EMERIMICINS II, III AND IV, ANTIBIOTICS PRODUCED BY *EMERICELLOPSIS MICROSPORA* IN MEDIA SUPPLEMENTED WITH *TRANS*-4-*n*-PROPYL-L-PROLINE

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Emerimicins II, III and IV are new antibiotics produced by *Emericellopsis microspora* when 4-*trans*-*n*-propyl-L-proline (propyl proline) is added in the fermentation medium. Emerimicins are similar to antiamoebin and stilbellin. However, all emerimicins have been differentiated from the latter antibiotics by comparison of tlc behavior and aminoacid composition. Propyl proline appears to induce the production of the new antibiotics since the aminoacid was not incorporated into the molecules of emerimicins II, III, or IV.

In the course of work related to the effects of environmental changes on antibiotic production, we observed that a microorganism identified as *Emericellopsis microspora**, grown in a complex medium, produced several antibiotics only when *trans*-4-*n*-propyl-L-proline was added to the medium. Three of these antibiotics named emerimicins II, III and IV** have been isolated crystalline and characterized. The production, isolation and characterization of these antibiotics is the subject of the present communication.

Experimental

Assay and Testing Procedures

Antibiotic production and purification was measured by a microbiological disc-plate assay procedure[1] with *Sarcina lutea* as the assay organism. Antibacterial activities were also determined by broth dilution methods described by LEWIS *et al.*[2]

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.

Spectroscopic Methods

Nuclear magnetic resonance spectra were obtained with a Varian A-60 spectrometer on solutions (*ca* 0.4 ml, *ca* 0.25 M) of the compounds in d_6-dimethylsulfoxide. Infrared spectra were obtained in mineral oil suspension.

Fermentation Procedures

Seed culture of *E. microspora* were prepared in a medium consisting of glucose monohydrate, 10 g/liter; Bacto peptone, 10 g/liter (Difco Laboratories, Inc., Detroit, Michigan, U. S. A.); and

* Taxonomic studies were done by Miss ALMA DIETZ of The Upjohn Company. The culture has been designated as *Emericellopsis microspora*, strain 333.

** The antibiotics are also known as antibiotics EM-2 (U-40588), EM-3 (U-40589), and EM-4 (U-40590), respectively.

Bacto yeast extract, 2.5 g/liter (Difco Laboratories Inc.). The cultures were incubated at 28°C for 96 hours on a rotary shaker. Fermentation medium consisting of glucose monohydrate, 45 g/liter; Buffalo starch, 40 g/liter (CPC International, Englewood Cliffs, New Jersey, U. S. A.); Black strap molasses, 20 g/liter (Knappen Molasses Company, Chicago, Illinois, U. S. A.); Wilson's s. p., granular peptone, 25 g/liter (Wilson Protein Technology, Division of Wilson Pharm. and Chem. Co., Calumet City, Illinois, U. S. A.); calcium carbonate, 8 g/liter; and potassium sulfate, 2 g/liter was adjusted to pH 7.2 and inoculated at a rate of 5% (v/v) with the 96-hour seed culture. Fermentations were incubated at 28°C on a rotary shaker. After 24 hours of fermentation time *trans*-4-*n*-propyl-L-proline was added to the fermentation at the level of 0.5 g/liter. Fermentations were then incubated and analyzed for antibiotic production at 48, 96, 144 and 192 hours after addition of propyl proline. Beers were harvested after a total fermentation time of 144~192 hours.

Isolation of Emerimicins II, III and IV

1. Filtration. Extraction of the Antibiotics

Fermentation broth (*ca* 10 liters), was filtered with the aid of diatomaceous earth. The filter cake was washed with one liter of water and the aqueous wash was combined with the clear filtrate.

The filter cake was triturated with 2 liters of absolute methanol, the methanolic extract was concentrated to dryness, and the dry residue was triturated with 1 liter of methanol. Insoluble material was bioinactive and was discarded. The filtrate was mixed with 5 liters of acetone. The precipitated material, 5.7 g, was also bioinactive. The new filtrate was then mixed with 20 liters of ether. The precipitate formed was isolated by filtration and dried (7.4 g). This material (Preparation A), a white powder, was found to contain emerimicins II, III and IV. Separation of these antibiotics was achieved by silica gel chromatogaphy as described below.

The clear filtrate-wash (*ca* 10 liters) was extracted twice with 2.5 liters portions of 1-butanol. The butanolic extracts were combined and concentrated to dryness. The residue was triturated with acetone. Insoluble material was isolated by filtration and dried (Preparation B, 6.4 g). This material, like the material obtained from the methanolic extract of the cake, contained emerimicins II, III and IV and was treated as described later.

2. Separation of Emerimicins II, III and IV

(1) Isolation of Crystalline Emerimicin IV. Silica Gel Chromatography of Preparation A (Methanolic Extract of the Cake):

A column was prepared from 1.8 kg of silica gel (Merck-Darmstadt Art 7034) packed in the solvent system consisting of chloroform-methanol (6 : 1, v/v). Preparation A, the material obtained by trituration of the filter cake with methanol, was dissolved in 500 ml of the solvent system and the solution was mixed with 100 g of silica gel. The mixture was concentrated to dryness and the obtained powder was added to the column. The column was then eluted with the chloroform-methanol solvent system. Fractions were analyzed for bioactivity and antibiotic composition. A total of 2,100, 20 ml-fractions were collected, then 15 fractions of 2 liters each. Selected fractions were analyzed for bioactivity against *S. lutea* and for antibiotic composition by tlc.

Fractions 1~939 were bioinactive and were discarded.

Fractions 940~1,350, containing emerimicin IV only, were concentrated to dryness to give 1.98 g of colorless crystalline material. Recrystallization of this material from 48 ml of methanol-water (1:1, v/v) gave 1.76 g of crystalline (colorless needles) emerimicin IV. Characterization of this antibiotic is described in another section of this paper.

Fractions 1,351~1,950 contained emerimicins IV and III. They were combined and the solution concentrated to dryness to give a colorless crystalline material. This material was not processed further at this point but it was combined with identical material obtained

from silica gel chromatography of preparation B. The combined preparation was then chromatographed for isolation of emerimicin III as described later.

Fractions 6~13 (2 liters each) containing emerimicin II were combined and concentrated to dryness to give 320 mg of amorphous colorless emerimicin II. This material was combined with emerimicin II obtained from silica gel chromatography of preparation B (butanolic extract, see above) and the combined preparation was treated as described later.

(2) Silica Gel Chromatography of Preparation B (Butanolic Extract of Clear Beer). Isolation of Crystalline Emerimicin II.

The procedure used was in general identical to that described above for the purification of preparation A. The column was prepared from 1 kg of silica gel in chloroform-methanol (6 : 1, v/v). Preparation B (obtained from the butanolic extract of the clear beer) was dissolved in 300 ml of methanol and 600 ml of chloroform and the solution was mixed with 50 g of silica gel. The mixture was concentrated to dryness and the obtained powder was added to the column. The column was eluted as described previously. Selected fractions were examined for bioactivity and antibiotic content. Fractions 270~600 contained 1.4 g of emerimicin IV which was recrystallized from 36 ml of methanol-water to yield 1.2 g of colorless crystals of emerimicin IV.

Fractions 601~1,165 contained both emerimicin III and emerimicin IV. These fractions were combined and concentrated to dryness. This material was treated as described below in the section dealing with isolation of emerimicin III.

Later fractions containing emerimicin II were concentrated to dryness yielding 260 mg of amorphous antibiotic. This preparation was combined with 320 mg of emerimicin II obtained in the chromatography of preparation A (*see* above). The combined preparation was dissolved in 10 ml of hot methanol. This solution was clarified by filtration and mixed with water to a final volume of 45 ml. Emerimicin II, crystallized in colorless needles, was isolated by filtration and dried; yield 280 mg. Characterization of emerimicin II is described in a later part of this communication.

(3) Isolation of Crystalline Emerimicin III. Silica Gel Chromatography,

The column was prepared from 1 kg of silica gel packed in chloroform-methanol (6 : 1, v/v). Preparations containing both emerimicins III and IV, obtained in the two chromatograms described above, were combined and the material obtained (*ca* 2 g) was dissolved in 200 ml of the solvent system. The solution was mixed with 20 g of silica gel, this mixture was concentrated to dryness and the powder obtained was added to the column. The column was eluted with the above-mentioned solvent system. Analysis of selected fractions showed that fractions 470~560 contained emerimicin IV. Fractions 570~610 contained emerimicin IV and traces of emerimicin III. Fractions 630~900 contained emerimicin III only. These fractions were combined and concentrated to dryness to give 650 mg of crystalline material. Recrystallization from methanol-water (10 : 35, v/v) yielded 400 mg of crystalline emerimicin III (colorless needles). Characterization of this material is described later.

Discussion and Results

Production of Emerimicins II, III and IV by *Emericellopsis microspora*

E. microspora produces no antibiotics related to emerimicins when grown in the medium described in this communication. However, addition of *trans*-4-*n*-propyl-L-proline (propyl proline) to the medium results in the production of emerimicins II, III and IV, and other compounds inhibiting the growth of the test organism (*Sarcina lutea*) used in the present studies. The time sequence of production of emerimicins was followed by tlc. A typical thin-layer chromatogram of the three emerimicins discussed in this paper is presented in Fig. 1. Emerimicin IV is the main antibiotic component of the fermentation and can be detected in

culture filtrates as early as 72 hours after addition of propyl proline. Emerimicin III which is produced in smaller quantities can also be detected early in the fermentation (72~96 hours after addition of propyl proline). Emerimicin II, in contrast to emerimicins III and IV, is produced late in the fermentation and can be detected 144 hours after addition of the aminoacid.

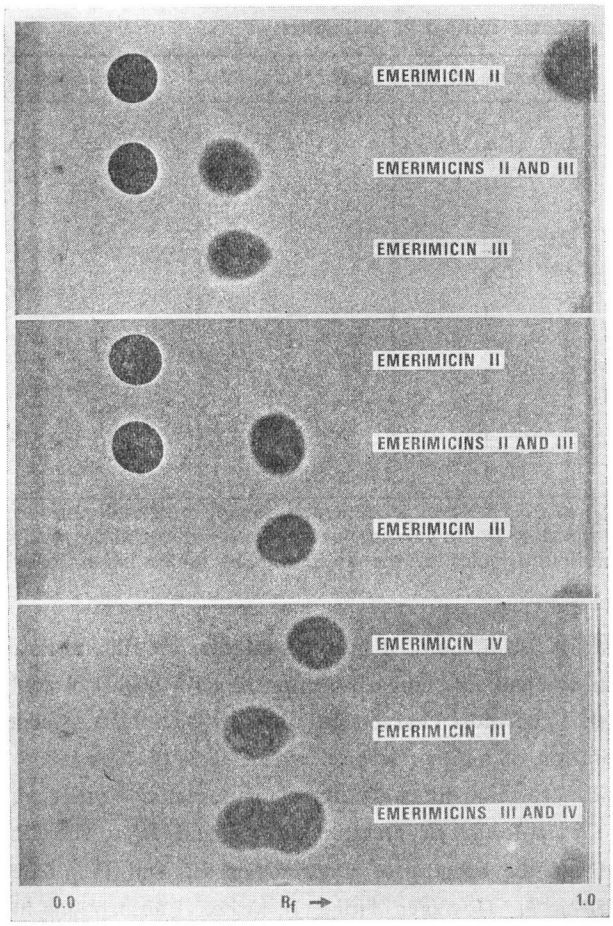
Propyl proline is not incorporated into the molecules of emerimicins II, III or IV (see aminoacid composition, Table 2). Information is not available at present on the role of propyl proline in stimulating the production of the emerimicins. It is of interest that the addition of either L-proline or 4-hydroxy-L-proline to the medium of *E microspora* did not stimulate the production of emerimicins.

Characterization of Emerimicins II, III and IV

All three emerimicins have been isolated as colorless crystalline materials (needles). Emerimicins II, III and IV are soluble in dimethylformamide, dimethyl sulfoxide and lower alcohols. They are less soluble in chlorinated hydrocarbon solvents and ethyl acetate. They are insoluble in acetone and ether and saturated hydrocarbon solvents.

Fig. 1. Thin layer chromatographic comparison of emerimicins II, III and IV

Upper: Emerimicin II
Emerimicin III
Middle: Emerimicin II
Emerimicin IV
Lower: Emerimicin III
Emerimicin IV



Some of the physical properties of emerimicins II, III and IV are presented in Table 1. All emerimicins are slightly dextrorotatory having specific rotations of +5° for II, +12° for III and +13.5° for IV. The compounds melt at temperatures over 200°C (Table 1) with decomposition. The UV spectra of emerimicin III and IV are almost identical with maxima of very low absorptivities at 253, 257, 264, and 267 nm. Emerimicin II shows a completely different UV spectrum with maxima at 273, 281 and 289 nm. The difference in the UV spectra between emerimicin II and emerimicins III and IV extends to other physical (IR, aminoacid camposition) and biological (antibacterial spectrum) properties.

Potentiometric titration showed the absence of any titrable group in all three antibiotics. The infrared spectra (in Nujol mull) of emerimicins

Tabbe 1. Physical properties of emerimicins

	Emerimicin II	Emerimicin III	Emerimicin IV
$[\alpha]_D^{25}$ (*c* 1, methanol)	+5°	+12°	+13.5°
Melting point (unc., dec.)	261°C	257°C	240°C
UV (methanol) λ_{max}, nm (a)	216 (sh) (24.02) 269 (sh) (2.67) 273 (2.81) 281 (2.97) 289 (2.62)	243 (sh) (0.48) 253 (0.43) 257 (0.46) 261 (sh) (0.43) 264 (0.44) 267 (0.41)	247 (sh) (0.26) 252 (0.24) 257 (0.26) 261 (sh) — 264 (0.26) 267 (0.15)
Potentiometric titration*	Neutral	Neutral	Neutral
IR (Nujol) (cm^{-1})	3400, 3292, 3060, 1650, 1636, 1538, 1455	3430, 3395, 3325, 3060, 1680, 1651, 1622, 1613, 1537, 1460	3420, 3390, 3330, 3290, 1690, 1660, 1620, 1535, 1460

* The antibiotics were titrated in 75% aqueous ethanol with either sodium hydroxide or hydrochloric acid.

Table 2. Relative aminoacid content of antibiotics

Aminoacid	Emerimicin II	Emerimicin III	Emerimicin IV	Antiamoebin
Lysine	1	—	—	—
Hydroxyproline	2	2	2	2
Threonine	1	—	—	—
Glutamic acid	2	1	1	1
Proline	1	—	—	1
Glycine	—	1	1	1
Alanine	—	1	—	—
α-Aminoisobutyric acid	1	—	1	1
Valine	*	1	1	*
Isoleucine	2	—	—	—
Leucine	1	1	1	1
Phenylalanine	—	1	1	1

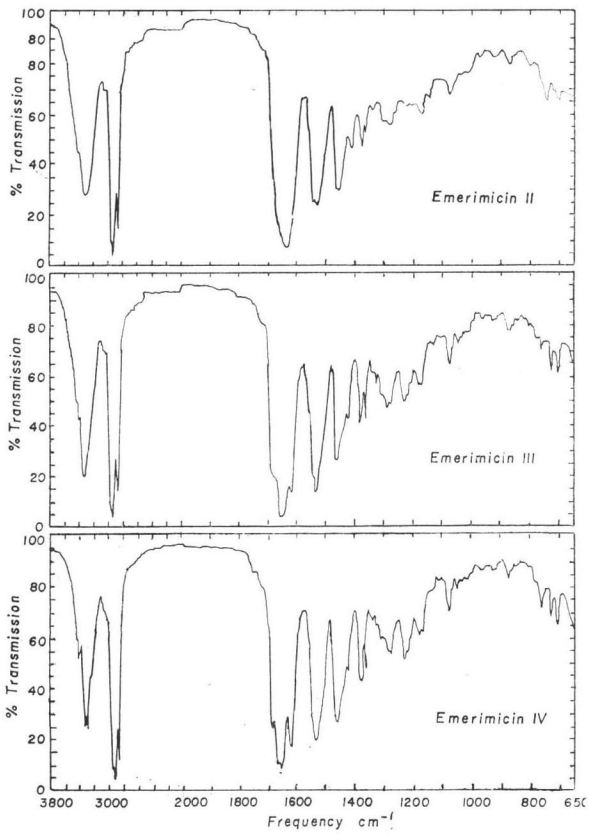
* A small amount of valine corresponding to 0.25 mole of leucine was found in the samples of emerimicin II and antiamoebin. We believe that valine is not present in these antibiotics and the results are artifacts. Aminoacids were determined on hydrolysates of the antibiotics by ARRO Laboratories, Joliet, Illinois, U.S.A.

III and IV (Fig. 2) are very similar so that it is difficult, if not impossible, to differentiate these two compounds using IR data. Both compounds show absorption at *ca* 3400~3420 cm^{-1} due to —NH or —OH stretching vibrations. The most characteristic absorption in the spectra is the absorption due to the stretching vibration of amide carbonyl which appears at *ca* 1680~1690 cm^{-1} (amide I). Another characteristic absorption at *ca* 1620 cm^{-1} is tentatively assigned to the presence of an aromatic system in the molecule of emerimicins III and IV. The IR spectrum of emerimicin II (Fig. 2) differs from the spectra of emerimicins III and IV. The amide I carbonyl absorption appears at 1650 cm^{-1}. However, amide II carbonyl absorptions in

all three emerimicins appear at 1535~1538 cm^{-1}.

The IR spectra of all three emerimicins suggested polypeptide type of antibiotics. This was confirmed by determination of the aminoacid content of these antibiotics. The aminoacid content of antiamoebin, an antibiotic described earlier[3], was also determined under the same conditions for comparison (see later discussion). As shown in Table 2, emerimicins III and IV differ from each other in that alanine, which is present in III, has been replaced by α-aminoisobutyric acid in IV. Emerimicin II differs from both emerimicins III and IV in that it contains lysine, threonine, proline and isoleucine, which are not present in either III or IV. On the other hand, emerimicin II does not contain glycine, valine and phenylalanine present in III and IV, and also does not contain alanine which is present in III. The aminoacids present in emerimicins II, III and IV contribute to *ca* 64, 63 and 59% of the weight of the antibiotics, respectively.

Fig. 2. Infrared spectra of emerimicins (in nujol mull)



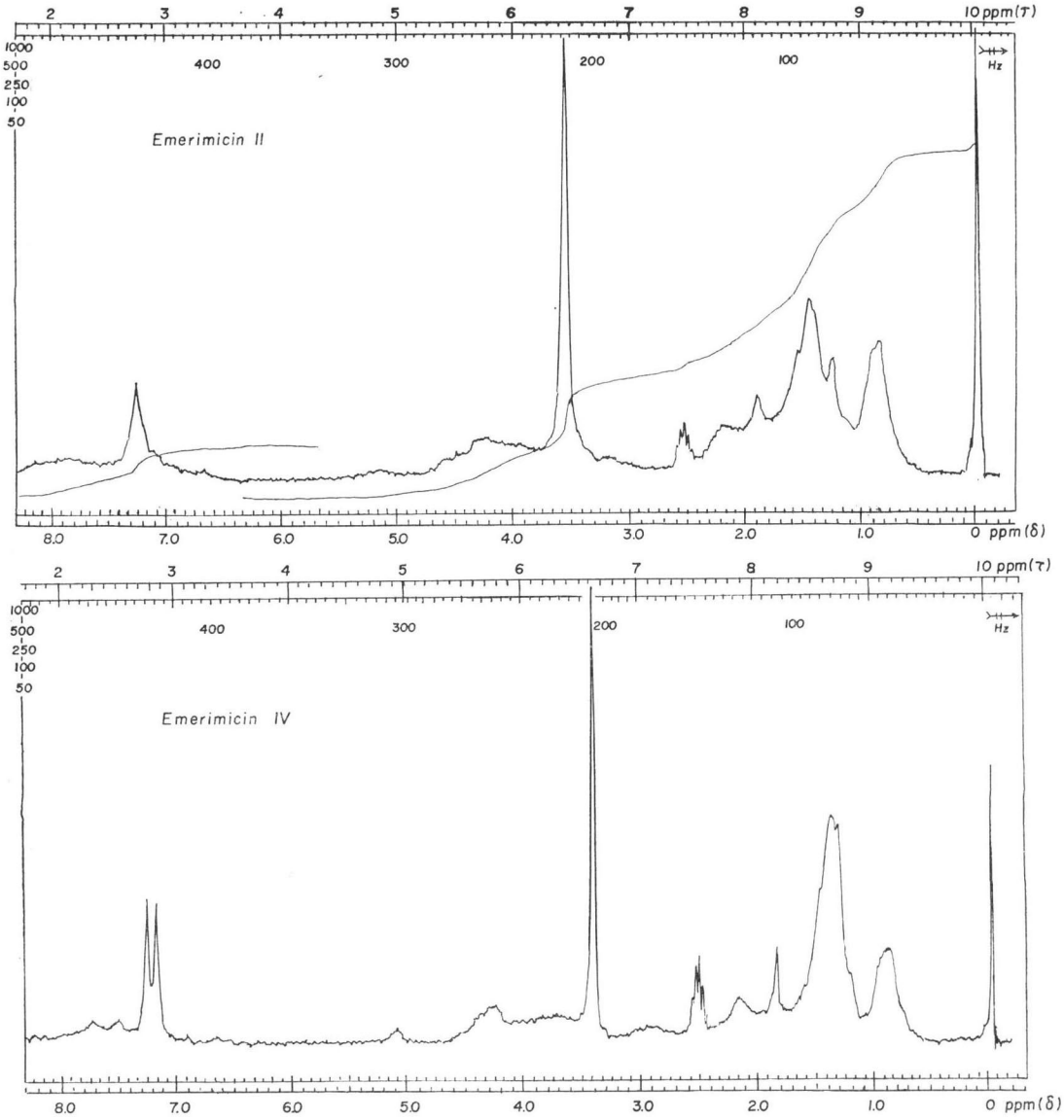
Analytical data (Table 3) obtained on emerimicins II, III and IV suggest the empirical formulas shown in Table 3. Aminoacid composition results (Table 2) indicale that emerimicins II, III and IV are compounds of high molecular weight. Conventional techniques (osmometric methods, mass spectrometry) were not helpful in determining the molecular weights of these antibiotics. However, the mole-

Table 3. Analytical data and molecular formulas of emerimicins

		Emerimicin II	Emerimicin III	Emerimicin IV
Anal. data	C	57.47	58.46	57.26
	H	7.70	7.78	7.45
	N	12.99	13.27	14.22
	O (diff)	21.87	21.49	21.08
	S	None	None	None
	Halogen	None	None	None
Calcd. elem. composition		$(C_{5.15}H_{8.28}NO_{1.46})_n$	$(C_{5.13}H_{8.18}NO_{1.41})_n$	$(C_{4.68}H_{7.30}NO_{1.29})_n$
Estimated mol. weight*		1929	1412	1705
Suggested mol. formula*		$C_{91}H_{146}N_{18}O_{26}$	$C_{68}H_{109}N_{13}O_{19}$	$C_{81}H_{127}N_{17}O_{22}$

* For discussion related to the estimation of the molecular weights and molecular formulas *see* text.

Fig. 3. Proton magnetic resonance spectra of emerimicins II and III (in d_6-DMSO)



cular size of emerimicins could be approximately estimated based on the aminoacid content results.

For example, emerimicin II would nave a minimum molecular weight of 1,234 if the aminoacids listed in Table 2 were the only moieties present in the antibiotic. However, we have determined that the aminoacids present in emerimicin II contribute to 64% of the weight of the antibiotic. We concluded, therefore, that the minimum molecular weight of emerimicin II is *ca* 1,929. Similarly, we estimated the approximate minimum molecular weights of emerimicins III and IV as being 1,412 and 1,705, respectively. On the basis of these assumptions we suggest the molecular formulas listed in Table 3 as approximate molecular formulas for emerimicins, II, III and IV.

Table 4. Antibacterial spectra of emerimicins* II, III and IV and antiamoebin

Test organism	Minimum inhibitory concentration (mcg/ml)			
	Emerimicin II	Emerimicin III	Emerimicin IV	Antiamoebin
Staphylococcus aureus UC 76	4	31	31	62
Staphylococcus aureus UC 552	8	31	62	125
Streptococcus hemolyticus UC 152	2	16	31	62
Streptococcus faecalis UC 3235	8	62	62	125
Escherichia coli UC 51	>500	>250	500	500
Proteus vulgaris UC 93	>500	>255	500	500
Klebsiella pneumoniae UC 57	>500	>250	500	500
Salmonella schottmuelleri UC 126	500	>250	500	500
Pseudomonas aeruginosa UC 95	500	>250	500	500
Bacillus subtilis UC 564	8	31	31	31
Diplococcus pneumoniae UC 41	2	31	62	125
Sarcina lutea UC 130	5	8	16	31

* Test method: Two-fold dilution endpoints in brain heart infusion broth; incubate at 37°C for 20 hours.

The proton magnetic resonance spectra of emerimicins II and IV (Fig. 3) offer little help in determining the nature of the antibiotics. The spectra are presented in this paper as an additional aid for the differentiation of emerimicins from other antibiotics.

Relation of Emerimicins II, III and IV to Other Antibiotics

Since emerimicins are produced by *E. microspora*, the three antibiotics were compared to other antibiotics produced by *Emericellopsis* species. Several investigators[4] have reported the production of β-lactam antibiotics, specifically penicillin N and cephalosporin C by different *Emericellopsis* species. Emerimicins, as evidenced by the data discussed in the characterization section, do not belong to the β-lactam family of antibacterial agents. COLE and ROBINSON[5] reported the production of 6-aminopenicillanic acid and two antibiotics designated emericellopsins A and B by *Emericellopsis minima*. Emericellopsin A was considered to be a penicillin since it was inactivated by β-lactamase. No chemical information is available on emericellopsin B. THIRUMALACHAR[3] described the production of antiamoebin by *Emericellopsis poonensis*, *Emericellopsis synnematicola* and *Cephalosporium pimprina*. Antiamoebin, like the emerimicins, is a neutral polypeptide[6] with properties resembling those of emerimicins and specifically emerimicins III and IV. All three emerimicins were, therefore, directly compared to antiamoebin. Thin-layer chromatography on silica gel using chloroform - methanol (6 : 1, v/v) or ethyl acetate - acetone - water (8 : 5 : 1, v/v) separated all emerimicins from antiamoebin. In addition the aminoacid composition of antiamoebin* was determined under identical conditions used for the emerimicins. As shown in Table 2 the aminoacid composition of antiamoebin is radically different from that of emerimicin II. Emerimicins III and IV do not contain proline, present in antiamoebin, and contain valine which is not present in antiamoebin. Furthermore, emerimicin III contains alanine not present in antiamoebin.

Another antibiotic resembling the emerimicins is stilbellin[7] produced by the fungus *Stilbella*

* The aminoacid composition of antiamoebin was found to be as reported by DESMHMUKH[6].

sp. This neutral polypeptide antibiotic has aminoacid composition identical to thal of antiamoebin and has been separated by tlc from the emerimicins, but not from antiamoebin.

Emericellopsis species have been reported[8] to produce cephalosporin P antibiotics which are steroid-like compounds and therefore different from emerimicins II, III or IV.

Biological Properties of Emerimicins II, III and IV

The *in vitro* antibacterial spectra of the emerimicins are presented in Table 4. The antibotics were active mainly against Gram-positive organisms. Emerimicins II, III or IV do not possess significant *in vitro* antifungal activity, but showed *in vitro* activity against selected protozoa (*ochromonas danica*, *Crythidia fasciculata*, and *Tetrahymena pyriformis*) at concentrations ranging from 50 to 100 mcg/ml. *In vivo* evaluation of emerimicins II and IV is in process.

Acknowledgment

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