UK-2A, B, C, and D, Novel Antifungal Antibiotics from *Streptomyces* sp. 517-02

VIII. Reactive Oxygen Species Generated by C9-UK-2A, a Derivative of UK-2A, in *Rhodotorula mucilaginosa* IFO 0001

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UK-2A was isolated from *Streptomyces* sp. 517-02, which has similarity to antimycin A_3 (AA) in structure and inhibitory activities toward electron transport at complex III in mitochondria and has a broad antifungal spectrum[1~3]. In an attempt to improve the duration of antifungal action of UK-2A, several UK-2A derivatives have been prepared by substituting a nine-membered dilactone ring of UK-2A with an *n*-alkyl or an isoprenyl moiety[4]. Previously, we reported a membrane injury caused by a UK-2A derivative, or C9-UK-2A in a strict aerobic yeast *Rhodotorula mucilaginosa* IFO 0001[5]. The patterns of efflux of potassium ions from the yeast cells and of release of enclosed chemicals from artificially prepared liposomes induced by C9-UK-2A were not as rapid as those by amphotericin B[5]. Therefore, the antifungal activity of C9-UK-2A could not be explained only by a nonspecific membrane injury, whose mechanism is still unclear[5].

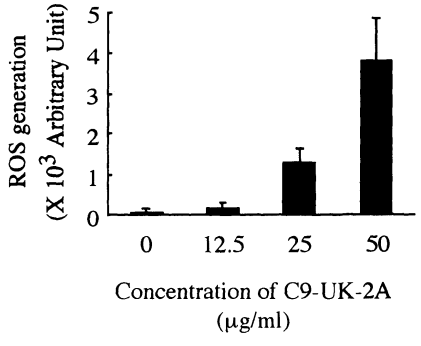
UK-2A did not stimulate the generation of cellular reactive oxygen species (ROS) in procine renal proximal tubule cells (LLC-PK1) whereas AA did[6]. This difference depends on the binding manner against dithionite-reduced cytochrome *b* of complex III[7] and might account for their different cytotoxicity[6]. During our preliminary experiments, AA also stimulated cellular ROS generation in *R. mucilaginosa* IFO 0001 as well as *Saccharomyces cerevisiae* IFO 0203 whereas UK-2A did not.

We tested the effect of C9-UK-2A on cellular ROS generation in *R. mucilaginosa* IFO 0001. C9-UK-2A was prepared and dissolved in *N,N*-dimethylformamide by a previously described method[4,8]. As shown in Fig. 1, C9-UK-2A generated the ROS nearly proportional to its concentration more than 12.5 μg/ml. Moreover, C9-UK-2A induced the generation time-dependently (data not shown).

Glutathione (GSH)[9], one of main antioxidants, is a largest quantity of intracellular SH compounds in budding yeast. To check whether C9-UK-2A gives oxidative stress against *R. mucilaginosa* IFO 0001 cells or not, we investigated GSH/GSSG ratio. In Table 1, GSH/GSSG ratio in the cells treated with C9-UK-2A was about 13% of that in control cells, indicating C9-UK-2A-generated ROS causes oxidation in *R. mucilaginosa* IFO 0001 cells. Its oxidizing activity was three times of that of H_2O_2.

Lipophilic antioxidant α-tocopherols including α-tocopherolacetate (α-TOH) are naturally occuring ones, which can easily penetrate the plasma membrane and protect free and membranous lipids from oxidative damages, such as lipid peroxidation[10]. Therefore, we examined the effect of α-TOH on the ROS generation

Fig. 1. Dose dependent effect of C9-UK-2A on ROS generation in *R. mucilaginosa* IFO 0001 cells in a Sabouraud dextrose (SD) broth.



The measurement of cellular ROS generation in *R. mucilaginosa* IFO 0001 was performed by a method dependent on intracellular deacylation and oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to the fluorescent compound 2′,7′-dichlorodihydrofluorescein as described by MACHIDA *et al.*[11]. Prior to the measurement of ROS generation, the cells (equivalent to 10^7 cells) were incubated in 1 ml of SD broth[5] at 30°C for 30 minutes with 0, 12.5, 25, and 50 μg/ml C9-UK-2A after pretreatment with DCFH-DA for 60 minutes. Values are means±standard deviations (n=3).

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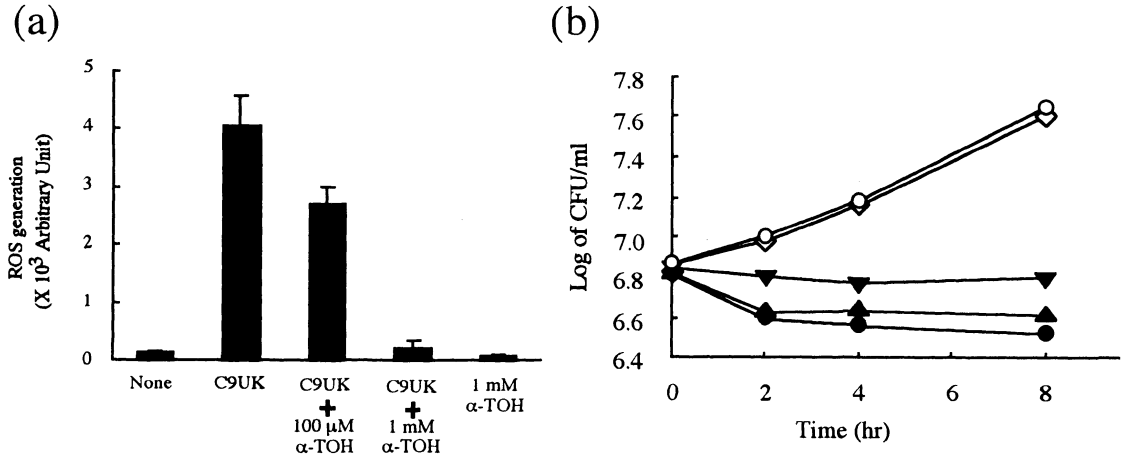
Table 1. Effects of C9-UK-2A and hydrogen peroxide (H_2O_2) on the total glutathione contents and the ratio of GSH (reduced form) to GSSG (oxidized form) in *R. mucilaginosa* IFO 0001 cells.

Addition	Conc.	Total glutathione (nmols /10^7cells)	GSH (nmols /10^7cells)	GSSG (nmols /10^7cells)	GSH/GSSG ratio
None	—	0.17	0.14	0.03	4.7
C9-UK-2A	25 μg/ml	0.13	0.05	0.08	0.6
	50 μg/ml	0.11	0.04	0.07	0.6
H_2O_2	2 mM	0.11	0.07	0.04	1.8

The total glutathione contents and the ratio of GSH to GSSG were measured by the method as descrived by MACHIDA *et al*[12]. *R. mucilaginosa* IFO 0001 cells (10^7cells/ml) were grown with or without each chemical at 30 °C for 1 hour in SD medium.

Fig. 2. Effects of α-TOH on ROS generation (a) and decrease in cell viability (b) caused by C9-UK-2A in *R. mucilaginosa* IFO 0001 cells.



(a); ROS generation was measured as described in Fig. 1. The yeast cells were pretreated with α-TOH for 15 minutes prior to incubation with C9-UK-2A. C9UK indicates incubation with 50 μg/ml C9-UK-2A. Values are means±standard deviations (n=3). (b); The cells were cultivated with 50 μg/ml C9-UK-2A (●), 50 μg/ml C9-UK-2A with 100 μM α-TOH (▲), 50 μg/ml C9-UK-2A with 1 mM α-TOH (▼), 1 mM α-TOH (◇), or without drugs (○) in a SD broth with shaking at 30°C. After incubation in SD broth in the presence of the agents indicated, CFU was determined by plating diluted sample on YPD agar plates followed by 48-hour incubation at 25°C before enumerating CFU.

induced by C9-UK-2A in *R. mucilaginosa* IFO 0001 cells (Fig. 2. (a)). α-TOH restricted the C9-UK-2A-generated ROS to 70% at 100 μM and completely suppressed it at 1 mM. Hydrophilic antioxidants, such as ascorbic acid, trolox and *N*-acetylcystein did not affect the C9-UK-2A-generated ROS (data not shown). It has been reported that fullerene and its derivative generated ROS on photoexcitation and induced significant lipid peroxidation/protein oxidation in membranes and these phenomena could be prevented by antioxidants[11]. Therefore, the protective effect of lipophilic antioxidants on C9-UK-2A-generated ROS in *R. mucilaginosa* IFO 0001 cells would suggest the possibility of peroxidation of membranes triggered by C9-UK-2A. On the other hand, a sesquiterpene dialdehyde, polygodial showed a membrane injury accompanying with ROS generation caused by depletion of

intracellular glutathione[12]. Polygodial-mediated depletion of glutathione was possibly dependent on a direct interaction between its enal moiety and the sulfhydryl group in glutathione by a Michael-type reaction[12]. As C9-UK-2A does not have any reactive moieties in the molecule, further studies are needed to reveal the correlation between the membrane injury and the ROS generation.

Previously, C9-UK-2A at 100 μg/ml was reported to show fungicidal activity after 48-hour treatment[5]. Here we examined the effect of α-TOH on decrease in cell viability caused by C9-UK-2A in *R. mucilaginosa* within 8 hours (Fig. 2(b)). α-TOH at 1 mM almost stopped the decrease in cell viability caused by C9-UK-2A at 50 μg/ml. In *S. cerevisiae* cells, α-TOH eliminated ROS generated by farnesol[10] and para-nonylphenol[13] accompanied by a recovery of the following growth arrest. Although a fungicidal nonyl gallate induced a membrane injury, α-TOH would not eliminate ROS generated or recover from lethality by its ester[14]. In this case, the attribution of the membrane injury to the fungicidal effect would be greater than that of ROS generation. α-TOH could not completely cancel the lethal damage caused by fungicidal drugs, such as nonyl gallate and C9-UK-2A.

The patterns of efflux of potassium ions from yeast cells and of release of enclosed chemicals from artificially prepared liposomes induced by C9-UK-2A were not as rapid as those by typical membrane damaging fungicides, such as amphotericin B[5], nonyl gallate[14] and polygodial[15]. In addition, the antimicrobial action of C9-UK-2A was limited to eukaryotic cells[5]. C9-UK-2A gradually decreased the number of CFU of *R. mucilaginosa* IFO 0001 at the concentration of 50 μg/ml (Fig. 2(b)). On the other hand, the typical membrane damaging fungicides did it rapidly. α-TOH almost stopped the decrease in cell viability caused by C9-UK-2A. These results indicate that antifungal activity of C9-UK-2A does not only depend on a membrane injury. C9-UK-2A stimulated ROS generation dose- and time-dependently. This generation was completely suppressed by 1 mM α-TOH (Fig. 2(a)). In spite of this fact, cell viability did not recover to control level. Thus, ROS production alone could not fully explain the fungicidal effect of C9-UK-2A. This UK-2A derivative might pull a trigger on lipid peroxidation mediated by ROS generation which might further accelerate the membrane injury.

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