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YCM1008A, a Novel Ca^{2+}-Signaling Inhibitor, Produced by *Fusarium* sp. YCM1008

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Abstract In the course of screening for drugs that suppress the Ca^{2+}-mediated growth inhibition in a yeast mutant, we found that the metabolite of *Fusarium* sp. strain YCM1008 inhibited Ca^{2+}-signaling. A novel pyrano-pyridone, YCM1008A was isolated from the fermentation broth using HLB column chromatography followed by HPLC, and the structure was elucidated by spectral analysis. YCM1008A suppressed Ca^{2+}-induced growth inhibition of the *Saccharomyces cerevisiae* (*Δzds1Δsyr1*) mutant.

Keywords YCM1008A, pyrano-pyridone, *Fusarium,* Ca^{2+}-signaling, yeast

Since the basic cellular functions are fairly well conserved from yeast to mammalian cells, it is possible to use the yeast *Saccharomyces cerevisiae* as a model microorganism for eukaryotes and a useful tool for drug screening. Cell-cycle progression in the G_2 period is specifically blocked by hyperactivation of the Ca^{2+}-signaling pathway in media with high concentrations of $CaCl_2$. Cell-cycle regulation *via* Ca^{2+} is affected by two parallel pathways, one is the calcineurin and the other is the Mpk1 MAP kinase cascade [1, 2]. These pathways together activate Swe1 to phosphorylate the Cdc28 cyclin-dependent protein kinase and suppress the transition to the M period of the cell-cycle, leading to G_2 arrest [3]. Miyakawa *et al.* established a screening assay system for drugs that suppress Ca^{2+}-induced growth inhibition [4] using the *Δzds1Δsyr1* yeast mutant. In fact, the calcineurin inhibitors FK-506 and cyclosporin A have been detected in this system, and MAP kinase inhibitors are also expected to be identified in this assay. In the course of this screening, we found that the metabolite of *Fusarium* sp. strain YCM1008 inhibited the Ca^{2+}-signaling, and we isolated a novel Ca^{2+}-signaling inhibitor, YCM1008A. In this paper, we describe its fermentation, isolation, and structure elucidation.

The strain YCM1008 was isolated from a soil sample collected in Indonesia and identified as a fungus, *Fusarium* sp.

A loopful of the strain YCM1008, grown on agar slant, was inoculated into 50-ml test tubes containing 10 ml of seed medium composed of 3.0% dried mashed potatoes, 10% glucose, and 0.5% yeast extract, in deionized water (pH 7.2 before sterilization). After the inoculated test tubes were incubated for 3 days at 25°C in a reciprocal shaker,

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3.3 ml of the seed culture was added to a 300-ml Erlenmeyer flask containing 50 ml of the fermentation medium composed of 3.0% sucrose, 2.0% soluble starch, 0.5% Ebios, 1.0% malt extract, 0.5% corn steep liquor, 20% V8 vegetable juice, 0.5% calcium carbonate in deionized water (pH 6.5 before sterilization). Fermentation was carried out for 5 days at 25°C with agitation of 200 rpm.

YCM1008A was purified from mycelia obtained by filtration of fermented broth (300 ml). The mycelia cake was extracted with 300 ml of methanol. The extract was diluted with 1.2 liters of deionized water and added to an HLB column (Waters). After washing the column with 20, 40, 60, 80% methanol, respectively, the active principles were eluted from the column with methanol. The active fraction containing YCM1008A was concentrated *in vacuo* to yield crude YCM1008A. This concentration was dissolved in methanol and purified by preparative HPLC using a column (ODS HG-5, Develosil) with 70% acetonitrile as elution solvent. Active fractions containing YCM1008A were combined and evaporated, and YCM1008A (1.0 mg) was obtained as pale yellow amorphous powder.

The *Δzds1 Δsyr1* cells were suspended at a cell concentration of 1×10^5 cells/ml in soft-agar medium then placed on YPD-agar plates containing 170 mM of $CaCl_2$. YCM1008A (3 nmol) dissolved in dimethylsulfoxide was spotted on top of the agar. The growth of *Δzds1 Δsyr1* cells was severely inhibited by $CaCl_2$. However, numerous microcolonies appeared after 2 days of incubation at 28°C around the spot loaded with YCM1008A, giving rise to a doughnut-like halo with a clear zone in the center (21 mm outside diameter and 7.2 mm inside diameter), thus indicating that YCM1008A inhibited Ca^{2+}-signaling and that it inhibited cell growth at high concentrations.

Table 1 Physico-chemical properties of YCM1008A

Appearance	Pale yellow amorphous
$[\alpha]_D^{23}$	−58.1° (*c* 0.03, MeOH)
Molecular formula	$C_{21}H_{29}NO_4$
FAB-MS *m/z*	382 $(M+Na)^+$, 360 $(M+H)^+$
HR FAB-MS	
Found	360.2177 $(M+H)^+$
Calcd	360.2175 (for $C_{21}H_{30}NO_4$)
TLC (Rf)	
$CHCl_3$-MeOH (97 : 3)[a]	0.3
Color reaction	
Positive	I_2
Solubility	
Soluble	MeOH

[a] Silica gel $60F_{254}$ plate (Merck).

Physico-chemical properties of YCM1008A are summarized in Table 1. The molecular formula was determined by high resolution FAB-MS as $C_{21}H_{29}NO_4$. The ^{13}C-NMR spectrum (Table 2) showed 21 signals assigned as follows: four methyls, one methoxy (δ 65.5), one methylene, four methines, seven sp^2 methines, and four quaternary carbons. Analyses of ^{1}H-^{1}H COSY (Fig. 1) revealed presence of three partial structures shown in Fig. 2. Furthermore, HMBC spectrum exhibited long-range correlation: from H-1′ to C2 (δ 84.9) and from H-2 to C1′ (δ 11.4) and C3′ (δ 132.4), indicating connection between the partial structures I and II. Configurations of the three double bonds in the acyl side chain of partial structure I were determined on the basis of coupling constants and NOE experiment. The coupling constants (11 or 15 Hz) of H-4′, H-5′, H-6′, and H-7′ and NOE data between H-1′ and H-4′, H-3′ and H-5′, H-4′ and H-6′, H-5′ and H-7′, and H-6′ and H-8′ showed that the three double bonds in partial structure I have the *E*-isomer. The coupling constant (7.9 Hz) of H-7 and H-8 showed that the double bond in partial structure III has the *Z*-isomer.

The HMBC spectrum exhibited long range correlations from H-7 to C5 (δ 160.8) and C8a (δ 163.8) and from H-8 to C4a (δ 112.5) indicated formation of a 6-membered ring

Table 2 NMR spectral data for YCM1008A

No.	δ_C[a] (ppm)	δ_H[b] (ppm, multi-, *J* in Hz)
2	84.9 d	4.39, 1H, d, *J*=11.3
3	35.7 d	1.92, 1H, m
4	63.0 d	4.66, 1H, d, *J*=3.0
4a	112.5 s	
5	160.8 s	
7	136.4 d	7.72, 1H, d, *J*=7.9
8	101.5 d	5.98, 1H, d, *J*=7.9
8a	163.8 s	
9	12.9 q	0.93, 3H, d, *J*=6.9
10	65.5 q	3.99, 3H, s
1′	11.4 q	1.76, 3H, d, *J*=1.1
2′	133.6 s	
3′	132.4 d	6.15, 1H, d, *J*=11.0
4′	126.8 d	6.41, 1H, dd, *J*=14.8, 11.0
5′	136.3 d	6.28, 1H, dd, *J*=14.8, 10.8
6′	130.4 d	6.14, 1H, dd, *J*=14.9, 10.8
7′	142.7 d	5.63, 1H, dd, *J*=14.9, 8.0
8′	40.0 d	2.10, 1H, m
9′	30.8 t	1.34, 2H, m
10′	12.1 q	0.87, 3H, t, *J*=7.4
11′	20.5 q	1.01, 3H, d, *J*=6.7

[a] ^{13}C-NMR spectra were recorded at 125 MHz in CD_3OD.

[b] ^{1}H-NMR spectra were recorded at 500 MHz in CD_3OD.

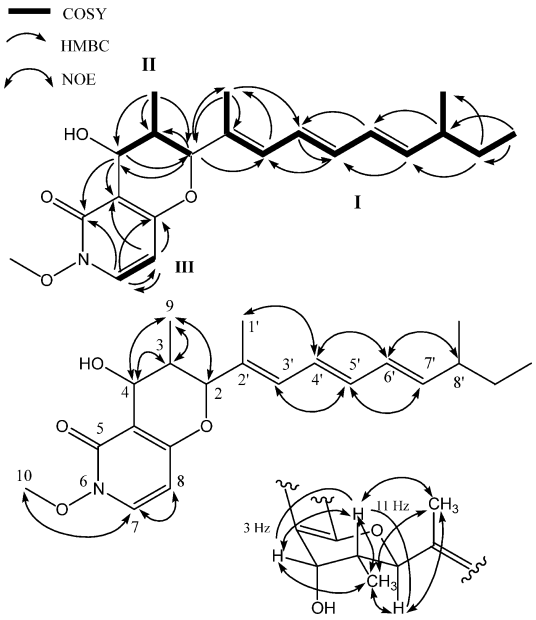

Fig. 1 Summary of COSY, HMBC, and NOESY data for YCM1008A.

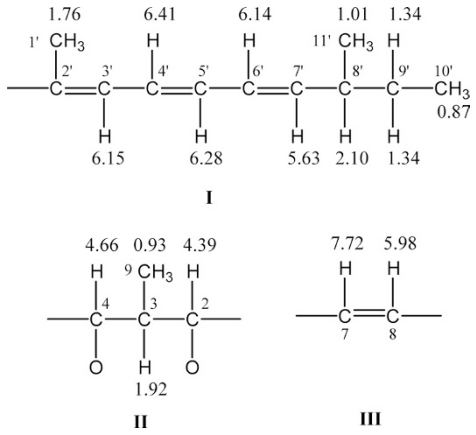

Fig. 2 Partial structures of YCM1008A.

with these carbons and partial structure III. NOE data between H-10 methoxy proton and H-7, absence of long range correlation from H-10 methoxy proton to H-7, and the chemical shift of C7 carbon (136.4 ppm) and C5 carbon (160.8 ppm) indicated these carbons were attached to the *N*-methoxy group. The long range correlations from H-4 to C4a and C5 show the connection between the partial structures II and III, and the chemical shifts of C2 carbon (84.9 ppm) and C8a carbon (163.8 ppm) indicate they are attached to oxygen forming a 6-membered ether ring system. Finally, detailed analysis of the HMBC and NOE spectrum, and taking account of the above three partial structures revealed the planar structure of YCM1008A as

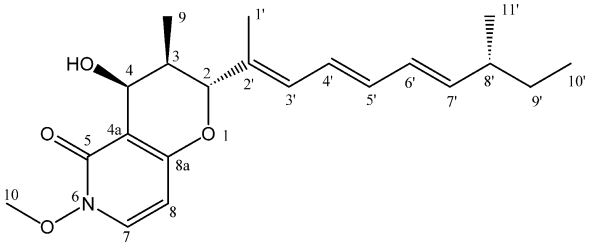

Fig. 3 Structure of YCM1008A.

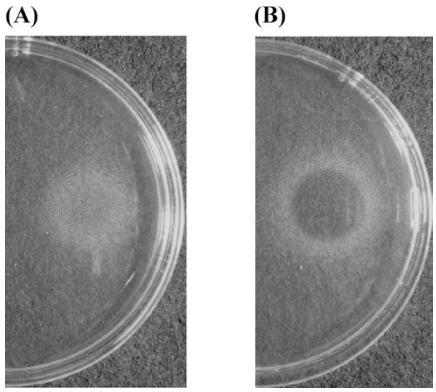

Fig. 4 Suppression of Ca^{2+}-induced growth inhibition of *Saccaromyces cerevisiae* mutants *via* synthetic YCM1008A (5 nmol) on YPD-agar plates containing 170 mM of $CaCl_2$.

(A) A doughnut-like halo in *Δzds1Δsyr1* mutant. (B) A doughnut-like halo in *Δzds1 Δsyr1 Δpdr1 Δpdr3* mutant.

described in Fig. 1.

The relative configuration of the 6-membered ether ring in YCM1008A was determined by coupling constants and NOE experiment as shown in Fig. 1. Observation of NOEs between H-4 and H-3, H-3 and H-9, H-4 and H-9, H-2 and H-9, and in addition a large coupling constant between H-2 and H-3 (J=11 Hz) indicated that H-2 and H-3 were of 1,2-diaxial orientation and that 9-CH_3 and H-4 were of equatorial orientation. These results reveal the relative configuration of the 6-membered ether group shown in Fig. 3. By total synthesis, the planar structure and relative configuration of YCM1008A were confirmed, and furthermore the absolute configuration of YCM1008A was also determined as 2*R*/3*R*/4*S*/8′*R*, respectively, as shown in Fig. 3 [5]. The synthetic compound indicated equipotent activity to that of natural product, YCM1008A in the east assay system described above. Namely, YCM1008A (5 nmol) gave a doughnut-like halo (24 mm outside diameter and 12 mm inside diameter) in *Δzds1 Δsyr1* mutant (Fig. 4A). Furthermore, YCM1008A (5 nmol) also afforded a larger doughnut-like halo (30 mm outside diameter and 16 mm inside diameter) in *Δzds1 Δsyr1 Δpdr1 Δpdr3* mutant (Fig.

4B). Two homologous genes, *PDR1* and *PDR3* encode major transcription factors that regulate the expression of various genes that function in the multidrug resistance [6]. Thus, the *Δzds1 Δsyr1 Δpdr1 Δpdr3* quadruplex mutant seems to be hypersensitive to YCM1008A. YCM1008A possesses a unique *N*-methoxy pyrano-pyridone structure.

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References

1. Nakamura T, Ohmoto T, Hirata D, Tsuchiya E, Miyakawa T. Genetic evidence for the functional redundancy of the calcineurin- and Mpk1-mediated pathways in the regulation of cellular events important for growth in *Saccharomyces cerevisiae*. Mol Gen Genet 251: 211–219 (1996)
2. Mizunuma M, Hirata D, Miyahara K, Tsuchiya E, Miyakawa T. Role of calcineurin and Mpk1 in regulating the onset of mitosis in budding yeast. Nature 392: 303–306 (1998)
3. Booher RN, Deshaies RJ, Kirschner MW. Properties of *Saccharomyces cerevisiae* wee1 and its differential regulation of p34CDC28 in response to G1 and G2 cyclins. EMBO J 12: 3417–3426 (1993)
4. Shitamukai A, Mizunuma M, Hirata D, Takahashi H, Miyakawa T. A positive screening for drugs that specifically inhibit the Ca^{2+}-signaling activity on the basis of the growth promoting effect on a yeast mutant with peculiar phenotype. Biosci Biotechnol Biochem 64: 1942–1946 (2000)
5. Tatsuta K, Yamaguchi T, Tsuda Y, Yamaguchi Y, Hattori N, Nagai H, Hosokawa S. The first total synthesis and structural determination of YCM1008A. Tetrahedron Lett 48: 4187–4190 (2007)
6. Miyamoto Y, Machida K, Mizunuma M, Emoto Y, Sato N, Miyahara K, Hirata D, Usui T, Takahashi H, Osada H, Miyakawa T. Identification of *Saccharomyces cerevisiae* isoleucyl-tRNA synthase as a target of the G_1-specific inhibitor reveromycin A. J Biol Chem 277: 28810–28814 (2002)