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SPECIAL FEATURE: BRIEF COMMUNICATION

YO-001A, a new antifungal agent produced by *Streptomyces* sp. YO15-A001

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Abstract

A new antifungal compound YO-001A was found from the culture broth of *Streptomyces* sp. YO15-A001, which was isolated from a soil sample collected in Toyama Prefecture. YO-001A was identified through morphological changes-based screening of the rice blast fungus, *Pyricularia oryzae* (*P. oryzae*). YO-001A is a new 26-membered macrolide of the oligomycin family, which exhibits potent antifungal activity against *P. oryzae* with an IC_{50} of 0.012 μM by disrupting mitochondrial respiration via inhibition of the F_OF_1-ATPase activity.

Rice blast disease, caused by the ascomycete fungus *Pyricularia oryzae* (*P. oryzae*), is one of the most damaging fungal diseases that leads to the reduction of rice production worldwide [1]. Several fungicides have been developed to control the disease; however, pathogenic fungi often acquire resistance to fungicides [2]. Thus, new antifungal agents need to be developed continuously to counter this disease.

Isono et al. at RIKEN previously carried out a screening for antifungal agents and identified polyoxins, nucleoside antibiotics, from the culture broth of *Streptomyces cacaoi* [3]. Polyoxins were then successfully developed as an agrochemical [4]. Polyoxins inhibit cell wall synthesis [5], leading to swelling of fungal cells; a characteristic morphological change caused by polyoxins [6]. Since then, we have continued the screening based on the changes in fungal cell morphology. Recently, we have constructed a database of the morphological changes in *P. oryzae* induced by the well-characterized antifungal agents and used it to screen for new fungicides [7].

In the course of screening, we found a new antifungal compound, *YO-001A* (Fig. 1a), from the culture broth of *Streptomyces* sp. YO15-A001. This strain was isolated from a soil sample collected at Tateyama, Toyama Prefecture, Japan. A BLAST search based on its 16S rRNA sequence revealed that YO15-A001 strain was designated as *Streptomyces* sp. A loopful of slant culture of YO15-A001 was inoculated into a 500-mL cylindrical flask containing 70 mL of SY medium (0.1% yeast extract (BD), 1% soluble starch (nacalai tesque), and 0.1% N-Z amine type A (Wako)). The seed culture was incubated at 28 °C for 3 days on a rotary shaker at 180 rpm. One milliliter of the seed culture was inoculated into five hundred milliliters cylindrical flasks containing seventy milliliters of oatmeal medium (6% oatmeal (Quaker Oats)), and then incubated at 28 °C for 4 days on a rotary shaker at 180 rpm. The culture broth (1.4 L) was adjusted to pH 9.0 with NaOH and extracted with EtOAc. The extracted oil sample was partitioned with Hexane/MeOH/H_2O (10:9:1). The MeOH layer was collected and evaporated in vacuo to yield 297 mg of the extract, which was then fractionated via centrifugal partition chromatography (CPC) with $CHCl_3$/MeOH/H_2O (5:6:4). The active fractions were refractionated via CPC with Hexane/EtOAc/MeCN (5:1:4) and further purified via reverse-phase high-performance liquid chromatography using a C18 column with a linear gradient of acetonitrile/H_2O to yield YO-001A (7.2 mg), having the following

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This article is dedicated to Dr Kiyoshi Isono with respect and admination for his achievement in antibiotics research

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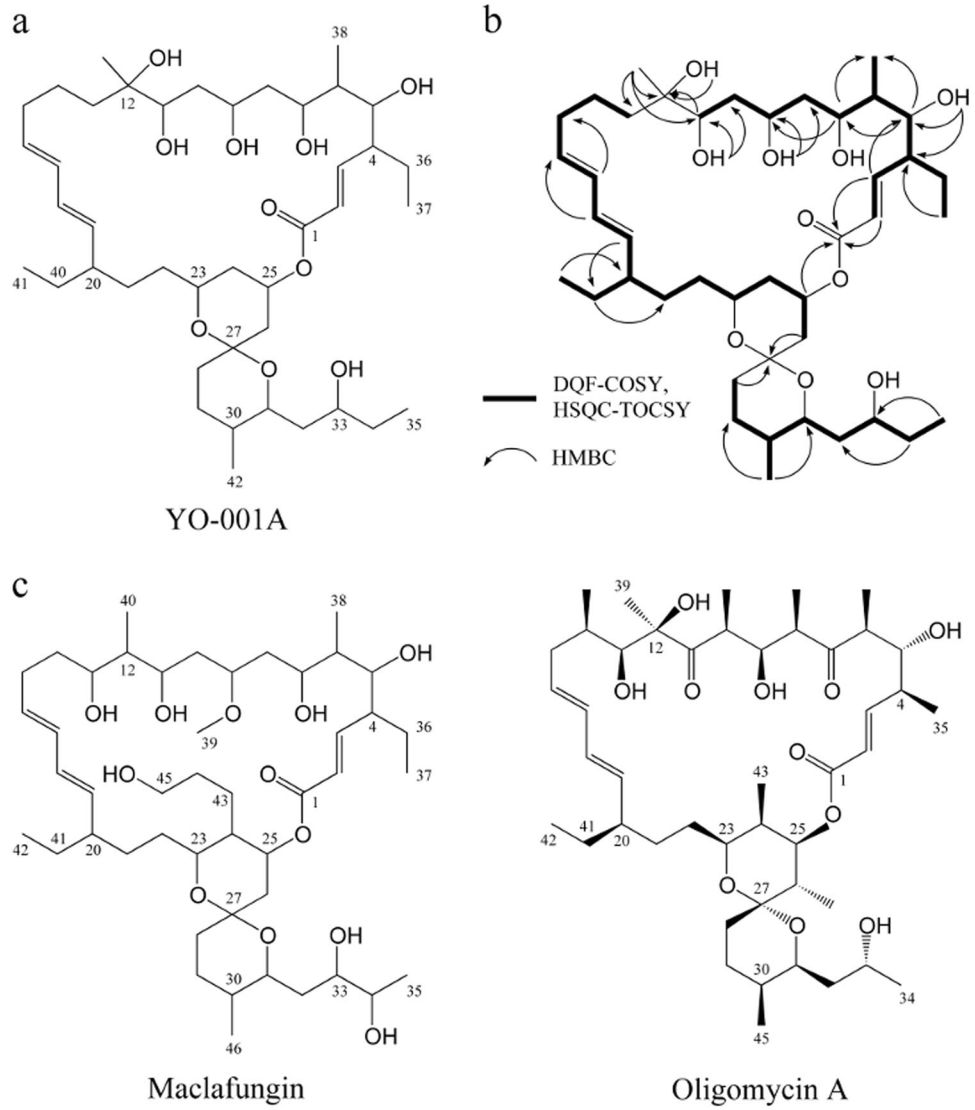

Fig. 1 **a** Structure of YO-001A. **b** Key 2D NMR correlations of YO-001A. **c** Structure of maclafungin and oligomycin A

characteristics: white solid; UV (MeOH) λ_{max} (log ε), 230 nm (4.67); $[\alpha]_{589}^{24}$, +14.9 (*c* 0.28, $CHCl_3$); IR (ATR) ν_{max}, 3421, 2960, 2935, 2873, 1697, 1452, 1386, 1265, 1070, 985, and 752 cm^{-1}; HR-ESI-MS *m/z*, 759.5022 $[M + Na]^+$ (calculated for $C_{42}H_{72}O_{10}Na$, 759.5018). IR spectra suggested a hydroxyl group (3421 cm^{-1}), alkenes (2960, 2935, and 2873 cm^{-1}), and a conjugated carbonyl moiety (1697 cm^{-1}). The ^1H NMR spectra showed six methyl signals containing one singlet signal, two doublet signals, and three triplet signals (Fig. S1 and Table 1). The ^{13}C NMR showed 42 signals containing six methyls, fifteen methylenes, eighteen methines, and three quaternary carbon moieties (Fig. S2 and Table 1). These signals were confirmed by ^{13}C DEPT spectra (Fig. S3). One characteristic quaternary carbon (δ_C 164.7) indicated the presence of an ester. Another quaternary carbon (δ_C 97.4) suggested the attachment of two oxygen substituents and two aliphatic carbon moieties, consistent with the spiroketal system. These partial structures suggested a potential similarity to maclafungin [8] and oligomycins [9, 10]. On comparing the ^{13}C NMR spectra of maclafungin with those of YO-001A, a significant upfield shift at C-9 from δ_C 81.0 to δ_C 74.6, a downfield shift at C-12 from δ_C 45.3 to δ_C 74.9, an upfield shift at C-13 from δ_C 75.2 to δ_C 38.3, an upfield shift at C-24 from δ_C 40.0 to δ_C 37.4, and an upfield shift at C-34 from δ_C 71.6 to δ_C 31.2 were observed (Table 1). These differences indicated the following changes in the attached functional groups: for C-9, from a methyl ether to a hydroxyl group; for C-12, from a methyl group to hydroxyl and methyl groups; for C-13, from a hydroxyl group to a proton; for C-24, from an alkyl chain to a proton; for C-34, from a hydroxyl group to a proton. The planar structure was identified through the interpretation of 2D NMR spectra (Fig. 1b and Figs. S4, S5, S6, S7, and S8). DQF-COSY and HSQC-TOCSY spectra proved three partial structures from C-2 to C-11, from C-13 and C-26, and from C-28 to C-35.

Table 1 ^{1}H and ^{13}C NMR spectra data of YO-001A in $CDCl_3$

YO-001A			Maclafungin [8]		
Position	δ_C, type	δ_H (J in Hz)	Position	δ_C, type	δ_H (J in Hz)
1	164.7, C	–	**1**	164.8, C	–
2	123.4, CH	5.72 (d, 15.5 Hz)	**2**	123.5, CH	5.70 (d)
3	148.1, CH	6.38 (dd, 10.5, 15.5 Hz)	**3**	148.4, CH	6.30 (dd)
4	48.7, CH	2.15 (m)	**4**	49.2, CH	2.11 (dq)
5	79.3, CH	3.69 (m)	**5**	78.6, CH	3.67(dd)
6	40.5, CH	1.24 (m)	**6**	40.7, CH	1.25 (m)
7	78.7, CH	4.16 (m)	**7**	78.8, CH	4.02 (dt)
8	41.7, CH_2	1.75 (m), 1.34 (m)	**8**	38.0, CH_2	1.51 (ddd), 1.45 (ddd)
9	74.6, CH	4.17 (m)	**9**	81.0, CH	3.71 (dt)
10	37.8, CH_2	1.49 (m)	**10**	38.0 CH_2	1.80, 1.35
11	78.8, CH	3.69 (m)	**11**	73.8, CH	3.37 (ddd)
12	74.9, C	–	**12**	45.3, CH	1.44 (tq)
13	38.3, CH_2	1.20 (m), 1.43 (m)	**13**	75.2, CH	3.47 (ddd)
14	22.1, CH_2	1.53 (m)	**14**	32.9, CH_2	1.67 (ddt), 1.42 (ddt)
15	33.2, CH_2	2.35 (m), 1.97 (m)	**15**	28.4, CH_2	2.23 (ddt), 2.22 (ddt)
16	131.1, CH	5.38 (ddd, 4.0, 10.5, 14.5 Hz)	**16**	131.0, CH	5.39 (ddd)
17	131.6, CH	5.97 (dd, 10.5, 14.5 Hz)	**17**	132.0, CH	6.03 (dd)
18	130.4, CH	5.90 (dd, 10.5, 15.0 Hz)	**18**	130.0, CH	5.89 (dd)
19	137.3, CH	5.23 (dd, 9.0, 15.0 Hz)	**19**	137.9, CH	5.31 (dd)
20	45.7, CH	1.81 (m)	**20**	45.4, CH	1.88 (m)
21	34.5, CH_2	1.47 (m), 1.29 (m)	**21**	30.8, CH_2	1.45 (dt)
22	31.0, CH_2	1.46 (m)[a]	**22**	29.4, CH_2	1.58 (ddd), 1.11 (dt)
23	67.1, CH	3.72 (m)	**23**	70.3, CH	3.74 (ddd)
24	37.4, CH_2	2.06 (m), 1.09 (m)	**24**	40.0, CH	1.83 (dq)
25	67.7, CH	5.21 (m)	**25**	70.9, CH	5.25 (dt)
26	40.7, CH_2	1.47 (m)	**26**	36.5, CH_2	1.74 (dd), 1.62 (dd)
27	97.4, C	–	**27**	97.5, C	–
28	30.0, CH_2	1.66 (m), 1.45 (m)	**28**	29.9, CH_2	1.56 (ddd), 1.38 (ddd)
29	26.5, CH_2	2.12 (m), 1.39 (m)	**29**	26.5, CH_2	2.06 (ddt), 1.33 (ddt)
30	30.7, CH	1.59 (m)	**30**	30.8, CH	1.54 (dtq)
31	67.2, CH	4.01 (dt, 11.0 Hz)	**31**	67.3, CH	3.98 (dt)
32	40.2, CH_2	1.64 (m), 1.23 (m)	**32**	37.2, CH_2	1.57 (ddd), 1.24 (ddd)
33	69.5, CH	3.76 (m)	**33**	72.9, CH	3.50 (ddd)
34	31.2, CH_2	1.48 (m)	**34**	71.6, CH	3.52 (quint)
35	9.7, CH_3	0.97 (t, 7.5 Hz)	**35**	19.8, CH_3	1.17 (d)
36	23.5, CH_2	1.99 (m), 1.19 (m)	**36**	23.8, CH_2	1.98 (ddq), 1.13 (ddq)
37	11.4, CH_3	0.83 (t, 7.0 Hz)	**37**	11.5, CH_3	0.74 (t)
38	3.8, CH_3	0.85 (d, 7.0 Hz)	**38**	4.1, CH_3	0.80 (d)
39	19.7, CH_3	1.09 (s)	**39**	55.9, CH_3	3.30 (s)
40	28.6, CH_2	1.41 (m), 1.23 (m)	**40**	12.9, CH_3	0.69 (d)
41	11.8, CH_3	0.82 (t, 7.5 Hz)	**41**	27.7, CH_2	1.33 (ddq), 1.23 (dquint)
42	11.3, CH_3	0.93 (d, 7.0 Hz)	**42**	12.1, CH_3	0.74 (t)
5-OH	–	3.79 (s)	**43**	17.7, CH_2	1.39 (m), 1.33 (m)
7-OH	–	4.38 (s)	**44**	32.9, CH_2	1.45 (dtt), 1.28 (dtt)
9-OH	–	4.59 (s)	**45**	62.6, CH_2	3.37 (dt), 3.35 (dt)
11-OH	–	3.40 (s)	**46**	11.3, CH_3	0.86 (d)
12-OH	–	No signal	**5-OH**	–	4.22 (br s)
33-OH	–	No signal	**7-OH**	–	4.31 (br)
			33-OH	–	2.22 (br)

[a]Overlapping signal

The linkage of partial structures was established by HMBC spectrum. The correlations from H-26 and H-28 to C-27 constructed the spiroketal system. A 26-membered macrocyclic lactone ring was suggested in accordance with HMBC correlations from H-39 to C-11, C-12 and C-13, and from H-25 and H-3 to the ester moiety at C-1 (δ_C 164.7). The presence of this macrocyclic lactone was confirmed by the degree of unsaturation of YO-001A (seven degrees of

unsaturation: three double bonds, one carbonyl, and three ring systems). Four of the six hydroxyl groups (5-OH, 7-OH, 9-OH, and 11-OH) were assigned via 2D NMR correlation spectroscopy; the remaining two were inferred via ^{13}C DEPT spectroscopy and chemical shift assignment. Finally, YO-001A was identified as a new 26-membered macrolide.

The relative configuration of 6,6-spiroacetal core structure (C-23 to C-32) was analyzed on the basis of ROESY correlations (Figs. S9 and S10). ROESY correlations between H-23/H-25, H-24/H-26, H-28/30-Me, H-29/H-31, and H-31/H-23 were observed. These ROE correlation patterns were similar to those of other oligomycins and neomaclafungins [11, 12], indicating the relative configuration of spiroacetal moiety in YO-001A as shown in Fig. S10.

Besides, coupling constants (14.5–15.5 Hz) of olefinic protons, the ROE correlations between H-2/H-4, H-16/H-18, and H-18/H-20, and strong IR absorption at 985 cm^{-1} suggested that all three double bonds were in the *trans* configuration; subsequent stereochemical investigations are currently underway.

Antimicrobial and cytotoxic effects of YO-001A were evaluated (Table 2). YO-001A inhibited the growth of *P. oryzae* ($IC_{50} = 0.012\,\mu M$), *A. fumigatus* ($IC_{50} = 0.42\,\mu M$), and *C. albicans* ($IC_{50} = 1.6\,\mu M$), displaying slight cytotoxicity to HeLa cells ($IC_{50} = 8.2\,\mu M$) and HL-60 cells ($IC_{50} = 5.8\,\mu M$). YO-001A was especially effective against filamentous fungi and the morphology induced by YO-001A was similar to that induced by oligomycin A. Based on the structural and functional similarities, YO-001A was considered to have F_OF_1-ATPase inhibitory activity. To test whether YO-001A interfered with mitochondrial respiration, a cell-based assay monitoring the Oxygen Consumption Rate (OCR) was performed using the Seahorse XFe96 Analyzer [13]. Our data showed that YO-001A markedly decreased the OCR in HeLa cells, indicating its inhibitory effects on mitochondrial respiration (Fig. S11, $IC_{50} = 0.0025\,\mu M$). Furthermore, on comparing the pattern of changes in OCR values via the Mito stress test, wherein oligomycin A, FCCP, and rotenone/antimycin A were added in cell culture media in a stepwise manner, we confirmed that its pattern of OCR variation is identical to that of oligomycin A (Fig. S11). Consequently, we examined its effect in vitro on F_OF_1-ATPase activity, using isolated bovine heart mitochondria and observed potent and concentration-dependent inhibition of F_OF_1-ATPase ($IC_{50} = 1\,\mu M$), similar to oligomycin A (Fig. S12). These results indicated that YO-001A inhibits F_OF_1-ATPase in cell-based and cell-free systems.

In this study, we report the new antifungal agent, YO-001A, as a new congener of 26-membered macrocyclic polyketides. Although YO-001A is similar to oligomycins and maclafungins, it is potentially interesting since there are no functionalities on C-13 and C-24 in its structure. Namely, C-13 in all reported 26-membered macrolides are hydroxylated, while that of YO-001A is not. OlmA5, a modular PKS synthesizing oligomycin, generates the C11–C13 carbon skeleton, and consists of acyltransferase, ketosynthase, β-ketoreductase, and inactive dehydratase (DH) domains [14], resulting in the hydroxyl group at C-13. Hence, we speculated that the corresponding module in *Streptomyces* sp. YO15-A001 may differ from that of oligomycin, containing active DH and additional enoylreductase in its biosynthetic cluster. We intend to further identify and analyze the functions of YO-001A biosynthetic genes.

Regarding the structure–activity relationships of oligomycins, Omelchuk et al. have semisynthesized 7,11-tetrahydrooligomycin A and revealed the importance of ketones in the 26-memberd ring [15]. So far, C7-C13 polyol region has been studied poorly in light of SAR partly due to the difficulty in site-selective chemical modification. Thus, when comparing with semisynthetic oligomycins and neomaclafungins, YO-001A might be a useful tool to study the SAR of this class of compounds.

Table 2 Biological activity (IC_{50}: μM) of YO-001A

	YO-001A	Oligomycin A
Fungi		
Pyricularia oryzae kita-1	0.012	<0.010
Aspergillus fumigatus Af293	0.42	0.18
Candida albicans JCM1542	1.6	0.83
Bacteria		
Staphylococcus aureus 209	>14	>3.0
Escherichia coli HO141	>14	>3.0
Mammalian cells		
HeLa	8.2	5.8
HL-60	5.8	>3.0
		(IC_{50} value: μM)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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