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# *p*-NITROPHENYL β-D-GLUCOPYRANOSIDURONIC ANALOGS AS POTENTIAL SUBSTRATES FOR β-GLUCURONIDASE

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***Dedicated to Professor Dr. Hans Paulsen on the occasion of his 75th anniversary***

**Abstract.** Three *p*-nitrophenyl-β-D-glycosides including gluco-hex-4-enopyranosiduronic, glucofuranosiduronic and xylofuranosiduronic moieties have been prepared. None of them appeared to be a better substrate for β-glucuronidase from bovine origin than usual *p*-nitrophenyl-β-D-glucuronide. © 1997 Elsevier Science Ltd.

For the last seven years, the strategy known as antibody-directed enzyme prodrug therapy (ADEPT) proposed for enhancing selective administration of anticancer drugs has been the subject of intense investigation.[1] In this two-step approach, prior to the administration of a prodrug, an enzyme is firstly specifically localized at the tumor cell surface by use of a conjugate monoclonal antibody-enzyme. Subsequently, a non-cytotoxic prodrug which is substrate for the enzyme, is injected to selectively generate the cytotoxic drug at the antigen-positive tumor cell surface. Immediate internalization is expected, minimizing toxic effects towards normal tissues.

Despite these improvements, the systems described so far have some major disavantages for clinical applications. Monoclonal antibody enzyme conjugates produced by chemical coupling have generally, as major drawback, a strong immunogenicity in man, due to the xenogenic origin of the antibody moiety and the enzyme. Consequently, repetitive applications in man are possible only to a very limited extent.[2]

Fusion proteins consisting of non-humanized binding moieties and xenogenic enzymes[3] produced by recombinant DNA technology will be immunogenic in man as well, with disavantages comparable to monoclonal antibody enzyme conjugates. This may be also the case with fusion protein incorporating humanized monoclonal antibody and xenogenic enzyme.[4]

This is the reason why, for the last four years, there has been a continuous interest in our group for finding prodrugs which are substrates for not very immunogenic fusion proteins, most probably allowing repetitive treatment cycle in man.

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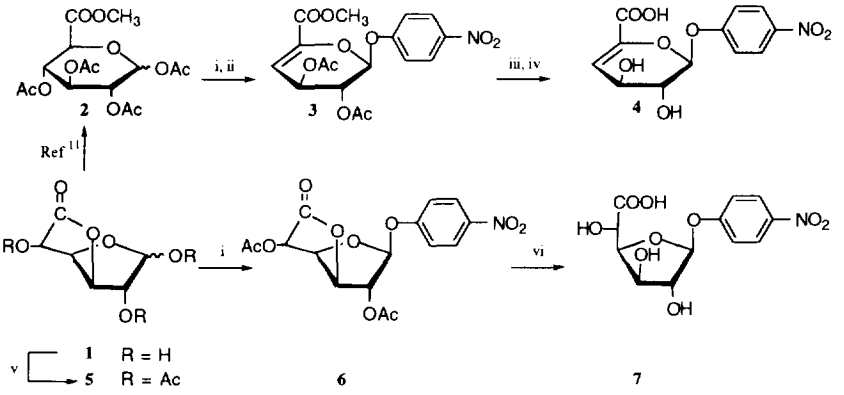
Such a fusion protein was prepared from a humanized CEA-specific binding region and a human glycosidase, like β-D-glucuronidase.[5] Among the large number of daunorubicin and doxorubicin prodrugs we have prepared,[6] the best results were obtained with the *o*-nitro-activated prodrug which displays very reduced toxicity, high stability in human plasma and suitable kinetics of cleavage *in vitro* in the presence of the fusion protein. Moreover, *in vivo* preclinical experiments have shown the therapeutic efficacy of such a system in nude mice bearing LoVo colon carcinomas for example.[7]

Despite these promising results, our system may have disavantages for clinical applications. One of them is the low turnover rate ($v_{max}$) of the human enzyme moiety and another is the high prodrug dose (250 mg/kg) needed to obtain significant catalysis in comparison to xenogenic enzymes having a high turnover rate and a low Michaelis-Menten constant ($K_m$). Based on *in vitro* cleavage experiments in buffer at pH 7.2, values for $K_m$ and $v_{max}$ for this fusion protein were[7] 1.3 mM and 635 nmol/min x mg at 37 °C, which is in full agreement with previous reports concerning other mammalian β-glucosidases.[8] Therefore, in order to decrease the amount of prodrug to be injected, we tried to reduce the $K_m$ of human β–glucuronidase by using modified glucuronic acid.

Although some experiments have been reported in the literature,[9] it remained to be proven whether a pyranose structure is required for hydrolysis by β-glucuronidase. This observation, associated with the fact that saccharo-1,4-lactone is a more potent competitive inhibitor[10] of β-glucuronidase ($K_i$ 5.4 x $10^{-7}$ M) compared to the corresponding saccharo-1,5-lactone ($K_i$ 8.4 x $10^{-4}$ M), led us to explore the potentiality of furanoside derivatives such as **7** and **11**, to be better subtrates. At the same time, we decided to evaluate the ability of the 4,5-unsaturated glucuronic acid analog, **4**, which is the by-product formed during deacetylation of the methyl per-*O*-acetyl-glucuronide, to be a substrate for the enzyme.



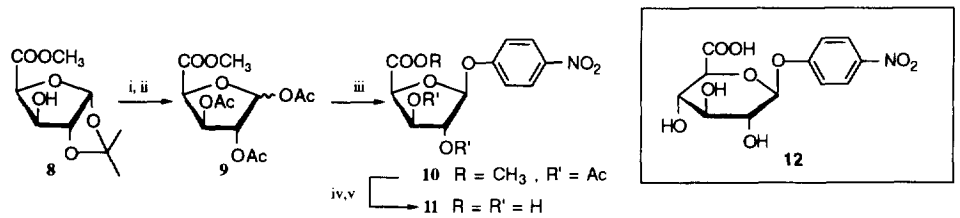

**Scheme 1** Reagents and conditions : i, $SnCl_4$, $HO\text{-}C_6H_4\text{-}NO_2$; ii, DBU, THF; iii, $NaOCH_3$ , $CH_3OH$; iv, NaOH, $CH_3OH$; v, pyridine , $Ac_2O$; vi, $(CH_3)_3COK$, acetone.

Synthesis of compounds **4** and **7** started from the D-glucurono-D-lactone **1**. Conversion of **1** (scheme 1) into **2** was performed according to the literature.[11] Condensation of **2** with *p*-nitrophenol in the

presence of $SnCl_4$, followed by treatment with DBU in THF, led to **3**[12] (14% overall yield) which was subsequently deprotected in a two-step sequence affording **4**.[13]

In order to obtain **7**, glucurono-D-lactone **1** was per-*O*-acetylated into **5** and further condensation with *p*-nitrophenol gave **6** in 30% yield. One-step deprotection of **6**[14] in the presence of potassium *tert*-butylate[15] afforded the target compound **7**[16] in 21% yield.

The β-D-xylofuranoside analog **11** was prepared (scheme 2) from the methyl (1,2-*O*-isopropylidene-α–D-xylofuranose) uronate **8**.[17] After peracetylation of **8** (pyridine-$Ac_2O$) and acetolysis ($Ac_2O$, AcOH, $H_2SO_4$) of the crude product, methyl 1,2,3-tri-*O*-acetyl-D-*xylo*-furanose **9** was isolated. Therefore, the same sequence of reactions as in the case of **4** (condensation with *p*-nitrophenol and two-step deprotection of **10**[18] with NaOMe in MeOH and then 2N aq. sol. of NaOH at 0 °C) led to **11**[19] in 12% overall yield for the three steps.




**Scheme 2** Reagents and conditions : i, pyridine, $Ac_2O$ ; ii, $H_2SO_4$, $Ac_2O$ ,AcOH , O°C; iii,$SnCl_4$, HO-$C_6H_4$ -$NO_2$; iv, $NaOCH_3$ , $CH_3OH$; v, NaOH, $CH_3OH$ ,$H_2O$ ,O°C.

The glycosides *p*-nitrophenylduronic acids **4**, **7**, **11**, **12**[20] were tested for hydrolysis with bovine β-D-glucuronidase.[21,22] The values obtained for the $K_m$ (mM) and $v_{max}$ (nmol/min x mg) were 0.6 and 230 for **12**, 4.5 and 100 for **4**, 10.25 and 70 for **7**, and 7.6 and 1 for **11**, respectively. This clearly indicates that furanoside analogs (**7** and **11**) are not better substrates than pyranoside derivative **12**. Since it has been reported that β-glucuronidase from human placenta is similar to β-glucuronidase from bovine liver[23], we may postulated that new compounds are also not better substrates than **12** for fusion protein.

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12. **3**: syrup; $[\alpha]_D^{20}$ -143° (*c* 1, $CHCl_3$); $^1H$ NMR (300 MHz, $CDCl_3$) δ 2.15 (s, 3H, OAc), 2.16 (s, 3H, OAc), 3.81 (s, 3H, $OCH_3$), 5.30 (m, 2H, H-2, H-3), 5.90 (d, 1H, J = 5.2 Hz, H-1), 6.30 (d, 1H, J = 4 Hz, H-4).
13. **4**: mp 195 °C (acetonitrile); $[\alpha]_D^{20}$ -2° (*c* 0.5, MeOH); $^1H$ NMR (300 MHz, $CD_3OD$) δ 3.85 (dd, 1H, J = 4.2, J'= 5.2 Hz, H-2), 4.15 (dd, 1H, J = J'= 4.2 Hz, H-3), 5.77 (d, 1H, J = 5.2 Hz, H-1), 5.90 (d, 1H, J = 4.2 Hz, H-4), 7.30 and 8.14 (2d, 4H, AB system, J = 7 Hz, H-Ar).
14. **6**: mp 177 °C (acetone); $[\alpha]_D^{20}$ -174° (*c* 1.05, $CHCl_3$); IR: ν 1811 ($cm^{-1}$, acetone); $^1H$ NMR (300 MHz, $CDCl_3$) δ 1.67 (s, 3H, OAc), 2.18 (s, 3H, OAc), 5.16 (d, 1H, $J_{2,3}$ = 5.2 Hz, J'= 5.2 Hz, H-2), 5.18 (d, 1H, $J_{3,4}$ = 7.3 Hz, H-4), 5.32 (dd, 1H,H-3), 5.55 (s, 1H, H-5), 5.92 (s, 1H, H-1), 7.08 and 8.21 (2d, 4H, AB system, J = 7 Hz, H-Ar).
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16. **7**: syrup; $[\alpha]_D^{20}$ -108° (*c* 1.3, $H_2O$); $^1H$ NMR (300 MHz, $CD_3OD$) δ 4.22 (m, 1H, H-4), 4.30 (bs, 1H, H-2), 4.37 (bs, 1H, H-5), 4.67 (m, 1H, H-3), 5.57 (s, 1H, H-1), 7.19 and 8.17 (2d, 4H, AB system, J = 7 Hz, H-Ar), 8.50 (s, 1H, COOH); MS (DCI/$NH_3$) *m/z* 333 $[M + NH_4]^+$, 194 $[M + NH_4 - HOArNO_2]^+$.
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18. **10**: syrup; $[\alpha]_D^{20}$ -153° (*c* 1.05, $CHCl_3$); $^1H$ NMR (300 MHz, $CDCl_3$) δ 2.11 (s, 3H, OAc), 2.17 (s, 3H, OAc), 3.65 (s, 3H, $OCH_3$), 5.10 (d, 1H, J = 6.5 Hz, H-3), 5.38 (s, 1H, H-4), 5.65 (d, 1H, J = 6.5 Hz, H-2), 5.80 (s, 1H, H-1), 7.15 and 8.22 (2d, AB system, J = 7 Hz, 4H-Ar); MS (DCI/$NH_3$) *m/z* 245 $[M + H - HOArNO_2]^+$.
19. **11**: syrup; $[\alpha]_D^{20}$ -146° (*c* 1, MeOH); $^1H$ NMR (300 MHz, $CDCl_3$) δ 4.35 (m, 2H, H-2, H-3), 4.95 (d, 1H, J = 5 Hz, H-4), 5.69 (s, 1H, H-1), 7.20 and 8.15 (2d, AB system, J = 7 Hz, 4H-Ar); MS (DCI/$NH_3$) *m/z* 317 $[M + NH_4]^+$, 300 $[M + H]^+$, 161 $[M + H - HOArNO_2]^+$.
20. *p*-Nitrophenyl-β-D-glucuronide **12** was obtained from Sigma. Choice of this compound has been based upon the fact that the corresponding β-D-glucuropyranosiduronic acid is one of the most commonly used reference substrate [see, for example, ref. 10 or Diez, T., Cabezas, J.A. *Eur. J. Biochem.* **1979**, *93*, 301-311] but also for the reason that the kinetics of cleavage has been found to be identical[6] for prodrugs including an *o*-nitro or a *p*-nitro substituted spacer.
21. Determination of $K_m$ and $v_{max}$ values were then made[22] at pH 5 at which the enzyme (β-glucuronidase (EC 3.2.1.31) from bovine liver) activity is optimum, over a 5-min period. This involved incubating the substrate in 0.7 mM acetate buffer at 37 °C at various concentrations from 0.25 -2.5 mM for the reference (*p*-nitrophenyl glycoside of glucuronic acid) and 1-20 mM for the tested substrates and then adding thermally equilibrated enzyme to start the reaction. After 5 min, this reaction was stopped by addition of gly-NaOH buffer solution and the concentration of phenolate released was determined by spectrophotometry at 405 nM. The Michaelis parameters $K_m$ and $v_{max}$ were obtained from the Line-weaver-Burk plot.
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