Video Article

Synthesis of Indoxyl-glycosides for Detection of Glycosidase Activities

Stephan Böttcher¹, Joachim Thiem¹
¹Department of Chemistry, Faculty of Sciences, University of Hamburg

Correspondence to: Joachim Thiem at thiem@chemie.uni-hamburg.de

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Abstract

Indoxyl glycosides proved to be valuable and versatile tools for monitoring glycosidase activities. Indoxyls are released by enzymatic hydrolysis and are rapidly oxidized, for example by atmospheric oxygen, to indigo type dyes. This reaction enables fast and easy screening *in vivo* without isolation or purification of enzymes, as well as rapid tests on agar plates or in solution (e.g., blue-white screening, micro-wells) and is used in biochemistry, histochemistry, bacteriology and molecular biology. Unfortunately the synthesis of such substrates proved to be difficult, due to various side reactions and the low reactivity of the indoxyl hydroxyl function. Especially for glucose type structures low yields were observed. Our novel approach employs indoxylic acid ester as key intermediates. Indoxylic acid esters with varied substitution patterns were prepared on scalable pathways. Phase transfer glycosylations with those acceptors and peracetylated glycosyl halides can be performed under common conditions in high yields. Ester cleavage and subsequent mild silver mediated glycosylation yields the peracetylated indoxyl glycosides in high yields. Finally deprotection is performed according to Zemplén.

Video Link

The video component of this article can be found at <http://www.jove.com/video/52442/>

Introduction

For a long time the production of indigo was an economically very important process. Before large scale chemical syntheses gave cheap access to indigo, precursors were obtained from natural sources since pre-Christian times. The cultivation of indigo providing plants (natural indigo) in Europe became unrewarding in the 17th century, as the amount of indigo precursors of the Indian indigo plant (0.2-0.8 %) is about 30 times higher. At the end of the 19th century chemical synthesis of indigo suppressed the conventional cultivation^{1,2}.

Indigo precursors occurring naturally in plants include Indican (1), Insatan A (2) and Isatan B (3) (**Figure 1**). All of them consist of an indoxyl motive linked to a glycosyl residue. Cleavage of the glycosidic linkage, for example by enzymatic hydrolysis, leads to release of indoxyl (4). Indoxyl itself is almost colorless, but can be rapidly oxidized to form an indigo dye (5). This sensitive reaction has been adapted in biochemistry, histochemistry, bacteriology and molecular biology for monitoring enzyme activities. Activity screening *in vivo* without isolation or purification of enzymes, as well as rapid tests on agar plates or in solution (e.g., blue-white screening, micro-wells) is possible. Depending of the residue (e.g., esters, glycosides, sulfates) linked to the indoxyl moiety, suitable substrates for different enzyme classes (e.g., esterases, glycosidases, sulfatases) have been developed³. In the following focus will be on formation and application of indoxyl glycosides.

Figure 1: Natural indigo precursors and formation of indigo dye by hydrolysis. [Please click here to view a larger version of this figure.](#)

The substitution pattern of the indoxyl moiety determines the color and physical properties of the resulting indigo dye. The most common substitution patterns are 5-bromo-4-chloro (abbreviated by X; greenish-blue), 5-bromo (blue) and 5-bromo-6-chloro (magenta), since these form the smallest dye particles, do not form granules and have the least diffusion from sites of hydrolysis. The last property is especially important for *in vivo* experiments³.

The first report of an indigogenic method for detection of esterase activity was published in 1951 by Barnett and Seligman, who employed indoxyl acetate and butyrate⁴. About one decade later the indigogenic principle was adapted for localization of mammalian glucosidase⁵. Up to now several indoxyl glycosides have been developed even though their synthesis proved to be difficult. Most syntheses are based on employing an *N*-acetylated indoxyl as acceptor and the respective glycosyl halide donor⁶⁻¹⁴. Glycosylation is performed in acetone with sodium hydroxide. Under these conditions a number of side reactions occur, decreasing the yield significantly. Especially for glucose type structures very low glycosylation yields were reported (e.g., 15% for (*N*-acetyl-5-bromo-4-chloro-indol-3-yl)-2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranoside⁶ and 26% for (*N*-acetyl-5-bromo-4-chloro-indol-3-yl)-2,3,2',3',4'-penta-*O*-acetyl- β -D-xylobioside¹⁴ in a more recent example). Through a novel approach, employing indoxyl acid esters, a considerable number of indoxyl glycosides were prepared in good yields (e.g., (*N*-acetyl-5-bromo-4-chloro-indol-3-yl)-2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranoside 57% yield).

The following protocol describes the straightforward synthesis of indoxyl acid allyl ester (5-bromo-4-chloro) and based thereon the synthesis of an indoxyl glycoside (X-Gal). A simple model experiment shows the enzyme reactivity of β -galactosidase employing X-Gal.

Protocol

General remarks:

All reactions were carried out using a fume hood and appropriate personal protective equipment. All reagents and solvents (p.a.; water content for dry solvents: MeCN <50 ppm; Et₂O <0.01%; CH₂Cl₂ <0.003%; THF <0.005%) were purchased from commercial sources and used as received. TLC was performed on Merck silica gel 60 F₂₅₄ plates. Compounds were detected by UV and/or by treatment with EtOH/H₂SO₄ (9:1)

and subsequent heating. Column chromatography was performed with Merck/Fluka silica gel 60 (230-400 mesh). Filtrations were performed using Büchner funnel and aspirator pump. Solvents were removed employing a rotary evaporator (40 °C, ~200 rpm). Products were dried in high vacuum (rotary vane pump). Reactions requiring dry solvents were carried out under argon atmosphere.

In the following the synthesis of X-Gal (16) employing 5-bromo-4-chloro-indoxyl acid allyl ester (12) is described. For further compounds with varied substitution patterns as well as methyl esters prepared according to this synthetic sequence see ref.¹⁷⁻¹⁹.

1. Acceptor: Synthesis of 5-Bromo-4-Chloro-Indoxyl Acid Allyl Ester¹⁸ (12)

1. *N*-Acetylation: Synthesis of *N*-(4-bromo-3-chloro-2-methylphenyl)-acetamide (6)

1. Dissolve 10.0 g (45.7 mmol) of 4-bromo-3-chloro-2-methylaniline in 30 ml dichloromethane.
Caution! Dichloromethane is harmful and cool the solution in an ice-bath.
2. Add 6.50 ml (68.7 mmol) acetic anhydride dropwise and allow the mixture to warm to room temperature. The mixture can be stirred for 8 hr or overnight. Caution! Acetic anhydride is corrosive and flammable.
3. Remove the solvent and recrystallize from 9:1 ethyl acetate/methanol (yield: 97% (11.6 g, 44.2 mmol), colorless solid).
Caution! Ethyl acetate is irritant and flammable. Caution! Methanol is toxic and flammable.

2. Oxidation: Synthesis of *N*-acetyl-5-bromo-6-chloro-anthranilic acid (7)

1. Reflux a mixture of 2.30 g (8.76 mmol) of 6 (product obtained from step 1.1) and 2.10 g (8.52 mmol) MgSO₄•7H₂O in 40 ml water in a 3-neck round bottom flask equipped with cooler and dropping funnel.
2. Add dropwise 50 ml saturated KMnO₄ solution in water to the reaction mixture over a period of 3 hr. Then heat for 2 additional hr under reflux, ca. 135 °C oil bath. Caution! Potassium permanganate is harmful and oxidising.
3. Cool to room temperature and filter the pyrolusite (MnO₂), which is formed during the reaction.
Caution! Pyrolusite is harmful.
4. Add hydrochloric acid (37%) to the filtrate until pH 1, then filter the product and dry in vacuum, (yield: 70% (1.80 g, 6.15 mmol), colorless solid).
Caution! Hydrochloric acid is corrosive.

3. Deacetylation: Synthesis of 5-bromo-6-chloro-anthranilic acid (8)

1. Dissolve 3.90 g (13.3 mmol) of 7 (product obtained from step 1.2) in 70 ml of 1 N aqueous sodium hydroxide solution and heat at ca. 100 °C (oil bath) for 5 hr.
Caution! Sodium hydroxide is corrosive.
2. Cool to room temperature and add hydrochloric acid (37%) until pH 1, then filter the product with a Büchner funnel and dry in vacuum, (yield: 88% (2.93 g, 11.7 mmol), colorless solid).

4. Anhydride formation: Synthesis of 5-bromo-6-chloro-isatoic anhydride (9)

1. Suspend 8.17 g (32.6 mmol) of 8 (product obtained from step 1.3) in 33 ml dry acetonitrile.
Caution! Acetonitrile is harmful and flammable.
2. Stir at room temperature and add 5.3 ml and a solution of 3.22 g (10.8 mmol) triphosgene in 18.5 ml dry dichloromethane dropwise simultaneously over a period of 30 min. Then heated for 3 hr at 50 °C (oil bath). Caution! Pyridine is harmful and flammable.
Caution! Triphosgene is toxic and can release phosgene.
3. Remove about 75% of the solvent and quench the reaction by adding 100 ml distilled water and filter your product. Wash with a small amount of cold dichloromethane.
4. Dry the product in vacuum, (yield: 88% (7.53 g, 27.2 mmol), colorless solid).

5. *N*-Alkylation: Synthesis of 5-bromo-6-chloro-*N*-[(methoxycarbonyl)allyl]-isatoic anhydride (10)

1. Dissolve 25 g (90 mmol) of 9 (product obtained from step 1.4) in 250 ml anhydrous dimethylformamide. and cool with an ice-bath.
Caution! Dimethylformamide is harmful and flammable
2. Add 1.15 equivalents of sodium hydride (60% in paraffin) portion wise.
Caution! Sodium hydride is corrosive and flammable; heavy reaction with water/ice.
3. After 30 min of stirring at 0 °C allow warming to room temperature and add 1.2 equivalents allyl bromoacetate dropwise.
4. After stirring for 5 hr quench the reaction by adding 500 ml distilled water and filter your product.
5. Wash with water (3 times, 100 ml) and dry the product in vacuum, (yield: 93% (31.5 g, 84.1 mmol), colorless solid).

6. Anhydride Opening: Synthesis of 5-bromo-6-chloro-*N*-(methoxycarbonylallyl)-anthranilic acid allyl ester (11)

1. Dissolve 10 g (27 mmol) of 10 (product obtained from step 1.5) in 100 ml allyl alcohol.
Caution! Allyl alcohol is harmful and dangerous for the environment.
2. Add 350 mg (8.70 mmol) sodium hydride (60% in paraffin) portion wise.
3. After stirring for 6.5 hr or overnight remove the solvent.
4. Subject the crude product to column chromatography (column 30 x 6 cm; 240 g silica) petroleum ether/ethyl acetate 2:1; R_F = 0.66), (yield: 80% (8.30 g, 21.3 mmol), yellow oil).
Caution! Petroleum ether is flammable, irritant and dangerous for the environment.

7. Dieckmann Condensation: Synthesis of 5-bromo-4-chloro-indoxyl acid allyl ester (12)

1. Mix 3.0 g (7.7 mmol) of 11 (product obtained from step 1.6) and 1.75 g (15.6 mmol) of potassium *tert*-butoxide in 100 ml dry diethyl ether.
Caution! Potassium *tert*-butoxide is corrosive and flammable.
Caution! Diethyl ether is harmful and extremely flammable.
2. Heat the mixture under reflux (cooler) for 2 hr at 40-45 °C.
3. Remove about 75% of the solvent, and add diluted hydrochloric acid (100 ml, 1 M).

Caution! Do not dry completely, decomposition can occur.

4. Filter the product and dry in vacuum, (yield: 84% (5.14 g, 15.5 mmol), colorless to slightly green solid).

2. Indoxyl-Glycoside: Synthesis of X-Gal¹⁷

1. Phase Transfer Glycosylation: Synthesis of (5-bromo-4-chloro-indox-3-yl)ic acid allyl ester) 2,3,4,6-tetra-O-acetyl- β -D-galactopyranoside (14)

1. Mix 400 mg (1.21 mmol) of the indoxyl acid allylester 12 (product obtained from step 1.7), 410 mg (1.21 mmol) tetrabutylammonium hydrogensulfate and 500 mg (1.21 mmol) of the donor 2,3,4,6-tetra-O-acetyl- α -D-galactopyranosyl bromide (α -acetobromogalactose) in 10 ml dichloromethane. Caution! Tetrabutylammonium hydrogensulfate is harmful.
2. Add 10 ml of an aqueous solution of potassium carbonate (1 M). Caution! Potassium carbonate is corrosive.
3. Stir at room temperature until complete consumption of the donor (TLC: petroleum ether/ethyl acetate).
4. Separate the organic phase (separatory funnel), dry over Na_2SO_4 and remove the solvent.
5. Subject the crude product to column chromatography (petroleum ether/ethyl acetate 1:1; R_F = 0.33). (yield: 86% (690 mg, 1.04 mmol), colorless solid).

2. Ester Cleavage and Decarboxylation: Synthesis of (N-acetyl-5-bromo-4-chloro-indol-3-yl) 2,3,4,6-tetra-O-acetyl- β -D-galactopyranoside (15)

1. Dissolve 600 mg (0.908 mmol) of 14 (product obtained from step 2.1) in 15 ml dry tetrahydrofuran. Caution! Tetrahydrofuran is harmful and flammable.
2. Add 800 μl morpholine and 105 mg (9.09 μmol) tetrakis(triphenylphosphine)palladium(0) and stir overnight at room temperature. Caution! Morpholine is flammable and corrosive.
3. Remove the solvent.
4. Add 400 mg (2.40 mmol) silver acetate, 800 mg (5.79 mmol) potassium carbonate and 10 ml acetic anhydride. Caution! Silver acetate is irritant and dangerous for the environment.
5. Heat at 90-100 °C for 20 min.
6. After cooling to room temperature dilute with dichloromethane.
7. Wash the mixture twice with water and once with a diluted NaHCO_3 solution (10%). Caution! Sodium bicarbonate is corrosive.
8. Dry the organic phase over Na_2SO_4 (filter) and remove the solvent.
9. Subject the crude product to column chromatography (PE/EA 1:1; R_F = 0.24), (yield: 88% (495 mg, 8.00 mmol), colorless solid).

3. Zemplén Deacetylation: Synthesis of (5-bromo-4-chloro-indol-3-yl)- β -D-galactopyranoside (16)

1. Dissolve 396 mg (0.436 mmol) 15 (product obtained from step 2.2) in 10 ml methanol.
2. Add a catalytic amount of sodium methanolate and stir overnight at room temperature. Caution! Sodium methanolate is flammable and corrosive.
3. Neutralize with Amberlite IR-120 H^+ (filter) and concentrate. Caution! Amberlite IR-120 H^+ is irritant.
4. Dry the product in vacuum, (yield: 87% (170 mg, 0.416 mmol), colorless solid).

3. Illustrating Model Experiment: Activity proof of β -galactosidase employing X-Gal

1. Prepare a 0.5 mM solution of X-Gal in 500 μl buffer (Tris-HCl 20 mM; pH 7.4; 50 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid; or any other suitable buffer for your enzyme) in a microcentrifuge tube (X-Gal can be dissolved in a few μl of dimethylsulfoxide).
2. Add 1 μl β ,1-3-galactosidase (EC 3.2.1.23 10,000 U/ml; or any other suitable β -galactosidase) and shake (600 rps) at 37 °C.
3. Follow the reaction. The indigo type dye is formed after a short period of time.

Representative Results

The very first syntheses of indicane and 5-bromo-indicane, were published by Robertson already in 1927 and 1929^{15,16}. By employing indoxyl acid methyl ester as acceptor, the reactive 2-position was blocked and thus side reactions were partially suppressed. Glycosylation in acetone/sodium hydroxide, following deprotection and decarboxylation (160 °C, acetic anhydride, 1 hr) and finally deacetylation yielded Indicane and 5-bromo-indicane. Based on this concept we developed an improved synthesis of indoxyl glycosides, employing indoxyl acid allyl esters as acceptors¹⁷⁻¹⁹. The acceptor synthesis starts with the respective substituted aniline derivative (5) (Figure 2). After acetylation of the amino function (6), oxidation with potassium permanganate yielded the N-acetylated anthranilic acid derivatives (7). Then the acetyl protecting group was removed (8) and by reaction with triphosgene the respective isatoic anhydride obtained (9). N-Alkylation could be easily performed by deprotonation with sodium hydride and subsequent treatment with allyl bromoacetate (10). The anhydride was then opened with allyl alcohol (11), and finally ring closure was performed in a Dieckmann condensation (12). With one exception (11), all compounds were purified by recrystallization, precipitation or washing. The indoxyl acid allyl ester was obtained in 30% overall yield (seven steps).

Figure 2: Synthetic sequence towards 5-bromo-4-chloro-indoxylic acid allyl ester. Reagents and conditions: (i) dichloromethane, acetic anhydride, 0 °C - rt, 5 hr; (ii) H₂O, MgSO₄, KMnO₄, reflux, 6 hr; (iii) sodium hydroxide (1 N), reflux, 5 hr; (iv) acetonitrile, dichloromethane, pyridine, triphosgene, 50 °C, 4 hr; (v) dimethylformamide, sodium hydride (60% paraffin), allyl bromoacetate, 0 °C - rt, 5 hr; (vi) allyl alcohol, sodium hydride, rt, overnight; (vii) diethyl ether, potassium *tert*-butoxide, reflux, 2 hr. [Please click here to view a larger version of this figure.](#)

After high yielding phase transfer glycosylation (14) of the indoxylic acid allyl ester (12) under common conditions with a peracetylated glycosyl halide (13), the allyl ester was cleaved selectively with a palladium catalyst and morpholine. The subsequent decarboxylation was performed under significant milder conditions (90-110 °C, 20-30 min) without purification of the starting material (15). Finally the acetyl protecting groups were removed (16) according to Zemplén employing sodium methanolate in anhydrous methanol. This way a three step glycosylation procedure gave the final indoxyl galactopyranoside component (16) in 66% overall yield. In a former attempt the synthesis of a corresponding indoxyl galactopyranoside resulted in 28% yield⁶.

Figure 3: Synthetic sequence towards X-Gal. Reagents and conditions: (i) dichloromethane, potassium carbonate (1 M), tetrabutylammonium hydrogensulfate, rt, 3-5 hr (TLC); (ii) (a) tetrahydrofuran, morpholine, tetrakis(triphenylphosphine)palladium(0), rt, overnight; (b) acetic anhydride, silver acetate, potassium carbonate, 90-110 °C, 20 min. [Please click here to view a larger version of this figure.](#)

Figure 4: Blue-white screening: An agar plate containing the respective indoxyl glycoside is treated with bacteria colonies expressing the corresponding matching enzyme. The released indoxyl is oxidized to give the indigo dye which occurs as blue spots. [Please click here to view a larger version of this figure.](#)

positive reaction

Figure 5: Enzyme activity monitoring in micro wells. The upper row contains 0.1 mM of X-glycoside, the lower row has a concentration of 1 mM of X-glycoside. The blue color occurs only in the wells containing the enzymes to cleave the glycosidic linkage of the X-glycoside. A concentration of 0.1 mM is already sufficient. [Please click here to view a larger version of this figure.](#)

Figure 6: Enzyme activity monitoring in Eppendorf tubes. After a short period of time the blue color evolves and is getting more intense. In the end the indigo dye precipitates. [Please click here to view a larger version of this figure.](#)

Discussion

Owing to poor yields and limitations, especially for glucose type structures and more complex saccharides, a novel synthetic approach towards indoxyl glycosides was developed. Indoxylic acid esters proved to be precious key intermediates and were obtained in a modular, scalable pathway. All steps are high yielding and due to cheap starting materials and easy workup multi-gram syntheses are possible. The advantage of the allyl ester approach is the blocking of the reactive 2-position. Thus yield decreasing side reactions are suppressed. Employing allyl esters allows selective cleavage, which is especially important on substrates containing a carboxyl function.

Phase transfer glycosylation of these acceptors was performed with the respective peracetylated glycosyl halide and succeeded in high yields under common conditions. Afterwards the allyl ester was cleaved selectively with a palladium catalyst, and the crude product was subjected to mild silver mediated decarboxylation. This is the key step of the synthesis. It was especially important to keep the reaction time short and the temperature as low as possible. The best conditions were found employing silver acetate. Finally the acetyl protecting groups were removed as usual according to Zemplén conditions. This way, we could obtain a number of indoxyl glycosides in the best yields to date.

A model experiment showed the enzymatic hydrolysis of the synthesized X-Gal employing a β -galactosidase. The formation of the blue dye in solution clearly indicates enzyme activity. Further elaborations of this concept could deal with α -glycosylations of the indoxylic acid allylesters.

Disclosures

The authors declare that they have no competing financial interests.

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References

- Clark, R. J. H., Cooksey, C. J., Daniels, M. A. M., Withnall, R. Indigo, Woad, and Tyrian Purple: Important Vat Dyes from Antique to the Present. *Endeavour*. **17**, 191-199 (1993).
- Hunger, K. *Industrial Dyes: Chemistry, Properties, Applications*. Weinheim Wiley-VHC (2003).
- Kiernan, J. A. Indigogenic Substrates for Detection and Localization of Enzymes. *Biotechn. Histochem.* **82**, 73-103 (2007).
- Barnett, R. J., Seligman, A. M. Histochemical Demonstration of Esterases by Production of Indigo. *Science*. **114**, 579-582 (1951).
- Pearson, B., Andrews, M., Grose, F. Histochemical Demonstration of Mammalian Glucosidase by Means of 3-(5-Bromoindolyl)- β -D-glucopyranoside. *Exp. Biol. Med.* **108**, 619-623 (1961).
- Anderson, F. B., Leaback, D. H. Substrates for the Histochemical Localization of some Glycosidases. *Tetrahedron*. **12**, 236-239 (1961).
- Van Dort, M. E., Lee, K. C., Hamilton, C. A., Rehemtulla, A., Ross, B. R. Radiosynthesis and Evaluation of 5-[¹²⁵I]iodoindolyl-3-yl- β -D-galactopyranoside ([¹²⁵I]IBDG) as a β -Galactosidase Imaging Radioligand. *Mol. Imaging*. **7**, 187-197 (2008).

8. Yoshida, K., Iino, N., Koga, I. Syntheses of Halogen Substituted β -D-Glucuronides and Their Hydrolysis by Rabbit Liver β -Glucuronidase. *Chem. Pharm. Bull.* **32**, 1759-1769 (1975).
9. Horwitz, J. P., *et al.* Substrates for Cytochemical Demonstration of Enzyme Activity I. Some Substituted 3-Indolyl- β -D-glycopyranosides. *J. Med. Chem.* **7**, 574-575 (1964).
10. Eschenfelder, V., Brossmer, R. 5-Bromo-indol-3-yl 5-Acetamido-3,5-dideoxy- α -D-glycero-D-galactononulopyranosidic Acid, a Novel Chromogenic Substrate for the Staining of Sialidase Activity. *Glycoconjugate J.* **4**, 171-178 (1987).
11. Fujii, I., Iwabuchi, Y., Teshima, T., Shiba, T., Kikuchi, M. X-Neu5Ac: A Novel Substrate for Chromogenic Assay of Neuraminidase Activity in Bacterial Expression Systems. *Bioorg. Med. Chem.* **1**, 147-149 (1993).
12. Berlin, W., Sauer, B. In situ Color Detection of α -L-Arabinofuranosidase, a "No-Background" Reporter Gene, with 5-Bromo-3-indolyl- α -L-arabinofuranoside. *Anal. Biochem.* **243**, 171-175 (1996).
13. Marmuse, L., *et al.* New Chromogenic Substrates for Feruloyl Esterases. *Org. Biomol. Chem.* **6**, 1208-1214 (2008).
14. Kaneko, S., Kiaoka, M., Kuno, A., Hayashi, K. Syntheses of 4-Methylumbelliferyl- β -D-Xylobioside and 5-Bromo-3-Indolyl- β -D-Xylobioside for Sensitive Detection of Xylanase Activity on Agar Plates. *Biosci. Biotechnol. Biochem.* **64**, 741-745 (2000).
15. Robertson, A. J. Syntheses of Glucosides. Part I. The Synthesis of Indican. *Chem. Soc.* 1937-1943 (1927).
16. Robertson, A., Waters, R. B. J. Synthesis of Glucosides. Part VII. The Synthesis of 6-Bromoindican. *Chem. Soc.* 2239-2243 (1929).
17. Böttcher, S., Thiem, J. Indoxyl Acid Esters as Convenient Intermediates Towards Indoxyl Glycosides. *Eur. J. Org. Chem.* 564-574 (2014).
18. Böttcher, S., Hederos, M., Champion, E., Dékány, G., Thiem, J. Novel Efficient Routes to Indoxyl Glycosides for Monitoring Glycosidase Activities. *Org. Lett.* **15**, 3766-3769 (2013).
19. Böttcher, S., Thiem, J. Facile Preparation of Indoxyl- and Nitrophenyl Glycosides of Lactosamine and Isolactosamine. *RSC Adv.* **4**, 10856-10861 (2014).