

## SNA-8073-B, a New Isotetracenone Antibiotic Inhibits Prolyl Endopeptidase

### I. Fermentation, Isolation and Biological Properties

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SNA-8073-B, an inhibitor of prolyl endopeptidase isolated from the broth filtrate of *Streptomyces* sp. SNA-8073, is a new isotetracenone antibiotic. It was purified by ethyl acetate extraction, silica gel column chromatography and high performance liquid chromatography on ODS column. SNA-8073-B has the molecular formula of C<sub>20</sub>H<sub>16</sub>O<sub>5</sub> and is a stereoisomer of SNA-8073-A (fujianmycin B, rubiginone A<sub>2</sub>). SNA-8073-B inhibited prolyl endopeptidase of *Flavobacterium* non-competitively (IC<sub>50</sub> = 8.9 μM) when Z-Gly-Pro-pNA was used as a substrate, but SNA-8073-A did not show any inhibition even at 60 μM.

Prolyl endopeptidase (PEP: post-proline cleaving enzyme, the latest name is prolyl oligopeptidase: EC 3.4.21.26) was first isolated from human uterus as an oxytocin-inactivating enzyme and cleaves peptide bonds at the carboxyl side of proline residues<sup>1~3</sup>). It is distributed in a wide range of species, especially reported to be in human brain<sup>4</sup>) and prolyl endopeptidase-like immunoreactivity was detected in the mouse hippocampus<sup>5</sup>). PEP, a new type of serine proteinases, has been proposed to play a role in degradation of proline containing biologically active peptides such as oxytocin, vasopressin, substance P, bradykinin, LH-RH, neurotensin and angiotensins<sup>6,7</sup>). Therefore, it is suggested to be closely related to the biological regulation of these peptides. Vasopressin has been suggested to be concerned with learning and memory processes<sup>8,9</sup>). PEP activity of ALZHEIMER's patients is significantly higher than normal<sup>10</sup>) and a putative amyloid A4-generating enzyme in ALZHEIMER's disease is identified as PEP<sup>11</sup>). Moreover it was reported that the neurodegenerative effects of β amyloid could be prevented by intracerebral or systematic administration of substance P<sup>12</sup>). Thus, specific inhibitors of PEP are expected to have anti-amnesic effects and its inhibitors have been synthesized as anti-amnesic drugs<sup>13,14</sup>). Many synthetic inhibitors have an aldehyde moiety in the C-terminal and the structure-activity relationships have been examined<sup>15,16</sup>).

In the course of screening for a new type of PEP inhibitor from Actinomycetes, we have already isolated a known antifungal and protein kinase inhibitor, staurosporine<sup>17</sup>) and a new cyclic peptide antibiotic named propeptin<sup>18</sup>). During further screening for PEP inhibitors, we found that *Streptomyces* sp. SNA-8073 produced a new isotetracenone antibiotic, SNA-8073-B, which inhibited PEP. In this communication, we report the fermentation, isolation, physico-chemical and biological properties of SNA-8073-B.

### Materials and Methods

#### Materials

Prolyl endopeptidase (*Flavobacterium*) and substrate (Z-Gly-Pro-pNA) were purchased from Seikagaku Kogyo Co., Ltd.

#### Microorganism

Actinomycete SNA-8073 isolated from a soil sample collected in Tottori-city, Tottori prefecture, Japan was used in this experiment.

#### Medium and Culture

The seed medium and the production medium consisted of glucose 2%, soluble starch 1%, meat extract 0.1%, dried yeast 0.4%, soybean flour 2.5%, NaCl 0.2% and K<sub>2</sub>HPO<sub>4</sub> 0.005%, pH 6.7. A loopful of the producing strain SNA-8073 from a slant culture was inoculated into a 500-ml volume Erlenmeyer flask containing 70 ml of

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the medium. The flask was incubated for 10 days at 27°C on a rotary shaker (200 rpm). Two milliliters of the culture was transferred into the same flask. The flask was then incubated for 3 day at 27°C on a rotary shaker. After incubation, 140 ml of the seed culture were transferred into a 10-liter jar fermenter containing 5 liters of the medium. The fermenter was operated for 216 hours at 27°C with agitation at 180 rpm and aeration of 5 liters/minute. The pH was maintained at 8.6.

#### Measurement of Enzyme Activities

PEP inhibition assay using cultured broth of Actinomycete and PEP inhibition activity of SNA-8073-B were measured as described previously<sup>17</sup>.

#### Physico-chemical Properties

Melting point was measured with a micro melting point apparatus (Yanagimoto Seisakusyo Co., Japan) and was uncorrected. EI-MS and HR-MS were carried out on a JEOL mass spectrometer DX-303. The optical rotation was determined with a Parkin-Elmer 241 polarimeter using a micro-cell. The UV spectrum was measured with a spectrophotometer U-3210 (HITACHI). The IR spectrum was recorded on a JASCO DIP-181 digital polarimeter.

## Results

### Producing Strain

Strain SNA-8073 did not produce characteristic spores on the aerial hyphae (ISP No. 7). The whole-cell hydrolysate of the strain showed that it contained

LL-diaminopimelic acid. Based on its characteristics, strain SNA-8073 is considered to belong to the genus *Streptomyces*<sup>19</sup>. *Streptomyces* sp. SNA-8073 has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan under the accession number FERM-P13007.

### Production and Isolation of SNA-8073-A and B

The time course of the production in a 500-ml volume Erlenmeyer flask is shown in Fig. 1. The maximum peak of SN-8073-B production in the flask was obtained at 6~7 days. The flow diagram for the isolation is shown in Fig. 2. The culture broth was centrifuged and the broth filtrate (3.8 liters) was obtained. It was extracted with ethyl acetate and evaporated to oily substance (830 mg, IC<sub>50</sub> = 34 µg/ml). Then it was dissolved in chloroform-methanol (20:1) and chromatographed on a silica gel column (2.5 × 23 cm) with chloroform-methanol (20:1 and 10:1). Active fraction was concentrated *in vacuo* and lyophilized. The crude powder (266 mg, IC<sub>50</sub> = 14 µg/ml) was dissolved in a small amount of methanol and final purification was carried out by preparative HPLC using Nucleosil 5C<sub>18</sub> (20 × 250 mm) with 55% CH<sub>3</sub>OH. After concentration and lyophilization, it gave SNA-8073-A and B as yellow powders. The yield of pure SNA-8073-A was 24.8 mg and B was 27.4 mg from 5 liters of the culture.

Fig. 1. Time course of fermentation of *Streptomyces* sp. SNA-8073 (500-ml volume Erlenmeyer flask containing 70 ml medium).

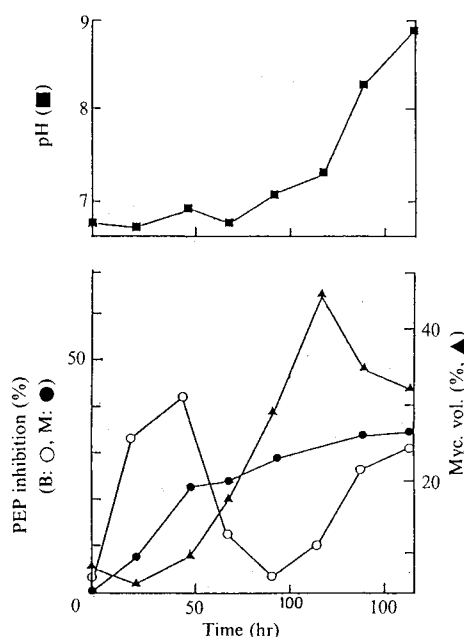


Fig. 2. Isolation procedure of SNA-8073-A and B.

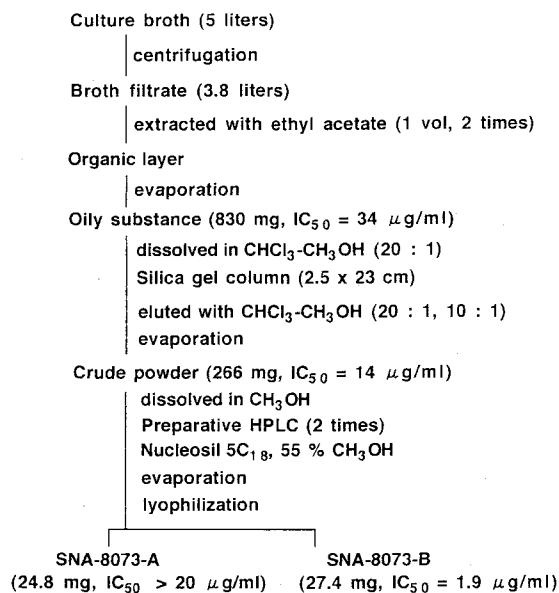


Table 1. Physico-chemical properties of SNA-8073-A and B.

	SNA-8073-A	SNA-8073-B
Nature	Dark yellow powder	Light yellow powder
Melting point	231~233 °C	174 ~176 °C
Molecular formula	C <sub>20</sub> H <sub>16</sub> O <sub>5</sub>	C <sub>20</sub> H <sub>16</sub> O <sub>5</sub>
EI-MS (m/z)	336	336
HREI-MS (m/z)	Found : 336.1014 Calcd. : 336.0998	Found : 336.0984 Calcd. : 336.0998
	for C <sub>20</sub> H <sub>16</sub> O <sub>5</sub>	for C <sub>20</sub> H <sub>16</sub> O <sub>5</sub>
[α] <sub>D</sub> <sup>24</sup>	+47° (c 0.141, CHCl <sub>3</sub> )	-106° (c 0.135, CHCl <sub>3</sub> )
UV λ <sub>max</sub> <sup>MeOH</sup> nm (ε)	264 (30400) 376 (5000)	263 (31200) 376 (5200)
IR ν <sub>max</sub> (KBr) cm <sup>-1</sup>	3520, 1690, 1660	3480, 1700, 1670
Solubility	MeOH, DMSO, CHCl <sub>3</sub>	MeOH, DMSO, CHCl <sub>3</sub>
TLC, Rf value *	0.65	0.65
HPLC, Rt (min) **	9.9	10.4

\* Silica gel 60 F<sub>254</sub> (Merck), CHCl<sub>3</sub>: MeOH (5 : 1).

\*\* ODS-1251-N (4.6 φ x 250 mm), 60 % MeOH, 0.8 ml/min, 220nm.

#### Physico-chemical Properties of SNA-8073-A and B

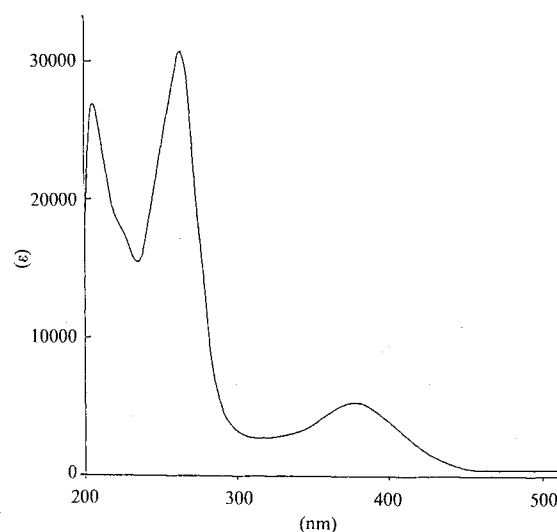
The physico-chemical properties of SNA-8073-A and B are summarized in Table 1. The molecular weights and formulae of both compounds were determined by EI-MS and HREI-MS. The UV and IR spectra of SNA-8073-B are shown in Figs. 3 and 4. SNA-8073-A and B are only different by melting point, optical rotation and retention time by HPLC. The <sup>1</sup>H and <sup>13</sup>C NMR spectra of SNA-8073-B are shown in Figs. 5 and 6. SNA-8073-A was identified as the known antibiotic fujianmycin B (rubiginone A<sub>2</sub>) via comparison with the NMR spectra (data not shown) and the physico-chemical properties described in the literature<sup>20,21</sup>.

The <sup>13</sup>C NMR spectra of SNA-8073-A and B were quite similar. In the <sup>1</sup>H NMR spectrum of SNA-8073-A, coupling constant value between H-3 and H-4 was 9.1 Hz showing it was *trans* configuration. On the contrary, coupling constant value between H-3 and H-4 in SNA-8073-B was 2.9 Hz showing it was *cis* configuration. These data suggested us that SNA-8073-A and B were diastereoisomers in C-3 and C-4 positions. Determination of the chemical structure of SNA-8073-B in detail will be reported elsewhere.

#### Biological Properties of SNA-8073-A and B

SNA-8073-B potently inhibited PEP in a dose dependent manner. The concentration of SNA-8073-B

Fig. 3. UV spectrum of SNA-8073-B (20 μg/ml in MeOH).



causing 50% inhibition of PEP was 3.0 μg/ml (8.9 μM). As shown in Fig. 7, though SNA-8073-B inhibited PEP non-competitively and the inhibitor constant (*K<sub>i</sub>*) was 2.7 μg/ml (8.0 μM), SNA-8073-A did not inhibit PEP even at 60 μM. SNA-8073-B did not inhibit other serine proteinases such as trypsin, chymotrypsin, plasmin, pancreatic kallikrein, thrombin and elastase at 10 μM. SNA-8073-B had weak antimicrobial activity against *Chlorella vulgaris* (10.7 mm) at 40 μg/disc by agar plate

Fig. 4. IR spectrum of SNA-8073-B (KBr).

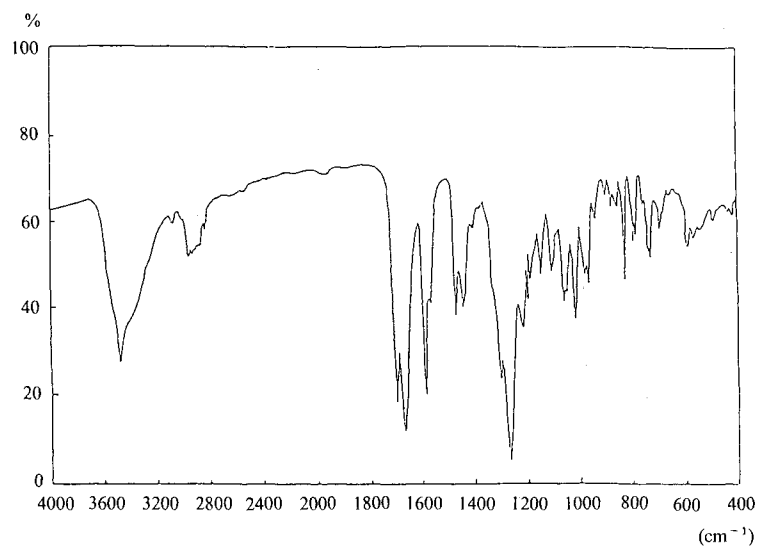
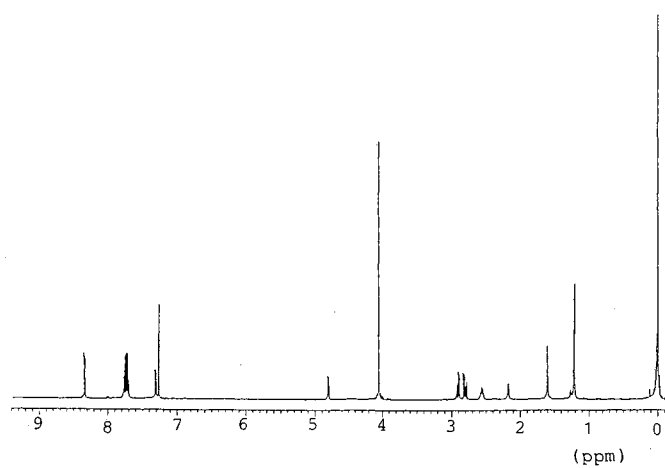
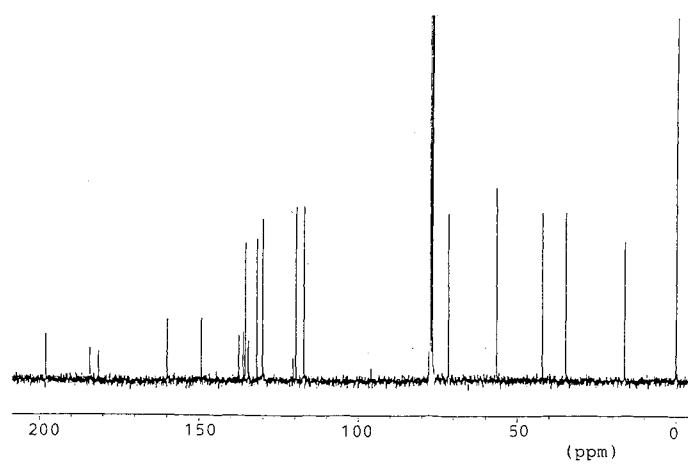
Fig. 5.  $^1\text{H}$  NMR spectrum of SNA-8073-B ( $\text{CDCl}_3$ , 400 MHz).Fig. 6.  $^{13}\text{C}$  NMR spectrum of SNA-8073-B ( $\text{CDCl}_3$ , 67.5 MHz).

Fig. 7. Dixon plot of the inhibition of prolyl endopeptidase by SNA-8073-B.

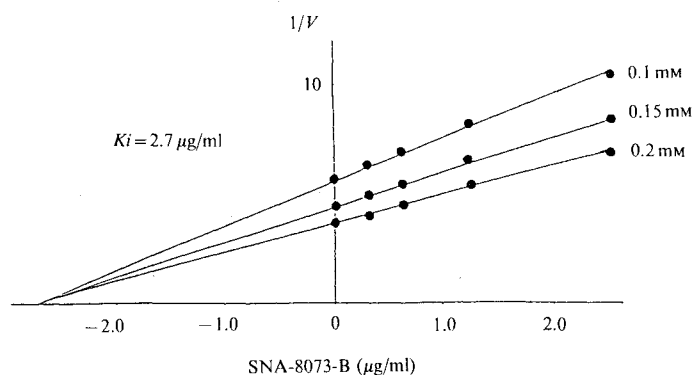
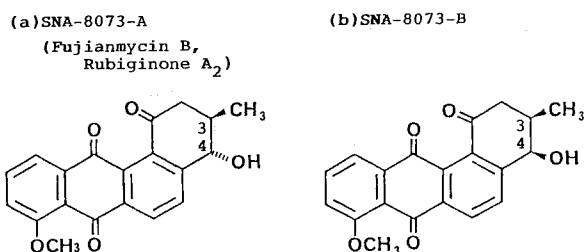


Fig. 8. Structures of SNA-8073-A and B.



method. No antimicrobial activity was detected against Gram-positive, Gram-negative bacteria and fungi even at 40  $\mu\text{g}/\text{disc}$ .

### Discussion

Many inhibitors of PEP such as Z-Pro-prolinol, Z-Pro-prolinal, Z-thiopro-thioproline and Z-thiopro-thiazolidine have been synthesized as anti-amnesic drugs<sup>13-16,22,23</sup>. Structure-activity relationships of inhibitors specific for PEP have also been investigated. On the other hand, known natural product inhibitors are only bacitracin<sup>24</sup>, staurosporine<sup>17</sup>, poststatin<sup>25</sup>, eurystatin<sup>26</sup> and propeptin<sup>18</sup>. Therefore, we screened other types of inhibitor from Actinomycetes and isolated a new type of isotetracenone inhibitor, SNA-8073-B. The strain also produced SNA-8073-A that was identical with known antibiotic fujianmycin B (rubiginone A<sub>2</sub>)<sup>20,21</sup> (Table 1). SNA-8073-B is a new compound which is the stereoisomer of fujianmycin B (rubiginone A<sub>2</sub>) at C-4 position (Fig. 8). Stereochemistry of the 4-OH has an important role in the PEP inhibition activity. It was first reported that the isotetracenone skeleton had an inhibitory effect against PEP.

### Acknowledgements

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