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ORIGINAL ARTICLE

Calpinactam, a new anti-mycobacterial agent, produced by *Mortierella alpina* FKI-4905

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Calpinactam, a new anti-mycobacterial agent, was isolated from the culture broth of a fungal strain *Mortierella alpina* FKI-4905 by solvent extraction, octadecyl silane column chromatography and preparative HPLC. Calpinactam was active only against *Mycobacteria* among various microorganisms, including Gram-positive and Gram-negative bacteria, fungi and yeasts. Calpinactam inhibited the growth of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* with MIC values of 0.78 and 12.5 μg ml^{-1}, respectively.
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Keywords: anti-mycobacterial peptide; calpinactam; fungal metabolite; mycobactin; tuberculosis

INTRODUCTION

Our research group has focused on the discovery of anti-infectives from microbial metabolites.[1–5] Tuberculosis (TB) is still the greatest single infectious cause of mortality in the world, together with human immunodeficiency virus and malaria.[6] Moreover, the spread of human immunodeficiency virus has resulted in an increase in the number of TB patients.[7] However, no new anti-TB agents have been developed for over 30 years, and only five anti-TB drugs can be used clinically at present. It is therefore increasingly urgent and necessary to discover anti-TB drugs with a new mechanism of action. As isoniazid and ethambutol, first-line anti-TB drugs, show selective inhibition against *Mycobacteria*, we have screened for microbial metabolites having selective inhibition against *Mycobacterium smegmatis* among 14 test microorganisms, including Gram-positive and Gram-negative bacteria, fungi and yeasts. By this screening system, we discovered and reported lariaitins, new lasso polypeptides, from actinomycete *Rhodococcus* metabolites.[8–10] During the continuous screening program, we isolated calpinactam (Figure 1), a new hexapeptide with a caprolactam ring at the C-terminal, from the culture broth of the fungal strain *Mortierela alpina* FKI-4905 (Figure 2). The structure elucidation of calpinactam is described in detail elsewhere.[11] In this report, the taxonomy of the producing strain, fermentation, isolation and biological properties of calpinactam are described.

RESULTS

Taxonomy of the producing fungus

Colonies on oatmeal agar medium were 80–86 mm in diameter after keeping for 7 days at 25 °C, the surface with white (a) floccose aerial mycelium, exudate lacking, the margin entire and soluble pigment not produced. Colonies on malt extract agar medium were 34–38 mm in diameter after keeping for 7 days at 25 °C, the surface with white (a) floccose aerial mycelium, exudate lacking, the margin irregular and soluble pigment not produced. Colonies on low-carbon agar medium were 50–68 mm in diameter after keeping for 7 days at 25 °C, the surface with white (a) floccose aerial mycelium, exudate lacking, the margin entire and soluble pigment not produced. In the malt extract agar medium, colonies showed no growth at 5 and 37 °C. The sporangiophores that arose from aerial mycelia were simple, not branched and with no columellae but with an apparent collar at the top, 100–300 μm, tall (Figure 2). Sporangia were nearly spherical, 15–30 μm in size, and appeared singly on top of the sporangiophore. Sporangiospores were ellipsoidal, smooth and 5–7.5×2.5–3.5 μm in size.

Furthermore, the total length of the ribosomal DNA (rDNA) internal transcribed spacer (ITS) (including 5.8S rDNA) of FKI-4905 is 648 base pairs. In a BLAST search using blastn from the National Center for Biotechnology Information,[12] the rDNA ITS sequence of FKI-4905 showed 99.2% similarity to that of *Mortierella alpina* CBS 224.37 (GenBank accession number AJ271630).

From the above morphological characteristics and the genomic result, the producing strain FKI-4905 was identified as *Mortierella alpina*.

Fermentation

A slant culture of the strain FKI-4905 grown on LCA was used to inoculate a 50-ml tube containing 10 ml of the seed medium (glucose 2.0%, polypeptone 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05%, yeast extract 0.2%, KH_2PO_4 0.1%, agar 0.1%, pH 6.0). The tube was shaken on a

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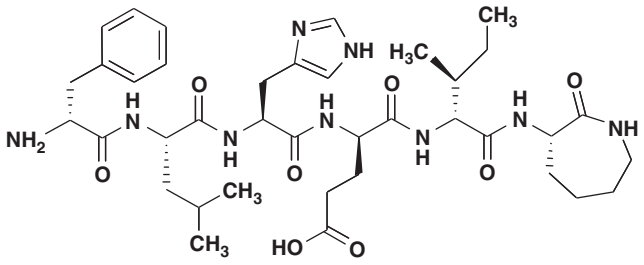

Figure 1 Structure of calpinactam.

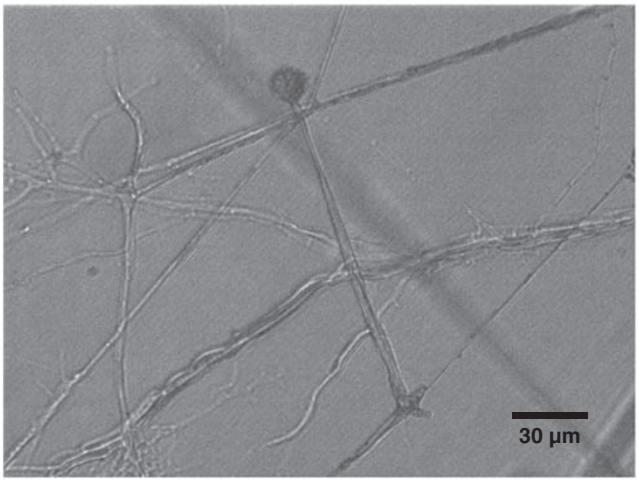

Figure 2 Photomicrographs of strain FKI-4905.

reciprocal shaker at 27 °C for 3 days. A 1-ml portion of the seed culture was then inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the production medium (soluble starch 3.0%, glycerol 1.0%, soy bean meal 2.0%, dry yeast 0.3%, KCl 0.3%, $CaCO_3$ 0.2%, $MgSO_4$ $7H_2O$ 0.05%, KH_2PO_4 0.05%, pH 6.5). The fermentation was carried out at 27 °C for 7 days. A typical time course of the fermentation is shown in Figure 3. Calpinactam was detected in the culture broth from day 3 after inoculation. The production reached a maximal level (88.5 μg ml^{-1}) on days 4 and 5, and kept almost the same levels at least on day 7.

Isolation

The whole broth (1.0 l) fermented for 4 days was extracted with an equal volume of ethanol. After the ethanol extracts were filtered and concentrated to remove ethanol, the aqueous resultant was diluted by adding distilled water (2.0 l) and applied on an octadecyl silane gel column (40 g, SSC-ODS-7515-12, Senshu Science, Tokyo, Japan). Materials were eluted stepwise with 30% CH_3CN, 60% CH_3CN and 100% CH_3CN (500 ml for each solvent). The 60% CH_3CN fraction showing the activity was concentrated to give a red brown material (140 mg). This material containing enriched calpinactam was finally purified with preparative HPLC (L-6200 system, Hitachi, Tokyo, Japan; column PEGASIL ODS (Senshu Science), 20×250 mm; solvent, a 40-min linear gradient from 20% CH_3CN to 40% CH_3CN; detection, UV at 210 nm; flow rate, 6.0 ml min^{-1}). Under the same conditions, calpinactam was eluted as a peak with a retention time of 31 min (Figure 4). The fraction was concentrated *in vacuo* and lyophilized to dryness to yield pure calpinactam (13.0 mg) as a white powder.

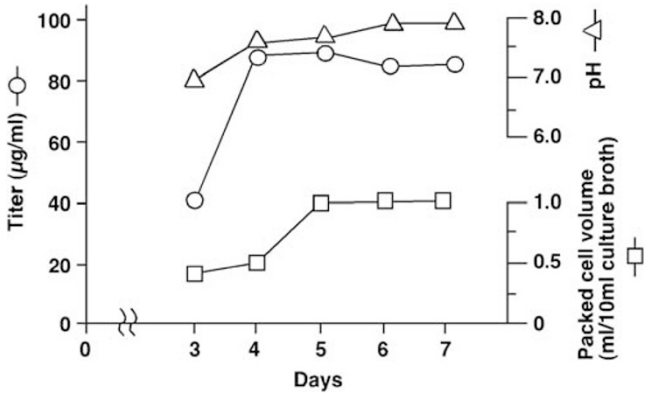

Figure 3 A typical time course of calpinactam production by *Mortierella alpina* FKI-4905. Titer (μg ml^{-1}), calpinactam (○). Packed cell volume (□), ml per 10 ml culture broth. pH (△).

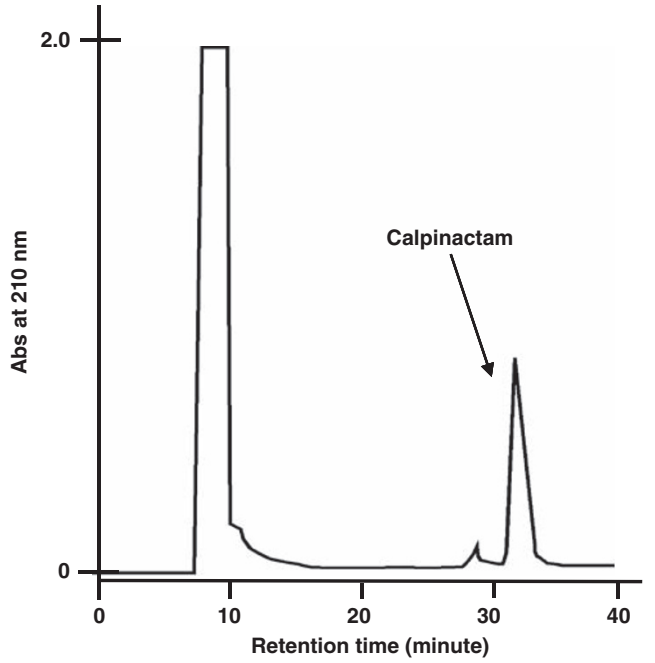

Figure 4 A chromatographic profile of calpinactam purification by preparative HPLC. Column, PEGASIL ODS 20×250 mm; solvent 20–40% aq acetonitrile containing 0.05% TFA; detection (0–40 min), UV at 210 nm; flow rate, 6.0 ml min^{-1}; sample, 30 mg of active materials (obtained through ODS column chromatography) dissolved in 300 μl DMSO.

Biological properties

The results by the paper disk method are summarized in Table 1. Calpinactam was only active against *Mycobacterium smegmatis* among 14 species of microorganisms tested, including *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Esherichia coli*, *Candida albicans* and *Saccharomyces cerevisiae* at 5 μg/6 mm disk. In a liquid microdilution method, the MIC value of calpinactam against *Mycobacterium smegmatis* was determined to be 0.78 μg ml^{-1}. Under the same condition, the MIC value of isoniazid was determined to be 1.56 μg ml^{-1}. Furthermore, calpinactam was found to inhibit the growth of *Mycobacterium tuberculosis* with an MIC value of 12.5 μg ml^{-1}.

Table 1 Antimicrobial activity of calpinactam

	Inhibition zone (mm)	
Test organism	*Calpinactam*	*Isoniazid*
Bacillus subtilis	—	—
Staphylococcus aureus	—	—
Micrococcus luteus	—	—
Mycobacterium smegmatis	22	31
Escherichia coli	—	—
Pseudomonas aeruginosa	—	—
Xanthomonas campestris	—	—
Bacteroides fragilis	—	—
Acholeplasma laidlawii	—	—
Pyricularia oryzae	—	—
Aspergillus niger	—	—
Mucor racemosus	—	—
Candida albicans	—	—
Saccharomyces cerevisiae	—	—
		5 μg/6 mm disk

DISCUSSION

Mycobacteria have a very unique cell wall structure. The whole genome sequence of *Mycobacterium tuberculosis*[13] revealed that there are a number of enzymes involved in biosynthesis of the cell wall. For example, mycolic acids, extremely long fatty acids, form a broad family of more than 500 closely related structures and comprise about 30% of the dry weight of *Mycobacterium tuberculosis*, and the microorganism has about 250 distinct enzymes involved in fatty acid metabolism. Thus, the biosynthetic pathways of the fatty acid and the cell wall have been considered to be attractive targets for anti-TB drugs. Isoniazid and ethambutol are first-line TB drugs, which inhibit cell wall synthesis; isoniazid inhibits mycolic acid synthesis by a blockade of Type II fatty acid synthase,[14] and ethambutol inhibits arabinogalactan mycolate synthesis by blocking arabinosyltransferase.[15] Several compounds, thiolactomycin, plantensimycin and platencin, have been isolated from actinomycete strains as inhibitors of type II fatty acid synthase.[16–19] Interestingly, calpinactam and lariatin,[9] on which we reported previously, have the same characteristics as the above-mentioned clinical agents, showing selective growth inhibition against mycobacteria (Table 1). Calpinactam has almost no cytotoxic activity against Jurkat cells up to at least 25 μg ml^{-1}. Calpinactam inhibited the growth not only of *Mycobacterium smegmatis* but also of *Mycobacterium tuberculosis*. Therefore, it might be plausible that calpinactam acts on the cell wall biosynthetic steps in mycobacteria. The mechanisms of action of calpinactam and lariatin are under investigation.

Calpinactam has a caprolactam moiety at its C-terminus although the structure will be elucidated elsewhere. The unique structure was seen in mycobacteria siderophores known as mycobactins, which are essential for iron transport in mycobacteria by Fe^{3+} chelating activity.[20] However, calpinactam is not expected to have iron chelating activity because neither hydroxamic acid nor 2-hydroxyphenyloxazoline moieties are present in its structure.

METHODS

General procedures

Fungal strain FKI-4905 was isolated from soil collected in Bonin Islands, Tokyo, Japan.[21] This strain was used for production of calpinactam. To measure the production of calpinactam in culture broths, the samples (ethanol extracts) were analyzed by an HP1100 system (Hewlett Packard Company, Palo Alto, CA, USA) under the following conditions: column, Symmetry (2.1×150 mm, Waters, Milford, MA, USA); flow rate, 0.2 ml min^{-1}; mobile phase, a 20-min linear gradient from 0% CH_3CN to 40% CH_3CN containing 0.05% H_3PO_4; detection, UV at 210 nm. Under these conditions, calpinactam was eluted as a peak with a retention time of 14.7 min.

Taxonomic studies

For taxonomic studies of the calpinactam-producing fungus, oatmeal agar (oatmeal powder 6.0%, agar 1.25%), malt extract agar (malt extract 2.0%, peptone 0.05%, dextrose 2.0%, agar 2.0%) and Miura's medium (low-carbon agar: glycerol 0.1%, KH_2PO_4 0.08%, K_2HPO_4 0.02%, $MgSO_4$ $7H_2O$ 0.02%, KCl 0.02%, $NaNO_3$ 0.2%, yeast extract 0.02%, agar 1.5%, pH 6.0) were used. On these media, the fungus was inoculated as 1-point cultures and cultured for 7 days at 25 °C (also at 5 and 37 °C on malt extract agar) in the dark. The color names and hue numbers were determined according to the procedure described in the Color Harmony Manual (Container Corporation of America, Chicago, IL, USA).[22] The morphological characteristics of the fungus were observed using a Vanox-S AH-2 microscope (Olympus, Tokyo, Japan).

To analyze the ITS sequence, genomic DNA of the fungus was extracted using the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Amplification of the rDNA ITS including the 5.8S rDNA was performed using primers ITS1 and ITS4 in a polymerase chain reaction thermal cycler Dice Mini Model TP100 (TaKaRa, Shiga, Japan).[23] The amplified polymerase chain reaction products were purified using a QIAquick, Polymerase Chain Reaction DNA Purification kit (Qiagen, Valencia, CA, USA). The amplified polymerase chain reaction products were used as a template and sequenced directly in both directions using primers ITS1, ITS2, ITS3 and ITS4 using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were further purified by ethanol/EDTA precipitation, and the samples were read on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). The contigs thus obtained were assembled using the forward and reverse sequences with the SeqMan and SeqBuilder programs from the Lasergene7 package (DNAStar, Madison, WI, USA). The ITS region rDNA sequence was compared with the database of the National Center for Biotechnology Information, USA. The ITS sequence of the strain FKI-4905 was deposited at the DDBJ with accession number AB516323.

Assay for antimicrobial activities

Antimicrobial activity against the 14 test microorganisms as listed in Table 1 was measured using paper disks (6 mm, ADVANTEC, Tokyo, Japan) containing a test sample according to our established method.[9]

Anti-mycobacterial activity was measured by the liquid microdilution method.[9] In this method, *Mycobacterium tuberculosis* H37Rv was adjusted to approximately 1.0×10^6 CFU ml^{-1} in a Middlebrook 7H9 broth (Difco, Becton Dickinson, Sparks, MD, USA) containing 0.05% Tween 80 and 0.5% glycerol. The culture broth (200 μl) was added to each well of a 96-well microplate (Corning Costar, Cambridge, MA, USA) with or without the test compounds. After incubation for 5 days at 37 °C, 12.5% Tween 80 (20 μl) and alamarblue (20 μl, Biosource, Camarillo, CA, USA) were added to each well. After overnight incubation at 37 °C, A_{570} and A_{600} were measured to determine the MIC.

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