Total Synthesis Facilitates *in vitro* Reconstitution of SgvP, the C–S Bond Forming P450 in Griseoviridin Biosynthesis

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ABSTRACT: Griseoviridin is a group A streptogramin natural product from *Streptomyces* with broad-spectrum antibacterial activity. A hybrid polyketide-non-ribosomal peptide, it comprises a 23-membered macrocycle, an embedded oxazole motif, and a macrolactone with a unique ene-thiol linkage Recent analysis of the griseoviridin biosynthetic gene cluster implicated SgvP, a cytochrome P450 monooxygenase, in late-stage installation of the critical C–S bond. While genetic and crystallographic experiments provided indirect evidence to support this hypothesis, the exact function of SgvP has never been confirmed biochemically. Herein, we report a convergent total synthesis of pre-griseoviridin, the putative substrate of P450 SgvP and precursor to griseoviridin. Our strategy features concise and rapid assembly of two fragments joined via sequential peptide coupling and Stille macrocyclization. Access to pre-griseoviridin then enabled *in vitro* validation of SgvP as the C–S bond forming P450 during griseoviridin biosynthesis, culminating in a 9-step chemoenzymatic synthesis of griseoviridin.

INTRODUCTION

Cytochromes P450 constitute a large family of heme-dependent enzymes that are involved in a multitude of oxidative transformations.[1] Among Nature's most ubiquitous oxidation catalysts, these enzymes are found across all domains of life, participating in fundamental biological processes such as drug metabolism,[2] xenobiotic degradation,[3] and natural product biosynthesis.[4] Indeed, P450s are known to mediate hydroxylation,[5] epoxidation,[6] *N*- and *S*-oxidation,[7] oxidative coupling,[8] and C–N/S bond formation,[9] among many other transformations.[10] This versatility has long attracted the attention of the scientific community, and owing to improved technologies for genome mining and bioinformatics,[11] the number of P450-encoding genes has increased drastically over the last decade.[12]

Likewise, advances in enzyme engineering and protein expression have generated myriad opportunities for the merger of biocatalysis and organic synthesis,[13] where P450s and other enzymes are employed in diverse contexts.[14] In the synthesis of complex natural products, for example, P450s are effective catalysts for regio-, chemo-, and stereoselective C–H oxidation.[15] This transformation is highly valuable since (1) the hydroxyl group serves as a key handle that enables further downstream functionalization, and (2) traditional methods struggle to match the selectivity of P450s in a variety of settings.[15] On the other hand, works by Arnold,[16] Narayan,[17] and others[18] have established powerful methods for C–C bond formation using engineered P450s or variants thereof, providing concise access to stereochemically enriched small molecules. Beyond C–O and C–C bonds, however, the biocatalytic repertoire of P450s remains largely underexplored.

In particular, a growing number of sulfur-containing secondary metabolites are thought to have arisen from P450-mediated C–S bond forming events.[9] Investigations into the biosyntheses of camalexin,[19] cyclobrassinin, and spirobrassinin[20] in plants uncovered several P450s responsible for intramolecular C–S bond installation (Figure 1A). More recently the intramolecular *S*-heterocyclization event in chuangxinmycin biosynthesis was attributed to P450 CnxD, its activity then confirmed through biochemical and crystallographic characterization.[21] In addition to their biosynthetic roles, P450s can also effect C–S bond formation with non-native substrates or via non-natural reactivity. Studies from Abe and coworkers have demonstrated the ability of P450 TleB, which performs intramolecular indole amination during indolactam V biosynthesis,[22] to instead catalyze C–S bond formation toward unnatural sulfur-containing indolactams.[23] Conversely, the Arnold lab reported an engineered P450 capable of lactone-carbene S–H insertion to produce α-thio-γ-lactones.[24] Nevertheless, while P450s are implicated in the construction of several other C–S bonds in natural products,[21] thorough characterization of these enzymes remains elusive.

Griseoviridin (**1**) is a sulfur-containing, group A streptogramin natural product isolated concurrently with the group B streptogramin viridogrisein from *Streptomyces griseoviridis* (Figure 1B).[25] Despite bearing no chemical resemblance, group A and B streptogramins are frequently isolated together due to colocalization of their biosynthetic gene clusters.[26] Group A streptogramins feature a hybrid polyketide-non-ribosomal peptide macrocycle and exhibit synergistic antibacterial activity when co-administered with group B streptogramins.[27] For instance, Synercid is a formulation of quinupristin, a group A derivative, and dalfopristin, a group B derivative, approved by the U.S.

Food and Drug Administration (FDA) for the treatment of vancomycin-resistant *Enterococcus faecium* (VREF) bacteremia.[28] In contrast to other group A streptogramins, such as the madumycins and virginiamycins, griseoviridin bears a unique nine-membered, vinyl thioether-containing macrolactone essential for biological activity.[29] A single prior synthesis of griseoviridin has been reported,[30] and the lactone motif remains challenging to access despite several published approaches (Schemes S1 and S2).[31]

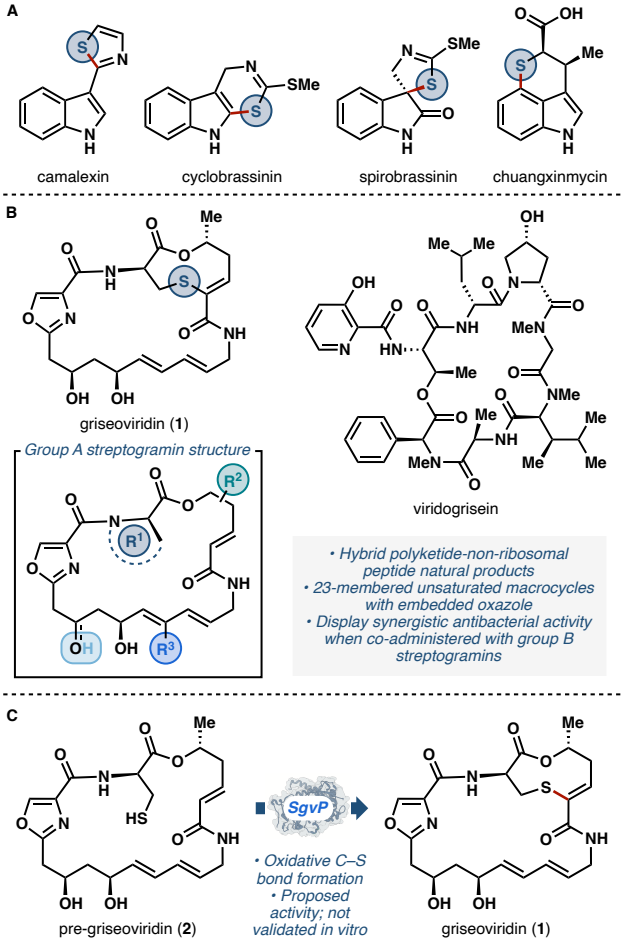


Figure 1. (A) Representative natural products containing C–S bonds installed by cytochromes P450. (B) Chemical structures of griseoviridin (**1**), viridogrisein, and related streptogramin antibiotics. (C) Proposed C–S bond formation during the final step of griseoviridin biosynthesis.

In 2012, Ju and coworkers identified the biosynthetic gene cluster responsible for griseoviridin production in *S. griseoviridis*.[32] Bioinformatic analyses revealed only one tailoring enzyme: SgvP, which was annotated as a cytochrome P450 monooxygenase with unknown function. Subsequent genetic[29] and structural[33] studies provided preliminary evidence that SgvP forges the key C–S bond of the macrolactone, though this activity was not confirmed *in vitro* (Figure 1C). Importantly, the presumed biosynthetic precursor to griseoviridin and putative substrate of SgvP – pre-griseoviridin (**2**) – was neither isolable nor identifiable in any capacity. Inactivation of *sgvP* in the producing strain instead led to accumulation of two desulfurized compounds, thereby precluding biochemical characterization and mechanistic studies. This observation also highlights some of the shortcomings of deciphering tailoring steps in natural product biosynthesis by purely genetic means, which are liable to polar effects and the whims of metabolic regulation.

Herein, we disclose a concise and scalable total synthesis of pre-griseoviridin (**2**) that features a convergent approach inspired by recent synthetic entries to streptogramin natural products.[34] Access to **2** not only facilitated analysis of P450 SgvP and *in vitro* confirmation of its role in C–S bond formation during griseoviridin biosynthesis, but also enabled a chemoenzymatic total synthesis of griseoviridin in nine linear steps. This work demonstrates the inherent potential of chemoenzymatic synthesis as a platform for evaluating biosynthetic hypotheses and enzyme activity.

RESULTS AND DISCUSSION

Synthesis of pre-griseoviridin. We envisioned that pre-griseoviridin (**2**) could be accessed via the suitably protected macrocyclic precursor **3** (Figure 2). Taking inspiration from Seiple and coworkers,[34] we partitioned **3** into two complex fragments, one bearing a carboxylic acid and a vinyl iodide (A, **4**) and the other an amine and a vinyl stannane (B, **5**). These fragments could feasibly be joined through sequential peptide coupling and palladium-catalyzed Stille macrocyclization. We opted for a *de novo* construction of the oxazole motif of **4**, which traces this compound back to a serine derivative and carboxylic acid **6**. Conveniently, **6** maps directly onto commercially available *syn*-1,3-diol **7**, an inexpensive (~$1.45/g) building block used in the synthesis of statin-based pharmaceuticals. On the other hand, we anticipated that **5** could arise from Steglich esterification of secondary alcohol **8** with a *D*-cysteine derivative, while the vinyl stannane could be introduced via functionalization of methyl ester **9**. In turn, **9** would be obtained from vinylmagnesium bromide addition to (*R*)-(+)-propylene oxide (**10**) and subsequent cross metathesis.

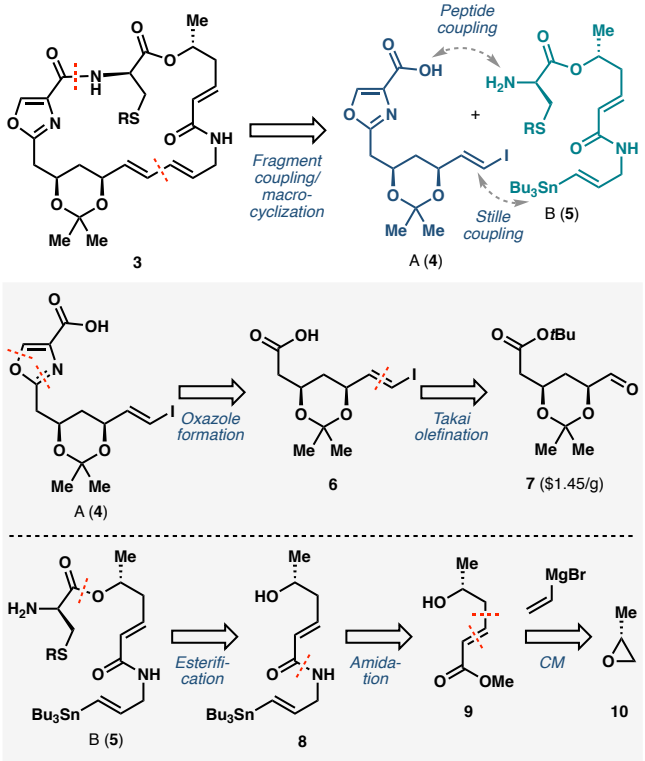


Figure 2. Retrosynthetic analysis of protected macrocycle **3**, the direct precursor to pre-griseoviridin (**2**).

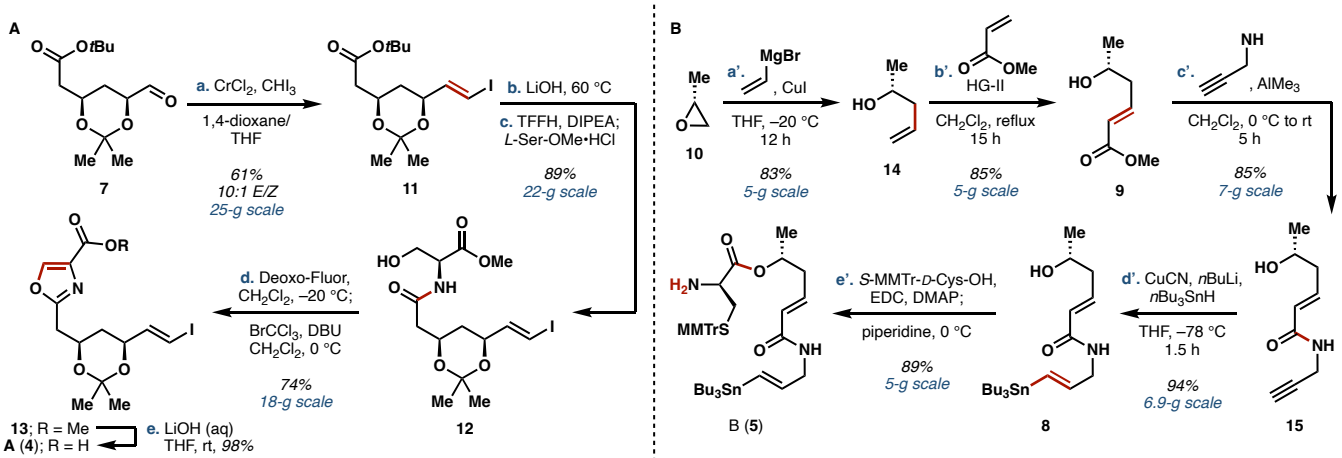
Synthesis of **4** commenced from *syn*-1,3-diol **7**, which was homologated to vinyl iodide **11** via the *E*-selective Takai olefination (Scheme 1A).[35] Though preliminary efforts gave high yields, a mediocre 2.5:1 *E*/*Z* ratio was observed (Table S1). We noted several reports touting the ability of 1,4-dioxane to improve *E*/*Z* selectivity in analogous systems.[36] Pleasingly, upon assessment of different mixtures of 1,4-dioxane and tetrahydrofuran (THF), the *E*/*Z* ratio increased to nearly 10:1. This procedure proved amenable to decagram scale synthesis, delivering over 20 g of **11** in a single batch. With **11** in hand, we initially targeted a two-step, one-pot approach to serinate **12**. Therein, treatment with thionyl chloride ($SOCl_2$) and catalytic *N*,*N*-dimethylformamide (DMF) furnished an intermediate acid chloride,[37] which was then trapped with the hydrochloride salt of *L*-serine methyl ester (*L*-Ser-OMe•HCl). While serinate **12** was isolated in acceptable yield, reaction efficiency suffered greatly upon scale-up, as the acetonide moiety is unstable to trace acid. To mitigate these issues, we opted for a stepwise workaround, first subjecting ester **11** to lithium hydroxide (LiOH)-mediated hydrolysis. The crude carboxylic acid was then converted to the acid fluoride *in situ* and coupled with *L*-Ser-OMe•HCl to provide serinate **12** in 87% yield on decagram scale. Furthermore, the two olefin isomers were separable at this juncture, enabling us to proceed with only the desired *E* vinyl iodide.

The stage thus set for oxazole formation, we converted **12** to the corresponding oxazoline by the action of Deoxo-Fluor, which provided a cleaner reaction than diethylaminosulfur trifluoride (DAST). Following oxidation with bromotrichloromethane ($BrCCl_3$) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), oxazole **13** was obtained in 79% yield over two steps. Upon optimization, this sequence was telescoped to a one-pot procedure without appreciable loss of yield or scalability. Finally, hydrolysis of the methyl ester afforded **4** in nearly quantitative fashion, completing a five-step synthesis in 38% overall yield.

In parallel, our synthesis of fragment B began with the known transformation of (*R*)-(+)-propylene oxide (**10**) to homoallylic alcohol **14** mediated by vinylmagnesium bromide and copper(I) iodide (CuI) (Scheme 1B).[38] Cross metathesis with methyl acrylate was achieved with Hoveyda-Grubbs second generation catalyst (HG-II), furnishing ester **9** in 85% yield. Then, treatment of **9** with propargylamine and trimethylaluminum ($AlMe_3$) resulted in the formation of propargyl amide **15**. Careful control of reaction duration and temperature was required to minimize elimination of the free secondary alcohol in the presence of strong Lewis acid. Copper-mediated hydrostannylation converted the terminal alkyne to vinyl stannane **8** in high yield, with nearly 20 g isolated from a single reaction. Lastly, the *D*-cysteine fragment was introduced as *S*-Trt-*N*-Fmoc-*D*-Cys-OH in a one-pot Steglich esterification/Fmoc deprotection sequence from secondary alcohol **8**, completing the synthesis of amine **S-1** in five steps and 50% overall yield (Scheme S3).

Having accessed fragments A and B, we focused our efforts on macrocycle formation. Fragment coupling was accomplished with hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU) and *N*,*N*-diisopropylethylamine (DIPEA), delivering the linear Stille coupling precursor **S-2** (Scheme S3). Next, a variety of conditions were explored to effect palladium-catalyzed Stille macrocyclization (Table S1). The traditional combination of triphenylarsine (Ph_3As) and Pd(0) failed to produce any appreciable amount of macrocycle **S-3**.[39] Similar results were observed when JackiePhos or various Buchwald palladacycle catalysts were used. Conversely, the Buchwald ligands XPhos and RuPhos in conjunction with Pd(0) gave 27% and 34% yield of **S-3**, respectively. In search of more efficacious conditions, we tested Fürstner's modified Stille protocol but saw substantial degradation regardless of reaction temperature.[40] Gratifyingly, when we omitted phosphine ligands altogether and utilized a simplified system of tris(dibenzylidineacetone)dipalladium(0) ($Pd_2(dba)_3$) and lithium chloride (LiCl), macrocycle **S-3** was cleanly generated in 64% yield. This result not only served as an example of successful macrocyclization – a notoriously difficult transformation to achieve with high efficiency[41] – but also brought us within one step of pre-griseoviridin.

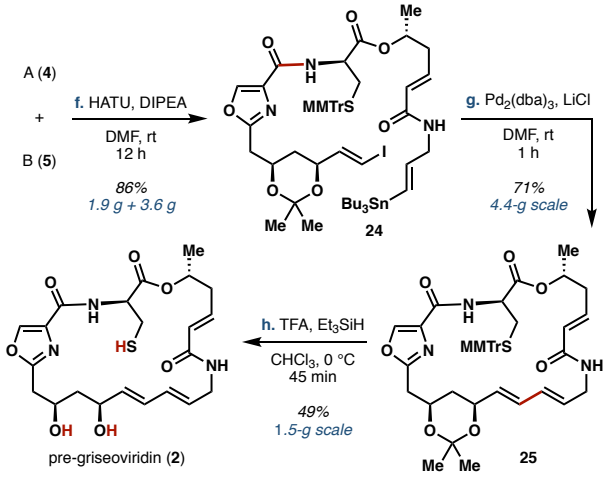
Scheme 1. A. Synthesis of Fragment A (4) from 1,3-Diol 7. B. Synthesis of Fragment B (5) from Chiral Epoxide 10.



Given the lability of trityl and acetonide protecting groups under acidic conditions, we envisioned concomitant liberation of the thiol and 1,3-diol in a single operation. Preliminary efforts examined various Brønsted and Lewis acids in a range of solvents (Table S2). It became immediately evident that acetonide deprotection was facile and rapid, often proceeding completely within minutes or even seconds. However, the trityl moiety was substantially more resilient, and the free 1,3-diol would eliminate long before any trityl deprotection was observed. To facilitate more targeted screening, we accessed free diol **S-4** by treating acetonide **S-3** with DOWEX cation exchange resin in methanol (MeOH). Again, no conditions were able to unmask the thiol, the reactions dominated by elimination and general degradation. We next assayed oxidative reagents, such as mercuric acetate ($Hg(OAc)_2$) or cuprous chloride (CuCl), seeking to deprotect the thiol of **S-3** selectively. Disappointingly, complex reaction profiles were consistently obtained, and no free thiol was formed.

At this point, we opted to exchange the trityl group for a more labile functionality – 4-monomethoxytrityl (MMTr), which is known to cleave more readily under acidic conditions due to increased cation stability.[42] To modify our synthesis of fragment B, Steglich esterification of **8** was performed with commercially available *S*-MMTr-*N*-Fmoc-*D*-Cys-OH (Scheme 1B). Further optimizations included the use of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) instead of diisopropylcarbodiimide (DIC) for esterification and piperidine instead of diethylamine for Fmoc removal. These modifications enabled preparation of MMTr-protected fragment B (**5**) in 89% yield on multigram scale. Coupling of **4** and **5** gave MMTr-protected Stille precursor **24**, which was subjected to the palladium-catalyzed macrocyclization conditions elucidated previously (Scheme 2). Similar yield (61%) was obtained, but closer inspection of the reaction profile suggested that an isomerized byproduct comprised a notable portion of the remaining mass. With this knowledge, we increased the equivalents of LiCl, which successfully attenuated isomerization and improved the yield of **25** to 71% on 4.4-g scale.

Scheme 2. Fragment Coupling and Deprotection Toward Pre-Griseoviridin (2).



Initial deprotection screening with **25** suggested nearly identical reactivity to **S-3**, wherein rapid acetonide deprotection would precede elimination and degradation without any trace of MMTr removal. However, treatment of **25** with trifluoroacetic acid (TFA) and triethylsilane (Et_3SiH) in chloroform ($CHCl_3$) produced a compound that had never been observed previously. Subsequent NMR and HRMS analyses confirmed this compound as pre-griseoviridin (**2**). This combination of TFA/Et_3SiH/$CHCl_3$ was subjected to extensive optimization, as even minute variations in temperature, equivalents, and duration were found to drastically impact reaction success (Table S4). Eventually, the finalized protocol afforded 49% isolated yield of **2** on gram scale. It should be noted that other thiol protecting groups were also screened without success. With deprotection accomplished, we achieved the first ever total synthesis and isolation of pre-griseoviridin (**2**) in 8 steps (longest linear sequence, or LLS).

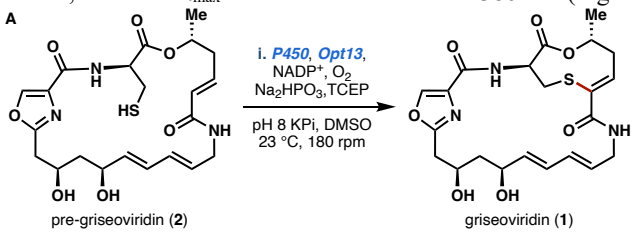
Reconstitution and analysis of P450 SgvP. With pre-griseoviridin (**2**) in hand, the proposed enzymatic C–S bond formation was initially benchmarked against several chemical oxidants. Treatment of **2** with *m*-chloroperbenzoic acid (*m*CPBA) led to an intractable mixture based on crude NMR analysis, though we were tentatively able to detect the presence of the sulfinic and sulfonic acids of **2** by LC-MS. On the other hand, a series of basic hydrogen peroxide (H_2O_2) conditions gave ester hydrolysis, *S*-oxidation, or broad degradation. Crucially, we did not observe the production of **1** in any of these attempts.

As prior reports have demonstrated the efficacy of self-sufficient P450-reductase chimeras for biocatalysis,[43] we commenced investigations into SgvP by fusing it with RhFRed, the reductase domain of $P450_{RhF}$, to generate a chimera referred to as SgvP-RhFRed (Figure 3A). Once solubility was confirmed via SDS-PAGE, overexpression of the *N*-His_6-tagged protein in *E. coli* aided by GroES/EL chaperones[44] gave sufficient yield of pure SgvP-RhFRed for preliminary studies. Initial incubation of **2** with SgvP-RhFRed in the presence of $NADP^+$ and sodium phosphite dehydrogenase Opt13 for NADPH regeneration[45] yielded disulfide as the only product based on LC-MS. Addition of tris(2-carboxyethyl)phosphine (TCEP) to the reaction mixture as a reductant effectively prevented disulfide formation, but no conversion to griseoviridin (**1**) was observed. Furthermore, varying the reaction buffer, pH, additives, and temperature had no effect on the outcome, nor did performing the reaction in crude lysates of *E. coli* expressing SgvP-RhFRed. A Genome Neighborhood Network (GNN) analysis of SgvP identified three homologs, named "FNS2", "NK82", and "YY64" (Figure S1), with high sequence identity (>80%) and general preservation of genomic context (i.e., acting in the production of griseoviridin-like compounds). These enzymes were subsequently assembled as the corresponding P450-RhFRed chimeras. As with SgvP-RhFRed, however, none of the homologs exhibited any activity with **2** in cell lysates across a range of conditions.

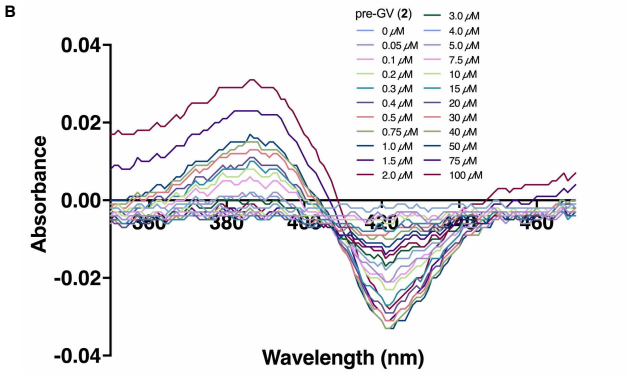
At this stage, we surmised that the pendant reductase domain could be interfering with P450 activity. Such concerns are well-documented in the study and application of P450-reductase chimeras, which can suffer from P450-reductase decoupling or unfavorable domain-domain interactions, among other issues.[43b] With this in mind, we switched our focus to an alternative reconstitution system comprising standalone SgvP and two different pairs of surrogate redox partners: spinach ferredoxin (Fdx)/ferredoxin reductase (Fdr) and putidaredoxin (CamB)/putidaredoxin reductase (CamA). To our delight, incubation of pre-griseoviridin (**2**) with SgvP, Fdx/Fdr, and Opt13 in the presence of $NADP^+$ and TCEP gave nearly full conversion to griseoviridin (**1**) in approximately three hours. We did not detect the presence of any apparent intermediates during the

course of the reaction. Analogous results were obtained in the presence of CamB/CamA, suggesting their viability as alternative redox partners for SgvP. Moreover, a preparative scale procedure with Fdx/Fdr successfully delivered 78% isolated yield of griseoviridin (**1**). Our findings not only provided the first biochemical confirmation that P450 SgvP installs the key C–S bond in griseoviridin biosynthesis, but also constituted the final step in an expedient chemoenzymatic synthesis of griseoviridin (9 steps, LLS).

In vitro reconstitution of SgvP provided an opportunity to perform further biochemical characterization of the enzyme. Upon incubating SgvP with pre-griseoviridin (**2**) at a wide range of concentrations, a characteristic type I binding spectrum was observed, in which λ_{max} shifted from 421 nm to 386 nm (Figure 3B). By performing nonlinear regression on the absorption difference between λ_{386} and λ_{421}, we calculated a dissociation constant (K_d) of 7.13 ± 0.39 μM for the SgvP–**2** complex (Figure 3C). Relative to other P450s from secondary metabolite biosyntheses, the K_d of SgvP is one order of magnitude higher than that of PtmO5 (K_d = 0.86 ± 0.07 μM)[46] and 2.5-fold lower than that of TleB (K_d = 18.9 ± 3.3 μM).[22] Furthermore, while previously characterized C–S bond forming P450s catalyze oxidative modification of electron rich aromatics, SgvP acts on an electron-deficient alkene, effecting macrocyclization to a 9-membered ring with high strain energy. Overall, this work is expected to lay the foundation for future studies into the mechanism of SgvP-catalyzed C–S bond formation, which is currently unelucidated.



Entry	P450	Reductase	Results
1	SgvP	RhF	*No reaction*
2	FNS2	RhF	*No reaction*
3	NK82	RhF	*No reaction*
4	YY64	RhF	*No reaction*
5	SgvP	Fdx/Fdr	*Full conversion, 78% yield*
6	SgvP	CamB/CamA	*Full conversion by LC-MS*



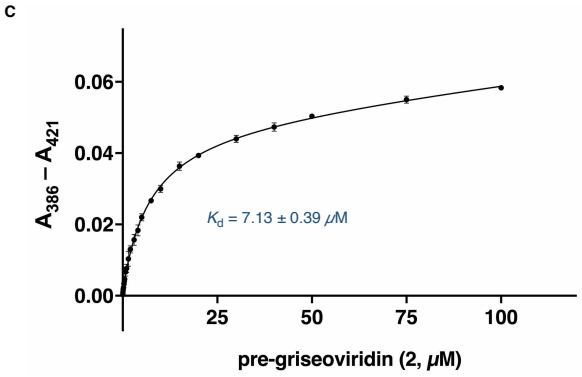


Figure 3. (A) Assessment of P450-catalyzed C–S bond formation *en route* to griseoviridin (**1**). (B) Type I binding spectra obtained from titration of SgvP with **2** (0 to 100 μM). (C) Determination of SgvP–**2** K_d via nonlinear regression of the difference in type I binding spectra ($\lambda_{386} - \lambda_{421}$) plotted against the concentration of **2**.

CONCLUSION

Implementing concise and convergent synthetic logic, we executed the first total synthesis of pre-griseoviridin (**2**), the putative substrate for P450 SgvP, in 8 steps (LLS) on multigram scale. Synthetic access to pre-griseoviridin then enabled (1) *in vitro* reconstitution of SgvP and confirmation of its role in C–S bond formation during griseoviridin biosynthesis, and (2) an expedient chemoenzymatic total synthesis of griseoviridin (**1**). This work exemplifies the complementary relationship between total synthesis, biocatalysis, and biosynthetic analysis, demonstrating that strategies and results from one field can greatly enrich the others. In particular, it highlights the unique role that synthetic chemistry can play in assisting biochemical studies and addressing knowledge gaps in biosynthesis that cannot be solved with genetic studies alone. We anticipate that this interdisciplinary approach will continue to create opportunities in each of these disciplines. Further investigations into SgvP, especially regarding the precise mechanism of C–S bond formation, are currently in progress.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

General materials and methods, synthetic procedures, biochemical procedures, NMR spectral data (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

CM, cross metathesis; dba, dibenzylidineacetone; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DIC, diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Fmoc, fluorenylmethoxycarbonyl; HATU; hexafluorophosphate azabenzotriazole tetramethyl uronium; HRMS, high resolution mass spectrometry; KPi, inorganic phosphate; LC-MS, liquid chromatography-mass spectrometry; LLS, longest linear sequence; *m*CPBA, *meta*-chloroperbenzoic acid; MMTr, 4-monomethoxytrityl; NADP+/NADPH, nicotinamide adenine dinucleotide phosphate oxidized/reduced; NMR, nuclear magnetic resonance spectroscopy; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCEP, tris(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid; TFFH, tetramethylfluoroformamidinium hexafluorophosphate; THF, tetrahydrofuran.

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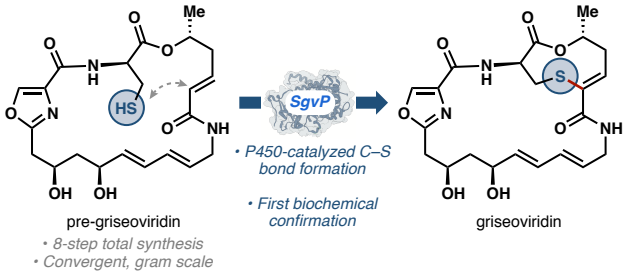

pre-griseoviridin

• 8-step total synthesis
• Convergent, gram scale

griseoviridin