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Size-controlled synthesis of $\beta(1\rightarrow4)$ -GlcNAc oligosaccharides using an *endo*-glycosynthase

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This work is dedicated to the memory of Hugues Driguez (1942-2021)

Abstract

Chitin and peptidoglycan fragments are well recognized as pathogen associated molecular patterns (PAMPs). Long-chain oligosaccharides of $\beta(1\rightarrow4)$ -linked *N*-acetyl-D-glucosamine (GlcNAc) units indeed activate plants and mammals innate immune system. However, the mechanisms underlying PAMPs perception by lysine motif (LysM) domain receptors remain largely unknown because of insufficient availability of high-affinity molecular probes. Here, we report a two-enzyme cascade to synthesize long-chain $\beta(1\rightarrow4)$ -linked GlcNAc oligomers. Expression of the D52S mutant of hen egg-white lysozyme (HEWL) in *Pichia pastoris* at 52 mg.L⁻¹ provided a new glycosynthase catalyzing efficient polymerization of α -chitintriosyl fluoride. Selective *N*-deacetylation at the non-reducing unit of the glycosyl fluoride donor by *Sinorhizobium meliloti* NodB chitin-*N*-deacetylase abolished its ability to be polymerized by the glycosynthase but not to be transferred onto an acceptor. Using NodB and D52S HEWL in

a one-pot cascade reaction allowed the synthesis on a milligram scale of chitin hexa-, hepta- and octasaccharides with yields up to 65% and a perfect control over their size.

Introduction

In all living organisms, carbohydrate-protein interactions are at the heart of important biological events including cell development and differentiation, response to bacterial and viral infections, cell adhesion during inflammation and cancer metastasis, modulation of the immune response and signaling.^[1] The lysine motif (LysM) domain is a carbohydrate-binding module conserved across all kingdoms — except Archaea — that plays a major role in these processes.^[2,3] LysM polypeptides, approximately 50 amino acids long, are found in secreted proteins and membrane receptors that bind to *N*-acetylglucosamine (GlcNAc)-containing glycans, such as peptidoglycan (PG) and chitin. Chitin, the homopolymer of $\beta(1\rightarrow4)$ -GlcNAc units, provides structural stability to fungal and insect cell walls. Peptidoglycan (PG), made up of polysaccharide chains with alternating $\beta(1\rightarrow4)$ -linked GlcNAc and *N*-acetylmuramic acid (MurNAc) residues cross-linked by short peptide bridges, forms a protective mesh-like layer outside the plasma membrane of most Gram-positive and Gram-negative bacteria.^[4] LysM domains were initially reported from enzymes (lysozyme, autolysins and transglycosylases)^[5-7] involved in bacterial cell wall biogenesis, modification and degradation. They are also found in a large number of proteins from insects, mammals and plants involved in defense against pathogens.^[8-10] Due to the unique occurrence of chitin and PG in harmful fungal and bacterial species, plants and animals have evolved defense mechanisms based on the perception of these polysaccharides or their fragments.^[11,12] Chitin oligosaccharides (COs) not only trigger immune response in plants, but also foster mycorrhizal associations that improve the uptake of soil nutrients by plants.^[13] Recently, plant

LysM domains have been shown to be a major determinant of specificity in immunity and symbiotic signaling.^[14]

Despite the wealth of knowledge on the biological roles of LysM proteins, little information is available on the mechanism underpinning LysM-carbohydrate interactions. The lack of oligosaccharide probes hampers functional and structural studies. In addition, the strength of LysM-carbohydrate interactions increases with oligosaccharide length, with a minimum of a few sugar units being required for productive binding.^[15] PG and chitin tetrasaccharides are the shortest reported active ligands *in vitro*.^[16] *In vivo*, COs having a degree of polymerization (DP) of at least six are able to trigger innate immune response in plants, the highest activity being observed with chitinoctose (CO-VIII).^[17,18] Thus, there is a need to obtain long-chain COs (i.e. having a DP > 5) and PG oligomers more efficiently. In addition to their value for basic research, long-chain COs have an agricultural potential as green organic fertilizers and PG oligosaccharides are desirable to study antibiotic resistance, a burden that is predicted to kill more people than cancer by 2050.^[19]

To date, efficient synthesis tools for the production of long-chain GlcNAc-containing oligosaccharides are scarce. Very few examples of synthetic PG oligosaccharides have been reported in the literature so far. Chemical syntheses of PG octasaccharides were only reported by Fukase and Fujimoto's group^[20] as well as Mobashery's group.^[21] Total syntheses of COs^[22] are more documented, however the numerous steps impede the access to long-chain oligomers. Most publications indeed report the access to tetra- or hexasaccharides and to the best of our knowledge, the preparation of chitosan dodecasaccharide by Kuyama *et al.* in 1993^[23] remained an isolated success until the efficient synthesis of a biotin-tagged heptasaccharide reported recently by Nifantiev's group.^[24] In 1998, COs up to the decasaccharide were produced through acid hydrolysis of chitin with a yield of 0.2-0.3% (weight fraction) for the longest oligomers.^[25] By far, the most widespread approaches for the

production of COs rely on enzymatic reactions. We recently reported the preparation of both short- and long-chain COs by optimized depolymerization of partially deacetylated chitin with lysozyme.^[26] The use of an amorphous water-soluble substrate has allowed overcoming the recalcitrance of crystalline chitin to enzyme treatments. However, there are very few similar examples in the literature. Instead, CO synthesis has mainly been explored using the transglycosylation activity of wild-type^[27] or mutant chitinases (i.e. glycosynthases).^[28–30] The preparation of long-chain COs by transglycosylation using wild-type chitinolytic enzymes is generally impaired by hydrolytic side reactions. Nonetheless, CO-VI and CO-VII have been synthesized from chitinbiose using a lysozyme in a buffer containing a high concentration of ammonium sulphate.^[31] Glycosynthases, which are glycoside hydrolases (GHs) whose hydrolytic activity has been suppressed by site-directed mutagenesis of the catalytic nucleophile residue, have been proven to be efficient tools for glycoside bond formation.^[32] Those derived from *endo*-GHs allow efficient synthesis of polysaccharides from an activated sugar donor.^[33] Depending on the mechanism of the GH from which it derives, i.e. whether it passes through an acyl-enzyme intermediate or not, glycosynthases require either an α -glycosyl fluoride, or an oxazoline donor, as in the GH family 18 (GH18) chitinases. Although *endo*-glycosynthases allow efficient synthesis of random-size polysaccharides, their use to obtain size-controlled oligosaccharides is not straightforward for two reasons. First, disproportionation side reactions with chitinase-derived glycosynthases occur on the synthesized product leading to oligosaccharide mixtures.^[29,30] Second, polymerization of the donor must be prevented to favor a stepwise and controlled sugar glycosylation. Strategies employing temporary protected donors with chemically or enzymatically labile groups have been successful.^[34,35] Despite some examples in the literature of glycosynthase-mediated CO syntheses, the efficient production of long-chain oligomers of chitin and PG with perfect control of the oligosaccharide DP has never been described.

Here, we report a straightforward synthesis of long-chain COs using a two-enzyme cascade reaction associating a lysozyme-derived glycosynthase and a chitinoligosaccharide-*N*-deacetylase in one pot. The former enzyme allows efficient glycosylation between an α -chitintriosyl fluoride donor and CO acceptors, and the latter prevents unwanted polymerization reactions (Figure 1).

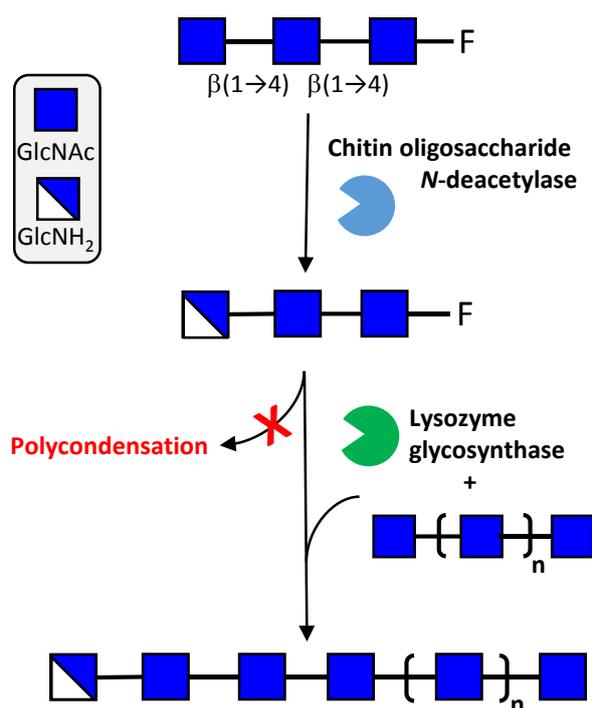


Figure 1. General strategy for the size-controlled synthesis of $\beta(1\rightarrow4)$ -linked *N*-acetyl-D-glucosamine oligosaccharides using a two-enzyme cascade reaction catalyzed by a chitin oligosaccharide-*N*-deacetylase and a lysozyme-derived glycosynthase.

Results and Discussion

Expression of HEWL mutants

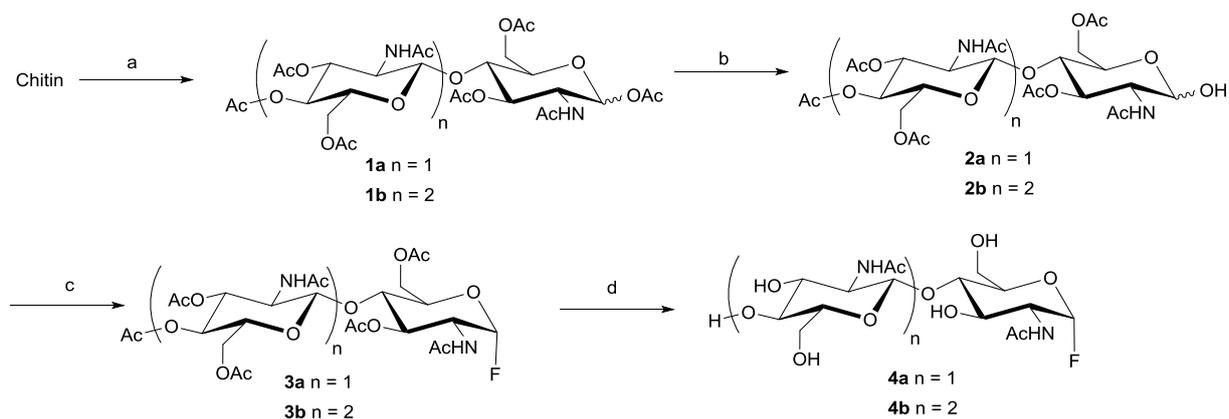
HEWL is a GH22 retaining hydrolase whose structure and mechanism have been extensively documented.^[36] In particular, the catalytic amino acids are the aspartic acid 52 and glutamate 35, which play the role of nucleophile and acid/base residues, respectively. The natural

substrate of HEWL is PG, but the enzyme also displays hydrolytic^[37] and transglycosylation^[38] activities on chitin, which should enable the synthesis of long-chain COs. Three HEWL mutants (D52A, D52S and D52N) were generated by site-directed mutagenesis. The replacement of the nucleophile by an alanine or a serine is a standard modification for designing glycosynthases.^[32,39] These modifications free up enough space in the active site for the incoming glycosyl fluoride. The asparagine mutant was intended to serve as control, because it should have neither hydrolytic nor synthetic activity due to excessive steric hindrance. The D52S HEWL mutant has previously been expressed with a yield of 12 mg.L⁻¹ in *Aspergillus niger* and its crystal structure^[40] has been solved; however its glycosynthase activity has never been demonstrated. In view of the poor expression of HEWL in *E. coli*^[41] and the complex genetic manipulation of filamentous fungi, we chose a yeast expression system. The wild-type HEWL (WT HEWL) and the three mutants were heterologously expressed in *P. pastoris* GS115 with a C-terminal His₆-tag. Flask cultures were carried out in two stages: accumulation of biomass with cultivation of the strain on a glycerol-rich medium followed by an induction phase on a methanol-rich medium. We optimized the production by varying the starting optical density (OD₆₀₀) and the amount of methanol added. The best conditions were obtained with induction started at OD₆₀₀ 30 and a daily addition of 3% (v/v) methanol at 20°C. With these conditions maintained for 5 days, the average yield of protein production reached 16 mg.L⁻¹ after purification. This yield is comparable to that previously reported for WT HEWL expression in *P. pastoris* GS115.^[42] To further increase the protein production, cell culture was next carried out in a bioreactor. Histidine was added to the growth medium to bypass *P. pastoris* GS115 auxotrophy. After 3 days of culture, a yield of up to 52 mg.L⁻¹ of protein was obtained after purification for WT HEWL as well as for the mutants (Figure S1). Although higher production yields have been reported for WT HEWL,^[42] significant proteolysis after a period of three days forced us to

stop the culture at this stage. The hydrolytic activity of the mutants was assessed in potassium phosphate buffer with a fluorescence resonance energy transfer (FRET) chitinpentaose probe (Figure S2). As expected, neither the D52A, the D52S nor the D52N mutant exhibited hydrolytic activity, and the wild-type and commercially available enzymes used as control showed comparable activity.

Glycosynthase activity of the HEWL mutants

α -Chitinbiosyl fluoride **4a** and α -chitintriosyl fluoride **4b** were synthesized to evaluate the glycosynthase activity of the mutants (Scheme 1). Disaccharide **4a** was previously prepared using a *Vibrio proteolyticus* chitobiose phosphorylase on α -fluorinated GlcNAc, albeit without information on process efficiency.^[43] In the present work, we developed a chemical synthesis starting from peracetylated CO-II **1a** and CO-III **1b**, which are easily accessible from chitin acetolysis.^[44] Selective anomeric deacetylation with hydrazine acetate^[45] afforded **2a** and **2b**, which upon fluorination with (dimethylamino)sulfur trifluoride (Me-DAST), produced a mixture of α/β fluorinated compounds. While preliminary experiments performed with (diethylamino)sulfur trifluoride (DAST) gave slightly colored products even after meticulous purification, the use of its methylated counterpart Me-DAST avoided this problem. Crystallization from methanol allowed direct isolation of pure α -chitinbiosyl fluoride pentaacetate **3a** at 23% yield. Pure α -chitintriosyl fluoride heptaacetate **3b** was obtained by crystallization at 30% yield, but after an additional isomerization step with hydrogen fluoride-pyridine.^[46] Final deprotection was achieved quantitatively using Zemplén deacetylation.



Scheme 1. Chemical synthesis of α -chitinbiosyl fluoride **4a** and α -chitintriosyl fluoride **4b**: a) Reference 44, b) $\text{NH}_2\text{NH}_2/\text{AcOH}$, DMF (**2a**: 75%, **2b**: 83%), c) Me-DAST, CH_2Cl_2 (**3a**: 23%) or Me-DAST, CH_2Cl_2 then HF-pyridine 7/3 (v/v) (**3b**: 30%), d) MeONa, MeOH (quant.).

The stability of **4b** in aqueous solution was assessed using ^1H NMR spectroscopy. At 37°C , **4b** was relatively stable as shown by the slow decrease of its H-1 signal even after 96 h (Figure S3). At 60°C , hydrolysis did not exceed 70% after 72 h confirming the good stability of the glycosyl donor. Next, polycondensation reactions of **4a** and **4b** were carried out to evaluate the glycosynthase activity of the mutants. These reactions were performed at 37°C in the protein purification buffer (50 mM Tris-HCl, 300 mM NaCl, 300 mM imidazole, pH 8, hereafter referred to as Tris-HCl purification buffer) and monitored using thin layer chromatography (TLC) and mass spectrometry. No detectable transglycosylation product was observed with the D52A and D52N mutants. We hypothesized that the high stability of the glycosyl fluoride was possibly a shortcoming for a glycosynthase-based strategy. The formation of insoluble products in the reactions containing the D52S mutant has eliminated this uncertainty by demonstrating the ability of this enzyme to polymerize **4a** and **4b**. Hydrolysis of the precipitates by *Bacillus circulans* WL-12 chitinase A1^[30] released chitinbiose as major product, confirming that the insoluble product was indeed chitin. Analysis of the crude polycondensation reaction mixtures by mass spectrometry showed the

formation of water-soluble COs (Figure 2). As expected, the reaction with α -chitintriosyl fluoride **4b** led to the formation of oligosaccharides with DPs of multiples of three exclusively, whereas products of even DPs were obtained from α -chitinbiosyl fluoride **4a**.

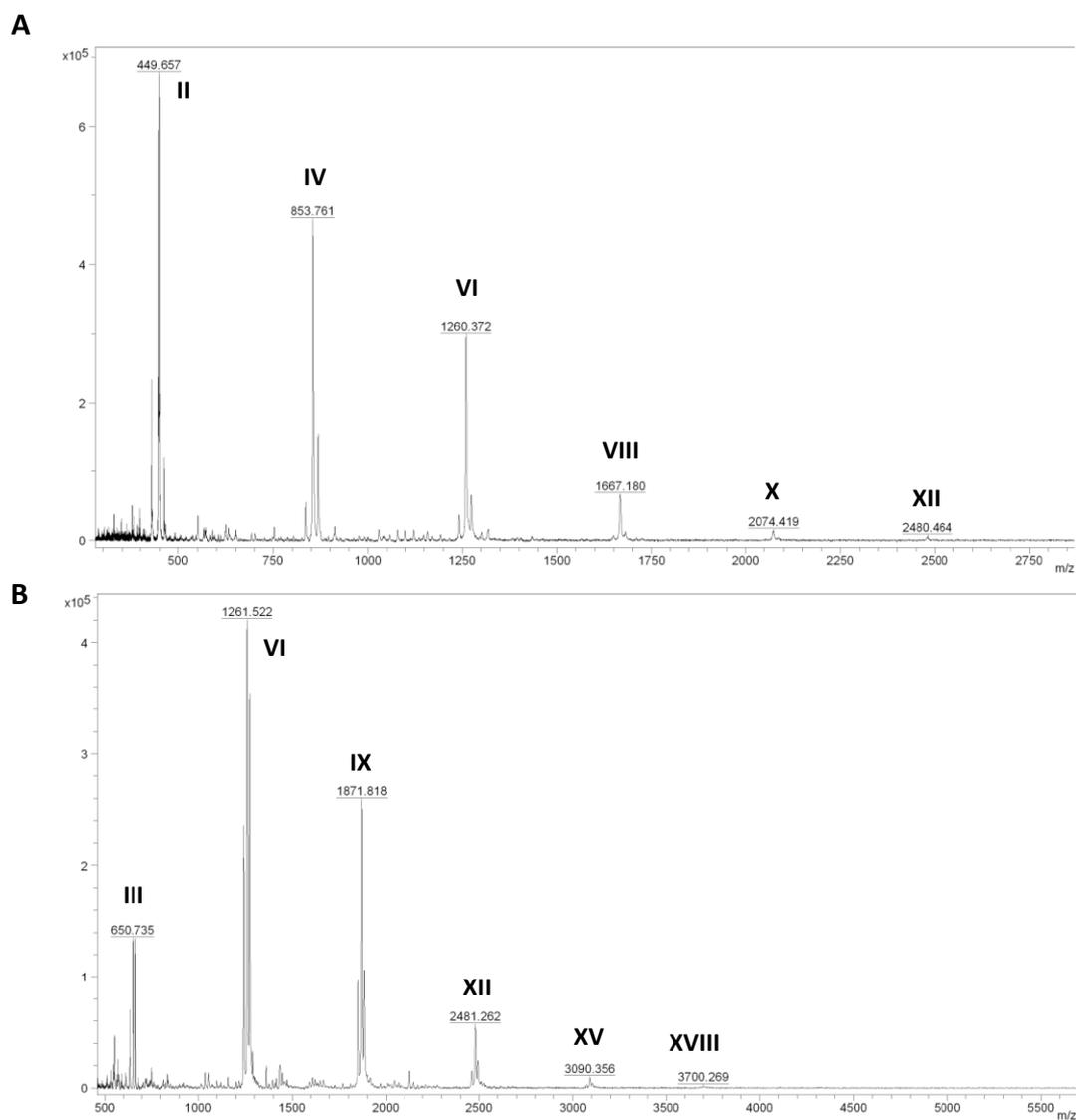


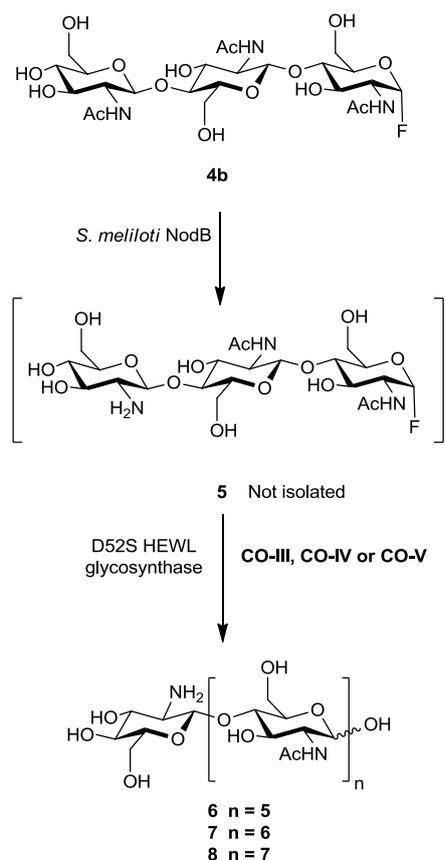
Figure 2. MALDI-TOF spectra of the polycondensation reactions catalyzed by HEWL D52S mutant using (A) α -chitinbiosyl fluoride **4a** and (B) α -chitintriosyl fluoride **4b**. Roman numerals indicate the degree of polymerization of the reaction products. Product masses (m/z) correspond to the $[M + Na]^+$ molecular adducts of COs.

Unlike family GH18 and GH20 glycosynthases,^[29,30] D52S HEWL did not display notable disproportionation activity, making it a promising tool for the synthesis of long-chain COs with perfect control of their size. Although the D52A mutant was expected to display glycosynthase activity, it has previously been observed that serine mutations sometimes outstrip those generated by the original alanine mutations.^[39] In the present case, one hypothesis is that the serine mutant has better affinity than the alanine mutant does for the donor, either in the ground state or in the transition state. The X-ray structure of the D52S mutant in complex with CO-IV showed that serine 52 in subsite -1 (D) creates favorable interactions with the oxygen atom at C1 in the α -configuration.^[40] This mutation also induces a sofa conformation of the sugar ring in subsite -1 (D) that approaches that of the transition state in the wild-type enzyme. In addition, a favorable electronic interaction of serine with glycosyl fluoride may occur.^[47] TLC monitoring of polycondensation reactions clearly showed better conversion efficiency for trisaccharide **4b** over the disaccharide **4a** (Figure S4). This result is consistent with a previous study reported by Yang *et al.* showing that HEWL has a 33-fold higher affinity for CO-III than for CO-II.^[48] Therefore, α -chitintriosyl fluoride **4b** was selected as the donor for the synthesis of long-chain COs in a cascade reaction with *S. meliloti* NodB chitin oligosaccharide *N*-deacetylase.

Size-controlled synthesis of long-chain COs

Taking advantage of the fact that a glucosaminyl unit (GlcNH₂) can be accommodated by HEWL in subsite -3 (B), but not in subsite +1 (E),^[49,50] we presumed that selective *N*-deacetylation of **4b** at the non-reducing unit would provide a non-polymerizable donor. Chitin oligosaccharide *N*-deacetylase NodB from *S. meliloti*, which displays the required specificity, was previously expressed in its active form in *E. coli* and efficiently applied to the synthesis of lipochitin oligosaccharides (LCOs).^[51] To develop a one-pot two-enzyme cascade with

NodB and D52S HEWL that avoids purification of the unprotected glycosyl fluoride, the conditions for NodB deacetylation were investigated. Temperature-induced denaturation of NodB was rejected to prevent the hydrolysis of the glycosyl fluoride. Instead, deactivation of the deacetylase was achieved by addition of tert-butanol to the reaction mixture. NodB is indeed irreversibly inactivated by the presence of 20% (v/v) of this alcohol (Figure S5) which offers the advantage of being easily evaporated under reduced pressure or by freeze-drying. Selective *N*-deacetylation of **4b** with NodB in 20 mM MOPS buffer pH 7.4 supplemented with 10 mM dithiothreitol and 1 mM manganese sulphate proceeded quantitatively affording trisaccharide **5** (Scheme 2). After NodB deactivation and solvent evaporation, crude fluoride **5** was glycosylated with chitintriose (CO-III), chitintetraose (CO-IV) or chitinpentaose (CO-V) using D52S HEWL in Tris-HCl purification buffer.



Scheme 2. One-pot two-enzyme cascade synthesis of chitin hexa-, hepta-, octasaccharide.

To optimize the reaction conditions, we decided to keep the reaction time (72 h) and the donor/acceptor ratio (2:1) constant. Only the temperature and the amount of glycosynthase were modified. The reactions were performed at 37°C and 60°C and the catalyst loading was varied from 0.3% to 1.2% with regard to the acceptor concentration. The reactions were conducted on an analytical scale (0.24 μmol acceptor) and monitored using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis and analytical high-performance size-exclusion chromatography (HP-SEC) (Figure 3 and S6).

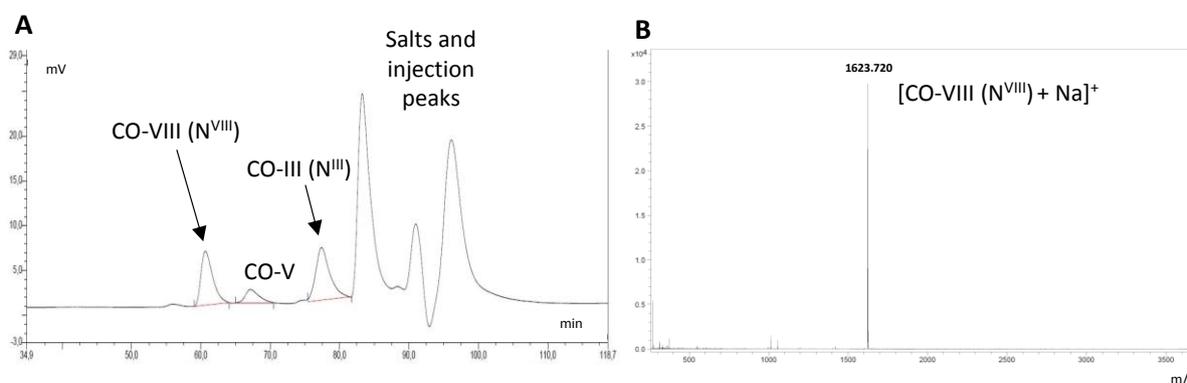


Figure 3. (A) Analytical HP-SEC chromatogram of D52S HEWL glycosynthase-mediated synthesis of **8** (2:1:0.006 donor:acceptor:D52S HEWL molar ratio, 60°C, 72 h). (B) MALDI-TOF mass spectrum of **8** after purification by analytical HP-SEC.

The reactions, which were carried out using CO-III, CO-IV and CO-V as acceptors, led to the formation of CO-VI (N^{VI}) **6**, CO-VII (N^{VII}) **7** and CO-VIII (N^{VIII}) **8** respectively as the unique glycosylation products. Neither disproportionation reactions nor product hydrolysis were observed. The yields of the reactions reported in Table 1 were determined using HP-SEC by quantifying the amount of products **6-8** against a calibration curve obtained with pure CO-VIII (N^{VIII}) (Figure S7) prepared by action of NodB on CO-VIII.^[26]

With the lowest amount of glycosynthase (0.3%), modest yields of 24 and 27% were observed for reactions conducted with **5** and CO-V at 37°C and 60°C, respectively (Table 1, Entries 1 and 2). Increasing the amount of enzyme led to significant conversion improvements. The formation of **8** increased from 41% yield to 64% using 0.6% (Entry 5) and 1.2% (Entry 6) of glycosynthase respectively. The degree of polymerization of the acceptor did not have a significant impact on the reaction since hexa- and heptasaccharides **6** and **7** were produced with a yield of 47 and 42% respectively (Entries 3 and 4) that is similar to that obtained for **8** under similar reaction conditions. Of note, the complex reaction media consisting of a mixture of Tris-HCl purification buffer and MOPS buffer only slightly or not at all influenced the glycosynthase activity. Attempts to run the reaction in phosphate buffer instead of Tris-HCl purification buffer did not lead to any further improvement.

Table 1. Effect of the temperature and glycosynthase:acceptor molar ratio on D52S HEWL glycosynthase-mediated synthesis of chitin oligosaccharides

Entry	Donor	Acceptor	Enzyme/Acceptor Molar ratio	Temperature	Product	Yield
1	5	CO-V	0.3%	37°C	8	24%
2	5	CO-V	0.3%	60°C	8	27%
3	5	CO-III	0.6%	60°C	6	47%
4	5	CO-IV	0.6%	60°C	7	42%
5	5	CO-V	0.6%	60°C	8	41%
6	5	CO-V	1.2%	60°C	8	64%

To confirm the efficiency of the process for the synthesis of long-chain COs, a final reaction was conducted on a scale of a few milligrams using the optimized conditions. Octasaccharide

8 was purified by preparative HP-SEC and isolated with a reproducible yield of 65%. In addition to mass spectrometry, ^1H and ^{13}C NMR spectroscopy (Figure S8 & S9) were used to characterize **8**. The ^{13}C spectrum of **8** is rather complex because of several overlapping signals. In contrast, the ^1H NMR analysis unambiguously confirmed the structure of **8**. The ^1H spectrum is indeed very similar to the one of tetra- N,N^2,N^3,N^4 -acetyl-chitinpentaose produced in *E. coli* using the chitin synthase NodC and the deacetylase NodB.^[52] Among the most representative signals, H-2 of the non-reducing unit produces a triplet at 2.71 ppm ($J = 8.0$ Hz). The anomeric protons appear as doublets with a coupling constant of 8.0 Hz between 4.50 and 4.71 ppm. A doublet at 5.20 ppm ($J = 1.7$ Hz) was assigned to the H-1 of the α -anomer reducing-end. Taken together, these data support the applicability of our method to obtain long-chain $\beta(1\rightarrow4)$ -GlcNAc-containing oligosaccharides in appropriate amounts for biological studies.

Conclusion

In this study, we designed a novel and efficient enzymatic synthesis route for obtaining long-chain $\beta(1\rightarrow4)$ -GlcNAc oligosaccharides. A glycosynthase derived from HEWL was produced at $52\text{ mg}\cdot\text{L}^{-1}$ in *P. pastoris*. The D52S HEWL mutant not only efficiently polymerized α -chitintriosyl fluoride, but it also led to the synthesis of COs perfectly controlled in size when used in a cascade reaction with NodB chitin oligosaccharide *N*-deacetylase. Oligosaccharides with a DP of up to eight (i.e. octasaccharide) with a GlcNH_2 at the non-reducing end were produced with a satisfactory yield of 65% over the two enzymatic steps. The non-reducing amino function offers a unique opportunity to introduce specific groups such as fatty acid chains for designing LCO analogues as well as chemical reporters or cross-linking groups for producing affinity-labelling probes. Acetylation of the non-reducing unit provides long-chain COs, which are important probes in the study of plant immunity. Finally, HEWL

glycosynthase offers a new perspective for the synthesis of PG oligomers. These molecules hold the key to progress in the understanding of PG metabolism, which is needed to tackle the issue of antibiotic resistance. New PG oligomers are currently being synthesized for this purpose using the D52S HEWL glycosynthase. Their preparation and use for the structural characterization of PG-binding proteins will be reported elsewhere in due course.

Experimental Section

General

Reagents for bacterial and yeast media were obtained from Euromedex (Mundolsheim, France) and Invitrogen (Cergy-Pontoise, France). Reagents for molecular biology were obtained from Invitrogen, Euromedex, Macherey-Nagel (Hoerd, France) and Thermo Fisher Scientific (Villebon sur Yvette, France). High and long fidelity polymerase enzyme mix was purchased from New England Biolabs (Evry, France) and the ligation mix was from Takara (Saint-Germain-en-Laye, France). Oligonucleotides were purchased from Eurofins MWG Operon (Germany). Chemicals were of reagent grade from Sigma-Aldrich (Saint Quentin-Fallavier, France) or TCI (Europe) and used directly without further purification.

Reactions were monitored using thin layer chromatography (TLC) on silica gel (Merck Silica gel 60 F254, Darmstadt). Carbohydrates were visualized with either 30% ammonium bisulfate or diphenylamine–aniline–phosphoric acid (4 g diphenylamine, 4 mL aniline, 2 mL HCl 37%, 20 mL 85% H₃PO₄ in H₂O, 200 mL ethyl acetate) staining solutions followed by heating with a heat gun. Silica gel chromatography purifications were carried out using wet-packed silica, Merck 0.04–0.063 mm. Mass analyses (ESI) were recorded with an ESQUIRE 3000+ (Bruker Daltonics, Wissembourg, France). MALDI-TOF spectra were recorded on a MALDI-TOF/TOF Autoflex Speed (Bruker Daltonics, Wissembourg, France) equipped with a laser emitting at 355 nm with a frequency of 250 Hz. 2,5-Dihydroxybenzoic acid (DHB) was used

as a matrix at a concentration of 50 g.L⁻¹ in water. COs samples were dissolved in water or dimethylsulfoxide and mixed with the same volume of matrix solution before air-drying at room temperature on the MALDI sample plate. HRMS spectra were recorded with a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Villebon sur Yvette, France).

NMR spectra were recorded at 298 K in CDCl₃, D₂O, or CD₃OD, with a liquid Bruker Avance III 400 MHz spectrometer or a liquid Bruker Avance III 500 MHz spectrometer (Bruker, Wissembourg, France). For ¹H NMR the solvent residual peaks of D₂O, CD₃OD, and CDCl₃ were used as internal standards at 4.78 ppm, 3.31 ppm, and 7.26 ppm, respectively. Data for ¹H NMR are reported in the conventional form: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (Hz), integration. Data for ¹³C are reported in terms of chemical shift (δ ppm). For ¹³C NMR the solvent residual peak of CDCl₃ was used as internal standard at 77.0 ppm.

Analytical size exclusion chromatography was performed with two Superdex peptide columns (10-300GL) (GE Healthcare, Uppsala, Sweden) in series using a refractive index detector RI Iota 2 (Interchim, Montluçon, France). Elution was performed with 0.1 M NaCl at a flow rate of 0.4 mL.min⁻¹. Preparative size exclusion chromatography was performed with three Hiload Superdex 30 columns (GE Healthcare, Uppsala, Sweden) in series using refractive index detection. Elution was performed with 0.1 M carbonate ammonium at a flow rate of 1.2 mL.min⁻¹.

Strains and plasmids

Escherichia coli (*E. coli*) TOP 10 (Invitrogen) cells were used for plasmid propagation. *Pichia pastoris* (*P. pastoris*) strain GS115 (*his4*) and the plasmid pPICZ α A used for HEWL glycosynthase production were from Invitrogen. The native HEWL gene is a kind gift from Dr Donald A. Mac Kenzie (Institute of Food research, Norwich UK).

Media composition

E. coli cells were cultured in low salt LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) to allow transformants selection on zeocin ($25 \mu\text{g}\cdot\text{mL}^{-1}$). *P. pastoris* was grown in yeast extract peptone dextrose medium (YPD, 1% yeast extract, 2% peptone, 2% dextrose), buffered glycerol-complex medium (BMGY, 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base with ammonium sulfate without amino acids, $4\times 10^{-5}\%$ biotin and 1% glycerol) or buffered methanol-complex medium (BMMY, 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base with ammonium sulfate without amino acids, $4\times 10^{-5}\%$ biotin and 3% (v/v) methanol). Fermentation basal salts medium consists of, per liter: 40.0 g glycerol, 0.93 g calcium sulfate, 18.2 g potassium sulfate, 14.9 g magnesium sulfate, 7 H₂O, 26.7 mL phosphoric acid (85%), 4.13 g potassium hydroxide, 10 g histidine, and 4.35 mL PTM1 trace salts. PTM1 trace salts contained, per liter: 6.0 g cupric sulfate, 5 H₂O, 0.08 g sodium iodide, 3.0 g manganese sulfate, H₂O, 0.2 g sodium molybdate, 2 H₂O, 0.02 g boric acid, 0.5 g cobalt chloride, 20.0 g zinc chloride, 65.0 g ferrous sulfate, 7 H₂O, 0.2 g biotin, and 5.0 mL sulfuric acid.

Construction of the wild-type hen egg-white lysozyme (HEWL) expression vector

Hen egg-white lysozyme gene was PCR amplified without its native signal peptide using forward (5'-CCCCTGGCTGCTGAATTCAAAGTCTTT-3') and reverse (5'-GGGTCTAGACGGCAGCCTCTGATCCAC-3') primers containing respectively EcoRI and XbaI restriction sites (underlined). The PCR product was cloned into the pPICZ α A vector between restriction sites EcoRI and XbaI in frame with an N-terminal peptide encoding the *Saccharomyces cerevisiae* α -factor secretion signal and a C-terminal peptide containing the c-myc epitope and a polyhistidine (6xHis) tag. The final construct (pPICZ α A-HEWL WT) was verified by DNA sequencing.

Preparation of HEWL mutants

Site-directed mutagenesis was performed on the pPICZ α A-HEWL WT vector. The aspartate 52 was mutated by Quick Change Mutagenesis® methodology using the following primers: 5'-GTACCG**CTT**ACGGAATCCTGCAGATC-3' and 5'-GATCTGCAGGATTCCGTA**AGCGG**TAC-3' for D52A, 5'-GTACCA**ACT**ACGGAATCCTGCAGATC-3' and 5'-GATCTGCAGGATTCCGTA**AGCGG**TAC-3' for D52N, 5'-CCGATGGGAGTAC**CTT**ACGGAATCCTGCAGATCAACAGC-3' and 5'-GCTGTTGATCTGCAGGATTCCGTA**AGAGG**TACTCCCATCGG-3' for D52S (mutated codon in bold, base pair substitution in italic). A PstI silent mutation (CTGCAG) was inserted for screening of positive clones. All sequences were confirmed by DNA sequencing.

Transformation of *P. pastoris* GS115

Electrocompetent *P. pastoris* GS115 cells were prepared according to the procedure described by Lin-Cereghino *et al.*^[53] The four pPICZ α A-HEWL vectors were linearized by digestion with PmeI. *P. pastoris* GS115 was transformed by electroporation using an electroporator set at 1500 V and 0.2 cm ice-cold cuvettes (Eppendorf, Montesson, France). Ice-cold 1 M sorbitol (1 mL) was immediately added to the cuvette and cells were incubated for recovery during 1 h at 30°C without shaking. YPD (1 mL) was then added and cells were shaken at 230 rpm at 30°C for one more hour. After incubation, they were plated on YPDS (YPD containing 1 M sorbitol) plates with 100 $\mu\text{g}\cdot\text{mL}^{-1}$ zeocin and incubated at 30°C for 3 days.

Enzyme production and purification

For small-scale production in flasks, a single transformant was grown in 10 mL of YPD medium at 30°C and 230 rpm for one day. BMGY medium (1 L) was inoculated with the pre-culture at $\text{OD}_{600} = 0.04$ and shaken at 30°C and 230 rpm until the OD_{600} reached 2 to 6 (approximately 16 h). The pellet was recovered by centrifugation (6000 rpm, 4°C during 10

min) and cells were then transferred to 200 mL of BMMY medium and the culture was incubated at 20°C in an orbital shaker (230 rpm) for 5 days, with a daily supplementation of 3% (v/v) methanol.

For large-scale production, fermentation was performed in a 3 L jar fermenter (Applikon biotechnology, Holland) under the control of temperature, pH, and dissolved oxygen. During fermentation, the temperature was maintained at 30°C or 20°C with a heating unit and a refrigerated circulating bath. The concentration of dissolved oxygen was maintained above 20%. The pH was adjusted to 5.0 through the addition of 35% ammonium hydroxide. The aeration rate was set on 5 L.min⁻¹. Fermentation was initiated by adding 100 mL of an inoculum grown overnight in BMGY medium (optical density of the inoculum around 6) to 900 mL of fermentation basal salts medium containing 4% glycerol, 1% histidine and 0.435% PTM1 trace salts at 30°C. After complete consumption of the glycerol in the medium, an adaptation batch phase was initiated at 30°C by the addition of a 200 mL solution containing 25% sorbitol, 2.5% methanol, 1% histidine and 2% PTM1 trace salts. When all the carbon sources were consumed (3-4 h), a methanol fed-batch phase was carried out at 20°C by the addition of 70% methanol containing 1% histidine and 1.2% PTM1 trace salts at a flow rate of 2.9 mL.h⁻¹ with a peristaltic pump. After one night, the feeding rate was increased to 5.7 mL.h⁻¹ for another day of induction. The total fermentation time was 72 h.

At the end of the expression period, the culture was spun down 10 min at 6000 rpm and 4°C. The pH of the supernatant was adjusted at 8.0 with 5 M sodium hydroxide. Supernatant was filtered through a 0.22 µm cellulose acetate membrane and HEWL was purified on a 1 mL Ni-NTA column (HisTrap FF crude, GE Healthcare, Buc, France) using NGC Chromatography system (Bio-Rad, Marnes-la-Coquette, France). The column was equilibrated and washed with 50 mM Tris-HCl buffer pH 8 containing 300 mM NaCl. Then HEWL protein was eluted with the same buffer containing 300 mM imidazole, later referred to as Tris-HCl purification

buffer, over 20 column volumes. The purity of the collected HEWL fractions was analyzed using 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining. Pure fractions were pooled. The enzyme was then stored in the Tris-HCl purification buffer. The protein concentration was determined using the Bradford assay with BSA as standard.^[54] The enzyme was then flash-frozen in liquid nitrogen, and stored at -20°C in the Tris-HCl purification buffer. The average yield of enzyme production was $16\text{ mg}\cdot\text{L}^{-1}$ in flask and $52\text{ mg}\cdot\text{L}^{-1}$ in bioreactor. All enzymes were stable under storage for months (-20°C).

Hydrolysis activity assay

To assess the hydrolytic activity of each mutant, a fluorescence resonance energy transfer (FRET) chitinpentaose probe was used. The synthesis and full characterization of this probe whose structure is shown in the Electronic Supplementary Material (Figure S2) will be reported in a forthcoming publication. Reactions were performed in 96-well plates (PS, non-binding, Greiner Bio-One, Germany) in $200\ \mu\text{L}$ total volume using 60 mM phosphate buffer pH 6. Reactions contained 2.4 nmol of FRET probe and 2.8 nmol of HEWL and were run at 30°C . Fluorescence was measured at 490 nm throughout time using a CLARIOstar (BMG Labtech, Champigny sur Marne, France) after excitation at 340 nm . As a positive control, commercial HEWL (Sigma Aldrich) was used.

Synthesis of 4a

Chitinbiose hexaacetate **1a** (345 mg , 0.51 mmol) prepared from chitin acetolysis^[44] was added to a solution of hydrazine acetate (57 mg , 0.6 mmol) in dry DMF (3.5 mL), and the resulting mixture was stirred for 2 h at room temperature. Another portion of hydrazine acetate (57 mg , 0.6 mmol) in DMF (0.3 mL) was added, and the solution was stirred for 15 min at room temperature, and then added dropwise to diethyl ether (150 mL) with continuous stirring. After filtration, the solid was subjected to flash-column chromatography [first toluene-acetone

(1:1.5), then (1:2)] giving α -chitinbiose pentaacetate **2a** (241 mg, 75%). Physical data agreed with those reported by Flitsch *et al.*^[55]

A solution of α -chitinbiose pentaacetate **2a** (328 mg, 0.52 mmol) in methylene chloride (25 mL) was cooled to -30°C , (dimethylamino)sulfur trifluoride (0.13 mL, 1.33 mmol) was added and the resulting mixture was stirred from -30°C to room temperature for 1 h. The solution was concentrated under reduced pressure, co-evaporated two-times with toluene, and the residue was subjected to flash-column chromatography [first CH_2Cl_2 , then CH_2Cl_2 -MeOH (9:1)] to give an α/β mixture of chitinbiosyl fluoride pentaacetate (149 mg). Crystallization from CH_2Cl_2 -MeOH gave pure α -chitinbiosyl fluoride pentaacetate **3a** (75 mg, 23%); m.p. 250°C (dec.); ^1H NMR (400 MHz, CDCl_3 - CD_3OD 2:1): δ 5.48 (dd, 1 H, $J_{1,\text{F}} = 52.7$, $J_{1,2} = 2.6$, H-1^I), 5.22 (dd, 1 H, $J_{2,3} = 10.2$ and $J_{3,4} = 9.5$, H-3^{II}), 5.17 (dd, 1 H, $J_{2,3} = 10.7$ and $J_{3,4} = 9.2$, H-3^I), 4.96 (t, 1 H, $J_{3,4} = 9.6$, H-4^{II}), 4.66 (d, 1 H, $J_{1,2} = 9.1$, H-1^{II}), 4.28 (dd, 1 H, $J_{5,6} = 1.1$ and $J_{6,6} = -11.9$, H-6a^I), 4.36 (dd, 1 H, $J_{5,6} = 4.2$ and $J_{6,6} = -12.4$, H-6a^{II}), 4.22 (ddd, 1 H, $J_{2,\text{F}} = 2.5$, H-2^I), 4.09 (1 H, H-5^{II}), 4.05 (1 H, H-6b^I), 3.98 (1 H, $J_{5,6} = 2.0$ and $J_{6,6} = -12.4$, H-6b^{II}), 3.81 (t, 1 H, $J_{3,4} = 9.5$, H-4^I), 3.69 (1 H, H-2^{II}), 3.67 (1 H, H-5^I), 1.96, 1.90, 1.88, 1.83, 1.82, 1.79 and 1.72 (7 s, 21 H, 7 COCH₃); ^{13}C NMR (100 MHz, CDCl_3 - CD_3OD 2:1) : δ 171.6, 171.4, 171.1, 170.8, 170.7, 170.6, 169.6 (7 COCH₃), 105.5 (d, $J_{\text{C,F}} = 222$, C-1^I), 100.3 (C-1^{II}), 74.7 (C-4^I), 72.0 (C-3^{II}), 71.3 (C-5^{II}), 70.6 (d, $J_{\text{C,F}} = 4$, C-5^I), 69.9 (C-3^I), 68.1 (C-4^{II}), 61.6 (C-6^I, C-6^{II}), 54.5 (C-2^{II}), 51.5 (d, $J_{\text{C,F}} = 24$, C-2^I), 21.9, 22.2 (2 NHCOCH₃), 20.0, 20.1, 20.2, 20.2, 20.3 (5 COCH₃); HRMS (ESI) m/z $[\text{M}+\text{Na}]^+$ calcd for $\text{C}_{26}\text{H}_{37}\text{FN}_2\text{NaO}_{15}$ 659.2076, found 659.2083.

Sodium methoxide (10 μL , 1 M in MeOH) was added to a solution of α -chitinbiosyl fluoride pentaacetate **3a** (4.2 mg, 6.6 μmol) in MeOH (1 mL). The solution was stirred for 1 h at room temperature and then neutralized with a strong acid cation-exchange resin (Amberlite® IRN-77 H⁺ form). After filtration and concentration under reduced pressure, α -chitinbiosyl fluoride

4a was freeze-dried (2.0 mg, 100%); ^1H NMR (400 MHz, D_2O): (*inter alia*) δ 5.58 (dd, 1 H, $J_{1,\text{F}} = 53.5$, $J_{1,2} = 2.5$, H-1^I), 4.5 (d, 1 H, $J_{1,2} = 8.3$, H-1^{II}); MS (ESI) m/z 449 $[\text{M}+\text{Na}]^+$, m/z 429 $[\text{M}-\text{HF}+\text{Na}]^+$.

Synthesis of **4b**

Chitintriase octaacetate **1b** (240 mg, 0.25 mmol) prepared from chitin acetolysis^[44] was added to a solution of hydrazine acetate (50 mg, 0.52 mmol) in dry DMF (1.6 mL), and the solution was stirred for 0.5 h at 50°C. The mixture was added dropwise to diethyl ether (50 mL) with continuous stirring. After filtration the solid was washed with diethyl ether and recrystallized from MeOH giving chitintriase heptaacetate **2b** as a mixture of anomers (191 mg, 83 %); MS (ESI) m/z 944 $[\text{M}+\text{Na}]^+$; HRMS (ESI) m/z $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{38}\text{H}_{56}\text{N}_3\text{O}_{23}$ 922.3299, found 922.3299.

A solution of chitintriase heptaacetate **2b** (230 mg, 0.24 mmol) in methylene chloride (11 mL) was cooled to -20°C, (dimethylamino)sulfur trifluoride (50 μL , 0.51 mmol) was added and the resulting mixture was stirred from -20°C to room temperature for 1 h. The solution was concentrated under reduced pressure and the residue was dissolved in a mixture of HF-pyridine [2 mL, (7:3)] cooled to -50°C and stirred at this temperature for 1 h. The mixture was diluted with CHCl_3 -MeOH [3 mL, (2:1)], and aq. ammonia (30%) was added dropwise until neutralization to pH 7. The resulting solution was diluted with CHCl_3 , washed successively with aq. potassium hydrogen sulfate, aq. sodium hydrogen carbonate and brine, dried (Na_2SO_4), and concentrated under reduced pressure. Flash-column chromatography [first CH_2Cl_2 , then CH_2Cl_2 -MeOH (9:1)] gave an α/β mixture of chitintriosyl fluoride heptaacetate (122 mg). Crystallization from CH_2Cl_2 -MeOH gave pure α -chitintriosyl fluoride heptaacetate **3b** (70 mg, 30%); m.p. 250°C (dec.); ^1H NMR (400 MHz, CDCl_3 - CD_3OD 2:1): δ 5.47 (dd, 1 H, $J_{1,\text{F}} = 52.6$, $J_{1,2} = 2.5$, H-1^I), 5.20 (dd, 1 H, $J_{2,3} = 10.4$ and $J_{3,4} = 9.5$, H-3^{III}), 5.17 (dd, 1 H, $J_{2,3} = 10.6$ and $J_{3,4} = 9.3$, H-3^I), 5.07 (dd, 1 H, $J = 8.5$ and 10.2 , H-3^{II}), 4.96 (t, 1 H, $J_{3,4} = J_{4,5} =$

9.6, H-4^{III}), 4.60 (1 H, $J_{1,2} = 8.4$, H-1^{III}), 4.53 (1 H, $J_{1,2} = 8.1$, H-1^{II}), 4.45 and 4.09 (2 H, H-6ab^I), 4.35 and 4.00 (2 H, H-6ab^{III}), 4.30 and 4.06 (2 H, H-6ab^{II}), 4.21 (ddd, 1 H, $J_{2,F} = 2.4$, H-2^I), 3.98 (dd, 1 H, H-5^{III}), 3.79 (t, 1 H, $J_{3,4} = 9.4$, H-4^I), 3.76 (1H, H-2^{II}), 3.69 (2 H, H-4^{II} and H-2^{III}), 3.60 (1H, H-5^I), 3.59 (1 H, H-5^{II}), 2.10, 2.09, 2.03, 1.99, 1.98, 1.96, 1.95, 1.9, 1.87, 1.86 (10 s, 30 H, 10 COCH₃); ¹³C NMR (100 MHz, CDCl₃-CD₃OD 2:1): δ 172.71, 172.63, 172.54, 172.10, 172.03, 171.76, 171.62, 171.54, 171.43 and 170.66 (10 COCH₃), 106.4 (d, $J_{1,F} = 213$, C-1^I), 101.3 (C-1^{III}), 101.1 (C-1^{II}), 76.7 (C-4^{II}), 75.7 (C-4^I), 73.5 (C-3^{II}), 73.3 (C-5^{II}), 72.9 (C-3^{III}), 72.2 (C-5^I), 71.4 (C-5^{III}), 70.9 (C-3^I), 69.0 (C-4^{III}), 63.3 (C-6^{II}), 62.5 (C-6^I), 62.4 (C-6^{III}), 55.4 (C-2^{III}), 55.1 (C-2^{II}), 52.5 (d, $J_{2,F} = 24$, C-2^I), 23.1 (2 NHCOCH₃), 22.8 (NHCOCH₃), 21.2 to 21.0 (7 OCOCH₃); HRMS (ESI) m/z [M+Na]⁺ calcd for C₃₈H₅₄FN₃NaO₂₂ 946.3075, found 946.3052.

Sodium methoxide (80 μ L, 1 M in MeOH) was added to a solution of α -chitintriosyl fluoride heptaacetate **3b** (9 mg, 9.7 μ mol) in MeOH-CHCl₃ (8 mL, 20:1). The solution was stirred for 6 h at room temperature and then neutralized with a strong acid cation-exchange resin (Amberlite® IRN-77 H⁺ form). After filtration and concentration under reduced pressure, α -chitintriosyl fluoride **4b** was freeze-dried (5.6 mg, 92%); ¹H NMR (400 MHz, D₂O): (*inter alia*) δ 5.63 (dd, 1 H, $J_{1,F} = 53$, $J_{1,2} = 2.8$, H-1^I), 4.60 (d, 1 H, $J_{1,2} = 8.1$, H-1^{II} or H-1^{III}), 4.59 (d, 1 H, $J_{1,2} = 8.4$, H-1^{II} or H-1^{III}); MS (MALDI-TOF) m/z 652 [M+Na]⁺.

Polycondensation reactions

In plastic microtubes, D52S HEWL (15 μ L of a 1.5 mg.mL⁻¹ solution, 1.36 nmol) in Tris-HCl purification buffer was added to **4a** or **4b** (0.38 μ mol) in a final volume of 31 μ L. The reactions were incubated at 37°C or 60°C and monitored by TLC using 7/3 acetonitrile/water (v/v) as eluting system. After 48 h the crude mixture was analyzed by MALDI-TOF.

Synthesis of 6, 7, 8

In plastic microtubes, *Sinorhizobium meliloti* (*S. meliloti*) NodB (39 μg , 0.76 nmol) was incubated with **4b** (0.3 mg, 0.48 μmol) in 20 mM MOPS buffer pH 7.4 (130 μL) supplemented with 10 mM dithiothreitol and 1 mM manganese sulfate. After 5 h at 37°C, the reaction was stopped by addition of tert-butanol (33 μL , 20 % (v/v)). After 2 h at room temperature, the solution was freeze-dried or concentrated under vacuum giving crude **5**. Acceptor (0.24 μmol) and D52S HEWL in Tris-HCl purification buffer (0.72, 1.44, or 2.88 nmol from a stock solution of 1.5 $\text{mg}\cdot\text{mL}^{-1}$) were then added to the crude donor **5**. The reaction (final volume of 32 μL) was incubated at 37°C or 60°C and monitored by TLC using a 1-butanol:ethanol:water (4:3:3 v/v) eluting system. After three days, the reaction was boiled for 10 min, and filtered through a 0.22 μm cellulose acetate filter prior HP-SEC analysis. Yields were determined based on HP-SEC standard curves of pure CO-VIII (N^{VIII}).

Preparative synthesis of **8** was performed with 3.0 mg (4.7 μmol) of **4b** using a 2:1:0.012 donor/acceptor/D52S HEWL molar ratio at 60°C for 72 h in a final volume of 313 μL . After purification by preparative size-exclusion chromatography, **8** was freeze-dried (2.6 mg, 65%); ^1H NMR (400 MHz, D_2O): δ 5.20 (d, 0.7H, H-1 α^{I}), 4.70 (m, 0.3H, H-1 β^{I}), 4.62-4.50 (m, 7H, H-1 $^{\text{II-VIII}}$), 3.96-3.40 (m, 44H, H-2 $^{\text{II-VII}}$, H-3 $^{\text{II-VIII}}$, H-4 $^{\text{II-VIII}}$, H-5 $^{\text{II-VIII}}$, H-6 $^{\text{II-VIII}}$), 2.71 (t, 1H, H-2 $^{\text{VIII}}$), 2.06-2.06 (m, 21H, $\text{COCH}_3^{\text{I-VII}}$); HRMS (ESI) m/z $[\text{M}+2\text{H}]^{2+}$ calcd for $\text{C}_{62}\text{H}_{104}\text{N}_8\text{O}_{40}$ 801.32477, found 801.32597.

Associated Content

Supporting Information: SDS PAGE analyses, Fluorescence spectra, TLC images of enzymatic reactions, HP-SEC chromatograms, mass and NMR spectra of the products.

Authors' Contributions

Antoine Rousseau: Conceptualization, Investigation, Writing - original draft, **Sylvie Armand:** Conceptualization, Supervision, **Sylvain Cottaz:** Conceptualization, Investigation, Supervision **Sébastien Fort:** Conceptualization, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing.

Conflicts of Interest

There are no conflicts to declare.

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Table of Contents/Abstract Graphic

An enzymatic cascade allowing the synthesis of long-chain $\beta(1\rightarrow4)$ -GlcNAc oligosaccharides perfectly controlled in size is reported. A hen egg-white lysozyme glycosynthase was developed to catalyze the glycosylation of α -chitintriosyl fluoride. Using this glycosynthase in concert with a chitin-oligosaccharide *N*-deacetylase allowed controlled glycosylation of the fluoride onto oligosaccharide acceptors without competing polycondensation reaction. This enzymatic cascade enabled the preparation of the important plant elicitors chitin hexa-, hepta- and octasaccharides with very good yields.

