Enzymatic Synthesis of Glycosides Using the β-Galactosidase of *Escherichia coli*: Regio- and Stereo-chemical Studies

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β-Galactosyl transfer from lactose to acceptor alcohols (*R*)-(−)-butan-2-ol, (*RS*)-butan-2-ol, (*S*)-(+)-propane-1,2-diol, (*RS*)-propane-1,2-diol, (*S*)-(+)-butane-1,3-diol, (*RS*)-butane-1,3-diol, propane-1,3-diol, (*S*)-(+)-isopropylideneglycerol (1,2-*O*-isopropylidene-*sn*-glycerol) and (*RS*)-isopropylideneglycerol (*rac*-1,2-*O*-isopropylideneglycerol) was studied, catalysed by the β-galactosidase (β-D-galactoside galactohydrolase EC 3.2.1.23) of *Escherichia coli*. Preference for galactosyl transfer to the *R*-enantiomers of chiral alcohols was observed, although selectivity was not pronounced. Higher selectivity for transfer to the primary hydroxy groups of the primary–secondary diols was observed. The results are interpreted in terms of a proposed active site model for the enzyme.

In the biosynthesis of glycosides and oligosaccharides, nucleotide sugars are substrates for enzymes that catalyse glycosidic bond formation.[1] The mechanism consists of nucleophilic substitution by the aglycone, with displacement of the nucleotide. The use of these systems in biotransformations has been limited by the restricted availability of the corresponding enzymes and substrates. However, an alternative is to use 'reverse hydrolysis', a process in which the hydrolytic action of glycosidases becomes reversed at high concentrations of mono- and oligo-saccharide hydrolysis products. The unfavourable equilibria of such reactions with respect to oligosaccharide formation are greatly improved by using a kinetically controlled procedure in which a glycoside is used as glycosyl donor (Scheme).[2] Since the product (**2**) is always a substrate for the

Scheme.

enzyme, the success of the procedure as a preparative method depends on the rate of hydrolysis of the product being slower than that of the glycosyl donor (**1**). These conditions in practice can be attained rather readily. As a consequence, there has been a steadily growing interest in the use of glycosidases for glycoside and oligosaccharide synthesis.[1,2] Major advantages of glycosidase-catalysed glycosyl transfer are that there is need for zero or only minimal protection and that there is complete control of the stereochemistry at the newly formed anomeric centre. However, a major remaining problem is that of regiocontrol with respect to the acceptor of glycosyl transfer. This is particularly important when the acceptor is a mono- or oligo-saccharide carrying multiple hydroxy functions. We report here initial experiments in a programme designed to investigate systematically the stereo- and regio-selectivity of the β-galactosidase of *Escherichia coli*. Galactosyl transfer to two chiral alcohols (butan-2-ol and isopropylidene glycerol), one achiral diol (propane-1,3-diol) and two chiral diols (propane-1,2-diol and butane-1,3-diol) was studied. The chiral alcohols and diols were examined both as a single enantiomer and as the racemate. In these experiments, lactose was used as the galactosyl donor, with the alcohol present in excess.

The substrates were dissolved in phosphate buffer, pH 7.3.

Table 1. Glycoside formation from lactose and various alcohols catalysed by the β-galactosidase from *Escherichia coli*.

Substrate	Yield of galactoside (%)[a]
(*RS*)-Butan-2-ol	32
(*R*)-(−)-Butan-2-ol	32
(*RS*)-Propane-1,2-diol	46
(*S*)-(+)-Propane-1,2-diol	51
(*RS*)-Butane-1,3-diol	61
(*S*)(+)-Butane-1,3-diol	63
Propane-1,3-diol	42
(*RS*)-Isopropylideneglycerol	47
(*S*)-(+)-Isopropylideneglycerol	47

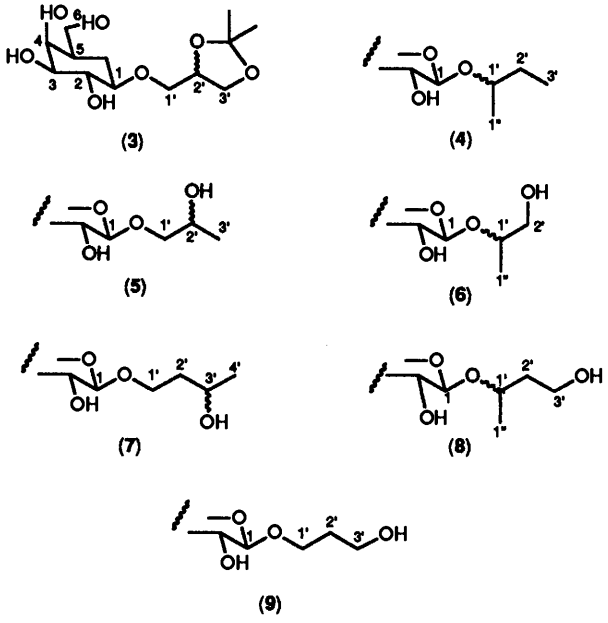
[a] Total yield (all isomers).

The enzyme was added in 50 unit amounts after 0, 3, and 6 h (150 units total). The reaction was followed by TLC and HPLC. In all experiments, most galactosyl transfer was to acceptor alcohol rather than to water, as indicated by the small quantities of free galactose produced. However, a significant proportion of galactosyl transfer was to glucose, with the production of allolactose (6-*O*-β-D-galactopyranosyl-D-glucose). The products were isolated and purified by HPLC, and characterised by ^{1}H and ^{13}C NMR (including DEPT experiments), and by mass spectrometry of the corresponding acetates. Isolated yields (with respect to lactose consumed) are given in Table 1. In all cases only the β-anomer was produced, as indicated by the absence of detectable signals attributable to the α-anomer in the anomeric region of the 400 MHz spectra of the products.

Galactosyl transfer to isopropylideneglycerol has been reported to be stereoselective.[3] However, we found, as have others,[4,5] that the diastereoisomeric products were formed in almost equal amounts, as indicated by the pair of doublets of nearly equal (within 3%) intensity in the ^{1}H NMR spectrum and which were attributable to the anomeric protons. A single doublet was observed in the spectrum of the product obtained from 1,2-*O*-isopropylidene-*sn*-glycerol. A similar result was obtained with racemic butanol. In this case, the signals in the ^{1}H NMR spectrum attributable to the anomeric protons were not resolved. However, the signals due to the methyl group attached to the carbinol carbon atom [C-1′ in (**4**)] were well separated. Comparison with the spectrum of the corresponding product

from (*R*)-(−)-butan-2-ol showed that the products from the *R* and *S* enantiomers were formed in the ratio 1.00:0.83.

With (±)-propane-1,2-diol, four products were formed. The major products (**5**) were expected to be the ones formed by



transfer to the primary hydroxy group of the diol. This was supported by the upfield shift of the signals due to the anomeric carbon atoms in the minor products (**6**) relative to those of the major isomer. Such a shift would be predicted on the basis of the γ-effect.[6,7] Assignment of signals attributable to the products from the *R* and *S* enantiomers of the substrate was again made by comparison with the product obtained from (*S*)-(+)-propane-1,2-diol. Integration of the signals due to the anomeric protons showed that selectivity with respect to the enantiomeric substrates was nearly the same. In transfer to the primary hydroxy group to give glycoside (**5**), the *R*-isomer was favoured over the *S* by a factor of 1.00:0.86, and the secondary hydroxy group [to give glycoside (**6**)] by a factor of 1.00:0.77. Overall, transfer to the primary hydroxy group was favoured over the secondary by a factor of 1.00:0.35. However, with (±)-butane-1,3-diol, selectivity was significantly different for the two modes. Selectivity for transfer to the primary hydroxy group over the secondary was overall 1.00:0.15. From the relative integrations of the signals due to the anomeric proton in the product (**7**) it was seen that at the primary hydroxy group, transfer to the *R*-enantiomer was favoured over transfer to the *S*-enantiomer by a factor of 1.00:0.99. However, the corresponding ratio for transfer to the secondary hydroxy group was 1.00:0.50, as indicated by the relative integrations of the signals attributable to the methyl group in the product (**8**), which were shifted downfield relative to the major isomer.

The selectivity of these reactions can be expressed in a different way. With propane-1,2-diol, and considering the *R*-enantiomer, selectivity with respect to transfer to the primary hydroxy group relative to the secondary was 1.00:0.37. For the *S*-enantiomer, the corresponding ratio was 1.00:0.33. However, for butane-1,3-diol, the corresponding ratios were, for the *R*-enantiomer, 1.00:0.20, and the *S*-enantiomer, 1.00:0.10. Thus in this case, selectivity was greater overall compared with propane-1,2-diol and greater for the *S*-enantiomer. When the *S*-enantiomers of propane-1,2-diol and butane-1,3-diol were used, the corresponding selectivities could be measured individually and were found to be 1.00:0.33 and 1.00:0.13 respectively, not significantly different from the ratios found for the racemates.

A different kind of selectivity was observed with propane-1,3-diol in that under the experimental conditions, single galactosyl transfer only to the substrate was observed, to give glycoside (**9**). There was no evidence of a digalactosyl product.

The regio- and stereo-selectivities of glycosyl transfer to simple alcohols by glycosidases has been little studied. Ooi *et al.*[8] used the β-galactosidase from *Aspergillus oryzae* to transfer glycosyl and galactosyl residues on to racemic alcohols and diols, but the enantioselectivities of the reactions were not reported. However, it was stated qualitatively that selectivity was high for transfer to primary as opposed to secondary hydroxy groups in primary, secondary diols. Gais *et al.*[9] observed high diastereoselectivities during galactosyl transfer to cyclic diols using the immobilised β-galactosidase from *Aspergillus niger*. Björkling and Godtfredsen[4] observed low diastereoselectivity (40%) during galactosyl transfer to 2,3-epoxypropan-1-ol, using the β-galactosidase from *Escherichia coli*. However, the most comprehensive study of structural factors relating to acceptor reactivity was made by Huber *et al.*,[10] who obtained kinetic data for a number of simple acceptor alcohols, monosaccharides and alditols. Since in our experiments the alcohol was present in considerable excess (over lactose), during the transglycosylation reactions, the results reflect a more or less uniform competitive situation for the duration of the reaction and are thus, if one ignores the possibility of selective product inhibition, comparable with the initial rate studies of Huber *et al.*

Huber *et al.* interpreted their results in terms of a hypothetical glucopyranose binding site (Figure). This models attack by the 6-hydroxy group of glucose, which would lead to allolactose, the major product of transgalactosylation with glucose as acceptor. In the absence of more precise structural data, this model must be regarded as tentative. However, if it is taken as a starting point for discussion, the corresponding conformations with respect to binding at the active site are (**10**) and (**11**) for attack by the primary and secondary hydroxy groups of (*R*)-butane-1,3-diol and (**12**) and (**13**) for the corresponding reaction modes for the *S*-enantiomer. These conformations have been chosen to mimic as closely as possible the conformation of the C-4 to C-6 component of glucopyranose (Figure), as this part of the molecule seems to be most important in relation to the recognition by the active site.[10] The relationship is indicated in structure (**10**) in which the numbering indicates the proposed

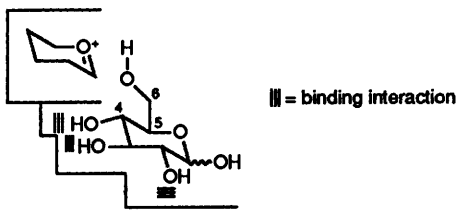


Figure.

Table 2. Accurate mass data for acetylated β-D-galactosides.

Galactoside	Formula of peracetylated product + NH_4	Accurate mass of peracetylated product + NH_4	
		Calculated	Observed
(**4**)	$C_{18}H_{32}O_{10}N$	422.2026	422.2050
(**5**) + (**6**)	$C_{19}H_{32}O_{12}N$	466.1924	466.1916
(**7**) + (**8**)	$C_{20}H_{34}O_{12}N$	480.2081	480.2085
(**9**)	$C_{19}H_{32}O_{12}N$	466.1924	466.1881

Table 3. ^{1}H NMR data for galactosides (3)–(9).

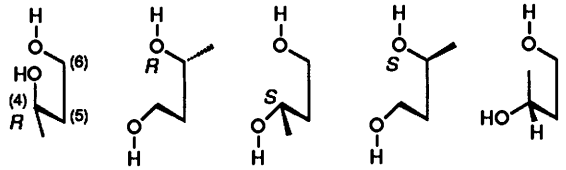
Galactoside	Configuration of aglycone	1-H	2′-H	3′-H	4′-H	1″-H
(3)[a]	*R*	4.343 (d, 7.78)[b]	—	—	—	—
(3)[a]	*S*	4.340 (d, 7.80)	—	—	—	—
(4)	*R*	~4.40 (d, 7.89)	1.517 (m)	~0.833 (t, 7.5)	—	1.12 (d, 6.19)
(4)	*S*	~4.40 (d, 7.89)	1.517 (m)	~0.833 (t, 7.5)	—	1.16 (d, 6.31)
(5)	*R*	4.34 (d, 7.81)	—	~1.104 (d, 6.54)	—	—
(5)	*S*	4.35 (d, 7.82)	—	~1.104 (d, 6.54)	—	—
(6)	*R*	4.41 (d, 7.89)	—	—	—	1.12 (d, 6.69)
(6)	*S*	4.47 (d, 7.87)	—	—	—	1.17 (d, 6.50)
(7)	*R*	4.33 (d, 7.90)	—	—	~1.15 (d, 6.31)	—
(7)	*S*	4.34 (d, 7.91)	—	—	~1.15 (d, 6.31)	—
(8)	*R*	~4.40 (d, 7.91)	—	—	—	1.17 (d, 6.27)
(8)	*S*	~4.40 (d, 7.91)	—	—	—	1.22 (d, 6.34)
(9)		4.33 (d, 7.90)	—	—	—	

[a] Isopropylidene methyl signals: δ 1.33, 1.40. [b] [Multiplicity, coupling constant (Hertz)].

Table 4. ^{13}C NMR data for galactosides (3)–(9).

Galactoside	(3) (2′-*R*)	(3) (2′-*S*)	(4) (1′-*R*)	(4) (1′-*S*)	(5) (2′-*R*)	(5) (2′-*S*)	(6) (1′-*R*)	(6) (1′-*S*)	(7) (3′-*R*)	(7) (3′-*S*)	(8) (2′-*R*)	(8) (2′-*S*)	(9)
C-1	103.9	103.8	101.7	102.8	103.7	104.1	101.9	103.2	103.6	103.7	101.8	103.3	103.6
C-2	71.6	71.5	71.6	71.8	71.6	71.7	71.6	71.8	71.6	71.6	71.8	71.8	71.5
C-3	73.4	73.5	73.8	73.7	73.5	73.5	73.6	73.5	73.6	73.6	73.7	73.6	73.5
C-4	69.4	69.4	69.4	69.4	69.4	69.4	69.4	69.4	69.4	69.4	69.4	69.4	69.4
C-5	75.9	75.9	75.8	75.8	75.9	75.9	75.9	75.9	75.8	75.8	75.8	75.8	75.8
C-6	61.7	61.7	61.7	61.7	61.7	61.7	61.8	61.7	61.7	61.7	61.8	61.7	61.7
C-1′	71.2	70.6	78.6	79.4	75.6	75.9	77.2	78.1	68.1	68.1	75.8	75.8	67.9
C-2′	75.4	75.1	30.0	29.1	67.1	67.4	66.1	65.6	38.4	38.5	39.4	38.9	32.2
C-3′	66.3	66.3	9.9	9.5	18.8	18.8	—	—	65.8	65.8	59.2	59.6	59.3
C-4′	110.9	110.8	—	—	—	—	—	—	22.9	23.0	—	—	—
C-5′/C-5″	25.0, 26.4	25.0, 26.4	—	—	—	—	—	—	—	—	—	—	—
C-1″	—	—	19.1	20.7	—	—	—	—	—	—	20.0	21.7	—
C-2″	—	—	—	—	—	—	16.3	17.8	—	—	—	—	—

relationship with corresponding positions in the bound glucose of the Figure.



(10) (11) (12) (13) (14)

For attack by the primary hydroxy group, it is conformation (**12**) of the *S*-enantiomer that resembles most closely that of the model (Figure). However, since there was essentially no discrimination between the *R*- and *S*-enantiomers, the alternative conformation (**14**) for the *R*-enantiomer must be equally acceptable. Although this places the methyl group in an 'axial' conformation in terms of the glucopyranose model, the only possible 1,3-interaction would be with a hydrogen atom at C-1. For attack by the secondary hydroxy group, the only forced difference in conformation for the two enantiomers is at C-3 [*cf.* (**11**) and (**13**)]. The preference for attack by primary as opposed to secondary hydroxy groups suggest that steric factors are important in transgalactosylation. However, it is known that the glucose binding site in β-galactosidase is highly hydrophobic.[10,11] The observed greater discrimination against attack by the secondary hydroxy group in the *S*-enantiomer suggests that the β-orientation of the 4-methyl group (**13**) is disfavoured for steric reasons, or that the 'α' orientation of the methyl group in conformation (**11**) of the *R*-enantiomer is favoured by hydrophobic interaction. It is possible that both factors operate simultaneously.

Interpretation of the results with propane-1,2-diol is less informative. The 1,2-diol structure mimics the C-4 to C-6 component of glucopyranose less well than butane-1,3-diol. Also, there is essentially no discrimination between the enantiomers. The only significant difference in the butane-1,3-diol case is that there is less discrimination against transfer to the secondary hydroxy groups. This would probably reflect the loss of a hydroxy group equivalent to that at C-4 in glucose rather than an improved binding for transfer to the secondary hydroxy group.

The conclusions of Huber *et al*,[10] although highly informative, did not have the benefit of product analysis. The importance of competing modes of reaction by polyhydroxylated substrates could not be taken into account. Our results do take this factor into account and also indicate that stereochemical discrimination is likely to be greatest for galactosyl transfer to secondary hydroxy groups. If used for biotransformations, with high enantioselectivity or diastereoselectivity as the goal, transgalactosylation is therefore likely to give better results in transfer to secondary alcohols. The limited evidence in the literature supports this conclusion.[4,9]

These investigations are currently being extended to more complex substrates.

Experimental

Analytical Procedures.—TLC was carried out using Kieselgel 60 F-254 plates with development in the solvent system propan-1-ol–nitromethane–water (10:9:3, v/v). Products were visualised by spraying the plates with a solution of phosphomolybdic acid in ethanol (70 g l^{-1}) and heating the plates at 150 °C for 5 min. HPLC was carried out using a Waters 6000A solvent delivery system with an R401 differential refractometer. A Magnasil 5H aminopropyl column (25 cm × 4 mm) was used with the solvent system acetonitrile:water (77:23 v/v). ^1H NMR was carried out at 220 MHz using a Perkin-Elmer R34 spectrometer, or at 400 MHz using a Bruker WH400 spectrometer. ^{13}C NMR spectra were determined at 100.62 MHz using the Bruker WH400 spectrometer. The solvent was D_2O. Mass spectra were determined using a Kratos MS80 mass spectrometer, in the chemical ionisation mode with ammonia as the ionising gas.

trans-β-*Galactosylation: General Procedure.*—The alcohol (6 mmol) and lactose (0.27 g, 0.75 mmol) were dissolved in 0.1M phosphate buffer, pH 7.3 (2.5 ml). β-Galactosidase from *E. coli* (Sigma, Grade X, 50 units) was added at t = 0, 3, and 6 h (150 units total). The mixture was incubated at 25 °C for 24 h, by which time TLC and HPLC examination showed that all of the lactose had been consumed. The reaction mixture was heated at 90 °C for 5 min and filtered. The filtrate was lyophilised and the product was purified by HPLC. Yields are given in Table 1, mass spectral data in Table 2, and NMR data in Table 3.

Acetylation of trans-*Galactosylation Products for Mass Spectrometric Analysis.*—The galactoside (10 mg) was treated with a mixture of pyridine and acetic anhydride (1:1 v/v; 2 ml) for 12 h at 20 °C. The reagents were removed under reduced pressure and the residue was submitted for mass spectrometric examination. The galactoside mixtures from (±)-butan-2-ol, (±)-propane-1,2-diol, (±)-butane-1,3-diol, and propane-1,3-diol were examined. Their accurate mass data are given in Table 2. ^1H and ^{13}C NMR data are given in Tables 3 and 4, respectively.

Acknowledgements

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