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STUDY OF ECDYSTERONE METABOLITES ISOLATED FROM RAT URINE

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Metabolites of ecdysterone have been isolated from rat urine. By a combination of electron-impact and secondary-ion mass spectrometries and with the aid of accurate measurements of ion masses the molecular masses have been determined and structures have been proposed for three new metabolites, a common feature of which is the reduction of C_6=O to a methylene group. Ecdysterone metabolite (2) has the structure of 2β,3β,14,20,22,25-hexahydroxy-5-cholest-7-ene; (3) is 2β,3β,14,20,25-pentahydroxy-5-cholesta-7,22-diene; and (4) is 2β,3β,14,20,22,25-hexahydroxy-5-cholestane.

Steroids form one of the most important groups of organic compounds. Among the various metabolites produced by plants a not unimportant place is occupied ecdysteroids found in animals (Invertebrata) and in plants. Structurally, these compounds are identical with or close to the hormones of the molting and metamorphosis of arthropods and, to all appearance, play an important role in the plant–insect ecological system. More than 150 ecdysteroids isolated from plants are known.

Ecdysteroids possess a high biological activity and a wide range of pharmacological effects [1]. An original tonic preparation ékdisten, the active principle of which is ecdysterone, has been created in the Institute of the Chemistry of Plant Substances, Academy of Sciences of the Republic of Uzbekistan, under the direction of N. K. Abubakirov. The study of the metabolism of ecdysteroids is arousing particular interest among chemists and biologists.

A general scheme of the metabolism in the organism of animals has been given in the literature [2-4]. Information on the metabolism of ecdysteroids is also given in a review by Lafont et al. [3], in which, together with diverse metabolic transformations, a specificity of the reactions for a given type of animals is noted. In particular, on the administration of 1 mg of α-ecdysone or ecdysterone to white mice the excrements collected during the 48 h after administration contained a certain amount of the initial substance. A whole series of metabolites, with the absence of polar conjugates, was determined by the reversed-phase HPLC method. Subsequent normal-phase analysis showed the predominant presence of metabolites not absorbing at 254 nm, which can be explained by the reduction of the keto group in ring *B*. In the review [3] it is stated that the structures of various metabolites have been determined with the aid of NMR and mass spectra. However, no concrete details are given.

In view of this, we have studied the metabolism of ecdysterone in the organism of white rats. The qualitative chromatography of an ethyl acetate fraction of the urine revealed five substances of ecdysteroid nature. Three of them formed the main components of the total ecdysteroids.

The IR spectra of metabolites (**2**)-(**4**) did not contain the absorption bands at 1640-1670 cm^{-1} that are characteristic for a Δ^7-6-keto group. The peak of the molecular ion had a very low intensity or was even absent because of the instability of the molecular ions of ecdysteroids on electron impact. In spite of the absence in the majority of electron-impact (EI) mass spectra of the peak of the M^+ ion, this method plays an important role in the prediction of the structures of ecdysteroids [5-7]. The key point here is the cleavage of the C-20-C-22 bond, giving, on the one hand, the ions *b* characterizing the composition of the steroid nucleus and the substituent at C-20, and, on the other hand, the C-23-C-27 chain (ions with *m/z* 99 and 81).

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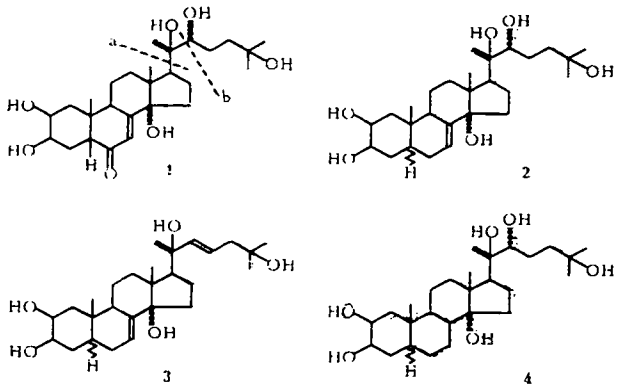
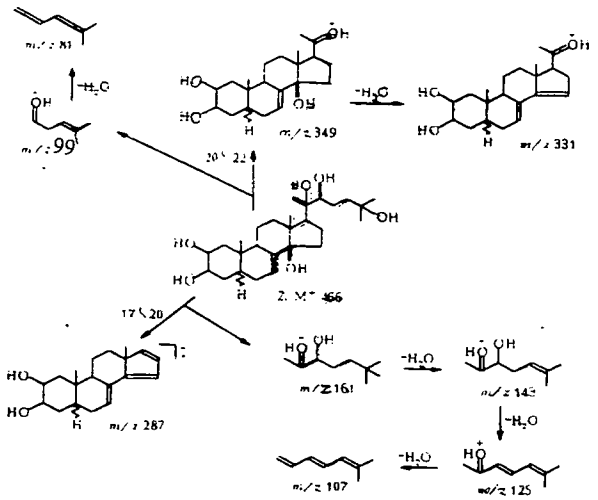


Ion *b* of the initial ecdysteroid (**1**) had the composition $C_{21}H_{31}O_5$ (*m/z* 363), while in the spectra of the metabolites (**2**) and (**3**) this ion had 349 a.m.u. and the composition $C_{21}H_{33}O_4$, and in the spectrum of metabolite (**4**) 351 a.m.u. and the composition $C_{21}H_{35}O_4$ (Schemes 1-3). The high degree of saturation of the ions with *m/z* 349 and 351 in comparison with the ion having *m/z* 363, and the decrease in the number of oxygen atom in their composition most probably shows reduction of the C_6=O group to CH_2 (**2**) and (**3**) and the additional reduction of the Δ^7 bond (**4**).



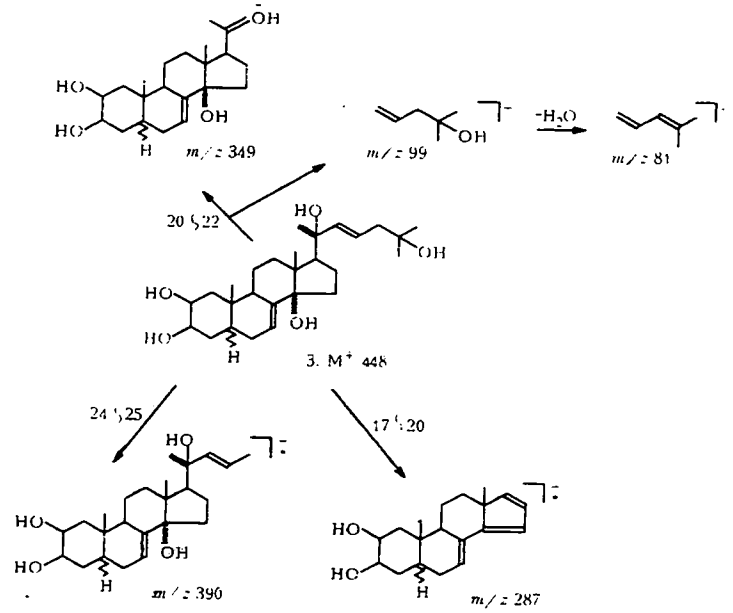
Scheme 1. Main routes of the mass-spectrometric decomposition of metabolite (**2**).

Alternative methods for the decrease in the number of oxygen atoms in the ions *b* (for example, the formation of 2- or 20-deoxyecdysteroids with simultaneous reduction of the Δ^7 bond) are unlikely, since in such cases one could observe the peak of the molecular ion [8]. In the spectra of metabolites (**2**)-(**4**), however, the M^+ peaks were absent. The peaks with the highest molecular masses in these spectra had *m/z* 448 (**2**), 433 (**3**), and 432 (**4**).

The key steroid fragment of second significance in the mass spectra of ecdysteroids is formed on the cleavage of the C-17-C-20 bond with the, as a rule, simultaneous elimination of a water molecule from C-14 [5-7]. The peaks of the corresponding ions are observed in the spectra of the metabolites – *m/z* 287 (**2**)-(**3**) and 289 (**4**) (see Schemes 1-3) and also in those of the products of their subsequent dehydration — *m/z* 269, 251 (**2**)-(**3**) and 271, 253 (**4**). This means that the 14-OH group is retained in the (**2**)-(**4**) molecules.

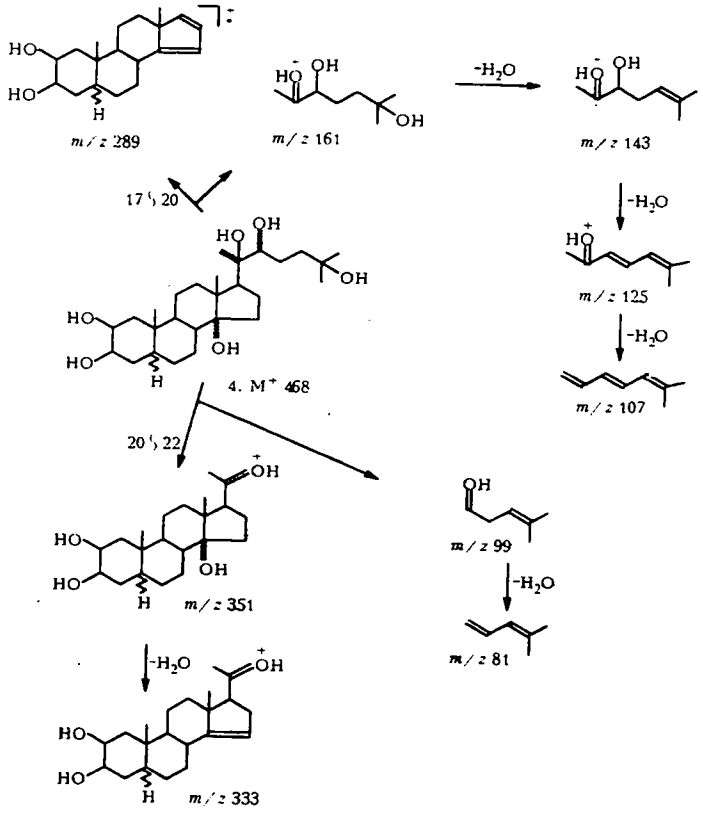
The presence of the OH group at C-20 was additionally confirmed by the absence of *b* + H ions (*m/z* 350, 352) corresponding to 20-deoxysteroids such as α-ecdysone [6, 8].

In all three spectra, the peaks of ions with *m/z* 99 and 81 were observed, but this does not permit an unambiguous determination of the structure of the C-22-C-27 chains of the metabolites (see Schemes 1-3). In view of this, in the first place, an independent method of establishing the molecular masses of the substances under investigation was necessary, and



Scheme 2. Main routes of the mass-spectrometric decomposition of metabolite (**3**).

for this we used secondary-ion mass spectrometry (LSIMS) [9, 10]. The LSIMS spectra were taken in a pure glycerol (Gl) matrix and in glycerol with added NaCl (Gl + NaCl). For control, we obtained the LSIMS spectra of ecdysterone under the same conditions.



Scheme 3. Main routes of the mass spectrometric decomposition of metabolite (**4**).

In the LSIMS (Gl) spectrum we observed the peak of the protonated molecular ion with *m/z* 481 and those of the products of its successive dehydration (*m/z* 463, 445, 427, 409). The intensities of the peaks of the type *b* ions was depressed, while the type *a* ions (*m/z* 303-301) retained the same positions and intensities as in the EI spectrum. In the LSIMS spectrum of ecdysterone (**1**) in the Gl + NaCl matrix the heights of the peaks of all the ions mentioned decreased and the 100% intensity peak became that of the $(M + Na)^+$ ion with *m/z* 503.

In the analogous spectra of the metabolites (matrix — Gl) we recorded the mass numbers of the $(M + H)^+$ ions with *m/z* 467 (**2**), 449 (**3**), and 469 (**4**), and, when Gl + NaCl was used as the matrix, those of the $(M + Na)^+$ ions with 489 (**2**), 471 (**3**), and 491 (**4**) a.m.u. All the spectra contained intense peaks of the fragments $(M + H - H_2O)^+$, while the peaks of the *a* and *b* ions were weak. In the LSIMS spectra of (**2**) and (**4**) there were the peaks of clusters containing a molecule of glycerol: $(M + Gl)^+$ and $(M + Na + Gl)^+$.

Thus, the molecular masses of the metabolites were 466 (**2**), 448 (**3**), and 468 (**4**); i.e., in compounds (**2**) and (**4**) the C-22−C-27 chain was identical with that of the initial ecdysterone (**1**). This conclusion was confirmed by an analysis of the fragments formed on the cleavage of the C-22−C-23 and C-23−C-24 bonds in the EI spectra of these metabolites (ions with *m/z* 379, 361 (**2**) and 381, 363 (**4**) — C-22−C-23 cleavage — and ions with *m/z* 356, 357 (**2**) and 358, 359 (**4**) — C-23−C-24 cleavage [8].

So far as concerns metabolite (**3**), the C-22−C-27 chain in its molecule must be dehydrated in some way: Δ^{22} or Δ^{25}. The EI spectrum of (**3**) lacked the peaks of ions characterizing the breakdown of the side-chain at the C-22−C-23 and C-23−C-24 bonds, while the peaks of a $(M - C_3H_6O)^+$ ion with *m/z* 390 and of its dehydration product with *m/z* 372 were present. This can only show the cleavage of the C-24−C-25 bond and, consequently, the impossibility of the presence of a Δ^{25} bond. It is interesting to note that an analogous process takes place in the formation of the LSIMS spectrum of (**3**), where there are the peaks of ions with *m/z* 391, 373, and 355. Activation of C-24−C-25 cleavage may take place with a Δ^{22} bond.

Thus, metabolites (**2**), (**3**), and (**4**) have the most probable structures of 2β,3β,14,20,22,25-hexahydroxy-5-cholest-7-ene; 2β,3β,14,20,25-pentahydroxy-5-cholesta-7,22-diene; and 2β,3β,14,20,22,25-hexahydroxy-5-cholestane.

EXPERIMENTAL

Thin-layer chromatography (TLC) was conducted on Silufol plates. For column chromatography we used silica gel L 100/160 μm (Czechslovakia). The solvent systems were chloroform−methanol (15:1) (1) and (9:1) (2). On TLC the ecdysteroid metabolites were detected by spraying with vanillin/sulfuric acid, followed by heating at 110-120°C for 2-5 min [11]. Mass spectra* were taken on a MKh-1303 instrument at an ionizing voltage of 50 V and a temperature of 100-140°C, and IR spectra on a UR-20 spectrophotometer in KBr. The elementary compositions of the ions were measured on a MKh-1310 mass spectrometer.

In the experiments we used 40 random-bred male white rats weighing 200-230 g. Ecdysterone in the form of a 2% aqueous solution was introduced into the stomach of each animal in a single dose of 50 mg/kg. The animals were placed in metabolism chambers and their urine was collected daily (every 24 h) over 10 days. For the whole of this time they were maintained on the usual vivarium ration.

Extraction of the Rat Urine. The rat urine (3.5 liters) was filtered and extracted with chloroform and then with ethyl acetate (4 × 1 liter). The total material obtained after evaporation of the solvents in vacuum was dissolved in 250 ml of methanol. After a day, crystals not containing ecdysterone metabolites deposited, and these were filtered off. (This procedure was repeated several times.) Evaporation of the solvent in vacuum led to the purified total extractive substances, containing five ecdysterone metabolites according to TLC (system 2).

The total metabolites were chromatographed on a column of alumina. Elution was performed first with system 1 and then with system 2. The eluates were monitored by TLC in system 2. This gave the purified ecdysterone metabolites.

The fractions containing the ecdysterone metabolites that were obtained after the distillation of the solvents were combined and rechromatographed on a column of silica gel. Elution with system 1 led to the isolation of a mixture of three substances. After repeated chromatography, substance (**1**) (1.5 mg) and (**2**) (2 mg) were obtained.

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*Electron-impact mass spectra were taken and elementary compositions were determined by Yu. M. Mil'grom, and secondary ion mass spectra were recorded by Ya. V. Rashkes.

Elution of the column with system 2 gave a mixture of substances (**2**) and (**3**). After preliminary purification it was rechromatographed on a column of silica gel. Elution with system 2 yielded substance (**3**) (3 mg). On further elution of the column with the same system the initial substance, ecdysterone (**1**), was obtained.

Ecdysterone (1), $C_{27}H_{44}O_7$.

Mass spectrum, LSIMS (Gl), *m/z* (%): 481 [$(MH)^+$, 141], 463 (27), 445 (26), 427 (18), 409 (8), 371 (9), 303 (17), 301 (26), 143 (39), 125 (31), 107 (38), 99 (100).

Mass spectrum, LSIMS (Gl + NaCl), *m/z* (%): 503 [$(M + Na)^+$, 100], 485 (10), 463 (8), 445 (10), 427 (7), 409 (9), 303 (10), 301 (12).

Substance 2, $C_{27}H_{46}O_6$.

Mass spectrum, *m/z* (%): 448 ($M^+ - H_2O$; 0.2), 433 (2), 430 (8), 415 (6), 412 (13), 402 (2), 397 (6), 379 (3), 361 (8), 357 (2), 356 (2), 349 (96), 331 (57), 315 (14), 313 (16), 303 (10), 299 (10), 297 (13), 287 (13), 269 (16), 251 (16), 161 (13), 143 (28), 125 (22), 107 (27), 99 (82), 81 (100), 69 (64).

Mass spectrum, LSIMS (Gl), *m/z* (%): 559 [$(MH^+ + Gl)^+$, 13], 467 [$(MH)^+$, 27], 449 (57), 431 (100), 413 (22), 349 (18), 333 (19), 331 (16), 315 (30), 303 (20).

Mass spectrum, LSIMS (Gl + NaCl), *m/z* (%): 581 [$(M + Na^+ + Gl)^+$, 28], 489 [$(M + Na)^+$, 89], 467 [$(MH)^+$, 33], 449 (61), 431 (100), 413 (70).

Substance 3, $C_{27}H_{44}O_5$.

Mass spectrum, *m/z* (%): 433 ($M^+ - CH_3$; 3), 430 (7), 415 (4), 397 (2), 390 (5), 372 (7), 349 (100), 331 (58), 313 (20), 287 (15), 269 (16), 263 (14), 251 (13), 199 (17), 129 (63), 111 (58), 99 (47), 81 (79), 69 (89).

Mass spectrum, LSIMS (Gl), *m/z* (%): 449 [$(MH)^+$, 28], 431 (100), 413 (25), 391 (18), 373 (58), 355 (55), 303 (25).

Mass spectrum, LSIMS (Gl + NaCl), *m/z* (%): 471 [$(M + Na)^+$, 8], 449 [$(MH)^+$, 25], 431 (100), 413 (24), 391 (17), 373 (44), 355 (42).

Substance 4, $C_{27}H_{48}O_6$.

Mass spectrum, *m/z* (%): 432 ($M^+ - 2H_2O$; 12), 417 (3), 414 (3), 399 (3), 381 (2), 359 (2), 358 (2), 351 (100), 333 (40), 317 (22), 315 (51), 299 (31), 297 (39), 289 (9), 287 (7), 271 (23), 253 (7), 161 (8), 143 (50), 125 (27), 107 (43), 99 (42), 81 (73), 69 (59).

Mass spectrum, LSIMS (Gl), *m/z* (%): 583 [$(M + Na + Gl)^+$, 8], 491 [$(M + Na)^+$, 36], 473 (29), 469 [$(MH)^+$, 15], 451 (32), 433 (100), 415 (24), 397 (22).

Mass spectrum, LSIMS (Gl + NaCl); *m/z* (%): 605 [$(513 + Gl)^+$, 23], 583 [$(491 + Gl)^+$, 23], 513 [$(M + 2Na-H)^+$, 42], 491 [$(M + Na)^+$, 100], 473 [$(491-H_2O)^+$, 24], 469 [$(MH)^+$, 12], 451 (26), 433 (77), 415 (22), 397 (18).

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