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

II. STRUCTURE DETERMINATION

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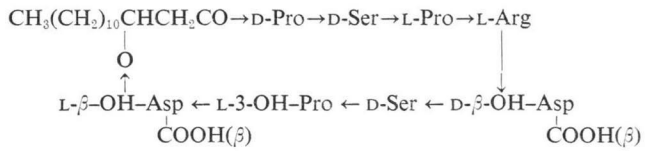
(Received for publication May 7, 1984)

Structure of a new antibiotic, empedopeptin, has been determined. It is a cyclic depsipeptide composed of two mol of D-serine and one mol each of β-hydroxytetradecanoic acid, L-arginine, D- and L-proline, L-3-hydroxyproline and D- and L-β-hydroxyaspartic acid. The sequence of these moieties was established by partial hydrolysis and mass spectral analysis of the antibiotic.

Empedopeptin is a new peptide antibiotic produced by a strain of *Empedobacter*. The taxonomy of the producing organism, the production, isolation and properties of the antibiotic were described in the preceding paper[1]. The experiments presented in this paper revealed that empedopeptin has the following structure:



General Structural Characteristics

Empedopeptin did not exhibit any absorption in the UV and visible regions. The molecular formula of $C_{49}H_{79}N_{11}O_{19}$ was assigned for the antibiotic on the basis of microanalysis, molecular weight determination (1,270 by osmometry, 1,250 by titration) and amino acid analysis. Empedopeptin gave a positive response to the SAKAGUCHI reagent but was negative in the ninhydrin and anthrone tests. The IR spectrum of empedopeptin showed a polyhydroxyl absorption at around 3350 cm^{-1}, an ester carbonyl at 1735 cm^{-1} and a broad, strong amide carbonyl band at around 1620~1680 cm^{-1}. The NMR spectrum indicated the presence of a triplet methyl and several methylene and methine protons. No aromatic or double bond proton signals were observed in the spectrum. Potentiometric titration indicated the presence of two carboxylic groups (*pKa'*: 3.0 and 4.1) and a guanidino group (*pKa'*: >11.0) in empedopeptin.

Complete Acid Hydrolysis of Empedopeptin

Empedopeptin was hydrolyzed with 6 N HCl in a sealed tube at 105°C for 16 hours. The reaction mixture was shaken with ethyl ether to extract an acidic lipophilic product (compound **VI**). The aqueous layer containing amino acid fragments was chromatographed on a column of Dowex 50W X4

† This antibiotic was originally called Bu-2517[10].
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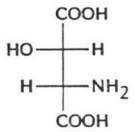
using increasing concentrations of hydrochloric acid (0.05~3.0 N) as eluant. Five amino acids, **I**, **II**, **III**, **IV** and **V** were eluted in that order and each amino acid was isolated as its crystalline hydrochloride. Three of them, amino acids **III**, **IV** and **V**, were identified as serine, proline and arginine, respectively, by their physico-chemical data.

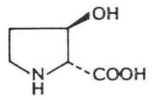
The IR and NMR spectra of amino acid **I** were consistent with those of *threo*-β-hydroxyaspartic acid which has been reported as an antimicrobial metabolite produced by *Arthrinium phaeospermum* and *Streptomyces* sp.[2]. The identity was established by direct comparison with an authentic sample. Amino acid **II** was determined to be 3-hydroxyproline[3] by its physico-chemical and spectral data. The small $J_{2,3}$ value (1.2 Hz) observed for **II** indicated a *trans* configuration for the amino acid. It was identified as *trans*-L-3-hydroxyproline by direct comparison with an authentic specimen prepared by acid hydrolysis of telomycin[4].



I

threo-ß-Hydroxyaspartic acid

(ß-OH-Asp)



II

trans-L-3-Hydroxyproline

(3-OH-Pro)

The molar ratio of these amino acids determined by an amino acid analyzer is shown in Table 1. The chirality of each amino acid was assigned from its optical rotational value together with the result of the amino acid oxidase reaction. The IR spectrum of **I** was superimposable with that of D,L-*threo*-β-hydroxyaspartic acid but was different from that of either the D or L form[2]. The acidic, lipophilic compound **VI** was treated with diazomethane to give a methyl ester which was purified by silica gel chromatography. The methyl ester showed the highest ion peak at *m/z* 257 (M^+-1) in the mass spectrum, suggesting a β-hydroxy C_{14} fatty acid ester. This, together with the NMR spectrum, indicated that **VI** was 3-hydroxytetradecanoic acid.

$$CH_3(CH_2)_{10}\underset{\underset{OH}{|}}{CH}CH_2COOH$$

VI 3-Hydroxytetradecanoic acid

Partial Acid Hydrolysis

Empedopeptin contains several β-hydroxyamino acid units whose amide linkages are known to be highly susceptible to acid cleavage through N→O acyl migration. When kept in 6 N HCl at 37°C for 72 hours, empedopeptin was hydrolyzed to yield several peptide fragments. After removal of acidic

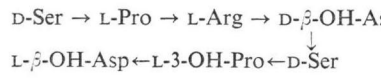
Table 1. Amino acids of empedopeptin.

	Amino acid	Molar ratio	$[\alpha]_D$ (in 5 N HCl)	Assigned chirality
I	β-Hydroxyaspartic acid	2	+1° (*c* 0.5)	1D+1L
II	3-Hydroxyproline	1	+14° (*c* 0.5)	1L
III	Serine	2	−8° (*c* 1.0)	2D
IV	Proline	2	−3° (*c* 1.0)	1D+1L
V	Arginine	1	+20° (*c* 0.5)	1L

Fig. 1. Structures of peptides **VII**, **VIII**, **IX** and **X**.

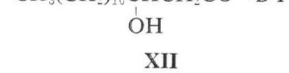
Peptide **VII** D-β-OH-Asp→D-Ser→L-3-OH-Pro→L-β-OH-Asp
VIII D-Ser→L-3-OH-Pro→L-β-OH-Asp
IX D-Ser→L-Pro→L-Arg→D-β-OH-Asp
X D-Ser→L-Pro→L-Arg

products by ethyl ether extraction, the hydrolysate was chromatographed on Dowex 50W X4 to obtain four major peptide fragments, **VII**, **VIII**, **IX** and **X**. Quantitative amino acid analysis established the amino acid composition of peptides **VII** (Ser, 3-OH-Pro, and 2 mol of β-OH-Asp), **VIII** (Ser, β-OH-Asp and 3-OH-Pro), **IX** (Ser, Pro, Arg and β-OH-Asp) and **X** (Ser, Pro and Arg). The chirality of these amino acids was assigned based on the specific rotation of each amino acid which was isolated by complete acid hydrolysis of the individual peptides. The *N*-terminal and *C*-terminal amino acids of these peptides were determined by the DNP method[7] and the Dakin-West degradation[8], respectively. Tetrapeptides **VII** and **IX** were degraded with phenyl isothiocyanate following EDMAN's method[5] with slight modifications. These studies established the order of amino acid linkages as illustrated in Fig. 1. Thus, a partial structure (**XI**) of empedopeptin was deduced as shown below:



XI

The acidic, solvent-extractable products obtained above were chromatographed on silica gel to yield a homogeneous crystalline compound **XII**. Compound **XII** displayed an amide carbonyl band at 1625 cm^{-1} in the IR spectrum and, upon complete acid hydrolysis, afforded D-proline and the C_{14} fatty acid **VI**. Therefore, the structure of **XII** was established as 3-hydroxytetradecanoyl-D-proline. The methyl ester of **XII** obtained by treatment with diazomethane showed the molecular ion peak at *m/z* 355 and several fragment ions which supported the assigned structure.



XII

Mild Alkaline Hydrolysis

When treated with 0.1 N NaOH at 5°C, empedopeptin was hydrolyzed to afford a bio-inactive compound designated as empedopeptinic acid. Complete acid hydrolysis of this compound in 6 N HCl yielded the same degradation products as those obtained from the intact antibiotic. The IR spectrum of empedopeptinic acid indicated the absence of the ester carbonyl band, and potentiometric titration revealed a new carboxyl group showing *pKa'* 2.4 in addition to the two carboxyl functions (*pKa'* 3.7 and 4.6) observed for empedopeptin. The above findings indicated that empedopeptin is a cyclic lactone which was cleaved by mild alkaline hydrolysis to yield a linear peptide, empedopeptinic acid.

Structures of Empedopeptin and Empedopeptinic Acid

Both empedopeptin and empedopeptinic acid were negative to ninhydrin, indicating that the carboxyl group of D-proline in **XII** must be linked to the *N*-terminal of **XI** in empedopeptin and empedopeptinic acid. Therefore, the structure of empedopeptinic acid is represented as shown below:

Table 2. Diagnostic fragment ions of empedopeptin and empedopeptinic acid.
(m/z, relative abundance in parenthesis)

	Empedopeptin	Empedopeptinic acid
VI→Pro→Ser→Pro	490 (2.3%)	508 (8.2%)
VI→Pro→Ser	393 (0.9%)	411 (1.7%)
VI→Pro	306 (2.2%)	324 (6.0%)
VI	209 (71.0%)	

VI→D-Pro→D-Ser→L-Pro→L-Arg
↓
L-β-OH-Asp←L-3-OH-Pro←D-Ser←D-β-OH-Asp
Empedopeptinic acid

The above peptide sequence was verified by the mass spectra of empedopeptin and empedopeptinic acid. Although molecular ions or larger peptide ions were not detected, the spectra clearly indicated the sequence of fatty acid (**VI**)→Pro→Ser→Pro. As shown in Table 2, fragment ions of empedopeptin were observed to arise from the hydroxy acid after dehydration in the mass spectrometer. On the other hand, the corresponding ions of empedopeptinic acid appeared 18 a.m.u. higher than those of empedopeptin, being consistent with the hydrated structure. This information, together with the carbonyl band observed at 1735 cm^{-1}, indicated that the hydroxyl group of **VI** should be esterified to form a cyclic lactone in empedopeptin[6]. As discussed earlier, the newly generated carboxyl group of empedopeptinic acid had pKa' 2.4 which was attributable to the α-carboxyl group of C-terminal L-β-hydroxyaspartic acid. Hence, the total structure of empedopeptin was determined as shown at the beginning of this paper.

Discussion

The present studies established that empedopeptin is an amphoteric, cyclic depsipeptide composed of 8 amino acid units and a fatty acid residue. In addition to D-serine, D- and L-proline and L-arginine, three unusual amino acids, *trans*-L-3-hydroxyproline and *threo*-D- and L-β-hydroxyaspartic acids, were identified as constituents of the antibiotic. Among a number of peptide antibiotics discovered so far, empedopeptin is a rare example of a depsipeptide antibiotic with an amphoteric nature. The presence of many hydroxyl groups is another structural feature of empedopeptin which makes the antibiotic highly soluble in water.

Experimental

Thin-layer chromatography (TLC) was performed on silica gel plate (Kieselgel $60F_{254}$, Merck) using the solvent systems shown below:

System No.	Solvent system
A-107	Phenol - H_2O (4: 1)
S-101	1-PrOH - Pyridine - AcOH - H_2O (15: 10: 3: 12)
S-114	MeOAc - 1-PrOH - 28% NH_4OH (45: 105: 60)
S-123	MeOH - 10% $AcONH_4$ - 10% NH_4OH (10: 9: 1)
S-126	1-PrOH - 10% $AcONH_4$ - AcOH (5: 5: 1)
M-101	1-BuOH - AcOH - H_2O (3: 1: 1)
SD-111	Petroleum ether - Et_2O (1: 1)
SD-112	C_6H_6 - EtOAc (1: 1)
PL-111	$CHCl_3$ - MeOH - 14% NH_4OH (4: 7: 2)

Gas chromatography and GC-MS were recorded on a Shimadzu GC-4BPT and a Shimadzu LKB-9000 apparatus, respectively, using OV-17 columns with temperature programming of 5°C/minute from 140°C.

Mild Alkaline Hydrolysis of Empedopeptin to Empedopeptinic Acid

A solution of empedopeptin (200 mg) in 40 ml of 0.1 N NaOH was kept at 5°C for 4.5 hours. The solution was acidified to pH 2.0 and extracted with three 50 ml-portions of 1-BuOH. The combined extracts were concentrated *in vacuo* to a sticky residue which was chromatographed on a column of Sephadex LH-20 (1.5×75 cm). The column was developed by 50% aqueous MeOH and the eluate monitored by TLC (M-101). Fractions which showed a spot at Rf 0.10, were combined and evaporated *in vacuo* to yield white solid of empedopeptinic acid (120 mg). Mp 222~225°C (dec); TLC Rf 0.10 (M-101) and 0.04 (S-114).

Anal Calcd for $C_{49}H_{81}N_{11}O_{20}\cdot 7H_2O$: C 46.32, H 7.54, N 12.13.
Found: C 46.36, H 6.70, N 11.51.

Complete Acid Hydrolysis of Empedopeptin

A solution of empedopeptin (1.0 g) in 50 ml of 6 N HCl was heated at 110°C for 17 hours in a sealed tube. After addition of 50 ml of H_2O the reaction mixture was extracted with three 100-ml portions of ethyl ether. Evaporation of the ethyl ether extracts afforded an oily residue (206 mg). The aqueous layer was concentrated *in vacuo* to a sticky solid of amino acid complex which was chromatographed on a column of Dowex 50W X4 (H^+ form, 1.5×50 cm) developing with an increasing concentration of HCl (0.05~3.0 N). Upon monitoring with ninhydrin test and TLC (A-107), the appropriate fractions were pooled and concentrated under reduced pressure. Amino acid **I** (167 mg) was eluted with 0.05 N HCl, **II** (95 mg) and **III** (153 mg) with 0.07 N HCl, **IV** (142 mg) with 0.3 N HCl and **V** (129 mg) with 3 N HCl.

Amino Acid **I**: Colorless needles from H_2O, mp >215°C (dec); $[\alpha]_D^{26.5}$ +1° (*c* 0.5, 1 N HCl); TLC Rf 0.03 (A-107) and 0.56 (S-123).

Anal Calcd for $C_4H_7NO_5$: C 32.22, H 4.73, N 9.39.
Found: C 32.52, H 4.58, N 8.95.

MS: *m/z* 149 (M^+). The IR and NMR spectra were identical with those of DL-*threo*-β-hydroxyaspartic acid[2].

Amino Acid **II**: Colorless needles from aqueous EtOH, mp >200°C (dec). $[\alpha]_D^{25.5}$ +14° (*c* 0.5, 5 N HCl); TLC Rf 0.34 (A-107) and 0.55 (S-123).

Anal Calcd for $C_5H_9NO_5$: C 45.80, H 6.92, N 10.68.
Found: C 45.82, H 6.90, N 10.41.

MS *m/z* 132 (M^++1), 86, 69. The spectral data were identical with those of *trans*-L-3-hydroxyproline[6].

Amino Acid **III**: Colorless solid; $[\alpha]_D^{26.5}$ −8° (*c* 1.0, 5 N HCl); Rf 0.11 (A-107) and 0.54 (S-123). Identified as D-serine by the IR, NMR and TLC.

Amino Acid **IV**: Colorless solid; $[\alpha]_D^{26.5}$ −3° (*c* 1.0, 5 N HCl); TLC Rf 0.47 (A-107) and 0.49 (S-123). Identified as DL-proline by the IR, NMR and TLC.

Amino Acid **V**: Colorless solid; $[\alpha]_D^{26.5}$ +20° (*c* 0.5, 5 N HCl); TLC Rf 0.06 (A-107) and 0.39 (S-123). Identified as L-arginine by the IR, NMR and TLC.

Oily residue obtained from the above ethereal extract was treated with diazomethane in ethereal solution. After evaporation of the solvent, the residual solid was chromatographed on a column of silica gel (1.4×32 cm). Elution was carried out with *n*-hexane and then with benzene and the eluate monitored by TLC (SD-111). Appropriate fractions were concentrated *in vacuo* to yield 46 mg of methyl ester of acid **VI**. Yellow oil; TLC Rf 0.30 (SD-111); GC Rt 11.6 minutes; GC-MS *m/z* 257 (M^+−1), 240 (M^+-H_2O), 227, 197, 185, 103, 74 *etc.* Identified as methyl 3-hydroxytetradecanoate by GC and ^1H NMR.

Mild Acid Hydrolysis of Empedopeptin

Empedopeptin (1.5 g) was hydrolyzed with 300 ml of 6 N HCl at 37°C for 72 hours. The solution was shaken with two 150 ml-portions of ethyl ether to extract lipophilic products. The aqueous layer

was concentrated *in vacuo* to a sticky residue (1.136 g) which was applied on a column of Dowex 50W X4 (H^+ form, $\phi 1.5 \times 60$ cm). Elution was performed with an increasing concentration of HCl and the eluate monitored by ninhydrin and SAKAGUCHI reagents and TLC (S-101 and S-126). Peptide **VII** (70 mg) was eluted first with 0.05 N HCl followed by **VIII** (173 mg) with 0.07 N HCl and **IX** (251 mg) with 2 N HCl. Peptide **X** (159 mg) was eluted with 3 N HCl solution. Amino acid contents of these peptides were determined by the amino acid analyzer after complete acid hydrolysis with 6 N HCl.

Peptide	TLC (Rf)		Amino acid content (in ratio)				
	S-101	S-126	I	II	Ser	Pro	Arg
VII	0.22	0.24	2.17	1.0	1.01	—	—
VIII	0.33	0.35	0.87	1.0	0.76	—	—
IX	0.22	0.25	0.92	—	0.76	0.75	1.0
X	0.45	0.38	—	—	0.91	0.89	1.0

The lipophilic products obtained in the above hydrolysis were treated with a large excess of diazomethane in ether. After removal of the solvent, the residue was charged on a silica gel column ($\phi 1.5 \times 20$ cm) which was eluted with benzene - EtOAc (2: 1). The elution was followed by TLC (SD-112) and appropriate fractions were evaporated *in vacuo* to give 64 mg of methyl ester of **XII**. Colorless oil; TLC Rf 0.20 (SD-112); MS *m/z* 355 (M^+), 337, 200, 128, 70, *etc.*; IR ν_{max}^{KBr} 1745 and 1625 cm^{-1}.

Complete Acid Hydrolysis of Peptides **VIII**, **IX** and **XII**

Peptides **VIII**, **IX** and **XII** obtained above were hydrolyzed with 6 N HCl at 110°C for 17 hours. The amino acids were isolated by Dowex 50W X4 chromatography and the chirality of each amino acid was determined by its specific rotational value as shown below.

Peptide	Amino acid isolated	$[\alpha]_D^{24 \cdot 5}$ (*c* 0.5, H_2O)	Chirality assigned
VIII	I	−12°	L
	II	−11°	L
	Ser	+5°	D
IX	I	+12°	D
	Ser	+3.2°	D
	Pro	−64°	L
	Arg	+9.8°	L
XII	Pro	+66°	D

Determination of *N*-Terminal Amino Acids of Peptides **VII**, **VIII**, **IX** and **X**[7]

An aqueous solution (1 ml) of each of the peptides (20 mg) and sodium bicarbonate (30 mg) was mixed with 2 ml of 5% dinitrofluorobenzene in EtOH and the mixture stirred for 2 hours in a dark room. The solution was diluted with H_2O (50 ml), washed with benzene to remove excess dinitrofluorobenzene and dinitrophenol, and then extracted with 1-BuOH at pH 2.0. Evaporation of the 1-BuOH extract afforded a yellow DNP-peptide which was purified by preparative TLC using solvent system of PL-111. The pure peptide was hydrolyzed with 6 N HCl and the DNP-amino acid produced was identified by comparison with an authentic sample by TLC and NMR. Peptide **VII** (DNP-I), **VIII** (DNP-Ser), **IX** (DNP-Ser) and **X** (DNP-Ser).

EDMAN Degradation of Peptides **VII** and **IX**[6]

Phenyl isothiocyanate (0.2 ml) was added to a solution of each of the peptides (5 mg) dissolved in 1.6 ml of dimethylallylamine buffer (pH 9.5). The mixture was warmed at 45°C for one hour under nitrogen atomosphere and then excess phenyl isothiocyanate removed by extraction with benzene (1 ml × 4). The aqueous layer was concentrated to dryness and the residue was dried over phosphorous

pentoxide at 60°C for one hour. The residue was taken up in 0.2 ml of trifluoroacetic acid and the solution kept at 40°C for 40 minutes under nitrogen atomosphere. After evaporation of trifluoroacetic acid, the residue was dissolved in 1 ml of 0.1 N HCl and the solution extracted with four 1 ml-portions of ether. Evaporation of the ethereal extracts afforded phenylthiohydantoin (PTH) amino acid. The aqueous layer was lyophilized to give a white solid of the remaining peptide which was used for the next cycle of EDMAN degradation. A part of the peptide fragment was hydrolyzed and the amino acids produced were analyzed by TLC. The PTH-amino acid obtained in each cycle of the reaction and the constituent amino acids of the remaining peptide fragments were determined as shown below.

Peptide	Step	PTH-amino acid	Amino acids of the remaining peptide				
			I	II	Ser	Pro	Arg
VII	0	—	2	1	1	—	—
	1	I	+	+	+	—	—
	2	Ser	+	+	—	—	—
	3	II	+	—	—	—	—
IX	0	—	1	—	1	1	1
	1	Ser	+	—	—	+	+
	2	Pro	+	—	—	—	+
	3	Arg	+	—	—	—	—

Acknowledgment

The authors are indebted to Prof. M. OHASHI of the University of Electrocommunication for mass spectroscpoic analysis and valuable discussions.

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