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Schima wallichii ssp. *Liukiuensis* 추출물 Sterol Glycoside의 *Candida* spp.에 대한 항균활성

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Antimicrobial Agent from *Schima wallichii* ssp. *liukiuensis* against *Candida* spp.

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ABSTRACT : This study carried out development of a natural antimicrobial agent from *Schima wallichii* ssp. *liukiuensis*. Compound I exhibiting potent antimicrobial activity against *Candida* spp. was isolated from the methanol extracts of *Schima wallichii* ssp. *liukiuensis*. The structure of I identified as a sterol glycoside consisted of a trisaccharide and α_1-sitosterol. Trisaccharide composed of L-rhamnose, D-galactose and D-glucose residues. The antimicrobial activity of I was selective on yeast rather than bacteria or other fungi. Compound I was demonstrated to be ineffective against toxicity to mouse liver cells where as protective to human dermal fibroblast cells at low concentrations. Thus, it is reasonable to expect a sterol glycoside (I) as a valuable alternative for synthetic antifungal.

Key Words **:** *Schima wallichii* ssp. *liukiuensis*, Antimicrobial, Sterol glycoside, *Candida* spp.

INTRODUCTION

Polyene antibiotics and synthetic azoles generally find application in the therapy for candidiasis, the choice is however largely dependent upon the extent of the disease (Mcginnis and Radali, 1991). These synthetic antifungal were introduced as general broad spectrum antimicrobials over 10 years ago. However, particular pharmaceuticals for candidiasis induced by various *Candida* spp. have not been found. The synthetic azoles and polyenes are susceptible to fungal attack hence solutions to treat fungal infections is an emerging problem (Pavese *et al*., 1999) Han *et al.* (2006) found that many edemic plants resources contain antioxidative and antibacterial substances.

Many plants produce low-molecular-weight compounds that inhibit the growth of microorganisms (Cho *et al*., 2007; Choi *et al*., 2008). These compounds may be preformed inhibitors that are present constitutively in healthy plants (phytoanticipins), or they may be synthesized in response to a pathogen attack (phytoalexins) (John *et al*., 1999). The antibacterial properties of individual components of a flavonoid (Moon *et al*., 1997), alkaloids (Verpoorte *et al*., 1978) and volatile oil (Yousef and Tawil, 1980) have been reported. Antifungal compounds are widely distributed among the higher plants, but very few have been evaluated for their activity against pathogenic yeasts. Choi *et al.* (1999a) screened 218 domestic and introduced tree species from Korea for phytochemicals with an antimicrobial activity

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using disc-agar plate diffusion method. Of all the species, only the extracts from *Schima wallichii* ssp. *liukiuensis* showed a potent antimicrobial activity than those of other tree species. These authors found that the butanol (BuOH) extracts of the *Schima wallichii* ssp. *liukiuensis* leaf and outer barks exhibited growth inhibitory action against *Candida* spp. and *Escherichia coli*.

Schima wallichii ssp. *liukiuensis* (Theaceae) is a large tree with evergreen broadleaves. It measures up to a height of 10-20 m with wide distribution in Southwest Japan, Taiwan and Indonesia. The chemical nature of the antimicrobial compound from *Schima wallichii* ssp. *liukiuensis* has also not been elucidated so far. This work reports the isolation and characterization of an antimicrobial compound against *C. albicans* from *Schima wallichii* ssp. *liukiuensis*.

MATERIALS AND METHODS

1. Plant material

The plant material of *Schima wallichii* ssp. *liukiuensis* was collected from Chollipo Arboretum, Korea. The plant is indexed our laboratory specimen collection.

2. Extraction, isolation and characterization of the antimicrobial compound

The plant material (1 ㎏ dry weight) was powdered and extracted 3 times with 70% (v/v) methanol (MeOH). The MeOH extract was evaporated *in vacuo*. The viscous concentrate obtained was further extracted successively with hexane, methylene chloride (CH_2Cl_2) and ethyl acetate (EtOAc). The aqueous phase was then partitioned between BuOH and H_2O. The BuOH-soluble phase was fractionated with a column chromatography. The column matrix was silica gel and the components were eluted using BuOH : glacial acetic acid : water (5 : 1 : 4) solvent system. The fraction with antimicrobial activity was refractionated by Sephadex LH-20 column chromatography using MeOH as mobile phase to yield I. The purity of I was assessed by HPLC system (TSP, Series P4000 pump) equipped with Lichrospher RP-C18 (3.2 × 250 ㎜, 5 ㎛, Merck) column and photodiode array detector. The solvent system chosen was MeOH : H_2O (70 : 30, v/v) with flow rate being 0.8 ㎖/min during analysis.

The structure of I was elucidated using NMR: 1D (Bruker AMX500, 500 MHz for ^{1}H NMR and ^{13}C NMR) and 2D (Bruker AMX500, 500 MHz for ^{1}H-^{1}H COSY, HMQC and HMBC), UV spectrophotometer (Shimadzu UV-260 spectrophotometer), FAB-MS (VG70-VSEQ, USA), GC-MS (JMS-AX505 WA) and FT-IR (FTS-60, Bio-Rad).

3. Analysis of composition of Compound I

Compound I (15 ㎎) was dissolved in 3 ㎖ of 1M HCl in MeOH and placed in an oven maintained at 100℃ for 2 h in an air atmosphere. After cooling, the solution was neutralized with Ag_2CO_3, and then filtrated and chromatographed on silica gel column. The sugar and aglycone fractions were eluted with solvent system comprising of chloroform ($CHCl_3$) and MeOH (19 : 1, v/v). Both fractions were later trimethylsilated for analysis by GC-MS to establish the aglycone and sugar structural relationship. The sugar composition of trimethylsilated fraction was analyzed by Gas Chromatography (GC, Agilent 6890N) with SP-2380 (0.25 × 30 ㎜) column, temperature of 230℃ for 10 min, ion source temperature; 250℃, detector temperature; 250℃ and carrier gas; N_2 (1.0 ㎖/min).

4. Antimicrobial assay

Antimicrobial activity of compounds against bacteria was carried out by the disc diffusion method (Pietro *et al*., 2000). The test organisms were *Escherichia coli, Salmonella typhimurium* SL 1102, *Staphylococcus aureus* IFO 12732, *Aspergillus niger* KTCC 6005, *Botrytis cinerea* KTCC 1937, *Candida tropicalis* KCTC 7901, *C. albicans* KCTC 7121 and *C. guilliermondii* KCTC 7144, from Korean Institute Science Technology Genetic Resources Center.

Test microbial strains were inoculated onto the Luria Bertani (LB) and Yeast Extract Peptone (YEP) medium without agar, and cultured at 37℃, 100 rpm for 12 h. To prepare solid agar plates, a 20 ㎖ of the autoclaved LB or YEP medium with 1.5% (w/v) agar was poured into a Petri dish (90 ㎜ × 20 ㎜).

The paper discs (8 ㎜ diameter) containing various concentrations of antimicrobial compound were placed on the agar plate for antimicrobial activity test. After 48 h of incubation at 37℃, the diameters of inhibitory zones were measured using an autocaliper (CD-15B, Mitutoyo Corp., Japan).

To determine the minimum inhibitory concentration (MIC) values, various concentrations of I were dispensed into wells of 96 microwell plates (Nunc, USA) and plates were air

dried in a cold room. The 24 h grown bacterial and yeast cultures were inoculated into these wells. After 24 h of incubation the growth of bacteria and yeast was measured by UV/VIS spectrophotometer at 660 ㎚. The bioassay of fungus was conducted as Yin and Cheng (1998) method with modification. Filter paper discs containing various concentrations of I were placed on the surface of the solid culture media. The results are expressed as the net zone of inhibitions after 5 days in cultures.

5. Cytotoxicity assay

Cytotoxicity of I was examined using mouse liver cells according to the method of Mosmann (1983) or human fibroblast and HaCaT dermal cells according to the method of Carmichael *et al*. (1987).

Lactate dehydrogenase (LDH) leakage was colorimetrically quantified in the cell culture medium of transiently transfected cells after the 24h treatment with test compounds according to the kit manufacturer instruction (CytoTox 96® Non-Redioactive Cytotoxicity Assay, Promega, Madison WI, USA).

6. Transmission electron microscopy

In order to determine the mechanism of action of the antimicrobial on *C. albicans* was incubated for 24 h with 40 ㎍/㎖ of I. The harvested cells were prepared for TEM by modified method of Lee *et al*. (2004) and examined with Transmission electron microscopy (JEM-2010, JEOL). The bacterial cultures treated with antimicrobial proteins or peptides for 30 min were mixed with an equal volume of 5% buffered glutaraldehyde (pH 7.4) and centrifuged (500 × g, for 20 min). The bacterial pellets were left in the fixative at 4℃ for 2 h, post-fixed in 2% osmium tetroxide, dehydrated in graded ethanol and embedded in Eponate 12 (Ted Pella, Inc. Redding, CA). Thin sections (70 ㎛) were mounted on coated specimen grids, contrasted with uranyl acetate and lead citrate and examined in a transmission electron microscope (CM120 BioTwin, FEI-Philips, Hillsboro, OR) operating at 80 ㎸.

RESULTS AND DISCUSSION

1. Elucidation of compound I

Antimicrobial activity of BuOH soluble crude fraction of *Schima wallichii* ssp. *liukiuensis* against the bacteria was observed at 1,000 ㎍/㎖ concentrations. The MIC of this BuOH fraction against yeasts; *C. tropicalis, C. albicans* and *C. guilliermondii* was also found to be 1,000 ㎍/㎖. A portion of the BuOH soluble crude fraction on chromatographic separation on silica gel column yielded five sub-fractions. Of the five fractions the second fraction showed significant antimicrobial activity. The second sub-fraction was further subjected to Sephadex LH-20 column chromatography to obtain I. Compound I was a white powder, with a UV absorption maxima of 218 ㎚. This compound could be detected as red color spots on TLC chromatograms by spraying with vanillin and sulfuric acid followed by baking.

Compound I displayed the following data on spectral analysis; IR (KBr): 3437, 2920, 2853, 1626 cm^{-1}; ^{1}H-NMR (CD_3OD, 500 MHz); δ 6.05 (1 H, m, olefin-H), 5.42 (1 H, m, olefin-H), 5.27 (1 H, br. s, rhm-1), 5.19 (1H, d, J = 7.0 Hz, gal-1), 4.46 (1 H, d, J = 7.2 Hz, glc-1), 4.26-3.07 (sugar-moieties), 1.93 (3H, d, J = 7.6 Hz, -CH_3), 1.26 (3H, d, J = 6.2 Hz, rhm-6), 1.07 (3H, d, J = 7.6 Hz, -CH_3), 1.02 (3H, d, J = 8.0 Hz, -CH_3), 0.87 (6H, d, J = 6.7 Hz, -CH_3), 0.80 (3 H, s, -CH_3) and 0.54 (3 H, s, -CH_3); ^{13}C-NMR (CD_3OD, 125 MHz): δ 144.6 (olefin-C), 138.0 (olefin-C), 130.2 (olefin-C), 126.4 (olefin-C), 105.8 (glc-1), 102.0 (rhm-1), 100.7 (gal-1), 81.1 (glc-2), 79.6 (C-3), 79.4 (gal-4), 78.1 (glc-3), 77.9 (glc-5), 76.9 (gal-5), 75.9 (gal-3), 73.8 (rhm-4), 72.6 (rhm-3), 72.6 (gal-2), 72.3 (rhm-2), 71.9 (glc-4), 70.3 (rhm-5), 63.5 (glc-6), 62.8 (gal-6), 56.6, 55.1, 47.6, 45.7, 42.4, 40.5, 40.2, 39.4, 37.9, 37.2, 33.6, 32.4, 30.7, 28.3, 26.9, 25.1, 24.8, 21.4, 21.1, 21.0, 20.4, 19.5, 13.0, 12.2, 12.1 (aglycone-moieties).

The IR spectral data displayed absorption peaks permitting the identification hydroxyl function (3,590-3,650 cm^{-1}), aliphatic single bond (2,920 cm^{-1}) and double bonds (1,626 cm^{-1}).

The ^{1}H-NMR spectrum of I showed signals for typical sterol methyls; two appeared as singlets at δ 0.80 and 0.54 and the other five as doublets at δ 1.93, 1.07, 1.02 and 0.87. The signals at δ 6.05 and 5.42 were assigned to olefin protons. Three hemiacetal signals were also noted at δ 5.27 (rhm-1), 5.19 (gal-1) and 4.46 (glc-1). And many oxygenated methine or methylene signals due to sugar moieties were observed between δ 4.26-3.07. This data indicated compound to be an unsaturated sterol glycoside with three sugars.

The ^{13}C-NMR spectrum of I showed signals for four olefin carbon signal at δ 144.6 (s), 138.0 (s), 130.2 (d) and

126.4 (d) indicating presence of two double bonds. Three anomeric carbon signals were observed at δ 105.8, 102.0 and 100.7 and fourteen oxygenated-carbon signals also were observed between δ 81.1 to 62.8 derived from three hexose sugars, one of which is 6-deoxy. The ^{13}C-NMR spectrum showed twenty-five carbon signals of aglycone between δ 56.6 to 16.3 as well as an oxygenated methine signal combining to sugar at δ 79.6.

The acid hydrolysis yielded sugars and an aglycone. The sugars were identified as D-glucose, D-galactose and L-rhamnose in a ratio of 1 : 1 : 1 based on GC analysis. The ^{1}H-NMR data and the calculation of coupling constants for anomeric protons indicated α configuration in L-rhamnose (br. s), and β configurations for D-galactose (J = 7.0 Hz) and D-glucose (J = 7.2 Hz). Linkage between the three sugars and an aglycone was established to be through C-3 hydroxyl group of aglycone and D-glucose, and C-2 hydroxyl group of glucose to D-galactose, and C-4 hydroxyl of galactose to L-rhamnose by comparison of chemical shifts from ^{13}C-NMR spectral data with those in literature. Most other signals in the ^{1}H- and ^{13}C-NMR data were unambiguously assigned through ^{1}H-^{1}H COSY (correlation spectroscopy) and HMQC (heteronuclear multiple quantum coherence) data.

In order to assign the aglycone, the hydrolysis products obtained above was trimethylsilylated and analyzed with GC-MS. The relative areas of each component in GC chromatogram and MS data indicated the aglycone to be composed of á$_1$-sitosterol. (Fig. 1).

Saponins are glycosylated triterpenoid, steroid, or steroidal alkaloid molecules which occur constitutively in many plant species and often exhibit antifungal activity (Osbourn, 1996). These molecules have an oligosaccharide chain, linked at the C-3 position, which may consist of up to five sugar molecules, usually glucose, arabinose, glucuronic acid, xylose or rhamnose (Marston, 1995). Some saponins also have an additional sugar moiety (normally one glucose) at C-26 or C-28 (Hughes, 1991). In our study, compound I consisted of aglycone that is linked to three sugars D-glucose, D-galactose and L-rhamnose.

2. Antimicrobial activity of compound I

Compound I on yeast and bacteria was evaluated for the antimicrobial activities. The MIC of I for yeast was 40 ㎍/㎖, indicating that this is more potent than BuOH soluble fractions. Compound I exhibited a potent antimicrobial activity against *C. albicans*. Treatment of yeast with I extended the lag phase of cell division by at least 15 h (Table 1,

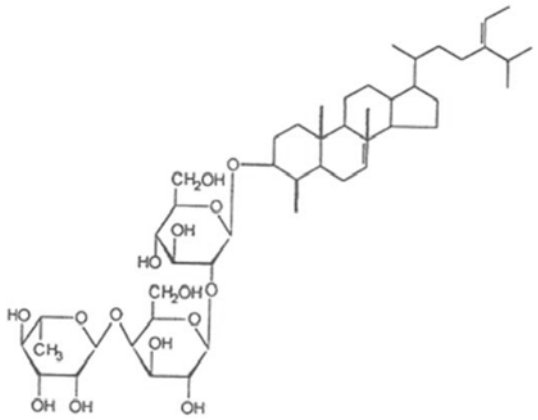

Fig. 1. Structure of I derived from *Schima wallichii* ssp. *liukiuensis*.

Table 1. Antimicrobial activities of compound I on bacteria, yeast and fungi.

Microorganisms	MIC (㎍/㎖)*
Bacteria	
Escherichia coli	1,250±250
Salmonella typhimurium	1,250±250
Staphylococcus aureus	1,250±250
Yeast	
Candida tropicalis	40±5
Candida albicans	40±1
Candida guilliermondii	50±10
Fungi	
Aspergillus niger	5,000±360
Botrytis cinerea	5,000±450

*The MIC (Minimum Inhibitory Concentration) values represent mean±standard deviation.

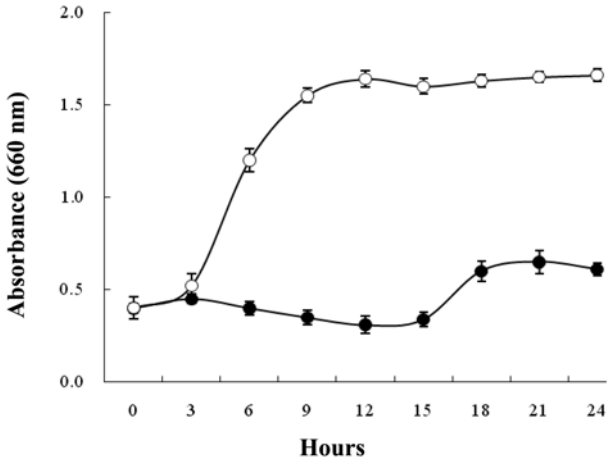

Fig. 2. Growth patterns of *C. albicans* after treatment with compound I (●: Presence of I, ○: Absence of I).

Table 2. Cytotoxicity of compound I on mouse liver cell.

Treatement (㎍/assay)		LDH activities (UNIT)*
No treatment		7±6
Compound I	10	25±2
	5	25±3
	0.5	17±4
CC14	10	341±59
	5	369±14
	0.5	339±55
0.5 mM CC14		305±47

*The LDH (Lactic Dehydrogenase) values represent mean±standard deviation.

Fig. 2). The MIC of I against *Candida* spp. was 40 ㎍/㎖ (Table 1). These MIC values are much lower than that of polylysine (5,000 ㎍/㎖), an artificial preservative used commercially.

The antimicrobial mechanisms of plant derived antimicrobials have not been thoroughly investigated. When microorganisms are treated with saponin, aggregation of the saponin and sterol complexes in the membrane was observed. These results due to the interactions mediated between the sugars in the saponin (Hughes, 1991; Keukens *et al*., 1995).

The sugar chain attached to C-3 is very critical for both the membrane-permeabilizing and antifungal properties of saponins, and removal of these sugar residues often results in the loss of biological activity (Wubben *et al*., 1996). Some pairs of steroidal glycoalkaloids that have a common aglycone but differ in the composition of their sugar chains show synergism in their membranolytic and antifungal activity, indicating that some kind of complementation occurs between carbohydrate moieties (Roddick *et al*., 1988). Since, compound I have three sugar moieties and a sitosterol its antimicrobial activity may be due to its structural similarity with saponins.

3. *In vitro* cytotoxicity to mouse liver cell and human fibroblast and HaCaT dermal cells

Safety and stability test of I derived from *Schima wallichii* ssp. *liukiuensis* was examined (Table 2). The cytotoxicity of I was evaluated against mouse liver cells. The lactic dehydrogenase (LDH) activity after CCl_4-treatemnt was markedly increased compared to normal cells, but these levels were independent of concentration of I employed. At levels up to 10 ㎍/㎖ no significant cytotoxic effect was noted. The treatment of cells with CCl_4 resulted in a marked increase of LDH activity. When active compound was applied to CCl_4 exposed cells, LDH activities increased indicating that compound I did not protect liver cells against toxic effects.

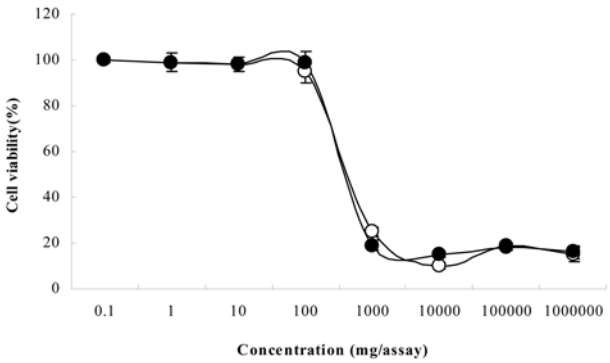

Fig. 3. Cytotoxicity of compound I for human fibloblast and HaCaT dermal cells (●: HaCaT cell, ○: Fibroblast cell).

The cytotoxicity of I was assessed on fibroblast and HaCaT cell lines (Fig. 3).

I (< 100 ㎍) did not induce any changes in cell viabilities. However, significant decreases in cell viability were observed at concentrations below 1000 ㎍. This result points out that lower concentration of I (< 100 ㎍) is non-toxic for human fibroblast and HaCaT dermal cells.

The antimicrobial activity of I appeared to be stable under the changes of pH, temperature, light quality, gas and metal ions. Previously study, the mutagenicity of I to *S. typhimurium* TA100 was also not found (Choi *et al*., 1996b).

4. Ultrastructure of *C. albicans* on treatment with compound I

On treatment of *C. albicans* with I, the structural changes were prominent in organelles. *C. albicans* usually has a thick three layered wall and its cytoplasm is densely packed with ribosomes (Fig. 4A). *C. albicans* treated with I, exhibited changed cell wall structure. The ultra structure of the cell displayed thin and broken cell wall (Fig. 4B) and a large vacuole (Fig. 4C).

The major mechanism of antifungal activity of saponins is apparently due to their ability to form complex with sterols in fungal membranes and to cause loss of membrane integrity (Keukens *et al*., 1995), although the precise mechanism is not fully understood. Electron microscopic analysis and electrical conductivity measurements suggest the formation of transmembrane pores (Seeman *et al*., 1973), although steroidal glycoalkaloids have been proposed to

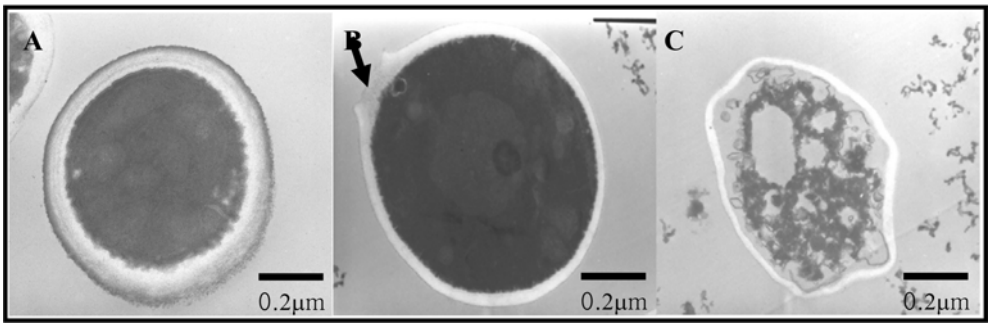

Fig. 4. Transmission electron micrographs of *C. albicans* treated with compound I. A: Normal cell, B: The cell wall damaged by compound I (arrow), C: Dead cell after treatment with I.

interfere with membrane integrity by extracting sterols from membranes (Keukens *et al*., 1995; Seeman *et al*., 1973). In this study, the antimicrobial effect of Compound I is presumed to be through it's interfere with sterols in *Candida* membrane.

Of the fifteen yeast genera, *Candida* spp. is an important pathogen of humans and animals (Gentles and Touche, 1987). The superficial *Candida* mycoses constitute a real public health hazard and prove costly in terms of medical expertise and money spent on treatment that is often ineffective (Hurley, 1980) Pietro *et al*. (2000) reported the nystatin was most effective against the yeast isolates from human body. The nystatin, which is a member of the polyene group, inhibits growth of the isolated pathogenic *Candida* spp. at concentration ranging from 10 to 20 ㎍/ℓ. Here the antimicrobial activity of I is comparatively lower than that of nystatin. However, the Compound I is extremely important since it is safe, plant-derived, and highly hydrophobic compound. In conclusion, these finding suggests that compound I has an inhibitory effect on *Candida* spp. Thus, it is reasonable to expect this as a valuable alternative for synthetic antifungals. Compound I also could be developed into a natural preservative for food and pharmaceutical industry. This material can be widely used as preservative for cosmetics owing to its low toxicity against human dermal cells and its potent antimicrobial activity. In the near future, further work is needed to understand the precise mechanism of action, biochemical and physiological activities and *in vivo* applications of I.

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