

Elucidation of Antifungal Drug Action by High Resolution GC/MS

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Overview

Metabolic profiling of yeast sterols was performed to precisely determine enzymatic targets for new potential antifungal drugs. Targeted analysis of the relative levels of ergosterol biosynthesis pathway intermediates was combined with an untargeted approach, empowered by accurate mass high resolution GC/Q-TOF technology, to obtain the most comprehensive results. Full scan electron ionization (EI) spectral information was complemented with MS/MS accurate mass product ion data to confirm the identity of the compounds accumulated in yeast as a result of drug treatment.

Introduction

Budding yeast *Saccharomyces cerevisiae* is widely used as a model genetic organism because of its simplicity and availability of strains with individual deletions in all of the genes in its genome. To evaluate new antifungal agents that target a key component of yeast membrane (ergosterol), complementary genetic and analytical approaches were utilized. As a first-tier high throughput approach Haploinsufficiency Profiling (HIP) screening was performed. HIP involves the growth of gene-depleted yeast against a drug where resulting sensitivity of the strain suggests that the enzyme product of heterozygous locus is being a target of the inhibitor. Furthermore, an analytical approach involving accurate mass high resolution GC/Q-TOF metabolic profiling of yeast sterols was used to specifically identify enzymatic targets of the potential drugs.

Based on the relative levels of targeted intermediates of the ergosterol biosynthesis pathway as well as accumulated untargeted metabolites in drug-treated *versus* untreated samples, the mechanisms of several potential antifungal therapeutic agents affecting sterol metabolism in yeast were proposed. In addition, the MS/MS accurate mass product ion spectra were used in conjunction with the Molecular Structure Correlator (MCS) tool for structural confirmation of some of the accumulated metabolites.

Experimental

Sample Preparation

Wild type yeast cultures (strain BY4743) were incubated with drug concentrations that inhibited growth by 10%. Yeast lipids were extracted by the Folch method (Folch et al., *J Biol Chem*, 1957, 226, 497). The lower chloroform aliquots were dried by speed vacuum, and the

Experimental

active functional groups were derivatized with 40 mg/mL hydroxylamine hydrochloride in pyridine followed by silylation using MSTFA + 1 % TMCS, prior to analysis by Agilent 7200 series GC/Q-TOF.

Analytical Conditions

This study was performed using an Agilent 7890 GC coupled to an Agilent 7200 series Quadrupole-Time-of-Flight (Figure 1). GC and MS conditions are described in Table 1.



Figure 1. 7200 series GC/Q-TOF system.

GC and MS Conditions:	
Column	HP-5 MS UI, 30 meter, 0.25 mm ID, 0.25 µm film
Injection volume	1 µL
Split ratio	20:1
Split / Splitless inlet temperature	250 °C
Oven temperature program	60 °C for 1 min 10 °C / min to 325 °C, 3.5 min hold
Carrier gas	Helium at 1 mL / min constant flow
Transfer line temperature	290 °C
Ionization mode	EI
Source temperature	230 °C
Quadrupole temperature	150 °C
Scan range	50 to 600 m/z
Spectral acquisition rate	5 Hz, collecting both in centroid and profile modes

Table 1. GC-MS conditions used in the study.

Data Processing

The chromatographic deconvolution was performed using MassHunter Unknowns Analysis software. Metabolites of interest were identified by comparison with the NIST11 mass spectral library. The multivariate software package Mass Profiler Professional (MPP) was used to determine compounds present at distinct levels in drug-treated vs untreated samples. Quantitation was performed using MassHunter Quantitative software B.05.

Results and Discussion

Proof of Concept

At the first stage of this study the analytical approach utilizing GC/Q-TOF was validated using two well-described antifungal drugs followed by the examination of novel drugs with potential therapeutic properties. Both Terbinafine and Fluconazole are widely used antifungal drugs that target distinct steps in the ergosterol biosynthesis pathway and whose mechanisms of yeast sterol pathway inhibition are well understood. Terbinafine inhibits the ERG1 gene product, squalene epoxidase, thus preventing the conversion of squalene to the next intermediate of the pathway. Therefore, squalene accumulation and depletion of downstream intermediates of the pathway is an expected outcome in this case. Data obtained by GC/Q-TOF were consistent with this expectation (Figure 2).

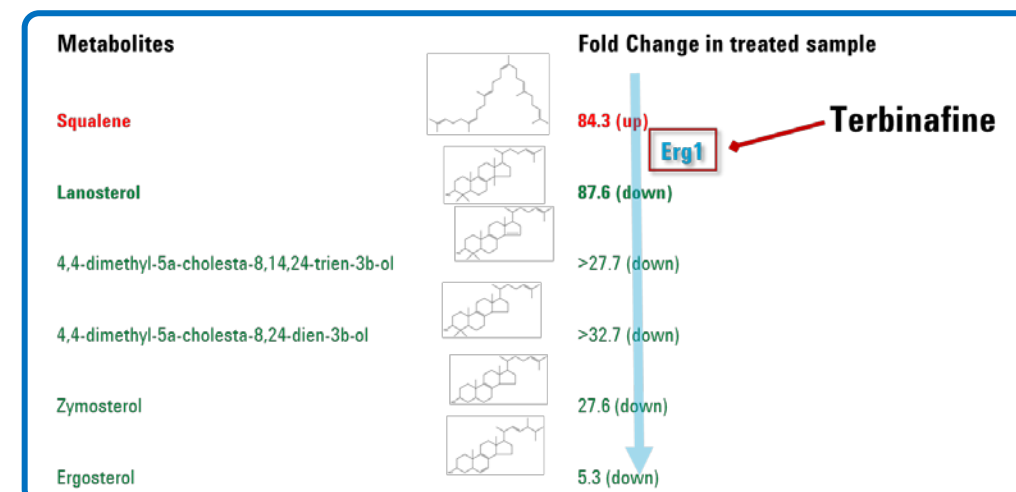


Figure 2. Squalene accumulation was observed in yeast treated with Terbinafine as compared to untreated control. The numbers represent fold change.

Similarly, in the case of an azole antifungal agent Fluconazole, that inhibits ERG11 gene product, the cytochrome P450 14 α -demethylase, or lanosterol demethylase, accumulation of lanosterol is expected.

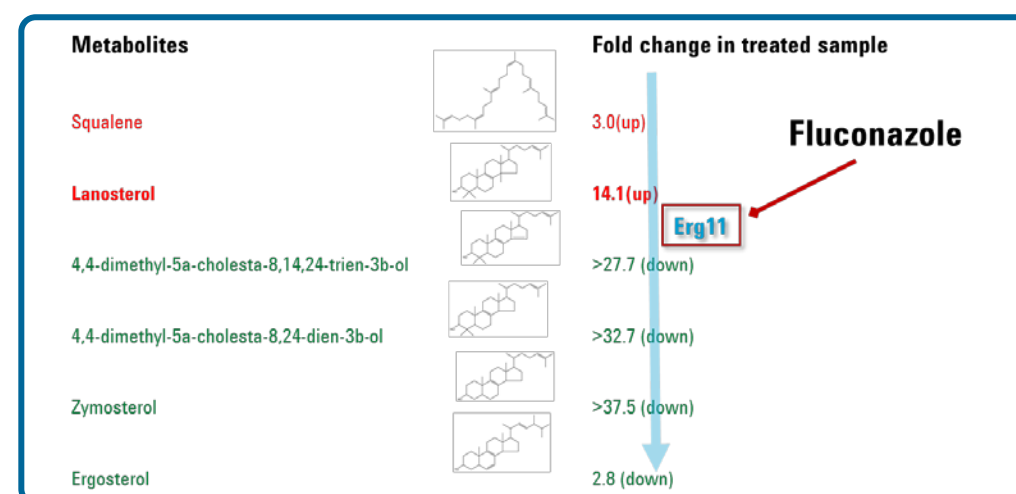


Figure 3. Significant accumulation of lanosterol as well as depletion of several targeted downstream metabolites was observed following Fluconazole treatment.

Unexpected Observation Revealed by Untargeted Approach

In addition to accumulation of lanosterol and depletion of other targeted downstream metabolites (Figure 3), untargeted approach revealed a buildup of several 14 α -methyl sterols that are not usually detected in yeast (Table 2).

Component (m/z @ RT)	Compound	Formula	MI of derivatized species (m/z)	Accurate mass of MI (m/z)	Mass error (ppm)
469 @ 28.08	14- α -methyl fecosterol	C ₂₈ H ₄₈ O	484.4095	484.4101	1.24
379 @ 28.26	14- α ,4- α -methyl zymosterol	C ₂₈ H ₄₈ O	484.4095	484.4107	2.48
467 @ 28.48	(3 β ,5 α)-14-methylergosta-8,24(28)-dione-3,6-diol	C ₂₈ H ₄₈ O ₂	572.4439	572.445	1.92

RT – Retention Time

MI – Molecular Ion

Table 2. Results of Untargeted Analysis of Fluconazole Treatment.

The empirical formulas of the accumulated 14 α -methyl sterols were determined based on EI fragmentation spectra and accurate mass information. Accumulation of 14 α -methyl sterols suggested that some of the downstream enzymes in the ergosterol biosynthesis pathway are rather “promiscuous” and are able to utilize sterols with an extra methyl group as their substrates.

Metabolic Profiling for Characterization of Potential Antifungal Drugs

A few potential inhibitors of ergosterol biosynthesis pathway such as Tolarol and a New Chemical Entity (NCE) were evaluated. Statistical analysis performed in MPP helped to easily identify specific steps of the pathway affected by a drug. In the case of NCE only 4,4-dimethyl-8,24-cholestadienol met the significance criteria for a biologically significant fold-change (Figure 4). When taking into account downregulation of the downstream intermediates (Figure 5), the results strongly suggested that the NCE specifically inhibited Erg25.

Tolarol treatment resulted in the accumulation of another sterol, 4 α -carboxy-4 β -methyl-5 α -cholesta-8,24-dienol, suggesting Erg26 being a specific target of Tolarol (Figure 6). This sterol was not originally targeted since it is a biologically unstable intermediate present only at trace levels and was revealed only in an untargeted approach.

Results and Discussion

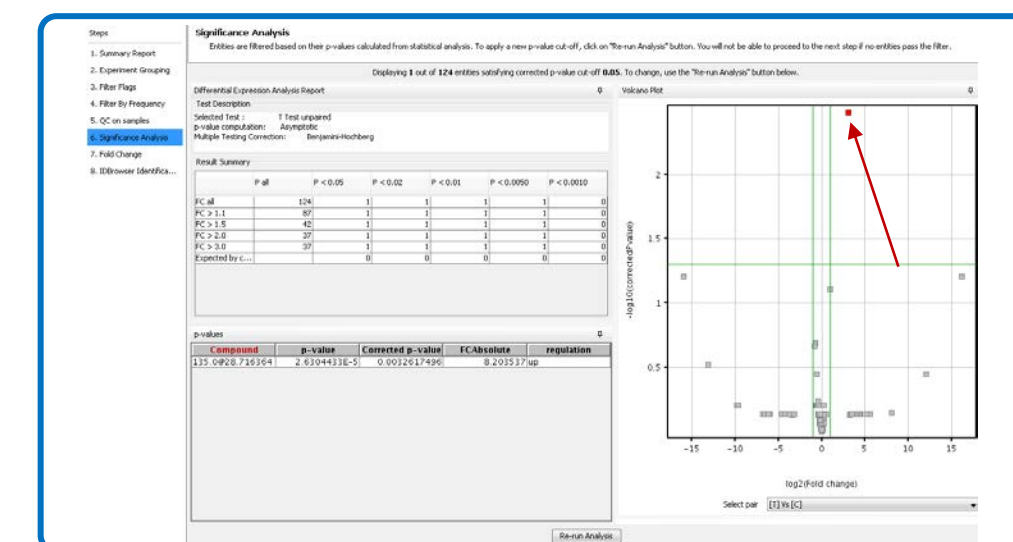


Figure 4. Significance analysis performed in MPP shows that only one compound (4,4-dimethyl-8,24-cholestadienol) is accumulated in response to treatment with NCE.

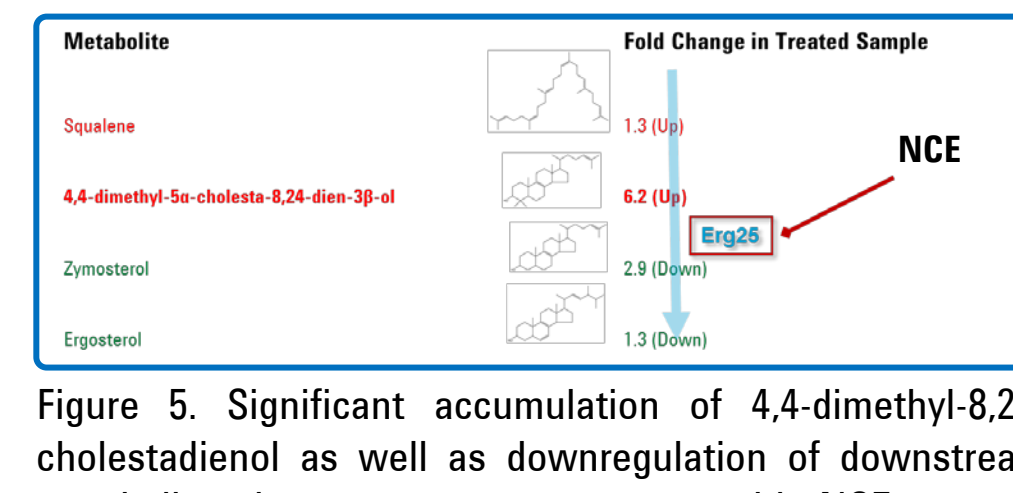


Figure 5. Significant accumulation of 4,4-dimethyl-8,24-cholestadienol as well as downregulation of downstream metabolites in response to treatment with NCE strongly suggest Erg25 as its target.

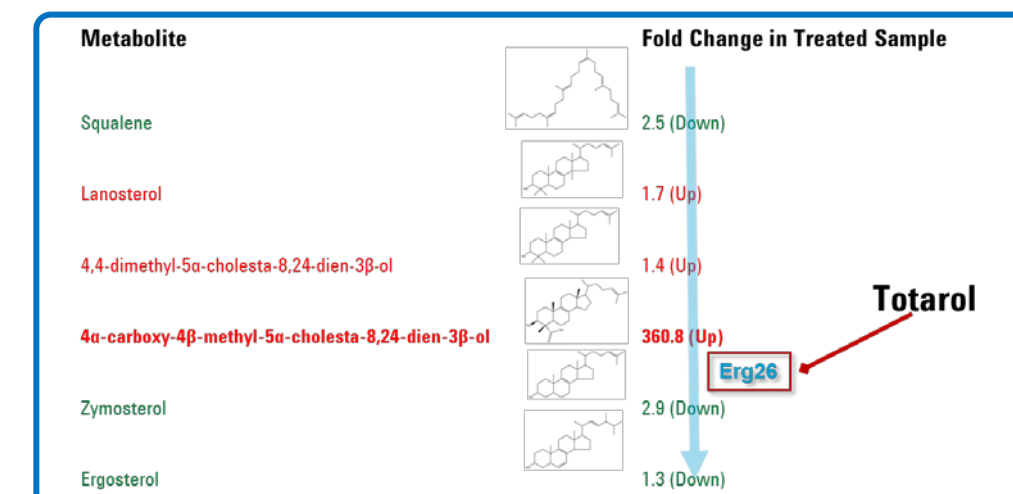


Figure 6. Buildup of 4 α -carboxy-4 β -methyl-5 α -cholesta-8,24-dienol is consistent with Erg26 being the specific target for Tolarol.

The structure of 4 α -carboxy-4 β -methyl-5 α -cholesta-8,24-dienol was confirmed using accurate mass product ion spectra information (Figure 7) since NIST library spectrum is not available for this compound.

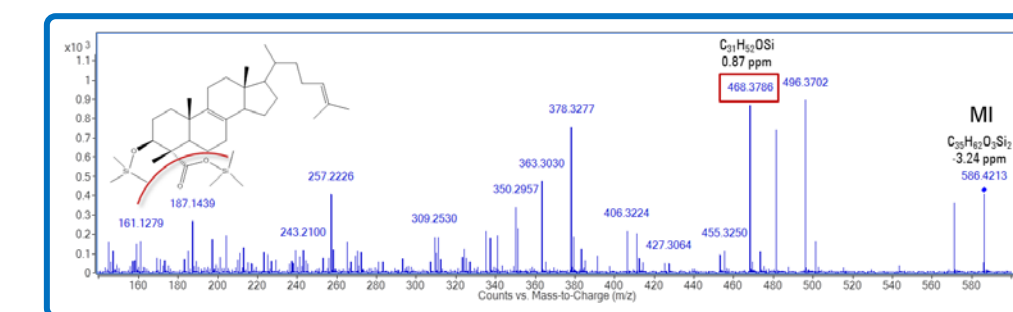


Figure 7. MS/MS product ion spectrum helps to confirm the structure of one of the compounds identified by untargeted approach, 4 α -carboxy-4 β -methyl-5 α -cholesta-8,24-dienol.

Molecular Structure Correlator (MSC) was further used to predict the substructures of the resulting MS/MS fragments and evaluate best candidate structures of accumulated compounds (Figure 8).

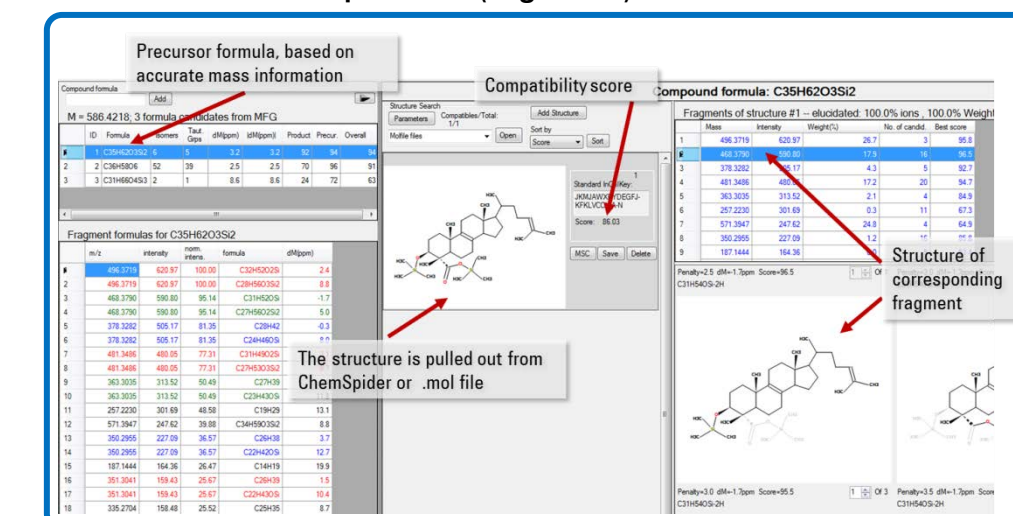


Figure 8. MSC helps to assign formulas to each accurate mass fragment from product ion spectrum. Compounds structures are extracted from ChemSpider database or from .mol files to visualize fragmentation and provide the score that reflects the probability of each fragment structure being formed. Finally, it assigns a compatibility score to each possible compound structure.

Conclusions

Metabolic profiling of yeast sterols is a powerful approach for identifying enzymatic targets of antifungal drugs, and can be used in combination with HIP to elucidate the mechanism of drug inhibition in more details. The accurate mass information and full spectrum sensitivity of GC/Q-TOF enabled reliable identification of targets as well as unknown compounds that accumulated as a result of the treatments. The accumulation of several intermediates of yeast sterol biosynthesis pathway in drug treated vs untreated samples helped to elucidate the mechanisms of several potential antifungal agents including NCE and Tolarol.