MELINACIDINS, A NEW FAMILY OF ANTIBIOTICS

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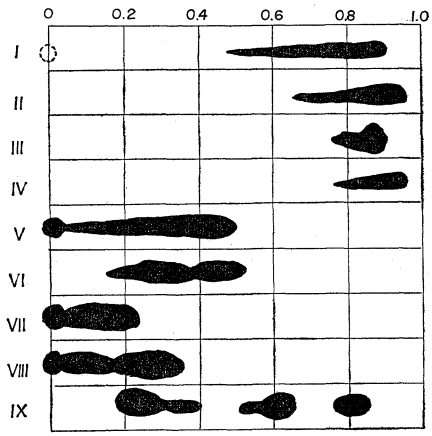
Melinacidin is a crystalline mixture of closely related antibacterial agents produced by *Acrostalagmus cinnabarinus* var. *melinacidinus*. Melinacidin inhibits a variety of Gram-positive bacteria *in vitro* but is found to be toxic and ineffective in the treatment of experimental bacterial infections in mice. Melinacidin which inhibits the growth of KB and L-1210 cells in tissue culture appears to belong to the "3,6-epidithiadiketopiperazine" group of antibiotics.

Melinacidins are new antibiotic substances produced by *Acrostalagmus cinnabarinus* var. *melinacidinus**. The paper-chromatographic pattern of the mixture of antibiotics produced by this organism and isolated by the procedures outlined in the following sections is shown in Fig. 1. The present communication describes the work which led to the isolation of the crystalline mixture of melinacidins as well as the physical, chemical and biological properties of this mixture which is called "melinacidin" throughout this paper. Individual components will be designated melinacidin, II, III, IV *etc.*, and will be described in later communications.

Fig. 1. Paper chromatographic pattern of melinacidin.

Solvent systems:
I. 1-butanol, water (84 : 16)
II. 1-butanol, water (84 : 16)+0.25% *p*-toluene-sulfonic acid
III. 1-butanol, acetic acid, water (2 : 1 : 1)
IV. 2% piperidine (v/v) in 1-butanol, water (84 : 16)
V. 1-butanol, water (4 : 96)
VI. 1-butanol, water (4 : 96), +0.25% *p*-toluene-sulfonic acid
VII. 0.5 M phosphate buffer pH 7.0
VIII. 0.075 N NH_4OH saturated with methyl isobutyl ketone, lower phase
IX. Benzene, methanol, water (1 : 1 : 2)



Experimental

Assay and Testing Procedures

Antibiotic production was measured by a microbiological disc-plate assay procedure[1] with *Bacillus subtilis*, growing in a synthetic medium, as the test organism. Samples of the mixture of antibiotics to be assayed were diluted with phosphate buffer, pH 7.85. The ingredients of the synthetic medium are presented in Table 1. The molten agar was seeded with an overnight culture of the microorganism and allowed to solidify. The fermentation broths or preparations to be tested were applied onto 12.7 mm paper discs and these were placed on the surface of the seeded agar and incubated at 37°C for 18 hours. A biounit is that amount of antibiotic which gives a 20-mm zone of inhibition under the assay conditions described above.

* The organism has been studied and classified by Dr. KURT E. WEINKE.

In vitro and *in vivo* antibacterial activities were determined by the methods described by LEWIS *et al*[2].

Antifungal tests were done on agar plates. Antibiotic was dissolved in fungal spectrum agar to give concentrations of 1,000, 100, 10 or 1 mcg/ml. Plates were inoculated by the cross-streak technique. Results are expressed as minimal inhibitory concentrations of antibiotic yielding total inhibition of fungal growth.

Inhibition of KB cell growth was measured by the method of SMITH *et al.*[3]

Table 1. Ingredients of synthetic assay medium*

$Na_2HPO_4 \cdot 7H_2O$	1.5 g
KH_2PO_4	4.3 g
$(NH_4)_2SO_4$	1.0 g
$MgSO_4$	0.1 g
Glucose	2.0 mg
Agar	15.0 g
Dist. water	1 liter
Metallic ion stock solution**	1 ml

* The pH is adjusted to 6.2

** Metallic ion stock solution: $NaMoO_4 \cdot 2H_2O$ 0.2 mg/ml, $CoCl_2$ 0.1 mg/ml, $CuSO_4$ 0.1 mg/ml, $MnSO_4$ 2.0 mg/ml, $CaCl_2$ 25.0 mg/ml, $FeCl_2 \cdot 4H_2O$ 5.0 mg/ml, $ZnCl_2$ 5.0 mg/ml.

Fermentation Procedures

Shake Flask-Fermentations. Seed cultures of *A. cinnabarinus* were prepared in a medium consisting of glucose monohydrate, 25 g/liter and Pharmamedia (Trader's Oil Mill Co., Fort Worth, Texas), 25 g/liter. The pH was adjusted to 7.2 with aqueous sodium hydroxide before sterilization. The cultures were incubated at 25°C for 72 hours on a rotary shaker (250 rpm, 6-cm stroke). Fermentation medium consisting of Blackstrap molasses (Knappen Milling Co., Augusta, Michigan), 25 g/liter and Yellow Cornmeal (Wilson's Corn Products Inc., Rochester, Indiana), 25 g/liter, was inoculated at a rate of 5 % (v/v) with the 72-hour seed culture. Fermentation flasks were incubated at 25°C on a rotary shaker as mentioned above. Antibiotic production was measured by the *B. subtilis* assay discussed in the previous section. Peak antibiotic titers were usually obtained after 120 hours.

Tank Fermentations. *A. cinnabarinus* was used to inoculate a series of 500-ml Erlenmeyer flasks, each containing 100 ml of preseed medium consisting of glucose monohydrate, 25 g/liter and Pharmamedia, 25 g/liter. The flasks were incubated at 25°C for 96 hours on a rotary shaker. Three flasks (300 ml) of this preseed culture were then used to inoculate a tank containing 250 liters of seed medium consisting of latose, 20 g/liter; glucose monohydrate, 5 g/liter; urea, 1 g/liter; KH_2PO_4, 0.5 g/liter; Distiller's solubles (Brown-Forman Distillers Co., P.O. Box 1080, Louisville, Kentucky, 40201), 40 g/liter; and UCON antifoam (Union Carbide, Chemical Division, 1042 West 7 Mile Road, Detroit, Michigan), 100 ml. The seed tank was agitated at an impeller speed of 280 rpm. Sterile air was supplied at the rate of 80 liters/minute and the tank was kept at 25°C for 48 hours. This seed tank was used to inoculate a tank containing 5,000 liters of the medium consisting of Blackstrap molasses, 25 g/liter; Yellow cornmeal, 25 g/liter and UCON, 5 liters. The fermentor was agitated at an impeller speed of 166 rpm and sterile air was supplied to the fermentor at the rate of 2,000 liters/minute. The fermentor was maintained at 25°C and the culture broth was harvested after 120 hours.

Isolation of Melinacidin

Extraction of Clear Broth. Whole broth (4,700 liters) obtained as described above, was filtered with diatomaceous earth. The clear beer was adjusted to pH 7.0 with aqueous sulfuric acid solution. Sodium chloride (*ca.* 200 kg) was added to the clear beer and this solution was then extracted three times with 1,200-liter portions of methylene chloride each. The methylene chloride extracts were combined and concentrated to a volume of 4 liters. This concentrate was added to 200 liters of Skellysolve B. The precipitated crude mixture of melinacidins was isolated by filtration and dried; yield 96.0 g. An equal amount of crude melinacidin can be isolated from the mycelial cake by trituration of the cake with methanol, concentration of the methanolic extract to an aqueous solution and extraction of this solution with methylene chloride.

Purification. Florisil Chromatography. The crude mixture of melinacidins, isolated as described above, was chromatographed over a column prepared from 4,200 g of Florisil packed in Skellysolve B. The preparation of melinacidins (96 g) was dissolved in methylene chloride and this solution was then mixed with 340 g of Florisil. The mixture was concentrated to dryness and the dry residue was added on the top of the Florisil column. The column was eluted with 5 liters of Skellysolve B, followed by 20 liters of acetone–Skellysolve B (30 : 70, v/v) mixture. Both fractions were found bioinactive and were discarded. The column was then eluted with 50 liters of acetone Skellysolve B (60 : 40, v/v). This eluate was concentrated to dryness to give 62.8 g of highly purified, colorless melinacidin.

Crystallization of Melinacidin. Two g of melinacidin obtained by the Florisil chromatography described above, was dissolved in 100 ml of acetone. The solution was clarified by filtration and then concentrated to dryness. The residue was triturated with 15 ml of 95 % aqueous ethanol. Crystallization started almost immediately. The crystals were allowed to stand at room temperature for 2 hours, then isolated by filtration, washed with 5 ml of 95 % ethanol and dried; yield 600 mg. Crystalline material obtained by this procedure was used for the biological studies and the characterization work reported in the next sections.

Results and Discussion

Characterization of Melinacidin

As mentioned earlier, culture filtrates of *A. cinnabarinus*, grown under the conditions described in detail in the experimental section, were found to contain several antibiotics. A typical paper chromatographic pattern of fermentation broth at harvest time is presented in Fig. 1. Two preparations (A and B) of crystalline melinacidin have been characterized. Paper-chlomatograms (Fig. 2) of either material showed the presence of four bioactive components well separated from each other. However the presence of additional activities with Rf values identical to those of any of the four antibiotics shown in Fig. 2 could not be excluded.

Melinacidin is a colorless crystalline (needles from ethanol) material soluble in chloroform, methylene chloride, dimethylformamide and dimethylsulfoxide. It is less soluble in acetone or 95 % ethanol and is essentially insoluble in water or saturated hydrocarbon solvents. Physical properties of melinacidin* are listed in Table 2. The antibiotic is neutral and strongly dextrorotatory ($[\alpha]_D^{25}$ of +736~786°). The ultraviolet spectra showed a shoulder at 241 nm and a single maximum at 300 nm. The infrared spectrum (Fig. 3) had absorptions at 3480, 3400, and characteristic bands at 1699 to 1635 cm^{-1} and at 1609 and 1599 cm^{-1}. Analytical data showed the presence of only C, H, N, O, S. Although calculation of an empirical formula from analytical data obtained on a mixture is meaningless, it was interesting to note that the analytical data suggested a formula of $C_{16}H_{17}N_3S_2O_4$ (calcd. C 50.66, H 4.48, N 11.08, S 16.89, O 16.89). The same formula has been postulated by GEIGER[4] for chetomin an antibiotic substance originally described by WAKSMAN and BUGIE[5] and by GEIGER *et al.*[6] Chetomin is reported to be produced by *Chaetomium cochliodes*[5,6]. However

* Preparative scale chromatography of crystalline melinacidin afforded small amounts of the individual components. The materials obtained had similar UV and IR spectra and similar antibacterial spectrum. This is taken as evidence that the four components present in crystalline melinacidin (Fig. 2) are closely related antibiotics.

Fig. 2. Paper chromatogram of crystalline melinacidin.

A, B: Two different preparations of crystalline melinacidin.
Solvent: System IX (See Fig. 1)
Antibiotics were detected by bioautography on *B. subtilis* seeded agar.

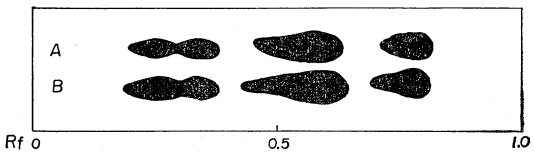


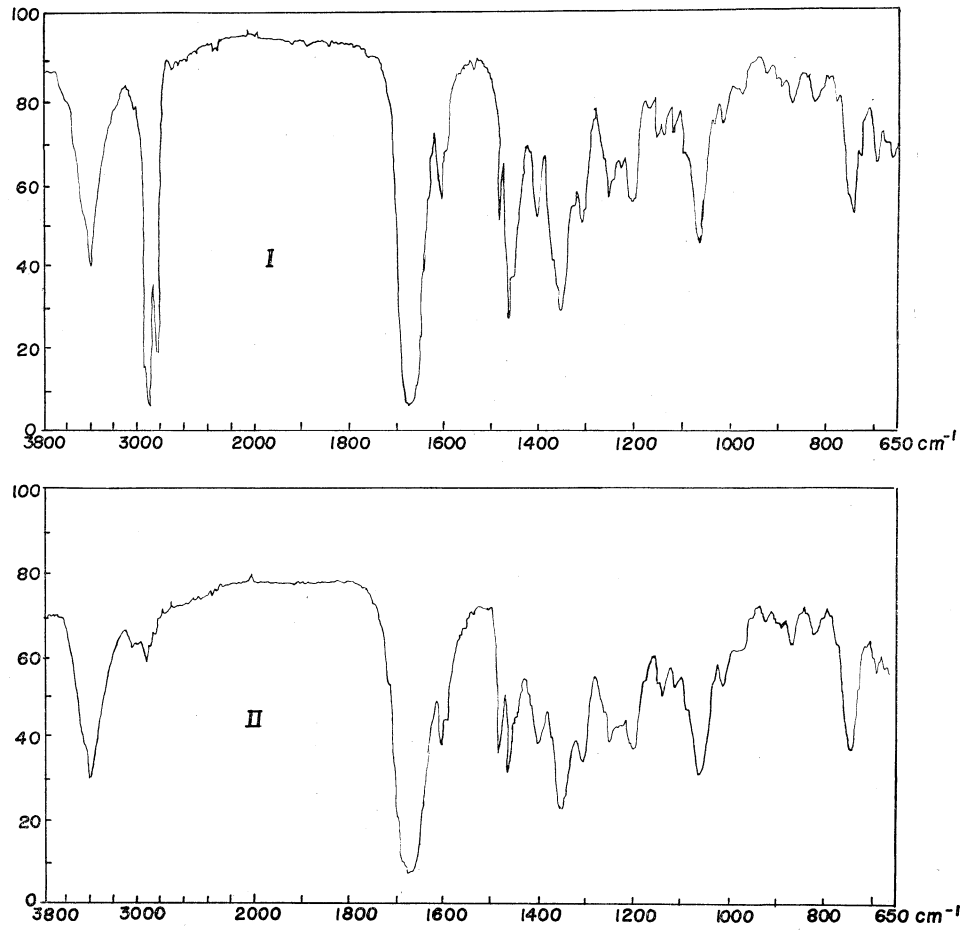
Table 2. Properties* of melinacidin

		Prep. A	Prep. B
Anal.	C	50.33	49.85
	H	4.32	4.21
	N	10.51	10.23
	O	16.52	15.47
	S	17.31	17.71
	Halogen	none	none
Melt. point		228~231°C (uncor.)	
Potent. Titration		Neutral	Neutral
$[\alpha]_D^{25}$		+736° (c 0.4, $CHCl_3$)	+786° (c 0.4, $CHCl_3$)
UV. (Methanol) λ_{max} (a)		241 (sh) (21) 300 (7.93)	241 (sh) (20) 300 (7.90)
Mol. Weight**		1,290	1,380

* The antibiotic composition of preparations A and B is shown in Fig. 2.
** The molecular weight was determined by vapor pressure osmometry in chloroform.

Fig. 3. Infrared spectra of crystalline melinacidin.

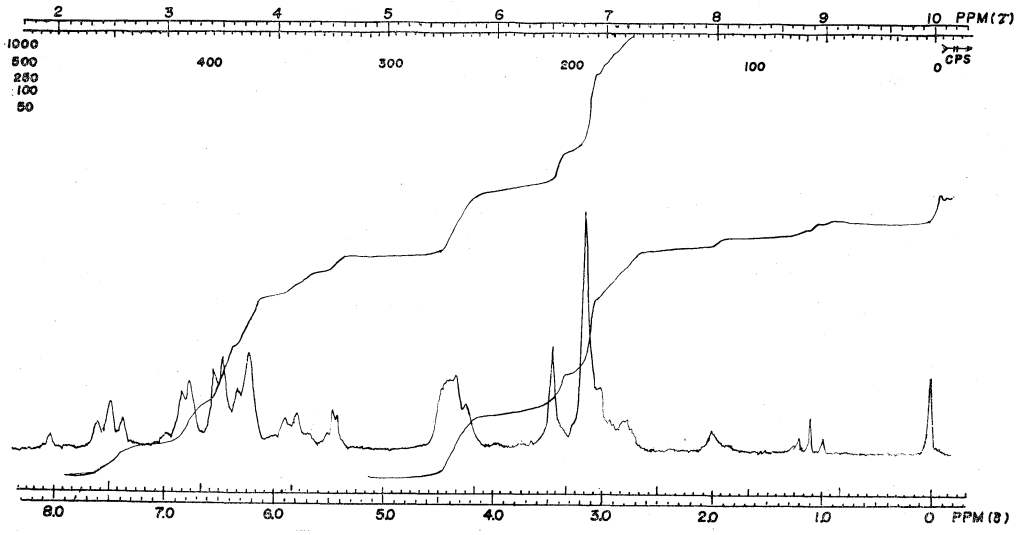
Upper (I) : Nujol mull
Lower (II) : KBr pellet



GEIGER[4] reports that *Verticillium cinnabarinum* known also as *Acrostalagmus cinnabarinus* has been found to produce chetomin. Since *A. cinnabarinus* produces melinacidin, crystalline as well as impure melinacidin were compared to chetomin* by thin-layer chromatography (silica gel G; toluene-ethyl acetate [1:1, v/v] or methylene chloride-ethyl acetate [70:30, v/v] as solvent systems). All components present in the melinacidin preparations separated from chetomin. The infrared spectra of melinacidin and chetomin were found to differ slightly and clearly indicated that melinacidin belongs to the "3,6-epidithiadiketopiperazine" group of antibiotics which in addition to chetomin includes gliotoxin[7], sporidesmins[8], aranotins[9], chaetocin[10], verticillin A[11], and possibly oryzachlorin.[12]

Fig. 4. Nuclear magnetic resonance spectrum* of crystalline melinacidin.

* NMR spectra were observed with a Varian A-60 spectrometer on solutions (*ca.* 0.4 ml, *ca.* 0.25 M) of the compounds in d_7-dimethylformamide.



Melinacidin can be differentiated easily from gliotoxin by paper chromatography. Sporidesmins are characterized by the presence of chlorine and two $-OCH_3$ groups. Melinacidin does not contain chlorine and its NMR spectrum (Fig. 4) shows the absence of $-OCH_3$ groups. Aranotins contain a $-OCOCH_3$ grouping, which, as shown by NMR is not present in melinacidin. Oryzachlorin can be eliminated from consideration since it contains chlorine. Chaetocin and verticillin A exhibit strong dextrorotatory properties [+789° (DMSO) for chaetocin, and +727.5° (dioxane) for verticillin A] and in this respect they resemble melinacidin (Table 2). All three materials, which are produced by related fungi, have similar infrared spectra but they can be differentiated from their NMR spectra**. Therefore the melinacidin

* Chetomin, sporidesmin and IR and NMR spectra of those compounds were kindly provided by Dr. A. TAYLOR, Atlantic Regional Laboratory, National Research Council, Halifax, Canada.

** We would like to express our thanks to Dr. Z. KIS, Chemical Research, Pharmaceutical Division, Sandoz Ltd., for the NMR spectrum of chaetocin and samples of the antibiotic. The NMR spectrum of verticillin has been published[8].

Table 3. *In vitro* antibacterial spectrum of crystalline melinacidin

Test organisms	Minimal inhibitory concentration (mcg/ml)
Bacillus subtilis UC-564	< 1.0
Staphylococcus aureus UC-70	< 1.5
Staphylococcus aureus UC-76	< 1.5
Streptococcus viridans UC-155	< 1.5
Streptococcus hemolyticus UC-152	< 1.5
Streptococcus faecalis UC-157	3.0
Escherichia coli UC-51	>50
Proteus vulgaris UC-93	25
Klebsiella pneumoniae UC-57	>50
Salmonella pullorum UC-267	25
Salmonella typhosa UC-215	>50
Salmonella paratyphi UC-263	>50
Pseudomonas aeruginosa UC-95	>50

Test method: Two fold dilution endpoints in brain heart infusion broth. Read after 20 hours of incubation.

Table 4. *In vitro* antifungal spectrum* of crystalline melinacidin

Test organisms	Minimal inhibitory concentration (mcg/ml)
Nocardia asteroides UC-2052	10
Blastomyces dermatitidis UC-1911	1000
Coccidioides immitis UC-1119	>1000
Geotrichum sp. UC-1207	>1000
Hormodendruw compactum UC-1222	>1000
Cryptococcus neoformans UC-1139	>1000
Histoplasma capsulatum UC-1220	>1000
Sporotrichum schenckii UC-1364	>1000
Monosporium apiospermum UC-1248	>1000
Trichophyton rubrum UC-1458	>1000
T. interdigitale UC-1399	>1000
T. violaceum UC-1459	>1000
T. asteroides UC-4775	>1000
T. mentagrophytes UC-4797	>1000
T. mentagrophytes UC-4860	>1000
Candida albicans UC-1077	>1000

* Agar dilution test: *see* Experimental.

antibiotics* are new bioactive materials closely related to the antibiotics mentioned in the present discussion.

Biological Properties of Melinacidin

The *in vitro* antibacterial spectrum of melinacidin is presented in Table 3. The antibiotic was found highly active against Gram-positive but not against Gram-negative bacteria (with the exception of *Proteus vulgaris*). However, melinacidin failed to protect mice infected with *Staphylococcus aureus* when administered subcutaneously at the maximum tolerated dose of 1 mg/kg. Of the pathogenic fungi tested *in vitro* (Table 4), melinacidin inhibited only *Nocardia asteroides* at 10 mcg/ml and *Blastomyces dermatitidis* at 1,000 mcg/ml. Melinacidin was found to inhibit the growth of KB cells in tissue culture[3] [ID_{50} (50 % inhibition of protein synthesis), 0.014 mcg/ml], and showed marginal *in vivo* activity in mice infected with Herpes virus. The mode of action of melinacidin has been studied by REUSSER[13]. It appears that the antibiotic blocks the synthesis of nicotinic acid and its amide in *B. subtilis* cells. Specifically melinacidin interferes with a reaction which occurs before the formation of quinolinic acid in the biosynthetic pathway leading to nicotinic acid.

Acknowledgements

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* Three components of melinacidin have been recently isolated crystalline, free from each other and have been partially characterized. All three are related to the "3,6-epidithiadiketopiperazine" antibiotics mentioned. However, they are different from all of them including chaetocin and verticillin A.

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