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Sulfated oligosaccharide cluster with polylysine core scaffold as a new anti-HIV dendrimer

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ABSTRACT

Polylysine-dendritic sulfated cellobiose **4** was synthesized by sulfation of polylysine-dendritic cellobiose, which was prepared from cellobiose and polylysine dendrimer generation 3. The degree of sulfation was 1.85 per glucose unit (maximum 3). The molecular weights measured by GPC were $\overline{M}_w = 12.4 \times 10^3$ and $\overline{M}_n = 10.4 \times 10^3$, suggesting that the sulfated **4** had a very compact structure. The anti-HIV activity of polylysine-dendritic sulfated cellobiose **4** was assayed in vitro by the MTT method, which indicated that the EC_{50} = 3.2 μg/ml for the 50% inhibitory concentration of virus replication, a concentration that is as high as that of the currently clinical used AIDS drug, ddC (EC_{50} = 3.51 μg/ml), and low cytotoxicity (CC_{50} = 1000 μg/ml). The blood anticoagulant activity determined by the method of the United States Pharmacopoeia was AA = 19.4 unit/mg, as the same as that of the sulfated polysaccharides, dextran sulfate (AA = 22.9 unit/mg) and curdlan sulfate (AA = 19 unit/mg). These results suggest that the biological activities were improved by a cluster effect of the sulfated cellobiose that originated from the dendritic structure because sulfated oligosaccharides have little anti-HIV activity. The structure of polylysine-dendritic sulfated cellobiose **4** was analyzed by NMR and FT-IR measurements.

1. Introduction

Since the antiviral activity of naturally occurring sulfated polysaccharides was first reported by Gerber in 1958 (Gerber, Dugene, Adams, & Sherman, 1958), many sulfated polysaccharides have been found to inhibit viral replication (Yoshida, 2001). In 1987, that a sulfated polysaccharide from sea agar, carrageenan, was found to effectively inhibit AIDS virus replication (anti-HIV activity) (Nakashima et al., 1987a, 1987b). The anti-HIV activity of sulfated polysaccharides was dependent on both the degree of sulfation and molecular weight (Yoshida et al., 1990). Sulfated polysaccharides with low molecular weights had low cytotoxicity but also low anti-HIV activity (Choi et al., 1996). Curdlan sulfates with molecular weights of more than $\bar{M}_n = 20 \times 10^3$ which were prepared by sulfation of a naturally occurring polysaccharide, curdlan, and consist of linear 1,3-β-linked glucose units, completely inhibited the infection HIV in MT-4 cells at a concentration as low as 3.3 μg/ml and inhibited intermediate blood anticoagulant activities (AA = 10–20 units/mg) (Yoshida et al., 1990). Although blood anticoagulant activity is another important biological activity of sulfated polysaccharides, higher activity was a side effect of the clinical use to treat AIDS for clinical use. Therefore, curdlan sulfate was a candidate for an AIDS drug (Gordon et al., 1994) because it has moderate blood anticoagulant activity. The anti-HIV activity of sulfated polysaccharides originated from the electrostatic interaction between negatively charged sulfate groups of sulfated polysaccharides and the positively charged envelope glycoprotein gp120 on the HIV surface (Uryu et al., 1992). This mechanism was elucidated in vivo by using recombinant gp120 (Jagodzinski et al., 1996) and in vitro by using NMR spectroscopy (Jeon et al., 2000).

Taking into account of the interaction mechanisms, ionic interactions play an important role in the enhancement of biological activities. Several papers on the development of carbohydrate clusters using dendrimers published (Newkome, Moorefield, & Vogtle, 2001). Dendritic compounds of polylysine or carbosilane core scaffolds with sialic acid on their surface were potent inhibitors of influenza viruses, depending on the cluster effects (Roy et al., 1993). An acyclic oligopeptide from HIV connects to cellobiose on the surface of a polylysine dendrimer (Baigude et al., 2005). This type of dendrimer may become an AIDS vaccine. Negatively charged sulfated oligosaccharides on the surface of the dendrimer were expected to have high biological activities due to both ionic interaction and the cluster effect on the positively charged surface proteins of HIV. We recently reported the synthesis of a new dendritic oligosaccharide

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consisting of a polylysine core scaffolding and cellobiose as a model oligosaccharide (Han et al., 2007a, b). In this paper, we describe the synthesis of a polylysine dendrimer with sulfated cellobiose and evaluate the anti-HIV and blood anticoagulant activities. The relationship between the structure and biological activities is described. The structure of polylysine-dendritic sulfated cellobiose **4** was determined by NMR and IR measurements.

2. Experimental

2.1. Synthesis of polylysine-dendritic sulfated cellobiose *4*

Polylysine-dendritic cellobiose **3** (0.25 g, 0.016 mmol), which was prepared by the method described in a recent paper (Han et al., 2007a, b) was dissolved in DMSO (20 ml) and then SO_3-pyridine (SO_3–Py) complex (0.94 g, 5.99 mmol) was added at room temperature. The mixture was stirred at 38 °C. After 2.5 h, the mixture was cooled to room temperature and then neutralized with 15% NaOH solution. The aqueous solution was dialyzed in a dialysis tube (3500 molecular weight cut-off) against deionized water for 1 day, and the dialyzate was freeze-dried to give 0.32 g of **4** with a molecular weight $\bar{M}_n = 10.4 \times 10^3$. The specific rotation $[\alpha]_D^{25} = -0.18^\circ$ (c1, H_2O). Found for C was 28.40, H; 4.91, S; 12.83%.

2.2. Anti-HIV assay

The anti-HIV activity of polylysine-dendritic sulfated cellobiose **4** was assayed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) method (Pauwels et al., 1988; Uryu et al., 1992). Cells for MT-4, which is a human T4-positive cell line carrying human T-lymphotropic virus type 1 (3.0×10^4/well, MOI: 0.01), were infected with HIV-$1_{HTLV\text{-}IIIB}$ 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 compound for 5 days at 37 °C in a CO_2 incubator. The viability of both HIV-1- and mock-infected cells was assayed spectrophotometrically by the reduction of MTT. The anti-HIV activity was represented by the EC_{50}, which is defined as the concentration of the test compound that decreased the HIV-induced cytopathic effect by 50%. The cytotoxicity CC_{50} was determined as the concentration of the test compound that was cytotoxic on 50% of MT-4 cells.

2.3. Blood anticoagulant assay (US Pharmacopoeia, 1985)

The blood anticoagulant activity of **4** was assayed by use of whole bovine plasma according to a modification of the United States Pharmacopoeia. The activity was calculated by comparison with that of standard dextran sulfate (Meito Sangyo Co., Ltd.) as AA = 22.7 unit/mg.

2.4. Measurements

^1H and ^{13}C NMR spectra were recorded on a JEOL JNM ECX-400 spectrometer at 400 MHz and 100 MHz at 40 °C in D_2O or DMSO-d_6 solvents, respectively. Chemical shifts are expressed as ppm downfield from 4,4′-dimethyl-4-silapentane-1-sulfonate (DSS) as an internal standard. Molecular weights of polymers were determined at 40 °C by an organic phase GPC (column; Tosoh TSK-gel G3000H_{XL}, G4000H_{XL}, and G5000H_{XL}, 7.6 mm × 600 mm × 3 eluted with $CHCl_3$) using polystyrene (Shodex standard SM-105) standards or an aqueous phase GPC (column; Tosoh TSK-gel G2500PW_{XL}, G3000PW_{XL}, and G4000PW_{XL}, 7.6 mm × 300 mm × 3 eluted with 66.7 mmol of phosphate buffer, pH = 6.68) with a Tosoh RI detector using pullulan standards. Infrared spectra were taken on a Shimadzu FT-IR 8300 spectrometer by a KBr pellet method. The MALDI-TOF-MS spectrum was measured by a Bruker Ultraflex III instrument with 337 nm nitrogen laser. A methanol solution of a mixture of 2,5-dihydroxybenzoic acid and 5-methoxysalicylic acid was used as a matrix.

3. Results and discussion

3.1. Synthesis of polylysine-dendritic sulfated cellobiose *4*

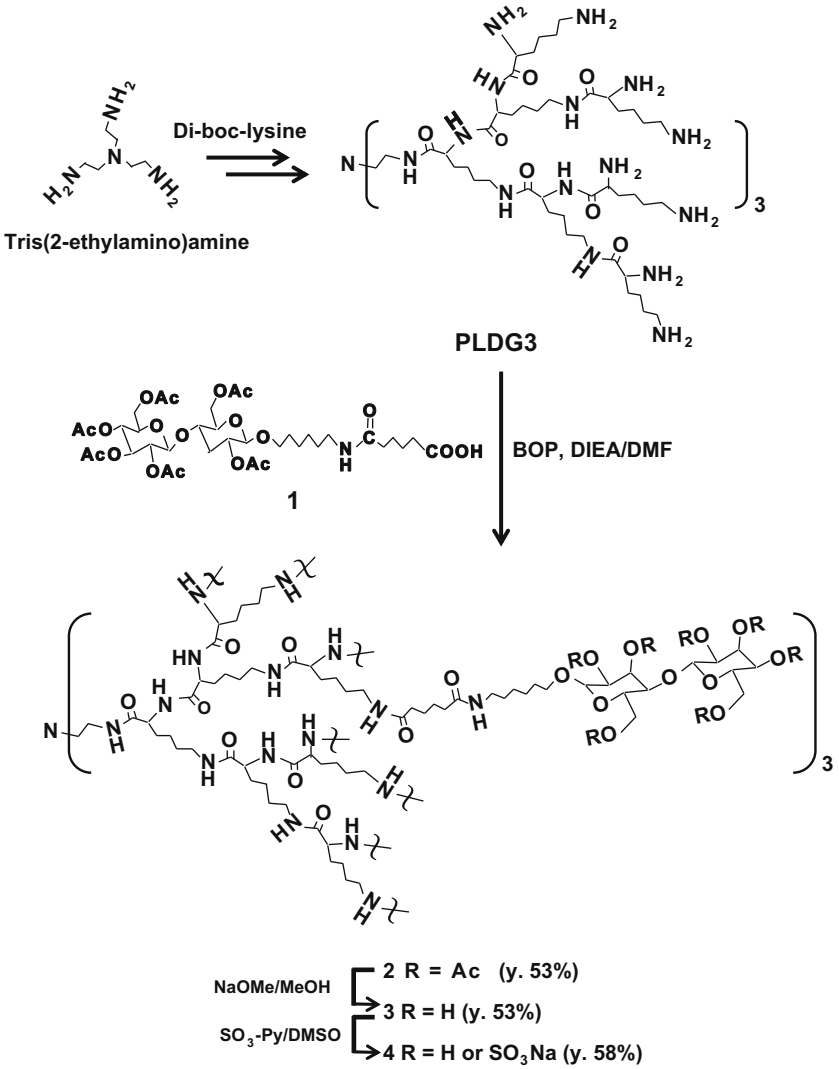
Scheme 1 shows the synthesis of polylysine-dendritic sulfated cellobiose **4**. We recently reported the synthesis of polylysine-dendritic cellobiose **3** from polylysine dendrimer generation 3 (**PLDG3**) with free amino groups (Han et al., 2007a, b), which was obtained from tris(2-ethylamino)amine as a trivalent core and subsequent repeated condensation and deprotection by di-boc-lysine. The carboxyl cellobiose portion **1** was prepared by the reaction of 1-bromoacetyl cellobiose and 1-aminobenzyloxycarbonyl-6-hexanol (Z-protected aminohexanol) and then condensation of adipic acid after deprotection of the Z-group. Finally, **PLDG3** was condensed with **1** to give polylysine-dendritic cellobiose **3** after deacetylation of **2**. Polylysine-dendritic sulfated cellobiose **4** was synthesized by the sulfation of **3** with SO_3-Py reagent in dry DMSO with 58% yield after purification. The degree of sulfation of a sugar unit was calculated to be 1.85 based on the result of the elementary analysis. Although the maximum degree of a sugar unit is 3, the complete sulfation of hydroxyl groups in poly- and oligosaccharides was difficult due to the steric hindrance.

3.2. Structure of polylysine-dendritic sulfated cellobiose *4*

As we reported recently, organic phase GPC and MALDI TOF MS measurements revealed polylysine-dendritic acetylated cellobiose **2** to have a very compact and homogeneous structure, in which the main m/z signal in the MALDI TOF MS was m/z = 19056.1 (calcd. M_w = 23137.9), suggesting that the acetylated **2** lacked several (5) units of **1**. The complete substitution of **1** with amino groups on **PLDG3** was difficult in the crowded structure of the core polylysine. The GPC measurements of **2** eluted with $CHCl_3$ as a solvent gave the molecular weights of $\bar{M}_w = 4.3 \times 10^3$ and $\bar{M}_n = 4.0 \times 10^3$, indicating the very narrow molecular weight distribution of $\frac{\bar{M}_w}{\bar{M}_n} = 1.08$. These results reveal that this dendrimer had a compact and homogeneous structure because the molecular weights measured by GPC indicated fairly low values. The real molecular weight of **2** was M_w = 23137.9. The long alkyl spacer between the dendrimer and sulfated cellobiose units gave the sulfated cellobiose moieties flexibility. Therefore, the long alkyl spacer plays an important role in the increase of the cluster effect of sulfated cellobiose on the surface of the dendrimer.

The FT-IR spectra of (A) polylysine-dendritic cellobiose **3** with free hydroxyl groups and (B) polylysine-dendritic sulfated cellobiose **4** are shown in Fig. 1. After sulfation, as shown in Fig. 1B, the strong band due to the hydroxyl stretching vibration around 3400 cm^{-1} decreased and a large $-SO_3$ stretching band appeared at 1230 cm^{-1}, suggesting that the hydroxyl groups in the cellobiose unit were replaced by sulfate groups.

Fig. 2 exhibits the ^{13}C NMR spectra of (A) polylysine-dendritic cellobiose **3** and (B) polylysine-dendritic sulfated cellobiose **4** in D_2O. In spectrum 1A, before sulfation, the absorptions due to the cellobiose unit appeared as several singlet peaks between 65 and 105 ppm. The signals of C1 and C1′ and C6 and C6′ in the cellobiose unit appeared as two sharp peaks around 107 and 65 ppm, respectively. The alkyl spacer and amide carbonyl carbons were absorbed as complex signals between 25 and 45 ppm and between 165 and 185 ppm, respectively. The absorption at 56 ppm was assigned to the methylene signal next to cellobiose unit marked "α". After



Scheme 1. Synthesis of polylysine-dendritic sulfated cellobiose.

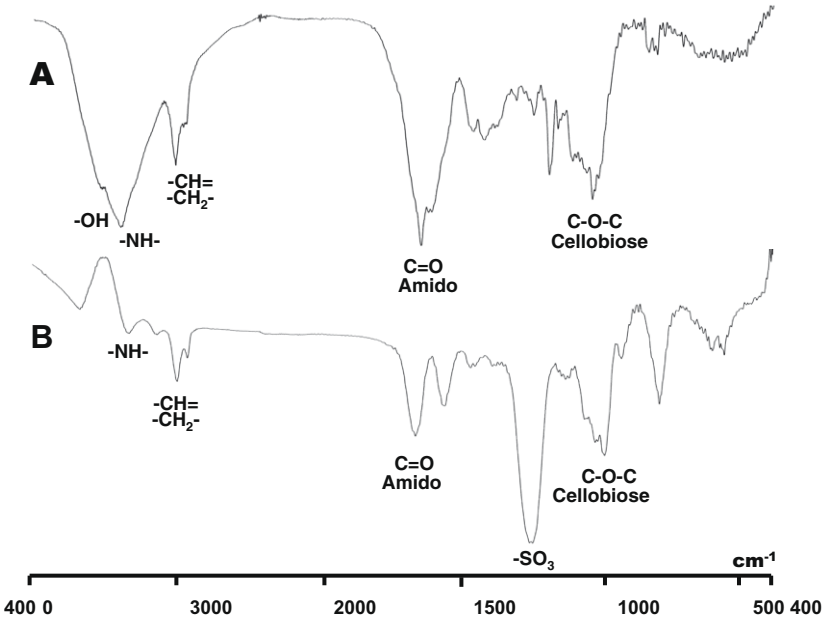


Fig. 1. FT-IR spectra of polylysine-dendritic cellobiose (A) before and (B) after sulfation.

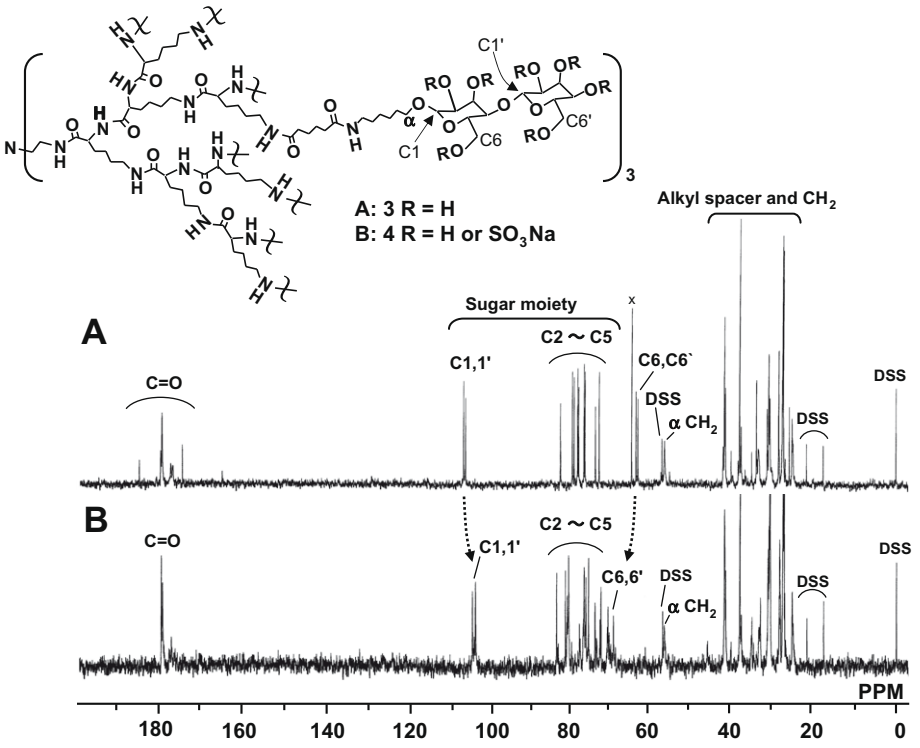


Fig. 2. 100 MHz ^{13}C NMR spectra of polylysine-dendritic cellobiose (A) before and (B) after sulfation in D_2O at 40 °C. DSS was used as an internal standard.

sulfation, as shown in spectrum 2B, the absorptions due to C2–C5′ signals between 69 and 85 ppm became complex due to the effects of incomplete sulfation. Comparing the chemical shifts of the C6 and C6′ around 65 ppm in spectrum 2A, the signals were shifted to 69 ppm, suggesting that the sulfate group was completely introduced at the C6 and C6′ positions, which have relatively lower steric hindrance than that of the C2–C4′ positions because the hydroxyl groups at the C6 and C6′ positions are primary alcohols. The degree of sulfation of 1.85 indicated that 62% of the hydroxyl groups in the cellobiose unit were replaced by sulfate groups. Taking into account the degree of sulfation and NMR spectra, the sulfate groups completely occupied the C6 and C6′ positions and partially occupied the C2–C4′ positions in the cellobiose units.

The molecular weights of **4** measured by an aqueous phase GPC were $\overline{M}_w = 12.4 \times 10^3$, $\overline{M}_n = 10.4 \times 10^3$, and $\overline{M}_w/\overline{M}_n = 1.20$ indicating that polylysine-dendritic sulfated cellobiose **4** had a compact structure compared with those of linear sulfated polysaccharides (Yoshida, 2001). On the other hand, the molecular weight of **4** increased to $\overline{M}_n = 10.4 \times 10^3$ from the $\overline{M}_n = 4.0 \times 10^3$ of **2**, suggesting that the molecular weight expansion of **4** was probably due to a mutual repulsion between negatively charged sulfate groups in the cellobiose units on the surface of dendrimer.

4. Biological activities

We have reported the relationship between the structure of sulfated polysaccharides and biological activities such as blood anticoagulant and anti-HIV activities, which closely depend on the degree of sulfation and molecular weights of sulfated polysaccharides (Yoshida, 2001). Sulfated oligosaccharides with lower molecular weights exhibited lower biological activities (Choi et al., 1996). On the other hand, dendrimers with sulfated oligosaccharides on the surface are expected to have high biological activities due to their cluster effects, which increase the interaction of carbohydrates with proteins of the surface on viruses. Table 1 shows the biological activities of polylysine-dendritic sulfated cellobiose **4**, whose activities are compared with those of sulfated polysaccha-

Table 1
Biological activities of polylysine-dendritic sulfated cellobiose **4** (PDSC).

Sample[a]		$\overline{M}_n^d \times 10^{3b}$	$[\alpha]_D^{25d}$ deg	Elemental analysis				EC_{50} (μg/ml)[e]	CC_{50} (μg/ml)[f]	AA[g] (unit/mg)
				C %	H	S	DS			
1	PDSC	10.4[c]	−0.18	28.40	4.91	12.83	1.85	3.2	>1000	19.4
2	Dextran sulfate	8.5	+92.1			18.4		0.84	101	22.9
3	Curdlan sulfate	79.0	+3.0			14.1		0.13	697	19
4	ddC							3.51	2537	
5	AZT							0.019	218	

[a] Standard dextran (H-39) and curdlan sulfates were used. ddC: dideoxycytidine, AZT: azidothymidine.
[b] Determined by GPC.
[c] $\overline{M}_w = 12.5 \times 10^3$, $\overline{M}_w/\overline{M}_n = 1.20$.
[d] Measured in H2O (c1).
[e] Anti-HIV activity denoted by 50% inhibitory concentration of virus replication.
[f] 50% Cytotoxic concentration on MT-4 cell.
[g] Blood anticoagulant activity.

rides and AIDS drugs for clinical use. EC_{50} and CC_{50} are the 50% inhibitory and 50% cytotoxic concentrations of drugs against the replication of MT-4 cells. An EC_{50} concentration shows higher anti-HIV activity. A large CC_{50} value means low cytotoxicity. Dextran and curdlan sulfates had the potent anti-HIV activities of EC_{50} = 0.84 and 0.13 μg/ml, respectively. Two AIDS drugs for clinical use, ddC (dideoxycytidine) and AZT (azidothymidine), also have the potent anti-HIV activities of EC_{50} = 3.51 and 0.019 μg/ml, respectively. It was found that polylysine-dendritic sulfated cellobiose **4** had the potent anti-HIV activity of EC_{50} = 3.2 μg/ml, which was as high as that of ddC, and no inhibitory effects on cell growth in concentrations higher than CC_{50} = 1000 μg/ml, suggesting low cytotoxicity.

In addition, because sulfated polysaccharides were characterized as blood anticoagulant agents, the anticoagulant activity (AA) of **4** was evaluated by the method of the US Pharmacopoeia, and showed almost the same blood anticoagulant activity at the concentration of 19.4 unit/mg as that of dextran sulfate (AA = 20.9 unit/mg) and curdlan sulfate (AA = 19 unit/mg). These results indicate that the cluster effect of sulfated cellobiose connected to the polylysine dendritic core structure played an important role in the high biological activities, because sulfated oligosaccharides have low anti-HIV and blood anticoagulant activities (Choi et al., 1996). Therefore sulfated oligosaccharides with a dendritic structure are expected to have higher biological activities due to the cluster effect and lower cytotoxicity.

In conclusion, polylysine-dendritic sulfated cellobiose **4** was synthesized by condensation of polylysine dendrimer generation 3 (**PLDG3**) with acetylated cellobiose **1** and subsequent deacetylation and sulfation. The degree of sulfation of **4** was 1.85 (maximum 3) and the structure was determined by ^{13}C NMR and FT-IR measurements. It was found that the sulfated cellobiose dendrimer **4** had anti-HIV activity as high as that of the AIDS drug, ddC and showed intermediate blood anticoagulant activity, equal to that of sulfated polysaccharides such as curdlan and dextran sulfates. These biological activities of **4** might originate from the cluster effects based on the dendritic structure. Although cellobiose was used as a model oligosaccharide in this work, further studies on the relationship between biological activities and sulfated oligosaccharide clusters with dendritic core structures are also under investigation.

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