Large Scale Production of Recombinant α-1,2-Mannosyltransferase from *E. coli* for the Study of Acceptor Specificity and Use of the Recombinant Whole Cells in Synthesis

Guido F. Herrmann,† Peng Wang,† Gwo-Jenn Shen,† Eduardo Garcia-Junceda,† Shaheer H. Khan,‡ Khushi L. Matta,‡ and Chi-Huey Wong*,†

Department of Chemistry, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037, and Department of Gynecologic Oncology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, New York 14263

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We report the first large scale heterologous expression of a recombinant yeast α-1,2-mannosyltransferase in *E. coli*. The enzyme was isolated from 10-L and 50-L fermentations, purified and used for mannosylation reactions. The specificity of the recombinant enzyme was extensively studied by using mannose derivatives, oligosaccharides, and analogs as acceptors, and the results show that the enzyme exhibits high activities toward ManOMe and disaccharides connected by an α-1,2-mannosidic linkage. The recombinant *E. coli* cells were also used as a catalyst for glycosylation reaction, and mannosylation of saccharides and glycopeptides proceeded in moderate to good yields.

Introduction

Due to their important functions in cell recognition events, oligosaccharides are attracting a growing interest as promising targets for the development of new pharmaceuticals.[1] The development of new methodologies that will provide oligosaccharides in large quantities[2,3] is one of the major goals in oligosaccharide synthesis. There is, however, no general and catalytic chemical method for the formation of glycosidic bonds.[3] On the other hand, enzymes have proven to be useful catalysts for the regio- and stereoselective synthesis of oligosaccharides.[4] *N*-Acetyllactosamine, for example, was synthesized on a large scale by using galactosyltransferase[5] or galactosidase.[6] Further sialylation of the disaccharide using sialyltransferase gave a trisaccharide as an intermediate of sialyl Lewis X, a potential antiinflammatory agent.[2] The major drawback of using glycosyltransferases in organic syntheses is the restricted availability of this class of enzymes.[7] Purification of glycosyltransferases from natural sources such as human milk is tedious and provides only limited amounts of enzyme. Recently, the genes of some glycosyltransferases have been cloned and the corresponding enzymes have been produced by heterologous expression in various host organisms.[8] No report was, however, described for the large-scale production of recombinant glycosyltransferases. This report is intended to address the practical issues of large-scale production and purification of a glycosyltransferase. Moreover, during the course of this study, we discovered that recombinant *Escherichia coli* cells expressing a glycosyltransferase exhibit glycosylation activity without prior cell disintegration or isolation of the periplasmic fraction. This observation led to the use of whole recombinant *E. coli* cells as a cheap, convenient catalyst source for large scale enzymatic syntheses of oligosaccharides and glycopeptides.

α-1,2-Mannosyltransferase (Mnt1p), a GDP-Man dependent Golgi glycosyltransferase from *Saccharomyces cerevisiae*,[9] is involved in the chain elongation of protein *O*-linked mannose chains.[10–12] The enzyme is distinguished from other α-1,2-mannosyltransferases found in yeast by its preference of ManOMe as an acceptor.[9] α-1,2-Mannosyltransferase catalyzes the mannosylation of protein *O*-linked mannobiose (eq 1) by using GDP-Man

† The Scripps Research Institute.

‡ Roswell Park Cancer Institute.

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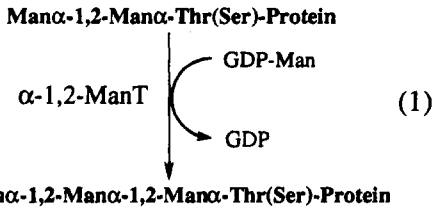
as a mannosyl donor. The gene encoding the α-1,2-mannosyltransferase (MNT1) was cloned and sequenced[9f] and is homologous to mammalian glycosyltransferases.



The enzyme contains a short *N*-terminal domain followed by a membrane spanning region and a large *C*-terminal catalytic domain. A soluble form of Mntlp (which is designated in this report as α-1,2-ManT), consisting of the catalytic domain of the enzyme, was produced in *E. coli* XL1-Blue strain.[13] The enzyme was used as a catalyst for mannosylation reactions coupled with cofactor regeneration *in situ* for large scale synthesis.[13] It was found that the recombinant α-1,2-ManT accepts mannose, mannobiose, and *O*-mannosylglycopeptides as substrates, and among them ManαMe is the best substrate.[13]

We report in this paper the optimization of heterologous expression of recombinant α-1,2-ManT in a 50 L fermentation and the purification of this enzyme. The substrate specificity of this enzyme was investigated with a number of mannose-containing oligosaccharides and glycopeptides. We also describe the use of recombinant *E. coli* cells as a new type of catalyst for enzymatic glycosylation reactions.

Results and Discussion

Large Scale Heterologous Expression of α-1,2-ManT. We confirmed in our initial shake flask experiments that the expression of α-1,2-ManT in *E. coli* is strongly dependent on the culture condition.[13] High yields of α-1,2-ManT were obtained when *E. coli* cells were grown in M9CA medium at 37 °C to an optical density of approximately 0.3 followed by induction of the enzyme expression with 5 μM isopropyl β-D-thiogalactopyranoside (IPTG) and a simultaneous switch of the temperature to 30 °C.[14] Under these conditions the enzyme was properly folded and secreted into the periplasmic space while no formation of inclusion bodies was observed (data not shown).

The expression of α-1,2-ManT was scaled up in two steps. In the first step, batch fermentations were conducted on a 10-L scale by employing the same culture conditions as that used in shake flask experiments (using M9CA medium and the induction was initiated at the early logarithmic phase with 5 μM IPTG). As these initial studies proved to be successful, we conducted fed-batch fermentations on a 10-L and a 50-L scale which employed a richer medium based on yeast extract.

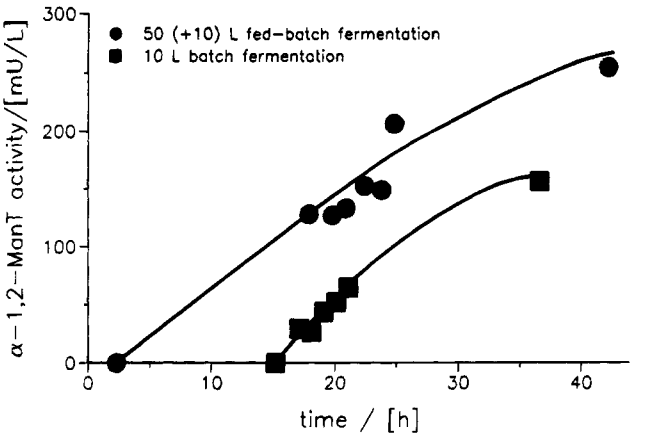


Figure 1. Heterologous expression of α-1,2-ManT in a 10-L batch fermentation (■) and in a 50-L fed-batch fermentation (●). For 10-L batch fermentation in M9CA medium, induction was done with 5 μM IPTG at t = 15.2 h (OD = 0.33) and a switch of temperature from 37 °C to 30 °C. The final α-1,2-ManT activity was 156 milliunits/L. For 50-L fed-batch fermentation in yeast extract medium, a 10-L medium containing 30 μM IPTG was added from t = 2.3 h (OD = 0.63) to t = 23.6 h (temperature was switched to 30 °C at t = 2.3 h). Cell yield was 19.5 g wet cells/L and the final α-1,2-ManT activity was 254.5 milliunits/L.

Figure 1 shows the production of α-1,2-ManT in a 10-L batch fermentation using M9CA medium and in a 50-L fed-batch fermentation using yeast extract medium. For the 10-L fermentation, the expression of α-1,2-ManT was induced with 5 μM IPTG when the temperature was shifted to 30 °C at the early logarithmic phase (OD = 0.33). Fermentation was finished after 35 h with a final α-1,2-ManT activity of 156 milliunits/L. A control experiment was conducted in a shake flask which resulted in the production of α-1,2-ManT at the level of 102 milliunits/L and a comparable cell yield (10-L batch fermentation: 1.6 g of wet cells/L; shake flask: 2.1 g of wet cells/L). As these initial batch fermentations indicated that the production of α-1,2ManT could be scaled up, we carried out another experiment to improve the cell yield and the concentration of α-1,2-ManT using a richer medium. A desirable higher cell yield was obtained in a 50-L fermentation using yeast extract medium, but formation of inclusion bodies occurred after induction with 5 μM IPTG. We then found that a slow feeding of the inducer IPTG in the fed-batch process prevented the formation of inclusion bodies. For a 50-L fermentation, the final IPTG concentration was adjusted to 5 μM by slow addition of IPTG dissolved in 10 L of fresh medium during a time period of 21 h. This process resulted in an improved cell yield (19.5 g/L) and increased α-1,2-ManT concentration (254 milliunits/L). A control experiment in a shake flask yielded 137 milliunits/L α-1,2-ManT and 4 g of wet cells/L. Comparison of the final α-1,2−ManT activity and cell specific α-1,2-ManT concentration (Table 1) indicates that cell specific enzyme concentration is higher in batch fermentations, whereas fed-batch fermentations yield higher cell mass and α-1,2-ManT activities.[15] In each fermentation, plotting of the

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Table 1. Comparison of Final Optical Density, α-1,2-ManT Activity, Cell Specific α-1,2-ManT Activity, and Growth Rate for Different Batch and Fed-Batch Fermentations

	batch fermentations		fed-batch fermentations	
volume of fermentation (L)	10	10	10 (+2)	50 (+10)
final optical density	1.47	1.39	5.52	13.1
final α-1,2-ManT activity (milliunits/L)	157	125	178	254
cell specific α-1,2-ManT concn[a] [milliunits/(OD × L)]	125	136	48.3	20.8
	(r = 0.97)	(r = 0.82)	(r = 0.97)	(r = 0.92)
growth rate μ (h^{-1})	0.074	0.068	0.14	0.097

[a] The cell specific α-1,2-ManT concentration provides information about the productivity of the recombinant *E. coli* cells and is obtained from the slope of the plot of α-1,2-ManT activity vs optical density. The corresponding correlation coefficients r are given in parentheses.

Table 2. Purification of α-1,2-ManT Obtained from Two 10-L Batch Fermentations

steps	volume (mL)	protein (mg)	activity (milliunits)	spec act. (milliunits/mg)	yield (%)
fermentation broth	20000	–	2850	–	–
periplasmic fraction	101	346	2000	6	70
$(NH_4)_2SO_4$ precipitation	6	53	989	19	35
mono Q column	24	4	682	166	24

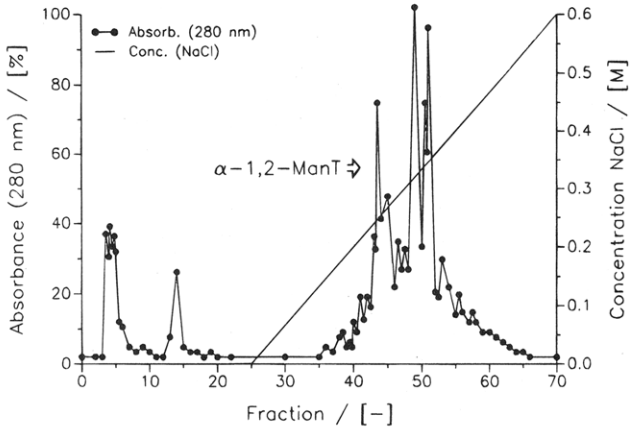

Figure 2. Purification of α-1,2-ManT by anion-exchange chromatography (Mono Q 10/10, 50 mM TRIS, pH 7.5, 26.35 mg of protein, 2.85 mL/fraction, 2.12 mL/min). ●: absorbance at 280 nm was recorded. The arrow marks the peak corresponding to α-1,2−ManT.

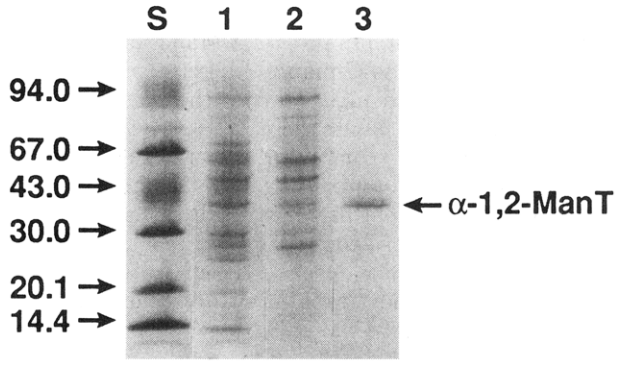

Figure 3. Purification of α-1,2-ManT, SDS-PAGE (coomassie blue staining); lane S: molecular weight standards; 94 kD: phosphorylase b; 67 kD: albumin; 43 kD: ovalbumin; 30 kD: carbonic anhydrase; 20.1 kD: trypsin inhibitor; 14.4 kD: α-lactalbumin. lane 1: periplasmic fraction. Lane 2: ammonium sulfate precipitation. Lane 3: anion-exchange chromatography.

concentrations of α-1,2-ManT as a function of optical densities resulted in a satisfactory linear correlation (data not shown), indicating that the expression of α-1,2-ManT is strictly growth associated.

Compared to the activity of Mnt1p in *S. cerevisiae* (0.2 milliunits/L),[16] a 1300- to 6000-fold overexpression has been achieved in the fed-batch fermentation which produced the enzyme up to 900 milliunits/L. In comparison, a homologous overexpression of the MNT1 gene in *S. cerevisiae* yielded an approximately 2.5-fold overexpression of Mnt1p.[9f]

Purification of α-1,2-ManT. Table 2 summarizes the purification of recombinant α-1,2-ManT. The periplasmic fraction of recombinant *E. coli* cells obtained from two 10-L batch fermentations was isolated by osmotic shock treatment to recover 70% of the enzyme activities. Isolation of the periplasmic fraction provided a straightforward separation of the enzyme from the *E. coli* cells. Subsequently, the proteins in the fraction were fractionated by ammonium sulfate precipitation, and the α-1,2-ManT was isolated in 35% overall yield. The enzyme was further purified by anion-exchange chromatography (eluted from a Mono Q column with a linear gradient from 0 to 0.6 M NaCl in 50 mM TRIS buffer) to give 4.1 mg of the enzyme with specific activity of 165.7 milliunits/mg (Figure 2). Figure 3 shows a SDS-PAGE of protein samples at different steps of purification. It is worthy to notice that only a faint band was detectable for the α-1,2-ManT in the periplasmic fraction, indicating the protein was not highly produced in *E. coli*.

In summary, this purification[8] protocol provides a convenient access to large amounts of α-1,2-ManT from the recombinant *E. coli*.

Acceptor specificity of α-1,2-ManT. Whereas most of the *N*-linked oligosaccharide structures of yeast *S. cerevisiae* have been determined,[17] little is known about the *O*-glycosylation pathway.[9-12] In the *O*-glycosylation, the first sugar is believed to be transferred from dolichol-

(15) These experiments suggest that the optimal conditions for a small-scale fermentation may not be applicable to large scale processes to produce large amounts of enzymes.

(16) Lewis *et al.*[9d] report the purification of Mnt1p from a 12-L fermentation of *S. cerevisiae*. After solubilizing the Mnt1p from crude microsomal extracts with Triton X-100 they obtained a total Mnt1p activity of 112 000 pmol/h. This corresponds to 1866 pmol/min (1.86 milliunits of Mnt1p). The initial Mnt1p concentration in the fermenter was approximately 0.155 milliunits/L.

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phosphate-mannose to serine or threonine in the endoplasmic reticulum,[10] followed by addition of α-1,2 and α-1,3 linked mannoses in the Golgi apparatus. The acceptor specificity of Mnt1p has been studied with purified enzyme obtained from solubilized yeast membranes.[9d] The enzyme transfers mannose to ManOMe (100% rel activity), mannose (15% rel activity) and α-1,2-mannobiose (42% rel activity).[9d] α-1,6-Mannobiose, α-1,6-mannotriose and α-1,6-mannotetraose are not substrates.[9d] These results were consistent with those obtained from the deletion mutants of *S. cerevisiae*, where the MNT1 gene was disrupted,[9e] and a decrease of activity in the transfer of mannose from GDP-Man to ManOMe as well to α-1,2- and α-1,3-linked methylmannobiosides was observed. We have reported[13] that the recombinant enzyme accepts ManOMe (**1**) (100% rel activity), mannose (**2**) (46% rel activity), methyl mannobioside (**12**) (62% rel activity), and *O*-mannosyl glycopeptides **23-27** (17−71% rel activity) as substrates. ManOMe derivatives with substituents at the 6 position are not substrates.[13]

This study further reveals that α-1,2-ManT accepts α-1,2, α-1,3-, and α-1,6 linked mannobiose derivatives as well as some linear and branched mannosyl trisaccharides as acceptors. Table 3 shows the relative activities of α-1,2-ManT toward compounds **1-27**[18] as acceptors.

Although α-1,2-ManT accepts mannose as a good substrate, the enzyme shows a very low activity toward modified monosaccharides **3-6** as acceptors. β-D-*p*-Nitrophenylmannoside (**5**) is the best acceptor among them with a relative activity of 0.5%. Compounds **3**, **4**, and **6** are modified at the 6 position and are not acceptable as substrates. This finding confirms our previous results[13] that α-1,2−ManT does not accept substrates with substituents at the 6 position of mannose.

In general, disaccharides with an α-1,2-linkage are good acceptors for α-1,2-ManT. Disaccharides **12**, **13** and **15**, for example, exhibit 39, 24, and 26% relative activity, respectively. Compound **15** contains a glucose residue at the reducing end, while **13** is substituted with a 6-deoxy-6-fluoro derivative at the reducing end. These findings show that α-1,2-ManT exhibits a relaxed substrate specificity toward the sugar at the reducing end of an α-1,2-linked disaccharide. However, replacing the mannose at the nonreducing end of an α-1,2-linked disaccharide with the 6-deoxy-6-fluoro mannose derivative (compound **14**) reduces the enzyme activity from 24% (for **13**) to 0.78%, indicating that α-1,2-ManT is very specific for mannose at the nonreducing end of disaccharides (see also acceptors **3**, **4**, and **6**).

α-1,3-Linked mannobiose **7** is a weak substrate, consistent with the observation with a *S. cerevisiae* deletion mutant,[9e] where a decrease of the transfer-activity toward α-1,3-linked mannobiosides was observed. In addition, if the hydroxy group at the 6 position of mannose at the nonreducing end is replaced by a fluoro group (compound **8**), the compound is not accepted by α-1,2-ManT.

Methyl 6-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (**9**) is not a substrate for either the deletion mutants of *S. cerevisiae*[9e] or purified Mnt1p.[9d] However, α-1,2-ManT accepts α-1,6 linked mannobioses **10** and **11** as weak substrates, indicating a broader specificity compared to Mnt1p.

All trisaccharides **16-19** are weak acceptors for α-1,2-ManT (<1% rel activity), which is in agreement with the results obtained from MNT1 mutant strains.[9e] It appears that Mnt1p and α-1,2-ManT prefer disaccharides as acceptors. Compound **16** is the only branched trisaccharide tested and shows a very low activity.

While 1-deoxymannojirimycin **21** is a weak inhibitor of α-1,2-ManT (32 mM **21** inhibts 65% of α-1,2-ManT activity), the corresponding deoxy thiosugar **22** is a substrate with 8.2% relative activity, and the lactam **20** exhibits 4.8% activity. In addition, α-1,2-ManT shows high activities toward the mannosylated glycopeptides **23-27**, suggesting that Mnt1p is involved in the elongation of protein-*O*-linked oligomannose-chains *in vivo*.

In summary α-1,2-ManT accepts ManOMe and the α-1,6-, α-1,3-, and α-1,2−linked mannobioses. It tolerates glucose and mannose derivatives at the reducing end. Mannosylated glycopeptides are good acceptors,[13,19] making α-1,2-ManT a useful tool to study the influence of sugar residues on the pharmaceutical properties of glycopeptides. In general, α-1,2-ManT shows a similar acceptor specificity to Mnt1p, with the exception that it weakly accepts some α-1,6-linked mannobiosides. Apparently, the acceptor specificity is not changed significantly by removing the *N*-terminal and the transmembrane domains from Mnt1p.

Use of Whole Recombinant *E. coli* Cells for Mannosylation Reactions. Although the use of whole cells in organic synthesis is well established,[20] no example has been reported so far concerning their use in glycosylation reactions.[19] To demonstrate the feasibility of the concept, we examined several acceptors for the enzymatic mannosylation reaction using GDP-Man as mannosyl donor (Scheme 1). The disaccharides α-1,2-mannobiose (**28**) (75% yield) and Manα1,2ManOMe (**29**) (53%), and the dimannosylated glycopeptides Cbz-Thr(αManα1,2Man)-OMe (**30**) (72%) and Boc-Tyr-Thr(αManα1,2Man)-Val-OMe (**31**) (42%) were obtained in moderate to good yields. Since inhibition of the reaction by the released GDP was not observed, there is no need to add alkaline phosphatase to decompose GDP. While it is not clear how mannosylation takes place, the cell walls of *E. coli* XL1-Blue strain used in this study might be permeable to allow the transport of products and substrates. Leakage of α-1,2-ManT from the periplasmic space of *E. coli* to the medium was excluded as no mannosylation activity was detected in the fermentation broth after separation of cells.

Conclusion

In summary, we have shown for the first time that a glycosyltransferase can be obtained on a large scale by

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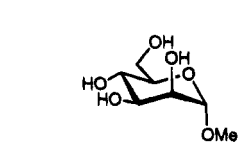
Table 3. Acceptor Specificity of α-1,2-ManT for Compounds 1-27. The Numbers in Parentheses are Relative Activities. Assay Conditions: 1-27: 1 mM GDP-Man, 20 mM Acceptor 1-22, 21.6 milliunits/mL α-1,2-ManT, 37 °C; 23–27: 3.3 mM GDP-Man, 150 mM Acceptor 23-27, 30 °C



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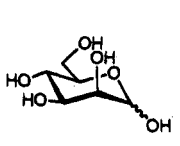

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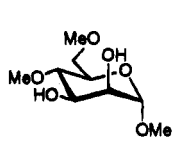

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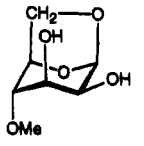

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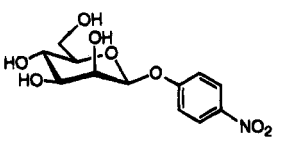

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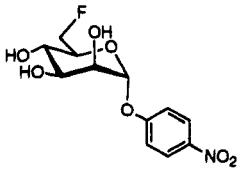

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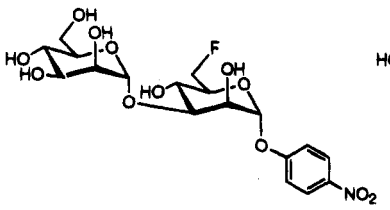

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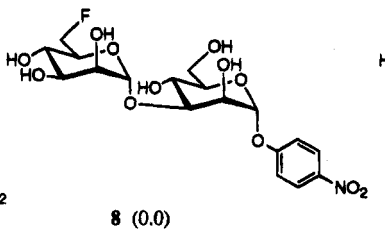

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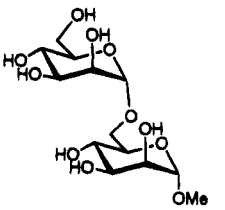

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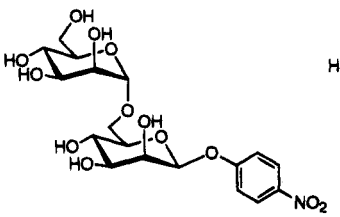

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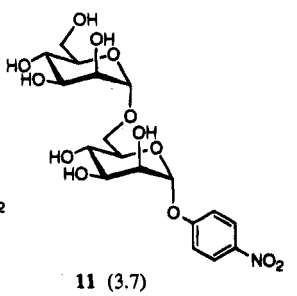

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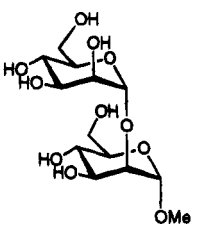

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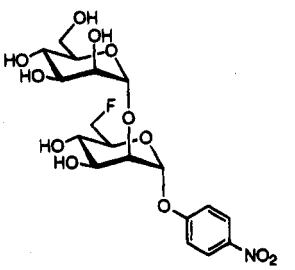

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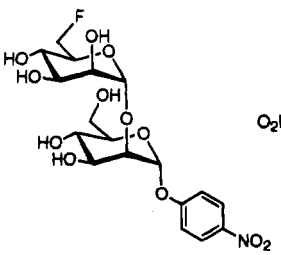

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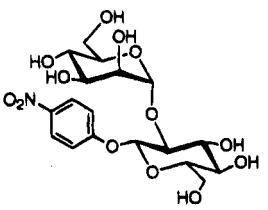

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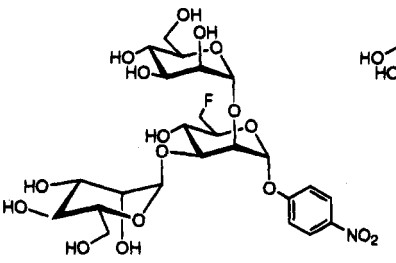

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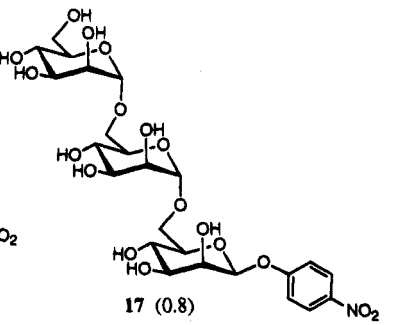

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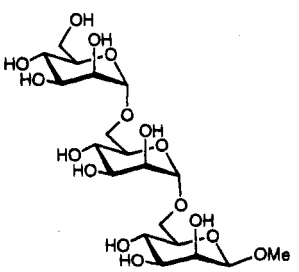

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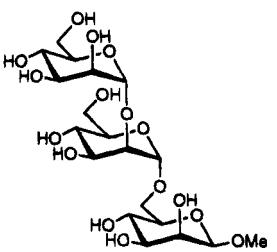

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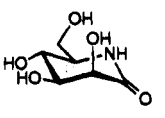

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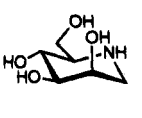

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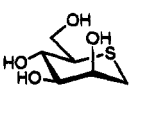

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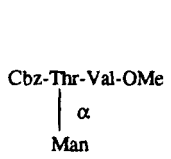

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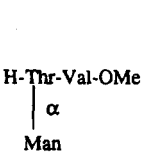

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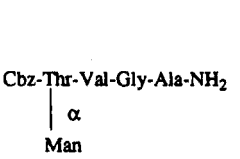

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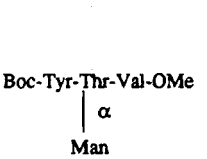

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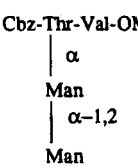
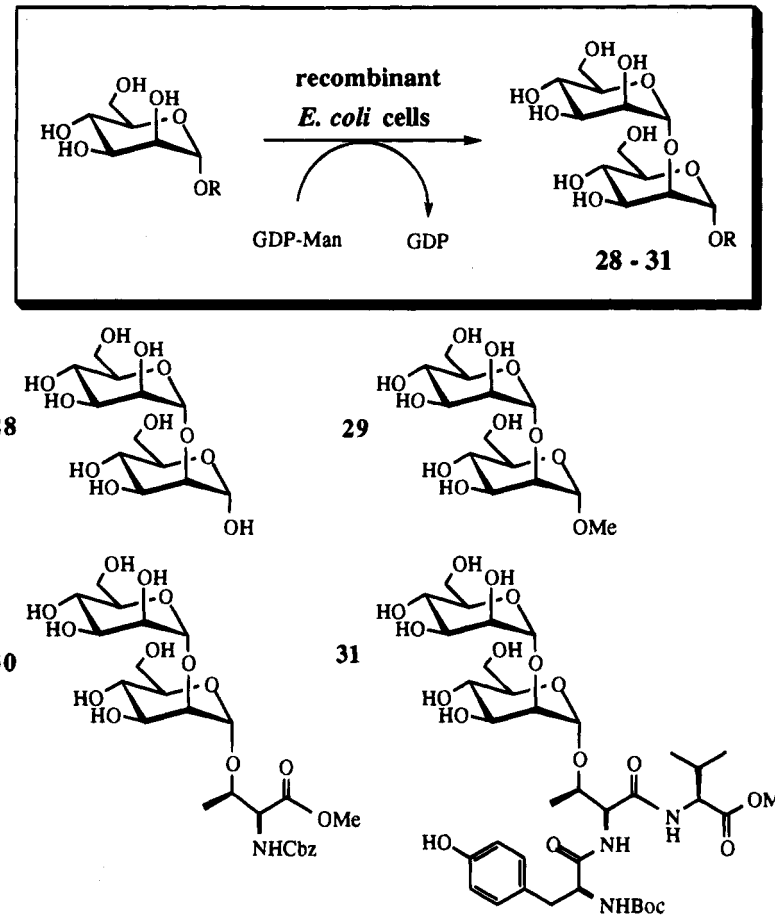


27 (17)

heterologous expression in *E. coli*. The level of the α-1,2-ManT produced could be remarkably increased by optimization of the fermentation condition. Analysis of the fermentation data demonstrate that the enzyme production is dependent upon cell growth. The broad acceptor specifity of recombinant α-1,2-ManT and its convenient

Scheme 1. Use of Whole Recombinant *E. coli* for the Synthesis of the Disaccharides α-1,2-Mannobiose (28) and Manα1,2ManOMe (29), and the Dimannosylated Glycopeptides Cbz-Thr(αManα1,2Man)OMe (30) and Boc-Tyr-Thr(αManα1,2Man)-Val-OMe (31)



access *via* the use of recombinant whole *E. coli* cells should make it a useful catalyst for enzymatic oligosaccharide synthesis. Work is in progress to prepare sufficient amounts of the enzyme for X-ray crystal structure determination and to alter the substrate and regiospecificity by site-directed mutagenesis.

Experimental Section

General. All chemicals were purchased from commercial sources as reagent grade. Silica gel 60 (Merck) was used for chromatography. The enzymatic activity of α1,2-mannosyltransferase was measured according to the reported protocol.[13,19] *E. coli* XL1-Blue strain harvesting the gene of α1,-2mannosyltransferase was developed in this laboratory and deposited with American Type Culture Collection (ATCC no. 77379).[13]

Fermentation of Recombinant *E. coli*. Fermentations of *E. coli* were performed in a 20-L Biostat E (B. Braun Biotech.) and a 150-L Biostat UE100 (B. Braun Biotech.). Batch-fermentations of *E. coli* were carried out by using M9CA medium.[13] For fed-batch fermentations the following medium was employed: 12.8 g/L $Na_2HPO_4 \cdot 7H_2O$, 3 g/L KH_2PO_4, 1.5 g/L NaCl, 6.2 g/L $(NH_4)_2SO_4$, 4.0 g/L yeast extract, 0.1 g/L vitamin B1, 5 g/L glucose, 0.24 g/L $MgSO_4$, 0.02 g/L $CaCl_2$, 20 μM $ZnSO_4$, 20 μM $MnSO_4$, 20 μM $CoCl_2$, 20 μM $CuSO_4$, 20 μM H_3BO_3, 0.1 g/L ampicillin. Fed-batch fermentations were fed by 20% (v/v) of the starting volume containing 100 g/L yeast extract, 50 g/L glucose, 0.22 g/L $CaCl_2$, 1 g/L $MgSO_4 \cdot 7H_2O$, 0.312 g/L vitamin B1, 0.1 g/L ampicillin, and 30 μM IPTG (final concentration of IPTG in the fermenter was 5 μM). Fermentations were started with an inoculum of 1% (v/v) *E. coli* in M9CA medium (OD of *ca.* 0.5). Fermentations were carried out at 37 °C and the temperature was switched to 30 °C after induction with IPTG. The system was maintained at pH 7 by adding 85% phosphoric acid or 40% NaOH. Agitation was set at 550 rpm. Partial pressure of oxygen was controlled by aeration with air (10 L/min) or with 70% oxygen (when cell density was higher than OD = 10). Excess foaming was prevented by adding DF 204 defoamer (PPG). Cells were separated after fermentation by using a centrifuge or a microfiltration unit (Pellicon 0.22 μm cassette filter, Millipore, Bedford, MA) followed by centrifugation.

Purification of α-1,2-ManT. The periplasmic part of *E. coli* cells (31.7 g wet weight, obtained from two 10 L batch-fermentations) was isolated by scaling up a procedure published previously.[13] The periplasmic component (101 mL) was dialyzed against 50 mM TRIS-buffer (4 L, pH 7.6) for 8 h at 4 °C. Subsequently, the periplasm was precipitated with ammonium sulfate (40–60%) at 4 °C and the resulting pellet was resuspended in HEPES-buffer (10 mM, pH 7.0). This solution was dialyzed extensively against 50 mM TRIS-buffer at 4 °C using Centripep-10 concentrators (Amicon, MA) to yield a clear protein solution (6 mL, 52.7 mg protein). Fast protein liquid chromatography was performed on a Pharmacia system with a 8-mL Mono Q 10/10 column. The protein solution obtained from the ammonium sulfate precipitation was applied in two runs to the column (each sample contains 26.35 mg protein) and eluted with a flow rate of 2 mL/min and a linear gradient of 0–0.6 M NaCl in TRIS-buffer (50 mM, pH 7.5). Fractions of 2.85 mL were collected. The desired fractions containing α-1,2ManT (24 mL) were dialyzed against HEPES buffer (10 mM, pH 7) at 4 °C and subsequently concentrated by using Centripep-10 concentrators (Amicon, MA) to yield pure α-1,2-ManT (4 mL, 4.1 mg of protein). SDS-PAGE was carried out on a Pharmacia Phast system by using PhastGels (gradient 8–25) with coomassie-blue staining.

Acceptor Specificity. Acceptors **1-27** were synthesized

and characterized as described previously or were obtained from Sigma: Methyl α-D-mannopyranoside (**1**) (Sigma); D-mannose (**2**) (Sigma); methyl 4,6-di-*O*-methyl-α-D-mannopyranoside (**3**);[18d] 1,6 anhydro-4-*O*-methyl-β-D-mannopyranoside (**4**);[18b] 4-nitrophenyl β-D-mannopyranoside (**5**);[18c] 4-nitrophenyl 6-deoxy-6-fluoro-α-D-mannopyranoside (**6**);[18a] 4-nitrophenyl 6-deoxy-6-fluoro-3-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (**7**);[18a] 4-nitrophenyl 3-*O*-(6-deoxy-6-fluoro-α-D-mannopyranosyl)-α-D-mannopyranoside (**8**);[18a] Methyl 6-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (**9**) (Sigma); 4-nitrophenyl 6-*O*-(α-D-mannopyranosyl)-β-D-mannopyranoside (**10**);[18c] 4-nitrophenyl 6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (**11**);[18e] methyl 2-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (**12**) (Sigma); 4-nitrophenyl 6-deoxy-6-fluoro-2-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (**13**);[18a] 4-nitrophenyl 2-*O*-(6-deoxy-6-fluoro-α-D-mannopyranosyl)-α-D-mannopyranoside (**14**);[18a] 4-nitrophenyl 2-*O*-(α-D-mannopyranosyl)-β-D-glucopyranoside (**15**);[18f] 4-nitrophenyl 6-deoxy-6-fluoro-2-*O*-(α-D-mannopyranosyl)-3-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (**16**);[18a] 4-nitrophenyl *O*-α-D-mannopyranosyl-(1-6)-*O*-α-D-mannopyranosyl-(1-6)-β-D-mannopyranoside (**17**);[18c] methyl *O*-α-D-mannopyranosyl-(1-6)-*O*-α-D-mannopyranosyl-(1-6)-β-D-mannopyranoside (**18**);[18c] methyl *O*-α-D-mannopyranosyl-(1-2)-*O*-α-D-mannopyranosyl-(1-6)-β-D-mannopyranoside (**19**);[18c] mannojirimycin lactam (**20**); 1-deoxymannojirimycin (**21**) (Sigma); 1,5-dideoxy-5-thio-D-mannopyranose (**22**).[18g]

α-1,2-ManT activity toward acceptors **1-22** was determined using the following assay-conditions: A solution containing 1 mM GDP-Man,[21] 15.6 nCi GDP-[^{14}C]Man, 20 mM acceptor **1-22** (compound **2**: 150 mM,[13] compound **21**: 32 mM), 50 mM HEPES (pH 7.2), 0.1% Triton X, 10 mM $MnCl_2$, phosphatase inhibitors[22] (4 mM ATP, 3 mM theophylline, 3 mM cimetidine), and 10 μL of purified α-1,2-ManT (0.864 milliunits) in a total volume of 40 μL was incubated for 58–60 min at 37 °C. Control reactions were carried out by omitting the acceptor **1-22**. By using purified α-1,2ManT no unspecific background reaction (hydrolysis of GDP-Man) could be detected. The assay was stopped by adding 400 μL of ice-cold QAE Sephadex A-25 in H_2O, 50% (w/v). The mixture was centrifuged for 10 min at 10000*g* and 200 μL of the supernatant was taken for radioactive counting.

Use of Whole Recombinant *E. coli* Cells.[19] Manα1,2Man (**28**):[23] To 1.5 mL of buffer (50 mM HEPES (pH 7.2), 0.1% Triton X, 10 mM $MnCl_2$, and phosphatase inhibitors[22] (5 mM ATP, 3 mM Theophylline, 3 mM Cimetidine)) was added GDP-Man (24.2 mg, 20 mM), Man (**2**) (72 mg, 200 mM), and recombinant *E. coli* cells (0.5 mL, 50% (w/w)). The mixture was stirred for 23 h at room temperature and the reaction was followed by TLC (system I, $CHCl_3$/MeOH/H_2O = 6/2.5/0.25 (v/v/v); R_f: **2** = 0.18, **28** = 0.08; system II, *i*PrOH/H_2O/NH_3 = 7/2/1 (v/v/v); R_f: **2** = 0.36, **28** = 0.26, GDP-Man = 0.16). Subsequently the *E. coli* cells were centrifuged at 10000*g* for 15 min. The pellet was washed twice with 3 mL of H_2O and the supernatants were combined with 40 mL of MeOH and stirred for 15 min. The precipitated proteins were spinned down at 10000*g* for 20 min, and the supernatant was lyophilized and applied to a Biogel P4 column (120 × 4 cm) with water as the eluent. The desired fractions were lyophilized to yield product **28** (10.3 mg, 75% yield). ^{1}H NMR data were consistent with those reported.[24] Compounds **29–31** were obtained following the same procedure. For details and spectroscopic data see ref 19.

Acknowledgment. We thank our colleagues Dr. Chris Fotsch and Wen-Chih Chou for providing compounds **20**, **21**, and **23**, and Su Chen for large-scale fermentations. The work was supported by the NIH (GM44154). G.F.H. wishes to thank the German Academic Exchange Service for financial support (Doktorandenstipendium HSPII/AUFE). E.G.-J. was supported by a fellowship from C.S.I.C. (Spain).

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