Synthesis, Enzymatic Hydrolysis, and Anti-HIV Activity of AZT−Spacer−Curdlan Sulfates

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ABSTRACT: An AIDS (acquired immunodeficiency syndrome) drug, azidothymidine (AZT), was bound by an ester bond onto curdlan sulfate having both anti-HIV activity and the property of accumulating in lymphoid tissues to produce a series of biodegradable AZT−aliphatic-dicarboxylate−curdlan sulfates (designated as AZT−spacer−curdlan sulfates). When the carbon number of the alkylene group was 2−12, AZT−spacer−curdlan sulfates exhibited high anti-HIV activities in the EC_{50} range of 0.04 − 0.21 μg/mL and low cytotoxicities of CC_{50} of more than 1000 μg/mL. AZT−dodecanedicarboxylate−curdlan sulfate with the highest anti-HIV activity was easily hydrolyzed by esterase−enzymatic hydrolysis to release free AZT. Furthermore, an acidically released AZT from curdlan sulfate exhibited its high anti-HIV activity. AZT−dodecanedicarboxylate−curdlan sulfate showed a low anticoagulant activity of 7 unit/mg.

Introduction

Although HIV (human immunodeficiency virus) patients may exhibit no symptoms for a long period, there is no corresponding viral latency. A continuous and high-level HIV replication has been observed in lymphoid organs throughout the course of HIV infection.[1,2] Meanwhile, the destruction of CD4 lymphocytes caused by HIV infection has been regarded to lead to an irreversible defect in the immune responses.[3] These findings imply that targeting of anti-HIV agents to lymphatic tissues is of much importance to inhibit HIV replication.

The therapeutic treatment by a protease inhibitor in combination with reverse transcriptase inhibitors has increased hope for HIV patients, because it can dramatically decrease the number of HIVs with a half-life of about 2 days in the blood.[4,5] However, it induced the emergence of a drug-resistant virus strain.[6,7] Thus, investigations on either a new anti-HIV agent possessing a different anti-retrovirus mechanism or a new drug combination have attracted extensive attention.[8−11] Curdlan sulfate, i.e., a sulfated polysaccharide consisting of β-1,3-D-glucan, has shown potent anti-HIV activity and low anticoagulant activity in vitro.[12−14] The phase I/II test of curdlan sulfate has demonstrated that intravenous administration of curdlan sulfate led to a dosage-related increase in the number of CD4 lymphocytes, which might indicate an effective treatment on AIDS patients.[15] No clinical side effect was observed at the dose level tested. It has been also found that curdlan sulfate is concentrated in organs such as lymph node and bone marrow.[16] Therefore, curdlan sulfate may be expected to act not only as an anti-HIV agent but also as a delivery carrier of AIDS drug into the lymphoid tissue, if a releasable drug is bound to the curdlan sulfate by a chemical bond.

In this study, AZT (azidothymidine)−spacer−curdlan sulfates that can release free AZT by enzymatic hydrolysis were synthesized. The relationship between enzymatic hydrolysis of AZT−spacer−curdlan sulfates and their structures was revealed. Anti-HIV and anticoagulant activities of AZT−spacer−curdlan sulfates were assayed in vitro.

Experimental Section

Materials and General Methods. AZT was purchased from Seikagaku Kogyo, Co. Commercial curdlan ($\bar{M}_n = 8.9 \times 10^4$, Wako Pure Chemical Ind., Ltd.), 4-(dimethylamino)-pyridine (DMAP), *N*,*N*-dicyclohexylcarbodiimide (DCC), sulfur trioxide-pyridine complex, succinic anhydride (Tokyo Kaisei Kogyo Co., Ltd.), glutaric anhydride, adipic acid, suberic acid, sebacic acid, 1,12-dodecanedicarboxylic acid (Nacalai Tesque Co., Ltd.), and dodecanedioic acid (DuPont) were used without further purification. Esterase (from porcine liver; minimum 150 units per mg protein (Biuret), Sigma) and lipase (from *Candida cylindracea*; 10.000 unit/mg, Seikagaku Kogyo, Co.) were purchased just before use. Dimethylsulfoxide (DMSO) and pyridine were distilled before use. Curdlan with a medium molecular weight ($\bar{M}_w = 3900$, $\bar{M}_w/\bar{M}_n = 1.58$) was prepared by hydrolysis of commercial curdlan ($\bar{M}_n = 8.9 \times 10^4$) in the presence of H_2SO_4 as catalyst, according to the method reported previously.[17] Molecular weight of the curdlan was obtained by measuring the molecular weight of peracetylated curdlan by means of GPC in chloroform solutions. AZT content in the AZT−spacer−curdlan sulfate was determined by a UV spectrometer by measuring maximum absorption at 268 nm. Mixtures of AZT and curdlan sulfate were used as references. Sample was measured in 0.1 mg/mL aqueous solutions at 25 °C. HMQC−FG $^1H-^{13}C$ 2D-NMR spectra were recorded with a JEOL LA500 NMR spectrometer. Samples were measured in DMSO-d_6 solution at 50 °C.

Synthesis of 5′-Acyl-AZT. Adipic acid (1.37 mmol) was added to a pyridine (5 mL) solution containing AZT (0.37 mmol), DMAP (0.82 mmol), and DCC (0.73 mmol) at room temperature. Then, the mixture was heated to 80 °C. The progress of the reaction was monitored by thin-layer chromatography (TLC) using mixtures of methylene chloride and ethyl acetate (1:2−5) as eluent solvents. After the reaction was completed, pyridine was evaporated under reduced pressure.

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The residue was purified by column chromatography over silica gel using mixtures of methylene chloride and ethyl acetate (1:2) and then methylene chloride and methanol (5:1) as eluents. A product of 3′-azido-3′-deoxy-5′-*O*-adipoyl-thymidine (5′-adipoyl-AZT) was obtained in 85% yield.

Other 5′-acyl-AZTs were synthesized by a few modifications of the above procedure.

Synthesis of AZT−Spacer−Curdlan. To a curdlan ($\bar{M}_w$ = 3900, 0.1 g) solution in DMSO (3 mL) were added 5′-adipoyl-AZT (0.09 g), DMAP (0.10 g), and DCC (0.20 g). The mixture was stirred overnight at room temperature. Then, cold ethanol was added until precipitates appeared. The precipitate was collected by centrifugation and washed with ethanol (30 mL) three times. White, powdery AZT−adipoyl−curdlan (0.08 g) was obtained by freeze-drying from water.

Other AZT−spacer−curdlans were prepared by the above method with a few modifications.

Synthesis of AZT−Spacer−Curdlan Sulfate. SO_3−pyridine complex (0.50 g) was added to an AZT−spacer−curdlan (0.10 g) solution in pyridine (10 mL). The mixture was stirred at 80 °C for 60 min. The pyridine was discarded after the reaction mixture was cooled to room temperature, and then the residue was neutralized by a saturated $NaHCO_3$ solution. AZT−spacer−curdlan sulfate was purified by dialysis against deionized water for 2 days.

Hydrolysis. Esterase hydrolysis of AZT−spacer−curdlan sulfates was carried out in a H_3BO_3−KCl−NaOH (pH = 8) buffer solution at 37 °C. Hydrolysis was initiated by adding esterase into a preheated AZT−spacer−curdlan sulfate (1.25 mg/mL) buffer solution at 37 °C. At various time intervals, an aliquot of the reacting mixture (0.5 mL) was taken out and added immediately into methanol (0.5 mL). A free AZT concentration was analyzed by HPLC (column, TSK gel ODS-80Ts QA; eluent, 7/3 H_2O/CH_3OH; detector, RI810). Lipase hydrolysis of AZT−spacer−curdlan sulfate was evaluated in pH = 6.8 phosphate buffer solution at 37 °C. The chemical hydrolysis was carried out in pH = 6.0−7.5 aqueous solution.

Dependence of AZT-releasing rate on esterase concentration and hydrolysis time was pretested. When the esterase concentration was more than 100 unit/mL and the hydrolysis time was limited to less than 6 h, the AZT-releasing rate obeyed a pseudo-first-order process. Therefore, the hydrolysis rate constants were obtained from the slope of semilogarithmic plots of the decay of AZT content in AZT−spacer−curdlan sulfate against hydrolysis time in the presence of an initial esterase concentration of 200 unit/mL, which was a saturated esterase concentration with a hydrolysis time of less than 6 h.[18]

Anti-HIV Activity Assay. The anti-HIV activity of AZT−spacer−curdlan sulfate was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) method.[19] An MT-4 cell (a human T4-positive cell line carrying human T-lymphotropic virus type 1) was infected with HIV-$1_{HTLV-IIIB}$ at the multiplicity of 0.01, and HIV-1- and mock-infected MT-4 cells were incubated in the presence of various concentrations of the test material for 5 days at 37 °C in a CO_2 incubator. The viability of both HIV-1- and mock-infected cells was assayed spectrophotometrically via the reduction of MTT. The anti-HIV activity is represented as EC_{50}, which denotes the concentration of the test material inhibiting the 50% infection of MT-4 cells from HIV-1. The cytotoxicity CC_{50} was determined by the 50% cytotoxic concentration of the test material on the MT-4 cell.

Anticoagulant Activity. Anticoagulant activity was evaluated by a modified United States Pharmacopoeia method using bovine plasma.[20] Dextran sulfate with an anticoagulant activity of 21.0 unit/mg was used as a reference sample.

Results and Discussion

Synthesis. To clarify the relationship between AZT-releasing rate in the presence of enzymes and structure of AZT−spacer−curdlan sulfate, AZT−spacer−curdlan sulfates having dioxyalkylene spacers between AZT and curdlan sulfate were prepared by a synthetic route as illustrated in Scheme 1. (Detailed data on preparation of AZT−curdlan sulfate is available by contacting the corresponding author.)

AZT was reacted with succinic anhydride in the presence of DMAP as catalyst in pyridine at room temperature for 18 h to give 5′-succinyl-AZT in 78% yield. Other 5′-acyl-AZTs were prepared by using DMAP in combination with DCC as catalysts. Reactions of AZT with adipic acid or suberic acid were carried at 80 °C, because adipic acid and suberic acid are insoluble in pyridine at room temperature. However, in the case of reactions with sebacic, dodecanedioic, and 1,12-dodecanedicarboxylic acids, the reaction was carried out at room temperature, because a large amount of byproduct was produced at 80 °C.

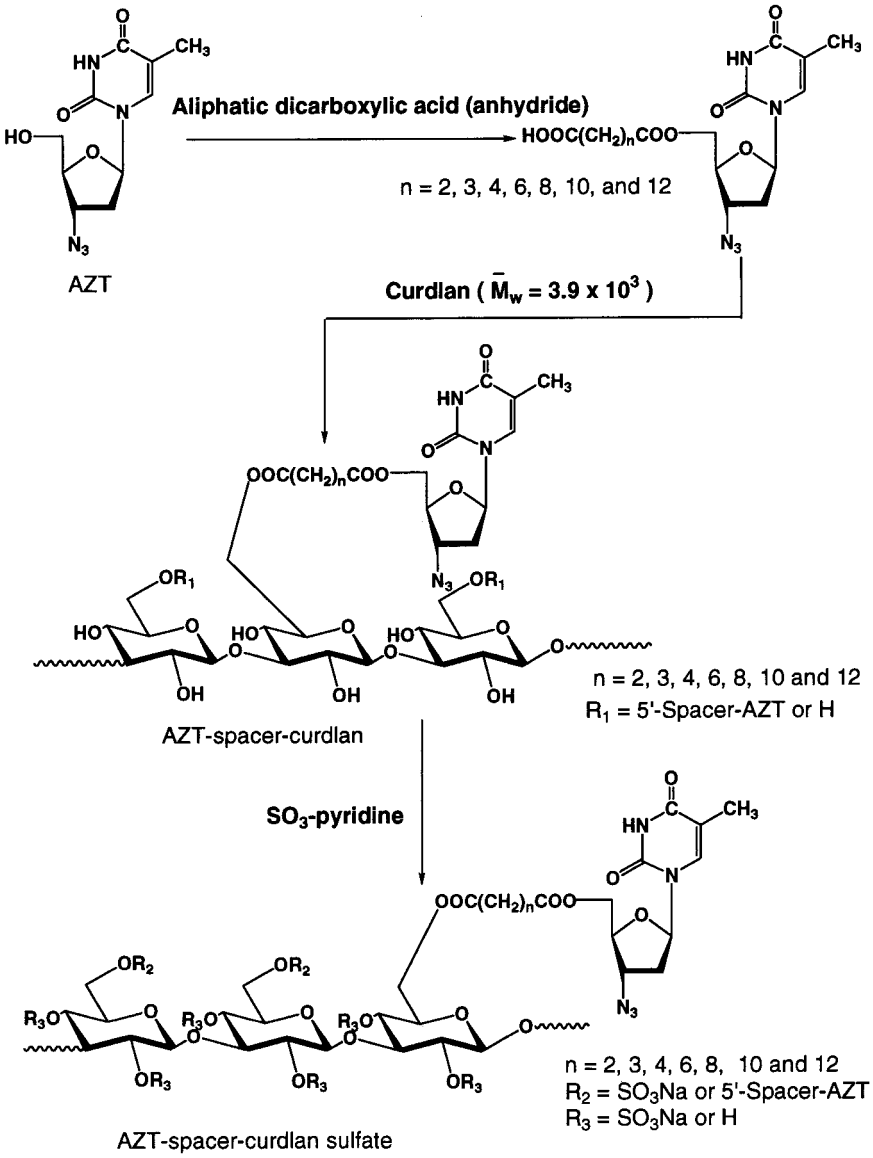
AZT was introduced into curdlan by esterification of 5′-acyl-AZT with curdlan in the presence of DMAP and DCC as catalysts in DMSO at room temperature. AZT content in AZT−spacer−curdlan sulfate was measured by UV spectroscopy after the following sulfation. It was in the range of 4.9−12.1%. The degree of AZT substitution based on a glucose unit was calculated by UV spectroscopy and elemental analysis. It was in the range of 0.09−0.17. The structure of AZT−spacer−curdlans was confirmed by HMQC−FG $^1H-^{13}C$ 2D-NMR spectroscopy, as showed in Figure 1. As reported previously, the esterification occurred predominately at the C6 position of curdlan.[17]

The AZT−spacer−curdlans were sulfated with SO_3−pyridine complex in pyridine at 80 °C to give AZT−spacer−curdlan sulfates. Sulfur content in the AZT−spacer−curdlan sulfate was determined by elemental analysis to be in the range of 10.1−17.7%. The degree of sulfation based on a glucose unit, which was estimated by elemental analysis in combination with UV spectroscopy, was 1.2 to 2.8.

Hydrolysis. To release AZT from curdlan sulfate by enzymatic hydrolysis, AZT−succinyl−curdlan sulfate was prepared in our previous work.[17] In this work, enzymatic hydrolysis of the AZT−succinyl−curdlan sulfate was first investigated. The results are shown in Figure 2. Low-molecular-weight 5′-succinyl-AZT was hydrolyzed with a half-life of 5.0 ± 0.1 h by esterase catalysis in a pH = 8 buffer solution at 37 °C. However, when 5′-succinyl-AZT was combined to curdlan sulfate to form a macromolecular AZT−succinyl−curdlan sulfate, the esterase did not release AZT from AZT−succinyl−curdlan sulfate even at high esterase concentrations over 24 h. It was suggested that steric hindrance originating from high-molecular-weight curdlan sulfate prevented the AZT moiety from reacting with the active center of esterase.

To overcome the hindrance between AZT and curdlan sulfate, various long alkylene groups were introduced between AZT and curdlan sulfate. Esterase hydrolysis constants of AZT−spacer−curdlan sulfates were evaluated in the presence of a saturated esterase concentration of 200 unit/mL at 37 °C for a hydrolysis time of 6 h. Results are shown in Figure 3. When the carbon number of alkylene group between AZT and curdlan sulfate was 2 (n = 2), no hydrolysis was observed. In the case of n = 4 and 6, less than 10% AZT was released within 6 h. Therefore, it was difficult to accurately calculate the hydrolysis rate constant. As n was increased to 8, 10, and 12, rate constants of AZT release by esterase hydrolysis increased with increasing carbon number of the alkylene group.

Scheme 1. Synthesis of AZT−Spacer−Curdlan Sulfates



Lipase hydrolysis of AZT−spacer−curdlan sulfates was investigated in a pH = 6.8 buffer solution at 37 °C. When the carbon number of alkylene group was 2, 4, and 6, no hydrolysis was observed even at a lipase concentration of more than 200 unit/mL over 24 h. In the case of *n* greater than 8, AZT−spacer−curdlan sulfates were hydrolyzed through lipase catalysis, and percentages of AZT released from AZT−spacer−curdlan sulfate increased with increasing carbon number of the alkylene group between AZT and curdlan sulfate. However, the AZT-releasing percentage by lipase hydrolysis was much lower than that by esterase at the same initial enzyme concentration of 80 unit/mL, as shown in Figure 4. Therefore, the esterase was more active than the lipase for releasing AZT from AZT−spacer−curdlan sulfate.

No free AZT was detected by HPLC after AZT−spacer−curdlan sulfates were incubated in a pH = 6.8−8 buffer solution at 37 °C for 24 h, indicating that the ester bonds between AZT and curdlan sulfate were stable in a moderate pH aqueous solution.

Taking into account that AZT−spacer−curdlan sulfates possess excellent stability in a neutral pH range and that they are hydrolyzed specifically through esterase catalysis to release free AZT, AZT−spacer−curdlan sulfates may be expected to act as a desirable AIDS drug delivery system and to sustain an AZT therapeutic concentration in living organs. Moreover, AZT release rate might be controlled by using different spacer lengths.

Anti-HIV and Anticoagulant Activities. Anti-HIV activity of AZT−spacer−curdlan sulfates was assayed by the MTT method in vitro using the MT-4 cell line and $HIV_{HTLV-IIIB}$ strain to give the EC_{50} value and the CC_{50} value. Because AZT was not released in a moderated pH solution in vitro, the anti-HIV activity of AZT−spacer−curdlan sulfate given by the MTT method must have presented the activity originating from curdlan sulfate moiety only.[17] Results of the anti-HIV activity are summarized in Table 1. It was found that anti-HIV activity of AZT−spacer−curdlan sulfate depended upon the carbon number of alkylene group between AZT and curdlan sulfate. When the carbon number of alkylene group did not exceed 10, AZT−spacer−curdlan sulfates exhibited high anti-HIV activities in the EC_{50} range of 0.10−0.21 μg/mL and low cytotoxicities of CC_{50} greater than 1000 μg/mL, which corresponded to the anti-HIV activity of highly active curdlan sulfate (EC_{50} = 0.17

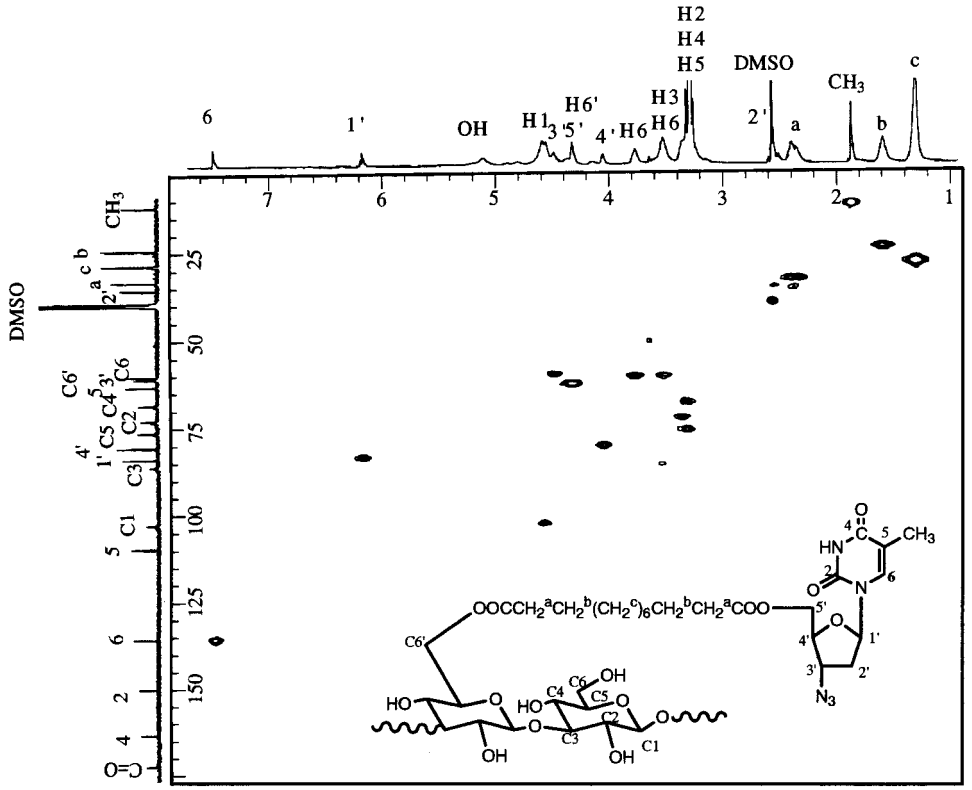


Figure 1. FMQC−FG $^{1}H-^{13}C$ 2D-NMR spectra of AZT−dodecanedioyl−curdlan with d_6-DMSO as solvent at 50 °C.

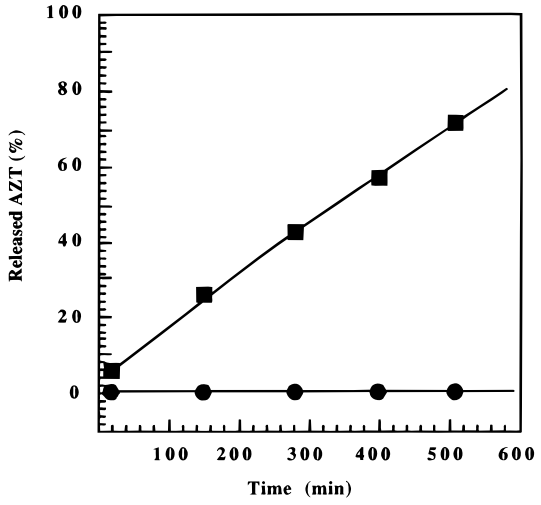


Figure 2. Esterase hydrolysis of 5′-succinyl-AZT (■ initial concentration, 0.69 mg/mL; esterase, 10 unit/mL) and AZT−succinyl−curdlan sulfate (● initial concentration, 2.86 mg/mL; AZT content in AZT−spacer−curdlan sulfate, 24%; esterase, 10 or 250 unit/mL) in a pH = 8 buffer solution at 37 °C.

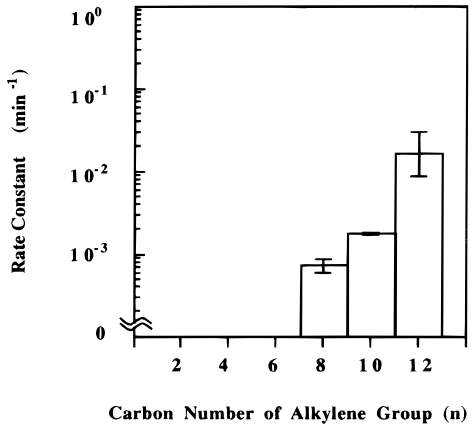


Figure 3. Relationship between the hydrolysis rate constant of AZT−spacer−curdlan sulfates by esterase catalysis and length of alkylene group between AZT and curdlan sulfate in a pH = 8 buffer solution at 37 °C.

μg/mL, CC_{50} > 1000 μg/mL). While the carbon number of alkylene group increased to 12, AZT−dodecanedicarboxylate−curdlan sulfate exhibited a much higher anti-HIV activity of EC_{50} up to 0.04 μg/mL and a low cytotoxicity of CC_{50} greater than 1000 μg/mL. It was assumed that a long hydrophobic alkylene branch introduced into a sulfated polysaccharide backbone played an important role in enhancing an anti-HIV activity. It was consistent with a high anti-HIV activity of sulfated alkyl oligosaccharides.[21,22]

To examine anti-HIV activity of the released AZT from curdlan sulfate, AZT−spacer−curdlan sulfate was incubated in an aqueous solution with a slightly acidic pH of 5.8−6.3 at 37 °C for 2 weeks to give a mixture of the released AZT and AZT−spacer−curdlan sulfate. For AZT−spacer−curdlan sulfates with n = 8 and 12, the weight ratio of the released AZT to the AZT−spacer−curdlan sulfate was determined by HPLC to be 1.2% and 1.8%, respectively. The anti-HIV activity was shown in Table 2. Mixtures of released AZT and AZT−spacer−curdlan sulfate exhibited much higher anti-HIV activities of EC_{50} showing 0.004 and 0.009 μg/mL than the initial activity of AZT−spacer−curdlan sulfate, indicating that the high activity was contributed mainly to the released AZT. In addition, for the mixtures of released AZT and AZT−spacer−curdlan sulfate, a much higher selective index (SI) than that for AZT was observed. This remains to be investigated further.

Anticoagulant activity possessed by various sulfated polysaccharides is regarded as a serious side effect for an AIDS drug. The effectiveness of many sulfated

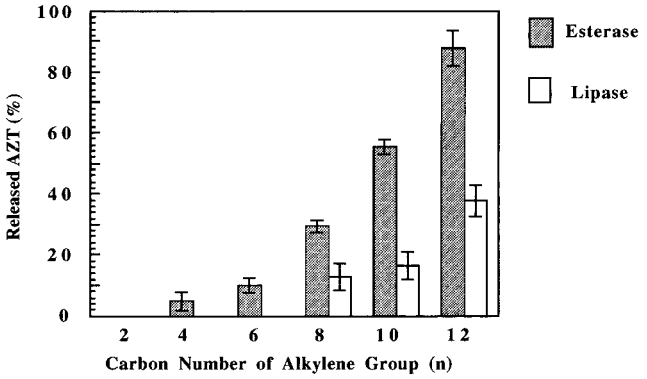


Figure 4. Percentage of released AZT from curdlan sulfates by esterase and lipase catalysis at 37 °C for 24 h (initial enzyme concentration, 80 unit/mL).

Table 1. Anti-HIV and Anticoagulant Activities of AZT−Spacer−Curdlan Sulfates

AZT (content)			S^d	$EC_{50}{}^e$	$CC_{50}{}^f$	SI	AA[g]
wt (%)[a]	$DS_{AZT}{}^b$	n^c	(%)	m/mL	μg/mL	CC_{50}/EC_{50}	unit/mg
4.9	0.09	2	17.0	0.11	>1000	>9 090	19
3.8	[h]	4	17.7	0.17	>1000	>5 880	[h]
12.1	0.15	4	10.1	[h]	[h]	[h]	11
3.3	[h]	6	17.2	0.21	>1000	>2 860	[h]
9.2	0.13	8	12.0	0.11	>1000	>9 090	12
10.6	0.17	10	11.1	0.15	>1000	>6 660	15
6.1	0.10	10	15.0	0.12	>1000	>8 330	[h]
7.5	0.11	12	12.5	0.04	>1000	>25 000	7
CS[i]				0.17	>1000	>5 880	
AZT (mM)				0.0002	6	30 000	

[a] AZT weight content in AZT−spacer−curdlan sulfate was determined by UV spectroscopy. [b] The degree of AZT substitution to a glucose unit of curdlan. [c] Carbon number of alkylene group between AZT and curdlan sulfate. [d] Determined by elemental analysis. [e] Anti-HIV activity: the drug concentration effective for 50% inhibition of virus infection in 5-day HIV-infected MT-4 cell culture. EC_{50} values represented 2 times the average data within error of 5%. [f] Cytotoxic effect: the drug concentration for 50% cytotoxicity in 5-day MT-4 cell culture. [g] Anticoagulant activity: the commercial dextran sulfate having an anticoagulant activity of 21.0 unit/mg as reference. AA values represented 3 times the average data within error of 10%. [h] Not measured. [i] Curdlan sulfate having weight-average molecular weight of 7.9×10^4 as reference.

Table 2. Anti-HIV Activity of Mixtures of Released AZT and AZT−Spacer−Curdlan Sulfate

AZT−curdlan sulfate[a]		released			SI
AZT (content) (%)[b]	n^c	AZT[d] (%)	$EC_{50}{}^e$ (μg/mL)	CC_{50} (μg/mL)	CC_{50}/EC_{50}
5.5	8	1.8	0.004	603	150 750
5.8	12	1.2	0.009	475	52 780
curdlan sulfate			0.17	>1000	>5 880
AZT (mM)			0.0002	6	30 000

[a] Incubated in aqueous solution at 37 °C for 2 weeks to give mixtures of free AZT and AZT−curdlan sulfate. [b] AZT content in AZT−spacer−curdlan sulfate complex was determined by UV spectroscopy. [c] Carbon number of the alkylene group between AZT and curdlan sulfate. [d] The weight ratio of released AZT to AZT−spacer−curdlan sulfate complex in the mixture of released AZT and AZT−spacer−curdlan sulfate was detected by HPLC. [e] Twice the mean values with error less than 5%.

polysaccharides as AIDS drugs in vivo has not been clarified.[23] Therefore, in this study, the anticoagulant activity of AZT−spacer−curdlan sulfate was estimated by a modified United States Pharmacopoeia method.[20] Results are shown in Table 1. When the carbon number of alkylene group between AZT and curdlan sulfate increased to 12, AZT−dodecanedicarboxylate−curdlan sulfate exhibited a lower anticoagulant activity of 7 unit/mg. Anti-HIV and anticoagulant activities of AZT−spacer−curdlan sulfate revealed that an alkylene group consisting of 12 carbons enhanced the anti-HIV activity and decreased the anticoagulant activity. It is assumed that the long alkylene group inserted between AZT and curdlan sulfate significantly improved the hydrophobicity of curdlan sulfate.

Conclusions

To release AZT in living organs through enzymatic hydrolysis, a long hydrophobic alkylene group was inserted between AZT and curdlan sulfate. Fortunately, the hydrophobic long alkylene group led to an increase in anti-HIV activity and a decrease in anticoagulant activity. Therefore, the AZT−spacer−curdlan sulfates may not only be expected to act as a desirable AIDS-drug delivery system, but also it may become a highly active anti-HIV agent.

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