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A STUDY OF COMPLEX MIXTURES OF NATURAL SUBSTANCES BY THE DEFOCUSING AND DADI METHODS.

III. COMPONENTS OF THE PROTECTIVE SECRETION OF THE BEETLE *Coccinella septempunctata*

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The composition of the protective secretion of the seven-spot ladybug *Coccinella septempunctata* L. (Coleoptera, Coccinellidae) has been studied by the methods of high- and low-resolution mass spectrometry. By determining the complete genetic link between the ions of a sample of the secretion by the methods of metastable defocussing and the direct analysis of daughter ions (DADI) the presence of the molecular ions of eight substances contained in the secretion has been shown. These components of the secretion have been identified as squalene, cholesterol, cholesta-3,5-diene, palmitic acid, and the alkaloids coccinellin, precoccinellin, and propylein, and a probable structure has been proposed for a base $C_{13}H_{16}N$.

The carnivorous beetle *Coccinella septempunctata* L. (Coleoptera, Coccinellidae, seven-spotted ladybug) is a useful insect in the fight against aphids, coccids, scale insects, and phytophagous mites. The beetle can consume every day, 68 adult lice or 175 larvae, and the larvae of the entomophage even more — 98 and 270, respectively [1].

Ladybugs are protected from their own enemies by an orange-colored lymphatic liquid of extremely pungent smell and with highly repellant properties for certain species of flies [2]. The alkaloids coccinellin and precoccinellin have been obtained previously by the extraction of a large number of seven-spot ladybugs [3-5]. The methodological procedures used in these investigations are specific only for the isolation of substances with a basic nature. Consequently, there is no information on the nature of the other components of the secretion. Continuing a series of scientific investigations [6], we have studied the composition of the protective secretion of this beetle in the native form.

The pattern of the low-resolution mass spectrum depends greatly on the temperature of recording the spectra of the sample, which is connected with the different volatilities of the components making up the composition of the secretion. Figure 1a-c gives mass spectra obtained at 50, 100, and 150°C, respectively. The determination of the complete genetic link between the ions with the aid of the methods of metastable defocussing and the direct analysis of daughter ions (DADI) [7, 8] showed the presence in the spectra of the peaks of the molecular ions of eight substances with m/z 410 (I), 386 (II), 368 (III), 256 (IV), 209 (V), 193 (VI), 191 (VII), and 189 (VIII) which had independent fragmentation pathways. The accurate m/z values of the peaks, determined by high-resolution mass spectrometry, and the elementary compositions of the molecular and some characteristic ions of substances (I-VIII) are given in Table 1.

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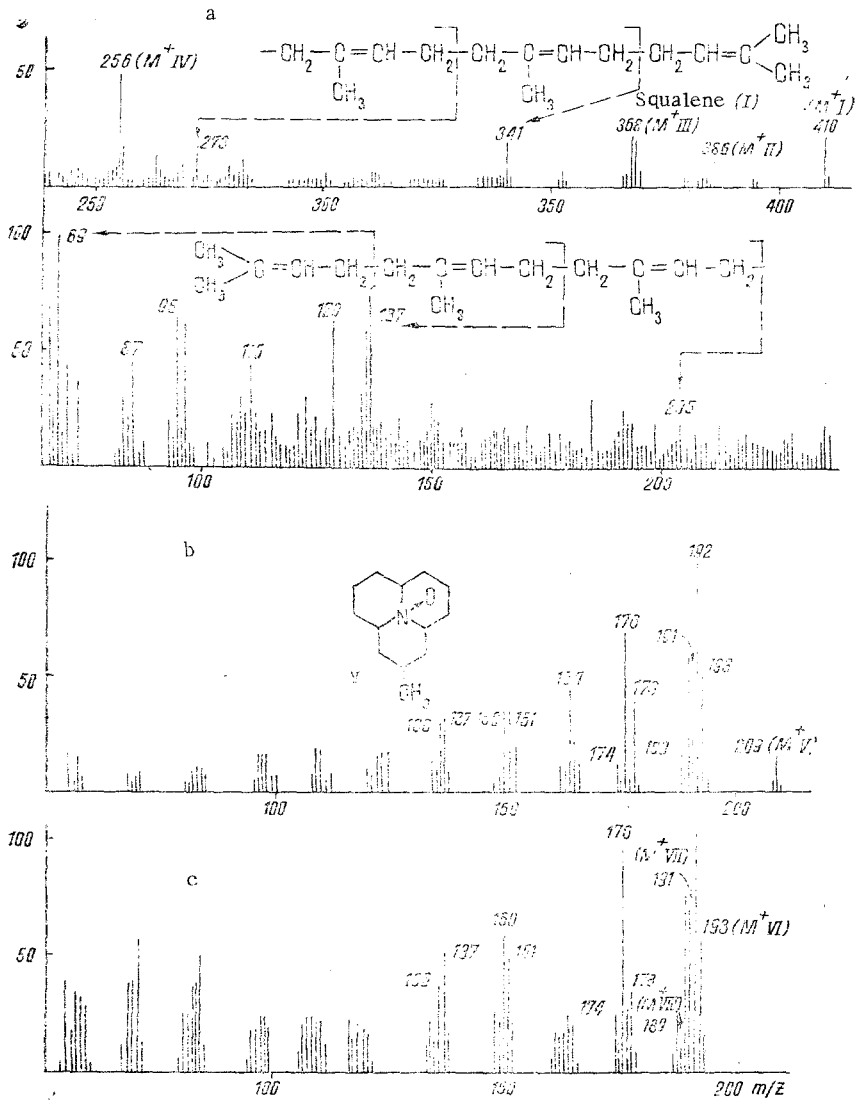

Fig. 1. Mass spectra of the protective secretion of the seven-spot ladybug taken at 50 (a), 100 (b), and 150°C (c).

TABLE 1. Accurate m/z Values of the Peaks Determined by High-Resolution Mass Spectrometry, and the Elementary Compositions of the Molecular and Some Characteristic Ions of Substances (I-VIII)

Accurate value of m/z	Elementary composition	Accurate value of m/z	Elementary composition
410,3887 M+I	$C_{30}H_{50}$	178,1588	$C_{12}H_{20}N$
386,3534 M+II	$C_{28}H_{46}$	176 1440	$C_{12}H_{18}N$
368,3434 M+III	$C_{28}H_{44}$	174 1281	$C_{12}H_{16}N$
341,3199	$C_{25}H_{41}$	171,1282	$C_{10}H_{19}O_2$
273,2208	$C_{19}H_{29}O$	157,1228	$C_9H_{17}O_2$
256 2389 M+IV	$C_{16}H_{32}O_2$	150,1286	$C_{11}H_{16}N$
227,2005	$C_{15}H_{25}O_2$	151,1358	$C_{10}H_{17}N$
209,1777 M+V	$C_{14}H_{23}ON$	148,1118	$C_{10}H_{14}N$
205,1949	$C_{15}H_{25}$	143,1063	$C_8H_{15}O_2$
199,1698	$C_{15}H_{23}O_2$	137 1329	$C_{10}H_{17}$
193,1830 M+VI	$C_{13}H_{23}N$	137,1200	$C_9H_{15}N$
191,1668 M+VII	$C_{13}H_{21}N$	136,1121	$C_9H_{14}N$
189,1514 M+VIII	$C_{13}H_{19}N$	129,0921	$C_7H_{13}O_2$
185,1549	$C_{11}H_{21}O_2$	69,0703	C_5H_9

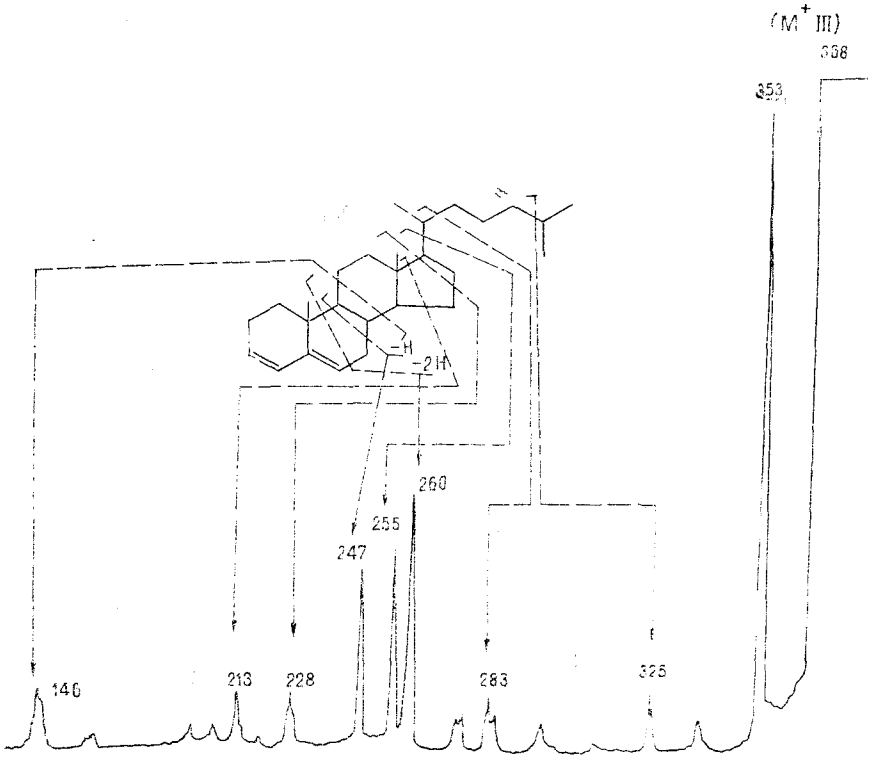

Fig. 2. DADI spectrum of the molecular ion of cholesta-3,5-diene (III).

Analysis of the results of high-resolution mass spectrometry of a sample and of the DADI spectrum of the molecular ion of substance (I) shows that the ion with m/z 410 is the precursor of daughter ions with m/z 395, 341, 273, 205, 137, and 69, arising through the ejection of methyl and isoprenyl radicals and of three molecules of isoprene C_5H_8. A comparison of these results with the literature [9, 10] enables substance (I) to be identified as squalene.

Squalene is a precursor of sterols and has been isolated from yeast [10] and plants [11]. This is the first time that it has been detected in the protective secretion of an insect. In connection with this, we may note that trans-farnesene — a biogenetic precursor of squalene — acts as an alarm pheromone in some species of aphids [12]. Since the ladybug feeds mainly on various species of aphids, the detection of squalene in its protective secretion is possibly a chemical manifestation of the ecological bond between these species existing in the relationship of prey and predator. It may be assumed that the organism of the beetle, acquiring trans-farnesene together with its food, converts it into squalene, which, apparently, plays a definite role in the transmission of the information necessary in inter- and intraspecies relations (for example, alarm or aggregation pheromone).

Substance (II) was identified from the DADI spectrum of its molecule as cholesterol. Some difficulties were presented by the identification of substance (III), the peak of the molecular ion of which is superposed on the peak of the dehydration ion $(M - H_2O)^+$ of cholesterol. At a high resolution, the peak of this ion is not resolved into other components, which shows the homogeneity of its composition (see Table 1); the ratio of the intensities of the peaks of the molecular and the dehydration ions in the mass spectrum of a native sample of cholesterol usually amounts to 2:1. In the mass spectra of the sample of protective secretion, however, it is 1:2. When the sample was present for a long time in the ion source of the instrument the peaks of the molecular and other fragmentary ions of cholesterol disappeared from the spectrum while the peaks of an ion with m/z 368 and of the other ions formed from it remained. The DADI spectrum of the molecular ion of substance (III) taken at the moment of absence of cholesterol from the sample under study is shown in Fig. 2. The elementary composition and a comparative analysis of the DADI spectra of the molecular ion of substance (III) and of the dehydration ion of a native sample of cholesterol showing their similarity, permitted the assumption that substance (III) is cholesta-3,5-

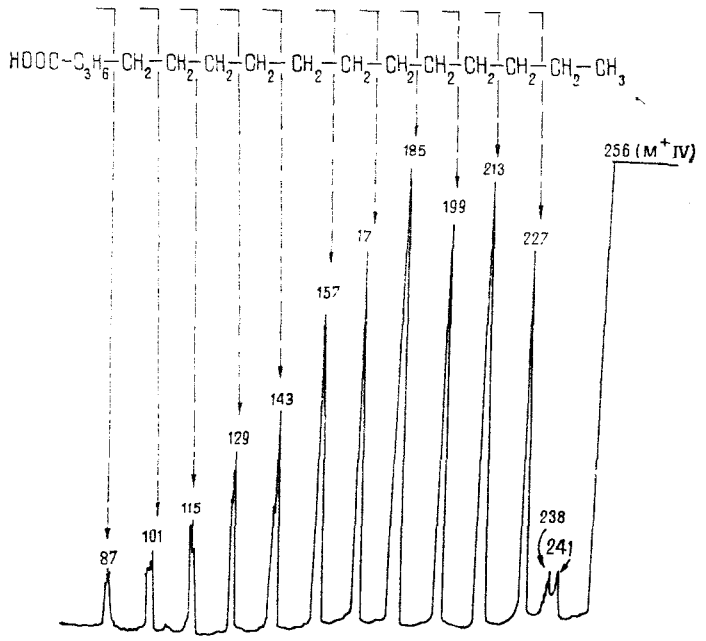

Fig. 3. DADI spectrum of the molecular ion of palmitic acid (IV).

diene. And in actual fact, the DADI spectrum of the molecular ion of the latter, obtained from cholesterol by a published method [13], proved to be identical with the DADI spectrum of the molecular ion of substance (III). The DADI spectrum of the molecular ion of cholesta-3,5-diene has a series of peaks of ions characterizing its structure. In this spectrum peaks of the ions arising in the ejection of a methyl radical, in the cleavage of the bonds of ring B and in the splitting out of the side chain (peaks of ions with m/z 353, 260, 247, and 235) have a high intensity (Fig. 2). The composition and DADI spectrum of the molecular ion of component (IV) proved to be identical with those of palmitic acid. The DADI spectrum of palmitic acid is characterized by peaks of low intensity with m/z 241 and 238, corresponding to the splitting out of a methyl radical and to the elimination of a water molecule, and also intense peaks of ions differing from one another by 14 mass units and corresponding to the sequence of methylene units of the molecule, while the intensities of these ions in the spectrum diminish as the bond cleaved approaches the carboxy group (Fig. 3).

As mentioned above, because of the different volatilities of the components of the beetle's secretion, at higher recording temperatures the peaks of the molecular and fragmentary ions of the nitrogen-containing substances (V-VIII) appeared in the spectrum with high intensity and the peaks of substance (I-IV) containing no nitrogen atoms disappeared (Fig. 4). In the DADI spectrum of the molecular ion of substances (V) with m/z 209, there is the peak of only one ion, corresponding to the splitting out of an oxygen atom. Then this ion with m/z 193 breaks down into ions with m/z 192, 178, 164, 150, and 137 by the loss of a hydrogen atom, of methyl, ethyl, and propyl radicals, and of a molecule of methylcyclopropane, respectively. These results permit substance (V) to be identified as the alkaloid coccinellin [3-5].

Although coccinellin is the main alkaloid of the protective secretion of the European seven-spot ladybug, in the secretion of the beetle that we studied it was by no means the main component, since its molecular peak appeared with a very low intensity and rapidly disappeared from the spectrum of the sample. And, conversely, the molecular ion of precoccinellin detected by the authors of the paper cited above as a minor component appeared with a very high intensity in the material that we studied. The DADI spectrum of the molecular ion of precoccinellin was identical with the spectrum of the daughter ion $(M - 16)^+$ of coccinellin (Fig. 4a).

Another feature of the mass spectrum of the protective secretion of the seven-spot ladybug that we studied is the appearance in in it of the peaks of the molecular and daughter ions of another two alkaloids – propylein and a new base with the composition $C_{13}H_{19}N$ (VIII). Propylein has been detected previously in the protective secretion of another species of beetle – the fourteen-spot ladybug (*Propylaea quatuordecimpunctata*) [14]. In the DADI spec-

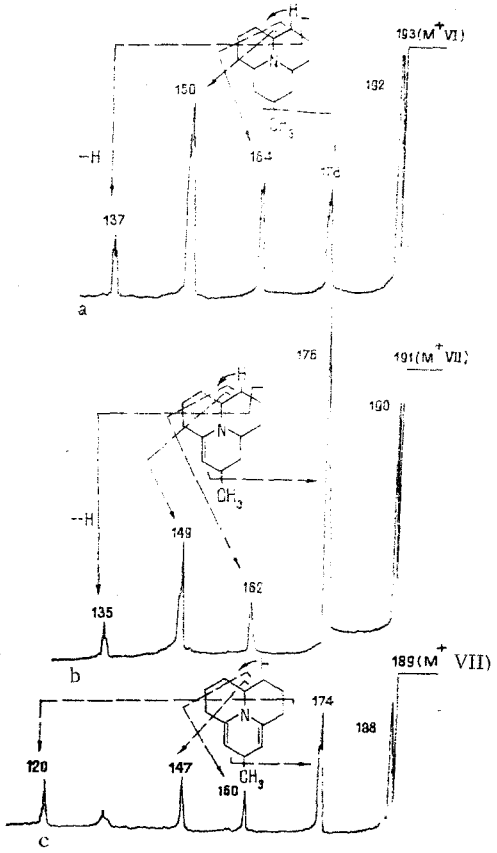

Fig. 4. DADI spectra of the molecular ions of precoccinellin (VI) (a), of proyplein (VII) (b), and of the new base (VIII) (c).

trum of the molecular ion of substance (VII) there are the peaks of the ions $(M-1)^+$, $(M-15)^+$, $(M-29)^+$, $(M-42)^+$, and $(M-56)^+$ (Fig. 4b).

Substance (VIII) has not previously been detected in the protective secretions of any species of ladybug. Its composition differs from that of (VI) by four and from (VII) by two hydrogen atoms. It can be seen from the DADI spectra of the molecular ions of substances (VI) and (VII) that they do not give a transition to the molecular ion of substance (VIII). This is also confirmed by the defocussing spectrum of the latter, showing the absence of a genetic link between these ions. The proposed structure for substance (VIII) is based on the presence in the DADI spectrum of its molecular ion of the peaks of ions with m/z 188, 174, 160, 147, and 120 arising on the ejection of a hydrogen atom, of methyl and ethyl radicals, and of the elements of rings A and B in the form of C_3H_6 and C_5H_9 groups, respectively (Fig. 4c). The high intensity of the peak of the $(M-15)^+$ ion in the spectra of (VII) and (VIII) can be explained by the rearrangement nature of the process of splitting out a methyl radical from the molecular ions of these substances caused by the dislocation of the double bond.

Thus, the combination of methods of metastable defocussing and direct analysis of daughter ions with low- and high-resolution mass spectrometries in the investigation of complex mixtures of natural substances such as is the protective secretion of the seven-spotted ladybug that we have studied has permitted without the use of chemical methods of isolation and separation, the identification of the components of the secretion as squalene, cholesterol, cholesta-3,5-diene, palmitic acid, coccinellin, precoccinellin, and propylein together with a new base for which it has been possible to suggest a probable structure.

EXPERIMENTAL

The spectra were obtained on a Varian MAT-311 mass spectrometer with a SS-100 MS computer data-processing system at temperature for the evaporation of the samples of 5-150°C, an energy of the ionizing electrons of 70 eV, an accelerating voltage of 2-3 kV, and a potential

of the energy analyzer of 505.5 V. The methods of calculating the masses of the daughter ions in the DADI spectra and of the ions of precursors on the defocussing spectra have been described previously [6, 8].

The samples of secretion were obtained from the beetle at the moment of reflex, release of orange hemolymph with the aid of a thin glass capillary, the contents of the capillary were rapidly transfered to a crucible containing glass wool and were introduced into the ion source through a direct-introduction system cooled with water at 20°C.

In the course of our study of the protective secretion of the beetle we established that the intensities of the peaks of the molecular ions of squalene (I) and of precoccinellin in the mass spectra of samples taken from the beetles in April and the beginning of May were 2-3 times greater than for those taken in June and July.

SUMMARY

1. The composition of the protective secretion of the seven-spot ladybug *Coccinella septempunctata* in the native state has been studied by the methods of low- and high-resolution mass spectrometries, metastable defocussing, and the direct analysis of daughter ions (DADI).

2. On the basis of an analysis of the spectra results obtained, the components of the secretion have been identified as squalene, cholesterol, cholesta-3,5-diene, palmitic acid, and the alkaloids coccinellin, precoccinellin, propylein, and a base of the composition $C_{13}H_{19}N$ for which the probable structure (VIII) has been suggested.

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