STUDIES ON NEW DEHYDROPEPTIDASE INHIBITORS

I. TAXONOMY, FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL PROPERTIES

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WS1358A1 (FR104007) and B1 (FR104008), new potent inhibitors of renal dehydropeptidase, were isolated from the culture broth of strain No. 1358 which was identified as Streptomyces parvulus subsp. In vitro inhibitory activities (IC50 value) of WS1358A1 and B1 against porcine renal DHP were 3 and 600 nM, respectively.

Renal dipeptidase, dehydropeptidase1,2) (DHP, EC.3.4.13.11.) hydrolyzes the β-lactam ring of the carbapenems and penems which are new classes of structurally novel β-lactam antibiotics3~5). The low recovery of the carbapenems in the urine of laboratory animal and the low efficacy against experimental infection are attributed to the metabolism of the antibiotics by DHP6).

Cilastatin7,8), an inhibitor of DHP, was synthetized by Merck’s researchers and has been clinically used in a new combination antimicrobial imipenem/cilastatin. In this combination, cilastatin has been proved to be effective in protecting imipenem, a derivative of thienamycin, from hydrolysis by DHP and restoring the urinary recovery of imipenem in human as well as in experimental animals9).

No specific inhibitor against DHP other than cilastatin has been yet reported. During our screening program searching for novel enzyme inhibitors, we recently found specific inhibitors against DHP, designated WS1358A1 and B1† (Fig. 1), from the fermentation products.

This paper describes taxonomic studies on the producing strain, fermentation, isolation and physico-chemical properties of these compounds. The structural elucidation of the compounds will be described in a succeeding paper10).

Materials and Methods

Taxonomic Studies

The producing organism, strain No. 1358, was isolated from a soil sample collected at Tochigi city, Tochigi Prefecture, Japan. The media and procedures used for cultural and physiological characterization of strain No. 1358 were described by Shirling and Gottlieb11). Each culture was incubated at 30°C for

† WS1358A1 and B1 have been reported as FR104007 and FR104008, respectively, in Eur. Pat. Appl. 276, 947, Aug. 3, 1989.
2 to 3 weeks before observation. The temperature range for growth was determined on yeast extract-malt extract agar using a temperature gradient incubator (Advantec Toyo Co.). The color names used in these studies were based on the Methuen Handbook of Colour. The chemical composition of the cell wall was analyzed by the methods described by Becker et al. and Lechevalier and Lechevalier. Utilization of carbon sources was examined according to the method of Pridham and Gottlieb.

Fermentation
A loopful of the slant culture of strain No. 1358 was inoculated to a 500-ml flask containing 160 ml of the seed medium composed of corn starch 1.0%, glycerol 1.0%, glucose 0.5%, cotton-seed flour 1.0%, dried yeast 0.5%, corn steep liquor 0.5% and CaCO₃ 0.2% (pH 6.5). The flask were shaken on a rotary shaker (220 rpm) for 3 days at 30°C. A 200-liter jar fermenter containing 150 liters of production medium composed of glucose 1.0%, glycerol 2.0%, cotton-seed flour 1.0%, soy bean meal 1.0%, dried yeast 0.5% and CaCO₃ (pH 7.0) was inoculated with 3 liters of the seed broth and cultured for 4 days at 30°C, aerated at 150 liters per minute and agitated at 250 rpm.

Enzyme Assay
The production and initial purification of WS1358 compounds were monitored with the enzyme assay to determine the inhibitory activity. Partially purified porcine renal DHP is able to hydrolyze the unsaturated dipeptide, glycyldehydrophenylalanine (GDP) as substrate. The activity of the enzyme was determined by observing the decline in absorbance of the substrate at 275 nm. Inhibitory effect of a compound on DHP activity was also measured in a similar way. Inhibition percent, I%, was calculated as follows, 

\[ I\% = \frac{(E - T)}{E} \times 100, \]

where E was DHP activity without the compound, T was DHP activity with the compound.

Materials
Partial purification of DHP from porcine kidney cortex was carried out using procedures slightly different from a previously reported method. Details will be described in a separate paper. GDP was synthesized by the method previously described. Cilastatin was purified from a commercially available drug, Zienam.

Results
Identification of Strain No. 1358
Morphological observations were made with a light and a scanning electron microscope (Fig. 2) on cultures grown at 30°C for 2 to 3 weeks on yeast extract-malt extract agar, oatmeal agar and inorganic salts-starch agar. The substrate mycelia were well developed and branched without fragmentation. The aerial mycelia branched monopodially, and formed spiral, sometimes looped, spore chains with 10 to 50 spore per chain. The spores had a smooth surface and were subglobose to oblong in shape with a size of 0.5 to 0.7 by 0.5 to 0.9 μm. These spore masses often appeared to coalesce in moist globules. Sclerotic granules, sporangia and zoo-spores were not observed. The cultural characteristics of strain No. 1358 are summarized in Table 1. The aerial mass color was brownish gray to grayish brown. Reverse side of growth was yellowish white to
Table 1. Cultural characteristics of strain No. 1358.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth</th>
<th>Aerial mycelium</th>
<th>Reverse side of substrate mycelium</th>
<th>Soluble pigments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract - malt extract agar</td>
<td>Abundant</td>
<td>White, brownish gray (6D2), grayish brown (7F3)</td>
<td>Grayish yellow (4B3)</td>
<td>None</td>
</tr>
<tr>
<td>Oatmeal agar</td>
<td>Moderate</td>
<td>Grayish brown (5F3)</td>
<td>Yellowish white (3A2)</td>
<td>None</td>
</tr>
<tr>
<td>Inorganic salts - starch agar</td>
<td>Abundant</td>
<td>Brownish gray (7D-E2)</td>
<td>Grayish yellow (4B3)</td>
<td>None</td>
</tr>
<tr>
<td>Glycerol - asparagine agar</td>
<td>Abundant</td>
<td>Brownish gray (6C2)</td>
<td>Yellowish white (4A2)</td>
<td>None</td>
</tr>
<tr>
<td>Peptone - yeast extract - iron agar</td>
<td>Moderate</td>
<td>None</td>
<td>Light yellow (4A4-5)</td>
<td>None</td>
</tr>
<tr>
<td>Tyrosine agar</td>
<td>Abundant</td>
<td>Orange gray (5B2), brownish gray (7E2)</td>
<td>Grayish orange (5B3)</td>
<td>Scant, grayish orange</td>
</tr>
<tr>
<td>Glucose - asparagine agar</td>
<td>Abundant</td>
<td>Brownish gray (6C-D2), grayish brown (7F3)</td>
<td>Yellowish white (4A2)</td>
<td>None</td>
</tr>
<tr>
<td>Nutrient agar</td>
<td>Moderate</td>
<td>None</td>
<td>Pale yellow (3A3)</td>
<td>None</td>
</tr>
<tr>
<td>BENNETT agar</td>
<td>Abundant</td>
<td>Dark brown (6F4)</td>
<td>Pale yellow (3A3)</td>
<td>None</td>
</tr>
<tr>
<td>Sucrose - nitrate agar</td>
<td>Abundant</td>
<td>None</td>
<td>Pale yellow (3A3)</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 2. Physiological characteristics of strain No. 1358.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature range for growth</td>
<td>16~33°C</td>
</tr>
<tr>
<td>Optimum temperature for growth</td>
<td>26~32°C</td>
</tr>
<tr>
<td>Liquefaction of gelatin</td>
<td>Weakly positive</td>
</tr>
<tr>
<td>Coagulation of milk</td>
<td>Negative</td>
</tr>
<tr>
<td>Peptonization of milk</td>
<td>Weakly positive</td>
</tr>
<tr>
<td>Hydrolysis of starch</td>
<td>Positive</td>
</tr>
<tr>
<td>Melanoid production</td>
<td>Negative</td>
</tr>
<tr>
<td>Decomposition of cellulose</td>
<td>Negative</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>Positive</td>
</tr>
<tr>
<td>NaCl tolerance</td>
<td>0~2%</td>
</tr>
</tbody>
</table>

Table 3. Utilization of carbon sources by strain No. 1358.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Utilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>+</td>
</tr>
<tr>
<td>Inositol</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>+</td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
</tr>
<tr>
<td>No addition</td>
<td>-</td>
</tr>
</tbody>
</table>

Grayish yellow. Melanoid and other soluble pigments were not produced. The physiological characteristics and utilization of carbon sources of strain No. 1358 are summarized in Tables 2 and 3. Hydrolyzed whole cell of strain No. 1358 contained L-L-diaminopimelic acid. Accordingly, the cell wall of this strain is believed to be of type I.

Based on the taxonomic properties described above, strain No. 1358 is considered to belong to the genus Streptomyces and to be a strain of the gray series of the PRIDHAM and TRESNER grouping. Strain No. 1358 was compared with Streptomyces species described in the literature. As a result, it was found that the strain proved to closely resemble Streptomyces parvulus IFO 13193 in detail. There, it was found that the properties of both strains were almost identical except for a few differences. Table 4 shows the differences between the two strains. These differences are not sufficient to consider that strain No. 1358 belong to a distinct species. So, it is considered to be proper that strain No. 1358 is a sub-species strain of S. parvulus. Therefore, this strain was designated as S. parvulus subsp. tochigiensis No. 1358. The strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan under the accession No. FERM BP-1638.
Table 4. Differences between strain No. 1358 and *Streptomyces parvulus IFO 13193.*

<table>
<thead>
<tr>
<th>No. 1358</th>
<th>IFO 13193</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature range for growth (°C)</td>
<td>16~33</td>
</tr>
<tr>
<td>NaCl tolerance (%)</td>
<td>2</td>
</tr>
</tbody>
</table>

Production of WS1358 Compounds

A typical fermentation profile for the production of WS1358 is shown in Fig. 3. The production of active compounds during the fermentation was monitored by measuring the inhibitory activity against DHP and assessed from the standard curve of the purified WS1358A1. The production as well as the cell growth was initiated on day 2 and rapidly reached a maximum of about 3.2 µg/ml on day 3.

Isolation and Purification of WS1358A1 and B1

The procedures of the isolation are summarized in Fig. 4. Throughout these purification procedures, the WS1358 compounds were monitored by an analytical reverse-phase HPLC instead of enzyme assay.

It is very difficult to isolate WS1358A1 directly from the fermentation broth by means of conventional methods, because it is only produced in a small quantity and has the same physico-chemical properties close to those of some impurities. However, we discovered that WS1358A1 can be reversibly transformed to the two compounds, designated A2 and A3, which are more lipophilic and more easily isolated from the fermentation broth than A1 is. Making use of this chemical transformation to purify WS1358A1, we converted A1 to A2 and A3 in the course of purification and finally converted the purified A3 to A1. More detailed mechanism of this chemical transformation will be described elsewhere.

The cultured broth was filtered with the aid of diatomaceous earth. The filtrate (390 liters) was adjusted to pH 10 with 6N NaOH and allowed to stand for 2 hours at room temperature (A2, A3 → A1 conversion). The resultant precipitate was filtered and discarded. The filtrate was passed through a column of Dowex 1X2 (Cl⁻ type). The active principle was eluted with 0.2M NaCl solution after washing the column with water and 0.1M NaCl solution. The eluate was adjusted to pH 2 with 6N HCl and adsorbed on activated carbon. The active principle was eluted with 25% aqueous methanol containing 0.5N NH₄OH after washing the column with water and 25% aqueous methanol. The eluate was concentrated *in vacuo* and adjusted to pH 2 with 6N HCl. The desalted eluate was applied on a column of an adsorption resin Diaion SP-207 and developed with water. The fraction containing WS1358A1 was eluted more slowly than the fraction containing WS1358B1.

WS1358A3

The former fraction of the Diaion SP-207 column chromatography was concentrated *in vacuo* followed
Fig. 4. Isolation procedure of WS1358 compounds.

Culture filtrate (390 liters)
  adjusted to pH 10, filtered

Filtrate
  Dowex 1×2 (Cl⁻)
    eluted with 0.2 M NaCl
    adjusted to pH 2
  Active carbon
    eluted with 25 % aq MeOH - 0.5 N NH₄OH
    concentrated
    adjusted to pH 2
  Diaion SP-207
    eluted with H₂O

neutralized
concentrated

Cellulose
  eluted with 75 % aq 2-ProH
  concentrated

DEAE Sephadex A-25 (Cl⁻)
  eluted with 0.2 M NaCl
  concentrated

Sephadex G-15
  eluted with H₂O
  concentrated

B1 (1.3 g)

neutralized
concentrated

Sephadex G-15
  eluted with H₂O
  freeze-dried

A3 (30.2 mg)
  dissolved in 0.5 N NaOH
  neutralized

DEAE Sephadex A-25 (Cl⁻)
  eluted with 0.2 M NaCl
  concentrated
  adjusted to pH 2
  Diaion SP-207
    eluted with H₂O
    neutralized
    freeze-dried

A1 (14.9 mg)
by the addition of an equal volume of 0.2 M NH₄H₂PO₄ buffer (pH 2.3). The resultant solution was passed through a column of Dowex 1X2 (Cl⁻ type) equilibrated with 0.1 M NH₄H₂PO₄ buffer and eluted with the same buffer. The active fraction containing WS1358A₁ was concentrated in vacuo and freeze-dried (A₁ → A₂ + A₃ conversion). The lyophilized material thus obtained was dissolved in deionized water. The solution was applied on a column of Diaion SP-207 and developed with water. WS1358A₂ and A₃ could be separated from each other in this column chromatography. The fraction containing WS1358A₃ was neutralized with 6 N NaOH and applied on a column of Sephadex G-15. The eluate was freeze-dried to give a white powder of WS1358A₃ (35.4 mg).

WS1358A₁

WS1358A₃ (30.2 mg) was dissolved in 0.5 N NaOH (3.5 ml) and allowed to stand for 30 minutes at room temperature (A₃ → A₁ conversion), then diluted with water after neutralization with 6 N HCl. The

**Table 5. Physico-chemical properties of WS1358A₁ and Bl.**

<table>
<thead>
<tr>
<th></th>
<th>A₁</th>
<th>B₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP (°C, dec)</td>
<td>98~100</td>
<td>92~93</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C₇H₉NO₇Na₂</td>
<td>C₆H₇NO₇Na₂</td>
</tr>
<tr>
<td>Elementary analysis</td>
<td>Caled: H 3.92, N 4.95, Na 16.24</td>
<td>Caled: H 3.37, N 5.20, Na 17.08</td>
</tr>
<tr>
<td>FAB-MS (m/z) for free acid</td>
<td>222 (M⁺ + 1)</td>
<td>208 (M⁺ + 1)</td>
</tr>
<tr>
<td>[a]D²² (H₂O)</td>
<td>-14.0° (c 0.9)</td>
<td>+2.5° (c 1.0)</td>
</tr>
<tr>
<td>IR v_max (KBr) cm⁻¹</td>
<td>3500~2500, 1660, 1580, 1360, 1160, 1100, 1000, 880, 800</td>
<td>3500~2500, 1660, 1620, 1580, 1400, 1380, 1200, 1130, 1100, 880, 800</td>
</tr>
<tr>
<td>Rf value (I)</td>
<td>0.43</td>
<td>0.40</td>
</tr>
<tr>
<td>(II)</td>
<td>0.56</td>
<td>0.46</td>
</tr>
</tbody>
</table>

* Silica gel TLC (Merck Art. No. 5715), solvent (I) BuOH- AcOH-H₂O (2:1:1), (II) 2-ProH-H₂O (65:35).

Fig. 5. IR spectra of WS1358A₁ and B₁ (KBr).

![IR spectra of WS1358A₁ and B₁ (KBr).](image-url)
resultant solution was applied on a column of DEAE-Sephadex A-25 (Cl⁻ type) and eluted with water, 0.1 M NaCl and 0.2 M NaCl solution. The active fraction was adjusted to pH 2 with 6 M HCl and concentrated in vacuo, then applied on a column of Diaion SP-207 and developed with water. The eluate was neutralized with 1 M NaOH and freeze-dried to give a white powder of WS1358A1 as Na salt (14.9 mg).

WS1358B1

The fraction containing WS1358B1 obtained by the 1st Diaion SP-207 column chromatography was neutralized with 1 M NaOH and concentrated in vacuo. The resultant solution was applied on a column of Cellulose CF11 and washed with 2-propanol, then developed with 75% aqueous 2-propanol. The active fraction was concentrated in vacuo and applied on a column of DEAE-Sephadex A-25 (Cl⁻ type). The active fraction was eluted with 0.2 M NaCl solution and concentrated in vacuo. The eluate was applied on a column of Sephadex G-15 equilibrated with water, then eluted with water. The desalted eluate was concentrated in vacuo to give the residue. The residue was recrystallized from aqueous methanol to give a white powder of WS1358B1 as Na salt (1.3 g).

Fig. 6. ¹H NMR spectra of WS1358A1 and B1 (400 MHz, D₂O).

![Fig. 6. ¹H NMR spectra of WS1358A1 and B1 (400 MHz, D₂O).](image)

Fig. 7. ¹³C NMR spectra of WS1358A1 and B1 (100 MHz, D₂O).

![Fig. 7. ¹³C NMR spectra of WS1358A1 and B1 (100 MHz, D₂O).](image)
Physico-chemical Properties of WS1358A1 and B1

Physico-chemical properties and spectral data of WS1358A1 and B1 Na salts are summarized in Table 5. The two compounds are soluble in water, slightly soluble in methanol and insoluble in acetone, ethyl acetate, chloroform and n-hexane. They give positive color reactions for ferric chloride, Ehrlich, ninhydrin and iodine vapor reagents, but not for Dragendorff, Molisch and cerium sulfate reagents. These data suggested that the compounds have N-hydroxyl group. The IR, $^1$H and $^{13}$C NMR spectra of Na salts are shown in Figs. 5, 6 and 7, respectively. The structures of WS1358A1 and B1 were deduced as shown in Fig. 1 on the basis of the physico-chemical and spectral data. Details of the structural elucidation will be described in the following paper$^{10}$.

Biological Properties of WS1358A1 and B1

WS1358A1 and B1 were found to strongly inhibit DHP from porcine kidney cortex. The IC$_{50}$ values of WS1358A1 and B1 were estimated to be about 3 and 600 nm respectively while that of cilastatin was 130 nm. They exhibited no antimicrobial activity at the concentration of 1 mg/ml by the disk diffusion assay method against Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Candida albicans and Aspergillus niger. The acute toxicity (LD$_{50}$) of WS1358A1 was > 1 g/kg when administrated intravenously to mice.

Discussion

The susceptibility of carbapenem antibiotics to metabolic inactivation by renal DHP is a major hindrance to the development of these antibiotics$^{24}$. There are two ways to overcome this problem. One way is to combine the carbapenem with a DHP inhibitor, the other way is to synthesize derivatives$^{25}$ of carbapenem which are resistant to enzymatic inactivation by DHP. Merck's researchers resolved the problem by synthesizing an DHP inhibitor, cilastatin$^{7}$. The antimicrobial combination imipenem/cilastatin has been proved to be very effective$^{9}$.

From the same point of view, we have been searching for novel DHP inhibitors of microbial origin in place of cilastatin. Recently we discovered potent DHP inhibitors, WS1358A1 and B1, produced by Streptomyces sp. They showed no cytotoxicity to the cultured cells (data not shown) and no toxicity to experimental animals.

In particular, WS1358A1 has more potent inhibitory activity than cilastatin and may have the potential to be developed in an antimicrobial combination with a carbapenem or penem antibiotic.

References


Kornerup, A. & J. H. Wanscher (Ed.): Methuen Handbook of Colour. Eyre Methuen Ltd., 1978


