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Notes

Pestalone, a New Antibiotic Produced by a Marine Fungus in Response to Bacterial Challenge

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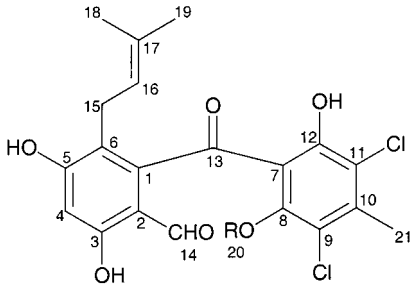
The isolation and structure determination of a new chlorinated benzophenone antibiotic, pestalone (**1**), is described. The new compound was produced by a cultured marine fungus only when a unicellular marine bacterium, strain CNJ-328, was co-cultured in the fungal fermentation. The fungus, isolated from the surface of the brown alga *Rosenvingea* sp. collected in the Bahamas Islands, was identified as an undescribed member of the genus *Pestalotia*. The structure of **1**, initially assigned with only modest confidence by combined spectral and chemical data, was confirmed by single-crystal X-ray analysis. Pestalone (**1**) exhibits moderate in vitro cytotoxicity in the National Cancer Institute's 60 human tumor cell line screen (mean $GI_{50} = 6.0\ \mu M$). More importantly, pestalone shows potent antibiotic activity against methicillin-resistant *Staphylococcus aureus* (MIC = 37 ng/mL) and vancomycin-resistant *Enterococcus faecium* (MIC = 78 ng/mL), indicating that pestalone should be evaluated in advanced models of infectious disease.

The development of resistance toward current antibiotics continues to be a significant problem in the treatment of infectious disease,[1−4] and therefore the discovery and development of new antibiotics is evolving as a high priority in biomedical research. As part of a continuing program to evaluate the drug potential of marine-derived microorganisms[5] and to develop new methods to enhance secondary metabolite production, we have begun exploring the use of mixed fermentation for the discovery of antibiotics that show efficacy against drug-resistant pathogens. In this report, we describe the isolation and characterization of pestalone (**1**), a new benzophenone antibiotic that exhibits potent antibacterial activity against drug-resistant bacteria. This antibiotic was obtained from the mixed fermentation of a marine deuteromycete (strain CNL-365, *Pestalotia* sp.) and an unidentified, antibiotic-resistant marine bacterium. It was not detected when either strain was cultured individually.

Mixed fermentation has been used in the food industry[6] and to enhance enzyme production;[7] however, it is not clear that this method has been used extensively by the pharmaceutical industry for secondary metabolite discovery. Given that antibiotics may be produced in nature to provide a competitive advantage,[8] it is possible that the pathways responsible for the biosynthesis of certain compounds are regulated by factors elicited by one microbe and detected by another. This proposal is supported by evidence that antibiotic production can be induced in response to microbial antagonism[9] and that the production of specific secondary metabolites can be increased up to 400-fold when



1 R=Me
2 R=H

strains are grown in the presence of an antagonist.[10] The isolation of pestalone (**1**) demonstrates that mixed fermentation can lead to the discovery of the new antibiotics and suggests that this method may have utility for drug discovery.

Pestalone (**1**) was isolated as yellow crystals which showed molecular ions at *m/z* 438/440/442 by LREIMS, which demonstrated the characteristic isotope molecular ions for two chlorine atoms in the molecule. The molecular formula was established as $C_{21}H_{20}O_6Cl_2$ by HRFABMS $[M + H]^+$ *m/z* 439.0731: calcd for $C_{21}H_{20}O_6{}^{35}Cl_2$ 439.0715 (11 degrees of unsaturation). The infrared spectrum of **1** showed typical absorptions for an aromatic aldehyde (1635 cm^{-1}), a ketone (1620 cm^{-1}), and hydroxyl groups (3225 cm^{-1}). The UV spectrum, which showed broad absorptions at 340 nm, confirmed the presence of an aromatic ring with an extended chromophore.

The ^1H NMR spectrum of **1** (Table 1) showed the presence of two olefinic methyl groups (δ 1.34, 3H, s; 1.48, 3H, s); one aromatic methyl group (δ 2.50, 3H, s); a methoxy group (δ 3.30, 3H, s); three exchangeable phenolic protons

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Table 1. ^{1}H and ^{13}C NMR Data for Pestalone (**1**) in Acetone-d_6

C no.	^{13}C NMR[a]	^{1}H NMR[b]	HMBC correlations
1	146.9		
2	113.3		
3	163.9	11.20 (s, OH)	103.2, 113.3, 163.9
4	103.2	6.60 (s)	26.1, 113.3, 117.4, 146.9, 163.9, 192.0
5	163.6	10.10 (s, OH)	103.2, 117.4, 163.6
6	117.4		
7	117.9		
8	157.1		
9	119.4		
10	145.5		
11	119.8		
12	158.4	13.20 (s, OH)	117.9, 119.8, 158.4, 202.4
13	202.4		
14	192.0	9.80 (s)	103.2, 113.3, 146.9, 163.9
15	26.1	3.10 (d, 6.9)	117.4, 123.0, 132.1, 146.9, 163.6
16	123.0	4.98 (t, 6.9)	17.6, 25.8
17	132.1		
18	17.6	1.34 (s)	25.8, 123.0, 132.1
19	25.8	1.48 (s)	17.6, 123.0, 132.1
20	61.9	3.30 (s)	157.1
21	19.2	2.50 (s)	119.4, 119.8, 145.9, 157.1, 158.4, 202.4

[a] Recorded at 100 MHz. Assignments are by DEPT, HMQC, and HMBC methods. [b] Recorded at 300 MHz. Assignments are by COSY, HMQC, and HMBC methods.

(δ 10.1, 1H, s; 11.2, 1H, s; 13.2, 1H, s); and an aldehyde proton (δ 9.80, 1H, s). The ^{1}H NMR COSY spectrum showed that two methylene protons (δ 3.10, 2H, bd, J = 6.9 Hz) were coupled to an olefinic proton at δ 4.98 (1H, t, J = 6.9 Hz). The olefinic proton showed further allylic coupling to two methyl groups at δ 1.34 and 1.48, thus indicating the presence of an isopentene functional group.

The ^{13}C NMR spectrum showed signals at δ 202.4 and 192.0, which were assigned to ketone and aldehyde carbonyls. Three methyl groups resonating at δ 17.6, 19.2, and 25.8; one methoxy carbon at δ 61.6; one methylene at δ 26.1; two olefinic carbons at δ 103.2 and 123.0; and 12 quaternary aromatic carbon signals were also observed. NMR experiments, including 2D COSY, HMQC, and HMBC, confirmed the presence of an isoprene fragment and linked this group to an aromatic ring with a phenolic carbon on the adjacent carbon (C-5). The chemical shift of the aromatic proton at C-4, and HMBC correlations of this proton to C-6 and C-2, established the presence of a pentasubstituted aromatic ring (C-1 to C-6) with the isopentenyl functionality at C-6. Establishing the substitution pattern of the second aromatic ring, which involved assigning the positions of a methyl group, two chlorine atoms, a methoxy group, and a phenolic hydroxyl group, was not accomplished with confidence. Although the substitution pattern could be assigned by analysis of carbon chemical shift data, the lack of substantive HMBC correlations made conclusive assignments impossible.

To confidently assign the substituent pattern on the second ring (C-7 to C-12) of pestalone (**1**), an X-ray diffraction experiment was undertaken which solved the structure in an unambiguous manner. The X-ray perspective drawing of the structure of pestalone is shown in Figure 1. Two intramolecular H-bonds exist between O(1)-H and O(6) of 2.62 Å and between O(5)-H and O(3) of 2.52 Å, and an intermolecular H-bond appears between O(2)-H and O(6) of 2.78 Å; symmetry transformations used to generate equivalent atoms: #1 $-x+2,y-1/2,-z+3/2$. These results explain the low chemical shifts of the three phenolic protons. The planes formed by the two rings were almost perpendicular in the crystal, indicating the restricted rotation of the C-1 to C-13 and C-7 to C-13 bonds. That pestalone did not adopt a fully planar structure was also shown in the ^{13}C NMR spectrum. The ketone signal at δ 204.4 indicated that there was little conjugation between the ketone and the two rings. However, the UV absorption at 340 nm and the planar relationship between the ketone and chlorine-containing ring (resulting from the H-bond between the ketone and the OH at C-12) did indicate extended conjugation through the ketone should be observed.

Although the structure of pestalone is relatively rare and unrelated to most other natural products, the C-20 desmethyl analogue **2** has been reported, but only in a patent, as a product of a fungal strain of the genus *Chrysosporium*. Compound **2** is reported to inhibit the enzyme testosterone-5α-reductase.[11]

Pestalone was not produced in control experiments in which the fungus and bacterium were cultured individually, suggesting that the production of pestalone is induced by bacterial competition. Neither the organic extract nor the cell-free broth of the bacterium were found to induce antibiotic production by the fungus, suggesting that regulation may not be chemically mediated. Interestingly, the production of pestalone could be induced, although in very low yield, in a pure culture of the fungus CNL-365 by the addition of ethanol (1% v/v) after the fermentation had proceeded for 24 h. This result clearly demonstrates that pestalone is a product of fungal biosynthesis in response to an external trigger. It is not produced by a mixed biosynthesis in which the fungus transforms a bacterial metabolite.

Pestalone (**1**) was found to exhibit moderate in vitro cytotoxicity in the National Cancer Institute's human tumor cell line screen (mean GI_{50} = 6.0 μM). More importantly, pestalone showed potent antibacterial activity against methicillin-resistant *Staphylococcus aureus*

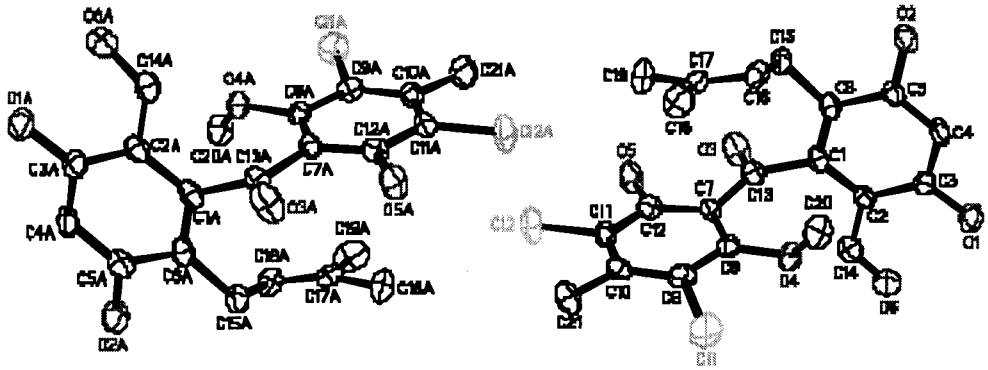

Figure 1. X-ray structure of pestalone.

(MIC = 37 ng/mL) and vancomycin-resistant *Enterococcus faecium* (MIC = 78 ng/mL). The potency of this agent toward drug-resistant pathogens suggests that pestalone should be evaluated in more advanced, whole animal models of infectious disease.

Experimental Section

Microbial Isolation and Identification. Fungal strain CNL-365 was isolated on medium YPG (1.0% glucose, 0.5% peptone, 0.5% yeast extract, 1.5% agar, 0.01% penicillin G/streptomycin sulfate, 100% seawater) from a sample of the brown alga *Rosenvingea* sp. collected in the Bahamas Islands in 1996. The alga was identified according to Littler et al.[12] Strain CNL-365 was identified (D. Porter, University of Georgia) based on morphological characteristics as a deuteromycete belonging to the genus *Pestalotia*. The bacterial strain (CNJ-328) used in the mixed fermentation was a Gram-negative unicellular bacterium that could not be identified by FAME (fatty acid methyl ester) analysis (Microbial ID, Inc., Newark, DE).

Mixed Fermentation of *Pestalotia* sp. Strain CNL-365 and Bacterium Strain CNJ-328. *Pestalotia* sp. strain CNL-365 was cultivated in 2.8 L Fernbach flasks (20 × 1 L) while shaking at 250 rpm at 25 °C in the seawater-based marine nutrient medium described above. After 24 h, 10 mL of a culture of the unicellular bacterium CNJ-328, grown in the same medium, was added to each flask. The mixed culture fermentation was allowed to proceed for an additional 6 days, after which the total culture volume was extracted with 25 L of ethyl acetate and the extract dried with anhydrous sodium sulfate.

Purification and Properties of Pestalone (1). The dried extract was concentrated under vacuum and, while monitoring in vitro cytoxicity against the human colon tumor cell line HCT-116, fractionated by reversed-phase C-18 silica flash chromatography using gradient elution (100% H_2O to 100% MeOH). Active fractions were combined and subjected to size exclusion chromatography (Sephadex LH-20) using isooctane−toluene−MeOH (3:1:1) as the eluent. The final purification of **1** was performed by normal-phase HPLC (Dynamax semi-preparative column, Si gel, 60 Å, 10 mm × 250 mm) with *n*-hexane−EtOAc (6:4), to yield 60.0 mg of pestalone (**1**). Pestalone, isolated as yellow crystals, mp 153−155 °C, showed the following spectral features: UV (CH_2Cl_2) λ_{max} (log ϵ) 340 (3.75), 281 (4.29), 238 (4.24) nm; IR film, (NaCl) ν_{max} 3225, 2943, 1635, 1620, 1383, 1251, 1205 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m*/*z* 438/440/442 $[M]^+$ (43, 29, 6), 423/425/427 $[M - CH_3]^+$ (60, 42, 9), 407/409/411 $[M - OCH_3]^+$ (59, 37, 8), 395/397/399 (100, 61, 12); HRFABMS $[M + H]^+$ *m*/*z* 439.0731 (calcd for $C_{21}H_{20}O_6{}^{35}Cl_2$, 439.0715).

X-ray Data. A single crystal (size 0.2 × 0.2 × 0.1 mm) was selected for the diffraction experiment. The data collection was performed at 173(2) K. The dimensions were $a = 8.4984(2)$ Å, $b = 14.4130(3)$ Å, $c = 32.6934(1)$ Å, and $\beta = 95.31(1)°$. Analysis of the diffraction pattern showed that the crystal belonged to the space group $P2_1/c$, with two molecules per asymmetric unit. The refined *R* factor was 6% for data with intensities greater the 2σ (see Supporting Information).

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Supporting Information Available: Crystallographic data for pestalone (**1**) have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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