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Inhibition and Substrate Specificity of Yeast Δ^{22}-Desaturase

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SUMMARY: Using yeast microsomes, 23-hydroxysterols were tested as intermediates in the formation of the sterol side Δ^{22}-double bond. No evidence could be found supporting a two-stage mechanism of desaturation via hydroxylation and dehydration. Sterols with various side chains were tested as substrates. Those with alkyl substituents in the 24-α position were poor substrates. A series of sterols, including cyclopropyl sterols, were tested as mechanism-based inhibitors without success. Inhibition was observed with an isocyano-sterol.

In our studies of the biosynthesis of cyclopropane-containing sterols from marine sponges (e.g. 1) we have recently proposed [1], and have subsequently shown [2], that the cyclopropane moiety arise from the enzymatic desaturation of a saturated sterol side chain. We have proposed that the sponge cyclopropyl sterol desaturase is a mutated Δ^{22} sterol desaturase because both reactions (Figure 1) are believed to involve a 23-carbocationic intermediate (2). In order to learn more about sterol side chain desaturases, we have carried out experiments on the substrate-specificity and inhibition of the Δ^{22} sterol desaturase of yeast.

No Δ^{22} sterol desaturase has yet been purified. Like fatty acid desaturases [3], Δ^{22} sterol desaturases require reduced nucleotide cofactor and oxygen, and remove two hydrogen atoms with syn stereochemistry - in fungi from the α-face [4], and in algae from the β-face [5] of the sterol side chain. On the basis of inhibitor tests, the yeast Δ^{22} sterol desaturase has been proposed to be a P-450 enzyme [6], while the fatty acid desaturase is known to contain non-heme iron [7]. The fatty acid desaturase was earlier thought to operate via a hydroxylation-dehydration sequence of reactions [8].

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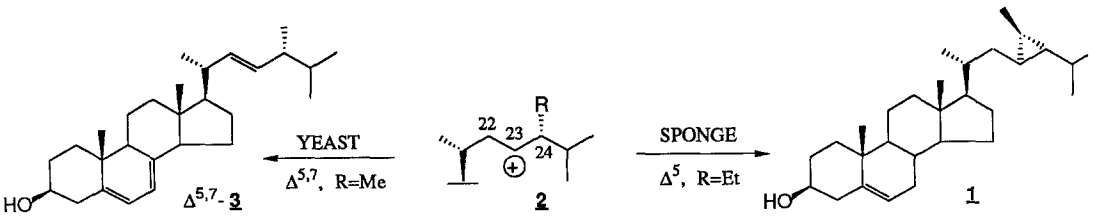


Figure 1

A similar sequence (4→5→3, Figure 2) has been proposed for the yeast Δ^{22} sterol desaturase based on the discovery of a 23-hydroxysterol (5) in a yeast mutant blocked in the Δ^{22} desaturase [9].

MATERIALS AND METHODS

Yeast microsomes: Aerobically adapted [10] baker's yeast (Saccharomyces cerevisiae) was put through a French Press (three times at 16,000 psi) in 100 mM sodium N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate buffer containing 2 mM magnesium sulfate, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride [11]. The broken cells were centrifuged at 10,000 x g for 10 min and the supernatant at 125,000 x g for 1 h. The high-speed pellet was resuspended in buffer and stored frozen in liquid nitrogen.

Enzyme assays: Yeast microsomes (40 µl), 3 mM NADPH (10 µl), and radiolabeled sterol (0.5-1 µCi in 10 µl 1% Tween 80) were incubated at 27°C for 4 h. The experiments were worked up and analyzed by reverse phase HPLC as previously described [11]. 22-Dihydrobrassicasterol (4) was tested under the assay conditions more than ten times. The other sterols were tested in single experiments. Inhibition was tested by addition of 2.5 µg of potential inhibitor (as 1% Tween 80 soln.), keeping everything else constant, and measuring the Δ^{22}-desaturation of radiolabelled 22-dihydrobrassicasterol (4).

Radiolabeled sterols: Synthesis of [3-^{3}H] 23-hydroxy sterols (5) is described in Figure 3. The same method ($NaBT_4$ reduction of the 3-ketones) was used for the other sterols. 22-Dihydrobrassicasterol (4) was obtained through hydrogenation of brassicasterol (3) i-methyl ether. Campesterol (8), clionasterol (12) and sitosterol (14) were similarly obtained from the i-methyl ethers of epicodisterol, clerosterol and stigmasterol (15), respectively. All radiolabeled (and "cold") sterols were purified by reverse phase HPLC with methanol [11].

23-Hydroxysterols: The known 23-hydroxy i-methyl ethers [12] were hydrolyzed to the diols (5):
(23R,24R)-Ergost-5-ene-3β,23-diol (5a): ^{1}H-NMR (400 MHz) δ($CDCl_3$) 5.351 (m, 1H, C6), 3.701 (m, 1H, C23), 1.005 (s, 3H, C19), 0.964 (d, J=6.4 Hz, 3H, C21 or 26 or 27 or 28), 0.936 (d, J=6.8 Hz, 3H, C21 or 26 or 27 or 28), 0.858 (d,

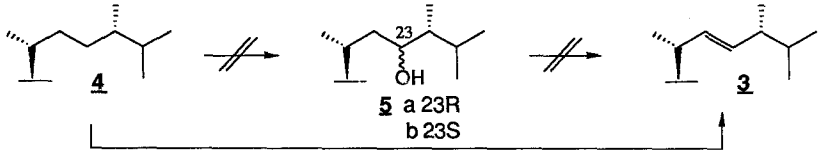


Figure 2

J=6.9 Hz, 3H, C21 or 26 or 27 or 28), 0.834 (d, J=6.9 Hz, 3H, C21 or 26 or 27 or 28), 0.712 (s, 3H, C18); high resolution mass spectrum, m/z (relative intensity) 416.3646 (M^+, $C_{28}H_{48}O_2$, 24) (calc. 416.3652), 401 (5), 398 (61), 383 (19), 365 (17), 327 (42), 213 (52), 159 (84), 133 (100), 81 (100).
(23S,24R)-Ergost-5-ene-3β,23-diol (5b): ^{1}H-NMR (400 MHz) $\delta(CDCl_3)$ 5.351 (m, 1H, C6), 3.610 (m, 1H, C23), 1.053 (d, J=6.5 Hz, 3H, C21 or 26 or 27 or 28), 1.005 (s, 3H, C19), 0.908 (d, J=6.8 Hz, 3H, C21 or 26 or 27 or 28), 0.840 (d, J=6.7 Hz, 3H, C21 or 26 or 27 or 28), 0.791 (d, J=6.9 Hz, 3H, C21 or 26 or 27 or 28), 0.693 (s, 3H, C18); high resolution mass spectrum, m/z (relative intensity) 416.3661 (M^+, $C_{28}H_{48}O_2$, 89) (calc. 416.3652), 401 (18), 398 (100), 383 (40), 327 (45), 300 (52), 213 (58), 145 (65), 133 (63), 107 (60).

(20S)-21-Isocyano-20-methylpregn-5-en-3β-ol (25): The acetate ester of (20S)-21-amino-20-methylpregn-5-en-3β-ol [13] was converted to the isonitrile via dehydration of the formamide [14]. The product was saponified with 3% KOH/MeOH and purified by silica gel TLC (C_6H_6/Et_2O 1:1). HPLC RT 12 min (MeOH); ^{1}H-NMR (400 MHz) $\delta(CDCl_3)$ 5.351 (m, 1H, C6), 3.361 (d, J=14.6 Hz, 1H, C21), 3.251 (dd, J=14.6, 6.4 Hz, 1H, C21), 1.139 (d, J=6.6 Hz, 3H, C22), 1.010 (s, 3H, C19), 0.703 (s, 3H, C18); high resolution mass spectrum, m/z (relative intensity) 341.2715 (M^+, $C_{23}H_{35}NO$, 49) (calc. 341.2717), 326 (100), 323 (49), 308 (78), 256 (50), 230 (97), 145 (5 (5 (96).

(24ξ)-24-Bromo-cholesta-5-en-3β-ol (24): (24ξ)-24-Hydroxycholesterol i-methyl ether was treated with phosphorus tribromide in pyridine to give (after deprotection) a mixture of the bromo compound and desmosterol (22). HPLC RT 38 min (desmosterol: HPLC RT 41 min); ^{1}H-NMR (400 MHz) $\delta(CDCl_3)$ 5.352 (m, 1H, C6), 3.973 (m, 1H, C24), 1.025 and 1.019 (d, J=6.6 Hz, 3H, C26 or 27), 1.006 (s, 3H, C19), 0.979 and 0.963 (d, J=6.4 Hz, 3H, C26 or 27), 0.938 and 0.925 (d, J=6.4 Hz, 3H, C21), 0.682 and 0.678 (s, 3H, C18); high resolution mass spectrum, m/z (relative intensity) 466.264748 and 464.265282 (M^+, $C_{27}H_{45}BrO$, 13 and 12) (calc. 466.263331 and 464.265378), 384 (73), 369 (63), 351 (33), 300 (34), 271 (100), 145 (33), 69 (29), 55 (29).

25-Methylcholesta-5,23(E)-dien-3β-ol (28): 25-Methyl-24-hydroxy-cholesterol tetrahydropyranyl ether [15] was dehydrated with phosphorus oxychloride in pyridine, and deprotected with 1% HCl in 90% methanol. HPLC RT 42 min (MeOH); ^{1}H-NMR (400 MHz) $\delta(CDCl_3)$ 5.407 (d, J=15.5 Hz, 1H, C24), 5.351 (m, 1H, C6), 5.255 (ddd, J=15.5, 8.0, 6.2 Hz, 1H, C23), 1.006 (s, 3H, C19), 0.990 (s, 9H, t-butyl), 0.886 (d, J=6.5 Hz, 3H, C21), 0.683 (s, 3H, C18); high resolution mass spectrum, m/z (relative intensity) 398.3549 (M^+, $C_{28}H_{46}O$, 17) (calc. 398.3549), 383 (20), 301 (51), 283 (62), 271 (63), 215 (47), 133 (55), 81 (69), 69 (77), 55 (100).

RESULTS AND DISCUSSION

We tested both the 23(R) and 23(S)-hydroxysterol side chains (5a and 5b) with yeast microsomes for conversion to Δ^{22} sterol. The Δ^5 sterol nucleus was used instead of the $\Delta^{5,7}$ nucleus ordinarily found in yeast, because of its greater stability and its synthetic advantages in terms of protecting group manipulation. Both 23-hydroxysterols (5) labeled with tritium in the 3-position were synthesized as shown for the 23(R) isomer (5a) in Figure 3. Upon incubation with yeast microsomes both labeled hydroxysterols (5) gave negative results. Furthermore, when the products of the enzymatic desaturation of dihydrobrassicasterol (4) were analyzed no 23-hydroxy sterols

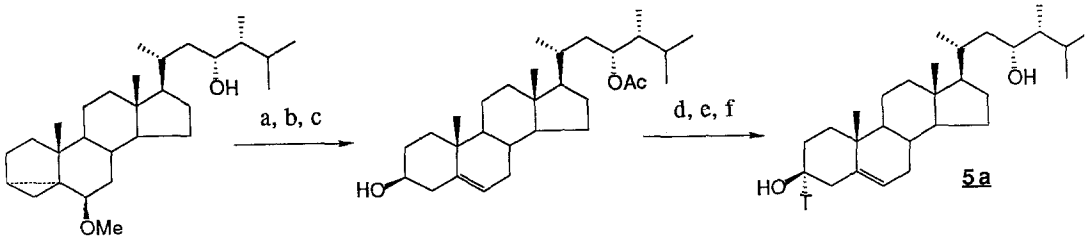
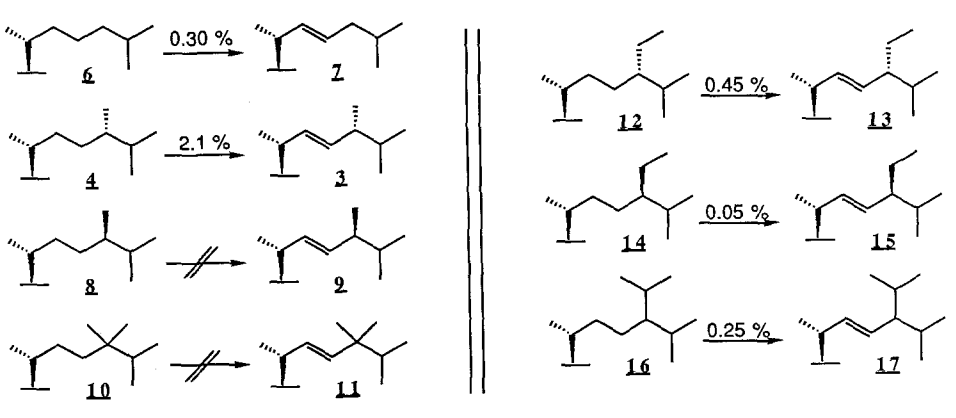


Figure 3. a) Acetic anhydride/ Pyridine; b) Trifluoroacetic acid/ Benzene; c) 5% Sodium hydroxide/ 10% Methanol; d) Pyridinium chlorochromate; e) Sodium borotritide; f) Lithium aluminum hydride.

(5) were detected. Based on these observations it is likely that the yeast mutation giving the 23-hydroxysterols (5) involves a shift in enzyme function from a desaturase to a hydroxylase rather than the loss of the dehydrase enzyme in a hydroxylase-dehydrase system.

Substrate specificity of the yeast enzyme was tested with a series of sterols with different side chains (Table 1). While $\Delta^{5,7}$ sterols are the major sterols in yeast and have been used in an earlier study of the yeast Δ^{22}-desaturase [6], their instability to light and air is problematic. We found Δ^{5} sterols to be utilized for Δ^{22}-desaturation (e.g. 4→3), but, unlike Δ^{7} sterols, not to undergo competing nuclear desaturation to the $\Delta^{5,7}$ compounds. Although we have not compared the relative efficiencies of the Δ^{5} and $\Delta^{5,7}$ nuclei in our Δ^{22}-desaturase assay, the results of the side-chain specificity experiments should be qualitatively similar. We found that

Table 1. Sterol side chain specificity of yeast Δ^{22}-desaturase (% conversion)



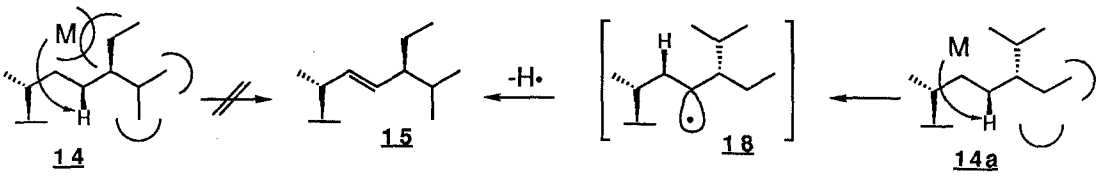
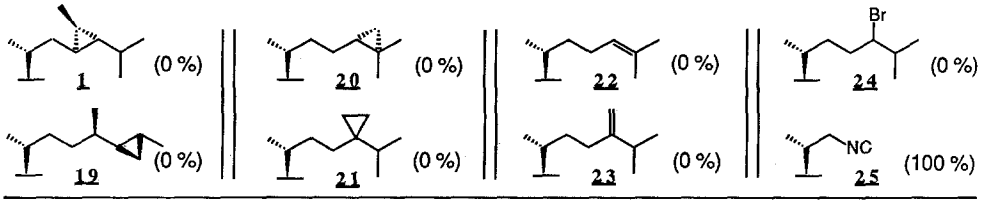


Figure 4. M = metalloenzyme reaction center.

compounds bearing an alkyl substituent in the 24α-position (8, 10, 14) were poor substrates. The small conversion of sitosterol (14) to stigmasterol (15) (sterols typical of higher plants) could be the result of the desaturation of an alternative enzyme-bound conformer (14a, Figure 4). The relative nonspecificity of the yeast Δ^{22} desaturase has biosynthetic implications for other organisms. The 24,24-dimethyl side chains (10 and 11) have recently been found in higher plants [16] and the 24-isopropyl side chains (16 and 17) are known from marine sponges and higher plants [17]. Δ^{22}-Cholesterol (7) and poriferasterol (13) are widely distributed in the marine environment. The specificity of the yeast desaturase for 24β-substituted side chains is probably related to the removal (in fungi [4]) of the hydrogen atoms from the α-face.

We thought that if a C-23 radical (e.g. 18) was an intermediate in the desaturation reaction, placement of a cyclopropane vicinal to the radical center might lead to enzyme inactivation via rearrangement to a new homoallylic radical [18]. This could provide a rationale for the biosynthesis of cyclopropyl sterols by sponges as chemical defenses. Several cyclopropyl sterols (1, 19-21), desmosterol (22) and 24-methylenecholesterol (23) (which could give rise to allyl radradicals), and 24-bromocholesterol (24, which could yield a bromine radical) were tested as inhibitors of the yeast Δ^{22} desaturase without success (Table 2). A positive control was provided by an isonitrile

Table 2. Inhibition experiments of desaturation of 4 to 5. (% inhibition at 150 μM inhibitor)



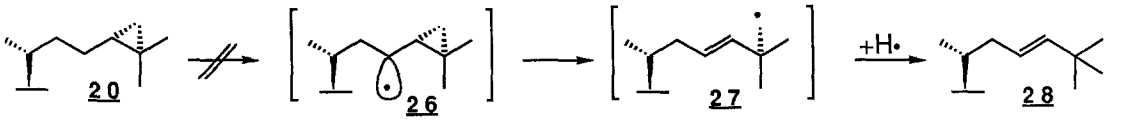


Figure 5

(25) containing sterol. This was designed to complex the metal at the active site and had a IC_{50} of 15 μM. Only the compounds with the cyclopropyl groups in the 24-position (20 [19] and 21 [20]) would be expected to undergo the methylcyclopropyl radical rearrangement (26→27, Figure 5). When 20 and 21 were assayed in radiolabeled form, no evidence could be found for either Δ^{22} desaturation or radical rearrangement.

These findings do not rule out the intermediacy of a radical species in the desaturation reaction. Even if the potential mechanism-based inhibitors were to serve as substrates for the enzyme and yield radical reaction intermediates, rearrangement of the radicals need not occur, and if it did, need not lead to enzyme inactivation. The possibility remains that cyclopropyl sterols could inhibit the Δ^{22} desaturase or other enzymes that produce side chain radical intermediates if they were accepted as substrates.

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