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Synthesis of betulinic acid acyl glucuronide for application in anticancer prodrug monotherapy

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ABSTRACT

The synthesis of 28-O-β-D-glucuronide betulinic acid, an acyl glucuronide derivative, was successfully carried out for the first time using commercially available peracetylated methyl glucuronate bromide under phase-transfer conditions. The target compound could be used in an anticancer prodrug monotherapy (PMT) strategy since it is non-cytotoxic, non-haemolytic, more water soluble than betulinic acid, it possesses a good in vitro stability in phosphate buffer and can be hydrolyzed in the presence of β-D-glucuronidase releasing in vitro betulinic acid, a promising anticancer agent.

Glucuronidation is one of the main phase 2 metabolic pathways by which the organism transforms a drug or a xenobiotic into a more water soluble substance allowing its detoxification and further excretion.[1] The glucuronidation reaction is catalyzed by enzymes of the family of uridine diphosphate glucuronosyltransferases (UDPGTs). Compounds featuring aliphatic alcohols and phenols are metabolized into *O*-glucuronide ethers, while those containing carboxylic acid functions are transformed into *O*-glucuronide esters, the so-called acyl glucuronides. Since most of the glucuronides exhibit a weaker biological activity than their corresponding aglycones, the glucuronidation is generally considered as an important detoxification metabolic process in mammals.[2] However, even if the glucuronide has no activity itself, it can undergo an enzymatic hydrolysis catalyzed by β-D-glucuronidase that subsequently releases the corresponding biologically active aglycone.[1] Furthermore, in some cases such as morphine-6-glucuronide, the glucuronidation can maintain or even increase the therapeutic effect of the drug.[3]

Several studies have revealed that acyl glucuronides are potentially active metabolites of carboxylic acid-containing drugs.[4] In fact, these compounds may be considered responsible for adverse side effects and the toxicity of such drugs.[5] Indeed, acyl glucuronides can undergo hydrolytic reactions, intramolecular rearrangements and intermolecular reactions with plasma proteins leading to covalent drug–protein adducts.[4,6] Therefore, the determination and characterization of acyl glucuronide metabolites have important toxicological implications during the clinical development of carboxylic acid-containing drugs.

Bevirimat or PA-457 (**2**, Fig. 1), namely betulinic acid 3β-*O*-(3′,3′-dimethylsuccinyl), is the first in the new class of HIV drugs called maturation inhibitors that specifically block a late step in processing of the HIV Gag protein.[7] Structurally, bevirimat (**2**) is a C-3 esterified derivative of the anticancer agent betulinic acid (**1**) that features two carboxylic acid groups. Recent studies have shown that bevirimat (**2**) is metabolized to two acyl monoglucuronides and one acyl diglucuronide after oral administration to rats, the monoglucuronide **4** (Fig. 1) being the major metabolite.[8,9] The naturally occurring lupane-type triterpenoid betulinic acid (**1**) is a promising anticancer agent[10] found in various fruits, vegetables and medicinal plants.[11] Betulinic acid (**1**) is currently undergoing phase II clinical trials for the treatment of melanoma.[12] Although metabolites of betulinic acid (**1**) have not yet been characterized, it may be hypothesized that acyl glucuronide **3** could be a major metabolic product. Therefore, it is of great importance to synthesize in adequate amounts the acyl glucuronide **3** in order to evaluate its biological activity and potential toxicity.

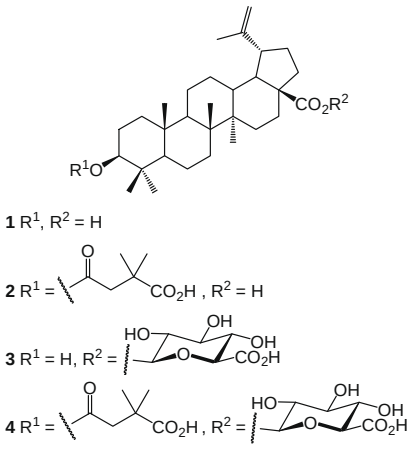

Figure 1. Structures of betulinic acid (**1**), bevirimat (**2**) and acyl glucuronides **3** and **4**.

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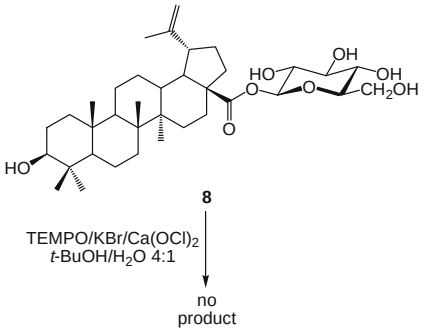
Anticancer compounds bearing a glucuronide moiety can also be considered as prodrugs.[1,13,14] Indeed, glucuronides can be selectively activated to the tumoural site since the enzyme β-D-glucuronidase is found at highly elevated concentrations in necrotic tumour tissue.[15,16] Moreover, β-D-glucuronidase is more active in cytoplasmic acidic pH, which is usually found in cancer cells.[17] The design of a suitable glucuronide prodrug must be based upon four criteria: (1) improved water solubility, (2) stability in blood, (3) decreased cytotoxicity and (4) susceptibility to enzymatic cleavage.[14] Thus, in this Letter, we report an efficient stereoselective synthetic strategy for the preparation of 28-*O*-β-D-glucuronide betulinic acid (**3**). In vitro stability in phosphate buffer, in vitro haemolytic and cytotoxic activities as well as enzymatic hydrolysis in the presence of β-D-glucuronidase were also investigated in order to determine if the acyl glucuronide **3** could be used in a prodrug monotherapy strategy (PMT).

Although the synthesis of *O*-glucuronide ethers has been frequently reported in the literature,[18–20] few studies have been undertaken on the chemical synthesis of acyl glucuronide derivatives.[5,21–23] A first approach developed by Juteau et al.[24] consists of coupling a carboxylic acid-containing compound with allyl glucuronate (**6**) via Mitsunobu conditions. The main advantage of this method is that the sugar is minimally protected, which considerably reduces the number of steps of the synthetic procedure. Thus, as depicted in Scheme 1, allyl glucuronate (**6**)[24] was obtained in 54% yield after treatment of D-glucuronic acid (**5**) with allyl bromide (AllBr) and potassium carbonate (K_2CO_3) in dimethylformamide (DMF). Compound **6** was then coupled with betulinic acid (**1**)[25] in the presence of triphenylphosphine (PPh_3) and diisopropylazodicarboxylate (DIAD) in anhydrous THF. Surprisingly, under these conditions, 28-*O*-α-D-glucuronide betulinic acid (**7**) was isolated in low yield (9%) as the major product instead of the allylated β-anomer. The configuration of the glycosidic linkage was confirmed by ^{1}H NMR (6.17 ppm, br s, H-1′).

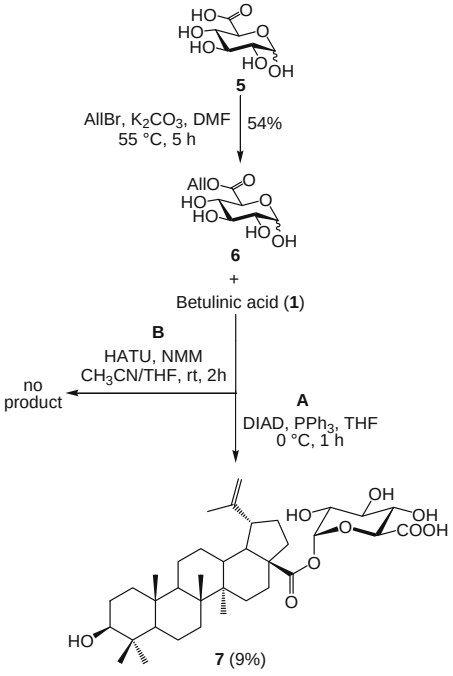
Therefore, we turned to another approach recently developed by Stachulski and co-workers[26,27] allowing the stereoselective formation of β-glucuronides. As shown in Scheme 1, the selective acylation of allyl glucuronate (**6**) with betulinic acid (**1**) was tried using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and *N*-methylmorpholine (NMM) in acetonitrile (CH_3CN). Unfortunately, as revealed by TLC and NMR analyses, no coupling product was formed during this reaction and triterpene **1** was almost completely recovered after silica gel column chromatography. Addition of THF in order to improve the solubility of both compounds **1** and **6** did not lead to better results.

We then chose to use the TEMPO-mediated selective oxidation[28] to achieve the preparation of acyl glucuronide **3**. As depicted in Scheme 2, this approach consists of selectively oxidizing the primary alcohol of the known 28-*O*-β-D-glucopyranoside betulinic acid (**8**)[29,30] in the presence of TEMPO/KBr/$Ca(OCl)_2$ under aqueous conditions (*t*-BuOH/H_2O).[31] Once again, using these conditions, no glucuronide was formed, probably due to a lack of solubility of glucoside **8** in the reaction medium.

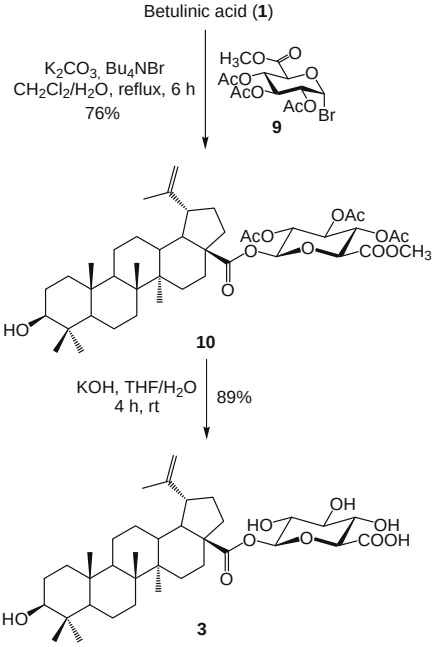
The successful synthesis of 28-*O*-β-D-glucuronide betulinic acid (**3**) was finally achieved under modified phase-transfer conditions.[32] Thus, as shown in Scheme 3, betulinic acid (**1**) was coupled



Scheme 2. Attempt to synthesize acyl glucuronide **3** via TEMPO-mediated selective oxidation of glucoside **8**.



Scheme 1. Attempts to synthesize acyl glucuronide **3** via Mitsunobu (**A**) and selective acylation (**B**).



Scheme 3. Successful synthesis of acyl glucuronide **3** under phase-transfer conditions.

with the commercially available methyl 2,3,4-tri-*O*-acetyl-1-bromo-D-glucopyranuronate (**9**) in the presence of potassium carbonate (K_2CO_3) and tetrabutylammonium bromide (Bu_4NBr) in a heterogeneous solution of CH_2Cl_2/H_2O to provide 28-*O*-(methyl-2,3,4-tri-*O*-acetyl-β-D-glucuronate) betulinic acid (**10**)[33] in good yield (76%). Subsequent simultaneous deprotection of acetyl groups and saponification of methyl ester under basic conditions (KOH, THF/H_2O) allowed the formation of the target acyl glucuronide **3**[34] (89%) in a pure β-linkage (1,2-*trans*-glycoside) as proven by ^1H NMR (5.55 ppm, d, $J_{1,2}$ = 8.1 Hz, H-1′). The strong HMBC correlation between the proton H-1′ and the carbon C-28 revealed that the reaction took place at the C-28 carboxylic acid position.

In order to determine if the acyl glucuronide **3** could be used as an anticancer prodrug in a PMT strategy, we investigated several parameters. Firstly, the in vitro cytotoxicity of glucuronide **3** was assessed against lung carcinoma (A549), colon adenocarcinoma (DLD-1) and normal skin fibroblasts (WS1) using both resazurin reduction test[35] and Hoechst assay.[36] As revealed in Table 1, the acyl glucuronide **3** did not exhibit any significant cytotoxic activity with an IC_{50} greater than 100 μM. The in vitro haemolytic activity of glucuronide **3** was also assessed against sheep erythrocytes. As for most of the lupane-type glycosides,[37] no haemolytic activity was measured for acyl glucuronide **3** (HD_{50} > 100 μM). Another essential parameter to evaluate for an anticancer prodrug is its stability in solution. To this end, the in vitro stability was determined by analytical RP-HPLC[38] after incubation at 37 °C of acyl glucuronide **3** in phosphate buffer (0.02 M; pH 7.2). As measured by Wen et al.[8] for the bevirimat glucuronide **4**, the acyl glucuronide **3** was quite stable, and no decomposition products were observed even after a one-week incubation time (168 h) in phosphate buffer. It is noteworthy that acyl glucuronide **3** was significantly more water soluble than betulinic acid (**1**) since it was possible to dissolve it at low concentrations (≈150 μM) in phosphate buffer. Finally, cleavage of acyl glucuronide **3** in the presence of *Escherichia coli* β-D-glucuronidase (4.3 U/mL) in phosphate buffer (0.02 M; pH 7.2) was measured by analytical RP-HPLC.[38] As shown in Figure 2, it is quite interesting that betulinic acid (**1**) was released at 75% in the solution after a 24 h incubation time at 37 °C. Increasing by 10-fold the concentration of β-D-glucuronidase (43 U/mL) led to the complete release of betulinic acid (**1**) after less than 1 h. Therefore, these results showed that the acyl glucuronide **3** is a good substrate for the enzyme β-D-glucuronidase.

In summary, for the first time, the chemical synthesis of 28-*O*-β-D-glucuronide betulinic acid (**3**) was carried out with success in a stereoselective and efficient manner under phase-transfer conditions. This novel methodology could be applied for the preparation of the major acyl glucuronide metabolite of bevirimat (**4**) or other carboxylic acid-containing drug metabolites. In view of the results, acyl glucuronide **3** could be used as an anticancer prodrug in a PMT strategy since it is non-cytotoxic, non-haemolytic, more water soluble than betulinic acid (**1**), quite stable in phosphate buffer and it can be hydrolyzed in the presence of β-D-glucuronidase releasing betulinic acid (**1**), a promising anticancer agent. Further studies will be undertaken in order to assess the in vivo antitumour potential of the acyl glucuronide **3** and results will be reported in due course.

Table 1
Cytotoxic and haemolytic activities of 28-*O*-β-D-glucuronide betulinic acid (**3**)

Cytotoxicity[a] (IC_{50}, μM)			Haemolysis[b] (HD_{50}, μM)
A549[c]	DLD-1[d]	WS1[e]	
>100	>100	>100	>100

[a] Resazurin reduction test[35] and Hoechst assay.[36]
[b] Sheep erythrocytes.
[c] Human lung carcinoma.
[d] Human colon adenocarcinoma.
[e] Human normal skin fibroblasts.

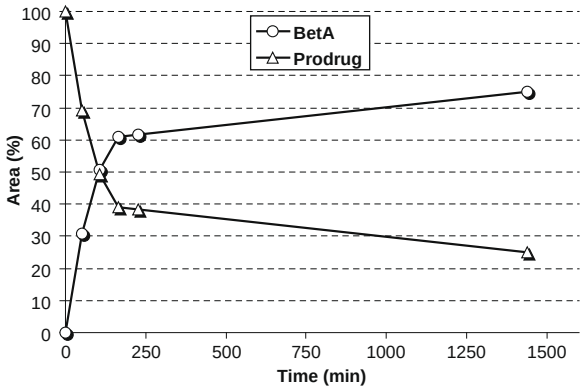


Figure 2. Enzymatic hydrolysis of prodrug **3** releasing betulinic acid (**1**, BetA) under the action of β-D-glucuronidase (4.3 U/mL) as measured by HPLC (relative percent area).[38]

Acknowledgements

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33. Typical procedure for the synthesis of 28-*O*-(methyl-2,3,4-tri-*O*-acetyl-β-D-glucuronate) betulinic acid (**10**): To a solution of **1** (1.0 equiv) and **9** (1.5 equiv) in CH_2Cl_2 (11.6 mL/mmol) were added K_2CO_3 (2.5 equiv) and Bu_4NBr (0.4 equiv). The mixture was stirred and refluxed for 6 h, then diluted with CH_2Cl_2 and the organic phase was washed with H_2O (2×) and brine (1×). The solvents of the dried solution ($MgSO_4$) were evaporated under reduced pressure, and the residue was purified by silica gel flash chromatography (hexanes/EtOAc 9:1 to 13:7) to afford **10** (76%) as a white crystalline powder. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 5.74 (d, J = 8.1 Hz, 1H, H-1′), 5.35 (m, 1H, H-3′), 5.25 (m, 2H, H-2′, H-4′), 4.73 (br s, 1H, H-29a), 4.60 (br s, 1H, H-29b), 4.16 (d, J = 10.0 Hz, 1H, H-5′), 3.73 (s, 3H, CO_2CH_3), 3.18 (dd, J = 11.4 Hz, J = 5.1 Hz, 1H, H-3), 2.94 (td, J = 11.1 Hz, J = 5.1 Hz, 1H, H-19), 2.05, 2.05, 2.02 (all s, 3× 3H, O(CO)CH_3), 1.67 (s, 3H, H-30), 0.96, 0.96, 0.89, 0.82, 0.75 (all s, 5× 3H, H-23, H-24, H-25, H-26, H-27). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 173.9, 170.0, 169.4, 168.9, 166.7, 150.1, 109.8, 90.8, 78.9, 74.0, 72.0, 69.7, 69.2, 56.8, 55.3, 50.5, 49.1, 46.7, 42.4, 40.6, 38.8, 38.7, 38.1, 37.2, 36.3, 34.4, 31.6, 30.2, 29.7, 28.0, 27.4, 25.4, 20.8, 20.6, 20.6, 20.5, 19.4, 18.3, 16.1, 16.0, 15.4, 14.7. HR-ESI-MS m/z 795.4280 $[M+Na]^+$ (calculated for $C_{43}H_{64}O_{12}Na$: 795.4290).
34. Typical procedure for the synthesis of 28-*O*-β-D-glucuronide betulinic acid (**3**): To a solution of **10** (1.0 equiv) in THF (148 mL/mmol) and H_2O (37 mL/mmol) was added KOH (6.0 equiv). The mixture was stirred at room temperature for 4.5 h, then the solution was acidified to pH 3–4 with 10% HCl. The solvents were evaporated under reduced pressure, and the residue was purified by silica gel flash chromatography (CH_2Cl_2/MeOH 9:1 to 2:3) to give **3** (76%) as a white amorphous powder. R_f 0.16 (CH_2Cl_2/MeOH 3:1); $[\alpha]_D$ −28.0 (c 0.2, MeOH); ^{1}H NMR ($CDCl_3$/MeOD 1:1, 400 MHz) δ: 5.55 (d, J = 8.1 Hz, 1H, H-1′), 4.72 (br s, 1H, H-29a), 4.60 (br s, 1H, H-29b), 3.79 (m, 1H, H-5′), 3.53 (m, 2H, H-3′, H-4′), 3.42 (m, 1H, H-2′), 3.15 (dd, J = 6.0 Hz, J = 10.0 Hz, 1H, H-3), 3.00 (td, J = 11.0 Hz, J = 4.5 Hz, 1H, H-19), 1.69 (s, 1H, H-30), 0.98, 0.95, 0.94, 0.83, 0.75 (all s, 5× 3H, H-23, H-24, H-25, H-26, H-27). NMR ^{13}C ($CDCl_3$/MeOD 1:1, 100 MHz) δ: 175.7, 175.6, 151.1, 110.0, 94.2, 77.3, 75.8, 73.1, 72.4, 79.2, 57.3, 56.0, 51.2, 50.1, 47.5, 43.0, 41.3, 39.4, 39.4, 38.6, 37.7, 36.8, 34.8, 32.3, 30.8, 30.1, 28.3, 27.4, 26.1, 21.4, 19.5, 18.8, 16.5, 16.2, 15.8, 15.1. HR-ESI-MS m/z 655.3804 $[M+Na]^+$ (calculated for $C_{36}H_{56}O_9Na$: 655.3817).
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37. Unpublished results.
38. RP-HPLC analyses were performed using Zorbax C18 column at a flow rate of 1 mL/min with a column temperature of 30 °C. Compounds were detected by UV absorption at 210 nm following this elution gradient: 30–100% B where B = CH_3CN + 0.1% HCOOH and A = H_2O + 0.1% HCOOH in 50 min.