Carbohydrate Research, 226 (1992) 239–246
Elsevier Science Publishers B.V., Amsterdam

Synthesis and antitumour activity of derivatives of curdlan and lichenan branched at C-6

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(Received May 23rd, 1991; accepted October 3rd, 1991)

ABSTRACT

Derivatives of curdlan and lichenan, linear (1→3)-β-D- and (1→3/1→4)-β-D-glucans, respectively, have been synthesised having α-L-arabinofuranosyl, α-L-rhamnosyl, β-D-glucosyl, and β-gentiobiosyl side chains attached at positions 6. These water-soluble derivatives, obtained by condensation of the 2,4- and 2,4-/2,3-di-*O*-phenylcarbamoyl derivatives of curdlan and lichenan, respectively, with appropriate ortho esters followed by saponification, were characterised by methylation analysis, g.p.c., and interaction with Congo Red. The curdlan derivatives and the lichenan derivative with few glucosyl branches were active against the Sarcoma 180.

INTRODUCTION

In recent years, much effort has been expended in seeking to elucidate structure–activity relationships of antitumour polysaccharides. The structure and conformation of the (1→3/1→6)-β-D-glucans SPG and lentinan have been studied extensively[1–6]. The existence of ordered structures, *i.e.*, single and triple helices, and a high molecular weight were thought to be essential for antitumour activity. However, Kraus *et al.*[7] demonstrated that (1→3/1→6)-β-D-glucans from various Phytophthora species, with a molecular weight of only 20 000 and no helical conformation, were active against the Sarcoma 180 and this was correlated with the degree of branching. A (1→3)-β-D-glucan backbone with single glusocyl units attached to positions 6 was the most active[8,9]. Matsuzaki *et al.*[10–15] synthesised branched derivatives of unprotected linear glucans, mannans, and glucomannans so that mono- and oligo-saccharides were attached non-regiospecifically.

The aim of our study was to investigate the necessity of a (1→3)-linked β-D-glucan backbone for the expression of antitumour activity, and the influence of sugars attached to positions 6. Curdlan, a (1→3)-β-D-glucan, and lichenan, a linear β-D-glucan with 33% of (1→3) and 66% of (1→4) linkages, were used as starting materials.

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0008-6215/92/$ 05.00

EXPERIMENTAL

Introduction of protecting groups[16]*: 6-O-Tritylcurdlan.* — A solution of curdlan (20 g, 123 mmol; purchased from Wako Pure Chemical Industry, Osaka) and trityl chloride (180 g, 646 mmol) in Me_2SO (120 mL) and pyridine (260 mL) was stirred for 1 h at 100°, then for 4 h at 70–80°. The yellow solution was diluted with hot MeOH (8 L) and the precipitate was washed thoroughly with MeOH to give the title compound (50 g, ~ 100%).

Anal. Calc. for $(C_{25}H_{24}O_5)_n$: C, 74.3; H, 5.94. Found: C, 74.1; H, 5.94.

2,4-Di-O-phenylcarbamoyl-6-O-tritylcurdlan. — To a solution of 6-*O*-tritylcurdlan (25 g, 61 mmol) in dry pyridine (400 mL) at 100° was added phenyl isocyanate (30 mL) with stirring. After 1 h, more 6-*O*-tritylcurdlan (61 mmol) and phenyl isocyanate (30 mL) were added, and the solution was stirred at 100° for 60 h, then diluted with MeOH (20 L) and sat. aq. NaCl (500 mL) to give the title compound (70 g, 88%).

Anal. Calc. for $(C_{39}H_{34}N_2O_7)_n$: C, 72.9; H, 5.30; N, 4.36. Found: C, 71.2; H, 5.45; N, 5.05.

2,4-Di-O-phenylcarbamoylcurdlan. — To a suspension of finely powdered 2,4-di-*O*-phenylcarbamoyl-6-*O*-tritylcurdlan (70 g, 109 mmol) in anhydr. MeOH (1 L) was added fuming HCl (10 mL), and the mixture was stirred for 15 h at room temperature. The product was collected, washed with MeOH and water, and dried. The procedure was then repeated. Complete detritylation was confirmed by methylation analysis of the product (40 g, 92%).

Anal. Calc. for $(C_{20}H_{20}N_2O_7)_n$: C, 60.0; H, 5.00; N, 6.9. Found: C, 58.1; H, 5.23; N, 6.9.

The corresponding derivatives of lichenan (purchased from Roth) were prepared analogously.

6-O-Trityl-lichenan. — Yield 84%.

Anal. Calc. for $(C_{25}H_{24}O_5)_n$: C, 74.3; H, 5.94. Found: C, 74.4; H, 5.96.

2,4-Di-O-phenylcarbamoyl-6-O-trityl-lichenan. — Yield 62%.

Anal. Calc. for $(C_{39}H_{34}N_2O_7)_n$: C, 72.9; H, 5.30; N, 4.36. Found: C, 71.2; H, 5.55; N, 4.50.

2,4-Di-O-phenylcarbamoyl-lichenan. — Yield 67%.

Anal. Calc. for $(C_{20}H_{20}N_2O_7)_n$: C, 60.0; H, 5.00; N, 6.9. Found: C, 58.1; H, 5.17; N, 6.85.

Ortho esters. — The ^{13}C-n.m.r. spectra (62.89 MHz) were recorded with a Bruker WM spectrometer for solutions of ortho ester (50 mg) in $CDCl_3$ (2 mL) at 33° with external Me_4Si. Melting points were determined on a Kofler hot-stage.

3,4,6-Tri-*O*-acetyl-α-D-glucose 1,2-(ethyl orthoacetate)[17] (**1**) and 3,5-Di-*O*-benzoyl-β-L-arabinofuranose 1,2-(methyl orthobenzoate)[18] (**4**) were prepared as described.

3,4-Di-O-acetyl-β-L-rhamnose 1,2-(ethyl orthoacetate) (**2**). — To a solution of 2,3,4-tri-*O*-acetyl-α-L-rhamnosyl bromide (10 g, 28.3 mmol) in 2,6-dimethylpyridine (32 mL) was added dry EtOH (1.72 mL, 37.4 mmol), and the mixture was kept for 18 h at 50°, then diluted with $CHCl_3$ (100 mL). The solution was neutralised by washing with 5M

HCl, then washed with sat. aq. $KHCO_3$ and cold water, and dried ($CaCl_2$), and the solvent was evaporated under reduced pressure. The residue was crystallised from aq. EtOH to give **2** (5 g, 55%), m.p. 87°, $[\alpha]_D$ + 21° (*c* 0.26, $CHCl_3$). ^{13}C-N.m.r. data ($CDCl_3$): δ 97.3 (C-1), 69.2 (C-2), 70.5 (C-3,5), 70.9 (C-4), 17.6 (C-6), 15.1 (CH_3CH_2), 58.1 (CH_3CH_2), 24.9 (CH_3CO_3), 124 (CH_3CO_3).

Anal. Calc. for $C_{14}H_{22}O_8$: C, 52.8; H, 6.9. Found: C, 52.9; H, 6.9.

3,4,2',3',4',6'-Hexa-O-acetyl-α-gentiobiose 1,2-(ethyl orthoacetate) (**3**). — To a solution of hepta-*O*-acetyl-gentiobiosyl bromide (7 g, 10.5 mmol) in 2,6-dimethylpyridine (20 mL) and EtOH (8.2 mL, 178 mmol) was added tetrabutylammonium bromide (1 g). The solution was kept at 60° for 18 h, then diluted with $CHCl_3$ (75 mL), washed with dil. HCl, sat. aq. $KHCO_3$, and cold water, and filtered, and the solvent was evaporated under reduced pressure. The residue was crystallised from aq. EtOH to give **3** (4.7 g, 67%), m.p. 172°, $[\alpha]_D$ + 2.0° (*c* 0.32, $CHCl_3$). ^{13}C-N.m.r. data ($CDCl_3$): δ 101.2 (C-1'), 96.9 (C-1), 15.3 (CH_3CH_2), 59.2 (CH_3CH_2), 20.6 (CH_3CO_3), 121.5 (CH_3CO_3).

Anal. Calc. for $C_{28}H_{40}O_{18}$: C, 50.6; H, 6.02. Found: C, 50.8; H, 6.04.

Condensation reactions. — Rigorously anhydrous conditions were essential.

A suspension of powdered di-*O*-phenylcarbamoyl derivative (1 g, 2.5 mmol) in chlorobenze (15 mL) was kept at 80° for 1–2 h. Various molar amounts of the sugar ortho ester (see Table I) were added and each suspension was distilled until the distillate was clear. After the addition of 2,6-dimethylpyridinium perchlorate (0.1 mmol), the mixture was boiled under reflux for the times given in Table I. Each suspension of a lichenan derivative was poured into MeOH (200 mL), and the precipitate was collected and dried.

The curdlan derivatives dissolved during the reaction. The chlorobenzene was distilled under reduced pressure, a solution of the residue in a small volume of acetone was poured into water (200 mL), and the product (80–100%) was collected and dried.

Saponification. — The acetyl and phenylcarbamoyl groups were saponified as described[16].

Methylation. — The procedure of Harris *et al.*[19] was used. The partially methylated alditol acetates were analysed by g.l.c.–m.s. with a linear temperature gradient 170→210° at 1°/min, then 210° for 10 min on a DB-1701-30W fused-silica column (30 m), in a Hewlett–Packard 5890A gas chromatograph with a mass-selective detector 5970B and an HP work station 300. The methylated alditol acetates obtained from the branched derivatives of lichenan were also analysed on a DB-225 fused-silica column (30 m) with a temperature program of 175° for 10 min, then 175→210° at 5°/min.

Congo Red assay. — The shift of λ_{max} of 0.38μM Congo Red (Sigma) was recorded with a Shimadzu double-beam UV 210-A spectrophotometer[29].

Antitumour tests. — Sarcoma 180 was kindly provided by Dr. Bogden (Mason Research Institute, Worcester, MA, U.S.A.). The tumour was maintained by routine passages (i.p.) of ascites fluid (5×10^6 tumour cells) inoculated into female BDF1-mice every week. Testing was performed by s.c. inoculation of 0.1 mL of ascites fluid (~ 5×10^6 tumour cells) into the right groin of female CD1-mice (10 mice/group). Solutions of the test samples in saline were injected i.p. daily from day 1 to 10, starting 24 h after

inoculation of the tumour. Tumour growth was controlled by measuring the tumour area (length × width) with a caliper every 10 days. The mice were sacrificed after 30 days and the tumours were excised and weighed. The rate of inhibition was calculated by comparing the tumour weight of the treated group to that of the untreated controls[7,20].

RESULTS AND DISCUSSION

The ortho ester method of glycosylation has been used extensively for the synthesis of branched derivatives of linear polysaccharides[10–15,21,22]. The most widely used reaction medium is boiling chlorobenzene with 2,6-dimethylpyridinium perchlorate catalysis, and the reaction is thought to involve the acyloxonium ion of the sugar ortho ester, leading stereospecifically to the *trans*-glycoside[23–26].

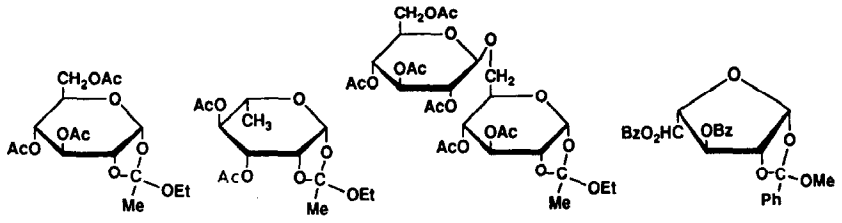
The novel sugar ortho esters **2** and **3** were obtained by reaction of the appropriate glycosyl bromide with ethanol, using established procedures (see Experimental section), and their structures were proved by methylation analysis and ^{13}C-n.m.r. spectroscopy. Thus, methylation analysis yielded 1,2-*O*-acetylated sugar alcohols, indicating the presence of the alkali-resistant ortho-ester structure[27]. These findings were supported by

TABLE I

Products of glycosylation of curdlan and lichenan at positions C-6

Branch	*O/G*[a]	*t*[b] *(min)*	*D.b.*[c] *(%)*
Lichenan			
Glucose	1.0:1.0	20	8
	1.0:1.0	40	30
	1.0:1.0	70	45
	1.0:1.0	60	25
	0.5:1.0	60	13
	0.25:1.0	60	4
Rhamnose	1.0:1.0	50	20
	0.5:1.0	40	10
Arabinose	0.5:1.0	40	10
Curdlan			
Glucose	1.0:1.0	60	63
	1.0:1.0	40	53
	0.8:1.0	40	28
	0.6:1.0	40	29
	0.4:1.0	40	16
	0.2:1.0	40	16
	0.1:1.0	40	9
Gentiobiose	1.0:1.0	60	29
Rhamnose	1.0:1.0	40	50
	0.6:1.0	40	35
Arabinose	1.0:1.0	60	53
	0.6:1.0	40	33

[a] Molar ratio of sugar ortho ester (O) and glucose residues in the starting polymer (G). [b] Reaction time. [c] Degree of branching.



1 2 3 4

the ^{13}C signals at 124.2 (**2**) and 121.5 p.p.m. (**3**) for the quaternary carbon atom of the ortho ester.

The sugar ortho esters were condensed variously with 2,4-di-*O*-phenylcarbamoylcurdlan and 2,4-/2,3-di-*O*-phenylcarbamoyl-lichenan in boiling chlorobenzene with 2,6-dimethylpyridinium perchlorate catalysis[21,22]. By varying the time of reaction and the molar ratio of the reactants, products with various degrees of branching were obtained (Table I). The extent of branching was determined by methylation analysis.

Comparison of the data for the lichenan and curdlan derivatives indicates that (1→3)-linked glucose residues were substituted more easily than the (1→4)-linked residues (up to 2.8-fold for a lichenan derivative with 8% of branches). Also, the lower the degree of branching, the higher was the molar ratio of (1→3/1→6)- to (1→4/1→6)-linked glucose residues in the lichenan derivatives (Table II).

Saponification of the above products gave water-soluble branched polysaccharides that were purified by chromatography on DEAE-Sephacel and dialysis. The molecular weights of these products were determined by g.p.c. on Superose TM 12, using pullulans as standard. The results (25 000 for the curdlan and 100 000 for the lichenan derivatives) indicated considerable degradation (*cf.*, 85 000 for curdlan and 300 000 for lichenan), possibly due to the amount of methyl sulfoxide in the solvent during the introduction of the protecting groups[28].

TABLE II

Ratio of (1→3/1→6)- and (1→4/1→6)-linked glucose in the lichenan derivatives

Branch	*D.b.*[a] *(%)*	*Molar ratio (1→3/1→6):(1→4/1→6)*
Glucose	8	1.4:1.0
	11	1.4:1.0
	20	1.0:1.0
	30	1.0:1.4
	50	1.0:1.6
Rhamnose	10	1.0:1.1
	20	1.0:1.4
Arabinose	10	1.0:1.0

[a] Degree of branching.

In order to detect highly ordered structures, the formation of complexes with Congo Red was investigated. The presence of a single helical polysaccharide shifts the λ_{max} of an aqueous solution of Congo Red to higher wavelength[6,29]. The synthetic branched derivatives of curdlan and lichenan shifted the λ_{max} from 498 to 509–512 nm. The single helices of the curdlan derivatives had the rhamnosyllichenan derivative were stable in sodium hydroxide up to 0.1M, whereas the Congo Red complexes of the other derivatives of lichenan were destroyed by the addition of sodium hydroxide (Fig. 1). It is not clear why the lichenan derivatives are able to form complexes with Congo Red, since this phenomenon was shown for (1→3)-β-D-glucans with or without side chains in position 6 only.

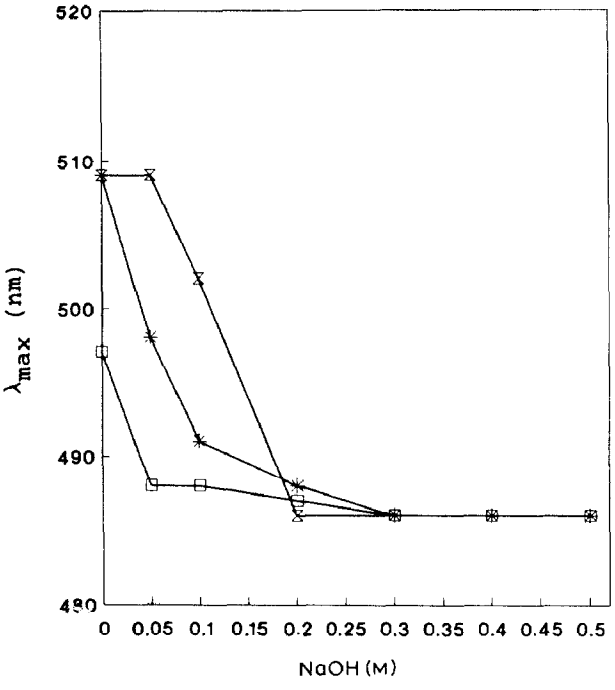


Fig. 1. Plot of λ_{max} against concentration of NaOH for the complexes of Congo Red with the polysaccharides with stable ( ) and sensitive (*) single helices (□ blank).

The antitumour activity of the synthetic branched polysaccharides was tested against the allogeneic Sarcoma 180 tumour. The results are given in Table III. The curdlan derivatives had inhibition ratios from 60 up to 100%, and the rhamnosyl and arabinosyl derivatives were the most active. The derivatives of lichenan showed no significant activity, except the glucosyl derivative with a low degree of branching. These findings indicate that a (1→3)-linked backbone is advantageous for an antitumour activity of β-D-glucans. The structure of the sugar in the branch seems to be less important, but the water-solubility conferred by the branching may be the most important prerequisite.

TABLE III

Antitumour activity of the branched derivatives of curdlan and lichenan against Sarcoma 180

Branch	*D.b.*[a] *(%)*	*Dose*[b] *(mg/kg)*	*Tumour weight (g)*	*Inhibition*[c] *ratio (%)*	*Significance*[d] *(p <)*
Curdlan					
Control			5.40		
Glucose	29	5	0.79	85	0.02
		25	2.20	59	n.s.
Gentiobiose	29	5	1.72	68	n.s.
		25	1.26	77	0.02
Rhamnose	33	5	0.39	93	0.01
		25	0.04	99	0.01
Arabinose	33	5	0.07	99	0.01
		25	0.04	99	0.01
Lichenan					
Control			4.03		
Glucose	8	5	2.17	46	n.s.
		25	0.48	88	0.01
	20	5	3.25	19	n.s.
		25	2.56	36	n.s.
	45	5	4.97	−23	n.s.
		25	2.48	39	n.s.
Control			5.40		
Rhamnose	10	25	5.98	−11	n.s.
Arabinose	10	25	4.95	8	n.s.

[a] Degree of branching. [b] Treatment was performed daily from day 1–10, i.p. [c] % = $(C - T/C) \times 100$, where C is the average tumour weight of the control group and T the average tumour weight of the treated group. [d] Evaluated according to the Student's t test ($p > 0.05$ = n.s.).

The finding that the derivatives of curdlan which showed antitumour activity had no ordered structures, such as triple helices, supports the findings of Kurachi *et al.*[30] and Kraus *et al.*[7] that highly ordered structures of branched (1→3)-β-D-glucans are not essential for an antitumour activity.

ACKNOWLEDGMENT

We thank the Fonds der Chemischen Industrie for financial support, and the Herrmann-Schlosser-Stiftung for a fellowship (to S.D.).

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