Carbohydrate Polymers **21** (1993) 283–288

Synthesis of sulfated derivatives of curdlan and their anti-HIV activity

Zenjiro Osawa, Tsubura Morota
Faculty of Engineering, Gunma University, Kiryu, Gunma 376, Japan

Kenichi Hatanaka, Toshihiro Akaike
Department of Biomolecular Engineering, Tokyo Institute of Technology, Midoriku, Yokohama, Kanagawa 227, Japan

Kei Matsuzaki*
Formerly, Faculty of Engineering, University of Tokyo, Bunkyoku, Tokyo 113, Japan

Hideki Nakashima, Naoki Yamamoto
Tokyo Medical and Dental University, Ochanomizu, Bunkyoku, Tokyo 113, Japan

Eiichiro Suzuki, Hiroshi Miyano, Tohru Mimura & Yutaro Kaneko
Ajinomoto Co., Inc., Kyobashi, Chuoku, Tokyo 108, Japan

(Received 17 February 1993; accepted 26 February 1993)

Sulfopropyl curdlan was synthesized, its structure was determined, and the anti-HIV activity was compared with that of standard curdlan sulfates obtained with piperidine *N*-sulfonic acid in dimethyl sulfoxide. It was shown that sulfopropyl curdlan exhibits weaker anti-HIV activity than curdlan sulfate. Curdlan sulfates were synthesized with a SO_3–pyridine complex in a heterogeneous phase. It was shown from ^{13}C-NMR spectra of acetylated curdlan sulfates that they had a different substituent distribution from standard curdlan sulfate. The cytotoxicity of the curdlan sulfates was attributed to their heterogeneous structure.

INTRODUCTION

It has been found that cellulose sulfate and curdlan sulfate with high degrees of substitution exhibit strong anti-HIV activity *in vitro* (Yoshida *et al.*, 1988; Kaneko *et al.*, 1990; Yamamoto *et al.*, 1990, 1991; Yoshida *et al.*, 1990). In this paper, in order to make clear the effect of the chain length of sulfate groups, i.e. the effect of the spacer, sulfopropyl curdlan having a trimethylene group as the spacer was synthesized and its anti-HIV activity was determined. Furthermore, in order to elucidate the effect of the sulfating condition either in a homogeneous or a heterogeneous phase, the structure and anti-HIV activity of the curdlan sulfates obtained with a SO_3–pyridine complex in a heterogeneous phase were compared with those of curdlan sulfates obtained with piperidine *N*-sulfonic acid in a homogeneous phase.

EXPERIMENTAL

Synthesis of sulfopropyl curdlan

So far, sulfopropylation of cellulose via alkali cellulose has been reported (Goethals & Natus, 1966). However, the application of this method to curdlan did not give products with a high degree of substitution (d.s.) Therefore, conversion of alcoholate of curdlan dissolved in dimethyl sulfoxide to sulfopropyl curdlan was attempted.

*To whom correspondence should be addressed at 4-10-10 Naritahigashi, Suginamiku, Tokyo 166, Japan

Carbohydrate Polymers 0144-8617/93/$06.00

As an example, synthesis of sulfopropyl curdlan SSC-8 is shown below. Curdlan (3 g) dissolved in dimethyl sulfoxide (300 ml) was reacted with sodium hydride (3 g) for 1 h at room temperature. Propane sultone (6 mole/AGU) was added and stirred for 12 h at room temperature. The reaction product was dissolved in iced water and precipitated into acetone–methanol. The precipitate was dissolved in deionized water and dialyzed against deionized water for 3–4 days. The solution was neutralized, concentrated under vacuum, and freeze-dried.

Samples SSC-1b, -5a, -8a and -8b were prepared by repeating the reaction to get samples with a high d.s.

Synthesis of curdlan sulfates with a SO_3–pyridine complex

Curdlan (1 g) was swollen in dimethyl formamide (50 ml) for 24 h at room temperature. Then a SO_3–pyridine complex (2–5 mole/AGU) was added to the swollen curdlan and the sulfation was continued with stirring for 4 h at room temperature. The reaction product was poured into water and neutralized with aqueous 1 N sodium hydroxide. The curdlan sulfate was precipitated with acetone and the precipitate was processed in the same method as for sulfopropyl curdlan.

Peracetylation of curdlan sulfate

Acetylation of curdlan sulfate was performed according to a method reported by Wolfrom and Spoors (1960) on chondroitin sulfate. Curdlan sulfate (1 g) was dissolved in anhydrous formamide (10 ml), and acetylation was performed by adding acetic anhydride (2·5 ml) in pyridine (10 ml) and keeping at room temperature for 12 h, when a further quantity (4 ml) of acetic anhydride was added and kept for 12 h. The reaction product was poured into a large quantity of ethanol. The precipitate was dissolved in water and dialyzed against distilled water.

Elemental analysis of sulfated curdlans

An elemental analysis (C, H and N) was carried out with a Yanaco CHN Corder MT-5 elemental analyser. The sulfur content was analyzed by burning the sample in a Pyrex tube with hydrogen peroxide aqueous solution and determining the sulfate ion produced with a Dionex ion chromatograph apparatus.

^{13}C-NMR spectroscopy

^{13}C-NMR spectra of sulfopropyl curdlan were recorded with a JEOL EX 270 spectrometer at 67·80 MHz in D_2O at 70°C. The repetition time was 2 s for non-gated decoupling and 10 s for gated decoupling. As the internal reference for chemical shifts, sodium 2,3-dimethyl-2-silapentane-5-sulfonate (DSS) was used.

^{13}C-NMR spectra of acetylated curdlan sulfate were recorded on 0·1 M NaCl solution in D_2O with a Bruker AMX 600 spectrometer at 150 MHz at 32°C. The repetition time was 3–4 s and the number of scans was 70 000.

Signal areas in ^{13}C-NMR spectra were determined by curve resolving using a computer program developed by Dr S. Ando (Ando, 1988).

Assay for anti-HIV activity

The assay for anti-HIV activity was carried out using the method described by Yamamoto *et al.* (1991).

RESULTS AND DISCUSSION

Synthesis and anti-HIV activity of sulfopropyl curdlan

The synthesis condition and results of the analysis of sulfopropyl curdlan are shown in Table 1. An example of a ^{13}C-NMR spectrum of sulfopropyl curdlan (SSC-8a) is shown in Fig. 1. From the methylene carbon (C8 or C9) absorptions of sulfopropyl group and C1 absorptions of anhydroglucose units, the d.s. of sulfopropyl curdlan was determined. The d.s. of sulfopropyl curdlan is also obtained from the elemental analysis and is shown in Table 1.

Table 1 shows that with repeating the reaction (SSC-1b, -5a, -8a and -8b), the d.s. increased from 0·4–0·8 to 2·4–3·2. A d.s. of more than 3 indicates that impurities containing sulfur may be included in the samples. The anti-HIV activity is expressed as EC_{50} (50% effective concentration of polysaccharide solution) and SI (selectivity index, CC_{50}/EC_{50}, where CC_{50} is 50% cytotoxic concentration of polysaccharide solution). In the table, the anti-HIV activity, determined at the same time, of standard curdlan sulfate (S content 14%, obtained with piperidine N-sulfonic acid) are also shown for comparison. The lower EC_{50} and the higher SI, the safer and the more effective the drugs. It is clear that sulfopropyl curdlan has weaker anti-HIV activity than standard curdlan sulfate, although sulfopropyl curdlans with a high d.s. (SSC-5a and -8b) show anti-HIV activity. It is concluded that the spacer group, i.e. the trimethylene group, weakens anti-HIV activity.

Synthesis of curdlan sulfate with a SO_3–pyridine complex and its structure and anti-HIV activity

Synthesis of curdlan sulfate with a SO_3–pyridine complex proceeds in a heterogeneous phase, in contrast to the synthesis with a SO_3–DMF complex in DMF or piperidine N-sulfonic acid in dimethyl sulfoxide (Yamamoto *et al.*, 1990, 1991; Yoshida *et al.*, 1990).

Table 1. Synthesis and anti-HIV activity of sulfopropyl curdlan[a]

Sample no.	Solvent	Mole ratio	Yield[b] (g)	S (%)	d.s.		EC_{50} (μg/ml)	SI
					EA[c]	NMR		
SSC-1	DMSO	6·0	0·63 (1·0)	6·4	0·60	—	—	—
SSC-1b	DMSO	30	0·34 (0·2)	16·0	3·67	—	5·32	> 188
SSC-5	DMF	6·0	1·00 (1·0)	7·5	0·73	0·86	—	—
SSC-5a	DMSO	7·5	1·00 (0·8)	13·9	2·46	3·0	1·95	> 465
SSC-6	DMF	6·0	1·42 (1·0)	8·3	0·8	—	1000	> 1
CS					1·5		0·18	> 5 700
SSC-8	DMSO	6·0	1·84 (3·0)	5·1	0·41	—	13·4	> 75
SSC-8a	DMSO	6·0	0·68 (0·5)	12·4	2·38	2·2	2·3	> 435
SSC-8b	DMSO	12·0	0·59 (0·5)	14·1	3·18	2·8	0·14	> 7 140
CS							0·018	> 55 600

[a] CS: Standard curdlan sulfate; EC_{50}: 50% effective concentration of polysaccharide solution; SI: CC_{50}/EC_{50}, where CC_{50} is 50% cytotoxic concentration of polysaccharide solution.
[b] Numbers in parentheses indicate the weight (g) of the starting polysaccharide.
[c] The degree of substitution determined from elemental analysis.

Table 2 shows the synthetic condition, the results of the elemental analysis and the anti-HIV activity of curdlan sulfates synthesized with SO_3–pyridine complex. It indicates that with an increasing mole ratio, SO_3/AGU, the d.s. increases.

Figure 2 shows a ^{13}C-NMR spectrum of sample PYC-4 as compared with that of DMFC-3 obtained with a SO_3–DMF complex. It is seen from the spectra that unsubstituted C6 carbon completely disappeared, indicating that the d.s. at C6 is 1·0. The spectrum of PYC-4, however, is different from that of DMFC-3 in C1 and C3 regions, and in the region of 78–84 ppm, which is attributed to the substituted C2 and C4 carbons, indicating that the substituent distribution is different between the two samples. Although assignment of the NMR spectra of curdlan sulfate was presented in a previous paper (Yamamoto *et al.*, 1990), it is not definite yet. Miyano and Suzuki (1992) reported on the distribution of substituents in curdlan sulfate obtained with piperidine *N*-sulfonic acid, and concluded that substitution at C4 is much lower than that at the C2 position, based on detailed NMR measurements using 2D-HOHAHA and 2D (both ^{1}H–^{1}H and ^{13}C–^{1}H)-NMR spectroscopy.

Tezuka *et al.* (1987, 1989, 1990*a*, *b*) reported that the substituent distribution of various cellulose ethers can be determined from the ^{13}C-NMR spectra of the carbonyl region in the acetylated derivatives of cellulose ethers. Therefore, the authors tried to obtain direct information on the distribution of substituents from acetylated curdlan sulfate. Peracetylation of chondroitin sulfate without a side reaction has been reported by Wolfrom and Spoors (1960). Curdlan sulfates were peracetylated by their method. Figure 3 indicates carbonyl carbon spectra of acetylated curdlan sulfates, ASC-307 (S, 14·1%) and ASC-006 (S, 12·0%) obtained with piperidine *N*-sulfonic acid in homogeneous phase.

The spectrum (Fig. 3(a)) of carbonyl carbon of acetylated ASC-307 exhibits three distinct peaks (A, B and C), which are assigned as shown in Table 3. There-

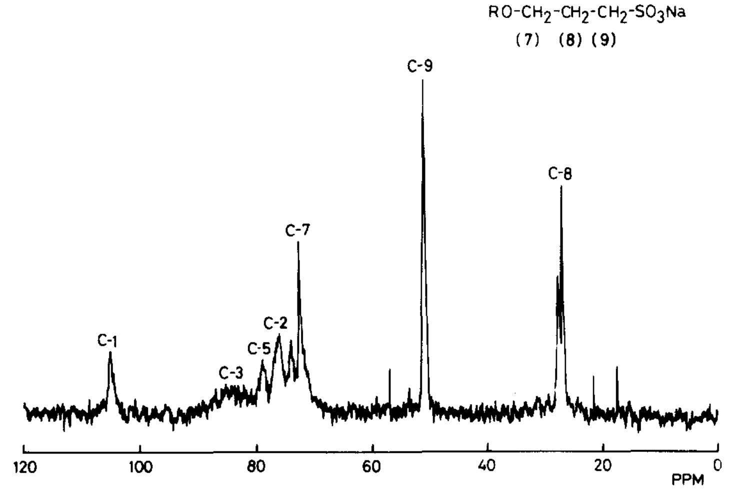

Fig. 1. ^{13}C-NMR spectrum of sulfopropyl curdlan SSC-8a.

Table 2. Synthesis and anti-HIV activity of curdlan sulfates obtained with a SO_3–pyridine complex, expressed as the number of viable cells and antigen-positive cells

Sample no.	SO_3/AGU	Yield (g)	S (%)	d.s. EA	Viable cells ($\times 10^4$)[a]		Antigen-positive[b]
					Uninfected	Infected	
PYC-2	2·0	1·55	10·4	0·93	119	1	90
PYC-2·5	2·5	1·57	13·6	1·45	—	—	—
PYC-3	3·0	1·77	14·6	1·75	5	2	1
PYC-4	4·0	1·82	15·6	1·98	3	7	1
PYC-5	5·0	2·24	16·1	2·10	6	3	1

[a] Uninfected = the number of viable cells after 6 days of culture of uninfected MT-4 cells on addition of curdlan sulfate (33 μg/ml); infected = the number of viable cells after 6 days of culture of HIV-infected MT-4 cells on the addition of curdlan sulfate (33 μg/ml).

[b] Percentages of antigen-positive cells.

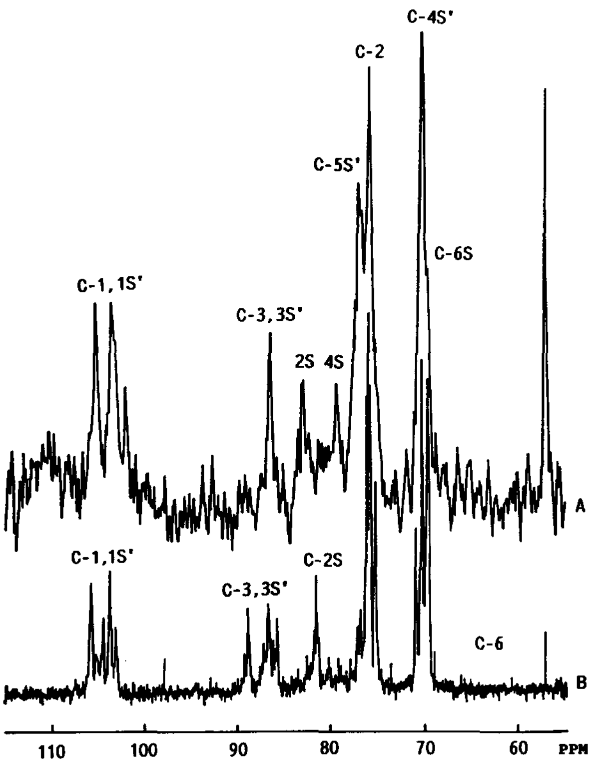

Fig. 2. ^{13}C-NMR spectra of curdlan sulfates. A, PYC-4 obtained with a SO_3–pyridine complex in pyridine; B, DMFC-3 obtained with piperidine *N*-sulfonic acid in DMSO. In the figure, S and S′ indicate substituted and neighbor-substituted carbons, respectively.

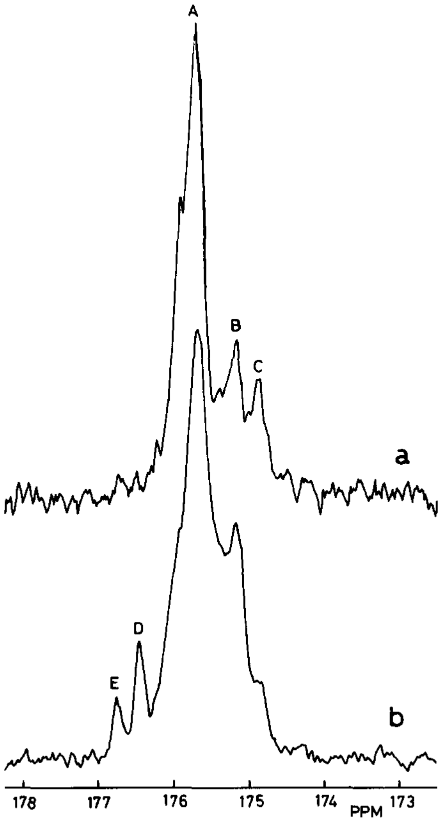

Fig. 3. ^{13}C-NMR spectra of acetyl carbonyl carbon in acetates of curdlan sulfates obtained with piperidine *N*-sulfonic acid in DMSO: (a) ASC-307; (b) ASC-006.

Table 3. Assignments and peak areas of ^{13}C-NMR absorptions in acetylated curdlan sulfates obtained in a homogeneous phase

Peak[a]	Chemical shift (ppm)	Carbon	Assignment	Peak area (%)	
				ASC-307	ASC-006
A	175·7	C4	2-AC, 4-Ac, 6-SO_4 + 2-SO_4, 4-AC, 6-SO_4	71·4	65·6
B	175·3	C2	2-AC, 4-Ac, 6-SO_4	18·2	21·1
C	175·0	C2	2-AC, 4-SO_4, 6-SO_4	10·4	4·1
D	176·5	C6	2-Ac, 4-Ac, 6-Ac	0	6·6
E	176·8	C6	2-SO_4, 4-Ac, 6-Ac + 2-Ac, 4-SO_4, 6-Ac	0	2·6

[a] Peaks A–E are indicated in Fig. 3.

fore, (A-B):C gives the ratio of substitution at C2 to that at C4 as 5·1:1. Thus, the degree of substitution at C6, C2 and C4 is obtained as 1·00, 0·38 and 0·07, as shown in Table 4, indicating the reactivity of the C4 hydroxyl group is about one-fifth that of the C2 hydroxyl group.

The spectrum (Fig. 3(b) of acetylated ASC-006 shows C6 carbonyl carbon peaks (D and E) as expected from its low sulfur content, and these are assigned as shown in Table 3. From the assignment and peak areas, the degree of substitution at C6, C2 and C4 is obtained as 0·88, 0·16 and 0·02, respectively, as shown in Table 4. Therefore, the reactivity of the C4 hydroxyl is about one-eighth that of the C2 hydroxyl in the early stages of the reaction.

The distribution of sulfate groups shown in Table 4 is different from those reported in the authors' previous paper (Yamamoto *et al.*, 1990); that is, substitution at the C2 position is much higher than that at the C4 position. The present results support the conclusion of Miyano and Suzuki (1992).

Figure 4 shows NMR spectra of acetates of PYC-2·5, -3 and -4 obtained with a SO_3–pyridine complex in a heterogeneous phase. It is seen from the figure that in the early stages of the reaction, the distribution of substituents is similar to that of curdlan sulfate obtained in a homogeneous phase, but as the reaction proceeds, signal area B attributed to C2 carbonyl carbon approaches that attributed to C4 carbonyl carbon (i.e. signal area A). The d.s. in curdlan sulfates obtained in the heterogeneous phase was estimated from the figure, based on the assumption that signal A is absorption due to C4 carbonyl carbon and signals B and C are due to C2 carbonyl carbon in acetylated curdlan sulfate (Tables 4 and 5).

Although the spectrum of acetylated PYC-4 shows the absence of C6 carbonyl carbon, the distribution of substituents is different from that of ASC-307, as is shown in Table 4. In a homogeneous phase, the C4 position is hardly substituted, while in a heterogeneous phase, as the reaction proceeds, the C4 position is sulfated as well as C2 position. This result suggests that in the heterogeneous phase, a helix or gel is formed by hydrogen bonds between C2 and C4 hydroxyl groups. It is deduced that C6 hydroxyl groups do not participate in helix formation. Therefore, the C6 position is easily sulfated but the helix domain does not react leaving microgel in which C2 and C4 hydroxyl group are not sulfated.

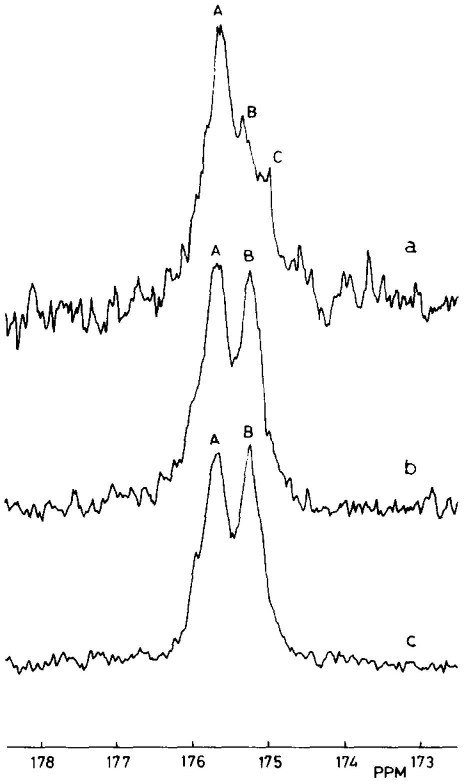

Fig. 4. ^{13}C-NMR spectra of acetyl carbonyl carbon in acetates of curdlan sulfates obtained with a SO_3–pyridine complex in pyridine: (a) PYC-2·5; (b) PYC-3; (c) PYC-4.

The anti-HIV activity of curdlan sulfates (PYC-3, -4 and -5) obtained with a SO_3–pyridine complex (Table 2) is high as expressed by low antigen-positive cell percentages after 6 days of incubation. However, the number of viable cells is very low, less than 10×10^4, indicating high cytotoxicity. As discussed above, the substituent distribution of curdlan sulfates obtained with a SO_3–pyridine complex in a heterogeneous phase is different

Table 4. Degree of substitution of each carbon in curdlan sulfates obtained in homogeneous and heterogeneous phases

Sample no.	Total d.s.[a]	Degree of substitution		
		C6	C4	C2
ASC-006	1·06	0·88	0·02	0·16
ASC-307	1·45	1·00	0·07	0·38
PYC-2·5	1·45	1·00	0·14	0·31
PYC-3	1·75	1·00	0·31	0·44
PYC-4	1·98	1·00	0·50	0·48

[a] d.s. obtained from elemental analyses.

Table 5. Assignments and peak areas of ^{13}C-NMR absorptions in acetylated curdlan sulfates obtained in a heterogeneous phase

Peak[a]	Chemical shift (ppm)	Carbon	Peak area (%)		
			PYC-2·5	PYC-3	PYC-4
A	175·6	C-4Ac	65·5	58	49
B	175·2	C-2Ac	22·9	42	51
C	175·0	C-2Ac	11·6	—	—

[a] Peaks A–C are indicated in Fig. 4.

from that of curdlan sulfates obtained in homogeneous phase, containing microgels in water.

It is supposed that MT-4 cells also interact with curdlan sulfate molecules as well as HIV virus, and are denaturated by the insoluble, associated structure in heterogeneous curdlan sulfates. Therefore, the formation of the heterogeneous structure in curdlan sulfates should be avoided to suppress cytotoxicity.

ACKNOWLEDGMENTS

The authors wish to thank Associate Professor Yasuyuki Tezuka, Department of Material Science and Technology, Nagaoka University of Technology for his advise concerning the peracetylation of sulfated polysaccharide. The authors are also grateful to Dr Takaya Sato, Tokyo Research Center, Nisshinbo Industries Inc., for carrying out the peracetylation of curdlan sulfates.

REFERENCES

Ando, S. (1988). *J. Assoc. Personal Computer for Chemists (Japan)*, **10**(2), 131.
Goethals, E.J. & Natus, G. (1966). *Makromol. Chem.*, **93**, 259.
Kaneko, Y., Yoshida, O., Nakagawa, R. *et al.* (1990). *Biochem. Pharm.*, **39**, 793.
Miyano, H. & Suzuki, E. (1992). *Anal. Sci.*, **8**, 561.
Tezuka, Y., Imai, K., Oshima, M. & Chiba, T. (1987). *Macromolecules*, **20**, 2413.
Tezuka, Y., Imai, K., Oshima, M. & Chiba, T. (1989). *Polymer*, **30**, 2288.
Tezuka, Y., Imai, K., Oshima, M. & Chiba, T. (1990*a*). *Carbohydr. Res.*, **196**, 1.
Tezuka, Y., Imai, K., Oshima, M. & Chiba, T. (1990*b*). *Makromol. Chem.*, **191**, 681.
Yamamoto, I., Takayama, K., Gonda, T. *et al.* (1990). *Brit. Polym. J.*, **23**, 245.
Yamamoto, I., Takayama, K., Honma, K. *et al.* (1991). *Carbohydr. Polym.*, **14**, 53.
Yoshida, O., Nakashima, H., Yoshida, T. *et al.* (1988). *Biochem. Pharm.*, **37**, 2887.
Yoshida, T., Hatanaka, K., Uryu, T. *et al.* (1990). *Macromolecules*, **23**, 3717.
Wolfrom, M.L. & Spoors, J.W. (1960). *J. Org. Chem.*, **25**, 308.