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Enzymatic syntheses and selective hydrolysis of *O*-β-D-galactopyranosides using a marine mollusc β-galactosidase

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Abstract—The use of crude extract of the hepatopancreas of *Aplysia fasciata*, a large mollusc belonging to the order Anaspidea containing a β-galactosidase activity, was reported for the synthesis of different galactosides. Good yields with polar acceptors and the uncommon β-1-3 selectivity in the transgalactosylation reactions with most of the acceptors were observed. A β-1-2 selectivity in the hydrolytic conditions was also observed and discussed.

O-β-D-galactopyranosyl moiety linked to xylopyranose ring represents an interesting disaccharidic template found in different oligosaccharides of biological interest. The β-1-2 interglycosidic linkage is present in the oligosaccharins, substances possessing hormone-like effects on plants[1] while the β-1-4 is found in the region between glycosaminoglycan (GAG) chains and protein parts, in serine-linked connective tissue proteoglycan. Interestingly this structure contains Gal-(β-1-3)-Gal as terminal disaccharidic unit.[2] In addition, free β-1-3 and β-1-4 Gal-Xyl disaccharides are useful substrates, as noninvasive diagnostic tool, for intestinal lactase, which is an enzyme involved in adult-type alactasia.[3] Chemical and enzymatic procedures were used for the preparation of these compounds, the former[3] being cumbersome due to numerous protection–deprotection steps. The enzymatic procedures are based on glycosyl hydrolases, able to catalyze the formation of glycosidic bonds in stereospecific manner, and on other enzymes such as glycosyl transferases.[4]

The marine environment furnished different sources of glycosyl hydrolases[5] and we are actively involved in the search for these enzymes from marine organisms;[6] the effort for the identification of different enzymes, each forming any desired glycosidic bond, being of current significance in this field.[7]

Recently we focused our attention on the sea hare *Aplysia fasciata* Poiret 1789, a large mollusc easily collectable and very common in Mediterranean habitats belonging to the order *Anaspidea*.[8]

We found a potent β-galactosidase activity in the hepatopancreas of this organism, an order of magnitude higher with respect to other mollusc extracts.[5] In the preliminary reactions performed, this catalytic activity attracted our attention in that it was able to synthesize β-galactosides in good yield with interesting regioselectivity towards secondary hydroxyl groups.[6]

In this communication we report on the synthesis of different β-galactopyranosides of xylose-containing molecules obtaining interesting compounds in high yield by simple procedures avoiding protein purification steps. The screening of acceptors has also been enlarged to hexopyranose structures and to a 1,2-unsaturated enol ether derivative (glucal).

The crude extracts from hepatopancreas[9] of *A. fasciata* contained different β-glycosyl hydrolases the most significant being the β-galactosidase activity (40% of the total β-D-fuco-β-D-gluco-β-D-galacto- and β-D-mannosidase activities). Lactose, *p*-nitrophenyl β-D-galactopyranoside and *o*-nitrophenyl β-D-galactopyranoside are substrates for the β-galactosidase; the latter

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Table 1. Syntheses conducted with crude extract of the hepatopancreas of the mollusc *Aplysia fasciata* with different acceptors

Donor	Acceptor	Products	EM[a]	Y[a] (%)
2-NP-Gal	D-Xylose	**1**, **2**	10	60
2-NP-Gal	Allyl β-D-xylopyranoside	**3**	0.5	12
2-NP-Gal	Allyl β-D-xylopyranoside	**3**, **4**	5	48
2-NP-Gal	Methyl β-D-xylopyranoside	**5**, **6**	10	75
2-NP-Gal	Benzyl α-D-xylopyranoside	**7**, **8**	5	33
2-NP-Gal	Benzyl β-D-xylopyranoside	**9**, **10**	5	30
2-NP-Gal	4-NP α-D-xylopyranoside	**11**, **12**	10	18
2-NP-Gal	4-NP β-D-xylopyranoside	**13**, **14**, **15**	10	50
4-NP-Gal	Glucal	**16**	5	35
4-NP-Gal	*N*-Acetyl-D-glucosamine	**17**	5	20
2NP-Gal	*N*-Acetyl-D-galactosamine	—	10	—
2NP-Gal	Methyl β-D-galactopyranoside	**18**, **19**	10	38
2NP-Gal	2NP-Gal	**20**, **21**	—	13 + 5
2NP-Gal	D-Galactose	**22**, **23**	10	75
2NP-Gal	**5**	**24**	0.5	3

[a] EM = acceptor/donor molar ratio; Y = yield. 2-NP-Gal = *o*-nitrophenyl β-D-galactopyranoside; 4-NP-Gal = *p*-nitrophenyl β-D-galactopyranoside.

resulted the best one (456 U/mg) while *p*-nitrophenyl β-D-galactopyranoside (156 U/mg) and lactose (3.5 U/mg) have intermediate and poor activity, respectively.

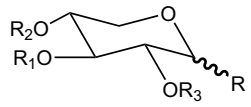
In Table 1 are reported the syntheses[10] conducted by using nitrophenyl β-D-galactopyranosides as donor and different xylose based acceptors. D-Galactopyranose based acceptors were also tested as well as a 1,2-unsaturated enol ether derivative (glucal). All the products (Fig. 1) were characterized as native or acetylated derivatives after chromatographic purification as single products or in mixtures. ^{1}H NMR spectra, DEPT and COSY and ^{1}H–^{13}C NMR correlations generally permit the assignments of chemical shifts of compounds obtained and the unambiguous structure assignment. In the COSY spectrum starting from the aglycon-linked or free sugar anomeric signals and following the correlations through pyranosidic protons it is easy to detect the position of glycosylation for the upfield shift of the signal due to the absence of acetyl group.

When the free sugar D-xylose was the acceptor in a reaction conducted using it in a 10-fold molar excess, good yield (60% with respect to the donor added) of the galactosides **1** (*O*-β-D-galactopyranosyl (1-3)-D-xylose, 60%) and **2** (*O*-β-D-galactopyranosyl (1-4)-D-xylose, 35%) was obtained. The minor β-1-2 isomer was also present in ca. 5%, as established by the integral of anomeric signals in the ^{1}H NMR spectra of acetylated derivatives.[11] The structure of the most abundant regioisomers **1** and **2** were secured by the analysis of diagnostic signals in the ^{13}C NMR spectra of native mixture (D_2O).[11] The yield of this reaction was satisfactorily high if compared to those obtained using other enzymes from different sources[12] in similar conditions. This mixture could be directly suitable for diagnostic purposes without further purification of single regioisomers.[11] As a comparison pure compound **2** was obtained by a chemical approach in 8.8% yield in seven reaction steps.[11]

When the anomeric hydroxyl group of D-xylose was protected, similar results were obtained. In the case of allyl β-D-xylopyranoside a single β-1-3 galactosyl derivative **3** was formed in 12% yield (with respect to the amount of the acceptor used) using the donor in molar excess (2:1). For the structural assignment of **3** diagnostic signals are the anomeric doublets of the xylose (ppm 98.5/4.52, J = 6.5 Hz) and of the galactose (ppm 101.1/4.60, J = 7.9 Hz), and the galactosylated C3/H3 of xylose found at 75.9/3.88 ppm). Two additional disaccharidic products due to the galactosylation of the donor and accounting for 14% yield, were also observed in this reaction. The yield of **3** can be increased to 48% by using a 5-molar excess of the acceptor obtaining in this case a mixture of galactosides in which **3** predominated (70%) with respect to other two regioisomers **4**, β-1-4 (29%) and trace amount (<1%) of the remaining β-1-2 compound.

The yield of reaction with alkyl aglycon could be further increased reaching a value as high as 75% increasing the molar excess of acceptor (methyl β-D-xylopyranoside) to 10-fold. Also in this case the products obtained were in the ratio (β-1-3, **5** and β-1-4, **6**) 73:26 as for allyl group. Unfortunately the overlap of diagnostic signals in the ^{1}H NMR spectrum of the mixture of acetylated derivatives hampered the direct unambiguous signal assignment of regioisomers, thus the structures of **5** and **6** were established by COSY spectra of purified acetylated derivatives. Starting from H1 of xylose at 4.40 ppm, H3 was found at 3.82 ppm (galactosylated position) for compound **5** and starting from H1 of xylose at 4.38 ppm, H4 was found at 3.81 ppm (galactosylated position) for compound **6**. No detectable presence of β-1-2 isomer was noticed.

Using benzyl α-D-xylopyranoside as acceptor a mixture of two galactosylated compounds (3:1) was obtained; the most abundant, **7** was the β-1-4 isomer.[6] The minor product was obtained in enriched form after extensive purification procedure and was characterized as β-1-3 isomer, **8**. The β-anomer of the same acceptor in the same conditions furnished a mixture of products in which the β-1-3 was the predominant isomer **9** (60%) over the β-1-4, **10** (40%).[13] The yields (ca. 33%) in both cases were moderate at 5-molar excess of acceptors.



1 R = OH; R1 = β-Gal; R2 = H; R3 = H
2 R = OH; R1 = H; R2 =β-Gal; R3 = H
3 R = O-β-allyl; R1 = β-Gal; R2 = H; R3 = H
4 R = O-β-allyl; R1 = H; R2 = β-Gal; R3 = H
5 R = O-β-methyl; R1 = β-Gal; R2 = H; R3 = H
6 R = O-β-methyl; R1 = H; R2 = β-Gal; R3 = H
7 R = O-α-benzyl; R1 = H; R2 = β-Gal; R3 = H
8 R = O-α-benzyl; R1 = β-Gal ; R2 = H; R3 = H
9 R = O-β-benzyl; R1 = β-Gal ; R2 = H; R3 = H
10 R = O-β-benzyl; R1 = H; R2 = β-Gal; R3 = H
11 R = O-α-p-nitrophenyl; R1 = H; R2 = β-Gal; R3 = H
12 R = O-α-p-nitrophenyl; R1 = β-Gal ; R2 = H; R3 = H
13 R = O-β-p-nitrophenyl; R1 = β-Gal; R2 = H; R3 = H
14 R = O-β-p-nitrophenyl; R1 = H; R2 = β-Gal; R3 = H
15 R = O-β-p-nitrophenyl; R1 = H; R2 = H; R3 = β-Gal

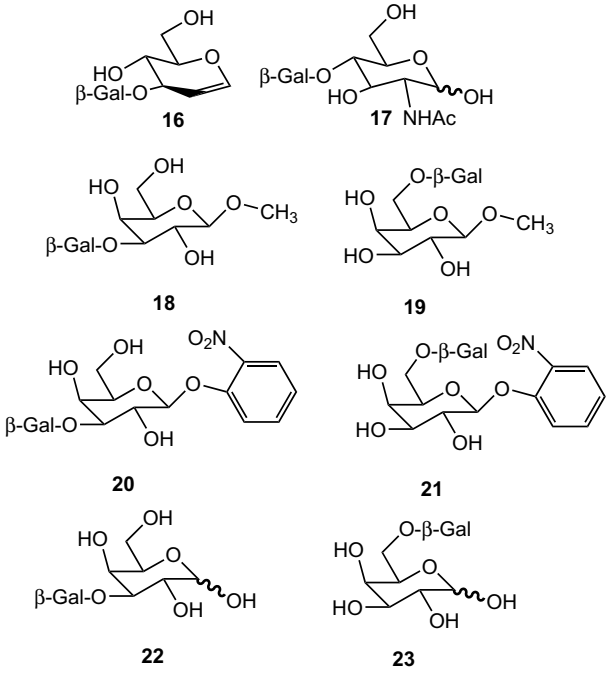


Figure 1. Galactosyl derivatives synthesized by β-galactosidase of *Aplysia fasciata*.

Using as acceptor *p*-nitrophenyl β-D-xylopyranoside (molar excess 10) the yield of reaction was 50% and all the three possible regioisomers were formed with the β-1-3 being predominant over the other two β-1-4 and β-1-2 (2:1:1, respectively). These three purified disaccharides were characterized as acetylated derivatives by COSY experiments. The spectra of **13** (β-1-3) and **14** (β-1-4) regioisomers are in accord to those reported.[13] The spectrum of β-1-2 regioisomer, **15** was characterized by the diagnostic xylose H2 signal at 3.94 ppm (Table 2). Compounds **13**, **14** and **15** after deprotection were used for the analysis of hydrolytic activity of the enzyme (see below). The α-anomer of the acceptor, *p*-nitrophenyl α-D-xylopyranoside, in the same conditions furnished 18% yield of a mixture of products containing the β-1-4 isomer **11** predominating (62%) over the β-1-3 **12** (38%) (Table 2).

Table 2. ^{1}H NMR signals (disaccharidic moiety) for **11**, **12** and **15**

Product	**11**[a]	**12**	**15**
Galactose			
1	4.07	4.61	4.67
2	5.43	5.14	5.20
3	5.05	5.00	4.99
4	5.44	5.35	5.36
5	3.43	3.87	3.88
6	4.14–4.02	4.06–4.01	4.02
Xylose			
1	5.53	5.65	5.52
2	5.12	5.61	**3.94**
3	5.94	**3.89**	5.14
4	**3.74**	5.12	4.90
5	3.60	3.85–3.63	4.14–3.64

In bold are the signals of galactosylated positions.
[a] In C_6D_6.

The anomeric control of regioselectivity observed with the latter two aryl acceptors is a well-known phenomenon reported for these enzymes.[14]

The predominant β-1-4 product obtained with the α-anomers was also previously detected[6] in the galactosylation of *N*-acetyl glucosamine **17** with a moderate 20% yield while the *N*-acetyl-D-galactosamine was not substrate at all even using it in 10-fold molar excess (Table 1). These results, compared with the clear capability of the enzyme for the transfer of galactose to methyl β-galactopyranoside and to *o*-nitrophenyl β-D-galactopyranoside itself, allow us to conclude that the *N*-acetyl group, more than the 4 axial hydroxyl group of *N*-acetyl-D-galactosamine, is responsible for the nonproductive orientation of this acceptor into the enzyme active site and for a different orientation of *N*-acetyl-D-glucosamine, which produce in fact the β-1-4 regioisomer.

Methyl β-D-galactopyranoside as acceptor furnished moderate–high yield of two products **18** and **19** (β-1-3:β-1-6, 81:19, respectively); the reaction with the aryl version of this galactose acceptor, *o*-nitrophenyl β-D-galactopyranoside, was less efficient both in terms of yield of **20** and **21** (13%) and selectivity: **20** β-1-3 (62%), **21** β-1-6 (38%). In the ^{1}H NMR and COSY spectra of acetylated derivatives of compounds **18** and **19** the signals of protons at galactosylated positions correlated with proper anomeric signals: **18** (3.83 ppm/4.29 ppm $J_{1,2}$ = 7.99 Hz), **19** (3.85–3.75 ppm/4.37 ppm, $J_{1,2}$ = 7.95 Hz). NMR data for compounds **20** and **21** are in accord to those reported.[15]

Reaction with free D-galactose furnished higher yield (75%). Three regioisomers were present in the purified disaccharidic fraction as established by ^{13}C and DEPT experiments; the β-1-3 and the β-1-6 regioisomers are in 1:1 ratio; a minor unidentified disaccharide, comigrating with the former, is also detectable.

Finally glucal can also be used as acceptor furnishing easy access to the β-1-3 isomer **16** (90% selectivity, 35% yield), which is synthetically interesting for the

great potential carried out by glucal moiety in chemical transformation reactions.[16]

Unfortunately modest yields of a mixture of trisaccharidic derivatives was obtained in the two cases reported in Table 1, namely the autocondensation of *o*-nitrophenyl β-D-galactopyranoside (5%) and the galactosylation of **5** (3%). The ESI-MS spectra (Q-Tof mass spectrometer) of these compounds after purification and acetylation account for their trisaccharidic nature; no further efforts were made for their structural characterization.

Although all these reactions were performed with crude extract of the hepatopancreas of *A. fasciata* the overwhelming presence of β-galactosidase activity, the regularity of the overall results and the observation of the known anomeric control of regioselectivity let us to consider some conclusions about yields and selectivity.

The results reported in Table 1 indicate a clear preference of the *Aplysia* enzyme for the galactosylation of polar acceptors. Owing to the specificity of acceptor site of most galactosidases for compounds with phenyl groups,[13] the yields obtained in the reactions using free or methyl derivative of xylose and methyl β-galactopyranoside and D-galactose, are interestingly high. In fact, for example, the enzyme from *A. oryzae* was reported to have a very low affinity for these polar acceptors thus resulting in low yield using the same acceptor excesses[17] and the *E. coli* β-galactosidase catalyzed the synthesis of **6** in 33% yield using, as in our case, 10-fold molar excess of methyl β-D-xylopyranoside.[13] Moreover no product formation was observed using β-galactosidase from bovine testes and a polar acceptor such as 2-deoxy-D-galactopyranose.[18]

Another interesting characteristic of this enzyme is the uncommon β-1-3 selectivity in the transgalactosylation reactions with most of the acceptors. Using free xylose or its β-allyl and methyl derivative the β-1-3 isomer was always selectively formed as in the case of methyl β-D-galactopyranoside and glucal. With β-aryl linked aglycons for both xylose and galactose this β-1-3 selectivity is again expressed although it is lost with α-anomers. However the influence of aryl groups as aglycones is not limited to the yield of reaction but also to the regioselectivity as shown comparing the results of the reactions using the *p*-nitrophenyl and benzyl xylopyranosides.

The easy enzymatic synthesis and purification of all galactosides of *p*-nitrophenyl β-D-xylopyranoside above reported (compounds **13**, **14** and **15**) prompted us to perform kinetic analysis in the hydrolytic conditions for these products. The hydrolysis reactions[19] formed *p*-nitrophenyl β-D-xylopyranoside and the rate of hydrolysis was compared for each isomer. The ratio of the hydrolysis rates $k_{1\text{-}2}/k_{1\text{-}4}$ resulted 4.1 while $k_{1\text{-}2}/k_{1\text{-}3}$ resulted 1.9; as a matter of fact after 3 h the β-1-2 isomer was hydrolyzed at an extent of ca. 60% while only 30% and 12% of β-1-3 and β-1-4 isomers were consumed, respectively, in the same conditions. These results could be of a certain interest from a physiological point of view since galactose was found to be β-1-2 linked to xylose (xyloglucan) and the enzymatic breakdown of ingested carbohydrate polymers has been shown to be very important in this context, in molluscs.[20] The β-1-2 specificity of this β-galactosidase in the hydrolysis reaction is also of interest in the field of characterization of large carbohydrate based polymer structures; a number of commercially available β-galactosidases tested for β-1-2 hydrolysis was in fact shown to be unsuccessful on this interglycosidic linkage.[21] The β-1-3 selectivity in the transgalactosylation reactions makes this enzyme very attractive as alternative catalyst in the synthesis of novel food components as also found for bovine testes β-galactosidase.[22]

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9. Twenty animals were carefully dissected to obtain 52 g of hepatopancreas, which were homogenized in ca. 2.5–3 vol/w of acetate buffer 50 mM pH 5; clear protein solution (170 mL 1.19 mg/mL) was obtained after centrifugation. After dialysis and ultrafiltration the crude extract contained a final concentration of 6 mg/mL of total protein. Activities were measured using nitrophenyl glycosides substrates as described in Ref. 6 or lactose. One unit corresponds to the amount of enzyme hydrolyzing one nanomol of substrate in 1 min/mg of total proteins.
10. The enzymatic syntheses were performed using 0.080–0.30 mmol of the donor dissolved with the molar excesses of acceptors as indicated (Table 1) in 1–10 mL of acetate buffer 50 mM pH 5.6. 3000 U/mmol of donor of β-galactosidase activity of the crude hepatopancreas extract were used as biocatalyst; the reactions were started in sealed vials incubating the mixtures under agitation at 30 °C up to total donor consumption (1–5 h; TLC monitoring $CHCl_3$/MeOH/H_2O 65:25:4 by vol or EtOAc/MeOH/

H_2O 70:20:10 by vol). The reactions were stopped by heating the mixtures at 90–100 °C for 5 min and the products purified by different procedures (reverse phase (RP18), water/methanol gradients; silica gel chromatography, $CHCl_3$/MeOH or EtOAc/MeOH gradients; Biogel P2, water; preparative TLC, EtOAc/MeOH/H_2O 70:20:10 by vol). The products were detected in TLC by α-naphthol reagent. Acetylation was performed overnight in Ac_2O/Pyr 1:2 at room temperature. NMR studies of acetylated derivatives were carried out using Bruker instruments 300 or 400 MHz in $CDCl_3$ or C_6D_6; D_2O was used for underivatized compounds.

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19. The hydrolysis reaction was conducted solubilizing the disaccharides (ca. 2.5 mM) in acetate buffer (1 mL) 50 mM pH 5.6 and adding equal amount of the enzyme (9000 U/mmol) for each compound. The *p*-nitrophenyl β-D-xylopyranoside liberated by the enzymatic hydrolysis was detected and quantitatively analyzed by HPLC (μBondapak-NH_2, acetonitrile/water 8:2, 1 mL/min, UV detection). Preliminary experiments with the extract secured that *p*-nitrophenyl β-D-xylopyranoside was not substrate for any enzymatic activity.
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