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# WF14861, a New Cathepsins B and L Inhibitor Produced by *Colletotrichum* sp.

## I. Taxonomy, Production, Purification and Structure Elucidation

Takanao Otsuka, Yuko Muramatsu, Tomoji Higaki, Tomoko Nakanishi, Shigehiro Takase, Hiroshi Hatanaka, Masanori Okamoto, Motohiro Hino and Seiji Hashimoto

Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-2698, Japan

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WF14861, a novel cathepsins B and L inhibitor, was obtained from the culture mycelium of a fungus strain *Colletotrichum* sp. No. 14861. Spectroscopic analysis showed that WF14861 consisted of *trans*-epoxysuccinic acid, L-tyrosine and spermidine. WF14861 inhibited cathepsins B and L selectively.

A variety of lysosomal cysteine proteases have been shown to be implicated in the catabolism of proteins and peptides. Among them, cathepsin B (EC 3.4.22.1) and cathepsin L (EC 3.4.22.15) which belong to the papain super family are thought to be involved in collagenolytic diseases such as chronic rheumatoid arthritis and osteoarthritis, *etc.*[1,2] In addition, cathepsin B participates in the process of antigen presentation to MHC class II and the formation of senile plaques in the brain of patients with Alzheimer's disease.[3,4] Recently, cathepsin K (EC 3.4.22.38) was identified as a new member of the papain super family and its crucial role in bone resorption was suggested.[5]

Cathepsins B, L and K inhibitors are therefore expected to be useful against these diseases. We screened microbial products for a new cathepsins B and L inhibitor. As a result of the screening, we isolated a new compound WF14861, 3-(*N*-(1-(*N*-(4-aminobutyl)-*N*-(3-aminopropyl)carbamoyl)-2-(4-hydroxyphenyl)ethyl)carbamoyl)oxirane-2-carboxylic acid, from the culture broth of a fungus *Colletotrichum* sp. No. 14861. This paper describes the taxonomy of the producing strain, production, purification and structure elucidation of this inhibitor. Biological properties of WF14861 both *in vitro* and *in vivo* are described in an accompanying paper[6].

### Materials and Methods

#### Materials

Human liver cathepsin B (Athens Research & Technology) and human liver cathepsin L (Calbiochem) were purchased. Enzyme activities were determined by following the release of 7-amino-4-methylcoumarin (AMC) from the fluorogenic substrate *N*-carbobenzoxy-L-Arg-L-Arg-AMC (Peptide Institute) for cathepsin B and *N*-carbobenzoxy-L-Phe-L-Arg-AMC (Peptide Institute) for cathepsin L. The fluorescence was determined using a Titertek Fluoroscan II spectrophotometer (Flow Laboratories). All other chemicals were of analytical grade.

#### Taxonomy

The fungal strain No.14861 was originally isolated from a soil sample collected at Kanoya City, Kagoshima Prefecture, Japan. The observations were made after 14 days of cultivation at 25°C. The compositions of malt extract agar and Czapek's solution agar were based on the JCM Catalogue of Strains[7]. The color names used in this study were taken from the Methuen Handbook of Colour[8]. The temperature range of growth was determined on potato dextrose agar. The morphological

characteristics were determined from the cultures on LCA plate by MIURA and KUDO[9].

Enzyme Assay

Cathepsin B inhibition was determined by the methods described by AHMED *et al.*[1] with some modifications. Briefly, inhibitors were serially diluted in 50 $\mu$l of 100 mM sodium phosphate buffer (pH 5.5) containing 10 $\mu$M *Z*-Arg-Arg-AMC, 5 mM dithiothreitol and 4 mM EDTA. The reaction was initiated by the addition of 50 $\mu$l of human liver cathepsin B (75 ng/ml). The mixture was incubated at 37°C for 60 minutes and the reaction was stopped by the addition of 100 $\mu$l of 100 mM sodium monochloracetate in a buffer containing 30 mM sodium acetate and 70 mM acetic acid (pH 4.3). The fluorescence of AMC was measured (excitation/emission; 360 nm/emission 460 nm). The fluorescence of the reagent blanks (without enzyme) was subtracted from test readings. Calibration was performed with different concentration of free AMC. All data are the averages of at least duplicate determinations. All reactions were performed in microtiter plates.

Cathepsin L inhibitory activities were similarly determined as cathepsin B using cathepsin L (final, 50 ng/ml) and *Z*-Phe-Arg-AMC.

Detection of WF14861

Detection of WF14861 from the fermentation broth and the fractions under purification was monitored by HPLC using a reverse phase column YMC-ODS-AM (AM303, 250 × 4.6 mm i.d., Yamamura Chemical). The solvent system was 10% aqueous methanol containing 0.05% TFA and the flow late was 1.0 ml/minute. The detection wave length was set at 220 nm.

## Results

### Identification of the Producing Strain

Cultural characteristics on various agar media are summarized in Table 1. This organism grew rapidly on various culture media, and formed black to brown colonies. Culture on malt extract agar spread broadly, attaining more than 7.5 cm in diameter. This colony surface was plane, thin, black at the center, yellowish gray in the outer area, and pale yellow at the edge. Conidial structures were formed abundantly. The reverse color was black at the center, dull green in the outer area, and greenish gray at the edge. The growth rate on Czapek's solution agar was similar to that on potato dextrose agar. The surface was plane, felty and gray. Some conidial structures were observed. The reverse was gray. This strain was able to grow at the temperature range from 4 to 35°C, with the growth optimum at 25 to 31°C.

Strain No. 14861 formed sporodochial conidiomata with setae on some agar media (Fig. 1), while it did not produce a teleomorph. The conidial structures were superficial, separate, consisted of phialidic conidiogenous cells and orange conidial mass. The conidiogenous cells were simple, aggregate, acrogenous, hyaline, smooth, cylindrical to clavate, $15\sim25\times3\sim5$ $\mu$m, and bearing conidial drop on the apex. The conidia were enteroblastic, phialidic, hyaline, orange in mass, smooth, one-celled, narrow cylindrical, with rounded apex and truncated base, and $6\sim18\times2.5\sim3.5$ $\mu$m in size. Setae were brown, smooth, thick-walled, with 3~5 septa, straight but frequently flexuous, subulate, with slightly swollen base, up to 200 $\mu$m long, 1~3 $\mu$m wide at tip and 3~6 $\mu$m wide at base. Vegetative hyphae were smooth, septate, hyaline and branched. The hyphal cells were cylindrical and 1~9 $\mu$m in width. Black sclerotia were sometimes observed.

On the basis of comparing the morphological characteristics with fungal taxonomic criteria by VON ARX[10], BARRON[11] and DOMSCH *et al.*[12], strain No.14861 was considered to belong to the hyphomycete genus *Colletotrichum* Corda 1832. Thus, we identified this isolate as one strain of the genus *Colletotrichum*, and named it *Colletotrichum* sp. No. 14861. The strain has been deposited to the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM BP-4688.

### Production

An aqueous seed medium (120 ml) containing 4.0% sucrose, 2.0% Pharmamedia, 1.0% dried yeast, 1.0% polypeptone, 0.2% $KH_2PO_4$, 0.2% $CaCO_3$ and 0.1% Tween 80 was poured into each of three 500-ml Erlenmeyer flasks and was sterilized at 120°C for 30 minutes. A loopful of strain No. 14861 from a mature slant, grown on YpSs agar at 25°C for 2 weeks, was inoculated into each of the seed flasks. The inoculated flasks were shaken on a rotary shaker at 25°C for 4 days with 5.1 cm throw at 220 rpm. The resultant seed culture (360 ml) was inoculated into 20 liters of sterile fermentation medium. The aqueous production medium consisted of 2.0% corn starch, 1.0% glycerin, 1.0% peanut powder, 0.5% corn steep liquor and 0.2% $CaCO_3$

Table 1. Cultural characteristics of the strain No. 14861.

| Medium | Cultural characteristics |
|---|---|
| Malt extract agar (Blaskeslee 1915) | G: Spreading broadly, more than 7.5 cm<br>S: Plane, thin, formed anamorph abundantly, black at the center, yellowish gray (2B2) in the outer area, pale yellow (1A3) at the edge<br>R: Black at the center, dull green (30D3) in the outer area, greenish gray (1B2) at the edge |
| Potato dextrose agar (Difco) | G: Spreading broadly, more than 7.5 cm<br>S: Felty, formed anamorph, grayish brown (6D3) at the center, black in the outer area, pale yellow (3A3) at the edge<br>R: Light brown (5D4) at the center, plive (1F4) in the outer area, yellowish white (1A2) at the edge |
| Czapek's solution agar (Raper and Thom 1949) | G: Spreading broadly, more than 7.5 cm<br>S: Plane, felty, formed anamorph, gray (1F1)<br>R: Gray (1F1) |
| Sabouraud dextrose agar (Difco 0190) | G: Spreading broadly, more than 7.5 cm<br>S: Felty to powdery, sulcate, formed anamorph, brownish gray (5E2) at the center, orange gray (6B2) in the outer area, brownish orange (6C3) at the edge<br>R: Gray (1F1) at the center, brownish gray (5F2) in the outer area, grayish brown (5D3) at the edge |
| Oat meal agar (Difco 1552) | G: Spreading broadly, more than 7.5 cm<br>S: Plane, felty, no anamorph observed, black<br>R: Olive (3D3) |
| Emerson Yp Ss agar (Difco 0739) | G: Spreading broadly, more than 7.5 cm<br>S: Plane, felty, no anamorph observed, brownish orange (7C3), pale yellow (4A3) at the edge<br>R: Brownish orange (6C3), yellowish gray (4B2) at the edge |
| Corn meal agar (Difco 0386) | G: Spreading broadly, more than 7.5 cm<br>S: Plane, thin, no anamorph observed, yellowish white (2A2)<br>R: Yellowish white (2A2) |

These characteristics were observed after 14 days of incubation at 25°C in the dark condition.

Abbreviation G: growth, measuring colony size in diameter, S: colony surface, R: reverse

was poured into a 30-liter stainless steel jar fermenter. The pH of the medium was adjusted to 6.0 with 6 N-NaOH solution prior to sterilization at 120°C for 30 minutes. The fermentation was carried out at 25°C for 6 days employing aeration at 20 liters/minute and stirring at 180 rpm.

## Purification

After the culture was terminated, the culture broth (25 liters) was filtered and 25 liters of methanol was added to the mycelial cake with stirring. The mixture was allowed to stand for 5 hours, and was filtered. The extract was concentrated to 2.5 liters of aqueous solution under reduced pressure and was washed with 2.5 liters of *n*-butanol. The water layer was passed through a cation exchange resin Amberlite IRC-50 ($H^+$ type, 2.5 liters) and washed with 5 liters of water and 7.5 liters of 0.6 N-$NH_4OH$. The desired substance was eluted from the column with 9 liters of 1.0 N-$NH_4OH$ and neutralized with 6 N-HCl. The eluate was adsorbed onto a polymeric adsorbent Diaion SP-207 (0.4 liters) followed

Fig. 1. Micrograph of conidiomata of *Colletotrichum* sp. No. 14861 grown on Miura's LCA plate.

Bar represents 10 $\mu$m.

by washing with 1 liter of water, 1.3 liters of 50% aqueous methanol, 1.1 liters of 90% aqueous methanol and 0.4 liters of 20% aqueous methanol. Active constituent was eluted from this column with 4 liters of 20% aqueous methanol containing 0.05% TFA. The eluate was concentrated to dryness to give 445 mg of residue. Forty milligram of the residue was dissolved in a small amount of water and was subjected to a reverse phase packed column YMC-ODS-AM (SH-343-5AM, Yamamura Chemical). The column was eluted with 10% aqueous methanol containing 0.1% TFA. The chromatography was repeated 10 times, and active eluates were combined, concentrated under reduced pressure and lyophilized to give 120 mg of WF14861 as pure powder.

Fig. 2. Structure of WF14861 (**1**).

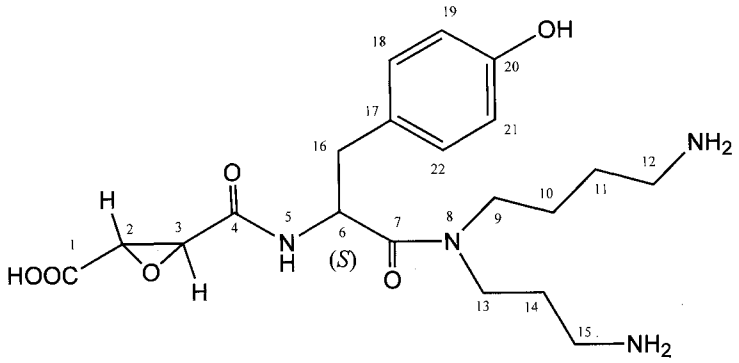



Fig. 3. Key FABMS/MS data of **1**.

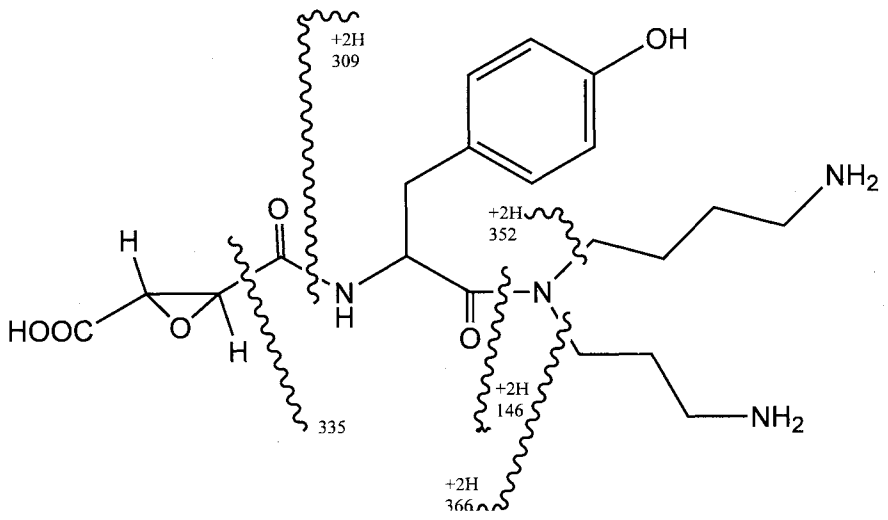
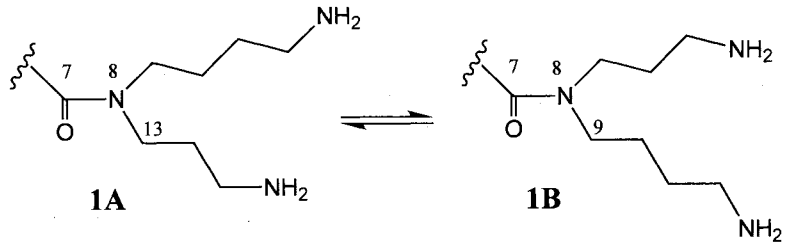


Fig. 4. Rotamers of **1A** and **1B**.




## Structure Elucidation

WF14861 (**1**) was obtained as a colorless amorphous powder with $[\alpha]_D$ +43° (*c* 0.5, MeOH). It was soluble in water and methanol but insoluble in chloroform and ethyl acetate. It showed positive color reaction to ninhydrin though negative to Sakaguchi. The molecular formula of **1** was determined to be $C_{20}H_{30}N_4O_6$ on the basis of HRFAB-MS ((M+H) *m/z* observed 423.2249, calcd 423.2244). The presence of a peptide bond was inferred from the IR absorption bands at 1630, 1520 $cm^{-1}$ and thus complete acid hydrolysis was carried out. The amino acid analysis of the hydrolyzate revealed the presence of tyrosine and spermidine. The chemical shifts at C-2 ($\delta_H/\delta_C$ 3.31/55.7) and C-3 ($\delta_H/\delta_C$ 3.52/53.9) and the small vicinal coupling constant ($J_{2\text{-H},3\text{-H}}$=2 Hz) are quite characteristic of *trans*-epoxysuccinic acid moiety. The sequence of linkage of the three components (*trans*-epoxysuccinic acid, Tyr and spermidine) was elucidated by the analysis of FAB MS/MS data as shown in Fig. 3. A detailed analysis of the $^{13}C$ NMR and DEPT NMR data ($CD_3OD$) revealed that **1** exists as an equilibrium mixture of two isomers (**1A** and **1B** (*ca.* 4 : 1)) (Table 2). This finding can be reasonably explained by a restricted rotation of the amide bond between N-8 and C-7 (Fig. 4). The assignment of the major isomer (**1A**) and minor (**1B**) was made by consideration of sterically induced upfield shifts of C-13 and C-9, respectively. The L assignment of the constituent Tyr was established by chiral TLC analysis of the acid hydrolyzate. From the above information, the structure was concluded to be 3-(*N*-(1-(*N*-(4-aminobutyl)-*N*-(3-aminopropyl)carbamoyl)-2-(4-hydroxyphenyl)ethyl)carbamoyl)oxirane-2-carboxylic acid.

Table 2. $^1H$ (400 MHz) and $^{13}C$ (100 MHz) NMR data for **1A** and **1B** in $CD_3OD$.

| Position | 1A | | 1B |
|---|---|---|---|
| | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 1 | 174.1 (s) | | 174.1 |
| 2 | 55.7 (d) | 3.31 (d) | 55.8 |
| 3 | 53.9 (d) | 3.52 (d) | 54.0 |
| 4 | 169.7 (s) | | 169.6 |
| 5 | | | |
| 6 | 52.5 (d) | 4.95 (m) | 52.1 |
| 7 | 173.9 (s) | | 173.2 |
| 8 | | | |
| 9 | 48.4 (t) | 3.21 (2H, m) | 46.7 |
| 10 | 26.8 (t) | 1.55 (m) | 27.9 |
| | | 1.30 (m) | |
| 11 | 25.7 (t) | 1.55 (2H, m) | 25.4 |
| 12 | 40.4 (t) | 2.88 (2H, m) | 40.4 |
| 13 | 44.1 (t) | 3.40 (2H, m) | 46.0 |
| 14 | 26.7 (t) | 1.83 (2H, m) | 25.8 |
| 15 | 38.0 (t) | 2.82 (2H, m) | 38.0 |
| 16 | 38.8 (t) | 3.01 (m) | 38.8 |
| | | 2.90 (m) | |
| 17 | 128.3 (s) | | 128.3 |
| 18, 22 | 131.5 (d) | 7.07 (d) | 131.6 |
| 19, 21 | 116.6 (d) | 6.75 (d) | 116.5 |
| 20 | 157.8 (s) | | 157.7 |

## Discussion

A variety of *trans*-epoxysuccinyl type cysteine protease inhibitors from fungal strains have been reported such as E-64 and CPI-1 to 5 from *Aspergillus* sp., cathestatin A and B from *Penicillium* sp., estatin A and B from *Myceliophthora* sp., TMC-52 A to D from *Gliocladium* sp. and so on[13~17]. These species belong to Hyphomycetes families. We isolated WF14861 from *Colletotrichum*

sp., which had been reported originally as a plant pathogenic fungus and a member of Melanconiales in the Coelomycetes. These results suggest that a variety of fungal strains produce *trans*-epoxysuccinyl type cysteine proteases.

WF14861 inhibited human liver cathepsin B and L with $IC_{50}$ value of $1.6 \times 10^{-8}$ M and $1.1 \times 10^{-9}$ M respectively. As we have evaluated it by several *in vitro* and *in vivo* assay systems, we will show the possible utility of WF14861 against protein digestive diseases, especially against bone destructing diseases, in an accompanying paper[6].

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