**Heterologous Over-expression of α 1,6-Fucosyltransferase
from *Rhizobium* sp. Application to the Synthesis of the
Trisaccharide β -D-GlcNAc(1 $\rightarrow$ 4) - [α -L-Fuc - (1 $\rightarrow$ 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 5 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 $\rightarrow$ 4)-[α -L-Fuc-(1 $\rightarrow$ 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 know the structural requirements for acceptors, the specificity of the enzyme was studied towards mono-, di- and trisaccharides structurally related to chitobiose. The enzyme is able to use, among others, the disaccharide *N*-acetyl lactosamine (**4**) as a good substrate and the monosaccharide GlcNAc (**8**) as a weak acceptor. Finally, several racemic polyhydroxylated indolizidines have been tested as potential inhibitors of the enzyme. The indolizidine **21** was the best inhibitor with an IC_{50} of 4.5×10^{-5} M and, interestingly, is the one that could better mimic 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 as their poor in vivo bioavailability, weakness of the protein-carbohydrate interactions and the 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 chemist mainly because their strict control over the stereo- and regioselectivity of the glycosidic bond formed, that have 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. This sugar-nucleotide is too expensive to be used in stoichiometric amount 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 glycosil donor.^[7] The other major drawback, that has not yet been solved, is the limited

availability of these enzymes. To our knowledge, only nine glycosyltransferases are commercially available and of these, 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, and they are it in amounts of 0.1 units. One alternative to overcome this problem is the cloning and over-expression of bacterial glycosyltransferases.^[8] Many bacterial glycosyltransferases are able to produce mammalian-like structures. Also, in microorganisms it is possible to find transferases with specificities not yet found in mammalian enzymes.^[9] On the other hand, bacterial proteins are expressed better in prokaryotic systems than those of mammals, 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 at high density on inexpensive substrates.^[10]

The recent efforts to sequence the complete genome of several microorganisms are making available a great number of genes putatively codifying for glycosyltransferases. In this sense, it is known that the rhizobia gene *nodZ* play a role in fucosylating the lipochitin oligosaccharide (LCO) signals 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 *nodz* genes from *Azorhizobium*

caulinodans,^[11c] *Bradyrhizobium japonicum*^[11a] and *Rhizobium* sp. NGR234^[13] have been cloned and sequenced, and the enzymes from *B. japonicum*^[14] and *Rhizobium* sp.^[15] have been identified as fucosyltransferases in an in vitro transfucosylation assay. In mammals, α 1,6-FucT catalyzes the transfer of fucose from GDP-Fuc to asparagine-linked GlcNAc of *N*-linked type complex glycoproteins and the enzymes from porcine^[16] and human^[17] have been cloned. 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 expressed in both hepatoma tissues and their surrounding tissues with chronic liver disease, but not in the case of normal 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**) whose structure has been elucidated by ¹H and ¹³C 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).

Results and Discussion

Cloning and expression of α 1,6-Fucosyltransferase.

The *nodZ* gene from *Rhizobium* sp. NGR234 was amplified by PCR using primers designed to specifically complement 15 bp at the 5' extremes of codifying and complementary DNA strains. The recognition sequence for the restriction enzymes Eco RI and Hind III was introduced in the amplification product during the PCR. The PCR amplification was quite specific, and only one band with the expected length (969bp) 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. Inc.) 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 Co.) and plated on LB-ampicillin plates. The presence of the *nodZ* 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 μ g/mL ampicillin. Since in the plasmid pKK1,6FucT, the expression of the recombinant enzyme 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 of 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 an usual problem when a protein is over-expressed, since the high production of protein does not allow its correct folding, causing its aggregation and precipitation. We tried to refold the inclusion bodies solubilizing the pellet with 8 M of urea^[20] or 6 M of guanidine, followed by a slow elimination of the denaturant agent by dialysis against buffer containing or not different folding aids.^[21] The encountered problems for the in vitro refolding of the inclusion bodies may be due to the presence of a 20 aminoacid fragment in the protein, near the C-terminal, that 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 slow the bacterial metabolism through fermentation engineering.^[23] We envisioned that the production rate of recombinant protein could be modulated by the moment 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). 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

(O.D.₆₀₀ = 3.4), the productivity per g of cells dropped, probably because some cells were already dead at the moment of the induction. In this way, we have been able to increase five times 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 $\rightarrow$ 4)-[α -L-Fuc-(1 $\rightarrow$ 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, a 10% of chitobiose remained in the reaction mixture (Figure 4, B). In spite of adding more fresh enzyme allowing the reaction to continue for 24 h, 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 a 84% overall yield. The ^1H NMR spectrum of **3** showed two doublets at 1.18 and 1.19 ppm assigned to the methyl group of the fucose residue (for the α - and β -anomers, respectively). The H-1 signal of the fucose was observed at 4.90 ppm as a doublet with a small $J_{1,2}$ (4.2 Hz), which was indicative of the α -configuration at the anomeric carbon. On the other hand, the ^{13}C NMR spectrum of **3** was in agreement with the previously reported one.^[24]

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]} The behaviour of both enzymes is similar, 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 very low reaction rate.

In order to study in more detail the acceptor specificity of the recombinant α 1,6-FucT, we have measured the initial rate of GDP release in the presence of different mono-, di- and trisaccharides as acceptor, using chitobiose as reference.^[25] Table 1 summarizes the results using di- and trisaccharides as acceptor. 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 the enzyme exhibited half of activity. The disaccharide having a L-fucose residue at the C-3 position of the GlcNAc (**5**),^[26] which in presence of the enzyme and GDP-Fuc lead to the release of GDP, showed a $V_{rel} = 31\%$. Similar result was obtained with its thio-analog **6**,^[27] which is in agreement with their conformational study,^[26] that 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 due to steric hindrance.

The enzyme also showed activity in presence of the monosaccharide GlcNAc (**8**), although with a V_{rel} of 18%. This

result differs from the 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 explain because in our case we have forced the reaction conditions using a higher amount of enzyme.

In view that the recombinant enzyme works on the monomer, we then examined how can affect to the reaction rate different modifications in the GlcNAc structure (Table 2). We found that the enzyme activity was rather influenced by the substituent at the anomeric position of the GlcNAc. With the methyl glycoside **9** there was a two-fold increase of activity. 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 was more evident when the *N*-acetyl glucosaminide derivative **12**, having a bulky Me₃Si group, was assayed since it was not substrate for the enzyme. Surprisingly, we have not found a significant difference between the β - (**10**) and the α -BnGlcNAc (**11**) ($V_{rel} = 51\%$ and $V_{rel} = 53\%$ respectively). From the comparison of the activity with GlcNAc **8** and with chitobiose **2** is possible to deduce that the presence of the GlcNAc moiety in the non-reducing end has a strong stabilizing effect in the complex enzyme-substrate. This effect is lesser, but already significant, when the non-reducing GlcNAc is substituted by Gal.

We have also studied the influence in the enzyme activity of the NHAc group and of the C-4 configuration in the reducing GlcNAc (Table 3). To our surprise, the acetamide group of the GlcNAc does not seem to be essential for

activity. Thus, the amino sugar **13** gave reaction rate similar to that of **8**. Some activity was also observed when the reaction was carried out with D-glucose **14** and the C-2 epimer **15**, although at lower reaction rates. Finally, we have tested the effect of the configuration change from *gluco* to *galacto* using GalNHAc (**16**) as acceptor. The enzyme is able to use **16** as a poor substrate ($V_{rel} = 9\%$), showing a important destabilizing effect of the ecuatorial OH group.

Inhibition studies with polyhydroxylated indolizidines.

The reactions catalyzed by glycosyltransferases^[29] and glycosidases^[30] have in common that both 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 developed during the process. Some naturally occurring polyhydroxylated indolizidines, such as castanospermine and swainsonine (Scheme 2), are among the most powerful glycosidase inhibitors, being widely assumed that the interesting biological profile of these aza-sugars is due to their ability to mimic the transition state of the glycosidase-catalyzed reaction.^[31] Recently, Carretero et al. described an efficient and stereoechemically flexible approach to the synthesis of polyhydroxylated indolizidines and analogues.^[32] As several of these compounds showed inhibitory activity against commercially available glycosidases,^[33] the racemic castanospermine stereoisomers **17-21**^[34] were also assayed 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 it is usual 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 μ M).

Interestingly, among the indolizidines assayed, the **21** is the only one with the same relative stereochemistry at the hydroxylic carbons than fucose. We speculated that this stereochemical analogy could be critical in order to attain a conformation^[35] that mimics the fucose moiety in the putative transition state of the enzymatic reaction (Scheme 3). As far as we know these are the first examples showing that polyhydroxylated indolizidines can act as inhibitors in glycosyltransferase-catalyzed processes, and not only in glycosidase-catalyzed reactions. These results open new potential applications of polyhydroxylated indolizidines and analogs as inhibitors in the biosynthesis of oligosaccharides.

Conclusions

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 how can be reduced the formation of inclusion bodies in vivo, 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 to synthesis of oligosaccharides has been also shown by the synthesis of the trisaccharide **3**. Finally, we have shown that castanospermine stereoisomers can act as inhibitors of this fucosyltransferase.

Experimental Section

General: ^1H 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 (GF₂₅₄ Merck) with fluorescent indicator and detection was carry out by charring with $\text{H}_2\text{SO}_4/\text{EtOH}$. 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.

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 Hind III were purchased from Boehringer Mannheim. 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), Triton X-100, Tween 20, GlcNAc (**8**), GlcNH₂ (**13**), Glc (**14**), MannNAc (**15**) and GalNAc (**16**) were obtained from Sigma. *N,N'*-Diacetylchitobiose **2**, *N*-Acetylglucosamine **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 μ L reaction mixture containing DNA (3 μ L) of *Rhizobium* sp. as template, water (70.5 μ L), buffer (100 mM Tris-HCL, 500 mM KCl, pH 8.0, 10 μ L), MgCl₂ (2 mM), dNTPs (200 μ M), primers (1 μ M) 1,6FucT-Nt (5'GCCGCGAATTCATGTACAATCGATAT3') and 1,6FucT-Ct (5'GCCGCAAGCTTTCAAGAGGCGGTATT3') and Taq DNA polymerase (2.5 U). The reaction was subjected to 30 cycles of amplifications. 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 pKK1,6FucT 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 Hind III (100 U) in 100 μ L 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 $250 \mu\text{g} \times \text{mL}^{-1}$ ampicillin. Ten colonies were randomly selected and grown up for screening of positive clones. The plasmid were 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 up on 100 mL of LB medium containing $250 \mu\text{g} \times \text{mL}^{-1}$ 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 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 the 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 ($3,000 \times g$, 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] To a suspension of cells in Tris buffer (8 mL/g cells, 50 mM pH 8.0) were added EDTA (50 mM, pH 8.2) and

lysozyme (2 mg/g cells). The suspension was gently stirred at room temperature during 1 hour, and the suspension was kept at 4 °C overnight. The preparation was gently sonicated for 40 s and cooled down in ice (4 times) to decrease viscosity. DNase (10 µg/g cells) and MgCl₂ (0.95 µg/mL of preparation) were added, and the mixture was refrigerated for 20 min. The mixture was then centrifuged for 30 min at 13,000 x g to separate the soluble proteins from the insoluble ones.

b) *B-PER™ Bacterial Protein Extraction Reagent*. One g 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 13,000 x g to separate the soluble from the insoluble proteins.

α 1,6-FucT activity assay: The enzymatic activity of α 1,6-FucT was assayed with a coupled enzymatic system, where 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), MnCl₂ (13 mM), ClK (50 mM), MgCl₂ (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). Acceptor was omitted for the blank. 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 μ mol 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} . Previously, was proved that the polyhydroxylated indolizidines did not inhibited the pyruvate kinase or the lactate dehydrogenase.

Synthesis of β -D-GlcNAc (1 $\rightarrow$ 4) - [α -L-Fuc- (1 $\rightarrow$ 6)] -D-GlcNAc (3) :

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), α 1,6-FucT crude preparation (1.4 mL, 93 mU) was added. Aliquots (200 μ l) were removed at different times to be analyzed by GC. The samples were heated at 100 $^{\circ}C$ during 10 min to stop the reaction. After lyophilization, the residue was treated with pyridine (5 μ l) containing benzyl β -D-xylopyranoside (1 mM) as internal standard, trimethylsilylimidazole (5 μ l) and heating at 60 $^{\circ}C$ for 30 minutes. GC analysis was carried out on a chromatograph Hewlett Packard 5890 Series II, with FID detector, using a SPB-1 capillary column (3 m, 0.25 mm id, and 0.25 μ m film); temperature program: initial temperature 195 $^{\circ}C$ during 5 min; rate 15 $^{\circ}C$ /min; final temperature 260 $^{\circ}C$. 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% yield). ^1H NMR (500 MHz, D_2O): δ = 1.18, 1.19 (2d, J (5'', 6'') = 6.6 Hz, 3H; H-6''), 2.01, 2.06 (2s, 3H each; 2 NHCOCH_3), 4.10 (m, 1H; H-5''), 4.62 (d, J (1', 2') = 8.0 Hz, 1H; H-1'), 4.71 (d, J (1, 2) = 8 Hz, <1H; H-1 β), 4.90 (d, J (1'', 2'') = 4.2 Hz, 1H; H-1''), 5.16 (d, J (1, 2) = 2.8 Hz, <1H, H-1 α). ^{13}C NMR (100 MHz, D_2O): δ = 16.5, 16.5 (CH_3), 23.1, 23.4 (2 NHCOCH_3), 91.7 (C-1 α), 96.2 (C-1 β), 100.6, 100.8 (C-1''), 102.4 (C-1').

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Legends of the Figures

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

Scheme 2. Conformation of indolizidine **21** that could mimic the proposed transition state^[29b] of the L-fucosyltransferase-catalyzed reaction.

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: 1,6FucT-Nt (5'GCCGCGAATTCATGTACAATCGATAT3') and 1,6FucT-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.

Figure 3. Growth curve for *E. coli* cells carrying on the pKK1,6FucT plasmid. Arrows indicate when the induction was done. The inserted table summarized the results obtained.

Figure 4. GC analysis of fucosylation-catalyzed 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.

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 $\rightarrow$ 4)-[α -L-Fuc-(1 $\rightarrow$ 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 IC₅₀ 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.

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 velocities with 52 μ M GDP-Fuc, 105 μ M acceptor and 3.3 mU of α 1,6-FucT

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 velocities with 52 μ M GDP-Fuc, 105 μ M acceptor and 3.3 mU of 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 velocities with 52 μ M GDP-Fuc, 105 μ M acceptor and 3.3 mU of α 1,6-FucT

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 μ M GDP-Fuc, 105 μ M chitobiose at pH 7.7 and 25 °C.

Scheme 1

Scheme 2

Figure 1

Figure 3

Figure 4

Graphical Abstract

The enzyme α 1,6-Fucosyltransferase from *Rhizobium* sp. has been overexpressed. The recombinant enzyme has been used for the synthesis of the trisaccharide (**3**). The specificity of the enzyme has been studied towards carbohydrates structurally related to chitobiose. The broad acceptor specificity of recombinant enzyme makes it a useful catalyst for enzymatic oligosaccharide synthesis. Finally, we have shown for the first time that castanospermine stereoisomers can act as inhibitors in glycosyltransferase-catalyzed processes.