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Activation of a limulus coagulation factor G by (1→3)-β-D-glucans

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ABSTRACT

Various oligo- and poly-saccharides differing in sugar compositions and types of linkage were examined for their ability to activate a limulus coagulation factor G, the first protease in an alternative coagulation cascade of the horseshoe crab, *Tachypleus tridentatus*, whose amebocytes have originally been known to contain a lipopolysaccharide(LPS)-driven pathway leading to the formation of coagulin gel. Linear and branched (1→3)-β-D-glucans and mixed linkage (1→3), (1→4)-β-D-glucans were found to exhibit the ability to activate factor G at concentrations of 10^{-8}–10^{-10} g/mL as assayed by amidolytic activity of the clotting enzyme. Laminaran oligosaccharides, laminaran dextrins (number-average mol. wt. ≦ 5800), and other polysaccharides including carboxymethylcellulose, amylose, starch, D-fructans, α-L-arabinan, β-D-xylans, (1→3)-β-D-galactan, water-soluble chitin derivatives, chondroitin, chondroitin sulfates, hyaluronan, keratan sulfate, heparins, heparan sulfate, and LPS's were virtually inactive as activators even at a concentration of 10^{-7} g/mL. The activating ability increased with increasing number-average mol. wt. (6800–216 000) of linear (1→3)-β-D-glucans. The apparent activating ability of gyrophoran, nigeran, and yeast α-D-mannan was largely abolished by digestion with a highly purified *Arthrobacter luteus* endo-(1→3)-β-D-glucanase, which provided supportive evidence for the activation to be ascribed to contaminating (1→3)-β-D-glucan(s). Possible participation of ordered structures of (1→3)-β-D-glucans in the activation of factor G is discussed.

INTRODUCTION

The amebocyte lysate of horseshoe crab hemolymph has been reported to contain a coagulation pathway that results in the coagulin clot formation in the presence of at least two discrete initiators of microbial origin, *e.g.*, lipopolysaccharides (LPS's; endotoxins) or (1→3)-β-D-glucans[1–3]. The pathway of clot formation appears to be operating as an hemostatic and defense system against infection by Gram-negative bacteria or eumycetes. Lipopolysaccharides serve as the initial activator for the enzyme system involving at least four steps of activation[4–6]. Morita *et al.*[7] have reported that water-soluble carboxymethyl(CM)-curdlan and -pachyman, lentinan, and sclerotan, at concentrations of 10^{-7}–10^{-8} g/mL, are effective to activate factor G, thus allowing an assay for the amidolytic activity of the clotting enzyme with (*tert*-butyloxycarbonylLeu)-Gly-Arg-*p*-nitroanilide as a substrate.

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However, the structural requirements for (1→3)-β-D-glucans and related polysaccharides in the coagulation system against eumycetes remains to be explored. We report herein the results of an extensive survey of carbohydrates differing in sugar composition, type of linkage, and degree of polymerization by monitoring the amidolytic activity of the clotting enzyme responsible for the final step of the coagulation cascade.

EXPERIMENTAL

Materials. — Linear (1→3)-β-D-glucans having number-average mol. wts. (M_n) ranging from 2370 to 9800 were prepared according to Ogawa *et al.*[8]. Upon formolysis with 90% formic acid at 85–90° for 20 min and subsequent repeated extraction with water, a water-soluble fraction (0.2 g) and an insoluble fraction (32.6 g) were obtained from curdlan (50 g), a linear (1→3)-β-D-glucan from *Alcaligenes faecalis* var. *myxogenes* (Wako Pure Chemical Industries, Osaka, Japan). Gel permeation chromatography (g.p.c.) of the soluble fraction with an LKB h.p.l.c. system (LKB Produkter AB, Bromma, Sweden) using water as the mobile phase at a flow rate of 0.5 mL/min on tandem columns (7.8 × 300 mm, each) of TSK gel G3000PW$_{XL}$ and G2500PW$_{XL}$ (Tosoh Corp., Tokyo, Japan) gave six components having the following M_n and polydispersities, as expressed in parentheses by the ratio of weight-average mol. wt (M_w) to M_n as indicated: M_n 2370 (1.20), 3400 (1.20), 4800 (1.20) 5800 (1.20), 6800 (1.20), and 9800 (1.22). A (1→3)-β-D-glucan having M_n of 145 000 (M_w/M_n 1.22) was isolated from the insoluble products by g.p.c. on the same column system, using 0.3M NaOH as a mobile phase. For the preparation of higher-mol.-wt. (1→3)-β-D-glucans, curdlan (0.2 g) was dissolved in 5mM NaOH (20 mL) and the solution, in an ice bath, was degraded with a sonicator (5202PZT, Ohtake Works, Tokyo, Japan) at 20 kHz and 80 W for 9 or 15 min[9]. Two samples of the resulting product were combined and subjected to g.p.c. on tandem columns (7.8 × 300 mm, each) of TSK gel G6000P$_{WX}$ and G5000PW$_{XL}$, which had been equilibrated with 0.3M NaOH, to give seven components with high homogeneity and low polydispersities (M_w/M_n). These were selected as standard samples of (1→3)-β-D-glucans having M_n 20 700 (1.27), 28 300 (1.18), 58 100 (1.29), 76 300 (1.26), 92 600 (1.23), 171 000 (1.19), and 216 000 (1.19).

A partially carboxymethylated curdlan was prepared by the procedure of Clarke and Stone[10], and its degree of etherification (d.e.) was determined by the $UO_2(NO_3)_2$ method[11]. Paramylon from *Euglena gracillis*[12] was a gift of Dr. B. A. Stone, Melbourne University, Australia; sclerotan from *Sclerotinia libertiana* was a gift of Dr. M. Kitahara, Gifu University[13]; and a levan from *Dactylis glomerate*[14] was a gift of Dr. S. Murakami, Saitama University. Gyrophoran, a (1→6)-β-D-glucan, was prepared from a lichen, *Gyrophora esculenta*[15]; an α-L-arabinan was purified from sugar beet pulp[16]; a (1→3)-β-D-galactan was a Smith degradation product of gum arabic[17]; and a (1→3)-β-D-xylan was prepared from fronds of *Caulerpa brachypus*[18].

The following glycans and lipopolysaccharides are commercial products: laminaran oligosaccharides (d.p. 2–7), amylose (Ex-I), pullulan, ethylene glycol-chitin, rooster

comb hyaluronan (Na salt, Artz), chondroitin (Na salt), chondroitin sulfates A, B, and C (Na salt), heparan sulfate (Na salt), and keratan sulfate (Na salt) (from Seikagaku Corporation); soluble starch and inulin (from Wako Pure Chemical Industries, Osaka); *Laminaria digitata* laminaran, bakers' yeast β-D-glucan, *Cetraria islandica* and *Usnea barbata* lichenans, barley β-D-glucan, dextrans of average mol. wt. 10 000, 40 000, 70 000, 500 000, and 2 000 000, *Aspergillus awamori* nigeran, *Saccharomyces cerevisiae* α-D-mannan, porcine intestinal mucosa and bovine lung heparins (Na salt) (from Sigma Chemical Co., St. Louis, MO, U.S.A.); *Eisenia araborea* laminaran, CM-cellulose (Na salt), and larch wood (1→4)-β-D-xylan (from Nakarai Chemicals, Kyoto); *Umbilicaria papullosa* pustulan (American Hoechst Corp., La Jolla, CA, U.S.A.); schizophyllan (Sonifilan, from Kaken Chemical Co., Tokyo, Japan); lentinan (from Ajinomoto Co., Tokyo, Japan); CM-chitins (Na salt, d. subst. 0.6 and 1.0; from Katokichi Co., Kagawa, Japan); LPSs from *Escherichia coli* 0111:B4, *Shigella flexneri* (from Difco Laboratories, Detroit, MI, U.S.A.), *Salmonella abortus equi* (from Sigma), *Pseudomonas aeruginosa* F-D Type-1, and *Salmonella minnesota* R595 (Re), and lipid A from *S. minnesota* R595 (from List Biological Laboratories, Inc., Campbell, CA, U.S.A.).

Determination of molecular weight. — Weight-average mol. wt. (M_w) and number-average mol. wt. (M_n) were determined by g.p.c. on size-exclusion columns (7.8 × 300 nm) of TSK gel PW_{XL} series by use of poly(ethylene oxides) (Tosoh Corp.) and poly(ethylene glycols) (Nakarai Chemicals) as mol. wt. standards and 0.3M NaOH as a mobile phase[19]. Glycans or the polymers were eluted from the columns at a flow rate of 0.5 mL/min at room temperature, and detected with a pulsed amperometric detector (Dionex Corp., CA, U.S.A.) or with an LKB differential refractometer. Polydispersity (M_w/M_n) was calculated from the values of M_w and M_n.

Assay for activating ability. — Factor G and the proclotting enzyme were prepared from the lysates of *T. tridentatus* amebocytes as previously reported[20]. The ability to activate factor G was monitored by measuring the amidolytic activity of the clotting enzyme arising from the proclotting enzyme by the action of activated factor G in the presence of the sample to be tested. The enzyme was assayed in a mixture (total volume, 200 μL) containing Tris·HCl buffer (20 μmol), pH 8.0, $MgCl_2$ (20 μmol) BocLeu-Gly-Arg-*p*NA (0.13 μmol), factor G (20 μL, A_{280} 0.9), proclotting enzyme (30 μL, A_{280} 2.5), and the sample. After incubation at 37° for 30 min, released *p*-nitroaniline was determined at A_{545} after conversion to the azo dye[20]. The carbohydrate samples were dissolved in water or 0.1–0.3M NaOH, centrifuged at 3000 r.p.m. for 10 min, and the supernatant solutions were serially diluted and assayed for enzyme activity directly or after neutralization with equivalent HCl. The sugar content of each sample was determined by the phenol–H_2SO_4 method[21] using D-glucose as the standard. The ability to activate factor G was defined as the minimum concentration (g/mL) of each sample required for the activation of factor G capable of yielding a clotting enzyme giving a defined amidolytic activity (ΔA_{545} 0.1).

Purification of an endo (1→3)-β-D-glucanase.— The endo-(1→3)-β-D-glucanase activity was assayed in a reaction mixture (total volume, 200 μL) containing sodium acetate buffer (10 μmol), pH 5.5, *L. digitata* laminaran (100 μg), and the enzyme

solution. After incubation at 37° for 60 min, the reducing sugar released was determined by the Somogyi–Nelson method[22]. One unit of activity is defined as the amount of enzyme capable of liberating 1 μmol of reducing sugar as D-glucose per min under the standard assay condition. Zymolyase-100T (153 mg), an endo (1→3)-β-D-glucanase preparation of *Arthrobacter luteus* (lot No. 108508, Seikagaku Corp.), was dissolved in a running solution and electrofocused at 1°, in an LKB 8101 column (110 mL) and a carrier ampholite, Pharmalyte (Pharmacia Fine Chemicals AB, Uppsala, Sweden) at a pH range of 3 to 10 and a constant power of 4 W for 46 h. Fractions (1 mL) were collected and monitored for enzyme activity. The active fractions electrofocused over a pH range of 5.8 to 6.2 were pooled and filtered through a Toyopearl HW50C column (2.2 × 46.5 cm, Tosoh Corp.) preequilibrated with 30mM $(NH_4)HCO_3$. The fractions (total A_{280} 4.77 and 37.9 units in 15.6 mL) containing the purified enzyme were pooled and stored at −20°. The activity of the enzyme toward (1→3)-β-D-glucans, other glycans, and laminarapentaose was expressed as the amount (μg) of reducing sugar (D-glucose equivalent) released from the substrate under the standard assay conditions, and as the relative activity as compared to that of *L. digitata* laminaran taken as unity (100). The following substrate specificities of the enzyme (2.11 milliunits), based on the amounts of reducing sugar (μg) released from each substrate and on the relative activities in parentheses, were observed: *L. digitata* laminaran, 22.8 (100); curdlan, 19.8 (86.8); *Eisenia araborea* laminaran, 5.60 (24.6); CM-curdlan, 2.09 (9.2); laminarapentaose, 35.5 (156). Laminaran oligosaccharides (d.p. 2–5) were identified as degradation products of curdlan at earlier stage of digestion upon g.p.c. using two tandem columns (7.8 × 300 nm) of TSK gel $G2500PW_{XL}$ and water as a mobile phase. No appreciable amount of reducing sugar was released from dextran (mol. wt., 500 000), CM-cellulose, amylose, pustulan, and nigeran, except for yeast α-D-mannan [0.06 (0.3)] after incubation with the purified enzyme preparation for 60 min.

RESULTS AND DISCUSSION

Sixty samples of poly- and oligo-saccharides and lipid A were investigated for their ability (expressed as minimum concentrations, g/mL) to activate factor G of the alternative coagulation pathway in the lysate of *T. tridentatus* amebocytes (see Table I). Curdlan and paramylon acted as potent activators at a concentration of 10^{-11} g/mL. Partially degraded curdlans having M_n ranging from 6800 to 216 000 activated factor G at concentrations 10^{-9}–10^{-12} g/mL. The most effective activator was a degradation product of curdlan (216 000), active at a concentration of 10^{-12} g/mL. On the contrary, water-soluble and lower-mol.-wt. (1→3)-β-D-glucans (laminaran dextrins having M_n's of ≦5800), and laminaran oligosaccharides had weak or no activating ability, although they are composed solely of consecutive (1→3)-β-D-glucopyranosyl residues. A product having mol. wt. of 6800 apparently has the critical mol. wt. limit for (1→3)-β-D-glucan capable of activating factor G at a concentration of 10^{-8} g/mL. The oligosaccharides having a d.p. of 2–7 were inactive even at a concentration of 10^{-6} g/mL. Alkali-soluble fractions obtained from mixed-linkage β-D-glucans, such as sclerotan and bakers' yeast

TABLE I

(1→3)-β-D-Glucans and related saccharides and their ability to activate a limulus coagulation factor G[a]

Saccharide	*Type of linkages*	*Mol. wt.*[b]		*Solvent for sample preparation*[c]	*Minimum concentration to activate factor G*[d] *(g/mL)*
		M_n	M_w/M_n		
D-Glucans					
Laminaran oligosaccharides (d.p. 2–7)	(1→3)-β-D	342–1153[e]		H_2O	$>10^{-6}$
Partially degraded curdlans	(1→3)-β-D	2370–5800	1.20	H_2O	$>10^{-6}$
	(1→3)-β-D	6800	1.20	H_2O	10^{-9}
	(1→3)-β-D	28 300	1.18	0.3M NaOH	10^{-10}
	(1→3)-β-D	216 000	1.19	0.3M NaOH	10^{-12}
Curdlan	(1→3)-β-D	>136 000	>2.76	0.3M NaOH	10^{-11}
Carboxymethylcurdlan (Na salt)[f]	(1→3)-β-D	>95 000	>2.80	H_2O	10^{-9}
Paramylon	(1→3)-β-D	>118 000	>1.51	0.3M NaOH	10^{-11}
Laminaria digitata laminaran	(1→6),(1→3)-β-D	5850	1.16	H_2O	10^{-8}
Eisenia araborea laminaran	(1→6),(1→3)-β-D	16 800	1.49	H_2O	10^{-8}
Sclerotan	(1→6),(1→3)-β-D	16 800	2.77	0.3M NaOH	10^{-10}
Schizophyllan	(1→6),(1→3)-β-D	76 800	1.64	H_2O	10^{-6}
Lentinan	(1→6),(1→3)-β-D	94 700	1.46	H_2O	10^{-9}
Bakers' yeast β-D-glucan	(1→6),(1→3)-β-D	>17 200	>12.89	0.3M NaOH[g]	10^{-11}
Cetraria islandica lichenan	(1→3),(1→4)-β-D	22 000	4.72	H_2O[g]	10^{-9}
Usnea barbata lichenan	(1→3),(1→4)-β-D	23 200	4.07	0.3M NaOH[g]	10^{-9}
Barley β-D-glucan	(1→3),(1→4)-β-D	>23 100	>5.39	H_2O[g]	10^{-8}
Carboxymetyl-cellulose (Na salt)	(1→4)-β-D	[h]		H_2O	$>10^{-6}$
Gyrophoran	(1→6)-β-D	5970	4.14	0.1M NaOH	10^{-10}
Nigeran	(1→3),(1→4)-α-D	>30 400	>3.62	0.1M NaOH	10^{-7}
Yeast α-D-mannan	(1→2),(1→3),(1→6)-α-D	14 500	1.70	0.3M NaOH	$>10^{-6}$
(1→3)-β-D-Galactan	(1→3)-β-D	[h]		0.1M NaOH	$>10^{-6}$
(1→3)-β-D-Xylan	(1→3)-β-D	[h]		0.3M NaOH	$>10^{-6}$

[a] Other saccharides having a lower ability are reported in the text. [b] For a solution in 0.3M NaOH. [c] Water-insoluble saccharides were dissolved in dilute NaOH solution, and used as the samples after neutralization with an equivalent of HCl. [d] The activating abilities were evaluated based on the minimum concentration (g/mL) required to convert factor G to the active form, which yield a defined amidolytic activity (ΔA_{545}/30 min = 0.1) of the clotting enzyme in the standard assay. [e] Absolute theoretical values. [f] D. subst. 0.63. [g] The samples used were the supernatant solutions after removal of the fractions insoluble in the solvents by centrifugation. [h] Not determined.

β-D-glucan, exhibited at a concentration of 10^{-10} g/mL an activating ability comparable with that of higher-mol.-wt. (1→3)-β-D-glucans, whereas *E. araborea* and *L. digitata* laminarans, lentinan, *C. islandica* and *U. barbata* lichenans, and barley β-D-glucan were relatively poor activators, acting at concentrations of 10^{-8}–10^{-9} g/mL. Hence, β-D-glucans containing (1→3)-β-D-glucopyranosyl residues fullfil the structural requirements for activation regardless of the branching and interspersing structures.

No appreciable activation could be detected with the following polysaccharides at concentrations $< 10^{-7}$ g/mL: CM-cellulose, amylose, starch, dextrans, pullulan, levan, inulin, α-L-arabinan, (1→4)-β-D-xylan, ethylene glycol-chitin, CM-chitins, hyaluronan, chondroitin, chondroitin sulfates A, B, and C, keratan sulfate, heparins, and heparan sulfate. Lipopolysaccharides from five Gram-negative bacteria and *S. minesota* R595 lipid A also were totally inactive, leaving no doubt regarding their inabillity to initiate the coagulation cascade involving factor G.

Fig. 1 illustrates the relationship between the M_n of linear (1→3)-β-D-glucans and the ability to activate factor G sufficient to increase a defined amidolytic activity (ΔA_{545} 0.1) of the clotting enzyme. The plot shows that the minimum concentration (g/mL) to activate factor G decreases with increasing mol. wt. (M_n).

Laminaran oligosaccharides (d.p. 2–7) and linear (1→3)-β-D-glucans of lower-mol.-wt. (laminarian dextrins with d.p. < 25; mol. wt. < 4068) were shown to be devoid of an ordered structure, whereas higher-mol.-wt. (1→3)-β-D-glucans tend to adopt a helical conformation in neutral or dilute alkaline solutions, or in suspensions[8,23]. Likewise, mixed-linkage (1→3), (1→4)-β-D-glucans from lichens and barley were shown to have similar patterns of repeating sequences and similar chain conformations corresponding to a right-handed, triple helix[24,25]. Schizophyllan, commercially available

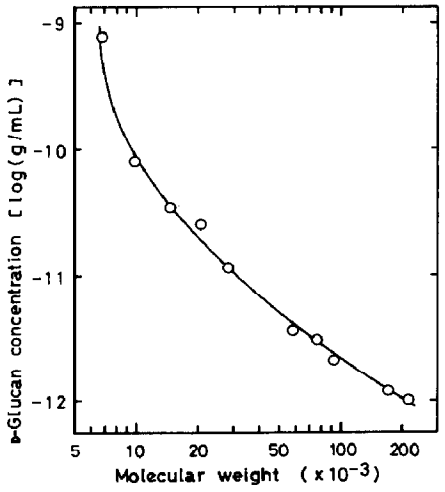

Fig. 1. Relationship between the number-average molecular weight of linear (1→3)-β-D-glucans and their ability to activate a limulus coagulation factor G. The activating ability was expressed as the concentration (g/mL) of each sample capable of increasing the amidolytic activity of the clotting enzyme to a defined level (A_{545} 0.1) as assayed under the standard conditions.

as Sonifilan, however, was found to be unexpectedly a poor activator despite its high mol. wt. (M_w) of ~450000. This low activating ability is unaccounted for as the water-soluble (1→3)-β-D-glucan, which bears (1→6)-β-D-glucopyranosyl groups at about every third residue along the main chain, has been reported to take the form of a triple helix[26]. Söderhäll *et al.*[27] have reported the inability of naturally occurring, branched (1→3)-β-D-glucans (*Laminaria hyperborea* laminarans G and M with a d.p. of ~20) to induce a gelation system in lysates of *Limulus polyphemus* amebocytes, whereas CM-curdlan acted as a potent activator. The failure of the laminarans to activate factor G in the coagulation system is likely to be due to their low molecular weigths, so that they are active only at unusually high concentrations.

Gyrophoran, a linear D-glucan, consisting of mainly consecutive (1→6)-β-D-glucopyranosyl residues, was found to activate factor G at a concentration of 10^{-10} g/mL. Significant activation of factor G was also observed with nigerian, an (1→3), (1→4)-α-D-glucan, and yeast α-D-mannan at concentrations of 10^{-7} and 10^{-5} g/mL, respectively. These unexpected results prompted us to examine the effect of an *A. luteus* endo-(1→3)-β-D-glucanase on these apparently active polysaccharides by measuring the amidolytic activity of the clotting enzyme. Thus, the activating abilities of the three polysaccharides were compared, curdlan and barley β-D-glucan being used as the controls. The data in Fig. 2 showed a rapid reduction of the activating abilities of the three glycans, as well as curdlan and barley β-D-glucan, at different concentrations as the enzyme digestion proceeded. These results implicated the enzymic degradation of contaminating (1→3)-β-D-glucan(s) in the apparently active glycans, leaving inactive low-mol.-wt. products with a simultaneous loss of their ability as an activator. Traces of activating ability remaining in larger amounts of curdlan, gyrophoran, nigeran, and yeast α-D-mannan could not be eliminated completely by the exhaustive enzymic digestion, possibly owing to product inhibition of the enzyme or a close association of (1→3)-β-D-glucan(s) with major polysaccharides, thereby hindering the enzyme attack. Rapid inactivation of curdlan and barley β-D-glucan by the enzymic degradation

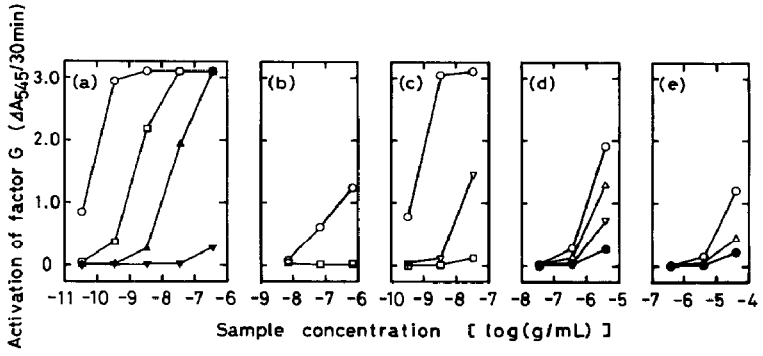

Fig. 2. The effects of *Arthrobacter luteus* endo-(1→3)-β-D-glucanase on curdlan and other glycans acting as the activator for a limulus coagulation factor G: (a) Curdlan, (b) barley β-D-glucan, (c) gyrophoran, (d) nigeran, and (e) yeast α-D-mannan. The samples of the glycans to be tested were prepared as described in the Experimental section. Incubation times with *A. luteus* endo-(1→3)-β-D-glucanase were as follows: (○), Control, 0 min; △, 10 min; ▽, 30 min; □, 60 min; ●, 90 min; ▲, 5 h; and ▼, 25 h.

provided an additional indication supporting the necessity of (1→3)-β-D-glucopyranosyl residues as well as of high degrees of polymerization for activation. Moreover, the results ascertained that the gel-forming ability of the lysate of *T. tridentatus* amebocytes in the presence of *S. cerevisiae* α-D-mannan, as reported by Mikami *et al.*[28], might be ascribed to contamination by small amounts of yeast β-D-glucan(s) in the sample. The lack of activating ability in (1→3)-β-D-galactan and (1→3)-β-D-xylan is indicative of the structural requirements for the activator in regard to the configuration at C-4 and the presence of a hydroxymethyl group at C-5 in D-glucopyranosyl residues.

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