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NOTE

Penicillide, a Nonpeptide Calpain Inhibitor, Produced by *Penicillium* sp. F60760

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Abstract Penicillide, having a 5*H*,7*H*-dibenzo[b,g][1,5] dioxocin-5-one skeleton, was isolated from the culture broth of *Penicillium* sp. F60760 as a nonpeptide inhibitor of calpain, a calcium-activated papain-like protease. The IC_{50} value for the effect of penicillide against m-calpaln was 7.1 μM. However, penicillide did not inhibit papain at a concentration of 200 μM. These results suggest that penicillide is a new class of nonpeptide calpain inhibitor having an eight membered lactone ring.

Key words: Calpain, nonpeptide inhibitor, penicillide

Calpain is a Ca^{2+}-dependent cysteine protease which is found in the microsomal and cytosolic compartments of most mammalian neurons and other cells [5, 17]. Calpain has two isoforms: calpain-I (or μ-calpain) and calpain-II (or m-calpain), which require low and high micromolar Ca^{2+} concentrations for activation. Increasing evidence now suggests that excessive activation of calpain could play a key or contributory role in the pathology of a variety of disorders, including cerebral ischaemia [9, 10], cataract [1, 6], myocardial ischaemia [18], muscular dystrophy [8, 15], and platelet aggregation [14]. Therefore, calpain inhibitors can be used for the treatment of neurodegenerative and muscular dystrophy diseases because of their therapeutic effects [20, 21].

In the course of screening for potential calpain inhibitors from fungal extracts, a strain of *Penicillium* sp. was found to exhibit activity in a Coomassie Brilliant blue G-250 dye based calpain-casein assay [3]. Activity-guided fractionation led us to the active compound. The compound was determined to be penicillide, a plant growth inhibitor with cytotoxicity [13], by spectroscopic methods. This compound is a new class of calpain inhibitors having a 5*H*,7*H*-dibenzo[b,g][1,5]dioxocin-5-one skeleton. This report presents the purification, identification, and biological activity of penicillide.

The producing strain F60760 was isolated from a soil sample and identified as a *Penicillium* sp. based on its cultural and morphological properties. The strain F60760 was grown for 10 days on a Difco potato-dextrose agar (PDA) plate at 25°C and maintained at -20°C. Seed inoculum from the plate was used to inoculate a seed flask containing 50 ml of culture medium (glucose 20 g, yeast extract 2 g, polypeptone 5 g, $MgSO_4$ 0.5 g, KH_2PO_4 1 g in 1 liter of distilled water). The seed culture was incubated at 28°C on a rotary shaker at 150 rpm for 3 days. For shake flask fermentation, 1 ml of seed culture was transferred to a 1 liter flask containing 150 ml of the above culture medium after which the flasks were incubated at 28°C for 5 days with agitation as above.

The active compound was isolated from the culture broth by calpain assay-guided fractionation. Two calpain assay systems were used in this study. One was a Coomassie Brilliant blue G-250 dye based calpain-casein assay system [3] and the other was a calpain-fluorogenic substrate assay system [11]. The casein-Coomassie blue microplate assay was carried out as follows. A mixture containing 0.5 mg/ml casein, 20 mM dithiothreitol (DTT), 50 mM Tris-HCl (pH 7.4), 25 mU calpain (Sigma, St. Louis, U.S.A.), and 4 mM $CaCl_2$ was added to a microplate well (200 μl). After incubation for 60 min at 25°C, 100 μl of the reaction mixture were transferred to another plate containing 80 μl of Bio-Rad Coomassie blue G-250 dye reagent (50% dilution with water) and then read on a Bio-Rad microplate reader (Model 3550, Hercules, U.S.A.) at 595 nm. For the fluorogenic calpain assay, *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (SLLVY-AMC, 13 μM) was

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incubated with 25 mU calpain in 20 mM DTT, 4 mM $CaCl_2$, and 50 mM Tris-HCl (pH 7.4) (100 μl) in a fluorescence-compatible microplate. Fluorescence of the liberated methylcoumarinamine (MCA) was monitored by a Perkin-Elmer fluorometer LS-50B (excitation at 345 nm and emission at 441 nm). To avoid artifacts such as fluorescence quenching by broth components in the fluorogenic calpain assay, a Coomassie Brilliant blue dye assay was concommitantly carried out.

A schematic diagram for the purification of the active compound is shown in Fig. 1. The mycelial 50% acetone extract and culture filtrate (900 ml) were combined and adsorbed on a Diaion HP-20 column. The active compound was eluted with 70% acetone and concentrated in a small volume. The aqueous residue was extracted with EtOAc. The EtOAc layer was dried in a rotary evaporator and the resulting materials were dissolved in a small volume of MeOH. The MeOH soluble compounds were fractionated by HPLC using a YMC-ODS-AM column, eluting with 50% acetonitrile at a flow rate of 1 ml/min. The HPLC fractionation yielded the pure active compound as a white amorphous powder (6 mg).

The molecular weight (MW) of the compound was determined to be 372 (M^+) by EI-MS. According to UV absorption at 280 and 201 nm, the chromophore of the compound is a substituted benzene ring; one of the substitutions is a phenolic hydroxyl, as demonstrated by the bathochromic shift in an alkaline solvent. From the MS and ^{1}H-and ^{13}C-NMR data, the molecular formula was deduced to be $C_{21}H_{24}O_6$. The ^{1}H-and ^{13}C-NMR data in $CDCl_3$ are summarized in Fig. 2. The ^{1}H-NMR spectrum showed 4 methyl proton signals at 0.93 (*dd*), 0.95 (*dd*), 2.20 (*s*), and 3.95 ppm (*s*, methoxyl). Signals

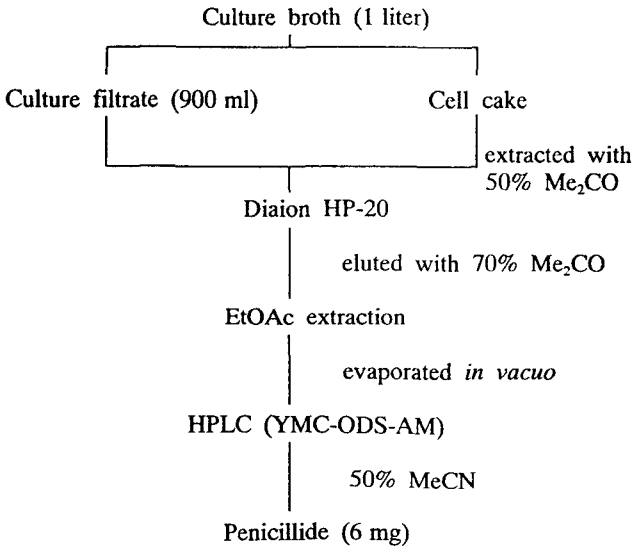

Fig. 1. Schematic diagram for purification of the calpain inhibitor from the culture broth of *Penicillium* sp. F60760.

of two independent pairs of aromatic protons were observed in this spectrum. Protons of the first pair (6.83 and 7.52 ppm) were in an *ortho* position (J=8.4 Hz), while those of the second (6.34 and 6.84 ppm) were *meta* arranged (J<2.0 Hz). The ^{1}H-^{1}H couplings suggested the presence of a 1-hydroxy-3-methylbutyl moiety. The ^{13}C-NMR spectrum showed signals of 21 carbons including 1 carbonyl carbon. Based on the above results, the compound was matched with penicillide through database analysis of fungal metabolites [18]. The chemical shifts of the purified calpain inhibitor in ^{1}H-and ^{13}C-NMR spectra are in good agreement with a literature data comparison of penicillide [12, 13]. The optical rotation $[\alpha]_D$ of the purified compound at 22°C is +3.0° (*c* 0.67 in MeOH; +4.9° in literature), suggesting that 1'-hydroxymethine has a *S*-configuration [2].

Penicillide is an eight-membered lactone with the structure 3-(1'-hydroxy-3'-methylbutyl)-11-hydroxy-4-methoxy-9-methyl-5*H*,7*H*-dibenzo[b,g][1,5]dioxocin-5-one. Penicillide has been isolated as a plant growth inhibitor, acyl-CoA: cholesterol acyltransferase (ACAT) inhibitor and cytotoxic agent [16].

It was found that the hydrolytic activity of m-calpain is inhibited by penicillide with an IC_{50} value of 7.1 μM

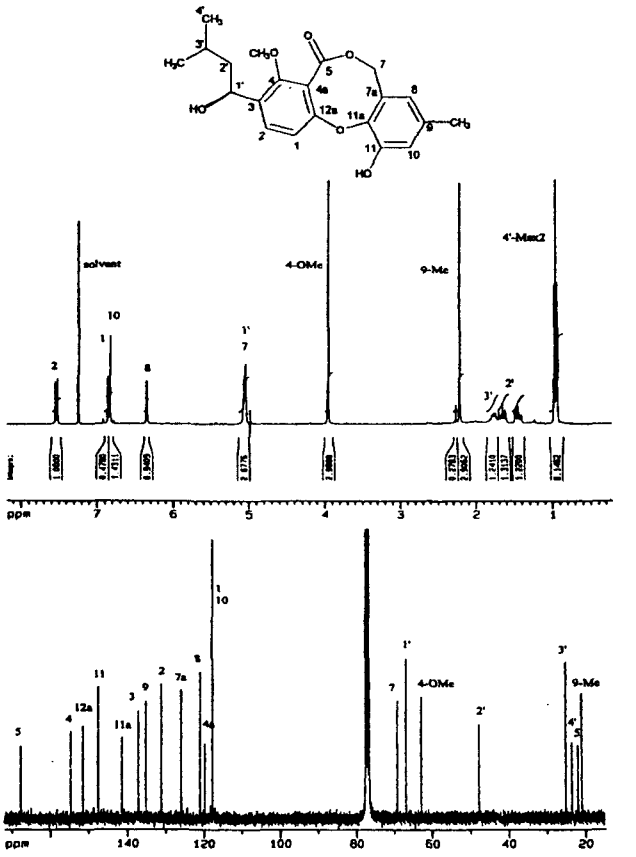

Fig. 2. Structure, ^{1}H-and ^{13}C-NMR spectra of penicillide in $CDCl_3$ at 300 MHz.

Table 1. Effect of penicillide on m-calpain and papain activity.

Protease	Substrate	IC_{50} (μM)
m-Calpain (13 mU/ml)	SLLVY-AMC	7.1
Papain (13.5 mU/ml)	BAPNA	>200

m-Calpain was assayed using SLLVY-AMC[1] as a substrate by reading MCA fluorescence with a Perkin-Elmer microplate fluorometer LS-50B and papain was assayed colorimetrically using BAPNA[2] as a chromophore substrate with microplate reader (Bio-Rad model 3550).
[1]*N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin.
[2]*Nα*-benzoyl-DL-Arg-*p*-nitroanillide.

(Table 1). However, penicillide did not inhibit papain, another Ca^{2+}-independent cysteine protease, at a concentration of 200 μM.

Almost all of the calpain inhibitors are active-site-targeted peptide inhibitors that contain epoxysuccinyls, aldehydes, halomethanes, diazomethanes, halohydrazides, or disulfides [20, 21]. Other calpain inhibitors are polypeptides such as calpastatin, a naturally occurring endogenous calpain inhibitor protein [7], and kininogen heavy chain analogue [4]. These peptide inhibitors have some problems such as membrane permeability and hydrolysis by cellular esterases or proteases [20]. These problems can be overcome by developing the hydrophobic nonpeptide inhibitors. A recent development has been the emergence of nonpeptide calpain inhibitors. These include an isocoumarin derivative which has only low affinity for calpain (calpain I IC_{50}=10 μM; calpaln II IC_{50} =120 μM) and is known to potently inhibit serine proteases [20]. However, penicillide is the first example of a nonpeptide calpain inhibitor containing a 5*H*,7*H*-dibenzo[b,g][l,5]dioxocin-5-one skeleton.

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