Obovatols, new chitin synthase 2 inhibitors of Saccharomyces cerevisiae from Magnolia obovata

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In the course of the search for inhibitors of ScCHS2 from natural sources, we have isolated a new type of chitin synthase 2 inhibitor, obovatol, which has a biphenol skeleton, from Magnolia obovata. Obovatol inhibited chitin synthase 2 activity of Saccharomyces cerevisiae with an IC_{50} of 38 µM. Its derivative, tetrahydroobovatol, inhibited chitin synthase 2 activity under the same conditions with an IC_{50} of 59 µM. These compounds exhibited no inhibitory activity for ScCHS3, and showed less inhibitory activity for chitin synthase 1 than for chitin synthase 2 (IC_{50} > 1 mM). These results indicated that obovatol and tetrahydroobovatol are specific inhibitors of ScCHS2. They also inhibited CaCHS1, which is structurally and functionally analogous to ScCHS2, with similar IC_{50}s to ScCHS2 (IC_{50} 28 and 51 µM, respectively). The compounds exhibited mixed competitive inhibition with respect to UDP-N-acetyl-D-glucosamine as substrate [inhibition constant (K) 21.8 µM for obovatol and 23.1 µM for tetrahydroobovatol]. Furthermore, they showed antifungal activities against various pathogenic fungi, with a particularly strong inhibitory activity against Cryptococcus neoformans (MIC 7.8 mg/L). The results indicate that obovatol and tetrahydroobovatol can potentially serve as antifungal agents.

Introduction

The need for more effective, novel antifungal agents has been increasing since most of the current antifungal antibiotics have associated problems. Amphotericin B has high toxicity and the azoles are fungistatic.1,2 Therefore, demand for development of new antifungal agents that have few side effects and specific activity against highly pathogenic fungi is greater than ever before. Chitin, the \( \beta \)-1,4-linked homopolymer of N-acetyl-D-glucosamine (GlcNAc), is an important structural component of the cell walls of nearly all zoopathogenic and phytopathogenic fungi, and plays a major role in determination of cell morphology.3 Although the content of chitin in the cell wall varies from species to species, its synthesis constitutes a model for morphogenesis and serves as a potential target in antifungal chemotherapy.4 Chitin is synthesized by chitin synthases 1, 2 and 3 in Saccharomyces cerevisiae.5,6 Chitin synthase 2 is an essential enzyme for primary septum formation and cell division,7,8 whereas chitin synthase 3 is responsible for chitin in the ring at bud emergence and in the lateral cell wall,9,10 and for the formation of glucan–chitin linkages.11 Chitin synthase 1 is a non-essential enzyme that repairs the damaged cell wall on cell division.6,12,13 Like S. cerevisiae, Candida albicans harbours three chitin synthases, 1, 2 and 3, which are analogous to the S. cerevisiae chitin synthases (ScCHS) 2, 1 and 3, respectively.14 Therefore, specific inhibitors of chitin synthases 2 and 3 from S. cerevisiae may be interesting lead compounds for developing effective antifungal drugs.

In the course of our continuous screening programme to find potent chitin synthase 2 inhibitors from wild and medicinal plant extracts, we found that the extract of leaves of Magnolia obovata inhibited chitin synthase 2 activity. The extract of bark of M. obovata has been used as a stomachic herb in Korea, China and Japan for hundreds of years,15 and the leaves have been eaten as a vegetable in Korea and Japan. A number of Magnolia species have been

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extensively examined for compounds with pharmacological, antimicrobial and pesticidal activity. However, this is the first report that the extract of the leaves of *M. obovata* could be used as a chitin synthase 2 inhibitor. Here, we report the isolation of obovatol and the synthesis of tetrahydroobovatol, and their inhibitory activities against chitin synthases of *S. cerevisiae* and *C. albicans* and whole cells of various pathogenic fungi.

**Materials and methods**

**Yeast strains and culture conditions**

The strains used in this study were *S. cerevisiae* YPH499 ((ura3-52 lys2-801amber ade20101chro trp1-Δ63 his-Δ200 leu2-Δ1),\(^\text{17}\) ECY38-38A(pAS6) (MATa chs1-23 chs2::LEU2 call1/csd2 ura3-52 trp1-1 leu2-2 pAS6)\(^\text{18}\) and ECY38-38A(pWJC6) (MATa chs1-23 chs2::LEU2 call1/csd2 ura3-52 trp1-1 leu2-2 pWJC6),\(^\text{18}\) which were used as sources of chitin synthase 1, 2 and 3, respectively. *S. cerevisiae* YPH499, the wild type for all three synthases, was grown in YEPD [1% yeast extract (Difco, Detroit, MI, USA), 2% Bacto peptone, 2% glucose]. *S. cerevisiae* ECY38-38A- (pAS6) and ECY38-38A(pWJC6), which can only over-express chitin synthases 2 and 3, respectively, were grown in YPG [1% yeast extract (Difco), 2% Bacto peptone, 2% galactose] at 30°C. In addition, the chitin synthase-deleted homologous mutant chs2Δchs3Δ (chs2Δ::hisG/chs2Δ::hisG chs3Δ::hisG) was used for *C. albicans* chitin synthase 1 (CaCHS1p) activity.\(^\text{14}\) The strain was grown in Sabouraud dextrose medium at 30°C.

**ScCHS1p, -2p and -3p assays**

The assays of chitin synthases 2 and 3 prepared from recombinant *S. cerevisiae* ECY38-38A(pAS6) and ECY38-38A(pWJC6), respectively, were conducted according to the method of Choi & Cabib.\(^\text{19}\) Cells suspended in 50 mM Tris–HCl (pH 7.5) containing 5 mM magnesium acetate were broken by vortex mixing with glass beads. Cell walls were sedimented at 4000 x g for 5 min and the supernatant fluid was centrifuged at 130 000 x g for 45 min. The membrane pellet was suspended in 50 mM Tris–HCl (pH 7.5) containing 33% glycerol used in the breakage, to a final volume of 1.6 mL/g (wet weight) of cells. Chitin synthase 2 activity was measured by the procedure described previously.\(^\text{19}\) For the proteolytic activation step, reaction mixtures contained 32 mM Tris–HCl (pH 8.0), 1.6 mM cobalt acetate, 1.0 mM UDP-[\(^\text{14}\)C]-N-acetyl-d-glucosamine (400 000 cpm/μmol; NEN, Boston, MA, USA), 2 mL of trypsin at the optimal concentration for activation (2.0 mg/mL), 20 μL of membrane suspensions and 14 μL of sample in a total volume of 46 μL. The mixtures were incubated for 15 min at 30°C. Proteolysis was stopped by adding 2 μL of a soybean trypsin inhibitor solution (4.0 mg/mL) at a concentration twice that of the trypsin solution used, and tubes were placed on ice for 10 min. GlcNAc was added to a final concentration of 32 mM, and incubation at 30°C was carried out for 90 min. For chitin synthase 3 activity,\(^\text{18,19}\) the assay was carried out as for chitin synthase 2, except that 32 mM Tris–HCl (pH 7.5) and 4.3 mM magnesium acetate were used. For chitin synthase 1 activity,\(^\text{19}\) reaction mixtures contained 37 mM Tris–HCl (pH 7.5), 0.12% digitonin, 4.8 mM magnesium acetate, 2 μL of trypsin (1.0 mg/mL), 6 μL of membrane suspension and 14 μL of the test sample in a total volume of 41 μL. After 15 min of incubation at 30°C, 2 μL of trypsin inhibitor (2.0 mg/mL) were added and the tubes were placed on ice. GlcNAc (32 mM) and 1.0 mM UDP-[\(^\text{14}\)C]-N-acetyl-d-glucosamine were added as for the chitin synthase 2 and 3 assays, and the mixtures were incubated for 30 min at 30°C. In all cases, the reaction was stopped by addition of 10% trichloroacetic acid and radioactivity of the insoluble chitin formed was counted after filtration through a glass fibre filter (GF/C; Whatman, Maidstone, UK). The concentration of protein was measured by the method of Lowry et al.\(^\text{20}\) Blank values were measured by addition of 25% aqueous MeOH instead of both enzyme and sample. Percentage inhibition of chitin synthase activity was calculated by subtracting the blank values from both control and test sample values.

\[
\% \text{Inhibition} = \left[ \frac{1 - \frac{\text{Sample (cpm)} - \text{Blank (cpm)}}{\text{Control (cpm)} - \text{Blank (cpm)}} \right] \times 100
\]

The chitin synthase 1, 2 and 3 activities of the enzyme were confirmed by positive control with polyoxin D and nikkomycin Z (Calbiochem, San Diego, CA, USA). Each isolated and control compound was solubilized in 25% MeOH and distilled water to make a stock solution (1 mg/mL), and an aliquot (14 μL) of the stock was used for each reaction to give a final concentration of 280 mg/L. The inhibitory activities were represented as average values in duplicates obtained from two independent experiments.

**CaCHS1p assay**

The preparation of microsome and chitin synthase 1 activity of *C. albicans* was conducted by a method described previously.\(^\text{21}\) For CaCHS1p activity, the assay was carried out similarly to that for ScCHS2p, except that 32 mM Tris–HCl (pH 7.5) and 2 mM cobalt chloride were used. Nikkomycin Z was used as a positive control for CaCHS1p inhibition. Each isolated and control compound was solubilized in 25% dimethyl sulphoxide and distilled water to make a stock solution (1 mg/mL).

**Isolation and purification of obovatol from *M. obovata***

We purified the active component from the extract of *M. obovata* using the inhibitory activity-guided fractionation method. The leaves of *M. obovata* were harvested in...
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Taejon, Korea and the isolation procedure of the active compound was the same as that reported previously.\(^2\) The dried and milled sample (1 kg) was soaked in chloroform–acetone (1:1, v/v, 5 L) at room temperature for 5 days. The extract was concentrated under reduced pressure, and purified by silica gel, C18 column chromatography and preparative thin layer chromatography (TLC). Finally, we purified the active compound by high-performance liquid chromatography (HPLC) (Phenomenex, Ultracarb 10 ODS; 250 × 21.2 mm at 285 nm, flow rate 3.5 mL/min) using a linear gradient rising from 80 to 90% methanol for 50 min. The *t*\(_R\) in HPLC of active compound (120 mg) was 35 min. The mass spectroscopic, infra-red spectroscopic and nuclear magnetic resonance data for the purified compound were in good agreement with the spectral data for obovatol (C\(_{18}\)H\(_{18}\)O\(_{3}\), mol. wt 282 kDa) published previously.\(^2\),\(^3\)

**Synthesis of tetrahydroobovatol**

The isolated obovatol (50 mg) was dissolved in 50 mL of tetrahydrofuran (THF) and added to palladium (Pd)–charcoal (100 mg). The mixture was stirred under a hydrogen atmosphere at room temperature, and allowed to stand overnight. The reaction mixture was then filtered through filter paper (Whatman No. 1). The filtrate was dried, washed twice with MeOH and re-dried *in vacuo* to dryness to give tetrahydroobovatol as red brown oil (45 mg; C\(_{18}\)H\(_{22}\)O\(_{3}\), mol. wt 286 kDa).\(^2\)

The structures of obovatol and tetrahydroobovatol are shown in Figure 1.

**Determination of MICs**

MICs were determined by a two-fold serial agar dilution method.\(^2\) Obovatol and tetrahydroobovatol were dissolved in 25% MeOH, and polyoxin D and nikkomycin Z were dissolved in distilled water. Human pathogenic fungi were grown on Sabouraud agar medium (Difco), and plant pathogenic fungi were grown on potato dextrose agar medium (Difco). *S. cerevisiae* ECY38-38A(pAS6) and ECY38-38A(pWJC6) were grown on YPG agar medium. The inoculum sizes of yeasts and spore-forming fungi were 10\(^3\) cfu/spot and 10\(^5\) spores/spot, respectively. Antifungal activity was observed after 24 h incubation at 30°C for yeasts and 48 h incubation for fungi at 25°C. The MIC was defined as the lowest concentration of antibiotic that completely inhibited the growth of the organism when compared with a control plate containing no antibiotics.

**Results**

**Selection of chitin synthase 2 inhibitors from plants**

One of the plant extracts, extract of *M. obovata*, strongly inhibited ScCHS2p activity (inhibitory activity 75% at a concentration of 280 mg/L) in our assay system and weakly inhibited the growth of *Cryptococcus neoformans* at 500 mg/L in an agar diffusion method (inhibition zone 12 mm). From this result, we selected *M. obovata* to isolate ScCHS2p inhibitors as antifungal agents and subsequently isolated and purified compounds inhibiting chitin synthase 2.

**In vitro inhibitory activities against ScCHS1p, -2p and -3p, and CaCHS1p**

Although several chitin synthase inhibitors have been isolated from microbes and higher plants,\(^2\)\(^6\)–\(^3\)\(^3\) obovatol and tetrahydroobovatol have not yet been reported as chitin synthase inhibitors. Chitin synthase inhibitory activities

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Figure 1. Structures of obovatol and tetrahydroobovatol.
were examined by measurement of the formation of chitin with UDP-[\textsuperscript{14}C]-N-acetyl-D-glucosamine. As shown in Figure 2 and Table 1, obovatol and tetrahydroobovatol strongly inhibited ScCHS2p activity in a dose-dependent manner with IC\textsubscript{50}s of 38 and 59 \textmu M, respectively. The IC\textsubscript{50} of both compounds for chitin synthase 2 represented 3.5 and 2.3 times stronger inhibitory activities, respectively, than that of polyoxin D. To examine whether the observed inhibition was specific for chitin synthase 2, the effects of these compounds on chitin synthases 1 and 3 from \textit{S. cerevisiae} were also determined. Results showed that these compounds had no effects on chitin synthase 3 activity, whereas they exhibited a weak inhibitory activity for chitin synthase 1 (IC\textsubscript{50} > 1 \textmu M). They also inhibited CaCHS1p with an IC\textsubscript{50} of 28 and 51 \textmu M, respectively, but these compounds showed weaker inhibitory activities than that of polyoxin D and nikkomycin Z. Based on these data, we consider that obovatol and tetrahydroobovatol are specific inhibitors for chitin synthase 2 from \textit{S. cerevisiae}. The activities of chitin synthases prepared from the yeast strains and the inhibitory activities of the compound were confirmed with polyoxin D and nikkomycin Z as positive controls, which exhibited chitin synthase 2 and 1 inhibitory activities with an IC\textsubscript{50} of 134.5 and 2 \textmu M in our assay systems, respectively (data not shown).

\textbf{Table 1. Effect of obovatol on chitin synthase isozymes in our assay system}

<table>
<thead>
<tr>
<th>Isozyme\textsuperscript{a}</th>
<th>Strain</th>
<th>obovatol</th>
<th>tetrahydroobovatol</th>
<th>nikkomycin Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>ScCHS total</td>
<td>YPH449</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>ND</td>
</tr>
<tr>
<td>ScCHS2p</td>
<td>ECY38-38A(pAS6)</td>
<td>38</td>
<td>59</td>
<td>355</td>
</tr>
<tr>
<td>ScCHS3p</td>
<td>ECY38-38A(pWJC6)</td>
<td>\textsuperscript{-}\textsuperscript{b}</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>CaCHS1p</td>
<td>\textit{chs2A chs3A}</td>
<td>28</td>
<td>51</td>
<td>15</td>
</tr>
</tbody>
</table>

\textsuperscript{a}pAS6 is a high-copy-number plasmid carrying \textit{CHS2} on a vector containing a \textit{TRP} marker. pWJC6 is a high-copy-number plasmid carrying \textit{CAL1/CSD2} (complete gene) under the control of the \textit{GAL1} promoter.

\textsuperscript{b}No inhibitory activity.

ND, not determined.

**Mode of inhibition of ScCHS2p by obovatols**

The mechanism of inhibition of ScCHS2p by obovatol and tetrahydroobovatol was investigated in a kinetic analysis of the inhibition with a Lineweaver–Burke plot. Double reciprocal plots of the data demonstrated that obovatol and tetrahydroobovatol acted as a mixed competitive inhibitor with respect to UDP-N-acetyl-D-glucosamine substrate (Figure 3). The \textit{K}\textsubscript{i} values of both compounds against UDP-N-acetyl-D-glucosamine were calculated to be 21.8 and 23.1 \textmu M, respectively.

**Determination of MIC**

As shown in Table 2, obovatol and tetrahydroobovatol exhibited low inhibitory activities (MIC 125–500 mg/L) against various plant pathogens including \textit{Rhizoctonia solani}, \textit{Colletotrichum lagenarium}, \textit{Pyricularia oryzae} and \textit{Botrytis cinerea}. In contrast, the compounds showed very
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Potent inhibitory activities (MIC 7.8–31.25 mg/L) against opportunistic human pathogens, including *Coccidioides immitis*, *C. neoformans* and *C. albicans*. The antifungal activities of obovatol against *Candida krusei*, *Microsporum canis* and *Trichophyton mentagrophytes* were very strong (MIC 31.2–62.5 mg/L), whereas tetrahydroobovatol exhibited a weak antifungal activity against these species (MIC 125–250 mg/L). In addition, obovatol and tetrahydroobovatol showed high inhibitory activities (MIC 15.6–62.5 mg/L) against various *S. cerevisiae*.

**Discussion**

We have isolated a neolignan compound, obovatol, from *M. obovata*. The neolignans magnolol, honokiol and obovatol are reported to have various biological activities, including anti-oxidative properties, and to have 5-lipoxygenase inhibitory, ACAT inhibitory, antifungal and antibacterial activities. Magnolol and honokiol in particular were reported to be candidate oral anti-septics, since they demonstrate not only good activity against periodontopathic microorganisms, but also a relatively low toxicity to human gingival tissue. In the present study, obovatol was found to have potent inhibitory activity against ScCHS2p and CaCHS1p. Although the effect of obovatol on CaCHS1p was 1.5 times weaker than its effect on ScCHS2p, obovatol and tetraobovatol clearly inhibited CaCHS1p. In addition, obovatol did not exhibit inhibitory activity against ScCHS3p, whereas it showed a weak inhibitory activity for ScCHS1p (IC50 > 1 mM). This indicates that obovatol and tetraobovatol are selective inhibitors of chitin synthases that belong to the family of ScCHS2p.

Recently, several types of compound affecting the chitin synthesis of *C. albicans* have been discovered. For example, protoberberine and HWY-289 inhibit chitin and sterol biosynthesis of *C. albicans*, and R0-09-3143 inhibits chitin synthase 1 of *C. albicans*. In *C. albicans*, obovatol showed a similar inhibitory activity to that of HWY-289, but a weaker activity than that of R0-09-3143 and nikkomycin Z. However, the inhibitory activity of obovatol on ScCHS2p was stronger than that of HWY-289, polyoxin D and nikkomycin Z. In *S. cerevisiae*, chitin synthases 1 and 3 are more sensitive to nikkomycin derivatives than chitin synthase 2. Nikkomycin showed inhibitory activity specifically for chitin synthase 3 in *S. cerevisiae*. Considering the fact that obovatols have biological activity such as ACAT, other than differential inhibitory activity on ScCHS2p and CaCHS1p, this compound or its derivatives may have multiple target sites. Thus, the antifungal activity of obovatol and its derivatives against various pathogenic fungi might be caused in part by potent inhibitory activities for SCCHS2 and CaCHS1, but it could be due to the inhibition of another cellular target alone or together with the inhibition of chitin synthase. However, such possibilities of the antifungal activity of these compounds still remain to be investigated. In *S. cerevisiae*, obovatol also transiently reduced the number of viable cells of ECY38-38A(pAS6), which can only overexpress chitin synthase 2, although it failed to kill these cells. The failure of obovatol to cause cell death of ECY38-38A(pAS6) is probably due to the low susceptibility of ScCHS2p to obovatol and also to the low solubility of the compound.

In order to obtain some information about structure–activity relationships in the hydroxybiphenyl compounds and structural requirements for more potent inhibitors of ScCHS2p, we synthesized tetrahydroobovatol possessing the methyl instead of methylene of obovatol, and its inhibitory activity was examined together with two neolignans, magnolol and honokiol. Tetrahydroobovatol...
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Table 2. In vitro antifungal activities of obovatol and tetrahydroobovatol against various pathogenic fungi (MIC, mg/L)

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Obovatol</th>
<th>Tetrahydroobovatol</th>
<th>Polyoxin D</th>
<th>Nikkomycin Z</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em> NRRL Y139</td>
<td>31.2</td>
<td>15.6</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> YPH499</td>
<td>31.2</td>
<td>31.2</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> ECY38-38A(pAS6)</td>
<td>31.2</td>
<td>62.5</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> ECY38-38A(pWJC6)</td>
<td>31.2</td>
<td>62.5</td>
<td>&gt;100</td>
<td>31.2</td>
</tr>
<tr>
<td><em>C. albicans</em> ATCC 10231</td>
<td>31.2</td>
<td>31.2</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>C. krusei</em> ATCC 6258</td>
<td>31.2</td>
<td>250</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>C. immitis</em> ATCC 34020</td>
<td>7.8</td>
<td>31.2</td>
<td>100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>C. neoformans</em> ATCC 36556</td>
<td>7.8</td>
<td>7.8</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>M. canis</em> ATCC 11622</td>
<td>31.2</td>
<td>250</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>T. mentagrophytes</em> ATCC 9533</td>
<td>62.5</td>
<td>125</td>
<td>&gt;100</td>
<td>100</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em> ATCC 16424</td>
<td>125</td>
<td>500</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td><em>B. cinerea</em></td>
<td>500</td>
<td>500</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td><em>C. lagenarium</em></td>
<td>125</td>
<td>125</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>P. oryzae</em></td>
<td>500</td>
<td>500</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>R. solani</em></td>
<td>125</td>
<td>125</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

ND, not determined. The experiment was repeated three times with essentially the same results.

showed a weaker inhibitory activity than obovatol against ScCHS2p (Figure 1 and Table 1). Interestingly, magnolol and honokiol exhibited no inhibitory activity for ScCHS2p at a concentration of 2 mM (data not shown). The results allow us to consider the possibility that the specific chitin synthase 2 inhibitory activity of neolignans may be dependent on the position of the hydroxyl group. To provide supporting evidence for such a notion, we carried out enzyme kinetics assays of obovatol and tetrahydroobovatol. The results demonstrated that both compounds inhibited chitin synthase 2 activity by mixed competition with UDP-N-acetyl-D-glucosamine, indicating that chitin synthase 2 is one of the cellular targets for the inhibitory activity of obovatol and tetrahydroobovatol.

Based on our present data, further analyses of structure–activity relationships of these compounds would make it possible to design novel synthetic antifungal agents. In this regard, we consider that obovatol and tetrahydroobovatol may be useful lead compounds for development of new antifungal agents through the control of chitin biosynthesis.

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