EMPEDOPEPTIN (BMY-28117)†, A NEW DEPSIPEPTIDE ANTIBIOTIC

I. PRODUCTION, ISOLATION AND PROPERTIES

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Empedopeptin is a new antibiotic produced by *Empedobacter haloabium* nov. sp. (ATCC 31962). It is a water-soluble depsipeptide antibiotic containing eight amino acid residues and a C_{14}-fatty acid moiety in the molecule. Although structurally unrelated, empedopeptin and vancomycin have similar antimicrobial spectra against aerobic and anaerobic Gram-positive bacteria including antibiotic-resistant strains. Empedopeptin is highly active *in vivo* in mice against systemic infections of *Staphylococcus aureus*, *Streptococcus pyogenes* and *Clostridium perfringens*. Empedopeptin is not absorbed orally.

In the course of our screening for new antibiotics, a new strain of *Empedobacter* species was found to produce a new peptide antibiotic which was named empedopeptin. It was extracted by 1-butanol from the fermentation broth and purified by chromatography. Empedopeptin inhibited aerobic and anaerobic Gram-positive bacteria including antibiotic-resistant strains of *Staphylococcus*. This paper reports the taxonomy of producing organism, fermentation, isolation, physico-chemical and biological properties of empedopeptin.

Producing Organism

An unusual bacterial strain No. G393-B445 which produces empedopeptin was isolated from a soil sample collected in Yamate-Dori, Tokyo, by means of a modified pollen bait technique[1]. This organism attached to and grew on a grain of pine pollen floating on the surface of soil-water suspension. Strain G393-B445 is a Gram-negative, asporogenic rod bacterium. The cells vary in shape from coccobacilli to slender rods and are motile with peritrichous flagella. The morphology of G393-B445 is summarized in Table 1. Strain G393-B445 gives vigorous growth on nutrient agar and YP medium*, and produces two types of colonies, R (rough) and S (smooth) forms. It is mesophilic, oxidative, alkali-sensitive and halophobic. The cultural and physiological characteristics of G393-B445 are shown in Table 2. The content of guanine and cytosine (GC content) of cellular DNA analyzed by the method of BENDICH[2] was 66.5 ± 1.5 mol%. The antibiotic sensitivity of G393-B445 shown in Table 3 was determined by the paper disc-agar diffusion method.

According to the descriptions in BERGEY's Manual 8th ed., 1974, strain G393-B445 resembles the

† This antibiotic was originally called Bu-2517[13].
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* YP medium: yeast extract 0.03%, peptone 0.1%, NaCl 0.01%, pH 6.6~6.8.

Table 1. Morphology of strain G393-B445.

Shape of cells:	Coccobacilli to slender rods. Occasional occurrences of partially swollen or curved filaments and vacuolated cells. Rounded ends. Occurring singly or in pair.
Size of cells:	$0.5\sim0.8\times1.0\sim3.0$ μm. Occasionally $5\sim10$ μm in length.
Spore:	Not formed
Motility:	Motile with peritrichous flagella. Concomitant formation of non-flagellated non-motile cells.
Number of flagella on a cell:	$4\sim10$
Fimbriae:	Scarcely borne
Gliding movement of single cells or cell mass:	None
Mode of reproduction:	Binary fission, not budding
Gram-stain:	Negative

Table 2. Cultural or physiological characterization of strain G393-B445.

Colony on nutrient agar: Occurrence of two forms of colonies, R and S

	Form	Surface	Edge	Elevation	Optical property	Color	Swarming
R	Irregular or wrinkled	Rough	Undulate	Raised and effuse	Translucent	Pale-yellow	Negative
S	Circular	Smooth	Entire	Raised	Translucent	Pale-yellow	Negative

Growth on nutrient broth:	Formation of pellicle. Slight turbidity. Flocculent sediment.	
Cellular carotenoid pigment:	Not detected (spectrophotometry)	
Growth temperature*:	Growth 7~42°C No growth 0°C or 45°C	
NaCl tolerance*:	Growth 1.0% NaCl or less No growth 2.0% NaCl or more	
NaCl requirement*:	None	
pH tolerance*	Growth pH 5.0~7.5 No growth pH 4.5 or less, and pH 8.0 or more	
Heating at 70°C for 10 minutes*:	No survival	
Growth on:	Anaerobic agar	−
	Glucose - ammonium - salts agar	+
	Bile - aesculin agar	−
	MacConkey agar	+
	NAC agar (Nalidixic acid - cetrimide agar)	−
Hydrolysis of:	Gelatin	+
	Casein	−
	Starch	+
	Agar	−
	Cellulose	−
	CM-cellulose	−
	Chitin	+
	Alginate	−
	Tween 20	+
	Tween 80	+
Oxidative acid production from:	Glucose	+
	Lactose	−
	Sucrose	+
	Maltose	+
Reactions:	Methyl red	−
	Voges-Proskauer	−
	Citrate alkalization (Simmons)	+
	Indole	−
	Pyocyanin-pyorubin (King's A Medium)	−
	Fluorescence (King's B Medium)	−
	Hydrogen sulfide	+
	Gas from glucose	−
	Gas from nitrate and nitrite	−
	Nitrite from nitrate	+
	Milk coagulation	−
	Milk peptonization	+
	Catalase	+
	Indophenol oxidase	very weak
	Urease	+
	Phenylalanine deaminase	−
	Phosphatase	+
	Deoxyribonuclease	+
	Hemolysis, rabbit blood	very weak

* YP medium was used.

Table 3. Sensitivity of strain G393-B445 to antibacterial agents (paper-disc method).

Antibiotics*	Amount (μg)/disc	Sensitivity**		
		Strain G393-B445	*Escherichia coli* NIHJ	*Staphylococcus aureus* 209P
Actinomycin D	10	I	R	S
Ampicillin	10	S	S	S
Chloramphenicol	30	S	S	S
Erythromycin	15	S	R	S
Benzylpenicillin	10***	S	I	S
Polymyxin B	300***	R	R	R
Streptomycin	10	S	I	I
Tetracycline	30	S	S	S

* Difco's antibiotic sensitivity discs.

** Determined on nutrient agar after 2-day incubation at 28°C. Abbreviations: S (sensitive), R (resistant), I (intermediate). Test procedure and sensitivity criteria follow those described in ref 14.

*** units.

species described in Section II of genus *Flavobacterium* which, however, involves heterogenous species. Several investigators[3,4,5] have recommended rearrangements of *Flavobacterium* species and proposed that Gram-negative, non-motile, non-gliding, non-spreading strains with low GC-content ($\leqq$40%) are to belong to the genus *Flavobacterium*, while Gram-negative, non-motile or motile, peritrichous species with high GC content (60~70%) are to be transferred to the genus *Empedobacter*. In view of the morphological and physiological characteristics of G393-B445 and the new taxonomic criteria described above, strain G393-B445 is considered to belong to the genus *Empedobacter*.

Strain G393-B445 was clearly differentiated from the six species of *Flavobacterium* described in the BERGEY's Manual on account at their physiological and biochemical properties. *Flavobacterium antibioticum* IFO-13715, which produces the peptide antibiotic BA-843[6], differs from strain G393-B445 in its non-motility, no growth at 37°C, negative hydrolysis of sucrose and other biochemical responses.

Strain G393-B445 has some similarities to a collagenolytic bacterium strain $CRZV_1$ described as *Empedobacter collagenolyticum*, but is differentiated from the latter in the motility, alkali-sensitivity, growth in glucose - ammonium - salts medium, phosphatase activity and Tween hydrolysis[7].

One of the common characteristics known for *Empedobacter* species is halotolerance (positive growth in 5.0% NaCl), whereas strain G393-B445 showed a distinct halophobic property. Therefore, strain G393-B445 was considered to be a new species of genus *Empedobacter* and designated *Empedobacter haloabium* sp. nov. The type strain is No. G393-B445 (single isolate); it was deposited in the American Type Culture Collection with the accession number ATCC 31962.

Antibiotic Production

A well-grown agar slant of strain G393-B445 was used to inoculate a seed medium composed of 2% soluble starch, 1% glucose, 0.2% meat extract, 0.2% yeast extract, 0.5% NZ Case (Humko Scheffield Chemical) and 0.2% $CaCO_3$, the pH being adjusted to 7.0 before sterilization. The seed culture was incubated at 28°C for 24 hours on a rotary shaker (250 rpm), and 5 ml of the growth was transferred to a 500-ml Erlenmeyer flask containing 100 ml of fermentation medium composed of 3% sucrose, 2% linseed meal, 0.3% $(NH_4)_2SO_4$ and 0.5% $CaCO_3$. The pH of the medium was adjusted to 7.0 before sterilization. The fermentation was carried out on a rotary shaker at 28°C and the antibiotic activity in fermentation broth followed by a paper disc-agar diffusion assay using *Bacillus*

subtilis PCI 219 as the test organism. The antibiotic production in shake flasks generally reached a maximum after 48~70 hours.

Fermentation studies were also performed in 20-liter jar fermentors which contained 10 liters of the production medium having the same composition as described above. The fermentors were operated at 28°C with stirring at 250 rpm for 20~23 hours.

Isolation and Purification

The harvested broth (37 liters, 250 μg/ml) was acidified to pH 3.0 and stirred with an equal volume of 1-butanol for 1 hour. The 1-butanol layer was separated and evaporated to dryness. The residue was dissolved in methanol (100 ml) and the solution diluted with acetone (1 liter) to precipitate a crude solid (21 g) which was chromatographed on a column of Dowex 1X2 (CH_3COO^- type, 800 ml). The column was developed successively with water (5 liters), 0.5 M ammonium acetate (5 liters) and a *1:1* mixture of 1.0 M ammonium acetate - methanol (5 liters). The bioactive fractions eluted with the methanolic ammonium acetate solution were pooled and concentrated *in vacuo* to a small volume (*ca* 80 ml). This solution was charged on a column of Diaion HP-20 (800 ml) which was developed with water (6 liters), 50% aqueous methanol (3 liters) and 80% aqueous methanol (5 liters), successively. Evaporation of bioactive fractions obtained from the 80% aqueous methanol eluate afforded pure preparation of empedopeptin as a white solid (3.80 g).

Physico-chemical Properties

Empedopeptin is a white amorphous solid with amphoteric nature showing *pKa*'s of 3.0, 4.1 and >11.0 in 50% aqueous ethanol. It is readily soluble in water at neutral and alkaline pH values (solubility: >10%). It precipitates out of the solution in the pH range of 2.4~4.2 and dissolves again below pH 2.0. Empedopeptin is also soluble in methanol, ethanol, aqueous dioxane, dimethylformamide and dimethyl sulfoxide. It is slightly soluble in 1-propanol, 1-butanol and dioxane and practically insoluble in other organic solvents. Empedopeptin showed a positive response to SAKAGUCHI reagent but was negative to the ninhydrin and anthrone reagents. Physico-chemical properties of empedopeptin are summarized in Table 4. The elemental analysis of the antibiotic did not allow an unambiguous deduction of the molecular formula due to hydration. The values, nevertheless, pointed towards $C_{49}H_{79}N_{11}O_{19}\cdot 5H_2O$ which was later confirmed by the structural studies described in the accompanying paper[8]. Empedopeptin did not exhibit absorption maximum above 210 nm in the UV spectrum. The IR spectrum (Fig. 1) showed a polyhydroxyl absorption at around 3350 cm^{-1}, an ester carbonyl at 1735 cm^{-1} and amide carbonyl bands at 1630 and 1540 cm^{-1}. The proton NMR spectrum of empedopeptin (Fig. 2) indicated a methyl group and several methylene and methine protons but no double bond proton.

Table 4. Physico-chemical properties of empedopeptin.

Nature:	White amorphous solid
Mp:	224~227°C (dec)
$[\alpha]_D^{26}$:	+9° (*c* 1.0, MeOH)
Microanalysis:	Calcd for $C_{49}H_{79}N_{11}O_{19}\cdot 5H_2O$; C 48.38, H 7.38, N 12.67
	Found; C 48.51, H 6.90, N 12.53
MW:	1,270 (osmometry), 1,250 (titration)
pKa' (in 50% EtOH):	3.0, 4.1 and >11.0
TLC (silica gel plate) Rf:	0.55 (10% $AcONH_4$ - MeOH, 1:1)
	0.23 (1-PrOH - H_2O - AcOH, 70:30:1)

Biological Properties

In Vitro Antibacterial Activity

The minimum inhibitory concentrations

Fig. 1. IR spectrum of empedopeptin (KBr).

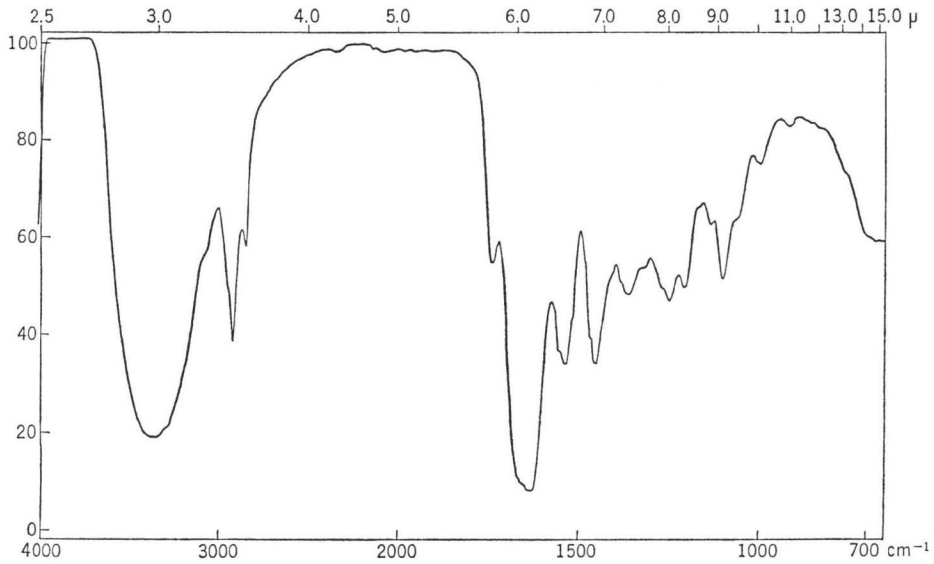
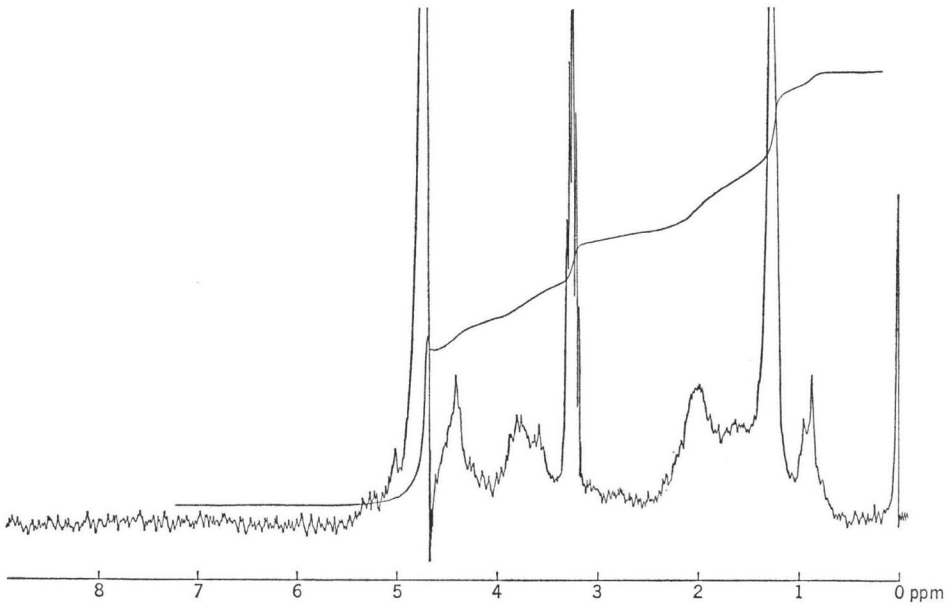


Fig. 2. NMR spectrum of empedopeptin in CD_3OD (60 MHz).



(MICs) of empedopeptin were determined by a serial agar dilution method. Mueller-Hinton agar (Eiken) was always used for aerobic bacteria, GC medium (Eiken) for fastidious organisms, and GAM agar medium (Nissui) for anaerobic bacteria. Table 5 shows *in vitro* antibacterial activities of empedopeptin in comparison with amphomycin, an amphoteric peptide antibiotic, and vancomycin. In general, the three antibiotics are similar in antibacterial spectra, inhibiting predominantly Gram-positive aerobic and anaerobic bacteria. The intrinsic activity of empedopeptin was at least 2-fold

Table 5. Antibacterial spectra of empedopeptin, amphomycin and vancomycin.

Test organisms	Test[a] medium	MIC (μg/ml)		
		Empedopeptin	Amphomycin	Vancomycin
Staphylococcus aureus 209P	A	1.6	3.1	0.4
S. aureus Smith	A	1.6	3.1	0.8
S. aureus BX-1633[b]	A	1.6	3.1	0.8
S. aureus A22421[c]	A	0.8	3.1	0.8
S. epidermidis A22547	A	1.6	6.3	1.6
Streptococcus pyogenes S-23	B	1.6	12.5	0.8
S. pneumoniae IID	B	0.8	25	0.8
S. faecalis A20687	A	6.3	12.5	3.1
Micrococcus luteus PCI 1001	A	<0.05	0.8	0.4
M. flavus D-12	A	<0.05	0.4	0.2
Bacillus subtilis ATCC 6633	A	0.8	1.6	0.1
B. anthracis IID-115	A	0.8	0.8	1.6
Escherichia coli NIHJ	A	>100	>100	>100
Klebsiella pneumoniae D-11	A	>100	>100	>100
Proteus mirabilis A9554	A	>100	>100	>100
Pseudomonas aeruginosa D-113	A	>100	>100	>100
Neisseria gonorrhoeae A15112	B	>100	>100	>100
N. meningitidis A20048	B	>100	>100	>100
Haemophilus influenzae A9729	B	>100	>100	>100
Clostridium perfringens A21284	C	1.6	6.3	0.8
C. difficile A21672	C	0.8	6.3	0.8
C. difficile A21675[d]	C	1.6	3.1	0.8
Peptococcus aerogenes ATCC 14963	C	3.1	50	1.6
Peptostreptococcus intermedius A21881	C	3.1	6.3	1.6
Propionibacterium acnes A21933	C	1.6	3.1	0.8
Bacteroides fragilis A22693	C	>100	>100	50
B. ovatus A22400	C	>100	>100	50
Fusobacterium necrophorum A20013	C	>100	>100	>100
Veillonella parvula A20010	C	3.1	12.5	3.1

[a] A: Mueller-Hinton agar (Eiken), B: GC medium (Eiken), C: GAM agar (Nissui).
[b] Penicillinase producer.
[c] Methicillin-resistant.
[d] Clindamycin-resistant.

Table 6. Antibacterial activity of empedopeptin and vancomycin against Gram-positive clinical isolates.

Test organisms	No. of strains	Geometric mean of MIC (μg/ml)	
		Empedopeptin	Vancomycin
Staphylococcus aureus[a]	24	0.9	0.5
S. epidermidis	18	3.1	1.9
S. epidermidis[a]	3	4.0	2.0
S. agalactiae	7	5.1	0.6
S. pneumoniae	8	0.3	0.5
S. pyogenes	7	0.3	0.5
S. viridans	5	2.5	0.7
Listeria monocytogenes	7	0.3	1.0
Clostridium difficile	10	4.0	1.2

[a] Methicillin-resistant.

Table 7. Resistance development of methicillin-resistant strains of *S. aureus*.

Strain No.	Resistance development after transfer (relative MIC increase)							
	Empedopeptin				Vancomycin			
	0	3	6	9*	0	3	6	9*
A9630	1	4	4	4	1	2	2	4
A15033	1	4	16	16	1	4	8	8
A15036	1	4	4	8	1	4	4	4
A15097	1	4	4	4	1	4	4	8

* Number of transfers in antibiotic containing medium.

Table 8. *In vivo* activity (mouse).

Test organisms	PD_{50} (mg/kg, im)		
	Empedopeptin	Amphomycin	Vancomycin
Staphylococcus aureus Smith	3.3	6.2	1.3
S. aureus BX-1633[a]	3.6	4.4	2.5
S. aureus A15097[b]	1.1	3.2	1.1
S. aureus A20609[b]	2.4	4.2	0.80
Streptococcus pyogenes A20201	0.94	2.5	0.74
S. pneumoniae A9584	0.82	—	0.82
Clostridium perfringens A9635	6.8	7.4	1.3

[a] Penicillinase producer.
[b] Methicillin-resistant.

higher than that of amphomycin and about one-half that of vancomycin.

Empedopeptin was compared with vancomycin for the activity against many strains of clinically important Gram-positive pathogens. As shown in Table 6, methicillin-resistant staphylococci were similarly susceptible to both antibiotics, vancomycin being approximately 2-fold more active than empedopeptin in terms of geometric mean MIC. Four strains of methicillin-resistant staphylococci were tested for the potential of resistance development to empedopeptin and vancomycin by successive subcultures in antibiotic-containing medium (Mueller-Hinton broth). As shown in Table 7, the pattern of resistance development was similar for both antibiotics and the increase of the MIC values was in the range of 4~16 fold after 9 transfers.

In Vivo Activity

The *in vivo* efficacy of empedopeptin was assessed in experimental infections of mice produced by strains of sensitive and resistant staphylococci, *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Clostridium perfringens*. Mice were challenged with a multiple of the lethal dose of the pathogens in a 5% suspension of hog gastric mucin (American Laboratory, Omaha, Neb.). Empedopeptin was dissolved in saline and administered to mice intramuscularly just before the bacterial challenge. The mice were observed for 5 days to determine the median protective dose (PD_{50}). Amphomycin and vancomycin were comparatively tested as reference antibiotics. As shown in Table 8, empedopeptin was more active *in vivo* than amphomycin but somewhat less active than vancomycin.

Blood Levels and Acute Toxicity

Blood levels were determined in mice following an intravenous or intramuscular administration

Table 9. Mouse blood level parameters.

Dose (mg/kg)	Route	Empedopeptin			Vancomycin		
		C_{max}[a]	$T_{1/2}$[b]	AUC[c]	C_{max}	$T_{1/2}$	AUC
30	iv	224	0.87	163	108	0.59	35
10	iv	70	0.81	39	55	0.41	10
30	im	48	1.8	137	27	0.83	32
10	im	14	1.6	40	8	0.50	8

[a] Peak blood level (μg/ml).
[b] Half life (hour).
[c] Area under the curve (μg·hour/ml).

of empedopeptin and vancomycin. Blood samples were collected from orbital sinuses and assayed by the paper disc-agar diffusion method using *Micrococcus luteus* PCI 1001 as the test organism. As shown in Table 9, empedopeptin was well absorbed parenterally and gave much higher and more sustained blood levels than vancomycin. Neither antibiotic was absorbed when administered orally. The intravenous LD_{50} of empedopeptin to mice was found to be 560 mg/kg, while no death occurred up to a dose of 1,600 mg/kg by im route.

Discussion

Empedopeptin is a new water-soluble, amphoteric antibiotic produced by *Empedobacter haloabium* nov. sp. It has a depsipeptide structure as reported in a subsequent paper[8]. Empedopeptin is active against a variety of aerobic and anaerobic Gram-positive bacteria both *in vitro* and *in vivo*.

Among a number of peptide antibiotics reported to date, empedopeptin has some similarities to amphomycin[9,10] in its amphoteric nature, to antibiotic BA-843[6] in the producing organism and some of constitutive amino acids, and to permetin A[11,12] in the cyclic depsipeptide structure. These antibiotics are, however, clearly differentiated from empedopeptin by chemical and biological properties.

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