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PAPER

Synthesis and evaluation of xylopyranoside derivatives as
“decoy acceptors” of human β -1,4-galactosyltransferase 7†Juan Francisco García-García,^a Guillermo Corrales,^a Josefina Casas,^b
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Proteoglycans (PGs), including heparan sulfate forms, are important regulators of tumor progression. In the PGs biosynthetic process, the core protein is synthesized on a ribosomal template and the sugar chains are assembled post-translationally, one sugar at a time, starting with the linkage of xylose to a serine residue of the core protein and followed by galactosidation of the xylosylprotein. Hydrophobic xylopyranosides have been previously shown to prime heparan sulfate synthesis, a property that was required to cause growth inhibition of tumor cells. To know if the antiproliferative activity of synthetic xylopyranosides is related to their ability to act as “decoy acceptors” of xylosylprotein 4- β -galactosyltransferase, we have heterologously expressed the catalytic domain of the human β -1,4-GalT 7 and studied the ability of a variety of synthetic xylopyranoside derivatives to act as substrates or inhibitors of the recombinant enzyme.

Introduction

Proteoglycans are highly active and complex biomolecules involved in a wide range of physiological and pathological processes through their interactions with several proteins.¹ They are composed of a core protein usually attached to the cell membrane and one or more glycosaminoglycan (GAG) chains attached at specific Ser residues of the core protein. GAGs are linear polysaccharides composed of alternating *N*-acetylated or *N*-sulfated glucosamine units and either glucuronic or iduronic acid. The GAG chains are linked to the protein core by a tetrasaccharide linker that is shared by all proteoglycans. The assembly of this tetrasaccharide is carried out in the Golgi and began with the transfer of a xylose residue to a specific Ser of the protein chain, catalysed by a xylosyltransferase and is continued by the sequential action of specific glycosyltransferases (Scheme 1).²

It is known for more than 30 years that β -D-xylopyranoside with hydrophobic aglycones can act as primers for GAG

Scheme 1 Biosynthesis of the proteoglycan tetrasaccharide primer.

biosynthesis and thereby inhibit the assembly of glycosaminoglycan chains on endogenous proteoglycan core proteins.³ Okayama *et al.*^{3a} named these compounds, that being substrates for a particular enzyme are able to inhibit a metabolic pathway, “decoy acceptors” to distinguish them from inhibitors, which are compounds that block the enzyme without being modified by it. This concept has been also applied to suppress the synthesis of the mucin chain in mucoproteins,⁴ to down-regulate the expression of sLex ligands⁵ or to restore the streptomycin antibiotic activity against aminoglycoside resistant *Escherichia coli* strains.⁶

Synthesis of GAG chain can be initiated by β -D-xylosides by joining the intracellular enzyme machinery at the second glycosyltransferase step (galactosyltransferase I, Scheme 1) and their ability to prime the assembly of GAG chains and their composition depends on the structure of the aglycone moiety.⁷ In most cases, xylosides prime chondroitin sulfate/dermatan sulfate (CS/DS) efficiently and heparan sulfate (HS) only weakly.^{7b} Xylosides containing polycyclic structures, such as naphthol-derivatives, will prime a mixture of both HS and CS/DS chains.^{7b,8} The xyloside-primed GAG chains

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† Electronic supplementary information (ESI) available: Cloning and purification of enzyme β -1,4-GalT 7, experimental details and Table S1 containing data of antiproliferative activity of xylopyranoside derivatives **4**, **7**, **8**, **9**, **13**, **14** and peracetates **24** and **25**. See DOI: 10.1039/c0mb00206b

can be retained inside the cells but in general are secreted into the medium in a nearly quantitative way, since the small hydrophobic aglycones are not able to tie the chains to cell membranes.

Therefore, xylopyranoside derivatives may be used to interfere with the proteoglycans biosynthesis and thus modulate various cellular activities, such as cell growth. In this sense, Esko and co-workers have shown that GAG-priming xylosides render tumor cells more sensitive to the cytostatic effects of α -difluoromethylornithine (DFMO), an inhibitor of *de novo* polyamine biosynthesis.⁹ Mani and co-workers, in a series of elegant papers,^{8,10} have shown that 2-(6-hydroxy-naphthyl) β -D-xylopyranoside (**14**) selectively inhibits the proliferation of transformed or tumor-derived cells both *in vitro* and *in vivo*. Also, treatment with this xyloside reduced the average tumor load by 70–97% in a SCID mice model.

The ability of the xylosides to prime the assembly of GAG chains—and hence their potential activity as antitumor agents—must be related to their ability to act as acceptors of the xylosylprotein 4- β -galactosyltransferase (β -1,4-GalT 7 in Scheme 1). Herein, we describe the heterologous expression in *E. coli* of a truncated form of the human β -1,4-GalT 7 and its biochemical characterization. Also, we report the synthesis of a family of xylopyranoside derivatives and the study of their ability to act as substrates or inhibitors of the recombinant β -1,4-GalT 7.

Results and discussion

Heterologous expression of a soluble form of human β -1,4-GalT 7 and its biochemical characterization

Galactosyltransferase I or β -1,4-GalT 7 (UDP-galactose:O- β -D-xylosylprotein 4- β -D-galactosyltransferase, EC 2.4.1.133) is involved in the synthesis of the common glycosaminoglycan-protein linkage region. In particular, it catalyzes the first galactosylation step of the xylose bound to the core protein of proteoglycans (Scheme 1). The gene codifying for this enzyme in humans was identified almost simultaneously by Almeida *et al.*¹¹ and Okajima *et al.*¹² Human β -1,4-GalT 7 is a Type II membrane protein. These proteins share a common structure comprised of a short N-terminal cytoplasmic tail, a membrane spanning region followed by a luminal stem region and a large C-terminal catalytic domain (Fig. 1).¹³

To facilitate general studies on enzymes, large-scale production systems are needed.¹⁴ Prokaryotic expression systems, and in particular *E. coli*, are the most attractive ones for large scale production of recombinant proteins because of their ability to grow rapidly and at high density on inexpensive substrates.¹⁵ However, when a heterologous protein is over-expressed in *E. coli* misfolding and aggregation happen frequently, driving the recombinant protein into inactive aggregates known as inclusion bodies. These limitations are especially important for eukaryotic proteins that require post-translational modifications such as glycosylation. A myriad of strategies have been developed in the last several years to increase the yield of soluble mammalian recombinant protein using *E. coli* as an expression host. Some of these strategies are: reduction of the recombinant protein production rate;¹⁶

Fig. 1 Schematic representation of the human β -1,4-GalT 7 topology.

increasing the cellular levels of molecular chaperones;¹⁷ to fuse the recombinant protein to a soluble fusion tag.¹⁸

Using the latter approach, soluble human β -1,4-GalT 7 has been lately expressed in *E. coli*. Lattard and co-workers expressed a truncated form of the enzyme—lacking the first 81 N-terminal amino acids that include the transmembrane and stem regions—fused with the MBP.¹⁹ On the other hand, Qasba and co-workers expressed the human β -1,4-GalT 7 using as fusion partner the human lectin galectin-1.²⁰ Very recently, Fournel-Gigleux and co-workers have reported the production of the wild-type β -1,4-GalT 7 and some mutants as truncated fusion proteins (lacking the 60 N-terminal amino acids) linked to GST.³⁰ These authors also have identified two functional regions critical for the organization of UDP-Gal binding. They also demonstrated the central role of Trp224 in governing interactions with both donor and acceptor substrates.

Several examples in the literature reported the soluble expression of the catalytic domain of glycosyltransferases (GT's).²¹ Since the human β -1,4-GalT 7 presents only one potential glycosylation site (Asn154), we decided to express the soluble domain of the protein (Fig. 1) in *E. coli* fused to a six His tag to allow its purification by immobilized metal affinity chromatography (IMAC). We took advantage of the presence of a unique recognition sequence for the endonuclease *Pst*I that allowed to cut between the aminoacids 49–50 of the enzyme and therefore to remove the transmembrane region and the cytoplasmic tail (for experimental details see ESI†).

Comparison of the graphic plots of Kyte–Doolittle hydrophobicity profiles of wild-type and truncated β -1,4-GalT 7 clearly shows that the transmembrane region had been eliminated (Fig. 2). Peptide mass fingerprinting from the SDS-PAGE band verified that the purified protein had the β -1,4-GalT 7 expected features. Nine peptides covering the major part of the amino acid sequence of the soluble domain were identified (Fig. 3). Almost all the predicted tryptic peptides with molecular masses falling in the analyzed m/z range were found in the peptide mass fingerprint of the β -1,4-GalT 7 soluble domain. Other unassigned peptides were identified using the FindMod tool.²² Thus, the peptide with $m/z = 1800.9094$ (dark gray in Fig. 3) was assigned to the

Fig. 2 Kyte–Doolittle hydropathicity profile of (A) wild-type human β -1,4-GalT 7 and (B) the truncated form of the enzyme.

Fig. 3 Peptide mass fingerprint of β -1,4-GalT 7 soluble domain incorporating a N-t His tag. The sequences of the identified peptides are shaded and underlined. Molecular mass of each peptide is indicated in Da.

peptide ERFEELLVFVPHMR included in the sequence of the soluble domain.

The activity of the β -1,4-GalT 7 soluble domain was initially assayed using UDP-Gal as the glycosyl donor and *p*-nitrophenyl- β -D-xylopyranoside (*p*-NO₂Ph- β -D-Xyl; **1**) as the acceptor (Scheme 2). The time course of the reaction was followed by HPLC. The formation of a new peak with a retention time of 10.2 min was observed. This peak showed a m/z of 432.1 ($M - H^+$), matching the mass of disaccharide **2**.

The recombinant β -1,4-GalT 7 soluble domain showed a Michaelis–Menten kinetic for *p*-NO₂Ph- β -D-xyl. The apparent K_M value (at saturating concentration of UDP-Gal) was 0.59 mM with an apparent k_{cat} of 49.8 s^{−1} (Table 1).

The behavior observed towards UDP-Gal was more complex since the soluble domain of the β -1,4-GalT 7 showed excess-substrate inhibition from a donor concentration of 1.25 mM (Fig. 4). This excess-substrate inhibition has been also described by Qasba and co-workers for the galectin1- β 4GalT7 fusion protein.²⁰ The calculated k_{is} for UDP-Gal was 21.1 mM.

Scheme 2

Table 1 Summary of the kinetic constants of the recombinant β -1,4-GalT 7 soluble domain

Substrate	K_M /mM	k_{cat} /s ^{−1}	k_{cat}/K_M s ^{−1} M ^{−1}	k_{is} /mM
<i>p</i> -NO ₂ Ph- β -D-Xyl (1) ^a	0.59	49.8	8.5×10^4	—
UDP-Gal ^b	0.1	51.4	55.6×10^4	21.1

^a Kinetic parameters obtained from fits to Michaelis–Menten eqn (1).

^b Kinetic parameters obtained from fits to eqn (2).

Fig. 4 Michaelis–Menten representation of the β -1,4-GalT 7 soluble domain activity (V) as a function of the UDP-Gal concentration, where the inhibition by substrate excess can be observed.

The K_M value obtained towards UDP-Gal for the soluble domain of the β -1,4-GalT 7, was comparable, although somewhat lower, to the data reported by Lattard and co-workers¹⁹ for the MBP- β 4GalT7 fusion protein (0.23 mM) and by Fournel-Gigleux and co-workers³⁰ for the GST- β -1,4-GalT7 (0.28 mM). However, the k_{cat} value obtained for the soluble domain was about 30-fold higher than that reported by Lattard and co-workers (Fournel-Gigleux *et al.* used 4-methylumbelliferyl- β -D-xylopyranoside as acceptor). These differences observed in the K_M and the k_{cat} values could be due to an entropy loss of the active site in the fused MBP- β 4GalT7 enzyme. Besides, it is necessary to have in mind that the reported data are apparent constants and it cannot be discarded that the differences observed may be due to the different conditions in which these values have been calculated in both works.

Synthesis of xylopyranoside derivatives

In Chart 1 the different xylose derivatives **3–14** used for the enzymatic study are shown.

A group of compounds containing a common *N*-(*O*-xylopyranosyl)-hydroxylpropylamide moiety with a variable acyl group (compounds **8–12**) was selected to compare the effect of aliphatic or aromatic groups on the activity of the xyloside as decoy acceptor. Acyl groups containing a carboxyl (**10** and **12**) were also included, since it is known that acidic amino acid residues are usually present near the glycosylation site of both HS and CS/DS.²³

Compounds **5–7** were obtained as previously described by one of us²⁴ and compound **14** was obtained following the procedure described by Mani *et al.*⁸

Chart 1

The synthesis of amides **8–12** was carried out from a common amine intermediate **17**, which was prepared by glycosidation of xylose tetracetate **15** with 3-hydroxypropionitrile followed by hydrogenation of the nitrile group (Scheme 3).

The reaction of **17** with different acylating reagents gave the corresponding amides **18–22** that were subsequently *O*-deacetylated to afford the targets **8–12**. For the synthesis of glycosyl amino acid **13**, xylose peracetate **15** was reacted with protected L-serine derivative **23** in the presence of TMSOTf, to give stereoselectively the β -glycoside **24** in 75% yield (Scheme 3). Subsequent *O*-deacetylation under mild conditions (KCN/MeOH) furnished **13**.

Study of the activity of the xylopyranoside derivatives as decoy acceptors of the recombinant soluble domain of the β -1,4-GalT 7

The activity as decoy acceptors of the xylopyranoside derivatives was evaluated by comparison of the catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) showed by the recombinant soluble domain of the β -1,4-GalT 7 towards the different xylosides. Since these xylopyranosides mimic the natural acceptor of the β -1,4-GalT 7, they may compete with it and act also as inhibitors of the enzyme. Therefore, another way to evaluate

Scheme 3

the activity of these compounds as decoy acceptors is to measure their ability to inhibit the formation of the natural reaction product. In our case, we evaluated their ability to inhibit (IC_{50}) the formation of **2** by the recombinant enzyme (Fig. 5).

The 2-(6-hydroxynaphthyl) β -D-xylopyranoside (**14**) was included in this study for comparative purposes. The results are summarized in Table 2.

As it has been commented above, Mani and co-workers⁸ have demonstrated that compound **14** is able to prime glycosaminoglycan synthesis. As expected, **14** was the substrate of the recombinant enzyme. However, like in the case of the UDP-Gal, **14** showed a strong inhibition by substrate excess ($k_{\text{is}} = 0.38 \text{ mM}$). Therefore, the kinetic parameters were obtained from fit data to eqn (2) (see Experimental). The calculated catalytic efficiency was slightly lower than with acceptor **1**. This difference can be attributed to the lower k_{cat} showed by **14**. According to the proposed hypothesis, since

Fig. 5 If the xylopyranoside derivative is a good substrate for the enzyme, then it can act as a decoy acceptor sequestering the enzyme activity and thus preventing the formation of the natural product. In this study, we have evaluated the ability of the xylosides **3–14** to prevent the formation of **2**.

Table 2 Evaluation of xylosides **3–14** as decoy acceptors of the recombinant human β -1,4-GalT 7^a

	K_M/mM	$k_{\text{cat}}/\text{s}^{-1}$	$k_{\text{cat}}/K_M/\text{s}^{-1} \text{mM}^{-1}$	IC_{50}/mM
14 ^{b,c}	0.58	30.2	52.1	0.30
3	N.D	N.D	N.D	> 20
4	N.D	N.D	N.D	> 20
5	0.37	60.7	164.0	0.95
6	0.28	57.6	205.7	1.26
7	0.74	87.4	118.1	0.52
8	0.75	41.0	54.7	3.69
9	0.75	64.3	85.7	2.46
10	0.56	47.2	84.3	3.96
11	0.53	78.7	148.5	1.04
12	0.40	106.3	265.7	0.61
13 ^c	0.53	152.0	286.8	2.39

^a All xylopyranosides have β configuration except for compound **3**, which was an α and β anomeric mixture. ^b Kinetic parameters obtained from fits to eqn (2). ^c Reaction contains 10% of DMSO.

xylopyranoside **14** is a good substrate for the enzyme, it can act as a decoy acceptor sequestering the enzyme activity and thus preventing the formation of **2**. In fact, **14** was able to inhibit the formation of **2** with an IC_{50} of 0.3 mM (Table 2). Although the IC_{50} value does not indicate the type of inhibition promoted by **14**, it seems clear that in this context the inhibition must be competitive. To substantiate this fact, we calculated the k_i for **14**. As expected, the data fit to eqn (3) that describes the competitive inhibition. The calculated k_i value (0.14 mM) was significantly lower than the IC_{50} value.

With respect to xylopyranosides **3–13**, only those lacking hydrophobic aglycone (compounds **3** and **4**) showed no activity with the soluble domain of the β -1,4-GalT 7. Their kinetic parameters were not calculated since the transglycosylation rates were in both cases lower than the 5% of the reaction rate of the recombinant enzyme with $p\text{-NO}_2\text{Ph-}\beta\text{-D-Xyl}$ (**1**) as acceptor. Moreover, they showed no inhibition even at concentration as high as 20 mM. All other xylosides were both substrates and inhibitors of the recombinant enzyme. The xyloside that showed the highest catalytic efficiency was the xylopyranosyl-serine derivative **13**. This result was not surprising since **13** was designed to mimic the natural acceptor of the enzyme, that is, a xylose bound to a serine of the protein core. Thus, the k_{cat}/K_M value for **13** was more than 5-folds higher than the k_{cat}/K_M for xyloside **14**, which showed the lowest value. Interestingly, the *S*-glycoside **7**, in which a sulfur atom has replaced the glycosidic oxygen atom, was a good

substrate for the recombinant enzyme with a k_{cat}/K_M of $118.1 \text{ s}^{-1} \text{mM}^{-1}$. This result is in agreement with earlier observations by Sobue *et al.* on the ability of phenyl β -D-thioxylopyranoside (**7**) to prime the synthesis of chondroitin sulfate chains.²⁵ *S*-Glycosides are interesting compounds since they are more stable against enzymatic cleavage than their *O*-glycoside counterparts²⁶ and, therefore, their bioavailability must be also higher.

Study of the antiproliferative activity of the xylopyranoside derivatives

To test if there is a relationship between the catalytic efficiency of the recombinant β -1,4-GalT 7 with the different xylopyranosides and their antiproliferative activity, we assayed selected xylosides against the human lung carcinoma cell line A 549. Xyloside **7** was chosen for its characteristic *S*-glycoside group, aliphatic xylosides **8** and **9** were selected as representatives of amide containing compounds, and, finally, the high k_{cat}/K_M value calculated for xyloside **13** prompted us to also test it. Xyloside **14** was included in the study as a positive control since its antiproliferative activity against this cell line has been already demonstrated.^{8a} Methyl D-xylopyranoside **4** was used as negative control since this compound was not the substrate for the recombinant enzyme (Table 2). Finally, the peracetylated derivatives of **4** (compound **25**) and **13** (compound **24**) were also included in the study since it has been described that peracetylated glycosides can penetrate more efficiently into the cells and once inside they can be deacetylated by endogenous carboxylesterases leading to the bioactive compound.⁵

As expected, xylosides **4** and its peracetylated derivative **25** were unable to inhibit the cell proliferation (Fig. 6 and Table S1 (ESI[†])). This lack of antiproliferative activity may be related to the inefficacy of **4** to be a substrate of the human β -1,4-GalT 7 and therefore to prime the synthesis of GAG.

Fig. 6 Effect of xylopyranoside derivatives **7** (●), **13** (■), **14** (○) and peracetate **24** (□) on the proliferation of human lung carcinoma cells A549. Values correspond to the mean of 3 different experiments performed in triplicate. For the sake of clarity only results from 4 compounds are shown. See Table S1 (ESI[†]) for antiproliferative values of xylosides **4**, **8**, **9**, and peracetate **25** (mean $\pm$ SD).

The only other tested xyloside that did not present anti-proliferative activity was the *S*-glycoside **7**. This result is in agreement with Mani's findings which showed that 2-naphthyl *S*- β -D-xylopyranoside was also unable to inhibit the growth of lung fibroblasts, lung carcinoma cells, or transformed endothelial cells.^{8a} The rest of xylopyranosides tested showed IC₅₀ values than 2-(6-hydroxynaphthyl) β -D-xylopyranoside (**14**). The xyloside that showed the lowest IC₅₀ (54 μ M) was the peracetate **24**. This value was lower than the IC₅₀ calculated for deacetylated **13** (88 μ M) and this difference may be explained because of a higher uptake of the peracetylated compound by the cell. Compounds **8** showed an IC₅₀ of 68 μ M, substantially lower than the IC₅₀ showed by xyloside **9**, in which the only difference is that the alkyl chain is four carbons longer. Finally, it is interesting to note that all the xylopyranosides that showed antiproliferative activity were cytotoxic at high concentrations. Xylosides **8** and **13** and peracetate **24** were cytotoxic at concentrations higher than 100 μ M, xylosides **9** and **14** exhibited cytotoxic effect at concentrations higher than 150 and 250 μ M, respectively, whereas xylosides **4**, **7** and peracetate **25** were not cytotoxic (Table S1, ESI†).

Experimental

Materials and general procedures

The clone IRAUp969H0626D containing the cDNA sequence of the human UDP-galactose:*O*- β -D-xylosylprotein 4- β -D-galactosyltransferase was purchased from imaGenes. The *E. coli* strain BL21(DE3) (Stratagene) was used as expression hosts and *E. coli* DH5 α (Promega) served as a host for plasmid maintenance. Restriction enzymes and T4-DNA ligase were purchased from MBI Fermentas AB and the pET-28b(+) expression vector was purchased from Novagen. DNase I was from Roche and lysozyme was from USB. Pyruvate kinase (PK) and lactate dehydrogenase (LDH) were purchased from Sigma-Aldrich. Isopropyl- β -D-thiogalactopyranoside (IPTG) was purchased from Applichem GmbH. A plasmid purification kit was from Sigma and DNA purification kit from agarose gels was from Eppendorf. All other chemicals were purchased from commercial sources as reagent grade.

UV/Visible spectra were recorded on a Spectra Max Plus 384 spectrophotometer at 25 °C. HPLC analyses were carried out on a chromatograph Dionex, Dual Gradient Pump, with a detector Diodo Array UV/VIS PDA-3000 (Dionex Co.). SDS-PAGE was performed in a Mini-Protean 3 Cell Electrophoresis Unit (BioRad) using 10% and 5% acrylamide in the separating and stacking gels, respectively. Gels were stained with Coomassie brilliant blue R-250 (Applichem GmbH). Electrophoresis was always run under reducing conditions, in the presence of 5% β -mercaptoethanol. Protein and DNA gels were quantified by densitometry using a GeneGenius Gel Documentation and Analysis System (Syngene). Nickel-iminodiacetic acid (Ni²⁺-IDA) agarose was supplied by Agarose Bead Technologies.

Optical rotations were determined on a Perkin Elmer 241 Polarimeter (λ = 589 nm, 1 dm cell). ¹H NMR spectra were registered at 500, 400 or 300 MHz, and ¹³C NMR were

obtained at 125 or 100 MHz using CDCl₃ or CD₃OD as a solvent at room temperature. Chemical shift values are reported in parts per million (δ). Coupling constant values (*J*) are reported in hertz (Hz), and spin multiplicities are indicated by the following symbol: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). Mass spectroscopy spectra were registered on a hp series 1100 MSD spectrometer.

Peptide mass fingerprint analysis

Peptide mass fingerprint analysis from the SDS-PAGE band corresponding to the recombinant β -1,4-GalT **7** was performed at the Proteomic Unit of the Spanish National Center of Biotechnology (CNB-CSIC). Samples were digested with sequencing grade trypsin O/N at 37 °C. The analysis by MALDI-TOF mass spectrometry produces peptide mass fingerprints and the peptides observed can be collated and represented as a list of monoisotopic molecular weights. Data were collected in the *m/z* range of 800–3600.

Synthesis of xylopyranose derivatives

2-Cyanoethyl β -D-2,3,4-tri-*O*-acetyl-xylopyranoside (16). To a solution of tetra-*O*-acetyl-xylopyranose (**15**, 5.0 g, 15.7 mmol) in CH₂Cl₂ (125 mL), 3-hydroxypropionitrile (1.3 mL, 18.8 mmol, 1.2 eq.) and BF₃·OEt₂ (1.86 mL, 15.7 mmol, 1 eq.) were added. The mixture was stirred for 1 h at room temperature under an argon atmosphere, then diluted with CH₂Cl₂ (50 mL) and washed with NaHCO₃ and H₂O. The organic layer was concentrated *in vacuo* and the resulting residue was crystallized from methanol to give **16** (4.3 g, 82%) as a solid. Mp: 161–165 °C. [α]_D²⁵: –57.1 (*c* 1.49, CH₂Cl₂). ¹H NMR (300 MHz, CDCl₃): δ 5.17 (t, 1H, *J* = 8.4 Hz), 4.98–4.88 (m, 2H), 4.56 (d, *J* = 6, 6 Hz), 4.15 (dd, *J* = 4.8, 12.0 Hz), 4.00 (dt, 1H, *J* = 5.7, 9.9 Hz), 3.72 (dt, *J* = 6.3, 9.9 Hz), 3.40 (dd, 1H, *J* = 8.4, 12.0 Hz), 2.63 (t, 2H, *J* = 6.0 Hz), 2.09 (s, 3H), 2.06 (s, 6H). ¹³C NMR (133 MHz, CDCl₃): δ 170.0, 169.8, 169.6, 117.2, 100.75, 70.8, 70.0, 68.5, 63.8, 61.9, 20.7, 19.0. Anal. calc. for C₁₄H₁₉NO₈: C 51.06, H 5.82, N 4.25. Found: C 50.86, H 5.78, N 4.16%.

3-Acetamido-propyl β -D-xylopyranoside (8). **16** (100 mg, 0.3 mmol) was dissolved in a EtOAc–MeOH solution (2 : 1, v/v, 9 mL) and Pd/C (50 mg) and trifluoroacetic acid (34 mL, 1.5 eq.) were added. The mixture was stirred for 18 h at room temperature under an H₂ atmosphere. After this time, the reaction mixture was filtered through Celite® and the solids were washed with a EtOAc–MeOH solution (2 : 1, v/v). The combined filtrate and washing were concentrated to give amine **17**. ¹H NMR (400 MHz, CD₃OD): δ 5.23 (t, 1H, *J* = 9.2 Hz), 4.98–4.82 (m, 2H), 4.61 (d, 1H, *J* = 7.3 Hz), 4.08 (dd, 1H, *J* = 5.5, 11.7 Hz), 3.94 (m, 1H), 3.69 (m, 1H), 3.47 (dd, 1H, *J* = 9.7, 11.7 Hz), 3.02 (t, 2H, *J* = 7.1 Hz), 2.02 (s, 3H), 2.01 (s, 3H), 2.00 (s, 3H), 1.98–1.88 (m, 2H). ¹³C NMR (100 MHz, CD₃OD): δ 171.5, 102.1, 73.2, 72.7, 70.4, 68.0, 63.3, 38.9, 28.6, 20.5.

17 (137 mg, 0.3 mmol) was dissolved in dry pyridine (2 mL) and acetic anhydride (2 mL) was added. The mixture was stirred for 18 h at room temperature, then concentrated and the residue was purified by column chromatography (EtOAc–MeOH, 20 : 0 $\rightarrow$ 20 : 1) to give **18** (130 mg, 100%).

^1H NMR (300 MHz, CD_3OD): δ 5.25 (t, 1H, J = 9.0 Hz), 5.05–4.79 (m, 2H), 4.60 (d, J = 7.3 Hz), 4.10 (dd, 1H, J = 5.1, 11.7 Hz), 3.88 (m, 1H), 3.66–3.39 (m, 2H), 3.31–3.19 (m, 2H), 2.07 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 1.95 (s, 3H), 1.88–1.70 (m, 2H).

18 (100 mg, 0.27 mmol) was dissolved in methanol (15 mL) and treated with 0.5 M NaOMe (5 mL) for 1 h at room temperature, then neutralized with Amberlite IR-120 (H^+) resin, and filtered. The solvent was evaporated to give **8** (67.3 mg, 98%). $[\alpha]_{\text{D}}^{25}$: -41.6 (c 1.25, H_2O). ^1H NMR (400 MHz, CD_3OD): δ 4.20 (d, 1H, J = 7.4 Hz), 3.92–3.82 (m, 2H), 3.57 (m, 1H), 3.47 (m, 1H), 3.36–3.12 (m, 5H), 1.92 (s, 3H), 1.78 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD): δ 172.1, 103.9, 76.3, 73.7, 70.0, 67.2, 65.8, 36.6, 29.1, 21.4. MS (ES) m/z (calc. 249.1): 250.1 ($M + 1$), 272.0 ($M + 23$). Anal. calc. for $\text{C}_{10}\text{H}_{19}\text{NO}_6$: C 48.19, H 7.68, N 5.62. Found: C 47.99, H 7.88, N 5.57%.

3-Hexanamido-propyl β -D-xylopyranoside (9). Amine **17** (274 mg, 0.6 mmol), obtained as described above, was dissolved in dry pyridine (2 mL) and hexanoic anhydride (278 μL , 2 eq.) was added. The reaction was stirred for 3 h at room temperature, then concentrated and the residue was purified by column chromatography (hexane–EtOAc 1 : 1) to give **19** (129 mg, 50%). ^1H NMR (300 MHz, CDCl_3): δ 5.95 (s, 1H), 2.15 (t, J = 9.0 Hz), 5.02–4.82 (m, 2H), 4.42 (d, 1H, J = 7.2 Hz), 4.10 (dd, 1H, J = 5.4, 11.7 Hz), 3.89 (m, 1H), 3.53 (m, 1H), 3.45–3.18 (m, 3H), 2.15 (t, 2H, J = 7.5 Hz), 2.05 (s, 3H), 2.03 (s, 6H), 1.82–1.68 (m, 2H), 1.59 (m, 2H), 1.37–1.21 (m, 4H), 0.88 (t, J = 6.6 Hz).

19 (100 mg, 0.23 mmol) was deacetylated under similar conditions as described for **18** to give **9** (64.2 mg, 91%). $[\alpha]_{\text{D}}^{25}$: -40.4 (c 1.53, H_2O). ^1H NMR (400 MHz, CD_3OD): δ 4.19 (d, J = 7.6 Hz), 3.91–3.81 (m, 2H), 3.56 (m, 1H), 3.36–3.12 (m, 5H), 2.16 (t, J = 7.2 Hz), 1.78 (m, 2H), 1.60 (m, 2H), 1.40–1.24 (m, 4H), 0.91 (t, 3H, J = 7.2 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 175.5, 103.9, 76.7, 73.7, 70.0, 67.2, 65.8, 36.4, 36.0, 31.3, 29.1, 25.6, 22.3, 13.1. Anal. calc. for $\text{C}_{14}\text{H}_{27}\text{NO}_6$: C 55.06, H 8.91, N 4.59. Found: C 54.78, H 9.06, N 4.52%.

Sodium *N*-[*O*-(β -D-xylopyranosyl)-3-hydroxypropyl]-succinamate (10). Amine **17** (274 mg, 0.61 mmol), obtained as described above, was dissolved in dry pyridine (2 mL) and succinic anhydride (60 mg, 2 eq.) was added. The mixture was stirred for 2 h at room temperature, then concentrated and the residue purified by column chromatography (AcOEt–MeOH, 10 : 0 $\rightarrow$ 10 : 1) to give **20** (387 mg, 75%). ^1H NMR (300 MHz, CDCl_3): δ 6.49 (s, 1H), 5.21 (t, 1H, J = 9.0 Hz), 5.02–4.86 (m, 2H), 4.42 (d, 1H, J = 7.3 Hz), 4.11 (dd, 1H, J = 5.1, 12.0 Hz), 3.91 (m, 1H), 3.56 (m, 1H), 3.42–3.26 (m, 3H), 2.66 (m, 2H), 2.56 (m, 2H), 2.06 (s, 3H), 2.03 (s, 6H), 1.78 (m, 2H).

20 (160 mg, 0.19 mmol) was deacetylated under similar conditions as described for **18** to give **10** (62 mg, 98%). $[\alpha]_{\text{D}}^{25}$: -31.9 (c 1.65, H_2O). ^1H NMR (400 MHz, CD_3OD): δ 4.20 (d, J = 7.8 Hz), 3.92–3.82 (m, 2H), 3.57 (m, 1H), 3.48 (m, 1H), 3.37–3.14 (m, 5H), 2.63–2.55 (m, 3H), 2.51–2.43 (m, 2H), 1.78 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD):

δ 175.1, 173.4, 103.8, 76.6, 73.7, 70.0, 67.2, 65.7, 36.5, 30.4, 29.1, 28.6. Anal. calc. for $\text{C}_{12}\text{H}_{20}\text{NNaO}_8$: C 43.77, H 6.12, N 4.25, Na 6.98. Found: C 43.08, H 6.12, N 3.90%.

3-Benzamido-propyl β -D-xylopyranoside (11). Amine **17** (274 mg, 0.6 mmol), obtained as described above, was dissolved in dry pyridine (2 mL) and benzoyl chloride (215 μL , 3 eq.) was added. The mixture was stirred for 18 h at room temperature, then washed with AcOEt and concentrated. The residue was purified by column chromatography (hexane–AcOEt 3 : 1 $\rightarrow$ 1 : 1) to give **21** (54 mg, 20%). ^1H NMR (300 MHz, CDCl_3): δ 7.77 (m, 2H), 7.53–7.38 (m, 3H), 6.69 (s, 1H), 5.18 (t, 1H, J = 9.0 Hz), 5.01–4.84 (m, 2H), 4.48 (d, 1H, J = 6.9 Hz), 4.08 (dd, 1H, J = 6.6, 5.1 Hz), 3.93 (m, 1H), 3.71–3.39 (m, 3H), 3.34 (dd, 1H, J 9.3, 2.4 Hz), 2.03 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 1.89 (m, 2H).

21 (54 mg, 0.12 mmol) was deacetylated under similar conditions as described for **18** to give **11** (37 mg, 99%). $[\alpha]_{\text{D}}^{25}$: -34.4 (c 1.42, H_2O). ^1H NMR (400 MHz, CD_3OD): δ 7.82–7.78 (m, 2H), 7.52 (m, 1H), 7.45 (m, 2H), 4.22 (d, 1H, J = 7.4 Hz), 3.92 (m, 1H), 3.85 (dd, 1H, J = 5.5, 12.3 Hz), 3.64 (m, 1H), 3.57–3.42 (m, 3H), 3.31 (m, 1H), 3.24–3.15 (m, 2H), 1.91 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD): δ 169.0, 134.6, 131.4, 128.4, 127.0, 104.0, 76.7, 73.8, 70.0, 67.4, 65.8, 37.1, 29.2. Anal. calc. for $\text{C}_{15}\text{H}_{21}\text{NO}_6$: C 57.87, H 6.80, N 4.50. Found: C 57.58, H 6.78, N 4.76%.

Sodium *N*-[*O*-(β -D-xylopyranosyl)-3-hydroxypropyl]-phthalamate (12). Amine **17** (274 mg, 0.6 mmol), obtained as described above, was dissolved in dry pyridine (2 mL) and phthalic anhydride (221 mg, 2 eq.) was added. The mixture was stirred for 2 h at room temperature, then concentrated and the residue was purified by column chromatography (AcOEt–MeOH 10 : 0 $\rightarrow$ 10 : 1) to give **22** (360 mg, 80%). ^1H NMR (300 MHz, CDCl_3): δ 5.14 (t, 1H, J = 8.8 Hz), 4.95–5.75 (m, 2H), 4.43 (d, 1H, J = 7.1 Hz), 4.02 (dd, 1H, J = 5.4 Hz, J = 11.7 Hz), 3.95–3.83 (m, 3H), 3.67–3.38 (m, 3H), 3.30 (dd, 1H, J = 9.5, 11.7 Hz), 2.01 (s, 3H), 1.99 (s, 3H), 1.98 (s, 3H), 1.87 (m, 2H).

22 (280 mg, 0.64 mmol) was deacetylated under similar conditions as described for **18** to give **12** (229 mg, 95%). $[\alpha]_{\text{D}}^{25}$: -24.3 (c 1.50, H_2O). ^1H NMR (300 MHz, CD_3OD): δ 7.96 (m, 1H), 7.68–7.48 (m, 2H), 7.43 (m, 1H), 4.23 (d, 1H, J = 7.6 Hz), 3.94 (m, 1H), 3.83 (dd, 1H, J = 5.4, 11.5 Hz), 3.67 (m, 1H), 3.52–3.12 (m, 11H), 1.98–1.84 (m, 2H). Anal. calc. for $\text{C}_{16}\text{H}_{20}\text{NNaO}_8$: C 50.93, H 5.34, N 3.71, Na 6.09. Found: C 51.03, H 5.61, N 3.54%.

***O*_γ-(2,3,4-tri-*O*-acetyl- β -D-xylopyranosyl)-*N*-Cbz-L-serine-methyl-ester (24).** *N*-Cbz-L-serine methyl-ester (**23**, 200 mg, 0.79 mmol) and tetra-*O*-acetyl- β -D-xylopyranose (**15**, 500 mg, 2 eq.) were dissolved in dry CH_2Cl_2 (15 mL), then molecular sieve (4 Å, 4.5 g) and TMSOTf (78 μL , 0.5 eq.) were added. The mixture was stirred for 2 h at room temperature under an argon atmosphere. The reaction mixture was further diluted with CH_2Cl_2 (20 mL) and neutralized with triethylamine. Evaporation of the solvent and purification by column chromatography (hexane–AcOEt 3 : 1 $\rightarrow$ 1 : 1) gave a residue fraction (340 mg) which was further purified by column chromatography (CH_2Cl_2 –AcOEt 9 : 1) to give **24** as a solid

(303 mg, 75%). Mp: 102–104 °C. $[\alpha]_{\text{D}}^{25}$: –29.4 (*c* 1.03, CH₂Cl₂). ¹H NMR (400 MHz, CDCl₃): δ 7.42–7.23 (m, 5H), 5.56 (d, 1H, *J* = 8.2 Hz), 5.12 (s, 2H), 5.09 (t, 1H, *J* = 7.8 Hz), 4.90–4.81 (m, 2H), 4.51 (m, 1H), 4.48 (d, 1H, *J* = 6.5 Hz), 4.22 (dd, 1H, *J* = 2.7, 10.1 Hz), 4.03 (dd, 1H, *J* = 4.7, 12.1 Hz), 3.79 (dd, 1H, *J* = 3.1, 10.1 Hz), 3.76 (s, 3H), 3.34 (dd, 1H, *J* = 7.8, 12.1 Hz), 2.04 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H). ¹³C NMR (100 MHz, CDCl₃): δ 170.3, 170.2, 170.1, 169.6, 156.1, 136.3, 128.8, 128.5, 128.4, 100.7, 70.7, 70.2, 69.2, 68.6, 67.4, 61.7, 54.4, 53.0, 21.0, 20.9, 20.8. MS (ES) *m/z* (calc. 511.2): 534.2 (*M* + 23). Anal. calc. for C₂₃H₂₉NO₁₂: C 54.01, H 5.71, N 2.74. Found: C 53.88, H 5.71, N 2.74%.

O-β-D-Xylopyranosyl-N-Cbz-L-serine-methyl-ester (13). To a mixture of KCN (6.5 mg, 0.09 mmol) and MeOH (10 mL) was added **24** (100 mg, 0.19 mmol). The mixture was stirred at room temperature until complete conversion into deacetylated xylopyranoside **13** (1 h), as indicated by TLC (AcOEt–MeOH 25 : 1, v/v). After this time an equimolar quantity of mixed Dowex® 50X4 (Na⁺) and Dowex® 1X8 (HCO₃[–]) resins were added, then the mixture was filtered and concentrated. The colorless residue was purified by column chromatography (AcOEt–MeOH 25 : 0 → 25 : 1) to give **13** (49.8 mg, 70%) as a solid. Mp: 42–46 °C. $[\alpha]_{\text{D}}^{25}$: –23.4 (*c* 1.13, MeOH). ¹H NMR (400 MHz, CD₃OD): δ 7.40–7.23 (m, 5H), 5.09 (s, 2H), 4.44 (m, 1H), 4.26 (dd, 1H, *J* = 3.7 Hz, *J* = 10.1 Hz), 4.16 (d, 1H, *J* = 7.7 Hz), 3.81 (dd, 1H, *J* = 5.3 Hz, *J* = 11.3 Hz), 3.74–3.67 (m, 4H), 3.43 (m, 1H), 3.27 (t, 1H, *J* = 8.8 Hz), 3.19–3.09 (m, 2H). ¹³C NMR (100 MHz, CD₃OD): δ 172.2, 158.7, 138.1, 129.47, 129.0, 128.9, 105.1, 77.6, 74.8, 71.0, 70.5, 67.8, 67.0, 55.7, 53.0. Anal. calc. for C₁₇H₂₃NO₉: C 52.98, H 6.02, N 3.63. Found: C 53.03, H 5.96, N 3.56%.

Enzyme activity assays

Galactosyltransferase activity of the recombinant β-1,4-GalT 7 was determined following the method previously described by Higuchi *et al.* with minor modifications.²⁷ Formation of disaccharide **2** was followed by HPLC with A_{300nm} monitoring in reaction mixtures of 1 ml containing sodium acetate buffer (25 mM, pH 5.0), MnCl₂ (20 mM), KCl (50 mM), UDP-Gal (50 μM), and **1** (0.5 mM). Aliquots were taken each 5 min and analyzed by HPLC using a Lichrosorb RP18 column. The mobile phase employed was TFA (0.1% in H₂O)–CH₃CN 17 : 3 (v : v) and the chromatography was run under isocratic conditions at 1 ml min^{–1} flow rate. Measurements of kinetic parameters for UDP-Gal were performed with 11–14 μg of purified protein at fifteen different UDP-Gal concentrations in the range 0.025–20 mM and with a fixed concentration of **1** of 10 mM. Assays to determine the kinetic parameters for *p*-Nph-β-D-Xyl **1** were performed with 7–8 μg of purified β-1,4-GalT 7 at 10 concentrations of the substrate (0.01–10 mM) under saturating concentrations of UDP-Gal (0.6 mM).

Activity of the recombinant β-1,4-GalT 7 with xylopyranoside derivatives **11** and **14** and their kinetic parameters were determined by HPLC as described previously. Activity with compounds **5–10**, **12** and **13** was measured spectrophotometrically in a coupled enzymatic assay, where the decrease of NADH absorbance at 340 nm is directly proportional to the

release of UDP during the galactosyltransferase-catalyzed reaction.²⁸ In these cases, activity assays were run at 25 °C following the decrease of absorbance at 340 nm ($\epsilon_{\text{NADH}} = 6220 \text{ M}^{-1} \text{ cm}^{-1}$) for 30 minutes in a 1 mL reaction mixture containing sodium acetate buffer (25 mM, pH 5.0), NADH (0.2 mM), PEP (0.7 mM), KCl (50 mM), MnCl₂ (20 mM), PK (6 U) and LDH (6 U). Substrate concentrations were varied between 0.01 and 10 mM. Activity with *p*-NO₂Ph-β-D-Xyl (**1**) was also measured by this method and the obtained results were consistent with those obtained by HPLC.

Inhibition of galactosyltransferase activity by the different xylopyranoside derivatives was analyzed in a 1 mL reaction mixture containing sodium acetate buffer (25 mM, pH 5.0), KCl (50 mM), MnCl₂ (20 mM), UDP-gal (50 mM), **1** (10 mM) and increasing concentrations of inhibitor. Accumulation of disaccharides was monitored by HPLC as described above.

Analysis of kinetic data

Kinetic constants were obtained using the built-in nonlinear regression tools in SigmaPlot 8.0. For the determination of apparent kinetic constants (variation of only one substrate), initial velocities (*V_i*) were fitted to the Michaelis–Menten equation (eqn (1)):

$$V_i = \frac{V_{\text{max}}^{\text{app}} \cdot [S]}{K_{\text{M,S}}^{\text{app}} + [S]} \quad (1)$$

Due to substrate inhibition, the apparent kinetic constants for UDP-Gal and xylopyranoside **14** were determined using the Michaelis–Menten equation for a mechanism that involves substrate inhibition (eqn (2)):

$$V_i = \frac{V_{\text{max}}^{\text{app}} \cdot [S]}{K_{\text{M,S}}^{\text{app}} + [S] + \frac{[S]^2}{K_{\text{IS}}^{\text{app}}}} \quad (2)$$

where $K_{\text{IS}}^{\text{app}}$ is the excess substrate apparent inhibition constant.

IC₅₀ for the different xylopyranosides was calculated from the four-parameter logistic regression model, which assumes symmetry around the inflection point of the standard curve. To calculate *k_i* for **14**, data were fitted to eqn (3) that describe the competitive inhibition:

$$V_i = \frac{V_{\text{max}}^{\text{app}} \cdot [S]}{K_{\text{M,S}}^{\text{app}} (1 + [I]/k_{\text{ic}}^{\text{app}}) + [S]} \quad (3)$$

where *[I]* denoted concentration of inhibitor and *k_{ic}* the competitive inhibition.

Antiproliferative activity studies

For the determination of the antiproliferative activity, A549 cells were seeded at 3000 cells per well in 96-well plates in media containing 10% of fetal bovine serum (FBS). After 4 h of plating, the cells were serum-starved for 24 h. Cells were then allowed to proliferate in media without FBS, containing 25 ng ml^{–1} of epidermal growth factor (EGF), in the presence of different concentrations of xylosides (330, 220, 147, 98, 65, 43, 29, 19 μM). Four days later, the number of viable cells was determined with the MTT test.²⁹

Conclusions

In conclusion, we have shown that it is possible to heterologously express the catalytic domain of the human β -1,4-GalT 7, soluble and stable enough to undertake *in vitro* studies with it. This achievement opens the possibility of developing an easy-to-use method to test the activity as decoy acceptors of natural and synthetic xylopyranosides. Since priming the synthesis of GAG is required but not enough for the antiproliferative activity of the xylosides,^{10a,b} it is not possible to establish a direct correlation between the kinetic parameters of the recombinant β -1,4-GalT 7 and the antiproliferative activity of the different xylopyranosides tested. On the other hand, preliminary results obtained in A549 cell line suggest that xylopyranosides **8**, **9**, **13** and **14** exhibit a promising antiproliferative activity.

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Notes and references

- For some reviews see: R. L. Jackson, S. J. Busch and A. D. Cardin, *Physiol. Rev.*, 1991, **71**, 481–539; B. Casu and U. Lindahl, *Adv. Carbohydr. Chem. Biochem.*, 2001, **57**, 159–206; R. Sasisekharan, Z. Shriver, G. Venkataraman and U. Narayanasami, *Nat. Rev. Cancer*, 2002, **2**, 521–528; U. Häcker, K. Nybakken and N. Perrimon, *Nat. Rev. Mol. Cell Biol.*, 2005, **6**, 530–541; J. R. Bishop, M. Schuksz and J. D. Esko, *Nature*, 2007, **446**, 1030–1037; N. S. Gandhi and R. L. Mancera, *Chem. Biol. Drug Des.*, 2008, **72**, 455–482 and references therein.
- N. B. Schwartz, *Trends Glycosci. Glycotechnol.*, 1995, **7**, 429–445; J. D. Esko and S. B. Selleck, *Annu. Rev. Biochem.*, 2002, **71**, 435–471; J. M. Whitelock and R. V. Iozzo, *Chem. Rev.*, 2005, **105**, 2745–2764.
- (a) M. Okayama, K. Kimata and S. Suzuki, *J. Biochem.*, 1973, **74**, 1069–1073; (b) N. B. Schwartz, L. Galligani, P. L. Ho and A. Dorfman, *Proc. Natl. Acad. Sci. U. S. A.*, 1974, **71**, 4047–4051; (c) H. C. Robinson, M. J. Brett, P. J. Tralaggaan, D. A. Lowther and M. Okayama, *Biochem. J.*, 1975, **148**, 25–34.
- S.-F. Kuan, C. J. Byrd, C. B. Baum and Y. S. Kim, *J. Biol. Chem.*, 1989, **264**, 19271–19277.
- A. K. Sarkar, T. A. Fritz, W. H. Taylor and J. D. Esko, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 3323–3327; A. K. Sarkar, K. S. Rostand, R. K. Jain, K. L. Matta and J. D. Esko, *J. Biol. Chem.*, 1997, **272**, 25608–25616; A. K. Sarkar, J. R. Brown and J. D. Esko, *Carbohydr. Res.*, 2000, **329**, 287–300; T. K.-K. Mong, L. V. Lee, J. R. Brown, J. D. Esko and C.-H. Wong, *ChemBioChem*, 2003, **4**, 835–840.
- M. Latorre, P. Peñalver, J. Revuelta, J. L. Asensio, E. García-Junceda and A. Bastida, *Chem. Commun.*, 2007, 2829–2831.
- (a) F. N. Lugenwa and J. D. Esko, *J. Biol. Chem.*, 1991, **266**, 6674–6677; (b) T. A. Fritz, F. N. Lugenwa, A. K. Sarkar and J. D. Esko, *J. Biol. Chem.*, 1994, **269**, 300–307; (c) F. N. Lugenwa, A. K. Sarkar and J. D. Esko, *J. Biol. Chem.*, 1996, **271**, 19159–19165; (d) B. Kuberan, M. Ethirajan, X. V. Victor, V. T. K. Nguyen and A. Do, *ChemBioChem*, 2008, **9**, 198–200.
- (a) K. Mani, B. Havsmark, S. Persson, Y. Kaneda, H. Yamamoto, K. Sakurai, S. Ashikari, H. Habuchi, S. Suzuki, K. Kimata, A. Malmström, G. Westergren-Thorsson and L.-Å. Fransson, *Cancer Res.*, 1998, **58**, 1099–1104; (b) K. Mani, M. Belting, U. Ellervik, N. Falk, G. Svensson, S. Sandgren, F. Cheng and L.-Å. Fransson, *Glycobiology*, 2004, **14**, 387–397.
- M. Belting, L. Borsig, M. M. Fuster, J. R. Brown, L. Persson, L.-Å. Fransson and J. D. Esko, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 371–376.
- (a) M. Jacobsson, U. Ellervik, M. Belting and K. Mani, *J. Med. Chem.*, 2006, **49**, 1932–1938; (b) R. Johnsson, K. Mani and U. Ellervik, *Bioorg. Med. Chem.*, 2007, **15**, 2868–2877; (c) F. Cheng, R. Johnsson, J. Nilsson, L.-Å. Fransson, U. Ellervik and K. Mani, *Cancer Lett.*, 2009, **273**, 148–154.
- R. Almeida, S. B. Levery, U. Mandel, H. Kresse, T. Schwientek, E. P. Bennett and H. Clausen, *J. Biol. Chem.*, 1999, **274**, 26165–26171.
- T. Okajima, K. Yoshida, T. Kondo and K. Furukawa, *J. Biol. Chem.*, 1999, **274**, 22915–22918.
- J. C. Paulson and K. J. Colley, *J. Biol. Chem.*, 1989, **264**, 17615–17618; K. J. Colley, *Glycobiology*, 1997, **7**, 1–13.
- A quite completed guide to select a method to produce recombinant proteins can be found in: S. Gräslund, P. Nordlund and J. Weigelt, *et al.*, *Nat. Methods*, 2008, **5**, 135–146.
- G. Hannig and S. C. Makrides, *Trends Biotechnol.*, 1998, **16**, 54–60; F. Baneyx, *Curr. Opin. Biotechnol.*, 1999, **10**, 411–421; W. Peti and R. Page, *Protein Expression Purif.*, 2007, **51**, 1–10; S. Zerbs, A. M. Frank and F. R. Collart, *Methods Enzymol.*, 2009, **463**, 149–168.
- F. Baneyx, in *Manual of industrial microbiology and biotechnology*, ed. in chief A. L. Demain and J. E. Davies, ed. G. Cohen, C. L. Hershberger, L. J. Forney, I. B. Holland, W.-S. Hu, J.-H. D. Wu, D. H. Sherman and R. C. Wilson, American Society of Microbiology, Washington D.C., 2nd edn., 1999, pp. 551–565; A. Bastida, A. Fernández-Mayoralas, R. G. Arrayás, F. Iradier, J. C. Carretero and E. García-Junceda, *Chem.-Eur. J.*, 2001, **7**, 2390–2397; A. Vera, N. Gonzalez-Montalban, A. Aris and A. Villaverde, *Biotechnol. Bioeng.*, 2006, **96**, 1101–1106; S. Sahdev, S. K. Khattar and K. S. Saini, *Mol. Cell. Biochem.*, 2008, **307**, 249–264; W. H. Brondyk, *Methods Enzymol.*, 2009, **463**, 131–147.
- A. Mogk, M. P. Mayer and E. Deuerling, *ChemBioChem*, 2002, **3**, 807–814; A. Bastida, M. Latorre and E. García-Junceda, *ChemBioChem*, 2003, **4**, 531–533; M. Martínez-Alonso, A. Vera and A. Villaverde, *FEMS Microbiol. Lett.*, 2007, **273**, 187–195.
- D. Esposito and D. K. Chatterjee, *Curr. Opin. Biotechnol.*, 2006, **17**, 353–358.
- F. Daligault, S. Rahuel-Clermont, S. Gulberti, M.-T. Cung, G. Branlant, P. Netter, J. Magdalou and V. Lattard, *Biochem. J.*, 2009, **418**, 605–614.
- M. Pasek, E. Boeggeman, B. Ramakrishnan and P. K. Qasba, *Biochem. Biophys. Res. Commun.*, 2010, **394**, 679–684.
- D. Aoki, H. E. Appert, D. Johnson, S. S. Wong and M. N. Fukuda, *EMBO J.*, 1990, **9**, 3171–3178; P. Wang, G.-J. Shen, Y.-F. Wang, Y. Ichikawa and C.-H. Wong, *J. Org. Chem.*, 1993, **58**, 3985–3990; A. Bastida, A. Fernández-Mayoralas and E. García-Junceda, *Bioorg. Med. Chem.*, 2002, **10**, 737–742; J. Egelund, B. L. Petersen, J. S. Motawia, I. Damager, A. Faik, H. Clausen, C. E. Olsen, T. Ishii, P. Ulvskov and N. Geshi, *Plant Cell*, 2006, **18**, 2593–2607; A. M. Swistowska, S. Wittrock, W. Collisi and B. Hofer, *Appl. Microbiol. Biotechnol.*, 2008, **79**, 255–261.
- E. Gasteiger, C. Hoogland, A. Gattiker, S. Duvaud, M. R. Wilkins, R. D. Appel and A. Bairoch, in *The Proteomics Protocols Handbook*, ed. J. M. Walker, Humana Press Inc., Totowa, NJ, 2005, pp. 571–607.
- Essentials of Glycobiology*, ed. A. Varki, R. Cummings, J. Esko, H. Freeze, G. Hart and J. Marth, Cold Spring Harbor Laboratory Press, New York, 1999, pp. 151–152; J. M. Whitelock and R. V. Iozzo, *Chem. Rev.*, 2005, **105**, 2745–2764.
- R. López and A. Fernández-Mayoralas, *J. Org. Chem.*, 1994, **59**, 737–745.
- M. Sobue, H. Habuchi, K. Ito, H. Yonekura, K. Oguri, K. Sakurai, S. Kamohara, Y. Ueno, R. Noyori and S. Suzuki, *Biochem. J.*, 1987, **241**, 591–601.

- 26 H. Driguez, *Top. Curr. Chem.*, 1997, **187**, 85–116; Z. J. Witczak and J. M. Culhane, *Appl. Microbiol. Biotechnol.*, 2005, **69**, 237–244.
- 27 T. Higuchi, S. Tamura, K. Takagaki, T. Nakamura, A. Morikawa, K. Tanaka, A. Tanaka, Y. Saito and M. Endo, *J. Biochem. Biophys. Methods*, 1994, **29**, 135–142.
- 28 S. Gosselin, M. Alhussaini, M. B. Streiff, K. Takabayashi and M. M. Palcic, *Anal. Biochem.*, 1994, **220**, 92–97; D. K. Fitzgerald, B. Colvin, R. Mawal and K. E. Ebner, *Anal. Biochem.*, 1970, **36**, 43–61.
- 29 T. Mosmann, *J. Immunol. Methods*, 1983, **65**, 55–63.
- 30 C. Bui, I. Talhaoui, M. Chabel, G. Mulliert, M. W. H. Coughtrie, M. Ouzzine and S. Fournel-Gigleux, *FEBS Lett.*, 2010, **584**, 3962–3968; I. Talhaoui, C. Buil, R. Oriol, G. Mulliert, S. Gulberti, P. Netter, M. W. H. Coughtrie, M. Ouzzine and S. Fournel-Gigleux, *J. Biol. Chem.*, 2010, **285**, 37342–37358.