Phenamide, a Fungicidal Metabolite from *Streptomyces albospinus* A19301

Taxonomy, Fermentation, Isolation, Physico-chemical and Biological Properties

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A new derivative of phenylalanine, phenamide, was discovered from the fermentation broth of an actinomycete identified as a member of the *Streptomyces albospinus* cluster. Phenamide was purified using successive C18 reverse phase and cation exchange chromatography. Its structure was determined by spectroscopic and chemical methods. Its molecular formula, $C_{14}H_{20}N_2O_3$, was determined by HRFAB-MS. Phenamide showed activity against *Septoria nodorum*, the causal agent of wheat glume blotch.

During the course of screening fermentation broths for antifungal activity, a derivative of phenylalanine, phenamide, was discovered (Fig. 1) which is active against *Septoria nodorum*. In this paper, we describe the isolation, taxonomy and fermentation of the producing organism, along with the purification, structure elucidation, synthesis, and physico-chemical and biological properties of pure phenamide.

Materials and Methods

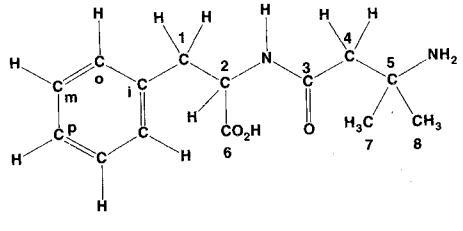
Isolation of the Organism

Strain A19301 was isolated from the soil sample collected in Anjozorobe, Madagascar, during attempts to isolate actinoplanetes by the chemotactic method described by VOBIS[1] using Czapek sucrose agar[1]. A pure culture of strain A19301 was preserved in the vapor phase of liquid nitrogen, as a mycelial suspension in nutrient broth (Difco) plus 10% (w/v) glycerol.

Taxonomic Examination

Morphological, cultural and physiological properties of strain A19301 were examined according to the methods described by SHIRLING and GOTTLIEB[2], and WILLIAMS *et al.*[3]. Detailed observation of mycelial and spore morphologies was performed by using a long working distance objective on a light microscope (Dialux 20, Leitz) and a scanning electron microscope (840, Jeol). Detection of diaminopimelic acid in the cell wall and whole-cell sugars was performed by using the methods of HASEGAWA *et al.*[4], and SCHAAL[5], respectively.

Fig. 1. Structure of phenamide.



Fermentation

In order to obtain a sufficient quantity of phenamide for structure elucidation, strain A19301 was grown in a 2-liter Erlenmeyer flask. Fermentation inoculum was prepared by inoculating a 250-ml flask containing 50 ml of seed medium composed of yeast extract 0.3% and Tryptone (Difco) 0.5%, with 1 ml of nutrient broth-glycerol suspension of mycelia and spores of strain A19301. After incubation at 30°C for three days on a rotary shaker at 250 rpm, the seed culture was inoculated into the 2-liter Erlenmeyer flask containing 1 liter of production medium, consisted of Maltrin M-100 (Grain Processing Corp.) 1.0%, soluble starch 0.5%, fish meal 0.5%, yeast extract 0.25%, tobacco hornworm (freeze-dried powder) 0.01%, $MgSO_4 \cdot 7H_2O$ 0.2%, KH_2PO_4 0.5%, K_2HPO_4 0.5%, $ZnSO_4 \cdot 7H_2O$ 0.005%, Fe-EDTA 0.0018% and Tween 80 0.0001%. The pH of the medium was adjusted to 6.8 before autoclaving. Antifoam P-2000 (Mazer Chemicals) was added to the medium at the rate of 0.1%. The fermentation was carried out on a rotary shaker at 30°C and 120 rpm, with 1 liter/minute aeration for 5 days. The harvested broth was passed through a 0.45 μm filter (Millipore), and the pH was adjusted to 7.0.

Analytical Procedures

A Varian 2010 HPLC with a model 2020 gradient controller and a model 2050 variable wavelength UV detector was used for all HPLC analyses with either a

Phenomenex 250×10 mm, 10 μ Zorbax ODS C-18 reverse phase column or a Whatman Magnum 9 SCX 250×10 mm, 10 μ HPLC column. Cation exchange chromatography was performed using BioRad AG 50W-X8, 50~100 mesh (H^+) with NH_4OAc prepared from acetic acid and ammonium hydroxide or NaH_2PO_4 from HPLC grade 85% H_3PO_4 and NaOH. The XAD-2 was Amberlite while bulk C-18 reverse phase flash resin was purchased from J. T. Baker Inc.

Physico-chemical Characteristics: The UV spectrum was recorded on a Hewlett-Packard 8450 A UV/VIS spectrophotometer, and Cl, El and FAB-mass spectra were obtained on a VG mass spectrometer. All NMR spectra were recorded on a Varian 300 MHz spectrometer in D_2O except for HMBC and HMQC experiments which were performed at 500 MHz.

Biological Assay

Botrytis cinerea, *Candida albicans* ATCC 10231, *Pyricularia oryzae* Cavara, *Sacchromyces cerevisiae* ATCC 2366, *Schizosaccharomyces pombe*, and *Septoria nodorum* (Berk.) Berk, were used in the antifungal screening assay. *Botrytis cinera*, *Candida albicans*, and *Saccharomyces cerevisiae* were grown on Vogal Salts medium[6] agar plates for two days at 25°C. *Pyricularia oryzae* was grown at 23°C for 7 days on V-8 agar medium[7]. *Schizosaccharomyces pombe* was grown at 30°C for 7 days on yeast extract dextrose agar containing yeast extract (Difco) 0.1%, dextrose (Sigma) 0.1%, and agar (Difco) 1.5%. *Septoria nodorum* was grown at 20°C, on media containing yeast extract (Difco) 0.4%, malt extract (Difco) 0.4%, sucrose (Sigma) 0.4%, and agar (Difco) 2.0% for ten days. Spore suspensions of *Botrytis cinerea*, *Pyricularia oryzae*, and *Septoria nodorum* were obtained by flooding the sporulating culture with 10 ml of sterile distilled water, and scraping across the agar surface, using a sterile loop, to suspend spores in the liquid. The spore suspension was filtered through sterile glass wool to remove mycelial fragments. Cell suspensions of *Candida albicans* and *Saccharomyces cerevisiae* were obtained by flooding the culture plates with 10 ml of sterile distilled water, and scrapping across the agar surface, using a sterile loop. Using a hemocytometer, the spore/cell concentration was adjusted with sterile distilled water to approximately 1×10^7/ml. In 24 well, flat bottom, sterile, polystyrene plates with lids (Corning) 800 μl of molten, sterile Czapek-Dox agar (Difco) amended with V-8 juice 2.0%, was added to 200 μl of fermentation sample for a final volume of 1 ml. Sample pH was adjusted to between 5 and 8, if necessary, prior to addition to the wells. Twenty μl of spore suspension was added to each well, and plates were incubated at a temperature depending upon the fungal species as described above. Plates were rated visually against an untreated control with 0 and 3 representing no inhibition and 100% inhibition of fungal growth, respectively.

Results and Discussion

Taxonomy of Strain A19301

The cultural and carbon/nitrogen utilization characteristics of strain A19301 are summarized in Tables 1 and 2, respectively. Under a light microscope strain A19301 showed well branched aerial mycelia bearing simple spiral chains (S)[2] of spores with 6~8 turns. Spores were bacilli shaped (0.6~0.75 $\mu\times$0.8~0.95 μ). Scanning electron microscopy demonstrated spiny spore surface ornamentation. The aerial mass color of strain A19301 was gray. Substrate mycelium was non-fragmenting. No diffusible pigment was formed. The whole-cell hydrolysate contained LL-diaminopimelic acid. Whole-cell sugars were found to be glucose and mannose. No diagnostic sugars were detected. Based on these data strain A19301 was classified as cell wall type I as described by LECHEVALIER and LECHEVALIER[8].

Physiological characteristics of strain A19301 are summarized in Table 3. There was no growth in the presence of 7% NaCl, or 0.01% sodium azide. Melanoid pigment was not produced on tyrosine agar nor on peptone iron agar.

Based on morphological and chemotaxonomic characteristics, strain A19301 was determined to belong in the genus *Streptomyces*. Studies of the spore surface

Table 1. Cultural characteristics of strain A19301.

Media*	Growth	Aerial mycelium	Soluble pigment	Colony reverse**
ISP No. 2	Good	Good (gray)	—	Orange yellow (71.m.OY)
ISP No. 5	Good	Good (gray)	—	Yellow olive brown (90.gy.Y.)
ISP No. 7	Good	Good (gray)	—	Orange yellow (77.m.y. br)
BENNET'S	Good	Good (gray)	—	Yellow olive brown (95.m.01Br)
DTA	Good	Good (gray)	—	Orange yellow (75.deep y Br)
GA	Moderate	Poor (white-gray)	—	Colorless (263)
OYEA	Good	Good (gray)	—	Yellow olive brown (91.d.gy.Y)

* ISP No. 2: yeast extract-malt extract agar; ISP No. 5: glycerol-asparagine agar; ISP No. 7: tyrosine agar; BENNET'S: yeast extract-beef extract agar; DTA: dextrose tryptone agar; GA: glucose asparagine agar; OYEA: oatmeal yeast extract agar.

** Colors were recorded as described by the Inter-Society Color Council, National Bureau of Standards.

ornamentation, carbon utilization and physiological characteristics suggest that it is a member of *Streptomyces albospinus* as described in BERGEY's Manual of Systematic Bacteriology[9].

Fermentation

The production level of A19301 was such that sufficient quantities of the active components from 1 liter fermentation were obtained. No further optimization was performed.

Biological Properties

Pure phenamide was active against *Septoria nodorum* at 500 ppm, and was inactive against other fungal species tested. However the crude filtrate of A19301 completely inhibited the growth of both *Septoria nodorum* and *Botrytis cinera* when 25 μl of crude broth was added to 1 ml of the medium containing spores in a 24 well plate. As described below the enhanced activity in the crude filtrate was found to be due to a known nucleoside toyocamycin[10].

Table 2. Carbon and nitrogen utilization of strain 19301.

Carbon sources utilized:	D-glucose, α-lactose, adonitol, D-galactose, salicin, (+)-mannose, D-fructose, D-mannitol, D-ribose, (+)-maltose, sucrose, myo-inositol, (+)-cellobiose
Carbon sources not utilized:	L-arabinose, dulcitol, inulin, α-(+)-melibiose, L-sorbose, D-xylitol, (+)-xylose, (+)-rhamnose
Nitrogen sources utilized:	L-valine, L-tyrosine, L-threonine, L-histidine, hydroxy-L-proline, L-glutamic acid
Nitrogen sources not utilized:	L-cystine, DL-α-amino-*n*-butyric acid

Table 3. Physiological features of strain A19301.

H_2S production	−
Nitrate reduction	−
Melanin production	−
Degradation of xanthine	+
hypoxanthine	+
pectin	−
arbutin	+
allantoin	−
lecithin	−
Growth in the presence of	
NaCl (7% w/v)	−
sodium azide (0.01%)	−
phenol (0.1%)	−
Resistance to rifampicin (50 μg/ml)	+
neomycin (50 μg/ml)	−
Antibiosis to *Streptomyces murinus* (ATCC 19788)	+
Aspergillus niger (ATCC 36233)	+
Bacillus subtilis (ATCC 6633)	+
Growth at 45°C temperature	−
25~37°C	+
Growth at pH 5.0~9.0,	+
4.0 and 10.0	−

Isolation

A total of 940 ml of fermentation broth was passed over XAD-2 (900 ml) and was washed sequentially with water, 1:1 water-MeOH, and MeOH. The 1:1 water-MeOH fraction was retained and chromatographed on a reverse phase flash column eluting with 10% increments of MeOH in water. Activity was noted in the 8:2 and 7:3 (water-MeOH) fractions. The 8:2 fraction contained 140 mg of material and was used for further purification work. This material was applied to AG50W X-8 cation exchange resin (50 g, NH_4^+ form) and was eluted with NH_4OAc (50 mM) in a pH step gradient (pH's 4.0, 5.0, and 6.0) using 100 ml volumes and 1 ml/minute flow rate collecting 2.5 ml fractions. The column was ultimately washed with 1.4 M NH_4OH (100 ml). Active fractions (No. 65~95) were pooled, evaporated and weighed giving 60 mg of material. This was applied to reverse phase HPLC using an isocratic 75:25 water-MeOH system, 3 ml/minute, to give 40 mg of 95% pure phenamide. The 7:3 water-MeOH fraction from reverse phase flash chromatography was similarly purified to obtain phenamide contaminated with the known nucleoside toyocamycin. Later work revealed that toyocamycin was present at approximately 1.5% in the phenamide obtained above. In order to remove traces of toyocamycin an HPLC cation exchange on a SCX column (Na^+ form) was used, eluting with a gradient of 1 mM NaH_2PO_4, pH 3.3, to 100 mM NaH_2PO_4, pH 3.3. After desalting on reverse phase HPLC, a pure sample of phenamide free of toyocamycin was obtained.

Physico-chemical Properties

Phenamide is a white solid. It is readily soluble in water, methanol and acetone with UV (water) λ_{max} 205 ($\log \varepsilon$ 3.70). The molecular formula of phenamide $C_{14}H_{20}N_2O_3$ (MW 264), was determined by high resolution FAB-MS (M+H) m/z 265.1551. The ^1H and ^{13}C NMR spectra of phenamide are shown in Figs. 2 and 3, respectively. The chemical shifts are also summarized in Table 4.

Structure Determination

Hydrolysis of a purified sample of **1** in 6 N HCl gave two materials which were analyzed by mass spectrometry. One product was identified as phenylalanine by its NMR and mass spectra as well as TLC. The second product displayed a simple NMR spectrum (2 singlets in a 3:1 ratio by integration) and an 118 ion by FAB^+ MS (M+H). The phenylalanine fragment was consistent with

Fig. 2. ^{1}H NMR spectrum of phenamide (300 MHz).

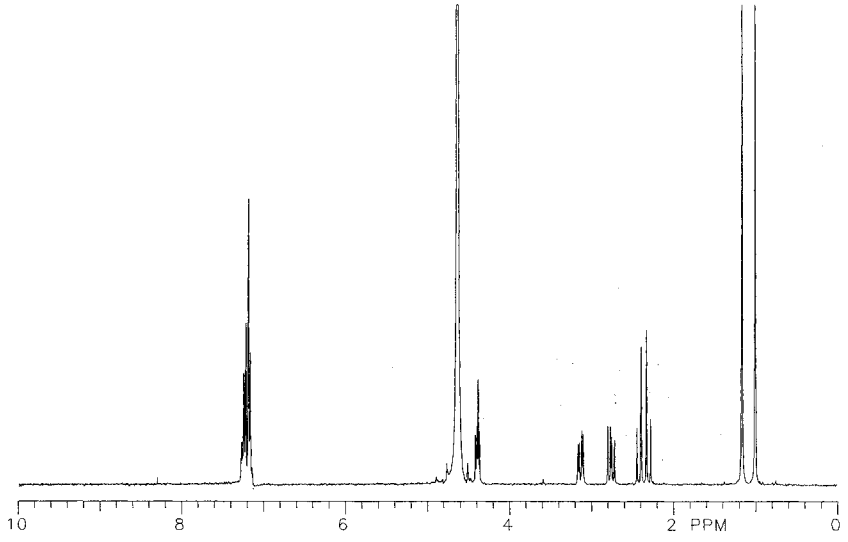


Fig. 3. ^{13}C NMR spectrum of phenamide (300 MHz).

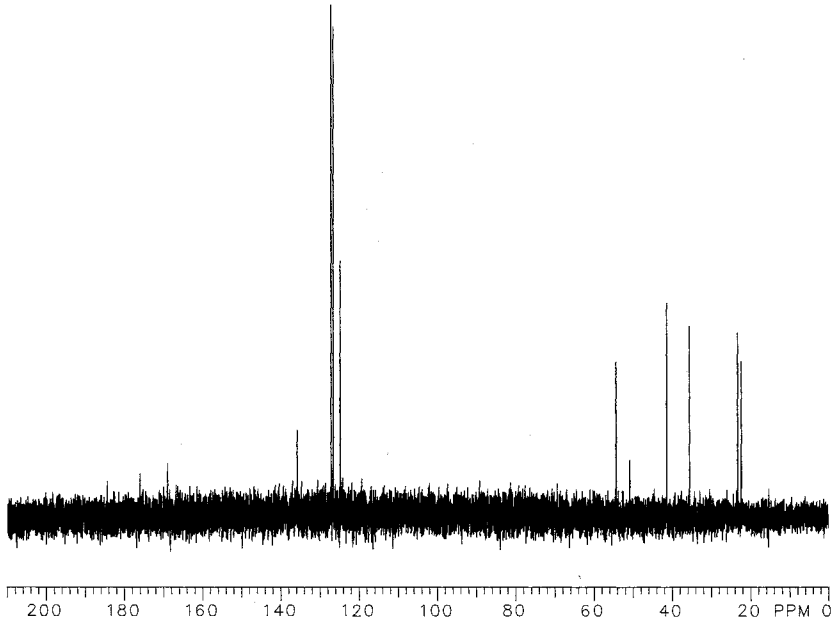


Table 4. NMR assignments (solvent D_2O).

Carbon number	^{13}C shift in ppm	^{1}H shift in ppm (J in Hz)
1	37.9	2.81 (9.9, 14.1); 3.17 (4.7, 14.1)
2	56.3	4.43 (4.7, 9.9)
3	171.4	—
4	43.5	2.35 (15.5); 2.48 (15.5)
5	53.2	—
6	178.1	—
7	24.7	1.04
8	25.7	1.21
i	139.2	—
o	129.5	7.17~7.33
m	128.8	7.17~7.33
p	126.9	7.17~7.33

the UV (λ_{max} 205 nm, log ε 3.7; and 258 nm, log ε 2.3) and many of the signals in the NMR and ^{13}C NMR spectra (Figs. 2 and 3). Combining the mass spectral data for **1** with the presence of phenylalanine, enabled us to assign a formula of $C_5H_{10}NO$ to the second fragment. Furthermore, the ^{1}H and ^{13}C NMR spectra (Table 4, and Figs. 2 and 3) can be explained by two methyls and a methylene.

Assignment of the linkage between phenylalanine and the $C_5H_{10}NO$ portion was determined using a series of HMQC and HMBC experiments. The HMQC experiment verified the assignments given in Table 4. The protons assigned to the methylene of the phenylalanine

fragment at 2.81 ppm (dd, $J=9.9$, 14.1 Hz) and 3.17 ppm (dd, $J=4.7$ and 14.1 Hz) show clear correlations in the HMBC to aromatic carbons at 129.5 ppm (*ortho* carbon) and 139.2 ppm (*ipso* carbon). Furthermore, cross peaks with carbons at 56.3 ppm (C-2, Fig. 1), and 178.1 ppm (carboxyl carbon, C-6, Fig. 1) are evident. The protons assigned to a methylene carbon on the $C_5H_{10}NO$ fragment (C-4, Fig. 1) at 2.35 and 2.48 ppm (AX system, $J=15.5$ Hz) give clear multiple bond correlations to the methyl carbons at 24.7 and 25.7 ppm, in addition to an amino bearing carbon (C-5) at 53.2 ppm, and a carbonyl at 171.4 ppm (C-3, Fig. 1). Most significant are the correlations noted for the methine proton on the phenylalanine portion (4.43 ppm, dd, $J=4.7$ and 9.9 Hz). Since this proton is coupled to both carbonyl carbons and to the *ipso* aromatic carbon, it strongly supports the structure shown. If the amide linkage were reversed, one would expect to see a correlation to only one carbonyl carbon. The correlations observed in the HMBC experiment are listed in Table 5.

Additionally, **1** was reacted with DMF-dimethyl acetal at 100°C for 30 minutes. Two products were detected by direct probe CI analysis with molecular weights of 333 and 288 (Fig. 4). Upon gc/ms analysis, only the lower molecular weight material was detected. The first material (MW 333) was consistent with an amidine structure predicted from **1**. The lower molecular weight material (MW 288), the sole product detected by gc/ms, was rationalized as a cyclization product involving the amidine and the amide linkage resulting in loss of 45 (loss of NMe_2, Fig. 4).

Table 5. HMBC correlations with jnxh=4.

H's at C-1 correlate to: C-2, C-*i*, C-*o*, C-6
H's at C-2 correlate to: C-1, C-*i*, C-3, C-6
H's at C-4 correlate to: C-3, C-5, C-7, C-8
H's at C's 7, 8 correlate to: C-4, C-5, C-7(8)

Fig. 4. Scheme for structure elucidation of phenamide.

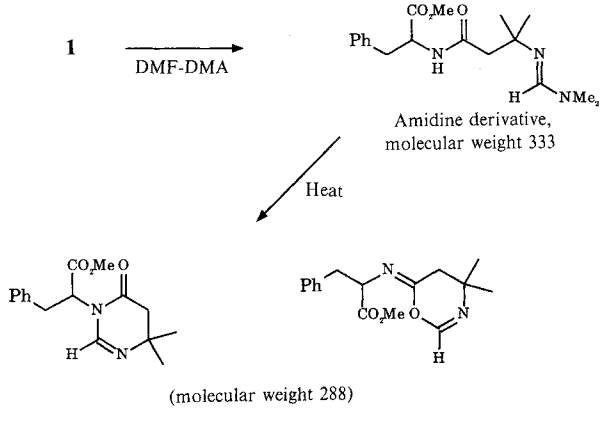
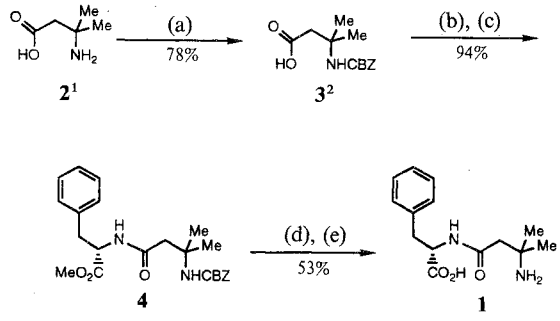


Fig. 5. Scheme for the synthesis of phenamide.



(a) Benzyl chloroformate, NaOH, acetone; (b) carbonyldiimidazole, THF; (c) L-phenylalanine methyl ester; (d) 1.0 N NaOH, MeOH (e) H_2, $Pd(OH)_2$, EtOH.

Synthesis

Additional proof of our structural assignment came upon comparison of the natural material with material synthetically derived from L-phenylalanine. The known 3-amino-3-methylbutanoic acid (**2**) was coupled with both D- and L-phenylalanine using standard blocking and deblocking chemistry (Fig. 5). The amino group of **2** was blocked with benzyl chloroformate, and subsequently coupled with the methyl ester of D- and L- phenylalanine using carbonyldiimidazole. Removal of the methyl ester with base and the Cbz group *via* hydrogenation gave **1** (Fig. 4) in 39% overall yield. Ultimately, the material derived from L-phenylalanine proved to be identical with the natural phenamide by comparison of their CD spectra. The biological activity of the D-isomer was weaker, with an inhibition of *Septoria nodorum* growth about half that of the natural product at 500 ppm.

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