IDENTIFICATION OF THE STEROLS OF THE YEASTS Saccharomyces cerevisiae AND Candida guilliermondii BY THE MASS-SPECTROMETRIC METHOD

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The sterol compositions of mutants of the yeasts of Saccharomyces cerevisiae and Candida guilliermondii have been characterized by the methods of GLC, TLC, UV spectroscopy, and chromato-mass spectrometry. The possibility has been shown of identifying yeast sterols on the basis of a comparison of the intensities of the peaks of the fragmentary ions having a common nature for all sterols ($m/z \geq 200$). A change in the sterol composition in mutant yeasts as compared with the initial strain has been detected which is obviously connected with the blockage of the routes for the biosynthesis of ergosterol in the yeast cell.

Chromato-mass spectrometry is one of the most sensitive and informative methods of identifying organic compounds, including sterols [1]. The interpretation of the mass spectra is based on the analysis of the fragmentation of the sterol molecule. Considerable advances in this field have been achieved in the interpretation of the structures of sterols having Δ^5 [2], Δ^7 and $\Delta^{5,7}$ [3, 4], and Δ^4 [5] double bonds in the nucleus.

In the present paper we discuss the identification of sterols isolated from strains of the yeasts, Sacch. cerevisiae and Candida guilliermondii. Both "wild" strains and also mutants resistant to polyene antibiotics – in our case, to nystatin – which have blocks in the biosynthesis of sterols are being analyzed. The conditions of production and the methods of selection has been described previously [6].

On the basis of the relative retention times in GLC, and also the results of UV spectroscopy and mass spectrometry, nine sterols accumulated by the strains have been identified; eight of them were detected in the yeast Sacch. cerevisiae and seven in C. guilliermondii. These were the following compounds: ergosta-5,7,22-trien-3β-ol (ergosterol) (I), cholesta-8,24-dien-3β-ol (zymosterol) (II), 22,23-dihydroergosterol (III), cholesta-5,7,24-trien-3β-ol (IV), cholesta-5,7,22,24-tetraen-3β-ol (V), ergosta-5,7,22,24-tetraen-3β-ol (VI), ergosta-8-en-3β-ol (VII), ergosta-8,24-dien-3β-ol (VIII) and 4,4,14-trimethylcholesta-8,22-diene-3β-ol (lanosterol) (IX).

The preliminary purification of the sterols was performed by thin-layer chromatography in a nonfixed layer of silica gel. The sterols were eluted as a single band with R_f 0.37 and, after elution with chloroform, they were analyzed in a gas–liquid chromatograph. Where the sterols were not separated completely in the course of GLC, the mixture was subjected to preliminary TLC on Silufol plates impregnated with a 10% aqueous solution of $AgNO_3$. Such chromatography permits sterols to be separated in accordance with their degrees of unsaturation [6]. Information on the GLC and TLC of the sterols is given below:

Sterol	Relative retention time, CGLC, OV-17	R_f (TLC with $AgNO_2$)
I	1,20	0,40
II	1,11	0,70
III	1,31	0,45
IV	1,19	0,35
V	1,22	0,35
VI	1,25	0,40
VII	1,30	0,65
VIII	1,33	0,80
IX	1,51	0,60

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The positions of the double bonds in a number of the sterols were determined by UV spectrophotometry.

$\Delta^{5,7}$-Sterols are characterized by absorption maxima at 271, 281, and 292 nm, [7], and $\Delta^{22,24}$-sterols by absorption maxima at 232, 238 sh., 247, 261, 271, 282, and 294 nm. Nonconjugated double bonds absorb in the wavelength interval of 200-220 nm, while a $\Delta^{8,14}$ system of double bonds has an absorption maximum at 251 nm [8].

The results of UV spectrometry and chromatography were provisional, the results of mass pectrometry forming the basis for identification.

The samples were introduced via a gas chromatograph under conditions selected previously. Since the work was performed with low-resolution mass spectra, fragmentary peaks with m/z values $\geq$ 200 were considered. This is connected with the fact that in the region of small masses the peaks of the ions have a complex nature, and identification on their basis appeared to us to be difficult. The mass spectra of 18 sterols of known composition were first recorded in order to study the main laws of the fragmentation of the sterol molecules. Nine of them have been published [10], and the other mass spectra are given in the present paper. This preliminary investigation enabled us to show that many peaks in the mass spectrum are common for the sterols considered, but their intensities depend on the positions of the double bonds in the molecule. Some of the most important peaks for interpretation are those of the $[M - R]^+$ and $[M - R - 2H]^+$ ions. Their intensities, and also the presence or absence of peaks corresponding to the breakdown of the side chain, permit the distribution of the double bonds between the nucleus and the side chain to be established, and has been reported previously [10]. The laws of the fragmentation of sterols studied during this work, and also an analysis of literature information have permitted the interpretation of the mass spectra of sterols of unknown composition isolated from yeast biomass.

The only sterol that was accumulated by the initial strain 2-P712 proved to be a sterol with a molecular weight of 396 amu. Its identification caused no difficulties, since the mass spectrum of this compound was completely identical with that of ergosterol, which we have considered previously [10].

Two sterols were isolated from the strain 1G-P188. One of them was also ergosterol. The mass spectrum of the second sterol, with a molecular ion with m/z 394 (VI) contained the peak of the fragmentary ion $[M - 59]^+$ (13%) that is characteristic for a $\Delta^{5,7}$-system of double bonds. A number of other characteristics – in particular, the maximum intensity of the fragmentary ion $[M - CH_3 - H_2O]^+$ and the high intensity of the other dehydration ions – also indicated the presence of a $\Delta^{5,7}$-system. Their high intensity in these compounds is connected, as it is assumed, with the formation of a stable conjugated trienic system increasing the resistance of these ions to electron impact. On the basis of the figures for the molecular ion, this compound had another two double bonds. Its mass spectrum included a fragmentary ion with m/z 271 (29), which probably corresponded to the elimination of the side chain, $[M - R]^+$. It may be assumed that these two bonds are present in the side chain. The hypothesis has been put forward that this compound is erosta-5,7,22,24(28)-tetraen-3β-ol.

In all mutants with respect to the gene Nys 3, the main sterol proved to be ergosterol. In addition to ergosterol, (VI) was also detected in them.

The main sterol in mutants with respect to the Nys 1 gene proved to be sterol with a molecular ion having m/z 384 (II). The molecule contained two double bonds.

The intensity of the molecular ion for this compound was the maximum, which is one of the characteristics of Δ^8-sterols. This assignment was also indicated by the high intensity of the peak of a $[M - CH_3]^+$ ion with m/z 369 (51%). Its mass spectrum also contained ions with m/z 273 (9) and 271 (20). As may be assumed, these values correspond to $[M - R]^+$ and $[M - R - 2H]^+$ ions. Consequently, one of the double bonds was present in the nucleus and the other in the side chain. The fact that a double bond was present in the Δ^8 position was also indicated by the lower intensity of the $[M - R - 2H]^+$ ion with m/z 271 (20) and of the $[M - R - H_2O]^+$ ion with m/z 255 (3), while, as reported in [7], for Δ^7- and Δ^5-sterols these ions have the maximum intensity. In the side chain, the double bond was present in position 24-25, as follows from the low intensity of the peak of the $[M - R]^+$ ion (it is relatively high for Δ^{22}-sterols) and the absence of the peak of an ion with m/z 301, which is characteristic for Δ^{23}-sterols [11, 17]) and corresponds to the allyl cleavage of the 20-22 bond. The facts given above permit the conclusion that sterol (II) was cholesta-8,3-dien-3β-ol (zymosterol).

The identification of sterols (IV) and (V) presented no difficulties since their mass spectra practically coincided with those of cholesta-5,7,24-trien-3B-ol and cholesta-5,7, 22,24-tetraen-3β-ol, studied previously.

Mutants with respect to the Nys 2 gene accumulated sterols with molecular ions having m/z 400 (VII), 298 (VIII), 396 (I), and 394 (VI). Compound (VII) had one double bond located in the tetracyclic nucleus, as could be judged from the m/z value of the $[M - R]^+$ ion of 273 and that of the $[M - R - 2H]^+$ ion, of 271. The absence of the peak of an ion corresponding to the cleavage of the nucleus at the 10−1, 5−10, and 7−8 bonds forced us to assume the presence of the double bond in the 7-8 or the 8-9 position. It is difficult to distinguish between Δ^7- and Δ^8-sterols solely from the form of their mass spectra, which is connected with the partial isomerization of such sterols in the inlet system of the mass spectrometer [3]. The high intensity of the peak of the fragmentary ions $[M - CH_3]^+$ (43%) in the case of compound (VII) is more characteristic for sterols with an 8−9 double bond in the nucleus than for Δ^7-sterols, since the methyl group in them is present in the allyl position with respect to the double bond.

Compound (VIII) had two double bonds of which one was present in the side chain. It is possible to establish its position on the basis of the peak of a fragmentary ion with m/z 314 (6%) present in the mass spectrum, corresponding to the allyl cleavage of a 22−23 bond, i.e., the double bond was located at the 24th carbon atom. Furthermore, the presence of a double bond just in the C-24 position was confirmed by a comparison of the GLC results with those given in the literature [12, 13]. The strongest peak in the mass spectrum of the sterol (VIII) was that of the molecular ion, and the peak of a $[M - CH_3]^+$ ion was also strong (58%). This type of mass spectrum is not characteristic for sterols having an unsaturated side chain and a 5−6 or 7−8 double bond in the nucleus, and all this forces us to assume that the sterol (VIII) was ergosta-8,24(28)-diene-3β-ol.

The identification of the other two sterols (I and VI) was not difficult. The first of them proved to be ergosterol, and compound (VI) differed from this by the presence of one more double bond in the side chain at the 24th carbon atom.

Mutants with respect to the Nys X gene, together with a small amount of ergosterol (3-5%), accumulate a sterol with a molecular weight of 398 (III). The molecule of this sterol contains two double bonds clearly identified as a $\Delta^{5,7}$-dienic system, i.e., it is characterized as 22,23-dihydroergosterol.

For the majority of the Candida guilliermondii mutants, as for the initial strain, the only sterol proved to be erogsterol. In several mutants (six strains), however, in addition to ergosterol ergosta-5,7,22,24(28)-tetraen-3β-ol, not present in the initial strain, was detected. Fourteen mutants also contained zymosterol, cholesta-5-7,24-trien-3β-ol, and cholesta-5,7,22,24-tetraen-3β-ol, in addition to ergosterol. In five mutants belonging to this group, a sterol with a molecular weight of 426 amu was also detected in small amounts (10-15%). Its mass spectrum coincided completely with that of lanosterol described in [10].

EXPERIMENTAL

Strains of the yeast Saccharomyces cerevisiae Petergof genetic lines were used, of the wild type − 2-P712 (MATα ade 2-163 his 7-1 lys 2-A12) and 1G-P188 (MATa hys 7-1 lys 2-A21) − and also the mutants hys 1, hys 2, hys 3, and hys X obtained from these strains, and strains of the yeast Candida guilliermondii VPS-569 and its mutants. The compositions of the media and the conditions of growth have been published previously [14, 15].

The sterols were isolated by the method of Breivik and Owades in Woods' modification [16].

GLC was performed on a Pye-Unicam chromatograph using a capillary column containing the phase OV-17 at a temperature of 250°C.

The mass spectra of the sterols were obtained on a LKB-2091 instrument at 70 eV, the temperature of the ion source being 250°C.

For all the sterols, the nine strongest peaks in the mass spectrum ($m/z \geq 200$) are given, the intensities being shown in percentages of the maximum peak.

Sterols of known composition: Cholesta-5,7,22,24-tetraen-3β-ol: M^+: 380 (95), 365 (7), 362 (20), 347 (100), 321 (8), 271 (27), 269 (32), 253 (63), 251 (80), 229 (8), 211 (54).

2. Ergosta-5,22-dien-3β-ol: M^+: 398 (53), 383 (9), 380 (11), 365 (8), 273 (5), 271 (47), 255 (100), 253 (3), 231 (21), 213 (46).

3. Lanosterol: M^+: 426 (45), 411 (100), 393 (52), 273 (9), 271 (9), 258 (10), 253 (17), 241 (16), 223 (10), 215 (11).

4. 5α-Cholestanol: M^+: 388 (78), 373 (37), 370 (5), 355 (19), 275 (4), 257 (8), 233 (100), 246 (25).

5. Cholesterol: M^+: 386 (87), 371 (49), 368 (52), 353 (46), 275 (100), 273 (33), 255 (41), 231 (35), 213 (59).

6. Cholesta-4,6-dien-3β-ol: M^+: 384 (44), 369 (5), 366 (100), 351 (14), 271 (4), 253 (15), 229 (7), 211 (9).

7. Cholesta-5,22-dien-3β-ol: M^+: 384 (63), 369 (11), 366 (14), 351 (8), 273 (12), 211 (43), 255 (100), 231 (11), 213 (38).

Sterols isolated from the mutant yeasts:

1. Ergosterol: M^+: 396 (72), 378 (11), 363 (100), 337 (24), 271 (27), 253 (38), 251 (18), 227 (8), 213 (13), 211 (15).

2. Zymosterol: M^+: 384 (100), 369 (51), 366 (6), 351 (10), 273 (9), 271 (20), 255 (3), 231 (22), 229 (25), 213 (24).

3. Ergosta-5,7-dien-3β-ol: M^+: 398 (62), 383 (11), 380 (19), 365 (100), 339 (29), 271 (28), 269 (4), 253 (30), 251 (25), 211 (39).

4. Cholesta-5,7,24-trien-3β-ol: M^+: 382 (69), 367 (9), 364 (20), 349 (100), 328 (31), 271 (36), 269 (18), 253 (30), 251 (83), 213 (24), 211 (48).

5. Cholesta-5,7,22,24-tetraen-3β-ol: M^+: 380 (98), 365 (7), 362 (25), 347 (100), 321 (9), 271 (29), 269 (33), 253 (67), 251 (83), 229 (4), 211 (52).

6. Ergosta-5,7,22,24-tetraen-2β-ol: M^+: 394 (86), 379 (3), 376 (23), 361 (100), 337 (13), 271 (23), 269 (77), 253 (25), 251 (31), 229 (6), 211 (46).

7. Ergosta-8-en-3β-ol: M^+: 400 (100), 385 (43), 382 (2), 367 (12),273 (41), 271 (3), 255 (11), 253 (5), 231 (14), 213 (8).

8. Ergosta-8,24-dien-3β-ol: M^+: 398 (100), 383 (58), 380 (3), 365 (17), 273 (5), 271 (48), 255 (13), 253 (4), 231 (19), 213 (11).

9. Lanosterol: M^+: 426 (41), 411 (100), 393 (49), 273 (11), 271 (10), 253 (19), 258 (11), 241 (16), 223 (13), 215 (13).

SUMMARY

The sterol compositions of strains of the yeasts Saccharomyces cerevisiae and Candida guilliermondii and a number of their mutants have been identified. The possibility has been shown of identifying yeast sterols from a comparison of the intensities of the peaks of the fragmentary ions having a common nature for all the sterols ($m/z \geq 200$).

A change in the sterol composition of yeast mutants as compared with the initial strains has been detected which is obviously connected with a blockage of the routes for the biosynthesis of ergosterol in the yeast cell.

The results obtained can be used for the directed microbiological synthesis of a number of biologically active substances.

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NEOCONVALLOSIDE – A CARDENOLIDE GLYCOSIDE FROM PLANTS OF THE GENUS Convallaria

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In a study of the epigeal part and seeds of Convallaria keiskei, C. majalis, and C. transcaucasica, in addition to lokundjoside, convalloside, convallotoxoloside, and neovallotoxoloside, we have isolated the previously unknown glycoside neoconvalloside, for which, on the basis of the physicochemical properties of the compound and of the products of its chemical transformations, the structure of strophanthidin 3-O-[O-β-D-glucopyranosyl-(1 → 2)-α-L-rhamnopyranoside has been established.

Continuing an investigation of the epigeal parts and seeds of the lilies of the valley Convallaria keiskei Miq., C. majalis L., and C. transcaucasica Utkin, in addition to lokundjoside, convalloside, convallotoxoloside, and neoconvallotoxoloside, we have isolated a cardenolide glycoside (I) with the empirical formula $C_{35}H_{52}O_{15}$. Its UV spectrum exhibits two absorption maxima, in the 220 and 303 nm regions (log ε 4.19 and 1.2, respectively), which are characteristic for the butenolide rings and aldehyde groups of cardenolides. The Mannich–Siewert hydrolysis of (I) (scheme) led to its cleavage into D-glucose (V), L-rhamnose (IV), and a number of products of aglycon nature, two of which had R_f values in various systems coinciding with those of strophanthidin (II) and of 5-anhydrostrophanthidin (III).

On the basis of the results obtained, it was possible to assume that the substance was convalloside [2]. However, in contrast to convalloside it was not hydrolyzed by the enzymes of the grape snail [3] or of Aspergillus oryzae [4], which may be connected with the order or position of attachment of the sugar residues in its molecule.

The stepwise hydrolysis of neoconvalloside with 0.1 N sulfuric acid led to the formation of convallotoxin (VI) and D-glucose (V). This showed that the D-glucose residue was the terminal residue.

In the products of the periodate oxidation of the glycoside (I) under investigation we detected L-rhamnose, which indicates the absence of a 1 → 3 bond between the sugar residues.

Thus, the most probable linkage of the D-glucose residue in neoconvalloside to the L-rhamnose residue is by a 1 → 2 glycosidic bond. To confirm this hypothesis we performed the reduction of the isolated glycoside (I) with sodium tetrahydroborate. As a result we

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