AQUASTATIN A, AN INHIBITOR OF MAMMALIAN ADENOSINE TRIPHOSPHATASES FROM *Fusarium aquaeductuum*

TAXONOMY, FERMENTATION, ISOLATION, STRUCTURE DETERMINATION AND BIOLOGICAL PROPERTIES

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A new inhibitor of mammalian adenosine triphosphatases, designated aquastatin A, has been isolated from a fungus identified as *Fusarium aquaeductuum*. The structure of this compound has been determined by MS and NMR analyses. It inhibits Na^+/K^+-ATPase with an IC_{50} value of 7.1 μM, and H^+/K^+-ATPase with an apparent IC_{50} value of 6.2 μM.

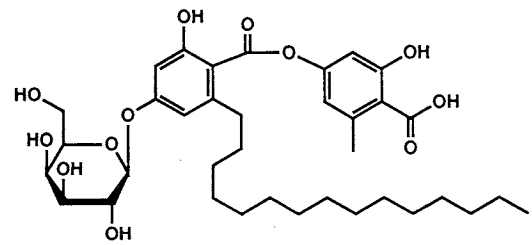
During the course of a screening for calcium channel blockers from microbial sources using a radioligand assay, a new compounds, designated aquastatin A (Fig. 1), was found to be produced by a fungus identified as *Fusarium aquaeductuum*. Spectroscopic data revealed that aquastatin A contains two aromatic rings and one galactose unit. Although aquastatin A showed negligible activity as a calcium channel blocker, it was found to inhibit mammalian adenosine triphosphatases (ATPase). In this paper we describe the taxonomy and fermentation of the producing organism, as well as the isolation, physico-chemical and biological properties, and the structure of aquastatin A.

Experimental

Discovery Screen and *In Vitro* Calcium Channel Antagonistic Effect

Originally, aquastatin A was found by a radioligand binding assay of ^{3}H-nitrendipine to porcine heart microsomes as described previously.[1] The *in vitro* calcium channel antagonistic effect was also measured as in the previous paper.[1]

Fig. 1. Structure of aquastatin A.



Analytical HPLC

Aquastatin A was separated on an analytical HPLC system with a C_{18} reverse phase column (type 8NVC184, 4 μm, 8.0 × 100 mm, Waters) and detected at 210 nm. The mobile phase was 60% acetonitrile - 40% triethylammonium phosphate buffer (0.1%, pH 3.2) with a flow rate of 2 ml/minute. The

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retention time of aquastatin A was 6.8 minutes.

Fermentation and Processing

All fermentations were carried out using GPMY medium consisting of glycerol 5.0%, potatoes 5.0% malt extract (Difco) 0.5% and yeast extract (Difco) 0.5% prepared in tap water without adjustment of pH. Sterilization was at 121°C for 30 minutes for both shake flasks and tanks. Five hundred-ml baffled Erlenmeyer flasks containing 100 ml of GPMY medium were inoculated with *Fusarium aquaeductuum* SANK 11089 grown on PDA agar medium for 7 days. The inoculated flasks were cultured on a rotary shaker (200 rpm) for 3 days at 26°C. Then, 300 ml of seed culture was transferred to a 60-liter tank containing 30 liters of same medium with 0.01% of CB-442 (Nippon Oils & Fats Co., Ltd.) antifoam. The tank operation was carried out for 7 days at 26°C, and antifoam was available on demand. The agitation speed was controlled to keep the concentration of dissolved oxygen at above 25% of saturation. The culture broth was filtered to obtain mycelial part. After filtration, aquastatin A associated with the mycelium (3.2 kg) was recovered by successive extraction with 10 liters of acetone and 5 liters of 80% aqueous acetone for 1 hour per extraction while stirring at room temperature. Combined extracts were concentrated under reduced pressure. The resulting water phase was adjusted to pH 3.0 with 6 N HCl and extracted twice with equivalent volumes of ethyl acetate. Broth filtrate (23 liters) was adjusted to pH 3.0 with 6 N HCl and extracted twice with 20 liters of ethyl acetate per extraction. The resulting organic phase was combined and concentrated to a syrupy residue under reduced pressure to give 20 g of the crude extract.

Isolation

Five g of the crude extract was dissolved in 30 ml of MeOH and was applied to a C_{18} reverse phase column (ODS-1050-20-SR 10 × 50 cm, Kurita Co., Ltd.) eluted with 70% acetonitrile-30% triethylammonium phosphate buffer (0.2%, pH 3.2) at 150 ml/minute. A differential refractometer (Waters 410) was used as the detector. After the peak fractions containing aquastatin A were combined, acetonitrile was evaporated and the residual solution was extracted twice with equal amounts of ethyl acetate. Evaporation of the ethyl acetate yielded aquastatin A as a white powder.

Preparation of 2,2′-*O*-Dimethyl Aquastatin A Methyl Ester

Aquastatin A (100 mg) was dissolved in MeOH (2 ml) and cooled to 0°C. To this solution was added CH_2N_2 in ether (3 ml). After the solution was stand for 2 hours at 0°C, the solvent was evaporated under reduced pressure. The residue was purified by preparative HPLC on a ODS column (Senshu H-5251, 20 × 250 mm) with aqueous 88% CH_3CN as mobile phase. After purification, 42 mg of 2,2′-*O*-dimethyl aquastatin A methyl ester was obtained. ^{13}C NMR (67 MHz, $CD_3OD + CDCl_3$ (4:1)) δ 169.7 (s), 167.8 (s), 161.1 (s), 159.4 (s), 158.7 (s), 153.6 (s), 144.2 (s), 138.7 (s), 122.3 (s), 117.2 (s), 116.1 (d), 110.5 (d), 103.7 (d), 102.4 (d), 99.4 (d), 76.9 (d), 74.6 (d), 71.9 (d), 69.9 (d), 62.2 (t), 56.5 (q × 2), 52.6 (q), 34.6 (t), 32.8 (t), 32.2 (t), 30.5 (m × 10), 23.4 (t), 19.6 (q), 14.4 (q).

Preparation of 2″,3″,4″,6″-*O*-Tetraacetyl-2,2′-*O*-dimethyl Aquastatin A Methyl Ester

To a solution of 2,2′-*O*-dimethyl aquastatin A methyl ester (20 mg) in a mixture of pyridine (1 ml) was added acetic anhydride (0.1 ml), and the mixture was stirred for 6 hours at room temperature. The reaction mixture was poured into ice water and extracted with ethyl acetate. The extract was washed with 0.1 N HCl and brine. Ethyl acetate was removed under reduced pressure to yield 22 mg of 2″,3″,4″,6″-*O*-tetraacetyl-2,2′-*O*-dimethyl aquastatin A methyl ester. ^1H NMR (270 MHz, $CDCl_3$) δ 6.68 (br s, 1H), 6.63 (br s, 1H), 6.48 (br s, 2H), 5.50 (m, 2H), 5.15 (dd, 1H), 5.10 (d, 1H), 4.20 (d, 2H), 4.10 (t, 1H), 3.91 (s, 3H), 3.86 (s, 3H), 3.83 (s, 3H), 2.67 (t, 2H), 2.32 (s, 3H), 2.20 (s, 3H), 2.09 (s, 3H), 2.06 (s, 3H), 2.02 (s, 3H), 1.62 (m, 2H), 1.25 (m, 24H), 0.88 (t, 3H).

Spectroscopic Method

The NMR spectra of aquastatin A and related compounds were recorded in $CDCl_3$ and methanol-d_4 (4:1) using JEOL JNM-GX-400 and/or JNM-GSX-500 spectrometers. FAB-MS and FAB-MS/MS spectra were obtained with a JEOL JMS-HX100 tandem mass spectrometer, which consisted of a converntional geometry double-focusing mass spectrometer (MS-I) followed by an electrostatic analyzer used as MS-II.

Xenon was used to provide the primary beam of atoms (6 KeV). The liquid matrix used for FAB ionization was 3-nitrobenzylalcohol. MS-MS spectra were obtained with the MS-II scanning under the conditions of activating the ions in the third field-free region by collisions with argon gas (sufficient to suppress the precursor ion beam by 20%) at a high collision energy (5 KeV).

Enzyme Assays

Assays of Na^+/K^+-ATPase

The activity of Na^+/K^+-ATPase from dog kidney (Sigma) was measured in a total volume of 1 ml containing 50 mM Tris-HCl (pH 7.5), 140 mM NaCl, 14 mM KCl, 5 mM $MgCl_2$ and 56 μg protein of the enzyme according to the procedure of JØRGENSEN.[2] The reaction was initiated by the addition of Na_2ATP (Sigma) and carried out at 37°C for 10 minutes. The reaction was terminated by the addition of 1 ml trichloroacetic acid (15%). The inorganic phosphate produced by the hydrolysis of ATP was assayed according to the method of FISKE and SUBBAROW.[3]

Assay of H^+/K^+-ATPase Activity

H^+/K^+-ATPase was prepared from porcine stomach as described by IM *et al.*[4] Briefly, membrane vesicles containing the H^+/K^+-ATPase were prepared by differential and zonal density gradient centrifugation. The microsomal fraction enriched in H^+/K^+-ATPase was obtained using a discontinuous sucrose gradient (75,000 × *g*, 60 minutes) and was stored at −80°C. H^+/K^+-ATPase activity was assayed in a total volume of 1 ml containing 70 mM Tris-HCl (pH 6.8), 2 mM Na_2ATP, 5 mM $MgCl_2$, and 20 ~ 40 μg protein of membrane vesicles with or without 10 mM KCl, in accordance with the procedure of NAGAYA *et al.*[5] After incubation at 37°C for 20 minutes, the reaction was terminated by the addition of 1 ml of trichloroacetic acid (10%) to which 100 mg of charcoal powder was added. The assay mixture was centrifuged for 10 minutes at 3,000 × *g*, and the supernatant was examined for inorganic phosphate content as described above.[3] The H^+/K^+-ATPase activity varied from 60 to 80 μmol Pi/mg protein/hour.

Biological Assays

Diuretic and Natriuretic Study

Aquastatin A was first dissolved at a concentration of 10% (v/v) in 0.5% carboxymethylcellulose suspension solution, then mixed with 25 ml of saline/kg body weight. Groups of 3 male Sprague-Dawley rats (230 ~ 240 g, Nihon Clare) were deprived of food but allowed free access to water for 24 hours before use. After administration of aquastatin A (100 mg/kg, po), 3 animals were placed in a metabolic cage and the study was carried out as described previously.[6]

Gastric Secretory Study

Groups of 3 male Sprague-Dawley rats (200 ~ 250 g, Charles River Japan) were also deprived of food but allowed free access to water for 24 hours before use. Under ether anesthesia, the abdomen was opened and the pylorus was ligated according to the method of SHAY *et al.*[7] Four hours after pyloric ligation, the animals were sacrificed by carbon dioxide inhalation and the gastric contents were collected and analyzed for volume and acidity. Acid concentration was determined by automatic titration of the gastric juice against 0.01 N NaOH to pH 7.0 (Comtite-8, Hiranuma). Titratable acid output was calculated as the volume times the acid concentration. Aquastatin A (100 mg/kg) or saline was given intraduodenally at the time of ligation at a volume of 0.1 ml/100 g body weight.

Results

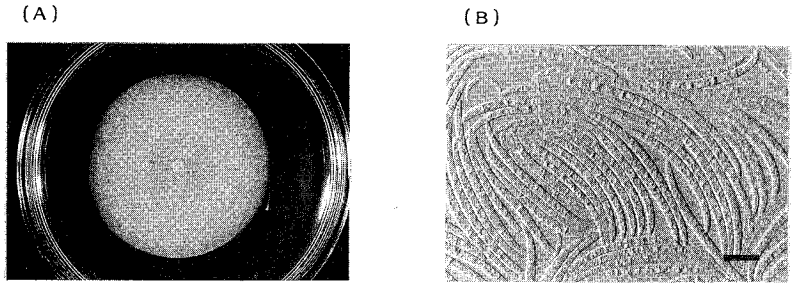
Taxonomy of Strain SANK 11089

The fungal strain SANK 11089, the aquastatin A producer, was isolated from slime fluxes collected in Karuizawa, Nagano Prefecture, Japan.

A colony on PDA is light yellow to deep orange in color, densely colored at the center; slow-growing;

Fig. 2. *Fusarium aquaeductuum* SANK 11089.

(A) Colony surface on PDA. Note the slimy appearance with no aerial hyphae. (B) Conidia formed on PDA. (scale bar: 10 μm).



reaching 3.5~4 cm in diameter in two weeks at 25°C. The colony surface is merismoid and wrinkled, to even slimy. Aerial mycelia are rare to absent (Fig. 2A). There is a tendency for hyphae on the surface of agar and aerial mycelium to form mycelial ropes with several strands. Conidia are borne directly from hyphae or from denticles formed laterally on hyphae. When the denticle elongates it is septated at the base and regarded as a phialide, which is rarely observed. Phialides are subulate, born singly or in groups, simiple or branched monophialides. Conidia show wide variation in shape; they are thin-walled, generally crescent to thread-like with pointed apical cells and indistinctly or apedicellate foot cells (Fig. 2B), to small and strongly curved. The conidia are mostly 30~65 μm × 2~3 μm in size. Chlamydospores are borne terminal or intercalary, formed singly or in chains, globose, smooth-walled, 4~7 μm in diameter.

The characteristics stated above agree fairy well with descriptions of *Fusarium aquaeductuum* (Radlk. et Rabenh.) Lagerh. var. *medium* Wollenw. aggr. given by BOOTH[8] and *Fusarium aquaeductuum* (Radlk. et Rabenh.) Lagerh. given by NELSON *et al.*[9] The taxonomical treatment of the species differs from author to author (for example, see SNYDER and HANSEN,[10] BOOTH,[8] NELSON *et al.*[9]), and taxonomy at the infra-specific level is not firmly established. Therefore, in these studies, the producing strain was identified as *Fusarium aquaeductuum* (Radlk. et Rabenh.) Lagerh, and has been deposited to the National Institute of Bioscience and Human-technology (formerly the Fermentation Research Institute), Agency of Industrial Science and Technology, Tsukuba, Japan, with the accession number of FERM BP-4203. Three other strains of *Fusarium aquaeductuum* were found to produce aquastatin A, and SANK 11089 produced the most among them.

Fermentation and Isolation

Fig. 3 shows a typical time course of the fermentation of *Fusarium aquaeductuum* SANK 11089 in a 60-liter tank. A maximum titer of 1,367 μg/ml was achieved after 168 hours. The fermentation broth was processed as described in the Experimental Procedure section. Approximately 250 g of the crude extract was obtained from a 30-liter fermentation yielding 31.6 g of aquastatin A after preparative HPLC.

Physico-chemical Properties

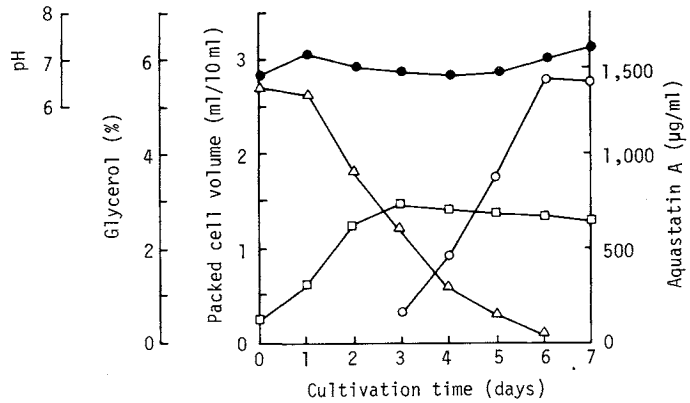
The physico-chemical properties of aquastatin A are summarized in Table 1.

Structure Determination

The molecular formula of aquastatin A was determined to be $C_{36}H_{52}O_{12}$ by HRFAB-MS ($(M+H)^+$ at m/z 677.3540, $(M-H)^-$ at m/z 675). The MS-MS spectrum of m/z 331 derived from the positive ion

Fig. 3. Time course of aquastatin A production.

○ Aquastatin A, □ P.C.V., ● pH, △ Glycerol.



For quantification of aquastatin A, whole broth containing mycelium was extracted with twice the volume of acetone, and the resulting supernatant was directly applied to analytical HPLC.

Table 1. Physico-chemical properties of aquastatin A.

Appearance		White powder
Molecular formula		$C_{36}H_{52}O_{12}$
FAB-MS (positive ion)		677 $(M+H)^+$
(negative ion)		675 $(M-H)^-$
HRFAB-MS (positive ion)	Found:	677.3540 $(M+H)^+$
	Calcd:	677.3537
UV λ^{EtOH} nm (ε)		213 (52,933), 265 (19,033), 307 (10,733)
UV λ^{EtOH} nm [+NaOH]		205, 265 (sh), 305
IR ν_{max} KBr (cm^{-1})		2923, 2851, 1665, 1613, 1247, 1070

FAB-MS of 2″,3″,4″,6″-*O*-tetraacetyl-2,2′-*O*-dimethyl aquastatin A methyl ester suggested that aquastatin A contained one galactose unit (Fig. 4). Furthermore, from the MS-MS analysis of $(M+H)^+$ and $(M-H)^-$ ions of aquastatin A and 2″,3″,4″,6″-*O*-tetraacetyl-2,2′-*O*-dimethyl aquastatin A methyl ester, it was apparent that aquastatin A contained two tetra-substituted benzene moieties (Fig. 5). Two sets of *meta* coupled aromatic protons ($J=2.4$ Hz) in the ^1H NMR spectrum of aquastatin A were consistent with the finding of the MS spectral analysis that aquastatin A would be an aromatic carboxylic acid consisting of two four-substituted aromatic rings connected by an ester linkage.

The substituents involved in aquastatin A could be identified in its ^1H NMR spectrum. Starting from a doublet at 4.97 ppm, a coupled seven-proton system in the range from 3.5 to 5.0 ppm could be identified. The analysis of their vicinal coupling constants suggested that this spin system corresponded to a β-galactopyroside. The final confirmation of the β-galactoside as a substituent was given by a comparison of the ^1H NMR spectrum of authentic 1-*O*-methyl galactose with that for the 1-*O*-methyl sugar obtained by the hydrolysis of aquastatin A. Mass spectral analysis of aquastatin A suggested that it contains a pentadecyl group. This is consistent with the NMR data. Specifically, in the ^1H NMR spectrum a triplet methyl signal at 0.88 ppm and two multiplet methylene signals at 1.64 and 2.93 ppms were found to couple with a group of methylene signals at *ca.* 1.3 ppm. In the ^{13}C NMR spectrum, a group of methylene triplets spaced between 28.9 and 36.4 ppms also supports the existence of the pentadecyl group. In addition, the presence of an aromatic methyl group was suggested by the presence of a singlet methyl signal at 2.63 ppm in the

Fig. 4. MS-MS spectra of *m/z* 331 derived from positive ion FAB-MS of (a) 2″,3″,4″,6″-*O*-tetraacetyl-2,2′-*O*-dimethyl aquastatin A methyl ester and (b) methyl-D-galactopyranoside 2,3,4,6-tetraacetate, respectively.

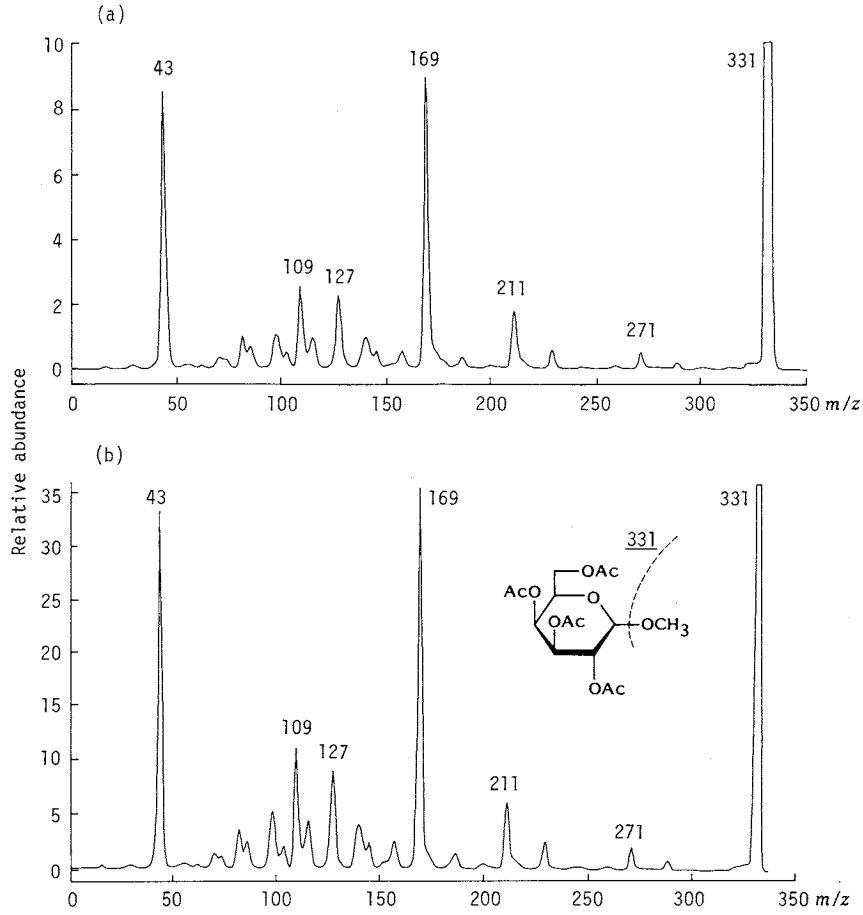
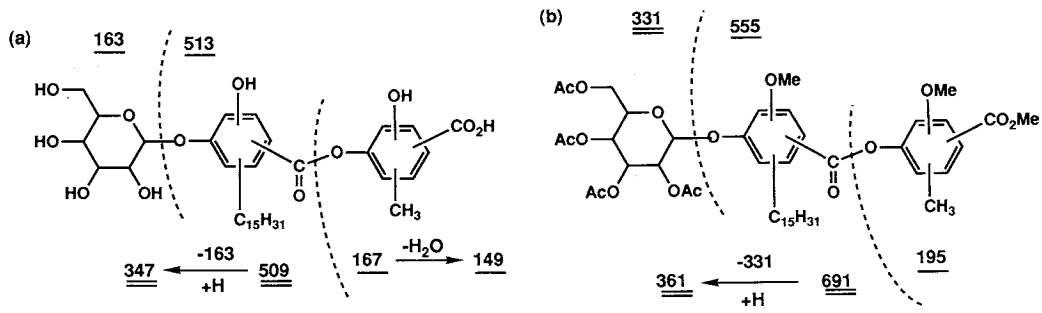
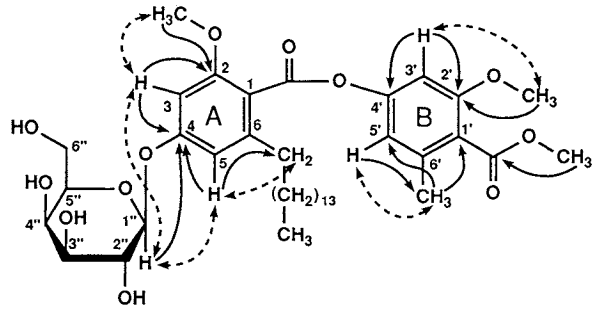


Fig. 5. Fragment ion derived from FAB-MS of (a) aquastatin A and (b) 2″,3″,4″,6″-*O*-tetraacetyl-2,2′-*O*-dimethyl aquastatin A methyl ester (——; negative ion, ═══; positive ion).



^{1}H NMR spectrum. The location of the substituents in the rings A and B were derived from the NOE difference spectra, and ^{1}H-^{13}C long range correlations by HMBC spectra as summarized in Fig. 6. The NOEs observed at 3-H and 5-H of ring A on irradiating the anomeric proton of the galactose moiety at

Fig. 6. The summary of NOEs and ^{1}H-^{13}C long range couplings observed in 2,2′-*O*-dimethyl aquastatin A methyl ester.



The double-headed arrow (←→) indicates the position where NOE was observed. The arrow (⟶) points the direction from ^{1}H to ^{13}C, between which the long range coupling was observed. The arrows connecting methoxy protons indicate the NOEs and the long range coupling observed in 2,2′-*O*-dimethyl aquastatin A methyl ester. The selected correlations are shown for clarity.

Table 2. ^{1}H NMR spectral data of aquastatin A.

Assignment	δ (ppm)[a]	J (Hz)
Ring A		
3	6.56 (d)	$J_{3,5}=2.4$
5	6.53 (d)	
6-Pentadecyl	0.88 (t),	
	ca. 1.3 (m)	
	1.64 (m),	
	2.93 (m)	
Ring B		
3′	6.68 (d)	$J_{3',5'}=2.4$
5′	6.59 (d)	
6′-Me	2.63 (s)	
Galactose		
1″	4.97 (d)	$J_{1'',2''}=7.8$
2″	3.85 (dd)	$J_{2'',3''}=9.8$
3″	3.63 (dd)	$J_{3'',4''}=3.4$
4″	3.99 (dd)	$J_{4'',5''}=0.7$
5″	3.72 (dt)	$J_{5'',6''a}=5.4$, $J_{5'',6''b}=6.5$
6a″	3.81 (dd)	$J_{6''a}$, $J_{6''b}=11.7$
6b″	3.85 (dd)	

[a] The chemical shifts were given referenced to internal TMS. The multiplicities of the signals are given in the parentheses.

Table 3. ^{13}C NMR spectral data of aquastatin A.

Assignment	δ (ppm)	Multiplicity
Ring A		
1	110.7	s
2	161.7	s
3	101.3	d
4	163.9	s
5	107.8	d
6	147.8	s
6-Pentadecyl	36.4, 31.8, 31.4,	t
	29.4～28.9,	t
	13.4	q
1-Carbonyl	168.8	s
Ring B		
1′	115.7	s
2′	164.1	s
3′	105.9	d
4′	153.3	s
5′	111.6	d
6′	143.8	s
6′-Me	23.3	q
1′-Carbonyl	172.9	s
Galactose		
1″	99.9	d
2″	70.4	d
3″	73.0	d
4″	68.2	d
5″	75.0	d
6″	60.6	t

The chemical shifts were given referenced to internal TMS.

4.97 ppm, while the NOE was observed at 5-H only on irradiating the terminal methylene signal of the pentadecyl group at 2.93 ppm. Thus, the galactose and the pentadecyl group were located at C-4 and C-6, respectively. The methyl group resonating at 2.63 ppm was attached at C-6′ of ring B by the NOE between 5′-H of ring B and this methyl singlet signal. The locations of the two hydroxy groups were identified in the methylated derivative of aquastatin A (2,2′-*O*-dimethyl aquastatin A methyl ester), where the signal enhancements of 3-H and 3′-H were observed on irradiating methoxy groups at 3.85 and 3.88 ppm, respectively. The confirmation of the substituents

and the complete assignment of all carbon signals of the aromatic rings were given by the ^{1}H-^{13}C long range correlations obtained by the HMBC spectra, as summarized in Fig. 6.

At this stage, the C-1 position of ring A was left as a site at which ring A is connected to ring B by the ester linkage, while the C-1′ and C-4′ positions remained as possible sites for the ester linkage in ring B. To account for the ^{13}C chemical shift pattern of rings A and B, which is characteristic for the *meta*-disubstitution of the –OR groups, it was uniquely determined that the ester linkage is formed between the C-1 of ring A and the C-4′ of ring B. The presence of a carboxylic acid group at the C-1′ of ring B was supported by the introduction of another methyl group (2′-*O*-Me) at this site, which was disclosed by the ^{1}H-^{13}C long range coupling from the methyl proton at 3.92 ppm to the carbonyl carbon at 168.8 ppm in methylated aquastatin A. Finally, the structure of aquastatin A was established as shown in Fig. 1. The ^{1}H and ^{13}C NMR data for aquastatin A are summarized in Tables 2 and 3.

Biological Properties

Aquastatin A inhibited ^{3}H-nitrendipine binding to porcine heart microsomes with an IC_{50} of 7.16 μM (4.84 μg/ml). However, aquastatin A only weakly inhibited the contraction of taenia induced by 1 mM Ca^{2+} (41% at 10^{-7} g/ml and 61% at 10^{-6}g/ml, respectively). No greater inhibition of contraction was achieved with higher concentrations of aquastatin A. By using the ^{3}H-nitrendipine binding assay, we previously reported the isolation of leualacin and folipastatin from microbial culture broths.[1,11] Leualacin inhibited the Ca^{2+}-induced contraction of taenia and lowered the blood pressure of spontaneously hypertensive rat (SHR).[1] Although folipastatin inhibited ^{3}H-nitrendipine binding to porcine heart microsomes more potently than leualacin, it did not inhibit the Ca^{2+}-induced contraction of taenia significantly, but inhibited the phospholipase A_2 purified from rabbit peritoneal exudate with IC_{50} value of 39 μM and suppressed the release of arachidonic acid from rat polymorphonuclear leukocytes with IC_{50} value of 24 μM.[11] Aquastatin A inhibited neither of them; namely, it inhibited the phospholipase A_2 only 49%

Table 4. Reversible inhibition of Na^{+}/K^{+}-ATPase by aquastatin A.

Concentration of aquastatin A (μM)		Inhibition (%)
At preincubation	At enzyme assay	
0	15	63.8
0	5	40.5
0	3	34.3
0	1	6.6
15	3	30.4
5	1	0.0

Enzyme assay was carried out as described in experimental procedure, except that the enzyme was preincubated with aquastatin A at 37°C for 5 minutes and the concentration of aquastatin A was lowered to one-fifth at enzyme assay.

Fig. 7. Inhibition of Na^{+}/K^{+}-ATPase from dog kidney (○) and H^{+}/K^{+}-ATPase from porcine stomach (●) by aquastatin A.

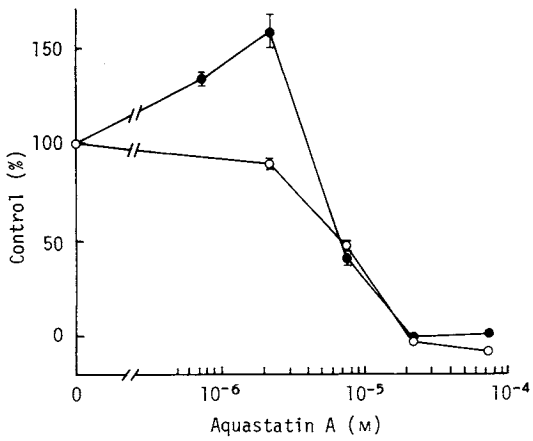


Table 5. Diuretic and natriuretic study of aquastatin A.

	Urinary volume	Total electrolyte, μEq/3 rats		
	ml/3 rats	Na^{+}	K^{+}	Cl^{-}
Control	6.3	649	297	876
Aquastatin A	10.0	1,340	640	1,690
(% of control)	(159)	(207)	(216)	(193)

even at 148 μM (100 μg/ml), and it did not suppress the release of arachidonic acid at all 74 μM (50 μg/ml). Thus far in testing, no antimicrobial activity has been detected at the concentration of 1 mg/ml.

The Na^+/K^+-ATPase and H^+/K^+-ATPase activities were measured in the presence of various concentrations of aquastatin A. Aquastatin A inhibited Na^+/K^+-ATPase activity in a concentration-dependent manner with an IC_{50} value of 7.1 μM. In the experiments shown in Table 4, Na^+/K^+-ATPase was preincubated with varying concentration of aquastatin A at 37 °C for 5 minutes, and the concentrations of aquastatin A were reduced to one-fifth at enzyme assay. As indicated, the inhibition of aquastatin A was completely reversible, as it depended on the concentration of the inhibitor at enzyme assay, not on the concentration at preincubation. The H^+/K^+-ATPase activity, on the other hand, was stimulated 34.2% as 0.7 μM and 58.8% at 2.2 μM, respectively. However, it was inhibited at a higher concentration of aquastain A with an apparent IC_{50} of 6.2 μM (Fig. 7). Slight natriuresis and kaliuresis were observed with oral administration of aquastatin A (Table 5). In a gastric secretory study, acid output was inhibited 26% by 100 mg/kg aquastatin A, but the value was not significant as compared to control groups.

Discussion

Two compounds having structures related to aquastatin A have been reported. Namely, NAKANISHI *et al.* isolated KS-501 and KS-502 from *Sporothrix* sp. as inhibitors of Ca^{2+} and calmodulin-dependent phosphodiesterase,[12,13] and YAGINUMA *et al.* isolated TPI from *Nodulisporium* sp. as an inhibitor of phosphodiesterase.[14] Aquastatin A inhibited these enzymes only weakly, and the IC_{50} values of calmodulin-dependent and independent phosphodiesterases were 58 μM and over 100 μM, respectively. These compounds have a common structure in which 2 aromatic rings are linked by an ester bond, and a sugar moiety is associated with one of them. The producers of the KS compounds and TPI are related in terms of conidiogenesis, but neither of them are related to *Fusarium aquaeductuum*. In addition, another related compound, 1-tetraacetylglycosidyl-2-hydroxy-3-pentadecylbenzene, was chemically synthesized, but its biological activity has not yet been described.[15]

Aquastatin A is a very hydrophobic compound. The stimulation of H^+/K^+-ATPase at a low concentration of aquastatin A can be explained by its detergent-like properties. It is possible that aquastatin A might not reach to its target *in vivo* because of poor solubility, which can explain its insignificant inhibition of acid output, although the possibility of poor intestinal absorption or rapid metabolism *in vivo* cannot be excluded.

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