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

II. PHYTOSTEROLS ACCOMPANYING β-SITOSTEROL

R. Razakov, A. K. Kasimov
Kh. A. Aslanov, and A. S. Sadykov

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Low- and high-resolution mass spectra and defocusing and DADI spectra of samples of β-sitosterol isolated from various plant material have been studied. The spectral information obtained has shown the presence in samples of β-sitosterol studied of minor accompanying components which have been identified as stigmasterol, campesterol, and cholesterol.

Phytosterols form a set of C_{27}-C_{29} sterols participating in the structure of cell membranes and in that part of plant metabolism which leads to the formation of phytosterols of higher plants the most widespread is β-sitosterol, which is frequently accompanied by a number of other minor sterols the isolation and identification of which by the usual method present great difficulties.

Continuing our investigation [1] of complex mixtures of natural substances by the defocusing and DADI methods [2, 3], we have studied a number of samples isolated from various plant materials and identified by all their constants [4] as β-sitosterol. Their mass spectra were identical. As an example we give the mass spectrum of a sample isolated from *Lagochilus inebrians* B. (family Labiatae) (Fig. 1). The establishment by the defocusing and DADI methods of a complete genetic link between the ions has shown that the mass spectrum of the sample contains, in addition to the peak of the molecular ion (M^+) with m/e 414 of β-sitosterol (I), the M^+ peaks of another three substances with m/e 412 (II), 400 (III), and 386 (IV) having independent fragmentation pathways.

Institute of Bioorganic Chemistry, Academy of Sciences of the Uzbek SSR, Tashkent.
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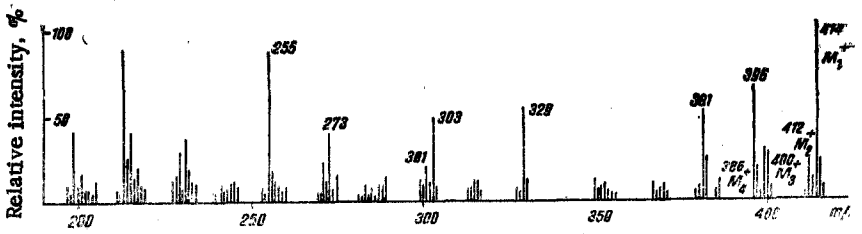

Fig. 1. Mass spectrum of a sample of β-sitosterol from *Lagochilus inebrians*.

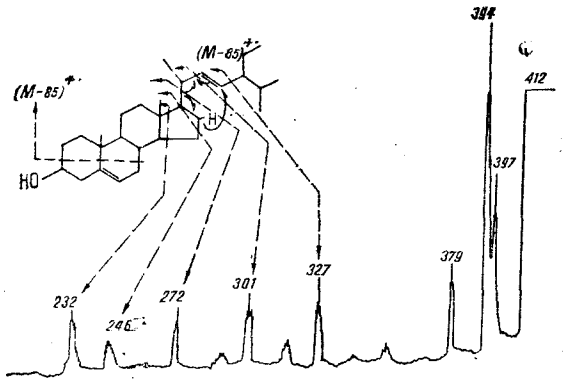

Fig. 2. DADI spectrum of the M^+ ion of stigmasterol (II).

The DADI spectrum of the molecular ion of (IV) (with m/e 386) proved to be identical with the spectrum of cholesterol [1]. The fact that substances (II) and (III) were 3-hydroxy-Δ^5-sterols was shown by the presence in the DADI spectrum of their molecular ions of the peaks of dehydration ions with m/e 394 (II) and 382 (III) corresponding to the peak of the $(M - 15)^+$ ion, and also of the peak of a number of ions with identical m/e values, such as 301, 246, and 232 (Figs. 2 and 3). The defocusing spectra of the latter confirm that the main precursors of these daughter ions were the molecular ions of (I-IV), as can be seen from the spectrum of one of them, namely, the ion with m/e 301 (Fig. 4).

In addition to the above-mentioned similarities, the DADI spectra of the molecular ions of (I-IV) also show some differences due to the structural features of the molecules. Thus, in the DADI spectrum of the molecular ion of substance (II) the peak of the ion formed by the cleavage of the C_{17}—C_{21} bond has a m/e value of 272, while the analogs of this ion in the spectra of (I), (III), and (IV) have a m/e value of 273. This shows the presence of a double bond at C_{22}—C_{23} in the (II) molecule causing, under the action of electron impact, the cleavage of the C_{17}—C_{20} bond with the migration of a hydrogen atom from C_{16} to C_{23} [5] (McLafferty rearrangement). The position of the C_{22}—C_{23} double bond is also confirmed by the peak of ions with m/e 301 and 327 arising from cleavages of C_{20}—C_{22} and C_{23}—C_{24} vinyl bonds.

Another freature of the DADI spectrum of the M^+ ion of (II) is that it includes the high-intensity peak of the $(M - 33)^+$ ion corresponding to the simultaneous splitting out of a methyl radical and a water molecule. The cleavage of this ion is also observed in the spectra of the M^+ ion of compounds (I), (III), and (IV), at least with a low intensity. The appearance of the process of the simultaneous splitting out of a methyl group and a water molecule is possibly connected to a greater degree with the presence of two allyl methyl groups at C_{10} and C_{20} in the (II) molecule. All these facts permit substance (II) to be identified as stigmasterol. We have detected stigmasterol previously in the protective secretion of an insect [1] in admixture with another sterol having the same composition of the molecular ion but differing from stigmasterol by the position of a double bond.

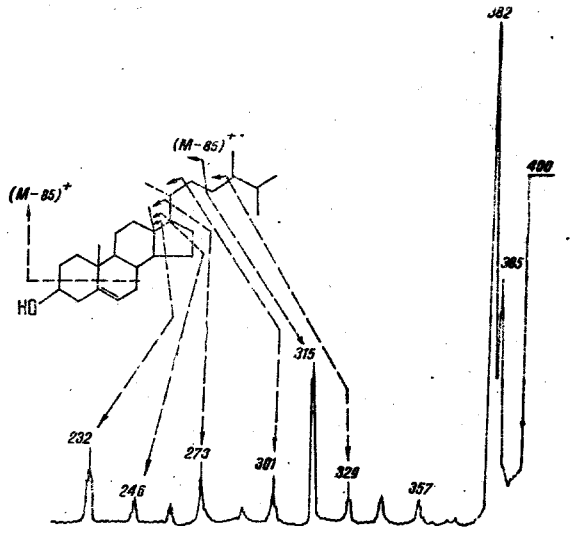

Fig. 3. DADI spectrum of the M^+ ion of campesterol (III).

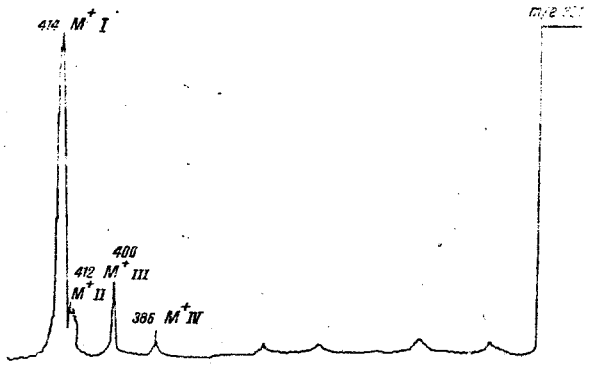

Fig. 4. Defocusing spectrum of the daughter ion with m/e 301.

The accurate m/e value of the molecular ion of (III) determined by high-resolution mass spectrometry was 400.3697 (calculated, 400.3693), which corresponds to the composition $C_{28}H_{48}O$. A comparative study of the DADI spectrum of substance (III) and the spectra of cholesterol (IV) and β-sitosterol (I) has shown that (III) differs from the latter by the presence of methyl group at C_{24} instead of the ethyl in (I) and the hydrogen in (IV). These facts permit the minor component with m/e for M^+ of 400 accompanying the sample of β-sitosterol as campesterol to be identified.

The DADI spectra of the molecular ions of (I-IV) each contain the peak of a daughter ion $(M - 85)^+$ of considerable intensity. Under high resolution, the peak of this ion splits into a doublet. The accurate m/e values, the elementary compositions, and the ratios of the components of these ions for substances (I-IV) are given in Table 1.

It can be seen from the Table that the $(M - 85)^+$ ion is formed both by the ejection of the elements of the side chain, giving oxygen-containing fragments, and by that of the elements of rings A and B with the simultaneous capture of two hydrogen atoms from the charged part of the molecule. The formation of the $(M - 85)^+$ ion without the retention of the oxygen atom is also observed in the spectra of 3-hydroxy-Δ^5-steroids [6].

Of the two competing pathways of the formation of the $(M - 85)^+$ ion, the pathway leading to the appearance of the oxygen-containing fragment is dominating in the fragmentation of the molecular ions of substances (I), (III), and (IV) in which the side chains do not contain

TABLE 1. Accurate m/e Values, Elementary Compositions, and Ratios of the Intensities of the Components of the $(M - 85)^+$ Ion for Compounds (I-IV)

Compound	Found	Calculated	Composition	Intensity ratio
β-Sitosterol (I)	329,2830	329,2835	$C_{23}H_{37}O$	
	329,3190	329,3198	$C_{27}H_{41}$	1:4
Stigmasterol (II)	327,2691	327,2679	$C_{23}H_{35}O$	
	327,3040	327,3042	$C_{24}H_{39}$	4:1
Campesterol (III)	315,2671	315,2679	$C_{22}H_{35}O$	
	315,3051	315,3042	$C_{23}H_{39}$	1:8
	301,2527	301,2523	$C_{21}H_{33}O$	
Cholesterol (IV)	301,2886	301,2886	$C_{22}H_{37}$	1:3

double bonds. Conversely, in the breakdown of the molecular ion of stigmasterol (II), which contains a C_{22}–C_{23} double bond, the route leading to the formation of the oxygen-containing fragment is predominant. The increase in the contribution to the $(M - 85)^+$ ion of a component containing no oxygen atom leads to a rise in the intensity of this peak in the DADI spectra of the molecular ions (I), (III), and (IV).

Thus, the specificity that has been found in the DADI spectra of the molecular ions of the compounds studied, in combination with the defocusing method and high-resolution mass spectrometry, has permitted the identification of the minor components accompanying β-sitosterol as stigmasterol, campesterol, and cholesterol.

EXPERIMENTAL

The spectra were recorded on a Varian MAT-311 instrument with an MS-100 computer data-processing system at an energy of the ionizing electrons of 70 eV, an evaporation temperature of the sample of 100°C, and a temperature of the ionization chamber of 150°C. In the DADI spectra the mass of each daughter ion (m_2) calculated from the formula

$$m_2 = m_1 - \frac{E_1}{E_0},$$

where m_1 is the mass of the precursor ion, E_1 is the electrostatic field strength at which m_1 is recorded, and E_0 is the initial value of the electrostatic field of the analyzer at which m_2 was recorded.

In the focusing spectra the mass of the precursor ion was found from the equation

$$m_1 = m_2 \frac{V_1}{V_0},$$

where V_1 is the accelerating voltage at which m_1 was recorded, and V_0 is the initial value of the accelerating voltage at which m_2 was recorded.

The values of E_0, E_1, V_0, and V_1 were measured by a digital voltmeter.

The samples of β-sitosterol studied had identical constants (mp 138°C, $[\alpha]_D^{25}$ + 37.8° ($CHCl_3$)) and gave a single spot in a thin layer of silica gel.

The accurate m/e values, the elementary compositions, and the ratio of the intensities of the components of the $(M - 85)^+$ ion for cholesterol were determined on a native sample of it.

SUMMARY

1. The high- and low-resolution mass spectra and the defocusing and DADI spectra of samples of β-sitosterol isolated from various plant materials have been studied.

2. On the basis of the spectral results obtained, the presence in the samples of β-sitosterol studied of minor accompanying components identified as stigmasterol, campesterol, and cholesterol has been established.

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A STUDY OF THE PRODUCTS OF THE REDUCTION OF THE ANTIBIOTIC REUMYCIN (6-METHYLPYRIMIDO[5,4-e][1,2,4]TRIAZINE-5,7-DIONE)

S. E. Esipov, N. A. Klyuev, L. A. Saburova, and V. M. Adanin

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The structures of the products of the reduction of the antibiotic reumycin (I), which is a specific autooxidizable acceptor of reducing equivalents from certain flavin dehydrogenases of the respiratory chain of the mitochondria of yeast and animal tissues, have been established with the aid of physicochemical methods (UV, IR, and PMR spectroscopy and mass spectrometry). In the determination of the structures of five reduction products of reumycin effective use has been made of high-resolution mass spectrometry and a consideration of the spectra of metastable ions (the DADI technique). It has been shown that only the asym-triazine ring in the initial (I) undergoes reduction and ring-opening.

It has been shown previously [1, 2] that the anticarcinogenic activity of the antibiotic reumycin (6-methylpyrimido [5,4-e][1,2,4]triazine-5,7-dione) [3,4] is connected with its capacity for oxidizing cytoplasmatic NADH. This leads to a decrease in the reduction potentials of the tumor cells and, as a consequence, to a retardation of their pathological growth. In this process, the antibiotic is reduced, accepting reducing equivalents from flavin dehydrogenases and transferring these equivalents to oxygen. In this connection, it is of interest to model the process of reducing reumycin as one of the possible mechanisms of the biological action of the antibiotic and to determine through what possible reduced forms it takes place.

The attempts made to obtain and isolate dihydro derivatives of antibiotics of the pyrimido[5,4-e][1,2,4]triazine series directly have proved unsuccessful because of their extreme lability and rapid oxidation by atmospheric oxygen [5, 6]. We have succeeded in obtaining stable acetyl derivatives of the reduction products of the antibiotic reumycin by performing catalytic hydrogenation with hydrogen in acetic anhydride. In this way we have isolated and characterized for the first time five acetyl derivatives of the reduction products of reumycin (see the scheme of isolation).

The structures of the products (I-V) isolated were determined on the basis of UV, IR, and PMR spectroscopy and low-resolution and high-resolution mass spectrometry (HRMS). The last method was the main one.

According to the results of HRMS, the empirical composition of the isolated compound (I) is described by the formula $C_8H_8N_5O_3$ (see the experimental part). The sequence of fragmentation of M^+ and the fragmentary ions was studied precisely from the spectra of the metastable ions (the DADI technique [7]). Their empirical compositions were checked with the

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