STUDIES ON SUBSTRATE SPECIFICITIES OF α-GLUCURONIDASES

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ABBREVIATIONS

DMF	*N*, *N*-dimethylformamide
GA-Glc	benzyl 4-*O*-α-D-glucopyranosyluronic acid-β-D-glucopyranoside
GA-GA	*O*-α-D-glucopyranosyluronic acid-α-D-glucopyranosiduronic acid
GA-X	*O*-α-D-glucopyranosyluronic acid-D-xylose
GA-2X	2-*O*-α-D-glucopyranosyluronic acid-D-xylose
GA-3X	3-*O*-α-D-glucopyranosyluronic acid-D-xylose
GA-4X	4-*O*-α-D-glucopyranosyluronic acid-D-xylose
GA-2Xn	2-*O*-α-D-glucopyranosyluronic acid-D-xylo-oligosaccharide
MeGA-2X	2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose
MeGA-2Xn	2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylo-oligosaccharide
PNP	*p*-nitrophenol
PNP-GA	*p*-nitrophenyl α-D-glucopyranosyluronic acid
PNP-GAase	*p*-nitrophenyl α-D-glucopyranosyluronic acid-hydrolyzing enzyme
SDS	sodium dodecyl sulfate
TMSOTf	trimethylsilyl trifluoromethanesulfonate

GENERAL INTRODUCTION

O-Acetyl-(4-*O*-methyl-glucurono)-xylan constitutes a considerable fraction of hardwoods. The macromolecule consists of about 200 xylopyranose units, linked together by β(1→4)-glycosidic bonds. About every tenth xylose unit is substituted by an α(1→2)-linked D-glucopyranosyluronic acid and 4-*O*-methyl-D-glucopyranosyluronic acid substituents. Seven out of ten xylose units contain an *O*-acetyl group at C-2 or at C-3.[1] The linkages of D-glucopyranosyluronic acid- and 4-*O*-methyl-D-glucopyranosyluronic acid-D-xyloses are mainly α(1→2), though Bishop has isolated α(1→3)-D-glucopyranosyluronic acid-D-xylose from wheat straw holocellulose.[2] The α(1→4)-linkage has not been reported so far.

α-Glucuronidase hydrolyzes the glycosidic linkages between α-D-glucopyranosyluronic acid and xylose or 4-*O*-methyl-α-D-glucopyranosyluronic acid and D-xylose, and there by liberates D-glucuronic acid or 4-*O*-methyl-D-glucuronic acid. There are some reports about the α-glucuronidase (refer to the review by Puls[1]), but it has not yet been registered in the Enzyme Nomenclature.

Roy and Timell reported that the sulfuric acid hydrolysis of hardwood xylan yields 2-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose (MeGA-2X) as the main acidic end product. They have concluded that the α(1→2)-glucopyranosyluronic acid linkage is hydrolyzed about 200 times slower than the β(1→4)-xylosidic linkage of xylobiose.[3]

In the enzymatic hydrolysis of glucuronoxylan, endo-β-xylanases hydrolyze xylan in a random action, and produce glucuronoxylo-oligosaccharides and xylo-oligosaccharides. These acidic sugars always carry the glucopyranosyluronic acid and/or 4-*O*-methyl-glucopyranosyluronic acid substituents at the non-reducing terminal xylose unit. This mode of

action of pure xylanases against various xylan has consistently been reported by many other workers.[4-7] The glucuronoxylo-oligosaccharides are hydrolyzed by α-glucuronidase to produce glucuronic acid and xylo-oligosaccharides, and then the resultant xylo-oligosaccharides are hydrolyzed by β-xylosidase to xylose. The stability of the linkage between the glucopyranosyluronic acid and xylose forms a bottleneck in the complete hydrolysis of xylans with enzyme. Thus α-glucuronidases are most important for maximizing the xylose yield in the enzymatic hydrolysis of xylan. [8]

Table 1 shows α-glucuronidase-producing organisms and their substrates. As shown in Table 1, many groups investigating α-glucuronidase have used their own substrates. Results of the independent studies are not always comparable, and some findings, which seem to be contradictory on the first glimpse, can be explained under consideration of the different substrates in use.

In this thesis, results of investigations on the substrate specificity of α-glucuronidases are described. In Chapter I, the synthesis of the regioisomeric α-D-glucopyranosyluronic acid-D-xylose (aldobiouronic acid) as substrates for studies of substrate specificity of α-glucuronidase is described. In Chapter II, the three basidiomycetes that produce α-glucuronidase are reported, and the substrate specificity of these glucuronidase is investigated by using the synthetic regioisomers. A new α-glucuronidase that hydrolyzes *p*-nitrophenyl α-D-glucopyranosyluronic acid (PNP-GA) is detected, purified, and characterized from commercially available snail acetone powder. The kinetics of the new enzyme are also described in Chapter III. The new enzyme is more active towards PNP-GA than other α-glucuronide.

Table 1. α-Glucuronidases and their substrates.

Organisms	Substrates	References
Agaricus bisporus *Trichoderma reesei*	MeGA-2X$_2$[a]	9
Streptomyces olivochromogenes	Wheat bran + Xylanase	10
Megalobulimus paranaguensis *Streptomyces olivochromogenes* *Dactylium dendroides*	Larchwood xylan, Wheat bran +Xylanase, *p*-nitrophenyl α-glucopyranosyluronic acid	11
Tyromyces palustris *Trichoderma* spp. *Polyporus versicolor* *Laetiporus sulphureus* *Agaricus bisporus* *Pleurotus ostreatus*	MeGA-2Xylitol[b]	12
Streptomyces spp	Larchwood xylan + Xylanase	13
Thermoascus aurantiacus	MeGA-2X$_3$[c]	14
Aspergillus niger 5-16	MeGA-2X[a]	15

[a] 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose.

[b] 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylitol.

[c] 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylotriose.

CHAPTER I

SYNTHESIS OF REGIOISOMERIC ALDOBIOURONIC ACIDS

SUMMARY

The three regioisomers of *O*-α-D-glucopyranosyluronic acid-D-xylose; (aldobiouronic acid, GA-X), 2-*O*-α-D-glucopyranosyluronic acid-D-xylose (GA-2X, **22**), 3-*O*-α-D-glucopyranosyluronic acid-D-xylose (GA-3X, **24**) and 4-*O*-α-D-glucopyranosyluronic acid-D-xylose (GA-4X, **26**), were stereoselectively synthesized to clarify the substrate specificity of α-glucuronidase. The regiospecifically protected precursors, namely benzyl 3,4-di-*O*-benzyl-β-D-xylopyranoside (**7**), benzyl 2,4-di-*O*-benzoyl-α-D-xylopyranoside (**9**), and benzyl 2,3-di-*O*-benzyl-β-D-xylopyranoside (**15**), were glycosylated with *O*-(benzyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranosyluronate) trichloroacetimidate (**20**) in the presence of trimethylsilyl trifluoromethanesulfonate as the catalyst.

INTRODUCTION

Glucuronoxylans are important components of plant hemicellulose. The (1→4)-β-D-xylopyranan backbone of these polymers carries occasional α-D-glucopyranosyluronic acid branches at the C-2 or C-3 position of the D-xylose residues.[16] Some of the α-D-glucopyranosyluronic acid residues are also methyl etherified at the C-4 position.[16] 2-*O*-(4-*O*-Methyl-α-D-glucopyranosyluronic acid)-D-xylose (MeGA-2X), 2-*O*-α-D-glucopyranosyluronic acid-D-xylose (GA-2X), and 3-*O*-α-D-glucopyranosyluronic acid-D-xylose have been isolated from the acid hydrolysate of glucuronoxylan.[2, 16] MeGA-2X has been synthesized by Kovác *et*

al.,[17] although the chemical synthesis of other aldobiouronic acids has not been described.

Uchida *et al.* have recently reported the substrate specificity of α-glucuronidase from *Aspergillus niger*.[15] This α-glucuronidase has the ability to hydrolyze the α(1→2) glycosidic bond between the D-glucuronic acid and D-xylose residues in MeGA-2X and GA-2X. However, it has not been demonstrated whether the enzyme can hydrolyze the α(1→3) and α(1→4) glycosidic bonds between the D-glucuronic acid and D-xylose residues. α-Glucuronidase is an important enzyme for the complete hydrolysis of plant hemicellulose and it is also necessary to clarify the substrate specificity of this enzyme. The regioisomers of aldobiouronic acid are needed for further studies on the substrate specificity of α-glucuronidase. Moreover, the synthesis of the regioisomers is also important for investigating the structure-function relationships of these uronides for biological activity. In this chapter, stereoselective synthesis of three *O*-α-D-glucopyranosyluronic acid-D-xylose regioisomers is described.

GENERAL METHODS

Melting points are uncorrected. Optical rotation was measured with a Jasco DIP-140 polarimeter as a solution in $CHCl_3$ at 24°C, unless noted otherwise. All solvents were used after being purified in the usual manner. Column chromatography and flash chromatography were carried out in columns of silica gel (Merck, 240-400 mesh). TLC was conducted on silica gel 60 F_{254} plates (Merck), and the products were detected either by UV light or by charring with H_2SO_4. NMR spectra were recorded with a Jeol JNM-EX270 or Bruker AM-500 NMR spectrometer as a solution in $CDCl_3$ or D_2O. The values for δ_H and δ_C are expressed in ppm downfield from tetramethylsilane as an internal standard for solutions in $CDCl_3$, and for

solutions in D_2O are expressed downfield from the signal for the 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt. FAB-MS was performed in the positive-ion mode on a Jeol HX-100 mass spectrometer operated at an accelerating voltage of 10 kV. Samples were dissolved in an aq. KCl solution and loaded onto a stainless-steel target with glycerol-thioglycerol as the matrix, argon being used as the bombarding gas.

RESULTS

Synthesis of D-xylose acceptors.

The D-xylose acceptor unsubstituted at the C-2 position, benzyl 3,4-di-*O*-benzyl-β-D-xylopyranoside (**7**), was prepared by the method of Kovác and Petráková (Scheme 1).[18] The D-xylose acceptor unsubstituted at the C-3 position, benzyl 2,4-di-*O*-benzoyl-α-D-xylopyranoside (**9**), was synthesized as described by Helm *et al.* (Scheme 2).[19] The D-xylose acceptor unsubstituted at the C-4 position was prepared according to the procedure of Takeo *et al.*[20] with slight modifications (Scheme 3). While they had used methyl β-D-xylopyranoside as the starting material, benzyl β-D-xylopyranoside (**10**) was used in the present study as the benzyl group at the reducing end can be deprotected more easily than the methyl group. Compound **10** was acetonized with 2-methoxypropene in *N,N*-dimethylformamide (DMF) in the presence of a catalytic amount of methanolic hydrogen chloride.[20] Without isolating 2,3-isopropylidene derivative **11**, the resulting compound was alkylated with allyl bromide-sodium hydride in DMF, and treated with dilute acid in acetone. Fractional crystallization of the product provided a 58% yield of pure benzyl 4-*O*-allyl-β-D-xylopyranoside (**13**). Benzylation of **13** gave 4-*O*-allyl-2,3-di-*O*-benzyl-β-D-xylopyranoside (**14**, 84%). The isomerization of the allyl group in **14** to the 1-

propenyl ether with tris(triphenylphosphine)rhodium(I) chloride[21] and 1,4-diazabicyclo[2.2.2]octane, and subsequent hydrolysis with dilute acid in aqueous acetone[22] gave benzyl 2,3-di-*O*-benzyl-β-D-xylopyranoside (**15**) in a 70% yield.

Synthesis of D-glucuronosyl donor.

As a glucuronosyl donor, *O*-(benzyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranosyluronate) trichloroacetimidate (**20**) was selected because **20** is more stable than the halogenated D-glucuronic acid derivatives and can be easily activated with a catalytic amount of various Lewis acids. The trichloroacetimidate was prepared by the reaction of benzyl 2,3,4-tri-*O*-benzyl-D-glucopyranuronate (**19**)[23] with trichloroacetonitrile and potassium carbonate (Scheme 4),[24] and β-isomer **20** was isolated by fractional crystallization in a 70% yield. The stereochemistry of **20** was confirmed to be β-form by the ^{1}H-NMR spectrum, which showed a doublet for the H-1 proton at δ 5.89 ppm ($J_{1,2}$ = 7.0 Hz).

Glycosylation and deprotection.

Glycosylation of **7**, **9** or **15** with **20** was carried out in the presence of trimethylsilyl trifluoromethane-sulfonate (TMSOTf)[25] as a catalyst in CH_2Cl_2 (Scheme 5). Glycosylation of trichloroacetimidate **20** proceeded stereoselectively to afford the corresponding α-glycosides, which were isolated by flash chromatography to give disaccharide derivatives **21α**, **23α** and **25α** in 60%, 44% and 53% yields, respectively. The β-isomers of these disaccharides were also isolated to give **23β** and **25β** in 23% and 6% yields, respectively. The purification of **21β** failed, but the ^{1}H-NMR spectrum of the crude disaccharide products containing **21α** and **21β** showed

the formation of **21β** in about a 15% yield (**21α**:**21β** = 4:1) by comparing the intensities of the newly formed anomeric signals. The structures of the glycosylated products were identified according to their ^{1}H- and ^{13}C-NMR spectra. The configuration at the newly formed anomeric center was assignable from the δ value for C-1' (α-form: **21α**, 95.5; **23α**, 98.0; **25α**, 99.2 ppm. β-form: **21β**, 101.4; **23β**, 103.9; **25β**, 102.3 ppm). Isolated **21α** and **25α** were deprotected by catalytic hydrogenation in the usual manner to give **22** (GA-2X) and **26** (GA-4X), respectively. On the other hand, **23α** was debenzylated by catalytic hydrogenation and then debenzoylated with NaOMe in MeOH to give disaccharide **24** (GA-3X). The structures of **22**, **24** and **26** were confirmed by their ^{1}H- (Fig.s 1, 2, and 3, respectively) and ^{13}C- (Fig.s 4, 5, and 6, respectively) NMR spectra, and by FAB-MS analyses (Fig.s 7, 8, and 9, respectively) were in good agreement with the proposed structure. The ^{1}H-NMR spectra showed that disaccharides **22**, **24** and **26** were present in the same anomeric ratio of α:β = 2:3 in a D_2O solution by comparing the intensities of the H-1' signals.

DISCUSSION

There are many reports about stereoselective glycosylations, but a few described about glycosylation of glycuronides (see some reviews [26-29]). I have tried some glycosylation reaction to synthesize aldobiouronic acid by using fluoride, chloride and thioglycoside derivatives, *etc*. The trichloroacetimidate (**20**) was more stable than the others and gave good yield (data not shown). The glycosylation reaction was stereoselective and gave corresponding α-anomer in 44 - 60% yields. The glycosylation products were easily converted into regioisomeric aldobiouronic acids.

In conclusion, the stereoselective synthesis of aldobiouronic acid regioisomers was efficiently achieved by the trichloroacetimidate method for the glycosylation reaction.

EXPERIMENTAL DETAILS

Benzyl 4-O-allyl-β-D-xylopyranoside (13). To a stirred suspension of benzyl β-D-xylopyranoside (**10**)[30] (3.20 g, 13.3 mmol) in dry DMF (10 ml) containing 10% HCl in MeOH (60 ml), 2-methoxypropene (3.20 ml, 33.4 mmol) was added slowly. The mixture was stirred for 1 h at room temperature, before the reaction mixture was diluted with $CHCl_3$ (100 ml) and washed with water (100 ml). The aqueous layer was extracted twice with $CHCl_3$ (50 ml), and the combined $CHCl_3$ solution was made slightly acidic by adding 10% HCl in MeOH (10 ml). After 10 min, the solution was successively washed with aq. $NaHCO_3$, water and brine, dried ($MgSO_4$), and concentrated to dryness. To a stirred solution of the residue in DMF (50 ml), washed NaH (0.64 g, 27.8 mmol) was added portionwise at 0°C, and the mixture was stirred for 30 min at 0°C. To the mixture, allyl bromide (3.50 g, 27.1 mmol) was added dropwise, before stirring for 2 h at room temperature. Upon completion of the reaction, the excess amount of NaH was decomposed by adding MeOH. Bulk solvent was removed by evaporation, the residue was dissolved in acetone (30 ml). To this mixture, 1 N aq. HCl (2.0 ml) was added, and the solution was boiled under reflux for 20 min. The resulting solution was neutralized with solid $NaHCO_3$, filtered through a Celite pad, and evaporated. The residue was crystallized from hexane-EtOH to give **13** (2.18 g, 58%), mp 54.5-55.5°C, $[\alpha]_D$ -104.5° (*c* 0.98, $CHCl_3$), R_f 0.17 (hexane-EtOAc 2:1). NMR ($CDCl_3$): δ_H 3.45-3.57 (3H, m, H-5ax., H-2 and H-4), 3.77 (1H, t, J = 5.8 Hz, H-3), 4.04-4.15 (3H, m, H-5eq. and $OCH_2CH=CH_2$), 4.62 (1H, d, J = 4.6 Hz, H-1), 5.26 (2H, m, $OCH_2CH=CH_2$), 5.91 (1H, m, $OCH_2CH=CH_2$); δ_C 60.5 (C-5), 70.7 ($OCH_2CH=CH_2$), 70.9 (C-2 and C-3), 76.5 (C-4), 101.2 (C-1), 118.1 ($OCH_2CH=CH_2$), 134.3 ($OCH_2CH=CH_2$). *Anal.* Found: C, 64.11; H; 7.11. Calcd. for $C_{15}H_{20}O_5$: C, 64.27; H, 7.15%.

*Benzyl 4-O-allyl-2,3-di-O-benzyl-β-D-xylopyranoside (**14**).* To a solution of **13** (2.00 g, 7.11 mmol) in dry DMF, washed NaH (0.50 g, 21.7 mmol) was added portionwise at 0°C. Benzyl bromide (3.60 g, 21.0 mmol) was then added dropwise to the mixture followed by stirring at room temperature for 20 h. After decomposing the excess amount of NaH by adding MeOH, most of the solvent was evaporated off, and the residue was partitioned with EtOAc-water. The EtOAc layer was washed with water and brine, dried ($MgSO_4$), filtered and concentrated. The residue was purified by chromatography (40 g of silica gel, hexane-EtOAc 10:1), and crystallized from hot hexane to give **14** (2.73 g, 84%), mp 61.0-61.2°C, $[\alpha]_D$ -12.6° (*c* 1.00, $CHCl_3$), R_f 0.37 (hexane-EtOAc 10:1). NMR ($CDCl_3$): δ_H 3.20 (1H, dd, J = 11.5 and 9.9 Hz, H-5ax.), 3.42 (1H, dd, J = 9.2 and 7.3 Hz, H-2), 3.47-3.59 (2H, m, H-3 and H-4), 4.02 (1H, dd, J = 11.5 and 5.1 Hz, H-5eq.), 4.16 (2H, m, $OCH_2CH=CH_2$), 4.47 (1H, d, J = 7.3 Hz, H-1), 5.23 (2H, m, $OCH_2CH=CH_2$), 5.9 (1H, m, $OCH_2CH=CH_2$); δ_C 63.9 (C-5), 71.1 ($OCH_2CH=CH_2$), 77.4 (C-4), 81.9 (C-2), 83.7 (C-3), 103.2 (C-1), 117.3 ($OCH_2CH=CH_2$), 134.7 ($OCH_2CH=CH_2$). *Anal.* Found: C, 75.89; H, 6.99. Calcd. for $C_{29}H_{32}O_5$: C, 75.63; H, 7.00%.

*Benzyl 2,3-di-O-benzyl-β-D-xylopyranoside (**15**).* A mixture of **14** (2.32 g, 5.34 mmol), tris(triphenylphosphine)rhodium(I) chloride (0.95 g, 1.03 mmol), and 1,4-diazabicyclo[2.2.2]octane (1.70 g, 15.2 mmol) in 10:3:1 EtOH-toluene-water (90 ml) was boiled for 9 h under reflux. The reaction mixture was concentrated to dryness, and the residue was extracted with $CHCl_3$ (100 ml). The extract was successively washed with cold dil. HCl, aq.

$NaHCO_3$, water and brine, dried ($MgSO_4$) and concentrated. To a solution of the residue in 9:1 acetone-water (20 ml), 1 N aq. HCl (0.20 ml) was added, and the solution was boiled for 10 min under reflux. After neutralizing the resulting solution with solid $NaHCO_3$, the mixture was filtered and concentrated. A solution of the residue in $CHCl_3$ was washed with water and brine, dried and concentrated. The residue was subjected to column chromatography (50 g of silica gel, benzene-EtOAc 10:1) to give **15** (1.59 g, 71%), mp 125.5-126.0°C, $[\alpha]_D$ -62.9° (*c* 1.00, $CHCl_3$), R_f 0.24 (hexane-EtOAc 2:1). NMR ($CDCl_3$): δ_H 2.44 (1H, s, OH), 3.29 (1H, dd, J = 8.6 and 11.6 Hz, H-5ax.), 3.42 (1H, t, J = 7.7 Hz, H-3), 3.43 (1H, dd, J = 6.2 and 7.7 Hz, H-2), 3.70 (1H, m, H-4), 4.04 (1H, dd, J = 4.7 and 11.6 Hz, H-5eq.), 4.57 (1H, J = 6.2 Hz, H-1); δ_C 64.4 (C-5), 69.1 (C-4), 80.1 (C-2), 82.0 (C-3), 102.7 (C-1). Anal. Found: C, 74.11; H, 6.74. Calcd. for $C_{26}H_{28}O_5$: C,74.26; H, 6.71%.

O-(Benzyl 2,3,4-tri-O-benzyl-β-D-glucopyranosyluronate) trichloroacetimidate (20). To a solution of benzyl 2,3,4-tri-*O*-benzyl-D-glucopyranuronate (**19**;[23] 1.50 g, 2.70 mmol) in dry CH_2Cl_2 (15 ml), K_2CO_3 (1.50 g, 10.8 mmol) and trichloroacetonitrile (1.50 ml, 15.0 mmol) were added, and the mixture was stirred under N_2 at room temperature for 4 h. The resulting solution was filtered through a Celite pad, and the insoluble material was washed with CH_2Cl_2. The filtrate and washings were combined and concentrated, and the residual syrup was crystallized from hexane-EtOAc to give **20** (1.46 g, 77%), mp 96.2-97.6°C, $[\alpha]_D$ +5.66° (*c* 1.12, $CHCl_3$), R_f 0.56 (hexane-EtOAc 2:1). NMR($CDCl_3$): δ_H 3.77 (2H, m, H-2 and H-4), 3.94 (1H, t, J = 9.0 Hz, H-3), 4.14 (1H, d, J = 9.7 Hz, H-5), 5.89 (1H, d, J = 7.0 Hz, H-1), 8.73 (1H, s, NH); δ_C 75.0 (C-5), 78.8 (C-2), 80.4 (C-4), 83.6 (C-3), 90.7 (CCl_3), 98.0 (C-1), 160.9

($C(=NH)CCl_3$), 166.1 (C=O). *Anal.* Found: C, 62.10; H, 5.15; N, 1.88. Calcd. for $C_{36}H_{34}O_7NCl_3$: C, 61.85; H, 4.90; N, 2.00%.

Glycosylation reaction. To a solution of glucuronosyl donor **20** (0.60 mmol) and xylose acceptor **7**, **9** or **15** (0.50 mmol) in dry CH_2Cl_2 (5.0 ml), 0.4 M TMSOTf in dry CH_2Cl_2 (70 ml) was added under N_2 at -20°C. The solution was stirred at -20°C for 30 min. Upon completion of the reaction, Et_3N was added to quench the catalyst, and the resulting solution was diluted with CH_2Cl_2, washed with water and brine, dried and concentrated. Subsequent flash chromatography of the residue gave each pure disaccharide derivative.

Benzyl O-(benzyl 2,3,4-tri-O-benzyl-α-D-glucopyranosyluronate)-(1→2)-3,4-di-O-benzyl-β-D-xylopyranoside (21α). The glycosylation product was purified by a flash chromatography (80 g of silica gel, benzene-EtOAc 20:1) to give compound **21α**. The formation of **21β** was confirmed by the ^{1}H-NMR spectrum of the crude glycosylated product. The α/β ratio of the disaccharide derivatives was estimated as 4:1 by comparing the NMR intensities of the newly formed anomeric signals. However, the isolation of **21β** failed, because complete separation of **21β** was limited at flash chromatographical techniques.

Compound **21α**: 287.1 mg, 60%, $[\alpha]_D$ +3.61° (*c* 1.00, $CHCl_3$), R_f 0.45 (hexane-EtOAc 3:1). NMR ($CDCl_3$): δ_H 3.26 (1H, m, H-5ax.), 3.52 (1H, dd, *J* = 3.5 and 9.8 Hz, H-2'), 3.66 (1H, dd, *J* = 9.2 and 9.9 Hz, H-4'), 3.68 (2H, m, H-3 and H-4), 3.81 (1H, dd, *J* = 7.5 and 9.3 Hz, H-2), 3.95 (1H, dd, *J* = 4.5 and 11.4 Hz, H-5eq.), 3.96 (1H, t, *J* = 9.4 Hz, H-3'), 4.61 (1H, d, *J* = 7.5 Hz, H-1), 5.70 (1H, d, *J* = 3.5 Hz, H-1'); δ_C 63.7 (C-5), 70.4 (C-2), 75.3 (C-4'), 78.5 (C-4), 79.0

(C-5'), 80.0 (C-2'), 80.9 (C-3), 81.3 (C-3'), 95.5 (C-1'), 103.4 (C-1), 170.2 (C-6'). *Anal.* Found: C, 74.67; H, 6.30. Calcd. for $C_{60}H_{60}O_{11}$: C, 74.59; H, 6.36%.

2-O-α-D-glucopyranosyluronic acid-D-xylose (22 , GA-2X). A solution of **21α** (280.0 mg, 0.29 mmol) in acetic acid (5 ml) was hydrogenated in the presence of 10% Pd-C (50 mg) at atmospheric pressure and room temperature for 24 h. The insoluble material was collected on a Celite pad and washed with MeOH, before the combined filtrate and washings were evaporated. The residue was purified by anion-exchange chromatography on DEAE-Sephadex A-25 (30 x 180 mm, Pharmacia) with a linear gradient of an $NH_4 \cdot HCO_3$ buffer (0.05 → 0.3 M). Fractions were assayed for total sugar concentration by the phenol-sulfuric acid method. The fractions containing **22** were combined and concentrated, and $NH_4 \cdot HCO_3$ was removed by evaporating water from the residue several times to give **22** (77.3 mg, 81%), $[\alpha]_D$ +98.8° (*c* 0.77, H_2O) [lit.[31] +88 - +98°]. NMR (D_2O, 500 MHz): δ_H 4.71 (0.4H, d, *J* = 10.1 Hz, H-5'α), 4.61 (0.6H, d, *J* = 10.2 Hz, H-5'β), 4.72 (0.6H, d, *J* = 7.9 Hz, H-1β), 5.11 (0.4H, d, *J* = 3.7 Hz, H-1α), 5.37 (0.4H, d, *J* = 3.7 Hz, H-1'α), 5.38 (0.6H, d, *J* = 3.6 Hz, H-1'β); δ_C (125 MHz): 63.5 (C-5α), 67.7 (C-5β), 79.3 (C-5'α), 81.5 (C-5'β), 92.4 (C-1α), 99.5 (C-1'α), 99.6 (C-1'α), 100.6 (C-1β). FAB-MS m/z: 365, ($[M+K]^+$).

Benzyl O-(benzyl 2,3,4-tri-O-benzyl-α- and β-D-glucopyranosyluronate)-(1→3)-2,4-di-O-benzoyl-α-D-xylopyranoside (23α and 23β). The glycosylation products were purified by a flash chromatography (80 g of silica gel, benzene-EtOAc 20:1) to give compounds **23α** and **23β**.

Compound **23α**: 216.7 mg, 44%, $[\alpha]_D$ +26.8° (*c* 1.07, $CHCl_3$), R_f 0.74 (benzene-EtOAc

15:1). NMR ($CDCl_3$): δ_H 3.37 (1H, dd, J = 3.3 and 9.6 Hz, H-2'), 3.53 (1H, t, J = 9.6 Hz, H-3'), 3.86 (2H, m, H-5ax. and H-4'), 4.03 (1H, dd, J = 5.9 and 10.9 Hz, H-5eq.), 4.43 (1H, d, J = 9.9 Hz, H-5'), 5.18 (1H, d, J = 3.6 Hz, H-1), 5.23 (1H, d, J = 3.3 Hz, H-1'), 5.27 (1H, dd, J = 3.6 and 9.6 Hz, H-2), 5.48 (1H, m, H-4); δ_C 58.8 (C-5), 70.9 (C-3), 72.1 (C-4), 72.3 (C-2), 74.4 (C-4'), 78.4 (C-5'), 79.5 (C-2'), 80.5 (C-3'), 95.2 (C-1), 98.0 (C-1'), 165.1 and 165.6 (C=O), 169.6 (C-6'). *Anal.* Found: C, 72.94; H, 5.64. Calcd. for $C_{60}H_{56}O_{13}$: C, 73.16; H, 5.73%.

Compound **23β**: 113.3 mg, 23%, $[\alpha]_D$ +20.6° (*c* 0.87, $CHCl_3$), R_f 0.66 (benzene-EtOAc 15:1). NMR ($CDCl_3$): δ_H 3.32-3.45 (2H, m, H-2' and H-4'), 3.74 (1H, dd, J = 8.9 and 9.6 Hz, H-3'), 3.80 (1H, t, J = 10.9 and 10.6 Hz, H-5ax.), 3.94 (1H, d, J = 9.9 Hz, H-5'), 4.10 (1H, dd, J = 5.6 and 10.9 Hz, H-5eq.), 4.73 (1H, d, J = 6.0 Hz, H-1'), 5.16 (1H, dd, J = 4.0 and 9.9 Hz, H-2), 5.19 (1H, d, J = 4.0 Hz, H-1); δ_C 59.1 (C-5), 70.3 (C-3), 74.0 (C-4), 74.6 (C-2), 74.7 (C-4'), 79.2 (C-5'), 81.6 (C-2'), 83.7 (C-3'), 95.1 (C-1), 103.9 (C-1'), 165.4 and 166.1 (C=O), 168.3 (C-6'). *Anal.* Found: C, 73.25; H, 5.79. Calcd. for $C_{60}H_{56}O_{13}$: C, 73.16; H, 5.73%.

3-O-α-D-glucopyranosyluronic acid-D-xylose (24, GA-3X). A solution of **23α** (210.0 mg, 0.21 mmol) in acetic acid (4 ml) was hydrogenated in the presence of 10% Pd-C (50 mg) at atmospheric pressure and room temperature for 24 h. After removing the catalyst by filtration, the filtrate was concentrated. The residue was dissolved in MeOH (5 ml), before 0.1 N NaOMe in MeOH (0.4 ml) was added to the solution. Upon completion of the reaction, the reaction mixture was neutralized with solid CO_2, and the solution was concentrated. The residue was purified by anion-exchange chromatography, as described for **22**, to give **24** (54.9 mg, 80%), $[\alpha]_D$ +19.5° (*c* 0.50, H_2O). [lit.[31] +18.5° (*c* 3.28, H_2O)]. NMR (D_2O, 500 MHz): δ_H 4.50

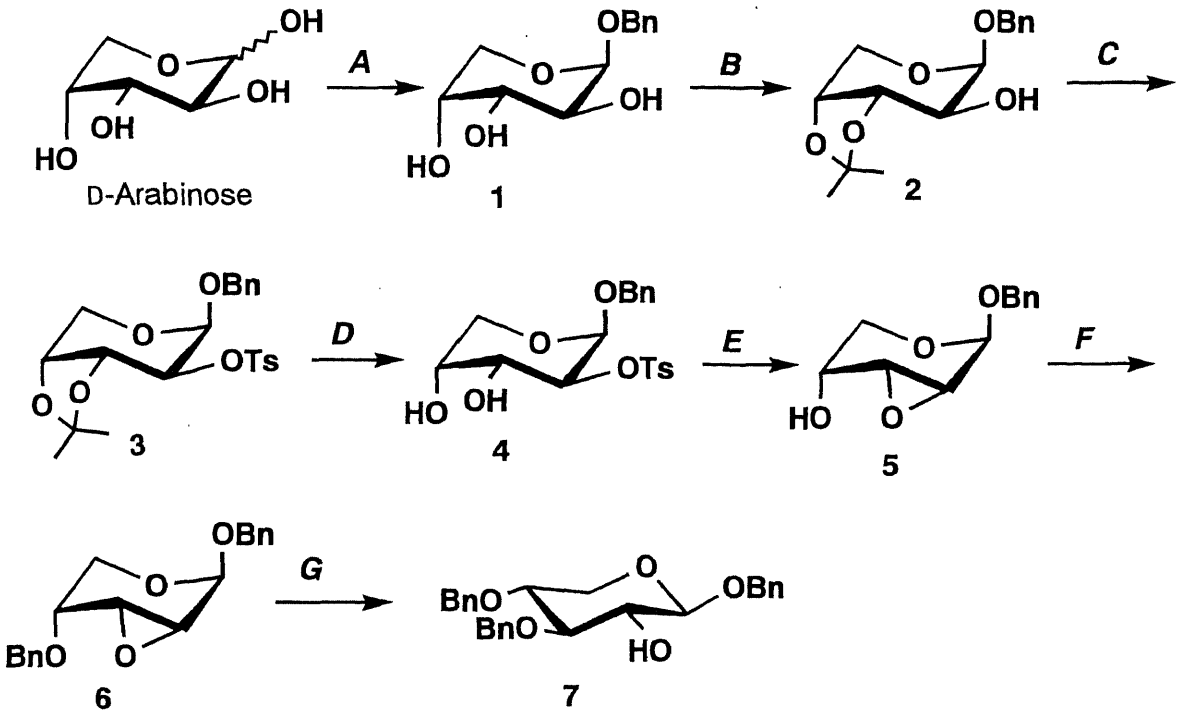
(0.4H, d, J = 10.1 Hz, H-5'α), 4.55 (0.6H, d, J = 10.2 Hz, H-5'β), 4.57 (0.6H, d, J = 8.0 Hz, H-1β), 5.18 (0.4H, d, J = 3.6 Hz, H-1α), 5.38 (0.4H, d, J = 4.4 Hz, H-1'α), 5.39 (0.6H, d, J = 4.0 Hz, H-1'β); δC (125 MHz): 63.7 (C-5α), 67.7 (C-5β), 82.1 (C-5'α), 84.4 (C-5'β), 95.1 (C-1α), 99.4 (C-1β), 101.5 (C-1'β), 101.7 (C-1'α). FAB-MS m/z: 365, ($[M+K]^+$).

*Benzyl O-(benzyl 2,3,4-tri-O-benzyl-α- and β-D-glucopyranosyluronate)-(1→4)-2,3-di-O-benzyl-β-D-xylopyranoside (**25α** and **25β**).* The glycosylation products were purified by a flash chromatography (80 g of silica gel, benzene-EtOAc 20:1) to give compounds **25α** and **25β**.

Compound **25α**: 253.6 mg, 53%, $[\alpha]_D$ +1.71° (*c* 0.53, $CHCl_3$), R_f 0.52 (benzene-EtOAc 15:1). NMR ($CDCl_3$): δ_H 3.30 (1H, dd, J = 9.8 and 11.1 Hz, H-5ax.), 3.45 (1H, dd, J = 9.0 and 7.6 Hz, H-2), 3.54 (1H, dd, J = 9.6 and 3.6 Hz, H-2'), 3.60 (1H, t, J = 9.0 Hz, H-3), 3.97 (1H, t, J = 9.6 Hz, H-3'), 4.15 (1H, dd, J = 5.5 and 11.1 Hz, H-5eq.), 4.23 (1H, d, J = 9.9 Hz, H-5'), 4.44 (1H, d, J = 7.6 Hz, H-1), 5.15 (1H, d, J = 3.6 Hz, H-1'); δ_C 64.5 (C-5), 71.1 (C-4), 78.8 (C-4'), 78.9 (C-5'), 79.3 (C-3), 80.8 (C-2'), 81.7 (C-2), 82.8 (C-3'), 99.2 (C-1'), 102.9 (C-1), 169.1 (C-6'). *Anal.* Found: C, 74.31; H, 6.21. Calcd. for $C_{60}H_{60}O_{11}$•0.5H_2O: C, 74.59; H, 6.36%.

Compound **25β**: 28.7 mg, 5.9%, $[\alpha]_D$ -23.8° (*c* 0.82, $CHCl_3$), R_f 0.45 (benzene-EtOAc 15:1). NMR ($CDCl_3$): δ_H 3.21 (1H, m, H-4), 3.44 (1H, t, J = 8.1 Hz, H-3), 3.44 (1H, t, J = 8.6 Hz, H-2'), 3.61 (2H, m, H-2 and H-4'), 3.83-4.06 (3H, m, H-3', H-5eq. and H-5ax.), 3.85 (1H, d, J = 9.9 Hz, H-5'), 4.53 (1H, d, J = 7.9 Hz, H-1), 4.86 (1H, d, J = 8.6 Hz, H-1'); δ_C 62.9 (C-5), 74.6 (C-4), 77.3 (C-4'), 79.4 (C-5'), 81.2 (C-3), 81.8 (C-2'), 82.0 (C-2), 83.8 (C-3'), 102.3 (C-1'), 102.8 (C-1), 168.1 (C-6'). *Anal.* Found: C, 75.29; H, 6.31. Calcd. for $C_{60}H_{60}O_{11}$: C, 75.30; H, 6.32%.

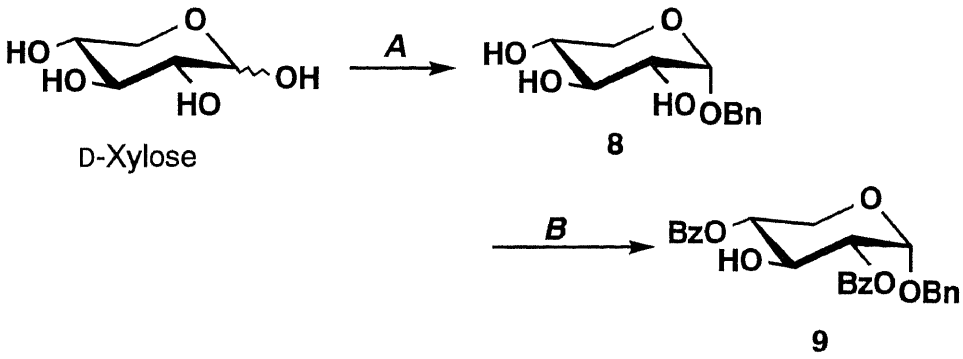
4-O-α-D-glucopyranosyluronic acid-D-xylose (26, GA-4X). Debenzylation of **25α** (250.0 mg, 0.26 mmol), in the same manner as that described for **22**, with 10% Pd-C (50 mg) in acetic acid (5 ml) at room temperature for 24 h, and anion-exchange chromatography of the crude product on DEAE-Sephadex A-25, as also described for **22**, gave **26** (72.4 mg, 85%), $[\alpha]_D$ +86.7° (*c* 0.54, H_2O). NMR (D_2O, 500 MHz): δ_H 4.57 (0.6H, d, *J* = 7.9 Hz, H-1β), 5.18 (0.4H, d, *J* = 3.7 Hz, H-1'α), 5.20 (0.6H, d, *J* = 3.7 Hz, H-1'β); δ_C (125 MHz): 62.6 (C-5a), 66.8 (C-5b), 81.0 (C-5'a), 81.1 (C-5'b), 94.6 (C-1a), 99.1 (C-1b), 102.7 (C-1'a), 102.7 (C-1'b). FAB-MS m/z: 365, ($[M+K]^+$).



Scheme 1. Synthesis of 2-Xylose Acceptor, **7**.

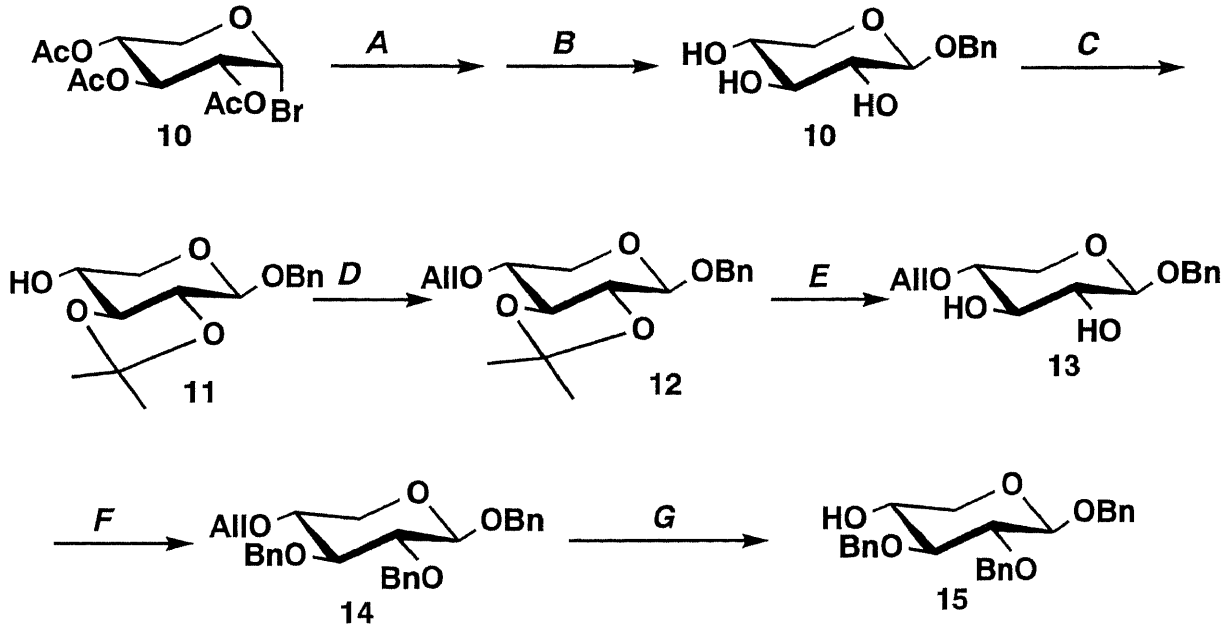
A : HCl, Benzyl alcohol, 80.4%. ***B*** : H_2SO_4, Acetone, $CaSO_4$. ***C*** : TsCl, Pyridine. ***D*** : HCOOH, Acetone, reflux, (***B*** -***D*** 80.0%). ***E*** : 0.2N NaOMe, 75%. ***F*** : BnBr, NaH, DMF, 80%. ***G*** : Benzyl alcohol, NaH, 80%.

Ts, *p* -toluenesulfonyl



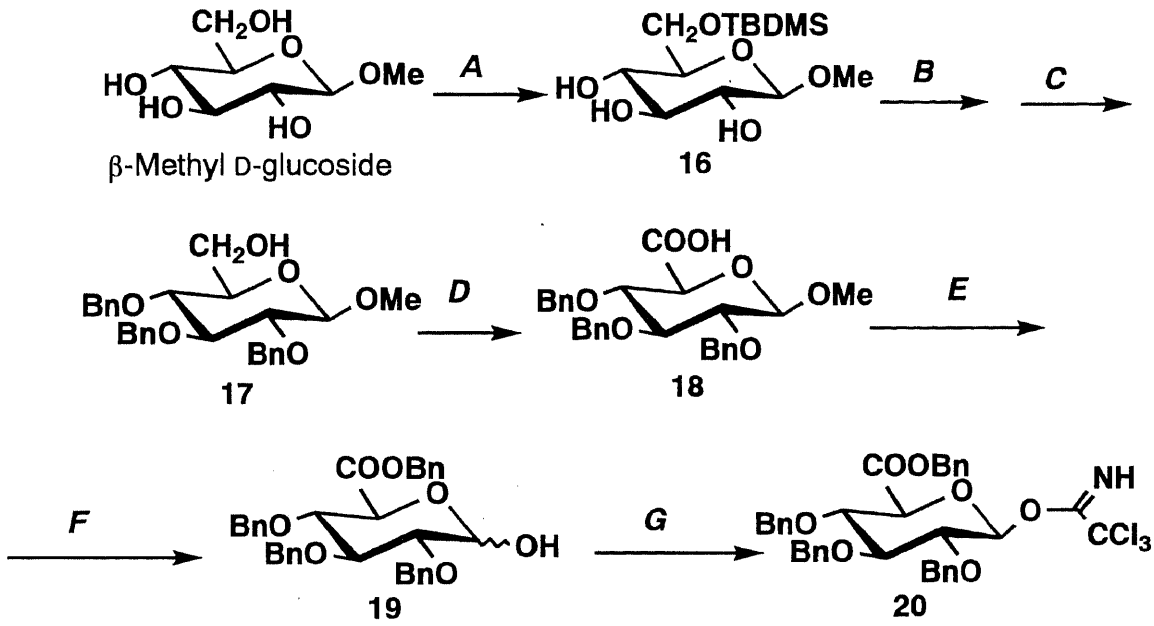
Scheme 2. Synthesis of 3-Xylose Acceptor, **9**.

A : Acetyl chloride, Benzyl alcohol, 40%. ***B*** : Dibutyltin oxide, Benzoyl chloride, Benzene, reflux, 85%.



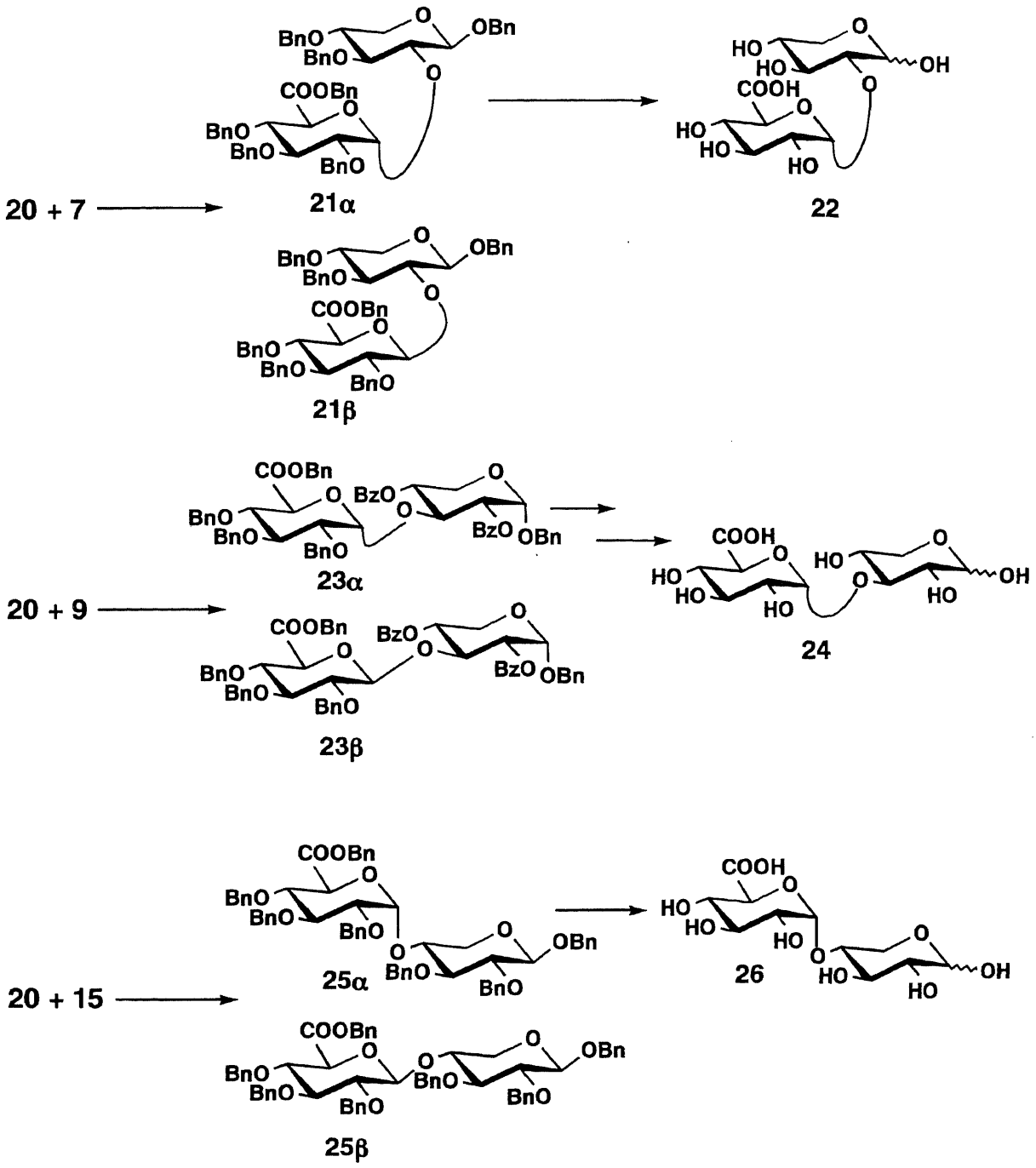
Scheme 3. Synthesis of 4-Xylose Acceptor, **15**.

A : Benzyl alcohol, Ag_2CO_3, I_2, Benzene. ***B*** : NaOMe, (***A*** -***B*** 81.6%).
C : 2-Methoxypropene, H^+, DMF. ***D*** : Allyl bromide, NaH, DMF. ***E*** : H^+, Acetone, reflux, (***C*** -***E*** 58.5%). ***F*** : BnBr, NaH, DMF, 64.8%.
G : 1. $(PPh_3)_3Rh(I)Cl$, DBO, 2. H^+, 45.9%.
DBO, 1,4-diazabycyclo[2,2,2]octane



Scheme 4. Synthesis of D-Glucuronosyl Donor, **20**.

A : $^{t}BuMe_2SiCl$, Imidazol, DMF, 77.5%. ***B*** : BnBr, NaH, DMF. ***C*** : Bu_4NF, DMF, (***B*** -***C*** 65.1%). ***D*** : $K_2Cr_2O_7$, H_2SO_4, Acetone, 76.9%. ***E*** : AcOH-TFA-H_2O, 100-105°C. ***F***: BnBr, Cs_2CO_3, DMF, (***E*** -***F*** 43.6%). ***G*** : CCl_3CN, K_2CO_3, CH_2Cl_2, -16°C, 80%.



Scheme 5. Synthesis of Regioisomeric Aldobiouronic Acids, **22**, **24** and **26**.

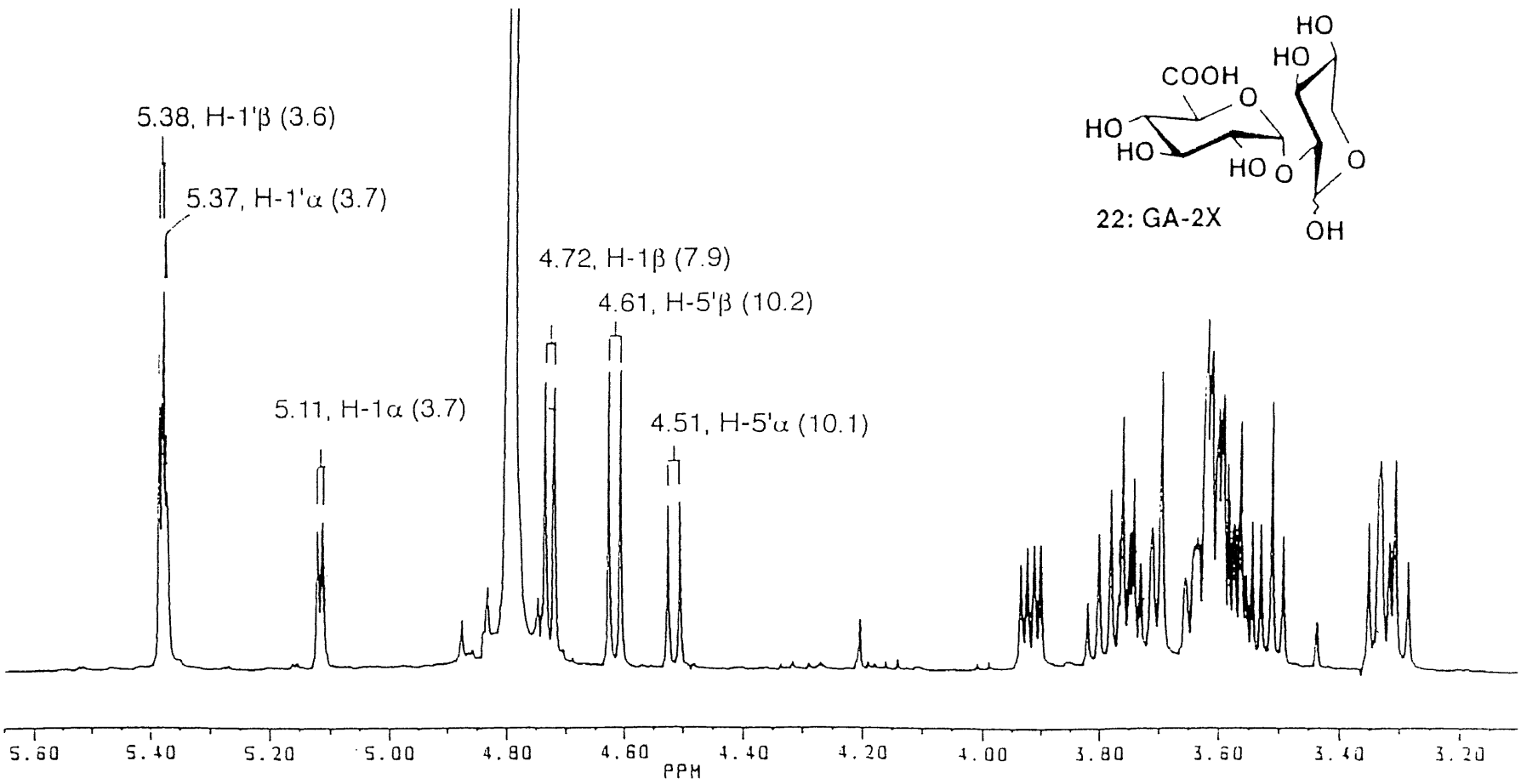


Fig. 1. ^{1}H-NMR Spectrum of GA-2X (**22**) in D_2O.

Values in parentheses are *J* in Hz.

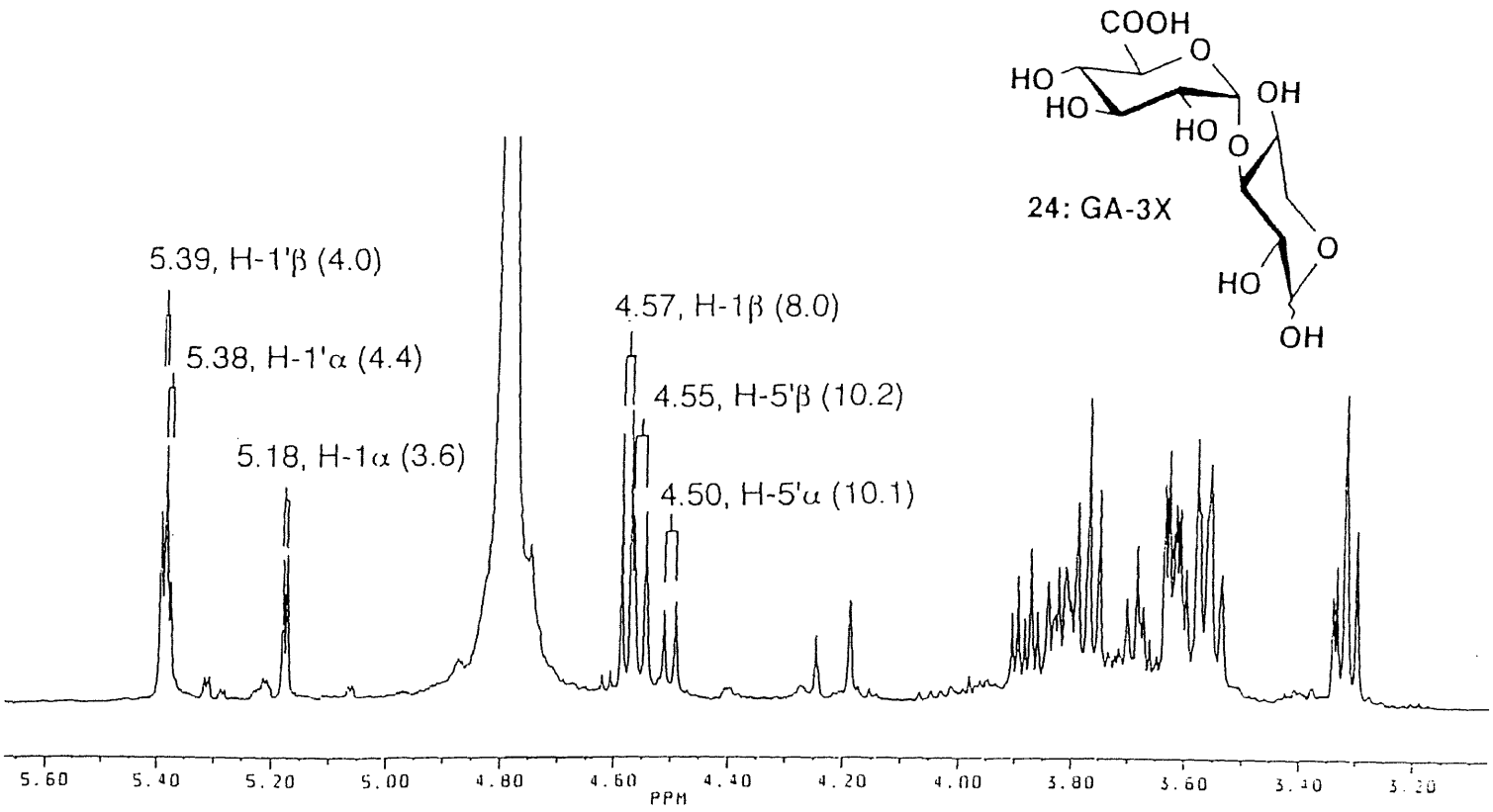


Fig. 2. ^{1}H-NMR Spectrum of GA-3X (**24**) in D_2O.

Values in parentheses are *J* in Hz.

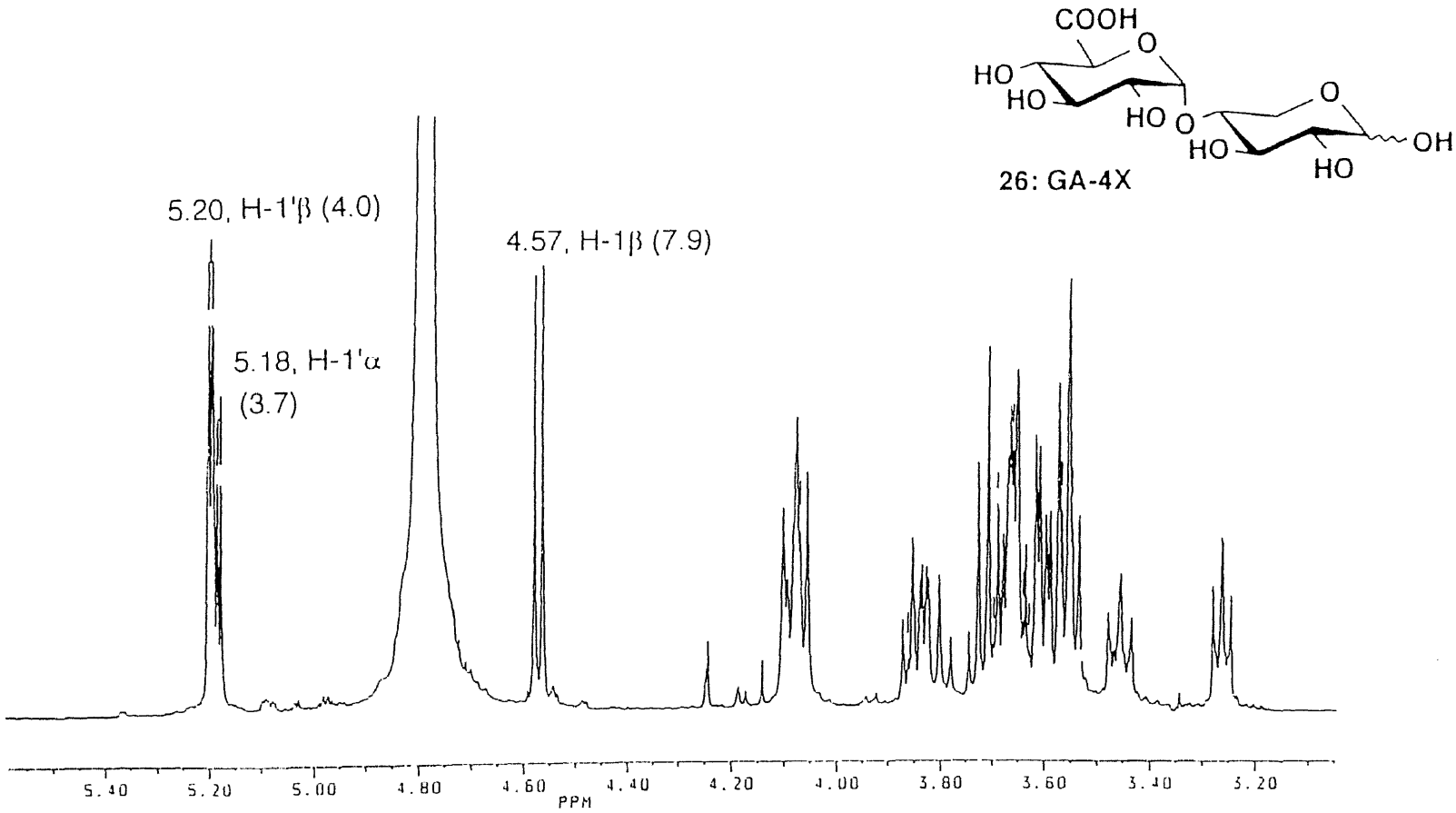


Fig. 3. ^{1}H-NMR Spectrum of GA-4X (**26**) in D_2O.

Values in parentheses are J in Hz.

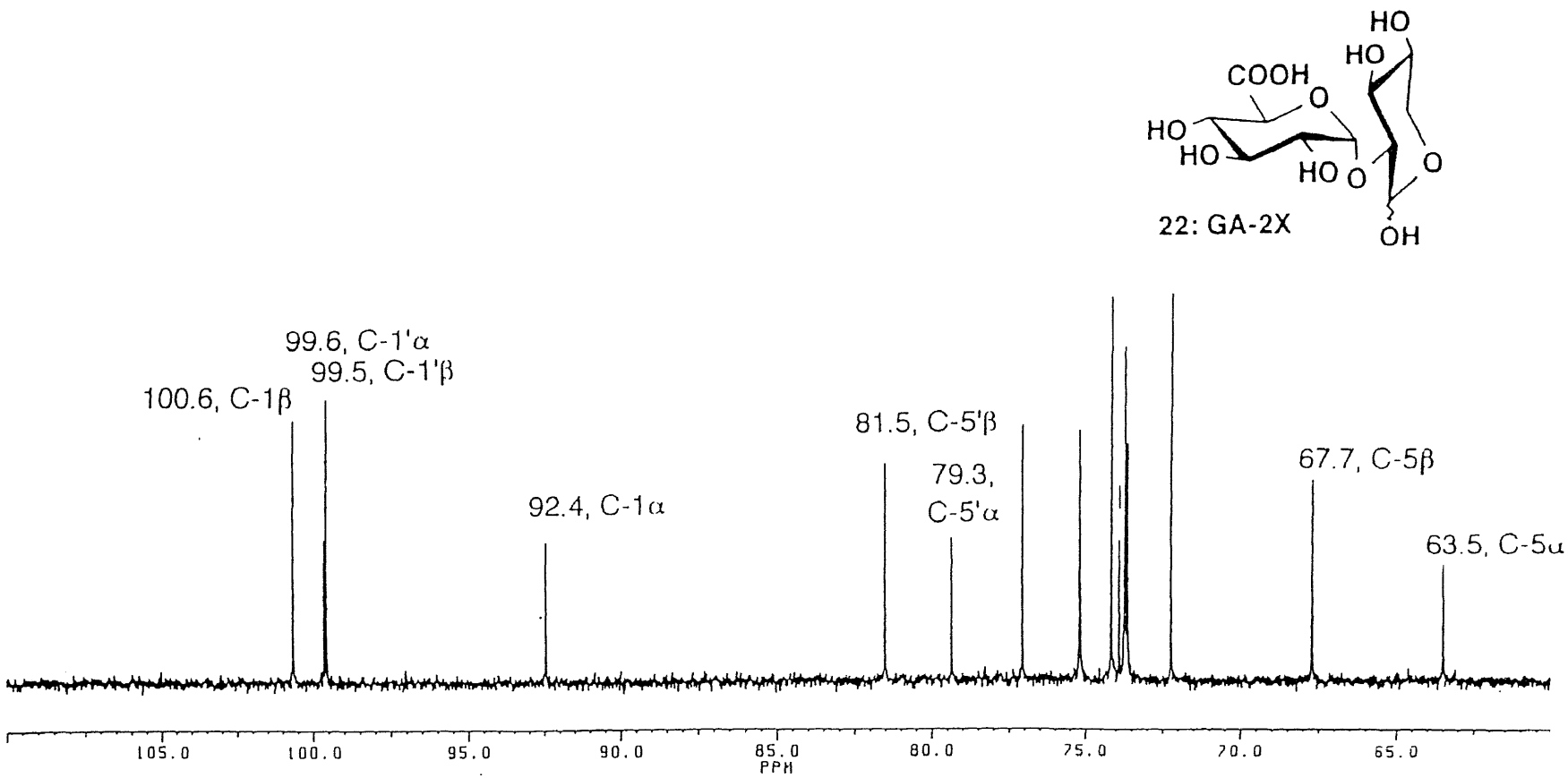


Fig. 4. ^{13}C-NMR Spectrum of GA-2X (**22**) in D_2O.

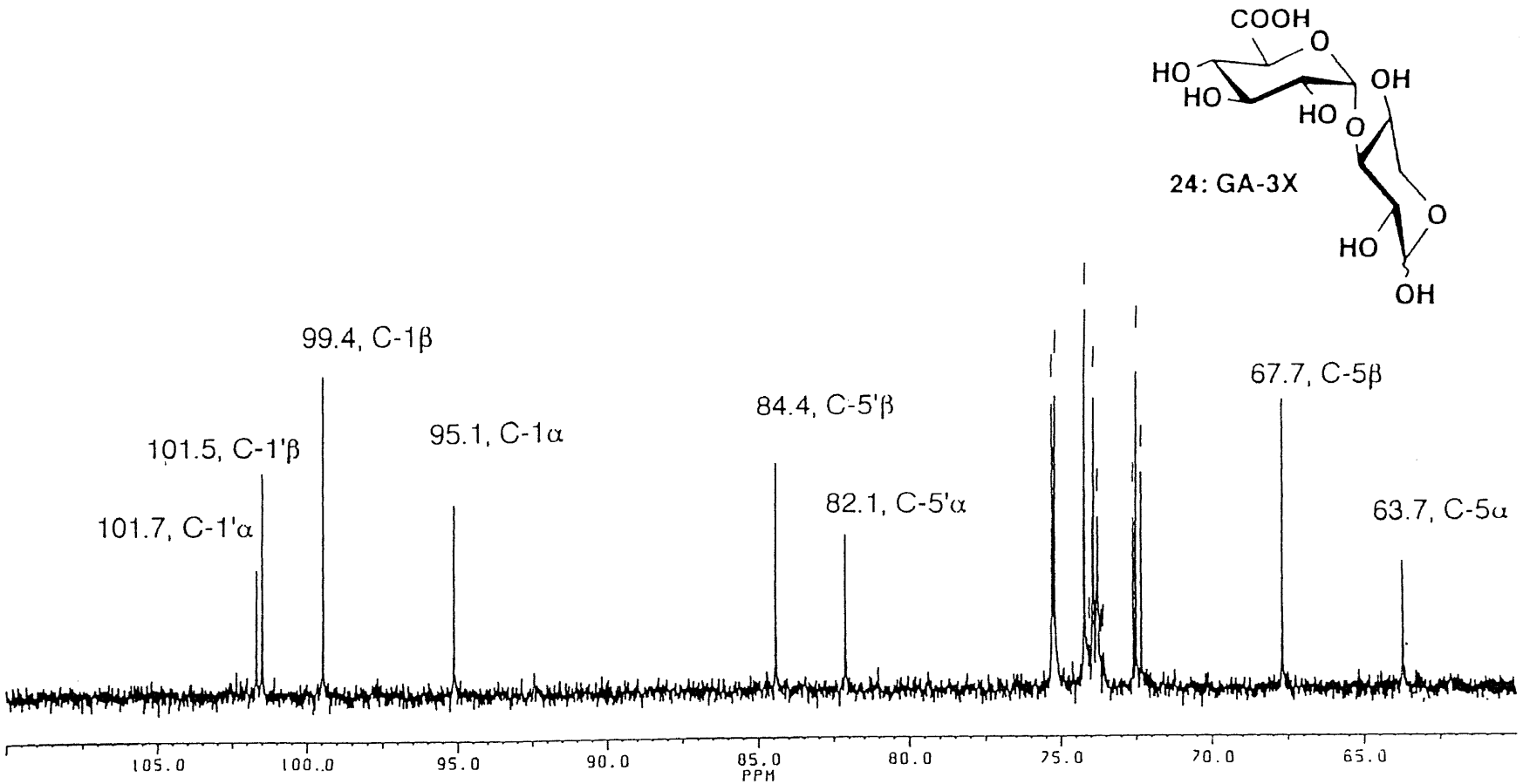


Fig. 5. ^{13}C-NMR Spectrum of GA-3X (**24**) in D_2O.

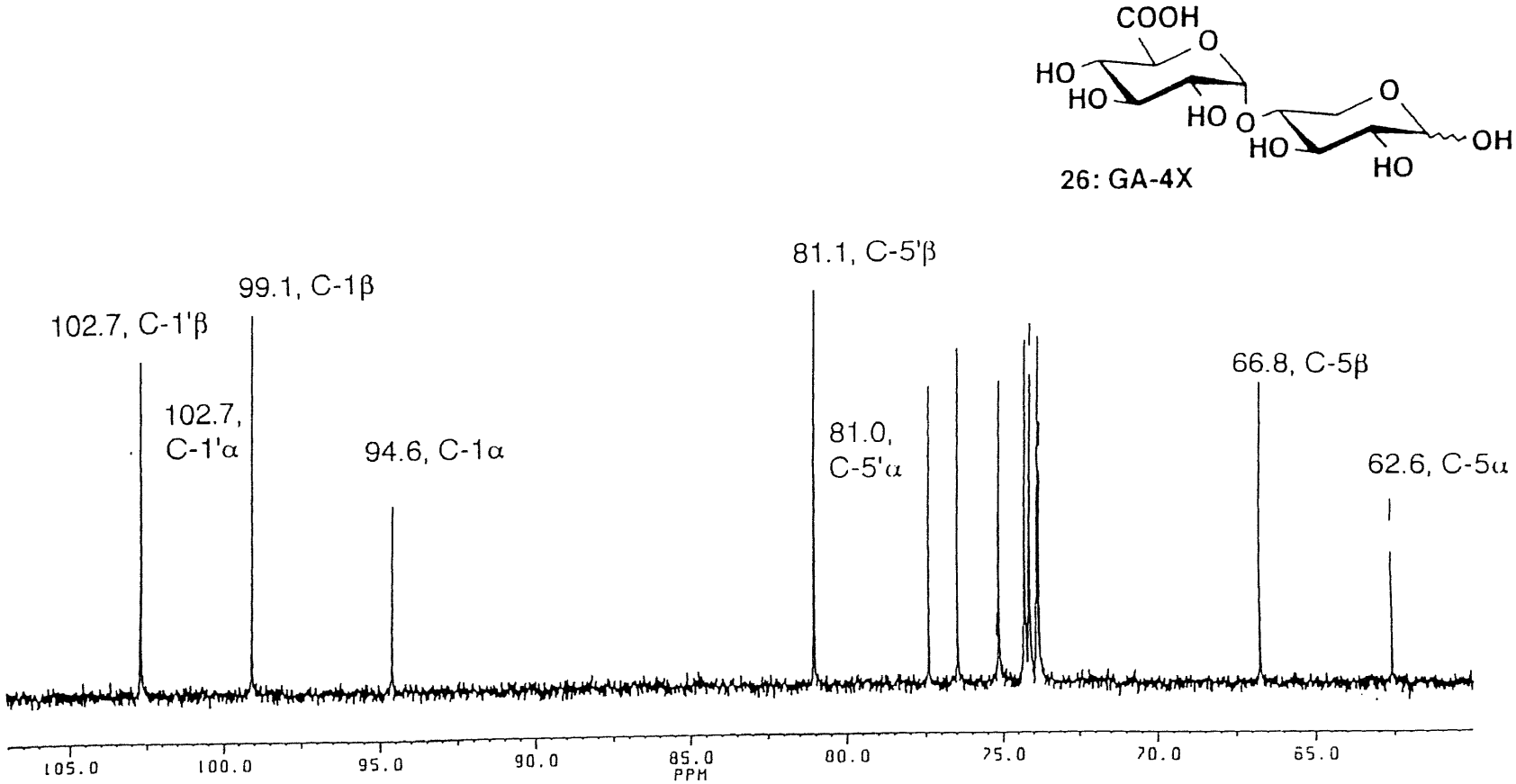


Fig. 6. ^{13}C-NMR Spectrum of GA-4X (**26**) in D_2O.

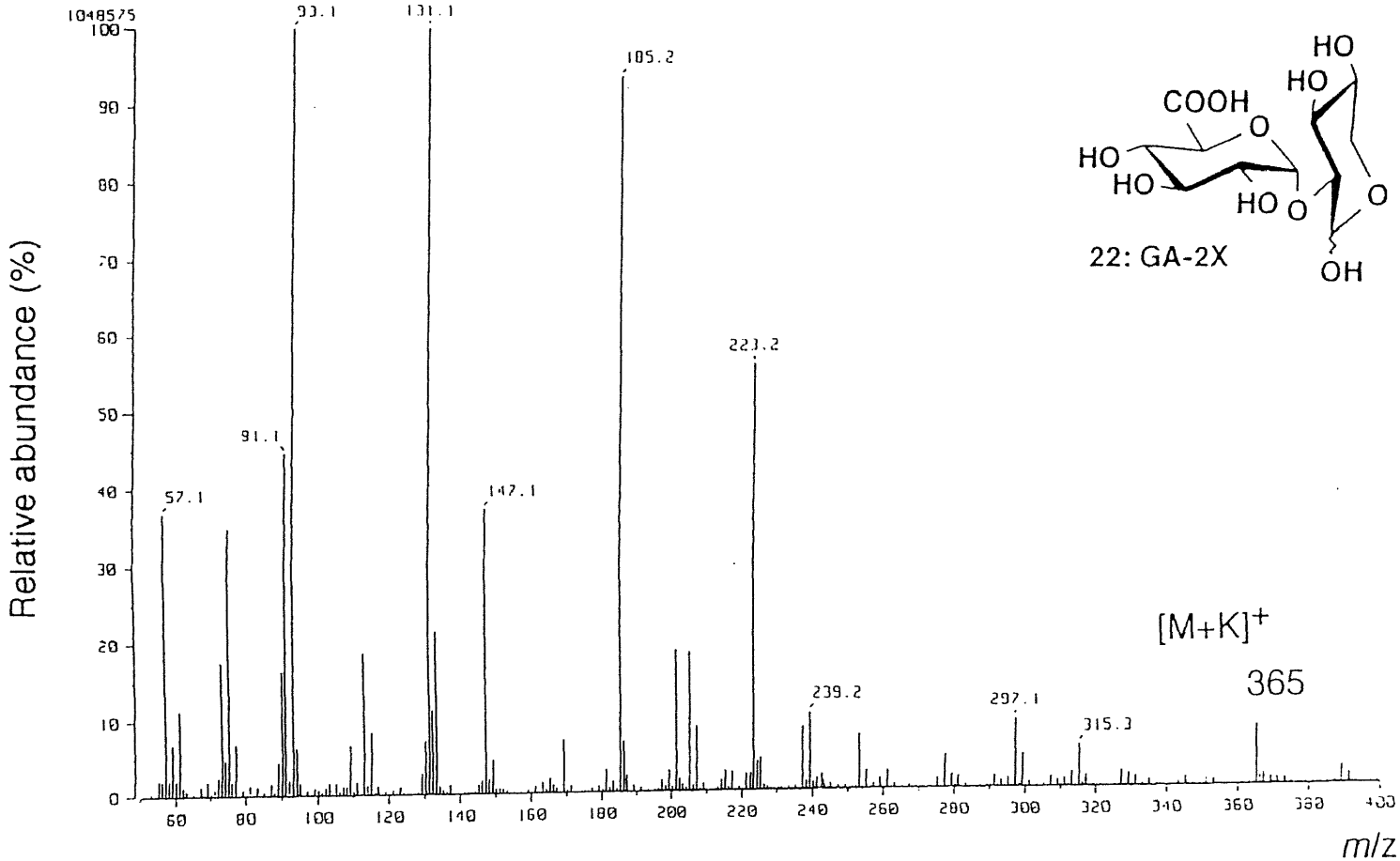


Fig. 7. FAB-MS Spectrum of GA-2X (**22**).

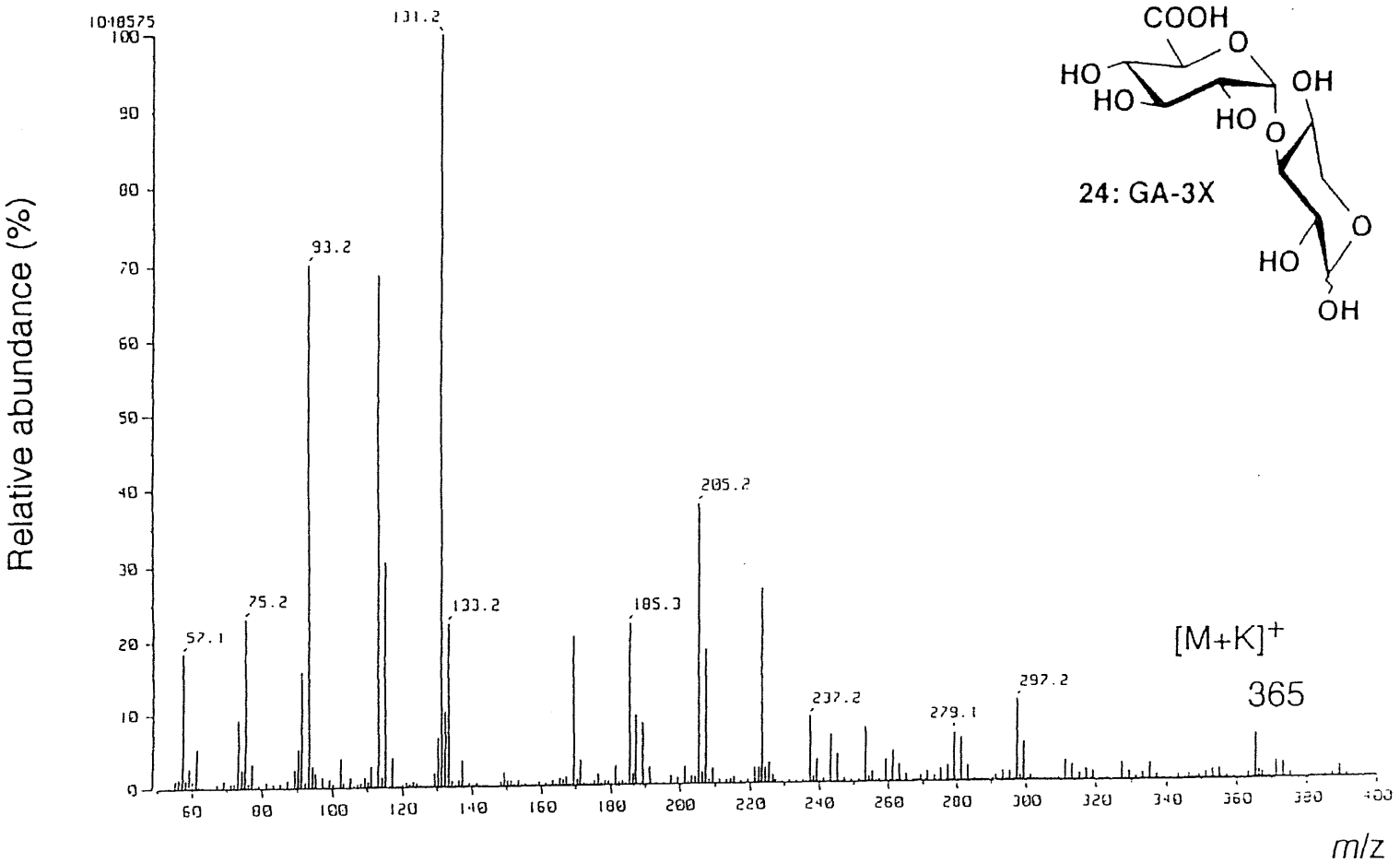


Fig. 8. FAB-MS Spectrum of GA-3X (**24**).

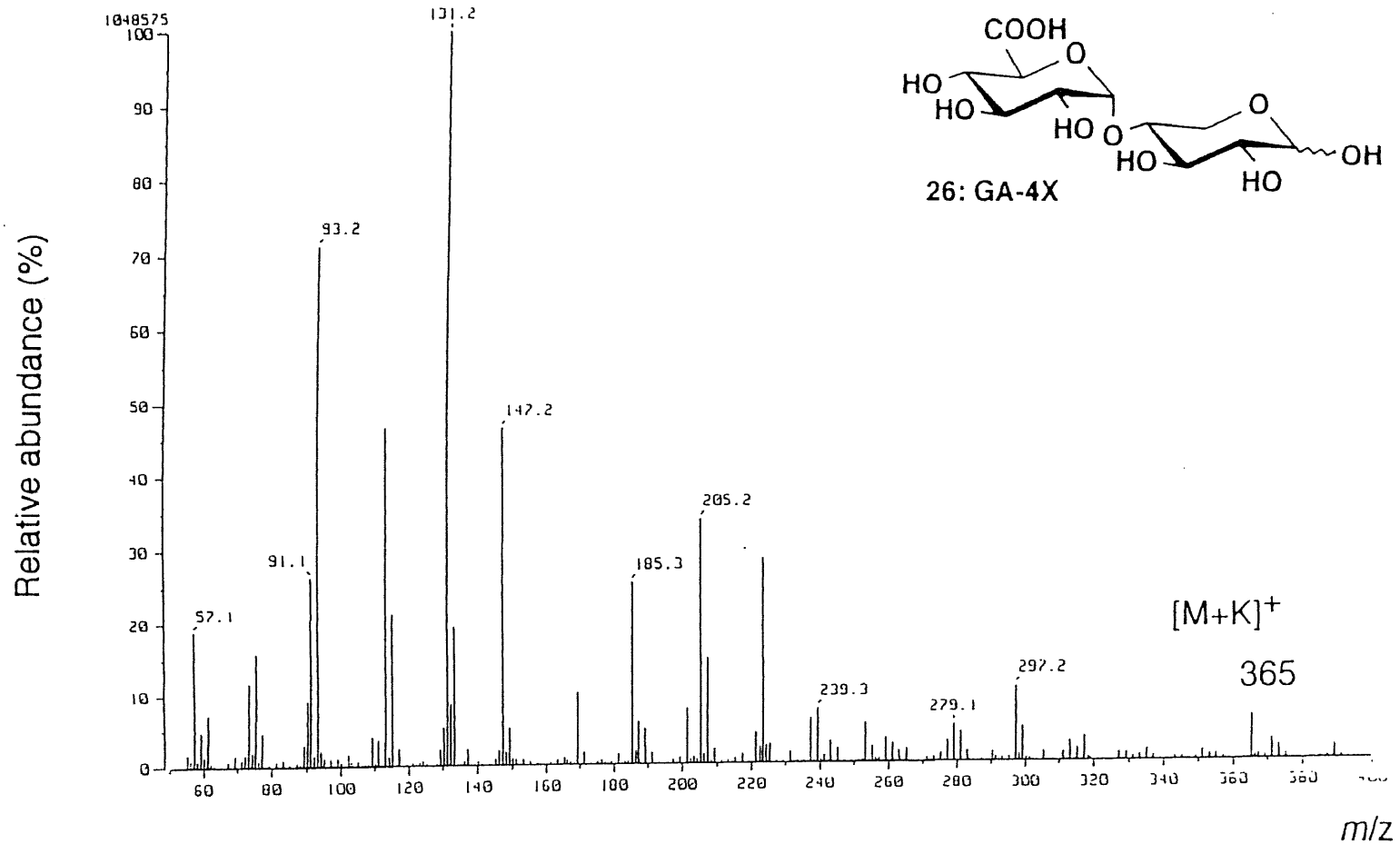


Fig. 9. FAB-MS Spectrum of GA-4X (**26**).

CHAPTER II

SPECIFICITIES OF α-GLUCURONIDASES FROM BASIDIOMYCETES AND *Aspergillus niger*

SUMMARY

The α-glucuronidase-producing ability of basidiomycetes (70 species) using 2-*O*-α-D-glucopyranosyluronic acid-D-xylose (GA-2X) and *p*-nitrophenyl α-D-glucopyranosyluronic acid (PNP-GA) as substrates for the enzyme reaction is investigated in this chapter. The crude enzyme preparations originating from five basidiomycetes, such as *Chlorosplenium aeruginosum, Coriolus pubescens, Coriolus versicolor, Irpex lacteus, Merulius tremellosus*, hydrolyzed GA-2X, but they did not hydrolyze PNP-GA. The results indicate that the two substrates are hydrolyzed by different enzymes.

In the previous chapter, the synthesis of three aldobiouronic acid regioisomers, namely GA-2X, 3-*O*-α-D-glucopyranosyluronic acid-D-xylose (GA-3X), and 4-*O*-α-D-glucopyranosyluronic acid-D-xylose (GA-4X) is described. In this chapter, those regioisomers are used to clarify the substrate specificities of α-glucuronidases from *Aspergillus niger* 5-16 and three basidiomycetes, namely *Coriolus pubescens*, *Irpex lacteus* and *Merulius tremellosus*. The enzyme from *A. niger* hydrolyzed only GA-2X, whereas the enzymes from the basidiomycetes hydrolyzed all three substrates. These results implicate that the α-glucuronidase from *A. niger* has strict substrate specificity towards the α(1→2)-glucopyranosyluronic acid-xylose linkage, and the α-glucuronidase from basidiomycetes may have broad substrate specificity.

INTRODUCTION

Many xylans in cell walls of plants have D-glucopyranosyluronic acid or its 4-*O*-methyl ether substituents attached to the C-2 position of xylose residues of xylan backbone as side-chains.[16] The bond between glucuronic acid and xylose residues is very stable against acid hydrolysis.[3] However, α-glucuronidase has the ability to easily hydrolyze the stable glycosidic linkage and liberates D-glucuronic acid. Therefore, the action mechanism of the enzyme is of considerable interest. Basidiomycetes play an important role in the decomposition of wood. In this connection, the present study was aimed to investigate the α-glucuronidase-producing basidiomycetes using 2-*O*-α-D-glucopyranosyluronic acid-D-xylose (GA-2X) and *p*-nitrophenyl α-D-glucopyranosyluronic acid (PNP-GA) as substrates of the enzyme reaction.

Recently, Uchida *et al.* have reported the substrate specificity of α-glucuronidase from *Aspergillus niger* 5-16.[15] The α-glucuronidase hydrolyzes 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose (MeGA-2X) and also GA-2X. However, it has not been shown whether the enzyme hydrolyzes α(1→3) and α(1→4) glycosidic bonds between D-glucopyranosyluronic acid and D-xylose residues.

Ishihara and Shimizu,[12] and Ishihara *et al.*[32] reported that some basidiomycetes produce α-glucuronidase. However, the substrate specificities of the enzymes from basidiomycetes have not been studied. Thus the substrate specificities of α-glucuronidases from *A. niger* and basidiomycetes using synthetic aldobiouronic acid regioisomers, GA-2X, GA-3X, and GA-4X as substrates for the enzyme reaction are studied presently.

MATERIALS AND METHODS

Basidiomycetes.

As shown in Table 2, seventy species of basidiomycetes were used for the test of α-glucuronidase-producing ability.

Enzyme preparation from basidiomycetes.

The seed cultures of basidiomycetes were injected into 100 ml of a medium composed of 1.0% hardwood xylan, 1.0% glucose, 1.0% peptone, 0.1% yeast extract, 0.1% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, in a 500-ml shaking flask and cultured at 25°C for seven days. The mycelia was collected by filtration, and homogenated with aluminum oxide and 0.1 M acetate buffer. The resultant homogenate was centrifuged (10,000 x *g*, 30 min), and the supernatant was used as the intracellular enzyme preparation (cell-free extract).

Enzyme preparation from Aspergillus niger.

A. niger 5-16 was cultured in 100 ml of a medium (pH 5.6) composed of 2.0% hardwood xylan, 0.6% peptone, 0.3% yeast extract, 1.0% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$, in a 500-ml shaking flask on a reciprocal shaker (125 oscillations per minute) at 35°C for 24 h. The culture broth was passed through filter paper (Toyo Roshi No. 2, Toyo Roshi Co. Ltd., Japan). The mycelia on the filter paper were mixed with the same weight of aluminum oxide and 100 mM acetate buffer (pH 5.0), and then homogenated in a porcelain mortar. The resultant homogenate was centrifuged (10,000 x *g*, 30 min), and the supernatant was dialyzed against 20 mM acetate buffer (pH 5.0) for two days. The resultant was centrifuged again, followed by the

lyophilization of supernatant. The resultant powder was dissolved in deionized water (10.0 mg/ 4.0 ml) and used as the intracellular enzyme solution.

Preparation of substrates.

GA-2X was prepared from the acid hydrolysate of cotton seed (kindly donated by Okamura Oil Mill, Ltd. Osaka, Japan), by the procedures reported by Yoshida *et al.*[33] and Matsuo *et al.*[7]

PNP-GA was synthesized by catalytic oxidation of *p*-nitrophenyl α-D-glucopyranoside according to the procedure of Marsh and Levvy.[34] Its physical data has been reported by Uchida *et al.*[15]

The aldobiouronic acid regioisomers (GA-Xs), GA-2X, GA-3X, and GA-4X (see Fig. 10), were synthesized as described in Chapter I.[35]

Enzyme assay.

The hydrolysis of GA-2X was assayed by the method of Uchida *et al.*[15] The reaction mixture, containing 0.1 ml of 2.0 mM GA-X solution, 0.3 ml of 100 mM acetate buffer (pH 5.0), and 0.1 ml of the enzyme solution, was incubated at 50°C for 30 min. After the inactivation of enzyme by boiling, the amount of glucuronic acid liberated from GA-X was measured by the Milner-Avigad method.[36] One unit was defined as the amount of enzyme which produced 1 μmol of glucuronic acid per min.

p-Nitrophenol released from PNP-GA was estimated as described by Uchida *et al.*[15] The reaction mixture, containing 0.5 ml of 2.0 mM PNP-GA, 0.4 ml of 100 mM acetate buffer (pH

5.0), and 0.1 ml of enzyme solution, was incubated at 50 °C for 1 h. The reaction was terminated by the addition of 1.0 ml of 0.2 M Na_2CO_3 solution, and *p*-nitrophenol released from the substrate was measured spectrophotometrically at 408 nm.

RESULTS

α-Glucuronidase-producing ability of basidiomycetes.

GA-2X-degrading activities in the extracellular and intracellular enzyme solutions were assayed by the method described above. The result of GA-2X-degrading enzyme productivity is shown in Table 2. Some of basidiomycetes such as *Chlorosplenium aeruginosum*, *Coriolus pubescens*, *Coriolus versicolor*, *Irpex lacteus*, and *Merulius tremellosus*, had greater GA-2X-degrading enzyme activity than others.

Substrate specificities of α-glucuronidases of basidiomycetes and Aspergillus niger .

Based on the result of above screening, three α-glucuronidase-producing basidiomycetes, *I. lacteus, M. tremellosus, and C. pubescence* were chosen. The crude enzyme preparations from the three basidiomycetes were used for investigating the substrate specificity of α-glucuronidase. The α-glucuronidases from them had different specificity compared with the enzyme from *A. niger*, because the preparations hydrolyzed almost similarly the three regioisomers of aldobiouronic acid (see Table 3). The results indicated that the basidiomycetes possess the abilities to hydrolyze the three regioisomers of the aldobiouronic acid.

Recently, Uchida *et al*. have reported that α-glucuronidase from *A. niger* 5-16 has strict substrate specificity toward α(1→2)-D-glucopyranosyluronic acid-D-xylose linkage, because

this enzyme hydrolyzed MeGA-2X and GA-2X, however was not effective to hydrolyze *p*-nitrophenyl α-D-glucopyranosyluronic acid and benzyl 4-*O*-α-D-glucopyranosyluronic acid-β-D-glucopyranoside.[15] They have also reported that the enzyme did not recognize the *O*-methyl group at the C-4 position of D-glucopyranosyluronic acid residue.[15] However, whether the enzyme hydrolyzes α(1→3)- and α(1→4)-D-glucopyranosyluronic acid-D-xylose linkages has not been studied. The substrate specificity of α-glucuronidase from *A. niger* using the three synthetic aldobiouronic acid regioisomers is investigated in the present study. The enzyme efficiently hydrolyzed the α(1→2) linkage of GA-2X, but poorly hydrolyzed the other linkages such as α(1→3) and α(1→4) (see Table 3). These results indicate that the α-glucuronidase from *A. niger* possess very strict substrate specificity toward the α(1→2)-D-glucopyranosyluronic acid-D-xylose linkage.

DISCUSSION

Ishihara and Shimizu,[12] and Ishihara *et al.*[32] have reported that the brown rotters (*Laetiporus sulphueus* var. miniatus and *Tyromyces palustris*) were strong 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylitol-degrading enzyme producers. We have found that most basidiomycetes can produce GA-2X-degrading enzyme (see Table 2).

Recently, Uchida *et al.*[15] have reported that the purified α-glucuronidase from *A. niger* 5-16 did not hydrolyze PNP-GA. It is clear that this phenomenon is due to the strict substrate specificity of the enzyme. Further, whether these basidiomycetes also produced PNP-GA-degrading enzyme was also investigated. Table 3 shows that none of tested basidiomycetes produce the enzyme.

Marsh and Levvy[37] have reported that the preparation of limpet (*Patella vulgata*) hydrolyzed the α-glucuronides of phenol, *p*-nitrophenol, (-)-menthol, and borneol, however it has not yet reported whether the preparation can degrade GA-2X such as aldobiouronic acid. The present study indicates that PNP-GA-degrading enzyme is either not present or is very rare on basidiomycetes. In general, many glycosidases are assayed with the corresponding *p*-nitrophenyl glycosides as the substrates of their enzyme reactions, but PNP-GA looks like it is unsuitable for the assay of α-glucuronidase. Because, the two substrates, GA-2X and PNP-GA, were not degraded together by any enzyme preparation, it can be said that the two substrates are hydrolyzed by different enzymes.

Recently, there were some reports about the isolation of GA-2X and GA-3X,[2, 31] but none described the presence of GA-4X. The unnatural aldobiouronic acid, GA-4X, was also hydrolyzed by the enzyme preparations from the three basidiomycetes. The α-glucuronidase from basidiomycetes may have broad specificity, but it is unclear at present whether the α-glucuronidases from basidiomycetes are the only enzyme class which has broad substrate specificity, or contain three enzymes which have strict substrate specificities.

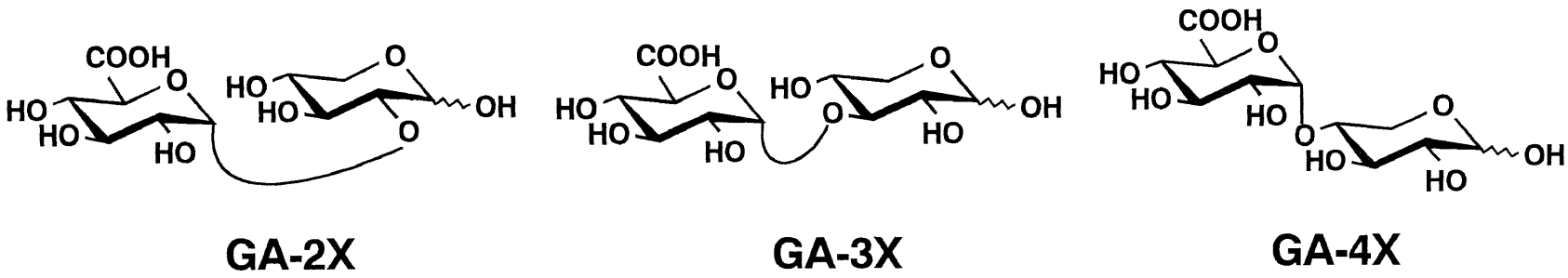


Fig. 10. Structures of Aldobiouronic Acid Regioisomers (GA-Xs).

GA-2X: 2-*O*-α-D-glucopyranosyluronic acid-D-xylose,
GA-3X: 3-*O*-α-D-glucopyranosyluronic acid-D-xylose,
GA-4X: 4-*O*-α-D-glucopyranosyluronic acid-D-xylose.

Table 2. Productivity of α-Glucuronidase of Basidiomycetes.

IFO Nos.	Names	GA-2X-degrading activity		PNP-GA-degrading activity	
		intra[a)]	extra[a)]	intra[a)]	extra[a)]
9075	*Agrocybe cylindracea*	n.d.	+	n.d.	-
31937	*Anellaria semiovata*	+	+	-	-
8440	*Asterophora lycoperdoides*	+	+	-	-
5949	*Auricularia auricula-judae*	n.d.	±	n.d.	-
30159	*Auriscalpium vulgare*	n.d.	±	n.d.	-
31652	*Bondarzewia montana*	n.d.	+	n.d.	-
6983	*Chlorosplenium aeruginosum*	+++	+	-	-
30567	*Clitocybe acromelalga*	n.d.	±	n.d.	-
9350	*Clitocybe nebularis*	n.d.	±	n.d.	-
4907	*Coriolus consors*	n.d.	±	n.d.	-
6479	*Coriolus hirsutus*	±	+	-	-
9782	*Coriolus pubescens*	+++	+++	-	-
9791	*Coriolus versicolor*	+++	+	-	-
8376	*Cortinarius cinnamomeus*	±	+	-	-
30259	*Crinipellis stipitaria*	+	+	-	-
30404	*Cryptoporus volvatus*	n.d.	+	n.d.	-
9076	*Cyathus stercoreus*	+	±	-	-
9789	*Cyclomyces fuscus*	+	±	-	-
30660	*Cymatoderma elegans*	±	+	-	-
6269	*Daedaleopsis tricolor*	+	+	-	-
7770	*Daldinia concentrica*	+	++	-	-
4959	*Favolus arcularius*	+	±	-	-
6897	*Filoboletus manipularis*	±	+	-	-
30224	*Flammulina velutipes*	±	+	-	-
30371	*Fomes fomentarius*	n.d.	+	n.d.	-
8705	*Fomitopsis pinicola*	+	+	-	-
30481	*Hebeloma radicosum*	+	+	-	-
4950	*Hirschioporus abietinus*	+	+	-	-
9788	*Inonotus cuticularis*	+	±	-	-
5367	*Irpex lacteus*	+++	+	-	-
8334	*Lactarius chrysorheus*	±	+	-	-
30745	*Laetiporus sulphureus*	++	+	-	-
8917	*Lampteromyces japonicus*	n.d.	+	n.d	-
30719	*Lentinus edodes*	++	+	-	-
30750	*Lentinus lepideus*	+	+	-	-
6513	*Lenzites betulina*	+	±	-	-
30380	*Lepista nuda*	n.d.	+	n.d.	-
8335	*Lyophyllum shimeji*	n.d.	+	n.d.	-
9618	*Macrolepiota procera*	±	+	-	-
9667	*Merulius tremellosus*	+++	+	-	-
9668	*Naematoloma sublateritium*	n.d.	+	n.d.	-
30386	*Onnia orientalis*	n.d.	+	n.d.	-

9784	*Oudemansiella mucida*	n.d.	+	n.d.	-
9785	*Oudemansiella radicata*	±	+	-	-
30362	*Panellus serotinus*	n.d.	+	n.d.	-
8994	*Panus rudis*	±	±	-	-
31249	*Phanerochaete chrysosporium*	±	+	-	-
9779	*Pholiota adiposa*	±	+	-	-
30265	*Pholiota aurivella*	+	+	-	-
30372	*Pholiota nameko*	+	+	-	-
30776	*Pleurotus ostreatus*	±	±	-	-
9005	*Podostroma cornu-damae*	n.d	±	n.d	-
30741	*Polyporellus brumalis*	±	+	-	-
31332	*Polyporus tuberaster*	+	+	-	-
4967	*Porodisculus pendulus*	+	+	-	-
30370	*Pseudohiatula ohshimae*	n.d.	+	n.d.	-
9596	*Psilocybe argentipes*	+	+	-	-
6495	*Pycnoporus coccineus*	+	+	-	-
30496	*Schizophyllum commune*	+	+	-	-
7651	*Stereum annosum*	+	±	-	-
4932	*Stereum frustulosum*	±	+	-	-
6520	*Stereum hirsutum*	n.d.	+	n.d.	-
6514	*Stereum roseum*	++	+	-	-
7548	*Stropharia aeruginosa*	n.d.	+	n.d.	-
6434	*Trametes albida*	n.d.	+	n.d.	-
4946	*Trametes gibbosa*	+	+	-	-
9315	*Tremella foliacea*	n.d.	+	n.d.	-
9317	*Tremella fuciformis*	n.d.	+	n.d.	-
30137	*Urnula craterium*	+	+	-	-
8826	*Wynnea gigantea*	+	+	-	-

Details of the measurements of GA-2X-degrading activities are described in Materials and Methods. ±, < 1.0×10^{-3}; +, 1.0×10^{-3} - 10.0×10^{-3}; ++, 10.0×10^{-3} - 40.0×10^{-3}; +++, > 40.0×10^{-3} unit/ml; n.d. not determined.

Details of the measurements of PNP-GA-degrading activities are described in Materials and Methods. +, PNP-GA was hydrolyzed; -, PNP-GA was not hydrolyzed; n.d., not determined.

[a] intra, intracellular enzyme; extra, extracellular enzyme.

Table 3. GA-Xs-degrading Activities of Some Enzyme Preparations.

Fungi	GA-2X-degrading activities ($x10^{-3}$unit/ml)	GA-3X-degrading activities ($x10^{-3}$unit/ml)	GA-4X-degrading activities ($x10^{-3}$unit/ml)
Aspergillus niger	76.7	2.0	0
Coriolus pubescens	10.5	11.7	12.8
Irpex lacteus	9.7	12.0	4.5
Merulius tremellosus	11.8	11.4	8.6

Details of the measurements of GA-Xs-degrading activities are described in Materials and Methods.

CHAPTER III

PURIFICATION AND CHARACTERIZATION OF α-GLUCURONIDASE FROM SNAIL ACETONE POWDER

SUMMARY

The *p*-nitrophenyl α-D-glucopyranosyluronic acid-hydrolyzing enzyme (PNP-GAase) from snail (*Helix pomatia*) acetone powder is purified by chromatographies on DEAE-cellulose, CM-Toyopearl, and Toyopearl HW-55F. The molecular weight of the enzyme is calculated as 180,000 by gel filtration chromatography with Superose 6, and 97,000 by SDS-polyacrylamide gel electrophoresis. The pI is calculated as 6.8 by isoelectric focusing. The enzyme showed the maximum activity at pH 3.0 and 50°C, and was stable at pH between 3.0-7.0 and up to 50°C. The enzyme activity was greatly inhibited by Hg^{2+}, *p*-chloromercuribenzoic acid, sodium dodecyl sulfate, and *N*-bromosuccinimide. The enzyme released D-glucuronic acid not only from PNP-GA but also from 2-, 3-, and 4-*O*-α-D-glucopyanosyluronic acid-D-xyloses, *O*-α-D-glucopyranosyluronic acid-α-D-glucopyranosiduronic acid, and benzyl 4-*O*-α-D-glucopyranosyluronic acid-β-D-glucopyranoside. The results suggest that the α-glucuronidase from snail acetone powder had a broad substrate specificity comparing with α-glucuronidases from *Aspergillus niger* and basidiomycetes.

INTRODUCTION

α-Glucuronidase is the most important enzyme for the increase of xylose yield in enzymatic and acid hydrolyses of xylan,[1)] because the α1→2 glycosidic bond of 4-*O*-methyl-D-

glucopyranosyluronic acid and D-glucopyranosyluronic acid side-chains is known to be stable to acid hydrolysis.[1, 3] Some of the enzyme have been purified from *Agaricus bioporus*,[38] *Aspergillus niger* 5-14,[15] *Thermoascus aurentiacus*,[14] and *Trichoderma reesei*.[32] In all of these investigations, 4-*O*-methyl-α-D-glucopyranosyluronic acid-(1→2)-D-xylo-oligosaccharide has been used as a substrate.

p-Nitrophenyl glycosides are generally used as substrates for the corresponding glycosidases.[39] Based on the anticipation that *p*-nitrophenyl α-D-glucopyranosyluronic acid (PNP-GA) may also be a substrate suitable for the assay of α-glucuronidase, the α-glucuronidase-producing ability of 70 species of basidiomycetes was tested. However, they failed to produce the PNP-GA-hydrolyzing enzyme (PNP-GAase) in Chapter II.[40] Fontana *et al.* have also reported that *Streptomyces olivochromogenes* do not have significant activity against synthetic PNP-GA.[11] Furthermore, they have described that fungal α-glucuronidase can not hydrolyze PNP-GA, and the snail enzyme was very active toward both configomers, especially the *p*-nitrophenyl β-D-glucopyranosyluronic acid. However, they have not purified the α-glucuronidase and have also not examined the enzyme activity toward other α-glucuronides such as aldobiouronic acid. Marsh and Levvy[37] also reported that the preparation from limpet (*Patella vulgata*) had PNP-GAase activity. However, they have also not purified or investigated the substrate specificity of the enzyme.

In this Chapter, the detection of PNP-GAase in extracts of commercially available acetone powders of abalone, limpet, and snail is described. The enzyme was purified from snail (*Helix pomatia*) and its properties are studied with particular reference to its substrate specificity.

MATERIALS AND METHODS

Chemicals.

Abalone, limpet and snail acetone powders used as enzyme sources were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). DEAE-cellulose (DE 52) was purchased from Whatman Paper Ltd. (Maidstone, England), CM-Toyopearl and Toyopearl HW-55F from Tosoh Co. (Tokyo, Japan), and *p*-nitrophenyl (PNP) glycosides from Sigma Chemical Co. PNP α-D-glucopyranosyluronic acid (PNP-GA), and *O*-α-D-glucopyranosyluronic acid- α-D-glucopyranosiduronic acid were synthesized[15] by catalytic oxidations of PNP α-D-glucoside and α,α'-trehalose (Nacarai Tesque Inc., Kyoto, Japan), as described by Marsh and Levvy,[34] and Goren and Jiang,[41] respectively. Benzyl 4-*O*-α-D-glucopyranosyluronic acid-β-D-glucopyranoside was synthesized by the method of Hirasaka.[42] The regioisomeric 2-*O*-, 3-*O*-, and 4-*O*-α-D-glucopyranosyluronic acid-D-xyloses were synthesized as described in Chapter I.[35] 2-*O*-(4-*O*-Methyl-α-D-glucopyranosyluronic acid)-D-xylose (MeGA-2X) was prepared by the method of Yoshida *et al.*[33]

Preparation of crude enzyme solution.

Each of the acetone powders (50 mg) described above was dispersed in 1.0 ml of McIlvaine buffer (pH 7.0) and the mixture was shaken at 4°C for 1 h. The resultant mixture was centrifuged at 7,000 x *g* for 10 min to remove insoluble materials, and the supernatant was used as a crude enzyme solution.

Enzyme assay.

For PNP-GAase assay, 0.1 ml of enzyme solution was added to a mixture of 0.5 ml of 2.0 mM PNP-GA and 0.4 ml of 0.1 M NaOAc-HCl buffer (pH 3.0). The reaction was done at 50°C for 10 min, and terminated by adding 1.0 ml of 0.2 M Na_2CO_3. The amount of *p*-nitrophenol released was measured at 408 nm. One unit of the enzyme activity is defined as the amount releasing 1 μmol of *p*-nitrophenol from PNP-GA per min under the conditions mentioned above. Other exoglycosidases were assayed with PNP glycosides[39] as substrates under the same conditions.

Electrophoretic analysis.

Native-polyacrylamide disk gel (7.5%) and sodium dodecylsulfate (SDS)-polyacrylamide gel (12 and 8.0%) electrophoresis were done as described by Reisfeld *et al.*[43] and Laemmli,[44] respectively. The isoelectric point of the purified enzyme was measured by isoelectric focusing with the Multiphor II system (Pharmacia) and Ampholine PAGplate (pH 3.5-9.5, Pharmacia). The proteins on the gel were stained with Coomassie Brilliant Blue R-250. An isoelectric focusing calibration kit (pH 3-10, Pharmacia) was used as the standard protein for pI measurement.

Measurement of protein .

Protein was measured by a BCA-Protein Assay (Pierce) with bovine serum albumin as a standard.[45]

Measurement of molecular weight.

The molecular weight of the purified enzyme was estimated by gel filtration on Superose 6 HR 10/30 (Pharmacia) and SDS-polyacrylamide gel electrophoresis. Molecular weight standards used for the gel filtration (Pharmacia) were catalase (232,000), aldolase (158,000), bovine serum albumin (67,000), and ovalbumin (43,000). SDS-PAGE molecular weight markers (Bio-Rad) used were myosin (200,000), β-galactosidase (116,250), phosphorylase b (97,400), bovine serum albumin (66,200) ovalbumin (45,000) carbonic anhydrase (31,000), soybean trypsin (21,500), and lysozyme (14,400).

Thin-layer chromatography (TLC).

TLC was done on a Merck Silica Gel 60 plate (0.25 mm thickness) with the solvent system of 1-butanol-acetic acid-water (2:1:1, v/v). Sugars on the plate were detected by heating at 140° C for 5 min after spraying with sulfuric acid.

Kinetic study of PNP-GAase toward some α-glucuronides.

Reaction mixture containing 50 μl of substrate solution of various concentration, 150 μl of 0.1 M NaOAc-HCl buffer (pH 3.0) and 50 μl of enzyme solution was incubated at 50°C for various times. The amount of glucuronic or 4-*O*-methyl-glucuronic acid released was measured by the Milner-Avigad method.[36] The initial velocity, υ, is expressed as μmol of glucuronic or 4-*O*-methyl-glucuronic acid liberated from the non-reducing end side of substrate / mg of protein / min.

RESULTS

Detection of PNP-GAase activities in acetone powders.

The α-glucuronidase-producing ability of 70 species of basidiomycetes using 2-*O*-α-D-glucopyranosyluronic acid-D-xylose (GA-2X) and PNP-GA as substrates was investigated. However, none of the tested basidiomycetes showed PNP-GAase-producing ability (Chapter II).[40] Marsh and Levvy have recently reported that the PNP-GAase activity is in the visceral hump of the common limpet (*Patella vulgata*).[37] Therefore, in this chapter, the acetone powders of abalone, limpet (*Magathura crenulata*) and snail (*Helix pomatia*) were used, and were assayed for their PNP-GAase activities (Table 4). Snail acetone powder was detected to harbour the highest PNP-GAase activity (0.149 units/ml). The study was furthered to purify this activity from snail acetone powder.

Purification of PNP-GAase from a snail acetone powder.

i) Extraction of PNP-GAase from snail acetone powder Snail acetone powder (10 g) was dispersed in 0.1 M Tris-maleic acid buffer (pH 7.0, 200 ml), and stirred at 20°C for 1 h. The resultant mixture was centrifuged at 10,000 x *g* and 4°C for 30 min. The supernatant (198.2 ml) was used as a crude enzyme solution.

ii) DEAE-cellulose column chromatography The crude enzyme solution was dialyzed against 0.05 M Tris-maleic acid buffer (pH 6.5). The dialyzed enzyme solution (205.5 ml) containing 3700 mg of protein was put onto a DEAE-cellulose column (5 x 30 cm) equilibrated with 0.05 M Tris-maleic acid buffer (pH 6.5), and the column was washed with the same buffer at a flow rate of 120 ml/h. The eluate was fractionated into 10-ml portions. The fraction showing

PNP-GAase activity (281.0 ml), tube numbers 21-47 (data not shown), was concentrated to 53.5 ml by ultrafiltration using an Amicon YM-10 membrane, and dialyzed against 0.02 M acetate buffer (pH 5.5).

iii) CM-Toyopearl column chromatography The PNP-GAase obtained in the above step (55.7 ml containing 59.2 units of PNP-GAase activities) was put onto a CM-Toyopearl column (2.8 x 50 cm) equilibrated with 0.02 M acetate buffer (pH 5.5). PNP-GAase was adsorbed onto the column, and the column was then washed with 5 bed volumes of the starting buffer. The enzyme in the column was eluted with a linear gradient of NaCl from 0 to 0.2 M (total volume, 1500 ml) in the same buffer, at a flow rate of 150 ml/h. The eluate was fractionated into 10-ml portions. The fraction showing PNP-GAase activity (97.8 ml, 23.7 units), tube numbers 71-81 (data not shown), was dialyzed against deionized water, and lyophilized.

iv) Toyopearl HW-55F gel filtration The lyophilized enzyme (11.8 units, the half of 23.7 units) was dissolved in 0.8 ml of 0.02 M acetate buffer (pH 5.5) containing 0.2 M NaCl, and put onto a column of Toyopearl HW-55F (2.5 x 60 cm) equilibrated with the same buffer. The enzyme was eluted with the same buffer at a flow rate 2.9 ml/h, and the eluate was fractionated into 2.0-ml portions. The PNP-GAase activity gave a single peak on the gel filtration (data not shown). The active fractions (10.0 ml), tube numbers 96-100, were pooled, dialyzed against distilled water, and lyophilized. The lyophilized enzyme was re-chromatographed onto the same column and under the same conditions. The PNP-GAase thus obtained gave a single band on both native-polyacrylamide disk gel electrophoresis (Fig. 11A) and SDS-polyacrylamide slab gel electrophoresis (Fig. 11B and C). The enzyme also appeared homogeneous by gel filtration chromatography on Superose 6 HR 10/30 (data not shown).

The steps for the purification of PNP-GAase are summarized in Table 5. The enzyme was purified about 132-fold, and the yield was 6.2%.

Molecular weight and pI of PNP-GAase.

The apparent molecular weight of PNP-GAase was 180,000 as estimated by Superose 6 HR 10/30 (Fig. 12). On the other hand, the molecular weight of the subunit was measured to be 97,000 by SDS-PAGE (Fig. 11B and C).

The pI of PNP-GAase was determined to be 6.8 by isoelectric focusing (data not shown).

Effects of pH and temperature on PNP-GAase activity.

The enzyme activity was measured in 0.05 M NaOAc-HCl and McIlvaine buffers of various pHs at 40° C (Fig. 13A). The enzyme was most active in NaOAc-HCl buffer of pH 3.0.

The enzyme solution was maintained at 30° C for 2 h in various buffers from pH 2 to 9 (Fig. 13B), and then the residual activity was assayed. The enzyme was stable at pH between 3.0 - 7.0.

The enzyme activity was measured in 0.05 M NaOAc-HCl buffer of pH 3.0 at various temperatures (Fig. 14A) and found to be most active at 50° C.

The enzyme solution was maintained at various temperatures from 30 to 70 ° C in 0.05 M NaOAc-HCl buffer of pH 4.0 for 2 h, and then the residual activity was assayed (Fig. 14B). The enzyme was stable up to 50° C.

Effects of chemicals on PNP-GAase activity.

The mixture containing the enzyme (0.04 units/ml) and the chemical (2.0 mM; except for *p*-chloromercuribenzoic acid, 0.2 mM) in 0.05 M NaOAc-HCl buffer (pH 4.0) was incubated at 30° C for 2 h, and the residual activity was then estimated (Table 6).

The metal ion, Hg^{2+}, strongly inhibited the PNP-GAase. Besides, *p*-chloromercuribenzoic acid, sodium dodecylsulfate, and *N*-bromosuccinimide were also strong inhibitors of the enzyme.

Substrate specificity of PNP-GAase.

i) Actions of PNP-GAase toward various PNP glycosides The PNP-GAase hydrolyzed only PNP-GA but not PNP β-D-glucopyranosyluronic acid, α-D-glucopyronoside, β-D-glucopyranoside, α-D-galactopyranoside, β-D-mannopyranoside, or α-D-xylopyranoside.

ii) Actions of PNP-GAase toward various α-glucuronides The hydrolyses of α-glycosidic bonds of PNP-GA, *O*-α-D-glucopyranosyluronic acid-α-D-glucopyranosiduronic acid (GA-GA), benzyl 4-*O*-α-D-glucopyranosyluronic acid-β-D-glucopyranoside (GA-Glc), α-D-glucopyranosyluronic acid-D-xylose (aldobiouronic acid) regioisomers, namely 2-*O*-, 3-*O*- and 4-*O*-α-D-glucopyranosyluronic acid-D-xyloses were also investigated. The reaction mixture containing each of the synthetic substrates and the enzyme were incubated at 40° C for 24 h. After the reaction, the enzyme was inactivated by heating, and the reaction mixture was treated with Amberlite 200-C (H^+ form, cation exchanger) to remove Na^+. The resultant hydrolysates were analyzed by TLC. Figure 15 shows the complete hydrolysis of the substrates by PNP-GAase.

iii) Hydrolytic activity and kinetic study toward some α-glucuronides. PNP-GA, GA-GA, GA-2X, and MeGA-2X were used as a substrate. As shown in Fig. 16, PNP-GA was readily hydrolyzed by snail PNP-GAase. GA-GA, GA-2X, and MeGA-2X were slowly hydrolyzed by the enzyme. GA-2X was hydrolyzed slightly faster than GA-GA and MeGA-2X. In addition, the relationship between substrate concentration and the enzyme activity was also investigated (data not shown). Michaelis constants (K_m's) and maximum velocities (V_{max}'s) were obtained by using Lineweaver-Burk plots. The K_m and V_{max} values for PNP-GA, GA-GA, GA-2X, and MeGA-2X were listed in Table 7A. The relative rate to the hydrolysis velocity for PNP-GA, which expresses the direct comparison of the velocity of cleavage of α-glucuronosyl linkage in each substrate, was estimated from each V_{max}. The relative V's determined were as follows: PNP-GA, 100; GA-2X, 2.77; MeGA-2X, 2.93; GA-GA, 0.47 (Table 7A).

DISCUSSION

α-Glucuronidase is an enzyme that hydrolyzes 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)- and 2-*O*-α-D-glucopyranosyluronic acid-D-xylo-oligosaccharides and then liberates 4-*O*-methyl-D-glucuronic acid and D-glucuronic acid.[1)] My earlier studies have manifested that several species of basidiomycetes produce α-glucuronidase hydrolyzing GA-2X, but none of them produce PNP-GAase.[11)] Fontana *et al.* reported that an enzyme preparation from snail juice had both *p*-nitrophenyl α- and β-glucuronidase activities, however, the enzymes have neither been purified nor characterized so far.[11)] Marsh and Levvy have also reported that the enzyme preparation of abalone has these enzyme activities.[37)]

The present study has detected the PNP-GAase activities in commercially available

acetone powders from abalone, limpet, and snail. The crude enzyme extract from snail acetone powder had the highest PNP-GAase activity. Thus the study was undertaken to purify and characterize PNP-GAase from the acetone powder.

The molecular weight of the purified PNP-GAase was estimated to be 180,000 by gel filtration (Fig. 12) and 97,000 by SDS-PAGE (Fig. 11B and C). These results suggest that PNP-GAase consists of two subunits. The optimum pH of the enzyme was found to be 3.0 (Fig. 13A) and the optimum temperature 50°C (Fig. 14A). In addition, the enzyme was stable at pH between 3.0 - 7.0 (Fig. 13B) and up to 50°C (Fig. 14B). Marsh and Levvy have reported some properties of the phenyl α-D-glucuronide-hydrolyzing enzyme from limpet tissue.[37] They have reported that the enzyme had highest activity at pH 3.2 and was stable at pH between 3.5 - 7.0. Thus it is likely that the reported enzyme is similar to snail PNP-GAase in enzymatic properties.

To date there are some studies on α-glucuronidase, however the substrate specificity of the enzyme remains unclear, because of the limitations in the preparation of α-glucuronide. In the present study, various α-glucuronides such as aldobiouronic acid regioisomers were prepared (Chapter I).[35] These compounds have enabled the study on substrate specificities. In Chapter II, *O*-α-D-glucopyranosyluronic acid-D-xylose and PNP-GA are shown to be hydrolyzed by different enzymes.[40] However, the PNP-GAase hydrolyzed not only PNP-GA but also regioisomeric GA-Xs, GA-GA, and GA-Glc. To the best of my knowledge, this is the first report indicating that α-glucuronidase hydrolyzes both PNP-GA and other α-glucuronides.

Some α-glucuronides were used as substrates for study of hydrolytic activity of PNP-GAase. The rate of hydrolysis for these glucuronides decreased in the following order: PNP-GA >> GA-2X > MeGA-2X = GA-GA. The K_m's for GA-2X and GA-GA were slightly higher than

that for PNP-GA, but the K_m for MeGA-2X was 100 times higher than that for PNP-GA. Uchida *et al.* reported that the K_m's for GA-2X$_3$ and MeGA-2X$_3$ of *A. niger* α-glucuronidase were 0.77 and 0.37 (CM-I, respectively), and 0.82 and 0.47 mM (CM-II, respectively).[15] These results indicates that the *O*-methyl group at C-4 of glucuronic acid residue causes the great decreasing of the affinity of the enzyme for the substrate. The V_{max} for GA-2X, MeGA-2X and GA-GA were greatly smaller than that for PNP-GA. These values are about only a few percent to compare with other glucuronidases (see Table 7B). Thus it can be concluded that PNP-GAase hydrolyzes specifically PNP-GA, but the enzyme acts slowly to other glucuronides such as GA-X.

In Chapter II, the substrate specificities of α-glucuronidases from *A. niger* and basidiomycetes are reported.[15, 46] The substrate specificities of the α-glucuronidases are summarized in Table 8. These results suggest that the α-glucuronidase of *A. niger* has a very strict substrate specificity as the enzyme hydrolyzed GA-2X only. In contrast, α-glucuronidase of snail acetone powder has broad substrate specificity.

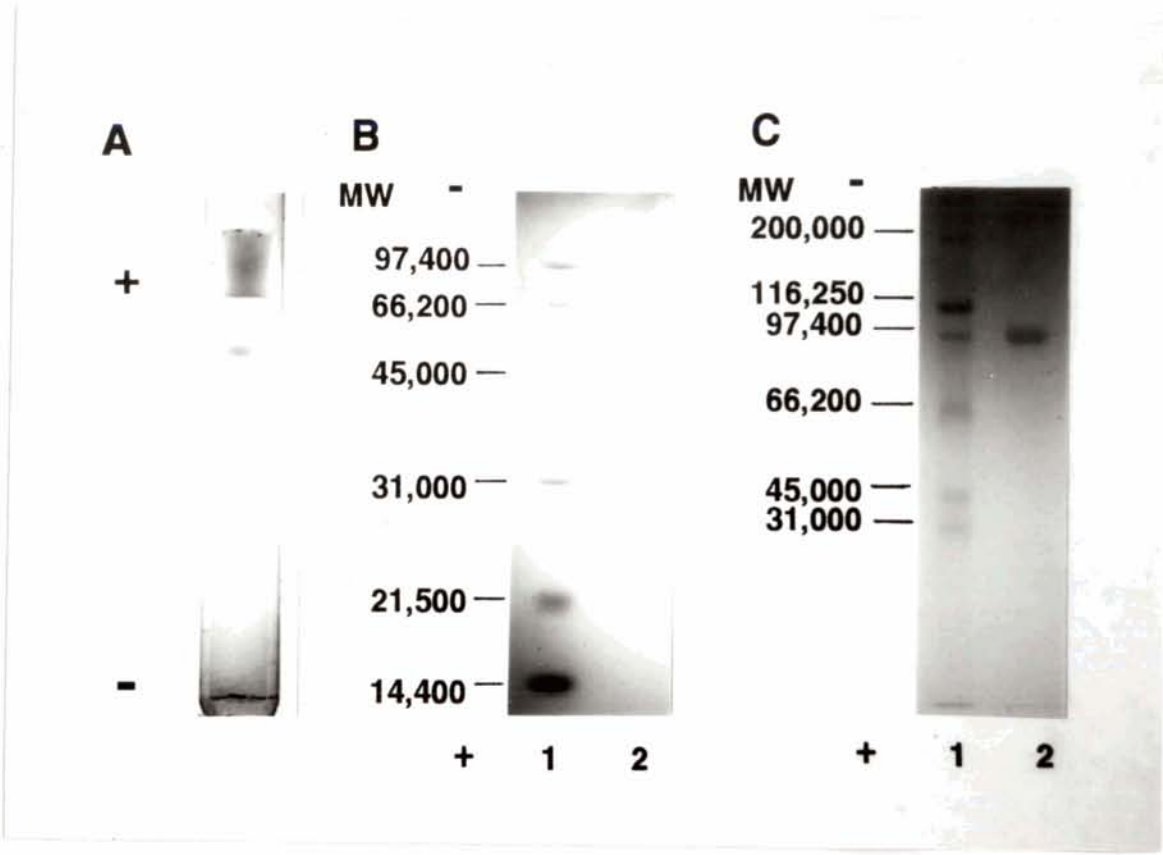


Fig. 11. Electrophoresis of PNP-GAase Purified from Snail Acetone Powder.
A, Native-polyacrylamide disk gel (7.5%) electrophoresis. The purified enzyme (10 μg) was electrophoresed as described in Materials and Methods. B and C, SDS-polyacrylamide slab gel (B, 15%; C, 8%) electrophoresis. Lane 1, molecular weight markers; Lane 2, purified PNP-GAase (5 μg). The conditions of the electrophoresis are described in Materials and Methods.

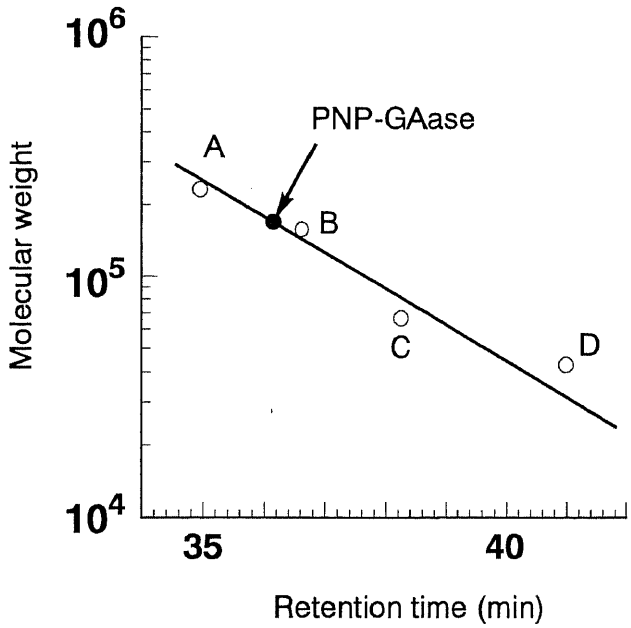


Fig. 12. Molecular Weight Estimation by Gel Filtration on a Superose 6 Column.
PNP-GAase (100 μl, 80 μg of protein) was put onto a column (10 x 30 cm) equilibrated with 0.05 M KH_2PO_4-NaOH (pH 7.0) containing 0.2 M NaCl. The enzyme was eluted with the same buffer at a flow rate of 0.5 ml/min. Molecular weight standards: A, catalase (232,000); B, aldolase (158,000); C, bovine serum albumin (67,000); D, ovalbumin (43,000).

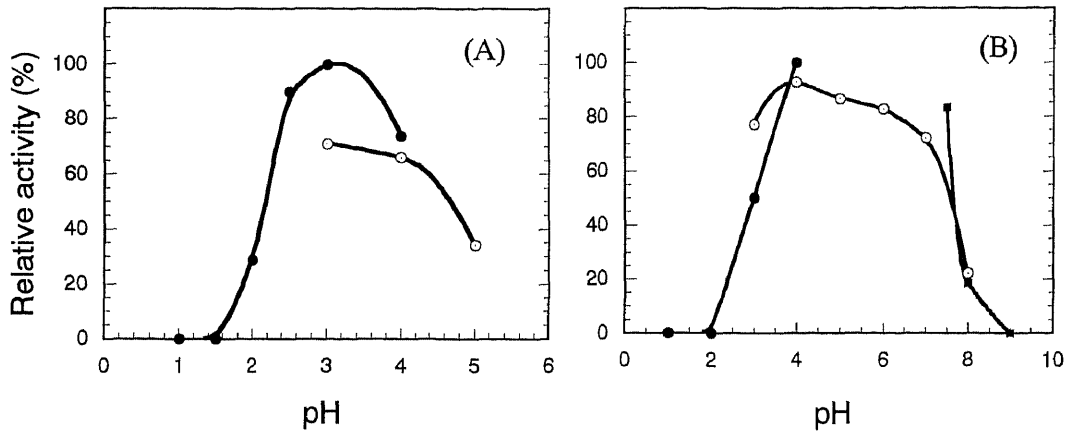


Fig. 13. Effects of pH on Activity (A) and Stability (B) of PNP-GAase.

The enzyme activity was assayed as described in Materials and Methods except for that the buffer. ●--●, 0.1 M NaOAc-HCl buffer; ○--○, McIlvaine buffer; ■--■, 0.1 M Tris-HCl buffer.

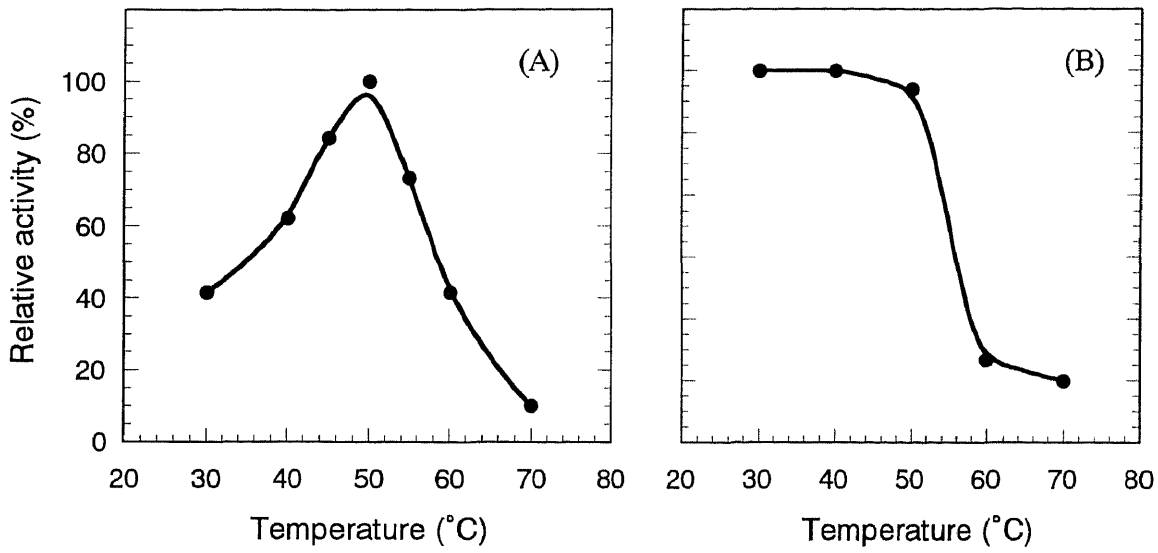


Fig. 14. Effects of Temperature on Activity (A) and Stability (B) of PNP-GAase.
The enzyme activity was assayed as described in Materials and Methods except for the temperature.

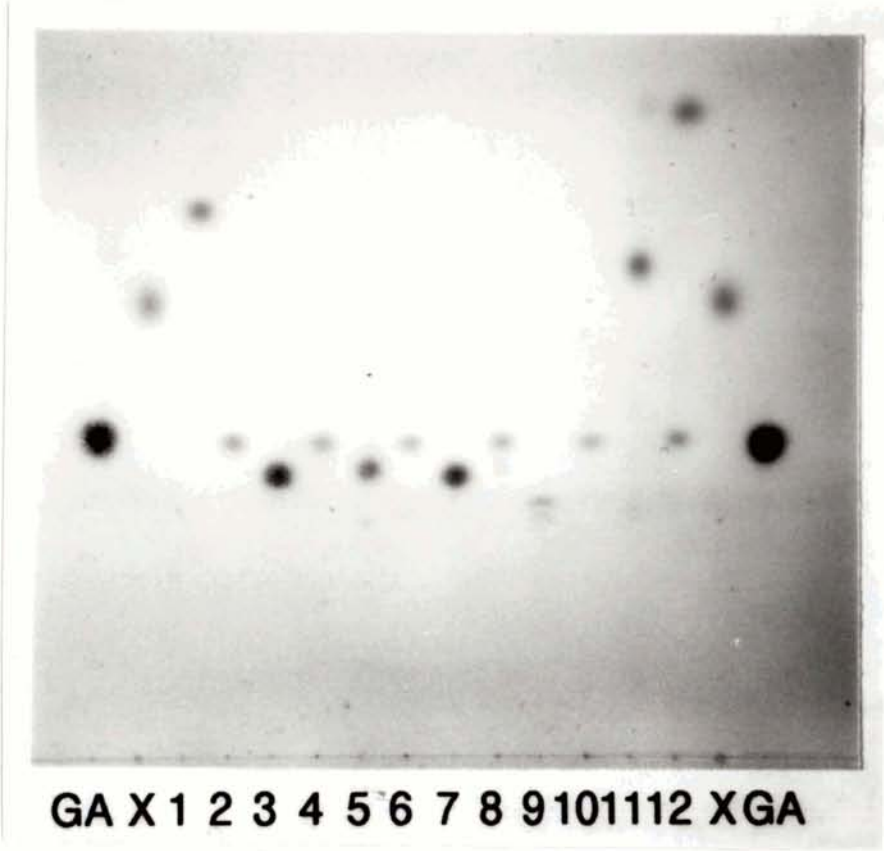


Fig. 15. Action of PNP-GAase toward Various Kinds of α-Glucuronide.

The enzyme solution (50 μl, 0.10 units /ml) was added to the same volume of substrate solution (2.0 mM), and the reaction was done at 40°C for 24 h. The resultant was deionized by Amberlite 200-C (H^+ form), and analyzed by TLC.

Lane GA, D-glucuronic acid; Lane X, D-xylose; Lane 1, PNP-GA; Lane 2, PNP-GA + enzyme; Lane 3, 2-*O*-α-D-glucopyranosyluronic acid-D-xylose (GA-2X); Lane 4, GA-2X + enzyme; Lane 5, 3-*O*-α-D-glucopyranosyluronic acid-D-xylose (GA-3X); Lane 6, GA-3X + enzyme; Lane 7, 4-*O*-α-D-glucopyranosyluronic acid-D-xylose (GA-4X); Lane 8, GA-4X + enzyme; Lane 9, *O*-α-D-glucopyranosyluronic acid-α-D-glucopyranosiduronic acid (GA-GA); Lane 10, GA-GA + enzyme; Lane 11, benzyl 4-*O*-α-D-glucopyranosyluronic acid-D-glucopyranoside (GA-Glc); Lane 12, GA-Glc + enzyme.

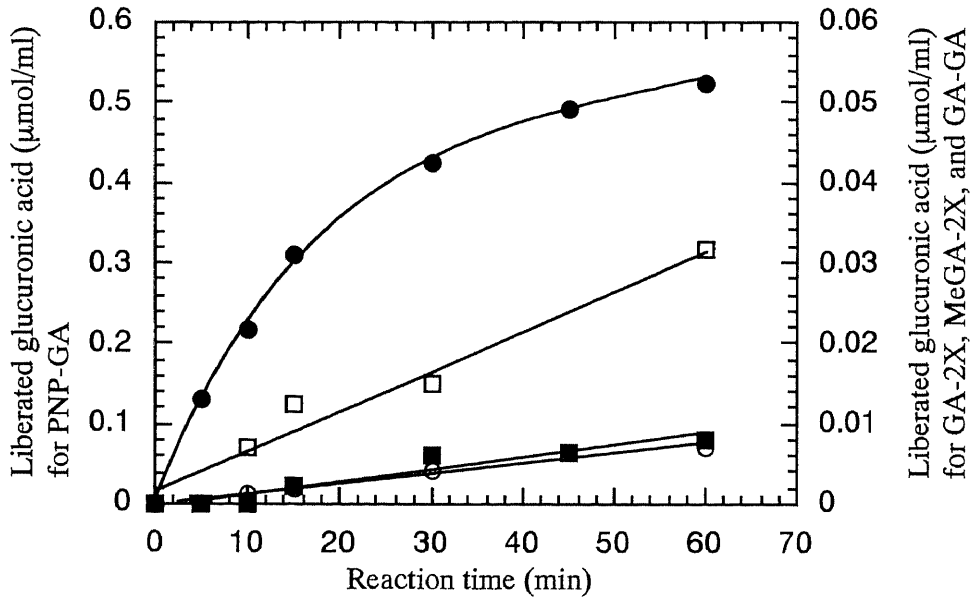


Fig. 16. Progressive Hydrolytic Activity of Snail PNP-GAase on α-Glucuronides.

Reaction mixture containing 50 μl of 5.0 mM substrate solution, 150 μl of 0.1 M NaOAc-HCl buffer (pH 3.0), and 50 μl of enzyme (2.0×10^{-3} mg) solution was incubated at 50°C. At the indicated time intervals, the reaction was terminated by adding copper solution, and then glucuronic acid or 4-*O*-methyl glucuronic acid liberated was determinded by the Miner-Avigad method.

●---●, PNP-GA; □---□, GA-2X; ■---■, MeGA-2X; ○---○, GA-GA.

Table 4. α-Glucuronidase Activities in Acetone Powders of Abalone, Limpet, and Snail.

	Abalone	Limpet	Snail
Activity (units/ml)	0.003	0.041	0.149

Enzyme solutions were prepared as described in Materials and Methods. Enzyme activities were measured in McIlvaine buffer (pH 4.0) at 40 °C.

Table 5. Summary of Purification of PNP-GAase from Snail Acetone Powder.

Step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (x 10^{-3}units/mg)	Yield (%)	Purification (-fold)
Crude extract	198.2	3700	79.5	21.5	100	1.0
DE 52 nonadsorbed	281.0	720	66.0	91.7	83	4.3
CM-Toyopearl 650M	97.8	20.1	23.7	1180	30	54.9
Toyopearl HW-55F 1st	10.0	6.16	12.4	2010	16	94.5
Toyopearl HW-55F 2nd	6.0	1.74	4.94	2840	6.2	132

Table 6. Effects of Chemicals on PNP-GAase Activity.

Chemical	Relative activity (%)
None	100.0
$FeCl_3$	125.4
$BaCl_2$	122.2
$CaCl_2$	121.7
$CuCl_2$	102.7
$PbCl_2$	102.1
$AlCl_3$	99.1
$NiCl_2$	97.3
$CdCl_2$	96.6
$MgCl_2$	90.8
$FeSO_4$	83.3
$ZnCl_2$	82.4
$MgCl_2$	81.2
$HgCl_2$	2.1
p-Hyroxylglyoxal	101.7
Trinitrobenzenesufonic acid	99.2
2-Mercaptethanol	94.7
EDTA	90.5
N-Ethylmaleimide	90.1
Monoiodoacetic acid	37.8
p-Chloromercuribenzoic acid	1.7
Sodium dodecyl sulfate	1.3
N-Bromosuccinimide	0.8

The effects of chemicals on the enzyme activity were measured using the various chemical compounds dissolved in 60 mM NaOAc-HCl buffer (pH 4.0). The concentration of the enzyme was made 0.04 units/ml, and residual activities were measured after treatment with these compounds at 30 °C for 1 h. The concentrations of the compounds were 2.0 mM except for *p*-chloromercuribenzoic acid (0.2 mM).

Table 7. Michaelis Constants (K_m) and Maximum Velocity (V_{max}) of snail (A) and *A. niger* (B) α-Glucuronidase for Some α-Glucuronides.

(A)

Substrate	PNP-GA	GA-2X	MeGA-2X	GA-GA
K_m (mM)	0.13	0.33	17.6	0.36
V_{max} [a]	3.21	0.089	0.094	0.015
Relative V [b]	100	2.77	2.93	0.47

(B)

Substrate	$GA\text{-}2X_3$		$MeGA\text{-}2X_3$	
	CM-I	CM-II	CM-I	CM-II
K_m (mM)	0.77	0.82	0.37	0.47
V_{max} [a]	5.20	17.1	1.42	4.68

[a] μmol of glucuronic and 4-*O*-methyl-glucuronic acid / min / mg of enzyme protein.

[b] Relative V caluculated from each V_{max} represents the relative velosity for cleavage of α-glucuronosidic linkage of non-reducing end side.

Table 8. Comparisons of Substrate Specificities of α-Glucuronidases.

Substrate	Snail acetone powder	*Aspergillus niger* 5-16	Basidiomycetes (*Merulius tremellosus*)*
PNP-GA	+	-	-
GA-2X	+	+	+
GA-3X	+	-	+
GA-4X	+	-	+
GA-GA	+	-	N.D.
GA-Glc	+	-	N.D.

+, hydrolyzed; -, not hydrolyzed, N.D., not determind.

Substrates are the same as listed in Fig. 15.

* I tested α-glucuronidase in cell-free extract of basidiomycetes, *Coriorus pubescense*, *Irpex lacteus*, and *Merulius tremellosus*.

GENERAL CONCLUSION

In the present study, the substrate specificities of α-glucuronidases are investigated.

In Chapter I, regioisomeric aldobiouronic acids, namely 2-*O*-α-D-glucopyranosyluronic acid-D-xylose (GA-2X), 3-*O*-α-D-glucopyranosyluronic acid-D-xylose (GA-3X) and 4-*O*-α-D-glucopyranosyluronic acid-D-xylose (GA-4X) were synthesized. These aldobiouronic acids were synthesized by block synthesis, as followings. The regiospecifically protected precursors, benzyl 3,4-di-*O*-benzyl-β-D-xylopyranoside (**7**, 2-xylose acceptor), benzyl 2,4-di-*O*-benzoyl-α-D-xylopyranoside (**9**, 3-xylose acceptor), and benzyl 2,3-di-*O*-benzyl-β-D-xylopyranoside (**15**, 4-xylose acceptor) were glycosylated with *O*-(benzyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranosyluronate) trichloroacetimidate (**20**, glucopyranosyluronic acid donor) in the presence of trimethylsilyl trifluoromethanesulfonate as the catalyst. These glycosylation gave stereoselectively α-products in yields ranging from 44-60%. The isolated α-isomers were deprotected to yield the target compounds. Their ^{1}H- and ^{13}C-NMR spectra and FAB-MS spectra were in good agreement with the proposed structure.

In Chapter II, three basidiomycetes, *Coriolus pubescens, Irpex lacteus* and *Merulius tremellosus*, producing α-glucuronidases were screened by using GA-2X as a substrate. However the fungi producing *p*-nitrophenyl α-glucopyranosyluronic acid-hydrolyzing enzyme (PNP-GAase) could not be detected. The cell-free extract of the α-glucuronidase-producing basidiomycetes hydrolyzed three regioisomers of aldobiouronic acid. While, the cell-free extract of *Aspergillus niger* 5-16 hydrolyzed only GA-2X. GA-3X and GA-4X were not hydrolyzed by the *A. niger* α-glucuronidase. These results indicated that α-glucuronidase produced by *A. niger* has strict substrate specificity, while the enzyme produced by basidiomycetes have broad

substrate specificity. However, it remains to be known whether the α-glucuronidases from basidiomycetes is the only one enzyme which has broad substrate specificity, or contain three enzymes which have strict substrate specificity for each other. The attempts to purify the enzyme from basidiomycetes were presently unsuccessful. Their α-glucuronidase are very labile, for example, the α-glucuronidase activity in the cell-free extract from *M. tremellosus* decreased to half in two days at 4°C in the extraction buffer. Ishihara *et al.*[32] also reported the α-glucuronidase from *Tyromyces palustris* was labile even in frozen storage.

In Chapter II, PNP-GAase-producing fungi remained undetectable. However, some acetone powders having the PNP-GAase activities were found. There is no report concerning the purification and characterization of PNP-GAase. In Chapter III, the enzyme was purified from snail acetone powder, since it had the maximum PNP-GAase activity among the samples studied. The PNP-GAase was purified by chromatographies on DEAE-cellulose, CM-Toyopearl, and Toyopearl HW-55F. The molecular weight of the enzyme was 180,000 by gel filtration chromatography with Superose 6, and 97,000 by SDS-polyacrylamide gel electrophoresis, and the pI was 6.8 by isoelectric focusing. The enzyme showed the maximum activity at pH 3.0 and 50°C, and was stable at pH between 3.0-7.0 and up to 50°C. The enzyme activity was greatly inhibited by Hg^{2+}, *p*-chloromercuribenzoic acid, sodium dodecyl sulfate, and *N*-bromosuccinimide. The enzyme released D-glucuronic acid not only from PNP-GA but also from 2-, 3-, and 4-*O*-α-D-glucopyranosyluronic acid-D-xyloses, *O*-α-D-glucopyranosyluronic acid-α-D-glucopyranosiduronic acid, and benzyl 4-*O*-α-D-glucopyranosyluronic acid-β-D-glucopyranoside. The results suggest that the α-glucuronidase from snail acetone powder had a broad substrate specificity comparing with α-glucuronidases from *Aspergillus niger* and

basidiomycetes.

Some α-glucuronides were used as substrates for study of hydrolytic activity of PNP-GAase. The rate of hydrolysis for these glucuronides decreased in the following order: PNP-GA >> GA-2X > MeGA-2X = GA-GA. The K_m values for PNP-GA, GA-2X, MeGA-2X and GA-GA were 0.13, 0.33, 17.6 and 0.36 mM, respectively. These results indicate that the *O*-methyl group at C-4 of glucuronic acid residue causes the decreased affinity of the enzyme for the substrate. The V_{max} for PNP-GA, GA-2X, MeGA-2X and GA-GA were 3.21, 0.089, 0.094 and 0.015 μmol of glucuronic acid formed / mg of enzyme protein / min. I conclude that PNP-GAase hydrolyzes specifically PNP-GA, but the enzyme acts slowly to other glucuronides such as GA-X.

It can be concluded from the present study that there are at least three type of α-glucuronidase: a) an α-glucuronidase that hydrolyze only GA-2X, such as *A. niger* α-glucuronidase, b) an α-glucuronidase that hydrolyzes regioisomers of GA-X but not hydrolyzes PNP-GA such as basidiomycetes α-glucuronidases, c) an α-glucuronidase that hydrolyzes specifically PNP-GA and slowly hydrolyzed other α-glucuronides.

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