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Synthesis and characterization of new chromogenic substrates for exoglycosidases: α-glucosidase, α-mannosidase, and β-galactosidase

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Abstract: Glycosides of 4-nitrocatechol (1,2-dihydroxy 4-nitrobenzene) with α-D-glucopyranose and α-D-mannopyranose were synthesized by the glycosylation of phenol with peracetylated sugars in the presence of $BF_3 \cdot OBu_2$. The glycoside of 4-nitrocatechol with β-D-galactopyranose was prepared by the glycosylation of this phenol as sodium phenoxide with tetra-*O*-benzoyl-α-D-galactopyranosyl bromide. The structure of the reaction products was confirmed by ^{1}H and ^{13}C NMR spectra and by chemical analysis. The latter consisted of acidic hydrolysis, followed by ethyl ether extraction and colorimetric determination of 4-nitrocatechol in the ether phase and application of the anthrone method for the sugar in the water phase. The synthetic glycosides were tested as substrates for enzymes from animal, vegetal, and microbial materials.

Key words: Glycosylation, 4-nitrocatechol-glycoside, exoglycosidase, enzymatic substrate

1. Introduction

4-Nitrocatechol (1,2-dihydroxy 4-nitrobenzene) primarily served as chromogen for the determination of sulfatases, the substrate being nitrocatechol sulfate (2-hydroxy 5-nitrophenyl sulfate).[1−5] 4-Nitrocatechol sulfate, 4-methylumbelliferyl sulfate, and 4-nitrophenyl sulfate are the substrates of choice for arylsulfatase determination.[6−10] Chromogens such as 2- and 4-nitrophenols and 4-methyl-umbelliferone (7-hydroxy-4-methyl-chromen-2-one) were used to construct substrates for glycosidases.[6,11−19] Other authors synthesized, to this aim, glycosides of 8-hydroxyquinoline,[20] cyclohexenoesculetin,[20,21] alizarin,[22] p-naphtholbenzein,[23] resorufin,[24] and fluorescein,[25] as well as of a series of indolyl derivatives.[26,27] In the present study, we synthesized glycosides of nitrocatechol in order to prevent the interference of natural fluorescent compounds and aromatic pigments from plant materials.[28,29] 4-Nitrocatechol sulfate is a specific inhibitor of the *Yersinia* protein tyrosine phosphatase and displays a more than 10 times higher selectivity towards this enzyme than to other mammalian protein tyrosine phosphatases.[30] Based on these results, a molecular dynamics simulation model was elaborated and used to study the docking of p-nitrocatechol sulfate with the *Yersinia* protein tyrosine phosphatase.[31] Many other inhibitors of protein tyrosine phosphatases were synthesized and their activity evaluated either experimentally or by molecular dynamics simulations.[32−35] The inhibitor target was the active

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site or the phosphotyrosine-binding pocket of these enzymes.[34,35] A new approach of phosphotyrosine-mediated recognition was disclosed by a series of experiments indicating the mimicking of tyrosine sulfate by the sulfogalactose moiety of sulfoglycosphingolipids.[36] 4-Nitrocatechol bears a widespread natural structural motif, 1,2-dihydroxy-benzene structure.[37] 4-Nitrocatechol proved to be an inhibitor of catechol-*O*-methyltransferase, and compounds with a similar structure—as entacapone, tolcapone, or nitecapone—revealed even higher inhibitory activity.[38] On the other hand, 4-nitrocatechol is an intermediate in the biological degradation of nitrobenzene and 4-nitrophenol by microorganisms, microsomes, and the digestive tracts of mammals.[39–44]

In this study, 3 new versatile substrates for exoglycosidases—α-glucosidases, α-mannosidases, and β-galactosidases—were synthesized by the glycosidation of 4-nitrocatechol. The structure of the glycosidation products was confirmed by ^{1}H and ^{13}C NMR spectra and by chemical analysis. Moreover, when tested with enzymes of animal, vegetal, or microbial origin, these glycosides proved to be remarkably good substrates.

2. Experimental

2.1. Materials and methods

The reagents, solvents, and chromatographic materials were of analytical grade. They were purchased either from Sigma or from Fluka. Biological materials of animal, vegetal, and microbial origin were used.

General. The ^{1}H and ^{13}C NMR spectra of synthesis intermediates and products were measured 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 ^{1}H and ^{13}C frequencies, respectively. The ^{1}H–^{1}H correlation spectroscopy (COSY) and ^{1}H–^{13}C heteronuclear multiple quantum coherence (HMQC) experiments were carried out with an inverse probe. The syntheses and separations were monitored using thin-layer chromatography performed on silica gel plates (Merck silica gel 60 F_{254} glass sheets). Ac(et)ylated glycosides migrated in the solvent system (SS) 1, chloroform–methanol (19:1), while deacylated glycosides migrated in chloroform–methanol–water 65:25:5 (v/v) (SS 2). Three types of visualization were used: (a) by dipping the plates in mostain, followed by heating; (b) under UV light; (c) by dipping the plates in a 1 M solution of NaOH in ethanol–water (1:1).[45] The separation of peracylated glycosides was performed with silica gel 60 (0.063–0.200 mm, Merck) column chromatography in a gradient of methanol in chloroform. Pure compounds were submitted to Zemplén saponification by heating for 1 h at 45 °C in 20 vols. of 0.2 M sodium methoxide. Any excess of alkalinity was removed by stirring with Dowex 50 WX2 (H^+) and the solution concentrated to dryness by Rotavapor. The residue was dissolved in a determined volume of water. When needed, purification of deacylated glycoside was achieved by silica gel column chromatography in a gradient of ethanol in 1,2-dichloroethane. The molar ratio of glycoside constituents was determined by boiling a small portion of glycoside for 2 h in 2 M HCl, followed by the partition in ethyl ether and water. In the water phase, sugar was determined by anthrone reaction and, in the ether phase, 4-nitrocatechol was determined colorimetrically by OD_{515} measurement.

General procedure for the preparation of 1a and 2a. Glycosylation donor, penta-O-acetyl α-D-glucopyranose (**1**) or -α-D-mannopyranose (**2**) (5 g, 12.8 mmol),[46,47] and 4-nitrocatechol (1,2-dihydroxy 4-nitrobenzene, **4**) (2 g, 12.89 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.[48,49] 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. The solution of the latter, containing **1a** or **2a**, was dried over $MgSO_4$, filtered, evaporated to dryness, and acetylated by stirring overnight with 10 volumes of Ac_2O/pyridine 1:2 (v/v). Any acetylation reagents were removed by Rotavapor and the residue was separated

by silica gel column chromatography. The protecting groups were removed by Zemplen saponification, the product being **1c** or **2c**.

2-Acetoxy-4-nitrophenyl (2,3,5,6-tetra-*O*-acetyl)-α-D-glucopyranoside (**1b**, 3.58 g, 6.78 mmol, 53%) was obtained as a greenish-yellow amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.18 (d, 3.6 Hz, 1H, 1-H), 5.30 (m, 2H, 2-H, 3-H), 5.13 (m, 1H, 4-H), 3.93 (m, 1H, 5-H), 4.27 (dd, 5.6 Hz, 6.8 Hz, 1H, 6a-H), 4.17 (dd, 10.0 Hz, 2.4 Hz, 1H, 6b-H), 7.12 (d, 9.2 Hz, 1H, 3'-H), 8.0 (d, 2.8 Hz, 1H, 5'-H), 8.12 (dd, 2.8 Hz; 6.4 Hz, 1H, 6'-H); 2.04, 2.06, 2.08, 2.09 (s, Me groups of Ac linked to sugar), 2.31 (s, Me group of Ac linked to 4-nitrocatechol). ^{13}C NMR (100 MHz, $CDCl_3$) δ 98.2 (1-C), 70.3 (2-C), 72.2 (3-C), 68.0 (4-C), 72.5 (5-C), 61.8 (6-C), 153.40 (1'-C), 142.77 (2'-C), 114.35 (3'-C), 139.87 (4'-C), 119.69 (5'-C), 122.81 (6'-C); 20.25, 20.56, 20.56, 20.61, 20.67 (Me groups of Ac linked to sugar and 4-nitrocatechol); 168.3, 169.4, 169.5, 170, 170.4 (>C=O groups of Ac).

2-Hydroxy-4-nitrophenyl α-D-glucopyranoside (**1c**, 1.98 g, 6.24 mmol, 92%), greenish-yellow amorphous solid, $[\alpha]_D^{24}$+42.6 (*c* 0.82 water). Anal. Calcd. for $C_{12}H_{15}NO_9$: C, 45.43; H, 4.76; N, 4.41. Found: C, 45.40; H, 4.83; N, 4.37.

2-Acetoxy-4-nitrophenyl (2,3,5,6-tetra-*O*-acetyl)-α-D-mannopyranoside (**2b**, 3.48 g, 6.59 mmol, 52%) was obtained as an amorphous greenish-yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.62 (d, 2.0 Hz, 1H, 1-H), 5.36 (m, 3H, 2-H, 3-H, 4-H), 3.98 (m, 1H, 5-H), 4.26 (dd, 7.6 Hz, 4.8 Hz, 1H, 6a-H), 4.04 (dd, 2.4 Hz, 10 Hz, 1H, 6b-H), 7.33 (d, 9.2 Hz, 1H, 3'-H), 8.03 (d, 2.8 Hz, 1H, 5'-H), 8.13 (dd, 2.8 Hz, 6.4 Hz, 1H, 6'-H); Me groups of Ac: 2.040, 2.077, 2.081, 2.211 (s, Me groups of Ac linked to sugar), 2.461 (Me groups of Ac linked to 4-nitrocatechol). ^{13}C NMR (100 MHz, $CDCl_3$) δ 95.9 (1-C), 68.8 (2-C), 68.5 (3-C), 64.9 (4-C), 70.4 (5-C), 61.8 (6-C), 152.1 (1'-C), 142.8 (2'-C), 114.8 (3'-C), 140.1 (4'-C), 119.3 (5'-C), 122.8 (6'-C); 20.25, 20.62, 20.66, 20.70, 20.79 (s, Me groups of Ac); 168.48, 169.63, 169.81, 169.94, 170.37 (>C=O groups of Ac).

2-Hydroxy-4-nitrophenyl α-D-mannopyranoside (**2c**, 1.82 g, 5.74 mmol, 87%), greenish-yellow amorphous solid, $[\alpha]_D^{24}$+164.4 (*c* 0.76 water). Anal. Calcd. for $C_{12}H_{15}NO_9$: C, 45.43; H, 4.76; N, 4.41; Found: C, 45.51; H, 4.75; N, 4.49.

General procedure for the preparation of 3b:[11] A solution of tetra-O-benzoyl α-D-galactopyranosyl bromide (**3**; 3 g; 4.55 mmol) in acetone (12 mL) was added to a solution of 4-nitrocatechol (**4a**, 0.77 g, 5 mmol) dissolved in 9.8 mL of 1 M NaOH.[50–52] The mixture was stirred overnight at room temperature to produce **3a**, and then neutralized with glacial acetic acid, evaporated to dryness, and peracetylated to give a mixture containing **3b**. The peracylated glycoside was separated by silica gel column chromatography and Zemplén saponification gave **3c**.

2-Acetoxy-4-nitrophenyl (2,3,5,6-tetra-*O*-benzoyl)-β-D-galactopyranoside (**3b**, 1.51 g, 1.95 mmol, 43%) was obtained as an amorphous greenish-yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.46 (d, 8.0 Hz, 1H, 1-H), 6.06 (dd, 3.6 Hz, 4.8 Hz, 1H, 2-H), 5.71 (dd, 3.6 Hz, 6.8 Hz, 1H, 3-H), 6.10 (dd, 2.4 Hz, 8.0 Hz, 1H, 4-H), 4.57 (m, 3H, 5-H, 6a-H, 6b-H), 7.90 (d, 2.8 Hz, 1H, 5'-H), 7.76 (d, 2.8 Hz, 1H, 6'-H); 2.12 (s, Me group of Ac); 7.238-8.127 (s or m, 23 H, phenyl groups of Bz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 99.3 (1-C), 67.7 (2-C), 71.3 (3-C), 68.9 (4-C), 72.4 (5-C), 62.2 (6-C), 153.5 (1'-C), 142.7 (2'-C), 115.4 (3'-C), 140.0 (4'-C), 119.6 (5'-C), 122.6 (6'-C); 19.97 (s, Me group of Ac); 128.23, 128.39, 128.47, 128.55, 128.68, 128.76, 128.82, 129.23, 129.73, 129.78, 130.09, 133.52, 133.61, 133.77, 133.87 (C atoms of Bz); 165.33, 165.42, 165.51, 165.92 (>C=O groups of Bz); 167.92 (>C=O group of Ac).

2-Hydroxy-4-nitrophenyl β-D-galactopyranoside (**3c**, 0.52 g, 1.64 mmol, 85%), greenish-yellow amorphous solid, $[\alpha]_D^{24}$ –43 (c 1.5 water). Anal. Calcd. for $C_{12}H_{15}NO_9$: C, 45.43; H, 4.76; N, 4.41; Found: C, 45.36; H, 4.79; N, 4.45.

Enzymatic test. About 2 decades ago we determined exoglycosidasic activities (β-hexosaminidase, β-galactosidase, α-galactosidase, β-glucosidase) in a series of animal tissues (brain, kidney, testis, seminal plasma), using the respective 4-nitrophenyl glycosides.[53–56] Moreover, we purified and characterized arylsulfatase from seminal plasma.[57] We applied the same protocol to demonstrate that glycosides **1c**, **2c**, and **3c** based on 4-nitrocatechol constitute excellent chromogenic substrates for exoglycosidases: the tissue was homogenized in 5–10 volumes of distilled water and then centrifuged at 10,000 $\times$ g. Equal volumes of supernatant solution, substrate solution (2–5 mg/mL, water), and buffer were incubated for variable times at 40 °C, stopped with 2 volumes of 0.5 M NaOH, and measured at 515 nm.[58] A portion of enzymatic solution was heated on a boiling water bath for 5 min, cooled, and incubated as before. A molar coefficient of 12,670 $cm^2 \times mol^{-1}$ was used for 4-nitrocatechol, in a strongly alkaline environment.[4] Protein was determined by the Lowry method by using a standard curve constructed with bovine serum albumin.[59]

3. Results and discussion

In a project connected with the elaboration of new compounds designed for the investigation of the metabolism of glycoconjugates, we decided to employ 4-nitrocatechol as a chromogen for exoglycosidase substrates. Hence, we synthesized and tested 3 new substrates, namely the corresponding glycosides 2-hydroxy-4-nitrophenyl α-D-glucopyranoside, α-D-mannopyranoside, and β-D-galactopyranoside.

O-Acetylation of D-glucose and D-mannose led to ring-closed penta-O-acetyl pyranosides **1** and **2**, respectively (Figure 1).[46,47] For the glycosidation with **1** and **2** the Helferich method with $BF_3 \cdot OBu_2$ as a promoter was chosen.[48,49] 4-Nitrocatechol **4** was used as it is a glycosyl acceptor.[45] In order to avoid or minimize the formation of a di-glycosylated product, an equimolar ratio of glycosyl donor and acceptor was used. Per-O-benzoylation of D-galactose led to galactopyranose, which was converted to bromide **3** (Figure 2) by reaction with HBr/AcOH.[50–52] 4-Nitrocatechol was converted to sodium phenoxide **4a** and then reacted with tetra-O-benzoyl α-D-galactopyranosyl bromide **3**.[11]

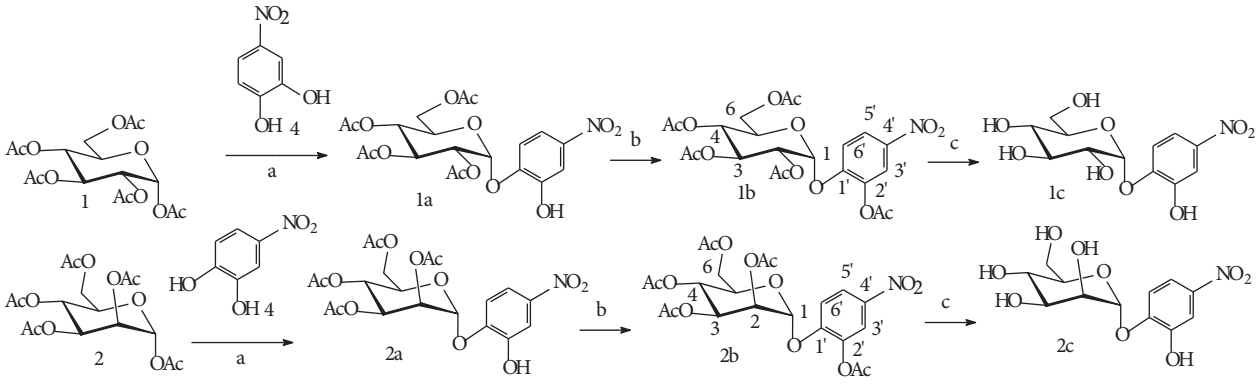

Figure 1. Synthesis of glycosides **1c** and **2c**. *Reagents and conditions*: (a) $BF_3 \cdot OBu_2$, CH_2Cl_2, rt; (b) Ac_2O, Pyr (**1b**: 53%; **2b**: 52%, 2 steps); (c) NaOMe, MeOH (**1c**: 92%; **2c**: 87%).

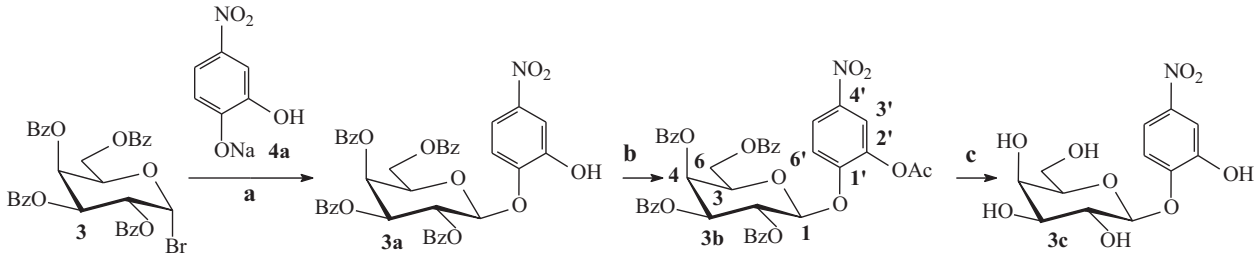

Figure 2. Synthesis of glycoside **3c**. *Reagents and conditions*: (a) NaOH (1 M), Me_2CO; (b) Ac_2O, Pyr (43%, 2 steps); (c) NaOMe, MeOH (85%).

Glycosylation products **1a**, **2a**, and **3a** were identified by TLC due to their yellow color in an alkaline environment.[45] Unreacted 4-nitrocatechol takes on a red color under these conditions.[60] After removal of unreacted 4-nitrocatechol by partitioning between aqueous $NaHCO_3$ and CH_2Cl_2, an acetylation reaction followed[61] and the ^1H and ^{13}C NMR spectra of glycosides **1b**, **2b**, and **3b** were recorded. The α-stereochemistry of the newly formed linkages in **1b** and **2b** was verified by the spectral data **1b**: δ 5.18 (d, 3.6 Hz, 1H, H-1) with 98.2 (C-1) and **2b:** δ 5.62 (d, 2.0 Hz, 1H, H-1) with 95.9 (C-1), respectively. For 4-nitrocatechol-galactoside **3b** β-stereochemistry was confirmed, as indicated by the spectral values: δ 5.46 (d, 8.0 Hz, 1H, H-1) and δ 99.3 (C-1).

Our previous results as well as those of others indicated that the methyl group of acetate residues linked to phenols showed a clear downfield shift (2.3 ppm) in ^1H NMR spectra in comparison with the same residue linked to sugars (about 2 ppm).[45,49] Values of around 2.3 ppm were clearly seen to be distinct in the ^1H spectra of the 3 glycosides synthesized in this study. The signals in ^{13}C NMR spectra (around 20 ppm) confirmed such results also for 2-acetoxy-4-nitrophenyl (2,3,4,6-tetra-*O*-benzoyl)-β-D-galactopyranoside **3b**. In this way, the monoglycosylation of 4-nitrocatechol was confirmed. Usually, the glycosylation of dihydroxy phenols (hydroquinone, resorcinol, 4,4'-dihydroxybiphenyl) by this method leads to complete glycosylation, i.e. diglycosylated phenol.[48] However, in the case of 4-nitrocatechol, the major products were monoglycosyl glycosides. This result could be attributed to the different nucleophilicity of the 2 hydroxy groups and/or to steric hindrance due to the vicinity of the 2 hydroxyl groups. The molar ratio, as determined by chemical means, between sugar and phenol was 1:1 for all 3 glycosides.

The glycosylation catalyzed by $BF_3 \cdot OBu_2$ gave 2-acetoxy-4-nitrophenyl (2,3,4,6-tetra-*O*-acetyl)-α-D-glucopyranoside **1b** in 53% yield and 2-acetoxy-4-nitrophenyl (2,3,4,6-tetra-*O*-acetyl)-α-D-mannopyranoside **2b** in 52% yield. The corresponding unprotected glycosides were obtained in 92% and 87% yields, respectively. The conversion of penta-*O*-benzoyl α,β-D-galactopyranose to **3** gave a 90% yield. The glycosylation yield was, in this case, 43% and unprotected glycoside **3c** was obtained in 85% yield.

This paper demonstrates that glycosides **1c**, **2c**, and **3c** based on 4-nitrocatechol constitute excellent chromogenic substrates for exoglycosidases. The values of enzymatic activities determined with 4-nitrocatechol-glycosides **1c**, **2c**, and **3c** (Table) are of the same order of magnitude as enzymatic activities determined with 4-nitrophenyl glycosides.[56] Boiling of the enzymatic solution abolished the biocatalytic activity. In the case of plants, we worked with germs after 48–72 h of imbibition. A β-glucosidase acting on a series of natural and artificial substrates was isolated from wheat seedlings.[62] In the same tissue we found α-glucosidase, α-mannosidase, and β-galactosidase. Leguminous plants are recognized as a source of α-mannosidase.[55,63,64] However, in bean germs (*Phaseolus vulgaris*) we found even higher activities for α-glucosidase and β-galactosidase. Furthermore,

a β-galactosidase,[65] α-glucosidase, and α-mannosidase were found in radish germs (Table). To the best of our knowledge, this is the first report in the chemical literature where glycosides based on 4-nitrocatechol were used as chromogenic substrates for exoglycosidases.

Table. Relative activities of crude enzymatic extracts from different living materials on synthetic glycosides: 2-hydroxy 4-nitrophenyl-α-D-glucopyranoside (**1c**), -α-D-mannopyranoside (**2c**), and -β-D-galactopyranoside (**3c**). The enzymatic activities were estimated as nmol $\times$ min^{-1} $\times$ mg protein^{-1}.

Nr. crt.	Biological source	Enzyme		
		α-Glucosidase	α-Mannosidase	β-Galactosidase
		Specific activity		
1.	Turkey (*Meleagris gallopavo*) testes	5.15	3.76	2.14
2.	Carp (*Cyprinus carpio*) liver	27.2	6.31	8.89
3.	Carp (*Cyprinus carpio*) spleen	29.9	23.6	7.13
4.	Carp (*Cyprinus carpio*) intestine	23	1.53	9.15
5.	Snail (*Helix pomatia*)	104.7	87.2	18.67
6.	Radish (*Raphanus sativus*)	12.9	73.7	3.62
7.	Bean (*Phaseolus vulgaris*)	29.5	9.4	25.8
8.	Chick pea (*Cicer arietinum*)	21.7	20.1	26.4
9.	Wheat (*Triticum vulgare*)	110.6	14.9	22.3
10.	Aubergine (*Solanum melongena*)	1.79	19.6	2.23
11.	Sugar beet (*Beta vulgaris*)	73.9	11.8	5.88
12.	Melon (*Cucumis melo*)	47.9	86.1	12.8
13.	Sorghum (*Sorghum saccharatum*)	7.6	5.19	2.12
14.	*Penicillium fellutanum*	18.9	6.12	3.14
15.	*Candida albicans* (ATCC[1] 10231)	2.79	n. d.*	n. d.
16.	*Bacillus subtilis* (ATCC[1] 6633)	1.17	n. d.	n. d.

*n. d. = not determined

Chromogenic and fluorogenic substrates have been used in various important activities. Detection, identification, and enumeration, including bacterial diagnostics, were based on glycosides constructed with a large variety of aglycons.[17,20–23,66–68]

Elaboration of enzyme mixtures with an optimum cellulosolytic activity, in view of industrial valorification of cellulose, was based on chromogenic and fluorogenic substrates, for instance, 4-methylumbelliferyl β-lactoside and p-nitrophenyl β-D-glucoside.[69–71] Transferase activity of exoglycosidases has become a beneficial technique in organic chemistry.[70,72,73] The conclusion drawn after synthesis and analysis of about 50 glycosides was that 4-methylumbelliferyl and resorufine β-D-galactopyranoside were less efficient donors in comparison with glycosides having a mononuclear aglycon.[73]

Enzymatic diagnosis of a series of metabolic diseases was based on chromogenic or fluorogenic substrates.[8,24,74–78] The activity of extracellular enzymes in aquatic habitats and in marine sediments; the variation in exocellular enzymatic activities in marine environments; the rate of metabolic circuit of organic matter, especially polysaccharides, in peatlands, normal soils, or lake sediments, and pelagic marine bacteria were also evaluated using chromogenic and fluorogenic enzymatic substrates.[16,28,79–81]

4. Conclusion

Three new substrates were synthesized by the glycosylation of 4-nitrocatechol, either with peracetylated monosaccharides in the presence of $BF_3 \cdot OBu_2$ as a promoter or with a perbenzoylated glycosyl bromide and

sodium phenoxide. The structure of the synthetic glycosides was confirmed by NMR spectroscopy. The 3 chromogenic glycosides proved to be remarkably good substrates for enzymes of animal, vegetal, or microbial origin.

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