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GALACTOSYLATION AND GLUCOSYLATION BY USE OF ß-GALACTOSIDASE

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Abstract: The transglycosylation activity of ß-galactosidase derived from *Aspergillus oryzae* and *Escherichia coli*, respectively, was examined in reaction systems containing up to 50% acetonitrile. Starting with *ortho*-nitrophenyl ß-galactoside (**1**), which functions both as donor and as acceptor, ß-Gal(1-6)ß-Gal-PhNO$_2$-*o* (**2**) and ß-Gal(1-3)ß-Gal-PhNO$_2$-*o* (**3**) were obtained. Under similar conditions the enzyme from *A. oryzae* converts *para*-nitrophenyl ß-glucoside (**5**) to ß-Glc(1-2)ß-Glc-PhNO$_2$-*p* (**6**) and α-Glc(1-4)ß-Glc-PhNO$_2$-*p* (**7**). Incubation of **1** and L-serine in the presence of the *A. oryzae* ß-galactosidase leads to ß-Gal-L-Ser (**4**).

Owing to multiple hydroxyl groups of similar reactivity and the essential requirement for stereoselective synthesis, controlled glycosylation remains a challenge to organic chemists. Even though well elaborated and still widely applied, classical chemical approaches inevitably require quite a number of protection, activation, coupling, and deprotection steps. In contrast to that, enzymes offer the opportunity of one-step preparations under mild conditions in regio- and stereoselective manner.[1] In particular glycosidases are already about to become common instruments in saccharide synthesis.[2]

Thus, ß-galactosidase from different sources is known to catalyze glycosylations by means of equilibrium- and kinetically controlled syntheses. Previously, using a continuous column reactor Wallenfels[3] applied the former method to the synthesis of mainly ß(1-6)-linked ß-Gal-GlcNAc isomers. More recently a similar reactor was described, which has been utilized for the formation of several ß-galactose containing di- and trisaccharides.[4] For nearly all acceptor saccharides tested different regioisomers were observed and separated by h.p.l.c. Kinetically controlled syntheses are usually performed in a batch reactor. For example Hedbys et al.[5] obtained ß-Gal(1-6)GalNAc in 20% yield using lactose as galactose donor. *para*-Nitrophenyl ß-galactopyranoside is known to be another effective donor; its ß-galactosidase-promoted reaction with ß-Gal-OMe yielded 22% ß-Gal(1-3)ß-Gal-OMe and 3% of the corresponding (1-6)-linked isomer.[6] Ooi et al. even succeeded in the preparation of some cardiac glycosides using the transfer potential of ß-galactosidase in aqueous-organic solvent mixtures.[7]

We now report the ß-galactosidase catalyzed syntheses of nitrophenyl disaccharide glycosides in a buffer-acetonitrile mixture. These compounds, due to their aromatic function are readily separated without further chemical modifications directly by gel permeation chromatography or by adsorption for instance on Amberlite XAD-4 resin.[8] Generally, in enzymatic glycosylation reactions isolation problems must be overcome, because the incubation

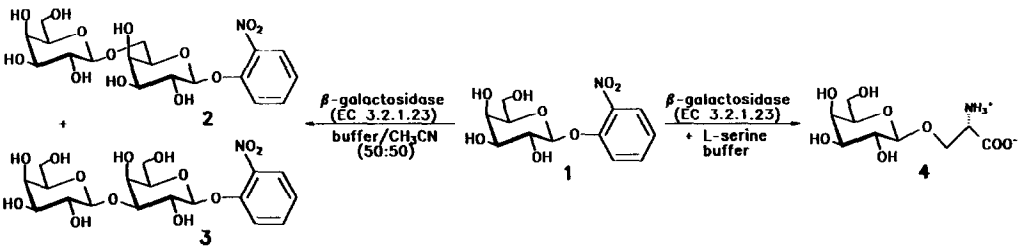
mixture contains not only donor and acceptor substrates but also diverse side products of the reaction and of hydrolysis. In addition to this the nitrophenyl group is useful for subsequent synthetic steps[9,10] or as marker to examine biological processes.[11]

Typically these transglycosylation experiments were conducted as follows: 1 mmol nitrophenyl glycoside was dissolved in a mixture of 2 ml acetonitrile and 2 ml buffer A (0.1 M KH_2PO_4, pH 5.0) or 2 ml buffer B (30 mM Na_2HPO_4, 1 mM $MgCl_2$, pH 7.3) in the case of *E. coli* ß-galactosidase. 1100 units of ß-galactosidase were then added, and the resulting solution was incubated at 20°C for 30 min with shaking. The reaction was terminated by boiling for 5 min. After centrifugation the supernatant was evaporated to dryness, dissolved in H_2O and again concentrated to remove *ortho*-nitrophenol. The removal of the *para*-isomer was best achieved by passing the solution through a column containing Dowex 1x2 resin. The residue was fractioned on Sephadex G-10, and thus even regioisomeric nitrophenyl disaccharide glycosides could be completely separated. The products were characterized by ^1H- and ^{13}C-n.m.r. spectroscopy (table 1).

Table 1. ^{13}C-n.m.r. chemical shifts of nitrophenyl disaccharide glycosides in D_2O.

compound	C-1	C-2	C-3	C-4	C-5	C-6
2						
ß-Gal-OPhNO$_2$-*o*	101.51	70.71	72.83	69.02	75.10	69.28
ß-Gal(1-6)	103.86	71.21	73.13	69.07	75.59	61.42
3						
ß-Gal-OPhNO$_2$-*o*	101.39	69.99	82.17	68.71	75.80	61.09
ß-Gal(1-3)	104.81	71.52	72.99	69.06	75.56	61.45
5						
ß-Glc-OPhNO$_2$-*p*	98.42	81.68	75.68	69.41	76.41	60.94
ß-Glc(1-2)	103.26	74.11	75.86	69.60	76.27	60.52

In this way, starting with *ortho*-nitrophenyl ß-D-galactopyranoside (**1**) *ortho*-nitrophenyl 6-O-(ß-D-galactopyranosyl)-ß-D-galactopyranoside (**2**) and *ortho*-nitrophenyl 3-O-(ß-D-galactopyranosyl)-ß-D-galactopyranoside (**3**) were obtained in amounts of 11 and 6% using ß-galactosidase from *A. oryzae* (Sigma) or 13 and 14% using the enzyme from *E. coli* (Sigma). In a very recent poster communication Martin-Lomas et al. reported on a



galactosylation of benzyl ß-xylopyranoside by use of ß-galactosidase.[12] In addition to ß-Gal(1-3/4)ß-Xyl-OBn compound **2** was obtained.

Interestingly, by serendipity in the case of incubations with *A. oryzae* ß-galactosidase the yields were observed to be almost doubled following addition of amphipathic co-substrates (table 2). Z-L-Ser-Bzl is more suitable than

Z-L-serine, because it could be more easily removed from the incubation mixture by filtration after evaporation and diluting in H_2O. At present we cannot offer an explanation for this phenomenon.

Table 2. Yields by addition of amphipathic co-substrates.

co-substrate	yield of **2**	yield of **3**
-	11 %	6 %
0.17 mmol Z-L-Serine	20 %	11 %
0.26 mmol Z-L-Ser-Bzl	21 %	10 %

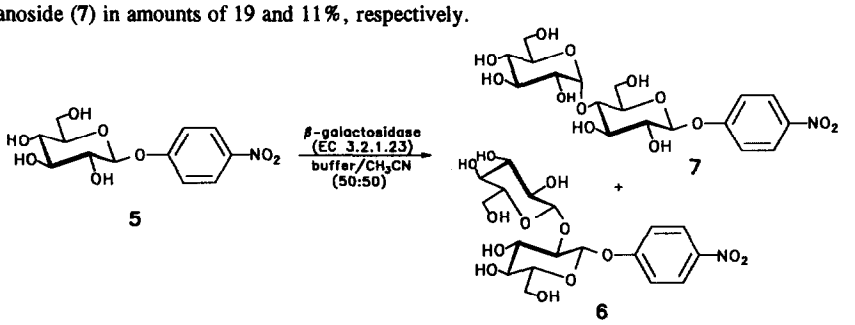
Previously the para-nitrophenyl analogue of compound **2** was prepared by classical methods starting from **1** and 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide (**8**) in three steps with 66% overall yield.[9] Although the enzymatic synthesis is less time-consuming, in this case it cannot compete with regard to yields. The situation is quite different for the synthesis of **3**, here the chemical approach required eight steps and yielded 5.5% related to **1** and **8** as educts.[13]

In order to get information about further galactosylation products, which did not carry a nitrophenyl group, the remaining fractions of the gel chromatography were peracetylated and subjected to h.p.l.c. (column: RP 18; solvent: hexane/ethyl acetate 1:1). Thus, in addition to mono- and oligosaccharides, four different disaccharide glycosides were isolated. However, it turned out, that only two of them were formed by the enzymatic ß-galactosylation. These were identified as both the anomers of ß-Gal(1-6)Gal. The others were found to be both the anomers of maltose, obviously generated by fragmentation of starch, which is a standardizing component of the utilized *A. oryzae* ß-galactosidase preparation. Amazingly there was not observed any formation of galacto disaccharides other than the (1-6)-linked species.

ß-D-Galactopyranosyl-L-serine **4** represents the linkage region of many O-glycosylated biologically active glycoproteins and thus its synthesis is of great interest. Just recently Cantacuzene et al.[15] reported the synthesis of amino blocked derivatives of **4** with ß-galactosidase. They failed to obtain the unprotected compound using lactose as donor substrate. For the preparation of ß-Gal-L-Ser (**4**) we chose a somewhat different approach: 0.25 mmol **1** and 7.5 mmol L-serine dissolved in 1 ml buffer A were preincubated at 37°C for 5 min, 1 ml buffer A containing 235 units ß-galactosidase from *A. oryzae* was then added, and the resulting mixture was allowed to react at 37°C for 10 min with shaking. The enzyme was denaturated by boiling. Afterwards the solution was centrifuged, and the concentrated supernatant was chromatographed on Sephadex G-10. The fractions containing **4** were pooled, concentrated and applied to h.p.l.c. (column: Shodex IONPAK S-802; solvent: H_2O). The pure product[16] was obtained in 10% yield.

Previously, there were reports that ß-galactosidase from *A. oryzae* was found to be also suitable for the synthesis of ß-glucosides.[7b] However, until now it remains unclear, whether the enzyme can accept glucose as a substrate or whether the enzyme preparation is contaminated by traces of ß-glucosidase. At present we tend towards the view that the enzyme is relatively unspecific particularly with regard to further observations to be reported in due course.[14]

Following the same procedure as described above, except for much higher incubation times and double the amount of enzyme, we started with **5** as donor substrate and 0.26 mmol Z-L-Ser-Bzl as co-substrate to obtain *para*-nitrophenyl 2-O-(ß-D-glucopyranosyl)-ß-D-glucopyranoside (**6**) and *para*-nitrophenyl 4-O-(α-D-glucopyranosyl)-ß-D-glucopyranoside (**7**) in amounts of 19 and 11%, respectively.



5 **7** **6**

In our opinion it is rather unlikely that the formation of the ß-maltoside **7** was catalyzed by the ß-galactosidase. For comparision it may be mentioned that an early synthesis of the mainly interesting product, the sophoroside **6** was performed in four steps with less than 2% overall yield.[10]

In conclusion, these examples demonstrate that especially the inexpensive ß-galactosidase from *A. oryzae* effectively catalyzes various glycosylations.

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16. ^{1}H-NMR data of compound **7**: (400 MHz, D_2O) δ = 4.35 (d, 1 H, H-1, $J_{1,2}$ = 8 Hz); 4.19 (dd, 1 H, H-ßa, $J_{ßa,ßb}$ = 11.5 Hz, $J_{ßa,\alpha}$ = 6 Hz); 4.00 (dd, 1 H, H-ßb, $J_{ßb,\alpha}$ = 3.5 Hz); 3.90 (dd, 1 H, H-α); 3.83 (dd, 1 H, H-4, $J_{3,4}$ = 3.5 Hz, $J_{4,5}$ < 1 Hz); 3.71 (dd, 1 H, H-6b, $J_{6a,6b}$ = 11.5 Hz, $J_{5,6b}$ = 8 Hz); 3.65 (dd, 1 H, H-6a, $J_{5,6a}$ = 4 Hz); 3.61 (ddd, 1 H, H-5); 3.57 (dd, H-3, $J_{2,3}$ = 10 Hz); 3.47 (dd, 1 H, H-2).

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