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Chemoreactive Natural Products Afford Resistance against Disparate Antibiotics and Toxins**

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Abstract

Microorganisms use chemical inactivation strategies to circumvent toxicity caused by many types of antibiotics, yet in all reported cases; this approach is limited to enzymatically facilitated mechanisms that each target narrow ranges of chemically related scaffolds. The fungal-derived shikimate analogues, perioxide and pericosine A, were identified as chemoreactive natural products that attenuated the antagonistic effects of several synthetic and naturally derived antifungal agents. Experimental and computational studies suggested that perioxide and pericosine A readily react via S_N2' mechanisms against a variety of nucleophilic substances under both in vitro aqueous and in situ coculture conditions. Many of the substitution products from this reaction were highly stable and exhibited diminished toxicities against environmental fungal isolates including the *Tolypocladium sp.* strain that produced perioxide and pericosine A.

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Graphical Abstract

The fungal-derived shikimate analogues, pericoxide and pericosine A, have been identified as archetypal chemoreactive natural products that neutralize the antagonistic properties of chemically-diverse, synthetic and naturally-derived antifungal agents and toxins.

Keywords

natural product; fungi; coculture; antibiotic resistance; isotope labelling; S_N2' reactions

Antibiosis is a type of antagonistic chemical exchange that lies at the heart of many microbe-microbe interactions. The regularity with which the genes responsible for biosynthesizing antibiotics/toxins^[1] are encountered in genomes lends support to the idea that natural product ‘chemical warfare’ is a widespread and perhaps essential feature of microbial community structure and function.^[2] In many cases, the direct detection of antibiosis within native microbial communities remains a challenge; however, the biological consequences of these antagonistic interactions – antibiotic and toxin resistance mechanisms – are abundant among both antibiotic producing and non-producing bacteria and fungi.^[3] The prevalence of antibiotic resistance genes throughout natural microbial populations suggests that antibiosis has long been an evolutionary driver in the refinement and proliferation of antibiotics.

Several types of antibiotic resistance mechanisms are recognized,^[4] including (i) membrane impermeabilization, (ii) expulsion via efflux, (iii) antibiotic inactivation, and (iv) modification of cellular targets. Concerning the antibiotic inactivation strategies, all currently known systems share two key features: the processes are enzymatically driven and the enzymes target narrow ranges of structurally related antibiotics. In this report, we provide evidence for a chemically driven (non-enzymatic) toxin inactivation system employed by a soil ascomycete. This process was serendipitously discovered shortly after we initiated further chemical studies of the shikimate-PKS-NRPS metabolite, maximiscin (**1**), which our group identified from *Tolypocladium* sp. Salcha MEA-2 (T1).^[5] These results led us to determine that the shikimate portion of **1** is incorporated via a substitution reaction involving a chemoreactive precursor metabolite. This natural product is reactive toward a broad range of exogenous antibiotic/toxic chemicals. Herein we describe the unique balance of electrophilic promiscuity and chemical stability exhibited by the chemoreactive *Tolypocladium* metabolite and its analogue, as well detail how they likely serve to protect the fungus from antibiosis.

Before discussing these new discoveries, it is important to draw the reader's attention to the fact that during the initial stages of our follow-up studies about the production of **1**, we were confronted with data that were at odds with our original hypothesis of the metabolite's absolute configuration (details of these studies are provided in the supporting information). The new results led us to realize that during the original VCD experiments, in which **1** was held for hours in DMSO with warming, the compound had rearranged into a new isomeric species, isomaximiscin (**2**) (Figure 1A). Despite this setback, the finding proved to be auspicious because it (i) provided an early clue regarding the remarkable process leading to the formation of **1**, (ii) enabled us to couple the spectroscopically-derived absolute configuration results for **1** to data reported for synthetically prepared **3** (Figure 1A),^[6] and (iii) led to our development of new investigational tools (i.e., ¹³C isotopic labeling analysis and ECD measurements; refer to supporting information) that proved useful for probing the reactivity spectrum of the precursors of **1** with a range of antibiotics and toxins.

Continuing with our exploration of fungus T1, a notable attribute of its behavior was the consistent production of secondary metabolites in response to the presence of cocultured microbial species.^[5] Further studies examining additional fungal coculture scenarios confirmed the robustness of this response (Table S17). For example, UPLC-ESIMSⁿ analysis of a coculture consisting of T1 and *Penicillium* sp. P1 provided evidence for a new compound that yielded ions at m/z 464.2301 ([M+H]⁺) and m/z 278.1763 ([M+H]⁺) (Figures 2E and 2F). Curiously, the difference between these two major ions (m/z 186.0538) was identical to the neutral loss due to cleavage of the shikimate analogue moiety, which we had previously detected during the MSⁿ analysis of **1** (Figures 2A and 2D). This alerted us to the possibility that the new coculture metabolite might also contain a shikimate analogue moiety. Scale-up preparation, purification, and structure determination revealed that the new compound was structurally related to **1** and was named pseudomaximiscin A (**4**) (Figure 2F). Similar to **1**, incubation of **4** in DMSO-*d*₆ led to its isomerization resulting in a ~1:1 equilibrium mixture containing its diastereomeric product, pseudomaximiscin B (**5**) (Figure 3).

Intrigued by the discovery of **4**, we reexamined the MSⁿ data from the fungal coculture experiments and noted that in addition to the recurring m/z 186 for several new metabolites, a second neutral loss of m/z 204 (Figure 2B) was apparent. Using these two parameters to filter the MSⁿ data, a neutral loss event of m/z 204 was identified for two metabolites that were generated when fungus T1 was cocultured with *Penicillium* P2 (Figure 2H). Scale-up production yielded the new compounds, mycophenolic acid 3-*O*-pericosine (**6**) and mycophenolic acid 16-*O*-pericosine (**7**). Whereas **6** was optically active ([α]_D²⁰ -126), **7** was not, indicating that it was a racemic mixture. Deliberate probing of all samples by UPLC-ESIMSⁿ and ion-selective MS revealed that the likely non-shikimate precursor metabolites of **4**, **6**, and **7** were coming from the coculture partners (Figure 3). For example, fungus P1, was determined to be the source of metabolite PF1140 (**8**),^[7] whereas fungus P2 made mycophenolic acid (**9**).^[8] Therefore, compounds **4**, **6**, and **7** were proposed to be chimeric metabolites made from the union of **8** or **9** with a yet undetermined chemoreactive compound from fungus T1.

To determine how the T1 culture facilitated this process, an experiment was conceived using P1-derived metabolite **8** as ‘bait’ in a chemoassay-guided process meant to uncover the origins of the shikimate analogue incorporation (Scheme 1). Initially, purified **8** was mixed with dialysate prepared from one-week-old T1 culture broth. LAESIMS monitoring of the reaction revealed that compound **4**, which yielded a $[M+H]^+$ quasi molecular ion peak at m/z 464.2301 was detectable with dialysate prepared both with large (1000 kDa) and small (0.5–1 kDa) average-molecular-weight cutoff membranes. This suggested that the reaction could occur in a cell-free environment by means of a non-enzymatic process. Next, T1 cultures were successively extracted with EtOAc and *n*-butanol. Whereas the remaining aqueous layer was inactive, samples from both organic layers were able to generate **4** upon the addition of **8**. Subsequent chemoassay-directed HPLC fractionation led to the purification of two shikimate analogues, including the new epoxide metabolite, pericoxide (**10**), from the EtOAc extract, as well as the known chlorinated compound, (+)-pericosine A (**11**),^[6] from the *n*-butanol extract. These results led us to determine that **10** and **11** were the probable precursors to the non-enzymatic formation of **1** in T1 cultures, not the 6-OH analogue as we had previously implicated.^[5]

With **10** and **11** identified as candidates for the formation of the cocultured-derived hybrid metabolites, the chain of antecedence linking the two compounds was called into question. UPLC-ESIMSⁿ analysis of the MeOH extract of the cell lysate of T1 revealed that neither **10**, **11**, nor any other hybrid metabolites (e.g. **1**) were detectable intracellularly, implying that both **10** and **11** were either formed extracellularly or sequestered and secreted from the cells upon their formation. Further examining the ¹³C-labeled **10** and **11** (prepared by feeding fungus T1 [U-¹³C₆]-D-glucose), it was determined that both compounds were present in the spent culture broth as single enantiomers exhibiting “type B” ¹³C-labeling pattern (Figure 1A and supporting information). Treatment of ¹³C-labeled **10** with NaCl in ddH₂O yielded **11** seemingly via an S_N2 mechanism (Figure 4A). ECD analysis showed that **11** prepared both from **10**, as well as directly from the fungal culture broth bore the same absolute configuration (Figure S126). These results implied that **11** was non-enzymatically produced from **10** within the T1 culture. This hypothesis was tested by preparing fungal culture broth for T1 using Millipore water and observing that the yield of **11** was strongly correlated with the quantity of NaCl or other Cl[−] sources that were added to the culture medium (Figure S124). Though the origin of **10** remains undetermined, a hint of the process involved in its formation can likely be gleaned from the biosynthesis of the chorismic acid derivative, cyathiformine A,^[9] (Figure S130).

The roles that **10** and **11** might play in the production of **1** were tested by assessing their reactivities toward pyridoxatin (**12**).^[5] The production of **1** was found to occur in Millipore water upon addition of **12** to both **10** and **11**. Manipulation of selected reaction conditions (i.e., solvent, temperature, and catalyst) confirmed that epoxide **10** was generally more reactive toward **12** than its halohydrin counterpart **11**. In all cases, enantiomerically pure **1** was obtained as the product indicating that a selective S_N2' mechanism was involved in the formation of **1** under both synthetic, as well as in situ culture conditions (Figure 4B).

To test the promiscuous reactivity of **10** and **11** toward other compounds, T1 cultures were treated with a panel of substrates that included chemically diverse functional groups: hydroxamic acids, phenols, carboxylic acids, alcohols, alkenes, amides, and amines (Table S14). Candidate products from each reaction were purified and their structures confirmed by HRESIMS and multidimensional NMR. In addition, T1 cultures were supplied with [U-¹³C₆]-D-glucose so that the labeling patterns of the resulting products (Figure 3) would afford insights regarding the probable substitution mechanisms involved in their formation (i.e., S_N1, S_N2, or S_N2') (Figure S125). Additionally, the absolute configuration of the C-6' position in each product was determined by comparing its experimental and theoretical ECD data (Figure S126). Based on these analyses, the stereoselectivities of the coupling reactions (particularly *anti*- vs *syn*-S_N2' mechanisms) were systematically assessed (Figure S125; details of the structure determination for the new compounds are provided in the supporting information).

The results demonstrated that **10** and **11** were decidedly reactive toward diverse chemical targets. Stereoselective S_N2' (*anti*-S_N2' for **10** and *syn*-S_N2' for **11**) reaction processes were observed involving all the hydroxamic acids, including PF1140 (**8**), ciclopirox (**13**), and SAHA (**15**), to give optically active products **4**, **14**, and **16**, respectively. A similar S_N2' reactivity pattern was observed involving the reaction of **10** and **11** with phenol-containing [3-OH of mycophenolic acid (**9**)] and secondary-amine-containing [anisomycin (**17**)] substrates. In contrast, reaction with the carboxylic acid moiety of **9** yielded racemic **7** (Figure S26). The 5'*R**6'*R** relative configuration of the product was assigned based on an examination of its ¹³C NMR chemical shifts in comparison with DFT calculated data (Figures S12–S14, details see SI). Whether product mixture **7** arose from competing reaction processes or a rearrangement remains unknown.

The primary amine tryptamine (**19**) was also administered to T1 resulting in the formation of products **20** and **21** and an unexpected novel product named mallimiscin (**22**) (Figure 3). Distinct *J*_{H-5',H-6'} values aided in determining the 5',6'-relative configuration (*trans* >9 Hz, *cis* ~5 Hz) of the products. Whereas **20** was obtained as an enantiomerically pure product, the diastereomers (**21**) were a racemate (Figure S27). Compound **22** was seemingly formed via a Maillard reaction of **20** with D-glucose, based on its ¹³C-labeling pattern, ROESY correlations, *J*_{H,H} coupling constants, and ECD calculation (Figures 3, S126, and S127, see SI for details).

To better understand the selectivity exhibited by **10** and **11** for several of the substrates, DFT calculations were employed to determine the energies of the transition states for *syn*-S_N2', *anti*-S_N2' and S_N2 reactions of **10** and **11** with the model hydroxamic acid **23**. The β-hydroxycarbonyl hydroxy group in **23** was estimated to have a p*K*_a of ca. 7–9 indicating that its anionic form may be present as the reactive nucleophile in aqueous media. The computational results (Figure 5) were in quantitative agreement with the experimentally observed regio- and stereoselectivities of the reactions of hydroxamic acids with both **10** and **11**. For the reaction of **23** with **11**, the *syn*-S_N2' transition state (and its corresponding activation energy) was energetically the lowest by a substantial margin. In contrast, the *anti*-S_N2' pathway was favored in the reaction involving epoxide **10** by a smaller margin, dependent on the computational method. Notably, each of the transition states identified in

these calculations suggest that tautomerization occurs between the C-2 carbonyl and N-OH groups.

The observed (and calculated) exclusively *syn*-S_N2' (for allyl-X) or *anti*-S_N2' (for vinyl oxirane) selectivity detected among some of these reactions has been found in other vinylogous nucleophilic substitution reaction systems, but there are also many exceptions, depending on the substrate, nucleophile and solvent.^[10] Numerous factors have been invoked to explain the observed selectivity in such reactions, including frontier orbital and coulombic interactions, conformational and steric effects, specific Lewis acid-base and H-bonding interactions, and solvation. The origin of the remarkable and divergent stereoselectivities in the reactions of these two allylic substrates is presently unclear and its elucidation will require additional experimental and computational investigation.

Reflecting on the potential biological roles of electrophilic **10** and **11**, we noted that the natural product precursors made by T1's partner fungus had possessed antifungal activities. This prompted us to ask the question what effect the addition of the shikimate analogue moiety had on the bioactivities of compounds **8**, **9**, **12**, and the other nucleophilic substances that we tested. A panel of fungi including four *Tolypocladium* spp. (T1–T4), four *Penicillium* spp. (P1–P4) and *Aspergillus niger* (A1) were selected for assessment. The test revealed a distinct trend in which the antifungal activities of the substrates were greatly diminished or abolished following the addition of the shikimate analogue moiety (Figure 6A). For example, both the P1-derived antifungal **8** and the synthetic antifungal **13** showed growth inhibition against all of the fungal strains with MIC values in the range of 1–50 μM, whereas their adducts, **4** and **14**, exhibited an average >5-fold decrease in potencies. To test the capacity of **11** to block the toxicity of **13** in real time, fungal cultures were preincubated with **11** and then treated with varying doses of **13**. This regimen of preadministering **11** afforded up to an 8-fold decrease in the MIC of **13** (Figure 6B). Our prior time-course studies examining the production of **10** and **11** showed that these metabolites rapidly accumulated in T1 cultures after 96 hours. We had also noted that the addition of NaNO₃ to the culture medium thoroughly abolished the formation of **10** and **11**. Using this information, we determined that fungus T1 was equally sensitive to the antifungal activities of **8** or **13** in both freshly prepared normal and NaNO₃-supplemented media during a 3-day test window. However, when 5-day-old T1 culture broth was used to prepare the test medium, the non-NaNO₃-supplemented T1 culture became increasingly resistant to **8** and **13** (Figure 6C).

In summary, these studies provide evidence for a new chemically facilitated mode of toxin resistance exhibited by a soil ascomycete. Whereas previously reported resistance mechanisms involving antibiotic modification depended on the enzymatic modification of target substrates, fungal metabolites **10** and **11** functioned as electrophilic warheads that were reactive to a wide variety of natural and synthetic organic substances. The actions of these metabolites appear to limit the deleterious effects of antibiotics/toxins against their microbial targets. Further studies investigating the steric and stereoelectronic regulation of this process are expected to provide inspiration for the creation of new biologically compatible toxin scavenging materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Structures for maximiscin (**1**) and its rearrangement artifact, isomaximiscin (**2**). ^{13}C isotope incorporation (accomplished by feeding $[\text{U-}^{13}\text{C}_6]\text{-D-glucose}$ to the fungus and analyzing the resulting $^1J_{\text{C-C}}$ coupling values) was used to monitor the origin of the C-2' position in **1** and the corresponding C-2 position for the key chemical degradation product, pericosine C (**3**). (B) Experimental and calculated ECD spectra (TD-DFT) were used to resolve the absolute configurations of **1** and **2**. The illustrated ^{13}C labeling and ECD methods developed during the structure revision process for **1** were instrumental for understanding the structures and mechanisms of formation for the compounds described in this study. Details of the rationale applied to the structure revision of **1**, along with the experimental methods created for this purpose are provided in the supporting information accompanying this report.

Figure 2.

UPLC-ESIMSⁿ profiling of selected secondary metabolites produced by *Tolypocladium* sp. T1 (C and D) and coculture of T1 with *Penicillium* sp. P1 (E and F) and P2 (G and H). (A and B) Proposed MSⁿ splitting patterns for shikimate substitution products. Selected ion traces for m/z 450.2, 464.2 and 507.2 are shown in C, E, and G, respectively. Their corresponding MSⁿ spectra are shown in D, F, and H (Figure 2H shows the MSⁿ spectrum of 6, which is identical to that of 7). *MS signals of unknown substances.

Figure 3.

Substrate transformation products obtained from *Tolypocladium* sp. T1. The ^{13}C -labeling patterns for the substrate-shikimate substitution products were generated by feeding T1 with $[\text{U-}^{13}\text{C}_6]\text{-D-glucose}$.

Figure 4. Electrophilic natural products from fungus T1. (A) ¹³C-labeling patterns for **10** and **11** generated by feeding T1 [U-¹³C₆]-D-glucose with NaCl present in the culture medium. (B) Selectivity and yields for the S_N2' coupling of **12** with **10** and **11**. The reaction rates of selected model substrates (**13** and **17**) were tested and the results provided in the SI (Figure S127).

Figure 5. Calculated transition states and relative free energies of activation for the reactions of **10** and **11** with 1-hydroxy-3-methyl-2-oxo-1,2-dihydropyridin-4-olate (**23**). ^aB3LYP/6-31G(d) was used to determine TS structures; free energies were obtained from single point calculations by M06-2X and MP2 (in parenthesis) methods and 6-311++G(d,p) basis set.

Figure 6.

(A) Comparison of the antifungal efficacy of the shikimate-substrate substitution products (**1**, **4**, **6**, **7**, **14**, and **18**) and their corresponding parent compounds (**8**, **9**, **12**, **13**, and **17**) against a panel of test fungi (T1–T4, P1–P4, and A1). (B) Co-treatment of fungi with **11** and **13** (fungi T1, T2, P1, P4, and A1) reduced the antifungal efficacy of **13**. (C) Effect of supplemental NaNO₃ in test medium on the antifungal efficacy of **8** and **13** against T1. Media types: PDB, potato dextrose broth; PDB-N, PDB plus 2 g/L NaNO₃; PDB 8:2, mixture of centrifuged broth of T1 grown in PDB for 5 days with fresh PDB at the ratio of 8:2; PDB-N 8:2, mixture of centrifuged broth of T1 grown in PDB-N for 5 days with fresh PDB at the ratio of 8:2.

Scheme 1.

Chemoassay-guided identification of shikimate analogues **10** and **11**. The culture broth of T1 was subjected separately to dialysis and partitioning and treated with the P1-derived metabolite **8**. LAESIMS was used to track the presence of **10** and **11** by monitoring the formation of **4** (m/z 464 Da).