CJ-21,058, a New SecA Inhibitor Isolated from a Fungus

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A new equisetin derivative, CJ-21,058 (I) was isolated from the fermentation broth of an
unidentified fungus CL47745. It shows antibacterial activity against Gram-positive multi-drug
resistant bacteria by inhibiting ATP-dependent translocation of precursor proteins across a
bacterial cell membrane.

Proteins that are destined to be translocated across or
inserted into the bacterial inner membrane are targeted to
transport sites by general export pathway1,2) and this is a
decisive step in the biosynthesis of most secretory proteins.
In a past decade proteins comprising the translocation
machineries have been purified and reconstitution of the
translocation activity from these proteins or a complex of
proteins has been successfully performed. Translocation of
proteins across the inner membrane is catalyzed by the
preprotein translocase. This is a complex, multisubunit
protein consisting of SecA, SecY, SecE, SecG, SecD, SecF
and YajC subunits. The core consists of an integral domain
composed of the SecY, SecE and SecG proteins and a
peripheral domain composed of a dimer of SecA.

SecA is a dimer of 102 kDa subunits that is found both
in the cytoplasm and bound to the inner membrane3-6). When
SecA is bound to the SecYEG complex, acidic
phospholipids and a precursor protein such as proOmpA
(the precursor of outer membrane protein A), SecA
becomes fully active as an ATPase7). SecA couples the
energy from ATP binding and hydrolysis to protein
translocation through repeated cycles of ATP-driven
membrane insertion and deinsertion of SecA8). So
inhibition of either association of multisubunit enzyme
complex or ATPase activity of SecA may lead to the new
class of antibiotics. Sodium azide and monoclonal
antibodies specific for SecA are reported to inhibit SecA
function9,10), but there is a clear need for other kinds of
SecA inhibitors with drug-like structures for the treatment
of bacterial infection.

In the course of screening for natural products possessing
pharmacological activities, an unidentified fungus CL47745
was found to produce SecA inhibitor. In this paper we
report the fermentation, isolation, structure elucidation and
biological activity of I.

Results

Isolation

The solid fermentation broth (1.5 liters) treated with the
same volume of EtOH overnight was filtered and
concentrated to an aqueous solution (100 ml). The solution
adjusted to 50% aqueous MeOH solution was applied
onto an ODS column (YMC-pack ODS-AM 120-S50,
26×50 mm, YMC Co. Ltd.) and eluted with 90% aqueous
MeOH after washing with 50% and 70% aqueous MeOH
(100 ml each). The 90% aqueous MeOH fraction was
evacuated to dryness to give a brown residue (1.63 g).
A part of the residue (270 mg) was applied to preparative
HPLC on an ODS column (YMC-pack ODS AM-343,
20×250 mm+20×50 mm, YMC Co. Ltd.) with MeCN-
0.05% TFA in H2O (9:1) at a flow rate of 10 ml/minute to
afford I (21.7 mg) as white powder.

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Physico-chemical Properties

Physico-chemical properties of I are summarized in Table 1. The compound is soluble in most of organic solvents, but insoluble in H2O and n-hexane. The molecular formula was determined to be C23H33NO4 by positive HRFAB-MS and NMR. The UV spectrum of I was comparable to that of equisetin (absorption maxima at 205, 252 and 293 nm), suggesting the presence of tetramic acid moiety.

Structural Elucidation

Since we have isolated equisetin11) and its new analog CJ-17,57212) from different microorganisms in the past, we instantly recognized that I is also a new equisetin analog by its closely related 1H and 13C NMR (Table 2) and UV data. Comparing NMR data to those of equisetin, I was supposed to have additional allylic methyl group at C-4 of equisetin. However, overall of 13C NMR signals are not perfectly correspondent to those of equisetin, to be confident to the structure. Hence, we also found another equisetin analog on the literature, named phomasetin13) which showed perfect match of 13C NMR data to those of I, except for additional 2 olefinic carbons on C-3 side chain. By this good agreement of 13C NMR data, along with COSY, HMQC and HMBC support, the structure of I was determined as shown in Fig. 1. trans-Geometry of C-13 olefin was confirmed by the coupling constant of H-14 (J=5.28 ppm, dq, J=14.9, 6.2 Hz) in methanol-d4 which was not clear in MeCN-d3.

1H NMR of I in various solvents showed many broadened and overlapped signals. Because of these reasons, many 1H NMR signals could not be read directly from its 1D-spectra. Chemical shifts of such signals in Table 2 were interpreted from 2D-spectra such as COSY and HMQC. After this interpretation, it became clear that

<table>
<thead>
<tr>
<th>Table 1. Physico-chemical properties of CJ-21,058 (I).</th>
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<tbody>
<tr>
<td>Appearance</td>
</tr>
<tr>
<td>Molecular weight</td>
</tr>
<tr>
<td>Molecular formula</td>
</tr>
<tr>
<td>HRFAB-MS (m/z)</td>
</tr>
<tr>
<td>Found:</td>
</tr>
<tr>
<td>Calcd.:</td>
</tr>
<tr>
<td>[α]D (23°C, MeOH)</td>
</tr>
<tr>
<td>UV λmax (MeOH) nm (ε)</td>
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<td>IR νmax (KBr) cm⁻¹</td>
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</table>

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<thead>
<tr>
<th>Table 2. 13C and 1H NMR assignment for CJ-21,058 (I) in MeCN-d3 (referred to δ 118.2 and 1.90 ppm for 13C and 1H, respectively).</th>
</tr>
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<tbody>
<tr>
<td>No.</td>
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<td>3</td>
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<td>4'</td>
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<td>5'</td>
</tr>
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<td>6'</td>
</tr>
<tr>
<td>7'</td>
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<tr>
<td>1-OH</td>
</tr>
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</table>

¹ Many of the 1H chemical shifts were interpreted from COSY and HMQC spectra.

Fig. 1. Structures of CJ-21,058 (1), equisetin (2), CJ-17,572 (3) and phomasetin (4).
all of $^1$H NMR chemical shifts of I could also be superimposed to those of phomasetin except for C-3 side chain. All of these correspondences, including some coupling constant data, let us assume that relative stereochemistry of I is same as that of phomasetin. But it should be further confirmed (NOESY of I at room temperature did not provide high-quality data, probably because of broadened and overlapped signals).

Biological Activities

SecA inhibitory activity of I was tested, comparing with equisetin and its derivative CJ-17,572. As shown in Table 3, only I showed SecA inhibitory activity at IC$_{50}$ with 15 $\mu$g/ml. In vitro minimum inhibitory concentrations (MICs) were determined by the broth microdilution method. Among three equisetin derivatives, I showed good growth inhibitory activity against multi-drug resistant *Staphylococcus aureus* and *Enterococcus faecalis* with MIC of 5 $\mu$g/ml but it has no activity against *Streptococcus pyogenes* and *Escherichia coli* like the other equisetin derivatives at 20 $\mu$g/ml (Table 4). Compound I showed moderate cytotoxicity against HeLa cell with IC$_{50}$ of 32 $\mu$g/ml.

Discussion

Compound I was isolated from the fermentation broth of an unidentified fungus CL47745 as a new equisetin derivative, having a hydrophilic tetramic acid moiety and a hydrophobic bicyclic moiety. A lot of microbial metabolites containing a tetramic acid moiety have been reported to show diverse biological activity\textsuperscript{14} and our present study provides the first example of natural product belonging to this novel class as an inhibitor of SecA. In our study, I inhibits SecA activity with an IC$_{50}$ of 15 $\mu$g/ml, while CJ-17,572 and equisetin showed 45% and 7% inhibitory activity, respectively at 20 $\mu$g/ml. These results suggest that it seems to have some correlation between SecA inhibitory activity and antibacterial activity. The structural difference between equisetin and I is a methyl group at C-4 position, indicating that it will be worthwhile to examine some SAR studies for SecA inhibitory activity and antibacterial activity. There has been reported that equisetin derivatives will decrease its antibacterial activity in the presence of 5% sheep blood owing to strong serum binding of the compound\textsuperscript{15}. *S. pyogenes* requires lysed horse blood in assay medium. This suggests that I lost its antibacterial activity in the assay medium of *S. pyogenes*.

Experimental

General

Spectral and physico-chemical data were obtained by the following instruments: UV, JASCO Ubest-30; IR, Shimadzu FTIR-8200PC; NMR, JEOL JNM-LA270; FAB-MS, JEOL JMS-700 (MStation); and Optical rotation, JASCO DIP-370 with a 5 cm cell. All FAB-MS spectra were measured using glycerol-matrix.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ ($\mu$g/ml)</th>
</tr>
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<tr>
<td>CJ-21,058 (I)</td>
<td>15</td>
</tr>
<tr>
<td>Equisetin</td>
<td>&gt;20 (7% inhibition at 20 $\mu$g/ml)</td>
</tr>
<tr>
<td>CJ-17,572</td>
<td>&gt;20 (45% inhibition at 20 $\mu$g/ml)</td>
</tr>
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</table>

Table 3. SecA inhibitory activity of equisetin derivatives.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>CJ-21,058 (I)</th>
<th>CJ-17,572</th>
<th>Equisetin</th>
<th>ERM</th>
<th>AZM</th>
<th>VAM</th>
</tr>
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<tr>
<td><em>Staphylococcus aureus</em> 01A1105</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>1.56</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em> 02C1068</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>0.39</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> 03A1069</td>
<td>5</td>
<td>20</td>
<td>20</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>12.5</td>
</tr>
<tr>
<td><em>Escherichia coli</em> 51A0266</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>100</td>
<td>1.56</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

ERM: erythromycin, AZM: azithromycin, VAM: vancomycin

Table 4. Antibacterial activity of equisetin derivatives.
Producing Microorganism

The strain CL47745 was isolated from a soil collected in Nagasaki, Japan. It was maintained on potato dextrose agar slant (Difco).

Fermentation

A vegetative cell suspension from the slant culture was inoculated into two 500-ml flasks containing 100 ml of a seed medium (potato dextrose broth 2.4%, yeast extract 0.5% and agar 0.1%). The flasks were shaken at 26°C for 4 days on a rotary shaker with 2-cm throw at 125 rpm to obtain seed culture.

This seed culture (5 ml) was used to inoculate into fifteen 500-ml flasks containing 100 ml of a production medium (glucose 1%, glycerol 6.6%, NZ Amine Type A 0.5%, ammonium sulfate 0.2%, defatted soybean meal 0.5%, tomato paste 0.5% and sodium citrate 0.2% and adjusted to pH 7.0) and 30 g buckwheat. Static fermentation was carried out at 26°C for 18 days.

HPLC Analysis

Analytical HPLC of equisetin derivatives were performed using an ODS column (YMC-pack FL-ODS3 AM, 4.6×50 mm, YMC Co. Ltd.) with MeCN-0.05% TFA in H2O (13:7) at a flow rate of 0.9 ml/minute. Under these conditions, equisetin, CJ-17,572 and I were eluted at the retention times of 4.8, 5.8 and 6.7 minutes, respectively.

SecA Activity

SecA, proOmpA and everted inner membrane vesicles (IMVs) were prepared from E. coli internally. Everted IMVs bear the heteromeric SecYEG.

To prepare the Malachite Green solution, one volume of 4.2% (w/v) ammonium molybdate in 4 M HCl was added to 3 volumes of 0.045% (w/v) Malachite Green in H2O. This solution was clarified on ice at least one hour stirring occasionally before filtering through # 3 paper (Whatman). Triton X-100 (1%, v/v) was added to this solution before use. Before assay, each substock solution of SecA, IMV and proOmpA was prepared. SecA substock (50 fold) contained 130 μg/ml SecA in 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM MgCl2, 100 μg/ml BSA and 1 mM DTT. IMV substock (25 fold) contained 1.87 mg/ml IMV in 50 mM HEPES, pH 7.6, 5 mM MgSO4 and 1 mM DTT. ProOmpA substock (50 fold) contained 300 μg/ml proOmpA in 8 M Urea, 50 mM Tris-HCl, pH 8.0 and 2 mM DTT. Master-mix was prepared on ice by mixing three substocks in 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM MgCl2, 1 mM DTT, 100 μg/ml BSA and 0.5 mM ATP. Ten μl of samples or sodium azide as a control inhibitor was placed in a 96 well microtiter plate and 40 μl of the master-mix was delivered to initiate the reaction. Plates were incubated at 30°C for 45 minutes. After the incubation, 200 μl of the Malachite Green solution was added and mixed well. Then, 25 μl of 34% (w/v) sodium citrate was delivered to arrest unwanted hydrolysis of ATP caused by HCl content of the Malachite Green solution. After an interval of 45 minutes at room temperature, plates were read spectrophotometrically at 690 nm (Wallac, ARVO). Percent inhibition of SecA was calculated as follows:

Inhibition (%) = \( \frac{1 - \frac{A_{690}(\text{sample}) - A_{690}(200 \text{mM NaN}_3)}{A_{690}(\text{control}) - A_{690}(200 \text{mM NaN}_3)}}{\times 100} \)

where A690 was the absorbance at 690 nm obtained on a counter.

Test Strains, Antibacterial Assay and Cytotoxicity

The MIC determinations of antibacterial activity and cytotoxicity test were performed according to the methods of our previous paper12).

References

9) OLIVER, D. B.; R. J. CABELLI, K. M. DOLAN & G. P.


