MERSACIDIN, A NEW ANTIBIOTIC FROM *BACILLUS*

FERMENTATION, ISOLATION, PURIFICATION AND CHEMICAL CHARACTERIZATION

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Mersacidin (**1**) is a new peptide antibiotic containing β-methyllanthionine. It is classified as a member of the proposed lantibiotic group of antibiotics, and is produced by a species of *Bacillus*. Mersacidin has a molecular weight of 1,824 ($C_{80}H_{120}N_{20}O_{21}S_4$). The antibiotic is active against Gram-positive organisms including methicillin-resistant *Staphylococcus aureus*, but has no activity against Gram-negative bacteria or fungi.

In our screening program for the discovery of novel antibiotics produced by eubacteria, we have isolated a new peptide antibiotic from fermentations of a *Bacillus* species HIL Y-85,54728. The antibiotic, mersacidin, is mainly active against Gram-positive bacteria, particularly *Staphylococcus aureus*, including methicillin-resistant strains[1~3].

Peptide antibiotics with some structural similarity to mersacidin have been reported including nisin[4], subtilin[5], cinnamycin[6], duramycin[7], actagardine[8], epidermin[9], gallidermin[10] lanthiopeptin[11] and pep-5[12]. All of these contain the amino acid lanthionine, and therefore are given the common name "lantibiotics" which has been proposed as a class of antibiotics.[13] Mersacidin has no lanthionine but contains methyllanthionine, and differs in other structural details from the previously reported antibiotics.

In this paper we report the production of mersacidin by fermentation, its isolation, purification, chemical characterization and biological activity. The structure elucidation will be reported separately.

Materials and Methods

Taxonomy Methods

The eubacterium producing mersacidin was isolated from a soil sample obtained in Mulund (salt pan), Maharashtra, India and was identified as a *Bacillus* species and given the number HIL Y-85,54728. It has been deposited at the Deutsche Sammlung fur Mikroorganismen (German Microorganism Collection) under the number DSM 4584. The isolation medium was composed of (g/liter): Proteose peptone 20, K_2SO_4 1.5, $MgSO_4 \cdot 7H_2O$ 1.5, glycerol 10 and agar powder 15, pH 6.8. The strain was maintained on nutrient agar medium of the following composition (g/liter): Peptone 10, beef extract 3, yeast extract 3, agar powder 15, pH 7.2 adjusted with 0.3 M NaOH solution. Demineralized water was used in the preparation of all media. The media were sterilized by autoclaving at 120°C for 20 minutes.

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Bacillus, Y-85,54728 is an aerobic, Gram-positive, non-vacuolated straight rod with an average size $0.8 \times 2.5 \sim 3.5\,\mu m$, occurring singly or in short chains of 2 to 3 rods. Rods are motile. Highly refractile endospores are produced under aerobic conditions, spores are oval to spherical, mostly terminal, sporangia are not swollen. For morphological studies the strain was grown on the maintenance medium cited above. It is catalase positive, reduces nitrate to nitrite and produces acid from glucose, xylose, arabinose, sucrose and mannitol. The biochemical tests were performed according to MACFADDIN[14].

On nutrient agar, the colonies are round with irregular margins, and have slightly raised centres, dull surfaces, dull pellicle on surface of broth and are wrinkled. Comparison with standard descriptions in BERGEY's Manual of Systematic Bacteriology[15] indicates that the strain belongs to the genus *Bacillus*.

Fermentation Methods

Bacillus sp. Y-85,54728 was cultured and maintained on nutrient agar slants as described earlier. Transfers were made every 2 weeks. A 20-hour old slant was used for inoculation of the fermenter.

Stage 1, Preparation of the Seed Culture in Shaken Flasks

The seed medium contained, in g per liter of demineralized water: Casamino acid 5, corn steep liquor 5, glycerol 20 and galactose 10. The pH was adjusted to 7.2 with 0.3 M NaOH. The medium was distributed in 100 ml quantities in 500-ml Erlenmeyer flasks and sterilized by autoclaving at 120°C for 20 minutes. Each flask was inoculated with a loopful of growth from a well grown agar slant (20-hour old) and the flasks were incubated at 28°C (±1°C) on a rotary shaker with a 4-cm throw at 220 rpm for 24 hours. This seed culture was used to inoculate smaller fermenters as described below.

Stage 2, Preparation of the Seed Culture in Small Fermenters

The medium contained, in g per liter of demineralized water: Sucrose 10, KH_2PO_4 0.9, Na_2HPO_4 3.83, $(NH_4)_2SO_4$ 2, Na-glutamate 0.02, $MgSO_4 \cdot 7H_2O$ 0.001, $MnSO_4 \cdot H_2O$ 0.004, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.001, $ZnSO_4 \cdot 7H_2O$ 0.004 and $FeSO_4 \cdot 7H_2O$ 0.005. The pH of the medium was 7.2 without adjustment.

Ten liters of the above medium containing 0.04% Desmophen (Type 3600, Bayer, Germany) as foam suppressant were prepared in a 15-liter stainless steel fermenter, sterilized at 121°C for 36 minutes, cooled and inoculated with 4% seed material from the above stage 1. Cultivation was carried out for 24 hours at a temperature of 28°C (±1°C) with a stirring speed of 150 rpm and aeration at 6 liters per minute. The culture grown for 24 hours was used to inoculate the production fermenter in stage 3.

Stage 3, Fermentation in Production Scale

100 liters of the medium, as used in stage 2, containing 0.06% Desmophen were prepared in a 150-liter fermenter, sterilized *in situ* at 121°C for 28 minutes and were inoculated at a 4% rate using the seed culture from stage 2. Cultivation was carried out at a temperature of 28°C (±1°C), stirring at 100 rpm, with aeration at 50 liters per minute. The fermenter was harvested between 60 to 72 hours depending on the antibiotic production which was monitored by testing the culture fluid against *S. aureus* 209P, *S. aureus* R 85 and *Alcaligenes faecalis* 187.

Isolation Methods

The culture broth (720 liters) was harvested after 45 hours and was separated from the mycelial cake by centrifugation. Mersacidin was present only in the culture filtrate (710 liters) which was passed through 30 liters of Diaion HP-20 resin (Mitsubishi Chemical Industries Limited, Japan). The column of HP-20 was washed with 212 liters of demineralized water followed by elution with 87 liters of 30% MeOH in water, 100 liters of 70% MeOH in water, and finally with 70 liters of MeOH. The eluates were collected in fractions of 1 liter in size and tested for the presence of mersacidin by monitoring their activity against *S. aureus* 209P. Mersacidin was eluted out in 42 liters of MeOH eluates which were concentrated under reduced pressure at 40°C to *ca.* 200 ml of a syrup which was triturated with 3×6 liters of petroleum ether (60~70°C) to give 61 g of crude mersacidin as a pale-yellow powder.

Crude mersacidin was subjected to medium pressure liquid chromatography on a 5×45 cm Labomatic glass column packed with SiO_2 (*ca.* 200 mesh) maintaining a flow rate of 55 ml per minute. The column was developed with acetonitrile, and then eluted with water-acetonitrile mixture using a gradient of water

in acetonitrile in steps of 2%. Mersacidin was eluted out with 10~13% of water in acetonitrile and was collected in 250 ml fractions which were monitored by UV detection at 220 nm and also by their bioactivity against *S. aureus* 209P. The eluates containing mersacidin were concentrated under reduced pressure at 40°C to give 13.5 g of a yellowish powder. This material was further purified by chromatography over SiO_2 using acetonitrile-water mixtures as eluents in a manner identical to as mentioned above, and 8.0 g of semipure mersacidin was obtained as an off-white powder. Final purification of mersacidin was effected by precipitation. The semipure antibiotic was dissolved in 50 ml of MeOH and precipitated by dropwise addition of water till precipitation was complete (*ca.* 50 ml). The precipitate was removed by centrifugation, re-dissolved in 25 ml of MeOH and reprecipitated with water (*ca.* 25 ml). The white precipitate of mersacidin thus obtained was collected by centrifugation, washed with 1:1 mixture of water and MeOH, and then dried over $CaCl_2$ under reduced pressure at 40°C to give 4.886 g of pure mersacidin as a white powder. The colored supernatants obtained during precipitation were concentrated under vacuum, and lyophilized to furnish 940 mg of semipure mersacidin which was recycled into the silica chromatography step.

Results and Discussion

Time Course of Antibiotic Production

One interesting aspect of mersacidin production is that its biosynthesis has been achieved in purely synthetic minimal medium with a supplement of 0.02% sodium glutamate (Medium for stages 2 and 3). This is in contrast to literature reports where production of lantibiotics like pep-5, epidermin and gallidermin could not be obtained in defined medium and the biosynthesis was strictly dependent on complex media ingredients like meat extract[16]. Moreover, with pep-5 and gallidermin it has been reported that production of these antibiotics and growth of the producing strains run parallel, reaching a peak within 12 hours. On the contrary, mersacidin is not produced in the active growth phase. HPLC analyses indicate that production starts only after 48 hours and increases steadily thereafter while the active growth phase is completed by 48 hours. However, as seen in Table 1, there is another antibiotic which is produced very early and detected in the bioassay. This is a second antibiotic other than mersacidin, which has better activity against *S. aureus* 209P compared to that against *S. aureus* R 85. Mersacidin has equal activity against both strains. This second antibiotic is produced very early (24 hours) and then declines. Detailed characterization of this antibiotic has not yet been carried out.

Physico-chemical Properties

Table 2 summarizes the physico-chemical properties of mersacidin. The HRFAB-MS analysis of mersacidin indicated two probable molecular formulae. The correct formula of $C_{80}H_{120}N_{20}O_{21}S_4$ could be established only after all the structural subunits were identified by ^1H NMR (Fig. 1) and ^{13}C NMR (Fig. 2) spectroscopy. Amino acid analysis of the 6 N HCl hydrolysate of mersacidin showed the presence of four glycines, two leucines, and one each of isoleucine, glutamic acid, phenylalanine, proline and valine, besides indicating cysteine. GC-MS analysis of the hydrolysate revealed β-methyllanthionine. The ^1H NMR spectra established the presence of three units of β-thioalanine,

Table 1. Production of mersacidin by *Bacillus* Y-85,54728 at different time intervals

Time in hours	Bioactivity, inhibition zone diameter (mm)[a]		Conc of mersacidin (mg/liter)[b]
	S. aureus, 209P	*S. aureus*, R 85	
24	22	11	—
36	21	12	—
48	20	14	30
60	16	15	170
72	13	16	180

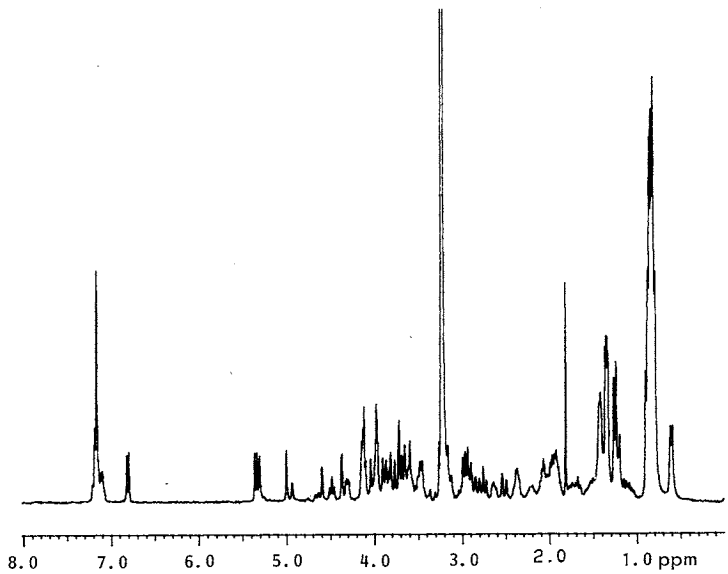
[a] Antibiotic activity was measured by the agar well method (50 μl of culture filtrate dispensed in wells of 6 mm diameter). Assay medium used, Antibiotic Medium No. 1, OXOID.

[b] Concentration of mersacidin was measured by HPLC, as described in Table 2.

Table 2. Physico-chemical properties of mersacidin.

Nature	White amorphous powder
Solubility	Soluble in MeOH; partly soluble in CH_3CN; insoluble in petroleum ether and water
MP	240°C (dec)
Mass spectrum	FAB-MS 1,825 $(M+H)^+$
Molecular formula	$C_{80}H_{120}N_{20}O_{21}S_4$
HRFAB-MS (*m/z*)	Found: 1,825.785 $(M+H)^+$ Calcd for $C_{80}H_{121}N_{20}O_{21}S_4$: 1,825.790
Stability	Stable under ambient conditions of temperature and pH (5.0~7.0)
$[\alpha]_D$	−9.38° (*c* 0.36, MeOH)
UV λ_{max} nm	207, 268 in MeOH (no acid or alkali shift)
TLC Rf	0.45; Merck silica gel plates 60 F_{254} (0.2 mm); butanol-acetic acid-water (4:1:1) solvent system; spot detected under a UV lamp at 254 nm
HPLC Rt	3.6 minutes [4×(30+250) mm 10 μ ODS-Hypersil column; eluent 70% MeOH in aqueous 0.02 M sodium phosphate buffer of pH 7.0; flow-rate 1.5 ml/minute; detection at 220 nm]

Fig. 1. ^1H NMR spectrum of mersacidin (300 MHz, CD_3OD, HDO signal suppressed).



four units of β-thio-α-aminobutyric acid, one dehydroalanine and an $-NH-CH=CH-S-$ residue. The structure of mersacidin was determined to be as represented by **1** in Fig. 3 based on extensive NMR analysis, chemical degradation, and MS/MS study of its desulfurized analogue. Mersacidin[1] is thus a linear peptide with four sulfide bridges, each encompassing one unit of alanine and α-aminobutyric acid (Abu), thus constituting three β-methyllanthionine moieties; the fourth one encompassing an Abu unit and an alanine which can be considered as having undergone an oxidative decarboxylation. Acetylation with Ac_2O-pyridine gave the *N*-acetyl derivative (molecular weight 1,866 by FAB-MS) which showed much weaker antibacterial activity.

The details of the structure elucidation of mersacidin have been reported elsewhere[1,17].

Antibacterial Activity

Mersacidin is active against Gram-positive organisms. The MIC values against a battery of bacterial strains are shown in Table 3 which also shows the comparative activity of vancomycin. Against almost

Fig. 2. ^{13}C NMR spectrum of mersacidin (100 MHz, CD_3OD).

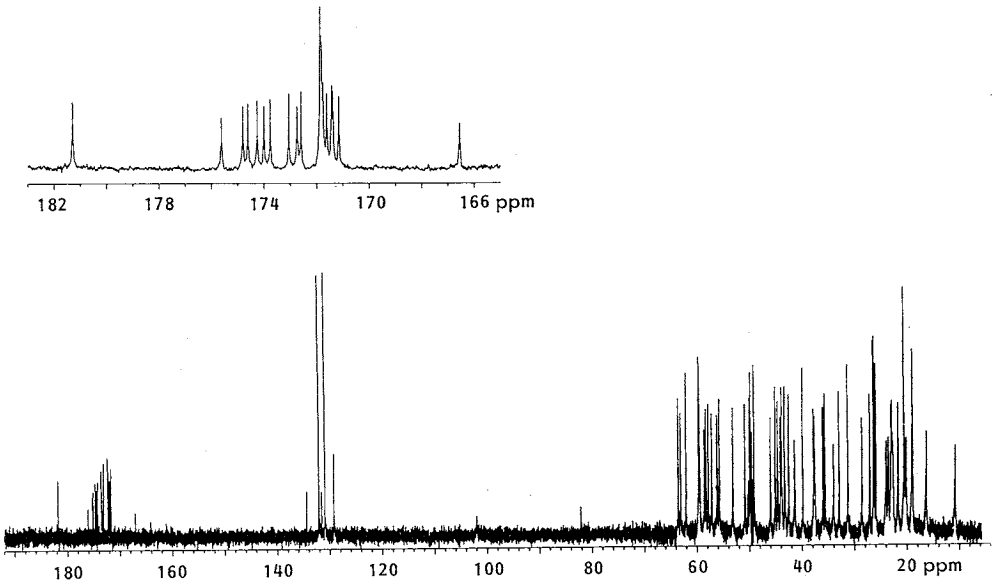


Fig. 3. Structure of mersacidin (**1**) (Z=H) and water soluble potassium mersacidin (**2**) (Z=K).

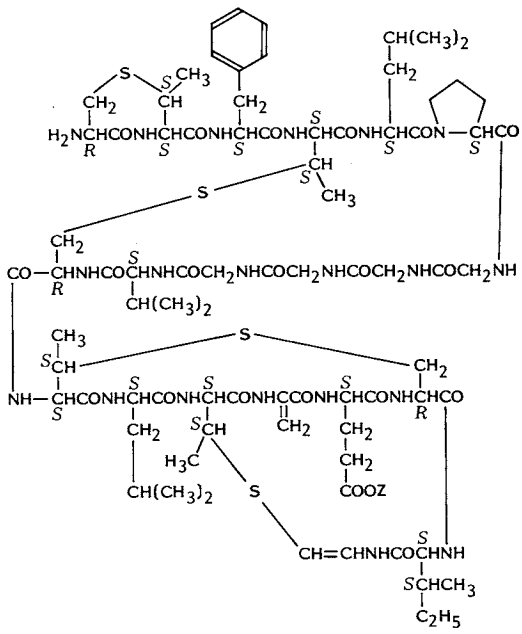


Table 3. Antibacterial activity of mersacidin.

Organisms	Minimum inhibitory concentration (μg/ml)	
	Mersacidin	Vancomycin
Staphylococcus aureus 209P	1.56	0.39
S. aureus R 85	0.78	0.78
S. aureus SG 511	0.78	0.39
S. aureus E 88	12.50	0.78
S. aureus 3066	25.00	0.78
S. aureus 20424	3.12	0.78
S. aureus 20240	6.25	0.78
S. aureus 503	1.56	0.78
S. aureus 710	0.78	0.39
S. epidermidis 823	6.25	0.78
Streptococcus faecalis 21777	50.00	3.12
S. pneumoniae A77	3.12	0.78
Bacillus subtilis ATCC 6633	0.78	0.39
Micrococcus luteus ATCC 9341	0.195	0.0975

all the strains tested, mersacidin showed much poorer activity than vancomycin. Mersacidin was also tested against Gram-negative bacteria and fungi where it was found to be inactive. However, mersacidin showed interesting activity in the *in vivo* system, details of which are presented in the accompanying communication.

Water-soluble Salts of Mersacidin

Mersacidin is insoluble in water. It can, however, be made water soluble in the form of its alkali metal salts, *e.g.* as the potassium salt. Thus when 50 mg (0.027 mmol) of mersacidin dissolved in 1 ml of pyridine was treated with a solution of 1.683 mg (0.030 mmol) of KOH in water at 0°C for 1 hour and then lyophilized, 49 mg of potassium mersacidin **2** was obtained as a crystalline white solid which was freely soluble in water upto 16% (w/v). FAB-MS using 3-nitrobenzyl alcohol as the matrix showed molecular ion peaks at 1,825.7, 1,863.7 and 1,902.6 corresponding to $(M+H)^+$, $(M+K)^+$ and $(M-H+2K)^+$ in ratios of approximately 1 : 2 : 0.8 where M is the molecular mass of mersacidin. It is likely that the potassium salt would be formed at the glutamic acid residue (Fig. 3) but this was not established. Addition of pyridine is an essential requirement, although its role in the formation of the potassium salt is not clear. The water soluble potassium salts of mersacidin showed biological activity comparable to that of mersacidin (data not shown).

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