Carbohydrate Research 430 (2016) 48–53

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Efficient chemoenzymatic synthesis of 4-nitrophenyl β-D-apiofuranoside and its use in screening of β-D-apiofuranosidases

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ARTICLE INFO

Article history:
Received 9 March 2016
Received in revised form 29 April 2016
Accepted 30 April 2016
Available online 3 May 2016

Keywords:
4-Nitrophenyl β-D-apiofuranoside
β-D-Apiofuranosidase
Aspergillus

ABSTRACT

4-Nitrophenyl β-D-apiofuranoside as a chromogenic probe for detection of β-D-apiofuranosidase activity was prepared in 61% yield from 2,3-isopropylidene-α,β-D-apiofuranose through a sequence of five reactions. The synthesis involves one regioselective enzymatic step—benzoylation of primary hydroxyl of 2,3-isopropylidene-α,β-D-apiofuranose catalysed by Lipolase 100T and stereoselective β-D-apiofuranosylation of *p*-nitrophenol using $BF_3{\cdot}OEt_2/Et_3N$. The product was used for screening of β-D-apiofuranosidase activity in 61 samples of crude commercial enzymes and plant materials. Fifteen enzyme preparations originating from different strains of genera *Aspergillus* display β-D-apiofuranosidase activity. The highest activity was found in Rapidase AR 2000 (78.27 U/g) and lyophilized Viscozyme L (64,36 U/g).
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1. Introduction

D-Apiofuranose (2-*C*-(hydroxymethyl)-D-erythrofuranose) is a branched monosaccharide relatively abundant in the plant kingdom. It is a natural component of rhamnogalacturonan II as a binding centre for borate ester bridge between two rhamnogalacturonan II chains.[1–4] D-Apiose also occurs as a structural constituent of plant defence substances,[5] and of different phenylpropanoid glycosides in medicinal plants such as isoliquiritin apioside,[6] luteosides,[7] kelampayosides[8] or cucurbitosides.[9,10]

β-D-Apiofuranosidases (apiosidases) are glycosidases cleaving β-D-apiofuranose residues from the side-chains of rhamnogalacturonan II, or from low molecular plant metabolites. They find practical use for example in wine technology as side-activity of industrial pectinases releasing volatile aroma substances from their glycosidic precursors.[11,12] Due to their relatively marginal role in food and beverage technologies, only a limited number of studies of apiosidases appeared, mostly from the laboratory of Günata,[11,13,14] who used 4-nitrophenyl β-D-apiofuranoside (**1**, Fig. 1) as a chromogenic probe for their assaying. They tested occurrence of this specific glycosidase in only a limited number of commercial enzyme preparations. Production of several of these enzyme preparations was abandoned or they have been renamed. For example, Günata et al. isolated this enzyme from Klerzyme 200, which is now produced under the label Rapidase AR-2000 (DSM, USA).

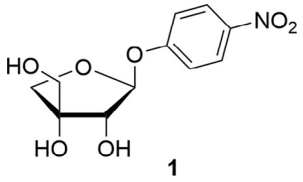

Fig. 1. Structure of 4-nitrophenyl β-D-apiofuranoside.

Our interest in straightforward synthesis of biologically active phenylpropanoid substances harbouring β-apiosyl moiety in the glycon part led us to an idea of enzymatic apiosylation of precursors of the target molecules. Since no pure apiosidase is in the market, extensive search for an inexpensive, easily available source of this enzyme has been started to provide it in reasonable amounts and costs without the necessity of microbial screenings, cultivations and enzyme preconcentration or purification. Our laboratory has a long-lasting experience in screening of interesting enzyme side-activities in present-day crude industrial enzymes or various plant materials and their use in synthetic procedures.[15–22] The purpose of this report is therefore to present our experience with screening of these crude enzyme materials for presence of β-apiosidase, including a new way of synthesis of its chromogenic substrate and modification of the current spectrophotometric method.

2. Results and discussion

2.1. Synthesis of chromogenic substrate

Synthesis of 4-nitrophenyl β-D-apiofuranoside **1** has been reported as early as in 1992, but no preparative details were given

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http://dx.doi.org/10.1016/j.carres.2016.04.030
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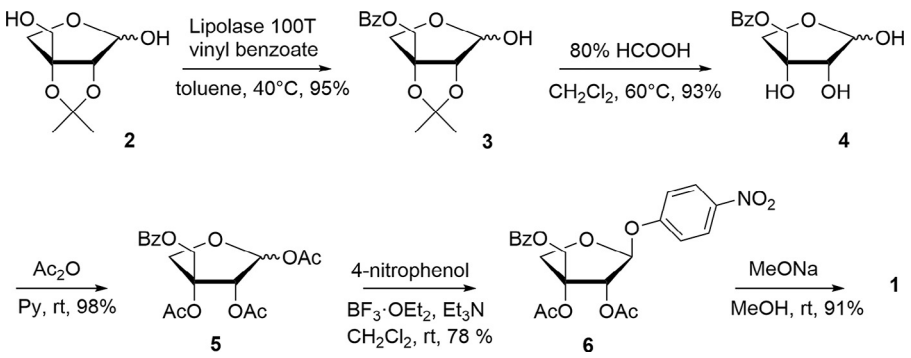


Scheme 1. Synthesis of β-D-apioside **1** from isopropylidenated apiose **2**.

other than a very general methodology.[14] The authors glycosylated 4-nitrophenol by per-O-acetylated D-apiose[23,24] under catalysis with *p*-toluenesulfonic acid. A mixture of α- and β-product (12% and 59%, respectively) was obtained and separated by column chromatography.

We have developed an effective, simple and selective multigram synthesis of 4-nitrophenyl β-D-apiofuranoside **1** from stable and crystalline 2,3-O-isopropylidene-α,β-D-apiofuranose (**2**, Scheme 1).

Obviously, 2,3-O-isopropylidene **2** has been already synthesized from D-mannose,[23–25] D-xylose,[26] L-arabinose[27] as starting compounds and its benzylidene analogue has been prepared from L-ribose.[10] We chose synthesis of **2** from D-mannose as the most convenient and verified way for preparative purposes, following Ho[23] and Hammerschmidt et al.[25]

The necessity of strongly acidic conditions for removal of stable 2,3-O-isopropylidene group of the apiofuranose ring limits usability of **2** for synthesis of apiofuranosides.[4,28] The transformation of **2** to peracylated apiofuranose derivative was therefore required. Compound **2** was firstly acetylated, but acetyl function at primary hydroxyl was rather unstable during removal of 2,3-O-isopropylidene moiety. Previous works[24,29] also reported moderate yields of this reaction (42% and 30%, respectively). We have therefore decided to introduce more stable benzoyl group to preserve the *erythro*-configuration of apiose after removal of isopropylidene (Scheme 1). Authors in the previous work[25] benzoylated **2** at low temperature from −78 °C to −65 °C for 16 hours using benzoyl chloride and pyridine in dichloromethane and obtained **3** in 71% yield. Deisopropylinated product **4** was acetylated without previous purification and **5** was obtained in 79% yield.

Regioselective enzymatic acylation by commercial lipases is becoming a common method in protection strategies of sugars.[30,31] After short screening of available lipases and reaction conditions, the benzoylation of **2** by Lipolase 100T[19] with vinyl benzoate in toluene was found as an appropriate method for preparative purposes. Compound **3** was isolated in 95% yield. After removal of 2,3-O-isopropylidene group from **3** by 80% formic acid, product **4** was obtained in 93% yield and conventionally acetylated in pyridine catalysed with 4-N,N-dimethylaminopyridine. We obtained benzoylated triacetate **5** as a promising donor for glycosylation of *p*-nitrophenol in almost quantitative yield.

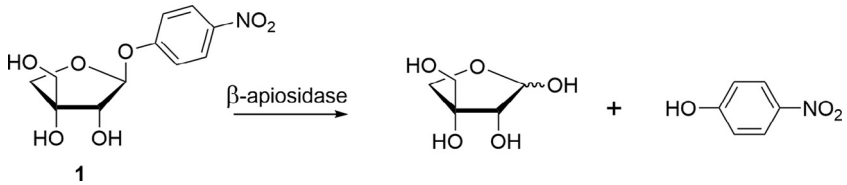
Acetoxy group has been a very convenient anomeric leaving group in glycosylation reactions. Furthermore, the neighbouring group effect of the 2-O-acyl moiety could be used for prevailing formation of β-anomer in 1,2-*trans* glycosylation. Generally, conventional glycosylation methods applied to furanosides showed low stereoselectivity. In our previous experiments, the donor **5** was directly used in glycosylation of *p*-nitrophenol catalysed by Lewis acids $SnCl_4$ and $BF_3{\cdot}OEt_2$. Mixtures of anomers were however obtained when only reaction temperatures and times were varied to optimize the reaction. The anomerization caused by acidic conditions was therefore suppressed by addition of Et_3N to the reaction.[32] Use of $BF_3{\cdot}OEt_2$ (10 equivs) and Et_3N (1 equiv) was found to be optimal and the reaction afforded stereoselectively the kinetically favoured β-glycoside **6** in 78% yield after isolation. Final deacylation under Zemplén conditions (MeONa/MeOH) afforded the target chromogenic substrate **1** in 91% yield.

2.2. Assay of β-apiosidase

The apiosidase assay introduced by Günata[14] is a simple, reliable method suitable for fast analysis of multiplex sets of samples. The principle of the method is notoriously known from routine assays of other glycosidases: 4-nitrophenol after its enzymatic release from **1** (Scheme 2) forms yellow coloration after alkalinization of the assay solution. We have slightly modified the method for our screening purposes, the modification being in different working wavelength (410 nm instead of 400 nm) and different stopping solution (saturated solution of borax instead of sodium carbonate). The main change lies in use of two working buffers to increase the chance for detection of the activity since glycosidases of various origins may have quite different pH range of activity.

2.3. Screening of β-D-apiosidase

Besides pectinases known for apiosidase content from previous studies (Rapidase AR-2000)[13,14] or from declaration of producer



Scheme 2. Principle of β-apiosidase assay.

Table 1
β-D-Apiosidase activity in liquid enzyme preparations

Declared activity	Commercial name	Source	Activity [U/mL]	
			pH 5.0	pH 6.5
Pectinases and polygalacturonases	Pectinex UF	*Aspergillus aculeatus*	1.34 ± 0.08	0.31 ± 0.03
	Ultrazym AFP	*Aspergillus sp.*	0.58 ± 0.05	0.34 ± 0.01
	TP714L	*Aspergillus sp.*	0.55 ± 0.03	0.38 ± 0.02
	Peclyve LVG	*Aspergillus niger*	0.03 ± 0.01	n. d.
	Pectinex Yield Mash	*not available*	n. d.	n. d.
	Pectinex Ultra Mash	*A. aculeatus*	n. d.	n. d.
	Rohavin LX	*not available*	n. d.	n. d.
	Rohavin MX	*not available*	n. d.	n. d.
β-Glucanases and cellulases	Viscozyme L	*A. aculeatus*	5.43 ± 0.29	3.79 ± 0.20
	Novozym 188	*A. niger*	2.04 ± 0.05	0.73 ± 0.08
	Depol 692L	*Aspergillus sp. & Trichoderma*	0.25 ± 0.02	0.06 ± 0.01
	Celluclast	*Trichoderma reesei*	n. d.	n. d.
Glucoamylases	Dextrozyme DX 1,5X	*A. niger (Bacillus subtilis)*	n. d.	n. d.
	Dextrozyme GA 1,5X	*A. niger*	n. d.	n. d.
	Spirizyme Ultra	*A. niger*	n. d.	n. d.
	Amylyve AG 400 L	*A. niger*	n. d.	n. d.
	AMG 300 L	*A. niger*	n. d.	n. d.
β-Galactosidase	Lactozym 3000 L	*Kluyveromyces lactis*	n. d.	n. d.
Earthworm hydrolases	Vermistimul	*Eisenia foetida*	n. d.	n. d.
	Protamyl Bio V	*Eisenia foetida*	n. d.	n. d.
	Protamyl Bio D	*Eisenia foetida*	n. d.	n. d.
	Drep A	*Eisenia foetida*	n. d.	n. d.

n. d. – not detected (detection limit = 0.03 U/mL).

(Lallzyme BETA), scale of various liquid and powdered commercial enzyme preparations as well as a small number of plant materials have been tested. Based on the mentioned literary data, enzymes originating from genus *Aspergillus* were preferentially selected for the screenings. For better comparison of results, we organized data from the screening to two sets – liquid and solid enzymes. Table 1 summarizes apiosidase activities displayed by liquid samples. The highest activity was found in crude β-glucanases Viscozyme L and Novozym 188. Less significant or minor levels of apiosidases were found in several pectinases and cellulases. In general, all products displaying β-apiosidase were products originating exclusively from *aspergilli*, mostly *A. niger* or *A. aculeatus*, but several *aspergilli* products, especially glucoamylases, did not possess this activity. This may be in accordance with the prior statement of Dupin et al.[14] about inducible character of this enzyme. The enzyme cocktails from fungi other than *aspergilli*, from yeasts or from earthworms *Eisenia foetida* did not display the apiosidase activity.

Similar pattern of β-apiosidase occurrence was observed among the powdered enzymes (Table 2) – all enzymes positive for this activity originate from genus *Aspergillus*, the highest level being found in Rapidase AR 2000, a pectinase from *A. niger*. Significant levels were found also in β-glucosidase for winemaking Lallzyme BETA, Amano α-galactosidase DS and pectinase Rapidase Expression. It is worth to point that three tested lipases from *A. niger* also possess levels of apiosidase, which are comparable with previously reported apiosidase source Ultrazym 100[13,14] (Table 2). Typically for fungal glycosidases, activity of the enzymes was higher at pH 5.

β-Apiosidase activity was not identified in plant materials rich for other glycosidases (including β-glucosidase and diglycosidases) such as lyophilized crude fruit juice and defatted seed meals or grape pressing cake. These results confirm the statement of Sarry et al.[33] that β-apiosidase is absent in whole grape berry pulp or berry skin, although the berry skin is a rich source of exoglycosidases involved in aroma development in must and wine.

The liquid glycanases Viscozyme L and Novozym 188 are inexpensive, widely used industrial enzymes. After their ultrafiltration and lyophilization, powdered catalysts were obtained, with activities of apiosidase on levels comparable to its content in Rapidase AR 2000, or Lallzyme BETA, respectively. This makes β-apiosidase available in high amounts and low cost for both scientific and industrial purposes.

3. Conclusion

We have developed an efficient chemoenzymatic synthesis of 4-nitrophenyl β-D-apiofuranoside **1** from readily available **2**. The preparation includes two important selective steps: enzymatic regioselective benzoylation of primary hydroxyl and optimized stereoselective 1,2-*trans*-glycosylation using $BF_3{\cdot}OEt_2$ as promotor and Et_3N for suppression of anomerization. The chromogenic apiosidase probe **1** was produced in 61% overall yield from **2** (40% in ten steps from D-mannose).

The probe was used to screen 61 crude commercial enzymes and other biological materials for presence of β-apiosidase. We have found that the enzyme is surprisingly abundant among enzymes available in the market. Only the fungal enzymes produced by *aspergilli* possessed the β-apiosidase activity, significantly high levels being found in Rapidase AR 2000, Lallzyme BETA, Amano α-galactosidase DS and ultrafiltered and lyophilized glycanases Viscozyme L and Novozym 188.

4. Experimental

4.1. General methods

The reactions were performed with commercial reagents and solvents purchased in satisfactory purity (min. 95%) from local subsidiaries of Sigma-Aldrich, Merck, Acrōs Organics, VWR, Mikrochem, Centralchem, Slavus. D-Mannose was obtained as a gift from Technikum, Institute of Chemistry, Slovak Academy of Sciences in purity above 98%. Solvents were refluxed for 2 h over an appropriate drying agent (dichloromethane—P_2O_5, methanol–Mg/I_2) under an argon atmosphere and subsequently distilled off and used in reaction. All reactions were monitored on TLC plates (Silicagel 60, F_{254}, 0.25 mm, E. Merck, Darmstadt, Germany). The spots of

Table 2
β-D-Apiosidase activity in powdered materials

Declared activity	Commercial name	Source	Activity [U/g]	
			pH 5.0	pH 6.5
Pectinases, polygalacturonases and β-glucanases	Rapidase AR 2000	*Aspergillus niger*	78.27 ± 3.98	46.85 ± 4.29
	Lallzyme BETA	*A. niger*	11.48 ± 0.63	6.77 ± 0.27
	Rapidase Expression	*A. niger*	6.93 ± 0.08	3.79 ± 0.40
	Ultrazym 100	*A. niger*	2.74 ± 0.16	1.33 ± 0.08
	Pentopan 500 BG	*Thermomyces lanuginosus*	n. d.	n. d.
	Ultraflo (lyof)	*Humicola insolens*	n. d.	n. d.
	Rohavine Color	Not available	n. d.	n. d.
	VERON 191	Not available	n. d.	n. d.
	Rohalase GMP	Not available	n. d.	n. d.
	Fungal pectinase (Fluka)	Fungal	n. d.	n. d.
	Fungal pectinase (Calbiochem)	Fungal	n. d.	n. d.
	Pectin esterase	*Lycopersicon esculentum*	n. d.	n. d.
	Rohament pectinase	Not available	n. d.	n. d.
α-Galactosidase	α-galactosidase DS	*A. niger*	11.19 ± 0.39	7.78 ± 0.42
β-Glucosidase	Lallzyme Cuvée Blanc	*A. niger*	4.85 ± 0.27	2.20 ± 0.19
β-Galactosidase	Fungal lactase	*Aspergillus oryzae*	n. d.	n. d.
	Lactase F	*A. oryzae*	n. d.	n. d.
Lipases	Lipolyve AN	*A. niger*	3.10 ± 0.11	2.34 ± 0.07
	Enzeco Lipase	*A. niger*	2.77 ± 0.14	1.73 ± 0.08
	Lipase A	*A. niger*	2.63 ± 0.07	2.22 ± 0.15
Protease	Prolyve PAC	*A. niger*	n. d.	n. d.
Lyoph. glycanases[a]	Viscozyme L	*A. aculeatus*	64.36 ± 4.76	30.86 ± 0.91
	Novozym 188	*A. niger*	15.81 ± 0.95	7.34 ± 0.22
Lyoph. crude fruit juices	campus tree	*Sophora japonica*	n. d.	n. d.
	rowan	*Sorbus aucuparia*	n. d.	n. d.
Defatted seed meals	buckwheat	*Fagopyrum esculentum*	n. d.	n. d.
	tartaric buckwheat	*F. tataricum*	n. d.	n. d.
	apricot	*Prunus armeniaca*	n. d.	n. d.
	peach	*P. persica*	n. d.	n. d.
	grape	*Vitis vinifera*[b]	n. d.	n. d.
Seed meal press cake	grape	*Vitis vinifera*[b]	n. d.	n. d.

n. d. – not detected (detection limit = 1.2 U/g).
[a] Ultrafiltered and lyophilised glycanases positive for β-apiosidase in liquid form.
[b] Mix of varieties Furmint and Lipovina.

compounds were detected with UV lamp (λ_{max} = 254 nm) or by charring the plates with 5% orcinol in 10% (*v*/*v*) ethanolic solution of H_2SO_4 by drying at ca. 200 °C. Column chromatography was performed on Silica gel 60 (0.035–0.070 mm, pore diameter ca. 6 nm, Acrōs Organics). Melting points were determined with a Kofler hotstage and are uncorrected. Optical rotations were measured with a Jasco P-2000 polarimeter at 20 °C in chloroform or methanol. NMR spectra were recorded at 25 °C on either Varian 400 MR or Bruker AVANCE III HD 400 MHz equipped with Prodigy CryoProbe. ^{1}H NMR spectra were recorded at 400 MHz and shifts are referenced to internal solvents or Me_4Si as internal standard, respectively. ^{13}C NMR spectra were recorded at 101 MHz and shifts are referenced to internal solvents. Chemical shifts are expressed in parts per million (d scale) downfield from tetramethylsilane and are referenced to the residual proton in the NMR solvent ($CDCl_3$: δ 7.26 ppm, δ 77.23 ppm and CD_3OD: δ 3.31 ppm, δ 49.15 ppm). For correct complete assignment of signals a two-dimensional homonuclear correlation technique (H-H COSY) and heteronuclear correlation technique (H-C HSQC) were performed. Mass spectra were measured on Orbitrap Velos PRO, Thermo Scientific with parameters HESI (heated electrospray): 50 °C, capillary temperature 275 °C, spray voltage +3.5 kV, auxilliary gas 0, sheath gas flow rate 15. Spectrophotometric assays were performed on UV-VIS Spectrophotometer UV–1800 (Shimadzu, Kyoto, Japan). Incubations in small volumes were performed in Eppendorf tubes on Thermomixer comfort (Eppendorf AG, Hamburg, Germany). Ultrafiltration was performed on filter VIVAFLOW 200 (10,000 MWCO PES) purchased from Sartorius Stedim Biotech (GmbH, Goettingen, Germany) with peristaltic pump Masterflex L/S (Cole-Parmer International, Vernon Hills, IL, USA). Lyophilization of the ultrafiltrated enzymes was performed on freeze dry system LABCONCO Freezone 18 overnight.

4.2. Biocatalysts

Crude commercial preparation Rapidase AR-2000 was a kind gift from Patrice Pellerin (DSM, USA), Rapidase Expression from O.K. SERVIS BioPro, s.r.o. (Czech Republic), local supplier of DSM, Lallzyme BETA and Lallzyme Cuvée Blanc were purchased from Lallemand (Canada). Enzyme preparations from Novozymes: Lipolase 100T, Viscozyme L, Novozym 188, Pectinex UF, Ultrazym AFP, Pectinex Yield Mesh, Pectinex Ultra Mash, Celluclast, Dextrozyme DX 1,5X, Dextrozyme GA 1,5X, Spirizyme Ultra, Lactozym 3000 L, AMG 300 L, Pentopan 500 BG and Ultraflo were gift from Marián Illáš (Biotech s.r.o., Slovakia), α-galactosidase DS, Lactase F and Lipase A were purchased from Amano Enzyme (USA). Peclyve LVG, Amylyve AG 400 L, Lipolyve AN and Prolyve PAC were gift from Lyven (France). Sample of Fungal lactase was a gift from Shin Nihon (Japan) and Enzeco Microbial Lipase Concentrated was a gift from Enzyme Development Corporation (USA). Rohavin LX, Rohavin MX, Rohavine Color, VERON 191, Rohament and Rohalase GMP were gift from Barentz Slovakia (local supplier of AB Enzymes GmbH, Germany), TP714L and Depol 692L from Biocatalysts (UK) and few preparations for wastewater treatment were purchased from Karel Pecl-EKOVERMES (Pustějov, Czech republic). Tomato pectin esterase was from Sigma and preparations of three fungal pectinases were from Sigma, Fluka and Calbiochem.

Plant materials as fruits of *Sophora japonica* and *Sorbus aucuparia* were harvested in local sources. Seeds of *Fagopyrum tataricum* var. Madawaska were kind gift from Iveta Čičová (Research Institute of Plant Production, Slovakia). Seeds of *Fagopyrum esculentum*, *Prunus armeniaca* and *Prunus dulcis* were purchased from the local supplier. Seed and pressing cake obtained after grape oil production were kind gift from Alžbeta Zlatnická (J. & J. Ostrožovič, Slovakia).

4.3. 5-O-Benzoyl-2,3-O-isopropylidene-D-apiofuranose (**3**)

To a solution of **2** (12 g, 0.08 mol) in toluene (1.2 L) was added vinyl benzoate (23.71 g, 0.16 mol) and Lipolase 100T (180 g). The reaction mixture was shaken at 40 °C and 200 rpm for 10 days. The reaction was stopped, the enzyme filtered off, washed with acetone, washings were combined with the filtrate and the solvents evaporated. The concentrated crude mixture was diluted with toluene and washed with saturated $NaHCO_3$. Organic fractions were dried over anhydrous sodium sulphate and concentrated *in vacuo*. The residue was purified by column chromatography (toluene/ethyl acetate = 5/1) to give **3** as yellowish-brown oil (22.84 g, 95%, α:β = 1:4), which upon crystallization gave **3** as white needles; Mp 71–72 °C (diethyl ether/hexane), lit.[25] mp = 64–67 °C; R_f = 0.52 (toluene/ethyl acetate, 1/1, *v/v*); $[\alpha]_D^{20}$ = –28.0 (c 1.0, $CHCl_3$), lit.[25] $[\alpha]_D^{20}$ = –31.2 (c 1.3, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) **3*α***: δ ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.47 (s, 3H), 1.59 (s, 3H), 3.68 (d, *J* = 10.6 Hz, 1H, H-4b), 4.01 (d, *J* = 10.6 Hz, 1H, H-4a), 4.02 (d, *J* = 11.8 Hz, 1H, OH), 4.47 (d, *J* = 3.3 Hz, 1H, H-2), 4.48 (d, *J* = 11.8 Hz, 1H, H-5b), 4.56 (d, *J* = 11.7 Hz, 1H, H-5a), 5.09 (dd, *J* = 11.7, 3.2 Hz, 1H, H-1), 7.44–7.48 (m, 2H, H-Ph), 7.57–7.62 (m, 1H, H-Ph), 8.02–8.04 (m, 2H, H-Ph); ^{13}C NMR (100 MHz, $CDCl_3$), **3*α***: δ 27.6 (CH_3), 27.8 (CH_3), 65.2 (C-5), 70.6 (C-4), 81.8 (C-2), 89.7 (C-3), 98.1 (C-1), 115.3 (C-Me2), 128.7 (2xCH-Ph), 129.8 (C-Ph), 129.9 (2xCH-Ph), 133.7 (CH-Ph), 166.2 (COPh); ^{1}H NMR (400 MHz, $CDCl_3$) **3*β***: δ 1.41 (s, 3H, CH_3), 1.50 (s, 3H, CH_3), 3.18 (d, *J* = 2.7 Hz, 1H, OH), 4.09 (d, *J* = 10.0 Hz, 1H, H-4_b), 4.17 (d, *J* = 10.0 Hz, 1H, H-4_a), 4.53 (s, 1H, H-2), 4.52 (d, *J* = 11.8 Hz, 2H, H-5_b), 4.64 (d, *J* = 11.7 Hz, 1H, H-5_a), 5.49 (d, $J_{H,OH}$ = 2.7 Hz, 1H, H-1), 7.42–7.46 (m, 2H, H-Ph), 7.54–7.59 (m, 1H, H-Ph), 8.07–8.09 (m, 2H, H-Ph); ^{13}C NMR (100 MHz $CDCl_3$) **3*β***: δ 27.6 (CH_3), 27.7 (CH_3), 66.1 (C-5), 74.6 (C-4), 87.3 (C-2), 90.4 (C-3), 102.3 (C-1), 114.0 (C-Me2), 128.6 (2xCH-Ph), 129.6 (C-Ph), 129.9 (2xCH-Ph), 133.4 (CH-Ph), 166.4 (COPh). ESI-HRMS $C_{15}H_{18}O_6Na$ m/z $[M + Na]^+$ Anal. Calcd. 317.09956; Found 317.09941.

4.4. 5-O-Benzoyl-D-apiofuranose (**4**)

Aqueous formic acid (30 mL, 80 wt %) was added to a solution of **3** (6.0 g, 0.02 mol) in dichloromethane (10 mL) and the reaction mixture was refluxed for 5.5 h at 60 °C. The reaction mixture was then concentrated under reduced pressure to about a half of a volume and several times co-evaporated with toluene until all formic acid was removed. After evaporation to dryness, the residue was purified by column chromatography (toluene/ethyl acetate = 1/4) to afford white amorphous solid **4** (4.73 g, 93%, α:β = 0.48:1); Mp = 138–143 °C (methanol); R_f = 0.31 (ethyl acetate), $[\alpha]_D^{20}$ = – 5.4 (c 1.0, methanol); ^{1}H NMR (400 MHz, CD_3OD) **4*α***: δ 3.98 (d, *J* = 5.9 Hz, 1H, H-2), 3.99 (t, *J* = 10.0 Hz, 2H, CH_2-4), 4.32 (d, *J* = 11.4 Hz, 1H, H-5a), 4.38 (d, *J* = 11.4 Hz, 1H, H-5b), 5.26 (d, *J* = 4.7 Hz, 1H, H-1), 7.51–7.45 (m, 2H, H-Ph), 7.64–7.58 (m, 1H, H-Ph), 8.10–8.03 (m, 2H, H–Ph); ^{13}C NMR (101 MHz, CD_3OD) **4*α***: δ 68.3 (C-5), 73.8 (C-2), 74.5 (C-4), 77.6 (C-3), 98.3 (C-1), 129.8 (2xCH-Ph), 130.9 (2xCH-Ph), 131.3 (C-Ph), 134.6 (1xCH-Ph), 168.0 (COPh); ^{1}H NMR (400 MHz, CD_3OD) **4*β***: δ 3.86 (d, *J* = 9.8 Hz, 1H, H-4b), 3.89 (d, *J* = 3.8 Hz, 1H, H-2), 4.17 (d, *J* = 9.8 Hz, 1H, H-4a), 4.38 (t, *J* = 11.8 Hz, CH_2-5), 5.22 (d, *J* = 3.8 Hz, 1H, H-1), 7.51–7.45 (m, 2H, 2xCH-Ph), 7.64–7.58 (m, 1H, 1xCH-Ph), 8.10–8.03 (m, 2H, 2xCH-Ph); ^{13}C NMR (101 MHz, CD_3OD), **4*β***: δ 67.8 (C-5), 74.5 (C-4), 78.7 (C-3), 79.3 (C-2), 104.3 (C-1), 129.8 (2xCH-Ph), 130.9 (2xCH-Ph), 131.3 (C-Ph), 134.6 (1xCH-Ph), 168.0 (COPh); ESI–HRMS $C_{12}H_{14}O_6Na$ *m/z* $[M + Na]^+$ Anal. Calcd. 277.06826; Found 277.06737.

4.5. 5-O-Benzoyl-1,2,3-tri-O-acetyl-D-apiofuranose (**5**)

Acetic acid anhydride (9.19 g, 0.09 mol, 6 equiv) and 4-N,N-dimethylaminopyridine (0.185 g, 0.0015 mol, 10 mol %) were added at 0 °C to a stirred solution of **4** (3.81 g, 0.015 mol) in pyridine (8.90 g, 0.113 mol, 7.5 equiv). The stirring was continued at r.t. for further 96 h. Reaction mixture was then poured on the mixture of crushed ice (60 g) and aqueous 1M hydrochloric acid (60 mL) under vigorous stirring. The resulting mixture after 1 h stirring was extracted with chloroform (3 × 25 mL). Combined organic extracts were washed with aqueous 1M hydrochloric acid (3 × 25 mL), saturated $NaHCO_3$ (3 × 25 mL) and the chloroform fraction was dried over anhydrous sodium sulphate. After filtration and evaporation to dryness, the residue was purified by column chromatography (toluene/ethyl acetate = 4/1) to give **5** as yellow oil (5.59 g, 98 %, α:β = 1:4); R_f = 0.53 ($CHCl_3$/MeOH = 9:1), R_f = 0.85 (CH_2Cl_2/MeOH = 18:1), R_f = 0.69 (toluene/ethyl acetate = 1:1), **5*β*** R_f = 0.43 (toluene/ethyl acetate = 2:1), **5*α*** R_f = 0.36 (toluene/ethyl acetate = 2:1), lit.[25] **5*β*** R_f = 0.22 (hexane/ethyl acetate = 3:1), **5*α*** R_f = 0.16 (hexane/ethyl acetate = 3:1); $[\alpha]_D^{20}$ = – 21.3 (c 1.0, $CHCl_3$), lit.[25] **5*β*** $[\alpha]_D^{20}$ = – 31.5 (c 0.7, CH_2Cl_2), **5*α*** $[\alpha]_D^{20}$ = 48.5 (c 0.9, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$), ***5α***: δ 2.05 (s, 3H, CH_3), 2.11 (s, 3H, CH_3), 2.12 (s, 3H, CH_3), 4.34 (d, *J* = 10.7 Hz, 1H, H-4b), 4.42 (d, *J* = 10.7 Hz, 1H, H-4a), 4.65 (d, J = 11.9 Hz, 1H, H-5b), 4.90 (d, *J* = 11.9 Hz, 1H, H-5a), 5.48 (d, *J* = 4.7 Hz, 1H, H-2), 6.42 (d, *J* = 4.7 Hz, 1H, H-1), 7.44–7.50 (m, 2H, H-Ph overlapping with ***5β***), 7.56–7.62 (m, 1H, H-Ph, overlapping with ***5β***), 8.01–8.04 (m, 2H, H-Ph); ^{13}C NMR (101 MHz, $CDCl_3$) ***5α***: *δ* 20.5 (CH_3), 21.1 (CH_3), 21.4 (CH_3), 63.8 (C-5), 72.3 (C-2), 72.8 (C-4), 81.2 (C-3), 94.4 (C-1), 128.7 (CH-Ph overlapping with ***5β***), 129.6 (C-Ph), 130.0 (CH-Ph overlapping with ***5β***), 133.6 (CH-Ph overlapping with ***5β***), 165.9 (COPh), 169.4 ($COCH_3$), 169.6 ($COCH_3$), 169.9 ($COCH_3$); ^{1}H NMR (400 MHz, $CDCl_3$), ***5β***: *δ* 2.07 (s, 3H, CH_3), 2.10 (s, 3H, CH_3), 2.13 (s, 3H, CH_3), 4.28 (d, *J* = 10.5 Hz, 1H, H-4b), 4.48 (d, *J* = 10.5 Hz, 1H, H-4a), 4.82 (d, *J* = 12.4 Hz, 1H, H-5b), 4.99 (d, *J* = 12.4 Hz, 1H, H-5a), 5.60 (s, 1H, H-2), 6.17 (s, 1H, H-1), 7.44–7.50 (m, 2H, H-Ph), 7.56–7.62 (m, 1H, H-Ph), 8.08–8.10 (m, 2H, H-Ph); ^{13}C NMR (101 MHz, $CDCl_3$), ***5β***: *δ* 20.7 (CH_3), 21.2 (CH_3), 21.2 (CH_3), 63.2 (C-5), 73.6 (C-4), 76.2 (C-2), 83.3 (C-3), 99.2 (C-1), 128.7 (2xCH-Ph), 129.9 (C-Ph), 130.0 (2xCH-Ph), 133.6 (CH-Ph), 166.1 (COPh), 169.2 ($COCH_3$), 169.3 ($COCH_3$), 169.8 ($COCH_3$); ESI–HRMS $C_{18}H_{20}O_9Na$ *m/z* $[M + Na]^+$ Anal. Calcd. 403.09995; Found 403.09975.

4.6. 4-Nitrophenyl 2,3-di-O-acetyl-5-O-benzoyl-β-D-apiofuranoside (**6**)

To a mixture of **5** (5.0 g, 0.013 mol) and 4-nitrophenol (2.7 g, 0.0195 mol, 1.5 equiv), a solution of triethylamine (1.32 g, 0.013 mol, 1 equiv) in dry dichloromethane (50 mL) was added under an atmosphere of argon. Then a solution of boron trifluoride diethyletherate (18.91 g, 0.13 mol, 10 equiv) in dry dichloromethane (30 mL) was added at room temperature over a period of 30 min. and then the solution was stirred for additional 1 h. The reaction mixture was then diluted with dichloromethane (70 mL) and saturated $NaHCO_3$ (100 mL) was added under stirring to the reaction mixture. After separation of phases, the organic fraction was dried over magnesium sulphate and the filtrate was concentrated under reduced pressure. The residue was chromatographed on silica gel (toluene/ethyl acetate = 5:1) to give the title compound **6** (4.66 g, 78 %), which upon crystallization from 2-propanol gave pale yellow solid; Mp = 127–129 °C (2-propanol); R_f = 0.61 (toluene/ethyl acetate, 1/1, *v/v*); $[\alpha]_D^{20}$ –117.3 (c 1.0, $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 2.10 (s, 3H, CH_3), 2.16 (s, 3H, CH_3), 4.37 (d, *J* = 10.6 Hz, 1H, H-5a), 4.51 (d, *J* = 10.6 Hz, 1H, H-5b), 4.93 (d, *J* = 12.4 Hz, 1H, H-4a), 5.02 (d, *J* = 12.4 Hz, 1H, H-4b), 5.76 (s, 1H, H-1), 5.79 (s, 1H, H-2), 7.11 (d, *J* = 9.2 Hz, 2H, 2xCH-NPh), 7.46–7.50 (m, 2H, 2xCH-Ph), 7.58–7.63 (m, 1H, CH-Ph), 8.09–8.11 (m, 2H, 2xCH-Ph), 8.20 (d, *J* = 9.2 Hz, 2H, 2xCH-NPh); ^{13}C NMR (101 MHz, CD_3OD): *δ* 20.7 (CH_3), 21.3 (CH_3), 63.5 (C-5), 73.8 (C-4), 76.9 (C-2), 83.5 (C-3), 104.1 (C-1), 116.6 (2xCH-NPh), 126.0 (2xCH-NPh), 128.7 (2xCH-Ph), 129.6 (C-Ph), 130.0 (2xCH-Ph), 133.6 (CH-Ph), 143.0 (C-NPh), 160.9 (C-NPh), 166.1 (COPh), 169.3 ($COCH_3$), 169.9 ($COCH_3$);

ESI–HRMS m/z $C_{22}H_{21}NO_{10}Na$ $[M+Na]^+$ Anal. Calcd 482.10577; Found 482.10518.

4.7. 4-Nitrophenyl β-D-apiofuranoside (**1**)

Compound **6** (4.66 g, 0.0099 mol) was dissolved in dry methanol (90 mL). Then 0.5 M sodium methanolate was added (21 mL, 0.0105 mol) and the reaction was stirred for 20 min at laboratory temperature. After this time the reaction mixture was neutralized with Dowex 50W–X8, filtered and evaporated to dryness. Crude product was dissolved in warm 2-propanol and placed in refrigerator for one day to afford pale yellow solid (2.46 g, 91 %); Mp = 109–110 °C (2-propanol), lit.[14] mp = 104–105 °C (Et_2O/MeOH/*n*–pentane); R_f = 0.46 (ethyl acetate); $[\alpha]_D^{20}$ = –186.1 (c 1.0, MeOH), lit.[14] $[\alpha]_D^{20}$ = –155.0 (c 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, CD_3OD): δ 3.62 (d, *J* = 11.4 Hz, 1H, H-5b), 3.65 (d, *J* = 11.5 Hz, 1H, H-5a), 3.91 (d, *J* = 9.8 Hz, 1H, H-4a), 4.12 (d, *J* = 9.8 Hz, 1H, H-4b), 4.30 (d, *J* = 3.0 Hz, 1H, H-2), 5.70 (d, *J* = 3.0 Hz, 1H, H-1), 7.17 (d, *J* = 9.2 Hz, 2H, 2xCH-NPh), 8.19 (d, *J* = 9.3 Hz, 2H, 2xCH-NPh); ^{13}C NMR (101 MHz, CD_3OD): δ 64.6 (C-5), 76.1 (C-4), 78.5 (C-2), 80.6 (C-3), 108.9 (C-1), 117.7 (2xCH-NPh), 126.8 (2xCH-NPh), 143.7 (C-NPh), 163.8 (C-NPh), ESI–HRMS $C_{11}H_{13}O_7Na$ m/z $[M+Na]^+$ Anal. Calcd. 294.05897; Found 294.05816.

4.8. Assay of β-apiosidase

An amount of 50 μL of diluted enzyme was mixed with 1 mL of 2 mM solution of the substrate **1** in 0.1 M McIlvaine buffer,[34] pH 5.0 and pH 6.5, respectively. Reaction mixture was incubated for 5 or 10 minutes at 1000 rpm and 37 °C. The hydrolytic reaction was terminated by mixing 200 μL of sample with 5 volumes of saturated solution of borax. For subtraction of spontaneous hydrolysis of substrate and background signal of enzyme solution (blank value), 500 μL of sample without enzyme was added into 5 volumes of saturated solution of borax followed by adding 25 μL of enzyme solution. The release of nitrophenol was measured at 410 nm and its respective amount was calculated from the calibration curve after subtraction of the blank value. One unit of enzyme activity was defined as the amount of enzyme catalysing release of 1 μmol of nitrophenol per minute under the described conditions.

Acknowledgements

This work was supported by the Slovak Research and Development Agency under the contract No. APVV-0846-12, the Scientific Grant Agency of the Ministry of Education of Slovak Republic and Slovak Academy of Sciences (VEGA) under the projects No. 2/0138/12 and No. 2/0157/16 and also the Research & Development Operational Programmes funded by the ERDF ("Centre of Excellence on Green Chemistry Methods and Processes", CEGreenI, Contract No. 26240120001 as well as the "Amplification of the Centre of Excellence on Green Chemistry Methods and Processes", CEGreenII, Contract No. 26240120025). The authors thank I. Uhliariková for NMR measurements and S. Vlčková for mass measurements.

Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.carres.2016.04.030.

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