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Glycoside hydrolases in *Aplysia fasciata*: analysis and applications

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Abstract

Glycosylation is considered to be an important reaction for the chemical modification of compounds with useful biological activities. Glycoside hydrolases are biotechnologically attractive enzymes which can be used in synthetic reactions for assembling glycosidic linkages with absolute stereoselectivity at an anomeric centre. Most of these enzymes are commercially available but there is great interest in the search for new biocatalysts with original catalytic characteristics. The marine environment has shown to be a very interesting source for new glycosyl hydrolases for both hydrolytic and synthetic aspects. In particular, *Aplysia fasciata* a marine herbivorous mollusc has been shown to be a potent producer of a library of glycoside hydrolases applied to the synthesis of glycosidic bonds. The impressive assortment of glycosidases in marine organisms clearly indicates that the potential biodiversity of these enzymes is still largely unexplored and that potential applications of biocatalysts from the sea will increase in the near future.

Introduction

Glycoside hydrolases (glycosidases) in the cells are responsible for the cleavage of glycosidic linkages, largely involved in nutrient acquisition. Exo-glycosidases can be also involved in glycan processing during *in vivo* glycoprotein synthesis.

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Abbreviations: COSY (Correlated Spectroscopy, 2D NMR), TOCSY (Total Correlation Spectroscopy), DEPT (Distortionless Enhancement by Polarization Transfer), pNP- (p-nitrophenyl), DMSO (dimethylsulfoxide), DMF (dimethylformamide), AZT (azidothymidine), HPAEC-PAD (high-pH anion-exchange chromatography with pulsed amperometric detection).

Alternatively different enzymes, called glycosyltransferases, are responsible *in vivo* for the synthesis of most cell-surface glycoconjugates (Davies and Henrissat 1995). Glycoside hydrolases are biotechnologically attractive enzymes which can be used in synthetic reactions for assembling a variety of glycosidic linkages with absolute stereoselectivity at anomeric centre (Trincone and Giordano 2006).

Speaking in general terms, glycosylation is considered to be an important method for the structural modification of compounds with useful biological activities. Glycosylation allows conversion of lipophilic compounds into hydrophilic ones, thus improving their pharmacokinetic properties and more effective drug delivery systems (prodrugs) can be obtained (Kren and Thiem 1997, Nikolakakis *et al*. 2003). Synthetic carbohydrate-based polymers have been used as coating agents and molecular recognition biomedical materials (Wang *et al*. 2002). Novel dietary carbohydrates have been introduced as food additives particularly in some European countries and Japan. Among these there are non-digestible oligosaccharides like inulin or sucrose-derived fructo-oligosaccharides, soy-derived galactosyl-sucroses and galacto-oligosaccharides derived from lactose, xylo-oligosaccharides and lactulose, which are increasingly being added to foods, particularly in some European countries and Japan (Voragen 1998). Finally we have to mention the synthesis of chromophoric oligosaccharides (such as nitrophenyl and 4-methylumbelliferyl glycosides and others) which are of widespread interest for the kinetic analysis of hydrolytic activities and to characterize the mode of action of particular enzymes (i.e., exo- or endo-glycosidases). Hence these compounds have proved to be a valuable tool in different fields of applicative interest such as clinical, biological and food chemistry (Borriss *et al*. 2003).

Most of the enzymes commonly used for all these purposes are commercially available but great interest rely upon the search for new enzymes with new catalytic characteristics. The glycosidase activities present in several dozen of enzymatic preparations have been reviewed (Scigelova *et al*. 1999). In addition, the marine environment has proven to be a very interesting source for new glycosyl hydrolases both for hydrolytic and synthetic applications (Westerop, 2003; Giordano *et al*. 2006).

Aplysia is a genus of sea hares belonging to the family *Aplysiidae*, containing different species of organisms. A*plysia fasciata* Poiret, 1789 which is is one of them, is very common in Mediterranean habitat. The dark brown body of this mollusc can grow up to 40 cm long, sometimes some whitish blotches are present. The parapodial lobes are well separated. The *Aplysiidae* are herbivorous, eating a variety of red, green or brown algae. They have been revealed to be a potent producer of a library of glycoside hydrolases applied in the synthesis of glycosidic bond (Giordano *et al*. 2004).

In this review a survey of glycoside hydrolases detected in *Aplysia fasciata* will be compiled focusing the attention on the characteristic of the enzymes from a synthetic point of view (donors, specificity, conversions) and on the products formed (acceptors, yields, regioselectivity). Analysis will be centered on (i) a β-galactosidase showing β-1,3 selectivity, presenting results for transgalactosylation of polar acceptors and antiviral nucleosides, (ii) an α-glucosidase with remarkable characteristics in synthesis of chromophoric di- and tri-saccharides and α-glucosides of maltose, sucrose and cellobiose and other interesting compounds in the food/pharmaceutical domain and (iii) a β-mannosidase with exo-acting action and transmannosylation capability.

Biochemical methodologies in the synthesis of oligosaccharides

Enzymatic strategies for high-yield and stereospecific construction of glycosidic bonds are based on the action of glycoside hydrolases (endo- and exo-glycosidases) and glycosyltransferases; different enzymes (aldolases, lipases, proteases, etc.) are also in use for the selective modification of the oligosaccharidic scaffold.

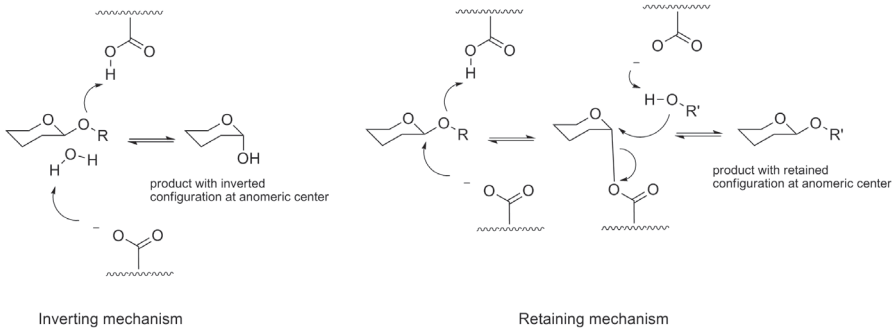


Figure 1. Inverting and retaining mechanisms of glycoside hydrolases. Inverting: the hydrolysis occurs via a double displacement mechanism which involves an oxocarbenium ion-like transition state: the product has an inverted anomeric configuration with respect to the substrate. Retaining: The reaction proceeds by a double-displacement mechanism in which a covalent glycosyl-enzyme intermediate is formed (glycosylation step) and then hydrolysed (deglycosylation step) in a general acid/base-catalysed process. Retaining glycosidases can transglycosylate by transferring the glycone of the glycosyl enzyme to an acceptor rather than to water (Crout and Vic 1998).

Glycoside hydrolases belong to inverting or retaining class according to their mechanism of action (*Figure 1*). As far as the utilization of glycoside hydrolases in the synthesis of glycosidic linkage is concerned, two general protocols are in use: the reverse hydrolysis procedure and the kinetic approach. The former, starting with free monosaccharide and an acceptor, is reported as an efficient and cost-effective methodology just for few enzymes and as a poor-yielding procedure for others (Crout and Vic 1998). The alternative kinetic approach is based on the use of a glycosyl donor which produces an intermediate glycosyl enzyme (*Figure 1*, retaining mechanism). In presence of a nucleophile other than water the ester is resolved in the products which are new glycosides and could be still substrates for the enzyme, hence the reaction must be carefully monitored to achieve good yield. In this context the use of *ad hoc* produced mutant enzymes, known as glycosynthases, has been introduced several years ago as an elegant approach to solve back-hydrolysis problems encountered using wild types biocatalysts. This topic has been extensively reviewed and reports of this technology (Perugino *et al*. 2004, Hanckock *et al*. 2006) were published. Synthesis of oligosaccharides by the glycosynthase enzymes, which do not hydrolyse the products and use inexpensive sugars, is an emerging alternative for a possible scale-up of oligosaccharide production.

The general stereochemical outcome of a glycosidase-based reaction is dependent on the nature of the enzyme, including its substrate preference towards the donor(s) and product(s) formed. Depending on the structure of active site, the biocatalyst can

accommodate certain sugar acceptor(s) and promote regiospecific formation of α- or β-glycosidic linkages. The impressive variety of available glycosidases clearly indicates that both the potential biodiversity of these enzymes (and of the derived glycosynthases) is still largely unexplored and that potential applications of these biocatalysts will increase in the near future starting from the knowledge of wild-type.

Marine ecosystem as a source for new glycoside hydrolases

Marine environment is by far an unexplored source of new enzymatic activitities and a detailed knowledge of marine biology and biochemistry together with genetic analysis will be fundamental for the exploitation of this source. In fact, the availability of genomes data will help to reveal novel and interesting glycoside hydrolases for scientific and practical applications. At the moment, the DNA characterization of only a few glycoside hydrolases from eukaryotic marine organisms (not included in genomic projects) are available: a β-1,3-glucanase from eggs of the sea urchin *Strongylocentrotus purpuratus* (Bachman and McClay 1996), an amylase from *Pecten maximus* (Le Moine *et al*. 1997), and an endo-β-1,4-glucanase from the blue mussel *Mytilus edulis* (Xu *et al*., 2001). In addition, genome projects (complete and in progress) concerning eukaryotic marine organisms represent only 10% of the total eukaryotic projects (about 400 according to the NCBI Genome database). On the contrary, microorganisms have been widely studied and the genome projects (complete and in progress) concerning marine bacteria and archaea from sea extreme environments are 800 ca. (NCBI Genome database).

The perusal of the literature led us to consider different facets for marine glycoside hydrolases. Scientific attention has been focused on biocatalysts from marine bacterial extremophiles because of their stability, the novelty of their catalytic qualities and the possible effortless expression of these proteins in an appropriate organism hosts (Tramice *et al*. 2007). Additionally, glycosynthase production starting from an endo-1,3-β-glucanase isolated from the thermophile *Pyrococcus furiosus* is also reported (van Lieshout *et al*. 2004) even though, as a glycosynthase, it showed poor performance in yield. An increasing exploitation of marine glycoside hydrolases, as resulting from our analysis, is in the expanding field of glycobiology. Among the examples reported, the marine α-galactosidase from *Pseudoalteromonas* (Bakunina *et al*. 1998), capable of removing the immunodeterminant sugar residues, and a sialidase from a *Pseudomonas* sp. used for the preparation of GM1 from a mixture of polysialogangliosides (Fukano and Ito 1997), are two exciting cases. Marine glycoside hydrolases have been applied also to the synthesis of glycosidic bonds although transglycosylation potential has been hardly studied for these enzymes. A factual account has been compiled (Andreotti *et al*. 2006) showing that only few type of enzymes have been investigated for synthesis (galacto- gluco- manno- and xylanases) and only few organisms analyzed. *Aplysia fasciata* is a mollusc which has revealed to be a potent producer of a library of glycoside hydrolases applied in the synthesis of glycosidic bond (Giordano *et al*. 2004). α-L-oligofucosides and α-D-oligoglucosides formed by the α-L-fucosidase extracted from digestive gland of the marine mollusc *Pecten maximus* (Berteau *et al*. 2002) and by α-D-glucosidases from *Aplysia fasciata* (Andreotti *et al*. 2006) and *Geobacillus* (Hung *et al*. 2005) are fascinating examples of marine enzymes for which a potent

transglycosylation activity has been observed. The production of oligoglucosides (up to tetra- and pentasaccharides) has been observed even in the presence of a high concentration of mono- and disaccharide acceptors. For *Geobacillus* enzyme it has been reported the preferential hydrolysis of α-1,4-glycosidic linkages of oligosaccharides in an exo-type manner. The analysis of multiproduct patterns of transglycosylation reactions with this enzyme shows an interesting characteristic: the first products of monoglucosylation would later serve as - or become a better - sugar acceptor.

Glycoside hydrolases present in *Aplysia fasciata*

Aplysia is a genus of sea hares belonging to the family *Aplysiidae* to which A*plysia fasciata* Poiret, 1789 belongs; this mollusc is very common in Mediterranean habitats, is herbivorous eating a variety of red, green or brown algae (Susswein 1984).

In searching for new marine glycoside hydrolases we focused our attention on three main parts of the animal: (i) the mantle and other external parts including oral tentacles, rhinophores, eyes, foot and parapodia, (ii) the hepatopancreas and (iii) other visceral mass including digestive, excretory, blood-vascular and reproductive system. Many glycosyde hydrolase activities were assayed by using chromophoric substrates of different sugars (fucose, galactose, glucose, mannose, N-acetyl glucosamine and xylose), obtaining interesting results. The extracts from hepatopancreas and from visceral mass resulted rich in glycoside hydrolases; their activities were in the range found in other marine molluscs (Kusaiykin *et al*, 2003). By contrast, the extract of external part did not hydrolyze any of the substrates tested. Interestingly the β-D-galactosidase and the α-D-glucosidase enzymes from hepatopancreas and visceral mass, respectively were present in large amount. An interesting β-mannosidase was also noticed. In the following paragraphs we present the results obtained in the isolation and applications of these biocatalysts with emphasis on products (reaction conditions, yields, regioselectivity).

α-D-GLUCOSIDASE

The ability of α-glucosidases to perform transglycosylation reactions is a relevant issue from the biotechnological point of view (in food industry, for production of glycoconjugates, etc.). Bacterial, fungal, animal and vegetal originating α-glucosidases were used to perform enzymatic synthesis of different α-glucosides of various molecular structures, from simple alkyl glucosides to different complex oligosaccharides used in industrial applications (Trincone and Giordano, 2006).

The α-glucosidase activity was the most abundant glycosyl hydrolase activity found in the visceral mass homogenate of *A. fasciata*. The purified enzyme gave only one band of 69 kDa on SDS-PAGE, while the molecular mass of the native enzyme was 255 kDa**,** indicating that this protein is a homotetramer as established by gel filtration on a Superdex-200 column. It showed a pH optimum at 5.8 and the optimum temperature at this pH was in the range 36–44 °C; pH 5.8 and 34 °C were identified as the best conditions for performing hydrolytic and transglycosylation experiments (Andreotti *et al*. 2006b). Solvent resistance of this α-glucohydrolytic activity was also studied in crude homogenate in the presence of 10% organic solvents. Good resistance of the

enzyme to DMSO (80% residual activity after 24 h), high sensitivity to acetonitrile and DMF (total loss of activity after few minutes) and an intermediate resistance to acetone (50% residual activity after 24 h) were recorded. These data are interesting in view of the low solubility of aryl substrates which can be used as donors in trans-glycosylation reactions and for the use of hydrophobic acceptors.

Starch, amylopectin, amylose, isomaltose, panose, pullulan and saccharose were not hydrolysed by this enzyme although a feeble reaction using trehalose was observed. Morevoer no enzymatic hydration of glucal (with formation of 2-deoxyglucose) was detectable. Nitrophenyl glucoside (pNP-α-D-Glc) and the α-maltoside (pNP-α-D-Glc-(1-4)-α-D-Glc) were highly hydrolysed while the β-form (pNP-β-D-Glc-(1-4)-α-D-Glc) was hydrolysed to a lesser extent.

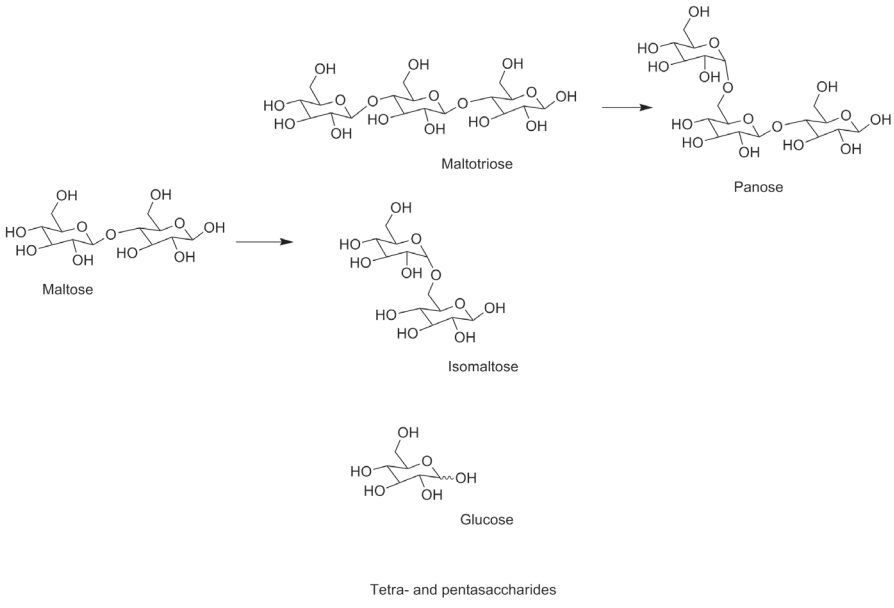


Figure 2. Bioconversion of maltose. Bioconversion of maltose performed by the pure enzyme produced the trisaccharide panose and the disaccharide isomaltose together with a smaller amount of tetra- and pentasaccharides. The time course for the bioconversion of maltose was studied using 93 mM maltose and 18 μg of pure protein per ml. Aliquots were withdrawn at different time intervals and analyzed by HPAEC-PAD using a CarboPacTM PA1 analytical column (Dionex). In these conditions, within the first hour of reaction ca. 60% of maltose was consumed forming maltotriose and panose; the concentration of maltotriose decreased to a very low value after 360 min as well as the concentration of the regioisomer panose increased to a plateau (after ca. 180 min), reaching a value of ca. 8 g/l in the reaction mixture. The disaccharide was identified as isomaltose (6-O-α-D-glucopyranosyl-d-glucose) by comparison of ^{1}H and ^{13}C NMR spectra of the acetylated derivative with an authentic standard sample. COSY, TOCSY and ^{1}H–^{13}C NMR correlation spectra of the acetylated derivative of the trisaccharide showed unambiguously the panose structure; tetra- and pentasaccharidic nature of the remaining products was established both by their Rfs in and by mass spectroscopy (ESI-MS positive ions) of acetylated derivatives.

The α-glucosidase from *A. fasciata* is very active on the α-1-4 glucosidic linkage as it was assessed by measuring the kinetic parameters for the hydrolysis of maltose (V_{max} 115 U/mg, K_M 5.70 mM, k_{cat} 489 sec^{-1}), pNP-α-D-Glc (k_M 0.26 mM, k_{cat} 163 sec^{-1}) and pNP-β-D-Glc-(1-4)-α-D-Glc (k_M 2.06 mM, k_{cat} 75.9 sec^{-1}).

The α-1-6 glucosidic linkage is only poorly hydrolysed as it was noticed that the specific activity for maltose was 40 times higher than that for isomaltose (V_{max} 2.9 U/mg by using 22 mM substrate). Moreover, according to the Michaelis constants for pNP-α-D-Glc and maltose, it could be suggested that the subsite + 1 of this enzyme has an affinity for the aryl group higher than for a glucosyl residue. Although the k_{cat} value for maltose was three times higher than that for pNP-α-D-Glc, the catalytic efficiency showed that *in vitro* the former is the preferred substrate for this enzyme (k_{cat}/k_M = 627 mM^{-1} s^{-1}). A still good catalytic efficiency was observed in the hydrolysis of pNP-β-D-Glc-(1-4)-α-Glc (k_{cat}/K_M 36.8 mM^{-1} s^{-1}), while no hydrolytic activity was detected when the pure enzyme was incubated in the presence of pNP-β-D-Gal or pNP-β-D-Man. This substrate specificity, together with the fact that this enzyme is not able to hydrate glucal, suggested that the enzyme from *Aplysia fasciata* may belong to family I type of α-glucosidases (Kimura, 2000) corresponding to family 13 of glycoside hydrolases GH (Kimura *et al*. 2004), however sequence similarity studies are necessary to establish it.

Maltose was a good substrate for this α-glucosidase, its bioconversion led to the formation of several products as shown in *Figure* 2, the most abundant being the trisaccharide panose and the disaccharide isomaltose whose structures were determined by NMR spectroscopy. Maltotriose was also a good substrate with a pattern similar to that observed using maltose: maltotetra- up to maltohexaose were found in the early stages of reaction but panose and isomaltose were later identified as end products also in this reaction. Using maltoheptaose the enzyme performed a reaction which is in agreement to the reaction of maltose.

Transglycosylation reactions using the aryl substrate pNP-α-D-Glc as donor are listed in *Table 1*; those conducted using maltose donor are listed in *Table 2*. Using both pNP-α-D-Glc or maltose different acceptors can be transglycosylated as reported: different aryl glycosides, cellobiose, saccharose, trehalose, isomaltose, glycerol, melibiose, pyridoxine and naringin.

When maltose was the donor and pNP-β-D-Glc was the acceptor, pNP-β-D-Glc-(1-4)-α-D-Glc was the sole product in the early stages of reaction (30 min), then isomaltoside derivative, pNP-β-D-Glc-(1-6)-α-D-Glc, was also formed and it accumulated up to the end when it became the most abundant isomer with a yield of ca. 15–20%. Using p-nitrophenyl β-cellobioside (pNP-β-D-Glc-(1-4)-β-D-Glc), the formation of a trisaccharidic product was observed while both α- and β-anomers of p-nitrophenyl galactopyranoside were not glycosylated in significative amount (*Table 2*). These results along with those obtained using chromophoric derivatives as donors (*Table 1*), suggest an intramolecular arrangement from maltotriose for the production of panose from maltose, instead of the alternative intermolecular direct α-1-6 glucosylation of maltose itself.

These findings, indicating the preferential enzymatic formation of α-1-4 linkages in the early stages of reaction and the accumulation of α-1-6 products, were confirmed by time course experiments (Andreotti *et al*. 2006b). Furthermore, as it is indicated by the absence of higher MW products in the maltoheptaose reaction, a possible molecular limit in the acceptor site of the enzyme is conceivable.

The reactions with cellobiose, saccharose, pyridoxine, naringin and 9-fluorenone derivatives have been studied in detail (*Table 2*). α-Glucosyl cellobioses have been recently produced from sucrose donor and cellobiose acceptor by alternansucrase

Table 1. Transglycosylation reaction performed by α-D-glucosidase of Aplysia fasciata.

Substrates		Reactivity	Notes
Donor	Acceptor		
PNP-α-D-Glc	PNP-α-D-Glc	+	Interconversion of regioisomeric disaccharides formed and their hydrolysis
PNP-ß-D-Glc-(1-4)-α-D-Glc	PNP-ß-D-Glc-(1-4)-α-D-Glc	+	Trisaccharide formation and hydrolysis to PNP-ß-D-Glc
PNP-α-D-Glc	D-Glc-(1-4)-ß-D-Glc, cellobiose	+	D-Glc-(1-4)-α-D-Glc-(1-4)-ß-D-Glc
PNP-α-D-Glc	α-D-Glc-(1-2)-ß-Fru	+	Erlose
PNP-α-D-Glc	α-D-Glc-(1-1)-α-D-Glc, threalose	+/-	Formation of trisaccharide(s)
PNP-α-D-Glc	D-Glc-(1-6)-α-D-Glc, isomaltose	+	Formation of trisaccharide(s)
PNP-α-D-Glc	α-cyclodextrin	-	
PNP-α-D-Glc	Ascorbic acid	-	
PNP-α-D-Glc	L-menthol	-	
PNP-α-D-Glc	Glycerol	+/-	Glucosyl glycerol
PNP-α-D-Glc	D-Gal-α-(1-6)-Glc, melibiose	+/-	Formation of trisaccharide(s)
PNP-α-D-Glc	D-Gal-ß-(1-4)-Glc, lactose	-	
PNP-α-D-Glc	Phenyl thiocellobioside	-	

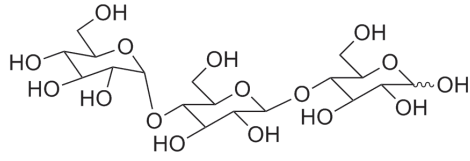
Table 2. Transglycosylation reactions performed by α-D-glucosidase of Aplysia fasciata using maltose donor and different acceptors.

Substrates		Reactivity	Notes
Donor	Acceptor		
Maltose	PNP-ß-D-Glc	+	PNP-ß-D-Glc-(1-4)-α-D-Glc then PNP-ß-D-Glc-(1-6)-α-D-Glc
Maltose	PNP-ß-D-Glc-(1-4)-ß-D-Glc	+	Formation of chromophoric trisaccharide
Maltose	PNP-ß-D-Gal	-	Trace amount of PNP-disaccharide
Maltose	PNP-α-D-Gal	-	Trace amount of PNP-disaccharide
Maltose	Pyridoxine	+	Formation of mono- and diglucosyl pyridoxine
Maltose	Naringin	+	Formation of mono- and diglucosyl naringin
Maltose	9-fluorenonecarboxyhydroxyesters	+	Formation of mono-, di- and triglucosyl derivatives
Maltose	9-fluorenone carboxyhydroxamides	+	Formation of mono-, di- and triglucosyl derivatives

(Morales *et al*. 2001) while glucosyl sucroses were also synthesised enzymatically by α-glucosidase from spinach (Sugimoto *et al*. 2003) and other enzymes.

By using the α-glucosidase from *A. fasciata* trisaccharides were obtained both with cellobiose and sucrose (*Figure* 3) (ca. 25% yield, ca. 4 g/1). The most abundant (ca. 90%) product (1, *Figure* 3) obtained from cellobiose was recognized as the α-1-4 glucosyl derivative by NMR spectroscopy. The sucrose was also α-glucosylated at position 4 of glucose unit forming erlose (4G-α-D-glucosyl sucrose, 2, *Figure* 3).

Glucosides of pyridoxine are more stable against light and heat than the aglycone (Kawai *et al*. 1971); these compounds and other derivatives are important molecules not only from the nutritional but also from the pharmaceutical perspective (Pham *et al*. 2003).



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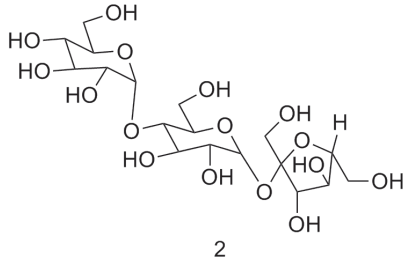

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Figure 3. Trisaccharides formed by transglycosylation using the α-glucosidase from *Aplysia fasciata.* The most abundant (ca. 90%) product, 1 from cellobiose was recognized as the α-1-4 glucosyl derivative, however other minor isomers were also present. The structure of 1 was assigned by (i) negative comparison with previously reported NMR data for α-1-2 and α-1-6 isomers and DEPT experiments (Morales *et al*. 2001), (ii) ruling out α-1-3 linkages from the absence of signals highly shifted (downfield O-glycosylation α-shift, >80 ppm for α-1-3 linkage) in ^{13}C NMRspectra and (iii) direct proof by two-dimensional NMR spectroscopy study of an acetylated derivative. Sucrose was also α-glucosylated at position 4 of glucose unit forming compound 2, erlose (4G-α-D-glucosyl sucrose) as could easily be established by comparison of the ^{13}C NMR signals (in D_2O) of our product with those reported for the erlose enzymatically prepared by using cyclodextrin glucosyl transferase (Martin *et al*. 2004). In the ^{1}H NMR spectrum it was easy to follow ^{1}H –^{1}H correlations of the pyranose ring starting from the anomeric signal of the glucose attached to fructose (HI 5.57/89.8) and showing the erlose structure by the presence of an H-4 signal at 3.98 ppm which was the most abundant. The mass spectra are in agreement with a trisaccharidic structure (989.4, $M^+ + Na^+$).

Glucosylation of pyridoxine by purified α-glucosidase from *A. fasciata* gave the products shown in *Figure* 4. The two monoglucosides 1 and 2, are synthesized in different proportion being 1 the most abundant (75%). The disaccharide mixture is almost totally (95%) composed by compound 3 (*Figure* 4). There is no doubt that the regioselectivity in the transglycosylation process is a characteristic of the marine enzyme and not due to the presence of other catalysts with different selectivity, an issue arising when using intact cells (Asano and Wada, 2003) or an heterogeneous protein

solution for transglycosylation. It has been also found that the observed selectivity is entirely expressed only during the two transglycosylation events and it is not due to a possible differential hydrolysis of regioisomeric products (Tramice *et al* 2006).

The enzymatic modification of naringin and other related compounds in citrus is of current biotechnological interest for food and pharmaceutical industries both for efficient and food-compatible reduction of bitter taste of juice and for modification of pharmacological activities of the molecule. Debittering grapefruit juices has been obtained by different techniques including transglycosylation reaction (Lee *et al*. 1999; Akiyama *et al*. 2000).

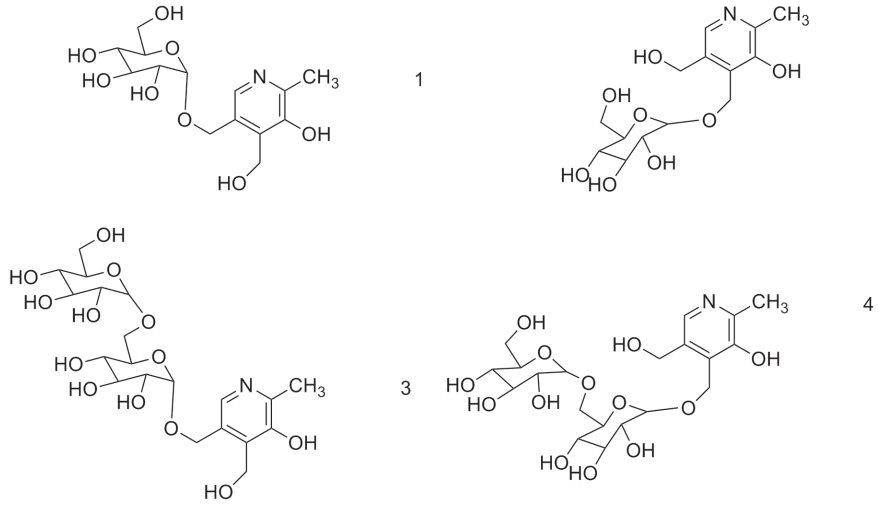
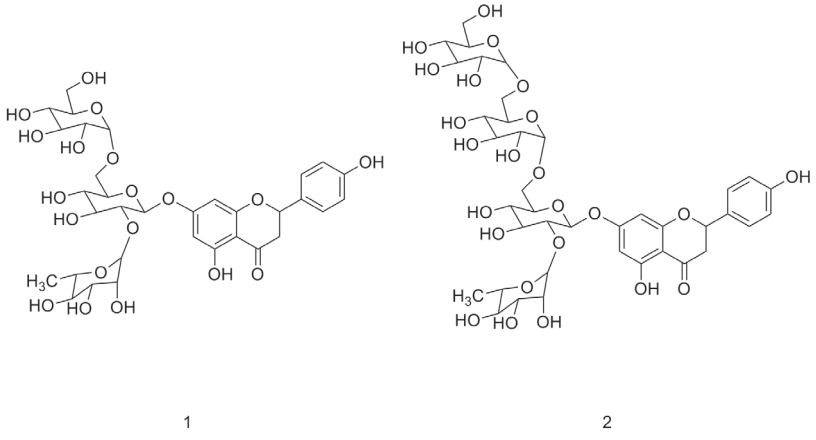


Figure 4. Pyridoxine glucosides obtained by transglycosylation using the α-glucosidase from *Aplysia fasciata.* The reaction was performed by adding pyridoxine in portions (keeping its concentration at 7.8 mg/ml as the highest level) to a 1 M maltose solution containing 15.5 μg enzyme/mmol of maltose, it was possible to add four aliquots of pyridoxine (during 55 h total reaction time) that were well consumed (81% conversion), forming monoglucosidic 1 and 2 (molar yield 40%, 72 mM, 24 g/l) and diglucosidic 3 and 4 (molar yield 41%, 71 mM, 35 g/l) and also traces of triglucosidic derivatives of pyridoxine.

Glucosylation of naringin by marine α-D-glucosidase from *Aplysia fasciata* is a very efficient process in terms of regioselectivity and yield of reaction. Only one out of eight possible mono-glucosylated derivatives have been isolated (1, *Figure* 5).

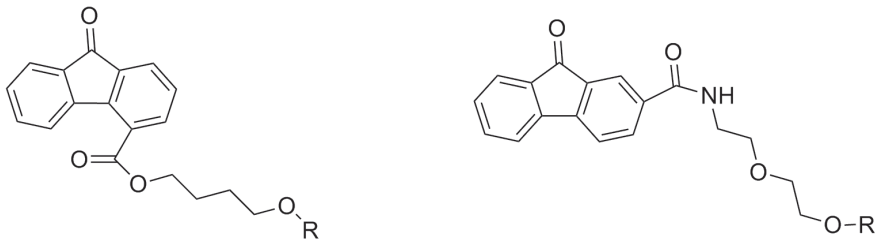
Further functionalization take place on the new α-glucose moiety just added, forming only isomaltosyl derivative 2 (*Figure* 5) of naringin which represents the end-product of glucosylation; it is in fact hydrolyzed very slowly in comparison to monoglucoside 1 (*Figure* 5). This glucosylation reaction can be conveniently conducted in a wide range of naringin content and almost complete conversion of naringin can be obtained. Remarkably, most of natural naringin naturally occurring in fresh grapefruit juice can be directly modified forming the two products shown in *Figure* 5 (Tramice *et al*. 2008).



1 2

Figure 5. Products formed by naringin glycosylation using α-glucosidase from *Aplysia fasciata*

In the framework of a research on the antiviral and immunomodulatory activity of tilorone congeners, two new series of compounds were prepared and pharmacologically explored: 9-fluorenone carboxyhydroxyesters and 9-fluorenone carboxyhydroxamides. Two representative members of these compounds, shown in *Figure* 6, were used as sugar acceptors in the transglycosylation reactions performed by α-glucosidase extracted from the marine mollusc *Aplysia fasciata*. The α-glucosidase provided several α-O-glucosides of 4-carboxyhydroxyester AG17 and 2-carboxyhydroxamide MG3 (*Figure* 6). The easy biocatalytic access to these derivatives is very interesting for the quick screening of the pharmaceutical profiles of the glucosides as modified by carbohydrate(s) moieties with respect to their parental aglycones) (Arena *et al*. 2007).



AG17A1 R = -α-(Glc)$_3$

AG17B1 R = -α-(Glc)-1,6-Glc

AG17C1 R = -α-(Glc)-1,4-Glc

AG17D1 R = -α-Glc

MG3A R = -α-(Glc)-1,6-Glc

MG3B R = -α-(Glc)-1,4-Glc

MG3C R = -α-Glc

Figure 6. Products formed by glucosylation of 9-fluorenone derivatives**.** Carboxyhydroxyesters, indicated as AG, and carboxyhydroxamides indicated as MG.

ß-D-MANNOSIDASE

Owing to steric and polar effects favouring the formation of α-anomer, the β-mannopyranoside linkage is one of the most difficult glycosidic bond to synthesize (Ennis and Osborn 2003), thus alternative biological methodologies, based on β-mannosidases, are very attractive. New β-mannosidase natural examples are needed also for advancement in the conformational study of the enzymatic transition state (Zechel *et al*. 2003) and for the possibility of genetic manipulation of such enzymes to produce efficient synthetic catalysts (mannosynthase) (Nashiru *et al*. 2001). Additionally β-mannosidases are of interest for the re-utilization of waste materials. In this context both the degradation of β-1,4-mannan to simple sugars, and the direct β-mannosidase-catalysed synthesis of alkyl β-D-mannosides from mannobiose by β-mannosidase have been studied (Itoh and Kamiyama, 1995).

β-D-Mannosidase activity was identified in the hepatopancreas of *Aplysia fasciata* and with higher specific activity in the visceral mass homogenate (Giordano *et al* 2004). The apparent molecular mass for the purified protein was 229 kDa as established by gel filtration on a Superdex-200 column, while the molecular mass as determined by SDS-PAGE was 130 kDa indicating that the purified enzyme is a homodimer. A $(\beta/\alpha)_8$-barrel fold was suggested for this protein. The enzyme showed an optimal activity at pH 4.5 (although a very high activity, 80-100%, was observed in the range 4.0-5.2) and 45 °C (Andreotti *et al*. 2005). Interestingly, this enzyme is not active at pH 6.7 in 50 mM Tris buffer, while, at this pH, only 30% of its maximum activity is recorded in phosphate buffer.

At the optimal pH the enzyme showed a half-life of 23 hours. The enzyme retains most of its activity (82%) after 24 hours incubation in the presence of 10% DMSO, while in the presence of the other co-solvents the activity quickly dropped down (a half-life of 1 hour was estimated in the presence of 10% acetonitrile, while in the presence of acetone the half-life was 8.5 hours). The K_M and V_{max} values for pNP-β-Man were determined to be 2.4 mM and 50.3 U mg^{-1} respectively. The K_M obtained was similar to those reported for β-D-mannosidase from *Helix pomatia* (1.43 mM, McCleary 1988), from *Thermotoga neapolitana* (3.1 mM, Duffaud 1997), and from *Homo sapiens* (2.2 mM, Noeske and Mersmann 1983), but slightly higher than those reported for *Thermobifida fusca* and other species (Beki *et al*. 2003). Assuming that the dimer is the smallest catalytic unit with a molecular mass of 229 kDa, a k_{cat} of 11,519 min^{-1} was calculated. According to these results, β-D-mannosidase from *A. fasciata* has a very good catalytic efficiency, in fact the k_{cat}/K_M obtained is 4800 min^{-1} mM^{-1} which is higher than those of analogous enzymes from other species: 1454 min^{-1} mM^{-1} for the *T. neapolitana* protein (Duffaud *et al*. 1997); 870 min^{-1} mM^{-1} for the *Aspergillus niger* protein (Bouquelet *et al* 1978).

This enzyme is also extremely specific for pNP-β–Man in fact it showed no activity on other substrates: pNP-β–Gal, -β–Glc, -β–GlNAc, and -β–Xyl, except for a barely detectable β-D-fucosidase activity. In contrast, β-D-mannosidases from *Pyrococcus* species exerted significant hydrolytic activity on pNP-β–Gal and -β–Glc (Bauer *et al* 1996, Kaper *et al* 2002). The high selectivity of *Aplysia* enzyme is a useful and needed characteristic when using a glycosydase for structural determination purposes.

Self-transfer of a β-mannosyl unit was observed when pNP-β-Man is treated with the β-D-mannosidase from *A. fasciata*, in fact the most abundant disaccharidic product obtained (10-15% yield) possessed the β-1-4 intermannosidic linkage as established

by the analysis of mono- and two-dimensional NMR spectra of products (pNP-β-Man-(1-4)-β-Man). The transmannosylation was studied using pNP-β-Man as donor and o-nitrophenyl α-D-2-deoxy-N-acetyl glucopyranoside as hetero-acceptor, the molar ratio used being 1:3. Two regioisomers (12% global yield) due to the β-mannosylation of the heteroacceptor were formed in an 85:15 ratio (by HPLC). The products were the β-1,4 and the β-1,6 derivatives as assessed by two-dimensional NMR spectroscopy.

β-D-GALACTOSIDASE

One of the most reported applications of β-galactosidases is the transfer of galactose from a donor to acceptors such as N-acetylglucosamine and N-acetylgalactosamine. Another interesting application of β-galactosidase is the formation of Gal-Xyl disaccharides since O-β-D-galactopyranosyl moiety linked to xylopyranose ring represents an interesting disaccharidic template found in different examples of biological interest. The β-1-2 Gal-Xyl interglycosidic linkage is present in the oligosaccharins, substances possessing hormone-like effects in plants (Fry, 1996); while the β-1-4 is found in the region between glycosaminoglycan (GAG) chains and protein parts in serine-linked connective tissue proteoglycan (Fukase *et al*. 1996). In addition, free β-1-3 and β-1-4 Gal-Xyl disaccharides are useful substrates, as non invasive diagnostic tool, for intestinal lactase which is an enzyme involved in adult-type alactasia (Rivera-Sagredo, 1992). Among other possible acceptors for galactosidases: galactosides are of particular interest as galactobiosides are present in various oligosaccharides with biological activities: the Gal β-(1-4)-Gal disaccharide is the major component of galactooligosaccharides promoting the growth of *Bifidobacteria* in human large intestine and the β-(1-6) regioisomer has been found to be responsible for binding of myeloma immunoglobulins (Onishi 1995).

The homogenate from hepatopancreas of *A. fasciata* contained different β-glycoside hydrolases, the most abundant being the β-galactosidase (40% of the total activity of β-D-fuco- β -D-gluco- β-D-galacto- and β-D-mannosidase (Giordano *et al*. 2004)). The purified enzyme is a homodimer whose molecular mass was 164 kDa as estimated by gel-filtration and 78 kDa by SDS-PAGE (Andreotti *et al*. 2007). β-D-Galactosidase activity measured as a function of pH revealed that the activity increases going from pH 5.5 to 2.3 and drastically drops at lower pHs. At pH 3.7 it was observed a maximum activity around 75 °C. The enzyme completely retains its activity after 23 h incubation at pH 4.7 in K-acetate buffer, while retained 81% of its activity when incubated at pH 3.9 in Na-citrate buffer for 23 h. At pH 2.8 in glycine-HCl, the half-life time was 0.6 h. At 50 °C in K-acetate buffer pH 4.5 the half-life was 15 h.

This enzyme is highly specific for pNP-β-D-galactopyranoside (pNP- β-Gal), being completely inactive on other substrates such as pNP-β-D-glucopyranoside, pNP-β-D-fucopyranoside, pNP- β-D-mannopyranoside, and pNP-β-D-xylopyranoside. The K_M and V_{max} values for pNP-β-Gal at 50 °C in 50 mM K-acetate pH 4.5 were determined to be 1.53 mM and 74.2 U mg^{-1} while those for oNP- β-Gal were 1.47 mM and 101 U mg^{-1}, respectively. Assuming that the dimer is the smallest catalytic unit with a molecular mass of 164 kDa, a k_{cat} of 203 s^{-1} was calculated for pNP-β-Gal and 276 s^{-1} for oNP-β-Gal. β-D-Galactosidase from *A. fasciata* is also able to hydrolyse the glycosidic linkage present in lactose although to a much lesser extent than those of pNP-β-Gal and oNP-β-Gal.

The results reported in *Table 3* indicate a clear preference of the *Aplysia* enzyme for the galactosylation of polar acceptors. Owing to the specificity of the acceptor site of most galactosidases for compounds with phenyl groups (Lopez *et al*. 2004), 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* has been reported to have a very low affinity for these polar acceptors thus resulting in low yield using the same acceptor excesses (Giacomini *et al*. 2002). The *E. coli* β-galactosidase catalyzed the synthesis of Gal-Xyl-OMe to a yield of 33% using, as in our case, a 10-fold molar excess of methyl β-D-xylopyranoside. Moreover no product formation was observed using β-galactosidase from bovine testes and a polar acceptor such as 2-deoxy-D-galactopyranose (Gambert 1997).

Another interesting characteristic of the enzyme from *Aplysia* is the uncommon β-1,3 selectivity in the transgalactosylation reactions with most of the acceptors (*Table 3*). Using free xylose or its β-allyl or methyl derivative, the β-1-3 isomer was always selectively formed. The same result was obtained by using 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 regioselectivity of reaction but also to the yield as shown comparing the results of the reactions using the p-nitrophenyl and benzyl xylopyranosides (Giordano *et al*. 2005).

Table 3. Transglycosylation reactions performed by ß-D-galactosidase of *Aplysia fasciata* using a 4-nitrophenyl ß-D galactopyranoside as donor and different acceptors.

Substrate acceptor	EM	Yield (%)	Interglycosidic linkage(s) in the products and ratio of regioisomers
D-Xylose	10	60	ß-1,3; ß-1,4 60:35 and minor products
Allyl-ß-D-xylopyranoside	0.5	12	ß-1,3
Allyl-ß-D-xylopyranoside	5	48	ß-1,3; ß-1,4 70:29 and trace ß-1,2
Methyl-ß-D-xylopyranoside	10	75	ß-1,3; ß-1,4 73:26
Benzyl-α-D-xylopyranoside	5	33	ß-1,4; ß-1,3 3:1
Benzyl-ß-D-xylopyranoside	5	30	ß-1,3; ß-1,4 6:4
4-nitrophenyl-α-xylopyranoside	10	18	ß-1,4; ß-1,3 62:38
4-nitrophenyl-ß-xylopyranoside	10	50	ß-1,3; ß-1,4; ß-1,2 2:1:1
Glucal	5	35	ß-1,3 90% selectivity
N-acetyl glucosamine	5	20	ß-1,4
N-acetyl galactosamine*	10	-	-
Methyl ß-D-galactopyranoside*	10	38	ß-1,3; ß-1,6 81:19
2-nitrophenyl ß-D galactopyranoside*	-		ß-1,3; ß-1,6 72:38
D-Galactose*	10	75	ß-1,3; ß-1,6 ca 50:50

*in these reactions 2-nitrophenyl ß-D galactopyranoside was used as donor

It is known that nucleoside analogues play an important role in antiviral and anticancer therapies. In addition it has been found that many natural antibiotics possessing significant antitumour and antiviral activities have the structure of a nucleoside connected to oligosaccharides (Takahashi, 1993). Several synthetic analogous of

naturally occurring nucleosides are clinically useful anticancer or antiviral agents (Kern and Estey, 2006). New glycosyl derivatives of AZT, the potent inhibitor of HIV replication, were also synthesized, and their pharmacokinetic profiles were evaluated (Bonina *et al*., 2002).

Enzymatic methods for the synthesis of nucleoside derivatives seemed to be advantageous over their chemical counterparts in terms of efficiency and stereo- and regioselectivity. o-Nitrophenyl β-D-galactopyranoside (oNP-β-Gal) was used as the glycosyl donor in reactions with 5 equivalents of nucleoside acceptors (uridine, fluorouridine, thymidine, adenosine, cytidine, 5-chlorocytosine arabinoside, 3'azido-3'-deoxythymidine) in the presence of the β-galactosidase from the hepatopancreas of *Aplysia fasciata*. Enzyme regioselectivity was extremely high, since in all reactions only the product of galactosylation at the 5' position of the nucleoside was observed (*Figure* 7). Reaction yields were satisfactory in most cases, and very high for uridine derivatives. In particular, 5'-O-β-galactosyl-5-fluorouridine (**2**, *Figure* 7), the galactosylated derivative of the anticancer drug fluorouridine, was synthesized with a 60% yield, and 5'-O-β-galactosyl-3'-azido-3'-deoxythymidine(**7**, *Figure* 7) , the derivative of the anti-HIV drug, was obtained in 43% yield (Andreotti *et al*., 2007). This was the first report dealing with a glycoside hydrolase used for the modification of nucleosides with such convenient yields.

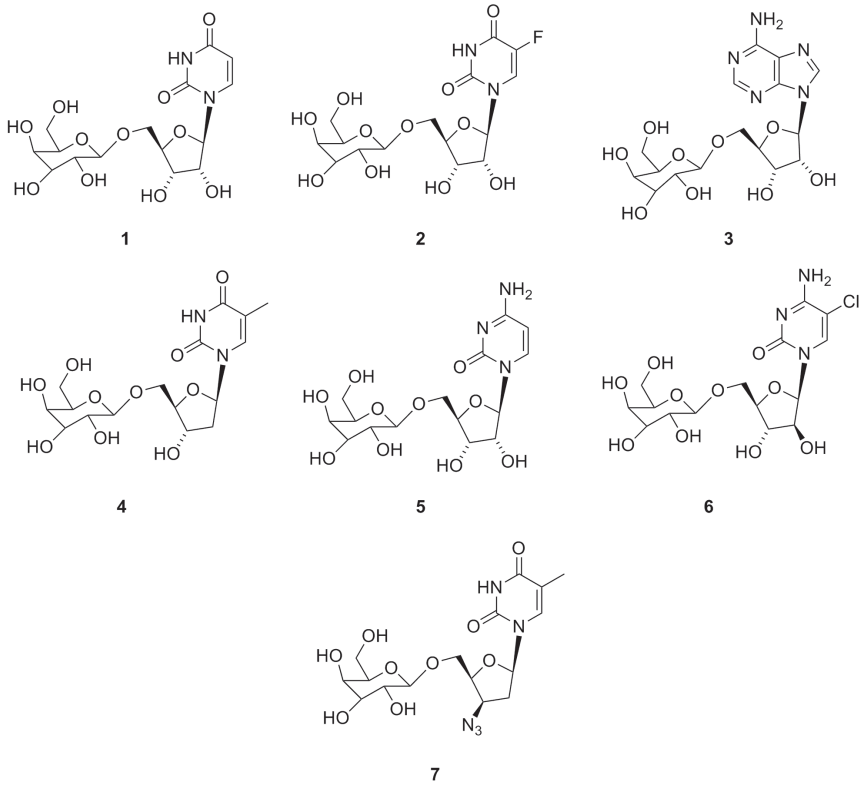


Figure 7. Structures of the reaction products obtained from galactosylation of nucleotide derivatives.

Conclusion

A very promising and quite unexplored field of application of marine glycoside hydrolases is the synthesis of the glycosidic bond. In some interesting cases found in the literature, a potent transglycosylation activity has been observed leading to the production of oligoglucosides (up to tetra- and pentasaccharides) even in the presence of a high concentration of mono- and disaccharide acceptors. One of these cases is the marine animal *Aplysia fasciata*. *Aplysia fasciata* is a mollusc which has shown to be a potent producer of a library of glycoside hydrolases applied in the synthesis of different types of glycosidic bonds. α-Glucosyl derivatives of different acceptors have been synthesized using the α-D-glucosidase activity found in visceral mass. We observed the formation of oligoglucosides with maltose alone, or heteroacceptors such as pyridoxine or derivatives of 9-fluorenone, etc. This is remarkable since it allow access to polyglucosides difficult to obtain by classical chemical procedures. The β-galactosidase and the β-mannosidase described above indicated the potentiality in synthesis of β-glycosidic linkages.

The β-mannosidase is an important enzyme from a variety of viewpoints. Its good catalytic efficiency as compared to analogous enzymes isolated from other sources is of particular interest: this characteristic coupled to the strict specificity for the β-mannosidic linkage and the exo-wise manner of hydrolysis, make this enzyme a candidate for a systematic study of its performance in the hydrolysis of galactosylated mannan fragments. Furthermore the ability of this biocatalyst for the construction of the synthetically difficult β-mannoside linkage, the regioselectivity to form mainly β-1-4 intermannosidic linkage and the possible use of N-acetylglucosamine based acceptor, are all interesting features for a thorough study of this enzyme under this point of view.

Yields have been extremely interesting for β-galactosidase used in the reaction with polar pyranosidic acceptors or nucleosides with antiviral activity. The use of other acceptors for this biocatalyst could reveal some interesting surprises due to the uncommon β-1,3 selectivity that has been observed.

In the expanding world of carbohydrate-active enzymes reports of glycosyltransferase and glycosyl hydrolase open reading frames frequently appear (Davies *et al*. 2005). In this context the sea environment and its marine biodiversity will play an important role as a source of useful biocatalysts in different fields of biotechnological relevance.

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