NOTES

Phevalin, a New Calpain Inhibitor, from a *Streptomyces* sp.

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Calpain is a Ca^{2+}-dependent cysteine protease which is found in the microsomal and cytosolic compartments of most mammalian neurons and other cells[1]. Calpain hydrolyses peptide bonds of proteins in the cytosol[2,3]. A large amount of research effort has been focused on this enzyme, since it potentially plays a central role in several physiological events, such as turnover of myofibrillar proteins, protein kinase C activation, cytoskeleton and cell membrane organization[2], neuropeptide metabolism[2], and activation of platelets[3]. Inhibitors of calpain prevent the breakdown of cytoskeletal proteins induced by calcium efflux in intact cells[4,5]. Consequently they could potentially be used in the treatment of neurodegenerative and muscular dystrophies diseases.

In the course of screening for potential calpain inhibitors from microbial extracts[6], an actinomycete strain, (SC433), exhibited activity in a calpain-casein assay using ^{3}H-casein as the substrate[7]. A new bioactive secondary metabolite named phevalin, was isolated from the ethyl acetate extract of this culture. This compound is a new pyrazinone calpain inhibitor derived from phenylalanine and valine. This report presents the production and structure determination of phevalin, mainly by detailed spectroscopic methods including MS, 1D and 2D NMR.

The producing culture was isolated from a soil sample collected in Taiwan and has been deposited in the Sterling Winthrop Culture Collection, Collegeville, PA, under accesion number SC433. The organism was identified as *Streptomyces* sp. based on the following: observation of mycelia and spore formation—light gray aerial mycelia with loosely spiral spore chains. The color of the aerial mycelia and spore mass was dark gray while the reverse side of colonial growth was dark gray to black with areas of brownish-gray. Cellular fatty acid analysis was performed (MIDI Laboratories, Newark, DE). Based on a similarity index comparing the fatty acid composition of references strains in the actynomycetes database,[8,9] SC433 was determined as belonging to the genus *Streptomyces*.

The culture SC433, was grown for 10 days on Difco ISP #2 agar at 27°C and maintained as frozen (−80°C) agar plugs in a freezing solution of 5% lactose and 10% glycerol. Seed inoculum from two thawed agar plugs was used to inoculate a seed flask containing a 30 ml aliquot of seed medium: glucose (Baker) 20 g, Pharmamedia (Traders) 15 g, yeast extract (Difco) 5 g, calcium carbonate 4 g (Mallinkrott), ammonium sulfate 3 g, zinc sulfate, 0.03 g in 1 liter of distilled water. The pH of the medium was adjusted to 7.0 before autoclaving. The seed culture was incubated at 27°C on an orbital shaker, (New Bruswick Sciences, model G 26), rotating at 220 rpm for 3 days. For shake flask fermentations, 1.5 ml of the seed stage was transferred to Fermentation Medium F (5% v/v) dispensed as 30 ml aliquots in 250-ml Erlenmeyer flasks. Fermentation Medium F had the following composition: glycerol (Mallinkrott) 20 g, dextrin (Difco) 20 g, soytone (Difco) 10 g, yeast extract (Difco) 3 g, ammonium sulfate 2 g, calcium carbonate 2 g in 1 liter of distilled water. The pH was adjusted to 7.0 before autoclaving. Flasks were incubated at 27°C and agitated as above for 6 days.

The crude ethyl acetate extract from 1 liter whole broth was fractionated by preparative HPLC using a Vydac column, eluting with a gradient 5% to 40% acetonitrile over 50 minutes, followed by a gradient 40% to 90% acetonitrile over 15 minutes in a solution 0.1% TFA/H_2O, at a flow rate of 8 ml/minute. Using the previous conditions, 5 mg of phevalin eluted with 25% acetonitrile in TFA/H_2O.

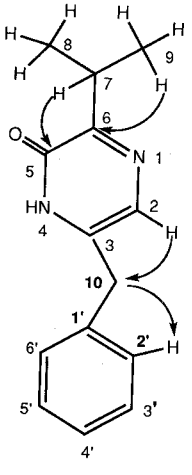
Phevalin showed a ninhydrin positive spot at Rf 0.6 on a thin layer reverse phase C18 chromatoplate with the solvent system, methanol - water - acetatic acid (1 : 1 : 0.1) The molecular formula of phevalin was established as $C_{14}H_{16}N_2O$ from FAB-HRMS (nitrobenzene matrix): MH^+ $m/z=229.1339$, found 229.1348 daltons. The UV (λ_{max} in methanol): 205 (2850), 325 (1296) nm; IR (KBr pellet): 3025 (m), 2970 (m), 1691 (s), 1621 (s), 1616 (m), 1457 (s), 1380 (s), 1358 (s) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 8.0 (br s, 1H, NH), 7.4 (m, 6H, H2′, H3′, H4′, H5′, H6′, H2), 3.9 (s, 2H, H10), 3.2 (septet, 1H, H7, $J_{7,8}=7$ Hz), 1.2 (d, 6H, H8, $J_{7,8}=7$ Hz); ^{13}C NMR ($CDCl_3$): δ 162.0 (C5), 157.1 (C6), 137.4 (C1′), 135.2 (C3), 129.4 (C3′, C5′), 129.3 (C2′, C6′), 127.9 (C4′), 123.4 (C2), 36.5 (C10), 30.0 (C7), 19.7 (C8).

The isopropyl group in phevalin was detected by ^{1}H NMR COSY, IR and MS/MS spectral data. ^{1}H NMR showed a methine proton (H7, δ 3.2) and two methyl groups (6H, H8, δ 1.2). These two groups were coupled to each other with a coupling constant of $J=7$ Hz, the methyl signal occurs as a doublet and the methine proton as a septet. IR spectrum showed absorptions of the gem-dimethyl groups (1380 and 1358 cm^{-1}). MS/MS showed the loss of 43 daltons from the molecular ion peak. The benzyl group in phevalin was indicated by ^{1}H NMR, COSY and by MS/MS spectral data. The aromatic

region of the 1D spectrum showed a monosubstituted ring (H2′, H3′, H4′, H5′ and H6′) overlapping with another proton and the aliphatic region showed a singlet. The long range COSY data showed the coupling of the methylene singlet (H10, δ 3.9) with two protons of the aromatic ring. MS/MS confirmed the presence of the benzyl group through loss of 91 daltons $(C_7H_7)^+$ from the molecular ion peak. The benzyl and the isopropyl groups are attached to a pyrazinone ring whose carbon chemical shifts are in good agreement to a literature data comparison of other disubstituted pyrazinones, 3,6-diisobutyl-2 (1H)-pyrazinone and 3,6-dimethyl-2 (1H)-pyrazinone[10]. C6 in phevalin (numbering system in Fig. 1) appears at δ 157.1 while in 3,6-diisobutyl-2 (1H)-pyrazinone appears at 156.5 and in 3,6-dimethyl 1 (1H)-pyrazinone appears at 153.6. C3 in phevalin appears at δ 135.2 while in 3,6-diisobutyl-2 (1H)-pyrazinone) appears at 137.7 and in 3,6-dimethyl 2 (1H)-pyrazinone appears at 134.5. The amide group was indicated in the IR spectrum (1621 cm^{-1}) and ^{13}C and DEPT showed a carbonyl carbon at 162.0 (C5). From 1D and HMQC data the olefinic proton resonated at δ 7.4 and is attached to a carbon (123.4 ppm) as indicated by HMQC. The imine was detected by IR (1616 cm^{-1}) and ^{13}C and DEPT showed that this carbon resonated at 157.1. Unambiguous assignment of the substitution of the pyrazinone ring was made by an HMBC experiment, confirming the isopropyl group substitution at C6 and the benzyl group at C3. Long range couplings were observed between the methyl protons of the isopropyl and the imine carbon and between the methine proton of the isopropyl and the amide carbonyl. Also, long range couplings were observed between the olefinic proton (H2) and the methylene carbon attached to the benzene ring (see Fig. 1).

Phevalin produced by actinomycete, is a 3-benzyl, 6-isopropyl disubstituted 2 (1H)-pyrazinone, presumably derived biosynthetically from phenylalanine and valine.

Fig. 1. Structure of phevalin and HMBC correlations.



Other microbial pyrazinones (from *Aspergillus* sp. *Streptomyces toxytricini*, *S. filipinensis* and *S. lavendurae*) have been previously described[11,12]. Of these arglecin[11] is the metabolite most studied so far due to its anti-arrhythmic properties. Phevalin inhibited calpain activity in the casein hydrolysis assay with an $IC_{50} = 1.3\,\mu M$. Other calpain inhibitors contain an oxirane group, such as E64, or an aldehyde group such as leupeptin[13], but this is the first example of a calpain inhibitor containing a pyrazinone ring. Furthermore, it has been reported that a leucine or a valine residue[14] may be important for binding at the P-2 subsite of calpain[14].

Experimental

High performance liquid chromatography (HPLC) was performed on a HPLC Waters 991 with a photo diode array detector. Mass spectra were acquired on a Finnigan MAT TSQ 70 Spectrometer (MS/MS), Kratos Profile Magnetic Sector (FAB MS) and V6 Analytical ZAB 2-SE high field (high resolution FAB MS). IR spectra were determined using KBr pellets on a FT IR IBM 30 S instrument. NMR spectra were acquired on a Varian Gemini 300, Varian Unity 500 MHz and JEOL GSX 270 MHz.

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