PYRIDOXINE AS A SUBSTRATE FOR SCREENING SYNTHETIC POTENTIAL OF GLYCOSIDASES

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The reactions of glycosidases with pyridoxine were used for testing their ability to make new glycosidic bonds. Of 35 glycosidases examined, some exhibited regiospecificity towards one primary alcoholic group; glycosylation of phenolic hydroxyl group was not observed. A series of new glycosides of pyridoxine, 2-acetamido-2-deoxy-β-D-glucopyranosides, α-D-mannopyranosides, and one α-D-galactopyranoside were prepared and completely characterized by MS and NMR.

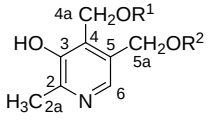
Key words: Pyridoxine; Glycosides; Glycosidases; Enzymatic glycosylation; Vitamins; Biocatalysis.

In plants, many compounds which are unstable, toxic, and hydrophobic or volatile, occur in a glycosylated form. These glycosylated compounds have excellent properties, such as a high stability, non-toxicity and water solubility. For instance, a major form of vitamin B_6 in plant-derived foods is 5a-*O*-(β-D-glucopyranosyl) pyridoxine[1]. Thus, glycosylation is considered to be an important biotransformation method for the formation of biologically important compounds possessing advantageous pharmacokinetic properties. Enzymatic glycosylation can be performed under milder reaction conditions as compared with harsh chemical methods. It enables avoiding toxic catalysts such as heavy metals and reduction of the number of reaction steps[2].

Regioselectivity of glycosidases, compared with, *e.g.*, glycosyltransferases, is substantially lower. Acceptor molecules bearing more OH groups often afford different products. Regioselectivity of glycosidases towards polyols

cannot be easily determined. Solution of this problem usually involves separation and identification of the product(s), which is time labor-consuming. Therefore, a simple and a cheap system providing information on the synthetic capability of various glycosidases and estimation of their regioselectivity is very important.

In this study, new *O*-glycosylpyridoxines were prepared and fully characterized. A range of glycosidases was tested using our new method employing pyridoxine (**1**) to demonstrate its suitability for screening synthetic potentials of these enzymes.



1; $R^1 = R^2 = H$
2a; R^1= 2-acetamido-2-deoxy-β-D-glucopyranosyl, $R^2 = H$
2b; $R^1 = H$, R^2= 2-acetamido-2-deoxy-β-D-glucopyranosyl
3; $R^1 = H$, R^2= α-D-galactopyranosyl
4a; R^1= β-D-galactopyranosyl, $R^2 = H$
4b; $R^1 = H$, R^2= β-D-galactopyranosyl
5a; R^1= α-D-mannopyranosyl, $R^2 = H$
5b; $R^1 = H$, R^2= α-D-mannopyranosyl

EXPERIMENTAL

NMR spectra were measured on a Varian VXR-400 spectrometer (399.952 MHz for ^1H, 100.58 MHz for ^{13}C) in D_2O at 25 °C. Carbon multiplicity was determined by APT (Attached Proton Test): the assignment was based on COSY, delayed-COSY, and HETCOR experiments performed using the manufacturer's software. HMBC (Heteronuclear Multiple Bond Correlation) experiments were perfomed on a Bruker DRX-500 spectrometer.

Mass spectra were recorded on a double-focusing instrument Finnigan MAT 95 (Finnigan MAT, Bremen, Germany) of BE geometry. The standard saddle field FAB gun (ion Tech Ltd., Teddington, U.K.) was operated at 2 mA current and 6 kV energy, using xenon as a bombarding gas (2 mPa). 3-Nitrobenzyl alcohol was used as a matrix. The calibration was performed with perfluorokerosene Ultramark 1600F (PCR Inc., Gainesville, U.S.A.) as a standard. The products of metastable decomposition in the first field-free region of the instrument were analyzed by daughter-ion-linked scan of $[M + H^+]$ ions (B/E constant), using the manufacturer's software. The collision gas (He) pressure was adjusted to 50% attenuation of the primary ion beam, with the collision cell voltage maintained at a ground potential.

Chemicals. 3-Nitrobenzyl alcohol (Aldrich, U.S.A.), 2,6-dibromoquinone-4-chloroimide (Avocado, U.K.), pyridoxine hydrochloride (Koch Light Lab.), 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (*p*NPβGlcNAc) (Sigma), and 4-nitrophenyl α-D-galactopyranoside (Fluka), 4-nitrophenyl β-D-galactopyranoside (Lachema, Brno, Czech Republic), mannose (Merck).

Chromatography

For TLC Kieselgel 60 F254 (Merck) and mobile phase **A** [CH_2Cl_2/MeOH/EtOH/H_2O (6 : 3.5 : 1 : 0.8, v/v/v/v), containing 0.5% (v/v) of concentrated NH_4OH] were used. Detec-

tion was done with UV light (254 or 350 nm) and by charring with 5% H_2SO_4 in EtOH. Pyridoxine and its compounds were detected by spraying with Gibbs' reagent[3] (methanolic solution of 2,6-dibromoquinone-4-chloroimide, 50 mg/100 ml).

Glycosides of pyridoxine were separated on a Bio-Gel P2 (Bio-Rad, U.S.A.) column (2.5 cm × 63 cm), equilibrated and eluted with 20 mM triethylammonium hydrogencarbonate buffer, pH 8.0 (flow rate 26 ml h^{-1}). The fractions (4 ml) were analyzed by TLC. The fractions containing pyridoxine glycosides were pooled and freeze dried.

The material after gel filtration was purified by flash chromatography on Chromagel 6–35 μm (SDS Peypin, France), using mobile phase **B** [CH_2Cl_2/MeOH (4 : 1, v/v) with 0.5% (v/v) of concentrated NH_4OH].

Enzymes and Production Microorganisms

The strains producing enzymes were obtained from the Culture Collection of Fungi (CCF), Department of Botany, Charles University, Prague and from the Culture Collection of the Institute of Microbiology (CCIM), Prague.

α-*Galactosidases (EC 3.2.1.22)* were from *Aspergillus terreus* (CCF No. 55) and *A. oryzae* (CCF No. 147). Cultivation proceeded as described previously[4] with 0.2% raffinose as an inducer. The ammonium sulfate precipitate of the culture supernatant (60% saturation) was used. Commercial α-galactosidase was from *A. niger* (Sigma).

β-*Galactosidase (EC 3.2.1.23)* from bovine testes was prepared according to the literature[5], while that from *Sulfolobus solfataricus* was a kind gift from Dr A. Trincone (University of Napoli, Italy). Commercial β-galactosidases were from *A. oryzae* and *E. coli* (both from Sigma), *A. oryzae* (Kojin Co., Ltd., Tokyo, Japan); lactases were from *Kluyveromyces lactis* Godo-YNL (Godo Shusei Co., Ltd., Tokyo, Japan), *Bacillus circulans* Biolacta (Daiwa Kasei Co., Ltd., Osaka, Japan), *Penicillium sp.* K-I (Chemical Industry Co., Ltd., Iwata, Shizuoka-ken, Japan), *A. oryzae* Sumilacto L (Shin Nihon Chemical Co., Ltd., Anjo, Aiachi-ken, Japan), *Aspergillus sp.* lactase Y-AO (Yakult Honsha Co., Ltd., Tokyo, Japan); β-glucosidase (EC 3.2.1.21) was from almonds (Sigma); cellulases (EC 3.2.1.4) were from *Trichoderma viride* Onozuka R-10 and *T. viride* Onozuka RS (both from Yakult Honsha Co., Ltd., Tokyo, Japan), *A. niger* cellulase A and *T. viride* cellulase T (both from Amano Pharmaceutical Co., Ltd., Nagoya, Japan), *T. viride* Sumizyme C (Shin Nihon Chemical Co., Ltd., Anjo, Aiachi-ken, Japan), *A. niger* and *Aspergillus sp.* (both from Calbiochem.), *P. funiculosum* (Sigma); hemicellulase was from *A. niger* (Amano Pharmaceutical Co., Ltd., Nagoya, Japan); hesperidinases were from *A. niger* and *Penicillium sp.* (both from Sigma) and from *A. niger* (Tanabe Seiyaku Co., Ltd., Osaka, Japan); macerozyme from *P. decubens* (Sigma), macerozyme R-10 from *Rhizopus sp.* (Yakult Honha Co., Ltd., Tokyo, Japan); naringinase from *A. niger* (Tanabe Seiyaku Co., Ltd., Osaka, Japan); pectinase (EC 3.2.1.15) (pectolyase Y-23) from *A. japonicus* (Seishin Pharmaceutical Co., Ltd., Tokyo, Japan). The enzymes that are not declared as β-galactosidases or lactases contained β-galactosidase activity as a secondary activity or an impurity.

β-*N-Acetylglucosaminidases (EC 3.2.1.52)* were from *Aspergillus oryzae* (CCF No. 147; 1066), *A. terreus* (CCF No. 869), *A. niger* (CCIM K2). Cultivation and isolation was performed as described previously[6]. The ammonium sulfate precipitate (0–80% saturation) was used. Commercial β-*N*-acetylglucosaminidases from *A. niger* and from jack beans (*Canavalia ensiformis*) were obtained from Sigma.

α-*Mannosidase (EC 3.2.1.24)* was from *Canavalia ensiformis* (Sigma).

Other enzymes. Maltose phosphorylase (EC 2.4.1.8) was from *Lactobacillus brevis*, sucrose phosphorylase (EC 2.4.1.7) from *Leuconostoc mesenteroides* (both from Sigma); cyclodextrin glucosyltransferase (EC 2.1.4.19) from *Bacillus stearothermophilus* was a kind gift from Hayashibara Biochem. Lab., Okayama, Japan.

New Pyridoxine Glycosides. Analytical Experiments

The reaction mixtures contained pyridoxine hydrochloride (**1**, 20 mg, 0.097 mmol), respective 4-nitrophenyl α/β-D-glycopyranoside (20 mg) dissolved in sodium citrate–phosphate buffer (0.5 ml, 0.05 mol l^{-1}, pH 5.0), and the respective glycosidase (1–10 U). pH of the mixture was always adjusted before the enzyme addition with aqueous NaOH/HCl. The reactions were monitored by TLC (mobile phase **A**). The TLC spots of pyridoxine compounds were UV active (254 nm) and emitted dark blue fluorescence under UV light (350 nm) and gave positive reaction with Gibbs' reagent (blue spots). After 3 h at 24 °C, the reactions were stopped by heating at 100 °C (5 min), lyophilized and separated by gel chromatography. The monoglycosides (regioisomers) of pyridoxine were separated by flash chromatography. Results are summarized in Table I.

Preparation and Characterization of New Pyridoxine Glycosides

4a- and 5a-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl) Pyridoxine (**2a** and **2b**)

Pyridoxine hydrochloride (**1**, 200 mg, 0.97 mmol) and *p*NPβGlcNAc (160 mg, 0.47 mmol) were dissolved in Mc Ilvaine citrate–phosphate buffer (50 mmol l^{-1}, pH 5.0, 5 ml) and 65 U of β-*N*-acetylglucosaminidase from *A. oryzae* 1066 CCF was added. During the reaction *p*NPβGlcNAc was supplemented (total 60 mg, 4 × 15 mg each after 30 min). After 3 h at 24 °C, the reactions were stopped by heating at 100 °C (5 min) **2a** and **2b** were obtained after separation in 13.5% yield (related to the sugar donor).

4a-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl) pyridoxine (**2a**). ^{1}H NMR (D_2O + CD_3COOD): 1.902 s, 3 H (NAc); 2.589 s, 3 H (H-2a); 3.360 dd, ^{1}H, J = 8.9, J' = 9.9 (H-4′); 3.384 ddd, 1 H, J = 1.9, J' = 5.4, J'' = 9.9 (H-5′); 3.457 dd, 1 H, J = 10.3, J' = 8.9 (H-3′); 3.610 dd, 1 H, J = 8.5, J' = 10.3 (H-2′); 3.676 dd, 1 H, J = 5.4, J' = 12.4 (H-6′); 3.849 dd, 1 H, J = 1.9, J' = 12.4 (H-6′); 4.508 d, 1 H, J' = 8.5 (H-1′); 4.743 s, 2 H (H-4a); 4.936 d, 2 H, J = 12.9 (H-5a); 5.051 d, 2 H, J = 12.9 (H-5a); 8.130 s, 1 H (H-6). ^{13}C NMR (D_2O): 16.05 q (C-2a); 23.33 q (Ac); 56.72 d (C-2′); 59.43 t (C-5a); 61.93 t (C-6′); 63.07 t (C-4a); 71.09 d (C-4′); 74.77 d (C-3′); 77.37 d (C-5′); 101.92 d (C-1′); 131.03 d (C-6); 139.36* s (C-5); 139.76* s (C-4); 144.42 s (C-2); 153.82 s (C-3); 175.73 s (CO). FAB-MS (daughter ion linked scan of $[M + H]^+$); *m/z* (rel. int.%, [ion type]): 373 $[M + H]^+$, 355 (25, $[M + H - H_2O]^+$), 204 (25, $[C_8H_{14}NO_5^+]$), 170 (100, $[C_8H_{12}NO_3^+]$), 152 (28, $[C_8H_{10}NO_2^+]$).

5a-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl) pyridoxine (**2b**). ^{1}H NMR (D_2O + CD_3COOD): 1.987 s, 3 H (NAc); 2.620 s, 3 H (H-2a); 3.42–3.46 m, 2 H (H-4′ and H-5′); 3.533 dd, 1 H, J = 10.3, J' = 8.6 (H-3′); 3.710 dd, 1 H, J = 8.5, J' = 10.3 (H-2′); 3.731 dd, 1 H, J = 5.7, J' = 12.3 (H-6′); 3.916 dd, 1 H, J = 1.9, J' = 12.3 (H-6′); 4.591 d, 1 H, J = 8.5 (H-1′); 4.798 d, 2 H, J = 13.0 (H-5a); 4.951 s, 2 H (H-4a); 5.034 d, 2 H, J = 13.0 (H-5a); 8.070 s, 1 H (H-6). ^{13}C NMR (D_2O): 15.71 q (C-2a); 23.36 q (Ac); 56.74 d (C-2′); 58.41 t (C-4a); 61.97 t (C-6′); 66.74 t (C-5a); 71.14 d (C-4′); 74.79 d (C-3′); 77.28 d (C-5′); 101.85 d (C-1′); 131.73 d (C-6); 134.36 s (C-5); 142.32 s (C-4); 144.82 s (C-2); 154.49 s (C-3); 175.79 s (CO). FAB-MS (daughter ion linked scan of $[M + H]^+$); *m/z* (rel. int.%, [ion type]): 373 $[M + H]^+$, 355 (100, $[M + H - H_2O]^+$), 204 (10, $[C_8H_{14}NO_5^+]$), 170 (60, $[C_8H_{12}NO_3^+]$), 152 (30, $[C_8H_{10}NO_2^+]$).

TABLE I
Regioselectivity of glycosidases from different sources

Enzyme	Source of enzyme	pH[a]	Glycoside	
			C-4a	C-5a
α-Galactosidase[b]	*A. terreus*	5.0	–	+
	A. oryzae	5.0	–	+
	A. niger (Sigma)	5.0	–	+
	Bovine testes	5.0	+	+
	Sulfolobus solfataricus	5.0	+	–
β-Galactosidase[c]	*A.oryzae* (Sigma)	5.0	+	+
	E. coli (Sigma)	5.0	+	–
	A. oryzae (Kohjin)	5.0	–	–
	Kluyveromyces lactis	7.0[d]	+	–
	B. circulans (Biolacta)	6.5	+	–
Lactase[c]	*Penicillium sp.*	4.5	+	+
	A. oryzae (Sumilacto L)	5.0	–	+
	Aspergillus sp.	4.5	–	–
β-Glucosidase[c]	Almonds	5.0	+	+
	T. viride (Onozuka R-10)	4.5	+	–
	T. viride (Onozuka RS)	4.5	+	–
	A. niger (cellulase A)	4.5	+	+
Cellulase[c]	*T. viride* (cellulase T)	4.5	+	+
	T. viride (Sumizyme C)	4.5	+	–
	A. niger (Calbiochem.)	4.5	+	+
	Aspergillus sp. (Calbiochem.)	4.5	–	–
	P. funiculosum (Sigma)	5.0	+	+
Hemicelullase[c]	*A. niger* (Amano)	4.5	+	–
	A. niger (Sigma)	3.8	+	–
Hesperidinase[c]	*Penicillium sp.* (Sigma)	3.8	–	–
	A. niger (Tanabe)	3.5	–	–
Macerozyme[c]	*P. decumbens* (Sigma)	5.0	+	–
	Rhizopus sp. (Yakult)	5.0	–	–
Naringinase[c]	*A. niger*	3.8	+	+
Pectolyase[c]	*A. japonicus*	4.0	+	+
	A. oryzae	5.0	+	+
β-*N*-Acetylglucosaminidase[e]	*A. terreus*	5.0	+	+
	A. niger	5.0	+	+
	Canavalia ensiformis	5.0	+	+
α-Monnosidase[f]	*Canavalia ensiformis*	5.0	+	+

[a] 0.05 M Mc Ilvaine buffer. [b] α-Galactosides. [c] β-galactosides; the enzymes β-glucosidase, cellulase, hemicellulase, hesperinidase, macerozyme, naringinase, and pectolyase that are not declared as β-galactosidases or lactases contain β-galactosidase activity as a side activity or an impurity. [d] 0.1 M phosphate buffer. [e] 2-Acetamido-2-deoxy-β-D-glucosides. [f] Mannosides of pyridoxine were formed.

5a-*O*-(α-D-Galactopyranosyl) Pyridoxine (**3**)

Pyridoxine hydrochloride (**1**, 225 mg, 1.10 mmol) and 4-nitrophenyl α-D-galactopyranoside (85 mg, 0.28 mmol) were dissolved in Mc Ilvaine citrate–phosphate buffer (50 mmol l^{-1}, pH 5.0, 2.5 ml) and 20 U of α-galactosidase from *A. terreus* 55 CCF was added. After 3 h at 24 °C, the reactions were stopped by heating at 100 °C (5 min). Compound **3** was obtained after separation in 36.4% yield (related to the sugar donor). ^{1}H NMR (D_2O + CD_3COOD): 2.614 s, 3 H (H-2a); 3.69 m, 1 H (H-5′); 3.82–3.87 m, 4 H (H-2′, H-3′ and both H-6′); 3.94 m, 1 H (H-4′); 4.749 d, 2 H, *J* = 13.2 (H-5a); 4.881 d, 2 H, *J* = 13.2 (H-5a); 4.991 s, 2 H (H-4a); 5.032 d, 1 H, *J* = 2.6 (H-1′); 8.202 s, 1 H (H-6); ^{13}C NMR (D_2O): 16.64 q (C-2a); 57.58 t (C-4a); 62.38 t (C-6′); 65.92 t (C-5a); 69.42 d (C-4′); 70.48 d (C-3′); 70.67 d (C-2′); 72.63 d (C-5′); 99.59 d (C-1′); 133.85 d (C-6); 145.90 s (C-2). FAB-MS (daughter ion linked scan of $[M + H]^+$); *m/z* (rel. int.%, [ion type]): 332 $[M + H]^+$, 314 (100, $[M + H - H_2O]^+$), 170 (10, $[C_8H_{12}NO_3^+]$), 152 (18, $[C_8H_{10}NO_2^+]$).

4a- and 5a-*O*-(β-D-Galactopyranosyl) Pyridoxine (**4a** and **4b**)

The reaction mixture contained **1** (200 mg, 0.97 mmol), 4-nitrophenyl β-D-galactopyranoside (100 mg, 0.28 mmol) dissolved in Mc Ilvaine citrate–phosphate buffer (50 mmol l^{-1}, pH 5.0, 5 ml) and 65 U of β-galactosidase from *A. oryzae* (Grade XI, Sigma) was added. After 3 h at 24 °C, the reactions were stopped by heating to 100 °C (5 min). Compounds **4a** and **4b** were obtained after separation in 32.5% yield (related to the sugar donor). Compounds **4a** and **4b** were prepared previously using β-galactosidase from *Sporobolomyces singularis*[7]. ^{1}H and ^{13}C NMR data of our compounds **4a** and **4b** were identical to those published previously[7].

4a- and 5a-*O*-(α-D-Mannopyranosyl) Pyridoxine (**5a** and **5b**)

Pyridoxine hydrochloride **1**, (80 mg, 0.39 mmol), mannose (400 mg, 2.22 mmol) were dissolved in Mc Ilvaine citrate–phosphate buffer (50 mmol l^{-1}, pH 5.0, 1 ml) and 10 U of mannosidase from *Canavalia ensiformis* (jack beans) was added. During the reaction more mannose was supplemented (2 × 100 mg after 30 h). The reversed mannosylation was carried out at 38 °C and the reaction was stopped after 94 h by heating to 100 °C (5 min). Compounds **5a** and **5b** were obtained after separation in 2.3% yield (related to the sugar donor).

4a-O-(α-D-Mannopyranosyl) pyridoxine (**5a**). ^{1}H NMR (D_2O + CD_3COOD): 2.476 s, 3 H (H-2a); 3.65–3.80 m, 5 H (H-3′, H-4′, H-5′ and both H-6′); 3.934 dd, 1 H, *J* = 1.8, *J*′ = 3.4 (H-2′); 4.753 d, 2 H, *J* = 11.4 (H-4a); 4.784 s, 2 H (H-5a); 4.912 d, 2 H, *J* = 11.4 (H-4a); 4.993 d, 1 H, *J* = 1.8 (H-1′); 7.657 s, 1 H (H-6). ^{13}C NMR (D_2O): 16.90 q (C-2a); 60.12 t (C-5a); 62.05* t (C-4a); 62.05* t (C-6′); 67.91 d (C-4′); 71.26 d (C-3′); 71.79 d (C-2′); 74.38 d (C-5′); 101.78 d (C-1′); 138.14 s (C-5); 145.86 s (C-2). FAB-MS (daughter ion linked scan of $[M + H]^+$); *m/z* (rel. int.%, [ion type]): 332 $[M + H]^+$, 314 (22, $[M + H - H_2O]^+$), 170 (100, $[C_8H_{12}NO_3^+]$), 152 (30, $[C_8H_{10}NO_2^+]$).

5a-O-(α-D-Mannopyranosyl) pyridoxine (**5b**). ^{1}H NMR (D_2O): 2.501 s, 3 H (H-2a); 3.59–3.83 m, 5 H (H-3′, H-4′, H-5′ and both H-6′); 3.983 dd, 1 H, *J* = 1.8, *J*′ = 3.4 (H-2′); 4.692 d, 2 H, *J* = 11.6 (H-5a); 4.857 d, 2 H, *J* = 11.6 (H-5a); 4.868 s, 2 H (H-4a); 4.990 d, 1 H, *J* = 1.8 (H-1′); 7.767 s, 1 H (H-6). FAB-MS (daughter ion linked scan of $[M + H]^+$); *m/z* (rel. int.%, [ion type]): 332 $[M + H]^+$, 314 (100, $[M + H - H_2O]^+$), 170 (20, $[C_8H_{12}NO_3^+]$), 152 (20, $[C_8H_{10}NO_2^+]$).

RESULTS AND DISCUSSION

New Glycosides of Pyridoxine

β-Glucosylation of **1** was previously studied using β-glucosidases from various sources[8–10]. Two β-glucopyranosylpyridoxines, 5a-*O*-(β-D-glucopyranosyl) pyridoxine and 4a-*O*-(β-D-glucopyranosyl) pyridoxine were formed in germinating seeds of wheat, barley, and rice, whereas only 5a-*O*-(β-D-glucopyranosyl) pyridoxine accumulated in germinating soybean seeds. A series of other pyridoxine glycosides 5a-*O*-(β-D-fructofuranosyl) pyridoxine and 5a-*O*-(β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl) pyridoxine[11], 4a-*O*-(β-D-galactopyranosyl) pyridoxine (**4a**), 5a-*O*-(β-D-galactopyranosyl) pyridoxine (**4b**) and 4a-*O*-(β-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl) pyridoxine[7], 4a-*O*-(α-D-glucopyranosyl) pyridoxine and 5a-*O*-(α-D-glucopyranosyl) pyridoxine were prepared previously[12–14].

We have succeeded in extending the series of pyridoxine glycosides by the preparation of new compounds, *e.g.*, 4a-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl) pyridoxine (**2a**), 5a-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl) pyridoxine (**2b**), 5a-*O*-(α-D-galactopyranosyl) pyridoxine (**3**), 4a-*O*-(α-D-mannopyranosyl) pyridoxine (**5a**), and 5a-*O*-(α-D-mannopyranosyl) pyridoxine (**5b**) using glycosidases of various origin. Also alternative approaches were employed, *e.g.*, transglycosylation using activated sugar donors (4-nitrophenyl derivatives) and reverse glycosylation in the case of pyridoxine α-mannosylation.

All new pyridoxine glycosides exhibited the expected $[M + H]^+$ ions in their FAB mass spectra. Daughter scans of these ions contained fragments corresponding to the loss of water, the splitting of the glycosidic bond accompanied by transfer of hydrogen, and further loss of water. The ions *m/z* 204 complementary to the glycosidic bond breakdown were found with the compounds **2a** and **2b**. Sets of vicinal couplings, observed in their ^{1}H NMR spectra confirmed the nature of the attached sugars (all vicinal *J*'s large – glucopyranosyl; small $J_{4,5}$, medium $J_{3,4}$ – galactopyranosyl; medium $J_{2,3}$ – mannopyranosyl). Coupling constants $J_{1,2}$ served to assign the anomeric configuration: 7.7–8.5 Hz (**2a**, **2b**, **4a**, **4b**), all β-; 2.6 Hz (**3**), α-; 1.8 Hz (**5a**, **5b**), α-. To avoid problems with protonation–deprotonation at neutral pH and the loss of some carbon signals intensity caused by presence of small amounts of inorganic ions[15], the NMR spectra were run with few drops of CD_3CO_2D added. The signal pattern of both pyridoxine CH_2O groups ranged from an AB system to two-proton singlet. That one attached to C-5 could be identified by its long-range coupling to H-6 found in a long range

COSY (LR COSY) experiment[16]. The position of substitution is then derived using the downfield shift of the corresponding carbon with respect to the parent compound[7] **1**; the only prerequisite being the signal assignment. An independent proof used with compounds **2a** and **2b** was based on a HMBC experiment. Three-bond C,H couplings between sugar H-1 and pyridoxine CH_2O carbon as well as between CH_2O protons and sugar C-1 unambiguously determined the site of substitution. Furthermore, C-5a was identified by its coupling to H-6 in the same experiment. Other observed couplings verified the assignments of all signals. The chemical shifts of C-4a and C-5a glycosides were different and relatively constant within each group so that a simple inspection of ^{13}C NMR spectrum is sufficient to determine the compound type. However, the simplest differentiation of both regioisomers can be achieved by TLC in the presence and absence of borate: both pyridoxine (**1**) and 5a-*O*-glycosides form borate complexes (strong reducing of their R_F), whereas the 4a-*O*-derivatives not (the spots remained in the original position).

Besides the monoglycosides, also the formation of oligoglycosides of pyridoxine was observed; however these products were not isolated in sufficient amounts as to allow their spectral characterization.

Pyridoxine as a New Tool for Screening Glycosidases Having Synthetic Potencial

During our work with a number of different glycosidases, we realized that pyridoxine is an ideal substrate for testing the synthetic capabilities of glycosidases. It is a cheap and easily available compound. Easy identification of pyridoxine derivatives (TLC, UV active, dark blue fluorescence under near UV light, a typical blue colour with Gibbs' reagent) is an important advantage. Derivatives of **1** can be easily and selectively separated and detected and the pyridoxine molecule provides a good tool for estimation of tentative regioselectivity of glycosidases. Pyridoxine bears two quasi-equivalent hydroxymethyl groups and their different ability to be glycosylated was investigated.

Separation of glycosylated regioisomers of pyridoxine is well possible using borate. Compound **1** itself forms a complex with borate in which 3-OH and 4a-OH participate[17]. 4a-*O*-(Glycosyl) pyridoxines cannot form the borate complex because of the blocking 4a-hydroxymethyl group. Therefore, the position of their TLC spots did not change, when developed. The borate complex formed with 5a-*O*-(glycosyl) pyridoxines as well as with

free pyridoxine migrates slowly in the mobile phase **A** having R_F below 0.1, because of a substantial polarity increment. After stopping the reaction, borate was added to the sample of the reaction mixture and the pyridoxine–borate complex was immediately formed. Comparison of the TLC spots of the reaction mixture with and without borate enabled easy and quick assignment of the glycosyl position in the pyridoxine molecule.

Various glycosidases (β-*N*-acetylglucosaminidases, α-mannosidases, α-galactosidases, and β-galactosidases) from different sources (Table I) were tested for synthetic potentials and for their regioselectivity. The reactions of pyridoxine, 4-nitrophenyl glycoside and the respective enzyme (see analytical experiments) were monitored by TLC, the R_F of the reaction products with a tested enzyme and R_F of **2a**, **2b**, **3**, **4a**, **4b**, **5a**, and **5b** were compared. Glycosylated position of pyridoxine was assigned using the borate complex formation. The transglycosylation abilities of the some enzymes from different sources were compared (Table I).

All three α-galactosidases transferred regioselectively α-galactosyl residue from 4-nitrophenyl α-D-galactopyranoside to the 5-(hydroxymethyl) group of pyridoxine, so only 5a-*O*-(α-D-galactopyranosyl) pyridoxine was formed. Most other enzyme preparations catalyzed the transfer of glycosyl residues to mainly 4-(hydroxymethyl) group or to both 4- and 5-(hydroxymethyl) groups of pyridoxine.

While the substitution of the hydroxymethyl groups of pyridoxine was successful, transglycosylations of phenolic group using maltose phosphorylase (*L. brevis*), sucrose phosphorylase (*L. mesenteroides*), and cyclodextrin glucanotransferase (*B. stearothermophilus*) with maltose, sucrose, and dextrin as the substrates, respectively, did not work. Enzymatic glycosylation by the above enzymes of phenolic OH groups in various substrates (hydroxybenzenes, hydroxybenzoic acids, benzyl alcohol, hydroxy- benzyl alcohols, kojic acid, catechin) was described previously[18,19].

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REFERENCES

1. Nakano H., Gregory III J. F.: *J. Nutr.* **1995**, *125*, 926.
2. Křen V., Thiem J.: *Chem. Soc. Rev.* **1997**, *26*, 463.
3. Jork H., Funk W., Fischer W., Wimmer H.: *Thin-Layer Chromatography*, Vol. 1a, p. 252. VCH, Weinheim 1990.

4. Huňková Z., Kubátová A., Weignerová L., Křen V.: *Czech Mycology* **1999**, *51*, 71.
5. Hedbys L., Johansson E., Mosbach K., Swensson S.: *Biochem. Biophys. Res. Commun.* **1984**, *123*, 8.
6. Huňková Z., Křen V., Ščigelová M., Weignerová L., Scheel O., Thiem J.: *Biotechnol. Lett.* **1996**, *18*, 725.
7. Suzuki Y., Uchida K.: *Biochim. Biophys. Acta* **1992**, *1116*, 67.
8. Suzuki Y., Uchida K., Tsuboi A.: *Nippon Nogei Kagaku Kaishi* **1979**, *53*, 189; *Chem. Abstr.* **1979**, *91*, 191344.
9. Suzuki Y., Inada Y., Uchida K.: *Phytochemistry* **1986**, *25*, 2049.
10. Suzuki Y., Ishii H., Suga K., Uchida K.: *Phytochemistry* **1986**, *25*, 1331.
11. Suzuki Y., Uchida K.: *Biosci., Biotechnol., Biochem.* **1993**, *57*, 875.
12. Ogata K., Tani Y., Uchida Y., Tochikura T.: *J. Vitaminol.* **1969**, *15*, 142.
13. Suzuki Y., Doi Y., Uchida K., Tsuge H.: *Oyo Toshitsu Kagaku (J. Appl. Glycosci.)* **1996**, *43*, 369; *Chem. Abstr.* **1996**, *125*, 301365.
14. Suzuki Y., Doi Y., Uchida K., Tsuge H.: *Methods Enzymol.* **1997**, *280*, 66.
15. Hartman J. S., Kelusky E. C.: *Can. J. Chem.* **1979**, *57*, 2118.
16. Bax A., Freeman R.: *J. Magn. Reson.* **1981**, *44*, 542.
17. Scudi J. V., Bastedo A., Webb T. J.: *J. Biol. Chem.* **1940**, *13b*, 399.
18. Kitao S., Sekine H.: *Biosci., Biotechnol., Biochem.* **1994**, *58*, 38.
19. Kometani T., Terada Y., Nishimura T., Takii H., Okada S.: *Biosci., Biotechnol., Biochem.* **1994**, *58*, 1990.