Amphistin, a New Melanogenesis Inhibitor, Produced by an Actinomycete

NORIKO ARAI, KAZURO SHIOMI, SATOSHI TAKAMATSU, KANKI KOMIYAMA, MAYUMI SHINOSE, YŌKO TAKAHASHI, YOSHITAKE TANAKA, YUZURU IWAI, JING-RONG LIU† and SATOSHI ŌMURA*

Research Center for Biological Function, The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan
†Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences, 1 Tiantan Xili, Beijing 100050, China

(Received for publication July 4, 1997)

A new melanogenesis inhibitor, named amphistin, was isolated from the fermentation broth of an actinomycete strain KP-3052. Amphistin was purified from the culture filtrate by the combination of cation exchange, gel filtration, and aminosilyl silica gel chromatographic methods. The structure of amphistin was elucidated as γ-(β-histidinoalanino)homoalanine by NMR experiments including ^{1}H-^{15}N HMBC experiment and other spectroscopic analyses. Amphistin inhibited the melanogenesis of B16 melanoma cells at concentration of 6.8 μM.

Melanins are bathochromic aromatic polymers that are synthesized by a pathway involving the progressive oxidation of tyrosine. Inhibitors of melanin synthesis would be expected to be used in remedies for chromatopathy and UV-induced hyperpigmentation, and in anti-tanning cosmetics.

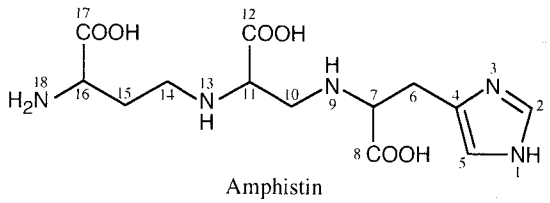
In the course of screening for melanogenesis inhibitor of microbial origin[1~3], we found a new inhibitor, which we named amphistin (Fig. 1). Amphistin was produced in the culture broth of an actinomycete strain KP-3052, which was isolated from a soil sample collected in China. In this paper we describe taxonomic studies of the producing strain and fermentation, isolation, structure elucidation, and biological activity of amphistin.

Materials and Methods

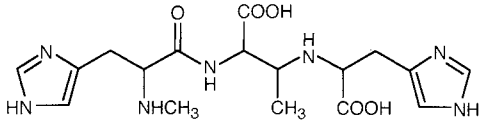
General

NMR spectra were obtained with a Varian Unity 400 spectrometer using D_2O as a solvent. Mass spectrometry was conducted on a JEOL JMS-AX505 HA spectrometer. UV and IR spectra were measured with a Shimadzu UV-240 spectrophotometer and a Horiba

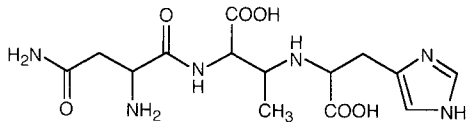
Fig. 1. Structures of amphistin and related compounds.



Amphistin



Feldamycin



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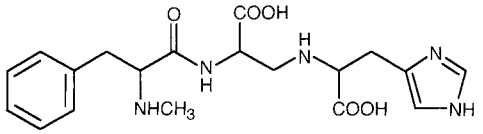
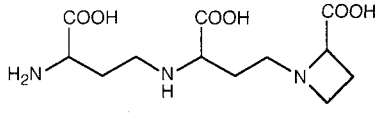


Melanostatin



Nicotianamin

FT-210 Fourier transform infrared spectrometer respectively. Optical rotation was recorded on a JASCO model DIP-181 polarimeter. Melting point was measured with a Yanaco micro melting point apparatus MP-S3.

Media

The seed medium was composed of potato starch 2.4%, glucose 0.1%, peptone (Kyokuto Pharmaceutical Industrial Co.) 0.3%, meat extract (Kyokuto Pharmaceutical Industrial Co.) 0.3%, yeast extract (Oriental Yeast Co.) 0.5%, and $CaCO_3$ 0.4%. The pH was adjusted to 7.0 prior to sterilization. The production medium was composed of Cultivater #100 (fish extract, Yaizu Suisankagaku Industry Co.) 0.1%, glucose 1.0%, yeast extract (Oriental Yeast Co.) 0.5%, and $CaCO_3$ 0.2%. The pH was adjusted to 6.5 prior to sterilization.

Taxonomic Studies

The morphological properties were observed with a scanning electron microscope (model S-430, Hitachi Co.).

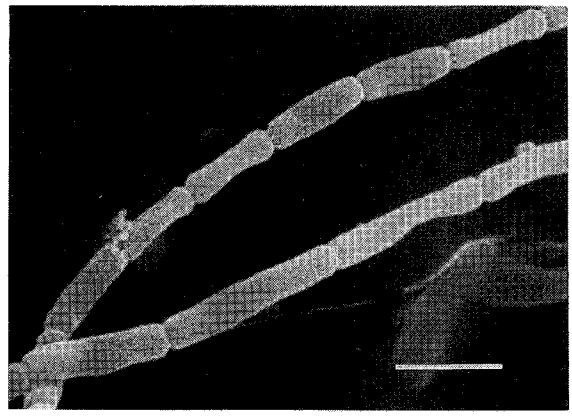
The isomer of diaminopimelic acid (DAP) was elucidated by the method of TAKAHASHI *et al.*[4]. Menaquinones were extracted and purified by the method of COLLINS *et al.*[5], and analyzed by HPLC using Capcell Pak C_{18} (Shiseido) eluted with methanol-2-propanol (70:30). To investigate the cultural characteristics and physiological properties, the International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB[6] and media recommended by WAKSMAN[7] were used. Cultures were observed after incubation at 27°C for two weeks. Color Harmony Manual, 4th Ed. (Container Corporation of America)[8] was used for color names and hue numbers. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium[9] containing 1% carbon sources.

Melanin Synthesis Inhibitory Activity Test

Stock culture of B16 melanoma cells were suspended in a monolayer in EAGLE's minimum essential medium containing 10% fetal calf serum and 0.1% glucosamine hydrochloride at 4.5×10^3 cells/ml. The cell suspension (6 ml) was poured into a 6-well culture plate (i.d. 35 mm, Corning) and incubated at 37°C in 5% CO_2-95% air atmosphere. After 5-days of incubation, each well was washed twice with HANK's solution (6 ml) and renewed with fresh medium containing various concentrations of amphistin or arbutin. Then, after 3-days of incubation, the adherent cells were washed with phosphate-buffered saline (1 ml) and scraped with a cell scraper (Coster). The cell suspension was centrifuged at 1,500 rpm for 10 minutes. The color and volume of resulting cells were compared with those of control.

Fig. 2. Scanning electron micrograph of spore chain of strain KP-3052 grown on inorganic salts-starch agar for 14 days.

Bar represents 1 μm.



Antimicrobial Activity Test

The antimicrobial spectra of amphistin were determined using i.d. 8×1.5 mm paper discs (Advantec Toyo Kaisha). Bacteria were grown on Mueller-Hinton agar medium (Difco) and fungi or yeast were grown on potato-broth agar medium. Antimicrobial activities were observed after 24 hours of incubation at 37°C for bacteria or longer incubation at 27°C for fungi or yeasts.

Results

Taxonomy of Producing Strain KP-3052

Morphological Properties

The vegetative mycelia grew abundantly on both synthetic and complex media, and showed fragmentation into bacillary elements. The aerial mycelia grew abundantly on inorganic salts-starch agar and glucose-asparagine agar. The spore chains were *Rectiflexibiles* type and each had more than 20 spores per chain. The spores were cylindrical in shape, 2.5×0.8 μm in size, and had a smooth surface (Fig. 2). Whirls, sclerotic granules, sporangia, and flagellated spores were not observed.

Chemical Composition

The DAP isomer in whole cell of strain KP-3052 was determined to be LL-type. Major menaquinones were MK-9 (H_4).

Table 1. Cultural characteristics of strain KP-3052.

Medium	Growth	Reverse color	Aerial mass color	Soluble pigment
Yeast extract - malt extract agar	Good, maize (2hb)	Gold (2lc)	Abundant, white (a)	None
Oatmeal agar[a]	Moderate, light ivory (2ca)	Light ivory (2ca)	Moderate, white (a)	None
Inorganic salts - starch agar[a]	Good, bamboo (2gc)	Light ivory (2ca)	Abundant, white (a)	None
Glycerol - asparagine agar[a]	Good, maize (2hb)	Maize (2hb)	Abundant, white ~ light ivory (a ~ 2ca)	None
Glucose - asparagine agar	Good, bamboo (2gc)	Maize (2ga)	Abundant, white (a)	None
Peptone - yeast extract - iron agar[b]	Moderate, bamboo (2gc)	Bamboo (2gc)	None	None
Tyrosine agar[a]	Good, bamboo (2fb)	Musterd gold ~ bamboo (2ne ~ 2fb)	Abundant, white ~ light ivory (a ~ 2ca)	None
Sucrose - nitrate agar[a]	Moderate, bamboo (2fb)	Bamboo (2fb)	Moderate, white (a)	None
Glucose - nitrate agar[b]	Poor, bamboo (2fb)	Bamboo (2fb)	None	None
Glycerol - calcium malate agar	Good, bamboo (2fb)	Pastel yellow (11/2fb)	Moderate, white (a)	None
Glucose - peptone agar	Moderate, pastel yellow ~ light wheat (11/2hb ~ 2ea)	Light wheat (2ea)	None	None
Nutrient agar	Moderate, bamboo (2fb)	Bamboo (2fb)	Moderate, white (a)	None

[a] Medium recommended by International Streptomyces Project.
[b] Medium recommended by S. A. WAKSMAN.

Table 2. Physiological properties of strain KP-3052.

Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Liquefaction of gelatin (21°C ~ 23°C)	+
Peptonization of milk (37°C)	+
Coagulation of milk (37°C)	+
Cellulolytic activity	−
Hydrolysis of starch	+
Nitrate reduction	−
Temperature range for growth	8°C ~ 38°C

+, Active; −, not active.

Table 3. Utilization of carbon sources by strain KP-3052.

D-Glucose	+
D-Fructose	+
L-Rhamnose	+
D-Mannitol	+
L-Arabinose	+
i-Inositol	+
Raffinose	+
D-Xylose	+
Sucrose	+
Melibiose	+

+, Utilized.

Cultural Characteristics and Physiological Properties

The cultural characteristics and the physiological properties are shown in Tables 1 and 2. The vegetative mycelia showed beige to yellow color on various media. The aerial mass color showed white. Soluble pigments were not produced. The utilization of carbon sources is shown in Table 3.

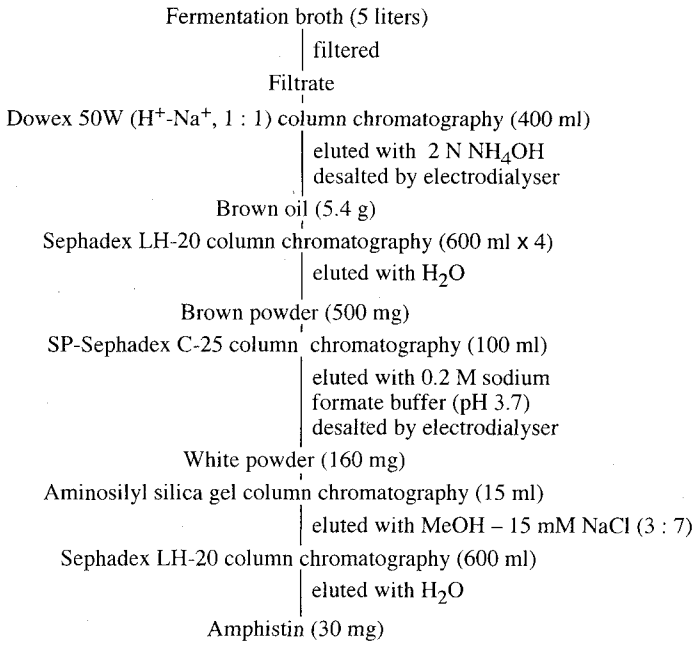
Although MK-9 (H_4) was detected in the strain KP-3052, we considered the strain as *Streptomyces*[10] or related genus tentatively. Further study for the strain is in progress. The strain has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology Japan, as Strain KP-3052 with an accession number of FERM P-14875.

Production and Isolation

A stock culture of strain KP-3052 grown on Seino agar was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium. It was incubated on a rotary shaker (210 rpm) at 27°C for 96 hours. One ml of the seed culture was transferred into each of fifty 500-ml Erlenmeyer flasks containing 100 ml of a production medium. The fermentation was carried out on a rotary shaker at 27°C for 144 hours.

Isolation procedure for amphistin is shown schematically in Fig. 3. The culture filtrate (5 liters) from the above fermentation was adjusted to pH 7.0 with HCl and applied on a Dowex 50W column (H^+ - Na^+, 1 : 1, 400 ml, Dow Chemical). After washing with 2 liters of 1 N NH_4OH, the active substance was eluted with 1 liter of 2 N NH_4OH. The eluate was desalted by a electro-

Fig. 3. Isolation procedure for amphistin.



dialyser (Micro acilyzer G3, Ashahi Chemical Ind. Co.) and lyophilized to give a brown oil (5.4 g). The oil was divided into four portions. Each portion was chromatographed over a Sephadex LH-20 column (Pharmacia, 600 ml) developed with H_2O. The active fractions of each chromatography were combined and concentrated to dryness *in vacuo* to give a pale brown powder (500 mg). The powder was dissolved in 0.1 M sodium formate buffer (pH 3.7) and adsorbed to a SP-Sephadex C-25 column (Pharmacia, 600 ml) equilibrated in the same buffer. After washing with the buffer, the active substance was eluted with 0.2 M sodium formate buffer (pH 3.7). The eluate was desalted by the electrodialyser and then concentrated to dryness *in vacuo* to give a white powder (160 mg). The powder was applied on an aminosilyl silica gel column (Chromatorex NH-DM1020, Fuji Silysia Chemical, 15 ml) equilibrated in methanol - 15 mM NaCl (6 : 4). After washing with methanol - 15 mM NaCl (6 : 4), the active substance was eluted with methanol - 15 mM NaCl (3 : 7). It was concentrated and applied on a Sephadex LH-20 column (600 ml), which was eluted with H_2O. Amphistin was eluted separately from salts and it was concentrated to yield 30 mg of a white powder. It showed a single spot on a silica gel TLC using a solvent system of 2-PrOH - pyridine - H_2O - AcOH (15 : 10 : 12 : 3) and detection with ninhydrin. The purity of amphistin was confirmed by NMR experiments.

Table 4. Physico-chemical properties of amphistin.

Appearance	White powder
Melting point	260°C (dec)
$[\alpha]_D^{25}$	$-9°$ (c 0.65, H_2O)
HR-FAB-MS m/z	
Calcd:	344.1570 $(M+H)^+$
Found:	344.1573 $(M+H)^+$
Molecular formula	$C_{13}H_{21}N_5O_6$
UV $\lambda_{max}^{H_2O}$ nm (ε)	220 (7500)
IR ν_{max} (KBr) cm^{-1}	1629, 1585
Solubility	
Soluble:	H_2O
Insoluble:	$CHCl_3$, acetone, DMSO
Color reaction	
Ninhydrin	Orange ~ yellowish pink

Physico-chemical Properties

The physico-chemical properties of amphistin are summarized in Table 4. The molecular formula of amphistin was deduced as $C_{13}H_{21}N_5O_6$ by HR-FAB-MS. Strong absorbances at 1629 and 1585 cm^{-1} in the IR spectrum suggested amine and carboxylate residues respectively. Chemical shifts in the ^1H and ^{13}C NMR of amphistin are shown in Table 5. The HMQC experiment revealed the connectivity of each proton and carbon.

Structure Elucidation

In the DEPT spectra, amphistin showed 4 methylene, 5 methine, and 4 quaternary carbon signals. Three partial

structures, $-CH_2-CH-$, $-CH_2-CH-$, and $-CH_2-CH_2-CH-$, were deduced from the ^1H-^1H COSY (Fig. 4). The HMBC experiment suggested that three carbonyl groups, C-8 (δ 180.1), C-12 (δ 176.5), and C-17 (δ 176.9), were connected to the respective methine carbons of the above partial structures, and that three aromatic carbons, C-2 (δ 119.5), C-4 (δ 133.8), and C-5 (δ 137.2), formed an imidazole ring (Fig. 4). Amphistin was only dissolved in H_2O and not in DMSO or acetone, so the active hydrogens were not observed in the ^1H-NMR. Therefore, the ^1H-^{15}N HMBC experiment was carried out to gain information on the nitrogens. ^{15}N chemical shifts were obtained by f_1 projection of the HMBC and shown in Table 5. In the HMBC experiment, long-range ^1H-^{15}N couplings were observed as shown in Fig. 5, which confirmed imidazole nitrogens N-1 (δ 172.2) and N-3 (δ 178.0). The chemical shifts of N-9 (δ 39.0), N-13 (δ 47.7), and N-15 (δ 40.0) suggested that they are not amides but amines. The alignment of the three partial structures and the amines were suggested by long-range ^1H-^{15}N couplings between the amines and their respective adjacent protons. The remaining three oxygens were assigned to be connected to three carbonyls by their ^{13}C chemical shifts. Consequently, the structure of amphistin was elucidated as γ-(β-histidinoalanino)homoalanine (Fig. 1).

Table 5. The ^1H, ^{13}C, and ^{15}N NMR data of amphistin.

Position	^{13}C	^1H	^{15}N
1			172.2
2	137.3 d	8.35 s (1H)	
3			178.0
4	133.8 s		
5	119.5 d	7.07 s (1H)	
6	30.7 t	2.92 d (2H, J=6.3 Hz)	
7	65.3 d	3.36 t (1H, J=6.3 Hz)	
8	180.1 s		
9			39.0
10	49.8 t	2.92 d (2H, J=5.9 Hz)	
11	63.8 d	3.49 t (1H, J=5.9 Hz)	
12	176.5 s		
13			47.7
14	46.6 t	3.00 m (1H), 3.06 m (1H)	
15	30.9 t	2.04 m (2H)	
16	55.6 d	3.69 t (1H, J=6.3 Hz)	
17	176.9 s		
18			40.0

Biological Activities

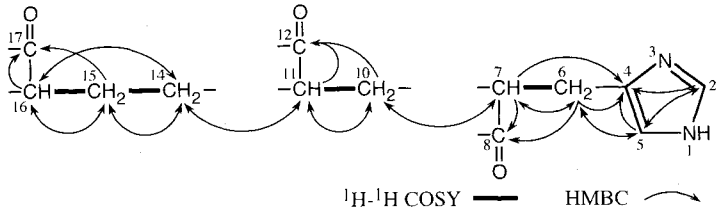
Melanin Synthesis Inhibitory Activity

The inhibitions of melanogenesis in growing B16 melanoma cells by amphistin and a known melanogenesis inhibitor, arbutin, are shown in Table 6. Amphistin inhibited the melanogenesis of B16 melanoma cells at the concentration of 6.8 μM without inhibitory effect on growth of the cells. On the other hand arbutin showed similar inhibition at the concentration of 69 μM. The IC_{50} values of amphistin against the growth of B16 melanoma cells were 55 μM.

Antimicrobial Activities

Amphistin weakly inhibited the growth of *Micrococcus luteus* PCI 1001, *Escherichia coli* NIHJ, *Pseudomonas*

Fig. 4. Partial structures of amphistin elucidated by ^1H-^1H COSY and HMBC.



Arrows of HMBC indicate the correlation from ^1H to ^{13}C.

Fig. 5. Structure of amphistin elucidated by ^1H-^{15}N HMBC.

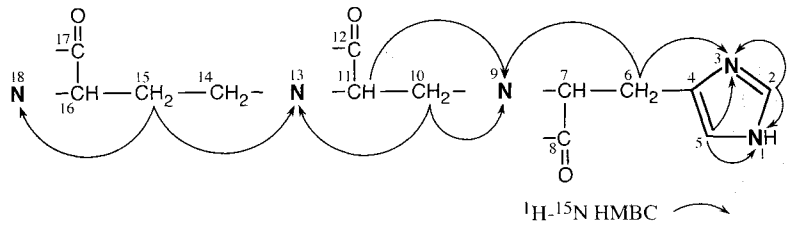


Table 6. Inhibitory effects of amphistin and arbutin on melanogenesis of B16 melanoma cells.

Final conc. (μM)	Amphistin	Final conc. (μM)	Arbutin
55	Toxic	551	Toxic
27	White	275	Yellowish white
14	White	138	Yellowish white
6.8	Gray	69	Gray
3.4	Black	34	Brown
1.7	Black	17	Black

aeruginosa P3, and *Staphylococcus aureus* FDA 209P in 10 μg/disc. But it did not inhibit the growth of *Bacillus subtilis* PCI 219, *Mycobacterium smegmatis* ATCC 607, *Xanthomonas oryzae* KB 88, *Acholeplasma laidlawii* PG 8 KB 174, *Candida albicans* KF 1, *Saccharomyces sake* KF 26, *Aspergillus niger* ATCC 6275, *Mucor racemosus* IFO 4581, *Bacteroides fragilis* ATCC 23745, and *Pyricularia oryzae* KF 180 in 20 μg/disc.

Discussion

In the course of our screening program for inhibitors of melanogenesis from microorganisms, we isolated a new compound named amphistin from the fermentation broth of an actinomycete strain KP-3052. Amphistin is a pseudotripeptide and is structurally related to feldamycin[11,12], FR-900490[13], and melanostatin[14] (Fig. 1). They also inhibited melanogenesis[14] and have three amino acids bonded by an amide bond and a N–C bond. In contrast, all amino acids in amphistin are bonded by N–C bonds. A metal chelating pseudotripeptide, nicotianamin, also has N–C bonds solely[15,16].

Amphistin inhibited the melanogenesis in B16 melanoma cells at the concentration of 6.8 μM without an inhibitory effect on growth of the cells. The inhibitory activity was similar to those of melanostatin and feldamycin[14]. Feldamycin was suggested to inhibit tyrosinase activity through post-translational modification of the enzyme itself or of other modulatory protein[17]. Amphistin may cause the inhibition of melanogenesis by similar manner.

Recently arbutin has been widely used as a bleach products. Amphistin is about ten times more potent than arbutin in inhibition of melanogenesis. Therefore amphistin might be useful for remedy for pigmentation disorders.

Acknowledgment

We express our thanks to Ms. A. HATANO, Ms. T. SAKABE, and Ms. A. NAKAGAWA, School of Pharmaceutical Sciences, Kitasato University, for NMR experiments and measurements of mass spectra. We also thank Ms. A. MATSUI and Mr. Y. SHIMOJYO for their assistances throughout this work.

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