Articles

Prodrugs of Anthracyclines for Use in Antibody-Directed Enzyme Prodrug Therapy

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A series of new prodrugs of daunorubicin and doxorubicin which are candidates for antibody-directed enzyme prodrug therapy (ADEPT) is reported. These compounds (**25a,b,c** and **32a,b,c**) have been designed to generate cytotoxic drugs after activation with β-glucuronidase. As expected, recovery of the active drug was observed after enzymatic cleavage by *Escherichia coli* β-glucuronidase as well as by a fusion protein which has been obtained from human β-glucuronidase and humanized CEA-specific binding region. The six prodrugs are highly stable and are more than 100-fold less cytotoxic than doxorubicin against murine L1210 cell lines. The ortho-substituted phenyl carbamates **25a,b,c** are better substrates for β-glucuronidase than the corresponding para-substituted analogues. After taking into account additional factors such as stability in plasma and kinetics of enzymatic cleavage, we selected the *o*-nitro prodrug **25c** for clinical trials.

The clinical efficacy of most antitumor drugs is limited to varying degrees by side effects resulting from the lack of selectivity and the appearance of acquired resistance. Rational design to avoid these side effects includes strategies such as drug targeting and prodrug synthesis. Ideally, the activation of a prodrug should be restricted to the required site of action, the tumor cell.

In this regard, the combination of prodrugs with tumor-specific enzymes for use as therapeutic agents was reported in 1978 by Connors[1] and re-emphasized in the case of antitumor drugs by Bagshawe[2] and Senter,[3] who termed the approach "antibody-directed enzyme prodrug therapy (ADEPT)". The ADEPT strategy, which has been frequently and recently reviewed,[4−17] entails the use of *monoclonal antibodies* (MAbs) directed against a particular tumor and covalently bonded to a prodrug-cleaving enzyme. Thus, the antibody−enzyme conjugate is first injected and localized at the tumor cell surface antigen. Subsequently, the noncytotoxic prodrug is administered, and the cytotoxic species is released on the tumor cell surface.

In this context, a large number of prodrugs have recently been developed that can be transformed into active anticancer drugs by enzymes[18] of both mammalian and nonmammalian origin. Very recently, this concept was used to target farnesyltransferase inhibitors.[19]

For our part, we have been interested in developing prodrugs of the antitumor antibiotic doxorubicin[20] which should be converted into the corresponding drug in the presence of glycosidases such as α-galactosidase or β-glucuronidase. Actually, we started our preliminary investigations in the field of α-galactosyl derivatives. Taking into account the fact that 14-*O*-galactoside(or 14-*O*-glucuronide)−doxorubicin derivatives,[21] as well as epirubicin glucuronide,[22,23] were poor substrates for these enzymes, we decided to prepare prodrugs including a self-immolative spacer between the drug and the sugar moiety. Two types of prodrugs were synthesized bearing either an *o*(or *p*)-hydroxybenzylcarbamate linkage[24,25] or a bis-carbamate linkage[26] between the galactosyl residue and the doxorubicin. As expected, from these experiments it appeared that both spacers spontaneously decompose after enzymatic cleavage.[27] However, introduction of an electron-withdrawing substituent in the ortho (or para) position to the phenol was required[25] in the first case to start the self-immolative process.

Simultaneously, some of us tried to prepare a stable fusion protein, consisting of the humanized Fab fragment of the anti-CEA MAb BW 431 and a human lysosomal enzyme.[29] As the fusion protein prepared from α-galactosidase proved to be unstable whereas the corresponding fusion protein[28] including the human β-glucuronidase[29] as enzymatic component proved to be highly efficient, we turned our attention toward the glucuronyl prodrugs of doxorubicin. Considering that the doxorubicin−galactosyl prodrugs including a substituted hydroxybenzylcarbamate linkage ideally fulfill

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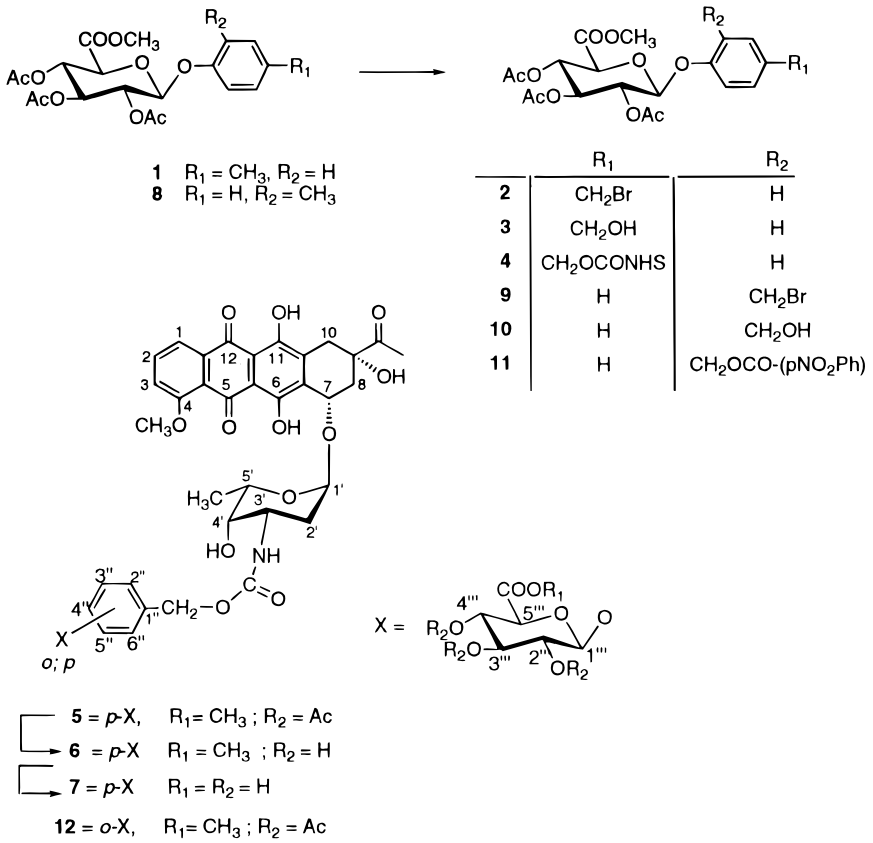
Scheme 1



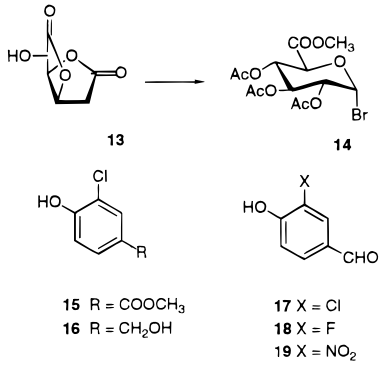
several of the prerequisite conditions (stability, low cytotoxicity, fast enzymatic cleavage...) for ADEPT, our next idea was to develop the corresponding prodrug analogues in the glucuronyl series. As in the case of galactosidase prodrugs, it was expected that the absence of high serum β-glucuronidase activities in humans should minimize premature activation of these new prodrugs. The generally low toxicity[30] of glucuronide prodrugs constitutes another advantage for their use.

Chemistry

The first two prodrugs (**7** and **12**) were prepared (Scheme 1) from the readily accessible methyl (*o*- or *p*-tolyl tri-*O*-acetyl-β-D-glucopyranosyl)uronates **1** and **8**.[31] Treatment of these compounds with *N*-bromosuccinimide under reflux and irradiation afforded **2** (75%) and **9** (80%) which were subsequently converted[32] to the corresponding alcohols **3** and **10** ($AgNO_3$, acetone$-H_2O$). These intermediates were, in turn, converted to their active esters **4** (52%) and **11** (73%) by the use of *N*,*N*′-disuccinimyl carbonate (DSC) or 4-nitrophenyl chloroformate, respectively. Coupling of **4** or **11** with daunorubicin was next carried out (65−70% yield) by reacting with daunorubicin (DMF, Et_3N) to give **5** or **12**. Deprotection of **5** was achieved in 50% overall yield following a two step procedure successively using NaOMe−MeOH (**5** → **6**) and BaO in MeOH (**6** → **7**). Glycoside **12** was only deprotected in situ during in vitro test of enzymatic behavior.

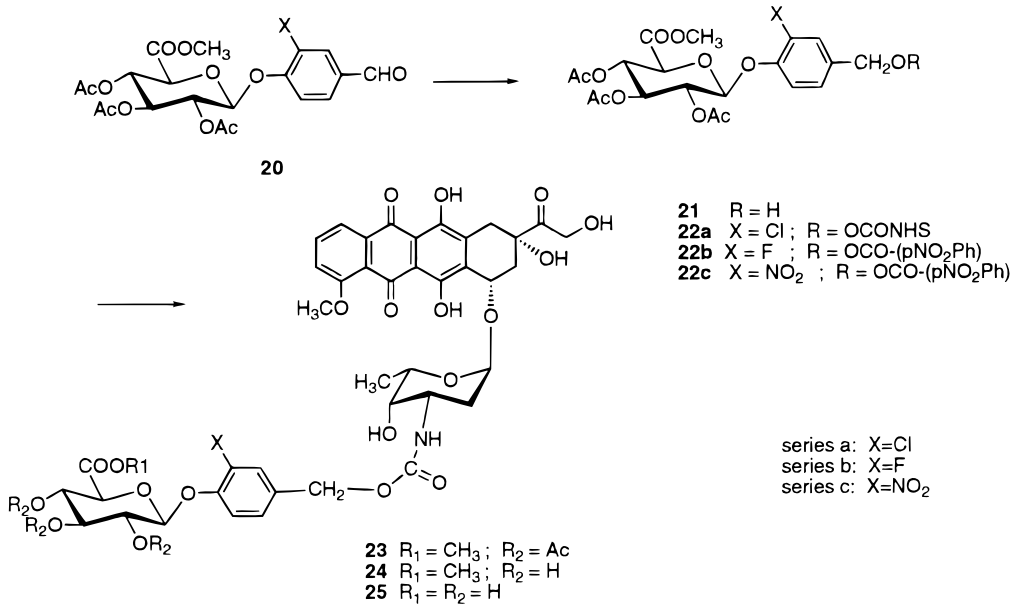
The general pathway (Scheme 3) for preparing prodrugs containing an ortho-substituted phenyl ring, i.e., **25a,b,c** involved the initial coupling of the methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate (**14**)

Scheme 2

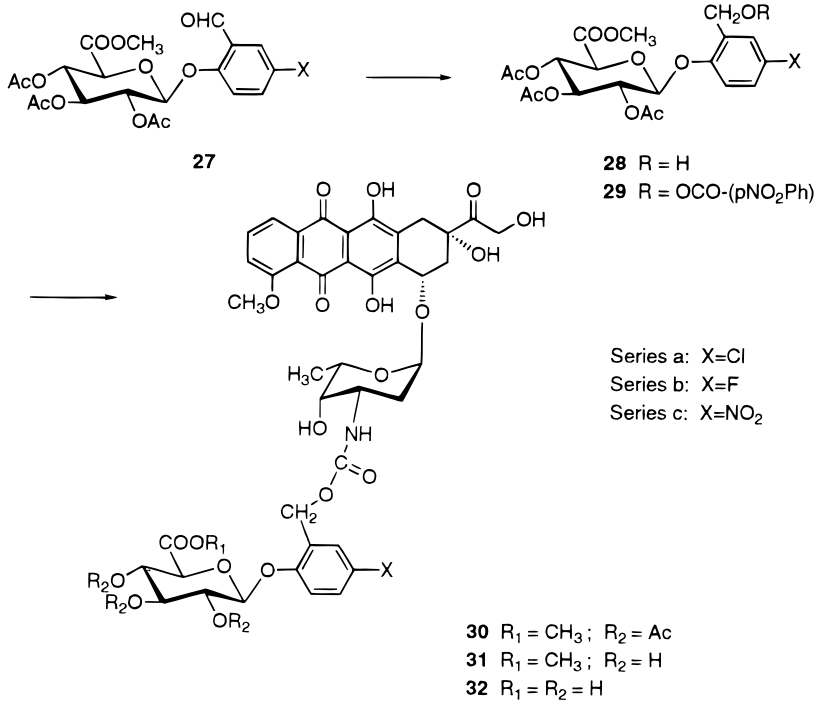


[easily prepared from D-glucuronolactone (**13**) by ring opening with MeONa, then peracetylation and bromination (HBr/AcOH)] with the phenol derivatives **17**−**19** (Scheme 2) in the presence of silver oxide. Whereas **18** and **19** were commercially available, the chlorophenol derivative **17** had to be prepared from the corresponding carbomethoxy analogue **15** by reduction (LAH, THF) to **16** (93% yield) and pyridium chlorochromate oxidation (PCC, EtOAc) of **16** to **17** (77% yield). The glycosides **20a,b,c** thus obtained under Königs−Knorr conditions were reduced with $NaBH_4$ in $CHCl_3$−*i*PrOH to afford the corresponding alcohol derivatives **21a,b,c**. Alcohol **21a** was activated as *N*-hydroxysuccinimido carbonate **22a** and **21b,c** as their 4-nitrophenyl carbonates **22b,c**. Coupling with doxorubicin led to glycosides **23a,b,c**. Deprotection, realized in two steps (NaOMe−MeOH at 0 °C, then 2 N aqueous NaOH), successively gave the partially deprotected and, then, the fully deprotected prodrugs **24a,b,c** and **25a,b,c**, respectively.

Scheme 3



Scheme 4



When the same sequence of reactions was applied (Scheme 4) to the phenolic regioisomers of **17**−**19**, this provided the corresponding para-substituted phenyl prodrugs **32a,b,c**.

Biological Data

All the prodrugs exhibit a strongly reduced cytotoxicity against the L1210 cell line compared to doxorubicin and, in this respect, are well-suited for use in the ADEPT concept. Even the most cytotoxic prodrug (Table 1), the *o*-nitro-substituted derivative **25c**, remains almost 100-fold less toxic (2.21 μM) than doxorubicin (0.03 μM). The hydrolysis of prodrugs **7** and **12** was not investigated in detail because the intermediate resulting after glucuronidase cleavage was not self-immolative.

Table 1. Growth Inhibition of L1210 Leukemia Cells in Vitro by Anthracycline Prodrugs and Doxorubicin

compd	IC_{50} (μM)[a,b]
7[c]	>10
12[c]	5.5
25a	17
25b	8.8
25c	2.21
32a	16.6
32b	31
32c	16.6
doxorubicin	0.03

[a] Drug concentration that inhibited cell growth by 50% compared to untreated control cultures. [b] MTT reduction. [c] After in situ deprotection.

In the presence of 0.1 μg/mL β-glucuronidase of *Escherichia coli*, ortho-substituted prodrugs **25a,b,c** and

Table 2. Kinetics of Enzymatic Hydrolysis[a,b]

	concentration (μg/mL)		
prodrug	prodrug	β-gluc[c]	halflife (min)
25a	200	0.1	55
25b	200	0.1	26
25c	200	0.1	37
32a	200	0.1	>240
32a	200	1	30
32b	200	0.1	30
32c	200	0.1	216

[a] In 0.1 M phosphate buffer, pH 7, + 0.01% BSA. [b] 37 °C. [c] β-Glucuronidase *E. coli* (Sigma, 8346853).

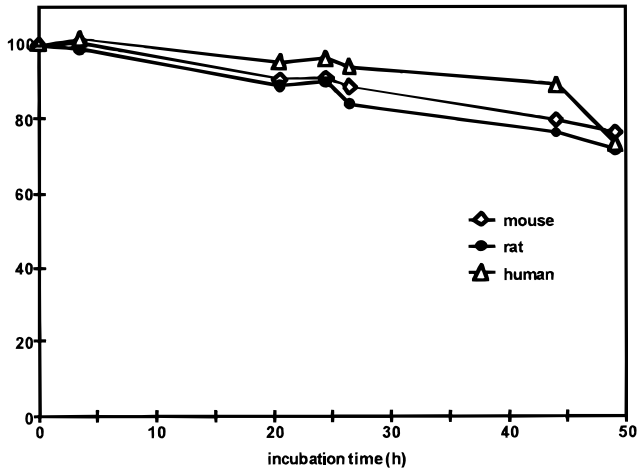


Figure 1.

the para-substituted prodrug **32b** were completely or almost completely hydrolyzed (Figure 1 in Supporting Information) within 150 min to generate free doxorubicin (Table 2). It must be noticed that, in the case of **32a,c**, the spacer was slowly removed. In both cases after 150 min, doxorubicin was detectable in relative low yield along with a significant amount of another product postulated as the intermediate doxorubicin−spacer. In the case of **32a**, kinetics of enzymatic hydrolysis may be improved by using a 10-fold higher concentration of enzyme.

From these data, it clearly appears, in relation with the kinetics of enzymatic hydrolysis, that ortho-substituted prodrugs are more conveniently hydrolyzed in the presence of β-glucuronidase of *E. coli* than the corresponding para-substituted analogues.

All the prodrugs are highly stable in plasma. Thus, when checked in the plasma after 24 h, prodrug **25c** was recovered in more than 90% yield and prodrugs **25a,b** in 80−85%. As an example, the stability of **25c** has already been reported (Figure 1).[35]

Discussion

The experimental results clearly indicate that prodrug **25c** is the most appropriate compound for a more complete biological evaluation. Consequently, compound **25c** has been prepared for clinical trials under the development name HMR 1826. The kinetics of cleavage of **25c** by fusion protein in vitro has also been studied. Addition of fusion protein to a solution of prodrug resulted in a quick disappearance of prodrug (Figure 2 in Supporting Information). Other experiments have shown[33,34] that superior therapeutic effects can be obtained by combination of fusion protein and HMR 1826 in LoVo colon carcinoma tumor-bearing nude mice. Moreover, LD_{50} values in mice (500−770 mg/kg) and in rats (370−950 mg/kg) as well as nondetectable toxicity in Cynomolgus monkeys using the LD_{10} equivalent dose of the mouse (60 mg/kg)[35] clearly demonstrate the low general toxicity of the prodrug. Double of the LD_{10} equivalent dose (120 mg/kg) is well-tolerated by the monkeys as well. Reduced cardiotoxicity may indeed be expected by combined application of fusion protein and prodrug, since there is at least a 5-fold lower drug concentration in normal tissues compared to that of the animals treated with doxorubicin and a 4−12-fold higher drug concentration in the tumor. This increased liberation in tumors also results in strong antitumoral effects even in cancers resistant to doxorubicin.[35]

Improved interest in HMR 1826 resulted from subsequent studies[36,37] supporting the hypothesis than human lysosomal β-glucuronidase becomes accessible to the prodrug in necrotic tumor cells, allowing a tumor-selective prodrug monotherapy (PMT). Since it is also known that β-glucuronidase, like others lysosomal enzymes, is released into the synovial fluid in inflammatory diseases,[38] efficacy of HMR 1826 has also been demonstrated in the arthritic rat model.[35] Such efficacy may also be expected in metastases of adenocarcinoma which are mediated in part by the release of this enzyme from the cancer cells.[39]

Experimental Section

Chemistry. Melting points were determined using an Electrothermal apparatus and are uncorrected. UV spectra were determined on a Varian-Cary/3E spectrophotometer. IR spectra were obtained with a Perkin-Elmer 1710 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded in the given solvent with a Bruker AC-250 spectrometer or with a Bruker AC500 spectrometer. Chemical shifts are reported as δ values in parts per million. The splitting pattern abbreviations are as follows: s = singlet, d = doublet, dd = double doublet, dt = double triplet, t = triplet, br = broad, m = multiplet. Routine chemical ionization (CI) mass spectra were recorded on a Nermag R 10,10C spectrometer. Other mass spectra, FAB and electrospray, were recorded on a JEOL JMS 700 or a Platform II Micromass spectrometer, respectively. Elemental analyses, performed by the Service de Microanalyze du CNRS (Vernaison-Lyon, France), were within 0.4% of the theoretical values calculated for C, H, and N. The thin-layer chromatographic analyses were performed using precoated silica gel ($60F_{254}$) plates, and the spots were examined with UV light and phosphomolybdic acid spray. Column chromatography was carried out on Merck silica gel (230−240 mesh). Extraction in the usual manner refers to washing the organic layer with water, drying it over $MgSO_4$, and evaporating the solvent under reduced pressure.

Methyl (4-methylphenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside)uronate (1): prepared according to Bollenback et al.;[31] mp 138−139 °C; ^{1}H NMR ($CDCl_3$) δ 7.12 (d, 2H, J = 8 Hz, Ar), 6.91 (d, 2H, J = 8 Hz, Ar), 5.35−5.25 (m, 3H, H-2, H-3, H-4), 5.08 (d, 1H, J = 7.5 Hz, H-1), 4.15 (m, 1H, H-5), 3.73 (s, 3H, $COOCH_3$), 2.30 (s, 3H, CH_3), 2.06, 2.05, and 2.04 (3s, 3 × 3H, OAc); lit.[31] mp 137−138 °C; $[\alpha]^{20}_D$ −31.3°.

Methyl (4-(Bromomethyl)phenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside)uronate (2). To a solution of **1** (5 g) in CCl_4 (100 mL) was added *N*-bromosuccinimide (2 g) prior to treatment under reflux and irradiation (1000 W) for 15 min. After cooling and filtration, the filtrate was concentrated to give 4.8 g of crude product which was purified by flash chromatography (hexanes−EtOAc, 80:20, v/v). Compound **2** was isolated in 75% yield as a crystalline compound: mp 144−146 °C; $[\alpha]^{20}_D$ +3° (*c* 0.85, $CHCl_3$); IR ($CHCl_3$) ν 1758 cm^{-1} (CO);

^{1}H NMR ($CDCl_3$) δ 7.33−6.89 (2d, 4H, Ar), 5.36−5.03 (m, 4H, H-1, H-2, H-3, H-4), 4.43 (s, 2H, CH_2Br), 4.16 (m, 1H, H-5), 3.66 (s, 3H, $COOCH_3$), 2.00 (s, 9H, 3 OAc); MS (CI) *m/z* 522, 520 $(M + NH_4)^+$. Anal. ($C_{20}H_{23}BrO_{10}$) C, H.

Methyl (4-(Hydroxymethyl)phenyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranoside)uronate (3). A solution of **2** (1.2 g, 2.3 mmol) in a mixture of acetone and water (400 mL, 1/1, v/v) was stirred at room temperature for 1 h in the presence of silver nitrate (660 mg, 3.9 mmol).[32] The resulting suspension was filtrated, and the filtrate was washed with water and brine. Evaporation of the organic layer under reduced pressure, followed by chromatography (hexanes−EtOAc, 2/1, v/v), afforded **3** (650 mg, 58%) which crystallized from MeOH: mp 134−136 °C; $[\alpha]^{20}_D$ −45° (*c* 0.5, $CHCl_3$); IR (KBr) ν 3456 (OH), 1758 (CO) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.20 and 6.86 (2d, 4H, Ar), 5.53−4.96 (m, 4H, H-1, H-2, H-3, H-4), 4.53 (s, 2H, CH_2), 4.20−3.96 (m, 1H, H-5), 3.63 (s, 3H, $COOCH_3$), 1.96 (s, 9H, 3 OAc); MS (CI) *m/z* 458 $(M + NH_4)^+$. Anal. ($C_{20}H_{24}O_{11}$) C, H.

4-*O*-((Methyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate)benzyloxycarbonyl)-*N*-succinimide (4). To a solution of **3** (170 mg, 0.39 mmol) in anhydrous dichloromethane (10 mL) containing Et_3N (54 μL, 0.39 mmol) was added *N*,*N*′-disuccinimidyl carbonate (197 mg, 0.77 mmol) in acetonitrile (8 mL). After stirring at room temperature for 24 h and filtration, the organic layer was concentrated under reduced pressure, affording **4** as a syrup (200 mg, 90% yield): $[\alpha]^{20}_D$ +3° (*c* 0.85, $CHCl_3$); IR ($CDCl_3$) ν 1747 (CO) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.44−6.96 (m, 4H, Ar), 5.44−5.13 (m, 6H, CH_2Ph, H-2, H-3, H-4, H-5), 4.24 (d, 1H, *J* = 9 Hz, H-1), 3.73 (s, 3H, $COOCH_3$), 2.85 (s, 2H, CH_2), 2.08 (s, 9H, 3 OAc); MS (CI) *m/z* 599 $(M + NH_4)^+$.

***N*-[4-*O*-(Methyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate)benzyloxycarbonyl]daunorubicin (5).** A solution of **4** (68 mg, 0.12 mmol) and daunorubicin (56 mg, 0.12 mmol) in *N*,*N*′-dimethylformamide (4 mL) was stirred for 15 min at room temperature in the presence of triethylamine (40 μL, 0.2 mmol). The reaction mixture was diluted with water (≈5 mL) and extracted with ether (200 mL). Evaporation of the organic solvent, followed by flash chromatography (hexane−acetone, 1:1, v/v), gave **5** (80 mg, 70%) as a syrup: $[\alpha]^{20}_D$ +112° (*c* 0.06, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 14.00 (s, 1H, PhOH), 13.33 (s, 1H, PhOH), 8.07 (d, 1H, *J* = 8 Hz, H-3), 7.83 (dd, 1H, *J* = *J*′ = 8 Hz, H-2), 7.43 (d, 1H, *J* = 8 Hz, H-1), 7.28 (d, 2H, *J* = 8 Hz, Ar), 6.98 (d, 2H, *J* = 8 Hz, Ar), 5.53 (d, 1H, *J* = 3.5 Hz, H-1′), 5.38−5.35 (m, 2H, H-3‴, H-4‴), 5.35 (d, 1H, *J* = 7 Hz, H-1‴), 5.30−5.25 (m, 2H, H-7, H-5‴), 5.14 (m, 1H, H-5), 5.00 (s, 2H, CH_2Ph), 4.19 (m, 1H, NH), 4.12 (s, 3H, OCH_3), 3.92 (br s, 1H, OH), 3.73 (s, 3H, $COOCH_3$), 3.70 (m, 1H, H-4′), 3.26 (d, 1H, *J* = 20 Hz, H-10a), 2.97 (d, H, *J* = 20 Hz, H-10b), 2.44 (s, 3H, $COCH_3$), 2.38 (d, 1H, H-8a), 2.18 (d, 1H, *J* = 16 Hz, H-8b), 2.07 (s, 9H, 3 OAc), 1.95 (dd, *J* = 12, *J*′ = 5 Hz, H-2′a), 1.77 (dd, *J* = 12, *J*′ = 5 Hz, H-2′b), 1.74 (s, 1H, OH), 1.32 (d, 3H, *J* = 6 Hz, CH_3-6′); MS (FAB) *m/z* 1016 $(M + Na^+)$. Anal. ($C_{48}H_{51}NO_{22}$) C, H, N.

***N*-[4-*O*-(Methyl *β*-D-glucopyranosyluronate)benzyloxycarbonyl]daunorubicin (6).** A solution of **5** (100 mg, 0.10 mmol) in 10 mL of a 0.1 N solution of sodium methoxide in methanol was stirred for 30 min at 0 °C and then neutralized by addition of Amberlite IRC 120 H^+ ion-exchange resin. Filtration followed by evaporation afforded 90 mg of crude compound which was purified by flash chromatography (CH_2Cl_2−MeOH, 9:1, v/v) to give 67 mg (78%) of **6** as a syrup: $[\alpha]^{20}_D$ +140° (*c* 0.02, MeOH); MS (FAB) *m/z* 890 $(M + Na^+)$. Anal. ($C_{42}H_{45}NO_{19}$) C, H, N.

***N*-[4-*O*-(*β*-D-Glucopyranosyluronic acid)benzyloxycarbonyl]daunorubicin (7).** A methanolic solution of **6** (10.8 mg, 0.012 mmol in 5 mL) was stirred at room temperature for 3 h in the presence of barium oxide (8 mg). As for **6**, the solution was neutralized with Amberlite and the filtrate was evaporated to dryness, thus affording **7** (7.8 mg, 78%) as an amorphous solid residue: $[\alpha]^{20}_D$ +2° (*c* 0.06, MeOH); ^{1}H NMR (400 MHz, pyridine) δ 7.97 (d, 1H, *J* = 10 Hz, H-3), 7.82 (dd, 1H, *J* = *J*′ = 10 Hz, H-2), 7.56 (d, 1H, *J* = 10 Hz, H-1), 5.50 (dd, 1H, *J* = 9, *J*′ = 6 Hz, H-1′), 5.13 (bs, 1H, H-7), 4.70−4.50 (m, 3H, H-5″, H-4″, H-3″), 4.05 (s, 3H, OCH_3), 3.60−3.40 (m, 2H, H-5′, H-4′), 3.05 (d, 1H, *J* = 19 Hz, H-10a), 2.92 (d, 1H, *J* = 19 Hz, H-10b), 2.35 (s, 3H, CH_3CO), 2.25−1.50 (m, 4H, CH_2-2′, CH_2-8); MS (FAB) *m/z* 876, $(M + Na^+)$.

Methyl (2-methylphenyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranoside)uronate (8): prepared according to Bollenback et al.;[31] mp 133 °C; $[\alpha]^{20}_D$ −38.5° (*c* 1, $CHCl_3$); IR (CH_2Cl_2) ν 1750 cm^{-1} (OAc); ^{1}H NMR ($CDCl_3$) δ 7.12 (m, 2H, Ar), 6.98 (m, 2H, Ar), 5.35 (m, 3H, H-2, H-3, H-4), 5.07 (d, 1H, *J* = 7.5 Hz, H-1), 4.18 (d, 1H, *J* = 8 Hz, H-5), 3.74 (s, 3H, CH_3), 2.17 (s, 3H, $ArCH_3$), 2.10, 2.07, and 2.05 (3s, 3 × 3H, OAc); lit.[31] mp 138−140 °C; $[\alpha]^{20}_D$ −40.6° (*c* 1, $CHCl_3$).

Methyl (2-(bromomethyl)phenyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranoside)uronate (9): obtained from **8** as described for the preparation of **2** and isolated as crystals in 80% yield after flash chromatography (hexanes−EtOAc, 2:1, v/v); mp 123 °C; $[\alpha]^{20}_D$ + 13° (*c* 1, $CHCl_3$); IR (CH_2Cl_2) ν 1755 cm^{-1} (OAc); ^{1}H NMR ($CDCl_3$) δ 7.32 (m, 2H, Ar), 7.05 (m, 2H, Ar), 5.38 (m, 3H, H-2, H-3, H-4), 5.23 (m, 1H, H-1), 4.65 and 4.35 (2d, 2H, *J* = 12 Hz, CH_2Br), 4.19 (d, 1H, *J* = 9 Hz, H-5), 3.74 (s, 3H, CH_3), 2.11, 2.07, and 2.05 (3s, 3 × 3H, OAc); HRMS (FAB) *m/z* calcd for ($C_{20}H_{23}BrO_{10}$ + Li)$^+$ 509.0634, found 509.0630.

Methyl (2-(Hydroxymethyl)phenyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranoside)uronate (10). Solvolysis of **9** (2.2 g, 0.43 mmol) under conditions similar to those described for the preparation of **3** from **2** afforded **10** (1.52 g, 80% yield) as crystals: mp 143−147 °C; $[\alpha]^{20}_D$ − 26° (*c* 0.9, $CHCl_3$); IR (CH_2Cl_2) ν 3540 (OH), 1760, 1225 cm^{-1} (OAc); ^{1}H NMR ($CDCl_3$) δ 7.34 (t, 1H, *J* = 7.5 Hz, Ar), 7.29 (d, 1H, *J* = 7.5 Hz, Ar), 7.11 (t, 1H, *J* = 7.5 Hz, Ar), 7.02 (d, 1H, *J* = 7.5 Hz, Ar), 5.35 (m, 3H, H-2, H-3, H-4), 5.15 (d, 1H, *J* = 7.2 Hz, H-1), 4.77 (d, 1H, *J* = 12.5 Hz), 4.40 (d, H, *J* = 12.5 Hz, CH_2OH), 4.13 (d, 1H, *J* = 9 Hz, H-5), 3.72 (s, 3H, CH_3), 2.11, 2.07, and 2.05 (3s, 3H, OAc); HRMS (FAB) *m/z* calcd for ($C_{20}H_{24}O_{11}$ + Na) 463.1216, found 463.1209.

4-Nitrophenyl 2-[Methyl (2,3,4-tri-*O*-acetyl-*β*-D-glucopyranoside)uronate]benzyl Carbonate (11). Pyridine (0.18 mL, 1.92 mmol) was added to a solution of **12** (283 mg, 0.64 mmol) and 4-nitrophenyl chloroformate (383 mg, 1.92 mmol) in EtOAc (2 mL). The mixture was stirred overnight at room temperature, diluted with dichloromethane, washed with an aqueous solution of $NaHCO_3$ and water, and dried over $MgSO_4$. Evaporation of the organic solvent under reduced pressure followed by flash chromatography (hexanes−EtOAc, 1:1, v/v) led to **11** (284 mg, 73%) as a crystalline compound: mp 117 °C; $[\alpha]^{20}_D$ +2° (*c* 1, $CHCl_3$); IR ($CHCl_3$) ν 1760 cm^{-1} (OAc); ^{1}H NMR ($CDCl_3$) δ 8.27 (d, 2H, *J* = 9 Hz, Ar), 7.39 (m, 4H, Ar), 7.10 (m, 2H, Ar), 5.30 (m, 6H, H-1, H-2, H-3, H-4, Ar−CH_2), 4.24 (d, 1H, *J* = 8.5 Hz, H-5), 3.74 (s, 3H, CH_3), 2.08, 2.07, and 2.05 (3s, 3 × 3H, OAc); HRMS (FAB) *m/z* calcd for ($C_{27}H_{27}NO_{15}$ + Na) 628.1278, found 628.1320.

***N*-[2-*O*-(Methyl (2,3,4-tri-*O*-acetyl-*β*-D-glucopyranoside)uronate)benzyloxycarbonyl]daunorubicin (12).** It was obtained by condensation of **11** with daunorubicin, as described for the preparation of **5**, and isolated in 65% yield as a crystalline residue: mp 153−155 °C; $[\alpha]^{20}_D$ +122° (*c* 0.4, $CHCl_3$); MS (FAB) *m/z* 1032 $(M + K^+)$. Anal. ($C_{48}H_{51}NO_{22}$) C, H, N.

3-Chloro-4-hydroxybenzylic Alcohol (16). To a stirred, ice-cooled solution of methyl 3-chloro-4-(hydroxymethyl)benzoate (**15**) (1.33 g, 7.1 mmol) in anhydrous THF (100 mL) was added $LiAlH_4$ (0.54 g, 14 mmol) in small portions. Two hours later, ice and a solution of 2 N NaOH (0.5 mL) were added to the mixture. After stirring for an additional 1 h and acidification by a solution of 10% HCl, the product was extracted with EtOAc. The organic layer was washed with saturated $NaHCO_3$ and dried over Na_2SO_4. Removal of the solvent under reduced pressure furnished a white solid that recrystallized from EtOAc as white needles: 1.05 g (93%); mp 123 °C (EtOAc); IR (KBr) ν_{max} 3452 (CH_2OH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.45 (d, 1H, *J* = 2 Hz, H-3), 7.20 (dd, 1H, *J* = 8, *J*′ = 2 Hz, H-5), 7.00 (d, 1H, *J* = 8 Hz, H-6), 5.55 (s, D_2O exch., 1H, OH-1), 4.60 (s, 2H, CH_2OH); MS (EI) *m/z* 158/160 $M^{+\bullet}$. Anal. ($C_7H_7ClO_2$) C, H.

3-Chloro-4-hydroxybenzaldehyde (17). To a stirred suspension of pyridinium chlorochromate (2.04 g, 9.46 mmol) in dry EtOAc (35 mL) was added a solution of **16** (1 g, 0.3 mmol) in dry EtOAc (15 mL) in one portion. The mixture was refluxed for 2 h, cooled, and diluted with dry ether and the supernatant decanted from the black gum. The insoluble residue was washed thoroughly three times with dry ether. The combined organic solutions were passed through a short column of Florisil, and the solvent was removed under reduced pressure. Column chromatography of the residue using cyclohexane/EtOAc (70/30, v/v) gave **17** (0.76 g, 77%) as crystals: mp 134 °C (EtOAc); IR (KBr) ν_{max} 1677 (CO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.90 (s, 1H, CHO), 7.90 (d, 1H, $J = 2$ Hz, H-2), 7.75 (dd, 1H, $J = 8$, $J' = 2$ Hz, H-6a), 7.15 (d, 1H, $J = 8$ Hz, H-6b); MS (EI) *m/z* 156/158 $M^{+\bullet}$. Anal. ($C_7H_5ClO_2$) C, H.

General Preparation of Prodrugs. (a) Coupling Reaction of Methyl Glucopyranosyl Bromide Uronate. To a solution of methyl (2,3,4-tri-*O*-acetylglucopyranosyl bromide)-uronate (10 mmol) in anhydrous acetonitrile (100 mL), were added silver oxide (10 g, 43 mmol) and corresponding substituted benzaldehydes (1.6 mmol) successively. After stirring for 4 h at room temperature and filtration, the filtrate was evaporated under reduced pressure and the residue was purified by flash chromatography, thus affording the aldehyde derivatives **20** and **27**.

(b) Reduction of Aldehyde Derivatives 20 and 27 into Alcohols 21 and 28. Compound **20** or **27** (10 mmol) was dissolved in a mixture of anhydrous chloroform (60 mL) and 2-propanol (15 mL). After addition of 1 g of silica gel HL60 and cooling to 0 °C, sodium borohydride (10 mmol) was added and the resulting suspension stirred to 0 °C for 45 min. Dilution with dichloromethane (100 mL) was followed by filtration on Celite, and the filtrate was washed with water and brine before evaporation under reduced pressure.

(c) Activation of a 4-Nitrophenyl Carbonate or *N*-Hydroxysuccinimido Carbonate and Coupling with Doxorubicin To Afford the Fully Protected Prodrugs 23a,b,c and 30a,b,c. A solution of methyl [(hydroxymethyl)halogenophenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside)uronate or methyl [(hydroxymethyl)nitrophenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside]uronate (**21** or **28**) (2 mmol) in anhydrous dichloromethane (80−100 mL) containing Et_3N (0.3 mL, 2.1 mmol) was added to a solution of *N*,*N*′-disuccinimidyl carbonate (DSC) (1.08 g, 4.2 mmol) or 4-nitrophenyl chloroformate (0.85 g, 4.2 mmol) in anhydrous acetonitrile (50 mL). After stirring under argon at 0 °C for 90 min, the solvent was evaporated. The crude product was added to a solution of doxorubicin (0.88 g, 1.6 mmol) in *N*,*N*′-dimethylformamide (40 mL) containing Et_3N (0.23 mL, 1.6 mmol), under argon. After stirring for 2 h at room temperature, the solvent was evaporated under reduced pressure and the residual product was subjected to column chromatography, affording **23** or **30**.

(d) Deacylation. To a solution of **23a,b,c** or **30a,b,c** (2 mmol) in an anhydrous mixture of *N*,*N*′-dimethylformamide (10 mL), methanol (50 mL), and THF (2.5 mL) was added a 1 N solution of NaOMe in MeOH (1 mL) at 0 °C, and the mixture stirred for 30−45 min. Thereafter, the solution was neutralized by addition of Amberlite IR $120H^+$ ion-exchange resin. Filtration followed by evaporation under reduced pressure of the filtrate afforded a crude residue which was purified by flash chromatography, giving **24a,b,c** or **31a,b,c**.

(e) Methyl Ester Cleavage. A solution of **24a,b,c** or **31a,b,c** (2 mmol) in a mixture of water and THF (80/140, v/v) was stirred in the presence of a 2 N aqueous solution of NaOH (2 mL, 4 mmol) at −10 °C. After 5 min, the excess of NaOH was neutralized as above and the solvent was evaporated, leading to the prodrugs **25** or **32**.

Methyl (4-formyl-2-chlorophenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside)uronate (20a): obtained from the aldehyde **17** and methyl (tri-*O*-acetyl-β-D-glucopyranosyl bromide)-uronate (**14**), purified by flash chromatography (cyclohexanes−EtOAc, 4:1, v/v), and isolated as a yellow amorphous powder; 1.48 g (92%); mp 124−126 °C (hexane); $[\alpha]^{20}_D$ −57° (*c* 0.5, $CHCl_3$); IR (KBr) ν_{max} 1760 (OAc), 1680 (CHO), 1235 (OAc) cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.90 (s, 1H, CHO), 7.92 (d, 1H, $J =$ 3 Hz, H-3′), 7.78 (dd, 1H, $J = 9$, $J' = 3$ Hz, H-5′), 7.30 (d, 1H, $J = 9$ Hz, H-6′), 5.37 (m, 3H, H-2′, H-3, H-4), 5.25 (d, 1H, $J =$ 6 Hz, H-1), 4.27 (d, 1H, $J = 8$ Hz, H-5), 3.75 (s, 3H, $COOCH_3$), 2.12, 2.09, and 2.06 (3s, 3 × 3H, OAc); MS (CI) *m/z* 490/492 $(M + NH_4)^+$. Anal. ($C_{20}H_{21}ClO_{11}$) C, H.

Methyl [4-(hydroxymethyl)-2-chlorophenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside)]uronate (21a): obtained by reduction of **20a** as a white amorphous powder; 4.40 g (73%); mp 138−140 °C (CH_2Cl_2); $[\alpha]^{20}_D$ −95° (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} 3510 (CH_2OH), 1760 and 1235 (OAc) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.38 (d, 1H, $J = 2$ Hz, H-3′), 7.25 (dd, 1H, $J = 8$, $J' =$ 2 Hz, H-5′), 7.18 (d, 1H, $J = 8$ Hz, H-6′), 5.35 (m, 3H, H-2, H-3, H-4), 5.04 (d, 1H, $J = 7$ Hz, H-1), 4.63 (s, 2H, CH_2OH), 4.15 (d, 1H, $J = 8$ Hz, H-5), 3.74 (s, 3H, $COOCH_3$), 2.12, 2.09, and 2.06 (3s, 3 × H, OAc); MS (CI) *m/z* 492/494 $(M + NH_4)^+$. Anal. ($C_{20}H_{23}ClO_{11}$) C, H.

***N*-[4-*O*-(Methyl (2,3,4-tri-*O*-acetyl-β-D-glucopyranoside)-uronate)-3-chlorobenzyloxycarbonyl]doxorubicin (23a).** After activation of **21a** (1 g, 2 mmol) as its *N*-hydroxysuccinimidocarbonate **22a** (1.3 g) according to the general procedure, **23a** was obtained by condensation of **22a** with doxorubicin (0.88 g, 1.6 mmol) and further purification by flash chromatography (CH_2Cl_2/MeOH, 95/5, v/v). It was isolated in 41% yield (0.69 g) as a red amorphous solid: $[\alpha]^{20}_D$ +260° (*c* 0.01, CH_3OH); IR (KBr) ν_{max} 3430 (OH, NH), 1725 (OAc), 1235 (OAc) cm^{-1}; MS (negative FAB) *m/z* 1042/1044. Anal. ($C_{48}H_{50}ClNO_{23}$) C, H, N.

***N*-[4-*O*-(Methyl (β-D-glucopyranoside)uronate)-3-chlorobenzyloxycarbonyl]doxorubicin (24a):** obtained from **23a**, purified by flash chromatography (MeCN/H_2O, 95/5, v/v), and isolated in 64% yield as a red amorphous powder; MS (FAB) *m/z* 940/942 $(M + Na^+)$. Anal. ($C_{42}H_{44}ClNO_{20}$) C, H, N.

***N*-[4-*O*-(β-D-Glucopyranosideuronic acid)-3-chlorobenzyloxycarbonyl]doxorubicin (25a):** obtained from **24a** in 96% yield as a red amorphous powder; $[\alpha]^{20}_D$ +140° (*c* 0.01, CH_3OH); IR (KBr) ν_{max} 3400 (OH, NH); ^1H NMR (DMSO-d_6) δ 7.88 (dd, 1H, $J = 8$, $J' = 2$ Hz, H-1), 7.79 (td, 1H, $J = 8$, $J' =$ 2 Hz, H-2), 7.43 (m, 1H, H-3), 7.36 (d, 1H, $J = 2$ Hz, H-3′), 7.20 (dd, 1H, $J = 8$, $J' = 2$ Hz, H-5′), 7.12 (d, 1H, $J = 8$ Hz, H-6′), 5.40 (dd, 1H, $J = 8$, $J' = 2$ Hz, H-1′), 5.15−5.00 (m, 5H, H-1‴, H-2″, H-3″, H-4″, H-7), 4.96 (s, 2H, CH_2OCO), 4.57 (s, 2H, CH_2-14), 4.54 (s, 1H, NH), 3.98 (s, 3H, OCH_3), 3.74 (s, 3H, $COOCH_3$), 3.70−3.50 (m, 3H, H-3′, H-4′, H-5‴), 3.00−2.80 (m, 2H, H-10a and b), 2.14 (m, 2H, H-8a, H-8b), 1.89 (m, 1H, H-2a), 1.65 (m, 1H, H-2b), 1.11 (d, 3H, $J = 6.5$ Hz, CH_3-6′); ^{13}C NMR (D_2O) δ 206.6 (C-13), 186.8 (C-12, C-5), 177.4 (C-6‴), 162.2 (C-4), 159 (CONH), 157.6 (C-6), 156.1(C-11), 153.9 (C-4″), 137.8 (C-1″, C-2), 134.8 (C-6a, C-12a), 133.9, 129.9, 124.5 (C-10a, C-2″, C-6″), 123.1 (C-3″), 121.3 (C-4a, C-1), 120.3 (C-5″), 118.2 (C-3), 112.1 (C-11a, C-5a), 103.0 (C-1′, C-1‴), 80.1 (C-9), 78.8, 77.6, 75.1, 74.1 (C-2‴, C-3‴, C-4‴, C-5‴), 71.9 (C-7), 71.6 (C-5′), 70.0 (C-4′), 67.6 (CH_2OCO), 66.8 (C-14), 68.5 (OCH_3), 49.5 (C-3′), 34.6 (C-8), 32.3 (C-10, C-2′), 18.5 (C-6′); MS (negative FAB) *m/z* 902/904. Anal. ($C_{41}H_{42}ClNO_{20}$) C, H, N.

Methyl (2-fluoro-4-formylphenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside)uronate (20b): obtained from **14** and **18** in 47% yield after purification and isolated as a crystalline compound; mp 151−153 °C; $[\alpha]^{20}_D$ −38° (*c* 0.2, $CHCl_3$); IR ($CHCl_3$) ν 1760 (F), 1698 (CHO), 1223 (F) cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.90 (d, 1H, $J = 2$ Hz, CHO), 7.70−7.65 (m, 2H, Ar), 7.60−7.30 (m, 1H, Ar), 5.38−5.30 (m, 3H, H-2, H-3, H-4), 5.25 (d, 1H, $J = 7$ Hz, H-1), 4.23 (d, 1H, $J = 9$ Hz, H-5), 3.73 (s, 3H, CH_3), 2.10, 2.07, and 2.06 (3s, 3 × 3H, OAc); MS (CI) *m/z* 474 $(M + NH_4)^+$. Anal. ($C_{20}H_{21}FO_{11}$) C, H.

Methyl (2-fluoro-4-(hydroxymethyl)phenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside)uronate (21b): obtained from **20b** in 66% yield; mp 90−92 °C; $[\alpha]^{20}_D$ −37° (*c* 0.2, $CHCl_3$); IR ($CHCl_3$) ν 3600 (OH), 1760 and 1225 (F) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.20−7.02 (m, 3H, Ar), 5.33−5.25 (m, 3H, H-2, H-3, H-4), 5.02 (d, 1H, $J = 7$ Hz, H-1), 4.62 (s, 2H, CH_2OH), 4.11 (d, 1H, $J = 9$ Hz, H-5), 3.75 (s, 3H, CH_3), 2.10 (s, 3H, OAc), 2.02 (s,

6H, 2 OAc); MS (CI) *m/z* 476 $[M + NH_4]^+$. Anal. ($C_{20}H_{23}FO_{11}$) C, H.

Methyl (2-fluoro-4-*p*-nitrophenyl carbonate 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranoside)uronate (22b): obtained from **21b**, following protocol as for preparation of **13**, and isolated in 66% yield after chromatography; mp 138−140 °C; $[\alpha]^{20}_D$ −27° (c 0.37, $CHCl_3$); IR ($CHCl_3$) *ν* 1760 (F), 1223 (F) cm^{-1}; ^1H NMR ($CDCl_3$) *δ* 8.29 (d, 2H, *J* = 9 Hz, Ar), 7.39 (d, 2H, *J* = 9 Hz, Ar), 7.25−7.15 (m, 4H, Ar), 5.36−5.31 (m, 3H, H-2, H-3, H-4), 5.23 (s, 2H, CH_2), 5.08 (d, 1H, *J* = 7 Hz, H-1), 4.15 (d, 1H, *J* = 9 Hz, H-5), 3.76 (s, 3H, CH_3), 2.10, 2.06, and 2.05 (3s, 3 × 3H, OAc); MS (CI) *m/z* 641 $[M + NH_4]^+$. Anal. ($C_{27}H_{26}FNO_{15}$) C, H, N.

***N*-[4-*O*-(Methyl (2,3,4-tri-*O*-acetyl-*β*-D-glucopyranoside)-uronate)-3-fluorobenzyloxycarbonyl]doxorubicin (23b):** obtained from **22b** and doxorubicin and isolated in 95% yield as a crystalline compound after purification by flash chromatography (toluene−acetone, 4/1 then 3/1, v/v); mp 147−149 °C; $[\alpha]^{20}_D$ +135° (*c* 0.02, $CHCl_3$); ^1H NMR ($CDCl_3$) *δ* 13.85 (s, 1H, PhOH), 13.06 (s, 1H, PhOH), 7.91 (dd, 1H, *J* = 8, *J′* = 2 Hz, H-1), 7.77 (dd, 1H, *J* = *J′*= 8 Hz, H-2), 7.40 (dd, 1H, *J* = 8, *J′* = 2 Hz, H-3), 7.13−6.98 (m, 3H, Ar), 5.50 (d, 1H, *J* = 1.5 Hz, H-1′), 5.32 (m, 4H, H-2‴, H-3‴, H-4‴, H-7), 5.19 (m, 1H, H-4′), 5.00 (d, 1H, *J* = 7 Hz, H-1‴), 4.95 (s, 2H, CH_2O), 4.75 (s, 2H, CH_2-14), 4.55 (s, 1H, NH), 4.13 (m, 2H, H-5′, H-5‴), 4.08 (s, 3H, OCH_3), 3.85 (m, 1H, H-3′), 3.72 (s, 3H, $COOCH_3$), 3.66 (s, 1H, OH), 3.26 (d, 1H, *J* = 19 Hz, H-10a), 2.98 (d, 1H, *J* = 19 Hz, H-10b), 2.33 (dd, 1H, *J* = 16, *J′* = 2 Hz, H-8a), 2.19 (ddd, 1H, *J* = 16, *J′* = 4, *J″* = 2 Hz, H-8b), 2.06, 2.04, and 2.03 (3s, 3 × 3H, OAc), 1.84 (ddd, 1H, *J* = 12, *J′* = 3, *J″* = 1.5 Hz, H-2′a), 1.29 (m, 1H, H-2′b), 1.10 (d, 3H, *J* = 6 Hz, CH_3-6′); LRMS (FAB) *m/z* 1028 $(M + H^+)$. Anal. ($C_{48}H_{50}FNO_{23}$) C, H, N.

***N*-[4-*O*-(Methyl *β*-D-glucopyranosyluronate)-3-fluorobenzyloxycarbonyl]doxorubicin (24b):** obtained from **23b** according to the general procedure, purified by flash chromatography (CH_2Cl_2−MeOH, 97:3 then 93:7), and isolated in 57% yield as crystals; mp 170−172 °C; $[\alpha]^{20}_D$ +209° (*c* 0.02, MeOH); IR (KBr) *ν* 3320 (OH, NH), 1725 (C═O), 1210 (F) cm^{-1}. Anal. ($C_{42}H_{44}FNO_{20}$) C, H, N.

***N*-[4-*O*-(*β*-D-Glucopyranoside)uronic acid)-3-fluorobenzyloxycarbonyl]doxorubicin (25b):** obtained from **24b** and isolated in 80% yield as crystals; mp > 350 °C; $[\alpha]^{20}_D$ −112° (*c* 0.016, H_2O); IR (Nujol) *ν* 3320 (OH, NH), 1720 (C═O) cm^{-1}; ^{13}C NMR (D_2O) *δ* 214.0 (C-13), 188.1 and 187.6 (C-5 and C-12), 180.3 (C-6‴), 162.2 (C-4), 158.3 (CONH), 156.4 (C-6), 155.9 (C-11), 150.8 (C-4″), 145.7 and 136.8 (C-1″ and C-2), 136.0 and 135.5 (C-6a and C-12a), 132.5 (2C) and 125.0 (C-10a, C-2″, and C-6″), 123 (C-3″), 120.7 and 120.2 (2C, (C-1, C-4a, and C-5″), 118.3 (C-3), 116.4 (C-11a), 111.8 (C-5a), 101.3 (C-1′ and C-1‴), 77.0, 75.3, 74.7, and 74.1 (C-2‴, C-3‴, C-4‴, C-5‴), 73.0 (C-7), 71.0 (C-5′), 68.9 (C-4′), 67.7 (CH_2OCO), 65.3 (C-14), 58.3 (OCH_3), 49.5 (C-3′), 37.3 (C-8), 37.2 (C-10), 31.2 (C-2′), 18.5 (C-6′); MS (FAB) *m/z* 910 $(M + Na^+)$.

Methyl (4-formyl-2-nitrophenyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranoside)uronate (20c): obtained in 90% from 4-hydroxy-3-nitrobenzaldehyde (**19**) and **14** in 89% yield after purification by chromatography (CH_2Cl_2/MeOH, 95/5, v/v) and recrystallization from dichloromethane; mp 172−173 °C; $[\alpha]^{20}_D$ +10° (*c* 1, $CHCl_3$); IR (syrup) *ν* 1760, 1230 (F) cm^{-1}; ^1H NMR ($CDCl_3$) *δ* 9.97 (s, 1H, CHO), 8.31 (dd, 1H, *J* = 7.5, *J* = 1.8 Hz, Ar), 8.08 (dd, 1H, *J* = 7.5, *J′*= 1.8 Hz, Ar), 7.49 (d, 1H, *J* = 7.5 Hz, Ar), 5.45−5.25 (m, 4H, H-2, H-3, H-4, H-5), 4.33 (d, 1H, *J* = 8.2 Hz, H-1), 3.71 (s, 3H, $COOCH_3$), 2.16, 2.12, and 2.07 (3s, 3 × 3H, OAc); MS (CI) *m/z* 501 $(M + NH_4)^+$. Anal. ($C_{20}H_{21}NO_{13}$) C, H, N.

Methyl (4-(hydroxymethyl)-2-nitrophenyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranoside)uronate (21c): obtained from the reduction of **20c** and isolated in 99% yield by crystallization from dichloromethane; mp 173−174 °C; $[\alpha]^{20}_D$ +10° (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) *δ* 7.65 (d, 1H, *J* = 1.8 Hz, Ar), 7.39 (dd, 1H, *J* = 7, *J′* = 1.8 Hz, Ar), 7.21 (d, 1H, *J* = 7 Hz, Ar), 5.35−5.02 (m, 4H, H-2, H-3, H-4, H-5), 4.62 (s, 2H, CH_2), 4.12 (d, 1H, *J* = 8 Hz, H-1), 3.63 (s, 3H, $COOCH_3$), 2.01 (s, 3H, OAc), 2.06 (s, 6H, 2 OAc); MS (CI) *m/z* 503 $(M + NH_4)^+$. Anal. ($C_{20}H_{23}NO_{13}$) C, H, N.

***N*-[4-*O*-(Methyl (2,3,4-tri-*O*-acetyl-*β*-D-glucopyranoside)uronate)-3-nitrobenzyloxycarbonyl]doxorubicin (23c):** obtained from **22c** (prepared from **21c** by the same protocol as for **22b**) and doxorubicin in 74% yield; mp 114 °C; $[\alpha]^{20}_D$ +121° (*c* 0.048, $CHCl_3$); IR ($CHCl_3$) *ν* 3457 (OH), 1760, 1225, 1040 (OAc) cm^{-1}. Anal. ($C_{48}H_{50}N_2O_{25}$ + 2 H_2O) C, H, N.

***N*-[4-*O*-(Methyl *β*-D-glucopyranosideuronate)-3-nitrobenzyloxycarbonyl]doxorubicin (24c):** obtained from **23c** in 95% yield after purification by flash chromatography (CH_2Cl_2−MeOH, 98/2 then 95/5); mp 150 °C; $[\alpha]^{20}_D$ +85° (*c* 0.048, THF); IR (KBr) *ν* 3416 (OH), 1735 (C═O), 1250 (Ar) cm^{-1}; MS (FAB) *m/z* 951 $(M + Na^+)$. Anal. ($C_{42}H_{44}N_2O_{22}$) C, H, N.

***N*-[4-*O*-(Methyl *β*-D-glucopyranosyluronate)-3-nitrobenzyloxycarbonyl]doxorubicin (HMR 1826) (25c):** obtained from **24c** and isolated (80% yield) as a crystalline compound; mp 210 °C; $[\alpha]^{20}_D$ −78° (*c* 0.05, H_2O); IR (KBr) *ν* 3540 (OH, NH), 1720 (C═O), 1620 (Ar) cm^{-1}; ^1H NMR (DMSO-d_6) *δ* 14.00 (bs, 2H, PhOH), 8.00−6.90 (m, 6H, Ar), 5.48 (s, 1H, H-1′ or 7), 5.23 (s, 1H, H-7 or 1′), 5.07 (d, 1H, *J* = 6.4 Hz, H), 4.98 (m, 2H, CH_2O), 4.59 (m, 2H, CH_2-14), 4,15 (m, 1H, H-5‴), 3.99 (s, 3H, OCH_3), 3,70 (m, 1H, H-3′), 3.30−3.10 (m, 5H, H-2‴, H-3‴, H-4‴, H-4′, H-5′), 2.96 (d, 1H, *J* = 19 Hz, H-10a), 2.91 (d, 1H, *J* = 19 Hz, H-10b), 2.20 (d, 1H, *J* = 11.5 Hz, H-8a), 2.13 (d, 1H, *J* = 11.5 Hz, H-8b), 1.87 (d, 1H, *J* = 13 Hz, H-2′a), 1.48 (d, 1H, *J* = 13 Hz, H-2′b), 1.13 (d, 3H, *J* = 6.5 Hz, CH_3-6′); ^{13}C NMR (D_2O) *δ* 206.5 (C-13), 186.8 (C-5, C-12), 177.4 (COOH), 162.2 (C-4), 159.0 (CONH), 157.6 (C-6), 156.1 (C-11), 151.8 (C-4″), 141.4, 138.5, 137.2, 135.6, 133.5 (C-6a, C-10a, C-12a, C-1″, C-3″, C-6″), 121.3 (C-1, C-4a), 120.3 (C-3, C-5″), 112.1 (C-5a, C-11a), 103.0 (C-1′, C-1‴), 78.8, 77.6, 74.9, 73.9 (C-9, C-2‴, C-3‴, C-4‴, C-5‴), 71.6 (C-7), 71.4 (C-5′), 70.0 (C-4′), 67.5 (CH_2O), 66.8 (C-14), 58.4 (OCH_3), 49.4 (C-3′), 34.6 (C-8), 32.2 (C-10, C-2′), 18.4 (CH_3-6′); MS (FAB) *m/z* 937 $(M + Na^+)$. Anal. ($C_{41}H_{41}N_2O_{22}Na$) C, H, N.

Methyl (2-formyl-4-chlorophenyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranoside)uronate (27a): obtained from **14** and **17** in 55% yield; mp 156−157 °C; $[\alpha]^{20}_D$ −39° (*c* 1, $CHCl_3$); IR (CH_2Cl_2) *ν* 1745 (OAc), 1680 (CHO) cm^{-1}; ^1H NMR ($CDCl_3$) *δ* 10.27 (s, 1H, CHO), 7.80 (d, 1H, *J* = 2.4 Hz, Ar), 7.54 (dd, 1H, *J* = 8.4, *J′* = 2.4 Hz, Ar), 7.11 (d, 1H, *J* = 8.4 Hz, Ar), 5.40−5.20 (m, 4H, H-1, H-2, H-3, H-4), 4.25 (d, 1H, *J* = 8.6 Hz, H-5), 3.74 (s, 3H, CH_3), 2.10, 2.09, and 2.08 (3s, 3 × 3H, OAc); DCI/NH_3 *m/z* 492 $(M + NH_4)^+$; HRMS (FAB) *m/z* calcd for $C_{20}H_{21}ClO_{11}$ + Li^+ 479.7757, found 479.7765.

Methyl (2-(hydroxymethyl)-4-chlorophenyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranoside)uronate (28a): obtained from **27a** in 90% yield; mp 120 °C; $[\alpha]^{20}_D$ −20° (*c* 0.9, $CHCl_3$); IR (CH_2Cl_2) *ν* 3600 (OH), 1750 (OAc) cm^{-1}; ^1H NMR ($CDCl_3$) *δ* 7.36 (d, 1H, *J* = 2.4 Hz, Ar), 7.21 (dd, 1H, *J* = 8.4, *J′* = 2.4 Hz, Ar), 6.94 (d, 1H, *J* = 8.4 Hz, Ar), 5.34 (m, 3H, H-2, H-3, H-4), 5.08 (d, 1H, *J* = 6.5 Hz, H-1), 4.73 and 4.43 (2d, 2H, *J* = 13 Hz, CH_2OH), 4.12 (d, 1H, *J* = 9 Hz, H-5), 3.71 (s, 3H, $COOCH_3$), 2.11, 2.07, and 2.05 (3s, 3 × 3H, OAc). Anal. ($C_{20}H_{23}ClO_{11}$) C, H.

Methyl (2-(*p*-nitrobenzyloxycarbonyl)-4-chlorophenyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranoside)uronate (29a): obtained in 66% yield from **28a**; mp 136 °C; $[\alpha]^{20}_D$ −145° (*c* 1, $CHCl_3$); IR (CH_2Cl_2) *ν* 1750 (OAc) cm^{-1}; ^1H NMR ($CDCl_3$) *δ* 8.29 (d, 2H, *J* = 9 Hz, Ar), 7.43−7.30 (m, 4H, Ar), 7.05 (d, 1H, *J* = 9 Hz, Ar), 5.36−5.14 (m, 6H, H-1, H-2, H-3, H-4, CH_2O), 4.21 (d, 1H, *J* = 8.8 Hz, H-5), 3.74 (s, 3H, $COOCH_3$), 2.08 (s, 3H, OAc), 2.06 (s, 6H, OAc). Anal. ($C_{27}H_{26}ClNO_{15}$) C, H, N.

***N*-[2-*O*-(Methyl (2,3,4-tri-*O*-acetyl-*β*-D-glucopyranoside)-uronate)-5-chlorobenzyloxycarbonyl]doxorubicin (30a):** obtained in 75% yield by condensation of **29a** with doxorubicin; mp 155−157 °C; $[\alpha]^{20}_D$ +40° (*c* 0.75, $CHCl_3$); IR ($CHCl_3$) 3442 (NH, OH), 1755 (C═O), 1720 (NHCO) cm^{-1}; MS (electrospray) *m/z* 1066/1068 $(M + Na^+)$, 1082/1084 $(M + K^+)$. Anal. ($C_{48}H_{50}ClNO_{23}$) C, H, N.

***N*-[2-*O*-(Methyl *β*-D-glucopyranosyluronate)-5-chlorobenzyloxycarbonyl]doxorubicin (31a):** obtained from **30a** in 55% yield as crystals; mp 158−160 °C; $[\alpha]^{20}_D$ +13.5° (*c* 0.037,

CH_3OH); IR ($CHCl_3$) ν 3450 (NH, OH), 1720 (C=O) cm^{-1}; MS (electrospray) *m/z* 917/919 ($M + Na^+$), 940/942, 956/958 ($M + K^+$).

***N*-[2-*O*-(Glucopyranosideuronic acid)-5-chlorobenzyloxycarbonyl]doxorubicin (32a):** obtained from **31a** in 70% yield; mp 210−212 °C; $[\alpha]^{20}_D$ +8° (*c* 0.07, H_2O); IR ($CHCl_3$) ν 1755 (OAc), 1720 (NHCO) cm^{-1}; ^{13}C NMR (D_2O) δ 206.6 (C-13), 187.2 (C-5), 186.8 (C-12), 178.5 (COOH), 163 (C-4), 160 (C-6, C-11), 158.7 (CONH), 155.3 (C-2″), 137.3 (C-2, C-12a), 136.9 (C-6a), 133 (C-4″), 130.8 (C-6″), 129.5 (C-10a), 126 (C-5″), 123.5 (C-1″), 122.5 (C-1), 121.7 (C-4a), 117.6 (C-3), 114.1 (C-3″), 113.3 (C-5a, C-11a), 103.6 (C-1‴), 103.1 (C-1′), 80, 78.7, 76, 75.1 (C-2‴, C-3‴, C-4‴, C-5‴), 72.5 (C-7), 71.2 (C-5′), 71.0 (C-4′), 70.9 (CH_2O), 66.8 (C-14), 59.5 (OCH_3-4), 50.7 (C-3′), 34.9 (C-10), 35.2 (C-8, C-2′), 19.6 (CH_3-6); MS (negative mode, electrospray) *m/z* 902 ($M - H^-$).

Methyl (4-fluoro-2-formylphenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside)uronate (27b): 30% yield from the condensation of **14** with 5-fluorosalicylaldehyde (**18**); mp 153−155 °C; $[\alpha]^{20}_D$ −28° (*c* 0.02, $CHCl_3$); IR ($CHCl_3$) ν 1760 (F), 1690 (CHO), 1210 (F) cm^{-1}; ^1H NMR ($CDCl_3$) δ 10.30 (d, 1H, J = 2 Hz, CHO), 7.53 (dd, 1H, J = 8, J' = 3 Hz, Ar), 7.25 (m, 1H, Ar), 7.15 (dd, 1H, J = 8, J' = 4 Hz, Ar), 5.40−5.25 (m, 3H, H-2, H-3, H-4), 5.18 (d, 1H, J = 8 Hz, H-1), 4.19 (d, 1H, J = 10 Hz, H-5), 3.75 (s, 3H, $COOCH_3$), 2.12−2.02 (3s, 3 × 3H, OAc); MS (CI) *m/z* 474 ($M + NH_4$)$^+$. Anal. ($C_{20}H_{21}FO_{11}$) C, H.

Methyl (4-fluoro-2-(hydroxymethyl)phenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside)uronate (28b): obtained in 44% yield from **27b**; syrup; $[\alpha]^{20}_D$ −31° (*c* 0.64, $CHCl_3$); IR ($CHCl_3$) ν 3080 (OH), 1760, 1250, 1045 (F) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.12 (m, 1H, Ar), 7.00−6.95 (m, 2H, Ar), 5.40−5.26 (m, 3H, H-2, H-3, H-4), 5.06 (d, 1H, J = 7 Hz, H-1), 4.78 (dd, 1H, J = 13, J' = 5 Hz, CH_2O), 4.42 (dd, 1H, J = 13, J' = 9 Hz, CH_2O), 4.09 (d, 1H, J = 10 Hz, H-5), 3.73 (s, 3H, $COOCH_3$), 2.81 (dd, 1H, J = 9, J' = 5 Hz, OH), 2.13, 2.08, and 2.05 (3s, 3 × 3H, OAc). Anal. ($C_{20}H_{23}FO_{11}$) C, H.

Methyl (4-fluoro-2-hydroxy-*p*-nitrophenylcarbamate methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside)uronate (29b): obtained from **28b** in 66% yield; mp 152−154 °C, $[\alpha]^{20}_D$ −15° (*c* 0.066, $CHCl_3$); IR ($CHCl_3$) ν 1760, 1240, and 1044, (F), 1530 and 1330 (NO_2) cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.30 (d, 2H, J = 8 Hz, Ar), 7.42 (d, 2H, J = 8 Hz, Ar), 7.18 (dd, 1H, J = 8, J' = 2 Hz, Ar), 7.08−7.05 (m, 2H, Ar), 5.36−5.33 (m, 3H, H-2, H-3, H-4), 5.31 (d, 1H, J = 12 Hz, CH_2O), 5.11 (d, 1H, J = 12 Hz, CH_2O), 4.19 (d, 1H, J = 10 Hz, H-5), 3.75 (s, 3H, $COOCH_3$), 2.09, 2.07, and 2.06 (3s, 3 × 3H, OAc); MS (FAB) *m/z* 646 ($M + Na^+$). Anal. ($C_{27}H_{26}FNO_{15}$) C, H.

***N*-[2-*O*-(Methyl (2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl)uronate)-5-fluorobenzyloxycarbonyl]doxorubicin (30b):** resulted from the condensation of **29b** with doxorubicin and isolated in 97% yield after chromatography with toluene−acetone (4:1, v/v) as a crystalline compound; mp 140−142 °C; $[\alpha]^{20}_D$ +100° (*c* 0.01, $CHCl_3$); IR (NaCl) ν 3300 (OH, NH), 1700 and 1215 (OAc) cm^{-1}; MS (FAB) *m/z* 1050 ($M + Na^+$).

***N*-[2-*O*-(Methyl β-D-glucopyranosideuronate)-5-fluorobenzyloxycarbonyl]doxorubicin (31b):** obtained from **30b** in 58% yield after flash chromatography (CH_2Cl_2−MeOH, 97:3 then 93:7, v/v): mp 160−162 °C; $[\alpha]^{20}_D$ +150° (*c* 0.008, MeOH); MS (FAB) *m/z* 924 ($M + Na^+$). Anal. ($C_{42}H_{44}FNO_{20}$) C, H, N.

***N*-[2-*O*-(β-D-Glucopyranosideuronic acid)-5-fluorobenzyloxycarbonyl]doxorubicin (32b):** isolated in 45% yield as a syrup from **31b**; $[\alpha]^{20}_D$ −75° (*c* 0.02, H_2O); IR (Nujol) ν 3300 (OH, NH), 1700 (CO) cm^{-1}; ^{13}C NMR (D_2O) δ 206.8 (C-13), 187.1 (C-5), 186.6 (C-12), 178.5 (COOH), 162.7 (C-4), 160.1 (C-6, C-11), 158.5 (CONH), 152.8 (C-2″), 154.5 (C-5″), 137.2 (C-2, C-12a), 137.1 (C-6a), 130.5 (C-1‴), 129.5 (C-10a), 123.7 (C-1″), 122.4 (C-1), 121.5 (C-4a), 118.7 (C-4″), 117.8 (C-3), 117.5 (C-5″), 114.3 (C-3″), 119.1 (C-5a, C-11a), 103 (C-1′), 79.8 (C-9), 78, 77.5, 76, 75.5 (C-2‴, C-3‴, C-3′, C-5‴), 72.5 (C-7), 71.0 (C-5′, C-4′), 70.8 (CH_2O), 66.6 (C-14), 59.4 (OCH_3), 50.6 (C-3′), 34.7 (C-10), 35.0 (C-8, C-2′), 19.5 (C-6′); MS (negative mode, electrospray) *m/z* 886 ($M - H^-$). Anal. ($C_{41}H_{42}FNO_{20}$) C, H, N.

Methyl (4-nitro-2-formylphenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside)uronate (27c): prepared in 70% yield by condensation of **14** with 5-nitrosalicylaldehyde (**19**); mp 173−174 °C; $[\alpha]^{20}_D$ −52° (*c* 1, $CHCl_3$); IR (CH_2Cl_2) ν 1755 (OAc), 1690 (CHO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 10.32 (s, 1H, CHO), 8.67 (d, 1H, J = 3 Hz, Ar), 8.42 (dd, 1H, J = 9, J' = 3 Hz, Ar), 7.32 (d, 1H, J = 9 Hz, Ar), 5.4 (m, 4H H-1, H-2, H-3, H-4), 4.32 (d, 1H, J = 8.5 Hz, H-5), 3.73 (s, 3H, CH_3), 2.09 (s, 9H, OAc). Anal. ($C_{20}H_{21}NO_{13}$) C, H, N.

Methyl (4-nitro-2-(hydroxymethyl)phenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside)uronate (28c): obtained in 75% yield by reduction of **27c**; mp 147 °C; $[\alpha]^{20}_D$ −34° (*c* 0.7, $CHCl_3$); IR (CH_2Cl_2) ν 3 600 (OH), 1755 and 1230 (OAc) cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.27 (d, 1H, J = 2.7 Hz, Ar), 8.12 (dd, 1H, J = 9, J' = 2.7 Hz, Ar), 7.08 (d, 1H, J = 9 Hz, Ar), 5.32 (m, 4H, H-1, H-2, H-3, H-4), 4.72 and 4.61 (2d, 2H, J = 13.8 Hz, CH_2OH), 4.30 (d, 1H, J = 8.7 Hz, H-5), 3.72 (s, 3H, $COOCH_3$), 2.95 (bs, 1H, OH), 2.10, 2.08, and 2.07 (3s, 3 × 3H, OAc); HRMS (FAB) calcd for $C_{20}H_{23}NO_{13}$ + Na *m/z* 508.1058, found 508.1067.

Methyl 4-nitro-2-(hydroxymethyl)-*p*-nitrophenyl carbonate (29c): mp 154−155 °C; $[\alpha]^{20}_D$ −34.5° (*c* 1 $CHCl_3$); IR (CH_2Cl_2) ν 1760 (OAc) cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.26 (m, 4H, Ar), 7.43 (d, 2H, J = 8.3 Hz, Ar), 7.23 (d, 1H, J = 9.1 Hz, Ar), 5.36 (m, 6H, H-1, H-2, H-3, H-4, CH_2O), 4.36 (d, 1H, J = 8.7 Hz, H-5), 3.74 (s, 3H, $COOCH_3$), 2.11, 2.09, and 2.08 (3s, 3 × 3H, OAc); HRMS (FAB) calcd for $C_{27}H_{26}N_2O_{17}$ + Li *m/z* 657.1391, found 657.1391.

***N*-[2-*O*-(Methyl (2,3,4-tri-*O*-acetyl-β-D-glucopyranoside)-uronate)-5-nitrobenzyloxycarbonyl]doxorubicin (30c):** mp 162−167 °C; $[\alpha]^{20}_D$ +122° (*c* 0.5, $CHCl_3$); IR (CH_2Cl_2) ν1755 (OAc), cm^{-1}; HRMS (FAB) calcd for $C_{48}H_{50}O_{25}N_2$ + Li *m/z* 1061.2862, found 1061.2841.

***N*-[2-*O*-(Methyl β-D-glucopyranosyluronate)-5-nitrobenzyloxycarbonyl]doxorubicin (31c):** mp 172−173 °C; $[\alpha]^{20}_D$ 0° (*c* 0.02, CH_3OH); HRMS (FAB) calcd for $C_{42}H_{44}O_{22}N_2$ + Na *m/z* 951.2283, found 951.2274.

***N*-[2-*O*-(β-D-Glucopyranosideuronic acid)-5-nitrobenzyloxycarbonyl]doxorubicin (32c):** isolated in 55% yield as crystals; mp 260 °C; $[\alpha]^{20}_D$ +10° (*c* 0.02, H_2O); ^{13}C NMR (D_2O) δ 207 (C-13), 187.4 (C-5), 186.2 (C-12), 178.7 (COOH), 163.3 (C-2″), 162.8 (C-4), 160.3 (C-6, C-11), 158.4 (CONH), 139.6 (C-5″), 137.3 (C-2, C-12a), 137 (C-6a), 130.7 (C-3′), 129.5 (C-10a), 128.4 (C-4″), 123.5 (C-6″), 123.0 (C-1″), 122.6 (C-1), 121 (C-4a), 117.9 (C-3), 113.6 (C-3″), 113.1 (C-5a, C-11a), 103.6 (C-1‴), 79.9 (C-9), 81, 78, 77, 76.5 (C-2‴, C-3‴, C-4‴, C-5‴), 72.8 (C-7), 71.3 (C-5′, C-4′), 70.9 (CH_2O), 66.8 (C-14), 59.7 (OCH_3-4), 50.8 (C-3′), 34.3 (C-10), 35.2 (C-8, C-2′), 19.7 (C-6′); MS (negative mode, electrospray) *m/z* 913 ($M - H^-$).

Biological Tests. Stability of the Prodrug in Plasma. Plasma containing 0.02 M citrate obtained from humans, mice, or rats was added to a solution of prodrug (200 μg/mL) in 100 mM phosphate buffer, pH 7.35 (1:1), and incubated for various times at 37 °C. Prodrug concentrations were analyzed by HPLC on reversed-phase material (Nucleosil C18, 5-μm particule size, 120-mm length, 4.5-mm inside diameter) after an online-extraction procedure on a C 18-RP precolumn. Before sample injection, the precolumn was preconditioned with 2.5 mL of methanol and 1.5 mL of phosphate buffer, pH 6.0. After injection of the sample, the precolumn was washed with 1.5 mL of phosphate buffer, pH 6.0. Analytes retained on the precolumn were eluted by valve switching and connection of the precolumn to the mobile phase. Elution on the analytical column was performed using a linear gradient made of two components (A, 20 mM phosphate buffer, pH 3; B, acetonitrile) with the following time−concentration profile: 0 min, 75% A, 25% B; 20 min, 25% A, 75% B; 30 min, 25% A, 75% B. Before starting the next run, the column was allowed to equilibrate at starting condition for 5 min. Detection was performed by using a fluorescence detector (ex 494 nm, em 560 nm).

Kinetics of Enzymatic Hydrolysis of Prodrugs 25 and 32. 1. By *E. coli* β-glucuronidase: Hydrolysis of prodrugs **25** (**a,b,c**) and **32** (**a,b,c**) by *E. coli* β-glucuronidase (Sigma type X-A, G 7896) was investigated by incubating the prodrug at 200 μg/mL with the enzyme (0.1 μg/mL) in 0.1 M phosphate

buffer (pH 7) and 0.01% bovine serum albumin (BSA). Analysis was carried out with the following HPLC system using a Zorbax SB-C18 column (3.5 μm, 150 × 4.6 mm). Elution of the analytical column was performed using a linear gradient made of two components (A, 20 mM phosphate buffer, pH 3; B, acetonitrile) with the following time−concentration profile: 0 min, 75% A, 25% B; 4 min, 70% A, 30% B; 10 min, 55% A, 45% B; 15 min, 45% A, 55% B; 10 min time for equilibration of starting conditions. Detection was performed by using a fluorescence detector (ex 494 nm, em 560 nm) and a UV detector (254 nm).

2. By fusion protein: Prodrug **25c** (or HMR 1826) (335 μg/mL) in 20 mM phosphate buffer, pH 7.2, was incubated with fusion protein (1.6 μg/mL) at 37 °C. HPLC analysis was performed as above.

In Vitro Cytotoxicity of Prodrugs 25 and 32. Exponentially growing L1210 tumor cells at a density of 5×10^{-3}/mL in RPMI were incubated in a 96-well microtiter plate for 72 h (37 °C, 5% CO_2, 95% relative humidity) with various concentrations of prodrugs **25** and **32**. The control consisted of cells exposed to fresh medium only. Quadruplicate wells were prepared for each drug concentration and for the control. After 65 h, 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (or MTT) (2.5 mg/mL) in phosphate-buffered saline (PBS) was added. The MTT was reduced by viable cells to red insoluble formazan dye. After an additional 7-h incubation, the supernatant medium was carefully removed. The formazan dye was solubilized by adding 100 μL of dimethyl sulfoxide (DMSO) to each well using a multiscan extinction after incubation with doxorubicin or prodrugs over that of the control. The coefficient of variation for replicate experiments was <15%. From the dose−response curves, IC_{50} values were evaluated.

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Supporting Information Available: Kinetics of cleavage by β-glucuronidase of *E. coli* of the prodrugs **25a,b** and **32a,b,c** and ^{1}H NMR data for all compounds which do not appear in the Experimental Section (5 pages). Ordering information is given on any current masthead page.

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