PHENAZOVIRIDIN, A NOVEL FREE RADICAL SCAVENGER FROM *Streptomyces* sp.

TAXONOMY, FERMENTATION, ISOLATION, STRUCTURE ELUCIDATION AND BIOLOGICAL PROPERTIES

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Phenazoviridin is a newly discovered free radical scavenger from microorganisms. It was isolated from the culture of *Streptomyces* sp. HR04. The structure of phenazoviridin was determined as 6-(3-methyl-2-butenyl)phenazine-1-carboxylic acid 6-deoxy-α-L-talopyranose ester on the basis of its spectroscopic and physico-chemical properties. The novel substance showed strong inhibitory activity against lipid peroxidation in rat brain homogenate and exhibited antihypoxic activity in mice.

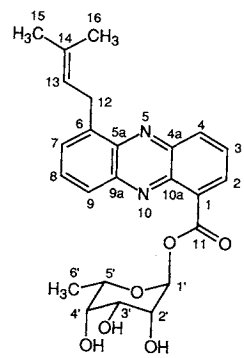
Generation of free radicals has recently been suggested to play a major role in the progression of a wide range of pathological disturbances including myocardial and cerebral ischemia[1,2], atherosclerosis[3], inflammation[4], renal failure[5] and rheumatoid arthritis[6]. Especially subsequent peroxidative disintegration of cells and organellar membranes has been implicated in various pathological processes[7]. In the course of our screening program for free radical scavenging substances from microorganisms, which are expected to be useful as therapeutic agents for these diseases, we isolated carazostatin[8], neocarazostatins[9], resorstatin[10] and pyrrolostatin[11], as reported previously. Further screening has resulted in the isolation of a novel substance, phenazoviridin (**1**), from the mycelium of *Streptomyces* sp. HR04 (Fig. 1). **1** has shown strong inhibitory activity against lipid peroxidation in rat brain homogenate. In this paper, we describe the taxonomy of the producing strain, as well as fermentation, isolation, physico-chemical properties, structure elucidation and biological properties of this compound.

Materials and Methods

Taxonomic Studies

The producing organism, strain HR04, was isolated from a soil sample collected in Kii peninsula, Japan. The media and procedures used for cultural and physiological characterization of strain HR04 were described by SHIRLING and GOTTLIEB[12]. Each culture was incubated at 27°C for 2 to 3 weeks before observation. The color names used in these studies were based on the Color Standard of Nippon Shikisai Co., Ltd.. Chemical composition of the cells was determined using the methods of LECHEVALIER and LECHEVALIER[13]. Detailed observation of mycelial and spore morphologies was performed

Fig. 1. Structure of phenazoviridin (**1**).



with the use of a light microscope and a scanning electron microscope (Hitachi S-800).

Fermentation

One loopful of a culture from an agar slant of *Streptomyces* sp. HR04 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of seed medium composed of glucose 2.5%, soybean meal 1.5%, yeast extract 0.2% and $CaCO_3$ 0.4% (pH 6.2 before autoclaving). The flask was shaken on a rotary shaker (200 rpm) for 3 days at 27°C. One ml of the seed culture was inoculated into 500-ml Erlenmeyer flasks each containing 100 ml of the seed medium. A total of 150 flasks were shaken on a rotary shaker (200 rpm) for 5 days at 27°C.

Isolation

Fifteen liters of cultured broth were filtered to yield the mycelium. The mycelial cake was extracted with 30 liters of acetone, and the extract was concentrated to 5 liters. The aqueous concentrate was extracted with 10 liters of EtOAc. The organic layer was dried with anhydrous Na_2SO_4 and then concentrated to give 1.47 g of oily material. This crude extract was dissolved in $CHCl_3$ and chromatographed on a silica gel column (Wako Pure Chemical Industries, Ltd., Wakogel C-200) using $CHCl_3$ - MeOH (80 : 1) as a mobile phase. Active fractions were pooled and evaporated to give 66.6 mg of a crude mixture, which was further chromatographed on a Sephadex LH-20 column developed with MeOH. After concentration of the active fractions, 52.8 mg of a crude powder was recovered and then chromatographed on a silica gel column using $CHCl_3$ - MeOH (30 : 1) as the mobile phase. Active fractions were concentrated, and 45.0 mg of pure **1** was finally obtained as a pale greenish powder.

Analytical Procedures

TLC: The content of **1** during purification was monitored by silica gel TLC (Kieselgel 60 F_{254}, E. Merck) developed with $CHCl_3$ - MeOH (10 : 1). Spots were detected by spraying with molybdatophosphoric acid.

Physico-chemical characteristics: IR and UV spectra were recorded with a Jasco A-3 spectrophotometer and a Hitachi U-3200 spectrophotometer, respectively. The ^1H and ^{13}C NMR spectra were measured with a Jeol JNM-GX500 spectrometer in CD_3OD solution. The MS was recorded with a VG Analytical AutoSpecQ mass spectrometer in FAB mode. Optical rotation was determined using a Jasco DIP-140 spectropolarimeter at 589.6 nm and a 10 cm cell.

In Vitro Inhibitory Activity against Lipid Peroxidation

Rat brain homogenate was prepared according to the method of KUBO *et al.*[14] with some modification. In brief, a male Wistar rat weighing about 300 g was sacrificed by decapitation. Whole brain except cerebellum was immediately homogenized with a teflon homogenizer for 30 seconds in 15 ml of an ice-cold 100 mM phosphate buffer (pH 7.4). The test mixture consisted of 0.5% (w/v) of rat brain homogenate, 100 μM of sodium ascorbate and 10 μM of $FeSO_4 \cdot 7H_2O$ as initiators for generation of oxygen radicals, and a sample of the test compound dissolved in MeOH. The mixture was incubated at 37°C for 1 hour with reciprocal agitation. Malondialdehyde (MDA) was stoichiometrically formed in the reaction mixture depending on the concentration of lipid peroxides. MDA thus formed was further allowed to react with thiobarbituric acid[15] for spectrophotometric quantification at 532 nm. Percent inhibition was calculated as follows; $[1-(T-B)/(C-B)] \times 100$ (%), in which T, C and B are A_{532} readings of the drug treatment, the control (peroxidation without any drug) and the time 0 control (no peroxidation), respectively.

In Vivo Antihypoxic Activity

Male ddY mice weighing about 30 g were treated intraperitoneally with a sample of the test compound suspended into 10% cremophor EL (Sigma Chemical Co.). Thirty minutes after treatment, 2.7 mg/kg of KCN in saline was injected intravenously. Immediately after injection of KCN, untreated mice started seizuring and died within 30 seconds under a transient hypoxic state. Protective activity against hypoxia was expressed in terms of percent survival at 1 minute after KCN injection.

Alkaline Hydrolysis of **1**

To a solution of **1** (8 mg) in EtOH (3 ml) was added 0.1 N NaOH (0.2 ml), and the reaction mixture was stirred for 10 minutes at room temperature. After the addition of H_2O (30 ml), the mixture was acidified by a few drops of 0.1 N HCl and extracted with 33 ml of EtOAc. The organic layer was dried with anhydrous Na_2SO_4 and then concentrated. The crude material was chromatographed with a silica gel column using $CHCl_3$-MeOH (100:1) as the mobile phase, and 5.2 mg (98% yield) of 6-(3-methyl-2-butenyl)phenazine-1-carboxylic acid (**2**) was purified as a pale greenish powder: MP 171~173°C (dec); FAB-MS (M^+, *m/z*) 292; molecular formula $C_{18}H_{16}N_2O_2$; UV λ_{max} nm (ε) in MeOH 254 (76,900), 354 sh (12,500), 370 (17,900); IR ν (KBr) cm^{-1} 1720, 1520, 1450, 740; ^1H and ^{13}C NMR, Table 5.

Acid Methanolysis of **1**

1 (40 mg) was dissolved in 4 ml of 5% HCl-MeOH and refluxed for 2 hours. The reaction mixture was concentrated to dryness *in vacuo*. The residue was chromatographed with a silica gel column using hexane-acetone (2:1) as the mobile phase, and 7.8 mg (48% yield) of methyl 6-deoxy-α-L-talopyranoside was purified as a colorless oil: $[\alpha]_D^{25}$ −103° (*c* 0.39, MeOH); FAB-MS ($(M+H)^+$, *m/z*) 179; molecular formula $C_7H_{14}O_5$; IR ν ($CHCl_3$) cm^{-1} 3350, 2900, 1630, 1100, 1060, 1010, 960; ^1H NMR ($CDCl_3$) δ 1.31 (3H, d, J=6.5 Hz), 3.37 (3H, s), 3.67 (1H, m), 3.76 (1H, m), 3.78 (1H, m), 3.88 (1H, m), 4.76 (1H, br s); ^{13}C NMR ($CDCl_3$) δ 16.5 (q), 55.0 (q), 65.9 (d), 66.5 (d), 70.4 (d), 72.8 (d), 101.6 (d).

Results

Taxonomy of Strain HR04

Mature spore chains were looped or straight, and comprised 10 or more spores per chain on aerial mycelia. The shape of the spores was cylindrical or ovoid as shown in Fig. 2 (1.2~1.7×0.7~0.9 μm in size), and their surface was smooth. Fragmentation of vegetative mycelium, sporagia, whirls, sclerotia or other special structures were not observed. The cultural characteristics are summarized in Table 1.

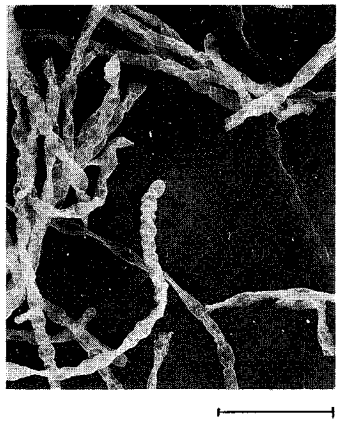


Fig. 2. Scanning electron micrograph of spore chains of strain HR04.

Bar represents 6 μm.

Table 1. Cultural characteristics of strain HR04.

Medium	Growth	Aerial mycelium	Reverse side color	Soluble pigment
Sucrose-nitrate agar	Poor	Poor, light olive gray	Light olive gray	None
Glucose-asparagine agar	Moderate	Moderate, yellowish white	Brown	None
Glycerol-asparagine agar (ISP No. 5)	Good	Good, yellowish white	Brown	Brown
Inorganic salts-starch agar (ISP No. 4)	Good	Good, light brownish gray	Dark reddish brown	Brown
Tyrosine agar (ISP No. 7)	Good	Moderate, greenish white	Dark reddish brown	Brown
Nutrient agar	Moderate	Poor, pale yellow	Pale yellow	None
Yeast extract-malt extract agar (ISP No. 2)	Moderate	Poor, dark yellow	Dark yellow	None
Oatmeal agar (ISP No. 3)	Good	Moderate, light olive gray	Brown	None

Table 2. Physiological characteristics of strain HR04.

Growth range (°C)	15~30	D-Xylose	±
Melanoid production on:		D-Glucose	+
Tyrosine agar	+	D-Fructose	+
Peptone-yeast extract-Fe agar	+	Sucrose	+
Tryptone-yeast extract medium	+	Inositol	±
Hydrolysis of starch	−	L-Rhamnose	±
Liquefaction of gelatin	−	Raffinose	+
Coagulation of milk	−	D-Mannitol	+
Peptonization of milk	+	D-Galactose	+
Reduction of nitrate	+	D-Sorbitol	±
Utilization of:[a]		D-Mannose	+
L-Arabinose	±	Maltose	+

+; Positive, ±; weakly positive, −; negative.
[a] Basal medium: PRIDHAM-GOTTLIEB's inorganic salts agar (ISP No. 9).

The mass color of the aerial mycelium was yellowish white to light brownish gray. The reverse side of the colony was brown to dark reddish brown. Melanoid pigment was observed in several media.

The physiological characteristics of strain HR04 are shown in Table 2. LL-Diaminopimelic acid was detected in whole cell hydrolysates of the culture. Based on these properties, strain HR04 was considered to belong to the genus *Streptomyces*. The cultural and physiological characteristics of this strain are similar to those of *S. cinnamonensis* according to ACTINOBASE, the database of actinomycetes (Japan Collection of Microorganisms, The Institute of Physical and Chemical Research), but a direct comparative study has not yet been performed. Further studies for species identification will be reported elsewhere. Strain HR04 has been deposited in the National Institute of Bioscience and Human-Technology (formerly the Fermentation Research Institute), Agency of Industrial Science and Technology, Tsukuba-shi, Japan, under the name of *Streptomyces* sp. HR04 and the accession number FERM BP-3904.

Fermentation

Since **1** was not produced in a jar fermentor under various culture conditions, the organism was fermented in flasks. The amount of **1** produced by *Streptomyces* sp. HR04 in a flask reached its maximum in about 5 days. **1** was present mainly in the mycelia.

Isolation

The isolation and purification procedure of the compound is summarized in Fig. 3. The mycelial cake was extracted with acetone followed by EtOAc, and the oily material obtained on evaporation of the organic extract was purified by column chromatography on silica gel and Sephadex LH-20. After concentration of the active fraction, pure **1** (45.0 mg) was obtained as a pale greenish powder.

Physico-chemical Properties

The physico-chemical properties of **1** are summarized in Table 3. It is soluble in lower alcohols, $CHCl_3$, acetone and EtOAc, but insoluble in H_2O and *n*-hexane. The Rf value of **1** on silica gel TLC developed with $CHCl_3$-MeOH (10:1) was 0.38. The molecular formula was determined to be $C_{24}H_{26}N_2O_6$ by HRFAB-MS. The UV and IR spectra suggested the presence of a phenazine chromophore[16] and an ester group (1740 cm^{-1} in the IR spectrum).

Structure Elucidation

The ^1H and ^{13}C NMR spectral data for **1** are shown in Table 4. ^1H NMR signals for six aromatic

Fig. 3. Isolation procedure of **1**.

Whole broth (15 liters)
- filtered

Mycelial cake
- extracted with acetone
- extracted with EtOAc
- concentrated *in vacuo*

Oily material (1.47 g)
- silica gel column chromatography
- eluted with $CHCl_3$ - MeOH (80 : 1)

Crude powder (66.6 mg)
- Sephadex LH-20 column chromatography
- developed with MeOH

Crude powder (52.8 mg)
- silica gel column chromatography
- eluted with $CHCl_3$ - MeOH (30 : 1)

Phenazoviridin (**1**, 45.0 mg)

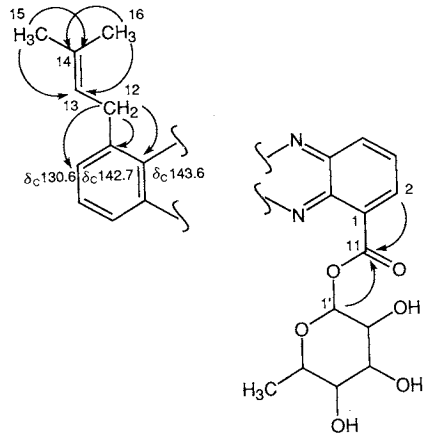
Table 3. Physico-chemical properties of **1**.

Appearance	Pale greenish powder
MP (°C)	71~73 (dec)
Molecular formula	$C_{24}H_{26}N_2O_6$
HRFAB-MS ($(M+H)^+$, m/z)	
Calcd:	439.1869
Found:	439.1876
UV λ_{max} nm (ε) in MeOH	253 (71,730), 348 (11,590), 366 (16,860)
IR (KBr) ν cm^{-1}	3400, 2980, 2920, 1740, 1530, 1420, 1270, 1120, 1020, 980, 940, 760
Rf [$CHCl_3$ - MeOH (10 : 1)]	0.38

Table 4. ^1H and ^{13}C chemical shifts of **1** in CD_3OD.

Position	^1H NMR δ_H (multiplicity, J=Hz)	^{13}C NMR δ_C (multiplicity)
1	—	132.9 (s)
2	8.22 (1H, dd, J=7.0, 1.4)	132.9 (d)
3	7.91 (1H, dd, J=8.6, 7.0)	130.7 (d)
4	8.30 (1H, dd, J=8.6, 1.4)	133.7 (d)
4a	—	143.2 (s)
5a	—	143.6 (s)
6	—	142.7 (s)
7	7.66 (1H, d, J=6.5)	130.6 (d)
8	7.81 (1H, dd, J=8.7, 6.5)	132.9 (d)
9	7.97 (1H, d, J=8.7)	127.9 (d)
9a	—	144.6 (s)
10a	—	140.9 (s)
11	—	166.7 (s)
12	4.07 (2H, d, J=7.5)	30.1 (t)
13	5.52 (1H, t, J=7.5)	123.4 (d)
14	—	134.7 (s)
15	1.81 (3H, s)	26.0 (q)
16	1.78 (3H, s)	18.1 (q)
1′	6.49 (1H, d, J=1.8)	97.6 (d)
2′	4.05 (1H, ddd, J=3.1, 1.8, 1.7)	71.2 (d)
3′	3.90 (1H, dd, J=3.1, 3.1)	67.1 (d)
4′	3.69 (1H, ddd, J=3.1, 1.7, 1.7)	74.0 (d)
5′	4.20 (1H, dq, J=6.5, 1.7)	71.4 (d)
6′	1.33 (3H, d, J=6.5)	17.0 (q)

Fig. 4. HMBC spectrum of **1**.



protons coupled with ^1H-^1H COSY spectral analysis indicated the presence of a 1,6- or 1,9-disubstituted phenazine ring in **1**. A connected coupling pattern from 1′-H to 6′-H revealed by the ^1H-^1H COSY experiment also showed the existence of a 6-deoxyhexopyranose moiety in **1**. The ^1H and ^{13}C NMR spectral analyses further indicated an 3-methyl-2-butenyl group and an ester carbonyl group as remaining partial structures in **1**.

The HMBC experiment revealed long range couplings of 2-H (δ_H 8.22) in the phenazine nucleus and 1′-H (δ_H 6.49) in the 6-deoxyhexopyranose moiety to the ester carbonyl carbon (C-11, δ_C 166.7) as shown in Fig. 4. These results indicated that the sugar unit was attached to C-1 of the phenazine ring through an

ester linkage. Since long range couplings were also observed from 12-H (δ_H 4.07) in the 3-methyl-2-butenyl group to phenazine carbons (δ_C 130.6, 142.7 and 143.6) as shown in Fig. 4, attachment of this group to either C-6 or C-9 of the phenazine nucleus was confirmed.

In order to determine the connected position of the 3-methyl-2-butenyl group to the phenazine nucleus, **1** was hydrolyzed in the presence of NaOH. The NMR spectral data of the hydrolysis product (**2**) isolated from the reaction mixture was consistent with the published data for 6-(3-methyl-2-butenyl)phenazine-1-carboxylic acid[16] as shown in Table 5. Thus, C-1 and C-6 in the phenazine nucleus of **1** were determined to be substituted by the ester carbonyl group and the 3-methyl-2-butenyl group, respectively.

The relative stereochemistry for the 6-deoxyhexopyranose moiety in **1** was determined by the NOE observations and coupling constants. The NOE observed between 3'-H and 5'-H indicated that they were axially oriented (Fig. 5). 2'-H and 4'-H must be in the equatorial orientation because of their small coupling constants ($J_{2'-3'}=J_{3'-4'}=3.1$ Hz and $J_{4'-5'}=1.7$ Hz) as shown in Fig. 5. Based on these results, the sugar moiety was identified as 6-deoxytalopyranose. Since the NOE was not observed either between 1'-H and 3'-H or between 1'-H and 5'-H, 1'-H must be equatorially oriented. Therefore, the anomeric configuration of **1** was established to be α.

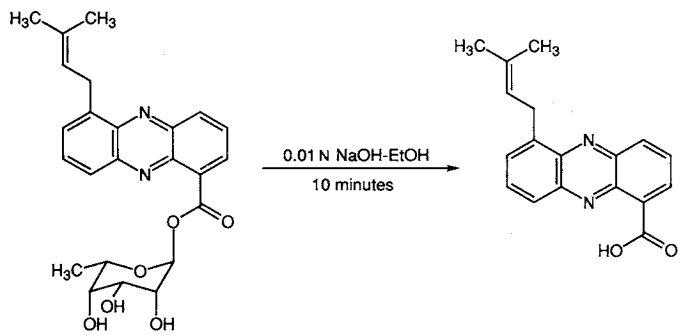
In an attempt to assign the absolute configuration, **1** was methanolyzed in HCl - MeOH to give methyl 6-deoxy-α-talopyranose. This compound was shown to possess the L configuration by comparison of the optical rotation, $[\alpha]_D=-103°$ (c 0.39, MeOH) measured in this study with that previously reported for methyl 6-deoxy-α-L-talopyranose ($[\alpha]_D=-117°$ (c 0.9))[17].

Table 5. The ^1H and ^{13}C NMR spectral data of **2** and 6-(3-methyl-2-butenyl)phenazine-1-carboxylic acid.

2
^1H NMR ($CDCl_3$) δ_H 1.81 (6H, s), 4.05 (2H, d, J=6.9), 5.47 (1H, t, J=6.9), 7.82 (1H, dd, J=7.0, 1.3), 7.90 (1H, dd, J=8.5, 7.0), 8.03 (1H, dd, J=8.5, 7.0), 8.19 (1H, d, J=8.5), 8.52 (1H, dd, J=8.5, 1.3), 8.97 (1H, dd, J=7.0, 1.3), 15.63 (1H, br s)
^{13}C NMR ($CDCl_3$) δ_C 18.0 (q), 25.8 (q), 29.8 (t), 120.3 (d), 124.9 (s), 128.0 (d), 130.1 (d), 131.6 (d), 131.8 (d), 134.9 (d), 134.9 (s), 135.4 (s), 137.1 (d), 139.1 (s), 139.1 (s), 143.0 (s), 144.5 (s), 166.1 (s)
6-(3-methyl-2-butenyl)phenazine-1-carboxylic acid[a]
^1H NMR ($CDCl_3$) δ_H 1.80 (6H, s), 4.02 (2H, d, J=7.3), 5.45 (1H, t, J=7.3), 7.82 (1H, dd, J=6.8, 1.1), 7.90 (1H, dd, J=7.3, 6.8), 8.03 (1H, dd, J=8.5, 7.3), 8.18 (1H, dd, J=7.3, 1.1), 8.52 (1H, dd, J=8.5, 1.5), 8.95 (1H, dd, J=7.3, 1.5), 15.56 (1H, br s)
^{13}C NMR ($CDCl_3$) δ_C 18.1 (q), 25.7 (q), 29.8 (t), 120.3 (d), 125.0 (s), 128.1 (d), 130.1 (d), 131.6 (d), 131.8 (d), 135.0 (d), 135.0 (s), 135.5 (s), 137.1 (d), 139.1 (s), 139.2 (s), 143.0 (s), 144.5 (s), 166.1 (s)

ppm (multiplicity, J=Hz).

[a] Cited from the data by TAX *et al.*[16].



Phenazoviridin (**1**) **2**

Fig. 5. The NOE (arrow) and coupling constants in the sugar moiety of **1**.

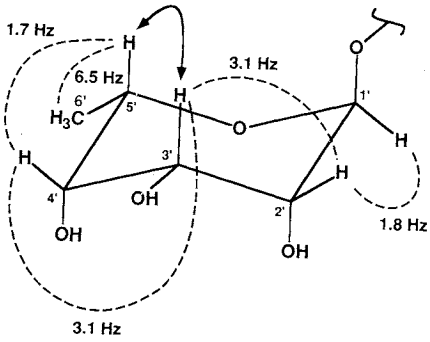


Fig. 6. Inhibitory activity of phenazoviridin on lipid peroxidation in rat brain homogenate.

● Phenazoviridin, ○ flunarizine 2HCl.

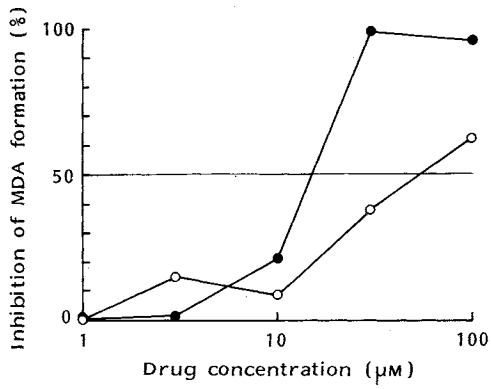


Table 6. Protective effect of phenazoviridin against KCN-induced hypoxia in mice.

Drug	Dose[a] (mg/kg)	N[b]	Survival	Survival rate (%)
Phenazoviridin	100	8	8	100
Indeloxazine HCl	50	16	15	94
Vehicle	—	13	1	8

[a] Each drug was administered intraperitoneally 30 minutes before injection of KCN (2.7 mg/kg, intravenously).
[b] The number of treated animals.

From these results, we determined the structure of **1** to be (6-deoxy-α-L-talopyranosyl) 6-(3-methyl-2-butenyl)phenazine-1-carboxylate as shown in Fig. 1.

Biological Activity

The inhibitory effect of **1** on lipid peroxidation in rat brain homogenate is shown in Fig. 6. The IC_{50} of **1** was 15.0 μM, indicating that the compound is more active than flunarizine (IC_{50}; 55.0 μM) which is a brain protective calcium antagonist with free radical scavenging activity[14], and α-tocopherol (IC_{50}; >100 μM) which is a well known antioxidant.

1 showed strong protective activity against KCN-induced acute hypoxia in mice when administered at 100 mg/kg intraperitoneally (Table 6). This activity was as strong as that of indeloxazine, a marketed brain protective agent.

1 has low acute toxicity; no death was observed after intraperitoneal injection to mice ($n=8$) with 100 mg/kg.

Discussion

In the course of our screening program for free radical scavenging substances from microorganisms, we isolated the compound named phenazoviridin from the culture of *Streptomyces* sp. HR04. Phenazoviridin has strong inhibitory activity against lipid peroxidation in rat brain homogenate. TAX *et al.* have reported the isolation and structure elucidation of phenazine-1-carboxylic acid and its derivatives as antimicrobial

agents from *Streptomyces cinnamonensis* ATCC 15413[16]. PATHIRANA *et al.* have also reported phenazine L-quinovose esters having antibacterial activity from marine *Streptomyces* sp.[18]. Phenazoviridin is structurally ralated to these compounds, for which, however, free radical scavenging activity has not been reported. Recently SHIN-YA *et al.* have isolated an unique compound named benthocyanin A from *Streptomyces prunicolor*[19]. The structure of this compound include a phenazine-like chromophore and the compound showed free radical scavenging activity. However, phenazoviridin is the first phenazine derivative with a sugar moiety that acts as a strong inhibitor of lipid peroxidation.

Production of large amounts of active oxygen radicals has been indicated in various pathological processes. Hydroxyl radicals (HO·) produced by FENTON's reaction[20] in the presence of Fe^{2+}, especially, are suggested to initiate lipid peroxidation and to lead to subsequent disintegration of the cell membranes. It has also been shown that lipid peroxidation of liver microsomes and mitochondria induced by ferrous ions and ascorbate decreases membrane fluidity[21]. Therefore, phenazoviridin is expected to be useful for the alleviation of tissue damages which are due to generation of free radicals and subsequent peroxidative disintegration of cell membranes.

In addition to its *in vitro* activity, phenazoviridin has shown a protective activity against acute hypoxia in mice induced by the injection of KCN. It has previously been reported that increased levels of lipid peroxides and reduced activities of antioxidative enzymes such as glutathione peroxidase were observed in brain due to the cyanide intoxication[22]. Thus, phenazoviridin may become one of the useful agents which are able to protect tissues such as brain from the peroxidative injury due to ischemia *in vivo*.

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