

Gerfelin, a Novel Inhibitor of Geranylgeranyl Diphosphate Synthase from *Beauveria felina* QN22047

I. Taxonomy, Fermentation, Isolation, and Biological Activities

SATOKO ZENITANI^a, SATOSHI TASHIRO^a, KAZUTOSHI SHINDO^b, KOJI NAGAI^c, KENICHI SUZUKI^c and MASAYA IMOTO^{a,*}

^a Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University,
Yokohama 223-8522, Japan

^b Department of Food and Nutrition, Japan Women's University,
2-8-1, Mejirodai, Bunkyo-ku, Tokyo 112-8681, Japan

^c Microbiology Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd.,
1-1-8 Azusawa, Itabashi-ku, Tokyo 174-8511, Japan

(Received for publication March 25, 2003)

A new compound, gerfelin, was isolated from a culture broth of *Beauveria felina* QN22047. It was purified by column chromatography on silica gel and by HPLC. Gerfelin has the molecular formula C₁₅H₁₄O₆. It inhibited synthesis of geranylgeranyl diphosphate, which was mediated by recombinant human geranylgeranyl diphosphate synthase (hGGPP synthase) *in vitro*. The inhibitory pattern of gerfelin was noncompetitive against isopentenyl diphosphate, and uncompetitive against farnesyl diphosphate.

Small GTP-binding proteins (Small GTPases) are well established as molecular switches that transduce an upstream signal to downstream effectors. Among them, Ras, Rho/Rac/Cdc42, and Rab proteins have sequences at their COOH termini that undergo posttranslational modifications involving the transfer of an isoprenoid moiety from farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP). These prenylation modifications are essential for small GTPases to function because they affect the attachment of the GTPase to the plasma membrane, which is crucial to their activation¹⁻⁴. Recently, the number of geranylgeranylated proteins has been shown to be larger than that of farnesylated proteins. Among geranylgeranylated proteins, Rho and Rac family proteins have an important role in cytoskeletal organization, cancer metastasis, and the proliferation of cancer cells. In addition, it has been shown that GGPP itself plays an important role physiologically, for example, GGPP inhibits the DNA binding activity of the nuclear hormone receptor LXR-RXR⁵, and is important for the survival of osteoclasts⁶. Therefore, a GGPP synthase inhibitor may be

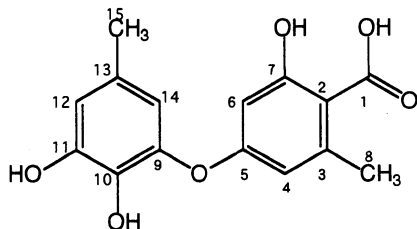
a useful tool with which to analyze the function of GGPP, and also an attractive drug to prevent and/or treat diseases caused by protein geranylgeranylation or GGPP itself.

GGPP synthase belongs to a subgroup of prenyltransferases, and are widely distributed among bacteria, plant and mammalian^{7,8}. The human GGPP synthase cDNA was cloned and encodes a protein of 300 amino acids which shows 16% sequence identity with the known human FPP synthase^{9,10}. Human GGPP synthase shows extremely high homology to other GGPP synthases of bovine, *Drosophila* and *N. crassa*⁸.

In the course of screening for an inhibitor of GGPP synthase, we found that a fungal strain, *Beauveria felina* QN22047 produced a novel compound named gerfelin. In this paper, the taxonomy of the producing strain, fermentation, isolation and biological activities of gerfelin are described. The structural elucidation of gerfelin will be described in an accompanying publication¹¹.

* Corresponding author: imoto@bio.keio.ac.jp

Fig. 1. Structure of gerfelin.



Materials and Methods

Taxonomic Studies

The following media were used for the identification of the fungus: potato dextrose agar (PDA), cornmeal agar (CMA), Miura's medium (LCA) and CZAPEK's agar. Morphological properties were observed under an optical microscope (Nikon OPTIPHOT-2).

In Vitro Geranylgeranyl Diphosphate Synthase Assay

Human geranylgeranyl diphosphate (GGPP) synthase cDNA (GenBank: DDBJ Accession No. AB019036)⁹⁾ was kindly provided by Dr. N. MISAWA, Central Laboratories for Key Technology, Kirin Brewery Co. Ltd. The human GGPP synthase cDNA was subcloned into pGEX2T, and the GST-fusion enzyme (GST-GGPP synthase) was expressed in *E. coli* DH5 α , and purified on glutathione-agarose beads (Sigma). The standard GGPP synthase assay mixture contained, in a final volume of 0.2 ml, 0.1 M Tris-HCl buffer (pH 7.5), 0.3 mM MnCl₂, 0.2 mM MgCl₂, 10 μ g/ml BHT, 47.9 ng/ml FPP, 0.81 nCi [¹⁴C] isopentenyl diphosphate (IPP) and an appropriate amount of purified GST-GGPP synthase in the presence or absence of test sample. The mixture was incubated at 37°C for 45 minutes and the reaction was terminated by the addition of 0.3 ml of 1-BuOH and mixed. The enzyme activity was evaluated for the radioactivity of the 1-BuOH extract of the reaction mixture. For product analysis, the reaction products in the 1-BuOH extract were resolved on a silica gel thin-layer chromatography plate using isopropyl alcohol: ammonia: water (6:3:1) as the developing solvent. The autoradiogram was analyzed using a Fuji Bas-5000 Bioimaging analyzer (Fuji film, Tokyo).

Results

Taxonomy of the Producing Strain

Strain QN22047 was isolated from a soil sample collected at Kajikazawa-chou, Yamanashi Prefecture, Japan. Cultural and morphological characteristics of this strain were as follows: colonies on PDA for 14 days at 24°C attaining 33 mm in diameter, forming floccose to velvety mycelium. The color of the colonies was pale yellow to pale purple. Reverse side of the colonies was yellowish brown. Soluble pigment was not observed around colonies. Hyphae of the aerial mycelium was hyaline, smooth-walled and creeping. Conidiophores were arisen from substrate and aerial mycelium, solitary or in small groups on PDA and LCA. Conidiogenous cells were not elongating, consisting of a swollen, flask-shaped or curved, 3~8 \times 2~3 μ m, mostly comprising 2 to 4 denticles. Conidia were single-celled, hyaline, smooth, subglobose, ellipsoidal or ovoidal, 3~4 \times 2.5~3 μ m. No chlamydospores were observed.

Based on cultural and microscopic characteristics described above, strain QN22047 was considered to belong to the genus *Beauveria* or related genera. Moreover, the characteristics above corresponded to the species description of *Isaria felina* (de Candolle: Fries) Fries by DE HOOG¹²⁾ which was a synonym of *Beauveria felina* (de Candolle: Fries) Carmichael. Therefore, we identified this strain as *Beauveria felina* and named it *Beauveria felina* QN22047. The strain has been deposited in the National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan, with the accession No. FERM P-19056.

Fermentation

A thawed suspension of strain QN22047 was used to inoculate in a 500 ml Erlenmeyer flask containing 100 ml of a seed medium composed of glucose 3%, corn steep liquor 1%, dry yeast 0.5%, Pharmamedia 0.5%, SIII meat 0.5%, and CaCO₃ 0.2% (adjusted to pH 6.5 before sterilization). The inoculated medium was incubated at 24°C for 5 days on a rotary shaker (200 rpm). Aliquots (3 ml) of this seed culture were added to a 500 ml Erlenmeyer flask containing 100 ml of a medium composed of glucose 3%, glycerol 2%, dextrin 1%, malt extract 1%, yeast extract 2%, tryptone 0.1%, NH₄NO₃ 0.1%, and KH₂PO₃ 0.1% (adjusted to pH 6.5). The inoculated medium was incubated at 24°C for 6 days on a rotary shaker (200 rpm).

Isolation and Purification

The culture broth (2.0 liters) at 6 days was separated into a mycelial cake and cultured filtrate (1.4 liters) by filtration. The cultured filtrate was extracted with EtOAc (1.4 liters). The extract was dried over anhydrous Na_2SO_4 and evaporated to dryness to give a brown oil. The brown oil was washed with 90% MeOH - Hexane, and its 90% MeOH residue was concentrated *in vacuo* to give a brown oil (2.6 g). Then, it was loaded on a silica gel column, and the active fraction was eluted with CHCl_3 -MeOH (100:5). The eluate was concentrated *in vacuo* to yield a light-brown powder (92.7 mg). It was further purified by HPLC (Capcell Pak C_{18} , column 4.6×150 mm) developed with 65% aq acetonitrile containing 0.2% H_3PO_4 . Active fractions were combined and concentrated *in vacuo* to yield a pure colorless powder of gerfelin (32.1 mg).

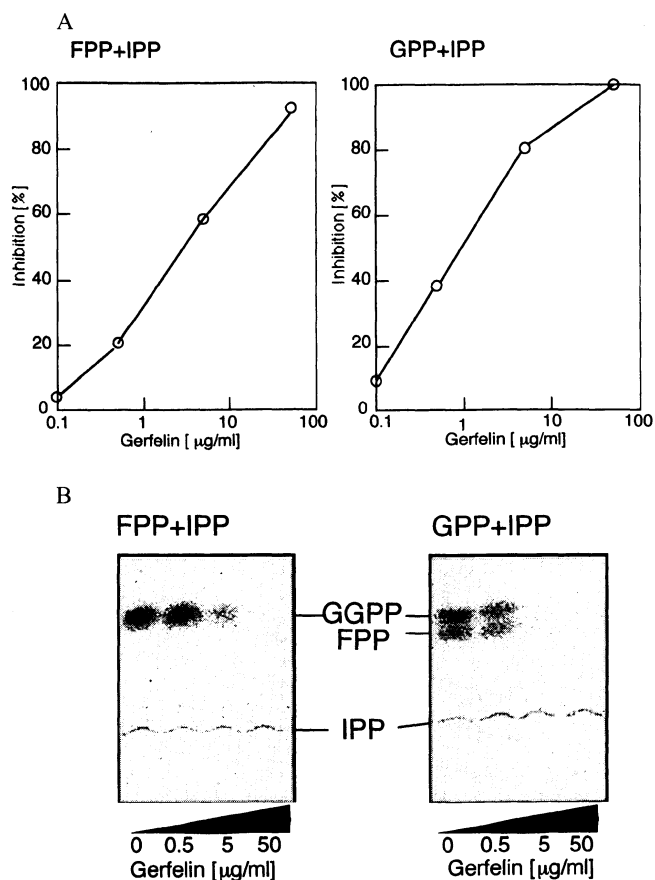
Inhibitory Activity of Gerfelin against GGPP Synthase

To examine the effect of gerfelin on GGPP synthase, we performed an *in vitro* GGPP synthase assay. As shown in Fig. 2A, gerfelin inhibited GGPP synthase activity with an IC_{50} of 3.5 $\mu\text{g}/\text{ml}$. Inhibition of GGPP synthase by gerfelin was further confirmed by thin-layer chromatography with autoradiographic detection of [^{14}C]GGPP (Fig. 2B). Because recombinant GGPP synthase was reported to catalyze the condensation of geranyl diphosphate (GPP) with IPP to give the GGPP through FPP¹⁰), we examined the effect of gerfelin on GGPP synthase using GPP instead of FPP as the substrate. As shown in Fig. 2B, although GGPP synthase produced not only [^{14}C]FPP but also [^{14}C]GGPP in this assay, gerfelin inhibited the formation of FPP as well as GGPP, and the IC_{50} value was about 2.9 $\mu\text{g}/\text{ml}$ (Fig. 2A).

Kinetic Analysis

Kinetic analysis revealed that the inhibitory pattern in the Lineweaver-Burk plot of gerfelin vs IPP was typical of noncompetitive inhibition, with each line meeting together on the x-axis, as shown in Fig. 3A. The inhibitory pattern of gerfelin vs FPP was uncompetitive (Fig. 3B). From the Dixon plot analysis, the K_i value of gerfelin against GGPP synthase was found to be about 5.5 $\mu\text{g}/\text{ml}$, as shown in Fig. 3C.

Fig. 2. Inhibition of human GGPP synthase by gerfelin *in vitro*.

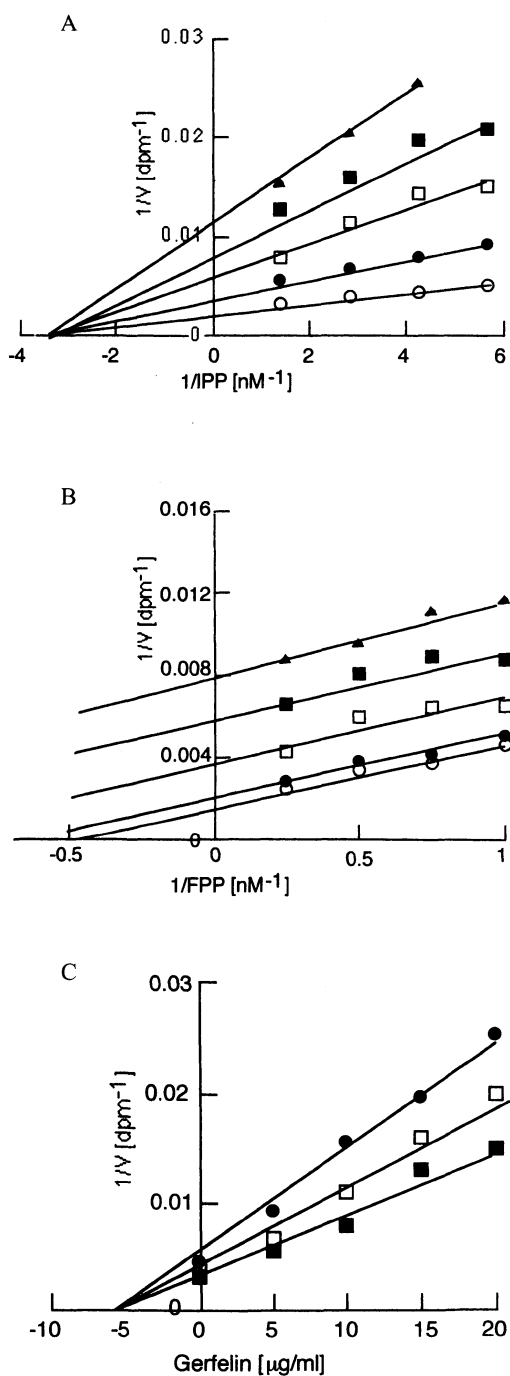


Recombinant GST-hGGPP synthase was incubated with [^{14}C] IPP plus FPP (left) or GPP (right) in the presence or absence of gerfelin. The reaction was terminated by the addition of 1-BuOH. The radioactivity of 1-BuOH fraction was measured (A). The reaction products were resolved by silica gel-TLC and visualized by autoradiography (B).

Discussion

The isoprenoid biosynthetic pathway is a key target for chemotherapeutic intervention in a number of diseases. Therefore, several effective and specific inhibitors of this pathway have been reported. For example, statins are an important class of inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. Terbinafine and squalastatin were reported to be an inhibitor of squalene synthase^{13,14}. Nitrogen-containing bisphosphonates such as alendronate have been reported to

Fig. 3. Kinetic analysis of gerfelin against GGPP synthase.



(A) Lineweaver-Burk plot of initial velocity vs varying IPP concentrations. Gerfelin concentrations are 0 (○), 5 (●), 10 (□), 15 (■), and 20 (▲) μg/ml.

(B) Lineweaver-Burk plot of initial velocity vs varying FPP concentrations. Gerfelin concentrations are the same as in (A).

(C) Dixon plot of initial velocity vs gerfelin concentration. IPP concentrations are 17.8 (●), 35.7 (□) and 71.3 (■) nM.

inhibit FPP synthase^{15,16}. More recently, azaprenyl diphosphates have been synthesized as inhibitors of GGPP synthase¹⁷. However, no GGPP synthase inhibitors of microbial origin have yet been reported. In the present study, we described the first natural inhibitor of GGPP synthase, gerfelin, isolated from the culture broth of *Beauveria felina* QN22047. It also inhibits the FPP synthesizing activity of GGPP synthase. Because human FPP synthase¹⁰ is a considerably different protein from GGPP synthase, we don't know at present whether gerfelin inhibits human FPP synthase.

In the process of isolating gerfelin, we also isolated a C10-deoxy derivative of gerfelin. This compound is identical to 4-carboxy-5,5'-dihydroxy-3,3'-dimethyl-diphenylether, which was originally isolated from an ethionine inhibited culture fluid of *Aspergillus fumigatus* DH413¹⁸. The C10-deoxy derivative of gerfelin showed only weak inhibitory activity against GGPP synthase (IC₅₀ value was 100 μg/ml), indicating that a hydroxy group at position C-10 is necessary for the inhibition of GGPP synthase. This finding is useful for developing gerfelin derivatives having more effective inhibitor activity against GGPP synthase.

Gerfelin would provide a valuable tool for further studies of the role of GGPP synthase in signaling pathways involved in cellular responses or diseases. Detailed studies of the biological activities of gerfelin *in vivo* and *in vitro* are in progress.

Acknowledgments

We thank Dr. N. MISAWA, Central Laboratories for Key Technology, Kirin Brewery Co. Ltd. for supplying human GGPP synthase cDNA. This study was partly supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

- 1) CASEY, P. J. & M. C. SEABRA: Protein prenyltransferases. *J. Biol. Chem.* 271: 5289~5292, 1996
- 2) GLOMSET, J. A. & C. C. FARNSWORTH: Role of protein lipidation reactions in programming interactions between Ras-related GTPases and cell membranes. *Annu. Rev. Cell Biol.* 10: 181~205, 1994
- 3) MAGEE, A. I.; C. M. NEWMAN, T. GIANNAKOULOS, J. F. HANCOCK, E. FAWELL & J. ARMSTRONG: Lipid modifications and function of the ras superfamily of proteins. *Biochem. Soc. Trans.* 20: 497~499, 1992
- 4) ZHANG, F. L. & P. J. CASEY: Protein prenylation: molecular mechanisms and functional consequences. *Annu. Rev. Biochem.* 65: 242~269, 1996
- 5) FORMAN, B. M.; B. RUAN, J. CHEN, G. J. SCHROEPFER, Jr. & R. M. EVANS: The orphan nuclear receptor LXR is positively and negatively regulated by distinct products

- of mevalonate metabolism. Proc. Natl. Acad. Sci. USA. 94: 10588~10593, 1997
- 6) WOO, J. T.; S. KASAI, P. H. STERN & K. NAGAI: Compactin suppresses bone resorption by inhibiting the fusion of prefusion osteoclasts and disrupting the actin ring in osteoclasts. J. Bone Miner. Res. 15: 650~652, 2000
- 7) JIANG, Y.; P. PROTEAU, C. K. POUTLER & S. J. FERRO-NOVICK: BTS1 encodes a geranylgeranyl diphosphate synthase in *Saccharomyces cerevisiae*. J. Biol. Chem. 270: 21793~21799, 1995
- 8) ZHU, X. F.; K. SUZUKI, K. OKADA, K. TANAKA, T. NAKAGAWA, M. KAWAMUKAI & K. MATSUDA: Cloning and functional expression of a novel geranylgeranyl pyrophosphate synthase gene from *Arabidopsis thaliana* in *Escherichia coli*. Plant Cell. Physiol. 38: 357~361, 1997
- 9) MISAWA, N.; H. OKAZAKI, Y. NOGUCHI, I. TATSUNO, Y. SAITO, T. YASUDA & A. HIRAI: Study on isolation of a geranylgeranyl pyrophosphate (GGPP) synthase cDNA and its expression—Development of a new assay system of gene functions. Proc. of the Japanese Conference on the Biochemistry of Lipids 41: 293~296, 1999
- 10) KUZUGUCHI, T.; Y. MORITA, I. SAGAMI, H. SAGAMI & K. OGURA: Human geranylgeranyl diphosphate synthase: cDNA cloning and expression. J. Biol. Chem. 274: 5888~5894, 1999
- 11) ZENITANI, S.; K. SHINDO, S. TASHIRO, M. SEKIGUCHI, M. NISHIMORI, K. SUZUKI & M. IMOTO: Gerfelin, a novel inhibitor of geranylgeranyl diphosphate synthase from *Beauveria felina* QN22047. II. Structural elucidation. J. Antibiot. 56: 658~660, 2003
- 12) DE HOOG, G. S.: The genera *Beauveria*, *Isaria*, *Tritirachium* and *Acrodontium* gen. nov. Stud. Mycol. 1: 1~41, 1972
- 13) WENTZINGER, L. F.; T. J. BACH & M. A. HARTMANN: Inhibition of squalene synthase and squalene epoxidase in tobacco cells triggers an up-regulation of 3-hydroxy-3-methylglutaryl coenzyme a reductase. Plant Physiol. 130: 334~346, 2002
- 14) DAWSON, M. J.; J. E. FARTHING, P. S. MARSHALL & M. V. HAYES: The squalenostatins, novel inhibitors of squalene synthase produced by a species of *Phoma*. J. Antibiotics 45: 639~647, 1992
- 15) BEEK, E.; E. PIETERMAN, L. COHEN, C. LÖWIK & S. PAPAPOULOS: Farnesyl diphosphate synthase is the molecular target of nitrogen-containing bisphosphonates. Biochem. Biophys. Res. Commun. 264: 108~111, 1999
- 16) KELLER, R. K. & S. J. FLIESLER: Mechanism of aminobisphosphonate action: Characterization of alendronate inhibition of the isoprenoid pathway. Biochem. Biophys. Res. Commun. 266: 560~563, 1999
- 17) SZABO, C. M.; Y. MATSUMURA, S. FUKURA, M. B. MARTIN, J. M. SANDERS, S. SENGUPTA, J. A. CIESLAK, T. C. LOFTUS, C. R. LEA, H. J. LEE, A. KOOHANG, R. M. COATES, H. SAGAMI & E. OLDFIELD: Inhibition of geranylgeranyl diphosphate synthase by bisphosphonates and diphosphates: a potential route to new bone antiresorption and antiparasitic agents. J. Med. Chem. 45: 2185~2196, 2002
- 18) YAMAMOTO, Y.; K. NITTA, Y. OOHATA & T. FURUKAWA: Studies on the metabolic products of a strain of *Aspergillus fumigatus* DH413. V. A new metabolite produced by ethionine inhibition. Chem. Pharm. Bull. 20: 931~935, 1972