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# THE REVISED STRUCTURE OF THE PEPTIDE ANTIBIOTIC ESPERIN, ESTABLISHED BY MASS SPECTROMETRY*

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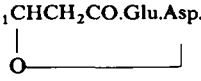
**Abstract**—The structures of the antibiotic esperin and of esperinic acid were re-examined using the techniques of N-permethylation and mass spectrometry. The structure of esperinic acid is revised to $RCHOHCH_2CO.Glu.Leu.Leu.Val.Asp.Leu.Leu(Val).OH$, where R is a mixture of $C_{10}H_{21}$, $C_{11}H_{23}$, and $C_{12}H_{25}$, and the C-terminal residue contains a 30% replacement by valine. Esperin (7) contains the same sequence with a lactone formed by the OH group of the fatty acid constituent and the carboxylic acid function of the aspartic acid residue.

ESPERIN is an antibiotic produced by *Bacillus mesentericus*,[1,2] for which structure **1** has been proposed.[3–5] Alkaline hydrolysis of this peptidic lactone produced a linear peptide derivative, esperinic acid, suggested to have structure **2**.[5] However, on the basis of differing characteristics of this acid and a synthetic sample of **2**, the correctness of structures **1** and **2** was questioned.[6] Thus, a reinvestigation of esperin and esperinic acid was undertaken with emphasis on the application of mass spectrometry.



$C_{10}H_{21}CHCH_2CO.Glu.Asp.Val.Leu.Leu.OH$

**1**

$C_{10}H_{21}CHOHCH_2CO.Glu.Asp.Val.Leu.Leu.OH$

**2**

*Fatty acid constituents.* An acid hydrolysis product of esperin, formerly thought to be 2-tridecenoic acid ($C_{12}H_{23}COOH$),[3–5] was re-examined by mass spectrometry. At least three homologs were found: $C_{12}H_{23}COOH$, $C_{13}H_{25}COOH$, and $C_{14}H_{27}COOH$, the latter being the most abundant. The unsaturation resulted from dehydration of the original hydroxyacyl peptide during hydrolysis. The location of the OH group at position 3 (as suggested in the structure **2**) of each fatty acid constituent could be confirmed by the mass spectrum of the methyl ester of another hydrolysis product in which the OH group was still present. The major peak in this spectrum at *m/e* 103 was due to the ion $^{\oplus}CHOHCH_2COOCH_3$.

*The esperinic acids.* When esperin was treated with sodium hydroxide, two different

* Part XVI in the series "Determination of amino acid sequences in oligopeptides by mass spectrometry"; part XV. D. W. Thomas, *Biochem. Biophys. Res. Commun.*, 33, 483 (1968).

compounds were produced: (a) hydrolysis with 1·5–2·0% NaOH in ethanol gave the crystalline sodium salt of an acid named esperinic acid in the previous report[5] and now given the name esperinic acid I ([α] +12·5°); (b) hydrolysis under more mild conditions (0·1 N NaOH) gave esperinic acid II ([α] −13°). The difference between these acids is revealed clearly by their ORD curves: the acid I gives a positive Cotton curve in methanol, whereas acid II and in fact esperin itself give negative curves.

*The structure of esperinic acid* II. Although mass spectra could be obtained of esperin and the esperinic acids, as well as of their methyl esters, little structure information could be derived because of the great complexity of these spectra. Thus, O,N-permethylation, a technique useful for simplifying the mass spectra of peptide derivatives,[7] was considered. Initial permethylation attempts with methyl iodide and silver oxide[7,8] gave only partial success, due to undesirable side reactions of the aspartic acid residue.[9] However, use of an alternative method employing methyl iodide and a methylsulfinyl carbanion conveniently eliminated this difficulty.[10–12] The mass spectrum (Fig. 1) of the resulting permethylated esperinic acid II clearly revealed the homologous fatty acid constituents discussed above, and in fact the entire amino acid sequence.

The spectrum of Fig. 1 may be thought of as representing six compounds: permethylated esperinic acid II itself (**3**), which is a mixture of three homologs, and another group of three homologs (**4**) which have lost one molecule of methanol (in the mass spectrometer) from the acyl constituent of **3**. Mass spectral fragmentation occurred predominantly at the peptide CO—NMe bonds; the resulting peaks, as outlined in structures **3** and **4** and in Fig. 1 (for the major homolog constituent only), delineated the sequence without difficulty.

An examination of relative peak intensities at the high mass end of the spectrum (Fig. 1) provided additional structure information: *m/e* 1130, 1161, and 1162 are more intense than *m/e* 1144, 1175, and 1176, respectively, whereas for all fragments

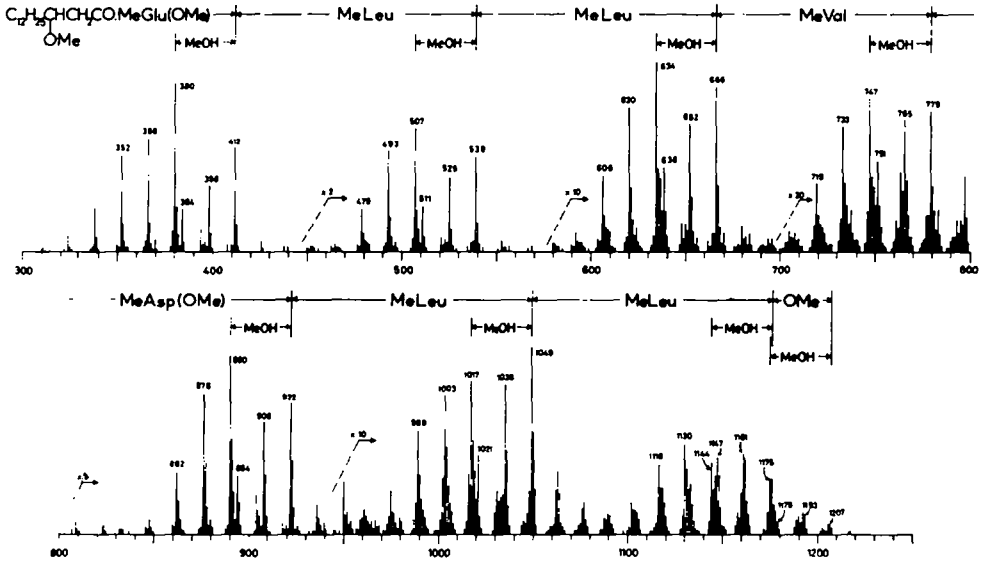



FIG. 1 Mass spectrum of permethylated esperinic acid II.

| RCHOMeCH$_2$CO.MeGlu(OMe) | MeLeu | MeLeu | MeVal | MeAsp(OMe) | MeLeu | MeLeu | OMe |
|---|---|---|---|---|---|---|---|
| R = $C_{12}H_{25}$ | 412 | 539 | 666 | 779 | 922 | 1049 | 1176 | 1207 |
| R = $C_{11}H_{23}$ | 398 | 525 | 652 | 765 | 908 | 1035 | 1162 | 1193 |
| R = $C_{10}H_{21}$ | 384 | 511 | 638 | 751 | 894 | 1021 | 1148 | 1179 |

**3**

| RCH=CHCO.MeGlu(OMe) | MeLeu | MeLeu | MeVal | MeAsp(OMe) | MeLeu | MeLeu | OMe |
|---|---|---|---|---|---|---|---|
| R = $C_{12}H_{25}$ | 380 | 507 | 634 | 747 | 890 | 1017 | 1144 | 1175 |
| R = $C_{11}H_{23}$ | 366 | 493 | 620 | 733 | 876 | 1003 | 1130 | 1161 |
| R = $C_{10}H_{21}$ | 352 | 479 | 606 | 719 | 862 | 989 | 1116 | 1147 |

**4**

which lack the C-terminal residue the homolog of *highest* mass is most abundant (e.g. see *m/e* 1049, 1035, 1021). This implies that the C-terminal leucine is replaced in part by valine (approximately 30%).

Use of deuteriomethyliodide[13] verified that all O,N-Me groups of **3** were introduced by the permethylation reaction, thus **5** is proposed as the structure of esperinic acid II, where homologue ratios were determined from the peaks at *m/e* 511, 525, and 539 of Fig. 1.

RCHOHCH$_2$CO.Glu.Leu.Leu.Val.Asp.Leu.Leu(Val).OH
R = $C_{12}H_{25}$ (45%)
R = $C_{11}H_{23}$ (35%)
R = $C_{10}H_{21}$ (20%)

**5**

Because the spectrum of permethylated esperinic acid II (Fig. 1) was not especially "clean" in the molecular ion region, there remained a slight uncertainty about the C-terminal residue. The correctness of structure **5** is however confirmed by two other experiments:

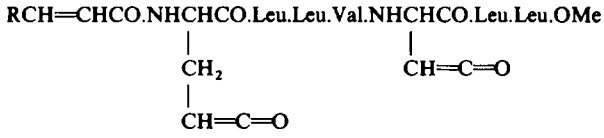
(a) Partial hydrolysis of esperinic acid II in hydrochloric and acetic acids, followed by permethylation, gave a single product (**6**) which lacked the homologous fatty acid constituents. The mass spectrum of **6** was very simple, with a single peak observed for each amide bond cleavage (as compared to the clusters of *six* peaks in Fig. 1), and with doublets in the molecular ion region which confirmed the valine/leucine mixture at the C-terminus.

| MeGlu.MeLeu | MeLeu | MeVal | MeAsp(OMe) | MeLeu | MeLeu(Val) | OMe |
|---|---|---|---|---|---|---|
| *m/e* 253 | 380 | 493 | 636 | 763 | 890 (876) | 921 (907) |

**6**

(b) A mass spectrum (Fig. 2) of esperinic acid II trimethyl ester (prepared with diazomethane) was particularly clean at the high mass end, the peaks of highest mass

being due to loss of water and two molecules of methanol from the molecular ion to give the following fragment:




R = $C_{12}H_{25}$ *m/e*: 1013
R = $C_{11}H_{23}$ *m/e*: 999
R = $C_{10}H_{21}$ *m/e*: 985

These peaks are accompanied by another series (*m/e* 957, 943, 929) which results by loss of a leucine side chain ($C_4H_8$) from each of these homologous fragments.

*Esperinic acid* I. The O,N-permethyl derivative of esperinic acid I gives a mass spectrum very similar to that of the corresponding derivative of acid II as discussed above. However, minor differences are observed in relative peak intensities for all fragmentations involving loss of methanol from the fatty acid constituent. Thus we suggest that the two compounds differ only in configuration at the *beta* carbon of the fatty acid constituent (a similar base-catalyzed inversion has also been observed with the mycolic acids[14]). This conclusion is consistent with the marked differences which may be observed in the ORD curves of the two esperinic acids.

*Esperin.* The mass spectrometric molecular weight (1063 and lower homologs) of esperin dimethyl ester is in accord with a lactone formed by the OH group of the fatty acid constituent and one of the three carboxylic acid functions of the esperinic acids **5**. Successive hydrazinolysis, Curtius rearrangement, and hydrolysis of esperin had

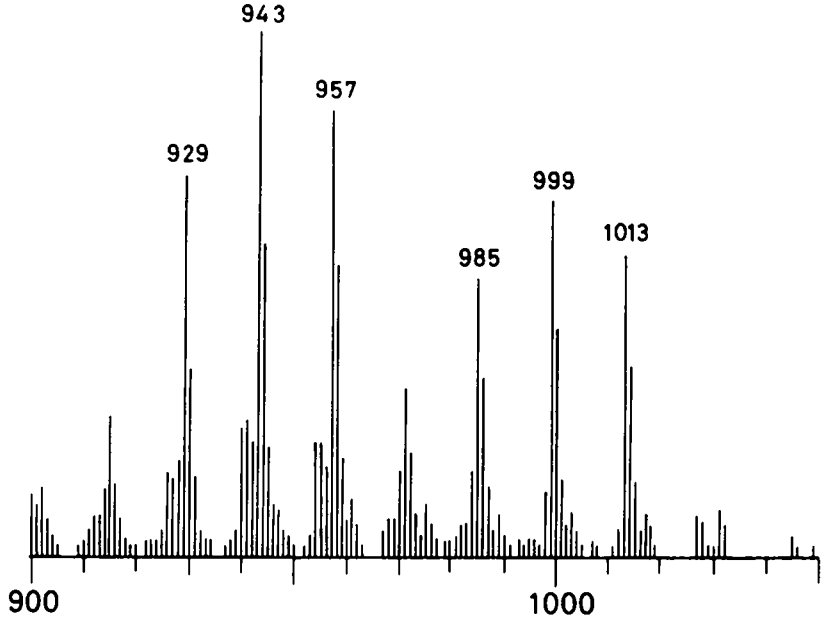
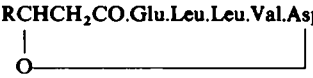



FIG. 2 Partial mass spectrum of esperinic acid II trimethyl ester.

transformed the aspartic acid residue to α,β-diaminopropionic acid;[5] thus the lactone involves the aspartic acid residue, and the revised structure of esperin may be represented by formula 7.




7

R = $C_{12}H_{25}$ (45%)
R = $C_{11}H_{23}$ (35%)
R = $C_{10}H_{21}$ (20%)

## EXPERIMENTAL

All mass spectra were determined with an AEI model MS9 mass spectrometer.

*Esperinic acid* I. Esperin (2 g) was dissolved in 10 ml EtOH and to this was added 2 ml 10% NaOH. After standing overnight at room temp, 1·7 g of a crystalline Na salt was obtained by filtration. Recrystallization from 70% EtOH gave needles, m.p. 269°. The free acid was obtained by acidifying with HCl. The resulting ppt was removed by filtration, dried, and recrystallized from acetone–pet. ether; m.p. 195°, $[\alpha]_D^{15}$ +12·5° (*c* 1·6 MeOH).

*Esperinic acid* II. Esperin (70 mg) was dissolved in 5 ml of 0·1 N NaOH. The soln was allowed to stand at room temp for 6 hr. The free acid II (55 mg) was obtained by acidifying with HCl; $[\alpha]_D^{27}$ −13° (*c* 0·6 MeOH).

*Permethylation of esperinic acid* II. NaH oil suspension (20 mg) was rinsed three times with ether. DMSO (0·2 ml) was added and maintained at 100° until $H_2$ evolution ceased, then cooled to room temp. Acid II (2 mg) and 0·2 ml MeI were added to this reagent and allowed to stand for 1·5 hr. The reaction mixture was added to 5 ml $CHCl_3$, then rinsed several times with water, dried, and evaporated. Without further purification the mass spectrum of Fig. 1 was obtained of this product.

*Partial hydrolysis of esperinic acid* II. Acid II (3 mg) was dissolved in 0·5 ml 1:1 12N HCl and glacial AcOH. After 6 hr at 50° the solvents were evaporated and the product was treated with $Ac_2O$ in MeOH, then permethylated as described above. A mass spectrum of this product revealed a mixture of unchanged esperinic acid and 6. The latter was isolated by TLC on silica gel.

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