Enzyme Library-enabled Chemoenzymatic Tropolone Synthesis

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Abstract

Enzymes can offer an enticing tool for building complex chemical scaffolds through succinct routes and under mild conditions. Yet, the common application of biocatalysts in organic synthesis is often hampered by unpredictable substrate scope and scalability challenges, deterring the planning of biocatalytic steps at the retrosynthetic planning stage. Herein, we detail a method using a sequence similarity network to curate a library of non-heme iron (NHI)-dependent enzymes capable of performing complexity generating biocatalytic transformations. In the course of this study, we probed the substrate scope of TropC-like enzymes to furnish a range of β-hydroxytropolone products. The potential to access diverse scaffolds was investigated and a variety of tropolone-containing molecules were prepared on milligram-scale. Furthermore, chemoenzymatically generated tropolones were transformed through a variety of chemistries to achieve the total synthesis of stipitaldehyde, an abbreviated formal synthesis of deoxyepolone B, and additional tropolone building blocks with a high density of functional handles. This work lays the foundation for using NHI enzymes in retrosynthetic planning of complex molecules and natural product analogues.

Introduction

Nature has an elegant way of creating complex natural products from a small pool of building blocks using a series of specialized proteins that can mediate highly substrate-, chemo-, site-, and stereoselective reactions.[1-4] Prior to the development of modern organic synthesis, nature was the sole source of all medicines and evolved to construct complex molecules through efficient biosynthetic routes (Fig. 1A).[5-6] Transformations in natural product biosynthesis are often performed with nearly perfect site- and stereoselectivity while channeling material toward a target product.[7-8] The substrate specificity observed in natural product biosynthesis provides an advantage in the synthesis of a single target molecule, but can hinder the application of biosynthetic enzymes beyond the synthesis of the specific natural product they were evolved to make. Although natural product biosynthesis is a finely tuned process, obtaining quantities of specific metabolites to support biological studies and serve as a primary source for commercial drugs can be an insurmountable challenge.[9]

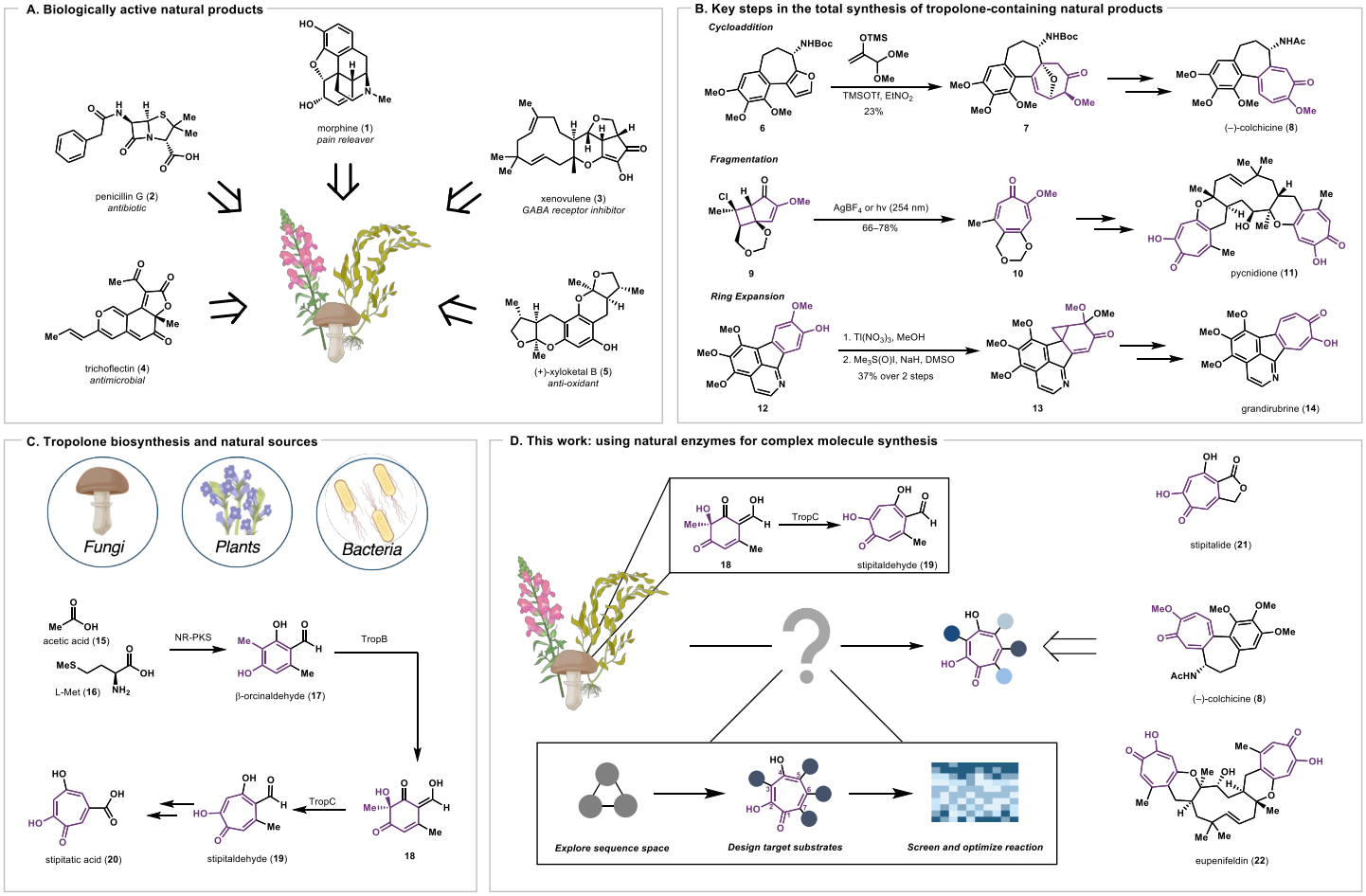

Figure 1. A. Biologically active natural products can be isolated from a variety of biological organisms. B. Key tropolone forming steps in the total syntheses of diverse tropolone natural products. C. Organisms natural tropolones can be isolated from (top) and the biosynthetic pathway of stipitatic acid in fungi (bottom). D. Proposed work whereby exploring enzyme sequence space and designing non-native target substrates can lead to a retrosynthetic strategy to reach diverse tropolone precursors to natural products and analogs.

To overcome the shortcomings of *in vivo* natural product biosynthesis, chemists often develop complementary approaches that rely on a breadth of established small molecule-mediated methods. Organic and inorganic reagent-based transformations can provide access to natural and unnatural complex molecules, greatly expanding the realm of attainable compounds.[5, 10] The utility of lab-based synthesis is highlighted by established approaches toward tropolone-containing natural products. For more than 50 years, chemists have sought ways to access these valuable scaffolds, characterized by their interesting physical properties and therapeutic potential.[11] It has been demonstrated that tropolones can behave as antifungicidal, anticancer, and antimitotic agents such as stipitaldehyde (**19**), eupenifeldin (**22**), and colchicine (**8**), respectively.[12-14] Furthermore, their metal chelating capacity positions them as superb iron transporters in iron-deficient cells and also as metalloprotein inhibitors.[12, 15] By taking advantage of classic cycloaddition strategies[16], fragmentation reactions[17], and Buchner cyclopropanations[18], amongst others[19], synthetic chemists have been able to access a small subset of these highly valuable molecules through intricate total syntheses, enabling their biological evaluation (Fig. 1B). Nonetheless, access to tropolones that vary in their substitution pattern and embedded functional groups can be a challenge that limits entry to this class of natural products. This is highlighted by the large number of tropolone-containing complex molecules that have not yet been synthesized, such as stipitalide[20] (**21**) and eupenifeldin (**22**).[21]

Emerging chemoenzymatic synthetic strategies offer an alternative approach to furnish complex molecules.[22-27] Biocatalytic transformations implemented in a chemoenzymatic synthesis enjoy the advantages observed in biosynthesis, notably performing highly-selective chemistry, whilst often circumventing the need for protecting groups.[27-29] Nonetheless, although chemoenzymatic synthesis has been fruitful in accessing a range of molecules[30-31], there are still challenges in chemoenzymatic routes to tropolone molecules, most likely due to numerous challenges regarding the general synthetic application of biocatalytic methods.[32-34] Notably, many classes of enzymatic reactions are largely underdeveloped on preparative-scale and have remained difficult to employ as intermediate steps in synthetic routes.[27, 29, 35-36] In addition to the practical limitations, enzymatic transformations remain difficult to incorporate into retrosynthetic planning, as substrate scope data beyond the native function of a biocatalyst is often not available.[23, 37-38] To date, biosynthetic pathways of several tropolone natural products have been fully elucidated from bacteria[39-40], plants[41], and fungi[42-44] (Fig. 1C). For example, Cox and coworkers discovered a two-enzyme cascade within the stipitatic acid (**20**) biosynthetic pathway responsible tropolone in fungi.[42] This two-step sequence proceeds from polyketide synthase-derived β-orcinaldehyde **17**, which is oxidatively dearomatized by a flavin-dependent monooxygenase (FDMO), TropB to afford **18**. Intermediate **18** then undergoes an oxidative ring expansion catalyzed by an α-ketoglutarate-dependent (α-KG) non-heme iron(II) (NHI)-dependent oxygenase, TropC, to yield stipitaldehyde (**19**). The direct conversion of an unprotected resorcinol to a tropolone product via this two-enzyme sequence provides a streamlined synthetic route for accessing tropolones. TropB has been well characterized and substrate scope investigations have been completed by our group and others, providing great precedent for accessing diverse dearomatized intermediates.[45-46] However, this potential remains untapped as the scalability and substrate scope of reactions with TropC remain unknown. To provide a platform to overcome the obstacles of incorporating biocatalysts at the stage of synthetic planning, experimental work is necessary to bridge the gap between biosynthetic data and retrosynthetic strategies. Here, we demonstrate our approach toward this end, targeting the chemoenzymatic synthesis of tropolone scaffolds (Fig. 1D). In an effort to reach synthons of tropolone-containing molecules, we developed a scalable, two-enzyme sequence inspired by the biosynthetic pathway of the natural product stipitatic acid (**20**) that can transform resorcinol substrates into tropolone products. This entails a method for chemoenzymatic-inclusive retrosynthetic planning and provides a chemoenzymatic route for accessing tropolone molecules in a streamlined manner.

Results and Discussion

Designing a diversity-oriented biocatalytic strategy

To access unnatural tropolone products, we designed a retrosynthetic approach dependent on the identification of tropolone-forming enzymes available in enzyme databases (Fig. 2A). It has been demonstrated by our lab and other groups that exploring enzyme sequence space can allow for the exploration of unknown enzyme reactivity.[47-49] Therefore, we hypothesized that natural enzymes with sequence similarity to TropC could be capable of performing the same biocatalytic transformation.

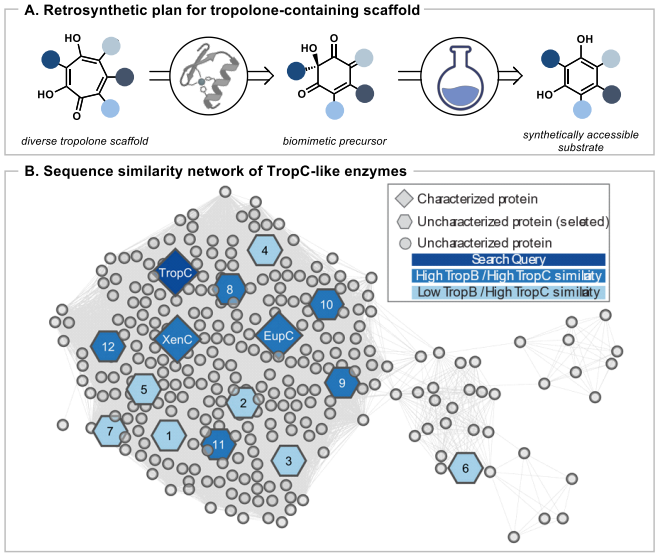

Figure 2. A. Proposed retrosynthetic approach to access diverse tropolone scaffolds from synthetically accessible substrates through a biomimetic oxidatively dearomatized precursor enabled by investigation of enzyme sequence space. B. Sequence similarity network of enzyme sequences closely related to the tropolone forming enzyme TropC from the biosynthetic pathway of the tropolone natural product stipitatic acid (**20**).

Towards building a library of TropC-like enzymes, we constructed a sequence similarity network (SSN) to generate a library of proteins with unknown chemical function. We first curated a subset of enzymes from the Joint Genome Institute (JGI) database that have high sequence similarity to TropC. To compliment this list, the amino acid sequence of TropC was used as a Basic Local Alignment Search Tool (BLAST) query sequence with an *e*-value of five to identify enzymes closely related to TropC within a wider enzyme sequence space to provide a broader representation of enzymes. Using the Enzyme Function Initiative – Enzyme Similarity Tool (EFI-EST) and the collected sequences, we constructed an SSN of the sequence space related to TropC-like enzymes (Fig. 2B).[50-51] Interestingly, three of these enzyme sequences had been previously identified as part of tropolone-containing natural product biosynthetic pathways (TropC, XenC, and EupC, involved in the biosynthesis of stipitaldehyde (**19**), xenovulene A (**3**), and eupenifelden (**22**), respectively).[46, 52-53] Because all three of these enzymes are proposed to generate the same tropolone intermediate **19**, we limited our selection to only TropC and XenC to reduce the potential redundancy of the generated enzyme library.

Additionally, we selected twelve TropC homologs with potential to perform ring expansion chemistry on diverse substrates. From the twelve homologs selected, we identified five enzymes (homologs 8-12) that were found within organisms that also contained sequences highly similar to TropB (>20% similarity), and seven (homologs 1-7) that were sequenced from organisms not containing enzymes with high similarity to TropB (see Supporting Information for additional detail and Fig. 2B). Although bioinformatic tools such as SSNs have been used to visualize trends in biocatalytic reaction data of various families of enzymes[54-55], to the best of our knowledge, within the class of α-ketoglutarate-dependent enzymes, SSNs have not been used to curate a selection of biocatalysts for reactions with diverse substrates. We were pleasantly surprised that all fourteen synthetic genes were successfully overexpressed in *E. coli* to afford soluble protein.

With our targeted enzyme library generated, we next sought to assess the viability of the proposed retrosynthetic plan by designing β-hydroxy tropolone biomimetic precursors with diverse functional handles strategically positioned at C-3, C-5, C-6, and C-7 (Fig. 1D). Importantly, none of the proposed substrates have been previously reported as native substrates in biosynthetic pathways or enzyme characterization databases.

Assessing the biocatalytic activity of curated library towards tropolone products

Upon curation of a library of biocatalysts and synthesis of diverse substrates, optimal conditions for analytical-scale reactions were investigated. Using the TropC native substrate **18** as a model substrate, we determined that conversion to tropolone product was possible with clarified TropC cell lysate in TES buffer with the necessary cofactors. Upon probing shaking and oxygenation conditions, we found that 200 µL reactions provided full conversion to stipitaldehyde (**19**) upon shaking at 300 rpm for 3 hours (Fig. 3A). It is worth noting that enzymatic ring expansion is not observed with TropC in whole *E. coli* cells and that the amount of product present in reactions decreases beyond 12 hours (see Supporting Information).

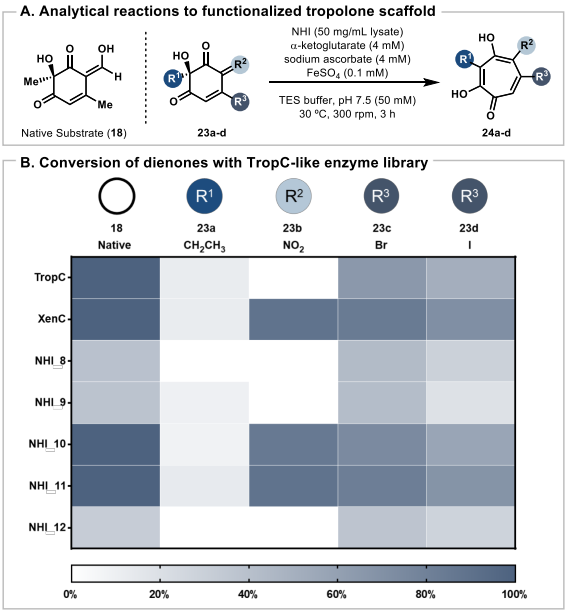

Figure 3. Conversion data from the screening of the top six enzymes identified to carry out ring expansion on representative non-native dearomatized substrates as compared to the query enzyme TropC.

With optimized reaction conditions in hand, we explored the compatibility of this one-pot, two-enzyme sequence with our representative resorcinol substrates (see Supporting Information). Upon reaction with TropB, we were able to access four of the proposed biomimetic precursors to various tropolone products (**23a-d**, Fig 3B). We explored various substrates with substitutions at the C6-position, but none were tolerated by the oxidative dearomatization step mediated by TropB (see Supporting Information). Due to instability upon isolation of the dearomatized species, stereochemistry is depicted as analogous to the TropB native substrate, but was not independently confirmed for each intermediate.[45, 52] Impressively, the compatible resorcinol substrates with variation at R^1 and R^3 were fully converted to their dearomatized dienone products, whereas substitution of the R^2 position with a nitro group furnished the respective dienone product in 63% conversion.

The biomimetic intermediates (see **23a-d**) accessed through biocatalytic oxidative dearomatization were then subjected to reaction conditions with each TropC-like enzyme. Six of the selected enzymes demonstrated activity with the substrates designed for ring expansion. XenC and five of the TropC homologs demonstrated complimentary or superior activity and substrate scope to TropC (Fig. 3B). Variation at the C-3, C-5, and C-6 positions were tolerated. Out of the enzymes tested, XenC offered the largest substrate scope and highest conversion to tropolone products, leading us to adopt it as the key enzyme to mediate enzymatic tropolone formation. These results provide context for the chemical utility of each enzyme and inform the planning of this biocatalytic transformation into retrosynthetic plans.

Scalability of NHI transformations and optimization

The diverse scaffolds accessible through the developed one-pot sequence provide the opportunity to investigate chemoenzymatic syntheses of tropolone-containing complex molecules. Therefore, to probe the synthetic utility of this tropolone-forming strategy, we explored the behavior of the two-enzyme sequence on preparative-scale. Through initial experiments, we were able to observe significant product formation on microgram-scale; however, the conversion on milligram-scale was not comparable. In addition, the isolation of the tropolone products from the biocatalytic reaction mixture proved challenging due to the tropolones' tendency to decompose upon chromatographic purification and insolubility in traditional organic solvents such as methanol, acetone, hexanes and dichloromethane.

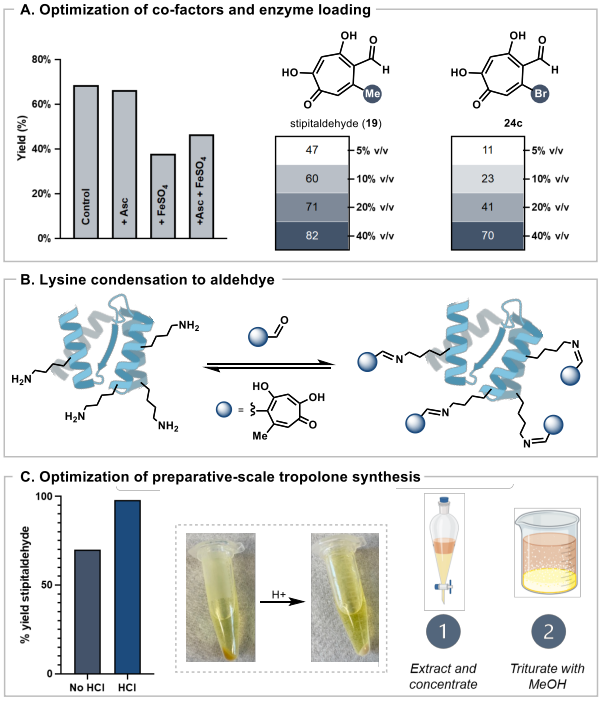

Figure 4. A. Reaction cofactor optimization for the ring expansion of stipitaldehyde on preparative scale. Conversion data determined after 1 h of reaction with crude cell lysate. Lysate concentration optimization of the native substrate **19** and substrate **24c** in the range of 5%–40% v/v crude cell lysate. Yield (for **19**) and conversion (for **24c**) determined after 1 h of reaction. C. Lysine residue condensation of aldehyde-bearing tropolones and optimization of tropolone isolation from biocatalytic reactions.

To address the dissonance in progression of the reaction on different scales, preparative-scale reaction conditions of the XenC step, including cofactor concentration, enzyme lysate concentration, reaction shaking speed, and reaction time, were optimized using **18** as a model substrate. A screen of traditional cofactors used with α-KG NHIs showed that addition of $FeSO_4$ to the reaction mixture significantly decreased the conversion to product. Furthermore, the addition of ascorbate had no significant effect unless in the presence of iron, where a slight increase in conversion was observed. In addition, increasing the concentration of lysate to 40% v/v provided a boost in yield from 47% to 82% on milligram-scale as determined by product calibration curve (see Supporting Information for additional detail). Gratifyingly, the lack of ascorbate and iron, and increased enzyme loading combined with reaction shaking speed of 300 rpm led to an increase in yield of stipitaldehyde (**19**, Fig. 4A). Applying these optimized conditions led to an increase in conversion for a breadth of substrates, for example substrate **24c**.

Upon attempting to isolate tropolone products from biocatalytic reactions, we observed that the calculated yields did not correlate with the amount of product that could be isolated. Notably, after quenching the reactions

and subjecting the mixture to centrifugation, the pelleted enzyme debris was a dark orange color, the characteristic color of tropolones. We hypothesized that inconsistencies in recovery were associated to the remaining tropolone interacting with the biological debris from the biocatalytic reaction through advantageous imine condensation with the aldehyde and the lysine residues on the enzyme (Fig. 4B). This hypothesis is supported by the 60% conversion to a tropolone-lysine imine adduct reactions producing stipitaldehyde, which also contained *N*-α-Boc-*L*-lysine methyl ester (see Supporting Information). Therefore, we surmised that addition of a strong acid could hydrolyze this bond and release the remaining tropolone. As expected, quenching the reaction with 6 M hydrochloric acid liberated the tropolone product and allowed for isolated yields greater than those observed under analytical- and unoptimized preparative-scale reactions. Specifically in the synthesis of stipitaldehyde (**19**), the isolated yield increased from 60% to >95% on milligram-scale (Figure 4C). Gratifyingly, quenching with acid and taking advantage of the innate solubility properties of tropolone products allowed for the isolation of product through trituration in a manner analogous to the 1993 Bristol-Meyers Squibb isolation of eupenifeldin (Fig. 4C).[56] Overall these efforts led to a scalable chemoenzymatic synthesis of the antifungal tropolone stipitaldehyde (**19**) in only three steps from commercially available methyl atratate (**SI-1**) and 82% overall yield.

Complexity building with functionalized tropolone scaffolds

With a scalable route to tropolone-containing molecules, we sought a greater understanding of innate tropolone reactivity and compatibility with complexity-building transformations. We selected a representative set of chemoenzymatically synthesized tropolones (**19**, **24b**, **24c**) to investigate the reactivity profile of these densely functionalized tropolone scaffolds. To begin, we investigated means to directly modify **19** in effort to generate derivatives of this compound that are valuable for natural product synthesis. Previously, Baldwin and coworkers demonstrated that thioether **25** was a stable precursor to the active diene in the hetero-Diels Alder reaction with humulene to synthesize (±)-deoxyepolone B.[57] With this precedent, thioether **25** serves as a promising building block towards the synthesis of related bioactive natural products including epolone B[58-59], pycnidione[60], neosetophomone B[59], and eupenifeldin.[57-61] However, previous construction of (±)-deoxyepolone required twenty synthetic transformations to access **25**.[57] We anticipated that accessing **25** would be possible through reduction of aldehyde **19**, and, after formation of the active *ortho*-tropoquinone methide (*o*-TQM) intermediate, nucleophilic attack by thiophenol. Pleasingly, our planned strategy was successful, enabling access to **25** in just four synthetic transformations. Additionally, we sought to facilitate aldehyde oxidation of **19** in effort to access the *ortho*-methyl benzoic acid motif in **26** that could be used in subsequent oxidations to yield stipitalide, a previously identified biosynthetic intermediate towards xenovulene A, a potent GABA-benzodiazepine inhibitor.[62] Under Pinnick conditions, we have demonstrated access to **26** in moderate yield.

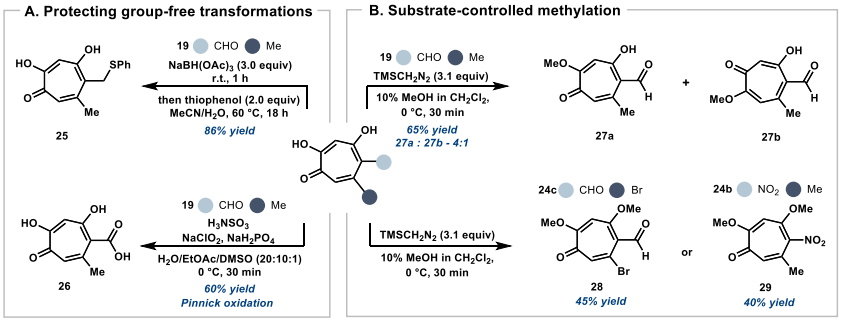

Figure 5. (A) Derivation of stipitaldehyde to synthetically valuable products possessing the free α-hydroxy enone motif. (B) Methylation of various tropolones obtained from two-enzyme cascade for increased substrate stability in purification and downstream transformations.

Although **19** could be directly transformed into compounds **25** and **26**, we found that, in general, the tropolone scaffold was challenging to isolate and had limited solubility in a number of common organic solvents, creating a challenge in performing downstream transformations. It has been postulated that the free α-hydroxy ketone moiety is a major contributor towards these observations.[17, 63-64] To overcome these challenges presented by the free hydroxyl moieties, we found that mono- or dimethylation of the α- and/or γ-hydroxy positions generated protected tropolones that were more amenable to downstream synthetic transformations. Typically, the ability of tropolones to tautomerize can add complication in tropolone natural product synthesis, often furnishing equal mixtures of isomers when exposed to methylating conditions.[17] Interestingly, we found that stipitaldehyde (**19**), when monomethylated using trimethylsilyldiazomethane, furnished the α-methyl ethers **27a** and **27b** in 65% yield and 4:1 selectivity favoring isomer **27a.** When addressing more functionalized tropolone scaffolds, bromotropolone **24c** was subjected to methylation conditions and provided solely the dimethylated product **28** in 45% yield. Similarly, nitrotropolone **24b** was treated to identical methylation conditions to furnish dimethylated tropolone **29** in 40% yield.

Conclusions

In this report, we demonstrate a strategy for bridging the gap between biosynthesis and chemical synthesis by taking a bioinformatics-based approach to profile the synthetic potential of a chemoenzymatic route. By building an enzyme library based on sequence similarity to a characterized enzyme, TropC, we developed the platform to explore the chemical space available to chemoenzymatic tropolone synthesis. The optimization of this chemoenzymatic platform has enabled the preparative-scale synthesis of tropolones and allowed for the investigation of tropolone chemistry. We anticipate that this strategy of canvasing the synthetic potential of biocatalytic reactions will accelerate the adoption of chemoenzymatic synthetic approaches by allowing for informed retrosynthetic planning that takes the best of what small and large molecule catalysts have to offer.

Methods

***Procedure for enzyme library curation*.** Sequence similarity networks (SSNs) were generated through EFI-EST using TropC as a BLAST query sequence with an e-value of 5 and an alignment score threshold of 100. The genes encoding *tropC*, *xenC,* and each of the 12 homologs were codon-optimized for overexpression in *E. coli* and synthesized by Twist Biosciences. The synthesized sequences were subcloned by Twist biosciences into a pET-28a vector containing the T7 expression system, kanamycin resistance, and N-terminal 6xHis-tag encoded upstream from the insert gene. Overexpression and preparation of clarified crude cell lysates of *tropC*, *xenC*, and *homologs 1-12* was accomplished utilizing previously reported methods.[26, 45]

In vitro analytical-scale reactions. Dearomatization reactions contained KPi buffer pH 8.0 (500 µL, 1 M), 5 mM substrate (500 µL, 1 M in DMSO), 1 mM NADP (100 µL, 100 mM), 10 mM G6P (200 µL, 500 mM), 5 U/mL G6PDH (500 µL, 100 U/mL), and 40 mg/mL TropB lysate (2 mL, 200 mg/mL) diluted with milliQ water (6.2 mL) to a final volume of 10 mL in an Erlenmeyer flask fitted with a breathable cover. These were then shaken at 125 rpm at 30 °C for 18 h and monitored for conversion to the intermediate product by UPLC-DAD analysis. After 18 h, the reaction was divided into individual 500 µL-scale ring expansion reactions with TropC, XenC, and the 12 homologs in 96-well plate format as follows: Each well contained 50 mM TES buffer pH 7.5 (25 µL, 1 M), 2 mM substrate (200 µL, 5 mM), 4 mM α-KG (20 µL, 100 mM), 4 mM sodium ascorbate (20 µL, 100 mM), 10 mM Fe_2SO_4 (10 µL, 10 mM), and 40 mg/mL NHI lysate (100 µL, 200 mg/mL) diluted with milliQ water (125 µL) to a final volume of 500 µL. 96-well plates were shaken at 300 rpm at 30 °C for 4 h and quenched by addition of 3 volumes of MeOH containing 500 µM pentamethylbenzene as an internal standard. Precipitated biological debris was pelleted by centrifugation of the 96-well plates (1250 x g, 20 min). The supernatant was analyzed by UPLC-DAD and conversion obtained by comparison of the dearomatized substrate and ring expanded product peaks.

Preparative-scale reactions. Preparative-scale reactions were conducted on 20-200 mg of substrate using the following conditions: dearomatization reactions contained KPi buffer pH 8.0 (50 mM final, 1 M stock[AN1]), substrate (5 mM final, 100 mM stock in DMSO), NADP+ (1 mM final,100 mM stock), sodium phosphite (10 mM final, 500 mM stock), PDH (10 μM final, 500 μM stock), and TropB lysate (40 mg/mL final, 200 mg/mL stock) diluted with milliQ water to the correct volume for substrate concentration in a flask of appropriate size (generally 2-3x the reaction volume) fitted with a breathable cover shaken at 125 rpm at 30 °C for 2 h or until substrate is completely converted as determined by UPLC-DAD analysis. Upon completion, TES buffer pH 7.5 (50 mM final, 1 M stock), α-KG (4 mM final, 100 mM stock), and XenC lysate (80 mg/mL final, 200 mg/mL stock) diluted with milliQ water to a final volume of 2.5x the previous reaction volume were added and the contents were shaken at 250 rpm and 30 °C for 1 h. The reaction was then quenched by the addition of 6 M HCl (5 vol %) and allowed to stir at room temperature for 30 min followed by extraction with 3 volumes of ethyl acetate. The combined organic extracts were dried over Na_2SO_4 and concentrated. Unless otherwise noted, the concentrate was then triturated with cold methanol to yield the tropolone products as a solid which were collected by filtration.

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Notes

The authors declare no competing financial interest.

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