Heterologous Over-expression of α-1,6-Fucosyltransferase from *Rhizobium* sp.: Application to the Synthesis of the Trisaccharide β-D-GlcNAc(1 → 4)-[α-L-Fuc-(1 → 6)]-D-GlcNAc, Study of the Acceptor Specificity and Evaluation of Polyhydroxylated Indolizidines as Inhibitors

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Abstract: An efficient heterologous expression system for overproduction of the enzyme α-1,6-Fucosyltransferase (α-1,6-FucT) from *Rhizobium* sp. has been developed. The gene codifying for the α-1,6-FucT was amplified by PCR using specific primers. After purification, the gene was cloned in the plasmid pKK223-3. The resulting plasmid, pKK1,6FucT, was transformed into the *E. coli* strain XL1-Blue MRF′. The protein was expressed both as inclusion bodies and in soluble form. Changing the induction time a five-fold increase of enzyme expressed in soluble form was obtained. In this way five units of enzyme α-1,6-FucT can be obtained per liter of culture. A crude preparation of the recombinant enzyme was used for the synthesis of the branched trisaccharide α-D-GlcNAc-(1 → 4)-[α-L-Fuc-(1 → 6)]-D-GlcNAc (**3**), from chitobiose (**2**) and GDP-Fucose (**1**). After purification, the trisaccharide **3** was obtained in a 84% overall yield. In order to elucidate the structural requirements for the acceptors, the specificity of the enzyme was studied towards mono-, di- and trisaccharides, which are structurally related to chitobiose. The enzyme uses, among others, the disaccharide *N*-acetyl lactosamine as a good substrate; the monosaccharide GlcNAc is a weak acceptor. Finally, several racemic polyhydroxylated indolizidines have been tested as potential inhibitors of the enzyme. Indolizidine **21** was the best inhibitor with an IC_{50} of 4.5×10^{-5} M. Interestingly, this compound turned out to be the best mimic for the structural features of the fucose moiety in the presumed transition state.

Keywords: α-1,6-fucosyltransferase · carbohydrates · enzyme catalysis · enzyme inhibitors · glycosyltransferases

Introduction

The broad range of biological functions in which carbohydrates are involved, mainly related to cell recognition events,[1] is indicative of their structural diversity. The growing interest in the use of carbohydrates as therapeutic agents[2] is hampered by several factors such as their poor in vivo bioavailability, weakness of the protein–carbohydrate interactions and lack of a general methodology for the stereo- and regioselective formation of the glycosidic bond. In the last years, glycosidases[3] and glycosyltransferases[4] have demonstrated to be useful tools for the synthesis of oligosaccharides. Glycosyltransferases have attracted the attention of chemists mainly because of their strict control over the stereo- and regioselectivity in the glycosidic bond formation, which led to the "one enzyme–one linkage" concept.[5]

However, the use of glycosyltransferases in synthesis faces some drawbacks. These enzymes use a nucleotide activated sugar as donor. The sugar–nucleotide donors are too expensive to be used in stoichiometric amounts in medium or large scale synthesis but, even more important, the nucleoside phosphate released during the reaction is a natural inhibitor of the glycosyltransferases. These problems can be avoided removing the nucleotide with alkaline phosphatase[6] or, in a more sophisticated way, by in situ regeneration of the glycosyl donor.[7] The other major drawback, which has not yet been solved, is the limited availability of these enzymes. To our knowledge, only nine glycosyltransferases are commercially available and of those, only the β-1,4 galactosyltransferase is available in amounts higher than 1 unit. Regarding fucosyltransferases, only α-1,3/4 and α-1,3 fucosyltransferases from human are commercially available in amounts of 0.1 units. One alternative to overcome this

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problem is the cloning and over-expression of bacterial glycosyltransferases.[8] Many bacterial glycosyltransferases are able to produce mammalian-like structures. Furthermore, it is possible to obtain transferases in microorganisms with specificities not yet found in mammalian enzymes.[9] On the other hand, bacterial proteins give enhanced expression in prokaryotic systems compared with mammalian systems, because they do not need to be glycosylated. For large scale production of recombinant proteins, prokaryotic expression systems, and in particular *Escherichia coli*, are the most attractive ones because of their ability to grow rapidly and in high density on inexpensive substrates.[10]

The recent efforts to sequence the complete genome of several microorganisms make a great number of genes available putatively coding for glycosyltransferases. For example, the rhizobia gene *nod*Z is responsible for the fucosylation of the lipochitin oligosaccharide (LCO) signal molecules.[11] NodZ proteins catalyze the transfer of an α-L-fucopyranosyl residue from GDP-β-L-Fucose (GDP-Fuc) to the C-6 position of the GlcNAc at the reducing end of the nodulation (Nod) factors.[12] The *nod*Z genes have been cloned and sequenced so far from *Azorhizobium caulinodans*,[11c] *Bradyrhizobium japonicum*,[11a] and *Rhizobium* sp. NGR234;[13] the enzymes from *B. japonicum*[14] and *Rhizobium* sp.[15] have been identified as fucosyltransferases both in in vivo and in vitro transfucosylation assays. In mammals, α-1,6-FucT catalyzes the transfer of fucose from GDP-Fuc to asparagine-linked GlcNAc of N-linked type complex glycoproteins. The enzymes have been cloned from porcine[16] and human so far.[17] α-1,6-Fucosylated N-glycans are present in many glycoproteins and are specially abundant in brain tissue.[18] In human liver diseases α-1,6-FucT is overexpressed in both hepatoma tissues and their surrounding tissues with chronic liver disease, not in healthy liver.[19]

Our lab is involved in a project aimed at developing over-expression systems for bacterial glycosyltransferases, in order to obtain efficient and cost-effective large scale production of these enzymes. In this work, we report the heterologous over-expression of the NodZ protein from *Rhizobium* sp. (EMBL/GenBanK accession number: AE000064) in *E. coli*. The recombinant enzyme has been used for the synthesis of the branched trisaccharide β-D-GlcNAc-(1$\rightarrow$4)-[α-L-Fuc-(1$\rightarrow$6)]-D-GlcNAc (**3**) which structure was confirmed by ^{1}H and ^{13}C NMR analysis. The glycosylation activity of the enzyme has been characterized studying its specificity towards different sugar acceptors. Finally, we report some preliminary results on the inhibition of the enzyme by several racemic castanospermine stereoisomers (1,6,7,8-tetrahydroxylated indolizidines).

Abstract in Spanish: *Se ha desarrollado un sistema de expresión heteróloga para la sobre-expresión del enzima α-1,6-Fucosyltransferasa (α-1,6-FucT) de Rhizobium sp. El gen que codifica para la α-1,6-FucT se amplificó mediante PCR utilizando primers específicos. Tras su purificación, el gen se clonó en el plásmido pKK223-3. El plásmido resultante, pKK1,6FucT, se transformó en la cepa de E. coli XL1-Blue MRF′. La proteína se expresó tanto soluble como en forma de cuerpos de inclusión. Modificando el momento de la inducción la cantidad de enzima expresada en forma soluble se multiplicó por un factor de cinco. De esta forma, se pueden obtener 5 unidades de enzima α-1,6-FucT por litro de cultivo. Un extracto crudo del enzima recombinante se utilizó para la síntesis del trisacárido β-D-GlcNAc-(1→4)-[α-L-Fuc-(1→6)]-D-GlcNAc (**3**), a partir de quitobiosa (**2**) y GDP-Fucosa (**1**). El trisacárido **3** se obtuvo, después de su purificación, con un rendimiento global del 84 %. Con el fin de determinar los requisitos estructurales de los aceptores, se estudió la especificidad del enzima hacia mono-, di- y trisacáridos estructuralmente relacionados con la quitobiosa. El enzima es capaz de usar, entre otros, el disacárido N-acetil lactosamina (**4**) como un buen sustrato y el monosacárido GlcNAc (**8**) como un aceptor débil. Por último, se han ensayado diversas indolicidinas polihidroxiladas racémicas como potenciales inhibidores del enzima. La indolicidina **21** fue el mejor inhibidor con una IC_{50} de 4.5×10^{-5} M, siendo a su vez la que mejor podría mimetizar las características estructurales de la molécula de fucosa en el estado de transición propuesto.*

Results and Discussion

Cloning and expression of α-1,6-fucosyltransferase: The *nod*Z gene from *Rhizobium* sp. NGR234 was amplified by PCR using primers designed to complement specifically 15 bp at the 5′ ends of codifying and complementary DNA strains. The recognition sequence for the restriction enzymes *Eco* RI and *Hin*d III was introduced in the amplification product during the PCR. The PCR amplification was rather specific, and only one band with the expected length (969 bp) was observed. The band was purified and double digested with the above-mentioned restriction enzymes and ligated with the digested vector pKK223-3 (Pharmacia Biotech.) to yield the plasmid pKK1,6FucT (Figure 1).

The plasmid encoding for the NodZ protein, was transformed in *E. coli* XL1-Blue MRF′ strain (Stratagene) and plated on LB-ampicillin plates. The presence of the *nod*Z gene in the transformed cells was checked by restriction analysis of the purified plasmids. Out of ten colonies selected, seven carried the desired insert. One positive colony was grown on LB medium containing 250 μg mL^{-1} ampicillin. Since the expression of the recombinant enzyme in the plasmid pKK1,6FucT is under the control of the strong *tac* promoter which is inducible by isopropyl-β-D-thiogalactopyranoside (IPTG), the induction was done in the early logarithmic phase with 1 mM IPTG. The expression of the recombinant enzyme was analyzed by sodium dodecyl sulphate–polyacrilamide gel electrophoresis (SDS-PAGE) of samples taken 5 h after induction (Figure 2).

An IPTG inducible protein matching the expected molecular weight of the α-1,6-FucT (36 kDa) could be observed in the soluble fraction and in the pellet (Figure 2, lanes 4 and 5, respectively). This result shows that although part of the recombinant enzyme is expressed correctly folded and soluble, the main part is segregated into insoluble aggregates known as inclusion bodies (I.B.). The formation of I.B. is a

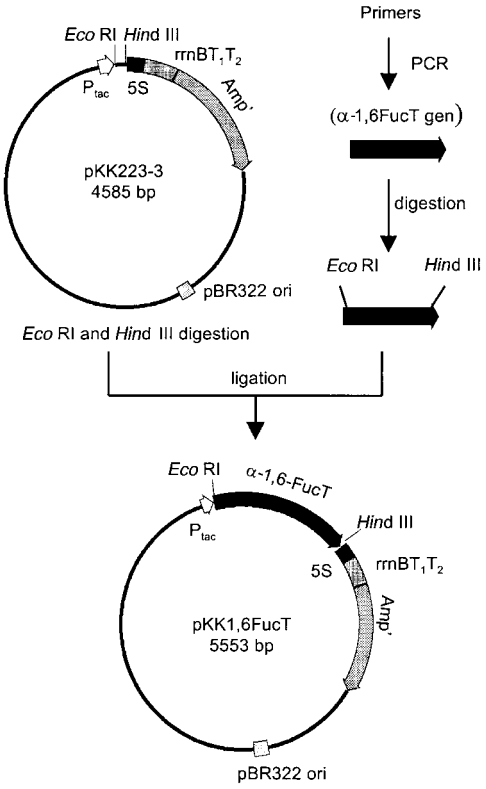

Figure 1. Construction of the pKK1,6FucT plasmid. The main characteristics of the expression vector are shown. The sequence of the primers used for the PCR were as follows: 1,6-FucT-Nt (5′GCCGCGAATTCATGTACAATCGATAT3′) and 1,6-FucT-Ct (5′GCCGCAAGCTTTCAAGAGGCGGTATT3′)

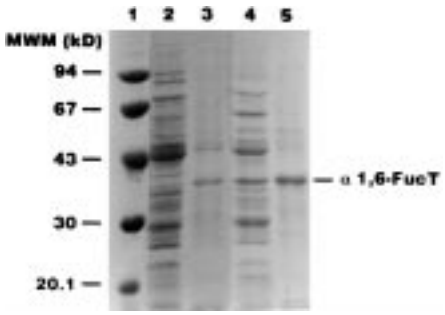

Figure 2. SDS-PAGE analysis of the recombinant α-1,6-FucT expression. Lane 1, molecular weight markers; lane 2, soluble fraction before induction; lane 3, pellet before induction; lane 4, soluble fraction 5 h after induction; lane 5, pellet 5 h after induction. For details see Experimental Section.

common problem when a protein is over-expressed, since the high production of protein does not allow its correct folding, and thus causes aggregation and precipitation of the protein. We tried to refold the inclusion bodies by solubilizing the pellet with 8 M urea[20] or 6 M guanidine, followed by a slow elimination of the denaturant agent by dialysis against buffer with or without different folding aids.[21] The problems encountered in the in vitro refolding of the inclusion bodies may be due to the presence of a 20 amino acid fragment in the protein near the C-terminal, which is assigned to a transmembrane region by the structure analysis program TMpred.[22] Another approach to reduce the in vivo formation of inclusion bodies is to slow down the bacterial metabolism through fermentation engineering.[23] We envisioned that the production rate of recombinant protein could be modulated during the time in which the induction is made, since the older the culture the slower the metabolism rate. We prepared different cultures that were induced throughout the growing curve (Figure 3).

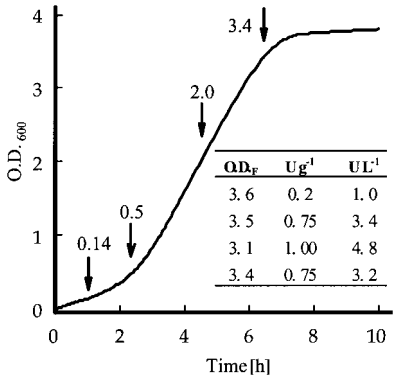

Figure 3. Growth curve for *E. coli* cells carrying on the pKK1,6FucT plasmid. Each arrows indicates a different experiment in which IPTG was added to promote induction. The inserted table summarized the results obtained.

In all the cases the final density of the culture was the same, but the production of soluble enzyme per unit of biomass increased when the induction was done in the later logarithm phase (insert in Figure 3). When IPTG was added at the beginning of the stationary phase ($OD_{600} = 3.4$), the productivity per g of cells dropped, probably because some cells were already dead at the time of the induction. In this way, we were able to achieve a five-fold increase in the expression of soluble recombinant α-1,6-FucT obtaining a final production of 5 U of recombinant enzyme per liter of culture.

Enzymatic fucosylation of chitobiose (2): A crude preparation of the recombinant enzyme was used for the synthesis of the branched trisaccharide β-D-GlcNAc-(1→4)-[α-L-Fuc-(1→6)]-D-GlcNAc (**3**), from chitobiose (**2**) and GDP-Fucose (**1**) (Scheme 1). Samples were drawn at different times and analyzed by GC (Figure 4). After 24 h, 10% chitobiose remained in the reaction mixture (Figure 4, B). In spite of adding more fresh enzyme for another 24 h reaction time, no further consumption of chitobiose was observed. The reaction was then stopped and the mixture eluted through a Sephadex G-10 column to obtain the trisaccharide **3** as α,β-anomeric mixture in an 84% overall yield. The ^{1}H NMR spectrum of **3** showed two doublets at $\delta = 1.18$ and 1.19 assigned to the methyl group of the fucose residue (α- and β-anomers, respectively). The H-1 signal of the fucose was observed at $\delta = 4.90$ as a doublet with a small $J_{1,2}$ (4.2 Hz), which indicates α-configuration at the anomeric carbon. On the other hand, the ^{13}C NMR spectrum of **3** was in agreement with the previously reported data.[24]

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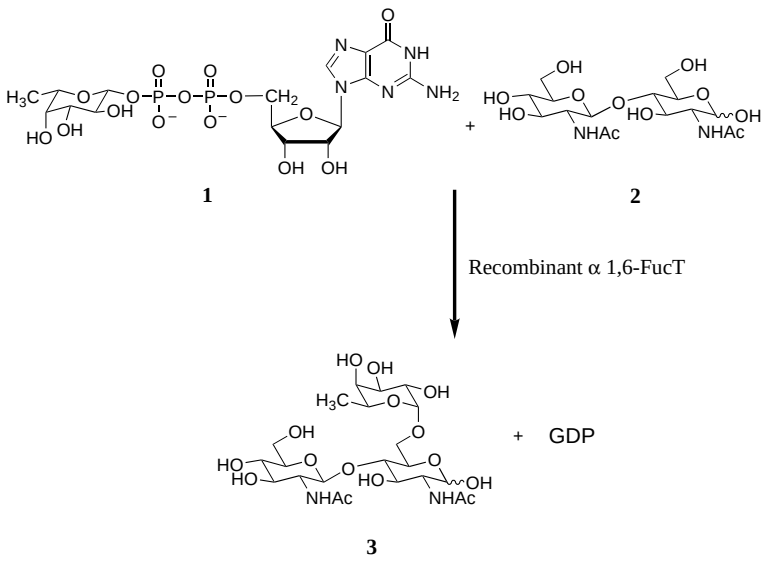


Scheme 1. Enzymatic fucosylation of *N*,*N*′-diacetylchitobiose using the recombinant α-1,6-FucT.

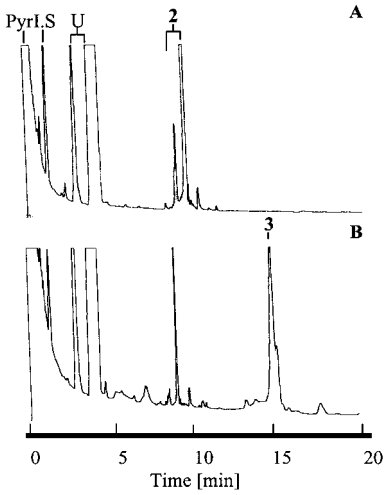


Figure 4. GC analysis of fucosylation-catalyzed reaction by the recombinant α-1,6-FucT. A) initial reaction mixture; B) reaction mixture after 24 h of reaction. Pyr: pyridine; I.S: benzyl β-D-xylopyranoside (internal standard); U: unidentified peaks (these two peaks were proved to come from the enzymatic extract); **2**: chitobiose (α, β mixture); **3**: trisaccharide.

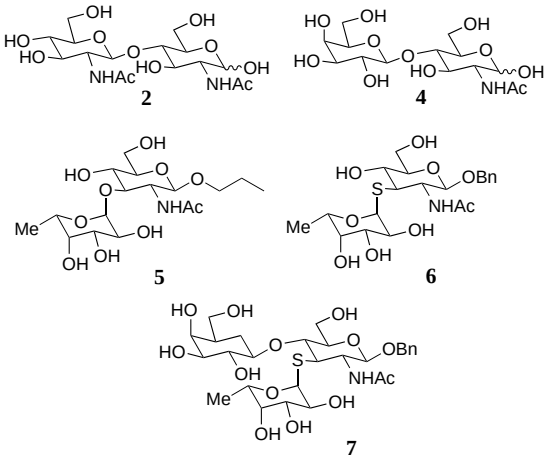
Acceptor specificity of α-1,6-FucT: In order to obtain information about the in vivo acceptor of the NodZ protein the acceptor specificity of the α-1,6-FucT from *Rhizobium* sp. and *B. japonicum* has been previously studied using chitin oligosaccharides of different length.[14, 15] Both enzymes give similar results, showing the highest reaction rates with hexa- and pentasaccharide oligomers. Quinto et al.[14] have also shown that the enzyme from *B. japonicum* is able to fucosylate the trisaccharide Lewis X, but at a very low reaction rate.

In order to study the acceptor specificity of the recombinant α-1,6-FucT in more detail, we measured the initial rate of GDP release in the presence of different mono-, di- and trisaccharides as acceptor, using chitobiose as a reference.[25] Table 1 summarizes the results using di- and trisaccharides as acceptors. The disaccharide *N*-acetyl-lactosamine (**4**), bearing a galactose residue instead of the terminal GlcNAc in chitobiose, was a good substrate ($V_{rel} = 56\,\%$), although only a 50% activity of the enzyme was observed. The disaccharide with an L-fucose residue at the C-3 position of the GlcNAc (**5**),[26] which in the presence of the enzyme and GDP-Fuc lead to the release of GDP, showed a $V_{rel} = 31\,\%$. Similar results were obtained with its thio-analogue **6**,[27] which is in agreement with the conformational study,[26] which concludes that the global three-dimensional shape of both compounds is fairly similar. On the other hand, the branched Lewis X thio-trisaccharide **7**[28] was not substrate for the enzyme probably a result of steric hindrance.

Table 1. Acceptor specificity of α-1,6-FucT for di- and trisaccharides.

Acceptor	V_{rel} [%][a]
2	100
4	56
5	31
6	36
7	0

[a] Relative reaction rate using 52 μM GDP-Fuc, 105 μM acceptor and 3.3 mU α-1,6-FucT.



The enzyme also showed activity in presence of the monosaccharide GlcNAc (**8**), although with a V_{rel} of 18%. This result differs from the results obtained by Quesada-Vincens et al.,[15] who reported that the α-1,6-FucT from *Rhizobium* sp. does not use GlcNAc as acceptor. This difference can be explained in terms of the enforced reaction conditions we used with larger amounts of enzyme.

In view that the recombinant enzyme works on the monomer, we then examined how the reaction rate is affect by different modifications in the GlcNAc structure (Table 2). We found that the enzyme activity was influenced signifi-

Table 2. Influence in the α-1,6-FucT activity of the substituent at the anomeric position of the GlcNAc.

Acceptor	V_{rel} [%] [a]
8	18
9	39
10	51
11	53
12	0

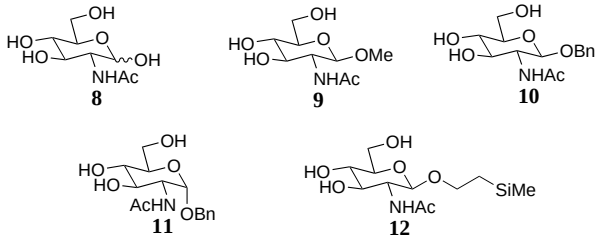
[a] Relative reaction rate using 52 μM GDP-Fuc, 105 μM acceptor and 3.3 mU α-1,6-FucT.

Table 3. Influence in the α-1,6-FucT activity of the NHAc group and C-4 configuration in the GlcNAc.

Acceptor	V_{rel} [%] [a]
8	18
13	18
14	9
15	12
16	9

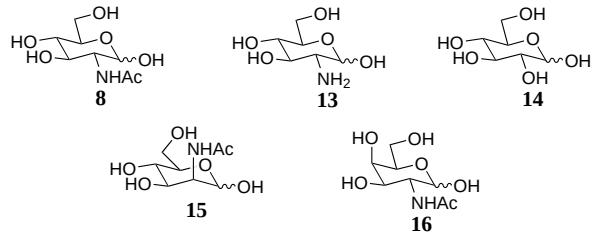
[a] Relative reaction rates using 52 μM GDP-Fuc, 105 μM acceptor and 3.3 mU α-1,6-FucT.

cantly by the substituent at the anomeric position of the GlcNAc. With the methyl glycoside **9** only a two-fold increase of activity was observed. The presence of an aromatic ring in the aglycon (compounds **10**[27b] and **11**) led to substrates with even higher activity than **9**. The influence of the aglycon nature became even more evident when the *N*-acetyl glucosamine derivative **12**, with the bulky Me_3Si group, was used



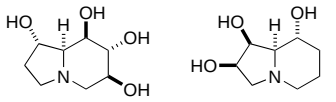
since this is not a substrate for the enzyme. Surprisingly, we have not found a significant difference between the β-**10** and the α-Bn-GlcNAc **11** ($V_{rel} = 51\,\%$ and $V_{rel} = 53\,\%$, respectively). By comparing the activity of GlcNAc (**8**) and chitobiose (**2**) it is possible to deduce that the presence of the GlcNAc moiety in the non-reducing end has a strong stabilizing effect on the enzyme – substrate complex. Although this effect is less important, it becomes significant, when the non-reducing GlcNAc is substituted by Gal.

We have also studied the influence of the NHAc group and the C-4 configuration in the reducing GlcNAc (Table 3) on the enzyme activity. Surprisingly, the acetamide group of the GlcNAc does not seem to be essential for the activity. Thus, the amino sugar **13** gave reaction rates similar to those of **8**. Some activity was also observed when the reaction was carried out with D-glucose (**14**) and the C-2 epimer, *N*-acetyl

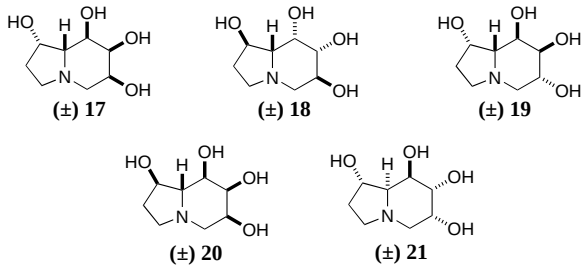


mannosamine (**15**), although at lower reaction rates. Finally, we have tested the effect of the configuration change from *gluco* to *galacto* using GalNAc (**16**) as the acceptor. The enzyme is able to use **16** but as a rather poor substrate ($V_{rel} = 9\,\%$), showing a important destabilizing effect of the equatorial OH group.

Inhibition studies with polyhydroxylated indolizidines: The reactions catalyzed by glycosyltransferases[29] and glycosidases[30] proceed through the cleavage of the bond between the anomeric carbon and the *exo*-anomeric oxygen atom, so that a positive charge at the anomeric carbon is formed during the process. Some naturally occurring polyhydroxylated indolizidines, such as castanospermine and swainsonine, are among



the most powerful glycosidase inhibitors. The interesting biological profile of these aza-sugars is said to be due to their ability to mimic the transition state of the glycosidase-catalyzed reaction.[31] Recently, Carretero et al. described an efficient and stereochemically flexible approach to the synthesis of polyhydroxylated indolizidines and analogues.[32] As several of those compounds showed inhibitory activity against commercially available glycosidases,[33] the racemic castanospermine stereoisomers **17** – **21**[34] were also investigated as possible inhibitors of the recombinant α-1,6-FucT (Table 4).



Although these structurally simple aza-sugars do not have any nucleotide or pyrophosphate moiety as commonly observed in glycosyltransferase inhibitors,[31b] we found that the indolizidines **17** – **20** showed a moderate inhibition of the recombinant α-1,6-FucT (IC_{50} from 1.67 to 3.37 mM), while the compound **21** proved to be a powerful inhibitor of this enzyme ($IC_{50} = 45$ μM). Interestingly, among the indolizidines

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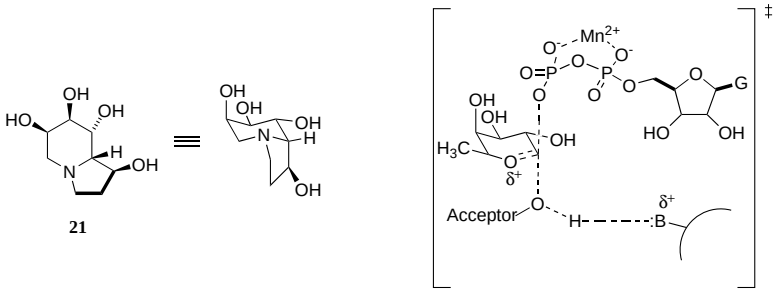
Table 4. Inhibition of α-1,6-FucT by castanospermine stereoisomers.

Inhibitor	IC_{50}[a] [mM]
(±) **17**	2.80
(±) **18**	2.35
(±) **19**	3.37
(±) **20**	1.67
(±) **21**	0.045

[a] Inhibitor concentration required to give 50% inhibition with 52 μM GDP-Fuc, 105 μM chitobiose at pH 7.7 and 25 °C.

studied, compound **21** is the only one with the same relative stereochemistry at the hydroxylic carbons as fucose. We speculated that this stereochemical analogy could be critical in order to attain a conformation[35] which mimics the fucose moiety in the putative transition state of the enzymatic reaction (Scheme 2). As far as we know these are the first examples showing that polyhydroxylated indolizidines can act as inhibitors both in glycosyltransferase-catalyzed processes and glycosidase-catalyzed reactions. These results open new potential applications of polyhydroxylated indolizidines and analogues as inhibitors in the biosynthesis of oligosaccharides.



Scheme 2. Conformation of indolizidine **21** which mimics the proposed transition state[29b] of the L-fucosyltransferase-catalyzed reaction.

Conclusion

In summary, we have developed an efficient heterologous expression system for the over-expression of the α-1,6-FucT from *Rhizobium* sp. We have shown the formation of inclusion bodies in vivo can be reduced by means of fermentation engineering. After optimisation of the expression conditions, we were able to obtain 5 units of soluble recombinant α-1,6-FucT per liter of culture. The broad acceptor specificity of recombinant enzyme makes it a useful catalyst for enzymatic oligosaccharide synthesis. Its applicability in the synthesis of oligosaccharides has also been shown by the synthesis of the trisaccharide **3**. Finally, we have shown that castarnospermine stereoisomers can act as inhibitors of this fucosyltransferase.

Experimental Section

General: ^{1}H NMR spectra were recorded on 400 MHz Inova-400 and 500 MHz Varian Unity spectrometers. ^{13}C NMR spectra were recorded at 100 MHz on 400 MHz Inova-400. TLC was performed on silica-gel plates (GF_{254} Merck) with fluorescent indicator and detection was carry out by charring with H_2SO_4/EtOH. UV/Visible spectra were recorded on a Perkin–Elmer Lambda 6 UV/VIS spectrophotometer at 25 °C. SDS-PAGE electrophoresis was performed in a Mighty-Small Mini-Vertical Electrophoresis Unit SE-250 (Hoefer Scientific Instruments). Protein concentration was determined using the Bio-Rad Protein Assay kit.

The pKK223-3 vector was obtained from Pharmacia Biotech. Inc.(Piscataway, NJ). Taq DNA polymerase was purchased from Ecogen. T4 DNA ligase was obtained from MBI Fermentas. Restriction enzymes *Eco* RI and *Hin*d III were purchased from Boehringer Manheim. Isopropyl-L-thio-β-D-galactopyranoside (IPTG) was purchased from Applichem. *Rhizobium* sp. NG234R strain was provided from the Microbiology Laboratory, ETSIA, Universidad Politécnica de Madrid (Spain). *E. coli* competent cells XL1-Blue MRF′ was purchased from Stratagene Co. (San Diego, CA).

Pyruvate kinase type II from rabbit muscle, L-lactic dehydrogenase type II from rabbit muscle, phosphoenol pyruvic acid (PEP), β-NADH, sodium lauryl sulfate, glycerol, polyethylene glycol (PEG), GlcNAc (**8**), $GlcNH_2$ (**13**), Glc (**14**), ManNAc (**15**) and GalNAc (**16**) were obtained from Sigma. *N*,*N*′-Diacetylchitobiose (**2**), *N*-acetyllactosamine (**4**) and benzyl 2-acetamide-2-deoxy-α-D-glucopyranoside (**11**) were obtained from Toronto Chemicals. GDP-β-L-fucose (**1**) was purchased from Oxford GlycoSciences. All other chemicals were purchased from commercial sources as reagent grade.

Amplification of α-1,6-fucosyltransferase gene: PCR amplification was performed in a 100 μL reaction mixture containing DNA (3 μL) of *Rhizobium* sp. as template, water (70.5 μL), buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.0, 10 μL), $MgCl_2$ (2 mM), dNTPs (200 μM), primers (1 μM) 1,6-FucT-Nt (5′GCCGCGAATTCATGTACAATCGATAT3′) and 1,6-FucT-Ct (5′GCCGCAAGCTTTCAAGAGGCGGTATT3′) and Taq DNA polymerase (2.5 U). The reaction was subjected to 30 cycles of amplification. The cycle conditions were set as follows: denaturation at 94 °C for 1 min, annealing at 55 °C for 1.5 min, and elongation at 72 °C for 1 min.

Construction of the pKK1,6 FucT vector: The α-1,6-fucosyltransferase gene obtained from the PCR was purified with the Wizard PCR Preps DNA purification system (Promega). The insert and the pKK223-3 plasmid were digested with *Eco* RI (100 U) and *Hin*d III (100 U) in 100 μL reaction mixtures following standard protocols.[36] After purification, the insert was ligated with the vector with T4 DNA ligase. The pKK1,6FucT expression vector constructed in this way was then transformed into *E. coli* XL1-Blue MRF′ competent cells and plated on LB agar plates containing 250 μg mL^{-1} ampicillin. Ten colonies were randomly selected and grown for screening of positive clones. The plasmid was purified by the Ultra Clean Mini Plasmid preparation kit (MoBio) and characterized by restriction analysis. One positive clone was selected and used for protein expression.

Expression of the recombinant α-1,6-FucT: The selected clone was grown on 100 mL LB medium containing 250 μg mL^{-1} ampicillin at 37 °C with shaking. When the cell growth reached an optical density at 600 nm ($O.D._{600}$) of 0.5, the temperature was switched to 30 °C and the culture was induced with 1 mM IPTG. Samples were taken at different times (3 h, 5 h and 24 h) after induction and the expression level was analyzed by SDS-PAGE using gels with 10% of polyacrylamide in the separation zone.

To study the influence of the induction time in the expression of the α-1,6-FucT in soluble form, the temperature was switched to 30 °C and 1 mM IPTG was added when the $O.D._{600}$ reached 0.14, 0.5, 2.0, and 3.4, respectively.

Preparation of cell-free extract (CFE): The culture broth was centrifuged (3000 g, 30 min, 4 °C), and the cell pellets were treated with lysozyme or with B-PER bacterial protein extraction reagent (Pierce) to separate the soluble proteins from the inclusion body.

a) *Lysozyme*:[37] EDTA (50 mM, pH 8.2) and lysozyme (2 mg g^{-1} cells) were added to a suspension of cells in Tris buffer (8 mL g^{-1} cells, 50 mM, pH 8.0).

The suspension was gently stirred at room temperature during 1 h, and the suspension was kept at 4 °C overnight. The reaction mixture was gently sonicated for 40 s and cooled down on ice (4 ×) to decrease viscosity. DNase (10 μg g^{-1} cells) and $MgCl_2$ (0.95 μg mL^{-1}) were added, and the mixture was refrigerated for 20 min. The mixture was then centrifuged for 30 min at 13000 g to separate the soluble proteins.

b) *B-PER* (*bacterial protein extraction reagent*): One gram of cells was suspended in 20 mL of B-PER reagent and stirred for 10 min. The mixture was then centrifuged for 15 min at 13000 g to separate the soluble proteins.

α-1,6-FucT activity assay: The enzymatic activity of α-1,6-FucT was assayed with a coupled enzymatic system, in which the decrease of NADH absorbance at 340 nm is directly proportional to the release of GDP during the fucosyltransferase-catalyzed reaction.[38] The activity was measured at 25 °C for 15 min in a final volume of 1 mL, containing Hepes (12 mM, pH 7.7), $MnCl_2$ (13 mM), KCl (50 mM), $MgCl_2$ (6.5 mM), PEP (0.7 mM), NADH (0.2 mM), pyruvate kinase (7.6 U), lactate dehydrogenase (18 U), chitobiose (105 μM), and GDP-Fucose (52 μM). The acceptor was omitted for the blank run. The assay was initiated upon addition of 50 μL of the α-1,6-FucT preparation and the decrease in the absorbance at 340 nm was monitored. For the study of the acceptor specificity chitobiose was substituted by compounds **4**–**16**. One unit of enzyme activity is defined as the amount that catalyzes the transfer of 1 μmol of fucose from GDP-Fuc to chitobiose per min.

Inhibition studies: Inhibition by polyhydroxylated indolizidines was studied using the activity assay described above. Compounds **17**–**21** were included in the reaction mixture before the addition of the α-1,6-FucT. Three different concentrations (0.01 mM, 0.1 mM, and 1.0 mM) of each compound were assayed to calculate the IC_{50}. Before performing these experiments, we proved that the polyhydroxylated indolizidines did not inhibited the pyruvate kinase or the lactate dehydrogenase.

Synthesis of β-D-GlcNAc(1→4)-[α-L-Fuc-(1→6)]-D-GlcNAc (3): Crude α-1,6-FucT (1.4 mL, 93 mU), prepared by method a) was added to a solution of *N*,*N*′-diacetyl chitobiose (6 mg, 0.014 mmol), GDP-Fucose (10 mg, 0.014 mmol), $MnCl_2$ (5 mg, 0.025 mmol) in Hepes (3 mL, 15 mM, pH 7.7). Aliquots (200 μL) were removed at different times and analyzed by GC. The samples were heated at 100 °C during 10 min to stop the reaction. After lyophilization, the residue was treated with pyridine (5 μL) containing benzyl β-D-xylopyranoside (1 mM) as internal standard, trimethylsilylimidazole (5 μL) and heating at 60 °C for 30 min. GC analysis was carried out on a Hewlett–Packard 5890 Series II, with an FID detector, using a SPB-1 capillary column (3 m, 0.25 mm ID, 0.25 μm film); temperature program: initial temperature 195 °C during 5 min; rate 15 °C min^{-1}; final temperature 260 °C. When the reaction was finished, the mixture was concentrated and passed through a Sephadex G-10 column using water as eluent. The fractions containing the trisaccharide were pooled out and dried to give **3** as a white powder (6.8 mg, 84 %). ^{1}H NMR (500 MHz, D_2O): $\delta = 5.16$ (d, $J(1,2) = 2.8$ Hz, <1H, H-1 α), 4.90 (d, $J(1'',2'') = 4.2$ Hz, 1H; H-1″), 4.71 (d, $J(1,2) = 8$ Hz, <1H; H-1 β), 4.62 (d, $J(1',2') = 8.0$ Hz, 1H; H-1′), 4.10 (m, 1H; H-5″), 2.06/2.01 (2s, 3H each; 2NHCOCH_3), 1.19/1.18 (2d, $J(5'',6'') = 6.6$ Hz, 3H; H-6″); ^{13}C NMR (100 MHz, D_2O): $\delta = 102.4$ (C-1′), 100.6/100.8 (C-1″), 96.2 (C-1 β), 91.7 (C-1 α), 23.1/23.4 (2NHCOCH_3), 16.5/16.5 (CH_3).

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