Cell Wall Active Antifungal Compounds Produced by the Marine Fungus *Hypoxylon oceanicum* LL-15G256

I. Taxonomy and Fermentation

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The cell wall targeted antifungal activity of *Hypoxylon oceanicum* LL-15G256 extracts resulted from the production of novel lipodepsipeptides and previously reported macrocyclic polylactones. In an optimized medium, titers of the lipodepsipeptide and the polylactones reached approximately 200~400 mg/liter and 25~50 mg/liter, respectively. The optimum fermentation temperature for production of 15G256γ was 28°C. Seawater appeared to have an inhibitory effect on metabolite accumulation at lower fermentation temperatures.

As part of our continuing efforts to discover new naturally occurring agrochemicals, fungal extracts were screened for cell wall antifungal activity using the *os-1* assay.[1] Structure elucidation of the bioactive components from fermentations of the unusual marine fungus *Hypoxylon oceanicum* LL-15G256 led to the identification of the polylactones α and β and the lipodepsipeptide γ.[2] *H. oceanicum* has not been reported to produce secondary metabolites; however, compound classes isolated from other *Hypoxylon* species include the cytochalasins,[3] butyrolactones,[4,5] coumarins,[6] diterpenes,[7] naphthalenones[7] and naphthoquinones,[8] none of which are reported to inhibit fungal cell wall formation. Microbial production of cell wall active antifungal agents such as cystargin,[9] echinocandin,[10] polyoxins[11] and nikkomycin[12] has been limited to terrestrial fungi and bacteria. We report herein on the taxonomy and fermentation of LL-15G256 and on the production of its antifungal metabolites. Structure elucidation and details of the bioactivities of these compounds are described in the accompanying papers.[2,13]

Materials and Methods

LL-15G256, a strain of the fungus *Hypoxylon oceanicum*, was isolated from mangrove wood (*Kandelia candel* (L.) Druce) in the intertidal zone of the subtropical mangrove in Shenzen, China.

Fermentation

For preparation of a seed culture, 1 ml of a cryopreserved culture was added to 50 ml potato dextrose broth in a 250 ml Erlenmeyer flask and incubated at 22°C, 200 rpm, 2″ throw for 3 days. Two and one-half ml of seed culture were transferred to 50 ml of production medium in a 250 ml Erlenmeyer flask and incubated as above for 7 days, unless otherwise indicated. The production medium consisted of Sabouraud Maltose broth (Difco) or GSP broth (10 g/liter glycerol, 5 g/liter soy peptone, 1 g/liter $CaCO_3$). Temperature and salinity studies were performed with GSP medium prepared in 90%, 20% or 0% seawater (Carolina Biological Supply Company, Burlington, N.C.).

For large scale isolation of the polylactones and lipodepsipeptides, cultures were grown in 2.8 liter

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Fernbach flasks (1 liter of production medium), or in 30 liter or 300 liter fermentors. Fernbach flasks were inoculated with a 5% volume of seed culture and incubated as described above. Fermentors were inoculated with a 10% volume of seed culture and incubated at 22°C, with an aeration rate of 1 liter air per liter of production medium. Thirty liter fermentors were stirred at 500 rpm, while 300 liter fermentors were stirred at 250 rpm. Fermentation broths were harvested after 5~7 days.

Cell growth was estimated by determining the wet weight of a cell pellet from 10 ml of fermentation broth obtained by centrifugation ($3000 \times g$, 10 minutes). Medium pH was measured using a Corning pH meter, model 125.

Extraction and Quantitation of α, β and γ Metabolites

Fermentation broths were adjusted to ~pH 5 with dilute HCl and extracted by shaking with an equal volume of ethyl acetate. Broth/ethyl acetate emulsions were partitioned by centrifugation ($3000 \times g$, 10 minutes), and the ethyl acetate fraction was removed. The solvent was evaporated, and the residue was resolubilized as a 10-fold concentrate in methanol.

Titers of α, β and γ were determined by reverse phase HPLC using a Hewlett Packard model 1090M liquid chromatograph with photodiode array detection and a VYDAC PROTEIN and PEPTIDE C_{18} reverse phase column. After injection of the extract, the column was eluted with 70% methanol/30% water in 0.1% TFA at 1 ml/minute. The eluate was monitored at 262 nm. Peak identity was accomplished by comparison of retention time and UV chromophore with those of α, β and γ standards. Metabolites were quantitated by comparison of peak areas to those of standards. See the accompanying paper for further details.[2]

Determination of Cell Wall Acting Antifungal Activity

Cell wall antifungal activity of whole broths or extracts was determined using the *os-1* screen.[1] Briefly, test samples were spotted onto an *os-1* assay plate, and the plate was incubated at 37°C. After 36 hours, opaque zones of growth inhibition were examined microscopically for protoplast formation (200X magnification, Nikon Diaphot inverted phase contrast microscope).

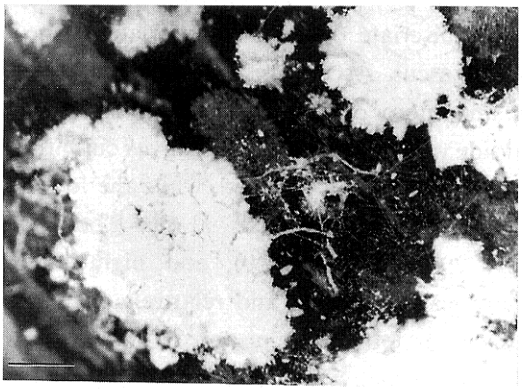
Results and Discussion

Taxonomic Identification

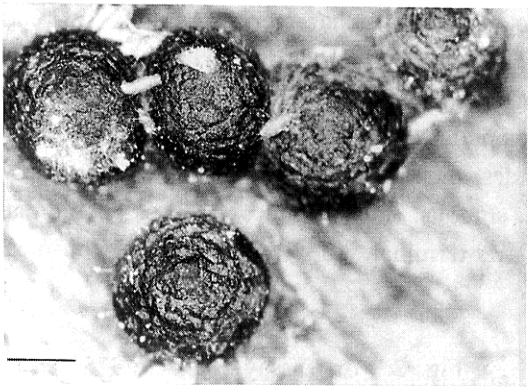
Culture LL-15G256 has been taxonomically identified as a strain of *Hypoxylon oceanicum* based on the lack of a well developed stroma, ascomata arrayed singly or in groups, ascospore measurements and its isolation from a marine subtropical habitat.[14] Colonies growing on oatmeal agar cover the medium surface in a Petri dish in 4 weeks. Growth is at first white, velvety, appressed, irregularly zonate, with dense, entire margins, then darkening on the zonation lines. The reverse side of the culture dish is uncolored.

Fig. 1.

(a) Hyaline colonies of the anamorphic stage of *H. oceanicum* on wood.



(b) Mature ascomata, solitary, black, leathery to carbonaceous on mangrove wood.



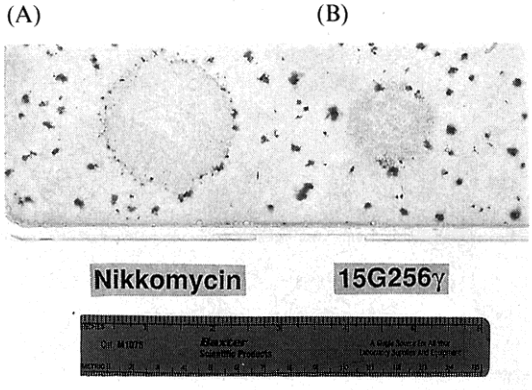
(c) Ascomata sectioned to show the thick peridial wall (arrowed P) and the gelatinous asci (arrowed).

On decorticated wood, ascomata (0.4~0.8 mm in diameter) are superficial, occasionally embedded at the base and pulvinate to hemispherical in shape. Ascomata are single or several coalesced, linear to suborbicular, and leathery in fresh material. When young, ascomata are covered with a whitish, hyphal layer bearing the anamorph stage (Figure 1a). At maturity, ascomata are thick-walled, black and carbonaceous (Figures 1b, 1c). Perithecial projections are generally inconspicuous and subglobose (0.4~0.6 mm in diameter). Asci are 8-spored, 177~219 μm, spore part 112~140 μm, stipe 37~79 μm; the apical apparatus is dark blue in Melzer's reagent, tapering cylindrical with a distinct apical rim (4.7~) 5.6~6.6×4.2~4.7 μm. Ascospores are uniseriate to obliquely uniseriate or partially biseriate at the upper end of the ascus. Ascospores are grey-olive to opaque brown and more or less inequilaterally ellipsoid; the ventral side varies in the degree of convex curvature, with the upper end broadly rounded, while the lower end is slightly pointed, (17.9~) 18.8~21.6 (~22.6)×8.5~9.9 μm (av. 20.7×9.3 μm, n/20), and bigluttulate. The ascospore wall is smooth and relatively thick, without appendages or loosening epispore. The ascospore germination slit, usually clearly seen on the dorsal side, is straight and conspicuous and is 1/2~3/4 total length of ascospore. Paraphyses are abundant, thread-like and septate.

The anamorph is characterized as follows. Conidium-bearing regions on the upper surfaces of stromata and on surfaces of colonies occur in small tufts independent of stromata. Conidiophores are in upright dense palisades, dichotomously branched several times from bases, sinuous, smooth and olivaceous. Conidiogenous cells are terminal, 10~15×2.5~3 μm, conspicuously geniculate, smooth-walled, bearing faint conidial secession scars on the twisted points. Conidia are produced holoblastically in sympodial sequence. Conidia are hyaline, smooth, obovate (4) 4.5~5.5 (~6)×2~3 μm, with flattened bases, indicating former points of attachment to the conidiogenous cell.[15] The anamorph can be referred to the mitosporic genera *Nodulisporium* (Preuss) and *Geniculosporium* (Chesters & Greenh). The anamorph stage has been observed in nature.[15]

Fig. 2. *os-1* assay of LL-15G256.

(A) Nikkomycin (50 μg). (B) 15G256γ (10 μg).



Biological Activity

Fermentation broths and extracts from LL-15G256 produced zones on *os-1* assay plates indistinguishable from those produced by the control compound nikkomycin or the purified antibiotics 15G256 α, β or γ (Figure 2). Microscopic examination of these zones revealed the extensive formation of protoplasts[13], suggesting the presence of compounds inhibitory to cell wall formation in *Neurospora crassa*.

Production of Metabolites α, β and γ

os-1 bioactivity of LL-15G256 resulted primarily from the macrocyclic polylactones α and β and the lipodepsipeptide γ (Figure 3), as described in the accompanying

Fig. 3. Structures of the macrocyclic polylactones α and β (A), and the lipodepsipeptide γ (B).

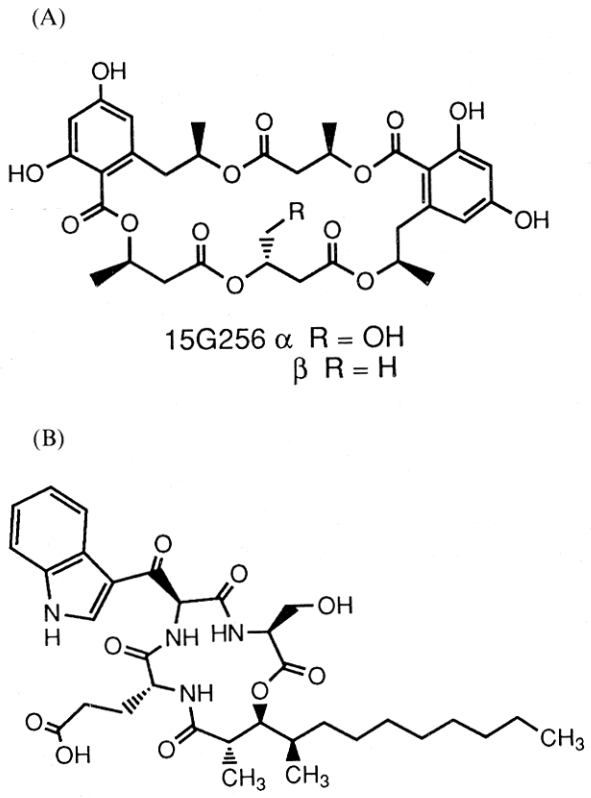
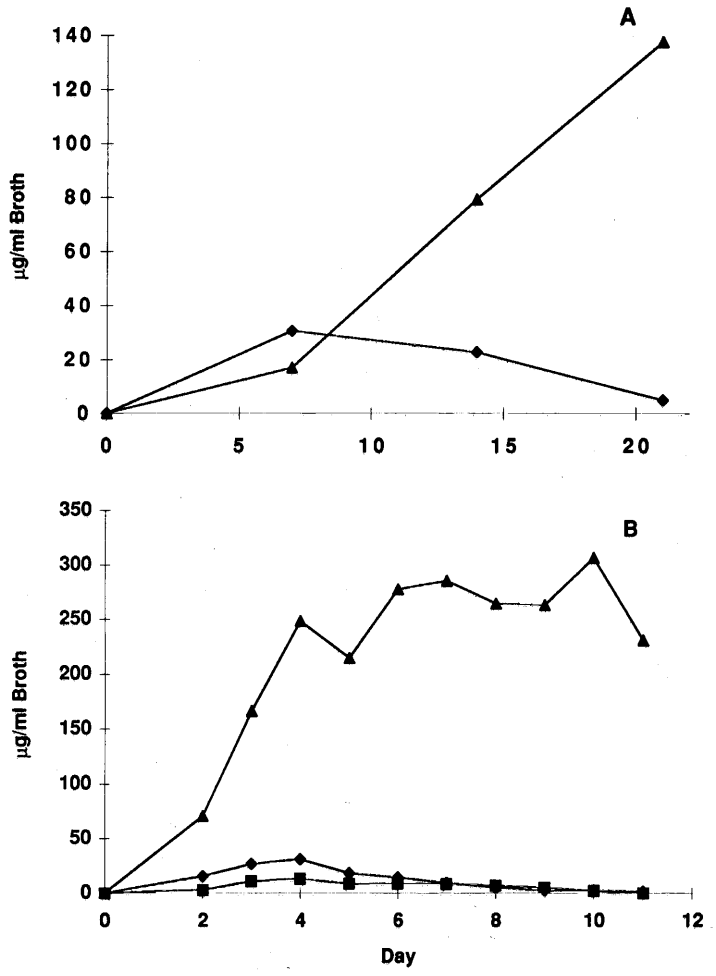


Fig. 4. Time course for the production of α, β and γ (μg/ml fermentation broth).

(A) LL-15G256 grown in Sabourauds Maltose broth. (B) LL-15G256 grown in GSP medium.
◆ α, ■ β, ▲ γ.



paper. Whereas the polylactones have been previously reported,[16] 15G256γ is novel[17] and is the focus of the studies described below. Arthrichitin, a fungal metabolite that may be identical to 15G256γ (no stereochemistry was described) was recently reported from the fungus *Arthrinium phaeospermum*.[18]

Fermentations of LL-15G256 initially utilized Sabouraud Maltose broth as a production medium. Titers of α peaked within 7 days, reaching approximately 30 μg/ml fermentation broth (Figure 4A). Titers of β, typically similar to those of α, were not recorded. Production of the γ component was frequently inconsistent and often continued beyond 21 days, reaching levels as high as 150 μg/ml.

Because of the erratic production and slow accumulation of the γ component, a medium study was performed. The medium selected, GSP, supported a more rapid, consistent production of the γ metabolite (Figure 4B), with titers peaking by days 6~8. Titers of γ were routinely higher than those observed in Sabourauds Maltose medium, with levels generally reaching 200~400 μg/ml broth. In contrast, titers of α and β remained unchanged, reaching levels similar to those previously observed. Growth in GSP medium was rapid, with cells reaching stationary phase by day 3 (Figure 5). By day 8, the extensive lysis of filaments was noted microscopically, and cell mass sharply decreased. Production of α, β and γ thus occurred in the stationary phase of growth, with titers peaking approximately 72 hours after maximum levels of growth were achieved. Growth and production characteristics in GSP medium with 30 liter and 300 liter fermentors were similar to those described above.

During growth in GSP medium, the broth pH remained neutral through day 5, after which an alkaline

Fig. 5. Growth and pH of LL-15G256 in GSP medium.

○ Cell pellet, ■ pH.

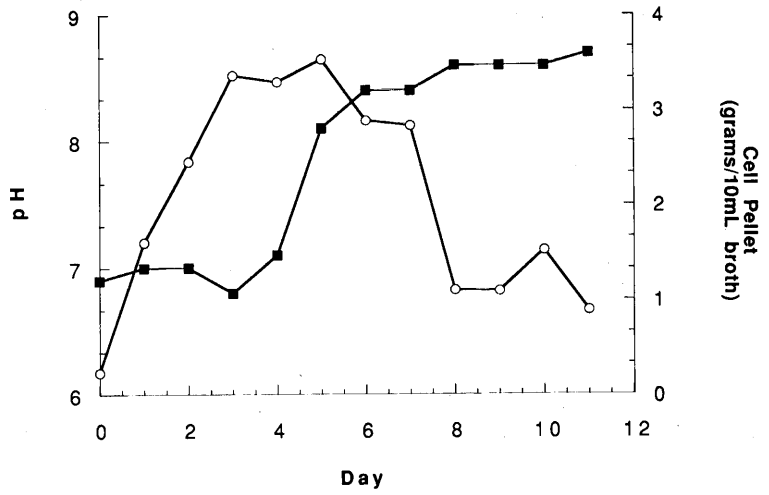
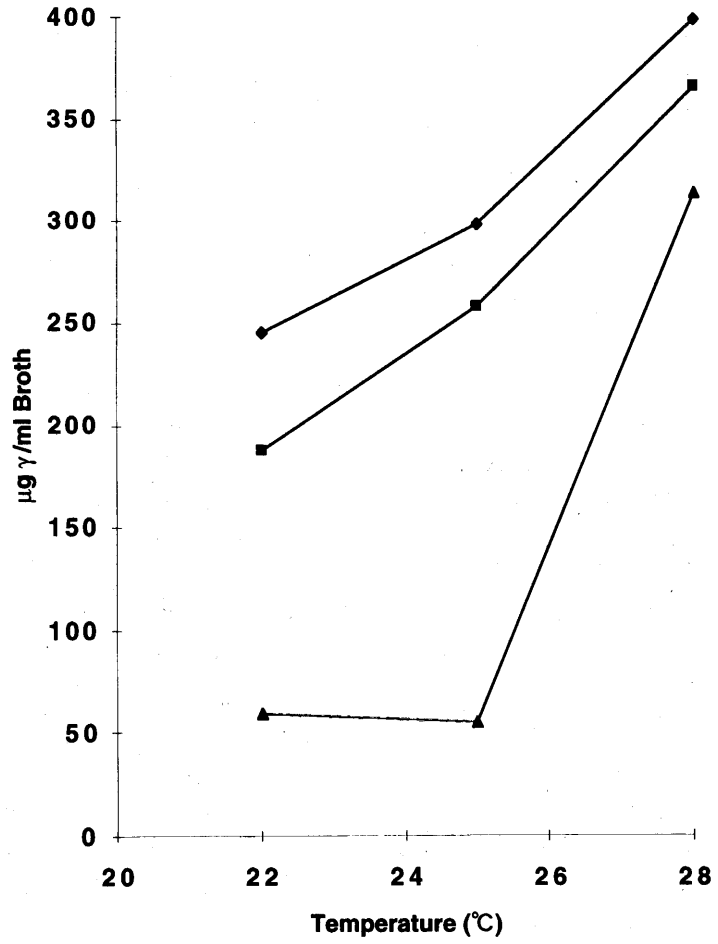


Fig. 6. Effects of seawater and temperature on the production of LL-15G256γ.

◆ 0% Seawater, ■ 20% seawater, ▲ 90% seawater.



shift was observed (Figure 5). The observed increase in pH on day 5 was associated with a rapid decrease in titers of the polylactones, which may reflect a chemical lability of these compounds under alkaline conditions. The resulting kinetics allowed for the exclusive accumulation of 15G256γ with minimum contamination from compounds α and β. During growth in Sabouraud Maltose broth, the pH remained at 5.0 +/− 0.5 throughout the fermentation. Under these conditions, the rate of decrease in polylactone titers was slower than was observed with GSP medium, possibly reflecting their greater chemical stability at this pH.

Although some information is available regarding the optimum growth conditions of marine fungi,[19] little is known regarding the conditions which affect the production of secondary metabolites. Two parameters, salinity and temperature, were selected for further study with LL-15G256. Initial experiments demonstrated decreased production of antifungal activity at 15°C and 35°C. Based on these results, the effects of varying seawater concentrations on the production of 15G256γ were determined in cultures grown at 22°C, 25°C or 28°C (Figure 6). A trend toward increased γ production was observed with increasing temperature and decreasing salinity, with maximum titers found at 0% seawater and 28°C. Thus, although this strain was isolated from a saline environment, under the conditions of this experiment salt appeared to depress secondary metabolite accumulation. In contrast, the optimum temperature for production (~28°C) was similar to the water temperatures of the mangroves from which this culture was isolated. Additional studies are necessary to further characterize the optimum conditions for production of metabolites α, β and γ.

To our knowledge, this is the first report of secondary metabolite production from *H. oceanicum*. The ability of this culture to produce two unrelated classes of compounds with the same activity appears to be unprecedented. All known species of *H. oceanicum* are saprophytes isolated from mangrove wood in tropical and subtropical regions,[19] an environment in which it may compete with other cellulolytic fungi such as *Aigialis parvus*, *Lophiostoma mangrovis* and *Julella avicenniae*.[19~22] The production of compounds inhibitory to cell wall biosynthesis of other filamentous fungi may provide a selective advantage for the growth of *H. oceanicum*.

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