Substrate-selective catalysis enabled synthesis of azaphilone natural products

Ye Wang,† Katherine J. Torma,*†† Joshua B. Pyser,*†† Paul Zimmerman,†* and Alison R. H. Narayan†* *

†Life Sciences Institute, ††Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109, USA.

ABSTRACT
Achieving substrate-selectivity is a central element of nature’s approach to synthesis; relying on the ability of a catalyst to discriminate, based on small structural changes, which molecules will move forward in a synthesis. This approach can be challenging to duplicate in the laboratory, but can be powerful when realized. In this work, substrate-selective catalysis is leveraged to discriminate between two intermediates that exist in equilibrium, subsequently directing the final cyclization to arrive at either the linear or angular tricyclic core common to subsets of azaphilone natural products. By using a flavin-dependent monooxygenase (FDMO) in sequence with an acyl transferase (AT), the conversion of several orcinaldehyde substrates directly to the corresponding linear tricyclic azaphilones in a single reaction vessel was achieved. Furthermore, mechanistic studies support that a substrate equilibrium together with enzyme substrate-selectivity play an import role in the selectivity of the final cyclization step. A panel of azaphilone natural products and derivatives thereof were synthesized using this strategy.

Introduction
Selectivity is a central consideration in planning a synthetic strategy toward a target molecule.† Therefore, highly selective transformations are of great value. However, in contrast to the tremendous development of catalysts for chemo-, site- and enantioselective reactions, the corresponding advances in the field of substrate-selective reactions have not been achieved. One major reason for this might be that it is often desirable to achieve generality in a catalytic reaction, with activity towards as many substrates as possible.2 Despite the dearth of substrate selective methods, when substrate-selectivity can be accomplished it can enable one-pot reactions and also discriminate between substrates in equilibrium (Fig. 1a).

Biosynthetic pathways provide a rich source of substrate-selective catalysts. Based on this selectivity, it is possible to access divergent pathways toward structurally distinct natural products from common intermediates that exist in equilibrium. Carbohydrates provide a classic example of this phenomenon, with an equilibrium between the cyclic and linear forms (Fig. 1b).3 With complementary substrate-selective enzymes, different forms of the sugar can be transformed in to related natural products. Specifically, α-galactose can exist in at least five forms which are all potential substrates for different natural products. For example α-galactitol, one natural product that leads to cataracts, is formed from aldo-α-galactose through a substrate enzymatic reduction.4 From the same equilibrium, α-Galp can undergo a substrate-selective reaction to form α-Glap-1-phosphate.5 Impressively, aside from the linear form Gla, all four other isomers are very similar (Fig. 1b). By leveraging this substrate-selective approach, these different forms of α-galactose can be used in the biosynthesis of diverse complex natural products. For instance, agolagastatin, is derived from galactose, by incorporating three distinct types forms of galactose.6 When equilibrating compounds possess functional groups with distinct reactivity (e.g. the formyl group of the linear carbohydrate), it is plausible to adopt this strategy in the laboratory using small molecule reagents or catalysts; however, if the reactivity of the equilibrating compounds is not distinct, this strategy becomes a challenge to implement in the lab. Biocatalysis provides an opportunity to implement substrate-selective strategies and access divergent endpoints from common equilibrating intermediates. To demonstrate the potential of this substrate-selectivity strategy, we envisioned a divergent chemoenzymatic approach toward tricyclic azaphilone natural product with two distinct core structures (Fig. 1c).
Substrate selectivity can be used to effectively and selectively make desired products. (a) Substrate selectivity allows for selective transformations for a mixture of intermediates in equilibrium. (b) Example of substrate selective transformations in nature with the sugar galactose. (c) Utilizing a substrate selective transformation to achieve different azaphilone natural products.

Tricyclic azaphilones are a subset of this expansive family of fungal natural products which can be classified into two categories: angular and linear (see 5 and 8, respectively, Fig. 2a).7 These tricyclic azaphilones are known fungal pigments which have garnered growing interest for their antimicrobial, cytotoxic, antioxidative, and anti-inflammatory activities.7 Most recently, azaphilones were discovered as potent inhibitors of SARS-CoV-2 spike protein binding to host ACE2 receptors.8 For example, the Monascus pigment, rubropunctatin (8), which is extracted from red mold rice, shows potent cytotoxic activity against human cervical carcinoma HeLa cells and was reported as a potential dual agent for cancer chemotherapy and phototherapy. In addition, rubropunctatin (8) has a favorable selectivity index, with an IC₅₀ value greater than 300 μM for immortalized human cervical epithelial H8 cells.9 These properties make tricyclic azaphilone natural products attractive synthetic targets, yet these molecules present significant challenge due to the difficulty in accessing either the linear or angular scaffolds in a selective manner.

To date, reported examples for selective tricyclic azaphilone cyclization rely on the innate reactivity of the substrate to dictate formation of either angular or linear tricyclic products, typically as mixtures of the two. In our investigation of the natural product, trichofilicin, we demonstrated the preference for formation of the angular product under a given set of conditions reported by Karuso, Franck and coworkers.10-12 In contrast, the original report by Karuso and Franck demonstrated that selectivity for the linear product could be achieved when the substrate contained less one degree of unsaturation.12 Comparison of these substrates demonstrates that the angular product was favored when the linear ketone was in conjugation with an enol functional group (see 11, Fig. 2b). In contrast, the linear product was formed from substrate 13, in which this conjugation was broken. Nature navigates this substrate-controlled selectivity by adjusting the oxidation state of the azaphilone core post-tricycle formation, as demonstrated in the biosynthesis of rubropunctatin.13 Interestingly, the analogous angular tricyclic azaphilone can form the substrate when conjugation exists between the linear ketone and the enol of the substrate (Fig. 2c).14 In both cases, the cyclization selectivity is substrate-controlled.

Figure 1. Substrate selectivity can be used to effectively and selectively make desired products. (a) Substrate selectivity allows for selective transformations for a mixture of intermediates in equilibrium. (b) Example of substrate selective transformations in nature with the sugar galactose. (c) Utilizing a substrate selective transformation to achieve different azaphilone natural products.
Toward designing a synthetic strategy that could allow for selective access to angular or linear tricyclic azaphilones, we envisioned use of this biomimetic control over lactone ring formation could allow for the construction of many valued natural products and their synthetic analogues from easily synthesized aromatic phenol precursors by only two steps. Thus, providing rapid access to this natural product class, and providing the opportunity for investigating the impact of the ring structure and other elements contribute to a given compound’s bioactivity. Herein, we report our progress toward these goals.

Figure 2. Tricyclic azaphilone natural products and methods to synthesize them. (a) Examples of linear and angular tricyclic azaphilone natural products. (b) Previous synthetic approaches towards tricyclic azaphilones (c) Biosynthesis examples of tricyclic azaphilones. (d) Substrate selective approach to selectively form the linear and angular tricyclic azaphilones.

Results and discussion

Reaction development. Based on the established access to angular azaphilone natural products from 11, we questioned if it could be possible to access linear tricyclic azaphilones from the same substrate (11), to arrive at natural products such as rubropunctatin (8). We postulated that access to the linear tricycle from a common intermediate would require some alteration of the bicyclic core prior to installation of the lactone through an acylation/Knoevenagel condensation strategy to access linear natural products, which to date have only been isolated from natural sources with no reported syntheses.

Through our work on biocatalytic oxidative dearomatization, we came to appreciate that under aqueous reaction conditions the direct product of dearomatization (3) is in equilibrium with condensation product 2. Extraction of the product into organic solvent and removal of water affords solely bicycle 2; however, if the open form (3) could be acylated, we anticipated that the subsequent cyclization would afford linear-type azaphilone products, due to the open form not having an electron donating group conjugated with the linear ketone. To realize linear product 4, suitable acylation conditions that display both (a) high substrate selectivity for the open o-quinol 3 (over the closed form) would be necessary and (b) high chemoselectivity for the tertiary hydroxyl group over the enolic
hydroxyl group in an aqueous phase. To this end, a small library of acyl transferases (ATs) was built based on their sequence similarity to MrPigD (PigD), an AT involved in the biosynthesis of rubropunctatin. Incubating each AT with α-quinol 3 and β-ketothioester 18 revealed that PigD was the best catalysts for the desired acylation.\(^{13}\)

Carrying out a one-pot, two-enzyme sequence, which combined the flavin-dependent monooxygenase (FDMO) AzaH-mediated oxidative deamorotization with PigD-catalyzed acylation, allowed for the conversion of orcinaldehyde substrate 3 (R1 = -CH=CHCH\(_3\)) directly to a tricyclic azaphilone product in a single reaction vessel without the need to isolate the deamorotized intermediate.\(^{13,15,16}\)

Upon isolation of the product, we confirmed that the linear azaphilone, rubropunctatin (8), was exclusively formed, achieving the first total synthesis of a linear tricyclic azaphilone natural product.

**Mechanism exploration.** We propose that the selective formation of the linear tricycle arises from the selectivity of PigD for acylation of the open form of the α-quinol (3). This is based on observations made during the optimization of the preparative-scale reaction of substrate 17. While optimizing the isolation conditions of 19, we observed that the solvent conditions used for the work up afforded two different products (Fig. 3a). When using ethyl acetate to extract, three characteristic protons of rubropunctatin were detected in the \(^1\)H NMR. However, if acetonitrile was used instead to quench the reaction, the crude NMR only showed two protons which can be assigned to the open form of the linear product 20 (Fig. 3b). The UPLC traces support 20 as the major product of this reaction, which slowly undergoes ring closure in the NMR tube. These quenching and work up conditions suggest that open form (3) is the substrate that PigD prefers.

To understand the factors that govern formation of the linear tricycle (4) over the angular product (1) we first sought to understand the innate reactivity of the acylation product. Toward this end, the protected β-keto bicyclic (closed) form of the deamorotization product (21) was synthesized. Then, under acidic conditions, 21 was deprotected to reveal β-ketoester 22, which was isolated and purified by prep-HPLC. Interestingly, under the same buffer conditions as used for the PigD acylation reaction, exclusively angular product was formed (Fig. 3c). This provides evidence against the linear product being formed selectively at room temperature due to being the kinetic product. To investigate the cyclization selectivity of the open form of the acylated material, the deamorotization product 25, which does not have the ability to adopt the closed bicyclic form, was elaborated through the same synthetic route. For this “open form” substrate, under the same conditions in buffer, the Knoevenagel condensation proceeded to afford a 72:28 ratio of products (26:27) favoring the product the corresponds to linear selectivity (Fig. 3d).

Figure 3. Assessing the cyclization selectivity of various acylated intermediates. (a) The two-enzyme sequence of AzaH followed by PigD affords the linear tricycle in its closed and open forms. (b) \(^1\)H NMR of the different extraction and quenching conditions. (c) The angular cyclization selectivity of the closed form substrate. (d) The linear cyclization selectivity of the open form substrate.
To further support the divergent cyclization selectivity from the open and closed forms of the acylated intermediate, a kinetic study was designed to assess the substrate selectivity of PigD. Under the standard telescoped reaction conditions, we can measure a rate of product formation forward from 28 that is 114 µM/min. To access the closed bicyclic form of the dearmatized product 29, the product of the dearmatization reaction was extracted into ethyl acetate and purified by prep HPLC, which induces the formation of the bicycle 29. From 29, a much slower rate, 1 µM/min, was measured in the PigD acylation reaction. We hypothesize that the difference in rates of acylation of the open and closed forms (see 28 and 29, respectively) is based on the substrate-selectivity of PigD. When an equivalent of the closed form compound 29 was added to the standard telescoped reaction conditions, the rate of the second step decreased to 50 µM/min. Based on this, PigD’s preferred substrate is the open form, and the closed form compound 29 could inhibit the acylation of the open form substrate 28. These data support PigD substrate-selectivity as the origin of the linear selectivity that can be uniquely achieved using this chemoenzymatic strategy.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Comparison of the initial velocity of PigD reactions with open and closed forms of substrate. (a) The kinetics of the open form substrate. (b) The kinetics of the closed form substrate. (c) The kinetics of equal amounts of open and closed substrates.

**Reaction scope.** With experimental support for the substrate-selectivity of PigD enabling the synthesis of linear tricycles, the next question rests in the substrate scope of this selective two-enzyme sequence. To answer this question, a range of substrates and thioester acyl donors were tested in this two-enzyme sequence. The incorporation of different groups specifically at R1 and R2 was designed to probe the diversity of groups at these positions which map onto the chemical diversity present among natural azaphilones or to provide functional handles for diversification at these positions. Overall, this strategy proved useful for accessing a range of tricyclic azaphilones with good selectivity for the linear tricyclic core over the angular core (88:12 to > 95.5:4). Further, the two-enzyme sequence displayed functional group tolerance in the resorcinol substrate (see Table 1) with the dearmatization step proceeding in 91-99% conversion and the PigD acylation affording conversions of 74-91%. In addition, various chain lengths on the thioester substrate were also tolerated for the one-pot sequence. Generally, the PigD acylation proceeded in low yield with a methyl ketone, with tricycle 35 detected in trace amounts. However, when the length of the R2 chain was increased to three carbons an increase in conversion to 17% was observed whereas the five to seven carbon chains afforded yields ranging from 73%-91% to deliver tricycles with good selectivity for the linear products 91:9-94:6 rr. However, when the chain length was increased further, solubility became an issue, leading to decreased conversion (28%).

Based on this analytic data, preparative-scale reactions were also tested on substrates with good conversions. Although the conversions for the biocatalytic sequence were high (73%-99%), the isolated yields were low (26-39%, Table 1). This observation
prompted an optimization of the work-up and isolation procedure from the two-enzyme sequence. Upon precipitation of protein and cellular debris during the reaction work-up, the intense red color of the pellet suggested that the characteristically red azaphilone product was also precipitating out of solution, potentially covalently linked to protein through condensation of free amino groups onto the azaphilone pyran ring.\textsuperscript{7,17,18} To solve this problem, a small molecule amine was added in an attempt to out compete the condensation with amino groups present in the protein or other biomolecules. After stirring for just five minutes with an amine, the product could be isolated in an improved yield of 69%. For alkynyl substrate 3 (R\textsubscript{1} = -CH=CHCH\textsubscript{3}), the unexpected thiol Michael addition side reaction also complicated scale-up, leading to variable yield confirmed isolated yield dependent on reaction time. To solve this problem, maleimide was used as a thiol scavenger to capture free pantetheine liberated over the course of the acylation reaction.\textsuperscript{19,20} After the addition of maleimide (2 equivalents), this byproduct was not detected, allowing for a 30% isolated yield of rubropunctatin with the second step conversions of 78%. Using an analogous approach, monascorubrin (9) was isolated in 19% yield over two steps with the second step conversions of 75%.

As many azaphilone natural products with variation at R\textsuperscript{2} which exceeds the substrate scope of PigD exist, we sought a thioester acyl donor that could allow for downstream functionalization.\textsuperscript{7} When ester 31 (R\textsuperscript{2} = OnBu) was tested as the acyl group donor, 82% conversion of the o-quinol intermediate was observed on analytical-scale. The resulting acylated intermediate displayed unique behavior. With a pKa of the 1,3-diester not low enough for the Knoevenagel condensation to spontaneously proceed under the enzymatic acylation conditions, the direct acylation product 43 observed as the major product. From 43, either the linear or angular tricycle could be selectively accessed. If 43 was treated with triethylamine, the cyclization from 43 was induced to afford the linear product following extraction into ethyl acetate. In contrast, direct extraction of 43 into ethyl acetate afforded the acylated bicycle 45 as major product, which upon treatment with Hünig’s base cyclized to give the angular tricycle 46. This further points to the substrate control as the origin linear selectivity rather than a cyclization that is dictated by the AT.
**Table 1.** Substrate scope of two-enzyme sequence to access linear tricyclic azaphilones.\(^a\)

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<tr>
<th>Substrate</th>
<th>Conversion</th>
<th>Yield (%)</th>
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<tbody>
<tr>
<td>30</td>
<td>74%</td>
<td>27%</td>
</tr>
<tr>
<td>19</td>
<td>91%</td>
<td>39%</td>
</tr>
<tr>
<td>33</td>
<td>99%</td>
<td>37%</td>
</tr>
<tr>
<td>34</td>
<td>85%</td>
<td>32%</td>
</tr>
<tr>
<td>8</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td>9</td>
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**Application.** With an established strategy for accessing tricyclic linear azaphilones, we sought to access additional natural products in this family beyond rubropunctatin (8) and monascorubrin (9). First, we investigated the reduction of the azaphilone core to access natural products such as monophilol B (47) and pitholide D (10).\(^{21,22}\) After exploring several reduction conditions, BH\(_3\)·DMS was identified as a broadly applicable reducing agent. For example, rubropunctatin (8) could be directly reduced to afford monophilol B (47) as single diastereomer in 77% yield. However, same reaction with gave an unexpected result, delivering a product that did not match the reported \(^1\)H NMR spectrum of the natural product pitholide D (10). Nearly all the \(^1\)H NMR peaks matched with the isolation paper, except for the methine at the newly set stereocenter, suggesting that a diastereomer of the pitholide D was synthesized in 73% yield. Since the \(^1\)H NMR did not match the reported data generated using the same reduction method for pitholide D (10) as monophilol B (47) which have the same reported relative configuration, it became clear that at least one of these structures had been misassigned (Fig. 5).\(^{21,22}\) The original structure determination paper assigned the relative stereochemistry based on an NOE signal between two functional groups in which the distance between the two group would be less than 5 Å whether the relationship between these groups was syn or anti. To further characterize our synthetic material, monophilol B was acylated with a 4-nitrobenzoyl group. An NOE signal between the ortho-proton on the 4-nitrobenzoyl group and the relevant methyl group was
detected, which supports that the methyl group and the hydroxyl group are arranged in a syn fashion. In addition, a number of natural products were accessed through amination rubropunctatin. For example, rubropunctamine and rubropunctin L-alanine were synthesized from the corresponding amine in high yields (89 and 90%, respectively).\textsuperscript{23-25} With synthetical natural products in hand, the absolute configuration of each compound was characterized by comparing the experimental optical rotations to those reported for the natural compounds. Because the enzymatic dearomatization with AzaH exclusively affords the R-product and the optical rotation measurements for each synthetic compound were opposite to those reported in the isolation papers, the absolute configuration of (−)-rubropunctatin and (−)-monascorubrin are S, not R as originally reported.\textsuperscript{25-27}

**Figure 5.** Transformation of common tricyclic azaphilone core into a variety of natural products and relate structural reassignments.

**Conclusion**

In summary, a substrate selective-strategy was developed to access linear tricyclic azaphilone natural products. Through a two-enzyme, one-pot sequence, linear tricyclic azaphilone scaffolds were built from readily available resorcinol starting materials. Specially, five linear azaphilone natural products were synthesized for the first time. In addition to this synthetic achievement, the origins of the observed selectivity were investigated to support that an enzyme, PigD, selectively acylates the open form of the substrate which controls the selectivity of the subsequent cyclization step to afford the linear azaphilone tricyclic core. This opens a new gate for divergent synthesis as well as retrobiosynthetic analyses of similar natural products.

**Corresponding Author**

*arhardin@umich.edu

**Notes**

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