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Structure of neutral branched xylooligosaccharides produced by xylanase from *in situ* reduced hardwood xylan

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Abstract

Two neutral oligosaccharides from *in situ* reduced hardwood xylan were isolated and completely characterized by methylation analysis and 2D NMR spectroscopy (COSY, HOHAHA, HMQC, HMQC–TOCSY, NOESY, and HMBC techniques). The oligosaccharides are the xylotetrasaccharide, 4-*O*-methyl-α-D-glucopyranosyl-(1→2)-D-xylotriose and the xylopentasaccharide 4-*O*-methyl-α-D-glucopyranosyl-(1→2)-D-xylotetraose. From reactivities observed with these compounds, the mode of action of β-xylanase is described. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: β-Xylanase; 4-*O*-Methylglucuronoxylan; Xylooligosaccharide

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1. Introduction

Oligosaccharides often possess some unique structural feature or unusual property that is of special value to the biological entity [1]. Our group has reported that acidic xylooligosaccharides affect the growth of tree tissue cultures [2] and the rooting of conifer cuttings [3]. In addition, our group has screened fungal β-xylanases with respect to the productivity of acidic xylooligomers using *in situ* reduced hardwood xylan as substrate [4]. The use of this substrate was expedient in defining the substrate specificity of the β-xylanases from the products of neutral and acidic branched xylooligomers [5]. Borohydride reduction *in situ* transformed 30% of 4-*O*-methyl-α-D-glucopyranosyluronic acid residues in the xylan into 4-*O*-methyl-α-D-glucopyranosyl residues. Among the fungal β-xylanases screened, fraction II of *Trichoderma viride* was the best producer of aldotetraouronic acid (4-*O*-methyl-α-D-glucopyranosyluronic acid-(1→2)-D-xylotriose). The β-xylanase produced neutral products, including two 4-*O*-methyl-D-glucose substituting xylooligomers, a tetrasaccharide (denoted as G-X$_3$) and a pentasaccharide (G-X$_4$), in addition to D-xylose, D-xylobiose and D-xylotriose.

This article describes the complete characterization of the neutral branched xylotetrasaccharide (G-X$_3$) and xylopentasaccharide (G-X$_4$) produced from the *in situ* reduction of hardwood xylan (Fig. 1). The NMR assignments of these oligomers

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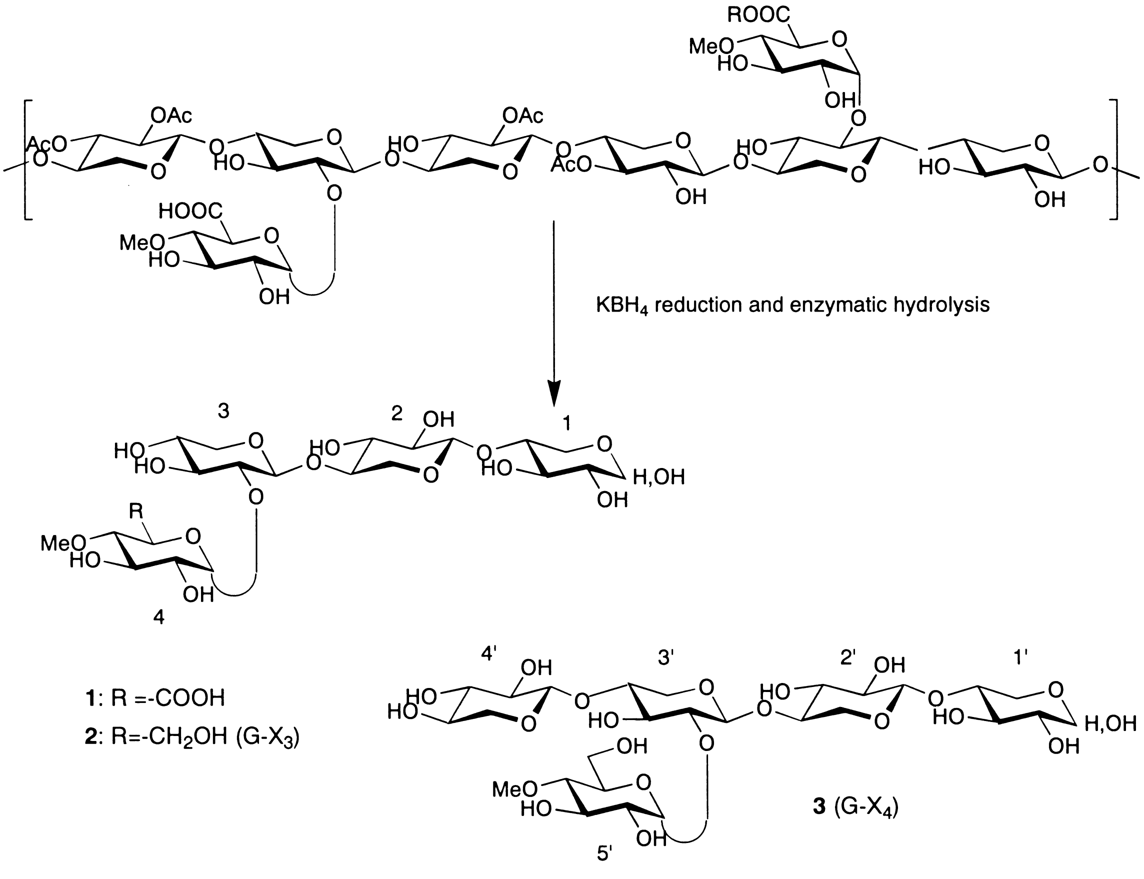


Fig. 1. Structures of G-X_3 and G-X_4.

will be useful as basic data for identification of analogous xylooligosaccharides [4].

2. Experimental

General methods.—Evaporation was conducted under reduced pressure at 40 °C using an apparatus for removing solvents from test tubes with filtered air [6]. Alditol acetates were prepared and analyzed as described previously [7], except that hydrolysis in 2 M CF_3CO_2H was done for 2 h, and acetylation of the alditols was performed for 20 min. GLC was performed on a Shimadzu GC 14A instrument equipped with a 30 m×0.25 mm SP-2330 fused silica column (Supelco) operating isothermally at 230 °C. Absolute configurations were determined as described previously [8]. Per-*O*-methylation was performed by a modification [9] of the method of Hakomori [10], and the per-*O*-methylated oligosaccharides and glycosyl alditols were purified as described previously [11].

Enzymatic hydrolysis of in situ *reduced 4-*O*-methylglucuronoxylan.*—*Betula platyphylla* (Skatchev) wood meal was reduced in 0.2 M potassium borohydride at pH 8 at room temperature for 24 h and washed with water. Xylan was extracted from the *in situ* reduced hardwood with 10% potassium hydroxide.

The *in situ* reduced 4-*O*-methylglucuronoxylan (23.0 g) was hydrolysed with the β-xylanase fraction II [4] of *Trichoderma viride* (137.2 mg) at 40 °C for 24 h. The hydrolyzate was separated into the neutral and acidic parts by use of a strongly basic anion exchanger (AG-1 ×8, OAc^-, Bio-Rad) after removal of the insoluble residue by filtration. The yields of neutral and acidic hydrolysis products were 13.0 and 2.1 g, respectively. From quantitative analysis of the enzymatic neutral and acidic hydrolyzate, the molar ratio of 4-*O*-methyl-D-glucosyl to 4-*O*-methyl-D-glucuronosyl residues in the *in situ* reduced hardwood xylan was estimated to be 1.00:2.46.

*Separation of 4-*O*-methyl-*D*-glucose substituting xylooligomers.*—The composition of the neutral products was analyzed by GPC on two Shodex Ionpak columns (KS-802, 0.8×30 cm, Showa Denko) connected in series. In addition to D-xylose, D-xylobiose and D-xylotriose, *T. viride* produced two 4-*O*-methyl-D-glucose substituted oligomers with retention times that differed from those of D-xylotetraose and D-xylopentaose. The neutral hydrolyzate (5.40 g) was ultrafiltered using an Amicon YC-05 membrane (cutoff molecular weight 500). The inner fraction (0.92 g), which did not pass through the YC-05 membrane, was subjected to preparative gel-permeation chromatography on two HiLoad Superdex 30 columns (1.6×60 cm, Pharmacia) connected in series. The gel chromatography was run at 50 °C using Milli-Q water as eluent at a flow rate of 0.5 mL/min. The yield of isolated 4-*O*-methyl-D-glucose-substituted oligosaccharides was 209.4 mg for the tetramer (G-X_3) and 40.0 mg for the pentamer (G-X_4).

Mass spectrometry.—GLC–MS was performed with a Shimadzu GC–MS QP2000A mass spectrometer. Per-*O*-methylated alditol acetates were separated as previously described [12]. FABMS was performed in the positive ion mode on a JEOL JMS-HX110A mass spectrometer operating at an accelerating voltage of 10 kV. Samples were dissolved in water and loaded on a stainless steel target with glycerol and thioglycerol as matrix. Xenon was used as the bombarding gas. Spectra were obtained by scanning the mass range from 100 to 1200 Da in 15 s and processing with the JEOL MS-NW7000/MP7010 data system.

NMR spectrometry.—NMR spectra were recorded at 25 °C with a JEOL ALPHA-500 FT-NMR (500 MHz). Samples were dissolved in D_2O (99.996 atom%), and ^1H and ^{13}C chemical shifts were measured relative to internal acetone (δ 2.23 and 31.01, respectively). One- and two-dimensional (2D) correlated spectroscopy (COSY), 2D *J*-resolved, 2D homonuclear Hartmann–Hahn (HOHAHA), and 2D nuclear Overhauser enhancement and exchange spectroscopy (NOESY), 2D heteronuclear correlated spectroscopy, and heteronuclear multiple bond correlation (HMBC), heteronuclear multiple quantum correlation (HMQC), HMQC total correlation spectroscopy (HMQC–TOCSY) were performed with a JEOL ALPHA-500 FT-NMR spectrometer. The normal-mode probe was used for HMBC.

3. Results and discussion

Characterization of G-X_3 and G-X_4.—Compositional analysis by GC of the alditol acetates showed that G-X_3 and G-X_4 contained xylose and an unknown sugar in a molar ratio of 3:1 and 4:1, respectively. The unknown sugar was identified as 4-*O*-methyl-D-glucose by means of methylation analysis and NMR spectrometry. Results of the methylation analyses are shown in Table 1. For G-X_3, two derivatives, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol (derived from branching glucopyranosyl residue), and 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylpentitol (derived from, nonreducing, internal and reducing xylopyranosyl residues) were obtained. For G-X_4, in addition to these two derivatives, 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylpentitol (derived from the nonreducing xylopyranosyl residue) and 1,2,4,5-tetra-*O*-acetyl-3-*O*-methylpentitol (derived from xylopyranosyl residue at the branching point) were obtained. Furthermore, the methylation analyses of the corresponding alditols of G-X_3 and G-X_4 were carried out. The highly volatile derivative, 4-*O*-acetyl-1,2,3,5-tetra-*O*-methylpentitol was obtained in common with G-X_3 and G-X_4. The compound indicates that the reducing-end residue of G-X_3 and G-X_4 is a 4-*O*-linked-D-xylopyranoside.

The positive-ion fast-atom-bombardment mass spectra (FABMS) of G-X_3 showed an intense peak

Table 1
Methylation analysis of G-X_3 and G-X_4 and their derived glycosyl alditols G-X_3(R) and G-X_4(R)

Glycosyl residue	Methylated positions	Corresponding units	Mole (%)			
			G-X_3	G-X_4	G-X_3(R) [a]	G-X_4(R) [a]
Xylopyranosyl	2, 3	Xy-1, Xy-2, Xy-3	71.8		55.8	
Xylopyranosyl	2, 3	Xy-1′, Xy-2′		37.3		27.9
Xylopyranosyl	3	Xy-3′		20.0		14.7
Xylopyranosyl	2, 3, 4	Xy-4′		25.5		24.7
Xylitol	1, 2, 3, 5	Xy-1, Xy-1′			21.1 [b]	14.1 [b]
Glucopyranosyl	2, 3, 4, 6	Glu-4, Glu-5′	28.1	17.3	23.1	18.6

[a] Prepared by $NaBD_4$ reduction of G-X_3 and G-X_4 before methylation.
[b] The highly volatile derivative was probably lost.

at *m*/*z* 591.2 that corresponds to its molecular ion. The FABMS of G-X$_4$ has an intense peak at *m*/*z* 723.3 that corresponds to its molecular ion, and a weaker peak at *m*/*z* 745.3 that corresponds to $(M+Na^+)$.

Analysis of G-X$_3$ and G-X$_4$ by ^{1}H and ^{13}C NMR spectrometry was carried out to specify the xylosyl residue which linked 4-*O*-methyl glucose, and to specify the anomeric configurations of the glycosyl residue. Assignments of signals were carried out using 2D NMR spectrometry and were based on published values for some related compounds [13–16]. Tables 2 and 3 give the complete assignments of the signals which were made by COSY, HOHAHA, HMQC, and HMQC–TOCSY spectroscopy. Fig. 2 shows an HMQC spectrum of G-X$_4$. The anomeric carbons of G-X$_4$ appear at δ 92.78, 97.26, 98.41, 102.18, 102.45, and 102.75 ppm, which were assigned as reducing α-D-xylopyranoside, reducing β-D-xylopyranoside, 4-*O*-methyl-α-D-glucopyranoside, internal β-D-xylopyranoside, internal branched β-D-xylopyranoside, and nonreducing β-D-xylopyranoside, respectively. The NOESY spectrum of G-X$_4$ shows an NOE between H-2 of the internal branched xylopyranose and H-1 of glucopyranose (Fig. 3a). The location of the internal branched xylopyranosyl residue was confirmed by HMBC analysis

Table 2
Assignments of signals in the ^{1}H NMR spectra[a] of G-X$_3$ and G-X$_4$

Compound and residue	Chemical shift (coupling constant)[b]								
	H-1	H-2	H-3	H-4	H-5a	H-5b	H-5	H-6	OCH$_3$
G-X$_3$									
Reducing α-D-xylose	5.19 ($J_{1,2}$ 3.66)	3.56	3.76	3.76	3.84	3.76			
Reducing β-D-xylose	4.59 ($J_{1,2}$ 7.32)	3.26	3.56	3.80	4.07	3.39			
Internal β-D-xylose	4.48 ($J_{1,2}$ 7.63)	3.29	3.57	3.82	4.17	3.45			
Nonreducing β-D-xylose (branching)	4.62 ($J_{1,2}$ 7.63)	3.40	3.50	3.64	3.99	3.31			
α-D-Glucose	5.30 ($J_{1,2}$ 3.66)	3.54	3.82	3.26			4.07	3.82	3.57
G-X$_4$									
Reducing α-D-xylose	5.19 ($J_{1,2}$ 3.36)	3.55	3.75	3.75	3.83	3.75			
Reducing β-D-xylose	4.59 ($J_{1,2}$ 7.62)	3.25	3.55	3.78	4.07	3.39			
Internal β-D-xylose	4.47 ($J_{1,2}$ 7.62)	3.31	3.59	3.81	4.15	3.44			
Internal β-D-xylose (branching)	4.64 ($J_{1,2}$ 7.63)	3.45	3.63	3.81	4.10	3.42			
Nonreducing β-D-xylose	4.47 ($J_{1,2}$ 7.63)	3.26	3.63	3.63	3.97	3.29			
α-D-Glucose	5.28 ($J_{1,2}$ 3.97)	3.52	3.81	3.24			4.09	3.81	3.57

[a] In D$_2$O.
[b] Values are chemical shifts relative to acetone-d_6 (δ 2.23 ppm).

Table 3
Assignments of signals in the ^{13}C NMR spectra[a] of G-X$_3$ and G-X$_4$

Compound and residue	Chemical shift[b]						
	C-1	C-2	C-3	C-4	C-5	C-6	–OMe
G-X$_3$							
Reducing α-D-xylose	92.76	72.08	71.69	77.30	59.6		
Reducing β-D-xylose	97.25	74.23	74.45	77.13	63.72		
Internal β-D-xylose	102.43	73.42	74.47	76.84	63.63		
Nonreducing β-D-xylose (branching)	102.43	77.30	75.19	70.13	65.73		
α-D-Glucose	98.30	72.08	73.34	79.70	71.34	60.66	60.66
G-X$_4$							
Reducing α-D-xylose	92.78	72.13	71.70	77.26	59.58		
Reducing β-D-xylose	97.26	74.75	74.66	77.15	63.74		
Internal β-D-xylose	102.45	73.45	74.47	76.77	63.56[c]		
Internal β-D-xylose (branching)	102.18	77.26	73.22	77.57	63.62[c]		
Nonreducing β-D-xylose	102.75	73.58	76.36	69.96	65.96		
α-D-Glucose	98.41	72.09	73.34	79.76	71.37	60.78	60.66

[a] In D$_2$O.
[b] Values are chemical shifts relative to acetone-d_6 (δ 31.01 ppm).
[c] These assignments may be interchangeable.

(Fig. 3b). The C-4 signal at δ 77.57 ppm was correlated with the H-1 signal of nonreducing xylopyranosyl residue. Thus, the branched xylose of G-X_4 is adjacent to the non-reducing xylose. The glucopyranosyl residue is substituted at O-4, as indicated by the C-4 signal at δ 79.76 ppm, which is deshielded relative to the corresponding carbon in methyl D-glucopyranoside that is expected to

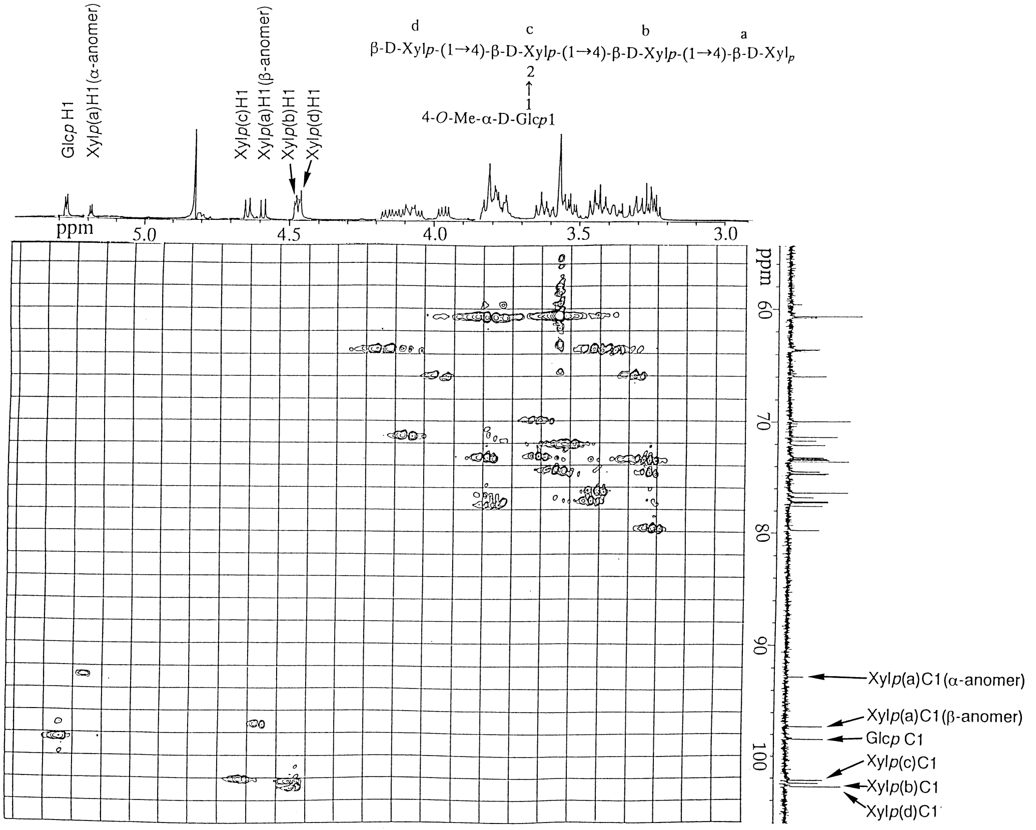
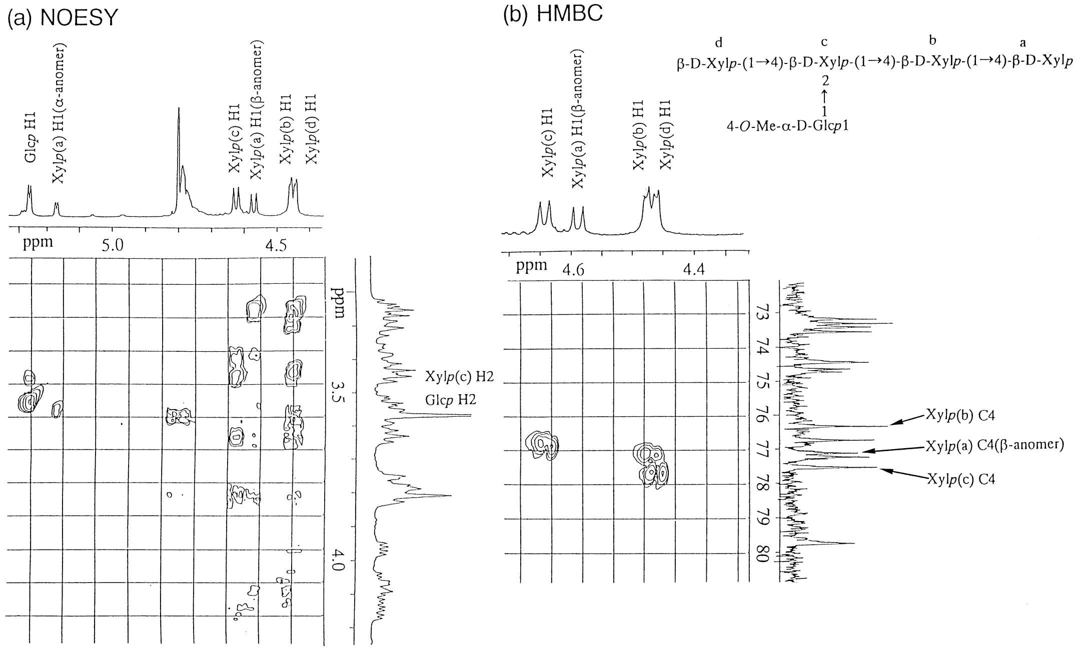


Fig. 2. HMQC spectrum of G-X_4.


(a) NOESY

(b) HMBC

Fig. 3. Spectra of G-X_4: (a) NOESY spectrum; (b) HMBC spectrum.

resonate at 70–72 ppm [15]. Final analysis identified the pentamer G-X_4 as β-D-xylopyranosy-(1→4)-*O*-[(4-*O*-methyl-α-D-glucopyr-anosyl)-(1→2)]-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-D-xylopyranose. This is the first report of the isolation and characterization of a pentasaccharide with this structure. In a similar way, G-X_3 was determined as 4-*O*-methyl-α-D-glucopyranosyl-(1→2)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosy-(1→4)-D-xylopyranose, which is identical to the neutral tetrasaccharide obtained by enzyme hydrolysis of a reduced 4-*O*-methyl-D-glucurono-D-xylan reported previously [5].

Structures of these hydrolyzates afford some information on the substrate specificity of β-xylanases on branched xylans. The presence of G-X_3 and G-X_4 suggests that the 4-*O*-methyl-D-glucose substituent on the *in situ* reduced D-xylan chain provides steric hindrance to the enzyme in accessing the two β-(1→4)-D-xylosidic bonds linked to the right of the substituted D-xylosyl residue. The D-xylosidic bond immediately to the left of the substituted 4-*O*-methyl-D-glucuronic acid (see structure) is easily cleaved by β-xylanases [17]. However, from formation of G-X_4, the 4-*O*-methyl-D-glucose substituent also seemed to reduce the enzymatic cleavage of the D-xylosidic bond immediately to the left (see structure) of the substituted D-xylosyl residue. Another instance of this mode of action of the enzyme is the recent isolation of the acidic xylooligosaccharides having a similar branched structure from enzymatic hydrolyzate of birch kraft pulp xylan [18]. Such branced oligosacchrides have also been isolated from the hydrolyzate of copra galactomannan by using *Streptomyces* β-mannanase [19,20].

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