SYNTHESIS OF CHROMOGENIC PROBES FOR DETECTION AND ASSAY OF DIGLYCOSIDASES

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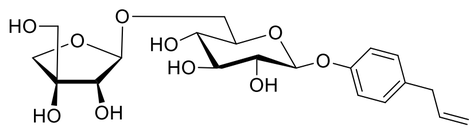
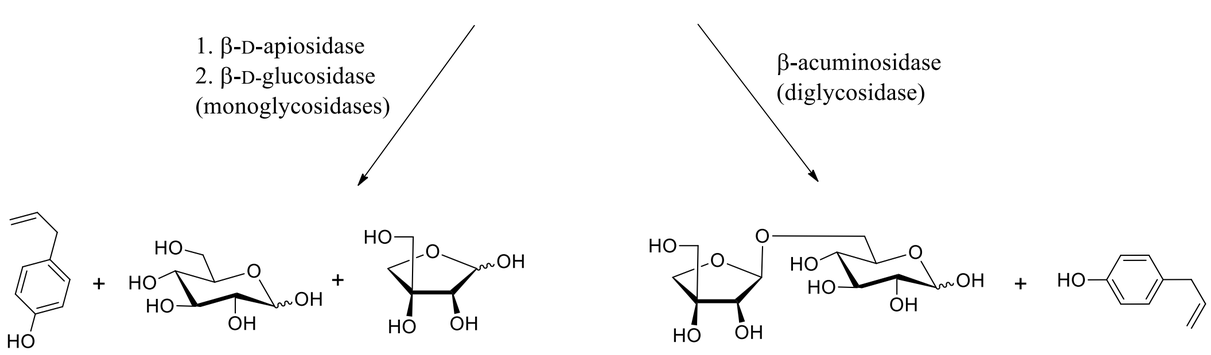
Introduction

Contrary to common glycosidases (monoglycosidases), diglycosidases do not hydrolyze the glycoside bond between the two monosaccharide units of natural diglycosides stepwise. Instead, they release the whole disaccharide in one step (Scheme 1).[1]

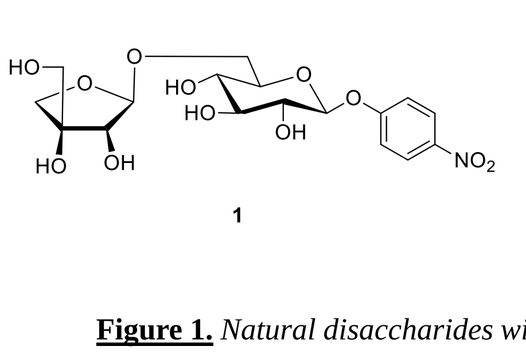




Scheme 1. *Comparison of stepwise degradation of a plant defense diglycoside furcatin by monoglycosidases β-D-apiosidase and β-D-glucosidase, vs. the straightforward furcatin hydrolysis by diglycosidase furcatin hydrolase (β-acuminosidase)*[2]

This unique property makes them very promising biocatalysts, not only in preparation of fine disaccharides, like rutinose, primeverose acuminose, robinobiose etc. from natural feedstocks, but also in synthesis of diglycosides via the transglycosylation process. These diglycosidases find use in beverage and food industry due to their ability to hydrolyze insoluble bitter flavonoids in citrus drinks or release aroma aglycons during wine or tea fermentation and fruit ripening.[3] Glycosides with chromogenic aglycone are convenient substrates for detection and quantification of activity of glycosidases in general, thanks to the possibility of colorimetric measurements. Since these substrates do not occur in nature, their chemical synthesis is the best option for further biotechnological research.

This work presents preparation of two chromogenic diglycosidase substrates - 4-nitrophenyl β-acuminoside **(1)** and 2-nitrophenyl β-robinobioside **(2)** (Figure 1)**.** These substrates are intended for use in screenings of acuminosidase and robinobiosidase and for assaying their activity.



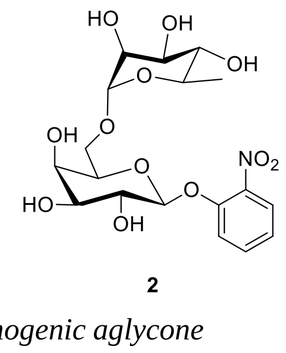
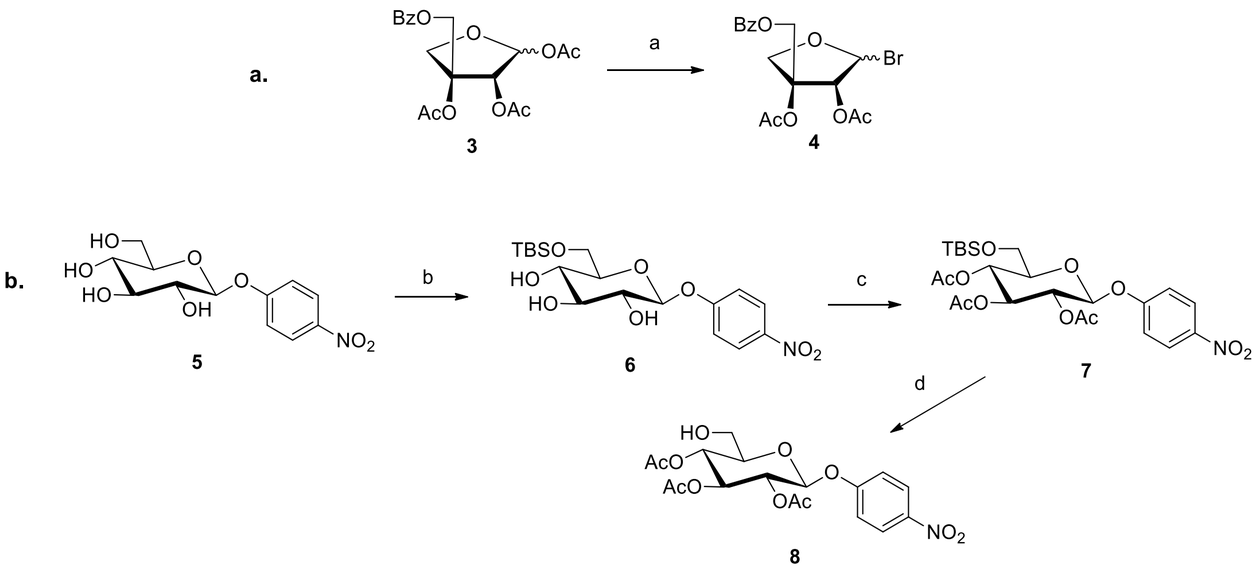


Figure 1. *Natural disaccharides with chromogenic aglycone*

Results and discussion

Acuminoside 1

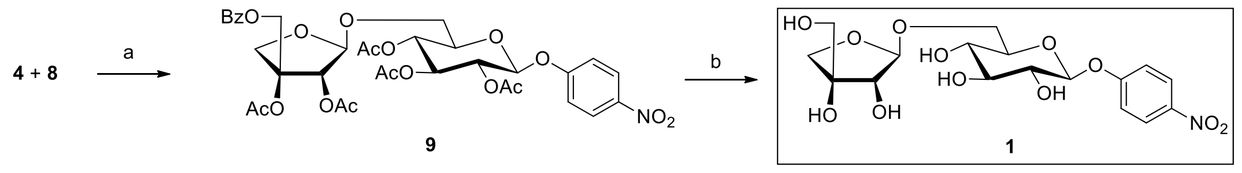
In our synthesis of 4-nitrophenyl acuminoside **(1)**, glycosyl donor was synthesized from 1,2,3-tri-*O*-acetyl-5-*O*-benzoyl-α,β-D-apiofuranoside (**3)** using TMS bromide as bromination agent, quantitively yielding apiofuranosyl bromide **4**, which was used in the glycosylation without isolation (Scheme 2a).[4] The glycosyl acceptor was prepared from commercially available 4-nitrophenyl β-D-glucoside (**5**). Reaction with TBS chloride yielded a 6-*O* silylated glucoside **6**, which was then subjected to protection of the remaining hydroxyl groups with acetic anhydride in pyridine resulting in fully protected glucopyranoside **7**. Final step of the preparation of the glycosyl acceptor was selective deprotection of the primary hydroxyl with silica supported perchloric acid. Catalytic acid conditions caused, that the reaction proceeded extremely quickly with nearly quantitative yield (Scheme 2b).



Scheme 2. *Reagents and conditions:* (**a**) TMSBr, CH_2Cl_2, 0°C→rt, 17 h, no isolation; (**b**) TBSCl, Et_3N, DMAP, THF, rt, 21 h, 97%; (**c**) Ac_2O, pyridine, rt, 1 h, 91%; (**d**) $HClO_4.SiO_2$, CH_3CN, rt, 15 min, 99%

References:
[1] Mazzaferro, L. S.; Breccia, J. D. *Biocat. Biotrans.* **2011**, *29*, 103-112
[2] a) Hemingway, K. M.; Alston, M. J.; Chappel, C. G.; Taylor, A. J. *Carbohydr. Polym.* **1999**, *38*, 283-286; Ma, S. J.; Mizutani, M.; Hiratake, J.; Hayashi, K.; Yagi, K.; Watanabe, N.; Sakata, K. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 2719-2729
[3] Ahn, Y. O.; Mizutani, M.; Saino, H.; Sakata, K. *J. Biol. Chem.* **2004**, *279*, 23405-23414
[4] Gillard, J. W.; Israel, M. *Tetrahedron Lett.* **1981**, *22*, 513–516.
[5] Zhang, J.; Kong, F. *J. Carbohydr. Chem.* **2002**, *21*, 89-97

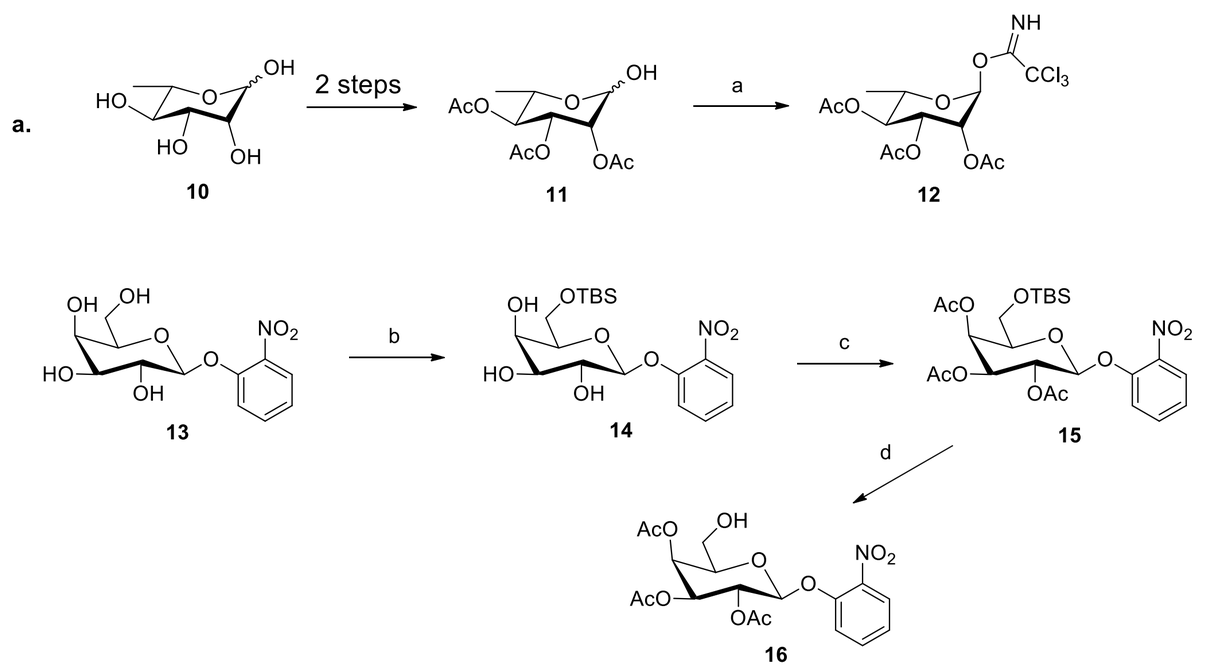
Glycosylation was carried out using Koenigs-Knorr conditions with zinc carbonate as promoter. Product **9** was obtained in 68% yield. Final deacetylation in Zemplén conditions afforded 4-nitrophenyl β-D-apifuranosyl-(1→6)-β-D-glucopyranoside (4-nitrophenyl acuminoside) (**1**) in 63% yield (Scheme 3). Both of these steps were problematic in terms of isolation due to the occurrence of remaining unreacted glycosyl acceptor. The reaction mixture after deacetylation, comprising of 4-nitrophenyl acuminoside (**1**) and 4-nitrophenyl β-D-glucoside (**5**), was treated with almond meal, which contains β-glucosidase, to selectively hydrolyze the unreacted 4-nitrophenyl β-D-glucoside to glucose, which allowed isolation of pure acuminoside **1.**



Scheme 3. *Reagents and conditions:* (**a**) $[ZnCO_3]_2.[Zn(OH)_2]_3$, PhMe, 4Å MS, Ar, 60°C, 2 h, 68%; (**b**) MeONa, MeOH, rt, 63%

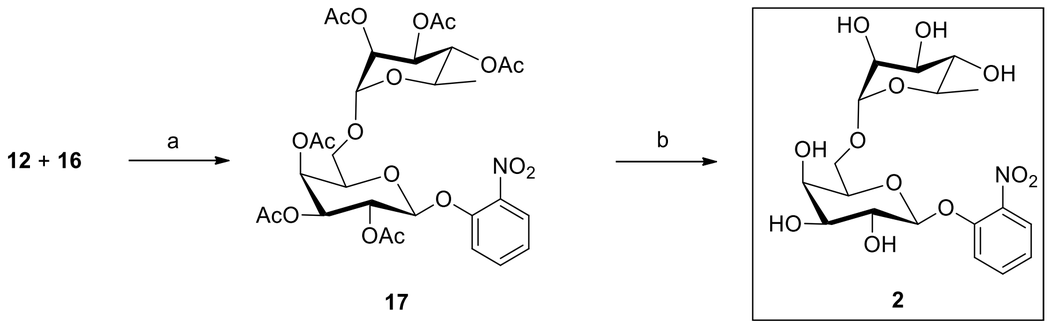
Robinobioside 2

The synthesis of robinobioside employed a different approach for glycosylation, since our attempts to use Koenigs-Knorr method were not successful. Addition of trichloroacetonitrile in basic conditions to 3,4,5-tri-*O*-acetylated rhamnopyranoside **11**, which was obtained from L-rhamnose **10** in two steps, afforded trichloroacetimidate **12** as glycosyl donor (Scheme 4a).[5] In the preparation of glycosyl acceptor **16**, we followed similar procedure to glucoside **8** – silylation-acetylation-desilylation sequence. Desilylation of primary hydroxyl group of galactoside **16** was tricky, due to the migration of acetyls from axial 4-OAc to primary hydroxyl 6-OH. Several desilylation methods were tried out, and only TBAF/HOAc conditions afforded satisfactory results. The reaction proceeded almost quantitatively, however a small amount of migration by-product was observed. Difficult separation of acetyl migration product entailed, that only a small amount of pure glycosyl acceptor was isolated (Scheme 4b).



Scheme 4. *Reagents and conditions:* (**a**) trichloroacetonitrile, DBU, CH_2Cl_2, rt, 3 h, 52%; (**b**) TBSCl, Et_3N, DMAP, THF, rt, quant. yield (**c**) Ac_2O, DMAP, pyridine, 0°C, 1,5 h, 77%; (**d**) TBAF, AcOH, DMF, rt, 3 h

Donor **12** and acceptor **16** were substrates ready for glycosylation in Schmidt conditions. Our attempts to use $BF_3.Et_2O$ as promoter provided unsatisfactory results. Reaction did not proceed after increasing the temperature and reaction time, or even adding excess $BF_3.Et_2O$. Changing the promoter to TMSOTf resulted in a near quantitative conversion (no unreacted glycosyl acceptor was observed on TLC) in 30 min at -20°C reaction temperature. Final deprotection of **17** with sodium methanolate afforded the final 2-nitrophenyl β-D-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (2-nitrophenyl robinobioside) (**2**) in 95% yield (Scheme 5).



Scheme 5. *Reagents and conditions:* (**a**) TMSOTf, DCM, 4Å MS, Ar, -20°C, 30 min, 87%; (**b**) MeONa, MeOH, rt, 95%

Conclusion

This work presents multistep gram-scale synthesis of two chormogenic glycosides derived from natural disaccharides acuminose and robinobiose. Two different ways of glycosylation had to be used as the key step in synthesis of the respective disaccharide motifs. The glycosylations proceeded stereoselectively in good chemical yields. Pure beta anomers were obtained, making these molecules ready as substrates for screening and assays of the demanded diglycosidases β-acuminosidase and β-robinobiosidase.

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