LEUALACIN, A NOVEL CALCIUM BLOCKER FROM *Hapsidospora irregularis*

I. TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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A new calcium blocker, designated leualacin, has been isolated from *Hapsidospora irregularis*. The compound inhibits the binding of ^{3}H-nitrendipine, a well known synthetic calcium blocker, to cardiac Ca channel in a competitive manner, although its structure is completely different from dihydropyridines.

Calcium blockers now in clinical use can be grouped mainly into three classes; dihydropyridines, benzodiazepines, and verapamil derivatives[1]. Considering the critical role of the cellular Ca concentration in biochemical processes, it is expected that Ca blockers with distinct structures and properties would be more effective and even have new pharmacological applications to the diseases such as thrombosis and atherosclerosis.

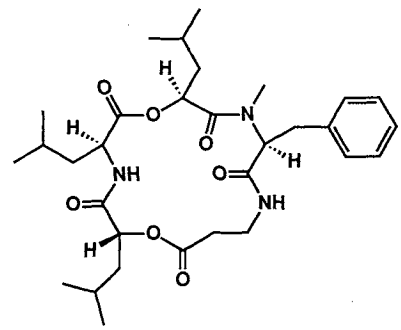
In this paper we report the isolation of leualacin†††, a new cyclic depsipeptide (Fig. 1). Although its structure is completely different from dihydropyridine-series Ca blockers, leualacin inhibits ^{3}H-nitrendipine binding to porcine heart membranes *in vitro*. Intravenous injection of leualacin at a dose of 100 mg/kg lowers the blood pressure of spontaneous hypertensive rats.

Materials and Methods

Discovery Screen

Leualacin was found by a radioligand binding assay of ^{3}H-nitrendipine to porcine heart microsomes by the method of GOULD *et al.*[2] with slight modification. Briefly, porcine heart was homogenized with Polytron in ice-cold 50 mM HEPES buffer, pH 7.4. The homogenate was centrifuged at $10,000 \times g$ for 10 minutes and the supernatant was centrifuged at $40,000 \times g$ for 20 minutes, and the pellets were resuspended in the same buffer and recentrifuged. The resulting pellets were finally resuspended into ice-cold 50 mM HEPES buffer and frozen at $-80°C$ until use. ^{3}H-nitrendipine (specific

Fig. 1. Structure of leualacin.



††† Patent K. HAMANO, K. FURUYA, K. TANZAWA, T. KAGASAKI, M. MIYAMOTO & T. KINOSHITA (Sankyo): New compound "Leualacin", its preparation and its use in the treatment of cardiovascular disorders. EP 358, 418 Mar. 14, 1990.

activity 80.9 Ci/mmol, NEN-DuPont), nifedipine (Sigma), and leualacin were dissolved in 100% ethanol and diluted with 50 mM HEPES buffer to the appropriate concentrations. Binding was carried out in a total volume of 1 ml containing 50 mM HEPES buffer, pH 7.4, 0.2 nM ^{3}H-nitrendipine, 22~36 μg microsomal proteins and each compound. After incubating for 20 minutes at room temperature, the reaction was terminated by rapid vacuum filtration over Whatman GF/C glass fiber filters using a Cell Harvester. The filters were rapidly washed 3 times with 2 ml of 50 mM phosphate buffer, pH 7.4. Nonspecific binding was defined as that which occurred in the presence of 400 nM nifedipine and was subtracted from total binding to give the specific binding.

Medium Used in Identification of Producing Strain

Medium used was WSH agar consisting of (%): Crushed oat meal (1.0), $NaNO_3$ (0.1), KH_2PO_4 (0.1), $MgSO_4 \cdot 7H_2O$ (0.1), and agar (2.0) without adjustment of pH (approximately 6.5).

Jar Fermentation and Processing

All fermentations were carried out using K-2 medium consisting of (%): Sucrose (2.0), potatoes (10.0), Casamino Acids (1.0), KH_2PO_4 (0.5), and $MgSO_4 \cdot 7H_2O$ (0.25), without adjustment of pH. Five hundred-ml baffled flasks containing 100 ml of K-2 medium were inoculated with the culture of strain SANK 17182 grown on WSH agar medium. The inoculated flasks were cultured at 26°C for 7 days on a rotary shaker at 200 rpm. Two hundred-ml seed culture was used to inoculate 15 liters of sterilized K-2 medium. The jar was stirred at 100~300 rpm with aeration at 0.50 v/v/m at 26°C for 7 days when it was harvested. After filtration, leualacin associated with the mycelium was recovered by its successive extraction with 35 liters of acetone and 15 liters of 80% aqueous acetone for 1 hour each with stirring at room temperature. Combined extracts were concentrated under reduced pressure. The resulting water phase was adjusted to pH 6.5 and extracted twice with equivalent volumes of ethyl acetate. Broth filtrate was adjusted to pH 7.0 and also extracted with ethyl acetate. The resulting organic phase was separately concentrated to a syrupy residue under reduced pressure.

Analytical HPLC

The analytical HPLC system consisted of a C18 reversed phase column (4 μm, 8.0 × 100 mm) eluted with acetonitrile - H_2O (7 : 3) at 2 ml/minute. Detection was by UV absorbance at 210 nm. The retention time of leualacin was 5.6 minutes.

In Vitro Ca Antagonistic Effect

Strips of taenia of 6~7 cm length, dissected from the caecum of male guinia pigs (250~300 g), were suspended in 20 ml of organ bath and isometric tension was measured[3]. The bathing medium was Ca-free, K-depolarizing Tyrode solution maintained at 35°C and gassed with 95% O_2 and 5% CO_2. After an equilibration period of 1 hour, Ca concentration in the medium was increased cumulatively (0.3~3 mM), and changes in tension were observed. The strips of taenia were then washed with Ca-free, K-depolarizing Tyrode solution for 30 minutes, followed by incubation with various concentration of leualacin or diltiazem for another 30 minutes. Ca-induced contraction were obtained in the presence of leualacin or diltiazem.

In Vivo Antihypertensive Effect

Male spontaneously hypertensive rats (SHR) at 15 weeks of age were anesthetized with pentobarbital (50 mg/kg body weight intraperitoneally). Catheters were inserted into the left femoral artery and vein for blood pressure measurement and drug injection, respectively. Blood pressure and heart rate were measured by connecting the artery catheter to a pressure transducer. Leualacin, dissolved in a saline solution containing 50% ethanol, was injected *via* the venous catheter and changes in blood pressure and heart rate were observed.

Results

Identification of Producing Fungus

The fungus SANK 17182, which produces leualacin, was freshly isolated from a soil sample collected

in Nepal. The mycological characteristics of this fungus are as follows. Colonies on WSH agar medium at 25°C growing restrictedly, 15 mm in 7 days, 35 mm in 14 days. The surface of the colonies are white (1-1-A)[4] to yellowish white (1-2-A) in color, thin, plane, with vegetative mycelium submerged, producing ascocarps as a black dot at the center of the colonies. Scanty growth was observed at 37°C.

Ascocarps are superficial to immersed in the medium, black, nonostiolate, glabrous, globose to subglobose, and 250~400 μm in diameter. Peridium of the ascocarps are more or less membranaceous, and dark green by transmitted light. Asci are 8-spored, evanescent, subglobose to ovoid, nonstipitate, and 8~15 μm in diameter. Ascospores are globose, olive-green to brown by transmitted light, dark green to black in mass, reticulate with low ridges, without germ pores or germ slits, and 1.0~6.0 μm in diameter including ridges.

Anamorph state is *Acremonium*; conidiophores are macronematous to micronematous, hyaline, short, unbranched, or rarely branched, septate, smooth-walled, tapering from the base to the apex, ending as a phialide. Phialides are slender, hyaline, 20~40 × 1.5~3.0 μm, and smooth-walled. Conidia are hyaline by transmitted light, pale pink to pale orange in mass, ovoid to ellipsoid, 3.5~10.0 × 2.0~3.5 μm, and collecting in wet masses at the tips of phialides.

From the mycological characteristics described above, the fungus was identified as *Hapsidospora irregularis* MALLOCH and CAIN[5], and has been deposited to Fermentation Research Institute, Agency of Industrial Science and Technology, Tsukuba, Japan, with the accession number of FERM BP-2511.

This species was originally isolated from a lawn grass compost heap in Canada[5] (ATCC 22087), and then from woodchuck dung in Canada (ATCC 22331), and from zebra dung in Tanzania

Fig. 2. *Hapsidospora irregularis* SANK 17182.

A) Asci (scale bar: 10 μm), B) scanning electron microscopic photograph of ascospores (scale bar: 3 μm), C) conidiophores and conidia (scale bar: 10 μm).

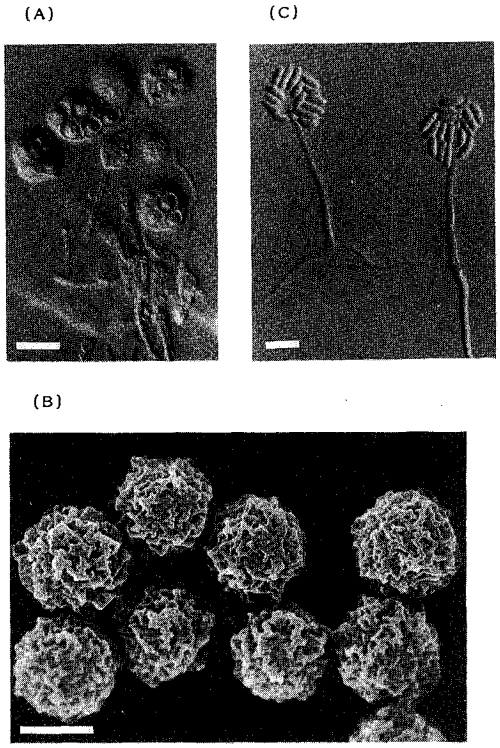
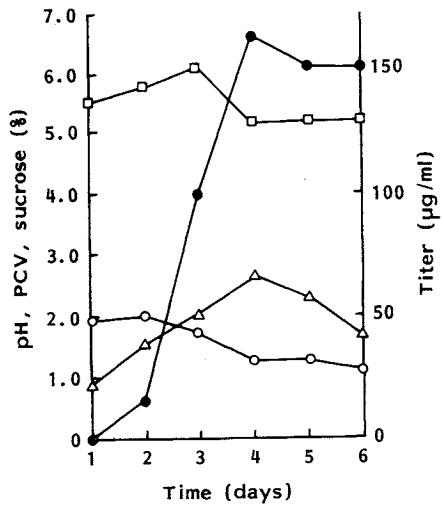


Fig. 3. Time course of leualacin production.

● Leualacin, ○ sucrose, △ PCV, □ pH.



For quantification of leualacin, whole broth containing mycerium was exracted with twice volume of acetone and the resulting supernatant was directly applied to analytical HPLC.

(ATCC 58423). Recently the species was reported by UDAGAWA[6] from soil collected in Nepal (80-NE-11). Of these strains tested for the production of leualacin, only one strain, ATCC 22331, produced the compound, but the amount produced was approximately 5% of that produced by SANK 17182 at most, and the result was not necessarily reproducible.

Fermentation

Fig. 3 shows a typical time course of the fermentation of strain SANK 17182 in 30-liter jar. A maximum titer of 160 μg/ml of leualacin was achieved after 96 hours. Approximately 80% of the compound was found to be associated with the mycelium.

Isolation

The chromatographic purification of leualacin is outlined in the flow diagram shown in Fig. 4. Principally, about 5 g of the crude extract was chromatographed on silica gel (Lobar column, Art. No. 10401 Lichroprep Si 60, size C, Merck) equilibrated in *n*-hexane - ethyl acetate (4 : 6) using the same solvent as the eluent. After fractionation according to the refractive index, the main fractions containing leualacin were concentrated and chromatographed in an approximately 180 mg portion with reverse phase HPLC (Senshu Pak ODS H-5251, 20 × 250 mm) using acetonitrile - H_2O (6 : 4) as the eluate at 7.0 ml/minute. The peak with retention time 28 minutes was collected, evaporated, and recrystallized with a mixture of acetone and *n*-hexane to yield 975 mg crystal.

Characterization

Leualacin appears as white needle crystal and its selected physico-chemical properties are summarized in Table 1. The compound is soluble in alcohols, acetone, ethyl acetate, acetonitrile, dimethyl sulfoxide,

Fig. 4. Isolation procedure for leualacin.

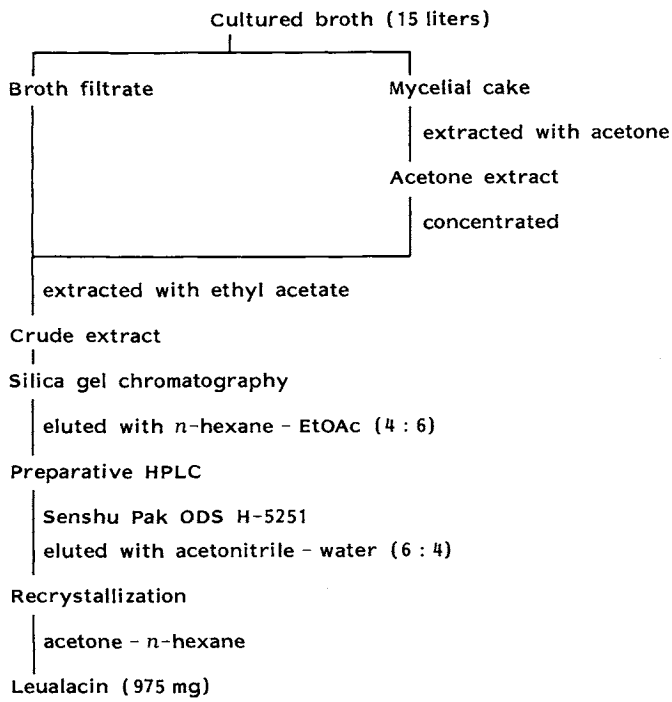


Table 1. Physico-chemical properties of leualacin.

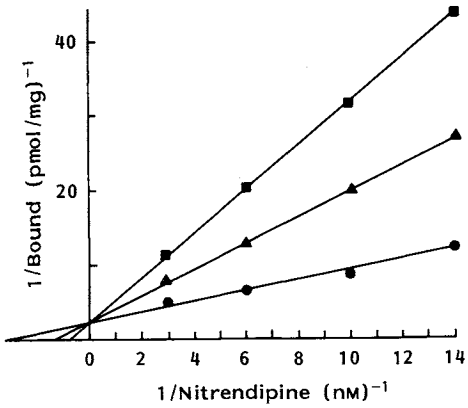
Appearance	White needles
MP	139~141°C
$[\alpha]_D^{25}$	−102° (*c* 1.14, MeOH)
Molecular formula	$C_{31}H_{47}N_3O_7$
Elemental analysis	
Calcd for $C_{31}H_{47}N_3O_7$:	
	C 64.90, H 8.26, N 7.32
Found:	C 64.95, H 8.33, N 7.21
EI-MS (*m/z*)	
Calcd for $C_{31}H_{47}N_3O_7$:	
	573.34143
Found:	573.33914
UV (MeOH)	End absorption
IR KBr (cm^{-1})	3326, 2961, 1741, 1681, 1638, 1512, 1255
Amino acid analysis	Leucine, *N*-methylphenylalanine, *β*-alanine

Table 2. Ca antagonizing effect in taenia caecum.

Concentration (g/ml)	Antagonizing rate (%)	
	Diltiazem	Leualacin
10^{-9}	—	0
10^{-8}	25	22
10^{-7}	98	73
10^{-6}	—	97

Fig. 5. Lineweaver-Burk plots of the inhibition of specific ^{3}H-nitrendipine binding by leualacin.

Specific nitrendipine binding was determined by incubating porcine heart membranes with increasing concentrations of ^{3}H-nitrendipine as described in the text in the absence (●) or presence of leualacin (1.75 μM, ▲ and 3.5 μM, ■).



and *N*,*N*-dimethylformamide, partially soluble in *n*-hexane and diethyl ether, but is almost insoluble in water. After TLC, the compound cannot be detected by UV nor does it give a positive reaction with ninhydrin. It does not char with sulfuric acid, and does not stain with iodine.

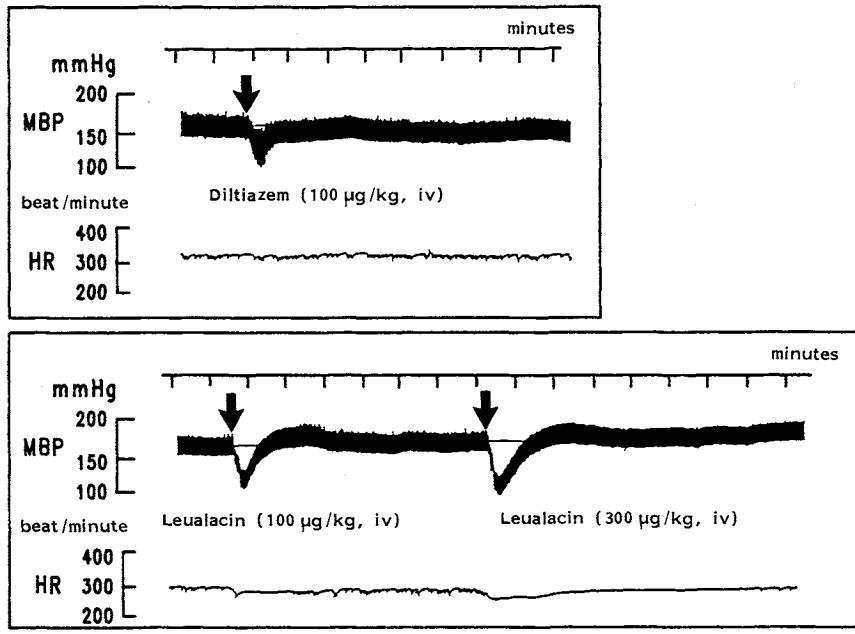
Biological Properties

Leualacin inhibited the specific binding of ^{3}H-nitrendipine to porcine heart microsomes. The IC_{50} value was 1.0 μg/ml (1.75 μM) in a standard assay condition. The mode of inhibition was competitive with respect to nitrendipine, as shown in Lineweaver-Burk Plots (Fig. 5). Almost all of the linear peptides derived from leualacin[7] showed negligible activity in ^{3}H-nitrendipine binding assay. Leualacin and diltiazem inhibited the contraction of taenia induced by 1 mM Ca (Table 2). The potencies of the two compounds were very close. As shown in Fig. 6, leualacin caused transient hypotension in SHR. The fall in mean blood pressure by leualacin was 44 and 57 mmHg for 100 and 300 μg/kg, respectively. Heart rate did not change markedly. Thus far in testing, no antimicrobial activity has been detected at the concentration of 1 mg/ml. No acute toxicity was observed up to 200 mg/kg po or 100 mg/kg ip in mice.

Discussion

Leualacin is a potent Ca blocker produced by the fungus *Hapsidospora irregularis* SANK 17182. Other deposited strains of *Hapsidospora irregularis* were not found to produce significant amount of leualacin. It specifically acts on the dihydropyridine-sensitive Ca channel, and has no activity against the N-type Ca channel (ODA *et al.*, unpublished observation). It inhibits the binding of ^{3}H-nitrendipine to porcine heart microsomes competitively with respect to nitrendipine, strongly suggesting that the binding site of leualacin is α1 subunit of the dihidropyridine-sensitive Ca channel[8], although direct binding has

Fig. 6. Effects of leualacin on blood pressure and heart rate in spontaneously hypertensive rats.



not yet been tested. Identification of the site of interaction, as carried out in case of nitrendipine and azidopine[9], will be informative for the future development of a new type of Ca blocker. A cyclic structure is an absolute requirement for the activity of leualacin. The potency of leualacin is of the same magnitude as diltiazem in systems such as inhibition of the contraction of taenia *ex vivo* (Table 2) or the fall in blood pressure in SHR by iv injection (Fig. 6).

Several plant metabolites have been reported to possess Ca antagonistic activity. They are trachelogenin, pachouli alcohol, capillarisin, osthool, and couromanocoumarin[10~12]. Some polypeptides, neurotoxins purified from snake venom, have also been reported to block cardiac Ca channels in a voltage-dependent manner[13,14]. Among the natural cyclic peptides, scytonemin A from a blue-green alga[15], was reported to be a Ca antagonist although no data is available as to the inhibition of ^{3}H-nitrendipine binding to microsomes by this compound. According to the published data, its potency is approximately half that of diltiazem on atria and only 2.5% that of diltiazem on rat portal vein Ca blocking. Scytonemin A also showed weak activity against a wide spectrum of bacteria and fungi, with an MIC in the range of 1 μg/ml, while leualacin showed no antimicrobial activity, even at a concentration of 1 mg/ml. These data suggest that the two peptides interact with Ca channel in different manners. More details of the structure-activity relationship of leualacin are now under investigation.

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