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Synthesis and antitumour activity of sulfoalkyl derivatives of curdlan and lichenan

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ABSTRACT

2-Sulfoethyl, 3-sulfopropyl, and 4-sulfobutyl derivatives of the (1→3)-β-D-glucan curdlan and the (1→3/1→4)-β-D-glucan lichenan have been synthesised. The substituents are located mainly at positions 6. The curdlan derivatives strongly inhibited the growth of the Sarcoma 180 tumour, whereas the lichenan derivatives were inactive, indicating that a (1→3)-linked β-D-glucan backbone is essential for activity.

INTRODUCTION

Numerous naturally occurring (1→3)-β-D-glucans, mostly branched at C-6 with glucose side chains, have high antitumour activity[1–4]. Although many chemical modifications have been made to these polysaccharides in seeking structure–activity relationships, the essential structures and conformations have not yet been defined fully. The high molecular weight and more highly ordered structures, *i.e.*, triple helices, are not as important as formerly assumed[5,6].

The influence of glycosidic and non-glycosidic side chains attached to linear polysaccharides[7–10] has been studied. Whereas the derivatives of (1→3)-β-D-glucans inhibited tumour growth, those of (1→4)-β-D-glucans or other polysaccharides had moderate or no activity.

The aim of the study now reported was to obtain water-soluble derivatives by sulfoalkylation of the (1→3)-linked β-D-glucan curdlan or the (1→3)/(1→4)-linked-β-D-glucan lichenan and to assess their antitumour activity.

EXPERIMENTAL

Syntheses[11]. — The molar ratios of NaOH and reagents are given in Table I. A solution of each derivative in water was neutralised with acetic acid and dialysed.

Sulfoethylation. — To a suspension of lichenan or curdlan in 2-propanol (10 mL) at 85° (reflux) was added a solution of NaOH and sodium 2-chloroethanesulfonate in water (2 mL) in two portions with an interval of 1 h. The mixture was then stirred and boiled under reflux for 3 h.

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Sulfopropylation. — To a solution of the polysaccharide in 25:3 2-propanol–water (15 mL) was added NaOH. The suspension was stirred at 50° for 1 h and a solution of 1,3-propanesultone in acetone (1 mL) was added. The mixture was stirred for 6 h at 50° and then left at room temperature overnight.

Sulfobutylation. — To a suspension of the polysaccharide in 25:3 2-propanol–water (15 mL) was added NaOH, and the suspension was stirred at 60° for 1 h. 1,4-Butanesultone was added, and the mixture was stirred for 6 h at 60°, then left at room temperature overnight.

Degree of substitution (d.s.). — The d.s. was determined as described by Casu and Gennaro[12].

For ^{13}C-n.m.r. spectroscopy and t.l.c., each polysaccharide derivative was hydrolysed with 2M trifluoracetic acid for 1 h at 121°. After evaporation of the acid, the products of hydrolysis were reduced with an excess of $NaBH_4$ in 2M NH_4OH for 1 h at 60°. The reaction was stopped by addition of acetic acid. The mixture was concentrated and boric acid was removed from the residue by repeated evaporation of MeOH therefrom. After treatment with Amberlite IR-120 (H^+) resin, a solution of the residue in water (30 mL) was freeze-dried.

The ^{13}C-n.m.r. spectrum (62.89 MHz) was recorded with a Bruker WM spectrometer for a solution of each reduced hydrolysate (50 mg) in D_2O (2 mL) at 33° with external Me_4Si. ^{13}C-N.m.r. data (D_2O): δ (unsubstituted carbon atoms of all products) C-1 62.8, C-2 73.1, C-3 70.0, C-4 71.5, C-5 71.4, C-6 63.1; δ (substituted carbon atoms) C-2 81.1–81.6, C-6 71.5–72; δ (substituents) C-1′ 50.8, C-2′ 66.4; C-1′ 48.2, C-2′ 24.6, C-3′ 70.7; C-1′ 50.9, C-2′ 20.8, C-3′ 30.4, C-4′ 70.6.

T.l.c. of the products was performed on Silica Gel KG 60, using EtOAc–acetic acid–MeOH–aq. 4.6% boric acid (55:11:20:15). R_F values of the substituted glucitol derivatives (relative to glucitol): 0.76, 0.68, 0.40 (only for lichenan derivatives), 0.17.

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[20].

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) into female BDF1-mice every week. Testing was performed by s.c. inoculation of 0.1 mL of ascites fluid ($\sim 5 \times 10^6$ tumour cells) into the right groin of female CD1-mice (10 mice/group). The test samples, dissolved in saline, were injected i.p. daily from day 1 to 10, starting 24 h after tumour inoculation. Tumour growth was controlled by measuring the tumour area (length × width) with a caliper every 10 days. Inhibition of tumour growth was calculated by comparing the average tumour area of the treated group to that of the untreated controls, determined at day 20 or 30, respectively[6,13].

Statistical analysis. — Statistical evaluation was performed using the Student's *t*-test (double-sided) at $p < 0.05$ level of significance.

RESULTS AND DISCUSSION

3-Sulfopropyl and 4-sulfobutyl ethers of polysaccharides can be obtained by reaction of the corresponding sultones in an alkaline medium[11,14,15]. The sulfoethyl derivative can be synthesised by reaction of 2-chloroethanesulfonic acid in strongly alkaline medium[11].

Sulfoalkylation is usually performed on suspensions in a water-miscible solvent such as 2-propanol. Since the reaction does not proceed in the absence of water[14], 2-propanol–water mixtures were used. The products obtained from lichenan or curdlan by reaction with 1,3-propanesultone, 1,4-butanesultone, and 2-chloroethanesulfonic acid are listed in Table I. It is probable that an excess of sodium hydroxide favours the formation of the corresponding ω-hydroxyalkane sulfonic acids. The sulfoalkyl polysaccharides gave clear solutions when heated at 100° in water for a short time. The degree of substitution (d.s.) was determined by acidimetric titration[12].

The pattern of substitution of the derivatives was investigated by ^{13}C-n.m.r. spectroscopy of the products of hydrolysis after borohydride reduction to the corresponding glucitol derivatives. The spectra indicated preferential 6-substitution (signal at 71.5–72 p.p.m.), and 2-substitution was confirmed by the signal at 81.1–81.6 p.p.m. Signals for 4-substitution in the products from curdlan and 3- and 4-substitution in the products from lichenan could not be assigned. However, t.l.c. of the reduced products of hydrolysis showed one (curdlan) and two (lichenan) weak spots, respectively, besides the expected spots for unsubstituted, and 2- and 6-substituted glucitol.

There is still controversy concerning the importance of helical structures for the biological activity of polysaccharides. In neutral aqueous solutions, the λ_{max} of Congo Red is shifted to higher wavelength in the presence of a polysaccharide with a single helical conformation[16]. No such shifts were observed with the sulfoalkyl derivatives.

TABLE I

Products of sulfoalkylation of curdlan and lichenan

Starting polymer	*Substituent*	*Molar ratio of reagent:NaOH:glucose*	*D.s.*[a]
Lichenan	Ethanesulfonic acid	2.5:4.5:1	0.27[b]
	Propanesulfonic acid	2.0:2.5:1	0.75
		4.0:5.0:1	0.70
		3.0:2.0:1	0.90[b]
	Butanesulfonic acid	2.5:4.5:1	0.40
		2.5:2.5:1	0.30
		2.5:3.5:1	0.55[b]
Curdlan	Ethanesulfonic acid	1.0:1.5:1	0.43[b]
	Propanesulfonic acid	3.0:2.8:1	0.45[b]
	Butanesulfonic acid	2.5:2.0:1	0.27[b]

[a] Degree of substitution. [b] Derivatives used in the antitumour assay

Derivatives of curdlan and lichenan with sugar branches at positions 6 usually adopt single helices in neutral solutions. Presumably, for the sulfoalkylated polysaccharide derivatives, electrostatic interactions of the sulfonic acid groups and the 2-substituent inhibit the formation of single helices.

The apparent molecular weight of the sulfoalkyl polysaccharides, determined by g.p.c. on Superose TM 12 using pullulan as standard, was >380 000, which may be higher than the actual value. In g.p.c., glucosaminoglycans[17,18] with anionic substituents are eluted too early in comparison with neutral standards, probably due to electrostatic interaction and solute–solvent interactions.

The 2-sulfoethyl, 3-sulfopropyl, and 4-sulfobutyl derivatives of curdlan and lichenan were tested for activity against the allogeneic Sarcoma 180 tumour, and the results are shown in Figs. 1–3. The lichenan derivatives were inactive, whereas the curdlan derivatives were highly active. The chain length of the substituent had no influence on the antitumour activity. Thus, in agreement with Kraus *et al.*[6], it appears that particular conformations are not important for antitumour activity, since the curdlan derivatives lack triple and single helical structures. Substitution is relevant only in so far as it increases the water-solubility, whereas the type of linkage in the main chain is essential for antitumour activity.

The results reported emphasise the importance of a (1→3)-linked β-D-glucan backbone for the expression of a high antitumour activity and support the findings

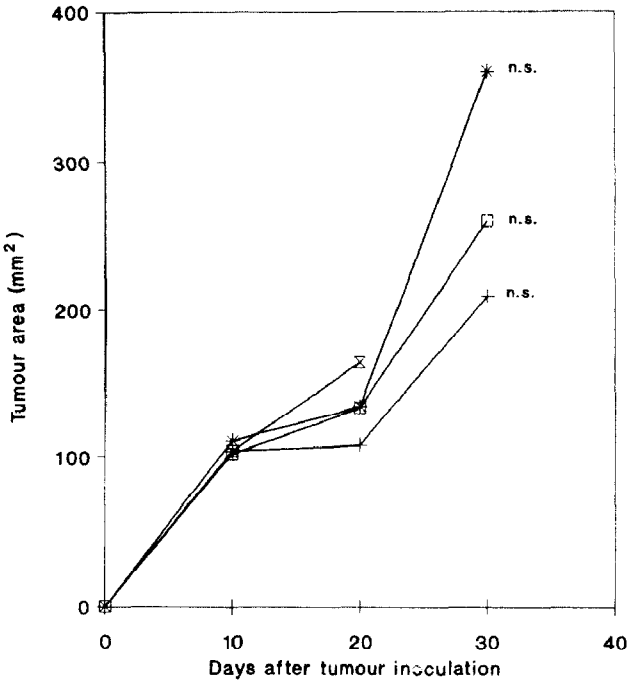

Fig. 1. Antitumour effect against Sarcoma 180 of sulfoalkylated lichenan derivatives at 25 mg/kg; □, sulfoethyl; +, sulfopropyl; *, sulfobutyl; n.s., not significant; ⧖, control.

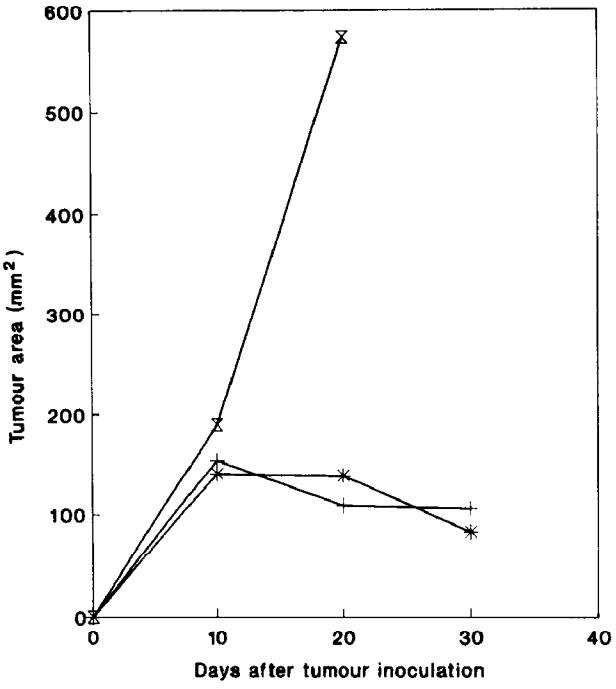

Fig. 2. Antitumour effect against Sarcoma 180 of sulfopropylated curdlan: +, 5 mg/kg; *, 25 mg/kg; , control; significant differences ($p < 0.0001$) to the control were determined at day 20.

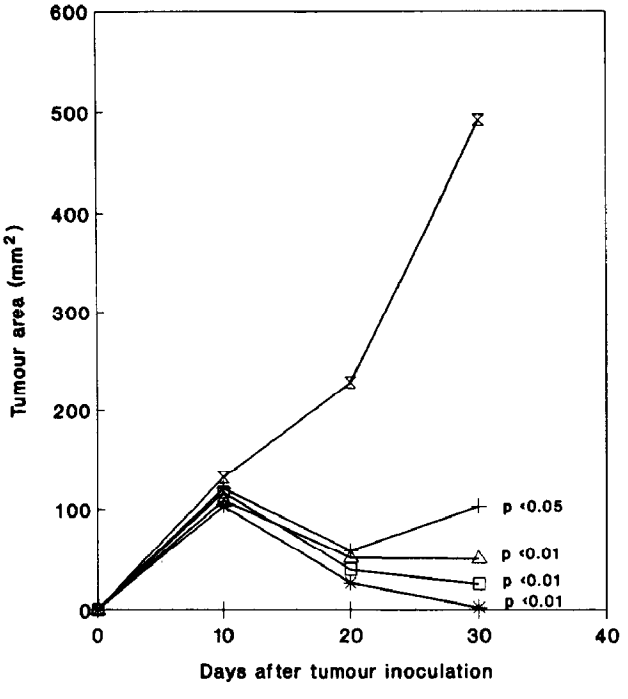

Fig. 3. Antitumour effect against Sarcoma 180 of sulfoethylated (+, 5 mg/kg; *, 25 mg/kg) and sulfobutylated (□, 5 mg/kg; △, 25 mg/kg) curdlan; ⧖, control; significant differences to the control were determined at day 30.

described by other workers[6,9,10,19]. A high proportion of (1→4)-linked β-D-glucose residues reduces the antitumour activity.

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