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A MASS SPECTROMETRIC STUDY OF NATURAL MIXTURES OF ENNIATIN ANTIBIOTICS

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The antibiotics enniatin A and enniatin B, which possess a high in vitro activity against various mycobacteria, have been isolated from the mycelia of a number of strains of Fusarium. These antibiotics have been ascribed the structures of the cyclotetradepsipeptides (I) and (II), respectively [4, 5].

The synthesis of compounds (I) and (II) that we have carried out has shown that these cyclotetradepsipeptides differ markedly in their physical properties from the natural antibiotics and possess no antimicrobial activity; consequently, formulas (I) and (II) do not correspond to the structure of enniatins A and B [6-8]. We assumed that the molecular weights of these compounds had been determined incorrectly and that they were really cyclohexadepsipeptides. In fact, the synthesized compounds (III) and (IV) proved to be completely identical with natural enniatins A and B [9-11]. The synthesis of enniatins A and B was performed simultaneously by Swiss workers [12, 13]. Thus it has been established that enniatins A and B correspond to formulas (III) and (IV).

Nevertheless, the hydrolysates of even the purest samples of natural enniatin A (III) contain N-methylvaline in addition to N-methylisoleucine. This has been explained previously by the assumption that enniatin A is contaminated with enniatin B (IV) which is extremely difficult to remove by recrystallization or by any other method [14]. However, another explanation is possible, which is that a natural mixture of the antibiotics contains substances with structures differing from enniatin A in one or two N-methylisoleucine residues having been replaced by one or two N-methyl-valine residues. We shall call the first of these compounds enniatin A_1(V) and the second enniatin B_1(VI).

L—Melle—D—Hylv
D—Hylv—L—Melle
(I)

L—MeVal—D—Hylv
D—Hylv—L—MeVal
(II)

L—Melle—D—Hylv—L—Melle
D—Hylv—L—Melle—D—Hylv
(III)

L—MeVal—D—Hylv—L—MeVal
D—Hylv—L—MeVal—D—Hylv
(IV)

L—Melle—D—Hylv—L—Melle
D—Hylv—L—MeVal—D—Hylv
(V)

L—Melle—D—Hylv—L—MeVal
D—Hylv—L—MeVal—D—Hylv
(VI)

Melle= —N(H$_3$C)—CH(CH(CH$_3$)C$_2$H$_5$)—CO—;

MeVal= —N(H$_3$C)—CH(CH(CH$_3$)$_2$)—CO—;

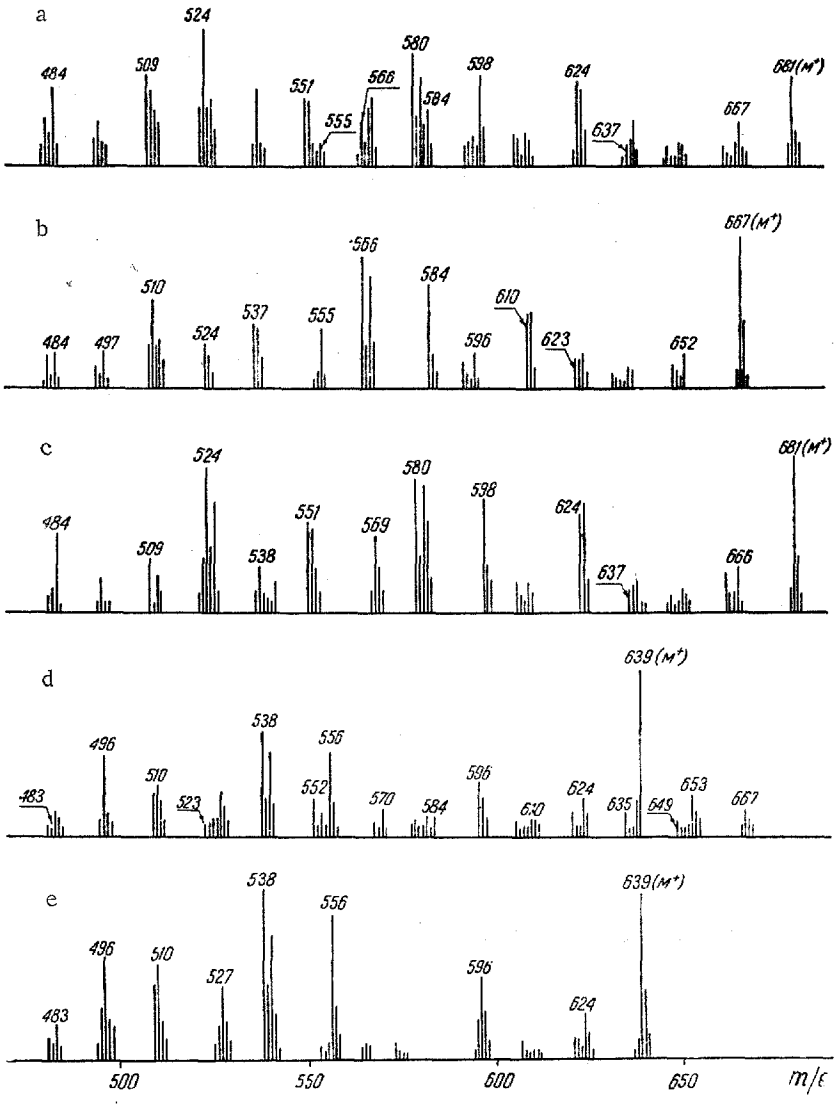
Hylv= —O—CH(CH(CH$_3$)$_2$)—CO—.

It is natural to assume that this type of compound is still more difficult to separate from enniatins A and B or to isolate in the individual state in view of the closeness of the physicochemical characteristics of all these substances. In actual fact, the compound that we synthesized possessing the structure of the hypothetical enniatin A_1(V) was practically identical to enniatin A in its physical properties and biological activity [11].

We have attempted to answer the question of the chemical nature of the minor component of natural enniatin A by mass spectrometry [the mass spectra were recorded on a RMU-6D instrument (Hitachi) at 250° C, the sample being introduced directly into the ion source]. The presence in the mass spectrum of natural enniatin A of a fairly strong peak with m/e 86 (amine fragment of N-methylvaline $H_3C-NH=CH-C_3H_7$) is an additional indication of the presence in the sample studied of a N-methlvaline residue. In the mass spectra of all the synthetic cyclohexadepsipeptides of regular structure that we have studied previously, in the group of peaks adjacent to the molecular peak the strongest (10-30% of the intensity of the molecular peak) is the peak $(M - 15)^+$, corresponding to the elimination of a methyl group [15]. Conversely, in the mass spectrum of a natural sample of enniatin A the strongest peak in this region is that with m/e 667 $(M - 14)^+$ (figure).

It appeared to us to be most logical to classify the ion corresponding to this peak not as one of the products of fragmentation of the molecular ion of enniatin A but as the molecular ion of enniatin A_1. If this assumption is correct,

in the mass spectrum of natural enniatin A we should find peaks corresponding to all five fragments of enniatin A_1. Let us take, for example, the amino (hydroxy) acid type of fragmentation which gives the fullest information on the struc-



Mass spectra of natural enniatin A (a), synthetic enniatin A_1 (b), synthetic enniatin A (c), natural enniatin B (d), and synthetic enniatin B (e).

ture of the compound studied [16]. The fragmentation of the synthetic cyclohexadepsipeptide having the structure of enniatin A_1 (V) of this type, beginning with the elimination of an α-hydroxyisovaleric acid residue with the capture of one hydrogen atom from the charged fragment takes place in three directions depending on which of the residues of this hydroxy acid is eliminated in the first act (see figure):

$$M^+ (667) \begin{cases} \rightarrow \text{Hylv (566)} \rightarrow \text{MeVal (453)} \rightarrow \text{Hylv (353)} \rightarrow \text{Melle (226)} \rightarrow \text{Hylv (126)} \\ \rightarrow \text{Hylv (566)} \rightarrow \text{Melle (439)} \rightarrow \text{Hylv (339)} \rightarrow \text{MeVal (226)} \rightarrow \text{Hylv (126)} \\ \rightarrow \text{Hylv (566)} \rightarrow \text{Melle (439)} \rightarrow \text{Hylv (339)} \rightarrow \text{Melle (212)} \rightarrow \text{Hylv (112)} \end{cases}$$

All these peaks appear in the mass spectra of a natural sample of enniatin A; at the same time, some of them (for example peaks with m/e 566 and 339) are completely absent from the mass spectrum of the synthetic antibiotic and other peaks are of considerably lower intensity (see figure). Similar evidence in favor of the identity of the cyclohexadepsipeptide (V) and enniatin A_1 can be obtained from an analysis of other types of fragmentation.

All the peaks mentioned above, including the molecular ion of enniatin A_1 (m/e 667) are present in the mass spectra of one of the samples of natural enniatin B (this sample, which had not been subjected to careful purification, was kindly given to us by Prof. Hardegger) (see figure). Of course, the intensities of these peaks are, as a rule, low because the content of enniatin A_1 in the sample studied was extremely small.

In addition, the mass spectrum of the same sample of natural enniatin B (see figure) has a peak (m/e 653) 14 mass units greater than the molecular peak of enniatin B. Since the intensity of this peak is approximately twice that of the molecular ion of enniatin A_1, this peak can be ascribed only to the molecular ion of enniatin B_1. The presence of the latter in a natural mixture of antibiotics is confirmed by an analysis of different routes for its fragmentation. Thus, for example, the hydroxy acid type of fragmentation must lead to the formation of the following fragments:

$$M^+ (653) \begin{cases} \rightarrow \text{HyIv (552)} \rightarrow \text{MeVal (439)} \rightarrow \text{HyIv (339)} \rightarrow \text{MeIle (212)} \rightarrow \text{HyIv (112)} \\ \rightarrow \text{HyIv (552)} \rightarrow \text{MeIle (425)} \rightarrow \text{HyIv (325)} \rightarrow \text{MeVal (212)} \rightarrow \text{HyIv (112)} \\ \rightarrow \text{HyIv (552)} \rightarrow \text{MeVal (439)} \rightarrow \text{HyIv (339)} \rightarrow \text{MeVal (226)} \rightarrow \text{HyIv (126)} \end{cases}$$

All the peaks corresponding to these fragments are present in the mass spectrum of a natural sample of enniatin B. At the same time, some of them (for example, the peaks with m/e 552, 439, and 226) are practically absent from the mass spectrum of the synthetic antibiotic (see figure). As in the case of enniatin A, an analysis of other types of fragmentation confirms the presence in the mixture of the individual compound enniatin B_1. The table gives the results of a determination of the accurate values of the masses of a series of fragments the peaks of which are observed in the mass spectrum of natural enniatin B and are absent from the mass spectrum of a synthetic sample of this antibiotic. As can be seen from the table, all these fragments are products of the degradation of the molecular ion of enniatin B_1.

The data given show that the mixture of antibiotics produced by various strains of Fusarium contains two other antibiotics besides enniatins A and B—enniatins A_1 (V) and B_1 (VI).

m/e	Mass of the fragment		Empirical formula of the fragment	Fragment
	found	calculated		
653	653.4248	653.4250	$C_{34}H_{59}N_3O_9$	Molecular ion (M^+) of enniatin B_1
610	610.3710	610.3702	$C_{31}H_{52}N_3O_9$	$M^+ - CHMe_2$
570	570.3727	570.3744	$C_{29}H_{52}N_3O_8$	$M^+ - O{=}C{=}C{-}CHMe_2$
423	423.2854	423.2859	$C_{23}H_{39}N_2O_5$	$N(Me){-}CH(CHMe_2){-}C{\equiv}O^+$ | $OC{-}CH(CHMe_2){-}O{-}CO{-}CH(CHMeEt){-}N(Me){-}CO{-}CH{=}CMe_2$
310	310.2017	310.2018	$C_{17}H_{28}NO_4$	$O{\equiv}C{-}CH(CHMe_2){-}O{-}CO{-}CH(CHMeEt){-}N(Me){-}CO{-}CH{=}CMe_2^+$
210	210.1499	210.1494	$C_{12}H_{20}NO_2$	$O{\equiv}C{-}CH(CHMeEt){-}N(Me){-}CO{-}CH{=}CMe_2^+$
100	100.1124	100.1126	$C_6H_{14}N$	$CH(CHMeEt){=}NH(Me)$

Any other structures for enniatins A_1 and B_1 are disproved both by the general nature of the mass spectra, which are typical for cyclohexadepsipeptides with regularly alternating amino acid and hydroxy acid residues and by biogenetic considerations. The results that we have obtained open up a route to the elucidation of the structure of antibiotics related to enniatin—lateritiins I and II, avenacein, sambucinin, and fructigenin [17]. This approach may also be used in the study of mixtures of other antibiotics.

The experimental work of the recording and calculation of the high-resolution mass spectra was kindly carried out on a MS-9 instrument by Dr. K. Vogler, Dr. W. Wetter, and P. Meyer (F. Hoffman-La Roche Co. Ltd., Chemical Research Department, Basle, Switzerland).

Conclusions

It has been shown that the natural mixture of enniatin antibiotics contains not only enniatins A and B but also two new substances, enniatins A_1 and B_1, which differ from enniatin A by the fact that in their molecules one or two N-methylisoleucine residues are replaced by one or two N-methylvaline residues.

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