Metabolic Profiling of Yeast Sterols Using the Agilent 7200 Series GC/Q-TOF System

Application Note

Metabolomics

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Abstract

Metabolic profiling of yeast sterols to precisely determine enzymatic targets for new potential antifungal drugs was performed using the Agilent 7200 series GC/Q-TOF System and Mass Profiler Professional (MPP) software. 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 acquisition electron ionization (EI) spectral information was complemented with MS/MS accurate mass product ion spectral data to confirm the identity of the compounds accumulated in yeast as a result of drug treatment.

Introduction

Budding yeast *Saccharomyces cerevisiae* is an attractive model organism because of its simplicity and the availability of strains with individual deletions in all of the genes in its genome. It was used in this study to evaluate new antifungal agents. This family of drugs often targets ergosterol, a key component of fungal membranes. Ergosterol performs a function similar to that of cholesterol in animal cells. However, despite the functional similarity between ergosterol and cholesterol, many of the enzymes involved in yeast sterol biosynthesis differ in their structure and enzymatic specificity from the corresponding enzymes in mammalian cells. Therefore, agents that inhibit the ergosterol biosynthetic pathway often have antifungal therapeutic potential. These agents can be identified using complementary genetic and analytical approaches. As a first-tier high throughput approach, Haploinsufficiency Profiling (HIP) screening [1] was performed. HIP involves the growth of gene-deleted yeast against a drug. Resulting increased sensitivity of the strain suggests that the product of a heterozygous locus might be a target of the inhibitor. Furthermore, an analytical approach involving accurate mass high resolution gas chromatography/quadrupole time-of flight (GC/Q-TOF) metabolic profiling of yeast sterols can be used in conjunction with genetics to specifically identify enzymatic targets of potential drugs.

Agilent Technologies

This application note describes the use of the 7200 series Q-TOF GC/MS System coupled to the Agilent 7890 Gas Chromatograph (GC) to reliably identify targets of emerging antifungal drugs through identification of the compounds accumulated as a result of drug treatment. The approach was first validated using two well-described antifungal drugs followed by the examination of novel drugs with potential therapeutic properties. The wide dynamic range of this instrument allowed for simultaneous quantification of lipid metabolites across a range of concentrations in the yeast cell.

Based on the relative levels of targeted intermediates of the ergosterol biosynthesis pathway as well as the 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 (MSC) tool for structural confirmation of some of the accumulated metabolites.

Experimental

Reagents and Standards

Standards and reagents	Source
Terbinafine	Sequoia Research Products
Fluconazole	Sigma-Aldrich
Totarol	Sigma-Aldrich
NCE 1181-0519	ChemDiv
MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide) + 1% TMCS (trimethylchlorosilane)	Sigma-Aldrich
Methoxyamine hydrochloride	Sigma-Aldrich
Anhydrous pyridine	Sigma-Aldrich

Instruments

This study was performed on a 7890A GC coupled to a 7200 series GC/Q-TOF. The instrument conditions are listed in Table 1.

Table 1. GC and MS Instrument Conditions

GC conditions	
Column	HP-5 MS UI, 30 m × 0.25 mm, 0.25 µm film (p/n 19091S-433UI)
Injection volume	1 µL
Split ratio	20:1
Split/Splitless inlet temperature	250 °C
Oven temperature program	60 °C for 1 minute 10 °C/min to 325 °C, 3.5 minutes hold
Carrier gas	Helium at 1 mL/min constant flow
Transfer line temperature	290 °C
MS conditions	
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
Collision energy in MS/MS mode	15 V

Sample Preparation

Six biological replicates consisting of 5 mL aliquots of wild type yeast cultures (strain BY4743) were incubated with drug concentrations that inhibited growth by 10% and harvested at OD_{600} around 1 (roughly 3×10^7 cells/mL). Yeast lipids were extracted by the Folch method [2]. The lower organic phase corresponding to 2.5 OD_{600} units of original culture was dried by speed vacuum, and derivatized with 40 mg/mL methoxyamine hydrochloride in pyridine followed by silylation using MSTFA + 1% TMCS mixture for a final volume of 100 µL. Of this solution, 1 µL was injected into the GC.

Data Analysis

Quantification was performed using Agilent MassHunter Quantitative software B.05. All chromatograms were deconvoluted using Mass Hunter Qualitative Analysis software B.05, and metabolites of interest were identified by comparison with the NIST11 mass spectral library.

The multivariate software package Mass Profiler Professional (MPP), was used for statistical evaluation of the data after deconvolution (data filtering, statistical significance, finding unique compounds in trace levels and visualization) to determine compounds present at distinct levels in drug-treated samples as compared to the control. This allowed the detection of several unexpected metabolites.

Results and Discussion

Data Analysis

Chromatographic deconvolution of GC/Q-TOF data can benefit from accurate mass information to differentiate between co-eluting compounds that have fragment ions of the same nominal mass. MassHunter Qualitative Analysis performs deconvolution in the four following steps:

1. **Noise Analysis** calculates a noise factor for each ion chromatogram.
2. **Perceive Compound** uses the noise analysis results along with retention time to determine a model peak shape for each chromatographic component.
3. **Spectral Deconvolution** takes a least-squares approach to creating a spectrum for each component.
4. **Compound Identification** compares each of these components against an EI spectral library.

One of the benefits of an unbiased approach is that deconvolution occurs without taking targeted ions into consideration. The peak shape information determined during the **Perceive Compound** step allows for the identification of components that have similar peak shapes and apexes in adjacent scans.

Peak deconvolution also helps distinguish different compounds with the same empirical formulas. Figure 1 illustrates three components identified as $C_{29}H_{48}O$, but only the peak in the right panel has an EI spectrum that matches the spectrum for 4,4-dimethyl-8,24-cholesta-dienol. Using the nine step guided workflow (see below), it was determined that 4,4-dimethyl-8,24-cholesta-dienol was the only one of these compounds that met the fold-change and significance criteria set using the Mass Profiler Professional (MPP) multivariate statistical software package.

Once peak deconvolution was completed, compound exchange file format (.CEF) files were generated. MPP was then used to determine those compounds whose concentrations changed significantly due to drug treatment. The MPP statistical evaluation steps were as follows:

1. Define the experiment type, workflow, and organism.
2. Select the data source as MassHunter Qualitative Analysis.
3. Import .CEF files from Qualitative Analysis.

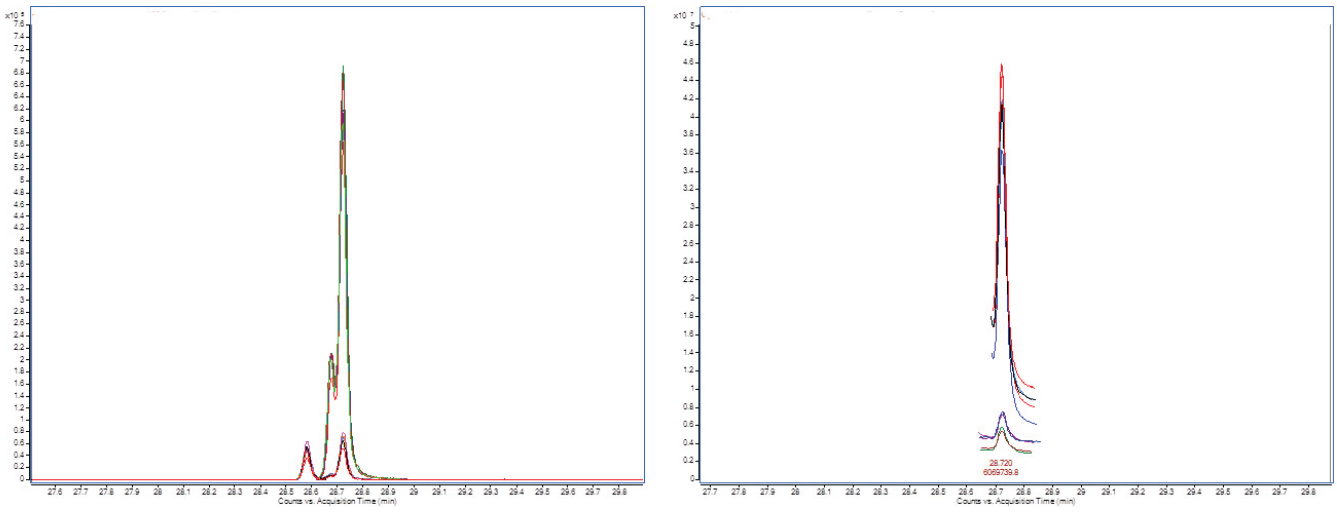

Figure 1. Chromatographic deconvolution helps to resolve co-eluting compounds that have the same empirical formulas. The left panel shows overlaid extracted ion chromatograms (EICs) revealing few co-eluting compounds having the same fragment ions. The right panel shows the Compound Chromatogram for one of these compounds, 4,4-dimethyl-8,24-cholesta-dienol, after MassHunter deconvolution was performed.

4. Set abundance and model ion filters.
5. Define retention and match factor alignment parameters.
6. Select calibration type.
7. Choose baseline correction.
8. Set condition filter flags.
9. Filter based on Volcano Plot. This combines the Student's t-test for significance ($p < 0.05$) with fold change threshold = 2.

Proof of Concept

Previously described antifungal drugs with well understood enzyme targets were utilized to demonstrate the utility of accurate mass high resolution GC/Q-TOF analysis for the metabolic profiling of yeast sterols. Both Terbinafine and Fluconazole are widely used antifungal drugs that target distinct steps in the ergosterol biosynthesis pathway and whose mechanisms of sterol pathway inhibition are well understood. Terbinafine inhibits the ERG1 gene product squalene epoxidase, thus preventing conversion of squalene to the subsequent intermediate of the pathway, squalene epoxide. This results in accumulation of squalene since yeast cells will keep producing it but fail to efficiently convert it to downstream intermediates. Fluconazole inhibits the ERG11 gene product, the cytochrome P450 14α-demethylase, or lanosterol demethylase. In this case, accumulation of lanosterol is expected. Changes in metabolite abundance in drug treated versus nontreated samples were evaluated for the pertinent sterols (Figures 2 and 3). As expected,

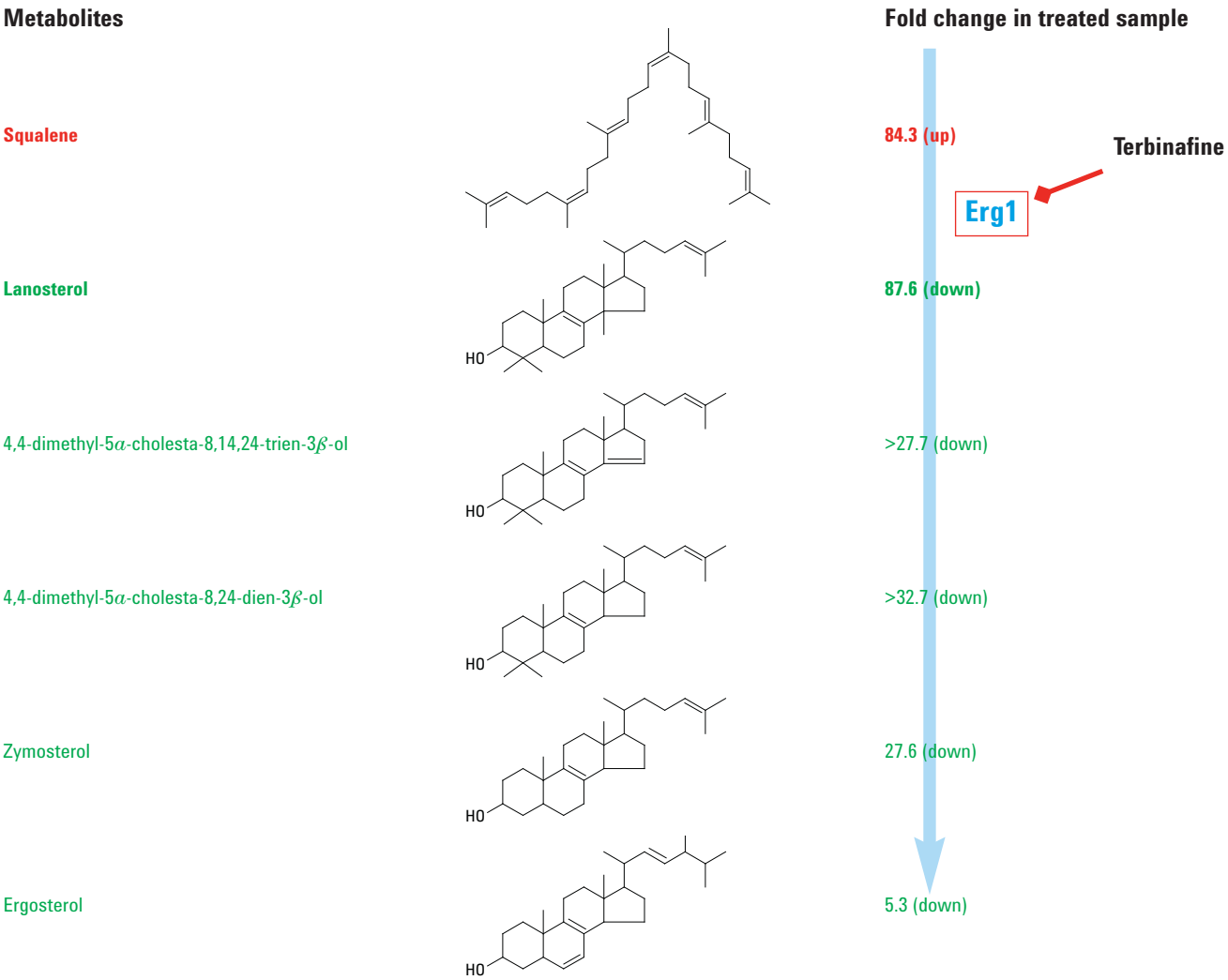

Figure 2. Fold-change in the abundance of various sterol metabolites as a result of Terbinafine treatment, including an accumulation of squalene due to the inhibition of the ERG1 gene product, squalene epoxidase.

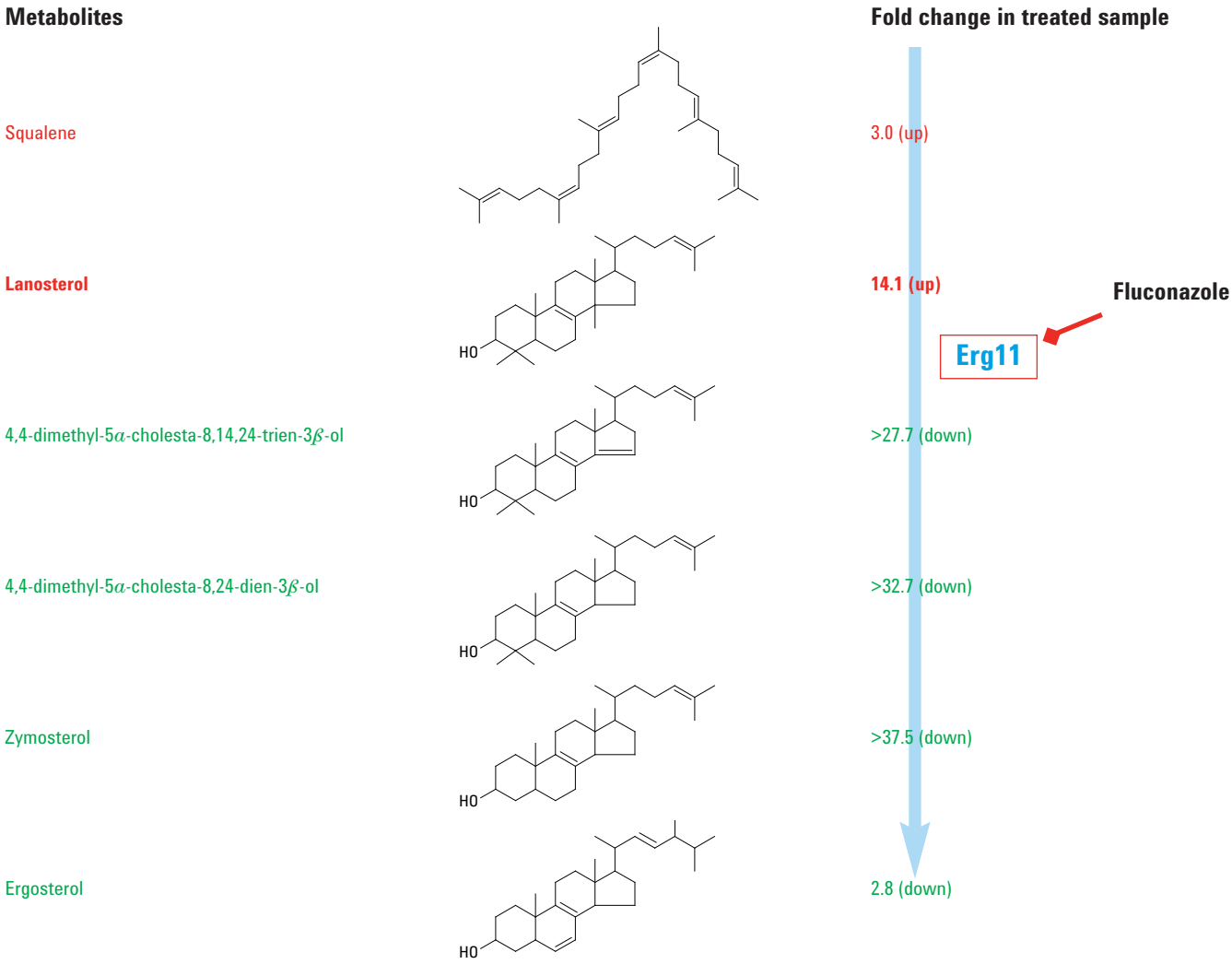

Figure 3. Fold-change in the abundance of various sterol metabolites as a result of Fluconazole treatment, including an accumulation of lanosterol due to the inhibition of the ERG11 gene product, 14 α-demethylase.

accumulation of squalene and lanosterol in Terbinafine- and Fluconazole-treated samples, respectively, was observed. Using MPP, it was revealed that Fluconazole treatment also resulted in accumulation of several 14α-methyl sterols because of the inhibition of 14α-demethylase activity. The results obtained by this untargeted analysis suggested that some of the downstream enzymes in the ergosterol biosynthesis pathway are rather "promiscuous", and thus can use accumulated sterols with an extra methyl group as their substrate. The empirical formulas of accumulated 14α-methyl sterols were determined based on EI fragmentation spectra and accurate mass information (Table 2). Accurate mass determination of fragment ions provided additional confidence in the identity of the compounds.

Table 2. Untargeted Analysis Results for Fluconazole Treatment

Component (*m/z* @ RT)	Compound	Formula	MI of derivatized species	Accurate mass of MI	Mass error (ppm)
469 @ 28.08	14-α-methyl fecosterol	$C_{29}H_{48}O$	484.4095	484.4101	1.24
379 @ 28.26	14-α, 4-α-methyl zymosterol	$C_{29}H_{48}O$	484.4095	484.4107	2.48
467 @ 28.48	(3β,6α)-14-methylergosta-8,24(28)-diene-3,6-diol	$C_{29}H_{48}O_2$	572.4439	572.4450	1.92

RT = retention time
MI = Molecular Ion

Metabolic Profiling for Characterization of New Potential Antifungal Drugs

A few potential inhibitors of the ergosterol biosynthesis pathway, such as Totarol and a new chemical entity (NCE) 1181-0519, were selected based on the results of genetic screening. To better understand the metabolic changes in yeast induced by these potential new drugs, both targeted and untargeted analyses were performed comparing the levels of metabolites accumulated after treatment to those present in the untreated control. In addition, MS/MS experiment results provided structural information essential for confirmation of the identity of accumulated metabolites as the result of the drug treatment. Results clearly identified the NCE 1181-0519 and Totarol targets as Erg25 and Erg26, respectively.

Statistical analysis performed in MPP helped to easily identify differentially regulated components (Figure 4).

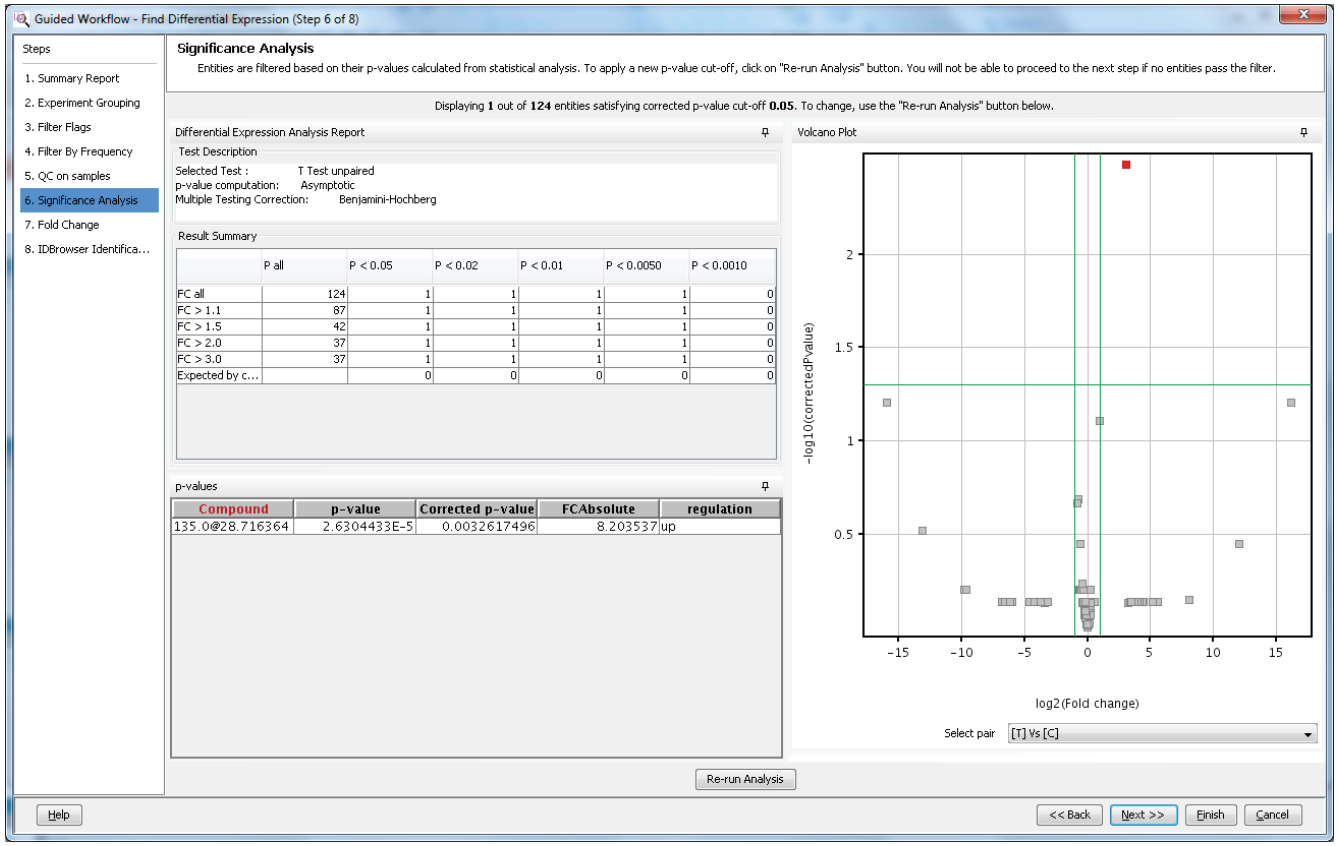

Figure 4. Significance analysis in MPP showing only one compound (4,4-dimethyl-8,24-cholestadienol) with a significant fold-change increase upon treatment with NCE 1181-0519.

In the case of NCE 1181-0519, only 4,4-dimethyl-8,24-cholestadienol meets the significance criteria of $p < 0.05$ for a biologically significant fold-change, suggesting that the NCE specifically inhibits Erg25. Depletion of downstream intermediates also strongly supports this conclusion (Figure 5).

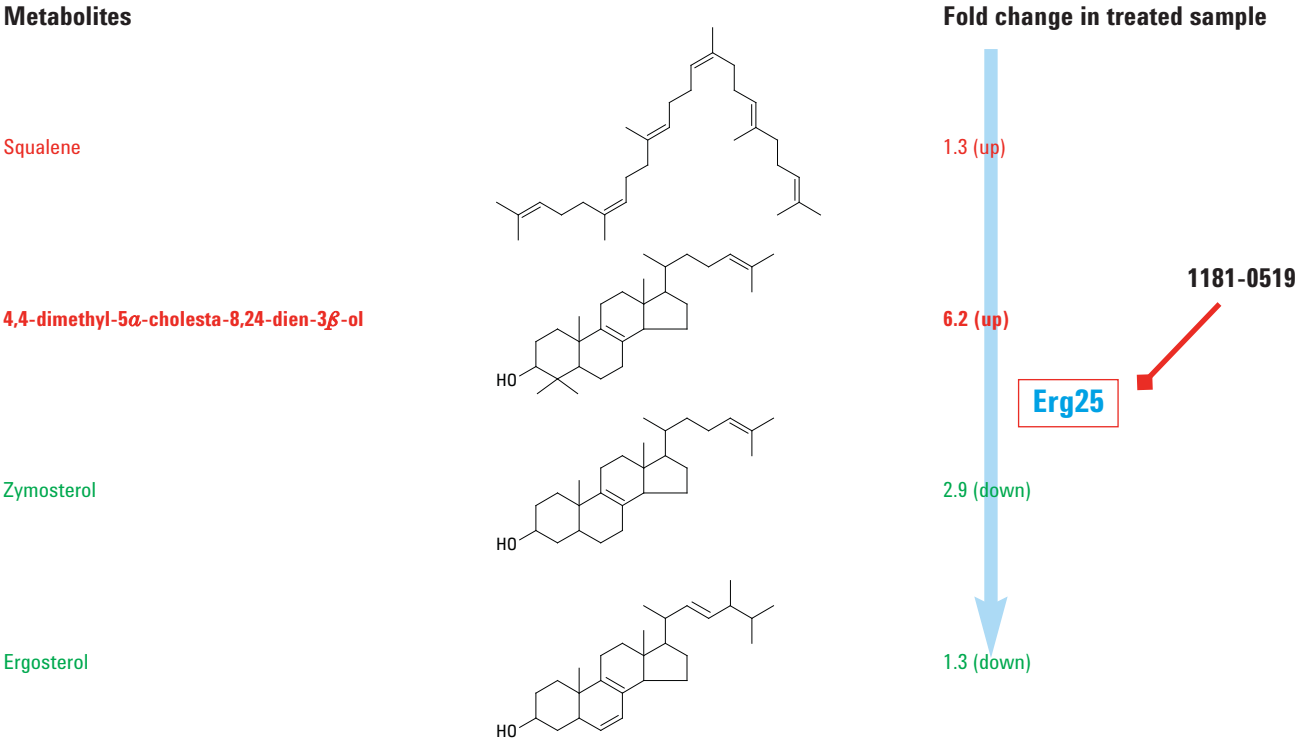

Figure 5. Fold-change in the abundance of various sterol metabolites as a result of Fluconazole treatment, including an accumulation of 4,4-dimethyl-8,24-cholestadienol and down-regulation of the downstream intermediates, confirming Erg25 as the drug target.

Totarol treatment resulted in the accumulation of 4α-carboxy-4β-methyl-5α-cholesta-8,24-dien-3β-ol which is consistent with Erg26 being a specific target of Totarol (Figure 6). Typically this compound is not detected, since it is a biologically unstable intermediate, and hence was not originally targeted in this experiment. Its accumulation upon Totarol treatment was only revealed using an untargeted approach. The structure of this metabolite was confirmed using accurate mass product ion spectra information (Figure 7). Molecular Structure Correlator software was further used to predict the structure of the resulting MS/MS fragments and to generate a compatibility score to evaluate best candidate structures of accumulated compounds (Figure 8).

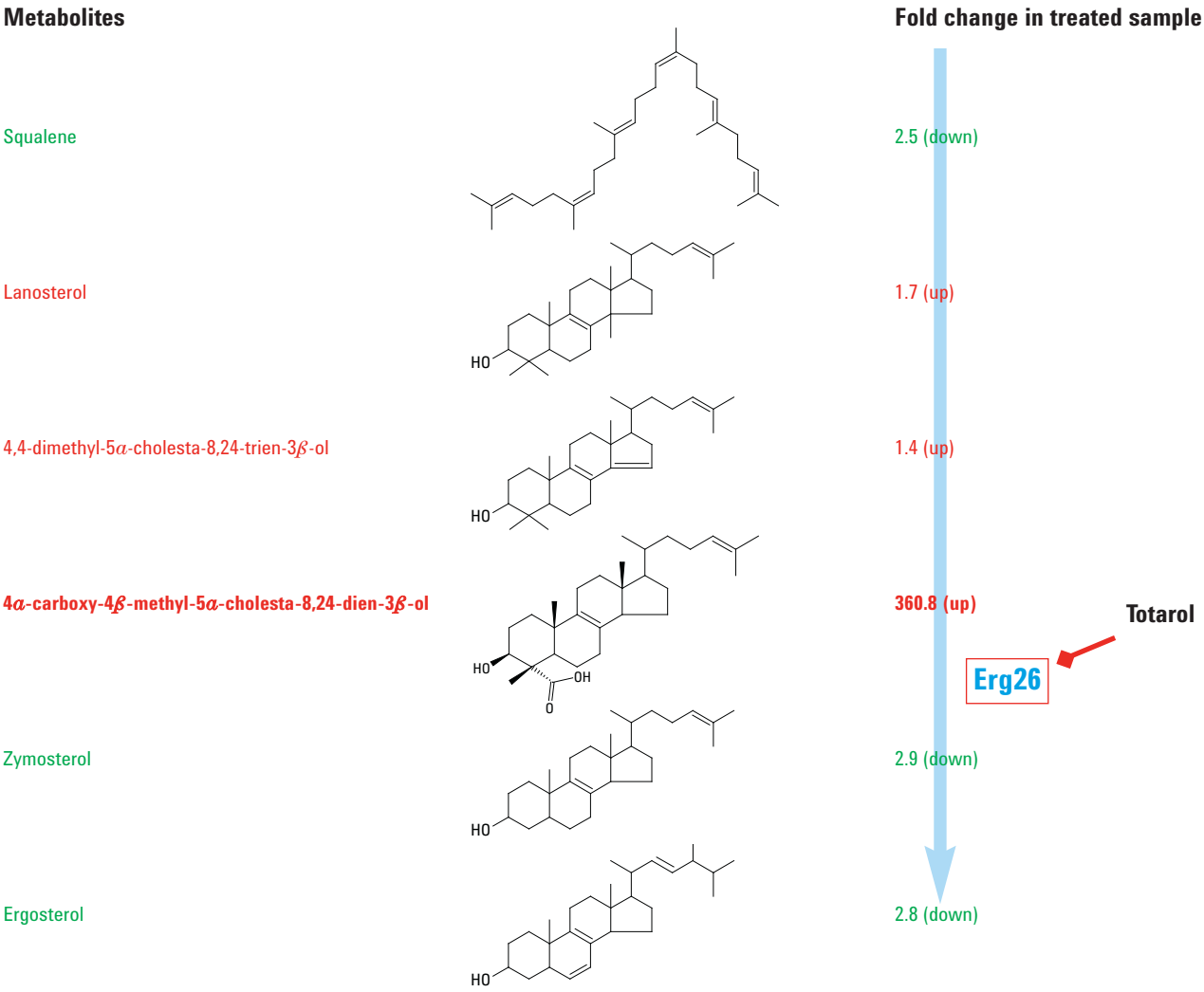

Figure 6. Fold-change in the abundance of 4α-carboxy-4β-methyl-5α-cholesta-8,24-dien-3β-ol as a result of Totarol treatment, consistent with Erg26 being the specific target for Totarol.

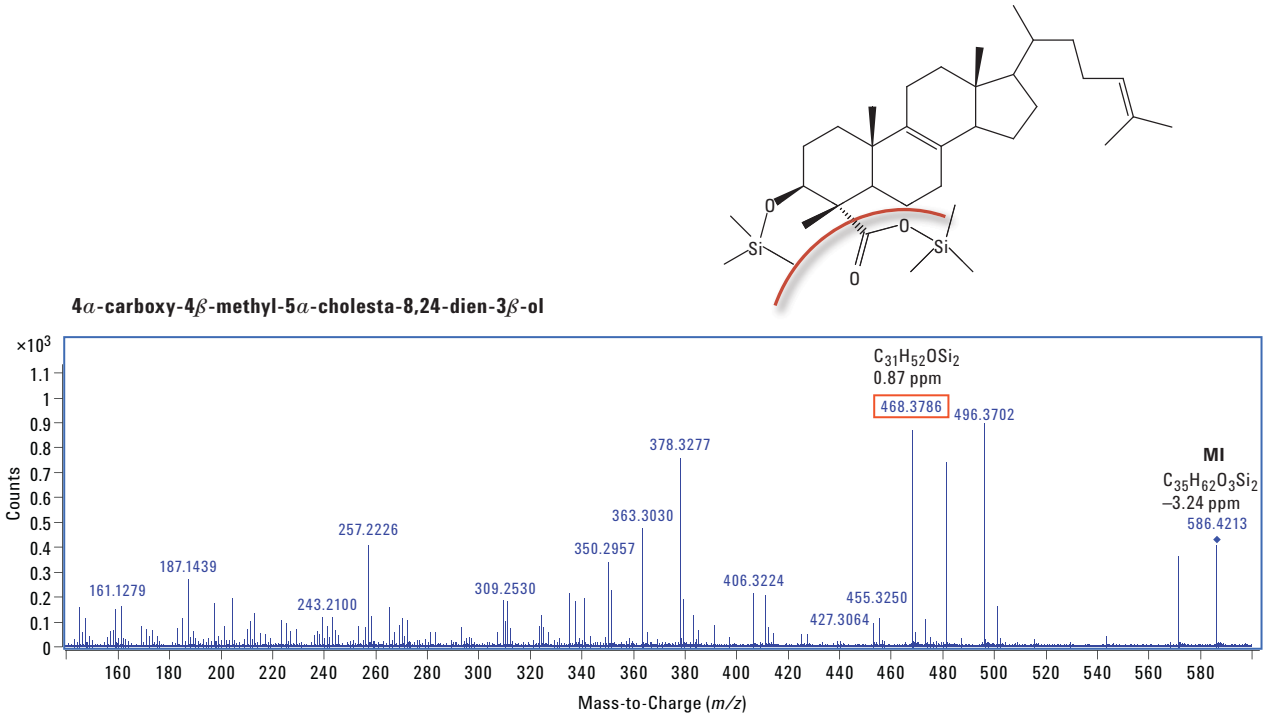

Figure 7. MS/MS product ion spectrum aids the confirmation of the structure of one of the accumulated intermediates identified by the untargeted approach, 4α-carboxy-4β-methyl-5α-cholesta-8,24-dien-3β-ol.

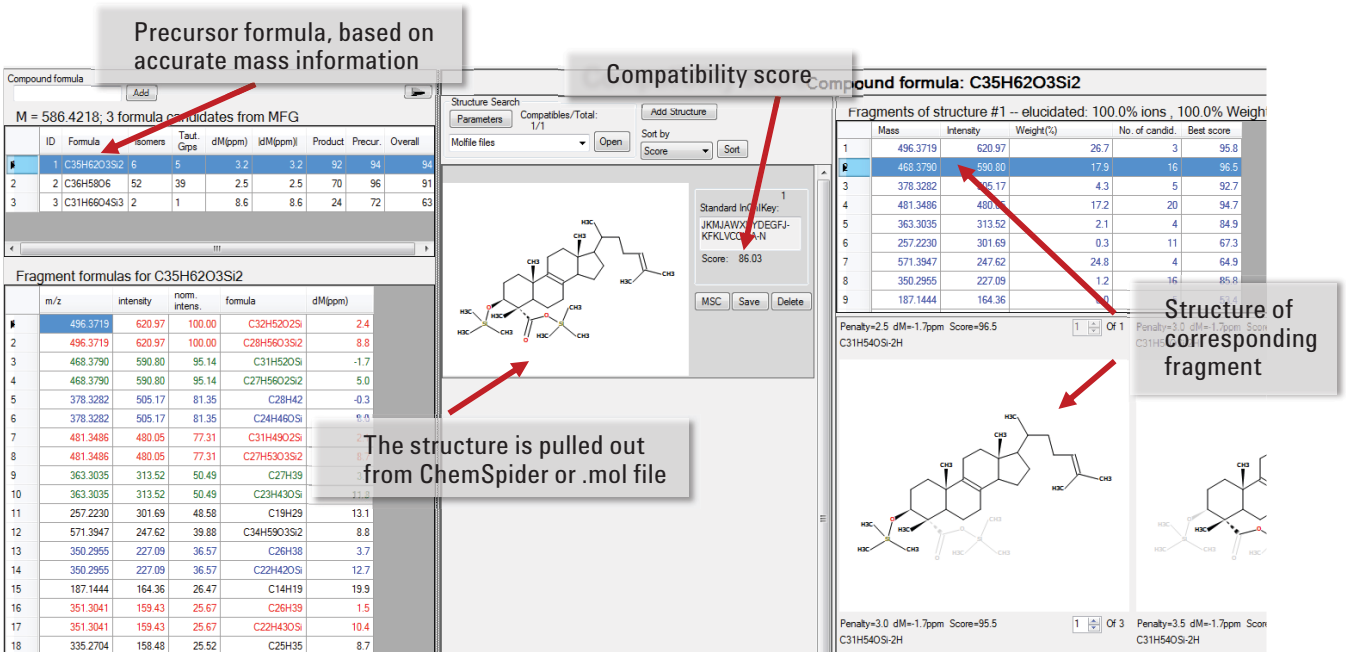

Figure 8. Molecular Structure Correlator assigns formulas to each accurate mass fragment of the MS/MS product ion spectra. The software tool mines the ChemSpider database. It can either evaluate all possible isomers or a specific compound by adding the structure via a MDL Molfile.

The exceptional sensitivity of the 7200 GC/Q-TOF in full acquisition mode helped to identify all necessary metabolites in one experiment, and thus played a crucial role in elucidating mechanisms of poorly characterized antifungal drugs. Figure 9 summarizes the results of the metabolic profiling of the two potential antifungal drugs, indicating genes ERG25 and ERG26 in the ergosterol biosynthesis pathway as the targets for NCE 1181-0519 and Totarol, respectively.

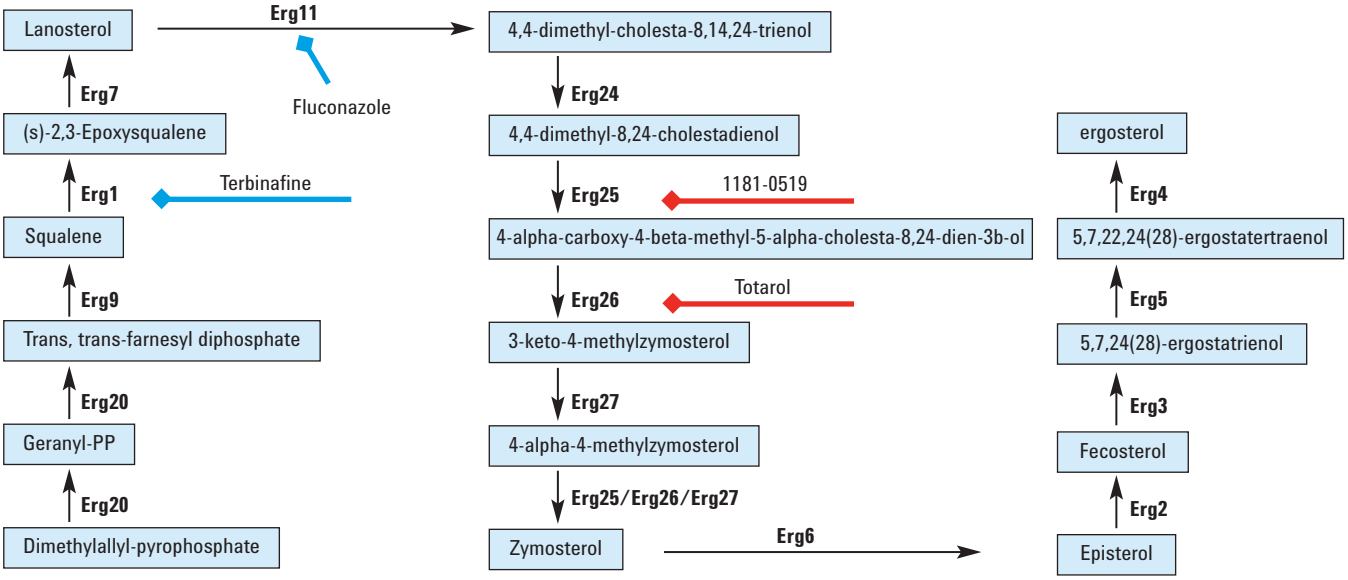

Figure 9. Diagram of the ergosterol biosynthesis pathway, indicating the gene targets (in red) that were determined in this study for NCE1181-0519 and Totarol.

Conclusions

Metabolic profiling of yeast sterols using the 7200 series GC/Q-TOF System is a powerful tool for identifying the enzymatic targets for putative antifungal drugs and can be used in combination with Haploinsufficiency Profiling to elucidate the mechanism of drug inhibition in more detail. The accurate mass information and full spectrum sensitivity of the 7200 GC/Q-TOF enable the reliable confirmation and identification of targets as well as unknown compounds accumulated as a result of the treatment. The dynamic range of up to five orders of magnitude allowed for simultaneous quantification of metabolites across cellular concentrations.

The mechanisms of several potential antifungal therapeutic agents, including NCE1181-0519 and Totarol, were elucidated using this approach. This was done by combination of both targeted and untargeted approaches in which levels of the intermediates were statistically evaluated using Mass Profiler Professional. In addition, the 7200 GC/Q-TOF can be used to confirm or elucidate structures of unknown metabolites using accurate mass MS/MS product ion spectrum capability in conjunction with the Molecular Structure Correlator tool.

References

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