MELINACIDINS II, III AND IV, NEW "3,6-EPIDITHIADIKETOPIPERAZINE" ANTIBIOTICS

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Melinacidins II, III and IV are new antibacterial agents produced by *Acrostalagmus cinnabarinus* var. *melinacidinus*. All three melinacidins belong to the "3,6-epidithiadiketopiperazine" group of antibiotics, and appear to be related to but distinct from chaetocin and verticillin A.

We have recently reported the isolation and physicohemical and biological properties of melinacidin[1], a crystalline mixture of related antibacterial agents produced by a fungus identified as *Acrostalagmus cinnabarinus**. Melinacidin isolated as colorless needles from ethanol, was found to contain several bioactive compounds. Paper chromatograms indicated at least four antibiotics to be present in the mixture[1]. However, the presence of additional activities with Rf values identical to those of any of the four antibiotics could not be excluded.

The present communication describes the isolation of three of the components of the mixture which have been designated melinacidin II, melinacidin III and melinacidin IV, respectively. The properties of these compounds and their relationship to other known antibiotics are also discussed in this paper.

Experimental

Assay and Testing Procedures

Antibiotic production and purification was measured by a microbiological disc-plate assay procedure[3] with *Bacillus subtilis* growing in a synthetic medium. Composition of this medium and details of the assay procedures have been reported by ARGOUDELIS and REUSSER[1].

In vitro and *in vivo* antibacterial activities were determined by the methods described by LEWIS *et al.*[4]

Antifungal tests were done on agar plates. Antibiotics were 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 or L-1210 cell growth was measured by the methods of SMITH *et al.*[5] and BUSKIRK[20], respectively.

Paper and Thin-Layer Chromatographic Procecdures

Melinacidins II, III und IV were differentiated from each other and from other related antibiotics by paper chromatography using benzene - methanol - water (1 : 1 : 2) as the

* The organism has been studied and classified by Dr. KURT WEINKE. Microbiological and mode of action studies[2] on these compounds were done by Dr. FRITZ REUSSER of the Infectious Diseases Unit of The Upjohn Company.

solvent system. Antibiotics were detected by bioautography on *B. subtilis*-seeded agar.

Thin-layer chromatograms were run on silica gel G using toluene - ethyl acetate mixtures (50 : 50 or 60 : 40 v/v) or methylene chloride - ethyl acetate (70 : 30 v/v) as solvent systems. Antibiotics were detected either by bioautography (see above) or by spraying with periodate-permanganate spray reagent.

Spectroscopic Methods

The infrared spectra were obtained in mineral oil suspension. Nuclear magnetic resonance spectra were observed with a Varian A-60 spetrometer on solutions (*ca* 0.4 ml, *ca* 0.25 M) of the compounds in D_6-dimethyl sulfoxide.

Preparation of Melinacidin

Fermentation as well as isolation procedures which yielded crystalline melinacidin have been reported by ARGOUDELIS and REUSSER[1]. The crystalline mixture obtained by these methods was used as the starting material for isolation of melinacidins II, III and IV.

Isolation of Melinacidins II and III

A column was prepared from 2 kg of silica gel (Merck-Darmstadt, 7734) packed in the solvent system consisting of toluene - ethyl acetate (3 : 1 v/v). Six grams of crystalline melinacidin[1] was dissolved in 2 liters of chloroform. This solution was mixed with 100 g of silica gel and the mixture was concentrated to dryness. The obtained powder was added on the top of the column and the column was then eluted with the solvent system described above. Fractions (2 liters each) were analyzed by bioactivity measurements, solids determination, UV spectra and thin-layer chromatography. The first six fractions were discarded. Fractions 7~14 were combined and this solution was concentrated to a volume of 25 ml. Crystalline colorless material, precipitated during concentration, was isolated by filtration and dried; yield 0.75 g. This material was found to contain melinacidin II.

Fractions 15, 16 and 17 were combined and concentrated to a volume of 25 ml. Crystalline material isolated (0.75 g) contained both melinacidin II and melinacidin III.

Fractions 18~29 were treated similarly to yield 2.5 g of material containing melinacidin III. Elution of the column with an additional 56 liters of selvent afforded materials which were mixtures of melinacidin III and other as yet unidentified components.

Isolation of Melinacidin IV

A. Counter Double Current Distribution: The solvent system used consisted of equal amounts of cyclohexane - ethyl acetate, 95% ethanol and water. The starting material, 5.0 g of crystalline melinacidin was dissolved in 500 ml of each phase. The two phases were separated and added in the twenty center tubes of an all glass counter double current distribution apparatus (100 tubes, 25 ml/phase). After 40 transfers, both the upper and lower phase coming out of the apparatus were collected in 25 ml fractions. A total of 90 transfers were run. Both the collected fractions and the tubes in the machine were analyzed for antibiotic content by solids determination, UV and thin-layer chromatography. Fractions containing melinacidin IV were combined and concentrated to dryness; yield, 3.1 g of material enriched in melinacidin IV. Melinacidin III and traces of melinacidin II were also present in this preparation.

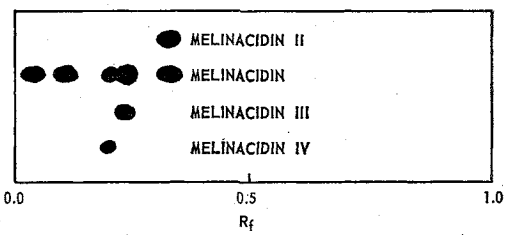
B. Countercurrent Distribution: The solvent system used was identical to that described above. The starting material, 3.0 g of the preparation obtained by the counter double current distribution described previously, was dissolved in 250 ml of each phase and addded in 25 tubes of an all glass countercurrent distribution apparatus (500 tubes, 10 ml/phase). The distribution was analyzed after 1,000 transfers by thin-layer chromatography. Tubes 260~300 were found to contain melinacidin IV; they were combined and the solution was concentrated to a volume of 10 ml. The precipitated crystalline melinacidin IV was isolated by filtration; yield 300 mg.

Results and Discussion

Isolation of Melinacidins II, III and IV

The thin-layer chromatographic comparison of melinacidin and melinacidins II, III and IV is presented in Fig. 1. As indicated in the thin-layer chromatogram melinacidin II is well separated from the other components, and therefore this antibiotic was isolated easily by silica gel chromatography as described in the experimental section. Crystalline melinacidin III was eluted from the silica gel column immediately after melinacidin II. Melinacidin IV, however, was eluted in mixture with melinacidin III and consequently a rather tedious procedure involving counter double current and counter current distribution was used for the isolation of this antibiotic. From the amount of the isolated melinacidins it is concluded that melinacidin III is produced in larger amounts followed by melinacidins II and IV.

Fig. 1. Thin-layer chromatography of melinacidins.*



* Silica gel G; Toluene-ethyl acetate (60 : 40). Spots were detected by periodate-permanganate reagent.

Characterization and Properties of Melinacidins

All three melinacidins have been isolated as colorless crystalline materials,

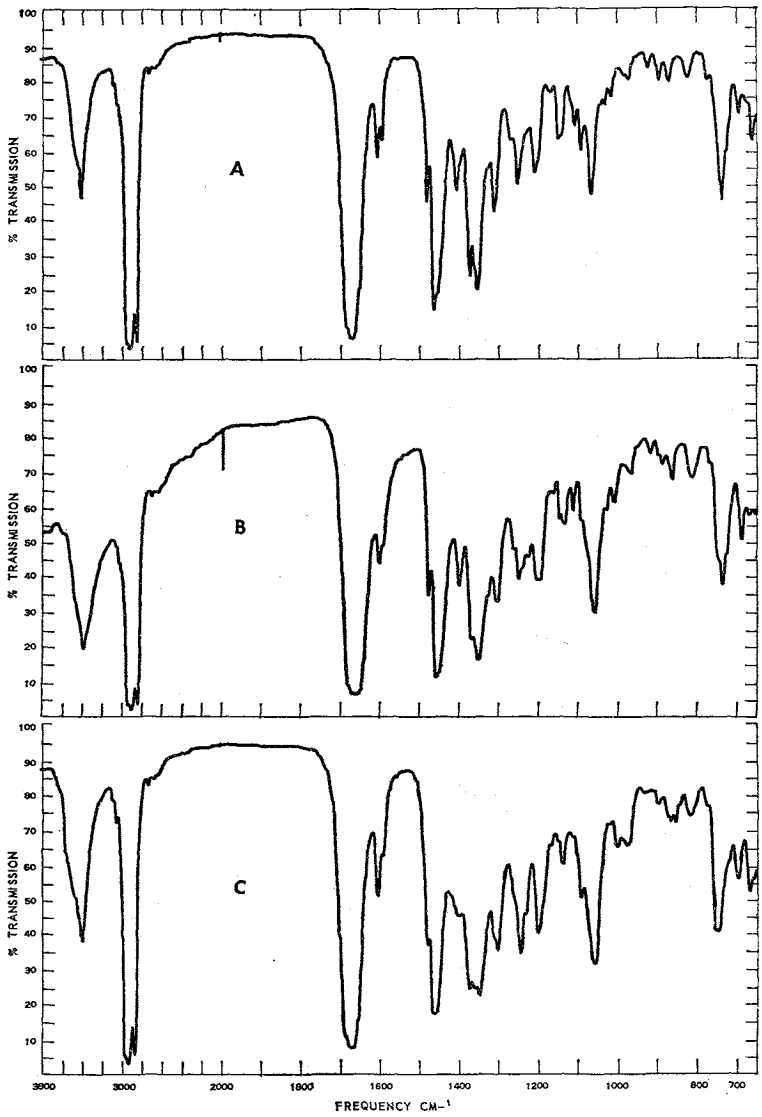
Table 1. Analytical data and molecular formulas of melinacidins

		Melinacidin	Melinacidin II	Melinacidin III	Melinacidin IV
Anal. Data	C	50.33	53.90	51.49	49.55
	H	4.32	4.64	4.15	4.13
	N	10.51	10.96	11.45	11.12
	O	16.52	—	—	—
	S	17.31	16.99	17.44	17.47
	Halogen	None	None	None	None
Calcd. emp. formula		—	$[C_{17}H_{17}N_3S_2O_3]_n$	$[C_{16}H_{15}N_3S_2O_4]_n$	$[C_{15}H_{15}N_3S_2O_4]_n$
Mol. weight (Vapor pressure osmometry, $CHCl_3$)		—	855	820	1200
Calcd. mol. formula		—	$[C_{17}H_{17}N_3S_2O_3]_2$	$[C_{16}H_{15}N_3S_2O_4]_2$	$[C_{15}H_{15}N_3S_2O_4]_3$
Calcd. mol. weight		—	750	758	1095

Table 2. Physical properties of melinacidins

	Melinacidin	Melinacidin II	Melinacidin III	Melinacidin IV
$[\alpha]_D^{25}$ ($CHCl_3$)	+736° (*c* 0.4) +786° (*c* 0.4)	+726° (*c* 0.5)	+776° (*c* 0.5)	+718° (*c* 0.5)
U.V. (Methanol) $\lambda_{max.}$, nm (a)	241 (sh) (21) 300 (7.9)	241 (sh) (21) 300 (7.3)	240 (sh) (21) 300 (7.5)	242 (sh) (15) 301 (6.0)
C.D. (Dioxane) $\lambda_{max.}$, nm ($[\theta]$)	—	236 (366,000) 272 (−21,000) 307 (80,300) 375 (−2,050)	237 (383,000) 271 (−21,600) 306.5 (87,600) 375 (−2,250)	237.5 (317,000) 272 (−15,200) 308 (63,000) 374 (−2,540)
I.R. (Nujol) (cm^{-1})	3480 (sh), 3400, 1685~1670, 1609, 1599 (sh)	3450 (sh), 3410, 1685~1670, 1605, 1595 (sh)	3480 (sh), 3405, 1685~1665, 1605, 1595 (sh)	3500 (sh), 3385, 1685~1665, 1605, 1595 (sh)

Fig. 2. Infrared spectra of melinacidins in nujol.
A: Melinacidin II. B: Melinacidin III. C: Melinacidin IV.



needles from ethanol and plates from toluene-ethyl acetate. They have a tendency to form solvates with several solvents including ethanol, toluene and benzene. The solvent could be removed with difficulty by drying for prolonged periods of time under high vacuum. Melinacidins II, III, or IV are soluble in chloroform, methylene chloride, dimethylformamide and dimethylsulfoxide. They are less soluble in ethanol and acetone and insoluble in water and saturated hydrocarbon solvents. Potentiometric titration using aqueous alcoholic KOH or HCl showed the absence of titratable groups.

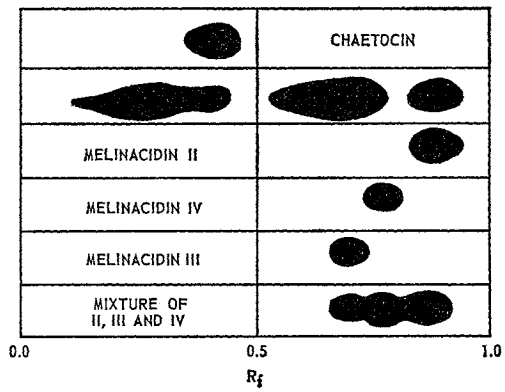
Analytical data obtained on dried samples of melinacidins II, III and IV (Table 1) suggest the empirical formulas of $[C_{17}H_{17}N_3S_2O_3]_n$ for melinacidin II, $[C_{16}H_{15}N_3S_2O_4]_n$ for melinacidin III and $[C_{15}H_{15}N_3S_2O_4]_n$ for melinacidin IV. The molecular weights*

* Attempts to determine the molecular weight by mass spectroscopy have not been successful.

determined by vapor pressure osmometry in chloroform were found to be 855, 820 and 1,200 for melinacidins II, III and IV, respectively. On the basis of these molecular weight values it appears that n=2 for melinacidins II and III and n=3 for melinacidin IV. Therefore, the molecular formulas of $C_{34}H_{34}N_6S_4O_6$, $C_{32}H_{30}N_6S_4O_8$ and $C_{45}H_{45}N_9S_6O_{12}$ are proposed as tentative molecular formulas for melinacidins II, III and IV respectively. In view of the unreliability of the vapor pressure osmometry method for determination of molecular weights and the extremely close properties of melinacidins it is possible that all three melinacidins have close molecular size and therefore melinacidin IV could have the molecular formula of $C_{30}H_{30}N_6S_4O_8$.

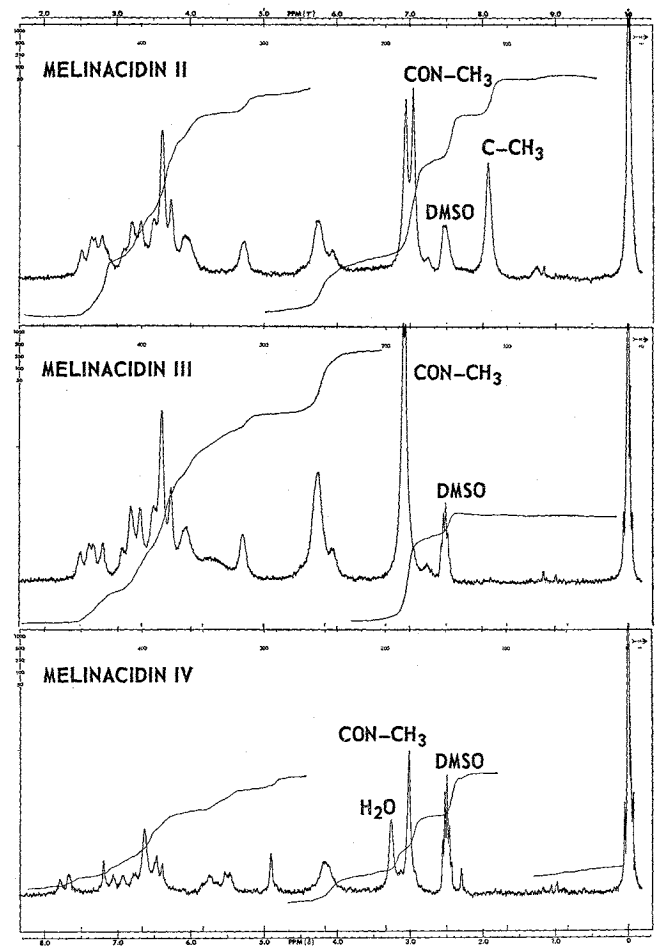
Some of the physical properties of melinacidins II, III and IV are presented in Table 2. All melinacidins are highly dextrorotatory having specific rotations of +726° for II, +776° for III and +718° for melinacidin IV. The UV spectra of the three antibiotics in absolute methanol are very similar and show a shoulder at *ca* 240 nm and a maximum at 300 nm. Circular dichroism curves of the three melinacidins are similar and show four characteristic Cotton effects at *ca* 236, 272, 307 and 375 nm. The infrared spectra of melinacidins II, III and IV (Fig. 2) are very similar so that it is difficult if not impossible to

Fig. 3. Paper chromatographic comparisons* of melinacidins and chaetocin.



* Solvent: Benzene-methanol-water (1:1:2)
Autibiotics were detected by bioautography on *B. subtilis*-seeded agar.

Fig. 4. NMR spectra of melinacidins II, III and IV.



differentiate these compounds using IR data. All compounds show absorption at 3480~3400 due to -NH- or OH stretching vibrations. The most characteristic absorption in the spectra is the absorption due to stretching vibration of amide carbonyl groups which appears at *ca* 1685~1665 cm^{-1}. Another characteristic absorption present in the spectra of all melinacidins is the weak absorptions at 1605~1595 cm^{-1} which is tentatively assigned to the presence of an aromatic system in the melinacidins molecule. The infrared spectra of melinacidins and in particular the amide carbonyl bands combined with the positive COTTON effect at 236~237 nm indicate the presence of a dioxopiperazine moiety[6,7,8] in melinacidins II, III and IV. Furthermore, the COTTON effects at 272, 307 and 375 nm are assigned to a disulfide chromophore[6,9] the presence of which is supported by the mass spectra [*m/e*, 64 due to loss of S_2[10]]. These data suggest that melinacidins II, III and IV belong to the group of "3,6-epidithiadiketopiperazine" antibiotics, which includes chetomin[11,12,13], gliotoxin[14], sporidesmins[15], aranotins[16], chaetocin[17], verticillin A[18], and possibly oryzachlorin[19]. We have mentioned in a previous communication in this series[1] that chetomin is reported to be produced by *Chaetomium cochliodes*[12,13]. However GEIGER[11] reports that *Verticillium cinnabarium* known also as *Acrostalagmus cinnabarinus* bas been found to produce chetomin. Since *A. cinnabarinus* produces melinacidin, crystalline melinacidin and melinacidins II, III and IV were compared with chetomin. All components present in the melinacidin complex were differentiated by TLC from chetomin. In addition melinacidins II, III and IV could be differentiated from chetomin* by TLC and by comparison of NMR spectra. In regard to the other antibiotics of this group melinacidins can be differentiated easily from gliotoxin by paper chromatography. Sporidesmins are characterized by the presence of chlorine and two $-OCH_3$ groups. Melinacidins do not contain chlorine and their NMR (Fig. 4) spectra show the absence of $-OCH_3$ groups. Aranotins contain a $-OCOCH_3$ grouping, which, as shown by NMR, is not present in melinacidins. 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 melinacidins II, III and IV which as we mentioned have similar specific rotations (Table 2). Both chaetocin and verticillin A and the three melinacidins are produced by related fungi have similar infrared spectra and similar circular dichroism curves (COTTON effects at 236, 272,

Table 3. *In vitro* antibacterial spectrum of melinacidins

Test organisms	Minimal inhibitory concentration (mcg/ml) Melinacidins		
	II	III	IV
E. coli UC 51	>100	50	100
K. pneumoniae UC 58	>100	50	100
S. aureus UC 70	1.0	1.0	1.0
S. aureus UC 76	1.0	0.1	0.1
P. vulgaris UC 93	10.0	10	50
Ps. aeruginosa UC 95	>100	>100	>100
S. hemolyticus UC 152	0.1	0.1	0.1
S. viridans UC 155	10	10	10
S. faecalis UC 157	10	10	10
S. typhosa UC 215	>100	50	>100
S. paratyphi UC 263	>100	>100	>100
S. pullorum UC 267	50	50	50

Test method: Tw fold dilution end points in brain heart infusion broth. Read after 20 hours of incubation.

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

307, 375 nm for milinacidins II, III and IV; 236, 272, 307 and 375 nm for verticillin A; and 239, 270, 304 nm for di-O-acetylchaetocin). Verticillin A has been differentiated from melinacidins by comparison of the published NMR spectrum of this antibiotic with the NMR spectra of melinacidins (Fig. 4). Chaetocin* is easily separated from melinacidins II, III, and IV by paper chromatography as shown in Fig. 3. In addition melinacidins are easily differentiated from chaetocin by comparison of the NMR spectra.

The NMR spectra of melinacidins II, III and IV are presented in Fig. 4. The spectra of melinacidins II and III are similar in all regions with the excption of a singlet present only in the spectrum of melinacidin II at *ca* δ 2.0 and assigned to C-CH_3 groups. As indicated in Fig. 4 the doublet or singlets at *ca.* δ 3.0 is assigned to -N-CH_3 amide groups present in all three melinacidins. At the present little can be said about the other absorptions in the spectrum with the exception that the complex absorption from δ 6.0 to 8.0 ppm is partly due to aromatic hydrogens.

The *in vitro* spectrum of melinacidins II, III and IV is presented in Table 3. Like most of the compounds of the "3,6-epidithiaketopiperazine" group of antibiotics the melinacidins are mainly active against Gram positive organisms. Melinacidins II, III and IV inhibited the growth of L-1210 cells in tissue culture[20] [LD_{50} (mcg/ml; 50 % inhibition of protein synthesis): melinacidin II, 0.0016; melinacidin III, 0.0038; melinacidin IV, 0.0140]; [LD_{90} (mcg/ml; 90 per cent inhibition of protein synthesis): melinacidin II, 0.0044; melinacidin III, 0.0062; melinacidin IV, 0.0170]. *In vivo* testing or toxicity data on these three compounds are not available at this time. However, melinacidin failed to protect mice infected with *Staphylococcus aureus* when administered subcutaneously at the maximum tolerated dose of 1 mg/kg[1].

As indicated in Figs. 1 and 3 melinacidin contains other bioactive materials in addition to melinacidins II, III and IV. Work on isolation of these compounds is in progress and will be the subject of future communications.

Acknowledgements

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* Samples of chaetocin and NMR spectrum of this compound were kindly provided by Dr. Kis of the Chemical Research, Pharmaceutical Division, Sandoz Ltd.

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