

ORIGINAL ARTICLE

Kribellosides, novel RNA 5'-triphosphatase inhibitors from the rare actinomycete *Kribbella* sp. MI481-42F6

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Yeast capping enzymes differ greatly from those of mammalian, both structurally and mechanistically. Yeast-type capping enzyme repressors are therefore candidate antifungal drugs. The 5'-guanine-N7 cap structure of mRNAs are an essential feature of all eukaryotic organisms examined to date and is the first co-transcriptional modification of cellular pre-messenger RNA. Inhibitors of the RNA 5'-triphosphatase in yeast are likely to show fungicidal effects against pathogenic yeast such as *Candida*. We discovered a new RNA 5'-triphosphatase inhibitor, designated as the kribellosides, by screening metabolites from actinomycetes. Kribellosides belong to the alkyl glyceryl ethers. These novel compounds inhibit the activity of Cet1p (RNA 5'-triphosphatase) from *Saccharomyces cerevisiae* *in vitro* with IC₅₀s of 5–8 μM and show antifungal activity with MICs ranging from 3.12 to 100 μg ml⁻¹ against *S. cerevisiae*.

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INTRODUCTION

Candidiasis is a fungal infection caused by yeasts of the genus *Candida*. *Candida* is the fourth most common cause of healthcare-associated bloodstream infections. These infections predominantly affect immunocompromised patients, including those with AIDS or cancer, or those who have received organ transplants. Currently, there are only a few systemic antifungal drugs (amphotericin B, flucytosine, several azole drugs and echinocandins) that are available for the treatment of such invasive candidiasis, and not all of these are satisfactory in terms of efficacy, toxicity, their antifungal spectra, or the possibility of drug resistance. There is thus an urgent need for the discovery and development of novel anti-*Candida* drugs, especially those which have a different mode of action from the currently available drugs.

The 5' guanine-N7 cap has been found to be an essential feature of all eukaryotic organisms examined to date and is the first co-transcriptional modification of cellular pre-messenger RNA.^{1–3} The first step of cap formation is the removal of γ-phosphate from the RNA 5'-triphosphate end of newly synthesized RNA to generate a diphosphate end by RNA 5'-triphosphatase. Then the GMP moiety of GTP is transferred to the 5'-diphosphate terminus by mRNA guanylyltransferase. After these two consecutive reactions, methylation at the guanine-N7 position catalyzed by mRNA (guanine-N7-) methyltransferase follows. *Saccharomyces cerevisiae* RNA 5'-triphosphatase (Cet1p) is a member of the divalent cation-dependent triphosphatase family observed in protozoa, eukaryotic viruses and fungi.^{4–7} The X-ray structure of Cet1p revealed the location of two independent active sites within parallel tunnels that are formed by

homodimerization of a domain.⁵ In yeast such as *S. cerevisiae* and *Candida albicans*, the triphosphatase and guanylyltransferase are encoded by distinct genes whose protein products form a non-covalent complex. By contrast, in mammals and plants, the triphosphatase and guanylyltransferase occur in a single-polypeptide chain. Although the guanylyltransferase domain is conserved across evolution, the triphosphatase domain is a metal-independent enzyme that shares structural homology with the cysteine phosphatase superfamily.^{8–10}

Thus, the capping enzymes of yeast differ greatly from the capping enzymes of mammals, both structurally and mechanistically, and inhibitors directed against Cet1p may have a fungicidal effect and may be active against clinically important pathogenic yeasts.

During the course of our screening program for new Cet1p inhibitors from metabolites of actinomycetes using a Cet1p enzyme assay system, we found that a strain of *Kribbella* sp. MI481-42F6 produced new antibiotics, namely kribellosides (Figure 1a,b). Kribellosides showed moderate RNA 5'-triphosphatase inhibitory activities with moderate anti-yeast activities against *S. cerevisiae*. In this paper, we describe the isolation, structural determination and biological activity of new RNA 5'-triphosphatase inhibitors, kribellosides.

RESULTS AND DISCUSSION

Taxonomy of the antibiotic-producing strain

Strain MI481-42F6 was isolated from a soil sample collected at Nerima-ku, Tokyo, Japan. This strain formed well-branched substrate

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This paper is dedicated to Professor Dr Satoshi Ōmura for his Nobel Prize in Physiology or Medicine 2015.

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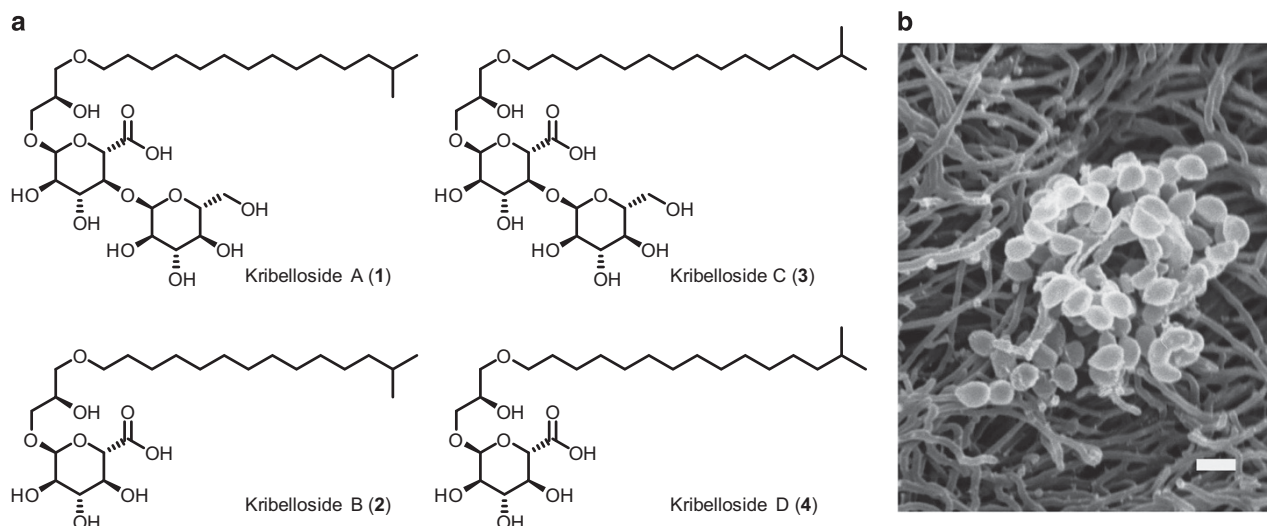


Figure 1 (a) Structure of kribbelosides A, B, C and D. (b) Electron micrograph of the kribbeloside producer *Kribbella* sp. MI481-42F6. *Kribbella* sp. MI481-42F6 was grown on oatmeal agar (ISP medium No. 3) for 14 days at 30 °C. Bar, 1 μ m.

mycelia and straight to flexuous aerial mycelia. Mature spore chains consisted of 8 to 10 or more spores. Each spore was oval in shape with a smooth surface and was $0.6 \times 0.8\text{--}0.9 \times 1.1 \mu\text{m}$ in size. The substrate mycelia were pale yellow to pale yellowish brown, whereas aerial mycelia were white. Diaminopimelic acid isomers were of the LL-type. The partial 16 S ribosomal RNA gene sequence (1450 bp) was determined and submitted to the GenBank/EMBL/DBJ database under accession number LC049957. The gene sequence showed high similarity with those of the genus *Kribbella*, such as *Kribbella aluminosa* (JCM 14599^T, 1419/1432 bp, T: Type strain, 99.1%)¹¹ and *K. solani* (JCM 12205^T, 1433/1453 bp, 98.6%).¹²

These phenotypic and genotypic data suggested that strain MI481-42F6 belongs to the genus *Kribbella*. Therefore, the strain was tentatively designated as *Kribbella* sp. MI481-42F6. Detailed taxonomic studies of strain MI481-42F6 are now underway.

Fermentation and isolation of kribbelosides

Yeast Cet1p requires magnesium ions for RNA 5'-triphosphatase activity, but has been reported to exert the nucleoside triphosphatase activity in the presence of manganese ions.¹³ Therefore, we monitored kribbelosides using an *in vitro* enzyme assay to determine their inhibitory effect on Cet1p nucleoside triphosphatase activity during the purification process. The fermentation of kribbelosides was performed by culturing in a 500-ml baffled Erlenmeyer flask containing 110 ml of a producing medium with rotary shaking. The fermentation broth (4 l) was separated into the mycelial cake and the supernatant by centrifugation. The mycelial cake was extracted with MeOH (1 l). The extract, including an active substance, was sequentially extracted with BuOH and a mixture of CHCl₃-MeOH-water (5:4:6) yielding a brown material (764 mg). The brown material was subjected to chromatography on a low-pressure reversed-phase (C₁₈) column and was developed with MeOH:ammonium carbonate (5 mM) at a ratio of 0:100, 60:40, 80:20 and 100:0. The active fractions were collected and further chromatography was performed by reversed-phase HPLC and developed with acetonitrile/ammonium carbonate (5 mM). The active fractions were collected and concentrated *in vacuo* to yield 79 mg of pure kribbeloside A(1), 83 mg of kribbeloside B(2), 26 mg of kribbeloside C(3) and 13 mg of kribbeloside D(4), respectively, as a colorless powder.

Physico-chemical properties and structure determination of the kribbelosides

The physico-chemical properties of compounds **1**, **2**, **3** and **4** are shown in Supplementary Table S1. The optical rotations of compounds **1–4** were $+103.8^\circ$, $+61.7^\circ$, $+95.7^\circ$ and $+50.4^\circ$, respectively (c 0.5, MeOH). Their UV spectra showed no characteristic absorption. The molecular formulas of compounds **1–4** were determined to be C₃₀H₅₆O₁₄, C₂₄H₄₆O₉, C₃₁H₅₈O₁₄ and C₂₅H₄₈O₉, respectively, by high-resolution electrospray ionization mass spectrometry (HRESI-MS, negative-ion mode), revealing a difference of CH₂ between **1**, **2** and **3**, **4** and C₆H₁₀O₅ between **1**, **3** and **2**, **4**. The LC-HRESI-MS indicated that **1** and **2** share the same in source fragment of m/z 303.2896 (C₁₈H₃₉O₃) and **3** and **4** possessed the fragment of m/z 317.3052 (C₁₉H₄₁O₃), as shown in Figure 2a.

The NMR data for the kribbelosides are summarized in Table 1. Compound **1** had 1 carbonyl, 2 anomers, 9 oxymethines, 1 sp³ methine, 4r oxygenated methylenes, 11 methylenes and 2 methyl carbons, as determined by ¹³C NMR and DEPT135 spectra. The ¹H/¹H COSY and TOCSY spectra of compound **1** established the five parts shown in Figure 2b. Correlations were observed from the oxymethine of δ_{H} 3.96 (H2) to the oxymethylene at δ_{H} 3.40, 3.83 (H1) and oxymethylene at δ_{H} 3.46, 3.49 (H3), suggesting a glyceryl moiety. The cross peaks observed from the anomer proton at δ_{H} 4.82 (H1') to oxymethine at δ_{H} 4.07 (H5') within three oxymethines (δ_{H} 3.51 (H2'), δ_{H} 3.91 (H3') and δ_{H} 3.71 (H4')), and from another anomer proton at δ_{H} 5.27 (H1'') to the oxymethylene at δ_{H} 3.65 and 3.81 (H6'') within four oxymethines (δ_{H} 3.37 (H2''), δ_{H} 3.62 (H3''), δ_{H} 3.26 (H4'') and δ_{H} 3.72 (H5'')) suggested two sugar moieties. The remaining portion might be a branched long alkyl chain from the oxymethylene at δ_{H} 3.46 (H4) to the terminal branched dimethyl protons at δ_{H} 0.88 (H17, 18) revealed by the 13-methyl-1-tetradecanol moiety. The connectivities of these parts were established by HMBC spectroscopy, the long-range couplings; H5'/C1' (anomer at δ_{C} 100.9) and carboxyl carbon at δ_{C} 175.2 (C6') and large proton-proton couplings from H2' to H5' (9.4–9.5 Hz) and ROEs between H3' and H5' revealed an α -glucuronic acid moiety. Long-range couplings, H5''/C1'' (anomer at δ_{C} 101.2) and large couplings from H2'' to H5'' (9.5 Hz) and ROEs between H3'' and H5'' revealed an α -glucose moiety. The HMBC correlation of H1''/C4' established an α -1, 4 bond between glucose and

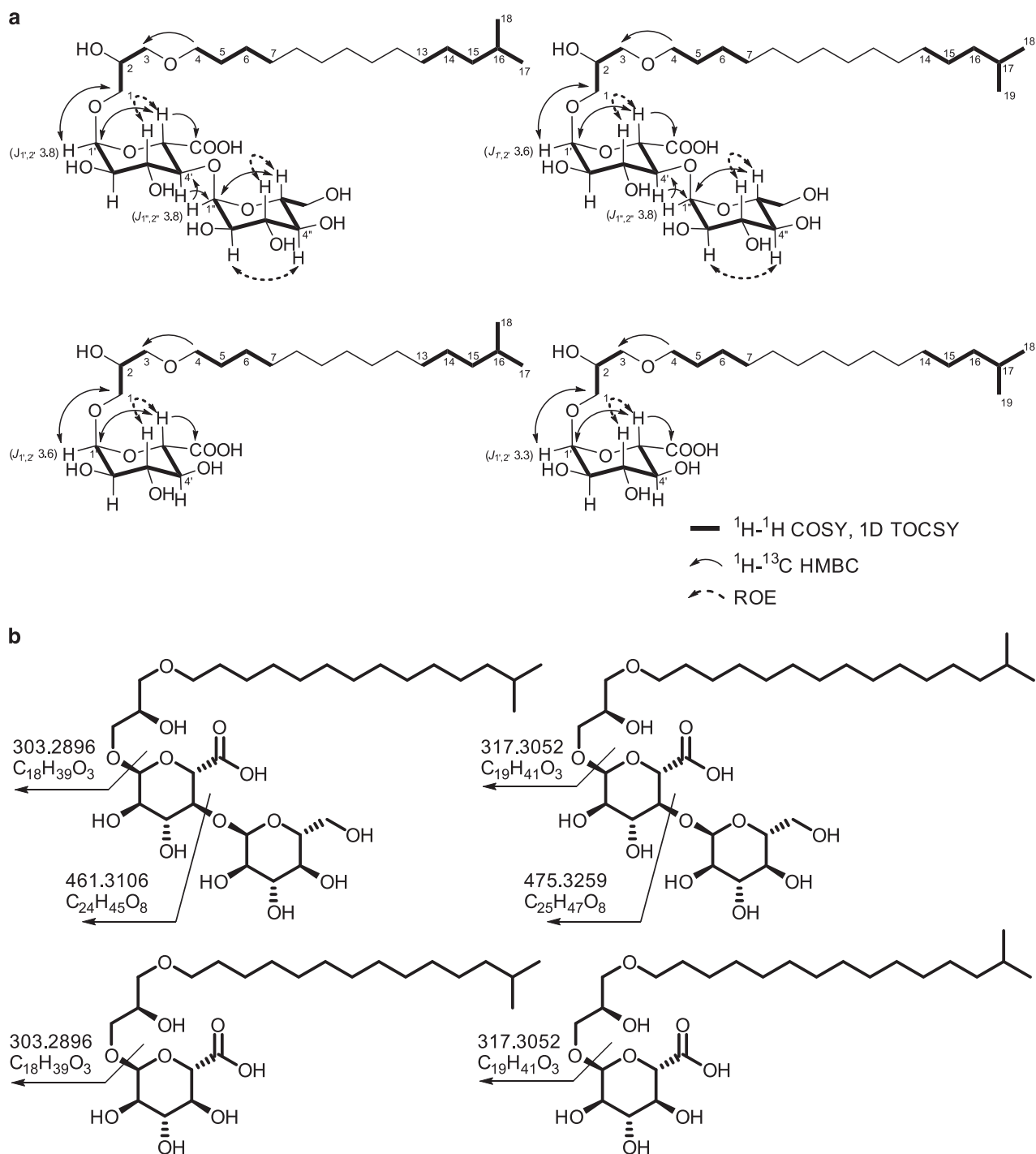


Figure 2 (a) COSY, HMBC and NOE analyses of the kribellosides. (b) ESI/MS fragment analysis of the kribellosides.

glucuronic acid. Furthermore, long-range connectivities were observed from H1' of the anomer proton of glucuronic acid to glyceryl oxymethylene of H1 and another glyceryl oxymethylene of H3 to H4 of the 13-methyl-1-tetradecanol moiety. Taken together, these results determined the planer structure of **1**. The length of the branched alkyl long chain was also established by the ESI/MS in source fragment (m/z 303.2896 ($\text{C}_{18}\text{H}_{39}\text{O}_3$)) as shown in Figure 2a.

The structures of compounds **2-4** were determined in the same manner and compared with the data for compound **1**. Comparisons of the molecular formula and the ^1H NMR spectra for **1** and **2** described

above suggested a lack of glucose moiety in compound **1**. Compound **2** was found to possess 1 carbonyl, 1 anomer, 5 oxymethines, 1 sp^3 methine, 3 oxygenated methylenes, 11 methylenes and 2 methyl carbons from the ^{13}C NMR and DEPT135 spectra. Furthermore, 1D and 2D spectral analyses revealed three parts, namely the glyceryl, 13-methyl-1-tetradecanol and glucuronic acid, that were the same as in compound **1**. The difference between compounds **1** and **2** was the higher field shifts from H2' to H5' of glucuronic acid, as observed in the ^1H NMR spectrum. The same in source fragments of ESI/MS spectra for compounds **1** and **2** also supported this structure

Table 1 ¹H and ¹³C NMR data for the kribbelosides in CDCl₃

Position	1			2			3			4					
	¹³ C (p.p.m.)	Multi- plicity	¹ H (p.p.m.)	Multi- plicity	¹³ C (p.p.m.)	¹ H (p.p.m.)	Multi- plicity	J in Hz	Multi- plicity	¹³ C (p.p.m.)	¹ H (p.p.m.)	Multi- plicity	J in Hz		
1	71.4	t	3.40	dd	7.3, 10.1	71.3	t	3.41	dd	7.3, 10.3	71.4	t	3.41	dd	7.3, 10.3
			3.83	dd	3.4, 10.1			3.84	dd	3.8, 10.3			3.84	dd	3.7, 10.3
2	70.8	d	3.96	m		70.8	d	3.95	m				3.94	m	
3	73.0	t	3.46	dd	5.2, 10.3	72.7	t	3.45	dd	5.6, 10.0	73.0	t	3.45	dd	5.7, 10.2
			3.49	dd	5.2, 10.3			3.49	dd	4.8, 10.0			3.48	dd	4.8, 10.2
4	72.7	t	3.46	m		73.0	t	3.45	m		72.7	t	3.46	m	
5	30.8	t	1.57	m		30.6	t	1.57	m		30.7	t	1.57	m	
6	27.2	t	1.34	m		27.2	t	1.35	m		27.2	t	1.36	m	
7	30.6	t	1.29	m		30.8	t	1.29	m		30.6	t	1.29	m	
8	30.7	t	1.29	m		30.8	t	1.29	m		30.8	t	1.29	m	
9	30.8	t	1.29	m		30.8	t	1.29	m		30.8	t	1.29	m	
10	30.8	t	1.29	m		30.8	t	1.29	m		30.8	t	1.29	m	
11	30.8	t	1.29	m		30.8	t	1.29	m		30.8	t	1.29	m	
12	30.8	t	1.29	m		30.7	t	1.29	m		30.8	t	1.29	m	
13	31.0	t	1.29	m		31.0	t	1.29	m		30.8	t	1.29	m	
14	28.5	t	1.29	m		28.5	t	1.29	m		31.0	t	1.29	m	
15	40.2	t	1.17	m		40.2	t	1.17	m		28.5	t	1.28	m	
16	29.1	d	1.52	m		29.1	d	1.52	m		40.3	t	1.17	m	
17	23.0	q	0.88	d	6.6	23.0	q	0.88	d	6.7	29.1	d	1.52	m	
18	23.0	q	0.88	d	6.6	23.0	q	0.88	d	6.7	23.0	q	0.88	d	6.6
19	-		-			-		-			23.0	q	0.88	d	6.6
1'	100.9	d	4.82	d	3.8	100.8	d	4.84	d	3.6	100.9	d	4.84	d	3.3
2'	73.2	d	3.51	dd	3.8, 9.4	73.9	d	3.44	m		73.2	d	3.52	dd	3.7, 9.6
3'	74.7	d	3.91	t	9.4	74.8	d	3.67	t	9.3	74.7	d	3.91	dd	9.0, 9.6
4'	73.9	d	3.71	t	9.4	73.5	d	3.44	t	9.3	73.9	d	3.72	dd	9.0, 9.8
5'	73.3	d	4.07	d	9.5	72.2	d	3.92	d	10.1	73.2	d	4.07	d	9.9
6'	175.2	s				176.7	s				175.3	s			176.0
1''	101.2	d	5.27	d	3.8						101.2	d	5.28	d	3.8
2''	74.2	d	3.37	dd	3.8, 9.5						74.1	d	3.38	dd	3.8, 9.7
3''	75.1	d	3.62	t	9.5						75.1	d	3.63	dd	9.1, 9.7
4''	71.6	d	3.26	t	9.5						71.6	d	3.26	dd	9.1, 9.6
5''	81.3	d	3.72	m							81.3	d	3.72	m	
6''	62.5	t	3.65	dd	5.0, 11.8						62.5	t	3.63	dd	4.8, 11.6
			3.81	dd	1.6, 11.8						3.81	dd		dd	2.0, 11.6

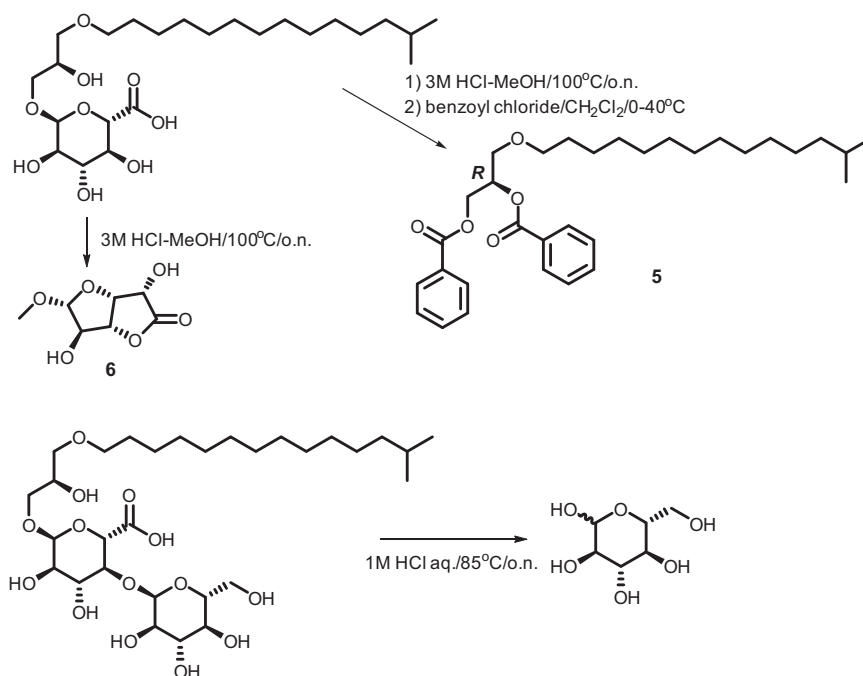


Figure 3 Degradation study of kribelloside A (1).

(Figure 2a). Thus, the structure of **2** was determined as shown in Figure 2. The structures of compounds **3** and **4** were similar to those of **1** and **2** with the exception of one methylene. The 2D analyses of compounds **3** and **4** revealed that they possessed 14-methyl-1-pentadecanol instead of the 13-methyl-1-tetradecanol moiety of compounds **1** and **2**. This finding was supported by MS/MS analysis. The structures of **3** and **4** were determined as shown in Figure 2b.

Stereochemistry of the kribellosides

The absolute chemistry of the C2 of glyceryl ether and the two sugar moieties of the kribellosides was determined by chemical degradations and their derivatives, as shown in Figure 3. Methanolysis of compound **2** indicated an aglycon and sugar moiety. The reaction mixture was partitioned by a solvent system of n-hexane-MeOH and the n-hexane layer containing aglycon was derivatized to dibenzoate **5**. Since the CD spectrum of compound **5** indicated a negative cotton effect, the C2 was determined to be *R* by the dibenzoate chirality method.¹⁴ The sugar moiety **6** described above was purified from the MeOH layer and identified as methyl- β -D-glucufuranuronic acid γ -lactone by comparing its NMR and CD spectra for the methanolysis product of D-glucuronic acid. Finally, the absolute chemistry of the glucose moiety in **1** was established to be *D* by the mutarotase-glucose oxidase method.¹⁵ Taking all of these results into account, the absolute chemistry of compounds **1** and **2** are shown in Figure 1. Compounds **3** and **4** showed the same stereochemistry, as indicated by the physico-chemical properties and the NMR data.

Kribellosides are novel compounds that belong to the ether lipids. Ether lipids are known to be ubiquitous in the cell membranes of mammals,¹⁶ marine organisms¹⁷ and archaea.¹⁸ These ether lipids, however, are 1-*O*-alkyl lipids, whereas the 3-*O*-alkyl lipids in kribellosides are rare. Therefore, the 1-*D*-glucuronosyl-*sn*-glycerol 3-alkyl ether is a unique structure among the ether lipids.

Table 2 Inhibitory activities of the kribellosides towards the RNA 5'-triphosphatase activities of Cet1p and hCap1

Compound	IC ₅₀ (μ M)		SI ^a
	Cet1p	hCap1	
Kribelloside A	6.8	26.1	3.8
Kribelloside B	6.3	17.4	2.8
Kribelloside C	3.6	16.1	4.4
Kribelloside D	4.5	9.3	2.1
Suramin	0.7	5.4	7.9

Abbreviation: SI, selectivity index.

^aThe SI as determined by the ratio of the IC₅₀ values between hCap1 and Cet1p.

Biological activities of the kribellosides

Cet1p inhibitory activity. We examined the inhibitory activity of kribellosides towards *S. cerevisiae* Cet1p using an *in vitro* enzyme assay. Since, suramin has been reported to inhibit Cet1p activity, and under our assay conditions it exhibited an IC₅₀ value similar to that previously reported (Table 2), confirming that our assay conditions with [γ -³²P]triphosphate-ended poly(A) were appropriate for evaluating the inhibitory effect of compounds on triphosphatase activity. Upon treatment with kribelloside A, the hydrolysis of the γ phosphate of triphosphate-ended RNA by Cet1p was inhibited in a dose-dependent manner, with an IC₅₀ of 6.8 μ M (Figure 4 and Table 2); whereas, kribellosides B, C and D inhibited Pi release from the RNA with IC₅₀ values of 6.3, 3.6 and 4.5 μ M, respectively. Furthermore, we evaluated the inhibitory potency of the compounds towards the RNA triphosphatase activity of human mRNA capping enzyme, hCap. Kribellosides A, B, C and D exhibited IC₅₀ values of 26.1, 17.4, 16.1 and 9.3 μ M, yielding selectivity indexes of 3.8, 2.8, 4.4 and 2.1, respectively (Table 2). These results indicated that kribellosides are novel inhibitors of *S. cerevisiae* RNA 5'-triphosphatase. Glycosyl-

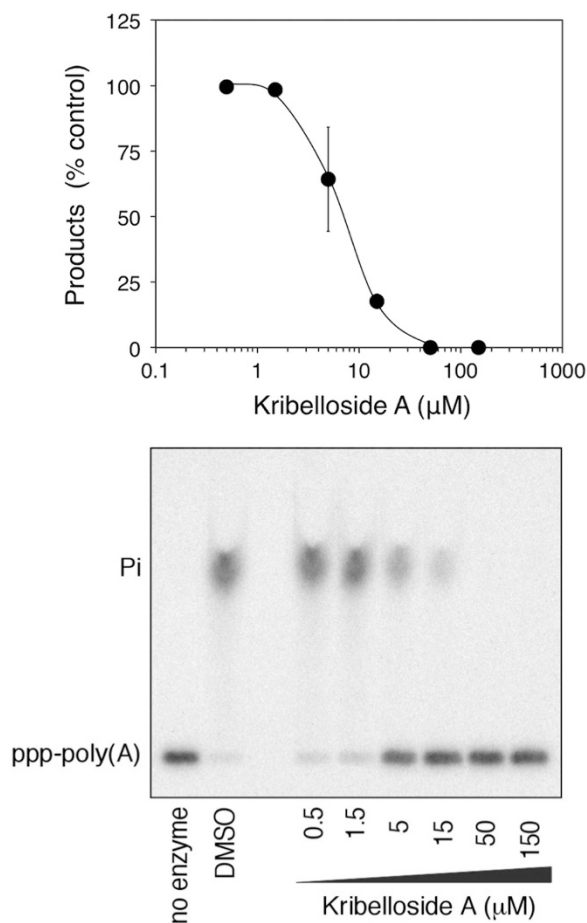


Figure 4 Inhibition of the RNA 5'-triphosphatase activity of Cet1p by kribelloside A. Triphosphatase activity by Cet1(201-549)p. The reaction was performed as described in the Materials and Methods using ^{32}P -triphosphate-ended poly(A). The reaction products were analyzed by PEI cellulose TLC (upper panel) and quantified by phosphorimaging (lower panel). The relative amount of the released $[\text{}^{32}\text{P}]\text{Pi}$ is shown as a percentage of that of the untreated control (DMSO). Results are shown as the means \pm s.d. from three independent experiments. DMSO, dimethyl sulfoxide; PEI, polyethyleneimine.

glycerol-alkyl ether has known biological activities, such as lipid hapten activity,¹⁹ antimicrobial activity²⁰ and the inhibition of cancer metastasis.²¹ The 1-D-glucuronosyl-sn-glycerol 3-alkyl ether of kribellosides might be advantageous in its selectivity for the yeast enzyme (Table 2).

Antimicrobial activity. We evaluated the antimicrobial activity of kribellosides against *S. cerevisiae*. The anti-yeast activities of kribellosides are shown in Table 3. Kribellosides exhibited weak anti-yeast activities against *S. cerevisiae* but no antibacterial activity against Gram-positive and Gram-negative bacteria (data not shown).

MATERIALS AND METHODS

General experimental procedures

The optical rotations of the isolated kribellosides were measured using a P-1030 polarimeter (JASCO, Tokyo, Japan). UV spectra were recorded using a U-2800 spectrophotometer (Hitachi High Technologies, Tokyo, Japan) and an FT/IR-4100 Fourier transform infrared spectrometer (JASCO) was employed. The ^1H and ^{13}C NMR spectra were measured using a JNM-ECA600 spectrometer (JEOL, Tokyo, Japan) at 25 °C using tetramethylsilane as an internal

Table 3 Antimicrobial activities of the kribellosides

Test organisms	MIC ($\mu\text{g ml}^{-1}$)			
	A	B	C	D
<i>S. cerevisiae</i> W303a	100	50	100	12.5
<i>S. cerevisiae</i> W303 α	100	50	100	25
<i>S. cerevisiae</i> SL101	50	50	100	12.5
<i>S. cerevisiae</i> HC101	50	25	50	12.5
<i>S. cerevisiae</i> BY25929	25	12.5	12.5	3.12

reference. The mass spectra were recorded using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). HPLC was detected using an Evaporative Light Scattering Detector (Alltech Associates, Inc., Deerfield, IL, USA).

Taxonomic studies of the producing strain MI481-42F6

Morphological properties were observed following incubation at 30 °C for 21 days on yeast extract-malt extract agar (ISP medium No. 2), oatmeal agar (ISP medium No. 3), inorganic salts-starch agar (ISP medium No. 4), glycerol-asparagine agar (ISP medium No. 5) and tyrosine agar (ISP medium No. 7). Detailed observation of mycelial morphology was performed using a scanning electron microscope S-570 (Hitachi High-Technologies) after strain MI481-42F6 had been incubated on ISP medium No. 3 at 30 °C for 14 days. The type of diaminopimelic acid isomers in the whole-cell hydrolysates was determined by the method of Stanek and Roberts.²² Total DNA of strain MI481-42F6 was prepared using a Genomic DNA Extraction Kit Mini (RBC Bioscience Co., New Taipei, Taiwan) according to the manufacturer's instructions. 16S rRNA (positions 31–1524, *Escherichia coli* numbering system)²³ was amplified by PCR and sequenced. A search for the most closely related sequences was performed using the BLAST algorithm in at the DNA Data Bank of Japan.

Fermentation of kribellosides

A slant culture of strain MI481-42F6 was inoculated into a 500-ml baffled Erlenmeyer flask containing 110 ml of a seed medium consisting of 2% (w/v) galactose, 2% (w/v) dextrin, 1% (w/v) Bacto Soytone (Becton Dickinson, Franklin Lakes, NJ, USA), 0.5% (w/v) corn steep liquor (Kogo starch, Chiba, Japan), 1% (w/v) glycerol, 0.2% (w/v) $(\text{NH}_4)_2\text{SO}_4$ and 0.2% (w/v) CaCO_3 in deionized water (pH 7.4 before sterilization). The seed culture was incubated on a rotary shaker (200 r.p.m.) at 30 °C for four days. The seed culture (2.5 ml) of the strain was then transferred into a 500-ml baffled Erlenmeyer flask containing 110 ml of a producing medium consisting of 2.0% glycerin, 2.0% dextrin, 1.0% Bacto Soytone (Becton Dickinson), 0.3% yeast extract (Nihon Pharmaceutical Co., Ltd, Tokyo, Japan), 0.2% $(\text{NH}_4)_2\text{SO}_4$ and 0.2% CaCO_3 in deionized water (pH 7.4 before sterilization). The fermentation was carried out on a rotary shaker (180 r.p.m.) at 27 °C for 7 days.

Purification of kribellosides

The fermentation broth (4 l) was separated into the mycelial cake and supernatant by centrifugation. The mycelial cake was extracted with MeOH (1 l). The MeOH solution was removed *in vacuo* to yield a brown oil. The brown oil, including an active substance, was extracted with BuOH and concentrated *in vacuo* to give 1.4 g of a brown oil. The 1.4 g of brown oil was extracted with a mixture of CHCl_3 -MeOH-water (5:4:6, 1 l). The upper layer was collected and concentrated *in vacuo* to dryness yielding a brown material (764 mg). The brown material was subjected to chromatography on a low-pressure reversed-phase (20 \times 250 mm, ODS-7515-12 A, Senshu Scientific co., Tokyo, Japan) column developed with MeOH:ammonium carbonate (5 mM) at a ratio of 0:100, 60:40, 80:20 and 100:0. The active fractions were eluted with a ratio of 80:20 and 0:100, and concentrated *in vacuo* to give a pale brown oil (427 mg). The active material was subjected to further chromatography on reversed-phase HPLC (Capcell Pak UG C18, 30 \times 250 mm, Shiseido Co., Ltd., Japan) developed with acetonitrile:ammonium carbonate (5 mM) at

5:95 (0 min) to 50:50 (60 min) and 50:50 (80 min) at a flow rate of 10 ml min⁻¹. The active fractions were collected and concentrated *in vacuo* to yield 79 mg of pure kribbeloside A (rt: 61–63 min), 83 mg of kribbeloside B (rt: 63–64 min), 26 mg of kribbeloside C (rt: 65–66 min) and 13 mg of kribbeloside D (rt: 67–68 min), respectively, as a colorless powder.

Analytical procedure

The kribbelosides in the fermentation broth and the various purification steps were monitored by reversed-phase HPLC and silica gel TLC. HPLC was performed on a reversed-phase HPLC column (Capcell Pak UG C18, 4.6 × 150 mm, Shiseido, Japan; mobile phase, acetonitrile:ammonium carbonate (5 mM) at a ratio of 45:55; flow rate, 1 ml min⁻¹; column temperature, 25 °C; detection, evaporative light scattering detector). Kribbelosides A, B, C and D were eluted at 6.5, 8.1, 10.3 and 12.8 min, respectively. A spot of each antibiotic on TLC plates was detected by TLC (Kieselgel 60 F254, Art. No. 5715, Merck) using a solution of molybdophosphoric acid and sulfuric acid in water. The resulting R_f values for kribbelosides A, B, C and D were 0.26, 0.36, 0.29 and 0.40, respectively, using the solvent system of CHCl₃:MeOH:H₂O at a ratio of 10:5:1.

(R)-3-((13-methyltetradecyl)oxy)propane-1,2-diyl dibenzoate (5)

Compound 2 (27.0 mg) was dissolved in 3 M of hydrogen chloride-methanol and stirred at 100 °C for 14 h. The solution was added to 6 ml of *n*-hexane and partitioned. The *n*-hexane layer was dried to yield 10.3 mg of crude aglycon. The methanol layer was dried and re-dissolved in 100 ml of methanol and 1.8 ml of acetonitrile and then centrifuged at 2500 r.p.m. for 5 min. The supernatant was concentrated and subjected to hydrophilic interaction liquid chromatography (PC HILIC, 20 × 250 mm, Shiseido, 97% acetonitrile). The eluent of fraction No. 18 containing aglycon was combined with the residue of the *n*-hexane layer, then evaporated *in vacuo* to yield 13.0 mg of crude aglycon. The residue was dissolved in 1.5 ml of dichloromethane and benzoyl chloride (14.3 mg, Tokyo Chemical Industry Co. Ltd.) and potassium carbonate (9.0 mg) were added and stirred at 0 °C to 40 °C gently for 13 h. The reaction mixture was filtered and subjected to preparative TLC (Kieselgel 60 F254, Art. No. 5715) developed with *n*-hexane:ethylacetate at a ratio of 2:1 to give 1.5 mg of compound 5 as a colorless syrup.

(R)-3-((13-methyltetradecyl)oxy)propane-1,2-diyl dibenzoate (5): colorless syrup; ¹H NMR (CDCl₃, 600 MHz) δ 8.05 (2H, m, Ph), 8.02 (2H, m, Ph), 7.56 (1H, m, Ph), 7.55 (1H, m, Ph), 7.43 (2H, m, Ph), 7.42 (2H, m, Ph), 5.59 (1H, m, H2), 4.68 (1H, dd, J = 3.7, 11.9 Hz, H1a), 4.61 (1H, dd, J = 6.5, 11.9 Hz, H1b), 3.79 (1H, dd, J = 5.3, 10.5 Hz, H3a), 3.76 (1H, dd, J = 5.2, 10.5 Hz, H3b), 3.50 (1H, m, H1'), 1.24 (1H, m, H2'), 1.31 (1H, m, H3'), 1.24 (16H, m, H4'-11'), 1.15 (2H, m, H12'), 1.51 (1H, m, H13'), 0.86 (6H, d, J = 6.6 Hz, H14', 15), ¹³C NMR (CDCl₃, 150 MHz) δ 166.3 (benzyl CO), 165.9 (benzyl CO), 133.14 (Ph), 133.09 (Ph), 130.0 (Ph), 129.9 (Ph), 129.8 (Ph), 129.8 (Ph), 129.7 (Ph), 129.7 (Ph), 128.4 (Ph), 128.4 (Ph), 128.4 (Ph), 128.4 (Ph), 71.9 (C-4), 71.1 (C-2), 69.0 (C-3), 63.7 (C-1), 39.1 (C-15), 30.0, 29.74, 29.69, 29.69, 29.61, 29.61, 29.5, 26.1 (C-7-14), 29.62 (C-5), 28.0 (C-16), 27.4 (C-6), 22.7 (C-17), 22.7 (C-18); HRESI/MS [M+Na]⁺ *m/z* 533.3227 (calcd for C₃₂H₄₆O₅Na, 533.3237).

Methyl β-D-glucofuranosiduronic acid γ-lactone from 2 and D-glucuronic acid (6 and 6')

The fraction No. 26 to 27 obtained by hydrophilic interaction liquid chromatography described above was evaporated *in vacuo* to give 2.0 mg of methyl-β-D-glucofuranosiduronic acid γ-lactone (6) as a colorless syrup: ¹H NMR (600 MHz, CD₃OD) δ 4.91 (1H, s, H1), 4.91 (1H, dd, J = 4.6, 6.5 Hz, H4), 4.80 (1H, d, J = 4.6 Hz, H3), 4.49 (1H, d, J = 6.4 Hz, H5), 4.18 (1H, s, H2), 3.33 (3H, s, OCH₃). ¹³C NMR (CD₃OD) δ 177.1 (C=O), 111.6 (C-1), 84.7 (C-3), 79.5 (C-4), 78.6 (C-2), 70.6 (C-5), 55.7 (OCH₃); HRESI/MS [M+Na]⁺ *m/z* 213.0372 (calcd for C₇H₁₀O₆Na, 213.0370).

As an authentic sample of compound 6, 38.0 mg of D-glucuronic acid (Tokyo Chemical Industry Co. Ltd.) was dissolved in 3 M of hydrogen chloride-methanol and stirred at room temperature for 1 h. The reaction mixture was concentrated and the residue was applied for hydrophilic interaction liquid chromatography using the same method described above.

Fractions No. 26 and 27 were collected and concentrated to dryness to give 16 mg of a colorless syrup of methyl-β-D-glucofuranosiduronic acid γ-lactone (6'): ¹H NMR (600 MHz, CD₃OD) δ 4.92 (1H, s, H1), 4.91 (1H, dd, J = 4.6, 6.4 Hz, H4), 4.81 (1H, d, J = 4.6 Hz, H3), 4.50 (1H, d, J = 6.4 Hz, H5), 4.18 (1H, s, H2), 3.33 (3H, s, OCH₃). ¹³C NMR (CD₃OD) δ 177.1 (C=O), 111.6 (C-1), 84.7 (C-3), 79.4 (C-4), 78.5 (C-2), 70.5 (C-5), 55.7 (OCH₃); HRESI/MS [M+Na]⁺ *m/z* 213.0373 (calcd for C₇H₁₀O₆Na, 213.0370).

Mutarotase-glucose oxidase method

Compound 1 (2.3 mg) was dissolved in 1 M HCl (1 ml) and the solution was heated at 85 °C for 12 h. The reaction mixture was dried to remove the acid. The residue was dissolved in water and washed with ethylacetate. The aqueous solution was evaporated *in vacuo*. The residue was dissolved in water (200 μl, 1 ×), and its concentration was halved (2 ×) for the sample solution. Standard D-glucose solutions (100, 500, 1000 and 2000 μg ml⁻¹) and negative controls of the L-glucose solution (1000 μg ml⁻¹) and D-glucuronic acid (1000 μg ml⁻¹) were prepared. The detection of D-glucose was performed using the mutarotase-glucose oxidase method.¹⁸ The sample, standard, negative control and blank solutions (each 20 μl) were added to 3 ml of glucose CII kit enzyme solution at 37 °C for 5 min. Then, the reaction mixtures were measured at an absorption of 505 nm. For the standard D-glucose solutions (0, 500, 1000 and 2000 μg ml⁻¹), OD values of 0, 0.097, 0.217 and 0.462 were recorded, and for the 1 × and 2 × sample solutions, OD values of 0.374 and 0.188 were recorded, respectively. No absorption was detected for the 1000 μg ml⁻¹ solution of L-glucose and D-glucuronic acid.

Screening system for RNA 5'-triphosphatase inhibitor

Nucleoside triphosphatase assay. The *S. cerevisiae* Cet1(201-549)p was expressed in *Escherichia coli* as an N-terminal hexahistidine (His)-tagged protein and purified by nickel-agarose and ion exchange chromatography. An nucleoside triphosphatase assay with Cet1(201-549)p was performed on a 96-well microplate for initial screening of metabolites from actinomycetes. Aliquots of 40 μl of a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 2 mM MnCl₂ and 8 nM Cet1(201-549)p were dispensed into each well, and then 5 μl of test sample was added to the mixture. After preincubation for 5 min, the reaction was started by adding 5 μl of 400 μM ATP. After incubation at 30 °C for 1 h, 75 μl of MicroMolar Phosphate Assay Reagent (ProFoldin, Hudson, MA, USA) was added, and the mixtures were incubated for 5 min. The absorbance at 620 nm was read using a plate reader (ARVOSX 1420 Multilabel counter; PerkinElmer, Waltham, MA, USA).

RNA 5'-triphosphatase assays for Cet1p and hCap

The hCap1 was expressed in *E. coli* as an N-terminal His-tagged protein and purified by nickel-agarose and ion exchange chromatography. RNA 5'-triphosphatase assays for Cet1p and hCap were performed as described previously with a minor modification.^{24,25} The reaction mixtures for Cet1p containing 50 mM Tris-HCl (pH 7.9), 0.5 mM MgCl₂, 40 μg of bovine serum albumin and 4 nM Cet1(201-549)p, and for hCap1 containing 50 mM Tris-HCl (pH 7.9), 2 mM DTT, 40 μg of bovine serum albumin and 0.23 nM hCap in a final volume of 10 μl were preincubated with various compounds for 10 min. The reaction was then started by adding 50 nM [³²P]-radiolabeled triphosphate-ended poly(A). After incubation at 30 °C for 10 min, the reaction products were analyzed by polyethyleneimine cellulose TLC with 0.5 M potassium phosphate buffer (pH 3.4). The TLC plate was exposed to an imaging plate and visualized using the phosphorimager Typhoon Variable Model Imager (GE Healthcare, Piscataway, NJ, USA), and the 50% inhibition concentration (IC₅₀) was calculated by four-parameter logistic curve fitting.

Antimicrobial activity

MICs were determined by the standard agar dilution method recommended by the Clinical Laboratory Standards Institute (CLSI) guidelines.²⁶ Bacteria were incubated on Mueller-Hinton agar (Becton Dickinson) at 37 °C for 18 h, whereas yeast were incubated for 42 h.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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