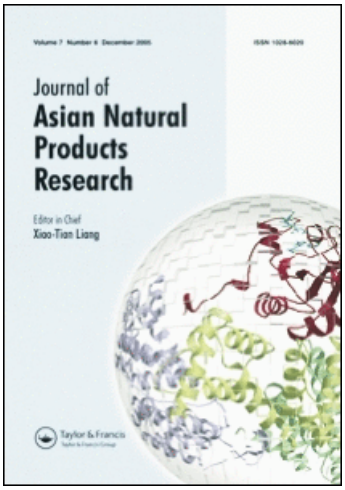

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

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To cite this Article Cui, Ya-Jun , Wang, Li-Da , Norton, Robert A. , Zheng, Jun-Hua and Guo, De-An(1999) 'Biotransformation of 24α-Methylcholesterol and 24β-Methylcholesterol by Yeast Mutant GL7', Journal of Asian Natural Products Research, 2: 1, 39 — 44
To link to this Article: DOI: 10.1080/10286029908039889
URL: http://dx.doi.org/10.1080/10286029908039889

JANPR, Vol. 2, pp. 39–44
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BIOTRANSFORMATION OF 24α-METHYLCHOLESTEROL AND 24β-METHYLCHOLESTEROL BY YEAST MUTANT GL7

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(Received 5 April 1999; Revised 17 April 1999; In final form 3 June 1999)

Incubation of 24α- and 24β-methylcholesterols with yeast mutant GL7 afforded their corresponding C-22-desaturated products under the catalysis of sterol $\Delta^{22(23)}$-desaturase. The metabolites were identified to be 22-dehydro-24α-methylcholesterol (2% yield from 24α-methylcholesterol) and 22-dehydro-24β-methylcholesterol (51% yield from 24β-methylcholesterol) respectively on the basis of their chromatographic and spectral properties. It was concluded that the sterol $\Delta^{22(23)}$-desaturase prefers the 24β-methyl sterols and is highly stereospecific.

Keywords: Biotransformation; Yeast mutant GL7; 24α-Methylcholesterol; 24β-Methylcholesterol; Sterol C-22-desaturase

INTRODUCTION

Ergosterol is the major sterol in majority of the advanced fungi. Since its discovery over 100 years ago, great progress has been made in the understanding of its structure, biosynthesis and function in fungi, particularly in yeast. It was thought to play two roles in the growth support of *Saccharomyces cerevisiae* (yeast): as a bulk membrane insert and to trigger hormonally cell proliferation [1–3]. The reaction mechanisms for the steps of

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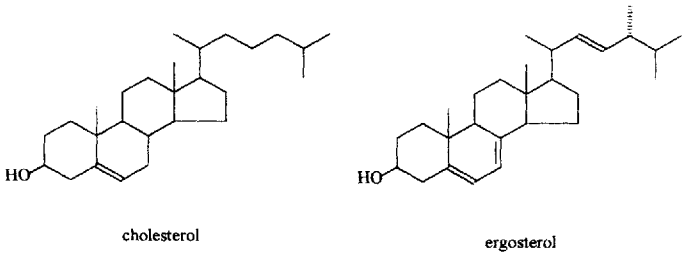

FIGURE 1 The structures of cholesterol (animal) and ergosterol (fungi).

ergosterol biosynthesis have been well approached. Several interesting aspects of sterol biosynthesis in yeast distinguish it from sterol biosynthesis in animals. Therefore these differences have been considered as the basis for developing antifungal agents that discriminate between cholesterol biosynthesis in animal and ergosterol biosynthesis in fungi. The obvious differences in the ergosterol structure from cholesterol are the C-5,7 double bonds of ring B, the additional unsaturation at C-22, and the methylation at C-24 (Fig. 1). Among these features, the C-22 unsaturation is a step in the ergosterol biosynthetic sequence regulated by the enzyme Δ^{22}-desaturase. We have reported earlier the biotransformation of 24-methylene cholesterol and sitosterol by using yeast mutant GL7 strain [4]. In order to further shed light on the substrate specificity of the enzyme, a pair of epimers, 24α-methylcholesterol and 24β-methylcholesterol, were fed to the cultures of yeast mutant GL7 to check the stereoselectivity and regiospecificity of Δ^{22}-desaturase.

EXPERIMENTAL SECTION

TLC was run on silica gel (Qingdao Marine Chemical Factory, Shandong Province, China) developed with benzene–ether (85 : 15). The C_{18} reversed-phase column (Zorbax and TSK Gel) were used for HPLC by respectively using pure methanol and MeOH–*i*-PrOH (4 : 1) as eluting solvents and detected by UV at 205 nm. The GLC was performed on a glass column packed with 3% SE-30. Operating conditions were as follows: column temperature – 245°C, detector temperature – 300°C, injector temperature – 275°C, helium gas flow rate set at 20 ml/min. Retention times in HPLC (α_c) and GLC (RRT_c) were relative to cholesterol. GC-MS was performed (by Dr. R.A. Norton) on a Hewlett-Packard 5890 series II gas chromatography at 70 eV. Temperature program: 170°C for 1 min, ramping to 270°C at 20°C/min, isothermal at 270°C for 3 min, ramping to 280°C at 2°C/min,

then isothermal at 280°C for 6 min. The capillary column on the GC-MS was a 15DB-MS (0.25 mm i.d., 0.25 μm film). The samples of 24α-and 24β-methylcholesterol are the gift from W. David Nes (Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas, USA). This pair of epimers can be distinguished based on their HPLC chromatographic and NMR spectral properties [5,6].

CULTURE CONDITIONS

Yeast mutant GL7 was a gift from W. David Nes. The mutant was grown on a medium containing 2% peptone, 1% yeast extract and 2% glucose. Experiments were carried out with 300 ml of medium in 1 l Erlenmeyer flasks. Sterol samples were dissolved in absolute ethanol (10 mg/ml), which was added to the autoclaved medium after dilution with Tween 80 (15 ml/l of medium). Sterol was added to the medium at a concentration of 5 ppm (5 mg/l). Cultures were inoculated with *ca.* 0.5×10^6 cells/ml and shaken continuously for 72 h at 180 rpm at 27°C.

ANALYSIS OF METABOLITES

The incubated cells were centrifuged for 15 min, weighed and saponified at reflux temperature for 30 min in aqueous methanolic KOH (10% KOH, 10% H_2O, and 80% MeOH, w/v/v) to produce a nonsaponifiable lipid fraction (NLF). The NLF obtained by dilution with water and extraction with petroleum ether was monitored by TLC and GLC, and then chromatographed on a preparative TLC plate. The band corresponding to 4-desmethyl sterols (by using ergosterol as a parallel standard) were scraped off the plate and eluted with acetone. The acetone extract was further purified to homogeneity by HPLC chromatography and identified by their chromatographic and spectral properties.

IDENTIFICATION OF METABOLITES

22(23)-Dehydro-24α-methylcholesterol (epibrassicasterol, Fig. 2) (**1a**): yield, 2% from 24α-methylcholesterol; m.p. 152–153°C (acetone); TLC (R_f), 0.18 (benzene/diethylether); GLC (RRT_c), 1.12; HPLC (α_c), 0.91 in Zorbax column and 0.82 in TSK Gel column; MS (*m/z*, rel. int.), 398 (M^+, 40), 383

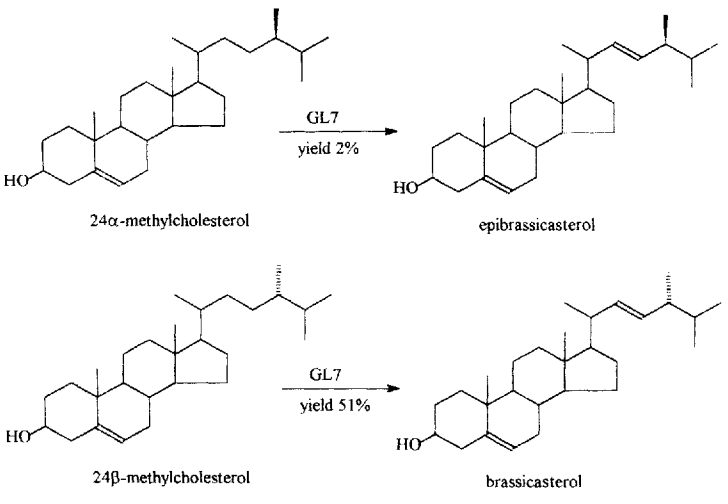

FIGURE 2 Biotransformation of 24α- and 24β-methylcholesterol by GL7.

($M^+ - CH_3$, 7), 380 ($M^+ - H_2O$, 6), 365 ($M^+ - CH_3 - H_2O$, 10), 337 (9), 300 (28), 271 (30), 255 (34), 229 (10), 213 (14), 159 (31), 69 (83), 55 (100); ^1H NMR (δ, ppm from TMS), 0.690 (3H, s, 18-CH_3), 1.006 (3H, s, 19-CH_3), 0.999 (3H, d, $J = 7.0$ Hz, 21-CH_3), 0.830 (3H, d, $J = 9.0$ Hz, 26-CH_3), 0.817 (3H, d, $J = 9.0$ Hz, 27-CH_3), 0.907 (3H, d, $J = 7.0$ Hz, 28-CH_3), 5.347 (1H, brd, 6-H), 5.156 (2H, brd, 22, 23-H). The above data agreed with those of standard epibrassicasterol.

22(23)-Dehydro-24β-methylcholesterol (brassicasterol, Fig. 2) (**2a**): yield, 51% from 24β-methylcholesterol; m.p. 141–142°C (acetone); TLC (R_f), 0.18 (benzene/diethylether); GLC (RRT_c), 1.12; HPLC (α_c), 0.91 in Zorbax column and 0.88 in TSK Gel column; MS (*m/z*), 398 (M^+, 50), 383 ($M^+ - CH_3$, 11), 380 ($M^+ - H_2O$, 6), 365 ($M^+ - CH_3 - H_2O$, 13), 337 (11), 300 (32), 271 (38), 255 (45), 229 (10), 213 (17), 159 (36), 69 (86), 55 (100); ^1H NMR (δ, ppm from TMS), 0.688 (3H, s, 18-CH_3), 1.006 (3H, s, 19-CH_3), 1.007 (3H, d, $J = 7.9$ Hz, 21-CH_3), 0.829 (3H, d, $J = 8.4$ Hz, 26-CH_3), 0.815 (3H, d, $J = 8.4$ Hz, 27-CH_3), 0.907 (3H, d, $J = 7.8$ Hz, 28-CH_3), 5.35 (1H, brd, 6-H), 5.18 (2H, brd, 22, 23-H).

RESULTS AND DISCUSSION

Saccharomyces cerevisiae, a single-celled eucaryote which can grow either with or without sexual reproduction, is an excellent tool for studies on the

biochemical role of sterols [7–10]. Elimination of oxygen can prevent the conversion of squalene to sterol in the sequence of ergosterol biosynthesis without inducing cell death. Therefore sterols with various structures can be administered and the effects on cell growth and other parameters can be approached without contamination from the endogenous sterol components. The GL7 mutant of yeast is a typical example that can be perfectly enlisted for this purpose. It is a sterol auxotroph which is defective at two places in the sterol pathway. The first defect is associated with oxidosqualene cyclase which catalyses the conversion of squalene oxide to lanosterol in yeast and other nonphotosynthetic living organisms. The outcome from this lesion is that no sterols were synthesized from this strain, therefore exdogenous sterols must be supplied in order to maintain the cell growth. The second defect has been identified at the point in the synthesis of porphyrins [11]. If ergosterol was supplemented to GL7, it can grow similarly to a wild-type strain that synthesizes primarily free ergosterol. After reconditioning of the cell culture several times, it becomes so-called "sterol adapted culture" [12]. The sterol adapted culture was employed for the biotransformation study because it retained auxotroph and possessed the complete enzyme systems that participate in the transformation of lanosterol to ergosterol.

A pair of diasteroisomers of sterols, 24α- and 24β-methylcholesterol, were fed to the above-mentioned culture of yeast GL7 mutant. The concentration of sterol administered was optimized and it was found that 5 ppm yielded the maximal cell growth rate. Therefore 5 ppm was used for all experiments. After 72 h of incubation the cells were harvested and sterol components were analysed and purified. It was found that the rates of conversion for both compounds are very different. In the case of 24β-methylcholesterol, over half of substrate administered was transformed, while 24α-methylcholesterol was hardly converted (about 2% by GLC quantitation). After careful analysis of the NLF fraction by various chromatographic and spectral techniques, it was clear that only one detectable metabolite was formed in both cases. Other than the difference in conversion rate, cell growth is much slower for 24α-methylcholesterol-fed cells than that for 24β-methylcholesterol. In each case, the corresponding C-22 desaturated metabolite was formed. One would expect some modification to happen for the nucleus such as forming $\Delta^{5,7}$-conjugated double bonds since ergosterol has this structural feature but this did not happen in the real system. This might be partly explained by the unidirectional biosynthetic sequence of ergosterol $\Delta^8 \rightarrow \Delta^7 \rightarrow \Delta^{5,7} \rightarrow \Delta^5$ in fungi, not the other way around. Therefore Δ^5-sterol substrate could not give rise to ergosterol.

From the biotransformation outcome, it was clear that only the C-22-desaturase in the normal pathway of ergosterol biosynthesis operated. The only difference of the substrates fed to GL7 lies in the configuration of C-24 methyl group. The C-22 desaturase has a high specificity to the stereochemistry of the alkyl groups at C-24. The β-methyl sterol yielded over 50% conversion to its Δ^{22}-metabolite while α-methyl sterol resulted in very little Δ^{22}-metabolite (about 2%). This phenomenon might be explained by that the natural ergosterol has a 24-methyl at β-configuration, therefore the enzyme binds the substrate with C-24 β-oriented methyl more tightly than that with α-oriented methyl. From above results, it could be concluded that in yeast 24β-methyl sterols induce greater Δ^{22}-desaturation and the enzyme involved is highly stereospecific.

Acknowledgements

This work was supported by Trans-Century Training Program Foundation for the Talents by the Ministry of Education. The authors are grateful to Dr. W. David Nes, Department of Chemistry and Biochemistry, Texas Tech University, USA for kindly providing samples of 24α- and 24β-methylcholesterol.

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