Biosynthesis of violacein: a genuine intermediate, protoviolaceinic acid, produced by VioABDE, and insight into VioC function†

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A biosynthetic intermediate of violacein produced by the mixed enzymes of VioABDE was elucidated to be 5-(5-hydroxy-1H-indol-3-yl)-3-(1H-indol-3-yl)-1H-pyrrole-2-carboxylic acid, named protoviolaceinic acid, indicating that VioC, responsible for the final biosynthetic step, works to oxygenate at the 2-position of the right side indole ring, and that the oxygenation reaction to form the central pyrrolidone core proceeds in a non-enzymatic fashion.

Violacein 1 and deoxyviolacein 2 are bluish-purple pigments produced by Chromobacterium violaceum, the bisindole core being closely related to those of rebeccamycin 3 and staurosporine 4. We have reported the biosynthetic studies of 1 since 1987.1 All the carbon, hydrogen and nitrogen atoms originated from two molecules of L-tryptophan (Trp).1 The most remarkable features are the 1,2-shift of the indole ring1 and the incorporation of three molecules of O2 into 1.16 Recent progress on the biosynthesis is remarkable. The first identification of the gene cluster for the biosynthesis of 1 was reported by August et al.2 by us (named pVBG04)3 (accession numbers: AF172851 and AB032799, respectively), and the complete genome sequence of the strain was later solved.3 Most recently, it was established that the gene cluster is composed of five ORFs (VioABCDE) as shown in Fig. 1, where VioE is essential for the production of 1, and the biosynthetic pathway is proposed to be VioA → VioB → VioE → VioD → VioC.4,5 From the biosynthetic studies on 3 and 4, VioA is assumed to be a L-tryptophan oxidase to give the imine of indole-3-pyruvic acid (IPAim), and VioB is presumed to condense two molecules of Trp; VioB has a highly homologous alignment (ca. 30%) to those of StaD and RebD, which produce chromopyrrolic acid5,6 with aid of StaO and RebO.6,7 However, 5 is not the biosynthetic intermediate of 1,14 thus the role of VioB has remained uncertain. No homologous protein can be assigned to VioE. Based on the finding that proviolacein 68 and prodeoxyviolacein 79 are produced by incubating Trp with VioABDE and VioABE, respectively,5,5 the recent papers suggested that VioE is responsible for the rearrangement reaction of indole ring.4,5 This finding also suggested that VioD catalyses the oxygenation reaction at the 6-position. 6 and 7 have been assumed to be the plausible intermediates, because oxygenation at the 16-position of 6 and 7 will lead to 1 and 2. However, we have failed to transform 6 and 7 into 1 and 2, respectively, despite carefully repeated experiments.16 Thus, 6 and 7 are not the genuine intermediates. No biosynthetic intermediate having a bisindole core has been uncovered hitherto. Herein, we report the first identification of a true intermediate of 1, called intermediate X (8), which is produced by incubating Trp with VioABDE in the presence of NADPH (NADP+ is essential to the production of 1).6. Furthermore, we describe the important finding that X is very labile to O2, resulting in the formation of a shunt product 6.

![Fig. 1](Image)

Our strategy to quest for the intermediate X was as follows. First, we examined whether X is extractable with organic solvent from the incubation mixture. The EtOAc-extract was added to VioC, giving rise to a purple color. Next, we examined when X was accumulated in a highest amount after incubating Trp with VioABDE in the presence of NADPH. Fig. 2 shows that the production of 1 reached a maximum after an incubation time of...
occurred by the exposure to O2. The TLC analysis clarified that significantly decreased, suggesting that a rapid decomposition solvent was evaporated to dryness, the production of decomposed at room temperature, but the decomposition was

produced. Intermediate followed by evaporation to a small volume (the solvent was never gas. The substitution with acetone-

and evaporated to a small volume by passing a stream of argon bubbling of O2 into the EtOAc-extract more significantly analysis revealed that a decreased production of the decomposed product was mainly decreased the production of O2.

remove the residual EtOAc. The 1D and 2D NMR spectra were found during the NMR measurements for 36 h, but gradual alteration was observed when measured at 25 °C. The 13C signal at the longer incubation times with VioABED; no detectable amount of 6 was added to VioC and further incubated in the presence of NADPH.

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Fig. 2 (Left) Production amount of 1, monitored at 570 nm in MeOH, against different incubation times (0.5–24 h) with VioABDE + NADPH. EtOAc was added to each of the mixtures incubated for different times. The EtOAc-extract, which was quickly evaporated into a small volume, was added to VioC and further incubated in the presence of NADPH. Longer incubation times (>5 h) with VioABED did not give a purple color. As shown right the production amount of 1 was significantly decreased and, in turn, formations of 6 and 7 were gradually increased, by the longer incubation times with VioABED; no detectable amount of 6 at 0.5 h, but a significantly high production of 6 after 24 h-incubation. Formation of 2 and 7 is due to the insufficient amount of VioD (Fig. S1 of ESI). 6 and 7 were produced from 8 and 9, respectively, by the oxidative decarboxylation reaction in a non-enzymatic manner (Schemes 1 and 2). (Right) SiO2-TLC of the samples described left, which developed twice with CHCl3 : MeOH (95 : 5).

1 h, but that prolonged incubation led to a significantly decreased production of 1, indicating that X is converted into other compounds during a prolonged incubation. The SiO2-TLC analysis revealed that a decreased production of 1 gave the higher production of 6, suggesting that 6 is produced from X.

Next, we tested the stability of X by estimating the amount of 1 produced. Intermediate X dissolved in the EtOAc gradually decomposed at room temperature, but the decomposition was suppressed at a low temperature of −20 °C. However, when the solvent was evaporated to dryness, the production of 1 significantly decreased, suggesting that a rapid decomposition occurred by the exposure to O2. The TLC analysis clarified that the decomposed product was mainly (EIMS, m/z 327, M+). The bubbling of O2 into the EtOAc-extract more significantly decreased the production of 1, further proving that X is labile to O2.

The EtOAc-extract from the incubation mixture of VioABE, which contains 9, was added to VioCD in the presence of NADPH, leading to the production of 1; thus the biosynthetic pathway of 1 was established to be VioA → VioB → VioE → VioD → VioC, as indicated by other workers.

VioA has a highly homologous alignment to 1-amino acid oxidase. We detected H2O2 with quinoneimine dye and phenol 4-aminooantipyrine peroxidase, and identified IPA as a hydroxyamine adduct. Our previous experiments using stable-isotopes demonstrated that the right side of 1 is constructed by direct incorporation of Trp (retention of C–C and C–N bonds in Trp side chain), and that IPA is not used for the construction of left side of 1, indicating that two molecules of IPAAmine are employed.

carboxyl group is substituted at the 11-position; the reaction of X with CH3N2 at 4 °C for 1 h gave the methyl ester (EIMS; m/z, 371, M+). The detailed NMR analyses of 8 measured at −10 °C led to the proposal that X was 5-(5-hydroxy-1H-indol-3-yl)-3-(1H-indol-3-yl)-1H-pyrrole-2-carboxylic acid, named protoviolaceinic acid 8. The methyl ester of 8 underwent no decomposition by the exposure to O2. Thus it turned out that 8 readily undergoes the oxidative decarboxylation to afford 6 in a non-enzymatic fashion. 9 (intermediate 2) was isolated from incubating Trp with VioABE in a similar way to 8. The detailed NMR analyses of 9 measured at −10 °C and those of the methyl ester (EIMS; m/z, 355, M+), at 25 °C unambiguously showed that 9 is the deoxy-derivative of 8. We propose to name 9 protodeoxyviolaceinic acid, which was successfully converted into 2 by incubating with VioC in the presence of NADPH. 9 was also converted into 7 (EIMS; m/z 311, M+ on the exposure to air. Thus, it is apparent that 6 and 7 are shunt products, which were generated via oxidative decarboxylation from 8 and 9, respectively. VioABED produced 8, while VioABE gave 9, indicating that VioD is a monooxygenase catalyzing the incorporation of O2 into 6-position of 9 to give 8.

Pyrole derivatives such as dimethylpyroles are sensitive to aerial condition and undergo autooxidation. Intermediates 8 and 9 could be allowed to react with triplet oxygen to form 1,4-adduct (Scheme 1), followed by decarboxylation to afford 10 and 11, respectively. Dehydration from 10 and 11 could provide 6 and 7, respectively. The methyl esters of 8 and 9 are stable to air oxidation, because decarboxylation of the methyl esters is impossible. It is to be noted that incorporation of O2 into 11-position of 6 and 7 proceeded in a no-enzymatic fashion.

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increased lability to O₂, compared to enzymatic oxidative decarboxylation reaction, leading to weak activity of VioD leads to the formation of 450 nm, which are characteristic of flavo-proteins. HPLC (C18) and ABCE produce position, yielding the putative intermediate monooxygenase catalyzing the hydroxylation reaction at the 16-

VioA and VioD also had FAD.

Scheme 2 (A) Biosynthetic pathway of Trp into 1 and 2. VioABCDE and ABCE produce 12 and 14, respectively, which undergo a non-enzymatic oxidative decarboxylation reaction, leading to 1 and 2. The weak activity of VioD leads to the formation of 2. The conversions of 6 and 7 into 1 and 2 (C) by VioC and that of 7 into 6 (D) never occurred by VioD. VioC cannot accept 6 and 7, and VioD also does not recognize 7.

for the bisindole core construction of 1 and 2. Structures of 8 and 9 further support our previous conclusion that Trp is directly incorporated into the right side of 1 and 2.1

Based on the BLAST search, VioC is highly homologous to p-hydroxybenzoate hydroxylase etc., and the characteristic motifs can be found such as GXGXXG, DG and GD sequences, which are responsible for binding to the cofactors of flavin and NAD(P)H.13 The electronic spectrum of VioC purified with Ni-NTA affinity column showed \( \lambda_{\text{max}} \) 448 nm and the supernatant, prepared by heating VioC at 100 °C for 10 min, had \( \lambda_{\text{max}} \) 370 and 450 nm, which are characteristic of flavo-proteins. HPLC (C18) analyses showed that the cofactor was FAD, but not FMN.13 VioA and VioD also had FAD.13 VioC is assignable to be a monooxygenase catalyzing the hydroxylation reaction at the 16-position, yielding the putative intermediate 12 (violaceinic acid, Scheme 2), which is a direct precursor of 1, because no enzymatic action occurs after the formation of 12 (VioC is the final enzyme). Trials to isolate 12 have been unsuccessful, because of the increased lability to O₂, compared to 8.11 However, the structure of 12 is rational and no other structure can be proposed from the following points: (1) VioC is a monooxygenase; (2) VioC accepts only 8 and 9 as the substrates, but not 6 and 7 (Scheme 2C); (3) furthermore, the reaction mechanism of 12 → 1 is identical to that of 8 → 6 (Scheme 1). 12 could undergo autooxidation to give 13,11 followed by the tautomerization process, affording 1 (Scheme 2A), thus 12 is the convincing structure. Again, it must be emphasized that addition of VioD into 7 and that of VioC into 6 never afforded 6 and 1 in our careful experiments, respectively (Schemes 2C and D), which are in sharp contrast to the previous reports.5,12 Together with all of the results, we propose the biosynthetic pathway of 1 as shown in Scheme 2A. Intermediate Y produced by VioAB has remained unsolved. VioE is assumed to be responsible for 1,2-shift of indole ring,4,5,13 but the exact function is still unknown, because intermediate Y has not been characterized.

In summary, we succeeded in the isolation of true intermediates 8 and 9. This allowed us to propose the definitive biosynthetic pathway (Scheme 2) and that the five enzymes VioA–E do not cover all the chemical reactions involved in the biosyntheses of 1 and 2. The oxidative decarboxylation reaction (12 → 1, 14 → 2) by a non-enzymatic process is required for the final biosynthetic step.

Notes and references


11. In the pyrrole autooxidation reaction, two indole rings act as electron donors, while O₃ works as electron acceptor. The electron density of 12 is higher than that of 8, due to the substitution with OH at the 16-position of the right indole ring. Thus, a more rapid formation of 13 occurred by the O₂ attack on 12, leading to the failure of isolation of 12. Furthermore, VioC requires O₂ for the catalysis, thus we cannot remove O₂ from the incubation system in order to trap 12.

12. Walsh et al. reported the successful conversions of 6 and 7 into 1 and 2 by VioC, and that of 7 → 6 by VioD, but our experiments clearly indicated that these conversions never occurred. Thus, the pathway proposed by them is erroneous.