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Structure and Total Synthesis of Fungal Calpinactam, A New Antimycobacterial Agent

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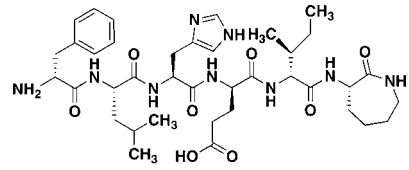
ABSTRACT



Calpinactam (1)

A new fungal metabolite designated calpinactam (1) was isolated from the culture broth of *Mortierella alpina* FKI-4905, and its structure was elucidated by spectroscopic analyses including NMR experiments. Calpinactam was found to be a hexapeptide with a caprolactam ring at its C-terminal. Its absolute stereochemistry was determined by amino acid analysis and total synthesis. Calpinactam selectively inhibited the growth of mycobacteria among various microorganisms. The MIC values of calpinactam against *Mycobacterium smegmatis* and *M. tuberculosis* were 0.78 and 12.5 μg/mL, respectively.

Our research group has focused on the discovery of anti-infectives from microbial metabolites.[1] Tuberculosis (TB) is still the greatest single infectious cause of mortality in the world, together with HIV and malaria.[2] Moreover, the spread of the HIV has increased the number of tuberculosis patients.[3] However, new anti-TB agents have not been developed for over 30 years, and only 5 anti-TB drugs, namely, isoniazid, ethambutol, rifampicin, streptomycin, and pyrazinamide, are in clinical use. It is increasingly urgent to discover anti-TB drugs with a new mechanism of action. Since isoniazid and ethambutol, first-line anti-TB drugs, show selective growth inhibition against *Mycobacteria*, we have screened for microbial metabolites that selectively inhibit the growth of *M. smegmatis* among 14 test microorganisms including gram-positive and gram-negative bacteria, fungi, and yeasts. By using this screening system,[4] we discovered and reported lariaitins, new lasso polypeptides, from the culture broth of actinomycete *Rhodococcus josti* K01-B0171.[4] During the continuation of the screening program, we have isolated calpinactam (**1**, Figure 1) from the culture broth of the fungal strain *Mortierella alpina* FKI-4905. From the various spectral analyses, the compound was found to have a hexapeptide skeleton with a caprolactam ring at its

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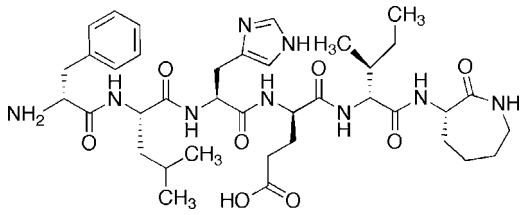

Figure 1. Structure of **1**.

C-terminal, which is seen in the structures of siderophore mycobactins.[5] In this study, the structure elucidation of **1** including its absolute stereochemistry and biological activity is described. *M. alpina* FKI-4905 was isolated from soil collected in the Bonin Islands, Tokyo, Japan. This strain was used for the production of **1**. The whole broth was cultured for 4 days (1.0 L), before being extracted with ethanol, and then the supernatant was filtered and concentrated under reduced pressure. The resulting aqueous layer was diluted by adding distilled water (2.0 L) and applied to an ODS gel column (40 g). The materials were eluted stepwise with 30, 60, and 100% CH_3CN (500 mL each). The 60% CH_3CN fraction, which showed activity, was concentrated to give a red brown material (140 mg). This material containing enriched calpinactam was finally purified using preparative HPLC under the following conditions: column, PEGASIL ODS (Senshu Sci. i.d. 20 × 250 mm); solvent, a 40 min linear gradient from 20 to 40% CH_3CN containing 0.05% trifluoroacetic acid; flow rate, 6 mL/min; detection, UV at 210 nm. The fraction eluted as a peak with the retention time of 31 min and was concentrated and lyophilized to dryness to yield pure **1** (13.0 mg) as a white powder.[14]

Calpinactam (**1**) showed a molecular ion peak at *m*/*z* 768 $(M + H)^+$ in FAB-MS, and the molecular formula $C_{38}H_{57}O_8N_9$ was assigned on the basis of HRFAB-MS [*m*/*z* 768.4411 $(M + H)^+$, Δ +2.4 mmu], requiring 15 degrees of unsaturation. The ninhidrin reaction and IR absorptions suggested the presence of amino and carbonyl groups in the structure. The ^{13}C NMR spectrum (in DMSO-d_6) showed 36 resolved signals, which were classified into four methyl carbons, 10 methylene carbons, eight sp^3 methine carbons, seven sp^2 methine carbons, two sp^2 quaternary carbons, and seven carbonyl carbons by analysis of its DEPT spectra. The ^1H NMR spectrum (in DMSO-d_6) displayed 51 proton signals, six of which were suggested to be NH protons (δ 7.79, 7.83, 7.92, 7.97, 8.42, and δ 8.57). Furthermore, analysis of the ^1H NMR spectrum in CD_3OD as a solvent revealed the presence of two new proton signals (δ 7.34 and δ 8.79) corresponding to aromatic protons of a heterocyclic ring. The connectivity of the proton and carbon atoms (Figure 2) was established by its HMQC spectrum. $^1H-^1H$ COSY and TOCSY analyses revealed the presence of the seven

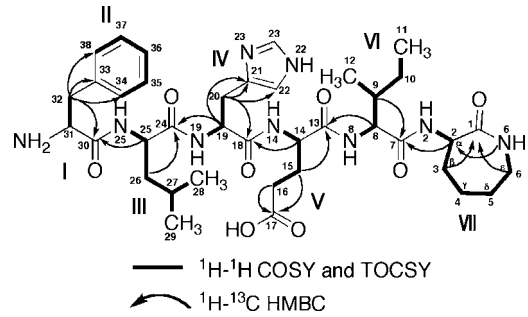

Figure 2. COSY, TOCSY, and HMBC correlations of **1**.

partial structures (I−VII,) as shown by the bold lines in Figure 2. Furthermore, $^{13}C-^1H$ long-range couplings of ^2J and ^3J were measured in the HMBC spectrum (Figure 2). First, the presence of six amino acid residues became clear due to the following observations: (1) The cross peaks from H_2-3 (δ 1.34 and δ 1.70) and H_2-6 (δ 3.02 and δ 3.14) to C-1 (δ 174.1) and the cross peak from NH-6 (δ 7.83) to C-2 (δ 51.4) showed a connection between the partial structure VII and the carbonyl carbon C1, indicating the presence of a caprolactam moiety derived from a lysine residue. (2) The cross peak from H-9 (δ 1.80) to C-7 (δ 169.8) showed the connection between the partial structure VI and the carbonyl carbon C-7, indicating the presence of an isoleucine residue. (3) The cross peaks from H_2-15 (δ 1.70 and δ 1.85) to C-13 (δ 171.1) and C-17 (δ 173.9) showed the connection among the partial structure V and the carbonyl carbons C-13 and C-17, indicating the presence of a glutamate residue. (4) The cross peak from H-26 (δ 1.26) to C-24 (δ 171.5) showed the connection between the partial structure III and the carbonyl carbon C-24, indicating the presence of a leucine residue. (5) The cross peaks from H_2-32 (δ 2.96 and δ 3.00) to C-30 (δ 167.9), C-33 (δ 134.9), C-34 (δ 129.5), and C-38 (δ 129.5) showed the connections among the partial structures I and II and the carbonyl carbon C-30, indicating the presence of a phenylalanine residue. (6) The residual molecular formula and the cross peaks from H-19 (δ 4.55) to C-21 (δ 130.5) and from H_2-20 (δ 2.82 and δ3.01) to C-21 and C-22 (δ 117.2) showed that a heterocyclic five-membered ring was formed among C-21, C-22, N-22, C-23, and N-23 to connect C-20 and C-21. Two singlet proton signals for H-22 and H-23 in the ^1H NMR and the chemical shifts of C-21, C-22, and C-23 (δ 134.2) supported the presence of an imidazole ring. Next, to determine the sequence of the six amino acid residues, the correlation from each α-proton to the adjacent carbonyl carbon observed in the HMBC spectrum was investigated. The respective cross peaks from H-25 (δ 4.26) to C-30, H-19 to C-24, H-14 (δ 4.36) to C-18, H-8 (δ 4.31) to C-13, and H-2 (δ 4.36) to C-7 indicated that the sequence from the N-terminal was phenylalanine, leucine, histidine, glutamic acid, isoleucine, and cyclized lysine. Taken together, the planar structure of calpinactam was elucidated as shown in Figure 2.

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To elucidate the absolute stereochemistry of **1**, amino acid analysis was carried out by the established method.[6] The acid hydrolysate of **1** was derivatized with *O*-phthalaldehyde (OPA) and *N*-acetyl-L-Cys to yield OPA derivatives, which were analyzed by HPLC (column, TSKgel Super-ODS (TOSOH, i.d. 4.6 × 100 mm); flow rate, 0.7 mL/min; detection, fluorescent intensity at 335 nm with excitation at 435 nm) using two following solvent systems: (1) A 60 min linear gradient from 0 to 20% MeOH and a subsequent 50 min linear gradient from 20 to 60% MeOH. (2) A 90 min linear gradient from 25 to 40% MeOH. Under the conditions of (1), the OPA derivatives prepared from authentic D/L-Glu, -His, -Phe, -Ile, -Leu, and -Lys were detected with retention times of 10.6/11.7, 34.4/32.7, 87.0/87.7, 91.0/88.0, 93.0/94.0, and 94.5/94.9 min, respectively. Under these conditions, the hydrolysate of **1** was analyzed to give D-Phe, L-Leu, L-His, D-Glu, D-Ile, and L-Lys. Regarding D-Ile, further analysis to differentiate D-Ile and D-*allo*-Ile was needed. Under the conditions of (2), authentic D-Ile and D-*allo*-Ile were eluted with retention times of 46.8 and 47.6 min, respectively, showing D-*allo*-Ile to be present in **1**. Therefore, the complete structure of **1** including its absolute stereochemistry was elucidated to be D-phenylalanyl-L-leucyl-L-histidyl-D-glutaminyl-D-*allo*-isoleucyl-L-caprolactam, as shown in Figure 1.

To confirm the complete structure of **1**, the total synthesis of **1** was carried out (Scheme 1). First condensation between a known ε-lactam **2**, which was readily derived from L-Lys, and Boc-D-*allo*-Ile-OH in the presence of EDCI, HOBt, and Et_3N provided dipeptide **3** quantitatively. The Boc deprotection of **3** by treatment with TFA was followed by a second condensation reaction with Boc-D-Glu(OBn)-OH under the same conditions as before, affording the tripeptide **4** in an 88% yield over two steps. Removal of the Boc group of **4** by TFA, a subsequent third condensation reaction with Fmoc-L-His(Trt)-OH, and deprotection of the Fmoc group by exposure to piperidine furnished the tetrapeptide **5** in a 91% yield over three steps. A fourth condensation reaction between **5** and Fmoc-L-Leu-OH followed by deprotection of the Fmoc group by piperidine afforded pentapeptide **6** in a 50% yield over two steps. The pentapeptide **6** was then condensed with Boc-D-Phe-OH to give hexapeptide **7** in a 69% yield. Finally, removal of the trityl and benzyl groups by hydrogenolysis and *N*-Boc deprotection by TFA furnished crude **1**, which was purified by HPLC to afford pure **1** in a 22% yield over two steps (not optimized). Synthetic **1** was identical to natural product **1** in all respects ($[\alpha]_D$, ^1H and ^{13}C NMR, IR, MS, inhibitory activity against *M. smegmatis*, and retention time in HPLC). This result shows that the foregoing results with regard to the amino acid analysis of natural calpinactam (**1**) were accurate.

Several groups of caprolactam-containing peptide-like compounds have been reported. (A) Mycobactins[7] and carboxymycobactins[8] produced by *Mycobacterium* spp. are well-known siderophores or iron chelators and play a role in iron transport during mycobacteria growth. (B) The formobactin,[9] nocobactins,[10] BE-32030,[11] and amamistatins[12] produced by *Nocardia* spp. were reported to show anti-lipid peroxidation activity or antitumor activity. All compounds belonging to (A) and (B) have two hydroxamic acid moieties and one 2-hydroxyphenyloxazoline moiety in their structures, which are expected to form extremely stable hexadentate

Table 1. ^1H (600 MHz) and ^{13}C NMR (150 MHz) Chemical Shifts of **1** in DMSO-d_6

position	δ_C	δ_H
1	174.1 s	–
2	51.4 d	4.36 (1H, m)
2-NH	–	7.79 (1H, d, J = 6.5)
3	31.2 t	1.34 (1H, m), 1.70 (1H, m)
4	27.6 t	1.60 (1H, m), 1.84 (1H, m)
5	28.8 t	1.17 (1H, m), 1.71 (1H, m)
6	40.7 t	3.02 (1H, m), 3.14 (1H, m)
6-NH	–	7.83 (1H, dd, J = 5.0, 7.0)
7	169.8 s	–
8	56.2 d	4.31 (1H, dd, J = 6.0, 9.0)
8-NH	–	7.97 (1H, d, J = 9.0)
9	36.6 d	1.80 (1H, m)
10	25.7 t	1.05 (1H, m), 1.27 (1H, m)
11	11.6 q	0.80 t (3H, d, J = 7.0)
12	14.5 q	0.77 d (3H, d, J = 6.0)
13	171.1 s	–
14	51.6 d	4.36 (1H, m)
14-NH	–	7.92 (1H, d, J = 8.0)
15	27.6 t	1.70 (1H, m), 1.85 (1H, m)
16	30.1 t	2.07 (2H, m)
17	173.9 s	–
18	170.0 s	–
19	52.2 d	4.55 (1H, dt, J = 7.5, 8.0)
19-NH	–	8.42 (1H, d, J = 7.5)
20	27.7 t	2.82 (1H, dd, J = 8.0, 16.0), 3.01 (1H, m)
21	130.5	–
22	117.2	7.34[a] (1H, s)
23	134.2	8.79[a] (1H, s)
24	171.5 s	–
25	51.2 d	4.26 (1H, dt, J = 7.0, 8.0)
25-NH	–	8.57 (1H, d, J = 8.0)
26	40.8 t	1.26 (2H, m)
27	23.8 d	1.16 (1H, m)
28	21.4 q	0.75 (3H, d, J = 6.0)
29	23.1 q	0.72 (3H, d, J = 6.0)
30	167.9 s	–
31	53.5 d	4.06 (1H, t, J = 7.0)
32	37.3 t	2.96 (1H, dd, J = 7.0, 14.0), 3.00 (1H, m)
33	134.9 s	–
34, 38	129.5 d	7.24 (2H, m)
35, 37	128.6 d	7.30 (2H, m)
36	127.2 d	7.26 (1H, m)

[a] ^1H NMR was measured in CD_3OD due to no signals being detected in DMSO-d_6. The value is shown here.

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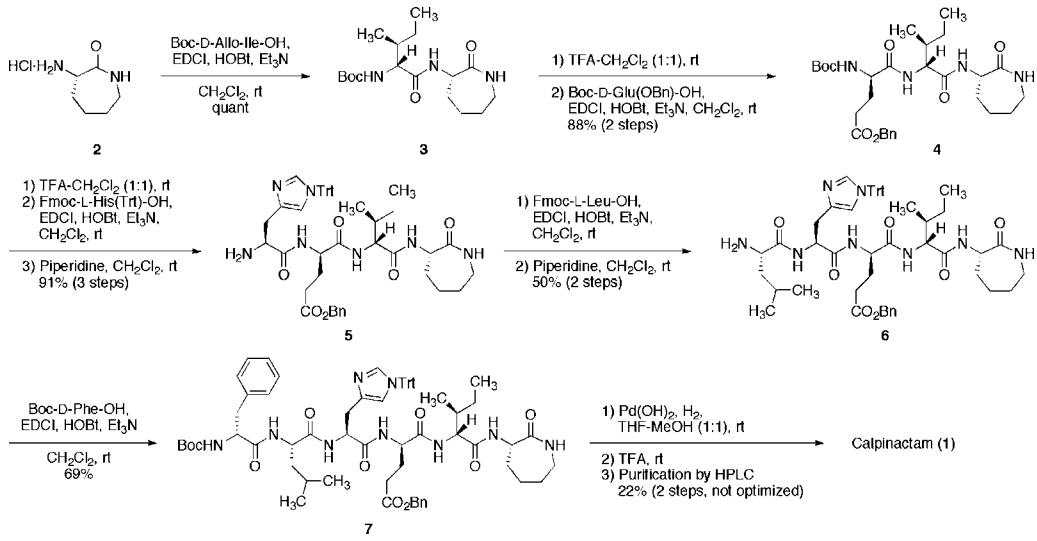
Scheme 1. Total Synthesis of **1**



iron(III) complexes. On the other hand, (C) VX1000, VVD130, and ZG3000, which are produced by the plant pathogenic fungus *Periconia circinata*, were reported to inhibit plant root growth.[13] These compounds have no structural characteristics showing iron chelating activity. Compound **1**, which was also isolated from a fungal strain, contains no hydroxamic acids or 2-hydroxyphenyloxazolines in its structure. In this sense, calpinactam belongs to the (C) group, although its core structures are quite different except for having a caprolactam moiety. Furthermore, several synthetic mycobactin analogues were reported to potently inhibit the growth of *M. tuberculosis*, probably as antagonists of mycobactin.[5] From the unique structure of **1**, the mechanism of action of **1** appears to be different from those of compounds belonging to (A) to (C), although this remains to be clarified.

The antimycobacterial activity of **1** was evaluated by a paper disk method and a liquid microdilution method.[4] In a paper disk method, the compound was selectively active against mycobacteria among various microorganisms including gram-positive and gram-negative bacteria, fungi, and yeasts. In a liquid microdilution method, **1** inhibited the growth of *M. smegmatis* and *M. tuberculosis* with MIC values of 0.78 and 12.5 μg/mL, respectively.

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Supporting Information Available: NMR spectra of natural calpinactam (**1**), and experimetnal procedures and characterization data of synthetic calpinactam (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) Calpinactam (**1**): white powder; $[\alpha]^{25}_{D}$ −23.0 (*c* 0.05, AcOH); IR (KBr) υ_{max} 3432, 3288, 1673, 1633, 1540, 1446 cm^{-1}; UV (MeOH) λ_{max} 201 (ε 11698); ^{1}H and ^{13}C NMR data (Table 1); FAB-MS *m/z* 768 (M + H)$^{+}$; HRFAB-MS *m/z* 768.4411 ((M + H)$^{+}$; calcd for $C_{38}H_{57}O_8N_9$, 768.4387).