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A new linker for glucuronylated anticancer prodrugs

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Abstract—The synthesis, enzymatic hydrolysis and self decomposition of model glucuronylated prodrugs, incorporating a new linker with different aryl substituents, have been studied. Determination of kinetic parameters (V_{max}, K_m and $t_{1/2}$) showed the important role of aromatic substitution in enzymatic recognition and linker decomposition.

1. Introduction

The design of produgs is currently an area of high interest in anticancer research. The lack of selectivity toward cancer cells may be reduced by site specific delivery of clinically used drugs which may be administered as non toxic derivatives. Several strategies to achieve this goal have been implemented using targeted (ADEPT,[1] GDEPT[2]) or endogenuous (PMT[3]) enzymes or via specific chemical activation (hypoxia selective agents[4]). The prodrug must fullfil some requirements such as a highly reduced toxicity compared to the parent drug, a large volume of distribution and a plasma good stability, an easy recognition by the enzyme and a fast liberation of the drug after activation to avoid diffusion away from the tumour site. Therefore, a large number of prodrugs have been designed in three parts, that is, trigger, linker and effector units, which are generally, though not always, distinct (Fig. 1).[5] The trigger and effector units respectively depend on the mechanism of activation of the prodrug and the targeted tumour cells. They are joined together by the linker unit which should release the drug, as fast as possible, after prodrug activation via a chemical breakdown. Thus, a careful design of the linker unit is crucial for prodrug efficiency.

Different types of linker have been designed based on elimination or cyclization processes.[6] Thus, Monneret et al.[7] have previously studied linkers based on 1,4- and 1,6-eliminations of an unstable carbamic acid after hydrolysis of a phenol glucuronide. This work led to the preclinical studies of HMR 1826 (Fig. 1) which has demonstrated a superior efficiency in vivo compared to doxorubicin toward several cancer cell lines.[8] Related linkers have been based on aniline derivatives and similar 1,4- and 1,6-elimination processes.[9] In this strategy, a glycoside is linked to an appropriate 4-amino benzyl alcohol via a carbamate function, the amino drug being then introduced via another carbamate.

In this paper, the synthesis and enzymatic cleavage of new linkers based on the elimination of a carbamic acid from a *para*-substituted 3-carbamoyl-2-aryl-propenal are described (Scheme 1). The latter could arise from the enzymatic hydrolysis of a β-glucuronide. Substitution of the aryl moiety should play a key role in the enzyme-catalysed hydrolysis (V_{max} and K_m) as well as in the β-elimination of the carbamic acid, which, after decarboxylation, will generate the amino drug (such as doxorubicin).

This mechanism is reminiscent of the biotransformation of felbamate, an anti-epileptic drug which has been withdrawn from clinical use due to associated toxicity such as aplastic anemia and hepatotoxicity. The metabolic pathway of this drug has been studied in detail by Mcdonald et al.:[10] after hydrolysis of one carbamate group, felbamate is oxidised in the liver to aldehyde **1** which is unstable ($t_{1/2}$ c.a. 30 s) and undergoes elimination to 2-phenylpropenal (atropaldehyde) **2** and mainly reversible cyclisation to oxazinone **3** (Scheme 2). The latter has a rather long half-life of 5 h and the subsequent slow release of the toxic atropaldehyde

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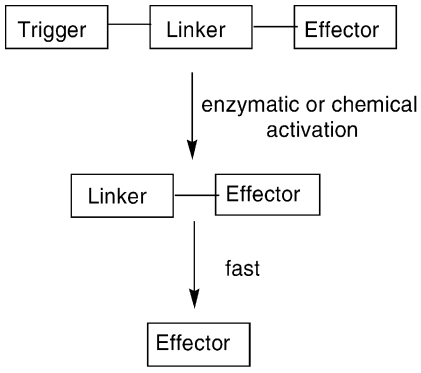


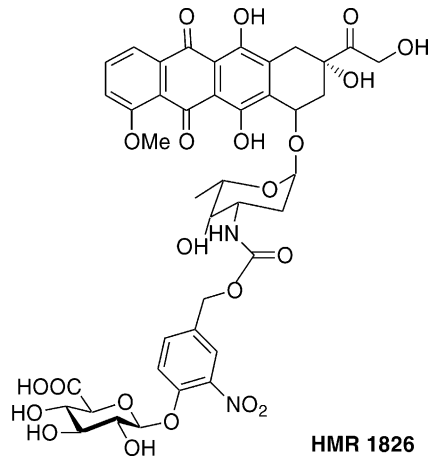
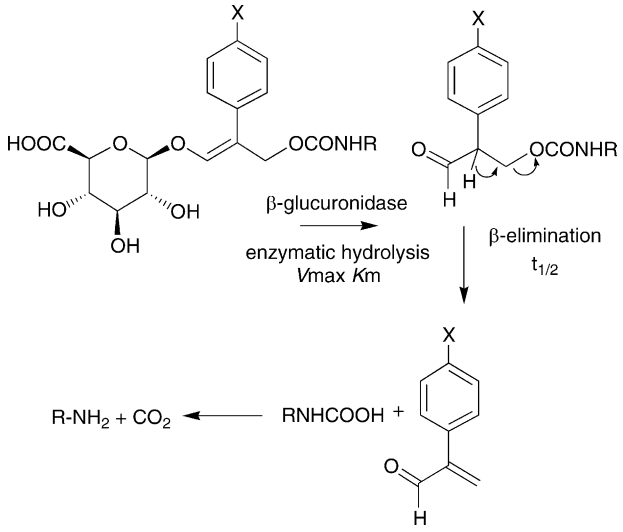
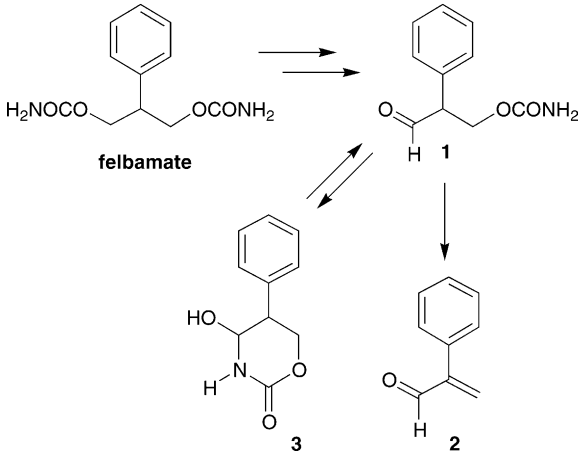


Figure 1. General principle of drug release; doxorubicin prodrug: HMR 1826.



Scheme 1. Prodrug decomposition.



Scheme 2. Metabolism of felbamate.

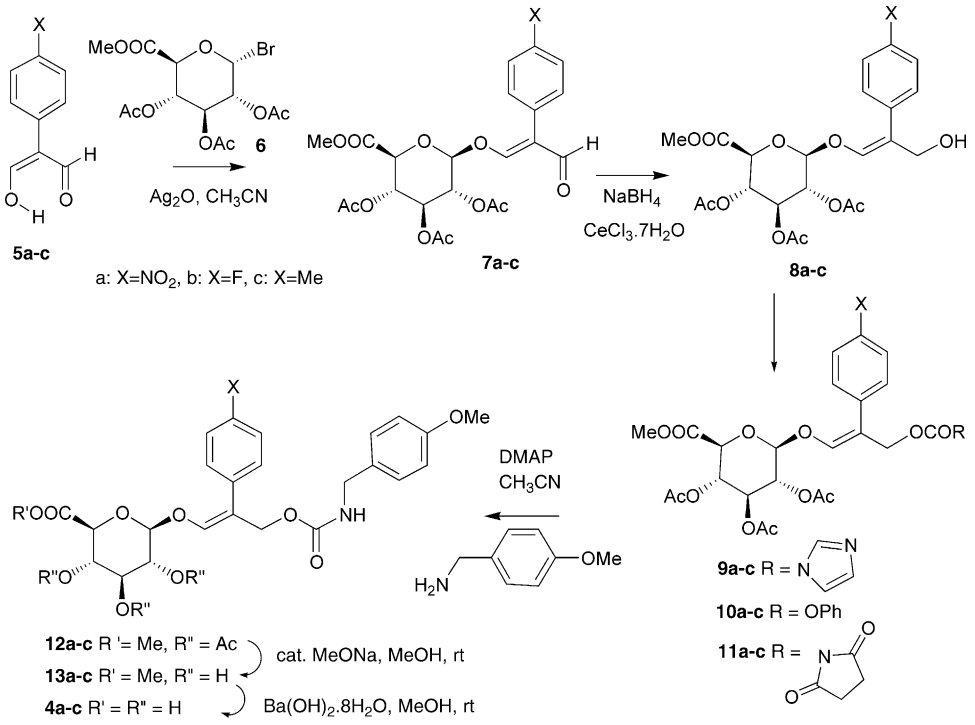
($GI_{50} = 4.1 \pm 1.1$ μM for HA1 cells) was assumed to result from this side reaction. It is also interesting to notice that acrolein ($GI_{50} = 4.9 \pm 0.9$ μM for HA1 cells) is released from the well known anticancer agent cyclophosphamide after bioactivation.

Thus, it seems worthwhile to design an anticancer prodrug based on such a linker since two toxic species, the drug (effector) and a 2-arylpropenal will be released near the tumor site. The synthesis, enzymatic hydrolysis and self decomposition of model compounds incorporating different aryl substituents have been first studied and are now reported.

2. Results

Three compounds have been prepared using 4-methoxybenzylamine as the model effector: **4a** (X = NO_2), **4b** (X = F) and **4c** (X = Me) (Scheme 3). The starting arylmalonaldehydes **5a–c** were either commercially available (**5c**) or prepared in two steps from the corresponding arylacetic acid according to the method of Arnold[11] (46-52% over yield). Glycosidation of **5a–c** with the known activated glucuronide **6**[12] was carried out in CH_3CN in presence of Ag_2O to afford β-glycosides **7a–c** (54–71%). As observed previously by Lubineau[13] in the case of 2-methylmalonaldehyde, only *E*-enol glycosides were obtained. Then, reduction with $NaBH_4$ in MeOH, in presence of $CeCl_3 \cdot 7H_2O$, afforded the corresponding alcohols **8a–c**. It should be noticed that the isolated yields for the nitro and fluoro derivatives were consistently higher (90–95%) than for the methyl derivative (44%) and that reduction is greatly improved in presence of cerium salts.[14] Then several activating groups were tested in order to introduce the carbamate moiety (Table 1). Variable results were obtained, both in the formation of a mixed carbonate or a mixed carbamate and in the displacement with the amine, depending on the X group. *N*,*N*-Carbonyldiimidazole and phenyl chloroformate turned out to be the reagents of choice for the first step but the latter proved to be less efficient than the former one for the second step of amine coupling, except for X = Me.

Finally, glucuronides **4a–c** were obtained in two steps: hydrolysis of acetate groups by the method of Zemplen (cat. MeONa in MeOH) to afford **13a–c** and then of the methyl ester with $Ba(OH)_2$ (61–66% overall).



Scheme 3. Synthesis of compounds **4a–c**.

Table 1. Results of alcohol activation and carbamate formation

Alcohol	Carbonate or carbamate (%)	Carbamate (%)
8a	**9a** (89)	**12a** (83)
	10a (99)	**12a** (47)
	11a (76)	(n.r.)[a]
8b	**9b** (94)	**12b** (44)
	10b (71)	**12b** (9)
	11b (n.r.) [a]	(n.r.)[a]
8c	**9c** (81)	**12c** (50)
	10c (78)	**12c** (65)
	11c (38)	(n.r.)[a]

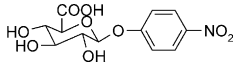
[a] No reaction was observed.

2.1. Enzymatic hydrolysis

First, model prodrugs **4a–c** were incubated in 0.02 M phosphate buffer (pH = 7) at 37 °C. After 48 h no decomposition of **4a–c** was detected. Then, enzymatic studies were conducted, in the presence of *Escherichia coli* β-glucuronidase to determinate kinetic parameters, V_{max} and K_m, of the glycosidic bounds hydrolysis (see Experimental). Results are reported in Table 2 with *para*-nitrophenol β-glucuronide as reference.

In a second step, we investigated the rate of the linker decomposition (Scheme 4). After addition of an excess of enzyme, the reaction was followed by HPLC. The intermediate compounds **14a–c** were immediately observed followed by the simultaneous formation of the expected amine and of a 2-arylacrolein. The half-life of the methyl intermediate **14c** was found to be much higher ($t_{1/2}$ = 13.5 h) than for the fluoro **14b** ($t_{1/2}$ = 5.3 h) and the nitro **14a** ($t_{1/2}$ = 1.6 h) intermediates.

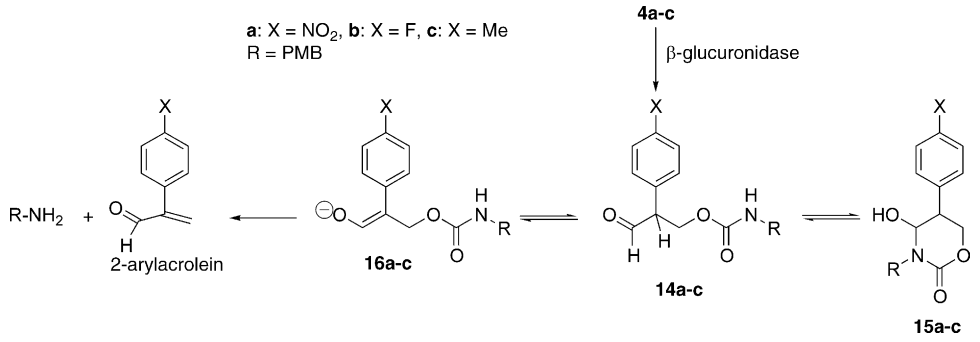
Table 2. Kinetic results of enzymatic hydrolysis

Glucuronides	K_m (μM)	V_{max} (μmol/min)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)
	610	77	46	75,400
4a	120	17.5	10.5	87,500
4b	1140	126	75.5	66,200
4c	504	4	2.5	4,950

3. Discussion

Prodrugs **4a** and **4b** are excellent substrates of the enzyme (high k_{cat}/K_m), comparable to the reference compound (*p*-nitrophenyl glucuronide) and much more better than **4c** (Table 2). These results are consistent with those obtained for phenol glucuronides: affinity and hydrolysis rate are enhanced with electron withdrawing group substitution on the aromatic ring.[15] The highest ratio k_{cat}/K_m (87,500 M^{-1} s^{-1}) of **4a** is due to a low K_m (120 μM) resulting from a strong enzyme affinity. Thereby, and despite a lower k_{cat} (10.5 s^{-1}), **4a** will undergo a relative fast hydrolysis at low concentrations of substrate and enzyme. Thus, **4a** can be considered as a good candidate for an ADEPT protocol. The interesting k_{cat}/K_m ratio (66,200 $M^{-1}.S^{-1}$) for **4b** is due to a high k_{cat} (75.5 s^{-1}), but at low concentration (like nearby tumours), **4b** is expected to be less efficient than **4a**. Introduction of a weak electron donating group in **4c** did not give any satisfactory results.

Determination of $t_{1/2}$ is also of great importance to evaluate the potential of such linkers for prodrug use. The slow half-life observed for intermediates **14a–c**



Scheme 4. Postulated decomposition of the linker.

should allow undesired diffusion of the drug from the tumour site. Results are similar to the reported half-life of felbamate metabolite **3** ($t_{1/2} = 5$ h)[10] which exists as an equilibrium with aldehyde **1** (Scheme 2). In our case, formation of oxazinones **15a–c** is also possible (Scheme 4). However, the 10 times acceleration of amine release on going from the methyl intermediate to the nitro one is consistent with the increase of acidity of the benzylic proton for **14a** (compared to **14b** and **14c**). The formation of N-substituted oxazinones **15a–c** may lower the concentration of the intermediate aldehyde **14a–c** (this process should be rather insensitive to the *para* substituent of the aryl group), but the rate limiting step may be formation of the enolate **16a–c**. On the one hand, the lower rate of elimination for such linkers, compared to aryl ones such as in HMR 1826, is thus mainly due (as shown in the aryl series) to the lower concentration of the enolate (compared to the phenate). On the other hand, a more hindered effector such as doxorubicin, could prevent oxazinone formation and thus higher rate of elimination may be observed.

4. Conclusion

Three glucuronylated prodrugs, incorporating a new linker, have been synthetised and evaluated with a model effector. Our synthetic strategy is suitable for any amine compound and connection with the linker is made by a stable (in vivo) carbamate. After enzymatic hydrolysis, drug release is observed via elimination of a carbamic acid from a substituted 3-carbamoyl-2-arylpropenal. Kinetic studies in phosphate buffer have pointed out the important role of the aromatic substitution in enzymatic recognition and linker decomposition. Our linker is of special interest as it behave as a dual-release drug. Further studies are now needed to improve the self decomposition process and evaluate (in vitro and in vivo) the potential of such linker in a drug targeting strategy.

5. Experimental

5.1. General methods

All solvents used were of HPLC quality and chemicals were of analytical grade. *E. coli* β-glucuronidase was purchased from Sigma-Aldrich.[16] Analytical HPLC was carried out using a Waters HPLC system with UV detection at 254 nm. The separation was performed on a reversed phase column (Discovery RP amide C16, 150×4.6 mm) using isocratic conditions (1 mL/min), eluent: 0.02 M phosphate buffer (pH = 3)–acetonitrile 60:40.

5.2. Stability of compounds in a buffer solution

A solution of 100 μg/mL of prodrug in 0.02 M phosphate buffer (pH = 7.2) was incubated at 37 °C. Aliquots were taken at various times and analysed by HPLC.

5.3. Enzymatic cleavage by *E. coli* β-D-glucuronidase

Hydrolysis was investigated by incubating a solution of 62.5 μg/mL of prodrug and 100 U/mL of *E. coli* β-D-glucuronidase in 0.02 M phosphate buffer (pH = 7.2) at 37 °C. Aliquots were taken at various times and analysed by HPLC.

5.4. Determination of K_m and V_{max} values

Experiments were run at different prodrug concentrations [80 to 160 μg/mL in 0.02 M phosphate buffer (pH = 7.2)] in presence of *E. coli* β-glucuronidase (100 U/mL). After incubation for 8 min at 37 °C, an aliquot was taken and analysed by HPLC and K_m and V_{max} values were calculated from the Lineweaver–Burk plot.

5.5. General procedure for preparation of *Z*-2-aryl-3-hydroxypropenal 5a,b[11]

5.5.1. *Z*-2-aryl-3-*N*,*N*-dimethylaminopropenal. To a vigorously stired solution of DMF (2.9 mL) at 0 °C, $POCl_3$ (2.8 mL) was added dropwise. After 5 min, the arylacetic acid (0.01 mol) in solution in DMF (5 mL) was carefully added. Then the reaction mixture was warm up to 70 °C overnight and then poured on ice.

For X = NO_2: after neutralisation by K_2CO_3, a solution of NaOH 50% (12 mL) was added and a precipitate was obtained upon cooling at 0 °C. The filtrate was dissolved in CH_2Cl_2 and recrystallized from Et_2O to give a yellow powder (65%).

For X = F: the solution was basified with K_2CO_3, 10 mL of toluene was added and the mixture was allowed to reflux during 1 h 30 min. The remaining solution was

extracted with toluene and the organic phases were washed with water. The combinated organic extracts were dried and concentrated in vacuo. The crude product was dissolved in a minimal amount of CH_2Cl_2 and recrystallized from petroleum ether to afford a white powder (68%).

5.5.2. Z-2-(4′-Nitrophenyl)-3-*N*,*N*-dimethylaminopropenal. Mp 131 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 2.89 (sl, 6H, $H_{N(CH_3)_2}$); 6.97 (sl, 1H, H_3); 7.38 (d, J = 9 Hz, 2H, $H_{2'}$); 8.21 (d, J = 9 Hz, 2H, $H_{3'}$); 9.12 (s, 1H, H_{CHO}); $[\alpha]_D = -16$ (c = 0.09; $CHCl_3$).

5.5.3. Z-2-(4′-fluorophenyl)-3-*N*,*N*-dimethylaminopropenal. Mp 80 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 2.82 (sl, 6H, $H_{N(CH_3)_2}$); 6.81 (sl, 1H, H_3); 7.03–7.13 (m, 4H, H_{arom}); 9.10 (s, 1H, H_{CHO}); $[\alpha]_D = -1$ (c = 0.30; $CHCl_3$).

5.5.4. *Z*-2-aryl-3-hydroxypropenal (5a,b). 25% NaOH (20 mL) was added to a solution of *Z*-2-aryl-3-*N*,*N*-dimethylaminopropenal (0.01 mmol) in ethanol (15 mL) and the reaction mixture was stirred at reflux for 3 h. Ethanol was removed under reduce presure and the residue was cooled down with ice to give a powder which was filtered and washed with CH_2Cl_2, then taken up in water and acidified by 6 N HCl to give a precipitate.

X = NO_2: the solid was disolved in acetone and recrystallized from ether to give **5a** as a light brown powder (71%).

X = F: **5b** was obtained as a white solid (77%).

5.5.5. Z-2-(4′-nitrophenyl)-3-hydroxypropenals (5a). Mp 223 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 7.45 (d, J = 9 Hz, 2H, $H_{2'}$); 8.28 (d, J = 9 Hz, 2H, $H_{3'}$); 8.76 (s, 2H, H_3, CHO); 14,67 (sl, 1H, OH); ^{13}C NMR ($CDCl_3$) δ (ppm): 113.2 (C-2); 124.5 ($C_{1'}$, $C_{3'}$); 126.4 ($C_{2'}$); 149.2 (C_3); 164.3 ($C_{4'}$); 187.5 (CHO).

5.5.6. *Z*-2-(4′-fluorophenyl)-3-hydroxypropenals (5b). Mp 163 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 7.10 (t, J = 8.5 Hz, 2H, $H_{3'}$); 7,24 (d, J = 6.5 Hz, 2H, $H_{2'}$); 8,60 (s, 2H, H_3, CHO); 14,29 (sl, 1H, OH); ^{13}C NMR ($CDCl_3$) δ (ppm): 116,1 (d, J = 21 Hz, $C_{3'}$); 117.5 (C_2); 128′4 (d, J = 8 Hz, $C_{2'}$); 129.5 ($C_{1'}$); 160.7 (C_3); 164.0 ($C_{4'}$); 180.9 (CHO).

5.6. General procedure for glycosidation

A solution of *Z*-2-aryl-3-hydroxypropenal **5** (0.01 mol), 1 equiv of bromoglucuronide **6** (3.97 g) and 1.5 equiv of freshly prepared Ag_2O in CH_3CN (20 mL) was stirred at 0 °C for 3 h. The reaction mixture was filtered through Celite and concentrated in vacuo. The residue was dissolved in CH_2Cl_2 and recrystallized from CH_3OH.

5.6.1. 2,3,4-Tri-*O*-acetyl-(2′-(4′-nitrophenyl)-3′-oxo-1′-*E*-propenyloxy)-β-D-methyl glucopyranosiduronate (7a). Yellow solid (71%); mp 166 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 2.02 to 2.06 (3s, 9H, HC$\underline{H_3}$CO); 3.75 (s, 3H, H_{OCH_3}); 4.29 (d, J = 9 Hz, 1H, H_5); 5.17 to 5.34 (2m, 4H, $H_{1,2,3,4}$); 7.47 (s, 1H, $H_{1'}$); 7.64 (d, J = 9 Hz, 2H, $H_{2''}$); 8.23 (d, J = 9 Hz, 2H, $H_{3''}$); 9.50 (s, 1H, H_{CHO}); ^{13}C NMR ($CDCl_3$) δ(ppm): 20.3–20.4–20.5 ($3C_{\underline{CH_3}CO}$); 53.2 (C_{OCH_3}); 68.4–70.2–70.6–73.0 ($C_{2,3,4,5}$); 100.8 (C_1); 123.1–123.3–130.3–130.5 ($C_{2',1''',2'',3''}$); 136.0 ($C_{4''}$); 147.0 ($C_{1''}$); 166.5–168.8–169.3–169.7 ($C_{CH_3\underline{C}O,\ \underline{C}OOCH_3}$); 189.3 ($C_{CHO}$); $[\alpha]_D$ −22 (c 0.10, $CHCl_3$); HRMS (ESI) calcd for $C_{22}H_{23}NO_{13}Na$ (M + Na +) 532.1067, found 532.1070.

5.6.2. 2,3,4-Tri-*O*-acetyl-(2′-(4′-fluorophenyl)-3′-oxo-1′-E-propenyloxy)-β-D-methyl glucopyranosiduronate (7b). White solid (60%); mp 163 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 2.02 to 2.07 (3s, 9H, $H_{CH_3}CO$); 3.76 (s, 3H, H_{OCH_3}); 4.24 (d, J = 9 Hz, 1H, H_5); 5.17–5.32 (2m, 4H, $H_{1,2,3,4}$); 7.08 (t, J = 9 Hz, 2H, $H_{3''}$); 7.29 (s, 1H, $H_{1'}$); 7.42 (d, J = 9 Hz, 2H, $H_{2''}$); 9.48 (s, 1H, H_{CHO}). ^{13}C NMR ($CDCl_3$) δ (ppm): 20.4-20.5–20.6 ($3C_{\underline{CH_3}CO}$); 53.2 (C_{OCH_3}); 68.5–70.1–70.7–73.0 ($C_{2,3,4,5}$); 100.5 (C_1); 114.9–115.2 ($C_{2',3''}$); 124.6 ($C_{2''}$); 131.2–131.3 ($C_{1'',4''}$); 162.3 ($C_{1'}$); 166.5–168.8–169.3–169.8 ($C_{CH_3\underline{C}O,\ \underline{C}OOCH_3}$); 190.3 ($C_{CHO}$); $[\alpha]_D$−3 (c 0.17, $CHCl_3$); HRMS (ESI) calcd for $C_{22}H_{23}O_{11}FNa$ (M + Na +) 505.1122, found 505.1122.

5.6.3. 2,3,4-tri-*O*-acetyl-(2′-(4′-methylphenyl)-3′-oxo-1′-E-propenyloxy)-β-D-methyl glucopyranosiduronate (7c). White solid (54%); mp 126 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 2.00–2.04 (3s, 9H, $H_{CH_3}CO$); 2.34 (s, 3H, HPhC$\underline{H_3}$); 3.73 (s, 3H, H_{OCH_3}); 4.26 (d, J = 8.8 Hz, 1H, H_5); 5.15–5.21–5.29–5.35 (2m, 4H, $H_{1,2,3,4}$); 7.17 (d, J = 8.1 Hz, 2H, $H_{3''}$); 7.28–7.30 (m, 3H, $H_{1',2''}$); 9.44 (s, 1H, H_{CHO}); ^{13}C NMR ($CDCl_3$) δ (ppm): 20.4 ($C_{\underline{CH_3}CO}$); 21.1 ($CPhC\underline{H_3}$); 52.9 (C_{OCH_3}); 68.4–69.9–70.6–72.7 ($C_{2,3,4,5}$); 100.3 (C_1); 125.1–126.0–128.5–129.0 ($C_{2',1'',2'',3''}$); 137.7 ($C_{4''}$); 162.4 ($C_{1'}$); 166.5–168.7–169.1–169.8 ($C_{CH_3\underline{C}O,\ \underline{C}OOCH_3}$); 190.8 ($C_{CHO}$); $[\alpha]_D$ +0 (c 0.23, $CHCl_3$); HRMS (ESI) calcd for $C_{23}H_{26}O_{11}Li$ (M + Li +) 485.1635, found 485.1653.

5.7. General procedure of reduction

To a stirred solution of aldehyde **7** (1 equiv) and $CeCl_3.7H_2O$ (1 equiv) in CH_3OH (7.5 mL/mmol) was added $NaBH_4$ (1 equiv). After 5 min, the reaction mixture was neutralised with 1 N HCl and extracted with CH_2Cl_2. The organic layer was dried over $MgSO_4$, filtered and concentrated in vacuo. Recrystallisation from AcOEt/Pet. ether gave alcohols **8**.

5.7.1. 2,3,4-Tri-*O*-acetyl-(2′-(4′-nitrophenyl)-3′-hydroxy-1′-E-propenyloxy)-β-D-methyl glucopyranosiduronate (8a). Yellow powder (95%); mp 206 °C, ^{1}H NMR ($CDCl_3$) δ (ppm): 2.00–2.04 (3s, 9H, $H_{CH_3}CO$); 3.75 (s, 3H, H_{OCH_3}); 4.18 (d, J = 8.8 Hz, 1H, H_5); 4.47 (s, 2H, $H_{3'}$); 4.92 (d, J = 7.4 Hz, 1H, H_1); 5.28–5.32 (2m, 3H, $H_{2,3,4}$); 6.76 (s, 1H, $H_{1'}$); 7.79 (d, J = 9 Hz, 2H, $H_{2''}$); 8.19 (d, J = 9 Hz, 2H, $H_{3''}$); ^{13}C NMR ($CDCl_3$) δ (ppm): 20.4 ($C_{\underline{CH3}CO}$); 53.1 (C_{OCH_3}); 63.6 ($C_{3'}$); 68.8–70.3–71.2–72.8 ($C_{2,3,4,5}$); 100.1 (C_1); 118.0 ($C_{2'}$); 123.3–128.8 ($C_{1'',2'',3''}$); 141.4 ($C_{4''}$); 144.2 ($C_{1'}$); 166.6–168.9–169.3–169.9 ($C_{CH_3\underline{C}O,\ \underline{C}OOCH_3}$); $[\alpha]_D$ −21 (c 0.18, $CHCl_3$); HRMS (ESI) calcd for $C_{22}H_{25}NO_{13}Na$ (M + Na +) 534.1224, found 534.1224.

5.7.2. 2,3,4-tri-*O*-acetyl-(2′-(4′-fluorophenyl)-3′-hydroxy-1′-E-propenyloxy)-β-D-methyl glucopyranosiduronate (8b). White powder (90%); mp 148 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.92 to 2.03 (3s, 9H, H_{CH_3CO}); 3.73 (s, 3H, H_{OCH_3}); 4.14 (d, J = 8.8 Hz, 1H, H_5); 4.31 (s, 2H, $H_{3'}$); 4.85 (d, J = 7.4 Hz, 1H, H_1); 5.13–5.29 (2m, 3H, $H_{2,3,4}$); 6.57 (s, 1H, $H_{1'}$); 7.00 (t, J = 8.8 Hz, 2H, $H_{3''}$); 7.53 (dd, J = 5.5–8.8 Hz, 2H, $H_{2''}$); ^{13}C NMR ($CDCl_3$) δ (ppm): 20.3 ($C_{\underline{C}H_3CO}$); 53.0 (C_{OCH_3}); 63.6 ($C_{3'}$); 69.0–70.2–71.4–72.5 ($C_{2,3,4,5}$); 99.9 (C_1); 114.7–115.0–119.2–129.8–129.9 ($C_{2',1'',2'',3'',4''}$); 141.2 ($C_{1'}$); 166.8–169.0–169.4–170.0 ($C_{CH_3\underline{C}O,\ \underline{C}OOCH_3}$); $[\alpha]_D$ +40 (*c* 0.12, $CHCl_3$); HRMS (ESI) calcd for $C_{22}H_{25}O_{11}FNa$ (M + Na +) 507.0750, found 507.0770.

5.7.3. 2,3,4-tri-*O*-acetyl-(2′-(4′-methyophenyl)-3′-hydroxy-1′-E-propenyloxy)-β-D-methyl glucopyranosiduronate (8c). White powder (44%); mp 124 °C, ^{1}H NMR ($CDCl_3$) δ (ppm): 1.92–2.03 (3s, 9H, H_{CH_3CO}); 2.33 (s, 3H, $HPhCH_3$); 3.74 (s, 3H, H_{OCH_3}); 4.12 (d, J = 8.8 Hz, 1H, H_5); 4.36 (s, 2H, $H_{3'}$); 4.83 (d, J = 7.4 Hz, 1H, H_1); 5.13–5.30 (m, 3H, $H_{2,3,4}$); 6.58 (s, 1H, $H_{1'}$); 7.14 (d, J = 8.2 Hz, 2H, $H_{3''}$); 7.44 (d, J = 8.2 Hz, 2H, $H_{2''}$); ^{13}C NMR ($CDCl_3$) δ (ppm): 20.5 ($C_{\underline{C}H_3CO}$); 21.2 ($CPhCH_3$); 53.0 (C_{OCH_3}); 63.9 ($C_{3'}$); 69.1–70.4–71.6–72.8 ($C_{2,3,4,5}$); 100.0 (C_1); 120.3 ($C_{2'}$); 128.0–128.9 ($C_{2'',\ 3''}$); 131.3–136.9 ($C_{1'',4''}$); 140.9 ($C_{1'}$); 166.8–169.0–169.3–170.0 ($C_{CH_3\underline{C}O,\ \underline{C}OOCH_3}$); $[\alpha]_D$ −34 (*c* 0.12, $CHCl_3$); HRMS (ESI) calcd for $C_{23}H_{28}O_{11}Li$ (M + Li +) 487.1792, found 487.1793.

5.8. General procedure for preparation of compounds 9a,b

To a solution of alcohol **8** (1 mmol) in dry dichloromethane (10 mL), carbonyldiimidazole (2 equiv) was added and the mixture was stirred for 30 min at room temperature. The solvent was removed in vacuo and the desired compounds were obtained after recrystallisation from CH_2Cl_2/Et_2O.

5.8.1. 2,3,4-Tri-*O*-acetyl-(2′-(4′-nitrophenyl)-3′-(*N*-imidocarbonyloxy)-1′-E-propenyloxy)-β-D-methyl glucopyranosiduronate (9a). Yellow powder (65%); mp 130 °C; ^{1}H NMR ($CDCl_3$): 2.00–2.04 (3s, 9H, H_{CH_3CO}); 3.76 (s, 3H, H_{OCH_3}); 4.19 (d, J = 9 Hz, 1H, H_5); 5.00 (d, J = 7.4 Hz, 1H, H_1); 5.19–5.37 (m, 5H, $H_{2,3,4,3'}$); 7.00–7.11 (m, 2H, $H_{2''',3'''}$); 7.35 (s, 1H, $H_{1'}$); 7.73 (d, J = 9 Hz, 2H, $H_{2''}$); 8.07 (s, 1H, $H_{1'''}$); 8.23 (d, J = 9 Hz, 2H, $H_{3''}$); ^{13}C NMR ($CDCl_3$) δ (ppm): 20.7 ($C_{\underline{C}H_3CO}$); 53.3 (C_{OCH3}); 68.6–68.8–70.4–71.2–73.0 ($C_{2,3,4,5,3'}$); 100.4 (C_1); 112.5 ($C_{2'}$); 117.1–123.8–128.6 ($C_{2'',\ 3'',2''',3'''}$); 131.0–137.2–140.4 ($C_{1'',4'',1'''}$); 146.6 ($C_{1'}$); 148.1 ($C_{OO\underline{C}N}$); 166.6–169.0–169.4–170.0 ($C_{CH_3\underline{C}O,\ \underline{C}OOCH_3}$); $[\alpha]_D$ −3 (*c* 0.15, MeOH); HRMS (ESI) calcd for $C_{26}H_{27}N_3O_{14}Na$ (M + Na +) 628.1391, found 628.1387.

5.8.2. 2,3,4-Tri-*O*-acetyl-(2′-(4′-methylphenyl)-3′-(*N*-imidocarbonyloxy)-1′-*E*-propenyloxy)-β-D-methyl glucopyranosiduronate (9b). White powder (94%); mp 131 °C; ^{1}H NMR ($CDCl_3$): 1.95–2.04 (3s, 9H, H_{CH_3CO}); 3.75 (s, 3H, H_{OCH_3}); 4.17 (d, J = 9 Hz, 1H, H_5); 4.92 (d, J = 7.3 Hz, H, H_1); 5.07–5.30 (m, 5H, $H_{2,3,4,3'}$); 6.82 (s, 1H, $H_{1'}$); 7.02–7.07 (m, 3H, $H_{2''',3''}$); 7.35 (s, 1H, $H_{3'''}$); 7.50 (t, J = 6.4 Hz, 2H, $H_{2''}$); 8.06 (s, 1H, $H_{1'''}$); ^{13}C NMR ($CDCl_3$) δ(ppm): 20.6 ($C_{\underline{C}H_3CO}$); 55.4 (C_{OCH_3}); 64.7 ($C_{3'}$); 69.0–70.4–71.4–71.8 ($C_{2,3,4,5}$); 100.2 (C_1); 114.4–122.1–125.2–129.2–129.3 ($C_{2,2''',3'',2''',3'''}$); 130.8 (C); 144.8 (C); 153.2 (C); 156.0 ($C_{1',1'',4'',1'''}$); 159.4 ($C_{O\underline{C}ON}$); 166.7–169.0–169.4–170.0 ($C_{CH_3\underline{C}O,\ \underline{C}OOCH_3}$); $[\alpha]_D$ −17 (*c* 0.26, $CHCl_3$); HRMS (ESI) calcd for $C_{26}H_{27}N_2O_{12}FNa$ (M + Na +) 601.1446, found 601.1449.

5.8.3. Preparation of 10c: 2,3,4-tri-*O*-acetyl-(2′-(4′-methylphenyl)-3′-(*O*-phenylcarbonyloxy)-1′-*E*-propenyloxy)-β-D-methyl glucopyranosiduronate. Alcohol **8c** (2.6 mmol, 1.26 g) was disolved in dichloromethane with pyridine (3 equiv 0.6 mL). The mixture was stirred at 0 °C and phenyl chloroformate (3 equiv, 0.98 mL) was added dropwise, the colorless solution became yellow and precipitated. After 30 min at 0 °C, the reaction was quenched with a saturated solution of $NaHCO_3$ and extracted with dichloromethane. After filtration with Na_2SO_4 and solvent evaporation, a yellow oil was obtained. Compound **10c** was obtained as a white powder after recrystallisation with AcOEt/pet. ether (1.225 g, 78%).

^{1}H NMR ($CDCl_3$): 1.95–2.05 (3s, 9H, H_{CH_3CO}); 2.35 (s, 3H, $HPhCH_3$); 3.73 (s, 3H, H_{OCH_3}); 4.14 (m, 1H, H_5); 4.88 (d, J = 7.3 Hz, H, H_1); 4.98 (s, 2H, $H_{3'}$); 5.18–5.30 (m, 3H, $H_{2,3,4}$); 6.75 (s, 1H, $H_{1'}$); 7.11–7.46 (m, 9H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ (ppm): 20.5 ($C_{\underline{C}H_3CO}$); 21.2 ($CPhCH_3$); 53.0 (C_{OCH_3}); 68.9 ($C_{3'}$); 69.7–70.3–71.4–72.8 ($C_{2,3,4,5}$); 100.1 (C_1); 115.3 ($C_{2'}$); 121.5–126.5–127.8–128.3–129.4–129.9–130.9–137.6 153.62 (C_{arom}); 144.4 ($C_{1'}$); 151 (C_{OCOO}); 166.7-168.9-169.3-170.0 ($C_{CH_3\underline{C}O,\underline{C}OOCH_3}$).

5.9. General procedure for *p*-methoxybenzyl amine coupling

To a stirred solution of activated alcohols **9a**,**b**–**10c** (0.32 mmol) in CH_3CN (4 mL), *para*-methoxybenzylamine (1 equiv) and DMAP (1 equiv) were added at room temperature and the solution was stirred for 24 h. The reaction was hydrolysed with a saturated solution of $NaHCO_3$ and extracted with CH_2Cl_2. The organic layers were dried with $MgSO_4$ and the solvents were removed in vacuo. The crude reaction mixture was purified by flash chromatography (eluent: MeOH/CH_2Cl_2 1:99).

5.9.1. 2,3,4-Tri-*O*-acetyl-2′-(4″-nitrophenyl)-3′-(4‴-methoxybenzylaminocarbonyloxy)-1′-*E*-propenyloxy)-β-D-methyl glucopyranosiduronate (12a). Red oil (83%); ^{1}H NMR (CD_3COCD_3) δ (ppm): 1.82–2.11 (3s, 9H, H_{CH_3CO}); 3.74–3.78 (2s, 6H, H_{OCH_3}); 4.18 (d, J = 9 Hz, 1H, H_5); 4.25 (d, J = 5.8 Hz, 2H, $H_{1'''}$); 4.80–5.00 (m, 3H, $H_{1,3'}$); 5.18–5.36 (m, 3H, $H_{2,3,4}$); 7.16 (d, J = 8.4 Hz, 2H, $H_{3'''}$); 7.70 (d, J = 8.7 Hz, 2H, $H_{2''}$); 8.15 (d, J = 8.7 Hz, 2H, $H_{3''}$); ^{13}C NMR (CD_3COCD_3) δ (ppm): 20.5 ($3C_{\underline{C}H_3CO}$); 44.4 ($C_{1'''}$); 52.7–55.2 ($2C_{OCH_3}$); 64.8 ($C_{3'}$); 68.8–70.2–71.1–72.6 ($C_{2,3,4,5}$); 100.1 (C_6); 113.7 ($C_{2'}$); 123.0–130.2 ($C_{2'',3'',3''',4'''}$); 141.0–158.0 ($C_{1',1'',4'',2''',5''',NCOO}$); 166.5–169.1–169.4–169.7 ($C_{CH_3\underline{C}O,\ \underline{C}OOCH_3}$); $[\alpha]_D$ −16 (*c* 0.09,

$CHCl_3$); HRMS (ESI) calcd for $C_{31}H_{34}N_2O_{15}Na$ (M + Na +) 697.1857, found 697.1857.

5.9.2. 2,3,4-Tri-*O*-acetyl-2′-(4″-fluorophenyl)-3′-(4‴-methoxybenzylaminocarbonyloxy)-1′-*E*-propenyloxy)-β-D-methyl glucopyranosiduronate (12b). Yellow powder (44%); ^{1}H NMR ($CDCl_3$) δ (ppm): 1.94–2.03 (3s, 9H, H_{CH_3CO}); 3.73–3.77 (2s, 6H, H_{OCH_3}); 4.13 (d, J = 9 Hz, 1H, H_5); 4.25 (d, J = 5.4 Hz, 2H, H_{CH2NH}); 4.74 to 5.29 (m, 7H, $H_{1,2,3,4,3'',NH}$); 6.67 (s, 1H, $H_{1'}$); 6.83 (d, J = 8.4 Hz, 2H, $H_{3'''}$); 6.99 (d, J = 8.4 Hz, 2H, $H_{3''}$); 7.15 (d, J = 8.4 Hz, 2H, $H_{2'''}$); 7.48 (d, J = 8.4 Hz, 2H, $H_{2''}$); ^{13}C NMR ($CDCl_3$) δ (ppm): 20.3–20.4–20.5 ($3C_{CH_3}CO$); 44.5 ($C_{CH_2}NH$); 52.9–55.3 ($2C_{OCH_3}$); 65.6 ($C_{3''}$); 69.4–70.5–71.5–72.6 ($C_{2,3,4,5}$); 100.2 (C_1); 114.0 ($C_{3'''}$); 114.2 ($C_{2'}$); 115.0 (d, J = 21 Hz, 2C, $C_{3''}$); 128.9 ($C_{2'''}$); 129.8 (d, J = 8 Hz, 2C, $C_{2''}$); 130.3 (d, J = 3 Hz, 1C, $C_{1''}$); 130.5 ($C_{1'''}$); 143.6 ($C_{1'}$); 156.3–160.1–163.3 ($C_{4''',4'',NCOO}$); 167.0–169.0–169.4–170.0 ($C_{CH_3CO,COOCH_3}$); $[\alpha]_D$ −30 (*c* 0.09, $CHCl_3$); HRMS (ESI) calcd for $C_{31}H_{34}NO_{13}FNa$ (M + Na +) 670.1912, found 670.1905.

5.9.3. 2,3,4-tri-*O*-acetyl-2′-(4″-methylphenyl)-3′-(4‴-methoxybenzylaminocarbonyloxy)-1′-*E*-propenyloxy)-β-D-methyl glucopyranosiduronate (12c). White powder (65%); ^{1}H NMR ($CDCl_3$) δ (ppm): 1.93–2.04 (3s, 9H, H_{CH_3CO}); 2.32 (s, 3H, H_{PhCH_3}); 3.73–3.78 (2s, 6H, H_{OCH_3}); 4.13 (d, J = 9 Hz, 1H, H_5); 4.25 (d, J = 5.4 Hz, 2H, $H_{CH_2}NH$); 4.82–5.28 (m, 7H, $H_{1,2,3,4,3',NH}$); 6.65 (s, 1H, $H_{1'}$); 6.83 (d, J = 8.4 Hz, 2H, $H_{3'''}$); 7.10–7.18 (m, 4H, $H_{3'',2'''}$); 7.40 (d, J = 8.4 Hz, 2H, $H_{2''}$); ^{13}C NMR ($CDCl_3$) δ(ppm): 20.3 ($3C_{CH_3}CO$); 21.0 (C_{PhCH3}); 44.3 (CCH_2NH); 52.8–55.1 ($2C_{OCH3}$); 65.5 ($C_{3''}$); 68.9–70.3–71.4–72.6 ($C_{2,3,4,5}$); 99.9 (C_1); 113.8 ($C_{3'''}$); 115.6 ($C_{2'}$); 127.7–128.6–128.7 ($C_{2'',3'',2'''}$); 130.5 ($C_{1'''}$); 131.2 ($C_{1''}$); 136.7 ($C_{4''}$); 143.2 ($C_{1'}$); 156.3–158.7 ($C_{4''',NCOO}$); 166.6–168.9–169.2–169.8 ($C_{CH_3CO,COOCH_3}$); $[\alpha]_D$ −28 (*c* 0.03, $CHCl_3$); HRMS (ESI) calcd for $C_{32}H_{38}NO_{13}Na$ (M + Na +) 666.2163, found 666.2166.

5.10. General procedure for deacylation

To a solution of fully protected glucuronide **12a–c** (0.35 mmol) in an anhydrous mixture of CH_2Cl_2/MeOH (8/16: v/v), a 0.32 M solution of MeONa in MeOH (1.08 mL) was added at 0 °C and the mixture was stirred for 2 h. Thereafter, the solution was neutralised by addition of Amberlyst IRC-50 ion-exchange resin. Filtration followed by evaporation under reduced pressure of the filtrate afforded a crude residue which was purified by flash chromatography.

5.10.1. [2′-(4″-Nitrophenyl)-3′-(4‴-methoxybenzylaminocarbonyloxy)-1′-*E*-propenyloxy]-β-D-methyl glucopyranosiduronate (13a). ^{1}H NMR (CD_3COCD_3) δ (ppm): 3.51–3.67 (m, 3H, $H_{2,3,4}$); 3.72–3.75 (2s, 6H, H_{OCH_3}); 4.04 (d, J = 9.5 Hz, 1H, H_5); 4.22 (d, J = 6.2 Hz, 1H, $H_{1'''}$); 4.90 (s, 2H, $H_{3'}$); 4.98 (d, J = 7.4 Hz, 1H, H_1); 6.70 (s, 1H, H_{NH}); 6.84 (d, J = 8.6 Hz, 2H, $H_{4'''}$); 7.13 (s, 1H, $H_{1'}$); 7.19 (d, J = 8.6 Hz, 2H, $H_{3'''}$); 7.96 (d, J = 9 Hz, 2H, $H_{2''}$); 8.17 (d, J = 9 Hz, 2H, $H_{3''}$); ^{13}C NMR (CD_3COCD_3) δ (ppm): 44.6 ($C_{1'''}$); 52.5 (C_{COOCH_3}); 55.4 (C_{OCH_3}); 65.4 ($C_{3'}$); 72.5–73.9–76.8–76.9 ($C_{2,3,4,5}$); 105.1 (C_1); 112.8 ($C_{2'}$); 114.5 ($C_{4'''}$); 123.8 ($C_{3''}$); 129.4–129.7 ($C_{2'',3'''}$); 132.5 ($C_{2'''}$); 143.4 ($C_{1'}$); 146.7 ($C_{1''}$); 150.6 ($C_{4''}$); 157.2 ($C_{5'''}$); 159.7 (C_{NCOO}); 169.6 (C_{COOCH_3}); $[\alpha_D]$ +6 (*c* 0.16, MeOH); HRMS (ESI) calcd for $C_{25}H_{28}N_2O_{12}Na$ (M + Na +) 571.1127, found 571.1190.

5.10.2. [2′-(4″-fluorophenyl)-3′-(4‴-methoxybenzylaminocarbonyloxy)-1′-E-propenyloxy]-β-D-methyl glucopyranosiduronate (13b). ^{1}H NMR (CD_3OD) δ (ppm): 3.33–3.60 (m, 3H, $H_{2,3,4}$); 3.71–3.72 (2s, 6H, H_{OCH_3}); 3.92 (d, J = 9 Hz, 1H, H_5); 4.12 (s, 2H, $H_{CH_2}NH$); 4.69–4.77 (m, 3H, $H_{3',1}$); 6.72 (s, 1H, $H_{1'}$); 6.79 (d, J = 8.5 Hz, 2H,$H_{3'''}$); 6.97 (t, J = 8.8 Hz, 2H, $H_{3''}$); 7.09 (d, J = 8.4 Hz, 2H,$H_{2'''}$); 7.60 (t, J = 8.8 Hz, 2H, $H_{2''}$); ^{13}C NMR (CD_3CD) δ(ppm): 44.8 ($C_{CH_2}NH$); 52.9 (C_{COOCH3}); 55.6 (C_{OCH3}); 67.4 ($C_{3'}$); 72.8–74.2–77.0–77.2 ($C_{2,3,4,5}$); 105.0 (C_1); 114.8 ($C_{3'''}$); 114.9 ($C_{2'}$); 115.5 (d, J = 21 Hz, $C_{3''}$); 129.5 ($C_{2'''}$); 131.2 (d, J = 8 Hz, $C_{2''}$); 132.6–132.8 ($C_{1'',1'''}$); 143.6 ($C_{1'}$); 158.7–160.0–161.3–162.4 ($C_{1''',4'',4''',COONH}$); 170.9 (C_{COOCH_3}); $[\alpha]_D$ +36 (*c* 0.08, MeOH); HRMS (ESI) calcd for $C_{25}H_{28}NO_{10}FNa$ (M + Na +) 544.1595 found 544.1591.

5.10.3. [2′-(4″-Methylphenyl)-3′-(4‴-methoxybenzylaminocarbonyloxy)-1′-*E*-propenyloxy]-β-D-methyl glucopyranosiduronate (13c). ^{1}H NMR (CD_3COCD_3) δ (ppm): 2.29 (s, 3H, H_{PhCH_3}); 3.47–3.59 (m, 3H, $H_{2,3,4}$); 3.70–3.74 (2s, 6H, H_{OCH_3}); 4.01 (d, J = 9.6 Hz, 1H, H_5); 4.21 (d, J = 6 Hz, 2H, $H_{CH_2}NH$); 4.78–4.86 (m, 3H, $H_{3',1}$); 6.60 (s, 1H, H_{NH}); 6.80–6.85 (m, 3H, $H_{1',3'''}$); 6.97 (d, J = 8 Hz, 2H, $H_{3''}$); 7.10 (d, J = 8.2 Hz, 2H, $H_{2'''}$); 7.55 (d, J = 8.1 Hz, 2H, $H_{2''}$); ^{13}C NMR (CD_3COCD_3) δ (ppm): 21.1 ($CPhCH_3$); 44.5 ($C_{CH_2}NH$); 52.4 (C_{COOCH_3}); 55.4 (C_{OCH_3}); 66.2 ($C_{3'}$); 72.5–73.9–76.9–77.0 ($C_{2,3,4,5}$); 105.0 (C_1); 114.3–114.5 ($C_{2',3'''}$); 128.8–129.3–129.4 ($C_{2'',3'',2'''}$); 132.6 ($C_{1'''}$); 133.4 ($C_{1''}$); 136.6 ($C_{4''}$); 147.0 ($C_{1'}$); 157.4 ($C_{4'''}$); 159.6 (C_{COONH}); 169.6 (C_{COOCH_3}); $[\alpha]_D$ +48 (*c* 0.11, MeOH); HRMS (ESI) calcd for $C_{26}H_{31}NO_{10}Na$ (M + Na +) 540.1846, found 540.1843.

5.11. General procedure for methyl ester hydrolysis

To a solution of methyl ester **13a–c** in methanol was added $Ba(OH)_2{\cdot}8H_2O$ (1.2 equiv) and the mixture was stirred for 4 h at room temperature. A white precipitate was formed and filtered. Purity and structure were confirmed by HPLC and SM analysis. ^{1}H NMR analysis of **4a,b** gave poorly resolved spectra.

5.11.1. [2′-(4″-Nitrophenyl)-3′-(4‴-methoxybenzylaminocarbonyloxy)-1′-*E*-propenyloxy]-β-D-glucopyranosiduronic acid (4a). $[\alpha]_D$ −6 (*c* 0.9, MeOH); HRMS (ESI) calcd for $C_{24}H_{26}N_2O_{12}Na$ (M + Na +) 557.1278, found 557.1272.

5.11.2. [2′-(4″-Fluorophenyl)-3′-(4‴-methoxybenzylaminocarbonyloxy)-1′-*E*-propenyloxy]-β-D-glucopyranosiduronic acid (4b). $[\alpha]_D$ −5 (*c* 0.9, MeOH); HRMS (ESI) calcd for $C_{24}H_{26}NO_{10}F_{Na}$ (M + Na +) 530.1018, found 530.1023.

5.11.3. [2′-(4″-Methylphenyl)-3′-(4‴-methoxybenzylaminocarbonyloxy)-1′-*E*-propenyloxy]-β-D-glucopyranosiduronic acid (4c). ^{1}H NMR (D_2O) δ (ppm): 2.35 (s, 3H,

H_{PhCH_3}); 3.36–3.60 (m, 3H, $H_{2,3,4}$); 3.83 (s, 3H, H_{OCH3}); 4.18 (s, 2H, $H_{\underline{CH}_2NH}$); 4.76 (s, 2H, $H_{3'}$); 6.82 (s, 1H, $H_{1'}$); 6.95 (sl, 2H, $H_{3'''}$); 7.10 (sl, 2H, $H_{3''}$); 7.24 (sl, 2H, $H_{2'''}$); 7.39 (sl, 2H, $H_{2''}$); $[\alpha]_D$ 0 (c 0.2, MeOH); HRMS (ESI) calcd for $C_{25}H_{29}NO_{10}Na$ (M + Na +) 548.1509, found 548.1494.

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