Stereochemical Studies of Enzymatic Transglycosylation using *Sulfolobus solfataricus*

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Stereochemistry of β-glycosyl transfer from phenyl β-D-galactoside, lactose, and phenyl β-D-glucoside to various 1,2-, 1,3-, and 1,4-diols, a secondary–tertiary diol, a cyclic diol, and a racemic alcohol has been studied using β-glycosidase activity in a crude preparation from the thermophilic archaebacterium *Sulfolobus solfataricus*. Good enantioselection for the galactosyl transfer to the secondary hydroxy group of different 1,2-diols has been observed. Good yields in comparison with enzymes from other sources, and results concerning the reaction's regioselectivity, have also been reported.

Glycosidases, enzymes capable of hydrolysing glycosidic bonds, are also used to synthesize different glycosides by means of two different methods: reverse hydrolysis and transglycosylation. Both these techniques have been used in this field by utilizing enzymes from different sources.[1] Alkyl β-glucosides, employed as biological detergents, have been synthesized using almond β-glucosidase immobilized on XAD-4.[2] A study of the transglucosylation activity of a lactase from *Kluyveromyces lactis* showed that primary and secondary alcohols can be used as substrates, but also that more hydrophilic alcohols, such as glycerol, cannot be glycosylated.[3] Enzymatic synthesis of glycosides appears to be interesting because this allows us the use of substrates with labile hydroxy groups. β-Galactosidase from *Aspergillus oryzae* has been used to produce several cardiac glycosides that are difficult to obtain by ordinary chemical methods.[4]

A β-galactosidase isolated from the thermophilic archaebacterium *Sulfolobus solfataricus* was recently used to synthesize gentobiose[5] and with the same enzyme different alkyl β-D-glycosides were obtained in good yield compared with the results reported using mesophilic enzymes.[6]

Stereochemistry of glycosyl transfer has been only poorly studied. Different commercially available enzymatic preparations have been used for the preparation of α- and β-monoglycosides of *trans*-cyclohexane-1,2-diol,[7] of mono-β-D-galactosides of *meso*-diols,[8] 2,3-epoxypropan-1-ol and *O,O*-isopropylideneglycerol.[9] However, apart from studies concerning the preparation of specific compounds, a more basic investigation has recently been started by Crout *et al.* (1990)[10] on the regio- and stereo-chemistry of β-galactosidase from *Escherichia coli* using five model compounds. The results showed a selectivity for galactosyl transfer to the primary hydroxy group in the case of primary–secondary diols while the enantioselection was not pronounced in the case of chiral alcohols.

Different stereochemistry for this reaction have been observed when using a crude homogenate of *Sulfolobus solfataricus* for the galactosyl transfer to propane-1,2-diol.[6]

In this paper we report the completion of these preliminary results, extending our investigation to different 1,2-, 1,3-, and 1,4-diols, with examples of a racemic alcohol, a secondary–tertiary diol, and a cyclic diol.

Results and Discussion

The products of enzymatic synthesis were purified and characterized by ^{1}H and ^{13}C NMR spectroscopy. Tables 1 and 2 report the signal assignments in the ^{13}C NMR spectra and the anomeric shifts in the ^{1}H NMR spectra of the glycosides, respectively. Assignments reported were made by DEPT experiments and by comparison with the literature values.[7,10] No evidence for α-D-glycosides was observed.

1,2-Diols.—Racemic mixtures of propane-1,2- (entry 1), butane-1,2- (2), and hexane-1,2-diol (5) were used for the synthesis of the corresponding β-D-galactosides by using phenyl β-D-galactoside. Four different compounds were obtained for each substrate due to the attachment of galactose to the primary (**1a**, **2a**, **5a**, Fig. 1) and secondary (**1b**, **2b**, **5b**, Fig. 1) hydroxy groups. All the substrates gave the corresponding galactosides at the primary hydroxy group as major products as indicated by the upfield shift of the signals due to the anomeric carbon atom.[10,11]

Integration of the signals due to the anomeric protons in the ^{1}H NMR spectra of the products permitted us to estimate the ratio of the galactosyl transfer to the primary hydroxy group with respect to the secondary hydroxy group. These data indicated that in all cases the ratio of the compounds **a** : **b** (Fig. 1) was 8:2. In the case of propane-1,2-diol, comparison with the literature chemical shifts permitted us to establish that no enantioselection had occured for the galactosyl transfer to the primary hydroxy group. Compounds **1a**, **2a** and **5a** are, in fact, racemic mixtures at the diol moiety. In contrast, good enantioselection was observed for the transfer to the secondary hydroxy group, compound **1b** being obtained with the (2*S*)-diol galactoside being the more abundant product (95:5), and compounds **2b** and **5b** (Fig. 1) were also obtained with one enantiomer being more predominant than the other (70:30).

Butane-1,3-diol.—Butane-1,3-diol was used for the synthesis of its corresponding β-galactosides (**3a** and **3b**, Fig. 1) and β-glucosides (**3c** and **3d**, Fig. 1) by reaction of phenyl β-D-galactoside and lactose, and phenyl β-D-glucoside, respectively as carbohydrate donors. Galactosyl and glucosyl transfer to the primary hydroxy group with respect to the secondary hydroxy group is the same as indicated by the integration of signals of the anomeric protons for the compound couples **3a**, **3b** and **3c**, **3d**. ^{1}H NMR spectroscopy of compounds **3a**, **3b** showed that galactosyl transfer to the primary and secondary hydroxy groups occurred without enantioselection. This result was supported by the integration of the anomeric signals of compound **3a** (Table 2) and by the integration of the signals of the methyl group in compound **3b** (δ 1.17 and 1.22 for *R*- and *S*-enantiomer, respectively) which were shifted downfield

Table 1 ^{13}C NMR signals of the compounds shown in Fig. 1

Compound[a]	**1a** *R*	**1a** *S*	**1b** *S*	**2a** *R*; *S*	**2b** *R*; *S*	**3a** *R*	**3a** *S*	**3b** *R*	**3b** *S*	**3c** *R*; *S*	**3d** *R*; *S*
Carbohydrate moiety											
C-1	103.8	104.2	103.3	104.1	103.9; 103.2	103.6	103.7	101.8	103.3	104.4; 104.3	102.35
C-2	71.6	71.7	71.8	71.6; 71.7	71.8	71.6		71.8		75.0	75.0
C-3	73.5		73.5	73.7	73.7	73.6		73.7	74.1	77.8	77.8
C-4	69.4		69.4	69.6	69.6	69.5		69.5		71.6	72.0
C-5	75.9		75.9	75.9	75.9	75.9		75.9		78.0	78.0
C-6	61.8		61.8	61.8	61.8	61.8		61.8		62.9	63.0
Alkyl group											
I	75.6	75.9	65.7	82.3; 82.4	63.8; 64.8	68.1		59.2	59.6	67.9; 68.0	59.8; 59.5
II	67.1	67.4	78.2	66.0	73.8	38.5		39.4	38.9	39.8	40.4; 40.9
III	19.0		18.0	26.7	25.0; 24.5	65.9		75.8	76.1	65.9	75.8; 76.1
IV				9.9	9.9	22.9	23.0	20.0	21.7	23.7	20.4; 22.4
V											
A, B											
VI											

Compound	**4a** *R*; *S*	**4b** *R*; *S*	**5a** *R*; *S*	**5b** *R*; *S*	**6a** *R*; *S*	**7a** *R*; *S*	**8a**	**9a** 1*R*,2R	**9a** 1*S*,2*S*
Carbohydrate moiety									
C-1	103.8	101.8; 103.8	104.6	104.1; 103.8	104.4; 104.2	104.1; 101.8	103.8	103.8	106.7
C-2	71.6	71.6	71.6; 71.7	71.8	71.7	71.8	71.8	75.0	76.2
C-3	74.0	74.1; 74.3	73.7	73.7	73.9	73.9; 74.0	73.9	78.8	78.7
C-4	69.5	69.5	69.6	69.6	69.5	69.6	69.7	72.0	71.9
C-5	76.0	76.0	75.9	75.9	75.1	76.0	76.0	78.0	78.7
C-6	61.8	61.8	61.8	61.8	61.4	61.8	61.9	62.8	62.9
Alkyl group									
I	71.1	63.5	75.0; 75.3	82.3; 82.4	75.6; 75.7	72.4	71.0	84.8	87.0
II	26.1	29.0	72.0; 72.1	64.3; 66.2	35.6; 35.5	50.0; 50.3	26.4	73.5	75.0
III	35.8	33.8	33.5[b]	31.5; 32.0[b]	26.5	76.1; 77.2	28.9	33.8	34.2
IV	68.4	75.8; 76.1	29.0[b]	29.0[b]	11.3; 11.4	21.5; 24.0	62.4	24.5	25.1
V	23.1	19.8	23.8[b]	23.8[b]	16.6			24.3	32.8
A, B						30.0 (30.5); 29.0			
VI			14.9	14.9				31.2	31.2

[a] *R* and *S* indicate the chiral centre of the diol moiety. *R*;*S* is used when the chemical shifts are not selectively assigned. [b] Not selectively assigned.

Table 2 Anomeric signals in the ^1H NMR spectra of the glycosides (for structures see Fig. 1)

1a	*R*	4.44	**1b**	*R*	4.48
	S	4.45		*S*	4.53
2a	*R*;*S*	4.38	**2b**	*R*;*S*	4.44; 4.49
3a	*R*	4.34	**3b**	*R*;*S*	4.41
	S	4.35			
3c	*R*;*S*	4.48	**3d**	*R*;*S*	4.55
4a	*R*;*S*	4.40	**4b**	*R*;*S*	4.47; 4.48
5a	*R*;*S*	4.31; 4.32	**5b**	*R*;*S*	4.41; 4.42
6a	*R*;*S*	4.30			
7a	*R*;*S*	4.48			
8a		4.35			
9[a]	1*R*2*R*	5.50			
	1*S*2*S*	5.18			

[a] Spectra in pyridine.

relative to the signals of the major isomer (δ 1.15). The same result was obtained in the case of glucosyl transfer.

Pentane-1,4-*diol*.—Pentane-1,4-diol used as substrate gave rise to the corresponding galactosides (**4a**, **4b** Fig. 1) in the ratio 7:3. Analysis of the not well resolved anomeric signals at δ 4.40 for *R*/*S* enantiomers of compound **4a** with respect to the better resolved signals at δ 4.47/4.48 for the *R*/*S* enantiomers of compound **4b** indicated that, for galactosyl transfer to the secondary hydroxy group of the pentane-1,4-diol, enantioselection between the two enantiomers is expressed by a factor of 70:30.

2-*Methylbutan*-1-*ol*.—2-Methylbutyl galactoside **6a** (Fig. 1) was formed with this substrate in an enantiomeric ratio 50:50 as indicated by spectroscopic studies.

2-*Methylpentane*-2,4-*diol*.—This compound was used for the synthesis of its corresponding galactosides since, possessing a secondary and a tertiary hydroxy group, it offers a different model for the study of regioselectivity. As expected the regioselection of the reaction in this case is well expressed in that the only product formed is compound **7a**, in which the galactose is attached to the secondary hydroxy group. Integration of the doublets at δ 1.28 and δ 1.31 attributable to the methyl groups of the *R*/*S* enantiomers of compound **7a** indicated that the two compounds were formed in the ratio 70:30.

Butane-1,4-*diol*.—Under the experimental conditions this compound formed the monogalactosylated product **8a** (Fig. 1); no evidence for digalactosyl transfer was observed.

trans-*Cyclohexane*-1,2-*diol*.—A racemic mixture of *trans*-

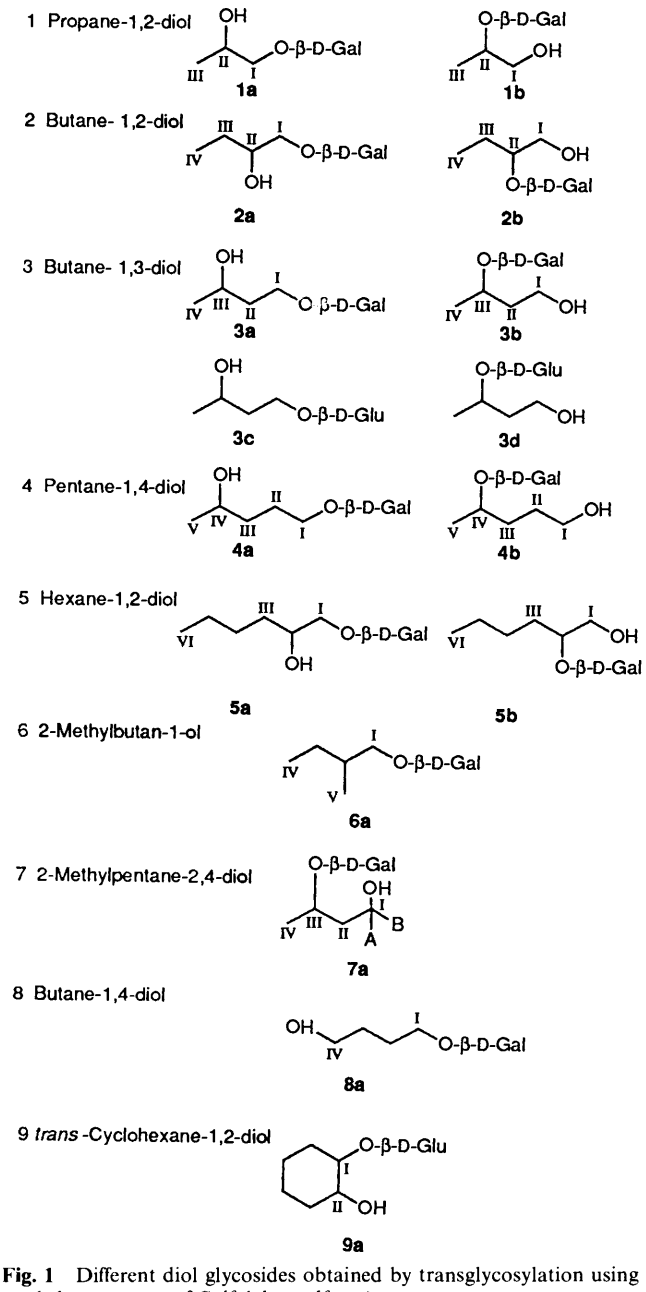

Fig. 1 Different diol glycosides obtained by transglycosylation using crude homogenate of *Sulfolobus solfataricus*

cyclohexane-1,2-diol was subjected to glucosyl transfer. A mixture of monoglucosylated diastereoisomers **9a** was formed (Fig. 1), which was in an enantiomeric ratio, at the diol moiety, of 50:50 as indicated by the integration of the anomeric signals for the 1*R*,2*R*- and 1*S*,2*S*-glucoside in the ^{1}H NMR spectrum.

Table 3 Total yield of the products shown in Fig. 1

Substrate	Yield (%)[a]
1 Propane-1,2-diol	85
2 Butane-1,2-diol	88
3 Butane-1,3-diol	87
4 Pentane-1,4-diol	90
5 Hexane-1,2-diol	60
6 2-Methylbutan-1-ol	90
7 2-Methylpentane-2,4-diol	35
8 Butane-1,4-diol	95
9 *trans*-Cyclohexane-1,2-diol	25[b]

[a] Isolated yield with respect to the glycosyl donor. [b] Using a 100 molar equiv. excess of alcohol.

Under our experimental conditions almost all compounds were isolated in ~90% yield with respect to the donor (Table 3) while a total yield of 30–60% for galactoside formation from lactose and different diols catalysed by β-galactosidase from *E. coli* has been reported.[10]

The enzyme involved in the glycosyl-transfer reaction with the crude homogenate of *Sulfolobus solfataricus* could be the β-galactosidase previously isolated and characterized from this microorganism.[12] This hypothesis is supported by the fact that the same results regarding the regio-and stereo-chemistry of the reaction were obtained in the case of butane-1,3-diol for both galactosyl and glucosyl transfer and by the fact that purified enzyme was used for the synthesis of gentobiose.[5]

Since the 1,3-diol structure mimics the C-4-to-C-6 component of the glucopyranose site hypothesized for the β-galactosidase of *E. coli*,[10,13] some hypotheses concerning three types of interactions of the substrate with the active site of the enzyme can be made for our compounds (Fig. 2 and Table 3).

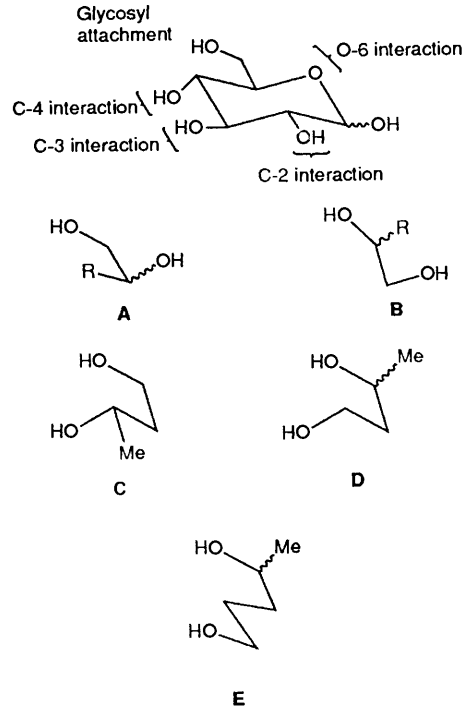

Fig. 2 Interactions of glucose and diols with the glucosyl site of the enzyme (see ref. 13)

For the transfer at the primary hydroxy group both enantiomers of the 1,2-diols could interact with the active site at O-6 relative to the glucopyranose component (**A**, Fig. 2). The 'better' reaction exhibited by the (*S*)-enantiomer of propane-1,2-diol, and probably by the same enantiomer for all 1,2-diols, for transfer at the secondary hydroxy group (**B**, Fig. 2) could be due both to the hydrophobic interactions of the alkyl chain linked to the alcohol moiety and to the steric hindrance in the (*R*)-enantiomer. Both factors may operate simultaneously, as also suggested for the β-galactosidase of *E. coli*.[10]

Both enantiomers of butane-1,3-diol can interact with the active site of the enzyme at C-4 of the glucopyranose component for transfer at primary (**C**, Fig. 2) and secondary (**D**, Fig. 2) hydroxy groups. The loss of enantioselection in this reaction could be due to the improved binding factor of the C-4 interaction.

Enantioselection was again observed for the interaction at C-3 for the galactosylation at the secondary hydroxy group of pentane-1,4-diol although it is not so pronounced as in the case of propane-1,2-diol, probably because of the major complexity of the molecule, folding itself into a shape more equivalent to the

Table 4 Possible interactions of the substrates with the glucosyl site[a] of the enzyme

	Interaction		
	C-3	C-4	O-6
Propane-1,2-diol			X,O
Butane-1,2-diol			X,O
Butane-1,3-diol		X,O	
Pentane-1,4-diol	X,O		
Hexane-1,2-diol			X,O
2-Methylpentane-2,4-diol	O		
Butane-1,4-diol	X		

[a] See ref. 13. X = Interaction for attack on the primary hydroxy group. O = Interaction for attack on the secondary hydroxy group.

cyclic structure of glucopyranose (**E**, Fig. 2) with respect to the parent structure of butane-1,3-diol (**D**, Fig. 2).

The regioselection of the enzymatic reaction expressed for the case of 2-methylpentane-2,4-diol, butane-1,4-diol, and *trans*-cyclohexane-1,2-diol is a valuable result from a synthetic point of view in that it permits the synthesis of monoglycosides of dihydroxyaglycones while avoiding difficult chemical protection–deprotection procedures.

In our case the use of a crude preparation of the enzyme to afford a good yield of glycoside products allowed the synthesis of carbohydrate derivatives, avoiding expensive and time-consuming purification of the enzyme, to be accomplished. The use of polyhydroxylated compounds as more complex substrates for the transglycosylation reaction, together with a thorough examination of stereochemistry with respect to *meso*-compounds, both with crude homogenate and purified enzyme, is of current interest in our laboratory.

Experimental

Chemicals.—Alcohols and carbohydrate donors were purchased from Aldrich and were of the highest available purity. Glass beads (type 1) were obtained from Sigma Chemical Company.

Cell Culture and Homogenate Preparation.—*Sulfolobus solfataricus* strain MT4 was isolated from an acid hot spring in Agnano, Naples.[14] The organism was grown at 88 °C in a 90 dm^3 fermenter with low mechanical agitation and an aeration flux of 30 cm^3 min^{-1} dm^{-3}. The standard culture medium contained KH_2PO_4 (3.10 g dm^{-3}), $(NH_4)_2SO_4$ (2.50), $MgSO_4 \cdot 7H_2O$ (0.20), $CaCl_2 \cdot 2H_2O$ (0.25) and glucose (3.0). The pH of the culture medium was adjusted to 3.5 with 0.1 mol dm^{-3} H_2SO_4. Cells were harvested in the stationary phase of growth by continuous flow on an Alfa-Laval model Lab 102 B-20 centrifuge. The pellet was washed twice with an iso-osmotic saline solution, pH 6, and collected by centrifugation at 3000 *g* for 30 min.

Frozen cells (100 g) were broken with glass beads (type 1) (100 g) and 10 mmol dm^{-3} Tris-HCl buffer (pH 7.5) (150 cm^3) plus 0.5 mol dm^{-3} KCl in a refrigerated Sorvall Omni-Mixer for 5 min at low speed and for 5 min at high speed. The glass beads were removed by low-speed centrifugation (150 *g* for 20 min) and the supernatant was centrifuged at 160 000 *g* for 60 min in a Spinco Ti 50-2 rotor. The supernatant after dialysis overnight against 10 mmol dm^{-3} acetate buffer (pH 5.5) (10 dm^3) represented the crude extract (570 mg of wet weight cells per cm^3; 7.2 mg of total proteins per cm^3).

Analytical Procedures.—TLC was carried out on Kieselgel 60 F_{254}, 0.2 mm (Merck) with $CHCl_3$–MeOH (8:2, v/v) as developing solvent. Spots were visualized by spraying the plates with α-naphthol reagent and then heating the plates at 150 °C for 5 min. ^1H and ^{13}C NMR spectra were run on a Bruker WH-500 spectrometer at 500 and 125 MHz, respectively, in $C^2H_3O^2H$–^2H_2O (1:1, v/v).

Enzymatic Synthesis of Glycosides.—In a typical experiment, an aliquot (0.2 mol) of the acceptor was solubilized in the crude extract (10 cm^3) and the donor (0.39 mmol) was added to this solution. The mixture was warmed to 75 °C and the reaction was monitored by TLC. After complete hydrolysis of the donor (1.5–3 h) the mixture was extracted with diethyl ether to remove phenol. The water phase was concentrated and the product was taken up in MeOH–water 8:2 (v/v), filtered, and evaporated at reduced pressure. The product was then subjected to column chromatography and eluted with a step gradient of MeOH in $CHCl_3$, 5% v/v increments.

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