EM 49, A NEW PEPTIDE ANTIBIOTIC

II. CHEMICAL CHARACTERIZATION

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EM 49 is a new, broad-spectrum peptide antibiotic. The products of acid hydrolysis have been identified as 2, 4-diaminobutyric acid, leucine, phenylalanine, and a mixture consisting chiefly of C_{10}- and C_{11}-β-hydroxy fatty acids. The antibiotic is a tetraäcidic base, having four free amino groups, which have been identified as the γ-amino groups of 2, 4-diaminobutyric acid residues. The nonintegral quantities of leucine and phenylalanine and the mixture of fatty acid obtained by acid hydrolysis indicated that EM 49 is a mixture of closely related compounds. That this is so has been verified by the separation of EM 49 by ion-exchange chromatography into four distinct fractions. The analytical properties of the mixture are in good agreement with those predicted from the products of hydrolysis of the individual ion-exchange fractions. We conclude that EM 49 is a complex of cyclic, homodectic octapeptide antibiotics that are monoacylated with β-hydroxy fatty acids.

EM 49 is a new broad-spectrum peptide antibiotic that is produced by *Bacillus circulans* ATCC 21,656. The isolation and preliminary characterization are described in Part I of this series.[1] In this paper, we describe experiments that further define the composition and chemical nature of the antibiotic.

The amino acids produced by acid hydrolysis of EM 49 were tentatively identified by quantitative amino acid analysis and by chromatographic comparison with authentic samples as 2, 4-diaminobutyric acid (2, 4-Dab), leucine, and phenylalanine.[1] To confirm these assignments, the amino acids were isolated by ion-exchange chromatography of the hydrolysate. Comparison with authentic materials by ir and nmr spectroscopy showed that the assignments are correct. Leucine hydrochloride, phenylalanine hydrochloride, and 2, 4-Dab dihydrochloride were obtained in a molar ratio of 2.55 : 0.44 : 5.01.

Optical rotation measurements at 589 and 224-225 nm were made to determine the configurations of the amino acids (Table 1). The rotation of the phenylalanine from EM 49 shows that it has the

Table 1. Molecular rotations of amino acids from EM 49

Amino acid	Measured [M]		[M] for optically pure material*	
	589 nm**	224~225 nm***	589 nm**	223~225 nm***
Phe	− 6.0°	+5204°	− 7.4°	+5180°
Leu	+ 4.0	+736	+21.0	+2770
2, 4-Dab	+19.2	+929	+35.9	+1780

* Molecular rotations of the optically pure amino acids at 589 nm are from ref. (2). The maximum rotations for Phe and Leu in the 223~225 nm region are taken from ref. (3). The molecular rotation of 2, 4-Dab at 225 nm was determined for material from Calbiochem, $[\alpha]_D^{25}$ +23.7° (*c*, 1 in 5 N HCl).

** *c*=1 in 5 N HCl.

*** *c*=0.1~0.2 in 0.5 N HCl.

L configuration. However, the rotation data indicate that EM 49 contains 2, 4-Dab and leucine in both the D and the L configurations. The optical purities of these amino acids are *ca.* 52 % and 23 %, respectively.

Acid hydrolysis of EM 49 yields, in addition to amino acids, an immiscible, oily fraction that was separated from the hydrolysis mixture by extraction with ether. When brief hydrolysis times (10~30 minutes) in 6 N HCl at 110°C were used, the extract consisted largely of a mixture of materials that could be assigned the partial structure $RCHOHCH_2CO_2H$ (R=*ca.* C_8H_{17}), on the basis of the nmr spectrum. Longer hydrolysis times (*e.g.* 17 hours) resulted in extensive destruction of the β-hydroxy fatty acids and formation of a mixture consisting largely of α, β-unsaturated fatty acids and butyrolactones.

Treatment of the β-hydroxy fatty acid mixture with diazomethane, and examination of the resulting mixture of methyl esters by gas chromatography, showed two major components comprising *ca.* 65 and 24 % of the mixture, respectively. There were also two minor components, each comprising about 6 % of the mixture. One of these minor components is not a β-hydroxy fatty acid, because it is stable to prolonged hydrolysis.

The mixture of methyl esters and the mixture produced by trimethylsilation of the esters were examined by mass spectrometry. Data from three types of studies-electron-impact ionization, chemical ionization, and gas chromatography coupled with mass spectrometry (GC/MS)-are shown in Table 2. These studies provide empirical formulas for the two major β-hydroxy fatty acids. The 65 % component has the formula $C_{11}H_{22}O_3$ ($C_8H_{17}CHOHCH_2CO_2H$), and the 24 % component has the formula $C_{10}H_{20}O_3$ ($C_7H_{15}CHOHCH_2CO_2H$).

The hydroxyl group on the fatty acid moiety is unsubstituted in the antibiotic. This was

Table 2. Mass spectral data for fatty acid derivatives from EM 49

Substance*	Method		*m/e*	Interpretation*
Methyl ester mixture	High-resolution, electron impact		199.1685	M_a^+-HO
			198.1619	$M_a^+-H_2O$
			185.1557	M_b^+-HO
			184.1463	$M_b^+-H_2O$
	Chemical Ionization	NH_3	234	$M_a+NH_4^+$
			220	$M_b+NH_4^+$
		i-Butane	217	M_a+H^+
			203	M_b+H^+
	GC/MS**	13.6 min	198	$M_a^+-H_2O$
		11 min	184	$M_b^+-H_2O$
Trimethylsilated methyl ester mixture	Low-resolution, electron impact		273	$M_c^+-CH_3$
			259	$M_d^+-CH_3$
	GC/MS**	11.7 min	273	$M_c^+-CH_3$
		7.5 min	259	$M_d^+-CH_3$

* The methyl ester mixture consists chiefly of $C_8H_{17}CHOHCH_2CO_2Me$ (M_a) and $C_7H_{15}CHOHCH_2CO_2Me$ (M_b). The trimethylsilated methyl ester mixture consists chiefly of $C_8H_{17}CHO(SiMe_3)CH_2CO_2Me$ (M_c) and $C_7H_{15}CHO(SiMe_3)CH_2CO_2Me$ (M_d).

** Gas chromatography was done on a 50′×0.02″ Carbowax 20 MSCOT column programmed at 7.5°C per minute up to 130°C and holding that temperature for 2 minutes.

implied by the lack of ester-carbonyl absorption in the infrared spectrum of the antibiotic and was further supported by acetylation of the hydroxyl group of EM 49. The acetylated derivative has essentially the same biological properties as the parent antibiotic.

It was concluded from the molecular weight of EM 49 free base (*ca.* 1,080 by ultracentrifugation) and the equivalent weight (255 by titration with perchloric acid) that EM 49 is a tetraäcidic base[1]. To confirm this, and to determine the nature of the basic groups, EM 49 was treated with 2, 4-dinitrofluorobenzene. Acid hydrolysis of the resulting derivative, and quantitative amino acid analysis of the hydrolysate, showed that 2, 4-Dab, 2-amino-4-(2, 4-dinitroanilino)-butyric acid, leucine, and phenylalanine were present in a ratio of 0.9 : 3.9 : 2.6 : 0.6. From this finding, we concluded that each of four 2, 4-Dab residues in EM 49 has a free γ-amino group, but the fifth residue is acylated at both amino groups, indicating a cyclic peptide structure.

The presence of four basic groups in the EM 49 molecule was also demonstrated by the method of partial substitution[4,5,6]. Treatment of EM 49 with 2, 4-dinitrofluorobenzene, using 1.5 moles of the reagent per mole of EM 49, gave a mixture that was resolved by electrophoresis into four yellow spots and the unchanged antibiotic, which was detected by bioautography against *Escherichia coli*. The electrophoresis was done on Whatman 3 MM paper at 330 v in 85 % formic acid - acetic acid - formamide - water (6 : 4 : 5 : 5 by volume). All components of the mixture are sufficiently soluble in this system to move, either by electrophoresis or by electro-osmosis. Alizarin was used as an electro-osmotic indicator, and had the same mobility as the fully substituted antibiotic. The mobilities of the components relative to the tetrasubstituted and the unsubstituted antibiotics (Table 3) agree well with those calculated for a molecule with four amino groups.

The presence of *ca.* 0.5 mole of phenylalanine and *ca.* 2.5 moles of leucine per mole of EM 49 would be most simply explained if L-leucine and L-phenylalanine can indiscriminately occupy a particular site in the molecule. This type of substitution is not uncommon[7], and the specific substitution of D-phenylalanine for D-leucine describes the relationship between polymyxins B and E. This hypothesis is particularly attractive in that, if the two hypothetical peptide variants each contain an L- and a D-leucine residue, the observed rotation for the leucine that was isolated would be due entirely to the "replaceable" leucine. For equal quantities of the variants, the theoretical optical purity of the leucine would be 20 %, which is in good agreement with the measured optical purity (23 %).

The two hypothetical variants in amino acid composition and the presence of two major fatty acid residues led us to expect four major components in the EM 49 complex. Partial resolution of

Table 3. Electrophoretic mobilities of 2, 4-dinitrophenyl derivatives of EM 49

Derivative	Migration toward cathode relative to tetra-DNP-EM 49	Relative mobility	Calc'd mobility**
Tetra-DNP-EM 49	0.0 cm*	0.00	0.00
Tri-DNP-EM 49	1.6	0.25	0.25
Di-DNP-EM 49	2.9	0.45	0.50
Mono-DNP-EM 49	4.6	0.72	0.75
EM 49	6.4	1.00	1.00

* The tetra-DNP derivative migrated 1.8 cm toward the cathode, due to electro-osmosis.
** The calculated mobilities are based on the unsubstituted antibiotic having four amino groups.

the mixture was achieved by countercurrent distribution in *n*-propanol, *n*-butanol, 0.01 N HCl, acetic acid (50 : 75 : 144 : 6 by volume). However, the peaks were badly skewed, making good separation difficult. Much better results were obtained by ion-exchange chromatography on CM cellulose, eluting with NaCl solutions. By this technique, EM 49 was resolved into four fractions, designated α, β, γ and δ in the order of their elution. EM 49 reduces the surface tension of water

Fig. 1. Separation of the components of EM 49 by chromatography on CM cellulose

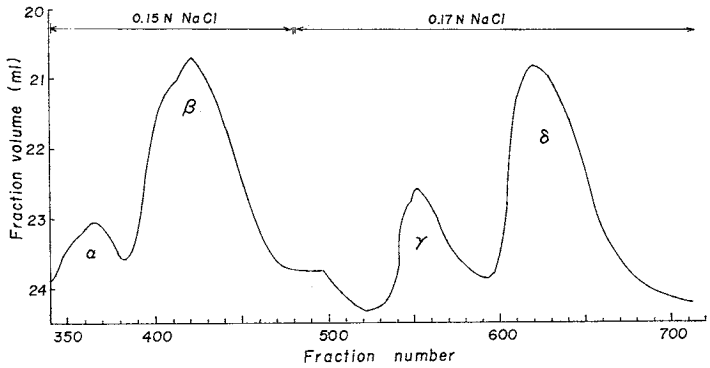

Table 4. Hydrolysis products from ion-exchange fractions of EM 49

Ion-exchange fraction	Acid	Number of residues			Theoretical empirical formula*
		2, 4-Dab	Leu	Phe	
EM 49 α	$C_{10}H_{20}O_3$	5	3	0	$C_{48}H_{91}N_{13}O_{10}$
EM 49 β	$C_{11}H_{22}O_3$	5	3	0	$C_{49}H_{93}N_{13}O_{10}$
EM 49 γ	$C_{10}H_{20}O_3$	5	2	1	$C_{51}H_{89}N_{13}O_{10}$
EM 49 δ	$C_{11}H_{22}O_3$	5	2	1	$C_{52}H_{91}N_{13}O_{10}$

* The sum of the hydrolysis products minus 9 moles of water.

Table 5. Elemental analyses of EM 49, its salts, and its derivatives

	C	H	N	Cl	S
EM 49 Found*	58.40	8.60	17.84	—	—
Calcd. for $C_{50.2}H_{91.5}N_{13}O_{10}$	58.13	8.89	17.56	—	—
EM 49 Hydrochloride Found*	50.80	7.96	15.18	12.01	—
Calcd. for $C_{50.2}H_{95.5}N_{13}O_{10}Cl_4$	50.96	8.14	15.39	11.99	—
EM 49 Helianthate Found*	56.18	6.92	15.60	—	5.73
Calcd. for $C_{106.2}H_{151.5}N_{25}O_{22}S_4$	56.47	6.76	15.50	—	5.68
EM 49 Phenylazobenzenesulfonate Found*	56.25	6.40	14.40	—	6.10
Calcd. for $C_{98.2}H_{131.5}N_{21}O_{22}S_4$	56.53	6.35	14.10	—	6.15
O-Acetyl-EM 49 Hydrochloride Found	51.42	8.20	15.16	11.29	—
Calcd. for $C_{52.2}H_{97.5}N_{13}O_{11}Cl_4$	51.17	8.02	14.86	11.58	—
EM 49-2,4-DNP Derivative Found	52.57	6.07	16.78	—	—
Calcd. for $C_{74.2}H_{99.5}N_{21}O_{26}$	52.37	5.89	17.29	—	—

* Ref. (1)

and, thus, provides a convenient means for its detection: when fractions containing a constant number of drops are collected, fractions containing the antibiotic have a smaller volume than those that lack it. A typical chromatogram is shown in Fig. 1. The peak shapes in the ion-exchange chromatograms suggest that further inhomogeniety remains, but the nature of this inhomogeniety is not known.

Amino acid analyses of the hydrolysates of fractions α and β show that they each contain five 2, 4-Dab and three leucine residues. Peaks γ and δ each contain one phenylalanine, five 2, 4-Dab, and two leucine residues. Examination of the lipid portions of the hydrolysates by gas chromatography (after conversion of the fatty acids to their methyl esters) showed that the more abundant fractions, β and δ, release the C_{11} acid, whereas the smaller fractions, α and γ, release the C_{10} acid and also the two other minor lipid components. Table 4 lists the ion-exchange fractions and their major hydrolysis products.

Each theoretical empirical formula listed in Table 4 is the sum of the formulas of the hydrolysis products, minus nine moles of water. Assuming that all of the ionic functionality and all of the hydrolysis products have been detected, EM 49 is a cyclic, homodectic octapeptide. A theoretical average empirical formula for the mixture derived from Table 4 (assuming a ratio for $\alpha : \beta : \gamma : \delta$ of 24 : 65 : 24 : 65) is $C_{50.2}H_{91.5}N_{13}O_{10}$. The elemental analyses of EM 49 and its salts and derivatives are in good agreement with compositions derived from this empirical formula (Table 5).

Experimental

Amino acid analyses were determined (method of SPACKMAN *et al.*[8]) on a Jeolco model JLC-5 AH amino acid analyzer. Infrared spectra were recorded on a Perkin-Elmer model 257 spectrometer. Nuclear magnetic resonance spectra were determined on Varian model T-60 and XL-100 spectrometers. Optical rotations at 589 nm were measured on a Perkin-Elmer model 141 polarimeter; those at 223~225 nm were measured on a Cary model 60 spectropolarimeter. Chemical ionization (CI) mass spectra were measured by Dr. R. C. DOUGHERTY, Florida State University, Tallahassee, on an AEI model MS-902 spectrometer equipped with a Scientific Research Inc. CI source, using *i*-butane or ammonia as the ionizing gas. Electron-impact mass spectra were obtained on an AEI model MS-902 spectrometer. An EAI model QUAD-300 mass spectrometer was used in combination with a Barber-Colman gas chromatograph for GC/MS.

Because EM 49 and its salts and derivatives are hygroscopic, elemental analyses for C, H, and N were done on anhydrous samples. Analyses for Cl and S were obtained on hydrated material and the values were adjusted to correspond to the anhydrous materials.

Hydrolysis of EM 49 and Isolation of the Amino Acids:

A solution of 4.48 g (dry weight) of EM 49 hydrochloride in 250 ml of 6 N HCl was refluxed under nitrogen for 16 hours. The solution was cooled and extracted with ether. Concentration of the ethereal solution gave 0.708 g of pale-yellow oil, consisting chiefly of α-β unsaturated fatty acids: nmr ($CDCl_3$) δ 7.00 (m), 5.73 ppm (d, J=16 Hz) and butyrolactones: ir ($CHCl_3$) 1760 cm^{-1}. The theoretical yield of $C_{11}H_{20}O_2$ (the principal component) from material having a molecular weight of 1183 is 0.697 g. The hydrochloric acid solution was taken to dryness *in vacuo*, giving 5.60 g of a slightly tan, crystalline solid.

A sample (1.044 g) of the hydrolysate was separated into its components by ion-exchange chromatography on a 2.5×19 cm column of Dowex 50 W-X 8 (H^+ form) resin. Elution with 0.36 N HCl gave leucine (effluent volume 1,800~2,900 ml) and, then, phenylalanine (4,400~5,800 ml). Further elution with 3.6 N HCl (225 ml) gave 2, 4-Dab. Concentration of the effluents *in vacuo* gave 322 mg of leucine hydrochloride, 66 mg of phenylalanine hydrochloride, and 723 mg of 2, 4-Dab dihydrochloride. Comparison of the nmr and ir spectra of the amino acids with those of

authentic materials confirmed their identities. Optical rotation data are given in Table 1.

β-Hydroxy Fatty Acid Moiety:

A solution of 4.4 g (anhydrous weight) of EM 49 hydrochloride in 250 ml of 6 N HCl was stirred at 107°C under nitrogen for 0.5 hour. The resulting mixture was cooled and extracted with ether. The ethereal solution was dried (Na_2SO_4) and concentrated *in vacuo*, giving 582 mg of an oil: nmr (CCl_4) δ 7.85 (broad, 2, $CO_2\underline{H}$ and O$\underline{H}$), 4.00 (unresolved multiplet, 1, C$\underline{H}$OH), 2.45 (doublet in dilute solution, 2, J=6 Hz, $C\underline{H}_2CO_2H$), 1.30 (envelope, *ca.* 11) and 0.90 ppm (m, 6); ir (CCl_4) 1707 cm^{-1}.

The mixture of β-hydroxy fatty acids was dissolved in ether and treated with excess diazomethane for 15 minutes at room temperature. Concentration *in vacuo* gave the methyl ester: nmr (CCl_4) δ 3.85 (m, 1, C$\underline{H}$OH), 3.67 (s, 3, OC$\underline{H}_3$), 3.20 (broad, 1, O$\underline{H}$), 2.37 (d, 2, J=6 Hz, $C\underline{H}_2CO_2Me$), 1.33 (envelope, *ca.* 11), 0.87 ppm (m, 6); ir (CCl_4) 3560 (—OH), 1730 cm^{-1} (ester carbonyl).

The methyl ester mixture was examined by gas chromatography on a 6′×1/4″ O.D. stainless steel column packed with 10% Carbowax 20 M on acid-washed, dimethyldichlorosilane-treated, 60 ~80 mesh Chromosorb W at 207°C, using He as the carrier gas at a flow rate of 90 ml/min, with detection by thermal conductivity. Four substantial peaks, with retention times of 8, 9.5, 11, and 12 minutes and relative intensities of 24, 6, 6, and 65, respectively, were observed. The material having the 11-minute retention time is not a β-hydroxy fatty acid methyl ester, because its intensity is independent of the hydrolysis time used for the antibiotic.

Mass-spectral data obtained for the methyl ester mixture and for the trimethylsilated methyl ester mixture (bistrimethylsilylacetamide-treated) are given in Table 2.

O-Acetyl-EM 49 Hydrochloride:

A solution of 2.5 g of EM 49 hydrochloride in 25 ml of trifluoroacetic acid was cooled to 0°C, treated with 1.47 ml of acetyl chloride, and stirred at 0°C for 1 hour. Addition of 125 ml of ether gave a precipitate that was washed with ether and dried *in vacuo*. This material was dissolved in a mixture of 50 ml of *n*-butanol and 50 ml of water, and the stirred mixture was adjusted to pH 12 with 5 N NaOH. The butanol phase was separated and washed twice with 0.01 N NaOH, once with 84 ml of 1 N HCl and twice with 0.36 N HCl. The butanol was removed *in vacuo* and the residue was converted to a powder by dissolving it in methanol, adding ethyl acetate, and removing the solvent mixture *in vacuo*. The resulting solid was dried for 2 hours at 0.02 mm and room temperature, and was then equilibrated with atmospheric moisture, giving 2.0 g of material containing 7.46% water: nmr (CF_3CO_2H) δ 2.23 ppm ($COC\underline{H}_3$); ir (KBr) 1720 cm^{-1} (shoulder, ester C=O); $[\alpha]_D^{23}$ —16.1° (*c* 1, DMSO). Calcd. for CH_3CO (M. W. 1,225): 3.51, Found: 3.19. The elemental analysis is given in Table 5.

2, 4-Dinitrophenyl Derivative of EM 49;

A mixture of 31 mg of EM 49, 1 ml of *n*-BuOH, 1 ml of methyl ethyl ketone (MEK), 0.2 ml of a 1 M solution of 2, 4-dinitrofluorobenzene in benzene, and 2 ml of a 10% aqueous $NaHCO_3$ solution was stirred at room temperature for 1.7 hours. The upper phase was separated and the lower phase was washed with several portions of ethyl acetate. The combined organic extracts were taken to dryness *in vacuo*. The residue was mixed with MEK, and insoluble material was removed by centrifugation and discarded. The supernatant was concentrated to dryness, and the residue was dissolved in a small quantity of acetone. Addition of benzene precipitated the product, which was washed with benzene and dried *in vacuo*, giving 35.1 mg of yellow powder. The crude product was purified by thin-layer chromatography on silica gel, using MeOH-$CHCl_3$ (1 : 9 by volume) as the developing solvent. A yellow band between R_f 0.5 and 0.7 was collected. The product was washed from the silica gel with acetone - MeOH (1 : 1 by volume) and obtained as a powder (30.4 mg) by precipitation from an acetone solution with benzene, as above. The elemental analysis is given in Table 5.

A 2.274-mg sample of the derivative in 1 ml of 6 N HCl was heated at 112°C for 12 hours. The

resulting solution was concentrated to dryness, and the residue was dissolved in 13.3 ml of 0.01 N HCl. Quantitative amino acid analysis of the solution showed 2, 4-Dab, 2-amino-4-(2, 4-dinitroanilino)-butyric acid, leucine, and phenylalanine to be present at concentrations of 99, 407, 275, and 63 nmol/ml, respectively. These concentrations were determined by using the hydrolysate of the 2, 4-dinitrophenyl derivative of polymyxin B as a standard. Also, 4-amino-2-(2, 4-dinitroanilino)-butyric acid[5] was prepared and shown to be well separated from 2-amino-4-(2, 4-dinitroanilino)-butyric acid under the analysis conditions employed[8,9]. No 4-amino-2-(2, 4-dinitroanilino)-butyric acid was detected in the hydrolysate of 2, 4-dinitrophenylated EM 49.

Partial 2, 4-Dinitrophenylation of EM 49:

To a solution of 0.48 g of EM 49 hydrochloride (1.6 meq) in 30 ml of 50 % aqueous ethanol was added 0.22 ml (0.60 mmol) of a 2.77 M solution of 2,4-dinitrofluorobenzene in ethyl acetate and 0.78g of powdered $NaHCO_3$. The mixture was stirred at room temperature in the dark overnight and was then taken to dryness *in vacuo*. For electrophoresis, the product was dissolved in 85 % HCO_2H - HOAc - $HCONH_2$ (3 : 2 : 5 volume) to give a concentration of 100 mg per ml, and 1 μl was spotted. Under the conditions used for electrophoresis (*vide supra*), adequate separation was achieved in 3 hours. Prior to bioautography, the electrophoretogram was dried at *ca.* 0.1 mm and 70°C for 2 hours. The results are shown in Table 3.

Separation of EM 49 Components by Ion-exchange Chromatography:

Ion-exchange chromatography of EM 49 hydrochloride was done on a 2.5×60 cm column of Whatman CM-52 cellulose, operated at 50°C. Best results were obtained by eluting the column with 0.15 N NaCl until the α and β fractions had emerged, and then increasing the concentration of NaCl to 0.17 N to elute the remaining material. A flow rate of 75 ml per hour was used and the eluant was saturated with helium at room temperature to prevent formation of bubbles on the column. The antibiotic was detected by monitoring the surface tension, as described above. A typical chromatogram is shown in Fig. 1. To recover the antibiotic from the effluent, portions of the effluent were extracted with 1/2 their volume of *n*-BuOH. The butanolic solutions were washed twice with equal volumes of 0.36 N HCl and were then evaporated to dryness *in vacuo*. The residues were dissolved in MeOH, precipitated with EtOAc, washed with EtOAc and ether, dried (75°C and 0.02 mm for 0.5 hour), and then equilibrated with atmospheric moisture. From chromatography of 500 mg of EM 49 hydrochloride, 36 mg of α, 117 mg of β, 55 mg of γ, and 95 mg of δ were recovered. The results of amino acid analyses of these fractions are summarized in Table 4.

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