaction mixture was accompanied by the formation of a product which first increased and then decreased in concentration with time (Figure 1). This suggests a chemically reactive entity which is removed from the system either by interaction with protein or by further decomposition. A product with identical chromatographic characteristics was formed from the hydrolysis of the daunomycin diester **1a** (R = H, n = 3) by carboxylate esterase (Scheme 1). The identity of this product (possibly **4**) has not been established, but clearly it is derived from the common aldehyde intermediate **3** (R = H, n = 3).

The cytotoxicity of **6a** was determined against human melanoma A375 cells in culture (Figure 2). In the absence of *E. coli* β-glucuronidase, the IC_{50} value of **6a** to the tumor cells after incubation at 37 °C for 72 h was 10^{-7} M. In the presence of the enzyme (125 units/mL), the IC_{50} value was 10^{-11} M, a 10000-fold increase in cytotoxicity. This is similar to the potency observed for **1a** against A375 cells.[3] Preincubation of **6a** with the enzyme for 4 h prior to mixing with the tumor cells did not change the cytotoxicity profile (data not shown) indicating that the active metabolite was stable in solution for this period. The IC_{50} value of 10^{-7} M for **6a** alone in the absence of added enzyme appears rather high, but this probably reflects some endogenous bioactivation, in either the tissue culture media or the tumor cells. Support for this interpretation is that structural analogues of **6a** that are incapable of bioactivation in tissue culture (data not reported here) generally give IC_{50} values of 10^{-5}−10^{-6} M. It should also be noted that since the difference in cytotoxicity between the prodrug and its active metabolite is at least 4 orders of magnitude, even 1% bioactivation would cause a minimum 100-fold increase in cytotoxicity.

The precise nature of the active metabolite(s) formed from the glucuronide and bis(acetoxy)acetal anthracycline prodrugs is unknown. However, certain conclusions might reasonably be drawn. Since both classes of prodrug are designed to give rise to aldehyde intermediates, and since both glucuronide **6a** and diester **1a** (n = 3) are cleaved enzymically to an apparantly single product with identical chromatographic characteristics, it is likely that aldehyde intermediates are formed initially. As we have suggested previously,[2] such aldehydes probably cyclize to carbinolamines that can then generate iminium ions capable of forming covalent adducts with DNA. Further studies addressing these considerations are underway.

In summary, we have prepared and characterized a daunomycin analogue glucuronide prodrug that is converted by β-glucuronidase to a metabolite that is at least 10000-fold more cytotoxic than the parent compound. This demonstrates that the formation of intensely

cytotoxic metabolites from bis(acetoxy)acetals, such as **1**, in the presence of carboxylate esterases is not limited to such structures but can be extended to related molecules that are biotransformed by other enzymes. This greatly extends the scope and therapeutic application of intensely potent anthracyclines and suggests the development of yet additional prodrug/activating enzyme combinations.

Experimental Section

Nuclear magnetic resonance spectra (^{1}H NMR) were recorded at ambient temperature on an IBM-Bruker Model NR/200 AF spectrometer in the Fourier transform mode, in $CDCl_3$, using tetramethylsilane as an internal standard. Mass spectral analyses were conducted at the University of Nebraska Center for Mass Spectrometry, Lincoln, NE. Elemental analysis were conducted at Galbraith Laboratories, Inc., Knoxville, TN. HPLC was performed on a Waters model 6000 chromatograph (Waters Assoc., Milford, MA) equipped with a Rheodyne model 7125 injector and a C-18 reverse-phase column (Phenomenex, Torrence, CA; μBondclone C-18 (10 μm, 150 × 3.9 mm i.d.) in series with a Guard-Pak column (Waters Assoc., Milford, MA; Bondapak C-18, 10 mm, 125 Å). Eluted products were detected with a Waters model 470 fluorescence detector with the excitation and emission wavelengths set at 495 and 595 nm, respectively. An HP 3395 B integrator (Hewlett-Packard Co., Ltd., Palo Alto, CA) was used to monitor and quantitate eluted peaks. All reactions were carried out in dry glassware and 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 30−40 °C. The homogeneity of the products was determined by ascending TLC on silica-coated alumina-backed plates (silica gel 60 F 254; Merck) using the following solvent systems: A, hexanes−ethyl acetate (1:1); B, chloroform−methanol (9:1); C, chloroform−methanol−water (13:6:1). Carbohydrates were detected by spraying the plates with 10% sulfuric acid and then heating at 150 °C for 3 min. Preparative separations were performed by column chromatography on silica gel (Merck; 230−400 mesh) or on thick layers (20 cm × 20 cm × 2 mm) of the same adsorbent. All chemical reagents were purchased from Aldrich Chemical Co., Milwaukee, WI.

4-Pentenal Diethyl Acetal (12). Method A. Boron trifluoride diethyl etherate (0.2 mL) was added, with stirring, to a solution of 4-penten-1-al (2.5 g, 29.8 mmol) in ethanol (150 mL). The mixture was refluxed for 10 min under nitrogen; then the solvent was removed under reduced pressure. The residue was taken up in dichloromethane (25 mL), and the solution was washed with 10% sodium acetate solution (15 mL) and water (2 × 10 mL). The organic layers were combined, dried over anhydrous sodium sulfate, and evaporated. The residue was chromatographed on a column of silica gel using chloroform−methanol (97:3) as eluent. 4-Penten-1-al diethyl acetal was obtained as an oil (4.5 g, 96%). ^{1}H NMR ($CDCl_3$): δ 5.92−5.70 (m, 1 H, H-4), 5.15−4.91 (m, 2 H, H-5), 4.51 (t, 1 H, J = 7 Hz, H-1), 3.78−3.42 (m, 4 H, 2 × CH_2), 2.25−2.08 (m, 2 H, H-2), 1.81−1.67 (m, 2 H, H-3), 1.27 (t, 6 H, J = 7 Hz, 2 × CH_3).

Method B. Amberlyst-15 (0.6 g) and molecular sieves, 4A (0.5 g), were added to a solution of 4-penten-1-al (1.0 g, 12 mmol) in dry ethanol (10 mL), and the mixture was kept at room temperature for 2 days. The solids were removed by filtration, and the liquid remaining after solvent evaporation was fractionated using a 10 cm Vigreux column to give **12** as an oil (bp 122−123 °C/0.5 mmHg). Yield: 1.5 g, 80%.

Methyl 1-*O*-[(1′*RS*)-1′-Ethoxypent-4-enyl]-2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate (13). Trimethylsilyl trifluoromethanesulfonate (1.0 mL of a 0.1 M solution in chloroform, 0.1 mmol) was added, with stirring, to a solution of **12** (1.25 g, 7.9 mmol) and methyl 2,3,4-tri-*O*-acetyl-1-*O*-(trimethylsilyl)-*β*-D-glucopyranuronate[16] (0.847 g, 2.1 mmol) in dry dichloromethane (10 mL) at −70 °C under a dry nitrogen atmosphere. The mixture was kept at −70 °C for 2 days; then triethylamine (1 mL) was added to quench the reaction. The cold solution was filtered through a short pad of silica gel which was then washed with ether. The filtrate was evaporated, and the residue was chromatographed on a column of silica gel using hexanes−ethyl acetate (2:1) as mobile phase. The eluted fractions were monitored by TLC in system A. The title compound (R_f = 0.67) was obtained as a semisolid (555 mg, 59%), ratio of 1′*R*:1′*S* = 1:1.4; 714 mg of unreacted **12** was recovered. ^{1}H NMR ($CDCl_3$): δ 5.86−5.71 (m, 1 H, H-4′), 5.34−5.20 (m, 2 H, H-3,4), 5.11−4.93 (m, 3 H, H-2, H-5′), 4.90−4.77 (m, 1.6 H, H-1, H-1′ (*S*)), 4.70 (t, 0.4 H, J = 6.0 Hz, H-1′ (*R*)), 4.06 (m, 1 H, H-5), 3.68 (s, 3 H, CO_2Me), 3.68−3.51 (m, 1 H, O*C*H_2CH$_3$), 3.51−3.34 (m, 1 H, O*C*H_2CH$_3$), 2.19−2.03 (m, 2 H, H-2′), 1.99, 1.98, 1.96 (3 s, 9 H, 3 Ac), 1.74−1.67 (m, 2 H, H-3′), 1.15 (t, 3 H, J = 7 Hz, CH_3). MS (FAB): *m*/*z* 447 (M + H)$^+$. Anal. ($C_{20}H_{30}O_{11}$) C, H.

Methyl 1-*O*-[(1′*RS*)-1′-Ethoxypent-4-enyl]-*β*-D-glucopyranosyluronate (14). Methyl 1-*O*-(1′-ethoxypent-4-enyl)-2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate (**13**) (400 mg, 1.25 mmol) was dissolved in dry methanol (10 mL), and 1.9 mL of a solution of sodium methoxide (42 mg, 0.78 mmol) in MeOH (10 mL) was added. The reaction mixture was maintained at ambient temperature for 3 h and then evaporated under reduced pressure. The residue was purified on a column of silica gel in dichloromethane−methanol (4:1) to give the title compound as a white semisolid (236 mg, 97.5%), ratio of 1′*R*:1′*S* = 1:1.4. The course of reaction and the column separation were monitored by TLC in system A (R_f = 0.07, **14**) or B (R_f = 0.53, **14**). ^{1}H NMR: δ 5.88−5.74 (m, 1 H, H-4′), 5.07−4.90 (m, 2 H, H-5′), 4.81 (t, 0.6 H, J = 6.0 Hz, H-1′ (*S*)), 4.70 (t, 0.4 H, J = 6.0 Hz, H-1′ (*R*)), 4.60 (d, 0.5 H, J = 7.5 Hz, H-1), 4.53 (d, 0.5 H, J = 7.5 Hz, H-1), 4.49 (br s, l H, OH), 4.25 (br s, l H, OH), 4.08 (br s, 1 H, OH), 3.84 (s, 3 H, CO_2Me), 3.91−3.47 (m, 6 H, H-2, H-3, H-4, H-5, O*C*H_2CH$_3$), 2.13−2.05 (m, 2 H, H-2′), 1.79−1.70 (m, 2 H, H-3′), 1.19 (t, 3 H, CH_3). MS (FAB): *m*/*z* 343 (M + Na)$^+$. HRMS calcd for $C_{14}H_{24}O_8Na$, 343.1233; found, 343.1376.

Methyl 1-*O*-[(1′*RS*)-1′-Ethoxy-4′-oxobutyl]-2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate (15a). A solution of methyl 1-*O*-(1′-ethoxypent-4-enyl)-2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate (**13**) (598 mg, 1.4 mmol) in dichloromethane (20 mL) was placed in a 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 the reaction was complete (until the mixture turned blue as a result of formation of the ozonide, approximately 5 min). The reaction mixture was then transferred to a round-bottomed flask containing sodium bicarbonate (50 mg) that had been cooled to −50 °C in a dry ice/acetone bath. Dimethyl sulfide (600 μL, 9.2 mmol) was added, and the mixture was warmed to room temperature and stirred for 6 days to reduce the ozonide to the corresponding aldehyde. During this period additional portions (3 × 200 μL) of dimethyl sulfide were added at 1−2-day intervals to the reaction mixture. When reaction was complete, as evidenced by TLC (system A, R_f = 0.5, **15a**), the mixture was filtered and excess dimethyl sulfide was evaporated. The residue was chromatographed on a column of silica gel using hexanes−ethyl acetate (2:1) as eluent. The title compound was obtained as a white semisolid (446 mg, 74%). ^{1}H NMR ($CDCl_3$): δ 9.72 (s, 1 H, CHO), 5.25−5.18 (m, 2 H, H-3,4), 5.09−5.01 (t, 1 H, H-2), 4.85−4.75 (m, 2 H, H-1, H-1′), 4.02−3.97 (m, 1 H, H-5), 3.73 (s, 3 H, CO_2Me), 3.65−3.39 (m, 2 H, O*C*H_2CH$_3$), 2.57−2.51 (m, 2 H, H-3′), 1.98−1.92 (m, 2 H, H-2′), 2.03, 2.02, 2.00 (3 s, 9 H, 3 Ac), 1.16 (t, 3 H, J = 7 Hz, CH_3). Anal. ($C_{19}H_{28}O_{12}$) C, H.

Methyl 1-*O*-[(1′*RS*)-1′-Ethoxy-4′-oxobutyl]-*β*-D-glucopyranosyluronate (15b). A solution of methyl 1-*O*-(1′-ethoxypent-4-enyl)-*β*-D-glucopyranosyluronate (**14**) (236 mg, 1.2 mmol) in dichloromethane (20 mL) was ozonized for 5 min as described for **15a**. The resulting ozonide was reduced with dimethyl sulfide (3 × 200 μL, 8.2 mmol) in the presence of sodium bicarbonate (100 mg) over a period of 7 days. The product was purified by column chromatography on silica gel using chloroform−methanol (9:1) as eluent (TLC in the same solvent system, R_f = 0.19). The title compound was obtained as a semisolid (183 mg, 77%), ratio of 1′*R*:1′*S* = 1:1.4. ^{1}H NMR ($CDCl_3$): δ 9.72 (s, 1 H, CHO), 4.87 (t, 0.6 H, H-l′ (*S*)), 4.77 (t,

0.4 H, H-l′ (*R*)), 4.64−4.49 (m, 1 H, H-1), 4.42−4.21 (2 br s, 2 H, 2 OH), 4.1 (br s, 1 H, OH), 3.97−3.40 (m, 9 H, H-2,3,4,5, COOMe, OCH_2CH_3), 2.61 (m, 2 H, H-3′), 2.07 (m, 1 H, H-2′), 1.21 (t, 3 H, OCH$_2CH_3$). MS (FAB): *m*/*z* 393 (M + Na)$^+$.

***N*-[(4″*RS*)-4″-Ethoxy-4″-[methyl 1‴-*O*-(2‴,3‴,4‴-tri-*O*-acetyl-*β*-D-glucopyranuronate)]butyl]daunorubicin (17a).** A solution of 1 M sodium cyanoborohydride in tetrahydrofuran (120 μL, 0.12 mmol) was added to a stirred solution of daunomycin hydrochloride (160 mg, 0.284 mmol) and methyl 1-*O*-(1′-ethoxy-4′-oxobutyl)-2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate (**15a**) (258 mg, 0.576 mmol) in acetonitrile−water (2:1) (40 mL). The mixture was maintained under a nitrogen atmosphere at room temperature in the dark. The progress of reaction was monitored by TLC in system B. After 2 h, another 16 mg of **15a** and 20 μL of the sodium cyanoborohydride solution were added. When reaction was complete, the solution was diluted with water (40 mL) and extracted repeatedly (10 × 40 mL) with chloroform−methanol (4:1). The product was purified by column chromatography on silica gel using chloroform−methanol (97:3) as eluent. It was isolated as a red amorphous film. The yield was 170 mg (76% based on reacted daunorubicin). ^{1}H NMR ($CDCl_3$) (free base): δ 8.02 (dd, 1 H, *J* = 7.8, 1.0 Hz, H-1), 7.82 (t, 1 H, *J* = 8.3 Hz, H-2), 7.39 (dd, 1 H, *J* = 8.3, 1.0 Hz, H-3), 5.53 (d, 1 H, *J* = 2.74 Hz, H-1′), 5.31 (s, 1 H, H-7), 5.29−5.18 (m, 2 H, H-3‴, H-4‴), 5.02 (t, 1 H, H-2‴), 4.82−4.57 (m, 3 H, H-1‴, H-4″, H-5‴), 4.10 (s, 3 H, 4-OCH$_3$), 4.03 (m, 2 H, H-5′, H-4′), 3.75 (s, 3 H, COOMe), 3.78−3.52 (m, 2 H, OCH_2CH_3), 3.45 (m, 1 H, H-3′), 3.24 (d, 1 H, *J* = 9 Hz, H-10b), 2.98 (d, 1 H, *J* = 9 Hz, H-10a), 3.61 (m, 1 H, H-1″), 2.40 (s, 3 H, 14-CH$_3$), 2.34 (m, 2 H, H-8b, H-8a), 2.21−1.94 (m, 2 H, H-2′a, H-2′b), 2.0 (s, 9 H, 3 Ac), 1.89−1.50 (m, 4 H, H-2″, H-3″), 1.39 (d, *J* = 6.5 Hz, 3 H, H-6′), 1.15 (m, 3 H, OCH$_2CH_3$). MS (FAB): *m*/*z* 960 (M + H)$^+$. HRMS: calcd for $C_{46}H_{58}O_{21}N$, 960.3502; found, 960.3493.

The remaining aqueous phase, which contained unreacted daunorubicin, was adjusted to pH 8 with saturated sodium bicarbonate and extracted with chloroform (5 × 50 mL). The combined chloroform extracts were washed with water (5 × 50 mL), dried over sodium sulfate, and evaporated. The product was converted to the corresponding hydrochloride by addition of the calculated amount of 1 M HCl in ether to the compound in chloroform−methanol solution; 28 mg of daunorubicin hydrochloride was obtained.

***N*-[(4″*RS*)-4″-Ethoxy-4″-(1‴-*O*-*β*-D-glucopyranuronate)-butyl]daunorubicin Sodium Salt (6a). Method A.** NaOH solution (0.1 N, 35 mL) was added to a solution of *N*-[(4″*RS*)-4″-ethoxy-4″-[methyl 1‴-*O*-(2‴, 3‴,4‴-tri-*O*-acety)-*β*-D-glucopyranuronate)]butyl]daunorubicin hydrochloride (**17a**) (106 mg, 0.106 mmol) in MeOH (27 mL). The mixture was maintained for 1 h at ambient temperature and was then added to a column of Amberlite IRC-50 CP cation-exchange resin in the H$^+$ form (6.75 g). The column was washed successively with water and MeOH until both eluents were colorless. The eluents were collected in flasks containing 0.1 M triethylamine acetate (23 mL, pH 7) maintained at 0 °C. After all the colored product had eluted, the contents of the flasks were analyzed by HPLC using acetonitrile−phosphate buffer, pH 7.0 (2:1), as mobile phase and TLC (system C). The products in both flasks were the same. The methanol solution was concentrated under reduced pressure and combined with the aqueous eluent. The solution was then frozen and lyophilized. The residue was taken up in water (2 mL) and applied to a column of weakly basic cation-exchange resin (Biorex 70, Na$^+$, 3.1 g). The column was washed with water until no more color eluted. The combined washings were extracted with ethyl acetate (5 × 150 mL) and the remaining aqueous layer was frozen and lyophilized to afford the title compound. The yield was 28.8 mg (31%). MS (FAB, glycerol): *m*/*z* 842 (M + H)$^+$. HRMS: calcd for $C_{39}H_{49}NNaO_{18}$, 842.2906; found, 842.2915.

Method B. Sodium methoxide solution (0.1 N) in dry MeOH (15 mL) was added to a solution of *N*-[(4″*RS*)-4″-ethoxy-4″-[methyl 1‴-*O*-(2‴,3‴,4‴-tri-*O*-acetyl)-*β*-D-glucopyranuronate)]-butyl]daunorubicin (**17a**) (150 mg, 0.15 mmol) in dry MeOH (2 mL). After 25 min at 0 °C, the reaction was complete as evidenced by TLC in systems B and C. The mixture was neutralized with Amberlite IR-120 and filtered, and the resin was washed with MeOH. The filtrates were combined and evaporated. The crude residue of **17c** was dissolved in MeOH (8 mL), and 0.1 N NaOH solution (4.5 mL) was added. The mixture was stirred at room temperature for 30 min. When reaction was complete (TLC in system C) the reaction mixture was neutralized with Amberlite IRC-50. The mixture was filtered into a flask containing ice-cold 0.1 M triethylamine acetate solution (20 mL), and the resin was washed with MeOH until colorless. The combined filtrates were evaporated at 0 °C in vacuo. The residue was dissolved in water (20 mL), and weakly basic cation-exchange resin (Biorex 70, Na$^+$, 3 g) was added. The mixture was filtered, and the resin was washed with water. The combined filtrates were extracted repeatedly with ethyl acetate until the ethyl acetate layer was colorless. The remaining aqueous solution was frozen and lyophilized. The title compound was obtained as a red amorphous powder. HPLC of the product using acetonitrile−phosphate buffer, pH 7.0 (2:1), as eluent indicated that it was 99.2% pure. The yield was 57.8 mg (43.8%).

***N*-[(4″*RS*)-4″-Ethoxy-4″-[methyl 1‴-*O*-(2‴, 3‴,4‴-tri-*O*-acetyl-*β*-D-glucopyranuronate)]butyl]doxorubicin Hydrochloride (17b).** This compound was prepared from doxorubicin hydrochloride (50 mg, 0.086 mmol), methyl 1-*O*-(1′-ethoxy-4′-oxobutane)-2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate (**15a**) (77 mg, 0.172 mmol), and sodium cyanoborohydride (20 μL of a 1 M solution in tetrahydrofuran, 0.02 mmol) in acetonitrile−water (2:1) (10 mL) as described for **17a**. When reaction was complete (as evidenced by TLC in system B), the solution was diluted with water (20 mL) and extracted repeatedly (10 × 20 mL) with chloroform−methanol (4:1). The combined extracts were dried and evaporated to give a red film which was purified by column chromatography on silica gel in chloroform−methanol (97:3). The product (23 mg) was dissolved in chloroform−methanol (4:1) (3 mL), and the calculated amount of 1 M hydrogen chloride in ether was added. The solution was evaporated and dried in vacuo to afford the title compound. It was stored under nitrogen in a tightly stoppered vessel at −78 °C in the dark. Yield: 25 mg, 56% (based on 25 mg of reacted doxorubicin). ^{1}H NMR ($CDCl_3$) (free base): δ 8.02 (dd, 1 H, *J* = 7.6, 1.0 Hz, H-1), 7.76 (t, *J* = 8.3 Hz, 1 H, H-2), 7.36 (dd, 1 H, *J* = 8.3, 1.0 Hz, H-3), 5.50 (s, 1 H, H-1′), 5.29 (s, 1 H, H-7), 5.27−5.12 (m, 2 H, H-3‴, H-4‴), 5.00 (t, 1 H, H-2‴), 4.83−4.69 (m, 4 H, H-4″, H-1‴, H-14), 4.12 (m, 1 H, H-5‴), 4.04 (s, 3 H, 4-OCH$_3$), 4.03−3.91 (m, 2 H, H-5′,4′), 3.75−3.36 (m, 3 H, H-3′, OCH_2CH_3), 3.71 (s, 3 H, CO$_2$-Me), 3.26 (d, 1 H, *J* = 10 Hz, H-10a), 3.02 (d, 1 H, *J* = 10 Hz, H-10b), 2.62 (m, 2 H, H-1″), 2.34 (m, 1 H, H-8a), 2.12 (m, 1 H, H-8b), 2.00 (3 s, 9 H, 3 Ac), 1.82−1.40 (m, 6 H, H-2″, H-3″, H-2′a, H-2′b), 1.36 (d, *J* = 6 Hz, 3 H, H-6′), 1.13 (m, 3 H, OCH$_2CH_3$). An amount of 25 mg of doxorubicin hydrochloride was recovered from the reaction mixture as described for daunorubicin (see **17a** preparation).

***N*-[(4″*RS*)-4″-Ethoxy-4″-(methyl 1‴-*O*-*β*-D-glucopyranuronate)butyl]doxorubicin (17d). Method A.** Sodium cyanoborohydride (1 M, 30 μL, 0.03 mmol) was added to a solution of methyl 1-*O*-(1′-ethoxy-4′-oxobutyl)-*β*-D-glucopyranosyluronate (**15b**) (25 mg, 0.078 mmol) and doxorubicin hydrochloride (38 mg, 0.065 mmol) in acetonitrile−water (2:1) (15 mL) under nitrogen. The mixture was stirred at room temperature in the dark for 2 h. When reaction was complete as evidenced by TLC (system C) or HPLC (using 0.05 M ammonium acetate−acetonitrile (6:4) as mobile phase), the solution was diluted with water (10 mL) and extracted repeatedly (10 × 10 mL) with chloroform−methanol (4:1). The combined extracts were dried and evaporated to give a red film. Preparative TLC of the product in system C indicated a mixture of several compounds. The compound with R_f = 0.42 was shown by NMR and MS to be the desired product. Yield: 1.8 mg, 3.2%. ^{1}H NMR (CD_3OD) (free base): δ 7.86 (m, 1 H, H-1), 7.82 (t, 1 H, H-2), 7.57 (m, 1 H, H-3), 5.49−5.44 (m, 1 H, H-1′), 5.05 (m, 1 H, H-7), 4.9−4.2 (m, 4 H, H-4″, H-1‴, H-14), 4.03 (s, 3 H, 4-OCH$_3$), 3.85−3.65 (m, 7 H, H-2‴, H-3‴, H-4‴, H-5‴, CO$_2$Me), 3.65−3.15 (m, 4 H, H-4′, H-5′, OCH_2CH_3), 3.15−2.55 (m, 5 H, H-10a, H-10b, H-3′, H-1″), 2.4−1.9 (m, 6 H, H-8a, H-8b, H-2′a, H-2′b, H-3″), 1.8−1.6 (m, 2 H, H-2″), 1.30 (d, *J* = 6 Hz, 3 H, H-6′), 1.18−1.08 (m, 3 H, OCH$_2CH_3$).

MS (FAB): *m/z* 850 $(M + H)^+$. HRMS: calcd for $C_{40}H_{52}NO_{18}$, 850.3192; found, 850.3189.

Method B. A solution of *N*-[4″-ethoxy-4″-[methyl 1‴-*O*-(2‴,3‴,4‴-tri-*O*-acetyl-*β*-D-glucopyranuronate)]butyl]doxorubicin (**17b**) (5.5 mg, 5.6 mmol) in dry MeOH (10 mL) was cooled to 0 °C, and a solution of sodium methoxide (1.04 mg, 19 mmol) in MeOH (10 mL) was added. The mixture was stirred at 0 °C for 3.5 h and then neutralized with Amberlite IRC-50. The filtered solution was evaporated to dryness and purified by preparative TLC in system C to give **17d**. Yield: 0.1 mg (2%).

Stability Studies of 6a in 0.05 M Phosphate Buffer, pH 7.4, in the Absence or Presence of *E. coli* β-Glucuronidase. The enzyme was purchased from Sigma Chemical Co., St. Louis, MO, and used as received. The specific activity of the preparation was 1000 units/mg of protein, where 1 unit is defined as the amount that will liberate 1.0 μg of phenolphthalein from phenolphthalein glucuronide per hour at 37 °C at pH 6.8. **6a** was dissolved in 0.05 M phosphate buffer, pH 7.4, at a concentration of 12 μM. One-milliliter aliquots of this solution contained in 5.0-mL screw-capped glass vials were incubated at 37 °C. At selected time intervals (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 9.0, and 20 h) aliquots (100 mL) were removed and analyzed immediately for parent drug by HPLC using, as mobile phase, a solution of CH_3CN−0.05 M phosphate buffer, pH 7.4 (1:1), at a flow rate of 1.0 mL/min. The retention time of the parent drug (**6a**) was 1.82 min. For the enzyme studies, 197 units of β-glucuronidase was used per μmole of substrate. The enzyme was added to 0.2 mL of the drug solution contained in 1.5-mL microcentrifuge tubes that were preequilibrated at 37 °C. At intervals of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, and 6.0 h, aliquots (100 mL) were removed and analyzed immediately by HPLC for parent compound and metabolite 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 5.76 min. The half-lives were determined by linear least-squares regression analysis of the pseudo-first-order reactions. An absorbance/concentration standard curve for **6a** was generated by HPLC analysis of solutions of concentration 12.0, 2.4, 1.2, 0.24, and 0.012 μM.

Stability Studies of 1a (n = 3) in the Absence or Presence of Porcine Liver Carboxylate Esterase. The enzyme was obtained from Sigma Chemical Co. and 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 EtOH per minute at pH 8.0 and 25 °C. Compound **1b** was dissolved in 0.05 M phosphate buffer, pH 7.4, at a concentration of 13 μM and then incubated in either the absence or presence of the enzyme. For the enzyme studies, 15 units of esterase/μmol of substrate was used. The incubates were analyzed by HPLC using CH_3CN−0.05 M phosphate buffer, pH 7.4 (1:1), as mobile phase at a flow rate of 1.0 mL/min. The disappearance of the parent compound was accompanied by a progressing increasing metabolite peak with a retention time of 5.76 min.

Cytotoxicity of 6a to Human Melanoma Cells in Culture. A375 human melanoma cells were grown in Hank's minimal essential medium (MEM; Gibco) containing 10% fetal bovine serum albumin (Atlanta Biologics). They were passaged weekly by trypsinization (Gibco) and plated at reduced density (50 000 cells/mL). The cells were routinely tested and found to be free of mycoplasma contamination. For these experiments, the cells were harvested by trypsinization and plated in 96-well plates at a density of 5000 cells/well in 100 μL of MEM culture media. The cells were allowed to adhere by incubation for 24 h at 37 °C in humidified 5% CO_2 and then treated in MEM solution with the following: (a) enzyme alone (*E. coli* β-glucuronidase, 125 units/mL), (b) **6a** alone at concentrations ranging from 10^{-5}−10^{-13} M, (c) **6a** plus enzyme (same concentrations as above) mixed immediately before adding to cells, and (d) **6a** plus enzyme (same concentrations as above) preincubated at 37 °C for 4 h before adding to cells. The plates were incubated for a further 72 h at 37 °C in a humidified CO_2 incubator. The wells were emptied, and the remaining cells were stained by addition of 100 μL of 0.5% crystal violet (Fisher Scientific) in 20% methanol (Fisher Scientific). After incubation for 20 min, the dye was removed and the wells were washed with deionized water. The cell-bound dye was solubilized in Sorenson's buffer (0.1 M sodium citrate, 50% (v:v) ethanol, pH 4.2) and quantitated on an ELISA microplate autoreader at 540 nm. The results were plotted as percentage of control cell survival versus molar concentration.

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