A GenoChemetic strategy for derivatization of the violacein natural product scaffold

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Abstract

Integrating synthetic chemistry with synthetic biology allows rapid access to xenobiotic compounds which may provide improved therapeutic activity. By supplementing an *Escherichia coli* strain expressing the violacein biosynthesis pathway with eight tryptophan substrate analogues or tryptophan halogenase RebH *in vivo*, 68 new-to-nature analogues of violacein were generated. Furthermore, 20 new derivatives were generated from brominated analogues via Suzuki-Miyaura cross-coupling reaction directly using the crude extract without prior purification. Herein, we demonstrate a flexible and rapid approach to access diverse chemical space that can be applied to a wide range of natural product scaffolds.

Main

Total synthesis of natural products and their analogues has often been challenging and costly due to their structural complexities[1]. However, greater chemical space may be accessed through utilisation of synthetic chemistry methodologies and reagents compared to enzyme-catalysed biosynthesis alone. Combining biosynthesis with chemical synthesis represents a powerful approach for rapidly generating new analogues of complex natural products and libraries of chemical derivatives suitable for screening and structure-activity relationship (SAR) assays[2,3], leading to discovery of new and more potent compounds. The generation of such analogues can enable improvement in bioactivity and bioavailability, as illustrated for example by analogues of the 'last-resort' antibiotic vancomycin, with >200-fold improvement of potency compared to vancomycin in resistant *Enterococci* strains[4]. In another study, the addition of a sterically unhindered primary amine group to a Gram-positive antibiotic deoxynybomycin expanded its antimicrobial activity to multi-drug-resistant Gram-negative pathogenic strains[5].

As a proof-of-concept, we have focused on the violacein biosynthesis pathway. Violacein (**1**),a violet pigment first isolated from the bacterium *Chromobacterium violaceum*, is part of the bisindole biosynthetic family that utilises L-tryptophan as the starting substrate. The violacein pathway is encoded within a conserved operon of five genes (*vioABCDE*), whose gene-products catalyse a 14-electron oxidative biosynthesis pathway[6] (**Figure S1**). The bisindole biosynthetic pathways have attracted considerable interest because of their therapeutic potential for medical applications, including antimicrobial, antiviral, trypanocidal and anti-tumorigenic properties[7]. The violacein biosynthetic pathway also produces deoxyviolacein (**2**) as a by-product, and the coloured properties of violacein and deoxyviolacein make them interesting targets for natural product pathway engineering, such as promoter library screening[8], CRISPR-based multiplex transcriptional regulation[9] or diverting pathway flux via RBS engineering[10].

A study on oxyviolacein, a hydroxylated analogue of violacein generated by feeding exogenous 5-hydroxy- L-tryptophan[11], showed bioactivity against a collection of pathogenic

bacteria and fungi strains[12] suggesting that violacein analogues may be a good starting point for developing more potent antibiotics. Although total chemical synthesis of violacein[13,14] and some substituted analogues[15,16] have been reported, pathway manipulation could represent a more flexible and rapid approach for generating violacein analogues. Other examples of using altered biosynthetic pathways for analogue generation, include using various enzyme homologues from bisindole pathways[17,18], interconversion of the biosynthetically related novobiocin and clorobiocin aminocoumarin antibiotics[19], and introduction of the halogenase from hormaomycin pathway to generate clorobiocin analogues[20]. In this study, we have applied such approaches to generate 86 new violacein or deoxyviolacein analogues via a combination of enzymatic halogenation of the starting substrate tryptophan, feeding different substrate analogues, and further derivatization using Suzuki-Miyaura cross-coupling directly in crude extracts (**Figure 1**).

VioA, the first enzyme in the violacein biosynthesis pathway, oxidises the substrate L-tryptophan to form the first pathway intermediate indole-3-pyruvic acid imine (IPA imine). In order to determine whether VioA would accept different tryptophan analogues as substrates, we purified VioA (**Figure S3**) and carried out VioA enzyme kinetic assays against various substrate analogues including L-tryptophan (TRP), 4-fluoro-DL-tryptophan (4FT), 5-methyl-DL-tryptophan (5MeT), 6-fluoro-DL-tryptophan (6FT) and 7-methyl-DL-tryptophan (7MeT). We found that VioA exhibited different enzyme kinetics against the substrate analogues tested with 5MeT showing the highest activity and 4FT the lowest (**Figure S4**). To determine if these differences were due to alterations in substrate binding, we next solved the crystal structures of VioA complexed with TRP (PDB **6G2P**), 4FT (PDB **6FW7**), 5MeT (PDB **6FW8**), 6FT (PDB **6FW9**) and 7MeT (PDB **6FWA**) with resolutions ranging from 2.4 Å to 3.0 Å (**Figure 2a, 2b**, data statistics in **Table S1**). Surprisingly, the structures show that the tryptophan analogues bind the VioA active site in different conformations, presumably to accommodate the substituted fluorine or methyl group at C4 to C7 positions (**Figure 2b**). However, the interatomic distances between respective Cα atom or amine nitrogen atom and N5 of FAD

cofactor is conserved for all the analogues (between 3.93 Å to 4.69 Å for Cα atom, 3.62 Å to 5.63 Å for amine nitrogen) and is similar to the TRP-bound structure (3.93 Å for Cα atom and 5.07 Å for amine nitrogen). This showed that all the analogues can bind VioA in a catalytically active, but slightly different conformation (**Table S2**). Interestingly, the hydrogen bonding between Arg64 and Tyr309 and the carboxylate group of all substrate analogues is conserved as are the positions of the other active site residues (**Figure 2b**). Of the four structures with tryptophan analogues, 4FT-bound VioA structure has the lowest resolution at 3.0 Å and weakest density around the 4FT ligand compared to the other structures (**Figure S6**), suggesting lower occupancy binding. This correlates with the observed poorer enzyme kinetics compared to wildtype. In addition, we observe two alternative conformers for the bound 6FT ligand demonstrating further conformational flexibility of tryptophan analogue binding. In summary our structural analyses show that a variety of tryptophan analogues can bind VioA in different conformations although the positions of active site residues and associated hydrogen bonding patterns are similar to TRP-bound VioA. Our data clearly show that the VioA active site can flexibly accommodate a variety of tryptophan substrate analogues for catalysis.

Having determined that VioA accepts various tryptophan analogues, we next investigated the potential for derivatizing violacein using a substrate feeding approach, using commercially available tryptophan analogues as starting precursors. This included 4-fluoro-, 5-fluoro-, 6-fluoro-, 5-chloro-, 5-hydroxyl-, 6-methyl- and 7-methyl-substituted tryptophan analogues. By adding the tryptophan analogues to cultures of *E. coli* cells that were expressing a synthetic violacein biosynthesis pathway *vioABCDE* (**Figure 2c**), a total of 62 new compounds were observed including structural isomers due to the asymmetry of the violacein core scaffold, demonstrating promiscuity of violacein biosynthesis enzymes (chemical structures in **Table S3**). The percentage compositions of the various violacein and deoxyviolacein analogues were calculated based on the extracted total ion chromatogram (TIC) peak area obtained from LC-MS/MS analyses (**Table S4**, **Figure 2d**). We then assayed the growth inhibitory activity of

the crude violacein extracts containing the various analogues and a control containing only the underivatized scaffolds against *Bacillus subtilis* cultures as a Gram-positive model organism. Using crude extracts of violacein analogues we observed few changes in growth inhibition compared to the extracts containing unmodified crude violacein (**Figures S9**). Interestingly, the 6MeT extract has about 4-fold higher IC50 compared to the 7MeT extract, where the only difference between these two samples is the position of methyl group attached to the indole ring, suggesting that the C6 substitution might be important for activity. In addition, cell extracts containing only deoxyviolacein did not show any significant inhibition towards *B. subtilis*, suggesting that the 5-hydroxyl group of violacein confers the antimicrobial activity, in agreement with previous studies[12,21,22].

Due to the promiscuity of violacein biosynthesis pathway enzymes, we rationalized that a combination of enzymes from closely related bisindole biosynthesis pathways (**Figure S2**) could generate compatible tryptophan analogues leading to other interesting new-to-nature analogues of violacein that might be otherwise difficult to synthesise. Rebeccamycin is a closely related bisindole where the biosynthetic pathway contains several genes including *rebO* and *rebD* which are homologous to *vioA* and *vioB* respectively. In addition, the pathway also consists of a tryptophan 7-halogenase RebH and its associated flavin reductase RebF that are responsible for generating 7-chloro-L-tryptophan from L-tryptophan via a chloramine-lysine intermediate[23,24]. As a proof-of-principle, we combined the rebeccamycin (Reb) and violacein (Vio) biosynthetic operons using the Golden Gate cloning kit EcoFlex[25] in order to generate C7-chlorinated violacein analogues which we were able to successfully produce in *E. coli* cells (**Figure 2e**) identified via LC-MS/MS (**Table S4**). We found that although the RebOD+VioCDE strain (lacking RebFH) did not produce any chlorinated analogues of violacein, it was able to functionally substitute VioAB to produce violacein and deoxyviolacein[6]. Next, by comparing RebFH+VioABCDE and RebFHOD+VioCDE (where RebOD replaced VioAB) strains, we observed that RebOD increased the proportion of chlorinated analogues from 6% to 33% when compared with VioAB (**Figure 2f**). This was likely due to the fact that

RebO has k_{cat}/K_m value 57-times higher for 7-Cl-L-tryptophan than that of L-tryptophan[26], thus RebO and RebD preferentially accept the 7-chloro analogue of L-tryptophan and IPA imine respectively, producing the 7-chlorinated intermediate for downstream enzymes in the violacein biosynthesis pathway. By replacing the weak promoter J23114 for RebF and RebH with a medium strength promoter J23108, we also observed a total analogue increase of nearly 30% (**Figure 2g**). This shows that it is possible to fine tune the proportion of chlorinated analogue by changing the promoter strength of pathway enzymes, and optimization of fermentation protocols such as length of incubation, temperature, media conditions might further increase both proportion and the total amount of chlorinated analogues[8].

Further derivatisation, through chemical cross-coupling, of new-to-nature, halogenated natural product analogues generated by a synthetic biological strain, was first demonstrated with pacidamycins[27]. We applied a similar strategy by first feeding 5-bromo-DL-tryptophan to *E. coli* cells expressing VioABCDE to generate 5'-bromo-violacein and 5'-bromo-deoxyviolacein which were detected by LC-MS/MS (**Table S4**). We then proceeded to screen a series of conditions that might facilitate Suzuki-Miyaura cross-coupling. However, due to low titres of 5'-bromo-violacein and 5'-bromo-deoxyviolacein available in the crude extract, isolation of products for further characterisation and bioassays of the cross-coupling product proved difficult (see SI Extended Data). We thus focused on systemically identifying conditions that would allow direct cross-coupling in crude extracts which identified optimal conditions for derivatization (**Tables S6-S8**). The corresponding cross-coupling products 5'-(*p*-tolyl)-violacein and 5'-(*p*-tolyl)-deoxyviolacein were detected in the cross-coupling mixture by LC-MS/MS (**Figure 3a**, **Table S5**). Having identified the optimised conditions for cross-coupling at low concentrations, we performed cross-coupling reaction with a selection of 15 aryl boronic acids chosen to sample a wide range of steric and electronic variations, which gave a total of 20 new cross-coupled analogues of violacein and deoxyviolacein (**Figure 3b**). We observed that while boronic acids with electron-donating groups (*e.g. p*-methoxyphenylboronic acid) gave better yield and MS/MS data were obtained, for those with electron-withdrawing groups

(*e.g. p*-formylphenylboronic acid), only MS data were obtained due to lower conversion and more difficult electrospray ionisation of corresponding violacein analogues (**Table S5**). Pleasingly, even boronic acid pinacol esters were successfully converted into the corresponding violacein product (*e.g. p*-cyanophenylboronic acid pinacol ester). In summary, the optimised conditions showed that a variety of cross-coupling analogues could be synthesised without the need for purification prior to cross-coupling, allowing for rapid access to potential cross-coupling products.

In conclusion, inspection of the biosynthetic assembly of the antibiotic violacein and crystal structure analyses of the first enzyme VioA, suggested that various tryptophan substrate analogues could be enzymatically processed. We utilized this finding and designed experiments in which we have powerfully combined biosynthesis and synthesis to enable rapid access to 68 new violacein and deoxyviolacein analogues only one of which has been described previously (oxyviolacein[11,12]). Furthermore, we have extended this portfolio of compounds further via Suzuki-Miyaura cross-coupling reactions on the new-to-nature natural products that have been generated, accessing an additional series of 20 violacein or deoxyviolacein coupling products. The ability to carry out these synthetic diversifications selectively on the halometabolites as components of a crude cell extract is exciting, opening the way for applying this approach to building of more diverse libraries of analogues. Although an initial growth inhibition screen of *B. subtilis* using crude extracts showed little change in activity compared with extracts containing violacein and deoxyviolacein, this is likely to reflect the increased lipophilicity (LogP) of the halogenated or methylated compounds, and could be potentially overcome using cross-coupling through the exploration of a series of more polar partners. Finally, the combination of synthetic chemistry with synthetic biology, provides a more sustainable approach with the potential for application towards generating analogues of natural products in order to access greater chemical diversity rapidly and predictably.

Data availability

The authors declare that data supporting findings in this study is included in this paper and its supplementary information file. Sequences of plasmids used for expressing the violacein and the rebeccamycin/violacein hybrid pathways are available in the supplementary information. Raw data for LC-MS/MS measurements are available upon request.

References

1. Kirschning, A. & Hahn, F. Merging chemical synthesis and biosynthesis: A new chapter in the total synthesis of natural products and natural product libraries. *Angew. Chemie - Int. Ed.* **51,** 4012–4022 (2012).

2. Goss, R. J. M., Shankar, S. & Fayad, A. A. The generation of “unNatural” products: Synthetic biology meets synthetic chemistry. *Nat. Prod. Rep.* **29,** 870 (2012).

3. Eichner, S. *et al.* The interplay between mutasynthesis and semisynthesis: Generation and evaluation of an ansamitocin library. *Angew. Chemie - Int. Ed.* **51,** 752–757 (2012).

4. Okano, A., Isley, N. A. & Boger, D. L. Peripheral modifications of [Ψ[CH2NH]Tpg4]vancomycin with added synergistic mechanisms of action provide durable and potent antibiotics. *Proc. Natl. Acad. Sci.* 201704125 (2017). doi:10.1073/pnas.1704125114

5. Richter, M. F. *et al.* Predictive compound accumulation rules yield a broad-spectrum antibiotic. *Nature* **545,** 299–304 (2017).

6. Balibar, C. J. & Walsh, C. T. In Vitro Biosynthesis of Violacein from L-Tryptophan by the Enzymes VioA−E from *Chromobacterium violaceum*. *Biochemistry* **45,** 15444–15457 (2006).

7. Durán, M. *et al.* Potential applications of violacein: A microbial pigment. *Med. Chem. Res.* **21,** 1524–1532 (2012).

8. Jones, J. A. *et al.* ePathOptimize: A Combinatorial Approach for Transcriptional Balancing of Metabolic Pathways. *Sci. Rep.* **5,** 11301 (2015).

9. Zalatan, J. G. *et al.* Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. *Cell* **160,** 339–350 (2015).

10. Jeschek, M., Gerngross, D. & Panke, S. Rationally reduced libraries for combinatorial

pathway optimization minimizing experimental effort. *Nat. Commun.* **7,** 11163 (2016).

11. Hoshino, T. & Ogasawara, N. Biosynthesis of violacein: Evidence for the intermediacy of 5-hydroxy-L-tryptophan and the structure of a new pigment, oxyviolacein, produced by the metabolism of 5-hydroxy-L-tryptophan. *Agric. Biol. Chem.* **54,** 2339–2346 (1990).

12. Wang, H. *et al.* Biosynthesis and characterization of violacein, deoxyviolacein and oxyviolacein in heterologous host, and their antimicrobial activities. *Biochem. Eng. J.* **67,** 148–155 (2012).

13. Petersen, M. T. & Nielsen, T. E. Supporting Information Tandem Ring-Closing Metathesis / Isomerization Reactions for the Total Synthesis of Violacein. *Org. Lett.* **15,** 1986–1989 (2013).

14. Wille, G. & Steglich, W. A Short Synthesis of the Bacterial Pigments Violacein and Deoxyviolacein. *Synthesis (Stuttg).* **2001,** 0759–0762 (2001).

15. Haun, M. *et al.* Bacterial chemistry. VI. Biological activities and cytotoxicity of 1, 3-dihydro-2H-indol-2-one derivatives. *Biol Res* **25,** 21–25 (1992).

16. McLaughlin, E. C., Norman, M. W., Ko Ko, T. & Stolt, I. Three-component synthesis of disubstituted 2H-pyrrol-2-ones: preparation of the violacein scaffold. *Tetrahedron Lett.* **55,** 2609–2611 (2014).

17. Sánchez, C. *et al.* Combinatorial biosynthesis of antitumor indolocarbazole compounds. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 461–466 (2005).

18. Du, Y. L. & Ryan, K. S. Expansion of Bisindole Biosynthetic Pathways by Combinatorial Construction. *ACS Synth. Biol.* **4,** 682–688 (2015).

19. Eustáquio, A. S. *et al.* Production of 8′-Halogenated and 8′-Unsubstituted Novobiocin Derivatives in Genetically Engineered Streptomyces coelicolor Strains. *Chem. Biol.* **11,** 1561–1572 (2004).

20. Heide, L. *et al.* Use of a Halogenase of Hormaomycin Biosynthesis for Formation of New Clorobiocin Analogues with 5-Chloropyrrole Moieties. *ChemBioChem* **9,** 1992–1999 (2008).

21. Jiang, P. X. *et al.* Pathway redesign for deoxyviolacein biosynthesis in Citrobacter freundii and characterization of this pigment. *Appl. Microbiol. Biotechnol.* **94,** 1521–1532 (2012).

22. Bilsland, E. *et al.* Antiplasmodial and trypanocidal activity of violacein and deoxyviolacein produced from synthetic operons. *BMC Biotechnol.* **18,** 22 (2018).

23. Yeh, E., Garneau, S. & Walsh, C. T. Robust in vitro activity of RebF and RebH, a two-component reductase/halogenase, generating 7-chlorotryptophan during rebeccamycin biosynthesis. *Proc. Natl. Acad. Sci.* **102,** 3960–3965 (2005).

24. Yeh, E., Blasiak, L. C., Koglin, A., Drennan, C. L. & Walsh, C. T. Chlorination by a long-lived intermediate in the mechanism of flavin-dependent halogenases. *Biochemistry* **46,** 1284–1292 (2007).

25. Moore, S. J. *et al.* EcoFlex: A Multifunctional MoClo Kit for E. coli Synthetic Biology. *ACS Synth. Biol.* **5,** 1059–1069 (2016).

26. Nishizawa, T., Aldrich, C. C., David, H. & Sherman, D. H. Molecular Analysis of the Rebeccamycin L-Amino Acid Oxidase from *Lechevalieria aerocolonigenes* ATCC 39243. *J. Bacteriol.* **187,** 2084–2092 (2005).

27. Roy, A. D., Grüschow, S., Cairns, N. & Goss, R. J. M. Gene Expression Enabling Synthetic Diversification of Natural Products: Chemogenetic Generation of Pacidamycin Analogs. *J. Am. Chem. Soc.* **132,** 12243–12245 (2010).

28. Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N. & Sternberg, M. J. E. The Phyre2 web portal for protein modeling, prediction and analysis. *Nat. Protoc.* **10,** 845–858 (2015).

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Author Information

Author Contribution

HEL, SJM, KMP, SVS, AMCO, RJMG and PSF designed the study, analysed the data, and wrote the manuscript. HEL performed experiments under guidance of SJM, KMP and PSF. AMCO performed cross-coupling reactions under guidance of SVS and RJMG. AMCO and SMC performed LC-MS/MS analysis. RMM assisted on VioA crystallography, data collection and structure refinement. All authors read and approved the final draft of the manuscript.

Notes

The authors declare no competing financial interests. For correspondence, please contact Paul S. Freemont at p.freemont@imperial.ac.uk.

Figures

Figure 1

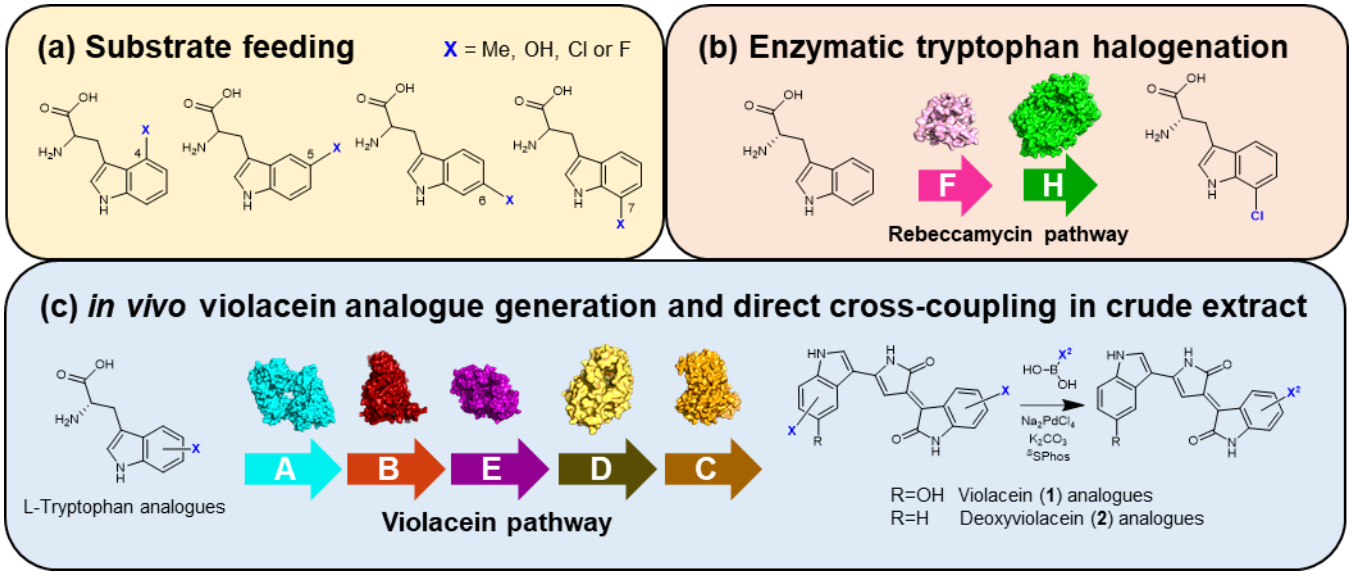
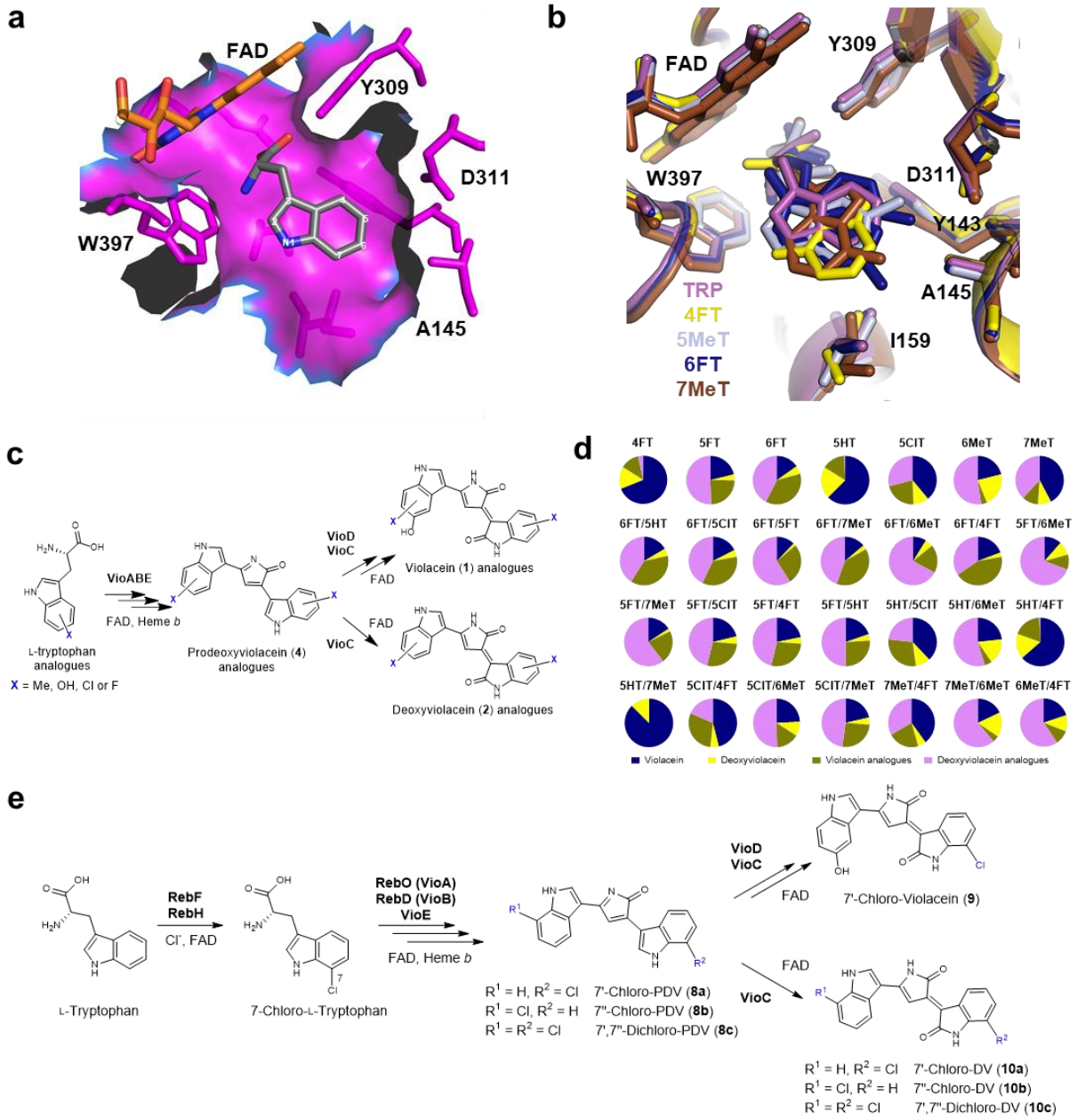


Figure 1. Schematics of GenoChemetic approach used in this study. Analogues of violacein (**1**) or deoxyviolacein (**2**) can be generated by either feeding synthetic tryptophan analogues to *E. coli* cells expressing the violacein biosynthesis pathway VioABCDE, or *in vivo* halogenation of tryptophan by expressing flavin reductase RebF and tryptophan halogenase RebH with the rest of violacein pathway. Further derivatization via Suzuki-Miyaura cross-coupling lead to new synthetic analogues. Available enzyme structures are shown and correspond to VioA, (PDB 6G2P); VioD (PDB 3C4A); VioE (PDB 3BMZ); RebH (PDB 2E4G) as well as models generated *in silico* by Phyre2[28] for VioB, VioC and RebF.

Figure 2



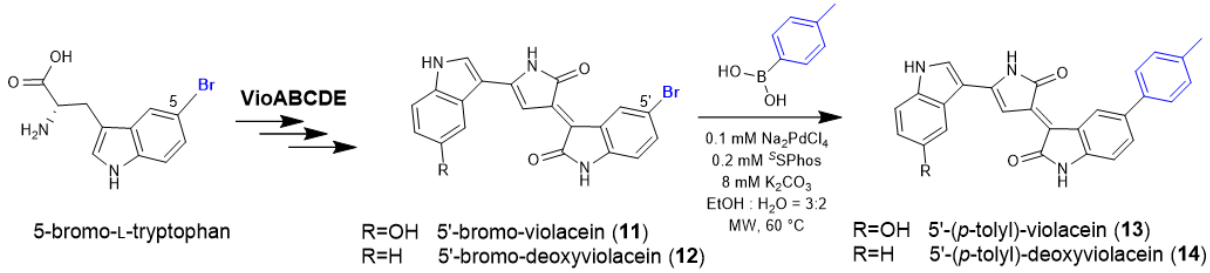
Pathway comb.	Rebeccamycin	Violacein
P1	RebOD	VioCDE
P2	RebFH	VioABCDE
P3	RebFHOD	VioCDE

Strain	RebF	RebH	VioD
S1	J23114	J23114	J23114
S2	J23108	J23108	J23108
S3	J23108	J23108	J23114

Figure 2. VioA structure and generation of violacein and deoxyviolacein analogues via substrate feeding and *in vivo* halogenation. **(a)** Binding site pocket showing space enclosed by residues (magenta) around L-tryptophan (grey with elemental colour). The carbon positions of tryptophan indole ring are labelled. **(b)** Superimposition of active sites of VioA structures complexed with L-tryptophan (TRP, magenta), 4-fluoro-L-tryptophan (4FT, yellow), 5-methyl-L-tryptophan (5MeT, light grey), 6-fluoro-L-tryptophan (6FT, dark blue) and 7-methyl-L-tryptophan (7MeT, brown) in stick representation. Side chains surrounding the tryptophan ligand are labelled. Note that 6FT has 2 conformers fitted in the density map (see Figure **S8**). **(c)** Schematics of substrate feeding leading to analogues generation. **(d)** Compositions of violacein (**1**), deoxyviolacein (**2**), violacein analogues and deoxyviolacein analogues based on extracted TIC peak area in crude extracts of *E. coli* cells fed with various tryptophan substrates. **(e)** Schematic of the hybrid rebeccamycin-violacein pathway enzymes that lead to 7-chloro analogues of violacein (**9**) and deoxyviolacein (**10a**, **10b**, **10c**). **(f)** Percentage compositions of 7-chloro analogues of violacein and deoxyviolacein extracted from different *E. coli* strains expressing three pathway combinations (P1, P2, P3) based on extracted TIC peak area. Data shows mean with n=2. **(g)** Percentage compositions of 7-chloro analogues of violacein and deoxyviolacein extracted from *E. coli* expressing RebFHOD+VioCDE with different combinations of medium (J23108) and weak (J23114) promoters controlling the expression of RebF, RebH and VioD based on extracted TIC peak area. Data shows mean with n=3. Relative analogues peak area show mean with SD, n=3.

Figure 3

a



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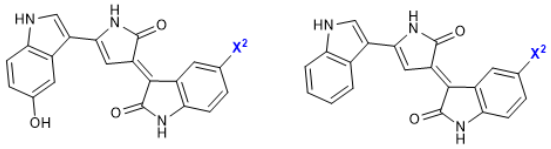


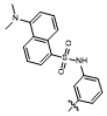
	Compound number	
X²	Violacein derivative	Deoxyviolacein derivative
	13	**14**
	ND	**54**
	ND	**55**
	56	**57**
	**58**	**59**
	ND	**60**
	61	**62**
	63	**64**
	ND	**65**
	66	**67**
	68	ND
	69	**70**
	ND	**71**

Figure 3. Further derivatisation of violacein and deoxyviolacein analogues via Suzuki-Miyaura cross-coupling reactions directly in crude extracts. **(a)** Optimised conditions for cross-coupling reaction directly in crude extracts. Coupling partner indicated is p-tolyl-boronic acid. **(b)** Compound numbers of full range of cross-coupled products as detected by LC-MS or LC-MS/MS (see **Table S5**). ND, not detected.