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WF14865A and B, New Cathepsins B and L Inhibitors

Produced by *Aphanoascus fulvescens*

I. Taxonomy, Production, Purification and Biological Properties

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WF14865A and B, novel cathepsins B and L inhibitors, were produced and isolated separately from the culture mycelium of a fungal strain *Aphanoascus fulvescens* No. 14865. Spectroscopic analysis revealed that both WF14865A and B were composed of *trans*-epoxysuccinyl moieties, 1-*H*-imidazole-2-ylamine, and isoleucine or leucine. These compounds inhibited human cathepsins B and L with IC_{50} values in the range of 8.4~72 nM *in vitro*. Though their *in vitro* properties were typical as *trans*-epoxysuccinyl type inhibitors, they exerted strong bone resorption inhibitory effects in low-calcium-diet-fed mouse model at 3.2~10 mg/kg.

It has been suggested that cathepsin B (EC 3.4.22.1) and cathepsin L (EC 3.4.22.15), lysosomal cysteine proteases, are involved in various pathophysiological processes. Among them, participations of these enzymes in osteoclastic bone resorption have been reported frequently[1~4]. In addition, suppression of these enzymes ameliorates the ischemic neuronal cell death[5], the invasion and metastasis of carcinoma[6] and the antigen processing in the immune response[4]. Furthermore, these lysosomal enzymes play a significant role in the process of muscle fiber destruction in inflammatory myopathy in concert with extralysosomal calpain[7]. In anticipation of the usefulness of cathepsins B and L inhibitor against these diseases, we screened new inhibitors against these enzymes from the products of microorganisms. As a result of the screening, we isolated new compounds WF14865A and B from the culture mycelium of a fungus *Aphanoascus fulvescens* No. 14865 (Fig. 1).

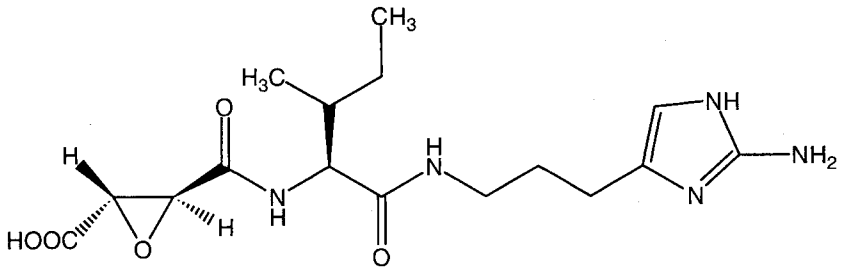
This paper describes the taxonomy of the producing strain, and the production and purification of WF14865A, 4-[3-[*N*-[[(2*S*,3*S*)-3-*trans*-carboxyoxiran-2-yl]carbonyl]-L-isoleucyl]aminopropanyl]-1*H*-imidazol-2-ylamine, and B, 4-[3-[*N*-[[(2*S*,3*S*)-3-*trans*-carboxyoxiran-2-yl]carbonyl]-L-leucyl]aminopropanyl]-1*H*-imidazol-2-ylamine. In addition, biological properties of these inhibitors will be shown in this paper. Structure elucidation and total synthesis of these inhibitors will be described in elswhere[8].

Materials and Methods

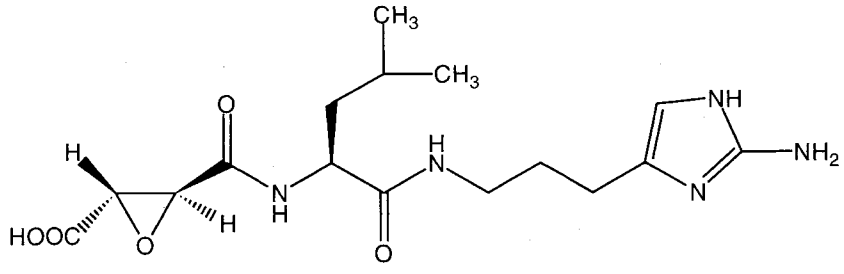
Materials

Human liver cathepsin B (Athens Research & Technology) and human liver cathepsin L (Calbiochem) were purchased. Inhibitions of these 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. Porcine μ calpain (Nacalaitesque), papain (Sigma), protein assay reagent (Bio-Rad) and BCA protein assay kit (PIERCE) were purchased. Fluorescein isothiocyanate-labeled collagen (FITC-labeled collagen) was obtained from Collagen Research Center. The low-calcium-diet for mice was obtained from CLEA Japan. Calcium concentration in the plasma was measured by the MXB method (Calcium E-test

Fig. 1. Chemical structures of WF14865A and B.



WF14865A



WF14865B

wako, WAKO). The fluorescence was determined using a Titertek Fluoroscan II spectrophotometer (Flow Laboratories). All other chemicals were of analytical grade.

Taxonomy

The observations were made after 14 days of cultivation at 25°C in the dark condition. The compositions of malt extract agar, Czapek's solution agar and MY20 agar were based on JCM Catalogue of Strains[9]. The color names used in this study were taken from the Methuen Handbook of Colour[10]. The temperature range of growth was determined on potato dextrose agar. The morphological characteristics were determined from the cultures on Emerson Yp Ss agar (Difco).

Enzyme Assay

The methods for measuring the inhibition of cathepsins B and L activity were described previously[11]. Briefly, inhibitions of human liver cathepsins B and L were determined using 10 μM *Z*-Arg-Arg-AMC or 10 μM *Z*-Phe-Arg-AMC in 100 mM sodium phosphate buffer (pH 5.5) with 5 mM dithiothreitol and 4 mM EDTA for 60 minutes at 37°C.

The liberation of AMC from the fluorogenic substrate *Z*-Phe-Arg-AMC by the mouse bone powder suspension was estimated as bone cathepsin activities. Precise methods for this assay had been described previously[11].

The inhibition of μ calpain was determined by casein degradation using a protein assay reagent (Bio-Rad). Papain inhibition was also determined by the degradation of casein using a BCA protein assay kit (PIERCE). Details for these assay methods were also described previously[11].

The inhibition of collagen degradation was determined using a bovine cathepsin B and FITC-labeled type I collagen in U-shaped microtiter plates. Twenty-five microliters of inhibitor was serially diluted in reaction buffer containing 5 mM dithiothreitol and 4 mM EDTA in 100 mM sodium phosphate buffer (pH 5.5). Fifty microliters of FITC-labeled collagen solution (1 mg/ml, dissolved in reaction buffer) and 25 μl of bovine cathepsin B (200 μg/ml) were added and incubated at 37°C for 18 hours. After the incubation, 100 μl of 70% ethanol in 170 mM Tris-HCl buffer (pH 9.5) containing 0.67 M NaCl were added and mixed vigorously. FITC-labeled collagen

was denatured by these procedures, whereas reaction product with FITC was still soluble. The U-shaped microtiter plate was centrifuged for 20 minutes at 2300 rpm in order to separate remaining substrate and reaction product. These procedures were performed on ice except for the time of enzyme reaction and the time of centrifugation at 4°C. One hundred microliters of supernatant was carefully withdrawn and transferred to another flat bottom microtiter plate and fluorescence intensity was measured (excitation/emission, 485 nm/538 nm).

All data are the averages of at least duplicate determinations.

Low-calcium-diet-fed Mouse Model

The low-calcium-diet-fed mouse model was performed by the method described previously[11]. Briefly, three week old female ICR mice were fed for 6 days with a 0.003% calcium diet. Ten mice were used for the control group and five mice were used for each drug-treated group. After the injection of sample, peripheral blood was withdrawn from retro-orbital plexus by heparinized capillary tubes under halothane anesthesia. Calcium concentration in the plasma was monitored at 0, 2, 4, 6 hours by calcium assay kit. Results were expressed as means±SE. Paired Student's t test was used for the statistical analysis of the results (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

Identification of the Producing Strain

The fungal strain No. 14865 was originally isolated from a soil sample collected at Kanoya City, Kagoshima Prefecture, Japan. Its mycological characteristics are described below.

Cultural characteristics on various agar media are summarized in Table 1. This organism grew very rapidly on culture media, and formed yellowish white colonies. Culture on Emerson Yp Ss agar spread broadly, attaining 6.5~7.0 cm in diameter within two weeks at 25°C. This colony surface was plane, thin, and pale red to white. Ascomata and conidial structures were observed. The reverse color was reddish gray to yellowish white. Colonies on corn meal agar grew rapidly, attaining 5.0~6.5 cm in diameter under the same conditions. The surface was plane, thin, powdery, and yellowish white. Some conidial structures were observed. The reverse was the same color of the surface. This strain was able to grow at the temperature range from 6 to 36°C, with the growth optimum at 26 to 31°C.

Strain No. 14865 formed cleistothecial ascomata and hyphal conidial structures on some agar media. The cleistothecia were superficial, smooth-walled, globose to subglobose, 130~290 μm in diameter, and grayish color at maturity. The asci were arranged in cleistothecia irregular, unitunicate, eight-spored, subglobose to ovoid, with evanescent wall, and 7.5~12 μm in diameter. Ascospores were lenticular, 3.5~5.5 μm in diameter, and covered by an irregularly reticulate arrangement of the ornamentation (Fig. 2). The conidial structures formed *Chrysosporium*-morph, consisting of aleurioconidia and arthroconidia. Terminal and lateral aleurioconidia were hyaline, smooth, one-celled, pyriform to clavate, with a truncated base, and 4.5~10×4~6.5 μm in size. Intercalary arthroconidia were hyaline, smooth, one-celled, cylindrical to doliform, truncated with both ends, and 5~12.5×2.5~5 μm. Vegetative hyphae were smooth, septate, hyaline and branched. The hyphal cells were cylindrical and 1~4.5 μm in width. Racket cells were sometimes observed.

On the basis of comparing the morphological characteristics with fungal taxonomic criteria by VON ARX[12], and DOMSCH *et al.*[13], strain No.14865 was considered to belong to the plectomycete genus *Aphanoascus* Zukal 1890. Moreover, the characteristics above corresponded to the species description of *Aphanoascus fulvescens* (Cooke) Apinis 1968 by DOMSCH *et al.*[13] and CANO and GAURRO[14]. According to the proposition by CANO and GAURRO[14], we decided the name described in our patents[15], *Anixiopsis stercoraria*, was a synonym of *Aphanoascus fulvescens*. In conclusion, we identified this isolate as one strain of *Aphanoascus fulvescens*, and named it *Aphanoascus fulvescens* No. 14865. The strain has been deposited to the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM BP-4689.

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. 14865 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. WF14865A and B were produced and isolated separately

Table 1. Cultural characteristics of strain No. 14865.

Media	Cultural characteristics
Malt extract agar*	G: Spreading broadly, 6.0-6.5 cm S: Circluar, felty, formed conidial structures, yellowish white (2A2) R: Pale yellow (4A3)
Potato dextrose agar (Difco 0013)	G: Spreading broadly, 6.5-7.0 cm S: Circular, felty, formed conidial structures, orange white (5A2) R: Light yellow (4A3)
Czapek's solution agar*	G: Rapidly, 4.0-5.0 cm S: Circular to irrerguar, plane, thin, formed conidial structures and immature ascomata, white (1A1) R: White (1A1)
Sabouraud dextrose agar (Difco 0190)	G: Spreading broadly, 6.0-6.5 cm S: Circular, felty, sulcate, formed conidial structures and immature ascomata, white (1A1) R: Light yellow (4A4)
Emerson Yp Ss agar (Difco 0739)	G: Spreading broadly, 6.5-7.0 cm S: Circular, plane, thin, formed conidial structures and ascomata, exudate, pale red (7A3), and white (1A1) at the margin R: Reddish gray (7B2), and yellowish white (3A2) at the margin
Corn meal agar (Difco 0386)	G: Very rapidly, 5.0-6.5 cm S: Irregular, plane, thin, powdery, formed conidial structures, yellowish white (3A2) R: Yellowish white (2A2)
Oatmeal agar (Difco 0552)	G: Spreading broadly, 6.0-6.5 cm S: Circular, cottony, formed conidial structures and ascomata, exudate, white (1A1) R: Orange white (5A2)
MY20 agar*	G: Rather restrictedly, 2.5-3.0 cm S: Circular, raised, felty to cottony, formed conidial structures, white (1A1) R: Pale yellow (4A3)
Abbreviation	G: growth, measuring colony size in diameter, S: colony surface, R: reverse.

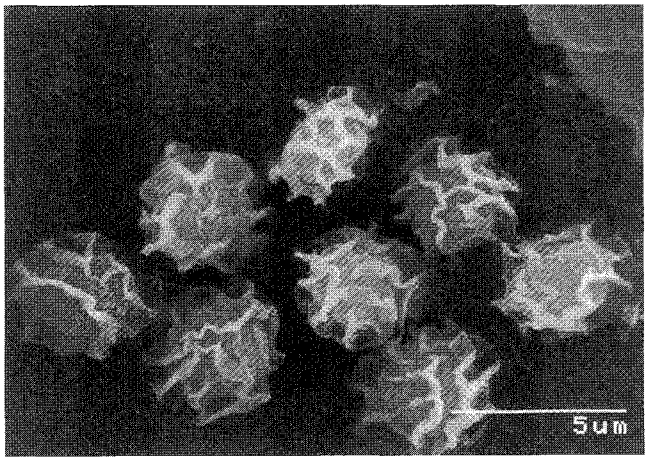
using same seed culture medium.

For the production of WF14865A, the resultant 360 ml of seed culture was inoculated into 20 liters of sterile fermentation medium. The aqueous medium consisting of 2.0% corn starch, 1.0% glycerin, 1.0% peanut powder, 0.5% corn steep liquor and 0.2% $CaCO_3$ 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 5 days employing aeration at 20 liters/minute and stirring at 180 rpm.

For the production of WF14865B, the aqueous medium consisting of 2.0% corn starch, 1.0% glycerin, 1.0% peanut powder, 0.5% corn steep liquor, 0.5% $(NH_4)_2SO_4$, 1.0% β-cyclodextrin and 0.2% $CaCO_3$. Another method for the production of WF14865B was based upon the methods of WF14865A.

The productions of WF14865A and B in the fermentations were monitored by HPLC using a reverse phase packed column YMC-ODS-AM (Yamamura Chemical, AM303). The solvent used for the detection of WF14865A was 25% aq. methanol containing 0.05% TFA.

Fig. 2. Scanning electron micrograph of ascospores with reticulate ornamentations of strain No. 14865 (on the Yp Ss agar plate, 25°C, 14 days).



WF14865B was detected using a mixed solvent acetonitrile - THF - water (12.5 : 2.0 : 85.5) containing 0.1% TFA. Each substance was detected at 210 nm.

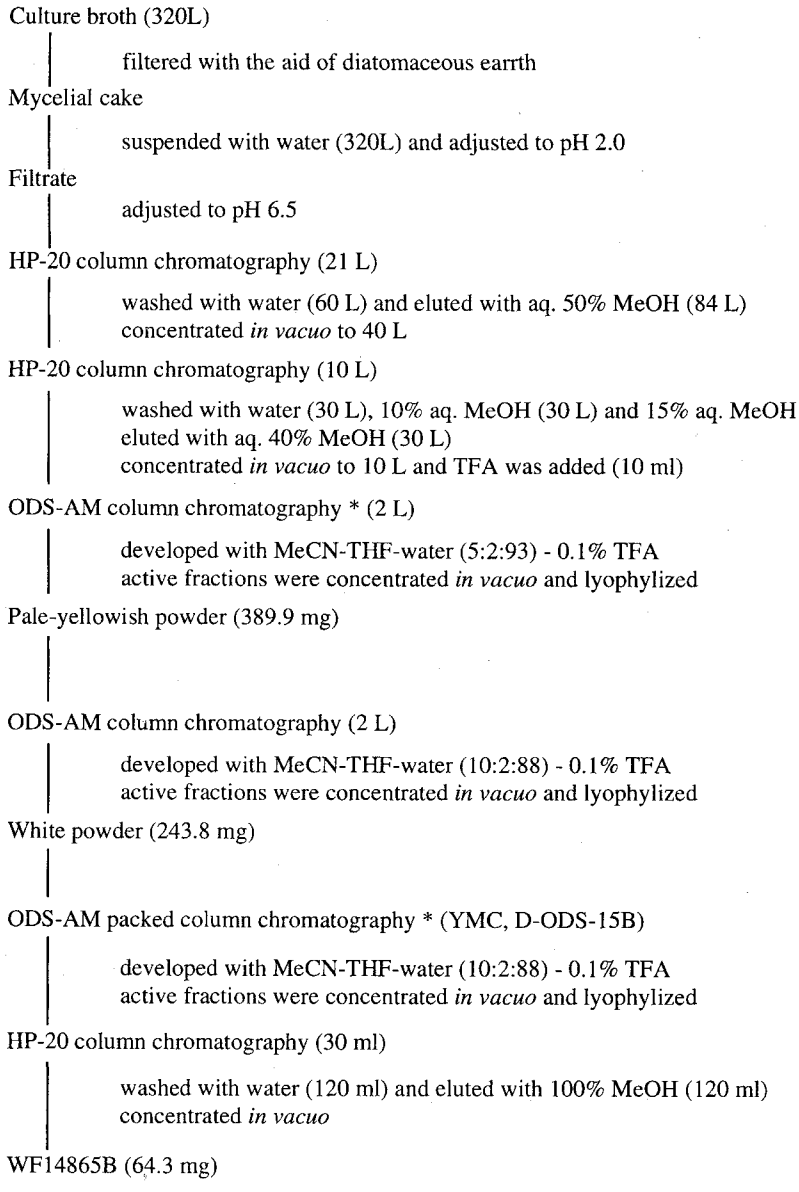
Purification

WF14865A was obtained by the following methods as described in Fig. 3. After the culture was terminated, the culture broth (40 liters) was filtered and 40 liters of methanol was added to the mycelial cake with stirring. The mixture was allowed to stand overnight, and was filtered. The extract was concentrated to be an aqueous solution under reduced pressure and was adsorbed onto polymeric adsorbent HP-20 (Mitsubishi Chemical, 1.5 liters). The column was washed with 4.5 liters of water, then the desired substance was eluted from the column with 50% of aq. methanol (6 liters). The eluate was concentrated to dryness (*ca.* 4.5 g) and dissolved in a small amount of water, and was subjected to reverse phase column chromatography YMC-ODS-AM (Yamamura Chemical, 2 liters). The column was developed with 10% of aq.

Fig. 3. Isolation procedure for WF14865A.

Culture broth (40 L)
↓ filtered with the aid of diatomaceous earrth
Mycelial cake
↓ extracted with MeOH (40 L)
Filtrate
↓ concentrated *in vacuo* (3 L)
HP-20 column chromatography (1.5 L)
↓ washed with water (4.5 L) and eluted with aq. 50% MeOH (6 L)
Eluate (ca. 4.5 g)
↓
ODS-AM column chromatography (2 L)
↓ developed with aq. 10% MeCN-0.1% TFA and concentrated *in vacuo*
Active fractions (831 mg)
↓
ODS-AM packed column chromatography (YMC, SH-343-5AM)
↓ developed with aq. 12.5% MeCN-0.1% TFA and concentrated *in vacuo*
Active fractions
↓
ODS-AM packed column chromatography (YMC, SH-343-5AM)
↓ developed with aq. 25% MeOH-0.1% TFA
concentrated *in vacuo* and lyophilized
WF14865A (11 mg)

Fig. 4. Isolation procedure for WF14865B.



* : Chromotographed twice using the same column and the same solvent.

acetonitrile containing 0.1% TFA. The fractions containing active substance were combined and were concentrated to dryness to give 831 mg of a crude residue. The residue thus obtained was dissolved in a small amount of developing solvent (100 mg/ml), consisting with 12.5% aq. acetonitrile containing 0.1% TFA, and was applied on a reverse phase packed column YMC-ODS-AM (Yamamura Chemical, SH-343-5AM). The column was developed with the solvent system as mentioned above and the active fractions were collected. The combined fractions were dried and redissolved in 25% aq. methanol containing 0.1% TFA and was applied to the YMC-ODS-AM packed column again. The column was developed with 25% aq. methanol containing 0.1% TFA. The active fraction was concentrated under reduced pressure and lyophilized to give 11 mg of WF14865A as pure powder.

WF14865B was purified by the following methods as described in Fig. 4. After the culture was terminated, the

Table 2. IC_{50} values of WF14865A, B and reference compounds against cysteine proteases ($\times 10^{-9}$ M).

	Human Cathepsin B	Human Cathepsin L	Mouse Cathepsins	Porcine μ Calpain	Papain
WF14865A	8.4	66	4.4	18000	650
WF14865B	13	72	NT	2000	390
WF14861	16	1.1	40	95000	850
E-64	55	68	6.1	4100	580
Chymostatin	8300	12	200	28000	86000
Leupeptin	19	1.5	8.0	1700	2200

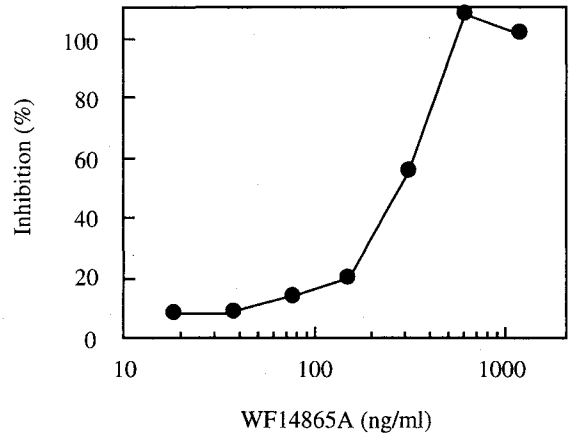
NT: Not tested

culture broth (320 liters) was filtered and 320 liters of water was added to the mycelial cake with stirring. As WF14865B could be extracted from mycelium by acid treatment or methanol extraction, the mycelial suspension was adjusted to pH 2.0 by conc. H_2SO_4 and allowed to stand for 5 hours. After the filtration of this suspension, the filtrate was adjusted to pH 6.5 by 6 N-NaOH and was adsorbed onto polymeric adsorbent HP-20 (21 liters). The column was washed with 60 liters of water, then the desired substance was eluted from the column with 50% of aq. methanol (84 liters). The eluate was concentrated to 40 liters under reduced pressure and adsorbed onto 10 liters of HP-20. The column was washed with 30 liters of water, 10% aq. methanol and 15% aq. methanol successively. The desired substance was eluted by 30 liters of 40% aq. methanol and concentrated to 10 liters under reduced pressure. After the addition of 10 ml of TFA, the concentrate was applied onto reverse phase column chromatography YMC-ODS-AM (2 liters). The column was developed with mixed solvent consisting of acetonitrile - THF - water (5 : 2 : 3) containing 0.1% TFA. The fractions containing active substance were combined, concentrated *in vacuo* and lyophilized. Crude yellowish powder thus obtained was dissolved in a small amount of water and rechromatographed using the same column and the same solvent system to give a 389.9 mg of pale-yellowish powder. This powder was dissolved in 4 ml of water and applied onto 2 liters of YMC-ODS-AM column and eluted with mixed solvent consisted with acetonitrile - THF - water (10 : 2 : 88) containing 0.1% TFA. Active fractions were mixed, concentrated *in vacuo* and lyophilized to give 243.8 mg of white powder. Crude WF14865B was dissolved in 2.5 ml of water and applied onto a reverse phase packed column (YMC, D-ODS-15B) and developed with the same mixed solvent used just above at 18 ml/minute. The desired substance was collected, concentrated *in vacuo* and lyophilized. The powder thus obtained was dissolved in a small amount of water and rechromatographed again using the same column and the same solvent. After the evaporation of organic solvent, the resultant aqueous solution was adsorbed onto 30 ml of the HP-20 column. The column was washed with water (120 ml) and eluted with 120 ml of methanol. The eluate was concentrated to dryness and suspended with ethanol. Following the dryness, 64.3 mg of pure WF14865B was obtained as white powder.

Biological Properties

IC_{50} values of WF14865A and B against cysteine proteases were listed in Table 2 with reference compounds including WF14861, E-64, chymostatin and leupeptin. Among them, WF14861 and E-64 contain *trans*-epoxysuccinyl moieties. WF14865A inhibited cathepsins B and L with IC_{50} values of 8.4×10^{-9} M and 6.6×10^{-8} M, respectively. Similarly, WF14865B inhibited cathepsins B and L with IC_{50} values of 1.3×10^{-8} M and 7.2×10^{-8} M, respectively. These compounds were more effective against cathepsin B than against cathepsin L. WF14861 was, however, more effective against cathepsin L than against cathepsin B. In the case of E-64, it inhibited both cathepsins B and L with similar IC_{50} values of 5.5×10^{-8} M and 6.8×10^{-8} M, respectively. These results suggest that the *trans*-epoxysuccinyl moiety is essentially common for the inhibitions of the cathepsins, though enzyme inhibitory potencies depend on peptide moieties. Similar to other *trans*-epoxysuccinyl inhibitors, WF14865A and B weakly inhibited porcine μ calpain and papain. Furthermore,

Fig. 5. Effect of WF14865A on FITC-labeled collagen degradation by cathepsin B.



neither compound inhibited bovine chymotrypsin nor human elastase even at 2.4×10^{-4} M (data not shown). WF14865A and B, however, inhibited bovine trypsin with IC_{50} values of 2.2×10^{-4} M and 2.9×10^{-5} M, respectively (data not shown). In any case, it may be stated that while WF14865A and B do not inhibit serine proteases strongly, they can inhibit cysteine proteases. The mode of inhibition of WF14865A and B,which has not been determined, might be noncompetitive, because the mode of inhibition of E-64 against papain had been suggested as noncompetitive[16].

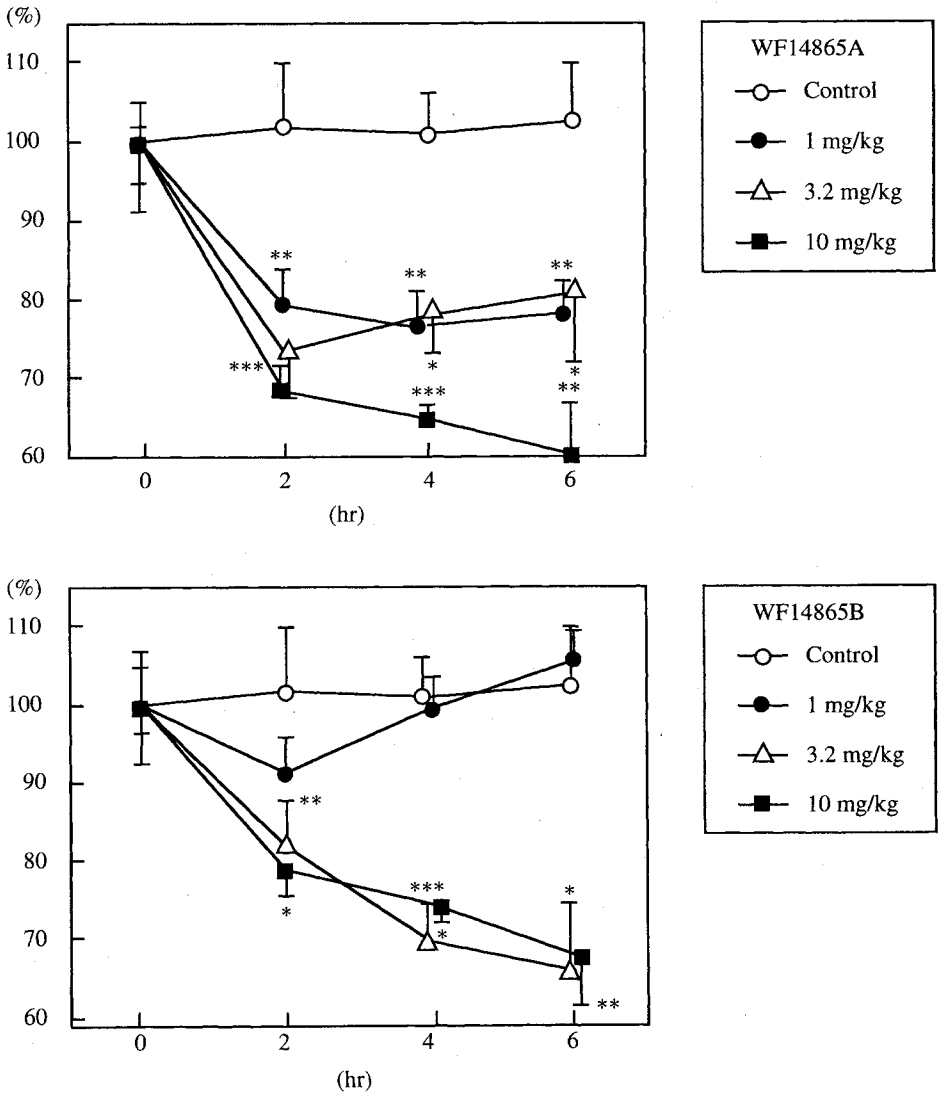
The substrates used above for the evaluation of WF14865A and B against cysteine proteases were synthetic. Accordingly, we adopted FITC-labeled type I collagen as a natural substrate. FITC-labeled type I collagen was originally used as a substrate for collagenase[17]. After the confirmation that human and bovine cathepsin B could attack FITC-labeled collagen and liberate FITC into reaction buffer, we evaluated the effect of WF14865A on bovine cathepsin B. WF14865A inhibited bovine cathepsin B dose-dependently with an IC_{50} value of 9.5×10^{-7} M (Fig. 5). The effect of WF14865A in this assay system was similar to WF14861 (IC_{50}; 7.8×10^{-7} M).

In vivo effects of WF14865A and B were evaluated in a low-calcium-diet-fed mouse model (Fig. 6). Normal diet contains 1.25% calcium, whereas mice fed with a 0.003% calcium-diet for 6 days were used in this experiment. The maintenance of blood calcium level in calcium deficient mice has been regarded as a result of bone resorption. If bone resorption is inhibited by some agents, plasma calcium level should decrease in this model[18]. Recently, we have reported that 100 mg/kg of subcutaneous injection of E-64 and WF14861 lowered plasma calcium level to 91.15±1.84% and 84.0±4.87%, respectively, of the initial plasma calcium level in 6 hours[11]. Fig. 6 shows the effects of WF14865A and B in the low-calcium-diet-fed mouse model. Ten mice were used for the control group and five were used for each drug-injected group (s.c.). Paired Student's t test was used for the analysis of the results. Comparing to the initial plasma calcium level, the levels of the control group of animals didn't decrease significantly for 6 hours. The efficacies of WF14865A and B were similar, when 10 mg/kg of subcutaneous injections of WF14865A and B lowered the plasma calcium level to 60.10±6.56% and 67.90±4.59%, respectively, of the initial level in 6 hours. The potency of WF14865A was stronger than that of WF14865B, because the former significantly lowered plasma calcium level to 78.34±3.76% in 6 hours at the 1.0 mg/kg injected group, whereas the plasma calcium level of the latter was 105.6±9.87% at the same dose. The difference may suggest that WF14865B is metabolized in 2 hours to an inactive metabolite and 1.0 mg/kg is below the threshold required for activity. In addition, another possibility that may explain the reduction of plasma calcium level by WF14865A and B should be considered. For instance, the reduction of plasma calcium level may be caused by a cascade of events in the mouse and not simply cathepsin enzymatic inhibition. In contrast with WF14861 and E-64, the efficacies and potencies of WF14865A and B were increased, because WF14861 and E-64 lowered the plasma calcium level to 84.0±4.87% and 91.15±1.84%, respectively, in the 100 mg/kg injected group in 6 hours[11]. Repeated *in vivo* experiments revealed that these effects of WF14865A and B were reproducible (data not shown).

The clear explanation of the effects of WF14865A and B *in vivo* from our *in vitro* data is difficult to undestand. Species specificity can't explain these results because WF14865A and E-64 inhibit crude mouse cathepsins activity to a similar extent (Table 2). In addition, participation of cathepsin K can't explain the effects of WF14865A *in vivo*, because it may work *in vitro* where WF14865A and E-64 showed similar inhibitory effects against crude mouse cathepsins, which may include cathepsin K activity besides cathepsins B and L activities[19,20].

Acute toxicities of WF14865A and B were examined. Drugs were administered intraperitoneally (300 mg/kg) to five ICR mice (female, 5 weeks old). After one-week of observation, no abnormal symptoms were recognized in any group.

Fig. 6. Effect of WF14865A and B on mouse low-calcium-diet-fed model.



Statistically different from the initial plasma calcium concentration by paired Student's t test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Discussion

Two types of cysteine protease inhibitors from microbial origin had been reported. Peptide aldehyde inhibitors had been obtained from Streptomyces such as leupeptin and chymostatin[21,22]. On the other hand, *trans*-epoxysuccinyl peptide type inhibitors had been obtained from fungus strains such as E-64 and catestatins[23]. Many *trans*-epoxysuccinyl peptide type inhibitors have one bulky amino acid, *e.g.* tyrosine, phenylalanine, leucine etc., and a basic residue. We have reported cathepsin B and L inhibitor, WF14861, from fungal strain *Colletotrichum* sp. Like the others, WF14861 consisted of a *trans*-epoxysuccinyl moiety, an amino acid and an aminoalkyl moiety[23].

Though these molecules fall under categories of *trans*-epoxysuccinyl peptide type inhibitors, essentially WF14865A and B had a new type basic residue. WF14865A inhibits human liver cathepsins B and L with IC_{50} values of 8.4×10^{-9} M and 6.6×10^{-8} M, respectively. In addition, WF14865B inhibits human liver cathepsins B

and L with IC_{50} values of 1.3×10^{-8} M and 7.2×10^{-8} M, respectively. *In vitro* properties of these inhibitors were not remarkable as *trans*-epoxysuccinyl peptide type inhibitor, however, they exerted strong effects *in vivo* at 3.2~10 mg/kg. Accordingly, it is possible that a new basic moiety, 1-*H*-imidazole-2-ylamine, contributes to the strong effects *in vivo*.

WF14865A and B or their derivatives are promising candidates for the treatment of rheumatic arthritis, osteoarthritis, inflammatory myopathy and potentially, other conditions.

References

1) Maciewicz, R. A. & S. F. Wotton: Degradation of cartilage matrix components by the cysteine proteinases, cathepsins B and L. Biomed. Biochim. Acta 50: 561~564, 1991
2) Flipo, R.-M.; G. Huet, F. Maury, F. Zerimech, C. Fontaine, B. Duquesnoy, P. Degand & B. Delcambre: Cysteine-proteolytic activities (cathepsins B, L and calpains) in rheumatoid synovium. Arthritis & Rheum. 36: S252, 1993
3) Page, A. E.; M. J. Warburton, T. J. Chambers, J. A. Pringle & A. R. Hayman: Human osteoclastomas contain multiple forms of cathepsin B. Biochim. Biophys. Acta 1116: 57~66, 1992
4) Katunuma, N.; Y. Matsunaga, A. Matsui, H. Kakegawa, K. Endo, T. Inubishi, T. Saibara, Y. Ohba & T. Kakiuchi: Novel physiological functions of cathepsin B and L on antigen processing and osteoclastic bone resorption. Adv. Enzyme Regul. 38: 235~251, 1998
5) Yamashita, T.; Y. Kohda, K. Tsuchiya, T. Ueno, J. Yamashita, T. Yoshioka & E. Kominami: Inhibition of ischaemic hippocampal neuronal death in primates with cathepsin B inhibitor CA-074. Eur. J. Neurosci. 10: 1723~1733, 1998
6) Navab, R.; J. S. Mort & P. Brodt: Inhibitions of carcinoma cell invasion and liver metastases formation by cysteine proteinase inhibitor E-64. Clin. Exp. Metastasis 15: 121~129, 1997
7) Kumamoto, T.; H. Ueyama, R. Sugihara, E. Kominami, D. E. Goll & T. Tsuda: Calpain and cathepsins in the skeletal muscle of inflammatory myopathies. Eur. Neurol. 37: 176~181, 1997
8) Neya, M. & S. Takase: WF14865A and B, new cathepsins B and L inhibitors produced by *Aphanoascus fulvescens*. II. Structure elucidation and total synthesis. J. Antibiotics, in preparation
9) Nakase, T.: JCM Catalogue of Strains, 6th edition. Japan Collection of Microorganisms, the Institute of Physical and Chemical Research (RIKEN), Toppan, 1995
10) Kornerup, A. & J. H. Wanscher: Methuen Handbook of Colour, 3rd edition.Methuen, London, 1978
11) Otsuka, T.; Y. Muramatsu, K. Niikura, M. Okamoto, M. Hino & S. Hashimoto: WF14861, a new cathepsins B and L inhibitor produced by *Colletotrichum* sp. II. Biological properties. J. Antibiotics 52: 542~547, 1999
12) Arx, J. A. Von: The Genera of Fungi - Sporulating in Pure Culture, 3rd ed., J. Cramer, Vaduz, 1974
13) Domsch, K. H.; W. Gams & T.-H. Anderson: Compendium of Soil Fungi. Vol. 1. Academic Press, London, 1980
14) Cano, A. & J. Gaurro: The Genus *Aphanoascus*. Mycol. Res. 94: 355~377, 1990.
15) Otsuka, T.; T. Sato, H. Hatanaka, S. Takase & S. Kiyoto: A new compound WF14865, production thereof and use thereof. Japan Patent Application Laid Open 119983, 1996
16) Hanada, K.; M. Tamai, M. Yamagishi, S. Ohmura, J. Sawada & I. Tanaka: Isolation and characterization of E-64, a new thiol protease inhibitor. Agric. Biol. Chem. 42: 523~528, 1978
17) Nagai, Y.; H. Hori, Y. Sunada, K. Terado, R. Hashida & K. Miyamoto: A micro-assay method of collagenase activity and its application in the study of collagen metabolism in pathological tissues. Inflammation (Tokyo) 4: 123~130, 1984
18) Delaissé, J.-M.; Y. Eeckhout & G. Vaes: *In vivo* and *in vitro* evidence for the involvement of cysteine proteinases in bone resorption. Biochem. Biophys. Res. Commun. 125: 441~447, 1984
19) Inaoka, T.; G. Bilbe, O. Ishibashi, K. Tezuka, M. Kumegawa & T. Kokubo: Molecular cloning of human cDNA for cathepsin K. Biochem. Biophys. Res. Commun. 206: 89~96, 1995
20) Littlewood-Evans, A.; T. Kokubo, O. Ishibashi, T. Inaoka, B. Wlodarski, J. A. Gallagher & G. Bilbe: Localization of cathepsin K in human osteoclasts by in situ hybridization and immunohistochemistry. Bone 20: 81~86, 1997
21) Aoyagi, T.; T. Takeuchi, A. Matsuzaki, K. Kawamura, S. Kondo, M. Hamada, K. Maeda & H. Umezawa: Leupeptins, new protease inhibitors from Actinomycetes. J. Antibiotics 22: 283~286, 1969
22) Umezawa, H.; T. Aoyagi, H. Morishima, S. Kunimoto, M. Matsuzaki, M. Hamada & T. Takeuchi: Chymostatin, a new chymotrypsin inhibitor produced by Actinomycetes. J. Antibiotics 23: 425~427, 1970
23) Otsuka, T.; Y. Muramatsu, T. Higaki, T. Nakanishi, S. Takase, H. Hatanaka, M. Okamoto, M. Hino & S. Hashimoto: WF14861, a new cathepsins B and L inhibitor produced by *Colletotrichum* sp. I. Taxonomy, production, purification and structure elucidation. J. Antibiotics 52: 536~541, 1999