EM49, A NEW PEPTIDE ANTIBIOTIC

IV. THE STRUCTURE OF EM49

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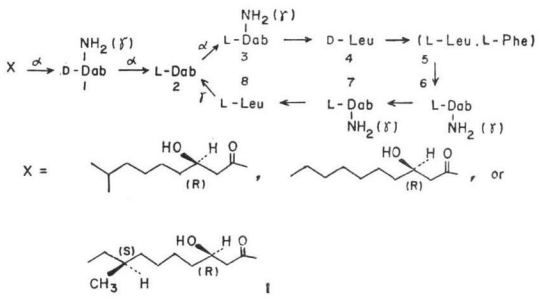
EM49 is a family of similar peptide antibiotics, each an octapeptide acylated with a β-hydroxy fatty acid. This paper describes the determination of the structure of the fatty acyl residue, the selective removal of this residue from the peptide portion of the molecule, and the sequential analysis of the peptide by the EDMAN method. The structure of EM49, **1**, is derived by this degradation.

EM49 is a family of peptide antibiotics that has a broad spectrum of antimicrobial activity[1,2]. A preliminary account of the chemistry of EM49[3] describes the separation of the family into four major components and the determination of the composition of each component with respect to amino acids and the nature of the fatty acid residue. Two different peptides are found: one comprised of five 2,4-diaminobutyric acid (Dab*), one Phe, and two Leu residues and the other comprised of five Dab and three Leu residues. Each of these variants is acylated with a C-10 or a C-11 β-hydroxy fatty acid.

EM49 has now been degraded by chemical methods and the total structure, **1**, derived. Because the differences in the components of the family had been established and because it is difficult to separate large quantities of the pure components, the degradation was carried out on the mixture. The general structure of the fatty acids produced by brief acid hydrolysis of EM49 has been shown by nmr and mass spectroscopy[3] to be $RCHOHCH_2CO_2H$ with $R=C_7H_{15}$ and C_8H_{17}.



This mixture has now been separated by preparative gas chromatography of the methyl esters into three pure components. An additional minor component, previously detected by analytical gas chromatography, was not present in the mixture used for the preparative separation. Retention times for the esters under conditions described in the experimental section and the quantities of the esters that were isolated are listed in Table 1. Elemental analyses of the pure methyl esters, listed in Table 2, confirm the empirical formulas previously derived for these esters.

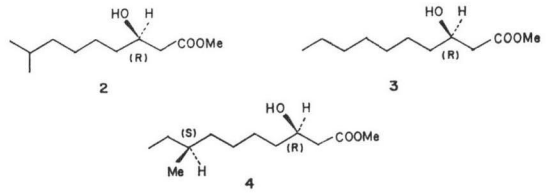
* The following abbreviations are used in this paper: Dab=2,4-diaminobutyric acid, DCC=dicyclohexylcarbodiimide, DCU=dicyclohexylurea, DNFB=2,4-dinitrofluorobenzene, DNP=2,4-dinitrophenyl, Leu=leucine, Phe=phenylalanine, PTC=phenylthiocarbamyl, PTH=3-phenyl-2-thiohydantoin, TFA=trifluoroacetic acid, TLC=thin-layer chromatography, Z=benzyloxycarbonyl.

Table 1. Fatty acid methyl esters from EM49

Structure	Retention time (min.)	Weight (mg)	$[M]_D^{22}$ ($CHCl_3$)	$[M]_D^{22}$ (MeOH)
2	10.6	15.1	$-38\pm1°$	$-1.5\pm0.3°$
3	12.4	7.9	$-37\pm2°$	$-2.6\pm0.6°$
4	17	60.1	$-22.6\pm0.2°$	$+13.9\pm0.1°$

Table 2. Elemental analyses of the fatty acid methyl esters from EM49

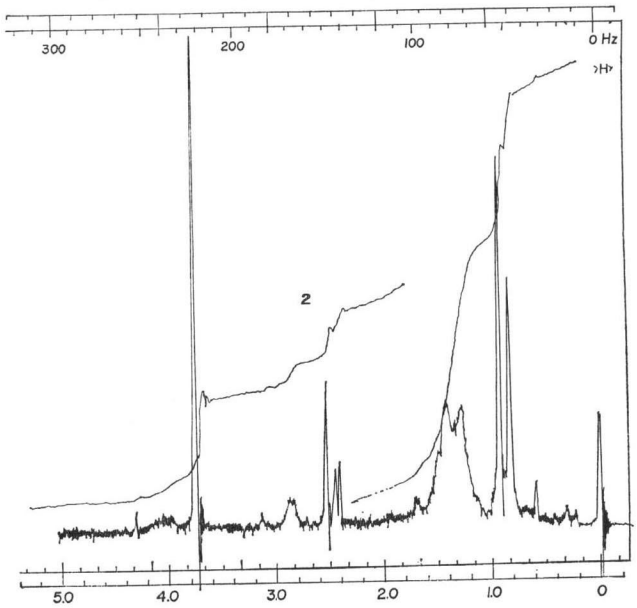
Structure	Formula	Calculated		Found	
		C	H	C	H
2	$C_{11}H_{22}O_3$	65.31	10.96	65.46	10.75
3	$C_{11}H_{22}O_3$	65.31	10.96	65.06	11.21
4	$C_{12}H_{24}O_3$	66.63	11.18	66.88	11.40

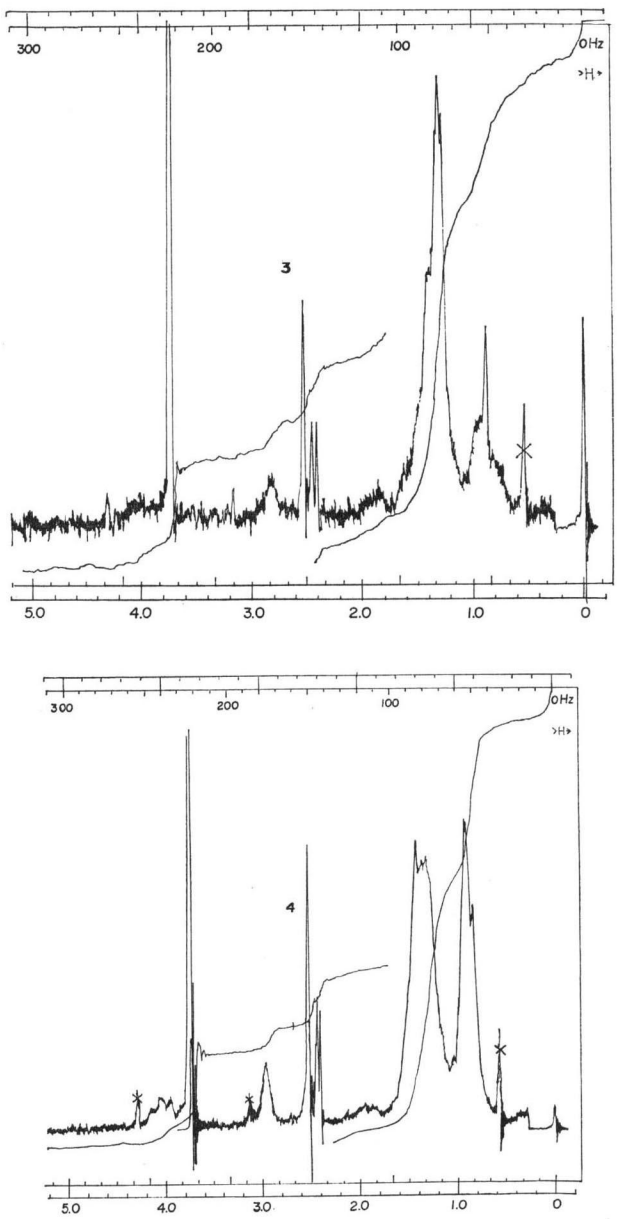


Except for configuration, structures **2** and **3** can be immediately assigned for the first two esters on the basis of the nmr spectra, Fig. 1. The structure of the third ester, **4**, can be tentatively assigned from its nmr spectrum. Although the nature of the terminus of the alkyl chains for **2** and **3** is apparent from the shape of the high-field nmr absorption, the anteiso terminus assigned to **4** is consistent with the nmr spectrum, but not proved by it, because methyl branching at other points along the chain would give nearly identical absorption in this region[4].

Optical rotations for the esters in methanol and chloroform are listed in Table 1. The

Fig. 1. Nmr spectra of fatty acid methyl esters from EM49 in $CDCl_3$



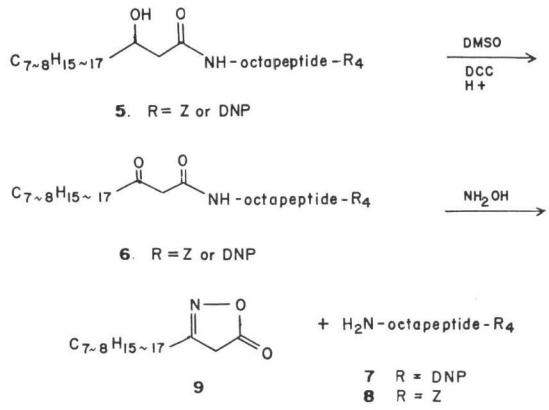


(R) configuration at the β-carbon atom for each of the esters can be inferred from the negative sign of the molecular rotation in $CHCl_3$[5,6,7]. The carboxylic acid corresponding to **3**, 3(R)-hydroxydecanoic acid, has previously been obtained from several other bacterial species[8]. Its absolute configuration was inferred from its optical rotation[6] and subsequently proved by synthesis of the optical antipode from a starting material of known absolute configuration[9]. The rotatory power of **2** and **3** is sharply reduced in methanol relative to that in chloroform. This effect has been previously observed[5,10] and probably results from the disruption, in methanol, of intramolecular hydrogen bonding between the β-hydroxyl group and the carbonyl oxygen that would confer a strong conformational preference in that part of the molecule.

Table 3. Optical rotations of DNP amino acids from EM49

DNP amino acid	EM49 residue number[a]	$[M]_D$[b]	Optical purity[c]
bis-DNP-D-Dab	1	+319°	89%
bis-DNP-L-Dab	2,3,6,7	−320°	89%
DNP-D-Leu	4	+135°	100%
DNP-L-Leu	5,8	−127°	94%

(*a*) See Structure **1**.
(*b*) Rotations were measured in glacial acetic acid.
(*c*) Calculated from optical rotations of DNP-L-amino acids reported in ref. 18.



The absolute value of the molecular rotation of **4** in $CHCl_3$ is smaller than that of **2** and **3** by 15°. This value is in excellent agreement with the limiting value (*ca.* 14°) of $[M]_D$ for long-chain anteiso acids[11,12]. The sign of this contribution to the rotation of **4** (+) allows assignment of the (S) configuration at C-8. The correlation between the sign of rotation and absolute configuration for anteiso acids has been well established by syntheses of a number of these acids, incorporating asymmetric centers of known configuration[11~16].

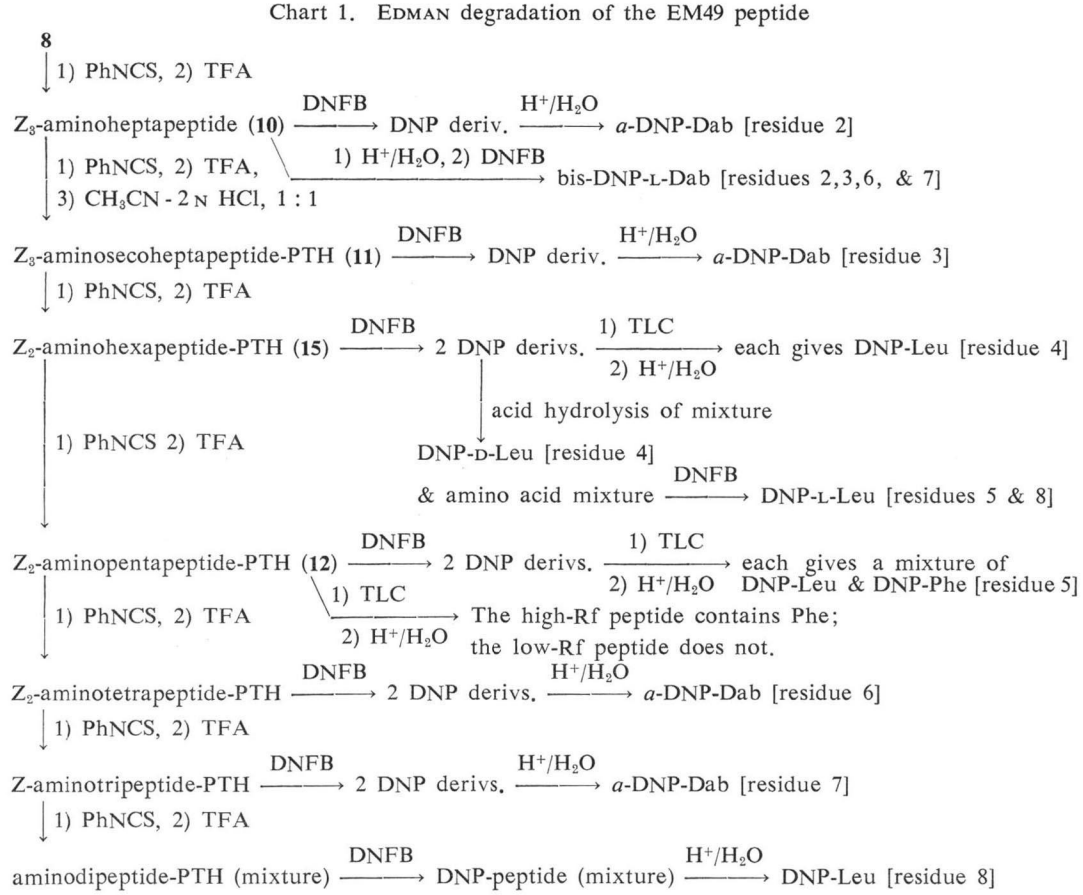
The +15° contribution to the optical rotation of **4** also constitutes substantial evidence that the methyl branch is at the ω-2 position. Branching closer to the middle of the chain (which would cause little change in the nmr spectrum) would result in a substantially smaller contribution to the optical rotation[11].

The presence of the hydroxyl group at the β-position of the acyl residue in EM49 provided convenient access to the peptide portion of the molecule by a reaction sequence developed for the degradation of peptides containing serine or threonine[17]. After the four amino groups of EM49 had been blocked, giving **5** with 2,4-dinitrophenyl (DNP) groups[3] or with benzyloxycarbonyl (Z) groups, the β-hydroxyl group was converted to a ketone, **6**, using the MOFFATT oxidation, and then the β-ketoacyl group was cleaved from the peptide with hydroxylamine to give derivatives **7** and **8** of desacyl EM49 and a mixture of isoxazolones, **9**, that was not further characterized.

Treatment of **7** with 2,4-dinitrofluorobenzene (DNFB), followed by acid hydrolysis of the peptide, gave bis-dinitrophenyl-2,4-diaminobutyric acid. Since the amino groups of EM49 had been previously shown to be the γ-amino groups of Dab residues[3], this result indicates that the fatty acyl group of EM49 is attached at the α-amino group of a Dab residue. The bis-DNP-Dab was isolated and purified by thin-layer chromatography, and the D configuration for this residue was established from the optical rotation in acetic acid (Table 3)[18]. Since the total Dab from EM49 has a predominance of the L configuration and an optical purity of 52%[3], the presence of a single D residue and four L residues is indicated. (The calculated optical rotation for such a mixture is 60% of that for pure L-Dab.) The optical purity of the total Dab and of the bis-DNP-Dab from the terminal amino acid residue may be slightly depressed from a small amount of racemization during hydrolysis.

The terminal amino acid was cleaved from **7** by the EDMAN method[19]. Treatment of the resulting heptapeptide with DNFB, followed by acid hydrolysis, gave α-DNP-Dab in addition

Chart 1. EDMAN degradation of the EM49 peptide



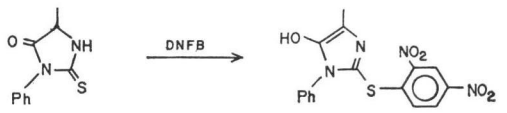
to γ-DNP-Dab. This established the second residue in the sequence as the trisubstituted (*i.e.* a ring-forming) L-Dab residue and also established the connection of the extracyclic chain at an α-amino group.

The EDMAN degradation of the EM49 peptide, starting with the benzyloxycarbonyl derivative, **8**, is shown in Chart 1. Removal of the terminal amino acid residue gave the Z_3-aminoheptapeptide, **10**, and end-group analysis of this peptide produced the expected α-DNP-Dab as the only DNP amino acid. The anticipated configuration (L) of the four Dab residues in **10** was also confirmed (Table 3) as outlined in Chart 1. The next cycle of the EDMAN degradation opened the peptide ring. The product of the reaction is a thiazolinone, as indicated by the development of a red color when a thin-layer chromatogram of the product was treated with dilute aqueous $AgNO_3$ and heated gently. This color reaction is also obtained for the various PTC peptides (without heat) and for thiazolinone amino acids cleaved from the peptide in the course of the degradation. In contrast, phenylthiohydantoins give a gray color when treated with $AgNO_3$ and heat. Treatment of the thiazolinone with aqueous acid converted it to the corresponding phenylthiohydantoin, **11**, which gave the typical gray color with $AgNO_3$.

Both the thiazolinone and **11** behave on TLC as a mixture of two components with slightly different Rf values, presumably because of the variation in structure at residue number 5, the position occupied almost equally by L-Phe and L-Leu. The only peptide intermediate in the

degradation sequence that was well separated by chromatography into components on the basis of variation in amino acid composition was the pentapeptide, **12**, in which residue number 5 is at the terminus. Hydrolysis (6 N HCl, 150°C, 48 hour) of the low-Rf component of **12** gave Dab and Leu (no Phe) in a ratio of 2.7 : 2.3 (calculated 3 : 2) and hydrolysis of the high-Rf component gave Dab, Leu, and Phe in a ratio of 2.8 : 1.2 : 1.0 (calculated 3 : 1 : 1).

The presence of the thiohydantoin is probably responsible for the chromatographic multiplicity observed for most of the DNP-peptides that were prepared after the opening of the peptide ring. To test for interference from this group, the PTH of alanine, **13**, was treated with DNFB under conditions used for the end-group analysis. This gave a substantial extent of reaction and, when the reaction time was increased to 16 hours, all **13** was consumed. The product was assigned structure **14** on the basis of its spectral properties and elemental analysis. The corresponding reaction of DNFB at the PTH group of the peptide intermediates is, therefore, probably the source of the multiplicity. However, this complication did not result in any ambiguity regarding the sequence of amino acids in the peptide. The DNP mixture derived from each peptide gave single DNP-amino acids upon hydrolysis, with the exception of the DNP mixture derived from the pentapeptide, **12**. This mixture was separated and each chromatographically homogeneous component yielded a mixture of DNP-Leu and DNP-Phe upon hydrolysis.



13 **14**

The configurations of the individual amino acid residues in EM49 (with the exception of Phe) were determined from the optical rotations of DNP-amino acids isolated in the course of the degradation, as shown in Chart 1. The DNP-amino acids were purified by thin-layer chromatography and the quantities used for rotation measurements were determined from the uv absorption spectra[18]. The values obtained are listed in Table 3. The configuration (L) of the Phe residue has been previously reported[3].

In addition to identification of the terminal amino acid residues by the SANGER method, the identities of the amino acid residues were also confirmed by conversion of the thiazolinones produced in the EDMAN degradation to the corresponding phenylthiohydantoins and chromatographic comparison with authentic materials. The results were entirely consistent with the conclusions derived by the SANGER method.

The final result of the degradation given above is structure **1**, which describes the members of the EM49 family. From structure **1**, it can be seen that the closest chemical relatives of EM49 are the polymyxins (including circulin A)[21,22,23]. The three fatty acyl residues in the EM49 complex have the same pattern of branching in the carbon chain as do the fatty acyl residues in the polymyxins (including the minor *n*-acyl component[23]). However, they are longer than the corresponding polymyxin acyl residues by two carbon atoms and have a β-hydroxyl group. It is interesting that this hydroxyl group occupies a position separated from the cyclic peptide portion by the same number of bonds as is the hydroxyl group of the extracyclic threonine residue found in all of the polymyxins. Yet, as pointed out previously[3], this hydroxyl group in EM49 is apparently unrelated to the antimicrobial activity since *O*-acetylation has little effect on this property.

The peptide portions of EM49 differ from the peptide portions of the polymyxins in the

Table 4. Sequences in common for EM49 and the polymyxins

EM49	Polymyxin A (M)	Polymyxin B	Polymyxin D	Polymyxin E (colistin)	Circulin A
acyl ↓ D-Dab 1 ↓ → Dab 2 ↓ Dab 3 ↓ D-Leu 4 ↓ Leu, Phe 5 ↓ Dab 6 ↓ Dab 7 ↓ Leu 8	acyl ↓ Dab ↓ Thr ↓ D-Dab ↓ → Dab Dab ↓ D-Leu ↓ Thr ↓ Dab Dab —Thr	acyl ↓ Dab ↓ Thr ↓ Dab ↓ → Dab Dab ↓ D-Phe ↓ Leu ↓ Dab Dab —Thr	acyl ↓ Dab ↓ Thr ↓ D-Ser ↓ → Dab Dab ↓ D-Leu ↓ Thr ↓ Dab Dab —Thr	acyl ↓ Dab ↓ Thr ↓ Dab ↓ → Dab Dab ↓ D-Leu ↓ Leu ↓ Dab Dab —Thr	acyl ↓ Dab ↓ Thr ↓ Dab ↓ → Dab Dab ↓ D-Leu ↓ Ileu ↓ Dab Dab —Thr

number of amino acid residues (eight and ten respectively) and in the absence of threonine from the EM49 peptides. However, a number of similarities are apparent. Both the polymyxins and EM49 contain cyclic heptapeptides, and the amino acid sequences in the EM49 peptide variants contain a number of coincidences with the amino acid sequences in the polymyxins. For example, the two L-Dab-L-Dab sequences in EM49 (positions 2~3 and 6~7 in structure **1**) are common to all of the polymyxins. A four-residue sequence, positions 1~4 in EM49, is also found in polymyxin A. The longest common sequence, comprised of the six amino acid residues in positions 2~7, is in polymyxin E (colistin) and those members of the EM49 family that contain Leu in position 5. These and other congruencies are summarized in Table 4. The Phe residue in position 5 of the variants containing this amino acid and the Leu residue in position 8 have not been found in the corresponding locations in the cyclic portions of any of the known polymyxins.

Although there are several structural correlations between EM49 and the polymyxins, the components of the EM49 family form a structural group that is distinct. This distinctiveness is supported by the broad antimicrobial spectrum of EM49 relative to those of the polymyxins and especially by the lack of cross-resistance between EM49 and polymyxin B[1,2].

Experimental Section

Nmr spectra were obtained on a Varian T-60 spectrometer. Chemical shifts are reported in δ units relative to tetramethylsilane as an internal standard. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter. Uv spectra were determined with a Perkin-Elmer Model 202 spectrometer. Melting points were measured with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Preparative gas chromatography was done on a Hewlett-Packard Series 5750 gas chromatograph. Preparative and thin-layer chromatography was carried out on Quantum Industries silica gel plates, eluting with $CHCl_3$-MeOH, 9:1 (system A);

$CHCl_3$-MeOH, 19 : 1 (system B); or with other solvent mixtures, as specified.

Fatty acid methyl esters from EM49: methyl 8-methyl-3(R)-hydroxynonanoate (**2**), methyl 3(R)-hydroxydecanoate (**3**), and methyl 8(S)-methyl-3(R)-hydroxydecanoate (**4**).

A sample of EM49 was hydrolyzed (6 N HCl, 110°C, 0.5 hour) and the resulting fatty acids were extracted with ether, converted to their methyl esters with diazomethane, and separated by gas chromatography at 210°C 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, essentially as described in Ref. 3. Table 1 lists the retention times, the combined quantities isolated from 13 separate 10 μl injections, and molecular rotations for the three esters. The elemental analyses are listed in Table 2.

Tetrabenzyloxycarbonyl-EM49 (**5**, R=Z)

A solution of 50 g of EM49 (0.143 equivalents based on the %Cl in the batch used) in 500 ml of methyl ethyl ketone and 1,000 ml of water was stirred vigorously. Powdered $NaHCO_3$, 30.6 g (0.365 mol), was added, followed by 0.146 mol of benzyloxycarbonyl chloride. After 1.5 hours, the reaction mixture was diluted with 500 ml of EtOAc. The upper phase was separated and washed with water, several portions of 0.01 N HCl, water (to neutrality), and saturated NaCl solution. The solution was dried ($MgSO_4$), concentrated *in vacuo*, and the residue converted to a powder by precipitation from EtOAc with hexane, giving 57 g of tetrabenzyloxycarbonyl-EM49.

For analysis, a sample was purified by TLC (system B, Rf 0.46 to 0.64) and reprecipitated from ethyl acetate with hexane.

Anal. Calcd. for $C_{82.2}H_{115.5}N_{13}O_{15}$*: C, 62.73; H, 7.40; N, 11.57
Found: C, 62.39; H, 7.53; N, 11.47

Tetra (DNP) desacyl-EM49 (**7**)

Tetra (DNP) EM49[3], 14.2 mg, was dissolved in 0.14 ml of dry DMSO and to the solution were added 0.0048 ml of pyridine, 0.0022 ml of TFA, and 38.6 mg of DCC. The resulting mixture was kept under argon in the dark for 17 hours and then diluted with EtOAc and treated with oxalic acid to destroy the excess DCC. DCU was filtered out and the filtrate washed with $NaHCO_3$ solution, water, 1 N HCl, water, saturated NaCl solution, dried (Na_2SO_4), and the solvent removed *in vacuo*. The residue was precipitated from acetone with benzene, giving 12.3 mg of the ketone, **6**, R=DNP. The ketone was chromatographically homogeneous (Rf 0.44 *vs* Rf 0.38 for the starting material in system A).

The ketone, 11.7 mg, was dissolved in a mixture of 0.5 ml of HOAc, 0.2 ml of MeOH, and 0.1 ml of H_2O. Powdered $NH_2OH \cdot HCl$, 5.2 mg, was added and the solution was heated at 70°C for 35 minutes. The solution was mixed with EtOAc and the product isolated as described above for the ketone. The resulting tetra (DNP) desacyl-EM49·HCl (**7**, 9.9 mg) was chromatographically homogeneous (Rf 0.10 in system A).

Tetrabenzyloxycarbonyldesacyl-EM49 hydrochloride (**8**)

Tetrabenzyloxycarbonyl-EM49, 56.7 g (36.1 mmol), was dried *in vacuo* and dissolved in 280 ml of dry DMSO. To the solution were added 2.92 ml (36.1 mmol) of dry pyridine, 1.34 ml (18.1 mmol) of trifluoroacetic acid, and 22.4 g (108 mmol) of DCC. The resulting solution was stirred for 3 hours at room temperature and then at 37°C overnight.

The mixture was diluted with ethyl acetate (280 ml), and a solution of 9.1 g (72 mmol) of oxalic acid in methanol was added to react with the excess DCC. The mixture was stirred for 0.5 hours and then filtered to remove DCU. The filtrate was washed with water, saturated $NaHCO_3$ solution, water, 1 N HCl, water, and saturated NaCl solution, and was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was precipitated from EtOAc with hexane, giving 55.9 g of the crude ketone, **6**, R=Z.

* The calculated empirical formula for **5**, R=Z is based on an average formula of $C_{50.2}H_{91.5}N_{13}O_{10} \cdot 4HCl$ for the EM49 family (ref. 3).

The ketone (54.3 g, 34.3 mmol) was dissolved in 900 ml of HOAc - H_2O - MeOH (1 : 1 : 2) at 70°C, and 4.78 g (69 mmol) of $NH_2OH \cdot HCl$ was added. The resulting solution was stirred at 70°C for 2 hours and then concentrated *in vacuo*. A solution of the residue in EtOAc was washed with water, several portions of 1 M Na_2CO_3 and with water to neutrality and was concentrated *in vacuo*. The residue was precipitated from CH_2Cl_2 with hexane, giving 49.2 g of the crude amine.

Bio·Rad AG50W-X2 resin, 200~400 mesh, H^+ form, 100 g, was washed with methanol and added to a solution of the crude amine in methanol. The mixture was stirred for 0.5 hour and then the resin was filtered out and washed with methanol.

The resin was stirred in a mixture of 1,000 ml of methanol, 1,000 ml of $CHCl_3$, and 500 ml of saturated, aqueous NaCl solution for 0.5 hour. The mixture was filtered, washing the solids (resin and NaCl) with $CHCl_3$. The lower phase was separated, washed with water, and concentrated *in vacuo*. The residue was precipitated from CH_2Cl_2 with hexane, dried *in vacuo*, and equilibrated with atmospheric moisture, giving 38.1 g of tetrabenzyloxycarbonyldesacyl-EM49·HCl containing 3.42 % water. A sample of the crude amine was also purified by a 28-transfer countercurrent distribution in heptane - benzene - EtOAc - 95 % EtOH - 0.01 N HCl (10 : 26 : 24 : 45 : 30) to give material for elemental analysis.

Analysis: Calcd. for $C_{71.5}H_{97}N_{13}O_{16}Cl$*: C, 60.05; H, 6.84; N, 12.73; Cl, 2.48.
Found: C, 60.33; H, 7.06; N, 12.93; Cl, 2.51.

Identification of the Terminal Amino Acid Residue of 7

Tetra (DNP) desacyl-EM49.HCl, 9.0 mg, was dissolved in 0.05 ml of methyl ethyl ketone, and to the solution were added 0.02 ml of a 2.8 M solution of DNFB in EtOAc and 4 drops of saturated aqueous $NaHCO_3$. The mixture was stirred in the dark for 2 hours, diluted with EtOAc, and the EtOAc solution washed with water and concentrated *in vacuo*. The residue was precipitated from acetone with benzene, giving 9.0 mg of a chromatographically homogeneous solid, Rf 0.35 (system A).

This solid, 8.4 mg, was hydrolyzed in 6 N HCl (1 ml) at 113°C for 17 hours. Extraction with a mixture of Et_2O - EtOAc (1 : 1) gave a yellow oil that was identified as bis-DNP-Dab by chromatographic comparison with authentic material[18]. The derivative was purified by preparative TLC on silica gel, eluting with EtOAc - MeOH, 3 : 1. A yellow band, Rf 0.43~0.53, was collected, dissolved in 1.20 ml of HOAc, and the optical rotation measured (α_D + 0.061°). A portion of the solution (0.100 ml) was concentrated to dryness and redissolved in 10 ml of 4 % aqueous $NaHCO_3$. The absorbance (0.564) at 358 nm (1 cm) and a molar extinction coefficient[18] of 2.95×10^4 were used to calculate the quantity (1.03 mg) of bis-DNP-Dab used in determining the rotation. From those data, a molecular rotation of +319° was calculated for the bis-DNP-Dab.

EDMAN Degradation of $(DNP)_4$desacyl-EM49 (7)

The $(DNP)_4$-peptide was treated with PhNCS, as described below for the degradation of the Z_4-peptide (**8**). The resulting PTC-peptide (Rf 0.60 in system A) was dissolved in TFA and heated at 40°C for 15 minutes. Addition of benzene precipitated the $(DNP)_3$-aminoheptapeptide, Rf (system A) 0.24.

A solution of the heptapeptide (52 mg) in 1 ml of methyl ethyl ketone - *n*-BuOH (1 : 1) was treated with DNFB (33 mg) and Et_3N (0.026 ml). After 1.5 hours, benzene was added to precipitate the resulting DNP-peptide, 46 mg, Rf 0.41 (system A). Hydrolysis (6 N HCl, 110°C, 17 hours) gave a mixture of α- and γ-DNP-Dab[20], as shown by TLC on silica gel plates that had been immersed in a 1 % solution of $Cu(OAc)_2$ in methanol and dried at 100°C. Elution of the plates with EtOAc - pyridine - HOAc - H_2O, (60 : 20 : 6 : 11) gave Rf values of 0.11 for α-DNP-Dab and 0.27 for γ-DNP-Dab.

Treatment of the heptapeptide with PhNCS gave a PTC-peptide, Rf 0.56 (system A), that

* The calculated empirical formula for **7**, R=Z is derived from the average formula of the two peptides represented by **1**, X=H.

gave only traces of amines when heated in TFA at 50°C for 0.5 hour. The bulk of recovered material was chromatographically indistinguishable from the untreated PTC-peptide.

EDMAN Degradation of tetrabenzyloxycarbonyldesacyl-EM49 hydrochloride (**8**)

The procedures used for the degradation of this peptide are adapted from those given by P. EDMAN in ref.[19] One gram (0.685 mmol) of **8** was dissolved in 5 ml of dry pyridine, and to the solution were added 0.95 ml (6.8 mmol) of Et_3N and 0.41 ml (3.4 mmol) of PhNCS. The solution was kept under Ar at 40°C for 1 hour and then the product was precipitated with heptane and reprecipitated from $CHCl_3$ with heptane until a powder was obtained. The resulting PTC-peptide was dissolved in 30 ml of TFA and kept under Ar at room temperature for 10 minutes. The solvent was then removed *in vacuo* and the residue reprecipitated from $CHCl_3$ with heptane until a powder (1.14 g) was obtained. A sample of this amine (0.25 g) was converted to the bicarbonate and was purified using Dowex 50W-X2 resin, as described above in the preparation of **8**. This gave 0.15 g of the heptapeptide **10**. Subsequent cycles of the EDMAN degradation were done on a reduced scale in essentially the same way, except that the intermediates were not purified. Solvent mixtures used for precipitating the intermediates were varied as necessary to obtain powders.

The configuration (L) of the four Dab residues in the heptapeptide, **10**, was confirmed by hydrolysis (6 N HCl, 110°C, 17 hours), conversion of the resulting amino acids to their DNP derivatives, and isolation of the bis-DNP-Dab by TLC. The optical rotation was measured as described above, with the same result, except that the sign of rotation was reversed (see Table 3).

The second cycle of the degradation was the only one that was exceptional. This cycle opens the peptide ring to give a phenylthiazolinone as part of the peptide. To convert the phenylthiazolinone, 121 mg, to the corresponding phenylthiohydantoin, **11**, it was heated in 24 ml of CH_3CN - 2 N HCl (1 : 1) at 80°C under Ar for 10 minutes. The CH_3CN was removed *in vacuo* and the product extracted into a mixture of EtOAc, MEK, *n*-BuOH and DMF. The solution was washed with water and taken to dryness *in vacuo*, giving 112 mg of **11**.

End-Group Analysis of Intermediates in the EDMAN Degradation of **8**

The N-terminal amino acids in the degradation intermediates were identified by the SANGER method. For example, the Z_2-aminohexapeptide-PTH, **15** (see Chart 1), 3.7 mg, was dissolved in 0.1 ml of MeOH and to the solution were added 0.01 ml of a 2.8 M solution of DNFB in EtOAc and 0.01 ml of Et_3N. After 1 hour at room temperature under Ar, the solvent was removed in a stream of Ar and the product was precipitated from acetone with heptane. Thin-layer chromatography of the product (system B) gave two yellow bands: Rf 0.64~0.75 (1.0 mg) and Rf 0.55~0.64 (1.4 mg). Each was hydrolyzed in 6 N HCl at 110°C for 17 hours. TLC of the hydrolysates (silica gel, eluting with $CHCl_3$ - MeOH - HOAc, 90 : 10 : 1) showed that each contained DNP-Leu, Rf 0.33, as the only DNP amino acid.

This particular sequence was repeated on a larger scale (starting with 15 mg of **15**) in order to get sufficient DNP-Leu to permit measurement of the optical rotation. The DNP-Leu was extracted from the hydrolysate with ether, purified by thin-layer chromatography and the rotation measured as described for bis-DNP-Dab, thus establishing the D configuration for residue number 4. After extraction of the DNP-Leu from the hydrolysate, the remaining Leu in the hydrolysate was isolated as the DNP derivative and the L configuration confirmed, as described above for the Dab residues in the Z_3-aminoheptapeptide, **10**.

The Reaction of PTH-ala, **13**, with DNFB

A sample of **13** was treated with DNFB, as descibed above for end-group analysis of **15**, except that the reaction time was extended to 16 hours. The product was isolated by preparative TLC (silica gel, eluting with benzene - ether, 2 : 1, Rf 0.2~0.6) and further purified by recrystallization from acetone-benzene mixture to give **14**: m.p. 234~236°C; nmr (acetone-d_6) δ 2.22 (s, 3), 7.54 (s, 5), 8~9 (m, 3), *ca.* 3 (very broad); uv max (95 % ethanol) 270 nm (ε 29,600).

Anal. Calcd. for $C_{16}H_{12}N_4O_5S$: C, 51.61; H, 3.25; N, 15.05; S, 8.61.
Found: C, 51.33; H, 3.32; N, 14.77; S, 8.47.

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