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Isolation, structure and antibacterial activity of pleosporone from a pleosporalean ascomycete discovered by using antisense strategy

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Abstract—Protein synthesis is one of the best antibacterial targets that have led to the development of a number of highly successful clinical drugs. Protein synthesis is catalyzed by ribosome, which is comprised of a number of ribosomal proteins that help the catalysis process. Ribosomal protein S4 (RPSD) is one of the proteins that is a part of the ribosomal machinery and is a potential new target for the discovery of antibacterial agents. Screening of microbial extracts using antisense-sensitized *rpsD Staphylococcus aureus* strain led to the isolation of pleosporone, a new compound, with modest antibacterial activities with MIC ranging from 1 to 64 μg/mL. This compound showed the highest sensitivity for *Streptococcus pneumoniae* and *Haemophilus influenzae*, and exhibited MIC's of 4 and 1 μg/mL, respectively. Pleosporone showed modest selectivity for the inhibition of RNA synthesis compared to DNA and protein synthesis, and showed activity against HeLa cells. Isolation, structure elucidation, and biological activity of pleosporone have been described.

1. Introduction

Antibiotic resistant bacteria continue to grow, particularly methicillin resistant *Staphylococcus aureus* (MRSA), and remain a serious threat to human lives.[1] There are a number of molecular targets that have been successfully utilized for the development of antibiotics. Protein synthesis is one of the highly effective antibacterial targets. Its inhibition continues to produce clinically useful antibiotics.[2,3] The examples of protein synthesis inhibitor antibacterial agents are: chloramphenicol, macrolides, aminoglycosides, tetracyclines, streptogramins, lincosamides, and oxazolidinones. Protein synthesis is catalyzed by ribosome which is comprised of two asymmetric macromolecular components, the large (50S) and small (30S) subunits. The large subunit consists of two ribosomal RNAs (rRNAs) 23S and 5S, and 34 unique ribosomal proteins (r-proteins), L1–L34.[4–6] The small subunit is composed of 16S rRNA and 21 r-proteins, S1–S21.[4–6] All but protein S1 of the small subunit appear to be essential for normal ribosomal functioning and protein synthesis. The catalytic power of the protein synthesis resides in the ribosome itself. Ribosomal proteins are known to help the ribosome to maintain its tertiary structure. Most of the drugs that are in clinical use today bind not only to rRNA but also to one or more r-proteins. The alteration of the rRNA binding to r-proteins inactivates the protein synthesis function. Therefore, it is expected that the selective alteration of the conformation of a particular r-protein, or the inhibition of the synthesis of an r-protein, would result in the loss of function, and may lead to the inhibition of the bacterial protein synthesis. Small ribosomal protein S4 is one of those proteins. It is encoded by *rpsD* gene in both Gram-positive and Gram-negative bacteria, conserved across bacterial species and essential for bacterial growth.[7,8]

We have recently reported a new method for the discovery of antibiotics by using antisense technology. We designed and applied a two-plate whole-cell differential

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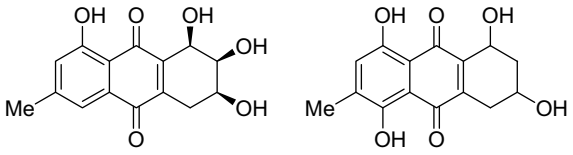
sensitivity screening assay using an antisense-sensitized *S. aureus* strain with a reduced expression of fabF/H gene and thus a reduced production of FabF/H proteins.[9,10] This led to the discovery of platensimycin and platencin, two novel and potent inhibitors of FabF and FabF/H enzymes with in vivo antibiotic activities.[11–14] To discover a compound that interacts with RPSD protein, a similar two-plate assay with a reduced expression of *rpsD* gene by antisense was developed and used for the screening of natural product extracts. This screening strategy led to the identification of lucensimycins A and B[15] from a strain of *Streptomyces lucensis* MA7349 and coniothyrione from a fungal strain of *Coniothyrium cerealis* MF7209.[16] Continued screening of the microbial extracts led to the identification of an extract produced by a fungal strain of Pleosporales that showed an activity in the *rpsD* two-plate differential sensitivity assay. Bioassay-guided fractionation of the extract led to the isolation of a new compound, pleosporone (**1**) which showed the differential sensitivity against rpsD strain, selectivity for RNA synthesis inhibition, and modest antibacterial activity against a number of Gram-positive and Gram-negative bacterial strains. The isolation, structure elucidation, and the biological activity of pleosporone are herein described.



2. Results and discussion

The producing organism (MF7028) was isolated as an endophyte from *Anthillis vulneraria* L. (Fabaceae) collected in Madrid, Spain, and its phylogenetic affinities were approximated by the sequencing of the internal transcribed spacers (ITS) region of the ribosomal DNA. To produce the secondary metabolites, the fungus was grown on a cracked corn medium and extracted with acetone. The acetone extract was concentrated and chromatographed on Sephadex LH20 followed by reversed-phase HPLC to afford pleosporone **1** (4.0 mg/L) as a yellow gum.

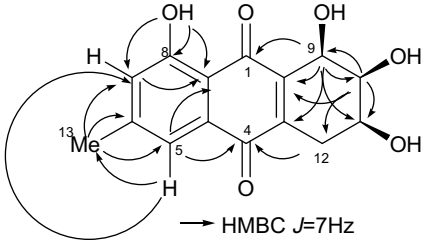


Figure 1. HMBC correlations of pleosporone (**1**).

Pleosporone (**1**) was assigned a molecular formula of $C_{15}H_{14}O_6$ by HRESI-FT-MS ($[M+H]^+$ obsd *m/z* 291.0860; calcd 291.0869) indicating the presence of 9 degrees of unsaturation. The UV spectrum of **1** showed absorption maxima at 215, 236, 251, 272, and 420 nm. The IR spectrum displayed absorption bands for conjugated ketones (1664 and 1638 cm^{-1}) and OH groups (3366 cm^{-1}). The ^{13}C NMR spectra (Table 1) displayed 15 signals thereby confirming the molecular formula. The presence of eight sp^2 carbons, including two proton-bearing and an oxygenated carbon (δ_C 160.2), two conjugated ketones, and an olefinic methyl group appearing at δ 21.5 indicated the presence of a naphthoquinone moiety.[17] HMBC correlations of the methyl protons (δ_H 2.37) to C-5 (δ_C 119.6), C-6 (δ_C 147.9), and C-7 (δ_C 123.4); H-7 (δ_H 7.14) to C-5, C-8a (δ_C 112.5), and C-13 (δ_C 21.5); H-5 (δ_H 7.34) to C-4 (δ_C 184.0), C-7, C-8a, and C-13; and OH (δ_H 12.0) to C-8 (δ_C 160.2), C-7, and C-8a further confirmed the naphthoquinone system (see Fig. 1). The remaining four aliphatic carbons, including three oxygenated methines appearing at δ_C 63.7, δ_C 65.6, and δ_C 71.5, and a methylene carbon appearing at δ_C 27.3, were present in the form of an isolated spin system apparent from the COSY spectrum. The naphthoquinone accounted for eight degrees of unsaturation. The remaining degree of unsaturation was fulfilled by the cyclization of the four aliphatic carbons into a six-membered fused ring. The ring system was confirmed by the key HMBC correlations of H-9 (δ_H 4.64) to C-2 (δ_C 141.3), C-3 (δ_C 144.7), and C-1 (δ_C 188.8); H-10 (δ_H 3.76) to C-2; H_2-12 (δ_H 2.72) to C-2, C-3, and C-4 (δ_C 184.0). The relative configuration at three oxygenated methines was determined by the measurement of the vicinal coupling constants. H-11 appeared as a doublet of doublet of doublets with a 2.1 Hz coupling with H-10 and larger couplings (J = 10 and 6 Hz) with both protons at C-12 indicating that H-11 must be oriented in such a way that it allows for an *anti*-relationship with one of the protons at C-12. H-11 showed only a weak coupling (J = 2.1 Hz) with H-10 which showed a slightly larger coupling (J = 3.3 Hz) with H-9. These couplings suggest that the cyclohexene ring adapts a pseudo chair conformation in which H-11 is axial, H-10 is equatorial, and H-9 is axial. Based on these data, structure **1** was assigned for pleosporone. Surprisingly, compound **2** isolated from *Penicillium islandicum* is the only compound reported that is closely related to **1**.[18]

3. Biological activity

Pleosporone was first evaluated in the antisense *rpsD* sensitized two-plate differential sensitivity assay. It showed a higher sensitivity for the sensitized strain than the control strain in a dose-dependent manner ranging from 1000 to 31.25 μg/mL exhibiting a small zone differential of ~2–3 mm at each dose with zone sizes of 21.8 and 19.2 mm at 1000 μg/mL against the antisense and the control strains, respectively. At the lowest concentration, a 9 mm faint zone of clearance was observed in the antisense plate but no zone of clearance was observed in control plate suggesting a MDC (minimum

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Assignment of pleosporone (1) in DMSO-d_6

#	δ_C	δ_H, mult, J in Hz	COSY	HMBC (H → C)
1	188.8			
2	141.3			
3	144.7			
4	184.0			
4a	131.6			
5	119.6	7.34, s		C-4, 7, 8a, 13
6	147.9			
7	123.4	7.14, s		C-5, 8, 8a, 13
8	160.2			
8a	112.5			
9	65.6	4.64, d, 3.2	H-10	C-1, 2, 3, 10, 11
10	71.5	3.76, dd, 3.2, 2.1	H-9, 11	C-2, 9, 11, 12
11	63.7	3.93, ddd,10.0, 6.0, 2.1	H-10, 12	
12	27.3	2.72, dd, 19.2, 6.0	H-11	C-2, 3, 4, 10, 11
		2.31, dd, 19.2, 10.0		C-2, 3, 11
13	21.5	2.37, s		C-5, 6, 7
OH		12.0, s		C-7, 8, 8a

detection concentration) of 31.25–62.5 μg/mL. Pleosporone showed a MIC value of 64 μg/mL against wild-type *S. aureus* strains with a MIC_{80} of 4–16 μg/mL (Table 2). It showed better activities against *Streptococcus pneumoniae* regardless of the medium used and showed a MIC value of 4 μg/mL. The activity against the other key pathogenic Gram-positive bacteria *Enterococcus faecalis* was similar to the activity against *S. aureus* (MIC 64 μg/mL). It inhibited the growth of one of the more promiscuous Gram-negative organisms, *Haemophilus influenzae* with a MIC value of 1 μg/mL but did not inhibit the growth of wild type *Escherichia coli*. When tested against the strains of *envA1* and *tolC* mutant *E. coli* strains with disrupted membrane and efflux pumps, it showed a MIC of value 16 μg/mL, confirming the presence of the biological target for this compound in *E. coli*. The lack of activity against wild type *E. coli* strain is likely due to either poor penetration or efflux. It did not inhibit the growth of *Candida albicans* at 64 μg/mL demonstrating a narrow selectivity for the bacterial strains over fungal strains. However, it showed cytotoxicity against Hela cells (IC_{50} 0.23 μg/mL). The inhibition of or interaction with RPSD protein is expected to show the inhibition of protein synthesis. However, pleosporone showed a slight preference (6- to 12-fold) of the inhibition of *S. aureus* RNA synthesis (IC_{50} 1.3 μg/mL) over DNA (IC_{50} 8.4 μg/mL) and protein synthesis (IC_{50} 15.4 μg/mL) (Fig. 2). This strain of *S. aureus* showed a MIC of value 3.13 μg/mL. The preferential inhibition of RNA synthesis over protein synthesis would suggest that this compound has another unknown mode of action in addition to the weak interaction with RPSD protein.

Table 2. Antibacterial activities of pleosporone (**1**)

Strains[a]	Phenotype	Strain #	MIC[b] (μg/mL)
S. aureus	$meth^S$	ATCC 29213	>64 (16)
S. aureus	$meth^S$	MB2865	64 (4)
S. aureus (+50% human serum)	$meth^S$	MB2865	>64
S. pneumoniae[c]	pen^S, $quin^S$, mac^S	CL2883	4
S. pneumoniae[d]	pen^S, $quin^S$, mac^S	CL2883	4 (2)
E. faecalis	van^S, mac^R	CL8516	64
Bacillus subtilis	wt	MB964	8
H. influenzae	Amp^S, $quin^S$, mac^S	MB4572	1
E. coli	Wt	MB2884	>64
E. coli envA1 tolC	envA1, tolC	MB5746	16
C. albicans	Wt	MY1055	>64
Hela tox (IC_{50})			0.23

[a] All strains were tested in Cation adjusted Mueller–Hinton Broth (CAMHB) medium unless mentioned otherwise under National Committee for Clinical laboratory Standards (NCCLS) guidelines.
[b] MIC (minimum inhibitory concentration), the numbers in parentheses show the concentration of compound that inhibit 80% of cell growth.
[c] CAMHB + 2.5% lysed horse blood medium.
[d] Isosensitet medium.

In this paper, the isolation and structure of pleosporone, a new tetrahydro-tetrahydroxy anthraquinone, from a Pleosporalean ascomycete using antisense differential sensitivity strategy have been described. It showed modest antibacterial activity exhibiting the highest sensitivities for one of the Gram-positive bacterial stains, *S.*

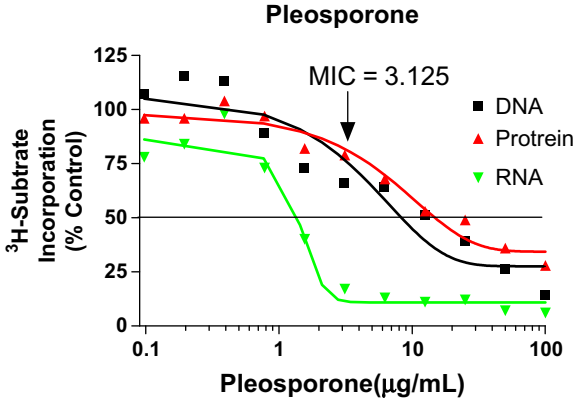


Figure 2. Inhibition of macromolecular synthesis by pleosporone.

pneumoniae and one of the Gram-negative bacterial strains, *H. influenzae*. It showed preferential inhibition of RNA synthesis.

4. Experimental

4.1. General experimental procedures

HP1100 was used for an analytical HPLC. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. The UV spectra were recorded in MeOH on a Beckman DU-70 Spectrophotometer. IR spectra were recorded on a Perkin-Elmer Spectrum One spectrometer. HRESI-MS were obtained on a Thermo Quest FT-MS spectrometer using electrospray ionization. The NMR spectra were recorded on a Varian INOVA 500 FT-NMR spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C in DMSO-d_6.

4.2. Producing organism and fermentation conditions

The producing organism (MF7028) was an endophytic fungus isolated from *Anthillis vulneraria* L. (Fabaceae) collected in San Sebastián de los Reyes (Madrid, Spain). The fungus was isolated using a surface sterilization technique previously described.[19] Agar cultures of the fungus failed to sporulate. Sequence analysis of the ITS region of the ribosomal DNA indicated moderate similarity (86–90%) with GenBank sequences from several taxa of ascomycetes in the *Pleosporales* (*Phoma betae* AY531685, *Leptosphaeria typharum* AF439465, and *Stemphylium solanii* AF203451). Based on the results from ITS sequence analysis, the fungus was characterized as an unidentified species of *Pleosporales*.

Plugs from potato dextrose agar (PDA, Difco) cultures were preserved in vials containing sterile glycerol (10%) at −80 °C. Inoculum was generated by inoculating agar plugs into a 250 mL Erlenmeyer flask containing 60 mL seed medium of the following composition in g L^{-1} distilled H_2O (corn steep powder, 2.5; tomato paste, 40.0; oat flour; 10.0; glucose, 10.0; $FeSO_4{\cdot}7\,H_2O$, 0.01; $MnSO_4{\cdot}4H_2O$, 0.01; $CuCl_2{\cdot}2H_2O$, 0.0025; $CaCl_2{\cdot}2$-H_2O, 0.001; H_3BO_3, 0.00056; $(NH_4)_6MoO_{24}{\cdot}4H_2O$, 0.00019; $ZnSO_4{\cdot}7H_2O$, 0.01). The pH was adjusted to 6.8 before autoclaving. The seed culture was incubated for 5 days at 22 °C on a gyratory shaker (220 rev min^{-1}) prior to the inoculation of production medium. Two milliliter of aliquots of the inoculum was used to inoculate 250 mL unbaffled Erlenmeyer flasks containing F1 production medium formulated as follows (per 250 mL flask): cracked corn (10 g) and base liquid (25 mL). Base liquid contained: ardamine pH (2 mg); KH_2PO_4 (1 mg); $MgSO_4{\cdot}7H_2O$ (1 mg); sodium tartrate (1 mg); $FeSO_4{\cdot}7$-H_2O (0.1 mg), and $ZnSO_4$ g·$7H_2O$ (0.1 mg). Production flasks were incubated statically at 22 ° C, 70% humidity for 28 days.

4.3. Extraction and isolation

Forty flasks (~1 L) were harvested, pooled and extracted with 1 L acetone by shaking on a platform shaker for 60 min. The 1 L acetone extract was concentrated to dryness. The residue was re-dissolved in MeOH, fractionated through gel filtration on a 2 L Sephadex LH-20 column and eluted with MeOH at a flow rate of 20 mL/min affording three adjacent active fractions (pooled weight 528.8 mg) eluting at the end of the first column volume. A 10% aliquot of the active fraction was further chromatographed by a preparative reversed-phase HPLC using a Zorbax RX C_8 (21.2 × 250 mm) column eluting with a 40 min gradient of 10–95% aqueous CH_3CN containing 0.1% TFA at a flow rate of 10 mL/min. The lyophilization of fractions eluting at 18 min afforded 0.4 mg of compound **1** as a yellow oil. Repeated HPLC of the remaining material provided another 3.6 mg of the material (yield 4 mg/L). The HPLC $[\alpha]_D^{25}$ +12.0° (*c* 0.5, 1:1 $CH_3OH/CHCl_3$); UV (CH_3OH) λ_{max} 215 (ε 28,675) 236 (9720) 251 (10,544) 272 (9512) 420 (2842) nm; IR (ZnSe) ν_{max} 3366, 2923, 1664, 1638, 1615, 1571, 1488, 1452, 1370, 1331, 1301, 1272, 1241, 1194, 1091, 1024, 978, 865, 752 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESI-FT-MS *m*/*z* 291.08598 (calcd for $C_{15}H_{14}O_6$+H: 291.08631).

4.4. RPSD two plate differential sensitivity assay

Staphylococcus aureus cells (RN450) carrying plasmid S1-782B bearing antisense to RPSD (*rpsD* AS-RNA strain) or vector (control strain) were inoculated from a frozen vial source into a tube containing 3 mL of Miller's LB Broth (Invitrogen) plus 34 μg/mL of chloramphenicol. Tubes were incubated at 37 °C at 220 rpm for 18–20 h. and kept at room temperature until use. Miller's LB broth was supplemented with 1.2% select agar (Invitrogen), 0.2% glucose, 15 μg/mL chloramphenicol, and 12 mM of xylose (only for the antisense strain). The OD_{600} of the culture was measured and 1:1000 of OD 3.0 inoculated. Next, 100 mL was poured into each NUNC plate and well-caster templates placed into the agar, and the agar was allowed to solidify. Then, 20 μL of test samples was added to the wells and the plates incubated at 37 °C for 18 h and the zones of inhibition measured. MDC (minimum detection concentration) values were determined by 2-fold serial dilution.

4.5. Antibiotic assay (MIC)

The MIC (minimum inhibitory concentration) against each of the strains was determined as previously described.[20] Cells were inoculated at 10^5 colony-forming U/mL followed by incubation at 37 °C with a 2-fold serial dilution of compounds in the growth medium for 20 h. MIC is defined as the lowest concentration of an antibiotic inhibiting visible growth.

4.6. Macromolecular synthesis inhibition

The assay was performed as previously described.[11,21] Briefly, mid-log (A_{600} = 0.5–0.6) *S. aureus* growth was incubated at an increasing concentration of each inhibitor at 37 °C for 20 min with 1 μCi/mL 2-[^{3}H]glycerol, 1 μCi/mL 6-[^{3} H]thymidine, 1 μCi/mL 5,6-[^{3}H]uracil, 5 μCi/mL 4,5-[^{3}H]leucine, or 5 μCi/mL 2,3-[^{3}H]alanine (or 2-[^{3}H]glycine) to measure phospholipids, DNA,

RNA, protein, and cell wall synthesis, respectively. Cell wall labeling with 2-[^{3}H]glycine (*S. aureus*) was performed in the presence of 100 μg/mL chloramphenicol, which prevents protein synthesis. The reaction was stopped by addition of the 10% trichloroacetic acid and the cells were harvested using a glass fiber filter (Perkin-Elmer Life Sciences, 1205-401). The filter was dried and counted with scintillation fluid.

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