The Use of Exoglycosidases for the Assay of Two New Enzymatic Substrates, β-D-xylopyranosyl-4-nitrocatechol-1-yl and α-lactosyl-4-nitrocatechol-1-yl

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Glycosylation acceptor, 4-nitrocatechol, has been prepared via 4-nitrocatechol sulfate (2-hydroxy-5-nitrophenyl sulfate). The two carbohydrates, D-xylose and lactose, were peracetylated and then served as glycosylation donors in a modified Helferich glycosylation method, by using $BF_3 \cdot OBu_2$ as a promotor. The new synthesized glycosides were crystallized from ethanol and then submitted to Zémplen saponification and separated by preparative thin layer chromatography (TLC). We have isolated two xylosides. Reaction mixture of lactoside proved to be unitary, a single product could be isolated. Small portions of the synthetic glycosides were re-acetylated and their ^1H and ^{13}C NMR spectra registered. The two separated xylosides were β- and α-xylopyranoside-4-nitrocatechol-1-yl. Being submitted to the action of an enzymatic extract from digestive tract of snail (Helix pomatia) only the β-anomer was susceptible to enzymatic hydrolysis. The isolated lactoside proved to be the α-isomer. Under the action of an enzymatic extract from wheat (Triticum aestivum) germs, it was sequencially cleaved, as indicated by a kinetic TLC analysis.

Keywords: β-D-xylopyranoside, α-D-xylopyranoside, α-lactoside, 4-nitrocatechol, β-xylosidase, β-galactosidase, α-glucosidase

Enzymatic substrates constitute a vast group of natural and synthetic compounds. A rule with few exceptions is that practically all organic compounds that enter a living organism, monocellular organisms inclusively, become enzymatic substrates. According to the latter assertion, all drugs synthetic or natural are included in enzymatic substrates.

Terms as nucleomics, proteomics, lipidomics, are currently used based on a hypothesis concerning the existence of a network of nucleic acids, proteins, lipids, respectively, in living organisms. Our opinion is that the term *substratomics* is even more justified than the above mentioned terms and a new science, *substratology*, will probably appear, similar in fact to molecular biology, protein science, lipidology.

The first chromogenic substrates elaborated and used *per se* were 4-nitrophenyl-sulfate, 4-nitrophenyl-phosphate, 4-nitrophenyl-glucoside, 4-nitrophenyl-galactoside [1,2]. Other chromogenic substrates – the β-glycosides of 4-nitrophenol with maltose, lactose, cellobiose and gentiobiose had been synthesized and characterized previously [3] but used to other purposes. Glycosides of 2-nitrophenol were also synthesized [4-6] and played an essential role in the assay of some exoglycosidases.

We have proved that two diastereomeric conjugates of 4-nitrocatechol and D-galactofuranose satisfied all requirements – stability in neutral and slightly acidic solutions among others, to serve as enzymatic substrates for exogalactofuranosidases [7,8]. Since we could not find these enzymes without some risk [9], we have elaborated an indirect test: β- and α-D-galactofuranosyl-4-nitrocatechol-1-yl were converted to α- and β-L-arabinofuranosyl-4-nitrocatechol-1-yl, respectively, by a system of redox reactions. The latter glycosides were submitted to the action of arabinofuranosidases from radish (*Raphanus sativus* L.) germs [10]. Synthesis and characterization of three glycosides based on 4-nitrocatechol and α-D-glucopyranose,α-D-mannopyranose and β-D-galactopyranose proved that they are stable, feasible and versatile substrates for exoglycosidases [11]. Subsequently, we have synthesized and assayed two other substrates - α-D-N-acetylmannopyranosaminyl-4-nitrocatechol-1-yl and α-L-rhamnopyranosyl-4-nitrocatechol-1-yl, and both were cleaved by enzymes from the snail (*Helix pomatia*) [12]. Moreover, the latter organism possesses hydrolytic activity on 3-*O*-methyl-β-D-glucopyranosyl-4-nitrocatechol-1-yl (D. P. Iga, unpublished data).

In this paper, the synthesis and characterization of three new enzymatic substrates based on 4-nitrocatechol is presented and cleavability of β-D-xylopyranosyl-4-nitrocatechol-1-yl by exoglycosidase from the digestive tract of snail (*Helix pomatia*) and of α-lactosyl-4-nitrocatechol-1-yl by exoglycosidases from wheat (*Triticum aestivum*) germs has been demonstrated.

Experimental part

Materials and methods

D-Xylose and lactose were from Sigma. All reagents used were of analytical grade being purchased from Fluka or from Merck. All solvents were distilled before use.

All reactions were followed by TLC analysis: acetylated glycosides were eluted in the solvent system (SS) 1, chloroform–methanol (19:1), while deacylated glycosides were eluted in chloroform-methanol-water 60:25:4 (v/v) (SS 2). Since lactosyl-4-nitrocatechol is relatively polar, in comparison with monoglycosides, other two SS were used for its migration: SS 3, chloroform-methanol-water 60/25/5 (v/v) or even SS 4, chloroform-methanol-water 60/35/8 (v/v). Three types of visualization were used [11]: (a) by exposing to UV light (380 nm); (b) by dipping the plates in

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a 1 M solution of NaOH in ethanol-water, 1:1 (v/v) for a few seconds and then removing the excess solvent by pressing the plates between filter paper; (c) by dipping the plates in Mo-stain, followed by heating at 200 °C. The peracetylated glycosides were separated by crystallization from ethanol. Pure compounds were submitted to mild alkaline hydrolysis with 0.2 M sodium methoxide and the excess of alkalinity was neutralized with glacial acetic acid. (Unreacted 4-nitrocatechol served as a *p*H indicator). Purification of deacetylated glycosides was accomplished by preparative TLC, the maximum amount loaded on a 20/20 cm plate being 50 mg. The molar ratio determination of glycosides constituents [7,8,10,11] was preceded by acidic hydrolysis (2 h in 2 N H_2SO_4) and partition between water and ethyl ether; in the ether phase 4-nitrocatechol was determined by alkalization (based on a molar coefficient of 12.670 $cm^2 \times mol^{-1}$ for phenol) and the sugar with anthrone reagent in the water phase.

The ^1H and ^{13}C NMR spectra of synthetic intermediates and products were acquired in $CDCl_3$ containing TMS. One-dimensional NMR experiments were performed on a Bruker Avance DRX 400 spectrometer using 400 and 100 MHz for the ^1H and ^{13}C frequencies, respectively. The ^1H-^1H correlation spectroscopy (COSY) and ^1H-^{13}C heteronuclear multiple quantum coherence (HMQC) experiments were carried out with an inverse probe.

Tetra-*O*-acetyl-αβ-D-xylopyranose (**A** and **B**) (fig. 1) [13,14]. D-Xylose (6.2 g; 40.75 mmol) was peracetylated by stirring on ice and then at room temperature with an excess of Ac_2O-pyridine 1/2 (v/v). The solvents and volatile reagents were removed by rotavapor in the presence of small volumes of toluene, and the residue, **A** and **B**, was repeatedly crystalized from ethanol (10.37 g; 32.6 mmol; 80 %).

^1H NMR. ($CDCl_3$; δ ppm; *J* Hz) (fig. 1): 5.741, (d, 6.8 Hz, 1H) (H-1, Xylpâ); 6.251 (d, 3.6 Hz, 1H) (H-1, Xylpα) 5.029 (m, 1H) (H-2); 5.194, (t, 8.0 Hz, 1H) (H-3); 4.95 (m, 1H) (H-4); 4.134 (1H) (H-5e); 3.528 (dd, 8.4 Hz, 3.6 Hz 1H) (H-5a).

Tri-*O*-acetyl-αβ-D-xylopyranosyl-4-nitrocatechol-1-yl-2-acetate (**1** and **2**, fig. 1) [11]. A mixture of **A** and **B** (5 g, 15.72 mmol), and 4-nitrocatechol (1,2-dihydroxy 4-nitrobenzene) (2.43 g, 15.67 mmol) were dissolved in 25 mL of CH_2Cl_2 and 2.53 g (12.8 mmol) of $BF_3{\cdot}OBu_2$ was added. The mixture was stirred for 2 days at room temperature and then partitioned 3 times between a saturated solution of sodium bicarbonate and CH_2Cl_2, in order to remove the unreacted 4-nitrocatechol. The organic solution was dried over $MgSO_4$, filtered, evaporated to dryness, and acetylated by stirring overnight with an excess of Ac_2O/pyridine 1:2 (v/v). Any acetylation reagents were removed by rotavapor and the residue was crystallized from ethanol, **1** and **2** (fig. 1) being obtained (2.93 g; 6.44 mmol; 41 %).

β-D-Xylopyranosyl-4-nitrocatechol-1-yl (1') and α-D-xylopyranosyl-4-nitrocatechol-1-yl (2') (fig. 1). The two anomers have been separated by preparative TLC, that was found by us to be an efficient, rapid and convenient method for diastereomers separation. The mixture of **1** and **2** (1.5 g; 3.29 mmol) was submitted to Zémplen hydrolysis by incubation with 0.2 M sodium methoxide followed by neutralization with acetic acid. The solution was evaporated to dryness and brought to constant weight in a dessicator to obtain a mixture of **1'** and **2'** (0.85 g; 2.96 mmol; 90 %). The mixture, as a concentrated solution in water-ethanol 1:1 (v/v), was applied on preparative plates (20 x 20 cm) and migrated with SS 2. The plates were visualized by UV light, marked, scraped and the silica gel washed in a small column with water-ethanol 1/1 (v/v). Amounts of the order of tens of mg have been obtained of the two diastereomers. They were peracetylated and their NMR spectra registered. The beta isomer **1** (fig. 1) had the following characteristics:

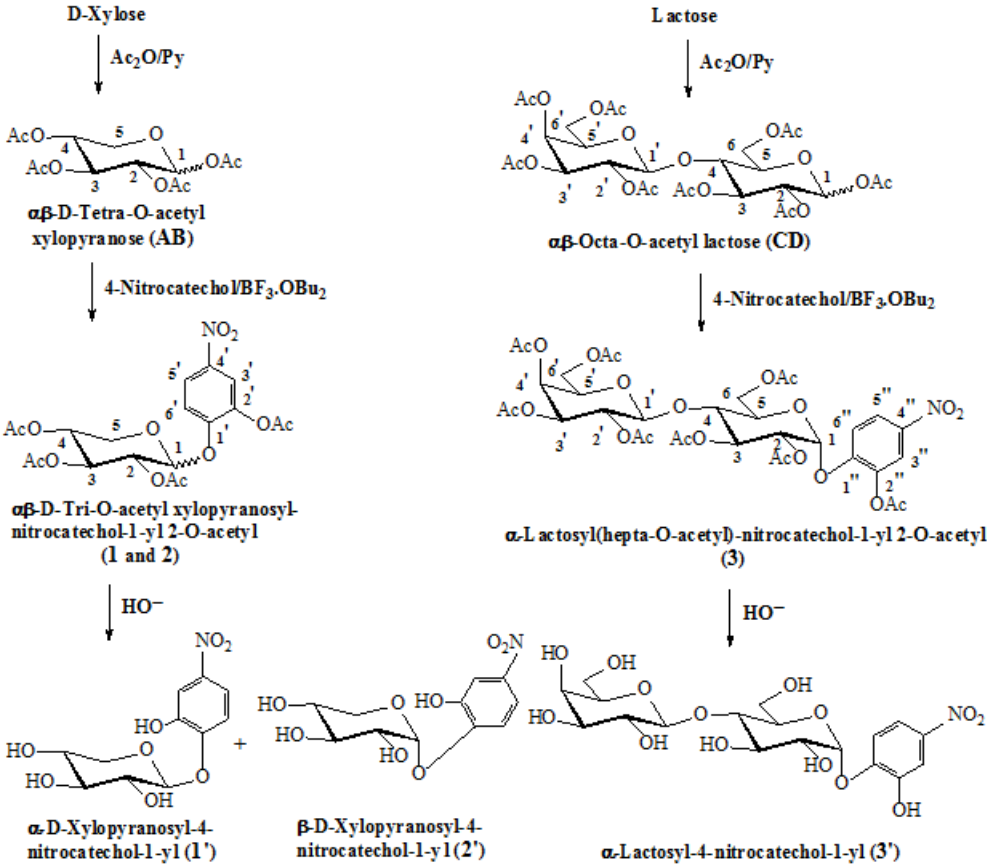

Fig. 1. New chromogenic substrates of 4-nitrocatechol to be assayed with exoglycosidases

^{1}H NMR. ($CDCl_3$; δ ppm; *J* Hz) 5.25 (d, 5.2 Hz, 1H) (H-1, beta linkage); 5.29 (1H) (H-2); 5.28 (1H) (H-3); 5.37 (1H) (H-4); 3.66 (1H) (H-5a); 3.99 (dd, 5.2 Hz, 6.0 Hz, 1H) (H-5e); 7.19 (1H) (H-3'); 7.97 (1H) (H-5'); 8.03 (1H) (H-6'); 2.01, 2.07, 2.09, 2.35 (s) (Me groups of Ac linked to sugar and 4-nitrocatechol).

^{13}C NMR. ($CDCl_3$; δ ppm; *J* Hz) (fig. 1): 98.9 (C-1); 71.0 (C-2); 68.8 (C-3); 68.1 (C-4); 62.7 (C-5); 146.2 (C-1'); 133.6 (C-2'); 111.2 (C-3'); 129.5 (C-4'); 118.6 (C-5'); 123.4 (C-6'); 20.2, 20.4, 20.6, 20.7 (Me groups of Ac linked to sugar and 4-nitrocatechol); 168.2, 169.8, 170.0, 170.1 (>C=O groups of Ac).

The alpha isomer **2** (fig.1) had the following characteristics:

^{1}H NMR. ($CDCl_3$; δ ppm; *J* Hz) 5.75 (d, 3.6 Hz, 1H) (H-1, alpha linkage); 4.92 (dd, 3.6 Hz; 6.8 Hz, 1H) (H-2); 5.60 (t, 10.0 Hz, 1H) (H-3); 5.05 (dd, 6.0 Hz, 4.0 Hz, 1H) (H-4); 3.60 (1H) (H-5a); 3.94 (dd, 6.0 Hz, 5.2 Hz, 1H) (H-5e); 7.22 (1H) (H-3'); 7.95 (1H) (H-5'); 8.06 (1H) (H-6'); 2.05, 2.07, 2.08, 2.44 (s) (Me groups of Ac linked to sugar and 4-nitrocatechol).

^{13}C NMR. ($CDCl_3$; δ ppm; *J* Hz): 95.2 (C-1); 70.4 (C-2); 68.7 (C-3); 68.4 (C-4); 59.7 (C-5); 147.9 (C-1'); 132.9 (C-2'); 110.9 (C-3'); 130.1 (C-4'); 118.5 (C-5'); 123.3 (C-6'); 20.2, 20.4, 20.6, 20.7 (Me groups of Ac linked to sugar and 4-nitrocatechol); 168.2, 169.7, 169.9, 170.1 (>C=O groups of Ac).

α-(Hepta-*O*-acetyl)lactosyl-4-nitrocatechol-1-yl (Fig. 1). Lactose (10 g; 29.23 mmol) was peracetylated by stirring overnight at room temperature with an excess of Ac_2O-pyridine 1/2 (v/v). The excess reagents and solvents were removed by rotavapor and the residue, mixture of **C** and **D**, was crystallized from ethanol (17.83 g; 26.3 mmol; 90 %). A mixture of **C** and **D** (6.5 g; 9.58 mmol), and 4-nitrocatechol (1,2-dihydroxy 4-nitrobenzene) (1.48 g, 9.6 mmol) were dissolved in 30 mL of CH_2Cl_2 and 2.18 g (9.5 mmol) of $BF_3 \cdot OBu_2$ was added by a syringe. The mixture was stirred for 2 days at room temperature and then partitioned 3 times between a saturated solution of sodium bicarbonate and CH_2Cl_2, in order to remove the unreacted 4-nitrocatechol. The organic solution was dried over $MgSO_4$, filtered, evaporated to dryness, and the residue, hepta-*O*-acetyl-lactosyl-4-nitrocatechol, crystallized from ethanol (2.59 g; 3.35 mmol; 35 %). Hepta-*O*-acetyl-lactosyl-4-nitrocatechol, 1.5 g (1.94 mmol) was submitted to Zemplen hydrolysis and the product (**3'**) separated by preparative TLC and acetylated to produce **3**.

^{1}H NMR. ($CDCl_3$; δ ppm; *J* Hz) (fig. 1): 4.56 (d, 7.6 Hz, 1H, â-linkage) (H-1); 5.11 (1H) (H-2); 5.15 (1H) (H-3); 5.36 (1H) (H-4); 3.94 (1H) (H-5); 4.01-4.17 (1H) (H-6a); 4.01-4.17 (1H) (H-6b); 5.36 (d, 3.6 Hz, 1H, á-linkage) (H-1'); 5.19 (1H) (H-2'); 5.47 (1H) (H-3'); 3.88 (1H) (H-4'); 3.97 (1H) (H-5'); 4.01-4.17 (1H) (H-6'a); 4.01-4.17 (1H) (H-6'b); 7.19 (d, 8.4 Hz, 1H) (H-3''); 7.95 (d, 2.4 Hz, 1H) (H-5''); 8.10 (1H) (H-6''); 1.96, 2.01, 2.03, 2.04, 2.07, 2.10, 2.15, 2.44 (s) (Me groups of –OAc linked to sugar and 4-nitrocatechol).

4-Nitrocatechol (1,2-dihydroxy-4-nitrobenzene). Synthesis of 4-nitrocatecholsufate (2-hydroxy-5-nitrophenylsulfate) [15] and its cleavage to 4-nitrocatechol, as well as its spectral properties [7] were described in detail elsewhere [10].

Enzymatic tests. Biological materials used for enzymatic extract preparation, degestive tract of snail (*Helix pomatia*) and germs of wheat (*Triticum aestivum*, 48 h at room temperature) were homogenized in cold water in the ratio 1/5 (w/v) and then centrifuged. Incubation mixture consisted of 0.25 mL glycoside solution (5 mM), 0.5 mL acetate buffer (0.03 M acetic acid, 0.07 M sodium acetate, 0.05 M sodium chloride, *p*H 5.0), 0.25 mL supernatant enzymatic solution, at 40-42 °C. Alternatively, total volumes of 20 mL containing the same constituents in the same ratio, were incubated for lactosyl-4-nitrocatechol, and periodically volumes of 1 mL were extracted and enzymatic processes blocked by five min heating on a boiling water bath. The products of the incubated mixtures were then analyzed by TLC in SS 3 and SS 4. Incubation lasted 30-60 min or even 48 h. The samples stopped by adding 2.5 mL 0.2 N NaOH were measured at 515 nm and a molar coefficient of 12,670 $cm^2 \times mol^{-1}$ was used for the calculation of 4-nitrocatechol concentration [11]. Protein concentration was determined by Lowry method [11] with bovine serum albumin as standard.

Results and discussions

Specific activities (nmol $\times$ min^{-1} $\times$ mg $protein^{-1}$) of 20.15 and 2.12 were measured for **1'** and **3'**, respectively, on the two tissues. They are of the same order of magnitude as enzymatic activities determined previously on other nitrocatechol glycosides [10-12].

Peracetylation of D-xylose at room temperature led to a mixture of **A** and **B** (fig. 1), as evidenced from its NMR spectra. Helferich glycosylation of 4-nitrocatechol with these peracetates produced two anomeric glycosides [16,17]. They could be separated by preparative TLC, and contained D-xylose and 4-nitrocatechol in 1:1 molar ratio. Glycosylation of 4-nitrocatechol with a mixture of **C** and **D** produced a glycoside migrating slower than monohexosyl-4-nitrocatechol. It contained 4-nitrocatechol and sugar (as a mixture of D-glucose and D-galactose) in 1:1 molar ratio. (In the case of lactose determination, a standard curve with lactose was constructed for anthrone reaction). An interesting observation has been made: a monohexosyl-4-nitrocatechol has been obtained, concomitantly with lactoside. This can be explained only admitting a limited glycolytic activity of promotor. ^{1}H NMR spectra of lactoside indicated it to be the alpha anomer **3** [18].

Production of 4-nitrocatechol glycosides, with either xylose or lactose, could be evidenced easily and directly by TLC as yellow bands, when visualization was made with sodium hydroxide. (Unreacted or acetylated 4-nitrocatechol gave rise to red bands in the same conditions). The main synthetic glycosides could be separated by preparative TLC. If larger amounts were needed, column chromatography could also be applied [11]. Glycosides colored in yellow in 0.2 M NaOH and this color was stable overnight at room temperature. In 2 N sulfuric acid at boiling, the synthetic glycosides were completely hydrolyzed, the reaction could be kinetically monitored. Friedel-Crafts glycosylation products (C-glycosides) would probably mimick in many regards normal glycosides. However, they cannot be hydrolyzed. NMR Spectra of peracetylated glycosides (fig. 1) contained simultaneously signals from both sugar and aglycon:

- **1**, 5.25 (d, 5.2 Hz, 1H) (H-1, beta linkage); 7.19-8.03 (4-nitrocatechol); 98.9 (C-1); 62.7 (C-5); 146.2 (C-1'); 133.6 (C-2');
- **2**, 5.75 (d, 3.6 Hz, 1H) (H-1, alpha linkage); 7.22-8.06 (4-nitrocatechol); 95.2 (C-1); 59.7 (C-5); 147.9 (C-1'); 132.9 (C-2'); 110.9 (C-3');
- **3**, 4.56 (d, 7.6 Hz, 1H, β-linkage, H-1); 5.36 (d, 3.6 Hz, 1H, α-linkage, H-1'); 7.19 (d, 8.4 Hz, 1H) (H-3''); 7.95 (d, 2.4 Hz, 1H) (H-5''); 8.10 (1H) (H-6'');

An interesting aspect linked with glycosylation of dihydroxy phenols (catechol, resorcinol, hydroquinone, 4-nitrocatechol, etc) is the possibility of di-glycosylation. In the latter case, the signals of methyl groups of acetate ester are exclusively aliphatic [19]. In case of

monoglycosylation, a *downfield* effect will be evidenced due to aromatic ester, as in 3-*O*-Ac-17α-17-β-D-(tetra-*O*-Ac)glucopyranoside [20]. We had distinct signals, all singlets, for the three glycosides that indicated monoglycosylation at: 2.35, 2.44, 2.44 ppm.

Some remarks should be made concerning chromatographic behaviour of 4-nitrocatechol glycosides: (A) anomers as β- and α-D-galactofuranosyl-4-nitrocatechol [7,8] present a difference of R_F values in the range of 0.05, similar to the double (twin) spot of cerebrosides [21]. However, due to their absorbance in UV, they can be traced and fully separated. (B) Mono- and dihexosyl-4-nitrocatechol constitute a homologous series and are clearly separated, as mono- and dihexosyl-ceramides [21].

D-Xylose is much more abundant in natural materials than other aldopentoses of biological interest, e. g., D-ribose. For this reason, chemical means have been elaborated for the conversion of D-xylose to D-ribose [22]. Xylans, heteropolymers of D-xylose, can be divided in two groups: (glucurono-)arabinoxylans and glucuronoxylans. Xylan from the cell walls of monocotyles (grasses and cereals) consist of linear chains of β-D-(1,4)-linked D-xylopyranosyl residues, which can generally be substituted with α-L-arabinofuranosyl at the 2-*O*- and/or 3-*O*-position(s) and α-D-glucopyranosyl uronic acid or its 4-*O*-methyl derivative at the 2-*O*-position [23,24]. D-Xylose is present in a large number of flavonoids [25], in a natural glycoconjugate of bilirubin [26] and in glycosaminoglycans from mammalia [27]. Aglycons for synthetic chromogenic substrates of D-xylose are especially 4-nitrophenol [28] and 4-methylumbeliferone [27].

Lactosyl, as lactosyl-ceramide, is present in a series of gangliosides [29]. Incubation of á-lactosyl-4-nitrocatechol with an enzymatic extract from wheat (*Triticum aestivum*) germs failed to produce chromogen at times of incubation of a few hrs. Therefore, incubations had to last overnight. Next day, free 4-nitrocatechol could be clearly identified. TLC analysis at variable times indicated, beside intact lactoside, α-glucopyranosyl-4-nitrocatechol. In a preceding paper [11], we have identified two enzymes in this biological material, α-glucosidase and β-galactosidase, by using suitable 4-nitrocatechol glycosides.

Glycosides based on di- and/or polyglycosides – prechromogenic, chromogenic or fluorogenic – are appreciably fewer than similar monoglycosides. α-Cellobiose or β-gentiobiose were linked to phenol [30], and α-maltose was linked to the following aglycons: phenol, *o*-cresol, *p*-(oxymethyl)-phenol, *p*-oxy-diphenyl, N-stearoyl-(*o*-aminomethyl)phenol [31]. 4-Methylumbelliferyl glycosides of cellooligosaccharides of the form MeUmb-β-(1-4β-D-Glcp)$_n$ (n=2-6) were used for the study of cellulolytic enzymes from *Trichoderma reesei* [32]. The same authors synthesized lactosyl-β-4-MeUmb. In this case, the enzyme cleaved lactosyl residue as a whole. To the same aglycon was linked â-cellobiose for the study of (1,4)-â-glucanase activity in lake sediments [33], while resorufin permitted fluorescence measurement with the same disaccharide glycoside [34].

An important question is, for the time being, unanswerable: are different enzymatic actions independent or we are measuring bi- or even polyfunctional enzmes? The answer could be given by working with one band proteins or with cloned enzymes. Proteins containing simultaneously α-L-arabinofuranosidase and β-D-xylosidase activity have been found in the following biological materials: radish (*Raphanus sativus* L.) seeds [35], *Thermomonospora fusca* BD25 [36], barley (*Hordeum vulgare* L.) seedlings [37], alfalfa (*Medicago sativa* L.) roots [38]. An enzyme capable of hydrolysing aryl-β-D-glucopyranosides and aryl-β-D-xylopyranosides has been isolated from *Stachybotrys atra* [39]. Moreover, bifunctional phenomenon has been demonstrated for enzymes of other classes than hydrolases: UDP-N-acetylglucosamine 2-epimerase (UDP-GlcNAc 2-epimerase, EC 5.1.3.14) and N-acetylmannosamine kinase (ManNAc kinase, EC 2.7.1.60) in rat liver [40] and a pyranose-furanose mutase in *Campylobacter jejuni* 11168 [41].

Conclusions

Helferich glycosylation of 4-nitrocatechol with tetra-*O*-acetyl-αβ-D-xylopyranoside in the presence of $BF_3{\cdot}OBu_2$ produced both anomeric glycosides.

Only β-xyloside was cleaved by an enzymatic extract from digestive tract of snail (*Helix pomatia*).

A similar glycosylation with octa-*O*-acetyl lactoside led to the α-lactoside.

α-Lactosyl-4-nitrocatechol-1-yl was cleaved sequencially by an enzymatic extract from wheat (*Triticum aestivum*) germs.

There was a good agreement between chemical assays and NMR spectra of synthetic glycosides, both indicated monoglycosylation.

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