Abstract: Plant research is hampered in several aspects by a lack of pure oligosaccharide samples that closely represent structural features of cell wall glycan. An alternative to purely chemical synthesis to access these oligosaccharides is chemo-enzymatic synthesis using glycosynthases. These enzymes enable the ligation of oligosaccharide donors, when activated for example as α-glycosyl fluorides, with suitable acceptor oligosaccharides. Herein, the synthesis of xylan oligosaccharides up to dodecasaccharides is reported, with glycosylnase-mediated coupling reactions as key steps. The xylo-oligosaccharide donors were protected at the non-reducing end with a 4-O-tetrahydropyranyl (THP) group to prevent polymerization. Installation of an unnatural 3-O-methylether substituent at the reducing end xylose of the oligosaccharides ensured good water solubility. Biochemical assays demonstrated enzymatic activity for the xylan acetyltransferase XOAT1 from Arabidopsis thaliana, xylan arabinofuranosyltransferase XAT3 enzymes from rice and switchgrass, and the xylan glucuronosyltransferase GU3 from Arabidopsis thaliana. In case of the glucuronosyltransferase GU3, MALDI-MS/MS analysis of the reaction product suggested that a single glucuronosyl substituent was installed primarily at the central xylose residues of the dodecasaccharide acceptor, demonstrating the value of long-chain acceptors for assaying biosynthetic glycosyltransferases.

Introduction

All plant cells are surrounded by a polysaccharide-rich wall that constitutes the majority of the terrestrial biomass on Earth and thus play important roles for both the environment and economy. The hemicellulose xylan is the second most abundant polysaccharide after cellulose in these walls, and plants incorporate approximately 10 billion tons of carbon each year into this polysaccharide alone. Xylans are a major component of everyday food and forage, playing a central role in human and animal health. As xylans constitute up to 30% of woody biomass, they are also highly relevant for the feed, paper, and biofuel industries. Specific non-food applications range from their use as oxygen barrier films for packaging to strengtheners in paper production. The molecular composition and primary structure of xylan polymers have a strong impact on the macroscopic properties of plant tissues, and can significantly vary between plant species and tissues. Xylans in land plants possess a common backbone consisting of β-1,4-linked O-xylopyranosyl residues (Figure 1A). In both hardwoods and softwoods, the 2-position of xylose may be appended with D-glucuronic acid or 4-O-methylated D-glucuronic acid substituents (glucuronoxylans). In contrast, the xylans present in grasses are mainly substituted with α-L-arabinofuranosyl residues in the 2- and/or 3-position, and are less frequently substituted with glucuronic acid (glucuronoarabinoxylans). Furthermore, most xylans are decorated with significant amounts of regularly or irregularly spaced acetyl groups. Like most polysaccharides, xylans are synthesized by glycosyltransferases (GTs) that use sugar nucleotides as donors to attach monosaccharides to the glycan acceptor. Direct biochemical evidence has demonstrated that the β-1,4-d-xylan backbone is synthesized from UDP-xylose by xylan synthase enzymes (XYS) that are classified as members of the GT47 family in the carbohydrate active enzyme (CAZy) database. The synthesis of complex xylans found in plants requires additional enzymes, and several responsible for catalyzing formation of most common substrates have been identified. For examples, ...
α-glucuronosyltransferases encoded by GLCA SUBSTITUTION OF XYLAN (GUX) genes from family GT8 add glucuronic acid (GlcA) to dicot xylan,[6b,10] xylan arabinosyltransferases (XATs) from family GT61 add arabinofuranose (Araf) to xylan in grasses,[9b,12] and xylan O-acetyltransferases (XOATs) from the Trichome Birefringence-Like (TBL) protein family acetylate xylan.[6b,10]

In the cell wall, the degree of polymerisation (DP) of a typical xylan polymer can reach hundreds of residues. Accordingly, during polysaccharide synthesis in the Golgi, the rapid addition of glycosyl and non-glycosyl substituents to the nascent backbone maintains solubility and inhibits assembly and interaction of the backbone residues to form aggregates.[13] As interest in elucidating the biochemical pathways responsible for cell wall polymers has grown, it has become clear that studies into the biochemical characterization of GTs that are responsible for catalyzing the addition of substituents to the polysaccharide backbone require longer, more biosimilar oligosaccharide fragments of the backbone as pseudo acceptor substrates.[14] However, the currently available oligosaccharides may be shorter or of similar length to the structural motif recognized by the respective GT, potentially yielding misleading biochemical results. Furthermore, the use of sub-optimal pseudo-substrates to study saccharide synthesis can limit or even prevent enzymatic activity, ultimately limiting information on the substitution patterns obtained.

Chemical synthesis in solution has provided xylan oligosaccharides up to a decasaccharide. Furthermore, using automated glycan assembly (AGA), linear xylan oligosaccharides up to an octasaccharide as well as different arabinosyl-substituted xylan oligosaccharides have been prepared. The synthesis of longer xylan oligosaccharides has not been reported. Removal of protecting groups following the assembly of long homo-oligomeric glycans can be problematic[15] due to limited solubility of intermediary formed semi-protected compounds that form supramolecular assemblies.[16] Enzymatic strategies may provide a solution to this issue, if the desired unprotected products are directly obtained from smaller reactants.

Here, we report the chemo-enzymatic synthesis of xylan oligosaccharides up to dodecasaccharides (DP12), with and without 3-O-methylation of the reducing end xylose, which enhances solubility for biochemical assays. Our strategy is based on glycosynthase-mediated coupling reactions of synthetic oligosaccharide fluorides with suitable oligosaccharide acceptors, providing a promising alternative to purely chemical synthesis. The prepared oligosaccharides were used as acceptor substrates in assays with both glycosyl- and acetyltransferases, demonstrating the importance of the substrate’s chain length (Figure 1B).

Results and Discussion

In addition to chemical synthesis, a plethora of enzymes can be used to form glycosidic bonds.[17] They provide precise stereo-selective control over the reactions and avoid the need for multiple protection and deprotection events. Particularly promising for the preparation of large homo-oligomeric glycan structures are glycosynthases derived from endo-glycosidases.[18] These enzymes are obtained by site-directed mutagenesis of the catalytic nucleophile residue in endo-glycosidases. Since the natural endo-glycosidases catalyze the hydrolysis of internal glycosidic bonds in oligo- and polysaccharides, their glycosynthase counterparts enable ligation of oligosaccharides. XynAE265G (also named XT6E265G) is a xylan glycosynthase that results from the substitution of the nucleophilic glutamic acid by glycine in the active site of the extracellular endo-1,4-β-xylanase of G. stearothermophilus (XT6; family GH10).[19] This modification maintains the topology of the active site that allows stabilization of the developing charges during transition state formation[20] but prevents cleavage of the glycosidic bond. When an α-xylosyl fluoride and a xylosyl...
acceptor are accommodated in the active site, their coupling reaction is catalyzed by the stabilizing interactions provided in the active site.

Starting from xylobioside building blocks (BBs) \(1^{[21]}\) and \(2\), long-chain xylan oligosaccharides, with or without a methylation at the reducing end xylose unit, were synthesized using chemoenzymatic iterative synthesis (Scheme 1). To generate disaccharide acceptors \(3\) and \(4\) for subsequent glycosynthase-mediated coupling reactions, the benzoyl esters in \(1\) and \(2\) were removed by methanolysis. Disaccharide α-xylosyl fluoride donor \(5\) was prepared by treatment of thioglycoside \(1\) with HF/py and THP- protection of the hydroxy group and careful removal of the benzoate esters using catalytic amounts of sodium methoxide in methanol at \(0\) °C. After workup with \(\text{Et}_2\text{O}/\text{H}_2\text{O}\) and lyophilization of the aqueous phase, xylosyl donor \(5\) was obtained. In the first of the three steps, excess HF/py-solution drives the reaction to the thermodynamically favored α-xyloside. Incubation of acceptors \(3\) or \(4\) with donor \(5\) and glycosynthase XynAE265G afforded tetra-xylosides \(6\) or \(7\) in good yields. As the products partly precipitated during the course of the reaction, pure product could be collected by centrifugation. Additional product remaining in solution was purified by size-exclusion chromatography (see Supporting Information). The THP-group

Scheme 1. A) Chemo-enzymatic synthesis of non-methylated and methylated dodeca-xylosides \(13\) and \(14\) using xylan glycosynthase XynAE265G. B) Synthesis of hexa-xylosides \(15\) and \(16\).
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prevented glycosynthase-catalyzed self-condensation of the donor.\(^{[21]}\) Subsequently, the corresponding tetrascaccharide donor (10) and tetrascaccharide acceptors (8 and 9) were prepared. Simple acidic treatment of 6 and 7 with AcOH (5% in water) provided deprotected acceptors 8 and 9. Formation of the tetrascaccharide donor 10 was realized by a four-step procedure, consisting of peracetylation with acetic anhydride in pyridine, fluorination using NIS and HF/py in DCM at -50°C to -5°C, with concomitant loss of the THP-group, re-installation of THP using dihydropyran (DHP) and catalytic amounts of CSA, and final removal of the acetyl groups by methanolysis at 0°C. Partial precipitation of the product upon neutralization of the basic solution provided crystalline fluoride donor 10. Without any further purification, careful concentration of the neutral solution provided the remaining fluoride donor 10. Alternative protecting groups to THP (PMB (p-methoxybenzyl), Nap (2-naphthylmethyl) and allyl) have been explored to avoid the necessity of protecting group re-installation after the fluorination step but did not provide superior results. Both PMB and Nap protecting groups were cleaved under fluorinating conditions (NIS and HF/py in DCM), even when lower temperatures were used, and the allyl group underwent iodofluorination.\(^{[22]}\) Glycosynthase-mediated coupling of donor 10 with acceptors 8 and 9 followed by THP-removal provided octa-xylotriosides 11 and 12, which were elongated to dodecasaccharides 13 and 14 with tetrascaccharide donor 10. Additionally, reaction of disaccharide donor 5 with tetrascaccharides 8 and 9 afforded hexasaccharides 15 and 16. As expected, the solubility of the oligo-xylotriosides decreased with increasing chain length, with the non-methyalted dodecasaccharide 13 being almost completely insoluble in pure water. The enzymatic coupling products still furnished with a THP group were consistently less water soluble than the respective oligosaccharides obtained after THP removal. THP-deprotection of the dodecasaccharides was possible in aqueous solution after prior lyophilization from DMSO.

The assembly of architecturally complex structures such as the plant cell wall depends on the action of glycan-biosynthetic enzymes, including glycosyltransferases, acetyltransferases, and methyltransferases. With the synthesized long-chain oligosaccharides in hand, the ability of different heterologously expressed xylan-modifying enzymes to add glycosyl- and acetyl-substituents to the xylan backbone was investigated. Methylated dodecasaccharide 14 was chosen as the acceptor substrate over the respective non-methylated oligosaccharide 13, due to its superior solubility in water. The following enzymes were investigated: i) the well-characterized acetyltransferase AtXOAT1 (from A. thaliana), which served as a proof-of-principle; ii) two arabinoxylosyltransferases from grasses, OsXAT3 (from Oryza sativa; rice) and PpXAT3 (from Panicum virgatum; switchgrass); and iii) glucuronosyltransferase AtGUX3 (from A. thaliana), whose activity had not been previously studied in vitro.

The catalytic domain of AtXOAT1, which acetylates the 2-position of xylose units in the backbone,\(^{[23]}\) was expressed in HEK293 cells following previously described protocols. Incubation of 14 with acetyl salicylic acid and XOAT1 for 16 h resulted in the formation of xylan oligosaccharides with five to ten acetyl groups attached to the xylose residues, as determined by MALDI mass spectrometry (Figure 2). Next, the catalytic domains of OsXAT3 and PpXAT3 were expressed. These enzymes belong to the GT61 family, containing several glycosyltransferases involved in the 3-O-Araf substitution of grass xylan, including multiple xylan arabinofuranosyl-transferases (XATs) from rice and wheat (OsXAT2-7, and TzXAT1-2\(^{[24,25]}\)). Here, we have demonstrated the ability of XAT3 to transfer Araf from UDP-Araf to long xylan oligosaccharides. While OsXAT3 transferred up to two Araf to synthetic dodeca-xyloside 14, PpXAT3 transferred up to five Araf substituents (Figure 2A), reflecting the ability of grass XATs to generate highly substituted arabinoxylan polysaccharides. Finally, we expressed for the first time the catalytic domain of AtGUX3. Substitution of xylan with glucuronic acid in dicot secondary cell walls requires two GT8 enzymes in Arabidopsis: GUX1 and GUX2, which were reported to generate strikingly different substitution patterns in vivo.\(^{[26]}\) More recently, GUX3 was identified as the only glucuronyltransferase required for glucuronolatation of Arabidopsis primary cell wall xylan.\(^{[27]}\) The comparison of xylan oligosaccharides released from cell walls of gux3 and gux1/gux2 knock-out mutants suggested that GUX3 produces a unique substitution pattern, in which every sixth xylose residue carries a glucuronic acid. However, biochemical characterization and confirmation of enzymatic activity for AtGUX3 had not been carried out. Incubation of dodecasaccharide 14 with the catalytic domain of AtGUX3 demonstrated that GUX3 was able to transfer one equivalent of GlcA from UDP-GlcA to the synthetic oligosaccharide acceptor 14 (Figure 2A).

To determine the positions along the backbone of the pseudo-acceptor to which GUX3 added the GlcA substituent, we used matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS)/MS. Our data shows that GUX3 specifically adds GlcA to one of the two middle xylose residues of the 12mer, i.e. the 6th or 7th xylose from the non-reducing end. This finding is based on the relative abundance of the corresponding B- and Y fragments\(^{[27]}\) (Figure 2B). These two types of fragments are the result of glycosidic bond cleavage. The resulting fragment towards the non-reducing end is the B-fragment, and the fragment towards the reducing end is the Y-fragment. The sub-index assigned to the letters B and Y represents the number of sugar units in the given fragment. Since the Y-fragments include an STot at the reducing end, they are readily identified and cannot be mistaken by internal fragments formed through cleavage of multiple glycosidic bonds. Given that the GlcA residue can be cleaved off during fragmentation, as observed for the unsubstituted Y\(_{th}\)-fragment (the most intense peak in the spectrum), the GlcA-containing Y-fragments (blue) represent the most reliable source of information. Of these, the smallest is the GlcA-substituted Y\(_{th}\)-fragment, suggesting that no GlcA-substitutions are present on the five xylose units towards the reducing end. Furthermore, unsubstituted Y-fragments with seven (Y\(_{7}\)) or more xylose units (red) were found to be of low abundance, suggesting that substitution is primarily present at the 6th or 7th xylose unit and not at the five xylose residues towards the non-reducing end. These observations are supported by the
Figure 2. MALDI-TOF MS analysis of glycosyltransferase reactions (16 h incubation) with oligosaccharide 14 as a glycosyl acceptor substrate (see Figure 1B for an illustration). A) The arabinosyltransferases OsXAT3 from rice and PvXAT3 from switchgrass show different degree of arabinosylation of the xylan 12mer. AtXOAT1 from Arabidopsis can catalyze the transfer of up to ten acetyl groups to the xylan backbone. Analysis of AtGUX3 reactions products showed the enzyme is able to catalyze the attachment of precisely one glucuronic acid to the xylo-oligomer. Indicated masses correspond to the Na$^+$ adducts. B) MALDI-TOF MS/MS analysis of the AtGUX3 product with the parent mass 1921. Y-fragments represent thioglycosides including the original reducing end (with STol), whereas the B-fragments include the non-reducing end of the 12mer. The Y12 fragment originates from the detachment of the GlcA residue during fragmentation. The proposed predominant glycosylation sites for GUX3 are indicated with a blue line. Indicated masses correspond to the Na$^+$ adducts of the fragments.
observed B-fragments. Unsubstituted B-fragments containing eight (B₈) or more xylose units (yellow) are substantially less abundant, while B-fragments with five (B₅) or less xylose units are present in both substituted (light blue) and unsubstituted form (yellow). The presence of small GlcA-substituted B-fragments (light blue) may be explained by the formation of internal fragments. Still, we cannot exclude the presence of small amounts of oligosaccharides with GlcA-substitution further towards the non-reducing end. Overall, our results suggest that GUX3 requires five unsubstituted xyloses adjacent to both sides of the glycosylation site to install a single GlcA residue, converting almost all of the acceptor to the glucuronosylated form. This is in contrast to the activity of the XOAT and XATs, which all add more than one substituent to the xyloglucaniser. This in vitro glycosylation pattern of GUX3 is in agreement with the pattern proposed to be produced in vivo. Our data therefore suggests that the GUX3 substrate specificity is sufficient to produce the xylan-substitution pattern with every 6th xylosyl residue decorated with GlcA that was observed using gux-mutant analyses. On the contrary, GUX1 generates specific acetylation patterns prior to glucuronosylation to guide the synthesis of the observed GlcA-substitution patterns in secondary cell wall xylan. Previous attempts to show activity of GUX3 using a xylan hexamer were unsuccessful. However, experiments with WT and mutant Arabidopsis microsomes from callus tissue indicated that GUX3 is involved in glucuronosylation of larger xylan oligosaccharides. It remained unclear, if GUX3 is an active glycosyltransferase or a rather scaffold protein in a xylan-synthesizing complex. Our finding of GUX3 activity on a xylan 12mer proves that GUX3 is active on its own and highlights the potential of using long-chain Oligosaccharides for biosynthetic studies.

Conclusion

We have performed the chemo-enzymatic syntheses of two xylan dodecasaccharides, with and without an artificial methyl group at the 3-position of the reducing end xylose residue. The glycosynthase XynAE265G efficiently catalyzed the reaction between two defined xylo-oligosaccharides, a glycosyl fluoride donor and a thioglycoside acceptor. To prevent polymerization, the 4-OH of the donor's non-reducing xylose residue was protected with a THP group. By applying further enzymatic coupling cycles, the presented strategy may provide an easy access to even larger xylan oligosaccharides for biosynthetic studies.

Experimental Section

General information: Solvents and reagents were used as supplied without further purification. Anhydrous solvents were taken from a dry solvent system (J.C.-Meyer Solvent Systems) or used directly from the supplier. Column chromatography was carried out using Fluka silica gel 60 (230–400 mesh). NMR spectra were recorded on a Bruker Avance III 600 or a Bruker 400 instrument spectrometer using solutions of the respective compound in CDCl₃, DMSO-d₆, or D₂O. NMR chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. H spectra were referenced to 7.26 (CDCl₃) and 0.00 (D₂O, external calibration to sodium 3-(trimethylsilyl)propane-1-sulfonate, DSS) ppm. 13C spectra were referenced to 77.16 (CDCl₃) and 67.19 (D₂O external calibration to 1.4-dioxane) ppm. High resolution mass spectra were obtained on a 6210 ESI-TOF mass spectrometer (Agilent) or a Micromass Q-TOF Ultima Global instrument. For filtration, syringe filters (RC, 0.45 μm) from Roth were used. Reactions were monitored using reverse phase HPLC with a C4 column (Jupiter, 150 x 2 mm, Phenomenex) in a Shimadzu LC-10 system that is coupled to a Shimadzu LCMS 2020 mass spectrometer and an Alltech 3300 Evaporative Light Scattering Detector (ELSD).

General procedure A for glycosynthase-mediated reactions:

- Xyllosyl fluoride donor (1.2 equiv.) and xylo-oligosaccharide acceptor (1 equiv.) were dissolved in phosphate buffer (100 mM; pH 7.4). XynAE265G mutations were introduced in phosphate buffer (100 mM; pH 7.4) and added to the reaction mixture. The reaction was allowed to react in a rotating shaker at 30 °C for 16 h and the desired product precipitated to give the final product. The cloudy reaction mixture was centrifuged, and the insoluble solid was dispersed twice in H₂O and centrifuged. The aqueous phases were combined and lyophilised. The solid obtained after lyophilisation was dissolved in a small amount of H₂O and purified by size-exclusion chromatography (SEC) using a Bio-Gel P-2 polyacrylamide gel with H₂O as an eluent. The compound obtained by SEC-purification was combined with the insoluble compound obtained after centrifugation to give the desired product as a white or colourless solid.

General procedure B for THP removal:

The THP-protected xylo-oligosaccharide product was dissolved in water. If necessary, a small amount of DMSO was added for complete dissolution of the starting material. AcOH was added and the reaction mixture was stirred for 16 h or until the reaction was complete, as monitored by thin layer chromatography (TLC) or liquid chromatography-mass spectrometry (LC–MS) using a C4 column (Phenomenex). The reaction mixture was then lyophilised, and the residue was purified, if necessary, by size-exclusion chromatography (SEC) using Bio-Gel P-2 polyacrylamide gel with H₂O as an eluent. The procedure yielded the desired product as a white or colourless solid.

Expression of the XynAE265G glycosynthase:

The expression of XynAE265G in E. coli was performed as previously described. In brief, the XynA gene, with Glu265 mutated to Gly, was expressed in E. coli BL21 (DE3) using the pET9d vector. The expression of the enzyme was induced with 0.5 mM isopropyl-β-D-thiogalactopyranosid (IPTG) and after cell lysis the cell debris was removed by centrifugation at 10000 g for 45 min at 4 °C. The supernatant could be directly used for the polymerization assay, because of the high expression rate of XynAE265G, with a single major band at the
Expression of the glycosyltransferases PoxAT3, OsXAT3 and AtGUX3 and the acetyltransferase AtXOAT1: The heterologous expression of the glycosyltransferases and the acetyltransferase (AtXOAT1-cat comprising amino acids 133–487) in HEK293F cells was performed as previously described.[23,26] Plant coding sequences were amplified from a cDNA template and cloned into mammalian expression vector pGEN2-DEST according to standard protocols.[26] Truncated versions of the coding sequences were amplified using the primers listed in Table 1 (Supporting Information) and cloned into the pDONR221 vector to generate Gateway entry clones. Using LR clonase (Invitrogen) the genes were then cloned into the pGEN2-DEST vector to generate an expression construct to yield recombinant proteins with an 8xHis tag, an AviTag, and “superfolder” GFP as an expression vector pGEN2-DEST according to standard protocols.[26] In brief, the cells were transfected and then the protein was secreted into the culture medium during several additional days of cultivation. Soluble secreted fusion proteins were purified from the clarified culture media using HisTrap HP columns (GE Healthcare) and dialyzed into 50 mM HEPES sodium salt buffer, pH 6.8, including 5% glycerol. The concentration of the resulting proteins was measured using a NanoDrop Microvolume Spectrophotometer based on their absorbance at 280 nm.

Enzyme assays: The enzyme assays were performed using the methylated xylan 12mer 14 as acceptor substrate with the following setup: i) for AtXOAT1: 0.5 mM oligosaccharide acceptor, 6 mM sodium acetate, 50 mM sodium chloride, 50 mM sodium phosphate, 1 mM UDP-glucuronic acid, 1 mM UDP-arabinose, 20 μM enzyme; ii) for AtGUX3: 0.5 mM oligosaccharide acceptor, 1 mM UDP-glucuronic acid, 10 μM enzyme. The reactions were adjusted to a final volume of 10 μL using 50 mM HEPES sodium salt buffer, pH 6.8. The reactions were incubated for 15 h at room temperature and then terminated by incubation for 10 min at 85 °C.

MALDI-TOF analysis: MALDI-TOF MS analysis was performed using an Autoflex Speed (Bruker Daltonics, Germany) in positive ion mode using FlexControl 3.4 software. To remove salts from the enzyme reactions, 10 μL of a suspension of AG 50W-X8 cation exchange resin (Biorad) in water was added before spotting the samples. After incubation for 10 min, the samples were centrifuged and 1 μL of the supernatant was spotted on a MALDI plate. After drying, 1 μL of a 2.5-dihydroxybenzoic acid matrix (DHB, 10 mg/mL in 50% acetonitrile/water) was added, and the plate was dried again. To identify the position of the GlcA residue in the AtGUX3 reaction product, MALDI MS/MS experiments were performed using the LIFT cell as described previously.[21] Fragment ions of the GlcA-substituted xylan 12mer (Na+ -adduct) were generated by laser-induced dissociation (LID) in positive ion mode. Typically, 500 shots were summed for MS (reflector voltage, lens voltage and gain respectively 21 kV, 8.8 kV and 1997 V) and 4000 shots were summed for MS/MS (reflector voltage, lens voltage and gain respectively 27 kV, 19 kV and 1944 V). Spectra were processed with the manufacturer’s software (Bruker Flexanalysis 3.3.80) using the SNAP algorithm.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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