N-ACETYL-L-PHENYLALANYL-L-PHENYLALANINOL A METABOLITE OF *EMERICELLOPSIS SALMOSYNNEMATA*

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(Received for publication July 3, 1975)

A new metabolite N-acetyl-L-phenylalanyl-L-phenylalaninol was isolated from culture filtrates of *Emericellopsis salmosynnemata* which produces zervamicins I and II. The structure was assigned from spectral properties and degradative studies.

In a recent communication[1] we reported the production of the antibiotics zervamicins I and II by *Emericellopsis salmosynnemata*. Subsequent studies indicated the presence of large amounts of a bioinactive metabolite in culture filtrates of *E. salmosynnemata*. The isolation of this material and determination of its structure as N-acetyl-L-phenylalanyl-L-phenylalaninol (**I**) are the subject of the present communication.

I was isolated as a colorless crystalline (needles) material, $[\alpha]_D^{25}$, $-40°$ (*c* 1, 95 % ethanol). Analytical data and high resolution mass spectrum indicated the molecular formula $C_{20}H_{24}N_2O_3$, Mol. weight, 340. UV absorptions 247(sh), 252, 258, 264 and 287(sh) nm indicated the presence of benzenoid systems in the molecule.[2] Infrared absorptions at 3300~3440 and 1645, 1542 cm^{-1} indicated -OH, -NH- and amide functions. Absorptions at 3030 and 1607 cm^{-1} are assigned to the aromatic system. Potentiometric titration showed the absence of titratable groups eliminating the presence of either -COOH or $-NH_2$. This is in agreement with a negative response to the ninhydrin reaction by **I**.

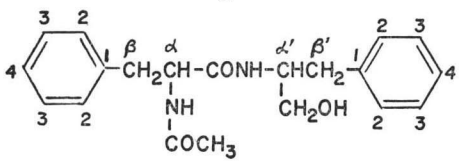
The nmr spectrum of **I** showed the presence of ten aromatic hydrogens centered at δ, 7.23 and an acetate methyl singlet at δ, 1.80. Fragments **II** and **III** (Fig. 2) were established by spin decoupling (Table 1).

The chemical shift of the two overlapping AB of ABX patterns assigned to the $-CH_2-$ groups of fragments **II** and **III** suggested the attachment of a phenyl group to the $-CH_2-$ of both **II** and **III**.

Consideration of the nmr, ir and other data discussed above indicates that the acetyl group is attached to the -NH- group of **III** while **II** and **III** are connected through an amide bond as shown in **I**.

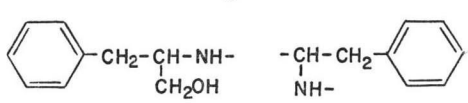
^{13}C-NMR data (Table 2) are in agreement with structure **I** for the metabolite produced by

Fig. 1.



I

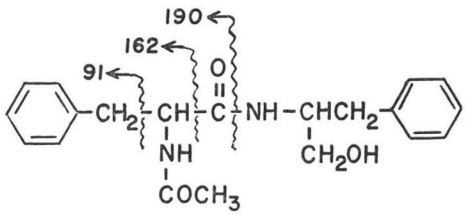
Fig. 2.



II **III**

E. salmosynnemata. Furthermore, high resolution mass spectra of **I** showed a fragmentation pattern (Table 3 and Fig. 3) consistent with the postulated structure.

Fig. 3.



Acid hydrolysis of **I** followed by Dowex-1 chromatography yielded L-2-amino-3-phenyl-1-propanol (L-phenylalaninol) isolated as colorless needles, $C_9H_{13}NO_2$, $[\alpha]_D^{25}$, $-21°$ (*c* 1, methanol) [reported $-23°$; $-25°$[3]]. The other moiety present in **I**, isolated by elution of the column with aqueous HCl as crystalline hydrochloride, $C_9H_{11}NO_2 \cdot HCl$, was identified as L-phenylalanine by ir, nmr, $[\alpha]_D$ and tlc comparison to an authentic sample. These results conclusively establish the structure of the metabolite as N-acetyl-L-phenylalanyl-L-phenylalaninol (**I**).

Table 1. Proton magnetic resonance absorptions[1] assigned to **II** and **III** (Fig. 2)

	Assignment	Area	Multiplicity[3]	Apparent coupling, Hz	Chemical[2] shift, ppm
Fragment II	CH_2	2	AB of ABX	$J_{AB}=-14$, $J_{AX}=6$, $J_{BX}=9$	2.87
	CH	1	m	$J_{CH\text{-}CH_2O}=5.1$	3.96
	NH	1	d	$J_{NH\text{-}CH}=8.5$	7.66
	CH_2-O	2	dd	$J_{CH_2\text{-}OH}=5.2$	3.34
	OH	1	t	—	4.79
Fragment III	CH_2	2	AB of ABX	$J_{AB}=-14$, $J_{AX}=6$, $J_{BX}=8$	2.80
	CH	1	dd of d	$J_{CH\text{-}NH}=8.7$	4.52
	NH	1	d	—	7.93

[1] Spectrum obtained in d_6-dimethylsulfoxide.
[2] Relative to internal TMS.
[3] Multiplicity: m=multiplet, d=doublet, dd=doublet of doublets, t=triplet, dd of d=doublet of doublet of doublets.

Phenylalaninol is present in antiamoebin,[3] emerimicin[4] and zervamicins (R. C. PANDEY and K. L. RINEHART, Jr., personal communication). N-Acetyl-L-phenylalanyl-L-phenylalaninol is the major secondary metabolite produced by *E. salmosynnemata* under the fermentation conditions used. It is estimated that *ca.* 150~200 g of material could be isolated from a 250-liter fermentation by extraction of the clear filtrate with 1-butanol and by trituration of the mycelial cake with methanol.

Experimental

Assay and Testing Procedures

Antibiotic production and purification was followed by a microbiological disc-plate assay procedure[5] with *Sarcina lutea* as the assay organism. Antibacterial activities were also determined by broth dilution methods described by LEWIS, *et al.*[6]

Thin-Layer Chromatographic Procedures

Thin-layer chromatograms were run on silica gel G using chloroform - methanol (6 : 1, v/v) as the solvent system. The antibiotics present in the fermentation or in preparations obtained during purification were detected by bioautography on *S. lutea*-seeded agar trays. Compound **I** was detected by development with a permanganate-periodate reagent.

Table 2. Carbon-13 magnetic resonance spectrum[1] of **I**

Chemical shift, δ[2]	No. of Carbons	Multiplicity[3]	Assignment[4]
22.69	1	q	$-CO\underline{C}H_3$
36.72	1	t	$C_6H_5-\underline{C}H_2$
37.96	1	t	$C_6H_5-\underline{C}H_2$
52.62	1	d	α-CH
54.29	1	d	α'-CH
62.44	1	t	CH_2-O
137.91, 139.03	2	2s	C-1
129.13	4	4d	C-2
128.02	4	4d	C-3
126.11, 126.43	2	2d	C-4
169.16	1	s	-C=O
170.83	1	s	-C=O

[1] Spectrum obtained in d_6-dimethylsulfoxide.
[2] Relative to internal TMS.
[3] Multiplicity in off-resonance proton-decoupled spectrum: q=quartet; t=triplet; d=doublet; s=singlet.
[4] Refer to structure **I**.

Table 3. High resolution mass spectrum of **I**

Found (*m/e*)	Calcd. for:	(*m/e*)	Assignment
340.1756	$C_{20}H_{24}N_2O_3$	340.1786	M+
322.1691	$C_{20}H_{22}N_2O_2$	322.1681	M+-H_2O
310.1672	$C_{19}H_{22}N_2O_2$	310.1681	M+-CH_2O
249.1234	$C_{13}H_{17}N_2O_3$	249.1239	M+-C_6H_5-CH_2·
207.11289	$C_{11}H_{15}N_2O_2$	207.11334	M+-(C_6H_5-CH_2· +CH_2=C=O)
190.08765	$C_{11}H_{12}NO_2$	190.0868	See Fig. 3
162.09197	$C_{10}H_{12}NO$	162.09188	See Fig. 3
120.0815	$C_8H_{10}N$	120.0813	162-(CH_2=C=O)

Spectroscopic Methods

Proton magnetic resonance spectra were recorded on a Varian XL-100-15 spectrometer operating at 100 MHz. Solutions (*ca.* 0.4 ml, *ca.* 0.25 M) of the compounds in d_6-dimethylsulfoxide were used.

Carbon magnetic resonance spectra were recorded on a Varian XL-100-15 spectrometer operating in the FOURIER transform (FT) mode. Pmr and cmr chemical shifts are reported as ppm relative to tetramethylsilane as internal standard.

High resolution mass spectra data were obtained on a Varian MAT CH5 DF mass spectrometer.

Fermentation Procedures

The procedures described by ARGOUDELIS, *et al.*[1] were used. Fermentations were analyzed for antibiotic production by bioactivity determinations. The presence of **I** was investigated by tlc. Beers were harvested after fermentation time of 192 hours.

Isolation Procedures

Filtration. Extraction of **I** and Zervamicins: Fermentation broth (*ca.* 250 liters) was filtered with the aid of diatomaceous earth. The clear filtrate was extracted three times with 80-liter portions of 1-butanol. The butanolic extracts were combined and concentrated to dryness. The obtained residue (327 g) contained **I** and several antibacterial agents including zervamicins **I** and **II**.

The mycelial cake was triturated with methanol. The methanolic extract was concentrated to dryness to give material containing mainly I and small amounts of zervamicins. Crystalline N-acetyl-L-phenylalanyl-L-phenylalaninol was isolated from this fraction by procedures similar to those described below.

Isolation of **I**: The residue obtained by extraction of the filtrate with 1-butanol (*ca.* 327 g) was triturated with 4 liters of chloroform. Insoluble material was separated by filtration and discarded. The filtrate was concentrated to dryness to give 230.0 g of material enriched in the zervamicin antibiotics and **I**. This mixture was then chromatographed over a column containing 7 kg of silica gel (Merck-Darmstadt Art 7034) packed in chloroform - methanol (6 : 1, v/v). The procedure described by ARGOUDELIS, *et al.*[1] was followed. **I** was eluted first in mixture with unidentified as yet antibacterially active metabolites followed by zervamicins. Fractions containing **I** were concentrated to dryness to give 95 g of colorless material. Trituration with the upper phase of the solvent system consisting of ethyl acetate - cyclohexane - 95% aqueous ethanol - water (1 : 1 : 1 : 1, v/v) yielded crystalline N-acetyl-L-phenylalanyl-L-phenylalaninol (75 g) which was recrystallized from 95% ethanol; $[\alpha]_D^{25}$ −40° (*c* 1, 95% ethanol); UV_{max} (95% ethanol)

247(sh) (ε 217), 252 (ε 302), 258 (ε 374), 264 (ε 278) and 287(sh) (ε 187) nm; ir (Nujol) 3440, 3300 (OH, NH), 3030, 1607 (aromatic), 1645 (amide I), 1542 (amide II) cm^{-1}.

Anal. Calcd. for $C_{20}H_{24}N_2O_3$: Mol. wt. 340.1786; C, 70.58; H, 7.05; N, 8.24.
Found: Mol. wt. 340.1756; C, 70.66; H, 7.10; N, 8.30.

Acid Hydrolysis of **I**. Isolation of L-phenylalaninol and L-phenylalanine hydrochloride

A solution of 10 g of **I** in 400 ml of 6 N aqueous hydrochloric acid was kept at reflux for 24 hours. The hydrolysate was concentrated to dryness. The residue was dissolved in 100 ml of water - methanol (1 : 1, v/v) and the solution was chromatographed over a column containing 200 ml of Dowex-1 (X-4) in the hydroxide form. The column was eluted with 700 ml water - methanol (1 : 1) followed by 0.5 N aqueous hydrochloric acid. Fractions were analyzed by tlc [silica gel; 95 % ethanol - water (75 : 25, v/v); spot development with ninhydrin]. L-Phenylalaninol, eluted with water-methanol, was isolated as colorless (needles) crystalline material (1.4 g) $[\alpha]_D^{25}$, −21° (*c* 1, methanol). High resolution mass spectrum indicated molecular formula of $C_9H_{13}NO_2$. L-Phenylalanine was eluted with 0.5 N HCl and was isolated as crystalline hydrochloride (1.8 g). $[\alpha]_D^{25}$, Ir, nmr, mass spectra and tlc were identical to that of authentic sample of L-phenylalanine hydrochloride.

Acknowledgment

The authors express their appreciation to Mr. K. J. GEIPEL for technical assistance, to members of the Physical and Analytical Chemistry Department of The Upjohn Company for analytical and spectra data and to the Fermentation Research and Development Unit of The Upjohn Company for large scale extractions.

References

1) ARGOUDELIS, A. D.; A. DIETZ & L. E. JOHNSON: Zervamicins I and II, polypeptide antibiotics produced by *Emericellopsis salmosynnemata*. J. Antibiotics 27: 321~328, 1974
2) SCOTT, A. I.: Interpretation of the ultraviolet spectra of natural products. Pergamon Press, New York, New York, 1964
3) DESHMUKH, P. V. & M. G. VAIDYA: L-2-Amino-3-phenyl-1-propanol (L-phenylalaninol) as a constituent of a fungal metabolite. Nature 217: 849, 1968
4) ARGOUDELIS, A. D. & L. E. JOHNSON: Emerimicins II, III and IV, antibiotics produced by *Emericellopsis microspora* in media supplemented with *trans*-4-*n*-propyl-L-proline. J. Antibiotics 27: 274~282, 1974.
5) HANKA, L. J.; M. R. BURCH & W. T. SOKOLSKI: Psicofuranine. IV. Microbiological assay. Antibiot. & Chemoth. 9: 432~435, 1959
6) LEWIS, C.; W. CLAPP & J. E. GRADY: *In vitro* and *in vivo* evaluation of lincomycin, a new antibiotic. Antimicr. Agents & Chemoth.-1962: 570~582, 1963