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Transfer activity of bovine liver β-glucuronidase: synthesis of disaccharides containing a β-D-glucopyranuronate unit

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Abstract—This paper deals with a study of the ability of a β-glucuronidase from bovine liver to catalyse transglucuronidation reactions. Our results show that when sodium (*p*-nitrophenyl β-D-glucopyranosid)uronate was used as a donor and as an acceptor, the sodium (sodium β-D-glucopyranosyluronate)-(1→3)-(*p*-nitrophenyl β-D-glucopyranosid)uronate and the (1→2) regioisomer were obtained in yields of 18 and 15%, respectively. A different regioselectivity was observed using *p*-nitrophenyl α-D-galactopyranoside as an acceptor leading to the synthesis of *p*-nitrophenyl (sodium β-D-glucopyranosyluronate)-(1→4) α-D-galactopyranoside and of the (1→2) regioisomer in yields of 16 and 21%, respectively.

1. Introduction

Recent understanding of the role played by oligosaccharides in biological processes, like their mediator function in molecular recognition, has stimulated the development of methodological approaches for the synthesis of new molecules for pharmaceutical and biotechnological applications.[1–3] Stereo- and regioselective syntheses of important oligosaccharides have been achieved through the enzymatic approach.[4–11] Two kinds of enzyme are able to catalyse the formation of the glycosidic bond: the glycosyltransferases and the glycosylhydrolases. Very curiously, this strategy has not been widely applied in the uronic series. Thus, whilst glucuronide prodrugs have recently been synthesized using UDP-glucuronosyltransferases as catalysts,[12–14] to our knowledge, the preparation of di- or tri-glucuronic acids by this method has not been described. The same is true for the transfer activity of 'retaining' glucuronidases. Some of the latter have been shown to catalyse reactions of a wide range of acceptors but these enzymes have not been widely used to catalyse the synthesis of uronic disaccharides.[15–17] Considering the potential applications of uronic acid derivatives in cancer therapy[18] and heavy metal detoxification of water,[19] we have undertaken a study of the synthesis of disaccharides using glucuronidases. The aim of this paper is to report on tests completed to ascertain the ability of bovine liver β-glucuronidase (E.C. 3.2.1.31) to catalyse the stereo- and regioselective synthesis of disaccharides. It should be pointed out that the synthesis of uronic acids can also be approached by means of the degradation of polyuronates catalysed by polyuronate lyases. We have recently shown that *exo*-polygalacturonate lyase from *Thermotoga maritima* was able to form (4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-(1→4)-(α-D-galactopyranosyluronic acid)-(1→4)-α-D-galactopyranuronic acid as a single compound from the degradation of orange peel polygalacturonate.[20]

2. Results and discussion

To our knowledge, only a few papers deal with the use of β-glycuronidases in transglycosylation reactions: Niemann et al.[16] have shown that purified rat liver or lysosomal β-glucuronidase can catalyse the regioselective synthesis of β-glucopyranosyluronic acid-(1→3) glycosidic linkages. More recently, Kuroyama et al.[17] have studied the hydrolysis of various β-glucopyranosyluronic acid (1→*n*) glycosides catalysed with a β-glucuronidase from *Aspergillus niger*. The selectivity of this enzyme was shown to be mainly of the (1→6) type. The authors also noted transfer activity for this glucuronidase, which led to the synthesis of β-glucopyranosyluronic acid glycosides of unknown structure. Our

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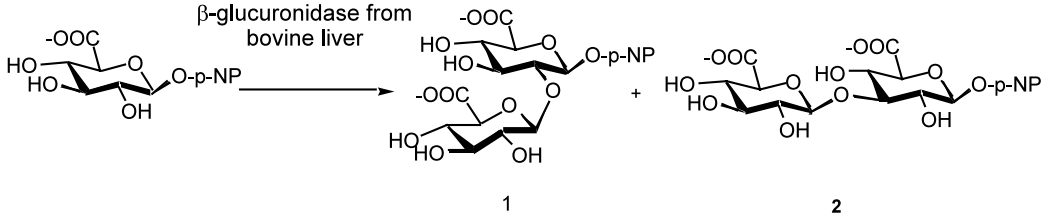
previous results concerning the transglycosylation reactions mediated by β- and α-galactosidases have shown that the condensation between an activated donor and an acceptor is very often in competition with the self-condensation reaction of the donor.[10,21] For this reason, we first decided to study the ability of bovine liver glucuronidase to catalyse the self-condensation of sodium (*p*-nitrophenyl β-D-glucopyranosid)uronate (*p*-NP β-D-GlcA). Thus, after incubation of a 80 mM solution of *p*-NP β-D-GlcA in the presence of the enzyme at room temperature for 5 h, NMR analysis of the mixture revealed the presence of remaining *p*-NP-β-D-GlcA (27%), sodium glucuronate (53%) and two self condensation disaccharides **1** (9%), **2** (11%). Considering that diglucuronides are synthesized from two equivalents of *p*-NP β-D-GlcA, the yields for **1** and **2**, calculated from the NMR spectra, are, respectively, 15 and 18% versus 44% for the glucuronate produced by the hydrolysis. The separation of the components was achieved by means of a Bio-Gel P2 column (see Section 3). The structure of compounds **1** and **2** was established by means of combined one- and two-dimensional NMR spectroscopy. Thus, disaccharides **1** and **2** are, respectively, sodium (sodium β-D-glucopyranosyluronate)-(1→2)-(*p*-nitrophenyl β-D-glucopyranosid)uronate and the (1→3) regioisomer (see Scheme 1). The ^{1}H NMR spectra of **1** is shown in Fig. 1.

The disaccharides **1** and **2** are new compounds. To our knowledge, only a few similar diuronates have already been synthesized and furthermore by means of a chemical approach. Thus, a method has been described to produce the preparation of a mixture of anomers of methyl-β-D-mannopyranosyluronic acid)-(1→3)-(D-mannopyranosid)uronic acid and of the (1→2) regioisomer.[22] In addition, with the work presented here, the self-condensation of a glycuronate donor induced by a glycuronidase enzyme has been highlighted for the first time.

The above results indicate that this glucuronidase has good transglycosylation ability, although the regioselectivity is not very high. Thus, we undertook the study of the condensation reaction using *p*-NP β-D-GlcA as a



Scheme 1. Self-condensation reaction catalysed by bovine liver β-glucuronidase.

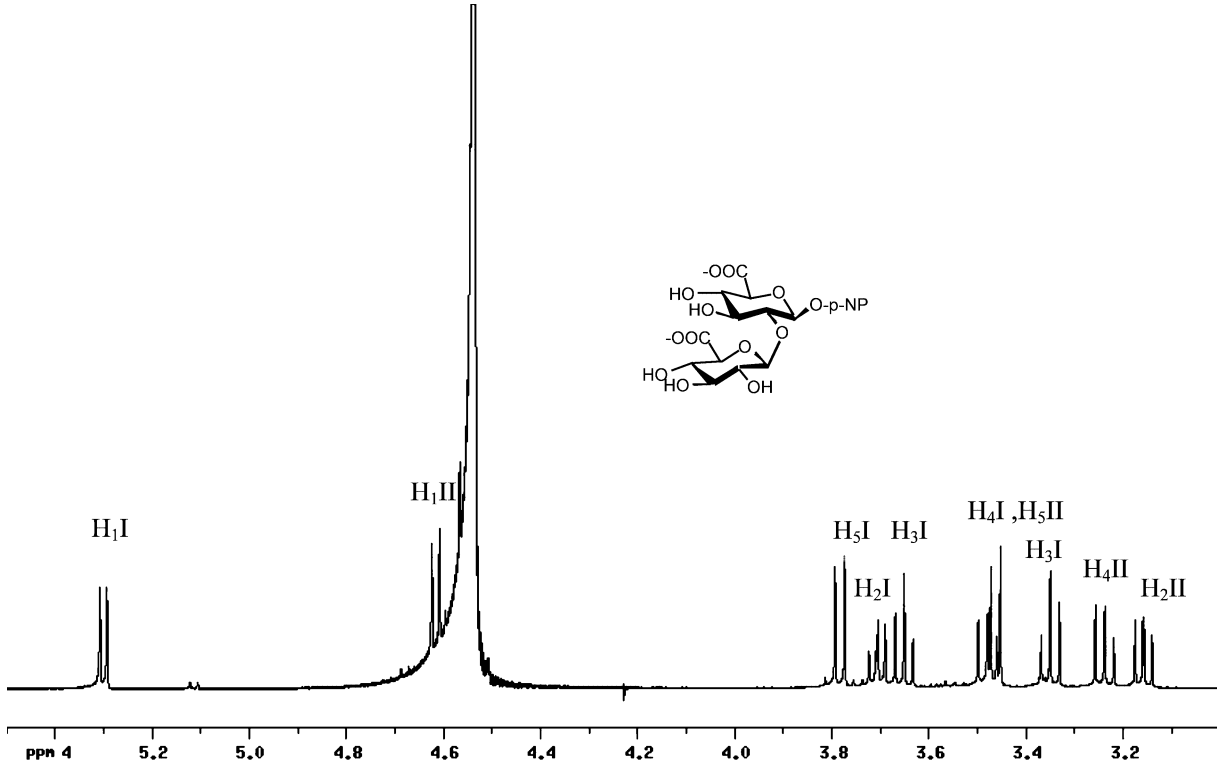
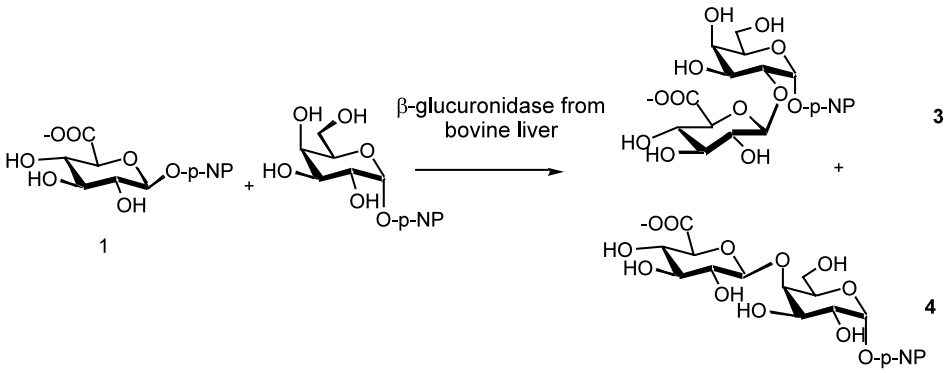


Figure 1. ^{1}H NMR spectra of disaccharide **1** (solvent D_2O, pD 8, aromatic proton resonances not shown, 500 MHz Bruker AX500 spectrometer).



Scheme 2. Transferase activity of β-glucuronidase from bovine liver.

donor and *p*-nitrophenyl α-D-galactopyranose (*p*-NP α-Gal) as an acceptor (Scheme 2).

The reaction conditions were adjusted in order to avoid the self-condensation reaction. As a consequence, the *p*-NP β-D-GlcA concentration chosen for the reaction was 10 mM. Thus, the incubation of a 1/4.5 mixture of donor/acceptor in the presence of the enzyme for 5.5 h yielded a reaction media composed of remaining *p*-NP β-Gal (6%), sodium glucuronate (57%) and two disaccharides **3** (21%) and **4** (16%), as determined by means of the relative integrations in the ^{1}H NMR spectrum of the mixture. It should be pointed out that the self-condensation products, disaccharides **1** and **2**, were not synthesized in the conditions used. In previous experiments performed with a longer incubation time (26 h) and higher enzymatic activity, other impurities were present and we were particularly surprised to identify galactose in the reaction mixture. An investigation of the behaviour of the β-glucuronosidase commercial preparation on a 45 mM solution of *p*-NP α-D-Gal by means of NMR spectroscopy indicated not only the formation of galactose but also the synthesis of self-condensation α-(1→6) and α-(1→3) disaccharides, the latter being identified by comparison with authentic samples synthesized previously in our laboratory.[21,11] This observation indicates that the commercial enzyme preparation used was contaminated with an α-galactosidase. Fortunately, these compounds were not detectable after 5 h reaction time. After purification over a Bio-Gel P2 column, **3** and **4** were separated and identified, respectively, as *p*-nitrophenyl (sodium β-D-

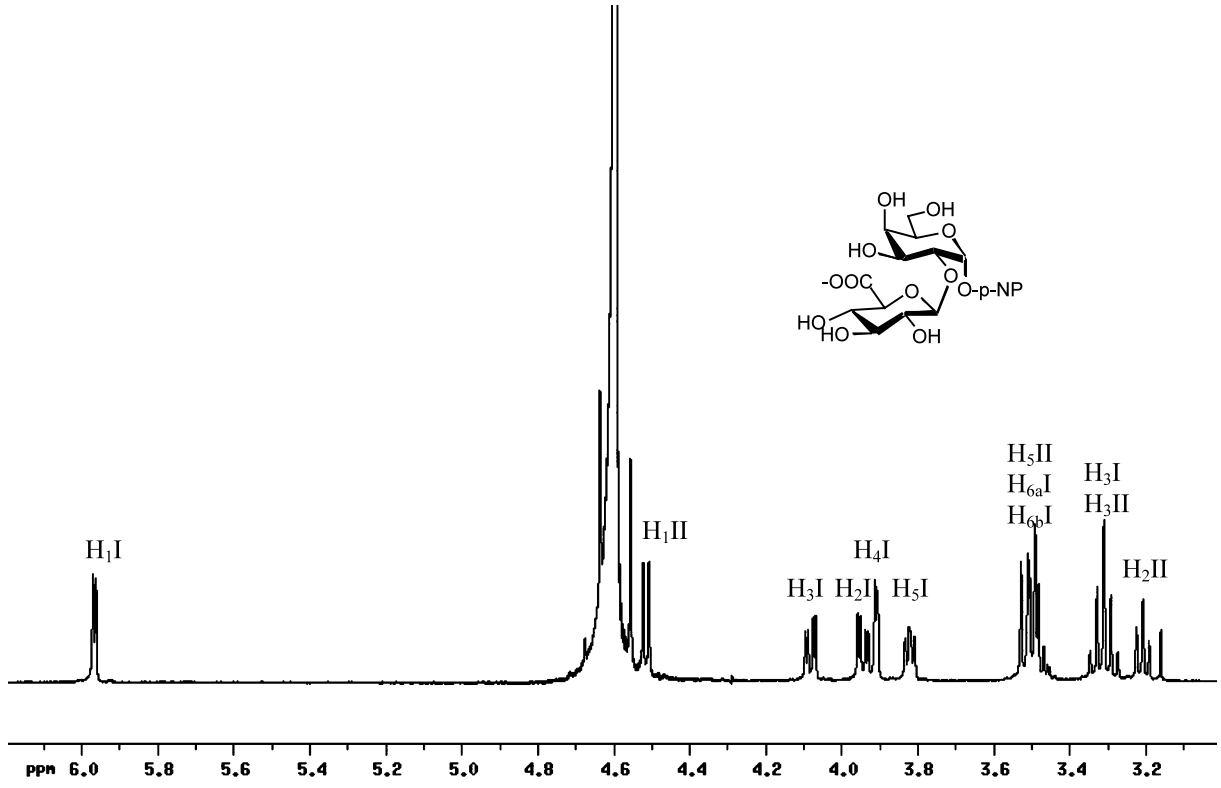


Figure 2. ^{1}H NMR spectra of disaccharide **3** (solvent D_2O, pD 8, aromatic proton resonances not shown, 500 MHz Bruker AX500 spectrometer).

glucopyranosyluronate)-(1→2) α-D-galactopyranoside and the (1→4) regioisomer by means of NMR spectroscopy (see Fig. 2). This method which leads to the synthesis of compound **4** is of great interest considering the biological occurrence of this type of structure.[23]

In summary, with this work we have shown that a retaining β-glucuronidase from beef liver was able to catalyse the synthesis of glucosuronic disaccharides using their good transfer ability (about 40% of transglycosylation products against 60% for hydrolysis). This enzyme induced moderate regioselectivity, since on one side (1→2) and (1→3) disaccharides were synthesized in nearly equal amounts in the self-condensation reaction. The same was true for the condensation reaction in the presence of *p*-NP α-D-Gal, which led to a (1→2) and (1→4) disaccharide mixture.

3. Experimental

Chemicals and bovine liver β-glucuronidase were purchased from Sigma and were used without further purification. The course of the reaction was monitored by means of TLC (pre-coated silica gel 60 sheets Merck F254) using eluent I ($CHCl_3$:MeOH:H_2O:AcOH, 12:12:1:1) or eluent II (*n*-BuOH:H_2O:AcOH, 3:3:2). The compounds of the reaction mixtures were separated on Bio-Gel P2 (molecular weight fractionation range: 100–1800 Daltons, Biorad) columns. ES-MS spectra were performed on a LC Quattro Micromass spectrometer. Complete analysis of the ^1H and ^{13}C NMR resonances and subsequent structure assignments were made using standard 2D sequences (COSY HH and HCOR correlations). The spectra were recorded with a Bruker AX500 spectrometer operating at 500 MHz for ^1H (solvent D_2O, chemical shifts in ppm quoted from the resonance of acetone at $\delta = 2.04$) and 126 MHz for ^{13}C (solvent D_2O, chemical shifts in ppm quoted from the methyl resonance of acetone at $\delta = 29.8$ ppm).

3.1. Synthesis of *p*-NP β-GlcA*p*-β-(1→2)-GlcA*p*, 1 and *p*-NP β-GlcA*p*-β-(1→3)-GlcA*p*, 2

p-NP β-GlcA (100 mg, 0.317 mmol) was dissolved in 4 mL of phosphate buffer (160 mM, pH 6.9). The pH (5.8) was adjusted to 5.2 with 2N HCl (25 μL) before adding 0.35 units of β-glucuronidase (amount calculated from the hydrolysis of *p*-NP β-GlcA at 25°C). The reaction was left 5.5 h at room temperature with stirring and was quenched by boiling for 10 min. *p*-NP was partially removed by washing twice with ether and the aqueous layer was evaporated under reduced pressure to give a solid. The latter was dissolved in water to be applied onto a 1.4×95 cm Bio-Gel P2 column. The compounds were eluted with water and 90 mL was discarded before collecting 1 mL fractions (7.5 mL/h). The course of the purification was monitored by TLC using *n*BuOH, H_2O, AcOH (3:3:2) as eluent ($R_f = 0.7$ and 0.61 for **1** and **2**, respectively). GlcA was first eluted, followed by **1**, **2**, and *p*-NP β-GlcA. The fractions containing pure **1** and **2** were evaporated. The overall yield for the two self-condensation products calculated from the integrations of the ^1H NMR spectra was 33%.

***p*-NP β-GlcA*p*-β-(1→2)-GlcA*p* 1**: The NMR parameters are given in Tables 1–3. ES-MS: 490 $(M-H)^+$, ***p*-NP β-GlcA*p*-β-(1→4)-GlcA*p* 2**: The NMR parameters are given in Tables 1–3, ES-MS: 490 $(M-H)^+$.

Table 1. ^1H chemical shifts of disaccharides **1**–**4** (solvent D_2O, chemical shifts quoted from methyl resonances of acetone, pD 8)

Compound	Unit	δ_{H1}	δ_{H2}	δ_{H3}	δ_{H4}	δ_{H5}	δ_{H6a}	δ_{H6b}	$\delta_{H\ aromatic}$
1	I	5.29	3.71	3.65	3.48	3.78			8.06/7.03
	II	4.61	3.16	3.35	3.24	3.46			
2	I	5.11	3.71	3.76	3.57	3.80			8.08/7.06
	II	4.68	3.24	3.36	3.36	3.57			
3	I	5.97	3.95	4.08	3.91	3.82	3.51	3.49	8.08/7.11
	II	4.52	3.20	3.33	3.29	3.52			
4	I	5.67	3.96	4.04	4.13	3.82	3.61	3.43	8.08/7.11
	II	4.55	3.24	3.35	3.35	3.52			

Table 2. ^1H coupling constants for disaccharides **1**–**4** (solvent D_2O, chemical shifts quoted from methyl resonances of acetone, pD 8)

Compound	Unit	$^3J_{12}$	$^3J_{23}$	$^3J_{34}$	$^3J_{45}$	$^3J_{56a}$	$^3J_{56b}$	$^2J_{6a6b}$
1	I	7.4	9.1	9.1	9.9			
	II	8.0	9.3	9.3	9.8			
2	I	7.8	9.1	9.1	10.0			
	II	8.0	9.0	n.m.	n.m.			
3	I	3.8	10.3	3.3	~0	5.3	7.4	−12.1
	II	8.0	8.6	9.2	9.4			
4	I	3.7	10.5	3	~0	6.6	6.6	−11.8
	II	8.3	8.3	n.m.	9.2			

Table 3. ^{13}C chemical shifts of disaccharides **1–4** (solvent D_2O, chemical shifts quoted from methyl resonances of acetone, pD 8)

Cmpd.	Unit	δ_{C1}	δ_{C2}	δ_{C3}	δ_{C4}	δ_{C5}	δ_{C6}	δ_{COOH}	δ_{C-O}*	δ_{C-H}*	δ_{C-N}*
1	I	97.7	80.9	74.4	70.5	75.0		175.2 or 174.3	142.6	126.0/115.9	161.5
	II	102.1	72.8	74.6	71.0	75.5		175.2 or 174.3			
2	I	99.1	72.6	82.6	70.0 or 75.7	76.3		175.7 or 174.8	142.6	126.1/116.5	161.7
	II	102.2	73.2	71.7 or 75.3	71.7 or 75.3	70.0 or 75.7		175.7 or 174.8			
3	I	95.8	77.4	67.8	68.7	71.6	60.5		142.0	125.5/116.8	160.7
	II	103.7	72.6	74.9	71.2	76.3		174.7			
4	I	96.6	68.0	69.4	76.7	71.1	59.2		142.0	125.6/116.9	161.5
	II	103.1	73.3	75.4 or 71.5	75.4 or 71.5	75.5		175.5			

* Aromatic carbons.

3.2. Synthesis of *p*-NP α-GlcA*p*-β-(1→2)-Gal*p*, 3 and *p*-NP α-GlcA*p*-β-(1→4)-Gal*p*, 4

A mixture of *p*-NP β-GlcA (109 mg, 0.35 mmol) and *p*-NP α-gal (430 mg, 1.43 mmol) was dissolved in acetate buffer (pH 5, 100 mM, 30 mL). The pH (4.8) was next adjusted to 5.2 by adding NaOH M (0.5 mL). Then, β-glucuronidase (0.95 units-amount calculated from the hydrolysis of *p*-NP β-GlcA at 25°C) was added and the reaction mixture was stirred at room temperature for 5 h. The reaction was quenched by boiling for 10 min. *p*-NP was partially removed by washing twice with ether and the aqueous layer was evaporated in vacuo. The solid obtained was dissolved in water to be applied onto a Bio-Gel P2 column (h = 95 cm, o.d. = 1.4 cm). The compounds were eluted with water and 90 mL was discarded before collecting 1 mL fractions (7.5 mL/h). Each fraction was checked by TLC using $CHCl_3$, MeOH, H_2O, AcOH (12:12:1:1) as eluent **3**, **4** (R_f = 0.48 and 0.55 for **3** and **4**, respectively). The order of elution for the components was: glucuronic acid, **3**, **4** *p*-NP α-GlcA and *p*-NP α-gal. The overall yield calculated from the integrations of the ^1H NMR spectra was 37%.

***p*-NP α-Glc*p*A-β-(1→2)-Gal*p*, 3**: The NMR parameters are given in Tables 1–3; ES-MS: 476 $(M-H)^-$, ***p*-NP α-Glc*p*A-β-(1→4)-Gal*p*, 4**: The NMR parameters are given in Tables 1–3; ES-MS: 476 $(M-H)^-$.

3.3. NMR parameters for compounds 1–4

The NMR parameters are listed in Tables 1–3.

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