Formobactin, a Novel Free Radical Scavenging and Neuronal Cell Protecting Substance from *Nocardia* sp.

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(Received for publication March 18, 1996)

Formobactin, a new free radical scavenger, was isolated from the culture of *Nocardia* sp. strain ND20. The structure of formobactin was determined to be a member of the nocobactin group antibiotics. Formobactin inhibited lipid peroxidation in rat brain homogenate. Formobactin also showed the activity to suppress L-glutamate toxicity in neuronal hybridoma N18-RE-105 cells.

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 diseases[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], pyrrolostatin[9] and phenazoviridin[10], as reported previously. Further screening has resulted in the isolation of a novel substance, formobactin (**1**), from the mycelium of *Nocardia* sp. ND20 (Fig. 1). **1** inhibited lipid peroxidation induced by free radicals in rat brain homogenate.

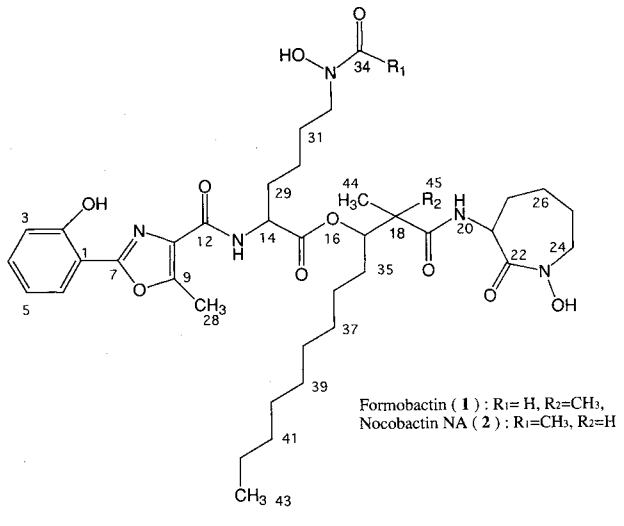
An excitatory amino acid, L-glutamate, which normally acts as a neurotransmitter in the major part of brain, induces neuronal cell death after brain-ischemic attack[11]. Recent studies have shown that oxygen radicals were produced through a variety of intracellular cascades in such events[12]. It was also reported that ischemic brain injury was prevented by some of free radical scavengers[13]. Therefore, we evaluated the inhibitory effect of **1** on the *in vitro* L-glutamate toxicity in neuronal hybridoma N18-RE-105 cells. **1** showed strong suppression of the cell death in this model. In this paper, we report taxonomy of the producing strain, fermentation, isolation, structural elucidation and these biological activities of **1**.

Materials and Methods

Taxonomic Studies

The producing organism, strain ND20, was isolated from a soil sample collected at Azumi in Nagano Prefecture, Japan. The media and procedures used for cultural and physiological characterization of strain ND20 were described by SHIRLING and GOTTLIEB[14]. 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[15]. Detailed observation of mycelial and spore morphologies was performed with the use of a light microscope and a scanning electron microscope (Hitachi S-800).

Fig. 1. Structure of formobactin (**1**) and nocobactin NA (**2**).



Spectral Analysis

Mass spectra were recorded with a JEOL SX-102A spectrometer in the FAB mode using α-thioglycerol as a matrix and polyethylene glycol as an internal standard. UV and IR spectra were obtained with a Hitachi U-3200 spectrophotometer and a JEOL Diamond 20 FT-IR spectrophotometer, respectively. ^{1}H and ^{13}C NMR spectra were measured with a JEOL JNM-A 500 spectrometer in DMSO-d_6 solution. Chemical shifts were given in ppm using TMS as an internal standard.

In Vitro Inhibitory Activity against Lipid Peroxidation

Rat brain homogenate was prepared according to the method of KUBO *et al.*[16] with some modification. In brief, a male Wistar rat weighing about 300 g was sacrificed by decapitation. The whole brain except cerebellum was immediately homogenized with a teflon homogenizer for 30 seconds in 15 ml of ice-cold 100 mM phosphate buffer (pH 7.4). The reaction mixture of the assay consisted of 0.5% (w/v) of the homogenate, 100 μM of sodium ascorbate as an initiator for generation of oxygen radicals and a sample dissolved in MeOH. If necessary, $FeSO_4$, which strongly promotes lipid peroxidation together with ascorbate, was added to the mixture. The mixture was incubated at 37°C for one hour under 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[17] 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 a drug) and the time 0 control (no peroxidation), respectively.

Cell Culture

N18-RE-105 hybrid cells[18] (mouse neuroblastoma clone N18TG-2 × Fischer rat 18-day embryonic neural retina) were grown in a DULBECCO's modified EAGLE's medium supplemented with 0.1 mM hypoxanthine, 0.04 mM aminopterin, 0.14 mM thymidine and 10% heat-inactivated fetal calf serum. The cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. Cells were plated at 6.25×10^3 cells/cm^2 for cytotoxicity studies. After culturing for 24 hours, the medium was removed and replaced with a medium containing 10 mM L-glutamate. Cytotoxicity was monitored after 24 hours by a phase-contrast microscope and quantified by the measurement of the cytosolic enzyme, lactate dehydrogenase (LDH), released into the culture medium from degenerated cells[19].

Results

Taxonomy of the Producing Strain

The aerial mycelium of the strain monopodially branched on the long main stem. The spore chains formed on the aerial mycelia contained 2 to 7 spores per chain. The spores were cylindrical ($0.8 \sim 1.3 \times 0.5 \sim 1.7$ μm in size) with smooth surface. The cultural characteristics of strain ND20 on various agar media are shown in Table 1. The color of the aerial mycelia was yellowish white to yellowish gray, while that of the reverse side of the colony was yellowish white to dull yellow orange. No water-soluble pigment was produced in any medium.

The physiological characteristics of strain ND20 are shown in Table 2. The whole-cell hydrolysate contained *meso*-diaminopimelic acid, arabinose and galactose. Analysis of whole-cell phospholipids revealed the presence of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol dimannoside but not phosphatidylcholine (phospholipid

Table 1. Cultural characteristics of strain ND20.

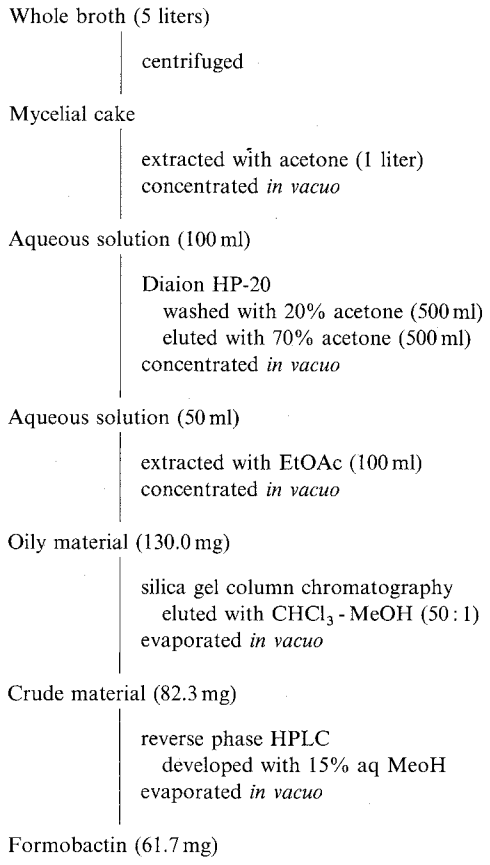
Medium	Growth	Aerial mycelium	Reverse side color	Soluble pigment
Sucrose - nitrate agar	Good	Good Yellowish white	Yellowish white	None
Glucose - asparagine agar	Good	Good Yellowish gray	Yellowish white	None
Glycerol - asparagine agar	Good	Good Dull yellow orange	Dull yellow orange	None
Inorganic salts - starch agar	Moderate	Poor Yellowish gray	Yellowish gray	None
Tyrosine agar	Good	Good Yellowish gray	Dull reddish orange	None
Nutrient agar	Moderate	Poor Pale orange	Pale orange	None
Yeast extract - malt extract agar	Good	Good Yellowish gray	Reddish yellow	None
Oatmeal agar	Moderate	Poor Greenish white	Greenish white	None

Table 2. Physiological characteristics of strain ND20.

Growth range (°C)	10~40
Melanoid production on:	
Tyrosine agar	+
Peptone-yeast extract-iron agar	−
Tryptone-yeast extract medium	−
Hydrolysis of starch	−
Liquefaction of gelatin	−
Coagulation of milk	−
Peptonization of milk	−
Reduction of nitrate	−
Utilization of:[a]	
L-Arabinose	±
D-Xylose	±
D-Glucose	+
D-Fructose	+
Sucrose	±
Inositol	±
L-Rhamnose	±
Raffinose	±
D-Mannitol	+
D-Galactose	+
D-Sorbitol	+
D-Mannose	+
Maltose	+
D-Ribose	+
Glycerol	+

+; Positive, ±; weakly positive, −; negative.
[a] Basal medium: Carbon utilization agar (ISP No. 9).

Fig. 2. Isolation scheme of **1**.



type PII according to the classification of LECHEVALIER *et al.*[20]). Mycolic acid methyl esters were detected on TLC developed with *n*-hexane-diethyl ether (4:1), having the same Rf as an authentic sample prepared from *Nocardia* sp. (ATCC 31319). The predominant isoprenoid quinone was tetrahydrogenated menaquinone with eight isoprene units (MK-8 (H_4)). These results indicate that strain ND20 belongs to the genus *Nocardia*. Further studies for species identification will be reported elsewhere. Strain ND20 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 *Nocardia* sp. ND20 and accession number FERM BP-4938.

Fermentation

Culture medium consisted of glycerol 1.5%, Pharma media 1.5%, sodium L-(+)-glutamate monohydrate 0.5% and NaCl 0.3%, pH of the medium was adjusted to 7.4 before autoclaving. A well grown agar slant of *Nocardia* sp. ND20 was used to inoculate into 500-ml Erlenmeyer flasks containing 100 ml of the medium. The flasks were shaken at 27°C for 5 days on a rotary shaker.

Isolation and Purification

Formobactin (**1**) was isolated according to the scheme as shown in Fig. 2. The fermentation broth (5 liters) was centrifuged to give a mycelial cake, which was extracted with acetone (1 liter). The extract was filtered and concentrated *in vacuo* to yield an aqueous solution (100 ml). The solution was applied to a Diaion HP-20 column (3 × 20 cm) which was washed with 20% acetone (500 ml). Then the active fractions were obtained by eluting with 70% acetone (500 ml) which was concentrated *in vacuo* to an aqueous solution (50 ml). The solution was extracted twice with EtOAc (100 ml). The organic layer was dehydrated over anhydrous Na_2SO_4 and then concentrated *in vacuo*. The residue (130 mg) was applied to a silica gel column (Silica gel 60, 2 × 15 cm) developed with $CHCl_3$-MeOH (50:1). The active fractions were further purified by preparative HPLC (CAPSELL PAK C18, i.d. 5 mm × 250 mm, 15% aq MeOH). The main peak was concentrated to give a white powder of **1** (61.7 mg).

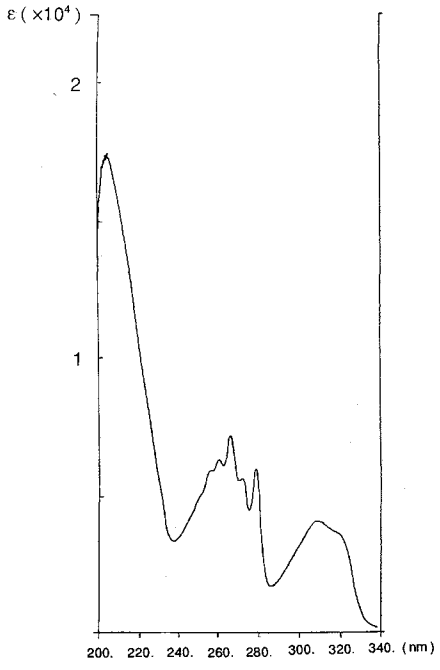
Physico-chemical Properties

The physico-chemical properties of **1** are summarized in Table 3. The molecular formula was established to be

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

Appearance	White powder
MP	68~72°C (dec.)
$[\alpha]_D^{25}$	−8.6° (*c* 1.0, MeOH)
Molecular formula	$C_{38}H_{57}N_5O_{10}$
HR FAB-MS *m/z* $(M+H)^+$	
Calcd:	744.4184
Found:	744.4191
UV λ_{max}^{nm} in MeOH (ε)	255 (5700), 260 (6200), 266 (7000), 272 (5600), 279 (5800), 308 (3800), 320 (3300)
IR (KBr) ν cm^{-1}	3400, 2929, 1743, 1666, 1660, 1650, 1641, 1633, 1513, 1205, 752

Fig. 3. UV spectrum of **1** in MeOH solution.



$C_{38}H_{57}N_5O_{10}$ by HRFAB-MS. The IR absorption spectrum indicated the existence of an ester carbonyl group (1743 cm^{-1}) and amide carbonyl groups (1666, 1660 and 1650 cm^{-1}). The characteristic UV spectrum (Fig. 3) showed that **1** belongs to the nocobactin group antibiotics, which were isolated as iron-chelating agents[21,22].

Structural Elucidation

The ^{13}C and ^1H NMR spectral data for **1** are summarized in Table 4. The ^1H NMR spectrum of **1** revealed the presence of amide signals (δ_H 7.48 and 8.90), 1,2-disubstituted benzene signals (δ_H 7.01, 7.07, 7.43, and 7.81), three singlet methyl signals (δ_H 1.06, 1.10, and 2.65) and one triplet methyl signal (δ_H 0.84). The ^{13}C NMR

Table 4. ^{13}C and ^1H NMR spectral data of **1**.

Position	δ_C	δ_H
1	110.4 s	
2	155.9 s	
2-OH		10.33 (s)
3	117.2 d	7.07 (d 7.8[b])
4	132.6 d	7.43 (t 7.8)
5	119.7 d	7.01 (t 7.8)
6	126.5 d	7.81 (d 7.8)
7	157.3 s	
9	152.4 s	
10	128.4 s	
12	160.8 s	
13-NH		8.90 (dd, 17.7, 7.9)
14	52.0 d	4.50 (br t 7.3)
15	171.6 s	
17	77.9 d	5.09 (d 9.7)
18	45.6 s	
19	173.3 s	
20-NH		7.48 (d 7.0)
21	50.9 d	4.41 (dd 10.4, 5.8)
22	168.6 s	
23-OH		9.76 (s)
24	52.3 t	3.50 (m), 3.88 (dd 15.5, 12.4)
25	25.6 t	1.30~1.50 (m), 1.51~1.71 (m)
26	27.0 t	1.51~1.71 (m), 1.85~1.95 (m)
27	30.0 t	1.51~1.71 (m), 1.71~1.83 (m)
28	11.4 q	2.66 (s)
29	29.9 t	1.85~1.95 (m)
30	22.7 t [22.9 t][d]	1.30~1.50 (m)
31	25.5 t [26.4 t][d]	1.50~1.71 (m)
32	45.5 t [48.9 t][d]	3.35~3.47 (m)
33-OH		9.49 (s), [9.94 (s)][d]
34	157.0 d [161.6 d][d]	7.90 (s), [8.23 (s)][d]
35	25.5 t	1.10~1.25 (m)
36	29.6 t	1.30~1.50 (m)
37	28.7 t[c]	1.10~1.25 (m)
38	28.7 t[c]	1.10~1.25 (m)
39	28.8 t[c]	1.10~1.25 (m)
40	28.8 t[c]	1.10~1.25 (m)
41	31.2 t	1.10~1.25 (m)
42	22.1 t	1.10~1.25 (m)
43	13.9 q	0.84 (t 7.0)
44	19.7 q	1.06 (s)
45	22.9 q	1.10 (s)

[a] Taken in DMSO-d_6.
[b] Coupling constants in J=Hz.
[c] The assignments may be interchanged.
[d] Observed as duplicate signals.

spectrum of **1** displayed three amide carbonyl signals (δ_C 160.8, 168.6, and 171.6), one ester carbonyl signal (δ_C 173.3) and nine aromatic signals (δ_C 110.4, 117.2, 119.7, 126.5, 128.4, 132.6, 152.4, 155.9 and 157.3). In addition, formyl amide signals were observed as duplicated ones owing to the restricted rotation (C-34, δ_C 157.0, 161.6; 34-H, δ_H 7.90, 8.23).

The characteristic UV spectrum of **1** suggested that **1** had an asteroidic acid moiety (partial structure A) which was the chromophore of nocobactin NA[21] (Fig. 4). This

Fig. 4. Asteroidic acid moiety (left) and a partial structure of didehydrotantazole A (right).

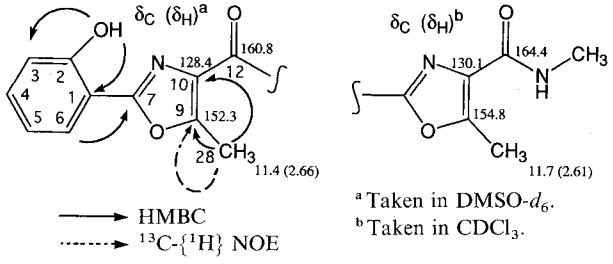


Fig. 5. Partial structures of **1** and ^{1}H-^{13}C long range couplings (arrows).

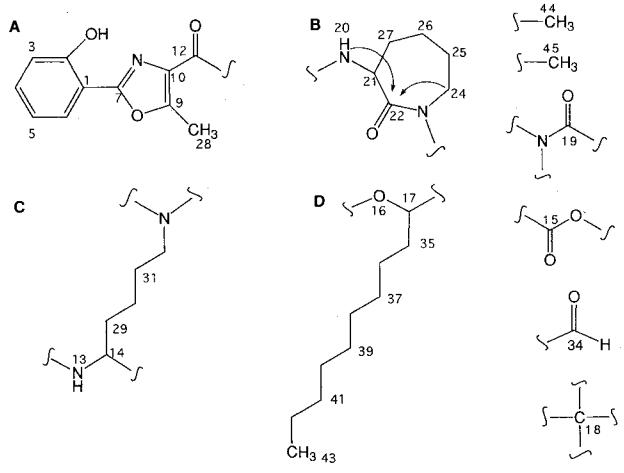
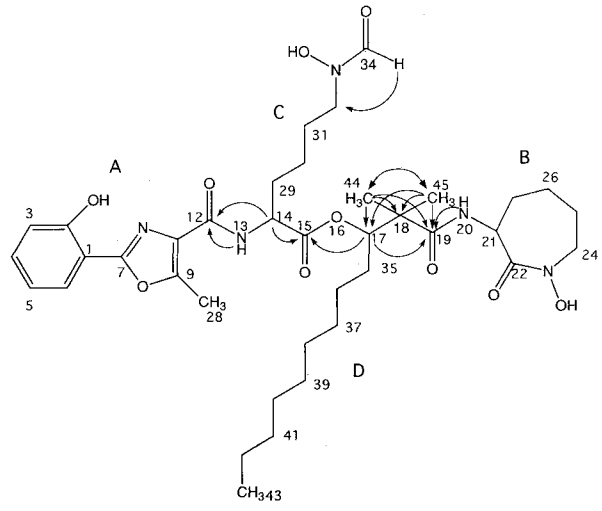


Fig. 6. ^{1}H-^{13}C long range couplings observed in the HMBC spectrum of **1** (arrows).



partial structure was supported by the HMBC experiment, which revealed long range couplings from phenolic proton 2-OH to quaternary carbons C-1 (δ_C 110.4) and C-3 (δ_C 117.2), and from 6-H to C-7 (δ_C 157.3). These correlations established that 2-hydroxyl benzene was attached to an oxazole moiety with a C-1 ~ C-7 bond linkage. Moreover, a methyl group (28-CH_3) was proved to be located at C-9 in the oxazole ring moiety by ^{13}C-{^{1}H} NOE observed from 28-H to C-9. The ^{13}C-NMR data of 4-methyl oxazole ring moiety was in good agreement with those of didehydrotantazole A[23] (Fig. 4). From these findings, partial structure A was deduced as shown in Fig. 5.

The connectivity of 20-NH ~ 21-H ~ 27-H ~ 24-H was revealed by an ^{1}H-^{1}H COSY spectrum. Long range couplings observed from 24-H and 20-NH to C-22 (δ_C 168.6) established the partial structure B as 2-amino-caprolactam (Fig. 5). Partial structures C and D were deduced by ^{1}H-^{1}H COSY and ^{1}H-^{1}H HOHAHA experiments as shown in Fig. 5. The functional groups consisting of two singlet methyls (C-44, δ_C 19.7 and C-45, δ_C 22.9), one amide carbonyl (C-19, δ_C 173.3), one ester carbonyl (C-15, δ_C 171.6), one formyl amide carbonyl [C-34, δ_C 157.0 (161.6)] and one quaternary carbon (C-18, δ_C 45.6) remained to be assembled.

Further structural elucidation was performed by an HMBC experiment. As shown in Fig. 6, a ^{1}H-^{13}C long range coupling from formyl proton 34-H to C-32 (δ_C 45.5) indicated that a formyl group was attached to partial structure C. Furthermore, the HMBC experiment also revealed long range couplings from 14-H and 17-H to C-15 (δ_C 171.6), which confirmed the linkages of the partial structures C and D through C-15. The amide carbonyl carbon C-12 (δ_C 160.8) was coupled to methine proton 14-H, which indicated that partial structure C was attached to partial structure A through an amide linkage. The correlations from tertiary methyls 44-CH_3 and 45-CH_3 to C-17 (δ_C 77.9) and C-18 (δ_C 45.6) indicated the attachment of two tertiary methyl groups at C-18 and established the linkage between C-17 and C-18. The amide linkage between C-21 in partial structure B and C-18 was determined by cross peaks from 20-NH, 44-CH_3 and 45-CH_3 to amide carbonyl C-19. From the above results, the connectivities of the partial structures were deduced as shown in Fig. 6.

Taking the molecular formura of **1** into consideration, two hydroxyl groups must be attached to the N-23 and N-33 positions.

Thus, the structure of formobactin (**1**) was deduced as shown in Fig. 1 except for the stereochemistry. The planar structure of **1** was closely related to that of nocobactin NA (**2**)[21]. The asteroidic acid (C-1 ~ C-12), lysine (N-13 ~ C-15 and C-29 ~ N-33), decanol (O-16, C-17 and

Fig. 7. Inhibitory effect of **1** on lipid peroxidation.

■ **1**, ○ BHT.

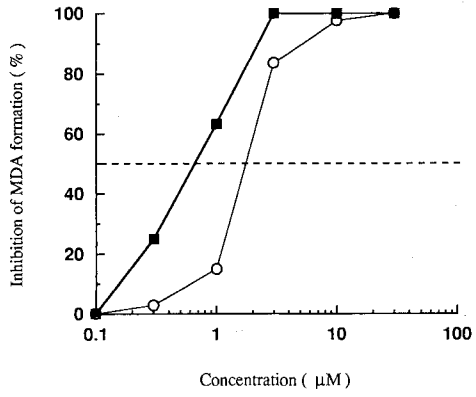


Fig. 8. Preventive effect of **1** on L-glutamate toxicity in N18-RE-105 cells.

□ **1**+BSO: 500 mM, ● **1**+L-glu: 10 mM.

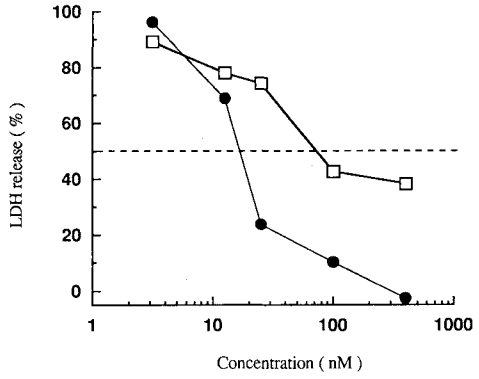


Table 5. Effect of Fe^{2+} addition (10 μM) on inhibitory activity of **1** and desferrioxamine on lipid peroxidation.

Compound	IC_{50} (μM)	
	$-Fe^{2+}$	$+Fe^{2+}$
1	0.65	3.10
Desferrioxamine	0.42	3.80

C-35～C-43) and 2-aminocaprolactam (N-20～C-27) moieties in **2** were preserved in the structure of **1**. Further experiments are in progress to determine the stereochemistry of **1**.

Biological Activities

The inhibitory effect of **1** on lipid peroxidation in rat brain homogenate is shown in Fig. 7. The IC_{50} of **1** was 0.65 μM, indicating that **1** was more active than butylated hydroxytoluene (BHT; 1.80 μM), a well known antioxidant. The activity of **1** was reduced in the presence of Fe^{2+} which acts as a promoter of lipid peroxidation (Table 5).

In addition, **1** showed a potent protecting activity to neuronal cells (Fig. 8). **1** reduced L-glutamate toxicity to N18-RE-105 cells in our evaluation system[24,25] (EC_{50}; 0.017 μM). An apoptotic death of these cells induced by an oxygen radical inducer, buthionine sulfoximine (BSO) was also suppressed by **1** (EC_{50}; 0.072 μM).

Discussion

In the course of our screening for free radical scavenging substances from microorganisms, we isolated a novel compound, formobactin from the culture of *Nocardia* sp. ND20. Since it had strong inhibitory activity against lipid peroxidation in rat brain homogenate, formobactin might be expected to be a therapeutic agent for ischemia, inflammation and so on.

Interestingly, the inhibitory activity of formobactin was reduced when Fe^{2+} was added to the reaction mixture. The presence of Fe^{2+} promotes lipid peroxidation by the formation of hydroxyl radicals (HO·) by FENTON's reaction as previously reported[26]. The similar reduction of the inhibitory activity against lipid peroxidation by the addition of Fe^{2+} was observed by desferrioxamine, a well known iron chelator (Table 5). The lipid peroxidation-inhibitory activity of desferrioxamine is due to suppression of HO· radical formation by chelation of intrinsic Fe[27]. Formobactin may also prevent the formation of HO· radicals by chelation of the intrinsic metal. This hypothesis is further supported by the structural characteristics of formobactin which belongs to the nocobactin group antibiotics isolated as iron-chelating agents[21,22].

The L-glutamate toxicity to N18-RE-105 cells depends on intercellular calcium concentration[28] and it is mainly caused by the inhibition of cysteine uptake[29]. Membrane depolarization caused by the addition of ouabain or the treatment of high concentration of KCl[30] reduced this toxicity. In addition, it is also reported that an increasing concentration of an endogenous antioxidative agent, such as glutathione causes the reduction of this toxicity[31]. Formobactin potently inhibited the toxicity in these cells. Since formobactin also suppressed the BSO toxicity, which resulted from the depletion of glutathione in the cell, it presumably acts as an inhibitor of excessive free radical formation and subsequent lipid peroxidation instead of glutathione. From these results, it is indicated that iron-catalyzed free radical formation and subsequent lipid peroxidation may participate in the L-glutamate toxicity in neuronal cells.

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