# Glycosidase-catalysed synthesis of oligosaccharides: a one step synthesis of lactosamine and of the linear B type 2 trisaccharide α-d-Gal-(1 → 3)-β-d-Gal-(1 → 4)-β-d-GlcNAcSEt involved in the hyperacute rejection response in xenotransplantation from pigs to man and as the specific receptor for toxin A from *Clostridium difficile*

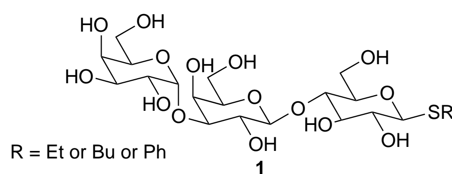
**Gabin Vic,† Chuong Hao Tran, Michaela Scigelova and David H. G. Crout***

*Department of Chemistry, University of Warwick, Coventry, UK CV4 7AL*

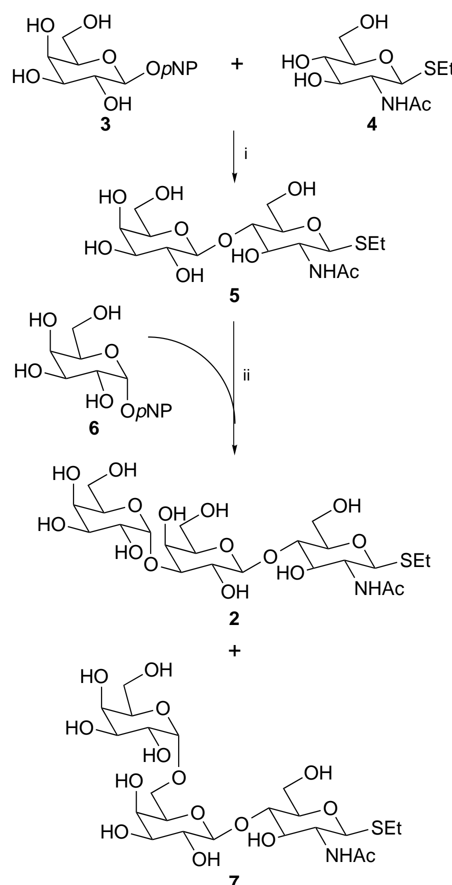
**Sequential use of the β-galactosidase from *Bacillus circulans* and an α-galactosidase from *Aspergillus oryzae* give the linear B trisaccharide 2, identified as the epitope that binds anti-Gal antibodies during pig-to-man xenotransplantation and as the receptor for toxin A from *Clostridium difficile*.**

There is at present a worldwide shortage of organs available for human transplantation. The use of pigs' organs would be a solution to this problem if the hyperacute rejection that occurs after such transplantation could be prevented.[1] Two trisaccharides **1** and **2** bearing the α-**d**-Gal-(1 → 3)-β-**d**-Gal terminus have been identified to play a fundamental role in the process of hyperacute xenograft rejection in xenotransplantation from pigs to man.[2] Both of these trisaccharides are specific antigens for the binding of xenoreactive antibodies of the recipient on endothelial cells in the graft which inevitably destroy it within a few hours. Moreover trisaccharide **2** is also known as the receptor for toxin A from *Clostridium difficile* which is the major cause of antibiotic-associated diarrhoea and the causative agent of pseudomembranous colitis (PMC) particularly in elderly patients.[3] The significance of these findings prompted the development of an enzymatic synthesis of these trisaccharides because the application of glycosidases in oligosaccharide synthesis avoids the need for protection-deprotection steps required in non-enzymatic syntheses.

In a previous communication[4] the enzymatic synthesis was described of several derivatives of the linear B type 6 trisaccharide **1** starting from alkyl 1-thio-β-**d**-lactopyranoside as acceptor and *p*-nitrophenyl α-**d**-galactopyranoside as donor using the newly discovered α-galactosidase II from *Aspergillus oryzae*.‡ Here we report the synthesis of the linear B type 2 trisaccharide **2** in two enzymatic steps (Scheme 1). The synthesis required first the synthesis of a glycoside **5** of *N*-acetyllactosamine. *N*-Acetylglucosamine has been synthesised using glycosyltransferases. The routes of Wong *et al.*[5] and of Thiem and Wiemann[6] both required the use of six enzymes because, in addition to the galactosyl transferase, an enzyme system for the regeneration of the galactosyl donor, UDP-Gal, was included. In a recent improvement of this procedure, Zervosen and Elling have reduced the number of enzymatic steps required to three by using sucrose synthetase to regenerate UDP-Glc.[7] However, reduction to practice of this scheme required the use of two further enzymes: dTDP-glucose 4,6-dehydratase to synthesise dTDP-6-deoxy-**d**-*xylo*-4-hexulose required for reactivation of UDP-Glc 4′-epimerase and invertase to hydrolyse the excess of sucrose remaining at the end of the reaction.




The method reported here is based on the use of the β-galactosidase from *Bacillus circulans* which was used by Sakai *et al.* to synthesise *N*-acetyllactosamine from lactose and *N*-acetylglucosamine.[8] Some 1,6-isomer was simultaneously formed and isolation of the product required extensive chromatographic purification. This method has been refined by using the thioethyl glycoside of *N*-acetylglucosamine as acceptor and *p*-nitrophenyl β-**d**-galactoside as donor. With these substrates the glycosylation was found to proceed with complete regiocontrol for formation of the β 1 → 4 isomer and isolation of the product was also greatly simplified.




**Scheme 1** *Reagents*: i, β-galactosidase from *Bacillus circulans*; ii, α-galactosidase II from *Aspergillus oryzae*

Ethyl 1-sulfanyl-(β-d-galactopyranosyl)-(1 → 4)-(2-acetamido-2-deoxy)-*O*-β-d-glucopyranoside **5** was obtained by incubation of *p*-nitrophenyl β-d-galactopyranoside **3** as galactosyl donor and ethyl 1-sulfanyl-(2-acetamido-2-deoxy)-*O*-β-d-glucopyranoside **4**[9] as acceptor using a crude β-galactosidase from *Bacillus circulans*.§ A simple procedure was followed: the donor **3** (0.55 g, 1.82 mmol) and the acceptor **4** (2.96 g, 11.15 mmol) in acetate buffer (pH 5.0, 50 mmol $dm^3$, 37 $cm^3$) were incubated with the crude β-galactosidase (3.8 mg, 7.4 U) at 22 °C for 7 h. After all of the donor has been consumed the reaction was stopped by heating at 100 °C for 5 min. *p*-Nitrophenol was extracted with diethyl ether (4 × 50 $cm^3$) and the aqueous layer was concentrated to a total volume of 12 $cm.^3$ This was applied in 4 $cm^3$ batches to a Biogel P2 column (120 × 2 cm) which was eluted with $H_2O$ (flow rate 0.6 $cm^3$ $min^{-1}$, fraction 6 $cm^3$) to give the disaccharide **5** in 50% yield (0.39 g). The structure of the product was confirmed by $^1H$ and $^{13}C$ NMR spectroscopy and mass spectrometry.[10]

Synthesis of the trisaccharide glycoside **2** was completed by transfer of the α-d-galactosyl residue (1 → 3) on to the disaccharide **5** using a partially purified α-galactosidase II from *Aspergillus oryzae*. This enzyme accepted *p*-nitrophenyl α-d-galactopyranoside **6** as an α-d-galactosyl donor giving a 1 : 0.9 mixture of the α 1 → 3 and α 1 → 6 linked trisaccharides **2** and **7**. These two trisaccharides were further purified to homogeneity by a combination of Biogel P2 and carbon–Celite column chromatography, and selective enzymatic hydrolysis. Thus donor **6** (79 mg, 0.26 mmol) and the acceptor **5** (0.40 g, 0.93 mmol) in citrate–phosphate buffer (pH 5.0, 50 mmol $dm^3$, 3.12 $cm^3$) were incubated with the partially purified α-galactosidase II (4 U) at 22 °C for 20 h. The reaction was stopped by heating at 100 °C for 5 min, the mixture was diluted with $H_2O$ (15 $cm^3$) and filtered. *p*-Nitrophenol was extracted with diethyl ether (4 × 10 $cm^3$). The aqueous layer was concentrated to 4 $cm^3$ and applied to a Biogel P2 column (120 × 2 cm) which was eluted with $H_2O$ (flow rate 0.6 $cm^3$ $min^{-1}$). The mixture of trisaccharides obtained in 32% overall yield (49 mg) was applied to a carbon–Celite column which was eluted with water–ethanol (75 : 25). The α 1 → 6 linked trisaccharide was eluted in the first fractions and a mixture of α 1 → 3 and a minor β linked trisaccharide were eluted in the following fractions. The presence of this β linked trisaccharide was due to the remaining β-galactosidase activity in the enzymatic preparation. The α 1 → 3 trisaccharide **2** was further purified to homogeneity by selective hydrolysis of the β linked trisaccharide using the β-galactosidase from *Bacillus circulans*¶ to give finally trisaccharide **2** (20 mg). The structure of **2** was confirmed by $^1H$ and $^{13}C$ NMR spectroscopy and mass spectrometry (Table 1). The method described here provides an easy access to this important trisaccharide but was not optimised. This may be realised by using the α-galactosidase II from *Aspergillus oryzae* free from β-galactosidase activity.

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**Table 1** Selected $^1H$ NMR data for trisaccharides **2** and **7**[a,b]

| Trisaccharide | 1-H | 1′-H | 1″-H |
|---|---|---|---|
| **2** | 4.61 (d, 10.28) | 4.50 (d, 7.62) | 5.10 (d, 3.65) |
| **7** | 4.65 (d, 10.62) | 4.43 (d, 7.63) | 4.97 (d, 2.98) |

[a] $^1H$ NMR was carried out at 400 MHz using a Bruker AC 400 spectrometer in $D_2O$. [b] [Multiplicity, coupling constant (Hz)].

## Footnotes

† Present address: Unilever Research, Biorecognition Department, Colworth House, Sharnbrook, Bedford, UK MK44 1LQ.

‡ Isolated from the partially purified β-galactosidase of *A. oryzae*, a gift from the Sigma Chemical Company Ltd.

§ A gift from Daiwa Kasei Co., Japan.

¶ The mixture of trisaccharide **2** and β linked trisaccharide (25 mg, 0.042 mmol) in acetate buffer (pH 5.0, 50 mmol $dm^3$, 2 $cm^3$) was incubated with the β-galactosidase from *Bacillus circulans* (8.0 mg, 15.5 U) at 22 °C for 1 h and purified using a Biogel P2 column.

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