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Bisabosquals, Novel Squalene Synthase Inhibitors

I. Taxonomy, Fermentation, Isolation and Biological Activities

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In the course of screening for yeast squalene synthase inhibitors, bisabosqual A was isolated from the culture broth of *Stachybotrys* sp. RF-7260. The related compounds bisabosquals B, C and D were also isolated from *Stachybotrys ruwenzoriensis* RF-6853. Bisabosquals inhibited squalene synthases. IC_{50} values of bisabosqual A against the microsomal squalene synthases from *Saccharomyces cerevisiae*, *Candida albicans*, HepG2 cell and rat liver were 0.43, 0.25, 0.95 and 2.5 μg/ml, respectively. Bisabosqual C exhibited inhibitory activities similar to bisabosqual A. Bisabosqual A showed broad spectrum antifungal activity *in vitro*.

Squalene synthase, the first committed enzymes of ergosterol biosynthesis, condenses two molecules of farnesyl pyrophosphate to give squalene. This enzyme has been purified from bakers yeast and characterized in detail[1]. At present, the genes encoding squalene synthases have been identified in *Saccharomyces cerevisiae*[2], human[3] and *Arabidopsis thaliana*[4]. Disruption of the squalene synthase gene by insertional mutagenesis indicated that yeast squalene synthase gene was a single copy gene that was essential for the growth of yeast[2]. We were interested in this enzyme, which regulates the flux of isoprene intermediates through the sterol pathway at unique branch points of isoprenoid biosynthesis. Squalene synthase inhibitors were expected to show antifungal activity. As a result of large-scale screening of fermentation cultures for yeast squalene synthase inhibitors, we found four new compounds, bisabosquals A, B, C and D from two fungal strains of *Stachybotrys*. In this paper, we describe the taxonomy of producing strains, fermentation, isolation and biological activities of bisabosquals. The physico-chemical properties and structure elucidation of bisabosquals are described in the accompanying paper[5].

Materials and Methods

Microorganisms

The fungal strain RF-7260 producing bisabosqual A was isolated from an unidentified decaying broad-leaved-tree leaf collected on Amami-oshima, Kagoshima Prefecture, Japan. The fungal strain RF-6853 producing bisabosquals B, C and D was isolated from an unidentified decaying broad-leaved-tree leaf collected in Sanda, Hyogo Prefecture, Japan.

Yeast Microsomal Extracts and Squalene Synthase Assay

Crude microsomal membrane fractions from mammalian cells were prepared as described by TAIT[6] and various yeast cells were prepared as described below. Yeast cells harvested and washed in 0.8% NaCl by centrifugation at 7,000×g were disrupted by vigorous mixing in a Vortex mixer with 0.45-mm glass beads in a breakage buffer [50 mM HEPES (pH 7.4), 4 mM $MgCl_2$, 1 mM EDTA (pH 8.0), 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride (PMSF), 2 mM benzamidine, 0.1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), 1 μg/ml leupeptin] on ice. Undisrupted debris was removed from the lysate by centrifugation at 10,000×g at 4°C. The

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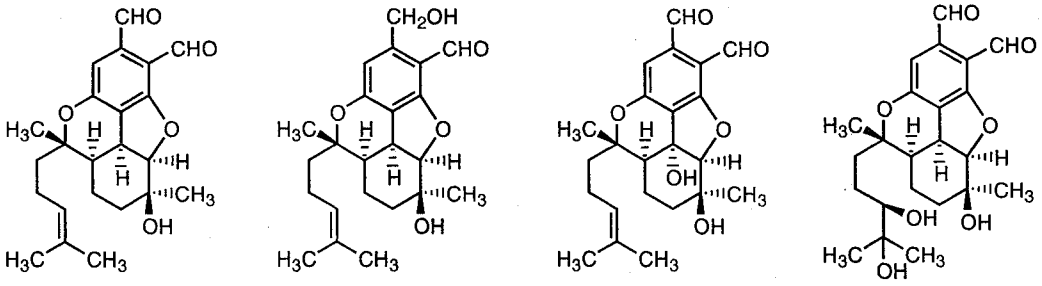
Fig. 1. Structures of bisabosquals A, B, C and D.



Bisabosqual A Bisabosqual B Bisabosqual C Bisabosqual D

supernatant was centrifuged at 4°C for 1 hour at 100,000×g to produce microsomal membranes as a pellet. The pellets were resuspended in a small aliquot of breakage buffer to adjust the protein concentration to 10 mg/ml and stored at −80°C as a stock solution until use. The enzyme stock solution was diluted to 0.1 mg protein/ml with the same buffer just before assay. Squalene synthase activity was assayed by a modification of the method described by TAIT[6]. Test compounds and enzyme solution suspended in 30 μl of 100 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM NADPH, 4 mM KF were mixed and preincubated at room temperature for 10 minutes in 96-well microtiter plates. The reaction was initiated by addition of 20 μl of water containing 1.85 Bq/ml [1(n)-^{3}H]farnesyl pyrophosphate (FPP, 629 GBq/mmol) or 46.2 KBq/ml [1-^{14}C]isopentenyl pyrophosphate (IPP, 2.11 GBq/mmol) purchased from Amersham Life Science (England). The reaction mixture was incubated for 20 minutes, and incubation was terminated by adding 50 μl of 2-PrOH. Five microliters of the reaction mixture was spotted on silicagel plates (Sigma T 6770) and dried completely. The plates were then washed in 200 ml of Tris base containing 1% SDS (pH 12.0) for 10 minutes with gentle shaking to remove unreacted substrates. The remaining radioactivity on the silicagel plate was measured by liquid scintillation method. The inhibition activity of bisabosquals was assessed at 50% inhibition concentration (IC_{50}).

Antifungal Activity

The antifungal activities of bisabosquals against various yeasts and filamentous fungi were measured by the micro-broth dilution method using 96-well microplates. A hundred microliters of yeast nitrogen base medium (YNB, Difco) containing 0.5% of glucose and 1×10^5 cfu/ml of various yeast cells or spores of filamentous fungi was mixed with 5 microliters of a test compound dissolved in DMSO. The concentration of the test compound required for 50% inhibition of cell growth (MIC_{50}) was measured at 595 nm by spectrophotometer (Bio-rad).

Fermentation for Production of Bisabosqual A

A slant culture of the strain RF-7260 was inoculated into a 2-liter Erlenmeyer flask containing 800 ml of medium consisting of glucose 2.0%, malt extract 3.0% and yeast extract 0.5%. The inoculated flask was incubated at 28°C for 5 days on a rotary shaker (180 rpm). The cultured broth was transferred into a 50-liter jar fermenter containing 30 liters of a production medium consisting of glucose 2.0%, glycerin 2.0%, beef extract 0.3%, yeast extract 0.2% and polypropyleneglycol (#2000) 0.04%. The fermentation was carried out at 28°C for 6 days with aeration of 30 liter per minute and agitation at 250 rpm.

Fermentation for Production of Bisabosqual B

A slant culture of the strain RF-6853 was inoculated into 500-ml flasks containing 100 ml of the seed medium consisting of glucose 2.0%, polypeptone 1.0%, beef extract 0.3%, yeast extract 0.2% and NaCl 0.1% (adjusted to pH 7.0 before sterilization). The flask was incubated on a rotary shaker (180 rpm) at 25°C for 5 days. The seed culture (4 ml) was transferred into thirty 500-ml Erlenmeyer flasks each containing 100 ml of a production medium consisting of glucose 2.0%, glycerin 2.0%, beef extract 0.3% and yeast extract 0.2% (adjusted to pH 7.0 before sterilization) and allowed to grow on a rotary shaker at 23°C for 10 days.

Fermentation for Production of Bisabosquals C and D

The slant culture of the strain RF-6853 was inoculated into fifty 50-ml test tubes containing 10 ml of a production medium consisting of sucrose 2.0%, glycerin 2.0%, beef extract 0.3% and yeast extract 0.2% (adjusted to pH 7.0 before sterilization). The culture was incubated on a rotary shaker (300 rpm) at 28°C for 10 days.

Results

Taxonomy of the Producing Strains

The morphological characteristics of the producing strains RF-7260 and RF-6853 are as follows. The colonies of RF-7260 cultured on Potato Carrot Agar at 25°C for 14 days reach 12~15 mm in diameter, with good conidiation, without aerial mycelia and are wet and pale yellow. The conidiophores are macronematous, mononematous, unbranched, 2~3 septate, smooth or rough, up to 160 μm in length, and 4.0~7.0 μm wide. Phialides are formed as a group of 6~8 at the apex of the conidiophore and are clavate, hyaline, smooth, 8.0~10.0×3.0~4.0 μm. The conidia are oblong with obtuse apices, sometime slightly curved, 9.0~11.0×3.0~4.0 μm, one-celled, smooth-walled, dark olive green and slimy in mass.

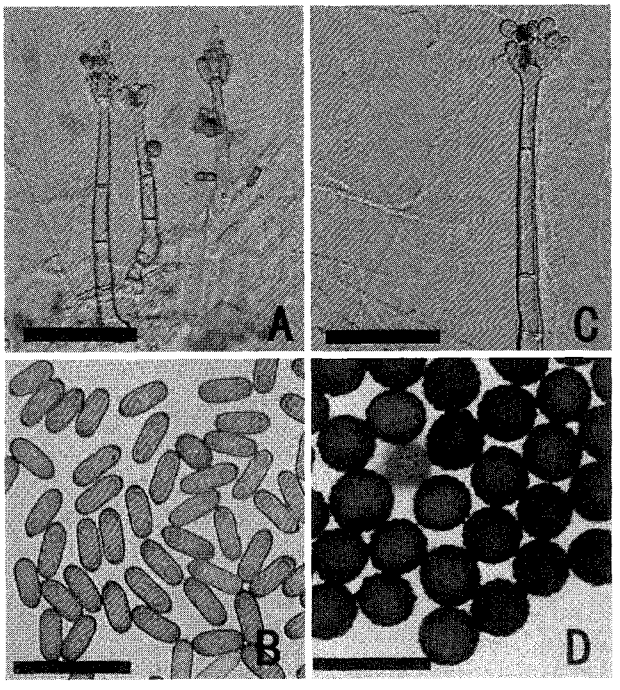
The colonies of RF-6853 cultured on Potato Carrot Agar at 25°C for 14 days reach 30~35 mm in diameter, with poor conidiation only on inocula, with poor aerial mycelia and are wet and hyaline. The conidiophores are macronematous, mononematous, unbranched, 2~5 septate, smooth-walled, up to 160 μm in length, and 5.0~10.0 μm wide. Phialides are formed as a groups of 5~7 and are clavate, hyaline, smooth, 8.0~10.0×3.0~4.0 μm. The conidia are obovate, more or less pointed at the base, one-celled, verrucose, 9.5~12.5×7.0~10.5 μm, dark olive green, and slimy in mass.

The morphological characteristics of the fungal strain RF-7260 are similar to those of *Stachybotrys longispora*[7] and *Stachybotrys queenslandica*[8]. However it is different from *Stachybotrys longispora* in the breadth of the conidia and different from *Stachybotrys queenslandica* in the size of conidiophores. We therefore identified the strain RF-7260 as *Stachybotrys* sp.

According to the morphological characteristics, the fungal strain RF-6853 was identified as *Stachybotrys ruwenzoriensis*[9~11].

The fungal strain RF-7260 and the fungal strain RF-6853 have been deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, under the accession numbers FERM P-14383 and FERM P-14384, respectively.

Fig. 2. Light micrographs of fungal strains RF-7260 and RF-6853.



A. Conidiophores and phialide of RF-7260 bar=50 μm.
B. Conidia of RF-7260 bar=20 μm.
C. Conidiophores and phialide of RF-6853 bar=50 μm.
D. Conidia of RF-6853 bar=20 μm.

Isolation of Bisabosqual A

The isolation procedure of bisabosqual A is shown in Fig. 3. The culture broth (25 liters) was stirred vigorously with EtOAc (20 liters) for 30 minutes and then separated into filtrate and mycelia by filtration. The filtrate was adjusted to pH 4.0 and then centrifuged and the supernatant was washed with water (5 liters). The EtOAc layer was evaporated *in vacuo* to dryness. The residue (50 g) was subjected to silica gel column chromatography (Silica gel 60, 1 kg, E. Merck), and eluted with *n*-hexane - EtOAc (6 : 4). The active fractions were combined and concentrated to dryness. The residue was dissolved in *n*-hexane - EtOAc and crystallized to give pale yellow plates of bisabosqual A (4.13 g).

Isolation of Bisabosqual B

The isolation procedure of bisabosqual B is shown in

Fig. 4. The culture broth (3 liters) was adjusted to pH 2.0 and then separated into filtrate and mycelia by filtration. The mycelia was extracted with 70% aqueous acetone (2 liters) and then filtered. The filtrate was evaporated under reduced pressure to give an aqueous suspension, which was extracted with EtOAc. The EtOAc layer was concentrated to dryness (2.53 g). A part of the residue (300 mg) was subjected to silica gel column chromatography (pre-packed column size B, LiChroprep Si60, E. Merck). The column was eluted with EtOAc - toluene (1 : 3). The active fraction was evaporated to give a white powder of bisabosqual B (146 mg).

Isolation of Bisabosquals C and D

The isolation procedure of bisabosquals C and D is shown in Fig. 5. The culture broth (500 ml) was separated into filtrate and mycelia by filtration. The mycelia were extracted with 70% aqueous acetone (1 liter) and then filtered off. The filtrate was evaporated under reduced pressure to give an aqueous suspension, which was extracted with EtOAc. The extract was evaporated *in vacuo* to dryness (5.97 g). A part of the residue (430 mg) was subjected to silica gel column chromatography (pre-packed column size B, LiChroprep Si60, E. Merck). The column was eluted with EtOAc - toluene (1 : 3). The active fraction was evaporated to give a white powder of bisabosqual C (67 mg).

The filtrate of the culture broth was extracted with EtOAc and the extract was concentrated to dryness (2.0 g). A part of the residue (400 mg) was subjected to silica gel column chromatography (pre-packed column size B, LiChroprep Si60, E. Merck). The column was eluted with

Fig. 3. Isolation procedure of bisabosqual A.

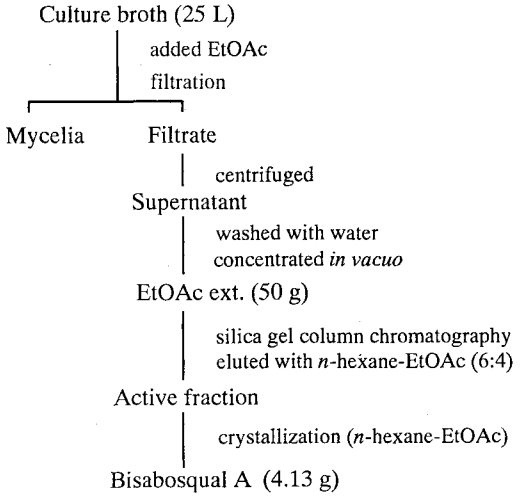


Fig. 4. Isolation procedure of bisabosqual B.

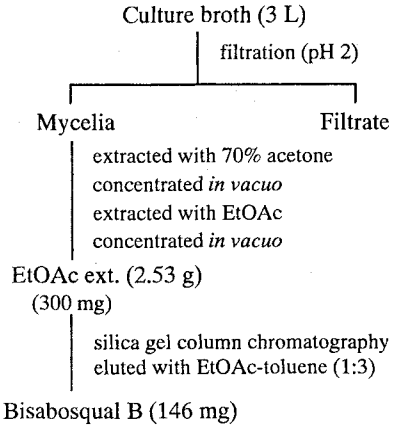
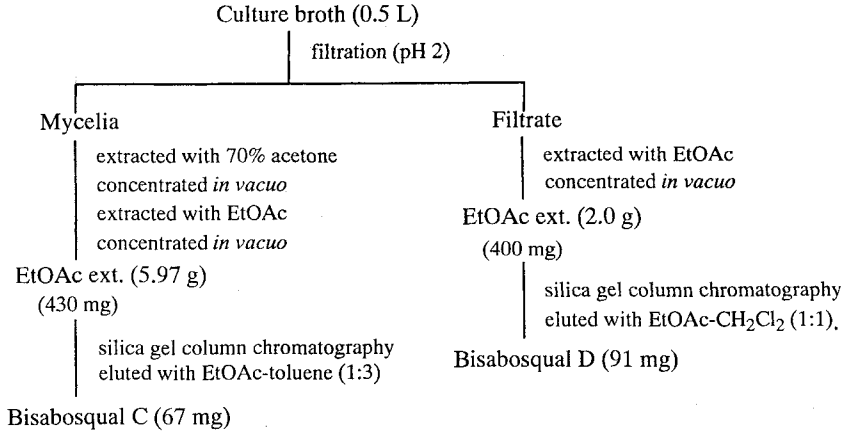


Fig. 5. Isolation procedure of bisabosquals C and D.



EtOAc-CH_2Cl_2 (1 : 1) and the active fraction was concentrated to give a white powder of bisabosqual D (91 mg).

Biological Activities

The inhibitory activities of bisabosquals against the microsomal squalene synthases are shown in Table 1. Bisabosqual A inhibited the microsomal squalene synthases from *Saccharomyces cerevisiae*, *Candida albicans*, HepG2 cell and rat liver with IC_{50} values of 0.43, 0.25, 0.95 and 2.5 μg/ml, respectively. Bisabosqual C also inhibited the microsomal squalene synthases to an extent similar to bisabosqual A.

The antimicrobial activities of bisabosquals were examined by micro-broth dilution method as shown in Table 2. Bisabosqual A showed broad antifungal activities against various yeasts, especially against *Cryptococcus neoformans*. The compound was not active against the dermatological fungi *Trichophyton asteroides* though it showed antifungal activity to pathogens of systemic fungal diseases. Bisabosquals B, C and D did not show any significant antifungal and antibacterial activities.

Table 1. *In vitro* inhibitory activities of bisabosquals A, B, C and D against microsomal squalene synthases.

Compound	IC_{50} (μg/ml)			
	SC	CA	HepG2	Rat liver
Bisabosqual A	0.43	0.25	0.95	2.5
Bisabosqual B	31.5	50	12	>100
Bisabosqual C	1.0	1.0	0.9	5.8
Bisabosqual D	18.5	12	5.1	37

SC: *Saccharomyces cerevisiae*, CA: *Candida albicans*.

Discussion

In this study, bisabosqual A and bisabosquals B, C and D were isolated from the culture broth of *Stachybotrys* sp. RF-7260 and *Stachybotrys ruwenzoriensis* RF-6853, respectively. Bisabosquals have novel *cis*-fused tetracyclic structures with a bisabolane-type sesquiterpene and phenol moieties[5]. Bisabosquals exhibited inhibition of fungal squalene synthase and weak antifungal activity *in vitro*. The inhibitory activities of bisabosqual D were 5 times weaker than those of bisabosqual A. This indicated that unsaturation in the side chain is better than the dihydroxy structure for the display of enzyme inhibitory activity. The 8′-hydroxy group of bisabosqual B diminished the enzyme inhibitory activity, suggesting that the aromatic aldehyde moiety might be important for the activity. The results of enzyme inhibitory activity indicated that the 5-hydroxy group of bisabosqual C was not essential for the inhibitory activity.

Squalestatins have been reported as potent squalene synthase inhibitors from natural sources, and their chemically modified derivatives show inhibitory activity against yeast microsomal squalene synthase and whole cell

Table 2. Antimicrobial activities of bisabosquals A, B, C and D.

Organisms	MIC (μg/ml)			
	Bisabosqual A	Bisabosqual B	Bisabosqual C	Bisabosqual D
Candida albicans IFO 1388	25	>100	>100	>100
Candida kurusei IFO 1063	12.5	>100	>100	>100
Candida glabrata IFO 0005	25	>100	>100	>100
Saccharomyces cerevisiae IFO 1346	25	>100	>100	>100
Cryptococcus neoformans IFO 0410	3.13	>100	>100	>100
Aspergillus fumigatus MA	25	50	>100	>100
Trichophyton asteroides AV	>100	>100	>100	>100

antifungal activity[12]. The squalene synthase inhibitory activities of these derivatives are approximately 500-fold more potent than bisabosqual A. On the other hand, NAKAYAMA *et al.*[13] recently reported that depletion of the squalene synthase gene did not impair growth of *Candida glabrata* in mice. This suggests that bisabosqual A would not be expected to show antifungal activity *in vivo*.

We consider that the bisabosquals could offer a useful lead structure for developing a novel class of therapeutic agents for treatment of hypercholesterolemia, because squalene synthase is also the key enzyme of cholesterol biosynthesis in mammals. However, such development remains to be investigated.

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