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# 2-Nitro and 4-nitro-quinone-methides are not irreversible inhibitors of bovine β-glucuronidase

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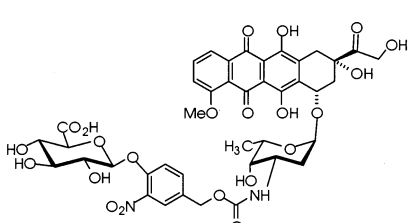
**Abstract**

4-Benzylamino-(and 4-chloromethyl)-2-nitro-β-D-glucuronides (**4**, **10**) and their 2-substituted-4-nitro regioisomers (**7**, **13**) were prepared by glycosidation of the 3-nitro-4-hydroxy- and the 2-hydroxy-5-nitro-benzylic alcohol, respectively, with a glucuronyl donor. Carbonate activation followed by reaction with benzylamine or methanesulfonyl chloride afforded, after complete deprotection, the target molecules **4**, **7**, **10** and **13**. These compounds have been synthesized to determine whether these molecules are (or not) glucuronidase inhibitors. After incubation with bovine liver β-glucuronidase, none of the cleavage products (the titled quinone-methides) showed to be irreversible inhibitors of this enzyme. © 2001 Published by Elsevier Science Ltd.

*Keywords:* ADEPT strategy; Doxorubicin; β-Glucuronidase; Quinone-methide

## 1. Introduction

Further improvements of cancer chemotherapy will certainly result from the discovery of new anticancer drugs but also, perhaps more efficiently, from site-specific targeting of known drugs. In this respect, several groups have searched for non-toxic prodrugs which may be activated at the tumor site by enzyme-catalyzed hydrolysis. The enzyme may be itself targeted by covalent binding to an antibody (ADEPT strategy)[1] or present in the tumor in higher concentration than in normal tissues. This is the case of β-glucuronidase, a lysosomal enzyme,[2] which has been shown to accumulate in necrotic areas surrounding the tumor.[3] This observation has led to the concept of prodrug monotherapy (PMT) recently introduced by Bosslet and co-workers.[4] In agreement with this hypothesis, superior efficiency against various tumors has been noted for HMR 1826,[5] a prodrug of the well-known anticancer agent doxorubicin.[6]



**HMR 1826**

A key feature of HMR 1826 is the introduction of a spacer group between the glucuronide moiety and doxorubicin, which is essential for enzyme recognition of the prodrug. After glycoside hydrolysis of this Type I derivative (Scheme 1), fast liberation of dox-

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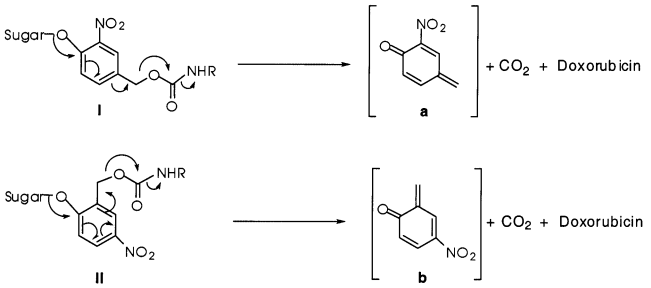
orubicin ($t_{1/2}$ 5 min) is expected to result from 1,6-elimination leading to an electrophilic *p*-quinone-methide **a** (Scheme 1).[7] 2-Nitro-4-hydroxymethylphenol has been characterized as the end-product of this process.[8] The same liberation of doxorubicin was also observed, albeit at a lower rate ($t_{1/2}$ ca. 300 min), with a Type II ortho-substituted spacer, leading to an *o*-quinone-methide **b**. In both cases, removal of the nitro substituent prevented drug elimination from the intermediate phenol under physiological conditions.



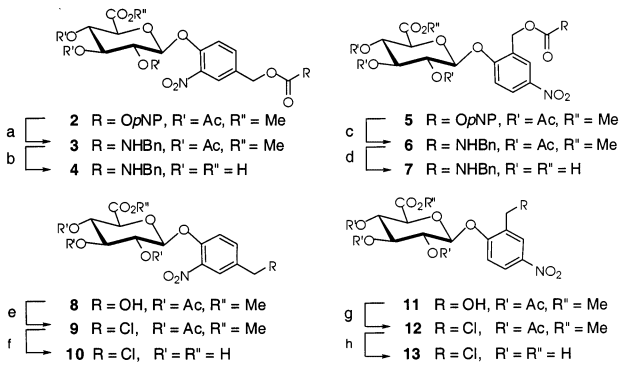

Scheme 1. $RNH_2$ = doxorubicin.

On the other hand, several quinone-methides have already been postulated as reactive intermediates in enzyme-activated irreversible glycosidase inhibition.[9] This is the case of the naturally occurring salicortin [(2-hydroxymethyl-phenyl) β-D-glucopyranosid], an inhibitor of *Agrobacterium faecalis* β-glucosidase[9] and of synthetic inhibitors such as 4-difluoromethyl-aryl β-D-glucopyranosides for β-glucosidase[10], 1,1′-difluoroalkyl α-D-glucopyranosides[11] and 2-chloromethyl-4-nitrophenyl α-D-glucopyranoside[12] for α-glucosidase. In the latter case, Haines and co-workers have shown that deletion of the nitro substituent leads to a much less potent inhibitor, in agreement with a slower rate of formation of the quinone-methide.

Taking these data into account, the question was to determine if para- or ortho-substituted hydroxybenzyl spacers I or II are (or not) really irreversible inhibitors of β-glucuronidase. A positive answer should be a hard restriction for further development of prodrugs incorporating such groups.

## 2. Results and discussion

In order to study this process, model compounds **4** and **7** were prepared from the known mixed carbonates **2** and **5**.[13] Condensation of the latter with benzylamine afforded **3** and **6** which were further fully deprotected to give **4** and **7** in one (*t*-BuOK, acetone) or two steps (MeONa, then NaOH), respectively. On the other hand, 4-chloromethyl-2-nitrophenyl and 2-chloromethyl-4-nitrophenyl analogs, **10** and **13**, were also prepared by treating the corresponding 4-hydroxymethyl and 2-hydroxymethyl derivatives **8** and **11**[13] by methanesulfonyl chloride in pyridine with subsequent one or two-step deprotection (Scheme 2).




Scheme 2. Reagents and conditions: (a) $NH_2Bn$ (1.3 equiv), $NEt_3$, $CH_2Cl_2$, 45%; (b) *t*-BuOK 0.5 M, acetone, 50%; (c) $NH_2Bn$ (1.5 equiv), pyridine, $CH_2Cl_2$, 60%; (d) NaOMe 1 M, $CH_3OH$, then NaOH 1 M, acetone 60%; (e) MsCl (1.5 equiv), pyridine, $CH_2Cl_2$, 50%; (f) *t*-BuOK 0.5 M, acetone, 35%; (g) MsCl (1.5 equiv), pyridine, $CH_2Cl_2$, 60%, (h) NaOMe 1 M, $CH_3OH$, then NaOH 1 M, acetone, 45%.

In order to determine their inhibitory activity and the type of inhibition, compounds **4**, **7**, **10** and **13** were incubated with bovine β-glucuronidase in conditions to show a time-dependent loss of activity. Surprisingly, we observed an increase of enzyme activity when compared to experiments without the supposed inhibitors. The only way to explain this result is to suppose that the quinone-methide intermediate is not an irreversible inhibitor of the β-glucuronidase from bovine liver. Therefore, during the incubation time, compounds **4**, **7**, **10** and **13** should be converted to an unstable para or ortho quinone-methide (Scheme 1(**a**, **b**)) and subsequently to a 4-hydroxymethyl-2-nitrophenol (or 2-hydroxy-

methyl-4-nitrophenol) which absorbed at 405 nm together with *p*-nitrophenol liberated by the enzyme from the *p*-nitrophenyl β-D-glucuronide.

To confirm such a hypothesis, the total enzyme activity was estimated by measuring the absorption at 405 nm, as a function of incubation time (15, 30, 45, 60 min), in three different ways:

- First, the enzyme alone was shaked at 37 °C. At various time, aliquots (50 μL) were incubated in the presence of the known substrate *p*-nitrophenyl β-D-glucuronide and the enzymatic test was performed as described in experimental section. In this experiment, the amount of *p*-nitrophenol liberated corresponds to the measured $OD_1$.

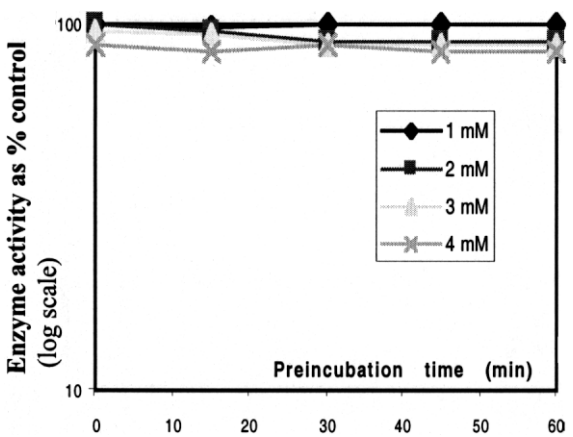



Fig. 1. Assay of time-dependent inhibition of bovine β-glucuronidase with compound **4** measured at different concentrations and plotted as a semi-logarithmic curve. Experimental conditions and expression of the residual activity as described in the text.

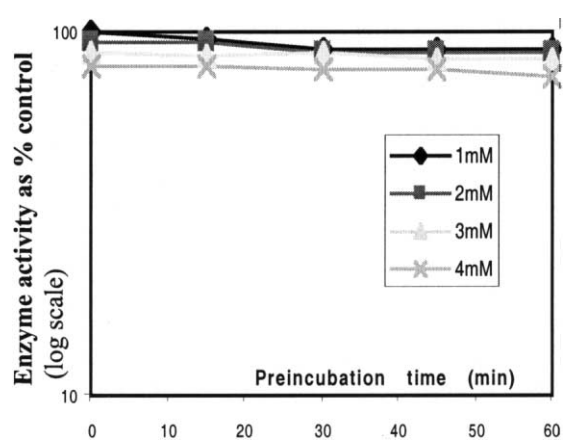



Fig. 2. Assay of time-dependent inhibition of bovine β-glucuronidase with compound **7**, as described for compound **4**.

- In the second experiment, the enzyme was incubated in the presence of variable concentrations of **4** or **7**. At different times, aliquots (50 μL) of the previous mixture were mixed with a fixed concentration of *p*-nitrophenyl glucuronide. In this instance, the $OD_2$ values correspond to the formation of both *p*-nitrophenol and 4-hydroxymethyl-2-nitrophenol from **4** (or the alternative isomer from **7**).
- Thirdly, the enzyme was also incubated in the presence of **4** (or **7**), but this time the enzymatic test was realized without addition of *p*-nitrophenyl glucuronide. Thus, $OD_3$ corresponds to the liberation of hydroxymethyl-nitrophenol only (or its regioisomer liberation).

From these experiments, the residual activity of the enzyme is expressed by calculating $(OD_2 - OD_3)/OD_1$. The resulting values and the corresponding time-dependent log's are given in Fig. 1 (for **4**) and Fig. 2 (for **7**). In the presence of **4** or **7**, no significant variation of enzyme activity was observed.

As depicted, neither **4** and **7,** nor their cleavage products, are enzyme inhibitors of bovine β-glucuronidase. One assumption is that, as soon as the intermediate quinone-methide is formed in solution, it is immediately quenched by nucleophilic addition of water, producing the release of the phenol (or phenate) at the enzyme active site. The commercially available bovine β-glucuronidase[14] was used in this study, because it has been reported that β-glucuronidase from human placenta[15] is similar to the bovine liver one. Therefore, it seems reasonable to assume that the HMR 1826 spacer will not interfere with the human enzyme.

## 3. Experimental

Optical rotations were determined with a Perkin–Elmer 241 polarimeter (589 nm) at 20 °C with a concentration expressed in g/100 mL. $^1$H NMR spectra were recorded using a Bruker WP-200 (200 MHz) and a Bruker AC-300 (300 MHz). Chemical shifts are expressed in ppm downfield from internal $Me_4Si$ with the notation indicating the multiplicity of the signal. For mass spectra, CI ($NH_3$) were

recorded with a Nermag R10-10C. Thin-layer chromatography (TLC) was performed on Silica gel $60F_{254}$ (E. Merck). Column chromatography was performed on $SiO_2$ (E. Merck, particle size 0.004–0.063 nm) using the flash technique. Preparations of compounds **2**, **5**, **8** and **11**[13] have already been reported.

*Methyl [(2-nitro-4-(*N*-benzylcarbamoyloxymethyl) phenyl) 2,3,4-tri*-O*-acetyl-β*-D*-glucopyranosid] uronate* (**3**).—To a solution of **2** (700 mg, 1.1 mmol) in $CH_2Cl_2$ (50 mL) were successively added benzylamine (160 μL, 1.4 mmol) and $Et_3N$ (500 μL). After stirring for 4 h at rt, the crude mixture was extracted with additional $CH_2Cl_2$ (200 mL) and the organic layer was washed with water and brine. The organic phase was dried, concentrated and the residue was chromatographed (1:1, cyclohexane–EtOAc) to give **3** (306 mg, 45%) as an oil: $[\alpha]_D^{20}$ +6.7° (*c* 1.8, $CHCl_3$); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 2.06, 2.07, 2.13 (3s, 3 × 3 H, OAc), 3.70 (s, 3 H, $OCH_3$), 4.20 (d, 1 H, $J_{4,5}$ 8 Hz, H-5), 4.40 (d, 2 H, *J* 5.6 Hz, C*H*$_2$–Ar), 5.10 (s, 2 H, $CH_2$–O–CO), 5.20 (d, 1 H, $J_{1,2}$ 6 Hz, H-1), 5.30 (m, 3 H, H-2, H-3, H-4), 7.30 (m, 7 H, Ar), 7.50 (d, 1 H, *J* 8 Hz, Ar), 7.80 (br s, 1 H, NH); MS (DCI/$NH_3$): *m*/*z* 636 $[M + NH_4]^+$.

*[2-Nitro-4-(*N*-benzylcarbamoyloxymethyl) phenyl] β*-D*-glucopyranosiduronic acid* (**4**).—To a stirred, ice-cooled solution of **3** (200 mg, 0.32 mmol) in acetone (10 mL) was added 1 mL of aq potassium *tert*-butoxide (0.5 N). The ice-bath was removed and the reaction mixture was stirred at rt for 3 h. The reaction was quenched by adding HCl (1 N) until neutralization. Evaporation of the crude product, followed by flash chromatography (9:1, MeCN–water) gave **4** (77 mg, 50%) as an oil: $[\alpha]_D^{20}$ +96.3° (*c* 0.6, water); $^1$H NMR (300 MHz, $CD_3OD$): $\delta$ 3.50 (m, 3 H, H-2, H-3, H-4), 3.90 (d, 1 H, $J_{4,5}$ 8 Hz, H-5), 4.20 (d, 2 H, C*H*$_2$–Ar), 5.00 (s, 2 H, $CH_2$–O–CO), 5.10 (d, 1 H, $J_{1,2}$ 7 Hz, H-1), 7.30 (m, 7 H, Ar), 7.50 (dd, 1 H, *J* 8 Hz, Ar), 7.80 (br s, 1 H, NH); Anal. Calcd for $C_{21}H_{22}N_2O_{11}$: C, 52.72; H, 4.64; N, 5.86. Found: C, 52.51; H, 4.94; N, 5.51.

*Methyl [2-(*N*-benzylcarbamoyloxymethyl)-4-nitrophenyl) 2,3,4-tri*-O*-acetyl-β*-D*-glucopyranosid] uronate* (**6**).—Prepared from **5** (500 mg, 0.77 mmol) with benzylamine (130 μL, 1.15 mmol) by the same protocol as for **3**. After chromatography, compound **6** (285 mg, 60%) was isolated as a white solid: mp 171–172 °C; $[\alpha]_D^{20}$ −57.4° (*c* 1, $CHCl_3$); $^1$H NMR (200 MHz, $CDCl_3$): $\delta$ 2.04, 2.07, 2.10 (3s, 3 × 3 H, OAc), 2.90 (d, 1 H, *J* 16 Hz, NH), 3.65 (s, 3 H, $OCH_3$), 4.30 (d, 1 H, *J* 9 Hz, H-5), 4.40 (d, 2 H, *J* 6 Hz, Ar–C*H*$_2$–N), 5.13 (d, 2 H, *J* 14 Hz, Ar–C*H*$_2$–O), 5.35 (m, 4 H, H-1, H-2, H-3, H-4), 7.15 (d, 1 H, *J* 8 Hz, Ar), 7.31 (m, 5 H, Ar), 8.20 (m, 2 H, Ar); MS (DCI/$NH_3$): *m*/*z* 636 $[M + NH_4]^+$.

*[2-(*N*-Benzylcarbamoyloxymethyl)-4-nitrophenyl] β*-D*-glucopyranosiduronic acid* (**7**).—Compound **6** (200 mg, 0.32 mmol) in anhyd MeOH (25 mL) was stirred for 4 h at 0 °C in the presence of NaOMe (20 mg, 3.7 mmol). Neutralization by addition of Amberlite IRC-50H$^+$ was followed by filtration and the filtrate was evaporated under reduced pressure. The crude product was dissolved in acetone (5 mL) containing 0.5 mL of a 1 M NaOH solution. The mixture was stirred for 15 min at rt, neutralized with a 1 M HCl solution and evaporated. The crude product was purified by chromatography (9:1, MeCN–water) to give **7** (92 mg, 60%) as a white solid: mp 149–150 °C; $[\alpha]_D^{20}$ −45.5° (*c* 1, $CH_3OH$); $^1$H NMR (200 MHz, $CD_3OD$): $\delta$ 3.30 (m, 2 H, H-1, H-5), 3.55 (m, 3 H, H-2, H-3, H-4), 4.32 (s, 2 H, C*H*$_2$–Ar), 5.13 (s, 1 H, NH), 5.28 (s, 2 H, Ar–C*H*$_2$–O), 7.00 (d, 2 H, *J* 8 Hz, Ar), 8.24 (m, 6 H, Ar), 10.80 (s, 1 H, COOH); Anal. Calcd for $C_{21}H_{22}N_2O_{11}$: C, 52.72; H, 4.64; N, 5.86. Found: C, 52.69; H, 4.74; N, 5.66.

*Methyl [(2-nitro-4-(chloromethyl) phenyl) 2,3,4-tri*-O*-acetyl-β*-D*-glucopyranosid] uronate* (**9**).—To a solution of **8** (500 mg, 1 mmol) in dry $CH_2Cl_2$ (25 mL) were added 0.4 mL (10 equiv) of methanesulfonyl chloride and 1.8 mL of pyridine. The mixture was heated under reflux for 3 h. After cooling to rt, the crude product was diluted with water (50 mL) and extracted with $CH_2Cl_2$ (150 mL). The combined organic layers were washed with water, brine and evaporated under reduced pressure. Purification by chromatography (1:1, cyclohexane–EtOAc), gave **9** (250 mg, 50%) as an oil; $[\alpha]_D^{20}$ +19° (*c* 0.5, $CHCl_3$); $^1$H NMR (300 MHz, $CHCl_3$): $\delta$ 2.08, 2.09, 2.16 (3s, 3 × 3 H,

OAc), 3.75 (s, 3 H, $COOCH_3$), 4.20 (d, 1 H, $J_{5,4}$ 9 Hz, H-5), 4.70 (s, 2 H, $CH_2$–Cl), 5.20 (d, 1 H, $J_{1,2}$ 7 Hz, H-1), 5.30 (m, 3 H, H-2, H-3, H-4), 7.35 (d, 1 H, *J* 10 Hz, Ar), 7.55 (dd, 1 H, *J* 10 Hz, *J* 1 Hz, Ar), 7.80 (d, 1 H, *J* 1 Hz, Ar); MS (DCI/$NH_3$): *m/z* 521 $[M + NH_4]^+$, 504 $[M + H]^+$.

*[2-Nitro-4-(chloromethyl) phenyl] β-*D*-glucopyranosiduronic acid* (**10**).—Obtained from **9** (250 mg, 0.49 mmol) as described for the preparation of **4** and after chromatography (9:1, MeCN–water) compound **10** (63 mg, 35%) was isolated as an oily residue: $[\alpha]_D^{20}$ −80° (*c* 0.9, $CH_3OH$); $^1H$ NMR (300 MHz, $CD_3OD$): δ 3.40 (m, 3 H, H-2, H-3, H-4), 3.80 (d, 1 H, $J_{5,4}$ 8 Hz, H-5), 4.60 (s, 2 H, Ar–C$H_2$–Cl), 5.10 (d, 1 H, $J_{1,2}$ 7 Hz, H-1), 7.40 (d, 1 H, *J* 10 Hz, Ar), 7.60 (dd, 1 H, *J* 10, *J* 1 Hz, Ar), 7.80 (d, 1 H, *J* 1 Hz, Ar); HRMS (FAB positive mode) Calcd for $C_{13}H_{14}ClNO_9$ $[M + H]^+$: 364.0436. Found: 364.0439.

*Methyl [2-(chloromethyl)-4-nitrophenyl) 2,3,4-tri-*O*-acetyl-β-*D*-glucopyranosid] uronate* (**12**).—Obtained from **11** (350 mg, 0.7 mmol) following the same protocol as for the preparation of **9**, the product was purified by column chromatography (1:1, cyclohexane–EtOAc) yield **12** (oil, 211 mg, 60%): $[\alpha]_D^{20}$ +2° (*c* 0.5, $CHCl_3$); $^1H$ NMR (200 MHz, $CDCl_3$): δ 2.05, 2.06, 2.11 (3s, 3 × 3 H, OAc), 3.74 (s, 3 H, $OCH_3$), 4.28 (d, 1 H, $J_{5,4}$ 9 Hz, H-5), 4.58 (s, 2 H, Ar–C$H_2$–Cl), 5.32 (m, 4 H, H-1, H-2, H-3, H-4), 7.38 (d, 1 H, *J* 9 Hz, Ar), 7.60 (s, 1 H, Ar), 7.85 (d, 1 H, *J* 2 Hz, Ar).

*[2-(Chloromethyl)-4-nitrophenyl] β-*D*-glucopyranosiduronic acid* (**13**).—Obtained from **12** (200 mg, 0.4 mmol), as described for **7**. Compound **13** (65 mg, 45%) was isolated as an oil: $[\alpha]_D^{20}$ −27° (*c* 1, $CH_3OH$); $^1H$ NMR (200 MHz, $CD_3OD$): δ 3.40 (m, 3 H, H-2, H-3, H-4), 3.75 (d, 1 H, $J_{5,4}$ 8 Hz, H-5), 4.55 (s, 2 H, Ar–C$H_2$–Cl), 5.10 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 7.35 (d, 1 H, *J* 9 Hz, Ar), 7.55 (s, 1 H, Ar), 7.80 (d, 1 H, *J* 2 Hz, Ar); Anal. Calcd for $C_{13}H_{14}ClNO_9$: C, 42.93; H, 3.88; Cl, 9.75; N, 3.85. Found: C, 43.11; H, 3.63; N, 3.71.

*β-Glucuronidase inhibition assay.*—*p*-Nitrophenyl β-D-glucuronide was purchased from Fluka. β-D-Glucuronidase (EC.3.2.1.3.1) from bovine liver (Sigma) was used in this study. Activity of β-glucuronidase was measured as follows: A freshly prepared solution of compounds **4**, **7**, **10** and **13** at different concentrations (100 μL; 5, 10, 20 and 40 mmol, respectively) was added to a solution of β-glucuronidase (200 μg protein) in 400 μL acetate buffer (0.07 M, pH 5); the solution was incubated at 37 °C. After 15, 30, 45 and 60 min, aliquots (50 μL) were taken from the incubation assay and added with 450 μL of *p*-nitrophenyl β-D-glucuronide (2.5 mM in 0.07 M acetate buffer, pH 5). The mixture was shaken at 37 °C for 15 min and the reaction was stopped by adding 500 μL of a glycine solution (0.4 M, pH 10.8). The activity was evaluated via measurement of the absorption of released *p*-nitrophenolate at 405 nm as a function of incubation time.[16]

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