Substrate structure and incubation-parameter-dependent selectivities in chiral discrimination of galactopyranosides by β-galactosidase hydrolysis

Barbara Werschkun,[a] Wilfried A. König,[a] Vladimír Křen[b] and Joachim Thiem *,[a]
[a] *Institut für Organische Chemie, Universität Hamburg, Martin-Luther-King-Platz 6, D-20146 Hamburg, Germany*
[b] *Institute of Microbiology, Academy of Sciences of the Czech Republic, Videňská 1083, CZ-14220 Prague 4, Czech Republic*

Mono-β-galactopyranosides of (±)-propane-1,2-diol, (±)-butane-1,3-diol, (±)-pentane-1,4-diol, (±)-butan-2-ol, (±)-pentan-2-ol and (±)-1,2-*O*-isopropylideneglycerol were synthesized by the Koenigs–Knorr reaction using hydroxycarbonyl compounds as precursors for the diolic substructures. Hydrolysis of 3-hydroxybutyl β-D-galactopyranoside **18** by β-galactosidases from *Escherichia coli*, *Aspergillus oryzae*, *Kluyveromyces lactis* and *Bacillus circulans*, respectively, resulted in each case in an enantiomeric enrichment of the released diol. This was most significant with the *E. coli* enzyme and increased with higher reaction temperature and shorter incubation periods. Under standardized conditions, cleavage of all synthesized galactopyranosides by this enzyme showed the highest stereoselectivity for butane-1,3-diol, butan-2-ol and isopropylideneglycerol with enantiomeric excesses in the range 60–75%. For compounds with substructural similarity to the natural substrate lactose, enhanced stereodiscriminations were expected. However, this could not be confirmed and instead a specific hydrophobic interaction is suggested to play a crucial role.

Introduction

As natural catalysts, enzymes can discriminate between chemical substances of even subtle distinctions and thus promote reactions in a chemo- and stereo-selective manner. This potential has been developed into a powerful means of obtaining optically pure chiral material and the separation of chemically produced stereoisomeric mixtures by the distinguishing action of hydrolytic enzymes such as amidases, acylases, carbonic acid ester hydrolases or lipases has become a widespread application.[1–4]

Glycoside hydrolases, however, in this respect have received little attention so far. Their use for the separation of enantiomers could principally proceed in two different ways: subjection of a chemically synthesized mixture of diastereoisomeric glycosides to enzymic hydrolysis might result in the release of only one enantiomer of the chiral aglycones, while the other remains in the form of an optically pure glycoside. The second possibility is to employ glycosidases for a stereoselective glycosyl transfer to only one enantiomer of a racemic alcohol mixture. The latter variant revealed the general ability of these enzymes to discriminate between different configurations in their substrate's aglycone moiety.[5–7]

Systematic studies of regio- as well as stereo-control of this reaction have been carried out by Crout *et al.*[8] with β-galactosidase from *Escherichia coli* and followed by Trincone *et al.*[9] who employed the β-galactosidase activity of a crude enzyme preparation from *Sulfobolus solfatarius*. For a variety of short-chain alcohols and diols, they found galactosyl transfer to result in diastereoisomerically enriched galactopyranoside mixtures. Dependence of the extent of stereoselection on the respective aglycones and preference for a certain configuration were explained by employing a model for the active site of the *E. coli* enzyme proposed by Huber *et al.*[10] in 1984. Accordingly, out of two enantiomers the one should be preferably converted which can best match the structure of glucopyranose as the natural aglycone substrate of the enzyme.

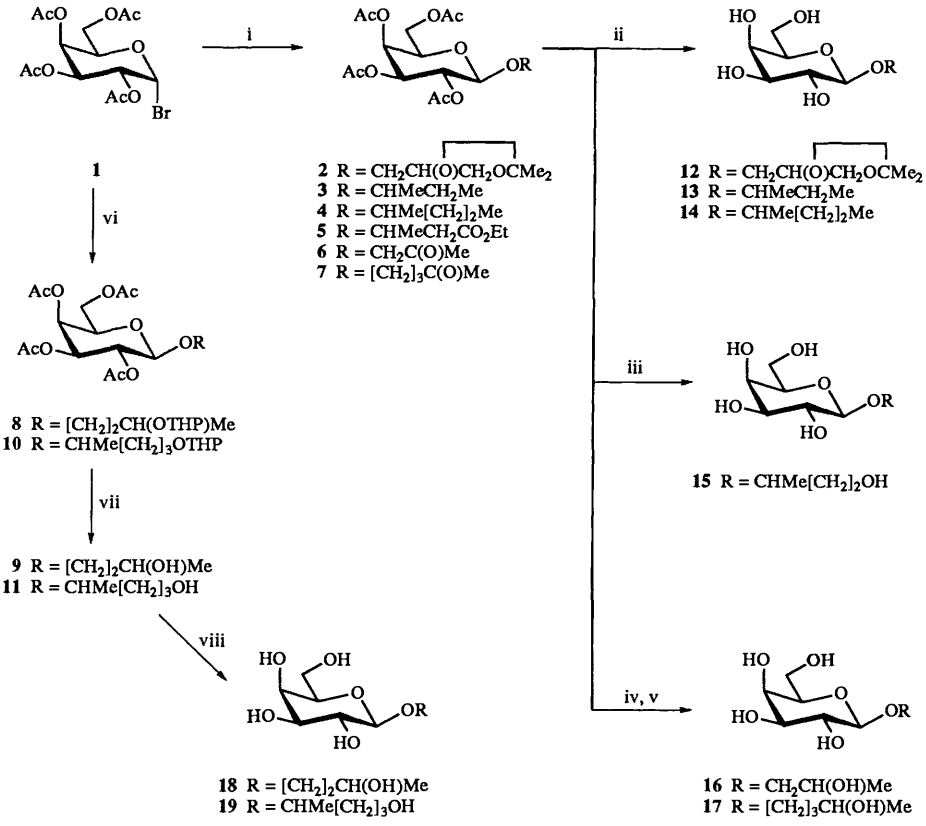
Although the diastereomeric excess achieved in those transglycosylation experiments was only moderate and did not exceed 40%, it encouraged us to exploit the stereochemical nature of the enzymic hydrolysis.

The stereoselectivity of a reaction is dependent on the difference between the free activation energy values for the two or more possible reaction pathways, from which the respective stereoisomeric reaction products emerge. Therefore, time and temperature are those parameters by variation of which the stereochemical outcome of the reaction may be controlled. This dependence can be expressed in equation (1), describing the

$$E = \exp(-\Delta\Delta G^{\ddagger}/RT) = \ln[1 - C(1 + ee(P))]/\ln[1 - C(1 - ee(P))] \quad (1)$$

selectivity, E, of a reaction wherein C stands for the conversion and ee(P) for the enantiomeric excess in the product.[11] If the selectivity for a reaction under a certain set of conditions is known, the degree of conversion necessary to obtain the desired extent of enantiomeric excess can be calculated. To increase the selectivity, either the reaction or the conditions must be changed. The former in the course of an enzymic process would mean to alter the substrate structure or to search for an enzyme which can be employed more efficiently.

The subject of our studies was to submit chemically synthesized galactopyranosides of several racemic alcohols to enzymic hydrolysis by β-galactosidases from different microbial sources, namely *E. coli*, *Aspergillus oryzae*, *Kluyveromyces lactis* and *Bacillus circulans*. In addition, the influence of incubation conditions on the stereoselectivity of the reaction should be elucidated by varying concentrations and activities, incubation periods, temperatures, and, if consistent with enzyme activity, solvents. Substrates were selected following the work of Crout *et al.*, in order to dispose of comparative data and adopting the general idea of mimicking the glucopyranose structure. Thus, in the galactopyranosides of propane-1,2-diol, butane-1,3-diol and pentane-1,4-diol the aglycone moiety is provided with a free hydroxy group at varying positions, which is omitted in the butan-2-ol and pentan-2-ol derivatives. Additionally, examination of 1,2-*O*-isopropylideneglyceryl galactopyranoside



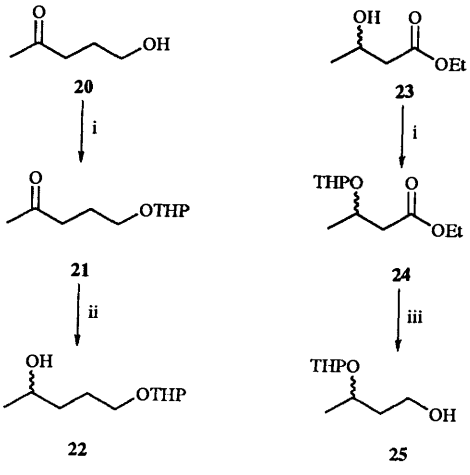
Scheme 1 THP = Tetrahydropyran-2-yl, NMP = *N*-methylpyrrolidine. *Reagents:* i, ROH, Ag_2O or Ag_2CO_3; ii, NMP, MeOH; iii, $LiAlH_4$; iv, $NaBH_4$; v, NMP, MeOH; vi, ROH, Ag_2CO_3; vii TsOH, MeOH; viii, NMP, MeOH

seemed to be of interest, since during transglycosylation experiments contradictory results had been obtained in the past.[6–8]

Results and discussion

Synthesis of the substrates was sought to be achieved by a simple glycosylation method applicable for all aglycone structures concerned. Utilization of β-pentaacetylgalactopyranose under Lewis acid catalysis[12] afforded the desired galactopyranosides in only moderate yields, accompanied by a significant amount of by-products which required laborious chromatographic purification. As catalysts, boron trifluoride–diethyl ether[13] as well as tin tetrachloride[14] were tested. Considerably better results could be obtained with peracetylated galactopyranosyl bromide **1** in a Koenigs–Knorr reaction.[15] The aglycones 1,2-*O*-isopropylideneglycerol, butan-2-ol and pentan-2-ol were thus easily converted into the corresponding acetylated β-galactopyranosides followed by deprotection with *N*-methylpyrrolidine (NMP) under very mild conditions[16] (Scheme 1). Direct glycosylation of diol, however, gave mixtures of both monogalactosylated regioisomers which could neither be separated by ordinary chromatographic means nor *via* subsequent esterification or bromide substitution of the free hydroxy group. Efforts to block the primary hydroxy group beforehand as acetate, benzoate, pivaloate or *tert*-butyldiphenylsilyl ether were unsuccessful even though selective acetylation of butane-1,3-diol had been described.[17] Instead, monohydroxycarbonyl compounds were used as precursors, reduction of which generated the other hydroxy function following the glycosylation step. By employment of the acetalisation–reduction sequences shown in Scheme 2, the same starting material could be used for the preparation of both isomers.

^{1}H NMR data of the prepared galactopyranosides are given in Tables 1 and 2. At the anomeric carbon atoms, β-configurations were observed, exclusively.



Scheme 2 DHP = 3,4-Dihydro-2*H*-pyran. *Reagents:* i, DHP, TsOH; ii, $NaBH_4$; iii, $LiAlH_4$.

In order to optimize the conditions for enhanced stereoselectivity of the enzymic process, a series of analytical experiments was performed. Formation of released galactose in our experiments was determined by the reductive conversion of 3,5-dinitrosalicylic acid into aminonitrosalicylic acid by the reducing sugar. This procedure had been developed to assay α-amylase activity,[18] but may as well be applied to the determination of galactose, as could be seen from the linearity obtained in the calibration curve. The stereochemical course of the reaction was monitored by analysis of the enantiomeric

Table 1 ^{1}H NMR data for acetylated galactopyranosides **2–7**, **9** and **11** (400 MHz; $CDCl_3$). Double entries correspond to separate signals observed for diastereoisomeric compounds or diastereotopic protons

Galactopyranoside	1-H	1′-H	2′-H	3′-H	4′-H	1″-H
2[a]	4.55 (d, 7.9)[d]	3.63–4.03 (m)	4.06–4.26 (m)	3.63–4.03 (m)		
	4.58 (d, 7.9)					
3	4.48 (d, 7.9)	3.66 (m)	1.56 (m)	0.89 (dd, 7.6, 14.2)		1.11 (d, 6.1)
	4.51 (d, 7.9)					1.23 (d, 6.1)
4	4.48 (d, 8.1)	3.66–3.80 (m)	1.47–1.63 (m)	1.28–1.45 (m)	0.89 (t, 7.1)	1.11 (d, 6.6)
	4.51 (d, 8.1)				0.90 (t, 7.1)	1.24 (d, 6.6)
5[b]	4.56 (d, 8.1)	4.05–4.25 (m)	2.36–2.79 (m)			1.18–1.31
	4.59 (d, 8.1)					
6	4.47 (d, 8.1)	4.10 (d, 16.5)		1.93–2.11 (s)[e]		
		4.20 (d, 16.5)				
7[c]	4.44 (d, 8.1)	3.51–3.57 (m)	1.77–1.93 (m)	2.51 (t)		
		3.85–3.91 (m)				
9	4.49 (d, 7.9)	3.90–4.20 (m)	1.67–1.77 (m)	3.65–3.73 (m)	1.20 (d, 6.6)	
11	4.42 (d, 7.9)	3.64–3.72 (m)	1.41–1.68 (m)	1.41–1.68 (m)	3.51–3.62 (m)	1.06 (d, 6.4)
	4.46 (d, 7.9)	3.74–3.87 (m)				1.20 (d, 6.4)

[a] Isopropylidene: δ 1.34, 1.42. [b] Ethyl: δ 1.18–1.31 (m), 4.05–4.25 (m). [c] 5′-H: δ 1.98–2.18 (s). [d] [Multiplicity, coupling constant (Hertz)]. [e] Among acetates.

Table 2 ^{1}H NMR data for galactopyranosides **12–19** (400 MHz; D_2O). Double entries: see Table 1

Galactopyranoside	1-H	1′-H	2′-H	3′-H	4′-H	1″-H
12[a,b]	4.16 (d, 7.1)	3.52–3.99 (m)	4.24 (m)	3.52–3.99 (m)		
13[a]	4.35 (d, 7.6)	3.79–3.90 (m)	1.50–1.60 (m)	1.00 (dd, 7.6, 14.8)		1.23 (d, 6.1)
			1.64–1.75 (m)			1.28 (d, 6.1)
14	4.32 (d, 7.6)	3.74–3.86 (m)	1.41–1.51 (m)	1.16–1.35 (m)	0.75 (dd, 7.1, 14.2)	1.04 (d, 6.1)
						1.09 (d, 6.1)
15	4.46 (d, 7.6)	4.00–4.13 (m)	1.69–1.90 (m)	3.62–3.81 (m)		1.22 (d, 6.6)
						1.28 (d, 6.6)
16	4.36 (d, 7.9)	3.48–3.89 (m)	3.97–4.04 (m)	1.12 (d, 6.1)		
17[c]	4.41 (d, 7.9)	3.41–3.71 (m)	1.34–1.51 (m)	1.23–1.32 (m)	3.41–3.71 (m)	
18	4.49 (d, 7.9)	4.04–4.15 (m)	1.81–1.95 (m)	3.71–3.91 (m)	1.30 (t, 6.6)	
19	4.50 (d, 7.9)	3.97–4.06 (m)	1.53–1.73 (m)	1.53–1.73 (m)	3.61–3.84 (m)	1.24 (d, 6.1)
						1.29 (d, 6.1)

[a] In [$^2H_4$] methanol. [b] Isopropylidene: δ 1.23, 1.24, 1.29 and 1.30. [c] 5′-H: δ 0.92 (d, J 6.6).

composition of the released aglycones by enantioselective gas chromatography using cyclodextrin stationary phases. Since its introduction in the late eighties, this technique has been developed into a powerful means for the resolution of stereoisomeric mixtures of a great variety of compound classes.[19] The amount of substance required is very small and sample preparation in the present case can be achieved most easily, since the alcohols and diols can be isolated from the product mixtures by simple extraction.

With the data for conversion of the reaction and enantiomeric excess of the products the selectivity was calculated by using equation (1) given above. The stereochemical outcome for one of the galactopyranosides as a model substrate had initially to be optimized. The optimized set of reaction parameters was subsequently to be applied to hydrolysis of other galactopyranosides in order to elucidate the dependence of the selectivity on the substrate structure.

3-Hydroxybutyl β-D-galactopyranoside **18** was chosen as a model substrate and was subjected to a diversity of hydrolytic conversions as specified in Tables 3 and 4. Among the four tested galactosidases, the one from *E. coli* clearly displayed the most pronounced steric discrimination and was therefore utilized for a wider array of investigations under different incubation parameters. As indicated above, the stereodifferentiation of the reaction occurs due to kinetic control and thus decreases with progressing incubation duration. This was unequivocally confirmed by all experiments, because the faster reaction resulted in a higher enantiomeric excess of the released alcohols, if the same conversion was reached after different periods of time by varying substrate concentration or enzyme activity. An increase of temperature accordingly also improved the selectivity. During transglycosylation experiments with β-galactosidase from *A. oryzae*, it had been observed that a considerable increase in stereoselection could be achieved when acetone was used as a co-solvent.[20] In the course of the hydrolytic reaction, however, this result could not be paralleled.

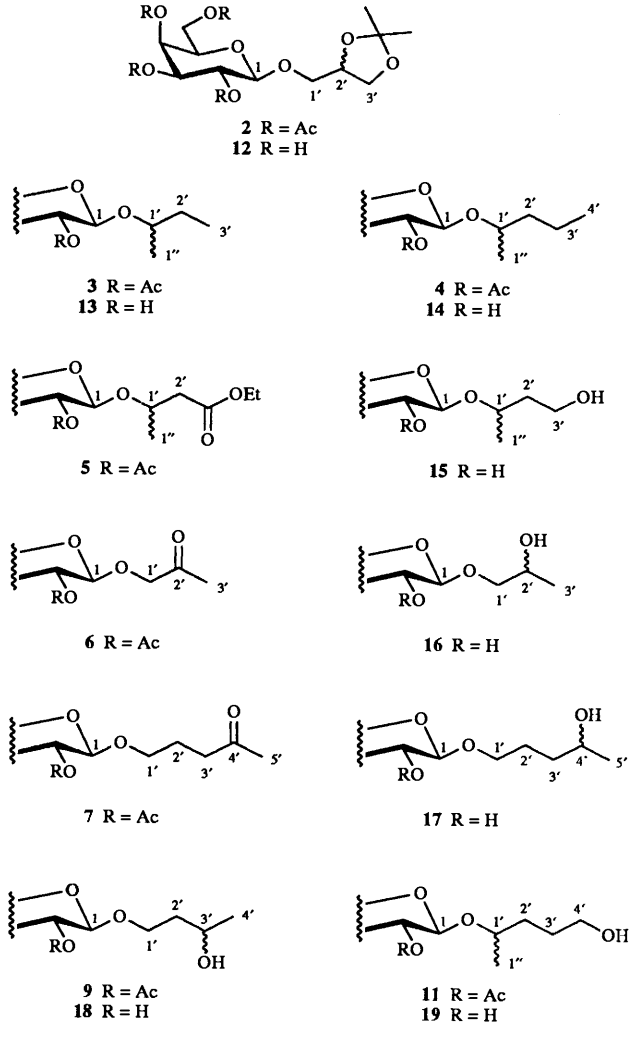
The comparative hydrolysis studies of the different galactopyranosides were thus carried out with the *E. coli* galactosidase. The results are summarized in Table 5. For a more accurate expression of the selectivity, the diastereoisomeric composition of the galactopyranosides had to be taken into account. For this purpose, the glycosidic bond was cleaved chemically and the resulting alcohol mixture was analysed to calculate the original diastereoisomeric excess. Thus, corrected selectivity values were obtained which exclusively represent the part of enantiomeric enrichment induced by the enzyme. Assignment of the absolute configuration was achieved by correlation with enantiomerically pure standards, which in the case of pentane-1,4-diol had to be synthesized according to the reaction sequence illustrated in Scheme 3.

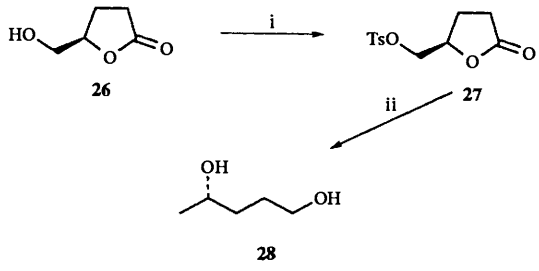
Regarding the extent of chiral discrimination obtained for the diverse aglycone structures, distinct differences can be seen. Despite occasional variations among the results obtained for the same compound, the substrates can be roughly divided into two groups. For the galactopyranosides of propane-1,2-diol (**16**), pentan-2-ol (**14**) and pentane-1,4-diol (**17** and **19**) only poor selectivities were achieved, with values below 2. The two

Table 3 Incubation of compound **18** with β-galactosidase from *E. coli* at various temperatures, substrate concentrations and activities

Temperature (T/°C)	Substrate concentration (μmol cm^{-3})	Enzyme activity (units cm^{-3})	Incubation period (t/min)	Conversion (C) (%)	Enantiomeric excess[a] [*ee*(P)] (%)	Selectivity (E)
40	4	0.8	15	22	56	4.1
40	4	0.8	60	67	22	2.3
40	4	0.5	120	67	14	1.7
40	4	0.4	180	67	13	1.6
40	8	0.8	15	3	48	2.9
40	8	0.8	30	7	54	3.5
30	4	0.8	15	15	42	2.6
30	4	0.8	30	27	32	2.2
30	4	0.8	45	38	13	1.4
30	4	0.8	60	51	8	1.3

[a] Enriched in (*S*)-enantiomer.





Scheme 3 *Reagents:* i, TsCl, pyridine; ii, $LiAlH_4$

diastereoisomers of the galactopyranosides of 1,2-*O*-isopropylideneglycerol (**12**), butan-2-ol (**13**) and butane-1,3-diol (**15** and **18**), on the other hand, were significantly discriminated between by the *E. coli* galactosidase. With selectivity values ranging from ~ 5 to 7, however, the conversion was still not specific.

In comparing these results to those obtained by Crout *et al.* with the same enzyme in the galactosyl-transfer reaction, we can conclude that in the case of propane-1,2-diol the extent of chiral discrimination is about the same. The preferred absolute configuration appears, surprisingly, to be opposite, namely (*R*) in the transglycosylation and (*S*) in the hydrolytic process. For the butan-2-ol and the butane-1,3-diol derivatives, however, considerably higher selectivities were obtained in the hydrolytic process. As for the absolute configuration, galactosyl transfer to butan-2-ol and to the secondary hydroxy group of butane-1,3-diol had shown preference for the (*R*)-enantiomer. The same could be observed during hydrolysis of the corresponding galactopyranoside mixtures. If the primary hydroxy group of butane-1,3-diol was involved in the glycosidic bond, however, the alcohol with (*S*)-configuration was preferably released during hydrolysis, whereas glycosyl transfer had shown virtually no selection. According to the initial presumption, the enzyme exhibited a higher degree of stereoselection during its hydrolytic action than could be achieved in transglycosylation. Since for the transglycosylation of isopropylideneglycerol contradictory results have been reported in the past,[6–8] it seems appropriate to emphasize that in the course of hydrolytic action the enzyme undoubtedly proved able to distinguish between the two enantiomers.

In contrast to our expectations and to the observations in the glycosyl-transfer reaction reported by Crout *et al.* and others, selectivity for the different aglycone structures during galactopyranoside hydrolysis was not found to be associated with their similarity to the glucopyranose structure. This was most clearly shown by the discrimination between the diastereoisomeric galactopyranosides of butan-2-ol. The distinguishing effect of the enzyme could be assumed to be based on hydrophobic interactions. Owing to this finding a detailed discussion of the results obtained for the different diol derivatives with respect to the position of the respective free hydroxy group in either configuration and its relation to the glucopyranose structure becomes unnecessary. Instead, it should be noted that butane-1,3-diol as the only significantly discriminated diolic aglycone has the same chain length as butan-2-ol. Further, the hydrolytic release of pentan-2-ol as a direct homologue of the latter did not result in a pronounced enantiomeric enrichment and thus the length of the alkyl chain

Table 4 Incubation of compound **18** (4 μmol cm^{-3}) at 40 °C with β-galactosidases from different microbial sources

Source of enzyme	Enzyme activity (units (units cm^{-3})	Incubation period (*t*/min)	Conversion (*C*) (%)	Enantiomeric excess[a] [*ee*(P)] (%)	Selectivity (*E*)
B. circulans	4	240	23	14	1.4
K. lactis	4	15	10	6	1.1
	4	30	17	10	1.2
	4	45	28	14	1.4
	4	60	35	5	1.1
A. oryzae	0.1	15	7	8[b]	1.2
	0.1	30	10	5[b]	1.1
A. oryzae[c]	0.5	30	19		
	0.5	60	15		not significant
	0.5	90	22		
	0.5	120	22		

[a] Enriched in (*S*)-enantiomer. [b] Enriched in (*R*)-enantiomer. [c] Using acetone as co-solvent (50%; v/v).

Table 5 Incubation of galactopyranosides **12**–**19** (4 μmol cm^{-3}) with β-galactosidase from *E. coli* (0.8 units cm^{-3}) at 40 °C

Galactopyranoside	Aglycone	Diastereoisomeric excess (%)	Conversion (*C*) (%)	Enantiomeric excess [*ee*(P)] (%)	Selectivity (*E*)	Corrected selectivity ($E_{corr.}$)
12[a]	Isopropylideneglycerol	22 (*S*)	40	46 (*R*)	3.6	7.0
			65	11 (*R*)	1.5	4.0
			7	61 (*R*)	4.3	7.5
13[b]	Butan-2-ol	20 (*S*)	14	51 (*R*)	3.3	4.8
			12	60 (*R*)	4.3	6.7
14[b]	Pentan-2-ol	11 (*R*)	8	22 (*R*)	1.6	1.3
			8	20 (*R*)	1.5	1.3
			9	21 (*R*)	1.6	1.3
16[a]	Propane-1,2-diol	23 (*R*)	63	5 (*R*)	1.2	0.5
			61	15 (*R*)	1.7	0.8
18[a]	Butane-1,3-diol	9 (*R*)	22	56 (*S*)	4.1	5.0
15[a]	Butane-1,3-diol	23 (*R*)	23	76 (*R*)	9.1	5.5
17[a]	Pentane-1,4-diol	0	41	24 (*R*)	1.9	1.9
19[a]	Pentane-1,4-diol	8 (*S*)	16	16 (*R*)	1.4	1.7
			24	16 (*R*)	1.4	1.7

[a] Incubation for 15 min. [b] Incubation for 30 min.

can be regarded as relevant for stereoselection. The specific interaction responsible for the discriminating process may be assumed to be of a kind that a three-carbon chain is too small to participate in it at all, whereas a five-carbon chain is already sufficiently long to allow each of two enantiomers to adopt a suitable conformation.

In summary, galactosidase-mediated hydrolysis of β-galactopyranosides led to significant enantiomeric enrichments in some of the released alcohols. Additional benefits should result from the observed substrate dependence, further elucidation of which can be expected to reveal valuable information concerning the nature of the active site and binding characteristics of the enzyme.

Experimental

General

Solvents designated as 'dry' were distilled prior to use: dichloromethane from calcium hydride; diethyl ether and tetrahydrofuran (THF) from sodium and benzophenone; methanol from magnesium. TLC was performed on silica 60 plates GF 254 (Merck). Spots were visualized by spraying with 10% sulfuric acid in ethanol (carbohydrates) and 1% anisaldehyde and 2% sulfuric acid in glacial acetic acid (non-carbohydrate compounds), respectively, and subsequent heating. Flash column chromatography (FCC) was carried out using silica 60 (0.04–0.63 μm, Merck). ^1H NMR spectra were recorded on a Bruker AMX-400 NMR spectrometer with tetramethylsilane as internal standard. Assignment of signals was supported by ^1H–^1H homonuclear chemical-shift correlation spectroscopy (COSY) experiments. Mp were determined with an Olympus BH polarization microscope and are uncorrected. Optical rotations were measured with a Perkin-Elmer polarimeter 243, with $[\alpha]_D$-values given in units of 10^{-1} deg cm^2 g^{-1}. UV spectral absorbance was measured in 1 cm quartz cuvettes using a Shimadzu spectrophotometer UV-160 A. Gas chromatography was carried out in a Fractovap 2150 (Carlo Erba) using a flame ionization detector and hydrogen as carrier gas (5×10^4 Pa). Recording was done with Merck-Hitachi integrators D-2000 and D-2500. Light petroleum refers to the fraction with distillation range 50–70 °C.

Standard procedure for the Koenigs–Knorr glycosylation

The reactions were performed in brown glass flasks. A mixture of silver carbonate (2 mol equiv.) or freshly prepared silver oxide (2 mol equiv.), the alcohol (1–5 mol equiv.) and the specified amount of magnesium sulfate in dry dichloromethane was stirred at room temperature. After 1 h, a solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide **1** (1 mol equiv.) in dry dichloromethane was added dropwise over a period of 0.5 h. Progress of the reaction was followed by TLC [light petroleum–ethyl acetate–methanol (10:10:1)]; the mixture was stirred at room temperature until consumption of the bromide was complete, then it was filtered, (if appropriate) washed with water to remove excess of alcohol and evaporated. The residue was purified chromatographically.

Standard deacetylation procedure

A solution of the acetylated compound in dry methanol was stirred with NMP (5%, v/v related to methanol) until the

reaction was complete (TLC, solvent will be specified). The solvent was then evaporated off and the residue was repeatedly co-distilled with toluene to remove the base, and was then purified either by crystallization or by chromatography.

Enzymes and buffer solutions

The following enzymes and incubation media were used: β-galactosidase from *E. coli* (Sigma, Grade VIII) in 0.1 mol dm^{-3} phosphate, pH 7.3, 10 mmol dm^{-3} magnesium chloride; from *A. oryzae* (Sigma, Grade XI) in 0.1 mol dm^{-3} phosphate, pH 5.0; from *K. lactis* (kindly donated by Godo-Shusei, Godo-YNL) in 0.1 mol dm^{-3} phosphate, pH 6.5, 0.1 mmol dm^{-3} manganese(II) chloride; from *B. circulans* (kindly donated by Daiwa Kasei, Biolacta FN5) in 0.1 mol dm^{-3} sodium acetate, pH 6.0.

Ethyl 3-(Tetrahydropyran-2-yloxy)butyrate 24

A solution of ethyl 3-hydroxybutyrate **23** (7.8 cm^3, 60 mmol), 3,4-dihydro-2*H*-pyran (12 g, 143 mmol), and toluene-*p*-sulfonic acid (PTSA) (100 mg, 0.5 mmol) in dichloromethane (150 cm^3) was stirred at 10–20 °C. After 1.5 h, the reaction mixture was diluted with dichloromethane (150 cm^3) and washed with a mixture of saturated aq. sodium hydrogen carbonate, saturated aq. sodium chloride, and water (1:1:2; 300 cm^3). Evaporation of the organic layer gave crude ether ester **24** as a yellow liquid (15.3 g), which was used for the subsequent reaction without further purification.

3-(Tetrahydropyran-2-yloxy)butan-1-ol 25

A solution of crude ester **24** (12.4 g) in dry diethyl ether (40 cm^3) was added dropwise to a suspension of lithium aluminium hydride (2.1 g, 55 mmol) in dry diethyl ether (90 cm^3). The mixture was refluxed and the course of the reaction was monitored by TLC [light petroleum–ethyl acetate (2:1)]. Upon completion, water was added carefully at 0 °C until all aluminium hydride was destroyed, the precipitated aluminium hydroxide was filtered off, and the filtrate was extracted with diethyl ether. Evaporation of the extracts and chromatographic purification of the residue with light petroleum–ethyl acetate (2:1) afforded a mixture (7.3 g, 70% for both steps) of the four diastereoisomers of compound **25** as a liquid, δ_H($CDCl_3$; 400 MHz) 1.18 and 1.29 (12 H, 2 d, 4 × Me), 1.45–1.89 (32 H, m, 16 × CH_2), 3.26 (4 H, br s, 4 × OH), 3.48–4.08 (20 H, m, 8 × OCH_2 and 4 × OC*H*MeCH_2) and 4.59 and 4.70 [4 H, 2 m, 4 × OC*H*(CH_2)O].

5-(Tetrahydropyran-2-yloxy)pentan-2-one 21

A solution of 5-hydroxypentan-2-one **20** (1 cm^3, 10 mmol), 3,4-dihydro-2*H*-pyran (2.2 cm^3, 25 mmol), and PTSA (13 mg, 0.1 mmol) in dichloromethane (25 cm^3) was stirred at 10–20 °C. After 1.5 h, the reaction mixture was diluted with dichloromethane (25 cm^3) and washed with a mixture of saturated aq. sodium hydrogen carbonate, saturated aq. sodium chloride and water (1:1:2; 80 cm^3). Evaporation of the organic layer gave crude product **21** as an orange-coloured liquid (2.8 g), which was used for the subsequent reaction without further purification.

5-(Tetrahydropyran-2-yloxy)pentan-2-ol 22

A solution of crude ketone **21** (2.8 g) in propan-2-ol (5 cm^3) was added in small portions to a suspension of sodium boranuide (200 mg, 5.3 mmol) in propan-2-ol (10 cm^3) and the mixture was stirred overnight at room temperature. Dil. hydrochloric acid was added carefully until all boranuide was hydrolysed. After extraction with dichloromethane and evaporation of the extracts, the residue was chromatographed with light petroleum–ethyl acetate (5:1) to yield a mixture (703 mg, 37% for both steps) of the four diastereoisomers of compound **22** as a faint yellow liquid, δ_H($CDCl_3$; 400 MHz) 1.13 (3 H, d, Me), 1.38–1.81 (10 H, m, CH_2), 2.25 (1 H, br s, OH), 3.30–3.47 (2 H, m, OCH_2), 3.67–3.83 [3 H, m, OCH_2 and MeC*H*(OH)CH_2] and 4.52 [1 H, m, OC*H*(CH_2)O] (since no separate signals could be observed, all four stereoisomers are reported as a single compound).

2,3-(Isopropylidenedioxy)propyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside 2

The standard glycosylation procedure was applied to silver oxide (4.64 g, 20 mmol), 1,2-*O*-isopropylideneglycerol (12 cm^3, 100 mmol) and magnesium sulfate (10 g) in dry dichloromethane (200 cm^3) and the galactopyranosyl bromide **1** (8.22 g, 20 mmol) in dry dichloromethane (50 cm^3). Chromatography with light petroleum–ethyl acetate–methanol (10:10:1) and then with light petroleum–diethyl ether (1:4) yielded a mixture (3.41 g, 37%) of the two diastereoisomers of compound **2** as a syrup which crystallised upon storage, mp 79–86 °C; $[\alpha]_D^{20}$ −6.8 (*c* 1.0, $CHCl_3$); δ_H see Table 1.

2,3-(Isopropylidenedioxy)propyl β-D-galactopyranoside 12

The standard deacetylation procedure was applied to compound **2** (3.13 g, 6.8 mmol) and NMP (2 cm^3) in dry methanol (50 cm^3) [TLC: dichloromethane–methanol (5:1)]. Chromatography with dichloromethane–methanol (5:1) yielded a mixture (1.76 g, 88%) of the two diastereoisomers of compound **12** as a yellow hygroscopic foam, $[\alpha]_D^{20}$ −8.0 (*c* 1.0, MeOH); δ_H see Table 2.

1-Methylpropyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside 3

The standard glycosylation procedure was applied to silver oxide (4.64 g, 20 mmol), butan-2-ol (9.2 cm^3, 100 mmol), and magnesium sulfate (10 g) in dry dichloromethane (200 cm^3) and bromide **1** (8.22 g, 20 mmol) in dry dichloromethane (50 cm^3). Chromatography with light petroleum–diethyl ether (2:3) yielded a mixture (5.31 g, 66%) of the two diastereoisomers of compound **3** as a syrup, $[\alpha]_D^{20}$ −11.2 (*c* 1.0, $CHCl_3$); δ_H see Table 1.

1-Methylpropyl β-D-galactopyranoside 13

The standard deacetylation procedure was applied to compound **3** (5.25 g, 13 mmol) and NMP (4 cm^3) in dry methanol (100 cm^3). Crystallization from ethyl acetate yielded a mixture (1.79 g, 58%) of the two diastereoisomers of compound **13** as a powder, mp 117–123 °C; $[\alpha]_D^{20}$ −22.1 (*c* 1.0, MeOH); δ_H see Table 2.

1-Methylbutyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside 4

The standard glycosylation procedure was applied to silver carbonate (2.76 g, 10 mmol), pentan-2-ol (5 cm^3, 50 mmol) and magnesium sulfate (5 g) in dry dichloromethane (40 cm^3) and compound **1** (4.11 g, 10 mmol) in dry dichloromethane (10 cm^3). Chromatography with light petroleum–ethyl acetate (2:1) yielded a mixture (1.85 g, 44%) of the two diastereoisomers of compound **4** as a syrup, $[\alpha]_D^{20}$ −12.0 (*c* 1.0, $CHCl_3$); δ_H see Table 1.

1-Methylbutyl β-D-galactopyranoside 14

The standard deacetylation procedure was applied to compound **4** (1.73 g, 4.1 mmol) and NMP (1.5 cm^3) in dry methanol (30 cm^3). Crystallization from ethyl acetate–light petroleum yielded a mixture (810 mg, 78%) of the two diastereoisomers of compound **14** as a powder, mp 124–134 °C; $[\alpha]_D^{20}$ −23.0 (*c* 1.0, MeOH); δ_H see Table 2.

Ethyl 3-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)butyrate 5

The standard glycosylation procedure was applied to silver carbonate (8.3 g, 30 mmol), ethyl 3-hydroxybutyrate **23** (8 cm^3, 60 mmol) and magnesium sulfate (10 g) in dry dichloromethane

(200 cm^3) and bromide **1** (9.4 g, 23 mmol) in dry dichloromethane (50 cm^3). Chromatography with light petroleum–ethyl acetate (2:1) yielded a mixture (4.2 g, 40%) of the two diastereoisomers of compound **5** as a syrup, $[\alpha]_D^{20}$ +2.1 (*c* 1.0, $CHCl_3$); δ_H see Table 1.

3-Hydroxy-1-methylpropyl β-D-galactopyranoside 15
A solution of compound **5** (2.77 g, 6 mmol) in dry THF (10 cm^3) was added dropwise to a suspension of lithium aluminium hydride (760 mg, 20 mmol) in dry THF (20 cm^3). After reflux of the mixture for three days [TLC: dichloromethane–methanol (4:1)], water was added carefully at 0 °C until all aluminium hydride was hydrolysed. The mixture was neutralized with dil. sulfuric acid, then was filtered and the filtrate was freeze dried. Chromatography of the residue with dichloromethane–methanol (4:1) yielded a mix-ture (252 mg, 17%) of the two diastereoisomers of compound **15** as a yellow syrup, $[\alpha]_D^{20}$ −15.6 (*c* 1.0, MeOH); δ_H see Table 2.

2-Oxopropyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside 6
The standard glycosylation procedure was applied to silver carbonate (2.76 g, 10 mmol), hydroxyacetone (3.5 cm^3, 50 mmol) and magnesium sulfate (5 g) in dry dichloromethane (40 cm^3) and bromide **1** (4.11 g, 10 mmol) in dry dichloromethane (10 cm^3). Chromatography with light petroleum–ethyl acetate–methanol (10:10:1) and dichloromethane–acetone (20:1) yielded compound **6** as a syrup, $[\alpha]_D^{20}$ −16.1 (*c* 1.0, $CHCl_3$); δ_H see Table 1.

2-Hydroxypropyl β-D-galactopyranoside 16
A solution of compound **6** (766 mg, 1.9 mmol) in propan-2-ol (4 cm^3) was added in small portions to a suspension of sodium boranuide (18 mg, 0.5 mmol) in propan-2-ol (1 cm^3). After stirring of the mixture at room temperature overnight, dil. hydrochloric acid was added carefully until all boranuide was hydrolysed. The mixture was extracted with dichloromethane and the extracts were evaporated. The remaining syrup was deacetylated with NMP (0.4 cm^3) in dry methanol (10 cm^3) according to the standard deacetylation procedure. Chromatography with dichloromethane–methanol (4:1) yielded a mixture (265 mg, 59%) of the two diastereoisomers of compound **16** as a faint yellow syrup which crystallized upon drying *in vacuo*, mp 154–169 °C; $[\alpha]_D^{20}$ −2.2 (*c* 1.0, water); δ_H see Table 2.

4-Oxopentyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside 7
The standard glycosylation procedure was applied to silver carbonate (1.66 g, 6 mmol), 5-hydroxypentan-2-one **20** (1.8 cm^3, 18 mmol) and magnesium sulfate (3 g) in dry dichloromethane (30 cm^3) and bromide **1** (2.47 g, 6 mmol) in dry dichloromethane (10 cm^3). Chromatography with dichloromethane–acetone (20:1) yielded compound **7** (833 mg, 32%) as a syrup, $[\alpha]_D^{20}$ −4.0 (*c* 1.0, $CHCl_3$); δ_H see Table 1.

4-Hydroxypentyl β-D-galactopyranoside 17
A solution of compound **7** (800 mg, 1.9 mmol) in propan-2-ol (4 cm^3) was added in small portions to a suspension of sodium boranuide (20 mg, 0.5 mmol) in propan-2-ol (1 cm^3). After stirring of the mixture at room temperature overnight, dil. hydrochloric acid was added carefully until all boranuide was hydrolysed. The mixture was extracted with dichloromethane and the extracts were evaporated. The remaining syrup was deacetylated with NMP (0.3 cm^3) in dry methanol (7 cm^3) according to the standard deacetylation procedure. Chromatography with dichloromethane–methanol (4:1) yielded a mixture (173 mg, 34%) of the two diastereoisomers of compound **17** as a yellow syrup, $[\alpha]_D^{20}$ −4.1 (*c* 1.0, water); δ_H see Table 2.

3-Hydroxybutyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside 9
The standard glycosylation procedure was applied to silver carbonate (8.3 g, 30 mmol), alcohol **25** (7 g, 40 mmol) and magnesium sulfate (10 g) in dry dichloromethane (200 cm^3) and bromide **1** (12.33 g, 30 mmol) in dry dichloromethane (50 cm^3). Some more polar side-products were removed by chromatography with light petroleum–ethyl acetate (2:1) and the remaining compound mixture, containing the THP ether **8**, was stirred with PTSA (75 mg, 0.4 mmol) in dry methanol (100 cm^3) for 1 h in order to remove the tetrahydropyranyl protecting group without hydrolysis of the glycosidic linkage. For work-up, the solution was diluted with dichloromethane (100 cm^3) and washed with a mixture of saturated aq. sodium hydrogen carbonate, saturated aq. sodium chloride and water (1:1:2; 200 cm^3). The solvent was evaporated off and the residue was chromatographed with light petroleum–ethyl acetate–methanol (10:10:1) to yield a mixture (3.74 g, 30%) of the two diastereoisomers of compound **9** as a syrup, $[\alpha]_D^{20}$ −10.1 (*c* 1.0, $CHCl_3$); δ_H see Table 1.

3-Hydroxybutyl β-D-galactopyranoside 18
The standard deacetylation procedure was applied to compound **9** (3.64 g, 8.7 mmol) and NMP (2 cm^3) in dry methanol (40 cm^3). Chromatography with dichloromethane–methanol (4:1) yielded a mixture (1.9 g, 86%) of the two diastereoisomers of compound **18** as a yellow syrup, $[\alpha]_D^{20}$ −2.2 (*c* 1.0, water); δ_H see Table 2.

4-Hydroxy-1-methylbutyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside 11
The standard glycosylation procedure was applied to silver carbonate (1.00 g, 3.6 mmol), alcohol **22** (680 mg, 3.6 mmol) and magnesium sulfate (1.5 g) in dry dichloromethane (20 cm^3) and bromide **1** (1.48 g, 3.6 mmol) in dry dichloromethane (5 cm^3). Subsequently, the same operations as described for the preparation of compound **9** were carried out on the THP ether **10** by using half the amounts of solvent and catalyst for deprotection. A mixture (384 mg, 25%) of the two diastereoisomers of compound **11** was obtained as a syrup, $[\alpha]_D^{20}$ −7.1 (*c* 1.0, $CHCl_3$); δ_H see Table 1.

4-Hydroxy-1-methylbutyl β-D-galactopyranoside 19
The standard deacetylation procedure was applied to compound **11** (364 mg, 0.8 mmol) and NMP (0.4 cm^3) in dry methanol (10 cm^3). Chromatography with dichloromethane–methanol (4:1) yielded a mixture (183 mg, 83%) of the two diastereoisomers of compound **19** as a yellow syrup, $[\alpha]_D^{20}$ −6.9 (*c* 1.0, water); δ_H see Table 2.

(*R*)-(−)-γ-(Toluene-*p*-sulfonyloxymethyl)-γ-butyrolactone 27
Toluene-*p*-sulfonyl chloride (1.9 g, 10 mmol) was added to a solution of (*R*)-(−)-γ-hydroxymethyl-γ-butyrolactone **26** (0.5 cm^3, 5.3 mmol) in pyridine (8 cm^3) at 0 °C. The mixture was stirred at that temperature for 1 h and was then stored in a refrigerator overnight. When the solution was poured onto an ice–water mixture, the product precipitated out, and after filtration and drying *in vacuo*, title compound **27** (1.19 g, 83%) was obtained as a slightly yellow powder, mp 81–85 °C; $[\alpha]_D^{20}$ − 41.4 (*c* 1.0, $CHCl_3$); δ_H($CDCl_3$; 400 MHz) 2.00–2.09 (1 H, m, CH_2), 2.24–2.32 (1 H, m, CH_2), 2.37 (3 H, s, Me), 2.41–2.58 (2 H, m, $COCH_2$), 4.04–4.14 (2 H, m, CH_2O), 4.59–4.65 (1 H, m, CH), 7.30 (2 H, d, aryl) and 7.73 (2 H, d, aryl).

(*S*)-(+)-Pentane-1,4-diol 28
A solution of γ-lactone **27** (1.1 g, 4 mmol) in dry THF (10 cm^3) was added dropwise to a suspension of lithium aluminium hydride (150 mg, 4 mmol) in dry THF (10 cm^3) at 0 °C. The mixture was stirred at room temperature and the reaction was followed by TLC [dichloromethane–acetone (7:1)]. Upon

completion, water was added carefully at 0 °C until all aluminium hydride was destroyed. The precipitated aluminium hydroxide was filtered off and the filtrate was extracted with ethyl acetate. Evaporation of the organic layer and chromatographic purification of the residue with dichloromethane–acetone (7:1) afforded the diol **28** (240 mg, 58%) as a viscous liquid, $[\alpha]_D^{20}$ +19.3 (*c* 1.0, $CHCl_3$); $\delta_H(CDCl_3$; 400 MHz) 1.16 (3 H, d, Me), 1.40–1.65 (4 H, m, CH_2), 2.60 (2 H, br s, OH), 3.55–3.66 (2 H, m, CH_2OH) and 3.73–3.82 (1 H, m, CH).

Enzymic hydrolysis of galactopyranosides 12–19

The substrate concentrations and enzyme activities as they are specified in Tables 3–5 were applied to a volume of 13.5 cm^3 of the appropriate buffer system. Likewise, an aliquot (1.5 cm^3) of solution was prepared as a blank by using previously denaturated enzyme. Reaction mixtures and blanks were incubated under conditions listed in Tables 3–5. Subsequently, they were heated in a boiling-water-bath for 5 min to denaturate the enzymes. In those cases where acetone was used as a co-solvent, denaturation was achieved by mixing with the same volume of 0.1 mol dm^{-3} aq. sodium carbonate.

Determination of conversion

Reagent. 1% (w/v) 3,5-Dinitrosalicylic acid, 30% (w/v) potassium sodium tartrate and 0.4 mol dm^{-3} aq. sodium hydroxide.

Procedure. Aliquots (3 × 0.45) cm^3 were taken from each incubation mixture and 1 × 0.45 cm^3 from each blank, diluted to 0.5 cm^3 with the appropriate buffer solution, and mixed with reagent (0.5 cm^3). The solutions were heated in a boiling-water-bath for 5 min and then stored at room temperature overnight. Aliquots (0.6 cm^3) of each sample and the blank were transferred to a quartz cuvette and diluted with water (2.4 cm^3). The absorbances were measured at 546 nm and correlated to a previously recorded calibration to calculate the galactose concentration by use of the photometer software. In those cases where acetone was used as a co-solvent, the aliquots taken from the incubation mixture and the blank were evaporated, and redissolved in buffer solution (0.9 cm^3) each. From these solutions, portions (0.45 cm^3) were diluted to 0.5 cm^3 with buffer solution, mixed with reagent (0.5 cm^3) and treated in the way described above.

Sample preparation for gas chromatography

For diols and isopropylideneglycerol. The incubation solutions were evaporated and the residues were extracted twice with dichloromethane (1 cm^3). The extracts were dried with molecular sieves (0.3 nm) for 0.5 h and exposed to a faint nitrogen flow until the volume was reduced to ≈0.2 cm^3.

For simple alcohols. The incubation solutions were saturated with sodium chloride and extracted twice with dichloromethane (1 cm^3). The extracts were dried and concentrated as described above.

Trifluoroacetylation

The concentrated sample solutions were mixed with trifluoroacetic anhydride (0.05 cm^3) and stored at room temperature for 0.5 h. After being washed with saturated aq. sodium hydrogen carbonate, the organic layers were dried with molecular sieves (0.3 nm) and, if necessary, concentrated.

Determination of diastereoisomeric excess in galactopyranosides 13–19

A solution of the galactopyranoside (10–20 mg, 40–80 μmol) in 1 mol dm^{-3} hydrochloric acid (2 cm^3) was refluxed until hydrolysis was complete [TLC: dichloromethane–methanol (4:1)]. After neutralization with aq. sodium hydroxide, samples for gas chromatography were prepared in the same way as described above for the incubation mixtures.

Determination of diastereoisomeric excess in galactopyranoside 12

Galactopyranoside **12** (5.4 mg, 18 μmol) and β-galactosidase from *E. coli* (0.7 units) in buffer solution (4.5 cm^3) were incubated at 40 °C until hydrolysis was complete [TLC: dichloromethane–methanol (4:1)]. Sample preparation for gas chromatography was carried out as above.

GC stationary phases

A 25 m fused-silica capillary column with heptakis-(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin/OV 1701 1:4 (w/w) was used to separate the enantiomers of 1,2-*O*-isopropylideneglycerol at 60 °C; of butane-1,3-diol (trifluoroacetate derivative) and pentane-1,4-diol (trifluoroacetate derivative) at 50 °C; of propane-1,2-diol at 40 °C; and of butan-2-ol under cooling with solid CO_2. A 25 m fused-silica capillary column with octakis-(6-*O*-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrin/OV 1701 1:1 (w/w) was used to separate the enantiomers of pentan-2-ol at room temperature.

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