Coniothyrione, a Chlorocyclopentandienylbenzopyrone as a Bacterial Protein Synthesis Inhibitor Discovered by Antisense Technology

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Bacterial protein synthesis inhibitors interact mainly with rRNA and to some extent ribosomal proteins, which are potential targets for developing new antibacterial agents. Specifically, the ribosomal protein S4 of the 30s ribosomal subunit known as ribosomal protein small-subunit D (rpsD) may be useful as a target. The antisense-*rpsD* gene-sensitized two-plate assay led to the discovery of a novel chlorinated cyclopentandienylbenzopyrone antibiotic, coniothyrione, $C_{14}H_9ClO_6$, isolated from *Coniothyrium cerealis* MF7209. It exhibited liquid MICs of 16−32 μg/mL against *Staphylococcus aureus*, *Bacillus subtilis*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Enterococcus faecalis* and >64 μg/mL against *Escherichia coli*. Isolation, structure elucidation, and antibacterial activity of coniothyrione are described.

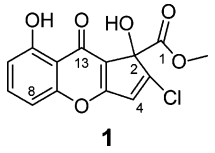
The incidence of infections by drug-resistant bacteria continues to grow and remains a serious threat to human lives. Protein synthesis is one of the most effective antibacterial drug targets, leading to a number of clinically useful agents.[1,2] These include macrolides, aminoglycosides, tetracyclines, streptogramins, lincosamides, and oxazolidinones. Protein synthesis is catalyzed by bacterial ribosomes, which are composed 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.[2−5] The small subunit is composed of 16S rRNA and 21 r-proteins, S1−S21.[3−5] Most of the drugs that are in clinical use today bind not only to rRNA but also to one or more r-proteins. Alteration of the binding to either rRNA or r-proteins inactivates the action of drugs.[2] Therefore, it is expected that selectively altering the conformation of a particular r-protein, or inhibiting the synthesis of an r-protein, would potentially result in the loss of function and may lead to the inhibition of protein synthesis. Small ribosomal protein S4 is one such protein which is encoded by the *rpsD* gene in both Gram-positive and Gram-negative bacteria.[6]

S4 is a primary protein that is required for functioning of the ribosome. Its structure appears to be conserved across bacterial species and, therefore, represents a broad spectrum target. In a genome-wide strategy for the identification of essential genes in *Staphylococcus aureus* using antisense, Forsyth et al.[7] showed that the *rpsD* gene function is essential for bacterial growth.

Recently, we reported the design and application of a two-plate whole-cell differential sensitivity screening assay using an antisense-sensitized *S. aureus* strain in which the FabF target was dialed down.[8,9] This led to the discovery of platensimycin, a novel and potent FabF inhibitor with *in vivo* antibiotic properties.[10] A similar two-plate assay[8] with a dialed down *rpsD* gene (S1-782B) by antisense using 12 mM xylose was used to screen natural product extracts.[11] This strategy led to the identification of an extract derived from a strain of *Coniothyrium cerealis* MF7209. Bioassay-guided fractionation of this extract led to the isolation of coniothyrione (**1**). The isolation, structure elucidation, and activities of this compound are described herein.

Table 1. ^{1}H and ^{13}C NMR Assignments of Coniothyrione (**1**) in DMSO-d_6

position	δ_C	δ_H, *J* in Hz	HMBC H → C
1	168.7		
2	79.8		
3	127.2		
4	143.1	7.20, s	C-2, 5, 14
5	164.5		
7	155.7		
8	108.1	7.22, dd, 8.5,1.0	C-10, 12
9	135.8	7.70, t, 8.5	C-7, 11
10	112.7	6.90, dd, 8.5,1.0	C-8, 12
11	160.6		
12	110.7		
13	176.0		
14	120.8		
15	52.9	3.64, s	C-1
11-OH		12.4, s	C-10, 11, 12



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One liter of fermentation broth (pH 4.4) was extracted with acetone, concentrated to an aqueous solution under reduced pressure, and chromatographed on an Amberchrom column followed by reversed-phase C_8 HPLC to afford 10 mg of **1** as a colorless, amorphous powder.

The UV spectrum of **1** showed absorption maxima at λ_{max} 270 and 344 nm. HRESIFTMS produced a formula of $C_{14}H_9Cl^{35}O_6$ (obsd *m*/*z* 309.0163, calcd for M + H, 309.0166). This formula was consistent with the ^{1}H and ^{13}C NMR spectra (DMSO-d_6), which showed the presence of nine hydrogens and 14 carbons (Table 1). The ^{1}H NMR spectra showed the presence of three aromatic protons, two of which were doublets of doublets resonating at δ_H 6.90 and 7.22 and one appearing as a triplet at δ_H 7.70. An olefinic proton singlet was observed at δ_H 7.20 and a methoxy singlet at δ_H 3.64. A chelated phenolic hydroxyl group was observed at δ_H 12.4 ppm.

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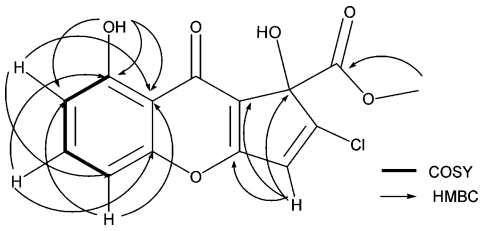

Figure 1. HMBC and COSY correlations of **1**.

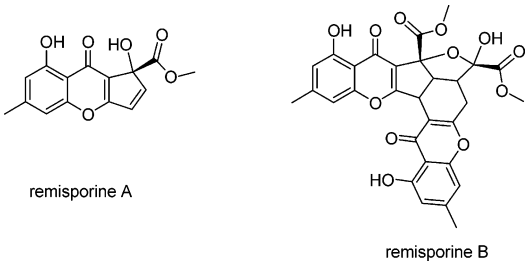

Figure 2. Structures of remisporines A and B.

The two doublets of doublets exhibited *ortho* and *meta* coupling. The COSY data indicated that the three aromatic protons were contiguous. Six-center hydrogen bonding between the phenolic proton and the carbonyl group indicated that the carbonyl is present in the adjacent ring at the *peri* position. The hydroxyl proton at δ_H 12.4 ppm disappeared when the NMR spectrum was recorded in CD_3OD.

The HMQC spectrum exhibited a correlation of the methyl group protons at δ_H 3.64 to δ_C 52.9 and produced an HMBC correlation to the carbonyl δ_C 168.7, suggesting the presence of a methyl ester group. The singlet proton at δ_H 7.20 (H-4) correlated to the carbon at δ_C 143.1. The olefinic proton H-4 showed HMBC correlations to quaternary carbons C-2 (δ_C 79.8), C-5 (δ_C 164.5), and C-14 (δ_C 120.8) and established the cyclopentandienol ring. HMBC correlations from H-8, H-9, H-10, and OH-11 established the substitution patterns of the aromatic ring A. Taken together various HMBC correlations coupled with the ^{13}C shifts of C-5, C-7, and C-13 (δ_C 176.0) allowed the assembly of a γ-pyrone ring fused between the aromatic ring and the cyclopentandienol ring. The presence of the chelated phenolic group, ^{13}C chemical shifts arguments, and lack of HMBC correlation of the olefinic proton (δ_H 7.20) to the carbonyl group allowed elimination of all other potential structures and helped establishing the structure **1** for coniothyrione.

An agar-based antisense-sensitized *rpsD* two-plate assay was developed similar to what was described for the *fabF* assay by Young et al.[8] except for substituting the *fabF* antisense strain with that of the *rpsD* antisense strain (S1 782B) and sensitization by 12 mM xylose. In this assay coniothyrione showed a zone of inhibition (ZOI) differential of 10 mm between the wild-type and antisense *rpsD S. aureus* strain at 16 μg/mL. Coniothyrione was further evaluated for its antibacterial potential against a small panel of key pathogenic microorganisms. It exhibited an MIC of 32 μg/mL against *Staphylococcus aureus*, 16 μg/mL each against *Bacillus subtilis* and *Haemophilus influenzae*, and 32 μg/mL each against *Streptococcus pneumoniae* and *Enterococcus faecalis*, and was not effective against the Gram-negative *Escherichia coli* (>64 μg/mL). In the presence of 50% human serum the MIC of **1** against *S. aureus* was greater than 32 μg/mL. Measurement of inhibition of macromolecular synthesis[12] in *S. aureus* indicated that **1** inhibited protein synthesis with an IC_{50} value of 5 μg/mL. However, it also inhibited DNA synthesis (IC_{50} 3 μg/mL), and thus its mode of inhibiting bacterial growth cannot be solely due to protein synthesis.

Coniochaetone B[13] and remisporines A and B[14] (Figure 2) were the only two compounds reported with structures similar to **1**, which were isolated from *Coniochaeta saccardoi* and *Remispora maritima*, respectively, and reported to have weak antibiotic activity. Neither of the two compounds showed antibacterial activity at 1 mg/mL; however, coniochaetone B exhibited antifungal activity. Remisporine A was stable long enough to allow for structure determination but dimerized rapidly to B. Apparently, the chlorine at C-3 in **1** stabilizes the molecule and prevents dimerization from occurring.

To summarize, we have described the discovery of a new natural product by using an antisense-based antibacterial assay. Coniothyrione exhibited moderate broad spectrum Gram-positive antibacterial activity. While it inhibited protein synthesis, the effect was not selective. Coniothyrione showed a ZOI differential in the antisense assay, indicating that it likely interacts with S4 protein, and inhibition of protein synthesis may be due to this effect.

Experimental Section

General Experimental Procedures. Optical rotation was recorded with a Perkin-Elmer 241 polarimeter. UV spectra were recorded on a Beckman DU-70 spectrophotometer. IR spectra were recorded with a Perkin-Elmer Spectrum One FT-IR spectrophotometer. All NMR spectra were recorded with a Varian Unity 500 (^1H, 500 MHz; ^{13}C, 125 MHz) spectrometer in DMSO-d_6. Chemical shifts are reported in δ (ppm) using residual solvent signals (^1H, 2.53 and ^{13}C, 39.51 ppm) as internal standards. $^1H-^1H$ COSY, DEPT, HMQC, and HMBC spectra were measured using standard Varian pulse sequences. LRMS data were recorded on an Agilent 1100 MSD with ES ionization. HRESIMS was obtained on a Thermo Finnigan LTQ-FTMS spectrometer. An Agilent HP 1100 instrument was used for analytical HPLC.

Fungal Material. The fungal strain was isolated from composted livestock manure collected in the province of Guadalajara, Spain. The fungus was induced to sporulate on oatmeal and potato-carrot agars. Identification was based on morphological analysis of pycnidia and the sequence of the ITS region of rDNA. Both morphology and sequence database searches led to the conclusion that MF7209 was conspecific with *Coniothyrium cerealis* (Pleosporales), an ascomycete commonly associated with decaying grasses in Europe

Fermentation. Seed culture was prepared by inoculation from frozen mycelium agar plugs in a 250 mL Erlenmeyer flask containing 60 mL of seed medium (KFA) of the following composition (in g/L): corn steep powder, 2.5; tomato paste, 40.0; oat flour, 10.0; glucose, 10.0; agar, 4.0; and trace elements solution, 10 mL ($FeSO_4 \cdot 7H_2O$, 1.0 g/L; $MnSO_4 \cdot 4H_2O$, 1.0 g/L; $CuCl_2 \cdot 2H_2O$, 0.025 g/L; $CaCl_2 \cdot 2H_2O$, 0.1 g/L; H_3BO_3, 0.056 g/L; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.019 g/L; $ZnSO_4 \cdot 7H_2O$, 0.2 g/L). The pH was adjusted to 6.8 by adding NaOH. The seed culture was incubated at 22 °C on a gyratory shaker (220 rpm) for 4−7 days, resulting in a pH of 7.0. The production medium (WS80) was prepared in 500 mL flasks with 120 mL of medium. WS80 was formulated as follows (in g/L): whole wheat flour, 50; D(+)-xylose, 40; and D(−)-fructose, 40. No adjustment was made to the pH. Fermentation flasks were inoculated with 4% of vegetative seed growth and were incubated at 22 °C, 220 rpm, and 70% humidity for 14 days. The final pH of the broth at harvest was 4.4.

Extraction and Isolation. A 1 L fermentation broth was extracted with 1 L of acetone and then concentrated to an aqueous slurry under reduced pressure and loaded onto a 60 cm^3 Amberchrom column in H_2O. The column was eluted with a liner gradient of H_2O/MeOH over 100 min at a flow rate of 5 mL/min followed by 20 min each of MeOH and acetone. The active fraction eluted in the 100% MeOH fraction and was concentrated to dryness, yielding 100 mg of material, and was further fractionated by RP HPLC using a Zorbax C_8 (250 × 21 mm) column. It was eluted at a flow rate of 12 mL/min with a linear gradient of 20−90% aqueous CH_3CN with 0.1% trifluroacetic acid (TFA) over 50 min. The active compound eluted at 39 min and was lyophilized to give 10 mg of coniothyrione (**1**).

Coniothyrione (1): powder; $[\alpha]^{23}_D$ −1.42 (*c* 0.1, MeOH); UV-(MeOH) λ_{max} 215 (log ϵ 3.99), 230 sh (3.84), 270 (4.08), 344 (3.43) nm; IR (ZnSe) ν_{max} 1745, 1644, 1618, 1597, 1465, 1202, 1125, 1035, 944 cm^{-1}; ^{13}C and ^1H NMR, see Table 1; ESIMS *m*/*z* 309 $[M + H]^+$; HRESIFTMS *m*/*z* 309.0163 (calcd for $C_{14}H_9Cl^{35}O_6$+H, 309.0166).

Antibiotic Assay (MIC). The MIC (minimum inhibitory concentration) against each of the strains was determined as previously described.[8] Cells were inoculated at 10^5 colony-forming units/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.

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Supporting Information Available: ^{1}H NMR, ^{13}C NMR, and UV spectroscopic data of coniothyrione. This material is available free of charge via the Internet at http://pubs.acs.org.

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