Isolation of an Antiviral Polysaccharide, Nostoflan, from a Terrestrial Cyanobacterium, *Nostoc flagelliforme*

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A novel acidic polysaccharide, nostoflan, was isolated from a terrestrial cyanobacterium, *Nostoc flagelliforme*. Nostoflan exhibited a potent anti-herpes simplex virus type 1 (HSV-1) activity with a selectivity index (50% cytotoxic concentration/50% inhibitory concentration against viral replication) of 13 000. Sugar composition and methylation analyses revealed that it was mainly composed of →4)-D-Glc*p*-(1→, →6,4)-D-Glc*p*-(1→, →4)-D-Gal*p*-(1→, →4)-D-Xyl*p*-(1→, D-GlcA*p*-(1→, D-Man*p*-(1→ with a ratio of ca. 1:1:1:1:0.8:0.2. Two pyridylaminated oligosaccharides were prepared by partial acid hydrolysis and pyridylamination. On the basis of MALDI-TOF-MS and NMR analyses, they were found to be *β*-D-Glc*p*-(1→4)-D-Xyl-PA and *β*-D-GlcA*p*-(1→6)-*β*-D-Glc*p*-(1→4)-D-Gal-PA. From these results, nostoflan might be mainly composed of the following two types of sugar sequence: →4)-*β*-D-Glc*p*-(1→4)-D-Xyl*p*-(1→ and →4)-[*β*-D-GlcA*p*-(1→6)-]-*β*-D-Glc*p*-(1→4)-D-Gal*p*-(1→. Besides anti-HSV-1 activity, nostoflan showed potent antiviral activities against HSV-2, human cytomegalovirus, and influenza A virus, but no activity against adenovirus and coxsackie virus was observed. Therefore, nostoflan has a broad antiviral spectrum against enveloped viruses whose cellular receptors are carbohydrates. Furthermore, nostoflan showed no antithrombin activity, unlike sulfated polysaccharides.

Cyanobacteria (blue-green algae) have been recently recognized as promising targets in the search for biologically active compounds.[1] Extant cyanobacteria dominate the microbial populations of many extreme environments, including soda lakes (*Spirulina*, *Cyanospira*), thermal springs (*Synechococcus*), and cold, dry polar deserts (*Chroococcidiopsis*). To grow in these extreme environments, the cyanobacteria produce various metabolites that are not present in higher plants. Furthermore, the cyanobacteria produce copious amounts of polysaccharides in the form of sheaths, slimes, and capsules. The function of these polysaccharides is thought to be mainly protective, and they may prevent the algae from dehydration. Calcium spirulan (Ca-SP) was isolated as an antiviral polysaccharide from *Spirulina platensis*, which grows in alkaline salty lakes.[2] Ca-SP is mainly composed of rhamnose, 3-*O*-methylrhamnose, uronic acids, and sulfates,[3] and it showed various biological activities including antithrombin via heparin cofactor II and endothelial cell proliferation inhibition.[4−7] Therefore, cyanobacteria could be candidates for supplying novel polysaccharides possessing various biological activities.

Nostoc, one of the most widespread genera of nitrogen-fixing filamentous cyanobacteria, is able to form macroscopic or microscopic colonies.[8] *N. flagelliforme*, one *Nostoc* species, grows in some arid and semiarid regions of China. The hot water extract from the alga has been reported to show antitumor activity, and this effect may be due to polysaccharides.[9] However, no information is available on the structure of polysaccharides from *N. flagelliforme*.

In the present study, we isolated and characterized a novel antiviral polysaccharide from *N. flagelliforme*. The antiviral spectrum of nostoflan was also investigated.

Results and Discussion

We attempted to isolate the antiviral component from *N. flagelliforme* by bioactivity-directed fractionation. The potency of antiviral activity was evaluated by the calculation of the selectivity index (SI), which was calculated as cytotoxicity (CC_{50})/antiviral activity (IC_{50}). In general, a substance was regarded as having antiviral activity when its SI was higher than 10, and that of acyclovir, a commercially available anti-HSV drug, was approximately 1000 in our assay system. When the hot water extract from *N. flagelliforme* was treated with 4 vol. of EtOH, potent anti-HSV-1 activity (SI = 310) was found in an insoluble fraction (NP). Further fractionation was performed by applying NP to ion-exchange column chromatography on DEAE Toyopearl 650M. Since the carbohydrate positive fraction (NPHM) showed an anti-HSV-1 effect (SI = 380), it was subjected to gel filtration on Sepharose 6B to obtain two fractions. The latter fraction showed anti-HSV-1 activity (SI = 6900) and was further purified by gel filtration on Sepharose 6B followed by Toyopearl HW 55F. A fraction (NPHM2b-2B) was finally obtained that showed extremely potent anti-HSV-1 activity (SI = 13 000) and was named nostoflan.

Nostoflan was eluted as a single peak, and its apparent molecular weight was estimated to be 2.11×10^5 by HPLC on TSK gel $GMPW_{XL}$ (Figure 1A). In addition, the electrophoretic pattern indicated that the electric charge of nostoflan was homogeneous (Figure 1B). These results revealed that nostoflan might be a homogeneous polysaccharide on the basis of molecular weight and charge distribution. Nostoflan had $[\alpha]_D$ +2.8° (H_2O, *c* 1.0).

A Bradford assay of nostoflan revealed that the polysaccharide does not contain a protein portion. Nostoflan was shown to contain 13.3% of uronic acids by colorimetric assay of uronic acids. On the other hand, no sulfate esters were present in nostoflan, since the S=O stretching absorption band was not observed at 1250 cm^{-1} in the IR

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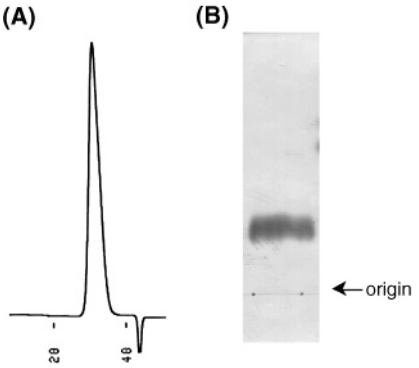

Figure 1. HPLC chromatogram (A) and cellulose acetate membrane electrophoretic pattern (B) of nostoflan.

spectrum and sulfur was not detected by X-ray microanalysis (data not shown).

The neutral sugars in nostoflan were glucose (42.8%), galactose (20.7%), xylose (29.9%), and mannose (6.6%). The only uronic acid residue identified was glucuronic acid. All these sugars were D-enantiomers since retention times of their derivatives on GC were identical to those of authentic sugars. When nostoflan and its carboxyl-reduced polysaccharide were submitted to methylation, varieties of methylated derivatives were obtained as shown in Table 1. From these data, nostoflan was considered as being mainly composed of 1,4-linked glucose, 1,4-linked galactose, 1,4-linked xylose, and 1,4,6-linked glucose residues. It was suggested that mannose residue was found to be present at the nonreducing end. In addition, an increase in the ratio of terminal glucose residue in C-6 reduced nostoflan suggested that the glucuronic acid residues were also at nonreducing ends.

Nostoflan was partially hydrolyzed with 0.2 M TFA at 90 °C for 2 h to obtain oligosaccharides for further analysis. The partial hydrolyzates were separated by Dowex 1 × 8 resin (acetate form) into neutral and acidic portions. Each fraction was pyridylaminated before applying to preparative RP-HPLC to obtain pyridylaminated oligosaccharides. As a result, two oligosaccharides, termed PA-1 and -2, were obtained from the neutral and acidic fractions, respectively. MALDI-TOF-MS of PA-1 gave a $(M + H)^+$ ion at 391.18. Therefore, PA-1 was suggested to be a pyridylaminated biose composed of hexose and pyridylaminated (PA) pentose [$(M + H)^+_{calc} = 391.39$]. The NMR resonance of PA-1 confirmed a disaccharide structure with glucose at the nonreducing end (Table 2). From the fact that the anomeric proton resonance of the residue was δ_H 4.64 (d, 7.8) and δ_C 104.6 ppm, the glucose residue was shown to be linked via β-glycosidic bonds. HMBC experiments provided a correlation between the xylose C-4 and glucose anomeric proton, indicating that the glucose residue was linked to the PA-xylose unit at the 4 position. Therefore, PA-1 was confirmed to be β-D-GlcAp-(1→4)-Xyl-PA (**1**). On the other hand, the molecular mass of PA-2 was 597.44 amu, as shown by MALDI-TOF-MS, which agreed with the calculated value of a pyridylaminated triose composed of a hexuronic acid and two hexoses [$(M + H)^+_{calc} = 597.54$]. The ^{1}H and COSY NMR spectra revealed that the pyridylaminated moiety in PA-2 was galactose (Table 2). The coupling constants for the remaining anomeric protons were greater than 7 Hz. Since these protons were correlated with carbons with chemical shifts greater than 105 ppm, it was concluded that glucose and glucuronic acid were linked via β-glycosidic bonds. Correlations between the glucose C-6 and the glucuronic acid H-1 and between the PA-galactose C-4 and the glucose H-1 indicated that the latter was linked to the glucopyranosyl unit at the 6 position and the glucopyranosyl unit at the 4 position of the PA-galactosyl residue, respectively. Thus, PA-2 was revealed to be β-D-GlcAp-(1→6)-β-D-Glcp-(1→4)-Gal-PA (**2**).

After our bioactivity-guided fractionation resulted in the isolation of an antiviral polysaccharide, its effects were examined on the growth of three cell lines and six viruses (Table 3). The cytotoxicities of nostoflan were very low, with CC_{50} values of 4900−7800, >10 000, and 8100 μg/mL for Vero, HEL, and MDCK cells, respectively. The IC_{50} values for HSV-1, HSV-2, HCMV, and influenza A virus replication were 0.37, 2.9, 0.47, and 78 μg/mL, respectively, under conditions in which the drug was added at the same time as viral infection (treatment A). The resulting SI values (CC_{50}/IC_{50}) indicated that nostoflan has potent antiviral activities against these enveloped viruses. However, when nostoflan was added after viral infection (treatment B), the activity was lower as compared with that from treatment A. On the other hand, nostoflan was inactive against nonenveloped viruses including adenovirus and coxsackie virus. When anti-HSV-1 activity of carboxyl-reduced nostoflan was evaluated, it was markedly less than that of native polysaccharide, the SI being 150.

It is well known that a lot of antiviral sulfated polysaccharides show antithrombin activity. Thus, it was examined whether nostoflan also exhibited antithrombin activity. When human thrombin (3.6 nM) and plasma heparin cofactor II (48.6 nM) were used, nostoflan did not show antithrombin activity even at a higher concentration of 500 μg/mL. In addition, no direct thrombin inhibition was also observed at the same concentration (data not shown). These results revealed that nostoflan does not possess antithrombin activity, unlike sulfated polysaccharides.

In this study, the active antiviral principle was isolated from a terrestrial cyanobacterium, *N. flagelliforme,* by successive hot water extraction, precipitation, anion exchange, and gel filtration chromatography. The active principle is an acidic but not sulfated polysaccharide. It has been reported that the polysaccharide from field-grown samples of *N. flagelliforme* is mainly composed of glucose, xylose, and galactose (approximately 2:1:1) with trace amounts of mannose and arabinose.[10] It is also reported that sugar compositions of the polysaccharides varied between field-grown and laboratory-cultured samples. In fact, a polysaccharide from laboratory-cultured *N. flagelliforme* was composed of glucose, galactose, mannose, and glucuronic acid with a similar ratio.

Structural characterization of an extracellular polysaccharide from *N. commune* DRH-1, a cyanobacterium closely related to *N. flagelliforme*, has been reported.[11] Its structure resembles nostoflan except for the presence of ribose and nosturonic acid (NosA, 3-*O*-[(*R*)-1-carboxyethyl]-D-glucuronic acid) as pendant residues. Both nosturonic acid and ribose residues were not detected even when nostoflan was methanolyzed and trimethylsililated as described by Helm et al. (data not shown), while its methylation analysis revealed the presence of glucuronic acid and mannose residues as pendant residues of nostoflan. Furthermore, oligosaccharides β-D-Glcp-(1→4)-Xyl, similar to PA-1, and β-D-NosA-(1→6)-β-D-Glcp-(1→4)-Gal (**3**), corresponding to PA-2, have been isolated.[11] Thus, the polysaccharides were slightly different from each other between *N. flagelliforme* and *N. commune* DRH-1 in pendant sugar residues.

In the present study, we showed that nostoflan has a broad antiviral spectrum against enveloped viruses (Table 3). These viruses are initiated by binding to carbohydrates on the host cell surface. Therefore, nostoflan is suggested

Table 1. Methylation Analysis of Nostoflan and Reduced Nostoflan

methylated sugar	deduced linkage	primary mass fragments (*m/z*)	native (mol %)	reduced (mol %)
2,3,4,6-Me_4 Glc[a]	t-Glc*p*	45,118,205,206	6.4 ± 0.87	13.5 ± 0.02
2,3,4,6-Me_4 Gal	t-Gal*p*	45,118,205,206	2.0 ± 0.30	1.3 ± 0.18
2,3,4,6-Me_4 Man	t-Man*p*	45,118,205,206	4.3 ± 1.38	2.9 ± 0.09
2,3,6-Me_3 Glc	4-Glc*p*	45,118,162,233,277	18.2 ± 1.78	17.7 ± 0.24
2,3,6-Me_3 Gal	4-Gal*p*	45,118,162,233,277	22.2 ± 2.35	16.7 ± 0.01
2,3-Me_2 Xyl	4-Xyl*p*	118,162,189,233	16.8 ± 0.53	19.4 ± 0.54
2,6-Me_2 Gal	3,4-Gal*p*	45,118,305	4.3 ± 2.08	3.5 ± 0.15
2-Me Xyl	3,4-Xyl*p*	118,261	5.8 ± 0.76	4.9 ± 0.35
2,4-Me_2 Glc	3,6-Glc*p*	118,189,234,305	2.3 ± 0.74	2.2 ± 0.21
2,3-Me_2 Glc	4,6-Glc*p*	118,162,261,305	17.7 ± 1.28	18.0 ± 1.26

[a] 2,3,4,6-Me_4-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol.

Table 2. Proton and Carbon Chemical Shifts (ppm) for Residues of Pyridylaminated Oligosaccharides

		1		**2**	**3**	**4**	**5**		**6**	
PA-1										
Glc	^{1}H	4.64 d (8.1)		3.33 dd (8.1, 9.4)	3.51 m	3.42 m	3.45 m		3.73 dd (5.4, 12.2)	3.89 m
	^{13}C	104.6		76.0	78.4	72.4	78.4		63.4	
Xyl[a]	^{1}H	3.57 m	3.62 m	4.08 m	3.83 m	4.00 m	3.80 m	3.86 m		
	^{13}C	47.2		72.2	73.5	82.8	63.3			
PA-2										
GlcA	^{1}H	4.57 d (9.1)		3.38 t (8,5)	3.56 t (9.0)	3.61 m	4.00 d (9.4)			
	^{13}C	105.9		75.6	78.1	74.2	77.6		175.5	
Glc	^{1}H	4.55 d (8.1)		3.33 t (8.6)	3.48 m	3.46 m	3.62 m		3.89 m	4.20 brd (10.2)
	^{13}C	105.5		76.0	78.4	72.5	77.5		72.4	
Gal[a]	^{1}H	3.59 m	3.64 m	4.32 m	3.92 m	3.93 m	4.04 m		3.72 dd (7.3, 11.5)	3.83 dd (6.4, 11.5)
	^{13}C	47.9		70.1	72.5	80.3	72.7		65.6	

[a] These are pyridylaminated residues. The spectra were recorded at 500 MHz in D_2O. Chemical shifts are relative to external tetramethylsilane at 0 ppm.

Table 3. Antiviral Spectrum of Nostoflan

virus (strain)	host cell	cytotoxicity (CC_{50}, μg/mL)	antiviral activity (IC_{50}, μg/mL)		selectivity index (CC_{50}/IC_{50})	
			A[a]	B[b]	A	B
HSV-1 (HF)	Vero	4900 ± 350	0.37 ± 0.07	>100 ± 0	13 000 ± 1900	<49 ± 3.5
HSV-2 (UW-268)	Vero	7800 ± 990	2.9 ± 0.36	7.7 ± 1.2	2700 ± 230	1000 ± 140
HCMV (Towne)	HEL	>10 000 ± 0	0.47 ± 1.0	14 ± 2.5	>21 000 ± 4700	>710 ± 120
influenza (NWS)	MDCK	8100 ± 860	78 ± 16	96 ± 12	104 ± 14	84 ± 9.3
adeno (Type 2)	HeLa	7200 ± 510	>500 ± 0	>500 ± 0	<15 ± 1.2	<15 ± 1.2
coxsackie (Conn-5)	Vero	7800 ± 990	>500 ± 0	>500 ± 0	<16 ± 2.3	<16 ± 2.3

[a] Nostoflan was added to the medium at the same time as viral infection for 1 h and throughout the incubation thereafter. [b] Nostoflan was added to the medium immediately after viral infection.

to be an inhibitor of virus−cell interaction. The hypothesis is consistent with the results showing that antiviral activity at treatment A was stronger than that of treatment B. So far, various sulfated polysaccharides have been reported to be active against different kinds of viruses,[12] but almost all of them have serious side effects, such as antithrombin activity. This restricts the clinical application of sulfated polysaccharides as antiviral agents. Although nostoflan is not a sulfated polysaccharide, it possesses strong antiviral effect without antithrombin activity. These findings emphasize that nostoflan has an advantageous characteristic as an antiviral drug candidate.

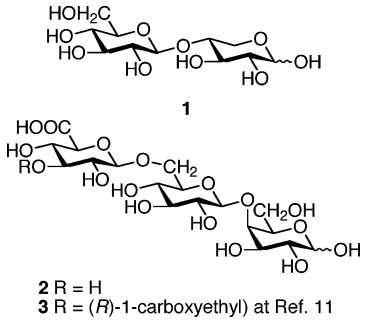

Figure 2. Structures of oligosaccharides.

Experimental Section

General Experimental Procedures. The optical rotation was determined in H_2O using a 1 cm light path length cell with a JASCO DIP-1000 digital polarimeter. The ^{1}H and ^{13}C NMR spectra were recorded using a Unity plus 500 spectrophotometer. Each sample was dissolved in 0.7 mL of 99.9% D_2O. All spectra were recorded at 45 °C, and solvent suppression in the homonuclear spectrum was achieved by presaturation during the recycle delay. COSY, TOCSY, HMQC, and HMBC experiments were also performed by using standard Varian pulse programs. All chemical shifts were relative to external tetramethylsilane. GC was carried out on a GL-Science GC-353 gas chromatograph equipped with FID, and N_2 was used as a carrier gas. GC-MS analysis was carried out on a JEOL JMS-GCmate, and the ionization potential was 70 eV. Metal elements were analyzed using a Hitachi X-650 scanning electron microanalyzer. MALDI-TOF-MS spectra were measured on a Bruker Autoflex spectrometer equipped

with a 337 nm N_2 laser; 1 μL of sample solution was mixed with an equal volume of matrix (2,5-dihydroxybenzoic acid in EtOH/H_2O, 1:1) and allowed to crystallize. HPLC was performed with a Shimadzu LC-6A HPLC system. Solvent evaporation was performed below 40 °C under reduced pressure.

Extraction and Isolation of an Antiviral Polysaccharide from *N. flagelliforme*. A terrestrial blue-green alga, *N. flagelliforme*, was collected in Alxa, Inner Mongolia, China, in the summer of 1996. The dried alga (100 g) was cut into small pieces and extracted three times with boiling water for 1 h. The combined extract was filtrated and concentrated in vacuo. After centrifugation, the supernatant was treated with 4 volumes of ethanol, and the precipitate was dried to give a dark green residue (NP, 9.8 g). NP (2.0 g) was dissolved in 600 mL of deionized water and centrifuged (3000 rpm × 5 min). The supernatant was dialyzed against tap water overnight followed by deionized water. The nondialyzed portion was lyophilized to give a green cotton-like residue (NPH, 1.16 g). The nondialyzate (NPH, 600 mg) was applied to a DEAE Toyopearl 650 M anion-exchange chromatography (5 × 15 cm, Tosoh). The column was eluted with a linear gradient of 0−0.5 M NaCl. Fractions of 15 mL were collected and monitored by UV detection at 260 nm for pigments and the phenol-H_2SO_4 method at 480 nm for carbohydrates.[13] Carbohydrate-positive fractions were combined, dialyzed, and lyophilized to yield a fraction termed NPHM (262 mg). Then, NPHM (500 mg) was dissolved in 0.01 M citrate buffer containing 0.1 M NaCl (pH 7) and applied to a Sepharose 6B column (4.4 × 89 cm, Amersham Pharmacia Biotech AB). The column was eluted with the same buffer to yield fractions termed NPHM1 (144 mg) and NPHM2 (264 mg). The latter eluate (NPHM2, 400 mg), which showed potent anti-HSV-1 activity, was rechromatographed under the same conditions to yield fractions NPHM2a (71 mg), NPHM2b (182 mg), and NPHM2c (65 mg). NPHM2b (200 mg) was further purified on the same column to give fractions NPHM2b-1 (131 mg) and NPHM2b-2 (57 mg). Finally, NPHM2b-2 (100 mg) was applied to a Toyopearl HW-55F column (2.4 × 86 cm, Tosoh) to give fractions NPHM2b-2A (35 mg) and NPHM2b-2B (36 mg).

Estimation of Homogeneity and Apparent Molecular Weight. Electrophoresis was performed on a cellulose acetate membrane (Separax, Jokoh) in 0.47 M formic acid/pyridine buffer (pH 3) at 0.5 mA/cm. The polysaccharide was stained with 0.25% toluidine blue and destained with 7% AcOH. The apparent molecular weight of nostoflan was estimated by HPLC analysis. The sample was analyzed by a TSK $GMPW_{XL}$ gel filtration column (7.6 mm × 30 cm, Tosoh) and eluted with 0.2 M NaCl at 0.5 mL/min. Commercially available pullulans (Shodex P-82, SHOWA DENKO) were used as standard molecular markers.

Reduction of Carboxyl Group of Uronic Acid. The carboxyl group of uronic acid in the polysaccharide was reduced according to the method of Taylor and Conrad.[14] Briefly, nostoflan (10 mg) in 10 mL of H_2O was adjusted to pH 4.75 with 0.1 N HCl, followed by adding 1 mmol of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and stirring. The reaction mixture was kept at pH 4.75 with 0.1 N HCl. After the hydrogen ion uptake ceased, 50 mmol of $NaBH_4$ was added at room temperature over a period of 1 h, and the pH was maintained at 7.0 by adding 4 N HCl. A drop of 1-octanol was added as an antifoaming agent. The mixture was dialyzed and lyophilized to obtain the carboxyl-reduced polysaccharide.

Chemical Analyses of Nostoflan. Carbohydrates were determined by the phenol sulfuric acid method.[13] Uronic acid content was determined by the *m*-hydroxydiphenyl method.[15] Protein content was determined by the Bradford method.[16] Sugar composition of nostoflan was determined as follows. A 1 mg sample was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 121 °C for 1 h. After removal of TFA under N_2 gas, the hydrolyzates were converted to alditol acetates, which were analyzed by GC using a DB-225 column (30 m × 0.32 mm i.d., J&W Scientific) at an oven temperature of 200 °C. The absolute configuration of the monosaccharides was identified on the basis of analysis of the trimethylsilyl ether of methyl 2-(polyhydroxyalkyl)-thiazolidine-4-carboxylates, as described.[17] Acid hydrolyzates of nostoflan (1 mg) were mixed with 0.5 mL of L-cysteine methyl ester hydrochloride in pyridine (6 mg/mL), and the reaction mixture was kept at 60 °C for 1 h. After the solvent was removed by flushing with N_2 gas, the trimethylsilylation reagent (TMS-HT, Wako) was added to the residue, which was kept at 60 °C for another 30 min. The precipitate was centrifuged off, and the supernatant was analyzed by GC using a DB-225 column (30 m × 0.32 mm i.d., J&W Scientific) at an oven temperature of 190 °C. Both D- and L-enantiomers of glucose, galactose, xylose, and mannose were used as standards. Methylation was performed according to the method previously reported.[18] The resulting methylated polysaccharide was hydrolyzed with 2 M TFA at 121 °C for 1 h and reduced with $NaBH_4$, and the alditols were then acetylated. The partially methylated alditol acetates were analyzed on a GC-MS using a SP-2330 fused silica capillary column (30 m × 0.32 mm i.d., Supelco) at an oven temperature from 160 to 210 °C at 2 °C/min, then to 240 °C at 5 °C/min. Identification of partially methylated alditol acetates was carried out from retention times relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and its fragmentation patterns.[19]

Partial Hydrolysis of Nostoflan. To obtain oligosaccharides, nostoflan was hydrolyzed with 0.2 M TFA at 90 °C for 2 h. After removal of TFA, the hydrolyzates were fractionated to neutral and acidic oligosaccharides by a Dowex 1-X8 column as described earlier.[20]

Pyridylamination of Oligosaccharides. Oligosaccharides were pyridylaminated according to the method of Hase et al.[21,22] After the removal of excess reagents by a Toyopearl HW-40F column (1.1 × 75 cm, Tosoh) eluted with 10 mM NH_4-OAc buffer (pH 6.0), fluorescence-labeled oligosaccharides were separated by reversed-phase HPLC on a Cosmosil 5C18-MS packed column (20 × 300 mm, Nacalai) using a water/MeOH gradient system.

Cells and Viruses. Vero, HEL, MDCK, and HeLa cells were grown in Eagle's minimal essential medium (MEM) (Nissui Pharmaceutical Co., LTD, Tokyo, Japan) supplemented with 5% fetal bovine serum (FBS). HSV-1 (HF strain), HSV-2 (UW-268 strain), and coxsackie virus (Conn-5 strain) were grown on Vero cells. HCMV (Towne strain), influenza A virus (NWS strain), and adenovirus (type 2) were grown on HEL, MDCK, and HeLa cells, respectively.

Antiviral Activity and Cytotoxicity. The plaque yield reduction assay for antiviral activity has been described previously.[23] Cell monolayers were infected with virus at 0.1 plaque forming unit (PFU) per cell for 1 h at room temperature. Virus yields were determined by plaque assay at 4-day incubation point for HCMV or at 1-day incubation point for HSV-1, HSV-2, influenza A virus, and coxsackie virus. In the case of adenovirus, cell monolayers were infected with virus at 0.01 tissue culture infectious dose ($TCID_{50}$) per cell. The production of adenovirus was determined by the presence of cytopathic effects in serially diluted samples, and the $TCID_{50}$ values were calculated according to Reed-Muench method. The 50% inhibitory concentration (IC_{50}) was obtained from dose−response curves. Nostoflan was added to the medium during viral infection and throughout the incubation thereafter (treatment A), or immediately after viral infection (treatment B). For cell growth inhibition studies, cells were incubated in 48-well plates at an initial density of 1.2×10^4 cells/well. After cells had been incubated for 8 h at 37 °C, polysaccharide was added and the incubation was continued for 3 days. Viable cell yield was determined by the trypan blue exclusion test. The 50% cytotoxic concentration (CC_{50}) was obtained from the dose−response curve.

Inhibition of Thrombin. The thrombin inhibition was measured using S-2238 (H-D-Phe-pipecolyl-Arg-*p*-nitroanilide) substrate. Human thrombin was incubated with or without nostoflan in 200 μL of 50 mM Tris-Cl (pH 8.4), 0.15 M NaCl, and 0.1% BSA at room temperature in the presence or absence of heparin cofactor II. After 5 min incubation, 50 μL of 1 mg/mL S-2238 was added. The residual thrombin activity was determined by measuring the change in the absorbance at 405 nm.

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