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Glycosidase-catalysed synthesis of α-galactosyl epitopes important in xenotransplantation and toxin binding using the α-galactosidase from *Penicillium multicolor*

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The α-galactosidase from *Penicillium multicolor* catalyses highly regioselective galactosyl transfer on to mono- and disaccharide acceptors that have a non-reducing terminal galactose unit to give products containing the α-D-Gal*p*-(1→3)-D-Gal*p* epitope found on pig tissue and which is responsible for the hyperacute rejection response in xenotransplantation of pig organs into man.

The α-D-Gal*p*-(1→3)-β-α-Gal*p* moiety of glycoconjugates has been found to be of considerable biological importance. It has attracted attention mainly because of its significance in the development of the xenotransplantation of animal organs into human patients,[1] a development driven on the one hand by the success of organ transplantation in the latter part of the twentieth century and on the other by an acute shortage of donor organs. Current research is directed towards the use of pig organs in humans (discordant xenotransplantation), as the pig is considered the best organ donor given the constraints imposed by the popular opposition to the use of organs from primates. The major problem with pig-to-man xenotransplantation is the extremely rapid human antibody-mediated hyperacute rejection that occurs following transplantation into human patients. The antigenic epitopes responsible for this hyperacute rejection response contain the α-D-Gal*p*-(1→3)-β-D-Gal*p* terminus and have been identified as α-D-Gal*p*-(1→3)-β-D-Gal*p*-R, α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-R, α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-R and α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-R, known collectively as 'α-Gal' epitopes.[2] These epitopes specifically bind to human anti-α-Gal antibodies during xenotransplantation.[3] They are found on tissues of almost all mammals except man and old world primates. Antibodies against the α-Gal epitope expressed in man comprise approximately 1% of circulating immunoglobulin G.[4] Recent investigations have shown that human anti-Gal antibodies can bind to, and be neutralised by, α-Gal-related oligosaccharides.[5] An α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p* trisaccharide, representing the natural α-Gal epitope, is capable of neutralising anti-Gal antibodies at low concentrations.[6] A further reason for interest in the α-Gal epitope is that it has been identified as a receptor for the toxin from *Clostridium difficile*, a micro-organism that is a major cause of antibiotic-associated diarrhoea and the causative agent of a serious condition, pseudomembranous colitis, in the elderly following antibiotic therapy.[7]

The possibility of utilising the adhesion of bacteria and viruses to specific carbohydrates has become an attractive goal in attempts to find alternatives to antibiotic therapy. Developments in this area have taken place against a background of increasing concern about the development of antibiotic resistance in important species of pathogenic bacteria.[8] An immobilised version of the *C. difficile* toxin receptor is in development for the treatment of *C. difficile*-associated diarrhoea.[9]

The use of α-Gal oligosaccharides for clinical applications would require large amounts of α-Gal oligosaccharides. Conventional synthesis of carbohydrates, although well developed over the last decade, requires multiple protection and deprotection steps.[10] Consequently, enzymatic synthesis is an attractive alternative for the synthesis of oligosaccharides.[11] Enzymatic syntheses of α-Gal oligosaccharides have been reported.[12] However, the α-galactosyl transferase method requires the use of a complex donor and is limited by the availability of the appropriate enzymes.[12*a*] The use of glycosidases has been reported but these have been limited by rather low yields and by the production of mixtures.[12*b–d*]

Here we report simple, regiospecific, glycosidase-catalysed syntheses of α-D-Gal*p*-(1→3)-β-D-Gal*p*-OMe and α-D-Gal*p*-(1→3)-α-D-Gal*p*-OMe disaccharides and α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p* and α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-GlcNA*cp* trisaccharides catalysed by an α-galactosidase from *Penicillium multicolor* discovered during a major screening programme for synthetically useful glycosidases.[13] The great advantage of this enzyme is that it is highly specific for α-(1→3) transfer and thus represents a considerable improvement over existing methods for the synthesis of α-Gal oligosaccharides.

Transfer of an α-galactosyl unit from *p*-nitrophenyl α-D-galactopyranoside **1** on to methyl β-D-galactopyranoside **2** using an α-galactosidase from *Penicillium multicolor* gave only one disaccharide α-D-Gal*p*-(1→3)-β-D-Gal*p*-OMe **3** in 43% yield [Scheme 1(*a*)]. Similarly, transfer of an α-galactosyl unit from donor **1** on to methyl α-D-galactopyranoside **4** gave the disaccharide α-D-Gal*p*-(1→3)-α-D-Gal*p*-OMe **5** in 46% yield [Scheme 1(*b*)]. These results suggest that by changing the conformation at the anomeric position in the acceptors **2** and **4** the regioselectivity of the transfer was not affected. This indicates that 'anomeric control', in which regioselectivity is influenced by the anomeric configuration in the glycosyl acceptor, does not operate in this system.[14] In both cases the product obtained was a (1→3)-linked disaccharide.

Transfer of the α-galactosyl unit from donor **1** on to lactose **6** gave again the (1→3)-linked product α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p* **7** in 25% yield [Scheme 1(*c*)]. Similarly transfer of α-galactosyl unit from the donor **1** on to lactosamine **8** gave the trisaccharide α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-GlcNA*cp* **9** in 32% yield [Scheme 1(*d*)].

In all these reactions the conversion (donor) was 40–50%. This was not taken into account in calculating yields. The incomplete consumption of donor indicated product inhibition. If this could be overcome, even higher yields should be obtainable. We found that the released *p*-nitrophenol inhibited the enzyme and showed that this inhibition could be largely overcome by operating in less concentrated solutions. Thus by decreasing the overall concentration of donor **1** (from 0.34 to 0.17 M) and the acceptor **8** (from 1.3 to 0.65 M), 90% conversion was achieved and the isolated yield of the trisaccharide **9** increased from 32% to 48%.

The α-galactosidase used in these experiments was isolated from Lactase P from *Penicillium multicolor* (KI Chemicals, Japan). The proteins from a crude extract were precipitated by EtOH (50% v/v) and separated by DEAE-Sepharose [equilibrated with potassium phosphate buffer (10 mM, pH 6.5) and eluted with a linear gradient of NaCl (0–0.8 M)] followed by

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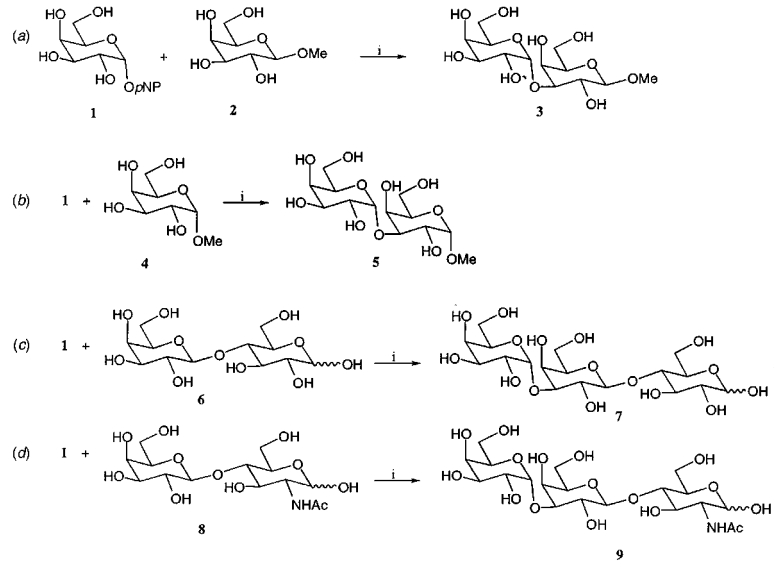

Scheme 1 *Conditions*: i, α-Galactosidase from *Penicillium multicolor; p*NP = *p*-nitrophenyl.

chromatography on ceramic hydroxyapatite [equilibrated with potassium phosphate buffer (5 mM, pH 6.5) and eluted with a linear gradient (5–400 mM) of potassium phosphate buffer (pH 6.5)]. Active fractions were concentrated using a Centriprep (Amicon) membrane with a 30 kDa cut-off to give a preparation [6.4 U cm^{-3}, 150 U (mg protein)$^{-1}$] free of β-galactosidase activity.

The method described here provides a remarkably simple one-step enzymatic synthesis of α-galactosyl epitopes and can be scaled up for clinical applications. Given the potential demand for α-Gal di- and tri-saccharides, the method described here offers an inexpensive and economical route to these compounds.

In a typical experiment *p*-nitrophenyl α-D-galactopyranoside **1** (0.1 g, 0.332 mmol) and *N*-acetyllactosamine **8** (0.5 g, 1.305 mmol) in citrate–phosphate buffer (0.05 M, 2 ml, pH 4.0) were incubated with the α-galactosidase from *Penicillium multicolor* (0.15 cm^3, 0.96 U) at 30 °C for 6 h. The reaction was stopped by heating in a boiling water bath for 5 min. Examination of the crude product by HPLC indicated that a single trisaccharide was formed. The product mixture was purified by carbon–Celite chromatography[15] to give trisaccharide **9** in 48% yield. The product was confirmed to be a single isomer by ^{1}H and ^{13}C NMR.

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