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Talosins A and B: New Isoflavonol Glycosides with Potent Antifungal Activity from *Kitasatospora kifunensis* MJM341

I. Taxonomy, Fermentation, Isolation, and Biological Activities

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Abstract In our screening program for new antifungal agents from microbial secondary metabolites, we isolated two new isoflavonol glycosides, genistein 7-α-L-6-deoxy-talopyranoside (talosin A) and genistein 4′,7-di-α-L-6-deoxy-talopyranoside (talosin B), from the culture broth of *Kitasatospora kifunensis* MJM341. The talosins exhibited strong antifungal activity against *Candida albicans*, *Aspergillus niger* and *Cryptococcus neoformans* with minimal inhibitory concentrations (MIC) in the range of 3∼15 μg/ml while genistein and genistein-7-glucopyranoside did not show antifungal activity at 100 μg/ml. These talosins are the first isoflavonol glycosides with a 6-deoxy-talose sugar component and they may be useful as antifungal agents with low toxicity because of no visible cytotoxicity against the human hepatic HepG2 cell.

Keywords *Kitasatospora kifunensis*, antifungal agent, isoflavonol glycoside, genistein, 6-deoxy-talose, talosin A, talosin B

Introduction

Although the number of potent bacterial pathogens has decreased markedly, opportunistic infections with *Candida* and *Aspergillus* have increased gradually. In addition, fungal diseases are increasing in immunocompromised hosts [1]. Such patients have a defect in their self-defense system as a result of AIDS or medication with immunosuppressive drugs, such as anticancer agents. Since the currently employed therapeutics have toxic side effects, the development of novel compounds, together with a search for new molecular targets, is greatly needed [2, 3]. Therefore, we screened microbial natural products from approximately 800 actinomycete strains isolated from soil in South Korea. In our screening program for new and low cytotoxic antifungal agents from microbial secondary metabolites, we isolated two new isoflavonol glycosides with potent antifungal activity, talosins A and B, from the culture broth of *Kitasatospora kifunensis* MJM341. Talosins A and B were determined to be genistein 7-α-L-6-deoxy-talopyranoside and genistein 4′,7-di-α-L-6-deoxy-talopyranoside, respectively (Fig. 1). The producing strain MJM341 was isolated from a soil sample collected from Gyeonggi province, Korea, and identified as *Kitasatospora kifunensis* based on a 16S rDNA sequence. *Kitasatospora kifunensis* is known to produce kifunensin as an immunomodulator [4], but there is no report of antifungal production from this species.

In this study we will demonstrate that talosin A and talosin B show strong antifungal activity, but no

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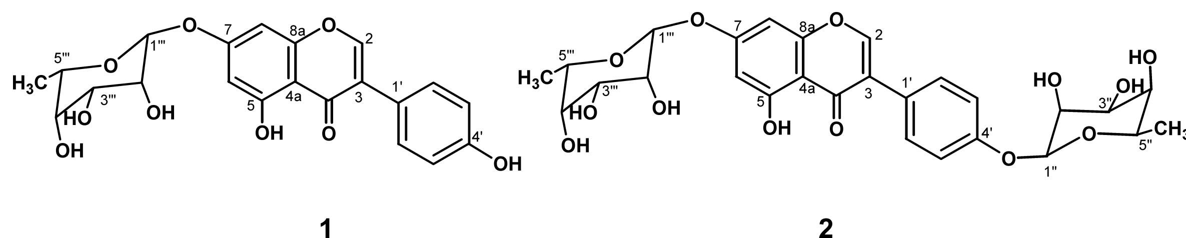


Fig. 1 Chemical structures of talosins A (**1**) and B (**2**).

cytotoxicity against the human hepatic HepG2 cell. In this paper, we describe the taxonomy, fermentation, isolation, and biological properties of the novel isoflavonol glycosides, and the structure determination will be described in a following paper [5].

Materials and Methods

Screening and Taxonomy

Strain MJM341 was isolated from a suspension of a soil sample inoculated onto a starch-casein KNO_3 agar (starch 1%, KNO_3 0.2%, K_2HPO_4 0.2%, NaCl 0.2%, casein 0.03%, $MgSO_4 \cdot 7H_2O$ 0.005%, $CaCO_3$ 0.002%, $FeSO_4 \cdot 7H_2O$ 0.001%, agar 1.8%) plates and incubated at 28°C for 2 weeks [6]. The soil samples were collected from the Gyeonggi province in South Korea. The isolates were individually maintained on starch-casein KNO_3 agar at 4°C and stored as a mixture of hyphae and spores in 20% glycerol at −80°C. Each isolated strain was cultured in a GSS liquid medium (soluble starch 1%, glucose 2%, soybean meal 2.5%, beef extract 0.1%, yeast extract 0.4%, NaCl 0.2%, K_2HPO_4 0.025%, $CaCO_3$ 0.2%, pH 7.2) at 28°C for 7 days. After clarification of the culture broths, the supernatant and methanol extract of mycelia were tested for antifungal activity and cytotoxicity.

The antifungal strains with the biggest inhibition zones and no cytotoxicity were identified by an analysis of the 16S rDNA sequence using primers fD1 and rP2 [7]. The amplified 16S rDNA gene fragment was ligated into a pGEM T-Easy vector and then transformed into *E. coli* DH5α. The plasmids, purified with a Wizard plasmid prep kit (Promega, USA), were sequenced. The sequence was aligned using CLUSTAL W software [8] and a phylogenic tree was constructed using the neighbor-joining method [9]. The morphological characteristics of the spores and mycelia grown on ISP4 for 14 days at 28°C were observed using a scanning electron microscope (S-3500N, Hitachi, Japan). The cultural and physiological characteristics were determined using the same methods of Shirling and Gottlieb [10]. Diaminopimelic acid (DAP) analysis was performed according to the procedure of Lechevalier [11]. The color of colony's surface was specified according to the symbols described in the Methuen Handbook of Colour [12].

Biological Assay

Antifungal activity was primarily evaluated by a paper disc assay against *Candida albicans* and the activity was determined by the diameter of the clear zone. The minimal inhibitory concentration (MIC) values against the pathogenic fungi were determined using the broth dilution method.

Cytotoxicity was tested with the human hepatic HepG2 cell which was maintained in RPMI 1640 medium (Life Technology, USA) containing 10% FBS and 20 μg/ml kanamycin. The CC_{50} values (50% cytotoxic concentration) against the HepG2 cell line was analyzed using an MTT assay after incubation at 37°C for 72 hours with an inoculum size of 10^4 cells/ml. The results were measured at 540 nm with a microplate reader (Bio-Rad Model 3550, USA).

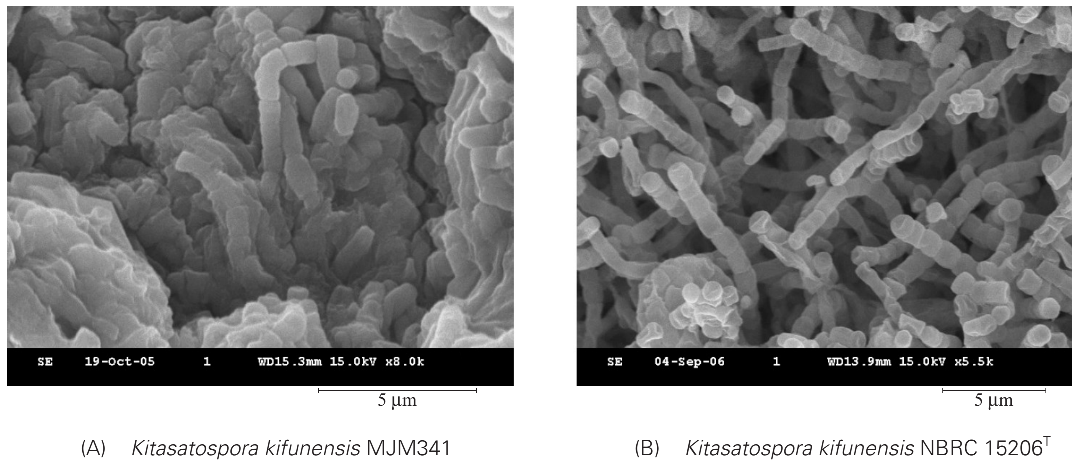
Fermentation

Kitasatospora kifunensis MJM341 was maintained on starch casein KNO_3 agar medium. A slant culture was inoculated into a 500 ml baffled flask containing 70 ml of Bennett's medium. The cultures were incubated on a rotary shaker at 200 rpm at 28°C for 24 hours. Five milliliters of the seed culture were inoculated into a 2-liter baffled flask containing 500 ml of the GSS medium to produce the antifungal compounds. The fermentation was carried out for 6 days under similar conditions.

Results

Taxonomy of the Producing Strain

The MJM341 strain was selected from approximately 800 actinomycetes collected from the Gyeonggi province in



(A) *Kitasatospora kifunensis* MJM341 (B) *Kitasatospora kifunensis* NBRC 15206^T

Fig. 2 Scanning electron micrograph of spore chains of *Kitasatospora kifunensis* MJM341 and *Kitasatospora kifunensis* NBRC 15206^T.

Table 1 Cultural characteristics of strain MJM341

Medium	Growth	Aerial mycelium	Reverse side color	Soluble pigment
Yeast extract - malt extract agar	Good	Yellowish white [3A2]	Yellowish brown [5D4]	None
Oatmeal agar	Good	Gray [4C1]	Brown [6F6]	None
Inorganic salts - starch agar	Good	Gray [4C1]	Pale yellow [3A3]	None
Glycerol asparagine agar	Good	Yellowish white [3A2]	Pale yellow [3A3]	None
Peptone - yeast extract - iron agar	Good	Pale yellow [3A3]	Pale yellow [3A3]	None
Tyrosine agar	Good	Yellowish white [3A2]	Grayish brown [6E3]	None

South Korea. Most of the culture broths with strong antifungal activity showed powerful cytotoxicity, but supernatant of strain MJM341 had no cytotoxic activity. This strain was identified as a *Kitasatospora kifunensis* species by a 16S rDNA sequence analysis with 99% identity. Consequently, we designated our strain as *Kitasatospora kifunensis* MJM341.

For the morphological characteristics of the MJM341 strain, the aerial mycelium of the cultured strain was extensively branched and consisted of straight chains with smooth-surfaced spores. The size of each rod-shaped spore was approximately 0.8×1.6 μm (Fig. 2). The culture characteristics are shown in Table 1. The color of the aerial mycelium was yellowish-white to gray on an ISP media No. 2∼7 and a soluble pigment was not observed. The chemical and physiological properties of strain MJM341 and *Kitasatospora kifunensis* NBRC 15206 are summarized in Table 2. Strain MJM341 was an unusal strain because it had a much higher ratio of LL-DAP to *meso*-DAP and galactose in whole cell hydrolysates, while *Kitasatospora kifunensis* NBRC 15206^T contained LL-DAP, *meso*-DAP and galactose. Strain MJM341 used most of carbohydrates and nitrogens with the exception of sodium-acetate. *Kitasatospora kifunensis* NBRC 15206^T utilized the major part of nitrogens, but it poorly used carbon sources.

Fermentation

Figure 3 shows the time course of talosin A and talosin B productions by the MJM341 strain in a 2-liter baffled flask, along with the cell growth. Maximum growth was observed on day 4 after the inoculation. Production of talosin B began after 24 hours and maximum production was reached on day 7, with a yield of 3.2 mg/liter. Talosin A was produced earlier than talosin B, but its production decreased transiently on day 5.

Purification

The entire culture broth (40 liters) was centrifuged (6,500 rpm, 15 minutes) to separate the mycelium and the supernatant. The supernatant was extracted with butanol (1 : 1, v/v) and the organic layer was evaporated to give an oily material (16.2 g). The oily material was then dissolved

Table 2 Chemical and physiological properties of strain MJM341 and *Kitasatospora kifunensis* NBRC 15206^T

Characteristics	*K. kifunensis* MJM341	*K. kifunensis* NBRC 15206^T
Wall chemotype	LL-DAP, *meso*-DAP, glycine	LL-DAP, *meso*-DAP, glycine
Whole-cell sugar pattern	galactose	galactose
Temperature range for growth (ISP2 No. 2)	15~37°C	15~37°C
Optimum temperature for growth (ISP No. 2)	20~33°C	20~33°C
Melanin pigmenet production		
Peptone - yeast extract - iron agar (ISP No. 6)	−	−
Tyrosine agar (ISP No. 7)	−	+
Degradation activity		
Casein	−	−
Starch	+	−
Gelatin	+	+
Enzyme activity		
Nitrate reduction	+	+
H_2S production	−	−
Lecithinase	+	+
Resistance to antibiotics (μg/ml)		
Neomycin (50)	−	−
Oleandomycin (100)	−	−
Streptomycin (100)	−	−
Tobramycin (50)	−	−
Growth in the presence of chemical inhibitors (% w/v)		
Crystal violet (0.0001)	+	+
Sodium azide (0.02)	+	−
Sodium chloride (4)	−	−
Antimicrobial activity		
Aspergillus niger	+	+
Bacillus subtilis	−	+
Candida albicans	+	+
Escherichia coli	−	−
Use of carbon sources (1.0% w/v)		
D-Glucose	+	+
D-Xylose	+	+
D-Fructose	+	±
D-Galactose	+	±
D-Arabinose	+	±
D-Mannose	+	−
L-Rhamnose	+	±
Na-acetate	−	−
Sucrose	+	±
Use of nitrogen sources (0.1% w/v)		
L-Asparagine	+	+
L-Methionine	+	+
L-Histidine	+	+
L-Valine	+	+
L-Threonine	+	+
L-Phenylalanine	+	+

+; positive, ±; slightly positive, −; negative

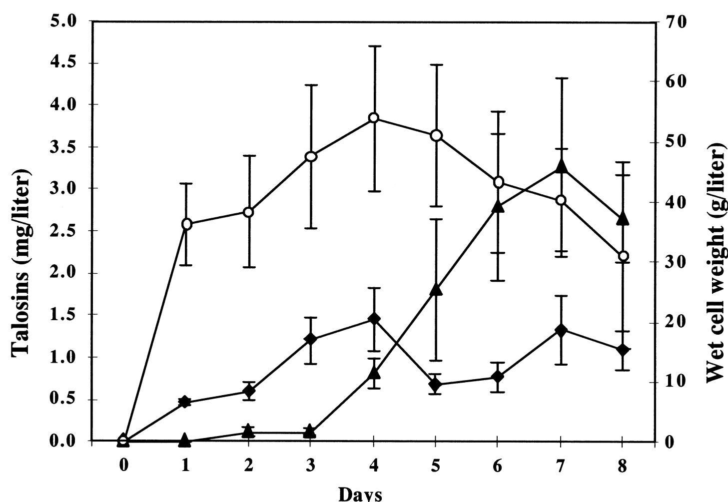


Fig. 3 Fermentation profiles for the production of talosins A and B.
◆: Talosin A; ▲: talosin B; ○: wet cell weight.

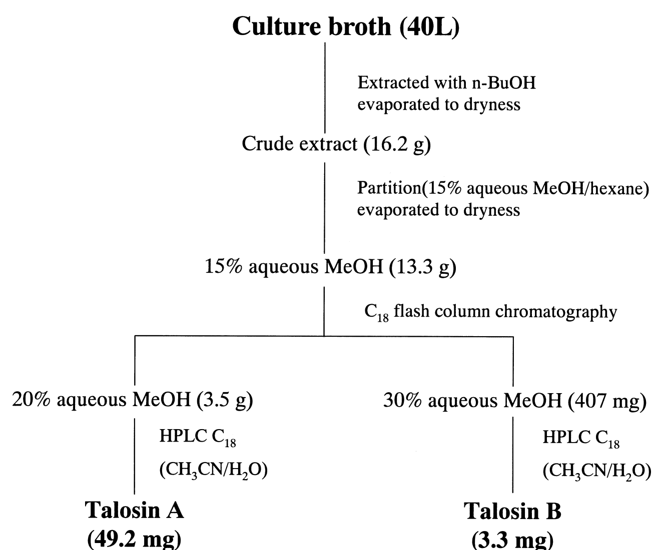


Fig. 4 Purification of talosins A and B from *Kitasatospora kifunensis* MJM341.

in 15% aqueous methanol and defatted by partitioning with *n*-hexane to give a solid extract. The biologically active 15% aqueous methanol layer (13.3 g) was separated using C_{18} reversed-phase vacuum flash column chromatography with sequential mixtures of MeOH and water as eluents (elution order: 50, 30, 20, 10% aqueous MeOH and 100% MeOH). The fraction (3.5 g) eluted with 20% aqueous MeOH that showed mild antifungal activity was separated by C_{18} reversed-phase HPLC (semi-preparative Symmetry RP-18 column, Waters, USA, solvent: 72% aqueous acetonitrile) to yield the active compound talosin A (49.2 mg). The fraction (407 mg) eluted with 30% aqueous MeOH that showed the highest antifungal activity was separated under similar conditions with 72% aqueous acetonitrile to yield the active compound talosin B (3.3 mg) (Fig. 4).

Antifungal Activity

The antifungal activities of talosin A and talosin B were determined as MIC against *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus niger*, which cause systemic mycosis. Talosin B was more potent than talosin A; the MIC values were 3～15 μg/ml, so the talosins are slightly weaker than amphotericin B in regards to antifungal activity [13]. Both of the talosins did not inhibit *Bacillus subtilis* and *Serratia marcescens* (data not shown)

Table 3 Antifungal activity (MIC) of talosins A and B

Test strains	Genistein (μg/ml)	Genistin (μg/ml)	Talosin A (μg/ml)	Talosin B (μg/ml)	Amphotericin B (μg/ml)
Candida albicans B 02630	>100	>100	15	7	0.1–1
Cryptococcus neoformans B 42419	>100	>100	7	3	0.1–1
Aspergillus niger ATCC 6275	>100	>100	6	3	1–2
Trichophyton mentagrophytes ATCC 9129	>100	>100	>100	>100	—

and the dermatomycosis pathogen *Trichophyton mentagrophytes*. Genistein without sugar and genistin (genistein 7-glucopyranoside) did not show antifungal activity at 100 μg/ml (Table 3).

Cytotoxic Activity

The cytotoxic acticities of talosins A and B were determined against the human hepatic HepG2 cell. The CC_{50} values were 100 μg/ml and the MCD (minimal cytotoxic density) was 50 μg/ml (Table 4). Thus, the two talosins did not have significant cytotoxicity.

Table 4 Cytotoxic activity of talosins A and B

	Amphotericin B (μg/ml)	Talosin A (μg/ml)	Talosin B (μg/ml)
MCD (minimal cytotoxic density)	1.20	50	50
CC_{50} (50% cytotoxic concentration)	1.56	>100	>100

Discussion

There is an increasing need for safe and effective antifungal agents because systemic mycosis has been a major cause of deaths in immunosuppressed patients in recent years [1]. New approaches for the treatment of invasive fungal infections are necessary because the incidence of infections has risen steadily since the 1970s [14]. Azole and amphotericin B are currently the most widely used antifungal agents, but these show serious cytotoxicity and side effects [15].

In the course of screening for new and safe antifungal agents, we discovered two new compounds; talosin A and talosin B. Based on physicochemical data, GC analysis of the sugar [16] and 2D-NMR spectra, these new compounds have novel structures. Talosins A and B are isoflavonol glycosides that consisted of genistein and 6-deoxy-talose. Talosin A is substituted by only one 6-deoxy-talose at the C-7 hydroxyl group and talosin B is substituted by two 6-deoxy-taloses at the C-4′ and C-7 hydroxyl groups of the aglycone. Thus, the structures of talosins A and B have been determined as genistein 7-α-L-6-deoxy-talopyranoside and genistein 4′,7-di-α-L-6-deoxy-talopyranoside, respectively (Fig. 1).

The stereochemistry of 6-deoxy-talose is different with regards to rhamnose at the C-4 position. It has been reported that *Aeromonas hydrophila*, *Burkholderia caribensis* and *Escherichia coli* contain 6-deoxy-talose as a constituent of O polysaccharide [17～19], and *Actinomyces bovis* has 6-deoxy-talose as a cell wall component [20]. However, talosins A and B are the first bioactive isoflavonols with 6-deoxy-talose as a sugar component.

Umezawa's group isolated genistein 4′,7-di-rhamnopyranoside and genistein 7-rhamnopyranoside, which were stereochemically different from talosins A and B, from *Streptomyces xanthophaeus* in 1979 [21]. They reported that these compounds showed no antifungal activities at 100 μg/ml, in contrast to our compounds [22]. Furthermore, genistein itself and genistin (genistein 7-glucopyranoside) did not inhibit yeasts and fungi at 100 μg/ml. Therefore, we propose that 6-deoxy-talose plays an important role in the antifungal effects.

Kitasatospora kifunensis MJM341 did not produce talosin A and talosin B in GSS medium without soybean meal, and we could not isolate a biosynthetic gene cluster of genistein from the *Kitasatospora kifunensis* MJM341 genome. Accordingly, it is suggested that strain MJM341 combined its own 6-deoxy-talose to the genistein of the soybean meal by glycosyltransferase. We are now attempting to obtain the gene cluster for the nucleotide diphosphate (NDP)-6-deoxy-talose biosynthesis for industrial applications [23].

Talosin B inhibits *Cryptococcus neoformans* approximately 3～30 more weakly than amphotericin B [13], but does not exhibit significant cytotoxicity (Table 4). Therefore, the side effects of the talosins may be remarkably low and they may have potential for therapeutic

applications. Pharmacokinetic tests may be necessary for the development of antifungal therapy products after obtaining large amounts of the talosins.

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