NATURAL PRODUCTS

On the Antibiotic and Antifungal Activity of Pestalone, Pestalachloride A, and Structurally Related Compounds

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ABSTRACT: Pestalone (1) is a prominent marine natural product first isolated by M. Cueto et al. in 2001 from a co-fermentation of a marine fungus with a marine bacterium. For more than 10 years, 1 had been considered as a promising new antibiotic compound, the reported MIC against methicillin-resistant *Staphylococcus aureus* (MRSA) being 37 ng/mL. After overcoming the limited availability of 1 by total synthesis (N. Slavov et al., 2010) we performed new biological tests, which



did not confirm the expected degree of antibiotic activity. The observed activity of pestalone against different MRSA strains was $3-10 \ \mu g/mL$, as determined independently in two laboratories. A number of synthetic derivatives of 1 including pestalachloride A and other isoindolinones (formed from 1 by reaction with amines) did not exhibit higher activities as compared to 1 against MRSA and a series of plant pathogens.

T he story of pestalone (1) started in 2001 in the context of a research program by Fenical and co-workers, who tried to trigger the expression of new antibiotics against drugresistant pathogens from marine microorganisms in response to bacterial challenge.¹ After co-culturing a fungus of the genus *Pestalotia* and a unicellular marine bacterium (strain CNJ-328) they isolated 1 as a new antibiotic agent. Structural elucidation revealed 1 to be a highly functionalized, chlorinated benzophenone derivative (Figure 1).²



Figure 1. The marine natural product pestalone (1).

Pestalone (1) was reported to exhibit strong antibiotic activity against methicillin-resistant *Staphylococcus aureus* (MRSA, minimum inhibitory concentration (MIC) = 37 ng/mL) and vancomycin-resistant *Enterococcus faecium* (VRE, MIC 78 = ng/mL). The authors even underlined that "the potency of this agent toward drug-resistant pathogens suggests that pestalone should be evaluated in more advanced, whole animal

models of infectious disease".¹ Due to its pronounced activity, **1** was highlighted in several publications as a promising new lead structure in the struggle against multiresistant microorganisms.³ Nevertheless, because of its limited availability, no further biological investigations of **1** have been reported so far. In 2008, Che and co-workers reported the discovery of the pestala-chlorides A–C (Figure 2) from the plant endophytic fungus *Pestalotiopsis adusta*.⁴ These compounds are structurally closely related to **1**, from which they may be biosynthetically derived. Recently, we demonstrated that **1** can be easily converted into pestalachloride A (*rac*-**2a**) by treatment with ammonia.⁵ The fact that this compound occurs in nature also as a racemic



Figure 2. The natural products pestalachloride A (*rac*-2a), pestalachloride B (3), and pestalachloride C (4).

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mixture indicates its nonenzymatic "biosynthesis" from pestalone (1).

Pestalachlorides A-C were reported to exhibit antimicrobial activity against certain plant pathogenic fungi (Table 1). The

Table 1. Activity (Minimum Inhibitory Concentration) of Pestalachloride A against Plant Pathogenic Fungi: *Fusarium culmorum, Gibberella zeae,* and *Verticillium aibo-atrum,* As Reported by Che⁴

	MIC (μ g/mL)			
compound	F. culmorum	G. zeae	V. aibo-atrum	
rac-2a	3.2	50.1	50.1	
3	20.1	5.0	5.0	
4	>100	>100	>100	

highest antifungal activity was observed for pestalachloride A (*rac*-2a) against *Fusarium culmorum* (MIC = $3.2 \ \mu g/mL$). In contrast, pestalachloride B (3) was most effective against *Gibberella zeae* (MIC = $5.0 \ \mu g/mL$) and *Verticillium aibo-atrum* (MIC = $5.0 \ \mu g/mL$), while no biological activity was observed for pestalachloride C (4) in these in vitro test systems.⁴

The limited availability of 1 from natural sources challenged efforts toward its chemical synthesis. A synthesis of deformylpestalone and some related analogues was achieved in 2003,⁶ and a first total synthesis was disclosed a little later.⁷ However, no further biological experiments were reported since then.

Recently, we developed an efficient total synthesis and were able to produce substantial amounts of pestalone (1), the identity of which was secured by X-ray crystal structure analysis.⁵ In the course of this work, we also demonstrated that 1 reacts with ammonia to give *rac*-2a or with primary amines to afford isoindolinones *rac*-2b and *rac*-2c, respectively. In addition we found that the conversion of 1 into pestalalactone *rac*-5 proceeds smoothly either under photochemical conditions or in the presence of a nucleophilic catalyst such as cyanide (Scheme 1).⁸

The availability of material enabled us to further explore the antibacterial potency of pestalone (1), the related isoindolinones (rac-2a-c), pestalalactone (rac-5), and analogues (Figure 3). For this purpose, we utilized a standardized broth microdilution method to determine the activity against three different clinically relevant *Staphylococcus aureus* strains, i.e., USA300 (epidemic community acquired MRSA), Mu50 (hospital acquired MRSA, vancomycin-intermediate *S. aureus*, VISA), *S. aureus* MW2 (community acquired MRSA), and one



Figure 3. Further synthetic analogues of pestalone (1) employed in this study.

reference strain, ATCC 29213. The results are shown in Table 2.

Table 2. Activity of Pestalone (1) and Analogues against Different S. aureus Strains

	MIC [μ g/mL] against different <i>S. aureus</i> strains ^{<i>a</i>}				
compound	USA300	Mu50	MW2	ATCC29213	
1	10	10		16	
rac-2a			10	10	
rac-2b			>50	>50	
rac-2c			>50	>50	
rac-5	25				
6	10-15	15-20			
7	30-35	30-45			
8	5-10	5-10			
9	7-10	7-10			
vancomycin	0.25 - 0.5	4	1	1	
^a 10 ⁵ cfu/mL; controls with vancomycin and 1.					

Our experiments revealed a significant biological effect of pestalone (1), pestalachloride A (*rac-2a*), and some analogues against methicillin-resistant *Staphylococcus aureus*, however, only at comparably high concentrations (MIC = 10 μ g/mL for 1). Pestalone (1) was found to be about 270 times less active then reported.¹ Pestalachloride A (*rac-2a*) also exhibited a certain activity (comparable to 1), in contrast to the N-alkylated derivatives (*rac-2b/c*), which were not active at any





^aConditions: (a) RNH₂, 1,4-dioxane/H₂O, AcOH; (b) cat. NaCN, DMSO (or hv, DMSO).

reasonable concentration. Pestalalactone (*rac*-5) displayed lower antibacterial potency (MIC = 25 μ g/mL). Deformylpestalone (8) showed a similar activity to 1, against both USA300 and Mu50 (MIC = 5–10 μ g/mL).

The activity of pestalone (1) was confirmed in an independent series of broth microdilution experiments employing other bacterial strains (Table 3). *Staphylococcus aureus* strain

Table 3. Activity of Pestalone (1) against DifferentPathogenic Bacteria

		MIC [μ g/mL]	
compound	B. subtilis 168	S. aureus SG511	MRSA LT-1334
1	1.6	3.1	6.25

SG511 was affected by 1 at a MIC of 3.1 μ g/mL, while the growth of a randomly chosen clinical isolate, MRSA LT-1334, was inhibited at a MIC of 6.25 μ g/mL. As a reference, a strain of *Bacillus subtilis* 168 was also tested (MIC = 1.6 μ g/mL). It was demonstrated again that 1 acts as an antibacterial agent, however, not at the very low concentrations originally reported.¹

We also examined the antifungal potency of these compounds against various plant pathogens.9 The results summarized in Table 4 indicate that the compounds behaved very differently. Pestalone (1) displayed significant antimicrobial activity against several of the microorganisms tested (7 out of 15 plant pathogens), while pestalachloride A (rac-2a) interfered only with Pyrenophora teres (ED₅₀ = 33.1 μ g/mL) and Fusarium culmorum (ED₅₀ = 31.9 μ g/mL) with moderate activities. (ED₅₀ = effective dose that causes 50% growth inhibition.) Pestalalactone (rac-5) selectively affected Phytophthora cryptogea (MIC = 18.9 μ g/mL), Trametes versicolor (ED₅₀ = 13.1 μ g/mL), and Pyrenophora teres (ED₅₀ = 4.3 μ g/mL). The dibromo derivative (7) was selective against the most sensitive organism, i.e., Fusarium culmorum (ED₅₀ = 0.014 μ g/ mL). While compounds 6 and 10 are active, only compound 11 showed no antimicrobial activities (at $\leq 50 \ \mu g/mL$).

In conclusion, we have demonstrated that the antimicrobial activity of synthetic pestalone (1) is significantly less than that reported for the natural product.¹ In addition, we demonstrated

that pestalalactone (*rac*-**5**), pestalachloride A (*rac*-**2a**), and related isoindolinones, which are readily formed from **1** under mild conditions, also do not exhibit a particular level of activity. Compounds **1**, *rac*-**2a**, and the deformyl derivatives **8** and **9** were found to be active against methicillin-resistant *Staphylococcus aureus* strains at concentrations of $3-10 \ \mu g/mL$ (MIC). The natural products **1** and *rac*-**2a** as well as a series of synthetic derivatives were also tested against a spectrum of plant pathogenic fungi. While **1** displayed the broadest activity, none of the compounds proved to be sufficiently active to qualify as a potential crop-protecting agent.

At this point we have no explanation for the discrepancy between our results and the reported data concerning the antibiotic activity of pestalone (1). Since no reference strain was used in the susceptibility testing of natural pestalone, it is possible that the employed isolate was particularly sensitive by chance. However, our data, which are based on repeated testing in different laboratories using both reference stains and clinical isolates, should reflect the susceptibility of *S. aureus* to pestalone rather precisely. Finally, having identified 1 as a reactive agent (e.g., forming isoindolinones with primary amines), we cannot exclude the possibility that a highly active derivate of 1 had formed and caused the adventitious antimicrobial effect.

EXPERIMENTAL SECTION

General Experimental Procedures. MIC determinations (Table 2) were carried out in microtiter plates according to CLSI standards with 2-fold serial dilutions of the compounds (Clinical and Laboratory Standards Institute; NCCLS, 2006: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard M7-A6). Strains were grown in Mueller–Hinton broth (Oxoid). Bacteria were added at an inoculum of 10^{5} cfu/mL in a final volume of 0.2 mL. After incubation for 24 h at 37 °C the MIC was read as the lowest compound concentration causing inhibition of visible growth. *S. aureus* reference strains were obtained from American Type Culture Collection (ATCC 29213) or from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (USA300, Mu50, MW2). The bacterial stocks were stored at -70° C and recovered on Mueller-Hinton agar a few days before experiments.

MIC determinations (Table 3) were carried out in 96-well microtiter plates according to CLSI standards with 2-fold serial

Table 4. Activity (ED ₅₀ Values) of Pestalone ((1), Pestalachloride A (<i>rac</i> -2a), an	nd Other Derivatives (see Figure 3) against
Different Plant Pathogens			

	$ED_{50} [\mu g/mL]$						
organism	1	rac-2a	rac-5	6	7	10	11
Phytophthora infestans	>50	>50	n.d.	n.d.	>50	n.d.	>50
Phytophthora cryptogea	33.9	>50	18.9	>50	>50	>50	>50
Pythium aphanidermatum	>50	>50	>50	44.4	n.d.	>50	>50
Alternaria mali	22.4	>50	>50	>50	>50	>50	>50
Botrytis cinerea	>50	>50	>50	>50	>50	>50	>50
Septoria tritici	26.7	>50	>50	>50	>50	>50	>50
Pyrenophora teres	2.2	33.1	4.3	5.4	>50	42	>50
Leptosphaeria nodorum	22.4	>50	>50	22.5	>50	>50	>50
Fusarium culmorum	0.12	31.9	>50	1.2	0.014	2.2	>50
Gibberella zeae	>50	>50	>50	>50	>50	>50	>50
Pyricularia oryzae	28.6	>50	>50	5	>50	12.6	>50
Ustilago avenae	>50	>50	>50	>50	>50	>50	>50
Aspergillus niger	>50	>50	>50	>50	>50	>50	>50
Trametes versicolor	>50	>50	13.1	>50	>50	>50	>50
Pseudomonas fluorescens	>50	>50	>50	>50	>50	>50	>50

dilutions of the compounds. Strains were grown and tested in halfconcentrated Mueller–Hinton broth (Oxoid). Bacteria were added at an inoculum of 10^5 cfu/mL in a final volume of 0.2 mL. After incubation for 24 h at 37 °C the MIC was read as the lowest compound concentration causing inhibition of visible growth.

In Vitro Test for the Calculation of the ED₅₀ Value with Microorganisms (Table 4). Wells of 96-well microtiter plates were filled with 10 μ L of a solution of the test compound in methanol together with the emulsifier alkylaryl polyglycol ether. Thereafter, the solvent was evaporated in a hood. At the next step, into each well 200 μ L of liquid potato dextrose medium was added that had been amended with an appropriate concentration of spores or mycelium suspension of the test fungus. With the aid of a photometer the extinction in all wells was measured at the wavelength of 620 nm. The microtiter plates were then transferred for 3-5 days onto a shaker at 20 °C and 85% relative humidity. At the end of the incubation time the growth of the test organisms was measured again photometrically at the wavelength of 620 nm. The difference between the two extinction values (taken before and after incubation) was proportional to the growth of the test organism. Based on the Δ extinction data from the different test concentrations and that of the untreated test organism (control) a dose-response curve was calculated. The concentration that is necessary to give 50% growth inhibition is defined and reported as ED₅₀ value in ppm (= μ g/mL).

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Notes

The authors declare no competing financial interest.

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