We have found that (--)-virgatinus and related compounds have antimicrobial and antifungal activity. To identify further biological activities of these compounds, we tested the activity of acridine orange efflux, which shows ionophore-like disruption of cellular ion homeostasis activity. After testing 31 compounds, we found that verrucosin and a related compound had disruption activity.

Key words: lignan; verrucosin; ion homeostasis; ionophore

In previous studies, we stereoselectively synthesized various tetra-substituted tetrahydrofuran lignans (Fig. 1) and studied their biological activities. (--)Virgatinus showed antifungal activity,\(^1\) and 9-O,9'-O-demethyl (++)-virgatinus and related compounds showed antibacterial activity.\(^2\) Although the precise mechanisms were unclear, 9-O,9'-O-demethyl (++)-virgatinus disrupted the membrane potential of Bacillus subtilis.\(^3\) This compound was considered an ionophore or an inhibitor of ion transport systems. Because ionophores or inhibitors of specific ion transport systems are useful tools to study cellular ion homeostasis, we tested to determine whether tetra-substituted tetrahydrofuran lignans have ionophore-like activity or disruption of the ion homeostasis activity against mammalian cells.

We purchased acridine orange and concanamycin A (ConA) from Wako Pure Chemical Industries (Osaka, Japan), and 2,4-dinitrophenol (DNP) from Nakarai Tesque (Kyoto, Japan). Tributyltin chloride (TBT), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), 4,4'-disothiocyanatostilbene-2,2'-disulfonic acid (DIDS), and 5-ethylisopropyl amiloride (EIPA) were from Sigma-Aldrich (St. Louis, MO). Tetra-substituted tetrahydrofuran lignans were synthesized following a protocol described previously.\(^1\)

The activity of acridine orange efflux was tested using the human cancer cell line MCF-7 (American Type Culture Collection, Rockville, MD) as the target cell. The cells were treated with each of the compounds at 37°C/95% air. After the culture medium was removed, 5 μM acridine orange dissolved in Hank’s balance salt solution (HBSS) was added, and the cells were incubated for 15 min at 37°C. The cells were then separated by centrifugation at 300 × g. The fluorescence intensity of the supernatant was measured at 526 nm with a fluorometer (FL2500; Hitachi, Tokyo). The excitation wavelength was 500 nm. Activity was interpreted by fluorescence intensity (FI) against dimethyl sulfoxide as control.

First we examined the activity of various ionophores and inhibitors against the membrane transporter system to disrupt the ion homeostasis of the cell. The activity of the compound in effluxing acridine orange, a cationic fluorescent dye, was observed in all the tested ionophores (Fig. 2A). TBT (0.5 μM), which has known ionophore\(^8\) and can increase the calcium concentration of the cytosol,\(^9\) has 3 times the activity of the control. The activities of 0.1 μM ConA and of 0.1 μM CCCP, inhibitors of vacuolar type H\(^+\)-ATPase and protonophore respectively, were more than 2 times that of the control. Ten μM EIPA, an inhibitor of the Na\(^+\)/H\(^+\) exchanger, 0.1 mM DNP, a protonophore, and 0.5 mM DIDS, an inhibitor of the anion transporter, showed moderate activity. This indicates that the method employed is effective in detecting disruption of ion homeostasis.

Then we tested the activity of 31 tetra-substituted tetrahydrofuran lignans previously synthesized by this assay method. We found that two compounds disrupted the ion homeostasis of the cell (Fig. 2B). One of these was verrucosin, previously reported to have been isolated from arilus of Virola oleifera.\(^10\) The other was a verrucosin-related compound, previously synthesized by us as Virg 12.\(^3\) Virg 12 was replaced at the residues of 7 and 7' positions by 3,4-methylenedioxyphenyl and 3,4-dimethoxyphenyl respectively (Fig. 1). We found no report of Virg 12 isolated from natural resources. The activities of verrucosin and Virg 12 were 1.6 times

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Note

Disruption of Ion Homeostasis by Verrucosin and a Related Compound

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Franklin Lakes, NJ) with Dulbecco’s Modified Eagle’s Medium (DMEM; Nissui Pharmaceutical, Tokyo), containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO\(_2)/95%\) air. After the culture medium was removed, 5 μM acridine orange dissolved in Hank’s balance salt solution (HBSS) was added, and the cells were incubated for 15 min at 37°C. The cells were washed 3 times with 10 mL of HBSS. The cells were stripped by trypsinization and suspended in HBSS at a concentration 2 × 10\(^6\) cells/mL. The cell suspension was treated with each of the compounds at 37°C for 15 min. The cells were then separated by centrifugation at 300 × g. The fluorescence intensity of the supernatant was measured at 526 nm with a fluorometer (FL2500; Hitachi, Tokyo). The excitation wavelength was 500 nm. Activity was interpreted by fluorescence intensity (FI) against dimethyl sulfoxide as control.

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Then we tested the activity of 31 tetra-substituted tetrahydrofuran lignans previously synthesized by this assay method. We found that two compounds disrupted the ion homeostasis of the cell (Fig. 2B). One of these was verrucosin, previously reported to have been isolated from arilus of Virola oleifera.\(^10\) The other was a verrucosin-related compound, previously synthesized by us as Virg 12.\(^3\) Virg 12 was replaced at the residues of 7 and 7' positions by 3,4-methylenedioxyphenyl and 3,4-dimethoxyphenyl respectively (Fig. 1). We found no report of Virg 12 isolated from natural resources. The activities of verrucosin and Virg 12 were 1.6 times higher than those of the control.
Fig. 1. Structure of Tetra-Substituted Tetrahydrofuran Lignans.
A. Basic structure of tetra-substituted tetrahydrofuran lignans and the lignans used in this study. Ar\textsubscript{1} = 3,4-methylenedioxyphenyl, Ar\textsubscript{2} = 3,4-dimethoxyphenyl. B. Structures of the lignans used in this study.

Fig. 2. Activities of the Various Inhibitors and Tetra-Substituted Tetrahydrofuran Lignans in Effluxing Fluorescent Dye, Acridine Orange.
Activity was determined as relative FI against the control (dimethyl sulfoxide). Values are presented as mean ± SD. A. Acridine orange efflux due to various inhibitors of the membrane transport system (n = 3). Lane 1, 0.5 μM TBT; lane 2, 0.1 μM Con A; lane 3, 0.1 mM CCCP; lane 4, 10 μM EIPA; lane 5, 0.1 mM DNP; and lane 6, 0.5 mM DIDS. B. Acridine orange efflux activity due to various lignans at 0.1 mM (n = 3). Lane 1, verrucosin; lane 2, Virg12; lane 3, Virg15; lane 4, Virg16; lane 5, Virg17; lane 6, Virg18; and lane 7, Virg23.
greater at a concentration of 0.1 mM (Fig. 2B). Virg 15, 16, 17, 18, and 23, all of which have the methoxy group at the 9 and 9′ positions (Fig. 1), showed no activity (Fig. 2B).

These results appear to indicate that the methyl groups at the 9 and 9′ positions are essential to the disruption activity. To clarify the essential role of the methyl groups, it is necessary to investigate the activity of compounds that change only the groups at the 9 and 9′ positions to other groups. Because there are only two compounds (verrucosin and Virg12) that have methyl groups at the 9 and 9′ positions among those we have synthesized to date, further compounds are needed to elucidate the roles of the benzyl groups at the 7 and 7′ positions. Our study to determine the structure-activity relationship of this compound is underway.

We also found that verrucosin and a related compound had the ability to disrupt ion homeostasis in cultured human cells by utilizing acridine orange. Next we will attempt to identify the target molecule and the target ion. Fluorescent sensors for specific ions should be useful tools in that study.11)

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