Glycoconjugate Journal 15, 757–767 (1998)

Hydrolase-catalyzed β-mannosylations

Norbert Taubken and Joachim Thiem*

Institut für Organische Chemie, Universität Hamburg, Martin-Luther-King-Platz 6, D-20146 Hamburg, Germany

Transmannosylations catalysed by β-mannosidase from snail viscera or β-galactosidase from *Aspergillus oryzae* were accomplished with 4-nitrophenyl β-D-mannopyranoside as donor substrate. With suitable hydrophobic acceptor molecules preferentially β1-4-linked disaccharides were obtained. The activities of both glycosidases in buffer cosolvent mixtures were determined, and conditions for their immobilization were elaborated and optimized. A model of the enzymic transfer mechanism is suggested.

***Keywords*: β-mannosidase, β-galactosidase, β-mannosylation, transfer mechanism, cosolvent, immobilization**

***Abbreviations*: DMF, dimethyl formamide; DMSO, dimethyl sulfoxide; Glyp, glycopyranosyl; GlypOMe, methyl glycopyranoside; GlypOpNP, 4-nitrophenyl glycopyranoside; TMS, tetramethyl silane; ax, axial; eq, equatorial**

Introduction

Whereas the formation of 1-2-cis-linked glycosides remains generally difficult, several attractive routes for the chemical synthesis of β-mannopyranosides have been introduced. Also some attempts which use enzymes for this purpose have been reported. By radioactive assays Flitsch *et al.* monitored β-mannosyl transfer catalyzed by mannosyltransferase [1]. We have described the formation of β-mannopyranosides on a preparative scale by the use of β-mannosidase from snail viscera (EC 3.2.1.25) [2] and also of β-galactosidase from *Aspergillus oryzae* (EC 3.2.1.23) [3]. Here we report on further studies with both β-mannosylating glycosidases. The yield of transfer products was optimized by the use of organic cosolvents and the influence of immobilization on transmannosylations was studied. By employing deoxygenated carbohydrates as acceptors the formation of excessive products was avoided. In addition to preparative goals, correlations between the properties of the acceptor molecule and the resulting transfer products was of interest for information concerning details of the enzymatic transfer mechanism.

Materials and methods

β-Mannosidase (EC 3.2.1.25; from snail viscera) and β-galactosidase (EC 3.2.1.23; from *Aspergillus oryzae*, grade XI, standardized with starch) were purchased from Sigma, Germany. Immobilizations were accomplished on CNBr-activated Sepharose 4B or on activated CH-Sepharose 4B (Pharmacia). Transfer reactions with β-mannosidase were incubated at pH 4.0 (0.1 M sodium citrate), with β-galactosidase at pH 5.0 (0.05 M sodium citrate, 0.05 M sodium phosphate). All reactions were monitored by (TLC) on silica gel FG_{254} (Merck, Germany; solvent: 2-propanol/ethyl acetate/water 4:7:2) with detection by UV light and charring with sulfuric acid at 130 °C. Free enzymes were deactivated by heating to 90 °C for 5 min. 4-Nitrophenol was removed by irreversible attachment to ion exchange resin, Dowex 1×8 (2×10 cm, Cl^- form; Serva, Germany; solvent: water) from the incubation mixtures. Gel permeation chromatography was performed on Fractogel TSK HW-40 (2×80 cm, Merck, solvent: water) or Biogel P-2 (2×110 cm, Bio-Rad, Germany, solvent: water). The flow rate was regulated by a perestaltic pump P 3 (Pharmacia). Peracetylated disaccharides were separated by flash chromatography on Silica gel 60 (200–400 mesh; Merck) or by HPLC (column: Nucleosil 100, 5 μm, 250×10 mm; Knauer, Germany; HPLC-pump 64 and differential refractometer 88; Knauer; eluent: petrol ether/ethyl acetate 5:6). A Beckman DU-62 spectral photometer was used for quantitative measurement of 4-nitrophenol. ^{1}H NMR spectra were recorded with a Bruker AMX-400 (400.13 MHz) instrument. To prove the β-configuration of the transfer products ^{13}C NMR spectra (100.67 MHz, internal standard: TMS) of compounds **8** and **13** were measured, and the $J_{C-1,H-1}$ coupling constants were determined by gated decoupling experiments [15]. For assignment of signals COSY experiments were performed. Spectral data are listed in Tables 1–4.

* To whom correspondence should be addressed.

Table 1. ^{1}H NMR data of peracetylated glycobiosides

Chemical shift δ/ppm (Coupling constant J/Hz)	*Manα1-6Manα1-OMe* ***(5Ac)***	*Glcβ1-6Glcα1-OMe* ***(6Ac)***	*Glcβ1-6Glcα1-OMe* ***(7Ac)***	*Manβ1-4Manα1-OMe* ***(8Ac)***	*Manβ1-2Glcα1-OMe* ***(9Ac)***	*Ara* ***(10Ac)***	*Manβ1-4 Ara* ***(12Ac)***
Reducing hexose							
H1	4.69	4.93	4.92	4.65	4.82	3.45/	3.45/
($J_{1;2}$)	(1.5)	(4.0)	(3.8)	(1.7)	(3.5)	3.97[a]	3.97[a]
H2	5.24	4.84	4.84	5.28	3.69	1.75/	1.72/
($J_{2;3}$)	(3.0)	(10.0)	(10.0)	(3.5)	(10.2)	2.03[a]	2.06[a]
H3	5.35	5.48	5.46	5.39	5.35	4.91	5.04
($J_{3;4}$)	(9.5)	(9.6)	(9.5)	(9.8)	(9.6)	(7.0)	(9.5)
H4	5.25	5.00	4.91	3.98	4.91	4.90	3.66
($J_{4;5}$)	(10.0)	(10.0)	(8.5)	(10.0)	(10.0)	(9.0)	(9.5)
H5	3.94	3.98	3.95	3.90	3.90	3.46	3.46
($J_{6S;6R}$)	(10.7)	(11.2)	(11.0)	(12.0)	(12.2)	(12.0)	(12.0)
H6S	3.57	3.55	3.91	4.33	4.00	4.03	4.35
($J_{5;6S}$)	(2.5)	(2.4)	(2.0)	(2.5)	(2.2)	(2.0)	(2.5)
H6R	3.78	3.74	3.55	4.28	4.20	4.17	4.16
($J_{5;6R}$)	(5.8)	(5.8)	(6.5)	(5.5)	(4.5)	(5.0)	(4.5)
OMe	3.42	3.43	3.39	3.37	3.37		
Non-reducing hexose							
H1′	4.87	5.12	4.55	4.72	4.66		4.74
($J_{1';2'}$)	(1.5)	(4.0)	(8.0)	(1.0)	(1.1)		(1.0)
H2′	5.28	4.86	5.01	5.41	5.34		5.41
($J_{2';3'}$)	(3.2)	(10.0)	(9.5)	(3.5)	(3.2)		(3.0)
H3′	5.34	5.47	5.21	5.04	4.94		5.04
($J_{3';4'}$)	(9.5)	(9.6)	(9.5)	(10.0)	(10.2)		(10.0)
H4′	5.29	5.05	5.08	5.20	5.15		5.21
($J_{4';5'}$)	(9.6)	(10.0)	(10.0)	(10.0)	(10.2)		(9.5)
H5′	4.09	4.10	3.70	3.64	3.56		3.63
($J_{6S';6R'}$)	(12.2)	(12.7)	(12.5)	(12.5)	(12.0)		(12.0)
H6S′	4.14	4.11	4.13	4.11	4.11		4.11
($J_{5';6S'}$)	(2.2)	(2.3)	(2.5)	(2.7)	(3.0)		(3.0)
H6R′	4.26	4.23	4.28	4.29	4.12		4.33
($J_{5';6R'}$)	(5.1)	(5.0)	(4.5)	(5.5)	(5.0)		(5.5)
OAc	2.16/2.16/ 2.11/2.06/ 2.04/1.99/ 1.98	2.11/2.10/ 2.08/2.05/ 2.02/2.01/ 2.00	2.09/2.07/ 2.05/2.03/ 2.02/2.00/ 1.99	2.17/2.14/ 2.13/2.09/ 2.05/2.04/ 1.99	2.10/2.08/ 2.03/1.98/ 1.96/1.94/ 1.92	2.03/ 1.97/ 1.96	2.18/2.13/ 2.09/2.09/ 2.04/1.99/

[a]Sequence: $\delta(H_{axial})/\delta(H_{equatorial})$. Coupling constants: $(J_{1ax;1eq}) = 12.0$; $(J_{1ax;2ax}) = 12.0–12.5$; $(J_{1ax;2eq}) = 2.0$; $(J_{1eq;2ax}) = 5.0$; $(J_{1eq;2eq}) = 1.0–1.5$; $(J_{2ax;2eq}) = 12.5–13.0$; $(J_{2ax;3}) = 9.0$; $(J_{2eq;3}) = 5.0$ Hz.

Activity of β-mannosidase in cosolvents buffer mixtures

A 45 µl aliquot (0.25 U) of the β-mannosidase suspension was made up to 1 ml with buffer (pH 4.0) and cosolvent and shaken at room temperature. Every other hour 0.2 ml of this mixture was added to a solution of ManOpNP (**1**; 0.1 µmol) in buffer (0.8 ml, pH 4.0). Ten minutes later 0.2 ml were diluted with aqueous sodium carbonate (0.5 ml, 0.1 M) and water (0.8 ml), and the extinction was measured at 400 nm.

Activity of β-galactosidase in cosolvents buffer mixtures

A suspension of β-galactosidase in buffer (20 µl, 5 U) was added to 3.0 ml of solvent mixtures (buffer: pH 5.0; cosolvent). Every other hour 0.4 ml of the incubation mixture was added to a solution of 2-nitrophenyl β-D-galactopyranoside in buffer (1.1 ml, 5 µmol, pH 5.0). After 10 min 50 µl were diluted with aqueous sodium carbonate (0.5 ml, 0.1 M) and water (0.95 ml), and the extinction was measured at 410 nm.

Table 2. ^{1}H NMR data of 4-nitrophenyl glycobiosides

Chemical shift δ/ppm (Coupling constant J/Hz)	*Manβ1-OpNP* ***(1)***	*Manα1-OpNP* ***(1α)***	*Manβ1-4Manβ1-OpNP* ***(13)***	*Glcα1-6Manβ1-OpNP* ***(15)***	*Manβ1-4Manβ1-OpNP* ***(17)***
Reducing hexose					
H1	5.56	5.82	5.60	5.61	5.83
($J_{1;2}$)	(< 1.0)	(2.0)	(< 1.0)	(< 1.0)	(1.5)
H2	4.28	4.25	4.36	4.30	4.32
($J_{2;3}$)	(3.1)	(3.6)	(1.6)	(4.0)	(3.6)
H3	3.82	4.12	4.00[a]	3.83[a]	4.27
($J_{3;4}$)	(9.1)	(9.7)		(9.7)	(9.2)
H4	3.74	3.81	3.80	3.83[a]	4.05
($J_{4;5}$)	(9.6)	(10.2)			(9.7)
H5	3.65	3.69	4.01[a]	3.83[a]	3.80[a]
($J_{6S;6R}$)	(12.7)	(12.2)		(11.5)	(∼ 12)
H6S	4.00	3.84	4.01[a]	4.03	3.79[a]
($J_{5;6S}$)	(2.0)	(2.2)		(3.0)	(∼ 2)
H6R	3.81	3.78	3.85	3.83[a]	3.81[a]
($J_{5;6R}$)	(6.0)	(5.6)	(5.1)		(∼ 5)
H_{Aryl}	8.29/7.26	8.31/7.14	8.32/7.29	8.31/7.28	8.33/7.34
($J_{Aryl;Aryl}$)	(9.0)	(9.6)	(9.6)	(9.2)	(9.7)
Non-reducing hexose					
H1′			4.83	4.99	4.81
($J_{1';2'}$)			(< 1.0)	(3.5)	(< 1.0)
H2′			4.15	3.56	4.10
($J_{2';3'}$)			(3.1)	(10.2)	(3.4)
H3′			3.73	3.76	3.71
($J_{3';4'}$)			(9.7)	(9.2)	(9.7)
H4′			3.64	3.42	3.62
($J_{4';5'}$)			(9.7)	(9.2)	(9.7)
H5′			3.52	3.65[a]	3.50
($J_{6S';6R'}$)			(12.2)		(12.2)
H6S′			4.02[a]	3.65[a]	4.01
($J_{5';6S'}$)			(2.0)		(2.0)
H6R′			3.81	3.83[a]	3.78
($J_{5';6R'}$)			(6.7)		(6.6)

[a] Assignment by COSY.

Immobilization at different pH

Buffers of different pH for the coupling of enzymes and for blocking of remaining active groups were prepared. The coupling buffers were 0.1 M KH_2PO_4: 0.5 M NaCl, pH 4.0, 5.0, or 6.0 (adjusted by aqueous HCl or NaOH), and 0.1 M $NaHCO_3$: 0.5 M NaCl, pH 7.0, 8.0. The blocking buffers were 0.5 M glycine: 0.5 M NaCl, pH 4.0, 5.0 or 6.0, and 0.5 M Tris HCl: 0.5 M NaCl, pH 7.0 and 8.0. CNBr- and CH-Sepharose gels (100 mg) were soaked in aqueous HCl (1 ml, 2 μM) for 15 minutes. All immobilizations were done at 4 °C and 20 °C. With CH-Sepharose the coupling and blocking processes took 2 h at 20 °C or 8 h at 4 °C. With CNBr-Sepharose the coupling and blocking took one hour at 20 °C; at the lower temperature the reactants were coupled for 4 h and blocked for 2 h.

Immobilization of β-mannosidase

The gel was washed with coupling buffer and subsequently incubated with the enzyme suspension (0.5 U, 90 μl) at the given pH. The liquid was filtered off, the gel washed with the corresponding blocking buffer and incubated with 0.5 ml of this buffer. Finally, it was washed with buffer (pH 4.0), and the remaining activity of the immobilized enzyme was determined by adding a solution of 4-nitrophenyl β-D-mannopyranoside (**1**) in buffer (1 ml, 10 μmol substrate, pH 4.0). Every two minutes 20 μl of this mixture were diluted with

Table 3. ^{1}H NMR data of peracetylated 4-nitrophenyl glycobiosides

Chemical shift δ/ppm (Coupling constant J/Hz)	*Manβ1-OpNP* ***(1Ac)***	*Manβ1-4Manβ1-OpNP* ***(13Ac)***	*Manβ1-3Manβ1-OpNP* ***(14Ac)***	*Glcα1-6Manβ1-OpNP* ***(15Ac)***	*Manα1-4Manβ1-OpNP* ***(16Ac)***	*Manβ1-4Manα1-OpNP* ***(17Ac)***
Reducing hexose						
H1	5.36	5.25	5.31	5.32	5.36	5.50
($J_{1;2}$)	(1.4)	(1.0)	(1.0)	(<1.0)	(<1.0)	(1.8)
H2	5.71	5.58	5.63	5.67	5.72	5.33
($J_{2;3}$)	(3.2)	(3.5)	(3.5)	(2.5)	(2.5)	(3.6)
H3	5.18	5.20	4.06	5.16[a]	5.18	4.98
($J_{3;4}$)	(9.6)	(9.2)	(9.2)		(8.0)	(10.0)
H4	5.33	3.98	5.16	5.16[a]	3.91	4.02
($J_{4;5}$)	(9.6)	(9.2)	(9.0)		(~ 9)	(10.0)
H5	3.91	3.80	3.89	3.92	3.74	3.86
($J_{6S;6R}$)	(12.4)	(12.2)	(12.2)	(~ 11)	(12.2)	(12.2)
H6S	4.33	4.34	4.23	3.48	3.99	4.22
($J_{5;6S}$)	(3.0)	(3.0)	(2.5)	(~ 2)	(2.5)	(~ 4)
H6R	4.22	4.23	4.30	3.87	3.83	4.17
($J_{5;6R}$)	(6.4)	(6.1)	(78.0)	(~ 7)	(5.5)	(~ 3)
H_{Aryl}	8.21/7.08	8.12/6.98	8.21/7.08	8.28/7.12	8.27/7.11	8.15/7.11
($J_{aryl;Aryl}$)	(9.6)	(9.6)	(9.7)	(9.5)	(9.6)	(9.2)
Non-reducing hexose						
H1′		4.69	4.74	5.08	4.84	4.69
($J_{1';2'}$)		(<1.0)	(<1.0)	(3.6)	(~ 1.0)	(<1.0)
H2′		5.37	5.33	4.86	5.28[a]	5.36
($J_{2';3'}$)		(3.2)	(3.2)	(10.2)		(3.6)
H3′		4.89	5.11	5.47	5.28[a]	5.52
($J_{3';4'}$)		(9.8)	(10.0)	(9.7)		(9.8)
H4′		5.16	5.22	5.01	5.28[a]	5.15
($J_{4';5'}$)		(9.6)	(9.7)	(10.2)	(9.5)	(10.0)
H5′		3.59	3.63	3.90	3.83	3.58
($J_{6S';6R'}$)		(12.2)	(12.2)	(12.2)	(12.2)	(12.2)
H6S′		4.09	4.14	3.97	4.00	4.04
($J_{5';6S'}$)		(3.1)	(3.0)	(2.0)	(2.5)	(2.5)
H6R′		4.24	4.30	4.03	4.10	4.26
($J_{5';6R'}$)		(5.6)	(5.0)	(4.2)	(4.5)	(5.6)
OAc		2.16/2.11/2.03/2.03/2.03/1.99/1.93	2.24/2.15/2.10/2.08/2.05/2.04/2.00	2.08/2.07/2.06/2.06/2.03/2.03/2.01	2.25/2.25/2.16/2.11/2.07/2.07/2.03	2.24/2.18/2.13/2.10/2.04/1.99/1.92

[a] Assignment by COSY

aqueous Na_2CO_3 (0.5 ml, 0.1 M) and water (1.0 ml), and the extinction was measured at 400 nm. For comparison, the same procedure was done with the free enzyme. For preparative purposes, β-mannosidase (5 U) was immobilized on CNBr-activated Sepharose 4B at pH 5.0 and room temperature according to the same procedure.

Immobilization of β-galactosidase

The procedure corresponds to the one described above, but only 20 µl of an enzyme suspension (7 U of β-galactosidase) and a solution of 4-nitrophenyl β-D-galactopyranoside in buffer (1 ml, 10 µmol, pH 5.0) were used. Preparative incubations were performed with the enzyme immobilized on CNBr-activated Sepharose 4B (200 mg β-galactosidase, pH 8.0, room temperature).

Transmannosylation of methyl α-D-glycopyranosides **2–4**

Saturated solutions of **1** (0.5 mmol, 150 mg) and the methyl α-D-glycopyranoside **2–4** (10.0 mmol) in buffer (pH

Table 4. ^{13}C NMR data of mannobiosides **8** (Manβ1-4Manα1-OMe) and **13** (Manβ1-4Manβ1-OpNP)

Hexose unit	*Chemical shift δ/ppm*[a]					
	8			**13**		
	Man	Man′	Δδ	Man	Man′	Δδ
C1	97.81	97.21	0.60	94.68	97.84	− 3.16
C2	69.49	67.83	1.56	68.13	67.30	0.83
C3	68.20	70.12	− 1.92	68.93	70.41	− 1.48
C4	72.47	65.40	6.93	72.75	64.32	8.43
C5	68.26	71.84	− 3.58	74.06	73.99	0.07
C6	62.19	61.98	0.21	58.64	57.99	0.65
$C_{Aglycone}$	OMe: 54.58 OAc: 20.24/20.20/20.15/ 20.06/20.04/19.94			OAryl: 159.06/140.05/ 123.68(2C)/ 113.91(2C)		
$J_{C-1/C-2}$ [Hz]	170.4	158.3		160.1	160.2	
configuration	*α*	*β*		*β*	*β*	

[a] Measured in ^2H_2O with acetone as internal standard ($\delta = 27.14$ ppm).

4.0, 10% DMF) were prepared and β-mannosidase suspension (5 U, 500 µl) added. After shaking for 15 h at 30 °C about 80% of **1** was converted, and the enzyme was inactivated by heating for 5 min to 90 °C. 4-Nitrophenol was removed by ion exchange resin, and the product disaccharides were separated by gel chromatography on Fractogel or Biogel P-2. This fraction was acetylated by the standard procedure with pyridine/acetic anhydride (2:1) and catalytic amounts of N, N′-dimethyl aminopyridine at room temperature. A partial separation was achieved by HPLC or flash chromatography on silica gel (20 × 1 cm, eluent: petrol ether/ethyl acetate 5:6). ^1H NMR data are listed in Table 1.

Synthesis of 1,5-anhydro-2-deoxy-D-arabino-hexitol (**10**) and 1,5-anhydro-1,3-dideoxy-D-erythro-hexitol (**11**)

3.4,6-Tri-*O*-acetyl-D-glucal (0.72 mg, 10 mmol) in degassed ethyl acetate (50 ml) was hydrogenated over platinum as catalyst (prepared from PtO_2, 30 mg) for 3 h at room temperature. The catalyst was removed by centrifugation, the solvent distilled off, and the residue separated by flash chromatography (silica gel, 15 × 6 cm, solvent: petrol ether/ethyl acetate 5:2). First was eluted a diacetate (1.00 g, 36%), which was deacetylated according to the standard procedure (methanol/sodium methanolate) to give compound **11** (0.58 g, 95%). Then a triacetate (0.71 g, 33%) was eluted which after deacetylation yielded **10** (0.36 g, nearly quantitative). The physical properties of **10** and **11** were in good agreement with data reported earlier [16].

Transmannosylation of anhydro deoxy hexitol **10**

β-Mannosidase (2 U, 0.36 ml) was added to a solution of **10** (360 mg, 2.4 mmol) and **1** (74 mg, 0.24 mmol) in 3 ml of buffer/DMF (10%, pH 4.0). During incubation at room temperature for 14 d additional enzyme (1 U) was added twice. The reaction was stopped as described above, and the product (**12**, 2 mg, 3% yield, yellow syrup) was isolated by ion exchange and gel permentation chromatography followed by peracetylation, and subsequent flash chromatography on silica gel (10 × 2 cm, solvent: petrol ether/ethyl acetate 1:1). The NMR data are listed in Table 1.

Transmannosylation by self-transfer reaction

Substrate **1** (in one case **1** + **1**α = 1:1) (1 mmol, 301 mg) was dissolved in a mixture of cosolvent and buffer (pH 4.0) and β-mannosidase (5 U, 0.9 ml suspension) was added to give saturated solutions (2.3 ml with 40% aqueous acetone, 4.5 ml with 40% aqueous acetonitrile; 2.1 ml with 50% aqueous DMSO). Incubations with β-galactosidase were performed in 50% of aqueous cosolvents (buffer: pH 5.0) with 1000 U of enzyme (free), or about 550 U of immobilized enzyme and 2.0 mmol of **1** (cf. Table 6). After 7 d the enzyme was removed, and separation by ion exchange and subsequently by gel permentation chromatography (twice at a very low flow-rate) led to isolation of the transfer products **13–17**. Products were detected photometrically at 298 nm. Details of the spectroscopic characterization are given in Tables 2 and 3.

Results and discussion

Activities of enzymes with organic cosolvents

Transfers on a preparative scale require sufficient enzyme activity for several hours at least. Thus, for both enzymes the best compatible cosolvents were checked by preliminary semi-quantitative tests and the hydrolytic activities determined photometrically with phenolates. Both hydrolases show remarkable activities in buffers containing DMF, DMSO, acetonitrile, or (only for β-mannosidase) acetone (Figures 1–4). Therefore, incubations for two and more days should be possible using buffers with up to 35% of DMF or up to 45% of DMSO, respectively. Previously, transgalactosylations with β-galactosidase in 60% or 70% aqueous acetonitrile were described by Ooi *et al.* [4] and Beau *et al.* [5], but, these were done with high enzyme concentrations in a very short time (min).

Immobilization

It was of interest to recycle these expensive enzymes and to influence the regioselectivity of the transfer reaction [6] by immobilization. For β-galactosidase the stabilizing starch was removed during immobilization [7]. Agarose matrices such as CNBr-activated Sepharose 4B and

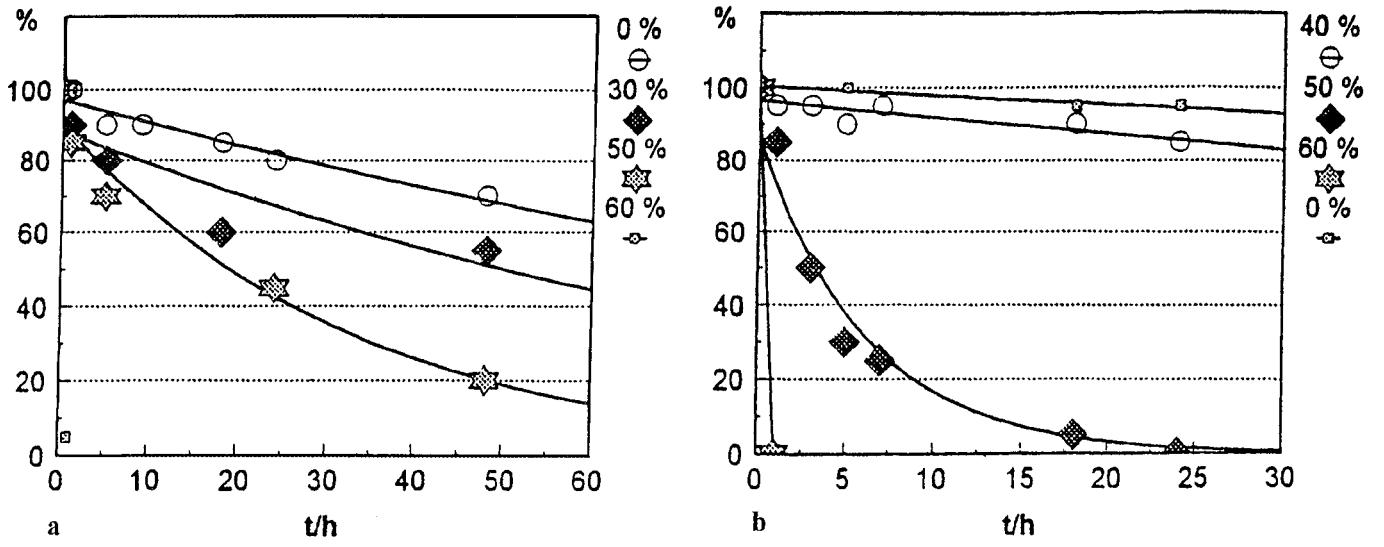


Figure 1. Decrease of activity in buffer/DMSO. **1a.** β-Mannosidase. **1b.** β-Galactosidase.

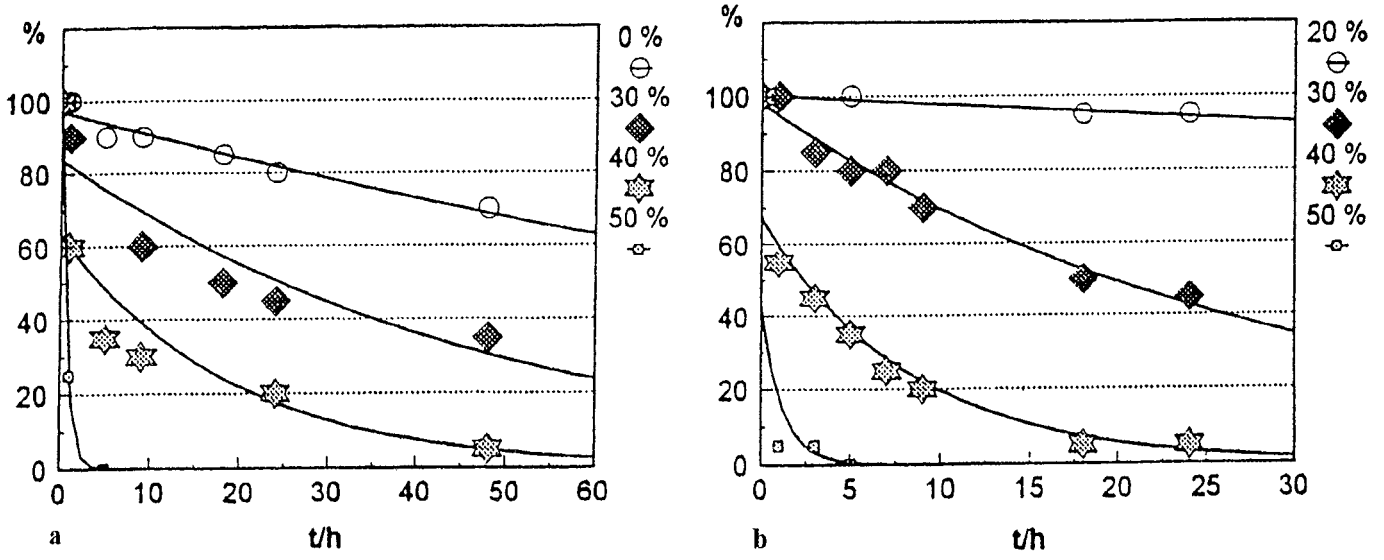


Figure 2. Decrease of activity in buffer/DMF. **2a.** β-Mannosidase, **2b.** β-Galactosidase.

activated CH-Sepharose 4B with a larger distance between reactive groups and the gel skeleton were selected, and the coupling reaction proved optimal at pH 6 for β-mannosidase and pH 7.5 for β-galactosidase (Table 5). The release of 4-nitrophenol dependent on the pH of the immobilization is shown in Figures 5 and 6. With CNBr-activated Sepharose the remaining activity was slightly better for both enzymes (about 55%). The best rates for immobilization of β-galactosidase were around pH 7–8 and for β-mannosidase about pH 5–6. Lowering the temperature (from 20 to 4 °C) had no significant but in most cases a slightly negative effect on the immobilization. Due to the much shorter incubation times, the immobilization of both enzymes at room temperature was more efficient and thus preferred.

Transmannosylations

As donor substrate for enzymic transmannosylations, 4-nitrophenyl β-D-mannopyranoside (**1**) was prepared as reported by Garegg *et al.* [8]. The sensitivity of the glycosidases and the solubility of the substrate determine the choice of the organic solvent system. Buffers containing between 10% (for methyl glycoside acceptors) and 50% (for self-transfer reactions) of cosolvents gave the best ratio between transglycosylation and hydrolysis. Apparently, in

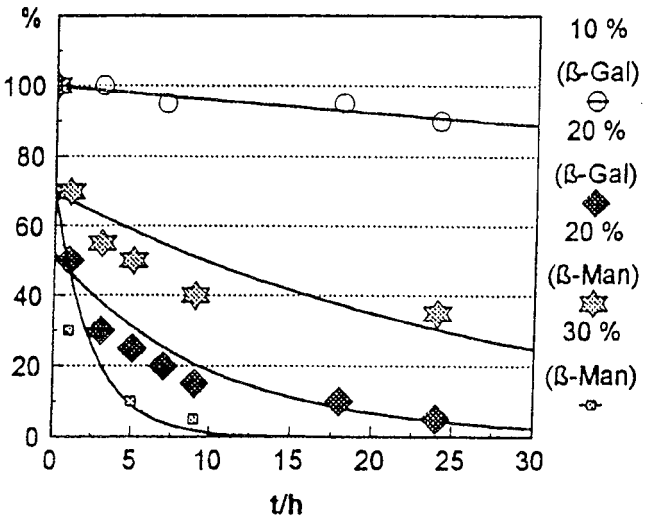


Figure 3. Decrease of activity in buffer/acetonitrile (*β*-mannosidase, *β*-Man;*β*-galactosidase, *β*-Gal).

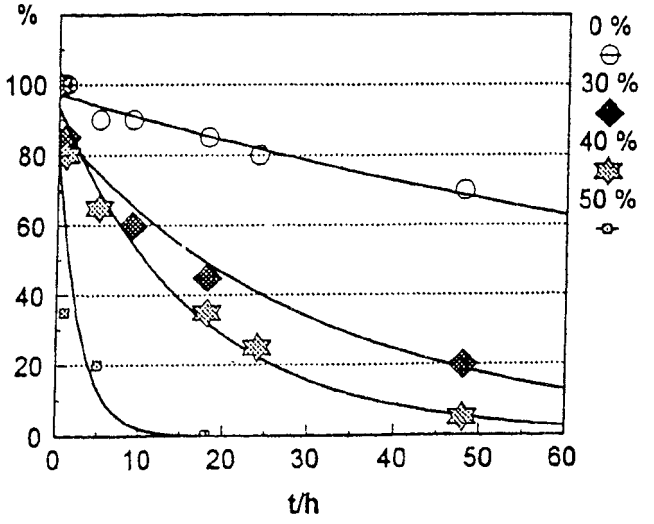


Figure 4. Decrease of activity in buffer/acetone (only *β*-mannosidase).

Table 5. Remaining activities of immobilized enzymes

Enzyme	*Matrix*	*at 4 °C*	*at 20 °C*
β-mannosidase	CH-Sepharose	40% (pH 6)	40% (pH 5.5)
	CNBr-Sepharose	25% (pH 5.5)	55% (pH 6)
β-galactosidase	CH-Sepharose	50% (pH 8)	50% (pH 8)
	CNBr-Sepharose	50% (pH 7.5)	55% (pH 7.5)

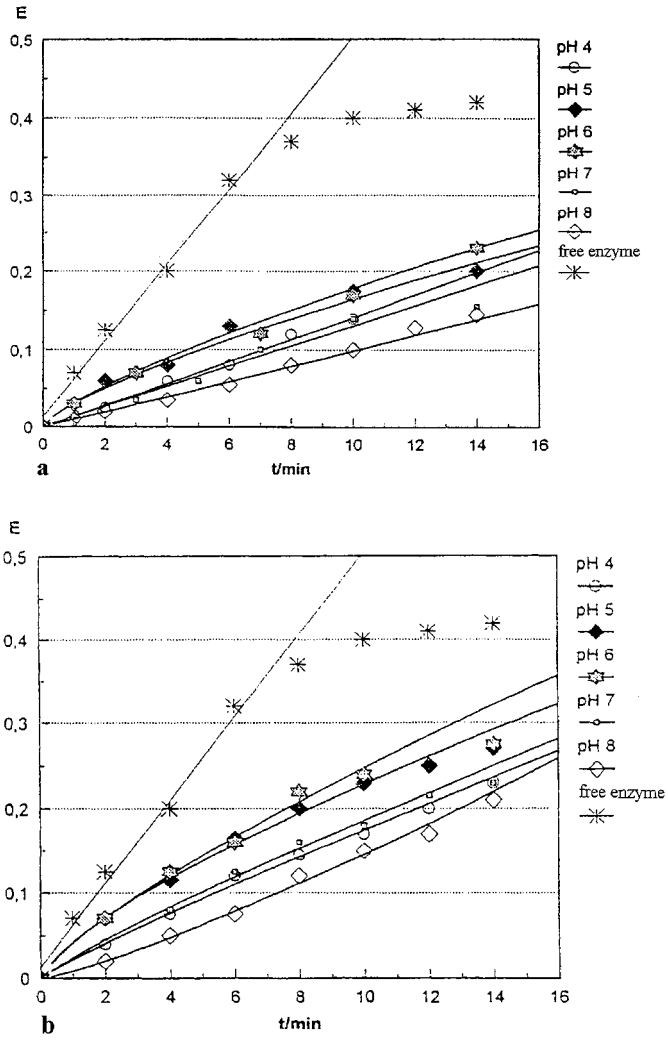


Figure 5. Release of 4-nitrophenol by immobilized *β*-mannosidase. **5a.** Immobilization on activated CH-Sepharose. **5b.** Immobilization on CNBr-activated Sepharose.

contrast to the transfer to alcohols [2, 3] transmannosylation of carbohydrates gave significantly lower yields. Whereas insufficient hydrophilicity can be ruled out it may be assumed that the newly formed disaccharides themselves are favorable substrates for cleavage by the glycosidases as confirmed by employing reversed hydrolysis conditions [9, 10].

The best results observed for transmannosylation of various methyl α-D-glycopyranosides (**2**–**4**) as acceptors with *β*-mannosidase from snail viscera are compiled in Table 6. Corresponding experiments with galactosidase had to be excluded because of its known side-activities towards other glycosides [3, 7, 11]. Partial separation of the peracetylated disaccharide fractions by flash chromatography or HPLC on silica gel and subsequent characterization by ^{1}H NMR showed a large number of products that exceed the number of possible *β*-mannosylations.

Some isolated products confirmed the presence of α-hydrolases during incubation. For example, Manα1-6Manα1-OMe (**5**) and Glcα1-6Glcα1-OMe (**6**) can only be formed if initially glycosidic linkages are cleaved and

subsequently α-glycosidases catalyse α-glycosylation by reverse hydrolysis. Even a β-glucosidase activity was proven by isolation and characterization of peracetylated Glcβ1-6Glcα1-OMe (**7**). Two products of β-mannosylation were determined by NMR methods to be Manβ1-4Manα1-OMe (**8**) and Manβ1-2Glcα1-OMe (**9**). The β linkage of **8** was clearly evident by the coupling $J_{C-1,1-H} = 158.3$ Hz [15]

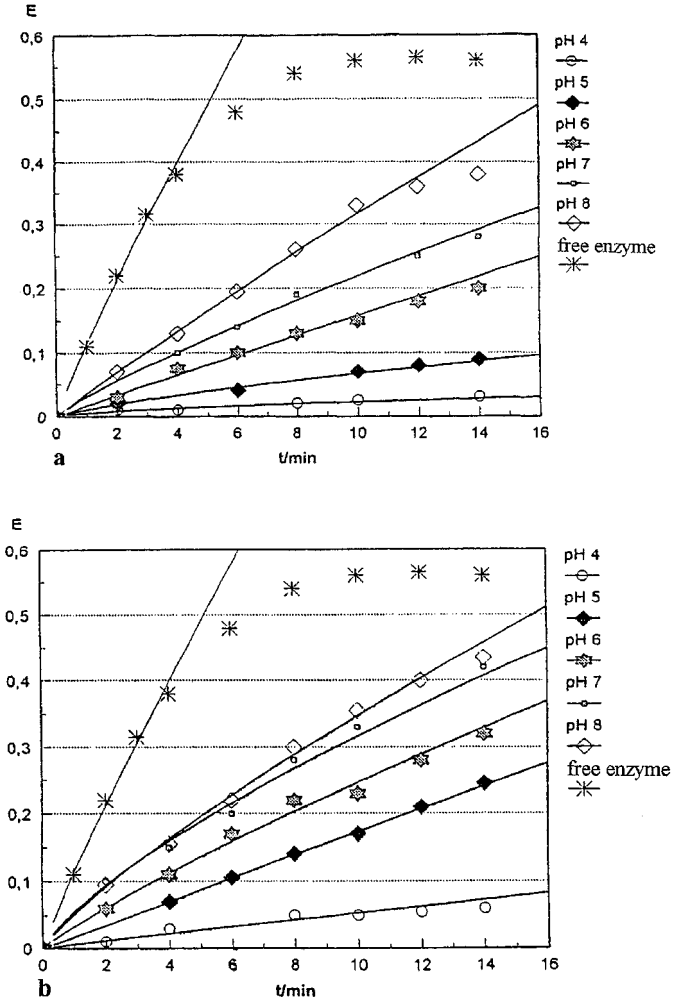


Figure 6. Release of 4-nitrophenol by immobilized β-galactosidase. **6a.** Immobilization on activated CH-Sepharose. **6b.** Immobilization on CNBr-activated Sepharose.

(see Table 4). Even the use of immobilized β-mannosidase did not enhance the yield of transfer products.

Further the β-mannosylation of deoxygenated glycitols was of interest. 1,5-Anhydro-2-deoxy-D-*arabino*-hexitol (**10**) and 1,5-anhydro-2,3-dideoxy-D-*erythro*-hexitol (**11**) were prepared by hydrogenation of peracetylated D-glucal. Incubation in aqueous DMF (10%) of **1** with β-mannosidase and a tenfold amount of **10** gave a single product in 3% yield which after peracetylation could be determined as β1-4 linked disaccharide **12**. The preference of a 1-4 over a 1-6 linkage demonstrates that the regioselectivity of β-mannosyl transfer does not reflect the reactivity of the hydroxyl group but rather specific enzymatic conditions. There was no transfer observed with the acceptor **11** and it may be assumed that the missing 3-OH group in **11** prevents mannosylation completely.

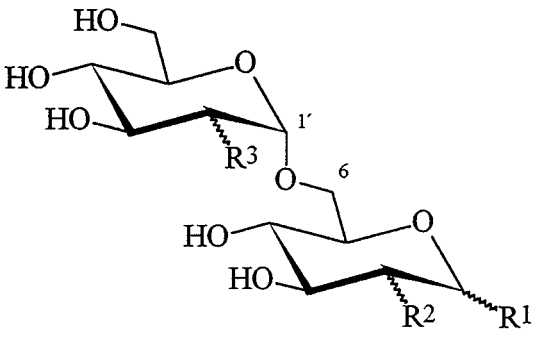
Since some glycosidases are well known to have higher affinity for hydrophobic acceptor glycosides [12], self-transfer reactions with the donor substrate **1** were examined. 4-Nitrophenyl mannobiosides were obtained (1.6%) with β-mannosidase in aqueous acetonitrile (40%). In aqueous acetone (40%) the yield was 0.6%, and with aqueous DMSO (up to 50%) hydrolysis was favored over transfer. Disaccharides were formed in every product fraction in minor amounts among which the β1-4-linked glycobioside **13** (Manβ1-4Manβ1-OpNP) was found to be the dominant product (80 to 85%). $J_{C-1,1-H}$ couplings of 160.2 Hz and 160.1 Hz in both mannose rings give evidence for the β-glycosidic linkages in **13**. As another side-product Manβ1-3Manβ1-OpNP (**14**) could also be isolated and characterized after peracetylation by ^{1}H NMR. In contrast to findings of Kyosaka *et al.* who described the formation of small amounts of β1-6- and β1-2-linked self-transfer products catalyzed by a β-mannosidase from guinea pig liver [13], none of these regioisomers were formed in significant quantities by the enzyme from snail viscera.

In 50% aqueous acetonitrile 3% of transfer products were obtained with the free β-galactosidase from *Aspergillus oryzae*. The main product (about 70%) was determined to be the α1-6 glucosylated derivative **15** (Glcα1-6Manβ1-OpNP) the formation of which is assumed to be due to the presence of starch and an α-hydrolytic side activity of the enzyme preparation. The mixtures of disaccharides obtained with immobilized enzyme after repeated separation

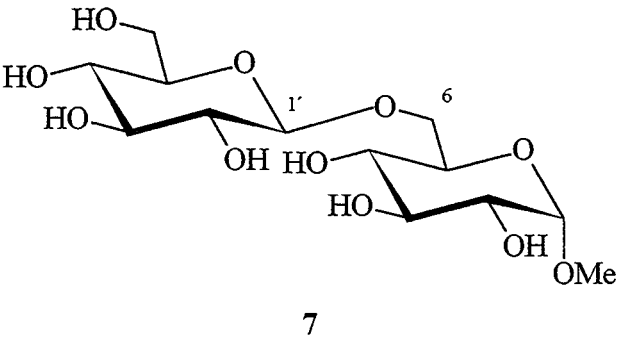
Table 6. Transmannosylation of methyl *a*-D-glycopyranosides with donor substrate *1*

Acceptor substrate	*Yield of products*	*Number of products*[a]	*Ratio donor/acceptor*	*Temperature*	*Best cosolvents*
Glc*a*1-OMe (**2**)	3%	13–14	1 : 20	30°C	10% DMF
Man*a*1-OMe (**3**)	4%	6	1 : 20	30°C	15% DMF
GlcNAc*a*1-OMe (**4**)	< 2%	> 7			

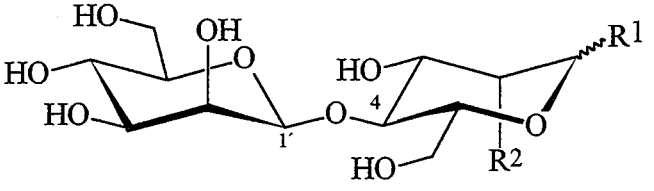
[a] TLC discrimination



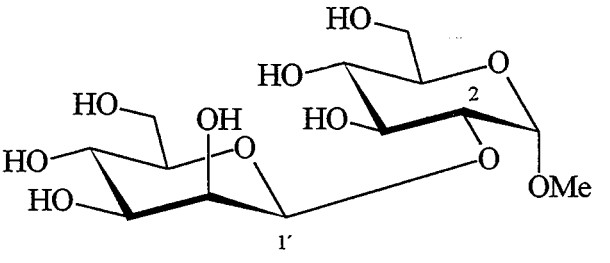
5 R^1 = αOMe, R^2 = R^3 = axOH
6 R^1 = αOMe, R^2 = R^3 = eqOH
15 R^1 = βOpNP, R^2 = axOH, R^3 = eqOH



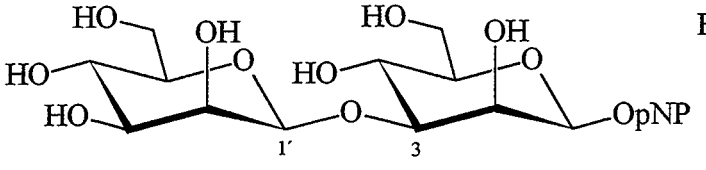
7



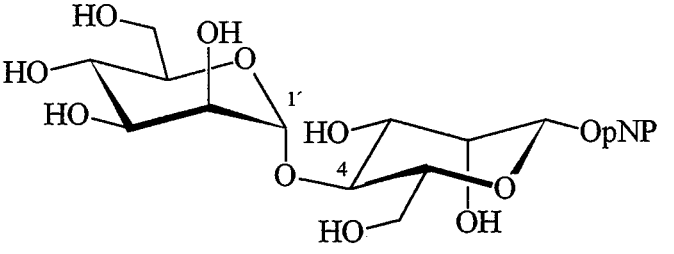
8 R^1 = αOMe, R^2 = OH
12 R^1 = R^2 = H
13 R^1 = βOpNP, R^2 = OH
17 R^1 = αOpNP, R^2 = OH



9



14



16

Figure 7. Glycobiosides.

by gel permeation chromatography are compiled in Table 7. NMR spectra of the subsequently peracetylated fractions gave evidence that β1-4- and β1-3-linked disaccharides **13** and **14** were the main products. The third product of transmannosylation was characterized after peracetylation as α1-4-mannosylated 4-nitrophenyl β-D-mannopyranoside **16** (Manα1-4Manβ1-OpNP). The products **13**, **14**, and **16** formed in a ratio of 2:1:1 with acetonitrile or DMF and 3:3:1 with DMSO as cosolvents, respectively.

To examine the influence of the anomeric configuration of the acceptor substrate an equal amount of 4-nitrophenyl α-D-mannopyranoside (**1**α) was added to the incubation mixture. The yield increased to 6% under the same conditions, and in addition to the same side-products equimolar amounts of **13** and Manβ1-4Manα1-OpNP (**17**) were obtained. Apparently, the enzyme does not differentiate the anomeric configurations of the acceptor substrate.

As observed by incubations with β-mannosidase, the amount of β1-4- and β1-3-linked self-transfer products dominated (>90%). Apparently, methyl glycosides **2–4** do not show specific orientations during the enzymic transfer process. However, the hydrophobic nitrophenol group allowed the acceptor to be linked to a mannosyl residue only from the opposite side. Even the hydrophobic area of

Table 7. Transmannosylation by self-transfer reaction of **1** with immobilized β-galactosidase

Cosolvent (50%)	*Volume (ml)*	*Turnover rate (after 2 w)*	*Yield*[a] *(%)*	*Products isolated*
CH_3CN	8.5	51%	5.4	**13** (50%): Manβ1-4Manβ1-OpNP **14** (21%): Manβ1-3Manβ1-OpNP **16** (20%): Manα1-4Manβ1-OpNP
DMF	9.5	68%	8.0	**13** (50%): Manβ1-4Manβ1-OpNP **14** (22%): Manβ1-3Manβ1-OpNP **16** (23%): Manα1-4Manβ1-OpNP
DMSO	11.0	87%	2.1	**13** (39%): Manβ1-4Manβ1-OpNP **14** (39%): Manβ1-3Manβ1-OpNP **16** (15%): Manα1-4Manβ1-OpNP

[a] based on turned over substrate

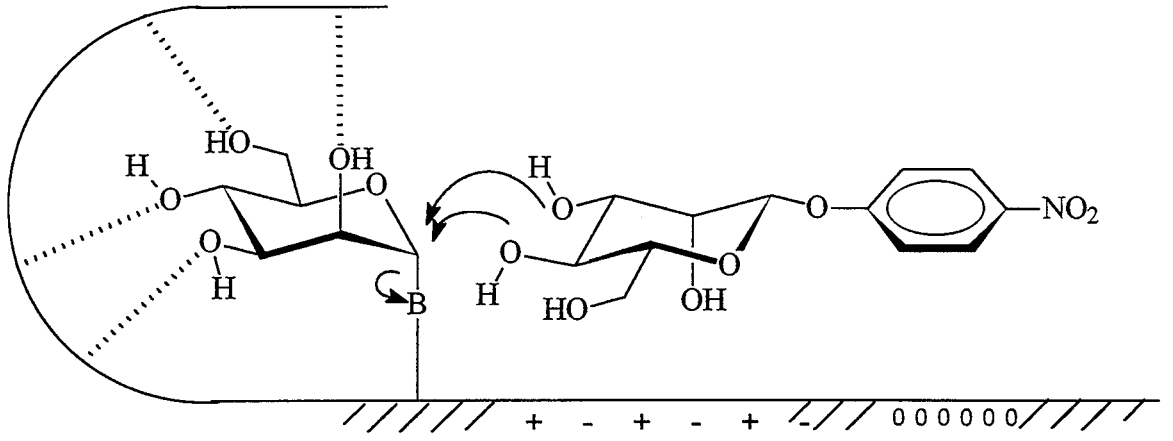


Figure 8. Schematic depiction of transmannosylation.

the anhydro deoxy hexitol **10** is sufficient for such a pre-transfer orientation. These findings support the assumption that there is an additional hydrophobic part of the acceptor binding-site at the catalytic centre of some glycosidases. Figure 8 depicts a scheme for transmannosylation as observed with the glycosidases studied, which is in good agreement with previous results on the transmannosylation of simpler hydroxyl compounds [2; 3]. The regiospecifity of the transfer is dependent on the structure of acceptor substrates and is illustrated in Figure 9. Even though these findings about enzymic transmannosylations are of general interest, the preparative use of such hydrolases for syntheses of mannosylated oligosaccharides is hampered by low yields of transfer.

Acknowledgments

Support of this work by the *Fonds der Chemischen Industrie* including a Kekulé stipend to Norbert Taubken is gratefully acknowledged.

References

1 Flitsch SL, Taylor JP, Turner NJ (1991) *J Chem Soc Chem Commun* 380–2.
2 Taubken N, Thiem J (1992) *Synthesis* **6**: 517–8.
3 Taubken N, Sauerbrei B, Thiem J (1993) *J Carbohydr Chem* **12**: 651–67.
4 Ooi Y, Hashimoto T, Mitsuo N, Satoh T (1984) *Tetrahedron Lett*. **25**: 2241–5.
5 Petit JM, Paquet F, Beau JM (1991) *Tetrahedron Lett*. **32**: 6125–8.
6 Svensson SCT, Thiem J (1990) *Carbohydr Res* **200**: 391–402.
7 Ooi Y, Hashimoto T, Mitsuo N, Satoh T (1985) *Chem Pharm Bull* **33**: 1808–14.
8 Garegg PJ, Iversen T, Norberg T (1979) *Carbohydr Res* **73**: 313–14.
9 Ajisaka K, Fujimoto H (1990) *Carbohydr Res* **199**: 227–34.
10 Ajisaka K, Fujimoto H, Nishida H (1988) *Carbohydr Res* **180**: 35–42.
11 Crout DHG, Singh S, Swoboda BEP, Critchley P, Gibson WT (1992) *J Chem Soc Chem Commun* 704–5.

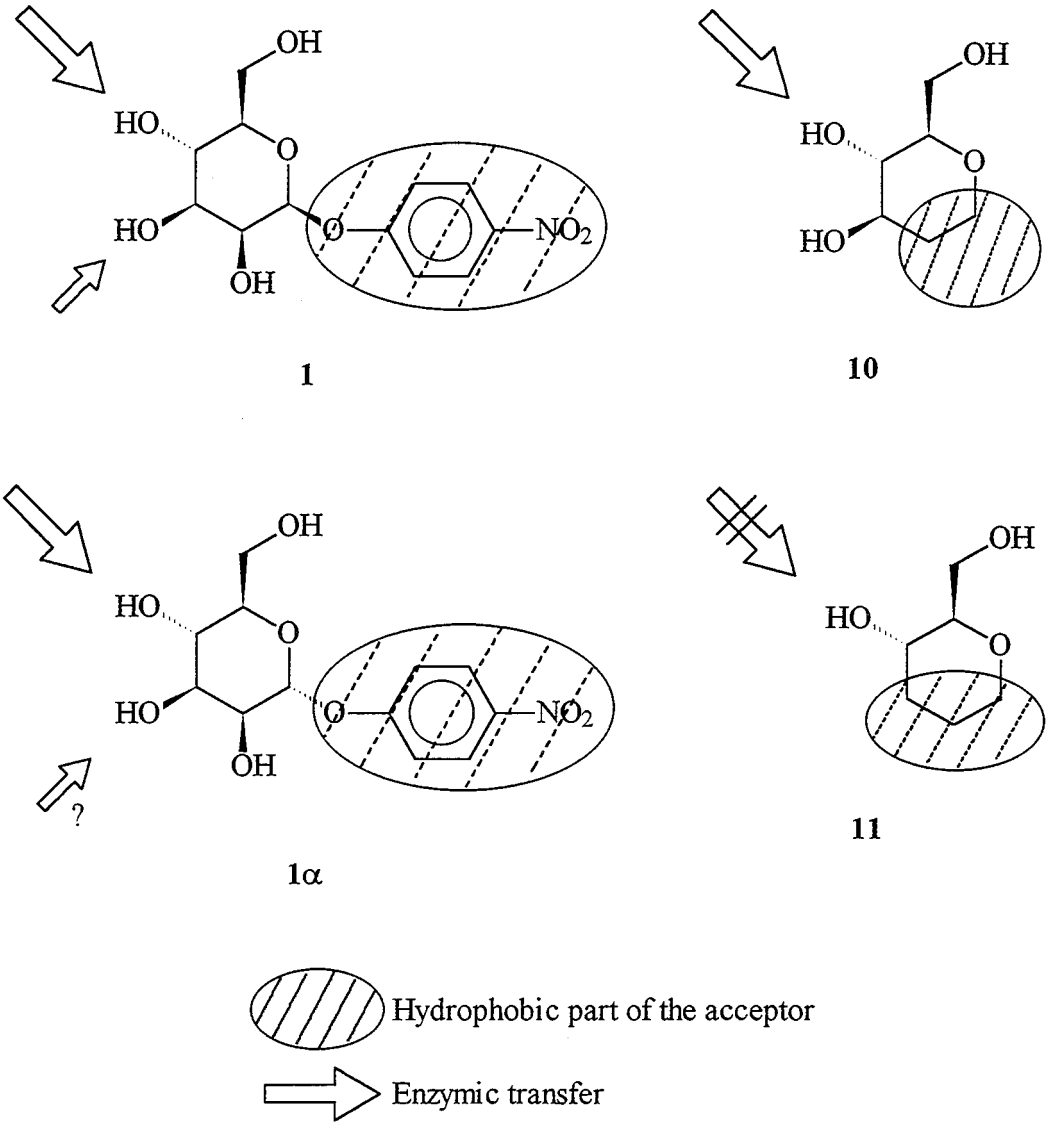


Figure 9. Regiospecifity of transmannosylation depending on the structure of acceptor molecules.

12 Huber RE, Gaunt MT (1983) *Arch Biochem Biophys* **220**: 263–71.
13 Kyosaka S, Murata S, Tsuda Y, Tanaka M (1986) *Chem Pharm Bull* **34**: 5140–3.
14 Nilsson KGI (1988) *Tibtech* **6**: 256–64.
15 Bock K, Lundt I, Pedersen C (1973) *Tetrahedron Lett* **13**: 1037–40.
16 Sathyanarayana BK, Stevens ES (1988) *Carbohydr Res* **182**: 179–96.

Received 2 September 1997, revised and accepted 26 February 1998