VINIGROL, A NOVEL ANTIHYPERTENSIVE AND PLATELET AGGREGATION INHIBITORY AGENT PRODUCED BY A FUNGUS, *VIRGARIA NIGRA*

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

TAKESHI ANDO, YASUHISA TSURUMI, NOBUTAKA OHATA, ITSUO UCHIDA, KEIZO YOSHIDA and MASAKUNI OKUHARA

Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3 Tokodai, Toyosato-machi, Tsukuba-gun, Ibaraki 300-26, Japan

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Vinigrol, produced by a fungal strain identified as *Virgaria nigra*, was extracted from the cultured mycelium, purified by solvent extraction followed by chromatography on silica gel and then isolated as crystals ($C_{20}H_{34}O_3$, mp 108°C). Vinigrol decreased arterial blood pressure of anesthetized normotensive rats dose-dependently when administered intravenously. Vinigrol inhibited platelet activating factor and epinephrine induced platelet aggregation.

In the course of our search for antihypertensive compounds of microbial origin, we reported the discovery of a very potent vasodilator, WS-1228 B[1] and a novel calcium channel blocker, amauromine[2]. Recently, the pharmacological characteristics of amauromine were studied in detail and it was established that it is a novel type of calcium channel blocker[3]. More recently we discovered the antihypertensive compound, vinigrol by testing culture broths using anesthetized normotensive rats. In this paper, we describe the taxonomy of the producing strain, the fermentation, the isolation and the physico-chemical and biological properties of vinigrol. Detailed studies of the biological and pharmacological characteristics of vinigrol will be discussed in a succeeding paper[4].

Materials and Methods

Fermentation

The growth of *Virgaria nigra* F-5408 on a mature slant culture was used to inoculate five 500-ml flasks each containing 100 ml of sterile seed medium composed of corn starch 1%, soluble starch 1%, glucose 1%, corn steep liquor 0.5%, dried yeast 0.5%, cotton seed flour 0.5% and $CaCO_3$ 0.2%. The medium was adjusted to pH 6.0 prior to the addition of $CaCO_3$. The flasks were shaken on a rotary shaker (220 rpm, 5.1-cm throw) for 3 days at 25°C. The contents of the flasks were used to inoculate 20 liters of fermentation medium in a 30-liter jar fermentor. The composition of the production medium was as follows: Glucose 3%, corn steep liquor 1%, cotton seed flour 0.5%, gluten meal 0.5%, soy bean meal 0.5% and dried yeast 0.5%. pH was adjusted to 6.5 and then $CaCO_3$ 0.2% was mixed before sterilization. Fermentation was allowed to proceed for 4 days at 25°C.

Blood Pressure and Heart Rate Measurement

Male Sprague-Dawley rats (350~400 g) were anesthetized with urethane (1 g/kg, ip). Arterial blood pressure was measured with a pressure transducer *via* a polyethylene catheter (PE50) that was inserted into the femoral artery. Blood pressure was recorded on a biophysiograph system 180 (Nihondenki-Sanei). The pulse pressure signal was used to trigger a tachometer for a measurement of heart rate. Active compound present in a fermentation broth or in a preparation therefrom was

detected by its hypotensive effect. To confirm the antihypertensive activity, test sample was injected intravenously through a polyethylene catheter inserted into a femoral vein.

Platelet Aggregation Test

Platelet aggregation was measured turbidimetrically with a platelet aggregation tracer (Niko Bioscience, Inc.) according to the method reported previously[5]. Briefly, rabbit blood was collected through a polyethylene catheter from the carotid artery of a male white Japanese rabbit into a plastic tube that contained one volume of 3.8% sodium citrate to nine volume of blood. After obtaining platelet rich plasma by centrifugation, the platelet number was adjusted to 5×10^5 cells/mm^3 with platelet poor plasma. Human blood was collected from the antecubital vein of healthy volunteers and platelet number was adjusted to 3×10^5 cells/mm^3. Platelet aggregation agents employed in this experiment were, epinephrine 0.4 mM plus ADP 0.4 μM, platelet activating factor (PAF) 20 nM, ADP 2.5 μM, bovine thrombin 0.5 U/ml and collagen (bovine achilles tendon) 10 μg/ml for rabbit platelet aggregation and epinephrine 5 μM, PAF 1.5 μM and ADP 2.5 μM for human platelet test, respectively.

Results

Identification of Strain F-5408

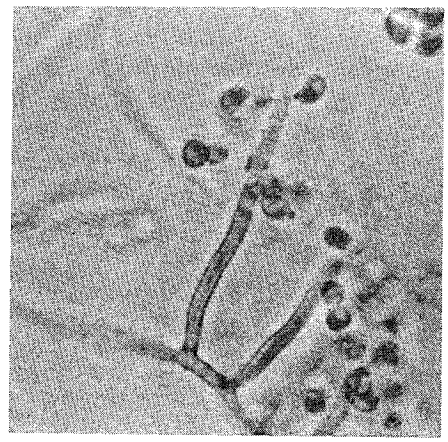
Strain F-5408 was freshly isolated from a soil sample collected at the foot of Mt. Aso, Kumamoto Prefecture, Japan.

On potato dextrose agar, this strain formed slow-growing and dark olive gray colonies, attaining 1.5 cm in diameter after 2 weeks at 25°C. This colony surface was raised, felty, thick and tough. The reverse of the colony was pale yellow brown. Conidial structures were abundantly produced on the surface. Its conidiogenesis was holoblastic and conidiophores developed sympodially (Fig. 1).

The conidiophores were erect, straight or flexuose, smooth, pale brown, 55~100 μm long and 2~3.5 μm thick. They were simple or branched under septum, and the terminals of each branches formed conidia-producing denticulate portions, measuring 10~15 μm long. The conidia were solitary, dry, aseptate, reniform, smooth, brown and 4~5×3~3.5 μm in size.

On the basis of the morphological observations, strain F-5408 was assigned to the hyphomycete genus *Virgaria* Nees, described as monotypic. The above mentioned characteristics corresponded with descriptions of the type species, *V. nigra* (Link) Nees et S. F. Gray by ELLIS[6] and MATSUSHIMA[7].

Fig. 1. Photograph of conidiophores and conidia of strain F-5408.



Isolation of Vinigrol

Fermentation broth (140 liters) was filtered with the aid of diatomaceous earth (Radiolite) and the mycelium was collected and then extracted with acetone (40 liters) by intermittent mixing. The acetone extract was concentrated *in vacuo* to aqueous solution (1.5 liters). After adjusting to pH 7.0, active component was extracted with ethyl acetate (3 liters). The extract was concentrated *in vacuo* to give an oily matter which was mixed with silica gel (250 g) and applied to a silica gel column chromatography

(1 liter). The column was washed with *n*-hexane and active compound was eluted with a mixture of *n*-hexane and ethyl acetate (1 : 2). Active fractions were combined and the organic solvent was removed by evaporation for further purification by repeated column chromatography on Lichroprep Si60 size B (E. Merck) with a mixture of chloroform and methanol (30 : 1) or *n*-hexane and ethyl acetate (1 : 1) respectively. Vinigrol was crystallized in a mixture of heptane and ethyl acetate. The yield was 445 mg.

Physico-chemical Properties of Vinigrol

Vinigrol is soluble in chloroform, ethyl acetate, methanol and insoluble in water. Color reactions of vinigrol are as follows; positive to cerium sulfate, phosphomolybdic acid reagents, and negative to ninhydrin, Dragendorff, ferric chloride and Molisch reactions. The IR ($CHCl_3$) and ^1H NMR (400 MHz, $CDCl_3$) spectra are shown in Figs. 2 and 3, respectively. The other physico-chemical properties of vinigrol are summarized in Table 1. The molecular formula, $C_{20}H_{34}O_3$, which was established from the elemental analysis and field desorption (FD) mass spectrum, indicated that vinigrol belong to a class of diterpenoid. The details of the chemical structure (Fig. 4) of this novel diterpene will be reported elsewhere in due course.

Effect on Arterial Blood Pressure and Heart Rate in Rats

The effect of vinigrol on arterial blood pressure was evaluated intravenously in urethane (1 g/kg, ip) anesthetized normotensive SD rats. The result is summarized in Table 2. Vinigrol reduced the systolic arterial blood pressure at 10~200 μg/kg and also decreased mean arterial blood pressure at 50~200 μg/kg in a dose-dependent manner.

The effect of vinigrol (100 μg/kg, iv) on mean arterial blood pressure and heart rate were estimated in a similar experiment and the result is shown in Table 3. Vinigrol reduced both mean arterial blood pressure and heart rate to similar degrees.

Fig. 2. IR spectrum of vinigrol ($CHCl_3$).

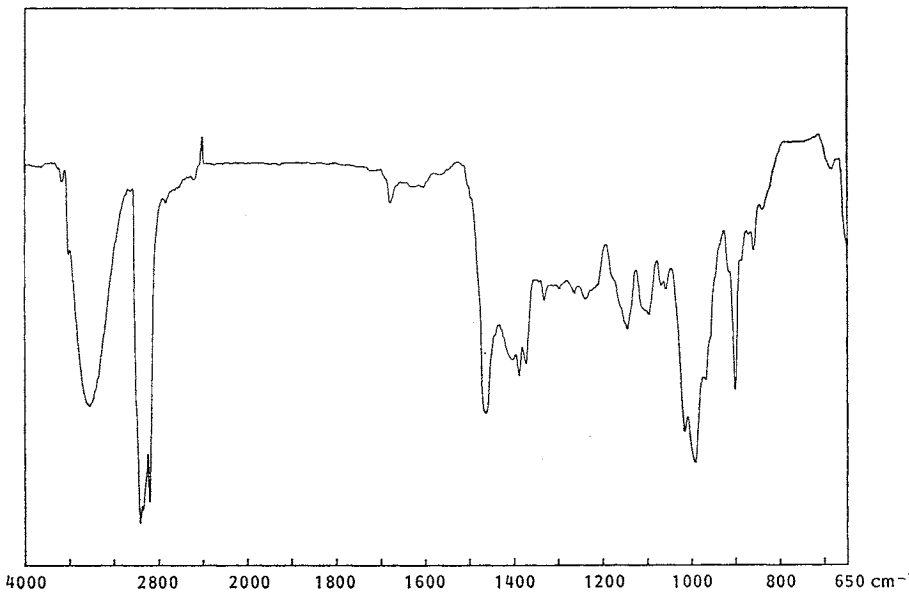


Fig. 3. ^1H NMR spectrum of vinigrol (400 MHz, $CDCl_3$).

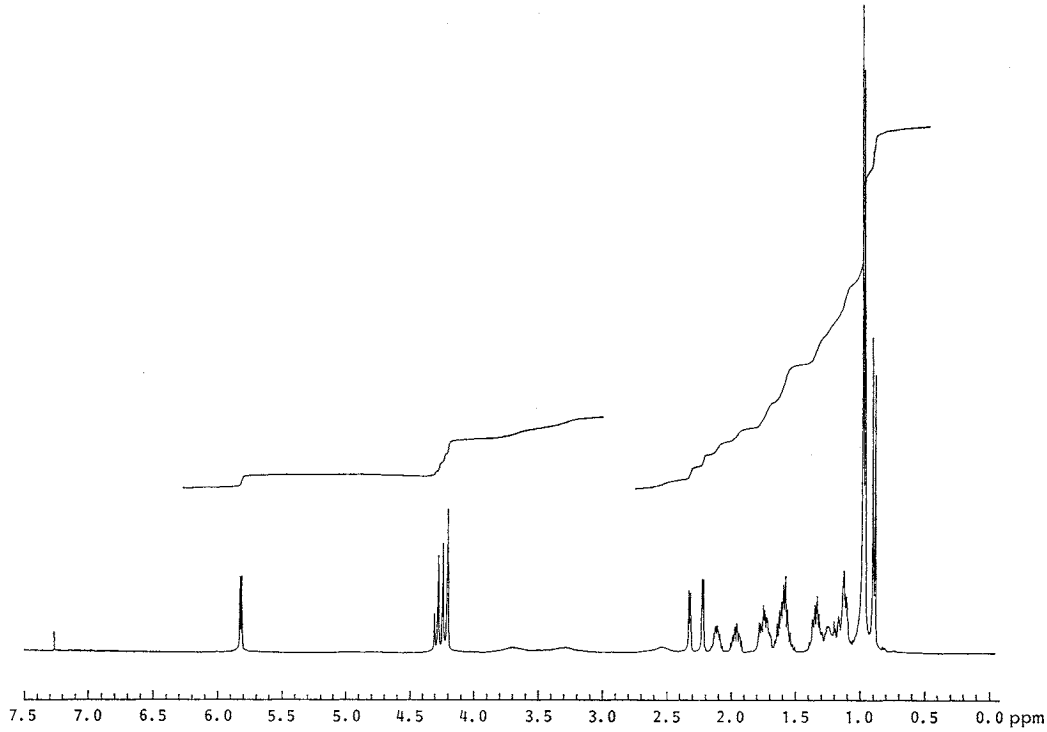
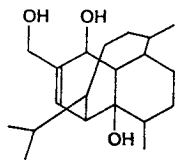


Table 1. Physico-chemical properties of vinigrol.

Appearance	Colorless prism
MP (°C)	108
$[\alpha]_D^{23}$ (*c* 1.05, $CHCl_3$)	−96.2°
UV (MeOH) nm (ε)	206 (13,000)
IR ($CHCl_3$) cm^{-1}	3450, 2960, 2940, 2870, 1670, 1460, 1380, 1370, 1140, 1100, 1010, 990, 970 (sh), 900
Molecular formula	$C_{20}H_{34}O_3$
Anal Found	C 74.20, H 10.23.
Calcd for $C_{20}H_{34}O_3$	C 74.49, H 10.63.
FD-MS (*m/z*)	323 $(M+H)^+$

Fig. 4. Structure of vinigrol.



Effect on Rabbit and Human Platelet Aggregation

The effect of vinigrol on rabbit and human platelet aggregation was estimated turbidimetrically. The IC_{50} values of vinigrol for several aggregation agents are summarized in Table 4. Rabbit platelet was not aggregated with epinephrine alone, but was induced to aggregate with a combination of epinephrine and low concentration of ADP[8].

Vinigrol inhibited the platelet aggregation induced with epinephrine or PAF. The IC_{50} values were 1.7×10^{-8} M and 4.4×10^{-7} M on rabbit platelet and 5.2×10^{-8} M and 3.3×10^{-8} M on human platelet for each aggregation agent, respectively. Vinigrol did not show any inhibitory activity on ADP, thrombin and collagen induced rabbit platelet aggregation and neither revealed any inhibitory

Table 2. Effect of vinigrol on systolic arterial blood pressure and mean arterial blood pressure in anesthetized SD rat.

Dose (μg/kg, iv)	Max ΔSBP		Max ΔMBP		Time to peak (minutes)	Duration (minutes)
	(mmHg)	Change (%)	(mmHg)	Change (%)		
10	-12 ± 0.63†	−8	NT	NT	5	4
25	-17 ± 0.0	−11	NT	NT	5	12
50	-18 ± 1.0	−12	-8.5 ± 0.87	−8	5	16
100	-29 ± 2.2	−26	-18.5 ± 1.7	−23	5	60
200	-27 ± 3.3	−26	-18.0 ± 0.88	−24	30	70

† Mean±SE. $n=4$.
NT: Not tested.

Table 3. Effect of vinigrol on mean arterial blood pressure and heart rate in anesthetized SD rat.

		Before	Time after administration (minutes)		
			2	10	30
Vehicle	MBP (mmHg)	87±5.3†	91±4.3	89±5.5	85±5.6
	Change (%)	0	5.2±2.4	2.4±3.3	−2.0±2.0
	HR (beats/minute)	360±21.7	361±17.9	361±19.2	352±18.3
	Change (%)	0	1.8±2.0	0.6±2.1	−2.0±1.4
Vinigrol	MBP (mmHg)	90±5.9	72±4.3[a]**	73±4.8**	77±6.3[c]
(100 μg/kg, iv)	Change (%)	0	−20.9±3.7[b]***	−20.1±3.9***	−15.5±4.4*
	HR (beats/minute)	358±16.0	280±12.1[a]***	301±13.5*	321±16.9[c]
	Change (%)	0	−21.2±3.5[b]***	−15.7±3.8**	−9.3±4.7[c]

† Mean±SE. $n=7$.
Students t-test; * $P<0.05$, ** $P<0.01$, *** $P<0.001$. [a] Against before (paired), [b] against vehicle (unpaired).
Vehicle; 10% PEG400 in 0.5% methyl cellulose solution (1 ml/kg, iv).
[c] Not significant.

effect on ADP induced human platelet aggregation at 1×10^{-6} M.

Vinigrol evoked aggregation of both rabbit and human platelet at higher concentrations. The minimum tolerated concentration for rabbit and human platelet was 6.2×10^{-6} M and 3.1×10^{-6} M, respectively.

Table 4. Effect of vinigrol on rabbit and human platelet aggregation.

Agonist	Rabbit PRP IC_{50} (M)	Human PRP IC_{50} (M)
Epinephrine	1.7×10^{-8}	5.2×10^{-8}
PAF	4.4×10^{-7}	3.3×10^{-8}
ADP	$>1\times10^{-6}$	$>1.0\times10^{-6}$
Thrombin	$>1\times10^{-6}$	NT
Collagen	$>1\times10^{-6}$	NT

$n=3\sim4$. NT: Not tested.
Agonist concentration: Rabbit; epinephrine 0.4 mM+ADP 0.4 μM, PAF 20 nM, ADP 2.5 μM, thrombin 0.5 U/ml, collagen 10 μg/ml. Human; epinephrine 5 μM, PAF 1.5 μM, ADP 2.5 μM.

Other Biological Properties

Antimicrobial activity of vinigrol was evaluated by the paper-disk diffusion assay. Vinigrol revealed a slight antimicrobial activity against *Bacillus subtilis* and *Staphylococcus aureus* at 10 mg/ml (growth inhibitory diameter was 12 mm), but was not effective against *Escherichia coli* and *Candida albicans* at the same concentration. Vinigrol showed cytotoxic activity on P388 cultured cells *in vitro* at $10\sim50$ μg/ml. LD_{50} of vinigrol was 23 mg/kg (ip) when tested in *dd*Y mice.

Discussion

Enzyme inhibitors, such as fusaric acid and oudenone which inhibited biosynthesis of catecholamines, have been identified as potent antihypertensive agents of microbial origin[9].

We published the discovery of several hypotensive vasodilators of microbial origin using a superfusion method of vascular smooth muscle preparation as a screening method[2,3].

Vinigrol was isolated as a very potent antihypertensive agent through our screening program by measuring rat arterial blood pressure directly. Vinigrol inhibited the platelet aggregation induced by epinephrine and PAF specifically at low concentration and provoked the platelet aggregation at higher concentration.

More detailed biochemical and pharmacological characterization will be described and discussed in the succeeding paper[4] to clarify the biological profile of this unique compound.

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